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Coping with stress: cellular relaxation techniques

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Proteins damaged by stressors such as heat, oxidizing conditions or toxic agents are deleterious to cells and must be properly taken care of. Accordingly, misfolded proteins trigger a cellular stress response that aims to either repair defective polypeptides or eliminate faulty elements when salvage is not possible. This stress response provides time for additional stressor-specific pathways that adapt the cell to the changed environment if necessary. Recent studies have investigated how proteins that frustrate the folding machinery are recognized and cleared from the cell. Surprisingly, these clearance mechanisms are not restricted to the protein level. The stress response can also eliminate the mRNA of polypeptides that are refractory to folding.

Introduction

The induction of a stress response was first observed in the early 1960s, when the larvae of *Drosophila melanogaster* flies were accidentally kept at an elevated temperature overnight. The next day, the salivary gland chromosomes of the larvae displayed an unusual puffing pattern, indicating that the elevated temperature had caused dramatic changes in the gene expression pattern [1]. This observation led to the discovery of the heat shock proteins (HSPs), a highly versatile group of proteins that is induced whenever cells are exposed to stressors such as heat, UV, oxidizing or alkylating agents, and even upon deprivation from nutrients or oxygen [2,3].

Most HSPs induced by the stress response are chaperones that protect native proteins from damage or refold defective polypeptides in an attempt to restore their native conformation. Because repair is not always an option, ubiquitin–protein ligases act in tandem with HSPs to monitor protein folding and label terminally misfolded polypeptides with a polyubiquitin chain for proteasomal destruction. Ubiquitination entails a reaction cascade that transfers activated ubiquitin from a ubiquitin-activating enzyme (E1) to a family of ubiquitin-conjugating enzymes (E2). The E2s cooperate with the large group of ubiquitin ligases (E3) to modify target molecules covalently with ubiquitin [4]. Notably, stress induces expression of components of the ubiquitin pathway, demonstrating that protein folding and degradation are also linked at the transcriptional level.

The step that determines if a deviant protein is refolded or degraded is termed 'molecular triage' [5]. Protein folding, molecular triage and degradation are the hallmarks of protein quality control (PQC) systems, which enable cells to sustain stress by enforcing the structural integrity of the cellular proteome after environmental insults. An additional branch of the stress response attenuates the translation of housekeeping proteins. This mechanism relieves the folding machinery from expendable proteins and ensures that the PQC systems can attend to proteins damaged by stressors. This arm is further supported by a pathway which degrades the mRNAs of secretory proteins that are prone to aggregation during maturation [6].

Maintaining cellular homeostasis during stress is not the only function of PQC systems. Organelles that actively support protein maturation harbour PQC systems, which degrade polypeptides that fail to fold. The importance of this task is underscored by a study which concludes that ~30% of all newly synthesized polypeptides are eliminated by PQC systems during or shortly after their synthesis, presumably because they are defective [7].

Because this study analysed the stability of proteins in amino acid-starved cells to improve the incorporation of radiolabelled amino acids, the authors might have observed a cellular response to nutrient deprivation. During acute starvation, cells degrade proteins more readily to maintain charged pools of tRNAs that are required for protein synthesis [8]. Although another study confirmed the high degradation of nascent polypeptides in unstarved cells [9], more work must to be done to characterize the stability of nascent polypeptides conclusively.

The cytosolic stress response

A timely activation of the stress response is mandatory to ensure survival after a disturbance of cellular homeostasis. Sensors that trigger a stress response reside in the cytosol and the lumen of the endoplasmic reticulum (ER).

Sensing stress in the cytosol

The cytosolic stress sensor is the heat-shock transcription factor 1 (HSF1) [10]. In yeast, HSF1 regulates nearly 3% of the genomic loci [11]. The main targets of HSF1 are chaperones of the Hsp70 and Hsp90 family and chaperonins. In the absence of misfolded proteins, chaperones bind HSF1 to keep it inactive. When these chaperones engage in the repair of damaged proteins, HSF1 is liberated and initiates the transcription of stress-responsive genes. Originally, it was thought that intramolecular interactions between HSF1 molecules lead to the formation of a HSF1 homotrimer, which constitutes the active

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transcription factor. Since then, two additional players have been identified that are also part of the activated HSF1 complex: the translation elongation factor eEF1A and a noncoding RNA called heat-shock RNA-1 (HSR1) [12]. The translational attenuation during stress might render eEF1A vacant and thus available for interaction with HSF1 and HSR1. This mechanism might link the initiation of the stress response to the decreased protein translation observed during stress. Reduction of HSF1 activity in *Caenorhabditis elegans* accelerates tissue aging, whereas HSF1 overexpression extends the life span of the worm, suggesting that a functional stress response promotes longevity [13].

