SUPPORTING INFORMATION for the article:

Biochemical and cellular analysis of human variants of the DYT1 dystonia protein, torsinA

CONTENT:

Supp. Figure S2. Representative FACS plots from sorted cells infected with lentivirus expressing torsinA wild-type and variants.

Supp. Figure S3. Protein folding is delayed after torsinA depletion.

Supp. Figure S4. ER morphology does not change when cells are incubated at 40°C in cells expressing variant forms of torsinA

Supp. Figure S5. Differential ER distribution of torsinAF205I in human fibroblast cells.

Supp. References

SUPP. METHODS

Cell Culture

Human control fibroblast line HF17 was generated from a skin biopsy under IRB approved guidelines [Breakefield et al., 1981]. We also used the human embryonic kidney fibroblast line 293T obtained from Dr. D. Baltimore (Rockefeller University, New York). Fibroblast cells and 293T cells were grown in Dulbecco's Modified Eagle Medium (Gibco, Rockville, MD, USA; Invitrogen Life Technologies, Denville, NJ, USA) supplemented with 10% fetal bovine serum and penicillin/streptomycin (Gibco, Invitrogen Life Technologies). In addition, we used the human neuroblastoma line BE(2)C (ATCC). BE(2)C cells were grown in a 1:1 mixture of complete serum-free/low protein medium (Cellgro, Manassas, VA) and F-12K nutrient mixture (Cellgro) supplemented with 10% fetal bovine serum and penicillin/streptomycin (Gibco, Invitrogen Life Technologies). When cells were transduced with siRNAs, these were delivered to cells by lipid based transient transfection using the X-tremeGENE transfection reagent (Roche Applied Sciences, Indianapolis, IN).

Antibodies and Reagents

Mouse antibodies included: mouse monoclonal D-M2A8 [Hewett et al., 2004] and polyclonal TA2 [Bragg et al., 2004], both specific for torsinA; as well as monoclonals against β-actin (Sigma-Aldrich, St. Louis, MO, USA), and protein disulfide isomerase (PDI; Enzo Life Sciences, Farmingdale, NY, USA). Dilutions of antibodies for western blots were: anti-torsinA (DM2A8 1:500 and TA2 1:1000), and anti-β-actin (1:2000). Dilutions of antibodies for immunocytochemistry were: anti-torsinA (TA2 1:500), anti-PDI (1:1000). HRP-conjugated secondary antibodies for rabbit IgG (1:5000) and mouse IgG (1:10000) (Cell Signaling, Danvers, MA, USA; GE Healthcare Life Sciences, Piscataway, NJ, USA) were used in immunoblot detection. Alexa Fluor CY3 anti-mouse IgG, Alexa Fluor 488 anti-mouse IgG, Alexa Fluor 647 anti-rabbit IgG and Alexa Fluor 555 anti-rabbit IgG (all from Invitrogen Life Technologies), and β-tubulin (9F3) rabbit mAb Alexa Fluor 647 (Cell Signaling) (1:250), were also used. All antibodies were used at a dilution of 1:1000 for immunocytochemistry analysis, except CY3 (1:500) and anti-rabbit Alexa Fluor 647 (1:250).

Immunocytochemistry

Cells infected with torsinA-IRES-mCherry lentivirus vectors were seeded on glass coverslips. Twenty-four hr after seeding, cells were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. Cells were then permeabilized with 0.3% Triton X-100 (Sigma-Aldrich) in PBS and blocked with 10% normal goat serum for 1 hr at room temperature. Cells were incubated with primary antibodies in PBS and washed 3 times for 5 min in RT. Cells were then incubated with fluorescent conjugated secondary antibodies or conjugates in PBS for 1 hr at 37°C. Coverslips were mounted on slides using ProLong Gold Antifade Reagent (Gibco, Invitrogen Life Technologies).

Protein Lysates and Western Blot

For western blots, cells were harvested and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 1% SDS) containing protease inhibitors (Roche Applied Science, Indianapolis, IN, USA) and protein concentration was estimated using the Bio-Rad Protein Assay and absorption spectrophotometry. Reduced Laemmli buffer (BioProducts Inc., Ashland, MA, USA) was added to the samples and they were boiled for 10 min. For analysis of torsinA knock-down, 30 μg protein was analyzed in each lane. Immunoreactive proteins were detected using enhanced chemiluminescence (ECL, Thermo Fisher Scientific) and visualized on HyBlot CL Autoradiography film (Denville Scientific Inc., Denville, NJ, USA). To obtain protein lysates under non-reducing conditions, cells were harvested, lysed, and resuspended with non-reducing Laemmli buffer (BioProducts Inc.). In addition, 10 mM N-ethylmaleimide (NEM) was added to these samples to reduce aberrant disulfide bond formation. ECL films were scanned densitometrically, and the optical density of bands was quantified using Image J version 1.38 software (http://rsb.info.nih.gov/ij/).

