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Interaction of TorsinA with Its Major Binding Partners Is Impaired by the Dystonia-associated ΔGAG Deletion*

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Early onset (*DYT1*) torsion dystonia is a dominantly inherited movement disorder associated with a three-base pair (ΔGAG) deletion that removes a glutamic acid residue from the protein torsinA. TorsinA is an essential AAA$^+$ (ATPases associated with a variety of cellular activities) ATPase found in the endoplasmic reticulum and nuclear envelope of higher eukaryotes, but what it does and how changes caused by the ΔGAG deletion lead to dystonia are not known. Here, we asked how the *DYT1* mutation affects association of torsinA with interacting proteins. Using immunoprecipitation and mass spectrometry, we first established that the related transmembrane proteins LULL1 and LAP1 are prominent binding partners for torsinA in U2OS cells. Comparative analysis demonstrates that these two proteins are targeted to the endoplasmic reticulum or nuclear envelope by their divergent N-terminal domains. Binding of torsinA to their C-terminal lumenal domains is stabilized when residues in any one of three motifs implicated in ATP hydrolysis (Walker B, sensor 1, and sensor 2) are mutated. Importantly, the ΔGAG deletion does not stabilize this binding. Indeed, deleting the ΔGAG encoded glutamic acid residue from any of the three ATP hydrolysis mutants destabilizes their association with LULL1 and LAP1C, suggesting a possible basis for loss of torsinA function. Impaired interaction of torsinA with LULL1 and/or LAP1 may thus contribute to the development of dystonia.

TorsinA is the causative protein in early onset torsion dystonia, also known as *DYT1* dystonia or Oppenheim Disease (1). The disease is characterized by severe and generalized abnormalities in motor control that typically begin during childhood (2). *DYT1* dystonia is an autosomal dominant disorder associated with a three-base pair (ΔGAG) deletion that removes one of a pair of glutamic acid residues (Glu-302/303) from near the C terminus of torsinA (3). We will refer to this mutant protein as torsinAΔE. TorsinA is expressed throughout the body, although its levels vary in different cell types and over the course of development (1, 4). TorsinA is an essential protein in the mouse, because Tor1A$^{-/-}$ mice die within a few hours of birth (5, 6). Because knock-in of torsinAΔE does not rescue these mice from perinatal lethality (5, 6), the disease-linked deletion is considered to be a loss-of-function mutation.

The cellular functions potentially ascribed to torsinA vary widely, but in general remain poorly understood. TorsinA resides within the lumen of the endoplasmic reticulum (ER)[2] and contiguous nuclear envelope (NE) (7–10). Based on its membership in the AAA$^+$ (ATPases associated with a variety of cellular activities) family of ATPases (1, 11) and the protein disaggregating activity of the most closely related AAA$^+$ protein ClpB/Hsp104, it seems likely that torsinA disassembles protein complexes or otherwise changes the conformation of proteins in the ER or NE. However, protein complexes acted upon by torsinA remain elusive, and definitive demonstration of torsinA activity is still lacking (12, 13). The NE is the favored binding site for a hydrolysis-deficient "substrate trap" torsinA mutant (14), and both expression of this substrate trap mutant and removal of torsinA by gene deletion perturb NE structure (5, 14). These observations point to a significant role for torsinA in regulating protein complexes within the NE. A candidate-based screen to determine whether any of a set of known NE proteins associate with torsinA uncovered an interaction with the inner nuclear membrane protein LAP1 (also known as TOR1AIP1) and a related protein in the ER, LULL1 (also known as TOR1AIP2 or NET9) (15). Nesprin-3, a resident of the outer nuclear membrane implicated in connecting the nucleus to the cytoskeleton, is another NE protein recently reported to interact with torsinA (16).

TorsinA has also been implicated in regulating the secretory pathway (17–20) and in modulating cellular responses to such insults as oxidative stress or aggregated proteins (21–23). Most studies of these effects have focused on differences between expressing wild-type torsinA and torsinAΔE. In a particularly striking set of studies, overexpressing torsinAΔE selectively impaired efflux of a secreted luciferase from cells (19). Importantly, this inhibitory effect was also seen in *DYT1* patient-derived fibroblasts (with one copy of the gene encoding torsinAΔE), and in this setting could be overcome by RNA interference-mediated removal of the mutant protein (20). Although it remains to be determined exactly how the ΔE deletion changes torsinA structure and function (see Refs. 24 and 25 for structural modeling), these results, together with its inability to rescue function in knock-in mice (5, 6), suggest that the torsinAΔE mutation causes both loss- and gain-of-function changes in torsinA, potentially explaining the autosomal dominant inheritance of *DYT1* dystonia.

