

## STATE-OF-THE-ART REVIEW

# Alternative systems for misfolded protein clearance: life beyond the proteasome

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cancer; chaperone; heat-shock protein; neurodegeneration; PQC; protein aggregate; protein degradation; protein triage; ubiquitin; ubiquitination

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Protein misfolding is a major driver of ageing-associated frailty and disease pathology. Although all cells possess multiple, well-characterised protein quality control systems to mitigate the toxicity of misfolded proteins, how they are integrated to maintain protein homeostasis ('proteostasis') in health—and how their disintegration contributes to disease—is still an exciting and fast-paced area of research. Under physiological conditions, the predominant route for misfolded protein clearance involves ubiquitylation and proteasome-mediated degradation. When the capacity of this route is overwhelmed—as happens during conditions of acute environmental stress, or chronic ageing-related decline—alternative routes for protein quality control are activated. In this review, we summarise our current understanding of how proteasome-targeted misfolded proteins are re trafficked to alternative protein quality control routes such as juxta-nuclear sequestration and selective autophagy when the ubiquitin–proteasome system is compromised. We also discuss the molecular determinants of these alternative protein quality control systems, attempt to clarify distinctions between various cytoplasmic spatial quality control inclusion bodies (e.g., Q-bodies, p62 bodies, JUNQ, aggresomes, and aggresome-like induced structures 'ALIS'), and speculate on emerging concepts in the field that we hope will spur future research—with the potential to benefit the rational development of healthy ageing strategies.

**Abbreviations**

ALIS, aggresome-like induced structure; ALS, amyotrophic lateral sclerosis; ATG8, autophagy-related protein 8; BAG1/3, BCL2-associate athanogene 1/3; CHIP/STUB1, carboxy-terminus of Hsc70 interacting protein/STIP1 homology and U-box containing protein 1; COPD, chronic obstructive pulmonary disease; CRED, chaperone- and receptor-mediated extracellular protein degradation; CUE, coupling of ubiquitin to ER degradation domain; DUB, deubiquitylase; ER, endoplasmic reticulum; ESCRT, endosomal sorting complexes required for transport; FDA, Food and Drug Administration (U.S.A.); HDAC6, histone deacetylase-6; HRD1, HMG-CoA reductase degradation protein 1; HSF1, heat-shock factor 1; HSP40/70/90, heat-shock protein 40/70/90-kDa; IPOD, insoluble protein deposit; JUNQ, Juxta-nuclear quality control compartment; LANDO, LC3-associated endocytosis; LC3, microtubule-associated protein 1A/1B-light chain 3; LIR, LC3-interacting region; LLPS, liquid–liquid phase separation; MTOC, microtubule organising centre; mTOR, mammalian target of rapamycin; NBR1, neighbour of BRCA1 gene 1; NDP52, nuclear dot protein 52-kDa; p62/SQSTM1, sequestosome-1 protein 62-kDa; p97/VCP, Vasolin-containing protein 97-kDa; PML, promyelocytic leukaemia protein; PQC, protein quality control; PSMD14, 26S proteasome non-ATPase regulatory subunit 14; SOD1, superoxide dismutase 1; TAME, targeting ageing with metformin; TAX1BP1, Tax1-binding protein 1; TOLLIP, Toll-interacting protein; TRiC/CCT, tailless complex polypeptide 1 ring complex/chaperonin containing tailless complex polypeptide 1; TRIM50, tripartite motif protein 50; UBA, ubiquitin-associated domain; UBD, ubiquitin-binding domain; UBL, ubiquitin-like domain; UBP, ubiquitin-binding protein; UBQLN1/2, ubiquilin-1/2; UCHL1, ubiquitin carboxy-terminal hydrolase L1; UPR, unfolded protein response; USP10, ubiquitin-specific peptidase 10; ZnF-UBD, zinc-finger ubiquitin-binding domain.

## Introduction

For a cell to function as a successful self-replicating system, it must maintain its constituent components in a robust state throughout its lifespan. Focusing on just the proteins, a eukaryotic cell contains anywhere between 100 million and 100 billion protein molecules [1]. Each protein must be synthesised with the correct amino acid sequence, folded into its correct 3D structure, and transported to the correct subcellular locations and protein complexes. If any of these processes fail, the system has the potential to break down. Although break-down sometimes results from the protein being unable to fulfil its function [e.g., in cystic fibrosis or chronic obstructive pulmonary disease (COPD)], the toxicity of misfolded or mislocalised proteins is predominantly attributed to interference with other critical cellular processes [2,3]. Almost all diseases where ageing is the major risk factor have been linked mechanistically to the accumulation of misfolded proteins. The most notable of these are neurodegenerative conditions where misfolded protein plaques are a core pathology (e.g., Alzheimer's disease, Parkinson's disease) [3,4]. Unsurprisingly, all cells evolved protein quality control (PQC) systems to minimise misfolding events and, in cases where misfolding is inevitable, to mitigate any damage they might cause [5,6]. The integration of PQC systems to balance the cell's protein production, folding, and clearance needs is referred to as the proteostasis network (Fig. 1).

In response to stimuli that increase a cell's misfolded protein load—referred to as 'proteotoxic stress'—the proteostasis network modulates one, some, or all of these PQC systems [7–9]. To reduce load on the network as a whole, global protein translation is reduced.

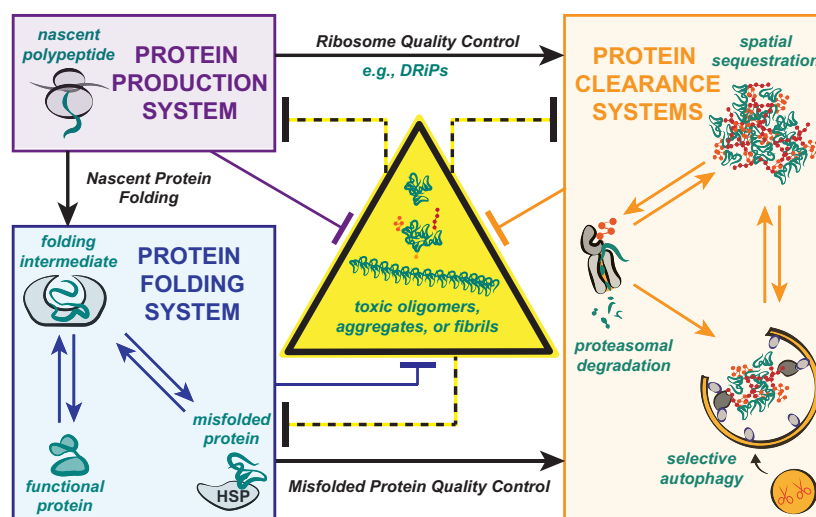
At the same time, translation of PQC system components (e.g., pro-folding molecular chaperones) is increased. Finally, protein clearance is initiated—both at the level of factors that target misfolded proteins to clearance systems, and by increasing flux through the clearance machineries themselves. In these ways, the proteostasis network attempts to alleviate rapidly any misfolded protein species it encounters before they interfere with normal cell function.

## A journey to the centre of the proteasome

A misfolded protein is initially recognised by the major components of the protein folding machinery: molecular chaperones. Often referred to as the first responders of proteotoxic stress responses [9], chaperones generally identify misfolded proteins on the basis of exposed hydrophobic sequence elements (which would be buried within a protein's core if correctly folded) and isolate them from the rest of the cellular milieu. Note that many of the stress-induced molecular chaperones—such as the highly abundant heat-shock proteins HSP70 and HSP90—are also required for the *de novo* folding of newly synthesised polypeptides from the ribosome. Indeed, nascent polypeptide chains are among the most sensitive to proteotoxic stress-induced misfolding [10].

Once bound to a misfolded protein 'client', the chaperone will attempt to aid refolding of the protein into its native, folded state. Failing this, however, the chaperone must filter the misfolded protein to the clearance systems of the network. Both pro-folding and pro-clearance roles of molecular chaperones are crucial for maintaining a robust proteome [11,12].

**Fig. 1.** Integration of PQC modules in the proteostasis network. Integration of different components within and between the protein production, protein folding, and protein clearance systems maintains proteome robustness by minimising the accumulation of toxic misfolded protein species. Proteotoxic stresses that overload the capacity of any individual system results in stress responses usually spanning multiple systems. If proteotoxic stress responses are impaired (e.g., during ageing, or following disease-associated mutations in amyloidogenic proteins), misfolded proteins accumulate and further interfere with PQC systems.



Misfolded protein transfer from the folding machinery to clearance systems is generally triggered by the chaperone-assisted recruitment of ubiquitin ligases [13]. These enzymes catalyse covalent attachment of one or more ubiquitin molecules onto a lysine (K) or N-terminal methionine residue in the misfolded protein [14]—although noncanonical ubiquitylation on other amino acids (e.g., serine) has also been identified [15,16]. Each ubiquitin can itself be further ubiquitylated on any of its seven surface lysines (in addition to its N-terminal methionine) to build a polyubiquitin chain. The residues through which the ubiquitin chain is linked encode the nature of the signal, thus determining the fate of the attached protein [17,18]. Chains linked through the lysine at position 48 in ubiquitin's amino acid sequence ('K48-ubiquitin') are generally considered the most effective signals for proteasomal degradation, although recent evidence implicates branched K11/K48-ubiquitin chains (i.e., where at least one ubiquitin moiety within the chain has extensions from both its K48 and K11 residues) in the clearance of various constitutive and stress-induced misfolded proteins [19–21].

