The Hsp70 chaperone network

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Abstract | The 70-kDa heat shock proteins (Hsp70s) are ubiquitous molecular chaperones that act in a large variety of cellular protein folding and remodelling processes. They function virtually at all stages of the life of proteins from synthesis to degradation and are thus crucial for maintaining protein homeostasis, with direct implications for human health. A large set of co-chaperones comprising J-domain proteins and nucleotide exchange factors regulate the ATPase cycle of Hsp70s, which is allosterically coupled to substrate binding and release. Moreover, Hsp70s cooperate with other cellular chaperone systems including Hsp90, Hsp60 chaperonins, small heat shock proteins and Hsp100 AAA+ disaggregases, together constituting a dynamic and functionally versatile network for protein folding, unfolding, regulation, targeting, aggregation and disaggregation, as well as degradation. In this Review we describe recent advances that have increased our understanding of the molecular mechanisms and working principles of the Hsp70 network. This knowledge showcases how the Hsp70 chaperone system controls diverse cellular functions, and offers new opportunities for the development of chemical compounds that modulate disease-related Hsp70 activities.

Ribosomal exit tunnel
A tunnel through which nascent polypeptide chains exit the large ribosomal subunit during protein translation.

Translocons
Complexes of proteins mediating the translocation of polypeptides across membranes.

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The Hsp70 chaperones function in a wide range of cellular housekeeping activities, including the folding of newly synthesized proteins, the translocation of polypeptides into mitochondria, chloroplasts and the endoplasmic reticulum (ER), the disassembly of protein complexes and the regulation of protein activity (FIG. 1). Furthermore, Hsp70s prevent the aggregation and promote the refolding of misfolded denatured proteins, solubilize aggregated proteins and cooperate with cellular degradation machineries to clear aberrant proteins and protein aggregates. Thus, Hsp70s act as sentinel chaperones, guarding cells from the deleterious effects of a wide range of proteotoxic stresses[1–5], pathophysiological conditions[6] and organismal ageing[7] that cause protein homeostasis imbalance. Not surprisingly, mutations in genes encoding components of the Hsp70 system cause several human diseases (Supplementary Table 1). Given this essential role in protein folding, it is implicit that Hsp70s must be able to act on a diverse range of substrates to adapt their numerous activities to fluctuating growth and stress conditions in the cell.

Genomes from bacteria to humans encode multiple Hsp70s (Supplementary Table 2). In humans, 13 Hsp70 homologues are expressed in distinct cellular compartments (cytosol, nucleus, ER and mitochondria), with levels of isoforms being regulated according to cellular needs (for example, growth and tissue-specific activities). Although they have some functional redundancy, Hsp70 family members also exhibit a high degree of specialization. Hsp70s can be distinguished by three main factors: first, differences in substrate recognition and allosteric regulation; second, adaptations imposed by target site localization, for example, Hsp70s binding at ribosomal exit tunnel and membrane translocons where nascent polypeptides emerge (FIG. 1); and third, the type of cooperation with the extensive network of co-chaperones of the J-domain protein (JDP) family (also referred to as DnaJ, Hsp40 (REF.[8])) and nucleotide exchange factors (NEFs) that control substrate presentation and release from Hsp70s[9,10] (FIG. 2). The functional diversity of Hsp70 members is further increased by cooperation with other cellular chaperone systems, including small heat shock proteins, chaperonins (bacterial and mitochondrial GroEL–GroES, eukaryotic TRiC/CCT), Hsp90 and Hsp100, and with protein degradation systems (FIG. 1).

Recent studies have provided crucial information on the mechanisms of the Hsp70 system, including insights into structural, kinetic and functional features of its network components. In this Review, we describe the structure and allosteric cycle of Hsp70 chaperones, the mechanism of their interaction with substrates and how the mechanistic aspects of the Hsp70 network are tied to distinct protein folding functions. We also highlight the structural features of Hsp70 co-chaperones, such as JDPs and NEFs, and detail the emerging working principles for the regulation of the Hsp70 network by these co-chaperones and other partner chaperones in the cell.

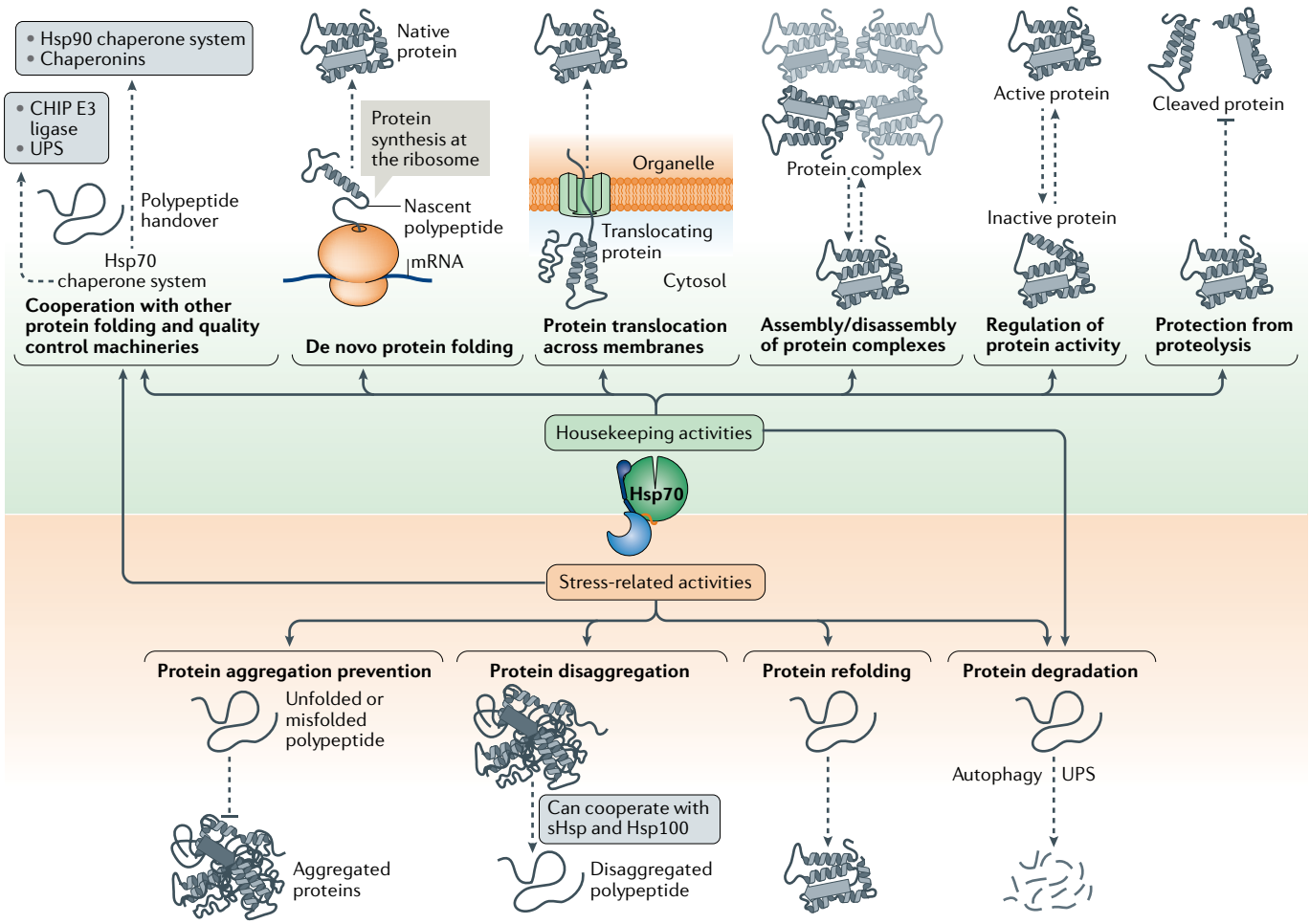


Fig. 1 | **The diverse housekeeping and stress-related activities of Hsp70s.** The Hsp70 chaperones participate in a wide range of cellular housekeeping functions (top), including the folding of newly synthesized proteins, the translocation of polypeptides into mitochondria, chloroplasts and the endoplasmic reticulum (ER), the assembly and disassembly of protein complexes, regulation of protein activity and assisting in protein handover to downstream chaperone machineries, such as the Hsp90 folding machinery and chaperonins. Furthermore, Hsp70s also participate in stress-related activities (bottom), such as preventing the aggregation of proteins, solubilizing aggregated proteins, promoting the refolding of misfolded or unfolded proteins and cooperating with cellular degradation machineries, such as autophagy and the ubiquitin–proteasome system (UPS), to clear aberrant proteins and protein aggregates. Hsp100, 100-kDa heat shock protein; sHsp, small heat shock protein.

Chaperonins
(Also referred to as Hsp60s). Molecular chaperones assembled into large double-ring complexes with a central cavity, creating an isolated compartment in which proteins fold and are protected from aggregation. Chaperonins include the bacterial and mitochondrial GroEL–GroES system and eukaryotic CCT/TRiC.

Hsp90
Hsp90 is a highly conserved, ATP-dependent molecular chaperone that has critical roles in protein maturation and folding. The wide range of Hsp90 substrates include, for example, kinases, transcription factors, steroid hormone receptors and E3 ubiquitin ligases.

The Hsp70 machine

Central to the chaperone function of Hsp70s is the allosteric control of their interaction with substrates, promoted by coordinated subdomain movements.

Domain structure of Hsp70. All Hsp70 family members share at least two of the four structural features of the archetype Hsp70, the bacterial DnaK: an N-terminal, 45-kDa nucleotide binding domain (NBD), followed by a 15-kDa substrate binding domain (SBDβ), a 10-kDa helical lid domain (SBDα) and a disordered C-terminal tail of variable length (FIG. 2a). In eukaryotic cytosolic and nuclear Hsp70s, the disordered tail ends frequently with a conserved charged motif (Glu-Glu-Val-Asp; EEVD) that interacts with specific cofactors[11] (FIG. 2a,e). The NBD has an actin-like fold consisting of four subdomains (IA, IB, IIA and IIB) arranged into two lobes separated by a deep cleft (FIG. 2b). In the catalytic centre at the bottom of this deep cleft, ATP coordinates all four subdomains, controlling lobe movements[12]. The SBDβ is built up of an eight-stranded β-sandwich containing the polypeptide binding cavity with its central hydrophobic pocket[13–15] (FIG. 2b). In the nucleotide-free and ADP-bound states, the SBDα docks onto the SBDβ to fully enclose this cavity. The NBD and SBDβ are connected by a conserved flexible linker that is essential for the allosteric mechanism that couples nucleotide binding and ATP hydrolysis to substrate binding[16–18] (FIG. 2a,c).

