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Molecular chaperones in protein folding and proteostasis

F. Ulrich Hartl¹, Andreas Bracher¹ & Manajit Hayer-Hartl¹

Most proteins must fold into defined three-dimensional structures to gain functional activity. But in the cellular environment, newly synthesized proteins are at great risk of aberrant folding and aggregation, potentially forming toxic species. To avoid these dangers, cells invest in a complex network of molecular chaperones, which use ingenious mechanisms to prevent aggregation and promote efficient folding. Because protein molecules are highly dynamic, constant chaperone surveillance is required to ensure protein homeostasis (proteostasis). Recent advances suggest that an age-related decline in proteostasis capacity allows the manifestation of various protein-aggregation diseases, including Alzheimer's disease and Parkinson's disease. Interventions in these and numerous other pathological states may spring from a detailed understanding of the pathways underlying proteome maintenance.

Proteins are the most versatile and structurally complex biological macromolecules. They are involved in almost every biological process. Mammalian cells typically express in excess of 10,000 different protein species, which are synthesized on ribosomes as linear chains of up to several thousand amino acids. To function, these chains must generally fold into their 'native state', an ensemble of a few closely related three-dimensional structures^{1,2}. How this is accomplished and how cells ensure the conformational integrity of their proteome in the face of acute and chronic challenges constitute one of the most fundamental and medically relevant problems in biology.

Central to this problem is that proteins must retain conformational flexibility to function, and thus are only marginally thermodynamically stable in their physiological environment. A substantial fraction of all proteins in eukaryotic cells (20–30% of the total in mammalian cells) even seem to be inherently devoid of any ordered three-dimensional structure and adopt folded conformations only after interaction with binding partners³. Aberrant behaviour of some of these metastable proteins, such as tau and α -synuclein, can give rise to the formation of fibrillar aggregates that are associated with dementia and Parkinson's disease. Thus, protein quality control and the maintenance of proteome homeostasis (known as proteostasis) are crucial for cellular and organismal health. Proteostasis is achieved by an integrated network of several hundred proteins⁴, including, most prominently, molecular chaperones and their regulators, which assist in *de novo* folding or refolding, and the ubiquitin–proteasome system (UPS) and autophagy system, which mediate the timely removal of irreversibly misfolded and aggregated proteins. Deficiencies in proteostasis have been shown to facilitate the manifestation or progression of numerous diseases, such as neurodegeneration and dementia, type 2 diabetes, peripheral amyloidosis, lysosomal storage disease, cystic fibrosis, cancer and cardiovascular disease. A major risk factor for many of these ailments is advanced age. Indeed, studies in model organisms indicate that ageing is linked to a gradual decline in cellular proteostasis capacity^{5,6}.

Here we discuss recent insights into the mechanisms of chaperone-assisted protein folding and proteome maintenance. We focus on how proteins use the chaperone machinery to navigate successfully the complex folding-energy landscape in the crowded cellular environment. Understanding these reactions will guide future efforts to define the proteostasis network as a target for pharmacological intervention in diseases of aberrant protein folding.

Fundamental role of molecular chaperones

Many small proteins refold after their removal from denaturant *in vitro*, in the absence of other components or an energy source. This signifies that the amino-acid sequence, encoded in the DNA, contains all of the necessary information to specify the three-dimensional structure of a protein¹. However, research over the past couple of decades has firmly established that in the cellular environment, many proteins require molecular chaperones to fold efficiently and on a biologically relevant timescale⁷. Why is this extra layer of complexity necessary?

Although small proteins may fold at very fast speeds⁸ (within microseconds), in dilute buffer solutions, larger, multidomain proteins may take minutes to hours to fold⁹, and often even fail to reach their native states *in vitro*. The folding of such proteins becomes considerably more challenging *in vivo*, because the cellular environment is highly crowded, with total cytosolic protein reaching concentrations of 300–400 g l⁻¹. The resultant excluded volume effects, although enhancing the functional interactions between macromolecules, also strongly increase the tendency of non-native and structurally flexible proteins to aggregate¹⁰. It seems likely, therefore, that the fundamental requirement for molecular chaperones arose very early during the evolution of densely crowded cells, owing to the need to minimize protein aggregation during folding and maintain proteins in soluble, yet conformationally dynamic states. Moreover, as mutations often disrupt the ability of a protein to adopt a stable fold¹¹, it follows that the chaperone system provides a crucial buffer, allowing the evolution of new protein functions and phenotypic traits^{11,12}.

Some basics on protein folding and how it can go awry

Because the number of possible conformations a protein chain can adopt is very large, folding reactions are highly complex and heterogeneous, relying on the cooperation of many weak, non-covalent interactions. In the case of soluble proteins, hydrophobic forces are particularly important in driving chain collapse and the burial of non-polar amino-acid residues within the interior of the protein (see ref. 13 for a discussion of membrane protein folding). Considerable progress has been made in recent years in understanding these reactions through biophysical experiments and theoretical analyses^{1,2}. In the current model, polypeptide chains are thought to explore funnel-shaped potential energy surfaces as they progress, along several

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downhill routes, towards the native structure (Fig. 1). Chain collapse and the progressive increase in the number of native interactions rapidly restrict the conformational space that needs to be searched en route to the native state. However, the free-energy surface that must be navigated is often rugged, which means that the molecules must cross substantial kinetic barriers during folding. As a consequence, partially folded states may become transiently populated as kinetically trapped species. Such folding intermediates are the rule for proteins larger than 100 amino acids (~90% of all proteins in a cell), which have a strong tendency to undergo rapid hydrophobic collapse into compact globular conformations¹. The collapse may lead either to disorganized globules lacking specific contacts and retaining large configurational entropy or to intermediates that may be stabilized by non-native interactions (misfolded states). In the former case, the search for crucial native contacts within the globule will limit folding speed, whereas in the latter, the breakage of non-native contacts may be rate-limiting¹ (Fig. 1). The propensity of proteins to populate globular intermediates with a high degree of flexibility may increase with larger, topologically more complex domain folds that are stabilized by many long-range interactions (such as α/β domain architectures). Such proteins are often highly chaperone dependent¹⁴.

