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Defining the Glycan Destruction Signal for Endoplasmic Reticulum-Associated Degradation

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Summary

The endoplasmic reticulum (ER) must target potentially toxic misfolded proteins for retrotranslocation and proteasomal degradation while avoiding the destruction of productive folding intermediates. For luminal proteins, this discrimination typically depends not only on the folding status of a polypeptide, but also on its glycosylation state. Two putative sugar binding proteins, Htm1p and Yos9p, are required for degradation of misfolded glycoproteins, but the nature of the glycan degradation signal and how such signals are generated and decoded remains unclear. Here we characterize Yos9p's oligosaccharide-binding specificity and find that it recognizes glycans containing terminal α1,6-linked mannose residues. We also provide evidence in vivo that a terminal α1,6-linked mannose-containing oligossacharide is required for degradation and that Htm1p acts upstream of Yos9p to mediate the generation of such sugars. This strategy of marking potential substrates by Htm1p and decoding the signal by Yos9p is well suited to provide a proofreading mechanism that enhances substrate specificity.

Introduction

The endoplasmic reticulum (ER) contains sophisticated quality control systems that monitor protein folding to ensure that only properly folded and oligomerized forms are transported forward through the secretory pathway (Anelli and Sitia, 2008; Fewell, 2001). Despite the highly specialized folding environment of the ER, inevitably some fraction of newly made polypeptides misfolds (Casagrande et al., 2000; Friedlander et al., 2000; Helenius and Aebi, 2001; Jensen et al., 1995; Travers et al., 2000; Ward et al., 1995). Such terminally misfolded forms are cleared from the ER through ER-Associated Degradation (ERAD) pathways in which they are first recognized and then retrotranslocated into the cytosol for destruction by the ubiquitin-proteasome degradation system (Nakatsukasa and Brodsky, 2008; Romisch, 2005).

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The cell must maintain a balance between overly promiscuous destruction of inherently slow-folding proteins or potentially functional mutants, while at the same time preventing the escape of toxic forms from the ER (Drumm et al., 1991; Sekijima et al., 2005). In order to achieve this balance, the ERAD machinery must accurately distinguish terminally misfolded proteins from abundant folding intermediates (Hebert and Molinari, 2007; Helenius and Aebi, 2001). The ERAD-L pathway, which degrades proteins that contain misfolded domains within the ER lumen (Huyer et al., 2004; Vashist and Ng, 2004), uses a bipartite recognition mechanism that interrogates both the glycosylation and the folding state of potential substrates (Denic et al., 2006; Gauss et al., 2006a; Knop et al., 1996b; Spear and Ng, 2005). This process is carried out by a large multi-protein complex that includes the E3 ubiquitin ligase Hrd1p (Bays et al., 2001; Deak and Wolf, 2001) and several transmembrane and cytosolic factors involved in extraction and ubiquitination of substrates (Carvalho et al., 2006; Denic et al., 2006; Gauss et al., 2006b). The luminal side of the complex contains a putative lectin, Yos9p, (Bhamidipati et al., 2005; Buschhorn et al., 2004; Kim et al., 2005; Szathmary et al., 2005) as well as Hrd3p (Gardner et al., 2000; Plemper et al., 1999), which recruits substrates based on the presence of misfolded domains. It has been recently shown that the disruption of the Yos9p sugar-binding domain or elimination of the sugar modification sites from the model ERAD-L substrate CPY* (CPY*0000) results in substrate stabilization (Finger et al., 1993; Knop et al., 1996b; Kostova and Wolf, 2005; Spear and Ng, 2005), however, CPY*0000 still efficiently interacts with Hrd3p in a manner that is dependent on folding status but not the glycosylation state of the substrate (Denic et al., 2006; Gauss et al., 2006a). These results suggest that recognition occurs as a multi-step process including the recruitment of misfolded proteins to the complex and a distinct commitment step where the presence of the glycans conveys information that is critical for substrate retrotranslocation and degradation (Denic et al., 2006; Gauss et al., 2006a).

