Published in final edited form as:

Biochem J. ; 476(11): 1653–1677. doi:10.1042/BCJ20170380.

Hsp70 molecular chaperones: multifunctional allosteric holding and unfolding machines

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Abstract

The Hsp70 family of chaperones works with its co-chaperones, the nucleotide exchange factors and J-domain proteins, to facilitate a multitude of cellular functions. Central players in protein homeostasis, these jacks-of-many-trades are utilized in a variety of ways because of their ability to bind with selective promiscuity to regions of their client proteins that are exposed when the client is unfolded, either fully or partially, or visits a conformational state that exposes the binding region in a regulated manner. The key to Hsp70 functions is that their substrate binding is transient and allosterically cycles in a nucleotide-dependent fashion between high-and low-affinity states. In the past few years, structural insights into the molecular mechanism of this allosterically regulated binding have emerged and provided deep insight into the deceptively simple Hsp70 molecular machine that is so widely harnessed by nature for diverse cellular functions. In this review, these structural insights are discussed to give a picture of the current understanding of how Hsp70 chaperones work.

Introduction

Molecular chaperones play key roles in maintaining cellular protein health, facilitating protein targeting, and ensuring high-fidelity protein biosynthesis. Central players among molecular chaperones are the 70-kDa heat-shock proteins, or Hsp70s, which occur in virtually all organisms and all cellular locations. While these chaperones are widespread and perform highly diverse functions, they share a common fundamental mechanism of action. Intensive study over the past decade has led to a much deeper understanding of the structural basis for the molecular mechanism of Hsp70. In turn, this understanding is elucidating the functional roles of Hsp70s and the nature of their partnerships with co-chaperones in the cell. Failures in protein homeostasis are now implicated in many diseases, and the resulting advances in understanding Hsp70s offer promise that they can be therapeutic targets to treat protein homeostasis pathologies. Several reviews on Hsp70s have been published in the last 5 years [1–8], and we point the interested reader to these. Here, we focus on the great strides

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This multipronged research activity on Hsp70s is exciting, and we look forward to a very fruitful future for research on Hsp70s.

Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

that have been made recently in the structure–function of Hsp70s and their interactions with co-chaperones and substrates.

Intramolecular allostery modulates Hsp70 substrate-binding affinities

Hsp70s are made up of an N-terminal 45-kDa actin-like nucleotide-binding domain (NBD) and a C-terminal 30-kDa substrate-binding domain (SBD). The SBD is composed of a β -sandwich subdomain (β SBD) with a canonical substrate-binding groove (which we will also refer to as the ‘canonical binding site’ for substrates), an α -helical lid subdomain (α -lid), and a disordered C-terminal region (Figure 1A). The NBD and SBD are connected by a conserved, largely hydrophobic interdomain linker. The functions of Hsp70s depend on an intramolecular allosteric mechanism that involves the binding and release of protein client modulated by ATP binding and hydrolysis. Much of the work elucidating the features of the allosteric mechanism of Hsp70s is based on a detailed study of the *Escherichia coli* Hsp70 DnaK. While recent work reveals evolutionary ‘tuning’ of allosteric properties of different Hsp70s [9], their overall mechanism is largely conserved, and so our general description is based on DnaK.

Hsp70s display high substrate-binding affinity in the ADP-bound state. In the ADP-bound state of DnaK, the NBD and SBD are largely independent of each other (Figure 1B) with the interdomain linker relatively exposed and dynamic as shown by a variety of observations ranging from high proteolytic susceptibility of the linker [10,11] to NMR analysis [12,13]. The ADP-bound state is referred to as ‘domain undocked’. In this state, the SBD adopts a closed conformation with the α -lid packed against the β SBD to form a stable β SBD- α -lid interface, resulting in a low substrate on/off rate and high binding affinity. In this state, the ADP-bound NBD has a subdomain arrangement that does not favor interaction with the hydrophobic segment of the interdomain linker and is incompatible with ATP binding/hydrolysis. Upon ATP binding, the chaperone undergoes massive domain rearrangement according to many biochemical observations [13–16] and more recently to solved X-ray structures [17,18] (Figure 1C). The α -lid detaches from the β SBD, and both SBD subdomains become docked onto the NBD to form a new NBD–SBD interface. As a result, the SBD adopts an open conformation with the high substrate on/off rates and low affinity. By virtue of ATP hydrolysis and nucleotide exchange, the Hsp70 cycles between these ADP- and ATP-bound states, visiting the allosterically active state in between, which leads to the cycle of substrate binding and release that is harnessed for all of the functions of Hsp70s.

The NBD consists of four subdomains, IA, IB, IIA, and IIB, which are located on two lobes, I and II (Figure 1D). Nucleotide (ADP or ATP) binds at the cleft formed between subdomains IB and IIB, and all four subdomains are involved in nucleotide coordination. Several studies have focused on allosteric conformational changes within the NBD [19,20]. NMR chemical shift perturbation studies of the NBD of DnaK in different nucleotide-bound states revealed rotation of subdomain IIB upon nucleotide dissociation [21]. Binding of ATP causes re-orientations of the subdomains and long-range perturbation along with the NBD subdomain interfaces, resulting in the binding of the interdomain linker to lobe IIA [21]. An NMR residual dipolar coupling study of Hsc70 NBD showed that subdomains IA and IIA could move with respect to each other by a 10° relative shearing motion, which is expected

to modulate their interaction with other domains and cofactors [20]. Additionally, a combined single-molecule force spectroscopy and theoretical simulation study suggests that the C-terminal helix of the NBD acts as molecular ‘glue’ mediating the coupling between lobes I and II [22]. Taken together, these results show that the subdomain re-orientations triggered by nucleotide binding play essential roles in NBD allostery.

In the high substrate affinity conformation of the SBD (when it is studied as an isolated domain or when the chaperone is in the undocked, ADP-bound state, Figure 1E), two antiparallel β -sheets and four upwards protruding loops ($L_{1,2}$, $L_{3,4}$, $L_{4,5}$, and $L_{5,6}$) make up the β SBD; the upper β -sheet consists of strands $\beta 1$, $\beta 2$, $\beta 4$, and $\beta 5$, and the lower one consists of strands $\beta 3$, $\beta 6$, $\beta 7$, and $\beta 8$. The substrate-binding groove is formed by $\beta 1$, $\beta 2$, $L_{1,2}$, and $L_{3,4}$. When ATP binds to the NBD, $\beta 8$ dissociates from $\beta 7$ in the lower sheet and hydrogen bonds with $\beta 5$ of the upper sheet in the β SBD [17,18,23]. The β SBD undergoes a seesaw-like conformational change from a high substrate-binding affinity conformation to one with lower substrate affinity [24]. NMR chemical shift perturbation analysis revealed that the $\beta 5/\beta 7/\beta 8$ hydrophobic cluster is a central hub that modulates the allosteric coupling within the SBD [24]. The α -lid subdomain is composed of helices α -A, α -B, and an α -bundle, which consists of three α -helices, α -C, α -D, and α -E. Several studies show that the α -lid is highly dynamic and could adopt heterogeneous conformations [14,25,26]. The α -lid covers the substrate-binding groove in the β SBD with the signature kink between α A and α B in the isolated SBD and undocked SBD structures. In the recent structures of the ATP-bound docked state of DnaK [17,18], the α -lid is detached from the β SBD, and α A and α B fuse into one long helix, which interacts with the NBD. The conformational properties of the α -lid can also be modulated by the nature of substrates. While the α -lid remains in a ‘closed’ conformation tightly associated with the β SBD when a peptide substrate is bound, it is quite dynamic [26] and it can also detach, thus allowing the binding of folded or large substrates [27–29]. (See fuller discussion in the section below on substrate binding.)

