Chapter 14
ER-associated Degradation and Its Involvement in Human Disease: Insights from Yeast

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14.1 Introduction

Proteins destined for the secretory pathway are synthesized on cytosolic ribosomes and transported, either co- or post-translationally, to the endoplasmic reticulum (ER) (Brodsky 1996). The ER is endowed with a quality control system that monitors newly synthesised proteins for correct chain folding and modification, as well as assembly into functional macromolecular complexes (Kopito 1997; Ellgaard and Helenius 2003; Sitia and Braakman 2003). Aberrant and misfolded proteins are retro-translocated to the cytosol, where they are ubiquitylated and eliminated by the 26S proteasome (Werner et al. 1996; Plemper and Wolf 1999; Hampton 2002; Kostova and Wolf 2003; McCracken and Brodsky 2003; Meusser et al. 2005). ER-associated protein degradation (ERAD) comprises at least three steps: (i) the recognition of a misfolded or unassembled polypeptide by the quality control system, (ii) its retro-translocation (or dislocation) into the cytosol across the ER membrane and (iii) ubiquitin-mediated degradation by the proteasome (Fig. 14.1). A second mechanism for dealing with misfolded proteins is termed the unfolded protein response (UPR), which increases the ER folding capacity and may trigger apoptosis (Ron and Walter 2007). A third mechanism is the sequestration of misfolded proteins in special subcellular compartments and autophagy (Klionsky 2007).

This review focuses on the budding yeast *S. cerevisiae*, a unicellular eukaryote that is highly amenable to genetic and biochemical analyses (Sommer and Jentsch 1993; Hampton et al. 1996; Hiller et al. 1996; McCracken and Brodsky 1996). Studies using yeast and mammalian cells have shown that the basic events of the ERAD pathway are conserved in eukaryotes (Ward et al. 1995; Lenk et al. 2002; Gnann et al. 2004). Hereafter, genes and proteins that are conserved between yeast and mammals, but have different names, will be prefixed by *y*- or *m*- (Table 14.1).

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C.J. Vidal (ed.), *Post-Translational Modifications in Health and Disease*, Protein Reviews 13,
DOI 10.1007/978-1-4419-6382-6_14, © Springer Science+Business Media, LLC 2011

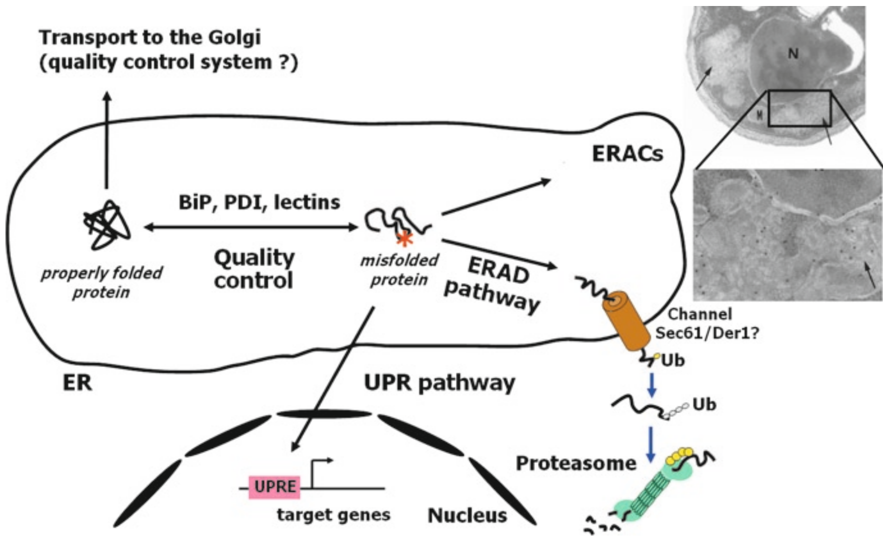

Fig. 14.1 Overview of ER protein quality control and proteasome mediated degradation. Newly synthesised secretory and membrane proteins are monitored for non-native conformation by a quality control system consisting of various ER folding sensors (y-Kar2p/m-BiP, PDI and lectin-like chaperones). Unfolded or misfolded proteins are transported across the ER membrane into the cytosol, where they are marked for proteasomal elimination by E3 ligase-mediated conjugation to ubiquitin (Ub). The accumulation of unfolded and misfolded proteins in the ER induces UPR, resulting in the activation of genes coding for ER chaperones and proteins involved in the secretory and ERAD pathways. Another mechanism for overcoming the toxicity linked to elevated amounts of misfolded proteins is their sequestration into ER-associated compartments (ERACs). The thin-section electron micrograph shows ERACs (*arrows*) in a yeast cell expressing a mislocalised variant of the plasma membrane H^+-ATPase (Pma1-D378N). An antibody conjugated to gold spheres (*black dots*) has been used to locate Kar2p molecules. The nucleus (N), mitochondria (M) and vacuoles can be seen (Courtesy of André Goffeau, 1995)

14.2 Recognition of Aberrant Proteins in the ER

Newly synthesised proteins undergo several post-translational modifications in the ER, including the removal of the signal sequence, disulphide bond formation, *N*-glycosylation and the addition of glycosylphosphatidylinositol. Protein folding involves ER-resident molecular chaperones of the heat shock protein (Hsp) 70 family, such as *y*-Kar2p/*m*-BiP (Kozutsumi et al. 1989; Normington et al. 1989; Rose et al. 1989) and its nucleotide exchange factor, *y*-Lhs1/*m*-ORP150 (Saris et al. 1997; Takeuchi 2006). Disulphide-bond formation is catalysed by protein disulphide isomerases (PDIs), including *y*-Pdi1p, 4 other non-essential Pdi1-related proteins (Norgaard et al. 2001) and *y*-Ero1p (Fassio and Sitia 2002; Gross et al. 2006). These oxidoreductases ensure that irreversible formation of disulphide bonds does not occur until protein folding is complete. However, no significant effects on ERAD are seen in various *PDI*-deleted strains (Norgaard et al. 2001). The attachment of

Table 14.1 Mammalian counterparts of the yeast ERAD components and associated diseases

