Subcellular Biochemistry 101

Adrienne L. Edkins
Gregory L. Blatch *Editors*

The Networking of Chaperones by Co-Chaperones

Third Edition

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Preface

We were somewhat surprised when Nature Springer invited us to consider producing a third edition of our book *The Networking of Chaperones by Co-chaperones*. However, the downloads and citation data revealed that the second edition of the book (published in 2015) had been very well received by the scientific community. We were pleased to learn that all contributed volumes published in 2015 within the Nature Springer Biomedicine/Lifesciences portfolio (except handbooks and encyclopaedias) accumulated on average only half of the downloads in comparison to our book. Based on the impressive performance of the book and given the important advances in our understanding of the structure and function of co-chaperones over the intervening period, we accepted the invitation. Indeed, the broader biological functions of co-chaperones are starting to emerge, as is our understanding of how they act as nodes to network and functionalize chaperones. In particular, the broader biological functions of these fascinating proteins suggest that their co-chaperone function may not always be their primary function. In the preface to the second edition of the book, we defined a co-chaperone as "a non-client protein that interacts with a protein chaperone and/or its client protein to regulate chaperone function". We wonder if there is a need to revise this notion of a co-chaperone, to better capture how these proteins functionally network chaperone machinery into the cellular ecosystem? The Hsp40/DnaJ proteins are a good example of a co-chaperone family whose biochemical and biological functions go well beyond the confines of this definition. This family exhibits considerable evolutionary radiation (especially in protozoan parasites!), with many more members than most other chaperone or co-chaperone families. In addition, many members are large proteins, with just the J domain (among many other domains) as evidence of their membership to the Hsp40/DnaJ family. Interestingly, it was recently proposed by a scientific consortium of experts in the field that this family of proteins be renamed J-domain proteins (JDPs), and this nomenclature is starting to gain traction in the literature (including our book). You will notice that the third edition has a completely new chapter addressing the exciting new theme of post-translational modification of co-chaperones and the contribution to the chaperone code. Furthermore, all the

other chapters are not simply updated chapters but substantially refreshed contributions, providing critical insights into the current status of the field. Together, the chapters comprehensively capture the roles of the major co-chaperone families under physiological and disease conditions, including the core and specialized co-chaperones of the major molecular chaperones Hsp70 and Hsp90, co-chaperones of chaperonins, and organelle-specific co-chaperone function. And so, it is our pleasure to present to you the third edition of our book, which we trust you will enjoy reading as much as we have enjoyed facilitating its creation.

Grahamstown, South Africa — Adrienne L. Edkins
Sharjah, UAE — Gregory L. Blatch

Acknowledgements

We would like to acknowledge everyone who played a role in this fascinating and creative book project. This high-quality third edition of our book would not have been a reality without the excellent ongoing commitment of the previous authors and the insightful contributions of all the new authors and the reviewers. We also received tremendous support from Nature Springer in the planning and rolling out of this book project. In particular, we are grateful to Dr. Miriam Sturm, who always provided prompt, expert advice, and guidance, including robust oversight of the peer review of the editor's chapters. As with the previous two editions of the book, we did not compromise on ensuring rigorous peer review of the chapters; however, given the COVID-19 context, the peer review process took longer than we anticipated. Hence, we would like to thank everyone for their patience at each stage of the book production.

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About the Editors

Adrienne L. Edkins is Associate Professor of Biochemistry at Rhodes University where she holds a South African Research Chair (SARChI) in Molecular and Cellular Biology of the Eukaryotic Stress Response from the Department of Science and Innovation/National Research Foundation. Her research interests focus on understanding molecular mechanisms by which molecular chaperones, particularly Hsp70–Hsp90 chaperone–co-chaperone complexes, regulate cellular processes critical in normal and pathological cell functions. She has been recognized for her research by the award of the Rhodes University Vice Chancellor's Research Award (2015), the national DSI South African Women in Science (SAWiSA) Award for Distinguished Young Scientist in the Natural/Engineering Sciences (2018), and a Newton Fund Advanced Fellowship from the Academy of Medical Sciences (2018–2021). She is Fellow of the Cell Stress Society International (FCSSI), Fellow of the African Academy of Sciences (FAAS), and Member of the Academy of Science of South Africa (MASSAf).

Gregory L. Blatch Professor, has worked in academia for over 30 years, and his contributions to the academy and the promotion of science more broadly have been recognized through a number of awards and fellowships (e.g. Senior Fellow of the Cell Stress Society International, FCSSI; Fellow of the Royal Society of South Africa, FRSSAf). He is currently Executive Dean of the Faculty of Health Sciences, Higher Colleges of Technology, UAE (2021-present). Previously, he served as Pro Vice-Chancellor (PVC) Research at the University of Notre Dame Australia (2017–2020) and remains associated as Emeritus Professor. He has developed teaching and research capacity and capability at departmental, school, faculty, and university-wide levels, with over 10 years' experience as Senior/Executive Leader. His personal research interests fall within the broad field of cellular stress biology, and he is an international leader for his work on the role of stress proteins in disease (e.g. cancer) and infection (e.g. malaria).

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Chapter 1
Nucleotide Exchange Factors for Hsp70 Molecular Chaperones: GrpE, Hsp110/Grp170, HspBP1/Sil1, and BAG Domain Proteins

Andreas Bracher and Jacob Verghese

Abstract Molecular chaperones of the Hsp70 family are key components of the cellular protein-folding machinery. Substrate folding is accomplished by iterative cycles of ATP binding, hydrolysis, and release. The ATPase activity of Hsp70 is regulated by two main classes of cochaperones: J-domain proteins stimulate ATPase hydrolysis by Hsp70, while nucleotide exchange factors (NEFs) facilitate the conversion from the ADP-bound to the ATP-bound state, thus closing the chaperone folding cycle. NEF function can additionally be antagonized by ADP dissociation inhibitors. Beginning with the discovery of the prototypical bacterial NEF, GrpE, a large diversity of nucleotide exchange factors for Hsp70 have been identified, connecting it to a multitude of cellular processes in the eukaryotic cell. Here we review recent advances toward structure and function of nucleotide exchange factors from the Hsp110/Grp170, HspBP1/Sil1, and BAG domain protein families and discuss how these cochaperones connect protein folding with cellular quality control and degradation pathways.

Keywords Disaggregase activity · Proteostasis · Protein structure · Protein quality control

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Introduction

Cells are confronted with a variety of adverse environmental conditions such as heat shock, oxidative injury, heavy metals, and glucose depletion as well as pathologic states such as inflammation, tissue damage, infection, ischemia, and reperfusion. To cope with this plethora of stresses, cells induce the expression of cytoprotective genes including heat shock proteins (Hsps). Many Hsps function as molecular chaperones that aid the folding, assembly, and targeting of their substrate proteins. Under stress conditions, chaperones shield denatured proteins from aggregation, disassemble protein aggregates, assist protein refolding, or target proteins to the degradation machinery in order to maintain protein homeostasis (proteostasis) in the cell (Hartl et al. 2011; Balch et al. 2008). Hsps can be classified into families based on their molecular mass: Hsp60, Hsp70, Hsp90, Hsp100, and small heat shock proteins. Importantly, these general molecular chaperones do not work by themselves but are dependent on an elaborate network of different cochaperones that control their functions and orchestrate the folding of proteins in the cell (Kim et al. 2013; Bukau et al. 2006). Within this proteostasis network, the Hsp70 system forms a central hub at the crossroads between the translation apparatus, specialized downstream chaperones, and the cellular degradation machinery. Hsp70 function, which is governed by its ATP hydrolysis activity, is regulated by cochaperones. In this review, we will focus on a specific group of Hsp70 cochaperones, the nucleotide exchange factors (NEFs). We will present the structures and molecular functions of NEFs and discuss their role in the cellular protein-folding and degradation machinery.

Hsp70 Architecture and Functional Cycle

Hsp70 was initially identified in the bacterium *Escherichia coli*, where it was named DnaK. Later, Hsp70 proteins were found to be conserved in eukaryotes as well (Gupta 1998). In eukaryotes, compartment-specific isoforms were identified in the cytosol/nucleus, endoplasmic reticulum (ER) lumen, and mitochondria. The human cytosol contains multiple Hsp70 paralogs, including constitutively expressed (Hsc70/HSPA8) and stress-inducible isoforms (Hsp72/HSPA1A/B). The ER-lumenal and mitochondrial forms are named BiP/Grp78/HSPA5 and mortalin/Grp75/HSPA9, respectively.

Hsp70 proteins share a conserved domain architecture containing two major domains (Fig. 1.1): an amino-terminal nucleotide binding domain (NBD) and a carboxy-terminal substrate-binding domain (SBD) (Mayer and Bukau 2005; Mayer and Gierasch 2019). The NBD is approximately 44 kDa in size and forms a bilobular structure that encloses a cleft with the nucleotide binding pocket at the bottom (Fig. 1.1c) (Flaherty et al. 1990). The structurally homologous lobes (I and II) of the NBD are subdivided into regions A and B. The SBD comprises of a

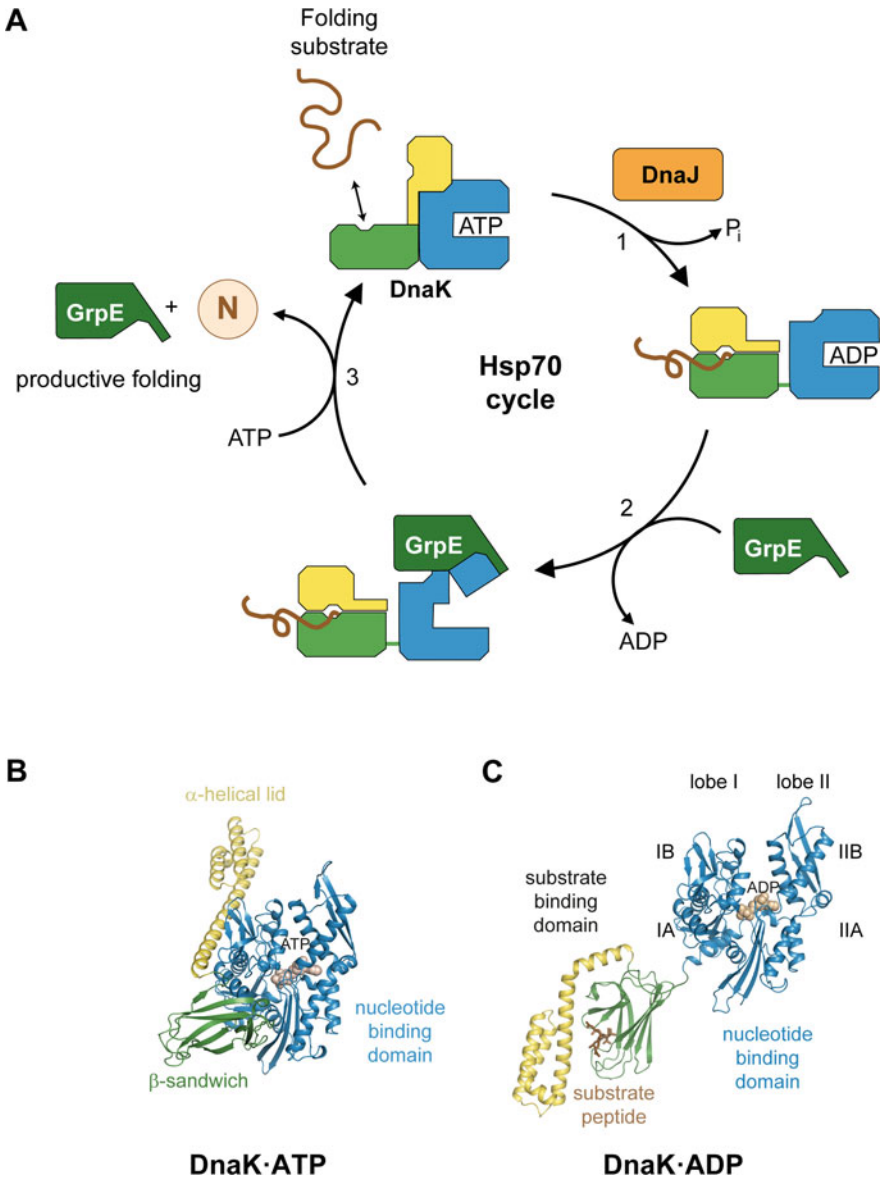

Fig. 1.1 DnaK structure and folding cycle. (**a**) Model for the Hsp70 folding cycle. The DnaK·ATP complex has weak substrate affinity. ATP binding to the NBD (blue) stabilizes a compact domain arrangement, which leaves the SBD (yellow and green) in an open conformation. This conformation exhibits dynamic interactions with the substrate (indicated in brown). ATP hydrolysis stimulated by DnaJ (1) causes a conformational change in the NBD that triggers the formation of the closed SBD conformation, which has higher affinity for the substrate, resulting in a stable substrate complex. The binding of the NEF GrpE (2) promotes a slight opening of the NBD, which results in the release of ADP from DnaK. The cycle is reset (3) when a new ATP molecule binds to the NBD, triggering the release of NEF and substrate. (**b**) Crystal structure of the DnaK·ATP complex. The peptide backbone is shown in ribbon representation, and the bound nucleotide as space-filling model [PDB code 4B9Q (Kityk et al. 2012)]. The nucleotide binding, β-sandwich, and α-helical domains are

β-sandwich subdomain with a groove that binds hydrophobic polypeptides and a carboxy-terminal α-helical "lid" that folds over the peptide binding site and facilitates high affinity substrate interaction (Zhu et al. 1996). The conserved hydrophobic NBD-SBD inter-domain linker plays an important role in conveying conformational information between the domains (Vogel et al. 2006; Swain et al. 2007).

Studies on DnaK from *E. coli* showed that Hsp70 functions through an ATP-dependent cycle (Fig. 1.1a). When ATP is bound to the NBD, the Hsp70 SBD transitions to a conformation with low affinity for the substrate (Fig. 1.1b) (Kityk et al. 2012; Qi et al. 2013). ATP hydrolysis induces a structural rearrangement in the NBD that detaches the SBD to assume a conformation with high affinity for segments in client proteins having five consecutive hydrophobic amino acid residues (Fig. 1.1c) (Rüdiger et al. 1997; Zhuravleva et al. 2012). Substrate binding increases the ATP hydrolysis rate of DnaK substantially. The spontaneous transition between the two states is slow as Hsp70 only has weak intrinsic ATPase activity. This prevents substrate-free cycling. The cycle is reset with the release of ADP and replacement with ATP, which releases the client protein for a new folding attempt.

DnaK, DnaJ, and GrpE: The Eubacterial Hsp70 System

For its proper functioning in protein folding, DnaK is dependent on the ATPase-stimulating cochaperone, DnaJ, and the nucleotide exchange function of GrpE (Fig. 1.1a). Although interactions with substrate protein trigger ATP hydrolysis in DnaK, meaningful folding rates with model proteins are only achieved in the presence of DnaJ, the prototypical Hsp40 protein (Laufen et al. 1999). Hsp40 and other J-domain proteins are reviewed elsewhere in the book. Because of DnaK's slow off-rate for ADP, the additional presence of GrpE is essential in *E. coli* to reset the Hsp70 folding cycle (Ang and Georgopoulos 1989). The combined action of the two cofactors is thought to drive the folding cycle of the molecular chaperone, resulting in repetitive rounds of substrate binding and release.

GrpE functions as the nucleotide exchange factor (NEF) for DnaK by stabilizing a NBD conformation with an open nucleotide binding cleft (Harrison et al. 1997) (Fig. 1.1a). The crystal structure revealed that subdomain IIB of DnaK is rotated outward in the complex, which weakens the contacts to ADP (Fig. 1.2).

The cytosol in *E. coli* comprises of two additional isoforms of Hsp70, HscA and HscC, and five more proteins containing a J-domain. These isoforms and their associated J-protein cofactors have more specialized functions than DnaK, such as

Fig. 1.1 (continued) indicated in blue, green, and yellow, respectively. (**c**) NMR model for the DnaK·ADP complex. In this state, the NBD and SBD are loosely associated [PDB code 2KHO (Bertelsen et al. 2009)]. The representation mode is the same as in panel B. The peptide NRLLLTG from the complex structure with the SDB alone [PDB code 1DKZ (Zhu et al. 1996)] is superposed

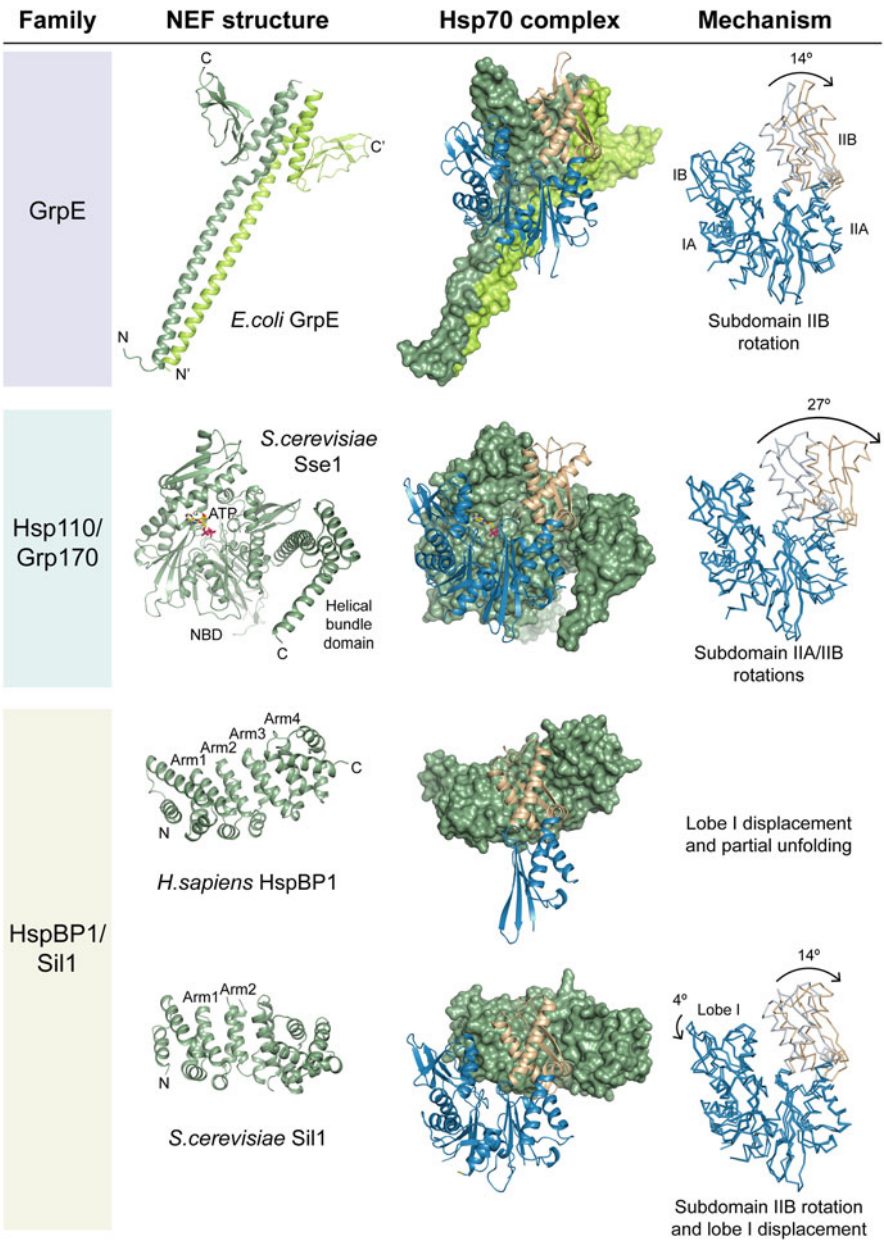

Fig. 1.2 Structure and mechanism of nucleotide exchange factors. Structures for the four NEF families are shown together with the respective Hsp70 complexes. The NEF is always shown in green, the Hsp70 NBD in blue with subdomain IIB highlighted in beige. On the right, the structure of the NBD in the complex is superposed with the ADP-bound conformation, and the putative nucleotide exchange mechanism indicated. For comparison, the structure of the NEF-antagonist Hip is shown. The drawings are based on the PDB coordinate sets 1DKG [GrpE·DnaK (Harrison et al. 1997)], 2V7Y [DnaK·ADP (Chang et al. 2008)], 3D2F [Sse1p·Hsp70 (Polier et al. 2008)], 1HPM [Hsc70·ADP (Wilbanks and McKay 1995)], 1XQS [HspBP1·Hsp70-lobeII (Shomura et al. 2005)], 3QML [Sil1p·Kar2p (Yan et al. 2011)], 1HX1 [Bag1·Hsc70 (Sondermann et al. 2001)], 3A8Y [Bag5·Hsp70 (Arakawa et al. 2010)], 3CQX [Bag2·Hsp70 (Xu et al. 2008)], and 4J8F [Hip·Hsp70 (Li et al. 2013)]

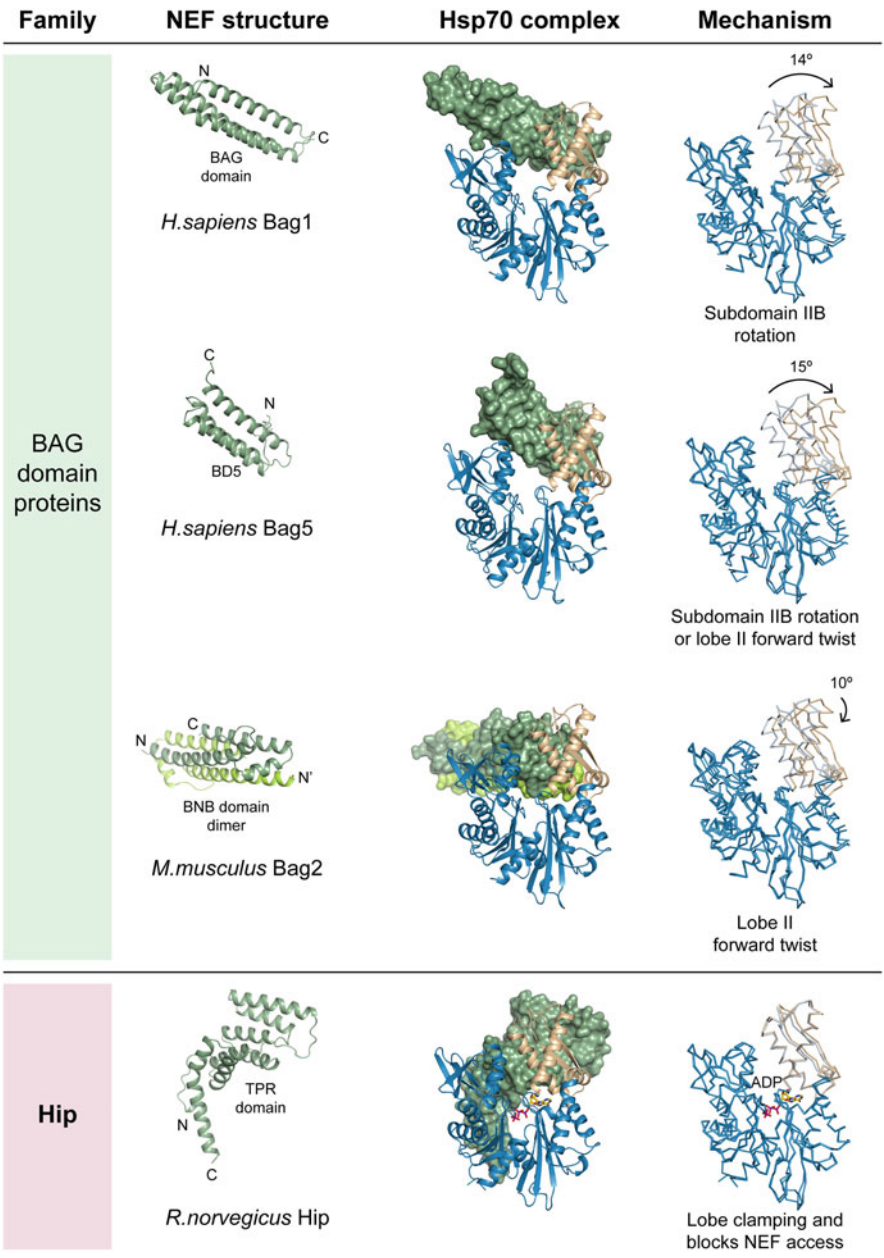

Fig. 1.2 (continued)

incorporation of Fe-S clusters into substrates using the IscU scaffold protein. In contrast, DnaK appears to be the more general-purpose protein-folding machine. Interestingly, functioning of HscA does not require GrpE (Brehmer et al. 2001).

The Evolution of the Eukaryotic Hsp70 Systems

In eukaryotes, close sequence homologs to GrpE are only found in mitochondria and chloroplasts, i.e., organelles of eubacterial origin, whereas orthologs to DnaK and DnaJ are found in the cytosol/nucleus and the ER lumen. These endosymbiont-derived organelles have thus preserved a eubacterial protein-folding machinery (homologs to GroEL, GroES, HtpG, and ClpA are further evidence for this), although the respective genes were eventually transferred to the host nuclear genome. The "paralogs" of DnaK, Hsp70, Hsc70, and BiP have somewhat different properties and are only found in eukaryotes. These proteins might thus have originated from an independent gene transfer to the archaeal progenitor of eukaryotes, perhaps of a more specialized isoform or without the NEF. Note that the genes of DnaJ and DnaK are often part of one operon in bacteria, whereas GrpE is independently transcribed. Consistently, archaea in general do not harbor components of the Hsp70 system, unless the presence of other typical bacterial genes suggests a relatively recent fusion event with a eubacterium. These archaeal Hsp70 proteins are clearly more closely related to their eubacterial counterparts than to the Hsp70 proteins of the eukaryotic cytosol and ER lumen.

For a long time, the eukaryotic Hsp70 proteins were assumed to require no NEF assistance. The measured ADP off-rates were at least one order of magnitude higher than for DnaK in *E. coli*. Hence, it came as quite a surprise when the first cytosolic NEF, Bag1, containing a BAG domain, was discovered (Höhfeld and Jentsch 1997; Takayama et al. 1999). Soon after, Sil1p and Fes1p of *Saccharomyces cerevisiae* were recognized as members of a second family of NEFs, the HspBP1/Sil1 proteins (Kabani et al. 2000, 2002b). Finally, the Grp170/Hsp110 family of Hsp70 homologs was identified as potent NEFs for Hsp70 in the ER lumen and cytosol, respectively (Dragovic et al. 2006a; Raviol et al. 2006b; Steel et al. 2004). The fascinating details of this discovery process were reviewed earlier (Brodsky and Bracher 2007).

It is now clear that under cellular conditions, the function of eukaryotic Hsp70 proteins is strongly dependent on nucleotide exchange factors. The combined deletion of the yeast Hsp110 homologs, Sse1p and Sse2p, is lethal (Raviol et al. 2006b; Shaner et al. 2004); the deletion of Fes1p results in a temperature-sensitive phenotype, suggesting severe problems in protein folding (Shomura et al. 2005; Kabani et al. 2000). The probable reason for the early misconception of NEF expendability is the presence of considerable amounts of inorganic phosphate (P_i) in cellular fluids (17–27 mM in *S. cerevisiae* according to ^{31}P-NMR measurements (Gonzalez et al. 2000). Additional binding of P_i lowers the spontaneous off-rate of ADP from eukaryotic Hsp70 by approximately one order of magnitude, apparently via reduced nucleotide binding domain (NBD) dynamics (Arakawa et al. 2011;

Gässler et al. 2001). Thus, the spontaneous off-rate of eukaryotic Hsp70 under physiological conditions is actually close to that of DnaK.

The Grp170/Hsp110 family of NEFs appears to be the most ancient and universal type of eukaryotic Hsp70 NEFs (Table 1.1). Coding sequences for presumed homologs have been identified in virtually every eukaryotic genome so far. Humans have three genes for cytosolic isoforms (Hsp105/Hsp110, Apg-1, and Apg-2) and one ER-lumenal isoform (Grp170); *S. cerevisiae* has two cytosolic (Sse1p and Sse2p) and one ER-resident form (Lhs1p). Grp170/Hsp110 family proteins are distantly related to eukaryotic Hsp70 and appear to have emerged from functional specialization of Hsp70 paralogs. The other NEF families, BAG domain proteins and Sil1/HspBP1 homologs, have rather generic structures frequently found in the eukaryotic proteome, specifically helix bundles and successions of armadillo repeats, respectively. Such scaffolds can rapidly (on an evolutionary timescale) adapt to a new function after a gene duplication event and have been employed over and over again in eukaryotic protein evolution. For example, helix bundles are also found in syntaxin SNARE proteins, and armadillo and HEAT repeat proteins in nuclear transport factors and β-catenin (Tewari et al. 2010). It is conceivable that BAG proteins have emerged multiple times, having short and long three-helix bundle structures (Bag1 and Bag4/Bag5), insertions, or four-helix bundle dimer structures (Bag2). Their few common signature residues have been forced by the evolutionary constraints of the binding partner, the NBD of Hsp70, which exhibits high surface conservation (details below). It moreover appears that the versions of the ER-lumenal NEF, Sil1, from yeast and animals have evolved independently: Although yeast Sil1p resembles the mammalian HspBP1 at the secondary and tertiary structure level (Shomura et al. 2005; Yan et al. 2011), it appears to employ a binding mode and mechanism of action distinct from mammalian Sil1, which acts more similar to HspBP1 (Hale et al. 2010; Howes et al. 2012). Consequently, the ancestry and exact functional role of BAG and Sil1/HspBP1 protein homologs in different species are difficult to rationalize on sequence data alone. Humans and *Arabidopsis thaliana* have five and seven known cytosolic BAG isoforms, respectively (Table 1.1); yeast has one ER-membrane-bound homolog, Snl1p, but exact functional homologs to Snl1p have not been identified in humans and *Arabidopsis* either (Sondermann et al. 2002; Takayama et al. 1999).

In addition to the emergence of three Hsp70 NEF families in multiple isoforms in eukaryotes, an even more dramatic expansion in J-domain protein diversity has occurred, resulting in approximately 50 isoforms in humans [see review in Kampinga and Craig (2010)].

Table 1.1 Eukaryotic NEFs for Hsp70 proteins

		Species	Name	Proposed functions	References
GrpE	Mitochondria	*S. cerevisiae*	Mge1p	Protein import and maturation	
		A. thaliana	*At*Mge1	Possible UV-B stress tolerance	Hu et al. (2012)
			*At*Mge2	Chronic heat stress tolerance	
		H. sapiens	GrpEL1/HMGE	Protein import and maturation	Srivastava et al. (2017)
			GrpEL2	Protein import and maturation	
	Chloroplast	*Chlamydomonas reinhardtii*	Cge1	Protein import, temperature tolerance, VIPP1 oligomer assembly	Liu et al. (2010), Willmund et al. (2007)
		Physcomitrella patens	*Pp*Cge1, *Pp*Cge2	Protein import and maturation	Shi and Theg (2010)
Hsp110/ Grp170	Cytosol/ nucleus	*Plasmodium falciparum*	*Pf*Hsp110c	Proteome stabilization in malarial fevers	Muralidharan et al. (2012)
		S. cerevisiae	Sse1p	Substrate binding, constitutive expression	Mukai et al. (1993)
			Sse2p	Stress-inducible isoform	
		S. pombe	Pss1	Ras1 GTPase signaling	Chung et al. (1998)
		N. crassa	Hsp88	Direct binding to small Hsp Hsp30	Plesofsky-Vig and Brambl (1998)
		A. thaliana	Hsp91/Hsp70-14	Possible role in thermotolerance	Jungkunz et al. (2011), Storozhenko et al. (1996)
		D. melanogaster	HSC70cb	Suppresses aggregation-induced toxicity along with DnaJ-1	Kuo et al. (2013)
		H. sapiens	Hsp105α,β/ Hsp110/HspH1	Disaggregase activity, substrate binding	
			Apg-1/Osp94/ HspA4L/HspH3	Spermatogenesis	
			Apg-2/HspA4/ HspH2	Spermatogenesis, disaggregase activity	
	ER	*S. cerevisiae*	Lhs1p	Protein translocation, lumenal folding, UPR, ERAD	

(continued)

Table 1.1 (continued)

		Species	Name	Proposed functions	References
		H. sapiens	Grp170/Hyou1/ Orp150	Response to oxygen deprivation	
HspBP1/Sil1	Cytosol/ nucleus	*S. cerevisiae*	Fes1p	Co-translational folding, degradation of misfolded proteins	
		A. thaliana	*At*Fes1A	Thermotolerance, response to salt stress	Zhang et al. (2010)
		H. sapiens	HspBP1	Spermatogenesis, Hsp70 inhibition?	
	ER	*S. cerevisiae*	Sls1p/Sil1p	Protein translocation, lumenal folding, UPR, ERAD	
		H. sapiens	Sil1/BAP	Neuronal morphology, migration and axon growth	
BAG-domain proteins	Cytosol/ nucleus	*S. cerevisiae*	Snl1p	Ribosomal editing, co-translational folding	
		S. pombe	Bag101/Bag-1A	Protein QC and degradation	
			Bag102/Bag-1B	Kinetochore integrity	
		A. thaliana	*At*Bag1–*At*Bag4	Plant programmed cell death	Kabbage and Dickman (2008), Doukhanina et al. (2006)
			*At*Bag5	Calmodulin signaling	
			*At*Bag6	Calmodulin signaling?	
		C. elegans	Bag-1	Protein QC and degradation?	
			Bag-2/unc-23	Muscle maintenance	
		D. melanogaster	Starvin	Muscle maintenance, recovery from cold stress	Coulson et al. (2005)
		H. sapiens	Bag1/Rap46/HAP	Apoptosis, protein QC and degradation	
			Bag2	CHIP Ub-ligase inhibition	
			Bag3/CAIR-1	Autophagy, cell adhesion and migration	
			Bag4/SODD	Apoptosis	
			Bag5	Parkin Ub-ligase inhibition, mitophagy	
	ER	*A. thaliana*[a]	*At*Bag7	UPR	Williams et al. (2010)

[a]*A. thaliana* has homologs to Sil1 (F4F15.90) and Grp170 (AtHsp70-17). These were however not characterized at the protein level yet

Molecular Structure and Function of Eukaryotic NEFs

Eukaryotic GrpE Homologs

Structural data for eukaryotic GrpE homologs are not yet available. Judging from sequence alignments, their structures are likely fairly similar to bacterial GrpE proteins, which have been solved for the homologs in *E. coli* (Harrison et al. 1997), *Thermus thermophilus* (Nakamura et al. 2010), and *Geobacillus kaustophilus* (Wu et al. 2012). All these proteins have dimeric two-domain structures composed of a coiled-coil helix bundle and a wing-like β-domain (Fig. 1.2). One β-domain engages in contacts with subdomains IB and IIB, assisted by additional contacts from the helix bundle, stabilizing a NBD conformation with an open nucleotide binding cleft. Opening is enabled by an outward rotation of subdomain IIB.

Simulations suggest a highly dynamic structure for the NBD of Hsp70 proteins, allowing shearing motions between the lobes and an outward rotation of subdomain IIB around an inbuilt hinge, which likely influence the nucleotide exchange rate (Ung et al. 2013). GrpE and the other Hsp70 NEFs appear to capture and stabilize open states in which a subset of the interactions between the NBD and ADP is disabled, thereby lowering ADP affinity. Substantial parts of the NBD contact area with GrpE become buried near the lobe interface in the ADP-bound conformation of DnaK, suggesting that GrpE captures open conformations, but cannot "force" the Hsp70 NBD to open. ATP binding induces a conformational change in the NBD of DnaK, displacing the binding sites on lobes I and II by inter-lobe shearing, resulting in a strongly decreased affinity for GrpE. In summary, both ADP and ATP compete with GrpE for binding to DnaK.

The Hsp110 Family of Nucleotide Exchange Factors

The Hsp110/Grp170 proteins belong to the Hsp70 protein family (Easton et al. 2000). Crystal structures of the yeast Hsp110 protein, Sse1p, revealed a shared domain composition comprising an N-terminal actin-type nucleotide binding domain, followed by a β-domain and an α-helix bundle (Liu and Hendrickson 2007; Polier et al. 2008; Schuermann et al. 2008). Hsp110 family protein sequences are however much less conserved than canonical Hsp70, with the greatest divergence found in the C-terminal domains. Backbone extensions compared to canonical Hsp70 proteins are found at the C-terminus and within the β-domain (Fig. 1.3). The Grp170 homologs have even larger extensions than cytosolic homologs and always bear N-terminal import and C-terminal ER-retention signal sequences (Table 1.1).

In the crystal structures of Sse1p, the α-helix bundle is associated with the flank of the NBD, resulting in a compact conformation (Fig. 1.2). The β-domain undergoes extensive interactions with the bottom of the NBD, but not with the α-helix bundle domain, which extends in the opposite direction. Sse1p exhibits a pronounced twist

of the NBD lobes, revealing a bound ATP molecule in the center. The crystal structure of the isolated NBD from the human Hsp110 isoform Hsp105 in complex with ATP exhibited a similar conformation (Gozzi et al. 2020). Structures of an ATPase-inactive DnaK mutant demonstrated that the binding of ATP induces a very similar conformation in canonical Hsp70 proteins (Kityk et al. 2012; Qi et al. 2013).

In the crystal structures of the complex, the NBDs of Sse1p and mammalian Hsp70 face each other in a pseudo-symmetrical fashion (Polier et al. 2008; Schuermann et al. 2008). The NBD of Hsp70 is captured in an open conformation by additional interactions of subdomain IIB with the α-helix bundle domain of Sse1p. In this conformation, ADP cannot simultaneously engage in direct interactions with all four subdomains and is thus more likely to dissociate, explaining the nucleotide exchange activity of Sse1p. The residues mediating key contacts to Hsp70 are conserved in all Hsp110/Grp170 proteins (Andreasson et al. 2010; Hale et al. 2010). Only the compact, ATP-bound conformation of Hsp110/Grp170 proteins provides the necessary geometry required for simultaneous interactions between NBD·NBD and α-helix bundle·subdomain IIB of Hsp110/Grp170 and Hsp70, respectively (Raviol et al. 2006b; Shaner et al. 2004; Andreasson et al. 2008).

Besides serving as essential NEFs for Hsp70, Hsp110/Grp170 proteins potently stabilize denatured proteins against aggregation (Goeckeler et al. 2002; Oh et al. 1997, 1999). The molecular basis for this holdase activity to buffer against proteostatic stress is still controversial. Canonical Hsp70 proteins stably interact with substrate proteins only in the ADP state, enclosing hydrophobic peptide segments between β-domain and α-helix bundle. While Sse1p appears to have no intrinsic ATPase activity—bound ATP survived in the crystallization experiments for weeks—ATPase stimulation by J-domain proteins has been observed (Mattoo et al. 2013; Raviol et al. 2006a). Consistently, binding of Sse1p and human Hsp110 to hydrophobic peptides has been reported, although with a preference for aromatic residues, which is in contrast to canonical Hsp70s that prefer aliphatic sidechains and prolines (Goeckeler et al. 2008; Xu et al. 2012; Rüdiger et al. 1997; Zahn et al. 2013). Because of their low sequence conservation in the β-sheet domain, Hsp110 orthologs may differ considerably in their substrate-binding properties. For example, Sse1p potently stabilizes the model protein firefly luciferase (FLuc) at 42 °C for subsequent refolding, while its close paralog, Sse2p, is inactive (Polier et al. 2010). The reason for this surprising difference seems to be that Sse1p unfolds partially at 37 °C with a concomitant increase in aggregation prevention capacity, while Sse2p is stable until 46 °C similar to the human Hsp110, Apg-2, which unfolds at 51 °C (Polier et al. 2010; Raviol et al. 2006a).

While the Hsp110 holdase activity appears to be important, its NEF function is critical (Raviol et al. 2006b; Shaner et al. 2004). Only mutant forms of Sse1p that abolish interactions with Hsp70 and nucleotide exchange were lethal in the *SSE1/SSE2* deletion background (Polier et al. 2008). Similar requirements were found for the "disaggregase" function of mammalian Hsp110, Hsp70, and Hsp40 (see below).

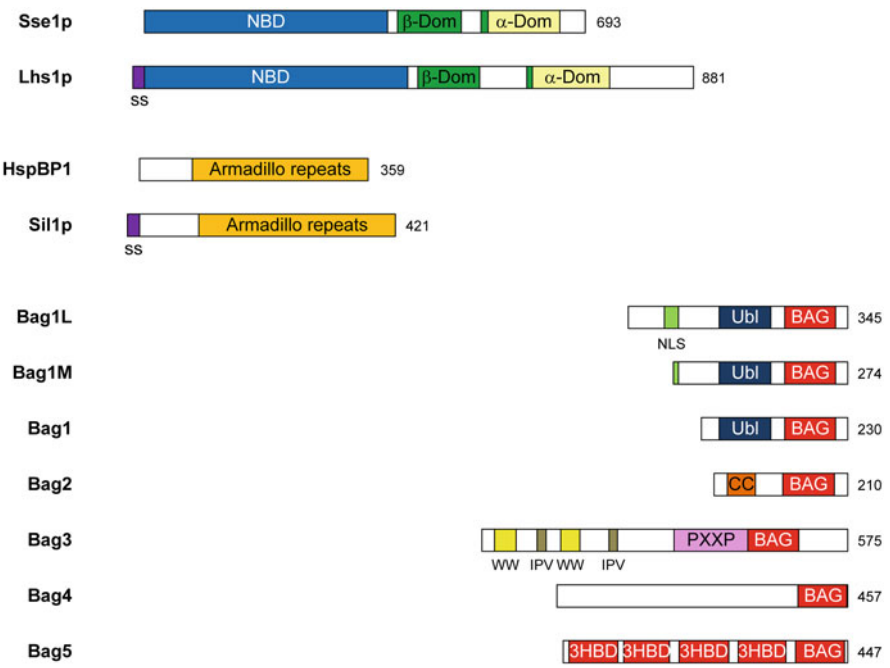

Fig. 1.3 Domain architectures of different NEF families. As examples for Hsp110 and Grp170 proteins, the yeast homologs Sse1p and Lhs1p are shown, respectively. Both consist of an N-terminal nucleotide binding domain (NBD, blue), a β-sandwich (β-Dom, green), and a α-helix bundle domain (α-Dom, pale yellow). SS indicates a signal sequence for ER import. The HspBP1/Sil family proteins have characteristic armadillo repeat folds (orange). All members of the BAG family in humans, Bag1–5, contain C-terminal Hsp70-binding BAG domains (red), but have otherwise divergent domain architecture. Bag5 has four additional BAG domains of unknown function. Bag1 isoforms contain ubiquitin-like domains (Ubl, dark blue), which might associate with the regulatory particle of the 26S proteasome. Bag2 contains a coiled-coil dimerization domain (CC, orange). Bag3 comprises multiple N-terminal sequence motifs, WW domains (WW, yellow), IPV sequence motifs (brown), and PXXP repeats (pink). Bag1L has a NLS sequence (light green) for nuclear targeting

Sil1/HspBP1 Homologs

HspBP1 (Hsp70 binding protein 1) is the mammalian homolog of the cytosolic Fes1p protein in *S. cerevisiae* (Kabani et al. 2002a, b; Raynes and Guerriero 1998). The ER-lumenal paralogs are named Sls1p/Sil1p or Sil1/BAP (BiP associated protein) in yeast and mammals, respectively (Kabani et al. 2000; Chung et al. 2002). Sil1 homologs occur almost ubiquitously in eukaryotes. Homologs to HspBP1 are found in most animal, plant, algal, and fungal genomes. Sil1/HspBP1 proteins are composed of a divergent N-terminal part of ~85 residues and a conserved C-terminal core domain, which alone is sufficient to mediate nucleotide exchange (Fig. 1.3). Crystal structures showed that the core domains of human

HspBP1 and yeast Sil1p consist of armadillo repeats flanked by capping helix pairs (Shomura et al. 2005; Yan et al. 2011) (Fig. 1.2). Surprisingly, the complex structures with the respective Hsp70 binding partner revealed distinct binding modes for the paralogs. The curved-shaped HspBP1 associates so extensively with subdomain IIB of the Hsp70 NBD that the bulk of the NEF clashes severely with lobe I, thereby destabilizing its fold, as judged from tryptophan fluorescence quenching and increased sensitivity against protease degradation (Shomura et al. 2005). Yeast Sil1p also embraces subdomain IIB, however using different molecular contacts, resulting in a distinct region covered by the NEF (Yan et al. 2011). This binding mode just induces an outward rotation of subdomain IIB and a slight sideway displacement of lobe I, more similar to the complexes with GrpE (Harrison et al. 1997) and the Hsp110 protein, Sse1p (Polier et al. 2008; Schuermann et al. 2008). The binding mode of animal and plant Sil1 appears to resemble HspBP1 closer than yeast Sil1p, as judged from mutational analysis (Hale et al. 2010; Howes et al. 2012). Interestingly, the flexible N-terminal extensions to the C-terminal core domain in HspBP1, Fes1p, and Sil1/BAP harbor sequence motifs that mimic a Hsp70 peptide substrate and increase the efficiency of Hsp70 substrate release upon nucleotide exchange (Gowda et al. 2018; Rosam et al. 2018). Removal of these elements from Fes1p causes a temperature-sensitive phenotype similar to the Fes1p deletion in *S. cerevisiae*, suggesting a crucial mechanistic role (Gowda et al. 2018).

BAG Domain-Containing NEFs

BAG (Bcl-2-associated athanogene) family proteins have a modular domain architecture comprising a conserved region of ~100 amino acids at the C-terminus, called the BAG domain (Takayama et al. 1999). At the N-terminal, diverse domains and sequence motifs were found for BAG domain proteins (Fig. 1.3). The human genome comprises six BAG family protein sequences, which were numbered Bag1–6 (Takayama and Reed 2001) (Table 1.1). This protein family is structurally and functionally quite heterogeneous and is discussed below. Only Bag1 and Bag3 appear to be conserved in most metazoans. Homologs have been described in the fruit fly, *Drosophila melanogaster* (Arndt et al. 2010), the nematode worm, *Caenorhabditis elegans* (Nikolaidis and Nei 2004), and the tunicate, *Ciona intestinalis* (Wada et al. 2006). Bag6 was later shown to lack Hsp70 NEF function (Mock et al. 2015).

The first structures to be solved were the BAG domain of Bag1 (Rap46) in isolation and in complex with the NBD of Hsc70, revealing a bundle structure with three long α-helices for the BAG domain (Sondermann et al. 2001; Briknarova et al. 2001) (Fig. 1.2). Interactions with α-helices 2 and 3 of Bag1 stabilize a conformational change in the Hsc70 NBD similar to the GrpE·DnaK complex (Harrison et al. 1997; Sondermann et al. 2001). Three different isoforms of Bag1 exist in cells, which are generated by alternative translation initiation from a single

mRNA (Fig. 1.3). All Bag1 isoforms contain a ubiquitin-like (Ubl) domain that serves as a sorting signal to facilitate interaction with the 26S proteasome (Alberti et al. 2003). The Bag1L isoform contains an additional nuclear localization signal (NLS) at the extreme N-terminus, whereas the other two isoforms are present in the cytosol (Takayama et al. 1998). Interestingly, the BAG domain shares binding sites with Hsc70 and Raf1, a stress-signaling anti-apoptotic kinase, and the two proteins bind Bag1 in a mutually exclusive manner (Song et al. 2001). The structure of the Ubl domain from mouse Bag1 has been solved by NMR, revealing the characteristic ubiquitin-like fold (Huang and Yu 2013). In mice, this domain of Bag1 mediates interaction with the cytoplasmic tail of the heparin-binding EGF-like growth factor (HB-EGF) precursor, thereby altering cell adhesion and secretion of the mitogen, HB-EGF (Lin et al. 2001).

Bag3 (CAIR-1) is expressed prominently in striated muscle tissue but is also necessary for development and blood cell formation. Bag3 deletion in mice resulted in severe myopathy (Homma et al. 2006) and loss of hematopoietic stem cells (Kwon et al. 2010). Interestingly, Bag3 is the only heat stress-inducible BAG domain protein (Franceschelli et al. 2008; Jacobs and Marnett 2009). Bag3 contains various sequence motifs and domains, such as WW domains, proline-rich repeats (PXXP), and IPV motifs, which mediate interactions with numerous partner proteins other than Hsp70. For example, the first WW domain was shown to interact with PXXP motifs at the C-terminus of PDZGEF2, a regulatory protein involved in cell adhesion (Iwasaki et al. 2010); two IPV motifs mediated binding to small heat shock proteins, Hsp27 (HspB1), αB-crystallin (HspB5), Hsp22 (HspB8), and Hsp20 (HspB6) as well as providing a physical link between Hsp27 and Hsp70 (Fuchs et al. 2010; Rauch et al. 2017). The PXXP repeats of Bag3 likely interact with SH3 domains found in regulatory proteins of cell adhesion and migration (Doong et al. 2000). These interactions link Bag3 to processes such as development, autophagy, and cytoskeletal organization [reviewed in Rosati et al. (2011)]. The complex of Bag3, Hsc70, and HspB8 was strongly implicated in macroautophagy (Arndt et al. 2010; Lamark and Johansen 2012), a process in which portions of the cytosol are engulfed by a membrane and digested. The complex appears to be involved in targeting aggregated proteins for degradation to aggresomes, which are microtubule-dependent collection points for such terminally misfolded proteins in the cell (Guilbert et al. 2018; Kopito 2000). Bag3 co-localizes with the ubiquitin adapter protein, p62/SQSTM1, a key regulator of the macroautophagy pathway (Gamerdinger et al. 2009). An association of Bag3 with the adaptor protein, 14-3-3γ, is dependent on phosphorylation at Ser136 and Ser173 and may serve to attach aggregates to the motor protein, dynein, that travels along microtubules (Xu et al. 2013). Macroautophagy appears to be vitally important for muscle maintenance. In *D. melanogaster* muscles, the Bag3 ortholog, starvin, is required for Z-disk maintenance through a process named "chaperone-assisted selective autophagy" (CASA) (Arndt et al. 2010). A complex of Bag3, Hsc70, and HspB8 is needed for autophagy of the large muscle protein filamin after mechanical tension-induced unfolding (Ulbricht et al. 2013). Autophagosome formation is dependent on the interaction of the Bag3 WW domain with the filamin-interacting protein synaptopodin-2. On the

other hand, Bag3 also stimulates filamin biosynthesis via sequestration of TSC1/TSC2—also mediated by the WW domain—relieving mTORC1 inhibition of translation (Kathage et al. 2017). Starvin biochemically and genetically interacts with the serine/threonine kinase, NUAK, which appears to regulate the process (Brooks et al. 2020). Besides its role in macroautophagy, the Bag3-HspB8 complex also appears to be involved in actin dynamics during mitosis (Fuchs et al. 2015). In addition to domains needed for targeting, Bag3 and Bag1 appear to have elements to enhance substrate release during nucleotide exchange outside of the BAG domain (Rauch et al. 2016). Mutations in Bag3 are associated with autosomal dominant forms of myofibrillar myopathy and dilated cardiomyopathy (Liu et al. 2021; Norton et al. 2011; Selcen et al. 2009). The disease-causing Bag3 mutation, P209L, causes the protein to misfold and form soluble oligomers, which recruit and stall Hsp70 via the BAG domain interaction, triggering widespread protein aggregation (Meister-Broekema et al. 2018).

Bag4 is alternatively named "silencer of death domains" (SODD) as it binds to the cytoplasmic regions of receptors that signal cell death, namely, TNFR1 and DR3, and prevents ligand-independent receptor signaling and apoptosis (Jiang et al. 1999). Surprisingly, NMR structures showed that the three-helix bundle in Bag4 is about 25 amino acids shorter than in Bag1, although it comprises the signature residues needed for interaction with Hsp70 proteins, suggesting that it might have evolved independently (Brockmann et al. 2004; Briknarova et al. 2002). Bag1, Bag3, and Bag4 have been shown to bind the anti-apoptotic protein Bcl-2 (Antoku et al. 2001). Together with their ability to interact with Hsp70, which also has an anti-apoptotic function, these proteins could be linked to mechanisms for apoptosis inhibition (Antoku et al. 2001). It is not known whether these BAG domain proteins can simultaneously bind Hsp70 and Bcl-2, but it has been hypothesized that these two proteins compete for binding as they have both been shown to interact with the BAG domain (Doong et al. 2002).

Among the Bag proteins, Bag5 is unique in containing five consecutive, short three-helix bundle domains similar in structure to the BAG domains of Bag3 and Bag4 (Arakawa et al. 2010). Of these, only the fifth functions as a BAG domain to bind the Hsp70 NBD and assist in its refolding of client substrates. The crystal structure of this domain with the Hsp70 NBD revealed two distinct conformations of the complex: one where the NBD is in an open state similar to the Bag1 complex and the other with a NBD exhibiting a binding pocket distorted by inter-lobe shearing (Arakawa et al. 2010) (Fig. 1.2). Both conformational states likely have reduced affinity for ADP. The functional consequences of the shorter BAG domain structures in Bag3, Bag4, and Bag5 are currently unknown. Interestingly, Bag5 was shown to associate with the E3-ubiquitin ligase, Parkin, modulating substrate protein ubiquitylation (Kalia et al. 2004). Complex formation with Bag5 moreover inhibits the association of Parkin with dysfunctional mitochondria, preventing mitophagy (De Snoo et al. 2019).

Bag2 is the most distantly related member of the BAG family. In the crystal structures, the predicted BAG domain adopted an unanticipated dimeric structure formed by pairs of long antiparallel helices intersected by a short additional helix

(Xu et al. 2008) (Fig. 1.2). Considering these differences from the canonical BAG domain and the low sequence homology, the respective fold was termed the "brand new BAG" (BNB) domain. Binding of Bag2 to Hsp70 also elicits a different conformational change in the NBD—a rotation of the entire lobe II (Fig. 1.2). The BNB domain was also implicated in substrate binding (Xu et al. 2008). Bag2 has clearly lower affinity for Hsp70 proteins than other NEFs (Rauch and Gestwicki 2014), but might compensate by being a dimeric protein with two Hsp70 interaction sites. Thus, Bag2 might be considered the most "eccentric" BAG family protein. Consistently, Bag2 was found to impair the function of the Hsp70-associated E3-ubiquitin ligase "carboxyl terminus of Hsp70-interacting protein" (CHIP), in contrast to Bag1, which seems to target substrate proteins for degradation in collaboration with CHIP (Arndt et al. 2005; Dai et al. 2005). Complex formation with CHIP and Hsc70 is dependent on an N-terminal coiled-coil region that forms a dimer structure on its own (Page et al. 2012). Overexpression of Bag2 inhibited CHIP activity and thereby stimulated chaperone-assisted maturation of the model protein cystic fibrosis transmembrane conductance channel regulator (CFTR) (Arndt et al. 2005). Employing a different mechanism, Bag2 has also been suggested to facilitate degradation of Tau, an aggregation-prone protein that accumulates in neurons of Alzheimer's disease patients (Carrettiero et al. 2009). The microtubule-tethered Bag2·Hsp70 complex was proposed to deliver Tau to the proteasome for degradation in a ubiquitin-independent manner.

In other organisms, only a few BAG domain proteins have been studied. Snl1p is the only known BAG domain-containing protein in *S. cerevisiae* (Table 1.1). This protein contains an N-terminal single transmembrane (TM) domain localizing it to the ER and nuclear membranes with the BAG domain facing the cytosol. Biochemical and genetic experiments have shown Snl1p to interact with cytosolic Hsp70s and components of the nuclear pore, respectively, but no phenotype could be associated with the deletion of Snl1p (Sondermann et al. 2002). Interestingly, a short lysine-rich motif at the beginning of the Snl1p BAG domain facilitates its interaction with intact ribosomes (Verghese and Morano 2012). This region, common with a *Candida albicans* homolog, which however lacks the TM region, is independent from the Hsp70 interaction region. It was proposed that the BAG homologs in fungi may serve a previously unknown role in protein biogenesis based on the recruitment of Hsp70 and ribosomes to the ER membrane.

Bag1 has two putative orthologs in the fission yeast, *Schizosaccharomyces pombe*, Bag101/Bag1A and Bag102/Bag1B. Both proteins have a Ubl domain and a C-terminal BAG domain and associate with the 26S proteasome and Hsp70, respectively. Interestingly, Bag102 contains an additional N-terminal single transmembrane helix localizing it to the ER/nuclear membrane, similar to Snl1p. It was found that Bag102 but not Bag101 was able to suppress the temperature-sensitive growth phenotype and the DNA segregation defect of a *spc7–23* strain containing a point mutation in a conserved kinetochore component, Spc7 (Kriegenburg et al. 2014). This suggests that the two BAG proteins in fission yeast have separate and specific cellular functions.

The crystal structure of the BAG domain of the *C. elegans* Bag1 homolog revealed a dimeric structure of two protomers forming mixed three-helix bundles (Symersky et al. 2004). A small β-sheet between helices 2 and 3 interferes with the formation of an intramolecular three-helix bundle in this ortholog. However, the function of this BAG domain protein as well as the putative Bag3 homolog, unc-23, remains poorly characterized.

In *D. melanogaster*, the Bag3 homolog, starvin, expressed in larval somatic muscles, was shown to be regulated in a highly developmental stage-specific manner. The name "starvin" was coined as this protein was essential for viability and was required by newly hatched larvae to ingest food and grow (Coulson et al. 2005). Starvin expression correlates with the response to cold exposure in *D. melanogaster*, but the precise role of this protein in the pathway is not known (Colinet and Hoffmann 2010).

Plants contain a large variety of BAG family proteins. Sequences for seven isoforms have been identified in the *A. thaliana* genome, named AtBAG1–7 (Kabbage and Dickman 2008). A comparative structural study on the AtBAG1–4 proteins, which share an architecture consisting of a Ubl domain and a BAG domain, showed that the respective BAG domains have short three-helix bundle structures similar to human Bag3, Bag4, and Bag5 (Fang et al. 2013). All the proteins lower the binding affinity of ADP with the NBD to a similar degree, suggesting functional redundancy. The structure of the complex of AtBAG1 with the NBD of human Hsp70 revealed for the first time Ubl and BAG domains in context, showing an extended conformation (Fang et al. 2013). The NBD conformation was similar to the Bag1 and Bag5 complexes, with the subdomain IIB rotated 15° away from the nucleotide binding site. AtBAG2 mutant plants are larger than wildtype counterparts, implicating a function of this isoform in regulating plant programmed cell death (PCD). Similarly, AtBAG4 confers tolerance to cold, drought, and salt stresses, apparently also by inhibiting PCD (Doukhanina et al. 2006). The AtBAG5–7 proteins contain an IQ motif adjacent to the BAG domain which is specific for binding to the ubiquitous calcium sensor, calmodulin (CaM). Structural and biochemical studies of AtBAG5 alone and in complex with CaM revealed that calcium-free CaM and Hsc70 bind AtBAG5 independently while calcium-saturated CaM and Hsc70 exhibit negative cooperativity for AtBAG5 binding (Li et al. 2016). The mitochondria-associated AtBAG5 was implicated in dark-induced leaf senescence (Li et al. 2016). AtBAG7 is the only known ER-lumenal BAG domain protein (Williams et al. 2010).

Thus, the diversity of BAG domain-containing proteins in cells appears to play a role in recruiting Hsp70 to specific locations and for specific functions. How the combinatorial assembly with multiple cochaperones governs the biochemical properties of Hsp70 will be a fertile field for further studies.

ADP Dissociation Inhibitors: Antagonists of Hsp70-NEF Function

It was recognized early on that the protein Hip (Hsc70-interacting protein, alternatively named ST13, suppressor of tumorigenicity 13) antagonizes Bag1, then the only known eukaryotic NEF (Kanelakis et al. 2000). Hip is present in protozoa, plants, and animals and is composed of a dimerization domain, a tetratricopeptide repeat (TPR) domain for interactions with the NBD of Hsp70, and a C-terminal DP domain (DP stands for Asp-Pro motifs) connected by flexible peptide linkers (Velten et al. 2002). The crystal structure of the core complex, consisting of the Hip middle domain and the NDB of Hsp70, showed that Hip slows ADP dissociation by forming a bracket over the nucleotide binding cleft (Li et al. 2013), functionally opposing NEFs (Fig. 1.3). Moreover, the binding area on the NBD overlaps substantially with the known contact regions for NEFs, indicating that interactions are mutually exclusive. The binding affinity between Hip and Hsp70 is however approximately two orders of magnitude lower than with NEFs, which exhibit dissociation constants of around 0.1 μM (Raviol et al. 2006b; Shomura et al. 2005; Sondermann et al. 2001). Therefore, Hip would only slow the Hsp70 cycle substantially when the mutual affinity is increased, for example, by additional interactions with Hsp70-bound substrates (via the DP domains of Hip) or simultaneous interaction with two Hsp70 molecules attached to the same client protein or aggregate. Such hallmarks might indicate that substrates require downstream chaperones like Hsp90 for folding or become hopeless clients to enter a Hsp90/Hsp70-based quality control pathway for damaged proteins (Wang et al. 2013).

Hsp70-NEF antagonists have also been discovered in different functional contexts. The fungal Hsp70-NEF antagonist, Sec72, another TPR-containing protein, is part of the Sec translocon in the ER membrane (Tripathi et al. 2017). Its interaction with the ribosome-binding Hsp70 isoform, Ssb, may contribute to recruitment of translating ribosomes to the translocon. Moreover, mesencephalic astrocyte-derived neurotrophic factor (MANF), a protein structurally unrelated to Hip, antagonizes the ER-lumenal Hsp70-NEFs by inhibiting ADP-ATP exchange and might stabilize certain BiP-client complexes (Yan et al. 2019).

Cellular Functions of NEF Proteins in *S. cerevisiae*

A comprehensive picture of NEF function is only available for one eukaryotic organism, *S. cerevisiae*. This budding yeast comprises seven NEFs, namely, the GrpE homolog, Mge1p in the mitochondria, Lhs1p (Grp170 homolog) and Sil1p in the ER lumen, and Sse1p and Sse2p (Hsp110 homologs), Fes1p (HspBP1 homolog), and Snl1p, the only known BAG domain protein, in the cytosol. These NEFs are associated with the isoforms of Hsp70 found in the mitochondrial matrix (Ssc1p and Ssq1p), the ER lumen (Kar2p), and the cytosol/nucleus (Ssa1–4, Ssb1/2).

Mge1p plays an important role in both import and maturation of mitochondrial matrix proteins encoded in the nucleus (Laloraya et al. 1995, 1994) and is therefore essential for yeast viability (Ikeda et al. 1994). During the final step of import through the inner mitochondrial membrane, Ssc1p and Mge1p form a complex with the import channel-associated protein, Tim44p (Horst et al. 1997). Protein import is furthermore dependent on the activity of the membrane-anchored J-protein complex, Tim16p/Tim14p (also known as Pam18p/Pam16p) (Mokranjac et al. 2006). The mechanism of protein import—ATP-hydrolysis-driven power strokes, entropic pulling, or a Brownian ratchet, which would only prevent back-sliding—is still under discussion. Overexpression of Mge1p leads to reduced pre-protein translocation into the mitochondria, likely due to excessive acceleration of nucleotide exchange and premature conversion of Ssc1p to the low affinity state, thus causing rapid release of the protein in transit (Schneider et al. 1996). In vitro studies have shown that ATP but not ADP effectively releases Mge1p interaction with mitochondrial Hsp70 (Miao et al. 1997). In the mitochondrial matrix, Mge1p furthermore helps to fold newly imported proteins. A strain harboring the temperature-sensitive allele, *mge1–100,* showed reduced rates of maturation of the Yfh1p protein, similar to the defect observed in a deletion strain for the mitochondrial Hsp70 isoform Ssq1p, suggesting a close relationship between the two proteins in substrate folding (Schmidt et al. 2001). Ssc1p and the much less abundant Ssq1p compete for binding to Mge1p (Schmidt et al. 2001). Overexpression of Mge1p increases the activity of Ssq1p, indicating that Mge1p availability is limiting for Ssq1p function. A role of Mge1p as a possible sensor of stress is attributed to the reversible cessation of the interaction between Mge1p and mitochondrial Hsp70s at heat shock temperatures and under conditions of oxidative stress (Marada et al. 2013; Moro and Muga 2006). Reversible methionine oxidation in Mge1p is thought to be regulated by the enzyme, Mrx2 (Allu et al. 2015). A similar sensor role has been discussed for bacterial GrpE (Nakamura et al. 2010). The loss of interaction with Hsp70 has been attributed to a transition from active dimer to inactive monomer.

The ER is a major folding compartment of the cell handling the folding, maturation, and post-translational modification of secretory and membrane proteins and is therefore rich in molecular chaperones. The ER stress response mediated through the "unfolded protein response" (UPR) pathway adapts the folding capacity to the protein load. Aberrant proteins are efficiently cleared by ER-associated decay (ERAD), a process which retro-translocates substrates into the cytosol for proteasomal degradation. Therefore, it is not surprising that the combined function of the ER-NEFs, Lhs1p and Sil1p, is essential and their double deletion is lethal (Tyson and Stirling 2000). Both proteins contribute to co-translational import and subsequent folding of proteins in the ER lumen together with the luminal Hsp70, Kar2p, and the translocon-associated J-domain protein, Sec63p. Both *LHS1* and *SIL1* gene expressions are up-regulated by the UPR, thus increasing the folding capacity of the ER. Deletion of either factor triggers the UPR. This may explain why the single deletions have comparatively mild phenotypes such as altered protein maturation in the ER lumen and increased ERAD. Lhs1p and Sil1p are however only partially redundant (de Keyzer et al. 2009; Tyson and Stirling 2000). Although Sil1p

appears to be about one order of magnitude more abundant than Lhs1p (but constitutes less than 0.1% of the Kar2p content) under normal growth conditions (Ghaemmaghami et al. 2003), *lhs1*Δ cells exhibit a slight import defect, as indicated by the accumulation of pre-proteins. Sil1p, but not Lhs1p, undergoes redox-dependent N-glycosylation, which apparently lowers its NEF activity in the absence of reductive stress (Stevens et al. 2017). The same redox sensor cysteines in Sil1p (Cys52 and Cys57) act as a reductant on Kar2p at Cys63, to switch from the holdase-only function of oxidized Kar2p to ATP-dependent cycling (Siegenthaler et al. 2017).

The cytosolic Hsp110 family proteins, Sse1p and Sse2p, are closely related, having 76% sequence identity (Mukai et al. 1993). Sse1p and Sse2p probably originated from a recent genome duplication event in *S. cerevisiae*. Sse2p is 10 times less abundant than Sse1p, although both proteins are expressed under normal conditions. Sse1p is the most abundant NEF in the yeast cytosol, but the concentration of cytosolic Hsp70 proteins is one order of magnitude higher. Under stress, *SSE2* gene expression is strongly induced by the heat shock response (HSR) pathway, while that of *SSE1* is only modestly increased (Mukai et al. 1993). The loss of Sse1p, but not Sse2p, renders cells slow growing, which is exacerbated by temperature stress (Liu et al. 1999). Overexpression of Sse1p also results in a slow-growth phenotype. The simultaneous deletion of both genes is lethal (Raviol et al. 2006b). Overexpression of the HspBP1 homolog Fes1p can partially compensate *sse1,2*Δ (Raviol et al. 2006b). Sse1p collaborates with both forms of cytosolic Hsp70, Ssa1–4, and the ribosome-associated, Ssb1/2, to fold newly synthesized proteins (Yam et al. 2005; Shaner et al. 2005). This is dependent on the ribosome-associated complex (RAC) (Koplin et al. 2010; Willmund et al. 2013), containing the J-protein, Zuo1p, and the type-I J-domain protein, Ydj1p, respectively (Shaner et al. 2006). Interactions of Sse1p with 1940 potential substrate proteins were listed in a proteomics survey (Gong et al. 2009), comprising a substantial part of the yeast proteome (~6600 proteins). Sse1p function also appears to have an impact on Hsp90 client proteins such as kinases and nuclear receptors (Goeckeler et al. 2002; Liu et al. 1999), probably by upstream client processing through the Hsp70 system. Specific examples are the growth control kinase, Sch9p (Trott et al. 2005), and the MAP kinase, Slt2p (Shaner et al. 2008), enabling Slt2p interaction with downstream effectors required for yeast cell wall integrity and morphogenesis. The NEF function of Sse1p for Ssa1/2 is required for proper distribution of the kinesin-5 motor during bipolar spindle assembly, thus preventing premature spindle elongation during mitosis (Makhnevych et al. 2012). Substrate binding via the SBD in Sse1 appears to be required only under heat shock conditions, as suggested by the growth defect of a quadruple mutant of the putative substrate-binding cleft ($Sse1_{sbd}$) in the absence of Sse1p and Sse2p at 37 °C (Garcia et al. 2017). Besides de novo protein folding, Sse1p is also deeply involved in cellular protein quality control, as shown by the impact of its absence on the proteasomal clearance of the von Hippel-Lindau (VHL) tumor suppressor protein (McClellan et al. 2005), a model substrate for the chaperonin, TRiC, which cannot stably fold in the absence of its complex partners Elongin-BC, and a folding-defective mutant version of the Hsp90 client protein

Ste11p (Mandal et al. 2010). Sse1p interacts with the 19S particle subcomplex of the proteasome and assists in the recruitment of Hsp70 and its substrates (Kandasamy and Andreasson 2018). Consistently, the proteasomal degradation of misfolded proteins via ubiquitin-dependent and ubiquitin-independent pathways was impaired in the absence of functional Sse proteins. The ability of Sse1p and Sse2p to interact with Hsp70 is also required for efficient protein disaggregation by the AAA protein, Hsp104, in collaboration with Hsp70 (Kaimal et al. 2017). In the absence of functional Sse proteins, recruitment of Hsp70 and Hsp104 to FLuc aggregates is impaired, and the disaggregation process is perturbed.

The cellular concentration of Fes1p, the yeast homolog of HspBP1, is ~five-fold lower than Sse1p, and its expression is up-regulated upon stress. Fes1p catalyzes nucleotide exchange on both Ssa- and Ssb-type Hsp70 proteins (Dragovic et al. 2006b; Kabani et al. 2002a) and associates with translating ribosomes (Kabani et al. 2002a). Fes1p and RAC appear to compete for binding to the Ssb proteins, perhaps indicating the necessity for sequential interactions—RAC and Ssb·ATP upon emergence of the nascent chain at the ribosomal exit channel and Fes1p and Ssb·ADP toward completion of translation (Dragovic et al. 2006b). The *FES1* transcript is subject to 3′ alternative splicing, which results in the cytosolic Fes1S isoform or the 12-residue longer Fes1L isoform that is targeted to the nucleus (Gowda et al. 2016). Fes1S is the predominant form, expressed at 2.5-fold higher levels. Both are equally active as Hsp70 NEFs. Deletion of *FES1* causes a growth defect under heat stress and a folding defect in the reporter protein, firefly luciferase (FLuc) (Ahner et al. 2005; Shomura et al. 2005). Fes1p binding to Hsp70, which is coupled to nucleotide exchange activity, and substrate displacement by the N-terminal RD domain are critical for its function, since the inactive, but structurally intact, mutant Fes1p (A79R/R195A) and the RD deletion, respectively, cannot complement the phenotype (Shomura et al. 2005; Gowda et al. 2018). Fes1p NEF activity might be regulated by reversible methionine oxidation (Nicklow and Sevier 2020). Interestingly, the armadillo repeat-containing NEF domain of Fes1p appears to have additional Hsp70-independent functions in the vacuole import and degradation (vid) pathway and in cell wall integrity (CWI) (Kumar and Masison 2019). Deletion of *FES1* induces a massive heat shock response with strong up-regulation of molecular chaperones under standard growth conditions, while *sse1*Δ triggers only a mild induction, suggesting a critical function of Fes1p in the heat shock factor (Hsf1) activation pathway (Abrams et al. 2014; Gowda et al. 2013). This might explain why the growth defect of *fes1*Δ is relatively mild compared to *sse1*Δ. Interestingly, Fes1p was implicated in the proteasome-mediated clearance of the constitutively misfolded proteins, DHFRmutC, DHFRmutD, and the protein fragment, Rpo41 (T920-L1217), but not in the clearance of folded proteins such as FLuc, Stp1p, and fructose-1,6-bisphosphatase 1 (Abrams et al. 2014; Ahner et al. 2005; Gowda et al. 2013). Nuclear Fes1L does not seem to contribute substantially to the clearance of constitutively misfolded proteins, even if they are targeted to the nucleus for degradation (Gowda et al. 2016). On the contrary, the misfolded test substrate, CpY*-GFP, a mutant form of carboxypeptidase Y fused to GFP, is efficiently degraded in *fes1*Δ cells (Abrams et al. 2014). In summary, Sse1p and

Fes1p both contribute to protein quality control, but with different specificities. This could be based on their distinct nucleotide exchange mechanisms or the additional holdase activity of Sse1p.

The BAG domain protein, Snl1p, is expressed at low levels similar to Sse2p. This ER-membrane protein interacts directly with the ribosome (Verghese and Morano 2012). Many of its surprisingly numerous interactors are integral membrane proteins, suggesting perhaps a role in faithful targeting of secretory proteins. Normally, these clients would not get in contact with cytosolic Hsp70 proteins.

The cytosolic NEFs of Hsp70 have a strong impact on the maintenance and propagation of prions in yeast. These fibrous polymeric forms of protein have a rather generic amyloid core structure, which is inherited in a non-Mendelian fashion by daughter cells. Interestingly, the fibers morphologically resemble pathologic protein deposits from amyotrophic lateral sclerosis (ALS) and Alzheimer's and Parkinson's disease in humans. The fibers grow by incorporation of unfolded monomers at their ends and by secondary nucleation, and multiply by fracturing into seeds. The AAA protein disaggregase, Hsp104, in collaboration with the Hsp70 system, contributes in a complex manner both to the fracturing and the disassembly of the filaments (Masison et al. 2009). The Hsp70 system might also erroneously deliver unfolded protein to the growing ends. Faithful maintenance of the [*PSI*$^+$] prion is dependent on the presence of *Sse1* (Fan et al. 2007; Sadlish et al. 2008). Complementation of the *sse1*Δ effect by overexpression of nucleotide-exchange active Sse1p mutants, Fes1p and non-membrane-associated Snl1p(ΔN), suggests that NEF function is the main requirement for propagation (Sadlish et al. 2008).

Aspects of NEF Function in Mammalian Protein Folding and Quality Control

Compared to yeast, much less is known about the integrated functions of mammalian NEFs, primarily due to the increased complexity of higher organisms. Mutations or deletions might affect specific cell types differentially or might prevent development to an adult organism. Simultaneous deletion of the Hsp110 isoforms, Apg-1 and Apg-2, in mice resulted in neonatal death (Mohamed et al. 2014); deletion of Apg-1 alone causes faulty spermatogenesis (Held et al. 2011). Deletion of the third isoform, Hsp105, causes no obvious defects (Nakamura et al. 2008), but truncation of the human Hsp105 gene by micro-satellite instability in intestinal cancer cell lines sensitizes these cells toward chemotherapy (Dorard et al. 2011). Knockout of the cytosolic NEF, HspBP1, in mice results in a defect in spermatogenesis causing male sterility (Rogon et al. 2014). HspBP1 expression in wildtype mice was strongest in testes, specifically in spermatocytes during meiosis. In the absence of HspBP1, the inducible, anti-apoptotic Hsp70 isoforms, HSPA1L and HSPA2, were downregulated, and spermatocytes underwent massive apoptosis. The knockout of the ER-lumenal HspBP1 homolog, Sil1, in mice causes the "woozy" phenotype

exhibiting ataxia and cerebellar Purkinje cell loss in the brain, apparently caused by an inadequate response to persistent ER stress (Zhao et al. 2005, 2010). Later in life, additional progressive loss of skeletal muscle mass and strength is observed (Ichhaporia et al. 2018). In Marinesco-Sjögren syndrome, characterized by myopathy and cataracts, in addition to ataxia, inactivating mutations are found in the Sil1 gene (Anttonen et al. 2005; Senderek et al. 2005). Deletion of Grp170/Hyou1 in mice is lethal (Kitao et al. 2004).

At high concentrations, NEFs inhibit Hsp70 by competing with nucleotide binding (Dragovic et al. 2006a; Rampelt et al. 2012; Gässler et al. 2001; Polier et al. 2008; Raviol et al. 2006b). It is unclear whether competition with J-domain proteins will also occur. The concentration of Hsp70 components determines if accelerated Hsp70 cycling will occur or if cycling is inhibited. Experiments employing overexpression of specific NEFs should be treated with caution for this reason. There are numerous reports about inhibitory effects of Bag1 and HspBP1 (Bimston et al. 1998; Raynes and Guerriero 1998), although these do accelerate ATP hydrolysis of Hsp70 in conjunction with Hsp40 at appropriate concentrations in vitro (Höhfeld and Jentsch 1997; Shomura et al. 2005). The expression levels vary between cell types, and cancer cells lines typically have abnormal chaperone levels.

Comparative studies with model proteins shed light on the differential effects of members of the different NEF families on the folding and degradation of specific proteins. Young and coworkers showed that the BAG domains of Bag1 (cBag1), HspBP1, and Hsp105 all trigger ATP hydrolysis by Hsc70/HSPA8 in the presence of the constitutive, cytosolic, type-I J-domain proteins, DNAJA1/Hdj2 and DNAJA2/Hdj3. However, only the combinations of Hsc70 and DNAJA2 with cBag1 or Hsp105 improved refolding of chemically denatured FLuc compared to the control without NEF (Tzankov et al. 2008). Protein folding however improved only in a narrow NEF/Hsc70 concentration range. Later, combinations of the NEF proteins, Bag1, Bag2, Bag3, and Hsp105, with the J-domain proteins, DNAJA1, DNAJA2, DNAJB1/Hdj1, and DNAJB4, were investigated, using the stress-inducible Hsp70 form, HSPA1A/Hsp72 (Rauch and Gestwicki 2014). All NEFs apart from Hsp105 accelerated ATP hydrolysis in the presence of the J-domain proteins. DNAJA1 was inactive in FLuc refolding in any combination, as noted before by the Young group. In the absence of phosphate, low concentrations of Bag1 and Bag3 together with the type-II J-domain proteins, DNAJB1 and DNAJB4, were most efficient in FLuc refolding. Type-II J-domain proteins have a slightly different domain composition than the type-I paralogs. In the presence of phosphate, the dependency on NEFs increased dramatically, and all combinations with Bag1–3 worked. Hsp105 did not increase FLuc refolding in any combination of the four J-domain proteins with HSPA1A. Taken together, this indicates that a combinatorial library of mammalian Hsp70 components might enable adaptation to a spectrum of substrates with different folding needs such as assistance by holdase activity and suitable cycling rates (Brehmer et al. 2001). In a comparative study, evidence for selective interaction of steroid receptors with the Hsp70 complexes of Bag1-M was found (Knapp et al. 2014). These receptors are prototypical Hsp90 client proteins that progress through the Hsp70 system beforehand. The respective HspBP1

complex with Hsp70 failed to interact with both the downstream factor, Hsp70-Hsp90-organzing protein (HOP) and Hsp90, as well as the (upstream) Hsp40 protein, DNAJB1/Hdj1 (the latter might however be an indirect effect).

The folding of the ABC transporter, cystic fibrosis transmembrane conductance regulator (CFTR), sheds light on the roles of NEFs in protein quality control and degradation. In addition to two transmembrane helix bundles, CFTR has large cytoplasmic domains, which on average require 10 min for folding, and employs the cytoplasmic Hsp70 and Hsp90 machineries (Wang et al. 2006). Mutations in CFTR are the molecular basis for cystic fibrosis in humans, causing a lack of functional protein in mucous membranes. Hsp105 appears to have a prominent role in early CFTR folding events and later at the epithelial membrane, employing its holdase activity (Saxena et al. 2012). Ineffective folding results in proteasomal degradation of most CFTR molecules before reaching the plasma membrane. CFTR is targeted for degradation by the dimeric E3-ubiquitin (Ub) ligase, CHIP (Meacham et al. 2001), which attaches to the C-termini of Hsp70 and Hsp90 and ubiquitylates client proteins (and Hsp70). In cells, most of the CHIP protein appears to be associated with Bag2 and Hsp70, which form a large complex that is Ub-ligase-inactive (Dai et al. 2005). Binding of Bag2 prevents association of CHIP with the E2 enzyme (Ub donor), UbcH5b (Arndt et al. 2010; Dai et al. 2005). In a ternary complex with Hsc70, HspBP1 also inhibits CHIP Ub-ligase activity, however by a different mechanism (Alberti et al. 2004), whereas Bag1 collaborates with CHIP in targeting substrate proteins for proteasomal degradation (Demand et al. 2001). Therefore, Bag2 and HspBP1 might help to keep CHIP in check while productive protein folding is ongoing. For the clearance of the disease mutant, F508Δ-CFTR, autophagy seems to play an important role, consistent with its increased recovery upon siRNA downregulation of Bag3 or the autophagy factor, ATG7 (Hutt et al. 2018).

Because of its considerable holdase capability, Hsp110 appears to play a special role among the NEFs. All Hsp110 isoforms were found attached to large aggregates of mutant superoxide dismutase (SOD1) that are a hallmark of Lou Gehrig's disease (also named amyotrophic lateral sclerosis (ALS)), a protein deposition disease in which the motor neurons degenerate (Wang et al. 2009). In vitro, Hsp105 can suppress mutant SOD aggregation to some degree (Yamashita et al. 2007). Apg2 and to a lesser extent Hsp105 and Apg1, but not Bag1, Bag3, Bag4, and HspBP1, preferentially interacted in vivo with overexpressed HSPA1A versus its close homolog HSPA1L, thereby suppressing aggregation of the SOD1 mutant, A4V, or the unstable GFP-FLuc DM mutant and triggering increased degradation—rather than the opposite effect (Serlidaki et al. 2020). A transgenic mouse lacking Hsp105 was shown to accumulate hyper-phosphorylated tau protein in an age-dependent manner, which in turn forms neurofibrillary tangles and causes neurodegeneration in tauopathies and Alzheimer's disease. This phenotype was comparable to mice deficient in Hsp70, confirming the role for Hsp70·Hsp110 complexes in maintaining tau in an unphosphorylated form during aging and central nervous system homeostasis (Eroglu et al. 2010). Hsp110 was also enriched in aggregates of an artificial model protein for protein deposition diseases, β23 (Olzscha et al. 2011). In a similar

functional role but with a distinct interaction partner, Hsp110 was required to suppress polyglutamine-induced cell death in a *Drosophila* model of polyglutamine (polyQ) diseases. This class of neurodegenerative diseases, which includes Huntington's disease, is characterized by cellular deposition of aggregated mutant proteins containing expanded polyQ regions. Together with the Hsp40 family member DNAJ-1, Hsp110 protected cells from neural degeneration, while either protein expressed alone had little effect, suggesting that an Hsp110·Hsp40 complex is required to maintain protein homeostasis (Kuo et al. 2013). The mammalian Hsp105α was found associated with deposits of polyQ-androgen receptor in spinal and bulbar muscular atrophy (Kennedy's disease) (Ishihara et al. 2003). Surprisingly, the β-sandwich subdomain of the SBD was expendable for the anti-aggregation activity of *Drosophila* Hsp110 in vitro, leading to the discovery of an additional substrate-binding site in the intrinsically disordered C-terminal region of the chaperone (Yakubu and Morano 2021).

Interestingly, Hsp110 along with Hsp70 and Hsp40 was found to form a disaggregase system capable of dissolving amorphous aggregates in mammalian cells that are resistant to Hsp70 and Hsp40 alone (Rampelt et al. 2012; Shorter 2011). This system seems to partially replace the function of ClpB/Hsp104 proteins found in bacteria, plants, and fungi, which together with the Hsp70 system remodel large aggregates and amyloids in an ATP-dependent process. Animals lack cytosolic ClpB/Hsp104 homologs. The most effective system for solubilization of aggregates of FLuc and GFP consisted of the isoforms Apg-2 and Hsc70 and a mixture of DNAJA2 and DNAJB1 (Nillegoda et al. 2015). Since the functional interfaces between Hsp110, Hsp70, and Hsp40 proteins are conserved, this suggests that specific interactions of all individual components with the substrate proteins are important for activity. Perhaps the substrate specificities of the chaperones have to be complementary, enabling simultaneous interactions with different regions in the substrate protein. Additionally, substrate interactions with small heat shock proteins increase the refolding yields. Co-aggregation with small heat shock proteins seems to make the aggregates more accessible to the disaggregase system. The disaggregase activity is dependent on Hsp70-binding and the NEF capability of the Hsp110 component. Other NEFs can partially substitute for Hsp110 in in vitro disaggregation reactions (Rampelt et al. 2012). The initial studies disagreed on the requirement for ATP hydrolysis by the Hsp110 component (Rampelt et al. 2012; Shorter 2011). Taken together the association of Hsp110 and Hsp70 with cellular aggregate deposits might be a sign for ongoing remodeling activity.

In vitro, the mammalian disaggregase complex of Apg-2, Hsc70, and DNAJB1 was capable of breaking down amyloid fibrils of α-synuclein, a protein found in Lewy body deposits in the brains of Parkinson's disease patients (Gao et al. 2015). Hsc70 and DNAJB1 bound to fibrils of diverse lengths; Apg-2 seemed to prefer shortened fibrils or oligomers. The breakdown of the fibrils seemed to operate by both fragmentation and depolymerization, as judged from electron microscopy and the kinetics of α-synuclein monomer emergence. Molecular dissection showed that DNAJB1 bound to the flexible C-terminal tails in fibril building blocks and recruited Hsc70, which recognized both motifs in the N-terminal tail and the amyloidogenic

core of α-synuclein (Wentink et al. 2020). This resulted in crowding of Hsc70 onto the fibrils. For the potentiation of amyloid disaggregation by Apg-2, its bulkiness seemed important, limiting access to the fibrils. The combined action of the cochaperones seemed to distribute Hsc70 on the fibrils to enable breakdown by entropic pulling. This disaggregation of amyloids in vivo might have deleterious side effects by generating amyloidogenic seeds, which can spread between cells in a prion-like manner (Nachman et al. 2020; Tittelmeier et al. 2020). It is unclear how the release of these seeds from cells might work. Slight overexpression of the Hsp110 isoform, Apg-1, reduced the load of α-synuclein aggregates and pathology in a mouse disease model (Taguchi et al. 2019).

Both Bag3 and Hsp110 are strongly up-regulated upon stress by the heat shock response. It is unclear to what extent autophagy and disaggregation/UPS-mediated degradation contribute to the recovery of the cell and the clearance of aggregated protein after heat shock. Another important aspect might be the cellular localization of respective proteostasis machineries. Hsp110 proteins are found both in the cytoplasm and nucleus, whereas Bag3 is found exclusively in the cytosol. Therefore, the two compartments might follow different strategies for recovery.

Conclusions

The Hsp70 system represents the central hub in the proteostasis network by interacting with polypeptides at various stages of their existence from birth to ultimate demise. The Hsp70 folding cycle is fine-tuned by cochaperones adapting it to the divergent folding requirements of individual substrates that are involved in various cellular pathways. Recent structural and biochemical evidence has shown that the different nucleotide exchange factors especially serve additional roles by linking Hsp70 to other branches of the proteostasis network. We now have first insights how functional diversity might be encoded in their distinct binding modes to Hsp70. What is still missing is the integration of all these processes. For this we would need good estimates for the local concentrations of the players, in healthy cells, and those under conditions of stress or disease. It will be exciting to dissect this complex interplay between NEFs, Hsp70, and client proteins.

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Chapter 2
Functions of the Hsp90-Binding FKBP Immunophilins

Nina R. Ortiz, Naihsuan Guy, Yenni A. Garcia, Jeffrey C. Sivils, Mario D. Galigniana, and Marc B. Cox

Abstract The Hsp90 chaperone is known to interact with a diverse array of client proteins. However, in every case examined, Hsp90 is also accompanied by a single or several co-chaperone proteins. One class of co-chaperone contains a tetratricopeptide repeat (TPR) domain that targets the co-chaperone to the C-terminal region of Hsp90. Within this class are Hsp90-binding peptidylprolyl isomerases, most of which belong to the FK506-binding protein (FKBP) family. Despite the common association of FKBP co-chaperones with Hsp90, it is abundantly clear that the client protein influences, and is often influenced by, the particular FKBP bound to Hsp90. Examples include Xap2 in aryl hydrocarbon receptor complexes and FKBP52 in steroid receptor complexes. In this chapter, we discuss the known functional roles played by FKBP co-chaperones and, where possible, relate distinctive functions to structural differences between FKBP members.

Keywords Co-chaperone · Immunophilin · FKBP · Hsp90 · Steroid hormone receptor · TPR domain

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Introduction

Immunophilins are a large, functionally diverse group of proteins that are defined by their ability to bind immunosuppressive ligands. The signature domain of the immunophilin family is the peptidyl-prolyl *cis-trans* isomerase (PPIase; also termed rotamase) to which the immunosuppressive drugs bind. Early investigations into the PPIase enzymatic activity led to the belief that the immunosuppressive drugs elicited their effects by inhibiting PPIase activity. However, some compounds binding the PPIase active site efficiently inhibit PPIase activity without inducing immunosuppression, so PPIase activity is not critical for immune responses. It is now known that effector domains on the immunosuppressive drugs project from the PPIase pocket. This allows the immunophilin-drug complex to bind tightly to and inhibit calcineurin or target of rapamycin, signal transduction proteins required for immune responses (see Hamilton and Steiner (1998) for a detailed review on the mechanisms by which immunophilins and their ligands suppress immune responses).

Since the initial identification of the immunophilin proteins, multiple family members have been identified in all major branches of life. Some immunophilins are small proteins containing only a single PPIase domain, while others are large, multidomain proteins containing one or more PPIase domains, as well as additional functional domains. Based on their ability to recognize different immunosuppressive ligands, the immunophilins are generally divided into two subfamilies: the FK506 binding proteins (FKBP), which also bind rapamycin, and the cyclosporin-A binding proteins or cyclophilins (CyP). Though the PPIase domains of FKBP and cyclophilins are structurally distinct and likely evolved independently, some members of either the FKBP or cyclophilin families contain a structurally similar tetratricopeptide repeat (TPR) domain that targets binding to heat shock protein 90 (Hsp90) (reviewed in Harikishore and Yoon (2015)).

Hsp90 is an abundant molecular chaperone that interacts with a broad array of protein clients that regulate numerous important cellular pathways. Among the known Hsp90 clients are transcription factors (e.g., steroid hormone receptors, heat shock transcription factor 1, aryl hydrocarbon receptor), both serine/threonine and tyrosine kinases (e.g., Raf and Src-related kinases), and key regulatory enzymes (e.g., nitric oxide synthase and telomerase). (A compilation of known Hsp90 clients maintained by Didier Picard at Univ. of Geneva can be accessed at: http://www.picard.ch/downloads/Hsp90interactors.pdf).

In concert with other chaperone proteins, Hsp90 facilitates client folding and proteolytic stability but can also promote client degradation. In the case of steroid receptors, Hsp90 and its associated co-chaperones also regulate receptor activity. Hsp90 binding to steroid receptors must be preceded by transient receptor interactions with Hsp40, Hsp70, and associated co-chaperones. Hsp90, which is recruited as a dimer in the latter stages of complex assembly, binds directly to the receptor ligand binding domain and stabilizes a receptor conformation that is competent for hormone binding. Proteins that are associated with Hsp90 in the functionally mature receptor complex are p23, a co-chaperone that stabilizes Hsp90 binding to receptor,

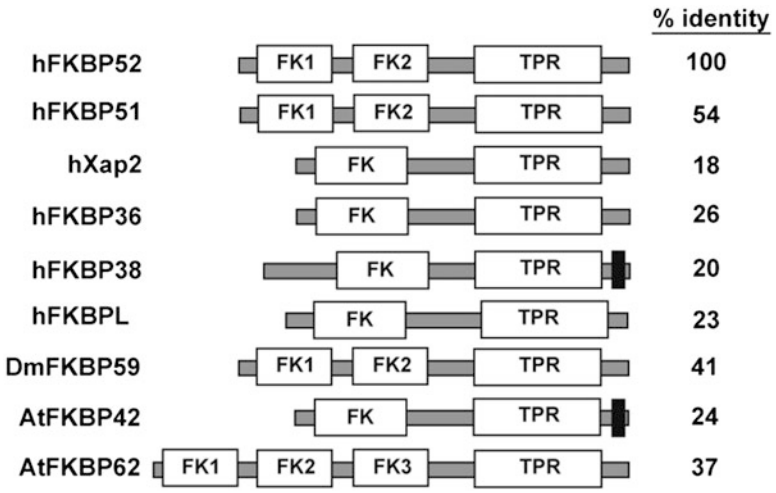

Fig. 2.1 Domain organization of representative Hsp90-binding FKBPs. TPR-containing FKBPs from vertebrate, insect, and plant sources were selected for comparison of domain organizations. The proteins are human FKBP52 (acc. # NP_002005), human FKBP51 (acc. # Q13451), human FKBPL (acc. # NP_071393.2), human Xap2 (acc. # O00170), human FKBP36 (acc. # NP_003593), human FKBP38 (acc. # NP_036313.3), *Drosophila melanogaster* FKBP59 (acc. # AAF18387), *Arabidopsis thaliana* FKBP42 (acc. # CAC00654), and *Arabidopsis thaliana* FKBP62 (acc. # AAB82062). The percent amino acid identity of each compared to human FKBP52 was determined from ClustalW2 alignments (http://www.ebi.ac.uk/clustalw). Each protein shown has at least one FKBP12-like domain (FK), which in some cases has peptidylprolyl isomerase activity and is the binding site for the immunosuppressant drug FK506, and one tetratricopeptide repeat domain (TPR), which is typically an Hsp90 binding site. The black box in the C-terminus of *At*FKBP42 is a transmembrane domain used for anchoring the protein to the plasma and vacuolar membranes

and any one of several TPR co-chaperones, including the immunophilin/PPIases FKBP52 (also termed p59, Hsp56, p50, HBI, FKBP59, and FKBP4), FKBP51 (also termed p54, FKBP54, and FKBP5), and CyP40, or the protein phosphatase PP5 (reviewed extensively in Cox and Johnson (2018)).

The domain organization for several FKBP co-chaperones is compared in Fig. 2.1. These co-chaperones compete for a common binding site in the C-terminal region of Hsp90 that includes the highly conserved -MEEVD sequence that terminates Hsp90. Co-crystallographic structures have shown how an MEEVD pentapeptide associates with the TPR binding pocket (Scheufler et al. 2000; Wu et al. 2004). In vitro affinity binding studies found that the specific binding of FKBP51 and FKBP52 to Hsp90 alpha or beta subunits was found to be regulated through affinity, and that the Met residue in the MEEVD motif in Hsp90 is critical for FKBP51/52 binding (Assimon et al. 2015). Although the TPR domains for each of these co-chaperones are structurally similar and interact in a similar manner with Hsp90, the client protein bound by Hsp90 can influence the rank order of co-chaperone recruitment to Hsp90-client complexes (reviewed in Riggs et al. (2004)). For instance, PP5 and FKBP51 are preferred components in glucocorticoid

receptor (GR) complexes, FKBP51 is preferred in progesterone receptor (PR) complexes, and CyP40 is relatively enhanced in estrogen receptor (ER) complexes (Silverstein et al. 1997; Barent et al. 1998). On the other hand, another TPR-containing FKBP, the hepatitis B virus protein X-associated protein 2 (Xap2; also termed AIP, ARA9, and FKBP37), shows little interaction with steroid receptors but is strongly associated with the aryl hydrocarbon receptor-Hsp90 complex (Ma and Whitlock 1997; Meyer et al. 1998). The distinctive patterns of preference for co-chaperone association in client complexes are one line of evidence that the co-chaperones bound to Hsp90 can also interact with the Hsp90-bound client.

In addition to FKBP52, FKBP51, and XAP2, several other FKBP family members contain TPR domains that are known or likely to bind Hsp90. FKBP36 is structurally similar to XAP2 but is required for male fertility and homologous chromosome pairing in meiosis (Crackower et al. 2003). FKBP38 is a unique family member that is anchored to the mitochondrial and endoplasmic reticulum membranes, and is involved in a variety of processes including protein folding and trafficking, apoptosis, neural tube formation, cystic fibrosis transmembrane conductance regulator (CFTR) trafficking, and viral replication (reviewed in Edlich and Lucke (2011)). FK506-binding protein like (FKBPL) protein is a divergent member of the FKBP family that can associate and functionally regulate steroid hormone receptors, has anti-angiogenic properties, has a role in the DNA damage response, and controls tumor growth (reviewed in Robson and James (2012)). *Drosophila melanogaster* expresses a TPR-containing immunophilin (*Dm*FKBP59) that has high similarity to FKBP52/51 in vertebrates (Goel et al. 2001; Zaffran 2000). Plants have several FKBP genes that encode TPR domains; for example, in *Arabidopsis thaliana* there are four such genes: *At*FKBP42, *At*FKBP62, *At*FKBP65, and *At*FKBP72 (Binder et al. 2008; Blair et al. 2013). Although prokaryotic and archaeal genomes also contain FKBP family members (Maruyama et al. 2004), none of these genes encode a TPR domain.

Structure/Function Relationships of Steroid Receptor-Associated FKBPs

X-ray crystallographic structures have been resolved for full-length FKBP51 and for overlapping fragments of FKBP52 (Fig. 2.2). Human FKBP51 and FKBP52 share 60% amino acid sequence identity and 75% similarity, and individual domains do not differ markedly between FKBP51 and FKBP52. Both share a similar TPR domain composed of three tandem repeats of the degenerate 34-amino acid motif, which is a typical characteristic of TPR proteins (Blatch and Lassle 1999). Each repeat adopts a helix-turn-helix conformation and adjacent units stacked in parallel to form a saddle-shaped domain with a concave binding pocket for Hsp90. In addition to the TPR domain, both FKBP51 and FKBP52 have two N-terminal domains, each of which is structurally similar to FKBP12. FK506-binding and

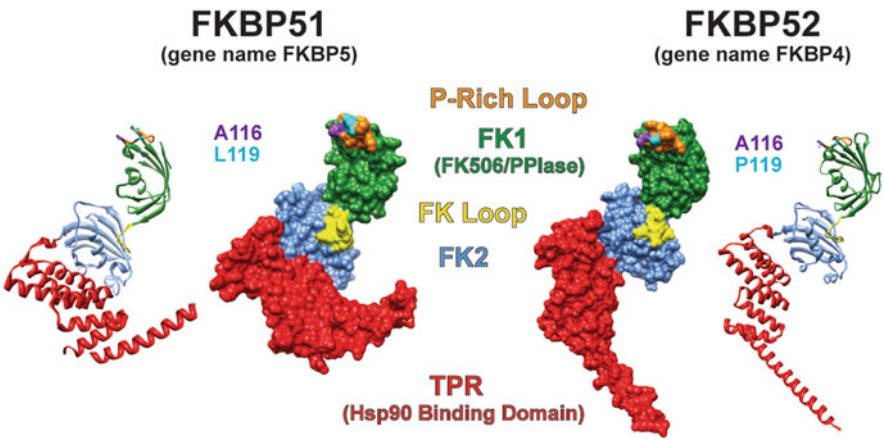

Fig. 2.2 Structural and functional characteristics of FKBP51 and FKBP52. Both ribbon and molecular surface depictions of the X-ray crystallographic structures for human FKBP51 (**a** protein data bank number 1KT0) and a composite of two partial structures for human FKBP52 (**b** protein data bank numbers 1Q1C and 1P5Q) are shown. In either protein the two FKBP12-like domains (FK1 and FK2, green and blue, respectively) are indicated, the first of which has FK506 binding and PPIase activities. PPIase activity is not required for receptor regulation. The proline-rich loop (orange) that overhangs the PPIase catalytic pocket is critical for FKBP52 function and is responsible for the functional difference between FKBP51 and FKBP52. Two functionally critical residues (A116 and L119 in FKBP51 and A116 and P119 in FKBP52) within this loop are highlighted. The FK1 domain, the proline-rich loop in particular, is hypothesized to serve as an interaction surface within the Hsp90-receptor heterocomplex. A loop structure containing a CKII phosphorylation site in the hinge region between FK1 and FK2 is pointed out (yellow). The C-terminal TPR domain (red) consists of three helix-loop-helix motifs that form the Hsp90 binding pocket. Structures of the individual domains are highly similar between the two proteins, but the angle between FK2 and TPR domains of FKBP51 is more acute and probably more constrained than in FKBP52. The FKBP51 and FKBP52 structure models shown were constructed using UCSF Chimera version 1.5

PPIase activities reside in the most N-terminal domain (FK1), which has a pocket and active site residues similar to FKBP12. Due to several amino acid differences, the second domain (FK2) lacks drug binding and PPIase activity (Sinars et al. 2003).

The most striking difference in crystal structures relates to apparent domain: domain orientations. The FKBP52 structure shown in Fig. 2.2 is a composite model derived from merging the separate FK1-FK2 and FK2-TPR structures. Thus, conclusions and ideas based on this composite structure must be viewed with caution. However, the composite model suggests that the FKBP52 TPR domain is aligned in a more linear fashion with the FK domains rather than in the kinked conformation seen with FKBP51 (Fig. 2.2). In fact, the static orientations shown in crystal structures are likely more dynamic in solution, but the different crystal orientations are perhaps telling. Amino acid side chains unique to FKBP51 form a salt bridge between FK2 and TPR that would stabilize the domain:domain interaction in FKBP51 relative to FKBP52, which lacks this salt bridge. The apparently

more malleable structure of FKBP52 might allow interactions within the receptor heterocomplex that are strained in FKBP51.

Significant progress has been made in understanding functionally important domains and residues on FKBP52 that contribute to the distinct ability to regulate steroid hormone receptor activity. Random mutagenesis studies in *S. cerevisiae* demonstrated that two point mutations (A116V and L119P) in the FKBP51 FK1 domain, which does not potentiate steroid hormone receptor activity under normal conditions, confer full receptor potentiating ability to FKBP51, similar to that of FKBP52 (Riggs et al. 2007). This suggests that FKBP51 and FKBP52 functionally diverged at some point in evolution by only a few residues. A previous study suggests that there are differences in conformational dynamics between FKBP51 and FKBP52 within the proline-rich loop (Mustafi et al. 2014). 15N NMR relaxation measurements demonstrated that only the proline-rich loop in FKBP51 displays significantly larger line broadening, which is completely suppressed in the presence of the L119P mutation. These data suggest not only that differences in the proline-rich loop confer distinct functions to FKBP51 and FKBP52, but also that the proline-rich loop is functionally important for FKBP52 regulation of receptor activity. The current hypothesis holds that the FKBP52 proline-rich loop serves as an interaction surface, and the interaction partner is likely the receptor hormone binding domain (Sivils et al. 2011; De Leon et al. 2011).

Bracher and colleagues demonstrate that the FK1-FK2 domains portray a flexible hinge that may account for regulatory differences between FKBP51 and FKBP52 (Bracher et al. 2013). It is hypothesized that the FK2 domain of FKBP52 contains an activation mechanism based on the calmodulin-binding motif at the C-terminus, yet this region is unable to bind FK506 and rapamycin and lacks PPIase activity (Chambraud et al. 1993; Pirkl and Buchner 2001; Rouviere et al. 1997).

FKBP51 and FKBP52 also differ in the hinge region connecting FK1 and FK2 domains (FK loop). The FK loop of FKBP52 contains a—TEEED-sequence that has been identified as an in vitro substrate for casein kinase II; the corresponding sequence in FKBP51, -FED-, lacks the threonine phosphorylation site. Phosphorylation of FKBP52 is potentially important since the phospho-protein is reported to lose Hsp90 binding (Miyata et al. 1997). This difference was further tested using comparative analysis of FKBP51 and FKBP52 FK linker sequences (Cox et al. 2007). While the phosphomimetic mutation T143E had no effect on FKBP52 binding to Hsp90 in this study, the mutation did abrogate FKBP52 regulation of receptor activity. It is predicted that phosphorylation of residue T143 in the FKBP52 FK linker reorients the entire FK1 conformation, thereby eliminating FK1 interactions with the receptor hormone binding domain.

Cellular and Physiological Functions of Hsp90-Associated FKBPs

FKBP52

FKBP52 is expressed in most vertebrate tissues and cell lines, although its expression can be upregulated by heat stress (Sanchez 1990), by estrogen in MCF-7 breast cancer cells (Kumar et al. 2001), and by the homeobox transcription factor HoxA-10 in the peri-implantation mouse uterus (Daikoku et al. 2005). FKBP52 associates with steroid receptor complexes in an Hsp90-dependent manner, but FKBP52 is not required in a defined cell-free assembly system for receptor to reach the mature conformation that is competent for hormone binding (Dittmar et al. 1996; Kosano et al. 1998). Nonetheless, FKBP52 in cells potentiates hormone-dependent reporter gene activation by GR (Riggs et al. 2003), AR (Cheung-Flynn et al. 2005), and PR (Tranguch et al. 2005). Potentiation of hormone signaling can be related to an increase in receptor affinity for hormone (Riggs et al. 2003; Davies et al. 2005), but there may be additional mechanisms by which FKBP52 enhances receptor activity.

