ERManI (Endoplasmic Reticulum Class I \( \alpha \)-MANnosidase) Is Required for HIV-1 Envelope Glycoprotein Degradation via Endoplasmic Reticulum-associated Protein Degradation Pathway*

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**Background:** HIV-1 envelope (Env) glycoprotein is targeted to endoplasmic reticulum (ER)-associated protein degradation (ERAD) pathway for degradation after infecting cells.

**Results:** ER class I \( \alpha \)-mannosidase (ERManI) interacts with Env and initiates this degradation process.

**Conclusion:** ERManI is essential for the Env degradation.

**Significance:** These findings define a novel endogenous and potential therapeutically applicable antiretroviral mechanism by targeting Env for degradation.

Previously, we reported that the mitochondrial translocator protein (TSPO) induces HIV-1 envelope (Env) degradation via the endoplasmic reticulum (ER)-associated protein degradation (ERAD) pathway, but the mechanism was not clear. Here we investigated how the four ER-associated glycoside hydrolase family 47 (GH47) \( \alpha \)-mannosidases, ERManI, and ER-degradation enhancing \( \alpha \)-mannosidase-like (EDEM) proteins 1, 2, and 3, are involved in the Env degradation process. Ectopic expression of these four \( \alpha \)-mannosidases uncovers that only ERManI inhibits HIV-1 Env expression in a dose-dependent manner. In addition, genetic knock-out of the ERManI gene MAN1BI using CRISPR/Cas9 technology disrupts the TSPO-mediated Env degradation. Biochemical studies show that HIV-1 Env interacts with ERManI, and between the ERManI cytoplasmic, transmembrane, luminal stem, and luminal catalytic domains, the catalytic domain plays a critical role in the Env-ERManI interaction. In addition, functional studies show that inactivation of the catalytic sites by site-directed mutagenesis disrupts the ERManI activity. These studies identify ERManI as a critical GH47 \( \alpha \)-mannosidase in the ER-associated protein degradation pathway that initiates the Env degradation and suggests that its catalytic domain and enzymatic activity play an important role in this process.

Viral Env glycoproteins bind to receptors and mediate the entry of virions into cells to initiate infection. Unlike viral structural and enzymatic proteins, Env is produced through the host secretory pathway, where Env is folded into a natural conformation in the ER and delivered to the cell surface (1). Notably, the efficiency of HIV-1 Env folding is very low: almost 85% Env proteins are retained in the ER and degraded (2–4). The degradation mechanism remained unknown until we recently demonstrated that Env is targeted to the ERAD pathway for degradation (5). ERAD is a host quality control mechanism for protein folding (6). It specifically delivers misfolded proteins to the SELIL-containing translocon pore complex on the ER membrane and elicits their retro-translocation to the cytoplasm and subsequent degradation by the ubiquitin/proteasome system.

Class I \( \alpha \)-mannosidases belong to the carbohydrate-active \( \alpha \)-enzymes (CAZy) GH47 (7), which consists of seven members: ERManI, EDEM1, EDEM2, EDEM3, and Golgi mannosidase IA, IB, and IC (8). Although the enzymatic activity of EDEM1, EDEM2, and EDEM3 has not been demonstrated in vitro, the others specifically cleave the \( \alpha \)-linked mannose residues during protein N-glycosylation. In addition, they also play an important role in the ERAD pathway.

\( \alpha \)-Glycosylation involves a number of enzymes and chaperones in the ER and requires the dedicated ERAD pathway to serve as surveillance system. When nascent glycoprotein precursors enter the ER lumen, they are covalently modified with

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\[^{3}\] The abbreviations used are: Env, envelope; ER, endoplasmic reticulum; ERAD, ER-associated protein degradation; CAZy, carbohydrate-active \( \alpha \)-enzymes; ERManI, endoplasmic reticulum class I \( \alpha \)-mannosidase; GH47, glycoside hydrolase family 47; EDEM, ER-degradation enhancing \( \alpha \)-mannosidase-like; NKR, CEM.NKR; TSPO, mitochondrial translocator protein; gRNA, guide RNA; KIF, kifunensine; CRISPR, clustered, regularly interspaced, short palindromic repeat; Cas9, CRISPR-associated-9; A3A, APOBEC3A; NHK, null Hong Kong; DPS, decapetase sequence.
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Schematic presentation of the N-linked core oligosaccharide structure. The core is composed of two N-acetylglucosamine (GlcNAc, blue squares), nine mannose (Man, green circles), and three glucose (Glc, red circles) residues. A, B, and C are three oligosaccharide branches. The ERManI preferred cleavage site is indicated.

