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Road to Ruin: Targeting Proteins for Degradation in the Endoplasmic Reticulum

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Abstract

Some nascent proteins that fold within the endoplasmic reticulum (ER) never reach their native state. Misfolded proteins are removed from the folding machinery, dislocated from the ER into the cytosol, and degraded in a series of pathways collectively referred to as ER-associated degradation (ERAD). Distinct ERAD pathways centered on different E3 ubiquitin ligases survey the range of potential substrates. We now know many of the components of the ERAD machinery and pathways used to detect substrates and target them for degradation. Much less is known about the features used to identify terminally misfolded conformations and the broader role of these pathways in regulating protein half-lives.

Proteins destined for secretion or insertion into the membrane enter the endoplasmic reticulum (ER) in an unfolded form and generally leave only after they have reached their native states. Yet, folding in the ER is often slow and inefficient, with a substantial fraction of polypeptides failing to reach the native state. Thus, the cell must continuously assess the pool of folding proteins and remove polypeptides that are terminally misfolded. This process of culling is critical to protect the cell from the toxic effects of misfolded proteins.

Remarkably, many proteins triaged as terminally misfolded are first removed from the ER via delivery (or dislocation) to the cytosol, where they are then degraded by the ubiquitinproteasome system (1). This process is commonly referred to as ER-associated degradation (ERAD)—an umbrella term that covers a range of different mechanisms. Once terminally misfolded proteins are distinguished from what are likely to be structurally similar folding species, they are extracted from the pro-folding chaperone machinery, delivered to a transmembrane complex that coordinates their dislocation and, finally, escorted to the proteasome for degradation (Fig. 1A).

A convergence of genetic and biochemical studies, including work in the budding yeast *Saccharomyces cerevisiae* and in metazoan systems, has led to the identification of many of the key components involved in substrate recognition and degradation. Characterization of these components has uncovered distinct and well-conserved ERAD pathways, but for only a limited number of model substrates. We still do not know all of the endogenous targets of ERAD or the relative importance of ERAD in the quality control of misfolded

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conformations versus a broader role in regulating the half-lives of proteins that have reached the native state.

E3 Ubiquitin Ligases: Central Organizers

At the center of all ERAD pathways are multi-protein transmembrane complexes formed around E3 ubiquitin ligases (2–4). The E3s have variable numbers of transmembrane domains and a cytosolic RING finger domain. They catalyze substrate ubiquitylation (5) and organize the complexes that coordinate events on both sides of and within the ER membrane. When overexpressed, the yeast Hrd1p protein—the prototypical ERAD E3—can autonomously carry out degradation of soluble substrates within the ER lumen (6). This ability implicates Hrd1p—and by inference other ERAD E3s—in the physical process of transporting substrates across the ER membrane. Yet, this step remains mysterious, and it is likely that other components also facilitate dislocation.

If the E3s can act alone, then why do they form large complexes? The E3s require a dynamic complement of adaptor proteins that facilitate substrate recognition and delivery while also regulating E3 activity. In fact, overexpression of Hrd1p without its adaptors is toxic to cells, apparently because of uncontrolled and inappropriate degradation of many proteins (2). Although we understand the role of these adaptors in specific systems, such as the delivery of glycoproteins to E3s, the broader role of adaptors in restricting E3 activity to legitimate substrates remains unclear.

Individual E3s can survey overlapping but distinct ranges of substrates with diverse topologies (those with misfolded domains in the ER lumen, membrane, or cytosolic compartments) (Fig. 1B) (3). The E3s implicated in ERAD include two proteins with distinct topologies in yeast, Hrd1p (5) and Doa10p (7), and many more in metazoans, such as HRD1, gp78, RMA1(RNF5), TRC8, and TEB4(MARCH IV) (8). Here, we will focus on the complexes formed around the best-characterized class of E3s, the HRD ligases, which include Hrd1p in yeast as well as HRD1 and gp78 in metazoans (Fig. 1, C and D).

