

The mammalian UPR boosts glycoprotein ERAD by suppressing the proteolytic downregulation of ER mannosidase I

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Summary

The secretory pathway provides a physical route through which only correctly folded gene products are delivered to the eukaryotic cell surface. The efficiency of endoplasmic reticulum (ER)-associated degradation (ERAD), which orchestrates the clearance of structurally aberrant proteins under basal conditions, is boosted by the unfolded protein response (UPR) as one of several means to relieve ER stress. However, the underlying mechanism that links the two systems in higher eukaryotes has remained elusive. Herein, the results of transient expression, RNAi-mediated knockdown and functional studies demonstrate that the transcriptional elevation of EDEM1 boosts the efficiency of glycoprotein ERAD through the formation of

a complex that suppresses the proteolytic downregulation of ER mannosidase I (ERManI). The results of site-directed mutagenesis indicate that this capacity does not require that EDEM1 possess inherent mannosidase activity. A model is proposed in which ERManI, by functioning as a downstream effector target of EDEM1, represents a checkpoint activation paradigm by which the mammalian UPR coordinates the boosting of ERAD.

Key words: Checkpoint activation, Endoplasmic reticulum-associated degradation, Unfolded protein response

Introduction

The secretory pathway provides a physical route through which newly synthesized proteins are eventually delivered to the eukaryotic cell surface (Ellgaard and Helenius, 2001). Conformational maturation, which transforms expressed genetic information into biological activity (Ellgaard et al., 1999; Gething and Sambrook, 1992; Sitia and Braakman, 2003), takes place in the endoplasmic reticulum (ER) in response to physical engagement with molecular chaperones (Helenius et al., 1997) (Fig. 1). As a rule, productive transport requires adoption of native structure (Klausner and Sitia, 1990). Terminally misfolded proteins and unassembled complexes are eliminated by a conformation-based quality control system designated 'ER-associated degradation' (ERAD) (Brodsky and McCracken, 1999). This series of requisite events that orchestrate the dislocation of ERAD substrates into the cytosol for degradation by 26S proteasomes (Bonifacino and Weissman, 1998; Plemper and Wolf, 1999) operationally expands the surveillance of eukaryotic genome expression beyond the nucleus.

Asparagine-linked glycosylation generates a scaffold by which a small ensemble of oligosaccharide-processing enzymes mediate the entrance of N-linked glycoproteins into both folding and degradation pathways (Helenius, 1994; Parodi, 2000; Spiro, 2000). Following co-translational N-linked glycosylation (Kornfeld and Kornfeld, 1985), the two outermost glucose units are hydrolyzed from asparagine-linked $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ by glucosidase I and glucosidase II (D'Alessio et al., 1999; Kalz-Fuller et al., 1995; Kornfeld and Kornfeld, 1985; Trombetta et al., 1996). The resulting monoglucosylated oligosaccharide functions as a ligand that is capable of promoting physical engagement with either calnexin or

calreticulin (Hammond et al., 1994; Labriola et al., 1999; Ou et al., 1993), which function as lectin-like members of the glycoprotein folding machinery (Suh et al., 1989). Disengagement coincides with removal of the remaining glucose unit by glucosidase II (Hebert et al., 1995). Post-translational reassembly is driven by UDP-glucose:glycoprotein glucosyltransferase (UGGT), which is capable of catalyzing the regeneration of the monoglucosylated appendage. This arrangement has been coined the 'calnexin cycle' (Zapun et al., 1997).

Opportunistic removal of α 1,2-linked mannose units in response to prolonged ER residence (Gonzalez et al., 1999; Tremblay and Herscovics, 1999) is diagnostic of the failure of a molecule to acquire native structure within a biologically relevant time frame (Fig. 1). The modified glycans promote extraction of glycoproteins from the calnexin cycle (Sousa et al., 1992), and, in combination with nonnative protein structure, are suspected to complete the formation of a proposed glycoprotein ERAD (GERAD) signal (Cabral et al., 2001). This bipartite nature (Wu et al., 2003) and slow rate of formation of the glycan-based signal component (Jakob et al., 1998), are predicted to underlie the mechanism by which slow-folding wild-type glycoprotein folding intermediates are protected from premature entrance into GERAD (Cabral et al., 2002).

The excessive accumulation of non-native proteins in the ER can evoke a coordinated adaptive signaling network, designated the unfolded protein response (UPR) (Ron and Walter, 2007), that minimizes the consequences of ER stress through three key proximal signal transducers. Signal transmission to the nucleus sequentially activates transcriptional programs that are capable of attenuating

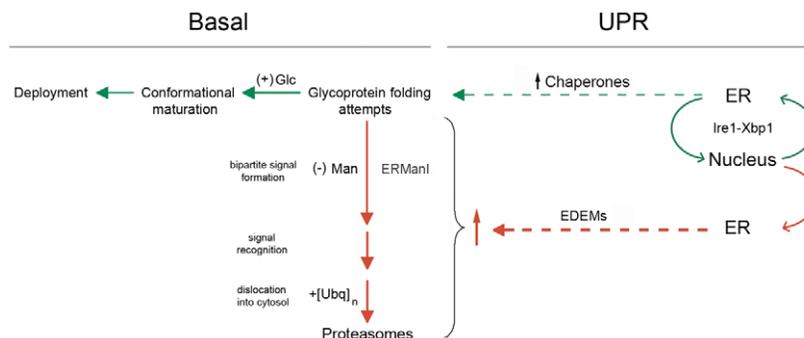


Fig. 1. The mammalian UPR enhances glycoprotein folding and ERAD. Newly synthesized glycoproteins undergo folding attempts in the ER. The re-addition of glucose (+Glc) to N-linked glycans can help promote conformational maturation that leads to deployment (unbroken horizontal green arrows). Under basal conditions, prolonged ER residence can lead to the removal of α 1,2-linked mannose units (-Man) by ERManI, which, together with nonnative protein structure, can initiate entrance into ERAD (unbroken vertical red arrows). A subsequent series of requisite events eventually leads to the addition of ubiquitin (+Ubq) to the polypeptide, which promotes dislocation into the cytosol and degradation by 26S proteasomes. The accumulation of unfolded proteins can evoke the UPR, which transmits signals between the ER and nucleus (curved arrows) to alleviate the situation. The Ire1-Xbp1 branch initially enhances protein folding through the transcriptional induction of ER chaperones (broken green arrow), and then boosts glycoprotein ERAD through a process that coincides with the transcriptional elevation of EDEMs.

protein translation (PERK) (Harding et al., 1999), augmenting the ER protein folding capacity (ATF6 and IRE1-Xbp1), and boosting the efficiency of ERAD (Ire1-Xbp1) (Li et al., 2000; Scheuner et al., 2001; Yoshida et al., 1998). Despite the wealth of information generated in recent years, the downstream events responsible for coordinating the enhanced efficiency of GERAD remain controversial (Hebert and Molinari, 2007; Kanehara et al., 2007) (Fig. 1). The enhanced efficiency of ERAD coincides with the elevated transcription of ER degradation-enhancing mannosidase-like proteins (EDEMs), which are members of the class 47 glycosylhydrolase family (Olivari et al., 2005; Yoshida et al., 2003). Although some EDEM orthologs in lower eukaryotes exhibit mannosidase activity (Banerjee et al., 2007), *in vitro* activity has not been reported for any of the mammalian orthologs (Hosokawa et al., 2001; Mast et al., 2005). Nevertheless, several are capable of enhancing the removal of mannose from asparagine-linked oligosaccharides and accelerating the proteasomal degradation of multiple N-glycosylated GERAD substrates upon experimental overexpression (Hirao et al., 2006; Hosokawa et al., 2001). However, ER mannosidase I (ERManI), which is another member of the class 47 glycosylhydrolase family, catalyzes the removal of one (Gonzalez et al., 1999) or multiple (Frenkel et al., 2003; Tremblay and Herscovics, 1999) α 1,2-linked mannose units from asparagine-linked $\text{Man}_9\text{GlcNAc}_2$, and can accelerate GERAD upon experimental elevation (Hosokawa et al., 2003; Wu et al., 2003). To further complicate matters, although Lederkremer and colleagues (Avezov et al., 2008) recently used RNAi technology to confirm the essential role played by ERManI in generating a glycan structure important for GERAD, the corresponding gene is not a transcriptional target of the UPR in mammalian cells (Hosokawa et al., 2001). Because ERManI catalyzes the rate-limiting step in GERAD in mammalian cells (Cabral et al., 2001) and in budding yeast (Jakob et al., 1998), the aforementioned observations raise the issue of whether, and how, its intracellular concentration might be elevated during the mammalian UPR to coordinate the enhancement of GERAD in higher eukaryotic cells.

