Review

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Hsp70-mediated quality control: should I stay or should I go?

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Abstract: Chaperones of the 70 kDa heat shock protein (Hsp70) superfamily are key components of the cellular proteostasis system. Together with its co-chaperones, Hsp70 forms proteostasis subsystems that antagonize protein damage during physiological and stress conditions. This function stems from highly regulated binding and release cycles of protein substrates, which results in a flow of unfolded, partially folded and misfolded species through the Hsp70 subsystem. Specific factors control how Hsp70 makes decisions regarding folding and degradation fates of the substrate proteins. In this review, we summarize how the flow of Hsp70 substrates is controlled in the cell with special emphasis on recent advances regarding substrate release mechanisms.

Keywords: disaggregation; Hsp40; J-domain protein; nucleotide exchange factor; protein degradation; protein folding.

Introduction

Proteins need to fold into defined three-dimensional structures to become functional and exhibit activity. During biosynthesis proteins populate unfolded conformations and are at a high risk of toxic misfolding and aggregation. Such protein misfolding is accelerated under stress conditions and modified by different metabolic regimes (Andréasson et al. 2019). To counteract protein misfolding and thereby safe-guarding proteostasis, the cell is equipped with a complex system that governs the folding status of the proteome. The core of the proteostasis system is a precisely coordinated network of molecular chaperones that suppress unwanted proteostasis disturbances by supporting protein folding and quality control (Kaushik and Cuervo 2015).

One of the most abundant molecular chaperones with high evolutionary conservation belongs to the 70 kDa heat shock protein superfamily (Hsp70). Hsp70 has a role in all stages of protein life from synthesis to degradation, and forms proteostasis subsystems in the cytoplasm, the endoplasmic reticulum (ER), mitochondria and chloroplasts (Figure 1). Hsp70 delays the folding of emerging polypeptides from the ribosomal tunnel exit until the sequence elements needed for assembly of a complete domain become exposed at the ribosomal surface (Döring et al. 2017; Teter et al. 1999; Willmund et al. 2013). Hsp70 also aids in *de novo* formation of protein complexes via folding of subunit interaction domains and protection of aggregation-prone subunits before assembly into complexes (Shiber et al. 2018). During protein translocation into mitochondria, chloroplasts and the ER, Hsp70 acts on both sides of the membrane. Cytosolic Hsp70 escorts proteins in a translocation-competent state and organellar Hsp70 binds emerging substrates at the pore and promotes the transport via a directional pulling mechanism (Craig 2018). Hsp70 also plays a key role in endocytosis in eukaryotic cells. Loading of Hsp70s onto the clathrin cage of endocytic membrane vesicles triggers their uncoating by steric interference or entropic pulling (Ungewickell et al. 1995; Schmid et al. 1984; Sousa et al. 2016). Hsp70 further determines the fate of its protein substrates, either by supporting their refolding or targeting them to degradation by the proteasome or the autophagic system (Gamerdinger et al. 2011; Lüders et al. 2000). Finally, Hsp70 family members are involved in regulatory circuits at the cellular level. Hsp70 plays a key role as suppressor of apoptosis, a regulated cell-death regime, by direct interaction with several apoptosis-inducing factors (Fourie et al. 1997; King et al. 2001; Li et al. 2000; Takayama et al. 2003). On the transcriptional level, Hsp70 directly participates in regulating the activity of transcription factors in response to stress. For example is Hsp70 a key regulator of the heat shock response, a stress-induced transcriptional program that controls the expression of many pro-survival genes during proteostasis perturbations (Lindquist 1986;

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Richter et al. 2010). Under physiological conditions, Hsp70 suppresses the function of the respective transcription factor (bacterial σ^{32} or eukaryotic Hsf1) by direct binding. However, under stress the accumulation of misfolded proteins leads to down-titration of available Hsp70, thus allowing these transcription factors to induce the heat shock response (Krakowiak et al. 2018; Masser et al. 2019; Peffer et al. 2019; Pincus 2020; Rodriguez et al. 2008; Shi et al. 1998). A related chaperone-titration mechanism involving direct Hsp70 binding controls the unfolded protein response in the ER (Amin-Wetzel et al. 2019; Bertolotti et al. 2000; Kopp et al. 2019). Thus, Hsp70s are at the centre stage of the proteostasis system from protein folding to transcriptional regulation.

The cellular roles of Hsp70 may appear to be quintessentially different but they all rely on an unifying molecular mechanism. This involves the interplay of the two intrinsic activities of Hsp70, namely ATPase activity and protein substrate binding. Together, they allow Hsp70 to function as an ATP-driven molecular protein-binding clamp that executes chaperone functions. Moreover, Hsp70 collaborates with different co-chaperones of the J-domain protein superfamily (alias Hsp40) to target and trap their substrates via ATP hydrolysis. The timing of substrate release is dictated by ADP-to-ATP exchange that is catalysed by nucleotide exchange factors (NEFs). Several co-chaperones leadingly bias the fate of a Hsp70 substrate by favouring refolding or degradation via feeding substrates into the respective downstream processes. In this review, we discuss the general concepts on how protein substrates are productively recognized and released by the Hsp70 chaperone subsystem.

The allosteric coupling of Hsp70

The sequences and structures of Hsp70s are highly conserved across species (Boorstein et al. 1994; Rosenzweig et al. 2019). All Hsp70s have two functional domains, a nucleotide-binding domain (NBD) at the N-terminus and a substrate binding domain (SBD) at the C-terminus that correspond to the two essential intrinsic activities (Figure 2A,B). These two domains are connected by a flexible interdomain linker. The NBD has an actin-like fold, composed of lobe I and II that are separated by a deep nucleotide-binding cleft with the catalytic centre at its bottom. The lobes are further divided into subdomain A and B. The SBD of Hsp70 is composed of two subdomains, SBDβ and SBDα. The SBDβ consists of eight β-strands organized into two β-sheets and contains a substrate-binding cavity

