PATHWAYS OF CHAPERONE-MEDIATED PROTEIN FOLDING IN THE CYTOSOL

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Abstract | Cells are faced with the task of folding thousands of different polypeptides into a wide range of conformations. For many proteins, the folding process requires the action of molecular chaperones. In the cytosol of prokaryotic and eukaryotic cells, molecular chaperones of different structural classes form a network of pathways that can handle substrate polypeptides from the point of initial synthesis on ribosomes to the final stages of folding.

MOLECULAR CHAPERONES
Proteins that help the folding of other proteins, usually through cycles of binding and release, without forming part of their final native structure.

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A central process in biology is the conversion of genetic information into the functional proteins that carry out the genetic programme. Ribosomes have an essential role in this process, as they translate mRNA into linear polypeptides. There is, however, a further step — the folding of the newly translated polypeptides into well-defined, three-dimensional conformations. Proteins that are known as MOLECULAR CHAPERONES are crucial for this final step in the readout of genetic information, which results in the formation of functional proteins.

The final native conformations of polypeptides are encoded in their linear sequences and the unassisted folding of isolated proteins has been carefully studied *in vitro*[1,2]. However, the situation in live cells is considerably more complex and is the focus of much research. In contrast to the conditions of *in vitro* refolding, the cellular environment is extremely crowded with high concentrations of proteins, nucleic acids and other macromolecules. The resulting EXCLUDED VOLUME EFFECT, or macromolecular crowding, has several consequences for the physical properties of the intracellular enviroment, and one of the most important is that intermolecular interactions become strongly favoured. For an unfolded polypeptide, this means that non-productive aggregation with other unfolded species — which is mediated by exposed hydrophobic residues and unstructured chain segments — can compete strongly with its folding to the native state[3].

The problem of protein aggregation is exacerbated by the fact that the formation of a stable TERTIARY STRUCTURE generally requires the presence of a complete protein domain as a folding unit (~100–300 amino acids). As a consequence, translating polypeptides populate non-native conformations until sufficient structural information is available for folding. The recently determined structure of a bacterial ribosome shows that the exit tunnel through which nascent polypeptides travel is too narrow to allow large structures to form[4], although some chain compaction could occur inside the ribosome because the tunnel might expand to a certain degree[5]. Therefore, polypeptide sequences that are part of a tertiary structural domain cannot be stably folded until the entire domain has been extruded from the ribosome. The exposure of extensive hydrophobic surfaces on the unfolded nascent chains early in their synthesis would render them prone to intermolecular aggregation, particularly considering the fact that numerous unfolded polypeptides are synthesized in close proximity to one another on ribosomes that are translating from a single mRNA.

To counteract these dangers, cells have developed a sophisticated system of molecular chaperones, which assist the folding of polypeptides as soon as they emerge from the ribosomal exit tunnel. The molecular chaperone proteins bind to the folding intermediates of polypeptides, which prevents their aggregation and, in some cases, actively helps them to fold through cycles of

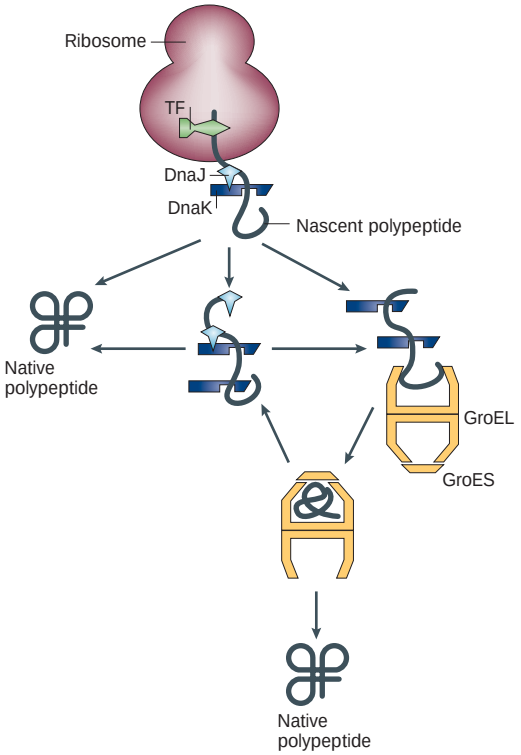


Figure 1 | **The chaperone network of the prokaryotic cytosol.** Nascent polypeptide chains are met by trigger factor (TF) as they emerge from the ribosome. The 70-kDa heat-shock protein DnaK, which is stimulated by its J-domain co-chaperone DnaJ, also binds nascent polypeptides. Newly synthesized polypeptides can fold spontaneously or can be assisted by DnaK. Alternatively, they can be passed to the GroEL–GroES chaperonin system for final folding, and, in some cases, might again interact with DnaK.

binding and release. Much valuable insight into the biochemical mechanisms of chaperones from different structural families has been gained (for a mechanistic overview, see BOXES 1–4). Furthermore, it is becoming increasingly clear that the different chaperone proteins cooperate with each other in living cells. In both prokaryotic and eukaryotic cells, the cytosol provides a well-developed network of chaperone pathways that can handle polypeptides at all stages of folding.

The importance of chaperones in promoting and maintaining the native conformation of cellular proteins is underscored by the toxic consequences of protein misfolding and aggregation. In several neurodegenerative disorders, including Parkinson's disease and Huntington's disease, the accumulation of protein aggregates accompanies neuronal death in specific brain regions, which leads to the irreversible neurological symptoms of these disorders. Molecular chaperones are a crucial, natural cellular defence against such toxicity [6,7]. So, understanding how they work, both individually and as part of an integrated team, is essential to address the root of protein-misfolding disorders.

The cytosolic chaperone pathways in prokaryotes and eukaryotes begin with ribosome-bound factors: the PEPTIDYL-PROLYL *CIS–TRANS* ISOMERASE trigger factor (TF) in *Escherichia coli*; the Ssb proteins of the 70-kDa heat-shock protein (Hsp70) family; the ribosome-associated complex (RAC) in *Saccharomyces cerevisiae*; and, possibly, the nascent-polypeptide-associated complex (NAC) in *S. cerevisiae* and mammals. These factors are structurally unrelated, but they all function during the translation of polypeptides. Proteins of the Hsp70 family, such as *E. coli* DnaK, are also important in chaperoning nascent polypeptide chains, and continue to assist in the folding of newly synthesized polypeptides together with the multimeric CHAPERONINS bacterial GroEL and the eukaryotic TCP1 ring complex (TRiC; also known as chaperonin containing TCP1 (CCT)). Eukaryotic GimC/prefoldin, which is not found in bacteria, binds to a subset of nascent chains and newly synthesized polypeptides, and cooperates with the chaperonin TRiC in their folding. Another subset of eukaryotic polypeptides uses chaperones of the 90-kDa heat-shock protein (Hsp90) family, as well as the Hsp70 family, to complete their folding. Several co-chaperones contribute to the chaperoning functions of eukaryotic Hsp70 and Hsp90 proteins and, in some cases, divert their activity towards other processes, such as protein sorting. Finally, the structurally related 104-kDa heat-shock protein (Hsp104) in *S. cerevisiae* and ClpB in *E. coli* can resolubilize aggregated polypeptides and pass them back into the chaperone network. These stages along the chaperone pathway are examined in this review, and are discussed according to the transitions and cooperations between them. The final section presents some thoughts on the intriguing similarities and differences between the prokaryotic and eukaryotic chaperone systems.

