Chapter 10
Hsp70: A Multi-Tasking Chaperone at the Crossroad of Cellular Proteostasis

Vignesh Kumar and Koyeli Mapa

Abstract Molecular chaperones are key components of protein quality control machineries in all biological systems. Members of Hsp70 group of molecular chaperones are one of the most commonly found chaperones that accomplish multitude of cellular activities in concert with its co-chaperones. Hsp70s are involved in almost all aspects of protein quality control starting from de novo protein folding, prevention of misfolded or aggregated protein formation to membrane translocation and degradation of terminally misfolded proteins. Barring few exceptions, all known Hsp70s accomplish cellular activities by consuming energies from ATP-hydrolysis by their ATPase activity. The ATP-hydrolysis-driven chaperoning activities of Hsp70s are always assisted and regulated by two groups of co-chaperones; J-domain proteins (JDPs) or Hsp40s and nucleotide exchange factors (NEFs), to accomplish cellular functions in physiological time frames. As the co-chaperones especially the JDPs outnumber the Hsp70s, it is thought that different co-chaperone networks actually bestow the multi-tasking ability to particular Hsp70. In this chapter, an overview of recent understanding of various cellular activities of Hsp70s assisted by its co-chaperones have been discussed to highlight the extent of diversity of cellular functions achieved by this group of molecular chaperones.

Keywords Heat shock proteins · Hsp70 · Molecular chaperones · Protein folding · Proteostasis

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Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ERAD	Endoplasmic reticulum associated degradation
FRET	Forster resonance energy transfer
Hsp	Heat shock protein
IMS	Inter-membrane space
JDP	J-domain protein
mtHSP70	Mitochondrial heat shock protein 70
NAC	Nascent polypeptide-associated complex
NBD	Nucleotide binding domain
NEF	Nucleotide exchange factors
PBD	Peptide binding domain
RAC	Ribosome associated complex
RNC	Ribosome nascent chain complex
SBD	Substrate binding domain
sm-FRET	Single molecule Forster resonance energy transfer

10.1 Introduction

Hsp70s are one of the most ubiquitous molecular chaperones present in almost all forms of life and accomplish a wide variety of cellular functions (Clerico et al. 2015; Hartl et al. 2011; Hartl and Hayer-Hartl 2009; Mayer 2013; Mayer and Bukau 2005; Zuiderweg et al. 2013). Although the primary sequences of Hsp70 chaperones have diverged significantly over evolution, the two-domain structure of the members belonging to Hsp70 group, remained conserved. All members of Hsp70s are known to harbour two functional domains, the N-terminal nucleotide binding domain (NBD) or ATPase domain and the C-terminal substrate or peptide binding domain (SBD/PBD), connected by a short (8–10 amino acids) linker sequence (Mayer 2013; Mayer and Bukau 2005; Zuiderweg et al. 2013). The nomenclature of domains is in corroboration with the functional roles they fulfil. The two domains allosterically regulate each other's activity achieving extremely diverse functions in the cells in collaboration with co-chaperones. The NBD dictates the substrate binding affinity of the SBD which in turn accelerates the inherent ATPase activity of the Hsp70-NBD and help in rapid hydrolysis of bound ATP. All crucial cellular activities of Hsp70s are all driven by allosteric communication between its domains leading to successful binding and timely release of substrates by the chaperone (Mayer 2013; Zuiderweg et al. 2013). Co-ordinated substrate binding and release is vital for Hsp70-driven cellular activities; this is possible due to extremely stringent regulation of the Hsp70 activity by the co-chaperones. Two groups of co-chaperones complete the Hsp70 chaperone activity, the J-domain proteins, in short JDPs and the

nucleotide exchange factors (NEF) (Bracher and Verghese 2015; Fan et al. 2003; Hartl et al. 2011; Kampinga and Craig 2010). JDPs constitute a wide variety of proteins and as the initially discovered members of this group of co-chaperones belonged to the molecular weight range of ~40 kDa, JDPs are also known as Hsp40s although many members especially the ones belonging to type III JDPs are much smaller in size (Fan et al. 2003; Kampinga and Craig 2010). The other group of co-chaperones are nucleotide exchange factors, in short NEFs, which facilitate exchange of ADP from the NBDs of Hsp70s, thereby initiating a new chaperone cycle (Bracher and Verghese 2015). In contrast to JDPs where all members harbour at least a J-domain consisting of helix-loop-helix structure with conserved tripeptide motif (HPD), NEFs vary starkly in their sequence as well as in structure. There are plenty of examples of a singleHsp70 being assisted by structurally different NEFs, although the specificity of NEFs towards particular functions of Hsp70s, remain largely enigmatic.

10.2 Domain Organization and Allosteric Communication in Hsp70 Chaperones

Hsp70 chaperones harbour an N-terminal ~45 kDa nucleotide binding domain (NBD) and a C-terminal ~25 kDa substrate binding domain (SBD). The two domains remain connected by a hydrophobic linker. The NBD is horse-shoe shaped and each lobe is subdivided into two sub-domains (altogether 4 sub-domains, IA, IB, IIA and IIB). The SBD has two prominent parts, the ß-sheet base and an α-helical lid like structure constituted of five α-helices (Mayer 2013; Mayer and Bukau 2005; Mayer and Kityk 2015; Zuiderweg et al. 2013). Helical lid is followed by a conserved intrinsically unstructured region with a hitherto unknown function. Hsp70 chaperones are known to exert their chaperoning activity by inter-domain allostery. The substrate binding affinity of the C-terminal SBD is dictated by the nucleotide bound status of the N-terminal NBD. Hsp70s bind substrates with a weak affinity in the ATP-bound state and the substrate-binding affinity increases drastically following the hydrolysis of ATP to ADP. On the other hand, substrate binding by SBD accelerates the ATPase activity of the NBD (Mayer 2013; Mayer and Bukau 2005; Mayer and Kityk 2015; Zuiderweg et al. 2013). This allosteric communication is the basis of successful chaperoning activity in all biological functions of the chaperone. An understanding of the mechanism of inter-domain allostery at the molecular details remained obscure due to unavailability of two-domain structures of the Hsp70 proteins for years. Ironically, the structure of isolated domains (NBD and SBD) of prototype Hsp70, DnaK (bacterial Hsp70), was solved long ago but structure of isolated domains could not explain the inter-domain communication that forms the basis of allosteric regulation. Simultaneously, many groups have shown that the nucleotide binding activity and the substrate binding activity of NBD and SBD respectively, remain intact by the isolated -domains although domains are either deficient or significantly less efficient in chaperoning