Adaptors control E3-substrate interactions

Adaptors are the peripheral components of the E3 complex that impart the rich substrate repertoire and stringent specificity of ERAD. Hrd3p in yeast and its metazoan counterpart SEL1L are the most thoroughly characterized adaptors (9). These proteins contain a single transmembrane domain, which in the case of Hrd3p is dispensable for function (5). Rather, the business end of the molecule is a large luminal domain composed of multiple tetratricopeptide repeats (TPRs) thought to facilitate protein-protein interactions (9). Hrd3p can bind potential substrates directly on the basis of their misfolded character (2, 9) and thus recruits misfolded proteins to the E3 ligase. Hrd3p/SEL1L also recruit other adaptors—such as the glycan-binding (lectin) protein Yos9p (9) in yeast and OS-9 and XTP3-B (10) in mammals—to the E3 complex. These lectins broaden the E3 substrate repertoire; their absence leads to a specific defect in glycoprotein degradation but does not affect degradation of other Hrd3p/SEL1L and HRD dependent substrates, such as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (11).

Housekeeping chaperones may serve as adaptors. The cytoplasmic Hsp70, Ssa1p, facilitates substrate interaction with Doa10p (12); the ER-resident Hsp70s, Kar2p in yeast and BiP in mammals, interact with Yos9p-Hrd3p (2) and OS-9/XTP3-B-SEL1L (13) in a stable manner, localizing them to the E3 complex. BiP may also help deliver substrates to E3s on the basis of its interactions with other ERAD components (14) and with substrates (15).

How is the activity of the E3s tuned to meet the cell's needs?

E3s are regulated by their intrinsic stability; for example, the Hrd1p E3 auto-ubiquitylates and thus triggers its own degradation (5). Hrd3p forms a stoichiometric complex with Hrd1p and inhibits this auto-ubiquitylation. The dual activities of Hrd3p as an adaptor and a regulator ensure that Hrd1p is active only in the presence of controlled substrate delivery. Although the ability to self-destruct appears to be conserved among E3s, their mechanisms of regulation may not be. In metazoans, SEL1L interacts with HRD1 but not gp78 (13), and HRD1 appears to stabilize SEL1L (in contrast to the relationship in yeast, in which Hrd3p stabilizes Hrd1p) (16).

The oligomeric state of Hrd1p modulates its activity and is regulated by Usa1p (6, 17), another member of the complex. Although Hrd1p oligomerization may be essential for its activity (6), Usa1p is dispensable for degradation of some substrates (3), and other regulators of Hrd1p oligomerization have not been identified. The regulation and role of oligomerization, especially in metazoans, which [with the possible exception of Herp (3)] lack a clear Usa1p homolog, awaits clarification.

Changes in relative adaptor abundance and expression level can tune E3 activity. Turnover of a number of upstream adaptors—including two regulators of glycoprotein deg radation, EDEMs and OS-9 (discussed in depth below)—may help set ERAD activity (18). In response to stress, the unfolded protein response (UPR) orchestrates increased expression of key ERAD components and thus facilitates the up-regulation of ERAD activity and capacity (19). In metazoans, different arms of the UPR up-regulate distinct sets of ERAD components (20, 21), suggesting that cells can dynamically alter the specificity of ERAD, not just its overall activity.

The Derlins: crucial but mysterious components

Perhaps the most enigmatic members of E3 complexes are the Derlins, of which the yeast Der1p is the founding member. Der1p is a multi-pass transmembrane protein that interacts with Hrd1p and Hrd3p (4) as well as with substrates (6). Although central members of the E3 complex, Derlins are dispensable for degradation of many substrates. At least part of their function is to act as adaptors.

