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Human OS-9, a Lectin Required for Glycoprotein Endoplasmic Reticulum-associated Degradation, Recognizes Mannose-trimmed *N*-Glycans*[S]

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In the endoplasmic reticulum (ER), lectins and processing enzymes are involved in quality control of newly synthesized proteins for productive folding as well as in the ER-associated degradation (ERAD) of misfolded proteins. ER quality control requires the recognition and modification of the *N*-linked oligosaccharides attached to glycoproteins. Mannose trimming from the *N*-glycans plays an important role in targeting of misfolded glycoproteins for ERAD. Recently, two mammalian lectins, OS-9 and XTP3-B, which contain mannose 6-phosphate receptor homology domains, were reported to be involved in ER quality control. Here, we examined the requirement for human OS-9 (hOS-9) lectin activity in degradation of the glycosylated ERAD substrate NHK, a genetic variant of α1-antitrypsin. Using frontal affinity chromatography, we demonstrated that the recombinant hOS-9 mannose 6-phosphate receptor homology domain specifically binds *N*-glycans lacking the terminal mannose from the C branch *in vitro*. To examine the specificity of OS-9 recognition of *N*-glycans *in vivo*, we modified the oligosaccharide structures on NHK by overexpressing ER α1,2-mannosidase I or EDEM3 and examined the effect of these modifications on NHK degradation in combination with small interfering RNA-mediated knockdown of hOS-9. The ability of hOS-9 to enhance glycoprotein ERAD depended on the *N*-glycan structures on NHK, consistent with the frontal affinity chromatography results. Thus, we propose a model for mannose trimming and the requirement for hOS-9 lectin activity in glycoprotein ERAD in which *N*-glycans lacking the terminal mannose from the C branch are recognized by hOS-9 and targeted for degradation.

Recognition and sorting of improperly folded proteins is essential to cell survival, and hence, an elaborate quality control system is found in cells. ER[4] quality control is well characterized with respect to the *N*-linked oligosaccharides regulating the folding and degradation of newly synthesized proteins in the ER (1). Immediately after polypeptides enter the ER, $Glc_3Man_9GlcNAc_2$ (G3M9) precursor oligosaccharides are covalently attached and subsequently processed. Terminally misfolded proteins are removed from the ER by the ERAD machinery (1–4). Aberrant conformers are recognized, retrotranslocated to the cytosol, and degraded by the ubiquitin-proteasome system (5, 6). Processing of mannose residues from the *N*-linked oligosaccharides acts as a timer for the recognition of misfolded glycoproteins in the ER lumen (1, 7). ER α1,2-mannosidase I (ER ManI) in mammals and ER α-mannosidase in yeast preferentially trim mannose residues from the middle branch of *N*-glycans, generating the $Man_8GlcNAc_2$ (M8) isomer B (M8B) (8). In mammals, further mannose processing is required as a signal for degradation (1, 9, 10), whereas the presence of M8B is sufficient to signal degradation in yeast (11). The postulated lectin EDEMs in mammals, their yeast homolog Htm1p/Mnl1p, and the yeast MRH domain-containing lectin Yos9p have all been proposed to recognize glycoproteins targeted for degradation (12).

The role of Yos9p in glycoprotein ERAD was identified using a genetic screen in *Saccharomyces cerevisiae* (13). Yos9p, a homolog of hOS-9, contains an MRH domain (14) and functions as a lectin. Yos9p recognizes substrates of the ERAD-lumenal pathway (15–17), generating a large ER membrane complex containing the Hrd1p-Hrd3p ubiquitin ligase core complex (18–20). The M8B and $Man_5GlcNAc_2$ (M5) *N*-glycans are predicted to function as ligands for Yos9p (17). Bipartite recognition of both glycan and polypeptide by Yos9p has also been reported (15).

Recent studies revealed that two mammalian MRH domain-containing lectins, OS-9 and XTP3-B, are ER luminal proteins

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[4] The abbreviations used are: ER, endoplasmic reticulum; ERAD, ER-associated protein degradation; MRH domain, mannose 6-phosphate receptor homology domain; EDEM, ER degradation enhancing α-mannosidase-like protein; ER ManI, ER α1,2-mannosidase I; NHK, α1-antitrypsin null (Hong Kong); α1-AT, α1-antitrypsin; FAC, frontal affinity chromatography; PA, pyridylaminated; M9, $Man_9GlcNAc_2$; G3M9, $Glc_3Man_9GlcNAc_2$; M8B, $Man_8GlcNAc_2$ isomer B; M8C, $Man_8GlcNAc_2$ isomer C; M7, $Man_7GlcNAc_2$; M6, $Man_6GlcNAc_2$; M5, $Man_5GlcNAc_2$; G1M8B, $Glc_1Man_8GlcNAc_2$ isomer B; hOS-9, human OS-9; siRNA, small interfering RNA; HEK, human embryonic kidney; G1M7, $Glc_1Man_7GlcNAc_2$; G1M8C, $Glc_1Man_8GlcNAc_2$ isomer C.

involved in ER quality control and form a large complex containing the HRD1-SEL1L ubiquitin-ligase in the ER membrane (21–24). The components of the complex are similar to yeast, suggesting evolutionary conservation, although the molecular mechanisms underlying the role of OS-9 and XTP3-B remain elusive. Studies using lectin mutants have suggested that the MRH domains are required not for binding to ERAD substrates but for interactions with SEL1L (21), which has multiple *N*-glycans (25, 26). Additionally, lectin activity appears to be dispensable for hOS-9 binding to misfolded glycoproteins (21, 24). Thus, to understand the role of hOS-9 in the ER quality control pathway, the specific carbohydrate structures recognized by the hOS-9 MRH domain need to be identified, and the requirement of the lectin domain in substrate recognition needs to be determined.

In the present study we demonstrate that the lectin activity of hOS-9 is required for enhancement of glycoprotein ERAD. We identified the *N*-glycan structures recognized by the recombinant hOS-9 MRH domain *in vitro* by frontal affinity chromatography (FAC). Using a model ERAD substrate, NHK (27), we show that the ability of hOS-9 to enhance ERAD *in vivo* depends on the oligosaccharides present on NHK, consistent with the FAC results.

EXPERIMENTAL PROCEDURES

Plasmid Construction—For bacterial expression, the hOS-9 MRH domain (Ile-98—Ile-244) was amplified by PCR and subcloned into the BamHI site of pET15b (Novagen). NHK (28) and NHK-QQQ (29) were used as model ERAD substrates and have been described previously. Hemagglutinin-tagged human ER α1,2-mannosidase I (30) and mouse EDEM3 (29) and FLAG-tagged hOS-9v1 and hOS-v2 (22) were used for overexpression experiments. The hOS-9 R188A mutant was constructed using a site-directed mutagenesis kit (Stratagene) to replace Arg (CGG) with Ala (GCG).