CHIP – more than a quality control ligase

In living cells, immature and ailing proteins colocalize with terminally misfolded polypeptides that must be degraded. In addition, misfolded proteins are structurally similar to folding intermediates that will mature properly. This raises the question of how quality control systems distinguish aberrant proteins from the pool of nascent specimen that will mature productively into their native fold. The ubiquitin–protein ligase carboxyl terminus of Hsp70-interacting protein (CHIP) is a key factor that controls the triage between protein folding and degradation in the cytosol of metazoans [14]. Architectural features of CHIP are a C-terminal U-box that confers the E3 activity and an N-terminal tetratricopeptide repeat domain that interacts with the C terminus of Hsp70 or Hsp90 chaperones. Thus, CHIP employs chaperones as adaptor proteins to survey the cytosol for folding-incompetent polypeptides. Whether CHIP recognizes a specific signature within its targets or if ubiquitination is a stochastic event is not understood because the link between misfolding, structural instability and degradation remains poorly characterized. A stochastic mechanism would preferentially degrade proteins that are difficult to fold because the chances of becoming ubiquitinated increase with the time spent by a substrate in an unfolded conformation, which has been observed for the chloride channel cystic fibrosis conductance regulator (CFTR). As a negative side effect of this mechanism, a fraction of folding-competent proteins will be degraded inadvertently.

CHIP is also active at the cytosolic face of the ER membrane. A mutation in CFTR prompts the ubiquitination of this plasma membrane protein by CHIP at the ER and the subsequent degradation of CFTR. To minimize promiscuous degradation of functional proteins, the destructive power of CHIP is tightly controlled. The cochaperone BCL2-associated athanogene 2 (Bag-2) binds to Hsp70 and abrogates the interaction between CHIP and its partner ubiquitin-conjugating enzyme UbcH5a. Consequently, mutant CFTR reaches the plasma membrane when Bag-2 is overexpressed [15,16]. In complex with Hsc70, the nucleotide exchange factor HspBP1 also inhibits the ligase activity of CHIP by an uncharacterized mechanism that rescues expression of a mutant form of CFTR [17].

After stress has ceased, an elegant system maintains the balance between the levels of Hsp70 and its substrates: when client proteins of Hsp70 are cleared from the cell, CHIP targets the chaperone for destruction. This mechanism reduces the cellular pool of Hsp70 in the absence of damaged proteins [18].

The stress response of the secretory pathway

In the ER, an abundance of chaperones and folding enzymes facilitate the maturation of polypeptides that enter the secretory pathway. Only those proteins that acquire their native fold are admitted to their dedicated site of action, whereas misfolded proteins and orphan subunits of oligomeric complexes are retained in the ER and ultimately degraded by cytosolic proteasomes.

Stress sensors in the ER

Misfolded proteins in the ER activate the stress sensor Ire1p, which elicits a transcriptional programme known as the unfolded protein response (UPR) [19] (Box 1). Targets of this interorganellar signal transduction pathway are factors required for the biosynthesis of phospholipids, glycosylation and protein folding, and components involved in the degradation of ER-resident proteins, totalling 5% of the yeast genome [20]. Developmental processes also depend crucially on Ire1p. Mice that cannot mount the UPR die as embryos owing to a diminished growth rate and prominent apoptosis of hepatocytes [21]. The notion that hepatocytes secrete up to 70% of all proteins synthesized explains the pivotal importance of the UPR for these cells [22]. Likewise, the UPR is essential, both for B cell development into plasma cells and for antibody secretion [23]. To avert stress, Ire1p adjusts the protein-folding capacity of the ER to the current demands. The luminal portion of Ire1p senses misfolded proteins and transmits ER stress to the nucleus via its cytosolic kinase and ribonuclease domain. In the presence of misfolded proteins, Ire1p oligomerizes and undergoes trans-autophosphorylation to activate its cytosolic endoribonuclease activity. The latter splices the precursor mRNA of the transcription factor Hac1p ['homologous to ATF/CREB1', the orthologue of the metazoan X-box-binding protein 1 (XBP1)], yielding the mRNA that encodes the potent activator of the UPR.