Activity/function	Yeast	Mammalian	Disease/disorder
E2s	Ubc6	UBE2J1, UBE2J2	Cystic fibrosis
	Ubc7	UBE2G1, UBEG2	
E3s	Hrd1-Hrd3	Synoviolin/HRD1-SEL1	Net sodium imbalance, rheumatoid arthritis, cystic fibrosis, accumulation of Pael-R
	Doa10	TEB4[a]	PFIC II
		RMA1, RFP2, Kf-1	Cystic fibrosis, PFIC II
Cdc48-Ufd1-Npl4 complex	Cdc48	p97 or VCP	Inclusion body myopathy with Paget disease of bone and frontotemporal dementia (Weihl et al. 2006)
Cdc48 receptor	Ubx2	VIMP[b]	
Dislocation pore?	Der1	DERLIN	Cystic fibrosis, ALS
	Usa1	HERP	Enhancement of β-amyloid protein generation (Sai et al. 2002)
Substrate adaptors for the proteasome	Dsk2	Ubiquilin1	Alzheimer's disease
	Rad23	HHR23A and B	Machado-Joseph disease

[a]Sequence identity found in the N-terminal RING-CH domain and an internal 130-residue block, the TEB4-Doa10 (TD) domain, that might be involved in retro-tranlocation (Wang et al. 2008)
[b]Sequence homology is restricted to the UBX domain

N-linked carbohydrates is also essential for the maturation of secretory and membrane proteins. The added glycans stabilize the protein conformation by interacting with the hydrophobic protein surface and also act as substrates for *m*-calnexin (*y*-Cne1p) and *m*-calreticulin, ER lectins that assist the folding of some glycoproteins (Song et al. 2001; Helenius and Aebi 2004). Note that evidence for a canonical calnexin cycle in yeast is lacking. It is also not known which of the several yeast peptidyl-prolyl isomerases (cyclophilins and FK506-binding proteins) are responsible for protein folding in the ER, as none of these are required for cell viability (Dolinski et al. 1997). The mammalian enzymes involved in the secretory protein factory have recently been reviewed (Anelli and Sitia 2008).

As folding sensors, ER luminal Hsp70s, lectins and PDIs are key components of the quality control system (Bukau et al. 2006). These molecular chaperones monitor the thermodynamic stability of non-native proteins and the presence of unpaired cysteine residues or immature glycans (Sitia and Braakman 2003; Krebs et al. 2004). Folding or assembly intermediates are retained in the ER and allowed to undergo further folding attempts. It is still not clear how molecules that have not had the time to fold are distinguished from those that have failed to fold after many attempts and must therefore be eliminated. According to the mannose timer hypothesis (Jakob et al. 1998; Nakatsukasa et al. 2001; Ellgaard and Helenius 2003), the terminal mannose residue of the $Man_9GlcNAC_2$ oligosaccharide on misfolded glycosylated proteins is removed by α1,2-mannosidase I (*y*-Mns1p), producing the sugar moiety $Man_8GlcNAC_2$. The mannosidase reaction is slow, so only terminally

misfolded proteins that spend an excessive amount of time in the ER will be trimmed. The Man_8-species are recognized by α-mannosidase-like lectins so they may be trimmed more extensively. However, as all folded glycoproteins also contain a Man_8 oligosaccharide, it seems likely that the signal for ERAD consists of a polypeptide determinant in addition to the glycan structure (Byrd et al. 1982; Spear and Ng 2005). The mannose timer hypothesis has been challenged in the fission yeast *Schizosaccharomyces pombe*, as ER mannose trimming does not appear to be required for ERAD (Movsichoff et al. 2005).

14.2.1 Model ERAD Substrates

Several model proteins have been used to identify the ERAD components in yeast. Hydroxy-methylglutaryl-CoA reductase (*y*-Hmg2p) catalyses the synthesis of mevalonate, a precursor of activated isoprenes and sterols. Following the accumulation of sterols and other isoprenoids, *y*-Hmg2p undergoes rapid ER-associated degradation (Hampton et al. 1996). Other ERAD substrates are mutant forms of secretory or membrane proteins that fail to fold properly, as judged by an altered trypsin digestion pattern. Transmembrane chimeras have also been constructed that contain misfolded segments exposed on the cytosolic side of the ER membrane, the luminal side or both. Two different ERAD pathways can be distinguished according to the topology of the misfolded lesion (Fig. 14.2). ER luminal proteins and membrane proteins with lesions in a luminal domain are degraded via the so-called ERAD-L pathway, whereas membrane proteins with cytosolic lesions are degraded via the ERAD-C pathway (Ahner and Brodsky 2004).

14.2.1.1 Soluble Luminal ERAD Substrates

A mutant form of vacuolar carboxypeptidase Y, named CPY*, is retained in the ER, as shown by the lack of Golgi-specific carbohydrate modification, and is rapidly degraded by the ubiquitin-proteasome system (Finger et al. 1993; Hiller et al. 1996). Retrograde transport of CPY* through the Sec61 translocon depends on Sc-Kar2p and the Hsp40 co-chaperones *y*-Jem1p and *y*-Scj1p (the role of *y*-Sec63p is controversial) (Plemper et al. 1997; Nishikawa et al. 2001). These may play an important role in degradation by remodelling the aberrant proteins into a partially unfolded or reduced form, more amenable for retro-translocation. A non-glycosylated version of CPY* is not degraded, suggesting a role for *N*-linked glycans in entry into the ERAD pathway (Knop et al. 1996). CPY* degradation is impaired by mutation of glucosidases I and II (Jakob et al. 1998; Hitt and Wolf 2004a) or of an α-mannosidase-like lectin, named Mnl1p or Htmp1 in yeast (Jakob et al. 2001; Nakatsukasa et al. 2001) or EDEM (ER degradation enhancing α-mannosidase-like protein) in mammals (Hosokawa et al. 2001; Molinari et al. 2003). Mutagenesis analysis has revealed that a single, specific *N*-linked glycan at the C-terminus of CPY is required for sorting

