The BiP molecular chaperone plays multiple roles during the biogenesis of TorsinA, a AAA+ ATPase associated with the neurological disease Early-Onset Torsion Dystonia*

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Background: The ΔE mutation in the AAA+ ATPase TorsinA is associated with the neurological disease Torsion Dystonia.

Results: BiP and its co-factors maintain TorsinA and TorsinAΔE stability, glycosylation, and solubility.

Conclusion: TorsinA/ΔE, a chaperone-like protein, requires the assistance of other chaperones to fold.

Significance: Therapeutics that modulate BiP may counteract TorsinAΔE-associated physiological defects.

SUMMARY

Early Onset Torsion Dystonia (EOTD) is a neurological disorder characterized by involuntary and sustained muscle contractions that can lead to paralysis and abnormal posture. EOTD is associated with the deletion of a glutamate (ΔE) in TorsinA, an ER resident AAA+ ATPase. To date, the effect of ΔE on TorsinA and the reason that this mutation results in EOTD are unclear. Moreover, there are no specific therapeutic options to treat EOTD. To define the underlying biochemical defects associated with TorsinAΔE and to uncover factors that might be targeted to offset defects associated with TorsinAΔE, we developed a yeast TorsinA expression system and tested the roles of ER chaperones in mediating the folding and stability of TorsinA and TorsinAΔE. We discovered that the ER lumenal Hsp70, BiP, an associated Hsp40, Scj1, and a nucleotide exchange factor, Lhs1, stabilize TorsinA and TorsinAΔE. BiP also maintained TorsinA and TorsinAΔE solubility. Mutations predicted to compromise specific TorsinA functional motifs showed a synthetic interaction with the ΔE mutation and destabilized TorsinAΔE, suggesting that the ΔE mutation predisposes TorsinA to defects in the presence of secondary insults. In this case, BiP was required for TorsinAΔE degradation, consistent with data that specific chaperones exhibit either pro-degradative or pro-folding activities. Finally, using two independent approaches, we established that BiP stabilizes TorsinA and TorsinAΔE in mammalian cells. Together, these data define BiP as the first identified TorsinA chaperone, and treatments that modulate BiP might improve symptoms associated with EOTD.
INTRODUCTION

In order to function, proteins need to acquire proper secondary and tertiary structures. Although the amino acid sequence of a protein is a major determinant of its final fold, most proteins acquire intermediate folding states during translation that require the help of molecular chaperones and folding enzymes (1-5). Chaperones not only maintain protein solubility and facilitate folding but they also recognize misfolded proteins and target them for degradation via protein quality control pathways (6-11). Indeed, cell survival depends on the efficiency of these pathways, as defects in pathway function are lethal when cells are exposed to various stresses. Moreover, misfolded proteins that are not cleared from the cell can severely impact cellular physiology: these proteins can aggregate, they can gain a dominant negative function, and/or they can deplete chaperones, thereby exacerbating the accumulation of misfolded proteins and compromising protein homeostasis, or "proteostasis" (12-15). Among all the proteins in the eukaryotic cell, approximately one-third enter the secretory pathway through the Endoplasmic Reticulum (ER) and either remain in the ER, are secreted from the cell, or are ultimately distributed among the plasma membrane, endosomes, vacuole/lysosome, or Golgi apparatus. In the secretory pathway, misfolded proteins are cleared through diverse mechanisms, beginning with ER Associated Degradation (ERAD), which represents a critical step in the protein quality control of secretory proteins (10,11,16).

A key chaperone that is required for protein folding in the ER is the luminal Hsp70, BiP/GRP78 (also known as Kar2 in Saccharomyces cerevisiae) (17-19). Kar2/BiP is also a major contributor to general ER homeostasis, not only by participating in protein folding and protein translocation, assembly, and quality control, but also by regulating calcium homeostasis and ER stress signaling, including the unfolded protein response (20,21). Kar2/BiP acts in conjunction with several co-factors that regulate its ATP hydrolytic activity, and, therefore, its ability to bind substrates (21). These co-factors include the Hsp40s Erdj3 and Erdj4 (or Scj1 and Jem1 in yeast), and the nucleotide exchange factors (NEFs) GRP170 and Sil1 (Lhs1 and Sil1, in yeast) (16,22-30). Perhaps not surprisingly, BiP has also been associated with human diseases, including cancer, diabetes, and neurological disorders, such as Parkinson’s disease and Retinitis pigmentosa. These attributes suggest that BiP is a therapeutic target for the treatment of these ailments (31-33).

Many neurological disorders in humans are associated with defects in protein folding, in the function of the secretory pathway, or in protein quality control (34,35), including Amyotrophic Lateral Sclerosis, and Parkinson’s, Huntington’s, Alzheimer’s, and prion diseases (36-40). Dystonia, the third most common movement disorder in humans, is also associated with defects in these pathways (41). Dystonia manifests as involuntary muscle contractions that can lead to paralysis and abnormal postures due to the simultaneous contraction of agonist and antagonist muscles (41,42). A common inherited primary dystonia is Early-Onset generalized Torsion Dystonia (EOTD, also known as childhood onset Torsion Dystonia). EOTD is associated with an autosomal mutation in DYTI (43,44). Although dominant, the mutation in DYTI has low penetrance, indicating the existence of other environmental and genetic factors that are critical for EOTD development (45). The average age of EOTD onset is ~ 13 years, usually beginning in the lower limbs and spreading to other parts of the body. Brain biopsies from those afflicted with EOTD indicate the presence of inclusion bodies and neuronal cell enlargement, without associated neurodegeneration or neuronal cell death (46,47). This lack of neurodegeneration suggests that therapeutic treatment for this chronic, yet progressive movement disorder is possible (41).

DYTI encodes TorsinA, an ER- and nuclear envelope (NE)-localized ATPase that belongs to the AAA+ ATPases superfamily. AAA+ ATPases are a diverse group of enzymes that contain characteristic ATP-binding and hydrolysis domains defined by the Walker-A, Walker-B, Sensor-I, and Sensor-II motifs (Figure 1A) (48). AAA+ ATPases can remodel protein complexes or alter protein conformation, and
many of them play important roles in dissolving aggregates and in protein quality control, including ClpA, FtsH, Hsp104, p97/Cdc48, and one subassembly embedded within the 26S proteasome (48-51). Indeed, TorsinA appears to function as a chaperone and participates in ER protein quality control and the secretory pathway (48,52-56). Until now, it was unknown whether TorsinA itself required the assistance of chaperones to aid in its maturation.

The most common DYT1 mutation associated with EOTD is the deletion of a single glutamate residue from the E302/E303 pair near TorsinA’s C-terminus (“TorsinAΔE”) (Figure 1A). Deletion of E302/E303 causes a variety of alterations in TorsinA’s properties, including protein stability, degradation, subcellular localization, conformational change, and molecular interactions (57-68). How these alterations lead to EOTD is undetermined, which precludes the rational design of targeted therapies to control EOTD progression or prevent the disease.

To identify chaperones that aid in TorsinA folding and that might be targeted to correct disease phenotypes associated with the ΔE allele, we co-opted the genetic tools available in the yeast S. cerevisiae and designed a new TorsinA and TorsinAΔE expression system. Yeast have been used to define the molecular basis underlying several human diseases, including Amyotrophic Lateral Sclerosis, anti-trypsin deficiency, cancer, and Huntington’s, Alzheimer’s, and Parkinson’s diseases, among many others (69-72). Using this system, we found that Kar2/BiP and its Hsp40, Scj1, as well as the Kar2/BiP-associated NEF, Lhs1, contribute to TorsinA and TorsinAΔE stabilization in the ER. We also found that Kar2/BiP plays a dual role in controlling TorsinA stability, depending on the presence of the ΔE mutation and/or of secondary mutations in unique functional motifs. Further, Kar2/BiP affects TorsinA and TorsinAΔE solubility and N-linked glycosylation, consistent with a role in mediating protein folding. Finally, by depleting BiP levels in a mammalian cell model, we confirmed that BiP facilitates TorsinA and TorsinAΔE biogenesis. Together, these data represent the first demonstration of the cellular chaperones that orchestrate TorsinA maturation.

**EXPERIMENTAL PROCEDURES**

**Plasmid and yeast strain construction**

The pcDNA3.1-TorsinA and pcDNA3.1-TorsinAΔE expression plasmids were kindly provided by Dr. Xandra Breakefield (65). The TorsinA and TorsinAΔE open reading frames (ORF) were subcloned into the yeast expression vector pRS426GPD (73) by double restriction enzyme digestion with EcoRI/XhoI (Fermentas, Thermo Scientific) of pcDNA3.1-TorsinA and pcDNA3.1-TorsinAΔE and ligation into EcoRI/XhoI-linearized pRS426GPD (Figure 1B). To construct HA-tagged TorsinA and TorsinAΔE vectors, a single HA tag was introduced at the C-terminus of TorsinA and TorsinAΔE by in vivo recombination in S. cerevisiae following a previously published protocol (74). Briefly, primers LZJB12 and 13, encoding the HA tag sequence (Table 1), were annealed and co-transformed into S. cerevisiae together with NotI-digested pRS426-GPD-TorsinA or TorsinAΔE. Recombined plasmids were extracted from S. cerevisiae and transformed into Escherichia coli DH5α for amplification.

Vectors containing the TorsinA genes with mutations in the N-linked glycosylation sites, pLuBr85 (TorsinA-N143Q), pLuBr86 (TorsinAΔE-N143Q), pLuBr87 (TorsinAΔE-N158Q), pLuBr100 (TorsinA-N158Q), and pLuBr106 (TorsinA-N143Q,N158Q), or at D216, pLuBr60 (TorsinA-D216H), and pLuBr61 (TorsinAΔE-D216H) (Figure 1A and Table 2) were made using Quikchange Lightning Site-directed mutagenesis Kit (Agilent Technologies) using primers LZJB21-24 or LZJB17-18, respectively, that were designed using the Quikchange Primer design application available on the website https://www.genomics.agilent.com (Table 1). pRS426GPD-TorA, pRS426GPD-TorAΔE, and pLuBr100 were then used as the template in a mutagenic PCR reaction using primers LZJB21-24 or LZJB17-18, respectively, that were designed using the Quikchange Primer design application available on the website https://www.genomics.agilent.com (Table 1). pRS426GPD-TorA, pRS426GPD-TorAΔE, and pLuBr100 were then used as the template in a mutagenic PCR reaction using primers LZJB21-24 to introduce the N143Q single mutation, and LZJB23 and LZJB24 to introduce the N158Q single mutation.
Mutations in the Walker-A (K108A) and -B (E171Q) motifs in TorsinA and TorsinA∆E were generated as above, with primer pairs K108A-F and K108A-R, and E171Q-F and E171Q-R (Table 1), respectively. The mammalian expression vectors pcDNA3.1-TorsinA and pcDNA3.1-TorsinA∆E were used as templates. These constructs were then subcloned into pRS426GPD through EcoRI/XhoI digestion and ligation to generate the yeast expression vectors containing a single mutation in the Walker-A motif (K108A; pLuBr20 and 23) or Walker-B motif (E171Q; pLuBr21 and 24) (Figure 1A and Table 2). Similarly, the TorsinA constructs lacking the N-terminal hydrophobic domain (Figure 1A), TorsinA-∆24-40 and TorsinA∆E-∆24-40, were subcloned by EcoRI/XhoI digestion and ligation from pcDNA3.1-TorsinA-∆24-40 and pcDNA3.1-TorsinA∆E-∆24-40 (75) into pRS426GPD, to generate pLuBr18 and pLuBr19, respectively (Table 2). The sequence integrity of all the subcloned DNA fragments and mutagenized sequences was verified by DNA sequence analysis using primers CG_GPDprom and LZJB5 which anneal at the GPD promoter and CYC1 terminator regions in pRS426GPD, respectively (Table 1 and Figure 1B).