Allosteric signal transmission between the Hsp70 NBD and SBD triggered by nucleotide or substrate binding has been extensively studied in recent years [26,30–34]. Allostery depends on an energetic balance among the interfaces formed between the α -lid and the β SBD, the α -lid and the NBD, the NBD and the β SBD, and the linker contacts with either the β SBD or the NBD. The allostery of Hsp70s thus results from an energetic tug-of-war between two orthogonal interfaces, the β SBD- α -lid interface formed in the undocked state, and the NBD-SBD interface formed in the docked state [34] (Figure 2A,B). Complementary to these observations, additional residues were identified to be part of an allosteric pathway between the NBD and SBD, based on mutagenesis results and details of the ATP-bound DnaK structure [35]. These authors proposed that binding of the interdomain linker to the NBD, and the rotation of the two lobes triggered by ATP-binding results in the interaction of two NBD residues, I168 and D328 with the β SBD; these residues act as triggers by their interaction with D481 and K414 in the SBD, which snaps the β SBD into a low substrate-binding affinity conformation [35].

While the energetic tug-of-war of interdomain interfaces determines whether the docked or undocked state of an Hsp70 is favored, communication between the domains is mediated by the two structural components that act as ‘allosteric couplers’: the interdomain linker and the

α -lid. Both are able to form favorable interactions with both the NBD and the SBD, and importantly, their tendency to interact with one or the other domain is modulated by the conformational state of the given domain. The interdomain linker contains a pre-dominantly hydrophilic N-terminal region, followed by a highly conserved hydrophobic sequence (³⁸⁹VLLL³⁹² in DnaK). In the undocked state, NMR residual dipolar coupling and paramagnetic relaxation enhancement suggested that the NBD and SBD experience restricted rotation around a 35° cone with respect to each other [12]. While the linker in the ADP-bound state is relatively flexible, molecular dynamics simulations from our laboratory suggest that it is made up of relatively structured regions with hinges between them, which restricts both the relative orientations and distances between the NBD and SBD [31]. In addition, there is transient interaction between the C-terminal region of the linker and SBD, and the interaction site on SBD is only available when SBD is in high substrate-binding affinity conformation [31]. In the ATP-bound state, the hydrophobic sequence of the linker binds to subdomain IIA of NBD to form an antiparallel β -strand structure [23,36,37]. Binding of the linker to the NBD is sufficient to stimulate ATP hydrolysis activity [13,38]. Upon the association of the interdomain linker with the NBD triggered by ATP binding, the allosteric signal is further transmitted towards SBD by favoring the formation of NBD contacts with lynchpin sites on the β SBD, disengaging SBD strand β 8 from strand β 7, and disrupting the hydrophobic arch over the substrate-binding cleft [24]. Thus, the interdomain linker can interact with either domain, depending on the nucleotide-binding state of the NBD, and contribute to the allosteric energy landscape by favoring docked or undocked states: It is a true allosteric coupler.

The other allosteric coupler in Hsp70s is the α -lid. The α -lid can form strong and specific interactions with the β SBD loops when the SBD is in its high substrate affinity state (and more favorably, when a linear substrate is bound), or with the NBD when it is in the ATP-bound conformation. As in the case of the linker, the preferences of this structural entity for interaction with either domain are modulated by the conformational state of the domain, and the energy of the conformational state of the linker is engaged with is influenced by the interactions the α -lid participates in. Specifically, when the α -lid is bound to the β SBD, it forms three salt bridges (D526-R445, D540-R467, and K548-D431 in DnaK [39]), and when the α -lid coalesces onto the NBD, a large packing interface (1170 Å²) forms [18].

An ‘allosterically active’ state of DnaK, with both ATP and peptide substrate bound, has been observed by using an ATP hydrolysis defective mutant, DnaKT199A, so that ATP remains bound, and adding a peptide substrate [26,34]. This state must exist transiently during the allosteric cycle of wild-type DnaK because both ATP and substrate are bound when the ATPase activity is elevated, and the substrate off-rate is enhanced (Figure 2C). NMR studies showed that in this allosterically active state, the SBD and NBD are largely dissociated from one another, the interdomain linker remains bound to NBD thereby stimulating the ATP hydrolysis activity, and the α -lid/ β SBD interface is at least partially formed [34].

While they share a common mechanism, Hsp70s display highly tunable allosteric landscapes [9,34,40]. Since allostery results from the energetic tug-of-war between the β SBD- α -lid interface and the NBD-SBD interface, evolutionary sequence changes at these two key

interfaces shift the equilibrium between docked and undocked conformations thus modulating Hsp70 functions [34]. Two human cytoplasmic Hsp70s, HspA1, and Hsc70, which are 46% identical in sequence to DnaK, favor domain docking in all nucleotide-bound states more significantly than DnaK does (Figure 3). While DnaK adopts a docked conformation only when ATP-bound, significant domain docking is observed for both HspA1 and Hsc70 in their ADP-bound states [9]. As a result, HspA1 and Hsc70 exhibit lower substrate-binding affinities and reduced substrate stimulation of their ATP hydrolysis rates compared with DnaK. In contrast, the endoplasmic reticulum (ER) Hsp70, binding-immunoglobulin protein (BiP), shows less favorable domain docking than DnaK: its ATP-bound state is heterogeneous with only half of the molecules adopting a domain-docked conformation [40]. In addition to evolutionary tuning, the allosteric landscapes of Hsp70s are also modulated by post-translational modifications, and this tuning of their allosteric behaviors is directly related to their physiological functions [40–42]. For instance, AMPylation of T518 biases BiP towards the domain-docked state, subsequently inactivating BiP and limiting its interaction with substrates [40,42]. Phosphorylation of HspA1 at T66 promotes domain undocking and increases substrate-binding affinity in the ATP-bound state [41]. A physiological result of this modification is that the interactions of HspA1 with components of mitotic spindles are stabilized and the localization to the spindle is favored.

Co-chaperones modulate the allosteric functions of Hsp70s

Hsp70s do not work alone. The allosteric functions of Hsp70s are under the influence of two partner classes of co-chaperones: J-domain proteins (JDPs) and nucleotide exchange factors (NEFs). While JDPs stimulate ATPase activity and drive multifunctionality of Hsp70s by targeting them to specific substrates and cellular locations, NEFs ensure the continuity of Hsp70s' ATPase cycle by promoting ADP to ATP exchange and substrate release.

The prokaryotic JDP, DnaJ, facilitates substrate delivery and activates Hsp70 ATPase rate

JDPs form a large and diverse family of proteins consisting of over 50 members in humans. All of them share a ~70 amino-residue characteristic J-domain, which adopts a four-helical bundle structure with a conserved His–Pro–Asp (HPD) motif located between helices II and III that has been shown to be crucial for stimulation of Hsp70 ATPase activity [37,43–45] (Figure 4A). Considerable research over many years and many laboratories have shown that JDPs directly bind Hsp70s [36,45], but the structural details of the interaction remained elusive for a long time due to its transient and dynamic nature. Multiple research groups addressed this question using various methods and, in some instances, produced contradictory models of the complex [46,47]. A recently published crystal structure of *E. coli* DnaK in the ATP-bound state complexed with the J-domain of DnaJ (Figure 4B) and supporting mutagenesis analysis has revealed the mechanism of JDP action and explained previously published biophysical and biochemical observations [36]. In this structure, the J-domain binds at the interface between the NBD and the SBD of DnaK, forming direct contacts with both domains. The conserved HPD motif protrudes towards the interdomain linker, which plays such a crucial role in the allosteric cycle of DnaK (*vide supra*) [31,34,38], and also directly interacts with the β SBD. The authors propose that the way the J-domain interacts with the Hsp70 accounts for its favoring of substrate-induced undocking

of the SBD from the NBD and the transmission of conformational changes in the substrate-bound β SBD to the NBD through the linker. These two complementary signals ensure the optimal positioning of the NBD lobes for ATP hydrolysis. Notably, residues of DnaK involved in the interaction with DnaJ are highly conserved, suggesting that the mechanism of JDP binding to Hsp70s is conserved from bacteria to humans.

In addition to stimulating the DnaK ATP hydrolysis rates, DnaJ is proposed to help recruit substrates to its partner Hsp70. Like DnaK, DnaJ binds substrates through short sequences enriched in hydrophobic residues, but in addition, its substrates were observed to be enriched in aromatic and polar residues [48].