Derlins contain a motif shared with rhomboid proteases, which recognize and cleave protein sequences within the membrane using hydrophilic residues within their transmembrane domains (22). Although Derlins lack catalytic sites, their membrane-embedded hydrophilic residues resemble potential substrate-binding residues in the rhomboids and could facilitate substrate interactions. Signal peptide peptidase may play a similar role in dislocation of major histocompatibility complex (MHC) class I products in cells that express the viral protein US2 (23).

Luminal ERAD substrates are typically more dependent on Derlins. Intramembrane binding to Derlin could thus help prime the initial passage of luminal substrates across the membrane, making them resemble misfolded transmembrane proteins that could proceed through Derlin-independent pathways. The connection between rhomboid-like motifs and ERAD is also apparent in another class of catalytically inactive transmembrane proteins, iRhoms, that promote ERAD of potential rhomboid substrates (24).

E3s connect luminal and cytosolic ERAD pathways

Although the cytosolic ubiquitylation of substrates commits them for degradation and is likely past the point of no return, substrates still require delivery to the proteasome. Ubiquitylated substrates can follow multiple dynamically controlled pathways within the

cytoplasm (1). A key cytosolic event is the extraction of substrates from the ER membrane, in which the AAA adenosine triphosphatases (ATPases) Cdc48p (p97 in metazoans) and in some cases the proteasome lid (25) play a central role. Once substrates are in the cytosol, p97 recruits peptide *N*-glycanase (PNGase) to the site of dislocation (26) to remove *N*-linked glycans from substrates before they enter the proteasome (27). In what may be an information-processing step involved in substrate targeting, p97 also recruits YOD1, a deubiquitylating enzyme involved in dislocation (28). Thus, degradation is tightly coupled to substrate dislocation in a complex manner.

How Are Substrates Delivered to E3 Complexes?

An early event in the decision to degrade a protein involves removing it from the folding machinery and delivering it to the E3 complex. We are now beginning to understand the mechanism of delivery for some substrates (Fig. 2).

Viral hijacking of the E3 machinery

Human cytomegalovirus (HCMV) down-regulates MHC class I heavy chains (MHC-I HCs) to avoid detection by the immune system. The HCMV proteins US11 and US2 directly deliver correctly folded MHC-I HCs to E3s to effect their disposal (Fig. 2A). US11 and US2 use different pathways, which require Derlin-1 (29) and signal peptide peptidase (23), respectively, to achieve the same goal. By hijacking the ERAD machinery, HCMV bypasses the requirement for substrate recognition and achieves degradation rates much faster than those of other ERAD substrates (30). The abbreviated pathway of HCMV-mediated ERAD has been a critical tool for characterizing events without the use of model misfolded substrates (29, 31) and underscores the utility of host-pathogen interactions in exploring ERAD pathways. Toxins such as ricin, cholera, and Shiga toxins, which likely exploit ERAD machinery to reach the cytoplasm after retrograde transport through the secretory pathway, are similarly useful investigative tools (32).

Regulated delivery of specific substrates

Degradation of yeast HMG-CoA reductase (Hmg2p), the rate-limiting enzyme in cholesterol synthesis, is an elegant example of physiological regulation of protein abundance via ERAD. When cholesterol levels are high, Hmg2p undergoes a lipid-induced structural change so that it resembles a misfolded protein, which is then targeted for destruction (Fig. 2B) (33). Thus, when Hmg2p is not needed, it is constitutively degraded. This involves direct recognition of Hmg2p transmembrane domains by Hrd1p, circumventing the need for many of the known Hrd1p adaptor proteins, including Der1p, Usa1p, and Yos9p.

Other components involved in sterol biosynthesis also use ERAD-based mechanisms of regulation. Mammalian HMG-CoA reductase is regulated via degradation but is delivered to the E3 via specific adaptor proteins (Insig-1 and -2) (34). Apolipoprotein B (apoB), which is involved in delivery of cholesterol to tissues, is cotranslationally degraded if not adequately loaded with cargo (35), and Erg3p, a component of the sterol biosynthesis pathway in yeast, is an endogenous ERAD substrate (36).