Hsp70 allosteric cycle. Hsp70 chaperone activity requires rapid association and timely release of substrates to prevent substrate aggregation and promote folding. This ability to associate with and dissociate from substrates in a controlled manner critically depends on an intricate allosteric mechanism that couples ATP hydrolysis in the NBD to substrate capturing by the SBD (FIG. 2b,d). In the ADP-bound state, the SBD binds to peptide substrates with high affinity (K_D = 0.1–1 μM) but very low

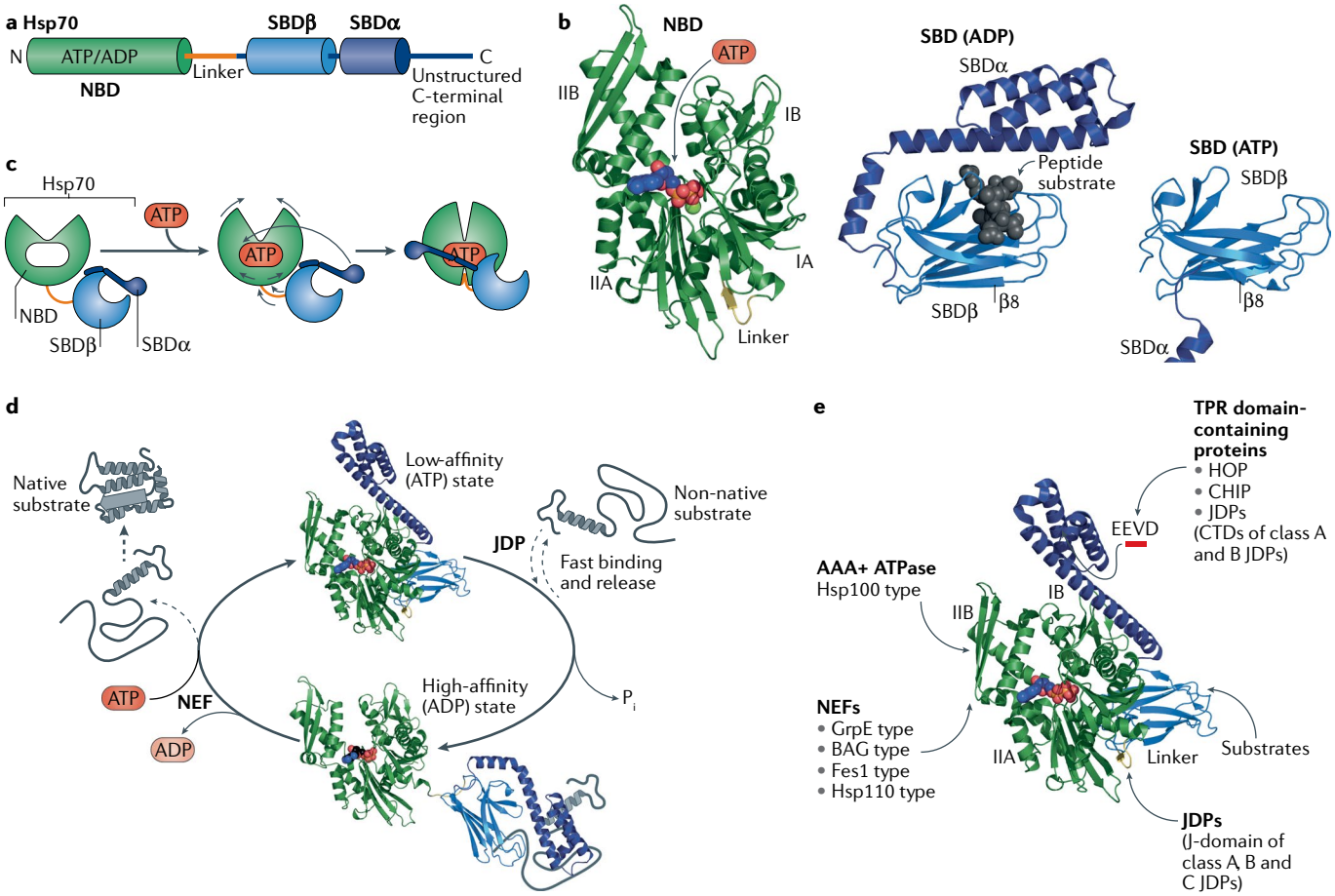


Fig. 2 | **Structure and functional cycle of Hsp70s. a** | Domain organization of Hsp70 chaperones. **b** | Cartoon representations of Hsp70s. Left, nucleotide binding domain (NBD) in the ATP-bound state with lobe subdomains IA, IIA, IB and IIB denoted and the linker inserted into the lower crevice (Protein Data Bank (PDB) ID: 4B9Q (REF.[167])). Middle, substrate binding domain (SBD) in the high-affinity state with a bound peptide in sphere representation (PDB ID: 1DKX (REF.[14])). SBDβ, light blue; SBDα, dark blue. Right, SBD in the low-affinity state with SBDα detached (only beginning of helix A shown for space reasons) and strand 8 (β8) of the lower β-sheet relocated to the upper β-sheet (PDB ID: 4B9Q (REF.[167])). **c** | Schematic representation of the ATP-induced conformational changes in Hsp70s. ATP binding to the NBD leads to rotation of the NBD lobes, opening of the lower crevice, insertion of the linker, detachment of SBDα from SBDβ and docking of SBDα and SBDβ onto the NBD. **d** | Chaperone cycle of Hsp70s. J-domain protein (JDP)-mediated binding of substrate proteins to Hsp70·ATP, in conjunction with a direct JDP–Hsp70 interaction, synergistically triggers Hsp70 ATP hydrolysis and transition to the ADP-bound state, which has high affinity for the substrate. Nucleotide exchange factors (NEFs) then induce ADP dissociation and rebinding of ATP, converting Hsp70 to the low-affinity state and consequently leading to substrate release. Subsequently, the released substrate can fold to the native state or, alternatively, re-enter the Hsp70 cycle. Hsp70 domains are coloured as in part **b**, with the client protein shown as a grey cartoon. **e** | Structure of Hsp70·ATP (PDB ID: 4B9Q (REF.[167])) highlighting the regions that interact with co-chaperones and substrates. CTD, C-terminal β-sandwich domain; SBDα, substrate binding domain α-helical lid; SBDβ, substrate binding domain; TPR, tetratricopeptide repeat.

association (approximately 10^4 M^{-1} s^{-1}) and dissociation (10^{-3} s^{-1}) rates. ATP binding weakens SBD–substrate interactions, increasing the K_D by around 10-fold by inducing a 100-fold and 1,000-fold increase in association and dissociation rates, respectively.

The binding of ATP to the catalytic centre leads to a rotation of the NBD lobes relative to one another, thereby opening a crevice at the bottom of the NBD for binding of the interdomain linker and association with the SBDβ (FIG. 2c). The SBDβ then clamps the NBD lobes, leaving the catalytic residues in a conformation unsuitable for ATP hydrolysis[15], which explains the low basal ATPase activity of Hsp70s (approximately 1 ATP molecule per 6–40 min). Substrate binding, in particular the insertion of a single residue of the client polypeptide (that is, the substrate) into the hydrophobic binding pocket of the SBDβ, triggers the release of the SBDβ and the α-helical lid from the NBD through a rearrangement of intramolecular hydrophobic and polar contacts[15,19]. The release of the SBDβ clamp allows the back-rotation of the NBD lobes towards a position suitable to promote ATP hydrolysis. Upon hydrolysis, the dissociated α-helical lid docks onto the substrate binding pocket of the SBDβ, preventing substrate dissociation[14,20]. The substrate binding-induced release of the SBDβ from the NBD, however, also leads to slipping of the linker out of the lower crevice, and as a consequence the linker can no longer keep the NBD lobes in the optimal position for

Autophagy
A highly regulated catabolic process in which cellular proteins and organelles are sequestered in a characteristic double-membrane vesicle called an autophagosome and are then degraded following vesicular fusion with a lysosome.

ATP hydrolysis. Hence, substrates alone only moderately stimulate the ATPase activity of Hsp70s. Recent findings have shown that the interaction of Hsp70 with the J-domain of a partner JDP prevents the aforementioned slipping of the linker from the NBD, enabling Hsp70 to transition to the 'fully competent' ATP hydrolysis competent state. It is therefore the combined action of the binding of a substrate to the ATP-bound (open) conformation of Hsp70 and the association of a J-domain that promotes maximal ATP hydrolysis (up to 15,000-fold above the basal activity for bacterial DnaK[21,22]). This increases the effective affinity for substrates by several orders of magnitude beyond the ADP-bound, so-called high-affinity (closed) conformation of Hsp70 (well into the low nanomolar K_D range) — a non-equilibrium property of the system called 'ultra-affinity'[23].

The other aspect of this allosteric mechanism, for the release of substrates, is triggered by ATP binding and involves several coordinated conformational changes within Hsp70. In the nucleotide-free state, the NBD and SBD are largely independent of one another except for the tethering mediated by the conserved linker[19] (FIG. 2c). Upon ATP binding, the SBDα and SBDβ separate from each other via a still unclear conformational trigger, and the SBDβ interacts with the NBD. This rearrangement opens up the substrate binding pocket, which leads to a 5- to 50-fold increase in substrate dissociation rates[24,25]. To achieve the >1,000-fold increase in substrate dissociation rates observed for full-length DnaK upon ATP binding[24], the SBDβ must undergo additional conformational changes. These changes are triggered by SBDβ interaction with the NBD, which induces a shearing movement in the β-sandwich, relocating strand 8 from the lower β-sheet to the upper β-sheet and opening the substrate binding channel, facilitating substrate release (FIG. 2b,c).

The elucidation of the allosteric mechanism suggests novel potential drug target sites. The ATP binding pocket of Hsp70s was traditionally considered a poor drug target site owing to the highly hydrophilic character of the binding pocket and the high affinity for ATP[26]. Also, the conservation of Hsp70s is so high that specificity of a drug for certain Hsp70s seems difficult to achieve. Allosteric sites seem to offer clear advantages and are beginning to be explored[27,28].

The Hsp70–substrate interaction

Hsp70s interact promiscuously with a broad range of substrate types, including nascent unfolded polypeptides emerging from the ribosome, folding intermediates, natively folded proteins (e.g. heat shock transcription factor), misfolded proteins as well as large assemblies that can be functional (e.g. clathrin-coated vesicles) or non-functional (e.g. protein aggregates) (FIGS 1,3,4). This versatility in substrate recognition raises the question of how the Hsp70s recognize such diversity of clients and protein conformations.