IRE1 prevents the synthesis of proteins refractory to folding

In yeast, *HAC1* pre-mRNA is the only identified target of Ire1p. Experiments with *Drosophila* S2 cells suggest that in metazoans, this is not the whole story [6]. Induction of the UPR in S2 cells induces the classical UPR targets. Unexpectedly, another group of mRNAs is degraded by a mechanism that depends on IRE1 but not the transcription factor XBP1. Targets are the mRNAs of certain proteins that traverse the secretory pathway without contributing to its fidelity, such as the mRNA of the matricellular glycoprotein secreted protein acidic and rich in cysteine (SPARC). The degradation mechanism involves two distinct endonucleolytic events, implying that a ribonuclease, perhaps IRE1 itself, catalyses this reaction. Thus, IRE1 can trigger the degradation of several mRNAs, possibly via its endogenous endoribonuclease activity. How can IRE1 recognize its target mRNAs? The degraded mRNA must encode an ER signal sequence because removal of this sequence abolishes the IRE1-dependent degradation of an mRNA reporter construct. Introduction of frame shifts

Box 1. Signalling pathways from the ER

The ATF6 pathway

In unstressed cells, immunoglobulin heavy chain-binding protein (BiP) masks two Golgi-localization sequences within the ATF6 protein (Figure Ia). In the presence of misfolded proteins (dark blue), BiP prefers unfolded substrates to ATF6, and releases the transcription factor precursor. Guided by its Golgi-localization sequence, ATF6 reaches the Golgi, where two proteases, S1P and S2P, sequentially cleave ATF6 to generate a luminal fragment and a cytosolic p90 fragment that constitutes the active form of the transcription factor. Active ATF6 (p90) is translocated to the nucleus, where it promotes the transcription of ER proteins that harbour an ERSE in their promoter region. ERSE elements are typically found in the promoter region of proteins that help to restore ER homeostasis. The active form of ATF6 is derived from an existing precursor protein, whereas XBP1 is generated more slowly because it requires translation. This difference enables a biphasic induction of the UPR. Initially, ATF6 induces chaperones to enhance the folding capacity of the ER. Persistence of misfolded proteins activates XBP1, which, in addition to promoting the ER folding machinery, unleashes a pathway that eliminates misfolded proteins from the ER [64]. Failure of the stress response to restore homeostasis leads ultimately to apoptosis [19].

The PERK pathway

When ER stress is low, PERK is in complex with BiP (Figure Ib). During stress, BiP engages in protein folding, which enables dimerization, phosphorylation and, thus, activation of PERK. Activated PERK phosphorylates eIF2α, which blocks recruitment of the charged initiator tRNA to the 40S ribosomal subunit and attenuates protein translation.

The XBP1 pathway

IRE1 interacts directly with misfolded polypeptides in the ER (Figure Ic). Recognition of a polypeptide chain by multiple IRE1 proteins might facilitate their oligomerization and activation. In metazoans, activated IRE1 processes the precursor mRNA of the transcription factor XBP1. The processed mRNA is translated into the active transcription factor XBP1, which enhances the transcription of genes with ER stress response element (ERSE) and UPR elements in their promoter region. UPR elements are found in the promoter region of proteins that are required for the turnover of proteins from the ER. IRE1 is depicted at the inner nuclear envelope but it might also reside at the ER membrane.