Cyclooxygenase-2 (Cox-2), a key enzyme in prostaglandin biosynthesis, uses an alternative strategy to control stability via ERAD. The C terminus of Cox-2, but not Cox-1, contains a 19-amino-acid sequence necessary and sufficient for degradation (37). This element not only presents an appealing minimal ERAD substrate but also hints at what could be a widespread mechanism for the control of protein turnover rates by a modular “degron” signal.

Glycans' signal-folding status

The most detailed and elaborate known mechanism for delivery of ERAD substrates is that for luminal glycoproteins (Fig. 2C). As nascent polypeptides are translocated into the ER, many are cotranslationally modified on specific asparagine (N-X-T/S) residues with a three-branch, high-mannose glycan (Glc_3-Man_9-$GlcNAc_2$) (Fig. 3A). Immediately upon transfer of the glycan to a protein, it is dismantled, one saccharide at a time, to yield specific structures recognized by different glycan-binding proteins. These modifications report on folding status and act as flags for degradation.

Soon after entry into the ER, proteins go through a deglucosylation/reglucosylation cycle on the A branch. This drives release and binding, respectively, to the chaperone lectins calnexin (CNX) and calreticulin (CRT), which together promote ER retention and folding (Fig. 3B) (38). As a protein attempts to reach its native state, mannosidases carry out competing reactions that remove mannose residues from the B and C branches and so decrease the likelihood of entry into further folding cycles. Removal of a C branch mannose by EDEMs (Htm1p in yeast) exposes a terminal α1,6–linked mannose that acts as a key signal for degradation (39–42).

Delivery of the substrate to the E3 does not require the terminal α1,6-mannose linkage, but this glycan is necessary for degradation. Although SEL1L/Hrd3p, a core member of the HRD complex, and the lectins OS-9/XTP3-B/Yos9p can bind misfolded proteins in a glycan-independent manner (2, 10, 21), degradation requires an additional, poorly characterized commitment step in which Yos9p/OS-9/XTP3-B recognizes the C branch α1,6-mannose linkage to effect degradation (Fig. 3C) (41, 43, 44). Thus, disposal of at least some unwanted glycoproteins requires both a misfolded conformation and the correct glycan signal.

Although the glycan-processing pathway can be conceived of as a linear pathway with multiple information processing steps, there are many possible branch points and detours that complicate this view. These include multiple glycan-independent roles for factors involved in glycan processing and recognition (2, 10, 21, 36, 45). The fact that the α1,6-linked mannose may reside both on substrates and on the adaptor proteins SEL1L/Hrd3p adds a further layer of complexity and affords a possible mechanism for regulating the activity of the HRD complex. The interactions between SEL1L and OS-9 (10) as well as EDEMs (45) require mannosidase-processed glycans on SEL1L. Given these observations, one interesting but speculative model is that the HRD complex is inactive when OS-9/Yos9p binds SEL1L/Hrd3p glycans but is activated in a hand-off mechanism when OS-9/Yos9p binds substrate glycans and releases SEL1L/Hrd3p.

Dismantling structures upstream of E3s

Processing of substrates upstream of the E3s is essential both for the recognition of misfolded proteins and for dismantling the compact folds of disulfide-bonded substrates to facilitate dislocation. ERAD may require isomerization of trans peptidyl-prolyl bonds to eliminate turns (46) as well as the reduction of disulfide bonds to separate non-native oligomers (14) or to recognize specific disulfide intermediates (15). ERdj5 interacts with EDEM1 and can reduce disulfide bonds of aberrantly linked proteins and accelerate ERAD when overexpressed (Fig. 2D) (14). Both its substrate-binding J domain and its disulfide-reactive thioredoxin domain are critical for ERdj5's activity. Other ER oxidoreductases also participate in ERAD, including the flavoprotein ERFAD (47) and protein disulfide isomerase (Pdi1p), underscoring the importance of disulfide modulation in this process. In yeast, Htm1p forms a disulfide-stabilized complex with Pdi1p (48), which is necessary for both maximal Htm1p activity as well as its stability (42). The known chaperone roles of

Pdi1p and ERdj5's binding partner BiP raise the possibility that these interactions may facilitate substrate recognition by EDEM1/Htm1p.