pre-assembled oligosaccharides on Asn residues in a consensus Asn-X-(Ser/Thr) motif (9). The N-linked oligosaccharides contain 14 sugars consisting of 2 N-acetylglucosamine (GlcNAc), 9 mannose (Man, 4 are α,1,2-linked), and 3 terminal glucose (Glc) residues distributed on three extended Man branches A, B, and C (Fig. 1). The sequential removal of the two outermost Glc residues on branch A by glucosidases I and II allows client proteins to interact with ER chaperones calnexin and calreticulin. In conjunction with other chaperones and thiol-disulfide oxidoreductases, precursors are folded and oligomerized into native proteins. During this process, ERManI cleaves the outermost Man residue on branch B on native proteins (Fig. 1). After further removal of the last Glc residue on branch A by glucosidase II, native glycoproteins are released from calnexin/calreticulin and transported to their final destinations. Noticeably, the glycoprotein folding in the ER is error-prone. If glycoproteins display non-native conformation, they are then reglucosylated by the UDP-Glc:unfolded glycoprotein glucosyltransferase and subject to additional rounds of re- engagement with the chaperone machinery until folding is achieved. However, if proteins are terminally misfolded, accumulation of misfolded proteins activates the unfolded protein response. Misfolded proteins are then guided to the ERAD pathway for degradation.

ERManI and EDEM1 play an indispensable role in ERAD. Genetic knock-out of the ERManI gene MANIBI orthologue Mns1p and EDEM1 orthologue Htm1p in Saccharomyces cerevisiae showed a clear involvement of these two genes in this pathway (10, 11). In mammalian cells an inhibition of ERAD is achieved by inhibiting the CAZy GH47 -mannosidase activity with kifunensine or by small interfering RNA-mediated gene knockdown (12–14). In addition, both ERManI and EDEM1 accelerate misfolded glycoprotein degradation in a dose-dependent manner (13–15). It has been suggested that ERManI extracts misfolded proteins from the calnexin/calreticulin cycle (16, 17), and misfolded proteins are targeted to the ER-derived quality control compartment where ERManI is enriched (12, 18). Although ERManI prefers to cleave the outermost Man residue on branch B, it may continue to cleave the other α,1,2-linked Man residues on branches A and C under conditions of overexpression (19). Thus, ERManI and possibly the EDEM proteins may catalyze more extensive demannosylation, which constitutes a signal of protein misfolding, resulting in misfolded proteins being degraded via ERAD.

Recently, we reported that the mitochondrial translocator protein TSPO induces HIV-1 Env glycoprotein degradation via ERAD in the human CD4+ T cell line CEM.NKR (NKR), resulting in a potent HIV-1 restriction (5). TSPO associates with the mitochondrial permeability transition pore complex by interacting with one of its component, the voltage-dependent anion channel protein (20). Mitochondrial permeability transition pore establishes the mitochondrial transmembrane potential (∆Ψm), which allows carrier proteins to exchange small molecules between the mitochondrial matrix and cytoplasm for energy production and controls the integrity of the mitochondrial membrane (21). The goal of this study was to elucidate how HIV-1 Env is degraded via the ERAD pathway, and we identified ERManI as a critical initiator for the Env degradation, resulting in inhibition of HIV-1 replication.

Experimental Procedures

Chemicals and Antibodies—Kifunensine, tunicamycin, anti-HA antibodies, anti-FLAG M2 antibodies, and anti-FLAG M2-agarose beads were purchased from Sigma. Lactacystin and anti-actin antibodies were purchased from Santa Cruz Biotechnology. The enhanced chemiluminescence detection kit was purchased from Amersham Bioscience. Monoclonal anti-glycolaldehyde-3-phosphate dehydrogenase antibodies were purchased from Meridian Life Science. Goat anti-human TSPO antibodies and monoclonal anti-MAN1B1 antibodies (3C2) were purchased from Novus. HIV-1 proteins were detected by antibodies from the NIH AIDS Research and Reference Reagent Program, and their catalogue numbers are 1513 (HIV-1 Gag), 526 (HIV-1 gp41), and 521 (HIV-1 gp120). Horseradish peroxidase-conjugated anti-rabbit, -goat, or -mouse immunoglobulin G second antibodies were purchased from Pierce.