Partially folded or misfolded states are problematic because they tend to aggregate in a concentration-dependent manner (Fig. 1). This is due to the fact that these forms typically expose hydrophobic amino-acid residues and regions of unstructured polypeptide backbone to the solvent — features that become buried in the native state¹⁵. Like intramolecular folding, aggregation is largely driven by hydrophobic forces and primarily results in amorphous structures (Fig. 1). Alternatively, fibrillar aggregates called amyloid may form, defined by β -strands that run perpendicular to the long fibril axis (cross- β structure). Although many proteins can adopt these highly ordered, thermodynamically stable structures under conditions *in vitro*¹⁶, the formation of these aggregates *in vivo* is strongly restricted by the chaperone machinery, suggesting that they may become more widespread under stress or when protein quality control fails. Importantly, the formation of fibrillar aggregates is often accompanied by the formation of soluble oligomeric states, which are thought to have key roles in diseases of aberrant folding¹⁶ (Fig. 1). The toxicity of these less ordered and rather heterogeneous forms has been suggested to correlate with the exposure of sticky, hydrophobic surfaces and accessible peptide-backbone structure that is not yet integrated into a stable cross- β core¹⁷. The soluble oligomers must undergo considerable rearrangement to form fibrils, the thermodynamic end state of the aggregation process, and may thus be comparable to the kinetically trapped intermediates in folding (Fig. 1). Notably, some common structural epitopes have been detected on the prefibrillar oligomers of different polypeptides¹⁸, but how these features are linked with toxicity is not yet understood. Such information is urgently needed to develop treatments for the numerous pathological states associated with protein aggregation.

Major chaperone classes

We define a molecular chaperone as any protein that interacts with, stabilizes or helps another protein to acquire its functionally active conformation, without being present in its final structure¹⁹. Several different classes of structurally unrelated chaperones exist in cells, forming cooperative pathways and networks. Members of these protein families are often known as stress proteins or heat-shock proteins (HSPs), as they are upregulated under conditions of stress in which the concentrations of aggregation-prone folding intermediates increase. Chaperones are usually classified according to their molecular weight (HSP40, HSP60, HSP70, HSP90, HSP100 and the small HSPs). They are involved in a multitude of proteome-maintenance functions, including *de novo* folding, refolding of stress-denatured proteins, oligomeric assembly, protein trafficking and assistance in proteolytic degradation. The chaperones that participate broadly in *de novo* protein folding and refolding, such as the HSP70s, HSP90s and the chaperonins (HSP60s), are multicomponent molecular machines that promote folding through

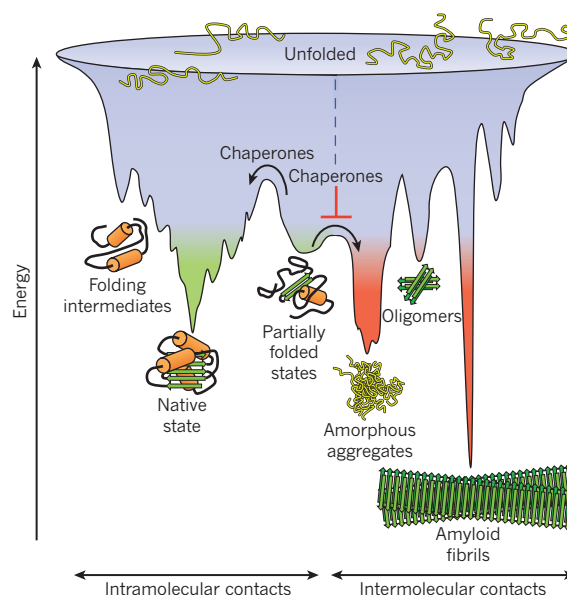


Figure 1 | Competing reactions of protein folding and aggregation. Scheme of the funnel-shaped free-energy surface that proteins explore as they move towards the native state (green) by forming intramolecular contacts (modified from refs 19 and 95). The ruggedness of the free-energy landscape results in the accumulation of kinetically trapped conformations that need to traverse free-energy barriers to reach a favourable downhill path. *In vivo*, these steps may be accelerated by chaperones^{39,41,42}. When several molecules fold simultaneously in the same compartment, the free-energy surface of folding may overlap with that of intermolecular aggregation, resulting in the formation of amorphous aggregates, toxic oligomers or ordered amyloid fibrils (red). Fibrillar aggregation typically occurs by nucleation-dependent polymerization. It may initiate from intermediates populated during *de novo* folding or after destabilization of the native state (partially folded states) and is normally prevented by molecular chaperones.

ATP- and cofactor-regulated binding and release cycles. They typically recognize hydrophobic amino-acid side chains exposed by non-native proteins and may functionally cooperate with ATP-independent chaperones, such as the small HSPs, which function as ‘holdases’, buffering aggregation.

In the ATP-dependent mechanism of chaperone action, *de novo* folding and protein refolding is promoted through kinetic partitioning (Fig. 2). Chaperone binding (or rebinding) to hydrophobic regions of a non-native protein transiently blocks aggregation; ATP-triggered release allows folding to proceed. Importantly, although the HSP70s and the chaperonins both operate by this basic mechanism, they differ fundamentally in that the former (like all other ATP-dependent chaperones) release the substrate protein for folding into bulk solution, whereas the cylindrical chaperonins allow the folding of single protein molecules enclosed in a cage. The two systems act sequentially, whereby HSP70 interacts upstream with nascent and newly synthesized polypeptides and the chaperonins function downstream in the final folding of those proteins that fail to reach native state by cycling on HSP70 alone^{20,21} (Figs 2 and 3). In the following sections, we will use the HSP70, chaperonin and HSP90 models to illustrate the basic mechanisms of the major cytosolic protein-folding machines. Client-specific chaperones that function downstream of folding in mediating the assembly of oligomeric complexes are not discussed (see, for example, refs 22 and 23).

The HSP70 system

The constitutively expressed (HSC70, also known as HSPA8) and stress-inducible forms of HSP70 are central players in protein folding and proteostasis control. Increasing HSP70 levels has also proven effective in preventing toxic protein aggregation in disease models²⁴.

