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Review

The Hsp70–Hsp90 Chaperone Cascade in Protein Folding

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Conserved families of molecular chaperones assist protein folding in the cell. Here we review the conceptual advances on three major folding routes: (i) spontaneous, chaperone-independent folding; (ii) folding assisted by repetitive Hsp70 cycles; and (iii) folding by the Hsp70–Hsp90 cascades. These chaperones prepare their protein clients for folding on their own, without altering their folding path. A particularly interesting role is reserved for Hsp90. The function of Hsp90 in folding is its ancient function downstream of Hsp70, free of cochaperone regulation and present in all kingdoms of life. Eukaryotic signalling networks, however, embrace Hsp90 by a plethora of cochaperones, transforming the profolding machinery to a folding-on-demand factor. We discuss implications for biology and molecular medicine.

Highlights

We discuss three productive pathways for protein folding in the cell: (i) spontaneous, chaperone-independent folding; (ii) Hsp70 cycling; and (iii) Hsp70–Hsp90 chaperone cascade.

Chaperone-assisted protein folding counts on the ATP-dependent action of Hsp70 and Hsp90 in the early stages in the folding reaction; protein folding proceeds subsequently in a chaperone-free fashion so that the global kinetics of the reaction remain unaltered.

Hsp90 has two defined functions: (i) the ancient, evolutionarily conserved function in protein folding downstream from Hsp70, independent of cochaperones; and (ii) the regulation of sophisticated signalling networks in the eukaryotic cytosol, finely tuned by a plethora of cochaperones.

Progress in understanding the Hsp70–Hsp90-assisted protein folding mechanism may inspire new therapeutic strategies for the treatment of proteinopathies.

Assisted Protein Folding

Molecular chaperones are crucial for maintaining the integrity of the **proteome** (see Glossary) in cells [1]. Diverse families of conserved molecular chaperones manage protein homeostasis from numerous fronts (Box 1). In particular, the highly abundant Hsp70 and Hsp90 families cooperate in a multitude of protein folding and maturation processes [2,3]. Successive action of chaperones with complementary activity is a general concept in protein quality control (PQC) (Box 2). What makes Hsp70 and Hsp90 unique is that they are the major conserved ATP-dependent chaperone machines, appearing together in most organisms and main cellular compartments. Hsp90 is dispensable in bacteria but essential and highly abundant in eukaryotes, representing 1–2% of the cytosolic proteome even under nonstress conditions [4,5].

Here we review recent progress that allows classification of the role of chaperones in three main folding paths, the function of Hsp90 in folding and regulation, the consequences of chaperone action in evolution, and the possible implications of drug development in protein folding diseases. We describe three main routes for protein folding in the cell: (i) spontaneous folding, without the aid of any chaperone; (ii) folding assisted in cycles of Hsp70 binding and release; (iii) folding along the Hsp70–Hsp90 cascade (Figure 1).

Productive Folding Pathways

Spontaneous Protein Folding

The native conformation of a protein is determined by its amino acid sequence [6]. The protein folding landscape resembles an energy funnel with the native state at a thermodynamic minimum [7,8]. The surface of this energy landscape is rugged, entailing trapping or 'frustration', which proteins need to overcome during folding. During this process, proteins might expose hydrophobic side chains destined to be buried inside the folded core or at domain interfaces that might engage in unproductive interactions [7] Small single-domain proteins such

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Box 1. PQC: Maintaining Proteostasis in the Cell

The PQC network is a complex system in the cell that strives to maintain each protein in a native, active conformation, providing robustness to the proteome in changing environments and cellular conditions. This network not only ensures the basal functionality of the proteome but is also in charge of responding rapidly to conditions of stress when many proteins become prone to misfolding and aggregation [85]. The PQC network is unique for each species and cell type and involves many mechanisms to adjust protein homeostasis, or 'proteostasis', to boost survival [86]. Protection of the proteome relies to a large extent on the action of molecular chaperones, but also on a large number of other proteins that contribute to PQC; these proteins include components of the proteolytic systems, RNA- and DNA- modifying enzymes, metabolic enzymes, and regulatory proteins such as kinases and transcription factors [1].

The **proteostasis network** dynamically adapts to meet the requirements of the cell. The response to proteotoxic stress in the cytosol is mainly at the level of transcription through heat-shock transcription factors (e.g., σ^{32} in prokaryotes, Hsf1 in eukaryotes), which bind to specific promoter sequences enhancing the expression of a large set of target genes [87,88]. These genes control the composition of the chaperone machinery and the Ub proteasomal system, helping to restore proteostasis in the cell by stimulating refolding or degradation processes [88].

The capacity of the PQC network is particularly challenged on ageing and disease, including neurodegenerative diseases, inherited diseases, and many forms of cancer. Restoring proteostasis using small-molecule regulators emerges as an attractive strategy for the prevention and treatment of protein folding diseases like Parkinsonism, Huntington chorea, Alzheimer's disease, amyloidosis, and many other amyloidosis [57].

as ribonuclease A, barnase, and chymotrypsin inhibitor 2 (CI2) overcome these traps spontaneously and efficiently by themselves [6,9].

Larger, multidomain proteins present a more complex topology and often fold slower, challenging spontaneous protein folding [10]. Nevertheless, unassisted protein folding is possible; even a paradigm chaperone substrate, the 60-kDa two-domain firefly luciferase refolds with up to 80% yield in highly diluted solution in the presence of detergent [11]. This indicates that complex proteins can also fold on their own, if aggregation and dead-end pathways are circumvented. However, larger proteins often fold inefficiently unless the conditions regarding protein concentration, temperature, and timescales are far from physiological requirements. Luciferase is a prime example that inside cells molecular chaperones are required for high folding yields [12,13]. The folding challenge increases with the complexity of the organism, as 70% of the proteome in higher eukaryotes is multidomain proteins [14].

