Supplementary Information

TorsinA participates in endoplasmic reticulum-associated degradation

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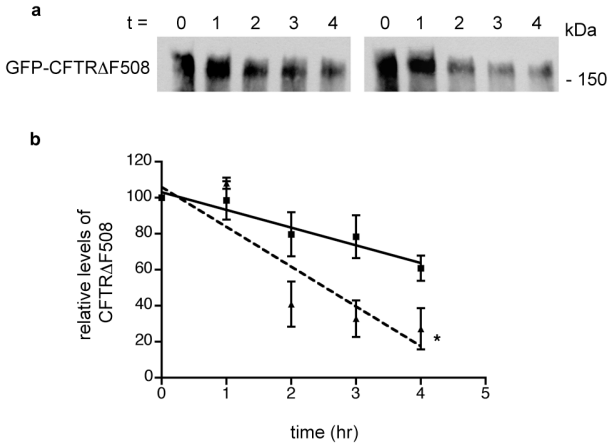
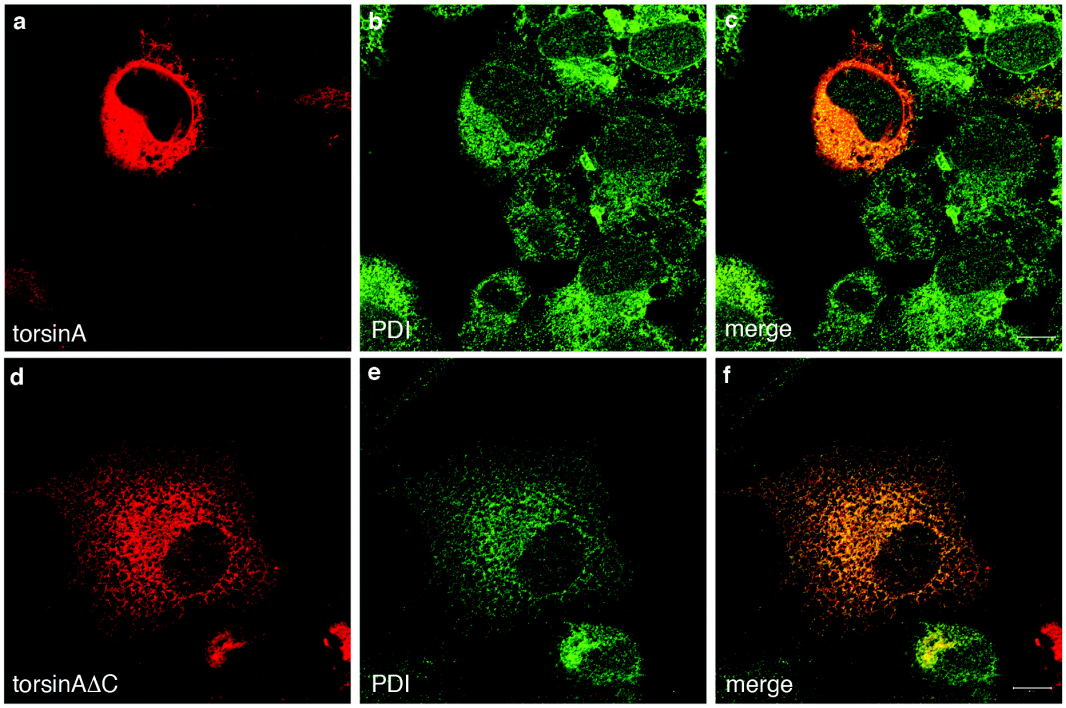
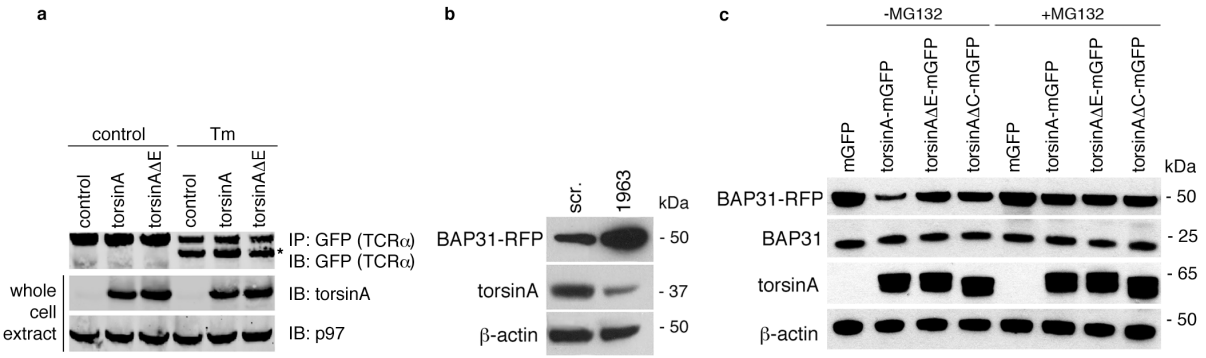