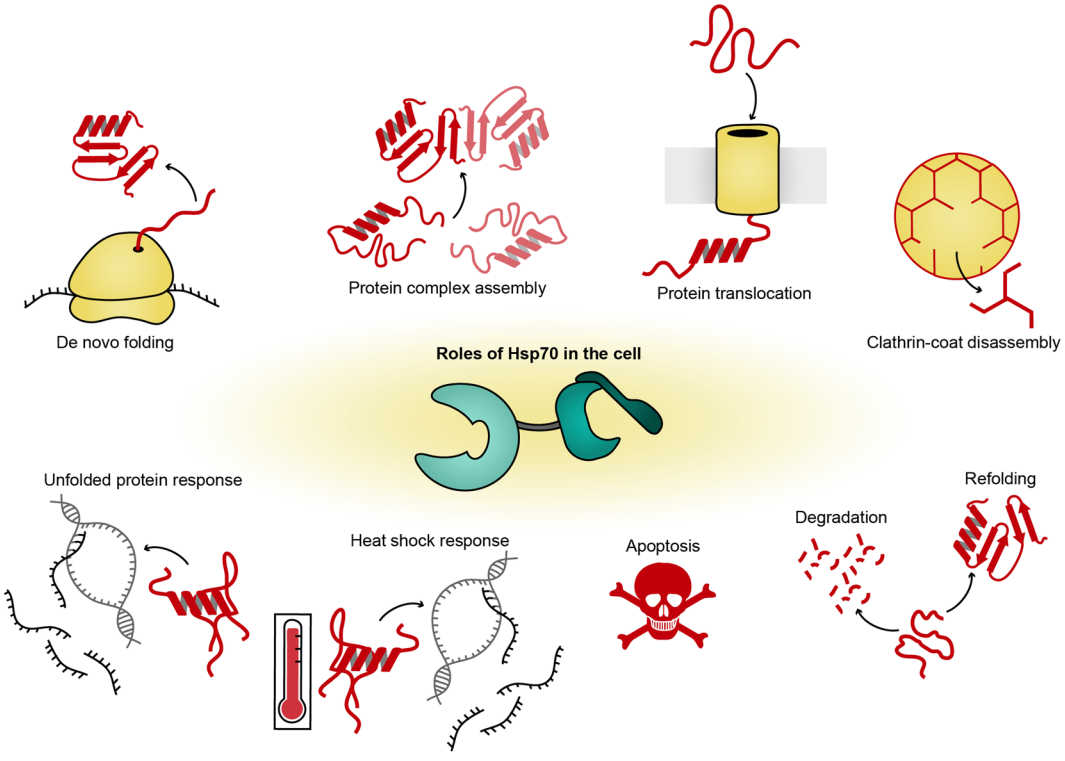

Figure 1: The diverse roles of Hsp70 in a cell. Schematic drawing of processes that are regulated by proteins of the 70 kDa heat shock protein (Hsp70) superfamily. While some functions are conserved between bacteria and eukaryotes, others (e.g. protein translocation) are solely found in eukaryotic organisms. Please see main text for details.

with a central hydrophobic pocket. The SBDα is an all-α-helical domain that covers the peptide-binding pocket as a lid (Flaherty et al. 1990; Kityk et al. 2012; Zhu et al. 1996).

Hsp70 ability to associate and dissociate from protein substrates in a controlled manner depends on an intricate allosteric mechanism. Conceptually, the activity of the NBD and the respective bound nucleotide control substrate binding and release in the SBD (Figure 2C) (Buchberger et al. 1995; Marcinowski et al. 2011; Rist et al. 2006; Swain et al. 2007; Vandova et al. 2020; Zhuravleva et al. 2012). In the ADP-bound state of Hsp70 (Figure 2C (i)), the NBD and the SBD behave as isolated domains without contact and the interdomain linker is exposed and flexible. The SBD is in a closed conformation, with the SBDα lid on top of the SBDβ subdomain. This results in high affinity for protein substrates and low substrate exchange rates. The increase in the effective affinity for substrates by several orders of magnitude beyond the ADP-bound state is a non-equilibrium property called ultra-affinity (De Los Rios and Barducci 2014). ATP binding to the NBD induces a drastic conformational rearrangement of Hsp70 (Figure 2C (ii)). The SBDα lid dissociates from the SBDβ, and both SBD subdomains and the interdomain linker bind to the NBD. This leaves the NBD in a conformation unsuitable for ATP hydrolysis, whereas the SBD adopts an open conformation with high substrate exchange rates and low substrate affinity. Upon protein substrate binding to the SBD (Figure 2C (iii)), the SBDβ and the SBDα lid are released from the NBD, again adopting a closed conformation. This, together with dissociation of the interdomain linker from the NBD, stimulates ATP hydrolysis. Finally, nucleotide exchange triggers the opening of the SBD and release of the substrate by immobilization of the SBDα lid, the SBDβ and the interdomain linker onto the ATP-bound NBD and the chaperone cycle can restart (Figure 2C (iv)) (Alderson et al. 2014; Bertelsena et al. 2009; Buchberger et al. 1995; Chang et al. 2008; Gragerov et al. 1994; Kityk et al. 2015; Kityk and Mayer 2018; Liu and Hendrickson 2007; Liu et al. 2020; Mayer and Gierasch 2019; Qi et al. 2013; Swain et al. 2007; Vogel et al. 2006; Zhuravleva and Gierasch 2011).

