Quality Control of Protein Folding in the Cytosol

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Article Contents

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In order to function properly, newly synthesised proteins must rapidly and efficiently attain their native conformations. If they fail to do so, the cell may be adversely affected due to loss of function or toxic gain of function effects of misfolded polypeptides. Effective quality control mechanisms to recognise and eliminate misfolded proteins are thus critical for cell viability. The primary means by which misfolded proteins are selectively removed from the cell is via the ubiquitin–proteasome system. Although much is known about regulated proteolysis, how any given protein, which could potentially misfold, is recognised and targeted for proteasome-mediated degradation has been challenging to decipher. Recent progress, much of it in yeast, has identified specific E3 ligases involved in this process, clarified or added to our knowledge of the roles of molecular chaperones, and identified multiple cellular locations where degradation, or failing that, aggregation, occurs.

Introduction

Establishing and maintaining an effective balance of protein synthesis, folding and degradation is critical to cell function and viability. Molecular chaperones are crucial to the successful triage of protein-folding challenges. In addition to their role in folding, chaperones assist the recognition and degradation of misfolded proteins by the ubiquitin–proteasome system (UPS). The numerous diseases linked to protein misfolding, among them a host of neurodegenerative disorders hallmarked by protein aggregation, highlight the importance of defining protein quality control mechanisms in the eukaryotic cytosol.

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The Role of Molecular Chaperones in Protein Folding

Newly synthesised proteins emerge linearly from the ribosome into the crowded intracellular milieu. Cellular proteins called molecular chaperones facilitate the rapid folding of newly synthesised polypeptides to the native state by preventing inappropriate or 'off-pathway' inter- and intramolecular interactions (Frydman, 2001). The action of most molecular chaperones is governed by adenosine triphosphate (ATP)-dependent cycles of nonnative polypeptide binding and release; importantly, chaperones no longer bind to their substrates once they attain their native, functional three-dimensional conformation. In addition to their role in *de novo* folding, chaperones protect proteins that unfold as a result of cellular stresses, such as chemical or temperature perturbation. In this case, chaperones serve to prevent nonnative protein aggregation and restore unfolded proteins to the native state once the stress has subsided. Indeed, many genes encoding chaperones are transcriptionally upregulated in response to such conditions, hence the moniker 'heat-shock proteins' (Hsps). **See also**: Chaperones, Chaperonin and Heat-Shock Proteins; Heat Shock Proteins (HSPs): Structure, Function and Genetics; Heat Shock Response; Protein Folding and Chaperones; Protein Folding *In Vivo*

In eukaryotes, one of the most extensively studied and well-understood chaperones is the 70 kDa heat-shock

protein (Hsp70). Hsp70 interacts with linear hydrophobic stretches of amino acids that are exposed during protein synthesis, but ultimately buried in the interior of a folded protein. Experiments in mammalian cells estimate that approximately 20% of newly synthesised polypeptides transit through Hsp70 upon synthesis (Frydman, 2001). In addition, Hsp70 associates with preproteins destined for translocation into the endoplasmic reticulum (ER), serving to maintain them in an unfolded conformation for transit across the ER membrane. Hsp70 function is often modulated by its interactions with other proteins or cochaperones, most notably Hsp40 s. From initial interactions with Hsp70, folding substrates requiring further conformational assistance may partition to other cellular chaperones, including those of the Hsp60 or Hsp90 classes (**Figure 1**). **See also**: Chaperones, Chaperonin and Heat-Shock Proteins; Heat Shock Response; Protein Translocation across Membranes

The Hsp60 chaperonins are a family of chaperones that form multisubunit double-ring structures. In eukaryotes, the cytosolic chaperonin TRiC (also called CCT) provides, via its central cavity, a protected environment in which proteins can fold, sequestered away from the crowded cytoplasm. TRiC, unlike the homomeric bacterial chaperonin GroEL, is a heteroligomeric complex made up of eight different subunits. This raises the possibility that the unique subunits contribute unique substrate-binding specificities, increasing the diversity and nature of TRiC-substrate interactions. The sequence or structural determinants that TRiC recognises in its substrates have remained elusive until recently. It is now clear that many TRiC substrates, including the von Hippel–Lindau (VHL) tumour-suppressor protein and numerous WD40 repeat proteins, contain regions of β-strand character that interact with TRiC (Spiess *et al*., 2004). Another interesting feature of some recently identified TRiC substrates is that they are components of multisubunit complexes, suggesting that TRiC primes its substrates for their subsequent interaction with other complex members. **See also**: Chaperones, Chaperonin and Heat-Shock Proteins; Heat Shock Proteins (HSPs): Structure, Function and Genetics; Protein Folding and Chaperones; Protein Folding *In Vivo*

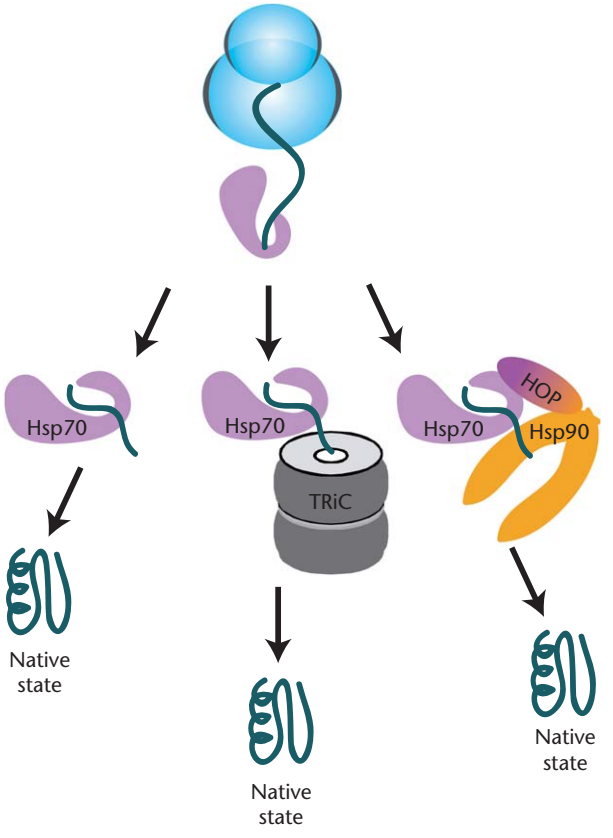

Figure 1 Pathways of chaperone-mediated protein folding in the eukaryotic cytosol. Newly synthesised proteins emerge linearly from the ribosome; exposed hydrophobic residues are prevented from off-pathway folding events by binding to Hsp70. The Hsp70 system is sufficient to fold some proteins (left), whereas other folding substrates require additional folding assistance from TRiC (centre) or Hsp90 (right).

