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Protein quality control in the endoplasmic reticulum

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Abstract

The topological barriers defined by biological membranes are not impermeable: from small solutes to intact proteins, specialized transport and translocation mechanisms adjust to the cell's needs. Here, we review the removal of unwanted proteins from the endoplasmic reticulum (ER) and emphasize the need to extend observations from tissue culture models and simple eukaryotes to studies in whole animals. The variation in protein production and composition that characterizes different cell types and tissues requires tailor-made solutions to exert proper control over both protein synthesis and breakdown. The ER is an organelle essential to achieve and maintain such homeostasis.

Introduction

Ever since its discovery as the site where secretory and membrane proteins start the journey to their final destination, the ER has attracted the attention of cell biologists and biochemists alike. Studded with ribosomes, the ER requires complex machinery to ensure proper docking of ribosomes, translocation of nascent polypeptide chains, and a suite of enzymes responsible for the introduction of the necessary co- and post-translational modifications. Nascent protein chains must fold as they roll off the ribosome, both in the cytosol and for new arrivals in the lumen of the ER. Given the enormous diversity of amino acid sequences and the protein folds they specify, a diverse set of chaperones and accessory proteins support these efforts. However, mistakes are unavoidable, and the resulting misfits must be removed to avoid the futile engagement of chaperones better deployed elsewhere or to prevent the assembly of defective protein complexes. Many of the individual steps that together comprise protein quality control in the ER have been expertly (and frequently) reviewed [1-4]. Here, we provide a more integrated view and also discuss what I consider to be the next frontier: *in vivo* models based on our current *in vitro* models of how cells control the quality of proteins that enter the ER.

Failure is an option

In a typical eukaryotic cell, polypeptide chain elongation proceeds at a rate of 4 to 10 amino acids per second. Misacylation of transfer RNAs (tRNAs) results in the incorporation of the "wrong" amino acid, and even properly acylated tRNAs may misread their appropriate codon, with a similarly disastrous outcome. Inappropriate termination yields partial products, the stability of which is usually compromised. These types of mistakes apply equally to proteins made in the cytosol and to proteins destined for entry into the ER. These are not the only types of mishaps that can befall a nascent chain: a failure to engage the necessary co-factors that participate in giving a protein its final shape may propel an otherwise error-free nascent chain onto a non-productive folding pathway, from which escape may not be possible. The rate at which misfolded proteins arise is, therefore, carefully matched with the degradative capacity of the cell, as is evident from the relative abundance of the cytosolic proteasomes, the main protease complex responsible for ATP-dependent proteolysis [5].

The chemical environment in which protein folding occurs and where mistakes in protein structure must be diagnosed and dealt with is very different for the cytosol

and for the ER. The lumen of the ER is a calcium-rich environment, whereas the cytosol is not: consequently, calcium-binding chaperones, such as calnexin and calreticulin participate in folding reactions in the ER. Many proteins that function in extracellular space are stabilized by disulfide bonds, the introduction of which occurs in the oxidizing environment of the ER lumen. Not surprisingly, disulfide bonds in cytosolic proteins are exceedingly rare.

The error rate in protein synthesis remains a controversial issue. Depending on the methodology used, defective ribosomal products may amount to a sizable fraction of newly synthesized material or represent only a small percentage of the primary translation product. Without taking sides in this somewhat contentious debate [6,7], we wish to point out that the number of mistakes probably, by far, exceed the error rate in DNA replication. The many sophisticated mechanisms available to repair DNA damage ensure fidelity of transmission of genetic information, but errors in protein synthesis are usually dealt with by complete destruction of the faulty product.