Substrate recognition by Hsp70. Early studies demonstrated that Hsp70s bind to various peptides that, like protein substrates, stimulate Hsp70 ATPase activity[29,30]. Research thereafter relied heavily on peptide models to understand the mechanistic principles underlying Hsp70 substrate recognition. Studies of DnaK peptide libraries indicated that DnaK prefers peptides with a five-residue hydrophobic core (with preference for aliphatic residues) that is flanked by positively charged regions[31]. Such motifs are abundant in protein sequences, but are largely buried in the hydrophobic core of native (that is, correctly folded) proteins, explaining why Hsp70s tend to promiscuously bind to unfolded or misfolded proteins.

The first Hsp70–substrate structure to be described was that of a model peptide (NRLLLTG) bound to DnaK. This structure revealed that the peptide binds in an extended conformation[14] to a hydrophobic cleft in the SBD and that the side chain of the central leucine residue of the peptide projects into a small hydrophobic pocket of the SDB (FIG. 2b). Over a stretch of five consecutive residues, the peptide backbone is further enclosed by the SBDβ cleft and stabilized through a network of hydrogen bonding and hydrophobic interactions and the helical lid of the SBDα, consistent with the biochemically determined binding motif. This peptide-binding configuration is evolutionarily conserved[32–36], but there is variability in the register and orientation of peptides bound to Hsp70s[37–39], indicating some degree of plasticity and promiscuity in substrate binding. When a complex between DnaK and a 53-residue polypeptide was analysed, the substrate had multiple binding sites for DnaK, generating a fuzzy promiscuous complex, thus suggesting that in the case of proteins multiple binding modes can exist in equilibrium[40].

Despite the SBD structure being conserved[32–36], Hsp70s originating from different organisms and compartments show some differences in their preference for substrates at the peptide level[29]. For example, whereas cytosolic Hsp70s preferably bind leucine-enriched peptide motifs (that is, rich in aliphatic residues), the ER homologue BiP (HSPA5) prefers motifs with aromatic residues[41]. Other striking examples are the *Escherichia coli* HscA and *Saccharomyces cerevisiae* mitochondrial Ssq1 homologues, both involved in the biogenesis of Fe–S cluster proteins, which preferentially recognize peptide sequences enriched in proline residues[42,43]. Also, *E. coli* HscC differs in substrate specificity from the other two *E. coli* Hsp70s, DnaK and HscA[44]. Differences in substrate specificity were furthermore reported for the canonical family members of the human cytosol, Hsp70 (HSPA1A) and Hsc70 (HSPA8)[45]. Although the diversity in substrate specificity among Hsp70s has not been investigated systematically, several findings indicate parameters that play a role. Part of the diversity in substrate specificity stems from alterations in the two residues (M404 and A429 in *E.coli* DnaK; A406 and Y431 in human HSPA1 and HSPA8) that form an arch over the bound peptide backbone[46], and from differences in the length of the substrate-enclosing loops[44]. The kinetics of substrate binding to the SBD may also influence substrate selection as it varies greatly between different Hsp70s, with the eukaryotic Hsp70 chaperones displaying higher substrate binding and release rates than their prokaryotic counterparts[47]. The NBD could also contribute to modulating functional specificities of Hsp70s. Interchanging a single amino acid residue in the NBD of *S. cerevisiae* Ssa1 and Ssa2 (A83 of Ssa1 and

Fe–S cluster proteins
Proteins containing iron–sulfur clusters, which are ubiquitous cofactors, complexed by inorganic sulfur and by the amino acid cysteine, that function as redox elements in electron-transfer reactions. Fe–S clusters are crucial for protein activity in many cellular contexts.

G83 of Ssa2) results in the switching of some biological activities of these Hsp70 chaperones[48]. This switch is independent of ATPase rates, suggesting that the NBD of Hsp70s plays a more complex role in determining substrate specificity. Overall, this spectrum of variations indicates that the functional diversity between the Hsp70 family members stems in part from differences in substrate binding specificities.

Substrate conformation and binding modes. An early study of a structurally unstable protein bound to DnaK indicated that the protein substrate is largely unfolded[49]. Likewise, single-molecule fluorescence and optical-tweezer force spectroscopy experiments detected largely unfolded conformations for Hsp70-bound proteins[50,51]. However, when DnaK interacts with a partially folded intermediate, the protein substrate becomes stabilized, preventing unfolding[51]. NMR studies demonstrated that substrates associated with DnaK constitute a conformationally heterogeneous but mostly unfolded ensemble[40,52–54], in which certain local structural propensities of the folded state are maintained[53]. During binding, Hsp70s seem to specifically disrupt tertiary, long-range contacts, enabling the formation of local structures[51,54]. Furthermore, Hsp70s selectively bind to substrate conformations that transiently expose hydrophobic binding motifs, shifting the equilibrium towards more unfolded conformations and thereby reshaping the folding energy landscape[55]. Thus, using the hydrophobic substrate binding pocket and the helical lid as interaction modules, bacterial Hsp70s associate with a broad range of non-native conformers from completely unfolded to folding intermediates.

The Hsp70-induced disruption of intramolecular contacts in substrates may be an efficient way of rescuing misfolded proteins from kinetic traps that would otherwise prevent folding and/or lead to aggregation. The partially unfolded state of substrates maintained by Hsp70 action may be a favourable starting point for refolding or presentation to cooperating chaperones such as Hsp90. The Hsp90 chaperones can bind to substrates in various conformational states and have been shown to stabilize their client proteins in a folding-competent state (for a review, see REFS[56,57]). In contrast to Hsp70s, Hsp90s do not bind to small linear sequence motifs but to a more extended surface of the substrate recognizing hydrophobic but also hydrophilic surface patches and thus preferentially bind near-native protein conformers[56,58]. Such substrate transfers occur frequently for late folding intermediates, and a large number of meta-stable proteins are regulated in activity and stability jointly by Hsp70 and Hsp90 (REFS[59,60]).

When binding to folded proteins, Hsp70s may function by recognizing peptide segments of a protein that form unstructured regions such as linkers or loops, whereas the rest of the protein remains folded. A prime example is the binding of mammalian Hsp70 to clathrin triskelions via an exposed QLMLT motif at the C-terminal unstructured tail of clathrin[61] (FIG. 4c). The interaction of DnaK with loop regions of *E. coli* heat shock transcription factor σ^{32} and plasmid replication protein RepE, and the interaction between BiP and immunoglobulin heavy chain, are other examples of such binding modes[62–66]. Interestingly, electron paramagnetic resonance and Förster resonance energy transfer studies showed that in these cases the SBDα lid of DnaK and BiP does not fully close over the engaged substrate[65–67]. Corroborating these findings, a recent single-molecule study found that, although the lid subdomain is completely closed when DnaK is in complex with a peptide, it does not close while binding to molten globule-like protein substrates[67]. Implicit in these findings is that the helical lid is able to directly contact bound substrates with structural folds. Whether it contributes directly to substrate selectivity, for example distinguishing partially folded from fully extended conformations, or perhaps even affects the conformation of bound substrates, is currently unknown. Furthermore, whether this binding mode, which has been described for DnaK and BiP, is a general feature of Hsp70 family members needs to be established.

The role of the highly disordered C-terminal tail of Hsp70, which directly precedes the lid domain also remains unclear. Mutating this region in DnaK results in substantial impairment of its protein refolding activity in vitro and reduces cellular thermotolerance in vivo[68]. It is possible that the disordered C-terminal tail serves as an additional interaction site for substrates or, alternatively, modulates the allosteric regulation of Hsp70s with their co-chaperones. This is partly corroborated by the observation that the GGAP repeat motifs in the C-terminal tail of the yeast Hsp70, Ssa1, contribute to substrate recognition[69].

The many functions of Hsp70s

Hsp70s function in de novo protein folding, membrane translocation, disassembly of protein complexes, prevention of aggregation, and solubilization and refolding of aggregated proteins (FIG. 1). To perform such various functions, Hsp70s can operate in different modes and cooperate with various other cellular machineries.

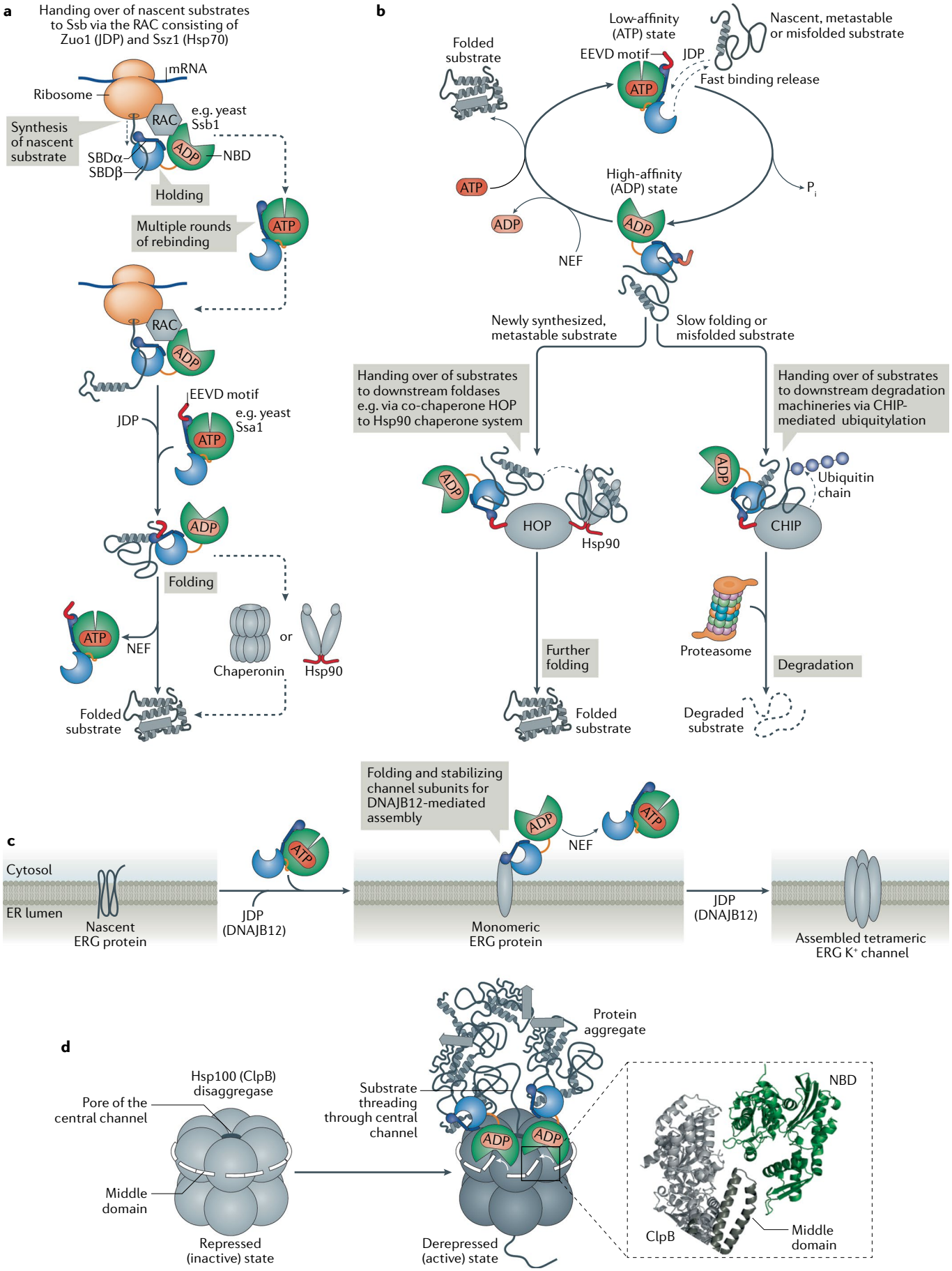
Hsp70 in de novo protein folding. The interaction with nascent proteins during translation, as they exit the ribosome, is one of the best established holding functions for Hsp70s. The proposed role of Hsp70s is to delay the folding of emerging polypeptides until the sequence elements required for folding a complete domain become exposed at the ribosomal surface, thus preventing the formation of non-native interactions and protecting the nascent chain from aggregating[1,70–72]. Some Hsp70s perform these functions by transient association with the ribosome, employing a specialized machinery for ribosome targeting (FIG. 3a). The prototype is the yeast Hsp70, Ssb, which is targeted to the peptide tunnel exit by the ribosome-associated complex. The ribosome-associated complex is composed of the JDP zuotin, which is stably complexed with the non-canonical Hsp70, Ssz1 (REFS[73,74]). Ssb interacts with a large spectrum of nascent polypeptides[75,76] by transiently binding to exposed hydrophobic stretches that are typically found in the core of folded protein domains[76]. Nascent proteins can bind Ssb through repeated cycles of association and dissociation during chain elongation[76]. In addition to Ssb, the canonical Hsp70s of yeast (Ssa1–Ssa4) can engage

