Cell Biology

UGGT1/2-mediated reglucosylation of *N*-glycan competes with ER-associated degradation of unstable and misfolded glycoproteins

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Abstract

Here we investigated how the fate (folding versus degradation) of glycoproteins is determined in the endoplasmic reticulum (ER). Monoglucosylated glycoproteins are recognized by lectin chaperones to facilitate their folding, whereas glycoproteins with well-trimmed mannoses are subjected to glycoprotein ER-associated degradation (gpERAD). Previously we elucidated how mannoses are trimmed by EDEM family members (George et al., 2020, 2021 eLife). Though reglucosylation by UGGTs (UGGT1 and UGGT2) was reported to have no effect on substrate degradation, here, we directly test this using genetically disrupted UGGTs. Strikingly, the results showed that UGGTs (mainly UGGT1) delayed the degradation of misfolded substrates and unstable glycoproteins including ATF6α. An experiment with a point mutant of UGGT1 indicated that the glucosylation activity of UGGT was required for the inhibition of early glycoprotein degradation. We further demonstrated the physiological importance of UGGT, since ATF6 cannot function properly without UGGTs. The fate of glycoproteins is determined by a tug-of-war between structure formation by UGGTs and degradation by EDEMs. Thus, our work strongly suggests that UGGTs are central factors in ER protein quality control via regulation of both glycoprotein folding and degradation.

eLife assessment

This **valuable** manuscript demonstrates that the glycosyltransferase UGGT slows the degradation of endoplasmic reticulum (ER)-associated degradation substrates through a mechanism involving re-glucosylation of asparagine-linked glycans following release from the calnexin/calreticulin lectins. The evidence supporting this conclusion is **solid** using genetically-deficient cell models and biochemical methods to monitor the degradation of trafficking-incompetent ER-associated degradation substrates, although the manuscript could be improved through additional studies directed towards defining potential functional differences between UGGT1 and UGGT2 and additional insights into the impact of UGGT on the nature of substrate glycosylation within the ER. This work will be of specific interest to those interested in mechanistic aspects of ER protein quality control and protein secretion.

Introduction

The endoplasmic reticulum (ER) plays an essential role in the cell as the site of biosynthesis of approximately one third of all proteins. Membrane and secretory proteins, after being newly synthesized on ribosomes attached to the ER membrane, are translocated to the ER in a translation-coupled manner (Brodsky and Skach, 2011). Post-translational modifications of proteins occurring in the ER include disulfide bond formation mediated by PDI (protein disulfide isomerase) family members and *N*-linked glycosylation of Asn in the consensus sequence consisting of Asn-X-Ser/Thr (X≠Pro). *N*-Glycan modifying a large number of proteins that enter the ER is converted from oligomannose type to complex type in the Golgi and is deeply involved in biological phenomena inside and outside the cell.

The structure of *N*-glycans plays a key role in protein folding and degradation in the ER (Berner et al., 2018; Ninagawa et al., 2020a). The *N*-glycan added to nascent proteins is composed of three glucoses, nine mannoses, and two *N*-acetylglucosamines (GlcNAc), and termed $Glc_3Man_9GlcNAc_2$ (G3M9), which is processed to GM9 by the action of glucosidase I and glucosidase II. Calnexin (CNX) and calreticulin (CRT) recognize glycoproteins with GM9 to facilitate productive folding (Lamriben et al., 2016) and then the last glucose is removed by glucosidase II. If glycoproteins with M9 attain their tertiary structure, they move on to the next compartment of the secretory pathway (Ninagawa et al., 2020a). If the protein portion does not form a proper structure, UDP-glucose glycoprotein glucosyltransferses (UGGTs: UGGT1 and UGGT2) re-add glucose to the glycoprotein (reglucosylation) for recognition by CNX/CRT to facilitate protein folding, collectively referred to as the “CNX/CRT” cycle (Lamriben et al., 2016; Sun and Brodsky, 2019). UGGT1 promotes substrate solubility (Ferris et al., 2013) and prefers proteins with exposed hydrophobic regions to folded proteins (Caramelo et al., 2004; Sousa and Parodi, 1995). UGGT1 has a homologue, UGGT2, whose glucosyltransferase activity is weaker than that of human UGGT1 (Ito et al., 2020; Takeda et al., 2014).

If glycoproteins with M9 are not folded correctly within a certain period, mannose residues are trimmed from M9 first by EDEM2-S-S-TXNDC11 complex to M8B (George et al., 2020; Ninagawa et al., 2014) and then by EDEM3/1 to M7A, M6 and M5 exposing α1,6-bonded mannose on the glycoproteins (George et al., 2021; Hirao et al., 2006; Ninagawa et al., 2014), which are recognized by the OS9 or XTP3B lectins for degradation (van der Goot et al., 2018). They are recruited to the HRD1-SEL1L complex on the ER membrane, retrotranslocated back to the cytosol,

and degraded via the ubiquitin-proteasome system (**Fig. 1A**). The series of these processes are collectively designated glycoprotein ER-associated degradation (gpERAD) (Ninagawa et al., 2020a; Smith et al., 2011).

However, how the fate of glycoprotein (folding versus degradation) is determined is not clearly understood, and remains one of the biggest issues in the field of ER protein quality control. One of the key enzymes for this appeared to be UGGTs. UGGTs have been shown to contribute to glycoprotein folding through its reglucosylation activity (Helenius and Aebi, 2004; Pearse et al., 2010; Sousa et al., 1992; Tessier et al., 2000), but did not appear to be related to ERAD, because it was previously reported that the presence of one glucose in the A branch of *N*-glycans was not involved in timing for substrate degradation (Tannous et al., 2015). MI8-5 Chinese hamster ovary cells are deficient in the dolichol-P-glucose–dependent glycosyltransferase termed Alg6, and therefore produce only glycoproteins with *N*-glycans lacking glucoses (M9). In these cells, *N*-glycans with one glucose are produced only by the action of UGGTs. In the experiment, the monoglucosylated state is maintained by treatment with the glucosidase inhibitor 1-deoxynojirimycin (DNJ). It was found that such trapping of glycoproteins in a monoglucosylated state did not affect their rate of disposal, while trapping of UGGT1 substrates for secretion in a monoglucosylated state with DNJ delayed their efflux from the ER. The degradation selection process seemed to progress in a dominant manner that was independent of glucosylation state of A-branch on the substrate glycans.

We consider that it has not been directly examined whether UGGTs-mediated reglucosylation of *N*-glycan is involved in degradation of glycoproteins in the ER. Here, we generated UGGT1-KO, UGGT2-KO and UGGT-double KO (DKO) cell lines to investigate this involvement. Unexpectedly, it was revealed that degradation of misfolded and unstable proteins was markedly accelerated in these KO cells. Our work thus dramatically demonstrates the importance of UGGTs in ER protein quality control via involvement in protein degradation as well as protein folding in the ER.

Results discussion

Generation of knockout cell lines of UGGTs

To explore the roles of UGGT1 and UGGT2 in ERAD, UGGT1-KO, UGGT2-KO and UGGT-DKO cell lines were generated in HCT116 diploid cells derived from human colonic carcinoma using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9. Gene disruptions of UGGT1 and UGGT2 were confirmed at the genome, mRNA and protein level (**Figure 1-figure supplement 1A-K**). The growth rate of the KO cell population was not significantly altered (**Figure 1-figure supplement 1L**).

Previously, the protein level of UGGT2 was estimated to be 4% relative to that of UGGT1 in HeLa cells (Adams et al., 2020; Itzhak et al., 2016). In our hands, the protein expression levels of UGGT2 in HCT116 and HeLa cells were found to be approximately 6.9% and 29.8% of that of UGGT1, respectively, as estimated by transfection of UGGT1-Myc3 and UGGT2-Myc3 (**Figure 1-figure supplement 2A**). Immunoblotting showed that ER stress marker proteins BiP, XBP1(S) and ATF4 were not induced in UGGT1-KO, UGGT2-KO or UGGT-DKO cells (**Figure 1-figure supplement 2B**). Both UGGT1 and UGGT2 are modified with several *N*-glycans, as indicated by their sensitivity to treatment with EndoH, which cleaves oligomannose-type *N*-glycans localized in the ER (**Figure 1-figure supplement 2C**).

In UGGT1-KO and UGGT-DKO cells but not in UGGT2-KO cells, the secretion efficiency of α1-antitrypsin (A1AT) and erythropoietin (EPO) determined by pulse-chase experiments using ^{35}S was decreased (**Figure 1-figure supplement 2DE**). In UGGTs-KO cells, maturation of hemagglutinin (HA) from oligomannose type to complex type was delayed, as reported previously (**Figure 1-**