Lentiviral Vector Preparation

Lentiviral vectors were prepared as previously described [Sena-Esteves et al., 2004]. In brief, 293T cells were co-transfected with the CSCW2-torsinA-IRES-mCherry plasmids, the lentivirus packaging genome CMVRD8.91 (from Dr. Didier Trono, Univ. Geneva, Switzerland) and envelope encoding plasmid (pVSV-G; provided by Dr. Miguel Sena-Esteves, UMassMedCtr). After 72 hr, lentivirus vector supernatant was harvested, concentrated by ultracentrifugation and

titered as transducing units (t.u.)/ml on 293T cells in the presence of 10 μg/mL polybrene (Sigma-Aldrich) by counting mCherry positive cells 48 hr post-infection. A typical titer was around 10^8 t.u./ml.

Lentiviral Infection

293T and BE(2)C cells were plated in 6-well plates at a confluency of 70-80% and transduced with lentiviral vectors at a multiplicity of infection (M.O.I.) of 20 t.u. per cell over 18 hr to achieve >90% infection. Infection efficiency was increased by centrifuging cells with lentivirus at 1800 × g for 90 min using a TTH-750 high-capacity swing-out rotor in a Sorvall Legend RT benchtop centrifuge (Thermo Fisher Scientific, Pittsburg, PA, USA). 293T and BE(2)C cells were transduced with lentivirus expressing torsinA constructs and FACS sorted for mCherry expression 72 hr after transfection and positive cell populations used for further analysis.

Lentivirus (LV)-Mediated Generation of mCherry-Expressing torsinA Clonal Cell Lines

At 3-day post-infection the neuroblastoma cells and fibroblasts were collected and centrifuged. The cells were isolated by FACS (DiVa Vantage Cell Sorter, BD Biosciences, San Jose, CA) as cell pools at a density of 3 to 4 × 10^3 cells/mL in ProCHO5. We gated for mCherry positive cells, we sorted only the high mCherry population (see Supp. Figure 2). To evaluate the stability of torsinA expression over time, the cells were analyzed by western blot for torsinA expression.

Adenoviral Infection and VSVG Trafficking

This protocol was performed as described [Ryan et al., 2012]. Briefly, 293T cells were transduced with an adenoviral vector expressing YFP-$VSVG_{ts045}$ [Keller et al., 2001] at an M.O.I. of 400 PFU/cell and incubated at 40.5°C for 16-20 hr during which time vesicular stomatitis virus glycoprotein G (VSVG) accumulated in the ER. Cells were then transferred to 32°C for 0, 10 or 20 min to allow VSVG to traffic to the Golgi complex. Cells were fixed at the end of the 32°C incubation periods with 4% paraformaldehyde. Cells were subsequently visualized and analyzed with a Carl Zeiss LSM 5 Pascal laser-scanning confocal microscope equipped with an LSM 510 META scan head (Carl Zeiss) or a LSM 510 meta confocal microscope and an EC Plan-Neofluar 40×/1.30 Oil DIC M27 objective. Images were analyzed using Zen 8.0 software (Zeiss, Toronto, ON, CANADA). VSVG was considered ER associated

prior to the appearance of punctate VSVG positive inclusions that are indicative of vesicles trafficking to the Golgi complex. The first appearance of punctate transport vesicles was used as conformation that protein had left the ER. VSVG was considered Golgi complex-associated once all VSVG was completely deposited within a discrete 20 μm^2 area. Data were expressed as a percent of total cell number in each condition. Blinded counts of 28-51 cells per condition were performed in each of three independent experiments.

siRNA Treatment.

We have previously shown torsinA knock-down with siRNA [Hewett et al., 2008; Nery et al., 2011]. Briefly, chemically stabilized siRNA targeting human torsinA message (siRNA1963 (1) and siRNA1958 (2), as well as scrambled control siRNAs were synthesized on an ABI3900 DNA synthesizer according to standard procedures and purified by AEX HPLC ([Peroz et al., 2009]; kindly provided by Alnylam Inc.). A concentration of 50 nM, siRNAs against torsinA or scrambled siRNA was used according to previous work [Hewett et al., 2008; Nery et al., 2011]. To knock-down torsinA, 293T cells were seeded onto 6-well plates 24 hr prior to transfection (80% confluency). The next day, cells were transfected with 50 nM siRNAs against torsinA using X-tremeGENE transfection reagent (Roche Applied Sciences, Indianapolis, IN), according to manufacturer's instructions. Forty-eight hr after transfection, the cells were infected with an adenoviral vector expressing temperature sensitive yellow fluorescent protein-VSVG, YFP-$VSVG_{ts045}$, as described in Methods.