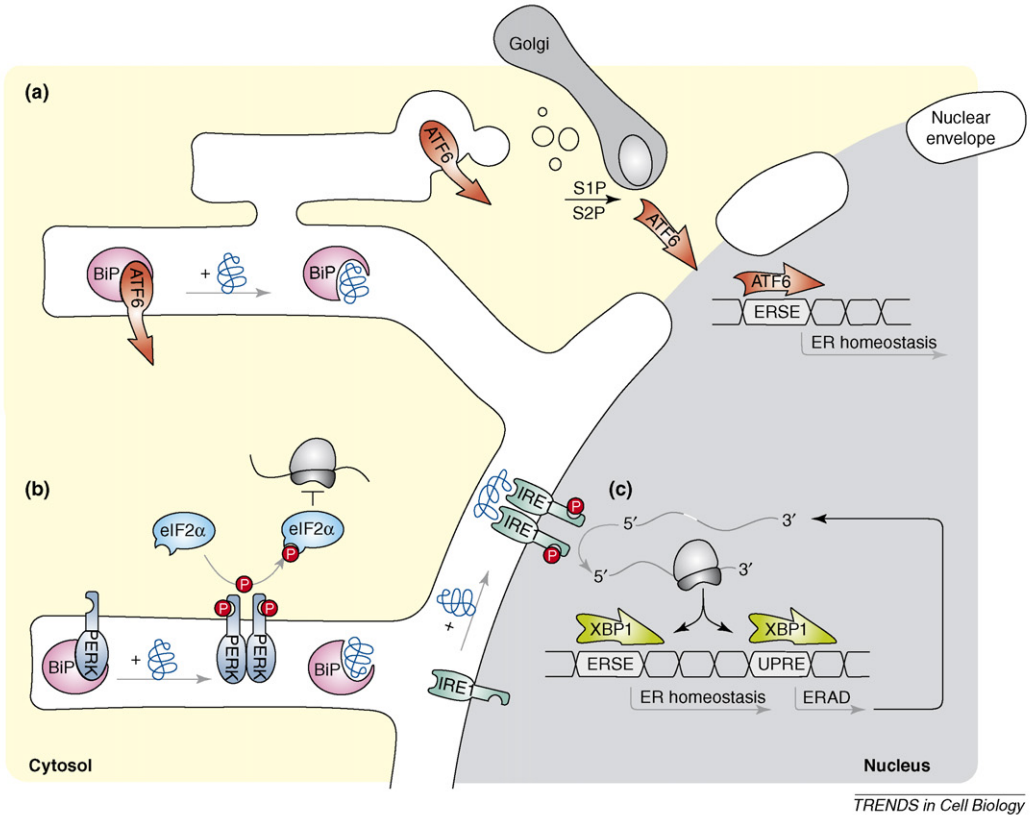

Figure I.

stabilizes an otherwise unstable message, suggesting that translation of the correct reading frame is also pivotal for mRNA silencing by IRE1. Therefore, unwanted polypeptides must be detected in the lumen of the ER. Based on its crystal structure, Ire1p itself might recognize targets of this pathway: Ire1p features a deep hydrophobic groove akin to the peptide-binding cleft of class I MHC molecules [24]. Mutations in the conserved amino acids that line this groove significantly impair Ire1p function. Therefore, Ire1p can bind misfolded proteins directly via its binding cleft and position its cytosolic ribonuclease domain in proximity to the ribosome that translates the corresponding mRNA to cleave the undesired message (Figure 1a). Because proteins recognized by IRE1 are likely to be refractory to folding, degradation of their mRNA should alleviate ER stress.

Cleaning the translocation pore

The degradation of mRNAs that encode secretory proteins might produce translocation-stalled polypeptides, if the