The Hsp90 chaperone is extremely abundant in the eukaryotic cytosol, comprising 1–2% of total protein. Hsp90 functions as a dimer in the final folding or activation states of its substrates, which include steroid hormone receptors and various signalling kinases. Interestingly, Hsp90 also interacts with HSF1, the transcription factor responsible for upregulating stress-inducible chaperones. When Hsp90 function is inhibited, or Hsp90 folding substrates are too numerous, HSF1 is released and free to activate the 'heat-shock' transcriptional response. Hsp90 generally receives its folding substrates from Hsp70 via a cochaperone Hsp Organising Protein (HOP; Sti1p in yeast) that can physically interact with both chaperones via tetratricopeptide repeat (TPR) domains specific for each chaperone. How Hsp90 recognises its substrates is still unclear and an active area of research. **See also**: Chaperones, Chaperonin and Heat-Shock Proteins; Heat Shock Response

Together, the Hsp70, TRiC and Hsp90 chaperones comprise the primary folding machinery in the eukaryotic cytosol (**Figure 1**). The function of these chaperones is based upon their ATP-dependent interactions with nonnative substrate proteins. The ability of chaperones to specifically interact with unfolded or otherwise nonnative proteins makes them prime candidates to also participate in the recognition and degradation of misfolded proteins.

Protein Misfolding and Disease

Despite the best efforts of molecular chaperones, not all newly synthesised or stress-denatured proteins are competent to attain their native states. 'Unfoldable' polypeptides may result from genetic mutation, post-transcriptional error, the absence of a necessary post-translational-binding partner or inherently challenging folding kinetics. These misfolded proteins can have damaging effects on the cell due to either loss of the function

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normally associated with that protein product or, conversely, misfolded conformers may have a cytotoxic gain of function. As such, protein misfolding lies at the core of numerous human diseases. For example, genetic mutations in the tumour-suppressor proteins p53 and VHL result in misfolding and proteolytic degradation, thus contributing to tumour development. Similarly, mutations that slow the folding kinetics of the cystic fibrosis transmembrane conductance regulator (CFTR) undermine the delivery of folded, functional CFTR to the plasma membrane, resulting in cystic fibrosis. **See also**: Protein Misfolding and Degradation in Genetic Disease

Protein misfolding also underlies a growing number of neurodegenerative diseases in which nonnative polypeptides gain a toxic function. Although the genetic mutations and affected proteins differ in these diseases, they all have in common the accumulation of intracellular aggregates, or inclusion bodies, of insoluble amyloid deposits consisting primarily of the disease-associated mutant protein. For instance, in autosomal dominant Parkinson disease, mutant alpha-synuclein conformers compromise cell viability and accelerate aggregate formation associated with disease pathogenesis. Heritable mutations in superoxide dismutase (*SOD1*), responsible for approximately 20% of familial amyotrophic lateral sclerosis (ALS) cases, represent a second example of misfolding resulting in a toxic gain of function. In addition, numerous neurodegenerative disorders are caused by the expansion of a polyglutamine (polyQ) tract, with a critical pathogenic threshold of approximately 40 repeats. Examples of aggregation-prone proteins with expanded polyQ tracts include Huntingtin (HTT), the causative agent of Huntington disease, the gene products underlying various types of spinocerebellar ataxia, and the androgen receptor associated with spinal and bulbar muscular atrophy. Despite a lack of primary amino acid sequence similarity, these disease-causing proteins adopt a toxic conformation that affects cell viability and results in their accumulation as heat and detergent-resistant aggregates (**Figure 2**). The reactivity of soluble oligomers of these various proteins with a single antibody that recognises amyloid oligomers suggests a common structural signature (Muchowski and Wacker, 2005). Of note, it is these soluble early intermediates in the aggregation pathway that appear to represent the toxic species, whereas the final amyloid deposits themselves are not pathogenic and may, in fact, be protective (Muchowski and Wacker, 2005). Importantly, protein aggregation diseases reflect a failing of the quality control system, either at the level of surveillance or elimination, and therefore a loss of balance between protein synthesis, folding and degradation. Regulated cellular responses to deal with protein aggregates exist in organisms from bacteria and lower eukaryotes to humans, suggesting that this is simply one natural outcome of proteostasis perturbation (Tyedmers *et al*., 2010). **See also**: Alzheimer Disease; Amyloidosis; Degradation of Misfolded Secretory and Membrane Proteins and Associated Diseases; Huntington Disease; Parkinson Disease; Protein Aggregation and Human Disorders; Protein Misfolding and Degradation in Genetic Disease; Trinucleotide Repeat Expansions: Disorders

Recently, efforts to characterise the intracellular fates of soluble intermediates versus terminally aggregated conformers of proteins have revealed a differential partitioning of these misfolded states to specific cellular locations. For example, proteins misfolded by virtue of temperature-sensitive mutation or the absence of a required post-translational binding partner preferentially