Proteolysis requires compartmentalization

Protein breakdown serves a number of important functions. Complete hydrolysis of peptide bonds yields free amino acids for re-use as building blocks in protein synthesis. Proteolysis likewise serves to remove proteins no longer wanted or no longer functional because of the damage they sustained, either in the course of translation or upon completion of their useful lifespan. Protease activity must be compartmentalized to avoid uncontrolled attack on otherwise perfectly functional proteins. Moreover, nascent chains that have yet to assume their final folded structure are more susceptible to proteolysis than their complete and fully folded counterparts, one more reason for compartmentalization. In the case of cytosolic proteolysis, the bulk of protein degradation is performed at the hands of the ubiquitin-proteasome system [5]. The architecture of the 26S proteasome is such that access to the chamber where peptide bond hydrolysis occurs is tightly regulated, requiring ubiquitylation and the concerted action of AAA-ATPases that are part of the proteasomal cap complex to gain entry. Proteolysis in the topological equivalent of extracellular space is confined mostly to the endolysosomal system. Nonetheless, the chaperone-dependent import of cytosolic proteins destined for lysosomal degradation has been reported [8]. Likewise, the autophagy pathways sequester not only damaged organelles, such as mitochondria, but also soluble cytoplasmic constituents for delivery to the lysosomal compartment by a process of fusion and dissolution of internal membranes [9]. The ER presents a special case, as—with the exception of mitochondria—it is the only intracellular membrane-delimited compartment highly active in protein folding. Although the ER was originally believed to be a possible site of the proteolytic removal of misfolded glycoproteins [10], the prevailing view now centers on the physical removal of such substrates from the ER, followed by the delivery to the cytosol for proteasomal degradation [1–4].

How to recognize a misfolded protein

Regardless of whether the nascent chain folds in the cytosol or in the lumen of the ER, how the cell distinguishes a terminally misfolded state from a conformation with remaining options for corrective action is not clear. For obvious reasons, analysis of misfolded proteins precludes the use of the standard highly resolving methods, such as X-ray crystallography or nuclear magnetic resonance and instead relies on rather crude parameters, such as differential susceptibility to proteases, differences in aggregation state, the occlusion or display of antigenic determinants unique to the folded protein or its misfolded counterpart, or even a reduction in protein half-life as an inferred trait characteristic of a misfolded protein. For ER-lumenal proteins, the calnexin/calreticulin cycle employs an ER-resident glucosidase and (uridine 5'-diphospho-glucose-4-epimerase) [UDP]-glucose glycoprotein glucosyltransferase. The latter preferentially re-glucosylates proteins that conform to a molten globule state, leading to a prolonged interaction with calnexin/calreticulin, assisted by oxidoreductases, such as ERp57 to enable additional attempts at reaching a fully native fold [11]. For proteins that span the membrane, defects or possible points of recognition as misfolded can arise in each of the compartments with which they interact: the cytoplasmic aspect, as is the case for the ΔF508 mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) chloride conductance regulator, in the transmembrane segment(s) (for example, by failing to associate with appropriate partner subunits, as is the case for isolated subunits of the T-cell receptor for antigens, and in the luminal domains). Yos9p (yeast) and OS9 (mammalian cells) are lectin-like molecules that also participate in the recognition of substrates to be targeted for degradation [1-4].

The unfolded protein response

The unfolded protein response (UPR) is a stereotypical set of transcriptional events that follow imposition of acute ER stress [12]. Exposure of cells to stressors, such as tunicamycin, a drug that blocks GlcNAc-1-phosphotransferase, the first enzyme in the synthesis of the lipid-linked N-linked glycan precursor, is a potent inducer of the UPR. Often overlooked is the fact that such treatment not only abolishes N-linked glycosylation, held responsible for the accumulation of proteins that misfold because of the