All S. cerevisiae strains used in this study are described in Table 3. To construct the strains containing a deletion of the PDR5 or PEP4 ORFs in the kar2-1 strain background, the pdr5Δ::KanMX and pep4Δ::KanMX deletion cassettes containing >200 bp of homology region upstream and downstream of each ORF were PCR amplified (Pfu Turbo, Agilent) from DNA extracted from the corresponding deletion mutant in the haploid BY4742 S. cerevisiae deletion strain collection (Open Biosystems, Thermo Scientific) using primer pairs LZJB69 and 70 (for PDR5) and LZJB34 and 35 (for PEP4) (Table 1), and transformation of these cassettes into the kar2-1 strain (Table 3). The correct genotype of the mutants was confirmed by PCR analysis of genomic DNA of strains resistant to the antibiotic geneticin sulfate G418 (Research Products International Corp.) using primer pairs LZJB69 and 70 (for PDR5) and LZJB34 and 35 (for PEP4), as well as the KanB primer which anneals to the KanMX gene (Table 1). The phenotypes of the constructed strains were verified as follows. Deletion of PEP4 was verified by a defect in the maturation of the vacuolar protease aminopeptidase 1 (Ape1) (76), and deletion of PDR5 was verified by a general increase in the level of ubiquitinated proteins in MG132 (Peptide Institute Inc., Japan) treated cells (77,78) (data not shown). All yeast transformations were performed using lithium acetate/PEG3350, and DNA extractions were performed following standard protocols (79,80).

**Media and growth conditions**

Yeast strains were grown at 26°-28°C on YPD medium (1% yeast extract, 2% peptone, 2% dextrose) or on synthetic complete (SC) medium lacking specific amino acids required for auxotrophic selection as previously described (81). For selection of KanMX expressing strains, YPD plates were supplemented with 250μg/mL G418.

**TorsinA secretion assays**

Overnight cultures of yeast cells grown at 26°-28°C in selective medium were spotted onto a nitrocellulose membrane. The membrane was layered on top of solid selective medium, and plates were incubated ~ 20 hrs at 30°C. To detect secreted protein or total cell protein (which includes the secreted material), we followed a previously published protocol (82). The membranes were then probed for the presence of TorsinA or G6PDH and developed as described below.

**Biochemical methods**

To assess protein stability, cells were grown overnight in selective medium at 28°C, diluted to an initial OD_{600} of 0.2 in fresh medium, and grown at the same temperature for ~ 6 hrs to an OD_{600} of ~ 1. Cycloheximide (CHX) (Sigma) was added to a final concentration of ~190 μg/ml and cells were incubated at 37°C for 90 min. A 1 ml aliquot was taken before adding CHX (time 0 min) and every 30 min after adding CHX. The cells were then pelleted by centrifugation at 18000g for 1 min at 4°C. The cell pellet was immediately frozen in liquid nitrogen. Samples were processed for western blot analysis as...
described below. To inhibit the proteasome, cells were pre-incubated in the presence of MG132 for 30 min at 37°C before addition of CHX.

Yeast protein extracts were prepared as previously described (83). Samples were resuspended in 50 μl trichloroacetic acid (TCA) sample buffer (80mM Tris pH 8, 8mM EDTA pH 8, 3.5% SDS, 5% glycerol, 0.4% Tris base, 0.01% bromophenol blue, and 4% fresh 2-mercaptoethanol), disrupted for 20 sec with a mechanical pestle, heated at 75°C for 5 min, resolved by SDS-PAGE, and fast-transferred to nitrocellulose membranes (Thermo Scientific Pierce Fast Semi-Dry Blotter).

Protein extractions from mammalian cells were performed as follows. Cells were washed with cold PBS, resuspended in RIPA buffer with protease inhibitors (Roche), rocked at 4°C for 40 min, and harvested. The cell suspension was then centrifuged at 18000g for 10 min at 4°C and the supernatant was used for western blotting.

The following antibodies were used for western blot analysis: rabbit polyclonal anti-TorsinA, produced by Cocalico Biologicals (Reamstown, PA), that was made against a soluble lumenal domain of human TorsinA (residues 41-332) which was expressed in Drosophila S2 cells and purified (75); mouse monoclonal anti-TorsinA D-M2A8 (Cell Signaling); rabbit polyclonal anti-G6PDH (Sigma-Aldrich, Saint Louis, MO); rabbit polyclonal anti-Kar2 (84); rabbit polyclonal anti-Pdi1 (a gift from V. Denic, Harvard University, Cambridge, MA); rabbit polyclonal anti-Sec61 (85); rabbit polyclonal anti-Bos1 (a gift from C. Barlowe, Geisel School of Medicine at Dartmouth University, Hanover, NH); mouse monoclonal horseradish peroxidase (HRP)-conjugated anti-HA (clone 3F10, Roche, Indianapolis, IN); goat polyclonal anti-GRP78 (C20, Santa Cruz Biotechnology); mouse monoclonal anti-β actin ab6276 (Abcam); rat monoclonal anti-Grp94 (9G10, Enzo Life Sciences); and sheep or goat HRP-conjugated anti-mouse, anti-rat, anti-goat or anti-rabbit IgG secondary antibodies (GE Healthcare, Waukesha, WI; and Cell Signaling). 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).

To monitor the acquisition of N-linked glycosylation, whole cell protein extracts were digested with 50 mU of Endoglycosidase H (Roche) for 1 hr at 37°C in the presence of 1mM PMSF and 100mM KOAc. Samples were resolved by SDS polyacrylamide gel electrophoresis (PAGE) and used for western blotting, as described above.

To measure protein extraction from microsomal membranes, ER-derived microsomes were prepared from cells grown overnight in selective medium at 28°C, diluted to an OD600 of 0.2, and grown at the same temperature for ~ 6 hrs to an OD600 of ~ 1.2. A total of 60 ODs of cells were harvested and frozen. The pellets were thawed on ice and resuspended in 600 μl of lysis buffer (20mM Hepes pH 7.4, 50mM KOAc, 2mM EDTA pH 8, 100mM sorbitol, 1mM dithiothreitol (DTT)). A total of ~ 500 μl of glass beads were added and samples were agitated on a Vortex mixer 7 times for 60 sec, with a 60 sec incubation on ice between each treatment. The lysate was collected in an Eppendorf tube and centrifuged twice at 3000 rpm at 4°C for 5 min to remove cell debris and unbroken cells. The supernatant was then centrifuged at 18000g at 4°C for 20 min to collect the microsomes, which were washed in 500 μl of Buffer 88 (20mM Hepes pH 6.8, 150mM KOAc, 250mM sorbitol, 5mM MgOAc) and centrifuged as above. Membranes were resuspended in Buffer 88 and the protein concentration was adjusted spectrophotometrically by measuring the OD280 in 2% SDS. Next, 20 μl of the isolated microsomes were resuspended in 1 mL of either Buffer 88 pH 6.8, 1% Triton X-100 (Sigma) in Buffer 88 pH 6.8, 0.1M freshly prepared sodium carbonate in water, 25mM CHES buffer pH 9.5, 25mM CAPS buffer pH 10.5, or 6M urea in 37.5mM Tris pH 8, 2mM EDTA. After the samples were incubated for 30 min on ice, the samples were centrifuged at 100,000g for 1 hr at 4°C in a Sorvall RC M120EX ultracentrifuge. The pellets were washed with 500 μl of Buffer 88, and re-centrifuged at 146000g for 15 min at 4°C. The
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supernatants were transferred to Eppendorf tubes, and total protein was precipitated by adding 110 μl of 100% TCA and incubating on ice for 15 min, followed by a centrifugation at 18000g for 10 min at 4°C. Pellets from samples containing Triton X-100 were washed with acetone. All pellets were resuspended in 35 μl of TCA sample buffer and proteins were resolved by SDS-PAGE and examined by western blotting. All buffers were supplemented with a proteinase inhibitor cocktail.

For protease protection assays, protease inhibitors were removed from the microsomes by washing them twice with 500 μl of Buffer 88. The washed microsomes were incubated on ice for 30 min in the presence or absence of 1% Triton X-100, followed by a 1 hr incubation on ice in the presence or absence of 50 μg/mL Proteinase K (Sigma). Proteins were precipitated with 500 μl of ice-cold 10% TCA and centrifuged at 18000g for 10 min at 4°C. Pellets were resuspended in 30 μl of TCA sample buffer. Samples were resolved by SDS-PAGE and analyzed by western blotting.

For protein co-immunoprecipitation studies, yeast cells were grown overnight in selective medium at 28°C, diluted to an OD600 of 0.2, and grown at the same temperature for ~ 6 hrs to an OD600 of ~ 1.2. A total of ~45 ODs of cells were harvested and frozen. The pellets were thawed on ice and resuspended in 600 μl of lysis buffer supplemented with 0.5% Triton X-100 and a protease inhibitor cocktail, and transferred to borosilicate tubes containing ~ 500 μl of glass beads. Samples were agitated on a Vortex mixer 9 times for 60 sec, with > 60 sec of incubation on ice between each treatment. The lysate was collected in an Eppendorf tube and centrifuged twice at 3000 rpm at 4°C for 5 min to remove cell debris and unbroken cells. The supernatant was further cleared by centrifuging twice at 18000g at 4°C for 20 min. The protein concentration was adjusted spectrophotometrically by measuring the OD280 of an aliquot in 2% SDS. Extracts were precleared by incubation for 1.5 hrs at room temp with 30 μl of Protein G-agarose (Roche), and then incubated overnight with 30 μl of Protein G-agarose (Roche) in the presence or not of 1 μl of anti-Kar2 antiserum. Beads were washed 4 times with 500 μl of wash buffer (20mM Hepes pH 7.4 (buffered with KOH), 150mM NaCl, and supplemented with a protease inhibitor cocktail). 30 μl of TCA sample buffer was added to the beads, and proteins were extracted by heating the beads for 5-7 minutes at 75°C. Samples were resolved by SDS PAGE, and analyzed by western blot.