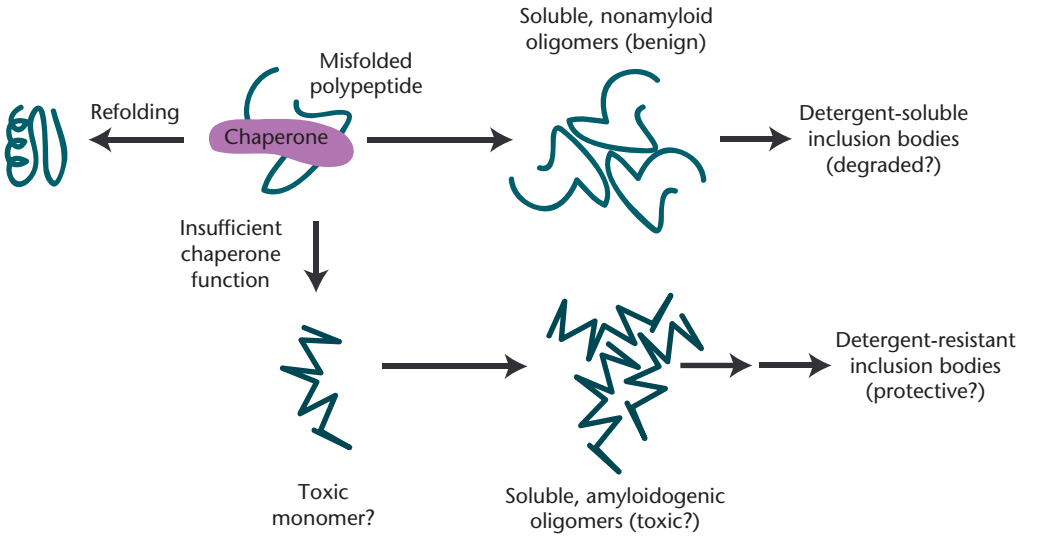

Figure 2 Molecular chaperones modulate misfolded protein conformations and degradation. Chaperones bound to misfolded polypeptides can first attempt refolding to the native state. Failing that, chaperones have the ability to modulate the conformation and subsequent oligomeric state of aggregation-prone proteins, facilitating the formation of nontoxic, benign conformations that may be subject to clearance by the UPS. In the absence of sufficient chaperone function, soluble toxic intermediates may adversely affect cell viability until ultimately sequestered in inclusion bodies.

localise to a juxtanuclear inclusion, whereas aggregation-prone disease model proteins, such as prions and HTT, localise as terminal insoluble deposits closely apposed to the vacuole (Kaganovich *et al*., 2008). This and other studies indicate that there are multiple cytosolic quality control fates for different types of conformationally challenged substrates.

Protein Degradation by the UPS

In eukaryotic cells, the primary route for protein degradation is the UPS (Ciechanover, 1998). Proteins are targeted for proteasome-mediated degradation by the covalent attachment of a polyubiquitin chain(s). Polyubiquitination of proteins is achieved by a multistep enzymatic process involving three distinct classes of proteins: the E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes (UBCs) and E3 ubiquitin ligases, which have substrate-specificity and facilitate ubiquitin transfer from E2s to target proteins. Moving up the hierarchy from ubiquitin activation to substrate attachment, there is an order of magnitude increase in the number of genes encoding the necessary enzymes. The human genome, for example, encodes eight E1s, 42 E2s and at least 600 known E3 ubiquitin ligases. Ultimately, the combinatorial possibilities of E2 and E3 enzymes allows for both incredible target diversity and specificity. Polyubiquitinated proteins are recognised and degraded by a large multisubunit protease known as the 26*S* proteasome. At the core of this complex is the 20*S* proteasome, a double-barrelled structure with protease activity localised in its central cavity. Proteolytic specificity is maintained by the presence of a 19*S* regulatory particle, or cap, on one or both ends of the 20*S* core particle. The 19*S* cap consists of a ring of si*x* AAA-ATPase subunits as well as several nonenzymatic proteins, some of which recognise and bind to polyubiquitin tags. Altogether, the components of the 19*S* cap facilitate the recognition and binding of polyubiquitinated species, and provide the energy to mediate their subsequent unfolding and translocation into the 20*S* proteolytic core for destruction. Also contributing to the overall balance of this process are deubiquitinating enzymes, the specific roles of which are becoming better understood (Reyes-Turcu *et al*., 2009). **See also**: Lysosomal Degradation of Proteins; Ubiquitin Pathway

Protein Quality Control: Recognition and Destruction of Misfolded Proteins

Molecular chaperones, together with the UPS, comprise a critical cellular surveillance system that recognises, targets and degrades misfolded proteins. The UPS is the major cellular pathway for the elimination of misfolded proteins. Accordingly, blocking proteasome function pharmacologically or genetically inhibits the clearance of misfolded proteins, eventually leading to the formation of intracellular aggregates. In fact, proteasome inhibition can also alter the localisation fate of misfolded cytosolic proteins (Kaganovich *et al*., 2008). In addition, proteasome inhibition results in the upregulation of stress-inducible molecular chaperones, presumably in an attempt to prevent the aggregation of accumulated misfolded proteins. The ultimate failure of chaperones and the UPS to effectively clear the cell of aggregation-prone species is reflected by the colocalisation of intracellular aggregates with chaperones and UPS components (Muchowski and Wacker, 2005; Tyedmers *et al*., 2010).

The regulated degradation of cellular proteins by the UPS is well understood. Proteins that must be expressed and then eliminated from the cell at precise times, such as cyclins or various transcription factors, contain inherent sequences, or 'degrons', that, in some cases upon modification and in others upon exposure, signal for their ubiquitination and degradation (Ravid and Hochstrasser, 2008). **See also**: Cell Cycle: Regulation by Cyclins; Ubiquitin Pathway

Since any protein could, in theory, become a 'misfolded protein' the cell must recognise and triage, this precludes the presence of a built-in destruction sequence in all possible proteins. Although it is well established that the yeast E2 families Ubc4/5 and Ubc6/7 are involved in the ubiquitination of misfolded proteins, how are such proteins first recognised as being misfolded? Some possible clues come from studies that identified short peptide sequences that conferred rapid proteasome-mediated degradation to normally stable proteins. Degron sequences identified using peptide-based screens in yeast include short hydrophobic stretches of amino acids (i.e. pentaleucine) as well as peptide sequences predicted to form amphipathic α-helices with exposed hydrophobic faces (as examples, PB29 and CL1, **Figure 3**; Sadis *et al*., 1995; Gilon *et al*., 1998). Intriguingly, the degradation of proteins fused to these degrons was dependent upon the Ubc4/5 and Ubc6/7 E2s, suggesting that these fusion proteins follow the ubiquitination pathway of misfolded proteins. Sequences having the properties of these degrons would most likely be found either buried in the interior of a folded protein, or could provide a surface for protein–protein interaction that occurs only when properly folded. The exposure of these sequences in the event of misfolding thus provides a signal that would otherwise be concealed. Molecular chaperones, which recognise and bind to exposed hydrophobic regions of nonnative proteins, are ideally suited to interact with these degron sequences. Recent sequence analyses of artificial degrons suggest that they represent frame-shifted sequences from existing yeast open reading frames (Metzger *et al*., 2008). Additional studies identified destabilising hydrophobic sequences that arise from reverse-translated complementary deoxyribonucleic acids or truncated coding regions, further supporting that substrates bearing 'misfolded protein degrons' can naturally occur *in vivo* (Heck *et al*., 2010; Rosenbaum *et al*., 2011; Fredrickson *et al*., 2011).