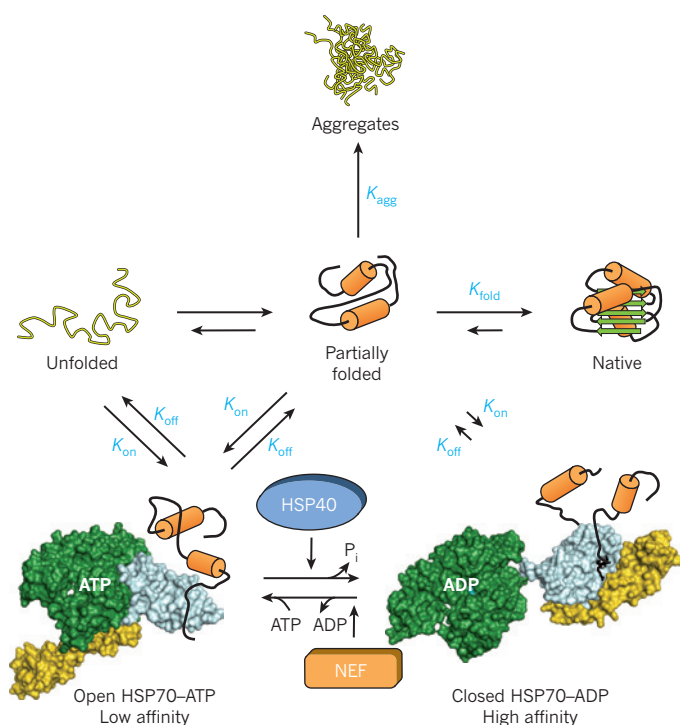


Figure 2 | The HSP70 chaperone cycle. HSP70 is switched between high- and low-affinity states for unfolded and partially folded protein by ATP binding and hydrolysis. Unfolded and partially folded substrate (nascent chain or stress-denatured protein), exposing hydrophobic peptide segments, is delivered to ATP-bound HSP70 (open; low substrate affinity with high on-rates and off-rates) by one of several HSP40 cofactors. The hydrolysis of ATP, which is accelerated by HSP40, results in closing of the α -helical lid of the peptide-binding domain (yellow) and tight binding of substrate by HSP70 (closed; high affinity with low on-rates and off-rates). Dissociation of ADP catalysed by one of several nucleotide-exchange factors (NEFs) is required for recycling. Opening of the α -helical lid, induced by ATP binding, results in substrate release. Folding is promoted and aggregation is prevented when both the folding rate constant (K_{fold}) is greater than the association constant (K_{on}) for chaperone binding (or rebinding) of partially folded states, and K_{on} is greater than intermolecular association by the higher-order aggregation rate constant K_{agg} ($K_{\text{fold}} > K_{\text{on}} > K_{\text{agg}}$) (kinetic partitioning). For proteins that populate misfolded states, K_{on} may be greater than K_{fold} ($K_{\text{fold}} \leq K_{\text{on}} > K_{\text{agg}}$). These proteins are stabilized by HSP70 in a non-aggregated state, but require transfer into the chaperonin cage for folding^{14,20}. After conformational stress, K_{agg} may become faster than K_{on} , and aggregation occurs ($K_{\text{agg}} > K_{\text{on}} \geq K_{\text{fold}}$), unless chaperone expression is induced via the stress-response pathway. Structures in this figure relate to Protein Data Bank (PDB) accession codes 1DKG, 1DKZ, 2KHO and 2QXL. P_i, inorganic phosphate.

The ATP-dependent reaction cycle of HSP70 is regulated by chaperones of the HSP40 (also known as DnaJ) family and nucleotide-exchange factors^{25,26}. Some of these factors are also involved in linking chaperone functions with the UPS and autophagy for the removal of misfolded proteins²⁷. Binding and release by HSP70 is achieved through the allosteric coupling of a conserved amino-terminal ATPase domain with a carboxy-terminal peptide-binding domain, the latter consisting of a β -sandwich subdomain and an α -helical lid segment⁵ (Fig. 2). The β -sandwich recognizes extended, ~seven-residue segments enriched in hydrophobic amino acids, preferentially when they are framed by positively charged residues³⁸. Such segments occur on average every 50–100 amino acids in proteins, and the exposure of these fragments correlates with the aggregation propensity of the protein²⁹. The α -helical lid and a conformational change in the β -sandwich domain regulate the affinity state for the peptide in an ATP-dependent manner⁵. In the ATP-bound state, the lid adopts an open conformation, resulting in high on

rates and off rates for the peptide. Hydrolysis of ATP to ADP is strongly accelerated by HSP40, leading to lid closure and stable peptide binding (low on rates and off rates for the peptide substrate) (Fig. 2). HSP40 also interacts directly with unfolded polypeptides and can recruit HSP70 to protein substrates^{20,26}. After ATP hydrolysis, a nucleotide-exchange factor binds to the HSP70 ATPase domain and catalyses ADP–ATP exchange, resulting in lid opening and substrate release. Release allows fast-folding molecules to bury hydrophobic residues, whereas molecules that need longer than a few seconds for folding will rebinding to HSP70, thereby avoiding aggregation. HSP70 (re)binding may also result in conformational remodelling, perhaps removing kinetic barriers to the folding process³⁰.

Proteins that are unable to partition to fast-folding trajectories after HSP70 cycling may be transferred into the specialized environment of the chaperonin cage for folding. Among these are several essential proteins, such as actins and tubulins³¹, which encounter high energetic barriers in folding and are completely unable to reach their native states spontaneously, even in dilute solution *in vitro*.

The chaperonins

Chaperonins are large double-ring complexes of ~800–900 kDa that function by globally enclosing substrate proteins up to ~60 kDa for folding. Group I chaperonins (also known as HSP60s in eukaryotes and GroEL in bacteria) have seven-membered rings in bacteria, mitochondria and chloroplasts, and functionally cooperate with HSP10 proteins (GroES in bacteria), which form the lid of the folding cage. The group II chaperonins in archaea (thermosome) and the eukaryotic cytosol (TRiC, also known as CCT) usually have eight-membered rings. They are independent of HSP10 factors.

The GroEL–GroES chaperonin system of *Escherichia coli* has been studied most extensively^{19,32} (Fig. 3). GroEL interacts with at least 250 different cytosolic proteins. Most of these are between 20 and 50 kDa in size and have complex α/β or $\alpha+\beta$ domain topologies, such as the TIM barrel fold^{14,33}. These proteins are stabilized by many long-range interactions and are thought to populate flexible, kinetically trapped folding intermediates exposing hydrophobic surfaces^{34,35}. The apical domains of GroEL present hydrophobic amino-acid residues for substrate binding in the ring centre. Subsequent folding depends on global substrate encapsulation by GroES (Fig. 3). GroES binding is ATP regulated and is associated with a marked conformational change of GroEL that leads to the formation of a cage with a highly hydrophilic, net-negatively-charged inner wall^{19,32,36}. Encapsulated protein is free to fold in this environment for ~10 seconds — the time needed for ATP hydrolysis in the GroES-bound ring (*cis* ring). Protein substrate leaves the cage after GroES dissociation, which is allosterically triggered by ATP binding in the opposite ring (*trans* ring). Not-yet folded substrate rapidly rebinding to GroEL for further folding attempts.

