Dystonia-associated mutations cause premature degradation of torsinA protein and cell-type-specific mislocalization to the nuclear envelope

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An in-frame 3 bp deletion in the torsinA gene resulting in the loss of a glutamate residue at position 302 or 303 (torsinA ΔE) is the major cause for early-onset torsion dystonia (DYT1). In addition, an 18 bp deletion in the torsinA gene resulting in the loss of residues 323–328 (torsinA Δ323–8) has also been associated with dystonia. Here we report that torsinA ΔE and torsinA Δ323–8 mutations cause neuronal cell-type-specific mislocalization of torsinA protein to the nuclear envelope without affecting torsinA oligomerization. Furthermore, both dystonia-associated mutations destabilize torsinA protein in dopaminergic cells. We find that wild-type torsinA protein is degraded primarily through the macroautophagy–lysosome pathway. In contrast, torsinA ΔE and torsinA Δ323–8 mutant proteins are degraded by both the proteasome and macroautophagy–lysosome pathways. Our findings suggest that torsinA mutation-induced premature degradation may contribute to the pathogenesis of dystonia via a loss-of-function mechanism and underscore the importance of both the proteasome and macroautophagy in the clearance of dystonia-associated torsinA mutant proteins.

INTRODUCTION

Dystonia is a movement disorder characterized by involuntary movements and prolonged muscle contraction, resulting in twisting body motions, tremor and abnormal posture (1). Early-onset generalized torsion dystonia (DYT1) is the most common and severe form of hereditary dystonia. DYT1 is autosomal dominant with a 30–40% penetrance, and the disease onset usually occurs between 1 and 28 years of age (1). Most cases of DYT1 dystonia are caused by an in-frame 3 bp (GAG) deletion in the gene-encoding protein torsinA, which results in the loss of a glutamate residue at position 302 or 303 (torsinA ΔE) (2). In addition, an in-frame 18 bp deletion in the torsinA gene resulting in the loss of amino acids F323-Y328 (torsinA Δ323–8) was found in three members of a single family with dystonia (3). Because of a concomitant mutation in the myoclonic dystonia (DYT11)-linked protein epsilon-sarcoglycan in two of these patients (4), it remains to be established whether the torsinA Δ323–8 mutation contributes to the pathogenesis of dystonia.

TorsinA is a 332 amino acid protein that is expressed in brain and multiple other tissues (5). It is unclear why mutations in torsinA manifest mainly as a neurological phenotype. TorsinA contains an N-terminal (NT) 20 amino acid endoplasmic reticulum (ER) signal sequence (6,7), followed by a 20 amino acid hydrophobic domain. Immunohistochemical studies in neurons reveal the presence of torsinA in the cytoplasm, neuronal processes and synaptic terminals (5,8–13). TorsinA is widely believed to reside in the lumen of the ER (14–20). In contrast to the ER localization of wild-type (WT) torsinA, torsinA ΔE mutant protein was reported to co-localize with nuclear envelope (NE) marker proteins (18–22), and overexpression of torsinA ΔE mutant may lead to the formation of cytoplasmic perinuclear inclusions (14,15,17,22,23). The effect of the torsinA Δ323–8 mutation on the localization of torsinA protein has not yet been determined.

Sequence analysis reveals that torsinA is a member of the AAA\(^+\) (ATPases associated with a variety of cellular activities) superfamily of ATPases (2,24,25). Members of the AAA\(^+\) family often have molecular chaperone activities and facilitate changes in protein conformation (26,27). They are involved in a wide variety of cellular processes, such as membrane trafficking and transcriptional regulation. The AAA\(^+\) proteins

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are known to oligomerize into multimeric complexes (26–33). Previous results regarding torsinA oligomerization have been controversial, with some showing that torsinA exists in a monomeric form (16) and others showing that torsinA oligomerizes (34,35). Moreover, whether dystonia-associated torsinA ΔE and torsinA Δ323–8 mutations affect the oligomerization of torsinA protein remains unclear.