Antibodies—The anti-SEL1L antibody (rabbit polyclonal) used for immunoprecipitation was kindly provided by Dr. Tatsuya Moriyama (Kinki University, Japan). Other antibodies were purchased as follows: anti-α1-AT (rabbit polyclonal, DAKO), anti-OS-9 (rabbit polyclonal, ProteinTech group), anti-SEL1L (mouse monoclonal, Lifespan), anti-actin (mouse monoclonal, Chemicon), anti-hemagglutinin (rabbit polyclonal, Santa Cruz Biotechnology), and anti-FLAG M2 (mouse monoclonal, Sigma).

siRNA Sequences—The siRNA duplexes targeting hOS-9 and the control siRNA were purchased from Invitrogen (Stealth™ siRNA). The three siRNAs for hOS-9 were: OS9-1, 5′-AUCCCUGAGUUGUUGAGCCCAAU-3′; OS9-2, 5′-GGAAACGCUGCUGUCCAGUUUGUUA-3′; OS9-3, 5′-GGAGGAGGAAACACCUGCUUACCAA-3′. The low GC (control-1) and medium GC (control-2) Stealth™ siRNA duplexes were used as negative controls.

Transfection of Plasmids—Plasmids were transfected into human embryonic kidney (HEK) 293 cells using FuGENE 6 (Roche Applied Science) according to the manufacturer's protocol. Lipofectamine RNAiMAX (Invitrogen) was used to introduce siRNA (30 nM) into cells. Cells were transfected with 1 μg of plasmid encoding NHK 24 h after introduction of the siRNA using the FuGENE 6 transfection reagent.

Cell Culture, Metabolic Labeling, and Pulse-Chase Experiments—HEK 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (100 units/ml penicillin G and 0.2 mg/ml streptomycin). Metabolic labeling and pulse-chase experiments were performed as described previously (22). Briefly, cells were labeled with 4.1 MBq/ml ^{35}S-Express Protein Labeling mixture (PerkinElmer Life Sciences) for 3 h or for pulse-labeling with 8.2 MBq/ml ^{35}S-Express Protein Labeling mixture for 15 min after incubation in medium lacking methionine/cysteine for 20 min. For the chase period, normal growth medium containing methionine and cystine-2HCl were added for the indicated times. To quantify the radioactivity of labeled proteins, SDS-PAGE gels were exposed to phosphorimaging and quantified using ImageQuant (STORM, GE Healthcare).

Immunoprecipitation and Western Blotting—Cells were solubilized in buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5) containing 1% Nonidet P-40 supplemented with protease inhibitors. After centrifugation at 12,000 rpm for 20 min at 4 °C, the supernatant was used for immunoprecipitation or Western blotting, as described previously (22). To detect coimmunoprecipitation of hOS-9 and SEL1L, cells were lysed in buffer containing 3% digitonin instead of 1% Nonidet P-40. Immunocomplexes precipitated with protein A- or protein G-Sepharose beads were incubated in Laemmli buffer (1×) at 65 °C for 15 min. Nitrocellulose or polyvinylidene difluoride membranes and Blocking-One solution (Nacalai Tesque, Japan) were used for Western blot analysis. Antibodies were diluted in phosphate-buffered saline containing 0.1% Tween 20 supplemented with 5% Blocking-One or in the Can Get Signal buffer (TOYOBO, Japan) and detected by ECL (GE Healthcare Biosciences).

Purification of the Recombinant hOS-9 MRH Domain—The hOS-9 MRH domain with a hexahistidine tag at the N terminus was expressed in *Escherichia coli* Origami (DE3) as inclusion bodies, which were solubilized with 6 M guanidinium chloride. The solution was diluted into a refolding buffer composed of 20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 5 mM glutathione reduced form, and 0.5 mM glutathione oxidized form. The refolded hOS-9 MRH domain was purified by gel filtration chromatography.

Frontal Affinity Chromatography—The recombinant hOS-9 MRH domain was immobilized on Ni^{2+}-Sepharose via its histidine tag according to the manufacturer's instructions (GE Healthcare). After immobilization, the Sepharose beads were packed into a high performance stainless steel column (4.0 × 10 mm, GL Sciences). FAC analyses were performed as described previously (31–33). K_a values were calculated and are presented as the mean ± S.D. of three independent experiments.

A pyridylaminated (PA)-oligosaccharide library was constructed as described previously (31). Each PA-oligosaccharide was dissolved at a concentration of 10 nM in 10 mM HEPES, pH 7.4, 150 mM NaCl, and 1 mM $CaCl_2$ and applied to the column at a flow rate of 0.25 ml/min at 20 °C. The elution profiles were monitored by fluorescence intensity at 400 nm (excitation at 320 nm). Retardation of an oligosaccharide compared with the