In the present study, we wanted to better understand the molecular basis for functional changes caused by the ΔGAG

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[2] The abbreviations used are: ER, endoplasmic reticulum; NE, nuclear envelope; GFP, green fluorescent protein; mGFP, monomeric GFP; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; SNARE, soluble NSF attachment protein receptors.

glutamic acid (ΔE) deletion. We began by identifying *de novo* torsinA interacting proteins in the cultured human U2OS cell line. After finding that the previously discovered transmembrane proteins LULL1 and LAP1 were the prominent binding partners in these cells (15), we proceeded to further characterize their interaction with torsinA and to explore how this is affected by the ΔE deletion. Our findings indicate that impaired or destabilized binding of torsinAΔE to LULL1 and LAP1 could provide a molecular explanation for a loss of function that contributes to *DYT1* dystonia.

EXPERIMENTAL PROCEDURES

Plasmids and Mutagenesis—Previously described torsinA expression constructs (wild-type and single mutants) include untagged torsinA in pcDNA3 (24); torsinA-His_6myc in pcDNA4/TO (24); and torsinA-green fluorescent protein (GFP) in pEGFP-N1 (14)). All GFP constructs used in this study were changed to monomeric GFP (mGFP) by QuikChange site-directed mutagenesis of L221K in GFP (40). E171Q, N208A, and K320M mutations were introduced into torsinAΔE by site-directed mutagenesis to create double mutants as indicated. LULL1-myc was made by PCR amplification of LULL1 (NM_145034, residues 1–470) from HeLa Quick-clone cDNA (Clontech) with primers containing HindIII and EcoRI restriction sites followed by ligation into pcDNA4/TO/MycHisC (Invitrogen). LAP1C-myc (NM_015602, residues 1–462) was created in the same way except with BamHI at the 3′ end. Expression vectors encoding the cytoplasmic fragments of LULL1 and LAP1C were prepared by amplifying the indicated fragments (LULL1-(1–217) and LULL1-(1–241); LAP1C-(1–217) and LAP1C-(1–240)) again with primers containing 5′ HindIII and 3′ EcoRI (LULL1) or BamHI (LAP1C) sites followed by introduction into pcDNA4/TO/MycHisC. Expression vectors encoding the lumenal fragments with the myc epitope tag and C-terminal KDEL ER-retrieval sequence were prepared by amplifying the lumenal fragments (LULL1 residues 236–470; LAP1C residues 237–462) with primers containing 5′ SalI and 3′ NotI restriction sites followed by subcloning into pCMV/myc/ER (Invitrogen). The LAP1C/LULL1 chimeric protein consists of the LAP1C nucleoplasmic domain (residues 1–216) fused to the LULL1 transmembrane and lumenal domains (residues 218–470) in pcDNA4/TO/MycHisC. A Nde1 restriction site was introduced into each fragment to ligate them together. The LULL1/LAP1C chimeric protein consists of the LULL1 cytoplasmic and transmembrane domains (residues 1 - 241) fused to the LAP1C lumenal domain (residues 237–462). In this case, a KpnI site was introduced to form the junction. The sequences of all coding regions were verified by nucleotide sequencing.

Cell Culture and Cell Lines—U2OS-T-Rex cells (Invitrogen) were grown in Dulbecco's modified Eagle's medium and 10% tetracycline-free fetal bovine serum (Atlanta Biologics). Tetracycline-inducible cell lines expressing wild-type torsinA-His_6myc, E171Q mutant torsinA-His_6myc, LULL1-His_6myc, or LAP1C-His_6myc were isolated as described previously (41) using hygromycin (50 μg/ml) and zeocin (100 μg/ml) for selection and stock maintenance. TorsinA cell lines with a low level of torsinA-His_6myc expression were used without added tetracycline to isolate endogenous binding partners. For tetracycline-induced expression (used with LULL1 and LAP1C stable lines) 1 μg/ml tetracycline was added at the time indicated. Transient transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Antibodies—The following antibodies were used: mouse anti-torsinA DMA28 (9), mouse anti-myc 9E10 (Developmental Studies Hybridoma Bank), rabbit anti-GFP (41), and mouse anti-LAP2 (BD Biosciences). Secondary goat anti-mouse and goat anti-rabbit antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 555 were purchased from Invitrogen. Secondary antibodies conjugated to horseradish peroxidase were purchased from Bio-Rad.