Gluc Activity

BE(2)C cells were co-transduced with lentiviral vectors encoding Gluc/GFP and Fluc/mCherry, each at a M.O.I. of 50. Twenty-four hr after transduction, cells were replated in 96-well plates (6×10^4). After 24 hr, Gluc activity was monitored in 20 μl media by adding 83 μM coelenterazine (Prolume Ltd./Nanolight, Pinetop, AZ, USA). The signal was measured for 10 sec and integrated over 2 sec using a Luminometer (Dynex Technologies, Chantilly, VA, USA). The Fluc activity was measured in lysed cells with 1 mM D-Luciferin and 500 μM Mg ATP under the same conditions.

Neurite Extension

The method described in Hewett et al. [2006] was used to evaluate neurite extension. Briefly, BE(2)C human neuroblastoma cells were plated into 6-well plates at 3×10^5 cells/well and grown, as described above. Cells were transduced with lentivirus vectors encoding torsinAwt or torsinA mutant/variants or non-transduced, and allowed to attach overnight. The next day cells were imaged to determine the average neurite length at baseline prior to differentiation using bright field optics at 20 × magnification. All cells were then placed in differentiation media supplemented with 5 μM blebbistatin (Sigma-Aldrich) and 10 μM all-trans retinoic acid (Sigma-Aldrich) for 3 days to promote elongation of neurites. Prior to and 3 days after differentiation, digital images were taken randomly from three fields per well at 20 × magnification, then analyzed blindly with respect to status using the MetaVue™ software to digitally trace and measure length of neurites. The average neurite length in each cell population at 24 hr post-differentiation was expressed as a proportional increase relative to the pre-differentiation baseline value set at 1. Data are presented as the mean proportional increase ± SEM from three independent experiments. Statistical differences among the means were determined, as described below.

Evaluation of Secretion of Super-folded GFP

293T cells were seeded onto 6-well plates 24 hr prior to transfection at 80% confluency. The next day, cells were transfected with 1 mg of an expression construct for secreted super-folded green fluorescent protein (sec-sfGFP; [Aronson et al., 2011]) using Lipofectamine reagent (Life Technologies/Invitrogen, Carlsbad, CA, USA), according to manufacturers' instructions. The media were collected at 10 hr after the transfection and proteins were precipitated with 10% trichloroacetic acid (TCA) for 1 hr, centrifuged and the pellet resuspended and boiled in SDS sample buffer for PAGE. Ten percent of the resuspension was analyzed by SDS-PAGE. The cells were washed with ice-cold PBS buffer, harvested in the same buffer, centrifuged, and lysed in RIPA buffer. Twenty micrograms of protein in cell lysates were analyzed by SDS-PAGE.

Confocal Microscopy

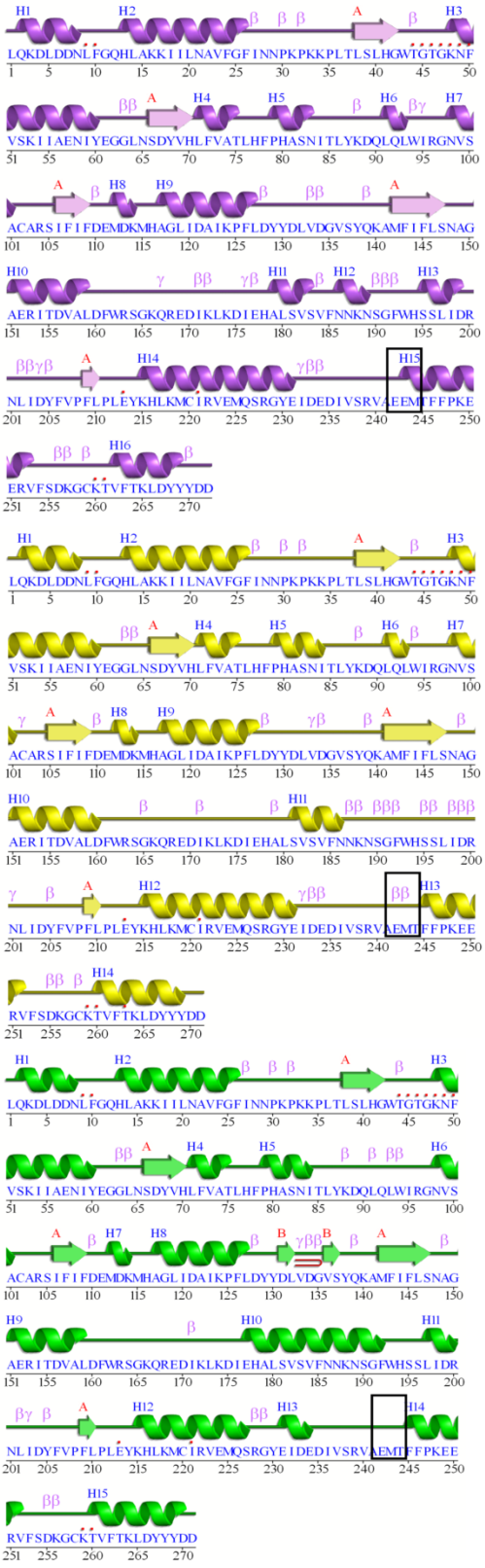
Laser scanning confocal imaging was performed using a Carl Zeiss LSM 5 Pascal laser-scanning confocal microscope. Images were acquired using a numerical aperture 1.4 PlanApo differential

