Bypass of glycan-dependent glycoprotein delivery to ERAD by up-regulated EDEM1

Efrat Ron, Marina Shenkman, Bella Groisman, Yana Izenshtein, Julia Leitman, and Gerardo Z. Lederkremer

Department of Cell Research and Immunology, George Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel

ABSTRACT Trimming of mannose residues from the N-linked oligosaccharide precursor is a stringent requirement for glycoprotein endoplasmic reticulum (ER)-associated degradation (ERAD). In this paper, we show that, surprisingly, overexpression of ER degradation-enhancing α-mannosidase-like protein 1 (EDEM1) or its up-regulation by IRE1, as occurs in the unfolded protein response, overrides this requirement and renders unnecessary the expression of ER mannosidase I. An EDEM1 deletion mutant lacking most of the carbohydrate-recognition domain also accelerated ERAD, delivering the substrate to XTP3-B and OS9. EDEM1 overexpression also accelerated the degradation of a mutant nonglycosylated substrate. Upon proteasomal inhibition, EDEM1 concentrated together with the ERAD substrate in the pericentriolar ER-derived quality control compartment (ERQC), where ER mannosidase I and ERAD machinery components are localized, including, as we show here, OS9. We suggest that a nascent glycoprotein can normally dissociate from EDEM1 and be rescued from ERAD by reentering calnexin-refolding cycles, a condition terminated by mannose trimming. At high EDEM1 levels, glycoprotein release is prevented and glycan interactions are no longer required, canceling the otherwise mandatory ERAD timing by mannose trimming and accelerating the targeting to degradation.

INTRODUCTION A crucial and obligatory step in endoplasmic reticulum (ER)-associated degradation of a misfolded glycoprotein in mammalian cells is the removal of three or four α1,2-linked mannose residues from its precursor sugar chains (Frenkel et al., 2003; Lederkremer and Glickman, 2005; Lederkremer, 2009; Aebi et al., 2010; Hebert et al., 2010). This process could be accomplished by ER mannosidase I (ERManI) by itself, through the high concentration of this enzyme in a pericentriolar subcellular compartment, the ER-derived quality control compartment (ERQC; Avezov et al., 2008), but might be aided by other mannosidases (Hosokawa et al., 2007; Olivari and Molinar, 2007).

An important player in this process is ER degradation-enhancing α-mannosidase-like protein 1 (EDEM1; or its yeast homologue Htm1), although the mechanism of its participation is still unclear (Kanehara et al., 2007; Olivari and Molinar, 2007; Aebi et al., 2010). EDEM1 was shown to bind endoplasmic reticulum-associated degradation (ERAD) substrate glycoproteins after their release from calnexin (Molinari et al., 2003; Oda et al., 2003), having also a chaperone-like function (Hosokawa et al., 2006). Because EDEM1 is homologous to α1,2-mannosidases but does not seem to have mannosidase activity in vitro, it was initially postulated that it may act as a lectin receptor, associating with N-linked sugar chains after the mannoside-trimming step (Hosokawa et al., 2007; Jakob et al., 2001). However, we have recently shown that EDEM1 associates with a glycoprotein substrate in the absence of the mannoside-Triming activity (Groisman et al., 2011). In fact, EDEM1 and its yeast homologue participate as mannosidases or cofactors in the trimming process in vivo (Olivari et al., 2006; Quan et al., 2008; Clerc...
et al., 2009; Hosokawa et al., 2010b). The lectin receptor role for the extensively trimmed species is now ascribed to OS9 and its functional homologue XTP3-B and the yeast homologue Yos9 (Hosokawa et al., 2010a). We have studied the influence of elevated EDEM1, a condition that exists during the unfolded protein response (UPR), on the targeting of an ERAD substrate glycoprotein to OS9 and XTP3-B and to degradation, processes that normally depend on trimming of mannose residues. We found that EDEM1 bypasses the mannose-trimming event and delivers the glycoprotein directly to late ERAD stages.

RESULTS

When EDEM1 is overexpressed, mannose trimming and ERManI are not required for ERAD of H2a

We have used here the uncleaved precursor of the asialoglycoprotein receptor (ASGPR) H2a, a well-studied model ERAD substrate. This glycoprotein is expressed naturally in hepatocytes as a membrane precursor that undergoes efficient cleavage, producing a 35-kDa secreted form (Tolchinsky et al., 1996). When H2a is expressed in other cell lines, such as NIH 3T3 or HEK 293, the membrane precursor is inefficiently cleaved and the uncleaved precursor, as well as most of the cleaved fragment, is completely retained in the ER and degraded by the ubiquitin–proteasome system (Shenkman et al., 1997; Kamhi-Nesher et al., 2001). ERAD of H2a requires the activity of α1,2-mannosidases (Ayalon-Soffer et al., 1999; Frenkel et al., 2003; Avezov et al., 2008), which can be blocked with the inhibitor kifunensine (Kif), as can be seen in the pulse-chase analysis experiment of Figure 1A (compare lanes 1–2 with lanes 3–4). The lower band of the precursor in lane 1 of Figure 1A represents underglycosylated molecules (one of the three possible glycosylation sites is not occupied). The small shift of H2a to a faster mobility, as seen in the chase (Figure 1A, lane 2), is due to mannose trimming, since blocking of this trimming abrogates the shift and stabilizes H2a (Figure 1A, lanes 3–4).