Hsp70 Binding-Release Mechanism

The Hsp70 chaperone is a major player in facilitating folding, including multidomain proteins, both during *de novo* folding and after heat stress [12,15]. Together with **J-domain proteins (JDPs)** and nucleotide exchange factors (NEFs), Hsp70 assists protein folding by ATP-regulated cycles of substrate binding and release [13,16,17]. The affinity of Hsp70 for its substrates is enhanced by hydrolysis of ATP and subsequent closure of the substrate-binding cavity, a process stimulated by JDPs (Box 3) [18,19]. Rebinding of ATP after NEF-stimulated ADP release reopens the substrate-binding cleft, granting substrate dissociation [15]. After release, either the protein folds on its own or Hsp70 rebinds.

Understanding the folding mechanism of Hsp70 requires a closer look at its client recognition mode. The hydrophobic properties of the conserved Hsp70-binding cavity allow the promiscuous recognition of short stretches of up to five hydrophobic residues, typically located in folded proteins in the hydrophobic core [20,21]. Binding of such core segments protects the protein from aggregation but also stalls the hydrophobic collapse. The specificity principle of Hsp70 chaperones is, in fact, that of a folding preventer. These

Glossary

CHIP: cochaperone that binds to both Hsp70 and Hsp90 through its TPR domain and functions as an E3 Ub ligase to regulate proteasomal degradation of chaperone client proteins. The name was coined as acronym for carboxyl terminus hsp70-interacting protein.
Cochaperones: nonclient regulatory proteins for the main chaperones, including Hsp70 and Hsp90, participating in their function and mediating the outcome of chaperone-assisted proteostasis. Although there are over 100 cochaperones identified in eukaryotes, many fall into two categories – 'J domain-containing cochaperones' and 'TRP cochaperones'.
Heat-shock proteins (HSPs): a group of proteins the cellular levels of which are increased in response to environmental, chemical, and physical stress and that limit the consequences of damage, facilitating cellular recovery. Many are members of conserved chaperone families such as Hsp70 or Hsp90 and assist in repair or targeting of proteins to degradation. NB: Not all HSPs are chaperones and vice versa.
Hop: cochaperone of the two major molecular chaperones Hsp70 and Hsp90 that facilitates their interaction and substrate transfer by acting as an adaptor between them. Hop can bind simultaneously to the C-terminal domain of both chaperones through its TPR domains. The name was coined as acronym for Hsp70/Hsp90 organising protein.
J-domain cochaperones: proteins containing a J domain that interact with Hsp70, catalysing the hydrolysis of ATP. Some JDPs can recognise client proteins on their own and may have potential aggregation-prevention activity, such as the bacterial DnaJ.
Proteasomal degradation: pathway through which most proteins are degraded; precisely, the Ub–proteasome pathway (UPP). It comprises a series of enzymatic activities that link Ub onto proteins that are then recognised by the 26S proteasome, a very large protease complex found in the cytoplasm and nucleus that degrades the proteins to small peptides.

hydrophobic Hsp70-binding stretches appear frequently in protein sequences – on average every 36 residues for the *Escherichia coli* Hsp70 DnaK – implying the likely binding of several Hsp70 molecules per substrate [20,22,23]. Thus, high Hsp70 levels could be detrimental for folding.

Hsp70 levels need to be stringently controlled *in vivo*. For example, expression of Hsp70 in otherwise unstressed cells has deleterious effects on *Drosophila* cell growth [24]. Abnormally elevated levels of Hsp70 expression are also a hallmark of malignancy in cancer tissues [25,26]. However, Hsp70 promotes folding of luciferase and other complex proteins *in vitro* and *in vivo* [12,15,27]. Given the high cellular concentration of Hsp70, binding to segments of the future core might locally beat substrate release; how can Hsp70 promote completion of the folding process?

Hsp70 does not directly fold proteins, in line with the concept of binding and release. Indeed, recent findings show that Hsp70 inhibits protein folding when present at physiological concentrations [28]. This is the case for luciferase but also for the ligand-binding domain of the glucocorticoid receptor. Fast rebinding of Hsp70 can lead to the binding of several Hsp70s to the same substrate molecule, which triggers an extended substrate conformation and hinders formation of the folding nucleus [22,28]. Recent single-molecule experiments suggest that in certain circumstances Hsp70 may also engage in additional interactions involving its helical lid domain more prominently than its hydrophobic substrate-binding pocket, which may also give this chaperone a possible role at later stages in the folding process [29]. It will be interesting to see how this activity complements Hsp70 binding to hydrophobic stretches at the ensemble level. The activity of Hsp70 to counter formation of the core of the folding protein implies that although binding and release cycles are a possible folding scenario, there are limitations, as high levels of Hsp70 prevent effective substrate release.

Together, these findings suggest the need for an additional cellular factor that makes Hsp70 chaperoning robust regardless of any fluctuations in cellular concentrations as may appear on heat shock and other cellular stress conditions.

Hsp70–Hsp90 Chaperone Cascade

Hsp70 and Hsp90 form an effective relay team, overcoming the limitations of the Hsp70 binding and release mechanism [28]. The two chaperone machines participate together in countless cellular processes. Hsp90 acts downstream of Hsp70 to improve folding and to optimise the maturation of key regulatory proteins [30–34]. Hsp70 and Hsp90 interact transiently to facilitate cooperation [32,35,36]. In eukaryotic cells, the **cochaperone** Sti1/**Hop** promotes the link between Hsp70 and Hsp90 by forming a physical bridge via simultaneous binding to the C termini of both Hsp70 and Hsp90 [31,37].

The substrate folding path leads via Hsp70 to Hsp90 and subsequently towards the native state [30,32,34]. This sequential interaction is conserved in the endoplasmic reticulum [38]. It is the substrate specificity that determines the order of action in the Hsp70–Hsp90 cascade [39]. Hsp70 binds early to core-forming segments that characterise nearly unfolded proteins whereas Hsp90 recognises late-folding intermediates [20,39,40]. Hsp90 makes use of an extended binding site, recognising scattered hydrophobic and charged patches typical of late folding stages [39,41–43]. In contrast to Hsp70, Hsp90 does not block folding. Rather, the substrate can reach the native state while bound to Hsp90 [31,34].