Immunoprecipitation—For identification of torsinA binding partners (Fig. 1), cells from three confluent 15-cm plates of torsinA(E171Q) or control U2OS cells were collected and solubilized in 1.5 ml of MPER buffer (Pierce) supplemented with 2 mM $MgCl_2$, 2 mM ATP, and 1× complete protease inhibitor (Boehringer). After an incubation of 30 min at 4 °C, samples were centrifuged to remove insoluble material, and 20 μl of c-Myc monoclonal antibody pre-conjugated to agarose beads (Pierce) was added followed by incubation for 4 h. Beads were washed 3× and bound material was eluted with three 20-μl batches of low pH elution buffer. Samples were neutralized with 3 μl of 1 M Tris, pH 9.5, and prepared for electrophoresis by adding 10 μl of non-reducing loading dye (Pierce). 50 μl were loaded and resolved on a 4–15% 1 mm Criterion gel (Bio-Rad), which was then fixed and stained with SYPRO Ruby (Invitrogen) according to the manufacturer's instructions.

For analysis of binding between variants of torsinA and LULL1 or LAP1C (see Figs. 3–5), LULL1-His_6myc or LAP1C-His_6myc expression was induced by adding tetracycline to stable cells cultured in 6-cm dishes, followed 6 h later by transient transfection of plasmid encoding the indicated torsinA protein. 18 h later, cells were scraped from the dish and solubilized in 500 μl of buffer containing 25 mM Hepes, pH 7.5, 20 mM NaCl, 2 mM ATP, 2 mM EDTA, 10 mM $MgCl_2$, and 0.5% CHAPS (w/v). Lysates were centrifuged to remove insoluble material, and 3–5 μg of anti-myc 9E10 monoclonal antibody was added. After 2 h of rotation at 4 °C, 15 μl of Protein G-Sepharose (Pharmacia) was added followed by rotation for 1 h. Unbound material was removed, and beads were washed 3×. Beads were then resuspended in buffer (100 μl for cells transfected with mGFP torsinA, 500 μl for cells transfected with untagged torsinA). All samples were then prepared for and analyzed by SDS-PAGE and immunoblotting. Immunoblots were processed and developed as described using Supersignal ECl reagent (Pierce) according to the manufacturer's directions.

Mass Spectrometry—Proteins were identified using in-gel trypsin digestion of excised bands followed by liquid chromatography-tandem mass spectrometry and analysis using Mascot (Matrix Science) at the Washington University Protein and Nucleic Acid Chemistry Laboratory.

Analysis of Protein Solubility—Stable cell lines were induced to express LULL1- or LAP1C-His_6myc for 18 h, followed by solubilization in buffer containing 20 mM Hepes, pH 7.5, 0.5% Triton X-100, and the indicated concentration of NaCl (0, 100, 250, and 500 mM). Samples were incubated for 30 min at 4 °C and then centrifuged at 18,000 × *g* for 30 min to separate solu-

ble from insoluble material. Insoluble material was resuspended in the same volume as the soluble fraction and dispersed by sonication. Equal volumes of soluble and insoluble material were resolved by SDS-PAGE and analyzed by immunoblotting.

Immunofluorescence—Immunofluorescence was performed on cells fixed in 3% paraformaldehyde in phosphate-buffered saline and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline. Samples were blocked with phosphate-buffered saline containing 10% goat serum, incubated for 1 h at room temperature with primary antibody (anti-myc 9E10; 1:1000), washed, and incubated with fluorescent secondary antibody. After washing, coverslips were mounted using Mowiol (Calbiochem). GFP fluorescence was visualized directly. Epifluorescence images were captured with a Leica Diaplan microscope using a 63 × 1.4-numerical aperture objective and a Zeiss AxioCam MRm. Figures were prepared by using Photoshop and Illustrator software packages (Adobe Systems, San Jose, CA).