activity in respect to the full length chaperones. This indicated the importance of inter-domain allosteric regulation for functionality of the chaperones. Due to dynamic nature of Hsp70 molecules, the X-ray crystal structures of the full length proteins remained unsolved for decades and came to light, fairly recently (Chang et al. 2008; Qi et al. 2013; Swain et al. 2007; Zhuravleva et al. 2012). In parallel, to capture this dynamic nature of Hsp70s, various Hsp70 proteins were subjected to different techniques in solution phase and indeed, the existence of conformational ensemble or co-existence of multiple conformational states in single allosteric states of Hsp70 family members came into light (Banerjee et al. 2016; Lai et al. 2017; Mapa et al. 2010; Marcinowski et al. 2011; Sikor et al. 2013). Many groups including ours have tried to understand the dynamic nature of Hsp70 molecules using state of the art techniques like single molecule FRET (FÖrster Resonance Energy Transfer) spectroscopy. Along with other techniques, Single molecule FRET experiments successfully revealed the structural heterogeneity in different biological states of various members of Hsp70 group of chaperones which remained so far masked in ensemble studies or in high resolution structural snapshots (Fig. 10.1).

Using biochemical and biophysical studies on purified Hsp70 chaperones, it was indicated that two types of prominent conformational changes take place during each allosteric chaperone cycle. The distance between domains changes drastically with ATP binding and hydrolysis and the α-helical lid of SBD opens or closes on top of the peptide binding pocket situated at ß-sheet base of SBD. Furthermore, the crystal structure of isolated peptide-bound SBD of DnaK (Zhu et al. 1996) demonstrated that the alpha helical lid closes on top of the substrate binding pocket of ß-sheet base. The state of lid in ATP-bound states were thought to be open from several other experimental evidences although the conformational heterogeneity, if any, remained unclear. Almost simultaneous to the discovery of two-domain structures of some members of Hsp70s, application of single molecule FRET on biologically relevant molecules became common. Some members of Hsp70 chaperones like bacterial Hsp70, DnaK, yeast mitochondrial Hsp70, Ssc1, yeast ER Hsp70, BiP were subjected to sm-FRET experiments to capture the conformational ensembles in various allosteric states (Banerjee et al. 2016; Mapa et al. 2010; Marcinowski et al. 2011; Sikor et al. 2013). From such single molecule FRET efficiency/ratio distribution plots, it became clear that Hsp70s are fairly dynamic molecules and contain inherent conformational heterogeneity in most of the functional states. Previous to this, the substrate-bound, lid-closed structure of isolated DnaK or in two-domain structure along in its ADP bound state hinted that the lid gets closed upon substrate binding (Bertelsen et al. 2009; Chang et al. 2008; Zhu et al. 1996). The lid-open structure of structural analogue of Hsp70s, Hsp110s showed the open state of lid in ATP state of the chaperone (Liu and Hendrickson 2007; Polier et al. 2008). It was expected from previous biochemical or biophysical studies that Hsp70 lids remain open in ATP-bound states. Although, the high resolution structures of full-length (without the C-terminal disordered region) Hsp70s in ATP state came after few years, sm-FRET studies had revealed the conformational ensemble in regard to the lid conformation. It was demonstrated that lid remains in open state in most of the molecules in ATP-bound state while the conformational states of lid in

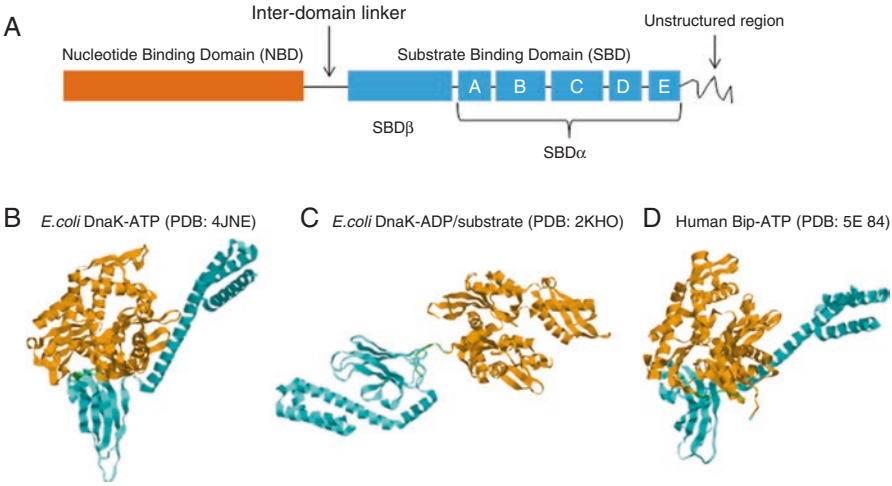

Fig. 10.1 Domain organization and structure of Hsp70 molecular chaperones: Panel A: Almost all known Hsp70 chaperones consist of two domains, the N-terminal nucleotide binding domain (NBD) and the C-terminal substrate binding domain (SBD). The SBD is divided into the beta-sheet base (SBDß) followed by alpha helical lid like structure (SBDα). The lid consists of 5 helices, A to E. The SBDα is followed by a disordered region. Panel B-C: Ribbon diagram of crystal and NMR structure of E. coli Hsp70, DnaK in the ATP and ADP/substrate bound states respectively. Panel D: Crystal structure of human BiP protein in the ATP-bound state. The NBDs have been coloured in orange, the inter-domain linkers in green and the SBDs have been coloured in cyan in all three structures

the ADP-bound conformation is quite heterogeneous (Banerjee et al. 2016; Mapa et al. 2010; Marcinowski et al. 2011). Additionally, Sm-FRET experiments also revealed that exclusively upon binding the substrates, lid of SBD closes significantly. This finding was a significant advancement from the previous knowledge of mere ATP-hydrolysis driven lid closure of Hsp70 molecules. Capturing the heterogeneity by sm-FRET proved that just ADP state is not sufficient to close the SBD lid. A recent study from our group had shown that the lid closure is not mandatory for substrate capture and the degree of lid closure depends on the nature of substrate bound (Banerjee et al. 2016).

Furthermore, the high resolution structures of full length (except the C-terminal unstructured domains) Hsp70s in ADP and ATP-bound states revealed the stark difference in domain interaction in the ATP state and the ATP-hydrolysed state. In the ATP-bound states NBD and SBD comes into close contact (domain-docked state) and the linker becomes buried, whereas in the ADP-bound states, the two domains are uncoupled exposing the linker (Bertelsen et al. 2009; Kityk et al. 2012; Qi et al. 2013). While probing the conformational ensemble for inter-domain interaction, we and others observed that in the ADP-bound state, an appreciable population of molecules remain in the domain-docked state which remain hidden in the crystal structures (Mapa et al. 2010; Marcinowski et al. 2011) In the ATP-bound state the interaction of two domains (or docking of SBD to NBD) was found for

most of the molecules (Mapa et al. 2010; Marcinowski et al. 2011). Upon substrate binding the two domains get uncoupled. Thus, by employing parallel approaches of solving of high resolution structures and subjecting the Hsp70 proteins for state of the art methodologies like single molecule FRET, our knowledge about the allosteric communication of these molecular machines has significantly enriched.