Although functioning at distinct organizational levels, the impaired progression of structurally aberrant proteins through the secretory pathway is broadly reminiscent of arrested cell cycle progression in response to damaged DNA (Zhou and Elledge, 2000).

To this end, we recently reported that human ERManI is subject to proteolytic downregulation under basal conditions (Wu et al., 2007) in a manner that is operationally similar to the regulation of checkpoint proteins that monitor DNA damage, except that lysosomal hydrolases contribute to proteolysis. Because many nuclear regulatory proteins are subject to checkpoint activation through impairment of proteolytic downregulation (Zhou and Elledge, 2000), herein we asked whether post-translational stabilization of ERManI might contribute to the coordinated induction between GERAD and the mammalian unfolded protein response. In the present study, several lines of experimental evidence are presented that support this hypothesis. Furthermore, the results of functional assays and co-immunoprecipitation studies are consistent with a checkpoint activation model in which ERManI is stabilized as a downstream effector target of EDEM1. How this observation represents a paradigm shift in the manner by which budding yeast coordinate the boosting of GERAD in response to ER stress is discussed, as is how reiteration of the checkpoint activation paradigm operationally expands the surveillance of genome expression in higher eukaryotes.

Results

ER mannosidase I is stabilized as part of the mammalian UPR

Our immediate objective was to test the prediction that the proteolytic downregulation of human ERManI is impaired as a component of the mammalian UPR. Because the endogenous protein concentration is below the level of immunological detection (Wu et al., 2007), the intracellular fate of the transfected recombinant human homolog was monitored by metabolic pulse-chase radiolabeling and immunoprecipitation in HEK293 cells. As previously reported for cultured hepatoma cells (Wu et al., 2007), an 82 kDa radiolabeled protein was synthesized during a 20-minute pulse with [^{35}S]methionine, and nearly the entire cohort of molecules was degraded during 90 minutes of chase (Fig. 2A, Co). To investigate the influence of ER stress on ERManI stability, the fate of the newly synthesized molecules was monitored under experimental conditions that evoke ER stress. For this, HEK293 cells were transfected with a DNA construct that encodes a natural genetic variant of human α 1-antitrypsin, designated null (Hong

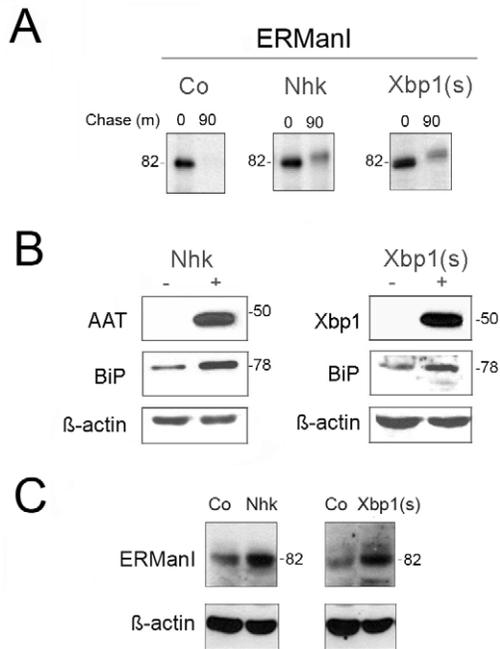


Fig. 2. Post-translational stabilization of transfected human ERManI during the mammalian UPR. (A) Metabolic pulse-chase radiolabeling and fluorographic detection of transfected human ERManI immunoprecipitated from HEK293 cells following 90 minutes of chase. Cells were either not transfected (Co) or were transfected with DNA constructs that encode either α 1-antitrypsin variant null (Hong Kong) (Nhk) or a spliced variant of Xbp1 [Xbp1(s)]. (B) Immunoblots for transfected α 1-antitrypsin variant null (Hong Kong) (Nhk), Xbp1(s) and endogenous BiP from Nonidet P-40 cell extracts under steady-state conditions (top panel) from HEK293 cells transfected with either NHK of Xbp1(s). Immunoblotting for endogenous β -actin served as loading control (bottom panel). (C) Immunoblot of transfected human ERManI from Nonidet P-40 cell extracts under steady-state conditions (top panel). HEK293 cells were transfected with ERManI, and endogenous EDEM1 co-transfected with DNA constructs that encode either α 1-antitrypsin variant null (Hong Kong) (Nhk), or a spliced variant of Xbp1 [Xbp1(s)]. Immunoblotting for endogenous β -actin served as loading control (bottom panel).

Kong) (Sifers et al., 1989), that misfolds following biosynthesis (Sifers et al., 1988), and has served as an inducer of the mammalian UPR (Yoshida et al., 2003). The steady-state concentration of molecular chaperone BiP was elevated when compared with cells transfected with ERManI alone (Fig. 2B, Nhk), indicating that the manipulation had induced the UPR. Under these conditions, a slower migrating radioactive species of transfected human ERManI was detected following a 90-minute chase (Fig. 2A, Nhk). To further corroborate the participation of the UPR, HEK293 cells were transiently co-transfected with a DNA construct that encodes a spliced variant of the X-box binding protein 1 (Xbp1) transcription factor, which constitutively activates the signaling pathway responsible for enhancing GERAD (Zhang and Kaufman, 2006). As before, the steady-state elevation of molecular chaperone BiP [Fig. 2B, Xbp1(s)] verified the induction of the UPR. Again, a slower-migrating radiolabeled species of transfected human ERManI was stabilized after 90 minutes of chase [Fig. 2A, Xbp1(s)].

ECL Western blotting was employed to investigate changes in the steady-state concentration of transfected human ERManI in NP-40 cell lysates. Co-transfection of ERManI with variant null (Hong Kong) or Xbp1 splice variant [Xbp1(s)] coincided with an increase in the steady-state concentration of transfected ERManI, when

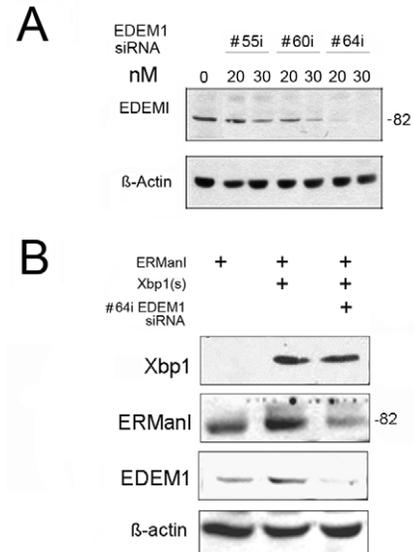


Fig. 3. Endogenous EDEM1 contributes to the UPR-mediated stabilization of ERManI. (A) Commercially available siRNA (Ambion) were tested for their ability to knock down the expression of endogenous human EDEM1 in HEK293 cells, as demonstrated by immunoblotting of Nonidet P-40 cell lysates. The siRNA identity and concentration are designated (top panel). (B) HEK293 cells were transfected with (+) either human ERManI alone, or in combination with Xbp1(s) and EDEM1 siRNA #64i, as depicted. Immunoblot of transfected Xbp1(s), transfected human ERManI and endogenous EDEM1 from Nonidet P-40 cell extracts under steady-state conditions. Immunoblotting for endogenous β -actin served as loading control.

compared with cells transfected with recombinant ERManI alone (Fig. 2C). However, the intensity of the 82 kDa form, rather than the slower-migrating species, was increased. Subsequent experiments failed to provide a conclusive explanation for this difference, although we cannot ignore the possibility that it may reflect the transient nature by which ERManI is modified. Importantly, because the human recombinant ERManI DNA construct used for the transfection experiments did not contain any inducible transcriptional elements or a 3'UTR, the increased intracellular concentration of the encoded protein implied that it had been subject to post-translational stabilization as a component of the mammalian UPR.

Evidence that EDEM1 contributes to post-translational stabilization of ERManI

Next, we sought to elucidate the mechanism by which the mammalian UPR mediates the post-translational stabilization of ERManI. We assessed the contribution of EDEM1 because of its transcriptional elevation as a component of the Ire1-Xbp1 pathway (Yoshida et al., 2003). Furthermore, experimental overexpression of EDEM1 accelerates the degradation of misfolded glycoproteins even in the absence of any detectable *in vitro* catalytic activity (Hosokawa et al., 2001). Our strategy was to ask whether RNAi-mediated knockdown of endogenous EDEM1 was capable of preventing the stabilization of transfected ERManI induced by Xbp1(s). Of the available purchased molecules designed to target human EDEM1 (Ambion), siRNA #64i was most effective in diminishing the endogenous EDEM1 levels in HEK293 cells (Fig. 3A). Next, HEK293 cells were transfected with combinations of DNA constructs that encode either recombinant human ERManI or

Xbp1(s), and with siRNA #64. Consistent with our hypothesis, ECL western blotting of NP-40 cell lysates demonstrated that siRNA #64i blocked the elevation of transfected ERManI caused by co-expression of Xbp1(s) (Fig. 3B). Importantly, ECL western blotting confirmed that transfection of Xbp1(s) elevated the intracellular concentration of endogenous EDEM1 above control, and that the effect was reversed with siRNA #64i (Fig. 3B).

