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TorsinA folding and *N*-linked glycosylation are sensitive to redox homeostasis

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ABSTRACT

The Endoplasmic Reticulum (ER) is responsible for the folding and post-translational modification of secretory proteins, as well as for triaging misfolded proteins. During folding, there is a complex yet only partially understood interplay between disulfide bond formation, which is an enzyme catalyzed event in the oxidizing environment of the ER, along with other post-translational modifications (PTMs) and chaperone-supported protein folding. Here, we used the glycoprotein torsinA as a model substrate to explore the impact of ER redox homeostasis on PTMs and protein biogenesis. TorsinA is a AAA+ ATPase with unusual oligomeric properties and controversial functions. The deletion of a C-terminal glutamic acid residue (ΔE) is associated with the development of Early-Onset Torsion Dystonia, a severe movement disorder. TorsinA differs from other AAA+ ATPases since it is an ER resident, and as a result of its entry into the ER torsinA contains two *N*-linked glycans and at least one disulfide bond. The role of these PTMs on torsinA biogenesis and function and the identity of the enzymes that catalyze them are poorly defined. Using a yeast torsinA expression system, we demonstrate that a specific protein disulfide isomerase, Pdi1, affects the folding and *N*-linked glycosylation of torsinA and torsinAΔE in a redox-dependent manner, suggesting that the acquisition of early torsinA folding intermediates is sensitive to perturbed interactions between Cys residues and the quality control machinery. We also highlight the role of specific Cys residues during torsinA biogenesis and demonstrate that torsinAΔE is more sensitive than torsinA when these Cys residues are mutated.

1. Introduction

The secretory pathway is a sequence of interconnected organelles that play a plethora of roles in eukaryotic cells. One of these critical roles is the post-translational modification (PTM), folding, and transport of proteins to intracellular organelles, to the plasma membrane, and to the extracellular space. The intricate and extensive membrane network of the secretory pathway also provides a platform to regulate signaling cascades and the function of other organelles. In addition, the first compartment in the secretory pathway, the Endoplasmic Reticulum (ER), is a major site of lipid synthesis and serves as a calcium reservoir [1–3]. Throughout the secretory pathway, dedicated chaperones and enzymes monitor the folding and degradation of proteins and the assembly of macromolecular complexes [4,5]. Thus, the function of the secretory pathway is essential for cell survival, and defects in resident components or mutations in substrates that transit through the secretory pathway can lead to a range of diseases [6].

One secretory pathway resident whose folding and function are perplexing is the AAA+ ATPase torsinA [7]. AAA+ ATPases are hexameric proteins with a variety of functions, but many participate in the unfolding of proteins for proteolysis, the dissolution of protein aggregates, and the assembly and disassembly of protein complexes [8,9].

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Indeed, torsinA forms a hexamer and appears to function as a chaperone and contribute to cellular protein quality control [10–14]. However, several features make torsinA a unique AAA+ ATPase [8,15]. First, torsinA and the other members of the torsin family are the only AAA+ ATPases located in the ER lumen [16,17]. Second, whereas most AAA+ ATPases are homohexamers, torsinA variably forms homohexamers [14,18,19], heterohexamers with its cofactors LULL1 and LAP1B [14,18,20–25] (Fig. 1A), or helical filaments [26]. TorsinA interaction with LULL1 and LAP1B is required for maximal ATPase activity and perhaps its chaperone-like activity [14,15,18,22,24,25,27]. Third, torsinA associates with the ER membrane through an N-terminal hydrophobic segment (Fig. 1B) [28]. This appendage retains torsinA in the ER and is required for hexamer formation [16,19,28–30]. Fourth, torsinA has at least one intramolecular disulfide bond (Fig. 1B), which is required for function [17,31–33]. And fifth, torsinA is a glycoprotein [34]. These intriguing features make torsinA an atypical AAA+ ATPase, which has complicated its purification and structural and functional characterization.

TorsinA is associated with the development of Early-Onset Torsion Dystonia (EOTD) [35,36]. EOTD is the most severe form of primary dystonia—the third most common movement disorder—with an average age of onset of ~13 years and the possibility of becoming generalized [37]. The most common mutation associated with EOTD is the deletion of a GAG codon near the 3′ end of the *DYT1* gene, which encodes torsinA. The mutation results in the loss of a single glutamic acid (ΔE) near the protein's carboxyl terminus [35,36]. The ΔE mutation leads to subtle conformational changes that cause multiple biochemical and cellular phenotypes. For example, the mutation causes torsinA to transit from the ER to the nuclear envelope, impacting torsinA stability and selection for different degradation pathways [13,30,38–40]. In addition, the ΔE mutation disrupts the interaction between torsinA and LULL1 or LAP1B, preventing oligomerization and compromising ATPase activity [19,25,26,32]. Further, the ΔE mutation impairs the degradation and trafficking of select membrane proteins, synaptic vesicle recycling, and dopamine neurotransmission [12,13,22,23,32,41–46]. Cells isolated from torsinAΔE-EOTD patients and a torsinAΔE mouse model also showed alterations in ER-associated degradation (ERAD) [13,47]. Collectively, these data highlight the prominent cellular defects associated with the expression of the torsinAΔE protein.

As with all proteins that enter the ER, torsinA folding and stability require ER-resident enzymes [11,30,48]. For example, the ER Hsp70 chaperone, BiP, which is involved in protein translocation, folding, and degradation in the ER, folds and stabilizes torsinA and torsinAΔE [30,49]. In addition, the presence of at least one disulfide bond and two *N*-linked glycans in torsinA indicate that the protein must engage protein disulfide isomerases (PDIs), the oligosaccharyltransferase complex (OST), and the chaperone network that monitors glycoprotein folding [4,6,50,51]. Disulfide bond formation is critical for the correct folding and function of the majority of secretory proteins [52–54], including torsinA [31,32]. Similarly, protein glycosylation is essential for protein folding and quality control, and the lack of glycosylation can accelerate protein degradation [50,55–59]. Notably, torsinA contains six conserved Cys residues (Cys-44, Cys-49, Cys-50, Cys-162, Cys-280, and Cys-319) and two fully occupied *N*-linked glycosylation sites (the N^{143}IT and N^{158}VS sequons; Fig. 1) located near the ATP binding and hydrolysis domains [8,17,28,33,34,60–62]. Cys-280 and Cys-319 form an intramolecular redox-sensitive disulfide bond (Fig. 1) [31,32]. Cys-319 is located within torsinA's non-canonical nucleotide-interacting Sensor-II motif, and nucleotide binding to Sensor-II is dependent on the redox status of Cys-319 [32] (Fig. 1). Disrupting redox sensing or nucleotide binding in this region also impairs torsinA interaction with the LAP1B and LULL1 co-factors [32]. In addition, mutating this C-terminal Cys alters the subcellular localization of torsinAΔE [32]. Moreover, glycosylation is required for torsinA expression and subcellular localization