Eukaryotic JDPs mediate multiple functions

JDPs became highly complex during evolution, enabling them to perform highly complex functions. Because of their complex evolutionary history and functional diversification, the classification and nomenclature of JDPs are a matter of some debate (a comprehensive recapitulation of the classification and functions of JDPs can be found in [49,50]). Traditionally, JDPs are subdivided into three major classes (A, B, C) based on the ancestral DnaJ of *E. coli* (Figure 4C). Class A and class B both have the N-terminal J-domain adjacent to the Gly–Phe-rich region, followed by two topologically similar twisted β -sandwich domains and a C-terminal dimerization domain. The only difference between class A and class B JDPs is that the class A members have a Cys-rich Zn²⁺-finger inserted into the first of the two domains; this domain is important for client binding and delivery [51,52]. In contrast, in class C JDPs, the functional J-domain can be present anywhere in the sequence and not specifically at the N-terminus. Class C JDPs include a wide variety of other functional domains along with the J-domain [50,53] and often perform very specialized functions. Most organisms express more JDPs than Hsp70s [49,50,54–56], which suggests that many Hsp70s will have multiple JDPs partners.

Like DnaJ, the eukaryotic JDPs are implicated in substrate binding as well as activation of their partner Hsp70's ATPase activity. Most JDPs, such as the JDPs of the ER lumen (ERdj4, ERdj5, and ERdj6) [57,58], display a broad substrate specificity ('broad binders'). Other JDPs have exquisite specificity for binding ('specific binders') like the specialized J-protein auxilin, which helps in the removal of clathrin coats from endocytic vesicles [59,60]. Interestingly, some JDPs fall at the interface of the 'specific binders' and 'broad binders'. For example, the metazoan JDPs, DnaJB6 and DnaJB8, bind Gln-rich sequences (polyQ, polyglutamine) [61]. In Huntington's disease, the binding of JDPs to the polyQ stretches of huntingtin protein has been reported to inhibit aggregation [62].

Among the class A JDPs, sequence divergence is restricted to the SBD at the C-terminus, which has consequently developed the ability to bind to different client proteins and contribute to the specificity of J-protein function [63]. The most abundant cytosolic class A JDP in *Saccharomyces cerevisiae* is Ydj1, which is involved in protein folding, trafficking and degradation [64–66]. The functional relevance of Ydj1 is supported by the observation that deletion of Ydj1 or introduction of a mutation within its conserved HPD motif (H34Q) results in a null phenotype (cell death) [64–67]. One of the most important class B JDPs in yeast is Sis1, which plays a critical role in the remodeling of yeast prion, aggregates working

in partnership with the Hsp70 Ssa1 and the AAA+ chaperone Hsp104 [68]. The remodeling of prion aggregates is dependent on the Sis1 J-domain. Importantly, studies have also shown that the mammalian JDPs, DnaJB6 and DnaJB8, are crucial for the remodeling of polyQ aggregates, again working with Hsp70 partners [69]. As in the yeast system, mutating the conserved HPD motif (H31Q) modulates the polyQ aggregation *in vivo* [61,70]. Class C JDPs are believed to interact with one or a small subset of Hsp70 substrates and perform more than one specialized function. The classic example is the ribosome-associated JDP of *S. cerevisiae*, zotin (Zuo1). Zuo1 is a positively charged, 433 residue-long protein that contains a classic J-domain. The different domains of Zuo1 are responsible for its association with the ribosome's 60S and 30S subunits, consistent with its proposed role of facilitating the folding of the nascent polypeptide [71]; this function is dependent on Hsp70 and relies on the Zuo1 J-domain. Zuo1 also plays an important role in imparting pleiotropic drug resistance to yeast cells in the ribosome-dissociated state. The C-terminal 69-residue domain of Zuo1 (Zuo1₃₆₅₋₄₃₃) is sufficient for inducing pleiotropic drug resistance in yeast cells. However, this fragment is dispensable for the ribosome-associated chaperoning function of Zuo1 [72–74].

It has become increasingly evident that failure to maintain cellular proteostasis by the Hsp70 system results in many diseases, and in some cases, the defect resides in the JDP co-chaperone. The specialized functions of JDPs, in contrast with the more diverse roles of the Hsp70 family members, account for the observation that over 50% of known 'chaperonopathies' are linked to mutations of the JDPs [49]. Mutations in JDPs have been implicated in neuro-, cardiac-, and motor-neuropathies [75–77]. Also, the regulation of the precise expression pattern of JDPs is important for cellular health, as pointed by the altered expression levels of JDPs in several types of cancers. For example, recent studies on breast cancer samples showed high expression levels of DnaJB1, DnaJB6, and low expression levels of DnaJB4 and DnaJB12 [78]. Also, the dysfunction of JDPs located at neuronal synapsis is associated with human neurodegenerative diseases. For instance, defects in many of the class C JDPs are linked with ataxia, phenylketonuria-related neurodevelopmental deficits, autosomal dominant adult neuronal ceroid lipofuscinosis, and other disorders; mutations in DnaJB2 are associated with distal motor neuropathy as well as spinomuscular atrophy and Parkinsonism, while defects in DnaJB6 are linked with limb-girdle muscular dystrophy type 1D [79–82].

NEFs accelerate ADP release and ATP binding

The exchange of nucleotide from the NBD of all Hsp70s is facilitated by widely divergent NEFs. While these co-chaperones are diverse, both in terms of their specific functions and their structures, the impact of their action on Hsp70s is strikingly similar. There have been several excellent reviews on Hsp70 NEFs published recently [83–85]. Here, we focus on recent advances that have enabled the structures of NEFs to be related to their functional partnership with Hsp70s.

The prokaryotic NEF GrpE as a paradigm—The single bacterial NEF GrpE was originally discovered in *E. coli* as a factor essential for DNA replication of bacteriophage λ and for cell viability at all temperatures [86–88]. Subsequently, GrpE was shown to

accelerate nucleotide exchange from ADP to ATP for the *E. coli* DnaK [89]. A crystal structure of GrpE in complex with the apo-NBD of DnaK was published in 1997 and revealed GrpE to be a homodimer consisting of two long N-terminal α -helices leading into a small four-helix bundle and two small β -domains [90] (Figure 5A). Although only one of the GrpE monomers directly interacts with a single NBD, dimerization of GrpE was shown to be essential for the stable association with DnaK [90,91]. Conformationally, the NBD in the complex with GrpE closely resembles the ADP-bound state with the exception of subdomain IIB's position, which has rotated 14° away from the nucleotide-binding site [90,92] (Figure 5B,C). Based on this structural difference, a mechanism for nucleotide exchange was proposed in which GrpE binding causes rotation of subdomain IIB, facilitated by NBD hinge residues located at the IIA/IIB subdomain interface, which opens the nucleotide-binding site [90,93,94] (Figure 5C). As a result, GrpE binding lowers the energy barrier required for nucleotide exchange and ultimately stimulates the dissociation of ADP from DnaK up to 5000-fold [95]. This mechanism was later determined to be universal among prokaryotic and all classes of eukaryotic NEFs (*vide infra*), as all of them primarily bind to subdomain IIB of the NBD causing its displacement and subsequent nucleotide release.

It is worth mentioning that because the crystal structure of a complex of GrpE and the NBD of DnaK was determined in the absence of ADP and the SBD of the chaperone [90], there is some debate regarding the physiological relevance of the crystal structure. These concerns are amplified, considering that the accuracy of the interface between GrpE and the NBD in the crystal structure has previously been contested [96,97]. Studies have demonstrated that mutations of select NBD residues at the binding interface observed in the crystal structure have no effect on the ability of the NBD to form a complex with GrpE [96]. At the same time, mutations/deletions to several NBD or GrpE residues, not located on the binding interface, prevent the association between the chaperone and its NEF [93,96,97]. Of particular interest is a highly conserved loop in bacterial NBD, which is located at the tip of subdomain IIB and whose deletion was shown to abolish the interaction between GrpE and DnaK [96]. In contrast with the observed 2:1 stoichiometry between GrpE and the NBD of *E. coli* DnaK, a crystal structure of full-length *G. kaustophilus* DnaK complexed with its corresponding GrpE revealed 2:2 stoichiometry [98]. In this structure, the NBD of each DnaK interacts with one of the GrpE monomers in a manner similar to what was previously reported [90] (Figure 5D). However, the interdomain linker of one of DnaK molecules from the quaternary complex is inserted into the substrate-binding pocket of DnaK from the second complex present in the asymmetric unit, which raises doubt about the physiological relevance of this arrangement. Moreover, this 2:2 stoichiometry has yet to be observed for *E. coli* as previous studies have shown 2:1 ratio even in the context of full-length proteins [99].