Overexpression of EDEM1 (which should mimic its high levels upon UPR), accelerated the degradation of H2a (Figure 1B), as we had seen before (Groisman et al., 2011). Surprisingly, upon overexpression of EDEM1, the degradation of H2a was no longer blocked by Kif (Figure 2A, compare lanes 4–6 with lanes 1–3). This is despite the shift to a slower mobility caused by Kif, which can be seen in the remaining H2a molecules (Figure 2A, compare lanes 5 and 6, and Supplemental Figure S1). The acceleration of ERAD by EDEM1 overexpression did not require the presence of ERManI, as its knockdown, which blocks the degradation in normal conditions, as we have shown before (Avezov et al., 2008), did not block it upon EDEM1 overexpression (Figure 2A, compare lanes 10–11 with lanes 7–8 and 4–5). Even upon ERManI knockdown plus cell treatment with Kif, there was no reduction in the EDEM1 acceleration of ERAD (Figure 2A, compare lanes 4–6 with lanes 10–12). In contrast to the results with EDEM1, acceleration of H2a degradation by ERManI overexpression was blocked by Kif, indicating that the effect of ERManI is dependent on mannose trimming (Figure S2). Dimers and higher oligomers of H2a can be seen in a nonreducing gel and accumulate upon Kif treatment of cells (Figure 3S, lanes 1–2). EDEM1 overexpression reduced the levels of these oligomeric forms both in the absence and in the presence of Kif, suggesting that it promotes their dissociation and degradation independently of the mannose trimming. This is consistent with a chaperone activity that had been proposed for EDEM1 in dissociating aberrant misfolded protein oligomers (Hosokawa et al., 2006; Olivari et al., 2006).

We have used HEK 293 cells, although the rate of degradation of H2a is relatively slow in these cells compared with that in other cell types. However, these cells allow efficient transfection, which is needed for simultaneous expression from several plasmids combined with short-hairpin RNA (shRNA) knockdown. We wondered whether the effect of EDEM1 overexpression also occurs in other cell types and for other substrates. To test this, we expressed unassembled CD38, another established ERAD substrate (Fang et al., 2001; Frenkel et al., 2003; Kondratyev et al., 2007), in NIH 3T3 cells. CD38 degradation could be blocked with Kif. Although EDEM1 overexpression accelerated the degradation of CD38 only modestly, it very effectively reduced the inhibition by Kif (Figure 2B).

Acceleration of ERAD caused by activation of the IRE1-dependent UPR pathway does not require mannosidase activity or ERManI

We then tested whether EDEM1 up-regulation during the UPR had a similar effect to that of direct overexpression. For this purpose, we could not use the classical UPR-inducing drugs (tunicamycin, dithiothreitol, etc.), which would directly affect the folding status of the substrate and also of EDEM1. Instead, we overexpressed the UPR sensor IRE1, a procedure that had been shown to activate the IRE1-dependent UPR pathway (Wang et al., 1998; Tiraphophon et al., 2000), which up-regulates the expression of EDEM1. IRE1 overexpression caused a much increased degradation of H2a already during the pulse, which was not blocked by Kif (Figure 3A). The reduction of
Overexpression of an EDEM1 mutant lacking the carbohydrate-recognition domain still accelerates ERAD in an ERManI-independent manner and abrogates mannose trimming dependence of substrate delivery to OS9 and XTP3B

As EDEM1 overexpression bypassed the requirement of mannose trimming for ERAD, we investigated whether its interaction with substrate glycoprotein sugar chains was at all involved in this process. For this, we aimed to overexpress a mutant EDEM1 that would not interact with sugar chains nor have any putative mannosidase activity. Several point mutants of EDEM1 had been made in conserved residues in the carbohydrate-recognition domain (CRD) that corresponds to the catalytic portion of homologous mannosidases (Olivari et al., 2006; Cormier et al., 2009). However, it is unclear whether any of these mutants is unable to associate to the sugar chains of the substrate; some may associate with an even stronger affinity than the wild-type protein (unpublished data). Therefore we constructed a mutant EDEM1 in which we deleted a segment encoding 156 amino acids, most of the CRD, and named it EDEM1ΔCRD (Figure 4A). EDEM1ΔCRD, expressed at a level similar to EDEM1 (Figure S4), still accelerated the degradation of H2a in an ERManI-independent manner (Figure 4B). Although no mannosidase activity was expected for EDEM1ΔCRD, it could eventually still modify in an indirect way the sugar chains of the substrate. We analyzed this possibility in a pulse-chase experiment with [2-3H]mannose as a precursor and with high-performance liquid chromatography (HPLC) analysis of H2a N-linked sugar chains. Whereas overexpression of wild-type EDEM1 caused trimming of H2a N-glycans to yield mainly Man₅GlcNAc₂, overexpression of EDEM1ΔCRD caused no major change in the sugar chain pattern compared with the mock-transfected cells, except for a certain delay in the trimming to Man₃GlcNAc₂, possibly due to competition with endogenous EDEM1 or ERMani (Figure 4, C–G).