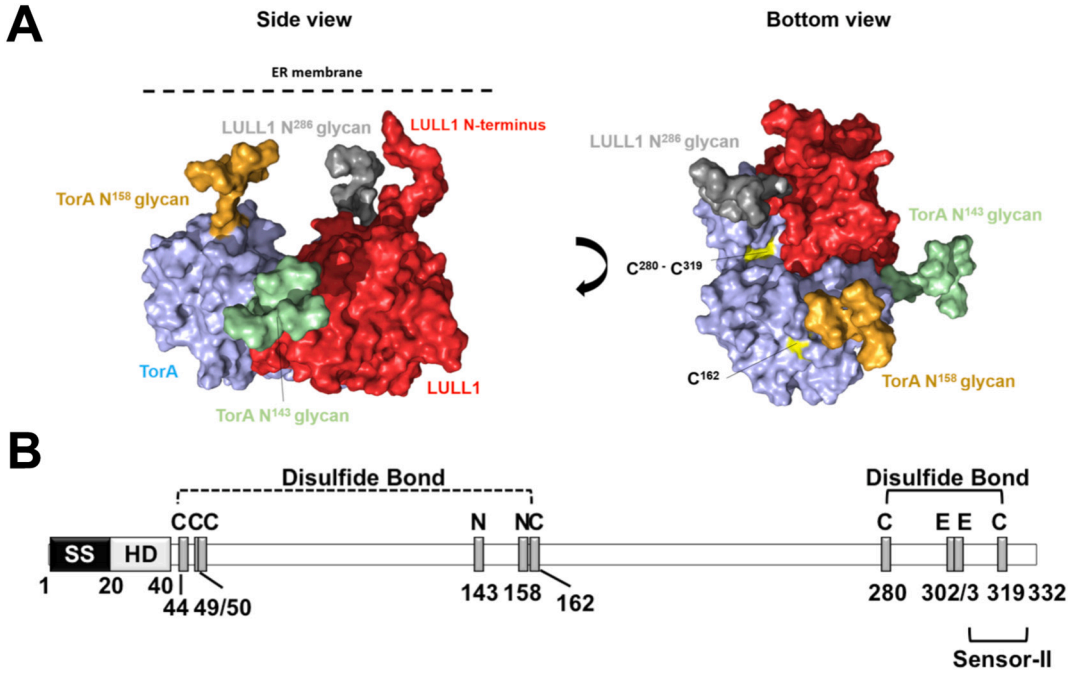

Fig. 1. TorsinA-LULL1 dimer structure and sites of post-translational modification. **A**) Structural modeling of glycosylated torsinA (violet) in complex with the C-terminal domain of its partner LULL1 (red). The type II membrane protein LULL1 is anchored to the ER membrane *via* a single transmembrane domain preceding the N-terminus in the crystal structure. High mannose glycans are shown at Asn-143 (green) and Asn-158 (orange) in torsinA, and at Asn-286 in LULL1 (grey) based on the high-resolution structure (PDB ID 5J1S) of the protein complex [23]. The positions of torsinA Cys-162, Cys-280, and Cys-319 are highlighted in yellow in the bottom view. The ER membrane is also indicated to provide spatial orientation. **B**) Schematic of the torsinA open reading frame showing the location of the six Cys residues and the two Asn residues to which *N*-linked glycosylation moieties are attached. Also shown are the signal sequence (SS), the membrane-embedded hydrophobic region (HD), a potential N-terminal disulfide bond (dashed line), the C-terminal disulfide bond, the Sensor II motif, and the position of the Glu-302/303 residues associated with Early-Onset Torsion Dystonia.

[17,30,60], and may regulate torsinA hexamerization [63]. Together, these data indicate that torsinA Cys and Asn residues play a critical role during torsinA biogenesis, yet a complete understanding of this role and a definition of the enzyme(s) and chaperones involved in torsinA disulfide bond formation, glycosylation, and glycan-dependent quality control is lacking.

PDIs form, isomerize, and reduce disulfide bonds in the ER, which help maintain protein conformation and structure, since improperly formed disulfide bonds can alter protein stability [51–53]. Some PDIs can also act as chaperones, recognizing hydrophobic regions within nascent proteins and shielding them to prevent protein aggregation [22,52,64–70]. PDIs can also act as pro-degradative factors, facilitating the ERAD of select substrates [71–75]. Indeed, prior work indicated that PDIs modulate torsinA and torsinAΔE turnover [76]. Because PDIs effect multiple cellular processes, including protein degradation, we hypothesized that these enzymes play a dual role during the biogenesis and degradation of torsinA and torsinAΔE in the ER, similar to BiP [30].

The human PDI family comprises 21 members, most of which are poorly characterized [77]. On the other hand, the yeast *Saccharomyces cerevisiae* has five well-characterized PDI family members [78–82]. Yeast are amenable to rapid genetic analysis, and because secretory pathway function and most quality control systems were first identified in this organism and are completely conserved in higher cells, studies in yeast have provided hints on the biogenesis of proteins underlying diverse human diseases [83–94]. Thus, to better understand the general roles of PDIs and ER redox homeostasis on protein folding, we used our established torsinA expression system in *S. cerevisiae* [30,76]. We then expressed torsinA and torsinAΔE in yeast cells devoid of select PDIs and performed biochemical studies to determine how these enzymes impact torsinA or torsinAΔE biogenesis and degradation. We show that Pdi1, the primary PDI in yeast, helps fold torsinA and torsinAΔE. We also show that torsinA *N*-linked glycosylation is sensitive to altered redox conditions and that Pdi1 facilitates redox-dependent *N*-linked glycosylation. Finally, we present data that Cys-319 is important for torsinA and torsinAΔE stability and that Cys-162 selectively stabilizes torsinAΔE. Together, these data highlight a major role for ER redox homeostasis and PTMs during the biogenesis of model ER resident proteins.

2. Materials and methods

2.1. Plasmid construction

All vectors used in this study are described in Table 1 and Supplementary Table S1. Vectors containing the torsinA genes with mutations in Cys residues were constructed as follows. pRS426-GPD-torsinA-C162S (pLuBr63) and pRS426-GPD-torsinA-C319S (pLuBr64) were constructed by subcloning torsinA-C162S and torsinA-C319S from pcDNA3.1-torsinA-C162S (pLuBr54) and pcDNA3.1-torsinA-C319S (pLuBr55) (a kind gift from H. C. Wu and M. Zolkiewski, Kansas State University) into pRS426-GPD by *Xho*I and *Eco*RI double restriction enzyme digestion and ligation. pRS426-GPD-torsinAΔE-C162S (pLuBr69) was constructed by subcloning a fragment containing the C162S mutation from pLuBr63 into pRS426-GPD-torsinAΔE (pLuBr280) by BamHI and *Sph*I double restriction enzyme digestion. To make pRS426-GPD-torsinAΔE-C319S (pLuBr68), the C319S mutation was introduced into pLuBr280 by site-directed mutagenesis, using Quikchange Lightning Site-directed mutagenesis Kit (Agilent Technologies) and mutagenic primers LZJB19 (GTTTTCTCAGATAAAGGCTCCAAAACGGTGTTCACCAAG) and LZJB20 (CTTGGTGAACACCGTTTTGGAGCCTTTATCTGAGAAAAC). Primers for site-directed mutagenesis were designed using the Quikchange Primer design application available on the manufacturer's website. TorsinA and torsinAΔE in all generated vectors were fully sequenced.

Table 1
Plasmids used in this study.

Plasmid	Alias	Reference
pRS426-GPD-TorsinA	pLuBr281	[30]
pRS426-GPD-TorsinAΔE	pLuBr280	[30]
pRS426-GPD-TorsinA-C162S	pLuBr63	This study
pRS426-GPD-TorsinAΔE-C162S	pLuBr69	This study
pRS426-GPD-TorsinA-C319S	pLuBr64	This study
pRS426-GPD-TorsinAΔE-C319S	pLuBr68	This study
pRS426-GPD-TorsinA-HA	pLuBr27	[30]
pRS426-GPD-TorsinAΔE-HA	pLuBr28	[30]
pRS426-GPD-TorsinA-N143Q,N158Q	pLuBr106	This study
pRS426-GPD-TorsinAΔE-N143Q,N158Q	pLuBr105	This study
pcDNA3.1-TorsinA-C162S	pLuBr54	This study
pcDNA3.1-TorsinA-C319S	pLuBr55	This study

2.2. Yeast strains, media, and growth conditions

All yeast strains used in this study are described in Table 2 and Supplementary Table S2 and were grown at 26–28 °C on YPD medium (1% yeast extract, 2% peptone, 2% dextrose), except where indicated, or on synthetic complete (SC) medium lacking specific amino acids required for auxotrophic selection as previously described [30].

2.3. Biochemical methods

Biochemical methods for cycloheximide chases to monitor protein stability, membrane extraction to monitor the strength of association with membranes, Endoglycosidase H (EndoH) digestion to monitor the acquisition of *N*-linked glycosylation, cellular protein extraction, and western blotting were previously described [30]. To test the effect of dithiothreitol (DTT), log phase cells were incubated with 10 mM DTT for 1 h at 28 °C before harvesting.