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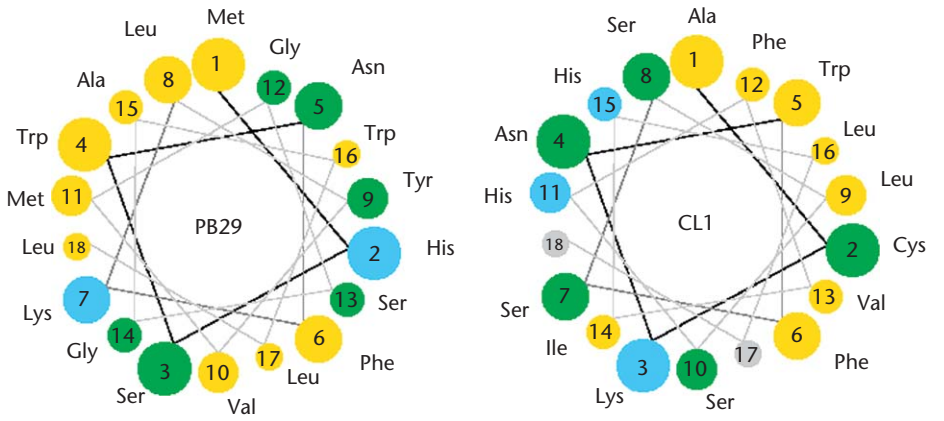

Figure 3 Artificial degron sequences projected as helical wheels show hydrophobic faces. The sequences of PB29 (MHSWNFKLYVMGSGAWLL; Sadis *et al.*, 1995) and CL1 (ACKNWFSSLSHFVIHL; Gilon *et al.*, 1998) are shown as helical wheels. Colour key: Yellow=nonpolar/hydrophobic; green=polar, uncharged; blue=basic. Helical wheels created at the following website: http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html

Chaperone-mediated Triage of Misfolded Proteins

Once misfolded proteins have been recognised, there are multiple ways for the cell to deal with these offending polypeptides (**Figure 2**). First, rescue attempts may be made in the hope of restoring misfolded conformers to a functional native state. Failing this, the cell could sequester or conformationally alter misfolded proteins, thus preventing potentially toxic activities and interactions. Finally, the misfolded species can be eliminated from the cell through proteolytic degradation. Molecular chaperones have been implicated in all three processes. Rescue attempts have been observed for Hsp70 and Hsp90, as well as the Hsp104 and Hsp26 chaperones, which seemingly cooperate to stabilise or 'hold' proteins during cellular stress and promote their renaturation once favourable conditions are restored.

Molecular chaperones also alleviate the toxicity resulting from aberrant protein conformations associated with neurodegenerative disease. In particular, Hsp70 overexpression suppresses cellular toxicity associated with the disease-causing proteins of Alzheimer disease (Aβ and TAU), Parkinson disease (alpha-synuclein), ALS (SOD1) and the numerous conditions arising from polyQ-expanded proteins. Initially, this was believed to be a consequence of chaperone-mediated prevention of inclusion body formation. However, in *Drosophila* models of Parkinson and polyQ-expansion diseases, Hsp70 overexpression suppressed toxicity and neurodegeneration without an apparent decrease in protein aggregation (Muchowski and Wacker, 2005). Subsequent research demonstrated that, *in vitro* and *in vivo*, Hsp70 and Hsp40 induce conformational rearrangements of polyQ-expanded HTT, which disfavour accumulation of specific types of soluble, fibrillar polyQ intermediates (Muchowski and Wacker, 2005). Recently, TRiC has also emerged as an important modulator of expanded polyQ toxicity. Intriguingly, numerous *in vitro* and *in vivo* experiments show, both visually and biochemically, that TRiC overexpression effectively reduces the overall number of insoluble cellular inclusions and modifies the oligomeric pathway taken by soluble polyQ monomers, ultimately resulting in reduced toxicity. A particularly interesting piece of data to come out of these studies is the observation that TRiC alone can inhibit the growth of amyloid fibrils, but is insufficient to conformationally modulate polyQ-expanded protein to more benign conformations; for successful inhibition of toxic conformers, TRiC requires the cooperation of the Hsp70/Hsp40 chaperones. Together, TRiC, Hsp70 and Hsp40 produce soluble polyQ oligomers of approximately 500 kDa, whereas reduced function of any one these chaperones results instead in alternative soluble, but amyloidogenic and presumably toxic, approximately 200 kDa species of polyQ oligomers (Behrends *et al.*, 2006). Thus, it appears that chaperones act to alleviate toxicity by modulating the conformation of potentially toxic oligomeric species, converting them instead into more benign, soluble intermediates (**Figure 2**). In addition to preventing aggregation or modulating conformations en route to aggregation, many chaperones also participate in protein disaggregation, which in some cases allows for successful protein refolding (Tyedmers *et al.*, 2010).

Ultimately, however, proteins that cannot be correctly folded must be targeted for elimination. A role for chaperones in the triage of misfolded proteins for degradation has been demonstrated in numerous studies. Hsp70 and Hsp90 are required for the degradation of CFTR and misfolded VHL, whereas the overexpression of Hsp70 and Hsp40 increases proteasome-mediated degradation of alpha-synuclein and proteins with expanded polyQ repeats (Muchowski and Wacker, 2005; McClellan *et al.*, 2005b). Whether the aforementioned effect of TRiC on

polyQ-expanded proteins extends to enhancing degradation rates is an open question. *In vitro* experiments demonstrate a requirement for Hsp70 in supporting the degradation of some misfolded proteins (Bercovich *et al*., 1997), whereas *in vivo* experiments in yeast implicate not only Hsp70, but also its Hsp40 cochaperone, Ydj1p (Lee *et al*., 1996; Park *et al*., 2007; Metzger *et al*., 2008). Recent studies also consistently reveal a role in cytosolic quality control for the Hsp70 cochaperone Sse1p, a yeast Hsp110 homologue which acts as a nucleotide-exchange factor for Hsp70 (McClellan *et al*., 2005a; Heck *et al*., 2010; Mandal *et al*., 2010; Prasad *et al*., 2010). More generally, a study examining the yeast transcriptional response to the overexpression of a cytosolic misfolded protein, the VHL mutant L158P, found that the profile of upregulated proteins mimicked that observed upon heat shock (Metzger and Michaelis, 2009). That is, cytosolic chaperone expression was induced, presumably to deal with the triage of L158P. Similar observations were made upon treating yeast with the proline analogue L-azedtidine-2-carboxylic acid (AZC), which results in a generic pool of newly synthesised misfolded proteins. Importantly, inhibition of the proteasome under such conditions resulted in decreased cell viability, suggesting that chaperone upregulation is indeed aimed at increasing proteasome-mediated degradation of misfolded proteins, as well as demonstrating the potentially toxic effects of misfolded protein accumulation in the cytosol (Metzger and Michaelis, 2009).