10.3 Role of Hsp70 in Quality Control of Nascent Chains

In eukaryotes, members of Hsp70 group of molecular chaperones are present in multiple intracellular compartments. In unicellular eukaryote model, yeast, there are 9 members (including two of the Hsp110s, Sse1 and Sse2) of Hsp70 family (Ssa1–4, Ssb1–2, Sse1–2 and Ssz1) that exist only in cytoplasm of the cell. While Ssa1 and its paralog Ssa2 are mainly engaged in various aspects of protein quality control in the cytosol under physiological scenario, Ssa3 and its paralog Ssa4 are thought be expressed under stress. Ssb1 and Ssb2, together known as SSBs are ribosome associated chaperones and their role have been described in details in the next section. Sse1 and Sse2 belong to Hsp110s and have been shown to act as nucleotide exchange factors for cytosolic Hsp70s, although various other roles in protein quality control have been ascribed to Sse1.

The role of Hsp70s in maintenance of protein quality control in eukaryotes starts at a reasonably early stage of a protein's life. Ribosome-bound Hsp70 (SSBs in yeast) with help of co-chaperones (RAC or Ribosome associated complex and NAC or Nascent polypeptide-associated complex) bind the newly emerging nascent chains as soon as they emerge through the ribosome exit tunnel (Gautschi et al. 2001; Hubscher et al. 2017; Preissler and Deuerling 2012; Rakwalska and Rospert 2004). Although the discovery of interaction of some eukaryotic Hsp70s with ribosome is rather old, the structural intricacies of Hsp70-ribosome interaction and its functional relevance has been unravelled only recently. In yeast, SSBs encompass two extremely identical ribosome-bound Hsp70 chaperones, Ssb1 and Ssb2, which differ only by 4 amino acids. In a recent study, it was shown that alpha helical regions in the lid domain of SSB's PBD contain a conserved positively charged surface which is essential for binding to ribosome and rRNAs. SSBs interact with two ribosomal proteins Rpl35 and Rpl39 that are situated near the ribosome exit tunnel explaining its capacity of binding nascent polypeptide chains just after emergence from the ribosome exit tunnel (Gumiero et al. 2016). Like other members of Hsp70 chaperone family, SSB follows the same trend regarding their dependence on the co-chaperones. For SSBs, a heterodimeric complex known as RAC (Ribosome Associated Complex) acts as a co-chaperone. Yeast RAC consists of a JDP called Zuo1 (a homologue of mammalian Zuotin) and an atypical Hsp70, Ssz1 (Gautschi et al. 2001; Gautschi et al. 2002; Leidig et al. 2013; Peisker et al. 2008). A subunit of RAC (J-domain protein Zuo1) helps to accelerate the ATPase rate of SSBs and thereby ensures tight binding with the nascent polypeptides (Fig. 10.2). RAC is essential not only for the acceleration of ATPase activity of SSB but also for efficient binding of SSB to ribosome (Gumiero et al. 2016). Absence of RAC (Δzuo Δssz1) or presence

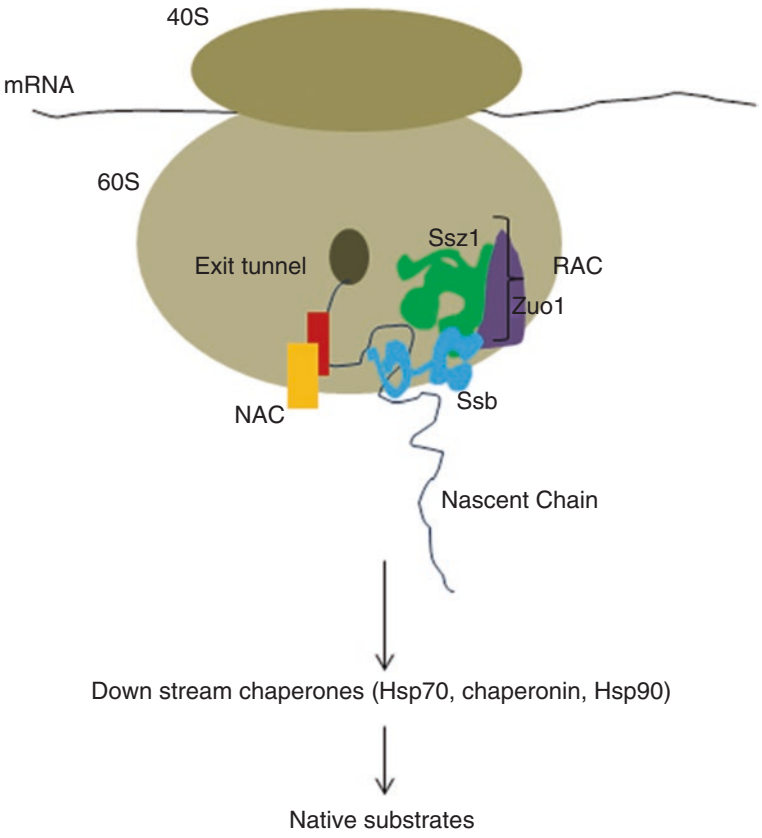

Fig. 10.2 Ribosome bound Hsp70 and its co-chaperones: Ribosome-bound Hsp70, Ssb interacts with nascent polypeptide chains soon after its emergence from ribosome exit-tunnel. RAC or ribosome associated complex consists of atypical Hsp70 protein Ssz1 and J-domain protein Zuo1. RAC acts as co-chaperone for Ssb and helps it in tight binding to the nascent chains. NAC or nascent chain associated complex also binds the nascent chain near the ribosome exit tunnel. The Ssb-RAC-NAC hands over the nascent chains to downstream chaperones to fold into native conformation

of a non-functional RAC (Zuo1 H128Q) leads to severe abrogation of SSB-ribosome interaction. The importance of SSB and its co-chaperones in chaperoning the nascent chains and in overall cellular protein homeostasis is further highlighted by the evidences showing significant aggregation of ribosomal proteins and ribosome biogenesis factors in cells deleted of SSBs and its co-chaperones (Koplin et al. 2010).