elimination of glycans at their usual positions but also affects the level of the isoprenoid alcohol, dolichol, that may affect membrane structure and function. Given the abundance of calcium in the ER and the important role of calnexin/calreticulin in glycoprotein folding, perturbation of calcium homeostasis, as produced by the calcium pump inhibitor thapsigargin, would be predicted to adversely affect the folding of numerous glycoproteins and hence the induction of the UPR. Finally, preventing the formation of disulfide bonds by the exposure of cells to the reducing agent dithiothreitol (DTT) would be predicted to have similar effects and indeed induces the UPR. Transcriptional control of the UPR is well understood and involves the kinase/RNAse Ire1, the transcription factor precursor ATF6, and the kinase proline-rich receptor-like protein kinase (PERK1; for review, see [12]), all of which—directly or indirectly—cause induction and accumulation of proteins held capable of matching the folding and degradative capacity of the ER with the amount of misfolded protein accumulated. The kinds of physiological stressors that would similarly cause induction of the UPR remain to be identified, and there are relatively few examples of a protein deliberately designed to misfold inducing a robust UPR. The action of Ire1 induces the formation of XBP1s, the unconventionally spliced form of the transcription factor XBP1. Equally important is the contribution of the Ire1-dependent decay of particular messenger RNAs (mRNAs) and not just the *de novo* induction of transcription of key UPR targets, to ER homeostasis. Strong inducers of XBP1s are polyclonal B cell activators that propel virgin B cells to become immunoglobulin-secreting cells [13]. Of note, the appearance of XBP1s precedes the onset of the massively increased rates of immunoglobulin secretion that characterize this transition [14], and thus this response is anticipatory rather than reactive, and might as well be an example of developmental regulation rather than damage control. Regardless of the distinctions between cause and consequence, many of the proteins implicated in the quality control of glycoproteins in the ER are upregulated when a UPR is induced, but it is by no means certain that their function is dedicated solely to protein quality control. Conversely, ablation of these quality control components may induce a UPR even in the absence of conventional UPR inducers such as tunicamycin, thapsigargin, or DTT.

Dislocation

Much of what we know about the quality control in the ER relies on the use of proteins that are misfolded because of mutations in their coding sequence. In yeast, a mutant version of carboxypeptidase Y, (CPY), has been a popular workhorse. The pioneering work of Wolf and colleagues [15] and Sommer and Jentsch [16] called attention to a pathway, counterintuitive at first, that invoked the extraction of a misfolded protein from the ER, its translocation across the ER membrane—a key step now commonly referred to as dislocation or retrotranslocation—followed by proteasomal proteolysis. Hampton and Rine [17] have provided a detailed picture of the behavior of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the analysis of which led to the discovery of Hrd1-Hrd3 in yeast. The removal of membrane proteins from the ER poses yet another conundrum: if a polypeptide is extracted with its hydrophobic transmembrane segment(s) intact for transfer to the proteasome, how are these membrane-anchoring segments shielded from aqueous solvent? The channel via which dislocation occurs remains a topic of interest, and perhaps more than one solution evolved to deal with the delivery of proteins from the ER to the cytosol, as discussed below.