RESULTS

Isolation of torsinA-interacting Proteins—Given the still unknown cellular function of torsinA, we set out to use an unbiased co-immunoprecipitation approach to identify proteins that bind to human torsinA in a cultured cell line with a well elaborated ER and a readily detectable level of endogenous torsinA. We made stable U2OS cell lines expressing wild-type or ATPase-deficient Walker B mutant (E171Q) torsinA tagged at the C terminus with His_6myc. To maximize recovery of interacting proteins, we selected cell lines that express tagged torsinA at levels comparable with those of endogenous enzyme (Fig. 1*A*). Immunostaining with a myc-specific antibody confirmed that wild-type torsinA localizes diffusely throughout the ER whereas the E171Q Walker B mutant is enriched in the NE (Fig. 1*B*), as expected based on previous work (14, 26). Because most AAA^+ proteins have the highest affinity for substrates and cofactors when ATP-bound (11), we focused on identifying proteins associated with the E171Q mutant.

To do this, we immunoprecipitated torsinA(E171Q)-His_6myc from an extract containing Mg^{2+} ATP and visualized both torsinA and co-precipitated proteins by SDS-PAGE and SYPRO-Ruby staining (Fig. 1*C*). Parallel samples from nonexpressing U2OS cells provided a control for nonspecifically recovered proteins. Of several bands unique to the torsinA(E171Q) sample, one migrating at ~90 kDa was identified as the glycoprotein chaperone calnexin by immunoblotting (data not shown). To identify other proteins, we performed in-gel trypsin digestion followed by liquid chromatography-tandem mass spectrometry mass spectrometry. Proteins for which we recovered more than three peptides are indicated next to the corresponding band in Fig. 1*C* and include LULL1 (also known as TOR1AIP2 or NET9) at ~70 kDa and Tor3A at ~50 kDa. Additional proteins identified by one or two peptides included, among others, LAP1B (also known as TOR1AIP1) and Tor1B. Although it is unlikely that these are the only proteins that bind torsinA, the fact that an unbiased analysis leads to two sets of proteins (LAP1-like transmembrane proteins and other torsin family members) already defined in tests of candidate binding partners (15, 27) indicates that these are likely to be of general importance. Our particularly efficient recovery of

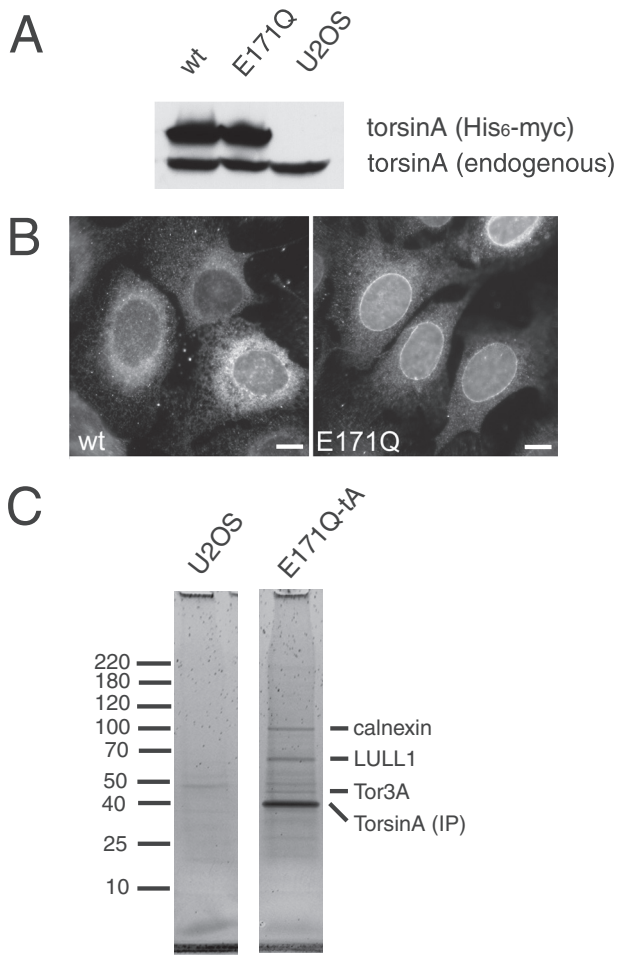

FIGURE 1. **Identification of torsinA-interacting proteins in U2OS cells.** *A*, immunoblot of torsinA in whole cell lysates of the indicated cells (parental U2OS-TRex cells, stable wild-type (*wt*)- or E171Q-torsinA U2OS cell lines) probed with an antibody specific for torsinA (*tA*) (DMA28 (9)). The faster migrating band corresponds to endogenous torsinA, and the slower band corresponds to His_6myc-tagged torsinA. *B*, immunofluorescence of wild-type or E171Q torsinA-His_6myc in stable U2OS cell lines visualized with anti-myc antibody. *Scale bar*, 10 μm. *C*, material immunoprecipitated with pre-conjugated Pierce myc resin from cells expressing torsinA(E171Q)-His_6myc or from control U2OS cells resolved by SDS-PAGE on a 4–15% gradient gel. Proteins were visualized by staining with SYPRO-Ruby. *Arrows* show proteins identified by immunoblotting (calnexin) or liquid chromatography-tandem mass spectrometry (LULL1 (14 peptides) or Tor3A (6 peptides)). Additional proteins identified by 1–2 peptides in the torsinA immunoprecipitate (*IP*) included LAP1B and Tor1B.