In addition to its nucleotide exchange activity, GrpE has been proposed to participate in substrate release from Hsp70s. The precise mechanism behind this function is not fully understood; however, it was suggested that an N-terminal disordered region of GrpE may assist in the dissociation of the substrate by either acting as a pseudo-substrate to prevent substrate re-binding or by binding close to the substrate-binding cleft of the Hsp70 thereby changing its conformation [90,99,100]. A model derived from electron microscopic analysis suggests that the α -helical tail of GrpE tips towards the SBD of DnaK, which might modulate substrate dissociation [99]. Finally, GrpE is widely acknowledged for its role as a

thermosensor within the Hsp70 chaperone system, as its conformational changes at physiologically relevant temperatures ranges regulate the chaperone system during heat shock [97,101,102]. The long α -helices of *E. coli* GrpE reversibly unfold with a melting transition midpoint of 48°C [103,104]. These temperature-induced conformational changes prevent the association between GrpE and DnaK, thus blocking the nucleotide exchange activity of the NEF and locking the chaperone in a high substrate affinity state [102]. In thermophilic bacteria, such as *T. thermophilus*, thermal transitions occur at higher temperatures to offset more extreme conditions [105].

Eukaryotic NEFs

In contrast with prokaryotes, eukaryotes have many NEFs, and they are associated with a variety of cellular functions. GrpE homologs in eukaryotes are found in mitochondria and chloroplasts [106–108]. There are multiple NEFs in the cytoplasm of eukaryotic cells, and they are classified into three structurally unrelated classes: BAGs, HspBP1/Fes1, and Hsp110s. Proteins from each class interact with both cytoplasmic Hsp70s, Hsc70, and HspA1, with no apparent preference (reviewed in [109]) and can be associated with specialized cellular processes (vide infra). In addition, two NEFs are localized to the ER where they regulate the functional cycle of the ER-resident Hsp70, BiP. One is an HspBP1 homolog called BiP-associated protein (Bap) in higher eukaryotes and Sil1 in yeast, and the other is an Hsp110-like protein called Grp170 (glucose-regulated protein of 170 kDa).

BAG (BCL-2-associated athanogene) proteins are the most structurally diverse family of NEFs. They contain a variety of domains that confer functional diversity to this family [110], but all share a characteristic BAG domain that was first described for BAG1 and is responsible for interaction with the NBD of Hsp70s [111]. Five out of the six BAG proteins in humans (BAG1–4 and BAG6) contain only one C-terminal BAG domain, while BAG5 has five BAG domains; only the most C-terminal has been shown to interact with Hsp70 [112]. Structurally, BAG domains form a three-helix bundle (Figure 6A) that directly interacts with subdomains IB and IIB of Hsp70s NBD leading to 14° rotation of the latter [111] (Figure 6D). Uniquely, the BAG domain of BAG2, called the ‘brand new BAG’ (BNB) domain, adopts a dimeric structure, and its interaction mode with the NBD is slightly different [113]. In addition to their interaction with the NBD, at least two BAG proteins, BAG1 and BAG3, have been reported to bind the SBD via a region that does not involve the BAG domain [114]. This interaction contributes to substrate release, similarly to the previous suggestion for prokaryotic NEF GrpE [100].

Presumably, an Hsp70 teams up with a given BAG depending on the particular function to be performed. BAG1, which has a ubiquitin-like domain, is involved in the degradation of misfolded proteins [115]. For example, BAG1 was found to promote binding of the leukemia-associated BCR-ABL protein to the proteasome [116] and, more recently, the Hsp70–BAG1 complex was shown to target mutant misfolded heart-specific potassium channels for proteasomal degradation [117]. BAG2, on the other hand, prevents uncontrolled degradation of proteins by inhibiting the E3 ubiquitin ligase CHIP (the carboxy terminus of Hsc70 interacting protein), which associates with Hsp70s and labels misfolded proteins for degradation [118,119]. A chaperone complex consisting of Hsp70, BAG3, and the small

heat-shock protein (sHsp) HspB8 was found to target mutant misfolded superoxide dismutase SOD1 and polyQ-containing huntingtin, associated with familial amyotrophic lateral sclerosis and Huntington disease, respectively, for macroautophagy, a degradation process responsible for removing large protein complexes and portions of cytoplasm [120–122]. Recently, BAG3 was also shown to act as a sensor of proteotoxic stress during proteasome failure [123] and as a scaffolding platform bringing Hsp70s and sHsps together [124]. BAG4 and BAG5 inhibit apoptosis, albeit through different mechanisms: BAG4 blocks downstream signaling from receptors of the tumor necrosis factor family [125], while BAG5 migrates to the ER during ER stress where it interacts with BiP and prevents apoptosis [126]. Finally, BAG6 controls targeting of newly synthesized proteins to the ER and is involved in ER-associated degradation (ERAD) [127,128]. How individual BAGs are selected by Hsp70s is not entirely clear, but it was recently shown that the affinities of BAG proteins to Hsp70s are not all the same [129]. The results in this study suggest that a selection process might depend on tightly balanced concentrations of BAG proteins in a way that higher concentration of the particular BAG protein at certain cellular conditions compensates for differences in their affinities for Hsp70s.

The cytosolic NEF HspBP1 (Fes1 in yeast) and its ER-resident homologue, Bap (Sil1 in yeast), represent the smallest family of eukaryotic NEFs. Structurally, both of these proteins are characterized by a conserved domain consisting of four Armadillo-like repeats [130], each containing three α -helices arranged in a loose triangle, flanked by longer α -helices at both ends (Figure 6B). Overall, the conserved domain adopts an elongated curved shape characterized by a right-handed superhelical twist with a concave surface that makes direct contacts with the NBD of an Hsp70 [131,132]. Interestingly, two different modes of interaction were reported for HspBP1 and Sil1 complexes with HspA1A and BiP, respectively. According to the crystal structure of HspBP1 with a fragment of HspA1A containing only lobe II of the NBD, the Armadillo-like domain wraps around subdomain IIB in a way that would cause its N-terminal capping α -helices to clash with NBD subdomain IB, but the steric clash does not happen since the interaction with HspBP1 causes partial unfolding of lobe I [131]. The Armadillo-like domain of Sil1 also wraps around subdomain IIB of BiP; however, in contrast with HspBP1, its N-terminal capping α -helices make only a few direct contacts with subdomain IB [132]. Moreover, Sil1 binding does not cause local unfolding of lobe I instead pushing lobe I and lobe II away from the nucleotide-binding pocket by $\sim 3.7^\circ$ and 13.5° , respectively, and thus disturbing ADP contacts with the NBD (Figure 6E). In addition to their NEF activity, this family of NEFs has been recently found to promote substrate release from Hsp70s. Both HspBP1 and Bap have flexible N-terminal regions that have been shown to act as a pseudo-substrate by binding to the substrate-binding pocket of the SBD and in so doing, facilitating substrate release [133,134]. The fact that prokaryotic GrpE and two of three families of eukaryotic NEFs have been implicated in substrate release suggests that this function may be a conserved characteristic of several families of NEFs in addition to their nucleotide exchange activity.