Ribosome-bound factors and Hsp70

Trigger factor and DnaK. In *E. coli*, TF is the first chaperone to meet nascent polypeptides as they emerge from ribosomes[8–10] (FIG. 1). TF has a peptidyl-prolyl *cis–trans* isomerase activity that is similar to animal IMMUNOPHILINS, and a micromolar affinity for unfolded polypeptide substrates[8,11]. In addition, TF docks specifically through a separate domain onto the ribosomal L23 protein and also contacts L29, both of which are near the polypeptide exit site of the large ribosome subunit[12–14]. Because TF is an ATP-independent chaperone, it does not actively assist folding through nucleotide-regulated cycles of polypeptide binding and release; *in vitro*, TF can even delay the refolding of proteins[15]. Therefore, TF is thought to function by scanning a nascent chain as it is extruded from the exit site and shielding hydrophobic stretches to keep them soluble. Active folding of the nascent and newly synthesized polypeptides can then be mediated by ATP-dependent chaperones, such as DnaK or GroEL. The significance of the peptidyl-prolyl *cis–trans* isomerase activity of TF remains unclear, as the prolyl isomerase domain is not essential for the chaperone function of TF[16,17].

Although it is not physically tethered to ribosomes by specific interactions, DnaK in *E. coli* also binds to ribosome-bound nascent polypeptides[18] (FIG. 1). Studies with purified proteins indicate that substrate polypeptides

EXCLUDED VOLUME EFFECT
Extremely high concentrations of inert macromolecules are thought to affect the thermodynamics of reactions between other macromolecules by reducing the available volume in the solution. This effect is observed as an increase in the rates and affinities of intermolecular binding reactions.

TERTIARY STRUCTURE
This term refers to the native three-dimensional conformation of a polypeptide, in which secondary (local) structure elements are packed against each other and specific contacts can form between sections of a polypeptide chain that are widely separated in the amino-acid sequence.

PEPTIDYL-PROLYL *CIS–TRANS* ISOMERASE
The folding of some proteins requires the rotation of a peptide bond that precedes a proline residue from the *trans* (extended) to the *cis* (bent) position, which normally takes place slowly. The peptidyl-prolyl isomerases catalyse this interconversion, which can increase the folding rate of some proteins.

CHAPERONINS
A family of chaperone proteins that have a characteristic double-ring structure. One class of chaperonin, which functions with a capping cofactor, is found in bacteria (for example, GroEL of *Escherichia coli*), and in the interior of mitochondria and chloroplasts. A second class of chaperonin, which functions without a capping cofactor, is found in the cytosol of eukaryotes (for example, TCP1-ring complex (TRiC)) and in archaea (thermosomes).

IMMUNOPHILINS
A family of intracellular eukaryotic proteins that contain a structurally related peptidyl-prolyl *cis–trans* isomerase domain and bind immunosuppressive drugs, such as FK506 (rapamycin).

Box 1 | The Hsp70 chaperones

The cytosolic 70-kDa heat-shock protein (Hsp70) chaperones (see table) are monomeric, and have an amino-terminal ATPase domain plus a carboxy-terminal polypeptide-binding domain. Hsp70 proteins recognize short hydrophobic polypeptide stretches that are in an extended conformation, and polypeptide binding is regulated by the nucleotide-bound state. In the ATP-bound state, DnaK — the *Escherichia coli* Hsp70 — exchanges polypeptide substrates rapidly, whereas it binds substrates stably in the ADP-bound state. The co-chaperone DnaJ can bind substrate polypeptides itself, and its J domain activates ATP hydrolysis by DnaK, which results in the transfer of polypeptides to DnaK. Nucleotide exchange, which is induced by the co-chaperone GrpE, returns DnaK to the ATP-bound state. Protein folding by Hsp70 involves ATP-dependent cycles of substrate binding and release[19,20]. However, optimal polypeptide folding might require the coordinated release of the polypeptide from several Hsp70 monomers, although, as yet, there is no evidence for such coordination.

Eukaryotic Hsp70 proteins follow a similar ATPase cycle. The DnaJ homologues (see table) also interact with polypeptides and stimulate ATP hydrolysis by their partner Hsp70 proteins[36,61,130]. Orthologues of GrpE are not found in the cytosol of eukaryotes, but nucleotide exchange by the 70-kDa heat-shock cognate protein (HSC70) can be stimulated by the structurally unrelated mammalian co-chaperone BCL2-associated athanogene-1 (BAG1) and its homologues[102,110–112]. Mammalian HSP70-binding protein (HSPBP1) and its *Saccharomyces cerevisiae* homologue Fes1 form another class of cytosolic Hsp70 nucleotide-exchange factors that are unrelated to GrpE[131,132]. The mammalian co-chaperone HSP70-interacting protein (HIP) counteracts BAG1 by stabilizing the polypeptide-binding ADP-bound state of HSC70 (REFS 133,134).

The Ssb class of Hsp70 proteins in *S. cerevisiae* are not regulated by the usual DnaJ homologues[130], but are thought to work together with zuotin, which is the DnaJ-related component of the heterodimeric ribosome-associated complex (RAC)[28,32]. Ssz1, which is the other component of RAC[25,28,32], is also a member of the Hsp70 family, although it diverges markedly from other Hsp70 proteins in its primary sequence[135]. Stimulation of the ATPase activity of Ssz1 by J domains has not yet been established. However, the ATPase domain of Ssz1 is necessary for its cellular function, unlike its peptide-binding domain[27].

Group or species	Hsp70	J-domain co-chaperone	Nucleotide-exchange factor
Escherichia coli	DnaK	DnaJ	GrpE
Mammals	HSC70	HDJ1 and HDJ2	BAG1* and HSPBP1‡
Saccharomyces cerevisiae	Ssa Ssb§	Ydj1 and Sis1 Zuotin§	Snl1* and Fes1‡ Not known

*BAG1 and Snl1 are homologous to each other, but are unrelated to GrpE. ‡HSPBP1 and Fes1 are orthologues, but are unrelated to GrpE, or to BAG1 and Snl1. §Ssb and zuotin interact with each other, but ATPase stimulation of Ssb by zuotin has not yet been shown; furthermore, zuotin does not seem to interact directly with substrate polypeptides.

that are transiently bound by the cofactor protein DnaJ are transferred onto DnaK. The DnaK–polypeptide binding reaction is coupled to ATP hydrolysis by DnaK, which is stimulated by the J DOMAIN of DnaJ[19,20] (BOX 1). It is therefore probable that DnaJ also interacts with nascent polypeptides to initiate DnaK binding[21], but that it is not recruited specifically to the ribosome itself. In *E. coli* strains in which the gene that encodes TF is deleted, DnaK can function as an effective substitute in the chaperoning of nascent polypeptides, and an increase in the number of nascent and newly synthesized polypeptides that are bound to DnaK is observed *in vivo*. However, the removal of both TF and DnaK severely diminishes the viability of *E. coli*, and results in the accumulation of misfolded, aggregated proteins[16,18,22]. Recent results show that this loss of viability can be circumvented if the bacterial cells are allowed to accumulate suppressor mutations; this process is facilitated by generating the combined gene deletions at 20°C and gradually adapting the cells to higher growth temperatures[16,23]. For certain multidomain proteins, TF and DnaK function together to delay the folding process relative to translation, and thereby prevent misfolding and aggregation during translation[24] (BOX 2).