Ssz1, the other component of yeast RAC, is a member of Hsp70 family although it has diverged significantly from typical Hsp70 features, both structurally and functionally (Conz et al. 2007; Huang et al. 2005). Ssz1 lacks the ATPase activity in vitro and the ATP binding activity has been shown to be dispensable for this unique Hsp70 (Conz et al. 2007; Huang et al. 2005). The substrate binding domain (SBD) of this protein is smaller in size compared to other Hsp70-PBDs and the

intrinsically unstructured region present at the extreme C-terminal part of most of the Hsp70s, is absent in Ssz1. It was shown that SBD of Ssz1 is essential for its interaction with its partner in RAC, Zuo1 and also play important role for its interaction with ribosome (Conz et al. 2007).

The functional significance of Hsp70-co-chaperone interaction with ribosome and nascent chains came pretty recently. In 2013, Judith Frydman's group had shown that ~70% of the nascent polypeptides interact with SSBs in yeast in a RAC dependent manner (Willmund et al. 2013). They had reported that the SSB-interacting nascent chains mainly belong to cytoplasmic and nuclear proteins, although nascent chains of some SRP (signal recognition particle)-independent ER targeted proteins were also found to interact with SSBs (Willmund et al. 2013). Very recently, Bernd Bukau's group has elegantly shown that not only the nascent chains of cytosolic and nuclear proteins but also a significant percentage of newly synthesized mitochondrial pre-proteins (~80%) interact with SSB (Doring et al. 2017). This finding is quite unexpected and worth exploring as absence of SSB will have significant impact on the biogenesis and functionality of this crucial organelle. Indeed, in this work, the authors have shown that the cells lacking SSB exhibit altered mitochondria dynamics and mass. Additionally, this study has also revealed that an appreciable percentage (~46%) of nascent chains of ER targeted proteins especially those which are translocated to ER without docking of RNC (Ribosome Nascent Chain) complex to ER-translocons, preferentially bind to SSB (Doring et al. 2017). Whether SSB-RNC complex works in collaboration with SRP for ER targeting of proteins or it serves as a parallel targeting pathway, remains to be explored. In summary, both canonical and non-canonical members of Hsp70 group of molecular chaperones play significant role in chaperoning of nascent chains and protein homeostasis as a whole.

10.4 Hsp70 in Mitochondria: A Multitasking Chaperone in Action

The multi-tasking nature of Hsp70 chaperones are extremely obvious in certain sub-cellular compartments like mitochondria where Hsp70 chaperones are involved in maintaining different aspects of protein homeostasis like membrane translocation, folding and assembly of newly imported unfolded precursor proteins (Neupert and Brunner 2002; 2007).

10.4.1 Role of Mitochondrial Hsp70 in Protein Translocation into Mitochondrial Matrix

Majority of biochemical activities carried out by mitochondria is located in its matrix. Most of the matrix proteins except those that are encoded by mitochondrial genome, are translocated across the outer membrane without expending energy but

has to cross the tightly gated inner membrane by an energy consuming process (Bolender et al. 2008; Kutik et al. 2009; Neupert and Herrmann 2007; Wiedemann and Pfanner 2017). Translocation across the inner mitochondrial membrane needs active participation of a molecular motor, also called the "import motor". The energy-driven translocase of inner mitochondrial membrane, also called TIM23 translocase, is operationally divided into membrane-embedded translocation channel and the channel associated import motor. The import motor consists of mitochondrial Hsp70 (mtHsp70) and its co-chaperones (Neupert and Herrmann 2007; Wiedemann and Pfanner 2017). In the import motor, through an ATP-dependent cycle of consecutive binding and release from the incoming precursor protein, mtHsp70 leads to vectorial movements of precursors into the matrix. In unicellular eukaryote, yeast, a J-domain like protein called Tim44 assists Hsp70 in import of precursor proteins across the tightly gated inner mitochondrial membrane. Tim44 is considered merely as a "J-domain like protein" because of absence of the classical J-domain with conserved HPD motif found in all designated JDPs essential for acceleration of ATPase activity of Hsp70s. Tim44 is a peripheral inner mitochondrial membrane protein and remains attached to the matrix side of the TIM23 translocase. It is thought to act as a membrane anchor for mtHsp70 to position the chaperone in the vicinity of the incoming precursor proteins through the channel of the TIM23 translocase (Blom et al. 1993). Several studies have demonstrated that interaction of Tim44 with mtHsp70 is highly dependent on the nucleotide-bound status of mtHsp70 (Kronidou et al. 1994; Rassow et al. 1994; Schneider et al. 1994). It was shown that Tim44-mtHsp70 forms a stable complex in the ADP state of the chaperone while the complex dissociates in presence of ATP (Rassow et al. 1994; Schneider et al. 1994; von Ahsen et al. 1995). As the Tim44-mtHsp70 interaction is similar in behaviour to substrate-mtHsp70 interaction regarding the nucleotide dependence, it is still enigmatic whether a ternary complex of Tim44-mtHsp70-substrate is formed at some point of translocation process or binding of Tim44 and substrates (incoming precursor proteins) to mtHsp70 are mutually exclusive in nature (Fig. 10.3).

There are two contrasting hypothesis regarding the working mechanism of mtHsp70 as a motor protein during translocation; the power-stroke model and the Brownian ratchet model (Neupert and Brunner 2002). According to Brownian ratchet model the pre-proteins undergo spontaneous Brownian motion within the translocase pore of the TIM23 translocase (Ungermann et al. 1994) and mtHsp70 binds and traps the segments of precursor proteins preventing its retrograde movement or backsliding into intermembrane space (IMS) or the cytosol (Neupert and Brunner 2002; Schneider et al. 1994). As has been shown for bacterial homolog DnaK, mtHsp70 binds sequence motifs constituted of ~7–10 amino acids in length which are enriched in hydrophobic residues in the core and are flanked by positively charged residues within substrates (Rudiger et al. 1997). Our previous data have shown that portions of precursors proteins which do not get recognised by mtHsp70, are preferentially bound by Tim44 thereby helping in unidirectional import towards the matrix. Thus, according to this model, no pulling force is exerted by the chaperone during the translocation process. Previous experiments also pointed towards more in favour of Brownian ratchet model. In an elegant experiment, by introducing Hsp70 disfavoured regions like polygycine or polyglutamine stretches