Other pathways to E3s

Substrates that are not glycosylated or that do not have misfolded domains within the ER lumen follow alternate pathways to E3s. Even though different pathways preferentially process certain substrates, the pathway and E3 used for a specific substrate probably depend on substrate load, adaptor availability, and the overall stress level of the ER.

In mammalian cells, degradation of some nonglycosylated proteins requires an E3 complex composed of HRD1, Derlin-1, and Herp, a specialized adaptor for nonglycoproteins (15). Herp is a transmembrane protein with a large cytosolic domain that binds p97 as well as components of the 26S proteasome, suggesting a possible link between protein dislocation and degradation (15). Although its primarily cytosolic location raises the question as to how nonglycosylated substrates are delivered to the E3 complex within the ER lumen, BiP might deliver misfolded substrates to E3s as well as serve as the primary folding chaperone for nonglycosylated proteins (15).

Via residues in its transmembrane domains, Hrd1p directly recognizes proteins that misfold within the ER membrane (11). Mutations within these Hrd1p intramembrane residues confer substrate-specific degradation defects (11). Given their hydrophilic character, some of these residues could act as sensors for misfolding within the hydrophobic environment of the membrane in a mechanism that could apply more generally to other E3s or to the Derlins.

Substrates with misfolded cytosolic domains prompt degradation via Doa10p, the only other ERAD-active E3 in yeast besides Hrd1p (7). Doa10p has broad substrate specificity, encompassing membrane proteins as well as soluble cytosolic and nuclear proteins and even exhibits some functional overlap with Hrd1p (7). Doa10p surveys this wide range of potential substrates without stably associated adaptors; the only other members of the core Doa10p complex are the E2 ubiquitin conjugating enzymes Ubc6p and Ubc7p (along with Cue1p, the membrane anchor for Ubc7p) (49). Without defined substrate adaptors, Doa10p may directly bind improperly exposed hydrophobic surfaces on the cytosolic surface of the ER membrane (49), or general cytosolic chaperones could act as adaptors.

Outlook

Great progress has been made in characterizing key ERAD pathways used for specific substrates. A fundamental question going forward is what biophysical, chemical, and structural properties characterize ERAD substrates. The absence of clear physico-chemical correlates of the misfolded state for any intracellular protein is a major impediment to experimental progress, and optical approaches (ideally single-molecule) that could accurately diagnose folding states of a given protein inside cells are needed. In both yeast and metazoan systems, the relatively slow rate of egress of dislocation substrates from the ER has hampered the establishment of robust in vitro assays for dislocation.

In addition to the physical properties of substrates, the time spent on folding and a substrate's precise position within the ER may be crucial determinants of ERAD recognition. Elucidating the basis of the slow degradation of soluble luminal substrates (~30 min in yeast and longer in mammalian systems) represents a critical technical and conceptual challenge in understanding substrate recognition. Key factors may be glycan modifications, in particular those imposed by the slow-acting ER mannosidase I, which are proposed to create a time window for folding before surveillance by ERAD components (38). However, this is

unlikely to be the full explanation because even if one bypasses the need for glycan processing, degradation remains slow (41).

The location of at least some of the degradation machinery may be distinct from the site of protein entry into the ER: Substrates may have to migrate to specialized subregions of the ER to access their paths to destruction, causing delays. For example, MHC-I HCs that fail to fold are relegated to specialized tubulovesicular areas of the ER (50). If the rate of migration to these subregions depends on the folding status of a protein, the ER could potentially fractionate proteins based on conformation and allow different-folding intermediates more or less time to reach the native state before encountering the ERAD machinery.