E3 Ubiquitin Ligases Involved in Cytosolic Quality Control

In 2001, two papers were published demonstrating the abiiity of a mammalian protein with E3 ligase activity, carboxy terminus of Hsp70 Interacting Protein (CHIP; Ballinger *et al*., 1999), to tip the balance for chaperone folding substrates from productive folding to degradation (Connell *et al*., 2001; Meacham *et al*., 2001). Intriguingly, CHIP contains chaperone-interacting TPR domains in addition to its RING finger-like U-box motif that conferes ubiquitin ligase activity. Thus, CHIP represented a viable link between chaperones attempting to fold a protein and the alternate fate of proteasome-mediated degradation. As CHIP expression was notably absent from certain tissue types and a homologue entirely missing in yeast, however, the identity (or identitites) of analogous or additional E3 ligases linked to misfolded protein degradation were not readily apparent. Recently, significant progress in the study of cytosolic quality control has been made in yeast, including the identification of multiple relevant E3 ubiquitin ligases.

The ubiquitin ligase Ubr1 is best known as a component of the N-end rule degradation pathway, in which the half-life of a protein is dependent upon the amino acid exposed at its amino terminus (Ravid and Hochstrasser, 2008). It was somewhat surprising, therefore, when several groups recently reported that Ubr1 also acts in the ubiquitination and degradation of misfolded cytosolic proteins (Eisele and Wolf, 2008; Heck *et al*., 2010; Nillegoda *et al*., 2010). *In vivo*, Ubr1-dependent degradation substrates include artificial fusion constructs containing carboxypeptidase Y (CPY, a vacuolar protease) lacking a signal sequence (Eisele and Wolf, 2008; Heck *et al*., 2010), newly synthesised protein kinases whose folding pathway has been compromised (Nillegoda *et al*., 2010), truncated cytosolic polypeptides lacking critical regions needed for correct folding (Heck *et al*., 2010), and heat-denatured fully mature proteins (Nillegoda *et al*., 2010). Further, yeast lacking Ubr1 are extremely susceptible to proteotoxic stress induced by growth in the presence of ethanol (Heck *et al*., 2010). Altogether, Ubr1 is now firmly established as an E3 ligase for cytosolic quality control in yeast. It will be interesting to see whether this is also the case in mammalian cells, a question surely under current investigation.

The E3 ligase San1 was originally identified as important for nuclear quality control (Gardner *et al*., 2005). Of course, misfolded proteins that end up in the nucleus are first synthesised in the cytoplasm, and thus should also be considered as substrates of cytosolic quality control mechanisms. Recently characterised substrates of San1 include a misfolded variant of GFP (Δ2GFP; Prasad *et al*., 2010), signal sequence-lacking mislocalised vacuolar proteases proteinase A (ΔssPrA; Prasad *et al*., 2010) or carboxypeptidase Y (ΔssCPY-GFP; Heck *et al*., 2010), as well as various unfoldable truncated or nonsensically coded polypeptides (Heck *et al*., 2010; Rosenbaum *et al*., 2011). San1 substrates accumulate in the nucleus in *san1Δ* yeast, emphasising that San1 is critical for degradation, but not for localisation/nuclear transport of its substrates (Prasad *et al*., 2010). Of note, while the nuclear accumulation of some overexpressed San1 substrates in *san1Δ* yeast is toxic, this toxicity is alleviated if nuclear localisation is prevented, indicating that it is specifically their nuclear aggregation that is toxic (Rosenbaum *et al*., 2011). This is perhaps due to the presence of other E3 ligases in the cytoplasm, such as Ubr1, that can compensate in such instances. Indeed there is evidence of overlapping function between San1 and Ubr1 (Prasad *et al*., 2010; Heck *et al*., 2010). For instance, cytoplasmically localised ΔssCPY-GFP gains dependence on Ubr1 for degradation although it normally prefers San1 (Heck *et al*., 2010). Forced localisation of San1 to the cytoplasm results in more rapid degradation of even ΔssCPY-GFP capable of nuclear localisation (Heck *et al*., 2010), indicating that it is, at least in part, the cellular location in which E3 ligases normally reside that dictates the specific population of misfolded substrates they encounter requiring ubiquitination. **See also**: Nuclear-Cytoplasmic Transport

The ER transmembrane E3 ligase DOA10 is known to participate in the ubiquitination of retranslocated misfolded ER proteins (ER-associated degradation). It is now clear that it is also required for the degradation of some misfolded cytosolic protein substrates (namely proteins

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bearing CL1 degrons; Ravid *et al.*, 2006; Metzger *et al.*, 2008).

The final recently characterised yeast E3 ligase to be discussed here is involved in the ubiquitination and degradation of proteins encoded by nonstop messenger ribonucleic acids (mRNAs). These proteins represent a class of cytosolic quality control substrates that likely have a common mechanism, namely pausing of the ribosome, to permit their recognition and subsequent degradation. The E3 ligase Ltn1 is found in association with ribosomes and can ubiquitinate stalled proteins (Bengtson and Joazeiro, 2010). That this is a physiologically relevant finding is supported by the facts that yeast lacking Ltn1 are hypersensitive to hygromycin B, which increases likelihood of stop codon readthrough (Bengtson and Joazeiro, 2010), and that mutations in mouse Ltn1 result in neurodegeneration (Chu *et al.*, 2009).

The identification of these yeast E3 ligases important for cytosolic quality control has not only increased our understanding of the mechanisms underlying this process, but made it clear that there is more than one cellular site where ubiquitination and degradation of misfolded proteins may occur. Aberrant proteins may be ubiquitinated even while still associated with ribosomes (Ltn1), in the cytoplasm following their translation (Ubr1), in localised sites of degradation at the ER membrane (DOA10), and following translocation into the nucleus (San1).

The Interface Between Chaperones and the UPS – Direct or Indirect?