The Hsp110s are the most abundant NEFs in eukaryotes. Hsp110s and Hsp70 share a common ancestor and are structurally homologous [135]. Like Hsp70s, Hsp110s consist of an N-terminal NBD and C-terminal SBD connected by a linker. While the NBD of Hsp110s closely resembles the NBD from Hsp70s, the SBD contains an insertion of unknown

function between strands $\beta 7$ and $\beta 8$, and an extended C-terminus, both shown to be disordered [136] (Figure 6C). In the Hsp70–Hsp110 complex, NBDs of both proteins tightly interact with each other in a way that subdomain IIB of Hsp70 is embraced by NBD subdomains IB and IIB and additionally by the SBD α -helical lid of the Hsp110 (Figure 6F). These extensive contacts cause a 27° outward rotation of the Hsp70 subdomain IIB. Importantly, the β SBDs of both proteins are located outside of the interaction interface with their substrate-binding sites, facing away from one another, which leaves them available for substrate binding [137,138]. Interestingly, available evidence suggests that despite the similarity of Hsp110 domain organization to that of Hsp70s, Hsp110s do not cycle through docked and undocked states to perform their functions like Hsp70s do. First, although ATP binding to the NBD of Hsp110s is required for their interaction with Hsp70 [139], ATP hydrolysis is not needed for their activity [140,141]. Second, the hydrophobic linker in Hsp70s is replaced by a positively charged linker in Hsp110s. This difference in sequences might prevent Hsp110's linker from sampling a set of conformations crucial for interdomain allosteric communication in Hsp70s [31]. Finally, peptide binding by the SBD of Hsp110s is characterized by extremely fast on and off rates, indicating that the SBD does not adopt a closed state and interacts with substrates only transiently [142].

Besides their NEF activity, Hsp110s have also been reported to act as 'holdases' by binding proteins and preventing their aggregation [143]. Indeed, as seen in the crystal structure, the substrate-binding pocket present in Hsp70s is preserved in Hsp110s [136] although with specificity for peptides enriched in aromatic residues [142]. How this holdase activity is achieved, however, is unknown since there is no evidence that Hsp110s can adopt a closed high-affinity state.

Most strikingly, Hsp110s are unique among other eukaryotic NEFs in that they facilitate protein disaggregation by Hsp70s in metazoans, which lack Hsp104, the Hsp70 disaggregation partner in yeast [140,144]. In fact, it was recently found that even when Hsp104 is present it requires an Hsp70–Hsp110 complex for both its recruitment to protein aggregates and its disaggregation activity [145]. In metazoans, the precise mechanism by which Hsp110s assist Hsp70s in disaggregation remains unknown. Since Hsp110s contain the SBD domain known to bind substrates, it is plausible that Hsp110s interact with client proteins along with Hsp70s to assist in the process. Conversely, a recent study showed that only their NEF activity is necessary for disaggregation [146], in which case the question arises why other classes of NEFs are unable to assist Hsp70s in protein disaggregation [147]. In addition, one study showed that Hsp110 could use ATP to unfold proteins independently from Hsp70s, but these results have not been confirmed or reproduced to date [148].

The guts of the Hsp70 allosteric machine: Hsp70 substrate interactions and how they relate to their cellular functions

What characteristics define an Hsp70 substrate?

As early as in 1985, it was proposed that Hsp70s bind to hydrophobic surfaces exposed in non-native proteins: Pelham et al. [149] observed that upon heat-shock, ribosomal proteins aggregate through hydrophobic interactions, and Hsp70, by binding tightly to the resulting

exposed hydrophobic surfaces, helped to disrupt the aggregates and release their substrates in a strictly ATP hydrolysis-dependent fashion. The hydrophobic surfaces proposed to be Hsp70 substrate interaction sites were later defined more precisely to be short sequences that we now know are typical sequences that bind to the SBD pocket. Peptide arrays and phage display studies identified stretches of hydrophobic residues and positive charges as the main sequence features of Hsp70 substrates [150–154]. Even though there was not a specific consensus sequence for binding identified, these sequence features allowed the development of algorithms that can predict putative binding sites within a protein's primary structure [153,154]. When the relevant algorithms are applied to the *E. coli* proteome, the frequency of appearance of the predicted DnaK-binding sites is remarkably high (one site every 40 residues, on average).

For the chaperone to bind, however, the sites on any given protein must be exposed, and accessibility constitutes the second major criterion for Hsp70 substrate selection. There are general situations where both criteria are met, and understanding these situations offers insight into the diverse functions of Hsp70s: (i) Polypeptide chains emerging from the ribosome during biosynthesis or emerging from translocation sites in membranes present potential binding sites until they are sequestered by structure formation during folding. (ii) Proteins that are partially unfolded or transiently visit the unfolded state, most often as a result of temperature stress, mutation, or lack of an obligatory partner in a complex. In some cases, folded proteins may display fluctuating accessible binding sites in their ensemble of populated states. (iii) Proteins that are largely folded but contain unfolded regions, often on termini or loops; in this class, the binding motifs generally serve as specific recognition sites (*vide infra* for examples of different types of Hsp70 protein substrates). Given these criteria, how many Hsp70 substrates are there in the cell? This point was extensively studied in the literature using a wide array of genetic and proteomics approaches. The results allowed not only the identification of multiple cellular substrates of the chaperone but also the characterization of the cellular effect of Hsp70 depletion, mutation, or overexpression in *E. coli* [155–157]. More recently, two studies sought to identify all physiological substrates bound to DnaK at a particular time. In one case, substrates were identified by pulling down the complexes with DnaK from *E. coli* and subjecting them to mass spectrometry [158]. In another case, chaperone effects on the folding of hundreds of cytosolic *E. coli* proteins were assayed using a reconstituted chaperone-free translation system. Addition of the DnaK system significantly augmented the amount of soluble protein produced for many of those tested [159]. These two reports show that numerous proteins with diverse functions, including both newly synthesized and pre-existing proteins, are substrates of DnaK.

The substrate-binding site of Hsp70s is promiscuously selective

The molecular details of the interaction between the canonical binding site of Hsp70s and substrates have been extensively characterized *in vitro* using short peptides as model substrates, which avoids the experimental challenges of working with large, unfolded proteins. This strategy has been exploited to obtain atomic-resolution structures of peptides bound to the canonical binding site of Hsp70. The first crystal structure [39] of the SBD of *E. coli* DnaK showed that the model peptide substrate NR (NRLLLTG) sits in a cleft in the β SBD, with the helical subdomain acting as a lid and covering the substrate (Figure 7).

Subsequently, multiple structural studies of DnaK/substrate peptide complexes by NMR and crystallography have established the generality of this mode of binding, regardless of the sequence of the bound peptides (see [2] for a summary). Moreover, structures of model substrates bound to Hsp70s from other organisms have revealed the same basic arrangement, pointing to the high conservation of this binding mode.

In Hsp70s, the binding groove is made up of five pockets in the β SBD, where the central pocket (termed the '0th position') has the highest stringency, and thus invokes the highest selectivity [15] (Figure 7). This position optimally accommodates Leu, and Ile, and less favorably Val and Phe. The other pockets in the groove can accommodate various substrate residues, although there is a bias against negatively charged residues, and there are slight preferences at each pocket. Taken together, the properties of the binding site lead to intriguing *selective promiscuity*. This feature provides Hsp70s with the ability to bind to various protein clients in the cell and perform a wide spectrum of functions.

The backbone of the bound substrate takes up an extended conformation with a gentle twist and extensive hydrogen bonding. While these features are remarkably common among all structures solved to date, there are some intriguing variabilities: For example, the NR model peptide can bind in a different register, hence shifting which pocket each residue occupies, some antimicrobial peptides can bind to the β SBD despite their lack of positive charges, and a few peptides have been observed to bind in the opposite orientation to the lion's share of examples [160,161].

A few studies have proposed that sites on the Hsp70 away from the canonical substrate-binding groove may be implicated in substrate binding, although the supporting evidence has usually been indirect, for example, by testing the impact of mutation or truncation of Hsp70s on its activities [162–166].