An unexplored key question is how WT and dystonia-associated mutant torsinA proteins are degraded in cells. Protein degradation serves as an important mechanism by which cells regulate the expression levels of specific proteins and consequently the cellular processes in which these proteins participate (36). In addition, selective degradation of misfolded proteins, which are generated by genetic mutations or oxidative damage, is essential for cell homeostasis (37). The ubiquitin–proteasome and macroautophagy (hereafter referred to as autophagy)–lysosome pathways are the two major intracellular proteolytic systems for the degradation of both normal cellular proteins and misfolded proteins (36,37). Whether one or both of these pathways are involved in WT or mutant torsinA protein degradation is unknown, and the effects of dystonia-associated mutations on torsinA protein stability remain undefined.

In this study, we characterized the localization, oligomerization and degradation of WT torsinA protein and investigated the effects of dystonia-associated torsinA ΔE and torsinA Δ323–8 mutations. Our results reveal that both dystonia-associated mutations cause neuronal cell-type-specific mislocalization of torsinA protein to the NE without affecting torsinA oligomerization and promote torsinA protein degradation by both the proteasome and autophagy–lysosome pathways. These findings provide new insights into the pathogenic mechanisms of torsinA ΔE and torsinA Δ323–8 mutations and have important implications for understanding and treating dystonia.

RESULTS

Analysis of torsinA localization reveals neuronal cell-type-specific enrichment in the NE

Given the widespread expression of torsinA (5), it is unclear why torsinA mutations lead to a neuronal phenotype. To determine whether there is a difference between torsinA localization in neuronal versus non-neuronal cell types, we used human HeLa cells, a well-established non-neuronal cell line (38), and human SH-SY5Y cells, a dopaminergic neuronal cell model (39). Double-labeling immunofluorescence confocal microscopic analysis was performed to determine the intracellular distribution of C-terminally HA-tagged WT torsinA in HeLa and SH-SY5Y cells. We found that torsinA WT protein exhibited extensive co-localization with the ER marker KDEL in both HeLa and SH-SY5Y cells (Fig. 1A). Both torsinA WT and KDEL immunoreactivities were predominantly localized to the ER, but a subset of the signal was also found at the NE (Fig. 1A).

Because of the direct continuity of the ER and NE membranes (40), it is difficult to find markers that are exclusively localized to the ER or NE. In addition, the amount of ER or NE membranes visible under the confocal microscope can vary greatly depending on the plane of focus. To control for these variables, we quantified the relative distribution of torsinA in NE and ER and compared the obtained NE/ER ratio with that of the ER marker KDEL from the same double-labeling experiments. The result showed no significant difference between the relative NE/ER distribution of torsinA WT and that of KDEL in HeLa cells (Fig. 1B), indicating that torsinA WT is primarily associated with the ER in these non-neuronal cells. In contrast, the NE/ER ratio of torsinA WT was significantly greater than the NE/ER ratio of KDEL in SH-SY5Y cells (Fig. 1B), indicating an enhanced preferential localization of torsinA WT to the NE compared with the ER marker KDEL in the neuronal cells. We found that the NE/ER ratio of KDEL in SH-SY5Y cells was significantly higher than the NE/ER ratio of KDEL in HeLa cells (Fig. 1B). To control for the cell-type variation in the relative NE/ER distribution of the ER marker, we determined the NE preference of torsinA by normalizing the NE/ER ratio of torsinA in HeLa or SH-SY5Y cells to the corresponding NE/ER ratio of KDEL in the same cells. Data represent mean ± SE from at least three independent experiments.

Figure 1. TorsinA is enriched in the NE in SH-SY5Y cells but not in HeLa cells. (A) HeLa or SH-SY5Y cells expressing C-terminally HA-tagged torsinA WT were stained with primary antibodies against HA and ER marker KDEL, followed by detection with secondary antibodies conjugated to TR (KDEL, red) or FITC (torsinA, green). Hoechst stain was used to visualize the nucleus. (B) Quantification shows the relative distribution of torsinA and KDEL in the NE versus the ER. Data represent mean ± SE from at least three independent experiments.

