Cellular strategies for controlling protein aggregation

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Abstract | The aggregation of misfolded proteins is associated with the perturbation of cellular function, ageing and various human disorders. Mounting evidence suggests that protein aggregation is often part of the cellular response to an imbalanced protein homeostasis rather than an unspecific and uncontrolled dead-end pathway. It is a regulated process in cells from bacteria to humans, leading to the deposition of aggregates at specific sites. The sequestration of misfolded proteins in such a way is protective for cell function as it allows for their efficient solubilization and refolding or degradation by components of the protein quality-control network. The organized aggregation of misfolded proteins might also allow their asymmetric distribution to daughter cells during cell division.

Conformer
One of many possible structural states from the same protein species.

Molecular chaperone
One of a group of unrelated proteins that interact with non-native polypeptides to assist in their folding, transport and assembly.

Heat shock protein
A protein that shows increased expression in stress conditions through a specialized heat shock-response element in the promoter of the corresponding gene.

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To be functional, most proteins must adopt a defined three-dimensional structure termed the native fold. Protein folding starts as proteins are synthesized at ribosomes and passes through structural intermediates before the native state is reached (FIG. 1). Some intermediates can be non-productive, such as misfolded conformers that are trapped in free energy minima. As the energy barriers that separate native and non-native conformations are usually small, even native proteins are at permanent risk of unfolding, especially under environmental stress conditions[1–3]. Folding intermediates, including misfolded conformers, typically expose hydrophobic residues that are normally buried in the native structure. Such hydrophobic surfaces are prone to triggering the aggregation of proteins. There seems to be a preference for the co-aggregation of the same type of protein[4,5]. However, one aggregating protein species can also influence the aggregation behaviour of another one[6], and different proteins can be found to co-aggregate, as was observed for aggregation-prone proteins containing polyglutamine stretches. This trapping of other proteins in aggregates was suggested to be one possible reason for the toxicity associated with neurodegenerative diseases that involves polyglutamine aggregation[7–9].

Preventing the accumulation of aggregation-prone misfolded proteins is the first and most effective intervention point to control protein aggregation. Cells of all kingdoms of life have evolved an elaborate protein quality-control system, which acts either to facilitate the folding or refolding of misfolded protein species by molecular chaperones or to remove them by proteolytic degradation, thereby preventing protein aggregation[10–14]. The main chaperone classes that prevent the accumulation of misfolded conformers include the heat shock proteins (HSPs) HSP60 and HSP70, which exhibit ATP-dependent refolding activities[11,15–17]. Misfolded proteins that are not refolded are generally turned over by either cytosolic ATP-dependent AAA+ proteases (for example, the 26S proteasome)[10] or acidic hydrolases after their transport into the lysosomal compartment[14,18–20]. A proper balance between these protein quality-control components is required for protein homeostasis, also referred to as proteostasis[21]. Protein aggregation seems to result from exhaustion of the above quality-control system.

In this Review, we summarize the basic principles of the cellular mechanisms that control protein aggregation and cope with aggregates. We focus on the organization of aggregates in specialized intracellular deposition sites, mechanisms to reverse protein aggregation and strategies to eliminate aggregates or retain them in the cells with lower life expectancies during cell division. We do not cover the details of the many folding diseases that are related to protein aggregation and mention various disease-related proteins or types of aggregates only in the context of the general principles of protein aggregation.

Conditions that result in aggregation

The quality-control system can adapt to the severity of protein damage through the induction of stress responses, which adjust the cellular levels of chaperones and proteases[10,13,22–24]. However, when the generation of misfolded proteins exceeds the refolding or degradative capacity of a cell, protein aggregates accumulate. This exhaustion of the cellular protein quality-control system

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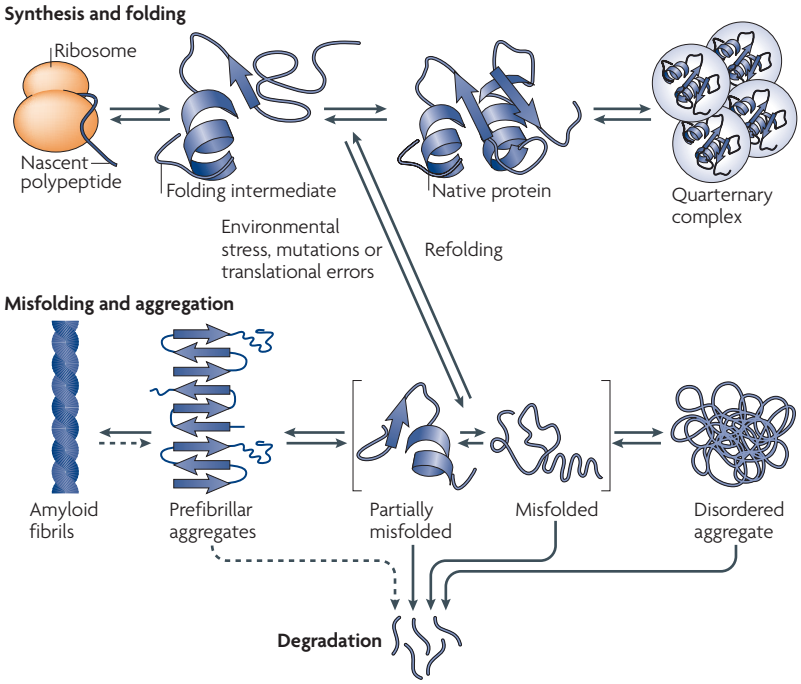

Figure 1 | **Overview of cellular protein aggregation.** A protein during and after its synthesis at the ribosome folds through different intermediates to its native, three-dimensional structure. Proteotoxic stresses, mutations in the synthesized protein or translational errors can cause protein misfolding. Once present, misfolded intermediates can be refolded to the native state or be degraded by different cellular proteolysis systems that prevent the accumulation of misfolded proteins. Once the quality-control network is overwhelmed — for example, through persisting harsh stress conditions, increased amounts of aberrant proteins or in aged cells — aggregates can form. Their formation can be guided by molecular chaperones. Forming aggregates can have varying degrees of structure, ranging from mostly unstructured, disordered aggregates to prefibrillar species and highly ordered β-sheet-rich amyloid fibrils. Disordered aggregates and intermediates during amyloid formation may be degraded. Arrows indicate a process that can include several single steps; dashed arrows indicate a process of minor significance.

can result not only from single, severe conditions, but also from the combination of different moderate conditions, which do not overwhelm the system on their own[4]. Various internal and external conditions have been identified and can be categorized into the four main classes described below.