In concordance with hormone binding affinity changes, domain-swapping experiments between GR and ER, which is not potentiated by FKBP52, demonstrated that FKBP52 potentiation is localized to the ligand binding domain of GR (Riggs et al. 2003). FKBP52-dependent potentiation of receptor activity is abrogated in point mutants that are defective for Hsp90 binding, and potentiation is blocked by the PPIase inhibitor FK506 (Riggs et al. 2003; Cheung-Flynn et al. 2005). One model to explain these findings is that Hsp90 recruits FKBP52 to the receptor heterocomplex such that the FK1 PPIase can effectively catalyze isomerization of one or more proline substrates in the receptor ligand binding domain. However, studies have shown that point mutations within the FKBP52 PPIase pocket that eliminate PPIase activity have no effect on FKBP52 potentiation of receptor activity (Riggs et al. 2007). Thus, FK506-mediated inhibition of FKBP52 function likely occurs through the inhibition of FK1 interactions as opposed to inhibition of PPIase enzymatic activity. As discussed above, the FKBP52 FK1 domain as a whole is functionally important and the proline-rich loop that overhangs the PPIase pocket could serve as a functionally important interaction surface that contacts the receptor hormone binding domain within the receptor-chaperone heterocomplex. A structure-based screen for small molecules targeting an alternative surface of the androgen receptor hormone binding domain identified a series of fenamic acid molecules that allosterically affect coactivator binding at the activation function 2 (AF2) site through interaction with a surface cleft termed binding function 3 (BF3) (Estebanez-Perpina et al. 2007). Steroid hormone receptor structural comparisons identified this region to be a highly conserved regulatory surface that could serve as a therapeutic target for hormone-dependent diseases (Buzon et al. 2012). Interestingly, mutations within the AR BF3 surface (F673P, P723S, and C806Y) result in increased dependence on FKBP52 for function. In addition, a drug termed MJC13 that specifically inhibits

FKBP52-regulated AR activity is predicted to target the BF3 surface (De Leon et al. 2011). Thus, the BF3 surface is a putative FKBP52 interaction and/or regulatory surface, and FKBP52 interaction with the receptor BF3 surface could allosterically affect receptor interactions at the AF2 site. In addition to the AR BF3 surface, studies suggest that the helix 1–3 (H1–H3) loop in the GR LBD is an important site of FKBP regulation. Glucocorticoid insensitivity in guinea pig has been linked to sequence differences in the H1–H3 loop and substitution of the guinea pig H1–H3 loop into rat GR resulted in increased FKBP51-mediated repression of receptor activity. It is hypothesized that changes in the H1–H3 loop result in changes within the GR-Hsp90 heterocomplex that favor FKBP51 repression over FKBP52 potentiation (Cluning et al. 2013).

Due to FKBP52's role as a known enhancer of AR transcriptional activity, and AR being a critical driver of prostate cancer development, a genomic profiling study recently sought out to understand the evolutionary mechanisms and genomic events leading to FKBP52's contribution to castration-resistant prostate cancer (CRPC). Using matched prostate cancer tumor tissues from both before and after castration, the tumor history of each sample was delineated via genomic profiling of DNA and found that chromothripsis resulted in amplification of FKBP52. Further, protein expression of FKBP52 in over 500 prostate cancer samples displayed increased levels in CRPC tissues when compared to those which were hormone-naïve and with elevated FKBP52 expression correlated with poor patient prognosis (Federer-Gsponer et al. 2018).

FKBP52 has been shown through in vitro studies to have a chaperone activity that is independent of Hsp90 binding or PPIase (Bose et al. 1996; Pirkl and Buchner 2001). Like Hsp90 and numerous other chaperone components, FKBP52 can hold misfolded proteins in a non-aggregated state that is amenable to refolding. The possibility that chaperone holding activity displayed by FKBP52 plays a role in altering receptor activity cannot be dismissed, but this appears unlikely since holding activity is highly redundant among chaperone components. Furthermore, holding activity, unlike FKBP52-dependent potentiation of receptor activity, is neither PPIase- nor Hsp90-dependent. Unfortunately, no one has identified an FKBP52 mutation that disrupts holding activity in a discrete manner.

In an effort to extend biochemical and cellular data to the physiological level, FKBP52 gene knockout (52KO) mice were generated independently by two groups (Cheung-Flynn et al. 2005; Yong et al. 2007). The mutant mice have striking reproductive phenotypes that can be attributed, at least in part, to loss of steroid receptor activity. Male 52KO mice are infertile and display abnormal virilization with persistent nipples, ambiguous external genitalia, and dysgenic seminal vesicles and prostate (Cheung-Flynn et al. 2005, Yong et al. 2007). These developmental defects are consistent with androgen insensitivity in these tissues. Testicular morphology, descent, histology, and spermatogenesis are normal and androgen production and release from testes is unimpaired; these developmental features are not highly androgen dependent. On the other hand, sperm isolated from the epididymis have abnormal tail morphology and reduced motility suggestive of a defect in sperm maturation within the epididymis, a process that is androgen dependent. Cellular

studies confirm that FKBP52 is required for full AR function, which provides a rational explanation for androgen insensitivity in tissues of 52KO males.

52KO females have no gross morphological abnormalities, yet are completely infertile (Tranguch et al. 2005). Oocyte formation and release are not markedly impaired, and oocytes are competent for in vitro and in vivo fertilization. Infertility is due, at least in part, to a maternal failure of embryonic implantation and uterine decidualization. During the early stages of pregnancy, the 52KO uterus does not display the usual molecular or physiological markers for implantation. These events are largely dependent on progesterone actions, and both molecular and cellular studies confirm that FKBP52 is required for full PR activity. Additionally, FKBP52 is related to the etiology of endometriosis given that 52KO mice display increased endometrial lesions, inflammation, cell proliferation, and angiogenesis, and FKBP52 protein levels are reduced in human endometrial tissues (Hirota et al. 2008).

Immunohistochemistry staining of granulosa cells (GCs) from rats with polycystic ovarian syndrome (PCOS) displayed higher expression of FKBP52, and culturing the GCs to overexpress or silence FKBP52 revealed a correlation between expression of AR, extracellular signal-regulated kinase (ERK), and phosphorylated ERK (p-ERK)(ref.). The mRNA expression of both FKBP52 and AR in GCs from rat ovaries with polycystic ovarian syndrome was found to be significantly increased when compared to the non-PCOS models. Interestingly, levels of p-ERK1/2 were also increased in the PCOS models. When FKBP52 was knocked down via siRNA in the GCs, levels of AR and ERK1/2 were found to be decreased; however, p-ERK1/2 was increased, indicating a potential AR regulation system through the MAPK/ERK pathway (Song and Tan 2019).

FKBP52 is critical for reproductive development and success in both male and female mice and its role can be traced to support of AR and PR function (Cheung-Flynn et al. 2005; Tranguch et al. 2005). Although GR-related phenotypes are not readily apparent, cellular and biochemical studies suggest that 52KO animals should display phenotypes related to reduced GR activity. Given that abnormal Mendelian ratios are not observed for heterozygous crosses, the 52KO phenotype does include partial embryonic lethality. This combined with the reproductive defects leads to difficulty in obtaining sufficient numbers of 52KO animals for experiments. Thus, heterozygous *fkbp52*-deficient mice (52+/−) were generated to determine the in vivo roles for FKBP52 in GR-mediated physiology. 52+/− mice displayed phenotypes associated with reduced GR signaling including increased susceptibility to high-fat diet-induced hepatic steatosis, hyperglycemia, hyperinsulinemia, and behavioral alterations under basal and chronic stress conditions (Wadekar et al. 2004; Warrier et al. 2010). To better understand the role of FKBPs in inflammation-associated depression, lipopolysaccharide treatment of both male and female Wistar rats resulted in exhibition of depressive-like behavior and further examination of GR regulation found that GR, FKBP51, and FKBP52 nuclear localization in the prefrontal cortex only occurred in the male rats as opposed to the female rats, with altered expression of the GR-regulated genes PTGS2 and BDNF (Brkic et al. 2016).

Although FKBP52 does not alter ER function in cellular studies and 52KO mice show no signs of estrogen insensitivity, FKBP52 expression is upregulated by estrogens and FKBP52 is overexpressed in breast tumors (Ward et al. 1999). In addition, the FKBP52 gene is methylated in ER-negative, but not in ER-positive breast cancer cells (Ostrow et al. 2009). Thus, a few studies have identified FKBP52 as a potential regulator of at least ER expression in breast cancer.

Despite the fact that FKBP52 was initially discovered in the immune system, it is ubiquitously expressed and particularly abundant in the central nervous system. Thus, it is not surprising that FKBP52 is involved in neurodegenerative tauopathies including Alzheimer's (AD) and Pick's disease, frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP), and progressive supranuclear palsy (Haelens et al. 2007; Hernandez and Avila 2007). The defining neuropathological characteristic of tauopathies is the aberrant aggregation of insoluble hyperphosphorylated microtubule-associated protein (MAP) tau within the neurons, which is termed neurofibrillary tangles (NFTs) and is also referred to as paired helical filaments (PHF) (Cao and Konsolaki 2011). Earlier studies have shown FKBP52's direct interaction with tau, particularly with its hyperphosphorylated form, has antagonistic effects on tubulin polymerization and microtubule assembly (Chambraud et al. 2007, 2010). In addition, FKBP52 has been shown to induce Tau-P301L oligomerization and assembly into filaments (Giustiniani et al. 2014). More importantly, knockdown of FKBP52 was shown to restore axonal outgrowth and branching caused by Tau-P301L expression, thereby validating FKBP52 as an attractive therapeutic target in tauopathies. FKBP52 is known to be involved in subcellular rearrangement. Studies by Quintá et al. demonstrated that the overexpression of FKBP52 can induce neuronal differentiation and neurite outgrowth (Quintá et al. 2010).

Previous reports have shown that copper (Cu) contributes to the neuropathology of AD by interacting with copper binding domains of amyloid precursor proteins (APPs) and beta-amyloid (Aβ) peptides causing the formation of amyloid plaques and disrupting metal ion homeostasis (Barnham and Bush 2008; Drago et al. 2008; Kong et al. 2007). FKBP52 is involved in the regulation of cellular Cu homeostasis by interacting directly with the copper transport protein Atox1 (Sanokawa-Akakura et al. 2004), which is part of the Cu efflux machinery in neurons. In addition, both genetic and cellular data in *Drosophila* suggest a novel role for FKBP52 in the regulation of intracellular Cu homeostasis via binding to APP, thus modulating the toxicity level of Aβ peptides (Sanokawa-Akakura et al. 2010).

S100A proteins belong to the EF-hand type calcium (Ca^{2+}) sensing protein family that are linked to regulation of various intracellular processes and are often expressed in a cell- and tissue-specific fashion (Santamaria-Kisiel et al. 2006; Wright et al. 2009). Based on biochemical evidence, it has been demonstrated that S100A1 and S100A6 interact with FKBP52 by competing with Hsp90 for the TPR domain in a Ca^{2+}-dependent manner (Shimamoto et al. 2010). Cellular data has linked S100A1's involvement in the neuronal cell dysfunction/death that occurs in AD by reducing APP expression and stabilizing the intracellular Ca^{2+} homeostasis (Zimmer et al. 2005). It seems that the function of FKBP52 can be regulated by Ca^{2+} homeostasis

within the cell leading to effects on the phosphorylation of tau and pathology in AD. Interestingly, a *Drosophila* orthologue of FKBP52 termed FKBP59 was found to interact with the Ca^{2+} channel protein TRPL in photoreceptor cells and to influence Ca^{2+} influx (Goel et al. 2001). Subsequent studies revealed that FKBP52 similarly interacts with a subset of rat transient receptor potential channel (TRPC) proteins that form Ca^{2+} channels in the mammalian brain (Sinkins et al. 2004). The C-terminus of FKBP52 contains a predicted calmodulin-binding domain, which enables the protein to bind to calmodulin-Sepharose in a Ca^{2+}-dependent manner, the biological function of which is still unknown (Silverstein et al. 1999). In addition to interacting with TRPC channels in the brain, FKBP52 was found to interact with TRPC3 channels in the heart using a yeast two-hybrid screen (Bandleon et al. 2019). In neonatal rat cardiomyocytes, downregulation of FKBP52 resulted in TRPC3-dependent hypertrophic response and this can be attributed to its PPIase activity, since overexpression of FKBP52 mutants lacking the PPIase domain displayed the same effect.

Apart from the well-established roles of FKBP52 in steroid hormone receptor function, FKBP52, as with other Hsp90 co-chaperones, has been identified in a variety of client-Hsp90 heterocomplexes such as those containing kinases, aryl hydrocarbon receptor, and heat shock transcription factor; however, many of these interactions might reflect passive, transient association of the protein with Hsp90 and have no functional impact on client activity. FKBP52 is also linked to various Hsp90-independent interactions. Aside from the aforementioned Hsp90-independent interactors, FKBP52 has been found to interact directly with the interferon regulatory factor 4 (Mamane et al. 2000), which regulates gene expression in B and T lymphocytes and forms a complex with tyrosine kinase receptor RET51, which is involved in the development and maintenance of the nervous system (Fusco et al. 2010) and FKBP associated protein 48 (Chambraud et al. 1996), which influences proliferation of Jurkat T cells (Krummrei et al. 2003). Each of these interactions was found to be disrupted by FK506 and to target the FKBP52 PPIase domain to specific proline sites in each partner protein. Phenotypes potentially related to these interactions have not yet been assessed in 52KO mice. Not only does FKBP52 interact with proteins, FKBP52 is also capable of directly binding adeno-associated virus DNA and regulating replication of the viral genome (Qing et al. 2001; Zhong et al. 2004). The relevant DNA binding site in FKBP52 has not been identified.

FKBP51

FKBP51/p54/FKBP54 was originally identified as a component of chicken PR complexes (Smith et al. 1990, 1993a, b) and is now known to assemble as an Hsp90 co-chaperone with all steroid receptors and other Hsp90-client complexes. FKBP51 is functionally similar in some ways to FKBP52; both have similar PPIase activity in the presence of model peptide substrates, both hold misfolded proteins in

a folding competent state, and they compete for binding a common site on Hsp90 (Nair et al. 1997; Pirkl et al. 2001). As noted above, the overall structural similarity of these FKBPs is consistent with these shared functional properties, yet their distinct effects on steroid receptor activity belie these similarities. In addition to the aforementioned structural differences between FKBP51 and FKBP52, another distinction is that the FKBP51 gene is highly inducible by glucocorticoids, androgens, and progesterone (Baughman et al. 1995; Kester et al. 1997; Zhu et al. 2001; Yoshida et al. 2002; Vermeer et al. 2003; Hubler et al. 2003; Febbo et al. 2005).

FKBP51 acts as an inhibitor of GR, PR, and MR function excluding AR. The first indication of its inhibitory role came from studies by Scammell and colleagues of glucocorticoid resistance in New World primates (Reynolds et al. 1999; Denny et al. 2000). In squirrel monkeys GR has a relatively low affinity for hormone yet the cloned monkey GR has an affinity similar to human GR in vitro. This observation led to a search for cellular factors in monkey cells that reduced GR binding affinity. A key factor identified was FKBP51, which is constitutively overexpressed in squirrel monkey cells as well as cells of other New World primates, all of which display some degree of glucocorticoid resistance. Human FKBP51 was also found to inhibit GR function but not to the degree of squirrel monkey FKBP51, which differs in amino acid sequence from its human counterpart at 15 of 457 amino acids. These differences are scattered fairly evenly along the sequence, and mapping studies have shown that amino acid changes in several domains contribute to the more potent inhibitory actions of squirrel monkey FKBP51 (Denny et al. 2005). Crystal structures for both human and squirrel monkey FKBP51 have been solved (Sinars et al. 2003); although functionally relevant structural changes are not yet apparent, comparison of these structures should ultimately help to understand why inhibitory potencies differ. The function of FKBP51 is dichotomous with respect to regulation of the steroid hormone receptors. In vitro experiments have shown that overexpression of human FKBP51 reduces glucocorticoid binding affinity and nuclear translocation of GR which forms an ultra-short negative feedback loop for receptor activity (Wochnik et al. 2005). This model is in agreement with the aforementioned data from squirrel monkeys that have a general resistance to glucocorticoids even though they express GR that has the full potential to bind cortisol with high affinity. Another interesting possibility by which FKBP51 decreases overall GR signaling is by promoting nuclear translocation of the transcriptionally inactive β isoform of GR (Zhang et al. 2008). Interestingly, FKBP51 has an opposing effect on AR; it increases the receptor signaling in prostate cancer cells. Using both recombinant protein- and cell-based assays, Ni et al. demonstrated that FKBP51 stimulates chaperone complex association with AR, which further enhances AR ligand binding and androgen-dependent transcription and cell growth, resulting in an ultra-short positive feedback loop (Ni et al. 2010).

In a yeast model for studying functional interactions between steroid receptors and human FKBPs, FKBP51 does not inhibit the activity of GR; however, FKBP51 can effectively reverse the potentiation of GR activity conferred by FKBP52 (Riggs et al. 2003). Therefore, FKBP51 acts as an antagonist of FKBP52. FKBP51 has also been shown to inhibit PR function (Hubler et al. 2003), presumably through a similar

inhibition of FKBP52-mediated potentiation. The mechanism by which FKBP51 antagonizes FKBP52's ability to enhance steroid receptor function is not understood. Other Hsp90-binding TPR proteins do not block FKBP52 actions, so it does not appear that competitive displacement of FKBP52 from receptor complexes by FKBP51 can fully account for antagonism. On the other hand, FKBP51 is known to preferentially associate with PR and GR complexes (Nair et al. 1997; Barent et al. 1998). Domain-swapping studies indicate that the FK1 PPIase domain partially contributes to antagonism but sequences in the FK2 and TPR domain also play a role (Riggs et al. 2003; Denny et al. 2005).

Given that FKBP51 gene expression is inducible by some steroid hormones and FKBP51 can both activate and inhibit receptor function, one can reasonably speculate that FKBP51 serves as a cellular modulator of hormone responsiveness. In cells unexposed to hormone, FKBP52 actions would predominate and promote a robust response to hormone. As a consequence, FKBP51 levels would rise and partially desensitize cells to a secondary hormone exposure in most systems excluding AR-mediated prostate cancer cells. These effects can be demonstrated in cellular models, but the physiological importance of this mechanism must be established with animal models. Toward this goal, FKBP51 gene knockout (51KO) mice were generated. Homozygous mutant animals are grossly normal and reproductively viable, so FKBP51 does not appear to be critical in the same physiological processes as FKBP52 (Storer et al. 2011). Nonetheless, modulatory actions of FKBP51 are relevant but subject to compensatory physiological mechanisms. Interestingly, double knockout of both FKBP51 and FKBP52 genes is embryonic lethal in mice, suggesting either that FKBP51 and FKBP52 have a critical, mutually redundant function or that FKBP51 and FKBP52 function in a common developmental pathway that requires the distinct actions of both immunophilins (unpublished observations).

The hypothalamic-pituitary-adrenal (HPA) axis controls stress response and is associated with susceptibility to depression as well as antidepressant efficacy (Touma et al. 2011; O'Leary et al. 2011). The HPA axis is regulated via negative feedback of GR activity and FKBP51. GR resistance is conferred by the overexpression of FKBP51, which is associated with an impaired negative feedback mechanism (Denny et al. 2005). Polymorphisms in the FKBP5 gene are associated with an increased susceptibility for depression, an increased response to antidepressants, and an increased risk of posttraumatic stress disorder in response to adverse early life events (Binder et al. 2004, 2008; Li et al. 2020). In addition, genotype-directed environment-induced gene programming through FKBP5 gene methylation was shown to mediate gene-childhood trauma interactions (Klengel et al. 2013). Studies have shown that FKBP51 is a modulator of the cortisol-HPA axis response to chronic stress and related psychiatric disorders (Hartmann et al. 2012; O'Leary et al. 2011; Tatro et al. 2009; Touma et al. 2011). Indeed, 51KO mice displayed diminished physiological and neuroendocrine response to the adverse effects of chronic stress with fast recovery from acute stress episodes (Hartmann et al. 2012). The null mice also showed reduced adrenal gland weight and lower levels

of basal corticosterone, suggesting an enhanced sensitivity of GR due to the loss of FKBP51 (Hartmann et al. 2012).

As aforementioned, aggregation of MAP tau into neurofibrillary tangles in neurons is the hallmark of tauopathies. In vitro studies demonstrated that PPIase activity of FKBP51 regulates and balances the phosphorylation state of tau for microtubule stabilization (Jinwal et al. 2010; Koren et al. 2011). Interestingly, knockdown of FKBP51 dramatically reduced tau levels, while inhibiting its PPIase activity led to increased stability and accumulation of phosphorylated tau (Jinwal et al. 2010). In addition, overexpression of FKBP51 prevented tau clearance and produced oligomeric tau in the brain, facilitating its neurotoxicity (Blair et al. 2013; Jinwal et al. 2010). Studies by Blair et al. demonstrated that upregulation of FKBP51 expression is attributed to a decrease in *FKBP5* methylation in which the process appears to be inversely proportional over time (Blair et al. 2013). This provides an explanation for the detection of increased FKBP51 protein levels in aged murine brains and the manifestation of depression and cognitive deficits in AD patients.

Aside from its role in steroid receptor function, FKBP51 has been identified in a wide array of Hsp90-independent complexes. Biochemical and cellular studies have demonstrated that FKBP51 inhibits apoptosis in irradiated melanoma cells (Romano et al. 2010), promotes dephosphorylation of Akt and downregulation of the Akt pathway (Pei et al. 2009), and is associated with polymorphisms in *fkbp5* as seen in affective and anxiety disorders (Binder 2009). Furthermore, FKBP51 has been shown to regulate NFκB pathways. FKBP51 was identified (Bouwmeester et al. 2004) by a proteomic approach in complex with IKKα, one of the serine/threonine kinases that stimulates phosphorylation and degradation of the NFκB inhibitor IκB. Knockdown of FKBP51 expression was shown to inhibit IKKα activation and thereby block TNFα-induced activation of NFκB, which confirmed the functional significance of FKBP51 in IKKα complexes. Perhaps related to FKBP51-dependent regulation of NFκB pathways, overexpression of FKBP51 has been correlated (Giraudier et al. 2002) with idiopathic myelofibrosis, a rare clonal stem cell disorder. Experimental overexpression of FKBP51 was subsequently shown to stimulate NFκB activity and, as a consequence, to increase secretion of pro-fibrotic TGF-β1 (Komura et al. 2005). IKKα had previously been shown to be an Hsp90 client (Broemer et al. 2004), so it is possible that, analogous to steroid receptor complexes, FKBP51 assembles with IKKα as a heterocomplex with Hsp90. Whether FKBP51 Hsp90 binding or PPIase is required for regulation of IKKα has not been determined.

TPR-Domain Immunophilins Regulate the Subcellular Localization of Soluble Proteins

Like almost all soluble proteins (RNA-binding proteins, cell proliferation regulators, transcription factors, enzymes, etc.), unliganded steroid receptors are constantly shuttling between cytoplasm and nucleus. Upon steroid binding, receptors

accumulate in the nuclear compartment, but a fraction of this pool escapes to cytoplasm and must be dynamically reimported (Fu et al. 2018; Yang et al. 1997). Receptors such as GR, MR, AR, and PR-B reside mostly in the cytoplasm in the absence of steroid, whereas others such as ER or PR-A are constitutively located in the nucleus, even in the absence of hormone. Regardless of their primary localization, all these receptors shuttle dynamically between both compartments (Elbi et al. 2004; Galigniana et al. 2010a) such that the final localization of a given receptor under a certain biological condition (i.e., mostly cytoplasmic or nuclear) is the result of the proper displacement of that dynamic equilibrium between both cellular compartments (Mazaira et al. 2021b). Accordingly, the presence of hormone favors the import-driven mechanism that results in the nuclear concentration of GR. Inasmuch as the transport mechanism operates bidirectionally, specific protein-protein interactions should be required to determine the direction of signal protein movement and the final dynamic equilibrium. In this sense, steroid receptors are associated with Hsp90•TPR-domain immunophilin complexes, a protein-protein association that regulates their subcellular localization. Thus, the presence of FKBP52 favors nuclear concentration of receptors, whereas FKBP51 favors the cytoplasmic accumulation.

The classical model heuristically posited during the early 1980s stated that after steroid binding, the receptor must dissociate from the Hsp90-based heterocomplex (a process referred to as "transformation") to become nuclear. Then, it was discovered that immunophilins are members of that GR•Hsp90 heterocomplex (Tai et al. 1992). *Perhaps o*ne of the most interesting findings related to the regulation of the subcellular localization of GR was the fact that PPIase domain of FKBP52 interacts with components of the dynein/dynactin motor protein complex (Galigniana et al. 2001). This led to the discovery that the Hsp90•FKBP52 heterocomplex works as a linker between the GR and the protein system that powers the retrograde movement of the receptor (Galigniana et al. 2010b; Wochnik et al. 2005) and also for its passage through the nuclear pore complex (Mazaira et al. 2020).

Figure 2.3 depicts a scheme of the FKBP52-dependent model for GR transport. Note that the cytoplasmic receptor has FKBP51 as prevailing immunophilin in the complex, which is exchanged by FKBP52 upon steroid binding (Davies et al. 2002; Gallo et al. 2007; Daghestani et al. 2012). Importantly, despite the similar structures of FKBP51 and FKBP52, the former immunophilin is not a relevant dynein interactor (Wochnik et al. 2005), which justifies the need for the protein exchange to release the inhibitory immunophilin FKBP51 when the receptor is stimulated since the active movement is mediated by dynein (Daghestani et al. 2012; Johnston et al. 2012; Galigniana et al. 2010b; Lukic et al. 2015; Vandevyver et al. 2012).

Because the domain of FKBP52 involved in dynein binding is the FK1 region of the PPIase domain, the potential interference of the macrolide FK506 was a likely possibility. However, neither dynein binding nor receptor retrotransport is prevented by FK506 and these events also occur with inactive point mutants of the PPIase domain. Therefore, it is regarded that such interaction does not involve or affect the FK506 binding pocket of the immunophilin. The crystallographic structure of the N-terminal domain of FKBP52 shows a pocket of β-sheets that forms a hydrophobic

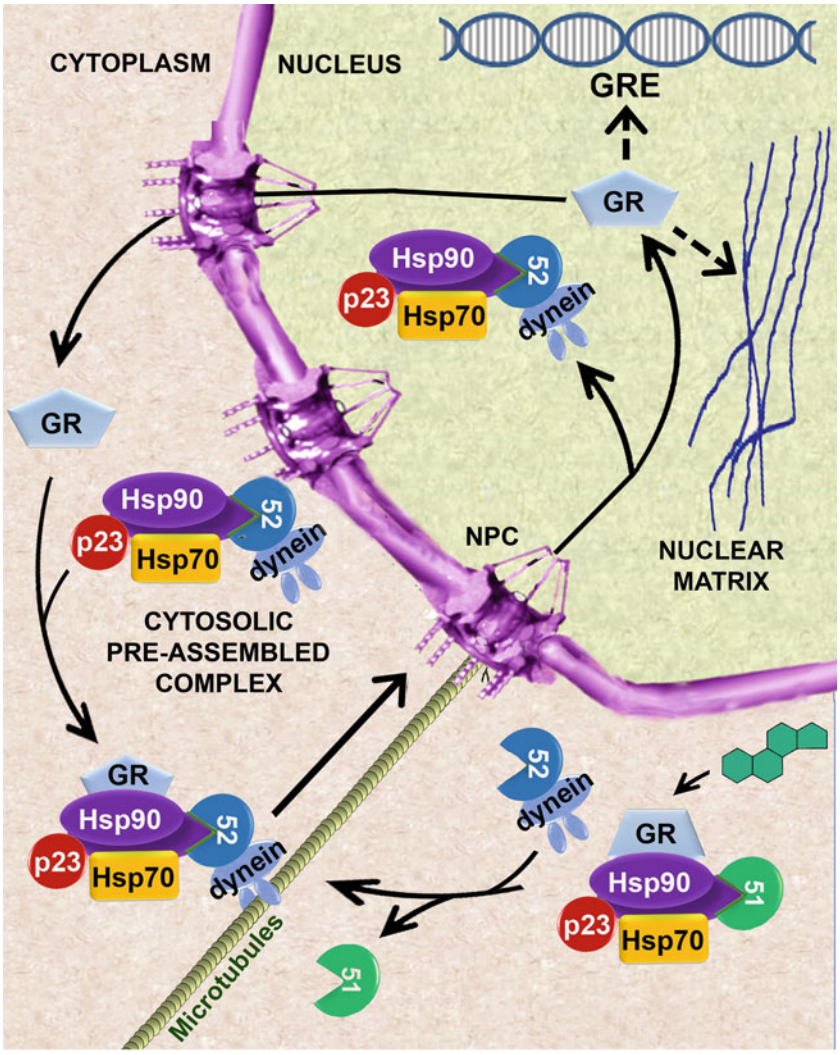

Fig. 2.3 TPR domain proteins regulate GR shuttling and the biological response. The scheme depicts the steps where TPR proteins exert regulation on the GR mechanism of action, i.e., the cytoplasmic assembly of the GR•Hsp90 heterocomplex, the cytoplasmic transport of GR towards the nucleus, its interaction with nuclear pore complex (NPC) factors, transcriptional activity upon GRE (glucocorticoid response elements) binding in target genes, GR association to nuclear matrix structures, and nuclear export. Note the exchange of immunophilins upon steroid binding and that FKBP51 is depicted as the preferred TPR-protein associated to the Hsp90 complex in the empty receptor. Transformation (i.e., Hsp90-complex dissociation) occurs in the nuclear compartment followed by receptor dimerization. The receptor is targeted to the promoter binding-sites to trigger the proper biological response and the heterocomplex is recycled

patch, which was assigned to be the potential binding site for the motor protein (Li et al. 2003). It is important to emphasize that this active transport via FKBP52•dynein machinery simply accelerates the nuclear accumulation of the client protein, but it is not an absolute requirement for this transport since after the disruption of the complex, the nuclear accumulation of the receptor takes place anyway, although the nuclear translocation rate is remarkably slower (∼50–70 min versus ∼4–6 min for the normal active transport) (Galigniana et al. 2010b; Silverstein et al. 1997).

A similar mechanism based on the FKBP52 complex has also been suggested for other primarily cytoplasmic receptors such as AR (Thadani-Mulero et al. 2014), PR (Banerjee et al. 2008), and MR (Piwien Pilipuk et al. 2007), as well as for other nuclear factors such as the ecdysone receptor (Vafopoulou and Steel 2012), p53 (Galigniana et al. 2004), the catalytic subunit of telomerase hTERT (Jeong et al. 2016), the diphtheria toxin (Schuster et al. 2017), NF-κB (Erlejman et al. 2014a), RAC3 (Colo et al. 2008), adeno-associated virus-2 (AAV) (Zhao et al. 2006), etc., just to mention a few relevant examples.

We refer to the GR•Hsp90•FKBP52•dynein heterocomplex as the "transportosome." When it was cross-linked and introduced into digitonin-permeabilized cells, all members of the transportosome molecular machinery were recovered in a complex in the nuclear compartment upon cell stimulation with steroid (Echeverria et al. 2009), suggesting that the transportosome is capable of translocating intact through the nuclear pore complex. This observation was confirmed for native complexes associated to the MR (Galigniana et al. 2010b), indicating that the ancient concept of "transformation" (i.e., dissociation of the Hsp90-based heterocomplex) is indeed a nucleoplasmic rather than a cytoplasmic event. This was confirmed using different methodologies (Grossmann et al. 2012; Presman et al. 2014). It is important to emphasize that, in contrast to the steps posited for the classic model of action in a heuristic manner, all the mechanistic steps described here have been experimentally supported for each individual step, and also predicts that other TPR-domain immunophilins may replace FKBP52 in the transport machinery. The Hsp90-binding immunophilin FKBPL/WISp39 also favors GR retrotransport in similar fashion as FKBP52 (McKeen et al. 2008).

Because the whole transportosome passes intact through the nuclear pore complex, it can be implied that the chaperones and immunophilins of the complex should interact with structures of the pore such as nucleoporins and importins. This prediction was experimentally demonstrated (Echeverria et al. 2009; Mazaira et al. 2020). Based on these observations, it was postulated (Galigniana et al. 2010a) that the permeability barrier of the pore is in part due to a sieve structure created by the reversible cross-linking between Phe and Gly (FG)-rich nucleoporin repeats, which create a three-dimensional meshwork with hydrogel-like properties (as described by Frey et al. (2006)). According to the novel model, nuclear transport receptors overcome the size limit of the sieve and catalyze their own nuclear pore passage by a competitive disruption of adjacent inter-repeat contacts, which transiently opens adjoining meshes. The chaperone complex would enhance the capability of the

receptor to overcome the resistance of the meshwork simply by accomplishing its standard chaperone role.

The overexpression of the TPR domain of the immunophilin impairs the nuclear import of the GR (Mazaira et al. 2020, 2021a), which is an expected effect for a disrupted transport mechanism that requires the involvement of the transportosome complex. Interestingly, the passage through the nuclear pore is also significantly delayed, suggesting an inefficient interaction of the receptor with nuclear import factors. Accordingly, the formation of functional complexes of nucleoporins and importins with Hsp90 and FKBP52 has been demonstrated (Echeverria et al. 2009; Mazaira et al. 2020). Moreover, the nuclear retention of the GR is shortened, and its nuclear export is also accelerated by the overexpression of the TPR peptide. This is the consequence of the capability of FKBP52 (and also PP5) to anchor the GR to the nucleoskeleton via NuMA (Nuclear Mitotic Apparatus) protein, a highly abundant component of the nuclear matrix where that serves a non-mitotic structural role and occupies the majority of the nuclear volume. It has recently been demonstrated that both FKBP52 and PP5 co-purify associated to nucleoskeleton and link the GR to other nuclear regions excluded from the transcriptionally active GRE foci (Mazaira et al. 2020, 2021a).

It is interesting to point out that molecular modeling showed that the isolated TPR peptide of PP5 overlaps the overall conformation of the 14–3-3σ monomer. Not surprisingly, 14–3-3σ functions in a similar manner to the transfected TPR peptide regarding the GR nuclear import and nuclear behavior (Mazaira et al. 2021a). On the other hand, the overexpression of FKBP51 also delays GR nuclear import due to the incapability of this immunophilin to recruit dynein and also expels the GR from the nuclear compartment, perhaps due to competition with FKBP52 for the nuclear anchoring sites. Similar observations and conclusions were also achieved for the role of FKBP52 in the mechanism of action of NF-κB (Erlejman et al. 2014a; Lagadari et al. 2016a) and hTERT, the catalytic subunit of telomerase (Lagadari et al. 2016b). The latter represents an interesting property since FKBP51 concentrates in the nucleus upon several stressing situations, whereas hTERT is exported to the cytoplasm and that fraction that is not degraded via proteasome moves into mitochondria (Lagadari et al. 2016b), a feature that has been associated with the resistance to apoptotic stimuli (Maida and Masutomi 2015; Lipinska et al. 2017) and may be part of a complementary mechanism for the anti-apoptotic actions reported for FKBP51 (Gallo et al. 2011; Lagadari et al. 2016b).

Xap2

Apart from the highly characterized steroid hormone receptor-associated FKBPs, several other TPR-containing FKBPs are present in higher vertebrates. As mentioned in earlier sections of this chapter, Xap2 is a TPR-containing immunophilin that is found almost extensively in AhR complexes. As the name implies, Xap2 also functionally interacts with the hepatitis B virus protein X (Kuzhandaivelu et al.

1996). Xap2 was shown to exert an inhibitory effect on both GR and ERα, but not ERβ activity, and may inhibit AR and PR as well (Cai et al. 2011; Laenger et al. 2009; Schulke et al. 2010). In addition, Xap2 is known to have functional interactions with peroxisome proliferator-activated receptor α (PPARα) (Sumanasekera et al. 2003) and thyroid hormone receptor β**;** however, these interactions have not been extensively characterized. AhR is a ligand-dependent transcription factor that mediates the physiological response to specific environmental contaminants termed polycyclic aromatic hydrocarbons, the most notorious of which is 2,3,7,8-tetrachlorodibenzo-p-dioxin. Similar to steroid receptors, AhR requires assembly with Hsp90 and p23 to achieve a mature ligand binding conformation (reviewed in Petrulis and Perdew 2002), although the AhR ligand binding domain is unrelated to steroid receptor ligand binding domains. AhR complexes also contain an FKBP component, but in this case, it is Xap2 rather FKBP52 or FKBP51.

As with FKBP51 and FKBP52, Xap2 has a C-terminal TPR domain that is known to facilitate binding to the MEEVD motif on Hsp90 (Carver et al. 1998) (Fig. 2.1). In addition, Xap2 contains one N-terminal FK domain that lacks drug binding and also likely lacks PPIase activity. Although the FK domain is not required for Hsp90 binding, it is required for an interaction with the AhR-Hsp90 complex that functionally influences receptor activity (Carver et al. 1998; Kazlauskas et al. 2002). In a cell-free assembly system that lacks Xap2, AhR is capable of assembling with Hsp90 and binding ligand, and upon ligand binding AhR is able to bind the AhR response elements on DNA (Meyer et al. 1998). Again, similar to FKBP52 or FKBP51 in steroid receptor complexes, Xap2 is not required for basal maturation of AhR activity, but in both yeast and mammalian systems, Xap2 can modulate AhR-mediated reporter gene expression (Miller 2002; Ma and Whitlock 1997; Meyer et al. 1998; Carver et al. 1998). By titrating the relative level of Xap2 protein in cells, AhR activity can be enhanced or decreased. For example, when Xap2 is expressed at a level two- to threefold higher than normal, binding of p23 in the AhR-Hsp90 complex is reduced (Hollingshead et al. 2004). Displacement of p23 by high levels of Xap2 would destabilize binding of Hsp90 to AhR and reduce the proportion of AhR in functionally mature complexes. Conversely, there is also evidence that at elevated Xap2 levels AhR is protected from ubiquitination and proteosomal degradation which would increase total AhR levels (Lees et al. 2003; Lapres et al. 2000; Meyer et al. 2000; Meyer and Perdew 1999; Kazlauskas et al. 2000). Finally, several studies suggest that Xap2 facilitates nucleocytoplasmic shuttling of AhR following ligand binding (Berg and Pongratz 2002; Petrulis et al. 2000; Kazlauskas et al. 2000, 2001; Petrulis et al. 2003).

The physiological relevance of Xap2 interactions with AhR complexes has not been examined in a whole animal model, but Xap2 could potentially influence any of several physiological and pathological pathways mediated by AhR. Mice that are homozygous for a disrupted AhR gene have many physiological and developmental defects; among these are immune system impairment, hepatic fibrosis, cardiac hypertrophy, impaired insulin regulation, and defects in ovarian and vascular development (Fernandez-Salguero et al. 1995; Lahvis et al. 2005; Thackaberry et al. 2003; Benedict et al. 2000). In addition, many of the toxic and teratogenic effects produced

by AhR ligands require an intact AhR signaling pathway (Mimura and Fujii-Kuriyama 2003; Fernandez-Salguero et al. 1996). For example, dioxin-induced defects in prostate development are absent in AhR knockout mice (Lin et al. 2002). In a conditional Xap2 hepatic knockout mouse model, AhR and Cyp1b1 levels were significantly reduced; however, Cyp1a1 and Cyp1a2 were induced to levels seen in wild-type mice in response to dioxin challenge (Nukaya et al. 2010). Development of a mouse strain lacking Xap2 would aid in determining the role Xap2 plays in these processes and might validate Xap2 as a potential target for therapeutic intervention. In addition to the above functional interactions, Xap2 has several other interacting partners including, but not limited to, PDE4A5 and 2A3, HSC70, TIF-2, TRβ1, RET, and TOMM20, thereby modulating a host of physiological functions (Reviewed in Trivellin and Korbonits (2011)). Examination of protein expression levels of Xap2 interacting protein, PDE4A4, and its closely related isoform PDE4A8, in human pituitary tissues and pituitary adenomas found that both interacting proteins were overexpressed in adenomas (Bolger et al. 2016).

FKBP36

FKBP36 (gene name FKBP6 in humans) is another TPR-containing FKBP that is structurally similar to Xap2, yet functionally distinct. FKBP36 has a single N-terminal FK domain and a C-terminal TPR domain. In vitro studies show that FKBP36 binds Hsp90 and can assemble with steroid receptor complexes (unpublished observation), but there is currently no evidence that FKBP36 alters receptor activity. FKBP36 mRNA is broadly expressed in vertebrate tissues with an exceptionally high level observed in the testis; male FKBP36 knockout mice lack sperm and FKBP36 was shown to be a critical component in meiotic synaptonemal complexes (Crackower et al. 2003). FKBP36 has been found to interact with Hsp72 which, in conjunction with clathrin, was found to be a critical element of male meiosis (Jarczowski et al. 2008). FKBP36 interacts with and inhibits GAPDH activity and expression (Jarczowski et al. 2009). FKBP36 forms a complex with Hsp90 and GAPDH and this complex may regulate GAPDH activity in a manner akin to FKBP/Hsp90/steroid receptor complexes (Jarczowski et al. 2009). FKBP36 can exert an effect on GAPDH in an Hsp90-independent manner by either directly inhibiting NAD+ binding to GAPDH or by decreasing GAPDH expression (Jarczowski et al. 2009). Patients with Williams syndrome, which is characterized by congenital cardiovascular defects, dysmorphic facial features, mental retardation, growth defects, azoospermia, and hypercalcemia, are typically haploinsufficient for FKBP6 (Meng et al. 1998); however, the contribution of FKBP6 deletion in this syndrome is not clear since several contiguous genes on chromosome 11, including genes for elastin and LIM-Kinase 1, are also deleted in these patients and clearly contribute to some phenotypic aspects.

FKBP38

FKBP38 (gene name FKBP8) contains a glutamate-rich domain, FK domain, three TPR domains, and a calmodulin-binding motif. FKBP38 is ubiquitously expressed in all tissues, with high expression in neuronal tissues. Among the FKBP family, FKBP38 is novel in several respects, including a unique C-terminal transmembrane anchor domain, used to localize FKBP38 to both the mitochondrial and ER membranes. Although FKBP38 contains a PPIase domain, PPIase activity is regulated. The structure of the PPIase domain is similar to the prototypical family member, FKBP12; however, there are important differences in the three-dimensional structure of the loop and the binding pocket of the active site (Maestre-Martinez et al. 2006; Kay 1996). The loss of several aromatic residues in the active site leads to lower PPIase activity, even upon activation, and low affinity for FK506 (Maestre-Martinez et al. 2006; Edlich et al. 2006). FKBP38 PPIase activation is dependent on the calmodulin-binding domain and calmodulin/Ca^{+2} binding stimulates PPIase activity (Edlich et al. 2005, 2007b; Maestre-Martinez et al. 2010).

FKBP38 participates in a number of cellular processes involving protein folding and trafficking, apoptosis, neural tube formation, CFTR trafficking, and viral replication (Edlich and Lucke 2011; Banasavadi-Siddegowda et al. 2011). FKBP38 interacts with the anti-apoptotic proteins Bcl-2 in regulating apoptosis and appears to have both pro- and anti-apoptotic activities that are likely tissue specific (Shirane and Nakayama 2004). In general FKBP38 anti-apoptotic activity appears to regulate apoptosis by transporting Bcl-2 to the mitochondrial membrane stabilizing Bcl-2 and inhibiting apoptosis (Shirane and Nakayama 2004). Two mechanisms on how FKBP38 protects Bcl-2 from degradation have been explored. One involves the interaction between FKBP38 and a caspase cleavage site located within Bcl-2 (Choi et al. 2010). When FKBP38 is associated with Bcl-2, access to the caspase cleavage site may be blocked, preventing caspase-mediated Bcl-2 degradation (Choi et al. 2010). The second mechanism is through an interaction between the S4 subunit of the 19S proteasome complex, thereby regulating proteasome activity. However, in neuroblastoma cells the active FKBP38/calmodulin/Ca^{+2} complex has a pro-apoptotic affect by interfering with the ability of Bcl-2 to interact with and block pro-apoptotic proteins (Edlich et al. 2005). In this case an interaction between Hsp90 and the FKB38/calmodulin/Ca^{+2} complex interferes with FKP38 pro-apoptotic activity, which could impede apoptosis (Edlich et al. 2007a). In addition to its broad anti-apoptotic roles, FKBP38 is known to be found in the outer mitochondrial membrane to function as an adaptor protein with anti-apoptotic activity. By way of an N-terminal region which interacts with LC3A, FKBP38 is able to effectively engage lipidated LC3A to damaged mitochondria to induce Parkin-independent mitophagy, without becoming degraded itself (Bhujabal et al. 2017). As an outer mitochondrial membrane protein, FKBP38 has been found to regulate cell death of CD8 T cells by associating with Bcl-2 and calcineurin to regulate Smyd1C, a histone methyltransferase mainly expressed in activated CD8 T cells (Nie et al. 2017). To further define the role of FKBP38 in mitophagy, studies

examining its effects in clearing damaged mitochondria in myocytes found that, while knockdown of FKBP38 in HEK293 or H9c2 myocytes did not result in altered mitophagy, it did produce an accumulation of misfolded protein aggregates in H9c2 myocytes (Misaka et al. 2018). Hearts from *Fkbp38* $^{-/-}$ mice which were subjected to transverse aortic constriction revealed elevated numbers of apoptotic cardiomyocytes along with increased levels of caspase-12 and endoplasmic reticulum stress markers, hinting at a potential protective role against hemodynamic stress.

FKBP38 is also implicated in the regulation of mTOR signaling through an interaction with Rheb (Rosner et al. 2003). mTOR regulates a wide range of cellular processes, including cell cycle and cell growth, in response to various conditions, including fluctuations in nutrient and energy levels, and growth factors (Yang and Guan 2007). The FKBP12/rapamycin complex interacts with and inhibits mTOR activity (Brown et al. 1994). However, FKBP38 interacts with and antagonizes mTOR in a rapamycin-independent manner (Bai et al. 2007). Overexpression of FKBP38 decreases the induction of mTOR-regulated genes, and siRNA-induced reduction of FKBP38 increased mTOR activity (Bai et al. 2007; Fu et al. 2015). Rheb disrupts the mTOR/FKBP38 complex by binding to FKBP38 in a nutrient-dependent manner leading to an induction of mTOR-responsive genes (Bai et al. 2007).

FKBP38 is also involved in neural tube formation as the loss of FKBP38 leads to gross abnormalities during embryonic formation of the nervous system (Wong et al. 2008). It has been speculated that this is due to deregulation of the Sonic hedgehog (SHH) pathway during neural tube formation, where FKBP38 is a SHH antagonist, and the loss of FKBP38 function leads to over-activity of SHH during development, resulting in neuronal malformation (Cho et al. 2008).

In addition to the regulatory role in response to nutritional conditions, FKBP38 is also involved in the cellular response to hypoxia. Hypoxia-inducible transcription factors (HIFs) are involved in the cellular response to low oxygen levels and, under normal conditions, are quickly degraded by prolyl-4-hydroxylase (PDH) enzymes (Wenger et al. 2005). FKBP38 interacts with PHD2 at the endoplasmic reticulum and mitochondrial membranes and regulates PDH2 activity through proteasomal degradation, thereby regulating HIF stability and downstream gene expression in response to hypoxic conditions (Barth et al. 2009).

FKBP38 is involved in CFTR synthesis and folding by negatively regulating CFTR synthesis and positively regulating folding (Banasavadi-Siddegowda et al. 2011). Knockdown of FKBP38 increased CFTR production, but reduced post-translational modification, resulting in a lower expression of functional CFTR (Banasavadi-Siddegowda et al. 2011). Interestingly, FKBP38 PPIase activity is required for the regulation of CFTR folding.

There is evidence of FKBP interaction with TRPC channels, and FKBP38 is not exempt. In human platelets, FKBP38 is believed to have a regulatory role in non-capacitative calcium entry through TRPC6 by working in tandem with FKBP25 (Lopez et al. 2015).

Finally, FKBP38 is required for replication of the hepatitis C virus (HCV). In HCV infection the viral nonstructural protein 5A (NS5A) has been shown to form a

complex with FKBP38 and Hsp90 at the mitochondrial and endoplasmic reticulum membranes (Wang et al. 2006). Either knockdown of FKBP38 with siRNA or inhibition of Hsp90 activity with geldanamycin results in decreased HCV RNA replication (Okada et al. 2004). In addition to HCV, FKBP38 was also found to interact with the NS5A protein of classical swine fever virus to promote viral replication, as shRNA knockdown of FKBP38 resulted in decreased viral replication and FKBP38 overexpression promoted expression of viral RNA (Li et al. 2016).

FKBPL

FKBPL shares the same general structure as other members of the FKBP family, including a TPR domain that facilitates Hsp90 binding and a PPIase domain, which lacks catalytic activity (Robson et al. 1999; Sunnotel et al. 2010). FKBPL was initially discovered while screening for genes that were protective against ionizing radiation (Robson et al. 1997, 1999). FKBPL is most closely related to the larger FKBP52 (26% identity) (Robson and James 2012). However, the PPIase domain only shares 17% identity with the FKBP52 PPIase region (Robson and James 2012). The FKBPL TPR domain shares 33% amino acid identity with FKBP52 and has the ability to interact with Hsp90 stabilizing steroid hormone receptor conformations as well as stabilizing newly synthesized p21 preventing its degradation (Robson and James 2012; Jascur et al. 2005). There is conflicting data on FKBPL and its role in conferring radiation resistance. Jascur et al. originally showed that, in response to high-dose radiation, the FKBPL/Hsp90/p21 complex stabilized p21 leading to G2 cell cycle arrest, which conferred a pro-survival effect. However, later data has demonstrated that there is a downregulation of p21 in response to radiation exposure and decreased p21 was involved in pro-survival after radiation exposure (Chu et al. 2004; Robson et al. 1999, 2000). In addition to radiation resistance, FKBPL plays a significant role in tumor progression (Robson et al. 1997, 1999, 2000; Jascur et al. 2005). In tumor cells FKBPL appears to participate in not only growth of the tumor, but also in the sensitivity of the tumor to various chemotherapeutic agents (Bublik et al. 2010). For example, high levels of GSTE-1 interact with the FKBPL/Hsp90/p21 complex, which leads to p21 stabilization, leading to resistance to the chemotherapeutic agent taxane (Bublik et al. 2010). Although the exact radio- and chemoprotective role of FKBPL needs to be elucidated, the data clearly show that FKBPL is an important factor in cell cycle progression, cell survival, and tumor progression.

Like other Hsp90-associated FKBP proteins, FKBPL also forms complexes with various steroid hormone receptors (reviewed in Erlejman et al. 2014b). FKBPL and Hsp90 appear to stabilize AR, ER, and GR/Hsp90 complexes (Sunnotel et al. 2010; McKeen et al. 2008, 2010). Similar to FKBP52, FKBPL affects the AR-dependent expression of prostate-specific antigen (Sunnotel et al. 2010). Sunnotel et al. demonstrated that two populations of azoospermic males had alterations in their FKBPL gene, which may alter FKBPL interaction with AR and contribute to infertility in the two populations. FKBPL was also shown to colocalize with the GR/Hsp90 complex

(McKeen et al. 2008). Dexamethasone treatment resulted in the colocalization of FKBPL and GR in the nucleus and the upregulation of GR-response genes in a prostate cancer cell line (McKeen et al. 2008). Translocation of the FKBPL/GR complex appears to be mediated by an interaction with dynamitin motor proteins, similar to the mechanism described for FKBP52 (McKeen et al. 2008).

FKBPL expression is regulated by estrogen and FKBPL functionally interacts with the ER/Hsp90 complex (McKeen et al. 2010). In addition, FKBPL expression correlates with breast cancer tumor growth as FKBPL and ER expression are inversely related; increased FKBPL levels lead to decreased ER expression (McKeen et al. 2010; Abukhdeir et al. 2008). Overexpression of FKBPL is associated with increased survival of untreated breast cancer patients and sensitizes cancer cells to the anti-proliferative effect of both tamoxifen and fulvestrant, which promotes increased recurrence-free survival (McKeen et al. 2011; Han et al. 2006). Interestingly, overexpression of related FKBP proteins in tumors is associated with a poor treatment outcome and prognosis (Romano et al. 2010; Solassol et al. 2011; Zhou et al. 2017). Conversely, increased levels of FKBPL correlate to a more positive response to treatment and a more favorable prognosis (McKeen et al. 2010, 2011; Han et al. 2006). Further examining the effects of FKBPL in breast cancer progression, McClements and colleagues found that FKBPL overexpression or treatment with FKBPL peptide derivatives in various ER+ and ER- breast cancer models (including in vitro, in vivo, and ex vivo) reduced the number of cancer stem cells in ER+ and ER- models and delayed metastasis in triple negative breast cancer models through downregulation of DLL4 and Notch4 (McClements et al. 2019). FKBPL stability is regulated by RBCK1, and as with FKBPL, RBCK1 is upregulated by estrogen and can interact with the FKBPL/ER/Hsp90 complex (Donley et al. 2013). Increased expression of both FKBPL and RBCK1 appears to correlate with increased survival; however, elevated RBCK1 levels reduce the efficacy of tamoxifen (Donley et al. 2013). The interactions leading to tumor survival and progression still need to be explored further.

Finally, FKBPL possesses anti-angiogenic properties (Yakkundi et al. 2013). In a mouse xenograft tumor model overexpression of FKBPL resulted in decreased tumor growth and tumor necrosis (Crabb et al. 2009). The anti-angiogenic effects of FKBPL are mediated through the N-terminal portion of the protein comprised of amino acids 34–58, termed peptide AD-01, which is currently being explored as a novel anti-angiogenic drug (Valentine et al. 2011; Yakkundi et al. 2013). FKBPL may also prove to be a valuable anti-angiogenic biomarker in cardiovascular disease (CVD). Increased plasma FKBPL was found in patients with CVD and to be a determinant of CVD, suggesting it's likely a novel mechanism in the development of CVD (Januszewski et al. 2020). Further, reduced plasma and placental CD44/FKBPL ratios were found to be indicative of preeclampsia risk in women at 15 and 20 weeks gestation, when compared to healthy controls (Todd et al. 2021). Exploiting FKBPL's role in inhibiting angiogenesis, siRNA targeting FKBPL is being explored as a means of wound therapy by promoting angiogenesis. In vivo wound healing studies using a bilayered patch impregnated with siFKBPL

nanoparticles displayed 326% increase in blood vessel density as compared to the untreated wounds (Mulholland et al. 2019).

Plant FKBPs

Hsp90-binding TPR immunophilins have been identified in all eukaryotes examined. A few examples of plant TPR-containing FKBPs are shown in Fig. 2.1. The TPR domain of each FKBP is very similar in amino acid sequence to that of vertebrate proteins; these are presumed to bind Hsp90, but that has not been determined in all cases. The plant and insect FKBPs contain one or more PPIase-related domain and can contain other functional domains. For example, *At*FKBP42 contains a C-terminal transmembrane domain that localizes the protein to the inner plasma membrane and the vacuolar membrane (Kamphausen et al. 2002; Geisler et al. 2003, 2004).

There is ample evidence to suggest that the plant and insect FKBPs are physiologically important. Mutations in *AtFKBP42* cause the severe developmental phenotypes termed *twisted dwarf 1* (*TWD*) (Geisler et al. 2003) and *ultracurvata* (*UCU2*) (Perez-Perez et al. 2004). The mechanism by which these phenotypes occur likely involves impairment of membrane transport of the growth hormone auxin, as *At*FKBP42 is known to interact with several ATP-binding cassette (ABC) transporters on the plasma and vacuolar membranes (Geisler et al. 2003, 2004; Liu et al. 2001; Geisler and Hegedus 2020; Bailly et al. 2014). Mutations in *AtFKBP72* result in a class of mutants termed *pasticcino* or *pas* mutants, which are characterized by a wide variety of developmental defects (Vittorioso et al. 1998). *At*FKBP42 has been found to play a crucial role in the biogenesis of auxin-transporting ABCBs, aiding in an early quality control step of nascent ABC transporters, and has recently been shown to be necessary for ABC-transporter trafficking to the plasma membrane following the endoplasmic reticulum (Geisler and Hegedus 2020; Zhu et al. 2016). Two Hsp90-binding TPR FKBPs in wheat, wFKBP72 and the heat shock-inducible wFKBP77, have been shown in transgenic plants to distinctively influence developmental patterns (Kurek et al. 2002). In *Arabidopsis*, FKBP15-1 and FKBP15-2 were found to influence nutrient absorption through negative modulation of lateral root development by inhibiting the catalytic activity of vacuolar invertase 2 (VIN2) via the FKBPs PPIase activity (Wang et al. 2020). Highlighting the various roles of plant FKBPs, crystal structures of another *Arabidopsis* FKBP, FKBP53, revealed the domains necessary to exert its functions as a histone chaperone—although its C-terminal domain displayed strong PPIase activity, the N-terminal domain contains a nucleoplasmin fold which interacts with H2A/H2B and H3/H4 oligomers (Singh et al. 2020). A recent study found that ectopic expression of an FKBP12 rice homolog, termed OsFKBP12, in *Arabidopsis* increased the plant's susceptibility to *Pseudomonas syringae* and the plant FKBP12 homolog was shown to be a negative regulator of salt tolerance (Cheung et al. 2020).

Summary

In addressing the physiological importance of PPIases, Heitman and colleagues (Dolinski et al. 1997) generated an *S. cerevisiae* strain that lacked all 12 PPIase genes in the FKBP and cyclophilin families; this pluri-mutant strain displayed some growth abnormalities but was viable, thus demonstrating that these genes collectively are non-essential in yeast. Nonetheless, it has become clear that the Hsp90-binding FKBP immunophilins, through interactions with steroid receptors, kinases, and other cellular factors, play important physiological and pathological roles in mammals. Significant progress has been made on the elucidation of these roles and the definition of underlying molecular mechanisms. This increased understanding of FKBP biology is beginning to drive the identification and development of specific inhibitors targeting individual FKBP immunophilins for the treatment of a variety of human diseases.

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Chapter 3
Hsp70/Hsp90 Organising Protein (Hop): Coordinating Much More than Chaperones

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Abstract The Hsp70/Hsp90 organising protein (Hop, also known as stress-inducible protein 1/STI1/STIP1) has received considerable attention for diverse cellular functions in both healthy and diseased states. There is extensive evidence that intracellular Hop is a co-chaperone of the major chaperones Hsp70 and Hsp90, playing an important role in the productive folding of Hsp90 client proteins, although recent evidence suggests that eukaryotic Hop is regulatory within chaperone complexes rather than essential. Consequently, Hop is implicated in many key signalling pathways, including aberrant pathways leading to cancer. Hop is also secreted, and it is now well established that Hop interacts with the prion protein, PrP^C, to mediate multiple signalling events. The intracellular and extracellular forms of Hop most likely represent two different isoforms, although the molecular determinants of these divergent functions are yet to be identified. There is also a growing body of research that reports the involvement of Hop in cellular activities that appear independent of either chaperones or PrP^C. While the various cellular functions of Hop have been described, its biological function remains elusive. However, recent knockout studies in mammals suggest that Hop has an important role in embryonic development. This review provides a critical overview of the latest molecular, cellular and biological research on Hop, critically evaluating its function in healthy systems and how this function is adapted in diseased states.

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Assisted Protein Folding by the Hsp70/Hsp90 Chaperone Complex

Living cells synthesise large amounts of protein in a short time. If the hydrophobic residues of proteins are exposed, they can aggregate with each other which could lead to precipitation (Martin 2004; Kampinga 2006). Specialised proteins, known as molecular chaperones, have evolved to prevent this from happening. They assist nascent or stress-denatured proteins in folding, conformational assembly, translocation and degradation (Ellis 1987; Welch 1991; Hendrick and Hartl 1995; Clarke 1996; Hartl 1996; Picard 2002; Wandinger et al. 2008; Taipale et al. 2010; Hartl et al. 2011). The heat shock proteins, Hsp70 and Hsp90, form an important molecular chaperone network required for folding and maturation of key regulatory proteins, many of which are signalling intermediates or transcription factors (Kimmins and MacRae 2000; Wegele et al. 2004; Carrigan et al. 2005). Whereas Hsp90 is primarily involved in conformational regulation and stabilisation of proteins that are almost completely folded, Hsp70 is required for earlier stages of assisted folding of nascent or denatured proteins (Whitelaw et al. 1991; Stepanova et al. 2000; Park et al. 2003; Pratt and Toft 2003; Citri et al. 2006).

Both Hsp70 and Hsp90 are dependent on ATP hydrolysis and association with a range of accessory proteins, known as co-chaperones, for chaperone activity (Nadeau et al. 1993; Jakob et al. 1996; Scheibel et al. 1997; Obermann et al. 1998; Panaretou et al. 1998; Prodromou et al. 2000; McLaughlin et al. 2004; Onuoha et al. 2008; Prodromou 2012). The Hsp70/Hsp90 protein folding cycle has been described for steroid receptors (e.g. progesterone and glucocorticoid receptors) and is widely accepted as the mechanism followed for most Hsp90 client proteins (Smith 1993; Dittmar et al. 1996; Johnson et al. 1998; Wegele et al. 2004; Li et al. 2012a; Alvira et al. 2014; Rohl et al. 2015a; Rohl et al. 2015b; Sahasrabudhe et al. 2017). The early stages of the chaperone-assisted folding cycle occur when Hsp70, together with one of the Hsp40 co-chaperone isoforms, captures nascent or denatured proteins. The next stage involves the formation of the intermediate complex, in which the client protein is transferred from the Hsp70 complex to the open Hsp90 complex. Hsp90 is constitutively dimerised at the C-terminus, while the N-terminal nucleotide binding domains (NBD) of the dimers are disassociated (resembling a “V” shape). This is followed by ATP binding to the nucleotide binding domain (NBD) of Hsp90. Subsequent conformational changes result in N-terminal dimerisation, docking of the middle domain and binding of the client protein. Hsp90 in this complex is in the closed conformation. Hydrolysis of ATP occurs, and the protein reverts to the open conformation and the client protein is released (Wegele et al. 2004; Wegele et al. 2006; Richter et al. 2008; Graf et al. 2009; Hessling et al. 2009). Progression through the different stages of this cycle is regulated by a variety of co-chaperones, including

Hsp70 interacting protein (HIP), C-terminus of Hsp70 interacting protein (CHIP), Hsp70-Hsp90 organising protein (Hop), activator of Hsp90 ATPase 1 (AHA1), CDC37 and p23 (Chen et al. 1996; Chang et al. 1997; Chen and Smith 1998; Johnson et al. 1998; Van Der Spuy et al. 2000; Angeletti et al. 2002; Richter et al. 2003; Lee et al. 2004; Hildenbrand et al. 2010; Sahasrabudhe et al. 2017). Hop and CDC37 are intermediate stage co-chaperones controlling entry of clients into the pathway, while p23 and AHA1 are involved in the later stages of the cycle involving client protein maturation (Li et al. 2012a). In this way, co-chaperones indirectly modulate the function of the Hsp70/Hsp90 complex by controlling the progression of client proteins through the chaperone cycle.

Hop (Hsp70-Hsp90 Organising Protein)

The Hsp70-Hsp90 organising protein (henceforth referred to as Hop; but also known as stress-inducible protein 1 [STI1], stress-inducible phosphoprotein 1 [STIP1] or p60) is a ubiquitous protein and one of the most widely dispersed co-chaperones of Hsp90 (Johnson and Brown 2009). First identified in yeast (Nicolet and Craig 1989), Hop has been demonstrated or predicted to be encoded in the genome of many organisms. This includes model organisms used for genetic studies of human disease: nematode (Song et al. 2009), fruit fly (Grigus et al. 1998; Adams et al. 2000), zebrafish (Woods et al. 2005; Tastan Bishop et al. 2013) and mouse (Blatch et al. 1997), as well as rats (Demand et al. 1998), frogs (Klein et al. 2002), fish (Andreassen et al. 2009), parasites (Webb et al. 1997; Hombach et al. 2013) and plants (Zhang et al. 2003; Chen et al. 2010). The gene and nucleotide sequence for Hop has also been identified in the genome and transcriptome of the Coelacanth (*Latimeria* spp.), an organism largely unchanged for millions of years (Amemiya et al. 2013; Tastan Bishop et al. 2013). The human homologue of Hop was isolated in 1992 (Honore et al. 1992). Despite the conservation of Hop in these species, there is some evidence that Hop is structurally and functionally different in different organisms. For example, Hop is an essential gene in the mouse (Beraldo et al. 2013), but not in yeast (Chang et al. 1997) and can be knocked out in adult human cell lines which retain viability (Bhattacharya et al. 2020).

Hop is predominantly a cytoplasmic protein but can also be found in the nucleus (Longshaw et al. 2004), Golgi apparatus (Honore et al. 1992), in the extracellular environment and associated with cell membranes (Hajj et al. 2013). Dogma suggests that the nuclear and extracellular Hop species derive from changes in the subcellular localisation of cytoplasmic Hop. Indeed, mammalian Hop contains a bipartite nuclear localisation signal (NLS) which has been proposed to facilitate translocation from the cytoplasm to the nucleus in response to stress. Hop also contains potential nuclear export signals, and inhibition of nuclear export enhances the nuclear localisation of murine Hop (Longshaw et al. 2004). Hop translocates to the nucleus during G1/S transition through phosphorylation by casein kinase II, whereas phosphorylation by cell division cycle 2 kinase retains Hop in the cytoplasm (Longshaw

et al. 2004; Daniel et al. 2008). In astrocyte cell lines, PIAS1 (protein inhibitor of activated STAT1) was identified as a nuclear retention factor for Hop (Soares et al. 2013). The mechanism by which Hop is transported to the plasma membrane and extracellular environment is currently undefined, although there is evidence for export of Hop from mouse astrocytes in exosomes derived from multivesicular bodies (Hajj et al. 2013) and Hop has been identified in extracellular vesicles released from mouse embryonic stem cells (Cruz et al. 2018).

Structure of Hop

Structurally, Hop is composed of repeating units of two different types of domain, namely, the tetratricopeptide repeat (TPR) motif and the aspartate-proline (DP) motif domains (also known as STI1 domains). Hop contains three TPR domains (designated TPR1, TPR2A and TPR2B), each of which is formed from three TPR motifs (Fig. 3.1). There are two DP domains, the DP1 and DP2 domains, which are positioned between TPR1 and TPR2A and C-terminal to TPR2B of Hop, respectively. The TPR domains of Hop are amongst the best characterised (Scheufler et al. 2000; Brinker et al. 2002; Odunuga et al. 2003; Odunuga et al. 2004; Onuoha et al. 2008). The TPR motif is a protein-protein interaction module that is found in a range of proteins, which are involved in diverse cellular processes, from transcription to protein degradation (Allan and Ratajczak 2011). The structure of the TPR domain consists of modules of anti-parallel α-helices arranged in tandem creating an amphipathic groove which is the main site of protein-protein interactions (Allan and Ratajczak 2011) (Fig. 3.1). In co-chaperones, TPR domains mediate the interaction with Hsp70 or Hsp90 by binding to the conserved C-terminal EEVD motif of the cytosolic isoforms of the chaperones. The TPR motif is not unique to Hop and other TPR-containing co-chaperones of Hsp70 and Hsp90 include, amongst others, CHIP, HIP, protein phosphatase 5 (PP5), cyclophilin 40 (Cyp40), FK506-binding protein (FKBP)51 and FKBP52 (Chen et al. 1998; Pratt and Toft 2003; Allan and Ratajczak 2011; Assimon et al. 2015).

Mutational studies in both yeast and murine systems have demonstrated that the TPR domains of Hop display different affinity for the Hsp70 and Hsp90 chaperones (Odunuga et al. 2003; Song and Masison 2005). Mutations in TPR1 but not TPR2AB impair Hsp70 binding, while the converse is true for Hsp90 binding. The ability of Hop to discriminate between Hsp70 and Hsp90 EEVD motifs is mediated by specific TPR residues which interact with residues immediately upstream of the EEVD (PTIEEVD in the case of Hsp70 and MEEVD in the case of Hsp90) (Scheufler et al. 2000; Odunuga et al. 2003; Carrigan et al. 2004). Hop is therefore differentiated from other TPR-containing co-chaperones in that its TPR domains can discriminate between Hsp70 and Hsp90 (Odunuga et al. 2003; Carrigan et al. 2004). Conserved residues in the TPR domains form a carboxylate clamp with the C-terminal aspartate of the EEVD motif in the chaperones. Adjacent residues in TPR1 and TPR2A promote high affinity binding to either the PTIEEVD peptide of

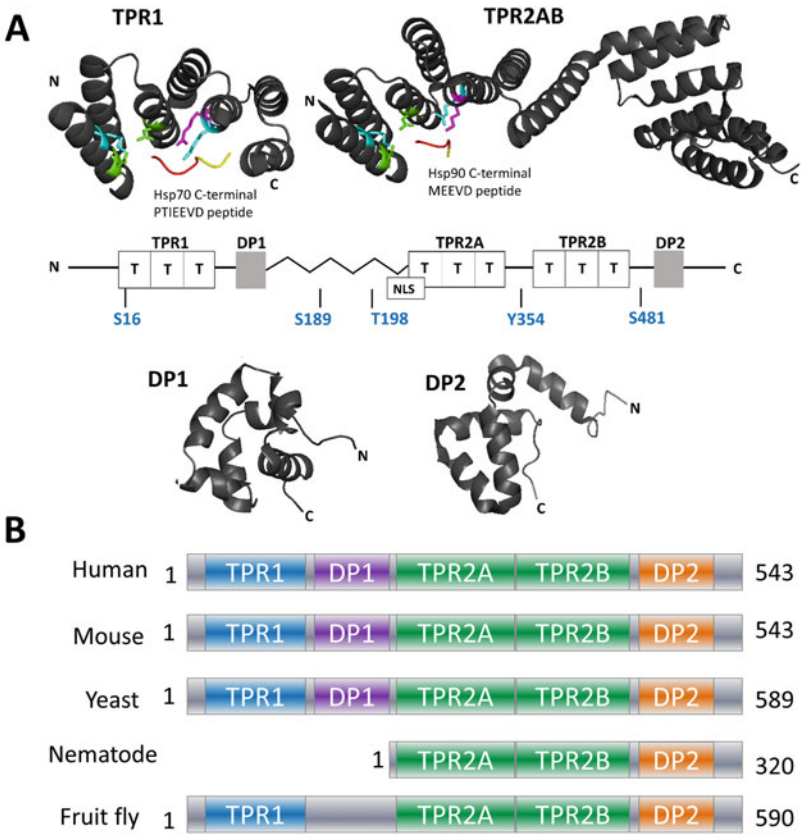

Fig. 3.1 Structural domains and architecture of Hop proteins. (**a**) Three-dimensional cartoon structures illustrate the interaction of the TPR1 and TPR2A domains of HOP with the C-terminal PTIEEVD (Hsp70) and MEEVD (Hsp90) motifs, respectively. The EEVD motif (red) and specificity residues (yellow) of each chaperone are shown as sticks. The two TPR-DP modules are connected by a long flexible linker. Conserved residues in the TPR1 and TPR2A domains of Hop facilitate binding to these chaperone motifs by forming a carboxylate clamp. Conserved residues involved in this interaction are represented as sticks and include lysine (cyan), asparagine (green) and arginine (magenta). Carboxylate clamp residue numbers for TPR1 and TPR2A are K8, N12, N43, K73, R77 and K229, N233, N264, K301 and R305, respectively. Phosphorylation sites of human Hop are in blue font. Images were generated using PyMOL (DeLano Scientific) with the PDB structure codes as follows: TPR1 (1ELW), TPR2AB (3UQ3), DP1 (2LLV) and DP2 (2LLW). (**b**) Comparison of Hop domain structure across model organisms. TPR1: tetratricopeptide repeat domain 1; DP1: aspartate-proline motif domain 1; TPR2AB: tetratricopeptide repeat domains 2A and B; DP2: aspartate-proline motif domain 2. The N-terminus is indicated by number 1, while the numbers at the C-terminus give the total number of amino acids in the proteins

Hsp70 or the MEEVD peptide of Hsp90, respectively (Scheufler et al. 2000; Brinker et al. 2002; Odunuga et al. 2003).

Hop binding to Hsp70 and Hsp90 is not restricted only to their respective C-terminal EEVD motifs. Hop inhibits the ATPase activity of Hsp90 by preventing

N-terminal dimerisation, by a mechanism that depends on the presence of TPR2A and TPR2B but does not require the MEEVD of Hsp90 (Richter et al. 2003; Schmid et al. 2012). Hop appears to interact with N-terminal regions of Hsp90, with residues in TPR2A (VISK, residues 334–337) and TPR2B (EIDQLYYKASQQR, residues 505–517) coming within 13 angstroms of residue 57 in the NBD during binding (Lee et al. 2012). This observation at first appears unlikely given that TPR2A is simultaneously involved in binding of the C-terminal EEVD motif of Hsp90. However, it is explained by the fact that the rate of Hop-Hsp90 binding is dependent on the length of the linker region between the C-terminal dimerisation domain of Hsp90 and the MEEVD (Lee et al. 2012; Schmid et al. 2012). This suggests a model in which the C-terminus of Hsp90 has conformational flexibility and can therefore support simultaneous interactions of Hop TPR2 with both the C-terminal and N-terminal domains. More recent evidence proposes a model for the Hsp70-Hop-Hsp90 cycle in which Hsp90 regulates the structural dynamics of Hop and coordinates its interactions with Hsp70, thus synchronising client transfer within the ternary complex (Rohl et al. 2015b). The TPR1-DP1 module of Hop is separated from the rigid TPR2A-TPR2B-DP2 module by an extended flexible linker (Rohl et al. 2015b). The C-terminal EEVD of Hsp90 interacts with Hop TPR2A, while the client-binding middle domain of Hsp90 makes contacts with Hop TPR2B (Lee et al. 2012; Schmid et al. 2012; Rohl et al. 2015b). Hsp70 demonstrates higher affinity for Hop TPR2B in the absence of Hsp90 but binds preferentially to Hop TPR1 when Hsp90 is present. Evidence suggests that Hsp90 binding induces structural rearrangements of Hop into a more open conformation. This makes TPR1 more accessible, and paired with the positioning of Hsp90 across TPR2A-TPR2B, it promotes preferential interaction of Hsp70 with TPR1 (Rohl et al. 2015b). The flexible linker of Hop brings the two Hsp70 binding sites on the separate TPR-DP modules into close proximity (Rohl et al. 2015b) and in this manner imparts a regulatory role reminiscent of the Hsp90 linker. This suggests a potential mechanism for client protein transfer in which the client-Hsp70 complex binds TPR1 and by structural rearrangements of the linker is positioned near to TPR2B, allowing client transfer to Hsp90 (Rohl et al. 2015b; Lackie et al. 2017; Reidy 2019; Lott et al. 2020).

In mammals, discrimination between TPR-containing co-chaperones by Hsp70 or Hsp90 depends on relative affinities and is regulated by phosphorylation (Muller et al. 2013). Phosphorylation of serine and threonine residues located close to the C-terminal EEVD motifs of Hsp70 and Hsp90 promotes association with Hop over CHIP. Therefore, the C-terminal phosphorylation of Hsp70 or Hsp90 controls the balance between Hop-based pathways (of finely controlled protein folding and degradation; Walsh et al. 2011; Bhattacharya et al. 2020) and CHIP-based pathways (of targeted protein degradation; Edkins 2016; Chap. 12). Phosphorylation of mammalian and yeast Hop has been reported at five and six different locations, respectively. The phosphorylation sites are in or adjacent to the Hsp70 binding regions of Hop (TPR1 and TPR2B) or in the flexible linker between the two TPR-DP modules (Fig. 3.1). Phosphorylation of human Hop at two of these sites (S189, T198) regulates shuttling of Hop between the nucleus and cytoplasm. Phosphorylation of

T198 disrupts the Hop/Hsp90 interaction which may assist in coordinating nuclear-cytoplasmic translocation (Daniel et al. 2008). Phosphorylation at the remaining sites (S16, Y354 and S481) reduces affinity of Hop for Hsp70 and impacts client activation (Rohl et al. 2015a). Furthermore, phosphorylation of the conserved tyrosine Y354, positioned between the rigid TPR2A and TPR2B domains, appears to be restricted to human Hop and is the only phosphorylation event that results in structural rearrangements of Hop, destabilising TPR2A-TPR2B and reducing interaction with Hsp70 (Rohl et al. 2015a).

The DP domains (also known as STI domains) are rich in aspartic acid and proline residues and adopt alpha helical structures (Fig. 3.1a). The DP domains are flexible structures of five to six alpha helices arranged in a hand-like structure incorporating a hydrophobic groove and often occur in pairs (Fry et al. 2021). There is sequence similarity between the DP2 domain of Hop and a C-terminal DP domain in HIP, although the two domains are not functionally equivalent (Nelson et al. 2003). The role of these DP motifs is less clear (Song and Masison 2005; Allan and Ratajczak 2011), although DP2 mutants showed reduced ability to bind HSP70 (Carrigan et al. 2004) and the DP2 segment is required for client activation in vivo (Carrigan et al. 2005; Flom et al. 2006; Schmid et al. 2012). The TPR1-DP1 module of Hop is directly involved in translocation of the client protein within the Hsp70/Hsp90 complex (Schmid et al. 2012), while the TPR2A-TPR2B-DP2 fragment is sufficient to support client activation in yeast (Schmid et al. 2012; Rohl et al. 2015b). However, the substitution of DP2 with DP1 in full-length Hop did not support client activation, despite structural similarity (Schmid et al. 2012). DP1 has also been demonstrated to bind the disordered N-terminal region of the prion protein with high affinity and specificity, an interaction that is also mediated by the TPR1 and TPR2A domains of Hop and does not require Hsp70 or Hsp90 (Maciejewski et al. 2016).

The overall structure of Hop as described above is conserved in the human, mouse and yeast proteins (Fig. 3.1b). The flexible linker joining the two TPR-DP modules appears to be necessary to promote a fully functional Hsp70-Hop-Hsp90 complex in TPR1-DP1-containing Hop orthologues. Deletion of the linker reduced ternary complex formation of Hsp70-Hop-Hsp90 in vitro and reduced client activation in yeast (Rohl et al. 2015b). Interestingly, not all Hop orthologues share this structure (Fig. 3.1). For example, Hop in *Drosophila* lacks the DP1 domain, while Hop/Sti1 in *Caenorhabditis elegans* lacks the TPR1 domain and the short linker region containing the DP1 domain that precedes the TPR2A domain. Nevertheless, Hop in *C. elegans* is able to bind both Hsp70 and Hsp90 via the TPR2AB domain, although unlike most organisms, the TPR domains of Hop in *C. elegans* do not discriminate between Hsp70 and Hsp90 (Gaiser et al. 2009). Hop in *C. elegans* is also subject to inhibitory phosphorylation at predicted sites within or adjacent to TPR2B and a unique site in DP2 (Zheng et al. 2020). This suggests that the transfer of client proteins between Hsp70 and Hsp90 chaperone systems and how they are regulated in these organisms may be different. Due to these differences, the study of Hop, especially using genetic approaches, has been limited to metazoans that are amenable to genetic manipulation.

Functions of Hop

The roles of Hop as a co-chaperone for the Hsp70/Hsp90 complex and as a ligand or receptor for the prion protein, PrP^C, are the best described. However, there is a growing body of literature that reports the involvement of Hop in cellular activities that appear independent of either chaperones or PrP^C (Table 3.1). It should be noted that many of these studies do not directly demonstrate that Hsp70 and Hsp90 are not involved, but rather fail to provide any evidence that they are involved. Therefore, it is possible that Hsp70/Hsp90, or indeed PrP^C, may fulfil yet undefined roles in these seemingly alternative functions of Hop. In vitro assays have demonstrated that Hop is able to bind a number of proteins via its TPR1, TPR2A or TPR2B domains, the former two domains containing the high affinity binding sites that are traditionally required for interacting with Hsp70 or Hsp90. Hop directly interacted with the cytoskeletal proteins tubulin (via TPR2B and possibly TPR1 domain) (Li et al. 2012b) and actin (via TPR2AB) (Beckley et al. 2020) as well as Rnd1, a small GTPase also involved in cytoskeletal dynamics (de Souza et al. 2014). The associated co-immunoprecipitation assays in these studies did not, however, rule out the possible involvement of Hsp70 and Hsp90 in these complexes. Hop was also found to interact directly with emerin, a nuclear protein, contributing to the structural dynamics of the nucleus via a mechanism that appears to involve Hsp70 but not Hsp90. This interaction is mediated by the TPR2A and TPR2B domains of Hop (Kituyi and Edkins 2018). While there is sufficient evidence of these interactions in vitro, the physiological significance of such interactions often remains to be validated in vivo. To clarify the network of proteins that interact with Hop independently of Hsp70 or Hsp90, wild-type Hop or a Hop double mutant that was unable to bind Hsp70 or Hsp90 was exogenously expressed in a HEK293T Hop knockout cell line model, and the Hop interactomes were compared (Bhattacharya et al. 2020). Immunoprecipitation and mass spectrometry analysis revealed that approximately 41.1% of differentially expressed proteins were only detected in cells expressing wild-type Hop, while 57.3% and 1.6% of proteins were enriched in cells expressing the wild-type or double mutant Hop, respectively (Bhattacharya et al. 2020). These data suggest that the vast majority of Hop interactions are mediated predominantly via Hsp70 and Hsp90. However, it should be considered that the point mutations of the two carboxylate clamp residues (K8A/K229A) in the mutant Hop may also impact binding of proteins other than Hsp70 and Hsp90. The primary binding site for PrP^C is directly adjacent the mutated K229 site, spanning one side of the TPR2A domain of Hop that forms part of the hydrophobic groove accommodating Hsp90 (Maciejewski et al. 2016). The potential DAYKKK actin-binding motif (residues 234–239) also resides within this region (Beckley et al. 2020). Single point mutations of carboxylate clamp residues have been shown to affect the global conformation of Hop (Carrigan et al. 2004; Carrigan et al. 2006) and the K8A and K229A mutations alone were found not only to impair binding of Hsp70 and Hsp90 as expected, respectively, but marginally reduced binding of the other chaperone to its respective site as well (Bhattacharya et al. 2020).

Table 3.1 Summary of the roles for Hop in human cellular function and disease

Condition	Hop status[#]	Model system	Cellular function*	Proposed biological mechanism*	References
Ageing	KO	*C. elegans* model	Stress response	Impairment of stress tolerance and fertility	Song et al. (2009)
Adenomyosis/endometriosis	UP (protein; serum)	Clinical adenomyosis/endometriosis versus matched healthy samples In vitro endometrial cancer cell line	Cell migration	Transcriptional regulation of MMP9 expression by Hop	Wang et al. (2018)
Angiogenesis	KD	HUVEC endothelial cell lines	Cell migration	Cytoskeletal dynamics (tubulin binding)	Li et al. (2012b)
Autism spectrum disorder	OE KD	Mouse models	OE—no major phenotype KD—attention deficits and hyperactivity	ND (possibly through regulation of Hsp70/Hsp90 or PrP^C levels)	Beraldo et al. (2015)
Stress resilience	KD	HEK293T cell line	Cell survival after stress	Regulation of HSF1 levels and activity	Chakraborty and Edkins (2020)
Bladder cancer	UP (protein, nucleus)	Clinical bladder cancer tissue samples	Cisplatin resistance	ND	Krafft et al. (2020)
Breast cancer	KD	Hs578T breast cancer lines	Cell migration	Cytoskeletal dynamics (actin binding)	Willmer et al. (2013)
	I	Isolated proteins and in vitro breast cancer cell lines	Cell growth	Disruption of interaction between Hop and Hsp90	Pimienta et al. (2011)
	UP (protein) KD	Clinical breast cancer tissues versus matched non-tumour samples In vitro breast cell lines	Proliferation, migration and reduced apoptosis	Activation of cell signalling pathways (JAK2/STAT3) (abrogated by KD)	Wu et al. (2018) Lin et al. (2021a)
Cervical cancer	KD	In vitro cervical cancer cell lines	Glycolysis and cell survival	Activation of cell signalling pathways – Wnt/β-catenin	Li et al. (2020)

(continued)

Table 3.1 (continued)

Condition	Hop status[#]	Model system	Cellular function*	Proposed biological mechanism*	References
				pathway via PKM2 and LDHA expression (abrogated by KD)	
Cholangiocellular carcinoma	UP (protein)	Clinical tumour versus non-tumour samples	ND	ND	Padden et al. (2014)
Colon cancer	CO	Clinical colon cancer versus non-tumour samples; in vitro cell lines	ND	Increased complex formation with Hsp90 and Hsp70	Kubota et al. (2010)
	UP (protein) S KD	Clinical colon cancer versus non-tumour samples In vitro colon cancer cell lines	Cell proliferation invasion (S), also migration and EMT (KD)	Activation of cell signalling pathways (JAK2/STAT3) (abrogated by KD)	Zhang et al. (2018); Xia et al. (2021)
Gastric cancer	UP (protein; serum) S I (antibody)	Clinical gastric cancer tissues versus matched non-tumour samples In vitro GC cell lines	Cell proliferation and decreased apoptosis, chemoresistance	Activation of cell signalling pathways (autocrine PLCγ1-ERK1/2) (abrogated by neutralising antibody)	Zhai et al. (2018)
	UP (protein) KD	Clinical gastric cancer tissues versus matched non-tumour samples In vitro GC cell lines; mouse model	Cell migration and invasion	Activation of cell signalling pathways (Wnt/β-catenin, MMP) (abrogated by KD)	Huang et al. (2018)
Glioblastoma	UP S	Glioma cell lines	Cell proliferation	Activation of cell signalling pathways (MAPK), potentially PrP^C-dependent	Erlich et al. (2007)
	S	Microglial and glioblastoma cell lines	Cell proliferation and migration	Activation of MMP (independent of PrP^C)	Fonseca et al. (2012)

	UP (protein) S I (peptide $Hop_{230–245}$) MUT ($\Delta Hop_{230–245}$)	Clinical glioblastoma tumour versus matched non-tumour samples In vitro glioblastoma cell lines; mouse model	Cell proliferation and survival; cognitive function	Activation of cell signalling pathways (PrP^C-PI3K-ERK1/2) (abrogated by disrupting Hop-PrP^C interaction)	Lopes et al. (2015)
	UP (protein) KD	Clinical glioblastoma tumour versus matched non-tumour samples In vitro glioblastoma cell lines; mouse model	Proliferation, invasion and reduced apoptosis	Activation of cell signalling pathways (TRAP1-AKT, MMP) (abrogated by KD)	Yin et al. (2019)
	S (recomb.); I (peptide $Hop_{230–245}$) KD	Glioblastoma stem-like cells	Proliferation and self-renewal; tumour growth	PrP^C dependent—activation of ERK1/2 signalling pathway	Iglesia et al. (2017)
Hepatocellular carcinoma	UP (protein)	Hepatocellular carcinoma clinical tumour versus non-tumour samples	ND	ND	Sun et al. (2007); Xu et al. (2018)
	UP (serum)		Microvascular invasion		Ma et al. (2020)
	UP (protein) KD/OE	Hepatocellular carcinoma clinical tumour versus matched non-tumour samples In vitro HCC cell lines	Cell growth, migration and colony formation	Activation of cell signalling pathways (β-catenin/TCF) (abrogated by KD)	Luo et al. (2018)
	UP (protein; serum) S I (antibody) KD	Hepatocellular carcinoma clinical tumour versus matched non-tumour samples In vitro HCC cell lines; mouse model	Cell growth and decreased apoptosis	Activation of cell signalling pathways (autocrine PI3K/AKT) (abrogated by KD/I)	Chen et al. (2017)

(continued)

Table 3.1 (continued)

Condition	Hop status[#]	Model system	Cellular function*	Proposed biological mechanism*	References
	UP (protein; serum) KD/I	Hepatocellular carcinoma clinical tumour versus matched non-tumour samples In vitro HCC cell lines	Metastasis; EMT (upon heat exposure)	MMP activation; shuttling of Snail1 into nucleus mediated by Hsp90/Hop complex (abrogated by Hop KD or inhibition of complex formation)	Su et al. (2018)
Leukaemia	UP	Bohemine-resistant versus Bohemine-sensitive lymphoblastic leukaemia cell lines	Drug resistance	ND	Skalnikova et al. (2011)
Lung cancer	UP (protein) KD	Clinical lung adenocarcinoma cancer tissues versus matched non-tumour samples In vitro lung cancer cell lines; mouse model	Cell proliferation, adhesion, migration and reduced apoptosis	Activation of cell signalling pathways (JAK2/STAT3) (abrogated by KD)	Guo et al. (2019)
Melanoma	UP (protein) KD/OE	Melanomas versus adjacent non-tumour tissues In vitro melanoma cancer cell lines; mouse model	Cell proliferation, migration, invasion and reduced apoptosis; tumour metastasis	Activation of cell signalling pathways (JAK2/STAT3) (abrogated by KD)	Sun et al. (2019b)
Oesophageal squamous cell carcinoma	UP (serum)	Clinical ESCC versus non-tumour samples	ND	ND	Xu et al. (2017)
Osteosarcoma	KD	CD133-positive osteosarcoma CSCs	Cell migration and invasion	MMP, Akt/ERK1/2	Wang et al. (2020a)
Ovarian/endometrial cancer	UP (protein; serum); S	Clinical ovarian cancer versus non-tumour samples In vitro ovarian cancer cell lines	Cell proliferation	Activation of cell signalling pathways (ERK)	Kim et al. (2010); Wang et al. (2010); Huang et al. (2016)

	UP (protein) S KD	Clinical ovarian cancer tissues versus matched non-tumour samples In vitro ovarian cancer cell lines, mouse model	Cell proliferation and invasion/migration; tumour growth	Activation of cell signalling pathways (ALK-SMAD-ID3) (abrogated by KD)	Tsai et al. (2012); Chao et al. (2013a); Cho et al. (2014)
	KD I (peptide 520)	In vitro ovarian and endometrial cancer cell lines Mouse model	Tumour growth	Activation of cell signalling pathways (JAK2/STAT3) (abrogated by peptide treatment) Peptide 520 derived from Hop DP2 domain disrupts Hop-Hsp90 interaction	Tsai et al. (2016)
Pancreatic cancer	UP (protein) KD	Clinical pancreatic cancer samples versus normal controls; invasive versus non-invasive clones of pancreatic cell line MiaPaCa-2 In vitro pancreatic cancer cell lines	Cell invasion/migration	Activation of cell signalling pathways (FAK/AKT/MMP) (abrogated by KD)	Walsh et al. (2009); Walsh et al. (2011); Jing et al. (2019)
	UP (protein) KD	Pancreatic clinical tumour versus non-tumour samples In vitro pancreatic cancer cell lines, mouse model	Cell proliferation	Activation of cell signalling pathways (c-Myc/hTERT/cyclin D1) via interaction with GOLPH3 (abrogated by KD)	Wang et al. (2020b)
Papillary thyroid carcinoma	UP (protein)	Clinical tumour versus non-tumour samples	ND	ND	Yuan et al. (2014)
Renal cell carcinoma	UP KD I (antibody) S	Clinical tissue samples – primary RCC versus bone metastatic tumours Bone-seeking OS-RC-2-BM5 and ACHN-BM5 cell	Cell proliferation and migration/invasion	Activation of cell signalling pathways (autocrine ALK2-SMAD1/5 pathway)	Wang et al. (2017)
			Differentiation		

(continued)

Table 3.1 (continued)

Condition	Hop status[#]	Model system	Cellular function*	Proposed biological mechanism*	References
		lines and parental cell lines, OS-RC-2 and ACHN; mouse monocyte-macrophage RAW264.7 cell line		Activation of cell signalling pathways (paracrine PrP^C-ERK1/2 pathway)	
Amyotrophic lateral sclerosis	KO/OE	Yeast model Neuronal SN56 and neuroblastoma N2a cell lines	Increased TDP-43 aggregation and toxicity rescued by moderate OE	ND	Lin et al. (2021b)
Alzheimer's disease (AD)	UP (compensatory?) KD S (rHop)	Human cortical tissue from AD sufferers/mouse models versus matched controls In vitro HEK293T cells, hippocampal neurons and slices	Increased AβO toxicity rescued by rHop treatment or Hop OE	AβO binding to PrP^C (abrogated by rHop treatment) α7nAChR-mediated signalling pathways	Ostapchenko et al. (2013); Maciejewski et al. (2016); Lackie et al. (2020a)
	KD/OE	*C. elegans*	Increased AβO toxicity	ND	Brehme et al. (2014); Lackie et al. (2020a)
	KD	*D. melanogaster*	Increased Tau toxicity	ND	Ambegaokar and Jackson (2011)
Cystic fibrosis	KD	CFBE41o − cells expressing ΔF508 CFTR cell line model	Mutant protein dynamics	Prevention of mutant CFTR variant (ΔF508) maturation	Marozkina et al. (2010)
Development	S?	Neural stem cell culture	Self-renewal and proliferation	Inhibition reduced neurosphere formation; self-renewal and proliferation	Lopes and Santos (2012)
				Autocrine Hop-PrP^C signalling	Lee et al. (2019)

	KD	Murine ESC culture	Pluripotency	Blocked embryoid body formation	Longshaw et al. (2009)
	KD (in ovarian germline nurse cells)	*D. melanogaster* model	Sterility	Inefficient piRNA biogenesis leading to transposon activation	Karam et al. (2017)
	KO	Mouse model	Embryonic development	Embryonic lethal (E10.5)	Beraldo et al. (2013)
	UP, S, I	Developing versus mature retinal tissues (rat)	Cell proliferation and death	Retinal development independent of PrPc	Arruda-Carvalho et al. (2007)
Huntington's disease	KD OE	Yeast model	Increased Htt toxicity (rescued by OE)	Hsp70 dependent	Wolfe et al. (2013)
	KD	*C. elegans*	Increased polyglutamine toxicity	ND	Brehme et al. (2014)
	KD	*D. melanogaster*	Reduced Htt toxicity	Possibly involves upregulation of Hsp70	Xu et al. (2019)
	KO	HEK293T and HCT116 cell lines	Reduced polyglutamine aggregation (HEK293T only)	Possibly Hsp90 dependent	Bhattacharya et al. (2020)
Memory	I (antibodies); UP	Intra-hippocampal infusion	Reduced performance	Short-term and long-term memory inhibited	Coitinho et al. (2007)
	OE (peptide 230–245)	Peptide 230–245 from Hop (including PrPC binding site)	Enhanced performance	ND (but involves PrP^C binding site)	
Neuronal functions	S	Mouse model and in vitro cell lines	Neuroprotection, neurite formation	PrP^C-dependent activation of signalling pathways, endocytosis	Lopes et al. (2005); Lima et al. (2007); Caetano et al. (2008); Roffe et al. (2010); Beraldo et al. (2018)
	KO/KD	MEF culture and hypomorphic mouse model	Neuroprotection	Possibly reliant on formation of Hsp70/Hsp90/Hop complex	Lackie et al. (2020b)

(continued)

Table 3.1 (continued)

Condition	Hop status[#]	Model system	Cellular function*	Proposed biological mechanism*	References
	OE	COS-7 and PC-12 cell lines	Neuritogenesis and cytoskeletal dynamics	Interaction with the GTPase, Rnd1	de Souza et al. (2014)
	S	Murine primary culture	Cell migration of neuroblasts	Extracellular interaction of Hsp70/Hop/Hsp90	Miyakoshi et al. (2017)

[#]KD: knockdown by RNA interference; S: secreted or extracellular Hop; KO: gene knockout: OE: Hop overexpressed (recombinant form); UP: Hop upregulated (endogenous form); CO: Hop in complex with Hsp90 and Hsp70; I: pharmacological inhibition; MUT: mutated. *ND: not determined

There is a report that Hop has independent ATPase activity (Yamamoto et al. 2014). Hop bound ATP with a similar affinity to Hsp90 and Hsp70 but hydrolysis of ATP took place at a slower rate than in the two chaperones. The ATPase activity of Hop was associated with the N-terminal regions of the protein, encompassing the TPR1, DP1 and TPR2A domains. While the DP1 domain was essential for ATPase activity, the mutation of a putative Walker B motif in this domain did not abolish the ATPase activity of Hop (Yamamoto et al. 2014). ATP binding by Hop induced a conformational change in the protein. The domains which display ATPase activity are those involved in binding both Hsp70 (TPR1) and Hsp90 (TPR2A), and therefore, it is plausible that the ATP-induced conformational changes may be involved in the transfer of client protein between Hsp70 and Hsp90. However, the consequences of this ATPase activity for the function of Hop have not been explored in any depth beyond this study and remain to be determined. Despite putative evidence of endogenous ATPase activity and the ability of Hop to directly bind and stabilise several proteins, there has been no report to date of Hop itself having independent chaperone activity.

Hop as a Co-chaperone for Hsp70 and Hsp90: A Shift in the Paradigm

Hsp90 substrates include a diverse set of proteins, many of which have been implicated in regulation of apoptosis (Samali and Cotter 1996; Mosser and Morimoto 2004; Lanneau et al. 2007), proliferation (Caplan et al. 2007; Lanneau et al. 2007; Dezwaan and Freeman 2008), autophagy (Agarraberes and Dice 2001; Qing et al. 2006; Joo et al. 2011; Xu et al. 2011) and cell cycle progression (Francis et al. 2006; Reikvam et al. 2009) as well as in tumorigenesis (Kamal et al. 2004; Muller et al. 2004; Whitesell and Lindquist 2005; Chiosis 2006; Neckers et al. 2007; Mahalingam et al. 2009; Trepel et al. 2010; Miyata et al. 2013; Lianos et al. 2015; Calderwood and Gong 2016; Graner 2016; Isaacs 2016; Jarosz 2016; Kumar et al. 2016; Vartholomaiou et al. 2016; Wong and Jay 2016; Calderwood 2018; Zuehlke et al. 2018). In early studies it was found that Hsp90 interacts with the yeast and vertebrate homologues of Hop in lysates of these cells (Chang et al. 1997). Deletion of the gene encoding Hop reduced the in vivo activity of the Hsp90 target proteins, glucocorticoid receptor (GR) and the oncogenic tyrosine kinase, v-Src (Chang et al. 1997). Hop was also shown to stimulate the refolding of luciferase by Hsp70 and a much more dramatic effect was seen when Hsp90 was also included (Johnson et al. 1998). This led to the conclusion that Hop is a general factor in the maturation of Hsp90 target proteins. Subsequently, it has been clearly demonstrated that Hop regulates the molecular chaperone activities of Hsp70 and Hsp90 and thus plays a crucial role in the productive folding of client proteins (Johnson et al. 1998; Kimmins and MacRae 2000; Wegele et al. 2004; Song and Masison 2005; Wegele et al. 2006; Kubota et al. 2010; Lee et al. 2012; Schmid et al. 2012; Rohl et al. 2015a;

Rohl et al. 2015b). Hop connects Hsp90 and Hsp70 in a ternary multichaperone complex, where it facilitates the transfer of client proteins from the early complex (Hsp70-Hsp40) to the intermediate complex (Hsp70-Hsp90) (Chen and Smith 1998; Johnson et al. 1998; Song and Masison 2005; Wegele et al. 2006). These client proteins include a variety of kinases, transcription factors and steroid hormone receptors, many of which are deregulated in cancer (Pratt and Toft 2003; Lee et al. 2004; Song and Masison 2005; Tan et al. 2011; Walsh et al. 2011; Ruckova et al. 2012; Taipale et al. 2012; Willmer et al. 2013). The central role of Hop in these processes is demonstrated by mutations in Hop that impair the client folding pathway (Song and Masison 2005; Reidy et al. 2018). Depletion of Hop levels using RNA interference leads to a dramatic reduction in the levels of obligate Hsp90 client proteins, HER2, Bcr-Abl, c-MET and v-Src (Walsh et al. 2011), while Hop knockout in mammalian cell lines led to selective loss of specific Hsp90 client proteins (Bhattacharya et al. 2020). Δ*sti1* yeast cells remained viable under optimal conditions but showed increased sensitivity to heat shock (Chang et al. 1997) and inhibition of Hsp90 (Song and Masison 2005) and were synthetically lethal when paired with Hsp90 mutants that reduced intracellular levels of Hsp90, or upon deletion of both Hsp90 genes (Chang et al. 1997; Flom et al. 2006). HEK293T cell lines in which Hop was depleted by shRNA showed reduced stress resilience which was linked to reduced levels of the stress-responsive HSF1 transcription factor that controls transcription of *HSP* genes (Chakraborty and Edkins 2020).

Hop has long been considered as an essential scaffold protein of the multichaperone complex (Dittmar et al. 1996; Chen and Smith 1998; Scheufler et al. 2000; Odunuga et al. 2003; Wegele et al. 2006; Alvira et al. 2014). However, in the prokaryotic *Escherichia coli* model, which lacks paralogues of Hsp90 co-chaperones, the *E. coli* Hsp90 (HtpG) and Hsp70 (DnaK) interact directly to facilitate client folding and maturation (Genest et al. 2011; Nakamoto et al. 2014; Genest et al. 2015). Mutational analysis identified a region of the M domain of HtpG to be important in the interaction with DnaK (Genest et al. 2015). Yeast Hsp90 (Hsp82) and Hsp70 (Ssa1) also interact directly in the absence of Hop (Sti1), via a region in the M-domain of Hsp82 that is homologous to that of HtpG (Chang et al. 1997; Flom et al. 2007; Kravats et al. 2018) . The human paralogues of Hsp90 that are restricted to the endoplasmic reticulum (Grp94) and mitochondria (TRAP1) have no known co-chaperones (Marzec et al. 2012; Huck et al. 2017), and both interact directly with their respective Hsp70 paralogue (Sung et al. 2016; Sun et al. 2019a). Direct interaction of human Hsp90β with the constitutive Hsc70 and stress-inducible Hsp70 was confirmed by in vitro pull-down assay of purified protein, while a yeast model demonstrated that Hsc70, but not Hsp70, was able to support the growth of Δ*sti* cells expressing Hsp90β as the sole source of Hsp90 (Reidy et al. 2018).

In the mouse, germline deletion of Hop is embryonic lethal (Beraldo et al. 2013). However, mammalian Hop can be knocked out using CRISPR in a range of human cancer cell lines which maintain viability (Bhattacharya et al. 2020). In human Hop null cell lines, Hop appears to mediate the proteostatic balance between protein folding via chaperone systems and degradation via the proteasome (Bhattacharya et al. 2020). In the absence of Hop, mammalian Hsp70 and Hsp90 interacted directly

in a prokaryote-like binary complex that promoted more efficient protein folding, compensating for reduced proteasome activity in these cells. Heat-inactivated luciferase refolding occurred at a higher rate in Hop-depleted cells and was reduced to wild-type levels upon inhibition of Hsp70 or Hsp90. This observation was supported by recovery experiments incorporating overexpression of wild-type Hop or TPR mutants unable to bind Hsp70 and/or Hsp90. Absence of Hop did not appear to affect steady-state levels of Hsp90 client proteins, nor did it affect client activation as there were no global differences observed in the level of active kinase clients between the wild-type and Hop-depleted HEK293T and HCT116 colon cancer cells. However, challenging the proteostatic balance in these cell lines by overexpressing the Hsp90 clients, glucocorticoid receptor (GR) and v-Src, resulted in the reduced accumulation and activity of these client proteins relative to that of wild-type cells. Overexpression of other Hsp90 client steroid hormone receptors, the oestrogen receptor α (ER) and progesterone receptor (PR), had only a moderate effect on corresponding protein level and activity in the Hop null background relative to wild-type cells. Interestingly, Hop deletion in A549 lung cancer cells reduced the accumulation and activity of endogenous GR, while the activity of exogenous GR and v-Src in *Δsti* yeast cells was reduced despite no difference in protein levels (Chang et al. 1997; Sahasrabudhe et al. 2017). The activity of the steroid hormone receptors ER, PR and mineralocorticoid receptor (MR) did not significantly change between *Δsti* and wild-type yeast cells (Sahasrabudhe et al. 2017). It was demonstrated in yeast that variants of GR and v-Src that were less dependent on Hsp90 for maturation no longer required Hop for their activity (Sahasrabudhe et al. 2017) (Sahasrabudhe et al. 2017) and evidence suggests that Hop might perform a more client-specific role than previously thought (Sahasrabudhe et al. 2017; Bhattacharya et al. 2020). It is possible that the observed effects of Hop depletion on obligate Hsp90 proteins may be dependent on the model system used and the intrinsic proteostatic buffering capabilities of different cell lines or organisms. Acute disruption of the cellular proteostatic balance by Hop knockdown or overexpression may yield different cellular responses as compared to the long-term effects of Hop knockout models, in which proteomic steady-state levels might be restored by alternative mechanisms. Inhibition of Hsp90 in the Hop-null background was synthetically lethal in mammalian cells (Bhattacharya et al. 2020), similar to observations in Δ*sti1* yeast. However, unlike Δ*sti1* yeast that demonstrated increased sensitivity to heat shock (Chang et al. 1997) and HEK293T with acute Hop depletion by shRNA (Chakraborty and Edkins 2020), Hop null mammalian cells were as comparably resilient to stress as wild-type cells. The Hsp70-Hsp90 interaction site was mapped to evolutionarily conserved residues shown to be important for the direct interaction of bacterial Hsp70/Hsp90. These residues were confirmed to be involved in interaction of human Hsp90α with Hsp70 in vitro and Hsc/Hsp70 in vivo, in Hop null cells (Bhattacharya et al. 2020).

