Mutant torsinA interferes with protein processing through the secretory pathway in DYT1 dystonia cells

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TorsinA is an AAA$^+$ protein located predominantly in the lumen of the endoplasmic reticulum (ER) and nuclear envelope responsible for early onset torsion dystonia (DYT1). Most cases of this dominantly inherited movement disorder are caused by deletion of a glutamic acid in the carboxyl terminal region of torsinA. We used a sensitive reporter, *Gaussia* luciferase (Gluc) to evaluate the role of torsinA in processing proteins through the ER. In primary fibroblasts from controls and DYT1 patients most Gluc activity (95%) was released into the media and processed through the secretory pathway, as confirmed by inhibition with brefeldinA and nocodazole. Fusion of Gluc to a fluorescent protein revealed coalignment and fractionation with ER proteins and association of Gluc with torsinA. Notably, fibroblasts from DYT1 patients were found to secrete markedly less Gluc activity as compared with control fibroblasts. This decrease in processing of Gluc in DYT1 cells appear to arise, at least in part, from a loss of torsinA activity, because mouse embryonic fibroblasts lacking torsinA also had reduced secretion as compared with control cells. These studies demonstrate the exquisite sensitivity of this reporter system for quantitation of processing through the secretory pathway and support a role for torsinA as an ER chaperone protein.

early onset dystonia | endoplasmic reticulum | luciferase | protein translation

Early onset torsion dystonia (DYT1) is a dominantly inherited movement disorder with reduced penetrance characterized by sustained, involuntary muscle contractions and abnormal posturing (1, 2). Symptoms appear to arise from altered neuronal circuitry in the brain rather than neuronal loss (3–5), and motor improvement can be achieved with deep brain stimulation (6, 7). The mutation underlying most cases of DYT1 dystonia is a GAG deletion in the DYT1 gene encoding torsinA, which results in a loss of a glutamic acid residue (ΔE) in the carboxy-terminal region (8, 9).

TorsinA is a member of the superfamily of ATPases Associated with a variety of Activities (AAA$^+$), a group of chaperone proteins involved in folding of proteins, assembly of protein complexes, and transport of cargo in cells (10, 11). Most torsinA within cells is localized in the contiguous lumen of the endoplasmic reticulum (ER) and nuclear envelope (NE) (12–16). Various potential functions for torsinA have been implicated in mammalian cells, including architecture of the NE (16, 17), neurite extension (18, 19), cell adhesion (18), protection from toxic insults and abnormal proteins (20–25), and processing of proteins through the secretory pathway (25–27). Many of these functions may involve a common denominator, regulation of protein processing in the ER/NE.

The secretory pathway controls processing of a variety of proteins destined for cell membranes, organelles, and the extracellular space (28). Proteins enter the secretory pathway through translocons in the ER membrane in close association with ER luminal chaperones, such as calnexin, immunoglobulin heavy-chain-binding protein (BiP), and protein disulfide isomerase (PDI) (29). After posttranslational modification, correct folding, and multimerization, secreted proteins leave the ER within vesicles, move on through the Golgi apparatus, and are incorporated into membranes and vesicles in cells or secreted. Entrance into and transit through the secretory pathway involves coordination between cytoskeletal elements and membrane components (30). Within the ER, misfolded or mutant proteins, high levels of some normal proteins, calcium depletion, and oxidative stress can trigger ER stress by means of the unfolded protein response (UPR) (31, 32). This response can lead to a delay in processing through the secretory pathway, increased levels of ER chaperone proteins, like BiP, and expansion of ER membranes as well as increased exit of abnormal proteins from the ER into the cytoplasm for degradation (31, 33–35).

In this study, we used a reporter, *Gaussia* luciferase (Gluc), a naturally secreted, highly sensitive luciferase (36) to monitor trafficking of proteins through the secretory pathway. Gluc alone or fused in-frame to a yellow fluorescent protein (Gluc-YFP) was used to monitor this pathway in primary fibroblasts from DYT1 patients and controls in culture. Levels of Gluc and Gluc-YFP luciferase activity in cells and media, and the intracellular location of Gluc-YFP were assayed after infection with lentivirus vectors encoding these reporters. In both DYT1 and control cells, processing of Gluc through the secretory pathway was confirmed, and torsinA was found to be associated with Gluc-YFP in cells. However, patient cells had a marked decrease in the rate of Gluc/Gluc-YFP secretion as compared with control cells. This appeared to be due to reduced function of torsinA as an ER chaperone protein, because mouse embryonic fibroblasts (MEFs) from homozygous torsinA knockout mice also showed reduced Gluc secretion as compared with MEFs from wild-type and heterozygous littermates.