Cell Lines—The human 293T cell line was purchased from ATCC. The human CEM-T4 T cell line and HIV-1 luciferase reporter GHOST cells were obtained from the NIH AIDS Research and Reference Reagent Program. The TSPO-KO 293T cell line A3 was reported before (5). The human CEM.NKR T cell line subclones N2-NP and N5-P were described before (22). CEM-T4, N2-NP, and N5-P cells were cultured in RPMI 1640 with 10% fetal bovine serum (HyClone). 293T and GHOST cells were cultured in DMEM with 10% bovine calf serum (HyClone).

Plasmids—The HIV-1 proviral vector pNL4-3 was obtained from the NIH AIDS Research and Reference Reagent Program. The HIV-1 luciferase reporter proviral vector pNL-Luc and the pcDNA3.1-TSPO-V5-His vector were described before (22, 23). Mammalian vectors expressing human ERManI, murine (m) EDEM1, mEDEM2, and mEDEM3 fused with a C-terminal HA tag were kindly provided by the Hosokawa and the Suzuki...
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laboratories. pCMV6-Entry vectors expressing human EDEM1, EDEM2, and EDEM3 with a C-terminal FLAG tag were purchased from OriGene. Vectors expressing human ERManI C-terminal deletion mutants FL-1–240 and FL1–240/ΔDPS were provided by the Sifers laboratory. The full-length human ERManI cDNA was subcloned into the pcDNA3.1 vector by replacing the APOBEC3G cDNA in the pcDNA3.1-A3G-HA-FLAG vector that expresses an in-frame C-terminal tandem arrayed HA-FLAG tag after HindIII/NotI digestion. The human ERManI single-point mutants E330A, R334C, E397K, D463A, C527A, C556A, and E599A were directly created in the pcDNA3.1-ERManI-HA-FLAG vector using QuikChange II Site-Directed Mutagenesis kit (Agilent Technologies). The pcDNA3.3-TOPO vector expressing human codon-optimized Cas9 was obtained from the Church laboratories. The pcDNA3.1-ERManI-HA-FLAG vector using QuickChange II Site-Directed Mutagenesis kit (Agilent Technologies). The pcDNA3.3-TOPO vector expressing human codon-optimized Cas9 was obtained from the Church laboratory through Addgene (24). To express MANIBI guide RNA (gRNA; see Fig. 5A), a 455-bp gBlock that contained the U6 promoter, 19-bp gRNA, gRNA scaffold, and termination signal sequences was ordered from Integrated DNA Technologies (IDT) and cloned into the pGEM-T Easy vector (Promega) after PCR amplification, according to the Church laboratory protocol (24).

Analysis of HIV-1 Infectivity—HIV-1 particles were produced from 293T cells after transfection with pNL-Luc and an ERManI expression vector. After being normalized by p24Gag ELISA, equal amounts of viruses were used to infect HEK293 cells. After 48 h of infection, cells were lysed, and viral infectivity was determined by measuring the cellular luciferase activity using a firefly luciferase reporter assay kit from Promega.

Immunoprecipitation—To determine ERManI and HIV-1 Env interaction, 293T cells were transfected with the HIV-1 proviral vector pNL4-3 and ERManI expression vectors that have a FLAG tag. After 48 h, cells were lysed with a buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA). The cytosolic fraction was rocked with anti-FLAG M2-agarose beads for 4 h at 4 °C. After extensive washing with phosphate-buffered saline, bead-associated proteins were detected by Western blotting.

Knock-out of MANIBI in 293T Cells by CRISPR/Cas9—A detailed protocol was described before (5). Briefly, 293T cells were transfected with a Cas9 expression vector and a MANIBI gRNA expression vector, and cloned by limiting dilution. Clones were screened for ERManI expression by Western blotting, and ERManI knock-out (KO) clones were identified. The MANIBI locus in these KO clones was further analyzed by PCR using ERManI-k0-S and ERManI-k0-A as a primer pair (see Fig. 5A), and sequenced. A verified MANIBI-KO clone E7 was finally identified.

