

Lectins sweet-talk proteins into ERAD

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How cells decide that a protein is misfolded is a mystery. The endoplasmic reticulum integrates N-linked glycosylation into the decision as to whether a protein is misfolded. The basic strategy of glycan-based recognition, previously identified in yeast, is conserved in mammals but is expanded, possibly to accommodate a more complex client portfolio.

The endoplasmic reticulum (ER) is a cellular factory that produces a wide range of proteins and lipids. These products are then transported to where they are needed, in the cell and beyond. Making accurate proteins is a challenge because folding and assembly take place at various sites within the lumen or membrane of the ER and in the cytosol. Furthermore, the specific and limited ER localization of many chaperones and modifying enzymes means that it is essential that molecules are not delivered to later compartments prematurely. For this reason, ER quality control (ERQC) mechanisms have evolved in all eukaryotes to monitor the folding and oligomerization states of nascent polypeptides. These systems retain proteins that are still in the process of folding in a particular compartment until folding is complete, and they target misfolded proteins for destruction by ER-associated degradation (ERAD) pathways.

ERAD mechanisms vary between organisms. The common link among all ERAD pathways is the final step: substrate degradation by the 26S proteasome. Mechanistically, all ERAD systems must have factors that recognize substrates, translocate them across ER membranes and target them to the proteasome, usually through polyubiquitination. The systems operating in yeast are the most basic, with two defined pathways organized around the two E3 ubiquitin ligases Hrd1p and Doa10p (ref. 1). The importance of these pathways is underscored by their conservation in all eukaryotes. Metazoans have evolved diverse ERAD pathways, with the complete list still to be revealed². Although the reasons for ERAD complexity continue to emerge, early indications are that different complexes are used to monitor different classes of substrate. The simplest version is found in yeast where the Hrd1p complex detects substrates with luminal or transmembrane lesions and the Doa10p com-

plex monitors the folding of cytosolic domains³. This arrangement might seem sufficient to cover all types of substrate. Therefore, understanding why mammals expanded this basic framework several-fold should provide a new insight into the evolution of complex biosynthetic pathways. Characterization of distinct Hrd1 complexes in mammals highlights one mechanism that has evolved to use different components to detect misfolded proteins.

N-linked glycans participate in the folding pathways of many glycoproteins⁴. Thus, when glycosylation was shown to directly affect folding quality control many people were interested. Aebi and co-workers were the first to observe that misfolded proteins bearing improperly processed glycans degrade poorly⁵. This led to the idea of a 'mannose timer' mechanism whereby proteins are free to fold until ER mannosidases begin trimming the glycans. In this model, processed glycans are used to indicate that the protein has exceeded its folding window and should be degraded. Currently, the best candidate for the interpreter of the glycan 'signal' is the Yos9p (Yeast osteosarcoma 9) family of lectin-like proteins⁶⁻⁹. Yos9p is a yeast luminal protein that is part of the Hrd1p complex^{3,10,11}. Although Yos9p can associate with misfolded proteins whether or not they have glycans attached, its ERAD function absolutely depends on the integrity of its glycan-binding MRH (mannose 6-phosphate receptor homology) domain^{7,8,12}. The identity of two metazoan homologues of Yos9p, OS-9 and XTP3-B, were known but their molecular functions were unclear. In this issue, Christianson *et al.*¹³ describe how they set out to determine whether and how they contribute to mammalian ERAD. In the course of their studies, they discovered that both OS-9 and XTP3-B are important for ERAD but form distinct complexes to target different substrates, thereby extending the theme of ERAD expansion in higher eukaryotes.

Christianson *et al.* determined that both OS-9 and XTP3-B are part of the mammalian Hrd1 complex, which strongly suggests they have roles in ERAD. Pull-down experi-

ments showed that both factors associate with SEL1L, the mammalian orthologue of yeast Hrd3p. SEL1L is a membrane protein with a large luminal domain that forms a 1:1 complex with Hrd1 (Fig. 1). This arrangement parallels that of the Hrd1p complex in budding yeast. Affinity-purification and mass-spectrometry experiments indicated that OS-9 and XTP3-B do not form complexes with one another. So, unlike in budding yeast, these data indicate the existence of distinct Hrd1 complexes, defined by the specific lectin factor bound to SEL1L. As there are significant differences between the two lectins (OS-9 has one MRH domain and XTP3-B has two), these data raise the possibility that different Hrd1 complexes handle different classes of substrate.