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Results

Gluc Assay for Protein Secretion. Protein secretion from human DYT1 and control primary fibroblasts was monitored after infection with a lentivirus vector encoding Gluc and the optimized blue fluorescent protein cerulean (37) under control of the CMV promoter. Levels of Gluc activity in the medium were proportional to cell number for both control and DYT1 cells (Fig. 1*A*), with DYT1 cells having a lower level of secretion as compared with control cells, average 0.5 and 1.5 relative light units (RLU) per cell per hour, respectively. Luciferase activity in the medium increased in a linear manner 24 to 72 h after infection, again with DYT1 cells having a lower rate of secretion (Fig. 1*B*). For both cell types, release of Gluc into the medium was blocked with brefeldinA,

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The authors declare no conflict of interest.

Abbreviations: DYT1, early onset torsion dystonia; ER, endoplasmic reticulum; Gluc, *Gaussia* luciferase; MEF, mouse embryonic fibroblast; NE, nuclear envelope; PDI, protein disulfide isomerase; UPR, unfolded protein response.

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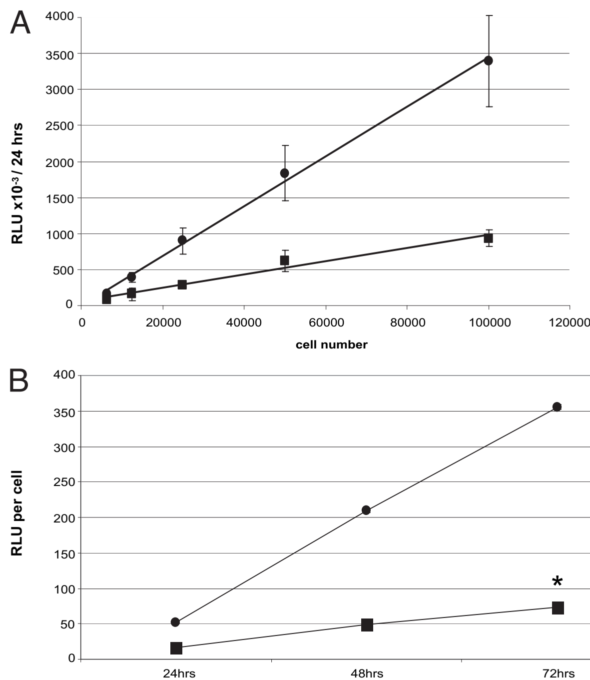

Fig. 1. Validation of Gluc secretion assay, linearity with cell number and time. Control (C2, circles) and DYT1 (D3, squares) fibroblasts were infected with a lentivirus vector encoding Gluc-IRES-cerulean to achieve infection of >90% cells. (*A*) Infected cells were plated 48 h after infection at different cell numbers per well, and luciferase activity in the medium was quantitated 24 h after plating. (*B*) Infected cells were plated at a density of 2.5×10^3 cells per well, and luciferase activity in the media was quantitated 24, 48, and 72 h after plating. Mean values are shown ± SD; comparison of control and DYT1 secretion at 72 h; *, $P < 0.001$.