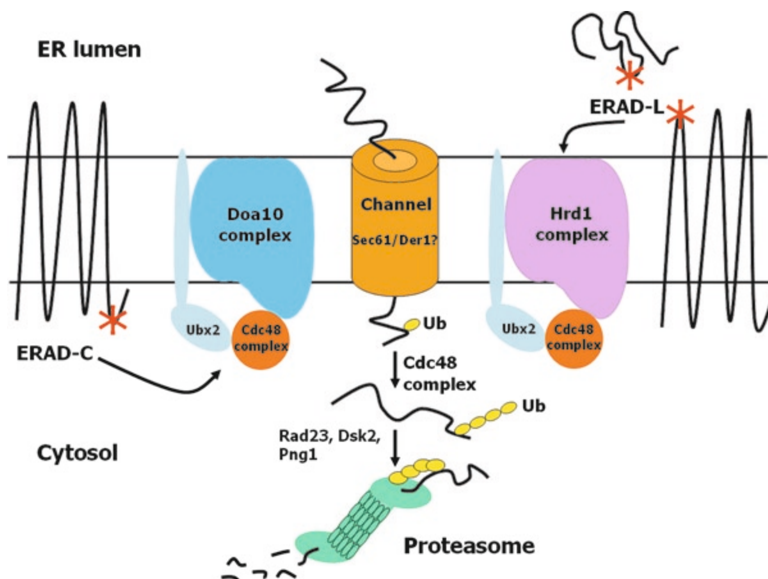

Fig. 14.2 Proteins with luminal and cytosolic lesions are targeted for proteasome-mediated degradation through distinct protein-ubiquitin ligase complexes. Membrane proteins with misfolded cytosolic domains are substrates of the ubiquitin ligase *y*-Doa10p (ERAD-C pathway), whereas membrane and soluble proteins with luminal misfolded domains are substrates of the ubiquitin ligase *y*-Hrd1p (ERAD-L pathway). A third checkpoint that screens transmembrane domains (ERAD-M) and can activate *y*-Hrd1p is not represented. Ubiquitylated substrates are extracted from the ER membrane by the Cdc48-Ufd1p-Npl4 complex, which is recruited to the ER membrane via its interaction with *y*-Ubx2p, and are delivered to the 26S proteasome by *y*-Rad23p and *y*-Dsk2p. The identity of the dislocation pore (*y*-Der1 or Sec61) is still a matter of debate

into the ERAD pathway, supporting the bipartite nature of the ERAD signal (Spear and Ng 2005).

Studies on mammalian cells have suggested that *m*-EDEM1 helps misfolded glycoproteins leave the calnexin/calreticulin cycle, where they are attempting to fold, and maintains substrate solubility for retro-translocation (Oda et al. 2003; Kanehara et al. 2007). *m*-EDEM associates with ER-resident *m*-BiP (Hsp70) and *m*-ERdj5 (Hsp40), a co-chaperone which cleaves the disulphide bonds of misfolded proteins (Ushioda et al. 2008). The related proteins *m*-EDEM2 and *m*-EDEM3 accelerate ERAD of a misfolded α1-antitrypsin variant (Mast et al. 2005; Hirao et al. 2006). In yeast, Htm1p functions as an α1,2-specific exo-mannosidase that generates the $Man_7GlcNAc_2$ oligosaccharide with a terminal α1,6-linked mannosyl residue on degradation substrates. Htm1p activity requires processing of the *N*-glycan by glucosidase I, glucosidase II and mannosidase I, resulting in a sequential order of specific *N*-glycan structures that reflect the folding status of the glycoprotein (Clerc et al. 2009). A second class of lectin-like ERAD factors consists of mammalian OS-9 and erlectin/XTP3-B proteins and yeast Yos9p, which contain a mannose-6-phosphate receptor homology domain (Buschhorn et al. 2004;

Bhamidipati et al. 2005; Kim et al. 2005; Szathmary et al. 2005). It has been proposed that *y*-Yos9p has a proof-reading function in scanning for the Man_8 determinant in substrates ready to be retro-translocated (Denic et al. 2006; Gauss et al. 2006a; Hirsch et al. 2006).

14.2.1.2 Membrane ERAD Substrates with a Misfolded Cytosolic Domain

y-Ste6p is an ATP-binding cassette (ABC) transporter that functions at the plasma membrane to export the **a**-factor mating pheromone from the cell (McGrath and Varshavsky 1989). Ste6p is slowly targeted for degradation in the vacuole through ubiquitin-mediated endocytosis (Kolling and Hollenberg 1994). However, a C-terminally truncated variant, Ste6-Q1249-X, is retained in the ER and degraded through the ERAD pathway (Loayza et al. 1998). *y*-Kar2p is dispensable, whereas the cytosolic Hsp70 *y*-Ssa1p chaperone and the Hsp40 co-chaperones *y*-Ydj1p and *y*-Hlj1p are not (Huyer et al. 2004b). *y*-Ydj1p is tethered to the ER membrane by prenylation, whereas *y*-Hlj1p is a C-terminally anchored ER membrane protein. These results indicate that ERAD of Ste6-Q1249-X employs different machinery from that of the soluble luminal substrate CPY*. *y*-Ssa1p and *y*-Ydj1p (but not *y*-Hlj1p) are required for the degradation of Ura3p-CL1, a cytosolic soluble protein with a transplantable degradation signal (Metzger et al. 2008). As its degradation also requires the ER-localized ubiquitin conjugating and ligase enzymes, it is likely that the cytosolic face of the ER membrane serves as a platform for the degradation of Ura3p-CL1 and other cytosolic misfolded proteins (Metzger et al. 2008).

y-Pma1p is a plasma membrane H^+-ATPase of the P-type family, which pumps protons out of the cell at the expense of ATP hydrolysis. The generated proton chemical gradient drives the uptake of nutrients and ions (Serrano et al. 1986). The Pma1-D378N variant is poorly folded and accumulates in ER-associated compartments (ERACs) prior to ER-associated degradation (Harris et al. 1994; Nakamoto et al. 1998). The mutant pump binds *y*-Esp1p, an ER membrane PDI that functions in protein quality control. The loss of *y*-Esp1 allows Pma1-D378N to travel to the plasma membrane (Wang and Chang 1999). No stabilisation of Ste6-Q1249-X is seen in cells lacking *y*-Esp1, indicating that *y*-Esp1p is a specific recognition factor (Huyer et al. 2004b).