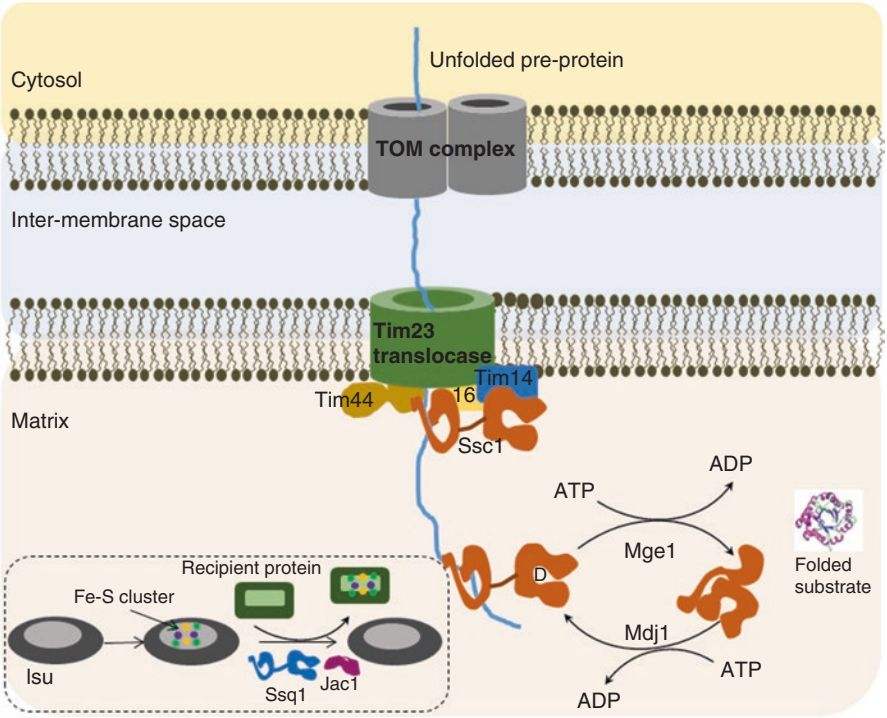

Fig. 10.3 Multifaceted role of mitochondrial Hsp70: Mitochondrial Hsp70 is engaged in variety of functions within the mitochondrial matrix. In yeast, S. cerevisiae, the main mitochondrial Hsp70 also known as Ssc1 is involved in protein translocation across the tightly-gated inner mitochondrial membrane and also in folding of unfolded precursors after their translocation into matrix. Several co-chaperones of Ssc1; Tim44, Tim14, tim16 which are also components of import motor of the TIM23 complex, are essential for protein import across the inner membrane. For folding function, separate group of co-chaperones are there, Mdj1 (J-domain protein) and Mge1 (NEF). Another mitochondrial Hsp70, Ssq1 is involved in Fe-S cluster biogenesis in mitochondrial matrix, Jac1 acts as a J-domain co-chaperone for Ssq1. T: ATP-state of Ssc1, D: ADP-state of Ssc1

in the pre-proteins, it was shown that the import of these proteins remain unaffected once it is initiated by the membrane potential across the inner mitochondrial membrane ($\Delta\psi$)(Okamoto et al. 2002). On the contrary, according to the power-stroke model mtHsp70 should actively pull the incoming polypeptide chain and the force required should be generated mostly by nucleotide induced conformational changes of the chaperone (Glick 1995; Matouschek et al. 2000). As there are ample contrasting results in favour of both the mechanism of mitochondrial protein translocation by import motor, there is a possibility that multiple modes of action of mtHsp70 exist for efficient translocation of mitochondrial precursor proteins towards matrix.

Following the discovery of Tim14 (Pam18) (Mokranjac et al. 2003; Truscott et al. 2003) as the typical J-domain co-chaperone of mtHsp70 and another structurally similar, closely associated protein Tim16 (Pam16) (Frazier et al. 2004; Kozany

et al. 2004; Li et al. 2004), the understanding of the sequence of events of import motor during protein import across inner mitochondrial membrane has become highly convoluted. From the high resolution structure of Tim14/Tim16 complex, it is clearly evident that the two proteins form a closely associated structure and this structure indicates the possibility of Tim16 as a controller of undue J-domain activity by Tim14 on mtHsp70, thereby preventing the formation of a 'stuck' translocase (Mokranjac et al. 2006). The human Tim14/Tim16 complex have also shown to be of similar three-dimensional structure indicating a conserved mechanism of mt-Hsp70-co-chaperone interaction even in higher eukaryotes (Elsner et al. 2009). It is important to note here that all high resolution structures of Tim14/16 complexes so far have given glimpses of the structural arrangements of soluble domains of these proteins although the membrane association of the co-chaperone complex or the sequence of events of interaction of Tim14/16 with Ssc1 and Tim44, remains elusive. It is important to emphasize that not only mtHsp70 but also all of its co-chaperones involved in import motor, are essential for viability of cells which clearly indicate the importance of such an orchestra of co-chaperones for mtHsp70 in fulfilling the protein translocation across inner mitochondrial membrane.

10.4.2 Mitochondrial Hsp70 in Protein Folding and Assembly of Proteins in Mitochondria

Following the translocation of newly imported precursor proteins, Hsp70 along with a new set of co-chaperones engage in folding of these unfolded precursors into their native structures. To assist in the folding activity of mtHsp70, Mdj1 acts as the JDP co-chaperone in yeast mitochondria (Prip-Buus et al. 1996; Rowley et al. 1994; Voos and Rottgers 2002; Westermann et al. 1996). Although it is thought that mtHsp70 carry out the folding of newly imported precursor proteins in the soluble phase of mitochondrial matrix, significant association of Mdj1 with the inner mitochondrial membrane raises some interesting possibilities (Duchniewicz et al. 1999; Mapa et al. 2010). Whether Mdj1 remain close to the import channel and helps mtHsp70 in folding of newly imported precursors without much delay, remains a question. Interestingly, despite the engagement of a plethora of JDPs and J-like proteins as co-chaperones, the nucleotide exchange activity on mtHsp70 is solely executed by Mge1 (Deloche et al. 1997; Laloraya et al. 1994; Miao et al. 1997; Sakuragi et al. 1999; Schmidt et al. 2001). It has been shown that Mge1 actually completes the chaperone cycle by dissociating the mt-Hsp70-JDP-substrate complex with an extremely fast reaction kinetics in presence of ATP (Mapa et al. 2010). Although the cytosolic Hsp70s interact with structurally diverse NEFs, mitochondrial Hsp70 is exclusively dependent on Mge1, the homologue of bacterial NEF, GrpE.

It is worth mentioning here that mtHsp70 (both Ssc1 and Ssq1 in *S. cerevisiae*), although harbour high homology to its bacterial counterpart DnaK, is more aggregation prone than DnaK. Interestingly, to chaperone this aggregation prone

Hsp70, a dedicated protein called Hep1 exists in mitochondrial matrix (Hsp70 escorting protein, also known as Zim17 or Tim15) (Sanjuan Szklarz et al. 2005; Sichting et al. 2005; Yamamoto et al. 2005). Hep1 interacts with the ATPase domain and linker region of yeast mtHsp70 (Ssc1) and prevents its aggregation (Blamowska et al. 2010). Recently, in mammalian cells, an orthologue of Hep1 has been discovered and was found to play critical role in solubility and maintenance of human mtHsp70 (Goswami et al. 2010). This example of chaperones for chaperones, especially for Hsp70s is quite unique and worth exploring for other aggregation prone chaperones.