Kinetic traps
Non-native low-energy conformations in which proteins can remain trapped when undergoing folding, preventing them from reaching their native fold.

Clathrin triskelions
Clathrin structures that consist of three heavy chains and three light chains. These triskelion-shaped trimeric complexes form a lattice-like coat that promotes engulfment of a membrane-derived vesicle during endocytosis.

Electron paramagnetic resonance
(EPR). A spectroscopic technique that detects materials and proteins that have unpaired electrons. EPR can be used to determine distances in molecules.

Förster resonance energy transfer
(FRET). A mechanism describing the distance-dependent energy transfer between two light-sensitive dipoles, a donor molecule and an acceptor molecule. FRET is used to investigate molecular interactions and to study changes in protein conformation and structure.


a
Handing over of nascent substrates to Ssb via the RAC consisting of Zuo1 (JDP) and Ssz1 (Hsp70)
mRNA
Ribosome
Synthesis of nascent substrate
RAC
e.g. yeast Ssb1
SBDα
SBDβ
ADP
NBD
Holding
Multiple rounds of rebinding
ATP
RAC
ADP
EEVD motif
JDP
e.g. yeast Ssa1
ATP
ADP
Folding
ATP
NEF
or
Chaperonin
Hsp90
Folded substrate
b
Low-affinity (ATP) state
Nascent, metastable or misfolded substrate
Folded substrate
EEVD motif
JDP
ATP
Fast binding release
ATP
High-affinity (ADP) state
P_i
ADP
ADP
NEF
Newly synthesized, metastable substrate
Slow folding or misfolded substrate
Handing over of substrates to downstream foldases e.g. via co-chaperone HOP to Hsp90 chaperone system
Handing over of substrates to downstream degradation machineries via CHIP-mediated ubiquitylation
ADP
HOP
Hsp90
ADP
Ubiquitin chain
CHIP
Further folding
Proteasome
Degradation
Folded substrate
Degraded substrate
c
Folding and stabilizing channel subunits for DNAJB12-mediated assembly
Cytosol
ER lumen
Nascent ERG protein
ATP
JDP (DNAJB12)
ADP
NEF
ATP
Monomeric ERG protein
JDP (DNAJB12)
Assembled tetrameric ERG K+ channel
d
Protein aggregate
Hsp100 (ClpB) disaggregase
Pore of the central channel
Substrate threading through central channel
Middle domain
Repressed (inactive) state
ADP
ADP
Derepressed (active) state
NBD
ClpB
Middle domain

◀ Fig. 3 | **Hsp70 array of functions. a** | The interaction of Hsp70 with nascent proteins during translation at the ribosome. Ribosome-associated Hsp70, Ssb1, is targeted to the ribosome exit channel by the ribosome-associated complex (RAC), composed of Zuo1 (J-domain protein (JDP)) and Ssz1 (Hsp70). Nascent proteins, exiting the ribosome, bind Ssb through repeated cycles of association and dissociation during chain elongation. Following their release from Ssb1, the newly synthesized polypeptides can either fold spontaneously or be transferred for further folding and maturation to other chaperones, such as the Hsp90 system or the chaperonins. **b** | Substrate handover from Hsp70 to cooperating protein quality-control machineries. During protein refolding, the client proteins, upon release from Hsp70, can either fold spontaneously or be transferred to downstream foldases (in the case of newly synthesized or metastable proteins) or to the protein degradation machineries (in the case of slow folding or misfolded substrates). The substrate handover is mediated via tetratricopeptide repeat (TPR) chaperone adaptors, such as HOP, which bridges Hsp70 and Hsp90 via its TPR domains, which bind the C-terminal EEVD motifs of the two chaperones (left). On the degradation pathway, CHIP associates with the EEVD motif of Hsp70 via its TPR domain, and at the same time recruits E2 ubiquitin conjugating enzymes to ubiquitylate the substrates and target the ubiquitylated substrates for degradation via the proteasome (right). **c** | Hsp70 activity in the assembly of the tetrameric ERG-type K^+ channel in the endoplasmic reticulum (ER) membrane. Following translation, the nascent ERG proteins are engaged by DNAJB12 and DNAJB14 JDPs, which activate cytosolic Hsp70 to fold and stabilize the ERG monomeric channel subunits. Next, DNAJB12 tetramerizes the subunits to form a functional K^+ channel. **d** | Hsp70 function in Hsp100-dependent protein disaggregation. Hsp100-type disaggregases are recruited by Hsp70 chaperones to the surface of protein aggregates. Through direct interaction with the regulatory middle domain of Hsp100s, Hsp70s activate Hsp100s, which then use an ATP hydrolysis-driven mechanism to break apart protein aggregates by threading them through their central pores. NBD, nucleotide binding domain; NEF, nucleotide exchange factor; SBDβ, substrate binding domain; SBDα, substrate binding domain α-helical lid.

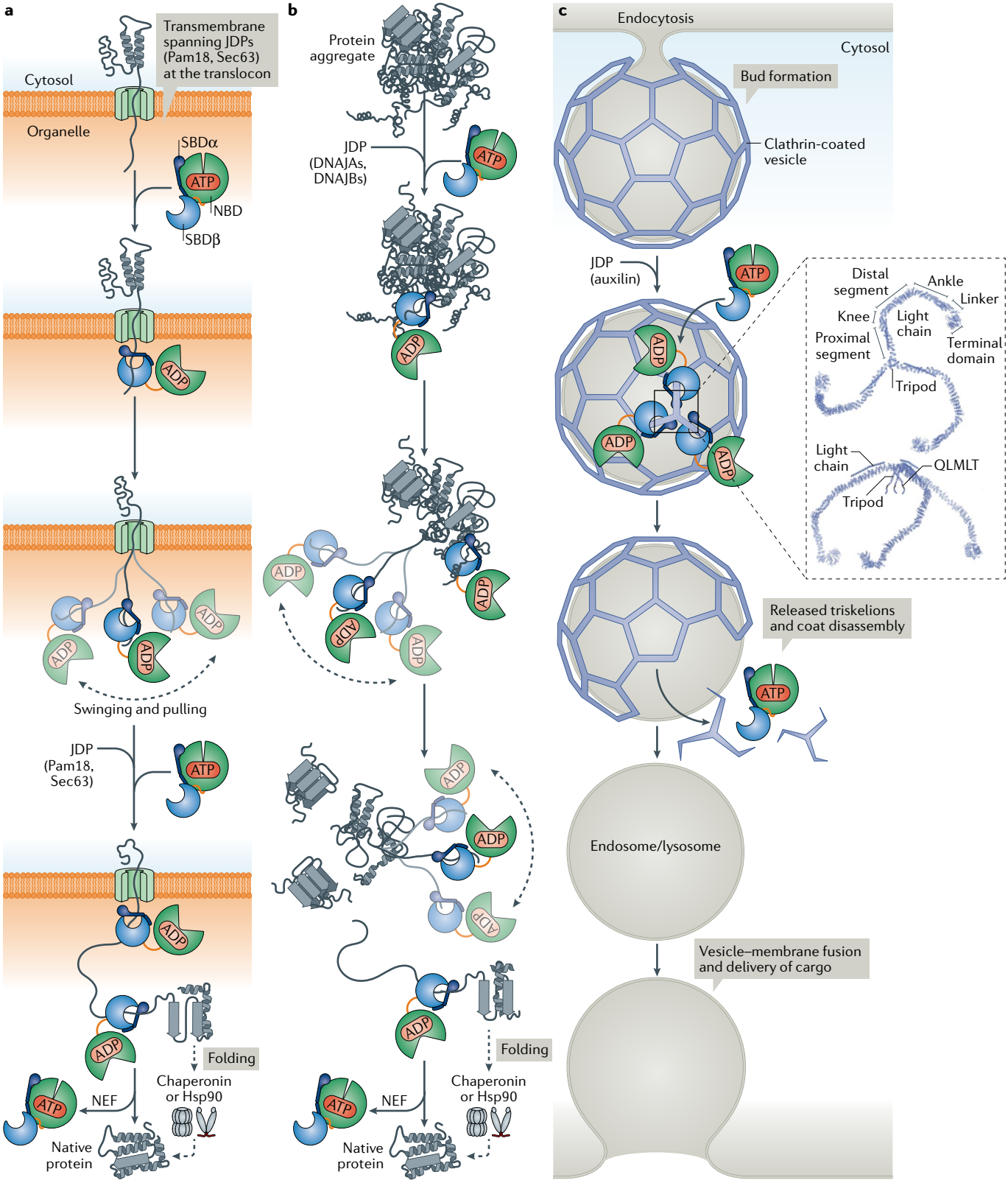
nascent chains as well[77], but it remains unclear whether Ssb and Ssa associate with the same or with distinct peptide stretches and nascent chain molecules (FIG. 3a).