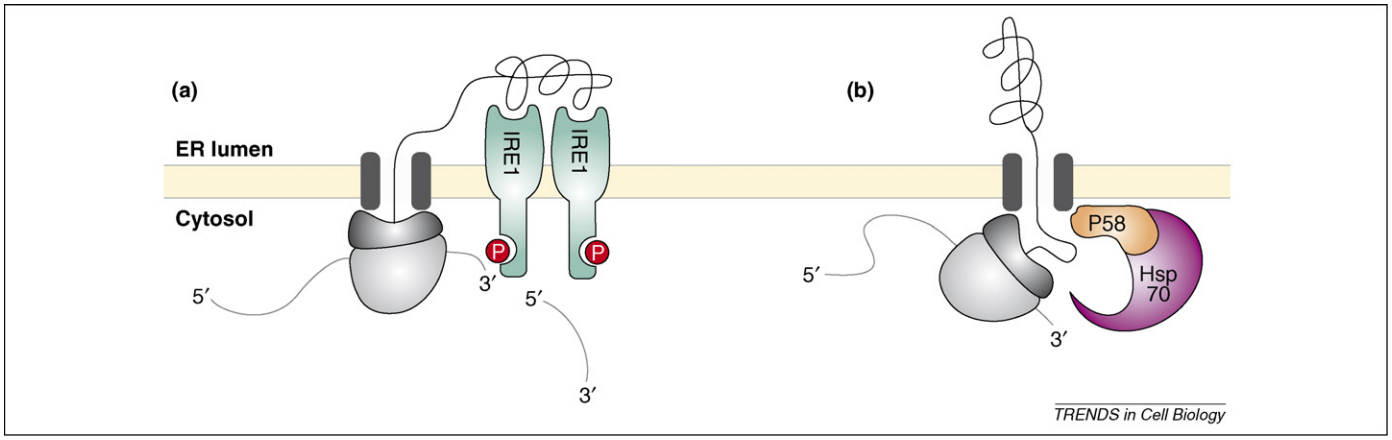

Figure 1. Terminating the production of proteins that challenge the ER. **(a)** Decay of mRNAs encoding secretory proteins. Targeted by its signal sequence, a polypeptide is co-translationally translocated into the lumen of the ER. Polypeptides with exposed hydrophobic patches, either because of limited luminal chaperone levels or because the polypeptide fails to fold, are recognized by IRE1. Although direct cleavage of mRNAs by IRE1p has not been demonstrated, binding of IRE1 to the polypeptide might position the ribonuclease domain of IRE1 in proximity to the translated mRNA and enable its cleavage. **(b)** Cotranslocational degradation of secretory proteins. A translocation stall destabilizes the junction between the ribosome and the translocon. P58 recognizes the arrested polypeptide chain and recruits Hsp70 to the translocon. Promoted by P58, Hsp70 extracts the arrested polypeptide from the translocon.

mRNA is destroyed before translation is completed. This requires a mechanism that removes arrested polypeptides from the translocation pore. Based on studies involving the mammalian apolipoprotein 100 (apoB100), a pathway has evolved that serves this purpose. apoB100 is the major structural component of lipoprotein particles [25]. To complete its synthesis, apoB100 must receive a lipid modification. When lipidation is blocked, an unusual pathway destroys apoB100. Supported by cytosolic chaperones of the Hsp70 and Hsp90 family, the proteasome degrades apoB100 cotranslocationally. Additionally, the UPR target $P58^{IPK}$ has a crucial role in this process. $P58^{IPK}$ is known to inhibit eukaryotic translation initiation factor 2α (eIF2α) kinases such as protein kinase R-like ER-localized eIF2α kinase (PERK), and can restore protein translation after ER stress has subsided [26]. Furthermore, $P58^{IPK}$ has a DnaJ domain, suggesting an additional role as a cochaperone. $P58^{IPK}$ is peripherally associated with the translocation machinery of the ER, where it can be crosslinked to apoB100 [27]. The crosslink is particularly efficient when lipidation is blocked; suggesting that destabilization of apoB100 facilitates its association with $P58^{IPK}$. In addition, overexpression of $P58^{IPK}$ enhances cotranslational degradation of apoB100. Both observations also apply to other secretory proteins. How does $P58^{IPK}$ gain access to arrested translocation substrates? If the luminal processing of a nascent polypeptide is delayed, continued protein synthesis might disrupt the junction between the ribosome and the Sec61 channel that translocates polypeptides into the ER lumen. Promoted by $P58^{IPK}$, Hsp70 chaperones could bind the arrested polypeptide and extract it by a mechanism that resembles protein import into the ER (Figure 1b). Likewise, a translation arrest might also destabilize the seal, render the stalled polypeptide accessible for $P58^{IPK}$ and initiate degradation of the stalled polypeptide.