We have recently shown that mannose trimming is required for substrate delivery in cells in vivo to XTP3-B (Groisman et al., 2011). In vitro, both OS9 and XTP3-B bind with high affinity to trimmed glycans and do not bind to untrimmed ones (Hosokawa et al., 2008; Quan et al., 2008; Yamaguchi et al., 2010). We tested the effect of EDEM1ΔCRD overexpression on the coimmunoprecipitation of H2a with XTP3-B. Whereas the association was much reduced by Kif treatment of cells, overexpression of EDEM1ΔCRD canceled the requirement of mannose trimming (Figure 5, top panel, compare lanes 5–6 with lanes 7–8). This was also true for the association of H2a with OS9, though the coimmunoprecipitation was much less robust in this case (Figure 5, top panel, compare lanes 1–2 with lanes 3–4). The overexpression of EDEM1ΔCRD also reduced the overall coimmunoprecipitation of H2a with XTP3-B or OS9, possibly by nonproductive binding of EDEM1ΔCRD to the substrate.

FIGURE 2: Overexpression of EDEM1 overrides the ERMani and mannose-trimming requirements for ERAD. (A) Similar to Figure 1 but performed with HEK 293 cells transiently cotransfected with the H2a-encoding vector together with either a control anti-lacZ shRNA-encoding pSUPER vector (lanes 1–3), or with the same vector encoding anti-ERMani shRNA (lanes 7–9), or with an EDEM1-HA-encoding vector (lanes 4–6), or with a combination of EDEM1-HA and anti-ERMani shRNA-encoding vectors (lanes 10–12). Cells were chased for the indicated times in complete medium in the absence or presence of Kif (100 μM; lanes 3, 6, 9, and 12). Note that here transfections were done with a higher amount of H2a-encoding vector than in Figure 1 (5 μg instead of 3 μg) to obtain higher expression and transfections were done with a higher amount of H2a-encoding vector together with either a control GFP-encoding vector (lanes 1–5) or with an EDEM1-HA-encoding vector (lanes 6–10).

H2a upon IRE1 overexpression was not due to slower synthesis; protein synthesis levels remained unchanged (Figure 3B). In these conditions, there was a robust increase in the levels of endogenous EDEM1 mRNA and also at the protein level (Figure 3, C–D).

To explore whether this effect of IRE1 overexpression was mediated by EDEM1, we performed an experiment where we combined overexpression of IRE1 and knockdown of EDEM1. As shown in the preceding section, IRE1 overexpression significantly increased the degradation of H2a, which was no longer blocked by Kif (Figure 3E, lanes 4–6). EDEM1 knockdown caused a strong stabilization of H2a (Figure 3E, lanes 7–9), as we had seen before (Groisman et al., 2011). Simultaneous overexpression of IRE1 and knockdown of EDEM1 partially decreased the IRE1-mediated acceleration of H2a degradation, and importantly, it restored the sensitivity to Kif (Figure 3E, compare lanes 4–6 with lanes 10–12 and graph). This result indicates that the effect of IRE1 in canceling the requirement of mannose trimming for the degradation was mediated by EDEM1. Figure 3F shows that EDEM1 up-regulation by IRE1 was significantly reduced by EDEM1 knockdown, but EDEM1 knockdown did not affect the increase in spliced XBP1 (XBP1s) mRNA levels, a direct indicator of the activation of the IRE1 branch of the UPR.
EDEM1 and OS9 localize to the ERQC and EDEM1 knockdown causes accumulation of the ERAD substrate at the ERQC

It had been observed that EDEM1 localizes mainly to vesicular structures (Zuber et al., 2007). Indeed, we could see that endogenous EDEM1 appeared in a distributed punctate pattern, partially colocalizing with H2a linked to monomeric red fluorescent protein (H2aRFP; Figure 7A, top panels). To inhibit degradation of the substrate upon proteasomal inhibition, both proteins concentrated and showed a significant colocalization at the juxtanuclear ERQC compartment (Figure 7A, bottom panels). Similar results, with even higher colocalization at the ERQC upon proteasomal inhibition were seen upon overexpression of EDEM1-HA (Figure 7B). We had observed the recruitment of the ERAD substrate and components of the ERAD machinery to the ERQC upon proteasomal inhibition or UPR induction (Kondratyev et al., 2007). OS9 also localized to the ERQC upon proteasomal inhibition (Figure 7C, bottom panels), but notably, OS9 also appeared concentrated in this juxtanuclear region in untreated cells (Figure 7C, top panels), similar to what we had seen for ERManI (Avezov et al., 2008).

Both ERManI and OS9 appear to be constitutive residents of the ERQC. They are both short-lived and OS9 was shown to be disposed of by an autophagic process (Bernasconi and Molinari, 2011).

 Altogether, these results suggest the ability of EDEM1 to target substrate proteins to OS9 and XTP3-B and to ERAD in a glycan-independent manner through protein–protein interactions.