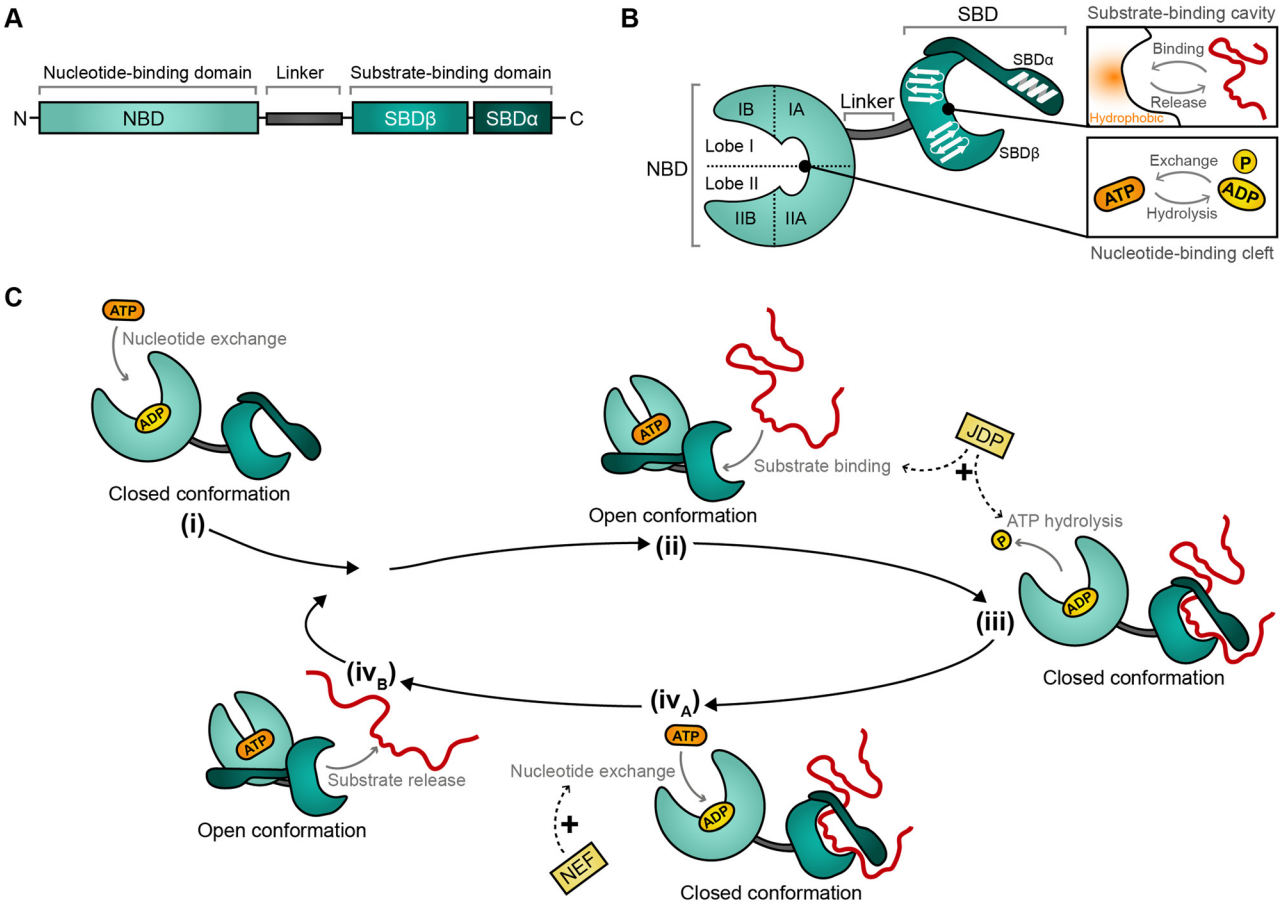

Figure 2: The allosteric cycle of Hsp70s. (A, B) Schematic representation of the main domains of Hsp70s including their structural features and respective functions. (C) The allosteric cycle of Hsp70 couples nucleotide binding and ATP hydrolysis to protein substrate binding and release processes. Conformational changes in Hsp70 govern this intricate mechanism and distinct co-chaperones of the J-domain protein (JDP) superfamily and nucleotide exchange factors (NEFs) speed up this process.

Increasing the efficiency of the Hsp70 allosteric cycle

Even though Hsp70 functions at the heart of central proteostasis processes, its autonomous chaperone activity is limited. Two classes of essential co-chaperones, J-domain proteins (JDPs, alias Hsp40s) and nucleotide exchange factors (NEFs), modify and enhance Hsp70 activity at different stages of the allosteric cycle (Figure 2C). JDPs stimulate ATP hydrolysis and bridge the substrate to Hsp70s by direct binding (Alderson et al. 2016; Kityk et al. 2018). NEFs control the timing and selectivity of substrate release by accelerating rates of ADP-to-ATP exchange and some subclasses block the instant rebinding of persistent substrates (Bracher and Verghese 2015; Gowda et al. 2018; Rosam et al. 2018).

JDP co-chaperones

JDPs are a heterogenous group of modular multi-domain proteins that have more diversity than Hsp70s and NEFs in each cellular compartment. The large sequence- and structure divergence of JDPs contribute substantially to the multifaceted roles of Hsp70s (Kampinga and Craig 2010; Kampinga et al. 2019). JDPs are characterized by a conserved α-helical hairpin domain with a signature tripeptide motif (HPD), the J-domain, that facilitates interaction with Hsp70s (Alderson et al. 2016; Cyr et al. 1992; Greene et al. 1998; Karzai and McMacken 1996; Kityk et al. 2018; Liberek et al. 1991; Russell et al. 1999). According to their domain composition, JDPs can be divided into three classes A, B and C. While this classification is used for nomenclature purposes, JDPs of the same class often display different biochemical functions and mechanisms of action (Cheetham and Caplan 1998; Hennessy et al. 2000; Kampinga and Craig 2010). The J-domain couples substrate binding to ATP hydrolysis and efficient trapping of the protein substrate (Kityk et al. 2018). The substrate-binding induced conformational changes in the SBDβ (Figure 2C (iii)) are transmitted to the J-domain, which arrests the NBD lobes and its catalytic residues in a position optimal for ATP hydrolysis. This combined action of protein substrate binding to ATP-Hsp70 and association of the J-domain promotes maximal ATP hydrolysis of Hsp70 (Kityk et al. 2018; Laufen et al. 1999; Russell et al. 1999). JDPs also serve as substrate-scanning factors for Hsp70s and their presence increases the number of Hsp70 binding sites in substrates (Misselwitz et al. 1998; Rüdiger et al. 2001; Suzuki et al. 2012). This is facilitated via increasing the apparent affinity of Hsp70 to suboptimal sites and induction of conformational changes in the substrate (Kellner et al. 2014; Rodriguez et al. 2008). In addition to their role in substrate delivery and increase of ATP hydrolysis, JDPs determine many Hsp70 functions by targeting the chaperone to distinct subcellular sites (Kityk and Mayer 2018).