Proteome: the totality of proteins that are present at a given time in a cell under defined conditions. Compared with the genome, the complexity of the proteome is higher due to alternative gene splicing and post-translational modifications.
Proteostasis network: cells possess a protein homeostasis network, a protein management system that keeps the cellular protein composition in a dynamic equilibrium through the coordinated regulation of gene expression, protein synthesis, protein folding and degradation, maintaining the health of the proteome and the organism.

Box 2. Chaperone Pathways

Successive and synergistic action of different chaperones provides a decentralised strategy to maintain protein homeostasis. From all chaperone pathways, the Hsp70–Hsp90 cascade stands out by combining two chaperone systems that are ATP dependent, evolutionarily conserved, and abundant in folding compartments. This box gives examples of other chaperone pathways.

In bacteria, nascent chains count on the assistance of the chaperone trigger factor (TF) at the exit from the ribosome, which prevents and reverses early misfolds until folding is achieved [89,90]. TF and DnaK have overlapping substrate pools and their combined deletion leads to large aggregation events in the cell [91]. Deletion of both TF and DnaK is synthetically lethal [92,93]. This does not necessarily require, however, that both chaperones act in a defined order to fold proteins, as DnaK may backup post-translationally if TF does not sufficiently function cotranslationally. Additionally, DnaK can cooperate bidirectionally with the chaperonin GroEL/ES, which is also an ATP-controlled chaperone [94–96].

To reverse aggregation, the molecular chaperone ClpB collaborates with DnaK, which assists the process both upstream and downstream of ClpB, allowing reactivation of aggregated proteins [70,97,98].

Small heat-shock proteins (sHsps) protect the cell from irreversible protein aggregation and favour aggregate solubilisation [99]. However, the association of sHsps with aggregates needs to be outcompeted by Hsp70, and subsequently assisted by Hsp100, for reactivation of aggregated proteins [100,101].

Protein translocation is assisted by the bacterial signal recognition particle (SRP) or on a different pathway, by SecA and SecB acting downstream of TF [102]. Subsequently, periplasmic chaperones such as Skp, FkpA, and SurA participate in the folding and assembly of envelope proteins and soluble periplasmic proteins emerging from the translocon [103]. Similarly, competition at the ribosome determines the compartment-targeting specificity of the nascent chain in eukaryotic cells [104].

Hsp90 optimises chaperoning downstream of Hsp70 by resolving the Hsp70-inflicted folding block [28]. Remarkably, this is the case even for luciferase, for which the binding and release paradigm was established. Binding to Hsp90 allows the completion of the substrate core and the attainment of the native state. Hsp90 takes over the client from Hsp70 in an ATP-dependent manner [28,34]. Consistently, Hsp90 ATPase activity is essential for Hsp90 function *in vivo* and critical for refolding of exogenous internalised substrates [44–46]. There are only certain bacteria without Hsp90, and some cellular compartments where Hsp90 is not essential. This implies that, although there may exist alternative folding paths, Hsp90 efficiently buffers detrimental effects caused by high Hsp70 levels, providing the cell with a robust folding machine [28].

The Hsp70–Hsp90 cascade does not change the Anfinsen folding principle [6]. All information defining the shape of the native state is encoded in the primary amino acid sequence and it is not the task of chaperones to change that. Despite dramatically increasing the folding yields, neither Hsp70 nor Hsp90 alters the global kinetics of the folding reaction [28]. For the folding reaction, the activity of the Hsp70–Hsp90 cascade is restricted to the early folding phase. ATPase activity of both Hsp70 and Hsp90 is required in the first seconds to minutes, but afterwards the protein enters a folding trajectory that does not require either chaperone (Figure 2) [28].

How does the Hsp70–Hsp90 cascade stimulate protein folding? We propose that the key of the mechanism could be the exposure of the client to a gradient of decreasing hydrophobicity by subsequent interaction with Hsp70 and Hsp90 (Figure 3). First, the highly hydrophobic binding cleft of Hsp70 binds to hydrophobic segments in the unfolded protein that are meant to form the folded core [20]. Thus, Hsp70 binding is incompatible with nucleus formation. Next, Hsp90 exposes the substrate to a large binding surface sprinkled with hydrophobic and charged residues [39]. The more hydrophilic Hsp90 shell stimulates formation of the nucleus, preparing the substrate for progression to the native state. The bipartite Hsp70–Hsp90 system resembles

