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Report

Regulation of the ER-Resident Mannosidase EDEM2 in HEK293 Cells

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EDEM2 plays an important role as the first enzyme that acts during mannose trimming of N-glycosylated proteins in the ERAD machinery. Although EDEM2 expression has been reported to be transcriptionally regulated by the IRE1-sXBP1 pathway, very little is known about how endogenous EDEM2 protein expression is regulated. In this work, three different ER stress inducers were used to treat HEK293 cells. Thapsigargan slightly increased both EDEM2 mRNA and protein levels in the cells. Treatment with MG132 did not increase the level of mature EDEM2 protein, and a truncated form of the protein appeared. In SEL1L-deficient cells, there was a slight increase in EDEM2 protein as well as in TXNDC11, a protein that has been reported to form disulfide bonds with EDEM2. On the other hand, EDEM2 protein level decreased in TXNDC11-deficient cells. DTT treatment decreased EDEM2 and TXNDC11 protein levels in a time-dependent manner. The decrease in EDEM2 protein after DTT treatment was attenuated by treatment of the cells with MG132 and by SEL1L deficiency. These findings demonstrate that endogenous EDEM2 protein is regulated posttranscriptionally and that it is in part an SEL1L-mediated ERAD substrate.

Key words EDEM2, ERAD, ER stress, SEL1L, TXNDC11

INTRODUCTION

Approximately one-third of newly synthesized proteins are processed within the endoplasmic reticulum (ER). Under normal conditions, correctly folded proteins are transported to their final destinations via the ER–Golgi pathway. During this process, specific asparagine residues in some proteins are labeled with $Glc_3Man_9GlcNAc_2$. Following the removal of two glucose residues by glycosidase I/II, the monoglycosylated form associates with calnexin/calreticulin; this promotes proper folding of the protein, and its terminal glucose is then released. On the other hand, misfolded proteins, even those that have passed through the calnexin/calreticulin cycle, are subjected to selective degradation.[1,2] It has been reported that several ubiquitin (Ub) ligases are embedded in the ER membrane[3] and that ER-associated degradation (ERAD) via these Ub ligases is responsible for the degradation of unfolded or misfolded proteins under normal/ER stress states and serves as a protein turnover process within the ER.[4,5] Degradation of incompletely folded N-glycosylated proteins via the SEL1L/Hrd1 complex, N-glycosylated protein recognition, retro-translocation, ubiquitination and degradation are the most studied parts of this process. During the recognition step, target N-glycosylated proteins are subjected to mannose trimming by ER mannosidase I or ER degradation-enhancing α-mannosidase-like protein (EDEM) family (EDEM1/2/3). ER mannosidase eliminates some mannose residues at the terminal of N-Glycan.[6] Following these events, lectin chaperones such as OS9 and XTP3-B recognize and bind to the 1,6-α-mannosyl linkage that is exposed after trimming of the mannose residues.[4,5,7] The target proteins, together with the lectin chaperones, are transferred to the ERAD complex and move from the ER to the cytosol in coordination with specialized proteins, including SEL1L, Hrd1 and the Derlin family. SL1L acts as a scaffold for the Hrd1-containing ERAD complex on the ER membrane, where Hrd1 ubiquitinates substrate proteins. In parallel, polyubiquitin chains are added to the target proteins by E3 Ub ligases (e.g., Hrd1 and gp78) in the same ERAD complex, resulting in discarding of the target proteins at the 26S proteasome at the degradation step.[3,4,5,8] EDEM2 is an important enzyme in ERAD; it functions at the earliest mannose trimming step, and it has recently been reported that disulfide bonding of EDEM to TXNDC11 is important for maintaining its activity.[9,10] Although many studies involving EDEM2 overexpression or knockdown have been conducted in an attempt to elucidate the ERAD mechanism,[11,12,13] little has been learned about the mechanism that regulates endogenous ERAD2 protein expression. We recently used genome editing of SEL1L and TXNDC11 in HEK293 cells to study the regulatory mechanisms of the ATF6/CREB3 family as ERAD substrates.[14,15,16] Based on these findings, in the present work we examined changes in endogenous EDEM2 expression under various conditions, including ER stress and ERAD dysfunction.

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MATERIALS AND METHODS

Materials Thapsigargin (Tg), tunicamycin (Tm), brefeldin A (BFA), cycloheximide (CHX), dithiothreitol (DTT) were obtained from Sigma–Aldrich (St. Louis, MO, USA). MG132 (MG) and concanamycin A (CMA) were obtained from Peptide Institute (Osaka, Japan) and from Wako (Osaka, Japan), respectively.

Construction of Plasmids gRNAs against human EDEM2 (5’-TTCCGGCTGCTCATCCCGCT-3’ and 5’-GCTGAGGCAGCAGCGCGCAC-3’ (EDEM2 KD)), human SEL1L (5’-GAGCTTGGCCTCGGCGTCCT-3’ (SEL1L KD#1)) and (5′-GCAGCAGCGTCAGCCCTATC-3′ (SEL1L KD#2)) and human TXNDC11 (5’-CCGGCCGCTGGCGCGCCATG-3’ and 5’-CAGCGCGCAGCCGAGCGCCA-3’ (TXNDC11 KD)) aligned with tracer RNA were inserted into a pcDNA3.1-derived vector containing a U6 promoter.[16,17] To prepare donor genes, a DNA fragment encoding the N-terminal region of human EDEM2 (107 bp from the translation start site), SEL1L (70 bp from the translation start site) or TXNDC11 (124 bp from 131 bp to 254 bp for TXNDC11 KD) was fused with a puromycin-resistance gene via IRES and inserted into a pGL3-derived vector. The hCas9 construct (#41815) used in this study was obtained from Addgene.[18]

Establishment of EDEM2-, SEL1L- and TXNDC11-Deficient HEK293 Cells EDEM2-, SEL1L- and TXNDC11-deficient HEK293 cells were established using the CRISPR/Cas9 system as described previously.[14,16,17] In brief, donor genes encoding human EDEM2, SEL1L or TXNDC11 N-terminus in a pGL3-derived vector, together with constructs for each gRNA and hCas9, were transfected into HEK293 cells, and cells were selected with the appropriate concentrations of puromycin. To establish EDEM2- and TXNDC11-deficient cells, two different constructs for guide RNAs were cotransfected together with the donor gene and hCas9 constructs.