A nonglycosylated substrate can interact with OS9 and XTP3-B and can be targeted to ERAD by overexpressed EDEM1

We then tested whether H2a could in effect associate with OS9 and XTP3-B through protein–protein interactions. This was done using a nonglycosylated version of H2a we have described before, H2aΔgly, which is also an ERAD substrate. In H2aΔgly all three N-glycosylation sites are mutated (H2aΔgly; Groisman et al., 2006). H2aΔgly showed significant interaction with XTP3-B and OS9 (Figure 6A). It had also been previously reported that OS9 and XTP3-B are able to interact with another nonglycosylated substrate, α1-antitrypsin NHKQQQ (Bernasconi et al., 2008; Hosokawa et al., 2008).

We then explored whether EDEM1 had an effect on the nonglycosylated substrate. Overexpression of EDEM1 caused a significantly accelerated degradation of H2aΔgly (Figure 6B).

These results indicate that overexpression of EDEM1 can indeed target an ERAD substrate without N-glycans, and that the substrate can associate to OS9 and XTP3-B through protein–protein interactions.
Accumulation of the ERAD substrate at the ERQC is dependent on ERManI and mannosidase activity. On ERManI knockdown or in the presence of inhibitors of α1,2-mannosidases, H2a distributes in a punctate pattern (Frenkel et al., 2003; Avezov et al., 2008). This is despite the fact that ERManI knockdown or mannos trimming inhibitors stabilize the ERAD substrate to the same extent as proteasomal inhibition. In contrast to ERManI knockdown, anti-EDEM1 shRNA caused H2aRFP accumulation in the ERQC (Figure 7, D–F). This suggests that the mannos trimming activity of ERManI is necessary for localization of the ERAD substrate to the ERQC, but the activity of EDEM1 is instead required for ERAD events occurring downstream of substrate accumulation in the ERQC.

**DISCUSSION**

Our results show that high levels of EDEM1 bypass the requirement of mannos trimming for glycoprotein ERAD. Although most of this study was done with H2a as a substrate, a similar effect on unassembled CD3δ (Figure 2) suggests a possible generality of this phenomenon. This finding has important implications, one being that the mannos trimming “timer,” which allows a time interval for newly synthesized glycoproteins to fold, will be effectively canceled upon EDEM1 up-regulation during the UPR. We can speculate that, under these conditions, nascent, still-unfolded proteins might be promptly sent to ERAD, as presumably they would not be distinguished from misfolded ones. This would clear the early secretory pathway of trafficking cargo. Another implication is that the mannos trimming of the N-glycans to Man$_5$-6GlcNAc$_2$ (Lederkremer, 2009) is not an absolute requirement for recognition or for physical movement of the glycoprotein during retrotranslocation leading to ERAD.

EDEM1 was initially postulated to be a lectin receptor, associating with N-linked sugar chains after the mannos trimming step (Hosokawa et al., 2001; Jakob et al., 2001). However, we recently showed that interaction of EDEM1 with a substrate glycoprotein is not affected by inhibition of mannos trimming or knockdown of ERManI (Groisman et al., 2011). This suggests that EDEM1 interacts with the substrate at an early stage in the quality control process, before the mannos trimming step. On the other hand, if EDEM1 were required in the quality control process only at an early stage, one would expect accumulation of the substrate at this early juncture as a result of its knockdown. This is what happens with
EDEM1 concentration is kept low by an autophagic process (Cali et al., 2008; Bernasconi and Molinari, 2011), except upon induction of the UPR. When the UPR is induced, there is overexpression of EDEM1 (Yoshida et al., 2003). IRE1 overexpression strongly up-regulated EDEM1 and led to enhanced ERAD, which was independent of mannoside trimming (Figure 3). Although IRE1 also induces expression of other chaperone and ERAD genes, combined IRE1 overexpression and knockdown of EDEM1 restored the dependence of ERAD on mannoside trimming, suggesting the effect was indeed mediated by EDEM1. On proteasomal inhibition, the ERAD substrate accumulates in the ERQC (Kamhi-Nesher et al., 2001; Kondratyev et al., 2007), colocalizing in these conditions with overexpressed EDEM1 (Figure 7). In normal conditions, most EDEM1 is seen in vesicles (Zuber et al., 2007), possibly on its way from the ERQC to degradation through an autophagic pathway (Cali et al., 2008). The localization of EDEM1 in the ERQC is consistent with a report that EDEM1 associates with a disulfide reductase, ERdj5 and with BiP, and that BiP dissociates from this complex at later stages (Ushioda et al., 2008). Indeed, BiP is mostly excluded from the ERQC (Kamhi-Nesher et al., 2001; Kondratyev et al., 2007).

No mannosidase activity was found for mammalian EDEM1 in vitro, but its overexpression does accelerate mannoside trimming in cells in vivo (Figure 4; Olivari et al., 2006; Clerc et al., 2009; Hosokawa et al., 2010b). The question remains whether EDEM1 itself has mannosidase activity and needs an activator or cofactor that is only present in vivo, as was recently shown for its yeast homologue Htm1 (Gauss et al., 2011), or if it accelerates mannoside trimming through an indirect effect, for example, by acting itself as a cofactor or causing increased delivery to the site of trimming, the ERQC. We can conclude from our results that if EDEM1 has intrinsic mannosidase activity, this function is not needed for its role in ERAD when it is up-regulated, in contrast to the requirement of mannosidase activity for the targeting to ERAD by ERMan1 (Figures 2 and S2). The acceleration of ERAD of a glycoprotein by overexpression of EDEM1ΔCRD (Figure 4) and of a nonglycosylated protein by wild-type EDEM1 (Figure 6) is clear-cut evidence that EDEM1 can function in a glycan-independent manner. This is consistent with the activity seen for some point mutants of EDEM1 (Olivari et al., 2006; Cormier et al., 2009).