Using short hairpin (sh)RNA-mediated knockdown experiments to assess the role of these lectins in ERAD, the authors observed a clear difference between the two factors. Reduction of OS-9 stabilized the well-established substrate NHK, a variant of α_1 -antitrypsin, and reduction of XTP3-B had no effect. The double knockdown showed no enhancement, suggesting that XTP3-B does not functionally compensate for OS-9. Curiously, both proteins can bind to NHK *in vivo*, yet only association with OS-9 triggers substrate degradation. The XTP3-B phenotype is reminiscent of Yos9p binding to a non-glycosylated variant of the luminal ERAD substrate CPY*, which is also non-productive. This view is supported by the ability of XTP3-B, but not OS-9, to bind to a mutant form of transthyretin (TTR), a non-glycosylated ERAD substrate. These results suggest that XTP3-B is involved in ERAD but has a client list distinct from that of OS-9. Attempts to find *bona fide* XTP3-B substrates have not yet been successful. Two other substrates, the T-cell receptor α subunit and mutant ribophorin I (RI₃₃₂) do not require either lectin for degradation.

The authors judiciously turned to a biochemical approach to learn more about the factors. S-tagged OS-9 and XTB3-B were affinity purified from cells and microsomes under native

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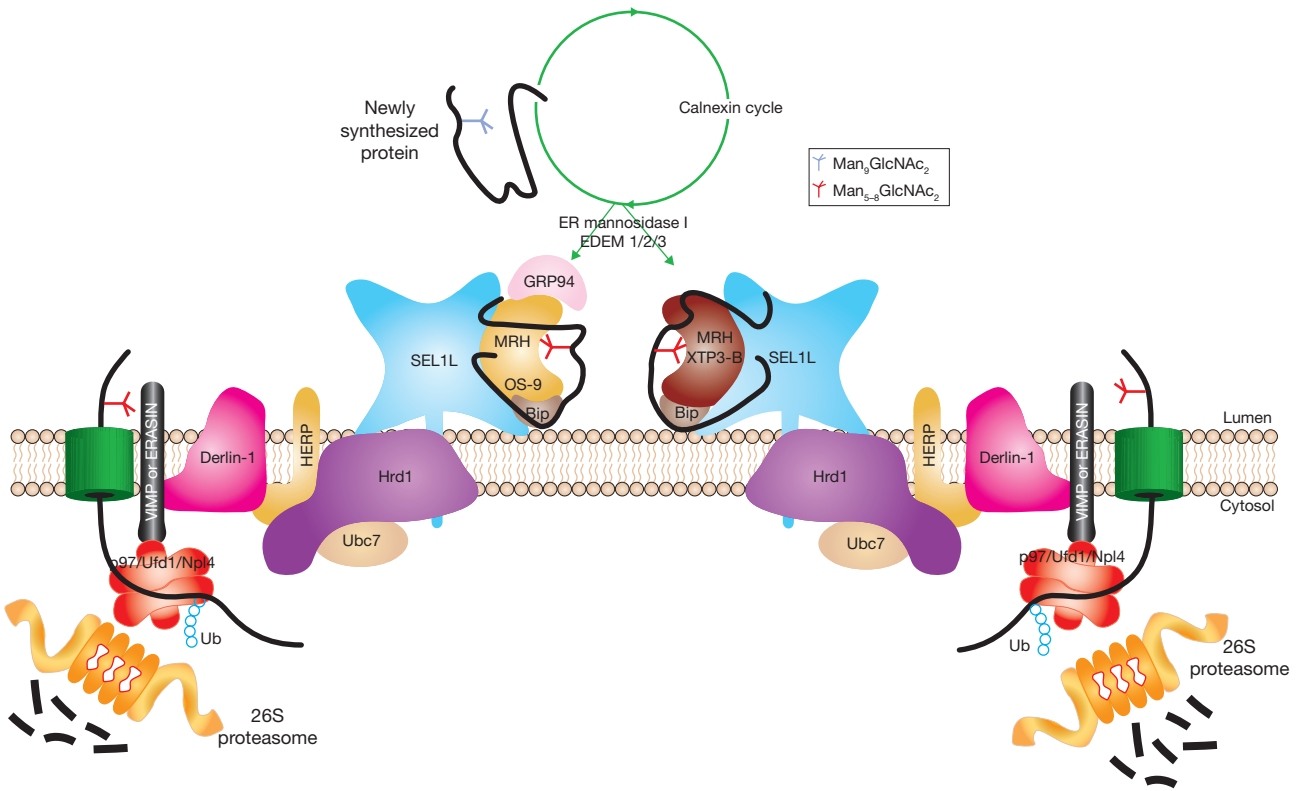