Taken together, the emerging evidence supports a conceptual change regarding the indispensable role of Hop within the Hsp70-Hsp90 chaperone complex (Sahasrabudhe et al. 2017; Kravats et al. 2018; Reidy 2019; Bhattacharya et al. 2020). It appears that the role of Hop may be regulatory rather than critical and it is

possible that the binary or ternary complexes of Hsp70/Hsp90 and/or Hop may perform specific roles under certain conditions, depending on the requirements of the cell.

Extracellular Hop Has Cytokine-Like Activity

Chaperones exist in the extracellular environment and play physiological roles such as modulation of the stress response and cell survival (Eustace and Jay 2004; Eustace et al. 2004; Arruda-Carvalho et al. 2007; Lima et al. 2007; Arantes et al. 2009; Santos et al. 2011; Tsai et al. 2012; Beraldo et al. 2013; Hajj et al. 2013; Carvalho da Fonseca et al. 2014; Li et al. 2014; Zhai et al. 2018). Hop is secreted by various cells types, including neuronal stem cells (Santos et al. 2011; Lee et al. 2019), microglia, astrocytes (Lima et al. 2007; Arantes et al. 2009) and cancerous cells such as gliomas (Erlich et al. 2007), ovarian cancer cells (Wang et al. 2010; Tsai et al. 2012), fibrosarcoma cells (Eustace et al. 2004), gastric cancer cells (Zhai et al. 2018) and hepatocellular carcinomas (Chen et al. 2017; Ma et al. 2020). Despite evidence of an extracellular Hsp90 complex, in the extracellular environment Hop appears to act more like a cytokine than a co-chaperone. Secreted Hop activates numerous different signalling pathways (Erlich et al. 2007; Caetano et al. 2008; Arantes et al. 2009; Beraldo et al. 2010; Wang et al. 2010; Tsai et al. 2012; Chao et al. 2013a).

Many, but not all, of the activities of extracellular Hop involve an interaction with normal cellular prion protein PrP^C. Extracellular Hop and PrP^C interact directly with each other via an interaction site that maps to residues 230–245 in Hop (encompassing the start of TPR2A domain) and 113–128 in PrP^C (Zanata et al. 2002). The TPR1 and DP1 domains have also been shown to associate with PrP^C (Maciejewski et al. 2016). The binding interface of TPR2A and PrP^C spans a hydrophobic groove of TPR2A that is involved in binding the Hsp90 C-terminus. Despite a partial overlap in the binding interfaces of PrP^C and Hsp90, both can bind Hop simultaneously. While the functions of the Hop-PrP^C complex appear independent of Hsp90, binding of PrP^C to Hop seems to promote recruitment of Hsp90 to the complex, potentially by exposing hydrophobic patches on PrP^C or by inducing conformational changes of Hop (Maciejewski et al. 2016). The Hop-PrP^C complex is important in a range of cellular processes such as cell growth, survival and differentiation. In particular, the interaction between Hop and PrP^C is linked to processes that involve neuronal development and cognitive function. Interestingly, these roles of Hop appear to be independent of the Hsp70/Hsp90 chaperones.

Hop-induced signalling was able to protect a range of neuronal cell types from apoptosis using mechanisms that were dependent on the presence of wild-type PrP^C (Zanata et al. 2002; Lopes et al. 2005; Arantes et al. 2009). Hop-PrP^C autocrine signalling in multipotent arachnoid-pia stem cells was involved in proliferation and self-renewal of cells (Lee et al. 2019). Hop expression was regulated by FOXC1, a transcription factor involved in many developmental functions (Lee et al. 2019). Studies using cells from PrP^C null mice have demonstrated that the effects of Hop on

neural stem cell renewal and differentiation (Santos et al. 2011; Lopes and Santos 2012), proliferation and survival (Lima et al. 2007), neuritogenesis (Lopes et al. 2005; Lima et al. 2007; Santos et al. 2013) and response to ischemic stress (Beraldo et al. 2013; Beraldo et al. 2018) are all dependent on an interaction with PrP^C. These interactions appear to have an important impact on cognitive functions, as disruption of the Hop-PrP^C interaction led to defects in memory and learning in rats (Coitinho et al. 2007), while Hop depletion led to hyperactivity and attention deficits in mice (Beraldo et al. 2015). Extracellular Hop also acts in a PrP^C-independent manner in certain cases. The control of retinal proliferation by extracellular Hop, for example, was found to be independent of PrP^C (Arruda-Carvalho et al. 2007), as are some of the functions of extracellular Hop in cancer (Fonseca et al. 2012; Tsai et al. 2012; Carvalho da Fonseca et al. 2014).

The effects of extracellular Hop appear to be mediated primarily by activation of downstream signalling pathways. Hop interacting with PrP^C or other receptors has been shown to induce activation of a range of signalling pathways, including SMAD (Tsai et al. 2012; Wang et al. 2017), ERK (Americo et al. 2007; Caetano et al. 2008; Wang et al. 2017), PKA (Chiarini et al. 2002; Zanata et al. 2002), JAK/STAT (Guo et al. 2019; Sun et al. 2019b; Xia et al. 2021) and PI3K/Akt (Erlich et al. 2007; Roffe et al. 2010; Chen et al. 2017) pathways. In this way, Hop appears to function like a classical cytokine, binding to a transmembrane receptor to induce cellular signalling cascades. A similar effect has been noted with extracellular chaperones like Hsp90, which are able to induce signalling from cellular receptors like LRP-1 (Tsen et al. 2013; Calderwood 2018). The studies on extracellular Hop are particularly interesting since there is limited information on the mechanism of export or the isoform specificity of extracellular Hop (Edkins et al. 2018). If indeed extracellular Hop is derived from intracellular Hop, then it begs the question of the mechanism and conditions under which Hop is exported from the cell. It is tempting to speculate that there may be alternative isoforms of Hop: one isoform that functions as the intracellular co-chaperone of Hsp70/Hsp90, the other, as an extracellular cytokine or receptor that associates with PrP^C.

Hop in Human Cellular Function and Disease

Cancer Cell Biology

Transformed cells rely on molecular chaperones together with co-chaperones to stabilise their mutant, unstable proteins (Soti et al. 1998; Tytell and Hooper 2001; Daugaard et al. 2005; Chiosis 2006; Boschelli et al. 2010). Numerous studies have demonstrated that Hop may regulate multiple biological processes in a range of cancer cell types and/or function as a biomarker (Table 3.1). In most cases, Hop levels are increased in cancer cells compared to normal cell equivalents, as well as being upregulated in metastatic, drug resistant or aggressive tumours (Walsh et al. 2009; Wang et al. 2010; Sims et al. 2011; Chao et al. 2013a; Chao et al. 2013b; Cho

et al. 2014; Van Simaeys et al. 2014; Yuan et al. 2014; Bertram et al. 2016; Chen et al. 2017; Wang et al. 2017; Xu et al. 2017). This was true of breast (Sims et al. 2011; Lin et al. 2021a), colon (Kubota et al. 2010; Xia et al. 2021), pancreatic (Walsh et al. 2009; Jing et al. 2019), thyroid (Yuan et al. 2014), oesophageal (Xu et al. 2017), ovarian (Wang et al. 2010; Tsai et al. 2012; Tsai et al. 2016), lung (Guo et al. 2019), hepatocellular carcinomas (Sun et al. 2007; Bertram et al. 2016; Chen et al. 2017; Luo et al. 2018; Ma et al. 2020), melanoma (Sun et al. 2019b) and glioblastomas (Lopes et al. 2015; Wang et al. 2017). Concomitant with the increased expression levels, Hop appeared to function to promote or support malignancy in tumours, while depletion of Hop levels in cancer cell lines was sufficient to ameliorate some of these cancer-promoting activities (Walsh et al. 2011; Li et al. 2012b; Willmer et al. 2013). Upregulated Hop expression in cancer may be regulated by oncogenic driver proteins. Inactivation of p53 combined with activation of RAS, a common event in cancer, was shown to upregulate Hop expression which suggests that Hop may form part of the cancer gene signature (Mattison et al. 2017).

The changes in Hop levels are associated with a major role for intracellular Hop in cellular functions relating to metastatic processes, such as cell migration and invasion. Depletion of intracellular Hop levels in endothelial (Bull et al. 2010; Li et al. 2012b) and breast cancer cells (Willmer et al. 2013) reduced pseudopodia formation and inhibited cell migration and polarisation. These effects were predicted to be via regulation of different cell processes, including a direct interaction with cytoskeletal proteins like actin and tubulin (Beckley et al. 2020). Hop also regulates the activity of specific proteins, such as matrix metalloproteinase 2 (MMP2), which are involved in the degradation of the extracellular matrix during cancer cell invasion (Eustace et al. 2004; Walsh et al. 2011). Hop was able to bind the MMP9 promoter, directly or as part of a complex, in endometrial cancer cells and stimulate MMP9 expression (Wang et al. 2018). Hop was also required in the nucleus for glycogen synthase kinase-3 beta (GSK3β)-mediated phosphorylation of lysine-specific demethylase 1 (LSD1), an integral epigenetic regulator implicated in tumour aggression (Tsai et al. 2018). The TPR1 and TPR2B domains of Hop interact with the AOL domain of LSD1, recruiting the protein to a complex that incorporates GSK3β (via Hop TPR2A and TPR2B), and Hsp90. GSK3β-mediated phosphorylation of LSD1 increased cell proliferation of ovarian and endometrial cells (Tsai et al. 2018), while cytosolic interaction of Hop with Golgi phosphoprotein 3 (GOLPH3) promoted cell proliferation in pancreatic ductal adenocarcinoma cell lines as a result of increased telomerase activity (Wang et al. 2020b). Yeast Sti1 participated in cytosolic transport of precursor mitochondrial proteins, potentially as part of an import complex, and was important for mitochondrial integrity (Hoseini et al. 2016). Nuclear structure dynamics relies on the stabilisation of the nuclear protein emerin by Hop that appears to be independent of Hsp90 (Kituyi and Edkins 2018), while Hop in complex with Hsp90 and Piwi supports genome integrity by involvement in piRNA biogenesis and transposon silencing (Gangaraju et al. 2011; Karam et al. 2017). Additionally, Hop is involved in cell-cycle-dependent transcriptional activation of histone H2B (Zheng et al. 2003) and aids in the removal of promoter-bound nucleosomes to allow

gene transcription (Floer et al. 2008), functions that appear to involve Hsp70 and/or Hsp90. Interestingly, the literature to date suggests that intracellular Hop does not seem to have a major role in cell proliferation, leading to the suggestion that intracellular Hop may be a selective target for inhibition of processes associated with metastasis (e.g. migration, invasion). These data are in contrast with the functions proposed for extracellular Hop. Interestingly, most studies on Hop function focus on depletion or knockout of Hop, which provides useful information, but is a rather blunt approach. Consequently, there are few studies focusing on the biological changes associated with finely tuning the levels of Hop above (overexpression) and below (underexpression) normal physiological/cellular levels, an approach that could potentially provide more meaningful information on the cellular role of Hop.

Extracellular Hop in cancer does not appear to induce a major migratory phenotype, but instead leads to an increase in cancer cell proliferation. Hop is secreted into the extracellular environment by a range of cell types, including ovarian carcinomas (Wang et al. 2010; Tsai et al. 2012) and glioblastomas (Erlich et al. 2007; Lopes et al. 2015; Wang et al. 2017). The ability of extracellular Hop to induce cell proliferation appears to be mediated by the ability of the co-chaperone to activate intracellular signalling pathways. In both glioma and ovarian cancer cells, Hop activated mitogenic pathways, including MAPK (Erlich et al. 2007), a major signal transduction pathway required for cell growth. The difference in biological response to intracellular versus extracellular Hop may, in part, be due to the involvement of PrP^C as a receptor, for which extracellular Hop is a major ligand. The proliferative effect of Hop in glioma occurs, at least in part via a PrP^C-dependent mechanism (Erlich et al. 2007; Lopes et al. 2015), which also supports self-renewal of glioblastoma stem-like cells and differentiation of osteoclasts (Iglesia et al. 2017; Wang et al. 2017), although PrP^C-independent growth stimulation has been observed in different cell lines (Fonseca et al. 2012; Carvalho da Fonseca et al. 2014; Lopes et al. 2015; Guo et al. 2019).

Many of the studies of the role of Hop in cancer do not include a direct analysis of the contributions to the phenotype of the chaperones Hsp90 and Hsp70. However, Hop has been shown to be constitutively incorporated into an Hsp90 complex in some cancer cells and many of the proteins affected by Hop inhibition or depletion are in fact client proteins of the Hsp90 complex (Kubota et al. 2010). Therefore, it is likely that many of the activities of Hop in cancer are linked to perturbations in the function of the Hsp70/Hsp90 complex. This conclusion is supported by the observations that compounds that disrupt interactions between Hop and the Hsp90 or Hsp70 chaperone are toxic to cancer cells (Ardi et al. 2011; Horibe et al. 2011; Horibe et al. 2012a; Horibe et al. 2012b, 2014). This apparent toxicity of inhibitors that disrupt the formation of the Hop-Hsp70/Hsp90 chaperone complex appears contradictory to the observation that cancer cell lines in which the Hop gene has been knocked out are still able to maintain viability (Bhattacharya et al. 2020). An explanation for these disparate observations could be attributed to promiscuity of inhibitors that are not sufficiently specific for the Hop-Hsp70/Hsp90 interaction (Edkins 2016) or cell-specific differences. Another consideration is the potentially

confounding effects of genetic compensation (El-Brolosy and Stainier 2017; Salanga and Salanga 2021). This increasingly documented cellular phenomenon describes the upregulation of alternative genes that functionally compensate for a loss-of-function mutation and can account for phenotypic differences observed in acute (knockdown/pharmacological inhibition) versus long-term (knockout) loss-of-function studies. Thus, a Hop null cell line model may have favoured the selection of viable cells that were able to compensate for the loss of Hop. This might also account for the observation that HEK293T Hop knockout cells were phenotypically similar to wild-type cells in terms of stress resilience (Bhattacharya et al. 2020), while acute Hop depletion by shRNA in HEK293T cells was associated with reduced stress resilience (Chakraborty and Edkins 2020).

Hop as a Therapeutic Target for Cancer

The link between Hop and oncogenic activity has led to the proposal that Hop itself may be a viable drug target for cancer (Edkins 2016; Darby et al. 2020). Indeed, studies in which Hop levels were reduced using RNA interference in cancer cells demonstrated that depletion of Hop could reverse oncogenic properties (summarised and referenced in Table 3.1). Despite this, there are currently no small molecule inhibitors that directly inhibit Hop. Currently, the most common strategy used for anti-cancer compounds is to inhibit the protein-protein interaction of Hsp90 and Hop, as an alternative to inhibiting Hsp90 (Ardi et al. 2011; Pimienta et al. 2011; Darby et al. 2020; Veale et al. 2020; Veale et al. 2021). Hsp90 is considered a promising drug target for cancer treatment because Hsp90 is the main chaperone required for the stabilisation of multiple oncogenic kinases (Reikvam et al. 2009). Overexpression of Hsp90 in cancer cells stabilises mutant oncoproteins, promoting cancer cell survival. Given that Hop is required for entry of these client proteins into the Hsp90 complex, targeting the interaction of Hop and Hsp90 is likely to inactivate client proteins. However, inhibition of Hsp90 (particularly by blocking the N-terminal ATP binding site) has been associated with unwanted compensatory upregulation of Hsp70, which can lead to drug resistance (Pimienta et al. 2011). Therefore, the targeting of protein-protein interactions with co-chaperones rather than ATPase activity has been considered as an alternative strategy for the treatment of cancer (Reikvam et al. 2009; Maciejewski et al. 2013; Darby et al. 2020; Veale et al. 2020; Veale et al. 2021).

Compounds specifically inhibiting the interaction of Hop with the Hsp70/Hsp90 complex have been identified. Introduction of a bioisosteric tetrazole moiety into the native MEEVD peptide resulted in robust inhibition of the interaction between the Hsp90 C-terminal domain and Hop TPR2A (Veale et al. 2020; Veale et al. 2021). A hybrid 12-amino acid peptide comprising a sequence based on the TPR2A region of Hop was designed to competitively inhibit the interaction between Hsp90 and Hop (Horibe et al. 2011). This peptide induced cell death in a range of cancer cell lines in vitro, as well as displaying anti-tumour activity in a pancreatic cancer xenograft model (Horibe et al. 2012a; Horibe et al. 2012b, 2014). The compound also showed

differential toxicity in that it did not affect the viability of normal cells, which might be attributed to the constitutive formation of the Hsp90 complex in cancer cells as opposed to normal cells (Kamal et al. 2003; Kamal et al. 2004; Echeverria et al. 2019). Unlike other inhibitors of the Hsp90 complex, this compound did not alter Hsp70 expression. In a similar approach, peptide LB76 was designed as a truncated, cyclic variation of the TPR peptide used by Horibe and colleagues, also targeting the MEEVD motif of Hsp90 (Horibe et al. 2011; Rahimi et al. 2018; Rahimi and McAlpine 2019). LB76 interfered with binding between Hsp90 and four TPR-containing co-chaperones tested, strongly inhibiting both Hop and Cyp40, as well as FKBP38 and FKBP51 to a lesser extent (Rahimi and McAlpine 2019). A peptide derived from the DP2 domain of Hop (peptide 520) was able to induce cell death by suppression of JAK-STAT signalling in ovarian and endometrial cancer cells lines, mimicking the effect of siRNA-mediated Hop knockdown. The Hop TPR1 domain was required for interaction with JAK2, while Hsp90/STAT3 interacted with Hop via the TPR2A-TPR2B-DP2 domains. Peptide 520 was able to disrupt the interaction between Hop and Hsp90 as well as JAK2 and STAT3 and was effective at reducing tumour growth in vivo (Tsai et al. 2016). It has also been possible to inhibit Hop interaction with Hsp90 via small molecules, like Sansalvamide A analogues (Ardi et al. 2011) and a compound termed C9 (1,6-dimethyl-3-propylpyrimido[5,4-e][1,2,4]triazine-5,7-dione) (Pimienta et al. 2011). The Sansalvamide A analogue bound Hsp90 at a region between the N-terminal and middle domains, inducing allosteric changes that blocked the binding of Hop (and two other TPR-containing proteins) to the Hsp90 MEEVD (Ardi et al. 2011). The compound C9 also blocked the interaction of Hsp90 with Hop in vitro. Six compounds containing a 7-azapteridine ring were similarly able to inhibit the interaction between Hsp90 and Hop (Yi and Regan 2008). Another effective small molecule inhibitor is the pyrimidine derivative, Y-632. This inhibitor does not bind directly to Hsp90 or disrupt its ATPase activity, but rather stimulates thiol oxidation of Hsp90. This interferes with Hsp90-Hop binding and client proteins are degraded via the ubiquitin-proteasome pathway. Y-632 was effective against imatinib-resistant cells expressing mutant Bcr-Abl in vitro and in mouse xenograft models (Wang et al. 2016), although the indiscriminate activity of this type of inhibitor may limit its progression as a lead compound (Darby et al. 2020). Most of these compounds were shown to have anti-cancer activity in cell lines, demonstrating that prevention of the interaction between Hsp90 and Hop may be a viable target for anti-cancer therapies (Yi and Regan 2008; Ardi et al. 2011; Pimienta et al. 2011; Edkins 2016; Dutta Gupta et al. 2019).

Recently, the first peptides designed to directly target the Hop-Hsp70 interaction were successful at inhibiting luciferase refolding in vitro (Zaiter et al. 2019). The success of one of these peptides, C1, was due to its ability to stabilise the Hop-Hsp70 interaction rather than inhibiting it, an effective strategy that is underrepresented in de novo compound design (Thiel et al. 2012; Andrei et al. 2017). The 5-amino acid peptide was designed based on the sequence of helix 3A of the Hop TPR1 domain and was small enough to hypothetically act as a minisolenoid, reinforcing the interaction between the helices of the Hop TPR1 domain and the C-terminal binding

domain of Hsp70. Interestingly, while C1 was able to bind both Hop and Hsp70, longer analogues consisting of 7 and 8 residues (SY7 and SY8, respectively) functioned as traditional inhibitors of this interaction, likely due to their larger size sterically preventing the simultaneous interaction of Hop and the respective compound with Hsp70 (Zaiter et al. 2019). To date, there has been no progression of Hop-Hsp70/Hsp90 inhibitors beyond proof of concept. Stabilising Hop-Hsp70/Hsp90 interactions offers another approach for compound design, and sequestering both proteins to limit their participation elsewhere in the cell may impart alternative therapeutic responses. The ability to specifically target Hop-chaperone interactions and accurately identify the on- and off-target effects within the complexity of protein networks remains a big challenge in Hop-directed drug design.

Developmental and Protein Folding Disorders

Hop has an established role in cellular development. Although human cell lines with CRISPR-mediated Hop knockout are viable (Bhattacharya et al. 2020), germline deletion of Hop in the mouse is embryonic lethal and Hop null mice fail to develop beyond E10.5 (Beraldo et al. 2013). Hop knockdown in ovarian germline nurse cells causes sterility in *Drosophila,* with eggs laid at a rate like those of wild-type flies but unable to hatch into larvae (Karam et al. 2017). Hop has also been linked with a role in embryonic stem cell biology in vitro. Transient silencing of Hop in embryonic stem cells led to a reduction in the ability to form embryoid bodies, suggesting a more differentiated phenotype (Longshaw et al. 2009; Prinsloo et al. 2009). This was attributed to a decrease in the phosphorylation and concomitant extranuclear accumulation of signal transducer and activator of transcription 3 (STAT3), a protein shown to interact directly with Hsp90 in vitro and in embryonic cells during leukaemia inhibitory factor (LIF)-induced pluripotency signalling (Setati et al. 2010; Prinsloo et al. 2012). Hop has also been identified in extracellular vesicles released from mouse embryonic stem cells, together with Hsp90 (Cruz et al. 2018). The role of Hop in stem cell biology supports a fundamental role for Hop in embryonic development. Hop is also required for neurosphere self-renewal and differentiation in neuronal cells which is linked to neuronal development and conceptual processes such as memory (Coitinho et al. 2007), attention span and hyperactivity (Beraldo et al. 2015). These findings are consistent with evidence that Hop interacts with Rnd1 GTPase to enhance neurite outgrowth in neuronal cell lines, leading to the proposal that Hop may be involved in neuronal development (de Souza et al. 2014).

Interestingly, linked to its role in foetal development through neuritogenesis, a decrease in Hop could be involved in autism spectrum disorders (ASD) (Braunschweig et al. 2013). The production of maternal IgG antibodies against several foetal brain antigens, including Hop, has been linked to ASD in the children born to these mothers. Children from mothers with specific reactivity to these had increased ASD-type stereotypical behaviours. It was suggested these antigens could

serve as a panel of markers for risk of maternal-autoantibody-related autism (Braunschweig et al. 2013). Heterozygous Hop knockout mice expressing reduced levels of Hop also demonstrated hyperactive behaviour with attention deficits. Mice overexpressing Hop displayed no distinct phenotype; however, the increased level of Hop was able to regulate the relative abundance of Hsp70 and Hsp90, while low levels of Hop could not. Whether this is relevant to the Hop-reduced phenotype observed remains to be investigated (Beraldo et al. 2015).

In Alzheimer's disease, soluble β-amyloid oligomers (AβOs) bind to PrP^C and trigger neurotoxicity. Hop was found to prevent the binding of AβOs to PrP^C, both in vitro and to mouse hippocampal neuronal PrP^C in vivo (Ostapchenko et al. 2013). Hop was able to prevent AβO-induced synaptic loss and neuronal death, and neurons that were haploinsufficient for Hop were more sensitive to AβO-induced death which could be rescued by treatment with recombinant Hop (Ostapchenko et al. 2013). The toxicity induced by AβOs could also be prevented by TPR2A (Ostapchenko et al. 2013; Maciejewski et al. 2016) and TPR2B but not DP1, despite all three domains binding with relatively high affinity to PrP^C as compared to full-length Hop (Maciejewski et al. 2016). The protective effect of increased levels of Hop was shown in primary hippocampal neuronal cultures overexpressing Hop. The addition of an antibody against Hop resulted in increased levels of cell death, indicative of the extracellular function of Hop (Lackie et al. 2020a). Further validation using a *C. elegans* model for AβO toxicity confirmed that increased levels of Hop and Hsp90 (overexpressed separately and in combination) reduced AβO-induced paralysis of worms, although this effect was not synergistic (Lackie et al. 2020a), while Hop depletion had the inverse effect (Brehme et al. 2014; Lackie et al. 2020a). Surprisingly, overexpression of Hop in a mouse model of Alzheimer's disease exacerbated plaque formation and promoted neurodegeneration. Overexpression of Hop was also associated with a compensatory increase in Hsp90β (Lackie et al. 2020a). In primary murine neurons the protection against AβO toxicity imparted by Hop was diminished by the addition of excess recombinant Hsp90, possibly due to sequestration of Hop by Hsp90 or interference with Hop-mediated PrP^C signalling (Maciejewski et al. 2016). The in vivo results suggest a more complex mechanism of disease presented in the mammalian system and aligns with the observation that Hop levels are upregulated in Alzheimer's disease patient brains (Ostapchenko et al. 2013; Lackie et al. 2020a). For more detailed information on the role of Hop and chaperones in neurodegeneration, the reader is referred to a comprehensive recent review (Lackie et al. 2017).

The role of Hop as a co-chaperone has linked it to disorders in which Hsp90 client protein stability or misfolding is a hallmark. The leading cause of cystic fibrosis is the presence of mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) protein. A variant of CFTR harbouring a phenylalanine deletion (CFTR ΔF508) has been shown to interact directly with Hop (Marozkina et al. 2010). Hop captures CFTR ΔF508 and prevents its maturation, thereby blocking its function. The maturation of CFTR ΔF508 could be rescued by treatment with S-nitrosoglutathione (GSNO), which reduced Hop levels, without affecting Hsp70 or Hsp90, a phenotype recapitulated by siRNA-mediated knockdown of Hop

(Marozkina et al. 2010). Hop has also been implicated in other protein conformational diseases, in which various proteins are converted into a common toxic conformational state similar to β-amyloid (Wolfe et al. 2013). Molecular chaperones have been found to suppress the toxicity of β-amyloid-like proteins by packaging the toxic proteins into protein-handling depots. Hop was found to be a component of the Hsp70/Hsp90 system in the control of spatial organisation of amyloid-like protein assemblies, leading to a suppression of toxicity by proteins such as the glutamine-rich yeast prion [RNQ+] and polyglutamine-expanded huntingtin (Htt103Q) in yeast (Wolfe et al. 2013). This observation was corroborated in a *C. elegans* model, in which Hop depletion was associated with increased polyglutamine toxicity (Brehme et al. 2014). Interestingly, the correlation between Hop depletion and Htt toxicity was contradicted by recent evidence that Hop knockout in a mammalian cell line (Bhattacharya et al. 2020) and Hop depletion in *Drosophila* (Xu et al. 2019) reduced aggregation of polyglutamine proteins. The studies in mammalian cell lines showed that Hop knockout reduced polyglutamine protein aggregation in HEK293T cells relative to their wild-type counterparts, but not in HCT116 cancer cells (Bhattacharya et al. 2020), while Hop depletion in *Drosophila* was associated with an increase in Hsp70 that possibly acted as a compensatory response more effective at preventing toxicity (Xu et al. 2019). It is difficult to unravel these varied responses without more empirical evidence, due to the level of complexity potentially imparted by differences in cell lines, Hop isoforms and the response of highly proliferative immortalised/cancer cell lines versus the global response of a whole organism. Interestingly, in a study investigating the relationship between Hsp90/Hop and the aggregation-prone Tar DNA-binding protein 43 (TBP-43), HEK293 cells displayed no significant change in TDP-43 aggregation or localisation upon Hop deletion and the effects of toxicity were therefore evaluated using mouse neuronal SN56 and neuroblastoma N2a cell lines (Lin et al. 2021b). TDP-43 is a predominantly nuclear protein associated with the neurodegenerative disorder amyotrophic lateral sclerosis, and Hop knockout and overexpression experiments in mammalian cell lines and yeast indicated that reduced levels of Hop were associated with reduced levels of TDP-43 but increased TDP-43 toxicity and cytoplasmic aggregation. TDP-43 levels were also reduced in Hop knockout mouse embryos (Lin et al. 2021b). TDP-43 appears to act as an Hsp90 client protein and its reduction in response to Hop depletion may be a consequence of modified chaperone function. TDP-43 was also reduced upon Hsp90 depletion and inhibition of Hsp90 chaperone activity in yeast. TDP-43 co-immunoprecipitated in a common complex with Hsp90, Hsp70 and Hop in SN56 mammalian cells, although Hsp70 and Hsp90 were able to interact with TDP-43 in the absence of Hop (Lin et al. 2021b). Interestingly, while moderate overexpression of Hop in yeast was able to rescue TDP-43 toxicity, both high overexpression and deletion of Hop increased TDP-43 toxicity. Similar effects were observed in partially differentiated mammalian N2a cells, although high overexpression of Hop did not affect TDP-43 toxicity. The dose-dependent effects of Hop suggest that a finely controlled balance is required between Hsp90 and Hop, in addition to other co-chaperones, to control TDP-43 toxicity (Lin et al. 2021b). These observations highlight the relevance of experimental approaches that

moderately adjust the levels of Hop in order to better understand its biological functions within a cell or organism.

Parasitic Diseases

Hsp70 and Hsp90 are considered drug targets for the treatment of infectious diseases like malaria and trypanosomiasis. Hop is conserved across species, including several parasitic organisms that cause disease in humans, such as *Plasmodium* and *Leishmania* species. Hop from *Leishmania donovani* is expressed during the amastigote stage (Joshi et al. 1993) which is important for adaption of the parasite to the human host (Morales et al. 2010). *Plasmodium falciparum* Hop (PfHop) shares a similar domain architecture with human Hop, and the residues that are known to be important in the interaction with Hsp70 or Hsp90 (Odunuga et al. 2003) are conserved. However, even though chaperone and co-chaperone systems are highly conserved, there is evidence that the proteins are sufficiently biochemically different to be considered as putative drug targets. The role of PfHop in facilitating the interaction of cytosolic PfHsp70-1 and PfHsp90 has been biochemically characterised (Gitau et al. 2012; Zininga et al. 2015). Furthermore, small-angle X-ray scattering (SAXS) analysis was used to study the structure of PfHop (Silva et al. 2020; Makumire et al. 2021), while the GGMP repeat residues have been found to regulate the interaction of PfHsp70-1 with PfHop (Makumire et al. 2021). These studies indicate that there are structural and functional features of PfHop proteins that are quite different to those of yeast and mammalian homologues. Consequently, antimalarial compounds could be designed to selectively target distinct regions of PfHop or the interface between PfHop and PfHsp70-1/PfHsp90 (Gitau et al. 2012; Zininga et al. 2015). Interestingly however, despite the presence of a divergent EEVN motif in the exported PfHsp70-x, human Hop was still able to interact with this PfHsp70-x isoform in vitro (Mabate et al. 2018), which suggests the possibility of heterocomplexes involving host and pathogen chaperones. Similarly, despite high structural and functional similarity, Hop from *Leishmania braziliensis* demonstrates low sequence conservation (Batista et al. 2016). Furthermore, deletion of specific residues in *Leishmania donovani* Hop blocked phosphorylation and led to parasite death (Morales et al. 2010). If these residues are unique to the parasitic Hop, they may indeed be targets for therapy. Furthermore, it may be relevant that the Hop interaction motif of Hsp90 which is crucial for survival of the parasite is MEQVD in *Leishmania* spp. instead of the MEEVD seen in the human host (Hombach et al. 2013). Hop knockdown and single-allele knockout in *Trypanosoma brucei and Trypanosoma cruzi,* respectively, were not sufficient to disrupt cell growth (Schmidt et al. 2018). However, Hop reduction impaired metacyclogenesis of *T. cruzi* in vitro (Schmidt et al. 2018). This differentiation process is usually prompted by stressed conditions and is vital for progression of the parasite to the infectious stage of its life cycle (Cruz-Saavedra et al. 2020). These findings offer a tentative route for

development of species-specific Hop inhibitors, depending on the role of Hop in parasitic life cycle progression.

Conclusion

While the exact biological function of Hop remains elusive, evidence from knockout studies in mammals suggests that it is important in embryonic development in this system at least. A role in development would be consistent with the reported link between Hop and cancer characteristics. The biological function of Hop will be system dependent, and while there are conserved features across species, the sequence and domain variations suggest that it could have been recruited by evolution for several different biological roles. The diverse functions of Hop in mammalian cells suggest that at least two major isoforms may exist, one intracellular and the other extracellular, although direct evidence for this has yet to be presented. Identification and elucidation of the molecular basis for these isoforms and their seemingly divergent cellular functions is an exciting area for future research. How has this dynamic scaffold protein been functionally adapted to such different roles and processes? More nuanced studies addressing how the concentration of Hop in time and space affects cellular function are needed to obtain meaningful information on its cellular and biological role. Such studies could determine the levels of Hop in different normal (body organs, cell types and subcellular compartments) and diseased systems, and then subtly adjust the levels of Hop to determine the effect on the equilibrium between folding and degradation of key functional marker proteins. A deeper structural and functional understanding of these Hop isoforms will assist research on the role of Hop in cancer. The intracellular isoform appears to be involved in processes important for successful metastasis, while the extracellular isoform appears to enhance proliferation of cancer cells. The identification of small molecules that can specifically disrupt Hop and its partner protein interactions are starting to emerge. These Hop modulators represent novel molecular tools for functional analyses as well as novel hit compounds for use in anti-cancer drug discovery research. In addition, the development of protein-protein interaction inhibitors for Hop may pave the way for a new class of chaperone regulators in the future. Finally, there is growing evidence that Hop has functions that may be independent of its major partner proteins (Hsp70, Hsp90 and PrP^{C}). Many of the recently defined activities of Hop, including ATPase activity, direct interaction and stabilisation of substrate proteins, are those that are more associated with chaperone function than co-chaperone function. As we learn more about this protein, it may be appropriate to evaluate if Hop is more than just a co-chaperone. This beckons a fresh approach to understanding the biological function of Hop, especially if its global biological function is in early development.

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Chapter 4
Specification of Hsp70 Function by Hsp40 Co-chaperones

Douglas M. Cyr and Carlos H. Ramos

Abstract Cellular homeostasis and stress survival requires maintenance of the proteome and suppression of proteotoxicity. Molecular chaperones promote cell survival through repair of misfolded proteins and cooperation with protein degradation machines to discard terminally damaged proteins. Hsp70 family members play an essential role in cellular protein metabolism by binding and releasing non-native proteins to facilitate protein folding, refolding, and degradation. Hsp40 (DnaJ-like proteins) family members are Hsp70 co-chaperones that determine the fate of Hsp70 clients by facilitating protein folding, assembly, and degradation. Hsp40s select substrates for Hsp70 via use of an intrinsic chaperone activity to bind non-native regions of proteins. During delivery of bound cargo Hsp40s employ a conserved J-domain to stimulate Hsp70 ATPase activity and thereby stabilize complexes between Hsp70 and non-native proteins. This review describes the mechanisms by which different Hsp40s use specialized sub-domains to direct clients of Hsp70 for triage between folding versus degradation.

Keywords Hsp70 · Hsp40 · Protein folding · Molecular chaperone · Protein triage

Introduction

The Hsp40 family of co-chaperone proteins plays a role in cell stress protection, folding of nascent polypeptides, protein translocation across membrane, refolding of denatured or aggregated proteins, modulation of amyloid formation, and protein triage (Kampinga et al. 2019). There are 50 *Hsp40* genes present in the human

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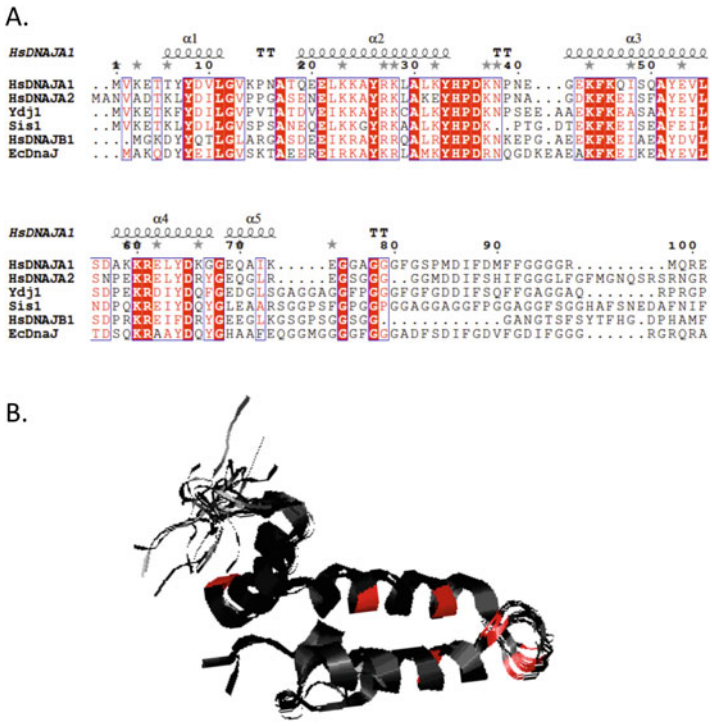

Fig. 4.1 The J-domain is responsible for regulation of Hsp70 function. (**a**) Sequence alignment of J-domains from bacterial (Ec), yeast (Ydj1 and Sis1), and human (Hs) Hsp40s. For reference: *Escherichia coli* DnaJ (UNIPROT P08622), *Saccharomyces cerevisiae* Sis1 (UNIPROT P25294) and YdJ1 (UNIPROT P25491), *Homo sapiens* DNAJA1 (UNIPROT P31689), DNAJA2 (UNIPROT O60884), DNAJB1 (UNIPROT P25685). (**b**) NMR solution structure of the J-domain from Sis1 (PDB 6D6X). Red denotes residues perturbed by the interaction with Hsp70. Those residues are in the HPD motif and in the interface between helices 2, 3, and 4

genome and 20 Hsp40s identified in the yeast genome. These proteins were identified by the presence of a conserved J-domain that stimulates the ATPase activity of the Hsp70 (Fig. 4.1). Type I (DNAJA) and Type II (DNAJB) Hsp40s also have the conserved ability to bind and deliver non-native proteins to Hsp70, which is essential for life (Johnson and Craig 2001; Lee et al. 2002; Summers et al. 2009).

Type I Hsp40s are descendants of bacterial DnaJ and contain the N-terminal J-domain, followed by a glycine/phenylalanine-rich region (G/F), a zinc finger-like region (ZFLR), and a conserved C-terminal domain (Kampinga and Craig 2010). Except for the absence of the ZFLR, the domain structure of Type II Hsp40s is like that of Type I Hsp40s. Type III Hsp40s contain the J-domain, not necessarily at the N-terminus, but none of the other conserved domains found in Type I or II Hsp40s. Instead, they often have specialized domains that localize them to certain areas of the cell and provide specificity in substrate binding. Type I and Type II Hsp40s contain a C-terminal dimerization domain, but this does not mean that all Hsp40s function as

dimers (Sha et al. 2000). The transmembrane Hsp40s DnaJB12 and DnaJB14, which lack a canonical dimerization domain, also form dimers (Sopha et al. 2012). So, in many instances dimeric Hsp40s interact with Hsp70, but a general requirement for dimerization in Hsp40 function has not been demonstrated.

Hsp40s are conserved across species and are found in organisms from bacteria to humans, and a variety of Type I and Type II, and Type III (DNAJC) Hsp40s are found in the same subcellular organelles where they can play specialized roles (Rosenzweig et al. 2019). To better understand the cellular processes that these chaperones facilitate, we must first understand the mechanism by which Hsp40s bind substrates and regulate Hsp70 function. It is also important to identify the protein quality control machinery for which Hsp40 and Hsp70 serve as client selectors or regulatory subunits. In the following sections, we will review the recently published genetic, biochemical, cell biological, and structural data that have helped elucidate the unique mechanisms that different Hsp40s use to maintain protein homeostasis.

A. Hsp70 Co-chaperone Activity of Hsp40s

The affinity of Hsp70 for polypeptides is regulated by its nucleotide-bound state. In the ATP-bound form, Hsp70 has a low affinity for substrate proteins. However, upon hydrolysis of the ATP to ADP, Hsp70 undergoes a conformational change that increases its affinity for substrate proteins (Fig. 4.2). Hsp70 goes through repeated cycles of ATP hydrolysis and nucleotide exchange, which permits cycles of substrate binding and release. Proteins that fold exit the cycle, whereas proteins that remain in a non-native state reenter the cycle to suppress their aggregation or promote delivery to degradation machinery (Bascos et al. 2017; Mashaghi et al. 2016; Schlecht et al. 2011).

The Hsp70 proteins are assisted and regulated by several different Hsp40 co-chaperones. These co-chaperones have been shown to not only regulate steps of the ATPase cycle of Hsp70 (Fig. 4.1), but they also have an individual specificity such that one co-chaperone may promote folding of a substrate while another may promote degradation. For example, the Hsp40 DNAJA1 assists Hsp70 in folding proteins, whereas DNAJB1 and DNAJB12 assist Hsp70 in the degradation of misfolded proteins (He et al. 2021; Hipp et al. 2019). The yeast Type II Hsp40 Sis1 functions in spatial protein quality control and promotes protective aggregation of amyloid-like proteins (Wolfe et al. 2013).

Hsp40 proteins that have polypeptide binding activity contain domains that have the general ability to bind non-native polypeptides such as DNAJA1 and DNAJB1 (Fig. 4.3) or have highly specialized substrate-binding domains (Kampinga and Craig 2010; Mayer and Gierasch 2019). The clathrin-binding DNAJC6 and the iron-sulfur cluster assembly factor DNAJC24 are examples of Hsp40s that bind specific clients (Rosenzweig et al. 2019; Thakur et al. 2012). The ability of Hsp40s to load substrates onto Hsp70 is common to most family members. Differences in client specificity exhibited by different Hsp40s enable Hsp70 to facilitate a broad

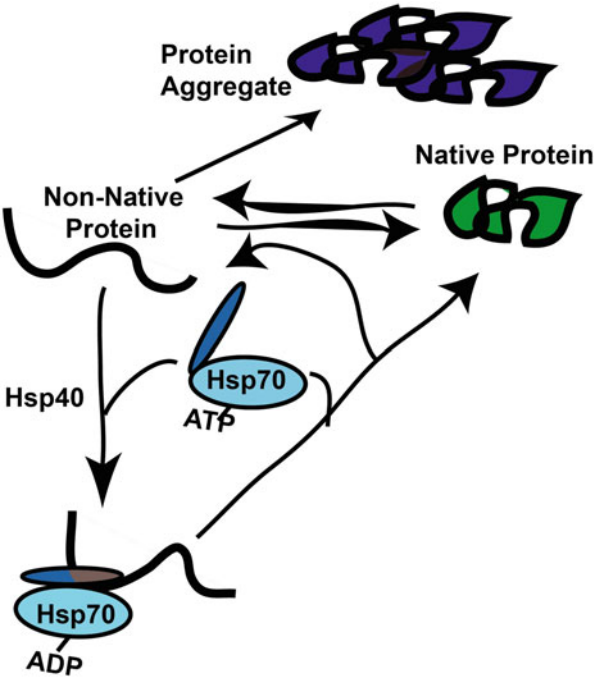

Fig. 4.2 The Hsp70-Hsp40 polypeptide binding and release cycle. Hsp70 requires the assistance of Hsp40s to stably bind non-native peptides. Hsp40s bind non-native proteins and upon their delivery to the Hsp70 SBD also stimulate Hsp70 ATPase activity. Repetitive cycles of polypeptide binding and release by Hsp70 suppress protein aggregation and thereby promote protein folding

array of cellular processes. The mechanism by which Hsp40s bind and interact with Hsp70s is discussed in a prior review (Mayer and Gierasch 2019).

B. How Does the J-Domain Regulate Client Binding by Hsp70

The recent crystal structure of the J-domain of *Escherichia coli* DnaJ in complex with the *E. coli* Hsp70 DnaK has provided insight into the mechanism by which the J-domain regulates Hsp70's ATPase activity and client binding (Kityk et al. 2018). The J-domain was found to interact with both DnaK's nucleotide-binding domain (NBD) and substrate-binding domain (SBD). It contacts the highly conserved interdomain linker that controls allosteric communication between DnaK's NBD and SBD. Mutations that hinder the ability of the J-domain to contact the SBD reduce the ability of Hsp70 to sense the presence of bound clients that stimulate ATP hydrolysis. The J-domain can directly influence Hsp70s ability to hydrolyze ATP and also enable Hsp70 to sense the presence of clients in the SBD (Kityk et al. 2018). This bipartite mechanism of action increases the efficiency of client transfer from Hsp40 to Hsp70.

New details about the mechanism for interaction of the J-domain with the HSP70 NBD are being revealed by nuclear magnetic resonance (NMR) studies. Chemical shift perturbation and intensity changes measured by NMR on the J-domain of Sis1

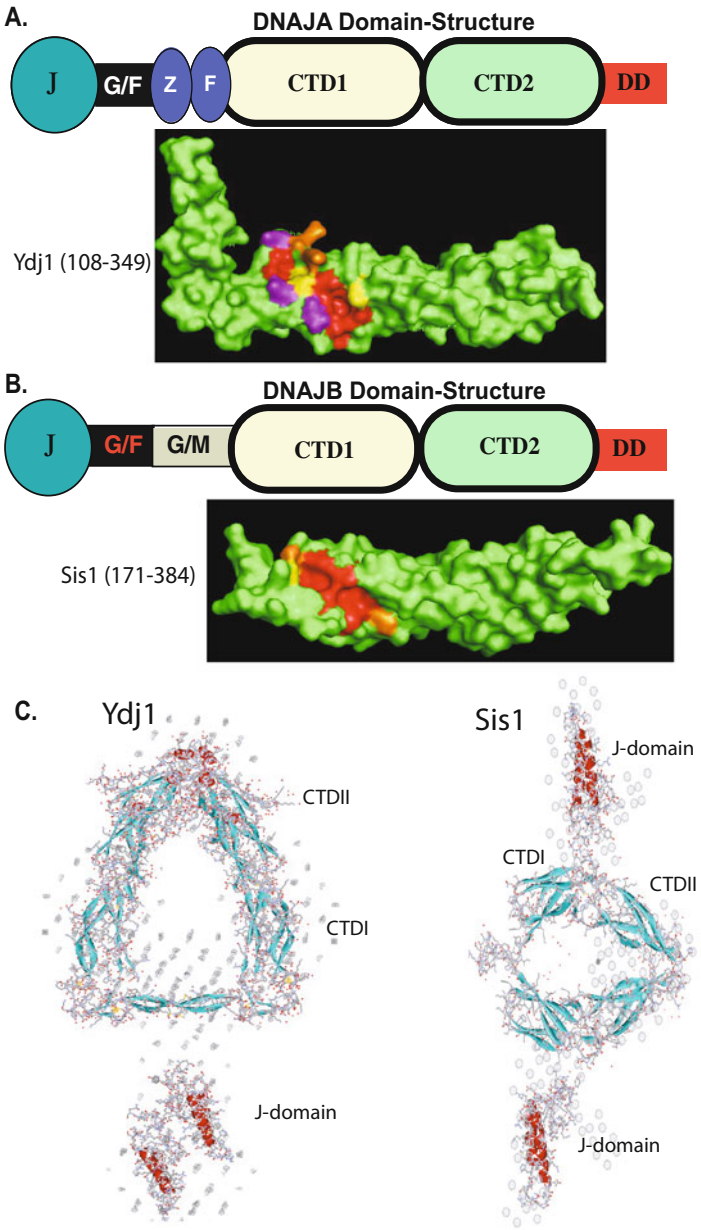

Fig. 4.3 Domain structures of DNAJA and DNAJB Hsp40 subtypes. (**a**) Schematic of the sub-domains within DNAJA Hsp40s with the X-ray structure showing the solvent-exposed surfaces of a yeast-Ydj1 fragment below. (**b**) Schematic of the sub-domains within DNAJB Hsp40s with the X-ray structure showing the solvent-exposed surfaces of a yeast-Sis1 fragment below. Colors denote the hydrophobic (red), polar (purple), basic (orange), and acidic (yellow). PDB file: Sis1: 1C3G and Ydj1: 1NLT_1

(residues 1–81) suggest that residues A29, H34, T39, F45, F52, and Y67 interact with Hsp70 (Pinheiro et al. 2019) (Fig. 4.1b). In Sis1 the residues 34–36 correspond to the HPD motif that is conserved in all Hsp40s. These data also indicate that the end of helix-2, the HPD, helix-3, and part of helix-4 are involved in binding to Hsp70. These data define aminos in the J-domain whose environment changes upon interaction with Hsp70 and define details about the J-domain surface that contact the Hsp70 NBD (Kityk et al. 2018).

C. Do Hsp40s Act as Chaperones?

It is established that Hsp40s specify Hsp70 function, but the manner by which Hsp40s bind and deliver substrates to Hsp40 is not completely understood (Fan et al. 2003; Summers et al. 2009). The first observations of intrinsic chaperone activity of an Hsp40 came from studying the bacterial Type I Hsp40 DnaJ (Langer et al. 1992) where purified DnaJ protein was shown to suppress the aggregation of denatured rhodanese. Subsequently, the yeast Hsp40 Ydj1 was shown to have the conserved ability to suppress protein aggregation (Cyr 1995; Lu and Cyr 1998).

Studies with the yeast Sis1 protein have shown that Type II Hsp40s can also bind chemically denatured luciferase and reduced α-lactalbumin and that this binding is dependent on specific residues within the C-terminal peptide-binding domain I (CTDI) (Lee et al. 2002; Sha et al. 2000). This ability of Sis1 to recognize and bind non-native polypeptides classifies Sis1 as a chaperone. However, Sis1 alone is not as effective of a chaperone as the Type I Hsp40s because Sis1 cannot prevent the aggregation of thermally denatured luciferase, nor does it hold the thermally denatured luciferase in a folding competent state. However, Sis1 is able to hold chemically denatured luciferase in a folding competent state (Lee et al. 2002) and also binds specific residues in yeast prions to promote prion propagation (Douglas et al. 2008). The human Hsp40, DNAJB1, also has the ability to bind non-native proteins and has the ability to recognize proline-rich regions of proteins (Lee et al. 2002). This appears to make DNAJB1 susceptible to inactivation by huntingtin protein (Park et al. 2013). The inactivation of DNAJB1 by huntingtin is associated with inhibition of the proteasome and may contribute to huntingtin toxicity (Park et al. 2013).

Crystal structures of the C-terminal domain (CTD) of both Ydj1 and Sis1 have been solved with each being subdivided into structurally similar CTDI and CTDII (Fig. 4.3) (Li et al. 2003; Li and Sha 2003; Qian et al. 2002; Sha et al. 2000). The crystal structure of YDJ1 complexed with a peptide substrate GWLYEIS bound to CTDI reveals two hydrophobic depressions that are involved in client binding. The X-ray crystal structure of Sis1 contains similar hydrophobic depressions in CTDI with mutational analysis revealing their importance in binding non-native clients (Fan et al. 2005; Lee et al. 2002). CTDII also contains hydrophobic depressions, but they are occupied by the side chains of hydrophobic residues from CTDI, so they were not initially implicated in client binding. Yet, data from recent NMR studies

suggest that both CTDI and CTDII function in the binding of non-native proteins (Jiang et al. 2019). DNAJA and DNAJB are suggested to function as multivalent polypeptide binding proteins.

C. Determination of Hsp70 Functional Specificity

Type I and Type II Hsp40s exhibit different substrate specificity and direct Hsp70 to perform different functions in vivo. *This may be the case for several regions.* The first is the hydrophobic depressions in CTDI of Type I and Type II Hsp40s are different (Fig. 4.3). Sequence analysis also reveals two possible regions that may also contribute to specifying clients bound by Type I and Type II Hsp40s. First, the G/F-rich regions of Ydj1 and Sis1 are different, with that of Sis1 containing a 10-residue long insert containing the amino acids, GHAFSNEDAF (Yan and Craig 1999). Second, as mentioned previously, the protein modules located in the middle of Ydj1 and Sis1 are different such that Ydj1 contains the ZFLR and Sis1 contains a G/M region.

There is also recent work that suggests that the specificity of Hsp40 recognition by an Hsp70 partner occurs through sets of residues that lie at the interface where helices II and III of the J-domain bind to Hsp70 (Faust et al. 2020). Thus, client recognition by different Hsp40s and selective recognition of the J-domain on different Hsp40s by Hsp70 provides layers of regulation for Hsp70 action in protein homeostasis.

D. Hsp40 Quaternary Structure

A common feature of Type I and Type II Hsp40s is that dimerization is important for them to function in vivo (Summers et al. 2009). There are no crystal structures of full-length Type I or Type II Hsp40s, but small-angle X-ray scattering (SAXS) and protein modeling have been used to build models of the quaternary structure of Type I and Type II Hsp40s (Borges et al. 2005; Ramos et al. 2008). These models suggest that there are substantial differences in the quaternary structure of the Type I and Type II Hsp40s that may help account for their ability to direct Hsp70 to perform different cellular functions (Fig. 4.3c). In Type I Hsp40 the interface between CTDI and CTDII and the ZFLR space the polypeptide binding pockets in CTDI and appear to impact the orientation for the J-domain relative to the long axis of the chaperone (Silva et al. 2011). In Type II Hsp40s CTDIs on the arms of the different dimers are closer together, and the J-domains are splayed to the side of the chaperone. It appears that J-domains can exist in a dimeric state, while the models depicted show the J-domains of Ydj1 and Sis1 as monomers. It is therefore possible that these models depict an inactive state of Ydj1 and Sis1 and that forms of these Hsp40s that regulate

Hsp70 ATPase activity undergo a conformational change to permit J-domain dimerization (Goodwin et al. 2014; Mokranjac et al. 2003).

Oligomerization has also been observed in Hsp40 proteins. Karamanos and co-authors solved the structure of the monomeric CTD domain of human DNAJB6B by NMR, showing that residues 190–230 form of a four-stranded beta-sheet while the C-terminus is likely disordered (Karamanos et al. 2019). The oligomeric interaction (2–40 subunits) likely involves beta-strand-beta-strand interactions as showed by cross-linking studies (Soderberg et al. 2018). A S/T-rich domain (residues 132–189) is important for the interaction as its presence shifts the equilibrium to oligomerization. DNAJB6 is suggested to fight the aggregation of polyglutamine-rich proteins that cause neurodegeneration. Whether the oligomers of DNAJB6 detected represent a storage form of this chaperone or a multivalent client binding form requires investigation.

E. ER-Transmembrane Hsp40s and Membrane Protein Quality Control

Most attention has been paid to functions of cytosolic Hsp40s in protein homeostasis. Yet, there are Hsp40s in the ER lumen and mitochondrial matrix that play a critical role in cell stress protection (Kampinga et al. 2019). There is also a subfamily of ER-transmembrane Hsp40s that contain a cytosolic J-domain and G/F-rich region and an ER-lumenal disordered region that play critical roles in membrane protein assembly, ER protein quality control (ERQC), and suppression of ER-stress-induced apoptosis (He et al. 2021; Sopha et al. 2017). DNAJB12 is a Type II Hsp40 whose deletion sensitizes cells to ER-stress-induced apoptosis because it functions with and ER-associated E3 ubiquitin-ligase GP78 to mediate the constitutive degradation for the BCL-2 homolog BOK (Fig. 4.4a). BOK has a short 15-min half-life and accumulates to levels that are sufficient to initiate mitochondrially induced apoptosis when DNAJB12 and the E3 ubiquitin ligase GP78 become saturated with misfolded clients (Llambi et al. 2016; Sopha et al. 2017).

DNAJB12 and Hsp70 can help degrade BOK because they serve as substrate selectors for several ERQC-E3 ligases with clients being rapidly ubiquitinated and retrotranslocated from the ER membrane to cytosol for proteasomal degradation (Grove et al. 2011) (Fig. 4.4a). There are instances where DNAJB12 and Hsp70 bind misfolded membrane proteins that contain stable tertiary structures that are resistant to retrotranslocation (Houck et al. 2014). This occurs due to inefficiencies in membrane protein folding where folding defects arise after the formation of disulfide bonds or partially assembled intermediates adopt a difficult-to-handle low free energy state (He et al. 2021; Houck et al. 2014). In these situations, DNAJB12 interacts with ER-associated autophagy initiation kinase complexes to mediate the focal activation of autophagy (He et al. 2021; Houck et al. 2014) (Fig. 4.4b). This mechanism drives the conversion of ER tubules containing ERAD-resistant

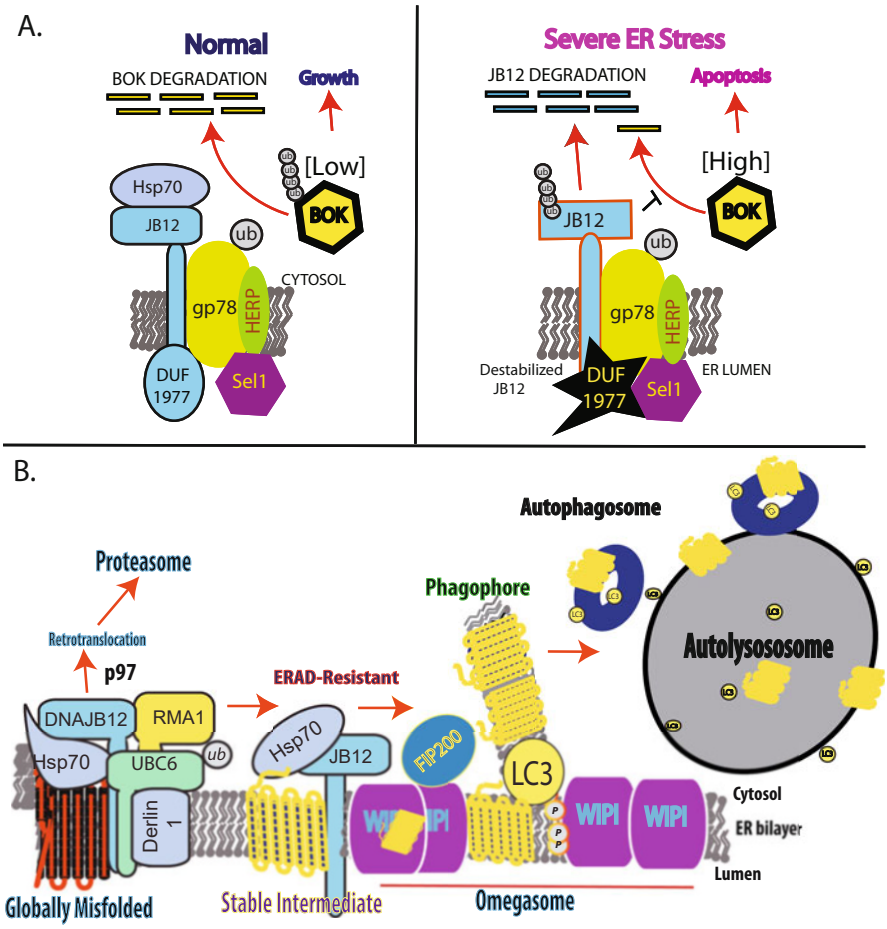

Fig. 4.4 ER-transmembrane JB12-dependent triage of membrane proteins for proteasomal versus lysosomal degradation. (**a**) Schematic depicting JB12/Hsp70 functioning with the ER-associated E3 ubiquitin ligase GP78/SEL1/Herp to select the BCL-2 family member BOK for constitutive proteasomal degradation (Llambi et al. 2016). JB12 is destabilized by severe ER stress. JB12 depletion causes BOK accumulation and induction of apoptosis. (**b**) Conformation-dependent triage of misfolded membrane proteins for proteasomal or lysosomal degradation. The E3 ubiquitin ligase RMA1/RNF5, E2 ubiquitin-conjugating enzyme UBC6, and Derlin-1 are components of an ERQC E3-ubiquitin ligase complex that utilizes JB12 and Hsp70 to select globally misfolded membrane proteins for proteasomal degradation (ERAD). Misfolded membrane proteins containing stable tertiary structure are resistant to extraction from the ER and are not delivered to the cytosolic proteasome. Long-term association of misfolded membrane proteins with JB12 drives interaction of JB12 with omegasomes and delivery of stable intermediates for degradation via ER phagy. The omegasome is an ER microdomain in which phagophores (autophagosome precursors) form. WIPI is a WD40 repeat protein that marks omegasomes and scaffolds autophagy initiation machinery. JB12/Hsp40 localizes in ER tubules with stable membrane protein intermediates that are intermingled with adjacent WIPI-decorated ER tubules

membrane proteins into phagophores that give rise to autophagosomes. In this way ERAD-resistant membrane proteins are cleared from the ER membrane and delivered to the lysosome. JB12 is an example of an ER-associated Hsp40 that interacts with cytosolic Hsp70 and different protein quality machines to triage nascent membranes, regulate autophagy initiation kinase activity, and suppress ER-stressed apoptosis.

F. Hsp40s into the Future

The Hsp40 family plays a central role in organismal vitality. Mechanisms for Hsp40 action in specification of Hsp70 function are being defined at cellular and atomic levels. Future Hsp40 studies will uncover mechanisms for regulating interactions between the J-domain and Hsp70, Hsp40 action in suppression of neurodegeneration, and triage of proteins between life and death. Use of super-resolution microscopy will define ER nanodomains where Hsp40s facilitate triage of misfolded membrane proteins. Quantitative proteomic studies will reveal how post-translational modification of Hsp40s regulates their dynamic interactions different cellular machinery. Genome-wide association studies are revealing genetic links between Hsp40 and disease. In order for the field to understand how the 50 different Hsp40 family members function to maintain protein homeostasis and suppress proteotoxicity, much work is still required.

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Chapter 5
Cdc37 as a Co-chaperone to Hsp90

Thomas L. Prince, Benjamin J. Lang, Yuka Okusha, Takanori Eguchi, and Stuart K. Calderwood

Abstract The co-chaperone p50/Cdc37 is an important partner for Hsp90, assisting in molecular chaperone activities, particularly with regard to the regulation of protein kinases. Analysis of the structure of Hsp90-Cdc37-kinase complexes demonstrates the way in which Cdc37 interacts with and controls the folding of a large proportion of intracellular protein kinases. This co-chaperone thus stands at the hub of a multitude of intracellular signaling networks. Indeed, the influence of Cdc37 reaches beyond the housekeeping pathways of protein folding into the regulation of a wide range of cellular processes. This co-chaperone has attracted attention as a potential intermediate in carcinogenesis. Cdc37 is an attractive potential target in cancer due to (1) high expression in a number of tumor types and (2) control of multiple signaling pathways. These properties indicate (3) a potential for selectivity due to its elevated expression in malignant cells and (4) robustness, as the co-chaperone may control multiple growth signaling pathways and thus be less prone to evolution of resistance than less versatile oncoproteins. Cdc37 may also be involved in other aspects of pathophysiology and has been shown to be secreted in exosomes. Protein aggregation disorders have been linked to age-related declines in molecular chaperones and co-chaperones. Cdc37 also appears to be a potential

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agent in longevity due to its links to protein folding and autophagy, and it will be informative to study the role of Cdc37 maintenance/decline in aging organisms.

Keywords Cdc37 · Hsp90 · Kinase · Chaperone · Cancer · Autophagy · Neurodegenerative

Introduction

For many of us, heat shock proteins (HSPs) were first encountered as dominant bands on SDS-PAGE gel analysis of protein extracts from heat-stressed cells, which could be readily visualized by a process as insensitive as Coomassie blue staining; they were apparently made in extraordinarily large amounts after the stress and became major bands. Their existence had been surmised from the studies of Ritossa in *Drosophila* and others who discovered the existence of heat shock-induced transcripts (Ashburner and Bonner 1979). The cellular functions of the HSPs were initially unknown. However, early studies of processes involved in Lamda bacteriophage replication in prokaryotes and clathrin cage uncoating in mammalian cells identified them as proteins that work on the structures of other polypeptides and sculpt the tertiary and quaternary structures of proteins and protein complexes (Polissi et al. 1995; Chang et al. 2002). The Lambda phage studies indeed indicated that a seventy kD *E. coli* protein called DnaK cooperated with other proteins DnaJ, GrpE, and GroEL to form a "chaperone machine" that promoted many aspects of Lamda phage replication (Polissi et al. 1995). DnaK was also found to have an ortholog in mammalian cells called heat shock protein seventy (Hsp70), DnaJ was the founder member of the J domain co-chaperone family (JDP), GrpE was a nucleotide exchange factor with functional homologs in mammals (*c.f.* BAG domain proteins), and GroEL turned out to be close relative of Hsp60 (Lindquist and Craig 1988; Hartl 1996; Lang et al. 2021; Frydman et al. 1992; Calderwood 2013). These studies in prokaryotes foreshadowed the identification of the conserved HSP families in mammals as well as the complex pathways of protein folding and maturation. The Hsp27, Hsp60, Hsp70, and Hsp90 families and TriC chaperonins are conserved throughout cellular life and collaborate to maintain the proteome in functional form (Frydman et al. 1992; Arrigo 2007; Rosenzweig et al. 2019; Neckers and Ivy 2003). Hsp70 and Hsp90 require families of accessory proteins known as co-chaperones to function at respectable rates in the cellular environment (Lang et al. 2021).

Protein folding has been proven to be a cooperative process, involving multiple chaperones and co-chaperones (Baker et al. 2018; Cox et al. 2007; Chen et al. 1998). An example of this is, the "folding cycle" of mammalian steroid hormone receptors such as the glucocorticoid receptor, from initial transcription/translation through intermediate folding states to mature receptors capable of binding the hormone ligand, traversing to the nucleus and initiating transcription. The cycle incorporates two main phases, including the binding of the nascent hormone, after translation by

Hsp70 and a member of the JDP family, Hsp40 in this case. This process results in the ATP-dependent sequestration of hydrophobic residues by Hsp70. The receptor cargo is then handed on to Hsp90 through the bridging protein HOP (Hsp70-Hsp90 organizing protein (Johnson et al. 1998)). Dissociation of Hsp70 from this complex then permits Hsp90 to apply the final folding steps of the protein, leading to a functional form. Hsp90 maturation of clients requires the co-chaperone p23 to stabilize the interaction; peptidyl-prolyl isomerases such as CYP40, FKBP51, and FKBP52 for proline isomerization; and nuclear localization and phosphatase 5 (PP5) for dephosphorylation of inhibitory modifications (Riggs et al. 2004; Conde et al. 2005). A similar folding cycle was also discovered for the maturation of many protein kinases, involving Hsp70- and Hsp90-mediated stages, but differing in that the essential co-chaperone for Hsp90 in this process was p50/CDC37, the main subject of this review (Shao et al. 2001; Kamal et al. 2004; Kamal et al. 2003; Calderwood 2015).

Cdc37 in the Chaperoning of Protein Kinase

Cell division cycle 37 (Cdc37) is a protein that assists in the three-dimensional folding of protein kinase domains along with Hsp90. Discovered in a yeast (*S. cerevisiae*) genetic screen, Cdc37 was found to be essential for G1 progression by facilitating the associations between cyclins and cyclin-dependent kinases (CDKs) (Reed 1980; Gerber et al. 1995). This 50 kD protein, often referred to as p50, was next found to directly interact with Rous sarcoma viral kinase $pp60^{v\text{-}src}$ and Hsp90. Cdc37 and Hsp90 were then shown to be required for the tyrosine kinase activity in fruit flies (*D. melanogaster*) (Gerber et al. 1995). It was then later confirmed that Cdc37 was the kinase-targeting co-chaperone of the Hsp90 chaperone machine that maintained Cdk4 levels in both fruit fly and mammalian cells (Fliss et al. 1997; Stepanova et al. 1996). Since then, Cdc37 has been found to interact with a large portion of mammalian "client" kinases. Cdc37 has also been shown to support androgen hormone receptor (AR) activity and endothelial nitric oxide synthase (eNOS) function, suggesting that the co-chaperone may possess properties beyond the folding of kinase domains (Rao et al. 2001; MacLean and Picard 2003).

Cdc37 is required for cell viability as it services a large portion of the kinase population that signals for growth and differentiation. Continuously needed to maintain the population of client kinases in a cell, Cdc37 and Hsp90 recognize and fold both nascent kinase polypeptides emerging from ribosomes and mature kinases that depend on Hsp90 and Cdc37 to maintain their day-to-day activity and stability. Protein kinases make up 1–2% of all the proteins encoded by eukaryotic genomes, making them the third most common protein domain in the human genome (Manning et al. 2002). This prevalence combined with the observations that aberrant kinase activity often leads to disease has led to the development of myriads of small molecules directed at kinases by the pharmaceutical industry.

Protein kinases function catalytically by binding ATP and covalently transferring the γ-phosphate residue to a serine, threonine, or tyrosine residue on the substrate protein. This event modifies the substrate protein's structural conformation which, in turn, affects its function, activity, localization, stability, and protein-protein interaction profile. This simple post-translational modification (PTM) allows for the transduction of information between and within cells regarding environmental conditions, stress, growth, differentiation, and the cell cycle. Transduction of information is propagated through signaling networks made up of tiers of kinases and effector substrate proteins such as factors that regulate gene transcription factors and protein translation. Kinase signaling throughout the network occurs in cascade-like manner with each kinase protein capable of phosphorylating numerous downstream substrates, allowing the original signal to be exponentially amplified. Each kinase may also phosphorylate different species of substrates, thereby broadening the effect of the original signal and creating cross-talk between signaling pathways. This multiplicity of substrates enables modulation of the signal through positive and negative feedback mechanisms. Throughout this process, Cdc37 and Hsp90 are both required to ensure the readiness and activity of the kinase signaling network by preventing the aggregation and degradation of protein kinases while maintaining the proper structure of their domains.

The overall protein fold of the kinase catalytic domain is remarkably conserved across eukaryotes. Made up of between 250 and 300 amino acids, the kinase domain consists of a two-lobed structure that can be flanked or split by a number of regulatory or structural domains. The smaller N-terminal lobe (NL) orients the ATP molecule and contains key catalytic residues, while the C-terminal lobe (CL) recognizes peptide substrates and also contains residues required for phosphorylation (Hanks et al. 1988). ATP molecules are cradled by the cleft created between the NL and CL. After substrate phosphorylation, the hydrolyzed ADP must be replaced with fresh ATP, and this exchange event requires the rearrangement of the NL-CL interface and exposure of the ATP-binding cleft. This extended conformation state of the kinase domain is recognized and bound by Cdc37 and Hsp90 (Verba et al. 2016). We depict the asymmetric structure of a cyclin-dependent kinase, CDK4, split and laced through the lumen of an Hsp90β dimer with Cdc37 straddling one Hsp90 protomer while binding each kinase lobe in (Fig. 5.1a, b). The most prominent structural motifs required for Cdc37 recognition/interaction include the αE-helix in the CL and the αC-β4 loop, αC-helix, and G-loop in the NL. Concomitantly, the Hsp90 dimer binds the extended β4–β5-sheet motif, as per nomenclature provided by Hanks and Hunter (Hanks et al. 1988). These interactions were crudely speculated/predicted by previous efforts (Prince and Matts 2004), but now the atomic structure provided by Verba et al. most beautifully models the stabilized unfolded state of protein kinases by the Hsp90-Cdc37 chaperone complex (Fig. 5.1) (Verba et al. 2016). Consideration of this structure suggests why kinases that are bound by Hsp90-Cdc37 are catalytically repressed and dissociation of the complex by Hsp90 inhibitors leads to transient activation prior to degradation (Gaude et al. 2012; Bendell et al. 2015).

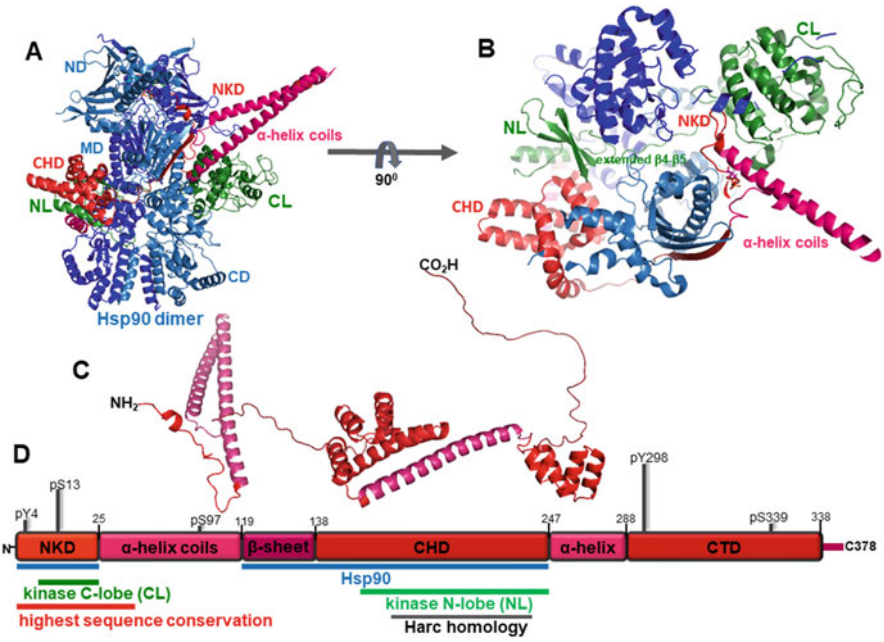

Fig. 5.1 Cdc37 structure and domain map. (**a**) Human Cdc37 asymmetrically interacts across Hsp90 dimer and binds cyclin-dependent kinase (Cdk4) (PDB:5FWM). (**b**) Top and cutaway view of Cdk4 extended through the lumen of Hsp90 dimer with Cdc37 binding each lobe of kinase domain. (**c**) AlphaFold predicted structure of free Cdc37 (AF-Q16543-F1-model_v1). (**d**) Cdc37 domain map. Cdc37: N-terminal kinase-binding domain (NKD), central Hsp90-binding domain (CHD), C-terminal domain (CTD), Hsp90: N-terminal ATP-binding domain (ND), middle domain (MD), C-terminal domain (CD), Cdk4: N-terminal lobe (NL), C-terminal lobe (CL)

The frequency and duration of the period a kinase domain occupies the unfolded/transition state vary among protein kinase family members as it is dependent on the overall structural stability of the NL-CL interface (Bendell et al. 2015; Taipale et al. 2012). This property suggests that there is no conserved primary amino acid sequence that is recognized by Cdc37 and Hsp90, but instead that recognition is dependent on exposure of the structural motifs bound by Cdc37 and Hsp90. The stability of the intramolecular interaction between the αC-β4 loop in the NL and the αE-helix in the CL is likely the most important factor influencing Cdc37 recognition. Indeed, analysis of mutations created in the laboratory and found in the clinic has suggested this (Xu et al. 2005; Nony et al. 2003). The αC-β4 loop typically contains a large amino acid such as a histidine (H) that inserts into a notch in the αE-helix allowing for a pivotal interface between the NL and CL. If the sequence/structure of the αC-β4 loop is too flexible or thermally unstable (Xu et al. 2005), then the NL-CL interface can come apart exposing the two motifs. Cdc37 contains a conserved HPN amino acid motif that mimics the αC-β4 loop and specifically probes the stability of the kinase domain as demonstrated by Keramisanou et al. (Keramisanou et al. 2016). The ability of Cdc37 to pry apart the NL-CL and expose the notch in the αE-helix is

key to determining whether or not a kinase will be recognized as a client. Moreover, this molecular mechanism suggests that Cdc37 acts as an ATP exchange factor for some client kinases (Eckl et al. 2015). This consideration also indicates why small molecule kinase inhibitors that bind and stabilize the ATP cleft reduce Cdc37 interaction and sensitivity to Hsp90 inhibitors (Giannini and Bijlmakers 2004).

Roughly 60% of all protein kinases tested were shown to interact with Cdc37 and Hsp90 (Taipale et al. 2012), while the remaining protein kinases that do not interact in this way likely possess stabilized domain structures, as seen with JNK (Prince and Matts 2005). Moreover, not all Cdc37-Hsp90 interaction dynamics with kinases are thought to be the same, as discussed here: (1) As a protein kinase is synthesized on the ribosome, it encounters a crowded cellular milieu (Ellis 2007). Cdc37 and Hsp90 ensure nascent kinase polypeptides become properly folded and are not allowed to aggregate. Once the kinase is folded and released, it may no longer require Cdc37 and Hsp90 interaction, such as observed with EGFR. (2) Some kinases, however, such as Her2/neu require continual Cdc37-Hsp90 support due to the unstable structure of their αC-β4 loop (Xu et al. 2005). Therefore, inhibition of Hsp90 can result in rapid loss of activity and subsequent degradation of these kinases. (3) Other kinases may become reliant on Cdc37 and Hsp90 once they are activated and need to repeatedly exchange ATP (Miyajima et al. 2013). Identifying such kinases, however, is challenging since their interaction with Cdc37 is stimulus-dependent and transitory, making it difficult to determine the correct experimental conditions. (4) Conversely, some kinases such as Erk5 may only bind Cdc37 and Hsp90 while inactive and dissociate upon activation (Erazo et al. 2013). (4) Lastly, mutated protein kinases often become increasingly dependent on Cdc37 and Hsp90 (da Rocha et al. 2005). This is observed in cancer with kinases that signal for cell proliferation. In this regard, mutations that induce increased kinase activity promote neoplastic transformation but often reduce the structural stability of the kinase. Furthermore, kinases may be mutated as a tumor develops resistance to a therapy, as was observed with a tyrosine kinase inhibitor (TKI) (Schwartz et al. 2015). Together these scenarios combined indicate why such a large portion of the encoded kinases in the human genome interact with Cdc37.

Structure and Interaction

Human Cdc37 is made up of 378 amino acids and consists of an extended structure with globular α-helical bundles in the central and C-terminal end of the protein as shown by cryo-EM and crystal structures (Verba et al. 2016; Roe et al. 2004). The predicted protein structure of Cdc37 by AlphaFold provides a similar structure (Fig. 5.1c) (Jumper et al. 2021). The N-terminus kinase-binding domain (NKD) of Cdc37 is the most conserved across evolution, containing S13 and the 20HPN22 motif responsible for probing the kinase CL. Phosphorylation of S13 by casein kinase II (CKII) is required for kinase recognition and stable assembly of the Hsp90-Cdc37-kinase complex (Shao et al. 2003). Phosphorylation of S13 on

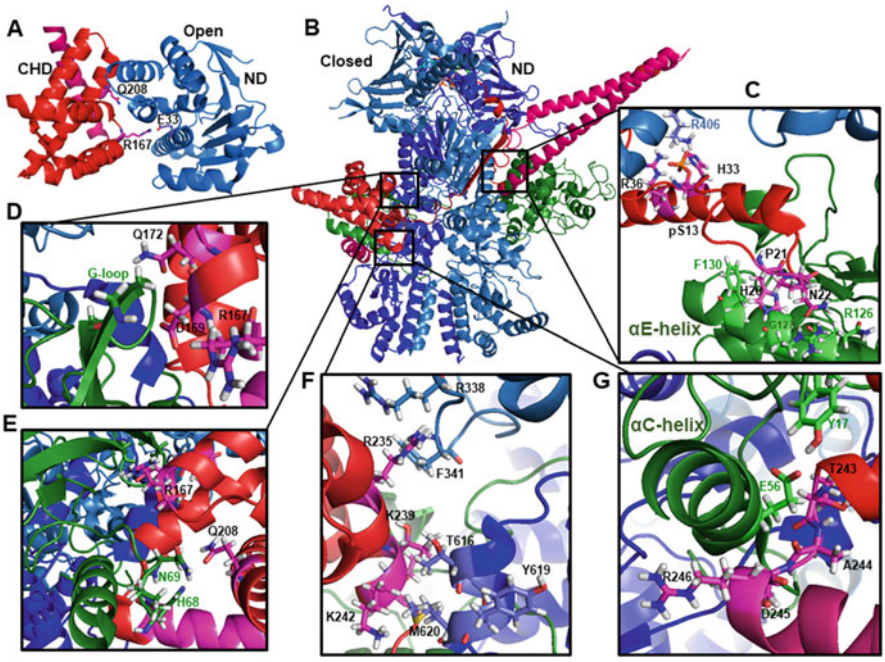

Fig. 5.2 Cdc37 molecular interactions. (**a**) Human Cdc37 CHD interacts with yeast Hsp90 ND in "open" client-loading state. Cdc37 R137 forms a salt bridge with catalytic residue E33 of Hsp90 (PDB: 1US7). (**b**) Cdc37 binds across Hsp90 with the CHD interacting with the MD in the "closed" client-holding state. (**c**) Phosphorylation of Cdc37 on S13 arranges residues H33 and R36 along with R406 on Hsp90. This allows the probing of the αE-helix notch in the CL of Cdk4 by the 20HPN motif of Cdc37. (**d**) Conserved residues Q172 and D169 of Cdc37 pinch the G-loop in the NL of Cdk4. (**e**) Q208 of Cdc37 interacts with 68HPN αC-β4 loop of Cdk4. (**f**) R235 of Cdc37 packs against R338 and F341 of the near Hsp90 protomer, while K339 and K342 of Cdc37 interface with T616 and M620 on the α-helix containing the phosphorylation site Y619 on the far Hsp90 protomer. (**g**) 243TADR of Cdc37 cradles the αC-helix of Cdk4 and orients catalytic E56

Cdc37 allows for electrostatic interactions between H33 and R36 of Cdc37 and R406 of Hsp90. This arrangement provides the rigidity for the 20HPN motif of Cdc37 to probe the αE-helix notch on Cdk4 (Fig. 5.2c). Dephosphorylation of S13 is performed by Hsp90-associated phosphatase, PP5, which allows for kinase release and Cdc37 dissociation (Vaughan et al. 2008). Another phosphorylation site exists at Y4 although the biological significance of this residue has not been elucidated. The NTD is followed by a coiled coil that extends away from the complex before transitioning into a β-sheet binding alongside the middle domain of Hsp90. According to the *PhosphoSitePlus* database, several PTMs exist in this region although not all of their biological roles have not been studied. However, it has been found that phosphorylation of S97 by PKA in response to non-steroidal anti-inflammatory drugs blocks Cdc37 interaction with Hsp90 leading to Axl kinase degradation and prevention of Zika virus cell entry (Pan et al. 2018).

Next, the central Hsp90-binding domain (CHD) of Cdc37 interacts with both the kinase NL and Hsp90. This domain shares homology with the co-chaperone Cdc7L1 also known as Harc (Scholz et al. 2001). Interestingly, the exact biological function and client set of Harc have not been fully explored. Depending on the conformational state, the central domain of Cdc37 binds Hsp90 in two different places. This effect is also observed for other co-chaperones such as Aha1, which promotes Hsp90's ATPase activity, and Hop, which coordinates client loading and inhibits Hsp90 ATPase activity in a similar manner to Cdc37 (Eckl et al. 2013). Further description of the ATP-fueled Hsp90 chaperone cycle and its numerous co-chaperones has been recently reviewed by our group and others (Lang et al. 2021).

In the "open" Hsp90 client loading state, Cdc37 binds the N-terminal ATP-binding domain (Fig. 5.2a) (Roe et al. 2004), while in the "closed" client holding state, Cdc37 binds the middle domain (MD) of Hsp90 (Fig. 5.2b) (Verba et al. 2016). The crystal structure generated by Roe et al. shows that human Cdc37 CHD inserts an arginine (R167) side chain into the ATP-binding pocket of yeast Hsp90 N-terminal domain, thereby locking Hsp90 into an "open" conformation (Roe et al. 2004). The amino acid sequence surrounding R167 is relatively conserved, including Harc. In the "closed" kinase-binding structure, however, the sequence surrounding R167 does not bind Hsp90 but instead interacts with the kinase NL. Here the kinase G-loop motif is grasped by amino acids D169 and Q172 (Fig. 5.2d), which are also conserved in Harc, suggesting it may also recognize a similar motif in non-kinase clients. Similarly, the Q208 residue of Cdc37 interacts with the "open" Hsp90 ND, but in the "closed" conformation where it interfaces with the αC-β4/HPN kinase motif (Fig. 5.2e). This property suggests that human Cdc37 has evolved to pivot between inducing the "open" Hsp90 client-loading state and the "closed" Hsp90 client-holding state, interacting with the same set of amino acid residues. However, what these interactions indicate, regarding the sequential steps and regulation of the Hsp90 chaperone cycle, deserves further investigation. It is also worth noting that Cdc37 mutants can stabilize client kinases without substantial CHD interaction with Hsp90 in cultured cells, suggesting the CHD is secondary to the conserved NKD in providing chaperone activity (Smith et al. 2015).

Cdc37 also interacts with each Hsp90 protomer and kinase exclusively in the "closed" state through a 15 amino acid helix-turn-helix motif. Here Cdc37 inserts a conserved R235 side chain into a pocket in the middle domain of the near Hsp90 protomer. This is followed by T238, K240, and K242 interaction with an α-helix in the C-terminal domain of the far Hsp90 protomer. Remarkably, phosphorylation of Hsp90 at Y619 in this α-helix has been found to dissociate client kinases from the chaperone complex (Fig. 5.2f) (Xu et al. 2012). Adjoining this interaction, amino acids 243TADRQ247 are observed to grasp the N-terminal end of the kinase αC-helix including the catalytic glutamate (56E) (Fig. 5.2g). This interaction further provides Cdc37 with its kinase-binding specificity and explains why antibodies that bind the αC-helix of kinases, such as Cdk2, disrupt Cdc37 and Hsp90 interaction (Prince et al. 2005). Notably, dimerization of Cdc37 is also predicted to occur

through residues within and downstream of this helix-turn-helix motif (Roe et al. 2004; Roiniotis et al. 2005).

The far C-terminal domain (CTD) of Cdc37 has been shown to be an α-helix bundle at the end the long α-helix extending out of the CHD. The exact function of this domain is not clear although it contains a phosphorylation site at Y298, suggesting that it may function as a scaffold for recruiting SH2 domain containing kinases, such as Yes, that regulate client kinase folding and dissociation through eventual phosphorylation of Hsp90 at Y197 (Xu et al. 2012). This domain is also phosphorylated by Ulk1 at S339 and results in disruption of kinase recruitment to the Hsp90-Cdc37 complex, suggesting that kinase maintenance might be reduced during autophagy (Li et al. 2017). The remaining C-terminal sequence of Cdc37 exists as an unstructured tail according to the structure predicted by AlphaFold (https://alphafold.ebi.ac.uk/entry/Q16543) (Fig. 5.1c, d).

Cdc37 in Cell Proliferation and Cancer

Molecular chaperones including heat shock proteins and co-chaperones are known to play key roles in the etiology of cancer (Ciocca et al. 2013; Calderwood et al. 2006; Calderwood and Gong 2016). As a cell cycle division protein, required to drive cell proliferation, it is probably not surprising that Cdc37 appears to play a positive role in tumorigenesis (Stepanova et al. 2000a; Gray Jr. et al. 2008) (Fig. 5.3). An early hint suggesting such a role was provided by the finding of a requirement for Cdc37, along with Hsp90 in the transforming functions of the viral oncogene p60/v-src (Dey et al. 1996; Perdew et al. 1997). More conclusive evidence for a transforming role for Cdc37 was next provided by the finding that overexpression of the *Cdc37* gene in transgenic mice could lead to elevated rates of prostate tumorigenesis, a process that was amplified by co-expression of the proto-oncogene c-Myc (Stepanova et al. 2000b). Consistently, increased expression of Cdc37 was found in prostate cancer cell lines (PC-3, LNCaP, and DU-145) as compared to normal prostate epithelial cells (RWPE-1) (da Rocha et al. 2005; Eguchi et al. 2019). Cdc37 has been recently shown to be transcribed by the zinc finger transcription factor MZF1 through binding to multiple motifs in the gene promoter (Eguchi et al. 2019; Eguchi et al. 2015) (Fig. 5.3a). MZF1 knockdown reduced prostate cancer growth and decreased Cdc37 expression in multiple prostate carcinoma cells (Eguchi et al. 2019). Interestingly, SCAN-D1, an endogenous inhibitor of MZF1 found abundantly in non-malignant prostatic epithelial cells, reduced Cdc37 expression and malignancy (Eguchi et al. 2019). Moreover, HSF1 the key transcription regulator of the HSR activates CDC37 expression in prostate cancer (Eguchi et al. 2020; Murshid et al. 2018). In addition to prostate carcinoma, other cancer types such as anaplastic large cell lymphoma, acute myeloblastic leukemia, multiple myeloma, and hepatocellular carcinoma have been shown to express high levels of Cdc37 (Gray Jr. et al. 2008). Indeed, Cdc37 appears to play a key role in fostering of anaplastic large cell lymphoma (Kuravi et al. 2019).

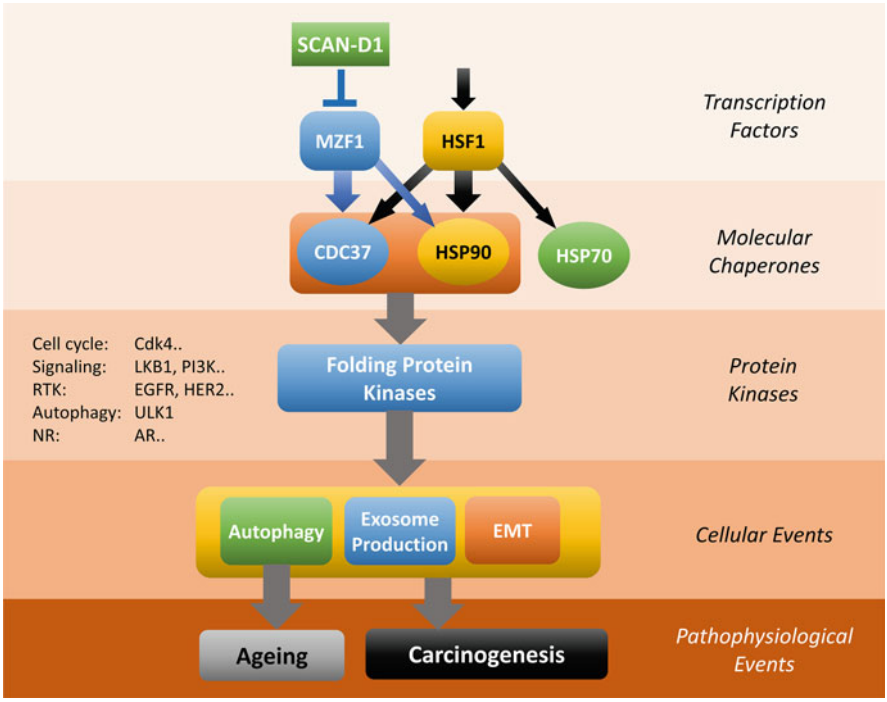

Fig. 5.3 (**a**) Pathophysiological cascade mediated by molecular chaperones. The Cdc37/Hsp90 chaperone complex promotes carcinogenesis by assisting folding of many protein kinases that promote EMT and exosome production. In prostate cancer, expression of Cdc37/Hsp90 is induced by oncogenic transcription factors such as MZF1 and HSF1 while suppressed by tumor suppressor SCAND1 (da Rocha et al. 2005; Schwartz et al. 2015). (**b**). Cdc37 controls a network of intracellular protein kinases. Cdc37 is able to influence a wide spectrum of protein kinases through their highly conserved catalytic domain. Depicted here are Cdc37 regulation of the ERK-MAP kinase pathway, the phosphatidylinositol-3-kinase (PI-3-K) pathway, Unc-51-like kinase (ULK-1) activity, and the activity of AR. In this way, Cdc37 is able to control a wide spectrum of intracellular metabolic pathways involved in cell growth, survival, autophagy, and carcinogenesis. AR seems to be exceptional in being a non-kinase client of Cdc37

However, the exact role of Cdc37 in promoting tumorigenesis remains difficult to tie down due to the large numbers of potential Hsp90/Cdc37 targets with potential roles in carcinogenesis. Nevertheless, probable candidates include the numerous kinase signaling pathways and the androgen receptor (AR) (Heinlein and Chang 2004). While most steroid hormone receptors require Hsp90 for optimal folding and activity, only AR appears to be dependent on both Hsp90 and Cdc37 (Fliss et al. 1997; Rao et al. 2001). Indeed, Cdc37 knockdown in AR^+ LNCaP cells was shown to lead to the loss of androgen-dependent AR-mediated transcriptional activity and to a reduction in target prostate-specific antigen (PSA) expression (Gray Jr. et al. 2007). It may be significant that Cdc37 has been found to be associated with the AR co-activating protein Vav3 (Wu et al. 2013). Disruption of AR-Vav3 interactions

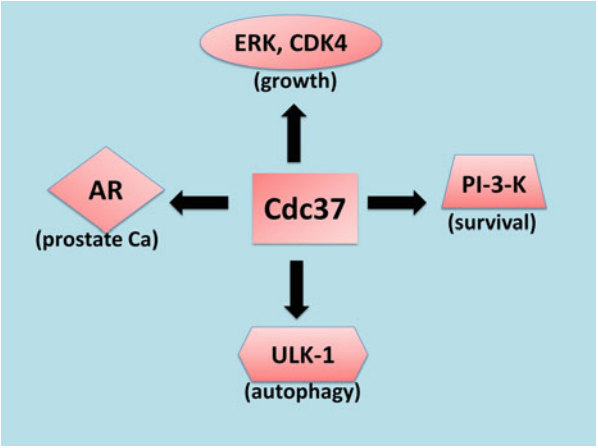

Fig. 5.3 (continued)

inhibited the co-activating effects of Vav3 (Wu et al. 2013). AR has been shown to be essential for the early stages in prostate tumorigenesis and to even play unpredicted roles in castration-resistant forms of PCa. Therefore, a role for Cdc37 in fostering AR activity and prostate carcinogenesis appears to be likely. Protein kinases within most pro-growth pathways are the preferred clients of Cdc37 (Gray Jr. et al. 2008). The pro-survival phosphatidyl-inositol-3 lipid kinase (PI-3 K) pathway plays a major driving role in prostate carcinogenesis often due to the inactivation of the lipid phosphatase PTEN tumor suppressor (Bitting and Armstrong 2013). Although PI-3 K is not a known client, knockdown of Cdc37 leads to reduction of PI-3 K downstream signaling through inhibition of Akt as well as phosphorylation of the S6 ribosomal protein and the mTORC1 nutrient-sensing complex (Gray Jr. et al. 2007). The mTORC1 pathway has been shown to play key roles in cancer progression by boosting the rate of translation in tumor cells and permitting elevated protein synthesis in such cells. Other potential Cdc37-dependent targets could include receptor tyrosine kinases such as EGFR and HER2/*neu* that are Cdc37 clients and could also play roles in prostate cancer (Calderwood, S. K. et al., in preparation), (Lavictoire et al. 2003).

Cdc37 may also play a role in the secretion from tumor cells of extracellular vesicles (EV) that promote tumor progression in surrounding cells. Triple depletion of Cdc37, Hsp90 alpha, and Hsp90 beta reduced the transforming potential of EVs released from prostate cancer and metastatic oral cancer (Ono et al. 2020). In addition, siRNA-based knockdown of Cdc37 significantly reduced the overall protein contents of prostate cancer cells and their EVs, suggesting that Cdc37 is broadly essential for proteostasis in the prostate carcinoma cells and EVs. Knockdown of Cdc37 significantly reduced the levels of CD9, one of the most established exosomal markers, indicating that Cdc37 is essential for exosome biogenesis and secretion. Moreover, triple depletion of Cdc37, Hsp90 alpha, and Hsp90 beta inhibited in vivo tumorigenesis of castration-resistant prostate cancer cells, which are AR-negative

prostate cancers (Eguchi et al. 2020). Cdc37 may thus contribute both to intracellular and extracellular components of oncogenic signaling.

Cdc37 and Cancer Treatment

The dependence of malignant cells, particularly prostate carcinoma cells, on Cdc37 suggests this molecule as a promising potential target. This approach would have the decided advantage of leading to multi-targeting and the potential for evasion of resistance, in contrast to targeting individual oncoproteins in which evolution of resistance is problematic. Cdc37 knockdown was shown to reduce proliferation to minimal levels in a range of malignant cell types (Gray Jr. et al. 2008; Gray Jr. et al. 2007; Smith and Workman 2009). A natural product-based drug has recently been isolated that can disrupt Hsp90/Cdc37 interactions. This compound celastrol might thus be employed for targeting Cdc37 activity in cancer (Salminen et al. 2010). However, this compound is somewhat lacking in specificity and was shown to directly inhibit both kinase activity and the function of the proteasome and to induce HSF1 activity (Calderwood 2013). Non-celastrol small molecule Cdc37 inhibitors have been described and may have potential in therapy (Wang et al. 2019; Huang et al. 2014). However, a group of small peptides predicted from structural studies to inhibit Cdc37-Hsp90 interaction proved non-toxic to human cancer cells (D'Annessa et al. 2020). No doubt future endeavors will lead to further Cdc37-targeted drugs. An indirect approach to targeting Cdc37 could be through reducing its gene expression in malignant cells by inhibiting MZF1 as described above (Eguchi et al. 2019). Moreover, recent drug development approaches such as molecular glues, proteolysis-targeting chimeras (PROTAC), and chaperone-mediated protein degradation (CHAMP) compounds may also find a way to take advantage of the intimate interactions between Cdc37, Hsp90, and protein kinases to induce targeted protein degradation of oncogenes.

Roles for Cdc37 in Autophagy and Protein Aggregation Disorders

Unsurprisingly, with its versatile role in promoting kinase activation, Cdc37 appears to also play roles in cell pathologies outside of cancer. The Hsp90-Cdc37 complex appears to participate in the upstream activation of autophagy, one of the primary pathways in protein quality control and longevity (Calderwood et al. 2009). Autophagy is a significant mechanism in protein homeostasis in that bulky protein aggregates or damaged organelles that cannot enter the lumen of the proteasome for proteolytic digestion can be enveloped by autophagosomes and broken down (Calderwood et al. 2009). The Cdc37-Hsp90 complex was shown to stabilize and

activate ULK1, a protein kinase that phosphorylates Atg1 as one of the first steps in initiating the autophagy pathway and in this way regulates mitophagy, a specialized autophagy-like process involved in breaking down damaged mitochondria (Joo et al. 2011) (Fig. 5.3b). Although once activated, ULK1 may shutdown further kinase maintenance and activation (Li et al. 2017). Cdc37-fostered autophagy may be important in neurodegenerative diseases such as amyotrophic lateral sclerosis and Alzheimer's disease that are components of the aging process, and chaperone complexes may be involved in clearance of misfolded proteins through the autophagy pathway (Jinwal et al. 2012; Lackie et al. 2017; Gracia et al. 2019). Furthermore, Cdc37's regulation of eNOS may also affect aging and ability to combat infection such as SARS-Cov2 (Harris et al. 2006; Guan et al. 2020).

Conclusions

Cdc37, as a major component of the Hsp90 complex that controls the folding of protein kinases in the cell, stands at the hub of a multitude of intracellular signaling networks (Gray Jr. et al. 2008; Karnitz and Felts 2007; Caplan et al. 2007) (Fig. 5.3). This folding function involves several intricate interactions and may be highly regulated by PTMS. The intracellular properties of Cdc37 thus reach beyond the housekeeping pathways of protein folding into a wide range of cell regulatory processes. Moreover, the role of extracellular Cdc37 and its association with malignancy deserves further attention as a possible cancer biomarker.

Due to its enabling influence, Cdc37, as described above, has attracted much attention as a potential intermediate in carcinogenesis, and indeed proof-of-concept cell line studies indicate that Cdc37 is required for cancer cell signaling and that quenching activity of the co-chaperone prevents malignant cell growth (Gray Jr. et al. 2008). Consequently, Cdc37 could continue to be an attractive target for combatting cancer as a result of it being expressed at high levels in several tumor types, and its targeting could provide a degree of specificity compared to normal cells. Cdc37 may also be associated with the activated forms of Hsp90 that are observed in cancer cells and that are thought to contribute to the retention of Hsp90 inhibitors in tumors (Kamal et al. 2003; Rodina et al. 2016). This property, combined with the fact that the co-chaperone is essential for key pro-growth signaling pathways, suggests that targeting Cdc37 may be relevant when addressing cancers that are driven by multiple oncogenic kinases and/or AR activity as a way to prevent the evolution of resistance by the tumor. Unfortunately, reliably and specific agents to target Cdc37 are currently not available.

Protein aggregation disorders have also been linked to molecular chaperones and to age-related declines in molecular chaperones and co-chaperones (Calderwood et al. 2009). Cdc37 appears to be a potential agent in longevity due to its links to protein folding and autophagy, and it will be informative to study the role of Cdc37 maintenance/decline in aging organisms. The development of agents that might

modulate Cdc37 activity levels may ultimately be called for to remedy aging-related shortfalls.

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Chapter 6
p23 and Aha1: Distinct Functions Promote Client Maturation

Maximilian M. Biebl and Johannes Buchner

Abstract Hsp90 is a conserved molecular chaperone regulating the folding and activation of a diverse array of several hundreds of client proteins. The function of Hsp90 in client processing is fine-tuned by a cohort of co-chaperones that modulate client activation in a client-specific manner. They affect the Hsp90 ATPase activity and the recruitment of client proteins and can in addition affect chaperoning in an Hsp90-independent way. p23 and Aha1 are central Hsp90 co-chaperones that regulate Hsp90 in opposing ways. While p23 inhibits the Hsp90 ATPase and stabilizes a client-bound Hsp90 state, Aha1 accelerates ATP hydrolysis and competes with client binding to Hsp90. Even though both proteins have been intensively studied for decades, research of the last few years has revealed intriguing new aspects of these co-chaperones that expanded our perception of how they regulate client activation. Here, we review the progress in understanding p23 and Aha1 as promoters of client processing. We highlight the structures of Aha1 and p23, their interaction with Hsp90, and how their association with Hsp90 affects the conformational cycle of Hsp90 in the context of client maturation.

Keywords Hsp90 · p23 · Aha1 · Hch1 · Glucocorticoid receptor

The Conformational Cycle of Hsp90

Hsp90 is an abundant molecular chaperone that is conserved from bacteria to humans (Chen et al. 2006; Taipale et al. 2010; Johnson 2012). For the maturation of a diverse set of several hundred client proteins, Hsp90 must undergo an ATP-driven chaperone cycle, comprising large conformational rearrangements (Fig. 6.1) (Weikl et al. 2000; Shiau et al. 2006; Cunningham et al. 2008; Echeverria et al. 2011; Taipale et al. 2012). Hsp90 homodimerization to a V-shaped dimer is mediated by the C-terminal domain (CTD) (Harris et al. 2004; Ali et al. 2006; Shiau

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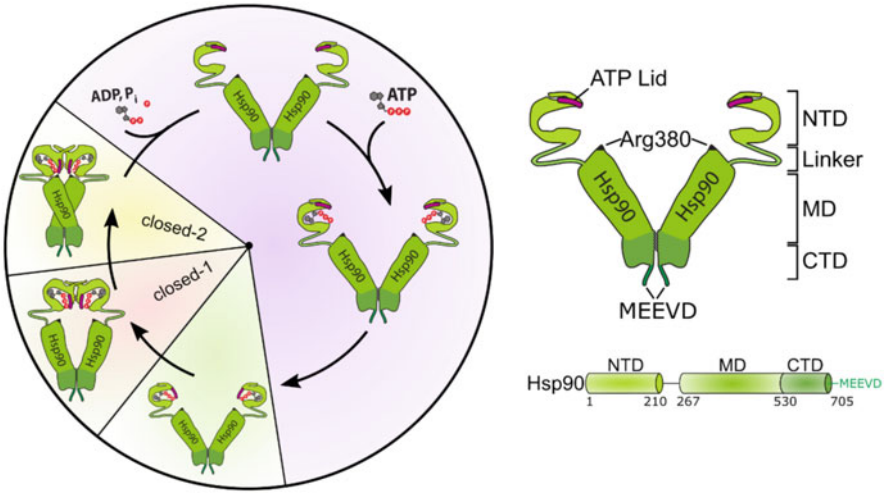

Fig. 6.1 The structure and conformational cycle of Hsp90. Hsp90 conformations that are populated during the ATPase cycle of Hsp90 are shown. After binding of ATP, the lid (pink) closes over the nucleotide-binding pocket. The N-terminal domains of Hsp90 dimerize forming the closed-1 state. Twisting and further compaction yield the ATPase-competent closed-2 state. In this state, the Arg380 residue of the Hsp90 MD contacts the bound ATP stabilizing the closed Hsp90 conformation and potentially catalyzing hydrolysis. After release of ADP and phosphate, the Hsp90 cycle is completed and Hsp90 returns to the open conformation. The domain architecture of yeast Hsp90 is shown on the right

et al. 2006; Wayne and Bolon 2007). Additionally, the C-terminal end of cytosolic eukaryotic Hsp90 contains the terminal MEEVD motif important for the binding of tetratricopeptide repeat (TPR)-containing co-chaperones (see below) (Scheufler et al. 2000; Schopf et al. 2017). The middle domain (MD) is implicated in client binding (Bohen and Yamamoto 1993; Nathan and Lindquist 1995; Kirschke et al. 2014; Lorenz et al. 2014; Karagoz and Rudiger 2015; Verba et al. 2016) and connected to the N-terminal domain by a large, charged linker (Hainzl et al. 2009; Tsutsumi et al. 2012; Zuehlke and Johnson 2012; Lopez et al. 2020). Nucleotides which regulate the conformational cycle are bound by the N-terminal domain (NTD) (Prodromou et al. 1997a, b; Panaretou et al. 1998; Hessling et al. 2009).