It was reported that overexpressed EDEM1 can form a complex with ERMan1, inhibiting the degradation of the latter and therefore boosting its levels (Termine et al., 2009). That might be another way to accelerate ERAD during the UPR, by indirectly increasing the trimming of mannoside residues, as ERMan1 transcription is not increased in the UPR (Avezov et al., 2008). Our results suggest that up-regulated EDEM1 can also directly deliver substrates to XTP-3B and OS9 and stimulate ERAD in an ERMan1- and mannoside trimming-independent manner. In these conditions EDEM1 and ERMan1 would act independently, which is consistent with the fact that overexpression of any of these proteins substantially accelerates ERAD (Figure 2; see Olivari et al., 2006) and Avezov et al. (2008). For some substrates in Saccharomyces cerevisiae, it was recently observed that the EDEM1 homologue Htm1 can also act independently of Mns1, the yeast ERMan1 homologue, but in this case the delivery to ERAD was still dependent on the mannoside-trimming activity (Hosomi et al., 2010). We can speculate that the EDEM1 substrate glycoprotein may be unable to dissociate and reenter the calnexin cycle in mammalian cells at high concentration of EDEM1 (e.g., upon UPR), whereas at low EDEM1 concentration it may be released and reenter this cycle as long as less than three mannoside residues have been trimmed (Figure 8). This newly acquired independence from the mannoside-trimming “timer” (Lederkremer and Glickman, 2005; Lederkremer, 2009; Aebi et al.,
FIGURE 6: Mutant nonglycosylated H2a also associates with XTP3-B and OS9 and EDEM1 overexpression accelerates its degradation. (A) Experiment similar to the one shown in Figure 5 but analyzing coimmunoprecipitation of S-tagged XTP3-B or OS9.1/2 with a mutant H2a with its three N-glycosylation sites abrogated (H2aΔgly). (B) Pulse-chase experiment, similar to the ones in Figures 1 and 2 but with HEK 293 cells transiently cotransfected with a vector encoding H2aΔgly together with either a GFP-encoding control vector (lanes 1–3), or with an EDEM1-HA-encoding vector (lanes 4–6).

2010), which we describe here for the first time, would be one more mechanism that the cell utilizes to accelerate ERAD under the UPR, in addition to the up-regulation of ERAD machinery components (Travers et al., 2000; Yoshida et al., 2003), preemptive proteosomal degradation (Kang et al., 2006), and the appearance of nonproteosomal degradation pathways (Shenkman et al., 2007b).

MATERIALS AND METHODS

Materials
Rainbow [14C]-labeled methylated protein standards were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Promix cell-labeling mix ([35S]Met plus [35S]Cys, > 1000 Ci/mmol) and Man (α-[2-3H](N)), 21 Ci/mmol) were from Perkin Elmer-Cetus (Waltham, MA). Protein A-Sepharose was from Repligen (Needham, MA). Lactacystin (Lac) and kifunensine (Kif) were from Cayman Chemicals (Ann Arbor, MI). N-carbobenzoxy-leucinyl-leucinyl-leucinal (MG-132) and proteasome inhibitors (R610, 200 nM; MG132, 20 μM) were from Calbiochem (La Jolla, CA). Protein A-Sepharose was from Amersham Biosciences (Piscataway, NJ). Protein A-Sepharose was from Clontech (Palo Alto, CA). Diamidophenylalanine (Dpa) was used for aliquots of the RT product was used for PCR with the following primers: CAATGAAGGAGAAGGAGAC and CAATGTGTCGCTGAGTCCTG for GAPDH, and TCTGCTGAGTCCTGAGCAG and GAAAGGGAGCCTGGTAAAGGAAC for spliced XBP1.

Antibodies
Rabbit polyclonal anti-H2 carboxy-terminal and anti-H2 amino-terminal antibodies were the ones used in earlier studies (Tolchinsky et al., 1996; Shenkman et al., 2000). R9, anti-C terminal polyclonal was used before (Frenkel et al., 2008). Rabbit polyclonal anti-EDEM1 and anti-OS9 were from Sigma and anti–mouse IgG and anti–rabbit IgG conjugated to HRP were from Jackson Labs (West Grove, PA).

Cell culture and transfections
Human embryonic kidney (HEK) 293 cells were grown in DMEM plus 10% fetal calf serum (FCS) and NIH 3T3 cells in DMEM plus 10% newborn calf serum. All cells were grown at 37°C under an atmosphere of 5% CO2.