Ribosome-associated complex and Hsp70. In the model eukaryote *S. cerevisiae*, nascent polypeptides are also met by binding factors on their emergence from the ribosomal exit tunnel. The stable RAC heterodimer consists of the DnaJ-related co-chaperone zuotin and the Hsp70-related Ssz1/Pdr13 (REFS 25,26; FIG. 2). Ssz1 is divergent from the other cytosolic forms of Hsp70 (REF. 25; BOX 1) and does not seem to interact directly with nascent chains. Also, the putative peptide-binding domain of Ssz1 is not essential for its cellular function, although it might have a secondary, regulatory role[27]. Zuotin contains an Hsp70-interacting J domain that is homologous to the one in DnaJ, as well as a separate domain that binds ribosomes, possibly through ribosomal RNA[28]. The Ssb Hsp70 proteins (Ssb1 and Ssb2) are the proposed partner chaperones of zuotin[28], and they are found both associated with ribosomes and free in the cytosol[29–31]. Deletion of Ssz1, or mutation of the zuotin J domain, apparently reduces Ssb binding to nascent polypeptide chains, which indicates that RAC might recruit Ssb to assist in the folding of nascent polypeptides[32]. Loss of the RAC components leads to cold sensitivity and sensitivity to translation inhibitors, which is similar to the phenotype that is caused by deletion

J DOMAIN
A conserved domain that stimulates ATP hydrolysis by chaperones of the 70-kDa heat-shock protein (Hsp70) family. It was first identified in the *Escherichia coli* co-chaperone DnaJ, but it is also found in DnaJ homologues in eukaryotes, as well as in several co-chaperones that recruit Hsp70 proteins for specific cellular processes.

Box 2 | Folding yield versus rate in bacteria

The roles of bacterial trigger factor (TF) and the 70-kDa heat-shock protein DnaK (with the J-domain co-chaperone DnaJ and the nucleotide-exchange factor GrpE) are well recognized in protecting nascent polypeptide chains from misfolding and aggregation[18,22]. However, the mechanistic consequences of these chaperone functions early in protein folding had not been examined until a recent study[24] investigated the effect of these chaperones on the *de novo* folding of two model multidomain proteins: firefly luciferase and *Escherichia coli* β-galactosidase. In the absence of these chaperones, the folding of both polypeptides follows a rapid, but inefficient, default co-translational pathway. Together, TF and DnaK improve the folding yields of both model proteins, but only by causing a shift towards a more post-translational folding mechanism. This effect involves the dynamic recruitment of further TF molecules to the translating ribosomes. Although β-galactosidase uses this chaperone-assisted, slower folding pathway optimally, the folding of luciferase remains inefficient. Evidently, the nature of TF and DnaK interactions with the nascent luciferase chain is incompatible with its efficient and natural co-translational folding mechanism in eukaryotic systems[24]. Therefore, despite considerable evolutionary conservation in individual chaperone classes, there might be important differences between prokaryotes and eukaryotes in the coupling of translation and folding.

Either TF or DnaK alone can assist the folding of newly synthesized proteins, but the absence of both leads to a severe loss of viability[18,22]. This growth defect can be overcome by adaptation of the *E. coli* cells, and other factors can, at least partially, substitute for TF and DnaK. One such factor seems to be the multimeric chaperonin GroEL–GroES, as overexpression of this chaperonin system restores viability[16,23]. It remains to be determined whether GroEL accomplishes this by binding nascent chains in place of TF and DnaK, or by helping to keep newly synthesized proteins soluble after they have been released from ribosomes. SecB, which is an ATP-independent chaperone that normally functions in secretion, can also functionally replace TF and DnaK when it is overexpressed; this chaperone interacts with nascent polypeptides both during and after their translation[136].

of the *Ssb* genes[27,32]. However, it is not yet clear how this phenotype is caused by a protein-folding defect.

The 70-kDa heat-shock cognate protein (HSC70), which is the constitutively expressed cytosolic Hsp70 protein in mammals, also binds nascent polypeptides and is thought to help in their co-translational folding[33–35] (FIG. 2). In some cases, HSC70 binding might be initiated by the DnaJ homologues HDJ1 (also known as 40-kDa heat-shock protein (HSP40) or DJB1) or the constitutive form HDJ2 (also known as DJA1; BOX 1), although the interaction of these cofactors is transient[34–38]. Similar to *E. coli* DnaK, mammalian HSC70 is not known to be physically anchored to ribosomes by specific interactions. Nevertheless, in *E. coli*, *S. cerevisiae* and mammals, Hsp70 chaperones are associated with nascent polypeptides and also with newly synthesized polypeptides after their release from ribosomes.

Nascent-polypeptide-associated complex. In *S. cerevisiae* and mammals, NAC is specifically bound to ribosomes and contacts nascent polypeptide chains as they emerge from the ribosomal exit tunnel[39,40]. NAC is a heterodimer that seems to influence the fidelity of the co-translational targeting of nascent chains to the endoplasmic reticulum[39]. Although NAC is not known to function as a chaperone in preventing polypeptide aggregation, a chaperone activity of NAC cannot be ruled out entirely. As NAC has no ATPase activity or homology to peptidyl-prolyl isomerases such as TF, its chaperone activity would probably involve the simple binding and protection of hydrophobic peptide stretches.

Hsp70 and chaperonins

DnaK and GroEL. After release from the ribosome, some newly synthesized polypeptides in *E. coli* have been observed to pass through both DnaK and the ATP-dependent, double-ring-shaped chaperonin GroEL[18,41,42] (FIG. 1). Unlike monomeric DnaK, which binds short, extended peptide stretches, the oligomeric 14-mer GroEL functions mainly by encapsulating a substrate polypeptide in a cavity that is capped by the GroES heptameric cofactor, which allows the completion of folding and prevents non-productive interactions with other unfolded polypeptides[19,20] (BOX 3). Consistent with this mechanism, GroEL has, so far, not been observed to associate with ribosome-bound nascent polypeptides, which could not be encapsulated. Furthermore, the containment mechanism of GroEL must function at later stages of protein folding, when a substrate polypeptide is in a compact non-native state. So, there are cases in which DnaK binds to early extended intermediates and GroEL functions subsequently to finish the folding of the later intermediate stages[43] (FIG. 1).