Indirect immunofluorescence microscopy

The preparation of yeast for indirect immunofluorescence was performed following a previously published protocol (86). Briefly, cells were grown overnight in selective medium at 28°C, diluted to an OD600 of 0.3 in fresh medium, and grown at 28°C for ~ 5 hrs to an OD600 of ~ 0.7. To fix the yeast, 37% formaldehyde was added (final concentration of 4%) and the cells were incubated for 10 min at 28°C with shaking. The cell suspension was centrifuged and the pelleted yeast were resuspended in 5 mL of KM solution (40mM KPO4 pH 6.5, 0.5mM MgCl2) with 4% formaldehyde and incubated for 1 hr at 28°C with shaking. The cells were washed twice with KM solution and once with KM solution supplemented with 1.2M sorbitol (KM+Sorbitol), and were resuspended in 500 μl of KM+Sorbitol. Next, the cell walls were digested with 30 μl of 10 mg/ml Zymolyase (20T, MP Biomedicals LLC) for no more than 25 min at 37°C, and the spheroplasts were washed with KM+Sorbitol buffer, resuspended in 500 μl of the same buffer and incubated overnight at 4°C. A 20 μl aliquot of the cell suspension was spotted on polylysine coated slides, and the cells were dehydrated with methanol/acetone, blocked with PBS pH 7.4, supplemented with 0.5% BSA, 0.5% ovalbumin, and 0.1% Triton X-100, and incubated at 37°C for 1 hr. Primary antibodies (mouse anti-HA 12CA5 (Roche) (1:100), or rabbit anti-Kar2 (1:250)) diluted in blocking solution were applied and the cells were incubated overnight at 4°C, before secondary antibodies (Alexa Fluor 488 anti-mouse and Alexa Fluor 569 anti-rabbit (1:250)) were added for 1 hr at room temperature. The slides were mounted using Prolong Gold Antifade reagent with DAPI (Invitrogen). Pictures were taken using a Leica TCS SP5 Confocal Microscope, 63x oil immersion objective, and analyzed with Adobe Photoshop (v.7.0) software.
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Analysis of TorsinA and TorsinAΔE stability and biogenesis in HeLa cells

HeLa Tet-on cells (Clontech, Mountain View, CA) were maintained in DMEM (Gibco) supplemented with antibiotics and 10% fetal bovine serum at 37°C in a 5% CO₂ humidified incubator. Vectors engineered for the transient expression of TorsinA/ΔE or C-terminal HA-tagged versions of TorsinA/ΔE (Table 2) were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The medium was changed ~ 3.5-4 hrs post-transfection. siRNA transfections were performed using RNAiMax (Invitrogen), and the medium was changed ~ 7 hrs post transfection. In these experiments, the TorsinA expression vectors were transfected into siRNA treated cells 24 hrs after the siRNAs had been introduced. The following Stealth siRNAs (Invitrogen) were used: HSS105077 and HSS179390 against BiP/GRP78, and Stealth RNAi siRNA Negative Control Medium GC (Invitrogen), at a concentration of 1.6nM. Protein extracts were prepared from cells incubated for another 24 hrs (a total of 48 hrs after siRNA transfection). Although higher efficiency of BiP knock-down can be achieved, our protocol was adjusted to minimize cell toxicity (2). As an alternate means to deplete the levels of BiP, we employed the AB5 subtilase cytotoxin (87).

To monitor TorsinA stability, pulse chase analyses were performed as previously published (63) and included a 30 min starvation and 15 min labeling with 200 μCi/well of [35S]Met/Cys (ICN Biochemicals) in a 6-well plate. Protein levels at the indicated time points were analyzed after immunoprecipitation with mouse monoclonal anti-HA 16B12 (Covance) and Protein G-agarose (Roche) using a Typhoon FLA7000 and associated software (v1.2) (General Electric). Where indicated, the active subtilase toxin, or an inactive mutant (SubA272B), were added to a final concentration of 0.5μg/ml during amino acid starvation, and were present throughout the labeling and chase steps.

Statistical analysis were performed using Student’s t-Test (Microsoft Excel Software), 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, since they reflect maximal differences between data sets.

RESULTS

A yeast model to study TorsinA and TorsinAΔE biogenesis

To express TorsinA and TorsinAΔE in S. cerevisiae we subcloned the ORF of TorsinA and TorsinAΔE into the multicopy yeast expression vector pRS426 (73), placing TorsinA under the control of the constitutive glyceraldehyde 3-phosphate dehydrogenase (GPD) promoter (Figure 1B). In this system, TorsinA and TorsinAΔE were expressed at similar levels and migrated by SDS PAGE as ~ 33 kDa species, which correspond to the monomeric forms of the proteins (Figure 1C). Endoglycosidase H (EndoH) treatment of TorsinA and TorsinAΔE protein extracts produced a faster migrating band, indicating that both TorsinA and TorsinAΔE were N-linked glycosylated in yeast, as previously shown in mammalian cells (65) (Figure 1C). Also in accordance with results in mammalian cells, TorsinA-HA and TorsinAΔE-HA were localized in the ER/NE compartments, as evidenced by co-localization with the ER chaperone, Kar2/BiP (Figure 1D). Similar results were obtained using sucrose density gradients with untagged TorsinA and TorsinAΔE, and through live cell fluorescence imaging of GFP-tagged forms of TorsinA and TorsinAΔE (data not shown).

TorsinA is a monotopic membrane protein (88) and behaves like a peripherally associated protein in mammalian cells (89). To determine if this was also the case in yeast we performed alkaline extractions using isolated, ER-derived microsomal membranes from yeast expressing TorsinA or TorsinAΔE, following a previously published methodology for TorsinA (89). All proteins remained associated with the microsomal pellet fraction at pH 6.8, as expected (Figure 2A, lanes 1-2 and 9-10). At pH 9.5 both Pdi1, a soluble
lumenal protein, and Kar2/BiP, a peripherally associated protein (90), were partially released into the supernatant. However, at pH 9.5 TorsinA and TorsinAΔE remained associated with the pellet (Figure 2A, compare lanes 3-4 and 11-12). At pH 10.5, Pdi1 was almost completely released into the supernatant, while a considerable fraction of TorsinA and TorsinAΔE remained associated with the pellet fraction, similar to Kar2/BiP (Figure 2A, compare lanes 5-6 and 13-14). At pH ~11.5, Pdi1 was completely solubilized, while both TorsinA and TorsinAΔE were almost completely extracted, similar to Kar2/BiP (Figure 2A, compare lanes 7-8 and 15-16, and Figure 7C). The transmembrane protein Sec61 remained associated with the pellet at each pH, as expected. Treatment with 6M urea efficiently released Pdi1, Kar2/BiP, and both TorsinA variants, but not Sec61, as expected (Figure 2A, lanes 17-20).

Together, these data indicate that TorsinA and TorsinAΔE are N-linked glycosylated, ER resident proteins that are peripherally associated with the ER membrane in yeast, as in mammalian cells. Because these basic cellular properties of TorsinA are conserved in yeast, we pursued subsequent studies to define how TorsinA and TorsinAΔE impact cellular homeostasis and which cellular factors regulate their protein stability.

TorsinA can modulate the ER stress response, and TorsinA overexpression leads to defects in ERAD and protein secretion (53,56,93-95). To test if TorsinA or TorsinAΔE expression in yeast induces a stress response or leads to any overt phenotype, we compared the behavior of cells expressing TorsinA, TorsinAΔE, or an empty vector control. Cells expressing TorsinA or TorsinAΔE showed no growth defect in the absence or presence of several ER stressors (including heat shock, tunicamycin, and DTT) compared to a strain transformed with an empty vector control (data not shown). Expression of TorsinA or TorsinAΔE in yeast also did not alter the heat shock response of wild-type cells incubated at 39°C, nor did it alter the unfolded protein response in wild-type cells incubated in the presence of tunicamycin (data not shown). These data are in-line with a recently published study in which the phenotypes of yeast expressing TorsinA were analyzed (96). In addition, the degradation of the ERAD substrates CPY* and CFTR was unaltered by the co-expression of TorsinA or TorsinAΔE. Finally no morphological changes were evident in the nuclear envelope of strains expressing TorsinA or TorsinAΔE, as determined by electron microscopy (data not shown) (53,97,98). These combined data indicate that TorsinA is not toxic when expressed in yeast.

The stability of TorsinA and TorsinAΔE depends on the ER chaperone Kar2/BiP

Most of the current knowledge on TorsinA is concentrated on the function and biochemical properties of TorsinA and on the effect the ΔE mutation has on these properties (59,99). However, nothing is known about whether molecular chaperones facilitate the folding, stabilization, and/or degradation of TorsinA and
TorsinAΔE. Using yeast as a model system we set out to identify which chaperones impact TorsinA and/or TorsinAΔE biogenesis.

A critical ER resident chaperone that plays a role in both protein folding and degradation is Kar2/BiP (17-19,100-102). To test the role of Kar2/BiP on TorsinA and TorsinAΔE stability we performed CHX chase experiments in a wild-type (KAR2) strain and in a Kar2/BiP mutant (kar2-1) strain. The kar2-1 allele carries a temperature sensitive mutation that compromises ERAD and protein folding due to a defect in the controlled release of bound peptide substrates (101,103-105). We first noted that there was no significant difference in the degradation of TorsinA and TorsinAΔE in wild-type cells (Figure 3A). These results are in accordance with experiments in mammalian cells showing identical TorsinA and TorsinAΔE turnover when measured during a short time course (63). However, in the kar2-1 strain both TorsinA and TorsinAΔE were significantly destabilized (55% vs. 81% TorsinA and 59% vs. 84% TorsinAΔE remained after 90 min in the mutant strain vs. the wild-type strain, respectively) (Figure 3A). The data suggest that Kar2/BiP helps maintain the stability of TorsinA and TorsinAΔE in yeast.

We noticed that TorsinA and TorsinAΔE expressed in the kar2-1 strain migrated as three distinct bands, with the highest molecular weight species migrating at the position of TorsinA expressed in the wild-type strain (Figure 3B and 3C). Since TorsinA can be N-glycosylated at two asparagines, N143 and N158 (Figure 1A, and see below) (65,106), we hypothesized that the faster migrating bands corresponded to mono- and un-glycosylated TorsinA. Indeed, the mobility of the middle band coincided with the mobility of the TorsinA-N143Q mutant, which can only be mono N-linked glycosylated at the N158 residue (~30 kDa) (Figure 3B). Similar results were obtained for TorsinAΔE (data not shown). This result indicates that, similar to mammalian cells, TorsinA and TorsinAΔE are N-linked glycosylated at both asparagines. Further, the migration of the lowest molecular weight species coincided with the mobility of EndoH-treated samples of TorsinA and TorsinAΔE expressed in both the KAR2 and kar2-1 strains and with the mobility of a TorsinA-N143Q,N158Q double mutant (~27 kDa) (Figure 3B). Therefore, a loss of Kar2/BiP function leads to inefficient N-linked glycosylation of TorsinA and TorsinAΔE. Importantly, the effect of the kar2-1 mutation on TorsinA is specific since the N-linked glycosylation of other substrates, such as CPY*, is unaffected by this mutation ((107) and data not shown).

Although the kar2-1 allele does not appear to affect protein translocation into the ER (101), it was formally possible that the mono- and un-glycosylated TorsinA species in the kar2-1 strain arise from defective translocation. To exclude this possibility we performed a protease protection assay in the KAR2 and kar2-1 strains expressing TorsinA and TorsinAΔE. If indeed the mono- and un-glycosylated TorsinA species in the kar2-1 strain were caused by a translocation defect, they would be exposed on the surface of the microsomes and would be protease-accessible even in the absence of detergent. However, each of the TorsinA and TorsinAΔE species was stable after protease treatment (Figure 3C, compare lanes 1 and 2, 4 and 5, 7 and 8, and 10 and 11). In contrast, addition of 1% Triton X-100 initiated TorsinA and TorsinAΔE degradation (Figure 3C, compare lanes 2 and 3, 5 and 6, 8 and 9, and 11 and 12). Kar2/BiP was also protected from protease in the absence of detergent but was clipped and migrated faster in the presence of detergent. The ER transmembrane protein Bos1, which exposes an epitope to the cytosol (108), was proteolyzed in the presence and absence of Triton X-100, as anticipated. These results indicate that the differentially N-linked glycosylated TorsinA and TorsinAΔE species are ER-encapsulated, and support a role for Kar2/BiP in TorsinA and TorsinAΔE N-linked glycosylation and stability. Because a loss of protein stability is associated with misfolding (109), our data strongly suggest that Kar2/BiP functions as a TorsinA chaperone.