While it is clear that the identification of misfolded forms critically depends on the glycosylation status of the misfolded protein, the nature of the glycan species that triggers destruction and how it contributes to specificity remains unclear. Previously, it has been shown that that the processing of the pre-assembled $Glc_3Man_9GlcNac_2$ oligosaccharide that is initially transferred to proteins at N-X-S/T sites is involved in ERAD recognition. This initial glycan is trimmed through the sequential action of two glucosidases and a mannosidase (Mns1p) to yield a $Man_8GlcNac_2$ species (Hebert et al., 2005; Helenius and Aebi, 2004). These trimming steps are required for ERAD-L, and it has been suggested that the period of time it takes to reach a certain glycan structure provides proteins a period of time to fold without risk of being degraded (Helenius, 1994; Hitt and Wolf, 2004; Jakob et al., 1998; Knop et al., 1996b; Wu et al., 2003). However, recent studies in yeast suggest that the above trimming steps occur rapidly on the scale of synthesis and degradation and thus are not well suited to increase specificity (Szathmary et al., 2005). Here we reveal that Yos9p recognizes trimmed glycans that expose a terminal α1,6-linked mannose and provide evidence that suggests that Htm1p, a mannosidase-like protein whose critical role in ERAD has been largely uncharacterized (Jakob et al., 2001; Nakatsukasa et al., 2001), is required to generate such sugars. Our results suggest a model where Htm1p “marks” misfolded glycoproteins by revealing a terminal α1,6-linked mannose that is recognized by Yos9p as the signal for degradation. This dual checking mechanism could provide increased specificity during ERAD.

Results

Yos9p structure and function does not depend on its N-linked glycans

A hallmark of Yos9p and its mammalian homologues is the presence of a mannose 6-phosphate receptor homology (MRH) domain (Munro, 2001). Mutations in this domain (e.g. R200A) that ablate the putative sugar-binding pocket disrupt Yos9p’s ability to support ERAD of glycoproteins suggesting that Yos9p acts as a lectin during substrate recognition (Bhamidipati

et al., 2005; Szathmary et al., 2005). In order to explore Yos9p's role as a lectin, we sought to produce biochemical amounts of Yos9p and directly evaluate its sugar-binding specificity.

Recombinant production of suitable quantities of Yos9p posed a technical challenge as Yos9 is glycosylated and contains many disulfide bonds. First, we mutated all the N-linked glycosylation consensus sites on Yos9p and found that glycosylation of Yos9p is not required for function, as the mutant protein fully supported ERAD of CPY* in vivo (Figure 1A). Attempts to produce native protein in *E. coli* yielded soluble disulfide cross-linked aggregates (Figure S1). We therefore developed an alternate expression strategy in which Yos9p was isolated under denaturing conditions from *E.coli* inclusion bodies, refolded under optimized conditions, and purified to apparent homogeneity (Figure 1B, S2). The refolded material appeared well-ordered by circular dichroism spectroscopy (Figure 1D), contained minimal intermolecular disulfide crosslinks (Figure 1B) and migrated as a single peak by gel filtration that is consistent with it being a trimer (Figure 1C). Using the same protocol, we also produced the R200A Yos9p MRH mutant. The R200A mutation did not disturb the folding or structure of Yos9p which is consistent with the proposal that this mutation interferes with ERAD function specifically by preventing sugar-binding rather than causing global unfolding (Figure 1D) (Bhamidipati et al., 2005; Szathmary et al., 2005).

Yos9p recognizes a terminal α1,6-linked mannose

We next determined the sugar-binding specificity of our recombinant Yos9p using frontal affinity chromatography (FAC), which provides a quantitative way to evaluate lectin-oligosaccharide interactions in solution. In this approach, protein is immobilized on a matrix and oligosaccharides, which are fluorescently labeled by pyridylamination (PA), are applied to the column of immobilized protein. The degree of retardation of the sugar, relative to a control sugar that is not recognized by the immobilized protein, provides a quantitative equilibrium measure of the binding affinity, with longer delays corresponding to tighter binding constants (See Figure 2 and Experimental Procedures) (Hirabayashi et al., 2003).

The FAC analysis revealed that Yos9p has a specificity for oligosaccharides containing a terminal α1,6-linked mannose which is not present in the initial N-linked glycan (see Figure 2A, 2B for schematics of high mannose sugars). Importantly, Yos9p's sugar specificity is dependent on an intact MRH domain, as the R200A lectin mutant shows no affinity for any of the glycans tested (Figure 2D). Yos9p has little affinity for the final trimming product of the two glucosidases (M9.1) or for the trimming product of Mns1p (M8.1) (Figure 2B)(Helenius and Aebi, 2004). In contrast, Yos9p recognizes species with the final mannose on the C branch removed to reveal a terminal α1,6-linked mannose. A comparison between M8.1 and M8.2 highlights the critical role of the α1,6-linked mannose, as both have eight mannoses arranged into three branches but only M8.2, which has the terminal α1,6-linked mannose, interacts with Yos9p. The α1,6-linked mannose seems to be necessary and largely sufficient for recognition by Yos9p because sugars containing this signal show significant affinity for Yos9p. The other residues seem to have relatively small effects on Yos9p binding with one prominent exception being the M5.1 species which has a higher affinity. How and if this species is generated under physiological conditions is unclear (see discussion).