To identify cysteines that had formed disulfide bonds, a previously published protocol that utilizes 4-acetamido-4-maleimidylstilbene-2,2-disulfonic acid (AMS) was used [95,96]. In brief, yeast cells were cultured in SC-URA medium until log phase (OD_{600} ~ 1) and a total of 4.0 OD_{600} of cells was harvested for each sample. The cells were washed with 10× TE buffer (100 mM Tris pH 9.5, 10 mM EDTA), and were then fixed with trichloroacetic acid (TCA) at a final concentration of 10% for 30 min on ice. After centrifugation at 14,000 rpm in a microcentrifuge at 4 °C, the cells were washed five times with 10% TCA and then once with 10× TE buffer before being resuspended in 150 µl 10× TE buffer containing a protease inhibitor cocktail. The yeast cells were then lysed with glass beads by four rounds of 1 min vigorous agitation on a Vortex mixer with a 1 min rest on ice between each step. Next, sodium dodecyl sulfate (SDS) was added to a final concentration of 0.5%. A sample of the lysate (10 µl) was added to 10 µl 10× TE buffer and incubated in the presence or absence of 10 mM DTT for 1 h at 42 °C (to examine the protein under reduced conditions). To identify the free thiols , 10 µl 50 mM AMS was added to the mixture. Under these conditions, the final concentrations of AMS and DTT were 16.7 mM and 6.7 mM, respectively. Next, the samples were incubated for 30 min at 30 °C and then for 15 min at 37 °C. Where indicated, samples were treated with EndoH for 1 h at 37 °C. Proteins were resolved on non-reducing 10% SDS polyacrylamide gels and used for western blot.

The following antibodies were used for western blot analysis in this study: mouse monoclonal anti-TorsinA D-M2A8 (Cell Signaling) at 1:1000 dilution; rabbit polyclonal anti-G6PD (Sigma-Aldrich, Saint Louis, MO) at 1:5000 dilution; rabbit polyclonal anti-Kar2 [97] at 1:5000 dilution; rabbit polyclonal anti-Pdi1 (a gift from V. Denic, Harvard University, Cambridge, MA) at 1:5000 dilution; rabbit polyclonal anti-Sec61 [98] at 1:5000 dilution; rabbit anti-HA (Sigma, H6908) at 1:1000 dilution; and sheep or goat HRP-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (GE Healthcare, Waukesha, WI; and Cell Signaling) at 1:10,000 dilution. Western blots were developed with Supersignal West Pico or Supersignal West Femto Chemiluminescent Substrate (Pierce) and images were visualized using a Kodak 440CF Image Station. The signal was quantified using ImageJ v1.46r (NIH, USA).

Table 2
Yeast strains used in this study.

Strain	Genotype	Source
KAR2	*MATa leu2-3,112 ura3-52 ade2-101*	[162]
W303	*MATα, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1*	Rothstein laboratory
eps1Δ	*MATα, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1, eps1::KANMX*	[74]
eug1Δ	*MATα, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1, eug1::KANMX*	[74]
mpd1Δ	*MATα, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1, mpd1::KANMX*	[74]
mpd2Δ	*MATα, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1, mpd2::KANMX*	[74]
M4492	*MATα pdi1::HIS3 Δeps1 Δeug1 Δmpd1 Δmpd2::G418 ura3 trp1 his3 [pBH1800 (MPD1 CEN TRP19)]*	[102]
SRH01	*MATα pdi1::HIS3 Δeps1 Δeug1 Δmpd1 Δmpd2::G418 ura3 trp1 his3 [pSG01]*	[74]

2.4. Glycoprotein structural modeling

The TorsinA-LULL1 complex structure (PDB ID 5J1S) [23] was downloaded from the protein data bank (https://www.rcsb.org/) and the stabilizing nanobody (VHH-BS2) required for crystallization was removed. Next, the torsinA-LULL1 heterodimer was subjected to *in silico* glycosylation using the CHARMM-GUI Glycan Modeler [99] to attach high mannose *N*-linked glycans to residues Asn-143 and Asn-158 of torsinA and Asn-286 of LULL1. Figures and animations were prepared using PyMOL (Schrödinger) and UCSF Chimera (http://www.rbvi.ucsf.edu/chimera).

2.5. Statistical analysis

Statistical analyses were performed using a one tailed Student's *t*-Test (Microsoft Excel), assuming equal variances. A p value <0.05 was considered significant. All statistical analyses of protein degradation assays were performed only with the values at the end of the chase, reflecting maximal differences between data sets. A minimum of two completely independent yeast transformations were performed, and for each transformation several independent colonies (each representing a biological replicate) were used. All cycloheximide chases were repeated at least four times with independent biological replicates from at least two completely independent yeast transformations. The number of independent yeast colonies/biological replicates is indicated in each figure.

3. Results

3.1. The torsinA and torsinAΔE proteins exhibit different redox-dependent conformational changes in yeast

To determine if torsinA forms disulfide bonds in yeast, we performed a thiol alkylation assay in protein extracts [95,96] (Fig. 2). To this end, whole cell extracts from yeast expressing torsinA or torsinAΔE were collected, treated or not with DTT to reduce any disulfide bonds, and then free thiols were alkylated with AMS in the presence of SDS. The proteins were subsequently analyzed by western blotting. The alkylation of each free Cys with AMS leads to a ~530 Da increase in molecular mass, delaying protein migration by SDS-PAGE. Pdi1 and CPY*, which contain 6 Cys residues/2 disulfide bonds and 12 Cys/5 disulfide bonds, respectively, were used as positive controls (Fig. 2 and data not shown). Unmodified torsinA showed the expected molecular mass of ~36 kDa (Fig. 2, lane 1). Addition of AMS in the absence of DTT led to an increase in torsinA mass from ~36 to ~37.5 kDa (Fig. 2, compare lanes 1–3). This shift indicated that torsinA had unpaired and exposed Cys residues in yeast, as seen in metazoans [31]. When torsinA was reduced with DTT and then alkylated with AMS, the majority of the protein migrated at ~38.5 kDa, indicating AMS alkylation of an additional two Cys residues that had formed a disulfide bond (Fig. 2, compare lanes 3 and 4). However, the ~37.5 band observed with AMS alone still remained after DTT+AMS treatment (Fig. 2, compare lanes 3 and 4). This incompletely alkylated species after DTT treatment may reflect an inherent structural property of torsinA (as discussed in [32]). In contrast, and as controls for this experimental regimen, both Pdi1 and CPY* were fully alkylated after DTT + AMS treatment (Fig. 2 and data not shown), as anticipated [100,101]. Nevertheless, these data indicate that torsinA forms one disulfide bond in yeast.

We next compared the disulfide bonds formed in torsinA and

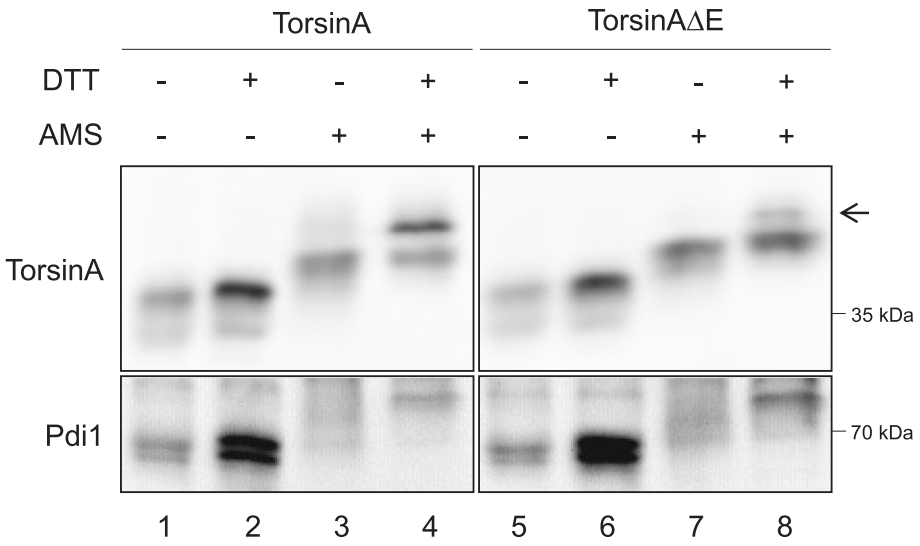

Fig. 2. The redox dependent alkylation of torsinA differs from torsinAΔE. Cell lysates from wild-type yeast expressing torsinA-HA and torsinAΔE-HA were incubated in the presence or absence of 10 mM DTT for 1 h at 42 °C. Available thiols were then modified with AMS. The samples were resolved by non-reducing SDS-PAGE and analyzed by immunoblot with anti-HA or anti-Pdi1 antibodies. Representative western blot images from one of two independent experiments are shown. The arrow denotes a species that differs between torsinA-HA and torsinAΔE-HA.

torsinAΔE in wild-type yeast (Fig. 2). TorsinA and torsinAΔE treated with only AMS migrated similarly (Fig. 2, lanes 3 and 7), suggesting that torsinAΔE also forms one disulfide bond. However, while ~61% of torsinA treated with DTT+AMS shifted to ~38.5 kDa, less than 5% of torsinAΔE migrated at this increased mass (Fig. 2, compare lanes 3 and 4 to 7 and 8, arrow). Thus, while torsinAΔE appears to have a similar number of unpaired free thiols as torsinA, reduced torsinAΔE is conjugated less efficiently to AMS compared to reduced torsinA. This result indicates that a subpopulation of torsinA and torsinAΔE, which is more prominent in torsinAΔE, is unavailable for alkylation, possibly as a result of a DTT-induced conformational change [32]. It is also possible that hypo-alkylation is amplified in torsinAΔE due to the reported C-terminal conformational defects in this mutant [22,23,32].