If as expected Kar2/BiP is a TorsinA chaperone, then both proteins should physically interact. To test this hypothesis we obtained whole cell extracts from wild-type cells, immunoprecipitated Kar2/BiP under native conditions, and probed for TorsinA and

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TorsinAΔE by western blot. Both TorsinA and TorsinAΔE co-precipitated with Kar2/BiP in the absence of crosslinking agents (Figure 3D). Only a relatively small fraction of TorsinA and TorsinAΔE co-precipitated with Kar2/BiP, suggesting a transient interaction. Prolonged Hsp70-substrate interactions normally lead to substrate degradation (110,111). However, transient interactions between chaperones and their substrates occur during substrate folding, as previously observed for Kar2/BiP (102,112,113). The combined data indicate that Kar2/BiP is a protein folding chaperone for TorsinA and TorsinAΔE.

**The ER luminal Hsp40 Scj1 and the nucleotide exchange factor Lhs1 also maintain TorsinA and TorsinAΔE stability**

Kar2/BiP function in yeast is augmented by the action of co-chaperones, including the Hsp40s Jem1 and Scj1 and the NEFs Lhs1 and Sil1 (21,114). Therefore, we compared TorsinA and TorsinAΔE stability by CHX chase analysis in a jem1Δ scj1Δ strain to the wild-type strain (jem1 SCJ1) (Figure 4A). Similar to the results observed in the kar2-1 strain (Figure 3A), TorsinA and TorsinAΔE stability were significantly decreased in the jem1Δ scj1Δ strain compared to the wild-type strain (55% vs. 84% TorsinA, and 62% vs. 80% TorsinAΔE remained after 90 min in the mutant and wild-type strains, respectively) (Figure 4A). To determine if one of the two Hsp40s played a more important role in stabilizing TorsinA and TorsinAΔE we performed chase analyses in the jem1Δ and scj1Δ single mutant strains. In agreement with previous reports showing a more prominent role of Scj1 than Jem1 during protein maturation (27,29), TorsinA and TorsinAΔE stability was exclusively SCJ1-dependent (89% vs. 48% TorsinA, and 83% vs. 46% TorsinAΔE remained after 90 min in the jem1Δ and scj1Δ strains, respectively) (Figure 4B). Interestingly, we observed a glycosylation defect in the jem1Δ scj1Δ mutant, but not in the jem1Δ or scj1Δ single mutants (Figure 4A and B). Therefore, while only Scj1 is required to stabilize TorsinA and TorsinAΔE, both Hsp40s support efficient TorsinA and TorsinAΔE glycosylation. These experiments suggest that TorsinA instability is independent of the glycosylation defect.

To test the role of the NEFs on TorsinA and TorsinAΔE stability we performed CHX chases in the lhs1Δ and sil1Δ strains (Figure 4C and data not shown). In accordance with a recent paper indicating a more prominent role for Lhs1 than Sil1 on ER protein biogenesis (115), the stability of TorsinA and TorsinAΔE was significantly decreased in the lhs1Δ strain compared to the wild-type strain (70% vs. 91% TorsinA, and 73% vs. 92% TorsinAΔE remained after 90 min in the lhs1Δ and the wild-type strains, respectively) (Figure 4C), whereas the sil1Δ deletion had no effect on TorsinA or TorsinAΔE stability (data not shown). In the absence of Lhs1 there was no effect on glycosylation (Figure 4C and data not shown), which is consistent with the proposal, above, that the defect in glycosylation is uncoupled from TorsinA stabilization. Together, these data strongly suggest that Kar2/BiP functions with a cognate Hsp40, Scj1, as well as a NEF, Lhs1, to facilitate the folding of TorsinA and TorsinAΔE in the ER.

Importantly, deletion of other ER chaperones had no impact on the stability or N-linked glycosylation of TorsinA and TorsinAΔE, indicating that these phenomena are not the result of a general ER stress response (Figure 5 and data not shown). For example, deletion of the chaperone-like lectins Cne1 and Yos9 had no effect on these TorsinA’s properties (Figure 5). Preliminary data obtained suggest that Mnl1 (the yeast EDEM1 homolog (116)) also has no effect on TorsinA or TorsinAΔE stability or glycosylation (data not shown). Mutations in other ER resident enzymes, such as select protein disulfide isomerases, also showed no effect on TorsinA or TorsinAΔE (data not shown). Further, similar to mammalian cells, the defects in N-linked glycosylation could also be induced by supplementing the growth medium with DTT (117), but this also occurred in an ire1Δ yeast mutant in which the unfolded protein response cannot be induced ((118) and data not shown). These results strongly support our conclusion that compromised TorsinA and TorsinAΔE stability and folding are direct consequences of the lack of Kar2/BiP, Scj1, and Lhs1 activity.
TorsinA and TorsinAΔE are degraded by the proteasome and vacuole when Kar2/BiP function is disabled

In mammalian cells, TorsinA and TorsinAΔE are degraded in the lysosome via autophagy, but TorsinAΔE is also degraded by ERAD (58,62). In order to determine the role of the proteasome and the vacuole—the lysosome equivalent in yeast—during the turnover of TorsinA and TorsinAΔE when Kar2/BiP function is compromised in yeast, we deleted PDR5 or PEP4 in the kar2-1 background. The PDR5 deletion allows for efficient MG132-mediated inhibition of proteasome function (119,120), and the PEP4 deletion inhibits nearly all vacuolar protease activity (121). We monitored TorsinA and TorsinAΔE degradation by CHX chase in the kar2-1 pdr5Δ strain treated with solvent (DMSO) or MG132 and found that both TorsinA and TorsinAΔE were partially, but significantly stabilized by MG132 (73% vs. 58% TorsinA, and 80% vs. 61% TorsinAΔE remained after 90 min in kar2-1 pdr5Δ cells treated with MG132 vs. DMSO, respectively) (Figure 6A).

To determine whether vacuolar proteases contribute to TorsinA and TorsinAΔE degradation, we performed a CHX chase in the kar2-1 pep4Δ strain (Figure 6B) and found that TorsinA was stabilized while TorsinAΔE was only partially, yet still significantly stabilized (91% vs. 55% TorsinA and 76% vs. 59% TorsinAΔE remained after 90 min in the kar2-1 pep4Δ and the kar2-1 PEP4 strains, respectively) (Figure 3A and 6B). Of note, TorsinA degradation in the kar2-1 strain was significantly more dependent on PEP4 than TorsinAΔE (p < 0.04), similar to what has been observed in mammalian cells (58,62). No evidence of TorsinA or TorsinAΔE aggregates was observed during chases in the absence of proteasome or vacuolar function (data not shown).

A defect in Kar2/BiP function alters TorsinA and TorsinAΔE membrane extraction

The Kar2/BiP-dependent defects in TorsinA and TorsinAΔE N-linked glycosylation and the observed destabilization of the proteins when Kar2/BiP function was curtailed suggest that this chaperone facilitates the folding of TorsinA and TorsinAΔE. One method to monitor a change in the folding of peripheral, membrane-associated proteins is to determine the relative strength of membrane association. In other words, defects in folding might lead to greater insolubility, which would be evidenced by reduced membrane extraction in the presence of chaotropic agents (29,101,102,122). To this end, we compared TorsinA and TorsinAΔE extraction from microsomes prepared from wild-type and kar2-1 strains after treatment with alkaline sodium carbonate buffer (pH ~11.5) (Figure 7A). As expected, TorsinA and TorsinAΔE associated with the pellet fraction after microsomes prepared from the kar2-1 strain were incubated at pH 6.8 (Figure 7A, lanes 1, 2, 5, and 6). While incubation with sodium carbonate released the majority of TorsinA and TorsinAΔE from the membrane in the wild-type strain (Figures 2A and 7C), in kar2-1 microsomes alkaline extraction was significantly less efficient (64% vs. 25% TorsinA, and 88% vs. 31% TorsinAΔE remained in the membrane fraction in the kar2-1 and wild-type strains, respectively; P < 0.001) (Figure 7A, lanes 3, 4, 7, and 8, and 7C). Interestingly, TorsinAΔE associated more avidly with the membrane than TorsinA in the kar2-1 strain (P < 0.02), suggesting that the presence of the ΔE mutation causes a subtle folding defect, which is exacerbated when Kar2/BiP is disabled.

Kar2/BiP helps maintain substrate solubility, and in its absence substrates such as pro-alpha factor, CPY*, and CPY oligomerize or aggregate (29,101,102). To test if decreased Kar2/BiP function also triggers TorsinA and TorsinAΔE aggregation, we extracted TorsinA and TorsinAΔE from membranes collected from wild-type and kar2-1 cells using the non-ionic detergent Triton X-100. In this experiment, TorsinA and TorsinAΔE were readily extractable by 1% Triton X-100 from microsomes prepared from wild-type cells (Figure 7B, lanes 1-4). However, in microsomes prepared from the kar2-1 strain there was a significant increase in the fraction of TorsinA and TorsinAΔE that remained associated with the pellet (51% vs. 14% TorsinA, and 48% vs. 18% TorsinAΔE in the kar2-1 vs. the wild-type strains, P < 0.008) (Figure 7B, lanes 5-8, and...
All of the differentially glycosylated species of TorsinA and TorsinAΔE in the kar2-1 strain were extracted to a similar degree both by detergent and alkali, indicating that the glycosylation state had no effect on TorsinA and TorsinAΔE solubility (Figure 7). Together, these results suggest that compromised Kar2/BiP function leads to folding alterations that decrease TorsinA and TorsinAΔE solubility, which in turn initiates TorsinA and TorsinAΔE aggregation. The data also provide further support for our hypothesis that Kar2/BiP is a TorsinA chaperone in yeast.

**Mutations in TorsinA functional motifs exhibit a synergistic interaction with the ΔE mutation and destabilize TorsinA**

Mutation of functional domains in TorsinA has provided valuable information on TorsinA function and on the role of the ΔE allele on disease-associated phenotypes. For example, mutations in TorsinA’s C-terminal cysteines prevent a redox-dependent conformational change in TorsinA that is critical for TorsinA function (63). The ΔE mutation yields the same phenotype in TorsinA as the cysteine mutant, suggesting that it also impacts local conformation (63). Furthermore, some intragenic mutations may mimic the effects of cellular or ER stresses in TorsinAΔE-expressing cells, potentially replicating events that lead to the manifestation of the DYT1 mutation and EOTD development. Indeed, mutations in the N-linked glycosylation sites (Figure 1A) can alter TorsinAΔE subcellular localization and inclusion formation in mammalian cells (106). Thus, an examination of secondary mutations in the context of the ΔE allele in yeast may unveil previously undiscovered ΔE phenotypes.