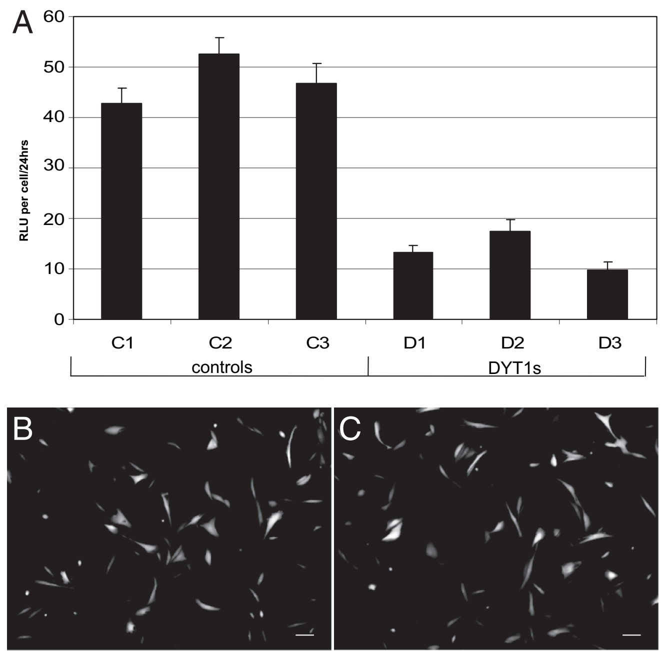

Fig. 2. Rate of Gluc secretion in DYT1 fibroblasts as compared with control fibroblasts. Cells from three DYT1 and three control lines were infected with the lentivirus vector encoding Gluc-IRES-cerulean and replated 48 h after infection at 2.5×10^3 cells per well. (*A*) Gluc activity 24 h after plating was determined in the medium as RLU per cell per 24 h. The experiment was repeated two times in triplicate for each cell line, shown as mean ± SD. The average mean of DYT1 lines and control lines was significantly different at $P <$ 0.004. (*B* and *C*) Examples of the high infectivity of control (C1) and DYT1 (D3) lines as assessed by fluorescence. (Magnification ×100.) (Scale bars, 10 μM.)

which blocks transit from the ER to Golgi, and with nocodazole which depolymerizes microtubules, as expected for processing through the secretory pathway [see supporting information (SI) Fig. 8].

Gluc Secretion in DYT1 and Control Fibroblasts. Levels of luciferase activity in the media were evaluated in three control lines and three lines from affected DYT1 patients after infection with the lentivirus vector encoding Gluc. Average Gluc activity in the medium of the DYT1 lines was ≈25% of that in control lines ($P < 0.004$) (Fig. 2*A*), with all lines being equally infectable with the lentivirus vector (e.g., Fig. 2 *B* and *C*). Thus, DYT1 fibroblasts, which express ΔE-torsinA and torsinA at endogenous levels, are compromised in their ability to release active Gluc into the medium as compared with control cells expressing only torsinA. Western blot analysis of total levels of immunoreactive torsinA in control and DYT1 lines showed comparable amounts in most lines (SI Fig. 9).

Fate of Gluc in DYT1 and Control Fibroblasts. To determine the intracellular fate of Gluc, Gluc was fused in-frame at its carboxy terminus to enhanced YFP (a derivative of GFP) (38). A decreased level of luciferase activity in the medium (by ≈30%) was found in DYT1 fibroblasts expressing this fusion protein, as compared with control fibroblasts (Fig. 3*A*). This difference in levels of luciferase activity in the medium was accompanied by a parallel decrease in the level of active luciferase within DYT1 cells as compared with control cells (Fig. 3*B*). In contrast, Western blot analysis of the Gluc-YFP protein in DYT1 and control cells revealed similar levels in both cell types (Fig. 3*C*), suggesting that the Gluc-YFP in DYT1 cells was less active than in control cells, possibly because of inefficient processing.

The cellular distribution of Gluc-YFP in control and DYT1 cells was similar as assessed by differential extraction and immunocytochemistry. Sequential extraction of cells with digitonin (cytoplasmic fraction), Triton X-100 (ER fraction), and scraping (residual proteins) was carried out by using GAPDH as a cytoplasmic marker and calnexin and torsinA as ER markers. In both cell types, marker proteins behaved as predicted, with essentially all of the 37 kDa torsinA band in the ER fraction (Fig. 4). Gluc-YFP was found predominantly in the ER fraction with <10% in the cytoplasmic fraction in DYT1 and control cells. Gluc-YFP was further confirmed to be predominantly within the NE/ER by immunocytochemical coalignment with PDI in digitonin-extracted control and DYT1 cells (Fig. 5). Further extraction with Triton X-100 to release ER proteins left only faint residual staining of Gluc-YFP for both cell types (data not shown).

Mutant torsinA in DYT1 cells might interfere with protein processing by reducing chaperone functions of torsinA. If torsinA is a chaperone protein. it would be expected to transitorily associate with Gluc-YFP in the ER. To evaluate this association, immune precipitation was carried out by using antibodies to torsinA in cells expressing Gluc-YFP or YFP, followed by Western blot analysis with antibodies to GFP (Fig. 6). An association between Gluc-YFP and torsinA was found for both DYT1 and control cells. As controls, no association was found between YFP and torsinA (Fig. 6), and when antibodies to GAPDH were used for immunoprecipitation, neither Gluc-YFP or YFP were detected by Western blot analysis (data not shown). To further elucidate a potential role for torsinA in protein processing, the Gluc secretion assay was carried out on MEFs cultured from individual embryos in matings of torsinA heterozygous knockout mice (39, 40). TorsinA$^{-/-}$ MEFs showed a marked decrease (≈50%) in secretion of Gluc (Fig. 7). Mutant torsinA might decrease chaperone function simply by being inactive, resulting in haploinsufficiency, but this does not seem to be