The first class comprises mutations that result in the sustained tendency of the affected proteins to misfold and aggregate. Such mutations are responsible for various 'conformational diseases', such as type II diabetes, Huntington's disease and familial forms (which are inherited and have a higher probability of developing in the affected family) of Parkinson's disease and Alzheimer's disease[21,25,26]. Moreover, mutations in components of the protein quality-control system can provoke protein aggregation. Examples are mutations in the genes encoding the small HSP (sHSP) α-crystallin, leading to cataract formation[27], and the E3 ubiquitin ligase Parkin, resulting in an early onset form of Parkinson's disease[28–30].

The second class comprises defects in protein biogenesis. These include translational errors, leading to the misincorporation of amino acids, and assembly defects of protein complexes, leading to the accumulation of non-complexed protein species that are frequently prone to aggregation[31,32].

The third class comprises environmental stress conditions, such as heat and oxidative stress. Excessive heat treatment at or above the upper temperature range for growth of a particular cell type leads to the bulk unfolding of cellular proteins. Whereas heat-induced unfolding of proteins may be reversible (see below)[33], oxidative stress can lead to several irreversible protein modifications by reactive oxygen species (ROS), including radical-induced fragmentation of the polypeptide backbone and the replacement of side chains of specific amino acid residues by carbonyl groups[34]. Carbonyl derivates can be generated either by a direct oxidative modification of Pro, Arg, Lys and Thr residues or in reactions of Lys, Cys and His residues with reactive carbonyl compounds on glycoxidation products, lipids and advanced glycation end products[35]. These irreversible modifications can then lead to misfolding and eventually aggregation. One possible reason for the accumulation of carbonylated proteins as aggregates may be that the carbonyl groups can further react with the α-amino group of Lys residues, thereby leading to cross-linked derivates that are resistant to proteolytic degradation by the proteasome[34].

The fourth class comprises protein aggregation in cells during ageing, which occurs at a slower pace. For example, the aggregation of polyglutamine model proteins in *Caenorhabditis elegans* and a misfolding-prone mutant of human superoxide dismutase 1 (SOD1) in mice is exacerbated during ageing[36,37]. As SOD1 mutants, in contrast to the wild-type protein, expose more hydrophobic surfaces and can be bound by chaperones, this example demonstrates how the quality-control system can become progressively exhausted during ageing[37,38]. Similarly, carbonylated proteins accumulate progressively to form visible aggregation foci in the cytoplasm in aged yeast cells[39]. These findings suggest a reduced capacity of ageing cells to eliminate misfolded protein species. Such a general decline in the capacity of cellular protein quality control during ageing was also suggested to be a reason for the late age of onset of Huntington's disease and many sporadic forms of Alzheimer's disease or Parkinson's disease. However, additional factors, including the unique combination of polymorphic variations in different proteins of an individual, may also influence its protein quality-control capacity[4,23]. In *C. elegans*, the general decline of protein quality control happens at an early stage of adulthood and thus is an early molecular event during cellular ageing in this organism[40].

Structural features of aggregates

Protein aggregates were initially classified, based on electron microscopy, as either apparently amorphous (for example, bacterial inclusion bodies generated on the overproduction of recombinant proteins) or amyloid-like. Meanwhile, it became apparent that such classification is an oversimplification as, for example, bacterial inclusion bodies have been demonstrated to contain amyloid-like structures[41]. Intramolecular β-sheets have now been recognized as a common structural element of aggregates and are shared by both

AAA+ protease
An ATP-dependent proteolytic machinery that acts in general and regulatory proteolysis.

E3 ubiquitin ligase
A component of the ubiquitin–proteasome system that delivers activated ubiquitin moieties to special substrates and thereby provides substrate specificity for ubiquitylation.

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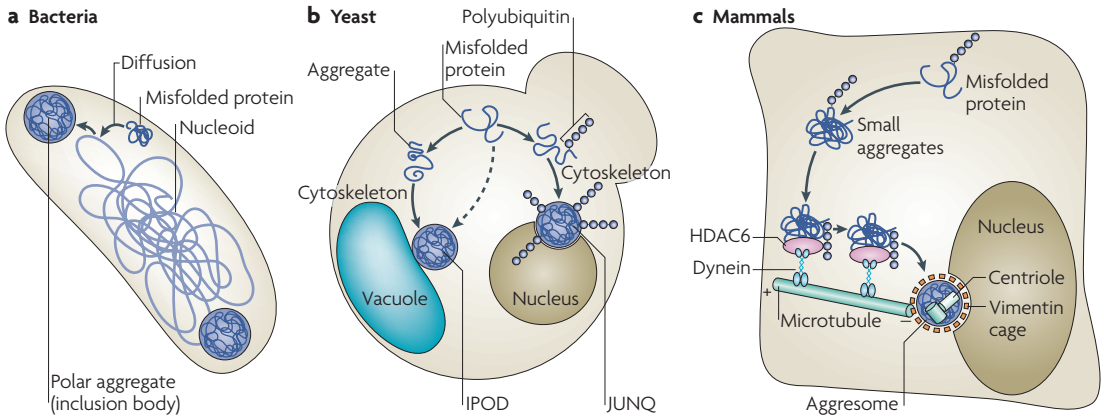

Figure 2 | **Pathways for the cellular sequestration of protein aggregates. a** | In bacteria, misfolded proteins can accumulate in inclusion bodies under different conditions, such as the heterogenous expression of proteins or stress. Inclusion bodies often form at the periphery of the cell. Nucleoid exclusion is sufficient to control the polar localization of aggregated proteins. Energy-driven active processes may contribute to the deposition of misfolded proteins in inclusion bodies. **b** | Yeast cells possess distinct protein quality-control compartments, the juxtanuclear quality-control compartment (JUNQ) and the perivacuolar insoluble protein deposit (IPOD). Soluble, misfolded, ubiquitylated proteins can be disposed at the JUNQ, whereas insoluble, terminally aggregated proteins can accumulate at a perivacuolar site. Disruption of the cytoskeleton disturbs the targeting to both compartments. **c** | JUNQ- and IPOD-like compartments have also been seen in mammals and are distinct from the perinuclear aggresomes where misfolded, ubiquitylated proteins accumulate. The aggresome is a vimentin-enwrapped structure located at an indentation of the nucleus surrounding the centriole. Aggresome formation requires the adaptor histone deacetylase 6 (HDAC6), which binds to ubiquitylated proteins on the one hand and the microtubule minus-end motor protein dynein on the other hand. Other sequestration pathways in yeast and mammals have been reported but are not shown.

apparently amorphous aggregates and amyloid fibrils. The degree of β-sheet organization is, however, variable in the different aggregate forms, with the highest one present in amyloid fibrils, in which the β-sheets run perpendicular to the fibril axis[1,25,42–44] (FIG. 1). The variability of protein aggregation is best illustrated by the finding that the morphology of protein aggregates generated from the same protein species can be diverse[45] and influenced by the denaturing conditions, which lead to different unfolding and aggregation pathways[6,46]. In an attempt to identify the underlying molecular differences in a recent study, different amorphous and fibrillar aggregates of the amyloidogenic amino-terminal fragment of the protein HypF were generated and compared by NMR techniques. These morphologically different forms have shared but also distinct segments of the protein sequence involved in the cross-β-sheet structural motif[46]. Furthermore, subtle structural differences in the interphase of interacting polypeptides that result in differences in the morphology and toxicity of the particular aggregate have been shown for some prion proteins[47–49].