Transient transfection of NIH 3T3 cells was performed using the FuGene6 reagent (Roche, Basel, Switzerland) according to the kit protocol or using an MP-100 Microporator (Digital Bio Tech, Seoul, Korea). ReddyMix (ABGene, Epsom, UK) was used for PCR. Reverse transcription (RT) was performed with a VersoTM cDNA kit (Thermo Fisher Scientific, Barrington, IL), using a mixture of random hexamer and anchored oligo-dT primers. An aliquot (10%) of the RT product was used for PCR with the following primers: CAATGAAGGAGAAGGAGAC and CAATGTGTCGCTGAGTCCTG for GAPDH, and TCTGCTGAGTCCTGAGCAG and GAAAGGGAGCCTGGTAAAGGAAC for spliced XBP1.
Subconfluent (90%) cell monolayers in 60-mm dishes were labeled with $[^{35}\text{S}]\text{Cys}$, lysed, and immunoprecipitated with anti-H2.

**FIGURE 7:** EDEM1 concentrates at the ERQC and is required for substrate accumulation at the ERQC. (A) NIH 3T3 cells were transiently transfected with a vector encoding H2aRFP. One day posttransfection, cells were incubated for 3 h without (top panels) or with (bottom panels) 25 μM lactacystin (Lac), fixed, permeabilized, reacted with rabbit anti-EDEM1 antibody and Cy2-conjugated goat anti–rabbit IgG, and visualized on an LSM confocal microscope.

Representative optical slices were selected. Overlap of Cy2 with RFP appears yellow. Scale bar: 10 μm. (B) Similar to (A), except that cells were cotransfected with vectors encoding H2aRFP and EDEM1-HA and stained with mouse anti-HA and fluorescein isothiocyanate (FITC)-conjugated goat anti–mouse IgG. (C) Similar to (B) but with cells transfected with vectors encoding S-OS9.1/2 instead of EDEM1-HA and reacted with rabbit anti-OS9 and Cy2-conjugated goat anti–rabbit IgG. (D) HEK 293 cells were transfected with an H2aRFP-encoding vector together with either a control pSUPER-retro-GFP or the same vector encoding anti-EDEM1 shRNA in addition to GFP. One day posttransfection, cells were incubated for 3 h with or without 10 μM Lac, fixed, and visualized in a fluorescence microscope. (E) The bar graph shows the percent of cells with H2aRFP accumulation in the ERQC (juxtanuclear concentration) relative to the total number of cells with GFP signal under each condition, (averages of 30 cells from three independent experiments similar to the one in (D) are presented; error bars are SDs). (F) In parallel, HEK 293 cells were transfected with either pSUPER-retro-GFP or the same vector encoding anti-EDEM1 shRNA in addition to GFP; RNA was extracted 24 h posttransfection and used for RT-PCR with primers for EDEM1 mRNA compared with GAPDH.
antibodies, as described previously (Tolchinsky et al., 1996; Shenkman et al., 1997). Labeling of CD38 with [35S]Met mix was done as described before (Frenkel et al., 2003). Kif (100 μM) was added to the cells after the pulse labeling, except where indicated. SDS–PAGE was performed on 10% or 12% acrylamide gels. The gels were analyzed by fluorography using 20% 2,5-diphenyloxazole and were exposed to Biomax MS film using a transcreen-LE from Kodak (Vancouver, British Columbia, Canada). Quantitation was performed in a Fujifilm FLA 5100 phosphor imager (Tokyo, Japan). Relative protein synthesis levels were measured by analyzing [35S]Cys incorporation in trichloroacetic acid precipitates of aliquots from labeled lysates, as described before (Shenkman et al., 2007a).

[2−3H]Man labeling and analysis of N-linked oligosaccharides
Subconfluent (90%) monolayers of cells in 100-mm tissue culture dishes were metabolically labeled for 60 min with 350 μCi/ml of [2−3H]Man, as described previously (Frenkel et al., 2003; Avezov et al., 2008). Cell lysis and immunoprecipitation were performed as for the [35S]-labeled samples. Endo-β-N-acetylglucosaminidase H treatment, high mannos N-linked oligosaccharide isolation, and separation by HPLC were as described before (Frenkel et al., 2003; Avezov et al., 2010) at a flow rate of 1 ml/min in acetonitrile/1% phosphoric acid (60/40 vol/vol ratio); fractions were monitored using a scintillation counter (Beckman Coulter, Brea, CA).

Coimmunoprecipitation and immunoblotting
Cell lysis was done in 1% NP-40, 50 mM Tris/HCl (pH 8), 150 mM NaCl, protease inhibitor cocktail (Roche) for 30 min on ice, and debris and nuclei were pelleted in a microfuge for 30 min at 4°C. The samples were immunoprecipitated with anti-H2a carboxy-terminal antibody and protein A-Sepharose. After overnight precipitation, the beads were washed three times with lysis buffer (diluted 1:5), which was followed by elution of the bound proteins by boiling with sample buffer containing β-mercaptoethanol at 100°C for 5 min.

Immunoblotting and detection by ECL were done as described previously (Kamhi-Nesher et al., 2001), except for exposure and quantitation in a Bio-Rad ChemiDocXRS Imaging System (Bio-Rad, Hercules, CA).

Immunofluorescence microscopy
The procedures used were as described previously (Kamhi-Nesher et al., 2001; Avezov et al., 2008). Treatment with Lac (25 μM) of cells on coverslips was done at 37°C in a CO2 incubator for 3–5 h. Confocal microscopy was done on a Zeiss laser-scanning confocal microscope (LSM 510; Carl Zeiss, Jena, Germany) as described before (Avezov et al., 2008). For epifluorescence, digital photography was done on a Leica DMRB fluorescence microscope.