Terminal α1,6-linked mannose containing N-linked glycans serve as the ERAD-L degradation signal

We next tested the functional significance of the terminal α1,6-linked mannose as an ERAD signal in vivo focusing on M7.1 because it is most likely to serve as a degradation signal as it requires minimal modification to the Mns1p produced M8.1 sugar (Herscovics, 2001). To test the role of M7.1, we took advantage of the previous observation by Aebi and coworkers that the M7.1 sugar could be produced in vivo using a series of genetic mutations in the asparagine-

linked glycosylation (ALG) biosynthesis pathway (Figure 3A top). Specifically, by simultaneously deleting *ALG9*, which leads to accumulation of M6.2 sugars, and then artificially bypassing the next step by over-expression of the *ALG12* mannosyltransferase, similar levels of M6.2 sugars and M7.1 sugars which contains a terminal α1,6-linked mannose are transferred to proteins (Figure 3A bottom) (Burda et al., 1999).

Analysis of ERAD-L in the *alg9*Δ /*ALG12* over-expression strain strongly supports the proposal that Man7.1 is productively recognized by Yos9p. As seen previously, deletion of *alg9* which produces M6.2 sugars that lack a terminal α1,6-linked mannose, results in stabilization of CPY* (Figure 3B and S3) (Jakob et al., 1998). By contrast, deletion of *alg9* together with overexpression of *ALG12* results in degradation of approximately fifty percent of CPY* (Figure 3B and S3). This result is consistent with the ratio of M6.2 and M7.1 sugars produced in the cell strongly suggesting that proteins with M7.1 are being subject to ERAD-L. Importantly, CPY* degradation in the *alg9*Δ/*ALG12* over-expression strain is dependent on presence of substrate glycans (Figure 3C), Yos9p (Figure 3D), and Der1p, another member of the Hrd1p complex (Figure 3E) (Gauss et al., 2006b; Knop et al., 1996a), indicating that degradation in this background goes through the classic ERAD-L pathway (Huyer et al., 2004; Kanehara et al., 2007; Kostova and Wolf, 2005; Spear and Ng, 2005; Vashist and Ng, 2004). Strikingly, in contrast to wild-type cells, we found that in the *alg9*Δ/*ALG12* over-expression background, Htm1p is dispensable for degradation (Figure 3F). An exposed α1,6-linked mannose on a second ERAD-L substrate, KHN, (Vashist et al., 2001; Vashist and Ng, 2004) also bypasses the need for Htm1p (Figure S4). Thus the presence of the M7.1 signal circumvents the requirement for Htm1p without bypassing the need for later-acting components like Yos9p and Der1p that are involved in reading out the signal and the ensuing steps leading to substrate degradation.

Discussion

Here we revealed the sugar-binding specificity of Yos9p, which together with functional studies, supports a model in which two key lectins, Htm1p and Yos9p, cooperate to enhance the specificity of the ERAD-L degradation system (Figure 4). This model builds on the previous observation that degradation by the Hrd1/Hrd3/Yos9 ubiquitin ligase complex requires a bipartite recognition of substrates involving both recognition of misfolded domains and glycans (Denic et al., 2006;Gauss et al., 2006a). Specifically, Hrd3p recruits potential substrates to the Hrd1p ligase complex based on the presence of misfolded domains. Yos9p then queries the glycans for what we have determined to be Yos9p's preferred oligosaccharide binding specificity, a terminal α1,6-linked mannose, through the action of Htm1p. Upon identification of an appropriate glycan signal, Yos9p commits the substrate for degradation. This model also provides an explanation for the previously enigmatic observation that ERAD-L occurs in *alg3*Δ strains even though several *ALG* mutants with less severe defects in the biosynthesis of N-linked glycans abrogate ERAD-L (Jakob et al., 1998), as the $Man_5GlcNac_2$ sugar produced in an *alg3*Δ strain also contains a terminal α1,6-linked mannose. Consistent with this, degradation of CPY* in the *alg3*Δ background does not require Htm1p (M. Aebi, personal communication and Figure S4).

While the enzymatic activity of Htm1p has not been directly examined, several observations suggest that Htm1p is required for generating the $Man_7GlcNac_2$ signal (either as an enzyme or as a cofactor) that is recognized by Yos9p. Jakob and colleagues reported that deletion of Htm1p results in reduced CPY*-Yos9ps interaction (Szathmary et al., 2005). Htm1p is homologous to α 1,2-mannosidases but lacks conserved cysteine residues that potentially contribute to enzymatic activity (Jakob et al., 2001; Lipari and Herscovics, 1996; Nakatsukasa et al., 2001). However, over-expression of the human Htm1p homologues, EDEM 1 and EDEM 3, which also do not contain the cysteine residues, leads to demannosylation suggesting that

they are active mannosidases (Hirao et al., 2006; Olivari et al., 2006). Additionally, Aebi and coworkers (personal communication) find that overexpression of Htm1p results in increased production of protein-bound $Man_7GlcNac_2$ oligosaccharide. Finally, we show that a yeast strain engineered to produce $Man_7GlcNac_2$ glycans as the starting sugar bypasses the requirement for Htm1p in Yos9p-mediated degradation of CPY*.