Finally, the ubiquitylated misfolded protein is trafficked to the 26S proteasome—an extraordinarily intricate, 2.5 megadalton multicatalytic protease responsible for the majority of selective misfolded protein degradation. Proteins that enter its central tunnel-like catalytic 20S core are digested relatively indiscriminately. However, access to the 20S core particle is gated at one or both ends by regulatory 19S particles [22,23]. It is these 19S regulatory particles that provide docking sites for ubiquitylated proteins—as well as conferring some degree of ubiquitin chain specificity—through its three resident ubiquitin receptors [23,24]. Once bound to one of these receptors, a series of highly choreographed structural rearrangements [23] in the regulatory particle results in threading of the misfolded protein through to the catalytic core of the proteasome, where it is digested into small peptides by subunits with trypsin-, chymotrypsin-, or caspase-like cleavage activities. These peptides are further digested by nonproteasomal endopeptidases and aminopeptidases to release individual amino acids [25]—which replenishes the amino acid pool, and could be used to synthesise the same protein, correctly folded.

### **Beyond the proteasome: alternative PQC systems for mitigating misfolded protein toxicity**

The events described above—from initial recognition of a misfolded protein by molecular chaperones, to

proteasomal degradation and subsequent recycling into its constituent amino acids—represent a well-regulated process that is efficient for most soluble substrates in the nucleus or cytoplasm. However, as the proteasome's core tunnel is relatively narrow, proteins that form energetically stable higher-order structures such as oligomers or aggregates often cannot be sufficiently unfolded to enter the core particle. These structures can clog-up proteasomes and prevent other, normally conducive substrates from being degraded—further increasing the misfolded protein load and proteotoxic stress. Gain-of-function proteasomal impairment by protein oligomers and aggregates is proposed as a root cause for toxicity in neurodegenerative diseases [5,8].

In addition to this biophysical limitation, numerous other scenarios and substrates pose challenges for proteasome targeting and/or degradation. Membrane-embedded misfolded proteins must first be extracted (usually by the ubiquitin-dependent AAA+ ATPase p97/VCP), and potentially ubiquitylated further, before they become competent proteasomal substrates. Mitochondria—organelles with two membranes, a very limited ubiquitylation machinery beyond the outer membrane, and an extreme redox environment—pose their own unique set of PQC challenges [26]. Furthermore, severe damage localised to subcellular regions or compartments (e.g., by reactive oxygen species) might necessitate degradation of all damaged macromolecules in the affected area—not just the proteins.

Finally, despite the substantial proportion of the proteome dedicated to molecular chaperones and the ubiquitin–proteasome system, acute environmental (e.g., heat shock or free radicals) or chronic genetic (e.g., chromosome duplication) stressors can still overwhelm the system's capacity and lead to an excess of misfolded proteins that must be dealt with by other systems to avoid proteostasis collapse.

For the rest of this review, we will focus on the current state of knowledge regarding re-trafficking of cytoplasmic misfolded proteins to alternative PQC systems in situations where proteasomal degradation routes are compromised (Fig. 2). Several excellent reviews exist for cross-talk between other aspects of the proteostasis network, including insights into the folding versus degradation decision in protein triage [13] and interaction between metabolic and proteotoxic stress responses [27,28]. In the context of cross-talk between proteasomal and autophagic systems, we draw attention to a recent therapy-minded review on exploiting this in Alzheimer's disease [29], as well as broader outlooks [30–32]. We also largely ignore the unfolded protein response (UPR) in the endoplasmic reticulum (ER) and other organelles—an equally

important proteotoxic stress response with broad-spanning implications in healthy and pathologic ageing research [33,34].

### **Autophagy: several routes to degradation by lysosomes**

Whereas the 26S proteasome is dedicated to digestion of polypeptide chains individually threaded through to its central pore, the lysosome is far more indiscriminate in its substrate specificity and mechanism of degradation, digesting virtually all macromolecules (including lipids and carbohydrates) in bulk as they enter the organelle. As such, lysosomes serve as recycling centres for much of the cell's contents and are key hubs for a host of signalling networks [35].

Misfolded proteins can be targeted to the lysosome through a broad range of pathways with varying degrees of overlap [32]. For globular cytoplasmic and nuclear proteins, this generally involves the autophagy system. The most widely studied context for autophagy is the self-eating 'macroautophagy' of bulk cytoplasmic regions by double-membraned structures called autophagosomes in response to starvation, thereby directly replenishing nutrient stores. However, macroautophagy can be made selective by using ATG8-family proteins (e.g., LC3, GABARAPs) tethered to autophagosomes as targeting platforms [36]. Ubiquitinated proteins are thus sent to autophagic structures via cargo receptors (e.g., p62/SQSTM1 [37]) that contain both ubiquitin-binding domains (UBDs) and LC3-interacting regions (LIRs). This 'selective autophagy' can function on individual misfolded proteins, or on higher-order oligomers or aggregates ('aggrephagy')—again, a distinct difference from proteasomal degradation. Therefore, a major role of selective autophagy under proteotoxic stress may be to serve as an insurance mechanism that catches escaped proteasomal substrates. In support of this hypothesis, selective autophagy cargo receptors tend to display a preference for binding ubiquitin chain linkages that are considered ineffective proteasome-targeting signals (discussed in later sections). Note that selective autophagy also works on whole organelles, including components of the proteostasis network (e.g., ribosomes, proteasomes, and parts of the ER) [38]. Some related processes such as microautophagy and chaperone-mediated autophagy are more limited in targeting only monomeric substrates [39].

It is beyond the scope of this review to delve into the numerous, highly regulated steps in autophagic degradation systems, or to describe the similarities and differences between the various types of autophagy in

any more than passing detail. We point to two reviews that highlight the truly impressive mechanistic detail the field has attained in the past two decades [40,41]. Although we will focus here on how autophagy mediates misfolded protein clearance in proteasome-compromised conditions, this pathway is certainly not limited to conditions of proteotoxic stress. It has important physiologic roles, especially at developmental landmarks that require extensive remodelling of the proteome [42]. Additionally, canonical and noncanonical autophagic degradation is a critical component of the host cell's immune response, although some pathogens hijack this response for their own benefit [43].

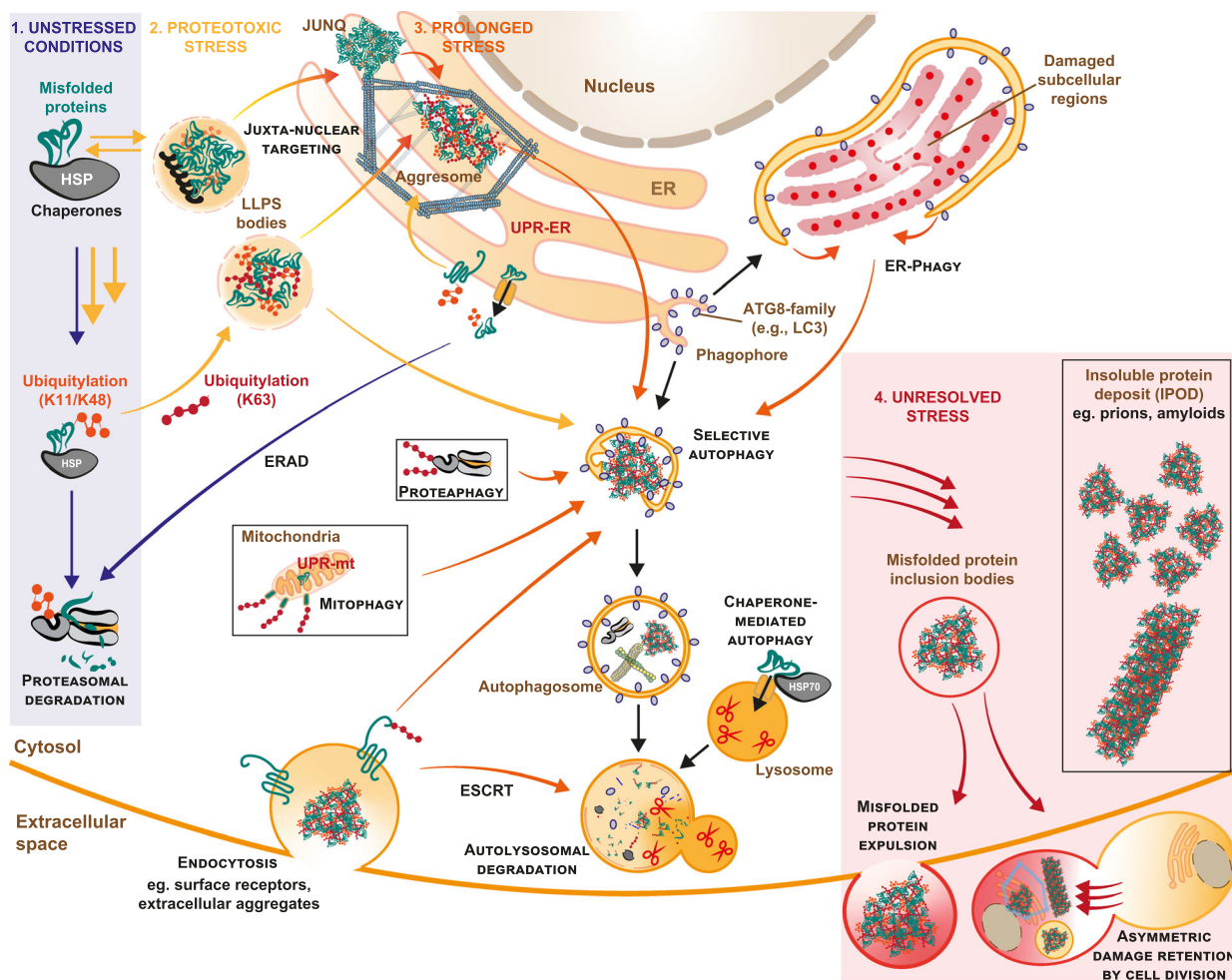
It is also worth noting that autophagy-independent lysosomal targeting routes exist for misfolded proteins—especially those in the endosomal and secretory systems. Cell surface receptors (e.g., receptor tyrosine kinases) are internalised and triaged at multivesicular bodies for recycling back to the surface, or for lysosomal degradation, by the ESCRT (endosomal sorting complexes required for transport) machinery—although the extent to which receptor misfolding or other damage is a factor in the recycling vs. degradation decision is unclear [44]. A PQC checkpoint was also discovered recently at the Golgi apparatus that prevents misfolded proteins and aggregates from proceeding on their secretory route, instead syphoning them off to the endosome-lysosomal system [45].

