

Chapter 7

Epithelial Ion Channel Folding and ER-Associated Degradation (ERAD)



Teresa M. Buck and Jeffrey L. Brodsky

Abstract Epithelial ion channels and transporters play critical roles in salt and water homeostasis. The expression, assembly, and trafficking of these proteins are tightly regulated at both the transcriptional and posttranscriptional levels. For example, ion channel residence at the plasma membrane is regulated in response to intra- and extracellular signals that can alter anterograde trafficking, endocytosis, and/or degradation. While regulation at the plasma membrane has been thoroughly characterized for a number of ion channels and transporters, the mechanisms underlying early events in the endoplasmic reticulum (ER) have only more recently been defined. Notably, all epithelial ion channels and transporters are synthesized at and insert into the ER membrane, at which time posttranslational modifications are added, the protein begins to fold, and subunits of oligomeric proteins assemble. However, before epithelial ion channels and transporters can traffic from the ER, they are subject to protein quality control “decisions,” which ensure that only properly modified, folded, and assembled subunits are directed to the Golgi apparatus and ultimately to the plasma membrane via vesicle intermediates. Here, we first describe the general steps to which ion channels and transporters are subject in the ER and highlight the factors that facilitate protein folding. We will also discuss the events that lead to the degradation of improperly modified, misfolded, and misassembled proteins in the ER. This pathway is known as ER-associated degradation, or ERAD. We will then provide specific examples of critical epithelial proteins that undergo these events in ER protein quality control.

Keywords Endoplasmic reticulum (ER) · ER-associated degradation (ERAD) · Kidney · Ion channels · Ion transporters · Molecular chaperones · ENaC · Na,K-ATPase · ROMK · V2R · NCC · Aquaporin-2

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7.1 Introduction

Approximately one-third of all cellular proteins, including all membrane proteins, transit through the secretory pathway (Ghaemmaghami et al. 2003). The translation of these secretory proteins begins in the cytosol. Signal recognition particle (SRP) binds to either an N-terminal signal sequence or the first transmembrane domain (TMD) of a nascent protein as it emerges from the ribosome and stalls translation (Walter and Blobel 1981; Halic et al. 2004, 2006). The SRP–ribosome nascent chain complex is transferred to the ER membrane by virtue of SRP binding to the SRP receptor, which facilitates ribosome docking to the Sec61 translocation channel in the ER membrane (Meyer and Dobberstein 1980; Gilmore et al. 1982). After the ribosome docks onto Sec61, translation resumes and secretory proteins are cotranslationally translocated into the ER lumen through Sec61 (Fig. 7.1) (Gorlich et al. 1992). For secretory proteins, the signal sequence may be cleaved by signal peptidase (Fig. 7.1a). For membrane proteins, such as epithelial ion channels and transporters, the TMDs move laterally through a Sec61 “gate” and are thus integrated into the lipid bilayer. Alternating TMDs act as either signal sequences or stop transfer sequences (Fig. 7.1b–c) (Egea and Stroud 2010). After membrane integration is complete, downstream events—such as protein folding, oligomeric protein assembly, and posttranslational modifications—occur. These events precede a quality control checkpoint which ensures that only properly folded proteins, and especially membrane proteins, can leave the ER and traffic to the plasma membrane (Barlowe and Helenius 2016).

7.2 Protein Folding in the Endoplasmic Reticulum

The integration of multi-pass ion-conducting membrane proteins is particularly challenging for the following reasons. First, ion-conducting pores are lined with polar residues that are embedded in the lipid bilayer. In fact, an analysis of all known TMDs predicts that ~30% are only marginally hydrophobic and may not integrate efficiently into the ER membrane (Hessa et al. 2007). Therefore, TMDs either from within the protein or from another subunit in an oligomeric complex are often required to stabilize polar TMDs (Beguin et al. 1998, 2000; Lu et al. 2000; Buck et al. 2007). Second, the final structure of a membrane protein requires the assembly of TMDs that might be synthesized early (i.e., at the N-terminus) and later (i.e., at the C-terminus). Thus, a TMD at the N-terminus might be orphaned for an extended time until it can assemble with a partner TMD. Third, the folding of a membrane protein takes place in three chemically distinct compartments: The ER lumen, which will ultimately be extracellular, the ER lipid bilayer, and the cytoplasm (Fig. 7.1). With few exceptions, it is generally unclear how the folding of a protein in these environments is coordinated. Finally, ribosomes synthesizing subunits of an oligomeric protein may not utilize adjacent Sec61 pores, so after membrane insertion each

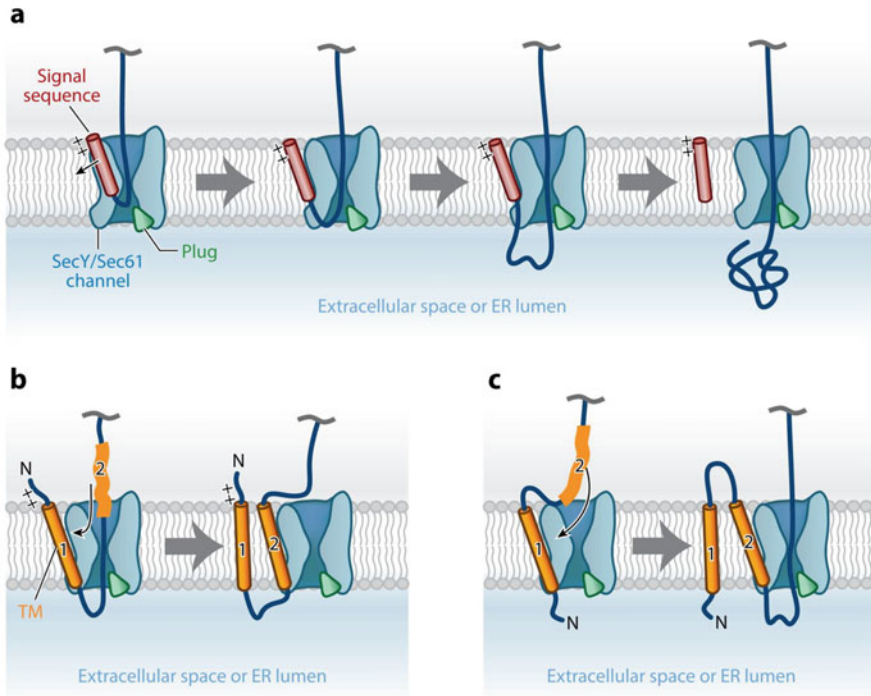


Fig. 7.1 Protein translocation. The ribosome nascent chain complex docks at the Sec61 channel. For soluble proteins (a) a signal sequence orients the polypeptide with positively charged amino acids facing the cytosol. The signal sequence is cleaved by signal peptidase prior to translocation of the nascent polypeptide chain into the ER lumen. For membrane proteins (b and c) the first transmembrane domain acts as a signal sequence to orient the N-terminus either toward the cytosol (b) or toward the ER lumen (extracellular) (c). Transmembrane domains move through a lateral gate in the Sec61 channel into the lipid bilayer. Subsequent transmembrane domains act as either stop transfers (b) or signal anchors (c) to position the more C-terminal transmembrane domains appropriately. Figure republished with permission of Rapoport et al. (2017). Permission conveyed through Copyright Clearance Center, Inc

subunit will need to diffuse in the plane of the ER to find one another. To overcome some of these hurdles, protein folding is aided by the contributions of other factors.

7.2.1 The Role of Molecular Chaperones in Protein Folding

Molecular chaperones bind nascent polypeptide chains as they emerge from the ribosome and facilitate folding, prevent aggregation, promote proper oligomerization, and/or trafficking from the ER. Chaperones continue to act often through cycles of binding and release until a mature protein conformation is reached. Chaperones