If molecular chaperones are indeed able to recognise misfolded proteins and facilitate their proteasome-mediated degradation, what is the mechanism by which chaperones interface with the UPS? The simplest possibility (**Figure 4**, left branch) is that molecular chaperones, through their ability to bind and release protein substrates concomitant with cycles of ATP binding and hydrolysis, serve to maintain nonnative polypeptides in a soluble state, allowing them to successfully partition to and engage with the ubiquitination machinery in lieu of aggregating. In this 'passive' scenario, the inability of the polypeptide to fold results in multiple rounds of chaperone binding and release. Prolonged cycling of the nonnative polypeptide with the bulk cytosol serves to increase the chance of substrate recognition by an E3 ubiquitin ligase. Chaperones also appear to facilitate the transport of nucleus-bound misfolded proteins, thus promoting their colocalisation with the nuclear quality control E3 ligase San1 (Prasad *et al.*, 2010).

This model assumes, of course, that the E3 enzyme can directly recognise and interact with its misfolded substrates. Interestingly, two mammalian ubiquitin ligases, Dorfin and NEDL1, selectively ubiquitinate mutant, but not wild-type, SOD1 (Miyazaki *et al.*, 2004). Furthermore, in yeast the ER transmembrane ligase DOA10

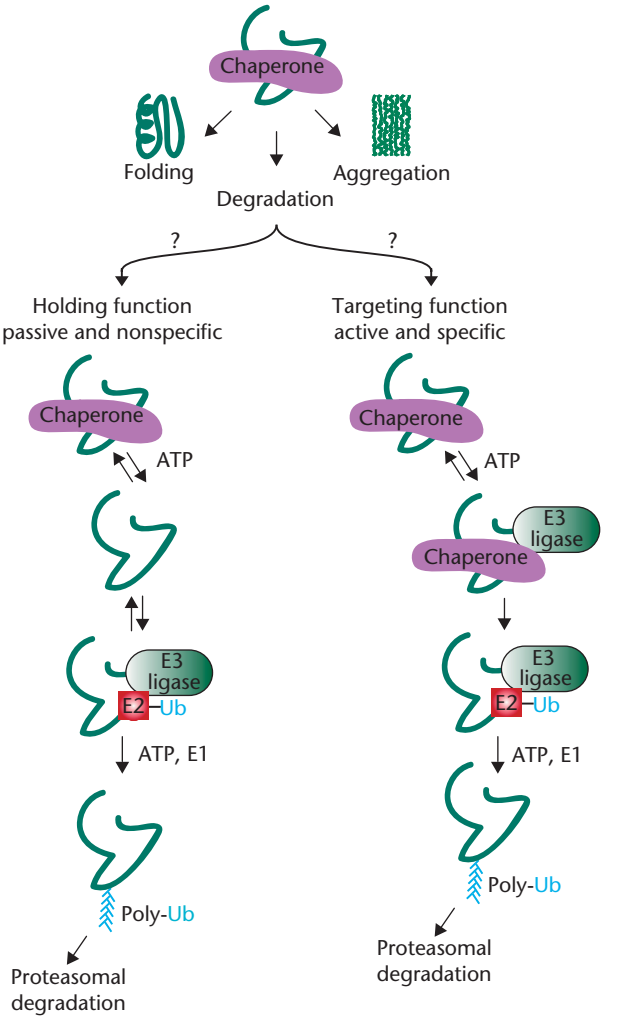

Figure 4 Models for chaperone function in protein triage. A nonnative polypeptide may either fold, aggregate or be targeted for degradation. To facilitate degradation, chaperones may act to prevent aggregation by virtue of repeated cycles of substrate binding and release. Eventually, the misfolded polypeptide is recognised by components of the UPS and committed to degradation (left branch). Alternatively, specific chaperones may interface with E3 ligases, thus actively participating in the targeting and degradation process (right branch).

differentially encourages the degradation of mutant, but not wild-type, cytoplasmic substrates (Ravid *et al.*, 2006). Another example is San1, which selectively acts only on mutant versions of its substrates (Gardner *et al.*, 2005). San1 has recently been shown to specifically recognise and bind to exposed hydrophobic substrate sequences directly via its own inherently disordered N- and C-terminal domains (Rosenbaum *et al.*, 2011; Fredrickson *et al.*, 2011). Finally, Ubr1 selectively ubiquitinates denatured, but not native, luciferase in *in vitro* assays containing E1, E2 and Ubr1, but lacking chaperones (Nillegoda *et al.*, 2010). It is important to note that another E3 was unable to ubiquitinate denatured luciferase in these experiments,

further supporting that Ubr1 can specifically recognise and interact with nonnative proteins.

As an alternative model, specific molecular chaperones may interact directly, or indirectly through an additional factor, with components of the UPS, thus effectively committing the conformational culprit to destruction by the proteasome. In this scenario, the ubiquitin ligase is recruited by the misfolded polypeptide–chaperone complex (**Figure 4**, right branch). A distinguishing feature of this model is that substrate recognition is provided by a specific chaperone or chaperones that physically interact with an E3 ligase. The only well-characterised E3 to date that fulfills this model is CHIP, which as mentioned above contains Hsp70 and Hsp90-interacting TPR repeats, as well as a U-box domain responsible for ubiquitin ligase activity. An additional physical link between chaperones and the UPS comes in the form of BAG1 and its homologues; Bcl-2-associated athanogene (BAG) domain-containing proteins act as nucleotide exchange factors for Hsp70 and, in some cases, also contain a ubiquitin-like (UBL) domain that binds to the 26*S* proteasome. CHIP, Hsp70 and another BAG family protein, BAG5, have also been linked to an E3 ligase, Parkin, whose mutation results in early onset of Parkinson disease (McClellan *et al*., 2005b). Although a direct physical link is not apparent, and although Ubr1 can selectively ubiquitinate misfolded proteins in the absence of chaperones, Hsp70 and the Hsp110 Sse1p greatly enhance its ability to do so (Nillegoda *et al*., 2010; Heck *et al*., 2010). In addition, the neuronal Hsp40-type protein HSJ1 stimulates both the ubiquitination and subsequent proteasomal localisation of Hsp70-bound proteins. Furthermore, Hsp90 itself associates with the proteasome in an ATP-dependent manner (Verma *et al*., 2000). Thus, there is extensive evidence supporting that direct interactions between specific chaperones and E3 ligases, and even with the 26*S* proteasome itself, mediate the recognition, ubiquitination and degradation of misfolded proteins. In further support of this second model, quality control analysis of misfolded VHL variants and newly synthesised kinases suggest that specific chaperones have an active role in selecting proteins for degradation (McClellan *et al*., 2005a; Mandal *et al*., 2010).