ACKNOWLEDGMENTS
We are grateful to R. Kopito and K. Nagata for reagents. The work was supported by grants from the Israel Science Foundation (1229/07) and German–Israeli Project Cooperation (DIP).

REFERENCES
Aebi M, Bernasconi R, Clerc S, Molinari M (2010). N-glycan structures: recognition and processing in the ER. Trends Biochem Sci 35, 74–82.
Avezov E, Frenkel Z, Ehrlich M, Herscovics A, Lederkremer GZ (2008). Endoplasmic reticulum (ER) mannosidase I is compartmentalized and required for Nglycan trimming to Man5-6GlcNAc2 in glycoprotein ER-associated degradation. Mol Biol Cell 19, 216–225.
Avezov E, Ron E, Izenshtein Y, Adan Y, Lederkremer GZ (2010). Pulse-chase analysis of N-linked sugar chains from glycoproteins in mammalian cells. J Vis Exp 38, 1899–1903.
Ayalon-Soffer M, Shenkman M, Lederkremer GZ (1999). Differential role of mannos and glucose trimming in the ER degradation of asialoglycoprotein receptor subunits. J Cell Sci 112, 3309–3318.
Bernasconi R, Molinari M (2011). ERAD and ERAD tuning: disposal of cargo and of ERAD regulators from the mammalian ER. Curr Opin Cell Biol 23, 176–183.
Bernasconi R, Pertel T, Luban J, Molinari M (2008). A dual task for the Xbp1-responsive OS-9 variants in the mammalian endoplasmic reticulum: inhibiting secretion of misfolded protein conformers and enhancing their disposal. J Biol Chem 283, 16446–16454.
Brummelkamp TR, Bernards R, Agami R (2002). A system for stable expression of short interfering RNAs in mammalian cells. Science 296, 550–553.
Cali T, Galli C, Olivari S, Molinari M (2008). Segregation and rapid turnover of ERAD1 by an autophagy-like mechanism modulates standard ERAD and folding activities. Biochem Biophys Res Commun 371, 405–410.
Christianson JC, Shaler TA, Tyler RE, Kopito RR (2008). OS-9 and GRP94 deliver the mannose-6-phosphate receptor subunits. Mol Biol Cell 19, 14422–14427.
Clerc S, Hirsch C, Oggier DM, Deprez P, Jakob C, Sommer T, Aebi M (2009). A dual task for the Xbp1-responsive OS-9 variants in the mammalian endoplasmic reticulum: inhibiting secretion of misfolded protein conformers and enhancing their disposal. J Biol Chem 283, 16446–16454.
Cormier JH, Tamura T, Sunryd JC, Hebert DN (2009). EDEM1 recognition and delivery of misfolded proteins to the SEL1L-containing ERAD complex. Mol Cell 34, 627–633.
Cali T, Galli C, Olivari S, Molinari M (2008). Segregation and rapid turnover of ERAD1 by an autophagy-like mechanism modulates standard ERAD and folding activities. Biochem Biophys Res Commun 371, 405–410.
Clerc S, Hirsch C, Oggier DM, Deprez P, Jakob C, Sommer T, Aebi M (2009). A dual task for the Xbp1-responsive OS-9 variants in the mammalian endoplasmic reticulum: inhibiting secretion of misfolded protein conformers and enhancing their disposal. J Biol Chem 283, 16446–16454.
Coomansky B, Bernards R, Agami R (2002). A system for stable expression of short interfering RNAs in mammalian cells. Science 296, 550–553.
Cali T, Galli C, Olivari S, Molinari M (2008). Segregation and rapid turnover of ERAD1 by an autophagy-like mechanism modulates standard ERAD and folding activities. Biochem Biophys Res Commun 371, 405–410.
Clerc S, Hirsch C, Oggier DM, Deprez P, Jakob C, Sommer T, Aebi M (2009). A dual task for the Xbp1-responsive OS-9 variants in the mammalian endoplasmic reticulum: inhibiting secretion of misfolded protein conformers and enhancing their disposal. J Biol Chem 283, 16446–16454.
Coomansky B, Bernards R, Agami R (2002). A system for stable expression of short interfering RNAs in mammalian cells. Science 296, 550–553.
Cali T, Galli C, Olivari S, Molinari M (2008). Segregation and rapid turnover of ERAD1 by an autophagy-like mechanism modulates standard ERAD and folding activities. Biochem Biophys Res Commun 371, 405–410.
Frenkel Z, Gregory W, Kornfeld S, Lederkremer GZ (2003). Endoplasmic reticulum-associated degradation of mammalian glycoproteins involves sugar chain trimming to Man6-5GlcNAc2. J Biol Chem 278, 34119–34124.

Gauss R, Kanehara K, Carvalho P, Ng DT, Aebi M (2011). A complex of Pdi1p and the mannosidase Htm1p initiates clearance of unfolded glycoproteins from the endoplasmic reticulum. Mol Cell 42, 782–793.