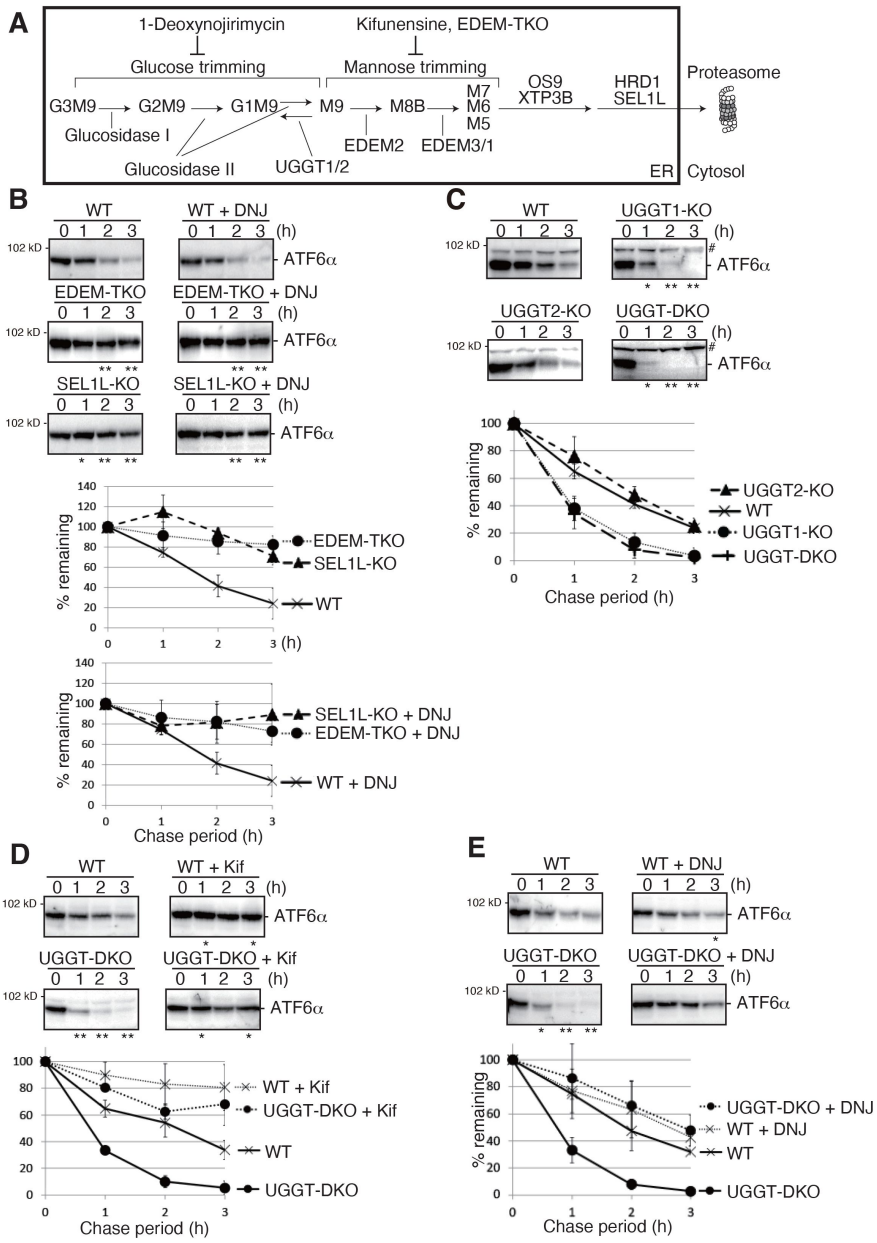

Fig. 1

Effect of UGGT1/2-KO on degradation of ATF6α

(A) Schematic presentation of *N*-glycan processing and gpERAD.
(B) Cycloheximide chase (50 μg/ml) to determine degradation rate of endogenous ATF6α in WT, EDEM-TKO and SEL1L-KO cells treated with or without 0.5 mM DNJ treatment. DNJ was added 2 hours before the addition of CHX. Endogenous ATF6α was detected by immunoblotting using anti-ATF6α antibody. The means from three independent experiments with standard deviations (error bars) are plotted against the chase period (n = 3). P value: *<0.05, **<0.01.
(C) Cycloheximide chase (50 μg/ml) to determine degradation rate of endogenous ATF6α in WT, UGGT1-KO, UGGT2-KO and UGGT-DKO cells (n=3) as in (B). # denotes a non-specific band. P value: *<0.05, **<0.01.
(D) Cycloheximide chase (50 μg/ml) to determine degradation rate of endogenous ATF6α in WT and UGGT-DKO cells with or without 10 μg/ml kifunensine (Kif) treatment (n=3), as in (B). Kifunensine was added 1 hour before the addition of CHX. P value: *<0.05, **<0.01.
(E) Cycloheximide chase (50 μg/ml) to determine degradation rate of endogenous ATF6α in WT and UGGT-DKO treated with or without 0.5 mM DNJ (n = 3), as in (B). DNJ was added 2 hours before the addition of CHX. P value: *<0.05, **<0.01.

figure supplement 2F) (Hung et al., 2022). These results confirmed the establishment of KO cells for UGGTs and show the expected phenotype being involved in the maturation of nascent polypeptides.

Effect of UGGT1/2 knockout on ERAD

We then examined the effect of UGGT1/2 knockout on ERAD. We previously showed that ATF6α functions as a UPR sensor/transducer but is a somewhat unfolded protein that is constitutively subjected to gpERAD (Haze et al., 1999; Horimoto et al., 2013). Degradation of ATF6α was blocked almost completely in SEL1L-KO and EDEM-TKO HCT116 cells (**Fig. 1B**), as was seen previously (Horimoto et al., 2013; Ninagawa et al., 2014); note that construction and characterization of SEL1L-KO cells were described in **Figure 1-figure supplement 3**. Strikingly, degradation of ATF6α was markedly accelerated in UGGT1-KO and UGGT-DKO cells but not in UGGT2-KO cells (**Fig. 1C**). These findings revealed for the first time that UGGTs are involved in not only folding but also ERAD. The fact that ATF6α was stabilized in UGGT-DKO cells treated with a mannosidase inhibitor, kifunensine, as in WT cells treated with kifunensine (**Fig. 1D**), showed that the acceleration of ATF6α degradation required mannose trimming.

We then examined the effect of DNJ, an inhibitor of glucosidase I and II (**Fig. 1A**). Stabilization of ATF6α in SEL1L-KO and EDEM-TKO cells was not affected by treatment with DNJ (**Fig. 1B**). This is because SEL1L is required for retrotranslocation (**Fig. 1A**), and because gpERAD requiring mannose trimming cannot work in EDEM-TKO cells, resulting in marked accumulation of M9 (**Figure 1-figure supplement 4A**); note that both retrotranslocation and mannose trimming are events downstream of glucose trimming (**Fig 1A**). The result obtained with EDEM-TKO cells indicates that ATF6α is still degraded via gpERAD requiring mannose trimming under the treatment with glucosidase inhibitor.

In contrast, degradation of ATF6α in WT cells was slightly delayed by treatment with DNJ, and accelerated degradation of ATF6α in UGGT-DKO cells was compromised by treatment with DNJ, so that the degradation rate of ATF6α became very similar in WT or UGGT-DKO cells after treatment with DNJ (**Fig. 1E**). This implies the importance of glucose trimming-mediated production rate of M9 for efficient gpERAD. Indeed, the increase in the level of GM9 and the decrease in the level of M9 were observed in WT and UGGT-DKO cells treated with DNJ (**Figure 1-figure supplement 4BC**). Nonetheless, ATF6α was not stabilized completely in WT or UGGT-DKO cells treated with DNJ (**Fig. 1E**), unlike in EDEM-TKO and SEL1L-KO cells (**Fig. 1B**). We consider it likely that this is because mannose trimming still occurred in the absence of glucose trimming, as evidenced by the increased detection of GM8 in cells treated with DNJ (**Figure 1-figure supplement 4BCD**), leading to recognition of GM7 by OS9/XTP3B for retrotranslocation (Refer to **Fig. 5**). These results clearly showed that UGGT1 is actively involved in gpERAD of ATF6α; UGGT1-mediated reglucosylation reduces the probability of the mannose trimming that is prerequisite for gpERAD.

We next examined the effect of UGGT1/2 knockout on degradation of NHK, a soluble gpERAD substrate. NHK with three *N*-glycosylation sites is severely misfolded because of its C-terminus truncation resulting from TC deletion in the α1-antitrypsin (A1AT) gene (Sifers et al., 1988). We found that degradation of NHK was accelerated in UGGT1-KO and UGGT-DKO cells but not in UGGT2-KO cells (**Fig. 2A**), similar to the case of ATF6α. Of note, this acceleration required glucosyltransferase activity, as introduction of myc-tagged WT UGGT1 but not its catalytically inactive mutant, D1358A, into UGGT1-KO cells by transfection decelerated degradation of NHK (**Fig. 2B**). In contrast, the degradation rate of NHK-QQQ, in which all three *N*-glycosylation sites in NHK were mutated (Ninagawa et al., 2015), was not affected by gene disruption of UGGT1 or UGGT2 (**Fig. 2C**).

We also found that degradation of CD3δ-ΔTM-HA, a soluble gpERAD substrate (Bernasconi et al., 2010), as well as degradation of its more severely misfolded form CD3δ-ΔTM-<33-7a-34>HA (Ninagawa et al., 2015) was accelerated in UGGT-DKO cells, albeit marginally (**Fig. 2DE**). Thus,