How might the requirement for the generation and recognition of a specific degradation glycan increase ERAD-L specificity? One possibility is that Htm1p acts as "timer," which acts independently of folding status of its substrate thereby providing all polypeptides with a protected window of time in which they can fold without risk of destruction (Helenius, 1994; Jakob et al., 1998; Wu et al., 2003). It is also possible that Htm1p is more sophisticated and that the presence of specific misfolded structures determines whether a substrate is marked by Htm1p. A precedent for such a mechanism is provided by the mammalian UDP-Glc:glycoprotein glucosyltransferase, which adds a glucose only to glycans proximal to a misfolded domain (Ritter and Helenius, 2000; Ritter et al., 2005; Trombetta and Helenius, 2000; Trombetta et al., 1991). Htm1p is also in a complex with Pdi1p whose chaperone activity could confer specificity based on substrate structure (Collins et al., 2007; Krogan et al., 2006). Either way, this modification of sugars adds another level of surveillance to the bipartate recognition of misfolded domains and sugar status by Hrd3p/Yos9p (Denic et al., 2006; Gauss et al., 2006a). The use of multiple query steps separated by irreversible steps would allow for a kinetic proofreading mechanism (Hopfield, 1974) ensuring enhanced specificity in the recruitment of misfolded proteins. In light of this model, it is intriguing that Yos9p shows its highest affinity for a specific $Man_5GlcNac_2$ species that is missing the two A- branch mannose residues, as this species has been observed in mammals (Avezov et al., 2008; Frenkel et al., 2003; Hosokawa et al., 2003; Kitzmuller et al., 2003; Lederkremer and Glickman, 2005). While it remains to be seen how or even if this species can be generated through additional sugar trimming in yeast, the enhanced affinity of Yos9p for the $Man_5GlcNac_2$ (M5.1) sugar species could provide a mechanism for preferentially degrading a subset of potential substrates.

On a practical note, a more sophisticated understanding of what constitutes a good ERAD substrate should enable a range of biochemical and structural studies to elucidate substrate recognition. In light of the multi-step nature of marking and decoding of ERAD-L substrates, it is perhaps not surprising that in vitro reconstitution of this process has proven so challenging. The discovery that substrate glycans containing a terminal α1,6-linked mannose such as $Man_7GlcNac_2$ are recognized by Yos9p should facilitate efforts to create a synthetic substrate with the correct glycan signal attached, thus bypassing the complicated upstream trimming steps. Furthermore, it should now be possible to monitor the specific recognition steps of ERAD-L in vitro. Several immediate questions emerge: How is Htm1p selecting its substrates? Are Yos9p and Hrd3p querying distinct structural features or are they simply double checking Htm1p's decisions? What is the mechanism of the commitment step once Yos9p confirms that the glycan is correct? Addressing these and related issues will provide a detailed molecular understanding of how and when the cell decides to commit ER proteins for destruction.

Experimental Procedures

Yeast Strains and Plasmids

All yeast strains are derivatives of S288c. Gene deletions, epitope taggings and promoter insertions were done using standard PCR based techniques. Details are available in the Supplemental Data section.

Cycloheximide Degradation Assays

Cycloheximide chase degradation assays were performed as previously described (Denic et al., 2006) with the exception that bands were visualized and quantitated using the LI-COR Odyssey system using an area of the blot with no specific signal as background. Following normalization to the hexokinase loading control, the values were plotted as averages ± standard error of the mean (SEM) with timepoint 0 set to 100%.

Yos9 Purification and Refolding

HIS-tagged Yos9p or Yos9 R200A was purified from Rosetta (DE3) pLysS inclusion bodies, solubilized in 8M urea and purified over a Ni-NTA agarose and a Source Q column. The protein was then refolded into 100mM Tris-HCL (pH 8.5) 150mM NaCl, 1mM CaCl2, 0.5M L-arginine, 5mM GSH, and 0.5mM GSSG at 4°C for 24 hours and subsequently purified over a Resource Q column before being buffer exchanged into 10mM HEPES, 1mM $Cacl_2$, 10% glycerol, and 150mM NaCl (pH 7.4). Further details are given in the Supplemental Data.

Frontal affinity chromatography

FAC analyses were carried out as previously described (Kamiya et al., 2008; Kamiya et al., 2005; Kasai, 1986). Details given in the Supplemental Data.