Protein substrates utilize the same binding mode as peptide models

The study of the atomic details of the binding interactions between Hsp70s and peptide substrates provided a necessary first step in understanding how Hsp70s bind to their cellular targets, which are generally full-length substrates. Recent advances in many experimental techniques, such as single-molecule fluorescence and high-resolution NMR, have allowed our structural investigation of substrate binding to Hsp70s to transition from peptides to larger proteins [2,5]. All cases studied to date show that Hsp70 utilizes the canonical binding site in the SBD to bind to accessible Hsp70 binding motifs in their client proteins, regardless of the overall conformation of the particular substrate [29,167–169].

A completely unfolded protein should expose one or more Hsp70 binding sequences that can be recognized and bound by the chaperone in the same manner as a peptide substrate. Indeed, several studies have identified and characterized by different methods Hsp70 chaperones bound to a variety of unfolded substrates. In some cases, the protein was engineered to be an unfolded model Hsp70 substrate; for example, a fragment of the staphylococcal nuclease that unfolds at 37°C [170], a slow-folding mutant of RNase H [171], truncated fragments of apomyoglobin [172,173], or rhodanese refolded in the presence of Hsp70 [174]. In other experiments, the whole conformational ensemble of a

substrate under conditions where unfolded states were in equilibrium with natively folded species was probed for Hsp70 binding: namely, the N-terminal domain of the *D. melanogaster* adaptor protein drk (drkN SH3) [175] or the small 53-residue human telomere repeat binding factor 1 (hTRF1) [176–178]. In all of these cases, the substrates were observed to be globally unfolded in the Hsp70-bound state [168,170,173–175,179–181] and to lack long-range interactions that might have been established in the unfolded state of the substrate in the absence of the chaperone [178]. Detailed analysis shows that the binding of the chaperone does alter the conformational ensemble of some substrates, like loss of some residual structure and long-range interactions [172,178], expansion of the unfolded state [174], or formation of the residual secondary structure away from the chaperone-binding site on the substrate [168,180]. Not surprisingly given the presence of multiple binding sites in several of the unfolded protein substrates, evidence has been presented that more than one chaperone-binding sites in the substrate can be simultaneously bound to Hsp70s [174,176,177]. The weak, transient, and cyclical nature of chaperone binding combined with the possibility of multiple binding sites generates a highly heterogeneous complex ensemble in these cases of fully or largely unfolded substrates in complex with Hsp70s.

Hsp70s can also bind to exposed chaperone-binding motifs on partially folded or near-native substrates [2,5] and in some cases, the substrate remains partially folded while bound [28,29,167]. There are fewer in-depth structural studies of these interactions, but it is clear that folded proteins can expose chaperone-binding sequences as they sample multiple conformations, in exposed unstructured loops or termini, and in sparsely and transiently populated unstructured states. These types of binding interaction have been described, for example, for the native DnaK substrate, σ^{32} [167], and the eukaryotic Hsp70 substrate, the glucocorticoid receptor (GR) [182] (see below for a detailed description of these substrates). In all cases, the result of the initial binding of Hsp70 to the exposed short binding motifs on partially unfolded substrates is the acquisition of more unfolded or less stable conformations of the clients that are then allowed to refold [183–185]), be targeted for degradation [167], or handed to other chaperone systems [182,186]. This type of binding is also used by Hsp70s to actively unfold substrates in an ATP-dependent fashion acting as an ‘unfoldase’ (for a comprehensive review see [3]). Nevertheless, whether by conformational selection or active unfolding, the consequence of Hsp70 binding in these cases is a shift to more unfolded states of the substrate.

One general finding is that Hsp70s accommodate more folded substrates by exploiting the dynamic nature of the α -lid, which can detach from the β SBD [17,18,26–29]. Moreover, the α -lid can directly interact with regions of the bound client. Interactions with the α -lid, together with the β SBD binding cleft, were shown to stabilize the DnaK-bound partially folded maltose-binding protein (MBP) and a monomeric variant of the replication initiation protein repE (RepE54), as the stabilization of these DnaK-bound substrates is abolished when the α -lid is truncated [28].

The last type of Hsp70 binding interaction with a protein substrate occurs when a binding motif is present on a well-folded substrate essentially as a tag to mediate chaperone binding, for example, at a terminus of the protein. The consequences of binding, in this case, are more straightforward, in that the ensemble of folded states of the substrate may not be

perturbed. Instead, the substrate may be delivered to a downstream chaperone or partner, or, as is postulated for clathrin (*vide infra*), disassembly of the substrate from a complex is facilitated by chaperone binding [187–189].

Hsp70 binding to protein substrates effectuates multiple biological outcomes

In the cell, Hsp70s perform a myriad of highly diverse functions by binding to their protein clients as described. In all cases, the ability of the chaperone to bind transiently (the residence time determined by allosteric cycling) to sequences that are usually sequestered within the folded state of a protein in some way confers an advantage to the given physiological system. This transient chaperone binding thus affects the substrate, whether it be delay of folding to prevent misfolding and enable translocation across a membrane, facilitation of productive folding, inhibition of aggregation and dissociation of aggregates, maintenance of an unfolded state for translocation across a membrane, hand-off to a downstream chaperone or degradation machine, and disassembly of specific complexes. In this section, we wish to paint a picture of how this simple binding/unbinding machine has been harnessed evolutionarily in such a wide array of functions. We will not give an encyclopedic description of Hsp70 functions, but rather select illustrative examples and relate them to the structural and mechanistic properties of Hsp70s described in the preceding sections (Figure 8).

Delay of folding to prevent misfolding and enable translocation across a membrane—Hsp70s have been implicated in the chaperoning of nascent chains in both prokaryotes [156,190] and eukaryotes [191]. In the case of eukaryotes, specialized Hsp70s have evolved that are dedicated to assisting protein biogenesis [191–193]. The *S. cerevisiae* system is particularly well studied: The Hsp70 homolog, Ssb, works together with a dedicated ribosome-associated complex (RAC) made up of the J-protein zotin (Zuo2) and a specialized Hsp70 homolog, Ssz1 to greet nascent chains while they are emerging from the ribosome and ensure the fidelity of protein folding while diminishing the risk of aggregation [194]. The association of Ssb with the ribosome is critical to its ability to perform a dedicated task in nascent chain folding [195]. Recently, *in vivo* Hsp70 has been reported to inhibit selective ribosome profiling has revealed exquisite details about the Ssb interactome and how specific substrate interactions integrate actions at the ribosome to modulate translation rates, facilitate folding, and support targeting to membranes [196].

Another critical cellular function that relies on the interaction of Hsp70s with unfolded polypeptides is the chaperoning of chains that are destined to cross membranes [197]. On the entry side of the membrane to be traversed, the targeted polypeptide must be unfolded, while on the exit side, the chain must be held to resist back-sliding and guided to avoid premature folding and prepare it for downstream chaperone interactions. These roles have been described for protein translocation into mitochondria [198], chloroplasts [199], and the ER [200].

Facilitation of productive folding—As described above, Hsp70s bind to unfolded substrates and generate a heterogeneous ensemble of conformations where substrates may undergo conformational sampling while chaperone-bound. In this way, substrates may be

helped to find an optimal folding pathway and to search a conformational space, also avoiding misfolded energy traps [175–177]. Additionally, simultaneous substrate binding to multiple DnaK molecules should lead to considerable substrate expansion as a way to convey the substrate through a productive folding pathway [174].

Details of the implications of partially folded or near-native substrates bound to Hsp70 were provided by a recent study that used optical tweezers to mechanically unfold MBP and RepE54 to test the impact of their interaction with DnaK [28]. While native MBP did not bind to DnaK, near-native states were stabilized against forced unfolding by binding to DnaK. Conversely, when MBP bound DnaK in a more unfolded state, the chaperone impaired substrate refolding. These provocative data were explained through a kinetic competition mechanism where unfolded DnaK-locked chains could not refold unless folding was initiated before chaperone binding, in which case they were then subsequently stabilized by DnaK and more resistant to mechanical unfolding.

To date, there is no direct evidence that the rate of folding of substrates is altered by Hsp70 binding. For example, the measured rate of refolding of both staphylococcal nuclease [170] and luciferase [181] has been reported to be the same in the absence of Hsp70 and after ATP-induced release from the chaperone. As the proposed influence of the chaperone on the unfolded ensemble suggests that it disfavors kinetically trapped misfolded states, acceleration of folding might occur. New observations from the Hartl laboratory indeed support this possibility (F. U. Hartl and M. Hayer-Hartl, personal communication, 2019).