Cellular aggregate deposition sites

How do cells deal with aggregated proteins? Compelling evidence, such as the deposition of protein aggregates at specific cellular sites, suggests that protein aggregation is a much more organized process than previously thought (FIG. 2). The sequestration of aggregated proteins can be viewed as a second cellular response that occurs when the quality-control system that refolds or degrades misfolded proteins in their soluble state has been overrun.

Directing aggregated proteins to specific compartments can protect the cellular environment from potentially deleterious protein species. As shown for certain amyloidogenic proteins, it becomes more and more evident that soluble oligomers themselves, rather than insoluble amyloids in their microscopically visible final state, are cytotoxic[25]. The formation of amyloid aggregates may even have a cytoprotective function[28,50–55]. Organizing protein aggregates might also facilitate the efficiency of aggregate removal in a subsequent phase[56–58]. Although the spatial sequestration of misfolded proteins seems to be a common strategy of all cells, the specific localization of deposition sites differs between organisms and depends on the particular aggregation-prone protein, the cellular compartment and the stress conditions causing protein misfolding. We do not cover the specific features of each aggregating species here, but we give some examples of more general, and better-characterized, deposition processes.

Deposition sites for protein aggregates in bacteria. Aggregates of endogenous proteins can form in bacteria, particularly under heat or oxidative stress conditions[59,60]. Furthermore, insoluble inclusion bodies frequently form in bacteria and also in eukaryotic cells that overexpress heterologous proteins[61,62]. Usually one or two inclusions form per cell, predominantly at the cell poles but also in mid- or quarter-cell positions, which are future sites for septation[63–65]. So far, the best quantitative analysis of protein aggregation has been established for heat-treated *Escherichia coli* cells. In *E. coli*, ~1.5–3% of total cytosolic proteins can aggregate and individual inclusion bodies contain ~2,400 –16,500 protein molecules. The number

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of proteins that are vulnerable to thermal unfolding and aggregation is surprisingly high, ranging from 150–200 individual protein species[65,66].

In *E. coli*, such aggregates are typically localized to the old cell pole for reasons that are still unclear[64,65] (FIG. 2a). The mechanism by which aggregating proteins reach the cell poles is controversial. One study claims that an active, energy-driven process is responsible for polar localization[67]. By contrast, a second study demonstrated that nucleoid occlusion is necessary and sufficient for controlling the polar localization of aggregated proteins, indicating a passive mechanism for aggregate sequestration[65].

Deposition sites for protein aggregates in yeast. Protein aggregation in eukaryotic cells is often studied in *Saccharomyces cerevisiae*[68]. When yeast cells are exposed to severe heat stress, aggregated proteins form multiple electron-dense foci of different sizes dispersed throughout the cytoplasm and nucleus, as visualized by transmission electron microscopy. No obvious specific compartmentalization was observed for this type of aggregate[33]. Notably, most of these aggregated proteins can be reactivated by chaperones during a recovery phase (see below).

Proteins that form aggregates in yeast and that are not refolded to the native state include oxidatively damaged proteins[35], proteins that are marked for degradation by ubiquitin[58], amyloidogenic proteins such as yeast prions[69,70] and polyglutamine model proteins (for example, Htt103Q — the first exon of huntingtin, with a stretch of 103 Gln residues)[71–73]. The pattern of aggregation of these protein classes is diverse and there may be various ways of organizing them. For example, it was shown for some substrates of each class that they can localize, at least partially, to one of two recently identified specialized quality-control compartments for the deposition of aggregated proteins[58] (FIG. 2b). One compartment adjacent to the nuclear membrane, termed the juxtanuclear quality-control compartment (JUNQ), transiently accumulates misfolded proteins that are ubiquitylated and are presumably substrates for proteasomal degradation. Substrates at the JUNQ are still mobile and exchange rapidly with the surrounding cytoplasm. The second compartment adjacent to the vacuole, termed the insoluble protein deposit (IPOD), harbours terminally aggregated, insoluble proteins, including carbonylation-sensitive proteins[74] and amyloidogenic proteins such as Htt103Q or the yeast prions [RNQ] and [URE3] (REF. 58). Strikingly, substrates for either compartment could be directed to the other compartment by experimentally manipulating the ubiquitin proteasome system. Impairing ubiquitylation of misfolded substrates — for example, through deletion of the E2 ubiquitin-conjugating enzyme pair Ubc4–Ubc5 — led substrates of the JUNQ to be directed to the IPOD, whereas introducing a ubiquitylation site into otherwise typical IPOD substrates allowed targeting to the JUNQ[58]. Thus, the overall picture is that ubiquitylated proteins that are usually substrates for proteasomal degradation can be stored reversibly in the JUNQ compartment when the capacity of proteasomal degradation is limiting. By contrast, terminally misfolded proteins that are not usually turned over by the proteasome are deposited more permanently at the IPOD. Furthermore, the IPOD could serve as an overflow compartment when the ubiquitin proteasome system is overwhelmed. This raises several intriguing questions, foremost of which is the mechanism by which the misfolded proteins are recognized, sorted and transported to the JUNQ and IPOD. It is also not clear what the fate of the aggregated proteins in the IPOD and JUNQ are. JUNQ- and IPOD-like compartments have also been observed in mammalian cells[58].