Quantitation of Protein and DNA Levels—Images from Western blots were quantitated using the ImageJ program. Protein expression levels were calculated and presented as relative values.

Results

TSPO Triggers Env Degradation via ERAD in the Human T Cell Line NKR—The human CD4+ T cell line NKR is non-permissive for HIV-1 replication due to TSPO overexpression, which causes rapid Env turnover by ERAD (5). This is further demonstrated in its permissive clone N5-P and non-permissive clone N2-NP, which were obtained by limiting dilution of NKR cells (22). N2-NP cells expressed significantly higher TSPO levels than N5-P (Fig. 2A), resulting in ~8-fold more TSPO expression (Fig. 2B). After HIV-1 infection, levels of Env expression were much lower in N2-NP cells than in N5-P cells (Fig. 2C), resulting in ~10-fold Env reduction (Fig. 2D). In addition, treatment of these infected cells with an ERAD inhibitor kifunensine (KIF) significantly increased the Env expression in N2-NP cells (Fig. 2, C and D); KIF also increased HIV-1 replication in N2-NP cells but not in N5-P cells (Fig. 2E). These results suggest that Env is degraded via ERAD, which is responsible for HIV-1 inhibition in N2-NP cells.

TSPO Triggers Env Degradation via ERAD in 293T Cells—To explore the mechanism of HIV-1 Env degradation by ERAD, the endogenous TSPO activity was further investigated in 293T cells. A3 is a clonal 293T cell line where the TSPO gene was knocked out by the advantageous “clustered, regularly interspaced, short palindromic repeat (CRISPR)/CRISPR-associated-9 (Cas9)” technology (5). When HIV-1 protein expression was compared in A3 and the wild-type (WT) 293T cells after transfection with the HIV-1 proviral vector pNL4-3, similar levels of Gag (p24, p55) were detected in both cell lines, but much more Env (gp41, gp160) proteins were detected in A3 than WT cells (Fig. 3A, lanes 1 and 10). In fact, a 4–8-fold higher Env expression was detected in A3 cells than in WT 293T cells after comparing serially diluted samples (Fig. 3A, lanes 3, 4, 10). Next these HIV-transfected cells were treated with increasing amounts of KIF, and the Env expression was determined. It was found that the Env expression was increased in a dose-dependent manner in WT cells (Fig. 3B). When levels of the increase were quantified, a maximal 4-fold increase was detected, which almost reached the Env expression levels in A3 cells (Fig. 3C). The same treatment did not increase the Env expression in A3 cells or the Gag expression in both A3 and WT cells (Fig. 3, B and C). These results further confirmed the TSPO activity in 293T cells.

Identification of ERManI from the ERAD Pathway That Inhibits HIV-1 Env Expression—KIF is an alkaloid that specifically inhibits CAZy GH47 α-mannosidases (25). Results that KIF rescues HIV-1 Env expression in both N2-NP and 293T cells suggest that these enzymes are involved in the Env degradation. Among the seven CAZy GH47 α-mannosidases, ERManI, EDEM1, EDEM2, and EDEM3 have been found to play a role in ERAD. To understand how they are involved in HIV-1 Env degradation, 293T cells were transfected with HIV-1 proviral vector pNL4-3 plus a human ERManI, murine (m) EDEM1, mEDEM2, or mEDEM3 expression vector or a human APOBEC3A (A3A) expression vector, which served as a control. After 48 h of transfection, protein expression was determined by Western blotting. It was found that although all these enzymes were expressed, only ERManI was able to inhibit the Env gp120 and gp41 expression (Fig. 4A, lane 4). Human EDEM proteins share an overall ~90% amino acid sequence identity with their murine orthologues (26). To confirm the lack of inhibitory activity of these EDEM proteins, human EDEM proteins were ectopically expressed with HIV-1 in 293T cells, and the Env expression was determined. The A3A protein was also used as a control in this experiment. Again, like their
murine orthologues and the A3A protein, these human EDEM proteins did not show any inhibitory effect on HIV-1 Env expression (Fig. 4, lanes 1, 2, and 3).