In our yeast system and in some mammalian cell studies, no difference was observed in the stability of TorsinA and TorsinAΔE (Figures 3-5, and (63)). However, TorsinAΔE showed enhanced membrane association compared to TorsinA in the kar2-1 strain (see above, Figure 7). This data suggests that lack of key chaperones can exacerbate subtle effects of the ΔE allele, which appear otherwise indistinguishable from the wild-type allele by our methods. We reasoned that TorsinAΔE instability may also be magnified if the ΔE allele is combined with additional intragenic mutations. Therefore, we set to explore the effects on TorsinA and TorsinAΔE stability when other mutations were engineered into distinct functional domains in TorsinA.

TorsinA has two asparagines to which N-linked glycans are added (N143 and N158; Figure 1A) (106,123). To examine how the presence of the ΔE mutation affects TorsinA stability in combination with mutations in the first (N143Q) or second (N158Q) N-linked glycosylation sites, we performed chases in a wild-type strain (KAR2) transformed with the TorsinA and TorsinAΔE alleles carrying either of these secondary mutations (Figure 8A). The N143Q mutation had no significant effect on the stability of TorsinA or TorsinAΔE (89% TorsinA-N143Q vs. 81% TorsinA, and 84% TorsinAΔE-N143Q vs. 84% TorsinAΔE remained after 90 minutes) (Figure 9B). The N158Q mutation also had no significant effect on TorsinA stability (74% of TorsinA-N158Q vs. 81% TorsinA remained at 90 minutes) (Figures 8A and 9B). However, the N158Q mutation significantly decreased TorsinAΔE levels (42% TorsinAΔE-N158Q vs. 84% TorsinAΔE remained at 90 minutes, P < 0.0004) (Figure 8A and 9B). These results are in accordance with data suggesting a more prominent role of glycosylation at position N158 in TorsinAΔE in mammalian cells (106), and indicate that glycosylation at position N158 is critical to stabilize TorsinAΔE, but not TorsinA.

The integrity of the Walker motifs is critical for the function of AAA+ ATPases (48,124-126). Indeed, mutations in the Walker motifs that are predicted to disrupt ATP binding (K108A) or hydrolysis (E171Q) (Figure 1A) (48,63,124,125,127) can alter TorsinA and/or TorsinAΔE subcellular localization, inclusion formation, and/or binding to interacting partners in mammalian cells (56,60,61,63,127-129). To test how the ΔE mutation affects TorsinA stability in the presence of mutations in the Walker motifs, we performed CHX chase experiments in a wild-type strain (KAR2) transformed with TorsinA and
TorsinAΔE alleles carrying either of these additional mutations (Figure 8C). The K108A mutation led to a significant decrease in the stability of both TorsinA and TorsinAΔE (40% TorsinA-K108A vs. 81% TorsinA and 20% TorsinAΔE-K108A vs. 84% TorsinAΔE remained after 90 minutes, P < 0.0007) (Figures 8C and 9B). On the contrary, while the E171Q mutation appeared to increase the stability of TorsinA (94% TorsinA-E171Q vs. 81% TorsinA remained after 90 minutes), it significantly destabilized TorsinAΔE (55% TorsinAΔE-E171Q vs. 84% TorsinAΔE remained after 90 minutes, P < 0.0007) (Figures 8C and 9B). Therefore, mutations in the Walker-B motif lead to opposite effects on TorsinA stability, depending on the presence of the ΔE mutation. Importantly, both the K108A and E171Q mutations had a greater destabilizing effect on TorsinAΔE than on TorsinA (p < 0.03) (Figure 9B). K108A-mediated destabilization in TorsinA and TorsinAΔE and the stabilizing effect of the E171Q mutation in TorsinA are in agreement with results for other AAA+ ATPases (124,126,130). Overall, these experiments support our hypothesis that the ΔE mutation harbors a subtle folding defect and that this defect is amplified in the presence of additional mutations that affect TorsinA conformation.

A naturally occurring polymorphism in ΔE carriers (D216 to H216) influences ΔE penetrance (Figure 1A) (131,132). The D216H mutation alters TorsinAΔE inclusion formation in mammalian cells and counteracts TorsinAΔE-associated ER stress in a Caenorhabditis elegans model (93,94). To test if the D216H mutation influences TorsinA and TorsinAΔE stability we performed CHX chases (Figure 9A). We found that introduction of the D216H mutation did not change the stability of glycosylation status of TorsinA or TorsinAΔE in a wild-type strain (85% TorsinA-D216H vs. 81% TorsinA, and 76% TorsinAΔE-D216H vs. 84% TorsinAΔE remained after 90 minutes) (Figure 9). Lack of Kar2/BiP significantly destabilized both TorsinA-D216H and TorsinAΔE-D216H (P < 0.02), but at similar levels than TorsinA and TorsinAΔE (66% TorsinA-D216H vs. 55% TorsinA, and 60% TorsinAΔE-D216H vs. 59% TorsinAΔE remained at 90 minutes in the kar2-1 strain) (Figure 9). We observed that TorsinA-D216H appeared somewhat more stable than TorsinAΔE-D216H in the wild-type strain (Figure 9), but this difference was not significant (p = 0.084). These results suggest that the synthetic effects of the D216H mutation observed in patients and C. elegans models are not caused by altered TorsinA stability.

Kar2/BiP plays a dual role during TorsinA and TorsinAΔE biogenesis

Loss of Kar2/BiP led to the formation of incompletely glycosylated forms of TorsinA and TorsinAΔE and to a decrease in protein stability (Figure 3). In our assays, all the differentially glycosylated species in the kar2-1 strain behaved similarly (Figures 3 and 7). However, our results also indicate that robust glycosylation is required for TorsinAΔE stability in a wild-type strain (Figure 8A). These observations can be reconciled by previous results in yeast that demonstrate that chaperone requirements can vary depending on a protein’s glycosylation state (133,134). Thus, we hypothesized that while Kar2/BiP is required for stabilizing fully glycosylated TorsinA and TorsinAΔE (Figure 3A), it is also required to degrade the mono- and un-glycosylated TorsinAΔE species. To test this model we performed CHX chases of TorsinA and TorsinAΔE containing the N143Q or N158Q mutations in the kar2-1 strain (Figure 8B and 9B). In agreement with our model, lack of Kar2/BiP function significantly stabilized TorsinAΔE-N158Q (69% TorsinAΔE-N158Q vs. 42% remained at 90 minutes in the kar2-1 or the wild-type strains, respectively, P < 0.002) (Figures 8B and 9). We also found that TorsinA-N143Q was significantly destabilized in the kar2-1 strain (75% vs. 89% TorsinA-N143Q remained at 90 minutes in the kar2-1 and the wild-type strains, respectively, P < 0.03) (Figures 8B and 9B). This result suggests that a lack of N-linked glycans at N143 in TorsinA does not impact the role of Kar2/BiP in maintaining TorsinA stability. On the contrary, although there was a clear trend toward lower stability in the kar2-1 strain, neither TorsinA-N158Q nor TorsinAΔE-N143Q stability was significantly altered in the kar2-1 strain (65% vs. 74% TorsinA-N158Q, and 76% vs. 85% TorsinAΔE-N143Q remained at 90 minutes in the
kar2-1 or the wild-type strains, respectively). This result suggests that either lack of N-linked glycans at N158 in TorsinA and at N143 in TorsinAΔE abrogates Kar2/BiP-dependence for stability, or that these species are protected from degradation in the absence of Kar2/BiP (Figures 8 and 9). Thus, combined with the data provided above (Figures 3, 8A, and 8C), Kar2/BiP can play either a pro-degradative or a pro-folding role depending on the glycosylation state of TorsinA and TorsinAΔE.

To determine if Kar2/BiP’s dual role was only associated with TorsinA’s glycosylation state or was more generally associated with protein folding, we measured the stability of TorsinA and TorsinAΔE Walker-A and Walker-B mutants in the kar2-1 strain (Figure 8D). Consistent with the acquisition of an altered conformation, the degradation of TorsinA-K108A, TorsinAΔE-K108A, and TorsinAΔE-E171Q was significantly attenuated in the kar2-1 strain (57% vs. 40% TorsinA-K108A, 66% vs. 20% TorsinAΔE-K108A, and 72% vs. 55% TorsinAΔE-E171Q remaining after 90 minutes in the kar2-1 and wild-type strains, respectively, P < 0.04) (Figure 8D and 9B). On the contrary, TorsinA-E171Q was significantly destabilized in kar2-1 yeast compared to the wild-type strain (59% vs. 94% TorsinA-E171Q remaining after 90 minutes in the kar2-1 and wild-type strains, respectively, P < 0.01) (Figure 8D and 9B). Taken together, these results indicate that Kar2/BiP plays a dual role during TorsinA degradation, which is linked to the folding or conformational states of TorsinA.

**BiP also promotes TorsinA and TorsinAΔE stability in mammalian cells**

To test if the mammalian BiP homolog also contributes to TorsinA and TorsinAΔE biogenesis in mammalian cells, we first compared the levels of TorsinA and TorsinAΔE in HeLa cells in which BiP expression was silenced using siRNA (Figure 10A). We designed a protocol using the BiP-specific siRNA 077 to obtain a reduction of BiP levels by ~ 55% compared to a control siRNA, in order to prevent defects in cellular viability caused by greater BiP silencing (data not shown). These data are consistent with previous observations that BiP levels cannot be reduced to < 40% of wild-type levels (2). Also consistent with previous data (135,136), the depletion of BiP was accompanied by the induction of Grp94, which is another major ER chaperone (Figure 10A). Under these conditions, the steady state levels of both TorsinA and TorsinAΔE were decreased by 70-75% compared to the negative control (Figure 10). Similar results were observed using another BiP-targeted siRNA (data not shown). These results suggest that BiP affects the stability of both TorsinA and TorsinAΔE in mammalian cells.

To confirm these data, we implemented a second approach to deplete BiP. To this end, we performed a TorsinA pulse-chase in HeLa cells transiently expressing TorsinA or TorsinAΔE in which BiP levels were acutely reduced by means of the AB5 subtilase cytotoxin, also known as the SubAB toxin (Figure 11). SubAB is a bacterial serine protease that cleaves and inactivates BiP rapidly and specifically (87). Indeed, only 45 min after the toxin was added to the culture medium (t = “0” minutes of the chase), BiP levels dropped to ~35% of the levels of BiP in cells treated with the catalytically inactive toxin, SubA A272 (Figure 11A). Moreover, BiP could not be detected by western blot in toxin-treated cells 1 hr into the chase, whereas the catalytically inactive toxin had no effect on BiP (Figure 11A). As anticipated, the reduction of BiP upon SubAB treatment also significantly destabilized TorsinA (26% vs. 44% of TorsinA remained after a 4 hr chase in cells treated with SubAB or SubA A272, respectively, P < 0.02) (Figure 11A and B). Similar results were observed if only the fully glycosylated bands were quantified (data not shown). Although the effect was less pronounced, there was also a difference in TorsinAΔE stability in the presence of active or inactive toxin (31% vs. 39% TorsinAΔE remained after a 4 hr chase in cells treated with SubAB or SubA A272, respectively, P < 0.02) (Figure 11A and B). We also noticed that the levels of un-glycosylated TorsinA and TorsinAΔE were higher at the beginning of the chase after toxin treatment compared to inactive toxin treatment or no treatment (72% vs. 53% of un-glycosylated TorsinA and TorsinAΔE in SubAB vs. SubA A272 treated cells or untreated cells, respectively).
Because the un-glycosylated species appears to mature into the fully glycosylated form of TorsinA, the more rapid disappearance of TorsinA is likely not due to the selective turnover of the un-glycosylated protein (Figure 11A). In fact, the unglycosylated form degraded similarly irrespective of whether the sample was treated with SubAB or SubA_{A272}B (data not shown).