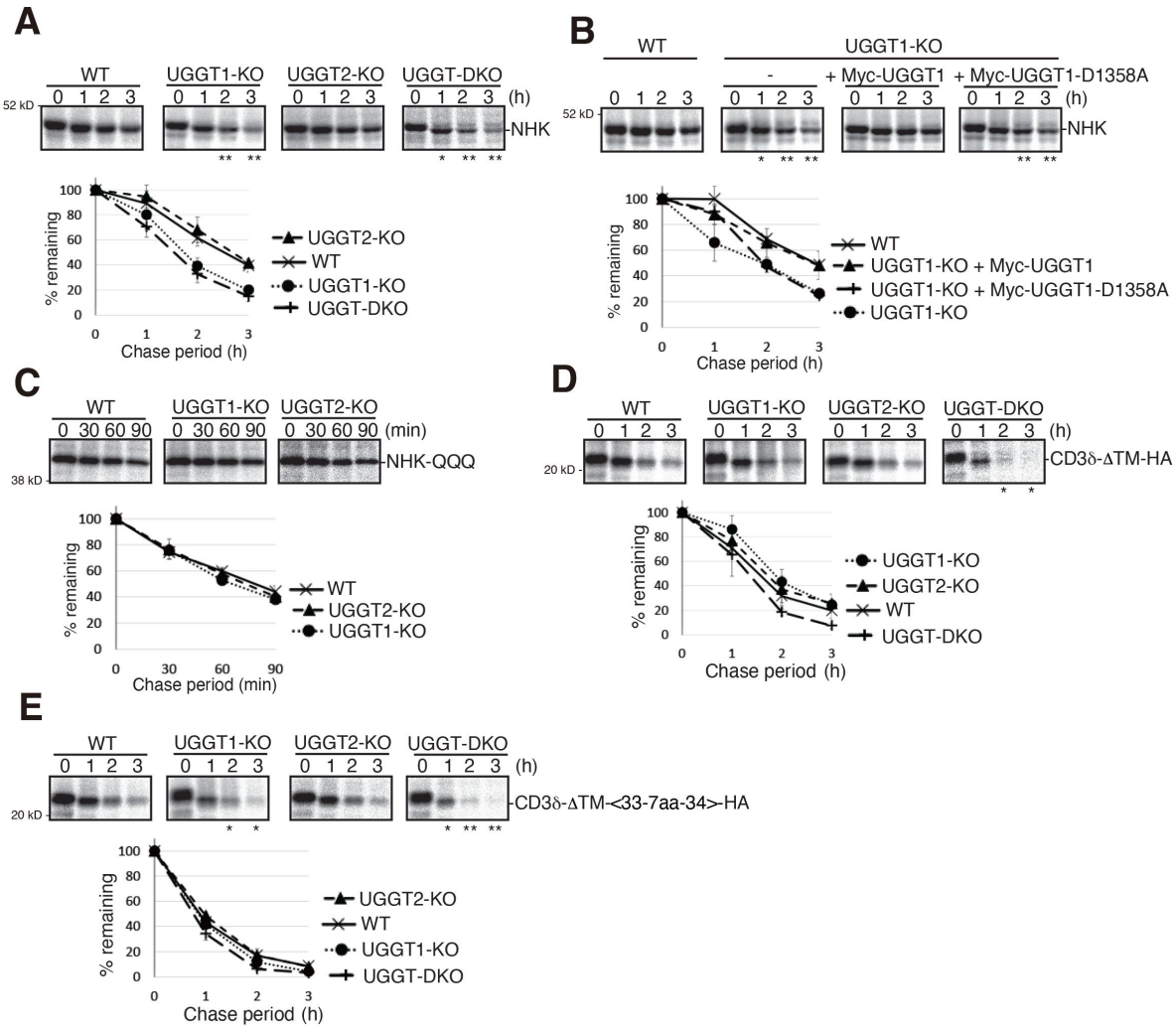

Fig. 2

Effect of UGGT1/2-KO on degradation of soluble ERAD substrates

(A) Pulse-chase and subsequent immunoprecipitation experiments using anti-α1-PI antibody to determine degradation rate of NHK in WT, UGGT1-KO, UGGT2-KO and UGGT-DKO cells transfected with plasmid to express NHK (n=3). The radioactivity of each band was determined and normalized with the value at chase period 0 h. The means from three independent experiments with standard deviations (error bars) are plotted against the chase period (n = 3). P value: *<0.05, **<0.01.
(B) Rescue experiments using WT and UGGT1-KO cells transfected with plasmid to express NHK together with or without plasmid to express Myc-UGGT1 or Myc-UGGT1-D1358A (n = 3), as in (A). P value: *<0.05, **<0.01.
(C) Pulse-chase and subsequent immunoprecipitation experiments to determine degradation rate of NHK-QQQ in WT, UGGT1-KO and UGGT2-KO cells transfected with plasmid to express NHK-QQQ using anti-α1-PI antibody (n=3), as in (A).
(D) Pulse-chase and subsequent immunoprecipitation experiments using anti-HA antibody to determine degradation rate of CD3-δ-ΔTM-HA in WT, UGGT1-KO, UGGT2-KO and UGGT-DKO cells transfected with plasmid to express CD3-δ-ΔTM-HA (n =3), as in (A). P value: *<0.05.
(E) Pulse-chase and subsequent immunoprecipitation experiments using anti-HA antibody to determine the degradation rate of CD3-δ-ΔTM-<33-7aa-34>-HA in WT, UGGT1-KO, UGGT2-KO and UGGT-DKO cells transfected with plasmid to express CD3-δ-ΔTM-<33-7aa-34>-HA (n =3), as in (A). P value: *<0.05, **<0.01.

UGGT-mediated reglucosylation affects the fate of unfolded, misfolded, and severely misfolded glycoproteins but not non-glycoproteins.

Effect of UGGT1/2 knockout on folded and functional proteins

We examined the effect of UGGT1/2 knockout on the stability of ER-localized endogenous proteins and found that they were stable both in WT and in UGGT-DKO cells no matter whether they were glycoproteins (Grp170, Sil1, and ribophorin I) or non-glycoproteins (CRT) (**Fig. 3ABCD**) and **Figure 3-figure supplement 1AB**). Similarly, the activity of the Golgi-resident glycosyltransferase GnT-V (MGAT5) with six putative *N*-glycosylation sites (Hirata et al., 2023) was similar in WT, UGGT1-KO, UGGT2-KO and UGGT-DKO cells (**Fig. 3E**).

Interestingly, however, a truncated version of rat ribophorin I lacking its C-terminal region (aa333-606), termed rRI332-Flag, (**Figure 3-figure supplement 1A**) (de Virgilio et al., 1998; Mueller et al., 2006) was unstable in WT cells compared with ribophorin I when expressed by transfection, and its degradation rate was accelerated in UGGT1-KO and UGGT-DKO cells (**Fig. 3D**, right panel).

EMC1 is a type I membrane protein with three glycosylation sites, and is involved in insertion of membrane protein (Jonikas et al., 2009; Shurtleff et al., 2018). Endogenous EMC1 was a relatively stable protein in both WT and UGGT-DKO cells, but when EMC1-ΔPQQ lacking the PQQ domain was transfected, the degradation rate of EMC1-ΔPQQ was accelerated in UGGT1-KO and UGGT-DKO cells (**Fig. 3F**), similarly to the case of ribophorin I vs rRI332-Flag. Thus, UGGT-mediated reglucosylation affects the fate of unstable proteins but not stable proteins.

UGGTs are required for the proper functioning of ATF6α

We finally investigated the physiological significance of preventing early degradation of substrates by UGGTs. Upon ER stress, a precursor form of ATF6α, designated ATF6α(P), is cleaved to the nucleus-localized form of ATF6α, designated ATF6α(N), to upregulate the ERSE and UPRE promoters, but not the ATF4 promoter (Mori, 2000; Yoshida et al., 1998). Degradation of ATF6α(P) was accelerated in UGGT1-deficient cells (**Fig. 1**), and accordingly the endogenous protein expression level of ATF6α(P) was decreased in UGGT1-deficient cells (**Fig. 4A**). Whereas degradation rate of ATF6α(P) was not changed in UGGT2-deficient cells, expression level of ATF6α(P) was decreased by a small amount (**Fig. 4A** $p=0.06$), suggesting that UGGT2 is also slightly involved in folding of ATF6α(P). The conversion from ATF6α(P) to ATF6α(N) is a marker for its activation. The amount of ATF6α(N) was decreased in UGGTs-KO at 1 hour after the treatment of thapsigargin (Tg), an inhibitor of the ER calcium pump (**Fig. 4BCD**). In addition, peak of activation was shifted from 1 hour to 4 hour after the treatment of Tg in UGGT1-KO cells. This can be explained by the rigid structure of ATF6α(P) lacking structural flexibility to respond to ER stress because the remaining ATF6α(P) in UGGT1-KO cells tends to have a more rigid structure that averts degradation, which is supported by its slightly weaker sensitivity to trypsin (**Figure 4-figure supplement 1A**). Then, we examined the effects of UGGTs on the activation level of ATF6α. In WT cells, the activity of ERSE and UPRE was upregulated 4.8 and 12.1-fold by Tg, respectively, an effect that was abolished in ATF6α-KO cells. In UGGTs-KO cells, the level of induction of the ERSE and UPRE reporters decreased (**Fig. 4EF**), but the reporter activity of ATF4 upon Tg treatment did not decrease in UGGTs-KO cells (**Fig. 4G**). These results clearly show that UGGT1 and UGGT2 are required for proper functioning of ATF6α.

Our results show that UGGTs contribute significantly to the timing of substrate degradation. Previously, in higher animals, the activity of UGGTs was thought to contribute to glycoprotein folding and secretion but not to glycoprotein degradation, based on experiments using a glucosidase inhibitor (Tannous et al., 2015). The reason why the degradation rate was not changed significantly by DNJ can be explained by the activity of UGGTs and the efficiency of mannose trimming. In WT cells, UGGTs prevent early degradation by adding a glucose to facilitate

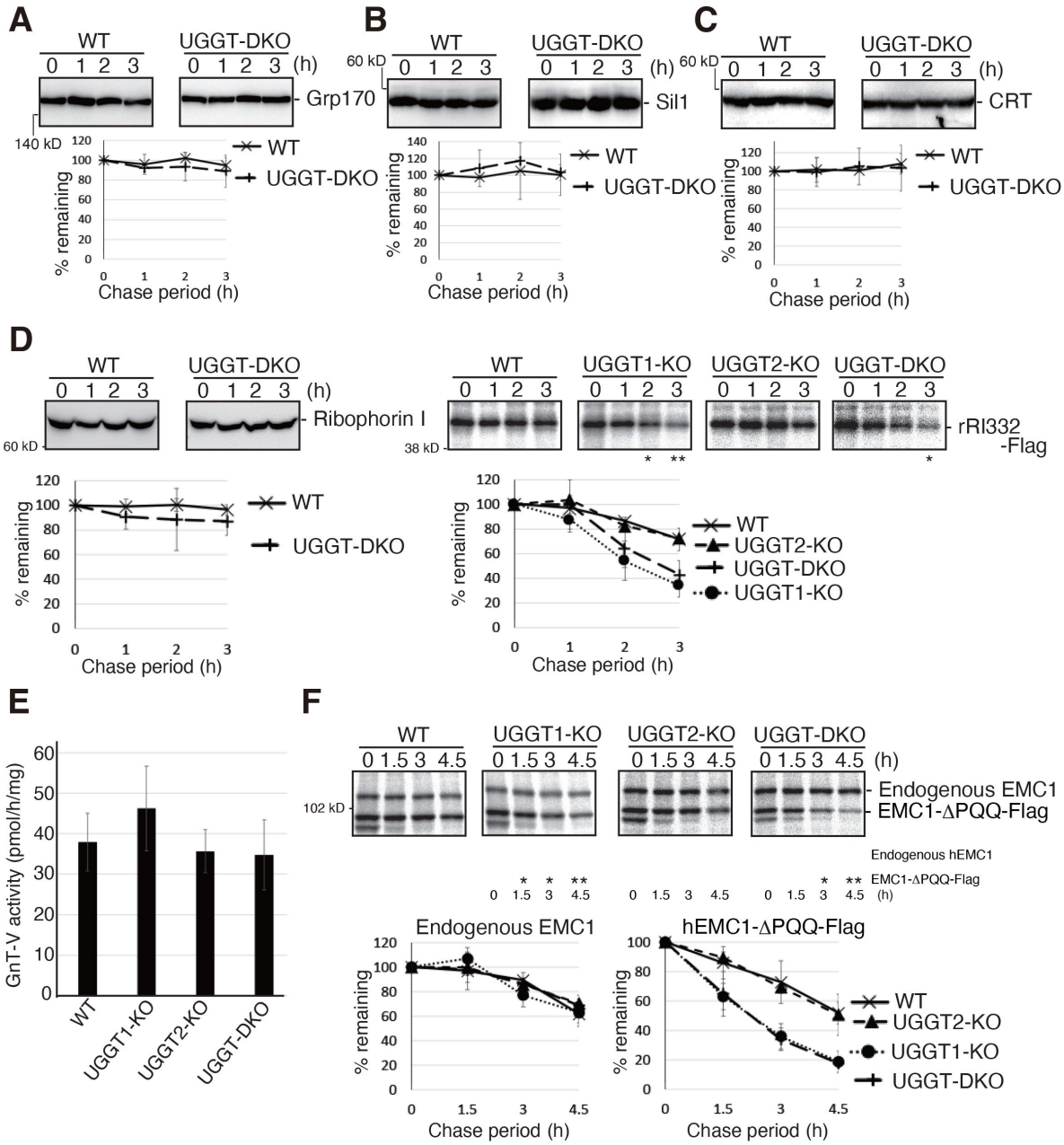

Fig. 3

Effect of UGGT1/2-KO on functional proteins in the secretory pathway and on their unstable mutants

(A)–(C) Cycloheximide chase (50 μg/ml) and subsequent immunoblotting experiments to determine the degradation rate of endogenous Grp170 (A), Sil1 (B) and CRT (C) in WT and UGGT-DKO cells using the respective antibody (n = 3).
(D) [left] Cycloheximide chase (50 μg/ml) and subsequent immunoblotting experiments to determine the degradation rate of endogenous Ribophorin I in WT and UGGT-DKO cells using anti-Ribophorin antibody (n=3), as in (A). [right] Pulse chase and subsequent immunoprecipitation experiments using anti-Flag antibody to determine the degradation rate of rRI332-Flag in WT, UGGT1-KO, UGGT2-KO and UGGT-DKO cells transfected with plasmid to express rRI332-Flag (n =3), as in **Fig. 3A**. P value: *<0.05, **<0.01.
(E) Determination of GnT-V activity in cell lysate of WT, UGGT1-KO, UGGT2-KO and UGGT-DKO cells (n=3).
(F) Pulse-chase and subsequent immunoprecipitation experiments using anti-EMC1 antibody to determine degradation rate of endogenous EMC1 and EMC1-ΔPQQ-Flag in WT, UGGT1-KO, UGGT2-KO and UGGT-DKO cells transfected with plasmid to express EMC1-ΔPQQ-Flag (n =3), as in **Fig. 3A**. P value: *<0.05, **<0.01.