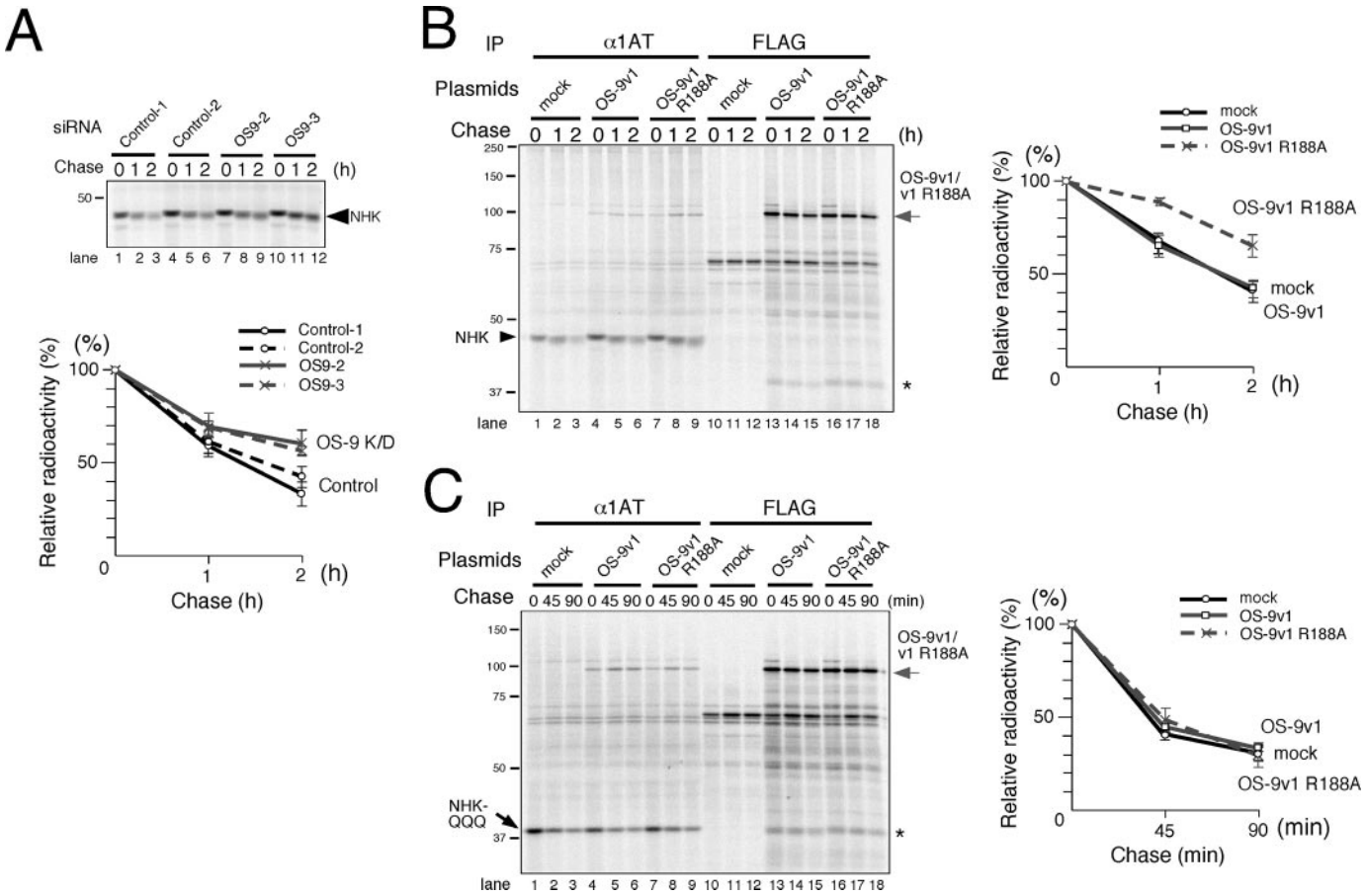

FIGURE 1. **The hOS-9 lectin domain is required for ERAD of the misfolded glycoprotein NHK.** *A*, inhibition of NHK degradation by hOS-9 knockdown. HEK 293 cells incubated with the indicated siRNA (30 nM) were pulse-labeled with [^{35}S]methionine/cysteine for 15 min and chased for the indicated times. NHK was immunoprecipitated with anti-α1AT, and samples were separated by SDS-PAGE. The relative radioactivity of NHK (*arrowhead*) was quantified and normalized using the level at the 0 h chase as 100%. *Error bars* indicate S.E. ($n = 3$ or 4). Two negative control siRNAs (*Control-1* and *-2*) and two specific siRNAs (OS9-2 and OS9-3) were used. *B*, degradation of NHK in the presence of the overexpressed hOS-9v1 R188A mutant. Cells were pulse-labeled as in *A*. Cell lysates were divided into aliquots and immunoprecipitated (*IP*) with anti-α1AT (*lanes 1–9*) or anti-FLAG (*lanes 10–18*). The *arrowhead* indicates the position of NHK, and the *arrow* indicates the position of the hOS-9v1-FLAG wild-type or R188A mutant. The *asterisk* denotes a hOS-9 fragment generated by overexpressing FLAG-tagged hOS-9. NHK was quantified and the data are presented in the *panel* on the *right* as the mean ± S.E. ($n = 3$). *C*, degradation of NHK-QQQ (*black arrow*) in the presence of the overexpressed hOS-9v1 or its R188A mutant. Quantitation is shown in the *panel* on the *right*.

control oligosaccharide (PA-GD1b-hexasaccharide) was estimated based on the difference in elution volume, V_f. Dissociation ($K_d = 1/K_a$) of the recombinant hOS-9 MRH domain and $Glc_1Man_7GlcNAc_2$-PA (G1M7) was determined by a concentration-dependent analysis using Equation 1, $[A]_0 \cdot (V_f - V_0) = B_t - K_d \cdot (V_f - V_0)$, where $[A]_0$, V_0, and B_t, are the initial concentration of the PA-oligosaccharide, the elution volume of the control sugar, and the total amount of immobilized lectin in the column, respectively. The elution profile was monitored by UV absorption at 300 nm to avoid possible quenching caused by the relatively high concentration of the PA-sugar. For determination of V_0, 50 μM *p*-nitrophenyl-β-D-galactopyranoside was used. The K_d was calculated based on the retardation $V_f - V_0$ measured at concentrations of 2–24 μM G1M7.

The relative affinity of each oligosaccharide was calculated under conditions where $[A]_0$ was negligibly small compared with the K_d using Equation 2, $V_f - V_0 = B_t/K_d$. To determine the V_0, Galβ1–3GalNAcβ1–4(Neu5Acα2–8Neu5Acα2–3)Galβ1–4Glc-PA (PA-GD1b-hexasaccharide) was used as the control sugar for the analysis of the hOS-9 MRH domain.

RESULTS

hOS-9 Lectin Activity Is Required for ERAD of NHK—To examine the role of hOS-9 in glycoprotein ERAD, we knocked-down hOS-9 in HEK 293 cells and examined the degradation of the lumenal ERAD substrate NHK. Pulse-chase experiments revealed a partial inhibition of NHK degradation when endogenous hOS-9 levels were reduced by siRNA (Fig. 1*A*), indicating that hOS-9 is involved in NHK ERAD. The inhibition was significant after a 2-h chase. NHK secreted into the medium was negligible under our experimental conditions (28) and was not affected by either knockdown or overexpression of hOS-9 (supplemental Fig. 1 and data not shown). Thus, we were able to evaluate the extent of NHK ERAD by its disappearance from cell extracts. All three of the siRNAs tested efficiently diminished hOS-9 accumulation within the cell (supplemental Fig. 2). However, the OS9-1 siRNA was excluded from subsequent experiments because it also reduced the expression of transfected hXTP3-B (data not shown). We used the two siRNAs specific for hOS-9 (OS9-2 and OS9-3) and two control siRNAs in the following experiments. The inhibition of NHK degrada-

tion by siRNA-mediated hOS-9 knockdown was rescued by transfecting siRNA-resistant hOS9 (supplemental Fig. 3).