14.2.1.3 Membrane-Associated ERAD Substrates with a Luminal Lesion

y-Pdr5p is a plasma membrane multidrug ABC transporter which mediates the efflux of a large variety of cytotoxic compounds (Balzi et al. 1994; Bissinger and Kuchler 1994). A mutant form, Pdr5-C1427Y (Pdr5*), is retained in the ER membrane and targeted for proteasomal degradation through the ERAD pathway (Egner et al. 1998; Plemper et al. 1998). There is no evidence that Pdr5* is misfolded, although its glycosylation pattern differs from that of intact *y*-Pdr5p (Plemper et al. 1998). It has been proposed that replacement of the cysteine residue affects the formation of disulphide bonds with other cysteine residues facing the ER lumen

(Egner et al. 1998). The chaperone lectin *y*-Htm1p, but not *y*-Kar2p, is involved in the degradation process (Plemper et al. 1998; Jakob et al. 2001; Gnann et al. 2004).

The CTG* chimera, consisting of CPY* fused to the green fluorescent protein (GFP) via a transmembrane segment from *y*-Pdr5p, is degraded through the ERAD pathway (Taxis et al. 2003). *y*-Kar2p is dispensable, whereas the cytosolic chaperone proteins *y*-Ssa1p, *y*-Hlj1p, *y*-Cwc23p and *y*-Jid1p are not. This indicates that the recognition of the misfolded CPY* moiety in CTG* is not the primary step targeting the protein chimera for ERAD. However, the misfolded CPY* domain might be responsible for a signalling event that recruits the cytosolic chaperones onto the stable GFP moiety for the unfolding of this domain to occur before proteasomal degradation (Ahner and Brodsky 2004).

14.2.1.4 Transmembrane Proteins Exposing Misfolded Domains to the ER Lumen and Cytosol

The use of different ERAD substrates in yeast has revealed the existence of the ERAD-C and ERAD-L checkpoints that monitor the folding of the cytosolic and luminal domains of newly synthesised proteins (see Fig. 14.2). What is the fate of a transmembrane protein with two lesions, one exposed to the ER lumen and the other to the cytosol? This question was addressed by analysing the fate of the KSS chimera, consisting of Ste6-Q1249-X fused to an ER luminal misfolded artificial domain. KSS turned out to be degraded as efficiently in cells lacking *y*-Mnl1p as in wild-type cells, even though the misfolded ER luminal domain confers Mnl1p-dependent degradation when transplanted to another stable transmembrane protein (Vashist and Ng 2004).

The use of modular ERAD substrates has shown that nascent transmembrane proteins are monitored by a two-step quality control system. The first, the ERAD-C checkpoint, is located at the cytosolic face of the ER membrane. If a lesion is detected, the membrane protein is ubiquitylated and degraded by the proteasome. If the cytosolic domains are found to be correctly folded, the transmembrane protein will pass to the second checkpoint, ERAD-L, which checks the luminal domains and targets misfolded proteins for degradation through a pathway shared by soluble secretory proteins (Vashist and Ng 2004). The existence of different ERAD pathways in mammalian cells has been recently analyzed using the plasma membrane V2R vasopressin receptor and three mutant forms that contain a misfolded cytosolic, luminal or intramembrane domain and are retained in different intracellular compartments (Schwieger et al. 2008). Unlike the wild-type protein, each variant is targeted for proteasomal degradation through a seemingly similar ERAD pathway involving *m*-DERLINs (see below) (Schwieger et al. 2008).

14.2.2 ER-Associated Ubiquitylation Machinery

Conjugation of ubiquitin to ERAD substrates occurs during their retro-translocation and is mediated by the E2 ubiquitin-conjugating (Ubc) enzymes *y*-Ubc6p, *y*-Ubc7p

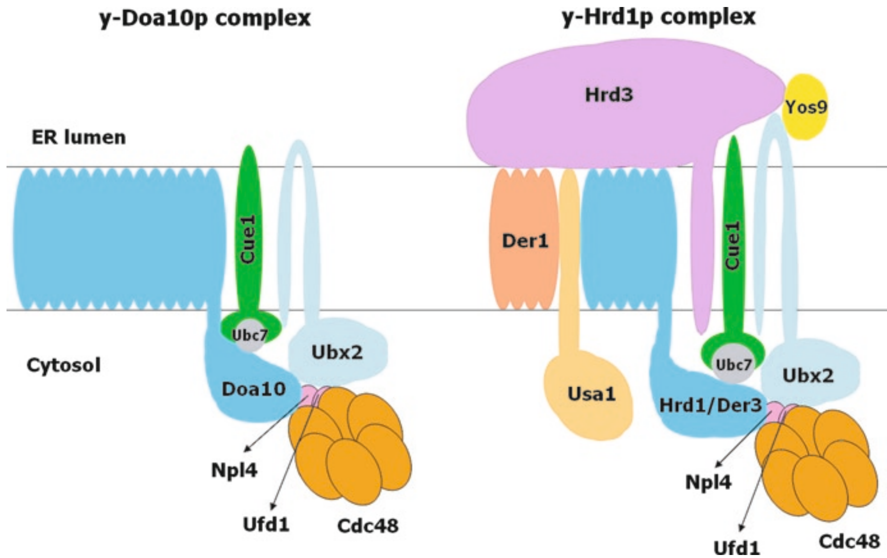

Fig. 14.3 Yeast ER-associated ubiquitin ligase complexes. The Doa10 complex of the ERAD-C pathway is composed of the ubiquitin ligase Doa10p, the ubiquitin-conjugating enzyme Ubc7p and its membrane-anchoring factor *y*-Cue1p. The Hrd1 complex of the ERAD-L pathway contains a large ER lumenal region consisting of *y*-Hrd3p and *y*-Yos9 that may serve as the receptor site for ERAD-L substrates. *y*-Usa1p interacts with *y*-Der1p, a putative constituent of the retro-translocation pore. Note that the ER membrane ubiquitin conjugating enzyme Ubc6p is not found in these complexes. *y*-Ubx2 acts as a membrane anchor of the Cdc48-Ufd1-Npl4 complex, which is involved in the extraction of ubiquitylated substrates from the ER membrane (Carvalho et al. 2006; Denic et al. 2006)

and *y*-Ubc1p (Hiller et al. 1996; Friedlander et al. 2000). The E3 ubiquitin ligases *y*-Hrd1p and *y*-Doa10p belong to the RING zinc finger domain family (Hampton et al. 1996; Plemper et al. 1998; Huyer et al. 2004b). They act as separate scaffolds for the assembly of components involved in substrate recognition, extraction and ubiquitylation for the ERAD-L and-C pathways (Fig. 14.3). In the case of some ERAD substrates, elongation of the polyubiquitin chain requires *y*-Ufd2p (Koegl et al. 1999; Richly et al. 2005; Nakatsukasa et al. 2008).