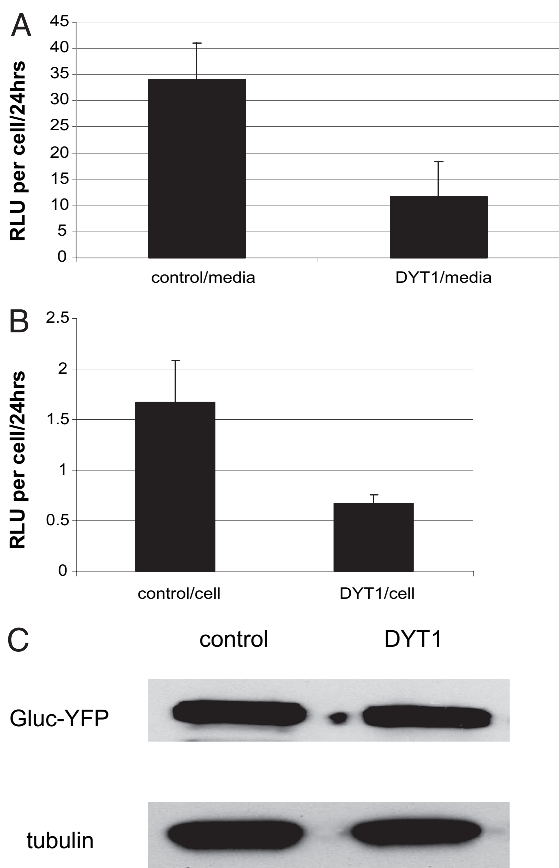

Fig. 3. Levels and distribution of Gluc-YFP in control and DYT1 media and cells. Cells were infected with lentivirus vector encoding Gluc-YFP, and, 24 h later, cells were plated at 2.5×10^3 per well. (*A* and *B*) Twenty-four hours after plating, luciferase activity was assessed in the media (*A*) or in living cells (*B*). In both control (C1) and DYT1 (D2) cells ≈5% activity was retained in cells with 95% in the media. (*C*) Western blot analysis indicated similar levels of Gluc-YFP protein in both cell types by using α-tubulin as an index of loading. Note that this was repeated in two additional DYT1 (D1, D4) and one control line (C2) with similar results.

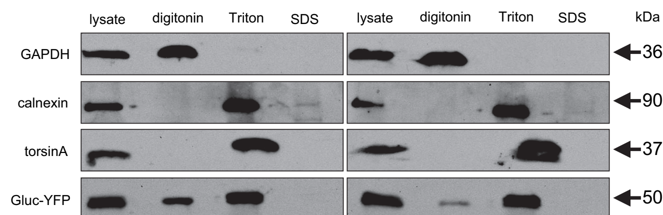

Fig. 4. Differential solubilization of control and DYT1 fibroblasts expressing Gluc-YFP. Twenty-four hours after infection with Gluc-YFP-IRES-cerulean lentivirus vector monolayers of control (C1) (*Left*) and DYT1 (D1) (*Right*) cells were homogenized directly (lysates) or sequentially extracted with digitonin (cytoplasmic fraction), Triton X-100 (ER fraction), and SDS/scraping (remaining proteins). Lysate and extracts were resolved by SDS/PAGE and immunoblotted for the cytoplasmic marker GAPDH, ER markers calnexin and torsinA, and the reporter protein Gluc-YFP. Note that this was repeated in two additional DYT1 (D2, D4) and one control lines (C2) with similar results.

the case because heterozygote knockout cells had near wild-type levels of Gluc secretion. Rather, ΔE-torsinA appears to inhibit torsinA activity, possibly by forming inactive multimers with torsinA (41, 42).

Mutant ΔE-torsinA protein might also sensitize DYT1 cells to the UPR, which can restrict protein processing in the ER (31, 43). The UPR can be induced by expression of abnormal proteins as well as agents, such as thapsigargin that interfere with protein processing in the ER (31). Because Gluc and Gluc-YFP are nonmammalian proteins expressed at relatively high levels after lentivirus vector infection, they might themselves induce an ER stress response. ER stress can be monitored by elevation in levels of the "master regulator" BiP (44). Both control and DYT1 cells showed comparable increases in BiP levels in response to thapsigargin and little to no increase in BiP because of expression of Gluc-YFP (SI Fig. 10).