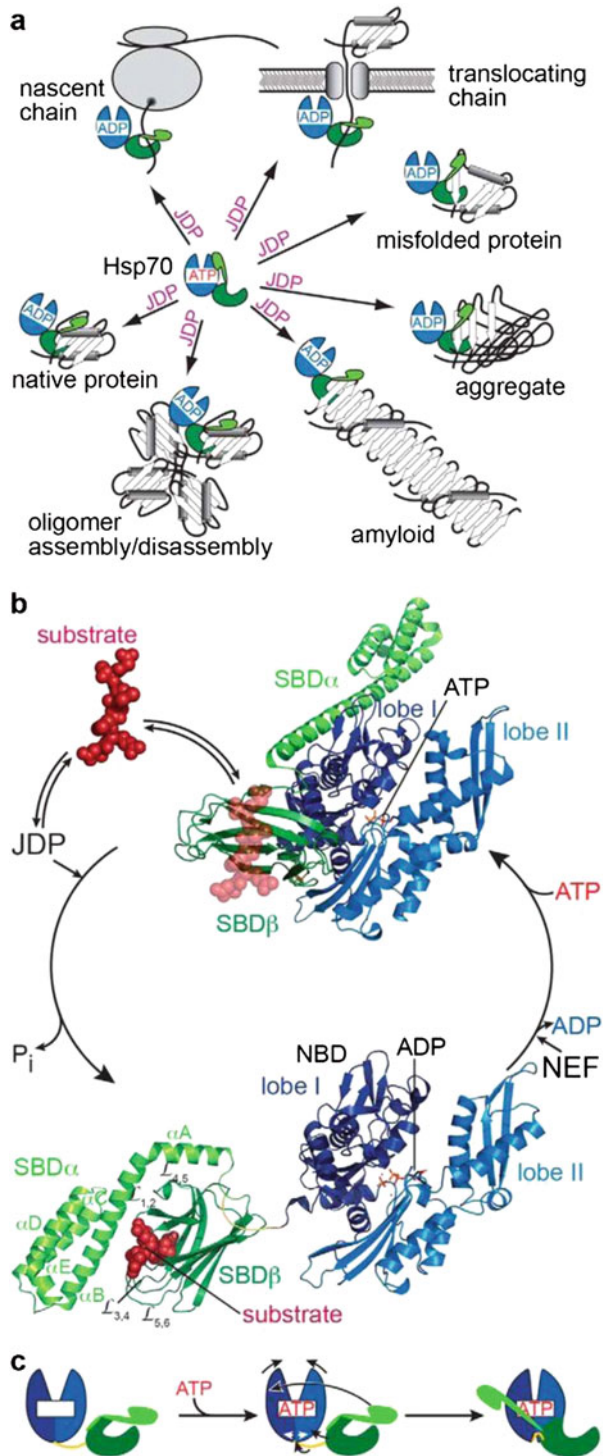
are a structurally diverse group of proteins that include Hsp70s, Hsp90s, lectins, thiol oxidoreductases, and small heat shock proteins (Buchner 2019; Genest et al. 2019; Lang et al. 2017), each of which are required for the quality control of ion channels and transporters.

One of the most abundant and promiscuous chaperones are the Hsp70s (Fig. 7.2). Hsp70s are located in both the cytosol and the ER and participate in ATP-dependent protein folding. Hsp70s are composed of an N-terminal nucleotide-binding domain (NBD) and a substrate-binding domain (SBD) composed of eight β strands and C-terminal helical “lid domain” that is connected to the SBD via a linker (Fig. 7.2b) (Pobre et al. 2019; Alderson et al. 2016). In the ATP bound state, Hsp70 binds weakly to substrates. ATP hydrolysis, stimulated by the J-domain containing Hsp40s (Fig. 7.2a) (Misselwitz et al. 1998; Kityk et al. 2018), results in a conformational change whereby the “lid” closes over the SBD, which results in a tightly bound state (Fig. 7.2c) (Mayer and Kityk 2015; Mayer 2018). In addition to stimulating Hsp70 ATPase activity, Hsp40s deliver substrate to the Hsp70 and may provide specificity (Fig. 7.2a). In humans, there are 13 Hsp70s and ~50 Hsp40s (Kampinga et al. 2009). After ATP hydrolysis, a second class of co-chaperones, the nucleotide exchange factors (NEFs), stimulate the release of both the hydrolyzed nucleotide (i.e., ADP) and substrate (Fig. 7.2b) (Bracher and Verghese 2015). If the protein fails to reach a mature confirmation, Hsp70 may reengage the substrate to support additional rounds of folding. Alternatively, Hsp70s can target misfolded proteins in the ER for degradation, which is described below.

7.2.2 *The Role of the Chaperone-Like Lectins in Protein Folding*

Another class of ER chaperones, the glycan-binding lectins, recognize proteins containing *N*-linked glycans. Glycans are appended cotranslationally to polypeptides by the oligosaccharyl-transferase, which appends a core-glycan (GlcNAc₂Man₉Glc₃) to an Asn in the sequence Asn-X-Ser/Thr (where X can be any amino acid except Pro) (Fig. 7.3a). The glycan is then trimmed by glucosidases to yield a mono-glucosylated glycan (GlcNAc₂Man₉Glc), which enables the binding of two lectins, calnexin (CNX) and calreticulin (CRT) (Fig. 7.3b). CNX is a type I membrane protein, and CRT is a soluble paralog. While CNX/CRT binding was initially thought to be entirely glycan dependent, later evidence indicated that CNX/CRT can bind polypeptides in the absence of glycans (Danilczyk and Williams 2001; Sandhu et al. 2007; Brockmeier et al. 2009). CNX/CRT can also recruit a thiooxidoreductase as well as a peptidyl-prolyl isomerase to respectively catalyze the formation of disulfide bonds in the ER and the interconversion of *trans*–*cis* Pro, both of which augment protein folding. In most cases, these events are sufficient to permit the mature protein to exit the ER after the action of another glucosidase. However, if folding is problematic, the UDP-glucose glycoprotein glucosyltransferase (UGGT1)

Fig. 7.2 Hsp70 cycle, cellular roles, and conformational changes. **(a)** Hsp40 J-domain proteins (JDPs) provide specificity for the diverse cellular roles and various substrates of Hsp70s. **(b)** Hsp70 ATPase/substrate binding cycle as described in the text. Substrate (dark red) is targeted to Hsp70 by JDPs. Structures of bacterial DnaK in the ATP bound open conformation (PDB 4B9Q) (Kityk et al. 2012) and the ADP-bound closed conformation (PDB 2KHO) (Bertelsen et al. 2009) bound to substrate (Zhu et al. 1996) are shown. Substrate-binding domain (SBD) is green. The nucleotide-binding domain (dark lobe I and light blue lobe II) linker between SBD and NBD is yellow. Nucleotide exchange factors (NEFs) promote release of ADP and ATP rebinding. **(c)** Model of Hsp70 conformational changes induced by ATP-binding and hydrolysis. Figure republished with permission of Mayer and Gierasch (2019). Permission conveyed through Copyright Clearance Center, Inc



acts as a protein folding sensor. This enzyme adds a single glucose back to the core glycan, preventing ER exit and allowing for CNX/CRT reassociation (Fig. 7.3b) (Cherepanova et al. 2016). In principle, the cycle of glucose re-addition by the glucosyltransferase, favoring CNX/CRT binding, and glucose removal could continue indefinitely if the protein has failed to mature. However, proteins that exit the CNX/CRT cycle prior to reaching a mature state can be modified by EDEM (ER degradation enhancing α -mannosidase-like protein) (Ninagawa et al. 2014), thereby generating a substrate that is targeted for ERAD (Figs. 7.3b–c and 7.4, step

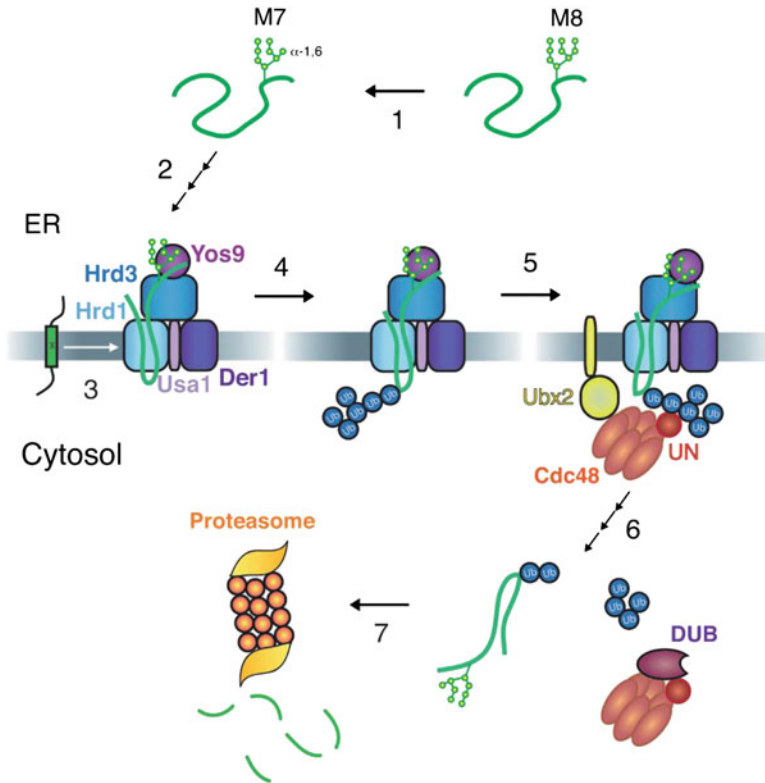


Fig. 7.4 ERAD-L/M pathway. *Step 1:* N-linked glycans are trimmed from 8 to 7 mannose residues (depicted as green circles). *Step 2:* The lectin Yos9 next binds to the terminal α 1,6-linked mannose residues and the misfolded protein is inserted into Hrd1 channel with assistance from Hrd3 and Der1 (see discussion of retrotranslocation for alternative scenarios). *Step 3:* A single spanning membrane protein (ERAD-M substrate) interacts with Hrd1 through misfolded TMDs. *Step 4:* Substrate is ubiquitinated by the E3 ligase Hrd1. *Step 5:* Cdc48 (p97 in mammals) binds Ubx2 and the Cdc48 cofactors, Ufd1/Npl4 (UN) bind to the ubiquitinated substrate. *Step 6:* The substrate is retrotranslocated from the ER to the cytosol using the energy generated by Cdc48 ATP-hydrolysis. A deubiquitinating enzyme (DUB) trims the ubiquitin chain triggering substrate release from the Cdc48 complex. *Step 7:* The substrate is degraded by the 26S proteasome. Figure republished with permission of Wu and Rapoport (2018). Permission conveyed through Copyright Clearance Center, Inc

1–2). Although this cycle has been established for several model proteins, it remains unclear whether all proteins, especially membrane proteins, are subject to this cycle. In addition, some proteins are not glycosylated or the glycans may not be accessible to the enzymes that regulate protein retention and ERAD. Recent evidence indicates that other lectins, XTP-3B and Os9, help “decode” substrates for ERAD (van der Goot et al. 2018).