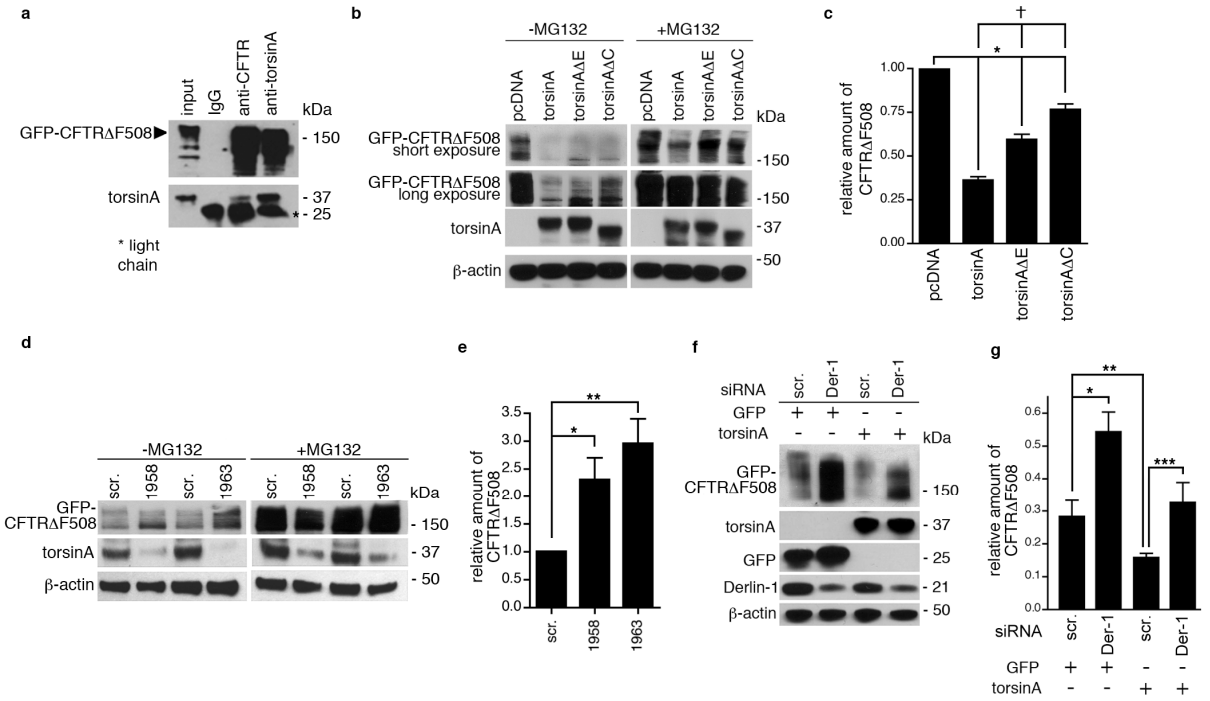
Supplementary Figure S1 | Enhanced torsinA expression increases the rate of CFTRΔF508 degradation. 293T cells which had been co-transfected with GFP-CFTRΔF508 and either control (pcDNA3.1) or a torsinA-expressing plasmid were treated with cycloheximide, followed by immunoprecipitation for GFP. (a) Immune precipitates were immunoblotted to determine relative levels of GFP-CFTRΔF508 levels over time. (b) Linear regression analysis showed that in the presence of overexpression torsinA the slope of degradation was -22.09 as compared to -9.84 for control transfected cells. Data is shown as the mean of three experiments $\pm$ S.D.; the difference in slopes was significant with * representing $p<0.013$ using the Student's t-test.



Supplementary Figure S2 | TorsinA C-terminal deletion perturbs ER morphology. COS-7 cells were transfected with expression cassettes for: (a-c) torsinA or (d-f) torsinAΔC and processed 48 h later for immunocytochemistry for torsinA (a, d) and PDI (b, e), with merged images (c, f), respectively. Magnification bar = 10 µm.