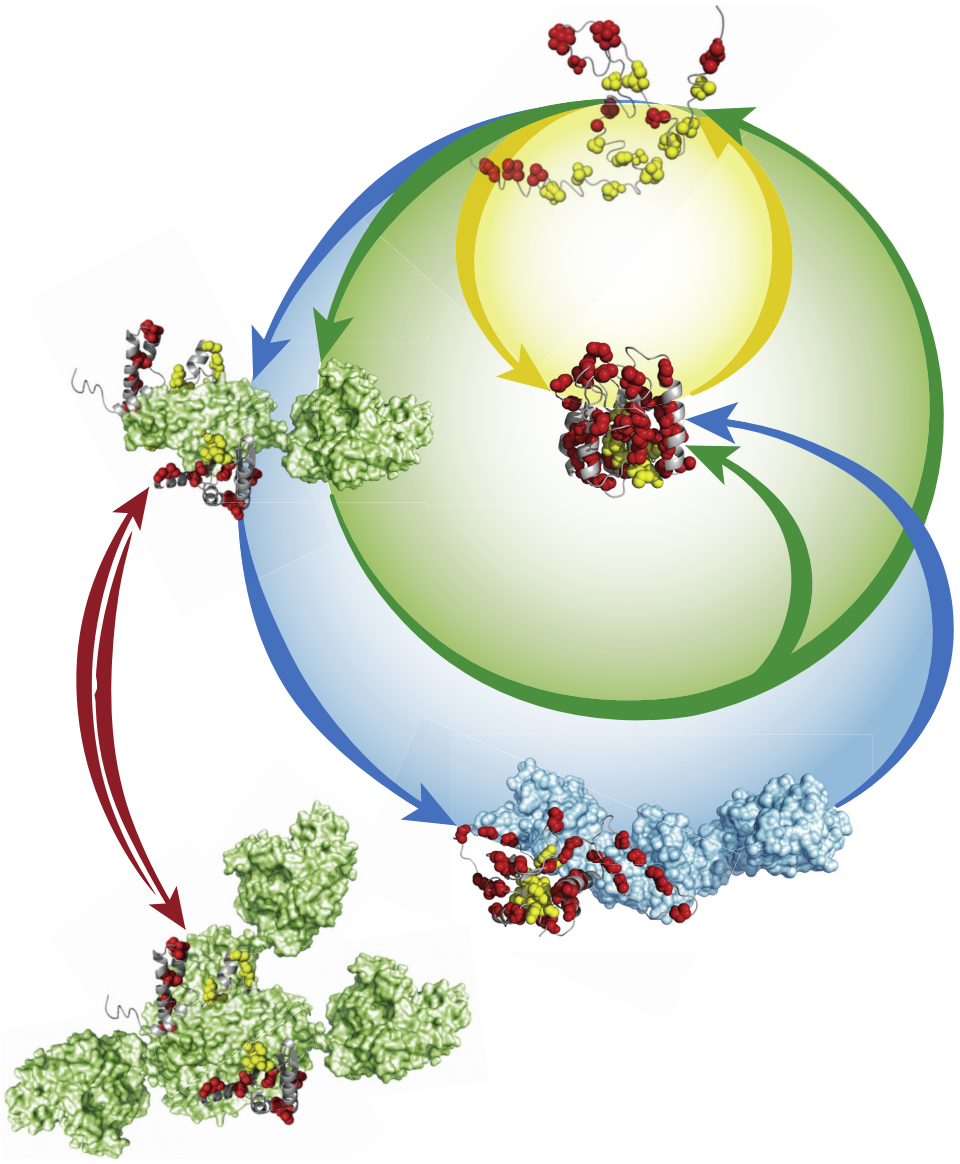

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Figure 1. Three Productive Folding Routes. Route 1: Spontaneous protein folding (yellow cycle). The folding intermediate reaches the native state without interference by chaperones. Route 2: Repetitive cycles of Hsp70 binding and release (green cycle). Hsp70 (green) binds fast to early folding intermediates. After release from Hsp70, the intermediate either folds to the native state in a chaperone-free manner or Hsp70 rebinds, starting another cycle. Binding of several Hsp70 s may result in an unproductive, dead-end equilibrium (red). Hsp70 substrate interaction is controlled by J-domain proteins and nucleotide exchange factor (not visualised). Route 3: The Hsp70–Hsp90 chaperone cascade (blue cycle). Hsp90 (blue) takes over the folding intermediate from Hsp70, shortcutting the Hsp70 cycling and preventing dead ends caused by multiple Hsp70 binding. The representations of Hsp70 and Hsp90 are based on structures in ADP-bound conformations and do not represent the conformational cycles (Box 3). The representation of the client is schematic (yellow, core-forming segments; red, noncore segments).

the mechanism of action of the chaperonin. The bacterial GroEL/GroES initially binds the substrate in a hydrophobic entry chamber that turns into a more polar folding chamber after ATP and GroES binding [47].

Box 3. Conformational Dynamics of Hsp70 and Hsp90

Hsp70 and Hsp90 are the main ATP-controlled chaperone families. Both are evolutionarily conserved from bacteria to humans and conformationally highly dynamic. Their ATPase domain controls drastic structural changes that are tightly coupled to their function (Figure I).

Hsp70 comprises a nucleotide-binding domain (NBD) and a substrate-binding domain (SBD), which is subdivided into a β-sandwich subdomain that harbours the polypeptide-binding cleft and an α-helical lid subdomain. Binding and hydrolysis of ATP in the NBD trigger allosteric conformational changes in the SBD that regulate Hsp70's affinity for the substrate [105]. Joint binding of the substrate and a JDP, in turn, stimulates Hsp70's ATPase activity [19]. In the ATP-bound state, the lid and β-sandwich subdomains of the SBD dissociate and dock onto the NBD. This opens the substrate-binding cleft, leading to high association and dissociation rates and lower affinity for substrates [15,106,107]. Substrate association with this open conformation and ATP hydrolysis dissociate the lid and β-sandwich subdomains from the NBD, resulting in trapping of the substrate [15,21].

Hsp90 is a homodimer that binds ATP in the N-terminal domain [108]. In ADP-bound and apo-states, Hsp90 is in a dynamic, extended conformation [109]. ATP binding favours closure by a second dimerisation site in the N-terminal domain [3,110]. The stringency of ATP-dependent closure varies between homologues; *Escherichia coli* Hsp90-ATP is predominantly closed while human Hsp90-ATP is predominantly open and closes only transiently [109]. Hsp90 can interact with substrates in both open and closed conformations [39,58].