*Significantly different from the NE/ER ratio of KDEL in SH-SY5Y cells (P < 0.05). #Significantly different from the NE/ER ratio of KDEL in HeLa cells (P < 0.05). (C) NE preference of torsinA was determined by normalizing the NE/ER ratio of torsinA in HeLa or SH-SY5Y cells to the corresponding NE/ER ratio of KDEL in the same cells. Data represent mean ± SE from at least three independent experiments. *Significantly different from HeLa cells (P < 0.05). Scale bar, 10 μm.
indicating a neuronal cell-type-specific enrichment of torsinA in the NE compared with the ER marker.

Next, we performed double-labeling immunofluorescence confocal microscopic analysis to examine the NE/ER distribution of endogenous torsinA in mouse embryonic fibroblasts and primary cortical neurons. We observed significant co-localization between endogenous torsinA and the ER marker KDEL in both fibroblasts and cortical neurons (Fig. 2A). Quantification analysis of the NE/ER distribution of torsinA and KDEL signal revealed no significant difference between the NE/ER ratio of torsinA and that of KDEL in fibroblasts (Fig. 2B), indicating that endogenous torsinA is primarily associated with the ER in these non-neuronal cells. In primary cortical neurons, however, the NE/ER ratio of endogenous torsinA was significantly greater than the NE/ER ratio of KDEL (Fig. 2B). After normalization to the NE/ER ratio of KDEL in the same cells, the NE preference of endogenous torsinA was increased from 0.97 ± 0.03 in fibroblasts to 1.58 ± 0.14 in cortical neurons. These data are consistent with the result of exogenous torsinA localization in HeLa and SH-SY5Y cells (Fig. 1) and provide additional support for a neuronal cell-type-specific enrichment of torsinA in the NE.

**Dystonia-associated mutations cause neuronal cell-type-specific translocation of torsinA from the ER to NE**

To determine the effects of dystonia-associated mutations on the subcellular localization of torsinA protein, we examined and compared the intracellular distribution of C-terminally HA-tagged torsinA WT, torsinA ΔE and torsinA Δ323–8 mutants in HeLa (Fig. 3A) and SH-SY5Y (Fig. 3B) cells by double-labeling immunofluorescence confocal microscopy. We found that, in HeLa cells, torsinA ΔE and torsinA Δ323–8 mutants had similar NE/ER ratios compared with torsinA WT and KDEL (Fig. 3C), indicating that dystonia-associated mutations do not affect the subcellular localization of torsinA in these non-neuronal cells. However, in SH-SY5Y cells, both torsinA ΔE and torsinA Δ323–8 mutants showed a significantly higher NE/ER ratio compared with KDEL (Fig. 3C). Moreover, the NE/ER ratio of both torsinA mutants was significantly greater than that of torsinA WT in SH-SY5Y cells (Fig. 3C). After normalization to the NE/ER ratio of KDEL in the same SH-SY5Y cells, torsinA ΔE and torsinA Δ323–8 mutants had an NE preference of 2.29 ± 0.13 and 2.22 ± 0.11, respectively (Fig. 3D), both of which represent a significant increase in the percentage of NE-associated mutant torsinA compared with the NE localization of torsinA WT. Together, these results indicate that dystonia-associated mutations induce translocation of torsinA from the ER to NE in neuronal cells but not in non-neuronal cells.

**Dystonia-associated mutations do not disrupt torsinA oligomerization**

Given our finding that dystonia-associated mutations selectively alter torsinA localization in SH-SY5Y cells but not in HeLa cells (Fig. 3) and our interest in addressing issues that are pertinent to the neuronal phenotype of dystonia, we focused the remainder of our studies on WT and mutant torsinA proteins in SH-SY5Y cells. Since torsinA belongs to the AAA family of ATPases and many AAA members oligomerize and are only functional in their oligomerized state (28,29,31), we performed co-immunoprecipitation experiments to examine the in vivo self-association of WT and dystonia-associated mutant torsinA in SH-SY5Y cells. As shown in Figure 4A, Myc-tagged torsinA WT co-immunoprecipitated with HA-tagged torsinA WT, but not with the HA vector alone, indicating the presence of torsinA WT oligomers in SH-SY5Y cells. We found that both dystonia-associated torsinA ΔE and torsinA Δ323–8 mutants were able to co-immunoprecipitate torsinA WT (Fig. 4B), demonstrating that the ability of torsinA to oligomerize is not altered by dystonia-associated mutations.
TorsinA oligomerization does not require its C-terminal region