LULL1 (Fig. 1*C*) indicates that this is a major torsinA-interacting protein in U2OS cells, although solubility issues probably limited the amount of LAP1 that we recovered. Because little is known about LULL1 other than that it shares sequence similarity with LAP1 (15), we decided to further characterize LULL1 and LAP1 and their relationship with torsinA. Interactions between torsinA and other torsins including Tor1B and Tor3A will be considered further elsewhere.

Subcellular Localization of LULL1 and LAP1 Is Determined by Divergent N-terminal Domains—LAP1 and LULL1 are type II single transmembrane domain proteins encoded by adjacent genes on human chromosome 1 (15). They are homologous to each other in their C-terminal lumenal domains and bind *via* these to torsinA (15). There are three splice variants of LAP1 (LAP1A, LAP1B, and LAP1C) with differing N-terminal initia-

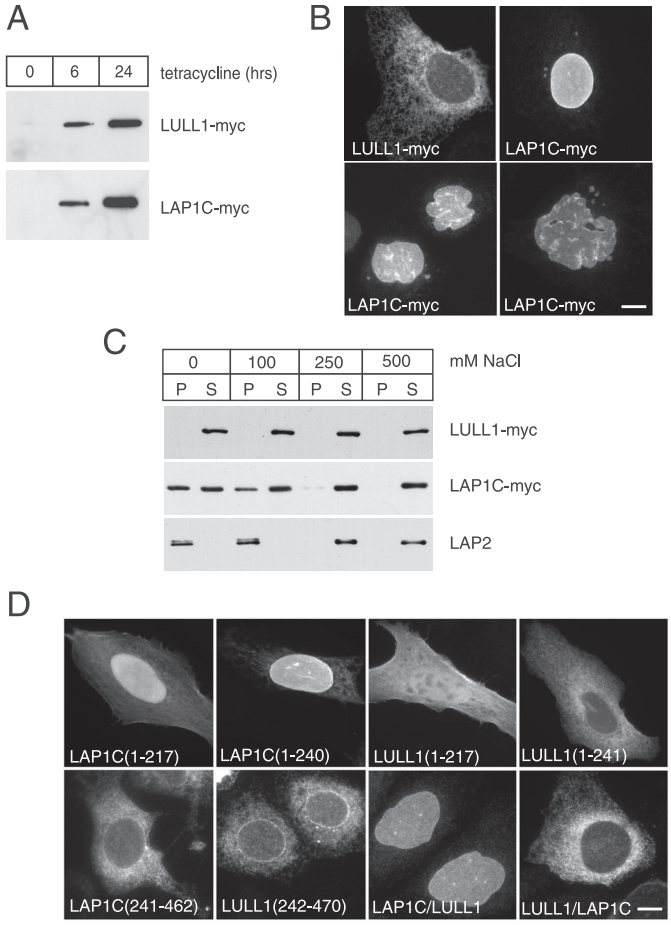

FIGURE 2. **Properties of torsinA-interacting proteins LAP1C and LULL1.** *A*, immunoblot of LULL1-His$_6$myc (*top*) or LAP1C-His$_6$myc (*bottom*) before and 6 or 24 h after adding tetracycline to stable U2OS cell lines. *B*, representative immunofluorescence of LULL1-His$_6$myc (*top left*) or LAP1C-His$_6$myc (*top right* and *bottom*) in stable U2OS cell lines expressing protein for 18 h. Nuclei are distorted in a large fraction of cells expressing LAP1C but not in cells expressing LULL1. *Scale bar*, 10 μm. *C*, solubilization of LULL1-His$_6$myc, LAP1C-His$_6$myc, or endogenous LAP2 in buffers containing 20 mM Hepes, 0.5% Triton-X100, and NaCl as indicated. Equal fractions of soluble and insoluble (*S* and *P*, respectively) fractions were resolved by SDS-PAGE and detected by immunoblotting with anti-myc antibody. *D*, immunofluorescence of LAP1C and LULL1 fragments and chimeric molecules. *Top panel*, LAP1-(1–217)His$_6$myc, LAP1-(1–240)His$_6$myc, LULL1-(1–217)His$_6$myc, and LULL1-(1–241)His$_6$myc. *Bottom panel*, LAP1-(241–462), LULL1-(242–470), LAP1/LULL1 chimera, and LULL1/LAP1 chimera. *Scale bar*, 10 μm.