Cell Culture and Treatment HEK293 cells were maintained in Dulbecco’s modified Eagle’s minimum essential medium containing 5% fetal bovine serum. The cells were seeded in 12-well plates and treated with Tg (0.01 μM), Tm (2 μg/mL), BFA (0.5 μg/mL), CMA (50 nM), MG132 (10 μM), CHX (20 μg/mL) or vehicle for the indicated times; the expression of the indicated genes and proteins was then measured using RT–PCR and western blot analysis.

Western Blot Analysis Cells were lysed in homogenization buffer (20 mM Tris-HCl (pH 8.0) containing 137 mM NaCl, 0.2 mM EDTA (pH 8.0), 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM PMSF, 10 μg/mL leupeptin, and 10 μg/mL pepstatin A) as described previously.[14-17] The protein concentrations of the lysates were determined using the Bradford assay, and equal amounts of lysate protein from different samples were separated by SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Burlington, MA, USA). The membranes were incubated with the indicated antibodies, and the target proteins were detected by enhanced chemiluminescence (ECL) (GE Healthcare, Buckingghamshire, UK) or enhanced chemiluminescence plus (ECL-plus) reagents (Life Technologies, Waltham, MA, USA). We used the following antibodies at the indicated dilutions: ATF6α (1:1200) (Proteintech, Rosemont, IL, USA); EDEM2 (1:1500) (Novus Biological, Centennial, CO, USA); GADD153 (1:1000) (Santa Cruz Biotech, Dallas, TX, USA); G3PDH (1:12000) (Proteintech); Herp (1:1000) (Cell Signaling Technology, Beverly, MA, USA); LC3 (1:3000) (MBL, Nagoya, Japan); OS9 (1:1000) (Cell Signaling Technology); SEL1L (1:1500) (Abcam, Cambridge, UK); and TXNDC11 (1:1200) (Abcam). The level of expression of each protein was analyzed using ImageJ software (National Institutes of Health, USA),[19] and the relative amount of each protein was calculated based on the G3PDH value obtained from an identical sample of cell lysate. Protein expression was normalized to the value obtained from untreated control cells.

Reverse Transcription-Polymerase Chain Reaction (RT–PCR) To estimate the mRNA expression level of each gene, we used RT–PCR as described previously.[14-17] Total RNA was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA). The extracted RNA was converted to cDNA by reverse transcription using a mixture containing DTT, dNTPs, random ninemers, RNaseOUT (Life Technologies), and Prime Superscript III RNase reverse transcriptase (Life Technologies) as was done previously in our laboratory.[14-17] Each cDNA sample was added to a PCR mixture containing primers, dNTPs, and Taq DNA polymerase (Taq PCR kit, Takara, Shiga, Japan) for amplification. The RT–PCR primers used in this study were as follows: EDEM2 sense primer, 5’-TGTCTGCTCATCTGCTCTCCAA-3’; EDEM2 antisense primer, 5’-CAACAATGAAGGTCCCAATCCC-3’; GADD153 sense primer, 5’-CGGAAACAGAGTGGTCATTC-3’; GADD153 antisense primer, 5’-TGCGTATGTGGGATTGAGGGTC-3’; G3PDH sense primer, 5’-ACCACAGTCCATGCCATCAC-3’; G3PDH antisense primer, 5’-TCCACCACCCTGTTGCTGTA-3’; TXNDC11 sense primer, 5’-GTGATAATACCAGCAAAGCC-3’; TXNDC11 antisense primer, 5’-TTTCTGCATTTCCCCTGGTT-3’; XBP1 sense primer, 5’-CGGCCTTGTGGTTGAGAA-3’; and XBP1 antisense primer, 5’-ACTTGTCCAGAATGCCCA-3’. The typical reaction cycling conditions were 30 sec at 96°C, 30 sec at 58°C, and 30 sec at 72°C. The reactions were terminated after 21–28 cycles of amplification, and the products were separated by electrophoresis on 1.5% agarose gels and visualized using ethidium bromide. The level of expression of each mRNA was analyzed using ImageJ software (National Institutes of Health),[19] and the relative amount of each mRNA was calculated based on the G3PDH value obtained from an identical sample of cDNA. Each mRNA expression was normalized to the value obtained from untreated control cells.