Apart from the translocation and folding function in mitochondria, mtHsp70 is also engaged in assembly of protein complexes like the Fe-S clusters (Delewski et al. 2016; Dutkiewicz et al. 2003; Schilke et al. 2006). For the Fe-S cluster formation, interaction of Hsp70 with the scaffold protein (Isu1, in yeast) aided by a specialized JDP co-chaperone is required (Fig. 10.3). Even among yeasts, *S. cerevisiae* and *S. pombe*, there is significant diversification of mtHsp70s. In case of *S. cerevisiae*, due to genome duplication, mtHsp70 or Ssc1 has a given rise to a specialized Hsp70 called Ssq1 in the mitochondrial matrix (Delewski et al. 2016; Dutkiewicz et al. 2003; Schilke et al. 2006). Ssq1 is specifically engaged in Fe-S cluster biogenesis in collaboration with JDP, Jac1 and has not been found to participate in protein translocation or folding in mitochondrial matrix (Kim et al. 2001; Schilke et al. 2006; Voisine et al. 2001). Interestingly, in *S. cerevisiae*, it has been shown that even after alanine substitution in HPD motif of Jac1, mutated Jac1 retains the capability to interact with Ssq1 and the cells remain viable. On the other hand, in *S. pombe* Ssc1 is the sole mtHsp70 and is involved in Fe-S cluster formation apart from membrane translocation and folding activities. In *S. pombe,* the alanine substitution of HPD motif of Jac1 is not at all tolerated and cells become inviable with such mutations in Jac1 (Delewski et al. 2016). Thus, it is interesting that either by increasing the co-chaperone repertoire or by increasing the chaperone number itself (e.g. by genome duplication), the multi-tasking ability of Hsp70 chaperones has been achieved in different organisms as well as in different sub-cellular compartments.

10.5 Hsp70 in ER Homeostasis

The glucose regulated protein 78 (GRP78) is a ER resident Hsp70 (Gardner et al. 2013; Gething 1999). Grp78 is also known as BiP (Binding immunoglobulin protein) and Kar2 in yeast. BiP or Grp78 protein is essential for protein folding and unfolded protein response (UPR) signalling in ER (Gardner et al. 2013; Lewy et al. 2017), additionally BiP plays crucial role in protein translocation into ER lumen(Dudek et al. 2015; Hassdenteufel et al. 2014; Zimmermann et al. 2011), Ca^{2+} homeostasis (Gething 1999; Hendershot 2004; Kania et al. 2015; Schauble et al. 2012) and protein degradation by ER-associated degradation (ERAD) (Casas 2017; Foufelle and Ferre 2007; Maattanen et al. 2010; McCaffrey and Braakman 2016;

Sano and Reed 2013; Wang et al. 2017). ER, being a folding and modification hub of membrane and secretory proteins, chaperones residing in ER play crucial role in maintenance of ER protein homeostasis in concert with ERAD machineries. BiP, being a member of Hsp70 family, consists of the typical nucleotide binding domain and the peptide/substrate binding domain. Additionally, for its ER localization, an ER retention signal (KDEL) is harboured by the protein at its extreme C-terminus. A recent crystal structure of human BiP in the ATP-bound state demonstrates the overall structural similarity with prokaryotic Hsp70 DnaK, although exhibiting sufficient differences in structural features, especially in the NBD-SBDα interface. The authors claimed that this NBD-SBDα interface is unique for eukaryotic cytosolic/ER resident Hsp70s and are in contrast to prokaryotic or mitochondrial Hsp70 (Yang et al. 2015). Although the domain-interface is important, it remains to be seen, if the evolution of the interface has indeed been functionally relevant in imparting specialized functions to the cytosolic and the ER counterparts.

Like other Hsp70s, BiP is assisted by members of two groups of co-chaperones, JDPs (Erdj1–7 in mammals) and NEFs like BAP (Sil1 in yeast), Grp170 (Lhs1 in yeast) etc. (Behnke et al. 2015) (Fig. 10.4). The partitioning of BiP between different activities in ER is thought to be guided by recruitment of specific JDPs during different activities. Although the role of all JDPs in folding and degradation are still not clear, JDPs involved in translocation are pretty specific, JDP like Sec63 is the one assisting BiP in protein translocation into ER lumen in yeast (Dudek et al. 2015; Hassdenteufel et al. 2014; Zimmermann et al. 2011). It is interesting to note here that, during Hsp70-motor mediated membrane translocation, presence of a co-chaperone like Tim44 (in case of inner mitochondrial membrane) or Sec63 (in ER membrane) as a membrane anchor for Hsp70s seems mandatory. Whether such membrane anchors actively participate in membrane translocation by holding or pulling the translocating chain, remains unanswered so far. Few more JDP co-chaperones of BiP have been shown to be involved in exclusive activities of the chaperone like Erdj3 and Erdj5 in mammals have been shown to be specifically involved in protein folding and degradation, respectively (Dong et al. 2008; Jin et al. 2008; Shen and Hendershot 2005).

BiP is highly expressed during ER stress and has been extensively used as ER stress marker for long time. It is also overexpressed in certain tumours associated with deregulation of glucose metabolism, glucose deficiency and hypoxia in tumour micro-environment. ER, as already mentioned is the main hub of folding of secretory and membrane proteins and as a result always experience presence of massive amount of unfolded or misfolded proteins inside the lumen. In physiological scenario, ER protein homeostasis machineries consisting of several chaperones like BiP, Grp94, ERp72, PDI, Grp170 etc. and the components of ERAD machinery are extremely efficient to prevent overloading of ER with misfolded or aggregated proteins. In situations where the ER proteostasis machinery is overwhelmed with misfolded or aggregated proteins, ER becomes stressed and a response pathway called Unfolded protein response (UPR) is elicited to restore the homeostasis.