7.3 Endoplasmic Reticulum-Associated Degradation

During ERAD, substrates are first recognized by molecular chaperones (Ismail and Ng 2006; Meusser et al. 2005; Vembar and Brodsky 2008; Hebert and Molinari 2012; Rutkevich and Williams 2011; Young 2014). Following recognition, an integral membrane ERAD substrate—such as an ion channel or transporter—is next ubiquitinated by a series of ER-associated ubiquitin ligases (Fig. 7.4, step 3–5) (Preston and Brodsky 2017). The protein is then “retrotranslocated” or removed from the ER and transported to the cytosol through the action of an energy requiring complex that associates with ubiquitinated proteins (Fig. 7.4, step 6) (Bodnar and Rapoport 2017a). Finally, the ubiquitinated substrate is delivered to and degraded by the cytosolic 26S proteasome (Fig. 7.4, step 7) (Hiller et al. 1996; Hampton et al. 1996; Raasi and Wolf 2007).

7.3.1 Recognition of ERAD Substrates

Chaperones recognize folding lesions that include exposed hydrophobic patches, unpaired charged residues within membrane domains, or discrete sequences termed “degrons” that target proteins for degradation. Extensive experiments to characterize the degradation pathway of a diverse group of ERAD substrates have led to their placement in three categories based on the site of the folding lesion: ERAD-L (ER lumen), ERAD-M (membrane), or ERAD-C (cytosolic) (Vashist and Ng 2004; Taxis et al. 2003; Carvalho et al. 2006). When examined using model substrates, each class requires a unique set of factors for ERAD targeting. We will briefly discuss these requirements here, which were developed using yeast, but we note that the rules positioning substrates into these unique groups do not necessarily hold up for membrane proteins in mammalian cells (Bernasconi et al. 2010).

ERAD-L substrates are soluble proteins and are recognized by ER luminal chaperones, including an Hsp70, BiP, along with Hsp40 and NEF cochaperones, select lectins, and thiol oxidoreductases. In addition to its role in protein folding, BiP identifies and targets ERAD-L substrates for ERAD and acts in conjunction with a subset of the seven Hsp40s (ERdj1-7) in the ER lumen (Pobre et al. 2019). For example, ERdj3 binds transiently to substrate through conserved, hydrophobic residues before delivering the substrate to BiP. Like most Hsp40s, ERdj3 is a

dimer and interacts with BiP through the 70 amino acid J-domain. Once identified, BiP directs misfolded proteins to the mannose trimming EDEM proteins (Oda et al. 2003) and then on to a membrane complex for retrotranslocation and ubiquitination.

Due to the fact that the folding lesion resides within the membrane, ERAD-M substrates generally require fewer chaperones for substrate recognition. BiP, Hsp40s, and lectins are often dispensable to target ERAD-M substrates for degradation (Gardner et al. 2000; Sato et al. 2009). ERAD-M substrates rely instead on the Hrd1 complex to recognize misfolded lesions within the membrane (Figs. 7.4 and 7.5) (Sato et al. 2009). In one model, the exposure of hydrophilic or charged residues within the membrane may act as a folding lesion, which is in contrast to the exposure of hydrophobic residues in soluble proteins (e.g., ERAD-L substrates) or the cytoplasmic domain in ERAD-C proteins. To date, the mechanisms that lead to the selection of ion channels and transporters that are targeted for ERAD have not been defined.

These substrates are integral membrane proteins with folding lesions facing the cytoplasm and are recognized and targeted for degradation by cytosolic chaperones. For example, a well-characterized ion channel target of the ERAD pathway is the Cystic Fibrosis Transmembrane Conductance Regulator, CFTR. Mutations in CFTR are responsible for Cystic Fibrosis and the most prevalent CFTR mutant, F508del, results in ER retention and premature degradation by the ERAD pathway (Cheng et al. 1990). The bulk of the CFTR protein resides in the cytosol and consequently cytosolic chaperones are required to target CFTR for ERAD, whereas the ER luminal chaperones are dispensable (Zhang et al. 2001). For example, cytosolic Hsp70 and sHsps are required for CFTR degradation (Rubenstein and Zeitlin 2000; Ahner et al. 2007) (also see Chap. 6 of this volume and Volume 3, Chaps. 15 and 16).

7.3.2 Ubiquitination of ERAD Substrates

The ubiquitination of an ERAD substrate occurs through a series of three enzymatic reactions carried out by an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme and an E3 ubiquitin ligase. The ubiquitin-activating enzyme catalyzes the formation of a high-energy thioester bond between the C-terminus of ubiquitin and an active site cysteine residue in an ATP-dependent reaction. The ubiquitin is then transferred to the active-site cysteine in an ubiquitin-conjugating enzyme. An E3 enzyme next facilitates the interaction between an E2 and the substrate and the transfer of ubiquitin to, most commonly, a Lys side chain. Ubiquitin conjugation onto Cys, Ser, Thr, or the N-terminus has also been observed (Shimizu et al. 2010; Swatek and Komander 2016). Polyubiquitin chains are formed when ubiquitin is appended to one of the seven Lys residues in ubiquitin (K6, K11, K27, K29, K33, K48, and K63). K48 polyubiquitin chains are the most common ubiquitin linkage for proteasomal degradation, and specificity of the linkage is provided by the E2. At least 4 ubiquitins in series are required for proteasome-

dependent degradation. There are ~100 ubiquitin ligases in yeast and ~600 in humans, which reflects the role of these enzymes in substrate recognition, whereas there are only ~38 E2s and 2 E1s in humans (Mehrtash and Hochstrasser 2018). Hrd1 and Doa10 are the dedicated E3s for the ERAD pathway in yeast, and conserved homologs (HRD1 and gp78, and TEB4/MARCH6, respectively) function similarly in human cells. Each of these ligases is an ER membrane protein with the catalytic “RING” domains deposited in the cytosol. Other mammalian E3s associated with the ERAD pathway include TRC8, THEM129, Rfp2, RMA1, RNF145, RNF170, and RNF185 (Stagg et al. 2009; van den Boomen et al. 2014; Younger et al. 2006; Lu et al. 2011; El Khouri et al. 2013; Jiang et al. 2018; Lerner et al. 2007).

To facilitate substrate ubiquitination, each ligase requires somewhat different ubiquitin-conjugating enzymes. Hrd1 acts with Ubc7 in yeast to ubiquitinate ERAD-L or ERAD-M substrates. Ubc7 is tethered to the ER membrane by a membrane protein, Cue1, which directly activates conjugating activity (Fig. 7.5) (Metzger et al. 2013). Hrd1 also forms oligomers that are stabilized by another

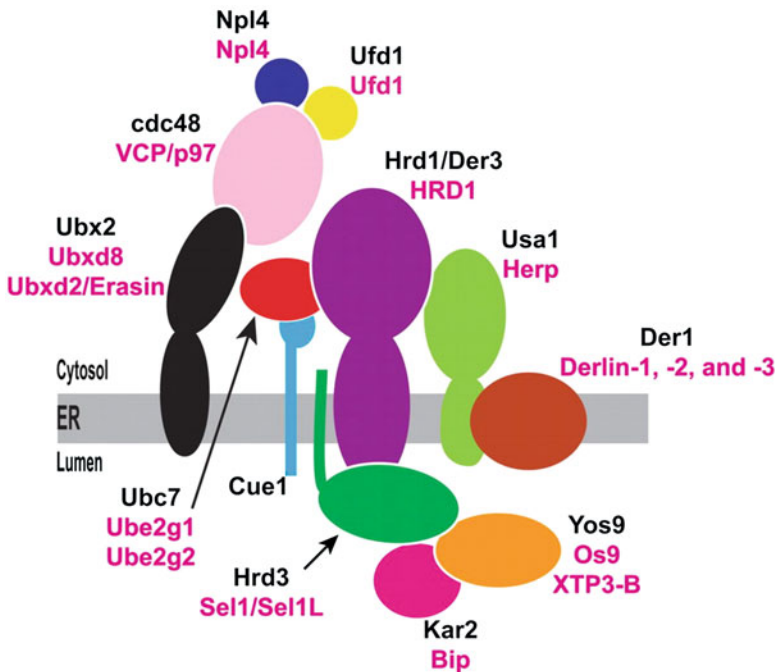


Fig. 7.5 Hrd1 E3 Ligase complex. Model of the *Saccharomyces cerevisiae* Hrd1 complex required for the degradation of ERAD-L substrates (black). Mammalian homologs are indicated in pink text. Key factors include the putative retrotranslocation channel, Hrd1, the adapter proteins, Usa1, Hrd3, and Der1, that complex with Hrd1, the Cdc48-Ufd1 complex (Npl4 is not shown) that retrotranslocates substrate, components of the ubiquitination machinery (Ubx2, Ubc7, Cue1) and chaperones, Kar2 and Yos9. Figure republished with permission of (Nguyen et al. 2009). Permission conveyed through Copyright Clearance Center, Inc