RESULTS AND DISCUSSION

EDEM2 plays an important role as the first enzyme in mannose trimming of N-glycosylated proteins in the ERAD machinery.[4,5,6] It has also been reported that transcription of the EDEM2 gene is upregulated by ER stress,[12] but few studies of endogenous EDEM2 mRNA and protein expression have been reported.[12,20,21] Thus, we first evaluated EDEM2 mRNA and protein expression in HEK293 cells after treatment with three different ER stress inducers: Tg, Tm and BFA (Fig. 1). As reported previously,[12] increases in EDEM2 mRNA were observed after stimulus with each individual ER stress inducer (Fig. 1A). TXNDC11 has been reported to form a disulfide bond with EDEM2 and to be involved in the mannosidase activity of EDEM2.[10] We recently reported that transcription of the TXNDC11 gene is regulated by the IRE1-sXBP1 pathway via the unfolded protein response element (UPRE) near the transcription start site.[16] The levels of TXNDC11, sXBP1

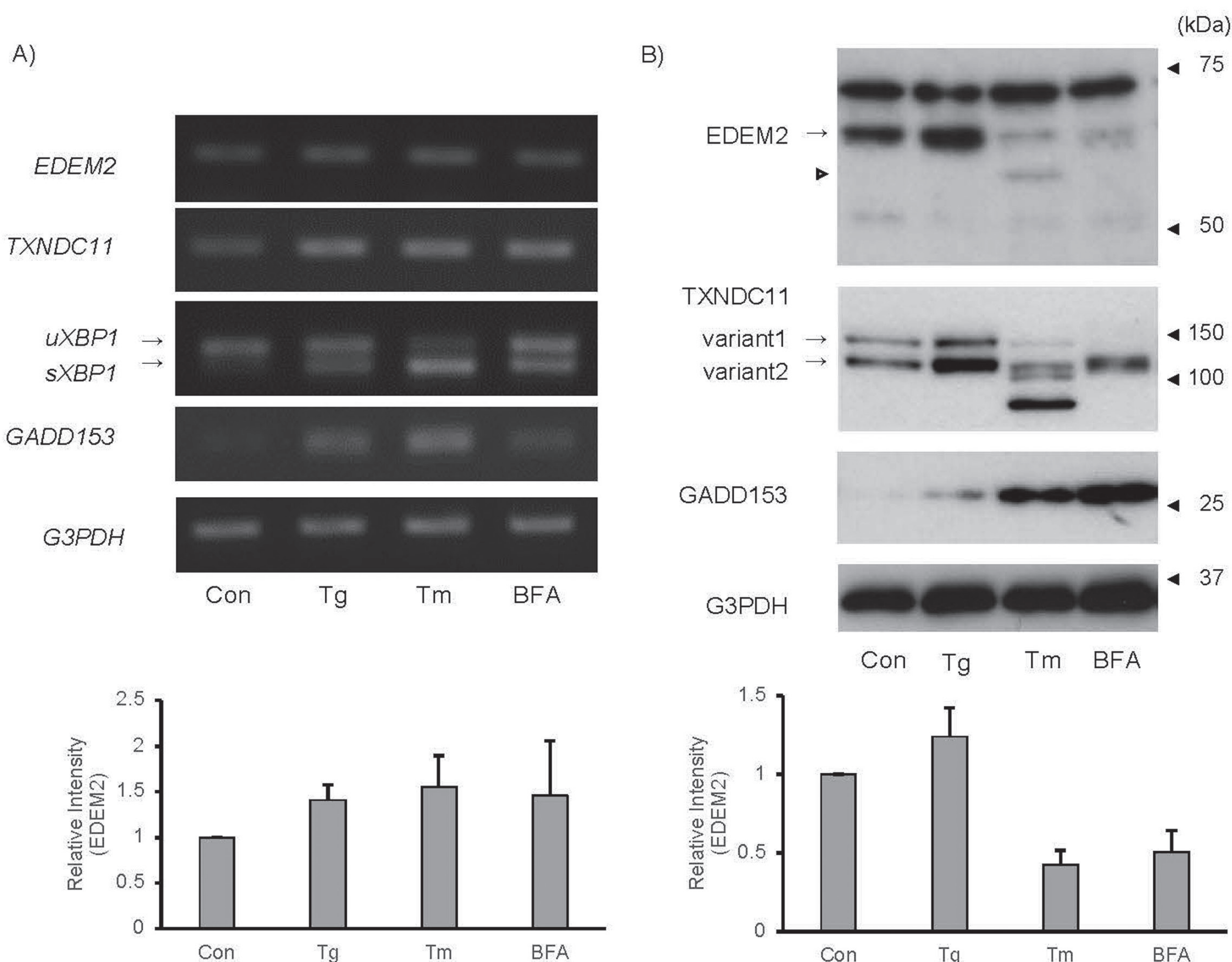

Fig. 1. Effects of ER Stress-Inducing Reagents on EDEM2 Expression in HEK293 Cells

Wild-type HEK293 cells were treated with Tg (0.01 μM), Tm (2 μg/ml), BFA (0.5 μg/ml) or vehicle (Con) for 6 h (A) or 24 h (B). The expression of the indicated mRNAs (A) and proteins (B) was measured as described in Materials and Methods. Representative results obtained for four independent cultures are shown. The amount of EDEM2 mRNA and protein in untreated cells was set at 1. An open arrowhead indicates the unglycosylated form.

and GADD153 mRNA were also increased after treatment with each individual ER stress inducer in this study. On the other hand, the effects of the individual ER stress inducers on EDEM2 protein expression were quite different. As shown in Fig. 1B, treatment of the cells with Tg for 24 h slightly induced EDEM2 protein expression in HEK293 cells. After treatment with Tm, low-molecular-weight EDEM2, which is thought to represent the unglycosylated form, was detected, and the level of expression of mature EDEM2 protein was correspondingly decreased. BFA treatment also reduced the level of mature EDEM2 protein in HEK293 cells, although the precise mechanism through which this occurred is unclear. On the other hand, the amount of TXNDC11 protein did not clearly decrease after stimulation with Tm or BFA. GADD153 mRNA and protein was respectively induced by each treatment (Fig. 1).