Direct evidence for a functional partnership between ERManI and EDEM1

In the next set of experiments, HEK293 cells were co-transfected with DNA constructs that encode EDEM1-HA or recombinant human ERManI to directly test the hypothesis that the former contributes to the stabilization of the latter. As before, recombinant human ERManI was synthesized as an 82 kDa protein during a 20-minute pulse with [³⁵S]methionine, and nearly the entire cohort was degraded during 90 minutes of chase (Fig. 4, top panel). In support of our hypothesis, co-transfection with EDEM1-HA led to the stabilization of a slower-migrating species of ERManI at the late timepoints (Fig. 4A, lower panel and graph). Its detection at the 90-minute time point in the absence of the 82 kDa band confirmed that it was generated post-translationally in response to co-transfection with EDEM1-HA.

Finally, co-immunoprecipitation studies were performed to determine whether the two proteins exist within the same complex. Immunocomplexes from co-transfected HEK293 cells were generated with antiserum against either ERManI or HA, resolved by SDS-PAGE and detected by ECL western blotting after transfer to nitrocellulose. No immunoreactive bands were detected in HEK293 cells transfected with empty vector (Fig. 4C, EV, top panels). By contrast, immunoreactive EDEM1-HA was detected as a component of the ERManI immunoprecipitate (Fig. 4C, Tr, top left panel), and immunoreactive ERManI was detected in the EDEM1-HA immunoprecipitate (Tr, top right panel), respectively. Taken together, these findings imply that the two proteins exist in the same complex. Although, it cannot be concluded whether the proteins are directly bound to one another, the experimental data provide physical evidence for the existence of a functional partnership.

Knockdown of ERManI, but not EDEM1, diminishes basal ERAD

Although α 1-antitrypsin variant PI Z misfolds following biosynthesis (Elliott et al., 1996) in response to a E342K substitution (Sifers et al., 1987), it fails to induce the UPR in any of the cell lines in which it has been expressed (Hidvegi et al., 2005; Lawless et al., 2004), including HEK293 (Cabral et al., 2002). Because variant PI Z is subject to proteasomal degradation in transfected HEK293 cells (Cabral et al., 2002; Wu et al., 2003) in the next set of experiments it served as a reporter protein to measure changes in the efficiency of ERAD under basal conditions and in response to the experimental overexpression, or knockdown, of ERManI or EDEM1. As previously described, the endogenous human ERManI concentration is below the level of immunological detection (Wu et al., 2007), forcing us to test the knockdown strategy with the transfected recombinant protein. Of the three commercially available siRNA molecules targeted against human ERManI (Ambion), #52i was most successful in knocking-down levels of the recombinant protein in transfected HEK293 cells (Fig. 5A, #52i, top panel). In all the remaining experiments, concentrations of siRNA were used that had been shown to consistently knockdown the target protein

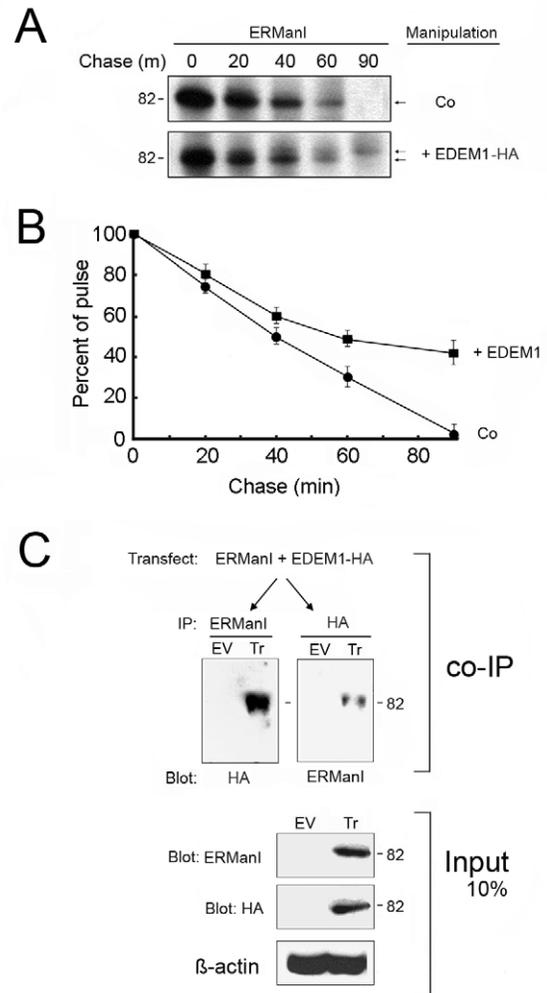


Fig. 4. Co-transfection of EDEM1-HA suppresses proteolytic downregulation of ERManI. (A) Metabolic pulse-chase radiolabeling and fluorographic detection of transfected human ERManI in HEK293 cells without additions (Co) or with (+) co-transfected EDEM1-HA. ERManI was immunoprecipitated from NP-40 cell lysates immediately after the pulse and after 20, 40, 60 and 90 minutes of chase. (B) Quantification of transfected recombinant human ERManI in control (Co) cells, and in those co-transfected with EDEM1 (+EDEM1). (C) HEK293 cells transfected with empty vector (EV) or co-transfected with ERManI and EDEM1-HA (from above). ERManI and EDEM1-HA were immunoprecipitated from equal aliquots of the Nonidet-P40 cell lysate and detected by immunoblotting with the opposite antisera (top panels). Immunoblotting of crude cell lysates detected proteins in the input. The immunoblot of endogenous β -actin demonstrates equal loading of cell lysates (bottom panels).

concentration. Furthermore, effects on PI Z disposal were not observed when the siRNAs were used at sub-optimal concentrations. The use of siRNA #52i to knock-down endogenous ERManI hindered the rate of basal PI Z degradation in metabolic pulse-chase radiolabeling experiments when compared with control (Fig. 5B, compare top and middle panels), especially at the earliest time points (Fig. 5C). Importantly, the manipulation also prevented the discrete electrophoretic mobility shift of radiolabeled variant PI Z in SDS-PAGE (Fig. 5B, middle panel), which is indicative of impaired N-linked oligosaccharide processing. By contrast, the use of siRNA #64i to knock down endogenous EDEM1 levels (Fig. 5B, bottom panel) had no demonstrable effect on the basal ERAD of variant

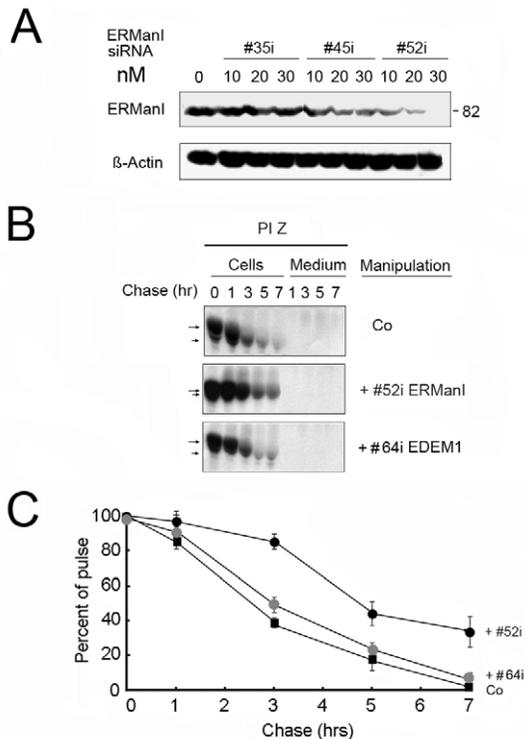


Fig. 5. Endogenous ERManI, but not EDEM1, contributes to the basal GERAD of α 1-antitrypsin PI Z. (A) Commercially available siRNA (Ambion) were tested for their ability to knock down the expression of transfected recombinant human ERManI, as demonstrated by immunoblotting of Nonidet P-40 cell lysates. The siRNA identity and concentration are designated (top panel). Immunoblotting for endogenous β -actin served as loading control (bottom panel). (B) Metabolic pulse-chase radiolabeling and fluorographic detection of transfected α 1-antitrypsin variant PI Z following immunoprecipitation from HEK293 cells (Co) (top panel). As indicated, cells were co-transfected with either #52i to knock down endogenous ERManI (middle panel) or #64i (from Fig. 3B) to knock down endogenous EDEM1 (bottom panel). The horizontal arrows indicate the migration of variant PI Z before and after the trimming of asparagine-linked oligosaccharides. (C) Quantification of intracellular PI Z in control (Co) cells, and in those treated with #52i or #64i at appropriate concentrations. The error bars represent one standard deviation, and represent data generated from at least five experiments.