3.2. Pdi1 stabilizes torsinA and torsinAΔE and is required for N-linked glycosylation

Since torsinA forms a disulfide bridge in yeast, PDIs are likely involved in torsinA folding. The five PDIs in yeast are Pdi1, Mpd1, Mpd2, Eug1, and Eps1, but only Pdi1 is essential for viability [78–82]. We first found that torsinA and torsinAΔE degradation was unaffected in strains lacking any one of the non-essential PDIs when compared to the wild-type strain through cycloheximide chases (Supplementary Fig. S1). Next, to test the effect of the essential Pdi1 enzyme on torsinA and torsinAΔE, we employed two previously published strains in which all the PDI-encoding genes are deleted (*pdi1*Δ *mpd1*Δ *mpd2*Δ *eug1*Δ *eps1*Δ) but growth is rescued by overexpression of either Mpd1 (strain M4492) or Pdi1 (strain SRH01) (Supplementary Fig. S1C) [102,103]. In this case, we observed a small but significant stabilizing effect on torsinA and torsinAΔE in the SRH01 (Pdi1 overexpressing) strain compared to the wild-type strain (81.6% torsinA in the wild-type *vs* 94.8% in the SRH01 strain remaining at 90 min, $p = 0.03$; and 77.7% torsinAΔE in wild-type *vs* 89.9% in the SRH01 strain remaining at 90 min, $p = 0.01$) and compared to the Mpd1-overexpression strain (M4492) (Supplementary Fig. S1). These results suggest that, in the absence of all other PDIs, Pdi1 overexpression stabilizes torsinA and torsinAΔE.

Interestingly, while torsinA and torsinAΔE appeared as the typical single band of ~36 kDa in SDS-PAGE (Fig. 3 and Supplementary Fig. S1), in strain M4492, which only expressed Mpd1, two additional torsinA and torsinAΔE species that migrated faster were observed (Fig. 3, compare lanes 1, 3, and 5, and 9, 11, and 13). This pattern was reminiscent of that observed in the BiP-deficient *kar2-1* yeast strain (Supplementary Fig. S2) as well as in other chaperone mutants which is caused by deficient *N*-linked glycosylation [30]. Therefore, to test whether the lack of Pdi1 led to torsinA hypoglycosylation, we deglycosylated protein extracts with Endoglycosidase H (EndoH), which removes high mannose *N*-linked glycans. We then compared torsinA migration by SDS-PAGE (Fig. 3, compare lanes 5 and 6, and 13 and 14). After EndoH digestion, the lower torsinA and torsinAΔE bands in the M4492 strain migrated similarly to unglycosylated torsinA, while the middle and top bands disappeared, indicating that they represented monoglycosylated and fully glycosylated torsinA and torsinAΔE, respectively (Fig. 3, compare lanes 5 and 6, and 13 and 14). These results indicated that Pdi1 supports maximal *N*-linked glycosylation of torsinA and torsinAΔE.

To determine whether other enzymes that control redox conditions in the ER might similarly affect torsinA glycosylation, we used the *ero1-1* mutant, which leads to defective PDI oxidation at the non-permissive temperature of 37 °C [100,104,105]. As shown in Supplementary Fig. S3, hypoglycosylation of both torsinA and torsinAΔE was only observed in the *ero1-1* strain but not in the wild-type strain when incubated at 37 °C, while full glycosylation was observed at 28 °C. Interestingly, hypoglycosylation was also observed in wild-type cells after DTT treatment (Fig. 3, lanes 7–8, and 15–16, and Supplementary Fig. S4). The effect of DTT on glycosylation was independent of the Unfolded Protein Response (UPR) as it also occurred in an *ire1*Δ strain (Supplementary Fig. S4A, B), which is unable to induce the UPR [106]. In contrast, Pdi1, which contains five sites for *N*-linked glycosylation [107,108], was unaffected by DTT treatment (Fig. 3, lanes 7–8 and 15–16). Together, these results suggest that the absence of Pdi1 alters torsinA and torsinAΔE *N*-linked glycosylation through a redox-dependent mechanism.

3.3. Pdi1 facilitates torsinA and torsinAΔE folding

We previously demonstrated that the ER-lumenal Hsp70 chaperone, BiP/Kar2, augments torsinA and torsinAΔE folding in yeast and mammalian cells and that defects in BiP/Kar2 lead to torsinA hypoglycosylation and aggregation [30] (also see Supplementary Fig. S2). Because the absence of Pdi1 also resulted in torsinA hypoglycosylation (Fig. 3), we hypothesized that the lack of Pdi1 would lead to a similar torsinA aggregation phenotype. To test this hypothesis, microsomes were prepared from the wild-type, M4492, and SRH01 strains expressing torsinA or torsinAΔE and microsomal proteins were extracted under different conditions (Fig. 4). Lumenal and peripheral proteins were extracted from microsomal membranes by incubation with Na_2CO_3 (pH 11.5–12) or 6 M urea, whereas integral membrane proteins were extracted with 1% Triton X-100 [30]. Extractable (S) and non-extractable/aggregated (P) proteins were then separated by ultracentrifugation and analyzed by SDS-PAGE and western blot. To verify extraction efficiency, BiP/Kar2, Pdi1, and Sec61 were used as controls. BiP/Kar2 is a peripherally associated protein and Pdi1 is a soluble lumenal protein, and both can be extracted from membranes at pH >11.5 and with urea [30]. On the contrary, Sec61 is an integral

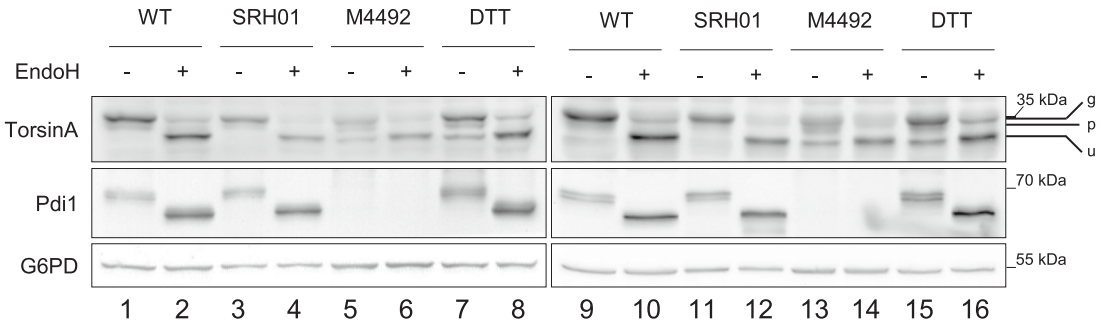

Fig. 3. Pdi1 facilitates torsinA and torsinAΔE *N*-linked glycosylation. Whole cell extracts from wild-type (W303), SRH01 (all PDI-encoding genes absent, but overexpressing Pdi1), or M4492 (all PDI-encoding genes absent, but overexpressing Mpd1) strains expressing torsinA (lanes 1–8) or torsinAΔE (lanes 9–16) were incubated with 10 mM DTT for 1 h where indicated and then treated with Endoglycosidase H (EndoH) or buffer for 1 h at 37 °C. G6PD served as a loading control and Pdi1 controlled for EndoH efficiency. After SDS-PAGE, resolved proteins were immunoblotted with anti-torsinA, anti-Pdi1, or anti-G6PD antibodies. Representative western blot images from one of two independent experiments are shown. "g" indicates the fully glycosylated form, "p" the partially glycosylated form, and "u" the unglycosylated form.