Binding of ATP to the NTD leads to the closing of the ATP lid—a helical segment in the NTD that locks the nucleotide in its binding pocket—and the association of the two NTDs accompanied by a partial closing of the Hsp90 dimer into the closed-1 state (Fig. 6.1) (Prodromou et al. 1997b, 2000; Richter et al. 2006, 2008; Hessling et al. 2009). Further compaction of the dimer including the twisting of the protomers yields the closed-2 state (Ali et al. 2006; Richter and Buchner 2006). In this state, a catalytic loop in the MD carrying the catalytic Arg380 residue (in yeast Hsp90) is repositioned and contacts the γ-phosphate of ATP (Ali et al. 2006; Mader et al. 2020). In this regard, Arg380 seems to enable ATP binding and to promote the closed state of Hsp90, since mutation of Arg380 inhibits Hsp90 closing

(Meyer et al. 2003; Cunningham et al. 2012; Schulze et al. 2016). Closing of Hsp90, i.e., the interaction of the N-domains and of the N- and M-domains, completes the Hsp90 "split ATPase" which is required for ATP hydrolysis (Meyer et al. 2003; Cunningham et al. 2008; Cunningham et al. 2012). The rate-limiting conformational changes required to form an ATPase-competent state render Hsp90 a very slow ATPase with hydrolysis rates of about 1 ATP/min and 0.1 ATP/min for yeast and human Hsp90, respectively (Panaretou et al. 1998; McLaughlin et al. 2006; Richter et al. 2008). Release of ADP and phosphate completes the cycle and induces the transition to the open state. While ATP hydrolysis by Hsp90 has long been considered essential for the function of Hsp90 in vivo, more recent data on Hsp90 mutants suggest that the ability of ATP to induce and populate different conformational states and the dwell time spent in specific conformations is important, but ATP hydrolysis is not necessarily required (Zierer et al. 2016). Notably, the general architecture of the Hsp90 ATPase cycle is conserved between isoforms and species, yet differences in the directionality, the population distribution of different states, and details of the hydrolysis mechanism have become evident (Southworth and Agard 2008; Graf et al. 2009; Mickler et al. 2009; Ratzke et al. 2012; Lavery et al. 2014; Huck et al. 2017).

The Hsp90 cycle is further regulated by different mechanisms including post-translational modifications (Mollapour and Neckers 2012; Backe et al. 2020), client binding (Rutz et al. 2018), subcellular localization, and the binding of co-chaperones (Biebl and Buchner 2019). The co-chaperome of Hsp90 has expanded from bacteria lacking co-chaperones over yeast encoding 12 co-chaperones to man where more than 25 co-chaperones have been identified (Johnson 2012; Li and Buchner 2013; Taipale et al. 2014; Woodford et al. 2016, 2017). Co-chaperones may bind Hsp90 in a conformation-selective way and either activate or deactivate the Hsp90 ATPase activity. Additionally, some co-chaperones function as selective recruiters of client proteins for Hsp90 (Grammatikakis et al. 1999; Taipale et al. 2012; Keramisanou et al. 2016). Notably, the network of Hsp90 co-chaperones is dynamic and reorganizes functionally to meet the requirements of Hsp90 clients in a client-specific way (Sahasrabudhe et al. 2017; Biebl et al. 2020). Within a network of loosely co-operating co-chaperones, for each client few tightly interacting modules are evident that promote client maturation (Biebl et al. 2020).

Due to the functional diversity of Hsp90 co-chaperones, they have been categorized structurally in two subgroups: (1) TPR-containing co-chaperones such as the Hsp70-Hsp90 organizing protein (Hop), peptidyl-prolyl-cis/trans-isomerases (PPIases), and protein phosphatase 5 (PP5) and (2) non-TPR-containing co-chaperones such as "activator of Hsp90 ATPase" (Aha1) and p23.

p23 and Aha1 have been studied intensely in the past. They have in common that their interaction with the NTD and MD of Hsp90 affects the Hsp90 ATPase and client binding, albeit in opposing ways. In this review, we will present progress made to elucidate the roles of p23 and Aha1 in the Hsp90 system.

The Hsp90 Co-chaperone p23

Discovery and Isoforms of p23

p23 was discovered in complexes of steroid hormone receptors (SHRs) and Hsp90 by the Toft lab in the 1990s (Smith et al. 1990; Johnson et al. 1994; Johnson and Toft 1995). Later p23 has been found in association with hepatitis B virus reverse transcriptase (Hu et al. 1997), telomerase (Holt et al. 1999), and other proteins (Nair et al. 1996; Xu et al. 1997). Since then, p23 has become one of the best studied Hsp90 co-chaperones (Sullivan et al. 1997; Weaver et al. 2000; Richter et al. 2004; Ali et al. 2006; Martinez-Yamout et al. 2006; McLaughlin et al. 2006; Echtenkamp et al. 2011; Karagoz et al. 2011; Li et al. 2011; Rehn and Buchner 2015; Biebl et al. 2021), and additional, Hsp90-independent functions have been found (Bose et al. 1996; Freeman et al. 1996; Echtenkamp et al. 2016; Wu et al. 2018). The yeast homolog Sba1 only shares 26% sequence identity with human p23 (Fig. 6.2a) (Johnson et al. 1994; Bohen 1998; Fang et al. 1998). Whereas p23 is not expressed in human heart and striated muscle, a second human isoform, Aarsd1 (formerly named tsp23), sharing 44% sequence identity with p23, is expressed solely in these tissues and was shown to be crucial for muscle differentiation (Freeman et al. 2000; Echeverria et al. 2016).

The Structure of p23

p23 is a small, acidic protein encoded by the PTGES3 gene in humans. Early circular dichroism studies suggested that p23 comprises a predominantly β-sheet--rich folded domain as well as a largely unstructured C-terminal tail. This tail is 56 amino acids long in human p23 and 93 amino acids in yeast p23 (Fig. 6.2b, c) (Weikl et al. 1999). This architecture was defined in more detail by X-ray crystallography and NMR spectroscopy (PDB: 2CG9, 1EJF) (Weaver et al. 2000; Ali et al. 2006; Martinez-Yamout et al. 2006; Biebl et al. 2020). The stably folded CS domain of p23 comprises a well-conserved 7-stranded β-sandwich fold similar to the α-crystallin fold of small Hsps (Fig. 6.2a, b) (Kim et al. 1998; Weaver et al. 2000; Garcia-Ranea et al. 2002; Ali et al. 2006). Interestingly, CS domains are also found in the co-chaperones Sgt1 and NudC (Zhang et al. 2010; Zheng et al. 2011).

Based on the amino acid composition, the C-terminal tail of yeast p23 can be divided in an acidic stretch adjacent to the core domain, followed by a GM/A-rich and a QL-rich stretch as well as a second, C-terminal acidic region. Yet the functional implications of these motifs have remained elusive (Fig. 6.2c). While the unfolded tail could not be resolved in crystal structures (Weaver et al. 2000; Ali et al. 2006), recent NMR studies suggest that the proximal region of the p23 tail has limited flexibility and forms contacts with the core domain (Biebl et al. 2020). This region contains residues that define the interaction with Hsp90 and the function of

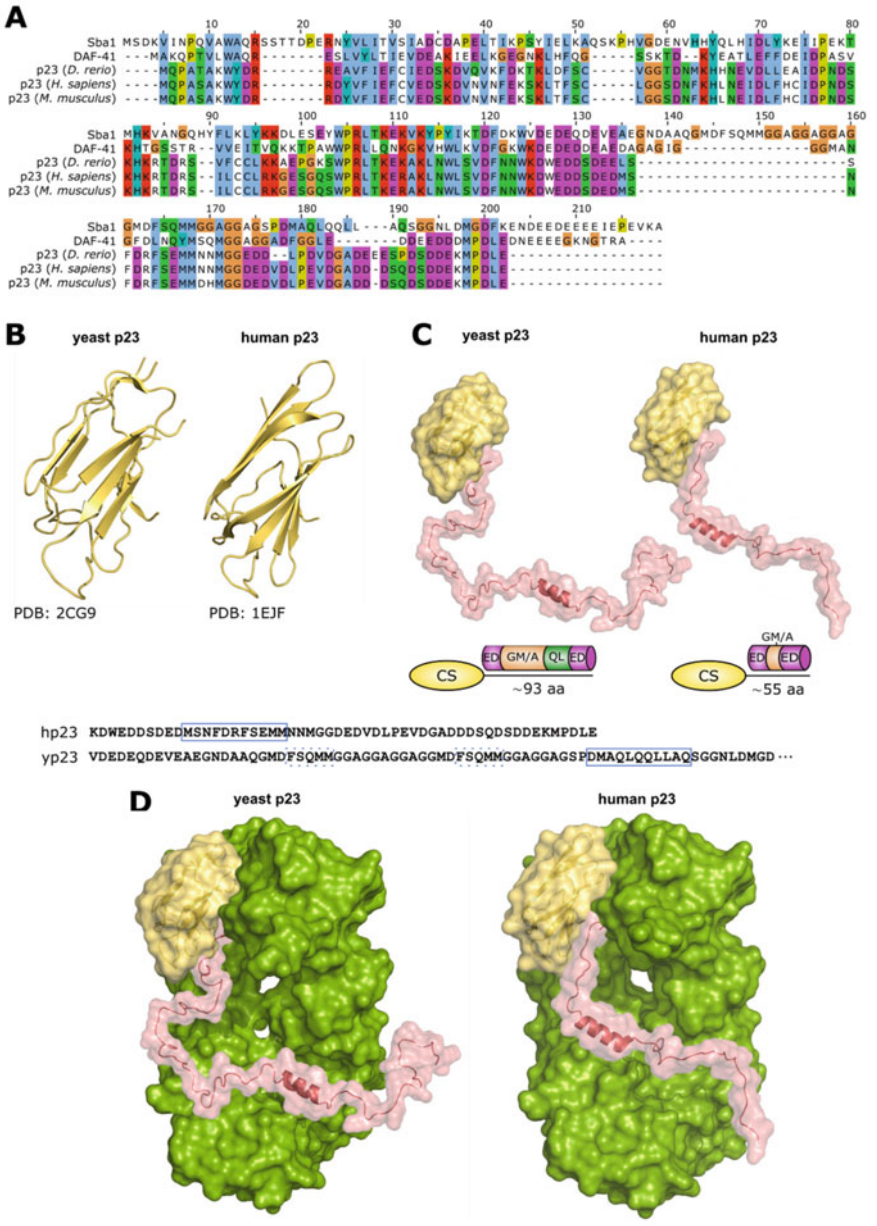

Fig. 6.2 p23 harbors a folded CS domain and a long, mostly unstructured tail. (**a**) The alignment of the indicated p23 homologs is shown (Sba1 = yeast p23; DAF-41 = nematode p23). Alignment was done using the Clustal Omega tool, and the ClustalX color code was applied in Jalview 2.11.1.3. (**b**) The folded CS domains of yeast p23 (PDB: 2CG9) and human p23 (PDB: 1EJF) are depicted. (**c**) The C-terminal unfolded tail which is not resolved in the structures shown in (**a**) has been modeled in PyMol 1.7.1.1. The location of the tail helix which directly interacts with GR (Noddings et al. 2020; Biebl et al. 2021) is indicated. The composition of the tail is shown as a cartoon (ED = glutamate- and aspartate-rich, GM/A = glycine, methionine/alanine-rich, QL =

p23 (see below) (Wochnik et al. 2004; Biebl et al. 2020). Notably, deletion of a major part of the tail does not interfere with Hsp90 binding but impairs the Hsp90-independent in vitro chaperone function of p23 (Bose et al. 1996; Freeman et al. 1996; Weikl et al. 1999; Forafonov et al. 2008). Recent data indicated the presence of α-helical segments within the tail of yeast and human p23 which seems to be important for client processing (Fig. 6.2c) (Seraphim et al. 2015; Biebl et al. 2020; Noddings et al. 2020).

The Interaction of p23 with Hsp90

Early work on Hsp90 revealed that complexes between Hsp90 and the progesterone receptor (PR) were stabilized by the addition of non-hydrolyzable ATP analogs such as AMP-PNP or by molybdate (Dahmer et al. 1984; Johnson and Toft 1995). It turned out that these conditions promote p23 binding (Johnson et al. 1994; Johnson and Toft 1995). Additionally, it was found that selective Hsp90 inhibitors such as geldanamycin disrupt p23 binding (Johnson and Toft 1995; Sullivan et al. 1997; Fang et al. 1998; Sullivan et al. 2002). Later work revealed that p23 is a conformational sensor of the closed-2 state of Hsp90 (Hessling et al. 2009; Li et al. 2011). p23 binds the closed Hsp90 state and contacts both protomers (Fig. 6.2d). Binding involves the Hsp90 ATP lid, the catalytic loop, and the N-terminal regions of the Hsp90 MD (Ali et al. 2006; Martinez-Yamout et al. 2006; Biebl et al. 2020). Notably, NMR spectroscopy also revealed transient contacts of the p23 C-terminal tail with an amphipathic helix protruding from the Hsp90 CTD that has been implicated in client binding (Fig. 6.2d) (Bohen and Yamamoto 1993; Nathan and Lindquist 1995; Kirschke et al. 2014; Lorenz et al. 2014; Karagoz and Rudiger 2015; Verba et al. 2016; Biebl et al. 2020).

Binding of p23 to Hsp90 partially inhibits the Hsp90 ATPase (McLaughlin et al. 2002; Panaretou et al. 2002; Richter et al. 2004; McLaughlin et al. 2006). In contrast to the well-known allosteric Hsp90 inhibitor Hop, which keeps Hsp90 in an open, ATPase-incompetent conformation, p23 locks Hsp90 in the closed state, implicating a different inhibitory mechanism (Chen and Smith 1998; Johnson et al. 1998; Prodromou et al. 1999; Richter et al. 2003). Two basic inhibition mechanisms are possible and still under discussion: Hsp90 could either be locked in a pre-hydrolysis state inhibiting the hydrolysis of ATP or ATP hydrolysis may occur, but release of

Fig. 6.2 (continued) glutamine- and leucine-rich). The sequences of the C-terminal tail of human p23 (hp23) and yeast p23 (yp23) are presented at the bottom: The α-helices interacting with the GR are highlighted by a solid box, and additional, predicted α-helices in the yeast sequence are indicated by dashed boxes. (**d**) Left: The binding of yeast p23 to yeast Hsp90 (PDB: 2CG9) is shown, and the C-terminal tail was modeled with PyMol 1.7.1.1. Right: The human p23 (PDB: 1EJF) core domain was aligned to yeast p23 bound in the crystal structure of yeast Hsp90 with yeast p23 (PDB: 2CG9), and the C-terminal tail was modeled using PyMol 1.7.1.1

ADP and phosphate required for the progression of the ATPase cycle may be inhibited. Evidence for both scenarios has been provided: NMR studies (Karagoz et al. 2011) suggested the inhibition of ATP hydrolysis, while the positioning of the catalytic loop found in the crystal structure of Hsp90 with p23 (Ali et al. 2006) and hydrogen exchange mass spectrometry favored a model of decelerated ADP release (Graf et al. 2014). In this context, two strictly conserved aromatic residues in p23 that lie in the proximal tail region were found to mediate binding to and the inhibition of Hsp90 (Wochnik et al. 2004; Biebl et al. 2020). Specifically, Trp124 in yeast and Trp106 in human p23 are pivotal to shift the conformation of the catalytic loop which prevents ATP hydrolysis. This finding favors a model in which p23 inhibits the ATP hydrolysis step (Biebl et al. 2020).

Two p23 molecules can bind the Hsp90 dimer in vitro (Richter et al. 2004; Ali et al. 2006; Karagoz et al. 2011). In the cellular environment, the concentration of p23 like that of all Hsp90 co-chaperones is much lower than that of Hsp90 (Ghaemmaghami et al. 2003; Finka and Goloubinoff 2013) suggesting that a 1:2 stoichiometry is more likely in vivo. Additionally, the Hsp90 co-chaperones and clients compete for binding sites on Hsp90, making a 2:2 stoichiometry for any co-chaperone unlikely in the cell (Lorenz et al. 2014; Noddings et al. 2020).

p23 and Hsp90 Client Maturation

The first implication of the Hsp90-associated function of p23 was linked to steroid hormone receptor maturation (Johnson et al. 1994; Johnson and Toft 1995). SHRs are a class of stringent Hsp90 clients that have been intensively studied in pioneering work by the Pratt, Toft, Smith, and Yamamoto labs (Smith et al. 1992; Smith 1993; Chen et al. 1996; Pratt et al. 1996; Dittmar et al. 1997; Dittmar and Pratt 1997; Pratt and Toft 2003; Picard 2006a). Reconstitution of a minimal chaperone system including Hsp40, Hsp70, Hop, Hsp90, and p23 allowed the activation of SHRs in vitro (Dittmar et al. 1996; Pratt and Dittmar 1998; Pratt and Toft 2003). In current models, clients are first bound by Hsp40 and Hsp70 (“early complex”) (Dittmar et al. 1998; Hernandez et al. 2002). Hsp70 together with Hsp40 induces local unfolding of the client, presumably to allow escape from kinetic traps in the folding pathway (Kirschke et al. 2014; Sekhar et al. 2016; Rosenzweig et al. 2017; Moran Luengo et al. 2018; Sekhar et al. 2018; Boysen et al. 2019; Dahiya et al. 2019). Hop functions as an adaptor between Hsp70 and Hsp90 by binding the EEVD motifs of both chaperones (“intermediary complex”) (Chen and Smith 1998; Johnson et al. 1998; Scheufler et al. 2000; Wegele et al. 2006; Schmid et al. 2012; Rohl et al. 2015). Additionally, Hop allosterically inhibits the Hsp90 ATPase and keeps Hsp90 in an open, client-accessible state (Prodromou et al. 1999; Richter et al. 2003). Optical trap experiments and a recent high-resolution cryo-EM structure of this “client loading complex” suggest that helix 1 of GR is undocked from the globular protein and threaded through the Hsp90 dimer orifice (Suren et al. 2018; Wang et al. 2020). This disrupts the integrity of the hormone-binding pocket of GR and inhibits

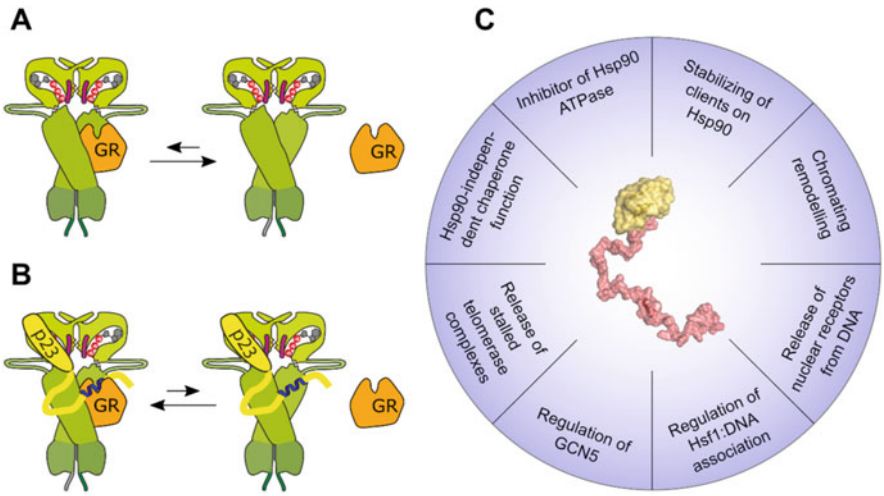

Fig. 6.3 p23 performs multiple functions. (**a**, **b**) The binding of GR to the closed Hsp90 conformation is shown in the absence and presence of p23. Binding of p23 to Hsp90 and GR prolongs the interaction of GR with Hsp90. (**c**) Different Hsp90-dependent and Hsp90-independent functions of p23 are shown. Note that the list is not exhaustive

hormone association. Release of the client from Hsp70 by transfer to Hsp90 releases the folding deadlock of the client (Kirschke et al. 2014; Moran Luengo et al. 2018; Boysen et al. 2019; Dahiya et al. 2019; Wang et al. 2020). Binding of ATP and p23 promotes the dissociation of Hop, the closing of the Hsp90, and the formation of a stable Hsp90:client:p23 complex ("late complex") (Dittmar et al. 1997; Li et al. 2011).

p23 was found to stabilize the "late complex" of Hsp90:SHR:p23 and to delay the release of the SHR, thus inhibiting the collapse of the hydrophobic hormone-binding pocket as a prerequisite for efficient hormone binding (Fig. 6.3a, b) (Dittmar et al. 1997). Additionally, it has been shown recently that p23 also directly contacts the bound GR (Fig. 6.3b) (Biebl et al. 2020; Noddings et al. 2020). In particular, an amphipathic helical element in the C-terminal tail of p23 binds a groove on the GR, which may contribute to the stabilization of the Hsp90:GR:p23 complex as shown by the Pratt and Toft labs. In the Hsp90:GR:p23 complex, the hormone-binding pocket is functional and the GR can bind hormone (Lorenz et al. 2014; Noddings et al. 2020). Notably, the association of the p23 helix with GR is also possible in the absence of Hsp90, suggesting that it may play a role in the Hsp90-independent chaperone function of p23 (see below) (Biebl et al. 2020). The helical elements in the p23 C-terminal tail are conserved between yeast and man. This suggests that the association of helical segments in the p23 tail with clients may be a universal element in the maturation of different clients. This is in line with the localization of the helical motifs in a flexible, dynamic region of p23 adapted to the diverse spatial requirements of different clients.

The importance of p23 for client maturation has been confirmed in the cellular environment. In particular, the relevance of p23 for the maturation of SHRs has been shown in mammalian cells as well as in yeast expressing mammalian SHRs (Bohen 1998; Fang et al. 1998; Knoblauch and Garabedian 1999; Freeman et al. 2000; Oxelmark et al. 2003; Oxelmark et al. 2006; Sahasrabudhe et al. 2017; Biebl et al. 2020). p23 is not essential in yeast and during prenatal development and morphogenesis in mice, yet p23 is strictly necessary for perinatal survival (Fang et al. 1998; Grad et al. 2006). The essential function of p23 in mice seems to be at least in part due to the effect of p23 on GR maturation, highlighting its central role in this process (Madon-Simon et al. 2017). In yeast, the knockout of p23 had a negative effect on GR, PR, the mineralocorticoid receptor (MR), androgen receptor (AR), and estrogen receptor (ER) (Sahasrabudhe et al. 2017). Additionally, the p23 knockout was also associated with the reduced maturation of v-Src kinase (Fang et al. 1998; Sahasrabudhe et al. 2017). Interestingly, for v-Src, the deletion of p23 in the yeast *sti1Δ* (yeast homolog of Hop) strain could rescue the detrimental effect of *sti1Δ* on kinase maturation, in line with the required balancing of the opposing effects p23 and Hop have on Hsp90 conformation (Biebl et al. 2020). Notably, p23 was found as one of only two co-chaperones in yeast that, when deleted, had a universal negative effect on client activation, whereas disruption of the majority of the co-chaperones had client-specific effects (Sahasrabudhe et al. 2017; Biebl et al. 2020).

Hsp90-Independent Functions of p23

Besides its crucial role as an Hsp90 co-chaperone, p23 has been found to have Hsp90-independent functions (Fig. 6.3c). These include intrinsic chaperone activity in vitro (Bose et al. 1996; Freeman et al. 1996; Weikl et al. 1999; Weikl et al. 2000) and a nuclear function in regulating chromatin and transcription factors (Freeman et al. 2000; Freeman and Yamamoto 2002; Echtenkamp et al. 2011, 2016). Intriguingly, the co-expression of p23 and SHRs in mammalian cells and yeast had different effects on the activity of distinct SHRs (Freeman et al. 2000; Morishima et al. 2003). This observation was shown to be at least in part due to effects of p23 on the transcriptional activity of SHRs downstream of Hsp90 (Freeman and Yamamoto 2002; Oxelmark et al. 2006; Toogun et al. 2007; Zelin et al. 2012; Echtenkamp et al. 2016).

The Intrinsic Chaperone Function of p23

An Hsp90-independent chaperone function of Hsp90 co-chaperones has first been shown in vitro for PPIases and p23 using different model substrates and monitoring the suppression of their unspecific aggregation (Bose et al. 1996; Freeman et al. 1996). Due to the ATP-independent nature of the interaction, a holdase effect of p23 is suspected as indicated by the formation of soluble high molecular weight

complexes between p23 and β-galactosidase that can be refolded by Hsp40 and Hsp70 (Freeman et al. 1996). The chaperone function of p23 has been mapped to the unstructured C-terminal tail (Weikl et al. 1999; Weaver et al. 2000). Yet, the exact motifs driving chaperone function have remained elusive, and a comprehensive knowledge of the function of the different tail regions is still missing. Recently, it was shown that helical motifs in the C-terminal tail of p23 directly bind GR, which increased GR function in vitro and in vivo (Fig. 6.3a, b) (Biebl et al. 2020; Noddings et al. 2020). Since a helix present in the C-terminal tail of yeast p23 also associates with and stabilizes GR expressed in yeast, client-specific co-evolution is unlikely. Hence, we hypothesize that the association of amphipathic helical structures in the C-terminal tail of p23 with substrates is a general feature. Since in a study using citrate synthase (CS) as a model substrate in vitro the C-terminal region of the tail lacking a helical motif seemed important for the chaperone function (Weikl et al. 1999), this could indicate that the conformational requirements of the client (e.g., opening of the hormone-binding pocket in GR vs. the suppression for aggregation in CS) dictate which part of the C-terminal tail is required for client chaperoning in the presence and absence of Hsp90.

The Nuclear Function of p23

While generally considered a cytosolic chaperone/co-chaperone system, both Hsp90 and p23 also localize to the nucleus under native and heat shock conditions (Carbajal et al. 1990; Morcillo et al. 1993; Picard 2006b; Tapia and Morano 2010; Ge et al. 2011). Early studies had shown that an excess of Hsp90 was negatively correlated with the binding of nuclear receptors to their cognate DNA and this effect could be suppressed by Hsp90 inhibition (Sabbah et al. 1996; Kang et al. 1999; Liu and DeFranco 1999). Freeman and Yamamoto showed by chromatin immunoprecipitation (ChIP) that p23 and to a smaller degree Hsp90 directly associate with GR response elements and promote the release of GR (Fig. 6.3c) (Freeman et al. 2000; Freeman and Yamamoto 2002). This answered the long-standing open question how the transcriptional activation of SHRs can be reduced within few minutes after hormone withdrawal. These findings are in line with a continuous recycling model, in which chaperones trigger the release of SHRs from the DNA and allow them to adjust to signal fluctuations in the cell quickly (Freeman and Yamamoto 2001). In this scenario, p23 collaborates with the acetyltransferase GCN5 (Fig. 6.3c) (Zelin and Freeman 2015). After p23 triggers the release of the DNA-bound protein, GCN5 inhibits re-association by acetylation. As a feedback mechanism, p23 negatively regulates GCN5 activity.

The release of transcription factors from DNA by chaperones may apply to more DNA-bound proteins beyond nuclear receptors. Recently, it was shown that Hsp90 and p23 promote the release of heat shock factor 1 (Hsf1)—the central regulator of the cellular heat shock response—from DNA as part of the regulatory mechanism (Zelin et al. 2012; Kijima et al. 2018) (Fig. 6.3c). Besides affecting the binding of nuclear receptors and transcription factors to their cognate DNA, Hsp90 and p23

have been found to affect other regulatory hubs of transcription, particularly chromatin remodeling (Gvozdenov et al. 2019). Mapping of nucleosome-depleted regions in the yeast genome revealed that Hsp90 triggers the release of "remodeling the structure of chromatin" (RSC) and additionally increases the chromatin remodeling activity of RSC (Fig. 6.3c) (Echtenkamp et al. 2016).

Intriguingly, p23 also affected the binding of telomerase to DNA and may facilitate the release of stalled telomerase complexes (Fig. 6.3c) (Toogun et al. 2007). Furthermore, the maturation of telomerase depends on p23 and Hsp90 (Holt et al. 1999; Forsythe et al. 2001). As p23 and Hsp90 were found in active telomerase complexes, this suggests a regulatory function (Forsythe et al. 2001; Keppler et al. 2006).

Concluding Remarks

Despite being known for more than 25 years, only now we begin to understand the mechanistic details of the interaction between Hsp90 and p23 and its function. Whereas Hsp90 co-chaperones generally exhibit client-specific effects, p23 is one of only two co-chaperones with a general function in Hsp90-dependent client processing. However, also Hsp90-independent functions have gained recognition, most importantly the intrinsic chaperone function of p23 and the role of p23 in the regulation of transcription and chromatin remodeling. Interestingly, the long, flexible tail of p23 seems to be involved in Hsp90-independent chaperoning but also interacts with Hsp90-bound substrates. While we now start to understand the function of specific parts of the tail such as helical segments found in yeast and human p23, the function of other tail regions is still elusive. Intriguingly, the p23 tail may even be involved in unrelated cellular processes: The proteolytic cleavage of the p23 tail after Asp142 by different caspases suggests that p23 may play a role during apoptosis (Gausdal et al. 2004; Mollerup and Berchtold 2005; Martini et al. 2017). Truncated p23 has reduced anti-aggregation capability and is degraded by the proteasome, suggesting that caspase-mediated cleavage of p23 contributes to the destabilization of proteins during apoptosis (Mollerup and Berchtold 2005). Hence, it is likely that new function of p23 will be discovered in the future, highlighting the crucial role of p23 beyond being one of the most important Hsp90 co-chaperones.

The Hsp90 Co-chaperone Aha1

Discovery and Isoforms of Aha1

While several Hsp90 co-chaperones have been identified as inhibitors of its ATPase, so far, Aha1 (Ahsa1 in humans) is the only known potent activator of the Hsp90 ATPase (Schopf et al. 2017). Aha1 consists of two domains, connected via a flexible

linker (Fig. 6.4a). While Aha1 is conserved from yeast to humans, *S. cerevisiae*, and several members of the *Saccharomycotina* subphylum, express "high-copy Hsp90 suppressor" (Hch1) as a homolog of Aha1 that has been discovered before Aha1 in an overexpression suppression screen for temperature-sensitive Hsp90 mutants by the Lindquist lab (Fig. 6.4b) (Nathan et al. 1999; Panaretou et al. 2002). The discovery of Hch1 paved the way for the identification of Aha1 due to the homology between Hch1 and the N-terminal domain (NTD) of Aha1 with a 36.5% sequence identity. Compared to Hch1, Aha1 carries an additional C-terminal domain (Panaretou et al. 2002). The expression of Aha1 and Hch1 is upregulated upon stress similar to established heat shock proteins (Gasch et al. 2000). Intriguingly, it has been suggested that Hch1 was functionally replaced in mammals during evolution by the phosphorylation of Tyr627 in human Hsp90 by Yes kinase (Xu et al. 2012; Zuehlke et al. 2017). Mammalian Aha1 which carries a short N-terminal extension of 22 amino acids that is missing in yeast has been shown to associate directly with substrate proteins and was suggested to funnel them toward ubiquitination and degradation (Fig. 6.4b) (Tripathi et al. 2014). In higher eukaryotes, Aha2 (Ahsa2 in humans) is expressed as a paralog of Aha1 with about 50% sequence identity, yet no published information about this protein is available.

The Structure of Aha1

As mentioned, Aha1 is organized into an NTD and a CTD connected by a flexible linker (Fig. 6.4a). The structure of the NTD was solved as a co-crystal with the Hsp90-MD, revealing a cylindrical structure (PDB: 1USU) (Meyer et al. 2004). The NTD consists of an N-terminal α-helix followed by a four-stranded β-sheet and a C-terminal α-helix (Meyer et al. 2004). Due to the significant similarity between the Hch1 and the Aha1 NTD, a similar structure of Hch1 is assumed. The structure of the Aha1-CTD has been solved by NMR (unpublished); it is composed of a β-sheet-rich structure and a C-terminal α-helix (Fig. 6.4a) (PDB: 1X53). Importantly, the connection of the Aha1-NTD and Aha1-CTD is flexible, and significant dynamics of Aha1 bound to Hsp90 is expected (Fig. 6.4c) (Koulov et al. 2010; Oroz et al. 2019).

The Interaction of Aha1 with Hsp90

The Aha1-NTD binds on an extensive surface along the Hsp90-MD contacting both Hsp90 monomers (PDB: 1USU) (Fig. 6.4c) (Meyer et al. 2004; Retzlaff et al. 2010; Oroz et al. 2019). Binding is governed mainly by polar interactions except for a hydrophobic patch interacting with the N-terminal region of the Hsp90-MD, in line with the salt sensitivity of the Aha1:Hsp90 interaction (Panaretou et al. 2002; Meyer et al. 2004). Binding of the Aha1-NTD to the Hsp90-MD leads to a conformational switch of the catalytic loop of Hsp90, positioning it in proximity of the nucleotide

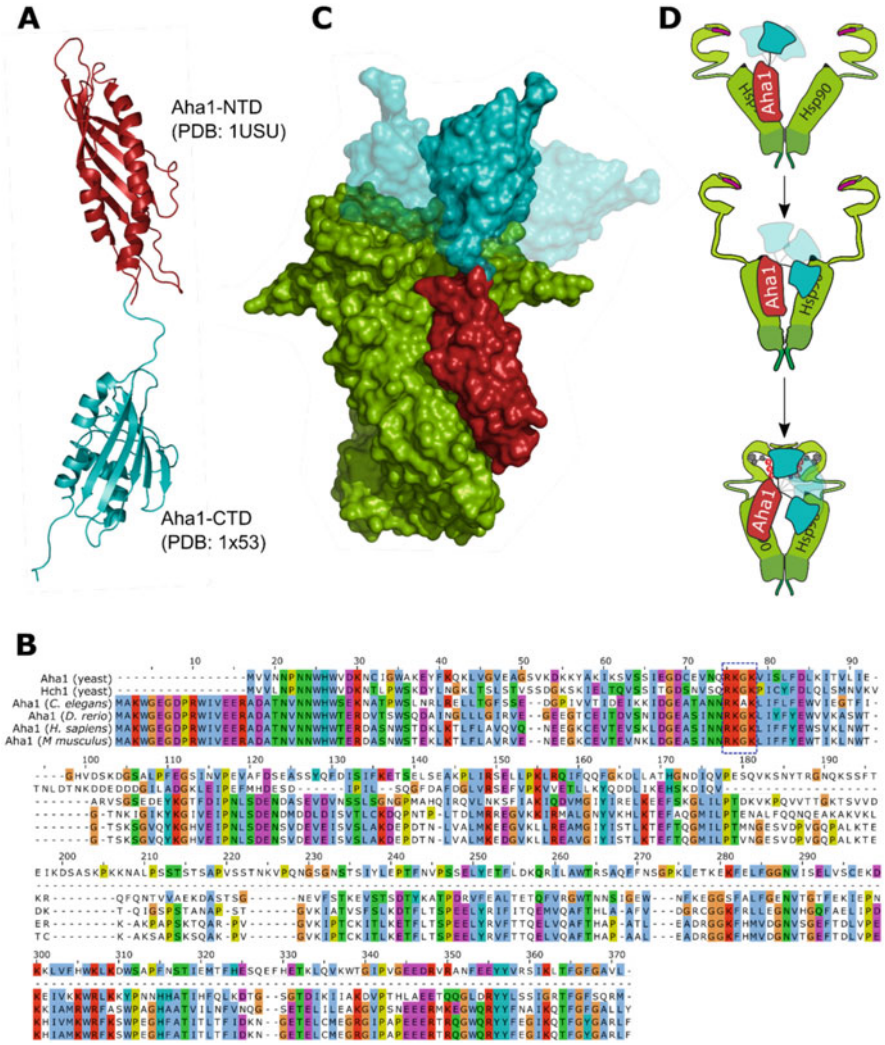

Fig. 6.4 Aha1 structure and Hsp90 binding. (**a**) The crystal structure of the Aha1-NTD (PDB: 1USU) and the NMR structure of the Aha1-CTD (PDB: 1x53) were connected via the unstructured linker between both domains using PyMol 1.7.1.1. (**b**) The alignment of Aha1 and Hch1 from different organisms is shown. The alignment was done using the Clustal Omega tool, and the ClustalX color code was applied in the Jalview 2.11.1.3 software. The conserved RKxK motif is highlighted by a dashed box. (**c**) Model of Aha1 binding to Hsp90. The crystal structure of the Hsp90-MD with the Aha1-NTD (PDB: 1USU) was aligned to the crystal structure of full-length yeast Hsp90 (PDB: 2CG9), and the NMR structure of the Aha1-CTD (PDB: 1x53) was modeled in PyMol 1.7.1.1. The dynamics of the C-terminal domain are indicated. (**d**) Different binding modes of Aha1 for Hsp90 are shown as a schematic model based on data from (Retzlaff et al. 2010; Oroz et al. 2019; Xu et al. 2019; Noddings et al. 2020). Transparent domains indicate conformational dynamics

bound to the Hsp90-NTD. This is supposed to contribute to the acceleration of ATP hydrolysis (Meyer et al. 2003; Meyer et al. 2004; Cunningham et al. 2012). While full-length Aha1 stimulates the ATPase rate of Hsp90 15- to 30-fold, the Aha1 CTD alone had no stimulatory activity (Panaretou et al. 2002; Meyer et al. 2004; Koulov et al. 2010; Retzlaff et al. 2010; Wolmarans et al. 2016). By contrast, the Aha1-NTD alone stimulates the ATPase activity by only 1.5- to 3-fold, similar to the activating effect of Hch1 (Retzlaff et al. 2010; Wolmarans et al. 2016). Intriguingly, the addition of the Aha1-CTD as an isolated domain increases the ATPase stimulation capacity of the Aha1-NTD (Retzlaff et al. 2010). In line with this notion, the affinity of the Aha1-NTD for Hsp90 ($K_D = 19$ μM) is increased by the presence of the Aha1-CTD in full-length Aha1 ($K_D = 4$ μM) (Retzlaff et al. 2010). Binding of the catalytic loop requires the interaction of the conserved RKxK motif in the N-terminal region of Aha1 and Hch1, yet surprisingly, the interactions of this motif seem to differ between Aha1 and Hch1 (Fig. 6.4b) (Horvat et al. 2014). While the motifs of both proteins interact with the Hsp90 catalytic loop, a mutation in the catalytic loop only affects the binding of Aha1 (Horvat et al. 2014). In line with the distinct interactions of the RKxK motifs in Aha1 and Hch1, the fusion of the Aha1-CTD to Hch1 could not increase the Hsp90 ATPase to levels of $Aha1^{WT}$ (Horvat et al. 2014).

Together, this suggests a specific synergistic function between the Aha1-NTD and Aha1-CTD, yet how these domains co-operate is not well understood. A conserved NxNNWHW motif is present in the Aha1-NTD and in Hch1 (Mercier et al. 2019). This motif was not resolved in the Hsp90-Aha1 co-crystal structure but is predicted to point toward the Hsp90-NTD (Meyer et al. 2004). A recent study showed that this motif is required for full ATPase activation, yet loss of the motif only played a role if the Aha1-CTD was present, suggesting that NxNNWHW is important for the interplay between the two Aha1 domains (Mercier et al. 2019). Additionally, this motif is suspected to alter the apparent affinity of Hsp90 for nucleotides. This is in line with NMR results showing Aha1-induced conformational changes near the nucleotide-binding site of Hsp90 (Oroz et al. 2019).

The mechanism of the Hsp90 ATPase stimulation by Aha1 is still under discussion but likely involves a multistep mechanism in which Aha1 binds Hsp90 in different binding modes (Fig. 6.4c, d) (Retzlaff et al. 2010; Wolmarans et al. 2016; Oroz et al. 2019; Xu et al. 2019). Aha1 binds the open and closed state of Hsp90, albeit with a higher affinity for the closed state (Li et al. 2013; Oroz et al. 2019; Liu et al. 2020). Under physiological conditions, the binding stoichiometry of 1:2 [Aha1:$Hsp90_{monomer}$] is most likely since Hsp90 is about 30-fold more abundant than Aha1 (Finka and Goloubinoff 2013), matching the finding that a single bound Aha1 leads to the maximal stimulation of the Hsp90 ATPase (Retzlaff et al. 2010). Notably, the ATPase activity is stimulated *in cis* and *in trans*, meaning that binding of Aha1 to one monomer can stimulate the ATPase activity in the opposite monomer (Retzlaff et al. 2010).

Recently, cryo-EM structures of Hsp90 in complex with Aha1 have been solved (Liu et al. 2020). In the resulting model, binding of Aha1-NTD to apo Hsp90 leads to a partial closing of the Hsp90 dimer, in line with previous results (Retzlaff et al. 2010; Oroz et al. 2019; Xu et al. 2019). In the cryo-EM structures, the Aha1-NTD

binds to one Hsp90 monomer, while the Aha1-CTD contacts and bridges both monomers. By contrast, previous biochemical and NMR analysis also indicated contacts of the Aha1-CTD with the Hsp90-NTD (Retzlaff et al. 2010; Oroz et al. 2019). In the semi-closed state, density for the Hsp90-NTD was missing, leading to the hypothesis that the Aha1-CTD clashes with the Hsp90-NTD, leading to an undocking of the Hsp90 NTDs from the MD (Fig. 6.4d). While surprising, this matches widespread chemical shift perturbations in the Hsp90-NTD after Aha1 binding found by NMR (Oroz et al. 2019) and optical tweezer experiments showing that the NTD easily undocks from the MD (Jahn et al. 2014). Additionally, large N-terminal rearrangements have been shown to be essential in the Hsp90 cycle (Daturpalli et al. 2017). In the model, the full closing of the Hsp90 monomers is induced by ATP binding, leading to a rearrangement of the Aha1-CTD to stabilize the fully closed Hsp90 state (Fig. 6.4a) (Koulov et al. 2010; Xu et al. 2019). Closing of the Hsp90 dimers is associated with a tilting of the Aha1-NTD, which then contacts the Hsp90-NTD (Fig. 6.4a) (Liu et al. 2020). In the proposed model, ATP hydrolysis is sequentially induced first in one monomer and then in the second monomer (Liu et al. 2020).

Importantly, the recruitment of Aha1 to Hsp90 is also regulated by post-translational modifications (Backe et al. 2020). Phosphorylation of Tyr313 and Tyr38 in human Hsp90 has been associated with enhanced Aha1 recruitment (Mollapour et al. 2010; Xu et al. 2012; Xu et al. 2019). Similarly, SUMOylation of Lys191 increases the recruitment of Aha1, while it suppresses the binding of p23, suggesting that a transient modification may be important for the progression in the Hsp90 cycle (Mollapour et al. 2014). By contrast, phosphorylation of Thr22 has been associated with reduced Aha1 binding (Mollapour et al. 2011). Notably, the different binding modes Aha1 samples on Hsp90 and which affect Hsp90 activity may be influenced by post-translational modifications (Dunn et al. 2015; Xu et al. 2019). Together, a complex regulatory layer of PTMs regulates the timely recruitment of Aha1.

Aha1 and Hsp90 Client Maturation

As the most potent activator of the Hsp90 ATPase and regulator of Hsp90 conformation, Aha1 should have a strong effect on client maturation. So far, depending on the cell type and organism under investigation as well as the choice of model substrates, contradicting results on the influence of Aha1 were obtained.

Early studies in yeast suggested that the knockout of Aha1 and Hch1 downregulated v-Src activity (Panaretou et al. 2002; Lotz et al. 2003). By contrast, in later studies, neither Aha1 nor Hch1 affected v-Src activity (Sahasrabudhe et al. 2017; Biebl et al. 2020). Similarly, early studies suggested that loss of Aha1 and Hch1 in yeast decreased the activity of GR (Harst et al. 2005). However, other studies exclusively found an activating effect of the Aha1 or Hch1 knockouts on different SHRs (Dunn et al. 2015; Sahasrabudhe et al. 2017; Zuehlke et al. 2017;

Biebl et al. 2020). In mammalian cells, overexpression of Aha1 seems to increase GR activity and a knockdown of Aha1 reduced GR activity (Harst et al. 2005). On the other hand, overexpression of Aha1 in mammalian cells did not affect v-Src activity but decreased the activity of Akt kinase and luciferase (Sun et al. 2012), matching the finding that overexpression of Aha1 and Hch1 in yeast led to reduced GR activity (Biebl et al. 2020).

Different results have also been obtained on the effect of altered Aha1/Hch1 levels on the sensitivity to Hsp90 inhibitors in mammalian cells and yeast. Knockdown of Aha1 by small interfering RNA (siRNA) led to an increase in sensitivity of mammalian cells accompanied by decreased client activity (Holmes et al. 2008). By contrast, deletion of Hch1, but not Aha1, increased sensitivity to Hsp90 inhibitors in yeast (Armstrong et al. 2012).

Interestingly, the knockdown of Aha1 in mammalian cells rescued the folding of mutant cystic fibrosis transmembrane conductance regulator (CFTR) Δ508 by increasing its dwell time on Hsp90 (Wang et al. 2006). The prolonged association with Hsp90 supposedly allows the mutant protein to overcome a kinetic folding barrier (Wang et al. 2006). Due to the prospect of rescuing the CFTR Δ508 mutant, inhibitors of the Hsp90-Aha1 interaction have been developed (Ghosh et al. 2015; Ihrig and Obermann 2017; Stiegler et al. 2017; Singh et al. 2020). In agreement with the findings for GR presented above, overexpression of Aha1 decreased the folding of the CFTR Δ508 mutant (Koulov et al. 2010). Notably, Aha1 mutants that bound less efficient to Hsp90 or mutants which increased the Hsp90 ATPase activity less potently were less efficient in inhibiting CFTR Δ508 folding (Koulov et al. 2010).

In line with this observation, competitive binding of Aha1 and clients to Hsp90 has been shown due to overlapping binding surfaces (Fig. 6.4c) (Meyer et al. 2003, 2004; Lorenz et al. 2014; Verba et al. 2016; Sahasrabudhe et al. 2017; Schopf et al. 2017). Furthermore, the solvent-exposed Trp300 residue in yeast Hsp90 has been implicated both in Aha1 and client binding (Meyer et al. 2004; Hawle et al. 2006; Rutz et al. 2018; Xu et al. 2019). Taken together these results imply that client proteins and Aha1 compete for binding to Hsp90. This raises interesting questions concerning the position of Aha1 in the chaperone cycle and its role in client processing. Aha1 may function as a general dwell time regulator for Hsp90 conformations and consequently as a "molecular referee" to shift the chaperone machinery toward productive folding by ridding it of clients trapped in unproductive folding pathways and resetting Hsp90 by inducing ATP hydrolysis (Koulov et al. 2010; Biebl et al. 2020). In line with this hypothesis, the affinity of Aha1 (K_D ~ 1–4 μM) (Meyer et al. 2004; Koulov et al. 2010; Retzlaff et al. 2010) and client proteins (K_D ~ 1–5 μM) (Muller et al. 2004; Hagn et al. 2011; Karagoz et al. 2014; Lorenz et al. 2014) for Hsp90 binding are similar. In addition, current models suggests that the open Hsp90 state is necessary for client binding, whereas Aha1 can bind to the open state and induces closing (Chen and Smith 1998; Retzlaff et al. 2010; Schmid et al. 2012; Kirschke et al. 2014). This supports the hypothesis that Aha1 may displace clients that have remained on Hsp90 for a longer time. Current data, however, is insufficient to decide if discrimination between productive and unproductive folding

pathways is possible or whether the prolonged association time of clients trapped in unproductive folding trajectories increases the chance of clearance by Aha1.

Integration of Aha1 and p23 into the Hsp90 ATPase Cycle

p23 and Aha1 affect the Hsp90 ATPase in opposite ways: p23 is an inhibitor of the Hsp90 ATPase, locking it in a closed conformation, in which the ATP hydrolysis or ADP and phosphate release are partially inhibited leading to approximately 50% Hsp90 activity (Panaretou et al. 2002; Richter et al. 2004; Siligardi et al. 2004; McLaughlin et al. 2006; Karagoz et al. 2011; Graf et al. 2014; Biebl et al. 2020). By contrast, Aha1 binds both the open and closed Hsp90 conformation, potently stimulating the ATPase rate and promoting N-terminal dimerization of Hsp90 (Panaretou et al. 2002; Koulov et al. 2010; Retzlaff et al. 2010; Horvat et al. 2014; Liu et al. 2020). While p23 remains associated when Hsp90 is bound to clients and even partakes in the stabilization of the bound client, Aha1 seems to bind competitively to clients in many cases (Koulov et al. 2010; Sun et al. 2012; Sahasrabudhe et al. 2017; Biebl et al. 2020). Due to their opposing functions, Aha1 and p23 are considered to associate with the Hsp90 cycle sequentially (Li and Buchner 2013).

In the currently accepted Hsp90 cycle, many clients are transferred to Hsp90 from the Hsp40 and Hsp70 system via the adaptor co-chaperone Hop (Fig. 6.5) (Chen and Smith 1998; Johnson et al. 1998; Scheufler et al. 2000; Wegele et al. 2006; Schmid et al. 2012; Rohl et al. 2015). A PPIase bound to the free MEEVD motif of the second Hsp90 monomer and Aha1 can function synergistically to release Hop (Li et al. 2013). Additionally, post-translational phosphorylation of Tyr313 on Hsp90 may promote the release of Hop and recruit Aha1 (Xu et al. 2019). This process is referred to as "co-chaperone switching." Notably, only full-length Aha1 can replace Hop, whereas the Aha1-NTD alone does not suffice (Wolmarans et al. 2016). In line with the "co-chaperone switching" hypothesis, a switching mechanism of Aha1 by displacing tissue inhibitor of metalloproteinase-2 (TIMP-2) from extracellular Hsp90 in the context of matrix metalloproteinase 2 (MMP2) maturation has been shown (Baker-Williams et al. 2019). By catalyzing the rate-limiting conformational rearrangements, Aha1 promotes the N-terminal dimerization of Hsp90 (Koulov et al. 2010; Retzlaff et al. 2010). How Aha1 is released from Hsp90 is still under discussion. Possibly, the affinity of Aha1 for Hsp90 changes throughout the different binding modes in which Aha1 engages with Hsp90. Additionally, phosphorylation of Aha1 by c-Abl kinase has been found to increase Aha1 affinity for Hsp90 but also poise Aha1 for proteasomal degradation (Dunn et al. 2015). The association of p23 and ATP aids in displacing Aha1 from Hsp90, yet whether a ternary Hsp90:Aha1:p23 complex is possible has remained controversial (Harst et al. 2005; Sun et al. 2012; Li et al. 2013). In this context, also the competition of other co-chaperones with Aha1 for Hsp90 binding may play a role in releasing Aha1, yet how this is regulated remains unclear (Woodford et al. 2016, 2017). Dynamic, secondary contacts of the p23 tail in the Hsp90 MD (Fig. 6.2) point toward

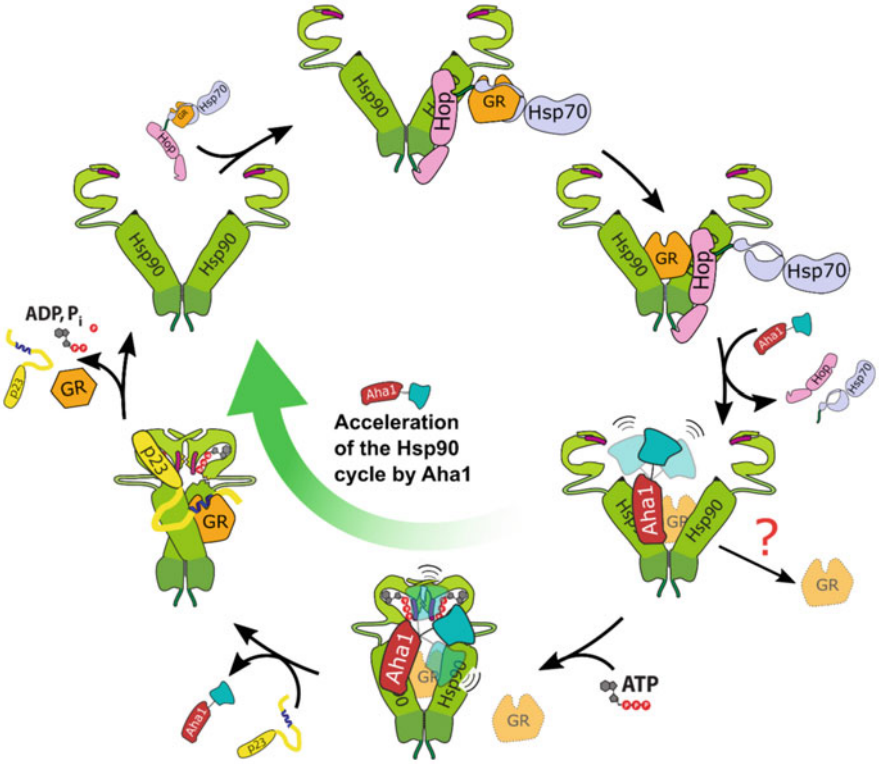

Fig. 6.5 p23 and Aha1 affect the Hsp90 cycle in opposite ways. A schematic model of the Hsp90 chaperone cycle is shown. Clients are recruited from the Hsp70 system via Hop. Binding of Aha1 and PPIases (not shown) contributes to the release of Hop and Hsp70. The possible release of clients by Aha1 is indicated by a red question mark and semi-transparent GR. In this case, Aha1 may help to free Hsp90 from clients that are trapped in unproductive folding pathways. Binding of Aha1 to Hsp90 promotes the closing of the Hsp90 dimers. The conformational dynamics of the Aha1-CTD is visualized as transparent alternative conformations. Subsequently, p23 aids in displacing Aha1 from Hsp90 and leads to a stabilized Hsp90:client:p23 client complex. After release of p23 and ATP hydrolysis, the mature client gets released, and Hsp90 transitions back into its open state

competition between p23 and Aha1 for Hsp90 binding (Biebl et al. 2021). Inhibition of the Hsp90 ATPase by p23 prolongs the completely closed state and the binding of clients to Hsp90 (Johnson and Toft 1995; Dittmar et al. 1997; Biebl et al. 2020; Noddings et al. 2020). To finish the cycle, ATP is hydrolyzed, ADP is released, and Hsp90 opens for another cycle.

Conclusion

p23 and Aha1 have opposing functions including the important regulation of the dwell time of clients on Hsp90. Whereas p23 prolongs the binding, Aha1 reduces the time a client is bound to Hsp90, seemingly competing with client binding at least in some cases. How these opposing effects integrate in the physiological environment to ensure efficient client maturation is still under investigation. It is not clear if the sequential action of Aha1 and p23 in the Hsp90 cycle observed in vitro is functionally important in the cellular environment. The cellular concentration ratio of p23 and Aha1 to Hsp90 in HeLa cells is about 1 (Hsp90) to 1/7 (p23) and 1/35 (Aha1) (Finka and Goloubinoff 2013), suggesting that binding of p23 to an Hsp90:client complex is more likely than the binding of Aha1 and the majority of Hsp90 cycles will take place in the absence of Aha1. This suggests that a sequential action of p23 and Aha1 is not generally necessary for the conformational cycle of Hsp90. Moreover, current evidence indicates that the acceleration of the Hsp90 ATPase cycle by promoting progression to the closed-2 state is not the only function of Aha1. The competition of Aha1 with client proteins for Hsp90 binding as well as the apparent inhibition of the maturation of some clients by Aha1 would agree with a model of low basal clearing function of Aha1 that frees Hsp90 from clients, yet the relevance of this proposed function and how this connects to the ATPase regulation by Aha1 is unknown. The presence of Aha1 and Hch1 as two structurally and functionally related homologs in yeast which form a genetic module and the emergence of a post-translational modification in humans that phenocopies the Hch1 function further indicate the importance of the action of Aha1 and Hch1 for the Hsp90 machinery. Future research will have to show how Aha1 and Hch1 in yeast or the phenocopying post-translational modification in humans cooperate to enhance client maturation and how the clearing of clients from Hsp90 is connected to the ATPase stimulating function of Aha1.

The discoveries made by many laboratories on the structure and function of p23 and Aha1 over the years have shown that the conformational cycle of Hsp90 is set up in a way that allows modulation at different steps by these co-chaperones and uncovered their intricate mechanisms. At the same time, the novel insight also revealed important questions on the mechanisms of action. Addressing these will further reveal the gearings of the complex Hsp90 machinery and how it exerts its central role in conformationally regulating proteostasis in the cytosol of eukaryotic cells.

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Chapter 7
Beyond Chaperoning: UCS Proteins Emerge as Regulators of Myosin-Mediated Cellular Processes

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Abstract The UCS (**U**NC-45/**C**RO1/**S**he4p) family of proteins has emerged as chaperones specific for the folding, assembly, and function of myosin. UCS proteins participate in various myosin-dependent cellular processes including myofibril organization and muscle functions, cell differentiation, striated muscle development, cytokinesis, and endocytosis. Mutations in the genes that code for UCS proteins cause serious defects in myosin-dependent cellular processes. UCS proteins that contain an N-terminal tetratricopeptide repeat (TPR) domain are called UNC-45. Vertebrates usually possess two variants of UNC-45, the ubiquitous general-cell UNC-45 (UNC-45A) and the striated muscle UNC-45 (UNC-45B), which is exclusively expressed in skeletal and cardiac muscles. Except for the TPR domain in UNC-45, UCS proteins comprise of several irregular armadillo (ARM) repeats that are organized into a central domain, a neck region, and the canonical C-terminal UCS domain that functions as the chaperoning module. With or without TPR, UCS proteins form linear oligomers that serve as scaffolds that mediate myosin folding, organization into myofibrils, repair, and motility. This chapter reviews emerging functions of these proteins with a focus on UNC-45 as a dedicated chaperone for folding, assembly, and function of myosin at protein and potentially gene levels. Recent experimental evidences strongly support UNC-45 as an absolute regulator of myosin, with each domain of the chaperone playing different but complementary roles during the folding, assembly, and function of myosin, as well as recruiting Hsp90 as a co-chaperone to optimize key steps. It is becoming increasingly clear that UNC-45 also regulates the transcription of several genes involved in myosin-dependent cellular processes.

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Keywords UCS · Myosin · TPR · Chaperones · Co-chaperones · Regulator · UNC-45

Introduction

UNC-45 is a prototype of a class of proteins known as the UCS (UNC-45 in *Caenorhabditis elegans*, CRO1 in *Podospora anserina*, and She4p in *Saccharomyces cerevisiae*) domain containing proteins (Hutagalung et al. 2002; Yu and Bernstein 2003). The UCS domain-containing proteins have emerged as essential for a wide spectrum of myosin-dependent cellular processes in many eukaryotes, ranging from fungi to humans (Table 7.1). They are necessary for important cellular processes such as myofibril and sarcomere organization, cell differentiation, embryonic development, cytokinesis, endocytosis, and syncytial-cellular stage transition (Hutagalung et al. 2002; Yu and Bernstein 2003; Odunuga and Epstein 2007; Ni and Odunuga 2015). UCS proteins can be divided into two sub-classes based on the presence or absence of an N-terminal tetratricopeptide repeat (TPR) domain (Das et al. 1998) in their structure (Table 7.1). The similarity between these two sub-classes is the presence of the canonical C-terminal UCS domain that is absolutely required for their interaction with myosins. UCS proteins that possess a TPR domain are referred to as UNC-45 proteins (Barral et al. 2002) (Fig. 7.1). While only one copy of the gene is found in invertebrates, vertebrates have two copies encoding differentially expressed isoforms (Price et al. 2002). Mutations in the UCS proteins result in various defective myosin-dependent processes such as cytokinesis in *Schizosaccharomyces pombe* (Balasubramanian et al. 1998), endocytosis and trafficking in *S. cerevisiae* (Jansen et al. 1996; Wendland et al. 1996; Gomez-Escalante et al. 2017), syncytial-cellular stage transition in *P.* anserina (Berteaux-Lecellier et al. 1998), and myofibril organization and cytokinesis in *C. elegans* (Epstein and Thomson 1974; Kachur et al. 2004). UNC-45 has been established as a chaperone for the motor domain of myosin (Barral et al. 2002; Bookwalter et al. 2014, 2017; Ni and Odunuga 2015). Other UCS proteins such as Rng3p in *S. pombe* (Mishra et al. 2005), She4p in *S. cerevisiae* (Wesche et al. 2003), and DUNC-45 (Yu et al. 2003) in *Drosophila melanogaster* have also been shown to interact with myosin and modulate its function. The interaction of UCS proteins is not limited to sarcomeric myosins alone; cytoskeletal myosins including both conventional and (filament assembling) myosin II and unconventional (non-assembling) myosins I and V are known to require UCS proteins for their functions (Barral et al. 2002; Wesche et al. 2003; Bookwalter et al. 2014, 2017; Ni and Odunuga 2015). Myosins require the UCS-containing chaperones due to their large size, complexity of their structures, and the need to form highly organized oligomeric assemblies which are sometimes composed of different isoforms and other associating proteins (Landsverk and Epstein 2005). Several studies have demonstrated that together with Hsp90, UNC-45 forms a functional ternary complex with myosin (Barral et al. 2002; Srikakulam and Winklemann 2004; Mishra et al. 2005; Liu et al. 2008; Srikakulam et al. 2008). Analysis of these previous and recent studies reveals that UNC-45 is the main

Table 7.1 UCS proteins and their characteristics

UCS proteins	Organisms	Myosin substrates	Loss-of-function phenotypes
TPR-containing UCS proteins (UNC-45 proteins)			
General cell (GC) UNC-45 (UNC-45A/a)	Vertebrates (mouse, human, zebrafish)	Cytoskeletal myosin II	Inhibition of cell proliferation and fusion
Striated muscle (SM) UNC-45 (UNC-45B/b, Steif)	Vertebrates (mouse, human, zebrafish, frog)	Sarcomeric and cytoskeletal myosin II	Loss of sarcomere organization
DUNC-45	*D. melanogaster* (fruit fly)	Sarcomeric and cytoskeletal myosin II	Embryonic and late larval stage lethality, reduced body size, and defects in motility
UNC-45	*C. elegans* (nematode)	Sarcomeric and cytoskeletal myosin II	Fewer thick filaments, myofibril disorganization, paralysis, and failure of embryonic cytokinesis
PUNC	*P. Falciparum*	Myosin XIV	Loss of cell motility
TgUNC	*T. Gondii*	Myosin XIV	Loss of cell motility
TPR-lacking UCS proteins			
She4p	*S. cerevisiae* (budding yeast)	Myosin types I and V	Loss of actin polarization, defective internalization of membrane, defects in endocytosis
Rng3p	*S. Pombe* (fission yeast)	Cytoskeletal myosin II	Defective actomyosin ring, failure of cytokinesis
CRO1	*P. anserina* (filamentous fungus)	?	Inability to form septum, defective syncytial-cellular transition

GC UNC-45: Price et al. (2002); Bazzaro et al. (2007); Comyn and Pilgrim (2012)
SM UNC-45: Wohlgemuth et al. (2007); Geach and Zimmerman (2010); Chen et al. (2012); Comyn and Pilgrim (2012)
DUNC-45: Yu et al. 2003; Lee et al. (2011b); Melkani et al. (2011)
UNC-45: Epstein and Thomson (1974); Venolia and Waterston (1990); Guo and Kemphues (1996); Barral et al. (1998); Venolia et al. (1999); Ao and Pilgrim (2000); Kachur et al. (2004, 2008)
She4p: Jansen et al. (1996); Wendland et al. (1996); Toi et al. (2003); Wesche et al. (2003); Lord et al. (2008)
Rng3p: Balasubramanian et al. (1998); Wong et al. (2000, 2002); Lord and Pollard (2004); Mishra et al. (2005); Lord et al. (2008); Stark et al. (2013)
CRO1: Berteaux-Lecellier et al. (1998)
***PUNC*:** Bookwalter et al. (2017)
TgUNC: Bookwalter et al. (2014); Frénal et al. (2017)

chaperone required for myosin folding (Kaiser et al. 2012; Bujalowski et al. 2014, 2015; Bookwalter et al. 2017; Lehtimäki et al. 2017; Hellerschmied et al. 2019; Gaziova et al. 2020) and that it recruits Hsp90 via its TPR domain to modulate myosin assembly, motility, and functions (Nicholls et al. 2014; Bujalowski et al. 2014, 2018).

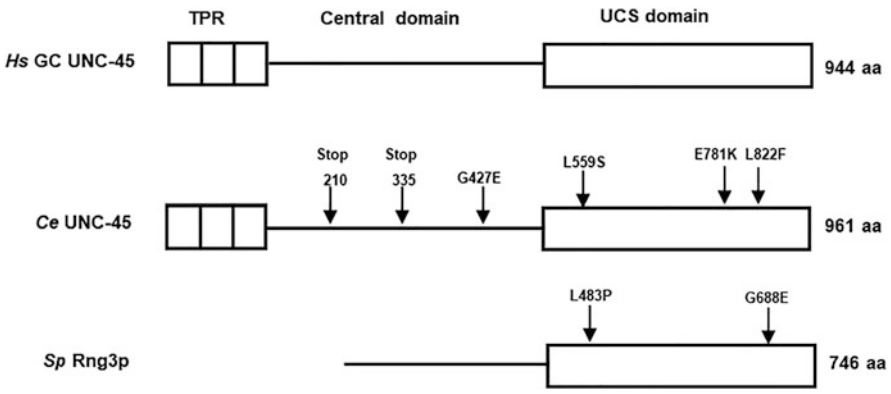

Fig. 7.1 Domain organization of homologs of UCS proteins: *Hs* GC UNC-45, *Ce* UNC-45, and *Sp* Rng3p represent the human general cell UNC-45A (NP_061141) (Price et al. 2002), *C. elegans* UNC-45 (NP_497205) (Epstein and Thomson 1974; Barral et al. 2002), and *S. pombe* ring assembly protein 3 (O74994) (Balasubramanian et al. 1998), respectively. The TPR and UCS domains are represented by small and large boxes, respectively. The horizontal line represents the central region. The positions of amino acid substitutions are indicated by vertical arrows

UCS Proteins Are Indispensable for Myosin Folding but not Hsp90

The myosin family is a large group of motor proteins that interact with actin, hydrolyze ATP, and produce movement along the actin filament. Myosins are involved in a broad spectrum of cellular processes that include cellular trafficking, phagocytosis, muscle contraction, cytokinesis, and cytoskeletal assembly. The full protein complement of a myosin is composed of two parts: the myosin heavy chains (MHC) and the myosin light chains (MLC). Typically, a myosin heavy chain is comprised of three functional regions: (1) a conserved (catalytic) motor or head that contains actin and ATP binding sites, (2) the neck domain which binds myosin light chains, and (3) the tail domain, which forms an α-helical coiled-coil rod in some myosin types and serves to anchor and position the motor domain to interact with actin. Myosin II includes the classical conventional myosin first isolated from muscle, but subsequently found in non-muscle cells and protists (Kuczmarski and Spudich 1980). The sarcomeric muscle myosin II is the only member of the myosin family that is assembled into thick filaments of skeletal and cardiac muscles. Myosin is a multidomain protein; therefore, its folding pathway is complex (Srikakulam and Winkelmann 1999). The myosin head itself contains multiple domains connected by flexible loops to form a globular structure (Rayment et al. 1993a; b), while the light chains and rod are simpler in structures (Atkinson and Stewart 1991; Saraswat and Lowey 1991). Myosin light chains and rod when expressed in bacteria fold into functionally active structures (Atkinson and Stewart 1991; Saraswat and Lowey 1991). Regardless of their origin, expression of myosin motors has proved difficult in bacteria; this may be due to the complex nature of their structure (McNally et al.

1988; Mitchell et al. 1989). Using the baculovirus expression system in insect cells, considerable success was achieved in expressing heavy meromyosin (HMM) from cytoskeletal types II, V, and VI and, to a limited extent, cardiac myosins (Trybus 1994; Sweeney et al. 1998; Wang et al. 2000). These HMMs have properly folded motor domains that are capable of binding actin (Trybus 1994; Sweeney et al. 1998; Wang et al. 2000). However, the fast, skeletal muscle myosin head was expressed in active form only in C2C12 mouse cell line suggesting that this myosin requires additional cofactor(s) which are present in myogenic cells for folding (Kinose et al. 1996; Chow et al. 2002). In vitro, chimeric fast skeletal muscle myosin head fused to green fluorescent protein (GFP) folds very slowly and transits through multiple intermediates in a temperature-dependent manner that strongly suggests a high susceptibility to off-pathway interactions and aggregations and hence the need for chaperone-assisted folding (Chow et al. 2002). Expression of the protein in vivo is cell-dependent: C2C12 myogenic cell lines yield a folded and active protein that exhibits Mg^{2+} ATP-sensitive actin-binding and myosin motor activity, while epithelia cell lines yield inactive protein aggregates (Chow et al. 2002). Myosins from *Toxoplasma gondii* and *Plasmodium falciparum* expressed in insect cells were found largely insoluble without the co-expression of the genus-specific UNC-45 genes (Bookwalter et al. 2014, 2017). These experimental evidences confirm that the myosin motor requires cell and/or genus-specific molecular chaperones to fold correctly under physiological conditions.

Genetic, biochemical, and structural studies have confirmed that UCS proteins, especially UNC-45, interact with Hsp90 chaperone (Barral et al. 2002; Mishra et al. 2005; Srikakulam and Winklemann 2004; Chadli et al. 2008; Liu et al. 2008; Srikakulam et al. 2008; Gazda et al. 2013). The TPR domain of UNC-45 preferentially and functionally binds to Hsp90 (Barral et al. 2002). Purified Hsp90, myosin, and UNC-45 can form the three possible binary complexes in pull-down assays (Barral et al. 2002). When expressed in striated muscle cells, UNC-45 was isolated as a stable complex with Hsp90 (Liu et al. 2008; Srikakulam et al. 2008). Hsp90 knockdown caused myofibril disorganization (Du et al. 2008; Gaiser et al. 2011) but did not imply a functional chaperone-client interaction between Hsp90 and myosin. Using the *unc-45* temperature-sensitive (*ts*) *C. elegans*, Frumkin et al. (2014) showed that knockdown of Hsp90 and its co-chaperones Sti-1, Aha1, and p23 resulted in myofibril disorganization and paralysis of the worms even at permissive temperature. The study by Frumkin et al. (2014) suggests that Hsp90 chaperone machinery is active in myosin folding and assembly when UNC-45 function is impaired but redundant under normal conditions. On the other hand, both in vitro and in vivo studies demonstrated that UNC-45 is the obligatory chaperone for myosin folding (Barral et al. 2002; Mishra et al. 2005; Srikakulam and Winklemann 2004; Liu et al. 2008; Srikakulam et al. 2008; Gazda et al. 2013; Bujalowski et al. 2014, 2015; Bookwalter et al. 2017; Lehtimäki et al. 2017; Hellerschmied et al. 2019; Gaziova et al. 2020). For example, experimental evidence showed that UNC-45's chaperoning activity on myosin heads is also necessary for myosin contractile function, actin interaction, and turnover in vivo (Du et al. 2008; Kachur et al. 2008; Shi and Blobel 2010; Gaiser et al. 2011; Melkani et al. 2011; Ni et al. 2011; Gazda

et al. 2013; Nicholls et al. 2014; Bujalowski et al. 2014, 2018; Iizuka et al. 2015, 2017). Interestingly, studies on *S. pombe* and *S. cerevisiae* showed that even the TPR-less Rng3p and She4p UCS proteins are found in complexes involving Hsp90 and myosin (Lord and Pollard 2004; Millson et al. 2005; Melkani et al. 2011). Hsp90 is capable of acting as a chaperone on its own without assistance from other proteins. However, there seems to be a distinction between TPR-less and TPR-containing UCS proteins on their dependence on Hsp90 for chaperoning activities. It appears that UNC-45 recruits and retains Hsp90 via its TPR domain primarily to maintain the integrity of myosin assemblies and regulate its contractile activity rather than for folding (Gaiser et al. 2011; Nicholls et al. 2014; Bujalowski et al. 2014, 2018). On the other hand, fungal TPR-less UCS proteins may not necessarily need to retain Hsp90 in close proximity to isolated myosin heads (Gazda et al. 2013).

Structural Organization and Versatility of UCS Proteins as Myosin Chaperones

Structurally, UNC-45 protein contains three domains: an N-terminal domain characterized by the presence of three TPR repeats (Figs. 7.1 and 7.2a), a central domain comprising of central and neck regions, and a C-terminal UCS domain (Shi and Blobel 2010; Lee et al. 2011a; b; Gazda et al. 2013). The TPR repeat is a degenerate motif that occurs in multiple copies in proteins and forms scaffolds that mediate protein-protein interactions (Sikorski et al. 1990; Das et al. 1998; Blatch and Lassle 1999; Scheufler et al. 2000). The TPR domain of UNC-45 preferentially binds Hsp90 over Hsp70 (Barral et al. 2002; Gazda et al. 2013; Haslbeck et al. 2013). Both the central and the UCS domains of *C. elegans* UNC-45 share strong sequence conservation only with other TPR-containing UCS proteins. Prior to determination of their three-dimensional structures, the UCS domain was identified based on the positions of *ts* mutations in *C. elegans* UNC-45 and the presence of blocks of sequence identity among UNC-45, CRO1, and She4p (Fig. 7.1) (Barral et al. 1998; Berteaux-Lecellier et al. 1998; Wong et al. 2000). It was later confirmed by the presence of analogous *ts* mutation in *S. pombe* Rng3p (Wong et al. 2000). The two UNC-45 null mutations that result in stop codons are both located in the central region, while three of the four UNC-45 *ts* mutations are found in the UCS domain. UNC-45 proteins share sequence conservation with fungal UCS proteins at key sites within the UCS domains. Apart from the easily recognizable N-terminal TPR domain (Fig. 7.2a and b), the rest of the protein is composed of repeating helices that organize themselves into irregular three-helix armadillo (ARM) repeats. An ARM repeat is a 40-amino acid long sequence comprising of three helices that mediates a variety of protein-protein interactions, for example, with β-catenin (Peifer et al. 1994). The central domain of CeUNC-45, which can be further divided into central and neck (or bend in She4p) regions, forms a rigid and somewhat flat backbone structure to which is attached the canonical UCS domain, a more

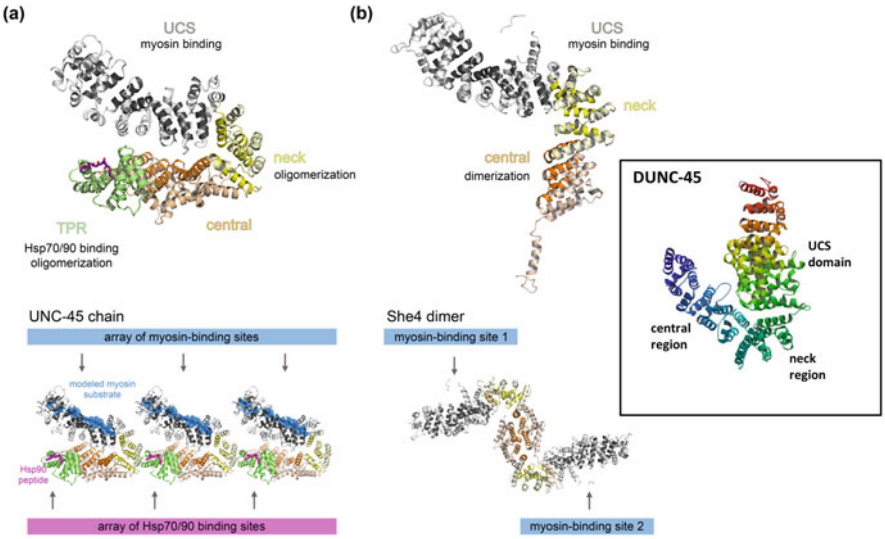

Fig. 7.2 Structures of representative UCS proteins. (**a**) Upper panel: cartoon representation of a protomer of *C. elegans* UNC-45 (PDB code: 4i2z) with co-crystallized Hsp90 peptide (magenta). Lower panel: UNC-45 chain formed by three protomers linked via a TPR domain-neck region interaction as seen in crystal lattice (Gazda et al. 2013). Co-crystallized Hsp90 peptide is shown in magenta, while a modeled myosin substrate based on beta-catenin/E-cadherin co-crystal structure (PDB code 1i7x) is shown in blue. (**b**) Upper panel: cartoon representation of a She4 monomer (PDB code: 3opb). Lower panel: dimer of She4 formed by central domain interaction as seen in the crystal packing (Shi and Blobel 2010). Insert: structure of *D. melanogaster* UNC-45 lacking the TPR domain (Lee et al. 2011a; b). Note: this figure, without the insert, was adapted from Hellerschmied and Clausen (2014)

cylindrical and flexible superhelix, on the C terminus. In the CeUNC-45 structure (Gazda et al. 2013; Hellerschmied et al. 2019), the rigid central-neck backbone thus provides binding grooves oriented in two directions: the TPR domain on the N-terminus to bind Hsp90 and the UCS domain on the C-terminus to bind myosin. In addition to providing rigidity to maintain the orientation of TPR and UCS domains, the central-neck domain serves as dimerization region in She4p (Shi and Blobel 2010) and possibly oligomerization in UNC-45 proteins (Fig. 7.2a and b) (Gazda et al. 2013). In She4p, the UCS domain groove can accommodate a 27-residue epitope, located in the ATP- and actin-binding region, of a myosin V from yeast with a binding affinity of approximately 1 μM (Shi and Blobel 2010). Previous biochemical study established that the TPR domain in UNC-45 binds Hsp90 peptide with a tenfold higher affinity than Hsp70 peptide (Barral et al. 2002), suggesting a more specific interaction with the Hsp90 molecular chaperone. These biochemical and structural studies have provided critical insights into the versatility of UCS proteins as myosin-specific chaperones in general and perhaps molecular explanation of the evolutionary requirement for TPR domain in UNC-45. In TPR-less homologs of UCS proteins, typified by She4p, the neck region

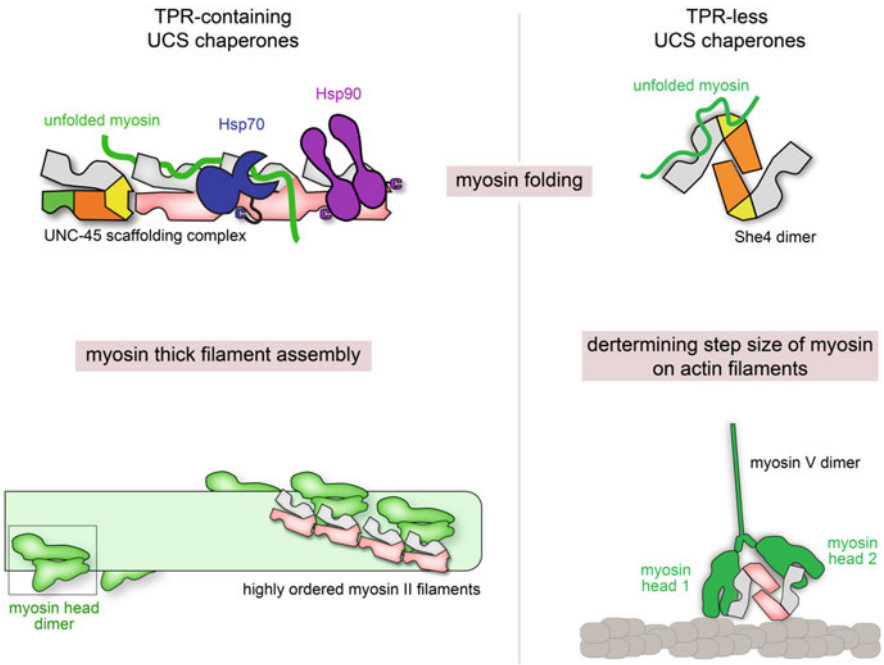

Fig. 7.3 Proposed models for myosin-specific chaperoning activities of UCS proteins. Left panel: TPR-containing UCS proteins, UNC-45, oligomerize to form scaffolds that serve as sites to recruit multiprotein chaperone complexes, comprising of Hsp90, Hsp70, and UNC-45 itself, that simultaneously assist in myosin folding and myofilament formation. Right panel: TPR-less UCS proteins, e.g., She4, act as adaptors that physically link two myosin heads to assist in folding them as well as regulate their step size along actin filaments. Note: this figure was adapted from Hellerschmied and Clausen (2014)

participates in dimerization such that two L-shaped She4p molecules form a Z-shaped zigzag molecule. In this dimeric form, She4p can act as adaptor that physically links two myosin heads to assist in folding them as well as regulate their step size along actin filaments (Fig. 7.3) (Shi and Blobel 2010; Hellerschmied and Clausen 2014). In this model, Hsp90 can bind in a TPR-independent manner to She4p-myosin complex to perform its chaperone function. On the other hand, the chaperoning activity of UNC-45 appears to be more sophisticated in higher eukaryotes in which highly organized myosin filaments have evolved (Gazda et al. 2013; Hellerschmied and Clausen 2014; Hellerschmied et al. 2019). Structural and biochemical analyses suggested that CeUNC-45 (Gazda et al. 2013) forms transient linear protein structures (essentially short filaments) by oligomerization that is mediated by a repeating TPR domain-neck region interaction (Gazda et al. 2013). The tilting of both the TPR and UCS domains at specific angles relative to the rigid central-neck domain enables two helices (7H2 and 8H2) in the neck region of one molecule of CeUNC-45 to bind to two helices (TPR3B and kinked helix) in the TPR domain of adjacent molecule in a defined array, simultaneously allowing for

interaction with Hsp90/or Hsp70 and myosin motors (Fig. 7.3). The resulting multimeric scaffolds can thus serve as sites to recruit multiprotein chaperone complexes that simultaneously assist in myosin folding, myofilament formation, and contraction (Gazda et al. 2013; Hellerschmied and Clausen 2014; Nicholls et al. 2014; Bujalowski et al. 2014, 2018). Interestingly, the length of the repeating unit in this multimer ("UNC-45 filaments") is highly similar to the repeating unit in the staggered arrangement of myosin heads in a thick filament (closest distance between adjacent double heads is 14.3 nm). Although staggering of myosin heads on the surface of thick filaments is dictated by the stagger of 98 residues of parallel myosin rods to optimize charge-charge interactions (McLachlan and Karn 1982), UNC-45 multimers may help stabilize this arrangement during thick filament and sarcomere assembly. UNC-45 multimers could also be recruited to and assemble onto established thick filaments to assist the refolding of damaged myosin heads. Further, it may serve to coordinate the activity of Hsp90 on the myosin heads (Hellerschmied and Clausen 2014; Gazda et al. 2013). Put together, these experimental observations provide strong evidence for the requirement for UCS proteins not only in the folding of myosin heads but also in myofibrillar assembly (Myhre et al. 2014) and regulation of myosin-dependent processes (Nicholls et al. 2014; Bujalowski et al. 2014, 2018; Lehtimäki et al. 2017).

UNC-45 Regulates Myosin Expression, Folding, Assembly, Contractile Function, and Degradation

In addition to its role as a chaperone for myosin, UNC-45 has emerged as a regulator of transcription of myosin genes in heart muscle by modulating the activity of its transcription factor, GATA-4 (Chen et al. 2012). In the study, Chen et al. demonstrated that UNC-45B and GATA-4 physically interacted in pull-down assay. In addition, UNC-45B was found to promote the transcriptional activity of GATA-4 when co-expressed (Chen et al. 2012). Additional studies (Odunuga and Anderson 2021) have shown that GATA-4 specifically binds to the UCS domain of UNC-45B, perhaps suggesting a chaperone-client relationship. The major revelation from these studies is that UNC-45, in particular UNC-45B, has the potential to exert both short-term (protein level) and long-term (gene level) controls over myosin and therefore muscle structure and function (Fig. 7.4, top panel). The observations also imply that UCS domain is versatile (due to its flexibility) and may have a narrow range of myosin-related protein clients, such as GATA-4, for its chaperone activity (Bujalowski et al. 2014; Hellerschmied et al. 2019; Gaziova et al. 2020). Thus, the UCS domain has been established as responsible for the chaperoning activity of UNC-45 without any significant contribution from the central and TPR regions. While not contributing to its chaperoning activity, the central domain of UNC-45B was found to specifically inhibit myosin power stroke step, thus blocking actin translocation (Nicholls et al. 2014; Bujalowski et al. 2014, 2018). Interestingly,

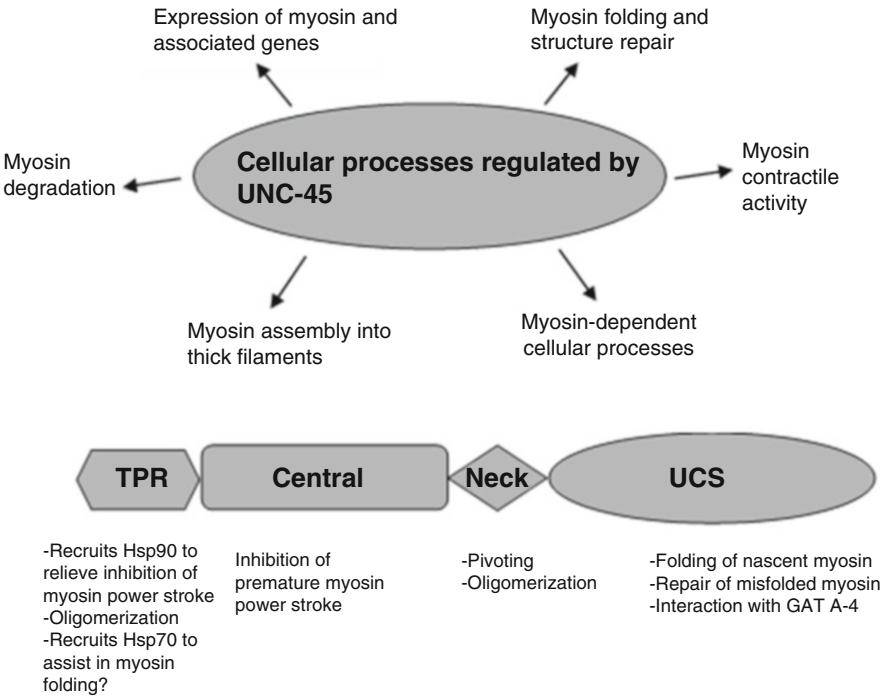

Fig. 7.4 Known functions of UNC-45 proteins. Top panel: A summary of myosin-dependent and associated cellular processes directly or indirectly regulated by UNC-45 proteins. Lower panel: specific functions of UNC-45 domains

this inhibition was relieved by Hsp90 (Nicholls et al. 2014; Bujalowski et al. 2018). It appears therefore that each domain of UNC-45B plays different but complementary roles during the folding and assembly of myosin. In a typical cycle of folding, assembly, and contraction, the UCS domain chaperones myosin head to fold correctly, while the central domain prevents premature myosin contractility to ensure proper myofibril assembly, and the TPR domain recruits Hsp90 to relieve myosin inhibition once properly assembled (Fig. 7.4, bottom panel). According to this model, under normal conditions, the UCS domain is bound to the myosin motor domain, and the TPR domain is bound to Hsp90, and yet the myosin head is active in pulling actin thin filaments. However, under stress conditions, Hsp90 detaches from the TPR domain, causing a conformational change in UNC-45 that allows the central domain to bind to the myosin neck resulting in inhibition of the myosin power stroke, while the UCS domain refolds the myosin head. When refolding is complete, Hsp90 rebinds the TPR domain, causing the central domain to release the myosin neck, thus allowing the myosin power stroke to resume. These recent findings strongly imply that UNC-45 is the main chaperone for folding and repair of myosin head while Hsp90 acts as a co-chaperone at specific steps (Fig. 7.4). UNC-45A has also been shown to regulate the expression of certain genes such as upregulating the

expression of glucocorticoid receptor (Eisa et al. 2019) and downregulating expression of endogenous retinoic acid receptor target genes (Epping et al. 2009). Interestingly, Hsp90 chaperone machinery has also been shown to modulate muscle differentiation suggesting a functional collaboration with UNC-45 in regulating myogenesis (Echeverría et al. 2016).

The regulation of the level of UNC-45 protein in muscle cells is important (Hoppe et al. 2004; Janiesch et al. 2007; Landsverk et al. 2007). Overexpression of UNC-45 in muscle cells resulted in increased myosin degradation and thus reduced or disorganized myofibrils (Janiesch et al. 2007; Landsverk et al. 2007). The chaperoning activity of UNC-45 on myosin seems to be dependent on regulation by the ubiquitin/proteasome system. A novel E3/E4-multiubiquitylation complex comprising of CDC-48, UFD-2, and CHN-1 proteins has been shown to link the turnover of both UNC-45 and myosin to functional muscle formation (Hoppe et al. 2004; Janiesch et al. 2007). Similar effects were observed in yeasts (Lord et al. 2008). From these studies, it is clear that, apart from its involvement in myosin folding, assembly, and functional muscle formation, UNC-45 is important in myosin degradation, muscle repair, and perhaps aging (Hoppe et al. 2004; Janiesch et al. 2007; Landsverk et al. 2007; Lord et al. 2008; Pokrzywa and Hoppe 2013).

UNC-45 Proteins in Invertebrates

Historically, the *unc-45* gene was originally identified in *C. elegans* through the recessive, *ts* mutant allele, *e286* (Epstein and Thomson 1974). *C. elegans* possesses only one copy of the *unc-45* gene. The *e286* mutant worms are paralyzed, with disorganized thick filaments in their muscles when grown at 25°C, but at the permissive temperature of 15°C, the worms display phenotypes essentially similar to the wild type (Epstein and Thomson 1974). These phenotypes can be reversed by temperature shifts in developing embryos and larvae but not in adult worms, implying that UNC-45 possesses a function essential for proper myofilament array formation (Epstein and Thomson 1974). Detailed genetic analysis revealed three additional recessive *ts* mutations and two lethal mutations in the *unc-45* gene (Venolia and Waterston 1990; Barral et al. 1998). All of the *ts* alleles, which show similar effects on myofibril formation, contain missense substitutions in the UCS domain of the UNC-45 protein (Barral et al. 1998). The lethal alleles each contain a stop codon located within the central region of the protein (Fig. 7.1) preventing further translation of the unc-*45* gene product (Barral et al. 1998). Genetic analysis confirmed functional relationships between the protein products of *unc-45* and of the *unc-54* and *myo-3* genes, which code for myosin heavy chains A (MHC A) and B (MHC B), respectively, that form homodimeric myosins in the body wall muscle of *C. elegans* (Waterston 1988). The *unc-45 ts* mutants directly affect MHC B, the major isoform, by generating an incorrectly folded MHC B which drastically reduces the number of intact thick filaments and therefore incapable of forming proper myofilament assemblies (Waterston et al. 1980; Waterston 1988). Null

mutations in the *unc-54* gene generate defects in muscle structure and functions similar to that of the *unc-45 ts* alleles, implying that the two genes may be epistatic (Waterston 1988). In the normal *C. elegans* thick filament, the two myosin isoforms are differentially assembled such that MHC B flanks a central myosin A zone (Schachat et al. 1977; Miller et al. 1983). However, in worms harboring the *unc-45 ts* mutant genes, this differential assembly is lost, and instead, there is a scrambling of the myosins (Barral et al. 1998), which might be due to improper folding of the myosins and consequent decreases in their concentrations (Hoppe et al. 2004; Janiesch et al. 2007; Landsverk et al. 2007). The lethal *unc-45* alleles cause arrest of development at the twofold embryonic stage resulting in inability to produce functional body wall muscle (Venolia and Waterston 1990). Interestingly, mutant worms lacking the essential *myo-3* encoded myosin heavy chain A, the minor isoform of myosins found in *C. elegans* body wall muscle, also manifest severely impaired thick filament assembly with little or no body movement (Waterston 1989). This observation suggests the necessity for myosin A in the formation of the nematode thick filament; moreover, MHC B cannot substitute for MHC A to reverse the phenotype (Waterston 1989). CFP tagged UNC-45 could be detected in all bands of the nematode muscle ultrastructure, and this fusion UNC-45 protein stably binds to MHC B containing bands, but nematode Hsp90 seems to function in the maintenance of muscle structures as a transiently accompanying diffusible factor with unstable association with the I-band and the M-line (Gaiser et al. 2011). In addition, decreased pharyngeal pumping in worms containing the *unc-45 ts* mutant genes suggests that myosins C and D, which are exclusively found in the pharyngeal muscles of *C. elegans* (Ardizzi and Epstein 1987), may be affected (Venolia and Waterston 1990). Localization by both antibodies and GFP tagging showed that UNC-45 protein is expressed in all *C. elegans* muscle cells at the adult stage (Venolia et al. 1999; Ao and Pilgrim 2000). In the developing body wall muscle of *C. elegans* larvae, UNC-45 is found in the cytosol, whereas in the mature adult muscle, it is localized to the A bands of the sarcomere, apparently chiefly with MHC B (Ao and Pilgrim 2000; Donkervoort et al. 2020). *C. elegans* UNC-45 also colocalizes in the cleavage furrow with the conventional cytoskeletal myosin II (NMY-2), a protein that plays an essential role during embryonic cytokinesis (Guo and Kemphues 1996). *C. elegans* germline and embryo have abundant UNC-45 protein, and RNA interference studies reveal that the UNC-45 protein (1) is maternally contributed—hence, rescue can occur to some extent—and (2) that it plays a role in cytokinesis in addition to muscle development (Kachur et al. 2004). The interaction between NMY-2 and maternally supplied UNC-45 has been further proven to be necessary for embryonic polarity establishment and germline cellularization (Kachur et al. 2008).

In *D. melanogaster* embryos, high levels of *dunc-45* (*D. melanogaster* homolog of *unc-45* gene) RNA were detected in mesodermal precursors to muscle, with accumulation in other tissues as well (Yu et al. 2003). This suggests that *dunc-45* gene product (DUNC-45) may be important in multiple cell types. Similar to *C. elegans*, *D. melanogaster* possesses only one copy of the *dunc-45* gene whose protein product, DUNC-45, has been shown to possess classical chaperone activity

(Melkani et al. 2010). DUNC-45 is constitutively expressed during development in *D. melanogaster* and peaks at pupation, when adult tissues are being formed. DUNC-45 associates with non-muscle myosin in embryonic blastoderm of 2-hour-old embryos, and then DUNC-45 enriches mostly in striated muscles of 14-hour old embryos, which is similar to muscle myosin (Lee et al. 2011a; b). Mutations in the *dunc-45* gene leading to DUNC-45 deficiency cause embryonic as well as late larval stage lethality, and reduced body size and defects in motility appear to be the results of embryonic body wall muscle dysfunction and defective myosin accumulation (Yu et al. 2003; Lee et al. 2011a; b). The *unc-45* knockdown experiment via RNA specifically targeted to *Drosophila* heart found that adult heart had been severely affected owing to reduced muscle contractility, decreased cardiac myosin accumulation, disassembled myofibrils, and myofibrillar disarray, while there were just mild cardiac abnormalities in third instar larvae and young pupal heart (Melkani et al. 2013). Severe cardiac problems develop during metamorphosis as *unc-45* knockdown after metamorphosis led to less serious phenotypes, which suggests that DUNC-45 is indispensable for myosin accumulation and folding during remodeling of the forming adult *Drosophila* heart (Melkani et al. 2011). It is also reported that co-expression of UNC-45 with expanded polyQ-72 (Huntington's disease-causing polyglutamine repeats which lead to protein unfolding in cardiac cells) in *Drosophila* heart could reverse polyQ-72-induced cardiac dysfunction such as protein aggregation and myofibril disassembly (Melkani et al. 2013). Thus, DUNC-45 is important for myosin to be fully functional through all stages of *Drosophila*'s life span.

UNC-45 Proteins in Vertebrates

Vertebrates have two copies of *unc-45*-like genes encoding distinct isoforms of UNC-45 (Price et al. 2002). The genes are designated as *UNC45A* and *UNC45B* in human and *UNC45a* and *UNC45b* in mouse, respectively. In mouse, *UNC45a* encodes an isoform that is expressed in multiple adult organs including the uterus, kidney, lung, and liver, hence the designation general cell (GC UNC-45 or UNC-45A) (Price et al. 2002). The second isoform encoded by *UNC45b* is found almost exclusively in the heart and skeletal muscles and was therefore designated as striated muscle (SM UNC-45 or UNC-45B) (Price et al. 2002). The two isoforms share 50–55% sequence identity within both human and mouse. There is >90% sequence identity among similar isoforms between these species. When compared with *C. elegans* UNC-45, both isoforms show 30–40% identity with the worm protein. In 8-day-old mouse embryo, UNC-45B is predominantly expressed in the contractile heart and is hardly found in other organs, whereas UNC-45A is diffusely expressed and later concentrates in regions of intense development such as the branchial arches and forelimb bud (Price et al. 2002).

Studies on *Danio rerio* (zebrafish) confirmed that UNC-45A has important function in pharyngeal arch and aortic arch development and is involved in the

pathogenesis of arteriovenous malformation (AVM) (Anderson et al. 2008). Knockdown of UNC-45B in zebrafish by morpholino oligonucleotide caused myofibril disassembly in the sarcomeres of the trunk muscle and the ventral displacement of jaw cartilages, thus demonstrating that the protein is required for skeletal, cranial, and cardiac muscle contraction (Wohlgemuth et al. 2007). Therefore, UNC-45B is necessary for zebrafish motility, and it is also essential for morphogenesis and function of the developing heart and jaw. Overexpression of *D. rerio* UNC-45B mimicked results of similar experiments in *C. elegans* as transgenic zebrafish embryos had defective myofilament assembly in skeletal muscles (Bernick et al. 2010). The defect by UNC-45B overexpression was shown to depend on the UCS domain but not the TPR domain since deletion of the UCS domain revoked myofibril disorganization by UNC-45B overexpression and deletion of the TPR domain had no effect (Bernick et al. 2010). Further studies on zebrafish revealed that the central region of UNC-45B mediated Z-line association and interacted with Apo2a (the cytidine deaminase Apobec 2a) (Etard et al. 2008, 2010). The interaction between UNC-45B and Apo2a is necessary for integrity of the myosepta and myofiber attachment in zebrafish, which is Hsp90-independent (Etard et al. 2010). The *apo2* mutant embryos share similar dystrophic muscle phenotypes with *unc45b* mutants but not with *hsp90α* mutants as *hsp90α* mutant had normal myosepta structure and a beating heart (Etard et al. 2010). Missense mutation of *Xenopus tropicalis* UNC-45B caused skeletal muscle myofibril disruption, paralysis, and heart beat problem, suggesting that UNC-45B participates in the Z-body maturation (Geach and Zimmerman 2010). A major implication of these studies is that loss of function of UNC-45 leads to defective muscle formation and thus myopathies (Etard et al. 2008, 2010; Geach and Zimmerman 2010). Recent studies revealed that *unc-45b* gene in zebrafish is under the control of a heat shock factor 1 (Hsf1) binding element found within the promoter region of the gene (Etard et al. 2015).

In C2C12 myogenic cells, only UNC-45A mRNA is detected in proliferating myoblasts, with the level decreasing as the cells progress to form myotubes (Price et al. 2002). In contrast, UNC-45B mRNA is detected only after the cells start fusing, peaking in young myotubes and dropping off as the myotubes age (Price et al. 2002). These observations in both mouse embryo and C2C12 myoblasts implicate stage-specific expression and functions of the UNC-45 isoforms in embryogenesis and muscle differentiation. The UNC-45A isoform is now known to be involved in cell division and related cytoskeletal formation (Iizuka et al. 2015, 2017; Short 2017; Lehtimäki et al. 2017; Habicht et al. 2019, 2021), while the UNC-45B isoform is involved in striated muscle differentiation and myofibril formation (Gazda et al. 2013; Hellerschmied and Clausen 2014; Nicholls et al. 2014; Bujalowski et al. 2014, 2018). Interestingly, UNC-45A has been reported to have higher apparent affinity and greater folding capability of smooth muscle myosin motor domain, compared to UNC-45B (Liu et al. 2008). Further experimental evidence that proved that the two isoforms of UNC-45 are functionally divergent was obtained from studies using zebrafish (Comyn and Pilgrim 2012). The authors found out that the singly homologous zygotic mutant *unc45b*$^{-/-}$ and the doubly homologous mutant *unc45b*$^{-/-}$/*unc45a*$^{-/-}$ displayed identical defects in cardiac and skeletal muscle and jaw

formation (Comyn and Pilgrim 2012). They therefore concluded that *unc-45a* did not appear to play an important role in muscle development and that the two gene paralogs are functionally divergent.

Two isoforms of UNC-45A were detected in several breast carcinomas: a 929-amino acid protein isoform and a 944-amino acid protein isoform, which differ by an N-terminal proline-rich 15-amino acid sequence (Guo et al. 2011). The protein level of the 929-amino acid protein was found to be threefold higher due to the 944-amino acid protein being degraded at a fivefold higher rate than the former by the ubiquitin-proteasome system (Guo et al. 2011). Immunoprecipitation experiments showed that both UNC-45A isoforms could interact with non-muscle myosin IIA, non-muscle myosin IIB, and Hsp90β, suggesting that both UNC-45A isoforms are capable of playing functional roles in cell motility (Guo et al. 2011).

Studies carried out on three UNC-45B recessive loss-of-function lines in C3H and C57BL/6 inbred mouse strains showed that these mutations caused arrest of cardiac morphogenesis at the formation of right heart structures and failure of contractile function (Chen et al. 2012). The authors also found out that UNC-45B was essential for sufficient accumulation and function of GATA-4 protein, a cysteine zinc finger transcription factor, in mouse embryonic cardiac morphogenesis (Chen et al. 2012). GATA-4 is a key transcriptional regulator for several cardiac genes including *α-MHC* (Molkentin et al. 1994). Pull-down experiments confirmed a direct physical interaction between UNC-45B and GATA-4 (Chen et al. 2012). Therefore, the heart-specific UNC-45B isoform may function as a molecular chaperone mediating contractile function of the sarcomere and gene expression in cardiac development. In addition, UNC-45B has been reported to be involved in lens development and pathogenesis of autosomal dominant juvenile cataract in humans (Hansen et al. 2014). It is hypothesized that developmental cataract might be induced by defective non-muscle myosin organization during maturation of the lens fiber cells, which is caused by UNC-45B mutation (Hansen et al. 2014). UNC-45 B has been shown to be directly involved in myopathy (Dafsari et al. 2019; Donkervoort et al. 2020). At the molecular level, these phenotypes are presumably due to defective myosin-UNC-45B interactions (Fratev et al. 2013; Hellerschmied et al. 2019; Gaziova et al. 2020). In recent studies, UNC-45A was revealed as a master regulator of cellular processes dependent on non-muscle myosin II such as NK-mediated cell toxicity (Iizuka et al. 2015) and neurite growth (Iizuka et al. 2017).

UCS Proteins in Yeasts, Fungi, and Apicomplexans

UCS domain proteins are present in *S. cerevisiae* (She4p) (Jansen et al. 1996; Wendland et al. 1996), *P. anserina* (CRO1) (Berteaux-Lecellier et al. 1998), and *S. pombe* (Rng3p) (Balasubramanian et al. 1998). Sequence similarity among the three fungal proteins and UNC-45 is restricted to the C-terminal UCS domain. Despite the lack of TPR motifs, the N-terminal sequence of fungal UCS proteins may contain sequences capable of recruiting chaperones (Young et al. 2003).

However, all three TPR-less UCS proteins are linked by their common association with cellular processes involving myosins. Although sequence similarity among the fungal UCS proteins is low (Lord and Pollard 2004), the *ts* mutations in *S. pombe*, like *C. elegans*, are in conserved residues (Fig. 7.1).

The *she4p* gene was identified and named differently in two independent screens in *S. cerevisiae* (Jansen et al. 1996; Wendland et al. 1996). The first screen was for the expression of the HO endonuclease in mother cells yielding the She4p-encoding gene (SHE: Swi5p-dependent HO expression) (Jansen et al. 1996) and the other for defects in endocytosis identifying the *dim1* gene (*dim*: defective internalization of membrane) (Wendland et al. 1996). Both null and *ts* mutants of the *she4p* gene caused defects in endocytosis and loss of actin polarization in the cell. Two-hybrid and biochemical experiments showed that She4p interacts, via its UCS domain, with the motor domains of conventional type II myosin (Myo1p) as well as unconventional types 1 (Myo3p/Myo5p) and V (Myo2p/Myo4p) myosins in an actin-dependent manner for proper endocytosis and cytokinesis to occur (Toi et al. 2003; Wesche et al. 2003). She4p was also reported to be important for myosin stability and interactions with actin (Lord et al. 2008). In addition, She4p interacts with Hsp90 in yeast two-hybrid assays (Millson et al. 2005). The She4p protein is composed of 789 amino acids and shares about 33% similarities with other fungal UCS proteins (Lord and Pollard 2004). Alanine mutagenesis of the phosphorylatable serine 18 residue in She4p rescued she4Δ mutant yeast cells from lysis at 45 °C (Gomez-Escalante et al. 2017), indicating that phosphorylation of the chaperone may be a negative modulator when the cells are under stress.