Whereas the ribosome-bound chaperone TF probably interacts generally with nascent polypeptides, GroEL functions in the folding of a subset of newly synthesized proteins[42,44]. Among the natural substrates of GroEL that have been identified so far are proteins with domains that combine α-helix and β-sheet structures: these seem to be preferred by the chaperonin relative to other folds, although not exclusively[44]. Proteins that cannot use GroEL for folding can still be kept in a soluble non-native state by this chaperonin, and other chaperones, including DnaK, might then assist in their folding[45] (FIG. 1). Both GroEL and DnaK are induced by stresses, such as heat shock[46], and together contribute to the refolding of pre-existing misfolded proteins[44,47]. Furthermore, DnaK can potentially bridge the co-translational function of the ribosome-bound TF and the post-translational function of GroEL[24,43]. Finally, GroEL can help in the folding of some proteins that are too large to be encapsulated[48] (BOX 3). In theory, GroEL could therefore interact productively with such proteins co-translationally, in a manner similar to DnaK, although this remains to be confirmed.

Hsp70 and TRiC. The eukaryotic chaperonin TRiC has a double-ring-shaped architecture that is related to that of GroEL, although there are certain differences in its structure and mode of function. Unlike the homo-oligomeric GroEL, the two rings of TRiC each contain eight different subunits[20,49,50] (BOX 3). Also unlike GroEL, TRiC has been observed bound to nascent polypeptide chains[34,38] (FIG. 2). Comparisons with the structurally related chaperonins from archaea indicated that TRiC also functions by enclosing substrate polypeptides in a cavity as part of an ATP-dependent cycle, although it does this without a GroES-like capping cofactor[51,52]. Recent biophysical experiments have confirmed that

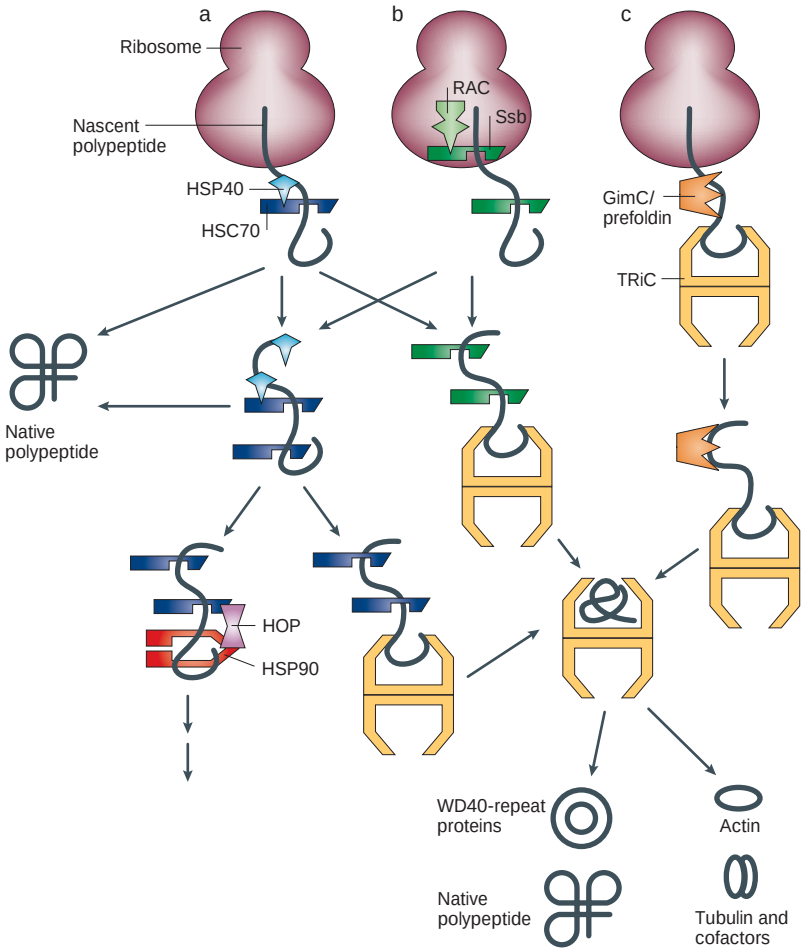


Figure 2 | **The early chaperone network of the eukaryotic cytosol. a** | In mammals, as nascent polypeptide chains emerge from ribosomes they are met by the 70-kDa heat-shock cognate protein (HSC70), which is stimulated by its cofactor the 40-kDa heat-shock protein (HSP40; which is also known as HDJ1) or by HDJ2. **b** | In *Saccharomyces cerevisiae*, the ribosome-associated complex (RAC) recruits Ssb, which is a 70-kDa heat-shock protein, to bind nascent chains. Some proteins that might be initially bound by Ssb are assisted in folding by Ssa, which is the *S. cerevisiae* homologue of HSC70. **c** | Actin and tubulin nascent chains are bound by GimC/prefoldin and the eukaryotic chaperonin TCP1 ring complex (TRiC), which assist them in folding to their native state. HSC70 (Ssa) and Ssb also cooperate with TRiC in the folding of different polypeptides, including several WD40-repeat proteins. Other newly synthesized polypeptides can fold spontaneously or be assisted by HSC70. Alternatively, they can be passed to the 90-kDa heat-shock protein (HSP90). The co-chaperone Hsp-organizing protein (HOP) links HSC70 to HSP90 for the subsequent folding or sorting of some polypeptide substrates.

TRiC uses such a mechanism, in which the opening and closing of the cage is mediated by the α-helical extensions of its apical domains[53]. Therefore, if a ribosome-bound polypeptide cannot be encapsulated, it is possible that TRiC might not complete the folding reaction at this stage and might function mainly to keep the polypeptide in a soluble state. It is also possible that TRiC might accomplish the folding of an individual domain of a multidomain protein by encapsulation, while the rest of the protein remains outside and extends through a gap in the apical domains[34,52]. Alternatively, the structural divergence of TRiC compared to GroEL might allow it to function without completely sequestering a protein[50].

WD40 REPEAT
A poorly conserved repeat sequence of 40–60 amino acids, which usually ends with Trp-Asp (WD). Several consecutive repeats fold into a circular structure, a so-called β-propeller, in which each blade is a four-stranded β-sheet. This domain is found in proteins that have various different functions.