### **The aggresome and other spatial PQC sites**

The aggresome was initially described by Kopito and colleagues as a juxta-nuclear, 'membrane-free, cytoplasmic inclusion containing misfolded, ubiquitinated protein ensheathed in a cage of [intermediate filaments]' [46]. The authors additionally identified that aggresomes formed specifically at the microtubule organising centre (MTOC) and that targeting of misfolded proteins to this site depended on intact microtubular transport. Although this initial paper used misfolded transmembrane proteins and focused on conditions of proteasome inhibition, the authors proposed that aggresome formation was 'a general response of cells which occurs when the capacity of the proteasome is exceeded by the production of aggregation-prone misfolded proteins'.

More than two decades of research into protein aggregation and clearance mechanisms has corroborated both the structural characterisations and functional inferences made in the original 1998 study. Aggresomes have been shown to accumulate misfolded proteins of diverse origins (e.g., cytoplasmic and ER





**Fig. 2.** PQC clearance routes for mitigating misfolded protein toxicity. For most soluble cytoplasmic or nuclear misfolded proteins that are recognised by molecular chaperones such as heat-shock proteins (HSPs), the preferred route of clearance is via the ubiquitin–proteasome system ('1. Unstressed Conditions', blue arrows). However, in conditions where this system is incapacitated or overwhelmed ('2. Proteotoxic Stress', yellow arrows), alternative PQC routes exist to ensure that misfolded proteins do not accumulate in the cell and potentially interfere with other critical processes. These misfolded proteins are concentrated into liquid–liquid phase-separated (LLPS) bodies and cleared by selective autophagy or sequestered at a juxta-nuclear site ('JUNQ'). Failure to clear proteins by any of the aforementioned routes ('3. Prolonged Stress', orange arrows) can trigger their accumulation at the aggresome, which can itself be cleared by selective autophagy. Note that other cellular macromolecules and organelles can also be targeted to selective autophagy via different Atg8 family-binding adaptors such as LC3 or GABARAPs. Protein misfolding within organelles (e.g., the ER or mitochondria) triggers the unfolded protein response (UPR), which can also initiate their degradation by proteasomes (e.g., ER-associated degradation ERAD) or selective autophagy (e.g., mitophagy, ER-phagy). An estimated 30% of cytoplasmic proteins also contain a KFERQ motif within their sequence that allows them to be inserted directly into the lysosome via the chaperone HSP70 and lysosomal receptor LAMP2A. Misfolded proteins of the endosomal system are internalised and targeted to the lysosome via K63-ubiquitin chains and the ESCRT machinery. External misfolded protein aggregates could also be endocytosed and targeted to the lysosome through cell surface receptor-mediated pathways such as LANDO ('LC3-associated endocytosis') and CRED ('chaperone- and receptor-mediated extracellular protein degradation'). If these PQC systems fail ('4. Unresolved Stress', red arrows), misfolded proteins accumulate in peripheral insoluble protein deposits (IPOD)—which are also sites of sequestration for disease-associated amyloidogenic/prion proteins. Although the IPOD is generally considered a terminal sequestration site, there is some evidence that aggregates within can be expelled by exocytosis. Additionally, asymmetrically dividing cells (e.g., budding yeast, stem cells) ensure proteome robustness of their progeny by retaining misfolded protein inclusions—including the aggresome and IPOD—in the mother cell.

proteins, with and without transmembrane domains), and with different reasons for misfolding [47,48]. It is also clear that formation of aggresomes is not contingent on direct proteasome inhibition, as shown in cells exposed to other stressors that increase misfolded protein load [49–51].

Of course, our understanding of the machinery involved in spatial sequestration has progressed substantially in the intervening years. Major developments include the following: central roles for the histone deacetylase HDAC6 and selective autophagy cargo adaptor p62/SQSTM1 in aggresome targeting and clearance [52,53]; the involvement of PQC systems such as molecular chaperones (e.g., the HSP40-HSP70-HSP90 machinery, TRiC/CCT, BAG3, small HSPs [47,54–57]), ubiquitin ligases (e.g., CHIP/STUB1, Parkin, HRD1, TRIM50 [54,58–60]), deubiquitylases (ataxin-3, UCHL1, USP10 [61–63]), ubiquitin-binding cargo adaptors (UBQLN1/2, p62/SQSTM1 [48,64,65]), the ubiquitin-dependent disaggregase p97/VCP [66,67], and proteasomes [56]; and the description of several ‘aggresome-like’ juxta- or intra- nuclear structures that may or may not be functionally analogous under different conditions, for different substrates, and between different species [68–72].

The last point has sparked spirited debate as to how the various protein-dense cytoplasmic inclusion bodies observed by different groups, in different conditions and cell types, are inter-related—or are indeed the same fundamental structure. Although the term ‘aggresome’ was initially used relatively indiscriminately to refer to any single perinuclear inclusion body in mammalian cells, the past decade has seen a shift in terminology that distinguishes between different types of inclusion bodies. A turning point came in 2008 with the discovery in single-celled budding yeast *S. cerevisiae* of a juxta-nuclear QC compartment (JUNQ), whose mammalian counterpart had many similarities with the aggresome [71,73–75]. A later study proposed that the yeast JUNQ actually forms inside the nucleus (an ‘intranuclear QC compartment’, or INQ) [76]. Note that the JUNQ and INQ are not necessarily mutually exclusive, as indicated by experiments confining the same model misfolded protein to the nucleus or cytoplasm via a nuclear localisation or nuclear export signal, respectively [19].

Regardless of which side of the envelope this inclusion is located, both the INQ and JUNQ are distinct from the peripheral cytoplasmic IPOD (insoluble protein deposit)—a site of terminal sequestration for prion or other amyloidogenic proteins that do not engage the PQC machinery in the same way as typical misfolded proteins [71,73,77,78]. Therefore, even lower eukaryotes possess several distinct spatial PQC sequestration sites. However, given the evolutionary distance

between budding yeast and mammalian cells, one must be careful when transferring insights from one system to the other.

For the purpose of this review, we define the aggresome from a broad functional perspective in mammalian cells, that is, a stress-induced, juxta-nuclear inclusion body that colocalises misfolded proteins, ubiquitin, molecular chaperones, and proteasomes. The mammalian JUNQ shares almost all of these features and is perhaps a more immature and transient form of the aggresome [74,75,79]. Similarly, we attempt wherever possible to distinguish between the aggresome and other ‘aggresome-like’ induced structures (ALIS). For example, although ALIS initially identified in dendritic cells (‘DALIS’) share many properties with aggresomes [68], they were subsequently shown to be the same as another ubiquitin-positive PQC structure: the p62 body [65].

Indeed, p62/SQSTM1 gets the second part of its name from its ability to bind polyubiquitylated proteins and sequester them in these cytoplasmic puncta. The puncta coalesce upon proteasome inhibition into a single structure first referred to as the ‘sequestosome’ in a December 1998 paper [80], in which the author posited ‘...that the p62 containing cytoplasmic structure is the compartment into which cytoplasmic multiubiquitinated proteins segregate, and that the degree of the segregation is maximized when the proteasome is malfunctioning’. It is possible that the larger compartment they described on proteasome impairment was the same structure that was named the aggresome by Kopito and colleagues in their paper published in the same month and year [46]. However, it is important to note that ALIS/p62 bodies do not invariably mature into aggresomes. Rather, these structures most likely represent cargo primed for selective autophagy, which would accumulate in aggresomes in conditions where PQC systems are overwhelmed.