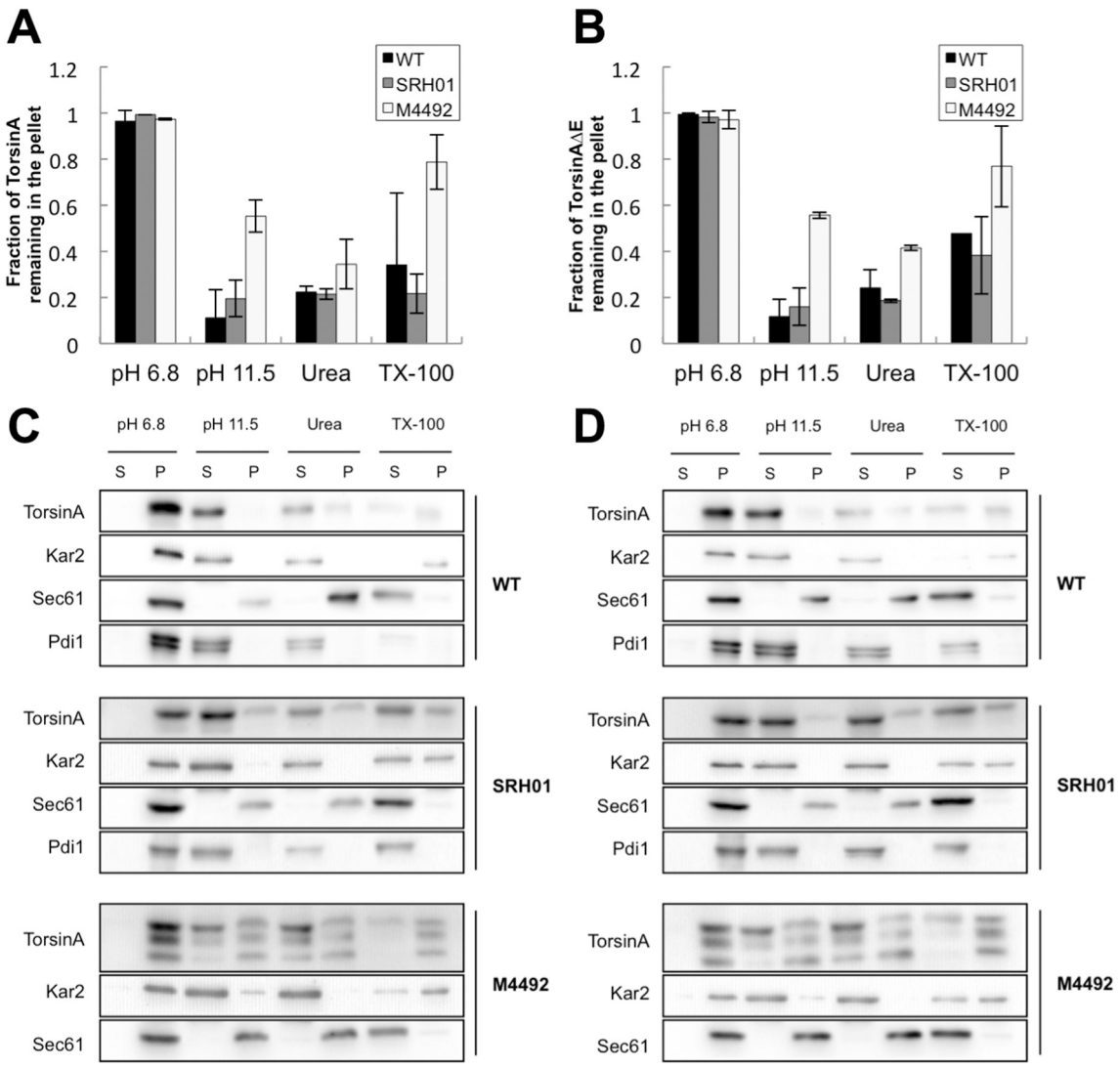

Fig. 4. Pdi1 maintains torsinA and torsinAΔE solubility. Membrane extractions were performed on microsomes from wild-type (W303), SRH01, and M4492 yeast strains expressing torsinA (**A**, **C**) or torsinAΔE (**B**, **D**). Proteins were extracted with 0.1 M Na_2CO_3 (pH 11.5–12), 6 M urea, or 1% Triton X-100 (TX-100), as indicated. Membrane-associated proteins and aggregates (P) were separated by ultracentrifugation, and proteins in the supernatant (S) were precipitated with TCA. After SDS-PAGE, resolved proteins were immunoblotted with anti-torsinA, anti-Kar2, anti-Sec61, or anti-Pdi1 antibodies. The graphs represent the means ± the range of the data from two independent experiments with one replicate per experiment. Representative western blot images from one of two independent experiments are shown.

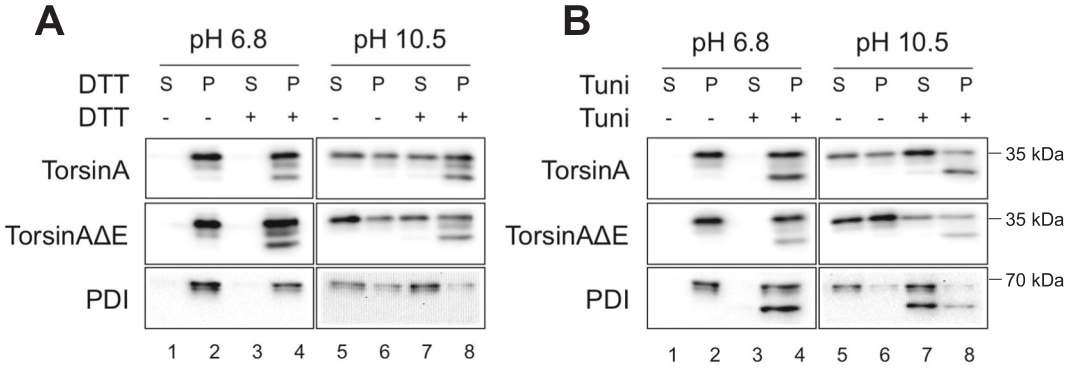

Fig. 5. Differential extraction of torsinA and torsinAΔE glycoforms from isolated membranes. Membrane extractions were performed on microsomes from a wild-type strain expressing torsinA-HA or torsinAΔE-HA incubated for 1 h with 10 mM DTT (**A**) or with 9 μg/ml tunicamycin (Tuni) (**B**). Proteins were treated with buffers at the indicated pH. Membrane-associated proteins and aggregates (P) were separated by ultracentrifugation, and proteins in the supernatant (S) were precipitated with TCA. After SDS-PAGE, resolved proteins were immunoblotted with anti-HA and anti-Pdi1 antibodies. Representative western blot images from one of two independent experiments are shown.

membrane protein [109] and is extracted by membrane-disrupting agents such as Triton X-100. As expected, all proteins in all strains remained membrane-associated at pH 6.8, since microsomes are intact at this pH [110] (Fig. 4). Also as expected, treatment with alkaline pH and urea efficiently released BiP/Kar2 and Pdi1, but not Sec61, which was released by Triton X-100 in all strains. In the wild-type strain, alkaline pH, urea, and Triton X-100 released the majority of torsinA and torsinAΔE into the supernatant (S) (Fig. 4, black bars), consistent with previous reports [16,29,30]. Similar results were obtained for extraction of torsinA and torsinAΔE in the SRH01 strain (Pdi1 overexpressing; Fig. 4, dark grey bars). In contrast, the extraction of torsinA and torsinAΔE expressed in M4492 yeast (Mpd1-overexpressing) was less efficient under all conditions (Fig. 4, white bars). As we had previously observed [30], torsinA and torsinAΔE were similarly extracted by all treatments (Fig. 4). These data were supported in a completely independent experiment, and the resulting quantification ± the range of the data is shown to facilitate data interpretation (Fig. 4A, B). These results indicate that Pdi1 helps retain torsinA and torsinAΔE in an aggregation-free state.

Interestingly, we noticed that torsinA and torsinAΔE extraction in the M4492 strain was affected by glycosylation. Specifically, glycosylated torsinA and torsinAΔE were extracted ~30–40% more efficiently under all conditions than the unglycosylated variants (Fig. 4). We also observed differential glycoform extraction in the *ero1-1* strain (data not shown) and in the wild-type strain treated with DTT or tunicamycin for 1 h (Fig. 5, compare the torsinA glycoforms in lanes 7 and 8). The effect of DTT on differential membrane extraction also occurred in the *ire1*Δ mutant (Supplementary Fig. S4C), indicating that the effect was UPR-independent and strongly suggesting that this phenomenon was a result of disulfide bond reduction. In contrast to the differential extraction observed for torsinA and torsinAΔE, hypoglycosylated Pdi1 was not differentially extracted under any condition (Fig. 5B, compare the two main glycoforms in lanes 7 and 8). Interestingly, hypoglycosylated torsinA was not differentially extracted in the Bip/Kar2 mutant (reference 30). These data suggest that the tendency of torsinA and torsinAΔE to aggregate depends on their glycosylation status, which requires Pdi1 and Ero1, and on the function of specific ER chaperones, such as BiP/Kar2, as previously shown [30]. These data further support the notion that Pdi1 aids torsinA and torsinAΔE folding. Furthermore, our data demonstrate that there is an UPR-independent link between ER redox homeostasis and glycoprotein folding.