Supplementary Figure S3 | Effect of overexpression of torsinA variants on the degradation of TCRalpha-YFP and BAP31-RFP. (a) 293T cells stably expressing TCRalpha-YFP were transfected with expression cassettes for torsinA or torsinAΔE in the absence or presence of tunicamycin (TM). Cells were harvested 72 h later and lysates were processed directly of following immunoprecipitation with antibodies to GFP (for TCRalpha-YFP). Whole cell extracts of immune precipitates were resolved by SDS-PAGE and immunoblotted using antibodies for GFP, torsinA and p97. (b) 293T cells were transfected with a scrambled (scr.) siRNA or an siRNA (1963) targeted to the torsinA mRNA. Lysates were resolved by SDS-PAGE and immunoblotted with antibodies to BAP31, torsinA and β-actin. (c) 293T cells were co-transfected with BAP31-RFP and torsinA-mGFP, torsinAΔE-mGFP, torsinAΔC-mGFP or control mGFP plasmid in the absence and presence of MG132. Cell lysates were resolved by SDS-PAGE and immunoblotted using antibodies to BAP31, torsinA and β-actin. A representative western blot is shown.



Supplementary Figure S4 | Downregulation of torsinA inhibits retro-translocation of the cholera toxin A1-chain – effect of MG132. After siRNA transfection with a scrambled (scr.) siRNA or an siRNA to torsinA mRNA, cells were intoxicated with wild-type CT for 45 min at 37°C in the presence and absence of the proteosome inhibitor, MG132 and then fractionated into cytosolic and membrane components which were resolved by SDS-PAGE. Immunoblotting was carried out with antibodies to CTA1, CTB, BiP, β-actin and Hsp90.

Supplementary Methods

Nematode experiments.

Plasmid constructs. For studies in nematodes full-length WT and CFTRΔF508 were PCR amplified using 5'-GGGGACAAGTTTGTACAAAGCAGGCTCCATGCAGAGGTCGCCTCTGG and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAAAGCCTTGTATCTTGCAC-3' from cDNAs provided by Dr. Elizabeth Stzul (University of Alabama at Birmingham). Expression plasmids were created with Gateway recombinational cloning (Invitrogen, Carlsbad, CA). The PCR products were initially recombined with the pDONR221 vector to create pENTRY clones.

Wild-type and mutant (ΔF508) CFTR were expressed under the promoter from the intestinal-specific type B carboxylesterase (*ges-1*) gene. Briefly, a Gateway (Invitrogen) recombination cassette was inserted downstream of the *ges-1* promoter in plasmid pJM16[61] to create the pDEST-JM16 vector. All pENTRY plasmids (described above) were then recombined into the pDEST-JM16 vector and these recombination plasmid products were then subsequently used to create transgenic worms.

C. elegans strains and protocols. Nematodes were grown and maintained using standard procedures. The starting strains used were *hsp-4*::GFP, WT torsinA [strain UA97 (*baIn16*)], and mutant torsinAΔE [strain UA98 (*baIn17*)][62]. For new strains generated in the course of this work, each plasmid was injected at a total concentration of 50 µg/ml. Wild-type or ΔF508CFTR,

each plasmid alone (50 µg/ml), were co-injected into the *hsp-4*::GFP strain with *unc54::*mCherry. For each plasmid, at least three stable lines were generated and analyzed. Representative lines were selectively integrated by UV irradiation using a UV crosslinker (Spectroline, Westbury, NY) either with 254 nm bulbs with an energy setting of 20 mJ/cm^2 or with 365 nm bulbs at 300 mJ/cm^2. After integration, each strain was outcrossed at least 5 times with *hsp-4*::GFP to get rid of potential random mutations generated by UV irradiation. The integrated transgenic lines were designated as follows: WT CFTR [UA151; P*ges-1*::CFTRWT, P*unc-54*::mCherry [*baIn27*]; *hsp-4*::GFP[*zcIs4*]] and CFTRΔF508 [UA152; P*ges-1*::CFTRΔF508, P*unc-54*::mCherry [*baIn28*]; hsp-4::GFP[*zcIs4*]].