NEF co-chaperones

NEFs control the precise targeting and timing of substrate release from Hsp70s by accelerating nucleotide exchange and securing that the chaperone does not rebind its protein substrate. Structures of complexes consisting of NEFs with isolated NBDs show that NEFs stabilize the nucleotide-free state of the NBD, thus explaining how they induce the opening of the nucleotide binding pocket (Bracher and Verghese 2015; Harrison et al. 1997; Polier et al. 2008; Schuermann et al. 2008; Sondermann et al. 2001). Nucleotide exchange is accelerated by binding to and stabilizing rarely populated Hsp70 NBD conformations resulting in opening of the Hsp70 nucleotide binding cleft. After ADP release, rebinding of ATP triggers opening of the Hsp70 SBD and promotes the removal of the substrate (Figure 2C (iv)). Four structurally unrelated protein families are known to perform NEF function. In prokaryotes, mitochondria and chloroplasts, the nucleotide exchange is regulated by GrpE and GrpE-like proteins, that depend on homodimerization for fulfilling their NEF function (Harrison et al., 1997; Packschies et al., 1997). In the eukaryotic cytoplasm, the nucleotide exchange is instead facilitated by either BAG, Hsp110/Grp170 or Armadillo-type NEF families. BAG-type NEFs contain additional interaction domains to localize them to specific subcellular structures, enabling timed transfer of substrate from Hsp70 to other protein quality control systems (Gamerdinger et al. 2011; Lüders et al. 2000; Rauch et al. 2017). Hsp110 and ER-resident Grp170 structurally belong to the Hsp70 superfamily, with a high sequence similarity in their NBD domain, while the SBD is less conserved. The structure of these NEFs resemble the ATP-bound open conformation of Hsp70 (Figure 2C) (Andréasson et al. 2008, 2010; Polier et al. 2008). Structural studies suggest a possible cooperation in substrate binding due to the close proximity between the respective SBDs of Hsp70 and Hsp110 when they form a NEF complex (Polier et al. 2008; Schuermann et al. 2008). Members of Armadillo-class NEFs rely on their core α-helical armadillo domain for Hsp70 nucleotide exchange, and a flexible N-terminal extension, called the release domain, ensures efficient liberation of protein

substrates from Hsp70 (Gowda et al. 2018; McLellan et al. 2003; Rosam et al. 2018). The release domain contacts the SBD of Hsp70 and hinders immediate rebinding of just released protein substrates. Similarly, the N-terminal helical extension of GrpE and unstructured tails of human BAG1 and BAG3 have been proposed to accelerate substrate release via contacting the Hsp70 SBD (Harrison et al., 1997; Rauch et al., 2016; Wu et al., 2012). Thus, NEFs combine the function of accelerating nucleotide exchange with securing that substrates remain liberated from Hsp70.

Divergent NEFs employ two principle mechanisms to accelerate nucleotide exchange. GrpE, BAG and Hsp110/Grp170 facilitate nucleotide release by stabilizing similar Hsp70 NBD conformations, involving tilting of the NBD lobe II outwards, thus opening the NBD structure (Andréasson et al. 2008; Harrison et al. 1997; Polier et al. 2008; Schuermann et al. 2008; Sondermann et al. 2001). In contrast, Armadillo-type NEFs instead associate with the lobe II subdomain IIB (Andréasson et al. 2008; Shomura et al. 2005; Yan et al. 2011). Both yeast Fes1 and human HspBP1 bind Hsp70 NBD lobe IIB, thereby displacing lobe I, which triggers almost global unfolding of the NBD (Andréasson et al. 2008; Shomura et al. 2005). Human Sil1 (BAP) similarly binds lobe IIB but destabilization of the Hsp70 NBD has not been observed and instead, a rotation of lobe I is visible in the structure (Yan et al. 2011). The conformational repertoire intrinsic to the Hsp70 NBD explains, how structurally distinct NEFs can induce similar conformational changes when bound to Hsp70.

Interestingly, several studies show that NEF-mediated substrate release can be downregulated during acute stress conditions (Grimshaw et al. 2001, 2003; Groemping and Reinstein 2001; Marada et al. 2013; Moro and Muga 2006; Nicklow and Sevier 2020; Stevens et al. 2017). This might result in prolonged association of Hsp70 with bound substrates during suboptimal folding conditions to limit further protein misfolding and aggregation. Thus, tuneable NEF-activity adds up another level of Hsp70-dependent proteostasis regulation.

Hsp70 substrate interactions

Hsp70 chaperone activity is based on its ability to bind to short peptide motifs that are found on average every 30–40 residues in virtually all proteins (Rüdiger et al. 1997b). Bacterial Hsp70 DnaK was shown to have about 700 *in vivo* substrates, both newly synthesized and pre-existing proteins (Calloni et al. 2012; Niwa et al. 2012; Rüdiger et al. 1997b). While DnaK binding stabilizes larger, multimeric proteins upon denaturing stress conditions, a certain subset of proteins is destabilized by DnaK association. These substrates are often smaller in size and usually monomeric, and are suggested to contribute to adaption to stress conditions (Zhao et al. 2019). The interactome of yeast Hsp70 Ssb1 and Ssb2 (Ssb1/2) comprises about 3,000 nascent proteins and is hence broader than originally anticipated. Ssb1/2 was shown to bind 80% of cytoplasmic and nuclear nascent proteins, 80% of mitochondrial nascent proteins and more than 40% of nascent ER proteins during transit through the cytoplasm (Döring et al. 2017). Even though the SBD is conserved (Cupp-Vickery et al. 2004; Morshauser et al. 1999; Pellecchia et al. 2000; Stevens et al. 2009), Hsp70s from different organisms or even different cellular compartments have somewhat divergent substrate preferences, which might be influenced by the kinetics of substrate binding to the SBD (Blond-Elguindi et al. 1993; Fourie et al. 1994; Gragerov and Gottesman 1994; Kluck et al. 2002; Rüdiger et al. 1997b, 2000; Schneider et al. 2016; Van Durme et al. 2009). In this respect, it is interesting to note that eukaryotic Hsp70s have higher substrate binding and release rates that their bacterial counterparts (Marcinowski et al. 2013). Nevertheless, both bacterial and eukaryotic Hsp70s display a broad substrate range, especially when nascent polypeptide chains are included in the analysis.