Finally, some combination of these two models is certainly possible. Perhaps specific chaperones and ubiquitin ligases can both independently recognise and directly interact with unfolded polypeptides, but direct transfer of the misfolded substrate from chaperone to ligase occurs without its release to the bulk solution.

Regardless of which model is ultimately correct, at some point an unfoldable polypeptide must be rerouted from folding attempts and instead targeted for degradation. One possibility is that chaperones that normally participate in the folding of a polypeptide also mediate its transfer to the UPS. For example, CHIP has been shown to enhance the degradation rates of Hsp70/Hsp90 folding substrates, including CFTR. Indeed, CHIP overexpression prevents folding, maturation and secretion of CFTR and instead diverts it from its chaperone-mediated folding pathway to proteasomal degradation, suggesting that E3 ligases can shift the balance from folding to degradation (Meacham *et al*., 2001). Another study addressing this possibility utilised VHL, a well-characterised folding substrate of Hsp70 and TRiC (McClellan *et al*., 2005a). Although TRiC function was not required for misfolded VHL degradation, the activity of Hsp70 was necessary. Hsp70 was not sufficient, however, as mutations inactivating Hsp90 or deleting Sse1p abrogated misfolded VHL degradation. Hsp90 does not participate in VHL folding; therefore, it seems that folding and quality control of a folding-challenged polypeptide may utilise distinct chaperone pathways, arguing for an active and specific role for chaperones in this process (**Figure 4**, right; McClellan *et al*., 2005a). For some newly synthesised kinases, the balance between their folding and degradation is tipped depending upon the presence or absence of certain chaperones; while Ydj1p participates in the early folding pathway of such kinases and thus protects against their degradation (Mandal *et al*., 2008), Sse1p instead promotes kinase degradation (Mandal *et al*., 2010). In another study the Hsp70 Ssa1p and cochaperone Ydj1p were the only chaperones required for ΔssCPY* degradation (Park *et al*., 2007). An intriguing possibility since this is a protein that would never utilise cytoplasmic chaperones for folding is that Ssa1p and Ydj1p directly transfer ΔssCPY* to the degradation machinery as an extension of their normal interaction with wild-type CPY to facilitate its post-translational translocation into the ER. Ssa1p and Ydj1p are also required for the degradation of degron-fused Ura3p (Ura3-CL1; Metzger *et al*., 2008). Altogether, the Hsp70 Ssa1p seems to be the chaperone most often required for the degradation of a wide range of cytoplasmic quality control substrates, including those destined for ubiquitination by Ubr1, San1 and DOA10 (Heck *et al*., 2010; Nillegoda *et al*., 2010; Prasad *et al*., 2010; Metzger *et al*., 2008), while Sse1p may play a key role in shifting the balance from folding to degradation pathways.

Of note, the balance between folding and degradation, or failing degradation, aggregation, can also be tipped by interfering with proteasome function or substrate ubiquitination. For instance, the rate of Ubr1-dependent substrate degradation is increased by overexpression of Ubr1 (Eisele and Wolf, 2008). For soluble folding intermediates in yeast, proteasome inhibition or deletion of relevant E2 UBCs relegated them to an insoluble, undegradable fate in a peri-vacuolar compartment instead of permitting their normal clearance from transitory cytosolic puncta or the juxtanuclear compartment (Kaganovich *et al*., 2008). On the other hand, enhancing the ubiquination potential of amyloidogenic substrates effectively relocalised them to the juxtanuclear compartment (Kaganovich *et al*., 2008), implying that enhancing UPS function overall could be useful to ameliorate diseases of protein aggregation. It will be interesting to learn and integrate the role(s) of chaperones in this locational triage into the overall picture of cytosolic quality control.

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Conclusions

Although it is clear that molecular chaperones play an important role in protein quality control in the cytosol, the precise details underlying the diversion of abnormal proteins from attempted folding to degradation remain unclear. There is evidence that some chaperones responsible for folding a protein can target it for degradation if it cannot fold; there is also evidence that chaperones that do not participate in the normal folding of a polypeptide help target it for destruction. Little to nothing is yet known about whether the numerous aggregation-prone proteins linked to neurodegenerative disease require chaperones for folding and, if so, which chaperones are involved. Addressing this deficiency has been hindered in part because, for most of those proteins, the normal cellular function is unknown, precluding the design of functional tests for folded, active protein. Determining whether chaperones normally assist the folding of the wild-type versions of proteins such as HTT will ultimately be important in elucidating the details of chaperone-mediated protein triage. Some progress to this end has been made by recent studies showing that TRiC interacts with both normal and pathogenic forms of HTT, and that TRiC, ribosomes and polyQ-expanded HTT appear to colocalise. These data suggest both an early interaction of TRiC and HTT and the possibility that TRiC may normally mediate HTT folding. This notion is further supported by the fact that polyQ repeats are capable of forming β-sheets, as TRiC is known to interact with its substrates via regions of β-strand character (Spiess *et al*., 2004).

