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Supplemental Data

Defining the Glycan Destruction Signal for Endoplasmic Reticulum-Associated Degradation

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Supplemental Experimental Procedures

Plasmids

Yos9p without glycans (Figure 1A) was expressed from a CEN/ARS plasmid made by using QuikChange mutagenesis (Stratagene) to change N to Q in the four N-X-S/T sites in the coding region of a C-terminal 3FLAG-tagged Yos9p expressed from its endogenous promoter (Denic et al., 2006).

For cycloheximide degradation assays, CEN/ARS CPY* and CPY*0000 plasmids were constructed with ~700 base pairs of the endogenous *PRC1* promoter, signal sequence, and the CPY* or CPY*0000 (made from pES150, a gift from Davis Ng, National University of Singapore) gene. CPY*/CPY*0000 was tagged with 3HA between the end of the coding region and the *PRC1* terminator as previously described (Bhamidipati et al., 2005). KHN (pSM70) was a gift from Davis Ng, National University of Singapore. For Figure 1A p316-BIPss-CPY*-3HA (Bhamidipati et al., 2005) was used.

C-terminally *HIS* tagged Yos9p was expressed in bacteria using pet23b-Yos9-6HIS (Figure 2B) constructed by amplifying the *YOS9* coding sequence without the signal sequence and inserting it into the *NdeI/XhoI* sites in pet23b. pet23b-Yos9 R200A-6HIS (Figure 2D) was made by subcloning the R200A mutation from pRS315-Yos9 R200A-FLAG3 (Denic et al.,

2006) into pet23b-Yos9-6HIS using *NcoI/PacI*. Pet23b-Yos9-8HIS (Figure 2C) was constructed by replacing the 6HIS with a GGS GGS linker and 8HIS tag.

All PCR derived inserts were checked by sequencing. The sequences of the plasmids are available upon request.

Strains

*yos9*Δ*::MET* was constructed as previously described (Bhamidipati et al., 2005). *yos9*Δ*::MET* and BY4741 (S288C, *MATa his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) were transformed with CPY* and either an empty vector or pRS315-YOS9-FLAG3 (Denic et al., 2006) with or without the glycosylation consensus sites to test whether the glycans on Yos9p are necessary for function (Figure 1A).

Strains for Figure 3 were made by crossing BY4741 with BY4742 (Brachmann et al., 1998) and subsequently deleting *ALG9* with the *Candida glabrata HIS3* prototrophic marker (Sakumoto et al., 1999), inserting the *TDH3* promoter in front of *ALG12* using a pFA6a-NATMX4-pTDH3 plasmid (a gift from D. Breslow, based on plasmids from (Longtine et al., 1998)) and deleting either *YOS9, DER1* or *HTM1* with the *Candida glabrata LEU2* gene using standard PCR-mediated methods at the genomic level. The diploid was then sporulated to obtain haploids that carried the desired combination of markers in the background of *Mat alpha his3*Δ*1 leu2*Δ*0 ura3*Δ*0 +/-lys2*Δ*0.* Strains for Figure S4 were made by taking the wild-type and single ERAD deletions from the above sporulation and deleting *ALG3* with the *Candida glabrata HIS3* prototrophic marker (Sakumoto et al., 1999). All strains altered by genomic insertions or deletions were checked by PCR.

Antibodies

The HA epitope was detected using 12CA5 monoclonal antibody (Roche) or HA.11 (Covance). Anti-hexokinase antibody was purchased from US Biologicals. Secondary antibodies labeled with IR800 dye and Alex Fluor 680 were purchased from Rockland Imunochemicals and Molecular Probes, Inc, respectively.