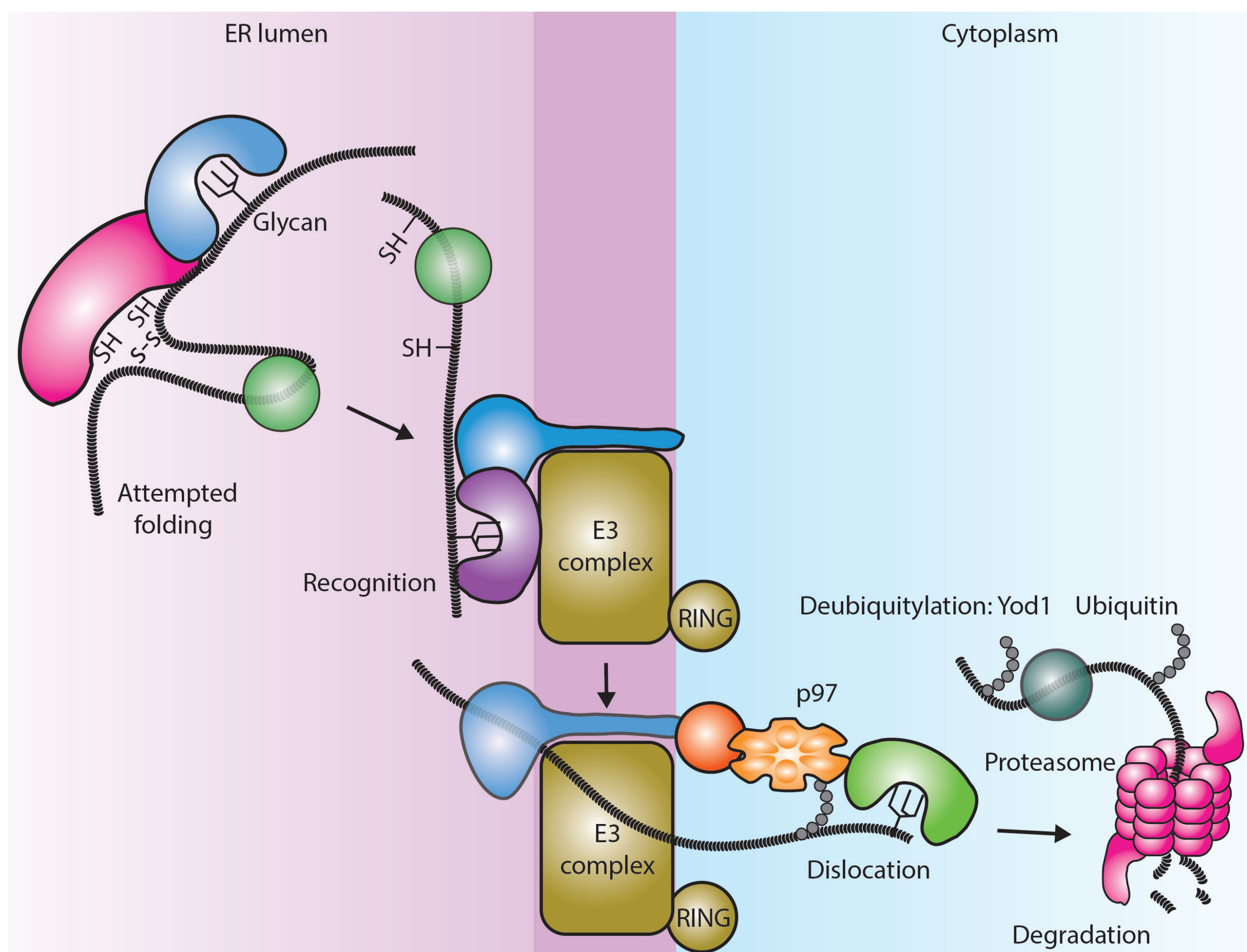
Model substrates and cell lines

Biochemical analysis of dislocation and associated events in mammalian cells has relied extensively on a few model substrates, considered to be misfolded: the cystic fibrosis-associated mutant version of the CFTR (ΔF508), variants of α1-antitrypsin, unpaired T-cell receptor subunits, variants of the asialoglycoprotein receptor, or the enzyme tumor necrosis factor-α-converting enzyme (TACE). Certain herpesvirus-encoded proteins such as the human cytomegalovirus (HCMV)-encoded US2 and US11 proteins apparently target properly folded class I major histocompatibility complex (MHC) products by exploiting the pathways that otherwise target misfolded proteins [1-4]. Although many of these studies exploit permanently established cell lines that express the degradation substrate(s) of interest, introduction of candidate substrates by transient transfection is commonly used as well. Based on a limited number of substrates, usually expressed and analyzed in easily manipulated cell lines in tissue culture, broad generalizations have come to dominate the field. Figures 1 and 2 provide a simplified schematic of some of the components for which there is a consensus as to their involvement in ER quality control. The often intimate connections between mitochondria and the ER [18] immediately suggest the possibility that transfer of proteins at these points of contact is also a point of quality control. Important areas that warrant further investigation include identification of natural, endogenous substrates of the degradation pathway [19] and events in the nuclear envelope, contiguous with the ER: we know little about how the cell manages selective protein turnover in the inner nuclear membrane and at the nuclear pore and the outer nuclear membrane.