membrane protein, Usa1, which enhances ubiquitination activity (Horn et al. 2009; Carvalho et al. 2010). In addition, oligomerization increases the activity of gp78, the mammalian homolog of Hrd1, and gp78 can build polyubiquitin chains on the E2, Ube2g2, which in vitro can be transferred *en bloc* to an ERAD substrate, thereby increasing ubiquitination efficiency (Li et al. 2007). It is not clear whether this occurs in vivo. In contrast, Doa10 functions with both Ubc6 and Ubc7. Unlike Ubc7, Ubc6 is a tail-anchored protein in the ER membrane. Doa10 ubiquitinates ERAD-C substrates but exceptions to this rule have been noted (Habeck et al. 2015). Recently, the role of a conserved 16 amino acid, C-terminal element (CTE) in Doa10 was identified. Mutations in the CTE block the ubiquitination and degradation of select Doa10-dependent substrates. Therefore, the CTE may be important for substrate recognition. Mutations in the CTE of the mammalian homolog, MARCH6/TEB4, resulted in a phenotype similar to the Doa10 mutant (Zattas et al. 2016). Even though the action of these enzymes on the degradation of several ERAD substrates has been noted, the rules that define mechanisms underlying E3-dependent substrate selection are ill defined.

7.3.3 Retrotranslocation of ERAD Substrates

The driving force for the extraction or retrotranslocation of proteins from the ER into the cytosol is provided by the p97 complex (or Cdc48 complex in yeast) (Bays et al. 2001; Jarosch et al. 2002; Rabinovich et al. 2002; Ye et al. 2001). p97/Cdc48 is a hexameric AAA⁺-ATPase and hydrolyzes ATP to spool proteins through a central aperture (Bodnar and Rapoport 2017b; Ripstein et al. 2017). Each p97/Cdc48 monomer contains two ATPase domains (D1 and D2) and an N-terminal domain (N). The two ATPase domains are stacked on top of one another. The N-terminal domains extend from the D1 ring, and movement of the N domains are coupled to ATP hydrolysis (Tang et al. 2010; Bodnar et al. 2018). p97/Cdc48 is involved in a variety of cellular activities that, in addition to ERAD, include mitochondrial associated degradation, removal of proteins from chromatin, membrane fusion, and vesicular trafficking (Xia et al. 2016). To date, ~38 p97 binding partners and cofactors have been identified, which dictate the cellular activity of p97 (Hanzelmann and Schindelin 2017).

During ERAD, the p97/Cdc48 cofactors—Ufd1 and Npl4—recognize polyubiquitinated proteins (Meyer et al. 2000, 2002). In addition to providing the driving force for retrotranslocation, the enzyme acts as an “unfoldase” (Bodnar et al. 2018). After recognition of a ubiquitinated substrate, ATP binds the D1 ring and the enzyme unfolds the protein using energy provided by ATP hydrolysis in the D2 ring. Partial trimming of the ubiquitin chain by a deubiquitinating enzyme may facilitate entry into the p97/Cdc48 central pore (Bodnar and Rapoport 2017b). ERAD remains proficient in a yeast strain lacking the deubiquitinating enzyme, suggesting that functional analogs are involved in retrotranslocation (Stein et al. 2014).

Does the retrotranslocation of an ERAD substrate require a dedicated channel? Early studies suggested that Sec61 (see, Fig. 7.1) is also required for the retrotranslocation and degradation of soluble ERAD substrates (Pilon et al. 1997, 1998; Wiertz et al. 1996; Plemper et al. 1997; Willer et al. 2008). However, later studies determined that Sec61 is dispensable for their retrotranslocation (Sato and Hampton 2006; Wahlman et al. 2007; Garza et al. 2009). In yeast, recent data strongly suggest that the E3 ubiquitin ligase, Hrd1, can also serve as a retrotranslocation channel for soluble substrates. For example, Hrd1 is necessary and sufficient for the retrotranslocation of an ER luminal protein *in vitro* (Carvalho et al. 2010), and the structure of Hrd1 displays channel-like properties (Schoebel et al. 2017). In fact, Hrd1 links ERAD-requiring proteins both in the ER lumen and in the cytoplasm (Nakatsukasa et al. 2013). Nevertheless, the solubilization and extraction of membrane proteins require associated proteins, such as Der1 and Dfm1 (Gauss et al. 2006; Stein et al. 2014; Willer et al. 2008; Garza et al. 2009; Mehnert et al. 2014; Neal et al. 2018).

Yeast Der1 (Derlin in mammals) is an ER resident transmembrane protein required for the degradation of both soluble and membrane proteins, i.e., ERAD-L and ERAD-M substrates (Knop et al. 1996; Vashist et al. 2001; Neal et al. 2018; Hitt and Wolf 2004; Vashist and Ng 2004; Baldrige and Rapoport 2016). Der1 coprecipitates with other proteins involved in the ERAD pathway, and the association between Hrd1 and Der1 is mediated by Usa1 (Fig. 7.5) (Horn et al. 2009; Mehnert et al. 2014). p97 also binds to Der1 in an Hrd1-dependent manner (Gauss et al. 2006), consistent with Der1's role as an accessory during retrotranslocation (Fig. 7.5). The relationship between Der1 was further delineated via a site-specific cross-linking study in which it was established that the transmembrane domains of Der1 are in direct contact with both Hrd1 and Hrd3 and the luminal domains and the transmembrane domains bind an ERAD substrate (Mehnert et al. 2014). Consistent with a direct role for Der1 in retrotranslocation, antibodies against Der1 inhibited the retrotranslocation of a soluble ERAD substrate that could be observed in real time (Wahlman et al. 2007). Together, Hrd3, which binds BiP (Mehnert et al. 2015), may recruit ERAD clients to Der1 (Fig. 7.5), and as the substrate emerges on the cytosolic face of the ER through the action of Hrd1, it is ubiquitinated by the cytoplasmic RING domain in Hrd1 (Mehnert et al. 2014).

Both Der1/Derlin and Dfm1 are members of the rhomboid protease family, which clip TMDs within membrane-spanning domains. However, both proteins lack critical catalytic residues and are therefore considered inactive rhomboids. A recent study indicated that rhomboids can distort and thin a lipid bilayer, suggesting that these proteins are unable to clip integral membrane ERAD substrates but instead help solubilize them prior to retrotranslocation (Kreutzberger et al. 2019). Consistent with this view, a genetic screen for ERAD effectors in yeast identified Dfm1 as being required for the degradation of integral membrane but not soluble ERAD substrates (Neal et al. 2018). Interestingly, yeast lacking Dfm1 rapidly acquire suppressor mutations that duplicate a region of the yeast chromosome that harbors the Hrd1 gene. Indeed, Hrd1 overexpression compensates for the deletion of Dfm1 (Neal et al. 2018). Together these data position a membrane complex, comprised of Hrd1 and

inactive rhomboids, as playing a central role in retrotranslocation, assisted by the action of the p97/Cdc48 ATPase (Fig. 7.5). It is currently unknown whether these factors are involved in the degradation of misfolded epithelial ion channels and transporters.

7.3.4 Degradation by the 26S Proteasome

Prior to proteasome-dependent degradation, accessory factors facilitate the processing and targeting of substrates to this multi-catalytic protease. First, N-linked glycans may be removed by the cytosolic N-glycanase, Png1. Png1 is recruited to the ER membrane by Cdc48 (Li et al. 2006; Suzuki et al. 2000), suggesting that it acts immediately after or with retrotranslocation. Second, Dsk2 and Rad23 harbor both ubiquitin association domains as well as ubiquitin-like domains, which allows them to shuttle substrates to the proteasome (Richly et al. 2005; Verma et al. 2004; Bard et al. 2018). In yeast, deletion of the genes encoding these factors has a minimal effect on ERAD, suggesting that they function with only a subset of substrates. Consistent with this view, mutations in the human Png1 homolog result in a catastrophic disease, which may arise from impaired mitochondrial function (Suzuki 2015; Kong et al. 2018).

The 26S proteasome is composed of the 20S core particle (CP) (Finley et al. 2016) and the 19S regulatory particle (RP). The 20S CP contains four stacked heteroheptameric rings. Two rings of β subunits are flanked by two rings of α subunits. Three out of the seven β subunits contain proteolytic activity (six total active sites). The 20S CP contains three protease activities, chymotrypsin-like, trypsin-like, and caspase-like that cleave after hydrophobic, basic, and acidic residues, respectively. The 20S core of the proteasome forms a chamber that reduces nonspecific degradation of cellular proteins (Bard et al. 2018).

The 19S RP is responsible for unfolding and translocating polypeptide substrates into the 20S proteolytic chamber. The 19S RP is made of a base and a lid. The base consists of a six subunit AAA-ATPase and non-ATPase subunits that have multiple sites for binding ubiquitin and ubiquitin-like proteins, such as Rad23 and Dsk2 (see above). The hexameric AAA-ATPase ring is at the center of the base and provides the “motor” necessary to translocate proteins into the 20S proteolytic chamber. The lid also contains deubiquitinating enzymes that remove ubiquitin chains prior to polypeptide translocation into the 20S CP.