The folding and dislocation pathways are fascinating not just because of their inherent attraction; they may also afford the possibility of intervention, genetically or with small molecules, to affect the outcome of the entire biosynthetic process. Where mutations produce proteins with mild defects that target them for degradation, despite being able to execute their assigned function [for example, the case of cystic fibrosis transmembrane conductance regulator (CFTR) (51)], interference in these pathways may help to restore the cell to relative health by pushing a larger fraction of the mutant protein into productive folding pathways.

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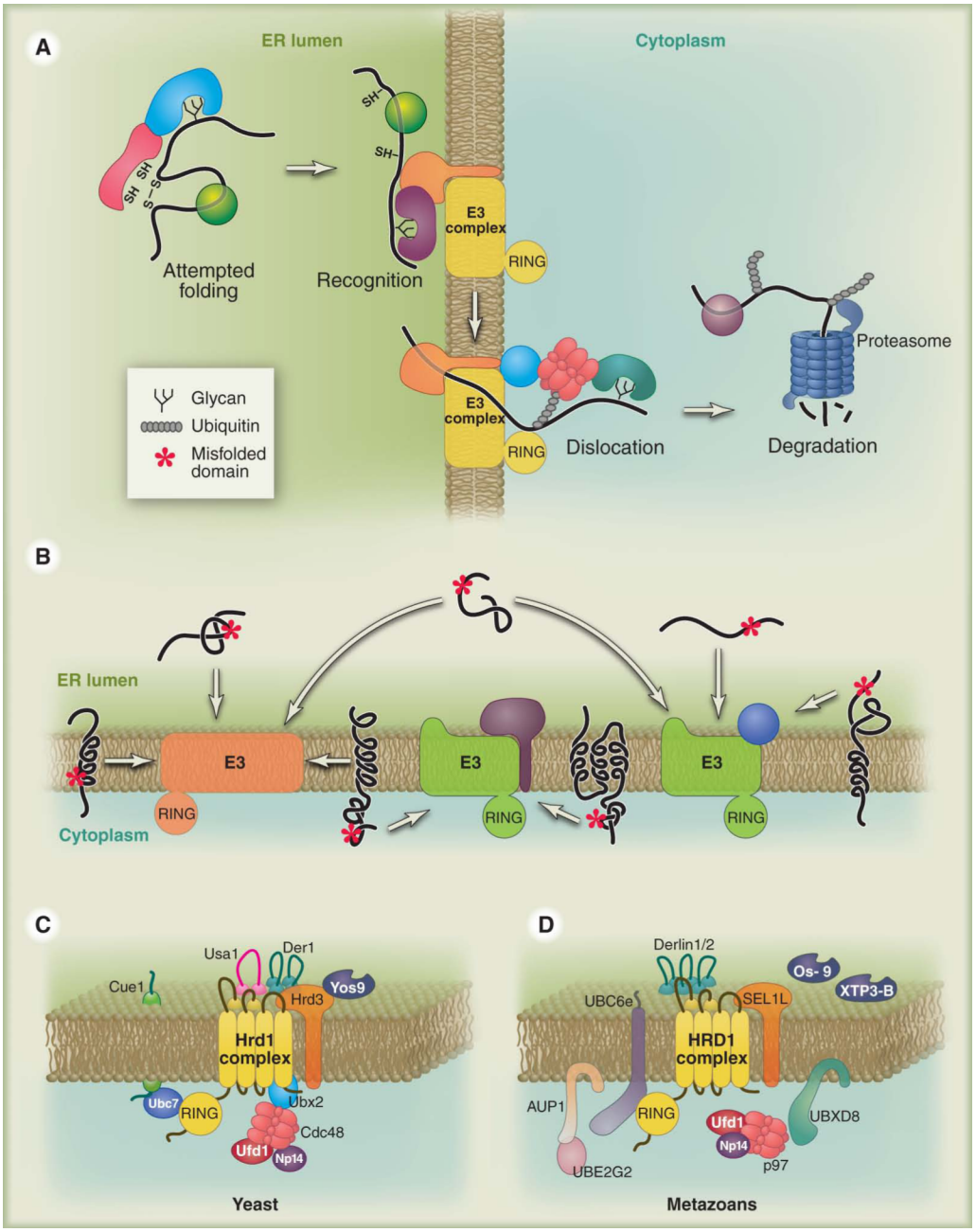

Fig. 1.