PI Z (Fig. 5C) or on its oligosaccharide trimming (Fig. 5B, compare top and bottom panels). Taken together, the data imply that ERManI plays a role in the disposal of variant PI Z under basal ERAD, and that EDEM1 is dispensable under these conditions.

Elevated ERManI accelerates ERAD in the absence of EDEM1
Our next objective was to exploit our functional assay to determine whether experimental elevation of ERManI is sufficient to accelerate the rate of PI Z disposal in HEK293. As before, the fate of variant PI Z was monitored by metabolic pulse-chase radiolabeling and immunoprecipitation. Co-transfection of HEK293 cells with recombinant human ERManI accelerated the rate of PI Z disposal (Fig. 6B), and this was accompanied by a faster rate at which the N-linked glycans were trimmed (Fig. 6A, compare top and middle panels). Importantly, incubation with #64i to knock-down endogenous EDEM1 failed to demonstrably hinder the capacity of ERManI to accelerate PI Z disposal (Fig. 6B) or to enhance the trimming of N-linked glycans (Fig. 6A, compare middle and bottom panels). This finding indicates that EDEM1

is dispensable in terms of the capacity of ERManI to accelerate PI Z disposal.

The capacity of EDEM1 to accelerate ERAD requires ERManI
Considering these findings, and our lines of evidence for the existence of a functional partnership, our next objective was to use the functional assay to determine whether elevated expression of EDEM1 was sufficient to accelerate PI Z disposal, or whether this capacity requires the existence of endogenous ERManI as our prior data suggest. Co-transfection of HEK293 cells with EDEM1-HA accelerated the rate of PI Z disposal (Fig. 6D), and this alteration included a faster rate at which the N-linked glycans were trimmed (Fig. 6C, compare top and middle panels). Consistent with our prior studies, incubation with siRNA #52i to knock-down endogenous ERManI was sufficient to ablate these changes (Fig. 6C, bottom panel) and significantly hinder the rate of PI Z turnover (Fig. 6D), supporting the role played by ERManI.

The functional partnership does not require that EDEM1 bear mannosidase activity

Next, we asked whether EDEM1 must exhibit mannosidase activity in order to either stabilize and/or form a complex with ERManI. Importantly, it was recently concluded that EDEM1 contains the amino acids necessary to support mannosidase activity (Olivari et al., 2006). Moreover, its elevated expression is capable of enhancing the trimming of N-linked glycans of multiple GERAD substrates, and site-directed mutagenesis of the putative active site was suggested to ablate the acceleration of N-linked glycan trimming (Olivari et al., 2006). However, considering the fact that α 1-antitrypsin variant null (Hong Kong) was used as substrate, the rate of degradation of which reflects the action of an enhanced ERAD system (Yoshida et al., 2003), we chose to re-assess this issue. For our purposes, glutamic acid at residue 220, which corresponds to an essential catalytic amino acid at residue 330 in human ERManI (Karaveg and Moremen, 2005), was mutated to alanine (to generate EDEM1(E220A)-HA) as a means to ablate any potential mannosidase activity associated with EDEM1.

First, we asked whether co-expression of EDEM1(E220A)-HA would influence the fate of newly synthesized variant PI Z in a manner that was similar to EDEM1-HA. Importantly, co-expression of EDEM1(E220A)-HA enhanced the degradation of radiolabeled PI Z (Fig. 7A, graph) and the trimming of its N-linked glycans (Fig. 7A, compare top and middle panels). Similar observations were made for EDEM1-HA (Fig. 7A, bottom panel, and graph). Even though the extent of glycan trimming was somewhat less for EDEM1(E220A)-HA, the results still imply that the molecule does not require inherent mannosidase activity to accelerate basal ERAD. Given these results, we then tested the prediction that EDEM1(E220A)-HA would impair the downregulation of co-expressed recombinant human ERManI. In pulse-chase studies, almost of the entire cohort of pulse-radiolabeled ERManI was degraded within 90 minutes of chase (Fig. 7B, top panel). In addition, another immunoprecipitated higher-molecular-weight species was detected at the 60 and 90 minute time points in HEK293 cells co-transfected with EDEM1(E220A)-HA (Fig. 7B, middle panel), and in cells co-transfected with EDEM1-HA (bottom panel). Moreover, the degree of stabilization was identical (Fig. 7B, graph).

In a final set of experiments, ECL western blotting was performed to determine whether EDEM1(E220A)-HA would exist in a complex with co-transfected recombinant human ERManI. Immunocomplexes were generated from co-transfected HEK293

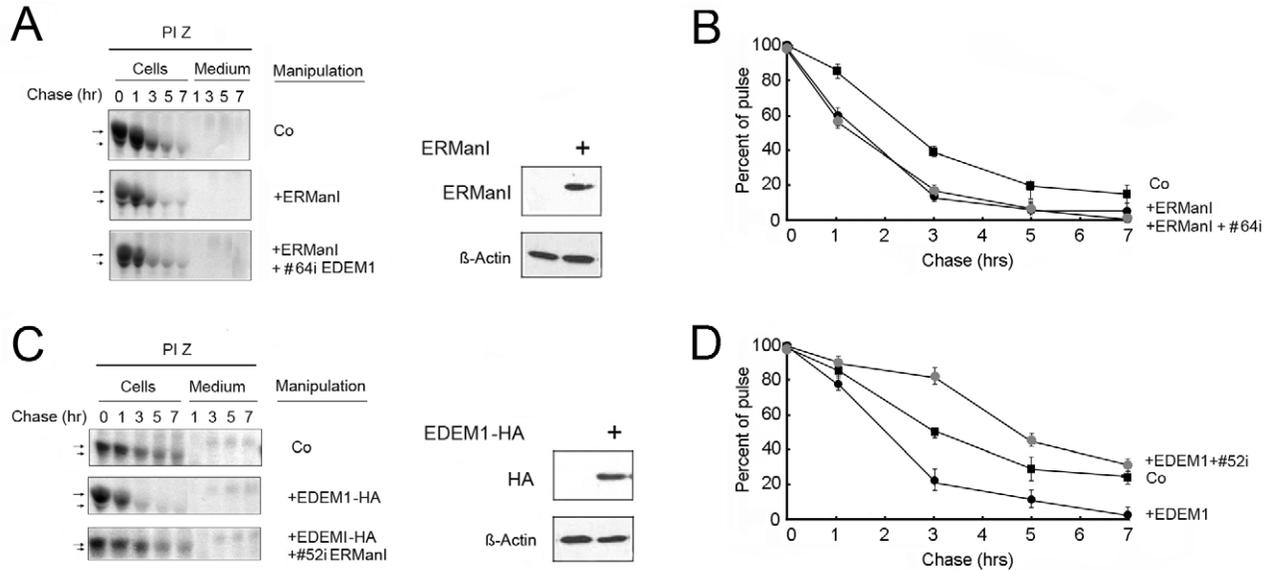


Fig. 6. Transfected EDEM1-HA requires endogenous ERManI to accelerate GERAD of variant PI Z. (A) Metabolic pulse-chase radiolabeling and fluorographic detection of transfected α 1-antitrypsin variant PI Z following immunoprecipitation from HEK293 cells (Co) (top panel). As indicated, cells were co-transfected with ERManI (middle panel) or with ERManI plus #64i to knock down endogenous EDEM1 (bottom panel). An immunoblot of transfected ERManI is shown. (B) Quantification of intracellular radiolabeled PI Z in control (Co) cells, and in those co-transfected with recombinant human ERManI (+ERManI) or co-transfected with ERManI and treated with #64i. (C) HEK293 cells were transfected with variant PI Z alone (Co), or co-transfected with EDEM1-HA (middle panel) or with EDEM1-HA plus #52i to knock down endogenous ERManI (bottom panel). The horizontal arrows indicate the migration of variant PI Z before and after the trimming of asparagine-linked oligosaccharides. An immunoblot of transfected EDEM1-HA is shown. (D) Quantification of intracellular PI Z in control (Co) cells, and in those transfected with EDEM1-HA, or with EDEM1-HA and treated with #52i. The error bars represent one standard deviation, and represent data generated from at least five experiments.

cells with antiserum against either ERManI or HA, resolved by SDS-PAGE and transferred to nitrocellulose. No immunoreactive bands were detected in untransfected HEK293 cells (Fig. 7C, top panel, lane 1). By contrast, immunoreactive EDEM1(E220A)-HA was detected as a component of the ERManI immunoprecipitate (Fig. 7C, top panel, lane 2), similar to EDEM1-HA (lane 3). Taken together, the results indicate that the capacity of EDEM1 to accelerate ERAD, via stabilization of ERManI, does not require intrinsic mannosidase activity.