3.4. The Pdi1-dependent effect on torsinA and torsinAΔE is independent of its ability to support disulfide bond formation

Our data linking the extent of torsinA *N*-glycosylation with protein solubility (Figs. 2–5 and Supplementary Figs. S3–4) suggest that Pdi1 helps maintain torsinA in an aggregation-free state and thus plays a key role during torsinA biogenesis. Since one of the main roles of PDIs is to oxidize ER lumenal Cys residues and reduce disulfide bonds [51,53], one model to explain our results is that Pdi1 is responsible for forming disulfide bonds in torsinA and torsinAΔE. To test the contribution of Pdi1 during disulfide bond formation in torsinA and torsinAΔE (Fig. 6), we again performed AMS crosslinking studies (*viz.*, Fig. 2).

To test whether Pdi1 supports torsinA and torsinAΔE disulfide bond formation, we performed the AMS assay comparing the wild-type, SRH01 (Pdi1-overexpressing), and M4492 (Mpd1 overexpressing) strains harboring torsinA or torsinAΔE (Fig. 6). To eliminate the confounding effect of hypoglycosylation in the M4492 strain (Fig. 3), we also deglycosylated the samples before SDS-PAGE with EndoH (Fig. 6B). Because no difference in torsinA or torsinAΔE gel migration was apparent between the strains (Fig. 6, compare lanes 2–4 to 5–7), these results suggest that overexpression of either Pdi1 or Mpd1 in a PDI-deficient background is sufficient for torsinA and torsinAΔE disulfide bond formation. This is consistent with previous work showing that Mpd1 supports the essential function(s) of Pdi1 in yeast [102,103]. Consequently, Pdi1 orchestrates torsinA stability and folding independent of disulfide bond formation. These results are also consistent with the previously described chaperone-like function of PDIs [71,111]. Nevertheless, it is formally possible that Pdi1 acts indirectly and instead impacts the function and/or stability of proteins required for torsinA folding and glycosylation.

3.5. Select cysteines are required to stabilize torsinAΔE

Our data show that a change in the redox state or lack of Pdi1 leads to altered torsinA folding and glycosylation (Figs. 3–5). In metazoans, point mutations in Cys-280 or Cys-319 that prevent the formation of the C-terminal disulfide bond alter torsinA's conformation and the subcellular localization of torsinAΔE [32]. Because changes in protein conformation and localization can also result in protein degradation [4,6,51,112], we hypothesized that the Cys-280-Cys-319 disulfide bond stabilizes torsinA. To test this idea, we analyzed the degradation profiles of torsinA-C319S and torsinAΔE-C319S in wild-type yeast cells (Fig. 7A). We also monitored the C162S mutant, which neither appears to participate in disulfide bond formation nor affects binding to interacting

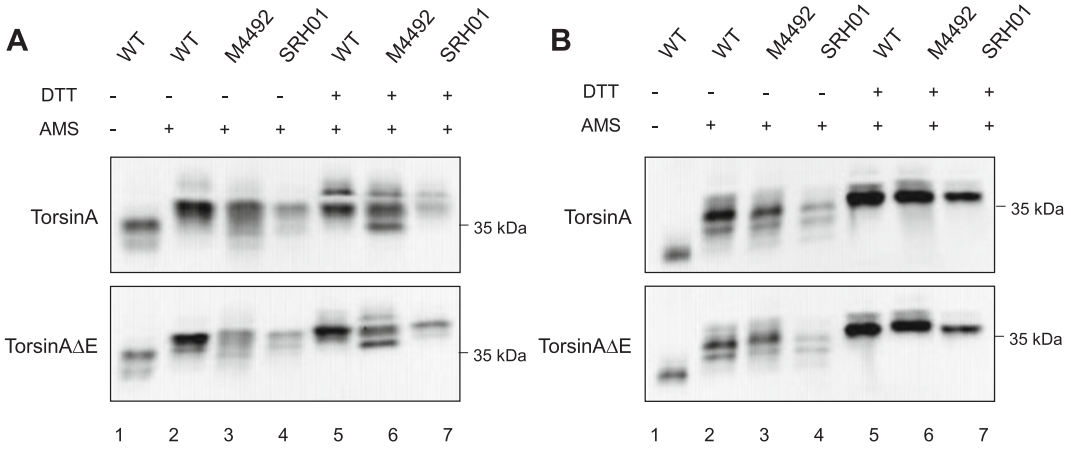

Fig. 6. Pdi1 and Mpd1 function redundantly to support torsinA disulfide bond formation. **A, B**) Cell lysates from wild-type, SRH01 (Pdi1-overexpressing), and M4492 (Mpd1-overexpressing) strains expressing torsinA-HA or torsinAΔE-HA were treated with AMS and/or DTT. **B**) Samples were treated with EndoH before analysis to eliminate the confounding effect of differences in *N*-linked glycosylation. After SDS-PAGE, resolved proteins were immunoblotted with anti-HA antibody. Representative western blot images from one of two independent experiments are shown.

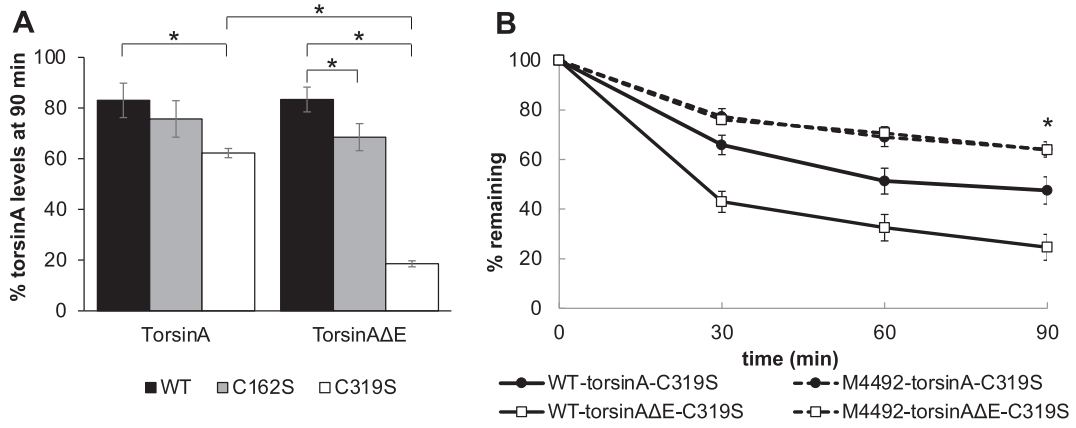

Fig. 7. Destabilizing effects of Cys residue mutations in torsinA and torsinAΔE. **A**) Cycloheximide chase analyses of wild-type yeast (KAR2) expressing torsinA or torsinAΔE (black bars), torsinA-C162S or torsinAΔE-C162S (grey bars), and torsinA-C319S or torsinAΔE-C319S (white bars). The graphs represent the means ± SEM at 90 min from at least 2 independent experiments with at least 2 replicates per experiment ($N = 5–14$). *$p < 0.05$. **B**) Cycloheximide chase analyses of wild-type (W303) (solid line) and M4492 (dotted line) yeast strains expressing torsinA-C319S (black circles) or torsinAΔE-C319S (white squares). The graph shows the means ± SEM of at least 2 independent experiments with at least 3 replicates per experiment ($N = 6–9$). *$p < 0.05$.