The machinery

As far as degradation of ER-resident misfits is concerned, we can distinguish three broad classes of events: (a) recognition and engagement of degradation substrates;

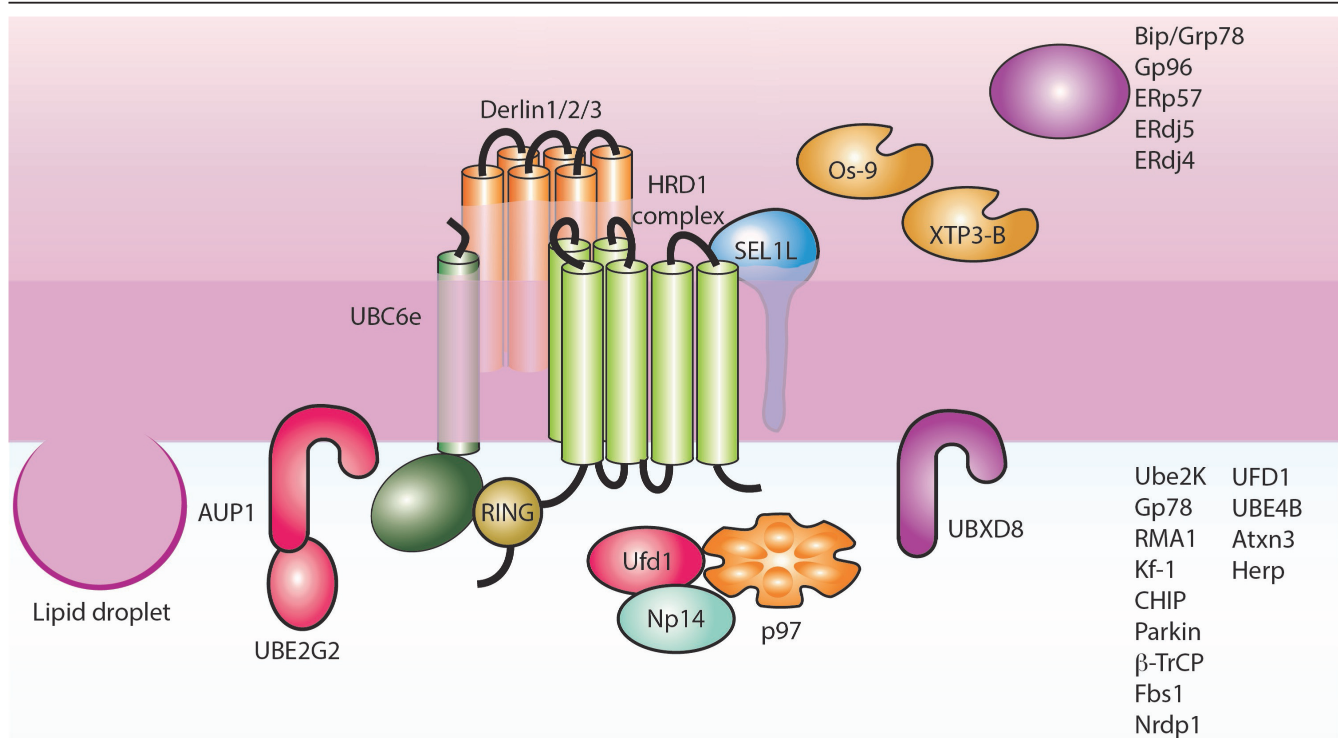
Figure 1. Overall scheme of a typical endoplasmic reticulum (ER) quality control pathway



Recognition of misfolded secretory proteins involves molecules that sense glycosylation and redox status. Transfer to an ER-resident ubiquitin ligase complex may require additional proteins. The mechanical force for dislocation can be provided by the AAA-ATPase p97. Cytosolically disposed glycans are removed coincident with or prior to transfer to the proteasome. Ubiquitin is removed and recycled prior to highly processive proteolysis by the 26S proteasome.