We then investigated the degradation and stability of the EDEM2 protein in HEK293 cells. After 6 h of treatment with the proteasome inhibitor MG132 (MG), the lysosome inhibitor concanamycin A (CMA), or the protein synthesis inhibitor cycloheximide (CHX), the level of EDEM2 mRNA was reduced by approximately 30% (Fig. 2A). These results differ from the reported effects of these agents on TXNDC11 mRNA expression;[16] the level of TXNDC11 mRNA was unchanged after treatment with MG or CMA and slightly increased after CHX treatment (Fig. 2A). On the other hand, TXNDC11 protein expression was slightly downregulated by 24 h of CHX treatment (Fig. 2B). Regarding EDEM2 protein expression, a low-molecular-weight band was observed after treatment of the cells with MG132 for 24 h (Fig. 2B). Since the bands about 50 kDa was observed in untreated EDEM2-deficient HEK293 cells (Fig. 3), it is likely that after MG132 treatment, the cleaved EDEM2 overlaps with nonspecific bands. CMA treatment increased LC3 expression but had no effect on EDEM2 protein expression (Fig. 2B). Our previous study showed that CHX treatment almost completely abolished ATF6/CREB3 family expression in HEK293 cells within 4 h,[15,16] but EDEM2 protein was reduced by about 50% even after 24 h of CHX treatment compared to that in untreated control cells (Fig. 2B). These findings suggest that the EDEM2 protein is a relatively stable protein, although it is partly subject to cleavage and degradation through a proteasome pathway. Since the anti-EDEM2 antibody used in this study recognizes the C-terminal 100 amino acid residues of human EDEM2, the low-molecular-weight EDEM2 that appeared in the MG132 treatment was deduced to be its C-terminal fragment.

We recently established HEK293 cells that are deficient in SEL1L, Herp, and TXNDC11 and analyzed the proteins pre-