Inhibition of aggregation and dissociation of aggregates—Not surprisingly, predicted Hsp70 binding sequences correspond closely with those that are predicted to be highly aggregation-prone. Thus, the sequestering of these short sequences by Hsp70s in unfolded, partially folded or misfolded proteins should inhibit aggregation (acting as a ‘holdase’) [201]. The early observation (described above) that deletion of DnaK caused widespread aggregation in *E. coli* [155] established that this Hsp70 plays a key role in preventing aggregation *in vivo*. In addition, *in vitro* studies have demonstrated inhibition of aggregation by the Hsp70 system using model substrates such as firefly luciferase, β -galactosidase, or rhodanese [202–205]. More recently, the ability of Hsp70s to inhibit the aggregation of disease-related proteins has been investigated [1,206,207]. For example, Hsp70 has been reported to inhibit α -synuclein aggregation *in vitro* by binding prefibrillar species [208,209], to block the early stages of Tau aggregation by suppressing the formation of critical nuclei [210], and to suppress the aggregation of the A β peptide [211]; both Tau protein and A β peptide aggregation have been associated with Alzheimer’s disease.

In addition, Hsp70 binding has been implicated in the dissociation of protein aggregates [212]. Hsp70s are envisioned to bind an aggregated substrate via an exposed favorable binding sequence at the surface of the aggregate [3,213–215]. Here, by ATP-fueled clamping of the substrate in the canonical binding site, local unfolding propagates and extracts the substrate from the aggregate’s surface. While the Hsp70 system has been reported to perform this function alone [216], the efficiency of disaggregation is greatly augmented by cooperation with other unfolding chaperones, like Hsp104/Hsp100/Hsp110 [3,212–214]. In the case of a polypeptide that is part of a large aggregate or refractory removal from the

aggregate, a mechanism called ‘entropic pulling’ has been invoked to explain the action of the Hsp70 system on the substrate [217,218]. This same model can be applied to the facilitation of polypeptide translocation across membranes and the tugging on nascent chains out of the ribosome exit tunnel [219,220]. By this model, the large Hsp70 molecules bound to a polypeptide on the surface of an aggregate (or at the exit of a channel) move with increased freedom away from the anchoring site by repulsion/collision with the aggregate (or the channel) creating an effective pulling force that removes the substrate [184,217,218].

Hand-off to a downstream chaperone or degradation machine—*In vivo* functions of Hsp70s frequently involve the partnering with downstream cellular machinery. A prime example is the hand-off of substrates from DnaK to the GroEL/ES chaperonin complex in *E. coli* [221]. Also, Hsp70s are known to bind to substrate proteins that display their chaperone-binding sequences as part of a regulatory process, or as they need to be processed in the cell (see [2,5]). The regulation of the stability of the *E. coli* σ^{32} by DnaK and DnaJ constitutes a paradigm for the interaction of Hsp70 chaperones with natively folded substrates and illustrates how such binding can be exploited physiologically to modulate the activity of a cellular factor [222–225]. The σ^{32} subunit of the RNA polymerase binds to the core enzyme in response to cellular stress and targets it to the promoters of heat-shock genes [226–228]. Under physiological conditions, the native state of σ^{32} is a substrate of DnaJ and DnaK [222–225], and chaperone binding renders σ^{32} susceptible to degradation by the protease FtsH. Upon heat shock, when DnaK/J are less abundant, σ^{32} is stabilized and is able to bind the RNA polymerase to mount a stress response. Many details of the interaction between DnaK with native σ^{32} as well as the impact of chaperone binding on σ^{32} structure were mapped at residue-level by Rodriguez et al. [167]. This work revealed that in native σ^{32} DnaJ and DnaK bind to different (proximal) sites that are transiently exposed in the substrate when it is not bound to the RNA polymerase. Initial binding of DnaJ to σ^{32} destabilizes the DnaK-binding site and facilitates its binding. Tight binding of DnaK destabilizes a region in the N-terminal domain of σ^{32} , which is the primary target for FtsH-mediated degradation.

In another well-characterized example, the ligand-dependent activation of the GR in the cell occurs as a result of the cooperation between the Hsp70 and Hsp90 systems [182,229,230]. The work by Kirschke et al. revealed the molecular details by which Hsp70 (in the presence of Hsp40 and ATP) binds to a folded form of the ligand-binding domain of the GR (LBDGR) hindering ligand binding, or actively removing it from its binding site, stabilizing an inactive form of the receptor. Hsp70 binding (but not Hsp40 alone) causes a local unfolding of LBDGR, enough to prevent ligand binding, and only after this ‘priming’ step, the Hsp90 system (Hop, p23) is able to rescue LBDGR from the Hsp70-mediated inactivation and promote ligand binding [182].

Disassembly of specific complexes—As described earlier, disassembly of the clathrin-coated vesicles during endocytosis is mediated by Hsc70 [183]. Clathrin in the lattice is first recognized by the specialized J-protein auxilin [185], which recruits Hsc70 to the site. Hsc70 binding to the QLMLT sequence at the C-terminal of clathrin results in the extraction of clathrin from the lattice and disassembly of the vesicle coat. Evidence supports

the action of Hsc70 on the clathrin triskelion to be a destabilization by binding to sites exposed by conformational distortions [187,188]. The ‘conformational selection’ and ‘entropic pulling’ mechanisms have been invoked to explain the molecular mechanism of action of Hsc70 in a disassembly process [184,189].

Final thoughts on the multifunctional Hsp70 molecular machine

It is impressive that the simple two-domain Hsp70 molecular chaperone has been recruited to participate in such a wide array of physiological functions throughout prokaryotes, archaea, and eukaryotes. The capacity to bind with nucleotide-modulated affinity to segments of polypeptide chain using sequences that discriminate fully folded proteins from partially folded species has endowed Hsp70s with a highly useful mechanism. The duality of their action implicates them in a multitude of cellular pathways: as holdases, they hold substrates for a residence time that can be modulated by ATP/ADP ratios and by co-chaperone availability, and as unfoldases, they cause their bound substrates to unfold by virtue of the geometric consequence of retaining a 7-residue segment in a clamped SBD binding site. Their binding site shows promiscuity, such that many, many client proteins can be recognized. Nonetheless, they are selective for hydrophobic stretches that would normally be sequestered, which is key to Hsp70 functions.

As we have discussed in this Review, Hsp70s are a result of evolutionary opportunism: the combination of an actin fold, which provides nucleotide *switchability*, and a novel fold that cradles the polypeptide under a regulatable lid. The deep understanding that has emerged from structural and biochemical studies of Hsp70s has provided mechanistic insight into how the allosteric communication between the two ligand-binding domains of Hsp70s is achieved by energetic competition between interfaces formed in the two alternative conformations accessible to each domain. A focus of future research will be the relationship of the tunable allosteric mechanism of Hsp70s to their functional diversification: are some Hsp70s tuned for specific functions, perhaps because of specific substrate repertoires?

This is indeed an exciting time to witness synergistic advances in the cell biology of Hsp70 functions, new discoveries of their relationship to many pathologies, and structural revelations about their molecular mechanism. While there have been major advances in recent years in our understanding of the molecular mechanism of Hsp70 chaperones, as described in this review, major questions remain about the physiological roles of these key players in protein homeostasis. Answering these questions will require a deeper understanding of the interactions of Hsp70s with their co-chaperones and substrates. The knowledge that eukaryotic Hsp70s may partner with multiple NEFs and JDPs adds to the complexity of the Hsp70 system and its capacity to be specialized for particular functions. Hsp70s themselves are tunable by sequence modification or through partnerships with diverse co-chaperones. In addition, in a cellular milieu, Hsp70s will see an array of potential protein substrates, the affinity of which may vary through a wide range. The result is that the selection of client interactions is non-random, and the dwell time of a given substrate on an Hsp70 will vary. Putting Hsp70 networks back into the complex, cellular environment will rely on clever methods of determining substrate partitioning.