Enclosing unfolded protein, one molecule at a time, avoids disruption of folding by aggregation or (re)binding to upstream chaperones. In addition, an effect of steric confinement probably modulates the folding-energy landscape. Although the chaperonin functions as a passive-aggregation prevention device for some proteins^{32,37}, encapsulation can also accelerate folding substantially^{37–39}. This rate acceleration may be due to steric confinement, entropically destabilizing collapsed yet flexible folding intermediates, and promoting their conversion to more compact, native-like conformations. As shown recently, the effect of the folding cage may be comparable to the role of disulphide bonds in restricting conformational space in the folding of secretory proteins³⁹. Furthermore, repeated unfolding events in successive binding and release cycles have been suggested to reverse misfolded, kinetically trapped states that are stabilized by non-native interactions^{40–42}. Thus, the chaperonins may be able to remove both entropic and enthalpic barriers in rugged free-energy landscapes of folding (Fig. 1).

TRiC, the group II chaperonin in the eukaryotic cytosol, consists of eight paralogous subunits per ring^{31,43,44}. All group II chaperonins deviate from GroEL in that their apical domains contain finger-like

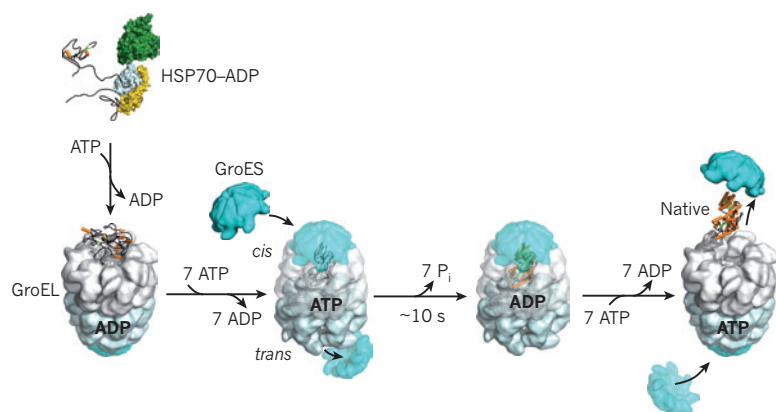


Figure 3 | Folding in the GroEL–GroES chaperonin cage. Substrate binding to GroEL (after transfer from HSP70) may result in local unfolding⁴². ATP binding then triggers a conformational rearrangement of the GroEL apical domains. This is followed by the binding of GroES (forming the *cis* complex) and substrate encapsulation for folding. At the same time, ADP and GroES dissociate from the opposite (*trans*) GroEL ring, allowing the release of substrate that had been enclosed in the former *cis* complex³² (omitted from the figure for simplicity). The new substrate remains encapsulated, free to fold, for the time needed to hydrolyse the seven ATP molecules in the newly formed *cis* complex (~10 s). Binding of ATP and GroES to the *trans* ring causes the opening of the *cis* complex. A symmetrical GroEL–(GroES)₂ complex may form transiently. Structural model is based on PDB accession 1AON.

protrusions, which act as an iris-like, built-in lid and replace the function of GroES. These segments open and close in an ATP-dependent protein-encapsulation cycle, similar in principle to that of GroEL–GroES⁴⁴. However, the TRiC reaction cycle is much slower than that of GroEL, probably providing a substantially longer period of protein encapsulation and folding in the cage⁴⁵. TRiC interacts with approximately 10% of newly synthesized cytosolic proteins, including actin and tubulins^{31,43}. Interestingly, TRiC also functions in preventing the accumulation of toxic aggregates by the Huntington's disease protein^{46–48}.

The HSP90 system

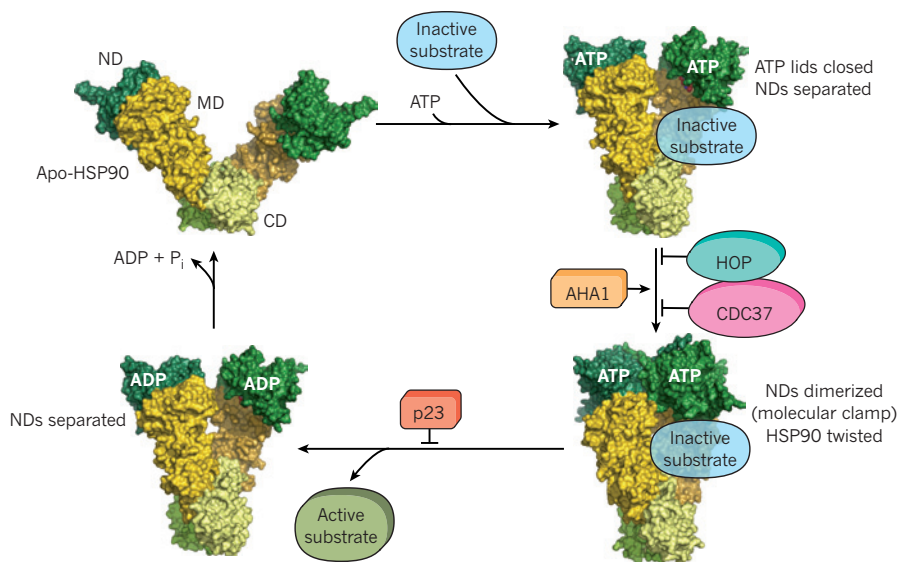
HSP90 forms a proteostasis hub that controls numerous important signalling pathways in eukaryotic cells⁴⁹. These pleiotropic functions include, among others, cell-cycle progression, telomere maintenance, apoptosis, mitotic signal transduction, vesicle-mediated transport, innate immunity and targeted protein degradation. Indeed, the evolution and maintenance of these functional networks is thought to depend on the ability of HSP90 to buffer the effects of structurally destabilizing mutations in the underlying protein complexes, thereby allowing the acquisition of new traits¹².