To examine the role of the lectin activity of the hOS-9 MRH domain, we constructed a mutant hOS-9 lacking lectin activity by mutating arginine 188 to alanine (R188A). Arg-188 corresponds to of Arg-200 of yeast Yos9p and is one of the conserved amino acids in the MRH domain. Mutation of Arg-200 abolishes lectin activity in Yos9p (17). The loss of glycan binding activity of the hOS-9 R188A mutant was confirmed by FAC analysis (see Fig. 3*C*). To determine the functional involvement of the hOS-9 MRH domain in the recognition of *N*-glycans on misfolded glycoproteins, we overexpressed the hOS-9 R188A mutant in HEK 293 cells expressing NHK. NHK degradation was partially inhibited by overexpressing the hOS-9v1 R188A mutant but not by overexpressing the wild-type hOS-9v1 (Fig. 1*B*), suggesting that an active lectin domain is necessary for ERAD of misfolded glycoproteins. We next examined the effect of hOS-9 R188A on the non-glycosylated ERAD substrate, NHK-QQQ. In contrast to NHK, NHK-QQQ degradation was not affected by overexpressing either the wild-type or the R188A mutant hOS-9 (Fig. 1*C*). To examine co-immunoprecipitation of NHK/NHK-QQQ and hOS-9v1/v1R188A, extracts of cells transfected with or without NHK/NHK-QQQ were immunoprecipitated with anti-α1-AT or with anti-FLAG as a control (supplemental Fig. 4). Both NHK and NHK-QQQ co-immunoprecipitated with hOS-9-FLAG (Fig. 1, *B* and *C*, *lanes 4–9*), suggesting that hOS-9 binds to the polypeptide in addition to binding carbohydrate moieties.

hOS-9 Lectin Activity Is Dispensable for SEL1L Binding—We next analyzed whether the interaction between hOS-9 and SEL1L is affected by mutation of the MRH domain. Co-immunoprecipitation experiments revealed that both of the two transcriptional variants of hOS-9 bind to SEL1L (Fig. 2*A*, *lanes 1–5*). The antibody specificity was confirmed by comparing immunoprecipitates with those of non-immune serum or IgG (supplemental Fig. 5). The levels of hOS-9 and hOS-9 R188A binding to SEL1L were similar, as were levels of binding by the two transcriptional variants, hOS-9v1 and hOS-9v2. Similar binding by the hOS-9 wild-type and R188A mutant to SEL1L was confirmed by Western blotting after immunoprecipitation (Fig. 2*B*, compare *lanes 7* and *8* and *lanes 9* and *10*). Taken together, these data demonstrate that the lectin activity of the hOS-9 MRH domain is necessary for ERAD of misfolded glycoproteins but is not required for binding to SEL1L.

Interaction of the hOS-9 MRH Domain with N-Glycans in Vitro—To determine the lectin specificity of the hOS-9 MRH domain, we analyzed oligosaccharide binding using a recombinant MRH domain by FAC *in vitro*. A bacterially expressed hOS-9 MRH domain with a hexahistidine tag was purified and refolded after denaturing in 6 M guanidinium chloride. The structure of the core *N*-linked oligosaccharide G3M9 is shown schematically in Fig. 3*A*. Elution profiles of two typical PA-oligosaccharides (M9 and M8C) from the wild-type OS-9 MRH domain are shown in Fig. 3*B*. The hOS-9 R188A mutant was unable to bind the glycans (Fig. 3*C*). The binding affinity (K_a) for each oligosaccharide is presented in Fig. 3*D*. These data clearly demonstrate that the hOS-9 MRH domain binds to *N*-glycans lacking the terminal mannose from the C branch

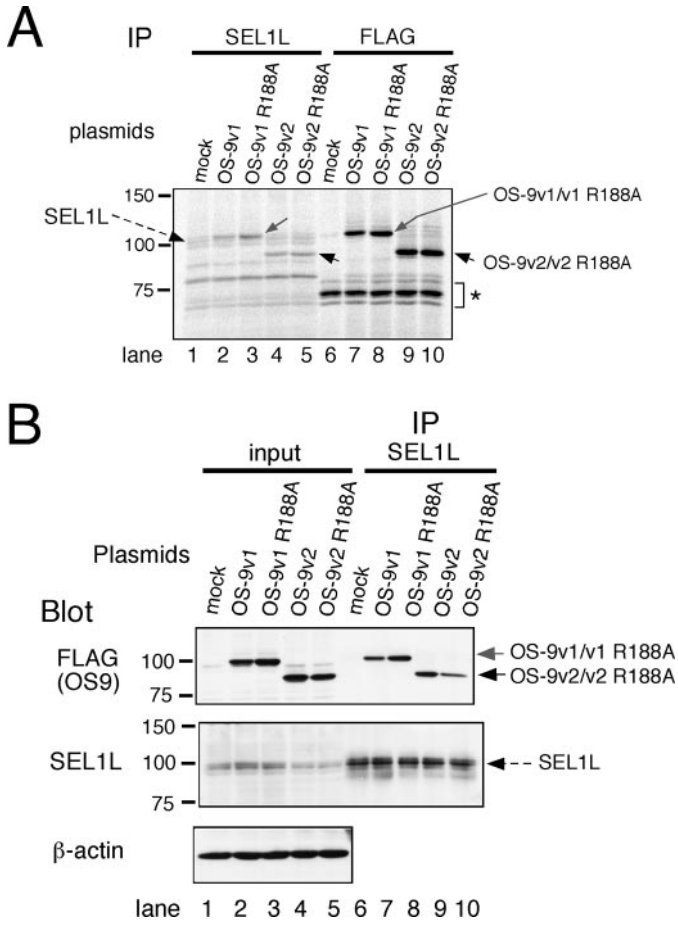

FIGURE 2. **Co-immunoprecipitation of hOS-9 with SEL1L.** *A*, co-immunoprecipitation of hOS-9 wild-type or the R188A mutant with SEL1L. HEK 293 cells were transfected with hOS-9v1-FLAG, v2-FLAG, or their respective R188A mutants, labeled with ^{35}S-Protein Labeling mixture for 3 h, and immunoprecipitated with anti-SEL1L (*lanes 1–5*) or anti-FLAG (*lanes 6–10*). The *asterisk* indicates a protein non-specifically precipitated by the protein A- or protein G-Sepharose beads. *B*, co-immunoprecipitation of hOS-9 or the hOS-9 R188A mutant with SEL1L was detected by immunoprecipitation (*IP*) followed by Western blot analysis. Cells were harvested in a buffer containing 3% digitonin, immunoprecipitated with anti-SEL1L, blotted, and then probed with anti-FLAG or anti-SEL1L (*lanes 6–10*). Aliquots (1/10 volume) of the cell lysate used for immunoprecipitation were Western-blotted to detect transfected hOS-9 and endogenous SEL1L (*lanes 1–5*). The *lower panel* shows β-actin as a loading control.