Given the involvement of the C-terminal region in the oligomerization of several AAA⁺ proteins (27), we next tested whether the C-terminal region of torsinA is required for its oligomerization. We made a torsinA NT expression construct (Fig. 5A) by deleting its C-terminal region (residues 189–332) and examined the ability of torsinA NT to bind torsinA WT by co-immunoprecipitation. The result showed that torsinA NT was able to bind torsinA WT in SH-SY5Y cells (Fig. 5B), suggesting that the C-terminal region of torsinA is not required for its oligomerization. Moreover, we found that the interaction between torsinA NT with full-length torsinA was not affected by dystonia-associated torsinA ΔE and torsinA Δ323–8 mutations (Fig. 5C), further supporting the results seen in Figure 4.

Dystonia-associated mutations destabilize torsinA protein

Next, we performed pulse-chase experiments to determine whether dystonia-associated torsinA ΔE and torsinA Δ323–8 mutations alter torsinA protein stability in SH-SY5Y cells. As shown in Figure 6A, torsinA WT had a half-life of ~80 h, indicating that torsinA is normally a very stable protein. In contrast, we found that >70% of torsinA ΔE and torsinA Δ323–8 mutant proteins had been degraded at the 24 h chase time point (Fig. 6A), suggesting that both dystonia-associated mutant proteins are considerably
less stable compared with WT torsinA. To further examine the effects of dystonia-associated mutations on torsinA turnover rate, we took a closer look at the first 24 h time period by performing additional pulse-chase experiments which measured the changes in WT and mutant torsinA protein levels every 6 h. We found the half-lives of torsinA

\[ \Delta E \] and torsinA

\[ \Delta 323–8 \] mutant proteins were reduced to \( \sim 18 \) h (Fig. 6B), further confirming that dystonia-associated mutations accelerate the degradation of torsinA.

Dystonia-associated mutations promote the degradation of torsinA by both the proteasome and autophagy–lysosome pathways

The ubiquitin-proteasome pathway is a major intracellular proteolytic system for the degradation of both normal cellular proteins and misfolded proteins (36,37). Misfolded ER proteins are usually retrotranslocated to the cytoplasm for the degradation by the proteasome through a process known as ER-associated degradation (ERAD) (41,42). Whether the proteasome participates in the degradation of misfolded NE proteins or normal ER and NE proteins remains unclear. To determine whether the proteasome is involved in the degradation of WT or mutant torsinA proteins, we first examined the effects of proteasome inhibition on the steady-state levels of torsinA WT, torsinA

\[ \Delta E \] and torsinA

\[ \Delta 323–8 \] in SH-SY5Y cells. We found that, in vehicle (DMSO)-treated control cells, the steady-state levels of both torsinA

\[ \Delta E \] and torsinA

\[ \Delta 323–8 \] mutant proteins were significantly (\( P < 0.05 \)) lower than the steady-state level of torsinA WT (Fig. 7), in agreement with the decreased stability of these torsinA mutants observed in the pulse-chase experiments (Fig. 6). The steady-state levels of torsinA

\[ \Delta E \] and torsinA

\[ \Delta 323–8 \] mutant proteins were significantly (\( P < 0.05 \)) increased by the treatment of cells with the proteasome inhibitor MG132 (Fig. 7). In contrast, proteasome inhibition by MG132 had no significant effect on the steady-state level of WT torsinA (Fig. 7). Moreover, pulse-chase analysis revealed that proteasome inhibition selectively delayed degradation of torsinA