Protein Purification

Yos9p protein was expressed in Rosetta (DE3) pLysS cells (Novagen) from pet23b-Yos9-6HIS, Yos9-8HIS, or Yos9 R200A-6HIS. Expression was checked by Coomassie staining and Western analysis using an anti-His antibody (Santa Cruz Biotechnology). Cells were harvested, washed in 10mM Tris-HCL (pH 7.9), resuspended in 50mM Tris-HCL (pH7.9), 1mM EDTA, 150mM NaCl, protease inhibitors and lysed with 100µg/ml lysozyme on ice for 30 minutes plus sonication. Inclusion bodies were pelleted and washed twice with 50mM Tris-HCL (pH 7.9), 1mM EDTA, 2% Triton X-100, and protease inhibitors, and once with 50mM Tris-HCL (pH 7.9), 0.5mM EDTA, and protease inhibitors. The inclusion bodies were then dissolved in Buffer U (25mM Tris-HCL, 8mM 2-mercaptoethanol, 8M urea, protease inhibitors (pH 8.1)), incubate for 1hour and then centrifuged. The supernatant was filtered and bound in batch to Ni-NTA agarose (Qiagen). Bound protein was washed over a column with Buffer U with 0.8M NaCl, followed by a no salt wash and elution with 200mM Immidizole. Pooled fractions containing Yos9p were applied to a Source Q column and eluted using 25mM Tris-HCL, 10mM DTT, 1mM EDTA, 7M Urea, and a gradient of 0-400mM NaCL at pH 8.1. Fractions with protein were pooled, concentrated, and buffer exchanged using a PD-10 column (GE Healthcare) to remove DTT and EDTA. The protein was then refolded by dilution from 5mg/ml into 100mM Tris-HCL (pH 8.5)

150mM NaCl, 1mM $CaCl_2$, 0.5M L-arginine, 5mM GSH, and 0.5mM GSSG at 4ºC over the course of 1.5 hours for a final concentration of 100µg/ml (Lilie et al., 1998; Rudolph and Lilie, 1996). Refolding was monitored by running a SDS-PAGE gel of timepoints treated with 10mM 4-acetamido-4'-maleimidylstilbene-2,2'- disulfonic acid (AMS) or N-ethylmaleimide (NEM). After 24 hours, the protein was concentrated and diluted with 50mM Tris-HCL and 1mM $Cacl_2$ (pH 8.1) to lower the salt concentration before being applied to a Resource Q column (GE Healthcare) and eluted using 10mM HEPES, 1mM $Cacl_2$, 10% glycerol, and a 0-400mM NaCl gradient at pH 7.4. Clean fractions were then combined, and buffer exchanged into 10mM HEPES, 1mM $Cacl_2$, 10% glycerol, and 150mM NaCl (pH 7.4). Final protein was checked for presence of aggregates by gel filtration on a Superdex 200 10/300 GL column (GE Healthcare) and a non-reducing SDS-PAGE gel.