Further, because of the acute nature of the subtilase treatment, the effect on TorsinA maturation and stability is unlikely to be caused by the upregulation of other chaperones. In contrast to the knock-down experiments, GRP94 levels did not vary during the >4 hr treatment with the toxin (data not shown). Taken together, the results using toxin-mediated BiP depletion are in accordance with both the results using RNAi-mediated depletion of BiP in mammalian cells and experiments in the yeast genetic system, and establish BiP as a TorsinA chaperone.

DISCUSSION

EOTD, one of the most common forms of inherited primary dystonias, is associated with a dominant ΔE302/303 mutation in the gene DYT1, which encodes the ER/NE localized AAA+ ATPase TorsinA (43,44). However, the variable phenotypic manifestation and low penetrance of the ΔE mutation (45) indicate that additional environmental or genetic factors must play a role in EOTD onset. One hypothesis is that ΔE impacts TorsinA folding, and thus modifies TorsinA’s half life, solubility, and/or interaction with chaperones, as evident in other genetic, “conformational” diseases (34). Cellular chaperone levels are sensitive to genetic and environmental insults. Thus, altering chaperone levels could augment the phenotypes associated with the ΔE mutation. By understanding the chaperone microenvironment required for the folding, stabilization, and degradation of wild-type TorsinA and of the disease-associated TorsinAAE variant, we hope to identify cellular factors that can be targeted with therapeutics. In this work we report for the first time on ER chaperones required for TorsinA and TorsinAAE biogenesis.

We discovered that Kar2/BiP is required to maintain the N-glycosylation and solubility of TorsinA and TorsinAAE in a yeast model (Figures 3 and 7), and that in the absence of Kar2/BiP, TorsinA and TorsinAAE are destabilized and degraded (Figures 3 and 6). In addition, we obtained preliminary evidence that the proteins are ubiquitinated (data not shown), which is in agreement with a recent study showing that the E3 ubiquitin ligase, FBG1, is involved in the degradation of TorsinA and TorsinAAE (137). We also found that Kar2/BiP plays a pro-degradative role when the stability of TorsinA and TorsinAAE is compromised (Figures 8 and 9). Finally, we provide evidence that BiP supports TorsinA biogenesis in human cells (Figures 10 and 11). Therefore, to our knowledge, BiP is the first ER molecular chaperone that has been reported to facilitate the maturation of TorsinA and TorsinAAE.

The ΔE mutation affects TorsinA’s redox sensitive conformational changes, potentially by altering the local structure of an alpha helix in the C-terminal subdomain (63,94). Nevertheless, several studies have failed to uncover significant structural differences between TorsinA and TorsinAAE, suggesting that the ΔE mutation confers a more subtle effect on TorsinA’s structure (91,94). This idea is supported by our observations that the ΔE mutation increases TorsinA membrane association in the absence of functional Kar2/BiP (Figure 7), and that the ΔE mutation and mutations in several functional motifs exhibit a synergistic interaction that affects TorsinA stability (Figures 8 and 9). These data also suggest that the effect of the ΔE mutation could be amplified through secondary genetic or environmental insults, potentially mimicking the sequence of events that is required to manifest the dominant but poorly penetrant ΔE mutation (45,91,94). Indeed, the secondary mutations we incorporated might mimic alterations in the availability or function of chaperones, enzymes, or interacting partners, which in turn could play a role in EOTD onset and/or progression.
BiP chaperones TorsinA maturation

Defects in N-glycosylation are associated with several human diseases (139-141). Interestingly, we found that defects in Kar2/BiP or its associated Hsp40s (Scj1 and Jem1) altered TorsinA and TorsinAΔE N-glycosylation, but this defect was uncoupled from destabilization (Figures 3 and 4). How do these chaperones impact N-linked glycosylation? The kar2-1 (BiP mutant) allele has not been shown to affect the glycosylation of other secreted substrates; therefore, a general effect on the glycosylation machinery can be ruled out. The efficiency of N-linked glycosylation is not only determined by the amino acid sequence to which the modification is appended (the NXS/T tripeptide motif) but is also affected by secondary and tertiary structure, hydrophobic collapse, and disulfide bond formation (142). Notably, TorsinA has two asparagines that can be glycosylated: N143 and N158 (Figures 1 and 3, and (65,123)). It is possible that the presence of the cysteine at position 162 (Figure 1A) may affect glycosylation at the second acceptor site, N158 VS by altering the local conformation through the formation of an intramolecular disulfide bond (143), or by forming a covalent bond with the oligossacharyltransferase during translocation (144). Any effect on glycosylation may be potentiated if the polypeptide is improperly folded due to compromised chaperone function, e.g., when TorsinA is expressed in the kar2-1 yeast. This hypothesis is supported by the observations that treatment with the reducing agent, DTT, or deletion of the N-terminal hydrophobic domain in TorsinA (Figure 1A), lead to a similar glycosylation defect observed in the kar2-1 strain (L.Z. and J.B., in preparation). Further, TorsinA interacts with the chaperone and lectin calnexin in mammalian cells (60). S. cerevisiae lacks a true calnexin (145). Calnexin and calreticulin have been shown to compete with BiP for binding to specific glycoproteins (112), but calnexin also binds non-glycosylated substrates (146). This may explain why compromised Kar2/BiP function in yeast leads to a co-translational folding defect (evidenced by the appearance of different glycosylated species) that is not observed in mammalian cells (Figures 3 and 9). Overall, our results indicate that TorsinA and TorsinAΔE glycosylation is sensitive to alterations in folding caused not only by the absence of specific chaperones but also by environmental insults and by intragenic mutations.

Protein aggregation can lead to profound effects on cell viability (147). A critical role played by molecular chaperones, including Kar2/BiP, is to maintain the solubility of nascent polypeptides until their final conformations are achieved (29,148). The membrane extraction of TorsinA and TorsinAΔE with carbonate or a non-ionic detergent was severely impaired in the absence of Kar2/BiP (Figure 7). In principle, this phenotype could be caused by a translocation defect, but translocation of the proteins into the ER was unaffected in the kar2-1 strain (Figure 3C). More likely, enhanced membrane association was caused by conformational changes that expose hydrophobic regions, which would enhance membrane association, or which could lead to aggregation (122). Alternatively, TorsinA and TorsinAΔE might associate with other proteins that aggregate in the kar2-1 strain (29). This model would be consistent with the proposed chaperone function of TorsinA (53-55,149), although no effect of TorsinA or TorsinAΔE on the ERAD of select substrates was observed in yeast (data not shown), and only terminally misfolded ERAD substrates have been reported to aggregate in the kar2-1 yeast (29).

The introduction of mutations in TorsinA and TorsinAΔE generally led to a more profound loss of stability in TorsinAΔE than in TorsinA (Figures 8 and 9). Lack of N-linked glycosylation at N158 specifically affects TorsinAΔE, both in mammalian cells and in our yeast model (Figure 8) (106). Defects in glycosylation can impact local folding, alter the interaction with lectins and chaperones, and reduce protein solubility (138). Thus, it is possible that the glycan at N158 is required for the interaction of TorsinA and TorsinAΔE with chaperones or lectins, which TorsinAΔE may require for folding. This effect, in turn, would lead to increased degradation of TorsinAΔE but not TorsinA. Alternatively, altered folding caused by the lack of a glycan at N158 may impact the ability of TorsinAΔE to form hexamers, which is the typical oligomeric state assumed by AAA+ ATPases, including TorsinA (48,61,150). These unassembled TorsinAΔE
monomers may be targeted for degradation via the ERAD pathway (10,151). This model may also explain why mutating the Walker-A motif triggers TorsinA and TorsinAΔE degradation whereas mutating the Walker-B motif stabilizes TorsinA (Figures 8 and 9). The K108A mutation in the Walker-A motif of AAA+ ATPases not only prevents ATP binding, it also impairs substrate interaction and hexamer formation (48,152). On the other hand, the E171Q mutation in the Walker-B motif locks the protein in an ATP- and substrate-bound conformation, and promotes hexamer stabilization (48,152). In the case of TorsinAΔE, the C-terminal conformational defect and the lack of binding to interacting partners required for ATPase activity (63,127) may prevent efficient hexamerization, a phenotype that could be aggravated by the E171Q mutation. This phenomenon would explain why the E171Q mutation destabilizes TorsinAΔE (Figure 9B).

Besides protecting nascent polypeptides and aiding in their folding, chaperones can also identify and target terminally misfolded intermediates for degradation (4,9). Thus, depending on the substrate, chaperones can have a pro-folding and/or a pro-degradative role (109). For example, mammalian Hsp/c70 is a key pro-folding factor for CFTR that can also act as a pro-degradative chaperone, targeting CFTR for ERAD (153). The loss of stability of TorsinA and TorsinAΔE containing additional mutations in functional motifs also uncovered a dual role of Kar2/BiP on the folding and stabilization of these proteins (Figures 8 and 9). Kar2/BiP normally aids in folding TorsinA and TorsinAΔE (Figures 3, 8-11), but is instead a pro-degradative factor when the stability and folding of TorsinA and TorsinAΔE are impaired by mutations in specific functional motifs (Figures 8 and 9). This dual role of Kar2/BiP is not unprecedented. Kar2/BiP functions as a pro-folding chaperone for the vacuolar protease CPY, but as a pro-degradative factor of the ERAD substrate CPY* (100,102). Even more, BiP has been shown to aid in the folding and assembly of monomers into oligomers, while simultaneously contributing to the degradation of the unassembled, potentially misfolded monomeric subunits (154-157). Thus, Kar2/BiP may help TorsinA and TorsinAΔE to fold (Figure 3) and hexamerize, while also contributing to the degradation of the TorsinA and TorsinAΔE monomers that fail to assemble into hexamers due to, for instance, the presence of the additional mutations mentioned above (Figures 8 and 9). Alternatively, Kar2/BiP may simply recognize misfolded features of the mutated TorsinA and TorsinAΔE and aid in their degradation. These results illustrate the complex relationship between chaperones and their substrate clients, and underscore the delicate equilibrium between folding and degradation that ultimately dictates the fate of folding intermediates.