Substrate handover to cooperating protein quality control machineries. After release from Hsp70s, the newly synthesized polypeptides can either fold spontaneously or be transferred for further folding and maturation to other chaperones, such as the Hsp90 system, as mentioned above, or the chaperonins GroEL-GroES (in bacteria and mitochondria) or CCT/TRiC (in archaea and the eukaryotic cytosol), the barrel-shaped chaperones that enclose folding proteins in their folding chamber[1,70–72] (FIG. 3a).

The co-chaperone Hop (known as Sti1 in yeast) facilitates substrate handover from Hsp70 to Hsp90 by bridging the two chaperones using its three tetratricopeptide repeat domains, as these domains bind the C-terminal EEVD motifs of the two chaperones[78,79] (FIGS 2e,3b). Native proteins are also transferred from Hsp70s to Hsp90 in order to regulate protein activity. Nuclear receptors (e.g. steroid hormone receptors), kinases (e.g. eIF2α-kinase, cyclin-dependent kinases), transcription factors (e.g. p53, HSF-1, pRb) and a diverse set of other proteins are examples where Hsp70 and Hsp90 together control the biological activity of target proteins through transient interactions[80]. If the handover to the Hsp90 chaperone is compromised, substrates can either rebind to Hsp70 or be targeted for degradation in an Hsp70-dependent manner. The co-chaperone CHIP[81] is an E3 ubiquitin-protein ligase, which competes with Hop for binding to the C terminus of Hsp70 and Hsp90 (REF.[82]) via its tetratricopeptide repeat domain (FIG. 3b). CHIP ubiquitylates the Hsp70-bound substrates and target them for degradation via the proteasome[83–85], thus connecting Hsp70 function to proteolysis (FIGS 1,3b). The ubiquitylation of Hsp70 substrates is believed to occur in a stochastic manner, preferentially targeting for degradation those substrates that spend a long time bound to Hsp70 and presumably cannot be refolded or transferred to Hsp90, for example, when Hsp90 is inhibited[82,84,85].

Hsp70 during protein translocation. Newly synthesized proteins destined for mitochondria, chloroplasts and the ER are guided to the respective membrane translocons via co-translational (predominantly to the ER) and post-translational (predominantly to mitochondria and chloroplasts) targeting pathways[3]. Hsp70 binds to the cytosolic precursors of these proteins to keep them in an unfolded state until they become captured by dedicated targeting machineries for delivery to the organelle-specific translocon channels in the outer membranes of mitochondria and chloroplasts and the ER membrane[3]. On the other side of the organelle membranes, the translocating polypeptides destined for the ER lumen, mitochondrial matrix and chloroplast stroma then bind to compartment-specific, translocon-associated Hsp70s that help to import the polypeptides and facilitate folding (FIG. 4a; insertion into the outer and inner membranes of mitochondria and chloroplasts or into the intermembrane space does not involve Hsp70s and is thus not discussed in this Review, see REFS[86,87]). The required pulling forces are generated as a result of the low intrinsic entropy state created by the limited freedom of movement of the Hsp70 molecules that are bound next to the membrane and the translocon. The inward movement of the translocating polypeptide increases the freedom of movement of the substrate-bound Hsp70, thereby increasing its entropy. This then generates a one-way pulling motion, as entropy can only ever spontaneously increase[3,88] (FIG. 4a). Therefore, through mere localized binding, Hsp70 can generate enough force[88] to drive protein translocation without the need for a fulcrum, a point of leverage, used by the chaperone to exert the inward pulling force.

Hsp70 in protein disaggregation. The reactivation of a heat-aggregated protein by Hsp70 was first reported for *E. coli* DnaK[89]. However, further analysis revealed that *E. coli* as well as yeast Hsp70 systems alone have very limited disaggregation ability[90]. Disaggregation is achieved more efficiently when Hsp70 recruits Hsp100-type, ring-shaped hexameric AAA+ disaggregases, for example, bacterial ClpB and yeast Hsp104 (REFS[91,92]), to the surface of protein aggregates (FIG. 3d). Through direct interaction with the regulatory middle domain of Hsp100s[93], Hsp70 is able to activate an ATP hydrolysis-driven mechanism in Hsp100 (REFS[94,95]) to extract trapped polypeptides by threading through a central pore, and thereby resolve aggregated proteins[96,97]. Hsp70 action may also alter the surface of aggregates by exposing or generating disentangled regions of trapped polypeptides for Hsp100 binding[98,99]. Hsp100-type disaggregases are found in bacteria, fungi, plants and unicellular parasites such as trypanosomes and plasmodium, but not in the cytosol or nucleus of metazoa[96].



In metazoa, Hsp70 family members cooperate with a specific subset of JDPs and NEFs to form a protein disaggregation machinery that solubilizes a wide range of amorphous and amyloid-like aggregates[100–103]. The activity of this machinery combats not only protein aggregation induced by acute stress but also chronic protein misfolding and/or the formation of protein aggregates that result from the decline or collapse in protein quality control pathways that occurs during cellular ageing and disease[104–106]. The central regulator of the

◀ Fig. 4 | **Entropic pulling in Hsp70 functions. a** | Translocation of polypeptide through a membrane by entropic pulling. The Hsp70 is targeted to bind to the incoming chain close to the membrane, leading to a restricted conformational freedom and a state of low entropy. Translocation of the polypeptide through the pore by Brownian motion increases the freedom of motion of the bound Hsp70 and thus the entropy of the system, providing a driving force for the reaction. **b** | Disassembly of protein aggregates by the Hsp70 machinery of metazoa through entropic pulling. **c** | Disassembly of clathrin cages by collision-pressure/entropic pulling. Targeted by the J-domain protein (JDP) auxilin (not shown), Hsp70 binds to a specific binding site at the flexible tail of the clathrin heavy chain at the vertices of the clathrin triskelia. Conformational confinement and Brownian motion exert a force on the cage, weakening the interactions and leading to disassembly. The inset shows a close-up view of the clathrin chain highlighting the helical tripod and the C-terminal QLMLT sequence[168]. NBD, nucleotide binding domain; NEF, nucleotide exchange factor; SBDβ, substrate binding domain; SBDα, substrate binding domain α-helical lid. Images in part **c** adapted with permission from REF.[168], Wiley.

heat shock response, the heat shock transcription factor Hsf1, which is also important for upregulating Hsp70, was shown to decrease in activity and/or abundance in ageing cells, especially in neurodegenerative and muscle-degenerative processes[107–109]. Such a decline in the Hsp70 levels could substantially effect cellular protein disaggregation capacity and other protein quality control functions associated with the Hsp70 chaperone system, allowing for aggregation of amyloidogenic proteins such as α-synuclein, Tau, huntingtin and ataxins — causative agents of age-related diseases such as Alzheimer disease, parkinsonism, Huntington disease, spinocerebellar ataxias and certain types of muscle dystrophy — to occur and persist[104–106]. In agreement, a recent study shows that the disaggregase activity of the constitutively expressed metazoan Hsc70 is vital for stress recovery in animals[110] and potentially important for reducing cytotoxicity associated with aggregate persistence. The force required for breaking open aggregates and releasing trapped polypeptides has been proposed to result from the thermodynamic terms of entropic pulling generated by Hsp70 and JDP binding and oligomerizing on the aggregate surface[2,62,100] (FIG. 4b).

Hsp70 activity in the assembly and disassembly of protein complexes. Hsp70 has been proposed to have a role in the assembly of protein complexes, but this role is mechanistically poorly understood and difficult to distinguish from the role of Hsp70 in de novo folding of proteins. For the de novo formation of protein complexes during protein synthesis in the cytosol of yeast cells, the action of Ssb guides the folding of subunit interaction domains and protects aggregation-prone subunits before their assembly into complexes, which occurs co-translationally[111]. Assembly of the tetrameric ERG-type K^+ channel in the ER membrane of *Caenorhabditis elegans* and mammalian cells requires an ER-associated Hsp70 machinery[112]. After translation, the nascent ERG proteins are engaged by DNAJB12 and DNAJB14, which are ER-localized transmembrane JDPs, which activate cytosolic Hsp70 to fold ERG monomers and prevent their degradation by the ER-associated protein degradation (ERAD) machinery. DNAJB12 then tetramerizes the subunits to form a functional K^+ channel, possibly in an Hsp70-independent manner[112] (FIG. 3c).

Several examples demonstrate a role for Hsp70 in dismantling large protein complexes, including viral capsids[113], the DNA pre-replication complex of bacteriophage λ[114] and the clathrin coat in eukaryotic cells[115]. Clathrin coat disassembly is the mechanistically best understood process (FIG. 4c). During endocytosis, the assembly of clathrin triskelion cages into polyhedral lattices drives membrane deformation and vesicle budding. Once fission of the vesicle has occurred, the clathrin cage must be rapidly shed to enable fusion of the vesicle with its target membrane and to recycle coat components for further rounds of vesicle budding. The uncoating of clathrin is mediated by Hsc70 and the specialized JDP auxilin (DNAJC6)[116]. Auxilin binds to clathrin trimers that hold the coat together, while at the same time recruiting Hsc70 (REF.[117]) to the QLMLT recognition motif of clathrin, which triggers cage disassembly[118,119]. Hsc70 binding shifts the equilibrium via conformational selection of clathrin states that are incompatible with the assembled triskelions, thereby disassembling and uncoating the vesicle. Such flexible tethering allows Hsc70s to generate a disassembling force through an intermolecular collision-pressure mechanism, whereby collisions between clathrin-coat walls and Hsc70s drive coats apart[115,120] (FIG. 4c). This molecular kinetics description of coat disassembly is complementary to the thermodynamic terms of entropic pulling, and the action of multiple Hsc70s accelerates the reaction[120].

Regulation by J-domain proteins

All Hsp70 chaperone activities require the regulatory and substrate targeting function of co-chaperones of the JDP family. JDPs are a heterogeneous group of modular multidomain proteins characterized by the J-domain, an α-helical hairpin domain approximately 70 amino acids long that is required to bind to Hsp70s and stimulate their ATPase activity[121].