Supplemental References

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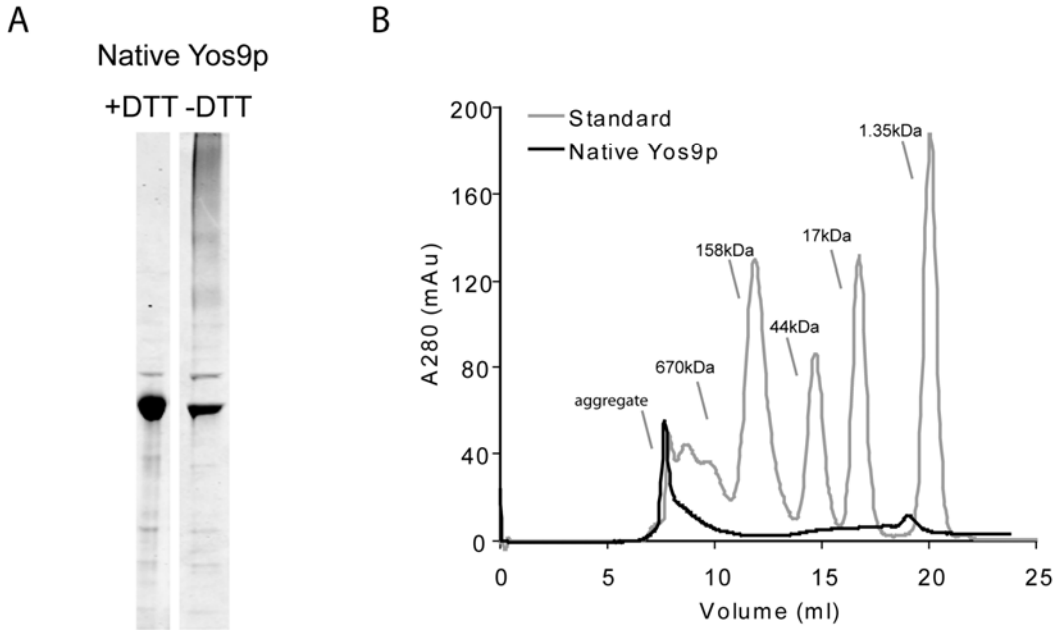
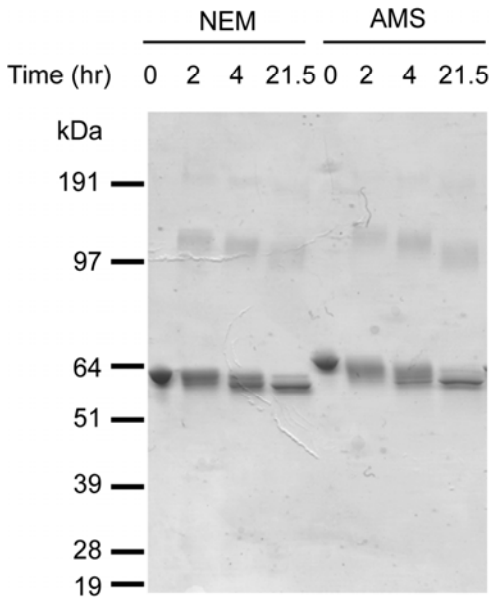


Figure S1. Native Yos9p forms disulfide-crosslinked aggregates
(A) Soluble Yos9p purified under non-denaturing conditions from *E. coli* was analyzed by SDS-PAGE with sample buffer containing either DTT or N-ethylmaleimide (-DTT).
(B) Elution profile for Yos9p purified natively from *E. coli* (black) and standards (gray) run on a gel filtration column in 20mM Tris-HCl (7.9), 150mM NaCl, 10% glycerol, 5mM GSH, and 1mM EDTA.



Figures S2. Refolding process of denatured Yos9p
Refolding of denatured Yos9p. To block free cysteines, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) or N-ethylmaleimide (NEM) were added to protein samples taken over time, resolved by SDS-PAGE and Coomassie stained. Note the time dependent shift of the protein. The protein was further purified to remove the higher molecular weight oligomers.

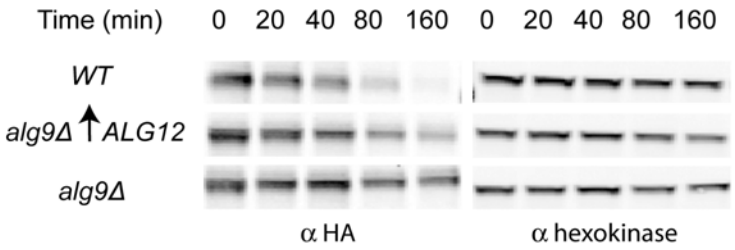


Figure S3. Production of $Man_7GlcNAc_2$ sugars results in degradation of CPY*
Degradation of CPY* in the indicated strain backgrounds were monitored and processed as in Figure 3B. A representative western blot is shown. Quantitation of the experiment is shown in Figure 3B.

Further Evidence that an exposed α1,6-linked mannose allows for Htm1p-independent ERAD of misfolded glycoproteins.

We tested a second ERAD-L glycoprotein substrate, KHN, to see if like CPY*, the Htm1p dependence could be bypassed by genetic manipulations that produced sugars containing an exposed α1,6-linked mannose. Because of the limited dynamic range of the *alg9Δ/ALG12* over-expression system, we used an *alg3*Δ to produce Man5.2 sugars with a terminal α1,6-linked mannose (Figure S4A)(Jakob et al., 1998). In agreement with observations from Aebi and coworkers (Clerc et al., 2009), we find that the loss of *ALG3* removes the requirement for Htm1p for the degradation of CPY* (Clerc et al. [2009] and Figure S4B). Similarly, we find that deletion of *ALG3*, eliminates the need for Htm1p in the degradation of KHN (Figure S4C). This is consistent with the hypothesis that a terminal α1,6-linked mannose is sufficient to allow for productive recognition by Yos9p although we have not directly tested Yos9p's ability to bind to M5.2 *in vitro*.