The mammalian aggresome. A specialized form of inclusion bodies in the cytoplasm of mammalian cells is termed the aggresome[75,76] (FIG. 2c). Aggresomes are not permanently present in the cell, but form in numerous disease states[62,77], as a result of the expression of several heterologous proteins — including cystic fibrosis transmembrane conductance regulator (CFTR)[76] and a green fluorescent protein (GFP)–p115 chimaera[75] — and on inhibition of the proteasome. Aggresomes localize to an indentation of the nuclear envelope at the microtubule-organizing centre (MTOC) and often surround the centriole. The exterior of aggresomes is sheeted by a cage-like shell formed by the intermediate filament vimentin. Their overall structure and size varies and depends on cell type and the aggregating substrate. Most aggresomes appear as a single sphere of 1–3 μm diameter or as an extended ribbon[62,75–77].

As shown for a GFP–p115 fusion protein, aggresome formation is initiated by the formation of smaller aggregates in the periphery, which then move in a dynein-based manner along the microtubule cytoskeleton to the final perinuclear site at the MTOC[75]. Although ubiquitylation of the substrate is generally considered to be a prerequisite for its recognition and transport to aggresomes, it could not be shown for all the substrates found in aggresomes, which leaves the possibility that signals other than ubiquitylation may also be involved[62,77]. The transport of aggregated proteins to aggresomes is mediated by histone deacetylase 6 (HDAC6), which functions as an adaptor that binds polyubiquitin chains of substrates and the microtubule motor protein dynein, thereby mediating the transport of polyubiquitylated cargo along microtubules towards the MTOC during aggresome formation[78] (FIG. 2c). The E3 ubiquitin ligase Parkin, which promotes the degradation of several substrates, has been suggested to recognize misfolded proteins and mark them by Lys63-linked polyubiquitylation[29]. This may provide one type of signal for HDAC6-dependent transport and sequestration of misfolded proteins to the aggresome under conditions of proteasomal impairment, for example in pathogenic situations[29,79].

Notably, HDAC6 has also been implicated in the regulation of cellular stress responses by being a component of a HDAC6–HSF1–HSP90 complex, which represses the stress response by trapping the transcription factor heat shock factor 1 (HSF1). The binding of HDAC6 to ubiquitylated aggregates may cause the release and activation of HSF1. This offers the possibility of coupling

Juxtanuclear quality-control compartment
A compartment for the deposition of soluble, misfolded proteins in yeast and mammals. The targeting of substrates to this deposition involves ubiquitylation.

Insoluble protein deposit
A perivacuolar quality-control compartment for the deposition of terminally aggregated proteins observed in yeast and mammalian cells. One group of substrate proteins accumulating here are aggregates of amyloidogenic proteins.

Ubiquitin proteasome system
A cellular quality-control system for the ubiquitin-dependent degradation of substrate proteins by the proteasome.

E2 ubiquitin-conjugating enzyme
A component of the ubiquitin proteasome system that harbours an activated ubiquitin moiety and cooperates with E3 ligases in substrate ubiquitylation.

Lys63-linked polyubiquitylation
A polyubiquitin chain in which the ubiquitin molecules are linked by the internal Lys63 residue of ubiquitin.

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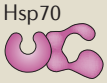
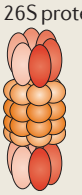
Table 1 | **Molecular chaperones and proteases implicated in protein disaggregation**

Chaperone	Organism	Structure and oligomeric state	ATP binding	Activity
ClpB or Hsp104 	Bacteria, yeast, plants and mitochondria of animals	Hexamer	Yes	Reactivation of aggregated proteins in cooperation with an Hsp70 chaperone system
Hsp70 	Bacteria, archaea and eukaryotes (cytosol, ER, mitochondria and chloroplasts)	Monomer	Yes	Prevention of aggregation, reactivation of aggregated proteins in cooperation with ClpB or Hsp104, and folding of newly synthesized proteins and misfolded protein species
sHSPs 	Bacteria, archaea and eukaryotes (cytosol)	8–24-mer	No	Prevention of irreversible protein aggregation
AAA+ proteases 	Bacteria and eukaryotes (mitochondria and chloroplasts)	Hexamer (for example, ClpA and ClpC) and heptamer (for example, ClpP)	Yes	Degradation of misfolded or aggregated protein species and of native proteins harbouring specific degradation tags
26S proteasome 	Eukaryotes (cytosol)	Hexamer (for AAA+ proteins) and heptamer (for α- and β-subunits)	Yes	Degradation of polyubiquitylated proteins (including misfolded and native proteins harbouring specific degradation tags)
VCP 	Eukaryotes (cytosol)	Hexamer	Yes	Degradation of misfolded ER proteins and membrane fusion

ER, endoplasmic reticulum; sHSP, small heat shock protein; VCP, valosin-containing protein.

the sensing of aggregated proteins by HDAC6 to an increased chaperone expression, thereby initiating a cellular response to counteract the accumulation of misfolded protein species[80].

Reversal of protein aggregation

Aggregation is not necessarily a dead-end situation for a protein *in vivo* as disaggregation followed by refolding of aggregated proteins has been observed in cells from diverse species, from bacteria to humans. Disaggregation is not achieved by a single activity, but by different cellular machineries, as summarized in TABLE 1.

Protein disaggregation by a bi-chaperone system. The reversibility of protein aggregation was first demonstrated for *S. cerevisiae*[33]. Heat-aggregated proteins in the cytosol and nucleus are reactivated by the cooperative action of the Hsp70 system, composed of Ssa1 and the J protein co-chaperone yeast DnaJ protein 1 (Ydj1), and the oligomeric, ring-forming AAA+ chaperone Hsp104 (REF. 81). A comparable activity exists in *E. coli*, provided by the bi-chaperone system of the corresponding orthologues of the Hsp104 (ClpB), Ssa1 (DnaK) and Ydj1 (DnaJ) chaperones[66,82]. Notably, each chaperone component on its own has only limited (the Hsp70 system) or no (Hsp104 and ClpB) disaggregation activity.

This bi-chaperone system is also found in the cytosol of most other bacteria, plants and several unicellular eukaryotes, and in mitochondria and chloroplasts of unicellular and multicellular eukaryotes. Its activity efficiently counteracts the damaging effects of severe stress situations, and the induction of its expression by a sub-lethal heat treatment even enables cells to transiently survive a subsequent, severe heat shock treatment that is normally lethal, a phenomenon referred to as thermotolerance[83–85]. Further experiments showed that for *E. coli* and yeast cells the main reason for the loss of viability under such severe stress conditions is the massive loss of protein activity by misfolding and aggregation and that thermotolerance requires the bi-chaperone mediated reactivation, rather than the degradation, of aggregated proteins[84,86].