Hsp70 binds protein substrates via short stretches of polypeptides with no stringent sequence specificity but with a preference for hydrophobic residues, especially aliphatic amino acids, as well as residues with positive charges, however with a bias against negatively charged amino acids (Figure 3) (Blond-Elguindi et al. 1993; Döring et al. 2017; Gragerov et al. 1994; Gragerov and Gottesman 1994; Rüdiger et al. 1997a, b; Schneider et al. 2016; Stein et al. 2019; Van Durme et al. 2009). A recent study showed that even though acidic or negatively charged residues are more efficient in inhibiting protein aggregation than positively charged amino acids, they are less compatible with the globular protein structure. Thus, the bias of Hsp70 towards basic residues might aid to prioritize binding to aggregation-prone protein sequences (Houben et al. 2020). The binding cleft of the Hsp70 SBD is composed of five binding pockets and the central groove accounts for the highest specificity in Hsp70 substrate binding (Figure 3A). Leucine residues fit optimal into this pocket, while isoleucine and valine can also bind. The remaining binding pockets can associate with a wider range of amino acids (Marcinowski et al. 2013). Interestingly, bacterial Hsp70 DnaK can bind substrate peptides in two isoenergetic orientations, the conventional forward and the reverse binding mode. Proline residues at central positions strongly favour the reverse orientation. Optimal interaction with

binding pockets –2 to 0 appear as the main determinant for directional preference (Tapley et al. 2005; Zahn et al. 2013). These characteristics of the Hsp70 SBD results in selective promiscuity, enabling the chaperone to bind diverse substrates and perform a wide spectrum of functions (Clerico et al. 2019). Peptides bind in extended conformation to the hydrophobic cleft in the SBD (Zhu et al. 1996). The peptide backbone is then enclosed by the SBDβ cleft and is stabilized by both hydrogen bonds and hydrophobic interactions, and the helical lid of SBDα. While the peptide-binding configuration is evolutionary conserved (Cupp-Vickery et al. 2004; Jiang et al. 2005; Morshauser et al. 1999; Pellecchia et al. 2000; Stevens et al. 2009), there is a variability on the register and orientation of peptides bound to Hsp70 (Clerico et al. 2019; Tapley et al. 2005; Zahn et al. 2013; Zhang et al. 2014). The peptide preferences of the SBD explain the selective promiscuity of Hsp70.

While model peptides are commonly used to elucidate molecular mechanisms of chaperone binding, Hsp70s encounter mainly protein substrates in their native environment and, most likely, unfolded nascent polypeptide chains at the ribosome dominate among those substrates. Unfolded proteins frequently expose multiple Hsp70 binding sites, and are recognized and bound in the same way as peptide substrates (Clerico et al. 2019). Hsp70 also interacts with partially folded intermediates, stabilizing the substrate and preventing further unfolding (Mashaghi et al. 2016). Hsp70 binding usually occurs via conformational selection of unfolded states rather than by unfolding partially folded species (Sekhar et al. 2018). When Hsp70 binds to folded proteins, it recognizes segments in unstructured regions of linkers and loops, while the rest of the protein remains folded (Böcking et al. 2011; Marcinowski et al. 2011; Rodriguez et al. 2008; Schlecht et al. 2011; Sekhar et al. 2018). In these cases, the SBDα lid does not close fully over the respective substrates (Banerjee et al. 2016; Marcinowski et al. 2011; Schlecht et al. 2011). Even though the SBDα lid is not closed completely over distinct clients, it might still form contacts with and cause conformational changes in the substrate, and is suggested to act as docking site for certain co-chaperones. In this line, a lidless bacterial Hsp70 DnaK was unable to refold a model substrate (Clerico et al. 2015; Laufen et al. 1999). Peptide substrates also stimulate the ATPase less efficiently than protein substrates and there is no synergism with JDPs observed (Figure 3B) (Laufen et al. 1999). Thus, Hsp70 substrate binding should typically be seen in the context of multiple binding sites of different affinities and concerted interactions with co-chaperones.

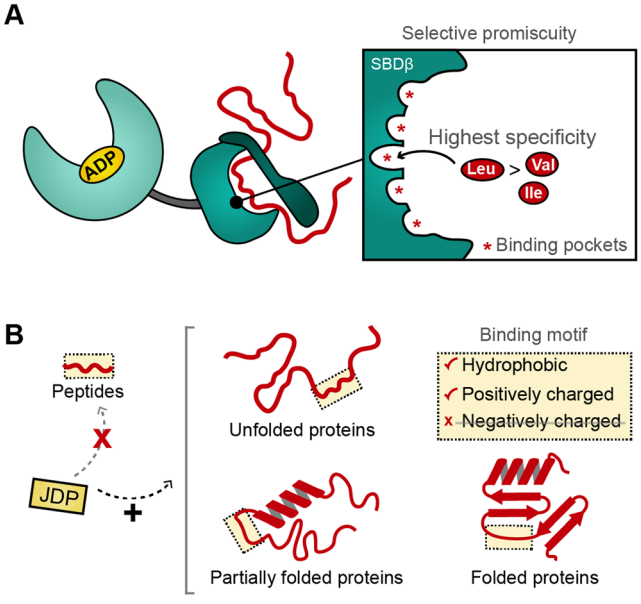

Figure 3: Protein substrate binding to Hsp70. (A) SBDβ has five binding pockets and the central binding cavity has the highest specificity. It binds favourably leucine (Leu), while also valine (Val) and isoleucine (Ile) can associate, resulting in selective promiscuity. (B) Main features of Hsp70 binding motifs. Hsp70 can recognize peptides and proteins in different folding states. Binding of Hsp70 to peptides does not show synergistic activation by J-domain proteins (JDPs).

Substrate release from Hsp70

Hsp70 cooperation with other cellular chaperone systems is required to maintain the intricate network of protein folding, unfolding, aggregation, disaggregation and degradation. One key step in this complex interplay is ensuring accurate timing and selectivity of substrate release to avoid futile cycles of Hsp70 re-binding. After protein substrate release from Hsp70, polypeptides either fold spontaneously or are transferred to other machineries that aid in downstream processing (Rosenzweig et al. 2019).