Discussion

ERAD functions under basal conditions to eliminate proteins unable to adopt native structure after translocation into the ER. However, the system is easily saturated, which can lead to the inappropriate accumulation of unfolded proteins and induction of the UPR (Travers et al., 2000). The accelerated rate at which misfolded proteins are selectively degraded, which is among the most fundamental requirements to restore ER homeostasis, is coordinated by the Ire1-Xbp1 branch of the mammalian UPR. However, elucidation of the exact mechanism by which this is accomplished has remained elusive because several class 47 glycohydrolase family members are capable of enhancing the efficiency of GERAD upon experimental overexpression (Hirao et al., 2006; Hosokawa et al., 2001; Mast et al., 2005; Molinari et al., 2003; Oda et al., 2003).

In the present study, our immediate objective was to focus on the capacity of the UPR to use human ERManI to boost GERAD because the gene for the human ortholog is not a transcriptional target for the mammalian UPR (Hosokawa et al., 2001). However, because it is subject to proteolytic downregulation (Wu et al., 2007), our initial experimental strategy involved the monitoring of ERManI turnover in response to ER stressors. Based on those data, we examined the

capacity of both endogenous and transfected EDEM1 to suppress ERManI downregulation. Subsequent experiments have revealed that the transient slower-migrating species stabilized by EDEM1 in pulse-chase experiments reflects the phosphorylation of ERManI (data not shown). Whether the modification is required for the interaction, or merely indicates that molecules destined for downregulation (Wu et al., 2007) are stabilized, is not yet known. Finally, because the PI Z variant of α 1-antitrypsin fails to induce the mammalian UPR (Cabral et al., 2002; Graham et al., 1990; Hidvegi et al., 2005; Lawless et al., 2004), it was used as a reporter assay to interrogate changes in the efficiency of basal GERAD in response to the overexpression or knockdown of suspected modifying components.

The simplest explanation for our findings is that a functional partnership exists between ERManI and EDEM1 to coordinate the boosting of GERAD as part of the mammalian UPR. The capacity of transfected EDEM1 to suppress the proteolytic downregulation of ERManI (Fig. 4) implies that the event is both necessary and sufficient to bypass the need for upstream stressor signals. The conclusion that ERManI can function as a downstream effector target of EDEM1 is not unreasonable because the luminal concentration of the former had already been shown to control the time allotted for non-native glycoproteins to remain in the ER prior to disposal (Wu et al., 2007). The partnership is suspected to allow the rate of mannose removal to operate over a range of conditions and on a specific timescale, commensurate with the overall folding capacity of the ER. This mechanistic insight does not lessen the role of EDEM1, but, rather, places it at center stage for propagating the signal initiated by Ire1-Xbp1 to suppress ER stress (Fig. 8). In addition, it is in agreement with the recent prediction (Rutkowski and Kaufman, 2007) that post-translational events will probably contribute to the completion of UPR signaling circuits.

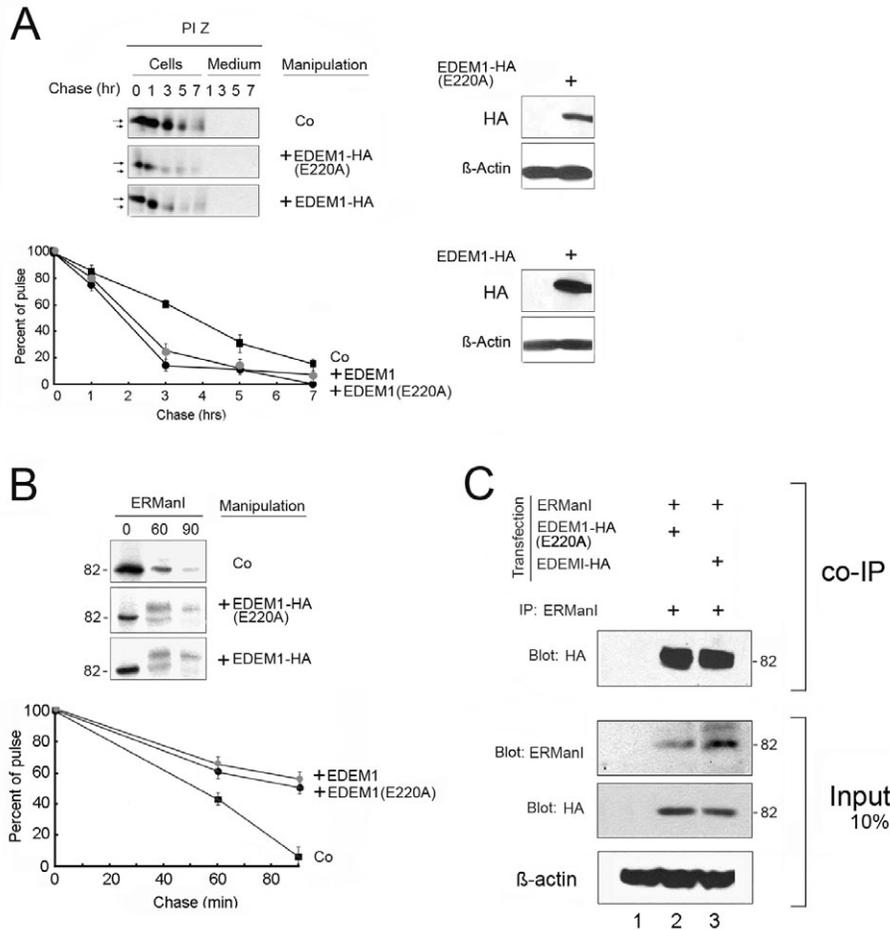


Fig. 7. EDEM1 does not require intrinsic mannosidase activity to support ERManI-mediated acceleration of GERAD. (A) Metabolic pulse-chase radiolabeling and fluorographic detection of transfected α 1-antitrypsin variant PI Z following immunoprecipitation from HEK293 cells (Co) (top panel), or co-transfected with either EDEM1(E220A)-HA (middle panel) or EDEM1-HA (bottom panel). The horizontal arrows indicate the migration of radiolabeled PI Z before and after the trimming of asparagine-linked oligosaccharides. Immunoblots for transfected EDEM1-HA (E220A) and EDEM1-HA are shown. A graph demonstrates the quantification of intracellular PI Z remaining in control (Co) cells, and in those transfected with either EDEM1(E220A) or EDEM1-HA. (B) Metabolic pulse-chase radiolabeling and fluorographic detection of transfected human ERManI in HEK293 cells without additions (Co) (top panel), or co-transfected with EDEM1(E220A)-HA (middle panel) or EDEM1-HA (bottom panel). A graph demonstrates the quantification of the intracellular transfected human ERManI remaining in control (Co) cells, and in those transfected with either EDEM1(E220A) or EDEM1-HA. The error bars represent one standard deviation, and represent data generated from at least five experiments. (C) HEK293 cells either not transfected (lane 1), or co-transfected with ERManI and EDEM1(E220A)-HA (lane 2), or with ERManI and EDEM1-HA (lane 3) (from B). EDEM1 was immunoprecipitated from Nonidet-P40 cell lysates and detected by immunoblotting with antiserum against the HA tag (top panel). Immunoblotting of crude cell lysates shows proteins in the input. The immunoblot of endogenous β -actin demonstrates equal loading (bottom panels).

The ability of co-expressed EDEM1 to suppress the downregulation of ERManI (Fig. 4A), and the necessity of ERManI in enabling EDEM1 to accelerate the disposal of variant PI Z (Fig. 6C) indicate that the two proteins are not functionally interchangeable. This conclusion is further supported by the observation that no inherent mannosidase activity associated with EDEM1 is required for its capacity to stabilize ERManI or accelerate GERAD through ERManI. Our contention is that the prior notion that EDEM1 possesses inherent mannosidase activity (Olivari et al., 2006) probably arose from the problematic use of monitoring α 1-antitrypsin variant null (Hong Kong), which constitutively activates the UPR (Yoshida et al., 1998). The capacity of transfected EDEM1 to further accelerate null (Hong Kong) degradation would be expected if the stabilization of ERManI is not fully saturated during the UPR. Finally, the use of EDEM1 to gradually increase ERManI levels should limit its action to terminally misfolded glycoproteins and prevent the inappropriate targeting of nascent wild-type glycoproteins into GERAD.