14.2.2.1 The y-Doa10 Complex

y-Doa10p (Degradation of soluble Matα2 transcription repressor), alias y-Ssm4p, is a 14-transmembrane segment protein of the ER/nuclear envelope, both termini facing the cytosol (Swanson et al. 2001; Kreft et al. 2006). *y*-Doa10p is part of a complex that includes both *y*-Ubc7p, which is tethered to the ER membrane via its association with *y*-Cue1p (Biederer et al. 1997), and the Cdc48-Ufd1-Npl4 complex, which is recruited to the membrane via *y*-Ubx2p (Schuberth et al. 2004; Neuber et al. 2005). The second ERAD E2 enzyme, *y*-Ubc6p, is anchored to the ER

membrane through its C-terminal hydrophobic tail. *y*-Doa10 is able to target cytosolic (Ura3p-CL1), nuclear (Matα2p) and ER membrane (Ste6-Q1249-X) proteins for proteasomal degradation (Metzger et al. 2008). The Cdc48-Ufd1-Npl4 complex facilitates the degradation of membrane-embedded Doa10 substrates, but is not required for soluble Doa10 substrates. Thus, while *y*-Doa10p ubiquitinates both membrane and soluble proteins, the mechanisms of the subsequent proteasome targeting differ (Ravid et al. 2006).

14.2.2.2 The y-Hrd1 Complex

y-Hrd1p (HmgCoA reductase degradation), alias *y*-Der3p (Degradation in the ER), is a 551 amino acid protein with six transmembrane spans and both termini facing the cytosol (Gardner et al. 2000; Deak and Wolf 2001). *y*-Hrd1p assembles with *y*-Ubc7p-Cue1p in a RING finger-dependent manner (Hampton et al. 1996; Friedlander et al. 2000). Physical association with *y*-Ubc1p and *y*-Ubc6p has been reported (Bays et al. 2001a). *y*-Hrd1p also interacts with the Cdc48 complex and *y*-Hrd3p, a membrane protein with a large ER-luminal domain that functions as a substrate receptor (Hampton et al. 1996; Gardner et al. 2000). In the absence of *y*-Hrd3p, *y*-Hrd1p is rapidly degraded by the ubiquitin-proteasome system (Plemper et al. 1999; Gardner et al. 2000). The interaction between *y*-Hrd1p and the Cdc48-Ufd1-Npl4 complex requires the membrane protein *y*-Ubx2p, which contains an N-terminal UBA domain, known to bind to the ubiquitin chain of ERAD substrates, and a C-terminal ubiquitin-like (UBX) domain, which binds Cdc48 (Decottignies et al. 2004; Schuberth et al. 2004; Neuber et al. 2005).

The transmembrane protein *y*-Der1p is linked to *y*-Hrd1p via *y*-Hrd3p and *y*-Usa1p, the latter of which has no known function (Carvalho et al. 2006; Gauss et al. 2006b). Deletion of the *USA1* gene is synthetically lethal with deletion of *IRE1*, which encodes a key player in the UPR (Carvalho et al. 2006). The lectin-like *y*-Yos9p physically and mechanistically interacts with *y*-Hrd1p by binding to a luminal domain of *y*-Hrd3 and to the chaperone *y*-Kar2p (Carvalho et al. 2006; Denic et al. 2006; Gauss et al. 2006a). The Kar2p-Yos9p-Hrd3p luminal complex is not disrupted by the loss of *y*-Hrd1p, but these proteins no longer interact with the Cdc48p complex (Carvalho et al. 2006; Denic et al. 2006).

14.2.3 Retro-translocation into the Cytosol and Delivery to the 26S Proteasome

The proteasomal degradation of ER luminal and membrane proteins requires their retrograde transport out of the ER back to the cytosol (see Fig. 14.2). The identity of the protein components responsible for retro-translocation is still debated, the potential candidates being the Sec61p translocation channel, Der1p and Doa10p (Nakatsukasa and Brodsky 2008).

Sec61p is an obvious candidate for the dislocation channel because of its role in the translocation of newly synthesised proteins in the ER. In mammalian cells, the Sec61 complex associates with MHC class I heavy chains that are targeted for ERAD by the human cytomegalovirus-encoded glycoprotein US2 (Wiertz et al. 1996). Studies in yeast also indicate that the Sec61 channel functions in the export of a misfolded secretory protein (CPY* and derivatives) or membrane protein (Pdr5*) for proteasomal degradation (Pilon et al. 1997; Plemper et al. 1997, 1998; Willer et al. 2008). Instrumental in these studies were the isolation of *sec61* alleles that are defective for protein export, but not for import, at an appropriate temperature (Pilon et al. 1997) and the modification of the translocation properties of model ERAD substrates (Willer et al. 2008). Formation of a disulphide-linked intermediate complex between Sec61p and a transmembrane ERAD substrate has been recently reported (Scott and Schekman 2008). These authors proposed that Sec61p acts directly in the retro-translocation of ERAD substrates or indirectly in remodelling membrane proteins before they are transferred to a separate ER channel for retro-tranlocation.

A role for Derlin-1 as the ER retro-translocation channel is based on its interaction with US11, a virally-encoded ER protein that targets MHC1 class I heavy chains for retro-translocation, and with VIMP, a membrane protein that recruits the cytosolic Cdc48-Ufd1-Npl4 complex to the ER membrane (Lilley and Ploegh 2004; Ye et al. 2004). Derlin-1 also facilitates the retro-translocation of cholera toxin (Bernardi et al. 2008). Real-time fluorescence analysis of pro-α factor retro-translocation in an *in vitro* mammalian system has shown a requirement for Derlin-1, but not Sec61α (Wahlman et al. 2007). The yeast Derlin-1 homolog (*y*-Der1p) is required for the efficient degradation of CPY* (Knop et al. 1996; Hitt and Wolf 2004b), but not of CTG* and other membrane ERAD substrates (Taxis et al. 2003; Huyer et al. 2004b; Vashist and Ng 2004). ERADication of these Der1p- and Sec61p-independent substrates requires *y*-Doa10p (Walter et al. 2001; Vashist and Ng 2004; Kreft et al. 2006), and it has been proposed that a subset of the Doa10 transmembrane segments might form a retro-translocation channel (Kreft et al. 2006).