Wild-type CFTR or ΔF508 male nematodes were crossed with hermaphrodites expressing either wild-type or torsinAΔE and grown at 20^{o}C. Worms containing double mutant genes, CFTRΔF508 plus torsinAΔE, were embryonic lethal, since no progeny were obtained (3 repeated crosses yielded the same result). Other crossed strains were viable. The crossed transgenic lines were designated as follows: wild-type CFTR with wild-type torsinA [UA153; P*ges-1*::CFTRWT, P*unc-54*::mCherry [*baIn27*]; *hsp-4*::GFP[*zcIs4*];P*ges-1*::torA WT[*baIn16*]] and CFTRΔF508 with wild-type torsinA [UA154; P*ges-1*::CFTRΔF508, P*unc-54*::mCherry [*baIn28*]; *hsp-4*::GFP[*zcIs4*];P*ges-1*::torA WT[*baIn16*]].

***Fluorescent analysis of hsp-4::GFP worms*.** ER stress was examined in late L4 stage animals that were transferred manually to NGM plates spread. Worms were mounted on a 2% agarose pad and analyzed with a CCD camera (Photometrics CoolSnapHQ) on a Nikon (E800) microscope at 40X magnification. GFP intensity was measured in pixels and assigned arbitrary

units (a.u.) from a 100 x 100 μm region of the anterior-most region of the intestine, directly behind the pharynx of each animal using MetaMorph software (Molecular Devices Corp., Sunnyvale, CA). For each strain and condition, at least 30 animals were quantitated in three independent replicates.

Real-time-PCR analysis. For each worm strain analyzed, 50 worms were placed into the cap of an RNase-free microcentrifuge tube containing 10 μl of M9. The worms were pelleted and lysed in Trizol (Invitrogen) by repeated freezing in liquid nitrogen and thawing at 42°C (four times). Phases were separated using 1-bromo-3-chloropropane (Sigma-Aldrich, St. Louis, MO) and the top phase was placed into a separate microcentrifuge tube and precipitated with isopropanol at -20°C for 1 h. Glycoblue (Ambion, Austin, TX) was added before isopropanol to a concentration of 50 μg/ml to allow for easy viewing of the RNA pellet. After precipitation, RNA was washed with 75% ethanol and resuspended in 10 μl RNase-free water. RNA was converted to cDNA using Superscript III RT (Invitrogen) and PCR was performed using Phusion Polymerase (New England Biolabs, Ipswich, MA) on a PTC-200 Thermo Cycler (MJ Research, Ramsey, MN). The following primers were used for detection of gene expression by PCR. For CFTR, the forward primer was 5'-ACAGTTTCCTGGGAAGCTTG-3', and the reverse primer was 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAAAGCCTTGTATCTTGCAC-3'. For *ama-1* (RNA polymerase II large subunit) loading control, the forward primer was 5'-CGAGTCCAACGTACTCTCC-3', and the reverse primer was 5'-GATGTTGGAGAGTACTGAGC-3'.

Statistical analyses. Comparisons were done on GFP pixel intensity between strains unless otherwise noted. For each assay, the data presented is the normalized fold change mean ± S.D. of three independent trials for every strain or condition unless otherwise noted. Each trial consists of the GFP intensity from 30 animals averaged. Normalization compares GFP intensity for all the samples (*hsp-4*::GFP alone or with torsinA and/or CFTR versions) divided by the average of *hsp-4*::GFP alone. Statistics were performed using ANOVA (www.physics.csbsju.edu/stats/anova.html) and post-test Bonferroni correction (http://www.graphpad.com/quickcalcs/posttest1.cfm). Statistical analysis for the nematode RT-PCR experiments was performed using Multi Gauge (Version 3.0). Normalization compares DNA band intensity for all the samples (*hsp-4*::GFP alone or with torsinA and/or CFTR versions) divided by the average of WT CFTR alone.

Mammalian cell culture experiments.

Plasmid expression constructs. The following expression plasmids were used: pcDNA3-torsinA and -torsinAΔE[63,64], a monomeric GFP vector (mGFP), torsinAmGFP or torsinAΔEmGFP vectors expressing fusion proteins[65] from Dr. Phyllis Hanson (Washington Univ., St. Louis, MO), GFP-CFTRΔF508 from Dr. Ron Kopito (Stanford University, Stanford, CA)[66]; TCRalpha-YFP[67], His-p97[68], Myc-VIMP[68], Hrd1-GFP and Hrd1-Myc[69].