interference contrast (DIC) 63× 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 far red fluorescence emissions were detected through BP 505-530, 560-615 and 650 nm filters, respectively. The different fluorochromes were scanned sequentially by using the multitracking function to reduce the chance of bleed through among fluorescent dyes.

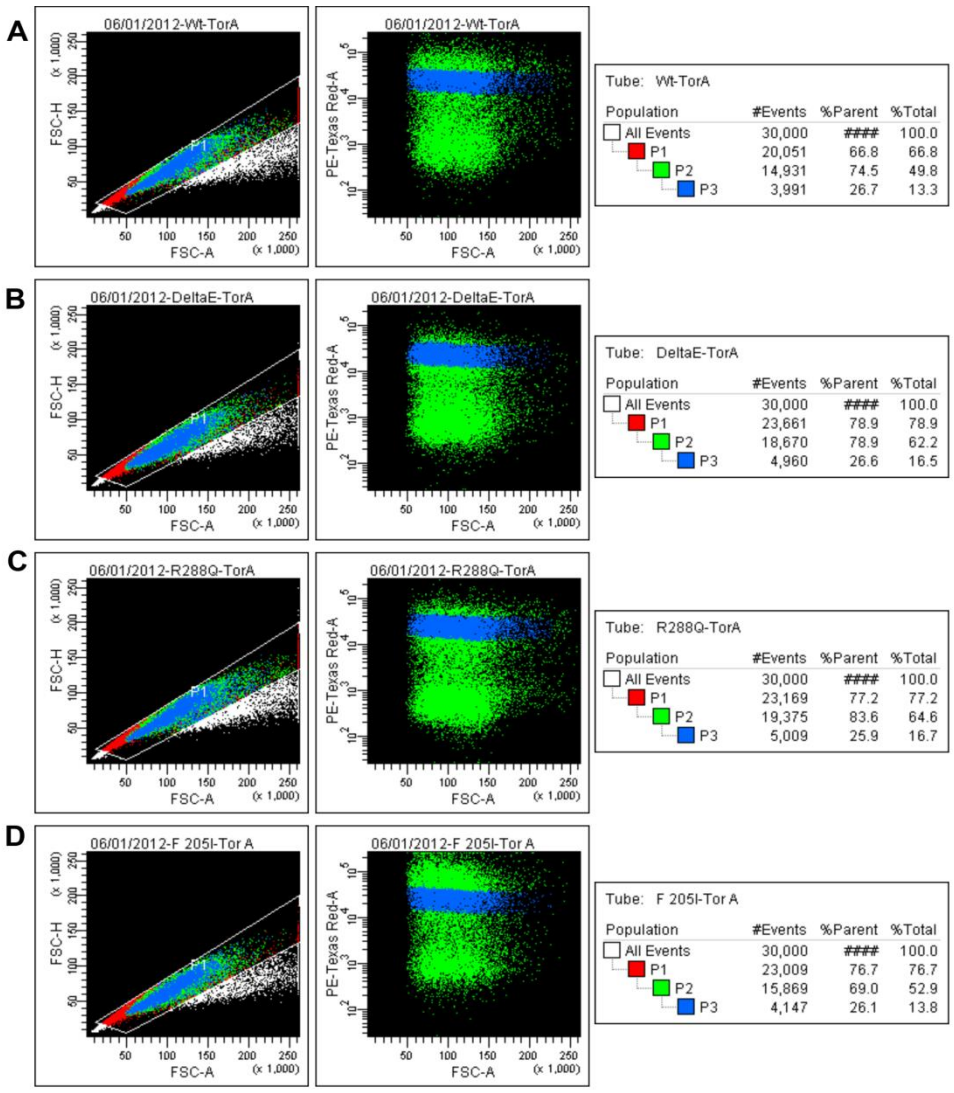
Electron Microscopy (EM)