Discussion

Findings in This Study. An assay incorporating a naturally secreted luciferase alone and fused in-frame to a fluorescent protein was used to evaluate the integrity of the secretory pathway in DYT1 cells. This assay proved highly sensitive and revealed a marked reduction in processing of this reporter in primary fibroblasts from DYT1 patients as compared with control cells. Specifically, expression cassettes for Gluc and Gluc-YFP delivered via a lentivirus vector revealed a 25–30% lower level of secretion of active enzyme by DYT1 patient fibroblasts (heterozygous for ΔE-torsinA and torsinA) as compared with control fibroblasts (homozygous for torsinA). This reduction appears to be due to a decrease in torsinA function in DYT1 cells because MEFs lacking torsinA (homozygous knockout) had a decreased rate of Gluc-YFP secretion as compared with wild-type and heterozygous MEFs. TorsinA coimmune precipitated with Gluc-YFP in control and DYT1 human cells, supporting a role as an ER chaperone protein in facilitating processing of proteins through the secretory pathway.

TorsinA in the Secretory Pathway. As an AAA$^+$ protein (10, 45), torsinA is predicted to determine the conformational state of proteins and their interactions with other proteins (11, 46). Thus, torsinA might participate in the quality control process of folding and assembly of proteins within the ER, as observed for other chaperone proteins (28, 29). This function is supported by the association between Gluc-YFP and torsinA in the ER and by inhibition in protein processing both in the presence of endogenous levels of ΔE-torsinA and in the absence of torsinA. Other studies have implicated torsinA in processing of proteins through the secretory pathway under conditions of overexpression of either torsinA or ΔE-torsinA (or their homologues). Overexpression of torsinA suppressed processing of membrane proteins, such as the dopamine transporter (DAT) and mutant forms of ε-sarcoglycan (26, 47). A chaperone function of torsinA was supported by the finding that a mutation in the Walker ATP site of torsinA blocked this inhibitory effect (26), with AAA$^+$ proteins typically interacting with their substrates in the ATP-bound state (48). Synthesis of a GFP-DAT fusion protein was also suppressed in nematodes overexpressing the homologue of torsinA, TOR-2 (25). Studies of overexpressed ΔE-torsinA are confounded by formation of ER-derived membrane inclusions (12, 13), which, for example, entrap vesicular monoamine transporter 2 in human neuroblastoma cells and restrict incorporation into synaptic vesicles (27). Although torsinA is clearly implicated in movement of proteins through the secretory pathway, its mode of action is still unresolved.

TorsinA and the ER Stress Response. The marked compromise in processing of Gluc/Gluc-YFP reporter proteins through the secretory pathway in DYT1 patient cells could also be caused by activation of the UPR through expression of mutant ΔE-torsinA and abnormal Gluc (Gluc-YFP) proteins (31). The formation of ER-derived membranous inclusions after overexpression of ΔE-torsinA (12, 13) might be related to the ER stress response, which causes ER membrane expansion (31, 49). However, high-level expression of ΔE-torsinA did not increase levels of the ER sensor BiP in glioma cells (50). If torsinA were a component of the ER stress response, its levels might increase in response to stress,

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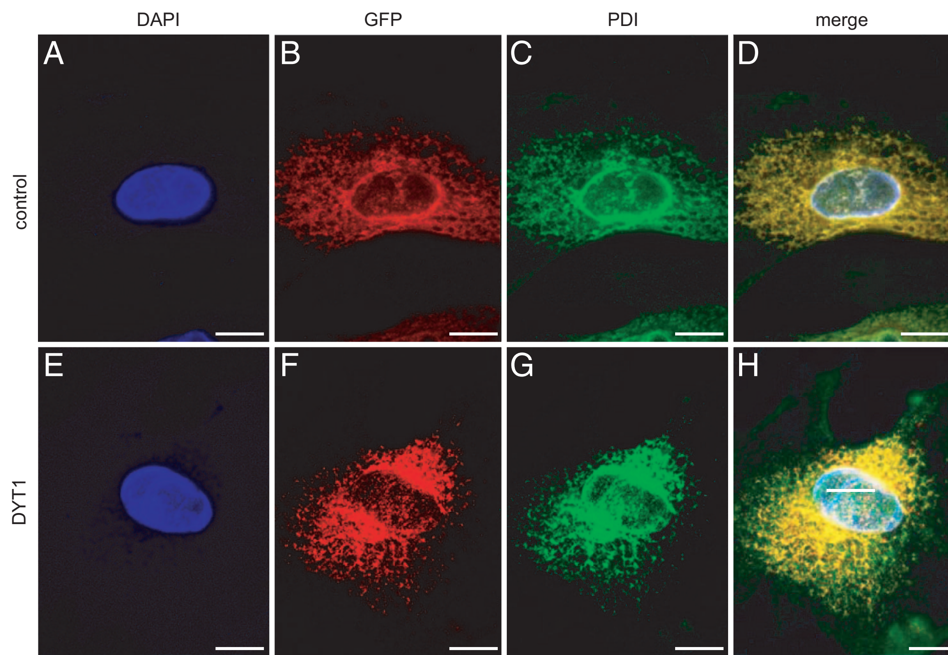