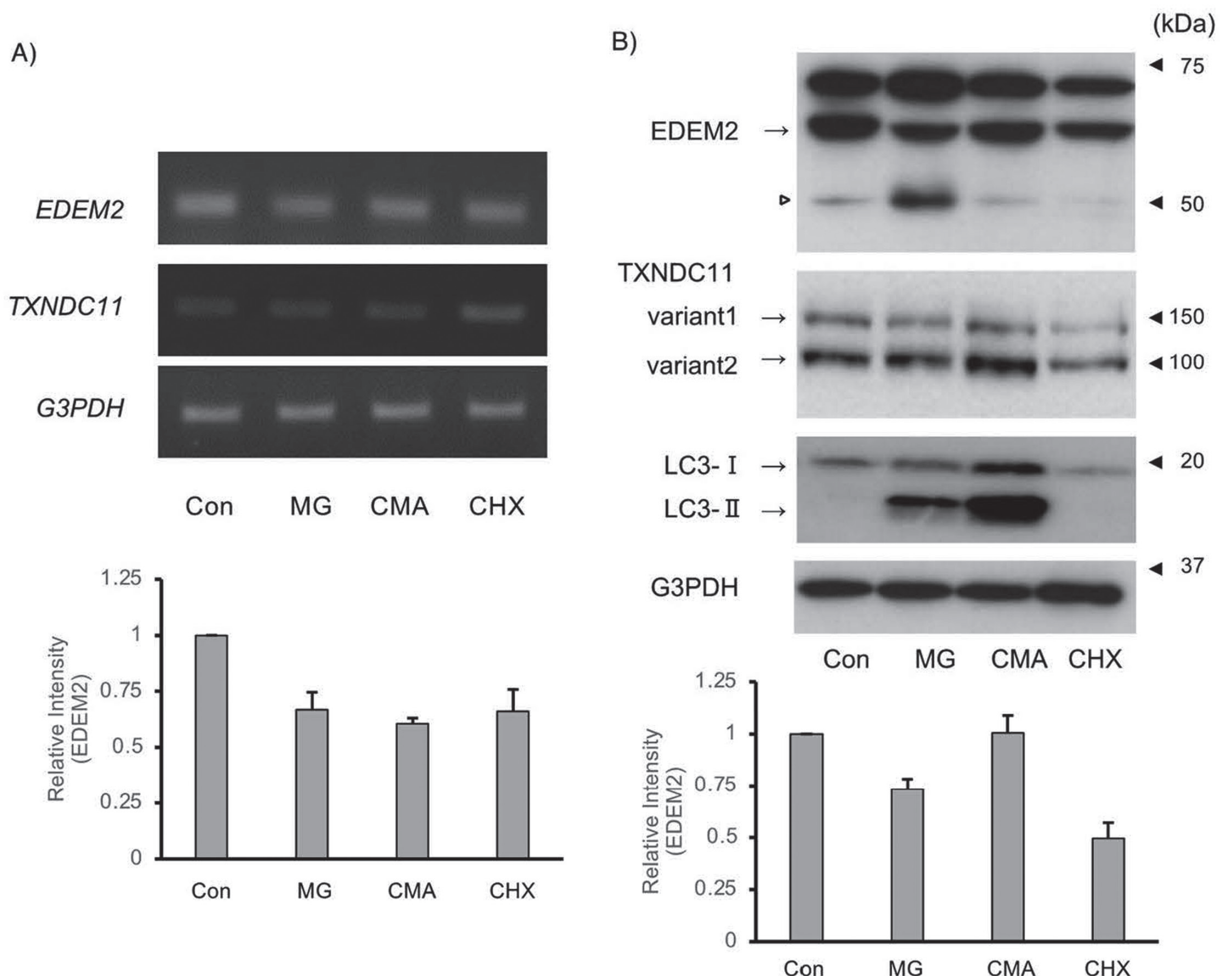

Fig. 2. Evaluation of the Stability of EDEM2 in HEK293 Cells

Wild-type HEK293 cells were treated with MG132 (MG) (10 μM), CMA (50 nM), CHX (20 μg/ml) or vehicle (Con) for 6 (A) or 24 (B) h. The expression of the indicated mRNAs (A) and proteins (B) was measured as described in Materials and Methods. Representative results obtained for four independent cultures are shown. The amount of EDEM2 mRNA and protein without treatment was set at 1. The bands indicated by an open arrowhead are considered truncated EDEM2 containing nonspecific bands.

sent in the ER of those cells, including members of the ATF6/CREB3 family.[14-16] As shown in Fig. 3, ERAD-related factors such as Herp, OS9, and TXNDC11 are highly expressed in SEL1L-deficient cells even under unstimulated conditions. Therefore, we examined changes in EDEM2 protein expression in SEL1L- and TXNDC11-deficient cells. EDEM2 protein expression was slightly increased in SEL1L-deficient cells compared to wild-type cells. In particular, EDEM2 mRNA was present in SEL1L-deficient clone #1 at levels slightly higher than those observed in the wild-type cells, but this did not occur in clone #2 (Supplementary Fig. 1A). In our previous study, the TXNDC11 protein level in SEL1L-deficient cells increased approximately 1.5-fold.[16] Both EDEM2 and TXNDC11 are thought to be regulated by the IRE1-sXBP1 pathway; however, there may be other regulatory mechanisms, such as posttranscriptional mechanisms. On the other hand, EDEM2 protein levels were markedly decreased in the TXNDC11-deficient cells (Fig. 3B) and did not correlate with the EDEM2 mRNA levels in the cells (Supplementary Fig. 1B). A decrease in the amount of EDEM2 protein was also observed in TXNDC11-deficient cells established using other gRNAs (data not shown).

George *et al.* reported that EDEM2 and TXNDC11 associate with each other via disulfide bonds.[10] Therefore, we attempted to determine whether the reducing agent DTT affects EDEM2 protein expression. As shown in Fig. 4A, treatment of wild-type HEK293 cells with 1 mM DTT decreased EDEM2 and TXNDC11 protein levels in a time-dependent manner. Since DTT, like Tg, Tm and BFA, is also used as an ER stress inducer,[22] we examined the mRNA levels of EDEM and TXNDC11 and found that both increased remarkably after DTT treatment (Supplementary Fig. 2). DTT treatment also induced GADD153 protein expression. The results suggest that DTT decreases EDEM2 protein expression in a post-transcriptional manner. Finally, we used our SEL1L-deficient cells (#2) to examine changes in EDEM2 protein levels after DTT treatment in the presence or absence of MG132 (Fig. 4B) and CMA (Fig. 4C). Interestingly, in wild-type HEK293 cells, the decrease in EDEM2 protein that normally occurred after DTT treatment was partially suppressed by MG132 treatment (Fig. 4B). On the other hand, in SEL1L-deficient cells, there was almost no decrease in EDEM2 after DTT treatment. The expression of c-Myc protein, a cytoplasmic/nuclear protein that is degraded by proteasome,[23] was markedly increased by MG132 treatment (Fig. 4B). This c-Myc expression was also decreased by DTT treatment. In addition to ERAD, a lysoso-