A number of new expression constructs were generated. The cDNA encoding full-length human BAP31 fused to RFP at its carboxyl terminus[70] was cloned in the BamHI site of the pcDNA3.1 vector (Invitrogen) (BAP31-RFP). mCherry-Derlin-1 fusion protein was generated by PCR-

cloning of mCherry cDNA (from Dr. Richard Tsien, UCSD, La Jolla, CA)[71] between the XhoI-EcoRV sites in pcDNA3.1 (Invitrogen), followed by insertion of the human Derlin-1 expression cassette[68] between the EcoRV-KpnI restriction sites. The 1 to 312 fragment of human torsinA was amplified by PCR using wild-type torsinA cDNA as a template with appropriate primers (available upon request) and cloned in the NheI-XhoI sites of the pcDNA3 vector generating the pcDNA3-torsinAΔC313-332 (torsinAΔC) construct. The pcDNA3-torsinAΔC-mGFP was generated by replacing the wild-type torsinA with the torsinAΔC cDNA into the EcoRI-XhoI restriction sites of the monomeric GFP constructs.

Antibodies. Mouse monoclonal antibodies included: D-M2A8 and D-M10 specific to torsinA[72], anti-RGS·His (Qiagen, Valencia, CA), monoclonal (Invitrogen) or polyclonal (Sigma-Aldrich) anti-myc (Invitrogen), anti-BAP31 (Alexis Biochemicals, Plymouth Meeting, PA), anti-p97 (BioLegend, San Diego, CA), anti-GFP (Invitrogen), β-actin (clone AC-15, Sigma-Aldrich), anti-Hrd1 (Novus Biologicals, Littleton, CO; anti-SYVNI, abnova), anti-CFTR M37A (Santa Cruz Biotechnology, Santa Cruz, CA) and goat monoclonal IgG (Abcam, Inc., Cambridge, MA). Polyclonal antibodies (rabbit unless stated otherwise) included: polyclonal torsinA TA913 specific to human torsinA[73] and TAB1 for human and rodent torsinA[72], specific to mouse torsinA (Abcam); as well as antibodies to PDI (SPA-891; Stressgen, Ann Arbor, MI); calnexin (calnexin; SPA 856; Stressgen), GFP (Molecular Probes, Eugene, OR; Invitrogen); Derlin-1 (raised against a peptide residues 238–251 in human Derlin-1)[74]; Hsp90, polyclonal (Sigma-Aldrich) or monoclonal (Invitrogen) Myc, and BiP (GRP78; Santa Cruz Biotechnology); CT antibody[75]; and polyclonal anti-mouse IgG (Abcam). Antibodies to VIMP, p97, ubiquitin, sec61α and ubiquitin were produced in Dr. Ye's laboratory[68,69].

Dilution of antibodies for western blots were: D-M2A8 (1:100, or 1:500 when torsinA was overexpressed), Derlin-1 (1:500), VIMP (1:300), p97 (1:500) rabbit or mouse GFP (1:2000), β-actin (1:3000), polyclonal BAP31 (1:1000), monoclonal BAP31 (1:1500), ubiquitin (1:1000), CT (1:5000), Hsp90 (1:500) and BiP (1:500).

siRNA. Chemically stabilized siRNA targeting both human torsinA and torsinAΔE messages (1958 & 1963) were synthesized on an ABI3900 DNA synthesizer according to standard procedures and purified by AEX HPLC[76]. Scrambled control siRNA were purchased from Santa Cruz (Cat #: sc-37007, http://www.scbt.com/product.php?datasheet=37007). The siRNAs Sense (5'-3'): GCUUAGCAAUGGAUAUGCAtt; antisense (5'-3'): UGCAUAUCCAUUGCUAAGCca targeting nucleotides 445-463 of Derlin-1 mRNA was a silencer custom-made siRNA from Ambion (Cambridgeshire, UK)[77]. To knock-down torsinA, COS-1 or 293T cells were seeded onto 6-well plates 24 h prior transfection (80% confluency). The next day, cells were transfected 50 nM siRNAs against torsinA or 100 nM siRNAs against Derlin-1, using X-tremeGENE transfection reagent (Roche Applied Sciences, Indianapolis, IN), according to instructions of the manufacturer. After 24 h, the cells were transfected with GFP-CFTRΔF508. The siRNA against Derlin-1 were purchased from Ambion (Cat# s35606).