The mechanism of protein disaggregation by this bi-chaperone system involves an essential activity of the Hsp70 system during the initial phases of the process[86,87] (FIG. 3). The binding of Hsp70 and J proteins to aggregates restricts the access of proteases to the aggregates and allows the transfer of aggregated polypeptides to the substrate-processing pore of ClpB or Hsp104 (REFS 88,89) (FIG. 3). These two functions combined provide a mechanism for pathway selection, in which the refolding pathway is preferred over the

J protein
A co-chaperone of HSP70 chaperones that harbours a characteristic domain called a J domain and is responsible for activating the ATPase activity of HSP70.

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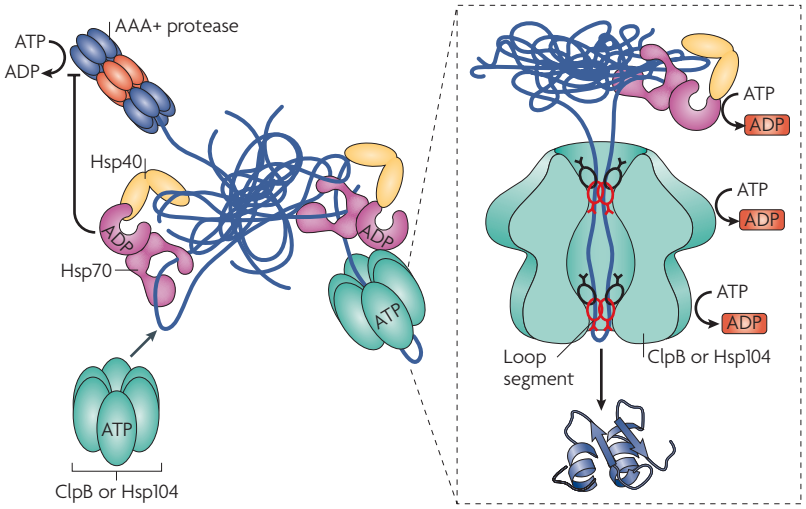

Figure 3 | **Protein disaggregation by the Hsp104–Hsp70 bi-chaperone system.** The 70-kDa heat shock protein 70 (Hsp70) system, consisting of the ATPase Hsp70 and a co-chaperone of the Hsp40 family, interacts first with protein aggregates. The initial binding of Hsp70–Hsp40 has two functions. First, it restricts access of AAA+ proteases to the aggregates and, second, it allows for substrate transfer to the ClpB (or the yeast homologue, Hsp104) disaggregase. Such transfer might require a physical interaction between the cooperating chaperones or be based on the Hsp70–Hsp40-mediated exposure of ClpB- or Hsp104-binding sites in the aggregate. ClpB or Hsp104-mediated disaggregation is regulated by a threading activity. Conserved aromatic residues (Tyr) are located on mobile loops at the central pore and contact substrate proteins. ATP binding and hydrolysis are suggested to cause conformational changes of the loop segments, generating a pulling force that drives the translocation of substrate segments through the ClpB or Hsp104 hexamer.

degradation pathway, with the concomitant benefit for cell survival during severe stress. The Hsp70- and J protein-mediated transfer of aggregated proteins involves the ClpB- or Hsp104-specific middle domain (termed the M domain) that is lacking in other AAA+ family members[88]. The precise mechanism of this chaperone interplay is still not well understood. DnaK may remodel protein aggregates leading to the exposure of substrate stretches that are recognized by ClpB or Hsp104. Alternatively, the cooperation may involve a direct physical interaction between both chaperones, a scenario that is supported by the fact that their cooperation in aggregate solubilization shows strong species specificity (that is, combinations of bacterial and yeast components are not active)[81]. After substrate transfer, ClpB or Hsp104 exert a threading activity leading to the one-by-one extraction of misfolded polypeptides from the aggregate[86,90]. Substrate threading is mediated by pore-located aromatic residues, which pull the substrate on ATP consumption into the central translocation channel[86,90,91] (FIG. 3). Notably, ClpB and Hsp104 are equipped with a remarkable flexibility, allowing them to thread internal aggregated segments of substrates into the central pore in a looped conformation. Substrate threading is arrested when ClpB or Hsp104 encounter a tightly folded domain leading to substrate dissociation, a process that ensures optimal substrate refolding[89]. This may involve additional assistance by Hsp70 chaperones and chaperonins.

The role of sHSPs in organizing and solubilizing protein aggregates. The chaperone-mediated protein disaggregation process is further facilitated by sHSPs that directly interact with aggregating proteins[92,93] (FIG. 4). For example, the *E. coli* sHSPs inclusion body-binding protein A (IbpA) and IbpB were initially identified by their tight association with bacterial inclusion bodies[94]. sHSPs are the most widespread molecular chaperones with an increasing number of family members in multicellular eukaryotes. They share the α-crystallin domain and also have variable extensions at the N and C termini[93]. Their synthesis and chaperone activity is often tightly controlled by temperature, allowing them to activate sHSP function on demand[92,95,96]. sHSPs bind tightly to misfolded protein species, resulting in the formation of sHSP–substrate complexes that do not release bound proteins spontaneously, thereby creating a reservoir of misfolded proteins during stress conditions. Such complexes are still aggregates, but of reduced size and altered composition[95,97]. It is tempting to speculate that sHSPs might even function to seed the aggregation of misfolded protein species, thereby controlling the aggregation process in the cell and potentially even directing aggregates to specific cellular sites.

sHSP-induced changes in aggregate architecture allow for more efficient disaggregation by the bi-chaperone system and contribute to the development of thermotolerance[98–100]. In addition, incorporation of sHSPs into aggregates allows for protein disaggregation by the Hsp70 chaperone system[97,101,102] (FIG. 4). The increased number of sHSP species in multicellular eukaryotes might potentially enable Hsp70 chaperones to work productively on aggregates even without the cooperation of an Hsp104-like AAA+ chaperone.

Other disaggregation activities. In bacteria, several additional AAA+ chaperones (for example, ClpA, ClpC and ClpE from *E. coli* and *Bacillus subtilis*), which act in proteolysis by complex formation with peptidases (such as ClpP) (see TABLE 1), have been shown to possess a disaggregation activity *in vitro*, underlining the unique capacity of the AAA+ protein family to act on aggregates[103,104]. However, their contribution to protein disaggregation *in vivo* has not yet been fully clarified.