Spontaneous protein folding following Hsp70 release

In contrast to the originally proposed model, where Hsp70 inhibits aggregation passively without influencing the folding pathways, accumulating evidence suggests folding bias and active substrate release from Hsp70 (Figure 4A). Bacterial Hsp70 DnaK combined with JDP DnaJ and NEF GrpE was shown to accelerate folding of the model substrate firefly luciferase (Imamoglu et al. 2020). Upon spontaneous folding, luciferase molecules populate

kinetically trapped misfolded states. Binding of several Hsp70 molecules to luciferase leads to steric repulsion and thus expansion of misfolded regions in the protein substrate, which resolves kinetically trapped intermediates (Imamoglu et al. 2020; Kellner et al. 2014; Sekhar et al. 2015, 2016). In line, a theoretical model proposed a faster release of Hsp70 from substrates in folding-competent states than in misfolding-prone conformations (Xu 2018). Hsp70 is described to reshape the folding energy landscape via shifting the equilibrium to more unfolded conformations upon selective binding to exposed hydrophobic stretches (Sekhar et al. 2016, 2017). Due to binding of several Hsp70 molecules to the respective substrate, long-range interactions between distal parts of the protein are disrupted and the formation of secondary structures is favoured (Sekhar et al. 2016). Further, the released substrate will begin its folding from an initial conformation that structurally resembles the Hsp70-bound state. The biased folding pathway originates from the asynchronous release of Hsp70 molecules from protein substrate (Rosenzweig et al. 2017; Sekhar et al. 2017). Due to several Hsp70 binding sites present in a substrate, Hsp70-bound proteins can form different secondary and tertiary structures. After substrate release, folding-incompetent proteins can re-enter the chaperone cycle by Hsp70 binding to another site, thus increasing the overall chance to avoid the same kinetic trap (Figure 4A). This explains, why proteins usually undergo multiple chaperone rounds until they reach their native state (Rosenzweig et al. 2017).

Combined analyses of several 3D structures show that Hsp70 exists also in a third fully-closed conformation (Kityk et al. 2012; Qi et al. 2013; Yang et al. 2015, 2017). This fully-closed form is suggested as the major conformation of the ATP-bound state that is incompatible with substrate binding. This ensures that the polypeptide substrate is released from Hsp70 and is not able to re-bind immediately. The action of a JDP is required to shift the fully-closed to the open conformation for further rounds of substrate binding. This active release of bound substrate and JDP regulation gives the chaperone cycle a direction instead of random oscillations between two nucleotide-bound states (Yang et al. 2017).

Hsp90-assistance in protein folding

The co-chaperone Hsp90 plays a key role in breaking up Hsp70-inflicted folding blocks (Morán Luengo et al. 2018). Productive folding stalls upon repeated Hsp70 binding to hydrophobic core-forming segments. The Hsp70 binding pocket is highly hydrophobic, while Hsp90 recognizes hydrophobic and hydrophilic surface patches, which preferentially occur in near-native folding conformers (Karagöz et al. 2014; Karagöz and Rüdiger 2015; Street et al. 2014; Taipale et al. 2012). The more polar Hsp90 binding site thus stimulates the substrate to complete its hydrophobic core (Biebl and Buchner 2019; Kirschke et al. 2014; Wegele et al. 2006). The decreasing hydrophobicity along the Hsp70-Hsp90 cascade might thus be crucial for spontaneous folding (Figure 4B). The co-chaperone Hop (Sti1 in yeast) facilitates the handover of partially folded intermediates from Hsp70 to Hsp90, and together with the protein substrate strengthens their direct interaction (Alvira et al. 2014; Doyle et al. 2019; Genest et al. 2011, 2015; Johnson et al. 1998; Kravats et al. 2017; Nakamoto et al. 2014; Scheufler et al. 2000; Wegele et al. 2006). This substrate transfers occurs frequently for late folding intermediates and a large number of meta-stable proteins are regulated in their activity and stability by Hsp70 and Hsp90 (Kirschke et al. 2014; Morishima et al. 2000). In the absence of Hsp90, substrates might escape Hsp70 folding deadlocks by other means. It is proposed that the new supply of nascent chains or unfolded proteins upon stress conditions down-titrate Hsp70 molecules, and thus lead to substrate release and either productive spontaneous folding or further downstream processing (Morán Luengo et al. 2018).

Linking futile protein folding with proteasomal degradation

If the handover from Hsp70 to Hsp90 is compromised, protein substrates either rebind Hsp70 for further chaperone cycles or are targeted for degradation in a Hsp70-dependent manner (Figure 4C). In metazoan, the co-chaperone CHIP docks directly to Hsp70 and competes with Hop for binding of Hsp70 and Hsp90 (Ballinger et al. 1999). CHIP ubiquitinylates Hsp70-bound substrates stochastically to mark them for proteasomal degradation (Connell et al. 2001; Meacham et al. 2001). Thus, substrates that spend an extended period of time at Hsp70, either due to unproductive repeated folding processes or absence of Hsp90, are preferentially targeted for degradation (Connell et al. 2001; Meacham et al. 2001; Stankiewicz et al. 2010). Additionally, the NEF BAG1 with its integral ubiquitin-like domain serves as proteasomal targeting signal that coordinates the transfer of Hsp70-bound substrates to the 26S proteasome for degradation (Demand et al., 2001; Lüders et al., 2000).

Hsp110 NEFs also contribute to Hsp70 triaging the fate of a protein substrate. Upon inhibition of yeast Hsp90, Hsp110 Sse1 is required for ubiquitination and thus