ALIS/p62 bodies appear not to rely on cytoskeletal networks, as they are not (a) localised to the MTOC, (b) surrounded by an intermediate filament cage, or (c) disrupted by microtubule- or F-actin-destabilising drugs [68–70]. These structures are also especially enriched in defective ribosomal products (‘DRiPs’). Therefore, ALIS/p62 bodies and aggresomes may serve slightly different functions in the mammalian cell, facilitating a division of labour for PQC of misfolded nascent polypeptides and mature proteins, respectively.

### Other clearance mechanisms

Although degradation by the proteasome or lysosome, or sequestration into aggresomes or related

structures are by far the most well-studied mechanisms for mitigating misfolded protein toxicity, there is a growing body of evidence to suggest that additional clearance mechanisms make important contributions to PQC.

For rapidly dividing cells, perhaps the simplest way to maintain proteome robustness in a population is by controlling the inheritance of damaged cellular components, including misfolded protein inclusion bodies. Asymmetric damage retention—conserved from single-celled bacteria [81] through to humans [75]—is a critical component of the rejuvenative properties of cell division, with important implications for maintaining somatic stem cell niches. It was also recently demonstrated that lysosomes and autophagic compartments are inherited asymmetrically in haematopoietic stem cells [82]. On the other side of the same coin, symmetrically dividing non-stem cells (e.g., fibroblasts during the wound-healing response) appear to rely on dispersing damage among their progeny to dilute the cytotoxic burden acquired by each cell. Indeed, according to the imperfectness model of ageing [83], dividing cells only actively clear more severe damage, relying on cell division to dilute minor imperfections. While aggregates, JUNQs, and IPODs—presumably representing ‘severe damage’ in this model—are asymmetrically inherited in eukaryotic cells [75,84–88], we currently do not know whether the same is true for misfolded proteins outside inclusion bodies that have not yet been cleared by PQC at the time of cytokinesis. For example, smaller cytoplasmic misfolded protein inclusion bodies known interchangeably as stress foci/CytoQ/Q-bodies (hereafter: Q-bodies) are not inherited asymmetrically in budding yeast and mammalian cells [74,84]. A provocative study even posits that larger inclusions are inherited asymmetrically in budding yeast purely as a result of the biophysical constraints on both bud-neck diffusion and mother cell growth, rather than active partitioning during division [89].

Of course, for the vast majority of cells in a fully developed metazoan, mitigating toxicity through division is not an option. This is especially true for neurons and cardiomyocytes, which need to maintain proteostasis for decades [12,90]. In addition to enhancing intracellular PQC systems, postmitotic cells such as neurons may employ misfolded protein expulsion as a mitigation strategy. The bulk of information for exocytosis as a misfolded protein clearance mechanism comes from studies into neurodegenerative amyloidoses and prionopathies, where secretion of such proteins is proposed to serve as a means of disease spread from neuron to neuron [91]. However, extracellular misfolded protein secretion is emerging as a general,

conserved piece in the PQC repertoire that acts on a variety of different substrates, in a variety of different cell types [92–96]. Misfolded protein secretion invariably involves members of the chaperone and ubiquitylation machineries, and becomes especially important in conditions of proteasome or autophagy dysfunction—placing it in a similar category to the other alternative PQC systems. The reverse route also exists as follows: misfolded extracellular proteins, including Alzheimer’s disease-linked amyloid- $\beta$ , can be lysosomally degraded via receptor-mediated endocytic pathways [97,98]. One such pathway, named ‘LANDO’ (LC3-associated endocytosis), employs several autophagy regulators (e.g., ATG4/5/7, Rubicon, Beclin1, VPS34) to recycle microglial amyloid- $\beta$  receptors and protects against extracellular amyloid- $\beta$  deposition, neuroinflammation, neuronal loss, and memory impairment in a mouse model of Alzheimer’s disease [98]. Incorporating these often-neglected protein clearance mechanisms into the emerging focus on cell nonautonomous PQC systems for organismal proteostasis [99] holds great potential for the next wave of research into neurodegeneration and healthy ageing.

### Determinants of the misfolded protein clearance route

It has long been accepted [100–103] that proteasomal degradation represents the preferred route of clearance for the majority of misfolded proteins, especially those that are short-lived and globular (i.e., not membrane-embedded). Longer-lived proteins (e.g., cytoskeletal components) and transmembrane proteins (e.g., cell surface receptors) are turned over by the lysosome. In cases where both of these clearance systems fail, misfolded proteins accumulate in aggregates that may be cleared by selective autophagy.

Studies quantifying the relative activities of different clearance systems indicate that this division of labour for short- vs. long-lived proteins represents trends rather than concrete rules, with several exceptions reported in both directions [104–106]. Furthermore, even in optimal cell growth conditions, lysosomes and proteasomes may contribute equally to total protein turnover [107,108]. Induction of stress, including common cell culture variations such as confluency and serum exhaustion, shifts the balance towards lysosomal degradation. It is likely that changing the balance between different clearance systems serve crucial physiologic purposes. To take one example, dermal fibroblasts—which spend most of their time in quiescence, with bursts of rapid yet tightly regulated proliferation during wound-healing—upregulate levels of macroautophagy and lysosome biogenesis

when in a contact-inhibited quiescent state [109]. The fibroblasts can therefore switch between relying on lysosomal degradation, or on dilution through rapid cell division, for quality control of their long-lived proteins—depending on their proliferative state. Such cell- and context-specific effects should be important considerations when measuring flux through protein clearance systems.

In a similar vein, misfolded protein accumulation into aggregates also appears to be an early PQC response, and not merely a back-up to catch misfolded proteins that escape ‘preferred’ clearance systems [6,87] (Fig. 3). For example, sequestration of misfolded proteins into Q-bodies occurs rapidly following proteotoxic stress in cells with fully functional proteasomal and lysosomal clearance systems [74,77,84]. Similarly, ALIS/p62 bodies are also formed in the absence of proteotoxic stress in a variety of mammalian cells [53]. Part of this discrepancy may exist due to the conflation of two distinct aggregation phenomena: (a) the active concentration of misfolded proteins by the PQC machinery into spatially constricted sites for further triage (e.g., Q-bodies, aggresomes, JUNQs, ALIS/p62 bodies), and (b) the active or passive sequestration of higher-order misfolded protein oligomers and fibrils into terminal deposits (e.g., the IPOD, amyloid inclusion bodies). Adding to this complexity is the ability of PQC factors themselves (e.g., p62/SQSTM1, small heat-shock proteins) to oligomerise in the presence of misfolded proteins—a phenomenon that may help concentrate the misfolded proteins via liquid–liquid phase separation (LLPS), discussed later. As much of the work on protein aggregation sites has been performed using microscopy-based readouts of fluorescently labelled misfolded proteins, it is difficult to distinguish between (a) and (b). Furthermore, the highly dynamic nature of (a) make them difficult to detect without some sort of PQC perturbation, perhaps explaining the conditions during which they were characterised in early studies. Deconvolution of such different types of aggregates will be an important step for clarifying the hierarchical nature of protein clearance—especially as erroneous accumulation of classical IPOD substrates (e.g., amyloidogenic proteins) in the juxta-nuclear sequestration site could represent a more important driver of protein misfolding disease pathology than the presence of aggregates *per se*.

### Ubiquitin-binding proteins as post-ubiquitylation triage factors

Although we mentioned that the proteasome’s cleavage preferences vary with the type of ubiquitin chain, perhaps a greater contributor to ubiquitin signal diversity

is the fact that differently linked ubiquitin chains recruit different ubiquitin-binding proteins (UBPs), with divergent downstream consequences [110]. Most UBPs themselves have multiple binding domains to recruit their own sets of effectors. Furthermore, it is common for UBPs to form dimers and/or higher-order oligomeric structures. When this UBP diversity is added to the potential for formation of ubiquitin chains of different lengths, linkages, and topologies [17,18], it is easy to imagine how incredibly complex signalling platforms could be generated on a single ubiquitylated substrate.