The events and components of ERAD. (**A**) The key steps of ERAD as shown for a luminal glycoprotein as applied to yeast or metazoans, and thus only the transmembrane E3 ubiquitin ligase complex is labeled along with its catalytic RING domain. (**B**) Overlapping substrate specificity of E3 ubiquitin ligases can be modulated by the presence of adaptors. Adaptors are depicted in purple and blue. (**C**) The core Hrd1p complex in yeast. (**D**) The core HRD1 complex in metazoans.

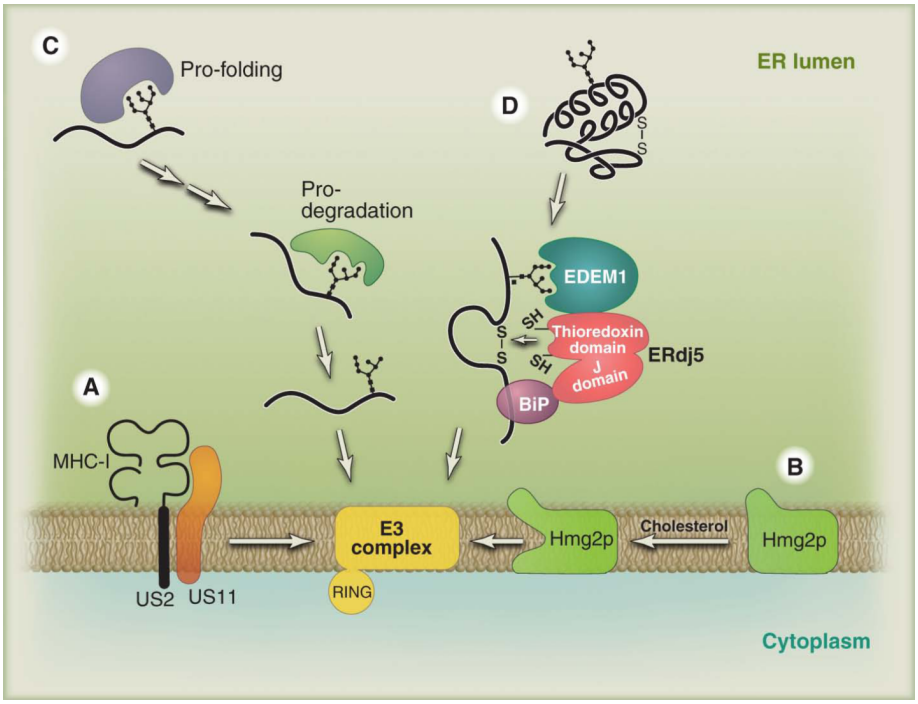

Fig. 2.

Different paths that substrates take to the E3 complexes. (**A**) Delivery of correctly folded MHC class I molecules to the E3 by US11. (**B**) Cholesterol-regulated recognition of Hmg2p by the E3 complex. (**C**) Removal of a glycoprotein from the folding machinery, and modification of its glycans followed by processing by prodegradation factors and delivery to the E3 complex. (**D**) Reduction of substrate disulfide bonds by the EDEM1-ERdj5-BiP complex in metazoans in preparation for translocation.