\[ \Delta E \] and torsinA

\[ \Delta 323–8 \] mutants, but not of torsinA WT protein (Fig. 8). Together, these results provide strong evidence supporting a role for the proteasome in the selective degradation of dystonia-associated mutant torsinA proteins.
The autophagy–lysosome pathway is another major intracellular proteolytic system for the degradation of both normal cellular proteins and misfolded proteins (36,37). This pathway involves the formation of a double-membrane structure called an autophagosome to engulf a portion of the cytoplasm and the subsequent fusion of the autophagosome with the lysosome to allow the degradation of its contents by lysosomal hydrolases. In contrast to proteasome-mediated degradation, which requires proteins to be first unfolded for entry into the 20S proteasome core particle, the autophagy–lysosome pathway is able to break-down completely folded proteins, misfolded and aggregated proteins, as well as entire organelles (43). To determine whether the autophagy–lysosomal pathway is involved in the degradation of WT or mutant torsinA proteins, we examined the effects of pharmacological inhibition of this pathway on steady-state levels of torsinA WT, torsinA ΔE and torsinA Δ323–8 in SH-SY5Y cells (Fig. 7). We assessed the changes in the steady-state levels caused by treatment with the following inhibitors: chloroquine and ammonium chloride (NH₄Cl), which inhibit the lysosomal proteases by raising the pH, and 3-methyladenine (3-MA), a specific inhibitor of autophagosome formation (44). We found that treatment with each of these three inhibitors significantly (P < 0.05) increased the steady-state levels of torsinA WT, torsinA ΔE and torsinA Δ323–8 proteins (Fig. 7), suggesting that the autophagy–lysosome pathway is involved in the degradation of WT torsinA as well as the dystonia-associated mutant torsinA proteins. In addition, our pulse-chase analysis revealed that the rates of degradation of torsinA WT, torsinA ΔE and torsinA Δ323–8 proteins in SH-SY5Y cells were significantly (P < 0.05) reduced by the lysosomal cysteine protease inhibitor E64 (Fig. 8), providing additional support for a role of the autophagy–lysosome pathway in the degradation of WT torsinA and dystonia-associated mutant torsinA proteins.

DISCUSSION

Given the widespread expression of torsinA in multiple tissues and cell types, it is unclear why genetic mutations in human torsinA result in mainly a neuronal phenotype (5). In addition, it is not understood why torsinA null and torsinA ΔE knock-in mice have abnormal NE membranes in neurons but not in non-neuronal cells (45). Previous studies have reported the mislocalization of torsinA ΔE to the NE in both neuronal and non-neuronal cell types (18,20,46,47). However, these studies did not provide a quantitative analysis of the relative NE/ER distribution which is crucial given the direct continuity between the NE and ER. In addition, the torsinA Δ323–8 mutation has not been included in these studies. Using quantitative double-labeling immunofluorescence confocal microscopic analysis of the relative NE/ER distribution, we found that WT torsinA shows a preferential localization to the NE in neuronal SH-SY5Y cells compared with torsinA WT in
non-neuronal HeLa cells. Similar preferential NE localization was also observed for endogenous torsinA in primary cortical neurons compared with endogenous torsinA in fibroblasts. The preferential NE localization suggests that torsinA may have a neuronal cell-specific role at the NE. In addition, our results indicate that the dystonia-associated torsinA ΔE and torsinA Δ323–8 mutations cause translocation of torsinA from the ER to NE in SH-SY5Y cells but not in HeLa cells. Together, these data suggest that the neuronal NE may be especially susceptible to torsinA dysfunction, which help explain the neuronal cell-specific phenotype of dystonia-associated torsinA mutations.

Previous studies regarding torsinA oligomerization have been controversial (16,34,35). Our data suggest that torsinA WT is capable of self-associating into an oligomeric complex. Because the C-terminal region of AAA+ proteins is thought to be necessary for oligomerization (27), it has been suggested that the dystonia-associated mutations may disrupt oligomerization. A previous study showed that torsinA ΔE mutation disrupted interaction with torsinA WT and torsinA ΔE and prevented the formation of a stable oligomeric complex (35). In contrast, Torres et al. (34) reported that the ΔE mutation appears to render torsinA more susceptible to oligomerization, and it has been shown that the presence of torsinA ΔE can increase the amount of torsinA WT in the NE (19). Our results indicate that neither of the dystonia-associated mutations disrupts the ability of torsinA to self-associate. To further analyze torsinA self-interaction, we created a deletion mutant that lacked the C-terminal region. The NT portion of torsinA was able to interact with torsinA WT as well as with both dystonia-associated mutant forms of torsinA, suggesting that the NT region of torsinA is sufficient for oligomerization.