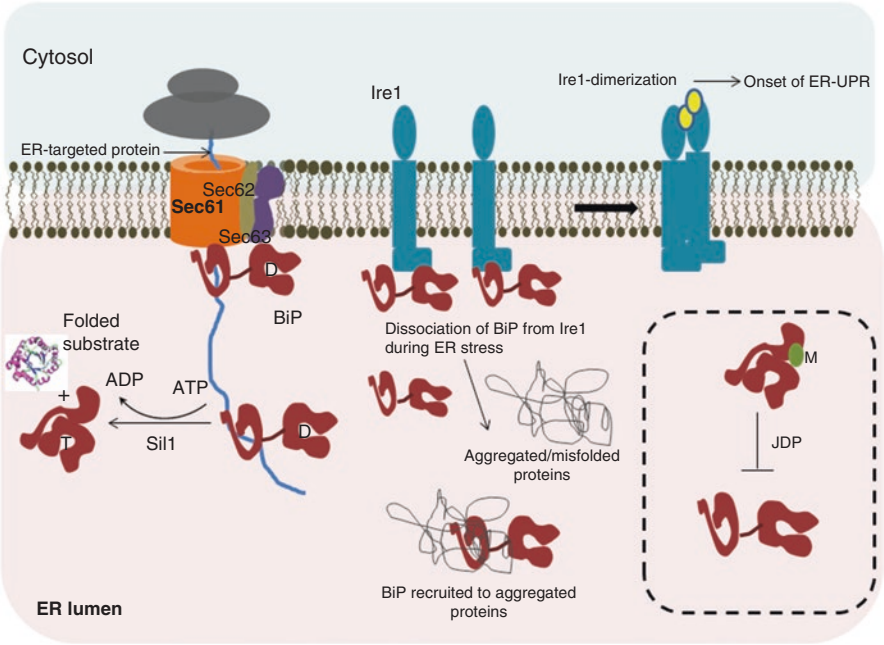

Fig. 10.4 BiP, a multi-tasking Hsp70 of ER: ER resident Hsp70, also known as BiP is involved in protein translocation into ER-lumen through Sec61 complex. It acts a motor protein for protein import through Sec61 complex. It is also involved in protein folding in ER and a major component to initiate the signalling cascade of ER-UPR (unfolded protein response). Here, the role of BiP in yeast ER-UPR has been shown. Upon accumulation of misfolded or aggregated proteins in ER lumen, BiP dissociates from ER-membrane protein Ire1 which is the sole sensory molecule for ER-UPR in yeast. Upon dissociation of BiP from Ire1, Ire1 auto-phosphorylates (shown in yellow circles) and dimerizes leading to its activation and initiation of ER-UPR signalling cascade. In mammals, a special modification called AMPylation (shown as green circle) takes place on BiP which makes it refractory to J-domain co-chaperone induced ATP-hydolysis. T: ATP-state of BiP, D: ADP-state of BiP, M: AMPylated site

10.5.1 Role of BiP in ER-Unfolded Protein Response

BiP plays a major role in initiating the signalling of ER-UPR (Foufelle and Ferre 2007; Gardner et al. 2013; Hendershot 2004; Lewy et al. 2017). In case of higher eukaryotes, there are three ER membrane embedded sensory molecules that sense the misfolding stress and initiate UPR signal. These proteins are Inositol requiring protein 1 (IRE1), PKR like ER kinase (PERK) and Activating transcription factor 6 (ATF6) (Gardner et al. 2013; Korennykh and Walter 2012; Walter and Ron 2011). All three UPR sensors have a cytosolic domain and an ER luminal domain. The luminal domains of these sensory molecules interact with ER-resident Hsp70, BiP. During the onset of ER-UPR, BiP dissociates from the luminal domains of UPR sensors leading to their activation and initiation of UPR signalling. In case of

IRE1 and PERK, BiP dissociation leads to the oligomerization of these proteins within ER membrane followed by phosphorylation of specific sites on the cytosolic domains of IRE1 and PERK leading to their activation (Fig. 10.4). Activated IRE1 exerts its endonuclease activity and splices the Xbp1 or Hac1 (yeast) mRNA and the protein products of spliced RNA act as a potent transcription factor to upregulate ER chaperones including BiP and components of ERAD machinery. PERK activation leads to a different response from IRE1 activation. Activated PERK leads to phosphorylation of EIF2α thereby abrogating the translation of new proteins and in turn decreases further protein load on ER. During generalized translation block, few genes are preferentially translated like ATF4 which acts as transcription factor to upregulate the genes for restoration of ER homeostasis. Similarly, displacement of BiP from the third UPR sensor ATF6, leads to its ER to Golgi transport and subsequent cleavage by Golgi specific proteases, SP1 and SP2. The cleaved cytosolic domain of ATF6 further acts as a transcription factor to bring back the ER homeostasis (Gardner et al. 2013; Korennykh and Walter 2012; Walter and Ron 2011). Thus, BiP not only binds to unfolded nascent chains, but also plays a crucial role in initiation of UPR signalling which is key in timely restoration of ER protein homeostasis.

10.5.2 BiP in Ca^{2+} Homeostasis in ER

Besides being a hub for post translational modification and folding of secretory and membrane proteins, ER also serves as the reservoir for cellular Ca^{2+} ions. The role of ER resident Hsp70 chaperone, BiP, in ER Ca^{2+} storage was discovered long ago (Lievremont et al. 1997). It was shown that increased BiP level leads to higher Ca^{2+} storage capacity of ER although the mechanism remained unclear. Rather recently, a novel interaction between BiP and sec61α was uncovered as the regulatory interaction for the BiP-mediated Ca^{2+} storage in ER (Schauble et al. 2012). The protein translocation channel or SEC complex situated in the ER membrane for translocation of ER targeted proteins also acts as a Ca^{2+} leak channel. Cytosolic protein Calmodulin prevents undue Ca^{2+} leakage by interaction with the IQ motif of the cytosolic domain of Sec61α protein of this complex. BiP was shown to play significant role in stopping of Ca^{2+} leakage from the ER luminal side. It was demonstrated that BiP interacts with a ER-luminal loop of Sec61α between its two transmembrane helices and closes the Sec61α channel from the open state. The conclusion came from the experiment where amount of BiP in ER lumen was artificially reduced either by genetic manipulation or by increasing the misfolded protein load in ER leading to increased Ca^{2+} release from ER. Furthermore, a mutation at the BiP-binding site of Sec61α (Y344H, originally found in diabetic animals) led to increased Ca^{2+} leakage from ER leading to apoptosis. This increased Ca^{2+} leakage by mutated Sec61α is independent of ER BiP levels and additional decrease in BiP level did not have added impact on the mutated SEC61 complex mediated Ca2+

leakage (Schauble et al. 2012). Altogether, BiP plays a crucial role in Ca^{2+} homeostasis in ER by preventing its undue leakage from ER lumen.