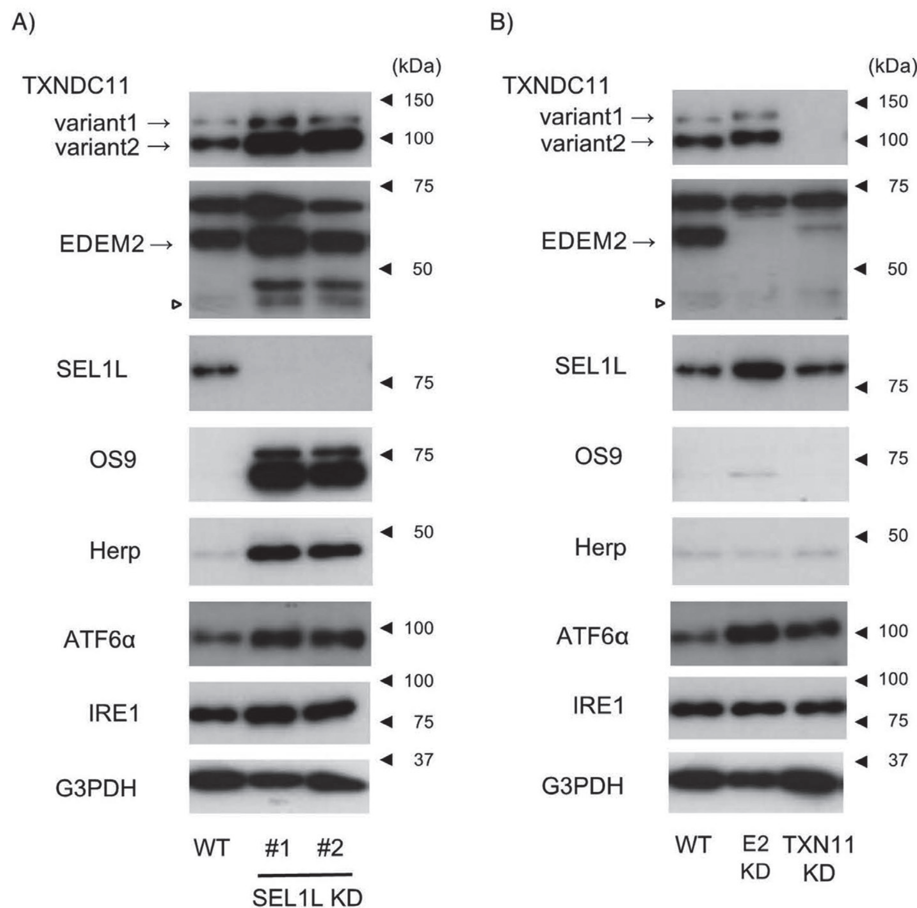

Fig. 3. Effect of SEL1L and TXNDC11 Deficiency on EDEM2 Protein Expression in HEK293 Cells

Expression of the indicated proteins in wild-type (WT), SEL1L (SEL1L KD)- (A) and in EDEM2 (E2 KD)- and TXNDC11 (TXN11 KD)-deficient HEK293 cells (B) was measured as described in Materials and Methods. Representative results obtained for 3 independent cultures are shown. The bands indicated by open arrowheads are considered truncated EDEM2 containing nonspecific bands.

mal degradation mechanism called ER-phagy also contributes to protein quality control in the ER.[24,25] However, CMA treatment did not affect EDEM2 protein expression in either wild-type or SEL1L-deficient cells although CMA treatment apparently increased LC3 expression (Fig. 4C).

EDEM2 plays an important role in mannose trimming, the first step in the degradation of N-glycosylated proteins in the ER.[4-6] This is also demonstrated by the increased expression of full-length ATF6 protein in our EDEM2-deficient HEK293 cells (Fig. 3B). The amount of full-length ATF6 protein in SEL1L- and EDEM2-deficient cells was comparable and higher than in wild-type HEK293 cells (Fig. 3), suggesting that the EDEM2-SEL1L pathway is important for the regulation of ATF6 protein within the ER. Our study and others show that ATF6 levels also increase under conditions of TXNDC11 deficiency.[10,16] On the other hand, SEL1L deficiency, but not EDEM2 deficiency, increased the levels of Herp, OS9 and TXNDC11 proteins under resting conditions (Fig. 3AB), suggesting that failure of ERAD substrates to be removed from the ER and ubiquitinated due to SEL1L deficiency has a greater impact on cells than does EDEM2 deficiency. Further studies are needed to determine the details surrounding the regulation of endogenous substrates by SEL1L and/or EDEM2 and the mechanisms by which the levels of these proteins are regulated. Interestingly, the amount of EDEM2 protein was reduced in our TXNDC11-deficient cells, and the molecular weights of EDEM2 and TXNDC11 appeared to shift slightly upward in the TXNDC11- and EDEM2-deficient cells (Fig. 3B). This indicates that the TXNDC11-EDEM2 complex affects the mannose trimming within the ER. A detailed analysis of the complex containing TXNDC11 and EDEM1/2/3 is necessary, since George *et al.* recently reported that TXNDC11 also interacts with EDEM1/3.[26]

The results of our experiments in which cells were treated with CHX indicate that the EDEM2 protein is relatively stable; although the results obtained in MG132-treated cells showed that EDEM2 is partially degraded via the proteasome after its cleavage, it is unclear where and how cleavage occurs (Fig. 2). We consider that further characterization is needed, as the truncated EDEM2 appeared to overlap with a nonspecific band in our experiments. On the other hand, DTT treatment decreased the expression of full-length EDEM2 and TXNDC11 protein in a time-dependent manner (Fig. 4A). DTT may have destabilized both proteins by affecting their disulfide bonds, but DTT treatment also reduces c-Myc protein (Fig. 4B). Therefore, the results obtained with DTT are not specific, and further studies are needed.

In this study, we used several reagents and ERAD-deficient cell lines to elucidate the posttranscriptional regulation of the EDEM2 protein. In particular, DTT treatment caused a