Confocal microscopy. Laser scanning confocal imaging was performed using a Carl Zeiss LSM 5 Pascal laser-scanning confocal microscope. Images were acquired using a 10x, 40x or 63x numerical aperture 1.4 PlanApo differential interference contrast (DIC) objective on an inverted microscope (Axiovert 200M; Carl Zeiss, Hamburg, Germany) equipped with an LSM 510

META scan head (Carl Zeiss). Argon ion (488 nm), HeNe (543 nm), or HeNe (633 nm), or Alexa Fluor (647) lasers were used for excitation. Green, red and blue fluorescence emissions were detected through BP 505-530, 560-615 and 650 filters, respectively. The different fluorochromes were scanned sequentially by using the multitracking function to avoid any bleed through among these fluorescent dyes. Brightfield images were obtained using the Argon 488 nm laser. Collected images were digitalized at a resolution of 8 bits into an array of 2048 × 2048 pixels. We also used an Olympus FluoView 1000 confocal microscope with independent photostimulation and imaging channels with the same lasers, as above, as well as a 361 nm UV laser, including a tunable two-photon source for reduced photodynamic damage. This microscope also has DIC optics and a photomultiplier collector for transmitted light imaging.

Cholera toxin retro-translocation assay. Retro-translocation experiments were modified and carried out, as previously described by Bernardi *et al*[78]. Briefly, COS-7 cells were plated in 6-well plates at a concentration of 8 x 10^4 cells/well. Cells were first transfected with siRNAs (1958 & 1963) for torsinA or a scrambled control siRNA (see above). Transfection was performed with X-tremeGENE siRNA transfection reagent (Roche Applied Sciences) according to the instructions of the manufacturer. Seventy-two h later, cells were incubated with 20 nM toxin (Calbiochem, San Diego, CA) in Hanks' Balanced Salts [HBSS+ modified (Sigma-Aldrich); without phenol red and sodium bicarbonate, 10 mM HEPES pH 7.4] for 45 min at 37ºC. Cells were detached with 0.25% trypsin with EDTA, washed and resuspended in 100 µl HN buffer [150 mM NaCl, 50 mM HEPES, pH 7.5, 0.5 mg/ml EDTA, Complete Protease Inhibitor Cocktail Tablet (Roche Applied Sciences)] incubated on ice for 10 min and centrifuged at 64,000 g (10 min at 4ºC). The supernatant was collected and the membrane pellet

resuspended in equal volume of HN buffer. Protein concentration of fractions was measured by BCA (Pierce, Rockford, IL). Aliquots of the cytosolic fraction (40 µg), the membrane fraction (4.5 µg) or whole cell lysate (20 µg) were loaded on SDS-PAGE and analyzed by immunoblots using antibodies against CT, Hsp90, β-actin and BiP, as previously described[79,80].

Fluorescence microscopy for cholera toxin in COS-1 cells. COS-1 cells were incubated with 20 nM CTB-Alexa Fluor 488 in HBSS at 37°C for 3 h, washed, fixed in 4% PFA for 15 min, and processed for immunofluorescence microscopy using standard methods (1:200 PDI pAb:Sigma). Cells were imaged using a Zeiss Axiovert 200M inverted microscope equipped with a CSU-X1 spinning disk confocal head (PerkinElmer, Waltham, MA) and a CascadeII cooled CCD camera (Roper Scientific, Trenton, NJ). For confocal imaging, the excitation source used was 50-mW solid-state lasers (473 nm [CrystaLaser] and 561 nm [Cobolt]), and intensity was controlled by an acoustic-optical tunable filter. 2D confocal sections of COS-1 cells were captured using a ×63 oil objective (plan apochromat, 1.4 NA; Nikon). Images were captured and analyzed using Slidebook 4.2 software (Intelligent Imaging Innovations). The fraction of cells showing CTB present in the ER was evaluated by visual inspection of confocal sections of samples (**Fig. 5 f**). Cells that exhibited CTB following the outline of the NE as defined by PDI immunostaining were considered positive for ER transport. Cells showing no detectable CT signal over the NE were considered defective in transport of CT to the ER. This method of analysis does not discern the intensity of CTB in the NE, only presence or absence.

ER trafficking assay of cholera toxin. The mutant toxin CTB-GS harboring glycosylation and sulfation motifs appended to the C-terminus of the B-subunit was used to gauge entry into the

TGN and ER of COS-1 cells, as previously described[81]. For isolation of total N-glycosylated CTB-GS, COS-1 cells transfected with scrambled or torsinA siRNA were intoxicated with 10 nM CTB-GS in HBSS for 3 h at 37ºC. Cells were washed and ConA sepharose resin was used to isolate glycosylated proteins from cell lysates, as previously described[82]. Post-ConA resin eluate from parallel control cells was treated with PNGase F per the protocol of the manufacturer (New England Biolabs) in order to confirm the glycosylated band. All samples were separated by SDS-PAGE and were either analyzed by immunoblot or Silver Stain Plus (BioRad, Hercules, CA).

Supplementary References

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