To verify the ERManI activity, 293T cells were transfected with fixed amounts of pNL4-3 and serially diluted ERManI expression vector, and levels of Env expression were deter-
mIned. It was found that ERManI could inhibit HIV-1 Env expression in a dose-dependent manner, suggesting that the Env inhibition is indeed caused by ERManI (Fig. 4C). In addition, these transfected cells were treated with KIF and a proteasomal inhibitor lactacystin. Both KIF and lactacystin were previously found to block the ERManI-mediated degradation of misfolded human α1-antitrypsin (A1AT) genetic variant-null Hong Kong (NHK) (13, 14). As expected, both KIF and lactacystin also rescued the HIV-1 Env expression (Fig. 4D). Moreover, the ERManI activity was further evaluated in a HIV-1 replication assay. HIV-1 reporter viruses were produced from 293T cells after ectopic expression of WT ERManI or its catalytically inactive mutant E330A (see below). After normalization of viral production by the Gag protein levels, equal amounts of HIV-1 were used to infect the GHOST cells, and viral infectivity was determined. It was found that unlike the E330A mutant, WT ERManI significantly reduced the HIV-1 infectivity (Fig. 4E). Taken together, these experiments identified ERManI as a potent CAZy GH47 α-mannosidase that strongly inhibits HIV-1 Env expression via the ERAD pathway.

**Knock-out of ERManI Disrupts TSPO Activity**—To demonstrate the role of ERManI in TSPO-induced Env degradation, the ERManI gene MAN1B1 was knocked out in 293T cells using the CRISPR/Cas9 technology (24, 27). MAN1B1 is located on human chromosome 9, which has 13 exons. A specific 19-nucleotide gRNA was designed to target the exon 4 and inactivate this gene (Fig. 5A). A clone E7, which did not show any ERManI expression, was identified by Western blotting (Fig. 5B). When an 83-bp DNA fragment was amplified from the targeted locus in E7 cells by PCR, a small deletion was identified (Fig. 5C). After cloning and sequencing the DNA fragment, a 5-bp deletion was found (Fig. 5A). These results demonstrate that MAN1B1 is successfully knocked out in these E7 cells.

Next, HIV-1 protein expression was compared in E7 and WT 293T cells after ectopic expression of TSPO. Cells were trans-
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A schematic illustration of MAN1B1. Numbers indicate the nucleotide or amino acid positions in the ERManI open reading frame. The intron 3–4 sequence is shown in lowercase, and the exon 4 sequence is shown in uppercase. The 19-bp gRNA target sequence is shown in green, and the protospacer-adjacent motif (PAM) is shown in red. The sense primer ERMani-ko-S and antisense primer ERMani-ko-A sequences were used to amplify this gene locus are underlined. A 5-bp deletion detected in MAN1B1-KO cells is boxed.

B

C

D

E

F

FIGURE 5. Role of the endogenous ERManI protein in the TSPO inhibitory activity. A, schematic illustration of \( MAN1B1 \). Numbers indicate the nucleotide or amino acid positions in the ERManI open reading frame. The intron 3–4 sequence is shown in lowercase, and the exon 4 sequence is shown in uppercase. The 19-bp gRNA target sequence is shown in green, and the protospacer-adjacent motif (PAM) is shown in red. The sense primer ERMani-ko-S and antisense primer ERMani-ko-A sequences were used to amplify this gene locus are underlined. A 5-bp deletion detected in MAN1B1-KO cells is boxed. B, analysis of the endogenous ERManI protein expression in three 293T clones (B4, E7, F7) isolated after transfection with Cas9 and MAN1B1 gRNA expression vectors by Western blotting. C, analysis of the \( MAN1B1 \) gene locus by PCR. An 83-bp DNA fragment was PCR-amplified from the \( MAN1B1 \) locus using primers ERMani-ko-S and ERMani-ko-A and analyzed by 10% TBE-polyacrylamide gel. M, marker. D and E, influence of \( MAN1B1 \) KO on HIV-1 Env inhibition. WT and E7 cells were transfected with indicated amounts of HIV-1 proviral vector pNL4-3 and TSPO expression vector in the absence (D) or presence (E) of an ERManI expression vector. Viral and cellular protein expressions were analyzed by Western blotting. F, quantification of the Env expression in D and E. The levels of HIV-1 gp120 expression in untransfected cells were set up as 100%, and the others were normalized and are presented as relative values. Error bars represent S.E. from three independent experiments.
whereas A3A, FL-1–240, or FL-1–240/ΔDPS could not (Fig. 6D). These results demonstrate that ERManI interacts with Env and suggest the luminal catalytic domain is involved in this interaction.