partners [31,32]. Thus, we anticipated no impact of the mutation on stability. In agreement with our hypothesis, the C319S mutation significantly destabilized both torsinA (83.0% torsinA *vs* 62.2% torsinA-C319S remaining at 90 min, $p = 0.038$) and torsinAΔE (83.4% torsinAΔE *vs* 18.6% torsinAΔE-C319S remaining at 90 min, $p = 5.5 \times 10^{-8}$) when expressed in wild-type yeast (Fig. 7A). Furthermore, torsinAΔE-C319S was significantly less stable than torsinA-C319S ($p = 1.95 \times 10^{-8}$ for torsinA-C319S *vs* torsinAΔE-C319S) (Fig. 7A). The C280S variants of torsinA and torsinAΔE were also less stable than the wild-type proteins (data not shown). These results suggest that lower stability is caused by the lack of the C280-C319 disulfide bond, but not by an alteration in the Sensor-II domain in which Cys-319 resides (Fig. 1B) [31,32]. Unexpectedly, however, the torsinAΔE-C162S mutant was also significantly less stable than torsinAΔE (83.4% torsinAΔE *vs* 62.5% torsinAΔE-C162S remaining at 90 min, $p = 0.034$, Fig. 7A). The C162S mutation did not affect torsinA stability (Fig. 7A). Since Cys-162 does not appear to form intramolecular disulfide bonds [31,32], it is possible that the mutation alters the local structure, leading to instability. Alternatively, the Cys-162 residue could participate in intermolecular disulfide bond formation or transiently form a disulfide bond during biosynthesis that is absent in the mature protein. Overall, these results show that Cys-319 is required for both torsinA and torsinAΔE stability and that Cys-162 selectively stabilizes torsinAΔE. We therefore propose that the torsinAΔE allele is more sensitive to defects in disulfide bond formation.

We previously observed that BiP/Kar2, a chaperone that stabilizes torsinA and torsinAΔE, was also required for the degradation of torsinA and torsinAΔE variants, such as the torsinAΔE-N158Q mutant in which the second sequon is mutated (Fig. 1) [30]. Thus, we hypothesized that Pdi1, which as noted above functions both in disulfide bond formation and as a chaperone [71,111,113,114], would also play a dual role during torsinA and torsinAΔE biogenesis and thus stabilize the proteins. As anticipated and as shown in Fig. 7B, the degradation of both torsinA-C319S and torsinAΔE-C319S was Pdi1-dependent, as both mutant proteins were significantly stabilized in the M4492 strain compared to the wild-type strain (47.5% in WT *vs* 64.1% in M4492 for torsinA-C319S remaining at 90 min, $p = 0.008$; and 24.6% in WT *vs* 63.8% in M4492 for torsinAΔE-C319S, $p = 1.1 \times 10^{-6}$).

4. Discussion

During secretory protein biogenesis there exists a complex interplay between disulfide bond formation, *N*-linked glycosylation, and protein folding. To better understand the machinery involved in these processes, we used an ER resident protein that undergoes each of these processes, torsinA, as a model substrate. In support of using this model substrate, accumulating evidence points to a role for ER redox homeostasis in regulating torsinA protein interactions and function [12,31,32,40,48,60,115–119]. Based on our work, we describe a previously unappreciated link between Pdi1, ER redox homeostasis, *N*-linked glycosylation, and torsinA and torsinAΔE folding and stability.

TorsinA has six conserved Cys residues whose roles are not completely clear (Fig. 1B). The two neighboring N-terminal Cys residues, Cys-49 and Cys-50, participate in a protease cleavage reaction that releases torsinA from the ER membrane [62]. In contrast, Cys-44 and Cys-162 were proposed to form an intramolecular disulfide bond, but this has not been demonstrated biochemically [31]. However, Cys-280 and Cys-319 form a redox sensitive intra-molecular disulfide bond required for protein function and subcellular residence [31,32]. Consistent with reports in mammalian cells [31,32], we found that torsinA also forms a disulfide bond in yeast (Fig. 2). We also found that DTT+AMS treatment led to the formation of two populations of torsinA and torsinAΔE, one that appears fully alkylated and one that is partially alkylated (Fig. 2). The partial alkylation phenotype could be due to low accessibility to AMS, to rapid reformation of the disulfide bond when DTT is quenched by AMS, or to a DTT-induced conformational change that alters Cys exposure and prevents AMS binding (Fig. 2). Regardless, the lack of full alkylation was more prominent in torsinAΔE (Fig. 2), suggesting that the ΔE allele has an additional effect on torsinA conformation. In fact, the ΔE mutation has been shown to alter the local structure of a C-terminal α-helix in torsinA [22,23], has a negative impact on redox-dependent C-terminal conformational changes in torsinA [32], and induces homo/heterodimerization *via* (most likely non-native) disulfide bridges [12,40,116,120]. Inaccessibility of Cys to alkylating agents has also been observed in other chaperones and ATPases [121,122]. Therefore, the yeast data align with the mammalian cell data, suggesting the continued use of yeast as a model for these studies. Furthermore, previous data in mammalian cells suggested that the torsinA C280S-C319S mutant was less stable and conformationally altered relative to the wild-type protein [32]. In agreement with these data in mammalian cells, we show that mutated Cys-319 altered both torsinA and torsinAΔE stability, displaying a more severe phenotype in torsinAΔE, and that mutation of Cys-162 selectively destabilized torsinAΔE (Fig. 7). Interestingly, our preliminary data suggests that mutating these Cys residues also affects torsinA and torsinAΔE expression levels after transient transfection into recipient human cells (data not shown). Our results suggest that Cys-162 and Cys-319 play a key role during torsinA folding and especially during the folding of torsinAΔE (Fig. 7).

Together, our results expand the repertoire of functions assigned to Cys residues in torsinA.

As might be expected, not all redox-related torsinA phenotypes in mammalian cells are recapitulated in yeast. For example, the higher molecular weight torsinAΔE species (~75–85 kDa) observed in non-reducing gels when lysates are prepared from mammalian cells [12,40,116] were not observed in yeast (data not shown). We also failed to observe the DTT-induced N-terminal torsinA proteolytic cleavage event that occurs in mammalian cells [62] (Supplementary Fig. S4). It is possible that these proteolytic species arise from interactions with mammalian-specific binding partners, from the fact that mammalian specific chaperones/enzymes assist in the formation of these species, or from the ability of yeast to rapidly destroy these species.

PDIs have enzymatic and chaperone functions and can participate in the biogenesis and/or degradation of non-native proteins in the ER [30,52,53,64–68,71–74,123]. Previously we showed that PDIs are pro-degradative factors for torsinA and torsinAΔE [76], and here we show that Pdi1 facilitates the degradation of torsinA Cys mutants (Fig. 7). However, we also show here that PDIs augment torsinA maturation, since they are required for torsinA and torsinAΔE *N*-linked glycosylation and help maintain solubility (Figs. 3–4). Therefore, the PDIs exhibit *bona fide* chaperone activity, as established in other systems [71,111].

Pdi1 is the only yeast PDI that is essential for growth [78–82,102,103], and we found that it was also the critical PDI to support torsinA folding (Figs. 2–4). The mechanism through which Pdi1 impacts torsinA biogenesis is not completely clear, but Pdi1 does not appear to be necessary for *de novo* disulfide bond formation (Fig. 6), likely due to PDI redundancy [102]. However, Pdi1 may help re-form the redox-sensitive C-terminal disulfide bond and eliminate non-native disulfide bonds formed during folding [32]. This conclusion is supported by the fact that mutating Ero1, which re-oxidizes Pdi1 [100,104,105,124], showed similar defects in folding and solubility as deleting Pdi1 (Supplementary Fig. S3 and data not shown). Interestingly, we also observed the same Pdi1-dependent defects in torsinA when ER redox conditions were altered with DTT (Figs. 3, 5, and Supplementary Fig. S4). Therefore, the lack of Pdi1, defects in Ero1 function, or DTT treatment each resulted in torsinA hypoglycosylation (Figs. 3, 5, and Supplementary Figs. S3, S4). DTT, but not other ER stressors, also altered the electrophoretic mobility of torsinA in mammalian cells [30,60,62,115,120]. Furthermore, DTT or protein expression in *ero1-1* or *pdi1*Δ yeast led to the presence of hypoglycosylated torsinA and torsinAΔE that was inefficiently extracted from membranes by alkaline pH, urea, or Triton X-100 (Figs. 4, 5, Supplementary Fig. S4, and data not shown). These data strongly suggest that redox-induced hypoglycosylation is accompanied by a conformational change that renders torsinA aggregation-prone, thereby linking environmental and enzymatic ER redox conditions to *N*-linked glycosylation and the maintenance of protein structure.