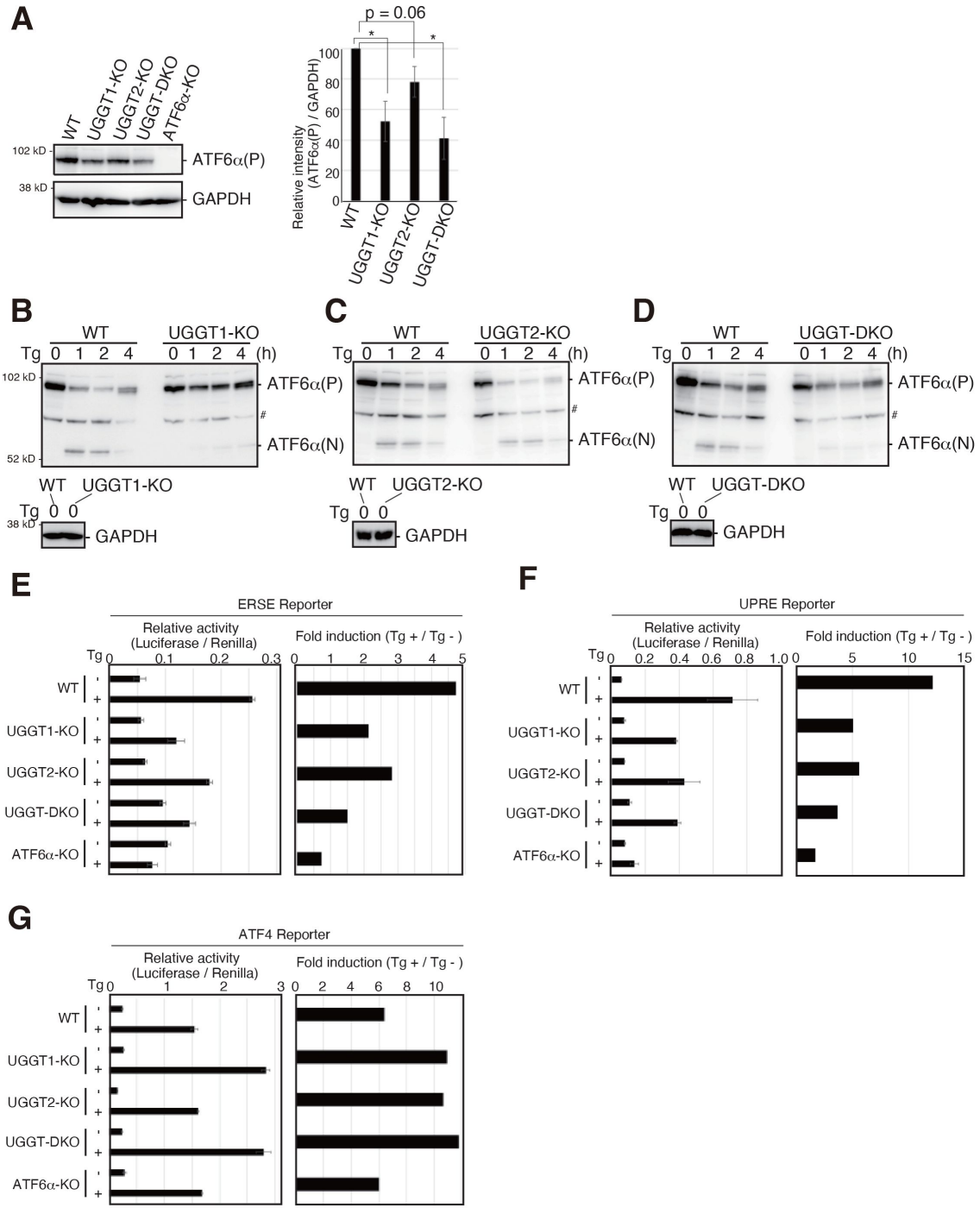

Fig. 4

Effects of UGGTs on unfolded protein response via ATF6α

(A) Immunoblotting to determine the protein level of ATF6α(P) in WT, UGGT1-KO, UGGT2-KO, UGGT-DKO, ATF6α-KO cells using anti-ATF6α antibody (n=3). P value: *<0.05.
(B–D) HCT116 cells cultured in 35-mm dishes were incubated in the presence of 300 nM thapsigargin (Tg) for the indicated periods. Endogenous ATF6α(P) and ATF6α(N) in WT was detected together with UGGT1-KO (B), UGGT2-KO (C), and UGGT-DKO (D), respectively. # indicates non-specific band.
(E-G) HCT116 cells transiently transfected with ERSE (E), UPRE (F) or ATF4 (G) reporters. Twenty-four hours after the transfection, cells were treated with 300 nM Tg for 6 h and harvested to determine luciferase activity (n=3). Relative activity of Luciferase and Renilla, and fold induction of Tg + and Tg – are shown.

further folding but mannose trimming occurs normally to promote degradation. In the presence of DNJ, the glycan-dependent folding pathway cannot work well to prevent early degradation, but mannose trimming does not occur at the normal rate due to a glucose residue on the A chain. Consequently, substrates in cells with DNJ treatment are degraded at approximately the same rate as ones in cells without DNJ. This is supported by our findings that there were no marked differences between WT and WT + DNJ, and UGGT-DKO + DNJ in the degradation of ATF6α (**Fig. 1E** and **Fig. 5**). In our experiments, we were able to evaluate the more direct effect of glucose reattachment by UGGTs on ERAD substrates and we found that UGGTs prevent early degradation of substrates.

This degradation-inhibitory effect of UGGTs, especially UGGT1, on early degradation was also observed for other model substrates in ERAD, such as NHK, CD3-δ-ΔTM, CD3-δ-ΔTM-<33-7aa-34>, EMC1-ΔPQQ and RI332, but not for native, stable glycoproteins, such as Ribophorin I, EMC1, Grp170 and Sil1. Glycoproteins with stable conformational states do not require UGGTs, but to unstable, unassembled, conformationally abnormal and severely misfolded proteins, UGGTs give opportunities for proper folding by CNX/CRT, inhibiting their early degradation in the ER. Therefore, we conclude that the capability of reglucosylation has a huge impact on determining the timing of substrate degradation, and UGGTs usually act to prevent early degradation. Given the delayed substrate degradation in the KO of EDEMs (George et al., 2020; Leto et al., 2019; Ninagawa et al., 2015; Ninagawa et al., 2014), it is further concluded that UGGTs play a "tug-of-war" with EDEMs regarding the fate of glycoproteins: whether to give them a chance to fold, or to degrade them (**Fig. 5**).

How many glycoproteins undergo glucose re-addition by UGGTs and how many times those glycoproteins have experienced the CNX/CRT cycle is a subject for future work. Our findings strongly suggest that UGGTs are not just a CNX/CRT safety net, but should be a central component in the *N*-glycan-dependent folding pathway competing with the EDEMs-mediated degradation pathway.

Materials and methods

Statistics

Statistical analysis was conducted using Student's t-test, with probability expressed as *p<0.05 and **p<0.01 for all figures.

Construction of plasmids

Recombinant DNA techniques were performed using standard procedures (Sambrook et al., 1989) and the integrity of all constructed plasmids was confirmed by extensive sequencing analyses. Using 3xMyc-Fw and 3xMyc-Rv primers, Myc3 fragment was obtained from ATF6α(C)-TAP2 (Myc3-TEV-ProteinA) (George et al., 2021) and inserted into A p3xFlag-CMV-14 expression vector (Sigma) at the site of BamHI to construct p3xMyc-CMV-14 expression vector. Full-length open reading frame of human UGGT1 or UGGT2 was amplified using PrimeSTAR HS DNA polymerase and a pair of primers, namely, UGGT1-cloningFw and UGGT1-cloningRv for UGGT1, and UGGT2-cloningFw and UGGT2-cloningRv for UGGT2, respectively, from a cDNA library of HCT116 which was prepared using Moloney murine leukemia virus reverse transcriptase (Invitrogen), as described previously (Ninagawa et al., 2014). Site-directed mutagenesis was carried out with DpnI to construct UGGT1-D1358A-Myc3 using UGGT1-D1358AFw and UGGT1-D1358Arv primers and DpnI. Partial open reading frame of Rat Ribophorin I was amplified using a pair of primers, namely, RatRI332-cloningFw and RatRI332-cloningRv, and inserted into p3xFlag-CMV-14 or p3xMyc-CMV-14 between the HindIII and KpnI sites to construct RI332-Flag or RI332-