Quality control in the ER

A PQC system in the ER redirects polypeptides that fail to fold or assemble properly to the cytosol for degradation by the proteasome by a process that is termed ER-associated degradation (ERAD) [28]. Secretory proteins can have defects in their luminal, transmembrane or cytosolic domain. Depending on the position of the lesion, different branches of the ER quality control system can be defined which ultimately all converge at the proteasome [29,30].

The Doa10p pathway

The Doa10p pathway is also designated ERAD-cytosolic (ERAD-C) because all characterized substrates of this pathway carry lesions in their cytosolic domains [30]. The defining member in yeast is the ubiquitin–protein ligase Doa10p, a multispanning transmembrane protein equipped with a cytosolically exposed 'really interesting new gene' (RING) domain. A candidate for the mammalian orthologue of Doa10p is the ubiquitin–protein ligase Teb4 [31,32]. Doa10p teams up with the E2s Ubc6p and Ubc7p to ubiquitinate its substrates. Certain cytosolic and nuclear polypeptides are also ubiquitinated by Doa10p [33,34]. ERAD-C dominates over other ERAD pathways and degrades substrates with multiple lesions in their luminal and cytosolic domains [30].

The HRD pathway

The second branch of the ER quality control system targets proteins with defective luminal domains, and is designated ERAD-L. Central to ERAD-L is the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase degradation (HRD) ligase with its two core subunits Hrd1p and Hrd3p (Figure 2) [35,36]. The membrane protein Hrd1p ubiquitinates misfolded proteins through its cytosolic RING domain, and Hrd3p is a type I transmembrane protein equipped with a large luminal domain. The partner E2s of the HRD complex are the cytosolic Ubc1p and Ubc7p. The latter is recruited to the ER membrane by the adaptor protein Cue1p [37]. Ultimately, all ERAD-L substrates arrive at the HRD complex – but how are these substrates recognized and conveyed to the ligase? The prototypical ERAD-L substrate in yeast is a mutant form (CPY*) of carboxypeptidase Y (CPY). The ER resident Hsp70 protein