The driving force for substrate retro-translocation can be derived from three non-mutually exclusive sources: chaperone-mediated extraction, (poly)ubiquitin-mediated ratcheting and proteasome-mediated retro-translocation (Tsai et al. 2002; McCracken and Brodsky 2003). Single-spanning membrane proteins, such as MHC class I heavy chain and the α subunit of the T cell receptor, may be completely dislocated from the membrane to the cytosol before their degradation by the proteasome. Polytopic membrane proteins may be processively dislocated and degraded from either the N- or the C-terminus by the proteasome. It is also possible that cytoplasmic loop(s) of the substrates may first be “clipped” by the endoproteolytic activity of the proteasome, then dislocated or extracted from the ER membrane (Nakatsukasa and Brodsky 2008). Retrograde transport of soluble proteins through the protein-conducting channel is conceptually more difficult. During import into the ER the N-terminal signal peptide targets these proteins to the ER membrane and into the opening in the translocon. In the ER lumen, the signal peptide is removed and the opening of the channel from the luminal side must therefore be triggered by a mechanism different from that used during protein import (Romisch 1999).

Ubiquitylated ERAD substrates are released from the ER by a soluble ubiquitin-specific chaperone complex consisting of Cdc48p, Ufd1p and Npl4p (Bays et al. 2001b; Hitchcock et al. 2001; Ye et al. 2001; Braun et al. 2002; Jarosch et al. 2002; Rabinovich et al. 2002). Yeast Cdc48p, named p97 or valosin-containing protein (VCP) in mammals, has two ATP-binding domains and two Second Regions of Homology (SRH) motifs (Frohlich et al. 1991). X-ray crystallographic studies showed six subunits assembled into a ring, the overall shape of which changes during the ATPase catalytic cycle (De LaBarre and Brunger 2003; Dreveny et al. 2004). A nucleotide-dependent conformational switch may apply tension to bound proteins and thereby allow polypeptide unfolding (Bukau et al. 2006). Cdc48p/p97 binds to the C-terminal domain of Ufd1p and to the N-terminus of the zinc-binding motif protein *y*-Npl4p (Meyer et al. 2000; Rape et al. 2001; Bruderer et al. 2004). The N-terminal domain of *y*-Ufd1, which is structurally similar to that of p97, and the Npl4 zinc finger contain binding sites for ubiquitin (Alam et al. 2004; Park et al. 2005). The yeast Cdc48-Ufd1-Npl4 complex is recruited to the ER membrane by the UBX domain-containing protein *y*-Ubx2p and associates with *y*-Der1p (Carvalho et al. 2006). Ubx2p also contains a ubiquitin-associated (UBA) domain that binds ubiquitin chains and may transport the ERAD substrates to Cdc48p (Schuberth et al. 2004; Neuber et al. 2005).

After their retro-translocation to the cytosol, the multi-ubiquitylated substrates are delivered to the proteasome by *y*-Rad23p and *y*-Dsk2p (Chen and Madura 2002; Funakoshi et al. 2002; Medicherla et al. 2004). These proteins have one or more UBA domains and a ubiquitin-like (UBL) domain, which is recognized by the proteasome subunit *y*-Rpn1 (Elsasser et al. 2002). Both the 26S proteasome and the E4 ubiquitin chain elongation factor *y*-Ufd2p associate with the *y*-Rad23 UBL domain in a mutually exclusive manner *in vitro* (Kim et al. 2004). *y*-Rad23p and Cdc48p have been shown to interact with a conserved peptide *N*-glycanase, Pgn1p, which is required for the deglycosylation of misfolded glycoproteins during proteasome-dependent degradation (Suzuki et al. 2000; Hirsch et al. 2004; Nita-Lazar and Lennarz 2005).

The multi-ubiquitylated versus de-ubiquitylated state of an ERAD substrate and its subsequent targeting to the proteasome are determined by a subtle balance between *y*-Ufd2p and *y*-Doa1p (a WD40 repeat protein controlling the cellular ubiquitin concentration) and y-Otu1p (a de-ubiquitinating enzyme). *y*-Doa1p and *y*-Otu1p bind to *y*-Cdc48p, enhancing their inhibitory effect on *y*-Ufd2p (Richly et al. 2005; Rumpf and Jentsch 2006). As the C-terminal end of *y*-Doa1p interacts with the UBX domain, it is possible that the binding of *y*-Ubx2p to *y*-Cdc48p is also inhibited by *y*-Doa1p (Decottignies et al. 2004).

HHR23, the human homolog of *y*-Rad23p, interacts with the second ubiquitin interaction motif of the human S5a subunit (*y*-Rpn10) of the 26S proteasome (Hiyama et al. 1999). It also interacts with the protease domain of ataxin-3, a de-ubiquitinating enzyme involved in the development of the neurodegenerative Machado-Joseph disease (Wang et al. 2000). Ubiquilins 1–4 are human homologues of *y*-Dsk2 that bind presenilins and are found in Lewy bodies and neurofibrillar tangles (Mah et al. 2000). It has been proposed that, under conditions of high levels of protein aggregation and

subsequent proteasome overloading, ubiquilin-1 interacts with Eps15, an essential component of the clathrin-mediated endocytosis pathway, to promote the trafficking of protein aggregates to the aggresome (Madsen et al. 2007).

14.3 Accumulation of Unfolded Proteins in Subcellular Compartments

14.3.1 The Unfolded Protein Response

The accumulation of unfolded proteins in the lumen of the ER induces a coordinated adaptive program called the UPR (Shamu et al. 1994; Chapman et al. 1998; Sidrauski et al. 1998) (see Fig. 14.1). *y*-Ire1p is a conserved membrane kinase and contains a luminal domain responsible for sensing misfolded proteins and cytosolic kinase and ribonuclease domains (Hampton 2003). In yeast, under normal growing conditions, the monomeric protein is bound to Kar2p in an inactive complex. In the presence of high amounts of unfolded or misfolded proteins, the Kar2p-Ire1p complex dissociates and Ire1p assembles into an oligomeric complex with kinase activity (Shamu and Walter 1996).