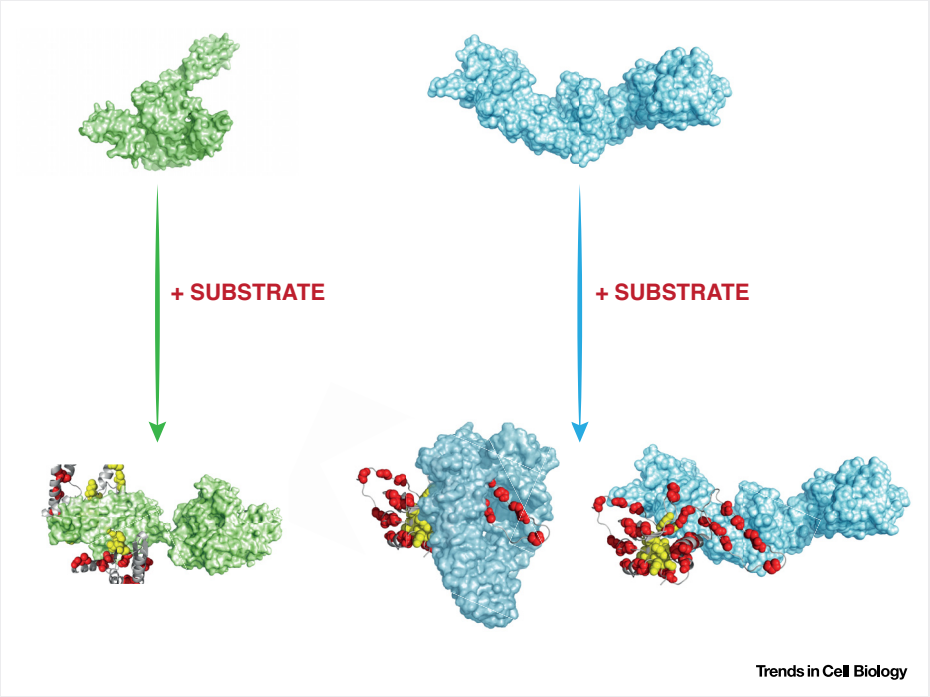

Figure I. Illustration of Nucleotide-Driven Conformational Changes of Hsp70 (Green) and Hsp90 (Blue).

Two Distinct Hsp90 Functions

The Hsp90 machine has two functions: (i) an evolutionarily conserved folding function downstream of Hsp70; and (ii) a specific regulatory activity adapted to specific needs in the eukaryotic cytosol.

Hsp90 in Protein Folding

E. coli Hsp90 improves folding downstream of Hsp70 in an ATP-dependent manner without specific cochaperones [28,32]. Such cochaperones have not been discovered yet and *E. coli*

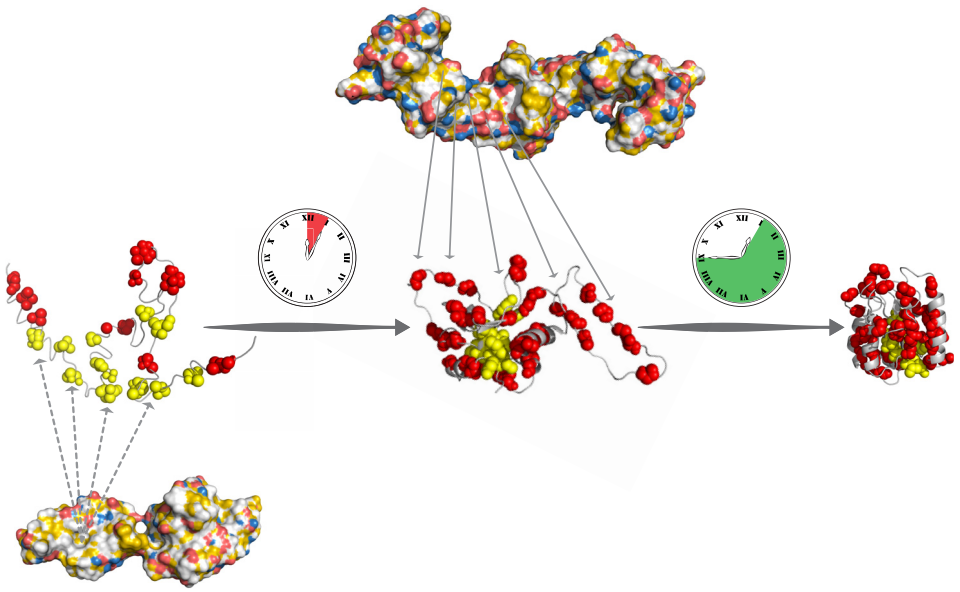

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Figure 2. Timing of the Hsp70–Hsp90 Cascade. Hsp70 and Hsp90 act fast and early in the folding path (chaperones are represented in YRB colours [115]: yellow, hydrophobic; red, negative charges; blue, positive charges). Hsp70 binds to hydrophobic residues of the folding intermediate that will later be part of the folded core of the protein (yellow). Hsp90 binds to the substrate downstream from Hsp70, offering a larger surface with both hydrophobic and charged residues that allows the formation of the protein core, which also prevents rebinding to Hsp70 (red). Afterwards, the substrate slowly reaches the native state on its own, without interference by chaperones.

Hsp90 is fully functional without any other regulatory component [28]. Notably, human Hsp90 also has full folding activity downstream of Hsp70, in the absence of the regulatory cochaperones such as p23, Aha1, Cdc37, and Cpr6 [28]. This is remarkable, as the ATPase activity of human Hsp90 in the cytosol is regulated by a plethora of cochaperones [3,48,49]. Although the

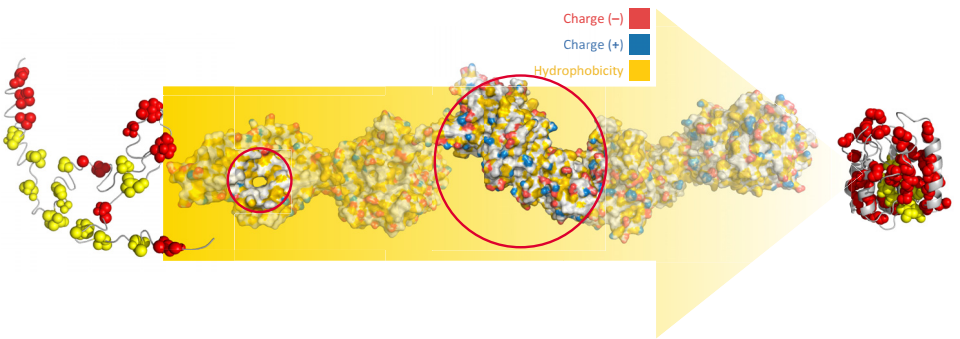

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Figure 3. Gradient of Decreasing Hydrophobicity in the Hsp70–Hsp90 Cascade. Hsp70 and Hsp90 expose the folding intermediate successively to a decreasingly hydrophobic environment. The earliest stages of the unfolded protein still expose hydrophobic stretches that will be part of the core of the folded protein (yellow). The highly hydrophobic substrate-binding pocket of Hsp70 chaperones is tailored for such stretches. It protects the substrate from aggregation, but also prevents the intermediate to complete the formation of its hydrophobic core. Hsp90 offers a larger, more extended binding surface with both hydrophobic and charged residues that allows core formation, which allows the intermediate to subsequently proceed towards the native state.

activity of Hsp90 in folding is strictly ATPase dependent, human Hsp90 has the same folding function as the bacterial homologue, in the absence of these cochaperones. This suggests that the Hsp90 function in folding downstream of Hsp70 is the ancient activity of this chaperone system.