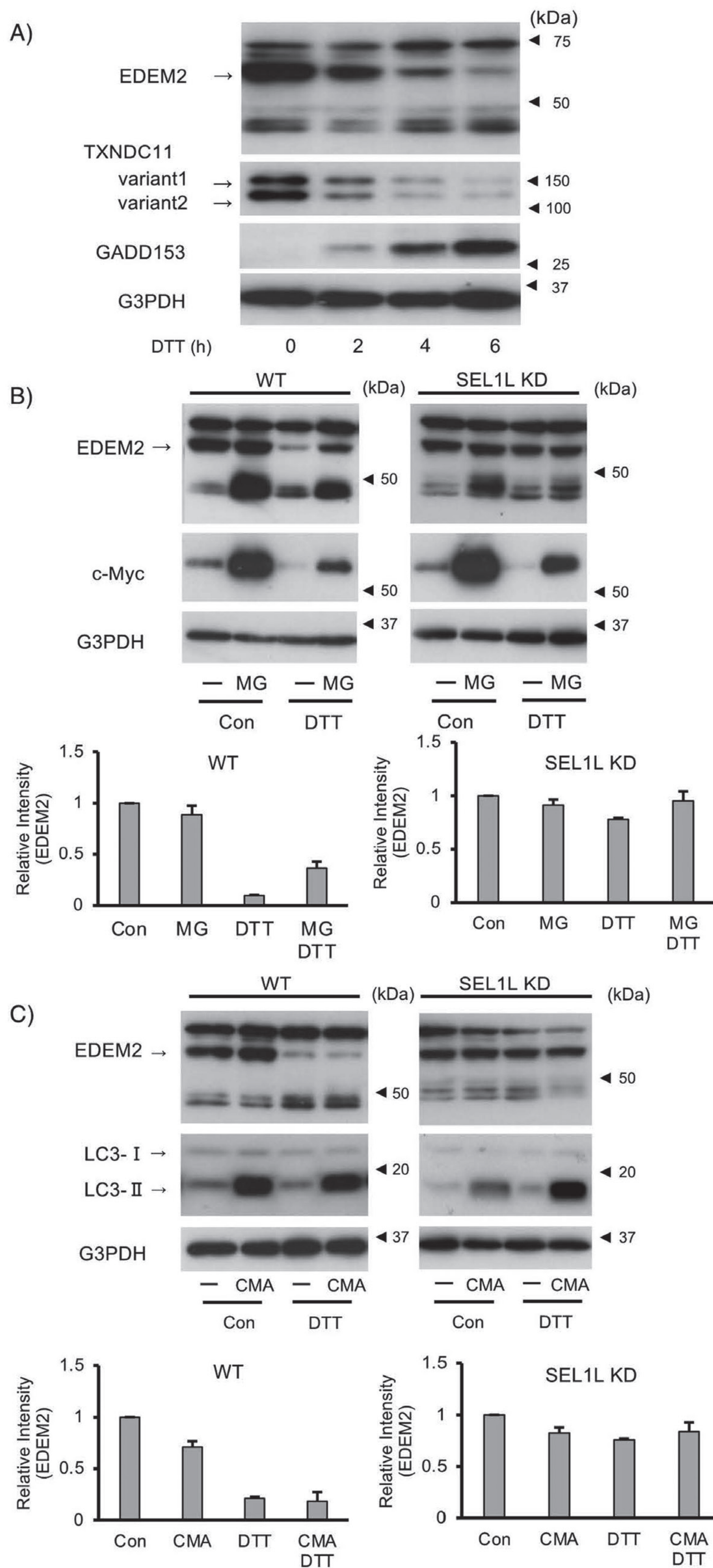

Fig. 4. Degradation of EDEM2 Protein Caused by DTT Treatment Is Dependent on SEL1L

(A) Wild-type (WT) HEK293 cells were treated with DTT (1 mM) for the indicated times. (B and C) Wild-type and SEL1L-deficient (SEL1L KD) HEK293 cells were treated with or without DTT (1 mM), MG132 (MG, 10 μM), CMA (50 nM) or vehicle (Con) for 8 h. The expression of the indicated proteins was measured as described in Materials and Methods. Representative results obtained for four independent cultures are shown. The amount of EDEM2 protein without treatment was set at 1.

decrease in EDEM2 protein expression, which was suppressed by MG132 treatment and SEL1L deficiency. According to Uniprot, EDEM2 does not have intramolecular disulfide bonds. Therefore, the downregulation of EDEM2 protein that occurs after DTT treatment may be the cleavage of the disulfide bond with TXNDC11. Future analysis of the effect of reducing reagents, including DTT, on the association of EDEM2 and TXNDC11 and the stability of unbound EDEM2 is needed. Recently, high expression of EDEM2 in several cancer cell lines, including glioma,[27,28,29] has been reported, although it is unclear whether high EDEM2 expression is related to cancer cell proliferation and poor prognosis via ERAD enhancement. Further investigations of this finding will contribute not only to the regulation of the basic molecular mechanisms of ERAD but also to tumor control.

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Conflict of interest The authors declare no conflict of interest.