Fig. 5. ER distribution of Gluc-YFP and PDI in control and DYT1 cells. Control (C1) and DYT1 (D1) cells were infected with Gluc-YFP lentivirus vector, and, 24 h later, cells were treated on ice with digitonin. Dual immunocytochemistry was carried out for Gluc-YFP (*B* and *F*) and the ER marker PDI (*C* and *G*) with merged images shown in the rightmost panels (*D* and *H*). Nuclei were stained with DAPI (*A* and *E*). (Magnification ×100.) (Scale bars, 10 μm.)

however, tunicamycin treatment of glioma cells did not increase levels of torsinA (50). In the present study, both control and DYT1 fibroblasts showed a comparable increase in BiP in response to thapsigargin, with little to no increase in response to Gluc. Thus, although torsinA may be involved at some level in the ER stress response, as are other ER chaperones, it does not appear to have a pivotal role.

Links Between the NE/ER and Secretory Pathway. Another function of torsinA appears to be in linking the NE and ER membranes to the cytoskeleton. TorsinA does not itself appear to span the NE/ER membranes and is presumed to move within the contiguous lumen of the NE and ER and associate with transmembrane binding partners in both domains (51). Participation of torsinA in a complex that links the outer nuclear membrane (ONM) to the cytoskeleton is supported by the involvement of a nematode homologue of torsinA, OOC-5 in nuclear rotation during early embryogenesis (52). A binding partner for torsinA in the NE is supported by findings that mutant forms of torsinA, both ΔE-torsinA and torsinA with a substrate trap mutation in the Walker ATP binding domain, cause accumulation of torsinA immunoreactivity in the NE, presumably because of prolonged interaction with substrate(s) there (16, 50, 53). Abnormalities in NE structure, i.e., altered spacing and formation of vesicles between the inner nuclear membrane and ONM have also been noted in cultured cells expressing these mutant forms of torsinA (16, 53) and in neurons from mice lacking torsinA or being homozygous for ΔE-torsinA alleles (17).

The coordinated movement of torsinA and vimentin in fibroblasts (18) suggests a possible explanation for mutant torsinA-induced alterations in the NE and compromise of protein processing. Vimentin is an intermediate filament protein abundant in fibroblasts, which forms a net around the NE and helps to define

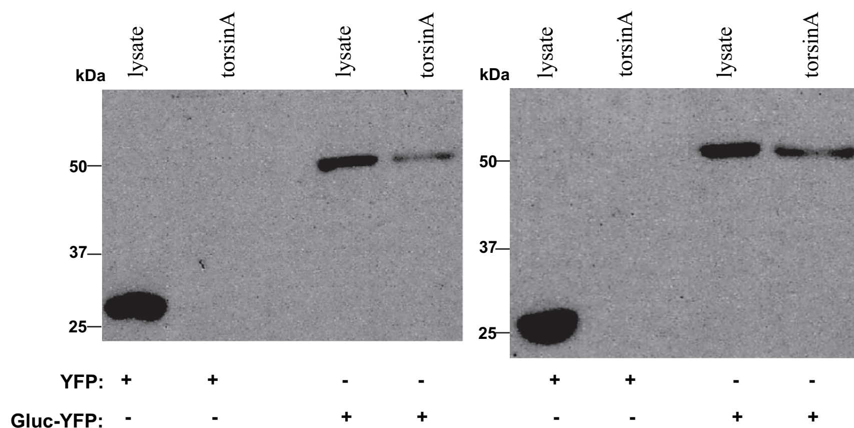

Fig. 6. Coimmune precipitation of torsinA and Gluc-YFP. Control (C4) (*Left*) and DYT1 (D2) (*Right*) cells were infected with lentivirus vector encoding Gluc-YFP-IRES-cerulean or YFP-IRES-cerulean (control), and, 72 h later, lysates were prepared and immune precipitated (IP) for torsinA. Lysates and torsinA-IP pellets were resolved by SDS/PAGE, and Western blotting was carried out with antibodies to GFP.