HSP90 functions downstream of HSP70 in the structural maturation and conformational regulation of numerous signal-transduction molecules, such as kinases and steroid receptors^{49,50}. It cooperates in this process with several regulators and co-chaperones, many of which use tetratricopeptide repeat (TPR) domains to dock onto HSP90. For example, the TPR protein HOP provides a direct link between HSP70 and HSP90, allowing substrate transfer⁵¹. Although the mechanism by which HSP90 and its cofactors mediate conformational changes

in substrate proteins is not yet understood⁵², recent crystal structures of full-length HSP90s provided long-awaited information^{53,54}. HSP90 functions as a dimer of subunits that are assembled by their C-terminal domains. An N-terminal domain binds and hydrolyses ATP and is joined to the C-terminal domain by a middle domain (Fig. 4). The middle domain participates in substrate binding and interacts with the co-chaperone AHA1. Similar to other chaperones, the HSP90 dimer undergoes an ATP-driven reaction cycle that is accompanied by considerable structural rearrangement²⁵ (Fig. 4). ATP binding leads to the dimerization of the N-terminal domains, forming the HSP90 'molecular clamp'. This results in a compaction of the HSP90 dimer, in which the individual monomers twist around each other. After hydrolysis, the ATPase domains dissociate, and the HSP90 monomers separate N-terminally. Various cofactors regulate this cycle: CDC37, which delivers certain kinase substrates to HSP90, inhibits the ATPase activity, and HOP inhibits N-terminal dimerization. AHA1 stimulates ATP hydrolysis, whereas p23 stabilizes the dimerized form of HSP90 before ATP hydrolysis. These factors are thought to adjust the kinetic properties of the cycle to achieve certain conformational transitions in HSP90-bound substrates, as well as their release from HSP90.

How HSP90 recruits different types of substrate protein with the help of various co-chaperones remains enigmatic. HSP90 appears to have several substrate-interaction regions, and the binding strength seems to be strongly influenced by the structural flexibility of the substrate⁵², in line with the proposed role of HSP90 as an evolutionary capacitor in protecting mutated protein variants from degradation¹². Because several HSP90 substrates are kinases with well-documented roles in tumour development, the inhibition of HSP90 with drugs

Figure 4 | ATPase cycle of the HSP90 chaperone system. Clockwise from top left, ATP binding to the N-terminal ATPase domain (ND) of apo-HSP90 induces a conformational change and the closure of the ATP lid in the ND. After lid closure, the NDs dimerize, forming the closed HSP90 dimer (molecular clamp) with twisted subunits. This metastable conformation is committed for ATP hydrolysis. After hydrolysis, the NDs dissociate. The inactive substrate molecule interacts mostly with the middle domain (MD) and is conformationally activated as HSP90 proceeds through the ATPase cycle. The cofactors CDC37, HOP, AHA1 and p23 accelerate or slow certain steps of the cycle. Structures relate to PDB accessions 2IQO, 2O1U, 2CG9 and 2O1V. CD, C-terminal ATPase domain.