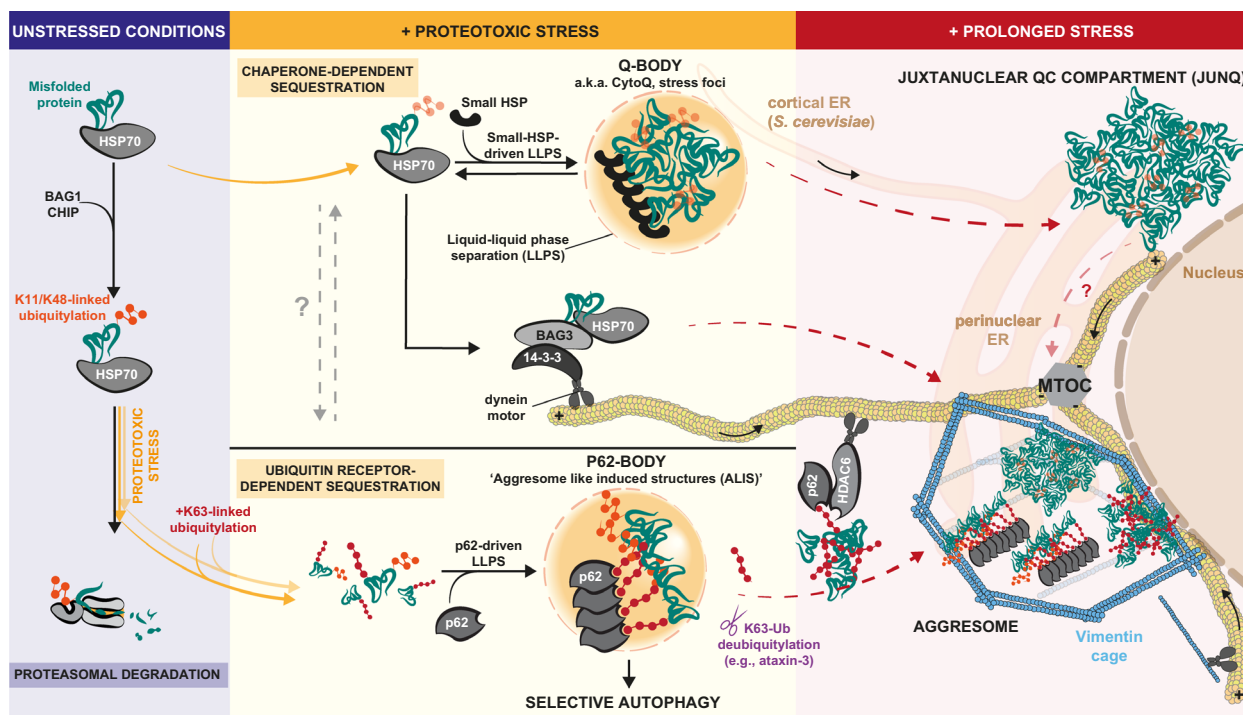
Molecular chaperones are considered the primary regulators of misfolded protein triage, which is generally described as a decision to send their clients to pro-folding *vs.* pro-clearance pathways. Of course, similar triage decisions must exist at several other stages in a misfolded protein’s journey—perhaps at every fork in the road.

The idea that UBPs function as chaperones is not new. Mammalian UBQLNs (‘ubiquilins’)—one class of UBPs with an N-terminal ubiquitin-like (UBL) domain and a C-terminal ubiquitin-associated (UBA) domain—can triage mitochondrial transmembrane proteins mislocalised to the cytoplasm [111]. However, this seems to be a more conventional chaperone role, where ubiquilins’ interactions with transmembrane clients permit productive membrane targeting events instead of ubiquitylation and degradation. Our focus here is on how UBP recognition of a ubiquitylated substrate can be a critical determinant of the substrate’s clearance route.

The domain structure of UBQLNs allows them to act as shuttle factors that bring ubiquitylated substrates to the proteasome [112]. All three widely expressed members of the UBQLN family colocalise with aggresome-like structures and are implicated in the pathology of protein misfolding diseases [48,64,113]. However, the exact division of labour between these different isoforms—and, indeed, between other UBPs—is unclear.

Some insights into the factors involved in interplay between UBPs might be provided by work in yeast. In addition to three UBL-UBA shuttling factors—Dsk2, Rad23, and Ddi1—yeast also express several proteins with CUE (‘*c*oupling of *u*biquitin to *E*R degradation’) domains, which are structurally and functionally related to UBA domains. One of these proteins, Cue5, also possesses an Atg8-interacting motif (the yeast equivalent of the LIR domain found in p62/SQSTM1 and other selective autophagy adaptors). Two papers from Lu and Jentsch [114,115] showed Cue5 to have a key role in the autophagic clearance of aggregation-





**Fig. 3.** Alternative PQC routes to juxta-nuclear sequestration sites. Under stress-free conditions, cells clear terminally misfolded proteins by the proteasome in a process directed by PQC machineries such as the molecular chaperone HSP70, co-chaperone BAG1, ubiquitin ligase CHIP/STUB1, and K11/K48-linked polyubiquitin chains. When the ubiquitin–proteasome system is compromised (e.g., by proteotoxic stressors, or direct proteasome inhibition), misfolded proteins are targeted to a juxta-nuclear PQC site such as the aggresome or JUNQ via chaperone- or ubiquitin-dependent routes. One chaperone-dependent route involves the concentration of misfolded proteins by liquid–liquid phase separation (LLPS) into Q-bodies (also known as CytoQ or stress foci)—a process that might be driven by the oligomerisation of small heat-shock proteins. Q-bodies can either be resolved by refolding of the misfolded protein cargo, or coalesced into the juxta-nuclear QC compartment (JUNQ). At least in the budding yeast *S. cerevisiae*, JUNQ formation requires an intact cortical ER network. An alternative chaperone-dependent route relies on the HSP70 cochaperone BAG3 outcompeting BAG1 for the misfolded protein substrate, which is consequently loaded onto microtubules via dynein motors and 14-3-3 adaptors for retrograde transport to the microtubule organising centre (MTOC). By contrast, the ubiquitin-dependent route involves K63-linked ubiquitylation that drives LLPS via p62/SQSTM1 binding and oligomerisation. These ALIS/p62 bodies are generally cleared by selective autophagy via p62/SQSTM1-mediated recruitment of LC3-positive autophagic structures. However, in situations where they cannot be cleared by this mechanism, ALIS/p62 bodies are targeted to the aggresome. Aggresome targeting required cleavage of K63-ubiquitin chains off misfolded proteins by deubiquitylases (e.g., ataxin-3). The resultant unanchored K63 ubiquitin chains are recognised and bound by HDAC6, which directly binds dynein for transport to the MTOC. The vimentin cage—important for proteasome targeting and asymmetric inheritance of the aggresome—is also formed at the juxta-nuclear site via dynein motor-driven transport.

prone proteins. The second of these studies demonstrated that the balance between Dsk2-mediated proteasomal targeting and Cue5-mediated autophagic targeting is dictated by the oligomerisation status of the two adaptors. Dsk2 functions mainly as a monomer or dimer, whereas Cue5 has the ability to form higher-order oligomers. Therefore, although Dsk2's UBA domain has a 10-fold higher affinity for ubiquitin than Cue5's CUE domain, the concentration of ubiquitylated proteins in aggregates combined with Cue5's oligomerisation propensity would increase avidity for Atg8 such that autophagosomes are recruited more effectively than proteasomes. This provides an

elegant biophysical solution where ubiquitylated proteins are sent to their most efficient clearance routes—that is, monomers to proteasomes, aggregates (that could clog-up proteasomes) to autophagy. The precise oligomer size at which the switch from Dsk2- to Cue5-dominance occurs is likely fine-tuned throughout evolution for different substrates and physiological contexts.

As one might expect, humans possess many more adaptors capable of binding ubiquitin and Atg8/LC3 [36]. Although there is some degree of redundancy between these adaptors, they also display distinct stress-specific activation and substrate preferences. A

recent study [116] demonstrated that p62/SQSTM1 forms higher-order oligomers that are nucleated by ubiquitylated proteins, and are inhibited upon recruitment of LC3, or in the presence of ubiquitin (both free ubiquitin, and various polyubiquitin chains). Of note, the evolutionarily related NBR1—known to dimerise with p62/SQSTM1—accelerated oligomerisation, whereas the unrelated autophagy adaptor optineurin had no effect. Revealing the precise physiologic function of such higher-order oligomers needs further work; however, it seems to be an essential feature of selective autophagy, as all eukaryotes possess at least one adaptor with oligomerisation capability [36].

### The common link: K63-ubiquitin chains as alternative PQC signals

The detection of a polyubiquitin ‘ladder’ on a protein of interest has commonly been used as a shorthand for assigning it as a proteasomal substrate. However, it is abundantly clear that polyubiquitylation can signal several non-proteasomal—indeed, non-proteolytic—fates for the protein to which it is attached [17,18]. The fate conferred is largely determined by how the polyubiquitin chain is linked together—often referred to as the ‘ubiquitin code’. Importantly, whereas K11- and K48-linked chains seem to be dedicated proteasomal targeting signals, almost all other linkages have been shown to signal different fates under different contexts. For example, K6-ubiquitin is important for both selective autophagy of mitochondria (‘mitophagy’) and the DNA damage response; K33-ubiquitin mediates Toll-like receptor signalling and post-Golgi protein trafficking [17,18].

The ubiquitin signal with the most fates identified to date is K63-ubiquitin, with roles in DNA damage repair, inflammation, intracellular trafficking, and different types of PQC [17,18]. Concerning this last point, K63-ubiquitin chains are associated with both misfolded protein aggregates and selective autophagy substrates [32,117]. The selective autophagy cargo adaptors are likely to be key effectors of this signal. p62/SQSTM1 can bind both K48- and K63-ubiquitin chains through its UBA domain; however, it appears to have a strong preference for K63-ubiquitin’s more extended, open conformation [118,119]. This preference for K63-ubiquitin and/or other extended chains (e.g., linear M1-ubiquitin) is a shared feature of most selective autophagy adaptors [32]. p62/SQSTM1 and the evolutionarily related adaptor NBR1 nucleate on K63-ubiquitin chains and form LLPS inclusion bodies that recruit autophagosomal membranes via interactions with LC3 [32,116,120–122]. Although the related

cargo adaptors NDP52, TOLLIP, TAX1BP1, and optineurin are more important in other types of selective autophagy (e.g., mitophagy, xenophagy) [36], they have all been shown to participate in clearance of misfolded protein inclusions to some extent [115,123–125]. Therefore, a major role of K63-ubiquitin chains on misfolded proteins could be as recruitment signals for selective autophagy cargo receptors. Whether the resultant punctate structures (e.g., ALIS/p62 bodies) are cleared by autophagy or accumulate in aggresomes would depend on the relative kinetics of protein misfolding, sequestration, and degradation.