Figure 1 ER-associated degradation via the Hrd1 complex. Newly synthesized glycoproteins enter the calnexin cycle for folding. If a protein becomes misfolded, ER mannosidase I and/or EDEM family proteins will trim mannose residues from $\text{Man}_9\text{GlcNAc}_2$ to leave $\text{Man}_{5-8}\text{GlcNAc}_2$ glycans. Two distinct complexes composed of either OS-9 or XTP3-B recognize substrates bearing trimmed glycans and transfer them to the Hrd1 complex. Substrates associated with BiP can be recognized by either OS-9 or XTP3-B, whereas those bound to GRP94 are specific for OS-9. The retrotranslocon pore is either formed by Derlin-1, which is linked to Hrd1 through HRP23 (Usa1p in yeast), or the Sec61 complex. Segments in the cytosol are ubiquitinated (shown as Ub) by Hrd1/Ubc7. ERASIN (Ubx2p in yeast) or VIMP recruits the p97 ATPase complex to the ER membrane and extracts the ubiquitinated substrate. Once in the cytosol, the substrate is degraded by the 26S proteasome.

conditions, and the identities of associated proteins were revealed using ‘shotgun’ LC-MS/MS. Although many proteins were detected, those scoring the highest included ribophorin I (with both factors), and the ER chaperones BiP (with both factors) and GRP94 (OS-9 only). The functional significance of the ribophorin I interaction is unclear, but the association with BiP is significant because the analogous association occurs in yeast and is relevant for ERAD¹¹. The interaction with GRP94 is particularly intriguing because of its preference for OS-9 and the lack of a yeast homologue. GRP94 is the ER version of the well-studied cytosolic Hsp90 chaperone. GRP94 shRNA knockdown experiments showed a marked defect in NHK degradation that provides strong support for its role in ERAD. The most tantalizing observation came from pull-down experiments. Although GRP94 comes down with OS-9, and OS-9 can pull-down SEL1L, GRP94 does not precipitate with SEL1L, suggesting that they do not simultaneously exist in the same complex. This raises

the possibility that GRP94 acts at an earlier step, possibly in facilitating substrate recognition and membrane targeting. Christiansen *et al.* suggest that GRP94 facilitates the transfer of substrate from OS-9 to SEL1L. In support of these ideas, the amount of GRP94 bound to OS-9 increases when either SEL1L or Hrd1 is reduced.

Christianson *et al.* have demonstrated that OS-9 and XTP3-B are functional homologues of Yos9p. Their study strongly emphasizes that the glycan-dependent mechanism of ERAD is robust and highly conserved. Interestingly, it may be the differences between the yeast and mammalian systems that are the most insightful. Sommer and coworkers¹⁰ previously reported that Yos9p is substoichiometric to the Hrd1 complex, suggesting that a subpopulation lacking Yos9p exists. This population would certainly be functional for substrates with membrane lesions such as the mutant Sec61-2 protein because strains lacking Yos9p degrade such substrates efficiently³. So, even in yeast, the lectin seems

to act as a modular substrate adaptor for the Hrd1 complex. In this view, the Hrd1 complex serves as a platform that organizes core ERAD functions including substrate dislocation (through the Derlins or Sec61 complex), ubiquitination (by Hrd1 and Ubc7) and membrane extraction (by p97). It seems that metazoans evolved multiple accessory factors, adding further layers of substrate specificity. For example, there are three variants of the EDEM (ER degradation enhancing α -mannosidase-like protein) ERAD factor and three variants of Derlin, a putative ERAD translocon protein^{2,13}. All of these factors are either associated with or functionally linked to the Hrd1 complex. There may be more factors yet to be discovered. By developing a modular system, a wider variety of substrates can be channelled to the same core complex, possibly from multiple folding pathways (Fig. 1).

It is likely that the evolutionary expansion of the number and complexity of secretory and membrane proteins necessitated the

simultaneous expansion of ERAD pathways in higher eukaryotes. The multiple ERAD E3 membrane complexes found in the mammalian ER seem to support that view². The report by Christianson *et al.* extends the theme and provides an excellent example of how a core ERAD complex organized by Hrd1 could increase its breadth of substrates by evolving additional substrate receptors.