Because BiP is associated with the development of diseases as diverse as cancer and Parkinson’s disease (20,31-33,158,159), there is growing interest in developing small molecule modulators of BiP. Chemical modulators of BiP activity have been developed and tested in vitro (87,160), and small molecules that enhance the activity of cytosolic Hsp70 exist (161), which may be adapted in the future to act on BiP. Alternatively, the expression levels of BiP can be modified, a procedure that has shown therapeutic potential both in vitro and in mouse models (162-166). Because our results demonstrate that BiP stabilizes TorsinA and TorsinAΔE—and because patients afflicted with EOTD are heterozygous for the ΔE mutation—the design of effective therapies may need to combine the modulation of BiP activity/expression levels with the modulation of secondary targets that selectively affect TorsinAΔE folding such as, for example, the glycosylation machinery (Figure 8). A deeper understanding of the molecular and environmental factors that alter the biochemical properties of TorsinA and of the factors that participate in TorsinA folding and degradation will provide new clues to define the etiology of EOTD and may identify new therapeutic targets to prevent or treat the disease.
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**FIGURE LEGENDS**

**Figure 1:** TorsinA and TorsinΔE are ER localized N-linked glycosylated proteins in yeast. (A) SS: signal sequence; HD: hydrophobic domain; C: cysteines; N143 and N158: N-linked glycosylated residues mutated in Figure 8; K108 and E171: residues mutated in Figure 8 that belong to the Walker motifs; D216: mutation that impacts ΔE penetrance; E302 or E303: glutamate residue deleted in patients with EOTD. Walker-A and –B, and Sensor-I and -II motifs (for ATP binding and hydrolysis) are indicated by black lines. (B) The ORFs of TorsinA and TorsinΔE were cloned into the multi-copy shuttle vector pRS426 and are under the control of the constitutive GPD promoter. (C) Protein samples were prepared from log phase wild-type yeast cells expressing TorsinA or TorsinΔE grown at 28°C and were treated with Endoglycosidase H (EndoH) as indicated. The glycosylated species migrate at ~ 33 kDa and the un-glycosylated species resolve at ~27 kDa. (D) Indirect immunofluorescence of yeast cells expressing TorsinA-HA and TorsinΔE-HA or empty vector control. The ER luminal chaperone Kar2/BiP and the nuclear concentrated dye DAPI were used as controls.

**Figure 2:** TorsinA and TorsinΔE peripherally associate with the yeast ER membrane through an N-terminal hydrophobic domain. (A) ER-derived microsomes were prepared from log phase wild-type yeast cells grown at 28°C that expressed TorsinA or TorsinΔE and were incubated in a pH 6.8 buffer, 25mM CHES pH 9.5 buffer, 25mM CAPS pH 10.5 buffer, 0.1M Na₂CO₃ pH ~11.5, or in 6M urea for 30 min in ice. After centrifugation at 100,000 g, aliquots from the supernatant (S) or the pellet (P) fractions were analyzed by SDS-PAGE and the presence of TorsinA and TorsinΔE were detected by western blot. Pdi1 is a lumenal ER protein, Kar2 is a peripherally associated ER luminal protein, and Sec61 is an integral membrane ER protein. (B) Nitrocellulose membranes spotted with overnight yeast cultures transformed with vector alone (V), or expressing TorsinA (T) or TorsinAΔE (Δ), were layered on top of solid selective medium and incubated for ~20 hrs at 30°C. To detect secreted proteins, cells were washed off the nitrocellulose membrane with water. To detect total protein (intracellular and secreted material), cells were lysed in situ and then washed. TorsinA and G6PDH were detected by western (“dot”) blot. Please note that secreted proteins yield a higher signal than their non-secreted counterparts in the total protein blot (82).

**Figure 3:** TorsinA and TorsinΔE stability in yeast depends on the ER chaperone Kar2/BiP. (A) Log phase yeast cells expressing TorsinA or TorsinΔE grown at 28°C were treated with cycloheximide (CHX), then incubated at 37°C for 90 min. Samples were taken every 30 min and the amount of TorsinA or TorsinΔE in each time point was quantified relative to that at the “0” min time point. Data represents the average +/- SE of at least 4 experiments with ≥ 2 independent replicates/experiment. The % of TorsinA or TorsinΔE remaining after CHX addition in wild-type KAR2 or kar2-1 yeast strains is graphed (* P < 0.04 for KAR2 TorsinA vs. kar2-1 TorsinA; P < 0.003 for KAR2 TorsinAΔE vs. kar2-1 TorsinAΔE). Representative western blots are shown. (B) TorsinA and TorsinΔE glycosylation is Kar2-dependent. Protein samples were prepared from log phase KAR2 and kar2-1 yeast strains as before, except that they were treated with Endoglycosidase H (EndoH) as indicated before western blot analysis.
Pdi1 is an N-linked glycosylated protein, whereas G6PDH is a cytosolic un-glycosylated protein. * Indicates a background band in the blot. (C) The TorsinA and TorsinAAE glycosylated species are ER embedded. Microsomes from KAR2 and kar2-1 strains expressing TorsinA and TorsinAAE were incubated on ice for 30 min in the presence or absence of Triton X-100, followed by a 1 hr incubation on ice in the presence or absence of protease K. Proteins were TCA precipitated, resolved by SDS-PAGE and analyzed by western blot. Note that the ER protein Kar2 is clipped by Proteinase K in the presence of Triton X-100. Bos1 is an ER transmembrane protein with an epitope exposed to the cytosol that is Proteinase K-sensitive even in the absence of Triton X-100. UC: uncleaved Kar2/BiP; C: cleaved Kar2/BiP. (D) Kar2/BiP co-precipitates with TorsinA and TorsinAAE. Whole cell extracts from KAR2 cells transformed with a vector control (V), or expression vectors for TorsinA (T) or TorsinAAE (ΔE) were immunoprecipitated under native conditions (0.5% Triton X-100) using anti-Kar2 antiserum. Immunoprecipitated material was resolved by SDS-PAGE and analyzed by western blot. Controls from precipitations in which primary antiserum was absent are also included (*““).
kar2-1 TorsinAΔE. (C) The graph shows the means +/- SE of the fraction of TorsinA or TorsinAΔE remaining in the pellet from at least 3 independent experiments.

Figure 8: Synergism between mutations in functional motifs and the ΔE mutation, and dual role of Kar2/BiP in TorsinA and TorsinAΔE degradation. (A) A defect in N-linked glycosylation causes the Kar2/BiP-dependent degradation of TorsinAΔE. CHX chases were performed as described before (Figure 3). KAR2 (A-C) and kar2-1 (B,D) strains expressing TorsinA or TorsinAΔE containing (A,B) N-Q mutations that prevent glycosylation at N143 and N158. (* P < 0.0004 for TorsinAΔE vs. TorsinAΔE-N158Q in the KAR2 strain); or (C,D) mutations in the Walker-A motif (K108A) or Walker-B motif (E171Q). (* P < 0.0007 for TorsinA vs. TorsinA-K108A in the KAR2 strain; P < 0.00001 for TorsinAΔE vs. TorsinAΔE-K108A in the KAR2 strain; P < 0.003 for TorsinAΔE vs. TorsinAΔE-E171Q in the KAR2 strain). Below are representative western blots used for quantitation. Experiments were done at least twice with ≥ 2 independent replicates/experiment.

Figure 9: The D216H mutation does not significantly affect TorsinA or TorsinAΔE stability. CHX chases were performed as described before (Figure 3). (A) TorsinA-D216H and TorsinAΔE-D216H degradation in KAR2 and kar2-1 strains. Representative western blots are shown below. Experiments were performed at least three times, with ≥ 2 independent replicates per experiment. (* P < 0.001 for KAR2 TorsinA-D216H vs. kar2-1 TorsinA-D216H; P < 0.02 for KAR2 TorsinAΔE-D216H vs. kar2-1 TorsinAΔE-D216H). (B) Comparative graph showing the effects of the mutations in Figures 8 and 9 on TorsinA and TorsinAΔE levels at the 90 min time point of the CHX chases in the KAR2 and kar2-1 strains. Specific mutations in the N-glycosylation, Walker-A, and B motifs, or D216H are indicated. * Other relevant P values not already described in Figures 3 and 8: P < 0.03 for TorsinA-N143Q in the KAR2 vs. kar2-1 strains; P < 0.003 for TorsinAΔE-N158Q in the KAR2 vs. the kar2-1 strains); P < 0.04 for TorsinA-K108A in the KAR2 vs. kar2-1 strains; P < 0.01 for TorsinA-E171Q in the KAR2 vs. kar2-1 strains; P < 0.0002 for TorsinAΔE-K108A in the KAR2 vs. kar2-1 strains; P < 0.03 for TorsinAΔE-E171Q in the KAR2 vs. kar2-1 strains.

Figure 10: siRNA-mediated knock down of BiP reduces the steady state levels of TorsinA and TorsinAΔE. HeLa cells were treated with siRNA against BiP for a total of 48 hrs, and were transfected with TorsinA and TorsinAΔE expression vectors 24 hrs before harvest. (A) Representative western blot and (B) graph showing the average +/- SE of the normalized levels of BiP, TorsinA and TorsinAΔE after treatment with a control siRNA or siRNA directed against BiP. Experiments were performed at least twice with 3 replicates per experiment. The graphed values were normalized as follows. The levels of BiP, TorsinA, and TorsinAΔE for each replicate in each experiment were normalized first to the corresponding actin levels. Then, this value from one siRNA control replicate was used to normalize the values of all other replicates. This procedure was performed for each TorsinA and TorsinAΔE experiment independently. Actin was used as loading control. Grp94 levels were also monitored by western blot.

Figure 11: BiP is required for TorsinA and TorsinAΔE stability in mammalian cells. HeLa cells transiently transfected with TorsinA or TorsinAΔE expression vectors were treated with 0.5μg/mL of an active SubAB or a catalytically inactive SubAΔ272B toxin for 45 min before a pulse chase was performed (t=0). (A) BiP and TorsinA levels were monitored at each time point by western blot (WB) and autoradiography, respectively. (B) Graph showing the average +/- SE of total TorsinA or TorsinAΔE remaining at the 4 hr time point in the presence of SubAB or SubAΔ272B toxin quantified relative to that at the “0” hr time point. (* P < 0.02 for TorsinA in cells treated with SubAB vs. SubAΔ272B). (C) Graph showing the average +/- SE of the relative levels of un-glycosylated/total TorsinA or TorsinAΔE at the “0” hr time point in the presence of SubAB or SubAΔ272B toxin. (* P < 0.002). Experiments were performed at least three times with at least one replicate/experiment.
### TABLES

**Table 1. Primers used in this study**

| Name      | Sequence 5’ to 3’                                                                 | Reference       |
|-----------|----------------------------------------------------------------------------------|-----------------|
| LZJB5     | CCTTCCTTTTCGGTTAGAGCGGA                                                           | This study      |
| LZJB12    | AC GGTTCAACCAAGTTAGATTACTACGATGATTACCCATACGATTTCCAGATTCACGCT                      | This study      |
|           | TGAGCGCGCCGCTGAGTCAGTGAATTAGTTATGTC                                              |                 |
| LZJB13    | TGACATAACTAATACATGACTCGAGCGGCCTCAACGCGTAATCTGGAACATCGTGATGGGT                      | This study      |
|           | AATCATCGTAGTAATAATCTAATCTGGAACACCCG                                              |                 |
| LZJB17    | GAGCAGAAAGGATCATACATGTTGCTTTGGATTTTC                                              | This study      |
| LZJB18    | GAAATCCCAAGCCACATGTTGATTCCTTTCTGCT                                               | This study      |
| LZJB21    | TTGCACTTTCCACATGCTTTCCAAATCACCCTTTGTAAGGATCAG                                    | This study      |
| LZJB22    | CTGATCCTTGTCAAAGGTTGATTTGTAAGCAATGTTGGAAGAAGTGCAA                                  | This study      |
| LZJB23    | GTTGTGGATTCGAGGCGCAATGAGTGCTGTGCGA                                              | This study      |
| LZJB24    | TCGCAGAGCCACTCACTCGCGCTCGAATCCACAAAC                                                | This study      |
| LZJB34    | AGTAAGAGATTTGGTAAATTCGCT                                                          | This study      |
| LZJB35    | AGTGTTCTATGGCTTTGGATTTTC                                                           | This study      |
| LZJB44    | TCAGTTGCGGGTGTCGATGG                                                              | This study      |
| LZJB45    | CGTTGATGTCCTCCTTAAGC                                                              | This study      |
| LZJB69    | TTGAAACGTAATCTGAGCAATACAAAA                                                       | This study      |
| LZJB70    | TCAACTAAATGCTGATGCGCTTAA                                                       | This study      |
| LZJB71    | TCCTAATATGATGTAATGGT                                                              | This study      |
| LZJB72    | TAACTATATCCATGCGTCC                                                              | This study      |
| CG_PDP prom | CCCTGAATTTATTTCCCTACTTTG                                                               | This study      |
| K108A-F   | GGACAGGCACCAGCAGCAATTTCCGTCAGCAAG                                                   | This study      |
| K108A-R   | CTTGCTGACGAAATTTTGCGCGGTGCTGTC                                                    | This study      |
| E171Q-F   | CCATCTTCCATATTTGATCAAATGGATAAGATGCATGCA                                              | This study      |
| E171Q-R   | GCATGCATCTTTATCCATTTGAGCAGATGG                                                   | This study      |
| KanB      | CTGCAGCGAGGAGCCGTAAT                                                               | (167)          |
Table 2. Plasmids used in this study