The *rng3* gene was identified in a large-scale screen for genes whose products function in cytokinesis (Balasubramanian et al. 1998). The gene encodes a protein of 746 amino acid residues. Actin ring formation was found defective in *S. pombe* cells harboring mutants of both *rng3* (*rng3–65*) and *rng5* (*rng5-E1*) which encodes for Myo2 (a type II myosin heavy chain), suggesting a functional interaction between the protein productions of the two genes. Null mutants in *rng3* resemble deletion mutants in *myo2*, while *ts rng3* mutants show strong adverse interactions with Myo2-E1 mutant myosin (Wong et al. 2000). Rng3p colocalizes with *myo2-E1* mutant myosin at the cell division site in an F-actin-dependent manner. More importantly, Rng3p has been shown to be necessary for the formation of progenitor "spots" that form the actomyosin ring assembly in interphase *S. pombe* cells (Wong et al. 2002). Maintenance of the myosin-containing spots, however, is independent of F-actin. While the actomyosin ring has a rapid turnover, the interphase spot does not, showing that this progenitor structure in the interphase is necessary to ensure proper assembly of the actomyosin ring and successful cell division. Recombinant full-length Rng3p or its UCS domain alone is necessary and sufficient to activate the actin-based motility of myosin in vitro and double its actin-activated Mg^{2+}-ATP activity (Lord and Pollard 2004; Lord et al. 2008). Although Rng3p is specifically necessary to maintain the activity of intrinsically unstable Myo2, it may possess the capability of responding to changes in the stability of other myosins (Stark et al. 2013). Rng3p was also found to associate with polysomes and bind to mRNAs encoding all types of myosin heavy chains, which suggest that Rng3p may be

involved in myosin folding cotranslationally (Amorim and Mata 2009). Whether Rng3p and other fungal UCS proteins require Hsp90 for their myosin-dependent functions is uncertain. However, in vivo, Swo1p (Hsp90 homolog in *S. pombe*) and Rng3p have been shown to be both required for Myo2 assembly in the contractile ring (Mishra et al. 2005). These observations suggest that some functional relationship exists between the *S. pombe* UCS protein and Hsp90.

The CRO1 protein of the filamentous fungus, *P. anserina*, is a 702-residue protein that is required for sexual sporulation (Berteaux-Lecellier et al. 1998). GFP tagging of the CRO1 protein reveals that it is a cytosolic protein expressed mainly at the beginning of the dikaryotic stage and at the time of ascospore maturation. The primary defect of null mutant allele of the gene, *cro1–1*, is the inability to form septa between the daughter nuclei after mitotic division. The mutant also results in abortive meiosis of resultant polyploidy nuclei and lack of progression from the syncytial (vegetative) state to the cellular (sexual) state (Berteaux-Lecellier et al. 1998). Unlike the wild-type fungal filaments, disorganization of the actin prevents microtubule disassembly.

Recently, UNC-45-like genes, *PUNC* and *TgUNC*, were isolated in the apicomplexans *Plasmodium falciparum* (Bookwalter et al. 2017) and *Toxoplasma gondii* (Bookwalter et al. 2014). In both studies, production of correctly folded and actin-gliding myosin in Sf9 cells was possible only when co-expressed with its genus-specific UNC-45-like protein. Both proteins share the same domain organization as other UNC-45 proteins suggesting their interaction with Hsp90 and myosin. Depletion of *TgUNC Toxoplasma* caused inability of the parasite to move and propagate due to myosin misfolding and scrambling (Frénal et al. 2017).

UNC-45 and Cancers

Experimental evidences are emerging that link UNC-45 to different cancers. UNC-45A was identified as a new factor to regulate Hsp90-dependent progesterone receptor (PR) chaperoning in a yeast two-hybrid screen (Chadli et al. 2006). UNC-45A can interact with PRs in vivo and in vitro. It was shown that UNC-45A inhibits the activation of Hsp90 by the co-chaperone Aha1 and blocks progression of PR chaperoning to its hormone-binding state in a cell-free system (Chadli et al. 2006). High-level expression of UNC-45A is associated with proliferation and metastasis of ovarian cancer, which is reversed in siRNA knockdown of the *unc-45a* gene (Bazzaro et al. 2007). UNC-45A has been shown to confer resistance to histone deacetylase inhibitors (HDACIs) and block retinoic acid-induced proliferation arrest and differentiation of human neuroblastoma cells (Epping et al. 2009). Human breast carcinoma and cell lines derived from breast carcinoma metastases also had enhanced UNC-45A mRNA and protein expression levels (Guo et al. 2011). RNAi knockdown of endogenously overexpressed UNC-45A caused significant reduction in proliferation and invasion in the most metastatic cell line (Guo et al. 2011). It appears that UNC-45A involvement in tumor pathogenesis and

proliferation occurs via its ability to destabilize microtubules (MTs), for example, during mitosis, and promote transcriptional activities of steroid hormone receptors in cancer cell lines (Eisa et al. 2019; Habicht et al. 2019, 2021; Mooneyham et al. 2019).

Conclusions and Future Work

UCS proteins have been established as indispensable chaperones that recruit Hsp90 as a co-chaperone to regulate myosin folding, assembly, functions, degradation, and associated cellular processes. In particular, UNC-45 has emerged as a master regulator of myosin-dependent cellular processes, not only by controlling myosin level but also by influencing expression of several proteins associated with these processes. Research has unraveled previously unknown roles of each of the domains of UNC-45, especially in myosin folding and assembly. Microtubule destabilization and regulation of gene expression are previously unknown functions of UNC-45 proteins and underbelly their roles in cell division, proliferation, and pathogenesis of various cancers. Future research in this field will probably focus on unraveling the mechanisms of UNC-45 chaperones' involvement in muscle repair, aging, and disease states such as chaperonopathies, myopathies, and cancer.

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Chapter 8
Chaperonin: Co-chaperonin Interactions

Aileen Boshoff

Abstract Co-chaperonins function together with chaperonins to mediate ATP-dependent protein folding in a variety of cellular compartments. Chaperonins are evolutionarily conserved and form two distinct classes, namely, group I and group II chaperonins. GroEL and its co-chaperonin GroES form part of group I and are the archetypal members of this family of protein folding machines. The unique mechanism used by GroEL and GroES to drive protein folding is embedded in the complex architecture of double-ringed complexes, forming two central chambers that undergo conformational rearrangements that enable protein folding to occur. GroES forms a lid over the chamber and in doing so dislodges bound substrate into the chamber, thereby allowing non-native proteins to fold in isolation. GroES also modulates allosteric transitions of GroEL. Group II chaperonins are functionally similar to group I chaperonins but differ in structure and do not require a co-chaperonin. A significant number of bacteria and eukaryotes house multiple chaperonin and co-chaperonin proteins, many of which have acquired additional intracellular and extracellular biological functions. In some instances, co-chaperonins display contrasting functions to those of chaperonins. Human HSP60 (HSPD) continues to play a key role in the pathogenesis of many human diseases, in particular autoimmune diseases and cancer. A greater understanding of the fascinating roles of both intracellular and extracellular Hsp10 on cellular processes will accelerate the development of techniques to treat diseases associated with the chaperonin family.

Keywords Chaperonins · Co-chaperonins · GroEL · GroES · Hsp60 · Hsp10 · Cpn60 · Cpn10

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Introduction

Chaperonins are ubiquitous ATP-driven protein folding machines characterised by a large multi-subunit ring structure. They prevent aggregation by binding non-native proteins and facilitate folding and unfolding of proteins. They form part of the Hsp60 family of heat shock proteins and are related by homology to the GroEL proteins of *E. coli* (Hartl and Hayer-Hartl 2002; Hemmingsen et al. 1988). The *E. coli* chaperonin GroEL and its co-chaperonin GroES are the quintessential members of this family of protein folding machines (Ellis and Hartl 1996; Hartl and Hayer-Hartl 2002; Horwich et al. 2007). The term 'chaperonin' (Cpn) was first coined in 1988 to represent this family of molecular chaperones after finding sequence similarity between Rubisco binding protein (now known as chloroplast Cpn60) and GroEL (Hemmingsen et al. 1988). The Hsp60 family of chaperones is one of the most abundant classes of molecular chaperone present in the plastids, mitochondria, and cytoplasm of all eukaryotes and eubacteria.

The terms GroEL and GroES were initially applied strictly to the two proteins found in *E. coli* and have been extended to include homologues from other bacterial species. The GroEL protein functions as a typical molecular chaperone as it binds and folds proteins, whilst GroES exhibits no autonomous role as a chaperone but modulates the activity of GroEL and is referred to as a co-chaperone. The term chaperonin is applied to bacterial proteins that are homologous to the *E. coli* GroEL and are also referred to as Cpn60, whilst co-chaperonins refer to homologues of *E. coli* GroES, also known as Cpn10. Although the mitochondrial homologues are called Hsp60 and Hsp10, the archaeal chaperonins are referred to as thermosomes (Trent et al. 1991). In the eukaryotic group, chaperonins found in the cytosol were first called TCP-1 and are now also called CCT (chaperonin-containing TCP-1) (Kubota et al. 1994), TRiC (TCP-containing ring complex) (Frydman et al. 1992), and c-cpn (Gao et al. 1992). The human HSP60/HSP10 proteins have been renamed HSPD/E (Kampinga et al. 2009). The chloroplast chaperonin is referred to as Cpn60 protein, and two types of co-chaperonins Cpn10 and Cpn20 are present (Koumoto et al. 2001). Prior to its recognition as chloroplast Cpn60, it was known as Rubisco-binding protein (Barraclough and Ellis 1980).

GroEL and GroES are essential molecular chaperones in *E. coli*, indispensable for viability at all temperatures (Ang and Georgopoulos 1989; Fayet et al. 1989; Tilly et al. 1981). Mitochondrial Hsp60 is similarly essential for the viability of *Saccharomyces cerevisiae* (Cheng et al. 1989; Rospert et al. 1993b), as are the group II CCT subunits (Lin and Sherman 1997; Stoldt et al. 1996). Mitochondrial Hsp60 inactivation results in embryonic lethality in mice (Christensen et al. 2010). Deletion of HSP60 in mouse cardiomyocytes resulted in heart failure due to impaired mitochondrial function (Fan et al. 2020). GroEL is critical for the correct folding of many proteins in the cell, under both normal and stress conditions. The folding of nascent polypeptides often requires the cooperation of both the Hsp70 and Hsp60 families, and these families are also responsible for most of the general folding events in the cell (Fink 1999; Hartl et al. 1992). Whilst CCT is not upregulated during heat shock

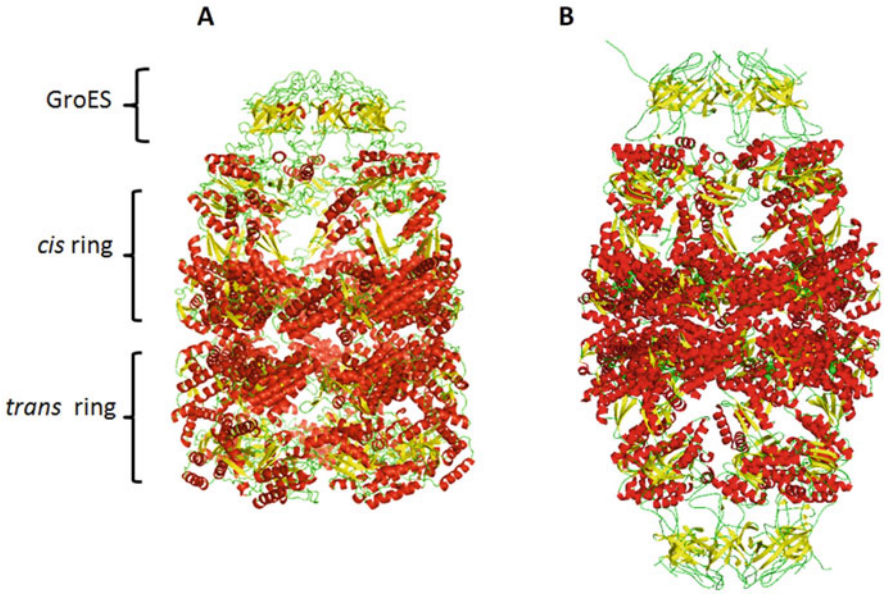

Fig. 8.1 The asymmetric GroEL/GroES complex comprises of two heptameric rings of GroEL stacked back-to-back with the GroES 'lid' bound to the *cis* ring to form a bullet-shaped complex, showing the side view (**a**), and the symmetric GroEL/GroES complex with two heptameric rings of GroEL stacked back-to-back with the GroES bound to both rings to form a football-shaped complex, showing the side view (**b**). The α-helices are shown in red and β-sheets in yellow. The images were generated using PyMol (DeLano Scientific) from coordinates in PDB: 1AON and 4PJ1

(Horwich et al. 2007), GroEL and mitochondrial Hsp60 are heat inducible. In addition to ensuring the correct folding of proteins, chaperonins play a role in the assembly of protein complexes (Seo et al. 2010), trafficking of proteins (Xu et al. 2011) and peptide hormone signalling (Sigal and Williams 1997).

The chaperonins share a common subunit organisation and structure. They are a family of ATPases consisting of twin heptameric rings stacked back-to-back to create a characteristic cylindrical structure and function by assisting in the folding of nascent and misfolded proteins (Hartl and Martin 1995; Houry et al. 1999). Each ring creates a large cavity for unfolded proteins to bind and undergo productive folding to the native state in a highly cooperative and ATP-dependent manner (Bukau and Horwich 1998; Hartl and Hayer-Hartl 2002). Co-chaperonins form a single heptameric ring of 10 kDa subunits and are present in all bacterial and eukaryotic organisms (Hartl 1996). The *E. coli* asymmetric GroEL/GroES complex consists of two stacked heptameric rings of GroEL capped by a single heptameric ring of GroES that forms the lid over the folding cage (Fig. 8.1). The GroEL ring that is bound to GroES and protein substrate is termed the *cis* ring, and opposite ring free of GroES is termed the *trans* ring (Fig. 8.1a) (Xu et al. 1997). Two folding mechanisms have been proposed for GroEL; these are termed the *cis* and *trans* mechanisms (named after the GroEL rings that are bound by GroES), with most

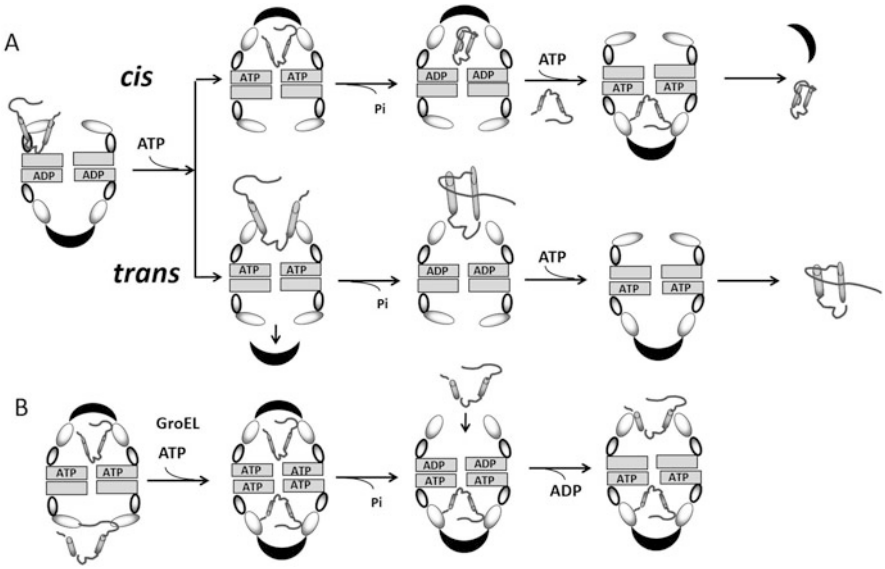

Fig. 8.2 GroEL-/GroES-mediated interaction cycle. The asymmetric (**A**) and symmetric (**B**) GroEL/GroES interaction cycles in the presence of substrate. The binding of ATP to the *cis* cavity causes conformational changes which allows the GroEL-bound substrate to follow either the *cis* or *trans* folding pathway, depending on the properties of the substrate (a). In the *cis* mechanism, the binding of substrate to one GroEL ring is followed by the binding of ATP and GroES to the *cis* ring. The substrate is released into the cavity closed by GroES and allowed to fold. ATP is hydrolysed, and the complex is ready to dissociate. The binding of ATP to the *trans* ring triggers release of substrate and dissociation of GroES from the *cis* ring and allows GroES to bind, releasing substrate into the cavity. Substrates that protrude outside the cavity and require only a portion of the protein to be folded, thus preventing GroES binding, follow the *trans* folding pathway. The subsequent binding of ATP and GroES to the *cis* ring releases the substrate. In the symmetric cycle, the two GroEL rings function simultaneously in substrate folding, with ATP hydrolysis resulting in GroES and substrate release (b). Encapsulation of substrate during the asymmetric folding allows a longer time for folding of the substrate, whilst a short residence time in the symmetric model increases the folding yields (Bigman and Horovitz 2019) [Adapted from Hayer-Hartl et al. (2016) and Kumar et al. (2015)]

GroEL substrates utilising the *cis* mechanism (Fig. 8.2) (Chaudhuri et al. 2001; Farr 2003). The functional cycle requires the binding of chaperonin 10 to one or both chaperonin rings which forms a lid-like structure on top of the cylinder when ATP is bound that causes the chamber to enlarge to allow for protein folding (Chandrasekhar et al. 1986). A vital part of the structure of each subunit is a flexible mobile loop that mediates binding to the chaperonin (Landry et al. 1996). The flexibility and the structure of the complex are conserved amongst co-chaperonins, and sequence variations impose differences in binding affinity (Richardson et al. 2001). Protein substrates first bind to the apical domain and are then dislodged and driven into the cavity by the binding of the co-chaperonin to the same area (Hartl and Hayer-Hartl 2002). The folding process is driven by the binding and hydrolysis of

ATP which triggers a complex set of allosteric signals both within and between the stacked rings (Gray and Fersht 1991; Todd et al. 1993). The *trans* mechanism has been proposed predominantly for large substrates that cannot enter the cavity, and partial substrate folding takes place through binding and release from the *trans* ring (Fig. 8.2) (Chaudhuri et al. 2001; Farr 2003). ATP-independent unfoldase activity has been reported for both GroEL and TRiC that allows the unfolded protein to fold outside the cavity (Priya et al. 2013).

The Gp31 protein from bacteriophage T4, a functional co-chaperonin that promotes the assembly of the T4 major capsid protein, can functionally substitute for GroES resulting in an increase in size and hydrophilicity of the enclosed chamber (Hunt et al. 1997; van der Vies et al. 1994). Another co-chaperonin from bacteriophage RB49, called CocO, is distantly related to GroES (Ang et al. 2001). Both of these bacteriophage co-chaperonins utilise host-encoded GroEL to assemble capsid proteins, and both proteins could functionally replace GroES in *E. coli* (Keppel et al. 2002). Interestingly, the first viral-encoded chaperonin was identified in the genome of *Pseudomonas aeruginosa* bacteriophage EL (Hertveldt et al. 2005) and later demonstrated to have functional properties similar to GroEL except that it does not require a co-chaperonin for activity (Kurochkina et al. 2012). The crystal structure of the GroEL homologue from the bacteriophage EL of *Pseudomonas aeruginosa* revealed that the chaperonin prevented protein aggregation without encapsulation and may represent an earlier version of the protein before the development of encapsulation (Bracher et al. 2020). A wide range of newly identified functions have been attributed to eukaryotic Hsp60, including roles in carcinogenesis, immunity, and cell signalling (Chandra et al. 2007). The roles played by both intracellular and extracellular forms of human HSP10 (HSPE) in pregnancy, cancer, and autoimmune diseases continue to receive attention (Corrao et al. 2010; Jia et al. 2011). The role of Hsp60 in promoting infection by hepatitis B virus (HBV), human immunodeficiency virus (HIV), and influenza A virus was reviewed by Wyżewski et al. (2018).

Whilst the *E. coli* chaperonins are encoded by only two genes, *groEL* and *groES*, both Cpn60 and Cpn10 found in green algae and plants are encoded by numerous genes (Boston et al. 1996; Hill and Hemmingsen 2001; Schroda 2004). Chloroplast chaperonins exhibit greater complexity than those found in bacteria and mitochondria with unique structures and functions (Vitlin Gruber et al. 2013a). It also appears that approximately 30% of bacteria encode more than one *groEL* gene (Hill and Hemmingsen 2001). The biological significance of several chaperonin genes has yet to be revealed (Lund 2009); however, the literature has expanded in recent years in this area of research. Cpn60 subunits with diverse expression profiles have evolved in chloroplasts, with the dominant subunits appearing to play housekeeping roles and minor subunits having more specialised functions, including the folding of specific proteins (Peng et al. 2011).

The chaperonins are subdivided into two distantly related groups. Group I chaperonins are found in eubacteria, mitochondria, and chloroplasts, of which GroEL from *E. coli* is the best studied and understood (Leroux 2001). Group II chaperonins are present in archaebacteria and in the eukaryotic cytosol (Frydman

2001; Horwich et al. 1993). Although both subgroups form ring-like structures with cavities for sequestered protein folding, group II chaperonins form heterooligomeric complexes (Archibald et al. 1999; Spiess et al. 2004). The group II chaperonins consist of two eight- or nine-membered rings consisting of one to three subunit types in the archaeal thermosome rings (Phipps et al. 1991), whilst TRiC/CCT rings consist of eight subunit types (Frydman et al. 1992; Spiess et al. 2004). An important difference between the two groups is the lack of a GroES homologue in the group II chaperonins (Horwich and Saibil 1998). Group I chaperonins utilise an independently expressed co-chaperonin that functions as a lid to aid the encapsulation of unfolded protein, whilst group II chaperonins have a built-in lid in the form of a particular α-helical protrusion and do not require additional protein subunits to function (Meyer et al. 2003; Vabulas et al. 2010). However, the activity of CCT is regulated by a number of co-chaperones, including prefoldin, phosducin-like proteins, and BAG3 (Fontanella et al. 2010; Martin-Benito et al. 2002; Stirling et al. 2006; Vainberg et al. 1998). Prefoldin produces tentacle-like coils that capture protein substrates and transfers them to group II chaperonins; further studies have highlighted the importance of human prefoldin in proteostasis and the development of various diseases (reviewed by Sahlan et al. 2018). In 2010, a third group was proposed in bacteria and are conserved in the genomes of eleven bacteria (Techtmann and Robb 2010). These novel chaperonins are capable of refolding denatured proteins in a GroES-independent manner. Group III chaperonins are highly divergent and distantly related to group I and group II, and they might represent an ancient horizontal gene transfer event from archaea to bacteria, and this revises the current paradigm for chaperonin classification (Techtmann and Robb 2010). The crystal structure of the thermophilic bacterial group III Cpn from *Carboxydothermus hydrogenoformans* revealed that it is mechanistically distinct from group I and II chaperonins, and further evidence suggests that groups I and II may have arisen from a group III Cpn (An et al. 2017).

To date, the structure and mechanism of chaperonin and co-chaperonin functions have centred on the GroEL and GroES system of *E. coli* (Hartl 1996; Hartl and Hayer-Hartl 2002; Horwich et al. 2007). This system has received the most attention and serves as a model for chaperonin and co-chaperonin interactions. The GroEL and GroES folding machine will be discussed in the following section with emphasis on the role of GroES. Group I chaperonins will be the focus of this chapter as the functional activity of group II chaperonins is not assisted by co-chaperonins. The biological impact of chaperonins extends beyond protein folding as they are the dominant immunogens present during human bacterial infections, and there is considerable interest in their role in cancer and autoimmune diseases (Kaufmann 1992; Wiechmann et al. 2017). Data on the extensive roles of both extracellular and intracellular Hsp10 has left no doubt that the functions of this protein extends beyond its role as a co-chaperonin, and these roles have been reviewed by (Corrao et al. 2010). Research on bacterial chaperonins has expanded in recent years as more bacterial genomes have been sequenced. Our understanding of co-chaperonins in other organisms and organelles is gaining momentum, and recent findings on bacterial and eukaryotic co-chaperonins will be addressed.

Activities of the *E. coli* GroEL/GroES Folding Machine

One of the most efficient chaperone systems is the well-characterised *E. coli* chaperonin machine composed of GroEL and its co-chaperonin GroES. Three different functions have been assigned to this folding machine, binding to non-native proteins preventing aggregation (Buchner et al. 1991), facilitating protein folding by encapsulating the protein in a sequestered environment (Weissman et al. 1995), and finally unfolding of kinetically trapped intermediates so that they can refold (Shtilerman et al. 1999; Sparrer et al. 1997; Sparrer and Buchner 1997; Todd et al. 1993). The *groE* genes of *E. coli* were the first chaperonin genes to be discovered. These genes were first identified when temperature-sensitive mutant strains of *E. coli* could not support the growth of bacteriophage λ (Georgopoulos et al. 1972); afterwards, it was determined that the two genes are encoded on the same operon *groE*. The importance of these GroEL and GroES proteins is emphasised by the fact that they are the only chaperones that are essential for the viability of *E. coli* at all temperatures (Fayet et al. 1989). Additionally, host GroEL and GroES play a role in both phage infection and defence strategies of the host (Ang et al. 2000), as well as protecting viral proteins at high temperatures (Chen et al. 2013). *E. coli* GroEL/ES was previously known to play a role in the regulation of sigma-32 by enhancing proteolysis (Guisbert et al. 2004). An additional proteolytic role was demonstrated whereby interaction with the cold shock RNA chaperone (CspC) lead to proteolysis (Lenz and Ron 2014).

It is estimated that under normal cellular growth conditions, 10–15% of all cytoplasmic proteins rely on GroEL in order to fold correctly, and this increases to 30% under conditions of stress (Ewalt et al. 1997). Many of the cytoplasmic proteins that interact with GroEL have been identified (Houry et al. 1999), and GroEL acts downstream of the *E. coli* molecular chaperones, DnaK (prokaryotic Hsp70), and trigger factor, in the folding of 10% of cytosolic proteins (Ewalt et al. 1997; Houry et al. 1999). The mechanism of action is different to that of Hsp70 as the protein is sequestered from its environment. In a proteomic study of *E. coli* proteins, ~250 different proteins interact with GroEL, of these ~85 proteins are dependent on GroEL for folding and 13 of these are essential proteins (Kerner et al. 2005). These 85 proteins were scrutinised further, and ~60% were found to be absolutely dependent on GroEL and GroES for folding, and an additional 8 proteins were classified as obligate substrates (Fujiwara et al. 2010). Most of the substrates are characterised by a size range of 20–50 kDa and complex α/β or α + β topologies and tend to populate kinetically trapped folding intermediates (Kerner et al. 2005).

Over the past 30 years, many researchers have demonstrated the abilities of the *E. coli* GroEL and GroES machine to bind and refold a wide range of aggregation-prone proteins both in vivo and in vitro. Early in vitro experiments demonstrating the abilities of *E. coli* GroEL and GroES to refold denatured proteins were carried out using heat-denatured Rubisco enzyme (Goloubinoff et al. 1989), and following this seminal paper, the GroEL-GroES cycle has been scrutinised in vitro. Chaperonins continue to also play an important role in recombinant protein production, and this

has been well documented in the literature. *E. coli* is a frequently used host, and the folding of proteins in the cytoplasm is assisted primarily by Hsp70 and Hsp60 (Vabulas et al. 2010). They aid in functional expression and retain solubility by assisting the refolding of aggregated target proteins. The chaperonin GroEL and its co-chaperonin GroES have been used extensively for this purpose and are often co-expressed with the protein of interest. Some of these proteins include malate dehydrogenase (Hartman et al. 1993; Ranson et al. 1997), citrate synthase (Buchner et al. 1991), rhodanese (Martin et al. 1991), carbamoylase (Sareen et al. 2001), and aconitase (Chaudhuri et al. 2001). The presence of *E. coli* GroEL and GroES significantly improved the yields of soluble protein in most instances; however, large amounts of the chaperonins are often required, exceeding endogenous concentrations. Extensive optimisation of the reaction conditions is also vital, and the requirements of each chaperonin are variable. A greater understanding of the effects of overexpressing chaperonins on cell growth, and conditions for optimum recombinant protein production, needs to be investigated (Gupta et al. 2006). Despite these drawbacks, the *E. coli* chaperonins have been used successfully in biotechnology for the production of a wide range of recombinant proteins. The co-expression of GroEL/ES appreciably enhanced the expression of human tumour necrosis factor, CD 137 ligand (Wang et al. 2012). The solubility of *Plasmodium falciparum* 1-deoxy-D-xylulose-5-phosphate reductoisomerase was significantly increased by the co-production of GroEL/ES (Goble et al. 2013). GroEL immobilised on a sensor has been developed to detect and quantify unfolded therapeutic proteins in solution (O'Neil et al. 2018).

Structure of GroEL and GroES

GroEL and GroES form both GroEL-GroES asymmetric bullet-shaped and GroEL-$GroES_2$ symmetric football-shaped complexes as one GroEL ring can bind to one GroES heptamer (Fig. 8.1). There has been much debate concerning which of these complexes is essential for protein folding and its mechanism of action (Bigman and Horovitz 2019; Taguchi 2015). The crystal structure of GroEL bound to GroES and ADP was resolved in 1997, which corresponded to the bullet-shaped complex (Xu et al. 1997). Nearly two decades later, the structure of the football-shaped complex was resolved (Fei et al. 2014; Koike-Takeshita et al. 2014). Several crystal structures of GroEL are available (Braig et al. 1994), including GroEL complexed with ATP (Boisvert et al. 1996), and a GroEL-peptide complex (Chen and Sigler 1999), as well as NMR (nuclear magnetic resonance) spectroscopy (Fiaux et al. 2002; Nishida et al. 2006) and cryo-electron microscopy structures (Chen et al. 2006; Ranson et al. 2006). Co-chaperonin structures alone have been reported for GroES (Boudker et al. 1997; Hunt et al. 1996; Seale et al. 1996).

The ability of GroEL and GroES to enhance protein folding is embedded in the unique quaternary structures of these proteins. The arrangement of the GroEL subunits results in an oligomeric structure consisting of 14 subunits arranged in two

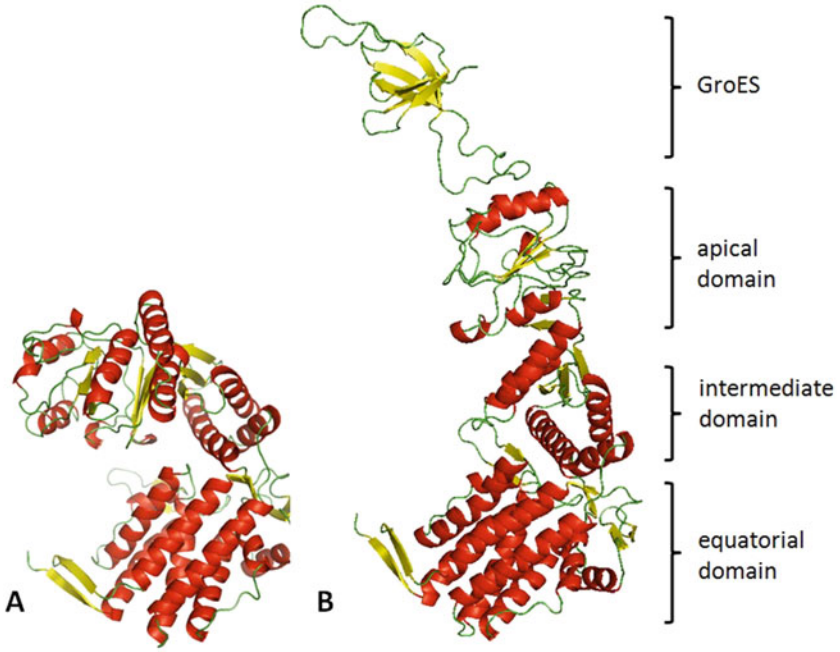

Fig. 8.3 Binding of GroES induces a large conformational change in GroEL. Each subunit of GroEL is divided into three distinct domains: an apical domain, an equatorial domain, and an intermediate domain that connects both domains. Unbound GroEL (**a**) undergoes large rigid body movements of the apical domain upon binding of GroES (**b**). Apical domain is twisted 90° relative to the open ring not bound to GroES. Alpha helices are shown in red and β-sheets in yellow. The images were generated using PyMol (DeLano Scientific) from coordinates in PDB: 1AON

inverted rings, whilst the GroES subunits are arranged into a single ring of 7 subunits, and both structures display sevenfold rotationally symmetric ring-shaped oligomers (Fig. 8.1). The GroEL subunits are composed mainly of α-helices, and the arrangement of the subunits into two stacked GroEL rings creates a central channel that is split into two functionally separate cavities at the ring interface (Braig et al. 1993, 1994). Each subunit is divided into three distinct domains: an ATP-binding equatorial domain that mediates interactions between subunits of each ring, a substrate-binding apical domain including co-chaperone binding sites, and an intermediate domain that connects both domains and transmits conformational changes generated by nucleotide binding between the equatorial and apical domains (Fig. 8.3) (Braig et al. 1994; Fenton et al. 1994). The apical domains are positioned on the outside of each ring, the intermediate domains are in the middle, and the equatorial domains are positioned at the interface of both rings. Coalescence of the disordered and flexible C-terminal segments of the subunits in each ring was determined to block the central channel at the equatorial domain causing discontinuity between the cavities turning them into two separate chambers for folding (Chen et al. 1994). An alternative model, based on molecular simulations, suggests that the non-native protein is

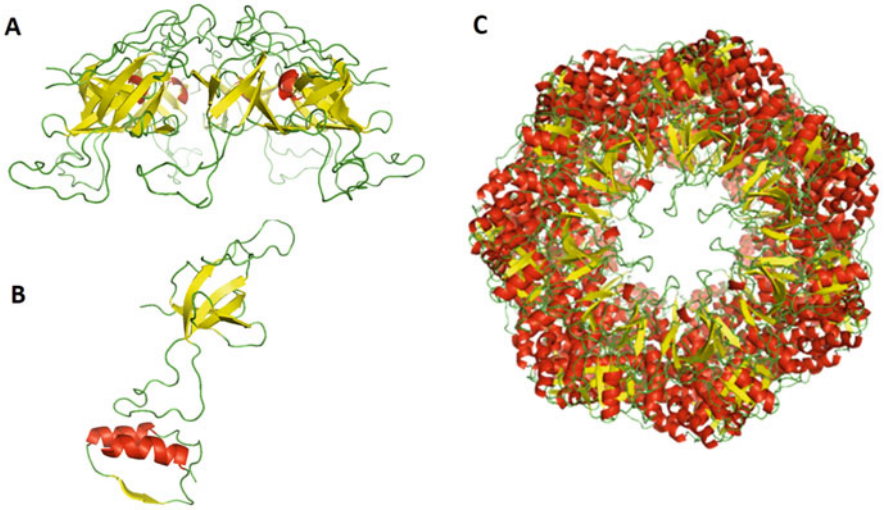

Fig. 8.4 Structure of GroES. Side view of GroES heptameric structure, as it occurs bound to GroEL and ATP, showing the flexible loops that interact with GroEL pointing downwards (**a**). The backbone structure of the GroES monomer interacting with the top of the apical domain of GroEL (**b**). A top view of the GroEL/GroES complex (**c**). Alpha helices are shown in red and β-sheets in yellow. The images were generated using PyMol (DeLano Scientific) from coordinates in PDB: 1AON

translocated from one barrel of GroEL to the next until it is fully folded, and this may account for the double-ring structure (Coluzza et al. 2006).

GroES is composed of a seven identical 10 kDa subunits that form a lid-like structure (Hunt et al. 1996; Mande et al. 1996). These subunits form an irregular β-barrel structure formed by five β-strands with anti-parallel pairing of the last β-strand of one subunit with the first β-strand of the following subunit (Landry et al. 1996). Each subunit includes two loop regions, one facing upwards that forms the roof of the lid and one extending downwards from the bottom of the lid that constitutes a highly flexible mobile loop 16 amino acids in length (Fig. 8.4) (Landry et al. 1993). Binding of GroES to GroEL is mediated by the seven flexible loops which are induced to form a β-hairpin structure upon formation of the GroEL/GroES/ATP complex (Fig. 8.4) (Richardson et al. 2001). Mutations in the mobile loop disrupted GroES binding to GroEL (Zeilstra-Ryalls et al. 1994). The contribution of the mobile loop was studied using a synthetic peptide resembling the loop, which lacked structure until induced to form the β-hairpin structure when bound to GroEL (Landry et al. 1996). The functional contribution of the flexibility of the mobile loop to chaperonin function was investigated by restricting the flexibility by the formation of disulphide bonds within the loop, and the results revealed that they play an important role in inducing substrate release into the cavity (Nojima et al. 2012).

The GroEL rings are subject to intra- and inter-ring allostery (Yifrach and Horovitz 1995). The two GroEL rings are staggered such that each subunit contacts two subunits on the other ring that facilitates negative cooperativity between rings (Braig et al. 1994; Roseman et al. 2001). A review of the unfolding and refolding of GroEL in the presence of ligands and different solvents has highlighted differences in behaviour between these two proteins (Ryabova et al. 2013). The crystal structure of the bullet-shaped GroEL-GroES-ADP complex revealed that the apical domains are twisted 90° relative to the open ring not bound to GroES (Fig. 8.3) (Roseman et al. 1996; Xu et al. 1997). The transmission of conformational changes between the apical and equatorial domains of GroEL via the intermediate domain is essentia, l as mutations in this domain compromised the folding capacities of GroEL/GroES (Kawata et al. 1999). Movement of the apical domains upon ATP binding shifts the hydrophobic GroES and substrate binding site from a position facing the cavity to an elevated and rotated position to facilitate the binding of the mobile loop of GroES to cap the folding chamber (Fig. 8.3). Mutational mapping revealed that there is an overlap between substrate and GroES binding to the hydrophobic binding site (Fenton et al. 1994). Another study suggested that rotation of the hydrophobic binding site weakens substrate binding (Ranson et al. 2001). However, mapping the trajectories of domain movements of the GroEL-ATP complex showed that the apical domains are linked by salt bridges that allow the binding sites to separate from each other in an extended conformation, at the same time maintaining the binding surface facing the cavity, providing a potential binding site for GroES which triggers a final rotation that provides the 'power stroke' to eject substrate in the chamber (Clare et al. 2012). The effects of interactions between the cavity wall of GroEL and rhodanese were investigated with the result that these interactions slowed down the folding rate of rhodanese (Sirur and Best 2013).

The Role of GroES in the Reaction Cycle

GroES functions as a co-chaperonin of GroEL to mediate the folding of unfolded or partially unfolded proteins. GroEL captures substrates at a site in the apical domain that exposes hydrophobic amino acid residues to facilitate substrate binding towards the ring centre (Fenton et al. 1994). GroES binds at the apical domain of ATP-bound GroEL, at a site that overlaps largely with the substrate binding site, and in doing so displaces the substrate into the binding cavity (Fenton et al. 1994). The distortion of the GroEL ring caused by the binding of GroES causes the hydrophobic residues that bind non-native protein to become inaccessible creating a hydrophilic lined cavity (Xu et al. 1997). The result is the eviction of the protein into the cage for folding, also referred to as the Anfinsen cage (Ellis and Hartl 1996). GroES then forms a lid over the central cavity entrapping the protein. GroES binding is faster than ATP-induced release of the substrate, and this provides a mechanism for the entrapment of proteins in the *cis* cavity (Burston et al. 1995).

Once the substrate is encapsulated in the chamber, the slow rate of ATP hydrolysis dictates the length of time for folding to take place (Fenton and Horwich 2003; Frydman 2001; Hartl and Hayer-Hartl 2002, 2009). GroEL exhibits weak ATPase activity that is lowered in the presence of GroES (Chandrasekhar et al. 1986; Goloubinoff et al. 1989). GroEL assists in the folding of non-native proteins with the consumption of ATP (Xu et al. 1997). Transformational changes in the *trans* ring caused by binding of substrate, ATP, and GroES to the *cis* ring result in the *trans* ring not being able to bind substrate (Tyagi et al. 2009). This phenomenon may be substrate specific, since in some cases GroEL was shown to bind two substrates simultaneously, one in each folding chamber (Llorca et al. 1997; van Duijn et al. 2007). ATP hydrolysis of the GroES-bound ring is required for the binding of ATP to the *trans* ring; negative cooperativity is displayed between the two GroEL rings which favours dissociation of GroES, ADP, and substrate from the *cis* ring (Rye et al. 1997). If the substrate is not folded correctly, it can rebind to another or the same GroEL for successive cycles of folding (Rye et al. 1997). GroES can now bind to the *trans* ring, and this ring then becomes the new *cis* ring in the subsequent round of substrate folding events. Thus both rings alternate to become the *cis* ring during folding cycles, and this has led to the term 'two-stroke engine' for the GroEL/GroES folding machine (Lorimer 1996; Xu and Sigler 1998).

The transition between the open conformation, that is receptive to protein binding, and the closed state, in which the protein is isolated, is induced by ATP binding and hydrolysis (Horovitz and Willison 2005). ATP binds with positive cooperativity within rings but with negative cooperativity between rings (Yifrach and Horovitz 1995). Allosteric transitions support the ATP-dependent control of the affinity of GroEL for its substrate and the subsequent folding (Roseman et al. 1996; Saibil et al. 1993; Yifrach and Horovitz 1995). ATP binding initiates bending and twisting of subunit domains that distort the ring structure and exposes the GroES binding sites. ATP binds to a ring with positive cooperativity, and movements of the interlinked subunit domains are concerted. In contrast, there is negative cooperativity between the rings, so that they act in alternation (Horovitz et al. 2001; Rye et al. 1997). GroEL/GroES-assisted protein folding is further complicated by the existence of both bullet-shaped (asymmetric) and football-shaped (symmetric) complexes. Computational analysis of GroEL substrates revealed that both complexes functionally co-exist with a shorter folding rate observed for certain substrates in the football complex, whilst low ATP concentrations favoured the bullet-shaped species (Fig. 8.2) (Bigman and Horovitz 2019). Asymmetric GroEL-GroES complexes appear to persist under physiological conditions as a result of negative cooperativity between GroEL rings, as nucleotide binding in one ring causes suppression of binding in the other ring (Gruber and Horovitz 2016; Inobe et al. 2008). However, the presence of a high concentration of substrate allows the symmetric cycle to dominate, suggesting that GroEL can switch between two types of reaction cycle (asymmetric or symmetric), possibly depending upon the substrate proteins [reviewed by Iizuka and Funatsu (2016)]. Using FRET assays, symmetric GroEL-$GroES_2$ were determined to be the folding functional form, and these reverted to asymmetric forms as protein substrate levels decreased (Yang et al. 2013). The

advantage of the symmetric complex is that protein folding can occur in both cavities of the football-shaped complex; in fact, the folding of GFP was determined to occur independently in both rings of the symmetric complex (Takei et al. 2012). In an effort to reconcile the two different types of complexes, transient ring separation caused by ATP binding to the *trans* ring allows the GroEL rings to function sequentially (Yan et al. 2018). Understanding the pathways of allosteric communication in GroEL has been the subject of intense research, and this was reviewed by Saibil et al. (2013) and more recently by Lorimer et al. (2018). A comprehensive review of the modes of action of the GroEL/ES folding machine revealed that the environment of the cage can accelerate the folding of some proteins (Hayer-Hartl et al. 2016). The results of numerous studies on the folding kinetics of the encapsulated protein substrate are available, and these vary due to differences in the properties of the protein substrate (Korobko et al. 2020). GroEL encapsulation repaired a folding defect of a maltose-binding protein with a destabilising mutation by restoring or re-establishing fast folding, suggesting an active role of GroEL in a compression effect in the cavity (Ye et al. 2018). Recently, it was determined that the protein stability of thermally unstable dihydrofolate reductase from *Moritella profunda* was greatly reduced after stable encapsulation by GroEL, resulting in unfolded protein that then had the option to fold again (Korobko et al. 2020).

In addition to its role as a lid for the folding chamber in the chaperonin complex, GroES controls the cooperativity by directing conformational changes in GroEL that are orchestrated by the seven mobile loops binding to each of the seven GroEL subunits, followed by release of substrate into the cage (Gray and Fersht 1991; Todd et al. 1994; Yifrach and Horovitz 1995). Interestingly, an alpha haemolysin nanopore was fused with the seven flexible loops of GroES allowing it to function as efficiently as the native GroES (Ho et al. 2015). GroES also plays a key role in controlling the competence and specificity of protein folding by GroEL (Richardson et al. 2001). Based on the GroEL-GroES-ADP complex, the binding of GroES causes large rigid body movements of the apical domains of GroES that result in doubling of the volume of the *cis* ring cavity compared to the *trans* ring (Fig. 8.3) (Xu et al. 1997). This increased volume is capable of binding a native protein of 70 kDa (Houry et al. 1999). Most of the *E. coli* proteins that require GroEL-GroES for folding are ~60 kDa, and larger proteins that cannot be accommodated within the folding cavity can be folded by binding to the uncapped *trans* ring of GroEL (Sigler et al. 1998). Binding of GroES causes a dramatic change in the walls of the cavity as the hydrophobic binding sites are rotated towards the interfaces of adjacent subunits and GroES resulting in a hydrophilic wall, and the intermediate domain twists downwards capping the nucleotide binding site (Xu et al. 1997).

Roles of Bacterial Chaperonins

Due to their importance in protein homeostasis, chaperonins are essential and universally distributed in all bacteria. Bacterial chaperonins are required for the correct assembly of the cell division apparatus (Ogino et al. 2004). In contrast to *E. coli* which possesses a single operon-encoded *groEL* gene with a *groES* gene, nearly 30% of all bacterial genomes contain multiple chaperonin genes (Lund 2009). The mycobacteria were the first bacteria revealed to have multiple chaperonins (Kong et al. 1993; Lund 2001). *M. tuberculosis* encodes two chaperonin genes, *cpn60.1* in an operon with the co-chaperonin gene *cpn10* and *cpn60.2* in a different position on the chromosome (Kong et al. 1993), whilst *M. smegmatis* has three copies of *cpn60* (Fan et al. 2012). In bacteria with multiple *groEL* genes, such as mycobacteria, the essential copy is unexpectedly often not the operon-encoded gene, and this has resulted in much interest and speculation about the functions of these additional chaperonins (Hu et al. 2008; Ojha et al. 2005). It is possible that one copy preserves the essential chaperone function, whilst the others diverge to take on altered roles (Lund 2001). Biophysical studies of the chaperonins from *M. tuberculosis* and *M. smegmatis* provide support of novel functions for Cpn60.1 as Cpn60.2 proteins assemble into oligomers and are able to replace GroEL in *E. coli* when co-expressed with GroES or the cognate Cpn10, whilst neither Cpn60.1 nor Cpn60.3 found in *M. smegmatis* could functionally replace GroEL (Fan et al. 2012). Based on the fact that Cpn60.1 appears to chaperone a discrete set of key enzymes involved in the synthesis of the complex cell wall and differences in protein sequence, this novel mycobacterial chaperonin may provide a unique target for drug development reviewed by (Colaco and MacDougall 2014). As part of the development of GroEL/ES inhibitors as potential antibiotics, clinically significant ESKAPE pathogen (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) GroEL/ES formed mixed complexes in the presence of *E. coli* GroEL/ES leading to a loss of viability in some cases, using chaperonin-deficient *E. coli* (Sivinski et al. 2021).

One of the five GroEL paralogs in *Sinorhizobium meliloti* is required for NodD protein folding (Ogawa and Long 1995), whilst *Bradyrhizobium japonicum* possess at least five *groESL* operons that can partially compensate for the lack of one or other genes (Fischer et al. 1993). These duplicated proteins have evolved specific roles in different bacteria, but the mechanism involved in functional divergence has not been elucidated (Wang et al. 2013). *Myxococcus xanthus* DK1622 displayed functional divergence with respect to substrate specificity, and this was as a result of differences in the apical and C-terminal regions of the two GroEL proteins (Wang et al. 2013). Interestingly, monomeric Cpn60 from *Thermus thermophilus* was able to support protein folding independently of both ATP and a co-chaperonin (Taguchi et al. 1994). The crystal structures of the *T. thermophilus* Cpn60/Cpn10 complex alone (Shimamura et al. 2003) and with bound proteins have been reported (Shimamura et al. 2004). Despite a destabilised structure, Cpn60 proteins from *M. tuberculosis*

also displayed activity in the absence of ATP or co-chaperonin (Qamra and Mande 2004). *Chlamydia* harbours three putative chaperonins, and ChGroEL has been associated with increased pathology and is the primary chaperonin, whereas the other two paralogues perform novel *Chlamydia*-specific functions during infection (Illingworth et al. 2017). Further functions of the multiple chaperonins in bacteria were reviewed by (Kumar et al. 2015).

Cpn60s are dominant immunogens present during bacterial infections. Moreover, Cpn60s of *M. tuberculosis* are potent inducers of host inflammatory responses and behave as antigens and cytokines (Qamra et al. 2005). The host immune response to exogenous chaperonins may be both protective and damaging (Ranford and Henderson 2002). It has been hypothesised that due to sequence conservation, the host immune response mounted against bacterial co-chaperonins may result in cross-reactivity to human Cpn60 causing an autoimmune reaction (van Eden et al. 1998). There is convincing evidence for the case in the development of atherosclerosis (Wick 2006). The roles of chaperonins in disease, including models and potential treatments, are addressed in a review by (Ranford and Henderson 2002).

Immunisation of mice with GroEL conferred full protection against *Bacillus anthracis* infection, whilst DnaK was ineffective (Sinha and Bhatnagar 2010). More recently GroEL was evaluated as an ideal vaccine candidate against *Streptococcus agalactiae*, responsible for significant economic losses in the fishing industry (Li et al. 2019). Extracellular leptospiral GroEL may play a role in the adhesion of leptospires to host tissues and induce cytokine secretion during infection (Ho et al. 2021).

Specific Functions of Bacterial Co-chaperonins

In addition to co-chaperonin activity, a number of diverse roles played by bacterial co-chaperonins are emerging, in particular during host-pathogen interactions. The possible reasons for numerous chaperonins in bacteria were reviewed by Lund in 2009, and the evolution of so many different functions is highlighted by Henderson and Martin (2011) (Henderson and Martin 2011; Lund 2009). Despite the conservation of the GroEL-ES system in prokaryotes, it is absent in several members of the class of mollicutes, which are bacteria lacking a cell wall (Schwarz et al. 2018). Most bacterial Cpn10 proteins are stimulators of the immune system, and the response varies between different species, with human and *E. coli* Cpn10 proteins being poor immunogens and *M. tuberculosis* and *M. leprae* Cpn10 proteins being strong immunogens (Cavanagh and Morton 1994). These proteins also play a role in apoptosis, cytokine secretion, and cellular growth and development (Cavanagh 1996). Cpn10 of *M. tuberculosis*, a secreted protein with cell signalling functions, is an important virulence factor during infection, and it plays a key role in the pathology of spinal tuberculosis by inhibiting the growth of osteoblasts (Meghji et al. 1997; Roberts et al. 2003). Structures have been reported for *M. leprae*, and *M. tuberculosis* Cpn10 proteins and immunodominant epitopes have been mapped

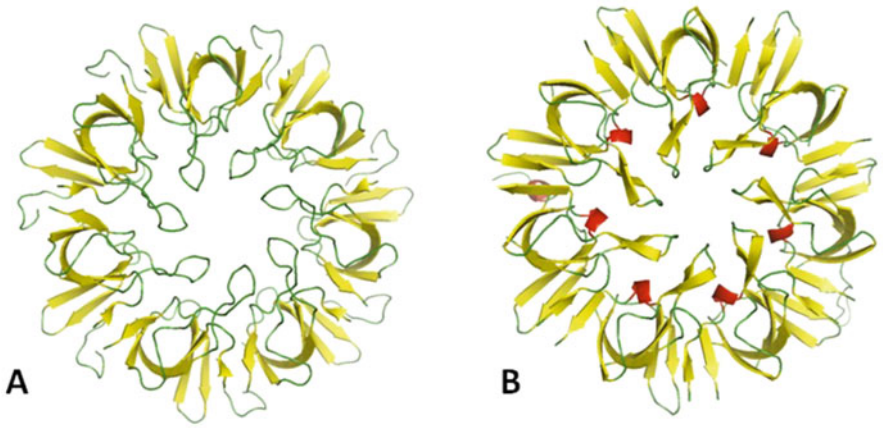

Fig. 8.5 The overall structures of *M. tuberculosis* Cpn10 (**a**) and *T. thermophilus* Cpn10 (**b**) conform to the GroES-fold. Differences are evident in the mobile loops, and a partially helical structure is present in the *T. thermophilus* Cpn10 monomer. Breaks are evident in the structures due to a lack of electron density in the highly flexible mobile loops. Alpha helices are shown in red and β-sheets in yellow. The images were generated using PyMol (DeLano Scientific) from coordinates in PDB: 1HX5 and WNR

to the mobile loop (Mande et al. 1996; Roberts et al. 2003). Further structural analysis of *M. tuberculosis* Cpn10, in the presence of divalent cations, showed the existence of a heptamer (Taneja and Mande 2001, 2002). The crystal structure of *T. thermophilus* HB8 Cpn10 showed disordered loops in five subunits (Numoto et al. 2005). Comparison of *M. tuberculosis* Cpn10 to that of *T. thermophilus* HB8 Cpn10 revealed a similar overall structure; however, the dome loops and mobile loops are different (Fig. 8.5). The Cpn10 from *Aquifex aeolicus* has a 25-residue C-terminal extension present in each monomer, that is absent from any other known Cpn10 protein, that is not essential for function but plays a role in preventing aggregation at high temperatures (Chen et al. 2008; Luke et al. 2005).

Roles of Eukaryotic Group I Chaperonins

In eukaryotes, group I Hsp60 is found in the mitochondria and also in chloroplasts of plants. It interacts with its co-chaperonin Hsp10 or Cpn10 to promote protein folding in the cell. Most mitochondria and chloroplasts in higher plants appear to possess multiple chaperonin subunit types (Hill and Hemmingsen 2001). A number of novel functions and interacting molecules have been assigned to Hsp60 (Czarnecka et al. 2006). Some of these are associated with carcinogenesis as its role in the survival and proliferation of tumour cells has increased (Cappello et al. 2008; Czarnecka et al. 2006). Human HSPD has received considerable interest as an anticancer drug target. It is highly expressed in ovarian tumours and knockdown of HSPD disrupted

mitochondrial functioning resulting in impeded cell proliferation (Guo et al. 2019). A review of small molecule modulators of Hsp60 function was conducted to identify potential anticancer drugs (Meng et al. 2018). The mitochondrial Hsp60 protein is essential for the folding of proteins imported into the mitochondria and prevention of denaturation during stress (Cheng et al. 1989; Levy-Rimler et al. 2001). They are also characterised by a host of additional functions, including extracellular functions. Hsp60 found in the cytosol and the extracellular space possesses various moonlighting functions (Henderson et al. 2013). Mutations of HSPD are linked to severe genetic diseases (Bross et al. 2007; Hansen et al. 2007; Magen et al. 2008). It also plays a role in the production of pro-inflammatory cytokines (Chun et al. 2010). In addition, it plays both pro-apoptotic and anti-apoptotic roles, depending on localisation (Knowlton and Gupta 2003; Xanthoudakis et al. 1999). The first assessment of proteins that interact with the human HSPD/E complex was conducted using HEK293 cells and revealed that half of the mitochondrial proteins associate with this complex (Bie et al. 2020). Not surprisingly, a number of these proteins are associated with human disease, whilst 19 highly abundant proteins occupied approximately 60% of the chaperonin capacity (Bie et al. 2020). A number of reviews have been written on Hsp60 chaperonopathies, diseases that arise from abnormal chaperonins (Cappello et al. 2008, 2011, 2013, 2014; Macario and Conway de Macario 2005, 2007). A review of Hsp60 in the pathogenesis of diabetes mellitus suggests that the chaperonin may provide the connection between mitochondrial stress and inflammation (Juwono and Martinus 2016). Future research is needed to understand the role of post-translational modifications of Hsp60 in chaperonopathies, and this is highlighted in a recent review by Caruso Bavisotto et al. (2020).

A structural study of HSPD/E revealed that both full football-shaped (double ring) and half-football-shaped complexes (single ring) are functional and co-exist, potentially forming two separate folding cycles that may be optimised for different substrate sets (Gomez-Llorente et al. 2020). The recent cryo-EM structure of apo HSPD1 reveals predominantly single ring assemblies and increased flexibility of the apical domain in comparison to GroEL (Klebl et al. 2021). The mitochondrial Hsp60 in mammalian cells is transformed to a double-ring structure in the presence of ATP and/or HSP10 (Levy-Rimler et al. 2001). X-ray crystallography confirmed that it is capable of forming a football-shaped complex in the presence of both HSP10 and ATP (Nisemblat et al. 2015). The mitochondrial chaperonin complex that is composed of a single ring of seven subunits and a ring of Hsp10 subunits cannot exploit binding of ATP to the *trans* ring as a mechanism for releasing *cis* GroES (Nielsen and Cowan 1998). This complex may have evolved an intrinsically lower affinity for the co-chaperonin, but the presence of a higher affinity mobile loop on Hsp10 may offset the low affinity (Nielsen and Cowan 1998). Despite the fact that mitochondrial Hsp60 can functionally replace GroEL, it is incapable of interacting with GroES (Nielsen et al. 1999). The elements that dictate the specificity of mitochondrial Hsp60 for Hsp10 appear to lie in the apical domain (Parnas et al. 2012). Analysis of in vivo substrates of yeast mitochondrial chaperonins revealed divergent chaperonin requirements, indicating that Hsp60 and Hsp10 do not always operate as a functional unit (Dubaquie et al. 1998). Yeast mitochondrial Hsp60 can bind to

single-stranded DNA in vitro and play a role in the structure and transmission of nucleoids (Kaufman et al. 2003). A number of parasites affecting human health have demonstrated an upregulation of Hsp60, which is possibly linked to diverse environmental conditions encountered during its transition from a mammalian to an insect vector (Maresca and Carratu 1992). Induction of Hsp60 was found to occur during the entire course of infection of *Trypanosoma brucei*, a protozoan parasite responsible for causing sleeping sickness in humans (Radwanska et al. 2000). The crystal structure of *P. falciparum* mitochondrial Cpn60 bound to ATP revealed that large conformational changes can occur in the apical domain regulating substrate binding, whilst a unique insertion in the equatorial domain increased interactions between the rings (Nguyen et al. 2021).

The chloroplast type I chaperonin complex (Cpn60) is structurally similar to GroEL and also forms two stacked heptameric rings (Tsuprun et al. 1991); however, these are composed of two different subunit types, Cpn60α and Cpn60β (Martel et al. 1990) which are ~50% identical to each other (Hill and Hemmingsen 2001). *Arabidopsis thaliana* encodes several Cpn60α and Cpn60β families and both are required for plastid division (Suzuki et al. 2009). A unique chaperonin subunit in *A. thaliana* confers substrate specificity, whilst the dominant subunits retain housekeeping functions (Peng et al. 2011). The unicellular green algae *Chlamydomonas reinhardtii* encodes three CPN60 subunits, CPN60α1, CPN60ß2, and CPN60ß2 (Schroda 2004). Hetero-oligomeric chloroplast chaperonins are unstable in the presence of ATP, and *C. reinhardtii* CPN60 subunits revealed ATP-induced disassembly (Bai et al. 2015). The crystal structure of apo *C. reinhardtii* CPN60ß1 appears similar to GroEL but with a larger binding cavity and a wider ATP binding pocket, which may justify the structural instability upon ATP hydrolysis (Zhang et al. 2016; Zhao and Liu 2018).

Specific Functions of Eukaryotic Group I Co-chaperonins

A single copy of the Cpn10 co-chaperonin is present in the mitochondria of yeast and mammals (Hansen et al. 2003; Rospert et al. 1993a). The chloroplast co-chaperonins are varied with *cpn10* encoding the conventional 10 kDa protein that is similar in structure and function to GroES, as well as *cpn20* encoding tandem fusions of Cpn10 domains that form tetrameric ring structures that function with GroEL and Cpn60 (Bertsch et al. 1992; Koumoto et al. 2001; Sharkia et al. 2003). Of the five co-chaperonin homologues present in *A. thaliana*, three reside in the chloroplast; Cpn10-2 and Cpn20 form functional homo-oligomers, whilst Cpn10-1 requires the integration of Cpn20 to form a functional hetero-oligomeric complex (Vitlin Gruber et al. 2014). Interestingly, *C. reinhardtii* has three co-chaperonins, Cpn10, Cpn20, and Cpn23, that are individually non-functional (Tsai et al. 2012). They are also structurally different, and the sequence encoding the roof-like ß-hairpin in the co-chaperonin complex is absent, though Cpn10 and Cpn23 maintain this sequence (Zhao et al. 2019). In studies using recombinant co-chaperonins of

A. thaliana and C. reinhardtii, hetero-oligomeric ring complexes formed by combinations of Cpn10, Cpn20, and Cpn23 were able to serve as co-chaperonins, in order to perhaps modify the chaperonin folding cage for specific client proteins (Tsai et al. 2012). The symmetrical match of Cpn60, with sevenfold symmetry, to the chloroplast co-chaperonin, may be solved by forming hetero-oligomers of Cpn20 and Cpn10 or by splitting the Cpn20 (Tsai et al. 2012). In fact, a symmetrical match is not an absolute requirement for chaperonin function, and the flexibility and plasticity of this interaction were demonstrated by forming concatamers of six to eight covalently linked 10 kDa domains and three to four covalently linked Cpn20 subunits, which could help the chaperonin to refold a denatured protein in vitro (Guo et al. 2015). A previous study determined that a minimum of four active GroES subunits are necessary to contact GroEL for the formation of a stable GroEL/ES complex, whilst five subunits allow for an active complex that can fold proteins (Nojima et al. 2008). Despite the fact that human mitochondrial Cpn60 can bind *A. thaliana* Cpn20, it does not lead to productive protein folding, demonstrating different modes of binding of co-chaperonins to chaperonins, some of which are not functional (Bonshtien et al. 2009). The high-resolution structure of *C. reinhardtii* hetero-oligomeric Cpn60, in complex with hetero-oligomeric chloroplast Cpn10, revealed that the overall structure was similar to that of GroEL/ES but with an uneven spread of roof-forming domains in the co-chaperonin and possible varied surface properties of the chaperonin that may enable the system to fold specific substrates (Zhao et al. 2019).

Just as Hsp10 may have many other roles in mammalian cells, it seems that Cpn20 may have many additional roles in plants. Analysis of the stromal proteome of *A. thaliana* chloroplasts indicates that the steady-state levels of Cpn20 exceed those required to function with Cpn60, implying that there may be further roles for Cpn20 (Peltier et al. 2006). Additional roles have been revealed in *A. thaliana*, including the identity of Cpn20 as a negative regulator of abscisic signalling (Zhang et al. 2013) and as a mediator of iron superoxide dismutase activity in chloroplasts (Kuo et al. 2013).

Whilst our understanding of the roles of HSPE in disease continues to receive research attention, little is known about the roles of its homologues in virulence and pathogenicity of protozoan parasites affecting human health, and they may interact with the human chaperone system. The first protozoan CPN10 protein characterised was from *Leishmania donovani* and was shown to interact with CPN60.2 with increased concentrations detected during the amastigote stage of the life cycle (Zamora-Veyl et al. 2005). Cpn20 proteins were known to exist only in chloroplasts; however, sequencing of the malarial genome revealed a single Cpn20 protein which correlates with the algal origin of the apicoplast (Janouskovec et al. 2010; Sato and Wilson 2005). Since the *P. falciparum* genome encodes only one *cpn20* gene, it functions as a homo-oligomeric co-chaperonin that can functionally replace GroES (Vitlin Gruber et al. 2013b). Characterisation of HSP10 from *Strongyloides ratti*, an intestinal nematode infecting humans, revealed a strong immunogenic response, and the inability to bind to *S. ratti* HSP60 provided evidence of a role in host-parasite interactions (Tazir et al. 2009).

The structure of HSPE has been solved, and mutations in the first and last β-strands altered both the oligomeric and folded states (Guidry et al. 2003). In contrast to HSPD, HSPE stimulates the production of anti-inflammatory cytokines and exerts immunosuppressive activity (Johnson et al. 2005). One of the first extracellular heat shock proteins to be isolated was a circulating immunosuppressive protein, termed early pregnancy factor (EPF), which was later identified as HSPE after the isolation and demonstration of its role as a co-chaperonin for Hsp60 (Cavanagh and Morton 1994; Morton et al. 1977). The isolation of EPF was also the first evidence that heat shock proteins could function as cell signalling agonists (Morton et al. 1977). EFP appeared in the maternal serum within 24 hours after fertilisation in some mammalians and has been found to exhibit growth factor qualities and anti-inflammatory properties essential for protecting the embryo from the mother's own immune system (Athanasas-Platsis et al. 2004; Morton et al. 1977; Quinn et al. 1990). The relationship between HSPE and EPF is discussed in a review by (Corrao et al. 2010). Recombinant HSPE has been used for the treatment of rheumatoid arthritis (Vanags et al. 2006) and multiple sclerosis (Broadley et al. 2009). HSP10 is essential for the regulation of histone transcription and cell proliferation (Ling Zheng et al. 2015). Selective overexpression of HSP10 in metastatic lymph modes suggest that it acts autonomously from HSP60 (Cappello et al. 2005). Elevated levels of Hsp10 correlate with poor prognosis in oral squamous cell carcinoma (Feng et al. 2017). Extracellular Hsp10 influences endothelial cell differentiation (Dobocan et al. 2009). There is growing evidence to suggest that extracellular Hsp10 plays an active role in cell signalling (David et al. 2013).

Conclusion

Despite the fact that HSPD/E can replace GroEL/ES, continued research has shown them to be mechanistically different. Divergence from the *E. coli* archetype is also apparent in chloroplasts and other bacteria. Defective chaperonins cause chaperonopathies. However, both wild-type and mutant Hsp60 are associated with a number of disease affecting human health, and the search will continue for small molecules that can modulate the activity of Hsp60 as therapeutic strategies. Research on the influence of the cellular environment on the GroEL/ES folding machine and factors affecting the rate of protein folding will continue to enhance our understanding of this system. The moonlighting functions of bacterial chaperonins and co-chaperonins will continue to evolve. The structural states of Hsp10, including mixed oligomeric or fragmented, appear to influence the function as well as location. Hsp10 often functions as an antagonist to Hsp60 and possibly other molecular chaperones. Further knowledge of the extracellular functions of Hsp10, including secretion pathways and cell signalling, will definitely be of benefit in the development of treatments for cancer and autoimmune diseases related to this protein.

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Chapter 9
Co-chaperones of the Human Endoplasmic Reticulum: An Update

Armin Melnyk, Sven Lang, Mark Sicking, Richard Zimmermann, and Martin Jung

Abstract In mammalian cells, the rough endoplasmic reticulum (ER) plays central roles in the biogenesis of extracellular plus organellar proteins and in various signal transduction pathways. For these reasons, the ER comprises molecular chaperones, which are involved in import, folding, assembly, export, plus degradation of polypeptides, and signal transduction components, such as calcium channels, calcium pumps, and UPR transducers plus adenine nucleotide carriers/exchangers in the ER membrane. The calcium- and ATP-dependent ER lumenal Hsp70, termed immunoglobulin heavy-chain-binding protein or BiP, is the central player in all these activities and involves up to nine different Hsp40-type co-chaperones, i.e., ER membrane integrated as well as ER lumenal J-domain proteins, termed ERj or ERdj proteins, two nucleotide exchange factors or NEFs (Grp170 and Sil1), and NEF-antagonists, such as MANF. Here we summarize the current knowledge on the ER-resident BiP/ERj chaperone network and focus on the interaction of BiP with the polypeptide-conducting and calcium-permeable Sec61 channel of the ER membrane as an example for BiP action and how its functional cycle is linked to ER protein import and various calcium-dependent signal transduction pathways.

Keywords Human endoplasmic reticulum · ATP/ADP exchange · Cellular calcium homeostasis · Calcium-dependent signal transduction · ER calcium leakage · ER energy homeostasis · ER protein import · J-domain proteins · Protein folding · ER-associated protein degradation

Introduction

In all nucleated human cells, the endoplasmic reticulum or ER forms a vast and dynamic membrane network (Palade 1975; English and Voeltz 2013; Nixon-Abell et al. 2016). The rough ER is studded with 80S ribosomes, which are engaged in the

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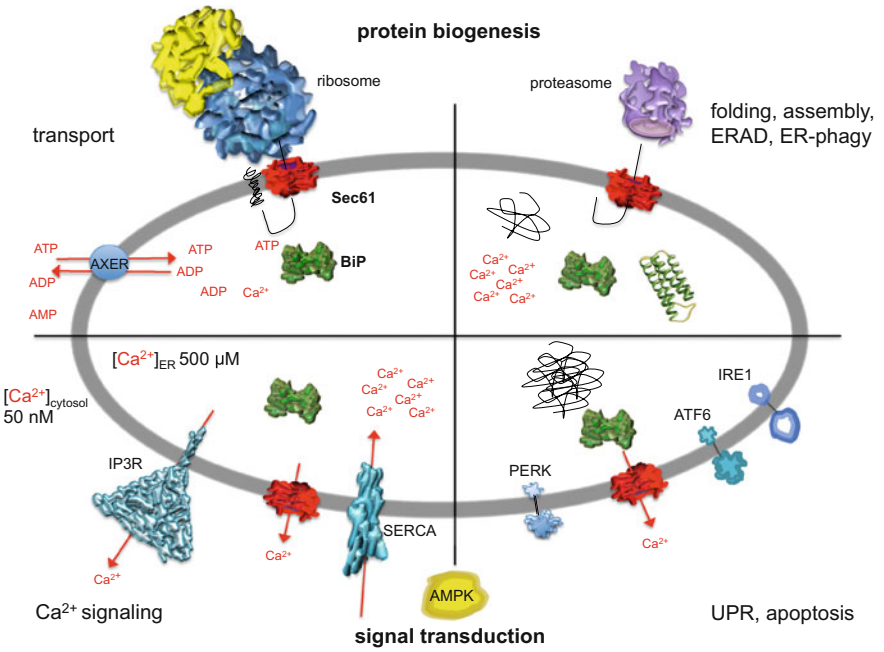

Fig. 9.1 Schematic and stylized cross section through the ER, highlighting the multiple roles of BiP and the Sec61 complex in protein biogenesis and signal transduction in human cells. We note that an artist's view is presented, rather than real structures. AMPK, AMP-dependent protein kinase; ATF6, activating transcription factor 6; AXER, ATP/ADP exchanger of the ER membrane; ERAD, ER-associated protein degradation; IP3R, inositol-1,4,5-trisphosphate receptor; IRE1; inositol-requiring enzyme 1; EIF2AK, eukaryotic translation initiation factor 2 alpha kinase; SERCA, sarcoendoplasmic reticulum calcium ATPase; UPR, unfolded protein response

biosynthesis of most secretory and many organellar proteins by cotranslationally inserting nascent polypeptides into the membrane or lumen of the ER, thus defining the one major function of the rough ER. The peripheral ER contacts the plasma membrane via the STIM and ORAI proteins (Feske 2019; Kappel et al. 2019), and the tubular ER contacts mitochondria via the ER-mitochondria encounter structures (ERMES complexes) of mitochondria-ER contact sites (MERCs) (Kornmann et al. 2009; Hayashi et al. 2009; Bakowski et al. 2012). These contacts play important roles in cellular calcium homeostasis and signal transduction, thus exemplifying the other major function of the mammalian ER.

As stated above, protein transport into the ER is the first step in the biogenesis of many proteins of eukaryotic cells (such as proteins of the ER, ERGIC, Golgi apparatus, endosome, lysosome, nucleus, lipid droplets, peroxisome, and plasma membrane) as well as of most extracellular proteins (Fig. 9.1, labeled "transport") (Blobel and Dobberstein 1975a, b). Typically, protein transport into the ER involves amino-terminal signal peptides in the precursor polypeptides and a complex machinery of transport components, most notably the heterotrimeric Sec61 complex in the

ER membrane and, at least in certain cases, the ER lumenal Hsp70-type molecular chaperone BiP and its Hsp40-type co-chaperone Sec63 plus the nucleotide exchange factor Grp170 (Görlich et al. 1992; Hartmann et al. 1994; Dierks et al. 1996 Lang et al. 2012; Haßdenteufel et al. 2018; Schorr et al. 2020). Protein transport into the ER was recently reviewed in more detail by Aviram and Schuldiner (2017), Gemmer and Förster (2020), and Lang et al. (2017, 2019) and will be discussed below, under the heading "The Dual Role of BiP and Its Co-chaperone Sec63 in Protein Import into the ER as an Example of Chaperone/Co-chaperone Action in the Mammalian ER."

Protein transport into the ER is followed by folding and assembly of the newly imported polypeptides (Fig. 9.1, labeled "folding, assembly") (Feige et al. 2010). Typically, folding and assembly of proteins involve some of the abovementioned components, i.e., the ATP- and calcium-dependent chaperone BiP and some of its co-chaperones, the ER-resident J-domain proteins (JDPs, most notably ERj3, ERj5, and ERj6), plus nucleotide exchange factors (NEFs) Grp170 and Sil1 (Haas and Wabl 1983; Bole et al. 1986; Weitzmann et al. 2007; Zahedi et al. 2009; Bulleid 2012; Behnke et al. 2016; Kampinga et al. 2018; Pobre et al. 2019). Up to a certain point, this machinery can also cope with misfolded and misassembled polypeptides within the ER by facilitating successive cycles of folding or assembly. Inspired by data on the cytosolic bacterial and human Hsp70/Hsp40 systems and taking into account the numerous observations of chaperone complexes in the ER lumen, it is tempting to speculate that, at least to a certain extent, the BiP/ERj system may even have the capacity to disaggregate aggregates of unfolded polypeptides (Tatu and Helenius 1997; Meunier et al. 2002; Nillegoda et al. 2015, 2017; Faust et al. 2020; Wentink et al. 2020). Excluding resident proteins of the ER, most of the correctly folded and assembled proteins are delivered to their functional location by vesicular transport (Schekman 2004, 2005; Sambrook 1990; Pelham 1990).

In the case of continuous misfolding or misassembly of polypeptides in the ER membrane or lumen, the respective polypeptides are exported to the cytosol and degraded by the proteasome (Fig. 9.1, labeled "ER-associated protein degradation or ERAD") (Smith et al. 2011; Bagola et al. 2011; Thibault and Ng 2012; Olzmann et al. 2012). Export of misfolded polypeptides from the ER lumen to the cytosol can involve some of the abovementioned components, such as the Sec61 complex and BiP and its co-chaperones (Pilon et al. 1997; Plemper et al. 1997; Schäfer and Wolf 2009). The general ERAD pathways, however, involve alternative export channels, such as Hrd1, and driving forces, as provided by the cytosolic ATPase p97 (Peterson et al. 2019; Schoebel et al. 2017; Vasic et al. 2020). This subject was expertly reviewed by Araki and Nagata (2011), Olzmann et al. (2012), and Ruggiano et al. (2014). Alternatively, whole sections of the ER can be subjected to autophagy of the ER, termed ER-phagy (Fig. 9.1, labeled "ER-phagy"), which typically involves the ER membrane protein FAM134B and phagophore-bound protein LC3 (Khaminets et al. 2015). However, this process may also involve the Sec63 interaction partner Sec62 or the ER-resident JDP ERj8 (Fumagalli et al. 2016; Yamamoto et al. 2020). Typically, however, Sec62 cooperates with LC3 in a more specialized kind of ER-phagy that was termed recovER-phagy, which was shown to act in ER turnover

during stress recovery, i.e., after the unfolded protein response or UPR solved the disturbance of ER proteostasis (see below). Briefly, the expanded ER as well as the high amount of ER lumenal chaperones has to be returned to a physiological level. Therefore, small vesicles derived from the ER membrane are engulfed by endolysosomes (Loi et al. 2019). For this purpose, Sec62 bears a LC3-interacting region (LIR motif) at its carboxy-terminus, which functions as a receptor for LC3. A similar mechanism may be involved in ER-phagy when misfolded polypeptides overwhelm the ERAD machinery and whole ER sections have to be sacrificed to protect the cell. We suggest that phosphorylation of the negative patch in the carboxy-terminus of Sec63 and/or Ca^{2+} binding to the EF hand in the carboxy-terminus of Sec62 may trigger dissociation of Sec62 from its interaction partners (Ampofo et al. 2013; Linxweiler et al. 2013) (Fig. 9.2). This subject was reviewed in more detail by Grumati et al. (2018) and Molinari (2020).

When protein misfolding or misassembly prevail, a complex signal transduction pathway is activated and, first, leads to an increase of the folding and degradation capacity of the ER and to a decrease of global protein synthesis (Fig. 9.1, labeled "unfolded protein response or UPR") (Gardner et al. 2013; Ron and Harding 2012; Ma and Hendershot 2001; Schroder and Kaufman 2005). In mammals, UPR involves the three ER membrane proteins PERK, ATF6, and IRE1, respectively, and their homologs (Fenech et al. 2020). These proteins comprise lumenal domains, which are distinct from J-domains but also interact with BiP, and cytosolic domains that attenuate global translation (PERK), induce selective transcription (ATF6, IRE1), or degrade ER localized mRNAs directly (IRE1) in the absence of available BiP. In addition, the interaction of BiP with the luminal domains of the UPR sensors IRE1 and PERK prevents the stimulation of BiP's ATPase activity, which typically occurs after interaction with J-domains. This novel cycle at the membrane located UPR sensors allows BiP to function as an ER stress sensor (Kopp et al. 2020; Amin-Wetzel et al. 2017). Interestingly, UPR is directly and functionally linked to ER protein import, i.e., one of the JDPs, namely, Sec63, as well as Sec61 complex itself interacts with IRE1 (Acosta-Alvear et al. 2018; Sundaram et al. 2017; Li et al. 2020), as does ERj4. The topic was scholarly reviewed by Walter and Ron (2011) plus Araki and Nagata (2011).

When protein misfolding and misassembly still persist, however, the programmed cell death pathway or apoptosis is activated in the respective cell to save the organism (Fig. 9.1, labeled "apoptosis) (Madeo and Kroemer 2009; Tabas and Ron 2011; Szegezdi et al. 2008). Among various other components (reviewed by Malhotra and Kaufman 2011), this switch involves efflux of calcium ions (Ca^{2+}) from the ER. Indirect evidence from various laboratories has first suggested that the Sec61 complex may contribute to the ER stress-induced Ca^{2+} leak (Lomax et al. 2002; van Coppenolle et al. 2004; Flourakis et al. 2006; Giunti et al. 2007; Ong et al. 2007; Lang et al. 2011). More recently, this concept was confirmed by the observations that the open Sec61 complex is Ca^{2+}-permeable and that silencing the *SEC61A1* gene in HeLa cells prevents a significant fraction of the Ca^{2+} leakage, in HeLa cells about 50% (Wirth et al. 2003; Lang et al. 2011; Erdmann et al. 2011; Schäuble et al. 2012; Gamayun et al. 2019). Under physiological conditions, BiP and

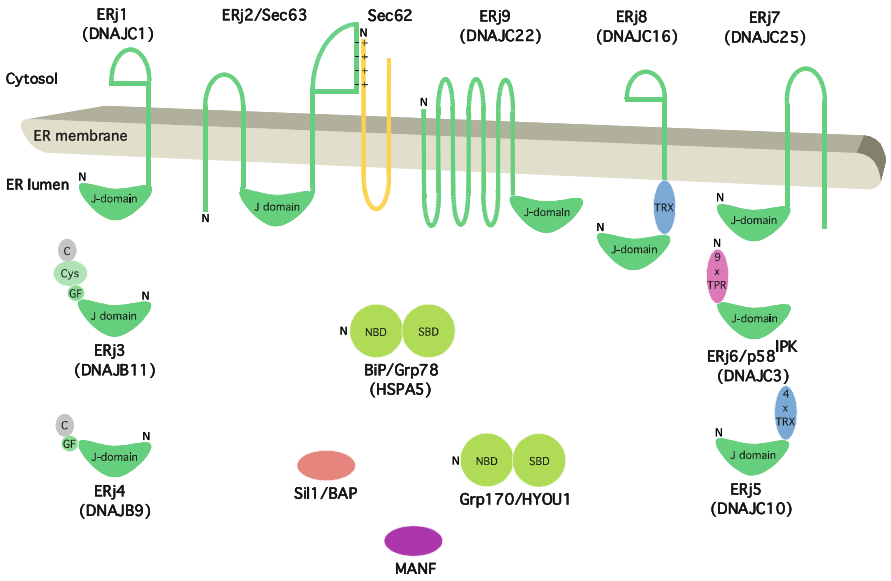

Fig. 9.2 Topology and domain organization of BiP and its co-chaperones and nucleotide exchange factors. We note that ERj1 and Sec63 both comprise large cytosolic domains that are structurally unrelated. In the case of ERj1, this domain is involved in ribosome binding (Blau et al. 2005; Dudek et al. 2005); the cytosolic domain of Sec63 is structurally related to certain helicases (Pena et al. 2009) and is involved in interaction with Sec62 via a cluster of positively charged amino acid residues in the amino terminal region of Sec62 and a patch of negatively charged amino acid residues in the most carboxy-terminal region of Sec63. The amino terminal region of Sec62 also comprises a ribosome binding site (Müller et al. 2010); the carboxy-terminal region of Sec62 harbors a LC3 binding site for its action in recovER-phagy (Fumagalli et al. 2016). The ER luminal domain of Sec63 can bind the Ca^{2+}-binding proteins calumenin and reticulocalbin (Tyedmers et al. 2005); the cytosolic domain of Sec63 contains a binding site for nucleoredoxin, which may link Sec63 to the Wnt signaling pathway (Müller et al. 2010). C, carboxy-terminal substrate-binding domain; Cys, cysteine repeat domain; GF, glycine-phenylalanine-rich domain; N, amino terminus; NBD, nucleotide-binding domain; SBD, substrate-binding domain; TPR, tetratricopeptide repeat; TRX, thioredoxin domain

two of its co-chaperones (ERj3 plus ERj6) are involved in limiting Sec61 complex-mediated Ca^{2+} leakage or passive Ca^{2+} efflux (Schäuble et al. 2012; Schorr et al. 2015). Therefore, it is tempting to speculate that the intrinsic Ca^{2+} permeability of the Sec61 complex and its regulation by BiP play an important role at the interface between protein biogenesis and Ca^{2+} homeostasis and, therefore, one aspect of ER stress-induced Ca^{2+}-dependent signaling in mammalian cells (summarized in Fig. 9.1). Since the more than thousand-fold Ca^{2+} gradient between ER lumen and cytosol allows Ca^{2+} to play its general role as a second messenger in cellular signaling (Berridge 2002; Rizzuto and Pozzan 2006), it is the function of the so-called sarcoendoplasmic reticulum calcium ATPase (SERCA) to counteract both the inositol-1,4,5-trisphosphate (IP3)- or ryanodine receptor-mediated Ca^{2+}

release and the passive Ca^{2+} efflux from the ER in order to maintain the Ca^{2+} gradient of the resting cell (Wuytack et al. 2002). Interestingly, one of the JDPs, namely, ERj5, "moonlights" as SERCA modulator, i.e., triggers the influx of Ca^{2+} via activation of the SERCA2 pump in a Ca^{2+}-dependent manner (Ushioda et al. 2016). Recent evidence suggests that the Sec61-associated Ca^{2+} leak is linked not only to the pathway of apoptosis activation but also to the cellular energy metabolism (Zimmermann and Lang 2020). As stated above, protein misfolding and misassembly present a burden to the BiP/JDP chaperone network, transiently causing a drop in the ER luminal ATP to ADP ratio plus sequestration of BiP and, therefore, increased Ca^{2+} leakage (Schäuble et al. 2012; Vishnu et al. 2014; Klein et al. 2018). As it turns out, Ca^{2+} leakage to the cytosol activates both increased ATP uptake and ADP release by the ATP/ADP exchanger in the ER membrane, termed AXER or SLC35B1, and activation of cytosolic AMP-activated protein kinase or AMPK, which among other things stimulates cytosolic glycolysis and reduces cap-dependent translation in the cytosol in order to save energy. Thus, the kinase AMPK provides yet another functional link between Sec61 complex and UPR and represents an additional Sec61-linked and Ca^{2+}-dependent signal transduction pathway (Klein et al. 2018). Furthermore, Ca^{2+} efflux via IP3-receptor, which is physiologically stabilized by BiP-free Sigma-1 receptor, into mitochondria via mitochondria-ER contact sites (MERCs), can activate the TCA cycle to promote mitochondrial respiration (Bravo et al. 2011; Hayashi and Su 2007). These two Ca^{2+}-dependent and energy metabolism-related signal transduction processes will be discussed in more detail below, under the heading "nucleotide transport into and out of the ER."

The Chaperone Network of the Mammalian ER

The mammalian ER contains molecular chaperones and folding catalysts in millimolar concentrations (Van et al. 1989; Bies et al. 1999, 2004; Weitzmann et al. 2007). Many of these molecular chaperones belong to the classical Hsp40, Hsp70, and Hsp90 protein families (Table 9.1 and Fig. 9.2). However, the ER also comprises a special class of molecular chaperones or lectins that are dedicated to the folding of glycoproteins. The mammalian ER contains a soluble lectin (calreticulin or CRT) as well as a membrane integrated lectin (calnexin or CNX), which upon palmitoylation associates with ribosomes (Degen and Williams 1991; Burns et al. 1992). The folding catalysts of the ER facilitate either the formation of disulfide bonds (protein disulfide isomerases or PDIs) or the isomerization of proline-containing peptide bonds (peptidyl-prolyl *cis/trans* isomerases or PPIases). The PPIases belong to either the cyclosporin A- or the FK506-sensitive protein family (cyclophilin or FK506-binding protein). All these chaperones and folding catalysts have been observed to be present in larger complexes in various combinations (Tatu and Helenius 1997; Meunier et al. 2002) and with other resident ER proteins that are involved in N- or O-glycosylation (UDP-glucose-glycoprotein-glycosyltransferase

Table 9.1 BiP and its interaction partners in the mammalian ER

Function	Protein (synonym)	Related human disease	OMIM	Mouse model	First reference
Hsp70-type Chaperone	BiP (Grp78, HspA5)	HUS	235400	Embryonic lethality or surfactant deficiency	Haas and Wabl (1983)
Hsp40-type co-chaperones (JDPs)	ERj1 (Htj1, DNAJC1, ERdj1)				Brightman et al. (1995)
	ERj2 (Sec63, ERdj2)	PLD, colorectal cancer	174050	Embryonic lethality	Skowronek et al. (1999)
	ERj3 (ERdj3, DnaJB11, HEDJ, Dj9)				Bies et al. (1999)
	ERj4 (ERdj4, DnaJB9, MDG1)			Postnatal lethality (surfactant deficiency)	Shen et al. (2002)
	ERj5 (ERdj5, DnaJC10, JPDI)			No phenotype	Hosoda et al. (2003) Cunnea et al. (2003)
	ERj6 ($p58^{IPK}$, DnaJC3, ERdj6)	Diabetes, neurodegeneration		Diabetic mouse	Rutkowski et al. (2007)
	ERj7 (Gng10, DnaJC25, ERdj7)				Zahedi et al. (2009)
	ERj8 (DNAJC16, ERdj8)				Yamamoto et al. (2020)
	ERj9 (DNAJC22, ERdj9)				Taipale, pers. commmun.
Nucleotide exchange factors (NEFs)	Grp170 (ORP150, HYOU1)			Embryonic lethality	Lin et al. (1993)
	Sil1 (BAP)	MSS	248800	Woozy mouse	Chung et al. (2002)
Additional BiP interactors	Sig-1R (Sigma-1 receptor)				Hayashi and Su (2007)
	MANF				Yan et al. (2019)
Additional Chaperones	Grp94 (CaBP4, ERp99, gp96, endoplasmin)			Embryonic lethality	Shiu et al. (1977)

(continued)

Table 9.1 (continued)

Function	Protein (synonym)	Related human disease	OMIM	Mouse model	First reference
	Calnexin (IP90, p88)			Postnatal lethality	Degen and Williams (1991)
	Calreticulin (CaBP3, ERp60)			Embryonic lethality	Burns et al. (1992)
UPR signal transducers	IRE1α/ß (ERN1/2)				Tirasophon et al. (1998)
	IRE2				Wang et al. (1998)
	ATF6α/ß				Yoshida et al. (1998)
	PERK (EIF2AK3, PEK)	WRS	226980	Diabetic mouse	Shi et al. (1998)
		Breast-cancer			Harding et al. (1999)
Sec proteins	Sec61α1	CVID, NP, TKD		Diabetic mouse	Görlich et al. (1992)
	Sec61ß	PLD			Hartmann et al. (1994)
	Sec61γ	Glioblastoma			
	Sec62 (TLOC1)	Prostate, lung, thyroid, breast, cervix, vulva, head and neck cancer			Mayer et al. (2000) Tyedmers et al. (2000)

or UGGT, SDF2L1) and calcium homeostasis (calumenin, reticulocalbin), respectively (Bies et al. 2004; Tyedmers et al. 2005).

The Hsp70/Hsp40 Chaperone Network of the Mammalian ER

Just like the bacterial cytosol or the mitochondrial matrix, the ER contains the typical Hsp70 triad, comprising the DnaK ortholog or Hsp70 (termed BiP in mammals) as well as the DnaJ orthologs or Hsp40-type co-chaperones, which stimulate the ATPase activity of BiP, and nucleotide exchange factors or NEFs, which catalyze the exchange of ADP for ATP (Tables 9.1, 9.2, and Fig. 9.2). BiP was discovered and termed immunoglobulin heavy-chain-binding protein for its role in immunoglobulin assembly. It is also known as glucose-regulated protein with a molecular mass of 78 kDa (Grp78) because it is overproduced under ER stress conditions, such as glucose starvation (Haas and Wabl 1983; Hendershot et al. 1988; Kassenbrock

Table 9.2 Properties of BiP and its co-chaperones, NEFs, and NEF antagonists. We note that the concentrations were determined by Hein et al. (2015). The kinetic data on full-length proteins and J-domains, respectively, were determined on a BIACORE 2000 system as described in the legend to Fig. 9.5. For the BiP/MANF interaction, the affinity was measured by microscale thermophoresis (Eesmaa et al. 2021). GST, glutathione S-transferase; ?, unknown; FL, full-length protein; J, J-domain

Protein	UPR controlled	Cellular function(s)	Concentration in HeLa cells (μM)	Recombinant protein (amino acid residues)	Rate constants for interaction with BiP in the presence of ATP	
					k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})
BiP	+	ERAD, folding, gating, transport, UPR	8.253	BiP-Hexahis (20–655)	–	–
ERj1	–	transcription, translation, transport	0.008	GST-J (44–140)	12.8×10^3	2.89×10^{-3}
ERj2	–	transport	0.168	GST-J (91–189)	1.49×10^3	3.53×10^{-3}
ERj3	+	ERAD, folding	1.001	GST-J (18–119)	2.53×10^3	4.56×10^{-3}
				GST-FL (18–336)	2.12×10^3	2.39×10^{-3}
ERj4	+++	ERAD, folding	0.012	GST-J (23–222)	1.09×10^3	4.86×10^{-3}
ERj5	+	ERAD, folding	0.043	GST-J (26–113)	0.60×10^3	3.52×10^{-3}
				GST-FL (26–793)	0.81×10^3	3.07×10^{-3}
ERj6	+	ERAD, folding	0.237	GST-J (392–498)	0.86×10^3	3.19×10^{-3}
				GST-FL (32–505)	0.63×10^3	2.55×10^{-3}
ERj7	+	Unknown	0.010	GST-J (39–149)	9.29×10^3	3.98×10^{-3}
ERj8	?	ER-phagy	0.024	–	Not determined	
ERj9	?	Unknown	Unknown	–	Not determined	
Grp170	+	Folding, NEF	0.923	Purified native protein	Not determined	
Sil1	–	NEF	0.149	GST-39–461	Not detectable	
MANF	+	NEF antagonist	1.092	25–182	K_D (M) 0.38×10^{-6}	

et al. 1988). BiP is the most abundant Hsp70-type molecular chaperone in the ER lumen even under non-stress conditions and depends on ATP and Ca^{2+} for its activity plus on trace amounts of additional ions and cofactors (reviewed by Dudek et al. 2009; Otero et al. 2010; Melnyk et al. 2015; Bandla et al. 2019; Pobre et al. 2019).

The Two Hsp70s and NEFs of the Mammalian ER

The ER-resident members of the Hsp70 triad have been shown to be able to perform the classical Hsp70 reaction cycle, thereby mediating the folding or assembly of newly synthesized and newly imported polypeptides as well as facilitating conformational changes and, therefore, the regulation of folded proteins (Fig. 9.3). Similarly to the bacterial cytosol and the mitochondrial matrix, there are two Hsp70-type chaperones in the yeast ER (Kar2p and Lhs1p) (Baxter et al. 1996; Craven et al. 1996; Hamilton and Flynn, 1996; Steel et al. 2004; De Keyzer et al. 2009) as well as in the mammalian ER (BiP and Grp170) (Haas and Wabl 1983; Bole et al. 1986; Munro and Pelham 1986; Hendershot et al. 1988; Lin et al. 1993; Kitao et al. 2004; Weitzmann et al. 2007; Mimura et al. 2007; Luo et al. 2006). Grp170, however, may also be referred to as a Hsp110 protein family member and "moonlights" as a NEF for BiP (Weitzmann et al. 2006). There is also a *bona fide* functional homolog to bacterial GrpE in the ER lumen (termed Sil1 or BAP in mammals) (Tyson and Stirling 2000; Chung et al. 2002; Zhao et al. 2005, 2009; Hale et al. 2010; Behnke et al. 2015), i.e., there is redundancy also at the level of the NEFs, which may explain the non-lethal phenotype of loss of Sil1 function that is associated with the neurodegenerative disease, Marinesco-Sjögren syndrome (Table 9.1, see below). The structures of the two cytosolic paralogs of the two ER-resident NEFs were already solved and revealed distinct interacting surfaces with the top of the amino-terminal nucleotide-binding domain (NBD) of the Hsp70 (Shomura et al. 2005; Polier et al. 2008); thus, the NEF binding sites on Hsp70 are different from the J-domain binding site, which resides at the NBD bottom (Yan et al. 2011). Based on these structural data, the two NEFs may even be able to bind simultaneously to BiP. Recently, the secreted neuroprotectant mesencephalic astrocyte-derived neurotrophic factor or MANF was shown to be retained in the ER to a certain extent, to interact with BiP (Yan et al. 2019, Eesmaa et al. 2021) as well as Grp170 (Eesmaa et al. 2021) and to affect ER proteostasis. It turns out that ER-resident MANF binds the NBD of ADP-bound BiP and, thereby, inhibits ADP release from BiP as well as ATP binding to BiP and concomitant substrate release. Therefore, MANF was suggested to contribute to ER proteostasis as a nucleotide exchange antagonist for certain BiP-substrate complexes (Fig. 9.3). Interestingly, MANF also acts as an anti-apoptotic regulator of the UPR (Eesmaa et al. 2021).

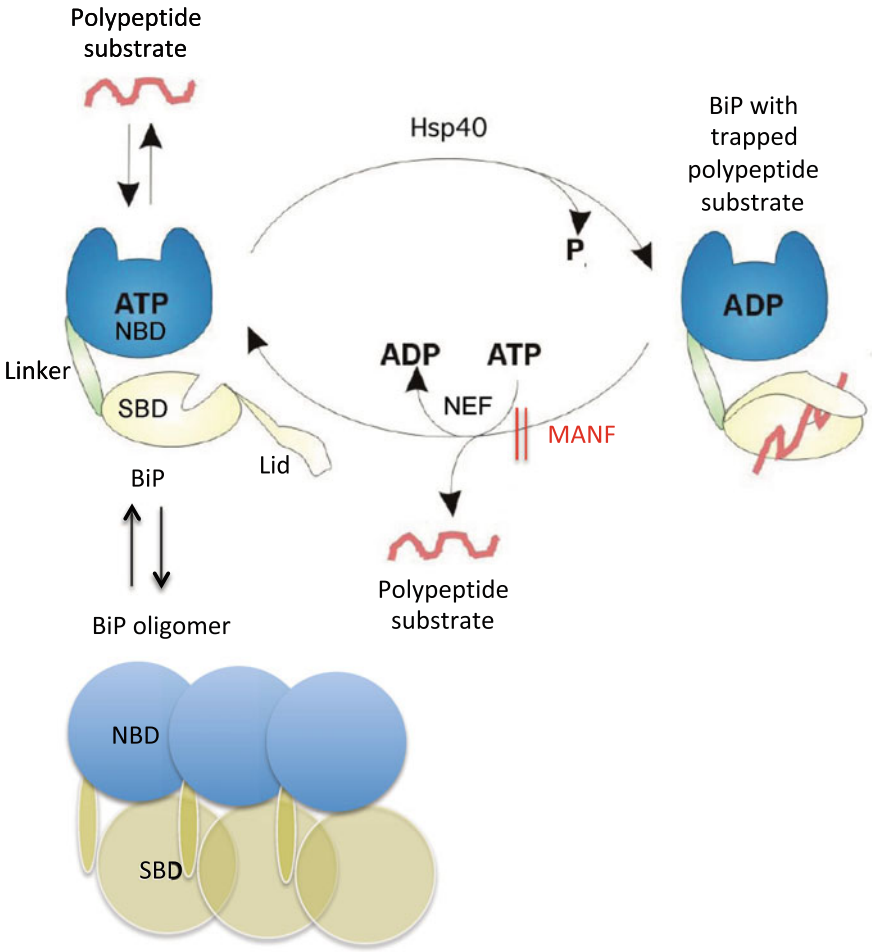

Fig. 9.3 BiP's functional cycle. See text for details. For additional details on the gymnastics of BiP and of Hsp70s, in general, see Awad et al. (2008), Kityk et al. (2015), Mashaghi et al. (2016), Preissler et al. (2015), Xu et al. (2016), and Zhuravieva and Gierasch (2015), and for details on the regulation of BiP, see Chambers et al. (2012), Freiden et al. (1992), and Perera et al. (2019). We note that BiP has a relatively high intrinsic ATPase activity compared to other Hsp70s and that MANF is an ATP-binding protein, too. MANF, mesencephalic astrocyte-derived neurotrophic factor; NBD, nucleotide-binding domain; NEF, nucleotide exchange factor; P_i, inorganic phosphate; SBD, substrate-binding domain

The Functional Cycle of BiP

Hsp70-type molecular chaperones, such as BiP, bind reversibly to substrate polypeptides via their carboxy-terminal substrate-binding domains (SBDs) (Fig. 9.3). Upon ATP hydrolysis, the carboxy-terminal extension of the SDB, termed the lid

domain, traps the substrate. Typically, BiP substrates are hydrophobic oligopeptides within loosely or unfolded polypeptides (Flynn et al. 1991; Blond-Elguindi et al. 1993). Binding of a substrate to the SBD inhibits unproductive interactions of the polypeptide and, thereby, favors productive folding and assembly, which occur concomitantly with release from BiP. In addition, BiP can also regulate the activities of folded polypeptides (e.g., Sec61α, see below). This binding and release of substrates by BiP are facilitated by interaction of its carboxy-terminal SBD and its amino-terminal nucleotide-binding domain (NBD), which are covalently linked via the linker region. NBD conformation and BiP's ATPase cycle are modulated by different Hsp70 interaction partners (Dudek et al. 2009; Otero et al. 2010; Melnyk et al. 2015; Pobre et al. 2019). The ATP-bound state of BiP has a low affinity for substrate polypeptides. In contrast, the ADP-bound state has a high substrate affinity. JDPs of the ER stimulate the ATPase activity of BiP and thereby favor substrate binding. NEFs of the ER lumen stimulate the exchange of ADP for ATP and thus induce substrate release. The latter can be antagonized by the regulatory protein MANF (Yan et al. 2019; Eesmaa et al. 2021).

The Co-chaperones of BiP in the Mammalian ER

In yeast, four JDPs or Hsp40-type molecular chaperones were discovered in the ER (Jem1p, Scj1p, Scj2p, and Sec63p) (Toyn et al. 1988; Rothblatt et al. 1989; Sadler et al. 1989; Schlenstedt et al. 1995; Brodsky et al. 1995; Eki et al. 1996; Nishikawa and Endo 1997; Silberstein et al. 1998). In mammalia, the ER-resident co-chaperone system is more complex. Nine different JDPs appear to reside in the human ER, although not necessarily simultaneously in the same cell type (Tables 9.1 and 9.2, Fig. 9.2). Until now, eight of these have been characterized in some detail and were termed ERj1 through ERj8 (or ERdj1 through ERdj8). As the name infers, ERjs or ERdjs are characterized by individual J-domains, which allow interaction with BiP via the bottom of its NBD, and contain four α-helices with a loop region containing a highly conserved tripeptide of histidine, proline, and aspartic acid (HPD motif) located between the second and third helix. They can be divided into membrane proteins with a lumenal J-domain and into lumenal proteins (Fig. 9.2). Furthermore, they can be classified according to the domains they have in common with the bacterial DnaJ protein (i.e., besides the actual J-domain) (Cheetham and Caplan 1998; Hennessy et al. 2005). Type I ERjs have four domains in common with bacterial DnaJ: the amino-terminal J-domain, the glycine-phenylalanine (G/F)-rich domain, the Zn-finger or cysteine repeat domain, and the carboxy-terminally located substrate-binding domain (ERj3). Type II ERjs contain three domains: an amino-terminal J-domain, a glycine-phenylalanine (G/F)-rich domain, and a carboxy-terminal substrate-binding domain (ERj4). Type III ERjs share with DnaJ only the J-domain and, typically, have more specialized functions as compared to type I and type II ERjs. Thus, only the type I and II ER lumenal ERjs ERj3 (Bies et al. 1999, 2004; Yu et al. 2000; Shen and Hendershot 2004; Jin et al. 2008, 2009) and ERj4

(Shen et al. 2002 Kurisu et al. 2003; Dong et al. 2008; Lai et al. 2012; Fritz et al. 2014) have the ability to bind substrate polypeptides and deliver them to BiP, analogous to the paradigm DnaJ in *E. coli*. However, the four thioredoxin (TRX) domains within ERj5 (Cunnea et al. 2003; Hosoda et al. 2003; Dong et al. 2008; Ushioda et al. 2008; Ladiges et al. 2005; Hagiwara et al. 2011; Oka et al. 2013), the two TRX domains in ERj8, and the tetratricopeptide repeat (TPR) domains in ERj6 ($p58^{IPK}$) (Kang et al. 2006; Rutkowski et al. 2007; Petrova et al. 2008; Dong et al. 2008; Svärd et al. 2011) may also function in substrate binding. So far, only ERj3, ERj4, ERj5, and ERj6 appear to be involved in protein folding under physiological as well as ER stress conditions and in ERAD (Table 9.2 and Fig. 9.2). This is consistent with the fact that these four ERjs are overproduced together with BiP under stress conditions, i.e., when there is an increased demand for chaperone activity or protein degradation by the proteasome (Table 9.2). In line with their classification as type III proteins, the other ERjs play roles in ER protein import (Sec63 and, possibly, ERj1) or ER-phagy (ERj8) or have a currently unknown function (ERj7 and ERj9) (Mayer et al. 2000; Tyedmers et al. 2000; Lang et al. 2012; Haßdenteufel et al. 2018; Schorr et al. 2020; Yamamoto et al. 2020). In addition to its potential – but far from proven – task in ER protein import, ERj1 may have regulatory roles that are related to transcription as well as to translation (Blau et al. 2005; Dudek et al. 2002, 2005; Zupicich et al. 2001; Benedix et al. 2010). The cytosolic domain of ERj1 has the ability to allosterically inhibit translation at the stage of initiation when its ER lumenal J-domain is not bound to BiP. Thus, ERj1 would be ideally suited to allow initiation of synthesis of precursor polypeptides on ER-bound ribosomes only when BiP is available on the other side of the membrane. Furthermore, ERj1 has all the features of a membrane-tethered transcription factor that can be activated by regulated intra-membrane proteolysis (Zupicich et al. 2001). The cytosolic domain has actually been shown to be able to enter the nucleus (Zupicich et al. 2001, Dudek et al. 2005). Last but not least, Sec63 and ERj3 plus ERj6 act as BiP co-chaperones in facilitating conformational changes and, therefore, regulation of the Sec61 complex, which will be discussed in detail in the two following sections.

We note that there are additional classifications discussed for JDPs, which are based either on the presence or absence of structural features of bacterial DnaJ or even on an evolutionary relatedness (Kampinga et al. 2018). According to the first and as a variation of the original classification (see above), class A and class B JDPs have their J-domains at the amino terminus, whereas class C JDPs can have them anywhere in the protein. Further distinguishing class A and B JDPs, in class A proteins, the J-domain is followed by a G/F-rich region of about 30 amino acid residues, two homologous ß-barrel domains (where the first one includes the Zn-finger or cysteine repeat domain), and a carboxy-terminal dimerization domain. Class B proteins also contain a G/F-rich region adjacent to the amino-terminal J-domain but do not contain the Zn-finger domain and do not necessarily contain a carboxy-terminal substrate-binding domain. Applying this classification to the ERjs of the mammalian ER, ERj3 is the only class A JDP in the ER, ERj4 is the only class B protein, and ERj1, Sec63, ERj5, ERj6, ERj7, ERj8, and ERj9 are all class C JDPs.