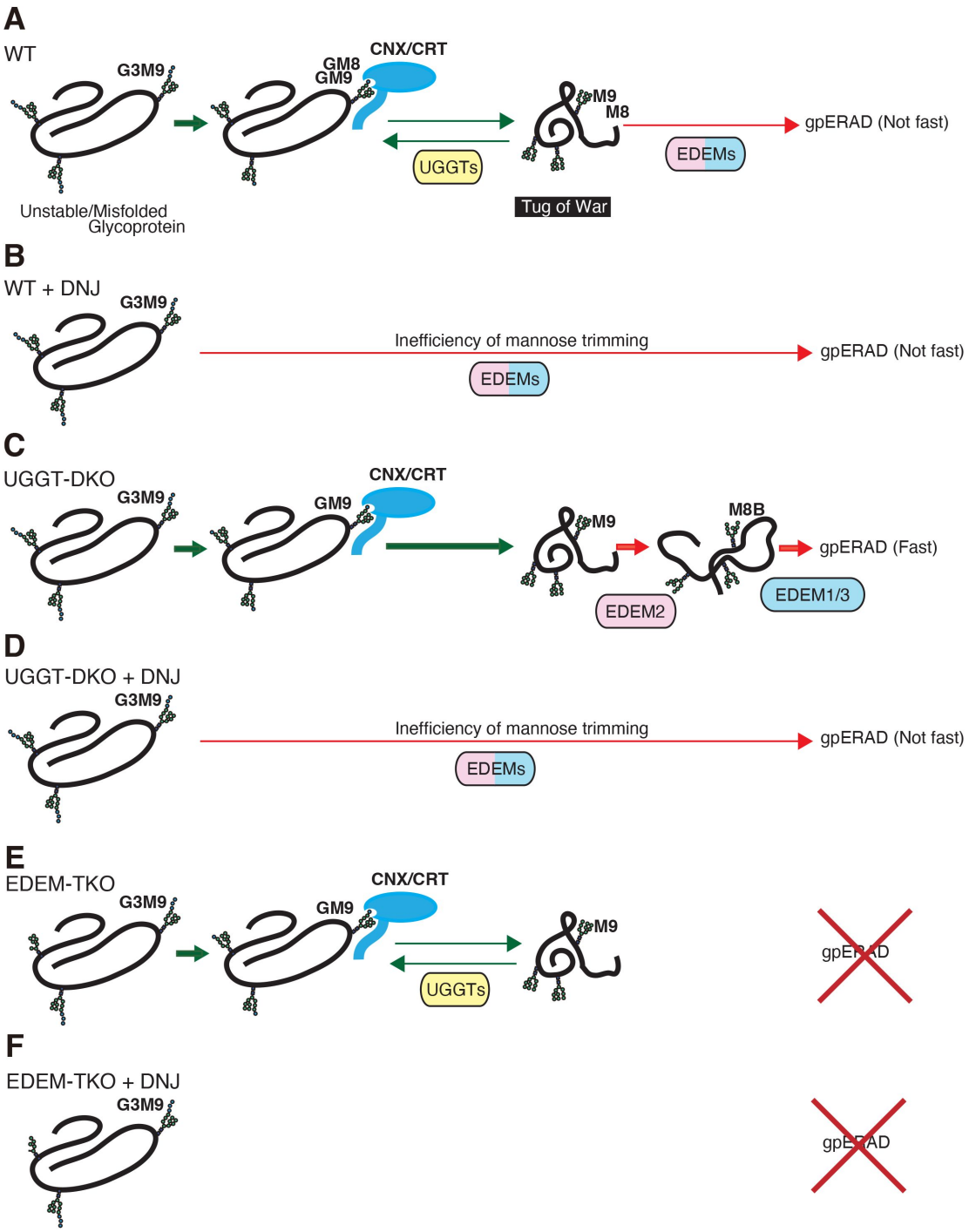

Figure 5

Model of behavior of glycoproteins in the ER

(A). In WT cells, glycoproteins with M9 or M8 are recognized by UGGTs to produce GM9 or GM8 to promote their folding by CNX or CRT, or by EDEMs to trim mannoses of *N*-glycan to expose the α1,6-bond of *N*-glycan in the C chain for gpERAD. Glycoproteins are caught in a tug-of-war between the fate of structure formation by UGGTs and degradation by EDEMs. (B). In WT cells with DNJ, glycoproteins are degraded by EDEMs-mediated gpERAD despite inefficiency of mannose trimming due to the presence of glucoses at A chain. (C). In UGGT-DKO cells, glycoproteins are subjected to premature degradation by EDEMs-mediated gpERAD. (D). In UGGT-DKO cells with DNJ, the almost the same situation as in (B) exists. (E–F). In EDEM-TKO cells, glycoproteins are not degraded via gpERAD.

Myc, respectively. Expression vectors of NHK, CD3-δ-ΔTM-HA, CD3-δ-ΔTM-<33-7aa-34>-HA, EMC1-ΔPQQ-Flag, A1AT and Hemagglutinin were described previously (Ninagawa et al., 2015; Ninagawa et al., 2014; Ninagawa et al., 2020b).

Cell culture and transfection

HCT116 cells (ATCC CCL-247) and HeLa cells (ATCC CCL-2) were cultured in Dulbecco's modified Eagle's medium (glucose 4.5 g/liter) supplemented with 10% fetal bovine serum, 2 mM glutamine, and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) at 37°C in a humidified 5% CO_2/95% air atmosphere. Transfection was performed using polyethylenimine max (Polyscience) according to the manufacturer's instructions. EndoH was obtained from Calbiochem; cycloheximide from Sigma; MG132 from Peptide Institute; and Z-vad-fmk from Promega. No mycoplasma contamination confirmed by MycoBlue Mycoplasma Detector (Vazyme).

Immunological techniques

Immunoblotting analysis was carried out according to the standard procedure (Sambrook et al., 1989) as described previously (Ninagawa et al., 2011). Chemiluminescence obtained using Western Blotting Luminol Reagent (Santa Cruz Biotechnology) was detected using an LAS-3000mini Lumino-Image analyzer (Fuji Film). The antibodies used are listed in Supplemental material. Anti-human ATF6α (Haze et al., 1999) and EMC1 (Ninagawa et al., 2015) antibodies were produced previously. Immunoprecipitation was performed using the described antibodies and protein G- or A- coupled Sepharose beads (GE Healthcare). Beads were washed with high salt buffer (50 mM Tris/Cl, pH 8.0, containing 1% NP-40 and 150 mM NaCl) twice, washed with PBS, and boiled in Laemmli's sample buffer.

N-glycan profiling

Pyridylamination and structural identification of *N*-glycans of total cellular glycoproteins were performed as described previously (Horimoto et al., 2013; Ninagawa et al., 2014).

Pulse-chase experiments

Pulse-chase experiments using 9.8 Mbq per dish of EASY-TAG EXPRESS Protein labeling mix [^{35}S] (PerkinElmer) and subsequent immunoprecipitation using suitable antibodies and protein G or A-coupled Sepharose beads (GE Healthcare) were performed according to our published procedure (Ninagawa et al., 2014).

CRISPR/Cas9 method to generate KO cell lines of UGGT1

Using the pair of primers UGGT1sgRNAFw and Rv, the sequences of the BbsI site of px330 (Addgene) was converted to that to express sgRNA for cleavage at exon 2 of the UGGT1 gene. PuroR and backbone fragment were amplified by PCR from DT-A-pA- loxP-PGK-Puro-pA-loxP (Ninagawa et al., 2014) using UGGT1-PuroFw and Rv primers, and UGGT1-BackboneFw and UGGT1-BackboneRv primers, respectively. Left and right arms were amplified by PCR from the human genome originated from HCT116 using UGGT1-LarmFw and Rv, and UGGT1-RarmFw and Rv. Four fragments were built up using an NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs) to create pKO-hUGGT1-Puro, which was transfected into HCT116 cells with sgRNA expression vector for UGGT1. Clones with puromycin (0.5 μg/ml) resistance were selected.

CRISPR/Cas9 method to generate KO cell lines of UGGT2

Using the pair of primers UGGT2sgRNAFw and Rv, the sequence of the BbsI site of px330 (Addgene) was converted to that to express sgRNA for cleavage at exon 4 of the UGGT2 gene. Hygro^r and backbone fragment were amplified by PCR from DT-A-pA- loxP-PGK-Hygro-pA-loxP (Tsuda et al., 2019) using UGGT2-HygroFw and Rv primers, and UGGT2-BackboneFw and UGGT2-BackboneRv primers, respectively. Left and right arms were amplified by PCR from human

genome originated from HCT116 using UGGT2-LarmFw and Rv primers, and UGGT2-RarmFw and Rv primers, respectively. Four fragments were built up using an NEBuilder HiFi DNA Assembly Cloning Kit to create pKO-hUGGT2-Hygro, which was transfected into HCT116 cells with sgRNA expression vector for UGGT2. Clones with hygromycin (300 μg/ml) resistance were selected.

TALEN method to generate KO cell lines of SEL1L

Platinum TALEN plasmid was constructed as described previously (Ninagawa et al., 2014; Sakuma et al., 2013). In brief, each DNA-binding module was assembled into ptCMV-153/47-VR vectors using the two-step Golden Gate cloning method. The assembled sequence was 5-TGCTGCTGTGTGCGGTGCTgctgagcttggccTCGGCGTCCTCGGGTCA-3, where uppercase and lowercase letters indicate TALEN target sequences and spacer sequences, respectively.

Reporter assay

Twenty-four hours after transfection, HCT116 cells cultured in a 24-well plate were washed with PBS and lysed in Luciferase Assay Lysis Buffer (Toyo Bnet). Luciferase activity was determined using PicaGene Dual-luciferase reporter assay reagent (Toyo Bnet). Relative luciferase activity was defined as the ratio of firefly luciferase activity to renilla luciferase activity. ERSE, UPRE and ATF4 reporters were described previously (Saito et al., 2022). Briefly, the ERSE reporter is pGL3-GRP78(−132)-Luc carrying the human BiP promoter, the UPRE reporter carries p5xUPRE-GL3 identical to p5xATF6GL3, and the ATF4 reporter carries the promoter region of murine ATF4 from position –261 to +124 (ORF starts at +1).

Measurement of glycosyltransferase activities

The experiment was described previously (Hirata et al., 2023). Briefly, 3 μl of the cell lysates was incubated in a total of 10 μl of a reaction buffer [125 mM MES (pH 6.25), 10 mM $MnCl_2$, 200 mM GlcNAc, 0.5% Triton X-100, and 1 mg/ml BSA] supplemented with 20 mM UDP-GlcNAc and 10 μM fluorescence-labeled biantennary acceptor *N*-glycan substrate GnGnbi-PA (PA, 2-aminopyridine) at 37 °C for 3 h. After the reaction, the sample was boiled at 99 °C for 2 min to inactivate the enzymes and then 40 μl of water was added. After centrifugation at 21,500 *g* for 5 min, the supernatants were analyzed by reverse-phase HPLC with an ODS column (4.6 x 150 mm, TSKgel ODS-80TM; TOSOH Bioscience). HPLC analysis was conducted in the isocratic mode in which 80% buffer A (20 mM ammonium acetic buffer (pH 4.0)) and 20% buffer B (1% butanol in buffer A) were loaded at 1 ml/min.

Trypsin digestion assay

The trypsin digestion assay was described previously (Ninagawa and Mori, 2016; Ninagawa et al., 2015).