Circular dichroism spectroscopy

Circular dichroism (CD) measurements were conducted on a Jasco J-725 spectropolarimeter (Jasco Inc., Japan) at room temperature using samples containing 0.05 mg/ml of Yos9p or its R200A mutant in 0.1 M sodium phosphate, at pH 7.4. Each spectrum was recorded as the average of four scans over the range 195 -250 nm with a step size of 0.1 nm and a bandwidth of 1.0 nm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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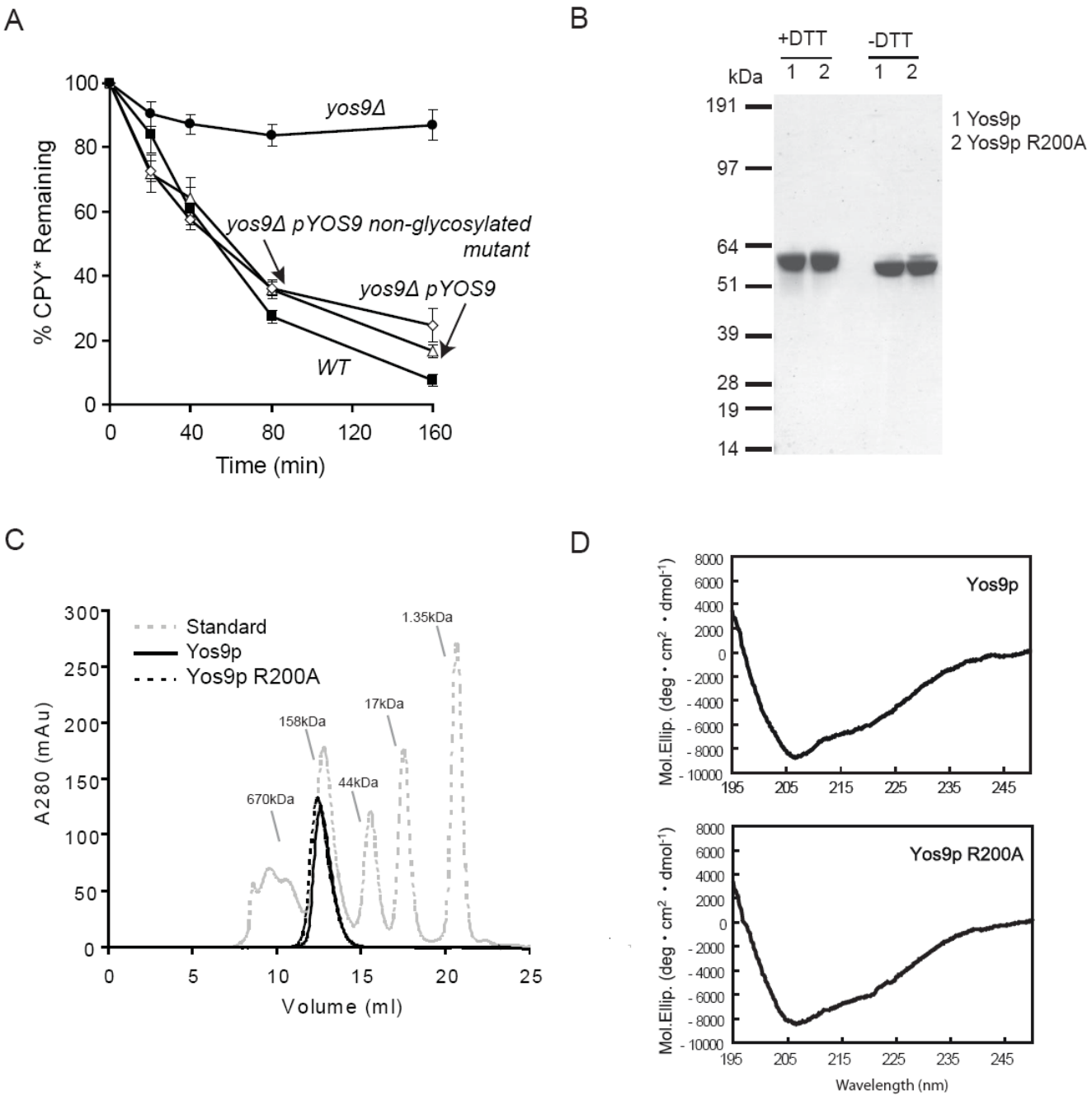

Figure 1. Purification of biochemical amounts of Yos9p from *E.coli*

(A) Degradation of CPY* in (■)wild-type (WT), and (●) a *yos9*Δ strain harboring an empty vector, and *yos9*Δ strain covered with a plasmid expressing YOS9-flag (△) or a Yos9p variant that is missing glycosylation sites (◇) was monitored by cycloheximide chase. Equal amounts of log phase cells were removed at the indicated times following addition of cycloheximide. Samples were resolved by SDS-PAGE and detected by Western analysis using anti-HA and anti-hexokinase antibodies. Each time point represents the average and +/- standard error of the mean (SEM) of 4 measurements (two independent experiments done in duplicate) and normalized to the hexokinase loading control.