Recently, two cryo-EM structures of a substrate-engaged human 26S proteasome were published (Dong et al. 2019; de la Pena et al. 2018). Based on data from these studies, updated molecular models for substrate processing by the proteasome were proposed. It was reported that the 19S RP can be found in up to seven unique allosteric conformations (Dong et al. 2019; de la Pena et al. 2018; Bard et al. 2018). These include conformational rearrangements of both the base and the lid domain of 19S RP in response to the nucleotide-bound state, the presence of substrate, occupancy of the deubiquitinating enzyme, and the occupancy of ubiquitin receptors.

Therefore, substrates are initially targeted through ubiquitin chains by ubiquitin receptors prior to an unstructured region of the substrate initiating ATP-dependent translocation into the CP. Substrate engagement triggers major structural and conformational changes to augment 19S RP-dependent peptide translocation (Bard et al. 2018).

7.4 Epithelial Ion Channels and Transporters Subject to ER Protein Quality Control

7.4.1 *The Na,K-ATPase*

The Na,K-ATPase pump is ubiquitously expressed in epithelia and can reside on either the apical or basolateral membrane. The Na,K-ATPase facilitates the transport of sodium and potassium across the membrane and generates ion gradients to maintain membrane potential and drive vectorial sodium-dependent transepithelial transport by pumping three Na⁺ ions into the extracellular space in exchange for two K⁺ ions (Jorgensen 1986). The Na,K-ATPase pump is a heterodimer composed of an α -subunit that contains the ion-transport activity, and a β -subunit that is essential for channel maturation and trafficking (Geering 2008). The α -subunit is a ten TMD-containing protein with N- and C-termini facing the cytosol (Blanco 2005). The β -subunit is a single TMD-spanning glycoprotein with a short cytosolic tail and large extracellular domain (Geering 2001; Blanco 2005). While some studies suggested alternative subunit ratios (Yoshimura et al. 2008; Clifford and Kaplan 2008) analysis of the high-resolution structures of the Na,K-ATPase confirms a 1:1 α : β stoichiometry (Morth et al. 2007; Shinoda et al. 2009). There are four isomers of the α -subunit (α_1 , α_2 , α_3 , and α_4) and three isoforms of the β -subunit (β_1 , β_2 , and β_3), and each show both tissue-specific and developmental differences in their expression patterns (Geering 2001; Blanco 2005). Each isoform also has unique kinetic properties and responds differently to substrate, ATP, and/or inhibitors (Blanco and Mercer 1998; Crambert et al. 2000). There is also evidence that the Na,K-ATPase is involved in cell adhesion and the maintenance of adherens junctions as the cytosolic domain of the pump binds E-cadherin and the ankyrin and spectrin cytoskeleton (Kizhatil et al. 2007; Nelson et al. 1990; Nelson and Veshnock 1987). The β -subunit dimerizes with β -subunits on opposing cells to maintain adherens junctions (Tokhtaeva et al. 2011; Padilla-Benavides et al. 2010). A more thorough discussion of the physiological roles and function of the Na,K-ATPase are provided in Volume 3, Chap. 1, of this series.

7.4.1.1 Assembly and ER-Associated Degradation of the Na,K-ATPase

In the absence of the β -subunit, the α -subunit is retained in the ER and targeted for ERAD (Beguin et al. 1998, 2000; Beggah et al. 1996, 1999). Several studies have provided insight into the mechanism by which the α -subunit is degraded (Beguin et al. 1998, 2000; Hasler et al. 2001). First, a series of truncated α -subunit TMD constructs were expressed in oocytes. Data indicated that the first four TMDs act alternatively as efficient “signal-anchor sequences” and “stop-transfer sequences” (see Fig. 7.1), thereby integrating TMD1-4 in the predicted topology (Beguin et al. 1998). However, efficient membrane integration of TMD6-10 required both C-terminal sequence information and the presence of the β -subunit. In the absence of the β -subunit, TMD7 was unable to act as a signal anchor sequence but remained in the cytosol. Therefore, the extracellular loop between TMD7-8, which contains a degradation signal (Beguin et al. 2000), is exposed to the cytosol, creating an unstable, immature protein (Beguin et al. 1998). Coexpressing the β -subunit promoted membrane integration of TMD7 and masked the degradation signal (Beguin et al. 2000). Membrane integration of the TMD7-8 pair resulted in proper localization of the TMD7-8 loop within the ER lumen and stabilized not only the full-length α -subunit but any construct containing at least TMD1-8. In the presence of the β -subunit, exposure of the degradation signal is only transient (Beguin et al. 1998, 2000). Thus, the β -subunit exhibits chaperone-like activity.

Although channel assembly is required for ER exit of the α_1 -subunit (Beguin et al. 1998, 2000; Beggah et al. 1996, 1999), whether oligomerization is a prerequisite for trafficking of the β -subunit is less clear (Noguchi et al. 1990). However, later studies established that all mature β -subunits were bound to α -subunits (Clifford and Kaplan 2008), and *Xenopus* oocyte β -subunits were ER retained in the absence of α -subunits (Ackermann and Geering 1990; Jaunin et al. 1992; Beggah et al. 1996). The α -subunit also appears to be the limiting factor for α - β assembly (Tokhtaeva et al. 2009). As with α -subunits, ER retained unassembled β subunits are rapidly degraded, and mutations in the β -subunit TMD at residues predicted to interact with α -subunit TMD7 or the β -subunit extracellular domain results in ER-retention of the β -subunit and reduced colocalization with the α -subunit (Shinoda et al. 2009). Corresponding mutations in the α -subunit similarly resulted in impaired assembly and ER retention of the subunits (Tokhtaeva et al. 2009). These studies are all consistent with an inability of the β -subunit to traffic to the Golgi in the absence of the α -subunit.

7.4.1.2 The Roles of Chaperones in Na,K-ATPase Regulation

The role of molecular chaperones in Na,K-ATPase folding, assembly, quality control, and trafficking is not well characterized, but some evidence exists that BiP and calnexin regulate Na,K-ATPase biogenesis (Beggah et al. 1996; Tokhtaeva et al. 2010). Moreover, the least stable isoforms of the α - and β -subunits associated

strongly with BiP, as did ER-retained subunits, whereas subunits with complex (Golgi appended) glycans did not coprecipitate with BiP. Consistent with these observations, coexpressing the α - and β -subunits reduced BiP association, presumably by promoting assembly, maturation, and trafficking of the Na,K-ATPase heterodimer (Beggah et al. 1996). These data strongly suggest that BiP is important either for Na,K-ATPase folding and assembly and/or for preferentially binding and targeting misfolded subunits for ERAD. These data are consistent with earlier data on yeast BiP, which is required for the folding and secretion of a wild-type version of a yeast glycoprotein; when mutated, BiP instead targets the substrate for ERAD (Simons et al. 1995; Plemper et al. 1997).

As described above, calnexin recognizes N-linked glycans and retains immature proteins in the ER. The three isoforms of the Na/K-ATPase β -subunit, β_1 , β_2 , and β_3 , possess 3, 8, and 2 glycosylation sites, respectively. Surprisingly, β_1 and β_3 subunit functions are not impaired when glycosylation is blocked (Takeda et al. 1988; Lian et al. 2006; Beggah et al. 1997; Zamofing et al. 1989). However, when glycosylation of the β_2 isomer is blocked, the protein is retained in the ER and fails to assemble with the α -subunit. All eight glycosylation sites are not required for trafficking of β_2 , but glycosylation sites two and seven are critical (Tokhtaeva et al. 2010). These data are consistent with studies in yeast that established a hierarchy of glycosylation sites in secreted proteins: Those that reside in hard-to-fold regions appear to be most vital for selecting the protein for ER retention and ERAD (Spear and Ng 2005). Consistent with these data, hydrophobic amino acids near glycosylation sites are required for calnexin binding and calnexin-mediated maturation of the β_2 -subunit (Tokhtaeva et al. 2010).

7.4.2 *The Epithelial Sodium Channel*

Epithelial Sodium Channel (ENaC) is formed by homologous α -, β -, and γ -subunits, and each subunit contains two transmembrane domains (TMDs), a large extracellular loop (ECL) and cytosolic N- and C-termini (Canessa et al. 1994a). ENaC is highly expressed in the lung, colon, and kidney epithelia where it reabsorbs sodium. In the kidney, ENaC is expressed on the apical surface of the distal convoluted tubule, connecting tubule, and collecting duct, where ENaC is responsible for Na⁺ reabsorption from urine and regulates salt and water homeostasis and therefore blood pressure (Rossier 2014; Bhalla and Hallows 2008; Snyder 2005; Palmer 2015). ENaC also provides the driving force for K⁺ secretion by acting in conjunction with the ROMK potassium channel. Gain of function mutations in ENaC lead to hypertension and Liddle's syndrome (Staub et al. 1996), and loss of function mutations lead to hypotension, salt-wasting and Pseudohypoaldosteronism Type 1 (PHA1) (Grunder et al. 1997; Bhalla and Hallows 2008). In the lung, ENaC is negatively regulated by CFTR. In the absence of CFTR, ENaC hyperactively reabsorbs Na⁺ which further dehydrates the airway mucosa and exacerbates cystic fibrosis symptoms (Mall et al. 2004; Hobbs et al. 2013; Gentsch et al. 2010;

Ismailov et al. 1996; Yan et al. 2004). ENaC trafficking and surface expression are highly regulated and have been extensively studied, which is discussed in Volume 3, Chap. 18 of this series.