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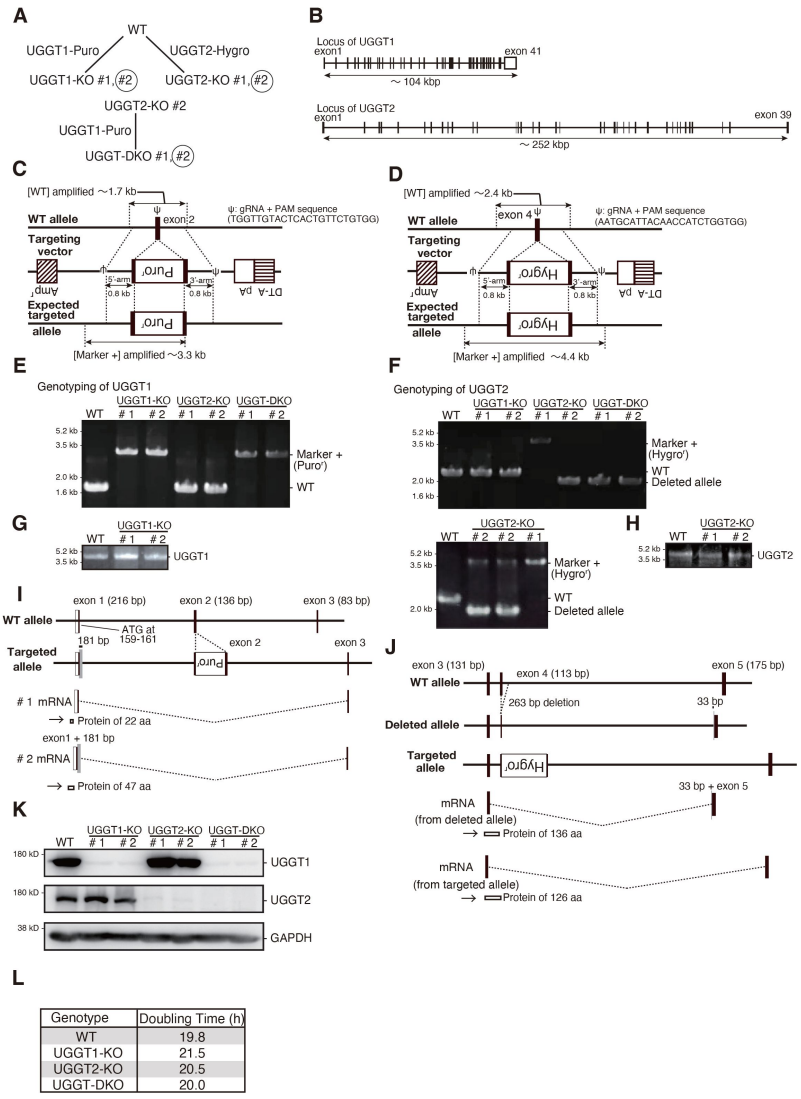
Abbreviations

- UGGT1
  UDP-glucose glycoprotein glucosyltransferase 1
- ER
  endoplasmic reticulum
- CNX
  Calnexin
- CRT
  Calreticulin
- gpERAD
  glycoprotein ER-associated degradation
- non-gpERAD
  non-glycoprotein ER-associated degradation
- EDEM
  ER degradation enhancing αmannosidase-like protein
- ATF6
  Activating transcription factor 6
- ATF4
  Activating transcription factor 4
- PDI
  Protein disulfide isomerase
- GlcNAc
  N-acetylglucosamines
- TXNDC11
  Thioredoxin domain-containing protein 11
- WT
  wild type
- DKO
  double knockout
- TKO
  triple knockout
- Puro
  puromycin
- Hygro
  hygromycin
- BiP
  immunoglobulin heavy chain binding protein
- EMC1
  ER membrane protein complex subunit 1
- EndoH
  endoglycosidase H
- DNJ
  1-deoxynojirimycin
- CHX
  cycloheximide
- ERSE
  ER stress responsive element

unfolded protein response element

- CRISPR
  Clustered regularly interspaced short palindromic repeats
- TALEN
  Transcription activator-like effector nuclease
- BLAST
  Basic Local Alignment Search Tool
- PQQ
  pyrrolo-quinoline quinone
- Tg
  thapsigargin
- XBP1
  X-box binding protein 1
- PERK
  Protein kinase R (PKR)-like endoplasmic reticulum kinase
- IRE1
  Inositol-requiring enzyme 1
- GAPDH
  Glyceraldehyde-3-phosphate dehydrogenase
- HA
  Hemagglutinin
- A1AT
  Alpha-1 antitrypsin
- NHK
  null hong kong
- EPO
  Erythropoietin
- SDS
  sodium dodecyl sulfate



Genotype	Doubling Time (h)
WT	19.8
UGGT1-KO	21.5
UGGT2-KO	20.5
UGGT-DKO	20.0

Figure 1-Figure supplement 1

Generation of UGGT1/2-KO HCT116 cells

(A) Strategy for obtaining UGGT1-KO, UGGT2-KO and UGGT-DKO cells from HCT116 cells. UGGT1-KO #2, UGGT2-KO #2 and UGGT-DKO #2 were mainly used in this report.
(B) Schematic presentation of UGGT1 and UGGT2 loci.
(C) Strategy of the CRISPR/Cas9-mediated targeting of exon 2 of the UGGT1 gene.
(D) Strategy of the CRISPR/Cas9-mediated targeting of exon 4 of the UGGT2 gene.
(E) Genomic PCR to confirm recombination of UGGT1 locus.
(F) Genomic PCR to confirm recombination of UGGT2 locus.
(G) RT-PCR to amplify cDNA corresponding to full length UGGT1 from WT and two independent UGGT1-KO cell lines (#1 and #2).
(H) RT-PCR to amplify cDNA corresponding to full length UGGT2 from WT and two independent UGGT2-KO cell lines (#1 and #2).
(I) Schematic presentation of UGGT1 mRNA expressed in two independent UGGT1-KO cell lines (#1 and #2) and their translational products. The sequence of the amplified cDNA in (G) was determined.
(J) Schematic presentation of UGGT2 mRNA expressed from the deleted and targeted alleles in UGGT2-KO cell lines and their translational products. The sequence of the amplified cDNA in (H) was determined
(K) Immunoblotting to determine endogenous protein expression of UGGT1 and UGGT2 in UGGT1-KO, UGGT2-KO and UGGT-DKO HCT116 cells using anti-UGGT1, anti-UGGT2 and anti-GAPDH antibodies.
(L) Doubling time of WT, UGGT1-KO, UGGT2-KO and UGGT-DKO HCT116 cells (n = 3).

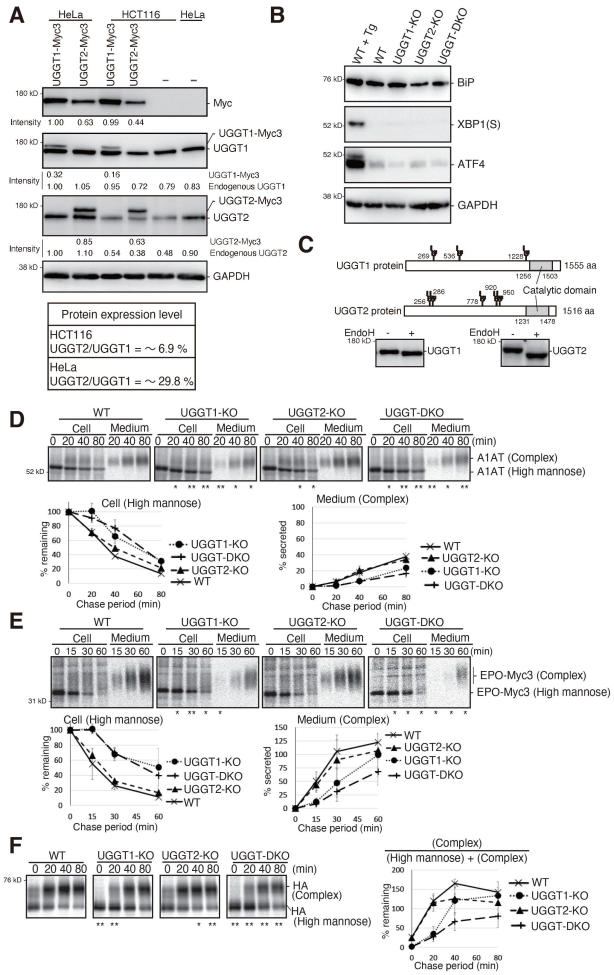
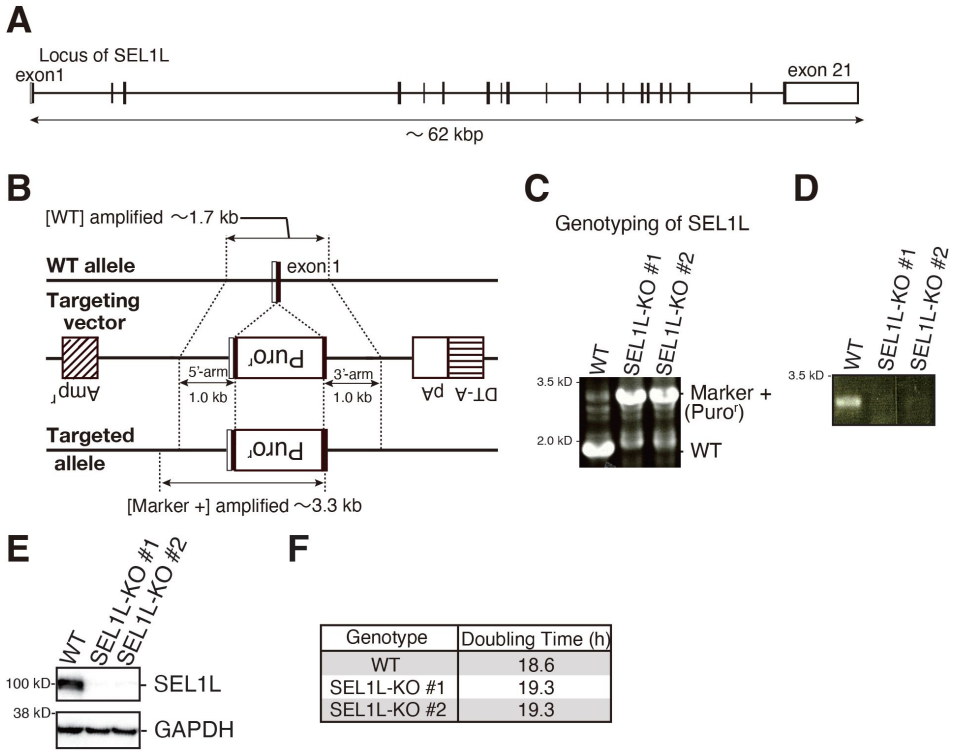