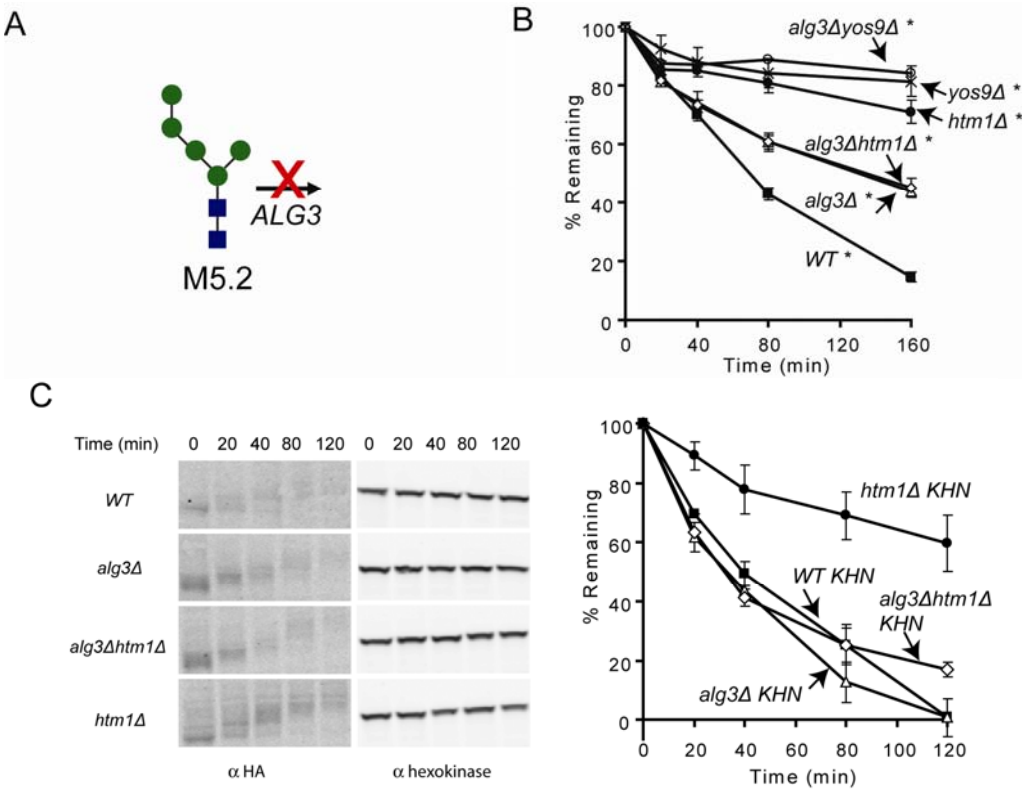


Figure S4. An exposed α1,6-linked mannose on CPY* or KHN results in bypass of *HTM1*
(A) A schematic representation of M5.2, a probably non-natural sugar produced by an *alg3*Δ strain. Mannose residues are represented as green circles and N-acetylglucosamine is represented by blue squares.
(B) Degradation of CPY* (depicted as a *) in (■) wild-type (WT), (△) *alg3*Δ, (◇) *htm1*Δ*alg3*Δ and (●) *htm1*Δ cells, (○) *yos9*Δ*alg3*Δ and (×) *yos9*Δ cells was monitored and processed as in Figure 1A.
(C) Degradation of KHN in (■) wild-type (WT), (△) *alg3*Δ, (◇) *htm1*Δ*alg3*Δ and (●) *htm1*Δ cells was monitored and processed as in Figure 3B. Representative gel shown (left).