The Dual Role of BiP and Its Co-chaperone Sec63 in Protein Import into the ER as an Example of Chaperone/Co-chaperone Action in the Mammalian ER

Protein import into the ER is mediated by the heterotrimeric Sec61 complex, which exists in a dynamic equilibrium between a closed and an open aqueous channel in the ER membrane (Fig. 9.4). Only the open conformation acts as a polypeptide-conducting channel; the closed conformation is necessary to allow preservation of the cellular Ca^{2+} homeostasis (Hamman et al. 1998; Alder et al. 2005). Mammalian BiP was shown to support both co- and post-translational ER import of certain precursor polypeptides into the ER via the Sec61 channel in two different ways. First, BiP acts as an allosteric effector of the Sec61 complex for channel opening by binding to the di-tyrosine motif-containing luminal loop 7 of Sec61α (Fig. 9.4) (Schäuble et al. 2012). Second, BiP works as a molecular ratchet on the incoming precursor polypeptide in transit through the Sec61 channel (Tyedmers et al. 2003).

BiP Can Support Opening of the Human Sec61 Channel for ER Protein Import

The structure of the Sec61 complex suggested a potential mechanism for opening of the Sec61 channel for polypeptide import (van den Berg, et al. 2004; Alder et al. 2005; Gumbart and Schulten 2007; Voorhees et al. 2014; Voorhees and Hegde 2016; Pfeffer et al. 2012, 2014, 2015, 2017; Zimmermann et al. 2011). Accordingly, binding of the ribosome primes the Sec61 channel for opening of the Sec61 channel for co-translational protein import, i.e., channel gating from the closed to the open conformation, and signal peptides of nascent presecretory polypeptides intercalate between the Sec61α transmembrane helices 2 and 7 and open the "lateral gate" of the Sec61 complex formed by these two transmembrane helices (van den Berg et al. 2004; Gumbart and Schulten 2007; Voorhees and Hegde 2016) (Fig. 9.4). It has been suggested that this intercalation represents the crucial reaction in the early phase of protein import. Next, the nascent chain can be fully inserted into the Sec61 channel. We suggested that the Sec62/Sec63 complex, on its own or in cooperation with BiP, in post-translational transport can prime the closed mammalian Sec61 complex for opening (Lang et al. 2012; Haßdenteufel et al. 2018, 2019). To a certain extent, this view was supported by the recent structural insights into the yeast SEC complex, i.e., the Sec61 complex together with the Sec62/63/71/72 complex, which visualized how in the post-translationally acting Sec61 complex the Sec62/63 subcomplex interacts with the cytosolic loops 6 and 8 on the cytosolic face of the Sec61 complex and how the ER luminal domain of Sec63 interacts with ER luminal loop 5 (Itskanov and Park 2019; Weng et al. 2021; Wu et al. 2019). The potential role of the yeast BiP ortholog Kar2p, however, was not addressed in these studies.

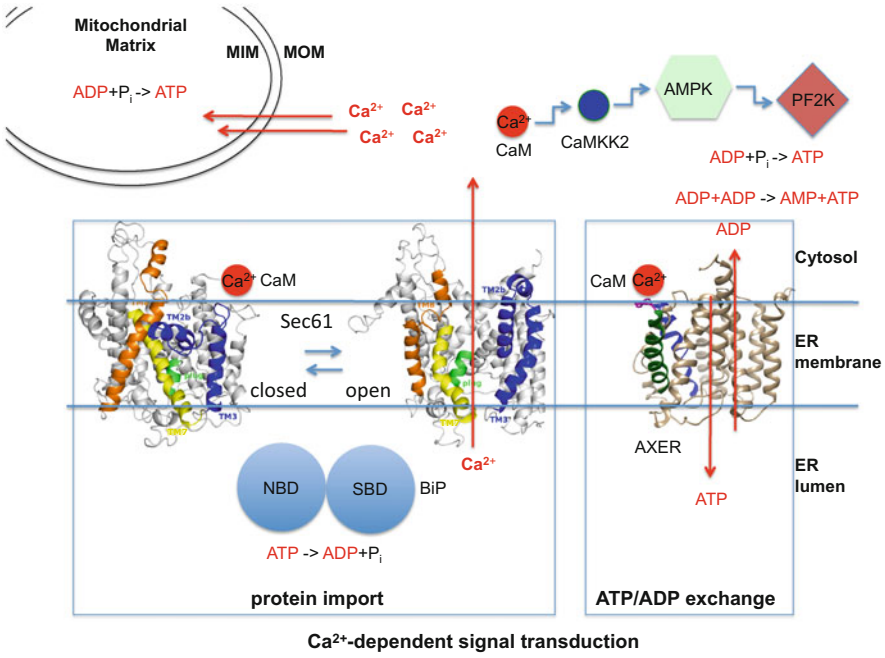

Fig. 9.4 Section of the ER membrane with a focus on ER protein import, Ca^{2+}-dependent signal transduction, and ATP/ADP exchange between ER and cytosol. See text for details. We note that Ca^{2+} enters mitochondria via voltage-dependent anion channels (VDACs) in the MOM plus the mitochondrial calcium uniporter (MCU) in the IMM and that ATP is exported to the cytosol via the mitochondrial ADP/ATP carriers (AACs, i.e., SLC25A4 and SLC25A5) in the IMM plus VDACs in the MOM (Baughman et al. 2011; Klingenberg 2008) and that the shown protein structures were modeled and do not represent PDB entries (Dudek et al. 2015; Klein et al. 2018). AMPK, AMP-dependent protein kinase; AXER, ATP/ADP exchanger of the ER membrane (shown on the far right), also termed SLC35B1; CaM, calmodulin; CaMKK2, Ca^{2+}-CaM-dependent protein kinase kinase 2; MIM, mitochondrial inner membrane; MOM, mitochondrial outer membrane; PF2K, 6-phosphofructo-2-kinase; P_i, inorganic phosphate; Sec61, Sec61 complex (shown in the closed conformation on the left and in the open state on the right); TM, transmembrane helix

Notably, a role of Sec63 and BiP in Sec61 channel opening was originally shown for the early phase of post-translational protein transport into the yeast ER (Brodsky and Scheckman 1993; Brodsky et al. 1995; Lyman and Schekman 1995, 1997). However, this idea was subsequently disputed by a model translocation reaction that allowed precursor movement through the yeast Sec61 complex in detergent solution (Matlack et al. 1997). We suggested that in this experimental system the otherwise BiP-dependent Sec61 channel gating may have been facilitated by the detergent. In any case, mammalian Sec62/Sec63 and BiP are required for the efficient import of small precursor proteins (such as preproapelin), which are, due to their short length, transported post-translationally (Haßdenteufel et al. 2018, 2019; Johnson et al. 2013; Lakkaraju et al. 2012). However, in contrast to its yeast ortholog Sec62p, the mammalian Sec62 protein experienced a gain of function, and, therefore, it is able

to interact with the ribosome near the ribosomal exit tunnel and to support, in collaboration with Sec63 and BiP, the co-translational transport of certain substrates, such as the precursors of ERj3 and prion protein (Müller et al. 2010; Ziska et al. 2019; Schorr et al. 2020). According to in vitro protein import studies, Grp170 may also be involved in Sec61 channel opening (Dierks et al. 1996). The idea is that some amino-terminal signal peptides may be "strong" enough to trigger Sec61 channel opening in co-translational import on their own, particularly after the ribosome has already primed the channel. However, precursor polypeptides with "weak" signal peptides appear to involve allosteric effectors of the channel in its opening and, therefore, in facilitating insertion of precursor polypeptides into the Sec61 complex, such as the abovementioned ER lumenal chaperone BiP and the Sec62/Sec63 complex (Table 9.1) (Lang et al. 2012; Schäuble et al. 2012; Schorr et al. 2020). We suggested that certain features of signal peptides may extend the "dwell" or "sampling" time of signal peptides on the cytosolic surface of the Sec61 channel and that BiP together with Sec63 and Sec62 can overcome this by facilitating Sec61 channel gating on the lumenal side (beautifully visualized by Zhang and Miller 2012). However, our work suggested that special features downstream of the signal peptides can also play a distinct role, which may be particularly relevant in co-translational import when a considerable stretch of a nascent precursor polypeptide accumulates at the interface between ribosome and Sec61 complex, i.e., prior to Sec61 channel opening, and in post-translational import (Haßdenteufel et al. 2018; Ziska et al. 2019; Schorr et al. 2020). In the case of BiP, it has been suggested that the di-tyrosine motif-containing minihelix within loop 7 of Sec61α plays a role in gating of the Sec61 complex from closed to open and that BiP binding to this minihelix may be required for some precursor polypeptides (Fig. 9.4). Thus, by providing the energy of binding, the ribosome and BiP may be able to "pull" transmembrane helix 7 from opposite ends to facilitate channel opening (Schäuble et al. 2012; Haßdenteufel et al. 2018; Ziska et al. 2019; Schorr et al. 2020). We favor this hypothesis because luminal loop 7 connects transmembrane helices 7 and 8 and is thus close enough to the "lateral gate" to influence gate movements. Thus, BiP together with Sec63 and Sec62 represents an allosteric effector of the Sec61 complex for channel opening. This view was supported by the observations that the murine diabetes-linked mutation of tyrosine 344 to histidine within the loop 7 minihelix destroys the BiP-binding site and, when introduced into HeLa cells, prevents in vitro transport of BiP-dependent, i.e., slowly gating precursor polypeptides in co- and post-translational ER import (Lloyd et al. 2010; Haßdenteufel et al. 2018; Schorr et al. 2020). In addition, similar experiments with mutant variants of Sec63 also highlighted the importance of the physical interactions between the Sec63 carboxy-terminus and Sec62 on the one hand and between the Sec63 J-domain and BiP on the other hand (Haßdenteufel et al. 2018; Schorr et al. 2020).

BiP Can Support ER Protein Import by Acting as a Molecular Ratchet on the Incoming Precursor Polypeptide

As soon as the incoming precursor polypeptide emerges from the Sec61 channel, BiP can bind to the precursor polypeptide in transit and act as a molecular ratchet, thus mediating completion of translocation or unidirectionality of import (Nicchitta and Blobel 1993; Tyedmers et al. 2003; Shaffer et al. 2005). At this stage, BiP binds the non-native precursor polypeptide as a substrate and prevents it from sliding back into the cytosol. That such a mechanism may indeed be at work came from protein import into proteoliposomes with ER membrane proteins and trapped avidin in the lumen where avidin could substitute for BiP in the case of biotinylated nascent precursor polypeptides (Tyedmers et al. 2003). It remains open, whether or not a single BiP molecule can first bind loop 7 of Sec61α and, subsequently, the incoming precursor polypeptide within one functional cycle (Schlecht et al. 2011). Notably, related questions were recently addressed for the bacterial Hsp70 DnaK by the creation of variants, which lack the lid domain (Mashaghi et al. 2016). Therefore, we designed two different BiP variants, which differ in length of the lid deletion and, so far, have only been characterized with respect to affinities for various ERjs via surface plasmon resonance spectroscopic analysis (Fig. 9.5). Besides this in vitro assay, we started to design protein-protein interaction studies in living cells with the help of a bimolecular luminescent complementation assay. As a proof of principle, preliminary results confirmed an interaction of Sec63 and BiP in living cells. Consequently, future experiments aim to address the interaction and interchangeability of BiP and its co-chaperones in live cells, i.e., under physiological conditions. Additional future experiments will have to address the question if the BiP variants are able to substitute for BiP in both aspects of ER import of BiP-dependent precursor polypeptides in human cells.

Interestingly, it has been shown that human ERj1 can complement the otherwise lethal deletion of Sec63p in yeast (Kroczynska et al. 2004). Therefore, ERj1 may be able to play a similar role as Sec63 in the mammalian ER, thereby providing at least partial redundancy for this essential function that may explain the non-lethal phenotype of putative loss of Sec63 function, associated with polycystic liver disease (Table 9.1, see below). ERj1 was observed in association with translating ribosomes (Fig. 9.2) (Dudek et al. 2002, 2005; Blau et al. 2005; Benedix et al. 2010). Therefore, we proposed that in the mammalian ER, two different membrane proteins provide J-domains in the neighborhood of translating ribosomes and Sec61 complexes and allow BiP to play its two roles in protein import, possibly in a substrate specific manner.

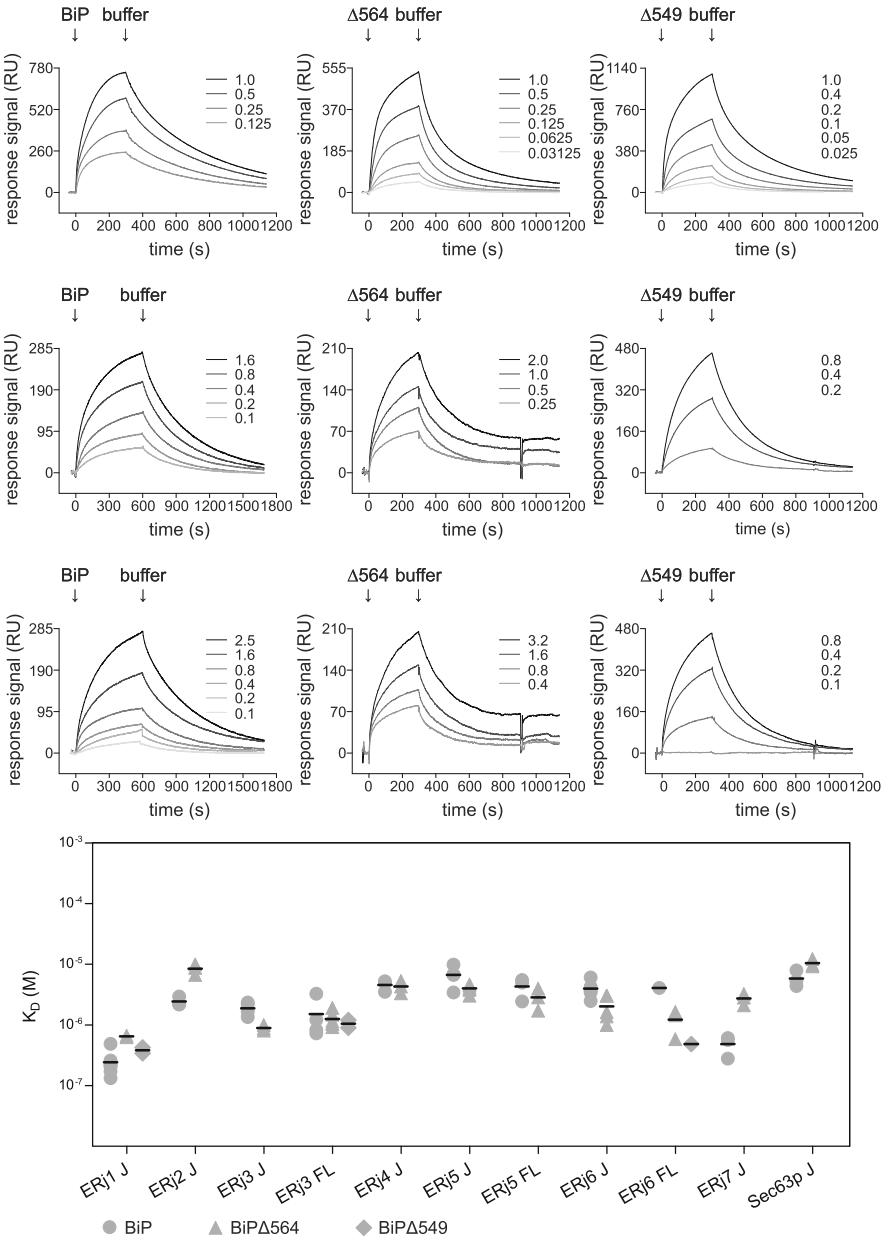

Fig. 9.5 Surface plasmon resonance spectroscopical measurements for the determination of kinetics of the interactions between BiP or two lidless variants and ERjs. Surface plasmon resonance spectroscopy was carried out in a BIACORE system as described (Schorr et al. 2015). Briefly, monoclonal goat anti-GST antibodies were immobilized on a sensor chip CM5 research grade by amine coupling according to the manufacturer's protocol. The chip was equilibrated with application buffer (150 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$ in sodium phosphate buffer, pH 7.4) supplemented with ATP (final concentration: 2 mM), BSA (final concentration: 5 mg/ml), and Tween 20 (final concentration: 0.1%), termed running buffer (flow rate: 15 µl/min). Purified

Closing of the Human Sec61 Channel for Preservation of Cellular Calcium Homeostasis

As already eluded above, the mammalian ER is also a central player in cellular calcium homeostasis and Ca^{2+}-dependent signal transduction (Figs. 9.1 and 9.4). It represents the major Ca^{2+} storage organelle in nucleated mammalian cells and allows the controlled release of Ca^{2+} from the ER upon hormone stimulation of a resting cell, e.g., via IP3 or ryanodine receptors (Berridge 2002; Clapham 2007). Subsequently, Ca^{2+} is pumped back into the ER by sarcoplasmic/ER Ca^{2+} ATPase (SERCA) to reestablish the steep ER to cytosol Ca^{2+} gradient (Wuytack et al. 2002). It had been known for quite some time that this gradient is constantly challenged by the so-called passive Ca^{2+} efflux from the ER. Therefore, SERCA has the additional task of counteracting this Ca^{2+} leakage. In addition, Ca^{2+} is taken up by mitochondria. In the course of the last 15 years, several proteins were linked to ER Ca^{2+} leakage, including the Sec61 channel (Lomax et al. 2002; van Coppenolle et al. 2004; Erdmann et al. 2011). Therefore, the Sec61 channel gating is tightly controlled (Fig. 9.4).

Originally, sophisticated biophysical measurements on ER-derived membranes established that closing of the aqueous Sec61 channel involves BiP and one of its JDPs, such as ERj4, in order to preserve the ER membrane permeability barrier (Hamman et al. 1998; Haigh and Johnson 2002; Alder et al. 2005). Subsequently, single-channel recordings from planar lipid bilayers characterized the Sec61 complex as a highly dynamic aqueous channel that is transiently opened by signal peptides within precursor polypeptides and is permeable to Ca^{2+} after completion of protein import (Wirth et al. 2003; Erdmann et al. 2011; Lang et al. 2011). The same experimental strategy showed that the Sec61 channel closes either spontaneously or as induced by binding of BiP or Ca^{2+}-calmodulin (Erdmann et al. 2011; Schäuble et al. 2012; Schorr et al. 2015). The fact that BiP is involved in closing the Sec61 channel was confirmed at the cellular level by combination of siRNA-mediated gene silencing or pharmacological manipulation and live cell Ca^{2+} imaging

Fig. 9.5 (continued) GST-ERj hybrids (termed full-length or FL) or GST-J-hybrid (termed J) was bound to the immobilized antibodies in the measuring cell. Similarly immobilized glutathione S-transferase (GST) in the reference cell served as a negative control. Subsequently, solutions containing the indicated concentrations of purified hexa-His-tagged hamster BiP or BiP variant (given in μM) were passed over the chip in the presence of ATP (indicated by arrows). At the indicated times, each BiP or variant application was followed by application of running buffer (indicated by arrows). The analysis was carried out employing a BIACORE 2000 system with BIA evaluation software version 2.2.4. The two lidless BiP variants comprised amino acid residues either 1 through 564 (termed Δ564) or 1 through 549 (termed Δ564), respectively, of BiP. The upper part of the figure shows examples of sensorgrams for ERj1 J-domain (upper rows), ERj3 full-length protein without signal peptide (middle row), and ERj6 full-length protein without signal peptide (lower row). The lower part shows the determined affinities (K_D values in mol/l) and the calculated mean value (short black line). Selected on and off rates are given in Table 9.2. FL, full-length protein; J, J-domain

(Schäuble et al. 2012). In addition, cytosolic Ca^{2+}-calmodulin was shown under similar conditions to contribute to Sec61 channel closing via an unrelated mechanism once Ca^{2+} has started to leak from the ER (Erdmann et al. 2011). During the last 10 years, additional siRNA-mediated gene silencing and live cell Ca^{2+} imaging experiments characterized the pair and possibly heterodimeric complex of ERj3 and ERj6, as co-chaperones of BiP, and Ca^{2+}-Sec62, as a cofactor of calmodulin, in Sec61 channel closure (Linxweiler et al. 2013; Schorr et al. 2015). Furthermore, the binding sites of BiP, Ca^{2+}-calmodulin, and Ca^{2+}-Sec62 were identified as the abovementioned di-tyrosine motif-containing minihelix within ER lumenal loop 7 of the Sec61 α-subunit and an IQ motif in the cytosolic amino terminus of the same subunit, respectively, and shown to be relevant to the described mechanisms by mutagenesis. Furthermore, the respective affinities of these interactions were determined by surface plasmon resonance spectroscopy and found to be physiologically relevant. Again, the idea is that binding of BiP to loop 7 of Sec61α provides binding energy for shifting the dynamic equilibrium of the Sec61 channel to the closed state. That such a mechanism may indeed be at work came from single-channel recordings where Fab fragments directed against loop 7 could substitute for BiP in channel closing (Schorr et al. 2015). In case of inefficient channel closure in intact cells, Ca^{2+} starts to leak from the ER into the cytosol and binds calmodulin, and Ca^{2+}-calmodulin is recruited to the IQ motif in the Sec61 α-subunit (Erdmann et al. 2011) (Fig. 9.4). Once again, the involved binding energy may favor channel closure. Binding of Ca^{2+}-calmodulin is supported by Sec62, which may have bound Ca^{2+} because of a putative EF hand within its cytosolic carboxy-terminal end (Linxweiler et al. 2013). Next, the Sec61 channel is closed, and Ca^{2+} leakage subsides. SERCA pumps Ca^{2+} back into the ER, calmodulin and Sec62 return to the Ca^{2+}-free forms, and the next protein import cycle can be initiated. When these mechanisms fail, however, the passive Ca^{2+} efflux of the ER membrane might actually represent part of a signaling pathway reporting about protein homeostasis and folding capacity within the ER lumen and eventually leading to apoptosis.

In addition, recent evidence provided further insight into the functional and regulatory role of ERjs and how they balance Ca^{2+} flux across the ER membrane. ERj5 triggers the ER influx of Ca^{2+} via activation of the SERCA2 pump in a Ca^{2+}-dependent manner (Schorr et al. 2015; Ushioda et al. 2016). Once Ca^{2+} levels in the ER are replenished, ERj5 is inactivated and forms oligomers. Interestingly, this circuit of Ca^{2+} flux across the ER membrane orchestrated by SERCA2 is tightly connected to the master regulator of the UPR, BiP, which, potentially in its function as classical chaperone, prevents oligomerization of ERj5 and hence inactivation of Ca^{2+} influx (Ushioda et al. 2016). Thus, BiP seems to fine-tune Ca^{2+} flux across the ER membrane in at least two ways.

Not All Hsp70s Are Created Equal: BiP and Kar2p Cannot Substitute for Each Other in Sec61 Channel Gating

First in vitro experiments addressing the potential interchangeability of Hsp70 proteins in ER protein import were carried out for Kar2p and cytosolic Hsc70 in yeast and did not observe any (Brodsky et al. 1993). Based on in vitro experimental results for the mammalian ER (Alder et al. 2005), even the yeast ortholog of BiP, Kar2p, is unable to substitute for BiP in both its actions on the Sec61 channel, i.e., facilitating channel opening for protein import and limiting Sec61-mediated Ca^{2+} efflux. Therefore, we attempted expression of the *KAR2* cDNA in the presence of the *BIP*-UTR siRNA in HeLa cells and observed that it was unable to rescue the *BIP* silencing phenotype; it did not restore the normal level of Ca^{2+} leakage although Western blot analysis showed that the *KAR2* expression plasmid restored an ER lumenal Hsp70 concentration comparable to control cells (Schäuble et al. 2012). Thus, the observed effect of BiP on cellular Ca^{2+} homeostasis was reproduced in intact cells and is highly specific for mammalian BiP.

Furthermore, we asked if the effect of the Y344H mutation in loop 7 in the same cellular model and the differential effect of BiP and Kar2p can be observed at the level of oligopeptide binding experiments. Therefore, we analyzed binding of BiP to the 15-mer of loop 7 that included the Y344H mutation in comparison to the corresponding wild-type peptide and Kar2p binding to the wild-type peptide. Replacement of tyrosine 344 by histidine led to significantly decreased BiP binding in peptide array assays and in surface plasmon resonance spectroscopic (SPR) analysis (Schäuble et al. 2012). In addition, purified native Kar2p appeared to bind less efficiently to the loop 7 15-mer as compared to BiP in an initial SPR analysis of the interaction. We note that there are 40% sequence identity between human and yeast 15-mer and 23% between the two entire loops 7. Thus, lower affinity for loop 7 of Sec61α appeared to be one possible reason for the inability of Kar2p to substitute for BiP in Sec61 channel closing in vitro as well as in intact cells. To more thoroughly address this issue, recombinant Kar2p was purified, and its interaction with human or yeast loop 7 15-mer was analyzed by SPR in comparison with BiP at various concentrations (Fig. 9.6). In this more recent data set, the affinities of Kar2p and BiP for the human loop 7 15-mer were indistinguishable, which argues against a differential substrate specificity of the two Hsp70s in the context of Sec61α. The different behavior of native versus recombinant Kar2p, which may be related to post-translational modifications, will have to be addressed in future work.

A priori, lack of co-chaperone interchangeability between BiP and Kar2p appeared as alternative or additional reason. Therefore, we measured the affinities of Kar2p for the mammalian ERjs and yeast Sec63 as a positive control via SPR analysis (Fig. 9.7). Since we already had observed co-chaperone specificity in channel opening versus closing, the focus was on Sec63 and the two lumenal ERjs, ERj3 and ERj6. In general, Kar2p had a more positive K_D value, i.e., lower affinity for mammalian JDPs as compared to BiP, which is consistent with the idea that the lack co-chaperone interchangeability between BiP and Kar2p is responsible

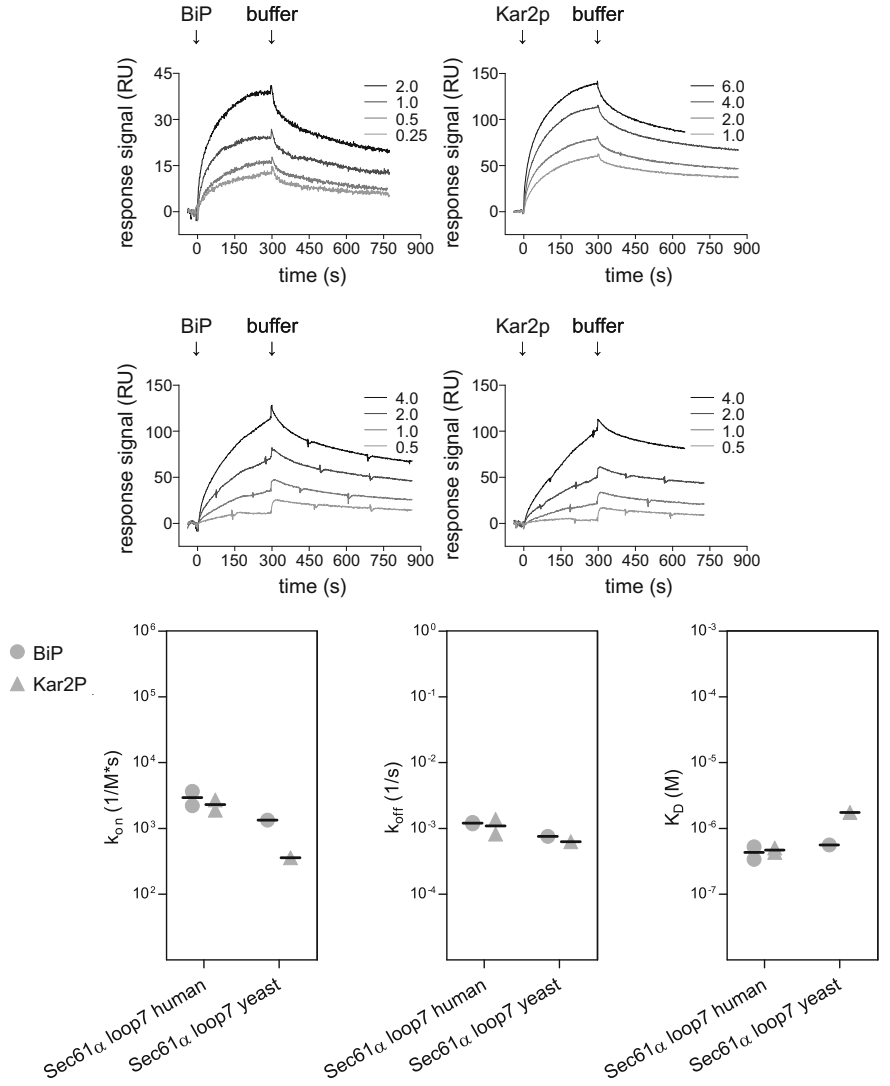

Fig. 9.6 BIACORE measurements for the determination of interaction kinetics for BiP or its yeast ortholog Kar2p and loop 7 of Sec61α. Surface plasmon resonance spectroscopy was carried out in a BIACORE system as described (Schäuble et al. 2012). Briefly, two oligopeptides corresponding to amino acid residues 339–353 (in single letter code: GGLCYYLSPPESFGS) of human Sec61α or residues 340–354 (SGLAYYIQPLMSLSE) of yeast Sec61α were synthesized with biotinylated dipeptide KG at the carboxy-terminus and immobilized on avidin sensor chips SA in the measuring cell; the reference cell contained peptide 325–339 of human Sec61α. The chip was equilibrated with application buffer (150 mM NaCl, 6.4 mM KCl, 2 mM $MgCl_2$ in HEPES/KOH buffer, pH 7.4) supplemented with surfactant P2 (final concentration: 0.005%), termed running buffer. Subsequently, solutions containing the indicated concentrations of purified hexa-His-tagged hamster BiP or yeast Kar2p (given in μM) were passed over the chip (indicated by arrows). At the indicated times, each BiP or Kar2p application was followed by application of running buffer (indicated by arrows). The analysis was carried out employing a BIACORE X instrument with BIA evaluation software version 2.2.4. The upper part of the figure shows sensorgrams for human 15-mer (upper

for Kar2p's inability to substitute for BiP in Sec61 channel gating. Furthermore, we performed the same measurements for two different BiP variants, which represent domain swaps between BiP and Kar2p. So far, the swap mutants have only been characterized with respect to affinities for some of the mammalian ERjs (Fig. 9.7). Although still incomplete, the data set points into the direction that the hybrid between BiP's NBD and Kar2p's SBD (termed BiP/Kar2p) behaves like BiP and that the reverse swap variant (termed Kar2p/BiP) behaves like Kar2p. This is exactly what one would expect since the J-domain binding site of Hsp70s resides at the NBD bottom. Future experiments will have to address the question if these variants are able to substitute for BiP in ER import of BiP-dependent precursor polypeptides in human cells.

Regulatory Mechanisms for the ER-Resident Hsp70/Hsp40 Chaperone Network

It has been known for some time that the genes of many of the protein transport components of the mammalian ER are under control of the unfolded protein response (see Table 9.2 for examples). In addition, there are splice variants for some of these genes according to the respective databases, and various miRNAs apparently target some of these same genes. Thus, additional genetic mechanisms are undoubtedly at work in regulation of the ER chaperone network and will have to be addressed in future research.

In addition, there is regulation at the protein level. In the case of mammalian BiP, AMPylation by filamentation-induced by cAMP domain protein (FICD, also termed HYPE) and BiP oligomerization were shown to be mechanisms for reversible inactivation of BiP when the concentration of unfolded polypeptides is low (Fig. 9.3) (Chambers et al. 2012). The equilibration between inactive oligomers and active BiP monomers was shown to be poised to buffer rapid fluctuations in unfolded proteins in the ER, e.g., induced by Ca^{2+} depletion, that cannot be attained by interconversions or the UPR (Preissler et al. 2015). Similarly, upon escape and retrieval, ER-resident proteins like Ero1α can be phosphorylated by the Golgi kinase Fam20C and influence folding capacity by PDI (Zhang et al. 2018).

Furthermore, various modifications have been observed for mammalian as well as yeast protein transport components, most notably phosphorylation. Cytosolic phosphorylation of mammalian membrane proteins ERj1 and Sec63 by CK2 was reported, but the functional consequences of these phosphorylations were not addressed (Götz et al. 2009; Ampofo et al. 2013). A first hint for the importance of CK2-dependent phosphorylation of components of the transport machinery may

Fig. 9.6 (continued) row) and yeast 15-mer (lower row). The lower part shows the determined affinities (K_D values in mol/l) and the calculated mean value (short black lines)

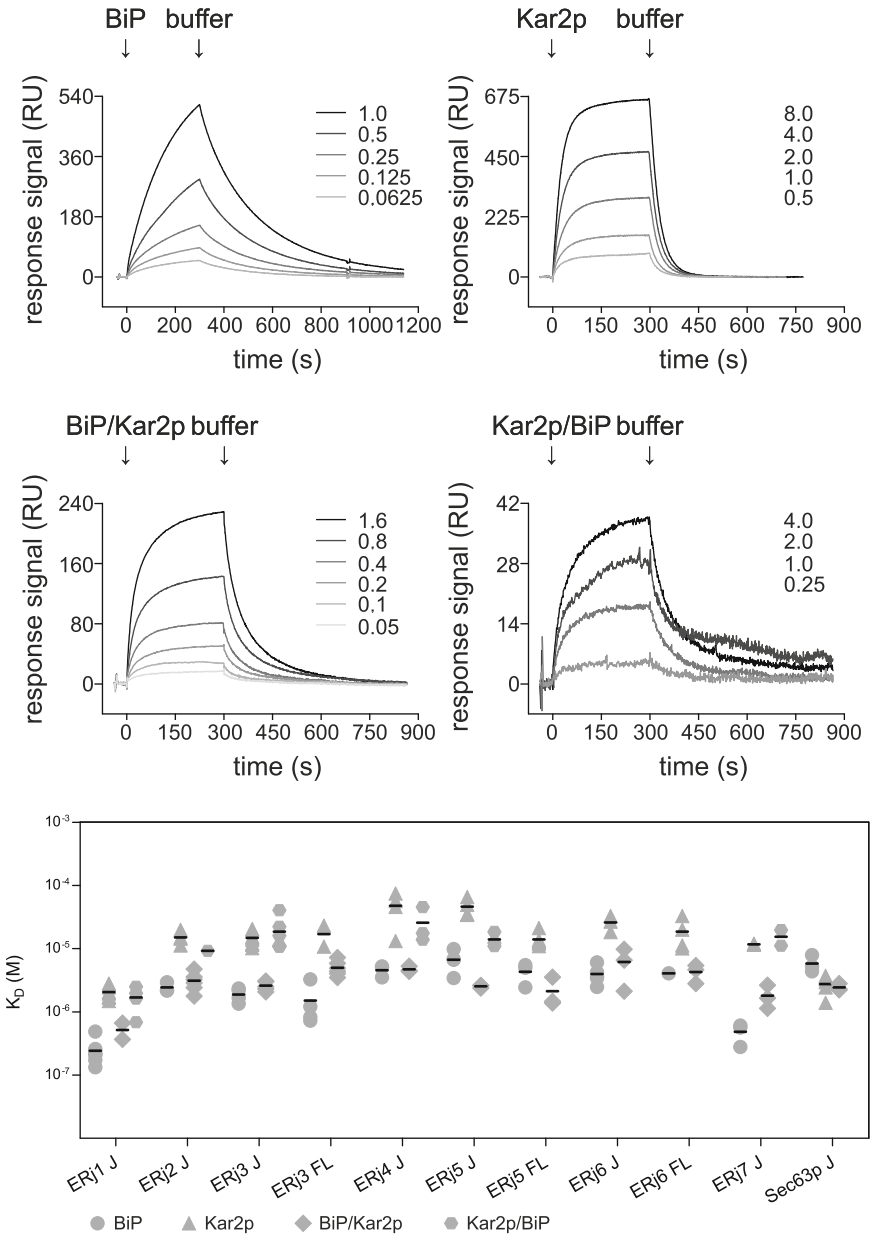

Fig. 9.7 BIACORE measurements for the determination of interaction kinetics for BiP, its yeast ortholog Kar2p, plus two domain-swap mutants thereof and ERjs. BIACORE analysis was carried out as described in the legend to Fig. 9.5. Here, solutions containing the indicated concentrations of purified hexa-His-tagged hamster BiP or yeast Kar2p (given in μM) were passed over the chip in the presence of ATP. At the indicated times, each BiP or Kar2p application was followed by application of running buffer. The two BiP/Kar2p domain-swap variants comprised either the NBD of BiP plus the SBD of Kar2p (termed BiP/Kar2p) or Kar2p-NBD plus BiP-SBD (termed Kar2p/BiP), respectively. The upper part of the figure shows examples of sensorgrams for ERj2 (Sec63) J-domain. The

come from studies in yeast (Wang and Johnsson 2005). The essential Sec63p is phosphorylated by the protein kinase CK2, and non-phosphorylatable Sec63p causes a protein translocation defect. More recently, phosphorylation of Sbh1p, the Sec61ß ortholog in yeast, was observed on threonine at position 5 and attributed to proline-directed kinase like Cdc28 (Soromani et al. 2012). However, in this case, mutation of this particular threonine did not have any consequences on ER protein import. Taken together, these findings suggest a general role of phosphorylation for a network of transport factors in regulation of protein translocation across the ER membrane.

The open questions are (i) if any of the observed phosphorylations is reversible, i.e. represents a molecular switch that responds to certain cellular conditions, (ii) what these cellular conditions may be that result in phosphorylations and de-phosphorylations, respectively, and (iii) how these reactions are regulated and executed. One idea is that phosphorylation of Sec63 allows regulated access of a subset of precursor polypeptides to the Sec61 channel and may, therefore, allow differential intracellular localization of certain polypeptides under certain conditions (such as cellular stress). ER import of ERj6 involves Sec62 and, by extrapolation, Sec63 (Schorr et al. 2020). We propose that this might explain the observed dual intracellular location of this protein (Rutkowski et al. 2007; Petrova et al. 2008).

Nucleotide Transport Into and Out of the ER

Pioneering work in the 1990s started to shed light on the interesting question of how ATP is imported into the mammalian ER. Despite its high energy demand, the ER harbors no system equivalent to the cytosol or mitochondria for ATP production or regeneration. While the original biochemical approaches established hallmarks of the ATP transport into the ER (including nucleotide selectivity and antiport mode), it was the more recent live-cell imaging approach employing sensitive, localized molecular nucleotide probes in combination with gene silencing in HeLa cells that identified the low abundant ATP/ADP exchanger in the ER membrane that was termed AXER (Klein et al. 2018). Here we focus on the regulation of AXER, which is also termed SLC35B1, as well as its partners acting in concert to orchestrate ER energy homeostasis (Fig. 9.4).

Two seemingly controversial concepts have emerged for the regulation of ATP homeostasis in the human ER. Based on experiments in HeLa cells, AXER was proposed to be part of a regulatory circuit and a Ca^{2+}-dependent signaling pathway, termed lowER (ER *low e*nergy *r*esponse), which was proposed to act as an immediate and locally confined response in the vicinity of the Sec61 complexes at the ER membrane, thereby guaranteeing sufficient ATP supply to the ER according to the

Fig. 9.7 (continued) lower part shows the determined affinities (K_D values in mol/l) and the calculated mean value (short black lines). Selected on and off rates are given in Table 9.2. FL, full-length protein; J, J-domain

following scenario (Klein et al. 2018) (Fig. 9.4). High ATP/ADP ratio in the ER allows BiP to limit Ca^{2+} leakage from the ER via the Sec61 channel (see above). Reduced ATP/ADP ratio due to increased protein import and folding or due to protein misfolding leads to BiP dissociation from the Sec61 channel and induces Ca^{2+} leakage from the ER and subsequent accelerated ATP/ADP exchange (Schäuble et al. 2012). Exported ADP can give rise to AMP in the cytosol, which may contribute to activation of AMP-dependent protein kinase or AMPK. Simultaneously, cytosolic Ca^{2+} can bind to calmodulin (CaM) (Erdmann et al. 2011) and activate AMPK via Ca^{2+}-CaM-dependent protein kinase-kinase 2 (CAMKK2). Finally, AMPK stimulates 6-phosphofructo-2-kinase (PF2K), which causes increased ADP phosphorylation in glycolysis, leading to ATP import into the ER via AXER. AXER itself also appears to be activated by Ca^{2+} efflux from the ER (Vishnu et al. 2014; Klein et al. 2018). Interestingly, mammalian AXER comprises an IQ motif in the cytosolic loop between transmembrane domains 2 and 3 and, thus, may well be regulated by Ca^{2+}-CaM. Subsequent normalization of the ER ATP/ADP ratio causes BiP to limit the Sec61-mediated Ca^{2+} leakage and thus inactivates lowER. SERCA pumps Ca^{2+} back into the ER lumen and balances the passive Ca^{2+} efflux, and protein phosphatase 2 (PP2) dephosphorylates AMPK. Note that activated AMPK was previously shown to lead to reduced cap-dependent translation and, therefore, ties the regulatory circuit lowER to the UPR (Preston and Hendershot 2013). LowER was proposed to represent the first and locally acting line of defense of a cell against ER stress (Klein et al. 2018).

Based on experiments in HeLa, INS-1, and CHO cells, AXER was proposed to also be part of a second and somewhat delayed but sustainable regulatory mechanism, termed *C*a^{2+}-*a*ntagonized *t*ransport *i*nto *ER* or CaATiER, which was supposed to guarantee sufficient and long-term ATP supply to the ER (Yong et al. 2019). According to the CaATiER model, the almost instantaneous increase in ER ATP levels after activation of lower was observed as a short-term and short-distance response to SERCA inhibition over a period of 5 minutes. This effect, however, was followed by a long-term decrease over the following 10 minutes, acting on long distance. These findings suggest the interesting possibility that there may actually be two phases associated with Ca^{2+}-coupled ER ATP homeostasis, a first phase that corresponds to lowER and a second one that was termed CaATiER. Such a biphasic regulatory scenario would be consistent with the observations that in HeLa cells the ATP for ER uptake is initially supplied mainly by anaerobic glycolysis and subsequently by oxidative phosphorylation. Therefore, we propose that lowER may describe the immediate and local regulation of ER homeostasis under physiological conditions, i.e., whenever the ATP to ADP ratio drops in the ER, to prevent problems of protein misfolding and that CaATiER may kick in during pathophysiological conditions such as protein misfolding, i.e., when the demand for ATP becomes particularly high.

Overall, the open questions are related to the proteins, which are responsible for the ATP supply of the ER, their regulation, and their potential interplay with the unfolded protein response (UPR). More precisely, the current questions are if there are more ATP/ADP exchangers than just AXER (SLC35B1) present in the ER

membrane and where this or these proteins are located, evenly distributed throughout, or enriched in contact sites to mitochondria (MERCs) (Klein et al. 2018; Yong et al. 2019). Furthermore, AXER structure needs to be determined as well as its molecular mechanism. In addition, future research needs to be directed toward unraveling AXER regulation as well as how these regulatory mechanisms are tied into the signal transduction mechanisms, which guarantee ATP homeostasis in the ER under physiological as well as stress conditions (Depaoli et al. 2019; Zimmermann and Lang 2020).

Human Diseases Related to ER Chaperones or the Sec61 Channel: On Chaperonopathies and Sec61-Channelopathies

As described above, the major ER chaperone BiP plus its co-chaperones and control elements play central roles in cellular protein and calcium homeostasis as well as in various signal transduction pathways. Although some components of this network have overlapping functions and, therefore, can partially substitute for each other, the precise functioning of this chaperone/co-chaperone network is of crucial importance for the health and survival of human cells, and acquired or genetically determined disruption of this network can lead to devastating human diseases or even death of the affected patients. Next, we summarize the current knowledge about these diseases and discuss them as chaperonopathies or Sec61-channelopathies.

Shiga toxigenic *Escherichia coli* (STEC) strains can cause morbidity and mortality in infected humans (Paton et al. 2006). Some of these pathogens produce AB_5 toxin or subtilase AB (SubAB) and are responsible for gastrointestinal diseases, including the life-threatening hemolytic uremic syndrome (HUS) (OMIM 235400). During an infection, the bacterial cytotoxin enters human cells by endocytosis, and retrograde transport delivers it to the ER. In the ER, BiP is the major, or possibly the only, target of the catalytic subunit A, which inactivates BiP by a single reaction of limited proteolysis within the linker region (Fig. 9.3). Eventually, most above-outlined BiP functions are lost, and the affected cells die. Therefore, HUS represents an acquired chaperonopathy.

Marinesco-Sjögren syndrome (MSS) (OMIM 248800) is a rare autosomal recessively inherited neurodegenerative disease, which represents an inherited chaperonopathy (Anttonen et al. 2005; Senderek et al. 2005; Howes et al. 2012). The hallmarks of MSS are cerebellar ataxia, cataracts, developmental and mental retardation, and progressive myopathy (Roos et al. 2014). The cause of the disease in the majority of MSS patients has been characterized as a mutation in the *SIL1* gene that results in mutated or truncated Sil1. Sil1 is a nucleotide exchange factor for BiP, and its role is to provide BiP with ATP (Weitzmann et al. 2006). Possibly because of the existence of an alternative NEF for BiP, i.e., Grp170, the loss of Sil1 function results in a reduction rather than a complete loss of BiP function. The possible consequences are as follows: (i) some precursor polypeptides may not be efficiently

transported into the ER, causing them to become accumulated or degraded in the cytosol; (ii) some proteins that are successfully transported into the ER may not be folded or assembled correctly, leading to accumulation of misfolded polypeptides in the ER and ERAD; (iii) some essential secretory or plasma membrane proteins may not reach their functional location, leading to secondary loss of function of BiP substrates; or (iv) Sec61 channel gating to the closed state may be compromised, thus leading to apoptosis (see below). Notably, we suggest that Grp170 deletion is lethal because, in contrast to Sil1, it also acts as a chaperone (Lin et al. 1993; Behnke et al. 2015).

Autosomal dominant polycystic liver disease (PLD) (OMIM 174050) is a rare human inherited disease that is characterized by the progressive development of multiple biliary epithelial liver cysts (Davila et al. 2004). This inherited chaperonopathy usually remains asymptomatic at young ages and manifests between the ages of 40 and 60 years. Liver function is usually preserved. A loss of Sec63 function has been postulated in several genetic mutations. Although no mechanism has been firmly established for PLD, the disease can be explained by a two-hit mechanism: patients with one inherited mutant allele and one wild-type allele may lose the wild-type allele in some liver cells through somatic mutation. A plausible scenario is that Sec63, possibly in collaboration with Sec62 and BiP, is essential for the ER import of a subset of non-essential secretory or plasma membrane proteins that are involved in the control of biliary cell growth or cell polarity. Thus, without functional Sec63, these proteins do not reach the correct location at the cell surface. This view was confirmed by recent results, and it was concluded that the secondary lack of polycystins 1 and 2 results in disrupted cell adhesion and, therefore, cyst formation (Fedeles et al. 2011; Lang et al. 2011). Alternatively to the two-hit scenario, the reduced levels of Sec63 in the presence of one wild-type allele may already be sufficient to cause disrupted cell adhesion. Since Sec63 is involved in Sec61 channel opening for ER protein import, PLD was also defined as Sec61-channelopathy (Haßdenteufel et al. 2014). It is possibly due to the existence of an alternative JDP for ER protein import, i.e., ERj1, that the loss of Sec63 function does not result in a lethal phenotype.

Obviously, however, the archetype Sec61-channelopathies are human diseases with mutations in one or both alleles of one of the three *SEC61* genes that have functional consequences in Sec61 channel gating (Haßdenteufel et al. 2014). Mammalian cells, which are highly active in protein secretion, may be particularly sensitive toward problems in Sec61 channel closure and, therefore, constantly on the verge to apoptosis. This has been seen associated with diabetes mellitus for the ß-cells of the mouse with the homozygous Sec61αY344H exchange and in human patients associated with dominant negative effects in the course of (i) hypogammaglobulinemia or common variable immunodeficiency (CVID) in plasma cells with the Sec61αV85D exchange (Schubert et al. 2018), (ii) tubulointerstitial and glomerulocystic kidney disease (TKD) in kidney cells with the Sec61αV67G or Sec61αT185A exchanges (Bolar et al. 2016), and (iii) neutropenia in neutrophils with the Sec61αV67G or Sec61αQ92R exchanges (Bolar et al. 2016; Van Nieuwenhove et al. 2020). The fact that ERj6 (DNAJC3) is involved

in Sec61 channel closure and that its absence in human patients, too, causes diabetes is in perfect line with this interpretation (Synofzik et al. 2014). However, efficient Sec61 channel closure is clearly not the only problem in the archetype Sec61-channelopathies; reduced ER protein import due to reduced levels of functional Sec61 complexes, i.e., haploinsufficiency, certainly also contributes to the respective disease phenotypes. Why each of these *SEC61A1* mutations affects mainly one or two organs is completely open.

In addition, professional secretory cells appear to be particularly sensitive to imbalances in the Sec62 to Sec63 ratio, which result in over-efficient Sec61 channel closure and, thus, a proliferative and/or migratory advantage that can lead to cancer, e.g., seen after overexpression of *SEC62* in prostate or lung cancer. Due to poor vascularization and the resulting hypoxia and glucose starvation, tumor cells are prone to ER stress and, therefore, UPR (Macario and Conway de Macario 2007; Aridor 2007). In cultured cells, BiP is one of the proteins involved in protecting cancer cells against ER stress-induced apoptosis (Fu et al. 2007). In addition to this general link between BiP and cancer, some of the abovementioned BiP interacting proteins have been connected to certain tumors.

Sec63 is an ER membrane-resident Hsp40 that, together with BiP, plays a role in gating of the Sec61 complex to the open state (Lang et al. 2012; Schäuble et al. 2012). The *SEC63* gene was found among the most frequently mutated genes in cancers that had deficient DNA mismatch repair, such as hereditary nonpolyposis colorectal cancer (HNPCC)-associated malignancies and sporadic cancers with frequent microsatellite instability (Mori et al. 2002; Schulmann et al. 2005). These genetic alterations may be associated with a more or less pronounced loss of Sec63 function and, thus, secondary loss of functions in analogy to the situation in PLD. This alone may contribute to tumorigenesis, or, alternatively, it may result in a non-physiological Sec62-Sec63 ratio (see below). This hypothesis is supported by a study on the gene expression signatures of sporadic colorectal cancers, which recognized the overexpression of *SEC62* as part of a 43-gene cDNA panel that was used for predicting the long-term outcome of colorectal cancer patients (Eschrich et al. 2005), and by the observation that simultaneous SEC*63* and SEC62 overexpression is a common feature of hepatocellular carcinoma (Casper et al. 2021).

Sec62 forms a complex with Sec63 and Sec61 and is also involved in Sec61 channel gating, both to the open and to the closed state (Linxweiler et al. 2013). Gene amplification at chromosome 3q25-q26 commonly occurs in prostate as well as several other cancers (such as cancers of the breast, cervix, head and neck, lung, thyroid, vulva). Mapping the 3q25-q26 amplification and identifying candidate genes with quantitative real-time PCR revealed that the *SEC62* (*TLOC1*) gene had the highest known amplification frequency (50%) in prostate cancer and was found to be upregulated at the mRNA and protein level in all tumors mentioned above (Jung et al. 2006; Greiner et al. 2011a, b; Linxweiler et al. 2012, 2013, 2016, 2017; Wemmert et al. 2016; Bochen et al. 2017; Takacs et al. 2019a, b, c; Körbel et al. 2018), and *SEC62* was characterized as a cancer driver gene (Hagerstrand et al. 2013). Thus, *SEC62* overexpression appears to be associated with a proliferative

and/or migratory advantage for various cancer cells, which may be due to the role of Sec62 in cellular calcium homeostasis, or in recovER-phagy, or in both. Thus, a Sec62-Sec63 imbalance appears to contribute to the development of various human malignancies.

Conclusions and Open Questions

As has been pointed out by many colleagues and on numerous occasions, the ER lumenal Hsp70-type molecular chaperone BiP is a master regulator of ER functions, such as protein import, protein folding and assembly, ER-associated protein degradation (ERAD), ER-phagy, and signal transduction (UPR, lowER) (Pobre et al. 2019). To do so, BiP depends on a whole set of distinct Hsp40-type co-chaperones (nine in humans), the ERjs or ERdjs, two different nucleotide exchange factors, NEFs (Sil1 and Grp170), and a NEF antagonist (MANF) for its various functions. Furthermore, a high ER lumenal calcium concentration and a constant supply of ATP to the ER are required for BiP function. Therefore, it does not come as a surprise that complete loss of BiP function is not compatible with life of human cells and, therefore, associated with a devastating infectious disease, hemolytic uremic syndrome or HUS. Since there is a certain redundancy in the ERjs and NEFs, loss of function of some of them is not necessarily lethal but, nevertheless, severe.

Because of the important possible medical implications, the burning open question in the field is whether this chaperone system can disaggregate protein aggregates, which are potentially toxic for cells and organisms (Bertolotti 2018; Hipp et al. 2019). Interestingly, a first study addressed the question if ß-sheet proteins, which are prone to form amyloid-like fibrils and undergo toxic aggregation in the cytosol, can do so within the ER of human cells (Vincenz-Donnelly et al. 2018). The observation was that toxicity was strongly reduced in the ER, even though the amyloidogenic ß-sheet protein reached very high concentrations. The protein was found to form soluble oligomers that were retained in the ER and to interact with several chaperones that were discussed here (such as BiP, CNX, Grp94, and Grp170). It would be interesting to determine if siRNA-mediated depletion of one or combinations of ERjs would allow toxic aggregation of the amyloidogenic ß-sheet protein in this model system.

However, the ER cannot prevent protein aggregation in general. Protein aggregation was found to be the reason for the severe form of α1-antitrypsin (A1AT) deficiency (Wu et al. 1994, Carrel and Lomas 2002). Briefly, mutations in the SERPINA1 gene, which codes for A1AT, can cause lung or liver disease in humans, i.e., emphysema or cirrhosis (OMIM 107400) (Bell and Carroll 1973). The first or late-onset form of the disease is more common and results from low levels of the elastase inhibitor in the blood plasma (age 20–50 years), and the second or early-onset form is the result of accumulation and aggregation of the mutant variants in the ER of liver cells, which interferes with the biogenesis of other proteins and, in general, full ER function. Children with the homozygous mutation p.E342K (the

Z-variant) can develop severe liver disease and liver failure, which was attributed to additional genetic factors, such as mutations in genes that code for ERAD components. Currently, autophagy-enhancing drugs are discussed as promising candidates for future treatment (Hidvegi et al. 2010). Based on the observation that mutant A1AT was found in association with BiP, Grp94, and Grp170 (Schmidt and Perlmutter 2005), consideration of chaperone-based alternatives or combinatorial therapeutic strategies seems to be warranted.

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Chapter 10
J-Domain Proteins Orchestrate the Multifunctionality of Hsp70s in Mitochondria: Insights from Mechanistic and Evolutionary Analyses

Jaroslaw Marszalek, Elizabeth A. Craig, and Bartlomiej Tomiczek

Abstract Mitochondrial J-domain protein (JDP) co-chaperones orchestrate the function of their Hsp70 chaperone partner(s) in critical organellar processes that are essential for cell function. These include folding, refolding, and import of mitochondrial proteins, maintenance of mitochondrial DNA, and biogenesis of iron-sulfur cluster(s) (FeS), prosthetic groups needed for function of mitochondrial and cytosolic proteins. Consistent with the organelle's endosymbiotic origin, mitochondrial Hsp70 and the JDPs' functioning in protein folding and FeS biogenesis clearly descended from bacteria, while the origin of the JDP involved in protein import is less evident. Regardless of their origin, all mitochondrial JDP/Hsp70 systems evolved unique features that allowed them to perform mitochondria-specific functions. Their modes of functional diversification and specialization illustrate the versatility of JDP/Hsp70 systems and inform our understanding of system functioning in other cellular compartments.

Keywords Molecular chaperone · Co-chaperones · Protein folding · Protein translocation · Iron-sulfur clusters · Protein evolution · Mitochondrial matrix

Introduction

Mitochondria are essential double-membrane organelles, divided into four sub-compartments: the outer membrane (OM), the intermembrane space (IMS), the inner membrane (IM), and the matrix. They are best known for their role in the

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energetics of eukaryotic cells, as they produce the majority of cellular ATP. However, mitochondria are also a major hub of cellular metabolism, including the biosynthesis of amino acids, lipids, heme, and iron-sulfur clusters (FeS) (Neupert 2016; Friedman and Nunnari 2014), and play important roles in cellular signaling pathways, programmed cell death, and protein quality control (Ruan et al. 2017; Pfanner et al. 2019). Not surprisingly, defects in mitochondrial functions lead to many diseases involving the nervous system, muscles, heart, and other organs and tissues (Russell et al. 2020; Nicolas et al. 2019; Palmer et al. 2020). The maintenance of healthy and metabolically active mitochondria requires action of molecular chaperones, including the most versatile of them—J-domain protein (JDP)/Hsp70 systems acting in the matrix (Srivastava et al. 2019; Vazquez-Calvo et al. 2020; Song et al. 2021).

Mitochondria originated from an endosymbiotic alphaproteobacterium engulfed by a primordial eukaryotic host, most likely related to the recently discovered Asgard archaea (Roger et al. 2017; Martijn et al. 2018; Zaremba-Niedzwiedzka et al. 2017). Appreciating their endosymbiotic origin is a key to understanding the cellular status and function of mitochondria, as major structural and functional rearrangements took place during transition from endosymbiont to organelle (Youle 2019). For example, transfer of genes encoding most mitochondrial proteins into the host genome enforced evolution of molecular machines necessary for import of proteins into mitochondria and their subsequent proper folding (Hansen and Herrmann 2019; Wiedemann and Pfanner 2017; Sloan et al. 2018; Craig 2018). Meanwhile, preservation of remnants of the endosymbiont genome in the form of mitochondrial DNA influenced the evolution of protein machines responsible for maintenance, propagation, and expression of mtDNA (Gilkerson et al. 2013; Neupert 2016). As mitochondria became a source of cofactors (e.g., FeS) needed for other cellular compartments, protein machines required for their biosynthesis and export have evolved (Dutkiewicz and Nowak 2018; Lill and Freibert 2020; Maio and Rouault 2022). In many cases, the functioning, assembly, and maintenance of these mitochondrial machines require participation of JDP/Hsp70 molecular chaperone systems (Srivastava et al. 2019).

In most mitochondria, a single Hsp70, mtHsp70, is involved in multiple processes, each orchestrated by a specific JDP. One, termed Mdj1 in *Saccharomyces cerevisiae* and Tid1 (DNAJA3) in humans, is a true jack of all trades. Mdj1/Tid1 is involved in folding of mitochondrial proteins (both those imported from the cytosol and those synthesized in mitochondria), as well as in protein refolding after stress. Mdj1/Tid1 also functions in maintenance and propagation of mtDNA. In mammals, including humans, Tid1 is implicated in many diseases, including cancer, cardiomyopathy, and neurodegeneration (Srivastava et al. 2019; Iosefson and Azem 2012). A more specialized JDP, termed Pam18 in yeast and DNAJC15/DNAJC19 in humans, is a key component of the protein import motor, enabling mtHsp70 binding to a polypeptide chain coming through the import channel of the inner membrane, into the matrix. A highly specialized JDP termed Hsc20 functions in the biogenesis of iron-sulfur clusters, enabling efficient FeS transfer onto recipient proteins. These four components were inherited from the bacterial ancestor of mitochondria. While,

Mdj1, Hsc20, and mtHsp70 are descendants of their functionally equivalent orthologs, Pam18 is most likely related to a bacterial JDP of unknown function—not surprisingly as the process of protein import to mitochondria evolved in the eukaryotic cell.

Yet, all JDP/mtHsp70 systems have diverged functionally from their bacterial ancestors, each evolving uniquely mitochondrial features and activities. However, one critical characteristic is shared, not only among these mitochondrial systems but also with other JDP/Hsp70 systems. Their participation in various functions is based on a single biochemical mechanism—the ability of Hsp70 to cyclically interact with a client polypeptide. This client-binding cycle is dependent on the ATPase activity of Hsp70, which is regulated by interaction with JDP and client.

In this chapter, we provide a current state of knowledge about the three mitochondrial JDP/mtHsp70 systems with a focus on mechanistic aspects of their function, particularly the client-binding cycle. Because a combination of mechanistic and evolutionary insights can be productively leveraged to understand molecular and functional properties, we also discuss the evolutionary history of these systems. Throughout, *S. cerevisiae* nomenclature is used as most detailed mechanistic studies have been performed using this organism.

The Mitochondrial Hsp70 - Client Polypeptide Binding Cycle

The structure of mtHsp70 is very similar to that of other members of the Hsp70 protein family. It consists of two domains: a ~ 44-kDa N-terminal nucleotide-binding domain (NBD) hydrolyses bound ATP to ADP and Pi, and a ~ 26-kDa C-terminal substrate-binding domain (SBD) consists of two subdomains (Liu et al. 2020; Mayer and Gierasch 2019; Clerico et al. 2019; Rosenzweig et al. 2019). The β-sandwich subdomain (SBD-β) contains the peptide-binding cleft, which contacts five to seven residue segments of client polypeptides that are overall hydrophobic in nature (Clerico et al. 2015, 2021). Almost every unfolded protein has multiple Hsp70 binding sites, because residues that, in the native conformation, constitute the hydrophobic core are exposed when unfolded. Evidence suggests that most proteins have an Hsp70 binding site every 30–40 residues (Rüdiger et al. 1997). SBD-β's interaction with client polypeptide is stabilized by the second subdomain—α-helical subdomain (SBD-α). SBD-α can fold back upon SBD-β, forming a "lid" over the peptide-binding pocket (Liu et al. 2020; Mayer and Gierasch 2019). The NBD and SBD are connected by a flexible, evolutionary conserved, linker which plays an important role in modulating interactions between domains (Clerico et al. 2019).

The client-binding/release cycle is controlled by the conformational state of Hsp70, which depends on ATP binding and hydrolysis (Fig. 10.1). In the ATP-bound state, the SBD and the interdomain linker are docked onto the NBD, restraining the α-helical lid and SBD-β such that the peptide-binding cleft is accessible to the client polypeptide. This "open" conformation allows for high on- and off- rates

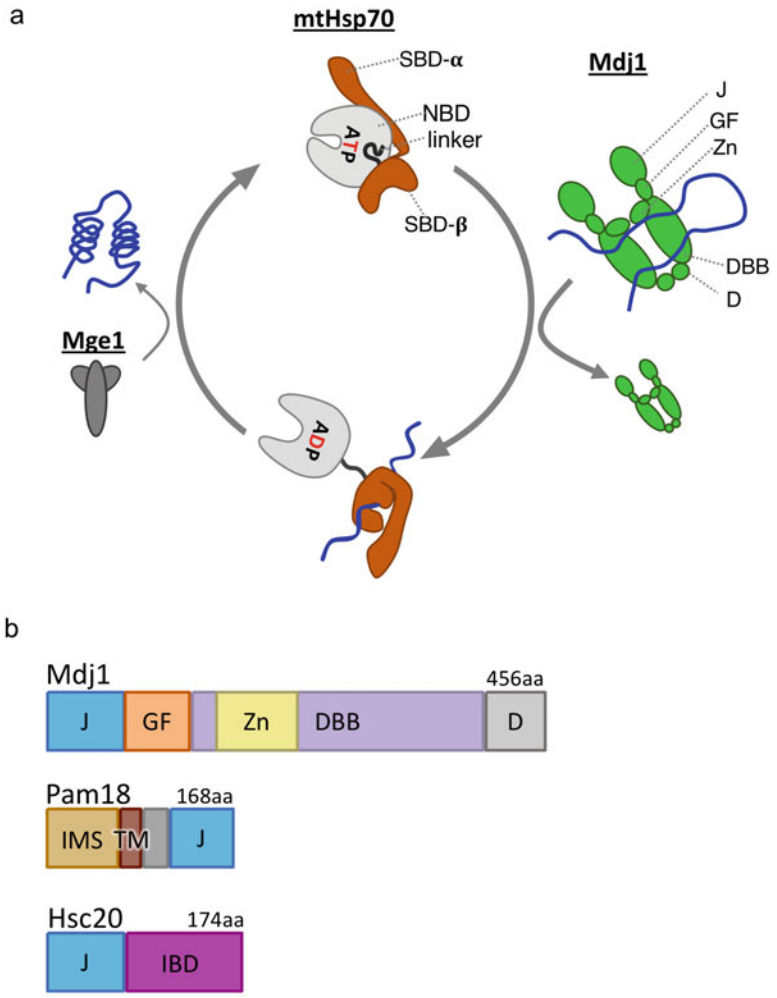

Fig. 10.1 The Hsp70 client-binding cycle illustrated by mtHsp70 interaction with JDP co-chaperone Mdj1 and nucleotide exchange factor Mge1. (**a**) Kinetics of Hsp70 interaction with a client polypeptide (blue) is driven by ATP binding and hydrolysis, followed by exchange of ADP for ATP. Hsp70 has two domains: a nucleotide-binding domain (NBD, gray), which binds and hydrolyzes ATP, and a substrate-binding domain (SBD, brown), which binds client polypeptide. SBD is composed of two subdomains: SBD-β contains a peptide-binding cleft; SBD-α constitutes a "lid" that can cover the peptide-binding cleft (bottom). When ATP is bound, Hsp70 is in the "open" or "docked" conformation (top). In this conformation, SBD-β, SBD-α, and the linker are docked to NBD, and the peptide-binding cleft is exposed resulting in high on and off rates of client binding. In the ADP bound conformation (bottom) NBD and SBD are undocked (connected only by a flexible linker); the lid, covers the peptide-binding cleft stabilizing client interaction. Mdj1 co-chaperone delivers client to mtHsp70 and stimulates its ATPase activity via the J-domain, which interacts with mtHsp70. Once substrate is delivered and ATP hydrolyzed to ADP, Mdj1 is released from the mtHsp70-client complex. Nucleotide exchange factor (NEF) Mge1 interacts with ADP-bound Hsp70 and facilitates the release of ADP and its exchange for ATP. Once mtHsp70 is back in the ATP-bound open conformation, client is released, completing the cycle. (**b**) Domain composition of the three JDPs functioning in *S. cerevisiae* mitochondrial matrix. Mdj1 is composed of five

of client binding. Hydrolysis of bound ATP to ADP triggers dramatic conformational changes. The SBD is released from the NBD leaving the two domains connected only by the extended linker. Unrestrained, the lid covers the peptide-binding cleft. Because most Hsp70s, including mtHsp70, bind adenine nucleotide with high affinity, a nucleotide exchange factor (NEF), which stimulates the release of ADP, is required to complete the cycle. Upon rebinding of ATP and thus transition of Hsp70 into the "open" conformation, the client is released, allowing the start of a new binding cycle. Mge1 (called GrpE in human cells) is the only NEF present in the mitochondrial matrix that functions with mtHsp70 (Nakai et al. 1994; Laloraya et al. 1994).

ATP hydrolysis is key to this cycle of mtHsp70-client interaction. Because Hsp70 has a very low intrinsic ATPase activity, a JDP co-chaperone, which stimulates ATPase activity, is required for cycle progression. JDPs are defined by the presence of a "J-domain" (Kampinga and Craig 2010; Craig and Marszalek 2017; Kampinga et al. 2019). This conserved domain consists of four α-helices, with helices 2 and 3 forming a fingerlike structure connected by a loop region. This loop invariantly contains a His-Pro-Asp (HPD) tripeptide, critical for stimulation its Hsp70 partner's ATPase. J-domains bind ATP-bound Hsp70. Residues of helices 2 and 3, as well as the HPD tripeptide, bind at the NBD/SBD-β/linker interface formed by Hsp70 in the "open," ATP-bound, conformation (Kityk et al. 2018; Tomiczek et al. 2020). Domain composition of JDPs is highly variable outside the J-domain. Many JDPs have domains that bind client (e.g., Mdj1 and Hsc20), thus allowing them to deliver client to Hsp70 for binding, while others have domains that anchor them to a particular cellular location (e.g., Pam18), increasing their local concentration for efficient interaction with their partner Hsp70 (Kampinga and Craig 2010; Craig and Marszalek 2017).

While obligatory, JDPs on their own stimulate Hsp70's ATPase activity only modestly. Hsp70 interaction with client, in the absence of a JDP, also only moderately stimulates the ATPase rate. It is the synchronized interaction of a J-domain and client polypeptide that results in rapid hydrolysis, fast conformational transition, and stable client binding. Interestingly, under such conditions, Hsp70 displays ultrahigh affinity for client, which is significantly higher than the affinities measured under the steady-state conditions for either ATP- or ADP-bound Hsp70 (Barducci and De Los Rios 2015). This phenomenon of ultra-affinity enables Hsp70s to interact with a

Fig. 10.1 (continued) domains. The N-terminal J-domain (J) interacts with mtHsp70 and stimulates its ATPase activity. Glycine-phenylalanine-rich region (GF) connects J-domain with a double β-barrel domain (DBB), which contains a zinc-binding domain (Zn). DBB domain binds client independently from Hsp70. Mdj1 functions as a dimer due to the C-terminal dimerization domain (D). Pam18 contains three domains, an N-terminal intermembrane space (IMS) domain, which interacts with the translocon; a transmembrane (TM) domain; which anchors it in the inner membrane (IM); and a C-terminal J-domain, which not only stimulates the ATPase of mtHsp70 but also interacts with the J-like domain of Pam16 (see Fig. 10.2). Mdj2 which has a similar architecture to Pam18, minus the IMS domain, is not shown. Hsc20 has just two domains, an N-terminal J-domain and a C-terminal Isu binding domain

wide range of clients, even those that do not have a "perfect" binding site. In summary, the client-binding cycle regulated by mitochondrial JDPs and NEF Mge1 enables mtHsp70 to function in processes that at the first glance have little in common—from protein folding and import to biogenesis of iron sulfur clusters (Rosenzweig et al. 2019; Liu et al. 2020; Clerico et al. 2019; Mayer and Gierasch 2019).

Mdj1: Multifunctional JDP that Evolved Mitochondria-Specific Functions

Mdj1/Tid1 is a multifunctional JDP, which partners with mtHsp70 and NEF Mge1. Both phylogenetic and functional evidence suggest that the entire Mdj1/mtHsp70/Mge1 chaperone system was inherited from the bacterial ancestor of mitochondria (Rowley et al. 1994; Deloche et al. 1997; Lisse and Schwarz 2000). This is not surprising, considering its important functions and ubiquitous presence in bacteria. A recent survey of 1709 bacterial genomes, spanning all major taxonomic groups, revealed that Mdj1 ortholog DnaJ and its Hsp70 partner DnaK are present in 99.9% of surveyed species (Barriot et al. 2020). DnaJ is the prototypical JDP—the first discovered. Its orthologs are classified as class I JDPs (Kampinga and Craig 2010). All, including Mdj1/Tid1, have an N-terminal J-domain, followed by a region rich in glycine and phenylalanine (i.e., a G/F region) and a double β-barrel segment with two structurally similar subdomains called CTD I and CTD II, a zinc-binding domain with four CxxCxGxG motifs extruding from CTD I (Fig. 10.1b). Class I JDPs function as dimers, formed via the C-terminal dimerization domain. While it is clear that the double β-barrel segment binds client polypeptide, the mode of binding and the role of the zinc-binding domain are not yet completely clear (Jiang et al. 2019). Also, the role of the G/F region in client-binding and/or progression of the ATPase cycle is not well understood.

In yeast, the Mdj1/mtHsp70(Ssc1)/Mge1 system performs functions similar to its bacterial DnaJ/DnaK/GrpE counterpart. In some cases, individual components can be exchanged between these systems, with only modest effects on functionality (Deloche et al. 1997; Lisse and Schwarz 2000), consistent with their evolutionary relatedness. Native mitochondrial proteins—both those encoded by mtDNA and synthesized on mitochondrial ribosomes and those imported from the cytosol—have been identified as clients requiring the Mdj1/Ssc1/Mge1 for their de novo folding (Westermann et al. 1996). Var1, a subunit of the mitochondrial ribosome encoded by mtDNA, has been studied in particular detail. Mdj1 and Ssc1 associate with nascent Var1; in the absence of functional Mdj1, Var1 readily aggregates (Westermann et al. 1996). In vivo, Mdj1 has also been found to be associated with native precursor proteins at the late stage of their import into the matrix, implicating the system in folding of newly imported proteins. Consistent with this notion, studies involving

import of model proteins into isolated mitochondria revealed a critical role of the Mdj1/Ssc1/Mge1 system in their productive folding (Liu et al. 2001).

The Mdj1/mtHsp70(Ssc1)/Mge1 system has also been shown to be involved in protection of mitochondrial proteins against heat-induced protein aggregation. For example, Mdj1 binds the imported thermolabile tester protein, firefly luciferase, thereby preventing its heat-induced aggregation (Prip-Buus et al. 1996). The system is also required for reactivation of thermally inactivated mitochondrial DNA polymerase Mip1, under both in vivo and in vitro experimental conditions (Duchniewicz et al. 1999; Germaniuk et al. 2002). However, despite the major role of the Mdj1/Ssc1/Mge1 system in both de novo folding and protection against heat-induced aggregation, it should be kept in mind that Mdj1/Ssc1/Mge1 does not function in isolation. Rather, it collaborates with other chaperone systems of the mitochondrial matrix. Mip1 serves as an example—reactivation requires collaboration with Hsp78 disaggregase, a mitochondrial homolog of bacterial ClpB belonging to the AAA+ protein family (Lewandowska et al. 2006).

Biochemical and biophysical studies, carried out in parallel to the in vivo/in organellar studies described above, indicated that Mdj1/Ssc1/Mge1 system functions in protein folding/refolding utilizing the canonical Hsp70 client-binding cycle—beginning with Mdj1/Tid1 binding a client protein, then delivering it to Ssc1/mtHsp70, and stimulating its ATPase activity—thus facilitate client capture; Mge1/GrpE then completes the cycle, initiating exchange of ADP to ATP, leading to the release of the client from Ssc1/mtHsp70 (Fig. 10.1) (Kubo et al. 1999; Liu et al. 2001; Iosefson et al. 2012). More recently, studies involving single-molecule Forster resonance energy transfer (FRET) revealed unprecedented details about the conformational dynamics of Ssc1 in the presence of Mdj1 and client peptide (P5) (Mapa et al. 2010; Sikor et al. 2013). These studies demonstrated that in the presence of Mdj1 and P5, fast conformational transition from the ATP-bound open state to the ADP-bound closed state, with fully undocked NBD and SBD domains and lid covering the client peptide, takes place upon nucleotide hydrolysis. Moreover, Mdj1 is released from the Ssc1-Mdj1-P5 complex once ATP is hydrolyzed to ADP and a stable Ssc1-P5 complex is formed.

While the Mdj1/mtHsp70(Ssc1)/Mge1 and bacterial system perform many similar cellular functions, one function sets the mitochondrial system apart—a direct role in the maintenance and propagation of the mitochondrial genome (Rowley et al. 1994). mtDNA most likely descended from the bacterial genome of the ancestral endosymbiont, but its current gene content is very limited, encoding only a small number of proteins (7 in yeast/13 in humans), as well as rRNA and a set of tRNAs (Roger et al. 2017). Despite the limited gene content, however, faithful maintenance and replication of mtDNA is critical for mitochondrial function (Gilkerson et al. 2013). A nucleoprotein complex termed the mitochondrial nucleoid is responsible for a wide range of mtDNA transactions required for propagation, expression, and maintenance of the mitochondrial genome (Gustafsson et al. 2016). Mdj1 and Ssc1 are among the many protein components of the mitochondrial nucleoid complex (Hensen et al. 2014; Miyakawa 2017; Bogenhagen 2012).

A strong link between Mdj1/mtHsp70(Ssc1) at the nucleoid and their involvement in the maintenance of mtDNA has been established in *S. cerevisiae*. Deletion or inactivation of Mdj1 results in loss of functional mtDNA even at optimal growth temperature (Duchniewicz et al. 1999), indicating that involvement of Mdj1 is not restricted to its role in reactivation of thermally inactivated mitochondrial DNA polymerase Mip1 (Germaniuk et al. 2002). Mdj1 binds mtDNA directly, and mutants defective in binding lose mtDNA, indicating that localization of Mdj1 to the nucleoid is critical for mtDNA maintenance (Ciesielski et al. 2013). However, binding to the nucleoid is not sufficient, as J-domain mutants defective in the Ssc1 interaction also lose mtDNA. Thus, both nucleoid localization of Mdj1 and its functional partnership with Ssc1 are required for mtDNA maintenance in yeast.

A link between Mdj1 and mtDNA maintenance has also been found in trypanosomes and human cells, suggesting that this function is evolutionary conserved. In *Trypanosoma brucei*, depletion of both mtHsp70 and the ortholog of Mdj1, mtHsp40, has been reported to impair replication of mtDNA (Týč et al. 2015). The human Mdj1 ortholog Tid1 has been found to be associated with the mitochondrial nucleoid and thus implicated in maintenance of mtDNA as well (Lu et al. 2006; Hensen et al. 2014). However, the molecular mechanism(s) behind Mdj1/mtHsp70 (Ssc1)/Mge1 function in maintenance of mtDNA remains to be elucidated. Mdj1's ability to directly interact with mtDNA suggests it may act as an "anchor," attracting Hsp70 and Mge1 to the mitochondrial nucleoid. What functions are executed by the Mdj1/mtHsp70(Ssc1)/Mge1 system within the nucleoid complex is not clear. One obvious possibility is that Mdj1/mtHsp70 system is involved in chaperoning specific protein(s) resident in the nucleoid complex. But other possibilities are suggested by the involvement of JDP/Hsp70 systems in replication of viral and plasmid DNAs (Konieczny and Zylicz 1999). For example, DNA replication of bacteriophage lambda depends on the bacterial host DnaJ/DnaK/GrpE system, which activates the replication process by remodeling multiprotein complex associated at the *ori* sequence of lambda DNA (Zylicz et al. 1989). It is important to stress that this remodeling requires DnaK's ATPase activity and is based on the cyclic interaction with a client protein.

A growing body of published data indicates that both Tid1 and mtHsp70 also have functions outside mitochondria related to signaling pathways involved in apoptosis, cell proliferation, and cell survival in mammals (Srivastava et al. 2019; Iosefson and Azem 2012). Tid1 is also implicated in the neuromuscular synapse formation (Song and Balice-Gordon 2008). These findings are very exciting but difficult to interpret mechanistically at this point in time as they have yet to be investigated with an eye toward addressing the question of involvement of a JDP/NEF-driven Hsp70-client interaction cycle. All studies of mitochondrial localized systems to date have pointed to the importance of the cycle in both protein folding/refolding and maintenance of mtDNA. Although it is possible that Tid1 and mtHsp70 have functions not dependent on the ATPase cycle, detailed mechanistic studies are needed to verify this notion.

Pam18: Specialized JDP that Functions in Protein Import

Pam18, also called Tim14, is the JDP of the Hsp70-based "import motor," critical for movement of proteins across the mitochondrial inner membrane into the matrix (Fig. 10.2) (Craig 2018; Hansen and Herrmann 2019; Wiedemann and Pfanner 2017; Mokranjac 2020). Unfolded polypeptides pass through a translocon formed by two integral membrane proteins, Tim17 and Tim23. The membrane potential (negative in the matrix compared to the intermembrane space) drives the positively charged targeting sequence across the membrane, but the motor localized on the matrix face of the inner membrane is required for transfer of the bulk of the protein. The fundamental mechanism behind the import motor function has been debated for decades – that is, whether "simply" Brownian motion or a "pulling force" driven by conformational changes in the motor (i.e., Hsp70) is responsible for movement. A proposed extension of the Brownian motion model has been gaining acceptance. This "entropic pulling" model posits that the necessary force is generated after the Hsp70-translocating polypeptide interaction occurs – by the restriction placed on movement of Hsp70 by the close proximity of the membrane/translocon (De Los Rios et al. 2006; De Los Rios and Goloubinoff 2016; Sousa and Lafer 2019). But regardless of the exact mechanism behind the force driving movement, the importance of multiple, sequential interactions of the translocating polypeptide in the

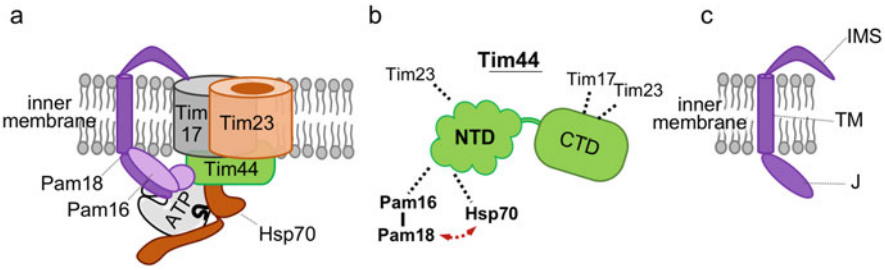

Fig. 10.2 The mtHsp70-based import motor. (**a**) The translocon through which proteins destined for the matrix pass is composed of two related integral membrane proteins, Tim17 and Tim23, with the later forming the channel. The membrane potential drives the targeting sequence across the membrane, but the remainder of the protein requires the action of mtHsp70. mtHsp70 associates with Tim44 when in its ATP-bound conformation, with the SBD-cleft easily accessible for binding an incoming polypeptide. The NEF Mge1 is not shown, but is necessary for nucleotide exchange after ATP hydrolysis. The sequential action of cycles of Hsp70 interaction drives the translocation of proteins from the IMS through the channel into the matrix. Tim44 acts as a hub—interacting with both mtHsp70(ATP) and Pam16 and the channel itself. Due to the Pam16-Pam18 interaction, Pam18's J-domain is in close proximity to Hsp70 allowing efficient ATPase stimulation. Pam18 also has a transmembrane domain and an intermembrane space (IMS) domain that interacts with Tim17. (**b**) Cartoon of Tim44. Tim44 has two domains: an intrinsically disordered N-terminal domain (NTD) and a structured C-terminal domain (CTD). Proteins that interact with each domain are indicated (dotted black lines), with the interaction of the J-domain of Pam18 with Hsp70 indicated by dotted red arrow. (**c**) Cartoon of Pam18. Pam18 has three domains: N-terminal intermembrane space (IMS) domain, transmembrane (TM) domain, and C-terminal J-domain (J)

peptide-binding cleft of Ssc1 are unquestioned. That this role of Ssc1 requires the canonical client interaction cycle requiring J-domain stimulation of ATPase activity and facilitated nucleotide exchange is also clear.

Not surprisingly, Pam18, like mtHsp70(Ssc1) and Mge1, is essential—as import of nuclear encoded proteins into the mitochondrial matrix is required for cell viability. Two other motor components, Tim44 and Pam16 (Tim16), are essential as well. Tim44 is the motor's hub, serving as the "connector" between the translocon and the motor (Fig. 10.2) (Larburu et al. 2020; Craig 2018). It is a modular protein, directly interacting with the translocon via its C-terminal structured domain and with the targeting sequence of the translocating polypeptide, Ssc1 and Pam16, as well as the translocon, via its intrinsically disordered N-terminus (Ting et al. 2014, 2017; Banerjee et al. 2015; Demishtein-Zohary et al. 2017). Considering the focus of this chapter, Pam16 is of particular interest. A segment of Pam16 is related to a J-domain in structure and sequence but lacks an HPD and does not stimulate Hsp70's ATPase activity. However, its J-like domain interacts with Pam18's J-domain forming a stable heterodimer (Mokranjac et al. 2005). This Pam18-Pam16 interaction positions Pam18 near Ssc1, likely via Pam16's interaction with Tim44 (Ting et al. 2017; D'Silva et al. 2008). It has been proposed that this interaction plays a role beyond simple positioning, that is, the dimer interface serves a regulatory function—but strong experimental support has yet to be obtained for this appealing idea (Pais et al. 2011; Mokranjac 2020). Regardless of the mechanistic functional details, Pam16 and Pam18 serve as examples of the evolution of specialized function of JDPs and of noncanonical functions.

It should also be noted that Pam18 is not simply a J-domain. The C-terminal J-domain is preceded by a single transmembrane domain. In addition, Pam18, though not most of its orthologs, also has an N-terminal intermembrane space segment, which interacts with Tim17 and, along with the Pam16 interaction, stabilizes its interaction with the translocon/motor (Fig. 10.1b/Fig. 10.2) (Frazier et al. 2004). Recent evidence indicates that the transmembrane segment also plays a role, interacting with the translocon and reducing lateral movement of translocating polypeptides into the membrane, thereby promoting movement into the matrix (Schendzielorz et al. 2018). In sum, the matrix Hsp70-based import motor is a finely tuned machine intimately integrated with other components of the inner membrane translocation apparatus.

Hsc20: Specialized JDP that Functions in Iron-Sulfur Cluster Biogenesis

One of the essential functions of mitochondria, which is dependent on the JDP/Hsp70 client-binding cycle, is the biogenesis of iron-sulfur clusters—ancient protein cofactors required for activity and/or structural stability of many proteins that function in mitochondria and other subcellular compartments (Lill and Freibert

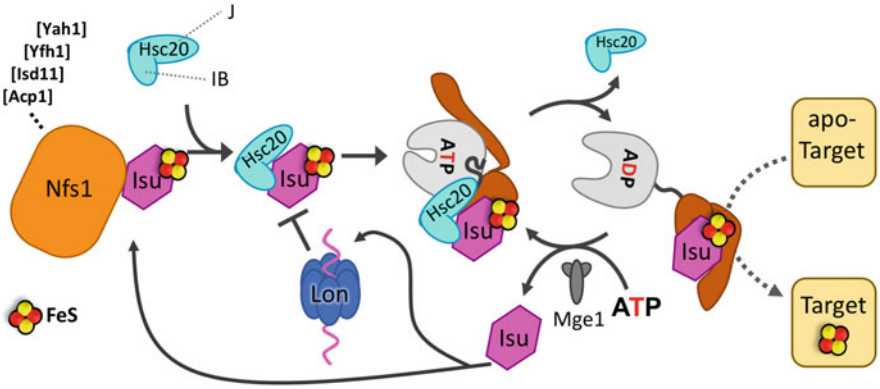

Fig. 10.3 The role of Hsc20/mtHsp70 system in mitochondrial biogenesis of iron-sulfur clusters (FeS). The cluster (FeS) is biosynthesized on a dedicated scaffold protein (Isu, pink) via cysteine desulfurase complex composed of Nfs1 (orange) and accessory proteins that interact with it (listed). Hsc20 (cyan), composed of an N-terminal J-domain (J) and a C-terminal Isu binding domain (IB), binds FeS-loaded Isu and delivers it to mtHsp70. Isu interaction with Hsc20 protects it against Lon (dark blue)-dependent proteolysis. Once the Hsc20-Isu complex interacts with ATP-bound Hsp70, its ATPase activity is stimulated triggering conformational changes; Hsc20 is released, and the Isu-Hsp70 interaction is stabilized. The cycle is completed by ADP/ATP exchange facilitated by Mge1, leading to release of Isu. FeS is transferred onto the target apo-protein during the ATPase-dependent step; the molecular mechanism underlying the FeS transfer is unknown

2020; Kim et al. 2015; Maio and Rouault 2020; Dutkiewicz and Nowak 2018; Srour et al. 2020). FeS proteins catalyze a variety of enzymatic reactions, function in electron transfer, act as regulatory factors detecting oxygen and iron, and are involved in the biogenesis of ribosomes and repair/replication of DNA (Rouault 2019). Among the three alternative FeS biosynthesis pathways present in bacteria, only one, ISC (iron-sulfur cluster), was inherited by mitochondria from their alphaproteobacterial ancestors (Roche et al. 2013). In the ISC pathway, FeS is first biosynthesized on a dedicated scaffold protein termed Isu and then transferred onto recipient proteins. The transfer requires Hsc20 (often called Jac1 in *S. cerevisiae*), its mtHsp70 partner, and NEF Mge1 (Fig. 10.3) (Dutkiewicz and Nowak 2018; Maio and Rouault 2022).

Hsc20 is one of the simplest and most specialized JDPs. It consists of just two domains: an N-terminal J-domain and a C-terminal Isu binding domain (Fig. 10.1b) (Vickery and Cupp-Vickery 2007; Ciesielski et al. 2012). No client proteins other than Isu have been identified. Similar to many other JDPs, Hsc20 interacts with Isu independently from its Hsp70 partner (Craig and Marszalek 2017). The interaction is unusual, in that it involves a large interface composed of evolutionary conserved hydrophobic and charged residues (Füzéry et al. 2008; Kim et al. 2009; Majewska et al. 2013; Ciesielski et al. 2012). Biochemical experiments revealed that the hydrophobic interactions contribute greatly to Hsc20-Isu complex stability (Füzéry et al. 2011; Ciesielski et al. 2012). Studies in *S. cerevisiae* demonstrated that the Hsc20/Isu interaction is critical in vivo. When either Hsc20 or Isu are severely

defective in this interaction, cells are inviable; when partially defective, cells grow slowly and have reduced activities of mitochondrial enzymes requiring FeS for catalysis (e.g., aconitase and succinate dehydrogenase).

Evidence indicates that formation of the Isu-Hsp70 complex, a reaction dependent on Isu delivery by Hsc20, is critical for FeS transfer from Isu onto recipient apo-proteins. This is a robust finding, as it is based on the analysis of three different Hsc20/Hsp70 systems. In most eukaryotic species, including humans, Hsc20 delivers Isu to multifunctional mtHsp70. However, in *S. cerevisiae* and closely related fungal species, mitochondria contain a duplicate of mtHsp70, termed Ssq1, which is specialized in FeS biogenesis (Schilke et al. 2006). Depletion of either Ssq1 or Hsc20 results in cluster accumulation on the Isu scaffold and reduced activities of mitochondrial enzymes that require FeS for catalysis, indicating that chaperones are required for FeS transfer, but not for cluster assembly on Isu (Mühlenhoff et al. 2003; Dutkiewicz et al. 2006; Uhrigshardt et al. 2010; Dong et al. 2017). Moreover, biochemical experiments involving the bacterial system, Hsc20 (HscB) and its Hsp70 partner (HscA), revealed that FeS transfer is accelerated in the presence of chaperones. Consistent with the canonical JDP/Hsp70 client-binding cycle, this acceleration is strictly dependent on ATP hydrolysis (Chandramouli and Johnson 2006; Bonomi et al. 2008, 2011).

The mechanism of Isu binding by Hsp70s involved in FeS biogenesis (mtHsp70, Ssq1, HscA) is conserved. All such Hsp70s interact with a single binding site on Isu, the LPPVK pentapeptide, localized on a flexible loop in close proximity to the conserved cysteine residues that coordinate a 2Fe-2S cluster (Hoff et al. 2003; Dutkiewicz et al. 2004; Vickery and Cupp-Vickery 2007; Schilke et al. 2006). Evidence suggests that the molecular mechanism of LPPVK binding is the same as that used in a typical Hsp70/client interaction (Clerico et al. 2015), involving conserved hydrophobic residues localized inside the peptide-binding cleft of the SBD-β subdomain (Knieszner et al. 2005). In fact, the LPPVK sequence is very similar to the consensus peptide sequence recognized by bacterial DnaK and other Hsp70s (Rüdiger et al. 1997; Clerico et al. 2015).

However, while it is clear that Isu is a client for the Hsc20/mtHsp70 system and that the formation of the mtHsp70-Isu complex is directly responsible for FeS transfer from Isu onto a recipient protein (Lill and Freibert 2020; Srivastava et al. 2019; Maio and Rouault 2020), the exact molecular mechanism underlying the transfer is not yet well understood. The prevalent model in the field proposes that Hsp70 binding results in a significant rearrangement of Isu structure such that it can no longer coordinate cluster and thus fosters cluster transfer (Bonomi et al. 2011). Such a mechanism would be consistent with the known ability of Hsp70 chaperones to remodel protein structures [e.g., during disassembly of clathrin-coated vesicles or amyloid disaggregation (De Los Rios and Goloubinoff 2016; Wentink et al. 2020)]. An alternative, less canonical, model is that Hsp70's role is "simply" to remove Hsc20 from Isu, as it was demonstrated that Isu-Hsc20 complex formation inhibits FeS transfer in the absence of Hsp70 (Iametti et al. 2015). According to this scenario, release of Hsc20 from Isu would be sufficient to initiate cluster transfer, either from the Hsp70-Isu complex or from free Isu released from Hsp70 upon ADP/ATP

exchange. Regardless of which idea is correct, Hsc20 and its interaction with FeS loaded Isu are critical for FeS transfer. Further experimental studies are obviously needed.

It is also important to note that the interaction of Hsc20 and Isu has consequences in the overall FeS biogenesis process other than cluster transfer. Residues of Isu involved in this interaction are also critical for its interaction with cysteine desulfurase Nfs1, an enzyme that provides sulfur for FeS assembly on Isu (Majewska et al. 2013) (Fig. 10.3). This overlap raises the intriguing possibility that the mutually exclusive binding of two proteins required for sequential steps in FeS biogenesis plays a regulatory role—driving the transition from FeS assembly to transfer. Moreover, Hsc20 and Nfs1 interaction protects Isu from proteolytic degradation by mitochondrial Lon protease (Pim1 in *S. cerevisiae*) (Ciesielski et al. 2016) (Fig. 10.3). Under physiological conditions, Isu has a very high turnover rate. However, under stress conditions, high levels of Isu are beneficial for cell survival (Song et al. 2012). Thus, Hsc20 may control the availability of FeS-loaded Isu upon lack of recipient proteins or under stress conditions (Ciesielski and Craig 2017).

Although the focus of this chapter is on mitochondrial functions of Hsc20 and its Hsp70 partners, we note that recently published data implicates Hsc20 in cytosolic FeS biogenesis—as suggested by protein-protein interactions identified using cellular pull-down assays in mammalian cells (Kim et al. 2018; Maio and Rouault 2020). Although these results are intriguing, all evidence obtained to date for mitochondrial and bacterial systems indicate that Hsc20 functions in the FeS biogenesis via an Hsp70-dependent Isu binding cycle. Thus, more data is needed to mechanistically understand this postulated cytosolic function.

Evolutionary Dynamics of Mitochondrial JDP/Hsp70 Systems

While in eukaryotic cells no genes encoding closely related proteins functioning outside mitochondria were identified for Hsc20 and Pam 18, this is not the case for Mdj1. Other class I JDPs function in the cytosol and ER (e.g., cytosolic Ydj1 and ER Scj1 of *S. cerevisiae*). This raised a question—how is Mdj1 related to other class I JDPs present in the eukaryotic cell? At least two scenarios were conceivable: either Mdj1 share the most recent common ancestor with eukaryotic class I JDPs or Mdj1 originated from the bacterial JDP present in the progenitor of mitochondria independently from class I JDPs present in the cytosol and ER. Phylogenetic analysis supports the latter scenario—independent bacterial origin of Mdj1 (Fig. 10.4). A phylogenetic tree of class I JDPs from bacteria, archaea, and all compartments of the eukaryotic cell recovers two most recent common ancestors of the eukaryotic JDPs—one for Mdj1s and the other for JDPs from the cytosol and ER (red and violet dots on Fig. 10.4, respectively). Separation of these two predicted ancestors by branches representing bacterial sequences supports the notion that Mdj1 originated

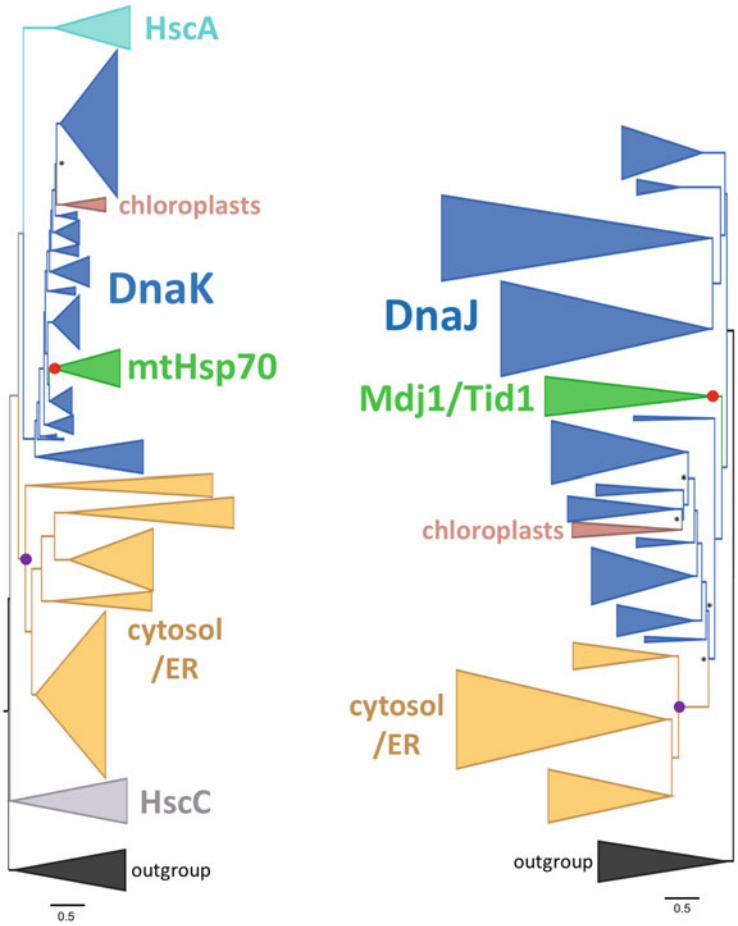

Fig. 10.4 Mdj1 and mtHsp70 are more closely related to bacterial DnaJ and DnaK than to their homologs from the cytosol and ER, as indicated by protein phylogeny. Maximum likelihood phylogeny of mtHsp70 (left) and Mdj1 (right) homologs from bacteria, archaea, and all subcellular compartments of the eukaryotic cell. Phylogenies were reconstructed based on the nucleotide-binding domain (NBD) for mtHsp70 homologs and J-domain/double β-barrel domains for Mdj1 homologs (3651 sequences for Hsp70 and 1510 sequences for Mdj1). The trees were reconstructed using IQtree with LG + I + G amino acid substitution model with 100 bootstrap trials. Bootstrap support below 50 (BS $<$ 50) is indicated with the asterisk (*). Mitochondrial mtHsp70 and Mdj1 (green), their homologs from the cytosol and ER (yellow), from the chloroplasts (pink), and from the prokaryotes (blue). Bacteria-specific Hsp70s HscA (cyan) and HscC (light gray). Outgroups (black) contain NBD sequences from proteins that do not belong to the Hsp70 family, e.g., actin (left) and J-domain/double β-barrel sequences from distantly related JDP homologs, e.g., bacterial CbpA (right). The most recent common ancestors of mtHsp70 and Mdj1 sequences are marked by red dots, while the most recent common ancestors of their homologs from the cytosol and ER are marked by violet dots. On both trees, these dots are separated by branches representing bacterial sequences indicating that mitochondrial mtHsp70/Mdj1 system originated from bacterial ancestor independently from the homologous systems functioning in the cytosol and ER

directly from the bacterial progenitor. Put another way, Mdj1 is more closely related to bacterial DnaJ than to other eukaryotic class I JDPs.

The coexistence of mtHsp70 with Hsp70 homologs present in the cytosol and ER also raises a question of its evolutionary origin. Similar to Mdj1, protein phylogeny of Hsp70 homologs from bacteria, archaea, and all compartments of the eukaryotic cell recovered two of the most recent common ancestors of eukaryotic Hsp70s—one for mtHsp70s and the other for Hsp70s from the cytosol and ER (Fig. 10.4). These two predicted ancestors are separated on the tree by branches representing bacterial DnaK sequences, indicating that mtHsp70 originated from the bacterial progenitor independently from the Hsp70s of the cytosol and ER. Therefore, mtHsp70 is more closely related to the bacterial DnaK than to its eukaryotic homologs. Such a pattern of relatedness is also consistent with the functional differences observed between Mdj1/mtHsp70 and JDP/Hsp70 systems present in the cytosol and ER, such as a unique, for eukaryotes, Mge1/GrpE NEF functioning with the mitochondrial system. Taken together, phylogenetic analyses and functional differences support a notion that Mdj1/mtHsp70 system was inherited from the bacterial progenitor of mitochondria and it is distantly related to JDP/Hsp70 systems present in other subcellular compartments.

The most interesting aspects of the evolutionary dynamics of mitochondrial JDP/Hsp70 systems are changes in Hsp70 partnership of Hsc20—first during evolution of mitochondria and then a second time in the fungal lineage to which *S. cerevisiae* belongs (Fig. 10.6). In bacteria, the Hsc20 ortholog HscB functions with HscA, an Hsp70 specialized in FeS biogenesis. However, there is no close relative of HscA in mitochondria (Huynen et al. 2001; Kleczewska et al. 2020). Its function in the FeS biogenesis was replaced by mtHsp70, which as discussed above descended from DnaK. It is worth emphasizing that DnaK not only is distantly related to HscA (Fig. 10.4) but also differs from it biochemically (Vickery and Cupp-Vickery 2007). DnaK requires NEF GrpE for the ADP/ATP exchange needed to complete the client-binding ATPase cycle, while HscA does not. HscA's low affinity for adenine nucleotides enables client release without the assistance of an NEF. Why HscA is not present in mitochondria is unclear—it either was lost during evolution of mitochondria (i.e., during massive transfer of endosymbiont genes into the host genome) or was never present in the ancestor of mitochondria. Indeed, a survey of bacterial JDPs revealed two species that harbor HscB but lack HscA (Barriot et al. 2020). It is tempting to speculate that in such species, HscB functions with DnaK in FeS biogenesis.

The second change in Hsc20/Hsp70 partnerships took place at the base of the *S. cerevisiae* lineage following duplication of mtHsp70 (Schilke et al. 2006; Kominek et al. 2013; Kleczewska et al. 2020). One duplicate, Ssc1, remained multifunctional, similar to its mtHsp70 predecessor. In contrast, Ssq1 evolved into the FeS biogenesis specialist, mimicking functional features of HscA and thus serving as an example of convergent evolution at the biochemical level. Ssq1 lost the ability to bind client proteins other than Isu (Schilke et al. 2006). However, similar to Ssc1, it requires NEF Mge1 to complete the Isu binding cycle (Dutkiewicz et al. 2003). Ssq1 also lost the ability to efficiently interact with the mitochondrial

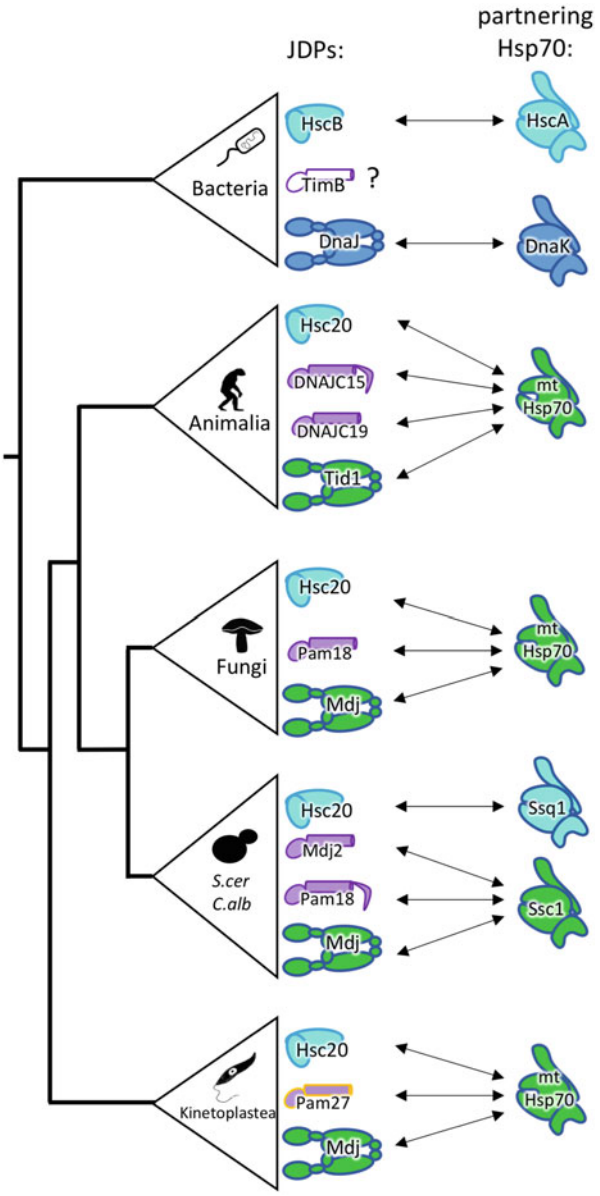

Fig. 10.6 Evolutionary dynamics of mitochondrial JDP/mtHsp70 systems. In bacteria, multifunctional DnaJ functions with DnaK, while FeS specialized HscB functions with dedicated Hsp70 (HscA), TimB, a putative ancestor of Pam18, functions with an unknown Hsp70 partner. In most animals and fungi, Mdj1 and Hsc20, descendants of DnaJ and HscB, respectively, function with a single mtHsp70, a descendant of DnaK. Pam18 duplicated at the base of the animal lineage (DnaJC15, DnaJc19 in humans) and in the subset of fungi (Pam18 and Mdj2 in *S. cerevisiae*). Postduplication DnaJC15 and Pam18 independently evolved a new N-terminal IMS domain. In a subset of fungi (*S. cerevisiae* (*S.cer*) and *Candida albicans* (*C. alb*) clade), mtHsp70 duplicated giving rise to multifunctional Ssc1 that cooperates with Mdj1 and Pam18/Mdj2 and FeS specialized Ssq1 partnering with Hsc20. In *T. brucei* and related Kinetoplastea species, TbPam27 replaced Pam18/Pam16 in protein import. Pam18 and Pam16 were retained in the genome, playing unknown role(s)

JDPs Mdj1 and Pam18 but interacts very efficiently with Hsc20 (D'Silva et al. 2003; Dutkiewicz et al. 2003; Pukszta et al. 2010; Delewski et al. 2016). Evidence suggests that evolution of the highly efficient Hsc20/Ssq1 interaction was accompanied by changes in structure and sequence of Hsc20's J-domain (Pukszta et al. 2010; Ciesielski et al. 2012; Tomiczek et al. 2020). This evolutionary story clearly illustrates that JDPs are capable of changing Hsp70 partners and govern their new

partner's functional specificity, regardless of whether the new partner is specialized (Ssq1) or multifunctional (mtHsp70). It also suggests that highly correlated changes of sequence and structure of JDP/Hsp70 pairs might be required to establish such functional partnership.