REFERENCES

1) Ellgaard L, Helenius A. Quality control in the endoplasmic reticulum. *Nat. Rev. Mol. Cell Biol.*, **4**, 181–191 (2003).
2) Vembar SS, Brodsky JL. One step at a time: endoplasmic reticulum-associated degradation. *Nat. Rev. Mol. Cell Biol.*, **9**, 944–957 (2008).
3) Ninagawa, S., George, G., & Mori, K. (2021). Mechanisms of productive folding and endoplasmic reticulum-associated degradation of glycoproteins and non-glycoproteins. Biochimica et biophysica acta. General subjects, 1865(3), 129812.
4) Hoseki J, Ushioda R, Nagata K. Mechanism and components of endoplasmic reticulum-associated degradation. *J. Biochem.*, **147**, 19–25 (2010).
5) Christianson JC, Ye Y. Cleaning up in the endoplasmic reticulum: ubiquitin in charge. *Nat. Struct. Mol. Biol.*, **21**, 325–335 (2014).
6) Ninagawa S, Okada T, Sumitomo Y, Kamiya Y, Kato K, Horimoto S, Ishikawa T, Takeda S, Sakuma T, Yamamoto T, Mori K. EDEM2 initiates mammalian glycoprotein ERAD by catalyzing the first mannose trimming step. *J. Cell Biol.*, **206**, 347–356 (2014).
7) van der Goot AT, Pearce MMP, Leto DE, Shaler TA, Kopito RR. Redundant and Antagonistic Roles of XTP3B and OS9 in Decoding Glycan and Non-glycan Degrons in ER-Associated Degradation. *Mol. Cell*, **70**, 516–530.e6 (2018).
8) Nakatsukasa K, Brodsky JL. The recognition and retrotranslocation of misfolded proteins from the endoplasmic reticulum. *Traffic*, **9**, 861–870 (2008).
9) Timms RT, Menzies SA, Tchasovnikarova IA, Christensen LC, Williamson JC, Antrobus R, Dougan G, Ellgaard L, Lehner PJ. Genetic dissection of mammalian ERAD through comparative haploid and CRISPR forward genetic screens. *Nat. Commun.*, **7**, 11786 (2016).
10) George G, Ninagawa S, Yagi H, Saito T, Ishikawa T, Sakuma T, Yamamoto T, Imami K, Ishihama Y, Kato K, Okada T, Mori K. EDEM2 stably disulfide-bonded to TXNDC11 catalyzes the first mannose trimming step in mammalian glycoprotein ERAD. *eLife*, **9**, e53455 (2020).
11) Tang HY, Huang CH, Zhuang YH, Christianson JC, Chen X. EDEM2 and OS-9 are required for ER-associated degradation of non-glycosylated sonic hedgehog. *PLoS One*, **9**, e92164 (2014).
12) Olivari S, Galli C, Alanen H, Ruddock L, Molinari M. A novel stress-induced EDEM variant regulating endoplasmic reticulum-associated glycoprotein degradation. *J. Biol. Chem.*, **280**, 2424–2428 (2005).
13) Mast SW, Diekman K, Karaveg K, Davis A, Sifers RN, Moremen KW. Human EDEM2, a novel homolog of family 47 glycosidases, is involved in ER-associated degradation of glycoproteins. *Glycobiology*, **15**, 421–436 (2005).
14) Oh-Hashi K, Takahashi K, Hirata Y. Regulation of the ER-bound transcription factor Luman/CREB3 in HEK293 cells. *FEBS Lett.*, **593**, 2771–2778 (2019).
15) Oh-Hashi K, Yamamoto A, Murase R, Hirata Y. Comparative Analysis of CREB3 and CREB3L2 Protein Expression in HEK293 Cells. *Int. J. Mol. Sci.*, **22**, 2767 (2021).
16) Murase R, Yamamoto A, Hirata Y, Oh-Hashi K. Expression analysis and functional characterization of thioredoxin domain-containing protein 11. *Mol. Biol. Rep.*, **49**, 10541–10556 (2022).
17) Oh-Hashi K, Sugiura N, Amaya F, Isobe KI, Hirata Y. Functional validation of ATF4 and GADD34 in Neuro2a cells by CRISPR/Cas9-mediated genome editing. *Mol. Cell. Biochem.*, **440**, 65–75 (2018).
18) Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM. RNA-guided human genome engineering via Cas9. *Science*, **339**, 823–826 (2013).
19) Schneider, C.A., Rasband, W.S., & Eliceiri, K.W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods*, **9**, 671–675 (2012).
20) Badiola N, Penas C, Miñano-Molina A, Barneda-Zahonero B, Fadó R, Sánchez-Opazo G, Comella JX, Sabriá J, Zhu C, Blomgren K, Casas C, Rodríguez-Alvarez J. Induction of ER stress in response to oxygen-glucose deprivation of cortical cultures involves the activation of the PERK and IRE-1 pathways and of caspase-12. *Cell Death Dis.*, **2**, e149 (2011).
21) Wu Y, Wang H, Xiang W, Yi D. EDEM2 is a diagnostic and prognostic biomarker and associated with immune infiltration in glioma: A comprehensive analysis. *Front. Oncol.*, **12**, 1054012 (2023).
22) Shen J, Prywes R. Dependence of site-2 protease cleavage of ATF6 on prior site-1 protease digestion is determined by the size of the luminal domain of ATF6. *J. Biol. Chem.*, **279**, 43046–43051 (2004).
23) Gregory MA, Hann SR. c-Myc proteolysis by the ubiquitin-proteasome pathway: stabilization of c-Myc in Burkitt's lymphoma cells. *Mol. Cell. Biol.*, **20**, 2423–2435 (2000).
24) Mochida K, Nakatogawa H. ER-phagy: selective autophagy of the endoplasmic reticulum. *EMBO Rep.*, **23**, e55192 (2022).
25) Chiritoiu M, Chiritoiu GN, Munteanu CVA, Pastrama F, Ivessa NE, Petrescu SM. EDEM1 Drives Misfolded Protein Degradation via ERAD and Exploits ER-Phagy as Back-Up Mechanism When ERAD Is Impaired. *Int. J. Mol. Sci.*, **21**, 3468 (2020).
26) George G, Ninagawa S, Yagi H, Furukawa JI, Hashii N, Ishii-Watabe A, Deng Y, Matsushita K, Ishikawa T, Mamahit YP, Maki Y, Kajihara Y, Kato K, Okada T, Mori K. Purified EDEM3 or EDEM1 alone produces determinant oligosaccharide structures from M8B in mammalian glycoprotein ERAD. *eLife*, **10**, e70357 (2021).
27) Sun Q, Zhu E. Molecular mechanism and diagnostic marker investigation of endoplasmic reticulum stress on periodontitis. *BMC Oral Health*, **23**, 135 (2023).
28) Wu Y, Wang H, Xiang W, Yi D. EDEM2 is a diagnostic and prognostic biomarker and associated with immune infiltration in glioma: A comprehensive analysis. *Front. Oncol.*, **12**, 1054012 (2023).
29) Li M, Keshavarz-Rahaghi F, Ladua G, Swanson L, Speers C, Renouf DJ, Lim HJ, Davies JM, Gill S, Stuart HC, Yip S, Loree JM. Characterizing the KRAS G12C mutation in metastatic colorectal cancer: a population-based cohort and assessment of expression differences in The Cancer Genome Atlas. *Ther. Adv. Med. Oncol.*, **14**, 17588359221097940 (2022).