It is remarkable that ClpB and Hsp104 homologues exist only in the mitochondria and chloroplasts of higher eukaryotes. Organisms that encode cytosolic ClpB and Hsp104 homologues lack large-scale mobility and cannot escape exposure to sudden and severe environmental changes such as thermal stress. The finding that the expression of Hsp104 leads to a substantial increase in the disaggregation activity in human cell lines and in *C. elegans* indicates that the disaggregation capacity is limited in such organisms[105,106]. Nevertheless, various studies showed that animal cells can solubilize aggregates, demonstrating the existence of a disaggregation activity in the absence of ClpB or Hsp104 (REFS 53,107,108). The identity of this activity is not yet clear, but may involve the action of Hsp70 chaperones and sHSPs.

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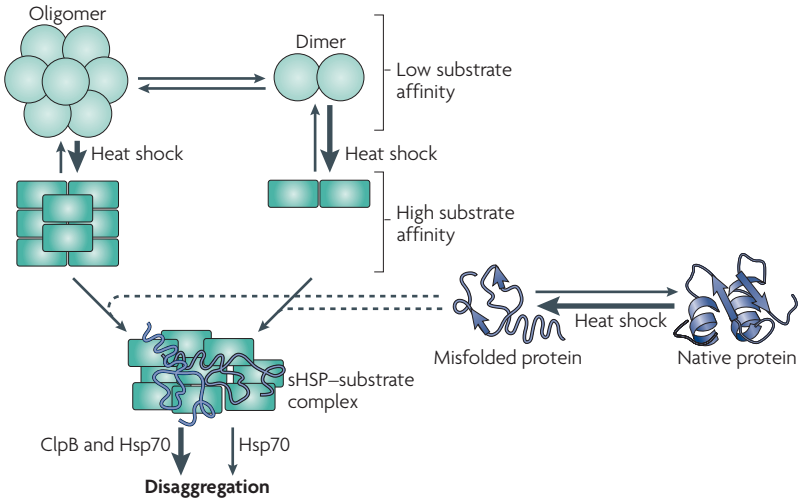

Figure 4 | **The role of small heat shock proteins in protein aggregation.** Small heat shock proteins (sHSPs) are in equilibrium between oligomeric structures and exchanging subunits. They can exist in two states, with a low and high substrate affinity, respectively. During heat shock, the equilibrium shifts towards the high affinity state, which can then form a stable complex with substrates, such as the misfolded proteins that arise during the heat shock. The stable sHSP–substrate complex is thought to prevent irreversible aggregation and can facilitate the re-solubilization of aggregated proteins by the bi-chaperone system, consisting of Hsp104 and the Hsp70 chaperone system, or by Hsp70 only.

The ATP- and ubiquitin-dependent AAA+ chaperone valosin-containing protein (VCP; also known as p97) is another candidate for exerting disaggregation activity in the cytosol of animal cells (see TABLE 1). Like ClpB and Hsp104, VCP contains two ATPase domains and acts in numerous cellular activities by cooperating with many adaptor proteins[109]. VCP mutations are linked to inclusion body myopathy with early-onset Paget's disease and frontotemporal dementia (IBMPFD) and loss of VCP function in mammalian cells leads to the accumulation of insoluble ubiquitylated proteins[110,111]. VCP also associates with polyubiquitylated aggregates generated on proteasomal inhibition, and their subsequent solubilization requires VCP activity[112]. Direct evidence for a disaggregation activity of VCP is, however, still missing. The potential function of VCP in aggregate clearance may relate to its role in aggresome formation[113]. Thus, VCP mutations can lead to changes in aggregate localization, thereby potentially affecting aggregate clearance.

Distinct roles of the ubiquitin proteasome system in formation and clearance of protein aggregates. Degradation by cellular proteases is an alternative route for the elimination of protein aggregates. In view of the limited refolding activity found in higher eukaryotes, the degradation route might have gained increasing importance in multicellular organisms. The 26S proteasome is the central cellular machine responsible for the degradation of soluble, misfolded proteins, thereby preventing protein aggregation (TABLE 1). Inhibition of proteasomal degradation can cause neurodegeneration, underlining the crucial function of the ubiquitin proteasome system in protein degradation[114, 115]. Misfolded proteins are recognized and marked for degradation by different specific E3 ubiquitin ligases, often in concert with Hsp70 chaperones. The most prominent examples of E3 ubiquitin ligases are C terminus of HSP70-interacting protein (CHIP) in mammals[116], Ubr1 and San1 in yeast[117–119,120], and HMG-CoA reductase degradation protein 1 (Hrd1; also known as Der3) and Doa10 for proteins derived from the endoplasmic reticulum[13].

A role of the ubiquitin proteasome system in the degradation of pre-existing protein aggregates is suggested by the presence of ubiquitylated proteins in protein inclusions, the frequent co-localization of the 26S proteasome with protein aggregates and the increased aggregate formation and delayed removal of aggregates on inhibition of proteasomal activity[57,76,115,121–124]. However, the involvement of the ubiquitin proteasome system in aggregate clearance may be less important than suggested by these observations. The 26S proteasome cannot degrade aggregates *in vitro*[125,126], and aggregates even reduce proteasomal activity *in vivo*[127] by irreversible sequestration of proteasomes or other effects[128,129]. Together, these findings do not support a major contribution of 26S proteasomes in the removal of pre-existing aggregates. They also indicate that the increased levels of aggregated proteins observed on proteasomal inhibition are a consequence of increased levels of misfolded proteins caused by substrate stabilization and the obstruction of other quality-control pathways.

Aggregate clearance by autophagy

Macroautophagy uses specialized, cytosolic, double-membrane structures that engulf substrates to form autophagic vesicles that ultimately fuse with the lysosome for degradation of their content[19,20]. It has traditionally been viewed as a rather unspecific degradative pathway, in which cytosolic contents and organelles are turned over in a non-selective manner. More recently, however, a form of selective macroautophagy has been identified as a major contributor in the clearance of misfolded and aggregated proteins in the cytosol of mammalian cells[14,18–20]. Initially, aggregates of proteins involved in neurodegenerative disease, such as α-synuclein or mutant huntingtin, were identified as substrates for this type of autophagy[130–132], which was regarded as a back-up system to complement proteasomal degradation when it is overwhelmed or incapable of dealing with specific aggregated substrates. In agreement with this, autophagy was also suggested to have a role in the clearance of aggresomes[130,131,133,134] (FIG. 5). Interestingly, one of the main players of aggresome formation, HDAC6, also controls a step that is essential for aggregate turnover by autophagy; the fusion of autophagosomes with lysosomes by the recruitment of the actin-remodelling machinery that is involved in this process[135]. An implication for autophagy in the clearance of misfolded proteins under more physiological conditions came from the observation that the conditional knock out of genes essential for autophagy, autophagy protein 5 (*Atg5*) and *Atg7*, in mouse liver and

Autophagosome
A double-membrane vesicle in the cytoplasm that includes intracellular components for lysosomal degradation.