K63-ubiquitin also links misfolded proteins to the microtubule and actin networks for trafficking to and from the aggresome, respectively. Initially, K63-ubiquitylation of PQC cargo (e.g., by the ubiquitin ligases CHIP/STUB1, Parkin, HRD1, or TRIM50) was proposed to serve as a direct signal for HDAC6 to tether them to dynein motors, thereby allowing retrograde transport of the misfolded proteins to the juxta-nuclear MTOC on microtubules [58–60,126]. However, subsequent work [127,128] indicates that HDAC6 actually binds to the C-terminal of unanchored K63-ubiquitin chains generated by the deubiquitylase ataxin-3—a protein that directly interacts with HDAC6, ubiquitin chains, and microtubules [61,129–132]. Taking these studies together, aggresomal targeting of misfolded proteins appears to require a round of K63-ubiquitylation and deubiquitylation. The purpose of such an intricate mechanism is unclear. A similar mechanism for generating unanchored K63-ubiquitin chains was later described for clearance of aggresomes during recovery from proteasome inhibition [133,134]. Note that an aggresome-targeting mechanism that requires unanchored ubiquitin chains implies that the misfolded protein cargo must already be in some sort of aggregate or inclusion body, as there would be nothing tethering a monomeric ‘free-floating’ misfolded protein to the HDAC6-dynein-microtubule assembly after the ubiquitin chain is cleaved from it. LLPS of misfolded proteins into ALIS/p62 bodies or Q-bodies—possibly triggered by oligomerisation of p62/SQSTM1 or small heat-shock proteins, respectively—could potentially solve this problem [57,121,122].

We should point out that chains of numerous other ubiquitin linkages colocalise with misfolded protein aggregates [18,135,136]. As discussed earlier, it is difficult to establish whether any of these chains play functional roles in aggresome formation or clearance. For example, the presence of K48-ubiquitin could be explained by the fact that aggresomes are often reported under conditions of proteasome inhibition; therefore, K48-ubiquitylated proteasomal substrates

would be targeted to the aggresome in these cells. Whether these proteasomal substrates require further modification with K63-ubiquitin chains (either on a different lysine residue on the substrate or off K48-ubiquitin to form K48-K63 branched chains) for targeting to aggresomal and/or autophagic structures has yet to be determined. Furthermore, ubiquitin-independent PQC routes involving p62/SQSTM1, NDP52, and optineurin have been reported [137–139]. How recruitment of adaptors and cytoskeletal networks is achieved in the absence of K63-ubiquitin needs further clarification.

### HDAC6 and p62/SQSTM1 in alternative PQC: more than just ubiquitin adaptors

We have described the key roles HDAC6 and p62/SQSTM1 play in alternative PQC by linking ubiquitylated proteins to the downstream targeting machinery. HDAC6 is predominantly considered to be an aggresome adaptor, whereas p62/SQSTM1 is a selective autophagy adaptor. At least functionally, this distinction appears rather arbitrary: Both proteins have been shown to play a role in aggresome targeting and selective autophagy. In fact, it is highly likely that HDAC6 and p62/SQSTM1 work together on this route. p62/SQSTM1 binds dynein independently of (and perhaps competitively with) HDAC6, and the absence of either protein impairs retrograde cargo trafficking to the MTOC [140]. The direct interaction between HDAC6 and p62/SQSTM1 appears to keep HDAC6's deacetylase activity in check—without it, microtubules become hypoacetylated [141], which, again, impairs retrograde transport [142].

Similarly, the absence of p62/SQSTM1 dysregulates HDAC6-mediated deacetylation of cortactin and the resultant remodelling of cortactin-actin bundles necessary for misfolded protein targeting from aggresomes to the autophagosome-lysosome machinery (Fig. 4) [133,141,143,144]. Note that HDAC6 may additionally exert its influence in autophagic clearance by deacetylating noncytoskeletal substrates, as exemplified by a study where HDAC6 inhibition with tubacin suppressed lipid-conjugated LC3 deacetylation upon serum starvation, resulting in impaired p62/SQSTM1-dependent selective autophagy [145].

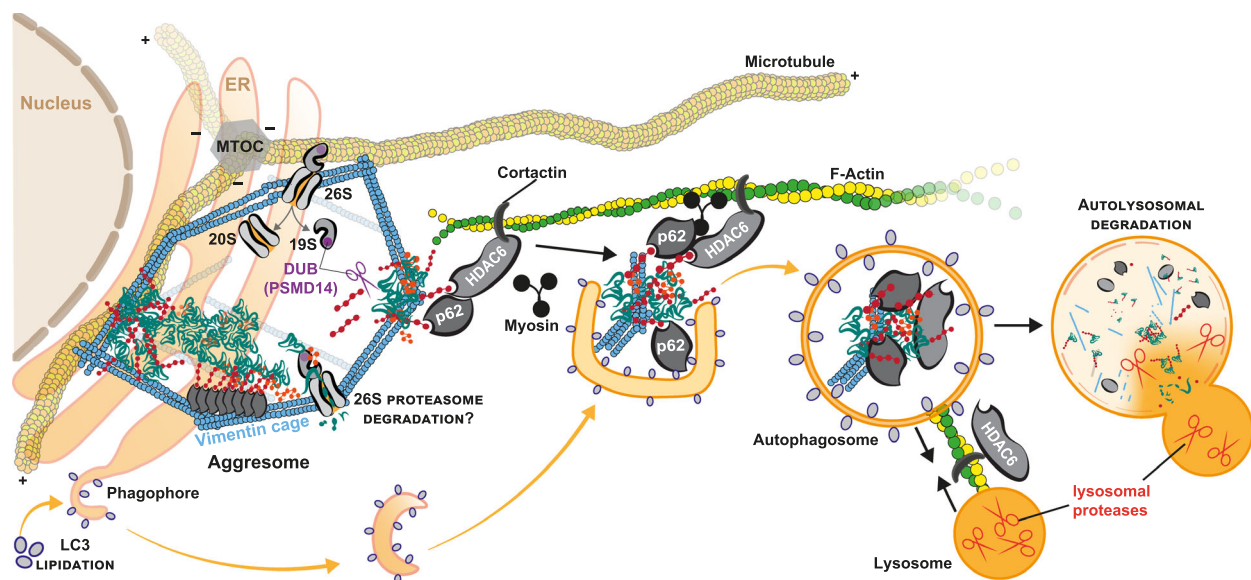
Outside of their direct role in this alternative PQC route, HDAC6 and p62/SQSTM1 can also alter the stability and/or activation of orthogonal PQC regulators. For example, HDAC6 has a broad range of cellular functions—both at the level of its histone deacetylation and chromatin remodelling activity, and through unrelated cytoplasmic interactions [146]. One

of its underappreciated roles is in activation of the heat-shock response [147,148]. Under nonstressed conditions (e.g., with a functional ubiquitin–proteasome system), HDAC6 forms a stable complex with HSP90, p97/VCP, and heat-shock factor 1 (HSF1). HDAC6 in this complex not only regulates HSP90's chaperoning activity through direct deacetylation [149], but also maintains HSF1 in an inactive state by preventing its trimerisation. Proteotoxic stresses including heat shock and proteasome inhibition trigger complex dissociation, perhaps because an increase in polyubiquitylated protein concentration outcompetes p97/VCP off HDAC6 [147,148]. As a result, HSF1 is released, and the heat-shock response transcriptional programme (e.g., expression of HSPs) is initiated.

### Ubiquitin-independent alternative PQC by BAG3

Like HDAC6, the HSP70 co-chaperone BAG3 is an evolutionarily conserved mediator of misfolded protein trafficking to both the aggresome and p62/SQSTM1-mediated selective autophagy [55,150]. One of six human HSP70-binding cochaperones of the *BCL2-associated athanogene* family, a cell's BAG3 expression in comparison with its BAG1 expression can be used as a rough guide to whether selective autophagy or the proteasome is the preferred route for misfolded protein clearance in that cell [150]. For example, BAG3 expression is induced following proteasome inhibition and leads to the targeting of K48-ubiquitylated proteins to selective autophagy [151]. The same study showed that BAG3 overexpression sequesters K48-ubiquitylated proteins in cytoplasmic puncta, supporting a model where BAG3's alternative PQC targeting is not merely a fail-safe in situations of proteasome insufficiency. These findings are especially relevant for improving our understanding of ageing-associated pathologies, as the BAG3-BAG1 ratio has been shown to increase in several healthy and pathologic ageing models [150].