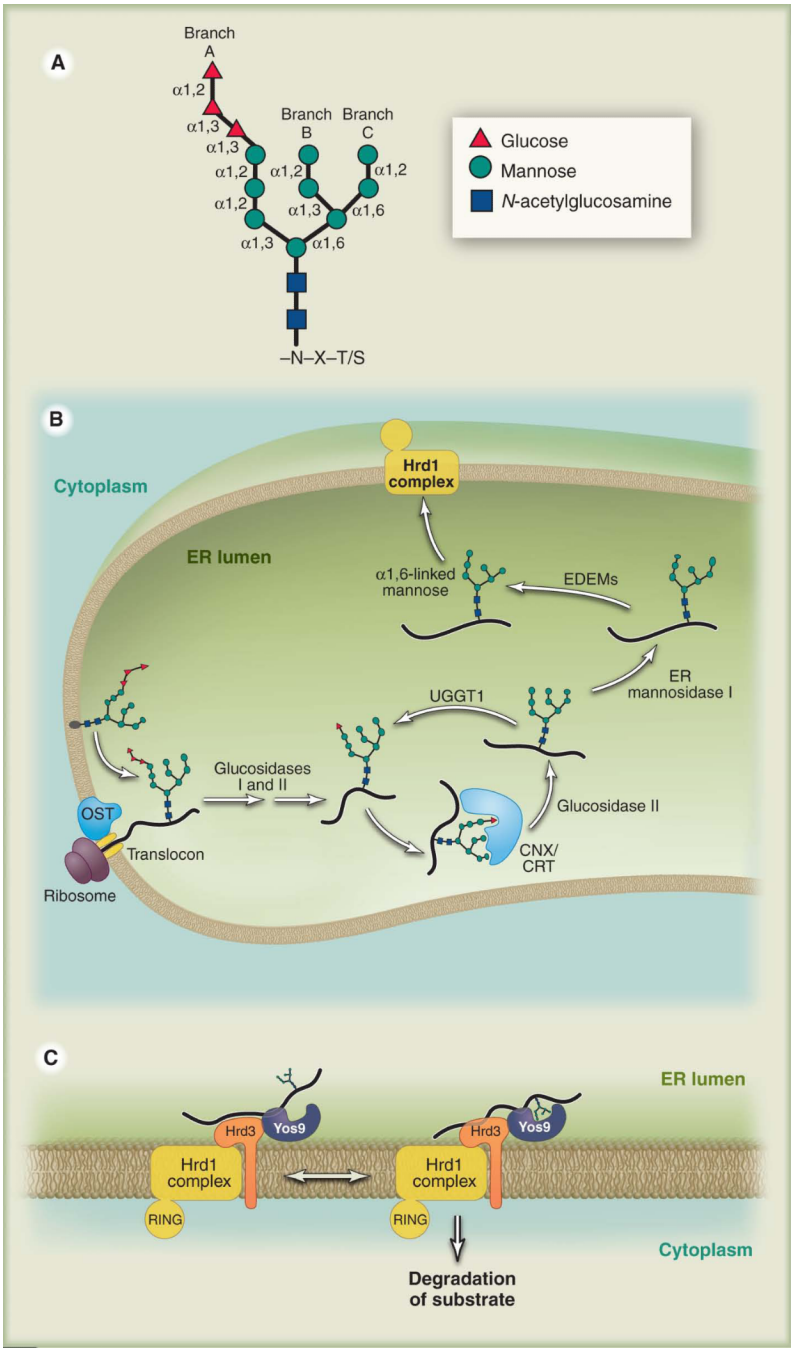

Fig. 3.

Targeting glycoproteins for degradation. (**A**) The initial high-mannose glycan appended to nascent polypeptides, Glc_3-Man_9-$GlcNAc_2$. (**B**) Metazoan glycan processing and delivery to the HRD1-ligase complex. (**C**) Luminal glycoprotein recognition by the Hrd3p-Yos9p complex in yeast. Only the complex in which Yos9p binds the glycan with the terminal α1,6-mannose linkage is capable of targeting substrates for degradation. In metazoan systems, it is thought that OS-9 may recognize glycans on SEL1L, as opposed to those on ERAD substrates, as discussed in the main text.