(G1M8C, G1M7, M8C, $Man_7GlcNAc_2$ (M7), $Man_6GlcNAc_2$ (M6) and M5) irrespective of the presence of glucose on the A branch or of mannose on branches other than the C branch.

To confirm the interaction of the hOS-9 MRH domain with specific *N*-glycans *in vitro*, we used an isothermal titration calorimetry analysis (supplemental Fig. 6). The hOS-9 MRH domain binds to M5 in an exothermic manner, whereas no interaction was detected between the MRH domain and M9. These results are consistent with the FAC data.

Dependence of hOS-9 Binding on N-Glycan Structures on NHK in Vivo—We next examined whether the effect of hOS-9 on NHK degradation depends on the specific oligosaccharides on NHK. As we reported previously, the oligosaccharides on NHK in cells overexpressing either ER ManI or EDEM3 are quite different (supplemental Fig. 7), although NHK degradation is enhanced to a similar extent (29, 30). EDEM3 is a member of the mammalian EDEM family (29, 34) and has α-manno-

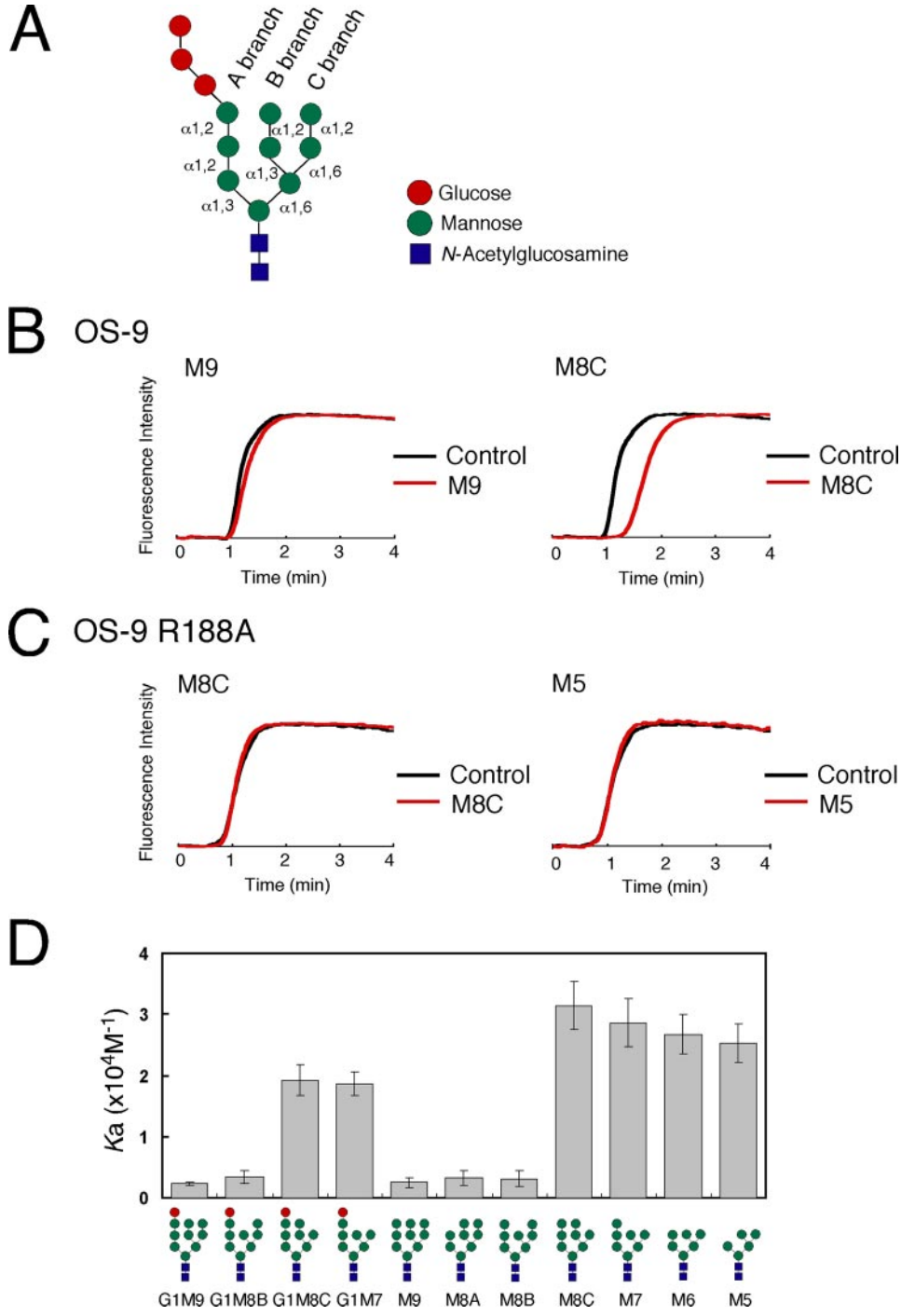

FIGURE 3. **The hOS-9 MRH domain interacts with *N*-glycans lacking the terminal mannose from the C branch.** *A*, the structure of the *N*-linked glycan G3M9 is shown schematically. The glycosidic linkages between mannose residues in each branch are indicated. *B*, elution profiles of the PA-oligosaccharides M9 and M8C from an affinity column presenting the hOS-9 MRH domain (*red lines*). The elution profile of a negative control glycan (PA-GD1b-hexasaccharide) is shown by the *black line*. *C*, elution profiles of the PA-oligosaccharides M8C and M5 from affinity columns presenting the MRH domain of the R188A mutant. Elution profiles of M8C and M5 are indicated by *red lines*, and that of a negative control glycan is shown by the *black line*. *D*, the affinity of the recombinant hOS-9 MRH domain for each oligosaccharide, as indicated by the K_a value, is presented as the mean ± S.D. ($n = 3$). *G1M9*, $Glc_1Man_9GlcNAc_2$; M8A, $Man_8GlcNAc_2$ isomer A.

sidase activity when overexpressed in mammalian cells (29). In ER ManI-transfected cells, most of the oligosaccharides on NHK are the $Glc_1Man_8GlcNAc_2$ isomer B (G1M8B) and M8B. Overexpression of EDEM3 increases M7 and M6 and decreases $Man_8GlcNAc_2$ (M8) on NHK.