For thin sections, cells at 80% confluency were transduced with lentivirus encoding torsinAwt and torsinA variants. Seventy-two hr after the infection cells were washed twice with 1× PBS and fixed in 2.5% glutaraldehyde 1.25% paraformaldehyde and 0.03% picric acid in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 hr at RT and washed in 0.1M sodium cacodylate buffer (pH 7.4). The cells were then post-fixed for 30 min in 1% osmium tetroxide (OsO4)/1.5% potassium ferrocyanide (KFeCN6), washed in water 3× and incubated in 1% aqueous uranyl acetate for 30 min followed by 2 washes in water and subsequent dehydration in grades of alcohol (5 min each; 50%, 70%, 95%, 2× 100%). Cells were removed from the dishes in propylene oxide, pelleted at 3000 rpm for 3 min and infiltrated overnight in a 1:1 mixture of propylene oxide and TAAB Epon (Marivac Canada Inc., St. Laurent, CANADA). The samples were subsequently embedded in TAAB Epon and polymerized at 60^{o}C for 48 hr. Ultrathin sections (about 80 nm) were cut on a Reichert Ultracut-S microtome, picked up onto copper grids, stained with lead citrate and examined in a JEOL 1200EX transmission electron microscope. Images were recorded with an AMT 2k CCD camera at the Harvard Medical School Electron Microscopy Core.