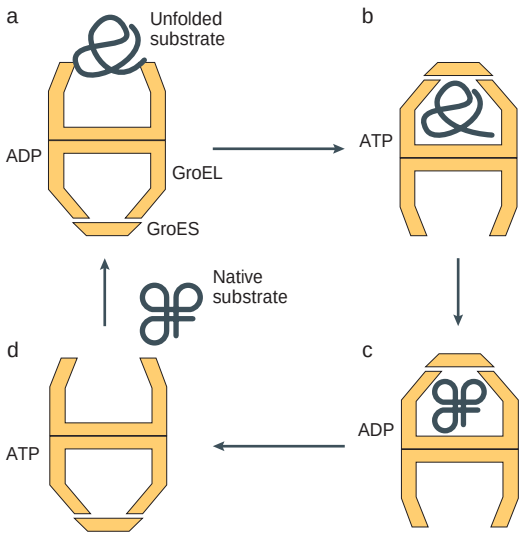
TRiC was originally characterized as being responsible for the folding of actin and tubulins[54,55] and, subsequently, for folding the Gα-transducin subunit, cyclin E and the von Hippel–Lindau (VHL) tumor suppressor[56–58]. Recently, TRiC in *S. cerevisiae* was shown to be required for the folding of several WD40-REPEAT proteins, which was the first identification of a structural class of proteins that is handled by the chaperonin. This class includes the Ste4 β-transducin subunit, the Cdc55 regulatory phosphatase subunit and the Pex7 peroxisomal import receptor[31], as well as the Cdc20 and Cdh1 activators of the anaphase-promoting complex[59]. As well as having cooperatively folded β-propeller structures, the WD40-repeat substrates of TRiC function as components of hetero-oligomeric protein complexes, and their strict dependence on TRiC might arise from a greater complexity in their folding requirements. Ssb seems to interact with these WD40-repeat proteins during their translation and continues to bind them post-translationally until they have completed their TRiC-mediated folding[31] (FIG. 2). Because the final interacting partners of β-transducin and Cdc55 (Gα-transducin and Sit4, respectively) are also handled by TRiC[31,56], a role for the chaperonin in complex assembly — as well as in subunit folding — might be envisioned. Ssa (including Ssa1 and Ssa2) is the *S. cerevisiae* equivalent of mammalian cytosolic HSC70 and stress-inducible HSP70, and it assists protein folding in cooperation with its DnaJ-related co-chaperones Ydj1 and Sis1 (REFS 60,61). It also works together with TRiC in the folding of the VHL protein[62]. Such cooperation between the Hsp70 chaperones and TRiC parallels the combined function of DnaK and GroEL, and is probably a feature that is common to the folding of many other polypeptides in eukaryotes.

GimC/prefoldin and chaperonins

The cytosolic chaperone Gim1–6 complex (GimC)/prefoldin is a complex of six different subunits in eukaryotes and has a related form in archaea, but not in bacteria[20,63,64]. GimC was originally described as supporting the folding and assembly of tubulins in *S. cerevisiae*[63], and the mammalian homologue prefoldin was reported to deliver unfolded actin to TRiC *in vitro*[64]. GimC is not ATP dependent and structural studies of archaeal prefoldin indicate that it recognizes substrates through the hydrophobic extensions at the end of its six coiled-coil domains[65]. These extensions also mediate weak interactions with TRiC, which might provide a mechanism for polypeptide transfer to the chaperonin[66].

GimC interacts with actin and tubulin nascent polypeptides during their translation[67] (FIG. 2). It also cooperates with TRiC in the post-translational steps of actin folding and helps to protect polypeptides from non-productive protein–protein interactions[68]. Interestingly, GimC seems to function mainly in the folding of actin and tubulin, and does not normally contribute to the TRiC-mediated folding of the WD40-repeat proteins in *S. cerevisiae*. Conversely, Ssb is involved in the productive folding of the WD40-repeat

Box 3 | The chaperonins



The bacterial chaperonin GroEL consists of two homo-heptameric rings, which form a barrel-shaped structure. Each subunit has an equatorial ATPase domain where the rings contact each other, and an apical substrate-binding domain at the open end of the barrel. Alternating ATP cycles in each ring control the conformations of the apical domains. The GroES co-chaperone is a homo-heptameric structure that can also bind to the GroEL apical domains. After substrate binding to one ring of GroEL (see figure, part **a**), ATP and GroES are bound by the same ring, which displaces the substrate into an enclosed cavity that is capped by GroES (part **b**). Sequestration of the substrate (part **c**) allows folding without the risk of non-productive intermolecular contacts, which could otherwise lead to aggregation. Dissociation of GroES after ATP turnover allows substrate release (part **d**)[19,20]. Some polypeptides require several cycles of binding and release to reach their native state. Certain polypeptides that are too large to fit inside the chaperonin cavity can also be bound by the ring of GroEL that is opposite the ring that is bound by GroES, and their folding can be assisted through cycles of such *trans* binding of GroES[48].

The eukaryotic chaperonin TCP1 ring complex (TRiC) is structurally related to GroEL, but has greater similarity to the chaperonins of archaea (thermosomes). TRiC has two rings of eight different subunits, which also have equatorial ATPase domains and apical substrate-binding domains[49]. Structural similarity to thermosomes indicates that extensions of the TRiC apical domains mediate the encapsulation of a substrate without the need for a GroES-like co-chaperone[51,52]. Similar to GroEL, ATP-dependent movements of the apical domains are involved in TRiC-mediated folding[50], as they bind substrate polypeptides and release them into the enclosed central cavity[53]. TRiC has no known co-chaperones that modulate its activity. Mammalian prefoldin binds TRiC with a comparatively weak affinity[66], but a regulatory function has not been observed. An open question is how important each of the eight TRiC subunits are for the folding of different proteins.

TRiC substrates, but not in the folding of actin and tubulin[31] (FIG. 2). It might be that GimC recognizes some specific features of its substrates in addition to exposed hydrophobic surfaces. This idea is consistent with electron-microscopy images of prefoldin bound to a compact folding intermediate of actin[66], and with recent biochemical experiments that used deletion mutants of prefoldin[69]. TRiC itself has also been analysed by electron microscopy and was found to be bound to compact folding intermediates of actin and tubulin[70,71], which is consistent with the post-translational completion of folding by GimC and TRiC[68]. It remains to be seen whether, and how, GimC/prefoldin works in the folding of other proteins.

HSC70 and HSP90

Protein folding. In eukaryotes, cytosolic HSC70 functions on certain polypeptides together with HSP90, which is the cytosolic member of the homodimeric ATP-dependent Hsp90 chaperone family (BOX 4). From yeast to mammals, HSP90 functions in the folding of a diverse set of proteins, including transcription factors, regulatory kinases and numerous other proteins that apparently lack common structural or functional features. The biochemical mechanisms of HSP90 are less well understood compared to HSC70, but it might function on more compact folding intermediates[72–74]. It is nevertheless clear that the ATPase activity of HSP90, which regulates the binding and release of polypeptide substrates, is essential for its function[75,76] (BOX 4). The diverse functions that are dependent on HSP90 in eukaryotes are reflected by the various phenotypes that are observed in genetic studies of *S. cerevisiae* and other model organisms[77].

Because HSP90 often works together with HSC70, the two chaperones might be considered as parts of a single multichaperone machinery. The activities of this HSC70–HSP90 machinery are modulated by a wide range of cofactor proteins that interact directly and specifically with either, or in some cases both, of the chaperones[72–74] (BOX 4). Several of these co-chaperones affect the ATPase cycles of HSC70 or HSP90 and thereby influence substrate binding by the chaperones. Among these are co-chaperones that can physically link HSC70 and HSP90 for the transfer of substrates between them (BOX 4). Another group of co-chaperones interacts preferentially with subsets of HSP90 substrates and might either target them to HSP90 or provide a specifically required chaperoning activity. A further group of co-chaperones recruits HSC70 or HSP90 to specialized cellular processes that are related to, but distinct from, protein folding — for example, protein translocation or degradation.