Based on the FAC analysis, the hOS-9 MRH domain is predicted to recognize oligosaccharides lacking the C branch terminal mannose and not to bind to M8B or G1M8B, which lack a terminal mannose residue from the B branch. Therefore, NHK degradation in ER ManI-overexpressing cells should not be affected by hOS-9 deficiency (supplemental Fig. 8). Indeed, siRNA-mediated knockdown of hOS-9 had little effect on NHK degradation when ER ManI was overexpressed (Fig. 4*A*, supplemental Fig. 9*A*). In contrast, NHK degradation was clearly inhibited by hOS-9 knockdown when EDEM3 was overexpressed (Fig. 4*B*, supplemental Fig. 9*B*). These data indicate that the effect of OS-9 knockdown on NHK degradation depends on the oligosaccharides present on NHK *in vivo* and suggest that the smaller M7 and M6 *N*-glycans are possible targets of hOS-9, whereas the M8B and G1M8B *N*-glycans are not recognized by hOS-9. In EDEM3-overexpressing cells, the electrophoretic mobility of NHK shifted during the chase under conditions of OS-9 depletion (Fig. 4*B*, *lanes 7–12*), further suggesting that NHK bearing mannose-trimmed *N*-glycans are targeted for degradation by hOS-9. When ER ManI was overexpressed, NHK degradation was partially inhibited by hOS-9 knockdown after a 2-h chase (Fig. 4*A*, $p < 0.05$, Student's *t* test, with the exception that the difference between the OS9-2 siRNA and the control-2 siRNA is not statistically significant). These results may reflect the small increase in M5-7 *N*-glycans, which are recognized by hOS-9 before degradation, on NHK in ER ManI-overexpressing cells at later times during the chase period (supplemental Fig. 8). Thus, we conclude that the G1M8B and M8B *N*-glycans are not recognized by hOS-9, whereas the smaller M7 and M6 oligosaccharides appear to be recognized by hOS-9.

DISCUSSION

In the present study we have demonstrated that hOS-9 recognizes oligosaccharides on the model ERAD substrate NHK

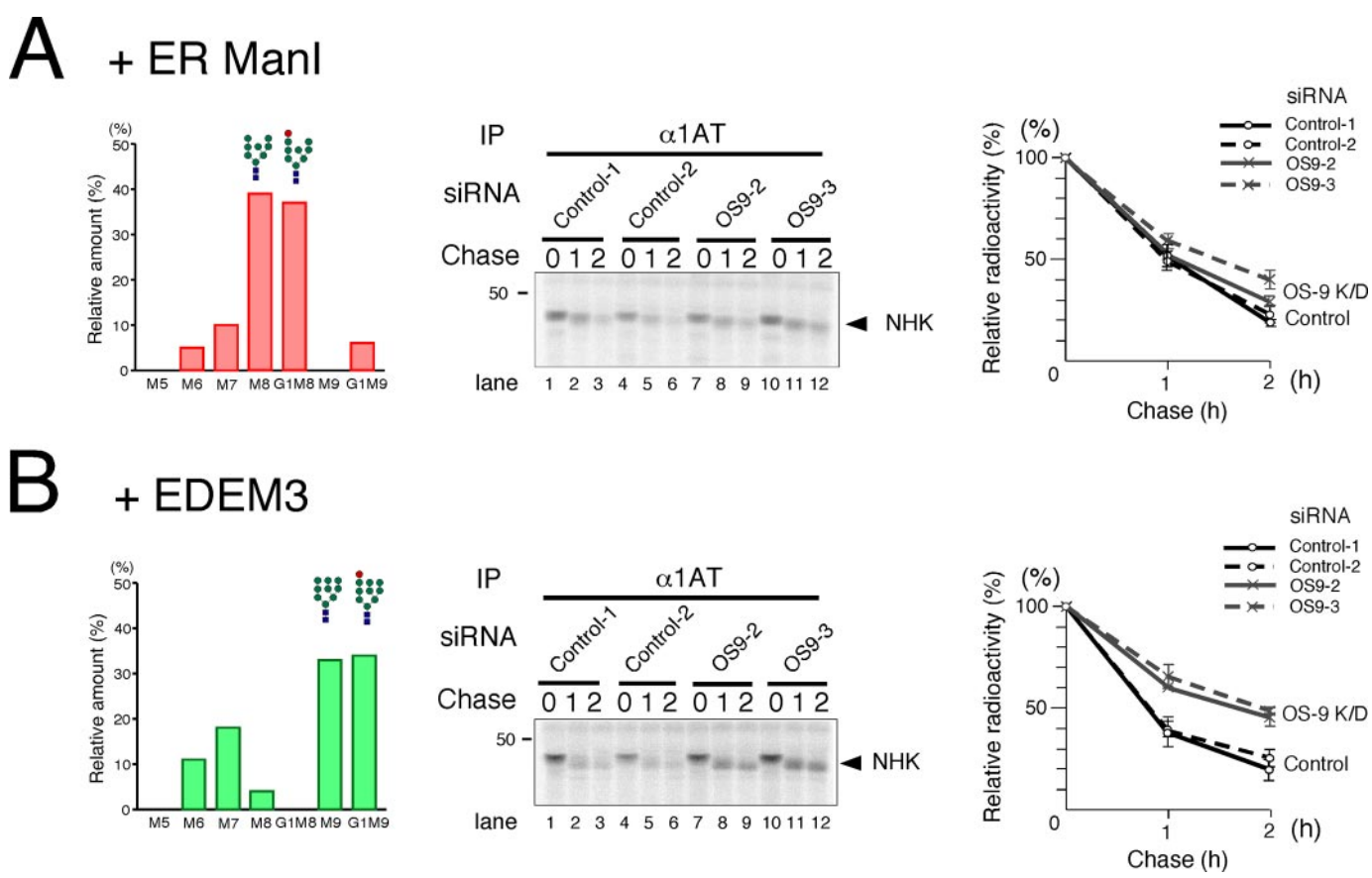

FIGURE 4. **Oligosaccharide structures modified by the overexpression of ER ManI or EDEM3 affect the degradation of NHK in hOS-9 knockdown cells.** *A*, ER ManI overexpression. Shown are oligosaccharides on NHK after a 1-h chase after pulse-labeling with [^{3}H]mannose in HEK 293 cells overexpressing ER ManI (*left panel*). NHK degradation in ER ManI-overexpressing cells treated with control or hOS-9 siRNA (*middle panel*) are shown. The data were quantified and presented as mean ± S.E. ($n = 3$ or 4) (*right panel*). *B*, EDEM3 overexpression. Oligosaccharide structures on NHK and NHK degradation were analyzed and quantified as in *A*. *IP*, immunoprecipitation; K/D, knockdown.