7.4.2.1 Posttranslational Modifications of ENaC

The ENaC subunits are modified by N-linked glycosylation, the formation of disulfide bonds, palmitoylation, acetylation, and proteolytic cleavage in the Golgi (Buck and Brodsky 2018). Interestingly, the α -, β -, and γ ENaC subunits are subject to different posttranslational modifications, even though they are ~30–40% identical (Bhalla and Hallows 2008; Canessa et al. 1994a; Mueller et al. 2010; Valentijn et al. 1998; Weisz et al. 2000; Snyder et al. 1994). For example, the α -, β -, and γ ENaC subunits contain 6, 13, and 7 sites for N-linked glycosylation, respectively (Buck and Brodsky 2018), and blocking glycosylation in any subunit altered channel conductance and Na^+ -dependent inhibition (Kashlan et al. 2018). Expression of β -subunits lacking glycans led to the most dramatic effect, with an overall reduction in Na^+ current of ~50–80%. Interestingly, the α -subunit was more sensitive to protease under these conditions. Although glycosylation of the α -subunit was previously shown to be dispensable (Canessa et al. 1994a; Snyder et al. 1994), other studies established that tunicamycin treatment, which blocks the addition of N-linked sugars, inhibited ENaC current (Kieber-Emmons et al. 1999). ENaC trafficking is also subject to Na^+ -dependent regulation: low Na^+ favors the maturation of glycans, consistent with trafficking to the later secretory pathway, whereas high Na^+ results in the acquisition of immature glycans (Heidrich et al. 2015). Because glycan maturation occurs in the Golgi under low Na^+ conditions (0 mM Na^+ in media), ENaC may follow an alternative trafficking pathway that bypasses the Golgi under conditions of high Na^+ (130 mM Na^+ in media) (Heidrich et al. 2015).

Members of the protein disulfide isomerase (PDI) family oxidize Cys residues within the ER to form disulfide bonds in nascent proteins (Galligan and Petersen 2012). The formation of disulfide bonds requires a thioredoxin motif (Cys-X-X-Cys), and to date 21 human PDIs are known (Galligan and Petersen 2012). Each ENaC subunit contains 16 conserved Cys residues, so perhaps not surprisingly each subunit in the channel forms several intrasubunit disulfide bonds, which are important for function (Buck and Brodsky 2018). For example, mutating the first or sixth Cys in α - or β ENaC reduces surface expression, but mutating any of the other Cys residues results in an increase in sodium current. Disulfide bonds are also required for self-inhibition in response to Na^+ (Sheng et al. 2007; Firsov et al. 1999).

In contrast to most PDIs that harbor two thioredoxin motifs, some family members contain only a single Cys-X-X-Cys motif. These PDIs are unable to catalyze the formation of disulfide bonds in substrates but can form covalent bonds to single Cys residues and thus rearrange existing disulfide bonds. One of these family members is ERp29, which promotes ENaC trafficking from the ER to the Golgi (Grumbach et al. 2014). Specifically, ERp29 promotes the association of β ENaC with a cargo

recognition component of the coat complex II (COPII), which regulates ER exit and thus channel maturation.

A third posttranslational modification to which ENaC subunits are subject is the conjugation of palmitate, which is transferred to a Cys, Ser, or Thr near a hydrophobic amino acid patch by a palmitoyl transferase (De and Sadhukhan 2018; Mueller et al. 2010; Mukherjee et al. 2014). Palmitoylation increases the net hydrophobicity of a substrate, and ~50% of all human ion channels are palmitoylated. The addition of palmitate can inhibit protein–protein interactions, act as a signal for endocytosis and block recycling to the plasma membrane (Shipston 2011; Nadolski and Linder 2007; Linder and Deschenes 2007; Fukata et al. 2016). Both the β ENaC (Cys43 and Cys557) and the γ ENaC (Cys33 and Cys41) subunits are modified (Mueller et al. 2010; Mukherjee et al. 2014). Treatment of cortical collecting duct cells with a palmitoyl transferase inhibitor reduced ENaC-specific Na^+ current (Mukherjee et al. 2017), and mutating the palmitoylation sites decreased ENaC channel open probability (P_o) and therefore reduced Na^+ current (Mueller et al. 2010; Mukherjee et al. 2014). Among the 23 mammalian palmitoyl transferases, coexpression of five (DHCC1, 2, 3, 7, and 14) increased Na^+ current, and ENaC coprecipitated with each of these enzymes (Mukherjee et al. 2017). All of the transferases are expressed in either the ER and/or the Golgi (Ohno et al. 2006), which raises the possibility that this modification affects early events during ENaC quality control.

Finally, each of the ENaC subunits is acetylated (Butler et al. 2015). Although protein acetylation is usually associated with histones, many other proteins are subject to this modification (Sadoul et al. 2008; Dragic et al. 2016). ENaC subunit acetylation increases both the cellular levels of the channel and the amount of ENaC present at the plasma membrane (Butler et al. 2015). Acetylation inhibitors increase ENaC current and expression, perhaps by inhibiting ubiquitination. Because ubiquitination of ENaC at the cell surface triggers endocytosis, these data suggest that acetylation and ubiquitination of Lys residues on the cytoplasmic tails of ENaC are antagonistic (Zhou et al. 2013; Oberfeld et al. 2011; Butler et al. 2015). In contrast, HDAC7 deacetylates ENaC early in the secretory pathway, because inhibition of HDAC7 increases the total ENaC pool, i.e., not just the population of surface-expressed ENaC (Butler et al. 2015). In the future, it will be important to determine if HDAC7-mediated deacetylation alters the targeting of ENaC for ERAD.

7.4.2.2 Regulation of ENaC by ERAD

Expression of all three ENaC subunits is required for efficient channel assembly and trafficking to the cell surface, and when the subunits are expressed individually they are rapidly targeted for ERAD (Snyder 2005; Staub et al. 1997; Valentijn et al. 1998; Asher et al. 1996; Masilamani et al. 1999). Moreover, even when all three subunits are present a significant percentage of the ENaC subunits continue to be degraded (Staub et al. 1997; Valentijn et al. 1998; Malik et al. 2001; Kashlan et al. 2007). To

identify factors required for ENaC degradation, a yeast ENaC expression system was developed (Mueller et al. 2007; Buck et al. 2010, 2017). Initial studies using the yeast model confirmed that ENaC is targeted for ERAD, and more specifically that ENaC degradation requires the proteasome and the Cdc48 complex (Kashlan et al. 2007) (see above). The sHsps, which prevent protein aggregation in an ATP-independent manner, are also required for α ENaC degradation (Kashlan et al. 2007). Importantly, overexpression of a mammalian sHsp in MDCK cells decreased ENaC expression, and co-expression of the sHsp with ENaC decreased Na^+ current and ENaC surface expression, consistent with results from the yeast model (Kashlan et al. 2007). Additionally, deletion of the ER luminal Hsp40s, Jem1, and Scj1, which normally interact with BiP, stabilized ENaC in yeast, and co-expression of the human homologs with ENaC in *Xenopus* oocytes also reduced ENaC current and surface expression (Buck et al. 2010). Surprisingly, ENaC degradation showed no dependence on the yeast BiP homolog, even though the Hsp40s played an important role in degradation (Buck et al. 2010). Because ENaC ubiquitination was reduced in the absence of the Hsp40s, the Hsp40s—but not BiP—appear to be required to recognize or target ENaC for degradation.

Although BiP does not regulate ENaC degradation in yeast, another Hsp70-like molecule, Lhs1, is present in the ER. Lhs1 is a member of the Hsp110/GRP170 family of proteins, which act as a NEF for Hsp70 (Baxter et al. 1996; Craven et al. 1996; Steel et al. 2004; Tyson and Stirling 2000). Similar to Hsp70, mammalian Lhs1 can also bind misfolded proteins (Behnke and Hendershot 2014) and acts as a “holdase” to prevent misfolded protein aggregation (de Keyzer et al. 2009). In the yeast model, we found that α ENaC degradation required this Hsp70-like NEF, and an unglycosylated species of α ENaC was preferentially stabilized (Buck et al. 2013). Consistent with these results, the mammalian Lhs1 homolog, GRP170, also promoted α ENaC degradation and preferentially bound to unglycosylated α ENaC in HEK293 cells (Buck et al. 2013). In contrast to strains lacking the Hsp40s, ubiquitinated α ENaC accumulated in the ER membrane when Lhs1 was deleted (Buck et al. 2017). These data suggest that Lhs1/GRP170 acts at a post-ubiquitination step and are consistent with this factor playing a role in α ENaC retrotranslocation. Similarly, a role for GRP170 in the retrotranslocation of a viral protein and a misfolded glycoprotein, NHK, from the ER was observed, but retrotranslocation was BiP-dependent in both of these cases (Inoue and Tsai 2015, 2016).