(b) translocation across the ER membrane, involving the components that constitute the dislocon; and (c) ubiquitylation/deubiquitylation reactions; ubiquitylation reactions may generate the necessary "handles" for exertion of physical force (for example, by the AAA-ATPase p97) to effectuate extraction of the substrate from the ER. Ubiquitylation/deubiquitylation reactions may obviously require different types of ubiquitin linkages and would necessitate involvement of distinct ubiquitin-conjugating enzymes and deubiquitylating enzymes. Typical approaches that implicate the involvement of key components involve co-immunoprecipitation experiments, often in conjunction with mass spectrometry, to validate protein-protein interactions [20], and gene disruptions (Table 1) or short hairpin RNA-based knockdowns of specific components or in genome-wide screens. The composition of this machinery is depicted in Figures 1 and 2. Key sensors of misfolded proteins include oxidoreductases and carbohydrate-binding proteins, not all of which have been identified. There is an important role for an ER-resident mannosidase, ER degradation-enhancing alpha-mannosidase-like protein 1 (EDEM-1). In addition, members of the heat shock protein (HSP) family, such as Bip and Gp96, together with their J-domain containing co-chaperones, have been implicated in substrate recognition. The levels of these sensors may themselves be controlled by the very same mechanisms that deal with misfolded proteins and thus may enable "tuning" of ER quality control [21]. In the absence of massive quantities of misfolded proteins, the need for sensors and other components of the quality control machinery is

Figure 2. Schematic of the dislocation-Ub ligase complex centered on Hrd1-Sel1L



The Hrd1-Sel1L complex interacts with the tail-anchored Ubc6e (Ube2j1), which serves as the E2-conjugating enzyme for Hrd1-Sel1L. Members of the heat shock protein (HSP) family, such as Bip/Grp78 and their co-chaperones (J-domain-containing proteins) together with the lectin-like molecule Os-9 and XTP3-B deliver misfolded substrates to the Hrd1-Sel1L complex for dislocation via the Hrd1-Sel1L-Derlin complex, followed by the extraction via p97 and its associated co-factors Ufd1 and Npl4. The mechanistic details of how other proteins participate in dislocation, such as UbxD8 and the lipid droplet-associated AUP1 protein, together with Ube2G2, a cytosolic E2-conjugating enzyme, remain to be worked out. The list of proteins on the right have all been implicated in endoplasmic reticulum (ER) quality control, as summarized in Table 1.

reduced, and a rapid switch must be possible should such accumulation occur. Transfer to the channels via which dislocation occurs may involve the channel proteins themselves, and proteins such as OS9, capable of recognizing not only misfolded glycosylated proteins but also proteins devoid of glycans. The identity of the channel itself remains ill defined, and it is likely that in fact multiple such channels exist. Strong biochemical evidence implicates Hrd1-Hrd3 (yeast) and Hrd1-Sel1L (mammals) as possible protein-conducting channels. Although Sec61 was initially considered a plausible candidate to facilitate translocation in reverse [22] export from the ER (instead of import into the ER), further experimental support for this notion has yet to emerge. The Derlins, discovered as proteins essential for dislocation, were also considered conduits to allow proteins to exit from the ER, and their proposed structural similarity to rhomboid intra-membranous proteases [23,24] is consistent with such a role. Moreover, for the HCMV-encoded immune evasion US2, an association with signal peptide peptidase was observed [25], suggesting possible analogies between intramembranous signal peptide recognition and handling transmembrane segments of proteins destined for extraction from the ER.

Lipids

Since lipids are not template-encoded and cannot be manipulated easily by genetic modifications, the participation of lipids in protein dislocation is an appealing proposition, proposed in a model that employs the genesis of lipid droplets [26]. Although this model has not met with experimental support in yeast [27], the metabolism of HMG-CoA reductase suggests the involvement of lipid droplets in ensuring its ubiquitylation [28]. This reaction requires AUP1, implicated in glycoprotein degradation in the HCMV immune evasion model [29].

Knockout models in mice are becoming increasingly manageable in terms of ease of production and tissue-specific control over the actual deletion events, relying on site-specific inducible recombinases, such as inducible Cre in combination with LoxP sites. Although deletion of the

Table 1. Mouse models affected in components of the endoplasmic reticulum quality control pathway

1. Recognition of substrate and 2. Possible connections to the dislocon

Bip/Grp78: embryonic lethal (a). Tissue-specific knockouts in adipocytes, prostate epithelial cells, or Purkinje cells produce severe cellular defects in the affected tissues [30-46].