Because EDEM1 does not play an obligatory role in basal ERAD, at least for variant PI Z, it is very unlikely that its capacity to boost glycoprotein degradation reflects an enhancement of the basal circuitry. In fact, recent studies suggest that ERManI might cooperate enzymatically with several Golgi α 1,2-mannosidases to target GERAD substrates for disposal (Hosokawa et al., 2007). Rather, it is entirely possible that EDEM1, upon its elevated

concentration, might also function as a chaperone (Hebert et al., 2005; Hosokawa et al., 2006; Molinari et al., 2002; Oda et al., 2003; Olivari et al., 2006) or even as a lectin (Sifers, 2003), as the mutation generated in the present study is not predicted to hinder either capacity. Alternatively, the detected partnership might broaden the mannosidase activity of ERManI and/or initiate its conversion to a lectin. Because our experimental design did not address these issues, additional studies are warranted to investigate each possibility and to determine whether EDEM1 and ERManI physically interact with one another or co-exist in a larger complex. Additional studies are also required to determine whether the roles of EDEM2 and EDEM3 are to operate in a manner that is similar to EDEM1, and can compensate for the loss of EDEM1.

The division of labor by which EDEM1 and ERManI operate to link GERAD with the UPR in higher eukaryotes reveals a level of complexity that apparently does not operate in budding yeast (Hebert and Molinari, 2007; Wang and Ng, 2008) where both of the corresponding homologs Htm1p and Mns1p (Burke et al., 1996; Jakob et al., 2001), are subject to transcriptional elevation (Oda et al., 2003; Travers et al., 2000). This operational distinction represents a mechanistic variation that is broadly reminiscent of nuclear checkpoint activation paradigms in which the proteolysis of key regulatory proteins controls progression through the cell cycle (Zhou and Elledge, 2000), albeit human ERManI is degraded by lysosomes (Wu et al., 2007). Although functioning at distinct

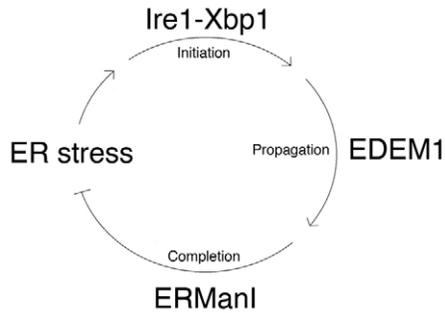


Fig. 8. Proposed anatomy of the Ire1-Xbp1 signaling circuitry as it relates to the enhancement of GERAD. The accumulation of unfolded proteins, which leads to ER stress, evokes the UPR, which transmits signals between the ER and nucleus to alleviate the situation. Key elements that contribute to the Ire1-Xbp1 signaling circuitry as it relates to the enhancement of GERAD include initiation, propagation and completion. Key components that contribute to the alleviation of ER stress, via the boosting of GERAD, are depicted.

organizational levels, these events attest to the role played by GERAD on the tempo-spatial surveillance of eukaryotic genome expression (Cabral et al., 2001; Ellgaard et al., 1999; Wu et al., 2007). Most importantly, by identifying the mechanism by which mammalian ERManI is regulated in response to ER stress, the new knowledge sets the stage for exploiting this interface as a potential target for the therapeutic intervention of conformational diseases of the secretory pathway (Balch et al., 2008; Molinari, 2007).

Materials and Methods

Routine materials and reagents

Routine chemicals and buffers were purchased from Sigma (St Louis, MO). Lipofectamine 2000 was a product from Invitrogen. The human embryonic kidney cell line was purchased from American Type Culture Collection. The generation of DNA expression constructs that encode human α 1-antitrypsin genetic variant null (Hong Kong) [hATNHK/pCDNA3.1Zeo(+)], variant P1Z [hATZ/pCDNA3.1Zeo(+)] and human ERManI [hERManI/pCDNA3.1Zeo(+)] have been previously reported (Wu et al., 2003). A plasmid that encodes a spliced variant of the X-box binding protein 1 transcription factor was a gift from Dr Tim Weaver (University of Cincinnati), and subcloned into the pCDNA3.1 Neo(+) to generate Xbp1(s)/pCDNA3.1Neo(+). The cDNA for hemagglutinin-tagged murine ER degradation-enhancing-mannosidase-like protein (EDEM1-HA), a generous gift from N. Hosokawa and K. Nagata (Kyoto University, Kyoto), was subcloned into the *Eco*RI site of pCDNA3.1Zeo(+) as described previously (Wu et al., 2003). A site-directed mutagenesis kit (Stratagene) used forward (cttgggaatgcatcgacgcttctgacctgacac) and reverse (gaacccttctgtagctcgaagacctggactgtg) primers (Invitrogen) to generate the E220A substitution in EDEM1-HA. Antibodies included an immunoglobulin fraction of rabbit polyclonal anti-human α 1-antitrypsin (Boehringer Mannheim), a rabbit polyclonal peptide-specific antiserum against human ERManI (ERMI-CC20) (Wu et al., 2003), a rabbit polyclonal antiserum against a 24 amino acid peptide homologous to the C terminus of human and murine EDEM1 (EDEM1hm24C) (Alpha Diagnostics), HA-probe (Y-11) rabbit sc-805 (Santa Cruz Biotechnology), a rabbit polyclonal Ab against β -actin (Sigma), and a peptide-specific rabbit polyclonal Ab against Xbp1 (Santa Cruz Biotechnology).

Transient transfection of HEK293 cells

The human embryonic kidney cell line HEK293 was grown as monolayers in standard growth medium (Cabral et al., 2002) and split into 100 mm dishes at a cell density of 70% confluence. The following day, cell monolayers were transfected according to the Lipofectamine 2000 protocol with the desired DNA construct(s). Qiagen-grade plasmid DNA (30 μ g) was diluted in 1 ml of OptiMEM media and combined with 30 μ l of Lipofectamine 2000 reagent in an equal volume of OptiMEM media. Incubation at room temperature allowed for the formation of the DNA-liposome complex, and the solution containing the DNA-liposome complex was added to cells. OptiMEM (3 ml) was added to the 100 mm dish, bringing the total volume to 5 ml. After 5 hours of incubation at 37°C, the media was removed and replaced with 10 ml of fresh OptiMEM media. Twenty-four hours after transfection cells from all the dishes were collected, mixed and sub-cultured at identical cell densities in either 60 mm or 100 mm dishes.

Western blotting

Intracellular proteins were detected by ECL western blotting of cell lysates as described (Wu et al., 2003). Approximately 48 hours post-transfection, 5×10^6 cells were lysed in 1 ml of buffered Nonidet P-40. Equivalent aliquots of the soluble fraction were resolved by SDS-PAGE and transferred to a nitrocellulose membrane before blotting as described in the text. Signals were detected with the recommended ECL western blotting reagents (Pierce).

Metabolic radiolabeling and immunoprecipitation

The rate of target protein degradation was monitored 48 hours post-transfection by pulse-chase metabolic radiolabeling with [35 S]methionine in combination with immunoprecipitation (Wu et al., 2003). Briefly, monolayers of semi-confluent cells were incubated for 1 hour at 37°C in complete media with or without specific inhibitors and then subjected to methionine starvation in methionine-free medium (Invitrogen) with or without specific inhibitors for an additional 30 minutes. [35 S]Methionine (Perkin Elmer) was added [0.150mCi (1mCi=37GBq) per 100 mm dish] during a 10-minute pulse, followed by up to 7 hours of chase in serum-free DMEM (Invitrogen) containing 0.2 mM unlabeled methionine with or without specific inhibitors at the same concentration used during preincubation and pulse. At designated timepoints, cells were lysed in Nonidet P-40 detergent. Immunoprecipitates were generated from the cell lysates as described (Wu et al., 2003; Wu et al., 2007), and resolved by SDS-PAGE for detection of radiolabeled proteins by fluorography. Quantitative analysis was performed by densitometric scanning by using the National Institutes of Health IMAGE program.