Discussion

In this report we studied the molecular mechanism of TSPO-induced HIV-1 Env degradation via ERAD and identified ERManI as a critical initiator for the degradation. Env is expressed through the classical secretory pathway, in which it needs to be properly folded in the ER (1). The Env folding involves cross-linking of 20 cysteine residues, which is dependent on heavy N-glycosylation and the most oxidizing redox status in the ER (31). It has been suggested that the oxidative protein folding in the ER is controlled by mitochondria, likely via regulating the ER redox status through releasing reactive oxygen species (32). Intracellular reactive oxygen species is mainly produced by mitochondria as a byproduct from energy production. Indeed, ER contains a specialized subcompartment that is called the mitochondrial-associated ER membrane, which physically connects ER to mitochondria (33). In mammalian cells, mitochondrial-associated ER membrane is supported by a protein complex consisting of voltage-dependent anion channel and several other proteins (34). As introduced earlier, TSPO is a mitochondrial protein (35) that interacts with voltage-dependent anion channel (20). We speculate that TSPO overexpression reduces the oxidative redox status in the ER, likely by blocking the mitochondria-ER communication, to interfere with HIV-1 Env folding. Accumulation of misfolded Env then activates unfolded protein response, resulting in recognition of these misfolded Env proteins by ERManI and their degradation via ERAD.
We found that the catalytic domain of ERManI plays an indispensable role in inhibition of HIV-1 Env expression. The structure of this domain shows an (ααααα)-barrel composed of 14 consecutive helices, and Glu-330, Asp-463, and Glu-599 were proposed as potential catalytic residues (36). Mutations of Glu-330, Asp-463, and Glu-599 caused 96.5%, 99.9%, or ~100% reduction in enzyme efficiency ($k_{cat}/K_m$), respectively (37). In addition, ERManI has two highly conserved cysteine residues Cys-527 and Cys-556, which are also conserved in three other Golgi CAZy GH47 α1,2-mannosidases, IA, IB, and IC, but not in EDEM proteins (36). The formation of a disulfide bond between these residues was demonstrated in the yeast Msn1, which was proposed to stabilize the protein (38). Moreover, R334C and E397K mutations are identified in nonsyndromic autosomal-recessive intellectual disability (NS-ARID) patients (39), and the R334C mutation is also found in the congenital disorders of glycosylation (40). The E397K mutation was found to reduce the ERManI expression, and the R334C mutation was found to reduce the enzyme efficiency by ~100% (39). We created seven ERManI mutants, E330A, R334C, E397K, D463A, C527A, C556A, and E599A, to inactivate these critical residues, and found that they all lost the Env inhibitory activity (Fig. 6B). In addition, we tested the activity of two previously reported catalytic domain deletion mutants, FL-1–240 and FL-1–240/ΔDPS. Although the FL-1–240 mutant still has the activity to trigger NHK degradation, the FL-1–240/ΔDPS mutant does not (30). Nevertheless, we found that they all lost the Env inhibitory activity (Fig. 6C). Together, these results demonstrate that the catalytic activity and the catalytic domain are required for the ERManI activity. The importance of the catalytic domain was further underscored from our investigation on Env-ERManI interaction. We found that WT ERManI could pull down HIV-1 Env, whereas both FL-1–240 and FL-1–240/ΔDPS mutants could not, suggesting that ERManI interacts with Env, and this interaction is dependent on the catalytic domain (Fig. 6D). Therefore, it is likely that Env cycles between the ER and Golgi and interacts with ERManI in a post-ER compartment, resulting in Env degradation.

Results from this report point out two remarkable differences in ERAD-mediated degradation of HIV-1 Env and misfolded host glycoproteins. First, although ectopic expression of EDEM proteins is able to accelerate the degradation of NHK and/or misfolded Env and required for N-glycan trimming to Man5–6GlcNAc2 in glycoprotein ER-associated degradation. Mol. Biol. Cell. 19, 216–225

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