Domain structure of JDP classes. Historically, JDPs have been divided into three classes (A, B and C) based on their similarity to *E. coli* DnaJ, the paradigm member of this protein family (FIG. 5a–c; Supplementary Table 2). The domain architecture of DnaJ, which defines class A JDPs, comprises an N-terminal J-domain, a glycine-phenylalanine-rich (G/F-rich) linker of unclear function, two C-terminal β-sandwich domains (CTD-I and CTD-II) involved in substrate binding, a zinc-finger-like region (ZFLR) inserted into CTD-I and a C-terminal dimerization domain (e.g. bacterial DnaJ; yeast Ydj1; human DNAJA1,2,3,4) (FIG. 5b). Class B JDPs were originally defined as having an N-terminal J-domain followed by a G/F-rich region and a C-terminal domain[122], which in many cases is homologous to the CTDs of class A JDPs and ends in a dimerization motif (e.g. bacterial CbpA; yeast Sis1; human DNAJB1,4,5,11) (FIG. 5a,b). Class C JDPs share only the J-domain with DnaJ (FIG. 5b). These highly diverse JDPs show drastic variations in length (for example, 100–4,600 amino acids) and associated domain/motif types (e.g. transmembrane helices, tetratricopeptide repeat domains, coiled coils, thioredoxin domains, GTP binding sites and protein kinase domains)[8].

Entropic pulling
A model describing the pulling forces generated by entropy change that are utilized by Hsp70 proteins to unfold protein aggregates, disassemble clathrin cages and translocate protein across membranes.

ERAD
(Endoplasmic reticulum-associated protein degradation). A process that mediates the recognition and retrograde delivery of aberrant (for example, misfolded) proteins from the ER into the cytosol for proteasome-mediated degradation.

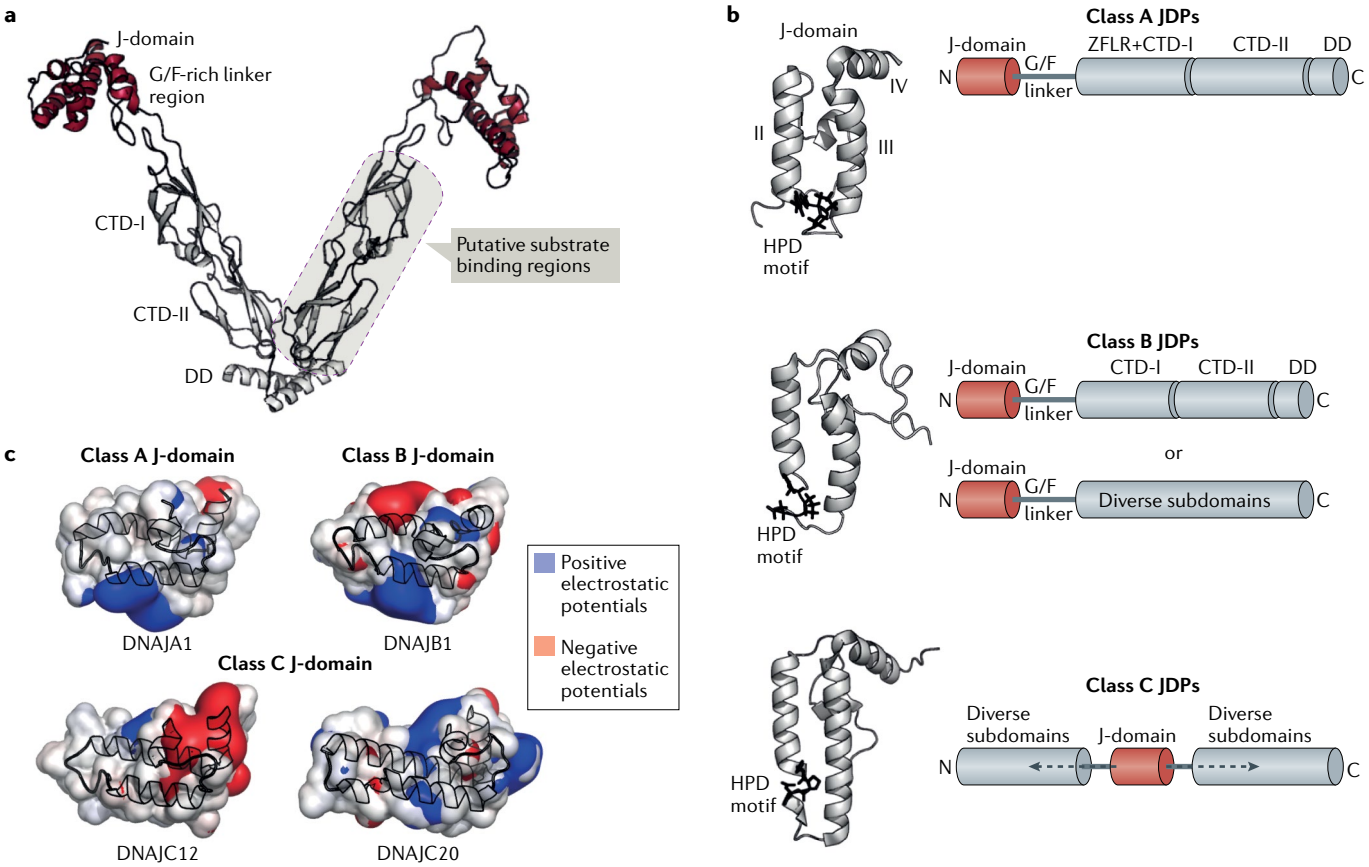


Fig. 5 | **Structure and domain organization of J-domain proteins. a** | Structure of bacterial (*Thermus thermophilus*) J-domain protein (JDP) DnaJ (PDB ID: 4J80), showing the J-domain in red and the potential substrate interaction surfaces highlighted in magenta. **b** | General domain organization of class A (top), class B (middle) and class C (bottom) JDPs with a cartoon representation showing the four-helical structure of the J-domain and highlighting the location of the conserved HPD motif residues. **c** | Electrostatic surface potentials of the J-domains in representative examples from each class of JDP (DNAJA1, PDB ID: 2M6Y; DNAJB1, PDB ID: 1HDJ; DNAJC12, PDB ID: 2CTQ; DNAJC20, PDB ID: 3BV0). CTD, C-terminal domain; DD, dimerization domain; G/F, glycine/phenylalanine; HPD, histidine-proline-aspartate; ZFLR, zinc-finger-like region.

Interaction of J-domain proteins with Hsp70. The interaction of JDPs with Hsp70 involves helices II and III of the J-domain and a connecting loop that contains the universally conserved and functionally essential histidine-proline-aspartate (HPD) motif (FIG. 5). The recently solved X-ray structure of the J-domain of *E. coli* DnaJ in complex with the ATP-bound (open) conformation of DnaK provided insight into the mechanism of J-domain action[123] (FIG. 6). The J-domain binds at the interface between the NBD and the SBDβ on top of the interdomain linker, forming polar and hydrophobic contacts between the J-domain and NBD–SBDβ interfaces as well as between the J-domain and linker–SBDβ interfaces, respectively. The HPD motif interacts with key residues of the allosteric network in Hsp70s, accessing two pathways of interactions that converge in the catalytic centre for ATP hydrolysis, explaining the ability of the J-domain to promote ATP hydrolysis. In addition, the J-domain contacts a series of residues in the SBDβ that are connected to the signal transmission pathway from the substrate to the NBD (FIG. 6a). Interfering with these contacts compromises the ability of substrates to stimulate the ATPase activity of DnaK in synergism with the J-domain binding. In essence, these allosteric pathways involving the J-domain couple substrate binding to ATP hydrolysis and efficient trapping of the substrate[123] (FIG. 6b). These investigations may open new routes for drug development to positively or negatively modulate the Hsp70 activity for treatment of neurodegenerative disease or cancer.

The residues in the J-domain and DnaK that form the interaction interface are highly conserved in evolution, whereas the electrostatic surface potentials of other parts of the J-domains show some differences (FIG. 5c). This conservation suggests that this mechanism is principally operational in all Hsp70–J-domain pairs, and explains why many mutations in the J-domain are linked to human diseases (Supplementary Table 1). J-domains known to interact with a specialized Hsp70, however, exhibit striking sequence divergence at distinct positions, with concomitant alterations in their cooperating Hsp70 partner. Hsp70 homologues that do not have a classical J-domain partner such as the ribosome-associated HspA14 (Ssz in yeast) or the cytosolic Hsp110 and ER Hsp170 members, which are both homologues of Hsp70s but act as NEFs for Hsp70s, show no