(B) Refolded recombinantly expressed Yos9p (1) and Yos9p R200A (2) was purified and analyzed by SDS-PAGE with sample buffer containing either DTT or N-ethylmaleimide (-DTT) and stained with Coomassie.
(C) Gel Filtration analysis of Yos9p (—), Yos9p R200A (- - -) and molecular size standards (gray).
(D) Circular Dichroism spectra were acquired of Yos9p (top) and Yos9p R200A (bottom) as described in the experimental procedures.

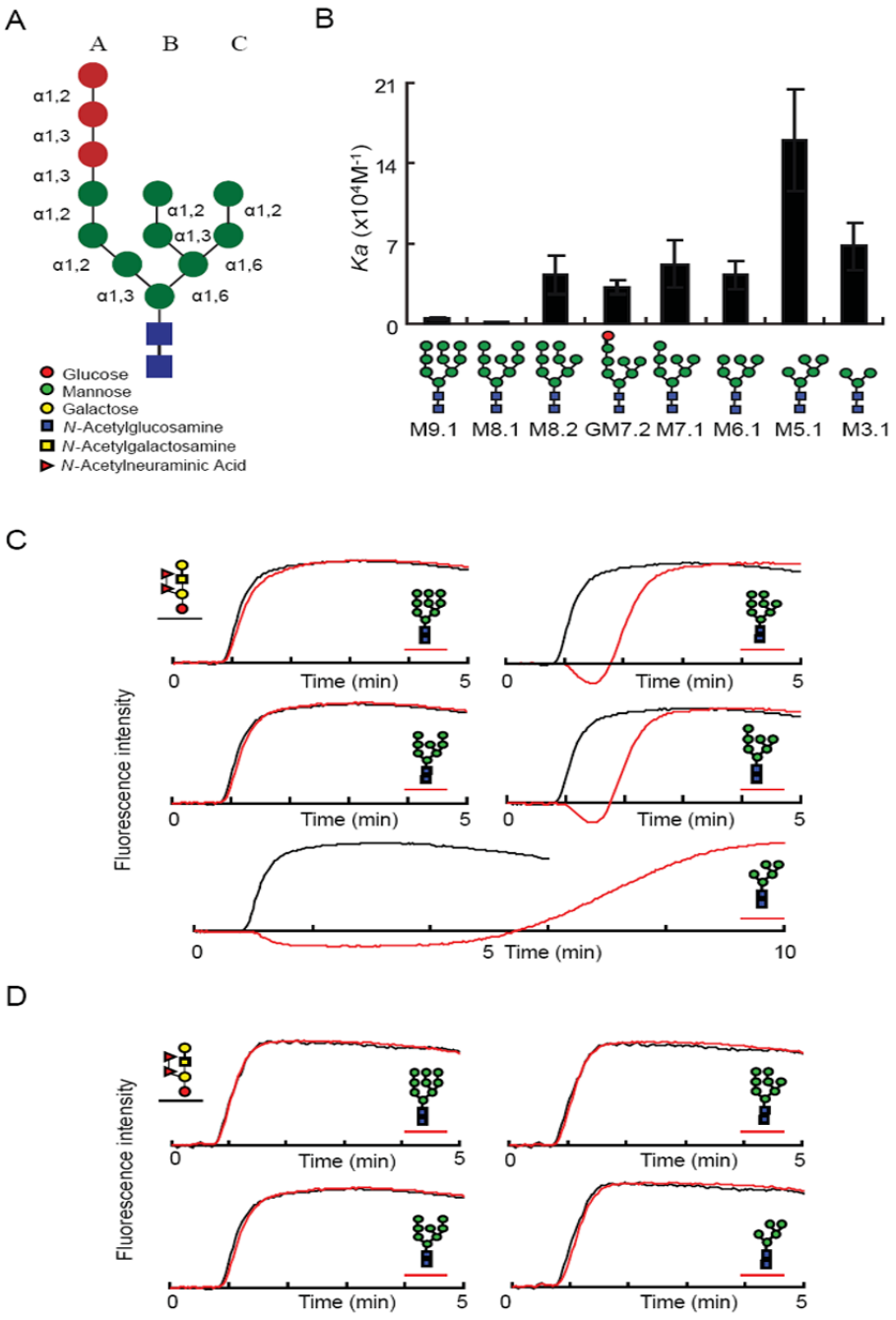

Figure 2. Yos9 recognizes glycans containing a terminal α1,6-linked mannose

(A) A schematic representation of the initial $Glc_3Man_9GlcNac_2$ N-linked sugar and the legend for each sugar moiety represented.