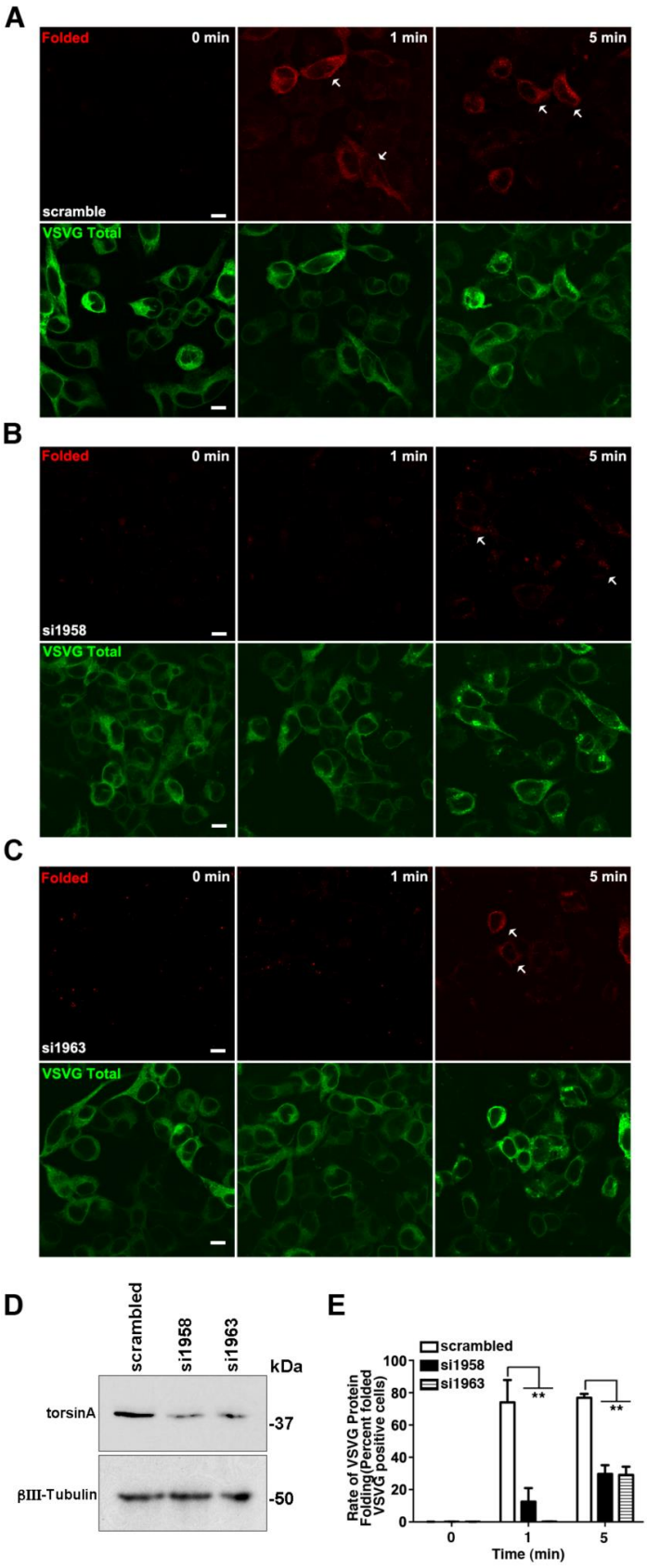
Supp. Figures



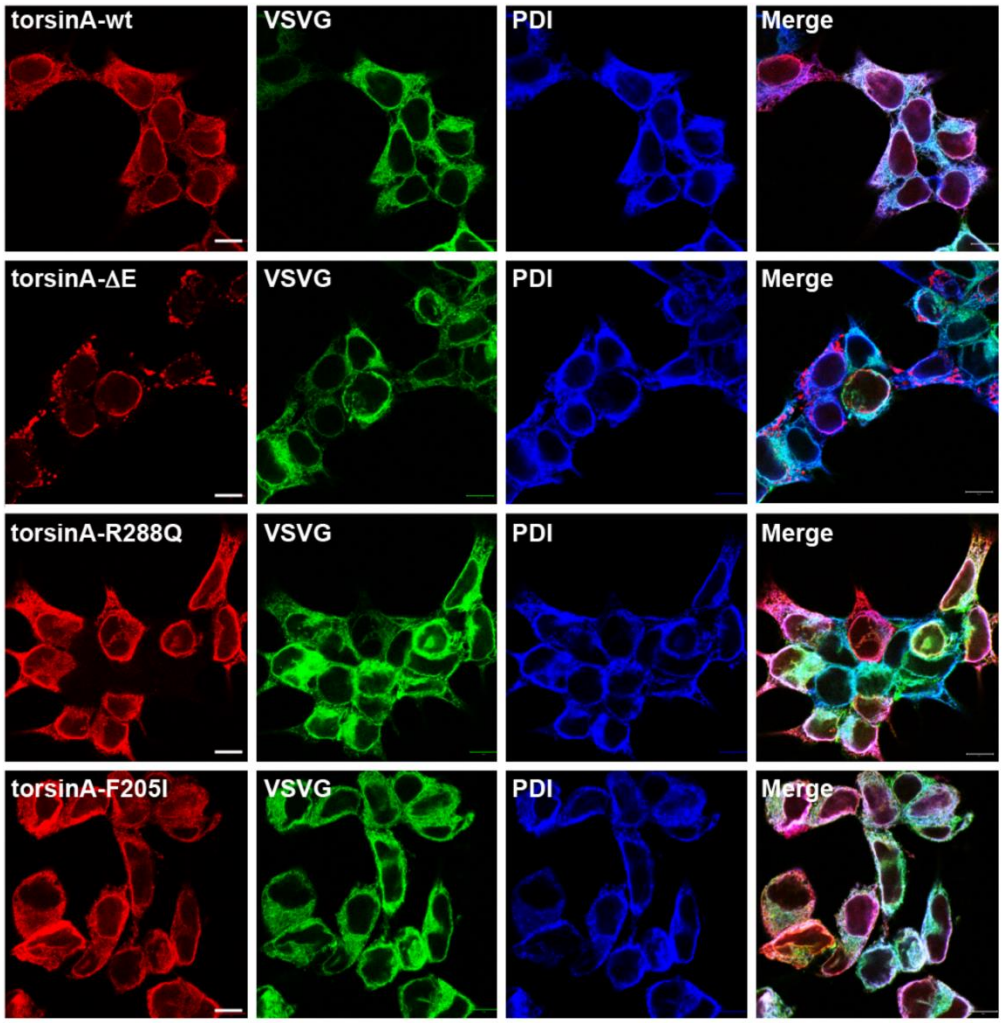
Supp. Figure S1. Predicted secondary structural elements of wild-type torsinA and mutants: delE302, and delE303. The figures represented a predicted (left) and the representative average conformation of the 8 to 20 ns production phase of the 20 ns MD simulations (right) of torsinAwt (purple), delE302 (green), and delE303 (yellow) mapped onto their amino acid sequences. This figure was generated with PDBSum (Tjaart et al., 2014; Laskowski, NAR, 2009). The rectangular boxes highlight the E302 and E303 residues in torsinAwt, and mutants del302 and del303, which occurs at the border between a loop and the penultimate helix (H15 in torsinAWT; H14 in Del302; and H13 in Del303) of the predicted structures.