In comparison to Mdj1 and Hsc20, the evolutionary origin of Pam18 was more difficult to track, because protein translocation function is unique to mitochondria. However, a conserved sequence motif present adjacent to the HPD of the J-domain of Pam18 (HPD-X-GGS) and the presence of 3, rather than 4, helices in the J-domain are features unique to Pam18, which are also present in a set of JDPs from alphaproteobacteria (Clements et al. 2009), as well as deltaproteobacteria and gammaproteobacteria (Fig. 10.5). For one of them, TimB from *Caulobacter crescentus*, functional studies demonstrated that its J-domain can functionally replace the J-domain of Pam18 upon introduction of a few sequence modifications (Clements et al. 2009). Thus, Pam18 likely originated from an uncharacterized bacterial JDP, with which it shares sequence and structure features. The origin of Pam16 was even more difficult to track. Pam18/Pam16 phylogeny (Fig. 10.5) rooted using their putative bacterial orthologs suggests that Pam16 emerged via an ancient duplication of Pam18. In support of this idea, all Pam16 sequences form a monophyletic group, which is more closely related to Pam18 than to their bacterial orthologs.

Another intriguing aspect of Pam18 evolution is the independent emergence of a new protein domain in two lineages following the Pam18 gene duplication. Independent duplications of Pam18 took place in animals and in a subset of fungal species closely related to *S. cerevisiae* (Fig. 10.5/Fig. 10.6). In both cases, one paralog evolved a new IMS domain, which contacts one of the subunits of the import channel (Fig. 10.2). However, a clear connection between IMS domains and function is not yet apparent. In *S. cerevisiae*, Pam18's IMS domain is required for growth under anaerobic conditions, but its truncation does not affect growth under standard conditions (Hayashi et al. 2011). The Pam18 duplicate, termed Mdj2, which has no IMS domain and is expressed at a lower level, can substitute for Pam18 when expressed at an appropriate level (Mokranjac et al. 2005). However, Mdj2 does not compensate for lack of Pam18's IMS domain in the absence of oxygen (Hayashi et al. 2011). In the case of animal duplicates, both paralogs DNAJC15, which has an IMS domain, and DNAJC19, which does not, are critical for protein import (Schusdziarra et al. 2013; Sinha et al. 2016). However, only DNAJC15 can functionally complement Pam18 in yeast. This complementation demonstrates the functional similarity of human and fungal orthologs. But though the presence of the IMS domain in DNAJC15 coincides with its ability to complement Pam18, further studies are needed to verify its functional importance.

Another interesting twist of Pam18 evolution is illustrated by *T. brucei.* Remarkably, in this parasitic species, unlike most eukaryotes which have two inner membrane translocon systems—Tim17/23 for the matrix-destined proteins and Tim22 for most inner membrane proteins—there is only one, which is related to Tim22. This system is not dependent on Pam18/Pam16 working with mtHsp70 for translocation of proteins into the matrix, but rather on an unrelated JDP termed TbPam27 (von

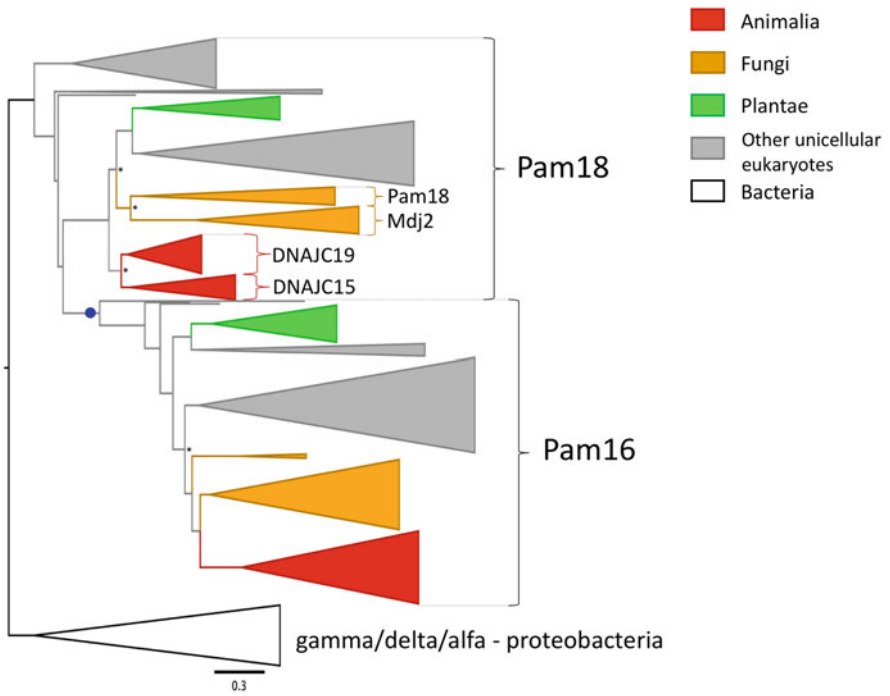

Fig. 10.5 Phylogenetic relationships among Pam18/Pam16 homologs. Maximum likelihood phylogeny of Pam16/Pam18 orthologs (457 sequences) retrieved from fungi (orange), animals (red), plants (green), and other unicellular eukaryote (gray) proteomes by probabilistic amino acid sequence profiles. Putative bacterial orthologs of Pam18/Pam16 (45 sequences) were identified in alphaproteobacteria, deltaproteobacteria, and gammaproteobacteria and were used as an outgroup. The tree was calculated using IQtree with LG + I + G amino acid substitution model with 100 bootstrap trials. Bootstrap support below 50 (BS $<$ 50) is indicated with the asterisk (*). The scale bar indicates substitutions per site. Bartlomiej Tomiczek unpublished results. All Pam16 sequences form a monophyletic group; a common ancestor is marked by blue dot. The Pam16 clade is separated from the outgroup by Pam18 sequences, suggesting that Pam16 emerged by Pam18 gene duplication. While Pam16 is encoded by a single gene in animals, fungi, and plants, Pam18 was duplicated at least twice—once early in animals (DNAJC15/DNAJC19) and once in a subset of fungi (*Saccharomycotina*, Pam18/Mdj2)

Känel et al. 2020; Schneider 2020; Mani et al. 2016). This radical replacement is surprising because it took place despite the fact that translocation of mitochondrial proteins is essential. Interestingly, the Pam18/Pam16 pair was retained, suggesting they carry out an important function(s). Indeed, ablation of Pam18/Pam16 results in growth arrest and slow accumulation of unprocessed cytochrome oxidase subunit 4 (Cox IV) (von Känel et al. 2020). Two scenarios can explain retention of Pam18/Pam16 in *T. brucei*; either (1) the pair has evolved a novel function in the *T. brucei* lineage during the course of evolution of the new TbPam27-based translocon or (2) the pair has retained an ancestral function unrelated to protein import, which is required in all eukaryotes, but not yet generally appreciated. Interestingly, the human

Pam18 ortholog DNAJC19 has been implicated in cardiolipin remodeling in the inner mitochondria membrane—a function likely unrelated to its role in the protein import (Richter-Dennerlein et al. 2014).

Taken together, the evolutionary histories of mitochondrial JDP/Hsp70 systems nicely illustrate two contrasting features of molecular evolution: on one hand, its predictability, recruitment of preexisting bacterial JDP and Hsp70 proteins combined with their functional divergence resulting in the emergence of highly sophisticated mitochondria-specific JDP/Hsp70 chaperone systems, and, on the other hand, its unpredictability, losses (HscA), replacements (DnaK replaced HscA; TbPam27 replaced Pam18), duplications (mtHsp70 duplicated to Ssc1 and Ssq1, independent duplications of Pam18), and convergent evolution (Ssq1 converged to HscA) among proteins constituting the JDP/Hsp70 systems. It is striking that these unpredictable changes took place despite the fact that these chaperones are engaged in essential mitochondrial processes.

Concluding Remarks

Mitochondrial JDPs very efficiently orchestrate the multifunctionality of their Hsp70 partners to perform specific roles required for proper functioning and maintenance of mitochondria. It is also clear that regardless of whether a given JDP/mtHsp70 system is involved in protein folding/refolding, maintenance of mitochondrial genome, and protein translocation or biogenesis of iron-sulfur cluster, the JDP-driven cyclic interaction with client polypeptide constitutes the mechanistic basis of its function. In this respect, mitochondrial JDP/mtHsp70 systems are similar to homologous systems functioning in other cellular compartments and in prokaryotes. Although, as discussed above, many details of the client-binding cycle have been revealed recently, there is still much to learn. Further mechanistic studies of mitochondrial JDP/mtHsp70 systems from various organisms, not only yeast and humans, have much to offer in terms of understanding how all JDP/Hsp70 systems function and evolve. Moreover, one can take advantage of the limited complexity of mitochondrial JDP/mtHsp70 systems and their well-defined functions to dissect functional interconnections among them.

Mitochondrial JDP/mtHsp70 systems also offer a unique opportunity to study noncanonical functions of their components. Although not a main focus of this chapter, we have noted a recent flow of publications reporting noncanonical roles for mitochondrial JDPs and their mtHsp70 partners, both inside and outside the organelle. A key question now is how many of these new functions are dependent on the client-binding cycle and how many are performed independently by the JDP or mtHsp70. In our opinion, JDPs are particularly well suited to perform roles independent from their partnership with Hsp70, as illustrated by Hsc20's functions at the early steps of the FeS biogenesis. Finally, mechanistic insights will hopefully lead to practical medical applications by connecting pathogenic mutations reported for

constituents of the human JDP/mtHsp70 systems with their effects on the progression of the client-binding cycles or on the noncanonical functions.

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Chapter 11
Impact of Co-chaperones and Posttranslational Modifications Toward Hsp90 Drug Sensitivity

Sarah J. Backe, Mark R. Woodford, Elham Ahanin, Rebecca A. Sager, Dimitra Bourboulia, and Mehdi Mollapour

Abstract Posttranslational modifications (PTMs) regulate myriad cellular processes by modulating protein function and protein-protein interaction. Heat shock protein 90 (Hsp90) is an ATP-dependent molecular chaperone whose activity is responsible for the stabilization and maturation of more than 300 client proteins. Hsp90 is a substrate for numerous PTMs, which have diverse effects on Hsp90 function. Interestingly, many Hsp90 clients are enzymes that catalyze PTM, demonstrating one of the several modes of regulation of Hsp90 activity. Approximately 25 co-chaperone regulatory proteins of Hsp90 impact structural rearrangements, ATP hydrolysis, and client interaction, representing a second layer of influence on Hsp90 activity. A growing body of literature has also established that PTM of these co-chaperones fine-tune their activity toward Hsp90; however, many of the identified PTMs remain uncharacterized. Given the critical role of Hsp90 in supporting signaling in cancer, clinical evaluation of Hsp90 inhibitors is an area of great interest. Interestingly, differential PTM and co-chaperone interaction have been shown to impact Hsp90 binding to its inhibitors. Therefore, understanding these layers of Hsp90 regulation will provide a more complete understanding of the chaperone code, facilitating the development of new biomarkers and combination therapies.

Keywords Hsp90 · Chaperone · Co-chaperone · PTM · Phosphorylation · Chaperone code · Hsp90 inhibitors

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Introduction

Heat shock protein 90 (Hsp90) is a ubiquitous and essential molecular chaperone. Approximately 300 client proteins depend on the 90-kDa chaperone for proper folding, stability, and activation (Schopf et al. 2017). Hsp90 clients include protein kinases, transcription factors, oncoproteins, and tumor suppressors (https://www.picard.ch/downloads/Hsp90interactors.pdf). Through a highly dynamic process known as the chaperone cycle, Hsp90 ATP hydrolysis is coupled to large conformational changes and consequent client chaperoning (Panaretou et al. 1998; Obermann et al. 1998; Schopf et al. 2017). This chaperone cycle is tightly regulated by a class of regulators known as co-chaperones. These proteins bind to distinct conformations of Hsp90 and regulate its progression through the chaperone cycle, client loading and release, and posttranslational modification (PTM) (Cox and Johnson 2018; Sahasrabudhe et al. 2017; Zierer et al. 2016; Rohl et al. 2013; Hohrman et al. 2021).

Cancer cells often rely on the Hsp90 chaperone machinery to support dysregulated proliferation and metastasis, making Hsp90 an attractive therapeutic target (Neckers and Workman 2012; Barrott and Haystead 2013; Rodina et al. 2016; Wang et al. 2016). Due to the breadth of the Hsp90 clientome, Hsp90 inhibitors can simultaneously disrupt numerous oncogenic pathways, making Hsp90 inhibitor development an area of intense focus. Despite promising preclinical results, Hsp90 inhibitors have yet to be approved for the treatment of human cancers. Notably, Hsp90 PTMs and co-chaperone dynamics modulate cellular sensitivity to Hsp90 inhibitors, suggesting that a comprehensive understanding of Hsp90 regulation is paramount to the clinical success of Hsp90 inhibitors (Walton-Diaz et al. 2013; Woodford et al. 2016a; Backe et al. 2020; Cloutier and Coulombe 2013).

The Chaperone Cycle

The Hsp90 chaperone cycle comprises an ordered series of conformational changes coupled to its ATPase activity (Graf et al. 2009; Mickler et al. 2009). Hsp90 consists of three structural domains (Ali et al. 2006; Verba et al. 2016). The amino-terminal domain (NTD) contains the nucleotide-binding pocket (Prodromou et al. 1997a, b), which is connected to the middle domain (MD) by a highly charged, flexible linker region (Tsutsumi et al. 2012; Hainzl et al. 2009; Jahn et al. 2014). Many Hsp90-interacting proteins bind to the Hsp90-MD, which also contains the catalytic loop that is required for ATP hydrolysis (Meyer et al. 2003; Biebl and Buchner 2019; Schopf et al. 2017). The carboxyl-terminal domain (CTD) contains the highly conserved, extreme C-terminal MEEVD sequence which is the docking site for tetratricopeptide repeat (TPR) domain-containing co-chaperones (Young et al. 1998; Carrello et al. 1999; Russell et al. 1999; Ramsey et al. 2000). Notably, the functional unit of Hsp90 is a dimer, and the CTD is the site of constitutive dimerization of the Hsp90 protomers (Harris et al. 2004; Prodromou and Pearl 2003; Wayne and Bolon 2007).

Apo-Hsp90 that is dimerized at the CTD adopts an open V-shaped conformation. Upon ATP binding to the nucleotide pocket of the NTD, Hsp90 undergoes large conformational rearrangements resulting in transient dimerization of the Hsp90 NTDs to form the "closed" conformation. Subsequent ATP hydrolysis causes a return to the open V shape, resetting the chaperone for the next cycle (Zierer et al. 2016; Mickler et al. 2009; Prodromou and Pearl 2003; Hessling et al. 2009; Neckers et al. 2009; Shiau et al. 2006). Interestingly, ATP binding in the NTD leads to conformational changes throughout the length of the entire protein (Cunningham et al. 2008). This ability has also been attributed to several PTMs of Hsp90, demonstrating the complex interdomain connectivity and communication throughout the Hsp90 protein (Rehn et al. 2020; Stetz et al. 2018; Xu et al. 2019).

To meet the differing needs of ~300 client proteins, the Hsp90 chaperone cycle is regulated by co-chaperones and PTMs. These regulators provide directionality to the cycle by altering conformational dwell time, coordinating assembly of chaperone-client complexes, and modulating Hsp90 affinity for ATP and ATP hydrolysis rate. The general progression of the cycle is well established and is outlined below and in Fig. 11.1.

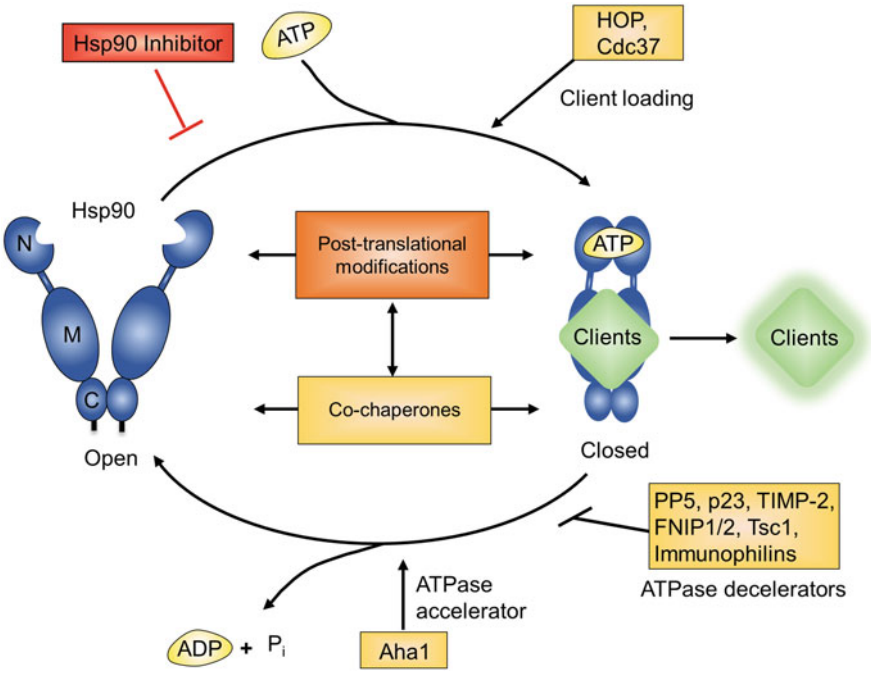

Fig. 11.1 The Hsp90 chaperone cycle. Open Hsp90 is dimerized only through contacts in the CTD. ATP binding and an ordered series of conformational changes allow Hsp90 to adopt a closed conformation, which is N-terminally dimerized. ATP hydrolysis leads Hsp90 to return to the open conformation and is ready to begin another chaperone cycle. This cycle allows for the activation of client proteins. Throughout the cycle, co-chaperones bind to Hsp90 and regulate its function. PTM of Hsp90 and PTM of co-chaperones provide further regulation of the chaperone cycle

An early event in the Hsp90 chaperone cycle is binding of the co-chaperone Hsp70-Hsp90-organizing protein (HOP) to the open conformation of Hsp90 via its TPR2A domain. HOP slows Hsp90 ATPase activity and helps transfer client proteins from the "early" chaperone Hsp70 to Hsp90 (Wegele et al. 2006; Li et al. 2011; Prodromou et al. 1999). HOP is then displaced by the co-chaperone activator of Hsp90 ATPase (Aha1) (Harst et al. 2005), which aids Hsp90 N-domain dimerization, thereby increasing the rate of ATP hydrolysis (Panaretou et al. 2002; Meyer et al. 2004; Retzlaff et al. 2010; Mercier et al. 2019). The co-chaperone cell division cycle 37 (Cdc37) assists in loading of protein kinase clients to Hsp90 (Keramisanou et al. 2016; Siligardi et al. 2002). Cdc37 co-chaperone function requires systematic phosphorylation and subsequent dephosphorylation by another Hsp90 co-chaperone protein phosphatase 5 (PP5) (Miyata and Nishida 2005; Vaughan et al. 2008; Bandhakavi et al. 2003). Late in the client maturation process, the co-chaperone prostaglandin E synthase 3 (p23) slows ATP hydrolysis by binding to and stabilizing Hsp90 in the closed conformation (Ali et al. 2006; Richter et al. 2004). Many of the co-chaperone/Hsp90 complex dynamics are heavily regulated by PTM. Regulation of co-chaperones' functions by PTM and the subsequent impact on Hsp90 function and downstream cellular processes will be discussed in detail below (Table 11.1).

Canonical Co-chaperones

Hsp70/Hsp90-Organizing Protein (HOP)

The co-chaperone HOP (stress-inducible protein 1, STI1, STIP1) catalyzes client protein transfer from ADP-bound Hsp70 to ADP-bound Hsp90 by binding each chaperone with a unique TPR domain (Chen and Smith 1998; Johnson et al. 1998; Odunuga et al. 2004; Brinker et al. 2002; Carrigan et al. 2004). Interestingly, HOP binds to Hsp90 with greater affinity than other TPR-containing co-chaperones (Hildenbrand et al. 2011). A high-affinity Hsp90/HOP complex may explain the observation that Hsp90 binding to HOP is mutually exclusive from some other co-chaperones including Aha1 and CHIP (Harst et al. 2005; Kundrat and Regan 2010a; Xu et al. 2019). Recent work has also demonstrated a critical role for HOP in assembly of the 26S proteasome, via its activity as an Hsp90 co-chaperone (Bhattacharya et al. 2020).

PTMs have been previously shown to regulate the formation of the Hsp70-HOP-Hsp90 chaperone heterocomplex, a critical initiation event in the Hsp90 chaperone cycle. HOP was shown to be phosphorylated by both Cdc2 (Cdk1) and casein kinase 2 (CK2), affecting HOP chaperone interactions and subcellular localization. Phosphorylation of HOP-T198 by Cdk1 resulted in cytoplasmic localization, whereas CK2 phosphorylation of HOP at S189 induced translocation to the nucleus (Fig. 11.2a) (Longshaw et al. 2004; Daniel et al. 2008). Interestingly, CK2 phosphorylation of the Hsp90 C-terminus (T725 and S726) enhanced binding to HOP and prevented the binding of CHIP (Muller et al. 2012), while the phosphomimetic

Table 11.1 Co-chaperone posttranslational modifications. Identified modification sites of co-chaperones are listed. When known, the modifying enzyme and impact on Hsp90 binding are shown. Increased binding to Hsp90 are identified by ↑, decreased ↓, and no change nc; no entry is provided in the table if not determined or examined

Co-chaperone	Modification	Residue	Enzyme	Hsp90 binding	References
HOP	Phosphorylation	S16		nc	Rohl et al. (2015)
HOP	Phosphorylation	S189	CK2	nc	Rohl et al. (2015), Longshaw et al. (2004)
HOP	Phosphorylation	T198	Cdk1	↓	Longshaw et al. (2004), Daniel et al. (2008), Rohl et al. (2015)
HOP	Phosphorylation	Y354		nc	Rohl et al. (2015)
HOP	Phosphorylation	S481		nc	Rohl et al. (2015)
Cdc37	Phosphorylation	Y4	Yes	↓	Xu et al. (2012).
Cdc37	Phosphorylation	S13	CK2, PP5	↑, ↓	Shao et al. (2003b), Bandhakavi et al. (2003), Miyata and Nishida (2005, 2007, 2008), Verba et al. (2016), Vaughan et al. (2008), Oberoi et al. (2016)
Cdc37	Phosphorylation	S97	PKA	↑	Pan et al. (2018)
Cdc37	Phosphorylation	Y298	Yes	↓	Xu et al. (2012), Bachman et al. (2018)
Cdc37	Phosphorylation	S339	Ulk1	nc	Li et al. (2017)
PP5	Ubiquitination	K185	VHL		Dushukyan et al. (2017)
PP5	Ubiquitination	K199	VHL		Dushukyan et al. (2017)
PP5	Phosphorylation	T362	CK1δ	nc	Dushukyan et al. (2017)
Aha1	Phosphorylation	Y223	c-Abl	↑	Dunn et al. (2015), Woodford et al. (2017)
p23	Phosphorylation	S113	CK2	↑	Kobayashi et al. (2004), Nakanishi et al. (2007)
p23	Phosphorylation	S118	CK2	↑	Kobayashi et al. (2004)
FKBP51	Acetylation	K28			Yu et al. (2017)
FKBP51	Acetylation	K155			Yu et al. (2017)
FKBP51	SUMOylation	K422	PIAS4	↑	Antunica-Noguerol et al. (2016)
FKBP52	Phosphorylation	T143	CK2	↓	Miyata et al. (1997), Cox et al. (2007)
SGTA	Phosphorylation	S305	Akt2		Moritz et al. (2010)
Sgt1	Phosphorylation	S249	CK2	↑	Prus et al. (2011)
Sgt1	Phosphorylation	S299	CK2	↑	Prus et al. (2011)
Sgt1	Phosphorylation	S331	Plk1	nc	Bansal et al. (2009), Liu et al. (2012a, b)
CHIP	Phosphorylation	S20	Cdk5, protein kinase G		Kim et al. (2016), Kim et al. (2018), Ranek et al. (2020)

(continued)

Table 11.1 (continued)

Co-chaperone	Modification	Residue	Enzyme	Hsp90 binding	References
FNIP1	Oxidation	C580			Manford et al. (2020)
FNIP1	Oxidation	C582			Manford et al. (2020)
FNIP1	Oxidation	C585			Manford et al. (2020)
FNIP1	Phosphorylation, O-GlcNAcylation	S938	CK2, PP5, OGT	↑, ↓	Sager et al. (2019)
FNIP1	Phosphorylation	S939	CK2, PP5	↑, ↓	Sager et al. (2019)
FNIP1	Phosphorylation	S941	CK2, PP5	↑, ↓	Sager et al. (2019)
FNIP1	Phosphorylation	S946	CK2, PP5	↑, ↓	Sager et al. (2019)
FNIP1	Phosphorylation	S948	CK2, PP5	↑, ↓	Sager et al. (2019)
FNIP1	Ubiquitination	K1119			Sager et al. (2019)
Tsc1	Phosphorylation	T310	Cdk1		Astrinidis et al. (2003, 2006)
Tsc1	Phosphorylation	S332	Cdk1		Astrinidis et al. (2003)
Tsc1	Phosphorylation	T417			Inoue et al. (2010)
Tsc1	Phosphorylation	S467	Plk1		Li et al. (2018b)
Tsc1	Phosphorylation	S487	IKKβ		Lee et al. (2007)
Tsc1	Phosphorylation	S511	IKKβ		Lee et al. (2007)
Tsc1	Phosphorylation	T578	Plk1		Li et al. (2018b)
Tsc1	Phosphorylation	T1047	Cdk1		Astrinidis et al. (2003)
TIMP-2	Phosphorylation	Y90	c-Src		Sanchez-Pozo et al. (2018)

HOP-T198E decreased HOP binding to Hsp90 (Daniel et al. 2008). HOP phosphorylation at residues S16, S189, Y354, and S481 was shown to regulate its interaction with Hsp70, but not Hsp90 (Daniel et al. 2008; Rohl et al. 2015). Interestingly, phosphorylation of HOP-S16, Y354, and S481 was also shown to reduce Hsp90-dependent GR activity (Rohl et al. 2015); however, the kinases regulating these phosphorylation events have yet to be reported (Fig. 11.2b). Hsp90α-T36 is also subject to phosphorylation by CK2, though its impact on HOP interaction has not been explored. Taken together, these data position CK2 as a master regulator of Hsp70-HOP-Hsp90 interactions (Lees-Miller and Anderson 1989; Mollapour et al. 2011a, b).

Cell Division Cycle 37 (Cdc37)

The kinase-specific co-chaperone Cdc37 facilitates recruitment and loading of kinase clients to the Hsp90 chaperone machinery (Siligardi et al. 2002; Roe et al.

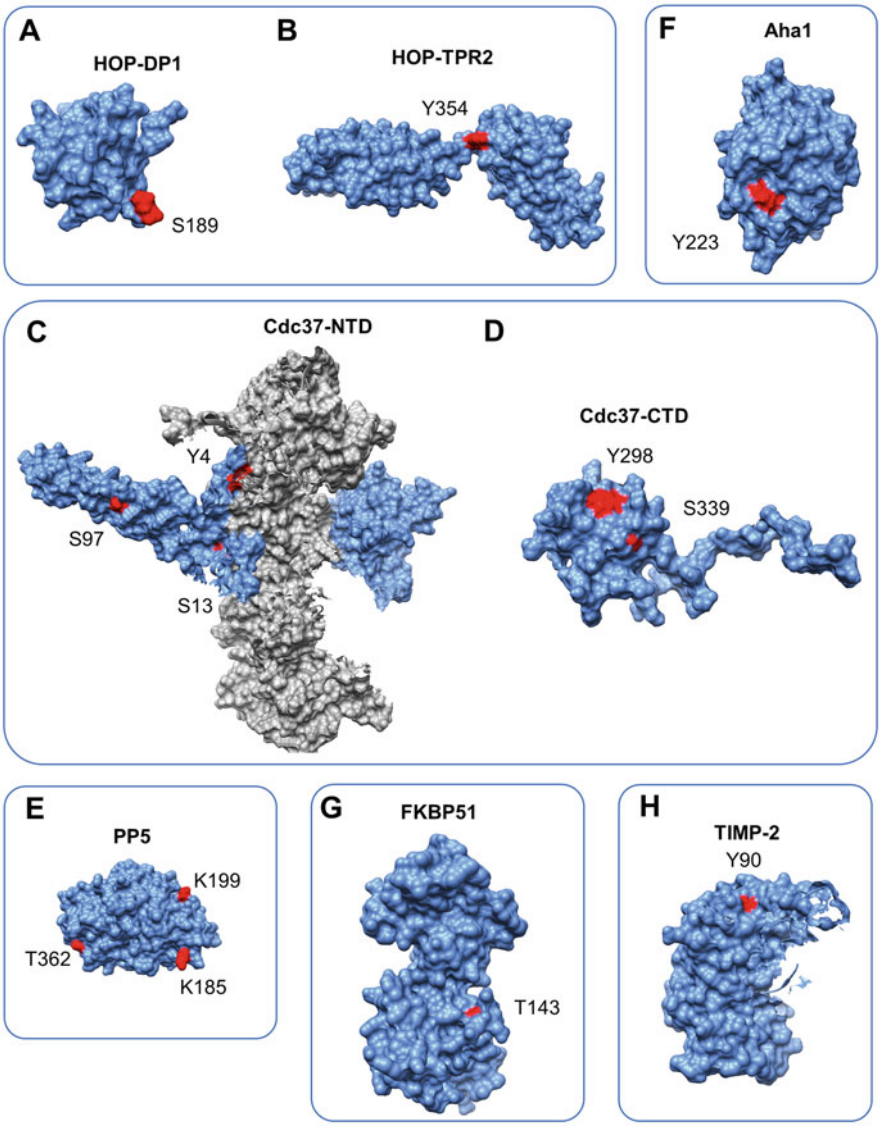

Fig. 11.2 PTM sites of Hsp90 co-chaperones. Surface structure of co-chaperones with confirmed sites of PTM highlighted in red. (**a**) HOP rich in aspartate and proline domain-1 (DP1), PDB ID, 2llv; (**b**) HOP-tetratricopeptide repeat domain-2 (TPR2), PBB ID, 3uq3; (**c**) Aha1, PDB ID, 1x53; (**d**) Cdc37 amino-terminal domain (NTD), PDB ID, 5fwp; (**e**) Cdc37-carboxy terminal domain (CTD), PDB ID, 2n5x; (**f**) PP5, PDB ID, 5hpe; (**g**) FKBP51, PDB ID, 5njx; (**h**) TIMP-2, PDB ID, 4ilw

2004; Verba et al. 2016). Cdc37 binds to the open conformation of the Hsp90 dimer which impedes Hsp90 conformational rearrangement, allowing time for client loading to the Hsp90 chaperone complex (Eckl et al. 2013; Vaughan et al. 2006; Shao et al. 2003a). Phosphorylation of the Cdc37-S13 by CK2 is required for Cdc37 co-chaperone activity (Fig. 11.2c) (Shao et al. 2003b; Bandhakavi et al. 2003); specifically, S13 phosphorylation is required for client recruitment and Hsp90 binding (Miyata and Nishida 2005, 2007, 2008). Mechanistically, S13 phosphorylation causes Cdc37 to adopt a more compact conformation with enhanced secondary structure. This is necessary for Cdc37 recognition of the unfolded kinase clients prior to binding Hsp90. Additionally, phosphorylation of Cdc37-S13 forms a salt bridge with Hsp90-K406, contributing to Cdc37-Hsp90 interaction (Verba et al. 2016). Subsequent dephosphorylation of S13 by protein phosphatase 5 (PP5) is required for client release from the chaperone complex, resetting Hsp90 to begin another chaperone cycle (Vaughan et al. 2008; Oberoi et al. 2016).

Cdc37 is also phosphorylated by protein kinase A (PKA) and the non-receptor tyrosine kinase Yes (Xu et al. 2012). Yes phosphorylates Cdc37-Y4 and Cdc37-Y298. Phosphorylation of Cdc37-Y298, and to a lesser extent Y4, causes dissociation of Cdc37 from the Hsp90/client complex (Fig. 11.2c, d) (Xu et al. 2012). Prior to dissociation, Cdc37-Y298 phosphorylation induces partial unfolding of the Cdc37-CTD (Bachman et al. 2018). Partial unfolding of Cdc37-CTD unmasks the polypeptide region of Hsp90 containing Y197, which is subsequently phosphorylated by Yes causing Cdc37 to dissociate from the complex and allow for progression through the chaperone cycle. PKA-mediated phosphorylation of Cdc37-S97 was subsequently found to be important for Cdc37/Hsp90 interaction and chaperoning of the kinase AXL. Interestingly, nonsteroidal anti-inflammatory drugs (NSAIDs) inhibited Cdc37-S97 phosphorylation leading to incomplete folding and eventual degradation of AXL by the co-chaperone CHIP (Fig. 11.2c) (Pan et al. 2018).

Posttranslational modification of Hsp90 also alters Hsp90/Cdc37 complex formation and chaperone function. Protein kinase A (PKA) phosphorylates Hsp90-T90 which perturbs the interaction between Cdc37 and Hsp90, leading to degradation of the kinase clients Src, Akt, and PKCγ (Wang et al. 2012). Interestingly, the Hsp90 client PKCγ phosphorylates three residues of Hsp90 and causes profound impacts on the chaperone cycle (Lu et al. 2014). Mutation of one of these residues, Hsp90-T115, to the phosphomimetic T115E, decreased interaction between Hsp90 and Cdc37. Further, Hsp90-T115E, Hsp90-T425E, and Hsp90-T603E all showed decreased binding to ATP and decreased ATPase activity. The authors propose a model where Hsp90:Cdc37 binds to newly synthesized and inactive PKCγ and progresses through the chaperone cycle leading to activation of mature PKCγ. Active PKCγ then phosphorylates Hsp90 triggering the release of the client from the chaperone complex. Notably, Hsp90-T115 was also shown to be phosphorylated by the mitotic checkpoint kinase, Mps1, which negatively impacts Cdc37 binding (Woodford et al. 2016c), and previous work has shown Cdc37 interaction with Mps1 is important for spindle pole body duplication (Schutz et al. 1997). Although the consequence of Hsp90-pT115 on Cdc37 function remains unknown, these works demonstrate the

importance of phosphorylation of both Cdc37 and Hsp90 to ensure efficient client chaperoning.

The co-chaperone activity of Cdc37 is critical to the activation of oncogenic kinase clients such as PKCγ, c-Src, and c-Abl (Gould et al. 2008; Dey et al. 1996; Keramisanou et al. 2016). Interestingly, Cdc37 expression is increased in some tumors, suggesting a direct role for Cdc37 in tumorigenesis (Stepanova et al. 2000; Zhu et al. 2018; McDowell et al. 2009). In contrast, Hsp90-Cdc37 also plays a role in starvation-induced autophagy through stabilization and activation of Unc-51 like autophagy-activating kinase (Ulk1) (Joo et al. 2011). Ulk1 was shown to phosphorylate Atg13 in an Hsp90-Cdc37-dependent manner, which led to Atg13 activation and release from the Hsp90-Cdc37-Ulk1 complex (Joo et al. 2011). Interestingly, Li et al. showed that phosphorylation of Cdc37 by Ulk1 enhances sensitivity of several colon cancer cell lines to Hsp90 inhibitors. Presumably after its activation by Hsp90-Cdc37, Ulk1 phosphorylates Cdc37-S339 leading to Hsp90 kinase client degradation (Fig. 11.2d). Ulk1 knockout (KO) DLD1 and HCT116 cells treated with an Hsp90 inhibitor (17-AAG or AUY922) were less sensitive to Hsp90 inhibition compared to Ulk1 WT cells (Li et al. 2017). Their finding supports a model by which Ulk1 phosphorylation of Cdc37 plays a role in proteasomal degradation of Hsp90 clients upon Hsp90 inhibition.

Protein Phosphatase 5 (PP5)

Protein phosphatase 5 (PP5) is a unique serine/threonine phosphatase and a member of the phosphoprotein phosphatase (PPP) family, which has recently been reviewed in great detail (Sager et al. 2020). PP5 regulates the chaperoning of numerous kinases and steroid hormone receptors and has been shown to work with the co-chaperones FNIP1 (Sager et al. 2019), FKBP51/52 (Gallo et al. 2007; Banerjee et al. 2008; Hamilton et al. 2018) and most notably Cdc37 (Vaughan et al. 2008; Oberoi et al. 2016; Prodromou 2017). Interaction of the amino-terminal TPR domain of PP5 with its C-terminal αJ helix locks PP5 in an auto-inhibited state, and association of the TPR domain with the Hsp90-MEEVD releases the auto-inhibition, enabling PP5 activation (Russell et al. 1999; Connarn et al. 2014; Haslbeck et al. 2015).

PP5-mediated dephosphorylation of Cdc37-S13 is a well-established mechanism to regulate the chaperoning of Hsp90 kinase clients (Vaughan et al. 2008). Mechanistically, it was shown that PP5 only dephosphorylates Cdc37 when both co-chaperones are bound to the same Hsp90 dimer (Vaughan et al. 2008). Interestingly, recent work has shown that an activating phosphorylation of PP5 allows for Hsp90-independent Cdc37 dephosphorylation (Dushukyan et al. 2017). Casein kinase 1δ (CK1δ) phosphorylates PP5-T362 in the absence of Hsp90, promoting its phosphatase activity and subsequent dephosphorylation of its substrates Cdc37 and GR (Oberoi et al. 2016; Dushukyan et al. 2017). Modulating PP5 activity also impacts Hsp90-binding affinity to its inhibitor ganetespib, likely via Cdc37

dephosphorylation, as expression of nonphosphorylatable Cdc37-S13A hypersensitized cells to the Hsp90 inhibitor geldanamycin (GA) (Oberoi et al. 2016; Vaughan et al. 2008). These works demonstrate a critical role for understanding the context-dependent modification of Hsp90 co-chaperones.

PP5 exhibits elevated expression in tumor cells and has an important role in tumorigenesis (Golden et al. 2008; Wang et al. 2015, 2018; Sager et al. 2020). Specifically, PP5 is overexpressed and hyperphosphorylated on T362 in clear cell renal cell carcinoma cells lacking the tumor suppressor ubiquitin ligase VHL (Dushukyan et al. 2017). Mechanistically, VHL ubiquitinates PP5-K185 and K199, leading to PP5 degradation and inactivation (Fig. 11.2e) (Dushukyan et al. 2017). Silencing PP5 using siRNA induces apoptosis, suggesting a pro-survival role for PP5 in cancer (Dushukyan et al. 2017). Accordingly, downregulation of PP5 has been also shown to facilitate apoptosis via G0/G1 phase cell cycle arrest (Wang et al. 2015; Zhi et al. 2015). PP5 depletion has the added effect of inducing Hsp90 hyperphosphorylation, which negatively regulates its chaperone function and the processing of client proteins (Wandinger et al. 2006). Taken together, these studies suggest that combined inhibition of Hsp90 and PP5 may show selectivity toward cancers addicted to Hsp90 client kinases.

Activator of Hsp90 ATPase 1 (Aha1)

Aha1 is a highly conserved 39-kDa protein that accelerates Hsp90 ATP hydrolysis by facilitating Hsp90 dimer N-terminal domain closure (Richter et al. 2008; Li et al. 2013; Retzlaff et al. 2010). Two-step binding of one Aha1 molecule to sites on both protomers of the Hsp90 dimer is sufficient to maximally stimulate Hsp90 ATPase activity (Retzlaff et al. 2010; Panaretou et al. 2002). Aha1 binding to Hsp90 impacts Hsp90-NTD and Hsp90-MD conformation, priming the catalytic Hsp90-R380 (yeast) for interaction with ATP, ultimately impacting client dwell time (Koulov et al. 2010; Meyer et al. 2003). Aha1 is the only co-chaperone known to accelerate Hsp90 ATPase activity in higher eukaryotes. Recently, Ids2 was identified as a co-chaperone for the yeast Hsp90 ortholog, Hsc82, which stimulates Hsc82 ATPase activity. Coordinated phosphorylation and dephosphorylation of Ids2-S148 by PKA and PP2C, respectively, was found to be imperative for Ids2 co-chaperone function (Chen et al. 2018). Notably, Ids2 does not have a known ortholog in higher eukaryotes.

Aha1 is recruited to Hsp90 by SUMOylation of Hsp90α-K191 on a single Hsp90 protomer (Mollapour et al. 2014). In addition to SUMOylation, phosphorylation, methylation, and acetylation of residues in Hsp90-NTD and Hsp90-MD have been shown to modulate the impact of Aha1 on Hsp90 activity (Mollapour et al. 2010, 2011b; Scroggins et al. 2007; Soroka et al. 2012; Xu et al. 2012, 2019; Rehn et al. 2020). These Hsp90 modifications have recently been reviewed in greater detail (Backe et al. 2020).

The tyrosine kinase c-Abl phosphorylates Aha1-Y223 promoting Aha1 association with Hsp90 and precedes Aha1 ubiquitination (Fig. 11.2f) (Dunn et al. 2015). The nonphosphorylatable Aha1-Y223F mutant did not stimulate the ATPase activity of Hsp90 nor form complexes with Hsp90, co-chaperones, or clients (Dunn et al. 2015). Notably, c-Abl-mediated phosphorylation of Aha1 displaces the co-chaperone Tsc1 from Hsp90 (Woodford et al. 2017). Mass spectrometric analysis revealed that phosphomimetic Aha1-Y223E preferentially interacted with proteins involved in metabolism, ribosomal components, and transcription and translation. Further, pharmacologic or genetic c-Abl ablation, and subsequent hypo-phosphorylation of Aha1-Y223, sensitized prostate cancer and renal cancer cells to Hsp90 inhibition (Dunn et al. 2015).

Aha1 expression is elevated in some cancers (Holmes et al. 2008). Moreover, knockdown of Aha1 increased cellular sensitivity to 17-AAG suggesting Aha1 levels could play a role in cellular response to Hsp90 inhibitors. Abrogating the function of the Aha1-Hsp90 complex might be beneficial in the context of the depletion of client proteins and kinase clients that are involved in tumor cell proliferation and survival. Several studies have examined the relationship between Aha1 expression and function and the efficacy of Hsp90 inhibitors. Aha1 overexpression in *L. donovani* increased radicicol (RD)-mediated inhibition of Hsp90, but not by geldanamycin (GA) (Bartsch et al. 2017). Conversely, Aha1 knockdown was shown to sensitize human colon cancer cells to the Hsp90 inhibitor 17-AAG (Holmes et al. 2008). The c-Abl inhibitor GNF-5, treatment of which led to hypo-phosphorylation of Aha1-Y223, hypersensitized tumors to Hsp90 inhibitors and caused increased apoptosis in prostate cancer and renal cancer cells (Dunn et al. 2015). Interestingly, the flavonoid TL-2-8 promotes breast cancer cell death, immature mitophagy, and downregulation of Hsp90 client proteins by disrupting the Aha1-Hsp90 complex (Liu et al. 2017). Aha1 has also been implicated in the increased production of pathogenic tau aggregates in Alzheimer's disease (AD). The Aha1 inhibitor KU-177 reduced the in vitro accumulation of toxic tau oligomers that correlated with (AD) progression (Shelton et al. 2017). These studies establish the importance of understanding the function and regulation of Aha1, as it shows promise as a therapeutic target in cancer and neurodegenerative disease.

Prostaglandin E Synthase 3 (p23)

There is a well-established role for the co-chaperone p23 in the maturation of Hsp90-dependent steroid hormone receptors (SHRs) such as glucocorticoid receptor (GR), estrogen receptor (ER), and progesterone receptor (PR) (Johnson and Toft 1995; McLaughlin et al. 2006; Graf et al. 2014; Xiao and Liu 2020). Upon ATP binding and Hsp90 N-terminal dimerization, p23 binds and stabilizes the closed, ATP-bound chaperone complex, providing the necessary dwell time for SHR maturation (Richter et al. 2004; Ali et al. 2006; McLaughlin et al. 2006; Martinez-Yamout et al. 2006; Woo et al. 2009; Blacklock and Verkhivker 2013; Cano et al. 2015; Borges et al.

2016; Gano and Simon 2010). Other co-chaperones including Aha1 and the p23-like Aarsd1L compete with p23 for binding to Hsp90 (Harst et al. 2005; Martinez-Yamout et al. 2006; Echeverria et al. 2016), while Hsp70/Hsp90-organizing protein (HOP) prevents the conversion of the ADP-Hsp90 to a state that can bind p23 (Johnson et al. 1998). Notably, recent work by Buchner's lab has provided evidence for Hsp90-independent chaperone functions of p23 (Biebl et al. 2021). The authors showed that a helical region in the unstructured tail of p23 is involved in direct interactions with the GR ligand-binding domain (LBD), which may stabilize the GR-LBD even in the absence of Hsp90. This is consistent with previous studies related to Hsp90-independent chaperone functions of p23 (Echtenkamp et al. 2011, 2016).

While posttranslational modification of Hsp90 regulates co-chaperone interaction (Backe et al. 2020), modification of the co-chaperones themselves provides an additional layer of regulation of the Hsp90 chaperone machinery. CK2-mediated phosphorylation of p23-S113 and p23-S118 is essential for p23 activation and association with Hsp90 (Kobayashi et al. 2004; Nakanishi et al. 2007). Interestingly, CK2 also phosphorylates the co-chaperones HOP (Lassle et al. 1997; Longshaw et al. 2000), Sgt1 (Bansal et al. 2009), Cdc37 (Shao et al. 2003b; Bandhakavi et al. 2003), FKBP52 (Miyata et al. 1997), and FNIP1 (Sager et al. 2019), as well as Hsp90 itself (Mollapour et al. 2011b, Lees-Miller and Anderson 1989), again highlighting the importance of CK2 activity to Hsp90 regulation (Miyata 2009).

Upregulation of p23 contributes to the progression of several cancers including prostate, breast, and lung as well as acute lymphoblastic leukemia (Mollerup et al. 2003; Oxelmark et al. 2006; Elmore et al. 2008; Reebye et al. 2012; Liu et al. 2012a; Cano et al. 2015). As p23 interaction with Hsp90 is nucleotide-dependent, ATP-competitive inhibitors such as geldanamycin (GA), macbecin, and radicicol (RD) could interfere with complex formation (Johnson and Toft 1995; Rehn and Buchner 2015). Concordantly, depletion of p23 hypersensitizes cells to Hsp90 inhibitors GA and RD (Forafonov et al. 2008; Bartsch et al. 2017). Interestingly, cancer cells treated with the C-terminal Hsp90 inhibitor gedunin induced caspase-dependent cleavage of p23 and cell death by apoptosis (Patwardhan et al. 2013).

Previous work has shown that treatment with the Hsp90 inhibitor 17-AAG shifts the binding of HDAC6 from Hsp90 to Hsp70, suppressing HDAC6 activity and promoting Hsp90 hyperacetylation (Kovacs et al. 2005). Hyperacetylation of Hsp90 decreases its affinity for ATP and subsequent p23 binding, resulting in the depletion of several Hsp90 client proteins (Kovacs et al. 2005, Scroggins et al. 2007, Rao et al. 2008, Yang et al. 2008). Taken together, targeting the Hsp90-p23 interaction can influence chaperone complex formation and drug sensitivity.

Immunophilins

Immunophilins are a class of peptidyl-prolyl cis/trans isomerases (PPIase) that include Cyp40, FKBP51, and FKBP52 and bind to Hsp90 through their TPR domain

(Pirkl et al. 2001). These co-chaperones can be found in Hsp90-steroid hormone receptor (SHR) maturation complexes along with the co-chaperone p23 (Faou and Tropschug 2003; Ratajczak et al. 2009, Zgajnar et al. 2019). Interestingly, immunophilins differentially regulate Hsp90 ATPase activity through the formation of distinct immunophilin-Hsp90-SHR complexes that lead to differential receptor function (FKBP51-GR, FKBP52-PR, Cyp40-ER) (Carrello et al. 1999; Davies and Sanchez 2005; Ratajczak et al. 2009).

Posttranslational regulation of immunophilin co-chaperones has recently been reviewed in detail (Daneri-Becerra et al. 2019) and will be contextualized here. FKBP51 binds to the open conformation of Hsp90 and decreases the rate of Hsp90 ATP hydrolysis (Oroz et al. 2018). Previous work has shown that FKBP51 is present in Cdk4-Hsp90-FKBP51 heterocomplexes that inhibit cell proliferation by preventing cyclin D1 binding and inhibiting Cdk4-T172 phosphorylation (Ruiz-Estevez et al. 2018). Interestingly, acetylation of FKBP51 at K28 and K155 promotes activity of the Hsp90 client Akt (Yu et al. 2017), suggesting a context-dependent role for FKBP51 in the regulation of Hsp90 client kinases. Although FKBP51 also appears subject to serine phosphorylation by PINK-1 (Boonying et al. 2019) and PKA (Toneatto et al. 2013), the effects of modification at individual phosphorylation sites have yet to be confirmed.

SUMOylation of FKBP51-K422 by the E3 SUMO ligase PIAS4 is essential for Hsp90 interaction and regulation of GR signaling (Antunica-Noguerol et al. 2016). Impairment of FKBP51 SUMOylation abolishes its interaction with both Hsp90 and GR, leading to recruitment of FKBP52 and nuclear translocation of GR (Antunica-Noguerol et al. 2016). Interestingly, SUMOylation of Hsp90 has been shown to recruit Aha1, thereby inducing closure of the Hsp90 dimer (Mollapour et al. 2014) and suggesting opposing roles for SUMOylation in the FKBP-mediated regulation of Hsp90-dependent GR transcriptional activity.

CK2-mediated phosphorylation of FKBP52-T143 attenuates its interaction with Hsp90 (Miyata et al. 1997), with a concomitantly impaired ability to activate GR (Fig. 11.2g) (Cox et al. 2007). Interestingly, phosphorylation of Hsp90 by CK2 has no impact on its interaction with FKBP52 (Miyata et al. 1997). Other works have shown that Hsp90-K292 and Hsp90-K294 acetylation reduced its interaction with FKBP52 (Scroggins et al. 2007; Prodromou 2016), highlighting the importance of PTM reversibility in the regulation of Hsp90-co-chaperone interactions.

SGTA

Small glutamine-rich TPR-containing protein alpha (SGTA) is an Hsp90 co-chaperone that is critical to cellular signaling in hormone-regulated tissues (Philp et al. 2013). Previous work has demonstrated that SGTA knockdown affects androgen receptor (AR) maturation and prostate cancer proliferation (Buchanan et al. 2007; Trotta et al. 2012, 2013; Paul et al. 2014). SGTA is also known to support the growth of non-small cell lung cancer though the stabilization of the

Hsp90 client PDGFRα (Moritz et al. 2010; Smyth et al. 2012). Phosphorylation of SGTA-S305, potentially by Akt2, augments this effect (Moritz et al. 2010). The impact of this modification on SGTA co-chaperone activity and Hsp90-binding dynamics remains under investigation.

Sgt1

Another TPR-containing Hsp90 co-chaperone, the unrelated suppressor of G_2 allele of SKP1 (Sgt1), is known to be regulated by phosphorylation (Gangula and Maddika 2017). Sgt1 dimerization is integral to kinetochore assembly, and this dimerization is antagonized by CK2 phosphorylation of Sgt1-S361 in *S. cerevisiae* (Bansal et al. 2004, 2009). The lack of Sgt1 dimerization precludes a functional Sgt1/Hsp90 complex which is necessary for kinetochore function and proper chromosome segregation. Interestingly, subsequent work has shown that nuclear translocation of Sgt1 is dependent on its phosphorylation at the same site, potentially by CK2, suggesting a complex regulatory role for CK2 phosphorylation of Sgt1 (Prus and Filipek 2011; Prus et al. 2011). Plk1 is a key mitotic regulatory kinase involved in kinetochore-microtubule attachment (Sumara et al. 2004). Sgt1-S331 (S361 in yeast) phosphorylation by Plk1 promotes kinetochore-microtubule attachment (Liu et al. 2012b), emphasizing a role for differential signaling input in the regulation of Hsp90 co-chaperone function.

C-Terminus of Hsc70-Interacting Protein (CHIP)

CHIP is a TPR-containing co-chaperone that acts as an E3 ubiquitin ligase, targeting unfolded proteins for proteasomal degradation (Edkins 2015). CHIP works in concert with Hsp70 and Hsp90 in the ubiquitination of client substrates such as CFTR, GR, ER, and p53 (Edkins 2015; Quintana-Gallardo et al. 2019). HOP and CHIP compete for binding to the Hsp70 and Hsp90 chaperones, providing a mechanism to control the balance between pro-folding and pro-degradation complexes (Stankiewicz et al. 2010; Muller et al. 2012; Edkins 2015).

Both CK2-mediated phosphorylation (Muller et al. 2012) and acetylation of Hsp90 (residues K69, K100, K294, K327, K478, K546, and K558) decrease Hsp90 binding to CHIP, suggesting these specific modifications represent pro-folding Hsp90 complexes (Scroggins et al. 2007; Yang et al. 2008). Indeed, it seems that CHIP participates in a negative feedback loop where CHIP ubiquitinates and targets HDAC6 for degradation, inducing Hsp90 hyperacetylation and attenuating CHIP-Hsp90 interaction (Rao et al. 2008; Cook et al. 2012). Interestingly, more than ten lysine residues on Hsp90 itself were identified to be targets of CHIP ubiquitination, resulting in proteasomal degradation of Hsp90 (Kundrat and Regan 2010b; Abu-Farha et al. 2011).

As a ubiquitin ligase, CHIP effector function is mediated by posttranslational modification. This suggests that posttranslational cross talk has an exaggerated impact on CHIP function and downstream targeting. In fact, CHIP-S20 phosphorylation by Cdk5 contributes to neuronal cell death via disruption of the interaction between CHIP and truncated apoptosis-inducing factor (tAIF) (Kim et al. 2016, 2018). A recent report shows that this residue can also be phosphorylated by protein kinase G, which enhances CHIP binding to Hsc70 and protein quality control following cardiac ischemic injury (Ranek et al. 2020).

New Co-chaperones

Folliculin-Interacting Proteins 1 and 2 (FNIP1/2)

FNIP1 and FNIP2 are large, multidomain proteins which have recently been identified as Hsp90 co-chaperones (Woodford et al. 2016b). Prior to the discovery that FNIP1/2 are co-chaperones, their functions were thought to be exclusive to mTOR regulation through stabilization of the tumor suppressor folliculin (FLCN) (Baba et al. 2006; Hasumi et al. 2008). Recently, a partial structure of FNIP2 was solved in complex with FLCN, Rag GTPase, and Ragulator complex. The structure shows that FNIP2 has two distinct domains, a longin and a DENN domain, and has several highly unstructured regions (Shen et al. 2019; Lawrence et al. 2019). FNIP1/2 play critical roles in cellular function and in disease, specifically cancer, through their association with FLCN (Hasumi et al. 2008, 2015; Baba et al. 2012). The carboxy-terminal end of FNIP1 interacts with Hsp90-MD and Hsp90-CTD, while FNIP2 interaction is confined to Hsp90-MD (Woodford et al. 2016b). The function of FNIP1/2 as Hsp90 co-chaperones provided an explanation for their protective role toward the tumor suppressor folliculin (FLCN) (Woodford et al. 2016b). FNIP1/2 also contribute to the chaperoning of additional Hsp90 kinase and non-kinase clients. The FNIPs are displaced from Hsp90 by Aha1 allowing the Hsp90/client complex to progress through the chaperone cycle (Woodford et al. 2016b).

FNIP1 co-chaperone activity is uniquely regulated by relay phosphorylation. CK2 phosphorylates FNIP1-S938 in the C-terminal domain leading to sequential phosphorylation of FNIP1-S939, FNIP1-S941, FNIP1-S946, and FNIP1-S948 (Sager et al. 2019). FNIP1 relay phosphorylation provides stepwise deceleration of Hsp90 ATPase activity and client activation. FNIP1 relay phosphorylation is reversed by the co-chaperone PP5. PP5 initially dephosphorylates FNIP1 on S948 residue which is essential for a complete dephosphorylation of all modified serine sites in a relay fashion. Dephosphorylation disrupts FNIP1 binding to Hsp90 and promotes O-GlcNAcylation of FNIP1-S938 (Sager et al. 2019). The addition of GlcNAc precedes ubiquitination of FNIP1-K1119 and subsequent proteasomal degradation (Sager et al. 2019).

FNIP1 is also ubiquitinated upon reductive stress. Under oxidative conditions, three cysteine residues in the middle, unstructured region of FNIP1 are oxidated

leading to FNIP1-mediated mitochondrial shutdown (Manford et al. 2020). Reductive stress reverses the oxidation of FNIP1 cysteine residues, allowing for the E3 ubiquitin ligase adaptor, FEM1B, to recognize FNIP1. FEM1B docks CUL2 onto FNIP1. CUL2, a component of an E3 ubiquitin ligase complex, polyubiquitinates FNIP1 leading to FNIP1 proteasomal degradation. FNIP1 degradation results in hyperactive mitochondrial function and increased ROS production. Fine-tuned oxidation of FNIP1 is therefore a key regulatory mechanism of mitochondrial function (Manford et al. 2020).

FNIP1 and FNIP2 are upregulated in a variety of cancer cell lines including breast, bladder, prostate, lung, colorectal, and renal cancer (Woodford et al. 2016b; Hasumi et al. 2008). FNIP1/2 also bind more to Hsp90 in these cancer cell lines compared to normal kidney (HEK293) cells. Further, FNIP1/2 protein levels were found to be higher in tumor tissue compared to normal from patient samples and upregulation of FNIP1/2 correlated with increased binding of Hsp90 to ganetespib (GB). Taken together, regulation of FNIP1 protein levels, via ubiquitination and degradation, in cancer is critical to sensitivity of Hsp90 to its inhibitors.

Tuberous Sclerosis Complex 1 (Tsc1)

Tsc1 was identified as an Hsp90 co-chaperone as a result of its known role in stabilization of its binding partner Tsc2 (Woodford et al. 2017; Benvenuto et al. 2000; Chong-Kopera et al. 2006). Notably, Tsc1 is also important for the stabilization and activation of many kinase and non-kinase clients. Mechanistically, the C-terminal portion of Tsc1 binds to the Hsp90-MD and decelerates Hsp90 ATPase activity. Additionally, Tsc1 serves as a scaffold to load Tsc2, and potentially other clients, onto the Hsp90 chaperone machinery. Interestingly, Tsc1 also decelerates Hsp70 ATPase activity; however, the impact of this on clients has not yet been determined (Natarajan et al. 2020). It is noteworthy that phosphorylation of Tsc1-T417 is required for interaction with Hsp70 (Inoue et al. 2010).

The Tsc1/Tsc2 complex has a well-established function inhibiting the mTOR pathway via Rheb inhibition. The observation that Tsc1 is phosphorylated during nocodazole-induced G2-M arrest led to the finding that Tsc1 is phosphorylated by cyclin-dependent kinase Cdk1 at T310, S332, and T1047. Mutation of these residues to nonphosphorylatable alanine causes increased suppression of p70S6K activity, suggesting that phosphorylation of these residues downregulates Tsc1 activity (Astrinidis et al. 2003). Interestingly, Tsc1-T310 required for Tsc1 binding to Plk1 (Astrinidis et al. 2006). Plk1 phosphorylates Tsc1-S467 and Tsc1-S578 leading to Tsc1 ubiquitination and proteasomal degradation. Cells expressing the phosphomimetic Tsc1-S467E/S578E show increased mTOR activity and hypersensitivity to mTOR inhibition by rapamycin (Li et al. 2018b). Notably, Plk1 is an Hsp90 client, and its stability is dependent on the co-chaperone Sgt1, whereas Plk1 expression is negatively correlated with Tsc1 expression (Simizu and Osada 2000; Martins et al. 2009; Astrinidis et al. 2006). Intriguingly, co-deletion of FNIP1 and

TSC1 promotes synergistic hyperactivation of mTOR and drives polycystic kidney disease (PKD) development (Centini et al. 2018). Together, the evidence shows complex regulation of mTOR activity and subsequent mitosis by posttranslational regulation of Tsc1.

Another mechanism of mTOR regulation through Tsc1 is the result of IKK-β-mediated phosphorylation of Tsc1-S487 and Tsc1-S511. IKKβ phosphorylation of Tsc1 suppresses Tsc1 activity, resulting in subsequent mTOR activation. Notably, the mechanism of Tsc1 suppression is similar to Plk1-mediated Tsc1 suppression. Mutation of Tsc1-S487 and Tsc1-S511 to phosphomimetic aspartic acid caused increased ubiquitination and a shorter half-life of Tsc1 (Lee et al. 2007). Tsc1-S511 was found to be hyperphosphorylated in breast cancer tissues with high IKKβ expression and promoted inflammation-mediated tumor angiogenesis. IKKβ phosphorylation of Tsc1-S511 also plays a role in insulin resistance (Lee et al. 2008). The diminished activity of Tsc1-pS511 results in hyperactivity of mTOR and downstream inhibitory phosphorylation of insulin receptor substrate 1 (IRS1) and decreased insulin response.

Although no studies have investigated the impact of posttranslational regulation of Tsc1 on its co-chaperone function, Tsc1 loss disrupts Hsp90 posttranslational regulation. Mutation and loss of function of Tsc1 leads to hypoacetylation of Hsp90-K407 and Hsp90-K419. Hypoacetylated Hsp90 has a higher ATPase activity and decreased affinity for Hsp90 inhibitors (Woodford et al. 2019). Hsp90 acetylation and drug binding can be restored by treating Tsc1 null cells with the HDAC inhibitor ACY-241, demonstrating a potential therapeutic utility in cancer.

TIMP-2

The tissue inhibitor of metalloproteinase-2 (TIMP-2) is a small extracellular protein responsible for regulating the activity of matrix metalloproteinases (MMPs) (Bourboulia and Stetler-Stevenson 2010; Brew and Nagase 2010, Olson et al. 1997). Together with its ability to impact angiogenesis, TIMP-2 is an established regulator of the tumor microenvironment (Kim et al. 2012; Remillard et al. 2014).

Recent work has demonstrated that TIMP-2 functions as a co-chaperone of extracellular Hsp90 (eHsp90) (Baker-Williams et al. 2019). Mechanistically, the NTD of TIMP-2 interacted with the MD of Hsp90, antagonizing eAha1 binding to eHsp90. The eHsp90-TIMP-2 chaperone complex was shown to mediate the inhibitory role of TIMP-2 toward its established extracellular target, the eHsp90 client MMP2 (Baker-Williams et al. 2019). In agreement, the presence of TIMP-2 sensitizes Hsp90 binding to its inhibitor, ganetespib (Baker-Williams et al. 2019), underscoring the importance of understanding TIMP-2-mediated regulation of eHsp90 chaperone function.

Previous work has also shown that extracellular phosphorylation of TIMP-2-Y90 by c-Src regulates its inhibitory activity toward MMP2 (Fig. 11.2h) (Sanchez-Pozo et al. 2018). This finding highlights the potential effect of co-chaperone

posttranslational modification on eHsp90 client activity; however, the impact of TIMP-2-Y90 phosphorylation on the direct regulation of eHsp90 chaperone activity remains unexplored.

Concluding Remarks

The Hsp90 chaperone machinery is a complex network of tightly regulated proteins which concertedly maintain a multitude of cellular pathways. Posttranslational modification of co-chaperones and Hsp90 itself provides regulation by controlling chaperone complex assembly, ATPase activity, and client activation to meet cellular requirements.

Hsp90 inhibitors have shown great promise for clinical utility. In fact, Taiho Pharmaceutical's Hsp90 inhibitor TAS-116 recently met its primary endpoint of prolonged progression-free survival in the phase III clinical trial CHAPTER-GIST-301 (Taiho 2021). However, reports of toxicity and induction of the heat shock response, as seen with other Hsp90 inhibitors, have led to the evolution of alternative approaches (Neckers and Workman 2012; Biamonte et al. 2010; Bagatell et al. 2000). Recent efforts toward inhibiting specific chaperone complexes have yielded encouraging results. In attempts to overcome the reported toxicity of Hsp90 inhibitors, Cdc37 and Hsp90/Cdc37 complex inhibitors have been developed (Wang et al. 2019; Zhang et al. 2008). The rationale for targeting Cdc37 was reviewed in detail and highlighted the importance of future investigation (Li et al. 2018a). Aha1-Hsp90 inhibitors have also been shown to selectively inhibit Aha1/Hsp90 complexes and hinder client activation (Stiegler et al. 2017). Another study showed that specific inhibition of Aha1/Hsp90 complexes was able to overcome the negative impact of Aha1 on maturation of mutant CFTR (Ihrig and Obermann 2017). Current developments toward chaperone complex inhibitors have been recently reviewed in detail (Gestwicki and Shao 2019; Serwetnyk and Blagg 2020). It is noteworthy that direct disruption of Hsp90 interaction with clients such as CDK4 by client-mimicking peptides has also been shown to be a promising strategy for selective Hsp90 kinase inhibition (Paladino et al. 2020). Treatment of clear cell renal cell carcinoma cell lines with CDK4 mimicking peptide caused dissociation of CDK4 from Hsp90 and apoptosis.

Several enzymes demonstrate the ability to modify a number of chaperone and co-chaperone proteins (Fig. 11.3). Hsp90-T90 phosphorylation by protein kinase A (PKA) has been shown to increase association with p23, PP5, and CHIP while decreasing interaction with HOP and Cdc37 (Mollapour et al. 2011b) and while also directly phosphorylating Cdc37 and FKBP51, suggesting bidirectional phosphorylation directs the formation of specific Hsp90 chaperone complexes (Wang et al. 2012). It is likely that other PTMs have a comparable effect on several co-chaperones simultaneously.

Similarly, it is common for a single enzyme to modify several co-chaperones with varied effects. The Hsp90 client Plk1 phosphorylates Sgt1, which had a positive

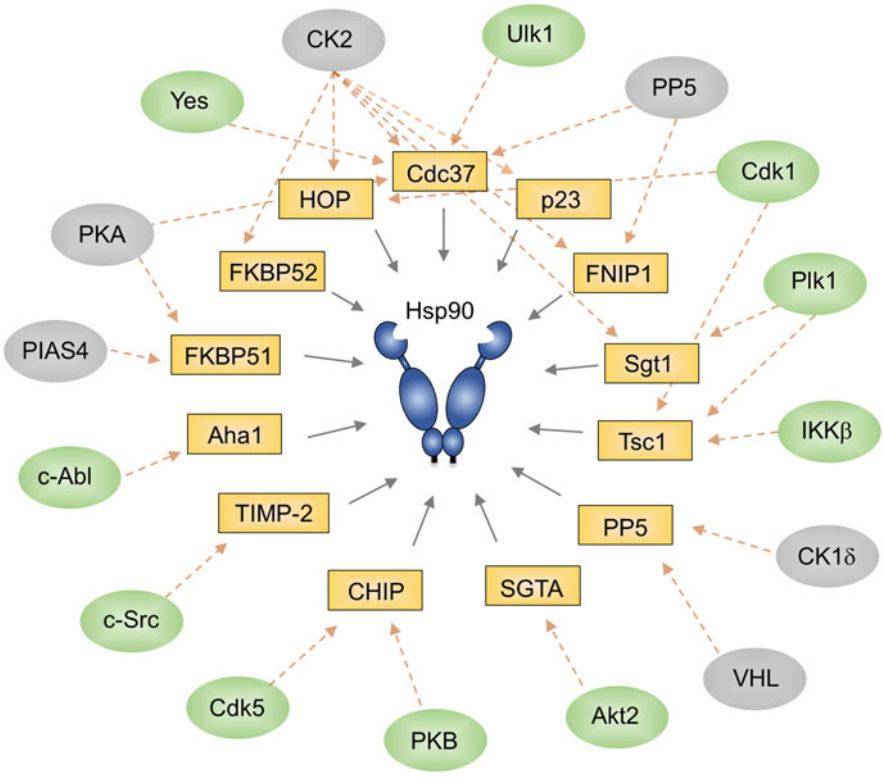

Fig. 11.3 A wide range of enzymes modify co-chaperones. Enzymes known to modify Hsp90 co-chaperones and alter their activity are represented in circles. Enzymes that are known Hsp90 clients are colored green. Co-chaperones discussed in this review are in yellow boxes

impact on Sgt1 function, whereas phosphorylation of Tsc1 by Plk1 led to its ubiquitination and degradation. CK2 phosphorylates several co-chaperones with greatly differing effects. Interestingly, phosphorylation of both HOP and Sgt1 by CK2 led to their translocation to the nucleus, while CK2 phosphorylation of Cdc37, p23, and FNIP1 enhances their binding to Hsp90 and is critical to their co-chaperone function. Notably, the opposite effect is observed when FKBP52 is phosphorylated by CK2. Phosphorylation is a frequent signal for co-chaperone/Hsp90 complex formation. It follows that PP5 may serve as common regulator of co-chaperone release from Hsp90, as PP5-mediated dephosphorylation of both Cdc37 and FNIP1 triggers their dissociation from Hsp90.

Most studies to date have focused on the impact of a singular PTM on a co-chaperone, leaving a gap in understanding of how the modification impacts the chaperone system as a whole. Stetz et al. thoroughly investigated a cross talk of structurally conserved Hsp90 PTMs and propose a model in which PTMs are allosterically coupled in an effector-sensor residue pair (Stetz et al. 2018). Further investigation into the commonality of this phenomenon or PTM cross-talk patterns

could yield insight into the impact of PTMs on overall changes in client chaperoning and subsequent cellular functions. A detailed characterization of the PTM makeup of specific Hsp90-co-chaperone-client complexes will provide the ability to identify unique druggable targets and a fundamental understanding of the chaperone code.

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Chapter 12
CHIP: A Co-chaperone for Degradation by the Proteasome and Lysosome

Abantika Chakraborty and Adrienne L. Edkins

Abstract Protein homeostasis relies on a balance between protein folding and protein degradation. Molecular chaperones like Hsp70 and Hsp90 fulfill well-defined roles in protein folding and conformational stability via ATP-dependent reaction cycles. These folding cycles are controlled by associations with a cohort of non-client protein co-chaperones, such as Hop, p23, and Aha1. Pro-folding co-chaperones facilitate the transit of the client protein through the chaperone-mediated folding process. However, chaperones are also involved in proteasomal and lysosomal degradation of client proteins. Like folding complexes, the ability of chaperones to mediate protein degradation is regulated by co-chaperones, such as the C-terminal Hsp70-binding protein (CHIP/STUB1). CHIP binds to Hsp70 and Hsp90 chaperones through its tetratricopeptide repeat (TPR) domain and functions as an E3 ubiquitin ligase using a modified RING finger domain (U-box). This unique combination of domains effectively allows CHIP to network chaperone complexes to the ubiquitin-proteasome and autophagosome-lysosome systems. This chapter reviews the current understanding of CHIP as a co-chaperone that switches Hsp70/Hsp90 chaperone complexes from protein folding to protein degradation.

Keywords CHIP · STUB1 · Co-chaperones · Ubiquitin · Proteasome · Lysosome · Autophagy

Introduction to Molecular Chaperones and Protein Degradation

A molecular chaperone is a protein that participates in the conformational regulation and folding of a range of substrate proteins, known as client proteins. Chaperones function under physiological conditions to maintain protein homeostasis and are also

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vital during or after stressful conditions to prevent or reverse the potentially disastrous consequences of protein aggregation for the cell (Agashe and Hartl 2000; Landry and Gierasch 1994; Hartl 1996; Welch and Brown 1996; Fedorov and Baldwin 1997; Ellis 1997). Many molecular chaperones are members of the heat shock protein family (HSP). In particular, Hsp70 and Hsp90 chaperones, as part of multiprotein complexes, regulate both de novo and stress-related protein folding and stability. Hsp70 and Hsp90 are ATP-dependent chaperones who participate in protein folding cycles that involve multiple phases of client binding and release (Wegele et al. 2004). Hsp70 captures hydrophobic regions in substrates and in collaboration with Hsp90 prepares client proteins for spontaneous folding (Morán Luengo et al. 2018). The activities of both chaperone complexes are dependent on interactions with several protein cofactors, known as co-chaperones. A co-chaperone is defined as a non-client accessory protein that lacks intrinsic chaperone activity but functions to modulate the activity of a known chaperone (Caplan 2003). Co-chaperones act at every stage of the chaperone folding cycle and control progression of the client protein through these cycles by a range of mechanisms, including regulation of ATPase activity, direct protein-protein interactions, and posttranslational modifications. Co-chaperones may be selective for one family of chaperones or may interact with multiple chaperone families (Li et al. 2012).

The Hsp70-Hsp40 chaperone complex is one of the main foldase complexes in the cell, participating in both de novo and stress-related protein folding (Landry and Gierasch 1994; Cheetham et al. 1994; Strickland et al. 1997; Hiromura et al. 1998). Hsp40 (DNAJ) co-chaperones deliver client proteins to Hsp70 and regulate the affinity of Hsp70 for these client proteins by stimulating the ATPase activity of Hsp70 (Cheetham et al. 1994). The stimulation of Hsp70 ATPase converts Hsp70 into the high-affinity substrate-binding form and leads to binding of the client protein by Hsp70 and prevention of misfolding or aggregation. This form of Hsp70 is ADP-bound and is stabilized by another co-chaperone, known as Hsc70/Hsp70-interacting protein (Hip). Client proteins are subsequently released from Hsp70 via nucleotide exchange, which returns the Hsp70 to the ATP-bound form, which has low affinity for the substrate. This stage is catalyzed by the GrpE (in prokaryotes) or BAG-1/HspBP1 (in eukaryotes) co-chaperones (Hohfeld 1998; Chang et al. 2010; Mao et al. 2006; Kabani et al. 2002).

A subset of client proteins will be passed from the Hsp70 chaperone complex to the Hsp90 chaperone complex. Hsp90 is also an ATP-dependent chaperone, but its function primarily relates to the maintenance of protein stability of labile client proteins prior to their activation (Prodromou et al. 1997; Panaretou et al. 1998) The Hsp90 complex is also able to stabilize several mutated client proteins, thereby preventing their degradation (Whitesell et al. 1998). Hsp90 is constitutively dimerized at the C-terminus and exists in an open conformation (resembling a V shape) when inactive (Ali et al. 2006). Once bound by Hsp90, the client protein then transitions through the Hsp90 cycle, a process which involves the sequential interaction with a range of co-chaperones (McLaughlin et al. 2002; Li et al. 2012). The early stages of Hsp90-mediated folding involve the transfer of client proteins between Hsp70 and Hsp90, regulated by the co-chaperone Hop (Frydman and

Hohfeld 1997; Brinker et al. 2002; Siligardi et al. 2004). Hop is a member of the TPR (tetratricopeptide repeat) domain containing co-chaperones that can simultaneously bind to both Hsp70 and Hsp90 (Prodromou et al. 1999). The TPR domain is a protein-protein interaction module that is found in a wide range of proteins (Allan and Ratajczak 2011). TPR domains are comprised of multiple copies of a TPR motif, which gives rise to a particular alpha helical structure. The TPR motif is loosely defined by a 34-residue degenerate consensus sequence (Tpr-Leu-Gly-Tyr-Ala-Phe-Ala-Pro). Therefore, while the primary sequence varies substantially between different TPR domains, the overall structure is conserved. Most TPR domains are comprised of three TPR motifs, each of which contributes six alpha helices, which pack together to form an alpha helical amphipathic groove. This groove is the site of interaction with the target peptide (Brinker et al. 2002). TPR-containing chaperones bind to the C-terminal EEVD motifs contained in both Hsp70 and Hsp90 (Odunuga et al. 2003; Blatch and Lassle 1999; Van Der Spuy et al. 2000). Hop binds to the complex as a monomer via one EEVD motif in the Hsp90 dimer, while the other EEVD motif may be bound by a peptidyl prolyl isomerase (PPIase) leading to the formation of the asymmetric intermediate complex. The binding of ATP and the interplay between Hop and the late co-chaperones p23 stimulate conformational change in Hsp90 to the closed conformation (Prodromou et al. 2000; Dahiya et al. 2022). While Hop is not essential for chaperone activity by the Hsp70-Hsp90 complex (Bhattacharya et al. 2020), Hop increases the efficiency of client transfer between the chaperones and hence optimizes progression of the client through the chaperone-mediated folding process (Dahiya et al. 2022). NudC is another co-chaperone that regulates client transfer between Hsp70 and Hsp90, but via a novel mechanism. NudC promotes transfer of Hsp40-bound client proteins directly to Hsp90 for activation by removing Hsp70 from Hsp40-Hsp70-client complexes (Biebl et al. 2022). ATP hydrolysis by Hsp90 is subsequently stimulated by Aha1, which returns Hsp90 to the open conformation and results in release of the client protein (Lotz et al. 2003). Co-chaperones like Hop, p23, and Aha1 are considered general co-chaperones, as they constitute the core co-chaperones required for the transition of general client proteins through the cycle. In addition, there exists a range of other co-chaperones that may associate with Hsp90 complex for specific functions, such as regulation of specific classes of client proteins (like Cdc37) or posttranslational modification (e.g., PP5) (Li et al. 2012).

The term chaperone is associated with protein folding. However, chaperones also participate in the degradation of proteins via the proteasome (Kriegenburg et al. 2012; Esser et al. 2004; Kettern et al. 2010; Kastle and Grune 2012) and lysosome (Agarraberes and Dice 2001; Kettern et al. 2010; Kaushik and Cuervo 2012). This role for chaperones is opposing, yet complementary, to their role in promoting protein folding and is consistent with a role as regulators of global protein homeostasis (Hohfeld et al. 2001; Imai et al. 2003). Molecular chaperones are thought to be able to identify and capture misfolded protein substrates for them to be directed to the proteasome (Bercovich et al. 1997; Kriegenburg et al. 2012; Meimaridou et al. 2009) or the lysosome (Agarraberes and Dice 2001; Kettern et al. 2010; Kaushik and Cuervo 2012). The involvement of chaperones, particularly Hsp70 and Hsp90, in

proteasomal- and lysosomal-mediated protein degradation is also regulated by co-chaperones, including the Hsp70/Hsp90 co-chaperone, carboxyl terminus of Hsp70-interacting protein (CHIP) (Murata et al. 2001; McDonough and Patterson 2003; Arndt et al. 2010; Ferreira et al. 2013; Guo et al. 2015; Maan and Pati 2018).

Protein Degradation via the Proteasome

The ubiquitin-proteasome system is a highly conserved mechanism through which eukaryotic cells facilitate the controlled enzymatic degradation of unwanted proteins (Amm et al. 2014; Ciechanover 1998) (Fig. 12.1). The ubiquitin and proteasome systems work in concert to regulate protein levels in eukaryotic cells (Roos-Mattjus and Sistonen 2004; Wolf et al. 2004; Lecker et al. 2006). The proteasome not only regulates the degradation of incorrectly folded proteins but is also important for the degradation of proteins whose expression needs to be temporally regulated (such as cell cycle kinases) (Wagner et al. 2011). The proteasome is composed of the 20S core proteasome and the 19S regulatory components. A single 20S core proteasome associates with two 19S regulatory particles to form the active 26S proteasome (Murata et al. 2009; Walz et al. 1998). Polyubiquitinated proteins targeted for degradation need to be deubiquitinated and delivered to the proteolytic active site of the proteasome that is buried within the 20S core particle (da Fonseca and Morris 2008; Heinemeyer et al. 2004).

Degradation of proteins by the proteasome is preceded by the conjugation of ubiquitin to the substrate via a series of sequential enzyme-catalyzed reactions (Fig. 12.1) (Hershko and Ciechanover 1998). Ubiquitin is initially activated by conjugation to a ubiquitin-activating enzyme (E1) in an ATP-dependent manner via a thioester linkage (Lee and Schindelin 2008). Ubiquitin is subsequently transferred via a ubiquitin-conjugating enzyme (E2) intermediate to the substrate protein targeted for degradation (Olsen and Lima 2013). This reaction is catalyzed by a ubiquitin ligase enzyme (E3) and results in the formation of a peptide bond between a glycine residue in the C-terminus of ubiquitin and lysine residues within the substrate protein (Scheffner et al. 1995; Wilkinson 2000). There are a range of different E2 and E3 isoforms that may combine for different substrate proteins, suggesting a diverse and discriminatory recognition system for ubiquitin conjugation (Spratt et al. 2012). This process may be repeated many times, often involving the conjugation of subsequent ubiquitin molecules to lysines within ubiquitin itself, leading to the formation of covalently linked polyubiquitin chains. A fourth enzyme may also be involved in this cascade. Known as E4, this protein acts as a ubiquitin chain elongation enzyme to catalyze the assembly of polyubiquitin chains on protein substrates (Koegl et al. 1999). While monoubiquitination may induce changes in activity or subcellular localization of proteins, the conjugation of a polyubiquitin chain to a substrate protein is required for degradation by the proteasome (Johnson et al. 1992). Ubiquitin contains seven lysines residues (K6, K11, K27, K29, K33, K48, and K63), in addition to its N-terminus, which act as potential sites of

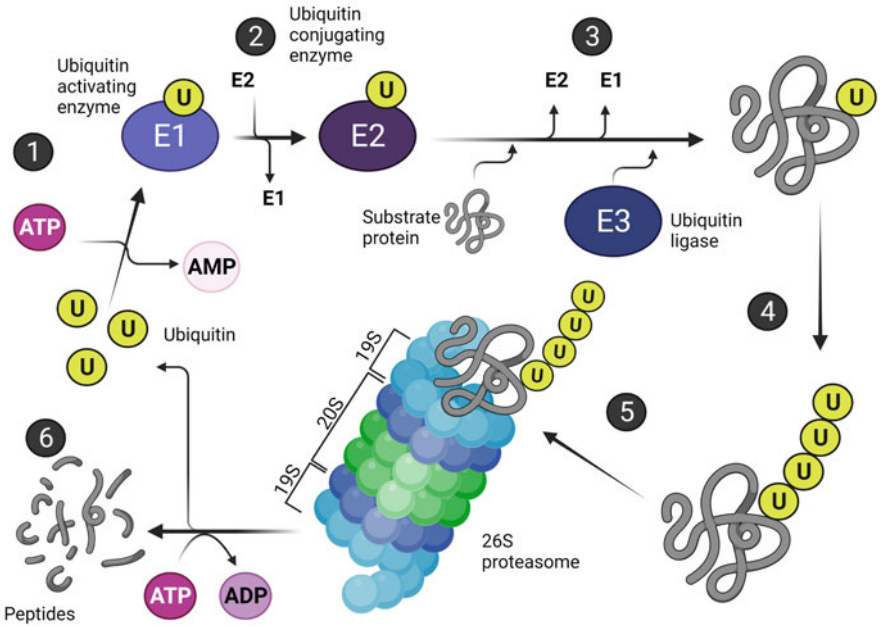

Fig. 12.1 Degradation of proteins via the ubiquitin-proteasome system. Conjugation of polyubiquitin chains to substrate proteins is catalyzed as part of an ATP-dependent enzyme-catalyzed cascade that precedes protein recognition and degradation by the proteasome. (1) The process is initiated by the activation of ubiquitin via conjugation to a ubiquitin-activating enzyme (E1) via a thioester bond. (2) The ubiquitin moiety is subsequently transferred to ubiquitin-conjugating enzyme (E2), which (3) subsequently forms a complex with a specific ubiquitin ligase (E3) and a substrate protein. (4) The E3 ligase transfers the ubiquitin to lysine residues within the substrate protein. This cycle is repeated multiple times to generate substrate proteins linked to polyubiquitin chains. (5) Polyubiquitination, particularly via K48 linkages, is the signal for transfer of substrate proteins to the proteasome. (6) At the proteasome, ubiquitinated substrates interact with the 19S regulatory particle which deubiquitinates them and passes them into the central cavity of the core 20S proteasome. Here, in the active site, proteins are degraded and peptides released. Ubiquitin molecules can subsequently be recycled. 19S, regulatory particle; 20S, core particle. Image created with BioRender

conjugation. The lysine residue involved in the bond can also impact on the outcome of ubiquitination (Hershko and Ciechanover 1998). K48-linked ubiquitin chains, where the covalent linkage of the ubiquitin chain is via the K48 residue of ubiquitin, are the canonical signal for proteasomal degradation (Jacobson et al. 2009). K48 linkage is regulated by members of the Ubc4, Ubc5, and Ubc7 E2 ubiquitin-conjugating enzymes. In contrast, K63 ubiquitination may have a regulatory function (Jacobson et al. 2009) and is catalyzed by the Ubc13 E2 in complex with other Ubc proteins (e.g., Uev1a) (Hofmann and Pickart 1999; Sun and Chen 2004). Most often, the selectivity of protein degradation is controlled by the E3 ligase. These E3 ligases integrate with the cellular molecular chaperone system, which is used as the recognition system of the misfolded substrate during this process. In this way, the

numerous different E3 isoforms, each of which may be specific for certain protein substrates, are able to utilize the innate ability of chaperones to capture a range of misfolded substrates, to target specific proteins for degradation (Kriegenburg et al. 2012; Esser et al. 2004).

Protein Degradation via Autophagy and the Lysosome

Protein degradation is also mediated via the lysosome in a process known as autophagy. Often activated under conditions of nutrient deprivation or cellular stress, autophagy is responsible for the catabolism of soluble proteins, insoluble protein aggregates arising from misfolding or pathological mutations and damaged organelles (like mitochondria and peroxisomes) (Groll and Huber 2003, 2004; Klionsky 2007; Mizushima 2007). There are three main autophagic pathways, namely, macroautophagy, microautophagy, and chaperone-mediated autophagy (Fig. 12.2).

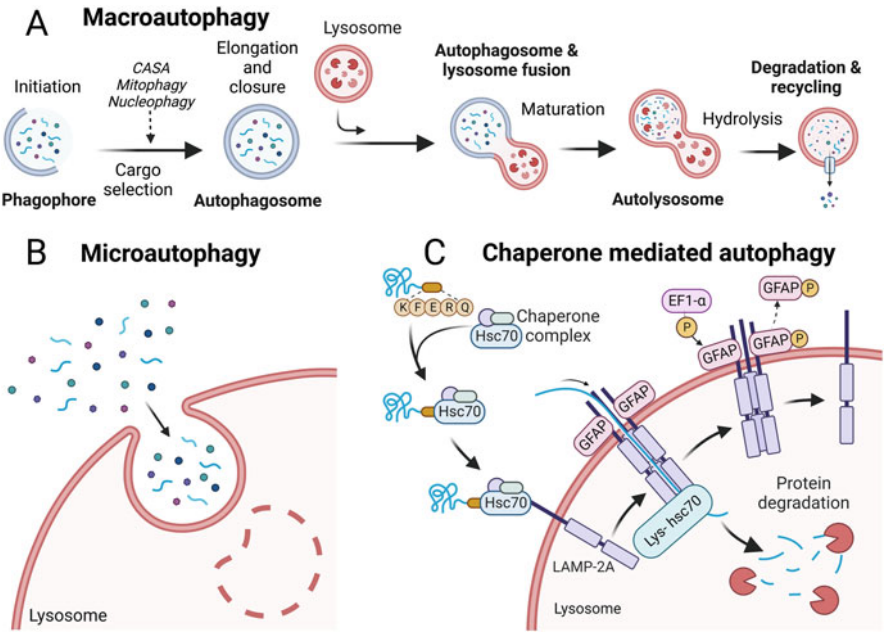

Fig. 12.2 Three main autophagy-lysosomal degradation pathways. Degradation of cellular cargo via the lysosome can occur via three main autophagic pathways. (**a**) In macroautophagy, the cargo for degradation is engulfed in LC3-positive phagophores which subsequently fuse with the lysosome to produce autophagosomes in which the contents are degraded. (**b**) In microautophagy, cellular cargo for degradation is taken up directly into the lysosome, and (**c**) in chaperone-mediated autophagy (CMA), a multichaperone complex recognizes a KFERQ-like motif in the cargo for degradation and delivers it to lysosomes where they are transported into the lysosome via LAMP2A and lysosomal-resident Hsp70. Image created with BioRender

These processes all converge on the lysosome but are differentiated by the nature of the substrate and the regulation and mechanism of substrate recognition (Shintani and Klionsky 2004; Mizushima et al. 2008; Bejarano and Cuervo 2010; Khandia et al. 2019; Park and Kang 2020; Aman et al. 2021).

In macroautophagy, the cytosolic cargo is compartmentalized into a double-membraned vesicle known as the autophagosome, which fuses with lysosomes to give rise to an autophagosome in which the cargo is degraded by lysosomal enzymes (Nakatogawa et al. 2009; Ravikumar et al. 2009) (Fig. 12.2a). Macroautophagy can be either selective or non-selective. Chaperone-assisted selective autophagy (CASA) is a form of macroautophagy where molecular chaperones recruit machinery to promote macroautophagy of ubiquitinated protein aggregates (Carra et al. 2008; Arndt et al. 2010). In addition, selective macroautophagy exists for organelles including mitochondria (mitophagy) (Lemasters 2005), peroxisomes (pexophagy) (Katarzyna and Suresh 2016), lysosomes (lysophagy) (Maejima et al. 2013), endoplasmic reticulum (ER-phagy) (Schuck et al. 2014), and the nucleus (nucleophagy) (Mochida et al. 2015).

Microautophagy involves non-selective lysosomal degradation, wherein damaged organelles or soluble proteins enter the lysosomes in the form of small vesicles derived from invaginations of lysosomal membranes (De Duve and Wattiuax 1966; Saito and Ogawa 1974; Sattler and Mayer 2000; Roberts et al. 2003) (Fig. 12.2b). In yeast, microautophagy is involved in clearance of damaged peroxisomes (micropexophagy) (Tuttle and Dunn 1995; Sakai et al. 1998), mitochondria (micromitophagy) (Campbell and Thorsness 1998), parts of nucleus/piecemeal microautophagy (PMN) (Roberts et al. 2003), ER (micro-ER-phagy) (Schuck et al. 2014), and lipid droplets (Van Zutphen et al. 2014).

Chaperone-mediated autophagy (CMA) is a specialized, selective lysosome-dependent proteolytic pathway for cytosolic proteins (Isenman and Dice 1989) (Fig. 12.2c). CMA is triggered upon exposure to cell stressors like starvation, hypoxia, and protein aggregation. Unlike macroautophagy, CMA does not require either autophagosomes or autophagolysosome formation, as the protein cargo is presented directly to the lysosome through Hsc70 complexes (which include Hsp90 and co-chaperones like Hop, Hip, Hsp40, and BAG-1) and LAMP2A (lysosome-associated membrane protein type IIA) (Agarraberes and Dice 2001; Bejarano and Cuervo 2010; Arias and Cuervo 2011; Tekirdag and Cuervo 2018). CMA activity is indicated by increased LAMP2A mRNA levels, co-localization of LAMP2A and Hsc70 and perinuclear LAMP2A-positive lysosomes (Patel and Cuervo 2015; Arias 2017). Substrates targeted for CMA contain a pentapeptide "KFERQ" motif where at least the Q amino acid must be present on either side of the pentapeptide together with a basic, acidic, and hydrophobic amino acid. Proteins targeted for CMA require at a least one KFERQ-like motif although multiple motifs are also observed (Cuervo et al. 2000). Hsc70 recognizes the KFERQ motif and directs the cargo to lysosomes (Chiang et al. 1989; Stricher et al. 2013). The KFERQ motif binds Hsp70 at a site distinct from the substrate-binding cleft and with significantly lower affinity than substrate-binding peptides (Taylor et al. 2018). Within the lysosomal lumen, lysosomal Hsc70 (lys-Hsc70) supports internalization of the substrate (Agarraberes et al.

1997), while Hsp90 also supports in cargo recognition, uptake, and translocation into lysosomes. Cytosolic Hsc70 promotes disassembly of LAMP2A from complexes, whereas Hsp90 stabilizes LAMP2A at the lysosomal membrane (Bandyopadhyay et al. 2008). Hsc70 also recycles LAMP2A when there is no substrate by causing destabilization of LAMP2A at the membrane interface (Bandyopadhyay et al. 2008). Ubiquitination is also used as a signal for controlling autophagy. K63 ubiquitination, of either substrates or autophagy-related proteins, is associated with increased autophagy, while K48 and K11 ubiquitination of autophagy intermediates results in proteasomal degradation and hence blocks autophagy (Chen et al. 2019). The primary mediator of K63 polyubiquitination is the E2 ligase Ubc13 which functions in concert with a range of E3 ligases, including CHIP (Zhang et al. 2005a, b; Hodge et al. 2016).

There is a cross talk between the different autophagic pathways and the ubiquitin-proteasome pathway. Chronic inhibition of CMA triggers macroautophagy (Massey et al. 2006), while acute inhibition results in dysfunctional macroautophagy (Massey et al. 2008). In contrast, blocking macroautophagy upregulates CMA (Kaushik et al. 2008). Acute proteasomal inhibition upregulates macroautophagy, while chronic inhibition of the proteasome results in deregulated macroautophagy (Ding et al. 2003; Iwata et al. 2005). Certain proteasomal subunits are also cleared by CMA which links CMA inhibition to a nonfunctional proteasome (Cuervo et al. 1995; Massey et al. 2008).

The Carboxyl Terminus of Hsp70-Interacting Protein (CHIP)

The carboxyl terminus of Hsp70-interacting protein (CHIP, also known as STIP1 homology and U-box-containing protein 1 or STUB1) has dual functions, one as a co-chaperone of Hsp70 and Hsp90 and the other as an E3 ubiquitin ligase to regulate degradation of chaperone client proteins (McDonough and Patterson 2003; Ballinger et al. 1999). In this way, CHIP is a major link between chaperone-mediated folding and protein turnover. CHIP is distinguished from the other Hsp90 co-chaperones in that it is primarily involved in tuning the chaperone complexes toward protein degradation, rather than supporting protein folding (Demand et al. 2001). The CHIP gene is conserved in a range of eukaryotes, being demonstrated, or predicted to exist in the genomes of the human, monkey, mouse, zebra fish, fruit fly, frog, and even the genome and transcriptome of the coelacanth (*Latimeria* sp.) (Tastan Bishop et al. 2013). CHIP knockout mice were viable and displayed normal development, suggesting that CHIP is not an essential gene (Morishima et al. 2008; Dai et al. 2003). However, CHIP-deficient mice developed severe phenotypes associated with premature aging including cardiovascular stress, motor malfunction, neurodegeneration, altered mTORC signaling, and early death (Lizama et al.

2018a, b). CHIP null mice showed wasting of the thymus, which is an indicator of reduced ability to cope with stress. The link between peripartum death of CHIP null mice and stress was subsequently supported by the fact that CHIP-/- mice were temperature-sensitive and that induction of stress in these animals induced apoptosis in multiple organs after challenge (Dai et al. 2003). Mice lacking CHIP also show cardiac effects, with increased arrhythmia, increased myocardial apoptosis, and incapability to induce HSP70 synthesis, although cardiac-restricted overexpression of CHIP was sufficient to alleviate the disease-related phenotypes (Zhang et al. 2005a, b).

CHIP translocates to the nucleus in response to oxidative stress (Ullah et al. 2020), while overexpression activated the stress response by specifically inducing trimerization and nuclear translocation of HSF-1 and activation of HSE containing stress-responsive promoters, like Hsp70 (Dai et al. 2003). This suggests that the role of CHIP is not exclusively linked to protein degradation but also involves regulation of the stress response. Notwithstanding its role in activation of the stress response, CHIP appears to be a master regulator of protein degradation via chaperones, although it is by no means the only co-chaperone associated with protein degradation. The Hsp40 isoform, Hsj1 (DNAJB2) (Chapple et al. 2004; Westhoff et al. 2005; Gao et al. 2011), and the nucleotide exchange factor, Bcl2-associated athanogene (BAG) proteins, have defined roles in proteasome-mediated protein degradation (Luders et al. 2000; Alberti et al. 2002, 2003; Elliott et al. 2007). Hop (STIP1), which competes with CHIP for Hsp90 and Hsp70 binding and has traditionally been considered a pro-folding cofactor, has also emerged as important for proteasomal function, since Hop knockout reduced proteasome activity in vitro (Bhattacharya et al. 2020). In addition, there are other E3 ubiquitin ligase proteins (e.g., Ubr1, Cul5, Parkin, Mdm2) that may associate with Hsp90 and/or Hsp70 chaperone complexes to target client proteins for ubiquitination and degradation (Nillegoda et al. 2010; Eisele and Wolf 2008; Ehrlich et al. 2009). These proteins can induce ubiquitination in the absence of CHIP, although there is also evidence that inhibition of these E3 ligases can affect protein folding and degradation even in the presence of CHIP. Functional redundancy exists between the E3 ligases and is possible that multiple members will collaborate as a complex to control degradation of specific proteins (Morishima et al. 2008).

In addition to regulating protein targeting to the proteasome, CHIP has also been linked to protein degradation by autophagy. The CHIP null mouse phenotype more closely recapitulates models of autophagy dysfunction than mild phenotypes observed with E3 ligase mutation (Lizama et al. 2018a, b). CHIP regulates autophagy via mechanisms that appear to be dependent on both ubiquitination and Hsp70 interaction. TFEB is a transcription factor that controls expression of genes linked to autophagy and lysosome synthesis that is negatively regulated by serine phosphorylation by mTOR and PPP3Cβ. Non-phosphorylated TFEB translocates to the nucleus to transcribe genes that promote autophagosome, lysosome, and mitochondrial synthesis. CHIP binds preferentially to phosphorylated TFEB, recruiting it for degradation via the ubiquitin-proteasome pathway (Rao et al. 2017; Sha et al. 2017). Starvation or mTOR inhibition dissociates CHIP from TFEB allowing TFEB

to accumulate and promote autophagy. CHIP-deficient MEF cells accumulate inactive phosphorylated TFEB, resulting in cytoplasmic accumulation and sequestering of non-phosphorylated TFEB into heterodimers producing a dominant negative effect. The subsequent reduction in TFEB activity prevents autophagy and mitochondrial biogenesis. This phenotype could be rescued by a constitutively active TFEB mutant in the CHIP-deficient background. In this way, CHIP regulates autophagy indirectly by controlling the proteasomal-mediated degradation of TFEB (Rao et al. 2017; Sha et al. 2017). CHIP depletion also perturbed autophagic flux due to an increase in PTEN levels and concomitant decreased ULK1 phosphorylation and reduced AKT/mTOR activity. While CHIP depletion stimulated autophagosome accumulation, CHIP loss blocked autophagosome-lysosome fusion leading to reduced p62 autophagic substrate processing. Consistent with this, CHIP-depleted cells were more sensitive to the autophagy inhibitor bafilomycin A1 (Guo et al. 2015).

Structure of CHIP

CHIP is a 35-kDa protein, expressed as a single isoform containing an N-terminal TPR domain together with a U-box domain linked via a long helical region (Ballinger et al. 1999). The crystal structure of murine CHIP was determined in 2005 by Pearl and colleagues (Zhang et al. 2005b). This structure demonstrates that CHIP is dimerized at the C-terminus via the U-box domains (Fig. 12.3). The murine CHIP homodimer from this study was shown to be asymmetrical, with the two monomers of the dimer adopting different structures. The structural difference in the two monomers is predominantly in the structure of a long helical region that links the TPR and U-box domains. This region, termed the helical hairpin, is formed from two continuous antiparallel alpha helices in an extended conformation in one protomer. In contrast, in the other monomer, the helical hairpin adopts a bent conformation, and the seventh helix is split into two helices (as opposed to being a single continuous helix) (Fig. 12.3). C-terminal to the helical hairpin domain is the U-box domain, which is composed of beta hairpins separated by alpha helices. Interestingly, the structure of the helical hairpin and U-box region of *D. rerio* CHIP presents a symmetrical dimer that differs from the asymmetric dimer observed in the crystal structure of the full-length mouse CHIP (Xu et al. 2006). This discrepancy may be a consequence of the absence of the TPR domains in this structure or may reflect the fact that crystal structures are static and may capture only one form of a dynamic structure. Consistent with this, studies demonstrate that the full-length human CHIP homodimer appears highly flexible in solution (Graf et al. 2010). Specific changes in the CHIP conformation were noted upon interaction with either chaperones or E2 enzymes. Binding of Hsp70/Hsp90 chaperones or chaperone-derived peptides to CHIP promoted stabilization of the TPR domains, while distinct changes were observed upon interaction with E2-conjugating enzymes (UbcH5a and Ubc13) (Graf et al. 2010).

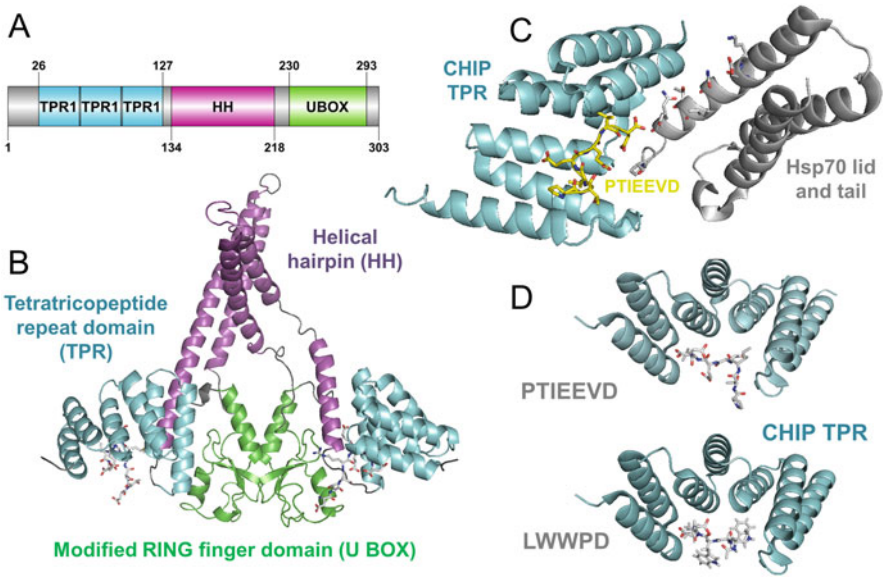

Fig. 12.3 Domain architecture and structure of CHIP. (**a**) Domain structure of CHIP showing the N-terminal TPR domain (composed of three TPR motifs) and the C-terminal U-box separated by the helical hairpin region. (**b**) The asymmetric dimer structure observed in the crystal structure of murine CHIP. The U-box domain (green) is the point of dimerization. The structure of the TPR (cyan, helices 1–6) and U-box domains are largely conserved between the two protomers. The helical hairpin (magenta, helices 7–8) region differs substantially. The C-terminal MEEVD peptide from Hsp90 is shown in a stick format and colored gray. (**c**) Binding of CHIP to the Hsp70 EEVD motif (yellow) and C-terminal lid domain (gray). Comparison of the binding modes of the (**d**) Hsp70 PIEEVD and LWWPD peptides to the TPR domain of CHIP. The images in (**b**–**d**) were generated using PyMOL (DeLano Scientific). PDB codes: 2C2L, 3Q49, 6NSV, and 4KBQ

Interaction of CHIP with Chaperones and E2 Ligases

The crystal structure of the CHIP dimer in complex with peptides from both Hsp70 and enzymes of the ubiquitin pathway demonstrates how the structure of CHIP has evolved to allow simultaneous interaction with chaperones and proteasomal substrates (Zhang et al. 2005a). The dimeric E3 ubiquitin ligase CHIP bound with its TPR domain to the C-terminus of molecular chaperones Hsp70 and Hsp90 and with its U-box region E2 ubiquitin-conjugating enzymes (Zhang et al. 2005b; Ballinger et al. 1999; Xu et al. 2008). This unique combination of domains allows CHIP to bind to both chaperones Hsp70 and Hsp90, via its TPR domain, and to interact with the proteasome or lysosome by acting as an E3 ligase using its U-box domain, effectively cross-linking the chaperones to the proteolytic machinery via ubiquitination of substrates for degradation.