The HSC70–HSP90 system has been most closely studied in the maturation of the steroid hormone receptors in animals — in particular, the mammalian

Box 4 | The HSC70–HSP90 system

The cytosolic 90-kDa heat-shock protein (HSP90) is a homodimer. Each subunit has an ATP-binding domain at the amino terminus, an extended central region and a dimerization site near the carboxyl terminus. The anti-tumour agents geldanamycin and herbimycin A both function by blocking the ATP-binding site on HSP90, which inhibits its function in polypeptide folding. In the ATP-bound state the amino termini can transiently dimerize, which is thought to close the structure around a substrate polypeptide. The opening of the structure after ATP hydrolysis is thought to release the bound substrate and allow the re-loading of substrate onto HSP90 (REFS 72,73,76,137). The exact mode of polypeptide binding by HSP90 remains to be determined, as does the mechanism by which HSP90 influences polypeptide conformations.

Specialized tetratricopeptide repeat (TPR)-clamp domains link a family of co-chaperone proteins to HSP90, the 70-kDa heat-shock cognate protein (HSC70) or both[72,73,138]. The mammalian co-chaperone Hsp-organizing protein (HOP; which is known as Sti1 in *Saccharomyces cerevisiae*) coordinates HSC70 and HSP90 through independent TPR-clamp domains[138], and is thought to promote the transfer of HSC70-bound polypeptides to HSP90 by slowing the HSP90 ATPase cycle[139]. The recycling of incompletely folded polypeptides from HSP90 onto HSC70 might be accomplished by the mammalian co-chaperone TPR2 (which is also known as TTC2 or DJC7), which interacts with both chaperones through TPR-clamp domains and also contains an HSC70-regulatory J domain[140]. TPR-clamp domains have been found in a growing number of co-chaperone proteins, which contribute different biochemical activities or link HSP90 or HSC70 to various cellular functions[72,73,102].

Other HSP90 co-chaperones are unrelated to the TPR-clamp proteins. The HSP90 cofactor p23 stabilizes the closed ATP-bound form of HSP90 on steroid receptors[74,137] and stimulates complex dissociation after ATP hydrolysis[76]. Activator of HSP90 ATPase (AHA1) stimulates ATP hydrolysis by HSP90 (REFS 141,142) and might also function in the turnover of bound polypeptides. CDC37, which cooperates with HSP90 in the folding of many kinases, also regulates ATP cycling by HSP90, which probably allows the binding of the substrate polypeptides[143].

TETRATRICOPEPTIDE REPEAT-CLAMP DOMAIN
(TPR-clamp domain). TPR motifs are 34-amino-acid degenerate repeat sequences. Some eukaryotic co-chaperones of the cytosolic 70- and 90-kDa heat-shock proteins (HSC70 and HSP90, respectively) contain specialized TPR-clamp domains, which consist of three TPR motifs and 'clamp' a conserved aspartate residue at the carboxyl termini of HSC70 and HSP90.

SARCOMERE
A specialized structure in striated muscle, in which actin filaments contact numerous molecules of myosin, the motor protein that makes up muscle thick filaments. The movement of actin and myosin filaments past each other provides the driving force for muscle contraction.

KINETOCHORES
Specialized regions on chromosomes that are connected to microtubules and motor proteins during cell division in eukaryotes. Kinetochores function in the separation of chromosome pairs.

SKP1–CULLIN–F-BOX UBIQUITIN-LIGASE COMPLEX
A conserved protein complex in eukaryotes that is named on the basis of three of its characteristic components. It transfers ubiquitin onto specific substrate proteins and polyubiquitylated proteins are targeted for proteasomal degradation. It functions in the regulated degradation of proteins and is also essential for cell division.

glucocorticoid receptor and the avian progesterone receptor. In this pathway, HSC70 functions at the initial stage, in which it binds the labile hormone-binding domain of the receptor, whereas HSP90 functions at the final stage, in which it allows the receptor to bind its hormone ligands[74] (FIG. 3). The DnaJ homologue HDJ1 (HSP40) apparently initiates the binding of a receptor by HSC70 (REF. 78). The co-chaperone Hsp-organizing protein (HOP) — which is a TETRATRICOPEPTIDE REPEAT (TPR)-CLAMP DOMAIN protein (BOX 4) — then, together with HSP90, forms a complex with the receptor. After HSC70 and HOP dissociate from the complex, the HSP90-binding co-chaperone p23 assists in the final folding steps of the receptor[74]. The 52-kDa FK506-binding protein (FKBP52; also known as FKB4), which is an immunophilin with peptidyl-prolyl *cis–trans* isomerase activity and a TPR-clamp domain, replaces HOP on HSP90 at this stage and enhances receptor folding[74,79].

Another well-documented set of HSP90-dependent substrates are regulatory serine/threonine and tyrosine kinases. The co-chaperone CDC37, which is conserved from yeast to mammals[80–82], works with HSP90 in the folding of a growing number of these kinases[73]; examples from recent reports include inhibitor of nuclear factor-κB kinase, Aurora B, protein kinase B/AKT and mitogen-activated protein kinase[83–86] (FIG. 3). Less is known about the sequence of chaperone interactions with these substrates or the contribution of other co-chaperones, although HSC70, HOP and p23 are detected in CDC37 complexes[87]. CDC37 apparently functions directly on its kinase substrates using a chaperone activity that is independent from that of HSP90 (REF. 88), although it cannot completely substitute for the HSP90 chaperone activity. Recent evidence in *S. cerevisiae* indicates that Cdc37 can partially replace HOP (Sti1) in supporting the binding of HSP90 (Hsc82/Hsp82) to kinase substrates, whereas cyclophilin, which is another TPR-clamp-containing peptidyl-prolyl *cis–trans* isomerase, is more stringently required[89]. Not all kinases require CDC37 for their folding — for example, CDC37 functions in the maturation of cyclin-dependent kinase-4 (CDK4) but not CDK2 (REFS 80,82) — and the properties that determine the dependence on CDC37 remain unknown.

Polypeptide folding by the HSC70–HSP90 machinery in other cases seems to involve substrate-specific co-chaperones. In the maturation of the mammalian dioxin receptor, which is a relative of the steroid receptors, the immunophilin-related cofactor XAP2/AIP functions with HSP90 instead of FKBP52 (REFS 90,91). The TPR-clamp co-chaperone UNC-45 recruits HSP90 apparently for the folding and assembly of myosin at the SARCOMERE in *Caenorhabditis elegans*, and presumably also in other animals[92] (FIG. 3). Another new family of HSP90 co-chaperones has recently been proposed: RAR1 and SGT1 are homologous plant proteins that might function with HSP90 in the maturation of the pathogen-resistance proteins RPS2 and RPM1 in *Arabidopsis thaliana*[93,94], and Pto, Rx and N in *Nicotiana benthamiana*[95,96]. It is not yet clear how RAR1 and SGT1 contribute to, or affect, the chaperone activity of HSP90. Although the pathogen-resistance signalling pathway is not present in yeast or animals, homologues of RAR1 and SGT1 have been found in these kingdoms. The *S. cerevisiae* homologue Sgt1 is involved in the cell-division regulatory functions of the KINETOCHORE and the SKP1–CULLIN–F-BOX UBIQUITIN-LIGASE COMPLEX[97], and Hsc82/Hsp82 (HSP90) has previously been implicated in Skp1 and kinetochore function[98]. Therefore, a cooperation between HSP90 and Sgt1 in the control of mitosis in yeast and animals might be an exciting possibility.