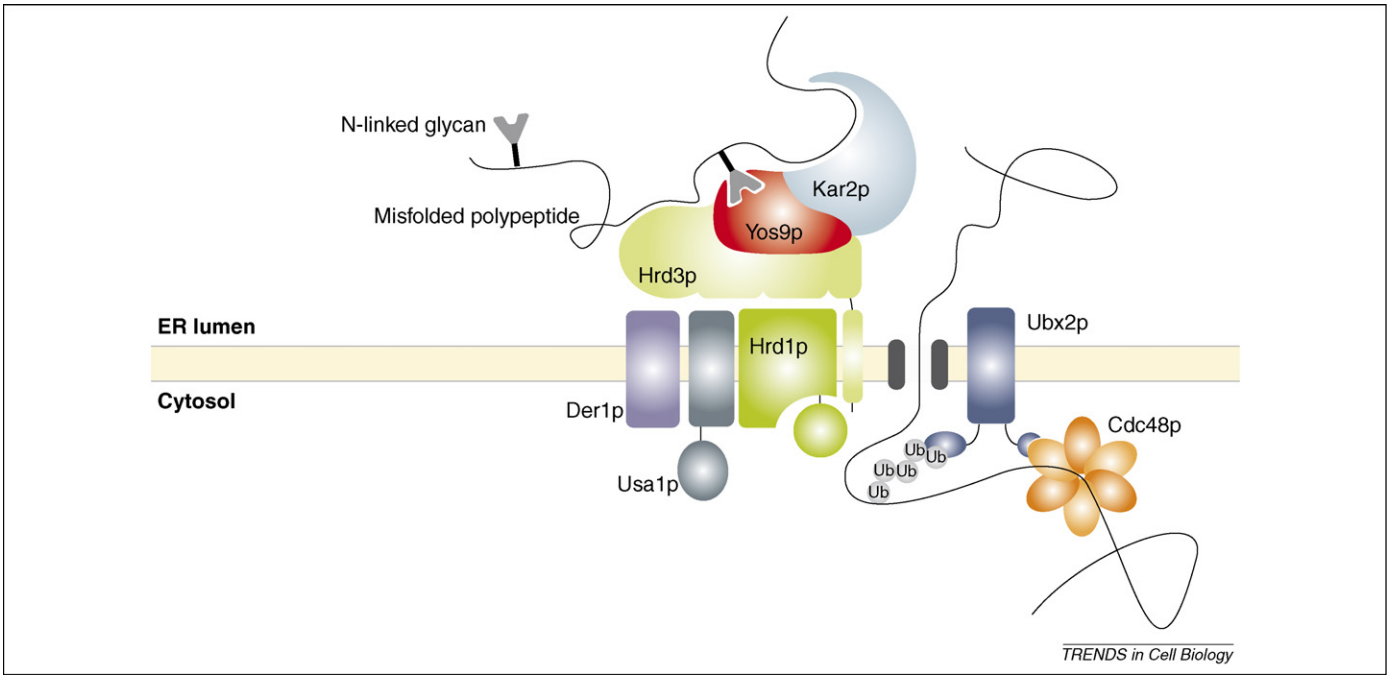

Figure 2. The core complex of the HRD ligase is composed of Hrd3p and Hrd1p. Hrd3p interacts with Yos9p and Kar2p to recruit misfolded glycoproteins to the ligase. All three proteins can bind misfolded proteins, yet their individual contributions to the process are not resolved. Der1p and Usa1p are required for ERAD-L [29]. Usa1p recruits Der1p to the ligase. The function of Der1p is not known but for both Der1p and Usa1p, it was proposed that they might constitute subunits of the export channel [29]. Hrd1p modifies partially translocated polypeptides with ubiquitin. Ubiquitinated substrates prompt Ubx2p to recruit Cdc48p to the ligase. Cdc48p mobilizes misfolded proteins from the membrane for degradation by the proteasome.

Kar2p partakes in the turnover of this soluble glycoprotein, suggesting a recognition mode that relies on hydrophobic patches [38,39]. The finding that ER mannosidase I is also required to degrade CPY* and other misfolded glycoproteins adds an additional layer of complexity to this process [40,41]. The mannose timer model explains this notion by proposing that Man_9 carbohydrates protect immature proteins from degradation. The trimming of Man_9 residues by ER mannosidase I yields Man_8 oligosaccharides and terminates the folding phase granted to maturing glycoproteins. The slow kinetics of ER mannosidase I could thus provide a time window for protein maturation. Because ER mannosidase I trims misfolded and properly folded glycoproteins equally effectively [42], a Man_8 moiety alone does not suffice to signal that a glycoprotein is terminally misfolded. In addition, a signature indicative of misfolding, perhaps hydrophobic patches, must be present on the glycoprotein to trigger degradation.

A key component of ERAD-L is the ER lectin Yos9p. It features a mannose receptor homology (MRH) domain that recognizes Man_8 bearing CPY*. Mutations in the sugar-binding site abrogate turnover of CPY*, although the association between Yos9p and CPY* persists, even when the substrate is not glycosylated [43,44]. The notion that the ER-resident member of the Hsp70 family Kar2p interacts with Yos9p [45] affords a possible clue for these seemingly contradictory observations: Kar2p might relay client proteins to Yos9p, or, alternatively, the chaperone might mediate an interaction between Yos9p and unglycosylated substrates. Because Yos9p and Hrd3p are organized in the same complex, Yos9p might select the substrates that are ubiquitinated by the HRD ligase [29,45,46].

This view is challenged by the finding that Hrd3p also binds CPY* but fails to degrade this substrate when Yos9p is absent [45,46]. Therefore, Yos9p might act as a gatekeeper that admits Man_8 proteins for degradation after their selection by Hrd3p. In this alternative model, Hrd3p binds potential substrates independently of their glycosylation status, yet it appears that hydrophobic polypeptides are preferentially selected at this stage [46]. Next, Yos9p tries to detect the presence of a Man_8 glycan on the bound substrate. Only the dual recognition of a Man_8-bearing glycoprotein by Hrd3p and Yos9p triggers its degradation.

Overexpression of Hrd1p in the absence of Yos9p and Hrd3p results in the promiscuous degradation of ER-resident proteins [45,47], confirming that Hrd3p and its associated factors ensure that only misfolded polypeptides are ubiquitinated by the ligase. How these misfolded proteins are delivered to the HRD complex is not clear. Yos9p and Kar2p might first recruit substrates to Hrd3p, or Hrd3p itself might sample the lumen of the ER for hydrophobic polypeptides. Because these mechanisms are not mutually exclusive, both pathways might exist to enable the recognition of a more diverse array of substrates.