Hsp90 is highly conserved and present in all kingdoms of life, including the main eukaryotic folding compartments [50]. The evolutionarily conserved function of Hsp90 evolved without developing a conserved set of regulatory cochaperones. The mitochondrial TRAP1 does not have any known cochaperones [51]. The endoplasmic Grp94 uses the CNPY family members CNPY3 and CNPY5/MZB1/pERp1 to act on Toll-like receptors and immunoglobulin, respectively [52–54]. These, however, are specific redox factors tailored to the specific situation in the endoplasmic reticulum and are not comparable with the ATPase cycle controlling cochaperones in the eukaryotic cytosol [55]. In line with this, most cytosolic Hsp90 cochaperones do not have a general function [49,56]. Taken together, these findings suggest a conserved Hsp90 role in protein folding free of cochaperone control (Figure 4A).

Hsp90 Regulatory Activity in Protein Maturation

The unique plethora of cochaperones in the eukaryotic cytosol implies evolved Hsp90 functions that are unique to this compartment (Box 4). The eukaryotic cytosol is rich in regulatory proteins that, for their activation, depend on cofactors and post-translational modifications. Before activation, these proteins are often less stable. For these clients, a fully efficient Hsp70–Hsp90 cascade would be counterproductive, as the substrate protein cannot reach a state of self-sufficient stability before either binding a cofactor or another activating modification. This Hsp90 function requires proper tuning, retardation, and stimulation to ensure that the substrate does not leave Hsp90 prematurely (Figure 4B). Inhibition of Hsp90 results in the degradation of many substrates, including newly made proteins and mature clients [3,57]. Degradation occurs, presumably, due to blocking of the regulatory action and failure of Hsp90 to further support the substrate in the premature state. The most prominent client classes are steroid hormone receptors and kinases [3,43].

In the case of steroid hormone receptors, Hsp90 binds to the apo state of the ligand-binding domain and its presence is required to maintain them in a nearly completely folded conformation, competent for ligand binding [3,34]. Binding of the hormone significantly stabilises the receptor and triggers receptor dissociation from Hsp90 and import into the nucleus [3,34]. Until this moment the Hsp90 activity needs to be retarded. Thus, this process is modulated by cochaperones.

Hsp90 interaction with kinases is also determined by the client thermodynamic properties. Hsp90 interacts with up to 60% of the human kinome together with the kinase targeting cochaperone Cdc37 [3,58,59]. Noticeably, Hsp90 is particularly recruited to those kinases with lower intrinsic stability and the presence of agents that stabilise kinases decreases their association with Hsp90 [59]. Hsp90 keeps the kinase in a semifolded state, ready for activation [58]. It should be noted that stable kinases that do not need Hsp90 have evolved and there is no reason to assume that the Hsp90 client kinases could not evolve into a stable conformation. However, the presence of Hsp90 may have reduced evolutionary pressure to increase stability for kinases at the cost of dynamics, in particular as many of them appear only at low levels. Other examples for Hsp90 substrates that are regulated by ligands or cofactors are the argonaute proteins involved in RNA biogenesis and inducible nitric oxide synthase (iNOS) [60,61]. Consistently, interaction of iNOS with Hsp90 is enhanced on depletion of its cofactor [61].