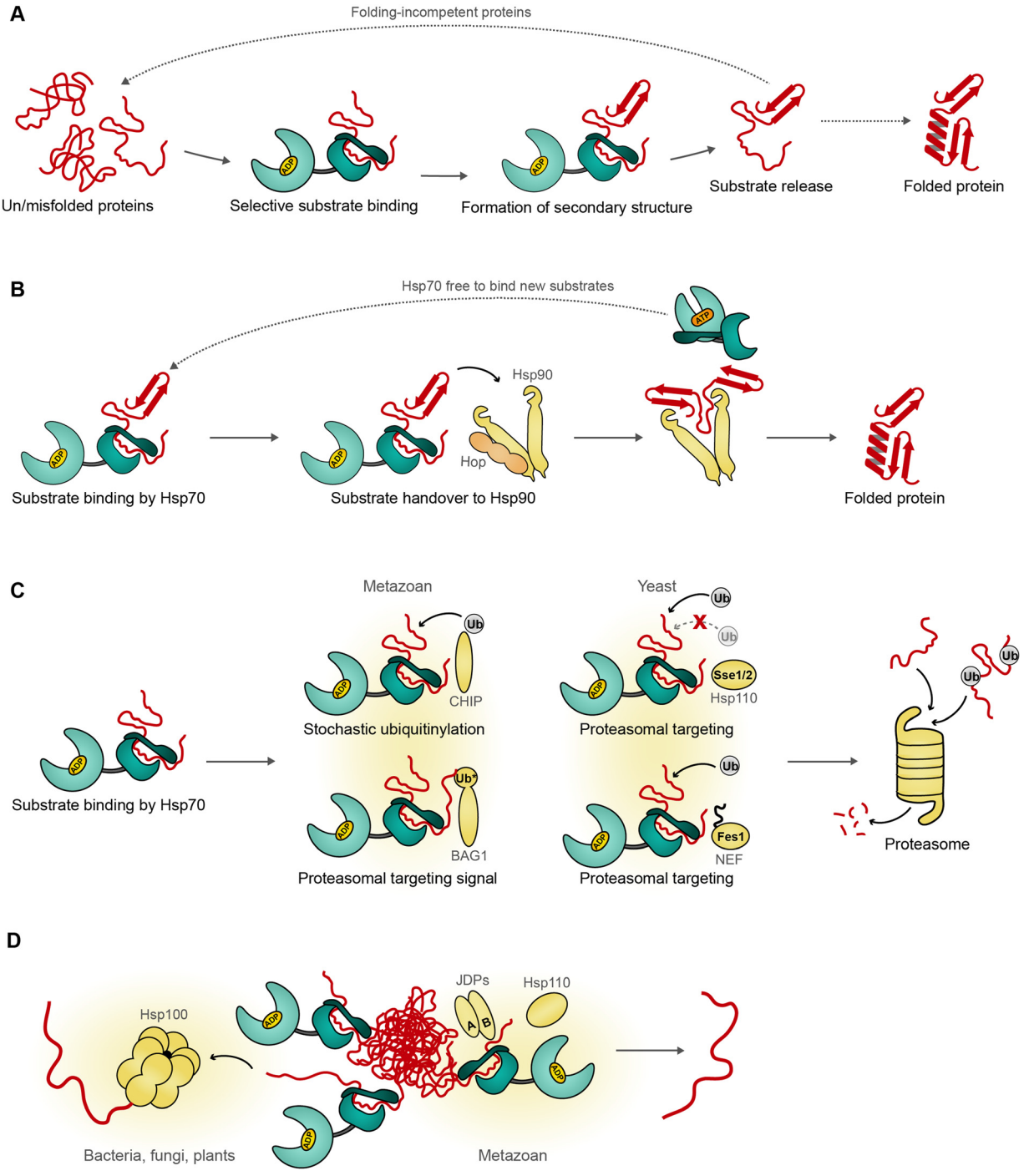

Figure 4: Substrate release mechanisms from Hsp70. (A) Hsp70 selectively binds to exposed unfolded stretches of protein substrates, which favours formation of secondary structure elements. Upon substrate release, proteins either fold spontaneously into their native 3D-structure or re-enter the chaperone cycle. (B) Substrates bound by Hsp70 are handed over to Hsp90 with the aid of the co-chaperone Hop. While Hsp70 is now free for further rounds of substrate binding, proteins bound by Hsp90 are frequently late folding-intermediates or near-native structures. (C) Substrates bound by Hsp70 can be targeted for proteasomal degradation either via ubiquitinylation (Ub) by the metazoan co-chaperone CHIP or via the ubiquitin-like domain (Ub*) of BAG1. Both Hsp110 Sse1/2 and the Armadillo-like nucleotide exchange factor (NEF) Fes1 regulate proteasomal targeting of folding-resilient substrates in yeast. (D) Hsp70 decoration of aggregates is the first step of disaggregation. While bacteria, fungi and plants rely on Hsp100 disaggregases, metazoan Hsp70 disaggregation capacity is activated by the NEF Hsp110 and J-domain proteins (JDPs) of class A and B.

targeting of a protein substrate for proteasomal degradation (Mandal et al. 2010). Further, yeast Hsp110 Sse1/2 is required to keep Hsp70-associated proteasome substrates soluble and interacts with the 19S regulatory particle of the proteasome. This enables a coordinated recruitment of the Hsp70-substrate complex to the 26S proteasome by employing Hsp110 as receptor that can act on both ubiquitinylated and non-ubiquitinylated cargo (Kandasamy and Andréasson 2018). Yeast Armadillo-class Fes1 also promotes proteasomal degradation of misfolded proteins. Proteins unable to refold undergo repetitive Hsp70 cycles until the Hsp70-substrate complex encounters Fes1. Fes1 triggers nucleotide exchange and secures the release of persistent protein substrates using its release domain, and thus is required to target proteins for degradation. Lack of Fes1 interferes with polyubiquitination of misfolded proteins and promotes their aggregation (Gowda et al. 2013). In these cases, NEFs go beyond their role in ending the state of ultra-affinity during the allosteric cycle of Hsp70, and instead abort unproductive folding cycles and target the substrate to proteasomal degradation.

Hsp70-mediated disaggregation

Cells induce controlled aggregation of proteins when the proteostasis system becomes overloaded (Ho et al. 2019). Upon restoration of proteostasis, Hsp70 is the priming factor for untangling protein aggregates, even though its disaggregation potential is limited and aggregate-bound Hsp70 requires assistance (Diamant et al. 2000; Weibezahn et al. 2004; Ziętkiewicz et al. 2004). Hsp70 coating of aggregates as the initializing step of protein disaggregation is highly conserved across all kingdoms of life (Figure 4D).