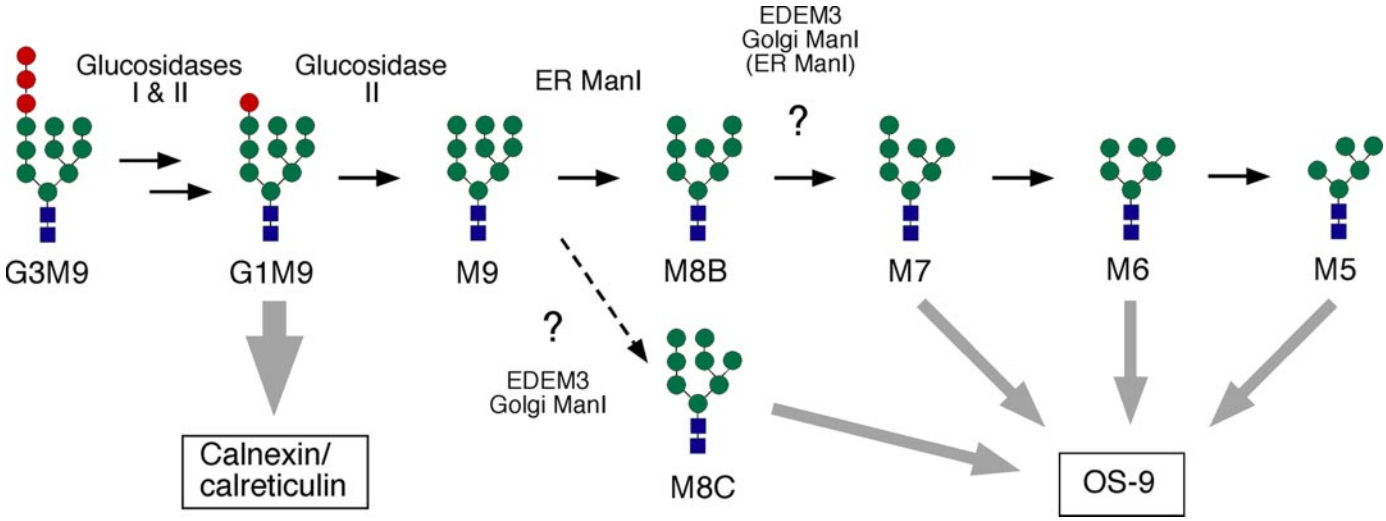

FIGURE 5. **Model for hOS-9 recognition of oligosaccharides on ERAD substrates *in vivo*.** *N*-Glycan processing by different enzymes and recognition of the ERAD substrate by the ER lectins calnexin/calreticulin and hOS-9 under physiological conditions *in vivo* are illustrated. *N*-Glycans that lack the terminal mannose from the C branch, most likely M7, M6, and M5, are recognized by hOS-9 and targeted for degradation. *G1M9*, $Glc_1Man_9GlcNAc_2$.

and that the lectin activity of hOS-9 is required for glycoprotein ERAD. By FAC analysis using a recombinant hOS-9 MRH domain *in vitro*, we showed that the absence of the terminal mannose from the C branch on *N*-linked oligosaccharides is the structural basis for recognition by the hOS-9 lectin domain. Our results here additionally suggest that hOS-9 interacts with the same carbohydrate structures *in vivo* on ERAD substrates in cells. This is the first study demonstrating that mammalian OS-9 is a lectin required for glycoprotein ERAD.

We first confirmed that the intact lectin domain of hOS-9 is required to enhance the degradation of misfolded glycoproteins. Both NHK and NHK-QQQ co-immunoprecipitated with hOS-9, and moreover, the hOS-9 R188A mutant bound to ERAD substrates as well. These observations are consistent with the results of previous studies (21, 24) suggesting that hOS-9 is capable of recognizing and binding to the polypeptide portion of misfolded proteins, similar to Yos9p (15). Importantly, degradation of NHK was partly inhibited in cells transfected with hOS-9 R188A, whereas degradation of NHK-QQQ was not affected. This result indicates that hOS-9 recognizes the carbohydrate moiety of the substrate at the same time and that an intact hOS-9 lectin domain is necessary to enhance glycoprotein ERAD. Thus, we hypothesize that the discrepancy between these results and those of a previous study may reflect whether the hOS-9 lectin mutant was used to detect a dominant-negative effect (this study) or to replace wild-type hOS-9 (24). Furthermore, we did not detect obvious secretion of NHK in the medium under our experimental conditions (Ref. 28 and supplemental Fig. 1), in contrast to the report by Bernasconi *et al.* (24). The higher level of NHK expression, under the transfection conditions used in the previous study, may enable some of the overexpressed substrate to escape the ER quality control mechanism in HEK 293 cells and be secreted. We reevaluated the binding of the hOS-9 lectin domain to SEL1L (21). Co-immunoprecipitation experiments showed similar binding by the wild-type and the R188A mutant hOS-9 to SEL1L, indicating that hOS-9 lectin activity is not essential for interactions with SEL1L. The use of a different detergent to detect the interaction may explain the discrepancy with the previous study (21).

FAC analysis clearly indicated that the hOS-9 MRH domain binds to *N*-glycans lacking the C branch terminal mannose residues. The specific binding of the hOS-9 MRH domain to specific *N*-glycan *in vitro* was also confirmed by isothermal titration calorimetry analysis. To analyze whether hOS-9 recognizes specific *N*-glycans *in vivo*, we modified the oligosaccharide structures on NHK by overexpressing ER ManI or EDEM3 (supplemental Fig. 7). In combination with siRNA-mediated hOS-9 knockdown, we showed that the G1M8B and M8B *N*-glycans on NHK, generated by ER ManI overexpression, are not recognized by hOS-9, whereas the M7 and M6 *N*-glycans, generated by EDEM3 overexpression, are possible targets of hOS-9. Recently, we identified the presence of a minor fraction of the G1M8C oligosaccharide on NHK in cells overexpressing EDEM1, suggesting that EDEM1 is capable of trimming mannose from the C branch.[5] Based on the structural similarities between EDEM1 and EDEM3 (29, 34), EDEM3 may also be capable of trimming mannose from the C branch. Thus, we propose a model for *N*-glycan recognition by hOS-9 *in vivo*, as shown in Fig. 5. After release from the calnexin/calreticulin cycle, mannose from the B branch of a terminally misfolded glycoprotein is trimmed by ER ManI. Further mannose trimming from the C branch then enables *N*-glycans to be recognized by hOS-9 and targeted for ERAD. There are several candidate enzymes capable of trimming the terminal mannose from the C branch on ERAD substrates *in vivo*. The Golgi α1,2-mannosidases may contribute through ER-Golgi recycling of the substrate (35, 36), and ER ManI may generate M5-7 *N*-glycans *in vivo* under conditions where substrates are incubated with high concentrations of ER ManI (30, 37), such as in the ER quality control compartment (38). EDEM3 may also be a candidate for mannose trimming *in vivo*, although its enzyme activity *in vitro* has not yet been demonstrated. The *N*-glycan structures most probably created on the misfolded glycoproteins and possible enzymes involved in this processing under physiological conditions are presented in Fig. 5 and differ from the *N*-glycan composition on NHK, as shown in Fig. 4. Thus, based on the present study, we propose that the MRH domain of hOS-9 recognizes *N*-glycans lacking the terminal mannose from the C branch both *in vivo* and *in vitro* and that the lectin activity of hOS-9 is required for glycoprotein ERAD.