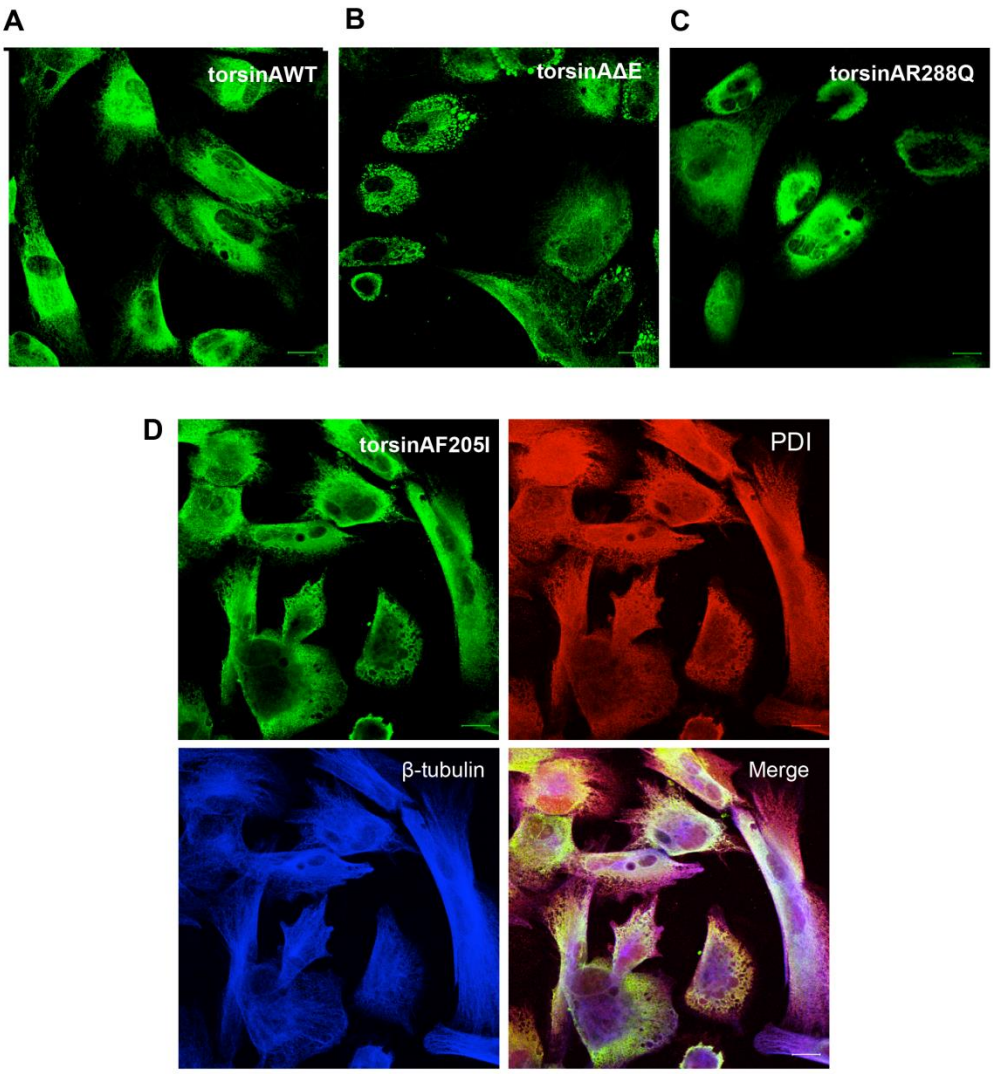
Supp. Figure S2. Representative FACS plots from sorted cells infected with lentivirus expressing torsinA wild-type and variants. Representative histograms are shown demonstrating the expression of mCherry by cells transduced with lentivirus expressing torsinAwt (A), torsinAE (B), torsinAR288Q (C), and torsinAF208I (D). Successful transduction was measured in terms of relative mCherry expression, compared with autofluorescence of noninfected control cell populations. For all pools we sorted cells with similar levels of mCherry expression. Representative results from multiple experiments are shown.


A
Folded
0 min
1 min
5 min
scramble
VSVG Total
B
Folded
0 min
1 min
5 min
si1958
VSVG Total
C
Folded
0 min
1 min
5 min
si1963
VSVG Total
D
scrambled
si1958
si1963
kDa
torsinA
-37
βIII-Tubulin
-50
E
scrambled
si1958
si1963
Rate of VSVG Protein Folding(Percent folded VSVG positive cells)
100
80
60
40
20
0
**
**
0
1
5
Time (min)