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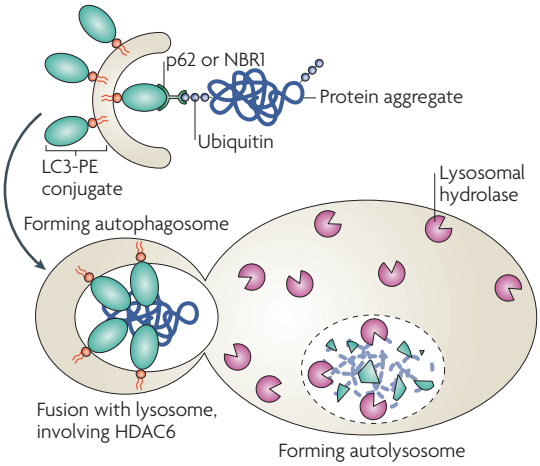

Figure 5 | **A model for the selective autophagic degradation of protein aggregates.** Ubiquitylation of protein aggregates is a signal that triggers binding of the adaptor proteins next to BRCA1 gene 1 (NBR1) or p62, which also binds light chain 3 (LC3; the mammalian homologue of autophagy protein 8 (Atg8)) conjugated with lipids in the double membrane of the forming autophagosome. This allows cargo to be included in the forming autophagosome, which subsequently fuses with lysosomal compartments to release its inner-membrane vesicle with included cargo for hydrolytic cargo degradation. HDAC6, histone deacetylase 6; PE, phosphatidylethanolamine.

brain led to hepatic dysfunction and a neurodegenerative disease phenotype, respectively. The knockout mice accumulated ubiquitin-positive aggregates that were not present in wild-type littermates[136–138]. Basal autophagy therefore seems to be of more biological importance under physiological conditions, as previously recognized. Furthermore, autophagy may also protect from neurodegeneration by reducing the levels of potentially toxic diffuse or oligomeric protein species. However, this hypothesis is still controversial[139].

The clearance of misfolded proteins through autophagy and the ubiquitin proteasome system is interconnected, as impairment of the ubiquitin proteasome system induces compensatory autophagy[114,130], the conditional knock down of autophagy in mice brains leads to the accumulation of ubiquitylated proteins[136–138] and genetic knock down of autophagy leads to the inhibition of proteasomal substrate turnover[139]. The targeting signal of ubiquitylated proteins for autophagy is still unclear. Initial evidence suggests that Lys63-linked ubiquitylation may promote the autophagic clearance of protein inclusions[29,79,140], but additional experimental evidence is needed to establish this.

In the search for a factor that would couple misfolded and aggregated ubiquitylated proteins to autophagy, p62 was suggested to function as a cargo receptor. p62 can bind ubiquitylated proteins through its ubiquitin-associated (UBA) domain and a component of autophagic vesicles, the mammalian Atg8 homologue light chain 3 (LC3), through its LC3-interacting region (LIR)[141,142] to control the packing of ubiquitylated substrates into autophagosomes (FIG. 5). p62 is a common component of aggregates of mutant huntingtin protein, Lewy bodies or neurofibrillar tangles[143–146]. The genetic ablation of p62 in ATG7-deficient mice suppressed the appearance of ubiquitin-positive protein aggregates in hepatocytes and neurons[147], which suggests that p62 also controls intracellular inclusion body formation. Another more recently identified protein that acts in the same pathway as p62 is next to BRCA1 gene 1 (NBR1). Similarly to p62, NBR1 binds polyubiquitin and LC3 on autophagic vesicles, thereby promoting autophagy of ubiquitylated substrates. Interestingly, recruitment of ubiquitin-positive cargo to the lysosome requires both p62 and NBR1 (REF. 148) (FIG. 5). Furthermore, the scaffolding protein autophagy-linked FYVE protein (ALFY; also known as WDFY3) was suggested to bridge cargo to the autophagic machinery by interacting with protein complexes containing p62 on the one hand and ATG5 on the other hand[149]. Taken together, these findings indicate an important role for the autophagy machinery in the specific, receptor-mediated clearance of misfolded and aggregated proteins[18,19,150].

Asymmetric aggregate partitioning

Although the sequestration of misfolded proteins in large, insoluble inclusions can have a protective role, the presence of protein aggregates is still a damage load for the cell. Aggregates could, for example, sequester other cellular proteins, including protein quality-control components, thereby impairing the overall cellular protein homeostasis and consequently lowering the fitness of the cell[4127,151–153]. The destiny of protein aggregates during cell division has therefore gained increasing attention. Are aggregates equally distributed between daughter cells or are they specifically retained in one cell leading to an asymmetric population of cells with high and low damage loads? Recent findings suggest that the strategy to inherit protein aggregates asymmetrically between two dividing cells is a general, ancient principle that can be found in organisms ranging from bacteria to mammals.

Aggregate segregation in E. coli. In *E. coli*, the accumulation of aggregated proteins correlates with increased cellular ageing[64,65,154]. The localization of aggregates at cell poles might enable bacterial cells to regain faster growth rates as it allows for the generation of damage-free cells by one or two cell divisions. Cell division can thereby act as a bypass mechanism to remove persisting protein aggregates. The asymmetry in the case of *E. coli* cells harbouring a single protein aggregate at the old pole allows for the generation of aggregate-free daughter cells on cell division (FIG. 6a). Importantly, the cells with old poles that carry the inclusion have reduced growth rates and a slower division time compared with the inclusion-free daughter cells[64,65]. Recent data demonstrate that abolishing the asymmetric distribution of protein aggregates diminishes growth rate and division speed differences between the *E. coli* cells emerging from a cell division[65]. This suggests that sibling-specific ageing may be the result of protein aggregate segregation.