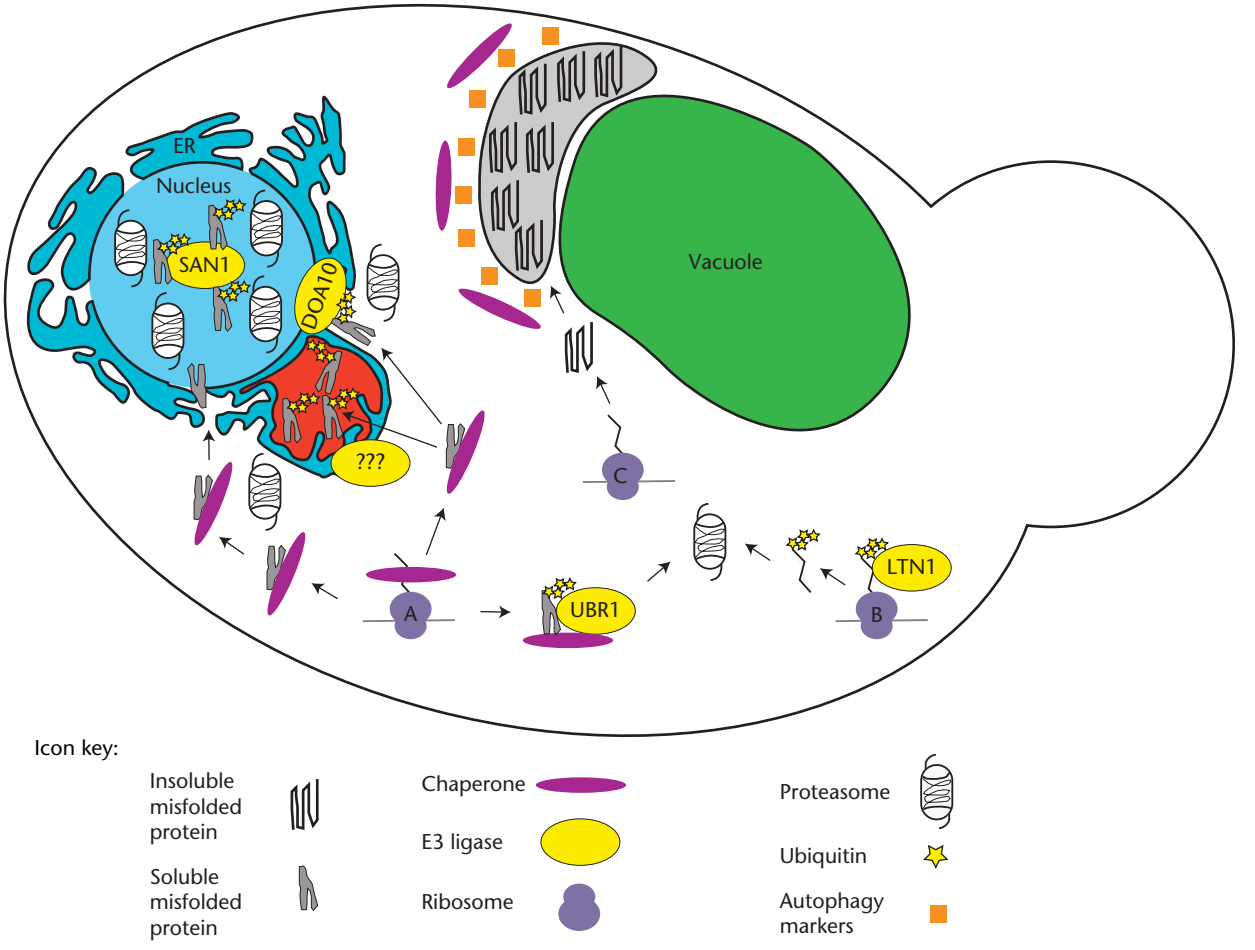

Figure 5 Schematic of cytosolic quality control pathways in yeast. Ribosome A depicts the synthesis of a soluble misfolded protein that, if it contains a nuclear localisation signal (NLS), will be targeted to the nucleus with the aid of chaperones, ubiquitinated by San1 in the nucleus, and degraded by nuclear proteasomes. If it lacks an NLS, or if its nuclear transport is otherwise compromised, it could instead be ubiquitinated, with the assistance of chaperones, by Ubr1 and degraded by cytosolic proteasomes. Alternatively, chaperones may target the soluble misfolded protein to the ER membrane for ubiquitination by DOA10 and degraded, or the protein may be ubiquitinated by an as yet unidentified E3 ligase and sequestered in a juxtanuclear ER membrane-bounded compartment (coloured in red). Whether such sequestered proteins are degraded within the compartment or must first exit is not clear at this time. Ribosome B depicts the stalled translational product of a nonstop mRNA, which is ubiquitinated by ribosome-associated Ltn1 and subsequently degraded. Ribosome C depicts the synthesis of an amyloidogenic protein fated for deposition as an insoluble aggregate in a peripheral perivacuolar compartment (colored in grey). It is unclear whether chaperones participate in delivering such proteins to this compartment, but disaggregase chaperones, such as Hsp104, colocalise with these compartments, as do protein markers for autophagy.

Also important in further elucidating cytosolic quality control will be in-depth characterisation of the interplay between the UPS and autophagic degradation pathways. Autophagy is a lysosome-mediated bulk degradation process that can remove cytoplasmic protein aggregates. Inhibition of the UPS has been shown to induce autophagy, and inhibition of autophagy contributes to neurodegeneration in mice and the accumulation of ubiquitin-positive aggregates in the brain. UPS inhibition induces the expression of p62/SQSTM1, which binds ubiquitin as well as autophagic proteins, thus providing a means to interact with ubiquitinated protein aggregates and direct them to degradation by autophagy (Bjørkøy *et al*., 2005). In the same study, reducing cellular levels of p62 increased the toxic effect of mutant HTT expression. This is likely due in part to inhibition of the UPS by increased protein aggregates not being cleared by autophagy. The interplay between these two systems becomes increasingly important with advancing age and the accompanying decline in the efficacy of cellular quality control systems. Age-dependent decreases in proteasome activity, as well as decreased chaperone induction from the HSF1-mediated pathway, together act to increase the presence of aggregation-prone misfolded proteins; these aggregation intermediates themselves further inhibit proteasome function. The use of autophagy to relieve some of the cytoplasmic aggregate burden may be enough to restore proteasome function to sufficient levels for cell viability. Of interest, the recently described insoluble protein deposits localised to the perivacuolar periphery colocalised with autophagic marker proteins (Kaganovich *et al*., 2008). It will be interesting to see further research on the possible cooperation of these two cytosolic degradation systems and whether molecular chaperones affect the degradation pathway taken by misfolded proteins.

In closing, although most research to date has focused on misfolded proteins linked to human disease, even seemingly normal proteins can have disastrous effects on the cell if cytosolic quality control is impaired. Intracellular accumulation of misfolded proteins not affiliated with disease can interfere with cell viability just as robustly as aggregation-prone proteins associated with neurodegeneration. This is the case in both the cytoplasm and nucleus (Metzger and Michaelis, 2009; Rosenbaum *et al*., 2011). Furthermore, the ability to adopt amyloidogenic structures is not restricted to disease-causing proteins, but is in fact a common feature of polypeptide chains (Stefani and Dobson, 2003). Thus, defining the detailed mechanistics of cytosolic protein quality control becomes increasingly important not only for understanding disease states linked to misfolded proteins, but how normal cellular function is maintained in the absence of evident disease risk. The plethora of recent data obtained using the yeast model system, particularly the identification of (1) numerous E3 ligases for cytosolic quality control, (2) multiple locations in which degradation may occur and (3) different sequestration locales for soluble versus insoluble protein aggregates, is summarised in **Figure 5**. Further characterisation in yeast as well as additional follow-up on these findings in mammalian cells will certainly prove useful in furthering our knowledge and understanding of cytosolic quality control systems.

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