Autophosphorylation and activation of the C-terminal endoribonuclease domain of *y*-Ire1p promotes the splicing of the pre-mRNA for the *y*-Hac1 (*m*-XBP-1) transcription factor with the help of the tRNA ligase *y*-Rgl1p. y-Hac1p activates the expression of target genes by binding to a conserved Unfolded Protein Response Element (UPRE) in their promoters (Cox and Walter 1996). The UPR alleviates stress by upregulating the expression of ER chaperones (*y*-Kar2p) and components of the ERAD pathway, such as *y*-Ubc7p, *y*-Hrd1p and *y*-Dfm1p (Der1-like-family-member) (Travers et al. 2000). *y*-Dfm1p binds to *y*-Cdc48p and has been identified in a protein complex containing the Cdc48p cofactors *y*-Ubx1p and *y*-Ubx7p (Hitt and Wolf 2004b). This suggests that the Der1p complex is involved in ERAD and the Dfm1p complex is involved in ER stress and homeostasis (Sato and Hampton 2006).

In addition to the IRE1 kinase, mammals utilize two other major transducers for sensing ER stress, the PERK eIF2a kinase, which attenuates protein translation in response to ER stress, and the ER transmembrane transcription factor ATF6. The accumulation of misfolded proteins allows ATF6 to reach the Golgi, where transmembrane proteases release a cytosolic transcriptionally active form, which enters the nucleus and induces the transcription of target genes (Wu and Kaufman 2006).

14.3.2 Proliferation of Subcellular Compartments

A second means of coping with non-native proteins in the ER is to sequester them in special sub-compartments (see Fig. 14.1). Overexpression of the ER membrane

3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase in yeast leads to the formation of stacked membrane pairs surrounding the nuclear envelope, called karmellae (Wright et al. 1988). A second type of ER sub-compartment, known as the ER-associated compartment (ERAC), consists of a network of tubulo-vesicular structures (Huyer et al. 2004a). ERAC formation is induced by the yeast plasma membrane H^+-ATPase variant Pma1-D378N and some, but not all, mutants of the pheromone ABC-transporter Ste6p (Wright et al. 1988; Supply et al. 1993; Harris et al. 1994; de Kerchove d'Exaerde et al. 1995; Loayza et al. 1998). The increased volume of the yeast ER may help to accommodate newly synthesized ER enzymes and inhibit the aggregation of unfolded proteins by reducing their concentration. The recently identified juxta-nuclear quality control (JUNQ) compartment serves as a temporary storage site for misfolded ubiquitylated proteins that cannot be folded or degraded because of the limited capacity of the ubiquitin-proteasome system under certain stress conditions (Kaganovich et al. 2008).

The term "macroautophagy" designates a cellular process leading to the nonselective sequestration of cytoplasmic components in double-membrane vesicles, called autophagosomes, for degradation in the vacuole/lysosome (Levine and Klionsky 2004). Activation of the UPR in yeast induces the formation of autophagosomes that selectively include ER membranes, their delimiting double membranes also being partially derived from the ER (Bernales et al. 2007). This so-called ER-phagy can remove damaged parts of the ER and participate in reducing the size of the ER back to normal once the folding stress is gone. ER-phagy could therefore represent an important degradative function of the UPR and be an integral player in achieving homeostatic control (Bernales et al. 2007). *y*-ATG19, a member of the cytoplasm-to-vacuole targeting (Cvt) pathway, increases the degradation of the misfolded Pma1-D378T form of the plasma membrane H^+-ATPase. Therefore, the efficient degradation of Pma1-D378T requires the cooperation of the ERAD and Cvt/autophagy pathways (Mazon et al. 2007).

14.4 ERAD Substrates Linked to Human Diseases

14.4.1 Regulation of Net Sodium Balance and Hypertension

Net sodium balance in humans is maintained through various ion transporters expressed along the entire nephron. Of these ion transporters, epithelial sodium channels (ENaCs), located along the aldosterone-sensitive distal nephron, play a pivotal role in the homeostasis of sodium balance. An increased abundance and activity of ENaCs in the plasma membrane causes hereditary hypertension, such as Liddle syndrome. The E3 ubiquitin ligase Nedd4-2 binds to the PY motif in the C-terminus of ENaC and catalyzes the ubiquitination of the NH_2 terminus for subsequent degradation (Staub and Rotin 2006). ENaC and Nedd4–2 are phosphorylation

substrates for the short-lived serum- and glucocorticoid-induced kinase (Sgk1), the expression of which is stimulated by aldosterone (Chen et al. 1999). Phosphorylation of Nedd4–2 results in the recruitment of 14-3-3 proteins to the phosphorylated sites, leading to a reduced interaction between Nedd4–2 and ENaC and causing reduced ubiquitylation of ENaC and its accumulation at the plasma membrane. Sgk1 is ubiquitylated by the action of UBE2J1-2 (UBC6), UBE2G1-2 (UBC7) and the E3 ligase HRD1 (synoviolin) (Arteaga et al. 2006). Sgk1 may be a key component of the cellular stress response, and HRD1 is upregulated to protect the cell against ER stress-induced apoptosis by degrading unfolded proteins accumulated in the ER (Arteaga et al. 2006). To be functional *in vitro*, HRD1/synoviolin requires UBE2G2, although its cognate E2 enzyme in cells has not been identified (Nadav et al. 2003; Kikkert et al. 2004; Omura et al. 2006).

14.4.2 Rheumatoid Arthritis

Rheumatoid arthritis is a disease associated with painful joints that affects approximately 1% of the population worldwide and for which no specific cure is available. Rheumatoid synovial cells produce large amounts of various proteins, such as cytokines and proteases, which might confer an autonomous proliferation property on the cells. The overexpression of HRD1/synoviolin results in a 'hyper-ERAD' state, allowing the cells to deal with the accumulation of unfolded proteins (about 30% of all newly synthesized, ER-sorted proteins) (Yagishita et al. 2008). Systemic overexpression of HRD1 in the mouse causes spontaneous arthropathy, with marked joint swelling, at 20 weeks of age in 30% of the mice analysed. Analysis of the affected joints shows bone destruction and severe synovial cell hyperplasia. HRD1-overexpressing mice exhibit pathologic features similar to those of rheumatoid arthritis. It has been hypothesized that the anti-apoptotic effects of HRD1 cause arthropathy by triggering synovial cell outgrowth (Amano et al. 2003).