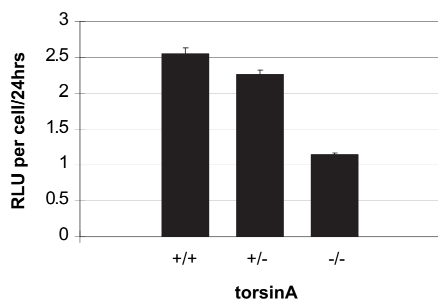

Fig. 7. Gluc secretion in torsinA knockout MEFs. MEFs in early passages were genotyped by genomic PCR as described (39). Cells were infected with lentivirus vector encoding Gluc-IRES-cerulean and replated 48 h later at 2.5×10^3 cells per well, and Gluc in media was assessed 24 h later. The experiment was repeated two times in triplicate for each genotype and the mean is shown ±SD. The mean values for torsinA$^{+/+}$ versus torsinA$^{-/-}$ cells were significantly different: *, $P < 0.001$.

nuclear shape and mediate nuclear movement (54, 55). The outer nuclear membrane as well as the reticular ER are sites of active protein translation (56, 30), with cytoskeletal elements involved in the organization of translational components (30, 57). For example, components of the translation elongation factor-1 complex appear to be associated with vimentin (58) and kinectin (59), and interference with either of these cytoskeletal elements can delay processing through the secretory pathway (J.Z. and X.O.B., unpublished data and ref. 59). Cytoskeletal elements are also critical in movement of vesicles between the ER and Golgi (60, 61). Thus, torsinA may participate in a link between the NE/ER and the cytoskeleton (62, 63) involved in synthesis and entry of proteins into the secretory compartment and/or transit from the ER to the Golgi.

Implications for Pathophysiology and Treatment of Dystonia. One of the enigmas of torsion dystonia is the healthy physical and mental condition of patients with the exception of loss of sensorimotor control (64, 65). If torsinA is important in modulation of the secretory pathway, and ΔE-torsinA interferes with this function, then mutant DYT1 carriers must have sufficient torsinA to carry out this function in most cells of the body. Complete loss of function of torsinA through homozygous knockout (or homozygosity for ΔGAG alleles) leads to death at birth in mice, apparently through malfunction in the central nervous system but with normal gross morphology (17, 39). TorsinA levels in the brain are highest in the perinatal period in rodents (66, 67) and humans (68), and compromise of torsinA function through expression of ΔE-torsinA may affect the development and function of neurons, for example in processing proteins, such as DAT. Such a situation might occur in dopaminergic neurons in the substantia nigra which have the highest levels of torsinA message expression in the human brain (69). This compromise in torsinA function by ΔE-torsinA may manifest as dystonia only when triggered by other factors, such as stress, hence the low penetrance of the ΔGAG mutation in DYT1.

DYT1 cells from DYT1 patients have a deficiency in processing of at least some proteins through the secretory pathway. This supports a role for torsinA as an ER chaperone protein in a dynamic complex of proteins that links the NE/ER/Golgi, cytoskeleton, and translational machinery. The Gluc secretory assay offers a simple, sensitive means to evaluate the integrity of the secretory pathway and to screen for drugs and agents that can facilitate protein processing in disease states.

Methods

Cell Culture. Fibroblast lines were generated from skin biopsies: human controls [HF6(C1), HF24(C2), HF18(C3), HF19(C4), HF17(C5)], and affected DYT1 ΔGAG carriers [HF48(D1), HF47(D2), FFF13111983(D3), FFF076111984(D4), HF60(D5)]. HF lines were generated in our laboratory (70), and FFF lines were obtained from Mirella Filocamo (L'Istituto Giannina Gaslini, Genoa, Italy). MEF cultures were prepared as described (71) from single embryos (embryonic days 13–14) of matings between heterozygous torsinA knockout mice (39, 40). Cells were grown in DMEM (GIBCO, Rockville, MD) as described (18). ER stress was induced by exposure to 10 nM thapsigargin for 16 h. To evaluate processing of Gluc-YFP through the secretory pathway, cells were exposed to 1 μg/ml nocodazole (Sigma, St Louis, MO) or 3 μg/ml brefeldinA during 24-h measurement of Gluc-YFP release.

Vectors and Expression Cassettes. Lentivirus vectors were derived from a self-inactivating lentivirus, CS-CGW (72). A cDNA encoding humanized Gluc (Nanolight; Prolume Pinetop, AZ) alone, Gluc fused in-frame at the carboxy terminus to enhanced YFP (Invitrogen, Carlsbad, CA), or YFP alone was inserted downstream of the CMV promoter, followed by an internal ribosome entry site (IRES) and the cDNA for cerulean (37) (from David Piston, Vanderbilt University Medical Center, Nashville, TN). Vectors were produced by cotransfection of 293T cells with the lentivirus packaging plasmid (pCMVR8.91), envelope coding plasmid (pVSVG), and vector construct yielding titers of 10^8 transducing units (tu)/ml (72).