RNA interference

RNAi targeted against either ERManI and EDEM1 were purchased from Ambion. Forty-eight hours post-transfection, cells were transfected with the appropriate siRNA using siPORT *Amine* (Ambion). Briefly, the siRNA (30 nM) was mixed with 100 μ l fresh OptiMEM I medium then combined with 8 μ l siPORT *Amine* in an equal volume of OptiMEM I medium. The siRNA-siPORT mixture was then incubated at room temperature. The siRNA-siPORT mixture (200 μ l) was added to cells in a single well of a six-well plate that already contained 2 ml of complete media. The effect of the manipulation was assayed 24 hours post-transfection. Cell lysates were prepared and resolved by reducing SDS-PAGE. Protein levels were monitored by ECL western blotting.

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References

- Avezov, E., Frenkel, Z., Ehrlich, M., Herscovics, A. and Lederkremer, G. Z. (2008). Endoplasmic reticulum (ER) mannosidase I is compartmentalized and required for N-Glycan trimming to Man₅GlcNAc₂ in glycoprotein ER-associated degradation. *Mol. Biol. Cell* **19**, 216-225.
- Balch, W. E., Morimoto, R. I., Dillin, A. and Kelly, J. W. (2008). Adapting proteostasis for disease intervention. *Science* **319**, 916-919.
- Banerjee, S., Vishwanath, P., Cui, J., Kelleher, D. J., Gilmore, R., Robbins, P. W. and Samuelson, J. (2007). The evolution of N-glycan-dependent endoplasmic reticulum quality control factors for glycoprotein folding and degradation. *Proc. Natl. Acad. Sci. USA* **104**, 11676-11681.
- Bonifacio, J. S. and Weissman, A. M. (1998). Ubiquitin and the control of protein fate in the secretory and endocytic pathways. *Annu. Rev. Cell Dev. Biol.* **14**, 19-57.
- Brodsky, J. L. and McCracken, A. A. (1999). ER protein quality control and proteasome-mediated protein degradation. *Semin. Cell Dev. Biol.* **10**, 507-513.
- Burke, J., Lipari, F., Igdoura, S. and Herscovics, A. (1996). The Saccharomyces cerevisiae processing alpha 1,2-mannosidase is localized in the endoplasmic reticulum, independently of known retrieval motifs. *Eur. J. Cell Biol.* **70**, 298-305.
- Cabral, C. M., Liu, Y. and Sifers, R. N. (2001). Dissecting glycoprotein quality control in the secretory pathway. *Trends Biochem. Sci.* **26**, 619-624.
- Cabral, C. M., Liu, Y., Moremen, K. W. and Sifers, R. N. (2002). Organizational diversity among distinct glycoprotein endoplasmic reticulum-associated degradation programs. *Mol. Biol. Cell* **13**, 2639-2650.
- D'Alessio, C., Fernandez, F., Trombetta, E. S. and Parodi, A. J. (1999). Genetic evidence for the heterodimeric structure of glucosidase II. The effect of disrupting the subunit-encoding genes on glycoprotein folding. *J. Biol. Chem.* **274**, 25899-25905.
- Ellgaard, L. and Helenius, A. (2001). ER quality control: towards an understanding at the molecular level. *Curr. Opin. Cell Biol.* **13**, 431-437.
- Ellgaard, L., Molinari, M. and Helenius, A. (1999). Setting the standards: quality control in the secretory pathway. *Science* **286**, 1882-1888.
- Elliott, P. R., Lomas, D. A., Carrell, R. W. and Abrahams, J. P. (1996). Inhibitory conformation of the reactive loop of alpha 1-antitrypsin. *Nat. Struct. Biol.* **3**, 676-681.
- Frenkel, Z., Gregory, W., Kornfeld, S. and Lederkremer, G. Z. (2003). Endoplasmic reticulum-associated degradation of mammalian glycoproteins involves sugar chain trimming to Man₆-5GlcNAc₂. *J. Biol. Chem.* **278**, 34119-34124.
- Gething, M. J. and Sambrook, J. (1992). Protein folding in the cell. *Nature* **355**, 33-45.