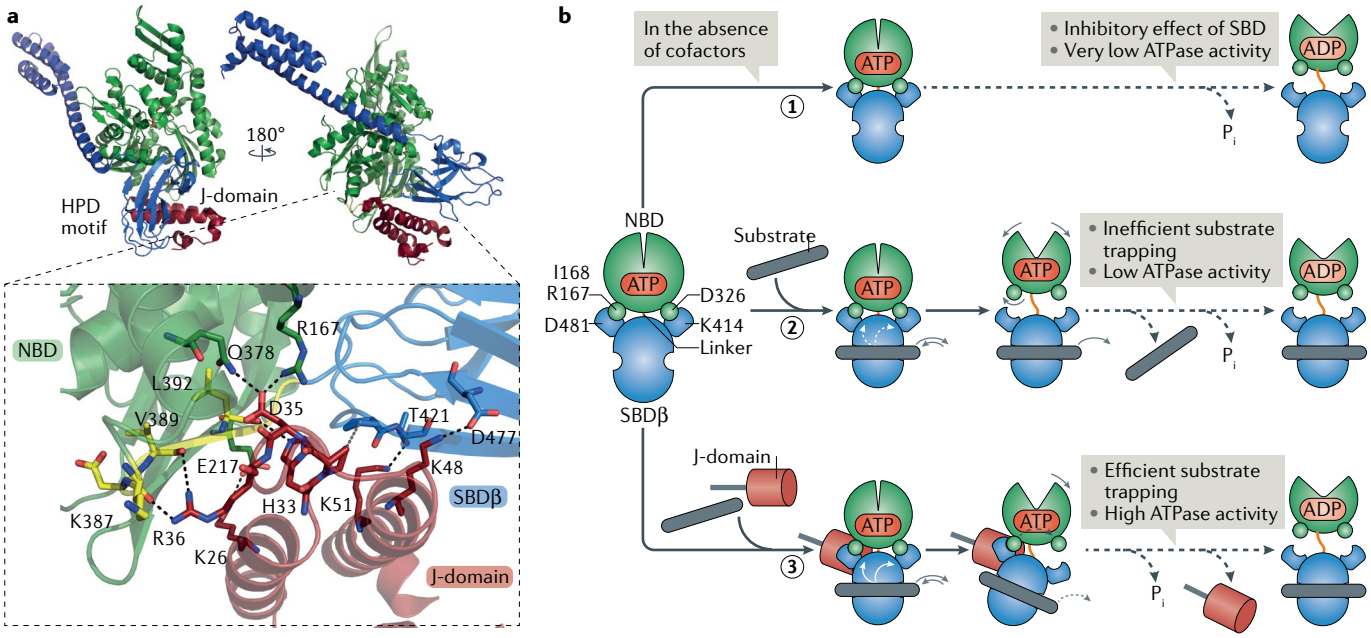


Fig. 6 | **J-domain protein binding and activation of Hsp70. a** | Structure of the J-domain of *Escherichia coli* DnaJ in complex with *E. coli* DnaK in the ATP-bound open conformation (PDB ID: 5NRO). Zoom image shows polar contacts between residues of the J-domain and residues of DnaK NBD (green), substrate binding domain (SBDβ, blue) and the conserved linker (yellow). **b** | Allosteric mechanism of substrate and J-domain regulated ATPase activity of Hsp70s. ATP binding induces the nucleotide binding domain (NBD) lobes to rotate into a conformation that keeps the catalytic residues in an ATP hydrolysis-incompetent conformation. The SBDβ docks onto the lobe-rotated NBD and clamps the conformation primarily through contacts of residues D481 with I168 and K414 with D326. (Step 1) In the absence of substrate and J-domain protein (JDP), the SBDβ only occasionally dissociates allowing back rotation of the lobes, resulting in very low basal ATPase activity of Hsp70s. (Step 2) Substrates stimulate the dissociation of the SBDβ from the NBD, thereby enhancing the ATPase activity. The linker, however, frequently slips out of the lower crevice, failing to arrest the lobes in the ATP hydrolysis-competent conformation. This results in only marginally increased ATPase activity and a fraction of the substrate (grey) dissociating before ATP hydrolysis. (Step 3) The J-domain makes the substrate-stimulated SBDβ dissociation more efficient, but prevents slipping of the linker from the lower crevice. This arrests the back-rotating NBD lobes in the ATP hydrolysis-competent conformation leading to efficient trapping of the substrate. HPD, histidine-proline-aspartate.

conservation in the interacting residues[123]. Interestingly, the paralogues of JDPs from the same organism thought to interact with the same Hsp70 family member deviate from the consensus sequence of interacting amino acids in a few residues[123] and show additional changes in exposed electrostatic potentials along helices II and III and the connecting loop[90]. This opens the possibility for a modulation of the J-domain–Hsp70 interaction, thereby establishing a gradient of interaction preferences within the network of JDPs.

Regions outside the J-domain can also interact with Hsp70, allowing for tuning of the allosteric regulation[124–126]. For example, the EEVD motif that is at the extreme C-terminal end of many eukaryotic cytosolic Hsp70s interacts with the CTDs of specific JDPs in yeast (Sis1) and humans (DNAJA2, DNAJB1). This interaction seems to activate the cooperative chaperone function of the JDP–Hsp70 machine[125,126]. Hsp70 proteins with a deleted EEVD motif cannot refold denatured firefly luciferase, a misfolded model substrate, in cooperation with the aforementioned JDPs, but function with other JDPs (Ydj1 and Xdj1 in yeast) that do not require the EEVD–JDP interaction for activity[125,126]. The interaction between Hsp70s and individual JDPs from classes A and B are therefore differentially regulated.

Interaction of J-domain proteins with substrates. The association of JDPs with substrates initiates the entire Hsp70 chaperone cycle and therefore is of crucial importance (FIG. 2d). The presence of JDPs has been shown to increase the number of Hsp70 binding sites in a given substrate[127,128]. This is most likely due to an increase in apparent affinity of Hsp70 (REF.[23]) to suboptimal sites and induced conformational changes in the substrate[50,64]. How the ATP-independent JDPs may affect the conformation of substrates is not clear, but it can be speculated that they selectively bind to those conformations that fit best their substrate interaction surface, thus selecting a subset of conformations from various conformations that coexist in equilibrium and interconvert constantly. This then implies that JDPs are capable of preselection and initial remodelling of substrates so that they are more accessible for the Hsp70 partner. Substrate handover from JDP to Hsp70 then requires that accessible chaperone binding motifs are within the spatial vicinity of the substrate polypeptide, to enable simultaneous binding of the substrate to a JDP and an Hsp70.

Class A JDPs and the subset of class B JDPs that have a homologous CTD are expected to bind substrates in a largely similar manner. Peptide library scanning experiments on bacterial DnaJ revealed that this class A

JDP recognizes a linear sequence motif consisting of a core of eight residues enriched in hydrophobic, in particular aromatic, residues[129]. A similar preference for aromatic residues was found for class A orthologues in yeast (Ydj1)[130,131] and human (DNAJA1)[132]. Class B Sis1 (yeast)[131] and ERdj4 (mammalian; DNAJB9)[133] also bind to peptides enriched in aromatic and hydrophobic residues, but display differences in selectivity in terms of relative contribution of positively charged versus large aliphatic versus aromatic residues.

The nature of the substrate binding sites within JDPs of classes A and B remains uncertain. A crystal structure of the CTD and ZFLR of yeast Ydj1 in complex with a substrate peptide revealed that the peptide binds in a groove formed between the two β-sheets of the β-sandwich of CTD-I, extending one of the two sheets[130]. Although this binding site was confirmed by mutational analyses of Ydj1 and Sis1 (REFS[134,135]), it is surprising that the binding affinity depends on polar contacts between the peptide backbone and the binding groove, but does not involve the only two exposed aromatic residues in the peptide. This is difficult to reconcile with the apparent binding preference of bacterial DnaJ (orthologue of yeast Ydj1) for aromatic residues and the finding that DnaJ does not distinguish between natural and unnatural peptides made of L and D-amino acids[129,136]. Further, a hydrogen exchange mass spectrometry analysis also shows that backbones of peptide substrates are not involved in DnaJ binding[64].

Compared with peptides that display binding affinities in the micromolar range, protein substrates bind to class A and B JDPs with nanomolar affinities[130,136,137], indicating that JDPs bind proteins by avidity through cooperative interaction of several low-affinity sites[138]. Similar to CTD- I, CTD-II could potentially bind to substrates due to the high structural homology between the two domains. Moreover, class A and B JDPs typically form homodimers in solution, thus potentially presenting four substrate-interacting surfaces. Moreover, the G/F-rich region adjacent to the J-domain may not only serve as flexible linker but also participate directly in substrate binding. Deletion or mutation of this conserved region reduces the affinity of JDPs for partially and fully folded substrates[139] and functionality in vivo[140,141]. The ZFLR of class A JDPs also contributes to substrate binding, as the ability of Hsp70–JDPs to prevent aggregation of misfolded proteins in both bacteria and yeast is lost when the first of the two Zn^{2+}-binding sites is mutated[142–144]. The second Zn^{2+}-binding site seems to be important for substrate transfer to Hsp70 (REFS[142,145]). Thus, the emerging picture is that class A and B JDPs have several distinct low-affinity interaction surfaces that enable highly dynamic binding to several different recognition motifs in the protein substrate. Such modes of interaction may be important to distinguish between native and misfolded or aggregated proteins, including amyloid aggregates, as the former displays only isolated exposed hydrophobic patches.

Despite many similarities in their mode of substrate binding, class A and B JDPs recognize distinct features in amorphous protein aggregates. Human DNAJA2 preferentially binds to and assists solubilization of small aggregates (approximately 200–700 kDa), whereas DNAJB1 prefers larger aggregates (approximately 700–5,000 kDa)[90,100]. Some specialized class B JDPs (DNAJB6, DNAJB8) contain a serine/threonine-rich region between the G/F-rich region and the CTD. This S/T-rich region is essential for the interaction with polyQ proteins and amyloid-β peptide (Aβ42), preventing the formation and elongation of amyloid fibrils[146–148]. Interestingly, DNAJB6 and DNAJB8 can function as stand-alone chaperones, and unlike other JDPs can achieve aggregation prevention of polyQ proteins without the aid of Hsp70 chaperones. Evidence for the G/F-rich region being required for the prevention of polyQ aggregation was also provided by the finding that many DNAJB6 mutations in this region display reduced aggregation prevention activities and thus are associated with heritable muscular diseases[149,150] (Supplementary Table 1). However, the structural details and exact mode of operation of these chaperones are unknown.

Class C members represent a multifunctional group of proteins able to couple with Hsp70 to exert activities not necessarily associated with general protein folding (for example, the pre-mRNA splicing factor DNAJC17/Cwc23). Owing to their heterogeneity, no general mode of interaction with substrates can be described for class C members, and they typically have small and unique interactomes (for example, clathrin-binding DNAJC6, also known as auxilin). Interestingly, some class C JDPs can fulfil cellular functions even in the absence of an intact J-domain (for example, Cwc23, whose J-domain, although functional, is dispensable for its function in pre-mRNA splicing[151]), suggesting that the J-domain was a late evolutionary addition to the protein.

Cooperation between J-domain proteins. Recent studies have revealed that class A and B JDPs in eukaryotes cooperate to increase the efficacy of Hsp70-mediated disaggregation of amorphous aggregates[90,100]. This cooperation is not required for Hsp70-mediated disaggregation of amyloid fibrils[102] or refolding of soluble misfolded proteins[100]. The two classes of JDP form transient complexes via intermolecular J-domain–CTD-I contacts, and structural (for example, electrostatic) features at these contacts ensure correct pairing of JDP family members[90]. Moreover, the J-domain surface involved in the interaction with the CTD-I is distinct from the surface interacting with Hsp70. Therefore, even a minimal complex between one class A JDP dimer and one class B JDP dimer would potentially present four J-domains, conceivably for the recruitment of multiple Hsp70s to local sites on protein aggregates, increasing Hsp70 activity. Most eukaryotic organisms contain several class A and B members that could potentially pair into distinct complexes to form a combinatorial network that considerably expands the range of substrates that can be targeted by this co-chaperone family.

Regulation by nucleotide exchange factors

NEFs mediate the opening of the Hsp70 nucleotide binding cleft to facilitate the release of ADP, which in turn allows the rebinding of ATP and promotes the release of substrates from Hsp70 (FIG. 2d). Four structurally

PolyQ proteins
Proteins containing a pathogenic elongation of a polyglutamine stretch caused by an increased number of CAG trinucleotide repeats. PolyQ expansions in huntingtin result in aggregation associated with Huntington disease. The length of the polyQ repeat is critical for disease onset.