BAG3's role in triggering the proteasome-to-autophagy switch appears to occur via tethering of misfolded protein cargo bound by HSP70 and/or small HSPs (e.g., HSPB8) to dynein-bound microtubules via 14-3-3 adaptors, thereby enabling retrograde transport to the aggresome (or related juxta-nuclear compartments) (Fig. 3) [150,152,153]. BAG3-dependent aggresome formation and clearance by selective autophagy requires the HSP70-interacting ubiquitin ligase CHIP/STUB1, although its ubiquitylation activity is dispensable here [54,55]. Disease-associated proline-209 mutations in BAG3 sever the link with selective autophagy, causing its clients and associated



**Fig. 4.** Molecular events in aggresomal protein clearance. Misfolded proteins at the aggresome are targeted to the selective autophagy machinery in a process involving p62/SQSTM1 and HDAC6. HDAC6-based remodelling of the actin-myosin network via its deacetylation of cortactin is essential for clearance, including the autophagosome-to-lysosome fusion step. The juxta-nuclear location of the aggresome ensures easy access to ER-budded membranes for autophagosome formation—as well as to lysosomes, which cluster around this site. Direct misfolded protein degradation by 26S proteasomes could also be a viable clearance route. Proteasomes may further contribute to clearance by generating more unanchored K63 ubiquitin chains by the deubiquitylase (DUB) PSMD14/Rpn11/Poh1 in the 19S subunit, which is activated following HSP90-mediated splitting of 26S proteasomes.

chaperones to accumulate terminally in the aggresome [153,154].

In light of the striking functional similarities between HDAC6- and BAG3-mediated alternative PQC, it is unclear why both pathways exist concurrently in the same cell. The fact that perturbing the activity of either protein alone has a clear effect on aggresome and/or selective autophagy trafficking indicates that they serve non-redundant functions. Perhaps the only major functional differences occur at the level of misfolded protein recognition. HDAC6 requires (unanchored) K63-ubiquitin chains for binding, whereas BAG3-mediated tethering to dynein motors appears to be ubiquitin-independent [55] (although most misfolded protein targets of BAG3 are ubiquitylated, by the HSP70-associated ubiquitin ligase CHIP/STUB1). Therefore, BAG3 could potentially target misfolded proteins that are refractory to polyubiquitylation (such as certain ALS-associated SOD1 mutants [155]). Conversely, BAG3 is recruited to misfolded proteins via HSP70—it cannot target non-HSP70 clients, or misfolded proteins that have escaped the HSP70 machinery (e.g., in times of extreme proteotoxic stress). HDAC6 does not have this limitation. Therefore, it is feasible that these two modules evolved independently under distinct selective pressures that would have otherwise led to proteostasis collapse.

## Emerging concepts in alternative PQC

### Juxta-nuclear sites as hubs enabling centralised PQC

The specific subcellular positioning of aggresomes and related structures ensures misfolded proteins are primed for engagement by various PQC systems. First, its colocalisation with the MTOC provides a mode for retrograde transport of misfolded proteins along the extensive microtubule network. Note that functional microtubule transport appears dispensable for targeting of Q-bodies to the JUNQ in budding yeast, but instead relies on tethering to an intact cortical ER [77] and/or actin [87]. This use of completely different systems to solve the same problem illustrates the broader point that the exact mechanisms for PQC may vary from cell-type to cell-type, and organism to organism. For example, not all cells possess the intermediate filament vimentin; however, oligodendroglial cells get around this problem by surrounding their aggresome with microtubules instead [156]. Regardless, the same principle holds true: Centralising the aggresome and JUNQ at a juxta-nuclear transport hub allows the use of pre-existing networks. The same transport networks can further be exploited for asymmetric inheritance of misfolded protein inclusions during cell division [87].



The juxta-nuclear sites also seem optimally positioned to receive misfolded proteins from different subcellular compartments, including the cytoplasm [52], ER [46,157], Golgi [158], mitochondria [159,160], and even the nucleus [161] (although nuclear misfolded proteins also readily accumulate in an intranuclear aggresome-like structure [76,158,162]). Similarly, clearance of the aggresome by selective autophagy is aided by its proximity to the ER (for a source of autophagosomal membranes), actin filaments (for transport through the autophagy–lysosome system), and lysosomes (whose clustering around the aggresome is required for efficient clearance) [163,164].

In addition to the ER, actin, and lysosome clusters, electron micrographs of the aggresome show that other organelles such as multivesicular bodies and mitochondria are seemingly ‘trapped’ at its periphery [46,159]. However, as with all correlative colocalisation-based observations in PQC, it is challenging to ascertain whether such compartments are substrates, bystanders, or active players in aggresome dynamics. For example, mitochondrial presence could be for (a) transfer of misfolded mitochondrial proteins; (b) disposal of damaged mitochondria via mitophagy; (c) a ready supply of ATP for various PQC processes; (d) apoptosis or inflammation responses if the proteotoxic stress exceeds PQC capacity; and/or (e) no productive reason, but rather as a coincidental passenger (which may nevertheless contribute to protein misfolding toxicity).

Centralising major spatial sequestration sites at the nexus of so many organelles would greatly simplify the cellular PQC burden by shuttling the majority of misfolded proteins detected by surveillance systems to a single site for downstream processing. This measure would allow cells to separate the resource-intensive tasks of misfolded protein identification and misfolded protein triage—with the former being performed *in situ* by the highly abundant and ubiquitous molecular chaperones, and the latter at the JUNQ and/or aggresome, harbouring a variety of downstream PQC effectors. Furthermore, targeting even globular cytoplasmic or nuclear misfolded proteins to a membranous site could effectively reduce a 3D problem to a 2D problem and potentially increase the avidity for crucial interactions with various parts of the PQC machinery. Such dimensionality reduction measures are illustrated by the handful of ER-integral ubiquitin ligases required for cytoplasmic misfolded protein ubiquitylation [19,21,165].

An additional advantage of aggresomal positioning in organelle-rich environments would be to place them at interorganellar membrane contact sites—which are emerging as critical centres for intracellular signalling

and homeostasis [166]. Maintenance and remodelling of these sites play an important role in the response to protein misfolding in the ER and mitochondria, protecting against proteotoxicity and contributing to lifespan extension [167–170]. Although underexplored for aggresomal clearance, several studies in budding yeast have reported the requirement for close contact with organelles, including lipid droplets and mitochondria, for clearance of various misfolded protein inclusion bodies [167,171,172].

### Phase separation as a feature of spatial sequestration

Although the aggresome is membrane-rich and surrounded by a vimentin cage, neither of these features are necessary for its formation. What, then, keeps misfolded proteins separated from the surrounding cytoplasm and prevents them interfering with essential molecular interactions? A growing body of work in recent years suggests that it is liquid–liquid phase separation (LLPS) that dictates formation and dissolution of such membrane-less compartments [173,174].

Simply put, LLPS describes the reversible demixing of a homogeneous fluid into two distinct liquid phases. In the context of intracellular biochemistry, LLPS usually involves dense yet mobile biomolecular condensates floating within the more fluid cytoplasmic or nucleoplasmic milieu. Several well-established intracellular structures—including P-bodies, stress granules, Cajal bodies, PML bodies, and the nucleolus—are now generally accepted as being membrane-less LLPS compartments [173,174]. Importantly, the LLPS properties play an essential part in their function, as demonstrated by perturbations that either prevent biomolecular condensate formation, or make them irreversible and more ‘gel-like’.

Phase separation enables the rapid assembly, propagation, and disassembly of functional modules based on shared structural features—making it an ideal property for the stress response machinery to adopt [174]. In addition to stress granules—which attenuate protein translation in response to various stresses—targeting of heat-shock-denatured misfolded proteins into the nucleolus via LLPS was identified recently as a means of preventing their irreversible and toxic aggregation [175]. Hyperosmotic stress results in the formation of a different nuclear LLPS body, which serves as a site for proteasomal degradation of misassembled ribosomal subunits [176].

The LLPS properties of aggresomes or JUNQs have yet to be explicitly demonstrated; however, many of

the compartments' described properties would be consistent with this notion. For example, fluorescence recovery experiments indicate that proteins within the JUNQ are highly mobile, whereas those within the IPOD—representing non-LLPS, irreversible, gel-like aggregates—are not [71,74]. Furthermore, ALIS/p62 bodies have already been shown to be phase-separated [121,122]. Other membrane-less punctate misfolded protein structures (e.g., Q-bodies) are likely to share this property.

Although the precise molecular requirements for LLPS transitions in PQC need further investigation, ubiquitin chains and ubiquitin-binding proteins play a critical role in formation of various types of stress granules, ALIS/p62 bodies, and hyperosmotic nuclear proteasome granules [121,122,176,177]. Dao *et al.* [177] propose a mechanism where UBQLN2 is recruited to LLPS stress granules via multivalent interactions involving its oligomerisation domain and intrinsically disordered region; subsequent binding to ubiquitin chains reverses UBQLN2's phase separation propensity and allows it to shuttle ubiquitylated substrates to the proteasome or selective autophagy [178]. Consistent with this model, toxic *UBQLN2* mutations found in amyotrophic lateral sclerosis and frontotemporal dementia impair the protein's LLPS properties [177,179]—although this is not sufficient to recapitulate fully the neuronal loss characteristic of the disease [180]. Alteration of LLPS dynamics by disease-associated amyloidogenic proteins has been added to the growing list of pathogenic drivers in neurodegeneration [181,182], potentially as a result of interfering with nucleocytoplasmic transport at the nuclear pore [183]—a multiprotein assembly that employs phase separation to moderate its permeability barrier [184]. The finding that targeting disease-associated IPOD substrates to the JUNQ impairs the mobility of other JUNQ-targeted misfolded proteins and chaperones, and eventually leads to cell death [73,185], suggests that a similar mechanism may be at play at this juxta-nuclear PQC site.