- Gonzalez, D. S., Karaveg, K., Vandersall-Nairn, A. S., Lal, A. and Moremen, K. W. (1999). Identification, expression, and characterization of a cDNA encoding human endoplasmic reticulum mannosidase I, the enzyme that catalyzes the first mannose trimming step in mammalian Asn-linked oligosaccharide biosynthesis. *J. Biol. Chem.* **274**, 21375-21386.
- Graham, K. S., Le A. and Sifers, R. N. (1990). Accumulation of the insoluble PiZ variant of human alpha 1-antitrypsin within the hepatic endoplasmic reticulum does not elevate the steady-state level of grp78/BiP. *J. Biol. Chem.* **265**, 20463-20468.
- Hammond, C., Braakman, I. and Helenius, A. (1994). Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. *Proc. Natl. Acad. Sci. USA* **91**, 913-917.
- Harding, H. P., Zhang, Y. and Ron, D. (1999). Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature* **397**, 271-274.
- Hebert, D. N. and Molinari, M. (2007). In and out of the ER: protein folding, quality control, degradation, and related human diseases. *Physiol. Rev.* **87**, 1377-1408.
- Hebert, D. N., Foellmer, B. and Helenius, A. (1995). Glucose trimming and reglycosylation determine glycoprotein association with calnexin in the endoplasmic reticulum. *Cell* **81**, 425-433.
- Hebert, D. N., Garman, S. C. and Molinari, M. (2005). The glycan code of the endoplasmic reticulum: asparagine-linked carbohydrates as protein maturation and quality-control tags. *Trends Cell Biol.* **15**, 364-370.
- Helenius, A. (1994). How N-linked oligosaccharides affect glycoprotein folding in the endoplasmic reticulum. *Mol. Biol. Cell* **5**, 253-265.
- Helenius, A., Trombetta, E. S., Hebert, D. N. and Simons, J. F. (1997). Calnexin and calreticulin and the folding of glycoproteins. *Trends Cell Biol.* **7**, 193-200.
- Hidvegi, T., Schmidt, B. Z., Hale, P. and Perlmutter, D. H. (2005). Accumulation of mutant alpha1-antitrypsin Z in the endoplasmic reticulum activates caspases-4 and -12, NFkappaB, and BAP31 but not the unfolded protein response. *J. Biol. Chem.* **280**, 39002-39015.
- Hirao, K., Natsuka, Y., Tamura, T., Wada, I., Morito, D., Natsuka, S., Romero, P., Seno, B., Tremblay, L. O., Herscovics, A. et al. (2006). EDEM3, a soluble EDEM homolog, enhances glycoprotein endoplasmic reticulum-associated degradation and mannose trimming. *J. Biol. Chem.* **281**, 9650-9658.
- Hosokawa, N., Wada, I., Hasegawa, K., Yorihuzi, T., Tremblay, L. O., Herscovics, A. and Nagata, K. (2001). A novel ER alpha-mannosidase-like protein accelerates ER-associated degradation. *EMBO Rep.* **2**, 415-422.
- Hosokawa, N., Tremblay, L. O., You, Z., Herscovics, A., Wada, I. and Nagata, K. (2003). Enhancement of endoplasmic reticulum (ER) degradation of misfolded Null Hong Kong alpha1-antitrypsin by human ER mannosidase I. *J. Biol. Chem.* **278**, 26287-26294.
- Hosokawa, N., Wada, I., Natsuka, Y. and Nagata, K. (2006). EDEM accelerates ERAD by preventing aberrant dimer formation of misfolded alpha1-antitrypsin. *Genes Cells* **11**, 465-476.
- Hosokawa, N., You, Z., Tremblay, L. O., Nagata, K. and Herscovics, A. (2007). Stimulation of ERAD of misfolded null Hong Kong alpha1-antitrypsin by Golgi alpha1,2-mannosidases. *Biochem. Biophys. Res. Commun.* **362**, 626-632.
- Jakob, C. A., Burda, P., Roth, J. and Aebi, M. (1998). Degradation of misfolded endoplasmic reticulum glycoproteins in *Saccharomyces cerevisiae* is determined by a specific oligosaccharide structure. *J. Cell Biol.* **142**, 1223-1233.
- Jakob, C. A., Bodmer, D., Spirig, U., Battig, P., Marciel, A., Dignard, D., Bergeron, J. J., Thomas, D. Y. and Aebi, M. (2001). Htm1p, a mannosidase-like protein, is involved in glycoprotein degradation in yeast. *EMBO Rep.* **2**, 423-430.
- Kalz-Fuller, B., Bieberich, E. and Bause, E. (1995). Cloning and expression of glucosidase I from human hippocampus. *Eur. J. Biochem.* **231**, 344-351.
- Kanehara, K., Kawaguchi, S. and Ng, D. T. (2007). The EDEM and Yos9p families of lectin-like ERAD factors. *Semin. Cell Dev. Biol.* **18**, 743-750.
- Karaveg, K. and Moremen, K. W. (2005). Energetics of substrate binding and catalysis by class I (glycosylhydrolase family 47) alpha-mannosidases involved in N-glycan processing and endoplasmic reticulum quality control. *J. Biol. Chem.* **280**, 29837-29848.
- Klausner, R. D. and Sitia, R. (1990). Protein degradation in the endoplasmic reticulum. *Cell* **62**, 611-614.
- Kornfeld, R. and Kornfeld, S. (1985). Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* **54**, 631-664.
- Labriola, C., Cazzulo, J. J. and Parodi, A. J. (1999). Trypanosoma cruzi calreticulin is a lectin that binds monoglucosylated oligosaccharides but not protein moieties of glycoproteins. *Mol. Biol. Cell* **10**, 1381-1394.
- Lawless, M. W., Greene, C. M., Mulgrew, A., Taggart, C. C., O'Neill, S. J. and McElvaney, N. G. (2004). Activation of endoplasmic reticulum-specific stress responses associated with the conformational disease Z alpha 1-antitrypsin deficiency. *J. Immunol.* **172**, 5722-5726.
- Li, M., Baumeister, P., Roy, B., Phan, T., Foti, D., Luo, S. and Lee, A. S. (2000). ATF6 as a transcription activator of the endoplasmic reticulum stress element: thapsigargin stress-induced changes and synergistic interactions with NF-Y and YY1. *Mol. Cell Biol.* **20**, 5096-5106.
- Mast, S. W., Diekmann, K., Karaveg, K., Davis, A., Sifers, R. N. and Moremen, K. W. (2005). Human EDEM2, a novel homolog of family 47 glycosidases, is involved in ER-associated degradation of glycoproteins. *Glycobiology* **15**, 421-436.
- Molinari, M. (2007). N-glycan structure dictates extension of protein folding or onset of disposal. *Nat. Chem. Biol.* **3**, 313-320.
- Molinari, M., Galli, C., Piccaluga, V., Pieren, M. and Paganetti, P. (2002). Sequential assistance of molecular chaperones and transient formation of covalent complexes during protein degradation from the ER. *J. Cell Biol.* **158**, 247-257.
- Molinari, M., Calanca, V., Galli, C., Lucca, P. and Paganetti, P. (2003). Role of EDEM in the release of misfolded glycoproteins from the calnexin cycle. *Science* **299**, 1397-1400.
- Oda, Y., Hosokawa, N., Wada, I. and Nagata, K. (2003). EDEM as an acceptor of terminally misfolded glycoproteins released from calnexin. *Science* **299**, 1394-1397.
- Olivari, S., Galli, C., Alanen, H., Ruddock, L. and Molinari, M. (2005). A novel stress-induced EDEM variant regulating endoplasmic reticulum-associated glycoprotein degradation. *J. Biol. Chem.* **280**, 2424-2428.
- Olivari, S., Cali, T., Salo, K. E., Paganetti, P., Ruddock, L. W. and Molinari, M. (2006). EDEM1 regulates ER-associated degradation by accelerating de-mannosylation of folding-defective polypeptides and by inhibiting their covalent aggregation. *Biochem. Biophys. Res. Commun.* **349**, 1278-1284.
- Ou, W. J., Cameron, P. H., Thomas, D. Y. and Bergeron, J. J. (1993). Association of folding intermediates of glycoproteins with calnexin during protein maturation. *Nature* **364**, 771-776.
- Parodi, A. J. (2000). Protein glycosylation and its role in protein folding. *Annu. Rev. Biochem.* **69**, 69-93.
- Plemper, R. K. and Wolf, D. H. (1999). Endoplasmic reticulum degradation: reverse protein transport and its end in the proteasome. *Mol. Biol. Rep.* **26**, 125-130.
- Ron, D. and Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. *Nat. Rev. Mol. Cell Biol.* **8**, 519-529.
- Rutkowski, D. T. and Kaufman, R. J. (2007). That which does not kill me makes me stronger: adapting to chronic ER stress. *Trends Biochem. Sci.* **32**, 469-476.
- Scheuner, D., Song, B., McEwen, E., Liu, C., Laybutt, R., Gillespie, P., Saunders, T., Bonner-Weir, S. and Kaufman, R. J. (2001). Translational control is required for the unfolded protein response and *in vivo* glucose homeostasis. *Mol. Cell* **7**, 1165-1176.
- Sifers, R. N. (2003). Cell biology: protein degradation unlocked. *Science* **299**, 1330-1331.
- Sifers, R. N., Carlson, J. A., Clift, S. M., DeMayo, F. J., Bullock, D. W. and Woo, S. L. (1987). Tissue specific expression of the human alpha-1-antitrypsin gene in transgenic mice. *Nucleic Acids Res.* **15**, 1459-1475.
- Sifers, R. N., Brashears-Macatee, S., Kidd, V. J., Muensch, H. and Woo, S. L. (1988). A frameshift mutation results in a truncated alpha 1-antitrypsin that is retained within the rough endoplasmic reticulum. *J. Biol. Chem.* **263**, 7330-7335.
- Sifers, R. N., Shen, R. F. and Woo, S. L. (1989). Genetic control of human alpha-1-antitrypsin. *Mol. Biol. Med.* **6**, 127-135.
- Sitia, R. and Braakman, I. (2003). Quality control in the endoplasmic reticulum protein factory. *Nature* **426**, 891-894.
- Sousa, M. C., Ferrero-Garcia, M. A. and Parodi, A. J. (1992). Recognition of the oligosaccharide and protein moieties of glycoproteins by the UDP-Glc:glycoprotein glucosyltransferase. *Biochemistry* **31**, 97-105.
- Spiro, R. G. (2000). Glucose residues as key determinants in the biosynthesis and quality control of glycoproteins with N-linked oligosaccharides. *J. Biol. Chem.* **275**, 35657-35660.
- Suh, K., Bergmann, J. E. and Gabel, C. A. (1989). Selective retention of monoglucosylated high mannose oligosaccharides by a class of mutant vesicular stomatitis virus G proteins. *J. Cell Biol.* **108**, 811-819.
- Travers, K. J., Patil, C. K., Wodicka, L., Lockhart, D. J., Weissman, J. S. and Walter, P. (2000). Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell* **101**, 249-258.
- Tremblay, L. O. and Herscovics, A. (1999). Cloning and expression of a specific human alpha 1,2-mannosidase that trims Man9GlcNAc2 to Man8GlcNAc2 isomer B during N-glycan biosynthesis. *Glycobiology* **9**, 1073-1078.
- Trombetta, E. S., Simons, J. F. and Helenius, A. (1996). Endoplasmic reticulum glucosidase II is composed of a catalytic subunit, conserved from yeast to mammals, and a tightly bound noncatalytic HDEL-containing subunit. *J. Biol. Chem.* **271**, 27509-27516.
- Wang, S. and Ng, D. T. (2008). Lectins sweet-talk proteins into ERAD. *Nat. Cell Biol.* **10**, 251-253.
- Wu, Y., Swulius, M. T., Moremen, K. W. and Sifers, R. N. (2003). Elucidation of the molecular logic by which misfolded alpha 1-antitrypsin is preferentially selected for degradation. *Proc. Natl. Acad. Sci. USA* **100**, 8229-8234.
- Wu, Y., Termine, D. J., Swulius, M. T., Moremen, K. W. and Sifers, R. N. (2007). Human endoplasmic reticulum mannosidase I is subject to regulated proteolysis. *J. Biol. Chem.* **282**, 4841-4849.
- Yoshida, H., Haze, K., Yanagi, H., Yura, T. and Mori, K. (1998). Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. Involvement of basic leucine zipper transcription factors. *J. Biol. Chem.* **273**, 33741-33749.
- Yoshida, H., Matsui, T., Hosokawa, N., Kaufman, R. J., Nagata, K. and Mori, K. (2003). A time-dependent phase shift in the mammalian unfolded protein response. *Dev. Cell* **4**, 265-271.
- Zapun, A., Petrescu, S. M., Rudd, P. M., Dwek, R. A., Thomas, D. Y. and Bergeron, J. J. (1997). Conformation-independent binding of monoglucosylated ribonuclease B to calnexin. *Cell* **88**, 29-38.
- Zhang, K. and Kaufman, R. J. (2006). The unfolded protein response: a stress signaling pathway critical for health and disease. *Neurology* **66**, S102-S109.
- Zhou, B. B. and Elledge, S. J. (2000). The DNA damage response: putting checkpoints in perspective. *Nature* **408**, 433-439.