(B) FAC analysis of Yos9p sugar binding specificity. Indicated PA-oligasscharides were tested for binding to Yos9p by FAC analysis. *Ka* values were determined as described in the supplemental experimental procedures and are mean ± S.D. of three independent experiments. Each glycan structure is detailed and given a code name beneath the chart.

(C-D) Elution profiles over time of fluorescently labeled (PA)-oligosaccharides applied over immobilized histidine tagged Yos9p (C, red) or R200A mutant (D, red) in comparison to a

negative control sugar (black). PA-glycans are schematically represented next to the corresponding elution profile.

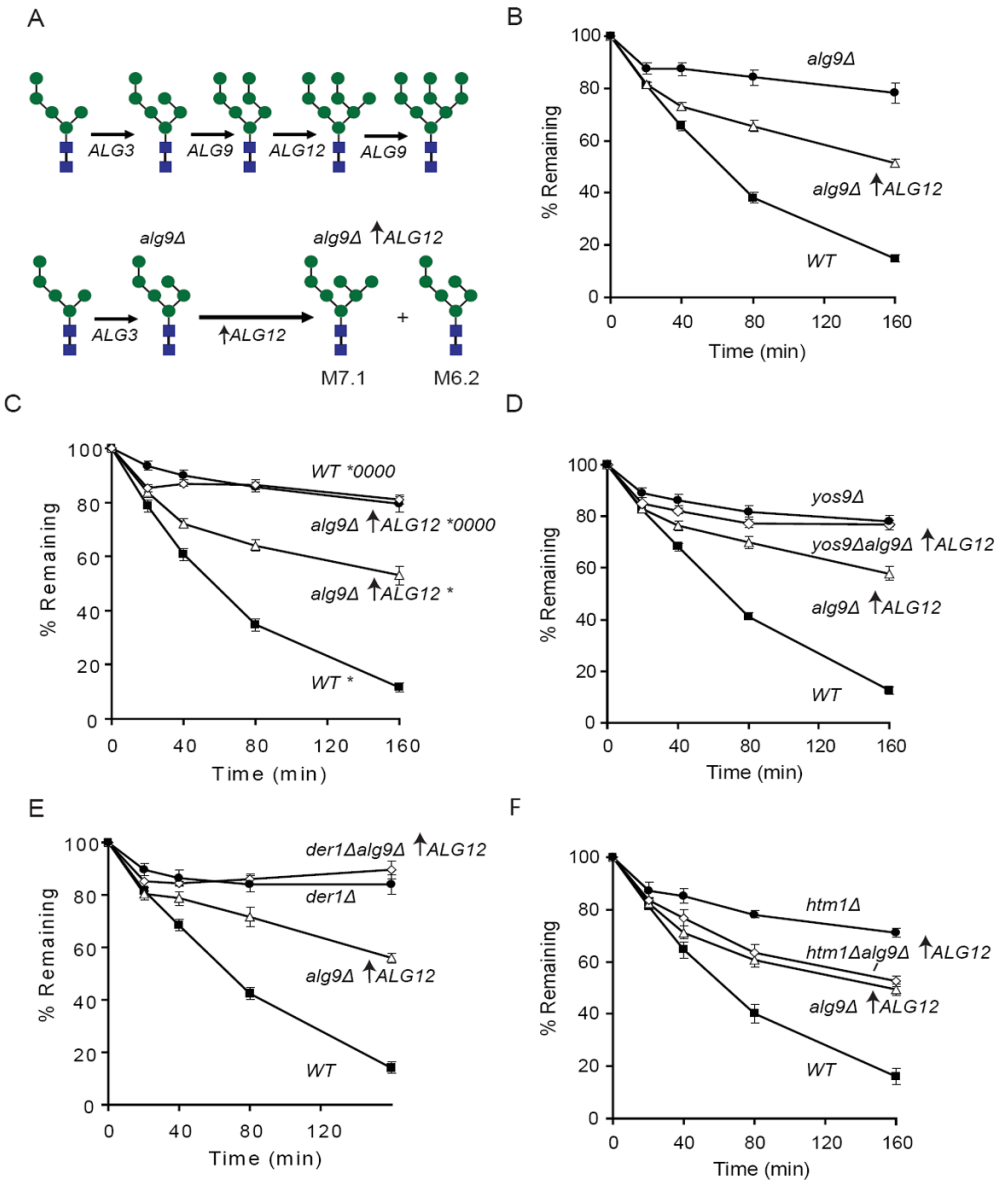

Figure 3. Production of $Man_7GlcNac_2$ sugars in vivo results in ERAD dependent degradation and bypass of *HTM1*

(A) Schematic representation of a portion of the asparagine linked glycosylation (ALG) pathway. Shown are the glycans produced in the wild-type (top) pathway and an *alg9*Δ over-expressing *ALG12* (upward arrow indicates TDH3 driven expression) strain (bottom). Mannose residues are represented as blue circles and N-acetylglucosamine is represented by blue squares.