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Aggregate segregation in yeast. Yeast cells can undergo a finite number of divisions before they die, and this is defined as their replicative lifespan. When a yeast cell divides, this replicative lifespan is also segregated asymmetrically, as the lifespan of the daughter cell is 'reset' to zero, whereas the lifespan of the mother cell depends on the number of divisions it has undergone. This led to the proposal that there must be one or more factors that accumulate and cause senescence when they reach a crucial threshold[155,156]. Protein aggregates, and particularly carbonylated proteins, have been suggested as such possible senescence factors[157]. Carbonylated proteins progressively accumulate over time in yeast and also in higher eukaryotes[35,155]. Yeast cells are able to segregate oxidatively damaged proteins in an asymmetric manner, leaving the daughter cell largely free of damaged proteins while the mother cell retains them. This asymmetry in damage distribution during budding is likely to have important implications in cellular ageing and the rejuvenation of progeny[39,157]. The mechanism of this specific damage retention and retrograde damage transport is largely unknown. However, the observation that the asymmetric distribution of aggregates, such as carbonylated proteins, towards the mother cell during cell division involves the actin cytoskeleton[157] (see below) and requires the cellular machinery for establishing actin-based cell polarity[158,159], suggests that these aggregates might somehow become tethered to actin cables (FIG. 6b). Interestingly, aggregates are not only retained in mother cells during division, but can even be retracted from the buds[158]. Another intriguing link is that Hsp104 plays a part in the establishment of cell polarity through genetic and physical interaction with components of the polarisome and the septin ring[159]. This raises the possibility of a regulatory link between formation of the bud and the quality-control system of protein aggregates.

Aggregate segregation in metazoans. Asymmetric distribution of misfolded and aggregated proteins has also been reported for mammalian cells, and in all cases the cell with the shorter life expectancy receives the protein aggregates[160,161]. In mammalian crypts of the small intestine from patients with a polyglutamine disease termed spinocerebellar ataxia type 3, the forming aggresomes segregate to the short-lived differentiated cell rather than to the intestinal stem cell[161]. Similarly, in a *Drosophila melanogaster* embryonic neuroblast model expressing a heterologous polyglutamine huntingtin fragment, the aggresomes segregate to the short-lived neuroblast rather than the long-lived ganglion mother cell[161].

The asymmetric distribution of aggresomes is thought to be coupled with the asymmetric inheritance of centrosomes[160–162]. Before division, the centrosome consists of a centriole and the pericentriolar material[163]. During cell division, after the centriole has duplicated, the pericentriolar material stays preferentially associated with the mother centriole, whereas the daughter centriole that separates and migrates to the opposite cell pole lacks any defined pericentriolar material until it has reached its destination[164–166]. Aggresomes form at the MTOC[62,77] and thereby occupy the same region as the pericentriolar material, which offers the possibility that the retention of both structures specifically in one cell (the mother cell) could be linked. Indeed, the inherited misfolded proteins destined for degradation localize to only one of the centrosomes[160]. However, the generality of such a possible mechanism is not yet clear. In yeast, for instance, the two protein aggregates described recently (IPOD and JUNQ) are inherited asymmetrically but neither are associated with the spindle pole body, which is the yeast equivalent of the mammalian centrosome[58]. It is likely that more species- and cell type-specific differences in the mechanisms of aggregate inheritance will be discovered.

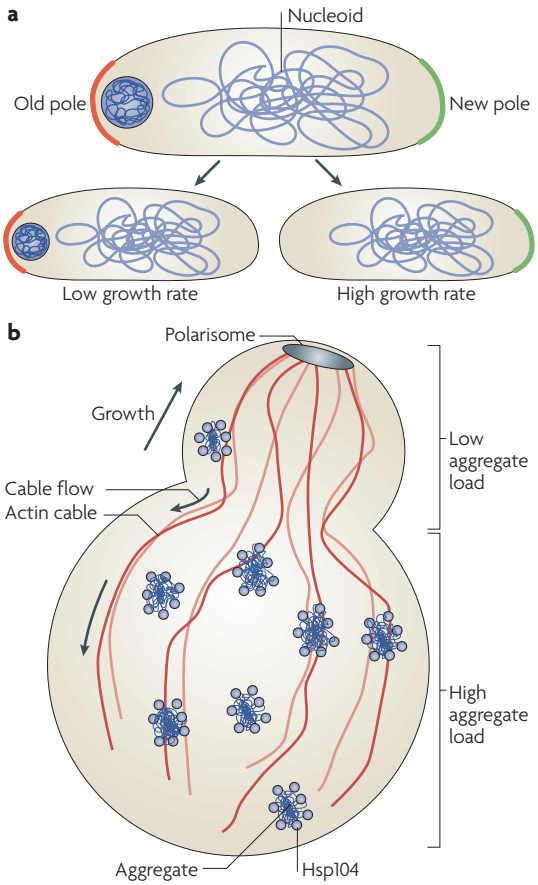

Figure 6 | **Asymmetric distribution of damaged proteins as a strategy for rejuvenating the progeny.** **a** | In *Escherichia coli* cells, inclusion bodies are localized to the old cell pole and consequently stay in the older cell after division, which displays a reduction in growth rate. Nucleoid exclusion is sufficient for this inclusion body localization. **b** | In yeast cells, diffuse carbonylated proteins, larger aggregates of carbonylated proteins and unspecified protein aggregates reside in the mother cell during cell division. Visible aggregates can even be transported back from the bud into the mother cell. Actin cables, the polarisome and 104-kDa heat shock protein (Hsp104) are required for this asymmetric distribution, which may be achieved by the tethering of the aggregates to actin cables by the action of Hsp104. Actin cable flow from the daughter cell to the mother cell results in the retention of tethered aggregates in the mother cell.

Polarisome
A protein complex involved in determining cell polarity by directing the localized assembly of actin filaments.

Septin ring
A ring-shaped structure that forms in the division plane at the future septation site.

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Concluding remarks

It is becoming more and more evident that protein aggregation in cells is not an uncontrolled and catastrophic process. Instead, directing protein aggregates to specific cellular sites can be viewed as a second line of active cellular defence that is evolutionarily conserved from bacteria to humans. Such specific localization sets the basis for aggregate removal by chaperones and proteolytic machineries or for the asymmetric distribution of aggregates on cell division. Both aspects will eliminate the damage load, thereby increasing the fitness of a cell population and re-establishing protein homeostasis. This would define protein aggregation as a novel facet of protein quality control. Understanding the molecular processes regulating this quality-control aspect may explain the pathological aspects of various diseases associated with protein aggregation and offer new targets for therapeutic intervention. Although different cells seem to share similar overall strategies to cope with protein aggregates, future research will need to establish whether components of specific aggregate deposition sites and mechanisms of substrate targeting, cellular organization and turnover in different organisms are conserved. Specific biochemical isolation strategies and genetic manipulations combined with high-resolution structure determination, such as cryoelectron tomography, will identify additional structural components of aggregate deposition sites and their molecular architecture. These studies will be a prerequisite for addressing additional questions regarding the regulation of these processes and the interplay with other systems in cellular protein quality control. The diversity of the different protein aggregates that have been investigated so far (for example, in terms of their structure and cellular localization) might argue in favour of substantial differences in the mechanisms involved.

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