Gp96: embryonic lethal. Tissue- or cell type-specific knockouts show severe phenotypes. Because gp96 is a co-factor essential for Toll-like receptor (TLR) expression, TLR-driven responses are compromised in gp96-deficent macrophages [47-64].

ERp57: embryonic lethal. ERp57-deficient B cells are largely normal [65-68].

ERdj5: ER stress in salivary gland [69]

ERdj4: perinatal death; constitutive induction of the unfolded protein response (UPR) in various tissues [70]

Sel1L: embryonic lethal. Induced deletion of floxed allele post-natally causes constitutive UPR, and death within 3 weeks, possibly because of nutrient malabsorption [71-74].

Derlin-1: embryonic lethal [75]

Derlin-2: perinatal lethality, constitutive UPR, mild cartilage defect [76]

Derlin-3: viable. Levels of Derlin-1 and -2 are decreased in pancreas [75].

Herp: increased levels of Hrd1. In various tissues, glucose tolerance is impaired [75].

3. Ubiquitylation and deubiquitylation

Ube2K: viable. Levels of caspase 12 are reduced in cortical neurons [77].

Hrd1: embryonic lethal, aberrant apoptosis. Hemizygous deletion causes multiple effects in models of acute liver toxicity (less fibrosis) [78-81].

Gp78: stabilization of CD82 and Insig-1 in gp78-deficient mouse embryo fibroblasts [82,83]

RMA1: Inducible transgenic overexpression causes weight loss and muscle wasting [84,85].

Kf-1: also called "hicky mouse". Anxiety-like behavior is increased [86].

CHIP: normal development. But perinatal lethality is approximately 25% [87-91].

Parkin: viable and fertile but with motor and cognitive defects [92-98]

β-TrCP: If exon 1 is deleted, the mouse model is viable, fertile, and superficially normal but with defects in retinal development. If exon 5 is deleted, fertility is reduced [99-105].

Fbs1: superficially normal but with age-related hearing loss [106,107]

Nrdp1: Transgenic overexpression produces less pro-inflammatory cytokines [108-110].

Smurf1: superficially normal with enhanced osteoblast activity [111]

UFD1: superficially normal [112]

UBE4B: embryonic lethal. Overexpression causes neural degeneration [113,114].

Atxn3: viable and phenotypically normal with increased tissue ubiquitin levels [115,116]

Yod-1: Inducible expression of a dominant-negative version (catalytically inactive) contributes to enhanced cross presentation [117].

Calreticulin, calnexin, and UDP-glucose glycoprotein glycosyltransferase (UGGT): calreticulin: heart, brain, and body wall defects; calnexin: early post-natal death and motor disorders; UGGT: embryonic lethal [118-120]

The approximate position of the listed components may be found in Figures 1 and 2.

genes for some of the ER quality control causes embryonic lethality (probably in accord with expectations), several prominent players can be eliminated without system-wide defects in protein management.

Below, we provide a brief list of the currently available (conditional) mouse null mutants or transgenic over-expressors and some of the phenotypes recorded for them, more fully described in the references cited. What this survey demonstrates is the essential nature of many of the components identified in the ER quality control pathway and the remarkable degree of tissue-specific aberrations in cellular physiology when using cell type- or tissue-specific knockouts. If we take into account the large variations in protein composition across different tissues and cell types and take note of the fact that, for many ubiquitously expressed genes, knockout phenotypes can be remarkably cell type- and tissue-specific, this should not come as a surprise. The remarkably coherent picture that has emerged from the study of a relatively limited number

of substrates, cell lines, and experimental tissue culture models will surely require adjustment in view of the continued investment in mouse models. Recent developments, such as cas9/CRISPR genome-editing methods will undoubtedly lead to a remarkable expansion and acceleration in this exciting field.

Abbreviations

CFTR, cystic fibrosis transmembrane conductance regulator; DTT, dithiothreitol; ER, endoplasmic reticulum; HCMV, human cytomegalovirus; 3-hydroxy-3-methylglutaryl-coenzyme A, HMG-CoA; tRNA, transfer RNA; UPR, unfolded protein response; XBP1, X-box binding protein 1.

Disclosures

The authors declare that they have no disclosures.

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