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A hypoxic twist in metastasis

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Hypoxia is a critical factor during tumour progression, regulating the expression of multiple factors implicated in tumour growth, epithelial to mesenchymal transition (EMT) and invasive cell behaviour. We now learn that the EMT inducer TWIST operates under the control of hypoxia signalling in the tumour microenvironment.

Hypoxia is an important factor in tumour development and progression¹. Cellular adaptation to hypoxia underlies critical steps in tumour progression, altering the expression of genes encoding products required for increased oxygen delivery, anaerobic metabolism and angiogenesis, among others. Intratumoural hypoxia has been also increasingly recognized as an important micro-environmental factor that promotes tumour aggressiveness and metastasis², through still poorly defined mechanisms. On page 295 of this issue, Wu and colleagues³ describe, for the first time, a link between hypoxia–HIF-1 α and TWIST upregulation that promotes EMT, invasion and metastasis.

The hypoxic response is mainly mediated by hypoxia induced factor-1 (HIF-1)⁴. HIF-1 is a heterodimeric protein consisting of a constitutively expressed subunit, HIF-1 β , and an oxygen-sensitive inducible subunit, HIF-1 α . Under normal oxygen tension (normoxia), HIF-1 α protein is hydroxylated by a family of oxygen-dependent prolyl hydroxylases (PHD1–3); this targets it for polyubiquitination by a protein complex containing von Hippel-Lindau protein (pVHL) and then degradation⁴. Under hypoxic conditions, prolyl hydroxylases are inactivated,

and HIF-1 α degradation is blocked; this allows HIF-1 α to accumulate and associate with HIF-1 β to form a functional transcription complex that triggers the transcription of a host of hypoxia-inducible genes⁴.

EMT — essential for several morphogenetic events during development — can be exploited by tumour cells to allow them to dissociate from neighbours and migrate⁵, and it often results in organ fibrosis⁶. EMT is regulated directly and/or indirectly by SNAI1, SNAI2 (Slug), some members of the bHLH family, such as E47/TCF3 and TWIST-1 (also known as TWIST), and ZEB factors (ZEB1/ δ EF1 and ZEB2/SIP1)⁷. Both SNAI1 and ZEB2 are important regulators of EMT, and it has been proposed that they are regulated by hypoxia during EMT in several models⁸. HIF-1 may control the expression of these genes: SNAI1, TCF3, ZEB1, and ZEB2 have been reported to be directly or indirectly regulated by HIF-1 in *VHL*-deficient renal carcinoma cells^{8,9}. Of note, regulators of SNAI1, such as lysyl oxidase like 2 (LOXL2) and related proteins, such as lysyl oxidase (LOX), have been also described as HIF-1 targets that are essential for EMT during hypoxia-mediated fibrogenesis and hypoxia-induced metastasis, respectively^{6,10}. However, the molecular mechanisms by which hypoxia induces EMT are still largely unknown.

Yang *et al.*³ bring light to how hypoxia results in EMT and metastasis. Through a series of elegant studies, the authors show that TWIST is a direct gene target of HIF-1 α and demonstrate

that TWIST mediates the invasion, migration and metastatic activity of different carcinoma cell types, including head and neck (HNSCC), breast and lung carcinoma. This regulatory mechanism seems to be relevant, at least in HNSCC, as TWIST is overexpressed in more malignant HNSCC tumours³.

TWIST is a bHLH factor that was first described as a master regulator of multiple developmental processes and induces EMT and metastasis¹¹. TWIST is expressed in several tumour types, and it is associated with more aggressive phenotypes and poor outcome⁷; however, it is still not well defined how TWIST expression is regulated and what its crucial target genes are. When overexpressed in polarized epithelial cells, TWIST induces EMT and transcriptional repression of E-cadherin¹¹, similarly to other EMT inducers (such as SNAI1/2, E47 and ZEB1/2)⁷. Overexpression of TWIST triggers the acquisition of invasive properties through induction of pro-migratory molecules, such as the cell adhesion protein N-cadherin¹², which also directly contributes to metastasis. Recently, a fascinating work by Ma *et al.*¹³ demonstrated that TWIST induces a microRNA molecule (miR-10b) that regulates *HOXD1* mRNA, thereby affecting downstream factors (such as RHOC) to control cell migration and malignant behaviour.

One open question in the field of EMT and tumour progression is the functional interaction between different EMT inducers. In this respect, the paper by Wu and colleagues

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