Gluc Activity. Cells were infected with lentivirus vector encoding Gluc (or Gluc-YFP) and cerulean at a multiplicity of infection = 50. Twenty-four to 48 h after infection, cells were replated in 12-well plates (25,000 cells per well). Luciferase activity was monitored in conditioned, cell-free medium (or in living washed cells) at varying time points after replating, typically over a 24-h period by using a luminometer (Dynex Technologies, Chantilly, VA) with 20 μM coelenterazine (Nanolight; Prolume) (36). Values are shown as mean ± SD, and significance was calculated by using the two-tailed Student *t* test (Excel, Microsoft, Redmond, WA).

Antibodies Used. Antibodies used were torsinA (D-M2A8; ref. 22); α-tubulin (DM1A; Sigma); GAPDH (Chemicon, Temecula, CA); GFP (Molecular Probes, Eugene, OR), PDI (SPA-891; Stressgen, Ann Arbor, MI), calnexin (SPA-856; Stressgen), and BiP (Grp78; SPA-826; Stressgen).

Differential Solubilization of Cells. Human fibroblast monolayer cultures were placed on ice and rinsed with PBS. Then a digitonin solution [150 μg/ml digitonin in 50 mM Hepes (pH 7.4)/100 mM KAc/2.5 mM MgAc] was added for 5 min, and the lysate (cytoplasmic proteins) were collected (73). After rinsing four times in PBS, a Triton X-100 solution [1% Triton X-100 in 50 mM Hepes (pH 7.4)/500 mM KAC/5 mM MgAC) was added for 5 min (ER proteins). Proteins in digitonin and Triton X-100 extracts were precipitated with 85% acetone. Remaining cell components were washed three times with PBS and scraped off the plate into PBS. Protein concentrations were determined by using the Coomassie plus protein assay (Pierce, Rockford, IL). Samples were resuspended in equal volumes and resolved by SDS/PAGE.

Immunocytochemistry. Cells were grown on coverslips and extracted with digitonin alone or digitonin and Triton X-100, as above, and then fixed with 4% paraformaldehyde in PBS (18). After rinsing with PBS, coverslips were incubated with 0.1% Nonidet P-40 in PBS for 20 min, followed by blocking with 10% goat serum (Vector Laboratories, Burlingame, CA) in PBS for 1 h. Nuclei were stained with 0.25 μg/ml DAPI (Sigma) for 5 min at room temperature. Cells were incubated with monoclonal antibodies to torsinA (1:1,000) and polyclonal antibodies to PDI (1:600) for 1 h at 37°C. Coverslips were washed with PBS and incubated with secondary antibodies conjugated to Cy3 affiniPure donkey anti-mouse, (1:1,000; Jackson ImmunoResearch, West Grove, PA) or Alexa Fluor 488 goat

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anti-rabbit (1:2,000; Molecular Probes) for 1 h at 37°C. Coverslips were mounted onto slides by using gelvatol mounting medium containing 15 μg/ml antifade agent 1,4-diazabicyclo(2.2.2)-octane (Sigma). Images were captured by using an inverted fluorescent microscope (TE 200-U; Nikon, East Rutherford, NJ) coupled to a digital camera.

Western Blot. SDS gel electrophoresis and protein transfer were carried out as described (18). Membranes were probed with antibodies against GFP (1:2,000), α-tubulin (1:10,000), GAPDH (1:4,000), calnexin (1:5,000), torsinA (1:100), and/or BiP (1:200) diluted in TBST and visualized with HRP conjugated to secondary antibodies and SuperSignal West Pico Chemiluminescent Substrate (Pierce). Secondary antibodies for Western blots were: sheep anti-mouse IgG-HRP (1:10,000) or donkey anti-rabbit IgG-HRP (1:10,000) (Amersham Pharmacia Biotech, Piscataway, NJ).

Immune Precipitation. Human fibroblasts (2×10^6) were lysed by resuspension in 1 ml of ice-cold RIPA buffer [150 mM NaCl/50 mM Tris (pH 7.5)/1% Nonidet P-40/0.5% deoxycholate/0.1% SDS) for 30 min. A portion of the lysates (450 μl) was incubated with 7 μl of D-M2A8 (anti-torsinA antibody) and 3 mg/ml Protein G agarose beads (Roche Applied Sciences, Indianapolis, IN) overnight at 4°C (12). Beads were washed three times with PBS and resuspended in SDS sample buffer (60 μl). Fifteen microliters of the original lysates and 30 μl of the immune precipitated resuspension were loaded onto gels for SDS/PAGE and Western blotting.

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