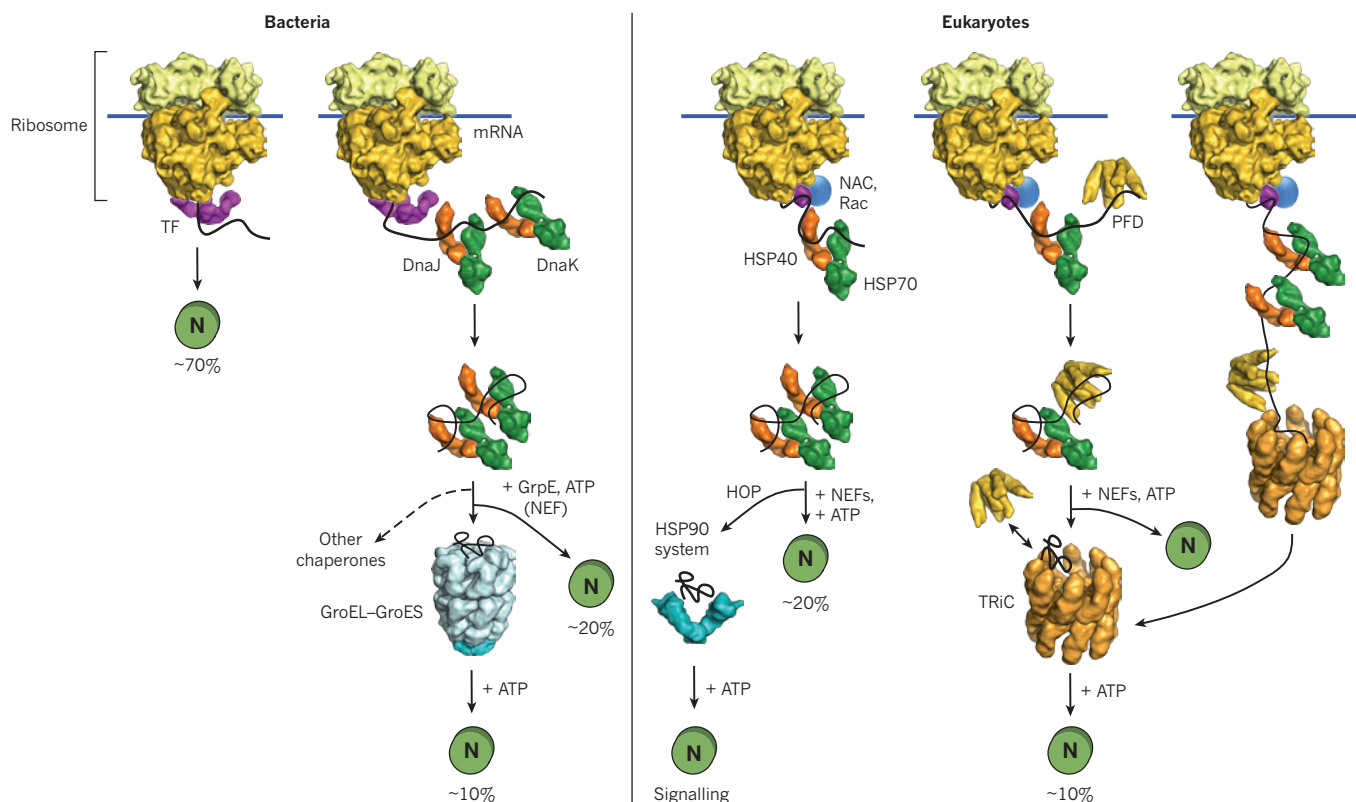


Figure 5 | Organization of chaperone pathways in the cytosol. In bacteria (left) and eukaryotes (right), chaperones that function in stabilizing nascent polypeptides on ribosomes and in initiating folding cooperate with machinery that acts downstream in completing folding^{7,19–21,31}. The number of interacting substrates is indicated as a percentage of the total proteome. The first category of factors includes chaperones that bind in close proximity to the ribosomal polypeptide exit site, such as trigger factor (TF) in bacteria and specialized HSP70 complexes (ribosome-associated complex (Rac) in *Saccharomyces cerevisiae*, MPP11 and HSP70L1 in mammalian cells) and nascent-chain-associated complex (NAC) in eukaryotes). These chaperones bind hydrophobic

chain segments. Non-ribosome-bound members of the HSP70 family (DnaK in bacteria and HSC70 in eukaryotes) function as second-tier chaperones for longer nascent chains, mediating co- or post-translational folding. They also distribute subsets of proteins to downstream chaperones, such as the chaperonins (GroEL in bacteria and TRiC in eukaryotes)^{14,20,21} and HSP90⁵². Substrate transfer from HSC70 to HSP90 is promoted by the coupling protein HOP. Dashed arrow indicates that pathway is not well established. Structures relate to PDB accessions 1DKG, 1DKZ, 2KHO, 1W26, 3IYF, 2AVY, 2AW4, 1FXK, 3LKX, 2IOQ, 2QWR and 1NLT. N, native protein; GrpE, protein GrpE; mRNA, messenger RNA; PFD, prefoldin.

such as geldanamycin has emerged as a promising strategy for the treatment of certain cancers⁵⁵. These drugs specifically inhibit the ATPase function of HSP90. They will probably prove useful not only in cancer therapy but also in the treatment of viral diseases, owing to the fact that various pathogenic viruses hijack the HSP90 system and use it for capsid assembly⁵⁶. However, the global inhibition of HSP90 is likely to result in a marked derangement of cellular circuitry, and it would be desirable to find ways to inhibit only specific aspects of HSP90 function.

From ribosome to folded protein

The vectorial synthesis of polypeptides on the ribosome has important implications in the folding process that are only partly understood. Key questions concern the stage at which the nascent chain begins to fold and the extent to which the translation process modulates the free-energy landscape of folding. In addressing these issues, it is useful to first consider small, single-domain proteins, which tend to fold spontaneously *in vitro*. The translation process for such proteins seems to increase the risk of misfolding and aggregation considerably, because an incomplete nascent polypeptide is unable to fold into a stable native conformation^{57,58} and the local concentration of nascent chains in the context of polyribosomes is very high. Furthermore, the exit channel of the large ribosomal subunit, which is ~100 Å long but at most 20 Å wide, is unfavourable to folding beyond α -helices and small tertiary elements that may begin to form near the tunnel exit^{59–61}; it

thus prevents the C-terminal 30–40 amino-acid residues of the chain from participating in long-range interactions that are necessary for cooperative domain folding. As a consequence, productive folding may occur only after the complete protein has emerged from the ribosome^{57,62}. Because translation is relatively slow (~4–20 amino acids s⁻¹), nascent chains are exposed in partially folded, aggregation-sensitive states for considerable periods of time. Moreover, non-native intrachain contacts formed during translation or interactions with the highly charged ribosomal surface could delay folding after completion of synthesis. For these reasons, nascent chains are thought to interact co-translationally with ribosome-bound chaperones, which inhibit their premature (mis)folding and maintain the nascent chain in a non-aggregated, folding-competent state (Fig. 5). For example, the bacterial trigger factor⁶³ binds to the small titin I27 chain (~120 amino acids) throughout translation⁶⁴, presumably delaying chain collapse until the complete β -sandwich domain has emerged from the ribosome and is available for folding. Moreover, the aggregation of nascent chains is disfavoured by the densely packed, pseudohelical arrangement of ribosomes in polyribosome complexes — an organization that maximizes the distance between nascent-chain exit sites on adjacent ribosomes⁶⁵.

Although single-domain proteins will reach their native state post-translationally, multidomain proteins may undergo domain-wise co-translational folding, as independently folding structural units (~50–300 amino acids in length) emerge sequentially from the

ribosome^{66,67}. This process avoids non-native interdomain contacts, thus smoothing the folding-energy landscape for large proteins^{66,68}. Sequential domain folding during translation, which is highly efficient on eukaryotic ribosomes, probably promoted the explosive evolution of complex multidomain proteins in eukaryotes^{66,68}. Co-translational folding is thought to be aided by the slower elongation speed of eukaryotic ribosomes (~ 4 amino acids s^{-1} in eukaryotes versus ~ 20 amino acids s^{-1} in bacteria) and as a result of various adaptations of the folding machinery. For example, eukaryotic ribosomes bind specialized HSP70 chaperone complexes (Fig. 5) and the binding and release of the canonical HSC70 from nascent chains may be coordinated with translation speed so as to support domain-wise folding. The eukaryotic chaperonin TRiC is recruited to nascent chains by HSC70 (ref. 69) and other upstream factors, such as prefoldin³¹, allowing co-translational folding. Moreover, fine-tuning of co-translational folding may be achieved by translational pausing at rare codons⁷⁰. Overall, the eukaryotic translation and chaperone machinery has been highly optimized through evolution, ensuring efficient folding for the bulk of newly synthesized proteins⁷¹.

The chaperone pathways operating in the endoplasmic reticulum (ER) follow analogous organizational principles, but specialized machinery is used in disulphide-bond formation and the glycosylation of many secretory proteins⁷².