| Details                  | Plasmid #   | Reference       |
|--------------------------|-------------|-----------------|
| pcDNA3.1-TorsinA         | pcDNA3.1     | (65)            |
| pcDNA3.1-TorsinAΔE       | pcDNA3.1     | (65)            |
| pcDNA3.1-TorsinA-HA      | pcDNA3.1     | (63)            |
| pcDNA3.1-TorsinAΔE-HA    | pcDNA3.1     | (63)            |
| pRS426GPD-TorsinA        | pLuBr281     | This study      |
| pRS426GPD-TorsinAΔE      | pLuBr280     | This study      |
| pRS426GPD-TorsinA-HA     | pLuBr27      | This study      |
| pRS426GPD-TorsinAΔE-HA   | pLuBr28      | This study      |
| pRS426GPD-TorsinA-N143Q  | pLuBr85      | This study      |
| pRS426GPD-TorsinA-N158Q  | pLuBr100     | This study      |
| pRS426GPD-TorsinAΔE-N143Q| pLuBr86      | This study      |
| pRS426GPD-TorsinAΔE-N158Q| pLuBr87      | This study      |
| pRS426GPD-TorsinA-N143,N158Q| pLuBr106  | This study      |
| pRS426GPD-TorsinA-D216H  | pLuBr60      | This study      |
| pRS426GPD-TorsinAΔE-D216H| pLuBr61      | This study      |
| pRS426-GPD-TorsinA-K108A | pLuBr20      | This study      |
| pRS426-GPD-TorsinA-E171Q | pLuBr21      | This study      |
| pRS426-GPD-TorsinAΔE-K108A| pLuBr23     | This study      |
| pRS426-GPD-TorsinAΔE-E171Q| pLuBr24     | This study      |
| pRS426-GPD-TorsinAΔ-24-40| pLuBr18      | This study      |
| pRS426-GPD-TorsinAΔE-Δ24-40| pLuBr19   | This study      |

Table 3. Strains used in this study

| Strain       | Genotype                                     | Reference       |
|--------------|----------------------------------------------|-----------------|
| BY4742       | MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0              | Open Biosystems |
| lhs1Δ        | MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 lhs1::KanMX | Open Biosystems |
| KAR2         | MATa leu2-3,112 ura3-52 ade2-101              | (103)           |
| kar2-1       | MATa leu2-3,112 ura3-52 ade2-101 kar2-1      | (103)           |
| kar2-1 pep4A | MATa leu2-3,112 ura3-52 ade2-101 kar2-1 pep4::KanMX | This study |
| kar2-1 pdr5A | MATa leu2-3,112 ura3-52 ade2-101 kar2-1 pdr5::KanMX | This study |
| JEM1 SCJ1    | MATa pcr1-l leu2-3,112 ura3-52 trp1-Δ901 his3-Δ200 lys2-801 | (29) |
| jem1Δ        | MATa pcr1-l leu2-3,112 ura3-52 trp1-Δ901 his3-Δ200 lys2-801 succ2-Δ9 GAL jem1::LEU2 | (29) |
| scj1Δ        | MATa pcr1-l leu2-3,112 ura3-52 trp1-Δ901 his3-Δ200 lys2-801 succ2-Δ9 GAL scj1::TRP1 | (29) |
| jem1Δ scj1Δ  | MATa pcr1-l leu2-3,112 ura3-52 trp1-Δ901 his3-Δ200 lys2-801 succ2-Δ9 GAL jem1::LEU2 scj1::TRP1 | (29) |
| CNE1         | MATa leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 can1-100 | (145) |
| Strain | Description |
|--------|-------------|
| cne1Δ | MATα leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 can1-100 cne1::LEU2 |
| CNE1   | MATα leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 can1-100 |
| cne1Δ | MATα leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 can1-100 cne1::LEU2 |
| yos9Δ | MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 yos9::KanMX |

Open Biosystems
Figure 4

A

B

C

% TorsinA remaining

Time (min)

0  15  30  60  90

0  15  30  60  90

0  15  30  60  90

JEM1 SCJ1 TorsinA
jem1Δ TorsinA
jem1Δ scj1Δ TorsinA
jem1Δ scj1Δ TorsinAΔE

jem1Δ TorsinA
scj1Δ TorsinA
jem1Δ TorsinAΔE
scj1Δ TorsinAΔE

jem1Δ TorsinA
scj1Δ TorsinA
jem1Δ TorsinAΔE
scj1Δ TorsinAΔE

% TorsinA remaining

Time (min)

0  15  30  60  90

0  15  30  60  90

0  15  30  60  90

LHS1 TorsinA
lhs1Δ TorsinA
LHS1 TorsinAΔE
lhs1Δ TorsinAΔE

LHS1 TorsinA
lhs1Δ TorsinA
LHS1 TorsinAΔE
lhs1Δ TorsinAΔE

% TorsinA remaining

Time (min)

0  15  30  60  90

0  15  30  60  90

0  15  30  60  90

JEM1 SCJ1
jem1Δ
scj1Δ
jem1Δ scj1Δ

jem1Δ
scj1Δ
jem1Δ scj1Δ

jem1Δ
scj1Δ
jem1Δ scj1Δ

TorsinA
G6PDH

TorsinA
G6PDH

TorsinA
G6PDH

TorsinA
G6PDH

TorsinA
G6PDH

TorsinA
G6PDH
Figure 5

### A

|       | TorsinA | TorsinAΔE |
|-------|---------|-----------|
| **CNE1** | | |
| Time (min) | 0 30 60 90 | 0 30 60 90 |
| TorsinA | [Image](#) | [Image](#) |
| G6PDH | [Image](#) | [Image](#) |

|       | TorsinA | TorsinAΔE |
|-------|---------|-----------|
| **cne1Δ** | | |
| Time (min) | 0 30 60 90 | 0 30 60 90 |
| TorsinA | [Image](#) | [Image](#) |
| G6PDH | [Image](#) | [Image](#) |

### B

|       | TorsinA | TorsinAΔE |
|-------|---------|-----------|
| **YOS9** | | |
| Time (min) | 0 15 30 60 90 | 0 15 30 60 90 |
| TorsinA | [Image](#) | [Image](#) |
| G6PDH | [Image](#) | [Image](#) |

|       | TorsinA | TorsinAΔE |
|-------|---------|-----------|
| **yos9Δ** | | |
| Time (min) | 0 15 30 60 90 | 0 15 30 60 90 |
| TorsinA | [Image](#) | [Image](#) |
| G6PDH | [Image](#) | [Image](#) |
Figure 6

A

% TorsinA remaining

Time (min)

0 30 60 90

DMSO TorsinA
MG132 TorsinA
DMSO TorsinAΔE
MG132 TorsinAΔE

kar2-1 pdr5Δ

MG132 Time (min) 0 30 60 90 +

TorsinA

G6PDH

kar2-1 pep4Δ

MG132 Time (min) 0 30 60 90 +

TorsinA

G6PDH

B

% TorsinA remaining

Time (min)

0 30 60 90

KAR2 PEP4 TorsinA
kar2-1 pepΔ TorsinA
KAR2 PEP4 TorsinAΔE
kar2-1 pepΔ TorsinAΔE

kar2-1 pep4Δ

Time (min) 0 30 60 90

TorsinA

G6PDH
Figure 7

A

| pH | T | ΔE |
|----|---|----|
| 6.8| S | P  |
| 11.5| P | S  |

| kar2-1 |
|--------|
| TorsinA |
| Pdi1 |
| Kar2 |
| Sec61 |

B

| KAR2 | kar2-1 |
|------|--------|
| T    | ΔE     |
| 6.8 S| P      |
| 11.5 P| S     |
| 6.8 S| P      |
| 11.5 P| S     |

| TorsinA |
| Pdi1 |
| Kar2 |
| Sec61 |

C

Graph showing the fraction of TorsinA remaining in the pellet under different conditions.
Figure 9

A

% TorsinA remaining at 90 min

0  20  40  60  80  100  120

Time (min)

KAR2 TorsinA D216H
kar2-1 TorsinA-D216H
KAR2 TorsinAΔE-D216H
kar2-1 TorsinAΔE-D216H

B

% TorsinA remaining at 90 min

WT  K108A  E171Q  N143Q  N158Q  D216H

TorsinA mutations

% TorsinA remaining

0  20  40  60  80  100  120

Time (min)

KAR2 TorsinA  KAR2 TorsinAΔE
kar2-1 TorsinA-D216H  kar2-1 TorsinAΔE-D216H

| KAR2    | TorsinA | TorsinAΔE |
|---------|---------|-----------|
| Time (min) | 0 30 60 90 | 0 30 60 90 |
| TorsinA  |         |           |
| G6PDH    |         |           |

| kar2-1  | TorsinA | TorsinAΔE |
|---------|---------|-----------|
| Time (min) | 0 30 60 90 | 0 30 60 90 |
| TorsinA  |         |           |
| G6PDH    |         |           |
Figure 11

A

|          | TorsinA | TorsinAΔE |
|----------|---------|-----------|
|          | SubA_{A272}B | SubAB | SubA_{A272}B | SubAB |
| Time (hrs) | 0 1 4 | 0 1 4 | 0 1 4 | 0 1 4 |
| BiP       | WB     | WB       | WB     | WB     |
| β-Actin   | WB     | WB       | WB     | WB     |
| Autoradiogram | WB | WB       | WB     | WB     |

B

C

% TorsinA remaining at 4 hrs

| TorsinA | TorsinAΔE |
|---------|-----------|
| SubAB   | SubA_{A272}B |

Fraction of un-glycosylated TorsinA at 0 hrs

| TorsinA | TorsinAΔE |
|---------|-----------|
| SubAB   | SubA_{A272}B |
The BiP molecular chaperone plays multiple roles during the biogenesis of TorsinA, a AAA+ ATPase associated with the neurological disease Early-Onset Torsion Dystonia

Lucia F. Zacchi, Hui-Chuan Wu, Samantha L. Bell, Linda Millen, Adrienne W. Paton, James C. Paton, Philip J. Thomas, Michal Zolkiewski and Jeffrey L. Brodsky

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