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REFERENCES

1. Helenius, A., and Aebi, M. (2004) *Annu. Rev. Biochem.* **73,** 1019–1049
2. Nakatsukasa, K., and Brodsky, J. L. (2008) *Traffic* **9,** 861–870
3. Anelli, T., and Sitia, R. (2008) *EMBO J.* **27,** 315–327
4. van Anken, E., and Braakman, I. (2005) *Crit. Rev. Biochem. Mol. Biol.* **40,** 191–228
5. Raasi, S., and Wolf, D. H. (2007) *Semin. Cell Dev. Biol.* **18,** 780–791
6. Meusser, B., Hirsch, C., Jarosch, E., and Sommer, T. (2005) *Nat. Cell Biol.* **7,** 766–772
7. Cabral, C. M., Liu, Y., and Sifers, R. N. (2001) *Trends Biochem. Sci.* **26,** 619–624
8. Herscovics, A. (2001) *Biochimie* (*Paris*) **83,** 757–762
9. Caramelo, J. J., and Parodi, A. J. (2008) *J. Biol. Chem.* **283,** 10221–10225
10. Lederkremer, G. Z., and Glickman, M. H. (2005) *Trends Biochem. Sci.* **30,** 297–303
11. Jakob, C. A., Burda, P., Roth, J., and Aebi, M. (1998) *J. Cell Biol.* **142,** 1223–1233
12. Kanehara, K., Kawaguchi, S., and Ng, D. T. (2007) *Semin. Cell Dev. Biol.* **18,** 743–750
13. Buschhorn, B. A., Kostova, Z., Medicherla, B., and Wolf, D. H. (2004) *FEBS Lett.* **577,** 422–426
14. Munro, S. (2001) *Curr. Biol.* **11,** 499–501
15. Bhamidipati, A., Denic, V., Quan, E. M., and Weissman, J. S. (2005) *Mol. Cell* **19,** 741–751
16. Kim, W., Spear, E. D., and Ng, D. T. (2005) *Mol. Cell* **19,** 753–764
17. Szathmary, R., Bielmann, R., Nita-Lazar, M., Burda, P., and Jakob, C. A. (2005) *Mol. Cell* **19,** 765–775
18. Carvalho, P., Goder, V., and Rapoport, T. A. (2006) *Cell* **126,** 361–373
19. Gauss, R., Jarosch, E., Sommer, T., and Hirsch, C. (2006) *Nat. Cell Biol.* **8,** 849–854
20. Denic, V., Quan, E. M., and Weissman, J. S. (2006) *Cell* **126,** 349–359
21. Christianson, J. C., Shaler, T. A., Tyler, R. E., and Kopito, R. R. (2008) *Nat. Cell Biol.* **10,** 272–282
22. Hosokawa, N., Wada, I., Nagasawa, K., Moriyama, T., Okawa, K., and Nagata, K. (2008) *J. Biol. Chem.* **283,** 20914–20924

23. Mueller, B., Klemm, E. J., Spooner, E., Claessen, J. H., and Ploegh, H. L. (2008) *Proc. Natl. Acad. Sci. U. S. A.* **105,** 12325–12330
24. Bernasconi, R., Pertel, T., Luban, J., and Molinari, M. (2008) *J. Biol. Chem.* **283,** 16446–16454
25. Lilley, B. N., and Ploegh, H. L. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 14296–14301
26. Mueller, B., Lilley, B. N., and Ploegh, H. L. (2006) *J. Cell Biol.* **175,** 261–270
27. Liu, Y., Choudhury, P., Cabral, C. M., and Sifers, R. N. (1999) *J. Biol. Chem.* **274,** 5861–5867
28. Hosokawa, N., Wada, I., Hasegawa, K., Yorihuzi, T., Tremblay, L. O., Herscovics, A., and Nagata, K. (2001) *EMBO Rep.* **2,** 415–422
29. Hirao, K., Natsuka, Y., Tamura, T., Wada, I., Morito, D., Natsuka, S., Romero, P., Sleno, B., Tremblay, L. O., Herscovics, A., Nagata, K., and Hosokawa, N. (2006) *J. Biol. Chem.* **281,** 9650–9658
30. Hosokawa, N., Tremblay, L. O., You, Z., Herscovics, A., Wada, I., and Nagata, K. (2003) *J. Biol. Chem.* **278,** 26287–26294
31. Kamiya, Y., Kamiya, D., Yamamoto, K., Nyfeler, B., Hauri, H. P., and Kato, K. (2008) *J. Biol. Chem.* **283,** 1857–1861
32. Kamiya, Y., Yamaguchi, Y., Takahashi, N., Arata, Y., Kasai, K., Ihara, Y., Matsuo, I., Ito, Y., Yamamoto, K., and Kato, K. (2005) *J. Biol. Chem.* **280,** 37178–37182
33. Kasai, K., Oda, Y., Nishikawa, M., and Ishii, S. (1986) *J. Chromatogr.* **376,** 33–47
34. Mast, S. W., Diekman, K., Karaveg, K., Davis, A., Sifers, R. N., and Moremen, K. W. (2005) *Glycobiology* **15,** 421–436
35. Hammond, C., and Helenius, A. (1994) *J. Cell Biol.* **126,** 41–52
36. Hosokawa, N., You, Z., Tremblay, L. O., Nagata, K., and Herscovics, A. (2007) *Biochem. Biophys. Res. Commun.* **362,** 626–632
37. Herscovics, A., Romero, P. A., and Tremblay, L. O. (2002) *Glycobiology* **12,** 14G–15G
38. Avezov, E., Frenkel, Z., Ehrlich, M., Herscovics, A., and Lederkremer, G. Z. (2008) *Mol. Biol. Cell* **19,** 216–225