Figure 1-Figure supplement 2

Generation and characterization of UGGT1/2-KO HCT116 cells

(A) Immunoblotting of cell lysates prepared from WT HCT116 and HeLa cells transiently expressing UGGT1-Myc3 or UGGT2-Myc3 using anti-Myc, anti-UGGT1, anti-UGGT2 and anti-GAPDH antibodies. The intensity of the lower left band in each panel is set to 1.00, and relative intensities are shown below. Anti-Myc antibody was used to estimate the ratio of the amounts of UGGT1-Myc3 and UGGT2-Myc3. Anti-UGGT1 antibody was used to estimate that of endogenous UGGT1 and UGGT1-Myc3. Anti-UGGT2 antibody was used to estimate that of endogenous UGGT2 and UGGT2-Myc3. These values can be used to calculate the relative amounts of endogenous UGGT1 and UGGT2. Approximate expression level of UGGT2 relative to UGGT1 in HCT116 and HeLa cells shown as % in bottom panel.
(B) Expression levels of BiP, XBP1(S), spliced form of XBP1, ATF4 and GAPDH determined by immunoblotting of cell lysates, which were prepared from WT, WT treated with 300 nM thapsigargin (Tg) for 6 h, UGGT1-KO, UGGT2-KO and UGGT-DKO cells.
(C) Schematic presentation of UGGT1 and UGGT2 proteins with potential *N*-glycosylation sites and catalytic domain indicated. Cell lysates were prepared from WT HCT116 cells, treated or not treated with EndoH, and analyzed by immunoblotting using anti-UGGT1 and anti-UGGT2 antibodies.
(D), (E) Secretion of A1AT (D) and EPO-Myc3 (E) in WT, UGGT1-KO, UGGT2-KO and UGGT-DKO HCT116 cells. Cells were transfected with plasmid to express A1AT or EPO-Myc3. Twenty-four hours later, cells were pulse labeled with ^{35}S-methionine and cysteine for 20 min and then chased for the indicated periods, followed by immunoprecipitation of A1AT or EPO-Myc3 from cells and medium using anti-A1AT or anti-Myc antibody, respectively, and then subjected to reducing SDS-PAGE and autoradiography (n=3). The amount of A1AT or EPO-Myc3 in WT cells at 0 min was set as 100 %. The means from three independent experiments with standard deviations (error bars) were plotted against the chase period.
(F) Maturation of HA in WT, UGGT1-KO, UGGT2-KO and UGGT-DKO HCT116 cells. Pulse-chase experiments were conducted using various cells transfected with plasmid to express HA, followed by immunoprecipitation of HA from cells using anti-HA antibody (n=3), as in (D). The immunoprecipitates were digested with EndoH prior to subjection to reducing SDS-PAGE. The amount of HA with high-mannose type *N*-glycan at time 0 h in WT cells was set as 100 %.



Genotype	Doubling Time (h)
WT	18.6
SEL1L-KO #1	19.3
SEL1L-KO #2	19.3

Figure 1-figure supplement 3

Generation of SEL1L-KO HCT116 cells

(A) Schematic presentation of SEL1L locus
(B) Strategy of the TALEN-mediated targeting of exon 1 of the SEL1L gene.
(C) Genomic PCR to confirm recombination of SEL1L locus.
(D) RT-PCR to amplify cDNA corresponding to full length SEL1L from WT and two independent SEL1L-KO cell lines (#1 and #2).
(E) Immunoblotting of SEL1L protein in WT and two independent SEL1L-KO cell lines (#1 and #2) using anti-SEL1L antibody.
(F) Doubling time of WT and two independent SEL1L-KO cell lines (#1 and #2).

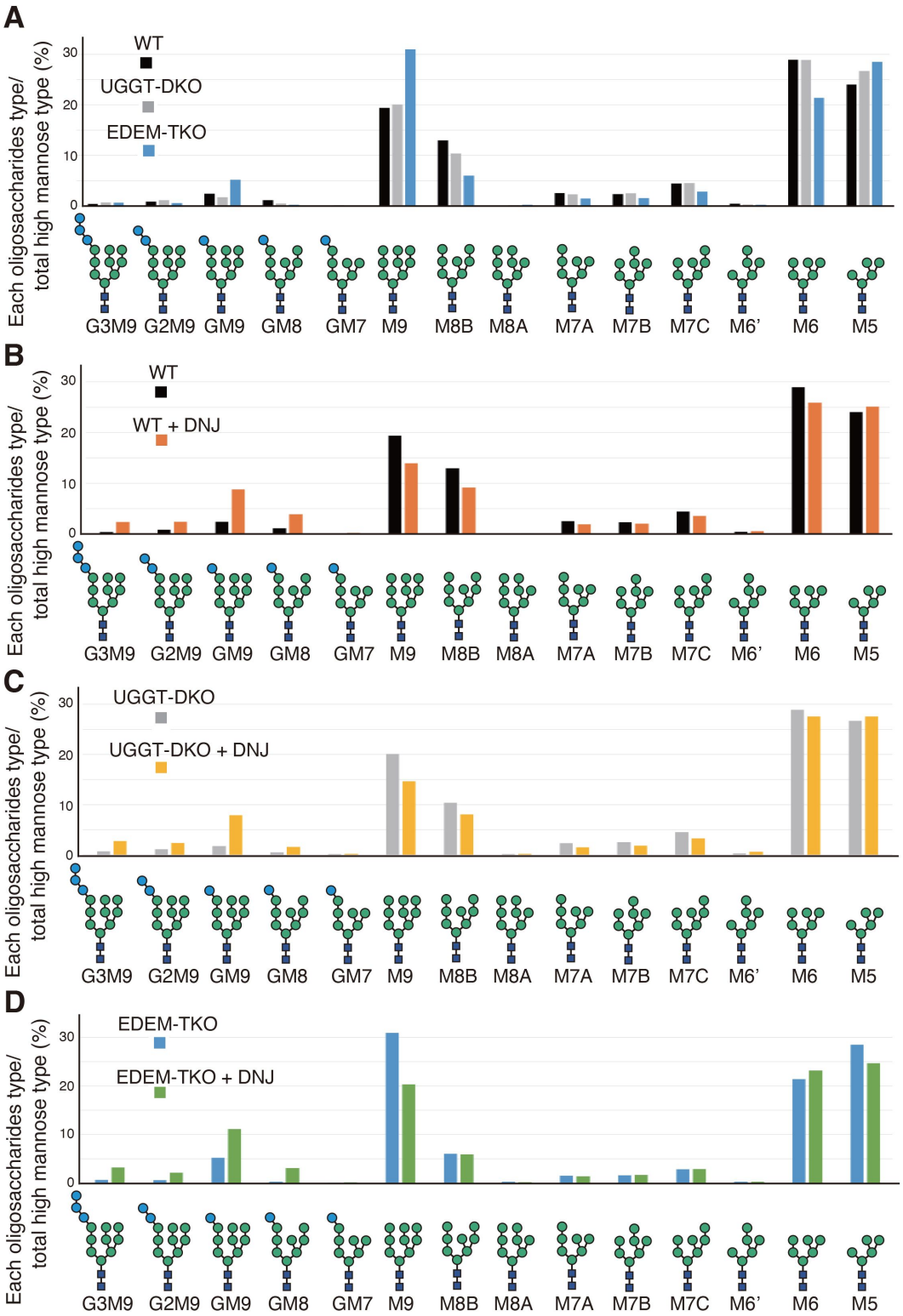


Figure 1-Figure supplement 4

N-glycan profiling of various types of cells treated or not treated with DNJ

(A)–(D) Isomer composition of high mannose-type *N*-glycans prepared from total cellular glycoproteins of WT, UGGT-DKO and EDEM-TKO cells (A), WT cells treated with or without 0.5 mM DNJ for 16 h (B), UGGT-DKO cells treated with or without 0.5 mM DNJ for 16 h (C), EDEM-TKO cells treated with or without 0.5 mM DNJ for 16 h (D). This experiment was completed once.

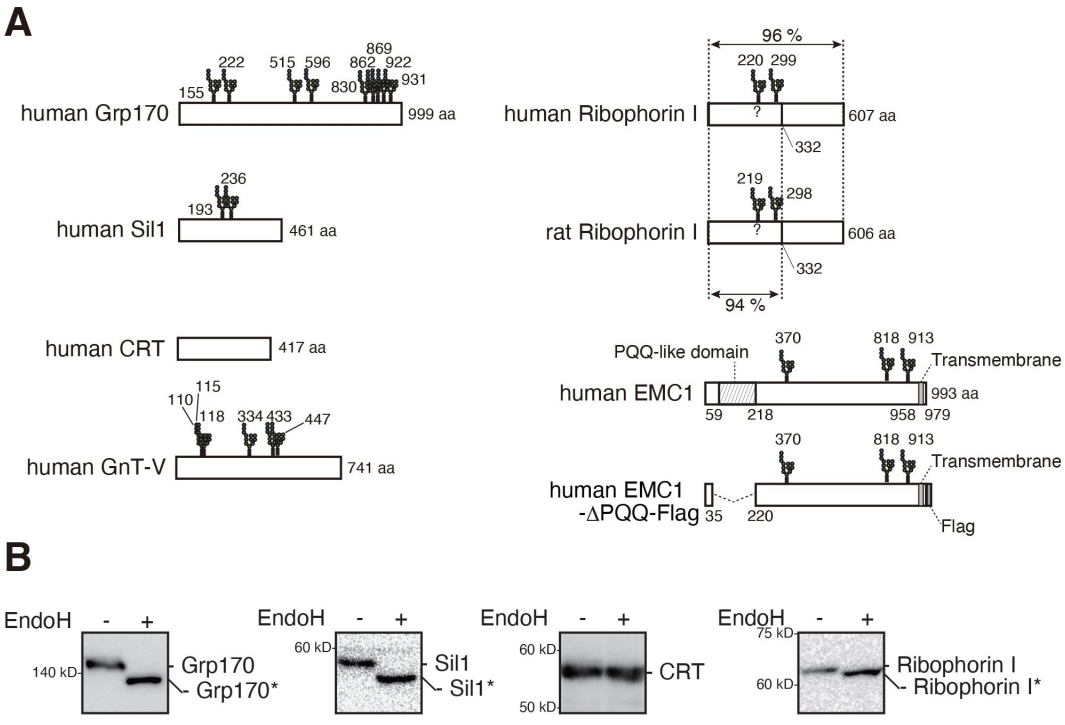


Figure 3-Figure supplement 1

Characterization of various proteins present in the secretory pathway

(A) Schematic structures of human Grp170, human Sil1, human CRT, human GnT-V, human Ribophorin I, rat Ribophorin I, human EMC1, and human EMC1-ΔPQQ-Flag with potential *N*-glycosylation sites indicated. It has not been confirmed whether two *N*-glycans are attached to human and rat Ribophorin I. Sequence identities between human and rat Ribophorin I are shown as percentages. rRI332-Flag lacks aa 333–606 of rat Ribophorin I.
(B) Immunoblotting with respective antibody of cells lysates prepared from WT cells and cells treated with or without EndoH treatment.