Hsp70s from bacteria, plants and fungi recruit Hsp100-type disaggregases to the surface of aggregates that thread protein substrates through their central pore in a rotary movement (Acebrón et al. 2009; Glover and Lindquist 1998; Goloubinoff et al. 1999; Winkler et al. 2012). The translocation mechanism requires close cooperation with Hsp70 to prevent this disaggregase from non-specific threading (Carroni et al. 2014; Glover and Lindquist 1998; Lipińska et al. 2013; Oguchi et al. 2012; Rosenzweig et al. 2013). Hsp70 modifies the surface of the aggregates by exposing disentangled regions of trapped polypeptides for Hsp100 binding (Ziętkiewicz et al. 2006). At the same time, Hsp70 coating of the aggregate surface restricts the access of other protein quality control machineries (Haslberger et al. 2008). Hsp100 selectively interacts with ADP-Hsp70, as association with the ATP-bound form is prevented due to steric clashes (Hayashi et al. 2017). This ensures that Hsp100 only associates with substrate-bound Hsp70. Further, simultaneous binding of two or more Hsp70 partners is required for Hsp100 activation, which is fulfilled at aggregate surfaces (Oguchi et al. 2012). Hsp100 recognizes exposed hydrophobic stretches of the aggregated proteins and actively displaces them from Hsp70 with pulling forces (Rosenzweig et al. 2013). Following substrate transfer, Hsp70 dissociates and restricts high disaggregase activity to initial strokes, thus some protein substrates are only partially threaded (Deville et al. 2017; Duran et al. 2017; Haslberger et al. 2008). This partial threading might enable Hsp100 to sense conformational states of aggregated substrates and stop threading when encountering tightly folded domains. Thus, Hsp100 might increase folding efficiency by avoiding complete exposure of the hydrophobic core of protein substrates. In this respect, Hsp100 disaggregases lighten the workload of Hsp70s by taking over entangled protein substrates and returning unfolded or partially folded intermediates for further processing (Figure 4D).

In contrast to the Hsp70-Hsp100 bi-chaperone machinery, metazoan Hsp70 standalone disaggregation activity is activated by a specific cast of JDPs and Hsp110 co-chaperones (Figure 4D) (Rosenzweig et al. 2019). Interestingly, the disaggregation capability correlates to the level of Hsp70 expression (Michels et al. 1997; Nollen et al. 1999). The force needed for disentangling aggregates and releasing trapped protein substrates is derived from entropic pulling by Hsp70 and JDP binding and oligomerizing on the aggregate surface (Sousa et al. 2016; Sousa and Lafer 2019). JDPs from classes A and B recognize differently-sized protein aggregates, thus enabling Hsp70 targeting to aggregates during different stages of the disaggregation process. These JDP classes further form transient complexes necessary for cooperative disaggregation (Nillegoda et al. 2015). Hsp110 is crucial for protein disaggregation in nematodes and mammals (Gao et al. 2015; Rampelt et al. 2012; Shorter 2011). As sub-stochiometric ratios of Hsp110–Hsp70 are optimal for disaggregation and increasing Hsp110 levels inhibits Hsp70-dependent refolding of aggregated proteins, a solely catalytic functions of Hsp110 as NEF is supported (Rampelt et al. 2012). Hsp110 might help Hsp70 tethering to aggregates, possibly explaining why other NEFs cannot directly substitute Hsp110 in efficient protein disaggregation (Garcia et al. 2017; Kaimal et al. 2017; Rampelt et al. 2012). Alternatively, the transient docking of Hsp110–Hsp70-substrate complexes has been proposed to provide an entropic pulling force that facilitates disentangling of the substrate (Gao et al. 2015; Shorter 2011). Even though Hsp70 alone is unable to conduct productive protein

disaggregation, its action is still at the starting point and thus essential for downstream processes.

Conclusion

Maintaining protein homeostasis requires an orchestrated cellular network of molecular chaperones. Thus, the activities and functions of the Hsp70 subsystem and associated factors are essential for maintaining cellular physiology and viability. In turn, their malfunction has detrimental consequences. Several human pathologies arise due to dysregulated proteostasis and the activity of the Hsp70 superfamily is linked to several human diseases, involving cancer and neurodegeneration (Albakova et al. 2020; Chiesa and Sallese 2019; Kityk and Mayer 2018; Liu et al. 2020; Zhao et al. 2017). In this line, the handover of protein substrates from Hsp70 to downstream machineries sets the course towards refolding or degradation, and is vital to prevent unproductive folding attempts upon repeated chaperone cycles. Even though the molecular principles of the allosteric cycle of Hsp70 have been elucidated in great detail, several *in situ* facets are still far from being understood. Even though structures of Hsp70s both in their apo- and in a complexed form have contributed to commonly-accepted models, they represent endpoint states that might diverge widely from the native cellular situation. In contrast to the three generally accepted conformations of Hsp70, the difference between high- and low-affinity state might not be the actual conformation but rather the frequency of transitions between them (Kityk et al. 2012; Mayer et al. 2000; Yang et al. 2017). Recent studies involving advanced NMR spectroscopy to study folding bias of Hsp70 impressively emphasized the need to integrate molecular dynamics into the pre-existing canonical models (Rosenzweig et al. 2017; Sekhar et al. 2015, 2017, 2018). The ability to study single states of substrate molecules out of a whole pool of different conformations will definitely promote our understanding on the cellular Hsp70-centered proteostasis system. Recent work evidenced that Hsp70 activity *in situ* differed vastly from established *in vitro* systems (Guin et al. 2019). Thus, the ability to study chaperone activity *in situ* might change the perspective on allegedly well-understood mechanisms. Further, even though Hsp70 function in triaging protein fate has been connected to downstream machineries and several apparently independent pathways have been elucidated (Liu et al. 2020; Mayer and Gierasch 2019; Rosenzweig et al. 2019), investigating a potential cooperation between them and unravelling hitherto unknown factors governing the inter-regulation will be an exciting task for future research.

Peptide and protein substrates are known to stimulate the activity of Hsp70 differently and only the latter trigger the cooperation with distinct co-chaperones (Laufen et al. 1999). Nevertheless, even though Hsp70s mainly deal with protein substrates in their native cellular environment, model peptides are indeed useful to investigate the principal molecular mechanisms and are almost irreplaceable for structure determination using X-ray crystallography. Model proteins that are unable to refold on their own, but emit quantifiable signals in their native state, e.g. firefly luciferase, have been widely employed to investigate folding in general as well as conditions favouring protein refolding over degradation. Further, these model substrates are characterized in greatest detail and the reaction environment around Hsp70 is thus controlled and absolutely predictable. These parameters indeed help to uncover and understand sophisticated cellular processes. Even though these experiments impressively contributed to our general understanding on Hsp70-substrate interplay in controlled *in vitro* conditions, studying these processes with native proteins *in situ* will substantially advance our understanding on Hsp70 substrate selection and release mechanisms.

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