Supp. Figure S3. Protein folding is delayed after torsinA depletion. 293T cells were transfected with torsinA siRNAs (si1958 and si1963) or scrambled siRNA as a control. Forty-eight hr after siRNA transfection, the cells were infected with a VSVG-expressing adenovirus and incubated at 40.5°C to block protein folding and allow VSVG to accumulate in the ER. Then cells were rapidly transitioned to 32°C to recover and permit protein folding prior to being fixed and antigenically labeled with a VSVG antibody specific to the folded form of VSVG. (A) Control siRNA treated cells display positive labeling for folded protein (top panel, red) relative to total VSVG (bottom panel, green) within 1 min of incubation at 32°C. (B) Cells treated with siRNA 1958 display reduced labeling for folded VSVG protein (bottom panel, green) within 1 min of incubation at 32°C and markedly lower labeling 5 min post-recovery, as compared to the control. (C) Under conditions of torsinA depletion by siRNA 1963, protein folding was markedly reduced and not evident until 5 min post-recovery. (D) Knock-down efficiency for torsinA was confirmed by western analysis 3 days after transfection. (E) Image analysis quantification of folded-VSVG positive cells relative to total-VSVG in cells transfected with scrambled (control), and siTorA (1958 and 1963) after 1 or 5 min at 32^{o}C. Decreased levels of torsinA are associated with impaired in VSVG folding (magnification bar = 10 μM; Student's *t* test **p<0.01; n=3).



Supp. Figure S4. ER morphology does not change when cells are incubated at 40°C in cells expressing variant torsinA forms. (A) 293T cells expressing variant torsinA forms do not show changes in the ER morphology. Cells were fixed after incubated for 16 hr at 40.5^{o}C. Maintenance of ER morphology was observed in all conditions (n=3) (magnification bar = 10 μM).



Supp. Figure S5. Differential ER distribution of torsinAF205I in human fibroblast cells. Fibroblast cells were infected with lentivirus expressing torsinAwt (A), torsinAE (B), torsinAR288Q (C), and torsinAF205I (D). Three days after transfected processed for immunocytochemistry for to torsinA (A-D). TorsinAwt and torsinAR288Q had no marked effect on its ER distribution, when torsinAΔ*E* tended to form aggregates. (D) Triple immunolabeling for torsinA, the ER marker PDI, and B-tubulin were performed in cells expressing torsinAF205I. Note when torsinAF205I was overexpressed, in most of the cells the ER assumed a 'honeycomb' vacuolar appearance.

Supp. References

Aronson DE, Costantini LM, Snapp EL. 2011. Superfolder GFP is fluorescent in oxidizing environments when targeted via the Sec translocon. Traffic 12:543-548.

Bragg DC, Slater DJ, Breakefield XO. 2004. TorsinA and early-onset torsion dystonia. Adv Neurol 94:87-93.

Breakefield XO, Braverman M, Riker DK, Giller ELJ. 1981. Catechol-O-methyltransferase activity in cultured human skin fibroblasts from controls and patients with dystonia musculorum deformans. J Neurosci Res 6:349-360.

de Beer TA, Berka K, Thornton JM, Laskowski RA. 2014. PDBsum additions. Nucl Acids Res 42:D292-296.

Hewett JW, Kamm C, Boston H, Beauchamp R, Naismith T, Ozelius L, Hanson PI, Breakefield XO, Ramesh V. 2004. TorsinB--perinuclear location and association with torsinA. J Neurochem 89:1186-1194.

Hewett JW, Nery FC, Niland B, Ge P, Tan P, Hadwiger P, Tannous BA, Sah DW, Breakefield XO. 2008. siRNA knock-down of mutant torsinA restores processing through secretory pathway in DYT1 dystonia cells. Hum Mol Genet 17:1436-1445.

Hewett JW, Zeng J, Niland BP, Bragg DC, Breakefield XO. 2006. Dystonia-causing mutant torsinA inhibits cell adhesion and neurite extension through interference with cytoskeletal dynamics. Neurobiol Dis 22:98-111.

Keller P, Toomre D, Diaz E, White J, Simons K. 2001. Multicolour imaging of post-Golgi sorting and trafficking in live cells. Nat Cell Biol 3:140-149.

Laskowski RA, Hutchinson EG, Michie AD, Wallace AC, Jones ML, Thornton JM. 1997. PDBsum: a Web-based database of summaries and analyses of all PDB structure. Trends Biochem 22:488-490.

Nery FC, Armata IA, Farley JE, Cho JA, Yaqub U, Chen P, da Hora CC, Wang Q, Tagaya M, Klein C, Tannous B, Caldwell KA, et al. 2011. TorsinA participates in endoplasmic reticulum-associated degradation. Nat Commun 2:393.

Peroz D, Dahimène S, Baró I, Loussouarn G, Mérot J. 2009. LQT1-associated mutations increase KCNQ1 proteasomal degradation independently of Derlin-1. J Biol Chem 284:5250-5256.

Ryan SD, Ferrier A, Kothary R. 2012. A novel role for the cytoskeletal linker protein dystonin in the maintenance of microtubule stability and the regulation of ER-Golgi transport. Bioarchitecture 2:2-5.

Sena-Esteves M, Tebbets JC, Steffens S, Crombleholme T, Flake AW. 2004. Optimized large-scale production of high titer lentivirus vector pseudotypes. J Virol Methods 122:131-139.