14.4.3 Cystic Fibrosis

Cystic fibrosis arises from the misfolding and premature degradation of the CFTR chloride channel, an ABC-transporter located in the plasma membrane of epithelial cells (Ward et al. 1995). An ER membrane-associated ubiquitin ligase complex containing the E3 RMA1, the E2 UBE2J1 and Derlin-1 cooperates with the cytosolic HSC70-CHIP E4 complex to promote the proteasomal degradation of CFTRΔF508, a mutant form in which phenylalanine 508 is deleted (Meacham et al. 2001; Younger et al. 2006). RMA1 can recognize folding defects in the mutated transporter coincidentally with translation, whereas CHIP appears to act post-translationally. CHIP reduces the ATPase activity of HSC70 and HSP70 and inhibits

the HSC70-HSP70 substrate-binding cycle (Meacham et al. 2001; Younger et al. 2006). CHIP activity is dependent on the C-terminal U box, a domain that shares similarity with yeast Ufd2p (Hoppe 2005).

14.4.4 Progressive Familial Intrahepatic Cholestasis Type II

Bile secretion is mediated by several ABC-transporters located in the canalicular membrane of the hepatocyte (Alrefai and Gill 2007). Progressive familial intrahepatic cholestasis type II (PFIC II) is associated with mutations in the bile salt export pump Bsep. Rat Bsep variants carrying PFIC II mutations are degraded by the proteasome through the ERAD pathway. The E3 ubiquitin ligases Rma1 and TEB4 contribute to the degradation of the G238V variant containing a cytosolic lesion, whereas HRD1 contributes to the degradation of a mutant lacking the luminal glycosylation domain (Wang et al. 2008).

14.4.5 Drug Efflux Pumps and Xenobiotic Metabolism

The human ABCG2 protein is a half ABC transporter bearing a single ATP-binding fold at the NH_2-terminus and containing six transmembrane domains. It is located in the plasma membrane, where it forms homodimers bound through disulphide-bonded cysteine residues. This efflux pump is suggested to be responsible for protecting the body against toxic xenobiotics and metabolites. The formation of an intra-molecular disulphide bond between Cys592 and Cys608 and *N*-glycosylation at Asn596 are critical check points for the stability and degradation of the *de novo* synthesized ABCG2 protein (Wakabayashi-Nakao et al. 2009). Non-synonymous single nucleotide polymorphisms, such as Q141K, F208S, and S441N, have also been found to greatly affect the stability of ABCG2 in the ER and to enhance the protein degradation rate via ubiquitination and proteasomal proteolysis (Wakabayashi-Nakao et al. 2009). The identity of the E3 ligase involved is unknown.

14.4.6 Accumulation of a G-Protein-Coupled Transporter and Neurodegeneration

Parkin, the gene product of *PARK2*, is an E3 ubiquitin ligase that is required for the degradation of several substrates, including Parkin-associated endothelin receptor-like receptor (Pael-R) (Imai et al. 2001). Accumulation of Pael-R in the ER of dopaminergic neurons induces ER stress leading to neurodegeneration (Imai et al. 2001). CHIP facilitates Parkin-mediated Pael-R ubiquitylation by promoting the dissociation of HSP70 from Parkin and Pael-R. CHIP also enhances the ability of

Parkin to inhibit cell death induced by Pael-R (Imai et al. 2002). Unfolded Pael-R interacts with HRD1, via its proline-rich region, and the disruption of endogenous *HRD1* by small interfering RNA induces Pael-R accumulation and caspase-3 activation (Omura et al. 2006). ATF6 overexpression, which induces *HRD1* through the UPR pathway, increases Pael-R degradation. These results suggest that in addition to Parkin, HRD1 is also involved in the degradation of Pael-R (Omura et al. 2006).

14.4.7 SOD1 and Amyotrophic Lateral Sclerosis

Human copper/zinc superoxide dismutase (SOD1) is responsible for destroying free superoxide radicals in the body. SOD1 is a soluble cytoplasmic protein that functions as a homodimer to convert naturally-occurring, but harmful, superoxide radicals into molecular oxygen and hydrogen peroxide. Mutations in the *SOD1* gene have been implicated as one of the causes of familial amyotrophic lateral sclerosis (ALS). In mouse motor neurons and human embryonic kidney cells expressing the G93A variant, mutant SOD1 protein (SOD^{mut}) interacts with the C-terminal cytoplasmic region of Derlin-1 and triggers ER stress by causing dysfunction of ER-associated degradation (Nishitoh et al. 2008). SOD^{mut} activates apoptosis signal-regulating kinase 1 (ASK1) by triggering ER stress-induced IRE1 activation in mouse motor neurons. Dissociation of SOD^{mut} from Derlin-1 protects motor neurons from SOD^{mut}-induced cell death. Furthermore, deletion of *ASK1* partially mitigates motor neuron loss *in vitro* and extends the life-span of SOD^{mut} transgenic mice. These results indicate that the interaction of SOD^{mut} with Derlin-1 is crucial for disease progression in familial ALS (Nishitoh et al. 2008).

14.5 Concluding Remarks

Eukaryotic cells have several quality control systems to ensure that only correctly folded proteins are transported along the secretory pathway. The use of modular ERAD substrates has shown that newly-synthesized ER-transmembrane proteins are monitored by a two-step quality control and targeted to the proteasome for degradation, through distinct pathways. The molecular mechanisms underlying retrotranslocation and the protein composition of the channel remain to be determined. Analysis of additional substrates and the identification of new factors that are required for regulation of ER-associated degradation will help to define pharmaceutical strategies to control the degradation of membrane transporters that are associated with human disease.

Acknowledgements We apologize to those whose work we were unable to cite due to space constraints. N. C is supported by a grant from the Fonds pour la formation à la Recherche dans l'Industrie et dans l'Agriculture (F.R.I.A.).

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