Amyloid fibrils
Protein fibril aggregates that can form in vitro or in vivo and contain characteristic cross-β-sheet motifs. Their formation is associated with neurodegeneration and diseases such as Alzheimer disease, Parkinson disease, Creutzfeldt–Jakob disease and type II diabetes.

unrelated protein families perform this function using distinct mechanisms, suggesting that proteins with nucleotide exchange activity have arisen multiple times during evolution (FIG. 7a; Supplementary Table 2). In prokaryotes, mitochondria and chloroplasts, nucleotide exchange in Hsp70s is regulated by GrpE, a homodimer that consists of an unusually long α-helical dimerization domain at the N terminus and a C-terminal β-sheet domain[152]. The GrpE dimer interacts with a single Hsp70 molecule, inserting its β-sheet domain to drive a wedge into the NBD of Hsp70 (REF.[152]). This complex formation induces a tilt of lobe subdomain IIB that results in an opening of the nucleotide binding cleft, thereby greatly reducing the affinity for nucleotide (FIG. 7b). The interaction also induces asymmetry in the GrpE dimer, explaining why the dimer can only bind one Hsp70 molecule at a time.

In the eukaryotic cytosol, nucleotide exchange is performed by three types of NEF belonging to the Bag, Hsp110 and Armadillo families. Although being structurally distinct, with little or no homology between them, and differing in their mode of binding to Hsp70s, they all function to stabilize the open conformation of the Hsp70 NBD. Bag-type NEFs contain a conserved three-helix bundle BAG (Bcl2-associated athanogene) domain[153] that binds to the IB and mainly IIB lobe subdomains of the Hsp70 NBD. This interaction locks the NBD in a conformation similar to that of DnaK in complex with GrpE, in which the IIB subdomain is tilted outwards[154] (FIG. 7b).

Hsp110-type NEFs belong to the Hsp70 superfamily, having high sequence similarity in the NBD whereas the SBD is less conserved. The structures of Hsp110s and the ER-localized NEF Grp170 are highly similar to the open conformation of Hsp70, with addition of a characteristic insertion of variable length in the β-sandwich and the C-terminal helical lid domain of the SBD. Hsp110s catalyse nucleotide exchange by a head-to-head interaction of their NBD with the NBD of Hsp70s, attaching to the side of subdomain IIB while anchoring to the remainder of the NBD[155,156], and tilting subdomain IIB outwards in a manner similar to GrpE (FIG. 7b).

Armadillo-type NEFs have a core domain composed of four α-helical Armadillo repeats, which is sufficient for Hsp70 binding. The ER-localized Armadillo-type NEF Sil1 wraps around subdomain IIB of the Hsp70 NBD and rotates this subdomain around one of its helices[157] (FIG. 7b).

In addition to inducing ADP release, enabling ATP binding and substrate dissociation, some NEFs prevent

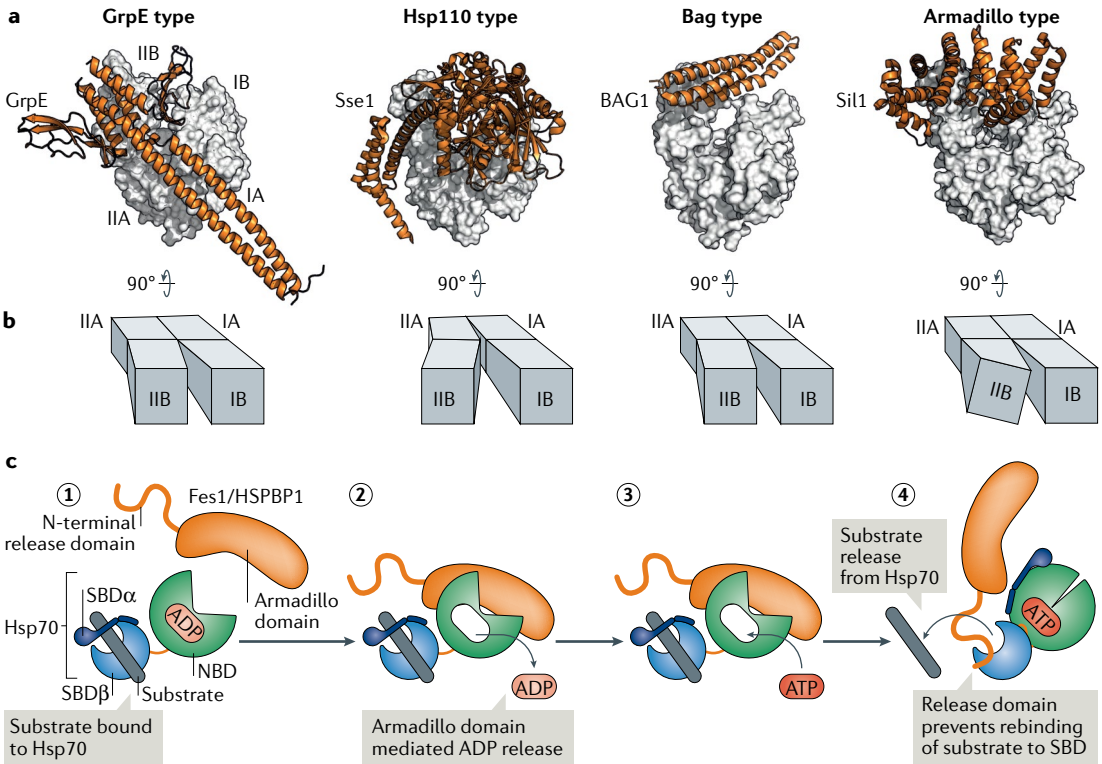


Fig. 7 | **Nucleotide exchange factors of the Hsp70 system. a** | Structural insights into the nucleotide exchange function of the different nucleotide exchange factors (NEFs). Crystal structures of GrpE-DnaK-NBD (PDB ID: 1DKG), Sse1-Ssa1-NBD (PDB ID: 3D2F), Bag-Hsc70-NBD (PDB ID: 1HX1) and Sil1-BiP-NBD (PDB ID: 3QML); Hsp70-NBD in surface representation (white) and NEF in orange. **b** | The relative motion of lobes I and II of the nucleotide binding domain upon NEF binding are shown schematically. **c** | Fes1/HSPBP1 favours substrate release by preventing rebinding of the substrate after nucleotide exchange. (Step 1) Recruitment of Fes1/HSPBP1 to substrate bound Hsp70. (Step 2) The Armadillo domain binds to lobe subdomain IIB to open the nucleotide binding cleft. (Step 3) After ATP binding, opening of the substrate binding cleft and substrate dissociation, (Step 4) the N-terminal unstructured segment of Fes1/HSPBP1 binds into the substrate binding pocket, preventing rebinding of the substrate. SBDα, substrate binding domain α-helical lid; SBDβ, substrate binding domain.

Ubiquitin adaptor
Multidomain protein that can bind ubiquitin chains on a substrate and the proteasome or autophagosome. p62 has a C-terminal domain that binds to polyubiquitylated substrates to deliver them to the proteasome (via its PB1 domain) or autophagosome (via its LIR domain).

the unproductive rebinding of the released substrate by directly blocking the Hsp70 substrate binding pocket[158,159] (FIG. 7c). Blocking substrate rebinding can be achieved as these NEFs contain structural elements that mimic motifs that are recognized by Hsp70s (e.g. the N-terminal release domain (RD) in Armadillo-type yeast Fes1 and human HSPBP1 (REFS[158,159]) and possibly the N-terminal helical extension in GrpE[152,160]), and therefore can occupy the substrate binding sites on the chaperone, preventing it from rebinding.

Nine different NEFs are expressed in the cytosol and nucleus of human cells that differ in affinity for Hsp70 and potency for nucleotide and peptide release, with Bag family members being the most efficient (ADP release rates enhanced up to 100-fold[161]) and Armadillo-type members being the least efficient (5- to 10-fold[162]). Such differences could potentially fine-tune the time needed for Hsp70 to complete one chaperone cycle to optimize handling of different substrates. Of note, some Hsp70s may even function independent of NEFs as they have high intrinsic ADP dissociation rates[163].

NEFs, in part, can control the precise targeting and timing of nucleotide exchange and concomitant substrate release from Hsp70s. Bag-type NEFs, for example, contain additional interaction domains that could localize them to specific subcellular structures, thereby enabling the timed transfer of substrates from Hsp70 to other protein quality-control systems. Such a mechanism is well documented for Bag family members. For example, BAG1 contains an integral ubiquitin-like domain that serves as a proteasomal targeting signal, promoting the transfer of Hsp70-bound substrates to the 26S proteasome for degradation[164], and BAG3 instead triggers the recruitment of the autophagic ubiquitin adaptor p62, and thus facilitates Hsp70-assisted substrate degradation through the autophagosome–lysosome pathway[165,166].

In light of these important observations, a fundamental question remains unanswered as to what determines the NEF selection for a given Hsp70–client complex, which potentially could determine the fate of the protein.

Conclusions and future perspectives

The multitude of functions performed by Hsp70s are specified through multilayered networks of JDP and NEF co-chaperones and the integration of other cooperating protein folding machineries, including Hsp90 and Hsp100, and cellular proteolytic systems. Across all kingdoms of life, cells encode multiple Hsp70 family members. The differential expression, abundance, subcellular localization and substrate specificity for distinct Hsp70 isoforms all contribute to diversification of the cellular functions of Hsp70s. Despite major progress made in recent years, not all known family members have been fully characterized, and thus a full appreciation of the spectrum of pathophysiological functions of the Hsp70 chaperones is still missing. Many mechanistic details of how the chaperone networks operate and are tuned to the cell needs under various growth and stress conditions also remain elusive, awaiting experimental investigation. How is the Hsp70 network altered during development or in pathological conditions such as cancer, neurodegeneration, autoimmunity or infections? For example, it is known that some members of the Hsp70 and JDP families are upregulated in cancer cells and upregulation of Hsp70 is associated with poor prognosis. But the mechanistic details and consequences of the remodelling of the Hsp70 network are still unclear. Therefore, a detailed analysis of the levels and activities of Hsp70 network components in these conditions and their interaction partners and substrates is necessary to elucidate which pathways need chaperone action and pose as potential drug targets. It will also be important to understand how mutations in genes encoding components of the Hsp70 system (Supplementary Table 1) can result in the development of a variety of human diseases.

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