Membrane extraction and destruction

Once admitted for degradation, a so-far-unidentified conduit exports substrates to the cytosol. Because the HRD ligase processes substrates on both sides of the membrane, the channel should be in close proximity to this complex. After ubiquitination of cytosolically exposed domains, Ubx2p recruits the ATPases associated with a variety of cellular activities (AAA) Cdc48p [p97 or valosin-containing protein (VCP) in mammals] and its cofactors Ufd1p and Npl4p to the ligase [48,49], where Cdc48p

releases the substrates from the membrane [50–54]. Cdc48p is composed of identical subunits assembled in a hexameric ring. Each subunit comprises an N-terminal domain that mediates interactions with ancillary factors, followed by two AAA domains that couple ATP hydrolysis to major conformational changes within the protein. Certain AAA ATPases, such as ClpB, translocate their substrates through the central pore of the ring to facilitate unfolding [55]. The crystal structure of the *Mus musculus* p97 suggests that a guanidyl-rich denaturation collar at the base of the ring denatures the substrates of p97 [56]. After Cdc48p has mobilized the polypeptide from the membrane, the ubiquitin-receptor proteins Rad23p and Dsk2p bind the polyubiquitinated substrates and escort them to the proteasome for destruction [57].

Conclusions

In addition to the described pathways, elements of a nuclear PQC system are emerging in yeast. Central to this system is the U-box containing ubiquitin–protein ligase San1p [58]. Currently, little is known about the substrate specificity of this pathway but lack of San1p manifests a stress response, implying a broader role in the recognition of aberrant nuclear polypeptides. So far, associated chaperones have not been identified, suggesting that San1p clients are degraded without an attempt at repair. Mitochondria also respond to stress. Expression of a mutant mitochondrial matrix protein induces mitochondrial stress proteins, mediated by the transcription factor C/EBP homologous protein 10 (CHOP), which also has a role in the UPR programmed cell death [59,60].

Cytosolic and ER-resident PQC systems cooperate to degrade certain substrates. The E3 protein-ligase RMA1 ('RING finger protein with a membrane anchor') is anchored in the ER membrane, where it supervises the folding of CFTR together with the cytosolic E3 CHIP [61]. Truncated versions of CFTR are recognized differently by CHIP and RMA1: CHIP targets predominantly the full-length protein, whereas RMA1 triages full-length and C-terminally truncated versions of CFTR. This indicates that RMA1 and CHIP act sequentially at two distinct checkpoints. RMA1 inspects N-terminal regions of CFTR cotranslationally, whereas CHIP targets preferentially full-length CFTR, demonstrating that these seemingly separate quality control pathways are linked.

Even in a perfect environment, cells are always exposed to a certain number of stressors. Consequently, basal levels of HSPs ensure that damaged proteins are either properly repaired or degraded. What causes protein damage in the absence of environmental stressors? With the evolution of aerobic metabolism, cells constantly produce reactive oxygen species, which are deleterious to almost all cellular molecules. Oxidative damage of proteins by free radicals is therefore an unavoidable by-product of the aerobic lifestyle. Moreover, as mentioned earlier, the largest source of misfolded polypeptides might be protein biogenesis itself.

Is stress only a threat to life? In flies and plants, it was shown that although stress is detrimental to the individual, it could be beneficial for the species [62,63]. Among many other targets, Hsp90 folds factors that orchestrate development. Minor genetic changes in these factors would lead to new morphological phenotypes, yet these mutations are buffered by Hsp90, which preserves the conformation of these mutated proteins, and thus their function. Therefore, Hsp90 is thought to serve as a 'capacitor' that compensates random genetic mutations. During stress, chaperones, including Hsp90, are occupied with the repair of damaged proteins, and the accrued genetic variations become effective. Although the changes that appear during stress are mostly deforming, certain variations can be advantageous. Surprisingly, once acquired, the morphological alterations persist in subsequent generations, even in the absence of stress. Thus, stress might aid evolution.

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