CHIP was originally identified as co-chaperone for Hsp70 and Hsp90 in a screen for novel TPR-containing proteins (Ballinger et al. 1999). CHIP interacts with the C-terminal EEVD motifs in both Hsp90 and Hsp70 via its TPR domains (Ballinger

et al. 1999), a feature in common with other TPR-containing co-chaperones, like Hop and PP5 (Allan and Ratajczak 2011; Brinker et al. 2002; Cortajarena and Regan 2006; Odunuga et al. 2003). The CHIP monomer contains only a single TPR domain which can bind indiscriminately to both Hsp90 and Hsp70. CHIP can bind two molecules of Hsp70 in a dynamic and flexible complex in which both CHIP and Hsp70 move independently of each other and is predicted to provide space to accommodate additional client proteins (Smith et al. 2013). CHIP binds to the extreme C-terminal residues (IEEVD) and residues in the lid region of Hsp70 (Fig. 12.3c) (Zhang et al. 2015). The IEEVD motif of Hsp70 was required for ubiquitination of Hsp70, and a reduction in the length of the C-terminal tail preceding the IEEVD led to reduced capacity to ubiquitinate Hsp70 (Smith et al. 2013), which is consistent with the involvement of Hsp70 lid regions in CHIP binding (Zhang et al. 2015). The optimal CHIP TPR-binding peptide was determined to be LWWPD. While the mechanism of binding of this peptide was consistent with the EEVD peptides, the LWWPD peptide bound significantly tighter than the Hsp70- and Hsp90-derived peptides (Fig. 12.3d) (Ravalin et al. 2019). This suggests that the EEVD motif is not optimized for binding to CHIP. Indeed, the glutamate residues in the EEVD motif were shown to be more important for the interaction with the CTD-I domain of type II (DNAJB) Hsp40 isoforms DnaJB1 and DnaJB4 than binding to CHIP (Johnson et al. 2022; Faust et al. 2020). Although the TPR motif is the chaperone-binding site of CHIP, there is evidence to suggest that allosteric interactions with the U-box domain are required for CHIP in association with Hsp70 (Matsumura et al. 2013). Despite this, there was a little difference in the affinity of binding to CHIP between full-length Hsc70 and the C-terminal IEEVD peptide (Smith et al. 2013). There are conflicting reports regarding the affinity of the interaction between CHIP and different chaperones, with reports that both Hsp70 binding (Kundrat and Regan 2010a) and Hsp90 binding to CHIP (Stankiewicz et al. 2010) are the greater affinity of the two. The most recent comparative study suggested that CHIP binds Hsp70 or Hsc70 more strongly than Hop or DnaJC7 and that the affinity of CHIP for C-terminal Hsp70-/Hsc70-derived peptides was twofold greater than C-terminal peptides derived from Hsp90α or Hsp90β (Assimon et al. 2015).

As an E3 ubiquitin ligase, CHIP also interacts with members of the E2 family of ubiquitin-conjugating enzymes during ubiquitination of substrates. These interactions between E2 and E3 proteins are highly specific and will ultimately determine the nature of ubiquitination that occurs, as well as the identity of the substrate protein. CHIP has been shown to interact with specific E2 from the UBCH5 family (which are involved in K48-mediated ubiquitination which promotes proteasomal degradation) (Cyr et al. 2002; Wiederkehr et al. 2002; Xu et al. 2008) and Ubc13 (which regulates K63 ubiquitination and has a regulatory role and a role in lysosomal degradation) (Alberti et al. 2002; Jiang et al. 2001; Murata et al. 2001; Zhang et al. 2005b; Ferreira et al. 2015; Maan and Pati 2018). CHIP also displays E4 ligase activity, in that it can catalyze the extension of polyubiquitin chains on substrate proteins (Murata et al. 2001, 2003; Jiang et al. 2001; Koegl et al. 1999). CHIP interacts with E2 enzymes via its U-box domain. The U-box is composed of

70 amino acids and is structurally similar to the RING finger domains found in other ubiquitin ligases (Ohi et al. 2003). U-box-containing ligases are distinct from HECT and RING finger E3 ligases and appear to associate almost exclusively with chaperones during ubiquitination of client proteins (Hatakeyama et al. 2004b, 2001; Kriegenburg et al. 2012).

CHIP binding can inhibit both Hsp90 and Hsp70, thereby preventing both protein folding by Hsp70 and conformational regulation of client proteins by Hsp90. CHIP blocked the Hsp40-mediated stimulation of Hsp70 ATPase activity and protein refolding and attenuated the function of Hip (Ballinger et al. 1999; Johnson et al. 2022). Previously, CHIP was thought to inhibit Hsp70 indirectly by regulating nucleotide-bound status of the chaperone (Ballinger et al. 1999). CHIP did not affect ATP or ADP association with Hsp70 but blocked the Hsp40-mediated stimulation of Hsp70 ATPase activity (Stankiewicz et al. 2010), which would indirectly promote an ATP-bound form of Hsp70, one which has low affinity for client proteins. However, more recent studies demonstrate that certain type II (DNAJB) Hsp40 isoforms bind via the Hsp40 CTD I domain to the Hsp70 EEVD motif (Faust et al. 2020). Therefore, CHIP may directly inhibit Hsp70 chaperone activity by competition with this class of Hsp40 for EEVD binding (Johnson et al. 2022). While chaperones may not be essential for CHIP-mediated ubiquitination, the efficiency of substrate ubiquitination is increased in ternary complexes containing CHIP, a substrate, and either the Hsp70 or Hsp90 chaperone. In these complexes, the substrate is positioned between the chaperone and CHIP, indicating a mechanism of translocation of substrate from chaperone to co-chaperone for degradation (Quintana-Gallardo et al. 2019).

CHIP also works in concert with certain members of the Hsp70 nucleotide exchange family of proteins, including known interactors and regulators of the UPS pathway (Meimaridou et al. 2009) like BAG-1 which interacts with the proteasome and induces CHIP-mediated degradation of classical client proteins (Demand et al. 2001; Alberti et al. 2004). BAG-1 could bind simultaneously to both Hsp70 and 26S proteasome (Luders et al. 2000). In doing so, BAG-1 induces the release of ubiquitinated client proteins from Hsp70 to the proteasome. Ubiquitination of Hsp72 by CHIP is negatively regulated by BAG-2 and HspBP1. In aging cells, senescence was associated with increased CHIP and BAG-2 but lower HSPBP1 levels. However, HSP72 ubiquitination was significantly reduced during aging suggesting that high BAG-2 levels can functionally counteract high levels of CHIP (Schönbühler et al. 2016). BAG-2 functions as an inhibitor of CHIP in a similar mechanism to HspBP1, by blocking CHIP's interaction with E2 ligase and thereby inhibiting ubiquitination (Arndt et al. 2005). BAG-5 also acts a negative regulator of ubiquitination and CHIP, leading to stabilization of mutant p53 and increased tumorigenesis (Yue et al. 2016).

Addition of CHIP is also sufficient to modify the co-chaperone complement of the Hsp90 complex and induce degradation of canonical Hsp90 client proteins (Connell et al. 2001). CHIP did not affect the ATPase activity of Hsp90 (Stankiewicz et al. 2010), but CHIP binding did reduce Hop binding and prevented binding of p23 completely (Ballinger et al. 1999). Displacement of p23 relies on the presence of the

CHIP TPR domains, while the ubiquitination of glucocorticoid receptor is U-box-dependent, demonstrating defined functions for the two different domains, but that cooperation between them is required for protein degradation. This is interesting, as p23 and CHIP bind to different termini of the chaperone. CHIP may antagonize the action of p23, thereby stabilizing the substrate protein within the complex for ubiquitination. CHIP activity does not require the N-terminal domain of Hsp90, and an interaction with the C-terminal region of Hsp90 is sufficient to allow CHIP-mediated ubiquitination of the client protein. This may suggest that client proteins that associate with different Hsp90 regions may be differentially susceptible to ubiquitination by CHIP.

The ability of CHIP to induce degradation requires the presence of the substrate protein in a denatured form, in addition to the chaperone, as demonstrated by the fact that denatured, but not native, luciferase could be ubiquitinated in vitro in the presence of Ubc4/5 (E2 enzyme) and the Hsp90 or Hsp70 complex (Murata et al. 2001). In this way, Hsp70/Hsp90 are involved in the recognition and delivery of substrates for ubiquitination. Indeed, binding of CHIP to the Hsp70 EEVD motif was favored when Hsp70 was in the ADP form, which has higher affinity for client proteins (Matsumura et al. 2013). In addition, Hsp70-bound peptides are preferentially targeted for degradation by CHIP compared to Hsp90-bound substrates (Qian et al. 2006; Stankiewicz et al. 2010). Therefore, the chaperones would act as sensors for denatured protein substrates which could subsequently be targeted for degradation by CHIP.

CHIP Substrates and Human Disease

Mutations in the STUB1 gene (which encodes CHIP) are linked with hereditary spinocerebellar ataxia autosomal recessive 16 (SCAR16) (Schisler et al. 2016; Pakdaman et al. 2017; Gazulla et al. 2018). SCAR16 is an autosomal recessive neurodegenerative disorder resulting in loss of cells of the cerebellum. This manifests in a range of neurological symptoms, including difficulty with coordination, walking, speaking, and cognitive decline. More recently, STUB1 mutations have been linked to an autosomal dominant neurodegenerative spinocerebellar ataxia (SCA48) (Genis et al. 2018; De Michele et al. 2019; Cocozza et al. 2020; Palvadeau et al. 2020), which shares many features with SCAR16, including substantive loss of cerebellar regions of the brain, movement abnormalities, and psychiatric disorders. This suggests that mutations in CHIP produce a spectrum of neurodegenerative conditions.

The role of CHIP as a major regulator of proteasome-mediated degradation has also been cemented by the identification of numerous protein substrates that are dependent on CHIP for proteasomal (Table 12.1) or lysosomal degradation (Table 12.2). One of the best described client proteins for CHIP is CFTR, upon which many of the early fundamental studies on CHIP function were performed. An updated list of CHIP substrate proteins destined for proteasomal degradation

Table 12.1 Selection of the proteins targeted for proteasomal degradation by CHIP

Substrate/client	Classification	Disease association	References
Androgen receptor (AR)	Receptor	Cancer	(Sarkar et al. 2014)
Cystic fibrosis transmembrane conductance regulator (CFTR)	Receptor	Cystic fibrosis	(Younger et al. 2004; Meacham et al. 2001)
Katanin-p60	Cytoskeleton		(Yang et al. 2013)
Profilin	Cytoskeleton		(Choi et al. 2014)
Tau	Cytoskeleton	Neurodegeneration	(Elliott et al. 2007; Dickey et al. 2007; Hatakeyama et al. 2004a; Petrucelli et al. 2004; Shimura et al. 2004)
Alpha-synuclein	Cytoskeleton	Neurodegeneration	(Kalia et al. 2011; Shin et al. 2005)
Keratin	Cytoskeleton		(Loffek et al. 2010)
Cytochrome P450, family 3, subfamily A, polypeptide 4 (CYP3A4)	Enzyme		(Wang et al. 2012)
Histone deacetylase 6 (HDAC6)	Enzyme		(Cook et al. 2012)
Nitric oxide synthase (NOS)	Enzyme	Neurodegeneration	(Chen et al. 2009; Peng et al. 2004)
NAD(P)H/quinone oxidoreductase 1 (NQO1)	Enzyme		(Tsvetkov et al. 2011)
Pyruvate kinase muscle isoform 2 (PKM2)	Enzyme	Cancer Diabetes	(Shang et al. 2017)
Transglutaminase 2 (TGM2)	Enzyme	Major autoantigen in celiac disease Cancer	(Liu et al. 2020)
Superoxide dismutase-2 (SODM)	Enzyme	Diabetes	(Zemanovic et al. 2018)
V-Erb-B2 avian erythroblastic leukemia viral oncogene homolog 2 (Her2)	Receptor	Cancer	(Zhou et al. 2003; Xu et al. 2002)
Interleukin-4 receptor subunit alpha (IL-4Rα)	Receptor	Cancer Inflammatory disorders	(Wei et al. 2014)
Phosphatidylinositol 3-kinase P85 (P13K)	Signaling intermediate	Cancer	(Ko et al. 2014)
Interferon regulatory factor 1 (IRF-1)	Signaling intermediate		(Gao et al. 2013)
Liver kinase B1 (LKB1)	Signaling intermediate	Cancer	(Gaude et al. 2012)
Phosphatase and tensin homolog (PTEN)	Signaling intermediate	Cancer	(Ahmed et al. 2012)

(continued)

Table 12.1 (continued)

Substrate/client	Classification	Disease association	References
Protein kinase B (PKB/Akt)	Signaling intermediate	Cancer	(Su et al. 2011)
TNF receptor-associated factor 2 (TRAF2)	Signaling intermediate		(Jang et al. 2011b)
Met receptor	Signaling intermediate	Cancer	(Jang et al. 2011a)
Immature BCR-ABL	Signaling intermediate	Cancer	(Tsukahara and Maru 2010)
MAPK/ERK kinase kinase 2 (MEKK2)	Signaling intermediate		(Maruyama et al. 2010)
Apoptosis signal-regulating kinase 1 (ASK1)	Signaling intermediate		(Gao et al. 2010; Hwang et al. 2005)
Leucine-rich repeat kinase 2 (LRRK2)	Signaling intermediate	Neurodegeneration	(Ding and Goldberg 2009; Ko et al. 2009)
Eukaryotic translation initiation factor 5A (eIF5A)	Transcription factor		(Shang et al. 2014)
Estrogen receptor (ER)	Transcription factor	Cancer	(Fan et al. 2005)
Glucocorticoid receptor (GR)	Transcription factor		(Wang and DeFranco 2005; Galigniana et al. 2004; Connell et al. 2001)
Tumor protein 53 (p53)	Transcription factor	Cancer	(Quintana-gallardo et al. 2019; Wang et al. 2011; Sisoula et al. 2011; Muller et al. 2008; Esser et al. 2005)
V-myc avian myelocytomatosis viral oncogene homolog (c-Myc)	Transcription factor	Cancer	(Paul et al. 2013)
Forkhead transcription factor p (FOXp)	Transcription factor		(Chen et al. 2013)
Hypoxia-inducible factor 1 alpha (HIF-1α)	Transcription factor	Cancer	(Luo et al. 2010)
Forkhead transcription factor 1 (FoxO1)	Transcription factor		(Li et al. 2009)
NFkB p65	Transcription factor	Cancer Immune disorders	(Wang et al. 2014)
Androgen receptor (AR) and variant AR-V7	Transcription factor Nuclear receptor	Cancer Drug resistance	(Liu et al. 2018)
Aryl hydrocarbon receptor nuclear translocator-like protein 1 (BMAL1)	Transcriptional activator	Mental and behavioral disorders	(Ullah et al. 2020)

(continued)

Table 12.1 (continued)

Substrate/client	Classification	Disease association	References
Peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC1-α)	Transcriptional coactivator	Diabetes Cardiovascular disease Neurodegeneration	(Schisler et al. 2016)
Eukaryotic translation initiation factor 5A-1 (eIF5A)	mRNA binding protein	Cancer	(Shang et al. 2014)

Table 12.2 Client proteins targeted for CHIP-mediated turnover by autophagy

Substrate/client	Classification	Mechanism	References
Aggregated p53; Δ133p53α	Transcription factor	Chaperone-mediated autophagy (CMA) Lys-63 ubiquitination	(Horikawa et al. 2014; Maan and Pati 2018; Wang et al. 2019)
Hypoxia-inducible factor 1 alpha (HIF1α)	Transcription factor	CMA Lys-63 ubiquitination	(Ferreira et al. 2013, 2015)
Myeloid zinc finger 1 (MZF1)	Transcription factor	Macroautophagy	(Luan et al. 2018)
Nuclear receptor subfamily 1 group I member 2 (PXR)	Nuclear receptor transcription factor	Macroautophagy	(Sugatani et al. 2016)
Receptor-interacting protein kinase-3 (RIPK3)	Signaling intermediate	Lysosome Ubiquitination	(Seo et al. 2016)
Bovine papillomavirus E5	Transmembrane viral oncoprotein	Selective macroautophagy Chaperone-assisted selective autophagy (CASA)	(Roperto et al. 2018)
Truncated tau (TauΔC)	Cytoskeletal protein	Macroautophagy	(Dolan and Johnson 2010)
Filamin	Cytoskeletal protein	Selective macroautophagy Chaperone-assisted selective autophagy (CASA)	(Arndt et al. 2010; Ulbricht et al. 2013)
Proteasomes	Protein complex	Selective macroautophagy (proteophagy)	(Choi et al. 2020)
Mitochondria	Organelle	Selective macroautophagy (mitophagy)	(Lizama et al. 2018a, b)
Peroxisomes	Organelle	Selective macroautophagy (pexophagy)	(Chen et al. 2020)

includes several transcription factors, signaling intermediates, and cytoskeletal or structural proteins (Table 12.1). The substrates, many of which are known client proteins of either Hsp90 or Hsp70, fulfill important roles in fundamental cellular processes. What is also striking is that many of these proteins are linked with diseases including cancer and neurodegenerative diseases like Alzheimer's disease. In particular, CHIP dysfunction is implicated in neurodegenerative diseases, where protein aggregates are a hallmark. CHIP may therefore of therapeutic value given the potential to regulate the degradation of disease-relevant proteins like tau or p53 (Hatakeyama et al. 2004a; Petrucelli et al. 2004; Shimura et al. 2004; Esser et al. 2005).

CHIP can bind directly to and ubiquitinate the protein tau, even in the absence of Hsc70 (Munari et al. 2022), actions that mediate the dissolution of tau aggregates (Hatakeyama et al. 2004a; Petrucelli et al. 2004; Shimura et al. 2004). CHIP recognizes and ubiquitinates an aggregation-prone proteolytic cleavage product of tau (tau^{D421}) which arises due to caspase activity (Ravalin et al. 2019). These tau aggregates are associated with the pathology of Alzheimer's disease (Kosik and Shimura 2005; Medeiros et al. 2011; Martin et al. 2011; Salminen et al. 2011). CHIP may also regulate the aggregation of tau via ubiquitination of other client proteins, like HDAC6 and Akt (Cook et al. 2012; Dickey et al. 2008). Therefore, overexpression of CHIP may represent a therapeutic strategy to prevent neuronal cell death and ameliorate the symptoms and onset of the disease (Dickey et al. 2007; Sahara et al. 2005). CHIP also controls the proteasomal degradation of several important oncogenic transcription factors, signaling intermediates, and metabolic enzymes, including p53, PTEN, Akt, c-Myc, and CYP3A (Paul et al. 2013; Kajiro et al. 2009; Ahmed et al. 2012; Wang et al. 2015). These classes of proteins often act as nodes for the activation of a host of downstream proteins in the cellular reactions that lead to oncogenesis. Therefore, CHIP may in fact indirectly regulate a larger cohort of cellular proteins via degradation of central transcription factors or signaling intermediates. Indeed, analysis of the function of CHIP in breast cancer has demonstrated that the protein can regulate cellular responses, many of which are considered cancer hallmarks. Overexpression of CHIP blocked oncogenic signaling pathways, inhibited cancer-associated processes like cell migration and anchorage-independent growth, and induced cell death. Conversely, depletion of CHIP protein levels increased tumor formation and metastasis in mouse models (Kajiro et al. 2009; Choi et al. 2014; Sarkar et al. 2014; Tingting et al. 2016; Xu et al. 2018; Park et al. 2019; Wang et al. 2020).

In addition to classical substrate proteins, CHIP also ubiquitinates the chaperones Hsp70 and Hsp90 on multiple solvent exposed but clustered lysine residues (6 in Hsp70 and 13 in Hsp90) (Kundrat and Regan 2010b). The polyubiquitination of these chaperones by CHIP occurs via K6, K11, K48, and K63 linkages. The canonical signal for protein degradation is ubiquitination via K48 linkages, and it is known that CHIP can mediate degradation of Hsp70 via this mechanism (Jiang et al. 2001). This reduction in Hsp70 plays a central regulatory role to return Hsp70 levels to basal after the induction of the stress response. However, noncanonical ubiquitin linkages (like K6, K11, and K63) have not been demonstrated to induce

proteasomal degradation but may mediate other functions. In some experiments, ubiquitination via K63 resulted in recruitment of Hsp70, Hsp90, and BAG-1 to the proteasome but did not lead to their degradation (Alberti et al. 2002; Jiang et al. 2001). This suggested that K63 linkage may be a proteasome-targeting sequence and represent a mechanism by which CHIP uses the chaperone to deliver its clients to the proteasome (Saeki et al. 2009; Chen and Sun 2009).

K63 ubiquitination by CHIP has also been linked to autophagic turnover of substrate proteins. Fewer substrates for CHIP-mediated autophagic degradation have been described, although several important disease-related chaperone client proteins are regulated in this manner (Table 12.2). Mutant p53 can be cleared by chaperone-mediated autophagy under both normal and hypoxic conditions (Vakifahmetoglu-Norberg et al. 2013). In esophageal cancer cell lines (ESCs), DpdtbA (Di-2,2'-pyridine ketone dithiocarbamate s-butyric acid) treatment induced ROS and promoted p53 turnover by a CHIP-regulated autophagy rather than MDM2-mediated ubiquitination (Wang et al. 2019). In this case, the CHIP-mediated autophagic clearance of p53 involved K63 ubiquitination and, although CHIP could bind non-aggregating mutant p53 via the TPR domain, autophagy occurred selectively for aggregated p53 which was bound by the E3 ligase domain of CHIP (Maan and Pati 2018). CHIP/STUB1 also binds the inhibitory p53 isoform Δ133p53α, and CHIP depletion promotes Δ133p53α clearance by autophagy leading to cellular senescence (Horikawa et al. 2014).

HIF1α, a cancer-related transcription factor regulating the cellular response to hypoxia, is a validated substrate for CMA and requires CHIP for lysosomal degradation (Ferreira et al. 2015). The HIF1α KFERQ motif is required by the CMA machinery for lysosomal degradation and occurs due to nutritional but not hypoxic stress. CHIP interacts with HIF1α and targets HIF1α for CMA-dependent degradation in a K63-dependent manner. Mutation of CHIP E3 ligase or TPR domains (interaction with Hsc70) abolishes lysosomal degradation of HIF1α (Ferreira et al. 2013, 2015).

Proteolytic processing of full-length tau (FL-tau) at Asp^{421} generates a truncated tau (tauΔC) with increased fibril formation and is linked to neurodegeneration. TauΔC is turned over faster than FL-tau, but interestingly the two forms of tau are degraded by different pathways via CHIP. While FL-tau was cleared by proteasomal degradation, tauΔC was degraded primarily by macroautophagy. CHIP bound tauΔC more efficiently than the FL-tau, resulting in increased tauΔC ubiquitination in vitro (Dolan and Johnson 2010).

CHIP can also mediate the degradation of damaged protein complexes and even organelles by autophagy (Table 12.2). Aggresome-associated proteasomes are degraded by macroautophagy in an SQSTM- and CHIP-dependent manner in a process known as proteophagy. In vitro studies using purified human proteasomes showed that CHIP catalyzed K63 ubiquitination of proteasomes and CHIP depletion reduced autophagic processing of the proteasome (Choi et al. 2020). CHIP in the mitochondria is important for the organelle integrity and functionality post-oxygen-glucose deprivation, as CHIP-deficient mitochondria show altered stress responses (Palubinsky et al. 2015). CHIP translocates to the mitochondria in response to

oxygen and glucose depletion, where CHIP regulates selective macroautophagy of damaged mitochondria (mitophagy), a function that requires intact E3 ligase and TPR domains. Consistent with this, CHIP depletion increases the number of deformed CHIP-associated mitochondria due to the inhibition of LC3B-mediated autophagy (Lizama et al. 2018a, b). Peroxisomes damaged by ROS produced through β-oxidation of fatty acids are first ubiquitinated followed by selective macroautophagy, known as pexophagy. CHIP, as part of a multichaperone complex including Hsp70, Hsc70, and Hsp40, was recruited to damaged peroxisomes and catalyzed subsequent ubiquitination and proteolysis by autophagy. RNAi-mediated CHIP depletion caused accumulation of ROS-stressed peroxisomes. Forced expression of CHIP in normal peroxisomes is sufficient to induce pexophagy (Chen et al. 2020). Ataxia-associated CHIP mutants N65S and A79D (in the CHIP TPR domain) showed reduced association with Hsp70 and hence failed to translocate to peroxisomes which may contribute to peroxisomal defects observed in ataxia patients (De Munter et al. 2015) and reinforce the importance of Hsp70 interaction for CHIP-mediated autophagy (Chen et al. 2020).

Ubiquitination of substrates by CHIP does not always lead to degradation. There are some examples that demonstrate a role for CHIP in noncanonical ubiquitination of substrates. One example is the protein sirtuin, which underwent noncanonical CHIP-mediated ubiquitination that culminated in its stabilization and promotion of DNA repair (Ronnebaum et al. 2013). CHIP also mediated T-cell activation by ubiquitination of CARMA1 (caspase recruitment domain (CARD) containing membrane-associated guanylate kinase protein 1), a receptor important in antigen receptor linked NF-kappaB signaling. The CHIP-mediated ubiquitination of CARMA1 via K27 was determined to be important for activation of this pathway (Wang et al. 2013).

Hsp70 and Hsp90: To Degrade or to Refold?

The chaperone folding and protein degradation pathways work competitively during protein homeostasis (Marques et al. 2006). The major question that remains unanswered is how is it determined whether proteins enter refolding or degradation pathways? Chaperone-mediated refolding by Hsp70 requires the Hsp40 co-chaperones and nucleotide exchange factors (NEFs). For a subset of Hsp90 protein clients, particularly steroid hormone receptors, pro-folding chaperone complexes are largely associated with the co-chaperone Hop, which facilitates entry of client proteins from the Hsp70 complex into the Hsp90 complex (Siligardi et al. 2004). However, it remains to be determined if Hop is required for general coordination of chaperone complexes for a wider range of clients. In contrast, Hsp70 and Hsp90 chaperone complexes containing CHIP are considered pro-degradation complexes. Hop and CHIP cannot bind simultaneously to the Hsp90 complex, which indicates that the complexes controlling either protein folding or protein degradation are mutually exclusive and possibly competitive (Kundrat and Regan 2010a).

Therefore, the simplest mechanism to control the choice of folding or degradation may be via regulation of the levels of the specific co-chaperone (Marques et al. 2006). Indeed, simply increasing the CHIP concentration by overexpression increased proteasomal degradation of client proteins, including hormone receptors (Connell et al. 2001; Adachi et al. 2007).

Hop and CHIP compete for binding to Hsp70 and Hsp90, which could determine whether pro-folding or pro-degradation complexes form. There is evidence that these associations are regulated by posttranslational modification of the C-terminal region of these chaperones. Phosphorylation of sites in the C-terminus of either Hsp70 or Hsp90 or the lid region of Hsp70 blocked CHIP binding and promoted association of Hop with the chaperones (Muller et al. 2013; Assimon et al. 2015; Zhang et al. 2015). This observation is particularly important in the context of cancer, where increased levels of phosphorylated Hsp70 and Hsp90 chaperones have been linked with high cell proliferation rates (Muller et al. 2013). Indeed, many of the kinases that phosphorylated Hsp90, including CK1, CK2, and GSK3β, are linked to the cell cycle or mitogenic signaling pathways. Hop has also been shown to be upregulated in numerous cancers, suggesting that increased levels of this co-chaperone may outcompete CHIP for chaperone-binding in these cells (Willmer et al. 2013; Ruckova et al. 2012). The net consequence of this would be to create a cellular environment that promoted protein folding over degradation. The fact that this is observed in cancer cells may explain the dependency of proteins on the Hsp70-Hsp90 folding complex and support the high cellular growth rates observed in most malignancies.

Early studies by the Regan group suggest that the switch to degradation of protein clients was largely determined by partitioning of the client protein between Hsp70 and Hsp90 chaperone complexes, which was controlled in part by the nature of complexes formed (Kundrat and Regan 2010a). Hsp70 and Hsp90 can both bind CHIP, but the greater affinity of the CHIP-Hsp70 interaction predicts that this is the dominant complex that mediates the degradation pathway (Kundrat and Regan 2010a). Therefore, the interaction between Hsp90 and CHIP is predicted to play a minor role in direct protein triage decisions. This is supported by the fact that CHIP prefers ubiquitination of Hsp70-bound client proteins compared to Hsp90-bound substrates (Stankiewicz et al. 2010). Rather, degradation of Hsp90 clients is proposed to be induced indirectly by inhibition of the Hsp90 complex, meaning that client proteins associate with the Hsp70 complex for an extended duration, leading to increased potential for degradation via CHIP (Kundrat and Regan 2010a). This is consistent with the observation that the time spent by the client protein in the Hsp70 complex determines its stability (Matsumura et al. 2013). Interestingly, the TPR-containing co-chaperone, DnaJC7, which catalyzes retrograde transfer of client proteins from Hsp90 back to Hsp70 (Brychzy et al. 2003), can interact directly with CHIP (Hatakeyama et al. 2004b). Hsp90 inhibition using compounds that induce the stress response (such as 17-AAG) leads to increased Hsp70 levels, which could subsequently associate with clients released from the nonfunctional Hsp90 complex and trigger their degradation via CHIP. The fact that CHIP is also known to induce

both the expression and the turnover of Hsp70 as part of the stress response supports the major role for Hsp70 over Hsp90 in this process (Qian et al. 2006).

The increase in substrate degradation in response to higher levels of CHIP could also be explained by the fact that increased levels of CHIP result in increased levels of Hsp70-CHIP degradation complexes to a concentration that exceeds Hsp70-Hop-Hsp90 folding complexes (Kundrat and Regan 2010a). However, complete Hop knockout in cell lines led to proteasomal defects, and consequently a basal level of pro-folding complexes are likely required for the formation of appropriate degradation complexes (Bhattacharya et al. 2020). The addition of CHIP to the Hsp90 complex induces a similar response to treatment with GA, promoting dissociation of stabilizing co-chaperones, like p23, and promoting proteasomal degradation of the client protein (Connell et al. 2001; Whitesell and Cook 1996). This suggests that, in addition to an increase in Hsp70-CHIP complexes, increased CHIP levels may also block Hsp90 complexes and push client proteins toward association with Hsp70. More recent studies, however, suggest that distinct chaperone complexes (termed the epichaperome) can be formed in cells with equivalent levels of chaperones due to biochemical differences (Rodina et al. 2016; Bolaender et al. 2021). In some cases, a strong networking of the Hsp70, Hsp90, and co-chaperone-chaperone complexes into higher-order integrated complexes was observed, while in others the Hsp70 and Hsp90 chaperone complexes are more loosely affiliated (Rodina et al. 2016; Bolaender et al. 2021). Therefore, the degree of biochemical networking between the Hsp70-Hsp90 chaperone complexes may also determine the outcome of chaperone complexes. While these studies did not detect CHIP as enriched in the epichaperome complexes studied, they do suggest a diversity in co-chaperone-chaperone complexes that is not necessarily dependent on the concentration of chaperones and implies that these complexes may not be mutually exclusive or competitive.

The balance between folding and degradation can also be controlled by naturally occurring regulators of CHIP (Fig. 12.4). The activity of CHIP is regulated by BAG isoforms (BAG-1, BAG-2, and BAG-3) and HspBP1, which are nucleotide exchange factors for Hsp70 (Kabani et al. 2002; Alberti et al. 2003). CHIP can bind directly to the proteasome (Connell et al. 2001; Meacham et al. 2001), or it may interact with the proteasome via BAG-1. BAG-1 binds simultaneously to CHIP and the 26S proteasome, thereby recruiting a complex that delivers CHIP-bound complexes to the proteasome (Luders et al. 2000). Interestingly, the association of BAG-1 with the proteasome is mediated in part by CHIP-mediated ubiquitination of BAG-1 (Alberti et al. 2002). BAG-3 favors recruitment of CHIP to the Hsp70 multichaperone complex which recruits the autophagic machinery for aggregated client protein degradation by the lysosome (Gamerdinger et al. 2011; Minoia et al. 2014). The BAG-1/BAG-3 ratio determines if degradation proceeds via the proteasomal or autophagic pathway (Gamerdinger et al. 2011; Minoia et al. 2014). BAG-2 and HspBP1 are negative regulators of CHIP activity (Alberti et al. 2004; Arndt et al. 2005). BAG-2 inhibits CHIP-mediated degradation by blocking the interaction of CHIP with the E2 ligase in the ubiquitination cascade (Arndt et al. 2005). HsBP1 interacts with the Hsp70 ATPase domain (Raynes and Guerriero

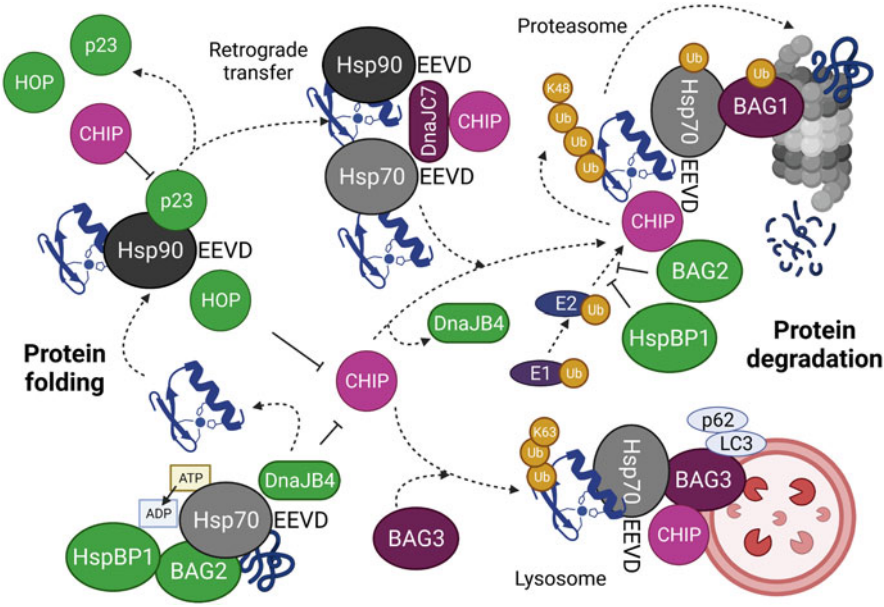

Fig. 12.4 Competition between co-chaperones regulates chaperone folding or degradation. Hypothetical model of the interplay between different co-chaperones to regulate either folding or degradation by Hsp70 and Hsp90 chaperones. Co-chaperones that promote folding as part of Hsp70-Hsp90 complexes are shown in green, while those that promote degradation are shown in purple. Hop and DnaJB4 compete directly with CHIP for binding to the Hsp70 EEVD motif. CHIP can displace DnaJB4, Hop, and p23 from folding complexes to promote degradation. DnaJC7 promotes retrograde transfer of client proteins from Hsp90 to Hsp70 and can bind CHIP. BAG-2 and HspBP1 inhibit CHIP-mediated ubiquitination by blocking E2 binding. BAG-1 recruits CHIP and Hsp70 complexes to the proteasome. BAG-3 recruits CHIP and Hsp70 complexes to the autophagic machinery to promote degradation of clients via the lysosome. Image created with BioRender

Jr. 1998) and induces conformational changes in the chaperone (McLellan et al. 2003), leading to binding of CHIP to the C-terminal site of Hsp70. This complex abolished the CHIP-mediated ubiquitination and degradation of the substrate protein, CFTR (Alberti et al. 2004). Hsp40 and client protein (steroid receptors) preferentially associate with BAG-1 over HspBP1. HspBP1, not BAG-1, also reduced binding of Hsp70 to client proteins and inhibited the activity of steroid hormone receptors at both high and low concentrations. In contrast, the effect of BAG-1 on steroid receptor function was concentration-dependent, being stimulatory at low levels of BAG-1 and inhibitory at higher concentrations of BAG-1 (Knapp et al. 2014). CHIP also competes directly with selected type II Hsp40 (DNAJB), like DnaJB4, for binding to the Hsp70 EEVD (Johnson et al. 2022). Whether DnaJB4 or CHIP is associated with Hsp70 influences the outcome. Addition of DnaJB4 to Hsp70-CHIP complexes reduced ubiquitination of the client protein tau in vitro, while addition of CHIP to DnaJB4-Hsp70 complexes reduced ATPase activity and

hindered refolding of luciferase (Johnson et al. 2022). Therefore, a range of structurally distinct co-chaperones can influence association of CHIP with chaperone complexes and hence fine-tune the complexes toward folding or degradation.

CHIP activity may also be regulated by cellular conditions that increase the concentration of CHIP substrates. This was demonstrated in *C. elegans*, where stress increased protein misfolding and resulted in competition for CHIP binding. This competition titrated CHIP away from native clients, with a consequent decrease in lifespan (Tawo et al. 2017). Another example is the appearance of previously hidden CHIP-binding sites by enzymes, such caspases. Caspases are cysteine proteases which preferentially cleave substrates at the C-terminal side of aspartate (D) residues, resulting in the production of novel C termini resembling CHIP TPR recognition sequences and hence increasing the concentration of CHIP substrates (Ravalin et al. 2019). Therefore, cellular conditions that enrich for CHIP-binding substrates may enable increased protein ubiquitination leading to enhanced proteolysis or altered degradation profiles.

In addition to deciding between folding and turnover, recent data suggest that CHIP-mediated ubiquitination can promote protein degradation by both the proteasome and the lysosome. In addition, CHIP regulates degradation by both CMA and macroautophagy, but the mechanisms determining which pathway is used are poorly defined. This reinforces CHIP as a key node in proteolytic processing by different cellular pathways and expands the proteolytic network regulated by CHIP. However, the signals required to promote CHIP-mediated autophagic degradation over CHIP-mediated proteasomal degradation still need to be described. From the identified autophagic clients, CHIP-mediated autophagic degradation involves K63 ubiquitination which likely arises from CHIP's ability to associate with specific E2 ligases like Ubc13 and appears to tentatively be associated with nutritional stress or substrates that are larger and insoluble. However, future work is needed to clearly define how CHIP clients are triaged to autophagy or the proteasome.

Conclusions

While the molecular mechanisms that are involved in defining the balance between protein folding and protein degradation are not fully understood, the existence of CHIP suggests that chaperones actively participate in protein degradation via the proteasome and lysosome. This suggests that an as yet undefined mechanism exists to determine which pathway, folding or degradation, should be followed under certain conditions. Recent studies have demonstrated that CHIP-mediated the degradation of a wide range of cellular proteins, which signifies a central role for this co-chaperone in protein degradation via multiple cellular proteolytic mechanisms. Many of these client proteins are important factors in a range of human diseases, an association that suggests CHIP may be a putative drug target. The potential applications of CHIP to human disease are likely to be largely restricted to those that

involve either the overexpression or the activation of CHIP. While some experiments demonstrate that CHIP depletion results in degradation of CHIP substrates, other reports demonstrate that CHIP clients remain unaffected in a CHIP depleted background. This hints at functional redundancy whereby other E3 ligase factors may compensate for the loss of CHIP. These data may also suggest that some substrates are more reliant on CHIP for their degradation, whereas others may be promiscuous with respect to the E3 ligase required for their degradation. The application of CHIP as a drug target will be limited until we are able to define the mechanisms which regulate whether chaperones function in protein folding or protein degradation and which degradation pathway is ultimately selected.

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Chapter 13
HSP70-HSP90 Chaperone Networking in Protein-Misfolding Disease

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Abstract Molecular chaperones and their associated co-chaperones are essential in health and disease as they are key facilitators of protein-folding, quality control and function. In particular, the heat-shock protein (HSP) 70 and HSP90 molecular chaperone networks have been associated with neurodegenerative diseases caused by aberrant protein-folding. The pathogenesis of these disorders usually includes the formation of deposits of misfolded, aggregated protein. HSP70 and HSP90, plus their co-chaperones, have been recognised as potent modulators of misfolded protein toxicity, inclusion formation and cell survival in cellular and animal models of neurodegenerative disease. Moreover, these chaperone machines function not only in folding but also in proteasome-mediated degradation of neurodegenerative disease proteins. This chapter gives an overview of the HSP70 and HSP90 chaperones, and their respective regulatory co-chaperones, and explores how the HSP70 and HSP90 chaperone systems form a larger functional network and its relevance to counteracting neurodegenerative disease associated with misfolded proteins and disruption of proteostasis.

Keywords HSP70 · HSP90 · Chaperone · Co-chaperones · Protein degradation · Protein quality control · Neurodegeneration

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Introduction

HSP70 and HSP90 are core components of an extensive molecular chaperone network that is essential for cell survival. They can perform multiple cellular roles as their ability to modulate protein-folding and conformational change is recruited to specific functions, including through their co-chaperones. This is exemplified by the HSP70 machinery where in humans approximately 50 co-chaperone J-domain proteins, with cell- and tissue-specific expression patterns, regulate the function and client protein interactions of 13 different HSP70s to facilitate diverse processes ranging from de novo protein-folding to clathrin-mediated vesicular trafficking (Kampinga and Craig 2010).

HSP70 and HSP90 have their own exclusive complements of co-chaperones that direct their function but are also directly linked by specific co-chaperones that can interact with both. The systems are also linked to protein degradation mechanisms through co-chaperones. This means that HSP70 and HSP90 can pass client proteins between them and triage them for degradation dependent on levels and activity of network components. This is most likely in combination with the folding state of the client. This flexibility in chaperone networks is particularly relevant in response to disruption of the proteome. This includes perturbations associated with protein-misfolding disease and in neurodegenerative diseases.

In this chapter, we focus on links between the HSP70 and HSP90 molecular chaperone networks and their roles in neurodegenerative diseases. Firstly, we describe the HSP70 and HSP90 chaperone machines, including cataloguing key co-chaperones. We then consider the role of these chaperones in protein quality control systems in the context of the maintenance of a functional proteome in health and disease. Finally, we consider the evidence for how HSP70 and HSP90 networks are linked in protein-misfolding neurodegenerations.

The HSP70 Chaperone Machine

The ability of HSP70 proteins to associate transiently with short hydrophobic peptide segments within their client proteins makes them versatile and provides them with a generalised ‘housekeeping’ chaperone function. However, through association with other chaperone machines such as HSP90 and HSP110, they are also involved in specific tasks (Mayer and Bukau 2005). Collectively, these functions include protein-folding and assembly of newly synthesised proteins, disassembly and refolding of misfolded or aggregated proteins, translocation across membranes and control of regulatory proteins (Bauer et al. 2002; Bukau et al. 2000; Hartl and Hayer-Hartl 2002; Mayer and Bukau 2005; Pratt and Toft 2003; Ryan and Pfanner 2001; Toft 1999; Young et al. 2003).

Homologues of HSP70 consist of two domains, a 45-kDa N-terminal ATPase domain (NTD) and a 25-kDa C-terminal substrate-binding domain (SBD) (Mayer

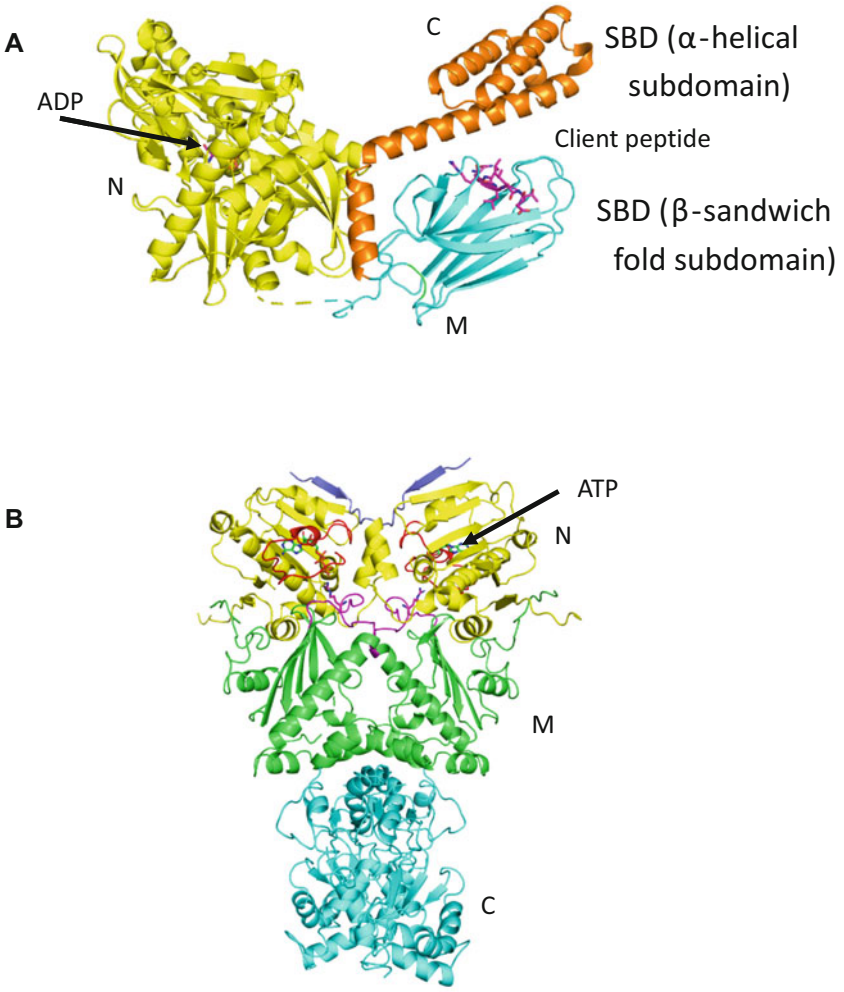

Fig. 13.1 Structural domains of HSP70 and HSP90. (**a**) HSP70 composite model from pdb structures 3FZF and 1DKX, showing the substrate-bound conformation of HSP70. Yellow, N-terminal ATP-binding domain (N); cyan, substrate-binding domain (M); and gold, substrate-binding domain helical lid (C). (**b**) PDB 2CG9 structure of HSP90. Yellow, N-terminal ATP-binding domain with ATP-bound and shown as green-coloured sticks; green, middle domain; cyan, C-terminal domain; blue, fragment of structure involved in the ß-strand exchange during N-terminal dimerisation; magenta, loop carrying the catalytic arginine residue (shown as magenta sticks); and red, the ATP lid. *N* N-terminal domain, *M* middle domain, and *C* C-terminal domain

and Bukau 2005). Nucleotide binds at the centre of the NTD, which consists of a two-lobed structure, divided into four subdomains, IA, IB, IIA and IIB. The SBD itself consists of two subdomains, the larger of which consists of a 15-kDa β-sandwich fold and the other a C-terminal α-helical subdomain (Fig. 13.1a). The α-helical subdomain is particularly important in switching conformation of the

chaperone between the high and the low substrate-binding state during the ATPase cycle of HSP70. The ATP state of HSP70 displays a low-affinity and fast exchange rates for substrates, whilst the ADP state shows high-affinity and low exchange rates. The cycle of binding and release of client substrate is itself regulated by J-domain-containing proteins, such as HSP40, which also target clients to HSP70, and by nucleotide exchange factors (NEFs), such as bag1, which are essential for displacing the tightly bound ADP from HSP70 (Kampinga and Craig 2010; Mayer and Bukau 2005; Radons 2016), although two specialised HSP70 isoforms, HscA and HscC, expressed in *Escherichia coli,* do not depend on GrpE (which functions as a NEF for DnaK) (Brehmer et al. 2001). However, during the ATPase cycle, the α-helical subdomain locks client substrate bound in the β-sandwich fold in the ADP state, whereas in the ATP state the α-helical subdomain docks onto the ATPase domain so opening up the substrate-binding site for release of the client protein. The interplay of HSP70s with co-chaperones determines the lifetime of the chaperone cycle (Zhu et al. 1996). In addition to J-domain-containing and NEF proteins, the extreme C-terminal IEEVD conserved motif of HSP70 can recruit a number of tetratricopeptide repeat (TPR) domain proteins such as HOP, CHIP, DNAJC7 and the mitochondrial import receptor subunit TOM34 (Assimon et al. 2015; Trcka et al. 2014).

It appears that the SBD of HSP70 proteins has high sequence conservation, and although differences exist between different HSP70 proteins, it is not clear what the significance of these are. Much of what is known about HSP70-client binding was gained form the X-ray structure of *E. coli* DnaK bound to a heptameric peptide (NRLLLTG) substrate in an extended conformation. The side chain of the central leucine was found bound to a hydrophobic cleft within the SBD of DnaK, whilst a further five residues are bound between the SBD cleft and the SBD helical lid and are stabilised by a network of both hydrogen bonding and hydrophobic interactions (Zhu et al. 1996). The configuration that peptides adopt when bound to HSP70s appears to be conserved (Cupp-Vickery et al. 2004; Jiang et al. 2005; Morshauser et al. 1999; Pellecchia et al. 2000; Stevens et al. 2003), but variability in both the exact register and orientation of binding has been observed (Clerico et al. 2015; Tapley et al. 2005; Zahn et al. 2013).

Despite the fact that the binding configuration is conserved, substrate preferences are noticeable between HSP70s from different organisms and even compartments (Fourie et al. 1994). For example, whilst cytosolic HSP70s preferably bind peptides rich in aliphatic side chains (such as leucine), endoplasmic reticulum (ER) homologues, such as HSPA5/BIP, prefer motifs containing aromatic residues (Gragerov and Gottesman 1994). In contrast, peptides containing proline are preferred by the *E. coli* HscA and *Saccharomyces cerevisiae* mitochondrial Ssq1 homologues (Dutkiewicz et al. 2004; Hoff et al. 2002), and yet other preferences have also been reported (Mok et al. 2018). It appears that this plasticity in binding specific substrate residues is mainly due to alterations in the two amino acid positions, represented by Met 404 and Ala 429 in *E. coli* DnaK and Ala 406 and Tyr 431 in human HSPA1/HSP70-1 and HSPA8/HSC70 (Rosenzweig et al. 2019; Rudiger et al. 2000), but also from differences in the length of loops that enclose the substrate

(Kluck et al. 2002). There is also evidence that the NTD of HSP70s may play a role in substrate specificity, but further work is required to understand the molecular details of this (Sharma and Masison 2011). Reiterative cycles of binding and release of the chaperone, regulated by an exchange factor, leads to folding of the client protein. This cycle is, however, strictly regulated by J-domain-containing proteins and NEFs that together regulate the rate of the folding cycle.

J-Domain Proteins J-domain-containing proteins form a heterogeneous class of multidomain proteins that interact with HSP70. These proteins share a common conserved sequence of approximately 70 residues that is often located at the N-terminus of the protein and has been named the J-domain. The best described mechanism for J-domain proteins is exemplified by the *E. coli* DnaJ protein. The canonical mechanism of HSP70 relies on an initial interaction with a J-domain protein, which stimulates the rate-limiting step of the HSP70 ATPase cycle in most cases (Karzai and McMacken 1996; Laufen et al. 1999; Liberek et al. 1991; Mayer and Bukau 2005). Binding of a J-domain-containing protein serves to deliver client proteins to the HSP70 chaperone but also prevents client protein aggregation prior to chaperone loading (Kampinga and Craig 2010). The stimulation of ATPase activity by a J-domain results in hydrolysis of ATP and the formation of a stable ADP-HSP70-client protein complex, in which the α-helical subdomain is locked over the bound client protein (Mayer and Bukau 2005). The activation of the ATPase activity of DnaK by DnaJ involves a complicated series of conformational couplings between the various domains of the chaperone and the co-chaperone, such that a synergistic effect leads to the hydrolysis of ATP by DnaK. Binding of a substrate to the hydrophobic SBD of DnaK involves coupling to the ATPase domain; this transmits binding to the catalytic centre (Laufen et al. 1999). Furthermore, the coupling activity of DnaJ is dependent on an interaction both with substrate and with the ATPase domain of DnaK (Karzai and McMacken 1996; Wall and Koger 1994). DnaJ itself interacts with substrates in a rapid and transient manner, which facilitates transfer to the DnaK SBD.

NEFs For most HSP70s, the dissociation of ADP from its tightly bound state within the ATPase domain involves an NEF. However, there are differences in the detailed mechanisms by which ADP is displaced form HSP70s; this typically appears to involve opening of the nucleotide-binding pocket and has been observed for both DnaK and bovine HSP70, whose respective NEFs are GrpE and bag1 (Gassler et al. 2001; Karzai and McMacken 1996). In common, it appears that all eukaryotic NEFs capture an open NBD conformation (Bracher and Verghese 2015; Rosenzweig et al. 2019).

GrpE, which represents the NEF in prokaryotes, mitochondria and chloroplasts, binds across the NBD of DnaK and literally drives a β-strand domain into the nucleotide-binding cleft (Harrison et al. 1997). This induces a tilt of subdomain IIB, opening up the nucleotide-binding pocket and reducing the affinity for ADP. In the cytoplasm of eukaryotic cells, the situation is more complex. Three structurally distinct families of exchange factor (BAG, HSP110 and armadillo) have been described that act on HSP70s to open the nucleotide-binding pocket (Sondermann

et al. 2001; Takayama et al. 1999). The BAG family of proteins use a conserved three-helix bundle to bind to both IB and IIB subdomains of HSP70s, which induces a tilt in these domains in a similar conformation to that induced by GrpE. HSP110-type of NEF actually belong to a HSP70 superfamily, in which the ATP-binding domain of both HSP110s and the ER homologue Grp170 resembles the ATP (open) state of the nucleotide-binding domain of HSP70s. The nucleotide exchange activities of HSP110s are initiated by a head-to-head interaction between the NBD of both HSP110 and HSP70 of their NBD resulting in an outward tilt of the HSP70 subdomain IIB in a similar manner to that seen for GrpE (Polier et al. 2008; Schuermann et al. 2008). Finally, the core domain of armadillo-type NEFs, which are composed of four α-helical armadillo repeats, binds HSP70s by wrapping around subdomain IIB of the HSP70 NBD. This causes rotation, rather than tilting, of the subdomain around one of its helices and thus weakens the binding for ADP (Yan et al. 2011). Another interesting feature of NEF is that some of them prevent rebinding of substrate to the SBD of HSP70 (Gowda et al. 2018; Rosam et al. 2018). This is achieved through structural elements able to mimic motifs that are recognised by the SBD of HSP70s and prevent unproductive rebinding. Examples include the N-terminal release domain found in the armadillo-type Fes1p protein of yeast and in human HSPBP1 (Gowda et al. 2018; Rosam et al. 2018) and perhaps with a helical extension at the N-terminal end of GrpE (Harrison et al. 1997; Wu et al. 2012).

Hsc70-Interacting Protein (HIP) HIP is a multidomain protein consisting of an N-terminal dimerisation domain, a predicted TPR middle domain, a highly charged segment and a C-terminal substrate-binding domain consisting of a GGMP repeat segment and a DP domain (Irmer and Hohfeld 1997; Li et al. 2013; Prapapanich et al. 1996). The DP domain is also known as the STI1 homology domain and is a region following the TPR domain rich in DP motifs. The TPR domain of HIP interacts with the NBD of HSP70s found in animals, plants and protozoa and delays the release of ADP form HSP70s (Hohfeld et al. 1995; Velten et al. 2000). It appears that HIP in collaboration with HSP70 and its DP domain has been implicated in glucocorticoid receptor (GR) binding (Li et al. 2013; Nelson et al. 2003; Schmid et al. 2012).

The crystal structure of the TPR domain of HIP in complex with HSP70 has been determined (Li et al. 2013). HIP was shown to have a strong preference for the ADP conformation of HSP70, preventing protein aggregation and perhaps promoting substrate degradation. The structure showed that the TPR domain of HIP binds across HSP70 and contacts the IA, IB and IIB subdomains of HSP70 and thus locks it in an ADP-bound conformation. The binding of HIP was also shown to be mutually exclusive to the NEFs BAG1, (Xu et al. 2008) HSP110 and HSPBP1, an armadillo-type NEF (Arakawa et al. 2010; Li et al. 2013; Polier et al. 2008; Schuermann et al. 2008; Shomura et al. 2005; Sondermann et al. 2001).

The HSP90 Chaperone Machine

HSP90 is a multidomain ATP-dependent dimeric chaperone (Ali et al. 2006). Whilst dimerisation is inherent through the C-terminal domains, the N-terminal domains undergo cycles of dimerisation and disassembly in response to ATP-binding and hydrolysis. The structural changes that take place within HSP90, in order to establish a closed catalytic state, are thought to occur simultaneously in a coordinated fashion and collectively represent the rate-limiting step of ATP hydrolysis (Schulze et al. 2016). This conformational cycle is responsible for the activation, via remodelling of the client protein structure (Noddings et al. 2022; Verba et al. 2016), of a vast array of client proteins, including proteins such as erbB-2, cyclin-dependent kinase 4 (CDK4), braf, structural proteins such as actin and tubulin and steroid hormone receptors. A full list of clients can be found at http://www.picard.ch/Downloads/HSP90interactors.pdf. The N-terminal domain has been shown in interactions with co-chaperones such as p23 (Sba1 in yeast), Cdc37 and Aha1, all of which are able to regulate HSP90 ATPase activity and therefore modulate the catalytic cycle of HSP90.

The middle and largest domain of HSP90 contains the catalytic loop arginine residue that interacts with the γ-phosphate of bound ATP and completes the catalytic unit (Fig. 13.1b). The structure and catalytic cycle of HSP90 has been reviewed in detail in a number of recent reviews (Genest et al. 2019; Hoter et al. 2018; Li and Buchner 2013; Prodromou 2012, 2016). Co-chaperones such as Cdc37, p23 and Aha1 have all been shown to interact with the middle domain and influence its ATPase activity through these interactions (Ali et al. 2006; Meyer et al. 2004; Roe et al. 2004). This is, at least in part, most likely achieved through interactions with residues on HSP90 preceding the catalytic loop of the middle domain. Structural work has revealed that kinases interact with the middle domain of HSP90; however, other clients may interact more extensively with the chaperone (Karagoz et al. 2014).

The C-terminal domain is inherently dimerised and contains a conserved MEEVD peptide motif that is responsible for the binding of TPR domain-containing co-chaperones. These include HOP (Sti1p in yeast), immunophilins (such as FK-binding protein (FKBP) 51 and FKBP52), protein phosphatase 5 (PP5), carboxyl terminus of Hsc70-interacting protein (Schipper-Krom et al. 2012), aryl hydrocarbon receptor-interacting protein (AIP), aryl hydrocarbon receptor-interacting protein-like 1 (AIPL1) and RNA polymerase II-associated protein 3 (RPAP3) to name but a few. These co-chaperones may be specific for particular client protein complexes and may impart additional enzymatic activities that help regulate the HSP90 complex and activation or degradation of client proteins (Prodromou 2012).

Cdc37 The Cdc37 co-chaperone is involved in delivering kinase client proteins to the HSP90 machinery. Remarkably, around 60% of the human kinome interacts with HSP90 through participation with Cdc37 (Taipale et al. 2012). Initial structural studies have shown that Cdc37 interacts with the N-terminal domain of HSP90 and inhibits the ATPase-driven cycle of the chaperone (Roe et al. 2004). In complex with CDK4, it was shown that the kinase was engaged with the N and middle domain

of HSP90, whilst Cdc37 engages with the N domains, and consequently the chaperone remained in an open state (Vaughan et al. 2006). In contrast, recent Cryo-EM studies of the HSP90-Cdc37-CDK4 complex have shown that Cdc37 can engage with HSP90 by binding at the interface of the N and middle domains of the chaperone (Verba et al. 2016). In this state, HSP90 is in a closed conformation, and the kinase is trapped in a partially unfolded state, bound across the HSP90 dimer. The two lobes of the kinase are separated between HSP90 and Cdc37, the latter of which mimics part of the CDK4 N-lobe to stabilise an open conformation of the kinase (Verba et al. 2016). Cdc37 phosphorylation at Ser 13 plays an important role in stabilising the closed conformation of the HSP90-Cdc37-CDK4 complex and is involved in contacts with residues of HSP90 within the helix immediately following the catalytic loop (Verba et al. 2016). The interaction helps stabilise the N-terminal fragment of Cdc37, which also interacts with the catalytic loop directly (Verba et al. 2016). This not only acts as an interaction hub stabilising the complex but may also influence the rate of ATP hydrolysis in the complex. Dephosphorylation of Ser 13 by PP5 has been shown to cause release of the protein kinase and disassembly of the complex (Vaughan et al. 2008). In contrast, Tyr 4 and Tyr 298 phosphorylation has been shown to lead to client dissociation (Xu et al. 2012).

p23 Unlike Cdc37, the co-chaperone p23 (Sba1p in yeast) is not involved in delivering clients to HSP90, but it appears to stabilise the chaperone-client complex in a closed state. p23 presents a characteristic cysteine- and histidine-rich domain known as the CS domain (CHORD and sgt1), an ~100 residue compact antiparallel β-sandwich formed by seven β-strands that shows structural homology to the core domain of several small HSPs and HSP90 interactors such as NudC, sgt1 and rar1 (Garcia-Ranea et al. 2002; Van Montfort et al. 2001; Weaver et al. 2000). p23 is involved in the maturation of protein clients, interacting with and stabilising the ATP-bound, closed state of the HSP90 dimer through its CS domain (Ali et al. 2006). Its unstructured C-terminal tail is responsible for inhibiting or downregulating the HSP90 ATP hydrolysis by most likely inducing structural changes in the catalytic loop at the NTD-MD interface of the closed dimer and consequently stabilising the interaction with the client (Ali et al. 2006; Biebl et al. 2021).

Aha1 The co-chaperone Aha1 consists of an NTD and C-terminal domain (CTD) connected by a 60-amino acid residue flexible linker. It remains the most potent ATPase activator of HSP90. Early structural studies showed that the NTD of Aha1 binds to the middle domain of Hp90 and influences the conformation of the catalytic loop, bringing about the acceleration of ATPase hydrolysis and thus the conformational cycle of HSP90 (Panaretou et al. 2002). This interaction represents the recruitment step for Aha1 into the HSP90 complex. It has been seen that Aha1 can accelerate the rate of HSP90 dimer closure and the CTD of HSP90 may play a major role in this, as it is known to interact with the N-terminal domain of the co-chaperone (Li et al. 2013). More recent cryo-EM studies have now shown that the C-terminal domain of Aha1 interacts with the middle domain of both protomers of the HSP90 dimer (Liu et al. 2020). The Aha1 CTD induces a semi-closed HSP90 conformation

together with the N-terminal domain of Aha1, but steric clashes within this conformation cause the NTDs of HSP90 to become undocked from their respective middle domains. The NTDs can now rotate and are now primed for ATP-binding and redocking in a conformation that allows N-terminal dimerisation of HSP90. This also causes the NTDs of Aha1 to tilt by around 30° to establish new interactions with the dimerised NTDs of HSP90. This not only stabilises the dimerised state of the NTDs of HSP90 but also facilitates ATP hydrolysis. However, it appears that the NTD of Aha1 only interacts with the lid region from only one of the NTDs of the HSP90 dimer. Thus, ATP hydrolysis is stimulated in that protomer alone, which establishes an asymmetric semi-hydrolysed state within HSP90. The open state of HSP90 and disassembly of the complex probably result following the hydrolysis of the second-bound ATP molecule by HSP90.

Within the HSP90-Aha1 closed state, there are a number of interactions that influence the stability and activity of the complex. In the tilted position, the conserved N-terminal motif, NxNNWHW, interacts across the dimerised N-terminal domains. This motif contacts the 'ATP lid' of one protomer, helps to stabilise the catalytic loop of the middle domain of HSP90 within the same protomer and probably provides additional stability to the β-strand exchange between the NTDs of HSP90, by binding to and locking the helix preceding the β-strand element. The deletion of the conserved NxNNWHW motif of HSP90 has been shown to reduce its ability to stimulate the ATPase activity of HSP90 and its ability to rescue the temperature-sensitive S25P mutation of yeast HSP90p (Mercier et al. 2019). A small α-helix (residues 163–170) at the extreme C-terminal end of the NTD of Aha1 also interacts with the 'ATP lid' of the same HSP90 protomer. Furthermore, the CTD of Aha1 helps to stabilise the N- and middle-domain interface of HSP90. Previous work has also shown that the F349A mutation, thought to destabilise the N-middle domain interface of HSP90, might be suppressed by Aha1 (Siligardi et al. 2004), and this suggestion has now been shown to be fully consistent with the structural model proposed for the HSP90-Aha1 complex.

RPAP3 and PIH1 Domain-Containing Protein 1 (PIH1D1) RPAP3 (Tah1p in yeast) and PIH1D1 (Pih1p in yeast) form a complex and act as a co-chaperone of HSP90 (Eckert et al. 2010; Millson et al. 2008; Morgan et al. 2015; Pal et al. 2014). Tah1p has also been shown to activate the HSP90 ATPase activity, but as a complex with Pih1p, it appears to inhibit activity, perhaps allowing client protein loading and complex formation (Eckert et al. 2010; Millson et al. 2008). The RPAP3 and PIH1D1 complex also forms part of the larger chromosome remodelling complexes, INO80 and SWR-C, by interacting with Rvb1p and Rvb2p (Martino et al. 2018; Rivera-Calzada et al. 2017). In this way, they may act as an adaptor between HSP90 and Rvb1/2p (RUVBL1/2 in humans) client proteins (Morgan et al. 2015; Zhao et al. 2008). In contrast to the inhibition of HSP90, the Tah1p-Pih1p complex activates the ATPase activity of Rvb1/2p complex (Rivera-Calzada et al. 2017).

Tah1 consists of five α-helices, which constitute a TPR domain and an unstructured C-terminal domain (Back et al. 2013; Jimenez et al. 2012; Millson et al. 2008; Pal et al. 2014). The TPR domain appears to be specific for HSP90 binding (Millson

et al. 2008). Unlike other TPR domains, Tah1p requires a helix swap, which involves the fifth α-helix between two Tah1p molecules, to create the normal binding environment for the methionine from the bound HSP90 MEEVD motif (Millson et al. 2008). This reconstitutes a TPR-binding site similar to that seen in other typical seven helix-containing TPR domain proteins. It has been suggested that dimerisation of Tah1p prevents other monomeric TPR domain proteins from simultaneously binding to HSP90 (Morgan et al. 2015). The TPR cleft of Tah1p also appears to be accessible and able to accept the MEEVD motif of HSP90 when bound within the R2TP (Rvb1p-Rvb2p-Tah1p-Pih1p) complex (Rivera-Calzada et al. 2017).

RPAP3 is considerably larger than Tah1p and appears to be recruited to the R2TP complex through a C-terminal RUVBL2-interacting domain that interacts with the ATPase face of the complex. It also contains two central TPR domains, which are equivalent to the dimerised TPR domains of Tah1p, whose binding maps to the opposite face of the R2 ring where it can interact with PIH1D1, as observed with the yeast R2TP complex (Martino et al. 2018). Similarly, as with Tah1p, there is an unstructured segment downstream of the TPR domains of RPAP3, represented by residues 400 to 420, which is sufficient to form a complex with PIH1D1.

PIH1D1 and Pih1p consist of two domains, an N-terminal PIH domain that recruits Tel2, through a phosphoserine-binding site, and a CS domain that interacts with the unstructured region of Tah1p and RPAP3 described above (Martino et al. 2018; Pal et al. 2014). The CS domain comprises of residues 264–344 forming a seven-stranded β-sandwich with the topology found in other HSP90 co-chaperones, such as p23/Sba1p (Ali et al. 2006) and Sgt1 (Zhang et al. 2008; Zhang et al. 2010). In contrast, the PIH domain consists of a twisted five-stranded β-sheet where strands four and five are traversed by a helix-turn-helix segment, and the other face of the β-sheet is traversed by a coil segment that extends from the end of β-strand 5 (Pal et al. 2014). An additional α-helix that connects β-strands two and three projects from the end of the sheet, packing against the larger of the other two helices. The interaction of Pih1 with Tel2 (and therefore the TTT complex) is through a casein kinase 2 phosphorylated motif in Tel2 (SELDpSDDEF) and the PIH domain (Horejsi et al. 2010; Pal et al. 2014).

The role of HSP70 in R2TP complexes is less well known, and direct interaction of RPAP3/Tah1 and HSP70 appears to be significantly weaker than with HSP90 (Millson et al. 2008). However, HSP70 has been shown to be associated with HSP90 (Wang et al. 2022) as well as the PAQosome (particle for arrangement of quaternary structure), which is a large multisubunit chaperone complex that interacts with several chaperones including HSP90, HSP70 and CCT and R2TP (Lynham and Houry 2018).

PP5 The PP5 Ser/Thr phosphatase plays an important role in the regulation of the HSP90 client kinase loading and unloading cycle. The HSP90 co-chaperone Cdc37 is phosphorylated on residue Ser 13 by casein kinase 2 and is then able to recruit kinases to HSP90 complexes (Miyata and Nishida 2004; Vaughan et al. 2006). Binding of PP5 to such Cdc37-kinase-HSP90 complexes results in dephosphorylation of Cdc37 at Ser 13 and leads to subsequent release of the kinase (Oberoi et al.

2016; Vaughan et al. 2008). PP5 is also found in HSP90 complexes with the co-chaperones FKBP51, FKBP52 and p23 in the chaperoning of steroid hormone receptors (Banerjee et al. 2008; Kaziales et al. 2020; Silverstein et al. 1997) and has been shown to directly dephosphorylate GR on several residues, modulating its activity (Dushukyan et al. 2017; Wang et al. 2007).

Immunophilins Immunophilins are peptidyl-prolyl cis-trans isomerases (PPIases) that catalyse and stabilise the cis-trans isomerisation of peptide bonds. PPIase proteins are classified into cyclophilins that bind cyclosporin and FK506-binding proteins. FKBP52, FKBP51 and Cyp40 are thought to catalyse and stabilise the cis-trans isomerisation of peptide bonds in the HSP90-steroid hormone receptor clients GR, oestrogen receptor and progesterone receptor as well as Tau (Jinwal et al. 2013; Lee et al. 2021; Nair et al. 1997; Ratajczak et al. 1993). Immunophilins are found together with p23 within HSP90-steroid hormone complex (Ebong et al. 2016; Johnson and Toft 1994; Nair et al. 1997). In the closed HSP90 complex, the TPR domain of FKBP51 is bound to the C-terminal domain of HSP90 (Johnson and Toft 1994; Noddings et al. 2020). The TPR cleft is directed away from the HSP90 molecule but is bound by the conserved DTSRMEEVD peptide motif of HSP90. Furthermore, the terminal helix of the TPR domain interacts between the terminal helices of the HSP90 protomers, and sequence conservation suggests that other immunophilin classes of TPR co-chaperones may collectively represent a conserved mechanism for HSP90-specific recognition. Binding within this C-terminal cleft of HSP90 appears to be favoured in its closed state. Further contacts between the C-terminal domain of FKBP and HSP90 occur in the connecting strand between helices 5 and 6 of FKBP51, including a direct backbone contact between N365 and N655 in HSP90. The FK1 domain is positioned adjacent to HSP90-client interaction sites (Genest et al. 2013; Verba et al. 2016) and may provide a means by which the PPIase activity of the FK1 domain may act on specific client proline residues. In contrast, the FK2 domain is inactive (Sinars et al. 2003) but helps transverse the middle domain of HSP90 so that the FK1 domain can dock correctly with the chaperone.

In yeast, Cpr6p and Cpr7p interact with the intact ribosome (Tenge et al. 2015). It has also been reported that an HSP90p-Cpr6p complex can interact with Ura2p, a protein involved in pyrimidine biosynthesis (Zuehlke et al. 2013). The structure of intact Cpr7p has been reported (Qiu et al. 2017). Cpr7p was also seen to interact with Ure2p and is required for the stability of its Ure3p prion derivative (Kumar et al. 2015). Together with the recruiter Hgh1p, Cns1p links HSP90 to translation elongation by chaperoning elongation factor 2 (Schopf et al. 2019).

AIP AIP consists of two domains, an N-terminal immunophilin-like domain and a C-terminal TPR domain (Linnert et al. 2012; Morgan et al. 2012). AIP interacts with a number of different chaperone proteins (HSP90, HSP70, TOMM20) that share a common conserved EEVD motif (DDVE in TOMM20) at the C-terminal end of these chaperones (Bolger et al. 2003; Scheufler et al. 2000; Zhang et al. 2005). Client proteins, amongst others, include nuclear receptors (aryl hydrocarbon (Ahr), oestrogen receptor α), phosphodiesterase 4A5 (rat isoform of human *PDE4A4*)

and PDE2A3, survivin, G proteins, ret. and Epstein-Barr virus nuclear antigen 3 (Trivellin and Korbonits 2011). Mutations in AIP have been linked to familial isolated pituitary adenomas (Chahal et al. 2010; Daly et al. 2007; Leontiou et al. 2008; Vierimaa et al. 2006), which leads to acromegaly and gigantism. The precise role in predisposition to pituitary adenoma is not well understood, but AhR may act as a tumour suppressor that becomes silenced (Heliovaara et al. 2009; Huang and Elferink 2005; Kolluri et al. 1999; Leontiou et al. 2008; Marlowe et al. 2004; Pang et al. 2008; Puga et al. 2000; Vierimaa et al. 2006).

AIPL1 AIPL1 shares 49% identity with AIP and similar to AIP is comprised of an N-terminal FKBP-like domain that lacks PPIase activity and a C-terminal TPR domain. AIPL1 has been shown to interact with both HSP70 and HSP90, and like other TPR domain co-chaperones, the terminal MEEVD and IEEVD motifs of HSP90 and HSP70, respectively, contribute to the interaction of AIPL1 with the chaperones (Hidalgo-de-Quintana et al. 2008). The interaction of AIPL1 with HSP90 is nucleotide-dependent (Sacristan-Reviriego et al. 2017), though the exact structural features mediating the AIPL1-HSP90 interaction have yet to be characterised. Interestingly, AIPL1 is expressed exclusively in the photoreceptor cells of the neurosensory retina and in the pineal gland, and AIPL1 mutations thus cause a severe and early onset inherited retinal degeneration, Leber congenital amaurosis (Sohocki et al. 2000; van der Spuy et al. 2002, 2003). To date, the only identified client protein for the AIPL1-HSP90 chaperone complex is retina-specific cGMP phosphodiesterase (PDE6), a critical component of the phototransduction cascade that catalyses the hydrolysis of cGMP to GMP upon light exposure, thus leading to the closure of cGMP-gated cation channels and membrane hyperpolarisation. In Aipl1 knockout or hypomorphic mice, all three subunits of the rod photoreceptor PDE6, including the catalytic α and β subunits and the inhibitory γ subunit, are translated; however, the PDE6 holoenzyme is misassembled, and the subunits rapidly degraded (Kolandaivelu et al. 2009; Liu et al. 2004; Ramamurthy et al. 2004). Similarly, AIPL1 is required for the proper assembly and stability of the cone photoreceptor PDE6 heterocomplex (Kirschman et al. 2010). Inhibition of HSP90 in the rat retina leads to the posttranscriptional loss of PDE6 (Aguila et al. 2014). Within the PDE6-AIPL1-HSP90 complex, the FKBP-like ligand-binding domain of AIPL1 is thought to interact with isoprenyl groups of the PDE6 catalytic subunits (Kolandaivelu et al. 2009; Majumder et al. 2013; Yadav et al. 2017), whereas the TPR domain of AIPL1 mediates the interaction with HSP90 (Hidalgo-de-Quintana et al. 2008; Sacristan-Reviriego et al. 2017), although an interaction between the PDE6 inhibitory γ subunit and the TPR domain has also been observed (Yadav et al. 2019). It is thought that through these interactions, AIPL1 mediates the specificity of HSP90 for the PDE6 client protein in retinal photoreceptors, and the folding, orientation and organisation of the AIPL1 domains are important for mediating this function (Gopalakrishna et al. 2016; Sacristan-Reviriego et al. 2017).

Sgt1 Sgt1 is an essential chaperone that can recruit HSP90 in to a range of cellular activities including Skp, Cullin, F-box-containing complex E3 ubiquitin ligases and

the kinetochore. It appears that in these pathways, Skp1, a small protein that heterodimerises with proteins containing the F-box motif, is associated with Sgt1 (Davies and Kaplan 2010; Willhoft et al. 2017). Sgt1 has also been implicated in the regulation of innate immunity systems in plants and animals. Sgt1 has been found to associate, together with HSP90, with plant R proteins and related animal Nod-like receptors. The crystal structure and stoichiometry of the core HSP90-Sgt1-Rar1 CHORD II domain complex in association with the HSP90 N-terminal domain have been determined (Siligardi et al. 2017; Zhang et al. 2008, 2010).

Major Co-chaperones Involved in Both HSP90 and HSP70 Complexes

Although many other co-chaperones are involved in the maturation and regulation of client proteins that are both HSP70 and HSP90-dependent, two major co-chaperones that work closely with the HSP70 and HSP90 chaperone systems are worthy of a mention here. The first is the adaptor HOP (Sti1p in yeast), which links the HSP70 and HSP90 systems together. The second is CHIP, which is utilised in client protein degradation by both chaperone systems (Fig. 13.2).

HOP HOP (Sti1p in yeast) is a TPR repeat-containing co-chaperone, which recognises the C-terminal EEVD motif in HSP90 and HSP70 (Brinker et al. 2002; Odunuga et al. 2003; Scheufler et al. 2000). HOP/Sti1p contains two Asp-Pro (DP)-rich domains, which are arranged as follows with the three TPR domains: TPR1–DP1–TPR2A–TPR2B–DP2. The TPR1 and TPR2A domains of HOP bind specifically to the C-terminal tails of HSP70 and HSP90, respectively (Brinker et al. 2002). HOP/Sti1p is able to bind simultaneously to HSP90 and HSP70 (Alvira et al. 2014; Lee et al. 2012; Rohl et al. 2015; Scheufler et al. 2000; Schmid et al. 2012) to facilitate client protein transfer. Clients include nuclear receptors (steroid hormone receptors), kinases such as eukaryotic initiation factor 2α-kinase, cyclin-dependent kinases and the p53, HSF-1, pRb transcription factors (Mayer and Bukau 2005).

HOP is a major TPR domain-containing co-chaperone that regulates HSP90's molecular chaperone function. HOP plays an important role in delivering steroid hormone receptors and other clients to HSP90 by working together with HSP70 and by stabilising the open conformation of HSP90 through inhibition of its ATPase activity (Jinwal et al. 2013; Kirschke et al. 2014; Prodromou et al. 1999; Rohl et al. 2015). Sti1p has been reported to be a dimer and a potent inhibitor of the ATPase activity of HSP90 (Prodromou et al. 1999). It has been proposed that HOP interactions with the middle domain of HSP90 may inhibit the conformational changes leading to the N-terminally closed state, but further evidence is required to substantiate these claims. In contrast, the effect of HOP or Sti1p on HSP70s has been reported as variable (Bhattacharya et al. 2020; Gross and Guerrieri 1996; Johnson et al. 1998).

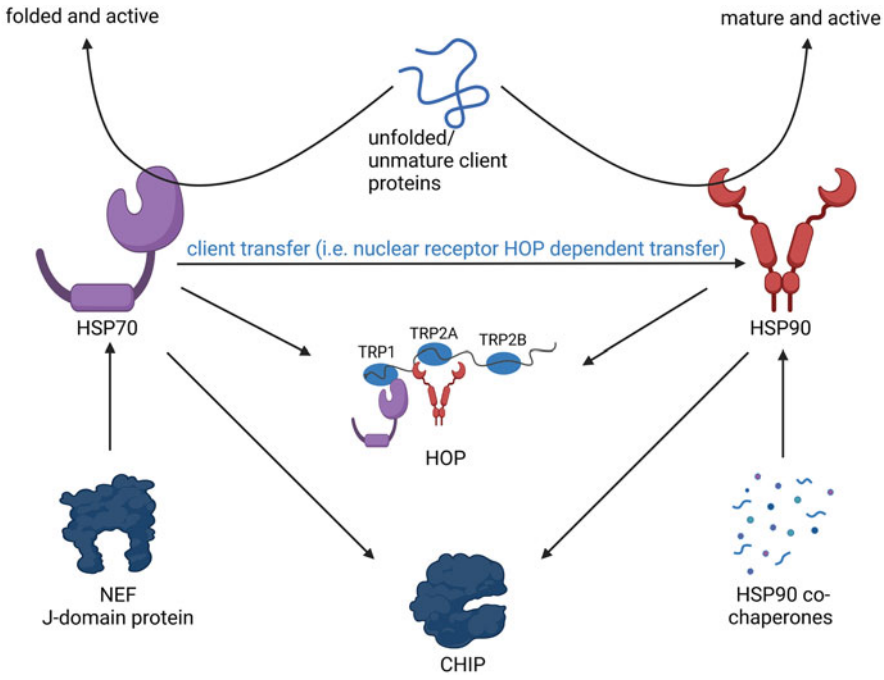

Fig. 13.2 Proteostasis by the HSP70 and HSP90 chaperone systems. Unfolded or unmatured client proteins are either folded or activated by HSP70 or HSP90 chaperones as required. If required by the client protein, HOP acts as a bridge allowing the transfer of the client to HSP90. Co-chaperones for the HSP70 and HSP90 systems are also shown, which aid the chaperone cycle. CHIP can access both chaperone systems to initiate protein degradation by ubiquitylation of client proteins that have stalled in the folding cycle. Figure created with BioRender.com

CHIP CHIP is a E3 ubiquitin ligase, which contains a TPR repeat domain able to interact with HSP70, but also HSP90 (Stankiewicz et al. 2010). CHIP is a dimeric protein of two identical polypeptide chains of ~35 kDa each containing an N-terminal TPR repeat domain and a C-terminal U box domain (Ballinger et al. 1999; Hatakeyama et al. 2001). The structure of CHIP has been determined (Zhang et al. 2005): CHIP displays an E3 and E4 ubiquitin ligase activity (Jiang et al. 2001; Murata et al. 2001), and it is thought to be involved in client protein quality control that facilitates switching between chaperone-mediated folding and maturation to a proteasome-mediated degradation involving lysine 48-linked polyubiquitylation (Cyr et al. 2002; Wiederkehr et al. 2002), although CHIP has been reported to autoubiquitylate and to facilitate nonlysine 48 polyubiquitylation (Alberti et al. 2002; Jiang et al. 2001; Murata et al. 2001). The ubiquitylation of HSP70 and HSP90 substrates targets them for proteolytic degradation by the proteasome (Connell et al. 2001; Edkins 2015; Hohfeld et al. 2001; Meacham et al. 2001). Thus, although both HSP70 and HSP90 are involved in protein-folding and regulation of clients, both send clients for degradation under appropriate conditions.

Details about the mechanism are enigmatic, but it is believed that CHIP might respond to substrates or client proteins that fail to fold or progress within the HSP70 and HSP90 chaperone systems (Connell et al. 2001; Meacham et al. 2001; Stankiewicz et al. 2010; VanPelt and Page 2017). Clients responding to CHIP-directed degradation include cystic fibrosis transmembrane conductance regulator, GR, the E2A transcription factor, tau, huntingtin (Htt) and ataxin, telomerase, apoptosis signal-regulating kinase 1, phosphatase and tensin homologue and p53 (Ahmed et al. 2012; Connell et al. 2001; Gao et al. 2010; Huang et al. 2004; Jana et al. 2005; Lee et al. 2010; Meacham et al. 2001; Petrucelli et al. 2004). Cryo-EM structures of HSP70-client and CHIP complexes show that the substrate is located between the chaperone and CHIP. This suggests that client protein is presented to CHIP and flexibility within such complexes is important for the ubiquitylation process (Quintana-Gallardo et al. 2019). Inhibition of HSP90 by ATPase competitive inhibitors, such as geldanamycin (GA), results in its client proteins being directed for proteolytic degradation of proteins such as ErbB-2 (Xu et al. 2002). Similarly, addition of CHIP to a HSP90-client complex promotes proteasomal degradation of the client protein. However, no physical link has been found between the HSP90-client protein complex and the proteasome (Connell et al. 2001; Whitesell and Cook 1996).

Disruption of Proteostasis and Disease

Given their importance in protein-folding and quality control, it is unsurprising that molecular chaperones and co-chaperones, including those of HSP70 and HSP90 networks, have been identified as key modulators of human-misfolding disease and in particular neurodegenerations (Barral et al. 2004; Bonini 2002; Muchowski and Wacker 2005). For example, FKBP51 is implicated in Tau pathologies (Chambraud et al. 2010; Jinwal et al. 2010) and a variety of other human diseases (Storer et al. 2011), and the HSP90 activator Aha1 drives production of pathological Tau aggregates (Shelton et al. 2017). The exact role that co-chaperones and chaperone systems play in disease is not always clear, and it may be that chaperone function itself is normal, but it is the dependency of mutant or dysfunctional client protein on such chaperone systems that ultimately leads to dysregulation of signalling pathways that cause disease.

Many neurodegenerative disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and polyQ expansion diseases, are characterised by conformational changes in disease-associated proteins that result in their misfolding and aggregation (Barral et al. 2004; Muchowski and Wacker 2005; Taylor et al. 2002). More specifically, although the majority of proteins fold via intermediate states, which can be kinetically stable and form amorphous aggregates, a subset of aggregation-prone proteins linked to neurodegenerative disease shares a propensity to assemble into ordered fibrillar aggregates, referred to as amyloid fibrils. These can be more stable than the native state of the

protein and protease-resistant. Structurally amyloid fibrils are characterised by a cross β-sheet conformation, with β-strands that run perpendicular to the fibril axis forming extended β-sheets (Dobson 2003; Klaips et al. 2018; Stefani and Dobson 2003). Although fibrillar aggregates are a pathological feature in the brains of patients with neurodegenerative diseases characterised by protein-misfolding, evidence suggests they are not the major culprit for neuronal toxicity. Instead, it appears that smaller soluble precursors of the fibrillar aggregates are key drivers of pathogenesis. These precursors are heterogeneous and thought to consist of both on and off pathway intermediates of amyloid fibrils, with variable toxicity (Soto and Pritzkow 2018). There are multiple examples of chaperone networks counteracting toxic misfolded protein species. These include HIP and HSP70 together, which can prevent cytotoxic aggregates of α-synuclein and polyQ-expanded androgen receptor (AR) that lead to neurological disease (Howarth et al. 2009; Roodveldt et al. 2009). Furthermore, even where there is no direct evidence for reduction in cytotoxic protein species, chaperone levels are frequently increased in neurodegenerative conditions. For example, upregulation of HSP90, sgt1 and CHP-1 (CHORD-containing protein1) has been seen in the majority of cases of PD and dementia with Lewy bodies (Bohush et al. 2019).

Another feature of amyloid fibrils is that they have the potential to nucleate subsequent fibril formation of their soluble constituent protein. Recently, it has been identified that this seeding activity can occur not just at the molecular level but between cells facilitating cell-to-cell spread and pathological transmission in misfolding diseases. There is significant evidence for HSP70 and HSP90 binding to amyloid fibril proteins. This includes the AD protein Tau, where HSP70 inhibits nucleation and elongation of fibrils and sequesters oligomers and fibrils into protective complexes (Kundel et al. 2018), whilst HSP90 promotes formation of small Tau oligomers yet inhibits formation of Tau fibrils (Weickert et al. 2020). Chaperones play multiple roles in neurons and other cells in the cellular defences against accumulation of misfolded proteins and aggregation (Ross and Poirier 2005) (Fig. 13.3). Aside from being essential for protein-folding and refolding (Bukau and Horwich 1998; Hartl and Hayer-Hartl 2002), they also function in disaggregation and triage of terminally misfolded proteins for degradation by the ubiquitin proteasome or autophagy-lysosome systems (Alberti et al. 2002, 2004; Chapple et al. 2004; Connell et al. 2001) (Fig. 13.3). This includes that chaperone may promote the sequestration of misfolded aggregated proteins into larger protein inclusions, with relatively decreased surface-to-volume ratios. This accumulation of the soluble oligomers into larger inclusions is thought to be neuroprotective, as it separates the toxic-soluble oligomers away from the cellular milieu and into an insoluble compartment that may also be more amenable to clearance by autophagic mechanisms (Arrasate et al. 2004; Cohen et al. 2006; Cuervo 2004; Cuervo et al. 2004; Kaganovich et al. 2008; Kopito 2000; Mannini and Chiti 2017; Sontag et al. 2017; Taylor et al. 2003).

Together, protein-folding and degradation pathways are integral to maintenance of cellular proteostasis (protein homeostasis) with a highly interconnected network of chaperones and degradation factors combating proteotoxicity. HSP70

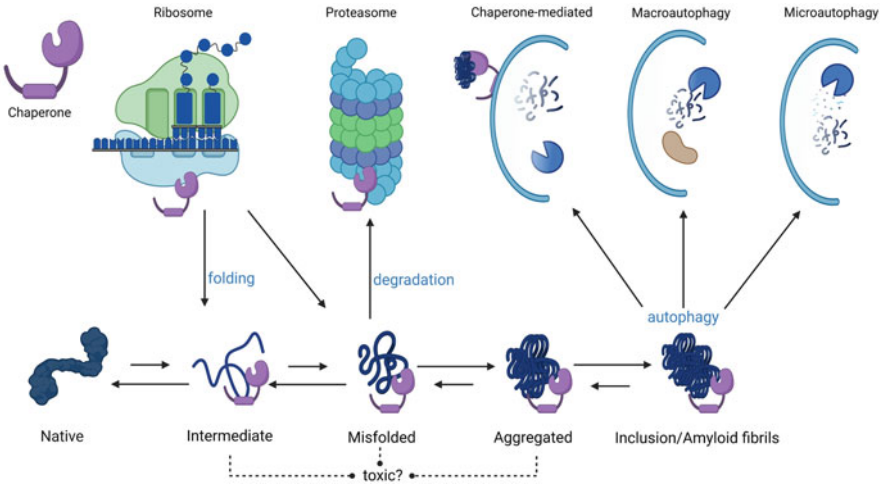

Fig. 13.3 Molecular chaperones in protein misfolding and aggregation. Molecular chaperones and their associated co-chaperones are essential in the cellular defences against protein aggregation. Molecular chaperone networks participate in protein folding and refolding, proteasome-dependent degradation and inclusion formation and lysosome-mediated autophagy. Figure created with BioRender.com

(Fernandez-Fernandez et al. 2017) and HSP90 (Taipale et al. 2010) can both be considered hubs in the proteostasis network. The HSP70 chaperone machinery can channel its clients along folding, refolding, maturation, disaggregation, and proteolytic pathways. This is in cooperation with its network of co-chaperones and other chaperone systems, including the HSP90 machinery (Sekhar et al. 2016). Indeed, dependent on chaperone-co-chaperone interactions and wider chaperone networking, HSP70 may act as a holdase, foldase or disaggregase and direct its clients to degradation by both the ubiquitin proteasome system (UPS) (Kapustian et al. 2013) and autophagy-lysosome pathways (Fernandez-Fernandez et al. 2017) (Fig. 13.3). In chaperone-assisted UPS degradation, HSP70- and HSP90-bound clients are ubiquitinated for sorting to the proteasome and subsequent degradation. This is mediated by CHIP, with a number of other co-chaperones, such as the J-domain protein DNAJB2/HSJ1 that acts to sort HSP70 clients to the proteasome (Westhoff et al. 2005). For chaperone-mediated autophagy (Karzai and McMacken 1996), which is a specific lysosome-dependent degradation pathway, cytosolic proteins destined for degradation are delivered directly into the lumen of lysosomes through a mechanism which involves interaction with HSPA8 (the constitutively expressed HSP70) and lysosome-associated membrane protein type 2 (LAMP-2A). HSPA8 binds clients with a KFERQ-like domain facilitating their targeting to the transmembrane domain of LAMP-2A which oligomerises to form a translocation complex taking the HSPA8 client into the lumen of the lysosome for degradation. There is evidence that other chaperones and co-chaperones play a role in regulating CMA as

DNAJB1/HSP40, HOP, HIP and bag1 have been reported to complex with HSPA8 on the lysosomal membrane (Fernandez-Fernandez et al. 2017). HSP70 and its co-chaperones also play roles in other forms of autophagy, including macroautophagy. For example, bag3 and HSP70 in conjunction with HSPB8 and the ubiquitin receptor p62/SQSTM1 target aggregation-prone proteins for macroautophagic degradation through a mechanism that requires the sequestration of misfolded proteins to inclusion bodies (Sturner and Behl 2017).

Impaired function of proteostasis networks is a common feature of neurodegenerative diseases (Klaips et al. 2018). This can occur through misfolded protein species interfering with the normal function of components of proteostasis networks. A key example is that specific oligomers of the proteins Aβ, α-synuclein and polyQ huntingtin (53Q), which aggregate in AD, PD and Huntington's disease, respectively, have a common conformation that has been shown to inhibit the proteasome (Thibaudeau et al. 2018). This is through an allosteric mechanism where misfolded oligomers bind the proteasome and stop normal functioning of the substrate gate that regulates entry to the degradation chamber. Thus, in misfolding disease, UPS can become compromised and/or overloaded, thereby promoting the special sequestration of ubiquitylated proteins to inclusion bodies. CMA may also be inhibited by misfolded proteins including acetylated Tau, which is elevated in Tau-mediated neurodegenerations (Min et al. 2010) and instead is cleared by other autophagic pathways (Caballero et al. 2021). The action of HSP70 in the clearance of potentially toxic protein species by targeting to UPS and autophagy-lysosome pathway is likely to reduce unwanted interactions between misfolded proteins and key cellular components such as transcription factors or other essential cellular proteins (Schaffar et al. 2004).

Despite redundancy and plasticity in proteostasis networks, maintaining the solubility and/or clearance of high levels of some aggregation-prone proteins places a burden on the total protein homeostasis machinery, leading to further disruption of proteostasis (Hipp et al. 2012; Schipper-Krom et al. 2012). Moreover, it is known that chaperone activity for HSP70, and other ATP-dependent chaperones, decreases during human brain ageing, which is consistent with prominent neurodegenerative disease being associated with old age (Brehme et al. 2014; Hipp et al. 2019). This suggests that levels of HSP70 chaperone activity may become limiting in age-associated neurodegenerative diseases. It is also consistent with data supporting HSP70 is neuroprotective. Indeed, overexpression of HSP70 and its co-chaperones is neuroprotective in multiple model systems supporting that chaperone activity may become limiting. This includes a reduction in Aβ plaque deposition in a mouse model of AD that was crossed with a mouse over expressing HSP70 (Hoshino et al. 2011). Modulation of the HSP70 machinery by altered co-chaperone expression has also shown to be neuroprotective. This includes that the transgenic overexpression of DNAJB2a in the R6/2 mouse model of HD led to improved neurological performance, significantly reduced mutant huntingtin (mHtt) aggregation and enhanced solubility dependent on DNAJB2a client binding, ubiquitin interaction and functional cooperation with HSP70 (Labbadia et al. 2012). Moreover, the overexpression of DNAJB2a in a mouse model of ALS was also shown to improve motor

performance and the survival of motor neurons at the late stages of disease progression (Novoselov et al. 2013).

Cooperation of HSP70 and HSP90 Chaperone Machines and Maintenance of Proteostasis in Neurodegenerative Disease

HSP90, HSP70 and their respective co-chaperones whilst acting as central hubs for protein-folding and turnover also function together in a multiprotein complex to drive the dynamic assembly and coordinate the stabilisation of HSP90 client protein heterocomplexes (Moran Luengo et al. 2019). Substrate recognition is mediated by HSP70 and HSP40, with the substrate passed from HSP70 to HSP90 via HOP (Fig. 13.2). Interestingly, a number of HSP90 client proteins are implicated in adult-onset neurodegenerative diseases, including AD, PD and polyQ expansion diseases (HD, spinal and bulbar muscular atrophy (SBMB), dentatorubral-pallidoluysian atrophy and spinocerebellar ataxias). These HSP90 client proteins include Tau (in AD), α-synuclein (in PD), Htt (in HD) and AR (in SBMB) amongst others, all critical proteins known to unfold and aggregate in intracellular inclusions that are the characteristic hallmarks of these neurodegenerative diseases (Kuiper et al. 2017; Mogk et al. 2018). HSP90 interacts with these metastable native or near-native clients to stabilise them against ubiquitin-mediated proteasomal degradation, thus facilitating their attainment of a functionally active conformation. However, in neurodegenerative diseases, the intrinsically unstable pro-aggregation segments of these client proteins can unfold to form soluble toxic oligomers before they aggregate into inclusions (Li et al. 2007; Outeiro et al. 2008). Evidence points to the accumulation of these soluble oligomers into larger inclusions being neuroprotective, with the sequestration of the neurotoxic species away from the cellular milieu and into an insoluble compartment to limit the cellular damage (Arrasate et al. 2004; Cohen et al. 2006; Kaganovich et al. 2008; Kopito 2000; Sontag et al. 2017; Taylor et al. 2003).

The quality control function of the HSP90/HSP70 chaperone machine regulates the turnover of aberrant HSP90 client proteins, with failure in this quality control function contributing to the aetiology of neurodegeneration (Balchin et al. 2016; Moran Luengo et al. 2019). When conformational instability of the native or near-native HSP90 client protein is extensive enough such that the client protein can no longer interact and cycle productively with HSP90, protein unfolding proceeds, and the client protein is degraded by the proteasome, which is the major route of client protein degradation. Chaperones recognise the unfolding client protein as a substrate for proteasomal degradation and direct the unfolded client protein to chaperone-dependent E3 ligases, such as CHIP (Connell et al. 2001; Schipper-Krom et al. 2012). The E3 ligases in turn target a ubiquitin-charged E2 enzyme to the substrate. The N-terminal TPR domain of CHIP interacts with both HSP90 and HSPA8/

HSP70, whilst the C-terminal U box interacts with the UBCH5 family of E2 ubiquitin-conjugating enzymes (Cyr et al. 2002). Overexpression of CHIP has been shown to increase the ubiquitylation and proteasomal degradation of many HSP90 client proteins (Adachi et al. 2007; Al-Ramahi et al. 2006; Jana et al. 2005; Petrucelli et al. 2004; Sahara et al. 2005; Saidi et al. 2015; Shimura et al. 2004; Shin et al. 2005; Zhang et al. 2020). However, there is evidence that there is functional redundancy of CHIP with other E3 ligases, as some HSP90 client proteins, including polyQ-expanded AR, are degraded at the same rate in CHIP−/− and CHIP+/+ cells (Morishima et al. 2008). Moreover, it has been shown that CHIP is functionally redundant with parkin, another HSP70-dependent ligase, against polyQ-expanded ataxin-3 (Morishima et al. 2008; Tsai et al. 2003). However, CHIP is thought to play a key role in the chaperone-dependent ubiquitylation and proteasomal degradation of unfolded client proteins (Al-Ramahi et al. 2006; Dickey et al. 2007, 2008; Jana et al. 2005; Kalia et al. 2011; Shimura et al. 2004; Shin et al. 2005; Zhang et al. 2020). Through this quality control function, the HSP90/HSP70 chaperone machinery plays an important role in triage decisions directing unfolded client proteins for proteasomal degradation. It has been proposed that the triage decision lies with HSP90, as once the unfolding of the client protein progresses to a state where it can no longer interact and cycle with HSP90, HSP70-mediated CHIP-dependent ubiquitylation and proteasomal degradation of the client protein are triggered and proceed unopposed (Pratt et al. 2015).

The model for HSP90/HSP70 coordination in protein quality control is supported by evidence in vitro and in vivo that show that inhibition of the HSP90 ATPase activity results in the rapid proteasome-dependent degradation of HSP90 client proteins. The ansamycin class of antibiotics (e.g. GA and herbimycin A) binds to the HSP90 nucleotide-binding pocket and acts as a nucleotide mimic, thus inhibiting the intrinsic ATPase activity, which is essential for HSP90 client protein heterocomplex assembly (Roe et al. 1999), and preventing the cycling of the client protein with HSP90. HSP90 inhibition leads to the degradation of the soluble client protein, thus preventing the formation of oligomers and aggregates, and the treatment of cell or animal models with HSP90 inhibitors has thus been shown to ameliorate neurotoxicity (Auluck et al. 2005; Thomas et al. 2006; Tokui et al. 2009; Waza et al. 2005). For example, HSP90 inhibitors were shown to promote the proteasomal degradation of polyQ AR in cellular models of SBMA and ameliorate polyglutamine-mediated motor neuron impairment in mouse models of SBMA (Thomas et al. 2006; Tokui et al. 2009; Waza et al. 2005). Moreover, GA was reported to reduce the formation of α-synuclein aggregates and α-synuclein-induced toxicity in a cell model of PD (McLean et al. 2004). Interestingly, the treatment of cells with pre-existing inclusions did not reduce inclusion formation, confirming that HSP90 inhibition leads to the targeted degradation of the soluble client protein but not aggregated client.

One mechanism whereby HSP90 inhibition is thought to prevent the formation of protein aggregates and alleviate neurotoxicity in neurodegenerative disease models is via induction of the heat-shock response (HSR) (Auluck and Bonini 2002; Hay et al. 2004; Sittler et al. 2001). Inhibition of HSP90 ATPase activity prevents the

cycling of HSP90 with heat-shock factor 1 (HSF1), which consequently trimerises and translocates to the nucleus where it upregulates the expression of chaperones including HSP70 and HSP40 (Kijima et al. 2018). The overexpression of HSP70 or its co-chaperone HSP40 in turn decreases the levels of aberrant proteins and is neuroprotective in models of neurodegenerative disorders, including PD, HD and SBMA (Adachi et al. 2003; Auluck et al. 2002; Bailey et al. 2002; Jana et al. 2000; Klucken et al. 2004; Kobayashi et al. 2000; Muchowski et al. 2000). Hence, it has been proposed that the activation or promotion of HSP70- and CHIP-dependent ubiquitylation could be beneficial in neurodegenerative disorders.

The HSP70 co-chaperone HIP stabilises HSP70 in its ADP-bound conformation which recognises unfolded substrates with high affinity, thus facilitating their HSP70-dependent ubiquitylation and degradation. It has been reported that HIP overexpression significantly reduced polyQ-expanded AR inclusion formation in a primary neuronal model of SBMA and promoted the CHIP-mediated ubiquitylation and proteasomal degradation of polyQ-expanded AR, thus reducing the formation of intranuclear inclusions (Howarth et al. 2009; Wang et al. 2013). HIP was moreover reported to mediate HSP70-dependent suppression of α-synuclein aggregation in vitro, whilst its knockdown exacerbated α-synuclein aggregation in a *Caenorhabditis elegans* model of inclusion formation (Roodveldt et al. 2009). YM-1 is a small molecule drug that selectively binds to the nucleotide-binding domain of ADP-bound but not ATP-bound HSP70, thus increasing the affinity of HSP70 for substrate proteins similar to HIP (Rousaki et al. 2011). YM-1 reduced oligomeric and aggregated polyQ AR, but soluble polyQ AR was unaffected indicating the preferential targeting of unfolded client protein (Wang et al. 2013). Hence, the rational design of small molecules to stabilise the ADP-bound conformation of HSP70 is a focus of ongoing research to manipulate HSP90/HSP70 quality control in neurodegeneration.

There is also evidence that the protein quality control function of the HSP90/HSP70 chaperone machine per se is involved in the clearance of abnormal client proteins in the absence of induction of the HSR. For example, the inhibition of HSP90 by GA inhibited polyQ AR aggregation and promoted the proteasomal degradation of polyQ AR in HSF1−/− cells (Thomas et al. 2006). In this model, polyQ AR aggregates were cleared in the absence of an HSR highlighting the coordination between HSP90- and HSP70-mediated degradations in the quality control of this substrate. However, the role of the HSR versus HSP90/HSP70 quality control per se in target protein turnover has been more difficult to dissect for other HSP90 client proteins. Full-length Htt and full-length polyQ-expanded mHtt are known HSP90 client proteins (Baldo et al. 2012). HSP90 inhibition induced the ubiquitylation and proteasomal degradation of mHtt in a manner that does not require upregulation of HSP70 through HSF1 activation (Baldo et al. 2012). However, Htt undergoes extensive N-terminal proteolytic cleavage, and it is not clear whether full-length polyQ-expanded mHtt or expanded proteolytic fragments thereof are the main culprits driving the toxic gain-of-function disease pathology. Many investigations of mHtt have been conducted with polyQ-expanded exon 1 of Htt, and the R6/2 mouse model, which expresses polyQ-expanded Htt exon

1, recapitulates many disease features of HD (Davies et al. 1997; Schilling et al. 1999). However, mHtt exon 1 fragments are not HSP90 client proteins, and their proteasomal degradation is entirely due to the HSF1-mediated stress response. Therefore, both the HSR and the direct HSP90/HSP70 quality control could play a role in abnormal client protein clearance in neurodegenerative diseases.

Conclusions

The molecular chaperones and their associated co-chaperones are of central importance to protein function from facilitating folding, transport and translocation, through functional maturation to the clearance of misfolded and aggregated species via the UPS, autophagy or lysosomal pathways. Failure of chaperones to fulfil these vital roles may ultimately contribute to a number of devastating human diseases, including neurodegenerative diseases. The coordinated activities of the HSP90 and HSP70 chaperone machineries and their respective co-chaperone networks are particularly important in the quality control of HSP90 client proteins implicated in the aetiology of neurodegeneration and other diseases. Therefore, the central importance of these molecular chaperones and their associated co-chaperones makes them a prime target for pharmacological intervention for the treatment of these diseases. In particular, targeting the quality control function of the HSP90/HSP70 chaperone machine with rationally designed small molecules might be a therapeutically valid approach to promote the degradation of critical unfolded proteins and ameliorate toxicity in neurodegenerative diseases.

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