Even though BiP is dispensable for ENaC biogenesis or quality control, the cytosolic Hsp70 promotes ENaC trafficking to the cell surface by enhancing ENaC association with COPII (Chanoux et al. 2012). Surprisingly, the closely related, constitutively expressed homolog of Hsp70, Hsc70, instead promotes ENaC degradation (Chanoux et al. 2013; Goldfarb et al. 2006). Although a mechanism for Hsc70-mediated degradation is unknown, the chaperone probably targets ENaC to the ERAD pathway. Consistent with these results, some stabilization of ENaC was noted when degradation was examined in yeast expressing a temperature-sensitive allele of a cytosolic Hsp70, Ssa1 (Buck et al. 2010).

Other modulators of ENaC stability and trafficking include PON2 (Paraoxonase-2) and Derlin-1 (Shi et al. 2017; You et al. 2017). PON2 is an ER-associated lactonase that is expressed in the ENaC-expressing principal cells of the distal nephron (Shi et al. 2017). The *Caenorhabditis elegans* PON2 homolog, MEC-6, promotes trafficking of an ENaC family member, the mechanosensitive degenerin, MEC-4 (Harel et al. 2004; Chelur et al. 2002). In transfected HEK293 cells, PON2 coimmunoprecipitates with ENaC, but in contrast to MEC-6, PON2 overexpression lowers ENaC currents and reduces ENaC surface expression (Shi et al. 2017). The reason that PON2 and MEC-6 appear to function antagonistically toward their respective sodium channels is unclear, but more work on these ill-defined chaperone-like proteins is essential. Similarly, overexpression of Derlin-1 promotes ENaC degradation in mammalian cells and associates with the channel (You et al. 2017). However, whether or not Derlin-1 serves as the ENaC retrotranslocation channel alone or in conjunction with other factors, perhaps gp78 or HRD1, must also be defined.

In yeast, ENaC degradation is dependent on both the ERAD-L/M E3 ubiquitin ligase, Hrd1, as well as the ERAD-C ubiquitin ligase, Doa10 (Buck et al. 2010). Whereas β ENaC is nearly completely stabilized in a yeast strain lacking both Hrd1 and Doa10, a significant portion of both α - and γ ENaC continues to be degraded. This result suggests that a third unidentified ubiquitin ligase targets α - and γ ENaC for degradation. Because Hrd1 and Doa10 play a role in ENaC degradation, ENaC may contain folding lesions in multiple cellular compartments or Hrd1 and Doa10 may compensate for one another. To date, it remains mysterious which E3 ubiquitin ligases act on misfolded forms of ENaC in the mammalian ER.

7.4.2.3 ENaC Channel Assembly and ER Exit

Assembly of the α -, β -, and γ ENaC subunits likely occurs in the ER, and indirect evidence supports this assumption. First, in yeast, where nearly all ENaC is retained in the ER, the ENaC subunits coprecipitate (Buck et al. 2010, 2017). Second, in mammalian systems, immature channels lacking complex glycans or Golgi-associated protease activation also associate with one another, suggesting assembly occurs in a pre-Golgi compartment (Hughey et al. 2003). Third, when constructs containing only single α ENaC domains were coexpressed with wild-type ENaC, channel trafficking was blocked (Bruns et al. 2003). These data imply that the single domain constructs assembled with wild-type ENaC subunits and prevented ENaC trafficking. While some heteromeric membrane proteins assemble in a defined order (Wanamaker et al. 2003; Adams et al. 1997; Feige et al. 2015), there is no evidence that ENaC assembly is also ordered. Nevertheless, intersubunit contacts between each of the ENaC domains is required for channel assembly, which was highlighted in the recent cryo-EM structure of the trimeric channel (Noreng et al. 2018). Assembly of ENaC TMDs is particularly important to reach a mature conformation. Evidence to support this view is derived from the expression of a series of chimeric α - β ENaC constructs, which were then coexpressed with wild-type α ENaC and

γ ENaC. It was established that interactions between the TMDs alone, and not interactions between the large ER loops, are required to evade chaperone-dependent degradation (Buck et al. 2017).

What is the signal for the assembled channel to exit the ER after assembly is complete? Early data indicated that the α ENaC subunit can form a homotrimer that inefficiently traffics to the plasma membrane in the absence of the β - and γ ENaC subunits, whereas the β - and γ ENaC subunits require the α -subunit for ER exit (Canessa et al. 1994b). Therefore, it was hypothesized that α ENaC possesses an ER exit signal (Canessa et al. 1994b; Mueller et al. 2007). Indeed, using a reporter protein fused to cytoplasmic segments of ENaC, a five amino acid motif was identified that acted as a trafficking signal (Mueller et al. 2007). The mechanism by which these residues promote ER exit, or whether a competing ER retention sequence is also present, is unknown.

7.4.3 Other Epithelial Ion Channels and Transporters Regulated by ERAD

7.4.3.1 Renal Outer Medullary Potassium Channel

Renal Outer Medullary Potassium Channel (ROMK) (Kir1.1/*KCNJ1*), is a member of the inwardly rectifying potassium channel family. ROMK is a homotetramer, and each ROMK subunit contains two TMDs, a potassium selectivity filter, and large cytosolic N- and C-terminal tails (Ho et al. 1993). Like ENaC, ROMK is expressed on the apical surface of epithelial cells in the distal nephron of the kidney. ROMK is responsible for potassium secretion and also regulates salt-water homeostasis. ROMK physiology, trafficking, and regulation are discussed in depth in Volume 3, Chap. 19, of this series.

To date, over 60 mutations have been identified in ROMK that result in Type II Bartter syndrome, a disease that is associated with early developmental defects and lifelong hypokalemic alkalosis (Welling 2016; Ji et al. 2008). N-terminal frameshifts result in a nonfunctional channel, but some mutations alter ion selectivity, gating, or ligand binding (Fang et al. 2010; Peters et al. 2003). Although it was unknown whether other mutations instead altered protein folding, potentially targeting the channel for ERAD, four mutations that cluster in a C-terminal immunoglobulin-like domain result in trafficking defects in oocytes and/or HEK293 cells (Fallen et al. 2009; Peters et al. 2003). Later, via the development of a yeast model, the same mutants were found to be selected for proteasome-dependent degradation in a p97/Cdc48- and Hsp70-dependent manner (O'Donnell et al. 2017; Mackie et al. 2018). The same mutants were also degraded in a proteasome- and p97/Cdc48-dependent manner in mammalian cells. It will be important to next define which of the other uncharacterized disease-causing mutants are also targeted for ERAD.

7.4.3.2 Thiazide-Sensitive Sodium Chloride Cotransporter

Sodium Chloride Cotransporter (NCC) is a member of the SLC12 cation chloride cotransporter family of electroneutral transporters and is a 12 TMD protein with a glycosylated extracellular loop and large cytosolic N- and C-termini (Castaneda-Bueno et al. 2010). NCC is expressed on the apical surface of the distal convoluted tubule and also regulates sodium reabsorption, thereby playing a major role in salt-water homeostasis and blood pressure regulation. The transporter is also a target for the thiazide diuretics. Mutations in NCC result in Gitelman Syndrome, which is typified by salt-wasting, hypokalemic alkalosis, hypomagnesemia, and hypocalciuria (Fulchiero and Seo-Mayer 2019). NCC is discussed in depth in Volume 3, Chap. 3, of this series.

Several characterized Gitelman Syndrome-causing mutations in NCC result in the production of an immature protein that contains only core N-linked glycans (Kunchaparty et al. 1999; De Jong et al. 2002; Sabath et al. 2004). To determine whether NCC is an ERAD substrate, and whether these mutations accelerated ERAD, a yeast expression system for NCC was developed (Needham et al. 2011). As anticipated, NCC was primarily ER localized, and degradation was slowed in the presence of a proteasome inhibitor. Further, NCC was ubiquitinated primarily by Hrd1 in an Hsp70-dependent manner. In mammalian cells, immature NCC was again ubiquitinated and targeted for proteasome-dependent degradation. Consistent with the data in yeast, Hsp70 overexpression increased the proportion of immature NCC and increased the degradation rate (Needham et al. 2011). A role for accelerated chaperone-dependent ERAD in Gitelman Syndrome was then verified via a mass spectrometry analysis. Major hits from this screen included Hsp90 and Hsp90 cochaperones, CHIP—an E3 ubiquitin ligase previously implicated in ERAD (McDonough and Patterson 2003)—an Hsp70/Hsp90 organizer protein, an Hsp40, as well as the known NCC effector, Hsp70 (Donnelly et al. 2013). Through the application of an Hsp90 inhibitor, it was established that NCC maturation depended on Hsp90 function (Donnelly et al. 2013). Notably, Gitelman syndrome mutants associated more strongly with Hsp70, Hsp40, and CHIP than wild-type NCC (Donnelly et al. 2013). The data are consistent with sequential NCC quality control in the ER, whereby the Hsp70 and Hsp90 chaperone complex acts sequentially to monitor NCC folding and ultimately promote channel trafficking (Donnelly et al. 2013).