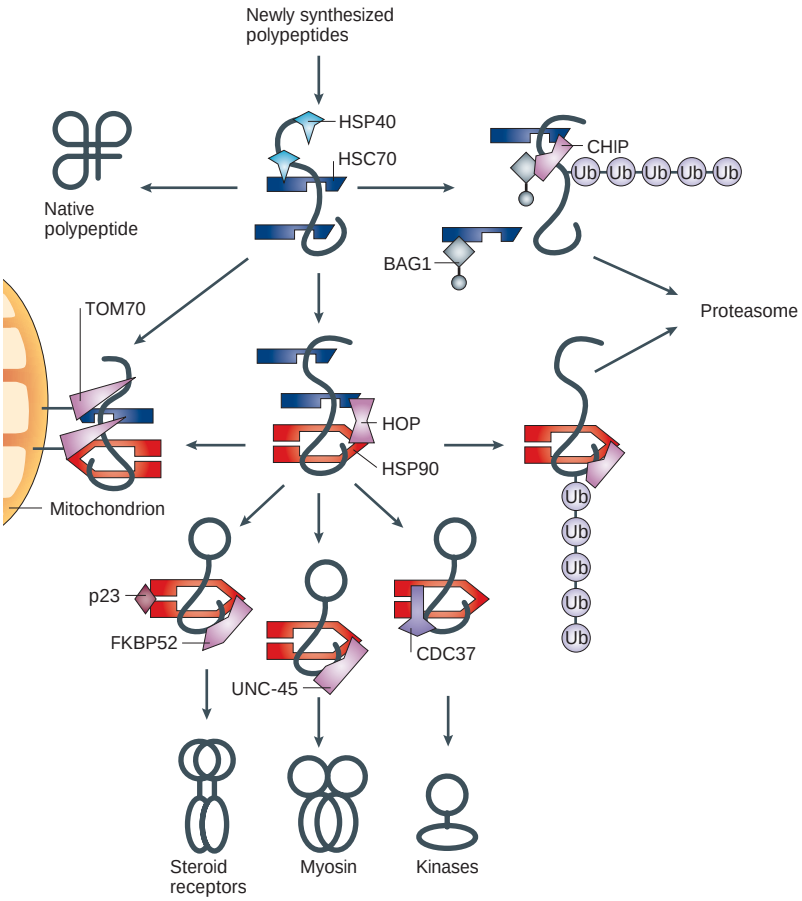


Figure 3 | **The late chaperone network of the eukaryotic cytosol.** Newly synthesized polypeptides are bound by the 70-kDa heat-shock cognate protein (HSC70), which works together with its 40-kDa heat-shock protein (HSP40 or HDJ1) or HDJ2 cofactor. Some polypeptides fold with the assistance of HSC70, whereas others are passed to the 90-kDa heat-shock protein (HSP90). The tetratricopeptide repeat (TPR)-clamp co-chaperone Hsp-organizing protein (HOP) organizes the transfer of animal steroid-receptor proteins from HSC70 onto HSP90 and might function similarly for other substrate polypeptides. Other TPR-clamp co-chaperones (shaded pink) help HSC70 and HSP90 in various functions. The immunophilin 52-kDa FK506-binding protein (FKBP52) functions in HSP90-dependent steroid-receptor folding, together with the HSP90 co-chaperone p23. UNC-45 functions with HSP90 in myosin folding. CDC37 is unrelated to the TPR-clamp cofactors and works with HSP90 in the folding of certain kinases. TPR-clamp interactions also recruit HSC70 and HSP90 for protein sorting. Some mitochondrial precursor proteins are delivered by HSC70 and HSP90 to the import receptor 70-kDa translocase of the outer mitochondrial membrane (TOM70) for import into the organelle. The U-box ubiquitin-ligase CHIP (carboxyl terminus of HSC70-interacting protein) contacts HSC70 or HSP90 to attach polyubiquitin onto substrate polypeptides, which results in these substrates being targeted to the proteasome for degradation. Finally, the HSC70-regulatory co-chaperone BCL2-associated athanogene-1 (BAG1) can assist in targeting the HSC70-bound polyubiquitylated polypeptides to the proteasome. Ub, ubiquitin.

A recent study reported that HSP90 that was recovered from tumour cells was largely contained in multiprotein complexes and that its ATPase activity was markedly higher than its activity in untransformed cells, in which HSP90 was mostly uncomplexed. The aberrant overexpression of HSP90-dependent oncoproteins, perhaps kinases or transcription factors, has been proposed to be the cause of this difference, which might explain the heightened sensitivity of tumour cells to HSP90-targeting drugs. Interestingly, the basic set of components that are involved in animal steroid-receptor maturation — HSP90, HSC70, HOP, HDJ1 and p23 — seem to be sufficient to form activated HSP90 complexes *in vitro*[99]. It is possible that these components comprise a central HSC70–HSP90 machinery, which might generally follow a sequential mechanism similar to that outlined for the steroid hormone receptors. Further co-chaperones might therefore modulate the central HSC70–HSP90 machinery to assist the folding of particular polypeptides. Consistent with this idea, *S. cerevisiae* has homologues of the five basic components, including p23 (Sba1)[72,73], although some other co-chaperones, such as FKBP52, are absent[79].

Protein sorting. In addition to its role in polypeptide folding, the HSC70–HSP90 machinery can be adapted by some co-chaperone proteins to function in protein sorting either to intracellular organelles or to the proteasome for degradation[100–102]. Mitochondrial proteins are mostly synthesized as precursor polypeptides in the cytosol and are imported post-translationally into the organelle[103]; HSC70, together with HDJ1 or HDJ2, is known to maintain their solubility[36,104,105]. In mammals, HSP90 and HSC70 have now been shown to stabilize some mitochondrial inner membrane proteins in a non-aggregated state in the cytosol and to mediate their targeting to the mitochondrial import receptor 70-kDa translocase of the outer mitochondrial membrane (TOM70). In both mammals and yeast, TOM70 recognizes the precursor-protein-binding chaperones through its co-chaperone TPR-clamp domain, and this docking step is important for the transfer of the precursors to the import receptor and the mitochondrial import machinery[106] (FIG. 3). Mitochondrial precursors with amino-terminal presequences rely on the main import receptor TOM20 and, in mammals, these precursors can be maintained in the cytosol by HSC70 and the co-chaperone XAP2/AIP, which can contact TOM20 directly[107]. In an independent pathway, Ydj1, which is the *S. cerevisiae* homologue of HDJ2, transiently associates with membranes through its covalently attached lipid moiety, and assists the HSC70 (Ssa)-mediated post-translational targeting of some proteins to both mitochondria and the endoplasmic reticulum[108]. Further interactions between the cytosolic chaperone systems and the targeting systems of other organelles remain to be explored.