10.5.3 Novel Regulatory Mechanism of BiP

Extensive research on BiP has revealed a number of post-translational modifications of the protein which have been demonstrtaed to play crucial role in regulation of its activity. A recent discovery has shown that BiP is AMPylated at a specific amino acid (Thr 518) by an enzyme called FICD in mammalian cells (Ham et al. 2014; Preissler et al. 2015a; Sanyal et al. 2015). This modification has profound effect on BiP activity. AMPylated BiP was shown to be completely resistant to stimulation by J-domain co-chaperones. On the other hand, the substrate binding affinity of AMPylated BiP remains unchanged although substrates are dissociated with a much faster off rate from the AMPylated BiP in comparison to the unmodified version. Interestingly, AMPylation of BiP mimics an ATP-bound state in the ADP-bound BiP leading to low affinity for substrates. This feature of AMPylated BiP is in compelling contrast to the high affinity states of ADP-bound unmodified BiP. On the other hand, AMPylation of the ATP- bound BiP renders it resistant to JDP co-chaperone-induced stimulation of ATPase activity (Fig. 10.4). Altogether, AMPylation of BiP leads to an inactive state of the chaperone which is probably a unique regulatory mechanism to wean BiP activity when the load of misfolded proteins decline in ER (Preissler et al. 2015b). There are reports that show multiple other post translational modifications (PTMs) of BiP influence the activity of the protein by several ways. For example, acetylation of BiP on lysine 585 is commonly found in various cancer cell lines when treated with HDAC inhibitor, vorinostat (Kahali et al. 2010). This PTM on BiP has a tremendous implication as the K-585 acetylated BiP is unable to interact with PERK, leading to induction of UPR and apoptosis (Kahali et al. 2010). Thus, such modifications of BiP if explored in details may have potential therapeutic application in future.

10.6 Role of Hsp70 in Protein Degradation

Apart from Hsp70's roles in various constructive activities related to protein homeostasis in different sub-cellular locations as mentioned in the previous sections, the chaperone is also involved in another extremely important aspect of protein homeostasis, the degradation of damaged, aggregated or terminally misfolded proteins from cellular milieu. Eukaryotic cells have several parallel pathways to eliminate unwanted or harmful proteins: Ubiquitin-proteasome system (UPS), autophagy (microautophagy and macroautophagy) and Chaperone mediated autophagy (CMA). In many of these vital pathways, some members of Hsp70 family have been shown to be involved in association with co-chaperones.

10.6.1 Hsp70 in UPS Mediated Protein Degradation

Ubiquin-proteasome system or UPS is a highly effective mechanism of degradation of mainly cytosolic proteins. UPS, as the name suggests consists of ubiquitin, a small protein that tags the proteins to be degraded whereas proteasome is a barrel shaped multicomponent protein complex harboring a cavity that acts as the site of degradation of ubiquitin-tagged proteins. The process of ubiquitination of proteins is quite elaborate and is achieved by an enzymatic cascade consisting of three groups of enzymes which work sequentially to tag a protein with ubiquitin (Amm et al. 2014; Ji and Kwon 2017). Three groups of proteins work in concert to achieve the ubiquitination of substrates; ubiquitin activating enzymes (E1), ubiquitin conjugating enzymes (E2) and ubiquitin ligases (E3) (Amm et al. 2014; Ji and Kwon 2017). To assist Hsp70s in the UPS mediated degradation process, a protein called CHIP (carboxy terminus of Hsc70 interacting protein) acts as co-chaperone. CHIP is a dimeric protein and harbours three domains, the N-terminal TPR domain which interacts with the conserved EEVD motif of Hsp70s and Hsp90s, the C-terminal domain U-box binding domain harbouring E3 ubiquitin ligase activity and an intervening charged domain. CHIP hinders the chaperoning activity of Hsp70 and Hsp90 and the substrates hold by these chaperones are positioned near the E2 enzymes by CHIP for successful ubiquitin tagging which is completed by its own E3 ligase activity (Edkins 2015; McDonough and Patterson 2003; Paul and Ghosh 2014). Another group of co-chaperone of Hsp70s called BAG proteins also play crucial role in UPS mediated protein degradation by Hsp70s (Doong et al. 2003; Elliott et al. 2007). BAG1 has been shown to acts as NEF for Hsp70 but also harbours an ubiquitin-like domain that interacts with proteasome and can deliver the Hsp70-bound substrate proteins for degradation by proteasome (Doong et al. 2003; Elliott et al. 2007).

10.6.2 Hsp70 in Chaperone Mediated Autophagy (CMA)

Chaperone mediated autophagy is a type of autophagy for efficient removal of misfolded or aggregated proteins from cytosol where the degradation prone proteins are translocated across the lysosomal membranes with the help of chaperones, mainly Hsp70s for degradation (Ciechanover and Kwon 2017; Jacob et al. 2017; Kettern et al. 2010; Li et al. 2011; Massey et al. 2006). In contrast to classical autophagy (macro or micro-autophagy) where the cargo is sequestered within autophagic vacuoles or autophagosomes followed by fusion of autophagosomes with lysosomes, CMA substrates with the help of cytosolic Hsp70s (Hsc70, HSPA8), interact with the lysosomal membranes protein (LAMP-2A) followed by translocation inside the lysosomal cavity. Presence of a specific sequence motif KFERQ is absolutely essential in CMA substrates to be degraded by this process. The cytosolic Hsc70 recognizes this motif on substrates and delivers the substrates to LAMP-2A (Rout et al.

2014). The interaction of Hsc70 is immensely important for CMA and is considered as one of the hallmarks of CMA substrates. Various proteins like transcription factors, lipid binding and calcium binding proteins, various enzymes and aggregation prone proteins like poly-Q-Huntingtin (are removed by this efficient degradation system (Arias and Cuervo 2011; Li et al. 2011; Qi and Zhang 2014). Once LAMP-2A binds substrates, it oligomerizes into ~700kDA complex for membrane translocation. Soon after the translocation of the protein substrate within the lysosomal cavity, LAMP-2A needs to revert to monomeric state for next round of binding and translocation of substrates. Lys-Hsc70 play a critical role in the rapid conversion of oligomeric LAMP-2A to its monomers and helps in next round of CMA-substrate binding and translocation within lysosomes (Arias and Cuervo 2011; Kaushik and Cuervo 2012).

10.7 Conclusions

In this chapter, we have summarized the roles of various members of Hsp70 chaperone family in diverse aspects of protein quality control. It is astonishing that despite similar structures, the members of this group can carry out such a varied function. It is quite evident from literature that the involvement of two groups of co-chaperones, especially the J-domain proteins impart the multi-tasking ability to Hsp70s. The mechanism of spatial segregation of co-chaperones within same subcellular location remains unanswered so far and needs to be explored in details. Apart from co-chaperones, modifications of Hsp70s and interaction with a number of other interacting partners, also help in achieving such diverse function.

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