7.4.3.3 V2 Vasopressin Receptor

The V2 vasopressin receptor (V2R) is a G-protein coupled receptor (GPCR) stimulated by arginine-vasopressin (AVP) (Birbaumer et al. 1992). AVP responds to either an increase in NaCl or a decrease in blood pressure and binds V2R, which resides in the basolateral membrane of principal cells in the distal nephron. Activation of V2R by AVP triggers AQP2 insertion in the plasma membrane and allows for

water reabsorption from urine. In the absence of functional V2R, AQP2 remains embedded in intracellular vesicles. Mutations in the V2R (*AVPR2* gene) cause X-linked nephrogenic diabetes insipidus (NDI) (Hoekstra et al. 1996), and >270 NDI-causing mutations have been identified (Milano et al. 2017). These mutations are responsible for ~90% of the inherited cases of NDI, while the other ~10% are caused by mutations in the AQP2 gene, as described below. Patients with NDI are unable to respond to AVP and produce copious volumes of urine (polyuria) and exhibit excessive thirst (polydipsia).

NDI-causing V2R mutants are either retained in the ER or Golgi, or they traffic to the plasma membrane and are defective (Milano et al. 2017). Among nine NDI-causing mutants, most are ER-retained and subject to ERAD (Robben et al. 2005). Later, three V2R mutants with alterations in the cytosolic portion of the protein (L62P), within the membrane (V226E), and within the lumen (G201D) were characterized (Schwieger et al. 2008). G201D localized throughout the secretory pathway, whereas the L62P mutant was found exclusively in the ER. The V226E mutant was visualized in both the ER and ER-Golgi intermediate compartment. Proteasome inhibition stabilized the unglycosylated and core glycosylated species of both the wild type and the mutant V2Rs, and all of the proteins were ubiquitinated to varying degrees. Consistent with V2R being a substrate for ERAD, the unglycosylated and core glycosylated V2R mutants coimmunoprecipitated with p97 and Derlin-1. Although the chaperone and ubiquitin ligase requirements for degradation were not addressed, this will be an important future area of research.

7.4.3.4 Aquaporin-2

Aquaporin-2 (AQP2) is responsible for water reabsorption in the distal nephron and is expressed on the apical surface of principal cells, where ENaC, V2R, and ROMK also reside. AQP2 is a six TMD homotetramer (Bai et al. 1996). Over 60 NDI-causing mutations in AQP2 have been described (Milano et al. 2017), many of which lead to protein retention in the ER (Lin et al. 2002; Lloyd et al. 2005; Tamarappoo and Verkman 1998; Marr et al. 2002; Iolascon et al. 2007). Some of these mutants function if they reach the cell surface, suggesting the folding defect is minor (Leduc-Nadeau et al. 2010; Marr et al. 2002). Interestingly, 11 of the 65 mutants are dominant (Kuwahara et al. 2001; Sohara et al. 2006; Asai et al. 2003; Mulders et al. 1998) and form heterotrimers with wild-type subunits that are either misrouted to the lysosome (Marr et al. 2002) or the basolateral membrane (Kamsteeg et al. 2003) or are retained in the Golgi (Mulders et al. 1998; Procino et al. 2003).

Perhaps not surprisingly, several of the AQP2 NDI-associated mutants are ERAD substrates, but there is a dearth of information on the factors that recognize and target AQP2 mutants for degradation. One study observed that, in contrast to wild-type AQP2, the glycosylated form of four AQP2 mutants was significantly more stable than the unglycosylated species. Therefore, lectins may be involved in AQP2 folding and quality control, but this has not been investigated (Buck et al. 2004). There is

also some evidence that the ERAD-associated E3 ubiquitin ligase, CHIP (see above), regulates AQP2 levels (Wu et al. 2018). AQP2 levels are higher in cells lacking CHIP, and CHIP seems to be required for both proteasomal and lysosomal AQP2 degradation. In an *in vitro* assay CHIP ubiquitinates AQP2, but Hsc70 blocks ubiquitination. AQP2 expression is also increased in a CHIP knockout mouse and water intake is lower, which is consistent with increased AQP2 activity (Wu et al. 2018). A more thorough discussion of AQPs is provided in Volume 3, Chap. 30, of this series.

7.4.3.5 Polycystin-2

Polycystin-2 (PC2) is a six TMD nonselective cation channel with cytosolic N- and C-termini (Mochizuki et al. 1996). The transmembrane domain of PC2 is homologous to polycystin-1 (PC1), which is a PC2 partner, and the TRP channels (Somlo and Ehrlich 2001). PC2 function is dependent on cellular residence. In the ER, PC2 acts as a Ca^{2+} release channel (Clapham 2003; Koulen et al. 2002), whereas at the plasma membrane PC2 may facilitate Ca^{2+} entry (Luo et al. 2003). In embryonic nodal cells and the primary cilia of renal tubular cells, PC2 acts as a flow sensor (McGrath et al. 2003), and when PC2 is inhibited the Ca^{2+} response to flow stimulation is absent (Nauli et al. 2003). Mutations in PC2—as well as PC1—are responsible for autosomal dominant polycystic kidney disease (ADPKD) (Torres and Harris 2006), which is the fourth leading cause of kidney failure.

Mounting evidence suggests that PC2 is targeted for ERAD. First, PC2 is ubiquitinated, and PC2 degradation is proteasome dependent (Liang et al. 2008). Second, PC2 degradation is dependent on p97, and PC2-GFP localizes to the ER and coprecipitates with p97 in MDCK cells. Liang et al. (2008) also determined that Herp, which may function similarly to the Hrd1-Der1 assembly factor, Usa1 (see above) and recruits substrates to this complex (Leitman et al. 2014), contributes to PC2 ERAD. It will be interesting to determine whether disease-causing mutations in PC2 enhance ERAD, as seen for several of the substrates discussed above, and which chaperones select PC2 for degradation.

7.4.3.6 The Sodium-Potassium Chloride Cotransporter-2

The sodium-potassium chloride cotransporter-2, NKCC2, is another renal protein that, when mutated, gives rise to a disease associated with altered salt-water balance (type I Bartter Syndrome). NKCC2, which is homologous to NCC (see above), resides in the thick ascending limb of the loop of Henle and plays a pivotal role in NaCl reabsorption. Prior work established that the protein is targeted for ERAD, which is consistent with its slow rate of ER exit (Zaarour et al. 2012). More recent evidence indicates that immature, ER glycosylated forms of the substrate are selected for degradation through the action of the ER lectin Os9 (Seaayfan et al. 2016). As in many of the examples outlined in this section, ongoing work on

NKCC2 will better define how the transporter is targeted for ERAD. A more thorough discussion of NKCC2 function is provided in Volume 3, Chap. 2, of this series.

7.5 Conclusions and Future Directions

In this chapter, we have provided an overview of the many hurdles that a nascent protein in the ER must overcome. We then outlined the chaperones, chaperone-like factors, and enzymes that impact protein folding and degradation, and focused on the mechanisms that lead to the selection, ubiquitination, and delivery of an ERAD substrate to the proteasome. As described in the preceding section, a growing body of literature indicates that TMD-containing proteins, many of which reside in epithelia, are targeted to this pathway. While disease-causing mutations may slow ER exit and hasten ERAD, even wild-type versions of these proteins fold inefficiently and are targeted for ERAD. Therefore, much has been learned from studying the biogenesis of both wild type and disease-associated mutants. As highlighted throughout the chapter the use of model systems has led to significant insights on ER quality control processes.

Although the field is in its infancy, there is growing interest in harnessing the knowledge obtained from studies on ER protein quality toward the development of therapeutics that correct misfolded epithelial proteins. Therefore, a better definition of how these varied ion channels and transporters assemble in the ER and traffic to the plasma membrane is imperative. Small molecules that target any of these steps might lead to the development of new therapeutic options.

For example, pharmacological chaperones are under development that target ER retained V2R mutants (Los et al. 2010). Like molecular chaperones, pharmacological chaperones are designed to assist protein folding. One class of these molecules are the nonpeptide vasopressin receptor antagonists, or vaptans (Francis and Tang 2004). Vaptans mimic the structure of AVP (Mouillac et al. 1995), are cell permeable, and thus bind the AVP binding site on V2R in the ER. Vaptans stabilize the mutant V2R allowing for maturation and trafficking while avoiding ERAD (Milano et al. 2017). Six vaptans can promote V2R trafficking, maturation, plasma membrane expression, and the AVP response (Robben et al. 2007; Morello et al. 2000; Wuller et al. 2004; Bernier et al. 2004). In small scale clinical trials for a vaptan, SR49059, patient urine volume was decreased while urine osmolality was significantly increased.

Perhaps the best example in which pharmacological approaches to target ERAD-associated diseases has been successful is Cystic Fibrosis. The F508del CFTR mutation, which targets this unfolded, ER-retained protein for ERAD, is significantly stabilized, can traffic to the apical membrane, and function in the presence of an FDA-approved drug combination (Lukacs and Verkman 2012). Current knowledge suggests these compounds bind directly to the mutated channel. One compound helps link two domains during channel synthesis, while the other compound binds to

the cell surface population of F508del CFTR and increases its open probability (Clancy 2018). While improvements in the efficacy of this treatment are actively being pursued, these data provide hope that other drugs that target misfolded ion channels and transporters can be identified. In the case of Cystic Fibrosis, a molecular definition of the cause of the disease was instrumental in developing this therapy. For the other diseases discussed in this chapter, we similarly propose that basic research will pave the way for future cures.

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