### Role of proteasomes in alternative PQC clearance routes

Based on what we have discussed until now, the autophagy–lysosome system would seem to be the predominant mode of clearance for aggresomal proteins. In conditions where the ubiquitin–proteasome system is compromised—the focus of our review—this conclusion is certainly well founded. However, here we briefly address whether proteasomes also contribute to aggresome clearance.

The colocalisation of proteasomes (both 19S and 20S particles) with the aggresomal site was an early observation [56]. However, as we described for mitochondria in an earlier section, ascribing a functional role for these proteasomes has proved difficult. For example, the (active or passive) sequestration of proteasomes in aggregates is considered a major contributor to the toxicity of amyloidogenic protein aggregates (e.g., in Huntington's disease), as it reduces the active pool of proteasomes available for global PQC [186]. Furthermore, a growing body of literature suggests that proteasomes are themselves substrates of spatial sequestration and clearance through selective autophagy ('proteaphagy') in response to a range of stresses, including proteasome inhibition [187–191]. A recent study [191] showed that inhibited 26S proteasomes are ubiquitylated with K63-chains by CHIP/STUB1, sequestered in the aggresome via the HDAC6-dynein route, and cleared by p62/SQSTM1-mediated selective autophagy. The authors demonstrated that clearance of the aggresome-targeted proteasomes after wash-out of the proteasome inhibitor was still dependent on functional autophagosome–lysosome fusion. Therefore, once at the aggresome, proteasomes are predominantly degraded by autophagy—even after the proteotoxic stress has been removed. Whether the same is true for misfolded proteins at the aggresome is worth exploring.

While it is unclear what proportion of proteasomes present at the aggresome are proteaphagy substrates, two studies by the Yao group describe how aggresome-localised proteasomes serve at least some functional significance for misfolded protein clearance [133,134]. However, this requirement was entirely independent of the 20S particle's catalytic activity, but rather attributed to unanchored K63-ubiquitin chain generation by the 19S-resident deubiquitylase PSMD14/Rpn11/Poh1. PSMD14 activation was dependent upon HSP90-mediated splitting 26S proteasomes into the 19S and 20S particles (Fig. 4).

Taking these observations together, it is possible to speculate a system where proteolytically inactive 26S proteasomes serve a rheostat function in aggresomal PQC. Their stress-initiated accumulation at the aggresome would generate unanchored K63-ubiquitin chains (either from surrounding misfolded proteins, or from the CHIP/STUB1-ubiquitylated proteasomes themselves), which trigger clearance of aggresomal fragments by HDAC6 and p62/SQSTM1-mediated selective autophagy. This mechanism would ensure a consistent supply of unanchored K63-ubiquitin, only exhausted once proteasomes stop being targeted to the aggresome—presumably indicating restoration of

active proteasome capacity. It will be important to establish the degree of overlap between misfolded proteins and proteasomes through this alternative PQC route. A proteomic analysis of isolated lysosomes in proteasome inhibitor-treated human HEK293 fibroblasts identified a strong enrichment in both proteasomal particles and misfolded proteasomal substrates [190]—although whether these were targeted there through the same route was not determined.

Despite these findings, we still cannot discount the possibility that proteasomes proteolytically degrade misfolded proteins at the aggresome and/or JUNQ. At least in budding yeast, clearance of the stress-induced JUNQ is predominantly dependent on proteasomal degradation, with autophagy playing a minor role. Whether this discrepancy with observations in higher eukaryotes is due to true differences between organisms, or instead an artefact of experimental design, is unclear. Whereas the yeast JUNQ has been described under a range of genetic and environmental stressors, most mammalian studies employ either proteasome inhibition or the expression of proteasome-resistant amyloidogenic proteins to trigger aggresome formation. Obviously, both of these triggers are not conducive to measuring proteasome-dependent clearance routes. Several studies have attempted to address this problem by removing proteasome inhibitors from the growth medium after aggresome formation [46,133,134]—however, uncertainty in the time taken to reduce the working intracellular inhibitor concentration and restore the functional proteasome pool make these wash-out experiments difficult to interpret. Therefore, work with aggresomes formed in conditions where proteasomes are not rendered non-functional would be more informative in addressing this question. To illustrate this point, aggresomes formed upon heat shock (a stress that does not inactivate proteasomes) were cleared predominantly by proteasomes via HSP70 and UBQLN2—and did not appreciably rely on autophagy [64].

The aggresomal enrichment of K63-ubiquitin chains—which are considered refractory to proteasomal degradation—also does not necessarily preclude the proteasomal degradation route. K63 chains behave similarly to K48 chains with regard to 26S proteasome binding ability and degradation rates *in vitro*; they are only refractory to proteasome targeting in cells (perhaps due to high-affinity interaction with K63-specific UBPs, such as the ESCRT-0 complex) [192]. Furthermore, K63 chains can trigger recruitment of K48-assembling ubiquitin ligases [193]. Substrates displaying such branched K63-K48 chains appear to be

perfectly competent for proteasomal targeting and degradation *in vivo* [193].

It is also important to remember that not all cells will have access to the same routes to, or capacities for, stress resilience. To take one example, a recent study in neuronal stem cells [194] identified for the first time a functional role for the vimentin cage that surrounds the aggresome: It was required for aggresomal clearance of misfolded proteins by binding proteasomes and localising them to the aggresome. Intriguingly, vimentin-knock-out neuronal stem cells upregulated autophagic flux to clear the aggresome. By contrast, another study [195] in HEK293 fibroblasts reported a suppression in autophagosome-to-lysosome fusion upon pharmacologic vimentin inhibition. Therefore, although vimentin function is important for aggresome clearance, how cells adapt to vimentin defects likely differs. Perhaps cells or tissues that have less efficient adaptive strategies are especially susceptible to proteostatic collapse-associated toxicity.

## Future perspectives

The mechanisms by which cells mitigate misfolded protein toxicity in conditions of acute or chronic proteotoxic stresses invariably require some degree of proteasome-independence. Technological and conceptual advances in intracellular molecular biology over recent years (e.g., super-resolution single-molecule tracking, linkage-specific ubiquitin quantification tools, biomolecular condensate probes) are paving the way to reveal several poorly characterised sections of the proteostasis field. Improving our understanding of alternative PQC has direct translational consequences. Proteostasis imbalance is a hallmark of ageing [196], with dysregulated cross-talk between PQC systems in ageing-associated disorders [30,31]. For example, numerous components of the protein clearance machinery—including the critical alternative PQC regulators p62/SQSTM1, HDAC6, BAG3, ataxin-3, UCHL1, Parkin, and UBQLN2—are strongly implicated in the pathology of neurodegenerative diseases [197]. Identifying mechanisms by which proteostasis can be restored could reveal novel therapeutic strategies for these diseases, as well as other ageing-associated disorders driven by proteostasis imbalances, such as type II diabetes, cardiovascular diseases, and several cancers [5].

With respect to cancers, the proteasome inhibitor bortezomib has been approved for clinical use for multiple myeloma and mantle cell lymphoma since 2003 and 2006, respectively. Both innate and acquired

resistance to proteasome inhibition is common and can occur through a variety of mechanisms, including modification of the 20S- $\beta$ 5 subunit (the direct target of bortezomib), depleting 19S particle expression, and induction of proteaphagy [198,199]. Targeting alternative PQC could be an effective means of countering at least some of these resistance mechanisms. For example, there are currently numerous clinical trials, across several haematological malignancies, combining HDAC6 inhibitors with bortezomib or the second-generation proteasome inhibitor carfilzomib [200]. However, given the broad-ranging cellular functions of HDAC6—potentially influencing cellular protein production, folding, trafficking, or clearance [146]—narrowing of the therapeutic window due to on-target toxicity would not be surprising. Small molecules that inhibit HDAC6's ZnF-UBD interaction with ubiquitin are a promising approach for specifically perturbing the misfolded protein trafficking function of HDAC6 [201].

Alternative PQC modulators also hold promise in the pursuit of interventions that improve our healthy lifespan, especially with the launch of the targeting ageing with metformin (TAME) study—the first FDA-approved clinical trial with ageing as its primary end-point [202]. Stimulating autophagy—one of the multiple modes of metformin action, via AMPK activation and mTOR inhibition—is among the most robust mechanisms for increased lifespan across a variety of organisms [203]. Furthermore, inhibitors of mTOR and HSP90 were recently identified as ‘senolytics’ [204,205], that is, agents with increased potency against senescent cells, the accumulation of which is linked to ageing-related organismal frailty. Therefore, mechanistic advances in our understanding of how different PQC systems are integrated in health, and how they become disintegrated during ageing and associated diseases, have the potential to drive therapeutic interventions in both pathologic and healthy ageing contexts.

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## Conflict of interest

The authors declare no conflict of interest.

## Author contributions

RSS conceived and wrote the first draft. HEJ and RSS edited the manuscript and composed the figures.

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