From the intense research effort on Hsp70 mechanisms and cellular roles in the recent past have emerged many opportunities to design or screen for modulators of Hsp70 function. We have not reviewed this body of work here, as there have been several recent reviews [231–238]. We direct the interested reader to these reviews, and we want to emphasize that the rich knowledge gained on the structure and mechanism of Hsp70s as well as the enhanced understanding of their functions have enabled either the rational design of inhibitors/activators as well as the design of assays to screen libraries for Hsp70 modulators. As a result, there are now numerous small molecules that can be used as leads for clinical therapies and as tools for dissection of Hsp70 functions.

Funding

The writing of this review was supported by a grant to L.M.G. from the National Institutes of Health (GM118161).

Abbreviations

βSBD	β-sandwich subdomain
Bap	BiP-associated protein
BAG	BCL-2-associated athanogene
BiP	binding-immunoglobulin protein
CHIP	carboxy terminus of Hsp70-interacting protein
drkN	terminal domain of the <i>D. melanogaster</i> adaptor protein drk
ER	endoplasmic reticulum
Fes1	factor exchange for SSA1 protein 1
GR	glucocorticoid receptor
hERG	human Ether a Go-go-Related Gene potassium channel
HspB8	heat shock protein B-8
HspBP1	Hsp70 binding protein 1
Hsp104	heat shock protein 104
Hsp110	heat shock proteins 110
Hsc70	heat shock cognate 71-kDa protein
JDPs	J-domain proteins
LBDGR	ligand-binding domain of the GR
MBP	maltose-binding protein
NBD	nucleotide-binding domain

NEF	nucleotide exchange factors
NMR	nuclear magnetic resonance
polyQ	polyglutamine
RAC	ribosome-associated complex
RepE54	monomeric variant of the replication initiation protein
SBD	substrate-binding domain
SOD1	superoxide dismutase
Zuo1	zuotin

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Figure 1. The structural arrangement of Hsp70 molecular chaperones.

(A) Schematic representation of the structural domains of Hsp70s. The sequence numbers are based on the *E. coli* Hsp70, DnaK. Structures of DnaK showing (B) the canonical ADP-bound undocked state (PDB ID 2kho [12]), and (C) the canonical ATP-bound docked state (PDB ID 4b9q [17]). The NBD is colored in blue. In the substrate-binding domain (SBD), the β SBD is colored green, α -lid in red, and the linker in orange. The bound nucleotide and the peptide substrate NRLLLTG are shown in purple. (D) Subdomain organization of the NBD of a representative Hsp70. The structure shown is ADP-bound Hsc70 (PDB ID 3hsc [92]). Subdomains IA, IB, IIA, and IIB are colored in yellow, green, blue, and red, respectively. The crossing α -helices are shown in cyan. (E) Structure of the DnaK SBD bound to the peptide NRLLLTG (PDB 1dkz [39]). The binding mode of peptide substrate is illustrated in Figure 7.

Figure 2. Hsp70 allostery is governed by the energetic competition between domain interfaces. (A) The β SBD- α -lid interface (red) formed in the undocked state of DnaK (PDB ID 2kho [12]) and (B) the NBD-SBD interface (blue) formed in the docked state of DnaK (PDB ID 4b9q [17]). (C) Schematic representation of the allosteric cycle of DnaK. Coloring follows the scheme described in Figure 1.

Figure 3. Evolutionary tuning of Hsp70 energy landscapes.

Schematic illustration of allosteric landscapes of HspA1, Hsc70 and DnaK in different ligand-bound states. ‘D’ represents the docked state; ‘U’ represents the undocked state; ‘P’ represents the partially docked state observed in ATP/peptide-bound DnaK, with the NBD and SBD largely undocked and with the linker still bound to NBD. The barrier heights and well depths are only qualitative. The figure is reproduced from [9].

Figure 4. Structural features of JDPs.

(**A**) NMR structure of the J-domain of *E. coli* DnaJ (PDB ID 1xbl [44]) indicating the four helices (α1, helix I; α2, helix II; α3, helix III; α4, helix IV) of the helical hairpin (in purple) and the His–Pro–Asp (HPD) motif (in cyan). (**B**) Structure of the ATP-bound open conformation of *E. coli* DnaK in complex with the J-domain of *E. coli* DnaJ (PDB ID 5nro [36]). The color scheme is the same as that for DnaK in Figure 1, and the J-domain is shown in magenta. The two orientations show that the J-domain binds at the interface between the NBD and the SBD of DnaK, forming direct contacts with both domains. (**C**) Domain organization and classification of J-proteins; JD, J-domain; G/F, Gly–Phe-rich region; Zn, Zn-finger; β1, first β-sandwich domain; β2, second β-sandwich domain; DD, dimerization domain. In class C JDPs, the functional J-domain can be present anywhere in the sequence and is not restricted to the N-terminus.

Figure 5. The prokaryotic NEF GrpE.

(A) Structure of *E. coli* DnaK GrpE (PDB ID: 1dkg [90]). (B) GrpE (green) in complex with the NBD of *E. coli* DnaK (blue). (C) Conformational changes in the NBD caused by GrpE binding. ADP-bound NBD is shown in light blue (homology model based on PDB ID 3hsc [92]) and GrpE-bound NBD in blue. The red arrow indicates the movement of subdomain IIB caused by GrpE binding. (D) Crystal structure of full-length DnaK from *G. kaustophilus* with its corresponding GrpE (PDB ID: 4ani [98]). The domains of DnaK are colored as in Figure 1.

Figure 6. Structural features of eukaryotic NEFs.

Structures of human BAG1 (A), yeast Sil1 (B), and the yeast Hsp110, Sse1 (C). Crystal structures of the complexes of these NEFs with their respective Hsp70s are shown below each isolated NEF structure, with an indication of what changes in the NBD conformation are caused by NEF binding: (D) bovine Hsc70 NBD-human BAG1 (PDB ID 1hx1 [111]); (E) yeast BiP NBD-Sil1 (PDB ID 3qml [71]); (F) yeast Hsc70-Hsp110 (PDB ID 3c7n [138]). The structure shown in (F) of the complex between Hsc70 and Hsp110 includes the SBD (shown in transparent surface and green ribbon). In (F), ADP*BeF₃ bound to Hsp110 and ADP bound to Hsc70 are colored in red.

Figure 7. The SBD of *E. coli* DnaK contains the canonical binding site.

(A) Representation of the crystal structure of the SBD of *E. coli* (gray) (PDB ID: 1dkz [39]) with bound NR peptide (NRLLLTG, in purple sticks). (B) Top view of the SBD canonical binding site, where the α-lid and residues 404–406 and 429–431 have been removed to visualize the bound NR peptide (residues labeled 1–7). The surfaces on the SBD contacted by the substrate peptide are colored according to the residue position in the substrate, and the numbers (–2 to +2) indicate the ‘positions’ relative to the ‘0th residue’, which corresponds to the SBD site that contacts the central residue of the peptide [39].

Figure 8. Examples of modes of Hsp70 binding to protein substrates.

(A) Hsp70s may bind to motifs exposed in unfolded proteins to facilitate their proper folding as they emerge from the ribosome, to keep them unfolded for translocation into organelles, to enable hand-off to downstream chaperones, or to prevent aggregation. (B) Hsp70s may bind to motifs exposed at the surface of misfolded or aggregated substrates. (C) Hsp70 may bind motifs that are available on folded or near-native proteins. This may occur when the protein transiently exposes the binding (left panel, for example, when DnaJ opens the DnaK binding site on σ^{32} [167], center panel, for example, when Hsp70 binds to an exposed site in the glucocorticoid receptor [182]) or when the binding site resides on in an unstructured region (right panel, for example, Hsc70 binding to the C-terminal motif of native clathrin in triskelion structures [187]). In all panels, the substrate is depicted in orange, the Hsp70 binding motif is shown as purple stars, and the Hsp70 colored as in Figure 1.