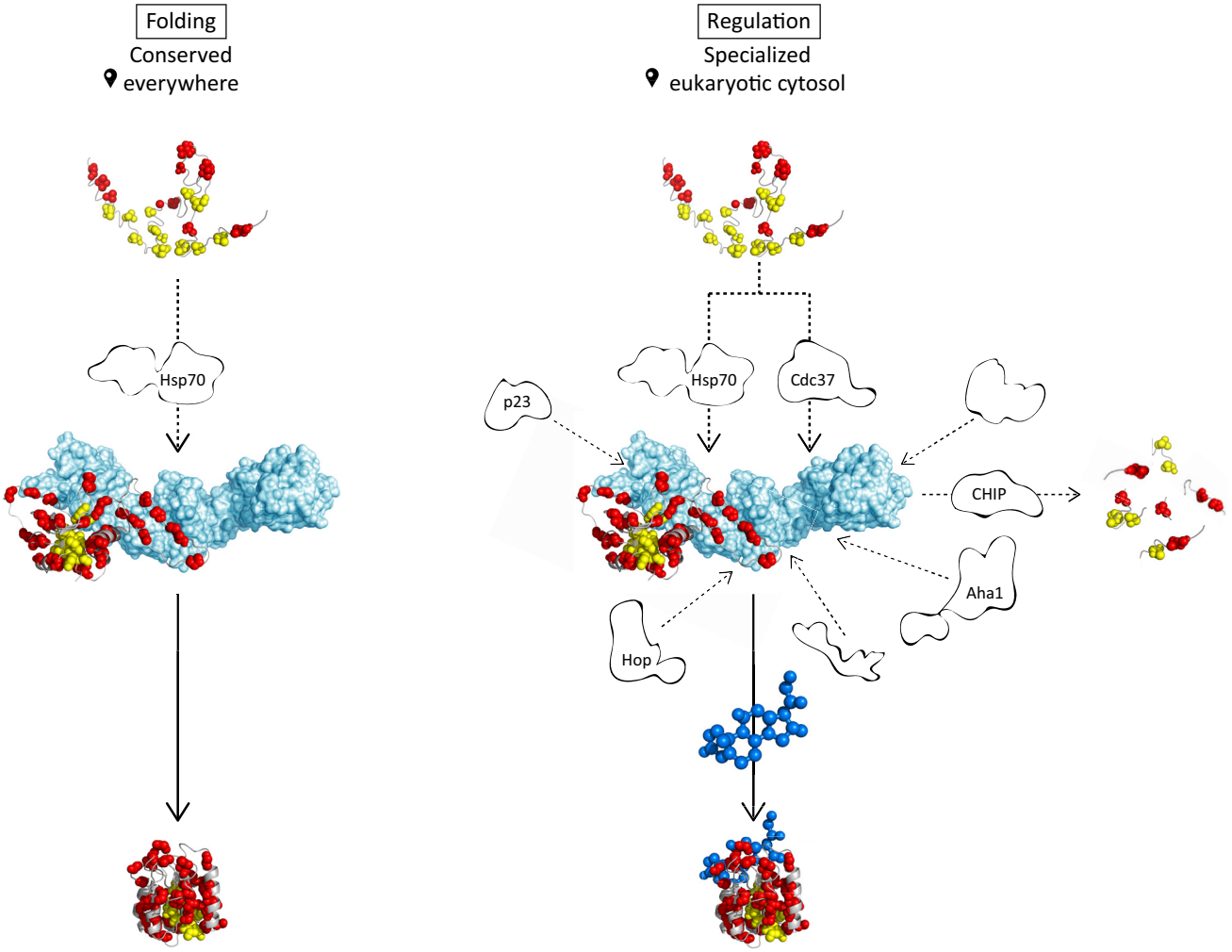

Figure 4. Evolutionary Branching of Hsp90 Functions. (A) The folding function of Hsp90 is evolutionarily conserved. Hsp90 acts downstream of Hsp70 in protein folding without the need of further cochaperones. (B) In the eukaryotic cytosol, Hsp90 acquired additional functions in regulation. Eukaryotic Hsp90 substrates are often regulatory proteins that do not acquire a stable and active conformation until either a cofactor is bound (mid-ocean blue) or another post-translational event such as phosphorylation occurs. Cochaperones such as p23 and Aha1 take care of the tuning up/down of the activity, delaying or accelerating it, or opening the way to degradation, depending on the presence of the cofactor needed for the active folded structure of the substrate. Cochaperones also control the influx of certain substrate classes; for example, kinases are targeted to Hsp90 by Cdc37. The cochaperone CHIP connects the Hsp90 machine to the degradation machinery.

Eukaryotic proteins are, in general, larger than bacterial proteins. It is possible that the complexity of substrates in the cytosol with special requirements has resulted in an increasingly complex Hsp90 chaperone machine to accommodate a more diverse clientele [62,63]; or, the other way around, a more elaborate Hsp90 machinery allowed the evolution of more complex proteins. However, folding of neither the complex 60-kDa luciferase nor the ligand-binding domain of the glucocorticoid receptor stringently depends on the presence of cochaperones. Also, in the endoplasmic reticulum some large and complex clients of the extracellular pathway such as immunoglobulins and extracellular domains for cell-surface proteins are able to fold although there are no known ATPase-regulating Hsp90 cochaperones [53].

Box 4. Cochaperones

The term cochaperone is used for nonclient proteins that interact with Hsp70 and Hsp90 regulating their activities and aiding in their function. Cochaperones are thus considered to be drivers of Hsp70 and Hsp90 functional diversity.

The crew of cochaperones assisting Hsp70 differs highly from that of Hsp90. The JDPs are crucial players in the Hsp70 ATP cycle, promoting ATP hydrolysis and targeting Hsp70 to its substrates [15]. *Escherichia coli* has six JDPs; humans have 54 [111]. The other main Hsp70 cochaperones, the NEFs, catalyse ADP release allowing ATP binding and substrate release [15]. In *E. coli*, GrpE is the only NEF present, as opposed to the several families of NEFs in eukaryotic organisms, which generates the supremely conserved Hsp70–JDP–NEF system that works together in a variety of processes [112].

In contrast to the high conservation of the Hsp70–JDP–NEF system, the number of cochaperones in the Hsp90 system is not conserved. So far, no cochaperones have been identified for Hsp90 in *E. coli*, TRAP1 in mitochondria, and Hsp90C in plastids. A wide array of Hsp90 cochaperones mainly evolved in the eukaryotic cytosol and are, with few exceptions, not essential for viability [3,48,49]. Hsp90 cochaperones bind in diverse regions and have different regulatory functions. The cochaperones p23 and Aha1 preferentially bind the dimerised N-terminal domain of Hsp90. p23 stabilises the closed conformation but inhibits the ATPase activity, whereas Aha1 stimulates ATP hydrolysis by Hsp90. Cdc37, a kinase-associated cochaperone, inhibits Hsp90 N-terminal dimerisation and ATPase activity.

A particularly interesting cochaperone family is the TPR domain-containing family, as they use the same recognition principle for both Hsp70 and Hsp90, and some even bind to both. They are unique to the eukaryotic cytosol and recognise the C-terminal EEVD sequence specific for eukaryotic cytosolic Hsp70s or Hsp90s [113]. One of them is Hop, which uses multiple TPR domains to bind Hsp70 and Hsp90 simultaneously [114]. Another example is the cochaperone CHIP that is able to bind either chaperone with its single TPR domain [64]. Other TPR-domain cochaperones preferentially recognise Hsp90 and compete for its binding site; for example, FKBP51, FKBP52, Cyp40, and PP5 [3].

Cochaperone evolution in higher organisms accounts for the differing needs in the range of biological processes that require Hsp70 and Hsp90 regulation and specificity.

Together, this shows that the need for cochaperones controlling the Hsp90 ATPase activity in the eukaryotic cytosol is linked to the appearance of regulatory clients that are not yet ready to complete folding. Client activation–retardation is a novel evolutionary feature unique to eukaryotic Hsp90.