N-glycans are bulky, hydrophilic molecules that fundamentally alter glycoprotein interaction with solvents, membranes, and other proteins [55,125]. Glycans not only influence protein function, but they also play an essential role in ER protein quality control [50,58,126]. Further, due to their location in the structure of torsinA (Fig. 1A), glycans may also modulate the formation of the active torsinA oligomer [63]. The susceptibility of torsinA glycosylation to alterations in the redox environment and the function of ER chaperone/enzymes suggest that this PTM could play a key role in torsinA biogenesis and function. Indeed, maximal steady-state expression levels of torsinA require occupancy of at least one *N*-linked glycosylation site in yeast, as well as in mammalian cells (data not shown). This result agrees with data showing the disappearance of torsinA after tunicamycin treatment in mammalian cells [60], and with reduced steady-state levels of torsinA and torsinAΔE when expressed in hypoglycosylating yeast strains [127] devoid of key glycosyltransferase enzymes (Supplementary Fig. S5). Moreover, Asn-158 is required for torsinAΔE stability in yeast, but does not—on its own—impact torsinA stability [30]. Similarly, Asn-158 helps maintain steady-state torsinAΔE levels in mammalian cells but does not, on its own, alter torsinA levels (data not shown). Given the high conservation of the Asn-158 sequon in torsinA across multiple species [63], its potential contribution to torsinA hexamerization [63], and the effect on torsinAΔE biogenesis [30,60], continued studies on the role of torsinA *N*-linked glycans is warranted.

TorsinA hypoglycosylation in response to a range of genetic and environmental insults is notable (Figs. 3 and 4, Supplementary Figs. S3, S5, and S6) [30]. These insults include the absence of functional Kar2 (BiP) or BiP-binding Hsp40s, or deficits in Pdi1, Ero1, Alg6, or Ost3, or after DTT treatment (Figs. 3 and 4, Supplementary Figs. S3 and S5) [30]. Interestingly, torsinA and torsinAΔE mutants lacking the hydrophobic domain are also hypoglycosylated (Supplementary Fig. S6), and the *kar2-113* strain that fails to induce the UPR even in the presence of tunicamycin [128] also hypoglycosylates torsinA (data not shown). Indeed, torsinA stability and glycosylation are not interdependent and do not correlate with the stress response (Figs. 3 and 4, Supplementary Figs. S3, S5, and S6) [30]. In addition, the loss of several other ER chaperones, which leads to increased ER stress, does not necessarily result in torsinA hypoglycosylation [30]. TorsinA glycosylation is also unaffected in mammalian cells in the presence of various stressors [17]. Therefore, the effects on torsinA glycosylation are not necessarily due to an elevated cellular stress response but they are instead rather specific. These data further highlight the value of using torsinA as a model protein to study protein folding mechanisms associated with *N*-linked glycosylation.

The interplay between disulfide bond formation and *N*-linked glycosylation has been well documented [102,129–135]. Some substrates handled by the ER quality control machinery (*e.g.*, CPY* and ApoB) do not require Pdi1 function to be glycosylated [103], but others (*e.g.* CPY) do [102]. Pdi1 is also required for full torsinA *N*-linked glycosylation, a function that cannot be fulfilled by Mpd1 (Fig. 3). However, Mpd1 expression is enough to ensure disulfide bond formation in torsinA (Fig. 6), suggesting that the hypoglycosylation defect in torsinA is not directly associated with disulfide bond formation. In line with this model, the Cys-280 and Cys-319 mutants were not hypoglycosylated (data not shown). There are several possibilities to explain this phenotype. First, torsinA hypoglycosylation may arise from Pdi1-dependent alterations in expression levels of glycosyltransferases, as proposed earlier [102]. Second, Pdi1 and the ER redox conditions may impact the function of specific glycosyltransferases. An ER protein complex that could be a key player in the redox-dependent glycosylation and folding of torsinA is the OST. The OST attaches *N*-linked glycans to proteins on Asn within the consensus sequence Asn-X_{+1}-Thr/Ser_{+2} (where X cannot be Pro) [136,137]. Mammalian OST forms two multi-protein complexes that function either co- or post-translocationally [138–141]. The post-translocational OST complex contains the subunits MAGT1 and TUSC3, which aid in sequon recognition and provide site-specificity [139,140,142–144]. MAGT1 and TUSC3 are thioredoxin-like enzymes that facilitate *N*-linked glycosylation by forming intermolecular disulfide bridges with substrates [141]. In contrast, the yeast OST only exists in one form and contains Ost3 and Ost6, which are the MAGT1 and TUSC3 orthologs [59,131,145] (Supplementary Fig. S5). Therefore, it is possible that the redox dependent activity of these subunits is required for redox-sensitive torsinA glycosylation, suggesting a model by which maintaining redox homeostasis in the ER becomes essential for *N*-linked glycosylation. Third, the redox-dependent effect on torsinA *N*-linked glycosylation could be due to unpaired or inappropriately paired torsinA Cys residues. For instance, a folding intermediate could form an intra- or intermolecular disulfide bond that modulates *N*-linked glycosylation by altering sequon accessibility to the OST. This transient disulfide bond would need to be reduced in order for torsinA to attain its native conformation, and PDIs might act upon this disulfide bond. In the presence of DTT, this bond may inefficiently form, thereby halting torsinA folding and leading to aggregation of hypoglycosylated folding intermediates with unpaired or inappropriately paired Cys residues.

Cys-162 is a prime candidate to generate this bond, since it is located four amino acids downstream of Asn-158, which becomes modified by a glycan (Fig. 1). Cys-162 is also exposed on the surface of the folded molecule (Fig. 1A and Supplementary Fig. S7, and [26]), and it may impact the post-translocational glycosylation of torsinA in mammalian cells [138–141]. Together, our results highlight the role of redox homeostasis on *N*-linked glycosylation and indicate that torsinA achieves its native conformation through a dynamic interplay between disulfide bond formation, *N*-linked glycosylation, and chaperone-assisted protein folding.

The ΔE mutation is autosomal dominant but has a penetrance of ~30%, indicating that other factors are required for disease onset [146,147]. The cellular and molecular defects leading to EOTD are unclear, including whether EOTD is the result of a dominant negative effect of the ΔE mutation [148] or triggered by torsinA haploinsufficiency [48]. While our results do not support either model, they may have implications for disease, as defects in the redox and glycosylation machinery could overlap with the presence of the ΔE mutation. Notably, genes involved in protein glycosylation account for ~2% of the human genome, ~50% of proteins are glycosylated, and defects in glycosylation are associated with an increasing number of diseases (>125 congenital disorders of glycosylation), some of which have an estimated prevalence of 1/10,000 [149]. Similarly, changes in redox homeostasis that can affect disulfide bond formation may trigger the onset of diseases, including neurological diseases [150–152]. Indeed, PDIs are involved in myriad human diseases, including cancer, neurological disorders (such as prion and Alzheimer's diseases, cerebral ischemia, and amyotrophic lateral sclerosis), and infectious diseases, and there are several small molecule modulators of PDI under development [113,114,153–158]. Furthermore, the modulation of chaperone function is an established therapeutic strategy that is being applied in the treatment of cancer and diseases arising from proteins with altered conformations [159–161]. Regardless, the next critical steps are to confirm the roles of PDIs and *N*-linked glycosylation on torsinA biogenesis in mammalian cells using refined genetic and pharmacological approaches, and to better define the degradation pathway(s) that lead to the loss of the many mutant versions of torsinA examined in this study. It will also be vital to determine whether the loss of specific disulfide bonds and *N*-linked glycans exert their effects through a common mechanism, perhaps *via* the alteration of common structural elements that expose buried hydrophobic motifs that trigger proteasome recruitment. Both goals will be pursued in future studies.

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CRediT authorship contribution statement

Jonas Honer: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Katie M. Niemeyer:** Validation, Formal analysis, Investigation. **Christian Fercher:** Investigation, Writing – original draft, Writing – review & editing, Visualization. **Ana L. Diez Tissera:** Investigation. **Noushin Jaberolansar:** Investigation, Writing – review & editing. **Yohaann M.A. Jafrani:** Investigation, Writing – review & editing. **Chun Zhou:** Investigation, Writing – review & editing. **Julio J. Caramelo:** Resources, Writing – review & editing, Supervision. **Annette M. Shewan:** Resources. **Benjamin L. Schulz:** Resources, Writing – review & editing, Supervision, Funding acquisition. **Jeffrey L. Brodsky:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition. **Lucía F. Zacchi:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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