The structural and functional consequences of torsinA Δ323–8 mutation remain mostly unknown. Owing to its co-occurrence with a mutation in another dystonia-related protein, epsilon-sarcoglycan (4), it is unclear whether Δ323–8 is actually a dystonia-causing mutation. In addition, its reported lack of apparent mislocalization phenotype has led to the suggestion that the Δ323–8 and ΔE mutant torsinA proteins may follow different pathogenic pathways (15). Our finding that both Δ323–8 and ΔE mutations in torsinA cause enhanced NE preference and premature degradation suggest that these two mutations share the same pathogenic pathway.

Many neurological diseases, including familial Alzheimer’s disease and Parkinson’s disease, have been linked to mutations in proteins that render them unstable and result in premature degradation and subsequent loss of function (48,49). Our data suggest that this could also be a pathogenic mechanism for DYT1 dystonia. Previous studies have shown that torsinA ΔE inhibits torsinA function (34,50–52) and that torsinA null and torsinA ΔE knock-in mice display similar phenotypes (45), suggesting that torsinA ΔE is a loss-of-function mutation. We found that, although the WT torsinA is a highly stable protein with a half-life of several days, both torsinA ΔE and torsinA Δ323–8 mutant proteins have significantly reduced half-lives, indicating a higher rate of turnover, which suggests that the torsinA mutant proteins are unstable or misfolded. Our data provide, for the first time, evidence that torsinA ΔE and torsinA Δ323–8 mutations cause premature degradation of torsinA and support a loss-of-function pathogenic mechanism for both dystonia-associated mutations.

The ubiquitin-proteasome and autophagy–lysosome pathways are the two main routes of protein degradation in cells (36,37). The proteasome pathway usually mediates selective degradation of short-lived regulatory proteins and misfolded proteins, whereas the autophagy–lysosome pathway is a relatively non-selective, bulk clearance system that is primarily responsible for the turnover of long-lived proteins as well as organelles (37,53). Our inhibitor studies reveal that WT torsinA is degraded through the autophagy–lysosome pathway but not the proteasome pathway, consistent with our finding that torsinA WT is a long-lived, ER/NE protein.

Although proteasome inhibition did not affect degradation of WT torsinA, it significantly delayed the degradation of both torsinA ΔE and torsinA Δ323–8 mutant proteins, indicating that dystonia-associated torsinA mutant proteins, but not WT torsinA, are selectively targeted to the proteasome for degradation. The mechanism(s) by which torsinA ΔE and torsinA Δ323–8 mutant proteins are specifically recognized and earmarked for proteasome-mediated degradation is unclear. One possibility is that torsinA ΔE and torsinA Δ323–8 mutations induce aberrant folding of torsinA protein, leading to exposure
of hydrophobic patches or unmasking of a cryptic degron. The hydrophobic patches or degron serves as a signal for recognition by an ER chaperone or E3 ubiquitin-protein ligase, which cooperates with other ERAD components to facilitate retrotranslocation from the ER to the cytosol and delivery of torsinA mutant proteins to the proteasome for degradation (41,42,54). Our data indicate that, in addition to proteasome-mediated degradation, the autophagy–lysosome pathway is also involved in the clearance of dystonia-associated torsinA ΔE and torsinA Δ323–8 mutant proteins. These results support the emerging view that the autophagy–lysosome pathway provides an alternative ER quality-control mechanism for the clearance of misfolded proteins in the ER lumen (55). In conclusion, our findings obtained from this study provide new insights into the pathogenic mechanisms of torsinA ΔE and torsinA Δ323–8 mutations and have important implications for developing novel therapeutic strategies to treat dystonia.