Hsp90 Regulatory Activity in Protein Degradation

An important regulatory switch for eukaryotic chaperones is decision-making between folding and degradation. Both Hsp70 and Hsp90 have a C-terminal recognition motif for tetratricopeptide repeat (TPR)-binding proteins, one of which is the E3 ubiquitin (Ub) ligase **CHIP**. Targeting substrates to degradation via CHIP is a general function of eukaryotic Hsp70 and Hsp90 [64,65]. One example is control of the NLR innate immunity receptors in an Hsp90–CHIP-dependent manner [66].

The linkage of chaperones to the degradation machinery is crucial for the management of proteins causing neurodegenerative diseases. A common feature of diseases such as Alzheimer, Parkinson, and Huntington is the aggregation of a protein into insoluble fibrils [67,68]. Hsp70 and Hsp90 interact with potentially nontoxic precursors: Tau in Alzheimer's disease, α-synuclein in Parkinsonism, and huntingtin in Huntington chorea [69–71]. Remarkably, the proaggregation segments of these fibril-forming proteins are all intrinsically disordered. Thus, none of them folds but all of them interact with Hsp70 and Hsp90 [69,71]. Significant conceptual progress comes from the control of the protein homeostasis of Tau. Hsp90 induces **proteasomal degradation** of Tau [72]. Still, Hsp90 recognises it as a *bona fide* client [39,41,72]. The cochaperone CHIP is crucial in ubiquitinating Tau to target it to proteasomal degradation [72]. Interestingly, Hsp70 also interacts with Tau [73]. It is unclear whether Hsp70 and Hsp90 act here as a cascade as in the folding reaction or whether they function independently of each other.

The cooperation of chaperones and proteases in controlling degradation is also known in bacteria; for example, the degradation of σ^{32} by the protease FtsH is Hsp70 dependent [74]. However, a function of Hsp90 in the bacterial degradation process is neither known nor expected. Potential clients for Hsp90-dependent degradation such as Tau or kinases are not present in bacteria. The evolution of the CHIP system in the eukaryotic cytosol may thus have been an essential requirement for this evolutionarily new Hsp90 function.

Chaperones' Role in Buffering Evolution

A particular challenge for the PQC system is buffering the consequences of mutations. Mutations may lead to a protein with increased or decreased function, but often with decreased stability. Hsp70 and Hsp90 control the protein-folding path at different stages, which gives them different roles in buffering the consequences of mutations at the protein level. Hsp90 stabilises metastable disease mutations that populate late folding stages [75]. Instead, Hsp70 predominantly binds to unfolded or partially folded proteins, targeting in particular hydrophobic stretches as they are required in the nucleation of the hydrophobic core [20,28]. Hsp70 can prevent the aggregation of proteins with reduced stability as consequence of severe mutations, which precludes them from folding into the active state [75,76]. At the stage of Hsp70 action, the client protein has not yet reached a sufficiently folded state that would allow selection based on its function. By contrast, Hsp90 is able to buffer mutations allowing them to function normally and to promote the acquisition of stabilising secondary mutations [77,78]. Under conditions that impair Hsp90 function, however, Hsp90 is inefficient and hidden abnormal phenotypes emerge [77,79]. In genetic disorders such as Fanconi anaemia, Hsp90 but not Hsp70 buffers the effect of some mutations helping them maintain the functionality. A reduction in Hsp90 availability brings out the detrimental effect of the mutations [75].

The specific ability of Hsp90 to buffer compromised late folding stages is also important in buffering the effect of silent mutations that alter the translation rate [80]. Hsp90 shows an effect in local translation kinetics, which is related to cotranslational folding, implying that Hsp90 buffers mutations not only at the protein level but also indirectly at the RNA level [80].

Concluding Remarks

The ability of Hsp90 to buffer the consequences of mutations makes it a key player in diseases such as cancer, promoting cell proliferation. Also, Hsp70, which is upregulated in most cancers, is thought to provide a survival advantage by interacting with components of both the apoptotic and the prosurvival pathway [57,81]. Tumour development relies on altered properties of mutated regulatory proteins, including several kinases, p53, SRC, and UHRF1 [82]. Tumour cells typically have disrupted proteostasis and therefore often elevated levels of **heat-shock proteins (HSPs)**, including Hsp70 and Hsp90 [57]. The Hsp70 and Hsp90 chaperone machineries integrate tightly in the tumour environment, promoting cell survival but also leading to an enhanced effect of the inhibitors [83]. Therefore, tumour cells have generally enhanced sensitivity to Hsp90 inhibitors [57,84].

Several inhibitors of Hsp90 have been tested in clinical trials. So far none have reached the clinic, reflecting that inhibition of chaperones is a drastic measure. Recent years have seen mechanistic progress in understanding the Hsp70–Hsp90 cascade, largely due to progress in understanding the function of Hsp90. Combining the progress in mechanistic understanding of chaperone action with the experience obtained with Hsp90 inhibitors may help to stimulate a new generation of therapies, not only for cancer but also for other

Outstanding Questions

What is the molecular mechanism of the substrate transfer from Hsp70 to Hsp90?

How is the life time of the Hsp90–client complex tuned?

How are clients released from Hsp90?

When and how does the Hsp90 system decide between folding and degradation of a bound client?

Can we improve the effectiveness of the Hsp70–Hsp90 cascade, and possibly modulate its function?

How do cochaperones switch Hsp90 between the regulatory state and the folding state?

Can we exploit the mechanistic progress on chaperone mechanism to tackle protein folding and aggregation diseases?

Can we treat Hsp70 and Hsp90 differently in 'gain-of-function' and 'loss-of-function' diseases?

What is the impact of ageing and disease on the Hsp70 and Hsp90 cascade?

How can proteins escape Hsp70- and Hsp90-dependent clearance in disease to aggregate into toxic species instead of being degraded?

misfolding diseases such as cystic fibrosis and neurodegeneration (see Outstanding Questions).

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