(B) Degradation of CPY* in a (■) wild-type (WT), (△) *alg9*↑*ALG12*Δ, and (●) *alg9*Δ strains in this and the following panels were monitored as in Figure 1A except that each time point represents the average and +/- SEM of at least 8 measurements (4 independent experiments done in duplicate).

(C) Degradation of CPY* in (■) wild-type (WT) and (△) *alg9*Δ↑*ALG12* (upward arrow represents TDH3 driven expression), or CPY*0000 in (●) wild-type (WT) and (◇)

*alg9*Δ↑*ALG12* cells. CPY* is represented as a * and non-glycosylatable CPY* is represented as *0000.

(D) Degradation of CPY* in (■) wild-type (WT), (△) *alg9*Δ↑*ALG12*, (◇) *yos9Δalg9*Δ↑*ALG12* and (●) *yos9*Δ cells.

(E) Degradation of CPY* in (■) wild-type (WT), (△) *alg9*Δ↑*ALG12*, (◇) *der1Δalg9*Δ↑*ALG12* and (●) *der1*Δcells.

(F) Degradation of CPY* in (■) wild-type (WT), (△) *alg9*Δ↑*ALG12*, (◇) *htm1Δalg9*Δ↑*ALG12* and (●) *htm1*Δ cells.

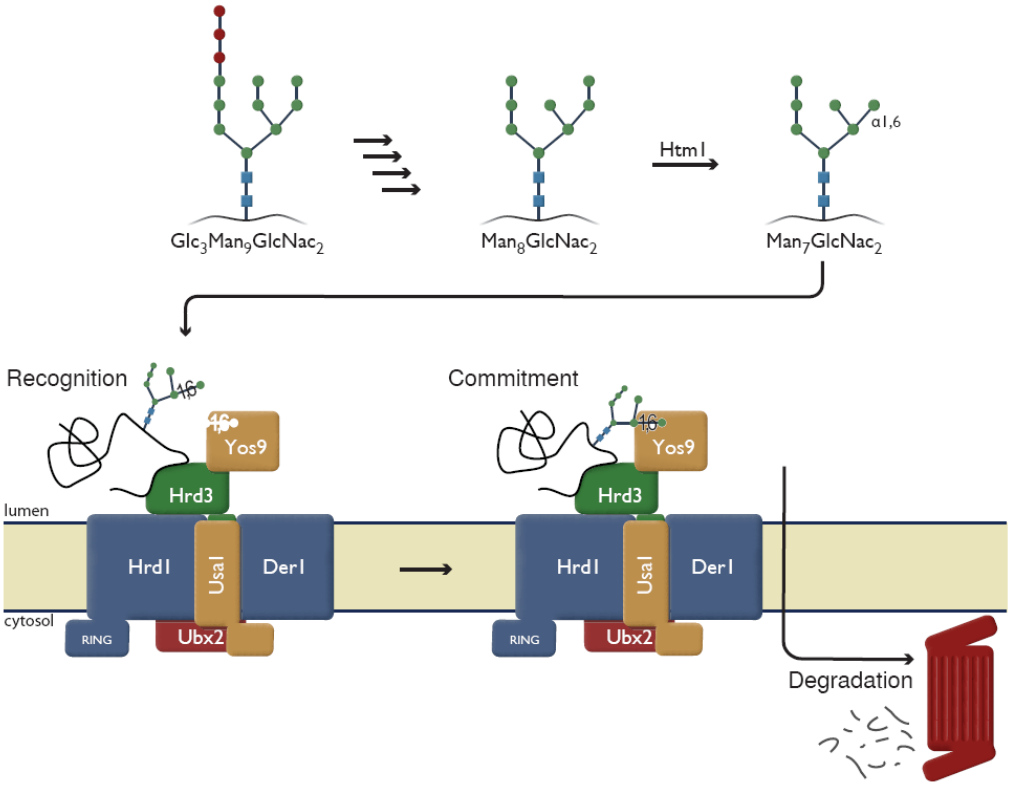

Figure 4. Model of dual recognition of substrates by ERAD

Glycan processing from the initial N-linked $Glc_3Man_9GlcNac_2$ to $Man_8GlcNac_2$ occurs by Glucosidase I, II and Manosidase I, respectively. Htm1p marks potential substrates by playing a role in the generation of $Man_7GlcNac_2$ (upper panel). Misfolded proteins are recruited to the Hrd1p complex by recognition of misfolded domains by Hrd3p. Yos9p queries the N-linked glycan and substrates are committed for degradation after Yos9p has identified the presence of a terminal α1,6-linked mannose. Note: Whether Htm1p is an enzyme or cofactor remains to be determined.