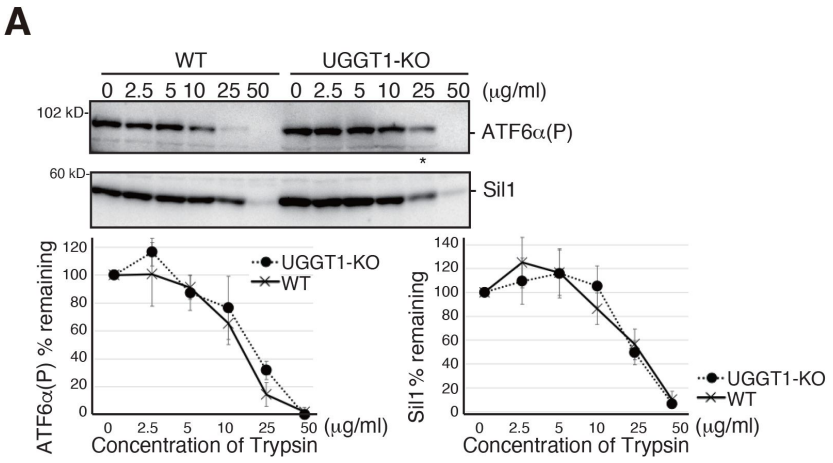


Figure 4-Figure supplement 1

Structural assessment of ATF6α in UGGT1-KO

(A) Trypsin digestion assay to evaluate the folding state of ATF6α(P) in WT and UGGT1-KO. The same amounts of proteins in cell lysate from WT and UGGT1-KO cells were treated with the indicated concentration of trypsin for 15 minutes at room temperature. Enzymatic activity of trypsin was terminated by addition of Laemmli SDS sample buffer containing 100 mM DTT and 10x protease inhibitor cocktail. Immunoblotting was conducted as in **Fig. 4A**, using anti-ATF6α and anti-Sil1 antibodies. In electrophoresis for the detection of ATF6α, the UGGT1-KO sample is loaded with twice as much protein as the WT sample.

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Reviewer #1 (Public Review):

Summary:
UGGTs are involved in the prevention of premature degradation for misfolded glycoproteins, by utilizing UGGT-KO cells and a number of different ERAD substrates. They proposed a concept by which the fate of glycoproteins can be determined by a tug-of-war between UGGTs and EDEMs.

Strengths:
The authors provided a wealth of data to indicate that UGGT1 competes with EDEMs, which promotes glycoprotein degradation.

Weaknesses:
Less clear, though, is the involvement of UGGT2 in the process. Also, to this reviewer, some data do not necessarily support the conclusion.

Major criticisms:

1. One of the biggest problems I had on reading through this manuscript is that, while the authors appeared to generate UGGTs-KO cells from HCT116 and HeLa cells, it was not clearly

indicated which cell line was used for each experiment. I assume that it was HCT116 cells in most cases, but I did not see that it was clearly mentioned. As the expression level of UGGT2 relative to UGGT1 is quite different between the two cell lines, it would be critical to know which cells were used for each experiment.

2. While most of the authors' conclusion is sound, some claims, to this reviewer, were not fully supported by the data. Especially I cannot help being puzzled by the authors' claim about the involvement of UGGT2 in the ERAD process. In most of the cases, KO of UGGT2 does not seem to affect the stability of ERAD substrates (ex. Fig. 1C, 2A, 3D). When the author suggests that UGGT2 is also involved in the ERAD, it is far from convincing (ex. Fig. 2D/E). Especially because now it has been suggested that the main role of UGGT2 may be distinct from UGGT1, playing a role in lipid quality control (Hung, et al., PNAS 2022), it is imperative to provide convincing evidence if the authors want to claim the involvement of UGGT2 in a protein quality control system.

In fact, it was not clear at all whether even UGGT1 is also involved in the process in Fig. 2D/E, as the difference, if any, is so subtle. How the authors can be sure that this is significant enough? While the authors claim that the difference is statistically significant (n=3), this may end up with experimental artifacts. To say the least, I would urge the authors to try rescue experiments with UGGT1 or 2, to clarify that the defect in UGGT-DKO cells can be reversed. It may also be interesting to see that the subtle difference the authors observed is indeed N-glycan-dependent by testing a non-glycosylated version of the protein (just like NHK-QQQ mutants in Fig. 2C).

To this reviewer, it is still possible that the involvement of UGGT1 (or 2, if any) could be totally substrate-dependent, and the substrates used in Fig 2D or E happen not to be dependent to the action of UGGTs. To the reviewer, without the data of Fig. 2D and E the authors provide enough evidence to demonstrate the involvement of UGGT1 in preventing premature degradation of glycoprotein ERAD substrates. I am just afraid that the authors may have overinterpreted the data, as if the UGGTs are involved in stabilization of all glycoproteins destined for ERAD.

3. I am a bit puzzled by the DNJ treatment experiments. First, I do not see the detailed conditions of the DNJ treatment (concentration? Time?). Then, I was a bit surprised to see that there were so little G3M9 glycans formed, and there was about the same amount of G2M9 also formed (Figure 1 Figure supplement 4B-D), despite the fact that glucose trimming of newly syntheized glycoproteins are expected to be completely impaired (unless the authors used DNJ concentration which does not completely impair the trimming of the first Glc). Even considering the involvement of Golgi endo-alpha-mannosidase, a similar amount of G3M9 and G2M9 may suggest that the experimental conditions used for this experiment (i.e. concentration of DNJ, duration of treatment, etc) is not properly optimized.

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Reviewer #2 (Public Review):

In this study, Ninagawa et al., shed light on UGGT's role in ER quality control of glycoproteins. By utilizing UGGT1/UGGT2 DKO cells, they demonstrate that several model misfolded glycoproteins undergo early degradation. One such substrate is ATF6alpha where its premature degradation hampers the cell's ability to mount an ER stress response.

While this study convincingly demonstrates early degradation of misfolded glycoproteins in the absence of UGGTs, my major concern is the need for additional experiments to support the "tug of war" model involving UGGTs and EDEMs in influencing the substrate's fate - whether misfolded glycoproteins are pulled into the folding or degradation route. Specifically, it would be valuable to investigate how overexpression of UGGTs and EDEMs in

WT cells affects the choice between folding and degradation for misfolded glycoproteins. Considering previous studies indicating that monoglucosylation influences glycoprotein solubility and stability, an essential question is: what is the nature of glycoproteins in UGGTKO/EDEMKO and potentially UGGT/EDEM overexpression cells? Understanding whether these substrates become more soluble/stable when GM9 versus mannose-only translation modification accumulates would provide valuable insights.

The study delves into the physiological role of UGGT, but is limited in scope, focusing solely on the effect of ATF6alpha in UGGT KO cells' stress response. It is crucial for the authors to investigate the broader impact of UGGT KO, including the assessment of basal ER proteotoxicity levels, examination of the general efflux of glycoproteins from ER, and the exploration of the physiological consequences due to UGGT KO. This broader perspective would be valuable for the wider audience. Additionally, the marked increase in ATF4 activity in UGGTKO requires discussion, which the authors currently omit.

The discussion section is brief and could benefit from being a separate section. It is advisable for the authors to explore and suggest other model systems or disease contexts to test UGGT's role in the future. This expansion would help the broader scientific community appreciate the potential applications and implications of this work beyond its current scope.

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Reviewer #3 (Public Review):

This manuscript focuses on defining the importance of UGGT1/2 in the process of protein degradation within the ER. The authors prepared cells lacking UGGT1, UGGT2, or both UGGT1/UGGT2 (DKO) HCT116 cells and then monitored the degradation of specific ERAD substrates. Initially, they focused on the ER stress sensor ATF6 and showed that loss of UGGT1 increased the degradation of this protein. This degradation was stabilized by deletion of ERAD-specific factors (e.g., SEL1L, EDEM) or treatment with mannose inhibitors such as kifunesine, indicating that this is mediated through a process involving increased mannose trimming of the ATF6 N-glycan. This increased degradation of ATF6 impaired the function of this ER stress sensor, as expected, reducing the activation of downstream reporters of ER stress-induced ATF6 activation. The authors extended this analysis to monitor the degradation of other well-established ERAD substrates including A1AT-NHK and CD3d, demonstrating similar increases in the degradation of destabilized, misfolding protein substrates in cells deficient in UGGT. Importantly, they did experiments to suggest that re-overexpression of wild-type, but not catalytically deficient, UGGT rescues the increased degradation observed in UGGT1 knockout cells. Further, they demonstrated the dependence of this sensitivity to UGGT depletion on N-glycans using ERAD substrates that lack any glycans. Ultimately, these results suggest a model whereby depletion of UGGT (especially UGGT1 which is the most expressed in these cells) increases degradation of ERAD substrates through a mechanism involving impaired re-glucosylation and subsequent re-entry into the calnexin/calreticulin folding pathway.

I must say that I was under the impression that the main conclusions of this paper (i.e., UGGT1 functions to slow the degradation of ERAD substrates by allowing re-entry into the lectin folding pathway) were well-established in the literature. However, I was not able to find papers explicitly demonstrating this point. Because of this, I do think that this manuscript is valuable, as it supports a previously assumed assertion of the role of UGGT in ER quality control. However, there are a number of issues in the manuscript that should be addressed.

Notably, the focus on well-established, trafficking-deficient ERAD substrates, while a traditional approach to studying these types of processes, limits our understanding of global

ER quality control of proteins that are trafficked to downstream secretory environments where proteins can be degraded through multiple mechanisms. For example, in Figure 1-Figure Supplement 2, UGGT1/2 knockout does not seem to increase the degradation of secretion-competent proteins such as A1AT or EPO, instead appearing to stabilize these proteins against degradation. They do show reductions in secretion, but it isn't clear exactly how UGGT loss is impacting ER Quality Control of these more relevant types of ER-targeted secretory proteins.

Lastly, I don't understand the link between UGGT, ATF6 degradation, and ATF6 activation. I understand that the idea is that increased ATF6 degradation afforded by UGGT depletion will impair activation of this ER stress sensor, but if that is the case, how does UGGT2 depletion, which only minimally impacts ATF6 degradation (Fig. 1), impact activation to levels similar to the UGGT1 knockout (Fig 4)? This suggests UGGT1/2 may serve different functions beyond just regulating the degradation of this ER stress sensor. Also, the authors should quantify the impaired ATF6 processing shown in Fig 4B-D across multiple replicates.

Ultimately, I do think the data support a role for UGGT (especially UGGT1) in regulating the degradation of ERAD substrates, which provides experimental support for a role long-predicted in the field. However, there are a number of ways this manuscript could be strengthened to further support this role, some of which can be done with data they have in hand (e.g., the stats) or additional new experiments.

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