Groismann B, Avezov E, Lederkremer GZ (2006). The Eδ ubiquitin ligases HRD1 and SCP7βz recognize the protein moiety and sugar chains respectively of an ER-associated degradation substrate. Isr J Chem 46, 189–196.

Groisman B, Shenkman M, Ron E, Lederkremer GZ (2011). Mannose trimming is required for delivery of a glycoprotein from EDEM1 to XTP3-B and to late endoplasmic reticulum-associated degradation steps. J Biol Chem 286, 1292–1300.

Hebert DN, Bernasconi R, Molinari M (2010). ERAD substrates: which way out? Semin Cell Dev Biol 21, 526–532.

Hosokawa N, Kamiya Y, Kato K (2010a). The role of MRN domain-containing lectins in ERAD. Glycobiology 20, 651–660.

Hosokawa N, Tremblay LO, Sleno B, Kamiya Y, Wada I, Nagata K, Kato K, Herscovich A (2010b). EDEM1 accelerates the trimming of α1,2-linked mannose on the C branch of N-glycans. Glycobiology 20, 567–575.

Hosokawa N, Wada I, Hasegawa K, Yorihuzi T, Tremblay LO, Herscovich A, Nagata K (2001). A novel ER α-mannosidase-like protein accelerates ER-associated degradation. EMBO Rep 2, 415–422.

Hosokawa N, Wada I, Nagasawa K, Moriyama T, Okawa K, Nagata K (2008). Human XTP3-B forms an endoplasmic reticulum quality control scaffold with the HRD1-SELI1 ubiquitin ligase complex and βP. J Biol Chem 283, 20914–20924.

Hosokawa N, Wada I, Natsuka Y, Nagata K (2006). EDEM accelerates ER-associated degradation of mammalian α1,2-mannosidases. Biochem Biophys Res Commun 332, 626–632.

Hosokawa N, Wada I, Nagata K, Yorihuzi T, Isogai T, Aebi M (2011). A complex of Htm1p, a mannosidase-like protein, is involved in glycoprotein degradation in yeast. EMBO Rep 12, 423–430.

Kamhi-Nesher S, Shenkman M, Tolchinsky S, Fromm SV, Ehrlich R, Weissman JS (2000). Masking of an endoplasmic reticulum retention signal by its presence in the two subunits of the asialoglycoprotein receptor. J Biol Chem 275, 2845–2851.

Shenkman M, Tolchinsky S, Kondratyev M, Lederkremer GZ (2007a). ER-associated degradation. Mol Cell 28, 24324–24334.

Shenkman M, Tolchinsky S, Lederkremer GZ (2007b). ER stress induces alternative nonproteasomal degradation of ER proteins but not of cytotoxic sols. Cell Stress Chaperones 12, 373–383.

Termine DJ, Moremen KW, Sifers RN (2009). The mammalian UPR boosts glycoprotein ERAD by suppressing the proteolytic downregulation of ER mannosidase I. J Cell Sci 122, 976–984.

Tirasophon W, Lee K, Callaghan B, Welihinda A, Kaufman RJ (2000). The endoribonuclease activity of mammalian IRE1 autoregulates its mRNA and is required for the unfolded protein response. Genes Dev 14, 509–516.

Shenkman M, Tolchinsky S, Lederkremer GZ (2007b). ER stress induces alternative nonproteasomal degradation of ER proteins but not of cytotoxic sols. Cell Stress Chaperones 12, 373–383.

termine DJ, Moremen KW, Sifers RN (2009). The mammalian UPR boosts glycoprotein ERAD by suppressing the proteolytic downregulation of ER mannosidase I. J Cell Sci 122, 976–984.

Tirasophon W, Lee K, Callaghan B, Welihinda A, Kaufman RJ (2000). The endoribonuclease activity of mammalian IRE1 autoregulates its mRNA and is required for the unfolded protein response. Genes Dev 14, 509–516.

Travers KJ, Patil CK, Wodicka L, Lockhart DJ, Weissman JS, Walter P (2000). Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. Cell 101, 249–258.

Ushioara R, Haseki J, Araki K, Jansen G, Thomas DY, Nagata K (2008). ERD5 is required as a disulfide reductase for degradation of misfolded proteins in the ER. Science 321, 569–572.

Wang XZ, Harding HP, Zhang Y, Jolicoeur EM, Kuroda M, Ron D (1998). Cloning of mammalian Ire1 reveals diversity in the ER stress responses. EMBO J 17, 5078–5077.

Yamaguchi D, Hu D, Matsumoto N, Yamamoto K (2010). Human XTP3-B binds to α1,α1-antritypsin variant null[α1,α1] via the C-terminal MRH domain in a glycan-dependent manner. Glycobiology 20, 348–355.

Yoshida H, Matsu T, Hosokawa N, Kaufman RJ, Nagata K, Mori K (2003). A time-dependent phase shift in the mammalian unfolded protein response. Dev Cell 4, 265–271.

Zuber C, Cormier JH, Guhl B, Santimaria R, Hebert DN, Roth J (2007). EDEM1 reveals a quality control vesicular transport pathway out of the endoplasmic reticulum not involving the COPII exit sites. Proc Natl Acad Sci USA 104, 4407–4412.