MATERIALS AND METHODS

Expression constructs and antibodies

Conventional molecular biological techniques (56) were used to subclone DNA fragments encoding torsinA WT, ΔE, Δ323–8 and NT into mammalian vectors expressing C-terminal HA, Myc or FLAG tags for transfection into cells. A rabbit polyclonal anti-torsinA antibody was raised against the NT region of torsinA and affinity-purified as described previously (57,58). Other antibodies used in this study are anti-KDEL (Stressgen); anti-β-actin (Chemicon); mouse monoclonal anti-HA (12CA5) and anti-Myc (9E10) (57). Horseradish peroxidase-conjugated secondary antibodies were used for immunoblotting (Jackson Immunoresearch Laboratories, Inc.). Flourescein isothiocyanate (FITC)- and Texas Red (TR)-conjugated secondary antibodies were used for immunofluorescence microscopy (Jackson Immunoresearch Laboratories, Inc.).

Cell transfections and co-immunoprecipitation

Transfections of HeLa and SH-SY5Y cells with the indicated plasmids were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Cell lysates were prepared from transfected cells and immunoprecipitations were carried out as described previously (49,57) using anti-HA or anti-Myc antibodies. Immunocomplexes were recovered by incubation with protein G-sepharose beads (Upstate). After washing, the immunocomplexes were analyzed by SDS–PAGE and immunoblotted with the appropriate antibodies and horseradish peroxidase-conjugated secondary antibodies. Results were visualized using enhanced chemiluminescence.

Primary cell culture

Primary cortical neuronal cultures were prepared from embryonic day 18 mice as described (59,60) and maintained in NeuroBasal Media (Gibco) supplemented with AraC (Sigma) for 3–7 days. Mouse embryonic fibroblast cultures were prepared from embryonic day 13 mice using a well-established method (61–63), and early passage cells were used for all experiments.

Immunofluorescence confocal microscopy

Cells were fixed in 4% paraformaldehyde, stained with appropriate primary and secondary antibodies and processed for

Figure 8. Degradation of torsinA mutants by both the proteasome and lysosome pathways. SH-SY5Y cells expressing HA-tagged torsinA WT (A), ΔE (B) or Δ323–8 (C) were pulse-labeled for 1 h with medium containing [35S]Met/Cys and chased with non-radioactive Met/Cys containing MG132 (closed circle), E64 (closed triangle) or vehicle control (open square) for the indicated time. Lysates were immunoprecipitated with anti-HA antibodies and detected by SDS–PAGE and autoradiography. Proteins levels were quantified using a PhosphorImager and plotted relative to the corresponding torsinA levels at 0 h. Data are shown as mean ± SE of the results from at least three independent experiments. The asterisks indicate a statistically significant (P < 0.05) increase in the level of torsinA in MG132-treated cells versus vehicle-treated controls. The plus sign indicates an increase approaching significance (P < 0.065) in the level of torsinA in MG132-treated cells versus vehicle-treated controls. The pound signs indicate a statistically significant (P < 0.05) increase in the level of torsinA in E64-treated cells versus vehicle-treated controls.
Quantitative analysis of the NE/ER distribution

Quantification of the NE/ER distribution of torsinA or KDEL was performed on unprocessed images of cells double-labeled for torsinA and KDEL using MetaMorph Imaging System Software (Molecular Devices). Using the region tool in the MetaMorph program, we outlined the whole cell and demarcated the NE area, which was clearly apparent in the KDEL staining of almost all images (64,65). The fluorescence in the cytoplasmic region outside of the NE area was defined as the ER staining. The amount of torsinA or KDEL fluorescence in the NE and ER of each cell was quantified with MetaMorph as described previously (64–66). The obtained NE/ER ratio of torsinA was compared with that of the ER marker KDEL from the same double-labeled cell. For each torsinA genotype (torsinA WT, ΔE or Δ323–8), 30–45 cells were randomly selected for analysis. Analysis was carried out in a blinded manner by an investigator without any knowledge of the torsinA genotype of the cells, and the result was confirmed by a second, independent investigator. Experiments were repeated at least three times, and the data were subjected to statistical analysis by unpaired Student’s t test.

Conflict of Interest statement. None declared.

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