Eukaryotic cells have a sophisticated system for protein degradation, in which multimers of the ubiquitin polypeptide are attached to proteins to target them for degradation by the proteasome. This system removes misfolded proteins from the cytosol and is also used to destroy native proteins for regulatory purposes[109] — the SKP1–Cullin–F-box complex that is mentioned above functions in regulated degradation. In mammals, the HSC70–HSP90 machinery interacts with the ubiquitin-mediated proteasome-degradation system through the co-chaperones CHIP (carboxyl terminus of HSP70-interacting protein) and BCL2-associated athanogene-1 (BAG1). The anti-apoptotic protein BAG1 regulates the ATPase cycle of HSC70,

and also contains a ubiquitin-like domain that mediates contacts with the proteasome[100,110–112]. CHIP contains a TPR-clamp domain that recognizes either HSC70 or HSP90, and a U-BOX-TYPE E3-UBIQUITIN-LIGASE activity that promotes the ubiquitylation and degradation of some HSC70–HSP90 substrate polypeptides[101,113,114] (FIG. 3). BAG1 and CHIP also interact with each other, and have been proposed to receive non-native polypeptides from the HSC70–HSP90 system and present them as ubiquitylated substrates to the proteasome for degradation[115]. Moreover, CHIP attaches atypical ubiquitin multimers onto BAG1, which apparently link this protein to the proteasome[116]. However, there are further functions of these co-chaperones that are unrelated to degradation. BAG1 promotes the activation of RAF1 kinase and the disruption of BAG1–RAF1-kinase complexes during heat shock reduces kinase activity, which contributes to cellular growth arrest[117]. Also, CHIP interactions with HSC70 are important for the activation of the heat-shock transcription factor HSF, which is necessary for the regulation of the heat-shock response and for protection from apoptosis[118]. Therefore, the protein-folding activity of HSC70–HSP90 can be modified by co-chaperones to give it roles in protein 'quality control', signal transduction and, potentially, many other processes.

Concluding remarks

The core of the cytosolic chaperone machinery — DnaK/HSC70 and the chaperonins — has been maintained from prokaryotes to eukaryotes. However, compared to bacteria, the range of folded polypeptides in the eukaryotic cytosol is more complex and, to handle such diverse conformational requirements, eukaryotes have expanded the core chaperone machinery that was inherited from bacteria. The archaeal chaperone GimC/prefoldin has been adapted in eukaryotes for the folding of cytoskeletal proteins that are not found in archaea[63,64]. The HSP90 homologue in bacteria (HtpG) is non-essential and has an accessory function under stress conditions[119], whereas eukaryotic cytosolic HSP90 is essential and fulfils numerous roles[120,121]. With the exception of DnaJ homologues, the various co-chaperones of HSC70 and HSP90 are not found in bacteria, but arose after the symbiotic development of eukaryotic cells. Although the biochemical mechanisms that are used by individual chaperones set limits with respect to the range of substrate proteins that they can assist in folding (as was observed in a recent mutagenesis study of GroEL[122]), the expansion of the chaperone systems in eukaryotes allows these limitations to be circumvented. Nevertheless, the essential principles of the chaperone networks are the same in prokaryotes and eukaryotes: chaperones bind to nascent polypeptides during synthesis and the polypeptides are then transferred between chaperones that cooperate to complete their folding.

One final comparison between prokaryotes and eukaryotes is instructive; that is, the re-solubilization of polypeptide aggregates by the AAA PROTEINS ClpB in *E. coli* and Hsp104 in *S. cerevisiae*. The disaggregation and unfolding activities of ClpB and Hsp104 are driven by their ATPase cycles, through a mechanism that is still under investigation[123]. In both cases, re-solubilization is optimal when the relevant Hsp70 chaperones (DnaK and Ssa, respectively) are also available to prevent re-aggregation and promote refolding[124,125]. Moreover, there is evidence that ClpB interacts directly with DnaK, perhaps to coordinate the activities of these proteins[126]. A common theme in these pathways, and those described above, is the central role of the Hsp70 chaperones. Conceptually, the Hsp70 proteins function at crossroads on the pathways of chaperone-mediated protein folding; they are universal and neutral, and receive newly synthesized polypeptides, stress-denatured proteins and polypeptides that have been re-solubilized from aggregates. In their various functions, Hsp70 proteins cooperate with other chaperones, including the ribosome-bound chaperones that handle nascent polypeptides in their first crucial folding steps, and the HSP90, GimC and TRiC systems that provide more specialized folding activities.

The chaperone pathways in both prokaryotes and eukaryotes share certain features with other networks of cellular processes, such as signal transduction or protein trafficking. One such trait is achieving a balance between specificity and redundancy. For example, DnaK is thought to compensate for a lack of TF in *E. coli*[18,22]. Also, in *S. cerevisiae* in which the *SSB* genes have been deleted, GimC can substitute in the chaperoning of the WD40-repeat substrates of TRiC, whereas other chaperones, including Ssa, cannot[31]. Another common feature is integrating chaperone function with other cellular processes, such as the regulation of transcription by HSP90 (REF. 127). As many of its substrate polypeptides are signal-transducing proteins, HSP90 might have a unique function in buffering genetic variability — a so-called CAPACITOR OF MORPHOLOGY function — with substantial effects on organism phenotypes[128]. Evidence for such genetic capacitance has been found in plants, as well as animals, which indicates a broad significance of this mechanism in evolution[77]. These are merely the more spectacular examples of how the control of protein conformation by the chaperone networks — as one of the most basic functions in biology — could influence processes at cellular, and higher, levels.

Note in proof

During the preparation of this review, Ferbitz *et al.*[129] published the crystal structures of *E. coli* TF and of the ribosome-binding domain of TF in complex with the archaeal *Haloarcula marismortui* ribosome. Using structural modelling, full-length TF is predicted to form a crescent shape that arches over the polypeptide exit site of the ribosome, with the ribosome-binding domain at one end of the crescent and the peptidyl-prolyl isomerase domain at the other. An emerging nascent polypeptide might therefore be shielded from aberrant interactions and might, perhaps, begin its folding in the protected space that is created by the TF arch. As folding proceeds, TF would eventually be released from the ribosome and the nascent polypeptide chain. The plausibility of this, and other, models for TF function awaits experimental validation.

U-BOX-TYPE E3 UBIQUITIN LIGASE
A class of ubiquitin-ligase domain that can transfer ubiquitin onto substrate proteins. It was first identified in the Ufd2 protein of *Saccharomyces cerevisiae*, and has since been found in several proteins from both yeast and mammals.

AAA PROTEINS
'ATPases associated with various cellular activities'. A superfamily of structurally related proteins (usually hexameric), a subset of which function to unfold proteins and a related subset of which function as proteases.

CAPACITOR OF MORPHOLOGY
The 90-kDa heat-shock protein (HSP90) has been proposed to buffer cryptic genetic variability by allowing mutated regulatory proteins to function normally. Phenotypes or morphologies that are associated with these mutations are only observed under stress conditions, when HSP90 function is reduced or overloaded, and favourable phenotypes can then be selected in a heritable manner. This mechanism is referred to as genetic capacitance.

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Competing interests statement
The authors declare no competing financial interests.

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