Proteome maintenance and the proteostasis network

Although it is generally accepted that the chaperone machinery is required for initial protein folding, we are only beginning to appreciate the extent to which many proteins depend on macromolecular assistance throughout their cellular lifetime to maintain or regain their functionally active conformations. Compared with prokaryotes, the proteomes of eukaryotic cells are highly complex, comprising a much greater number and diversity of multidomain proteins. In the dynamic cellular environment, these proteins constantly face numerous challenges to their folded states; these result from post-translational modifications (phosphorylation and acetylation), changes in cell physiology and alterations in the composition and concentration of small-molecule ligands that may influence protein stability⁴. Moreover, 20–30% of all proteins in mammalian cells are intrinsically unstructured³; that is, they may adopt defined three-dimensional conformations only after binding to other macromolecules or membrane surfaces. Such proteins probably require assistance to avoid aberrant interactions and aggregation,

particularly when their concentration is increased and they are not in complexes with partner molecules⁷³.

These considerations help to explain why cells must invest in an extensive network of factors, comprising ~ 800 proteins in human cells (~ 200 chaperones and co-chaperones and ~ 600 UPS and autophagy components), which cooperate to maintain the conformational integrity of the proteome and provide adaptation to changes in the environment. This proteostasis network integrates general and specialized chaperone components for proper protein folding and trafficking with the machinery for disaggregation and proteolytic degradation of irreversibly misfolded proteins (the UPS and the autophagy system) (Fig. 6). The remarkable complexity of the system arises from the expansion, in multicellular organisms, of the diversity of regulatory components for the major chaperone systems (HSP70 and HSP90)²⁶ and of factors functionally coupling these chaperones with the UPS and the autophagy system^{27,74,75}. For example, various HSP70 cofactors, such as the BCL2-associated athanogene (BAG) family proteins and certain HSP40s, contain ubiquitin-like or ubiquitin-interacting domains⁷⁴. The HSP70 and HSP90 cofactor known as carboxyl terminus of Hsp70-interacting protein (CHIP) has E3 ubiquitin ligase activity and channels certain mutant or damaged proteins towards proteasomal degradation⁷⁴. Notably, CHIP is only one of several hundred different E3 ligases, which reflects the enormous importance of proteolytic pathways for proteostasis and cell regulation. Interestingly, whereas the clearance of misfolded protein species by the UPS requires that these molecules are maintained in a non-aggregated state by chaperones, disposal by autophagy is thought to involve active mechanisms to force such molecules into larger, presumably less toxic, aggregates^{76,77}. These inclusions are often deposited at specific subcellular sites close to the microtubule-organizing centre, referred to as the aggresome⁷⁸.

The proteostasis network is regulated by several interconnected signalling pathways, some of which are stress responsive and ensure that cellular protein folding and/or degradation is adapted to avoid the accumulation of misfolded and aggregation-prone species (Fig. 6). These pathways include the cytosolic stress response and the unfolded protein response of the ER and mitochondria, as well as signalling pathways that control ribosome biogenesis and translational capacity (Box 1). How the inputs from these different branches are coordinated and fine-tuned is only partly understood, but proteostasis capacity and responsiveness to stress may vary considerably in different cell types⁷⁹.

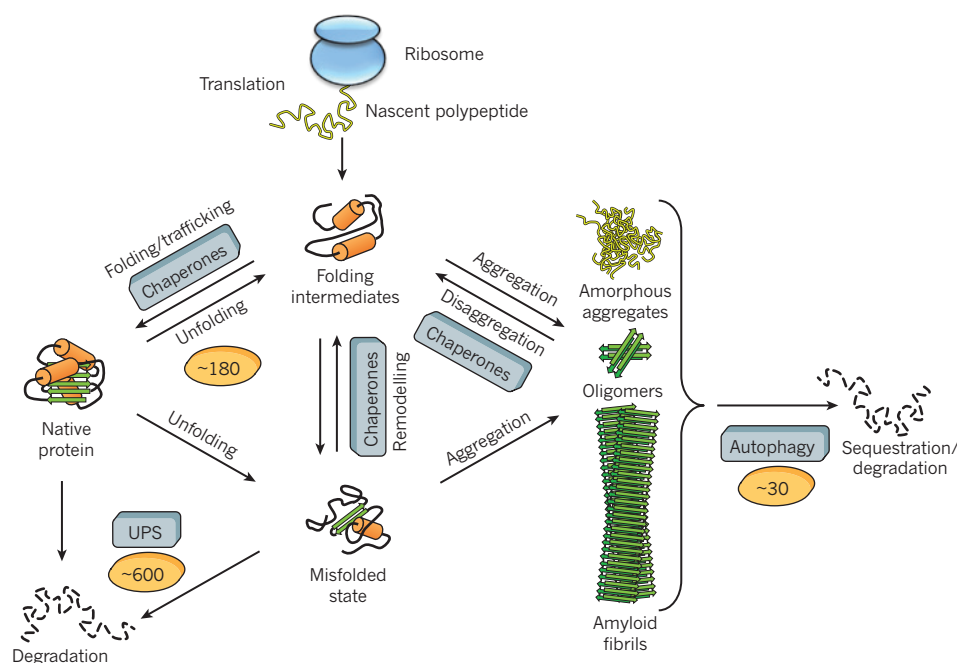


Figure 6 | Protein fates in the proteostasis network. The proteostasis network integrates chaperone pathways for the folding of newly synthesized proteins, for the remodelling of misfolded states and for disaggregation with the protein degradation mediated by the UPS and the autophagy system. Approximately 180 different chaperone components and their regulators orchestrate these processes in mammalian cells, whereas the UPS comprises ~ 600 and the autophagy system ~ 30 different components. The primary effort of the chaperone system is in preventing aggregation, but machinery for the disaggregation of aggregated proteins has been described in bacteria and fungi, involving oligomeric AAA⁺-proteins such as HSP104 and the *E. coli* molecular chaperone protein ClpB, which cooperate with HSP70 chaperones²⁵. A similar activity has been detected in metazoans, but the components involved have not yet been defined⁸³.

BOX 1

Signalling pathways in proteostasis and ageing

The expression of stress-inducible chaperone proteins (such as HSP70, HSP40, HSP90 and small HSPs) in the cytosol is governed by the heat-shock response⁹⁶. The genes encoding these proteins are transcriptionally regulated by the HSF-1 and FOXO (DAF-16 in *C. elegans*) transcription factors.

The unfolded protein response (UPR)⁷² of the ER adjusts the folding capacity of the secretory pathway by upregulating ER chaperones and/or attenuating protein synthesis by means of the transcription factors IRE1, PERK and ATF6.

The mitochondrial UPR^{97,98} is activated by conformational stress in mitochondria and increases resistance to oxidative damage.

Ageing and longevity pathways are coupled to the regulation of stress-protective pathways^{5,99}. Specifically, the upregulation of stress-protection factors such as chaperones by HSF-1 and FOXO is required for the lifespan-extending effect of mutations in the insulin and insulin-like growth factor I (IGF-I) receptor pathway. Autophagy, a process required for the recycling of organelles and the removal of large protein aggregates, is also necessary for lifespan extension and youthfulness in *C. elegans*. Autophagy is downregulated by the mammalian target of rapamycin (TOR) kinase when nutrients are plentiful⁹⁹ and is upregulated by FOXO⁸¹. Dietary restriction, which extends lifespan in model organisms, is also coupled with HSF-1 and FOXO activation^{81,100}.

Proteostasis collapse in ageing and disease

The accumulation of misfolded and/or oxidized proteins in cells during ageing is a challenge to the proteostasis system and eventually results in the deposition of aggregates, as shown in model organisms such as *Caenorhabditis elegans* and *Drosophila*^{80,81}. The inability of cells to restore normal proteostasis may result in disease, and even in cell death. Indeed, numerous diseases are now recognized to be associated with aberrant protein folding and are usually categorized as loss-of-function or toxic gain-of-function diseases, although specific pathological states often show elements of both groups. The former are generally caused by inherited mutations and include numerous disorders such as cystic fibrosis, lysosomal storage diseases and α 1-antitrypsin deficiency. The latter, gain-of-function disorders, include type 2 diabetes and the major neurodegenerative conditions (Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and Alzheimer's disease) and are either sporadic or caused by mutations that render specific proteins more aggregation prone. These gain-of-function diseases are typically age related and are caused by the accumulation of amyloid or amyloid-like aggregates of the disease protein. A plausible explanation for the late onset of these diseases is provided by recent evidence from model organisms that the signalling pathways that regulate proteostasis are integrated with the genetic and epigenetic pathways that control longevity^{82,83} (Box 1). Thus, the age-related decline in proteostasis and specifically in the inability to upregulate chaperones in response to conformational stresses would trigger disease manifestation and, in turn, accelerate proteostasis collapse^{81,84,85}.

Although the toxic principle operating in these disorders is far from understood, a consensus is emerging that soluble oligomeric aggregates, which may be 'on-pathway' or 'off-pathway' towards fibril formation, are the primary cytotoxic species^{16,18} (Fig. 1). One prominent hypothesis suggests that these oligomers expose promiscuous hydrophobic surfaces that can mediate aberrant interactions with several other proteins or with cellular membranes^{16,17}. In support of this proposal, a recent proteomics study in human cells showed that certain metastable proteins are targeted preferentially by such interactions, resulting in their co-aggregation with the amyloidogenic disease protein⁸⁶. The co-aggregating proteins are generally large in size and are enriched in intrinsically unstructured regions, properties that are coupled with a high degree of functionality. Accordingly, they tend to occupy essential hub positions in cellular protein networks, including transcriptional regulation, translation and maintenance of cell architecture, suggesting that their sequestration by the amyloid aggregates results in multifactorial toxicity. An interesting manifestation of this toxicity mechanism is the recent demonstration that aggregating mutant p53 may exert dominant oncogenic potential by sequestering wild-type p53 into co-aggregates, resulting in a complete loss of p53 function⁸⁷.

Aggregate toxicity may be exacerbated by the inability of affected cells to adequately respond to stress stimuli⁸⁶. This is consistent with

recent evidence that aberrantly folded protein species may interfere with central proteostasis functions, including protein folding and clearance mechanisms^{88,89}. Notably, the overexpression of members of the HSP70 system has been shown to inhibit the formation of toxic oligomers and to prevent the formation of amyloid aggregates for different disease proteins^{24,90,91}. In the case of polyglutamine-repeat proteins, which cause Huntington's disease and several related neurodegenerative disorders, HSP70 cooperates with the chaperonin TRiC to prevent the accumulation of potentially toxic oligomers⁴⁷, which is reminiscent of the functional cooperation between these chaperone systems in *de novo* protein folding.

On the basis of these findings, the pharmacological upregulation of chaperone function promises to open up new strategies for the treatment of numerous pathological states associated with aberrant folding and aggregation. Proof-of-principle experiments using small-molecule compounds to increase chaperone synthesis and rebalance proteostasis (for example, by activating heat-shock transcription factor-1 (HSF-1)-regulated pathways) have already demonstrated efficacy in loss-of-function and toxic gain-of-function disease models^{5,6,92,93}. Likewise, recently identified proteasome activators⁹⁴ have the potential to accelerate the clearance of toxic protein species, particularly when applied in combination with chaperone upregulation. Unlike conventional drugs, such 'proteostasis regulators' would not be disease-specific or protein-specific, and thus may be applicable to a whole group of related diseases — a new concept in medical practice.

Outlook

Studies over the past two decades have provided fascinating insight into the mechanics of chaperone-assisted protein folding, but there are still major gaps in our understanding of how the pathways of folding in the cell differ from those studied in the test tube. Progress is being held back by the problem that the sophisticated biophysical methods used to characterize folding intermediates *in vitro* are not easily transferable to the *in vivo* situation. Major innovation potential can thus be expected from the development of advanced imaging techniques, eventually allowing us to monitor conformational changes in a single polypeptide chain as it emerges from the ribosome, performs its biological function and is finally degraded in the living cell. Much research will also be stimulated by the emerging concept that molecular chaperones function as the central element of a much larger cellular network of proteostasis control, comprising, in addition, the protein biogenesis machinery as well as the UPS and the autophagy system. Unravelling the complex regulatory circuitry of this network and understanding why it loses its grip during ageing will pose a major challenge for years to come. Solving this problem will require a broad systems-biology approach relying on a combination of ribosome profiling, quantitative proteomics and computational modelling. How cells react to conformational stress or proteostasis deficiency at the proteome level is unclear. Key questions include determining how certain aberrantly folding proteins aggregate

into toxic species whereas others are degraded, how the composition of the proteosome changes during ageing, what the signature of a youthful proteome is, and how we can find ways to maintain it for longer as we age. Addressing these and related issues not only offers great opportunities for intervention with numerous, currently incurable diseases but will also eventually reveal the fundamentally important relationship between proteostasis and longevity. ■

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