tion sites that generate proteins with increasingly long N-terminal domains connected to a common transmembrane and lumenal domain (28, 29). LAP1 isoforms are abundant proteins that bind to A- and B-type lamins (30, 31) but how they contribute to NE organization and/or function is still unknown. LULL1 has been described as a diffusely distributed ER protein (15) and as a potential NE resident (32, 33). Neither LAP1 nor LULL1 have any predicted functional motifs. To understand how these proteins interface with torsinA and other cellular factors, we first studied their localization in U2OS cells.

After isolating tetracycline-inducible U2OS cell lines that express LAP1C- or LULL1-His$_6$myc (Fig. 2*A*), we found that LAP1C accumulates in the NE, whereas LULL1 is diffusely distributed throughout the ER and NE (Fig. 2*B*), consistent with most previous reports (15, 29). Interestingly, LAP1C concentrates in the NE even when highly overexpressed, suggesting that factors responsible for recruiting it there are not readily saturated. Overexpressed LAP1C induces abnormalities in the shape of the nucleus that sometimes include extensions of the NE into the nucleus (Fig. 2*B*, *lower panel*, and data not shown; note that extensions of the NE into the nucleus appear as bright areas over the nucleus in these widefield fluorescence images). In contrast, overexpressing LULL1 has no obvious effect on the morphology of either the nucleus or the ER (Fig. 2*B* and data not shown). As an independent measure of the relationship between these two proteins and the NE, we took advantage of the fact that proteins in the inner nuclear membrane that interact with lamins and/or chromatin typically resist extraction in low ionic strength buffers (30). LULL1 was completely soluble in a buffer containing only Triton X-100, whereas approximately half of the overexpressed LAP1C was insoluble under these conditions (Fig. 2*C*). Adding 250 mM NaCl to the buffer solubilized the remaining LAP1C. For comparison, an endogenous inner nuclear membrane protein, LAP2, in the same samples was insoluble in 0 or 100 mM NaCl and fully solubilized when 250 mM NaCl was added.

Why do LAP1 and LULL1 differ so strikingly in their subcellular localization? We separated N-terminal cyto- or nucleoplasmic domains from C-terminal lumenal domains and expressed each as a fragment with or without a transmembrane domain. The N-terminal LAP1C fragment concentrated in the nucleus without and in the NE with its transmembrane domain, whereas the same fragment of LULL1 distributed uniformly throughout the cell without and throughout the ER with its transmembrane domain (Fig. 2*D*). Lumenal fragments of both proteins (with C-terminal KDEL motifs) were present throughout the ER (Fig. 2*D*). Swapping N-terminal domains further confirmed that these are responsible for the different subcellular distribution of these two proteins (Fig. 2*D*). As a likely explanation for their distinct properties, we note that despite some sequence similarity, the N-terminal domain of LAP1C has a pI of ~9, whereas that of LULL1 has a pI of ~4. This difference in charge is maintained throughout evolution and in splice variants of LAP1 (LAP1A–C). Taking into account the known correlation between basic pI and localization to the inner nuclear membrane (34), we suggest that the charge within the N-terminal domains is likely to contribute to the fundamentally different distribution of these two proteins. Their shared ability to interact with torsinA will enable it to engage and affect distinct networks of proteins.

Effect of Mutations in Defined Nucleotide-interacting Motifs on torsinA Binding—In their description of the interaction between LAP1 or LULL1 and torsinA, Goodchild and Dauer (15) noted that the Walker B E171Q mutant interacted more efficiently with these proteins than did the wild-type enzyme, implying regulation by the nucleotide bound to torsinA. However, because the buffers used did not contain ATP and because it is yet to be determined exactly how torsinA responds to the presence or absence of ATP (13), other explanations for the observed differences are possible. Our recovery of LULL1 and LAP1 bound to Walker B mutant torsinA in Mg^{2+} ATP (Fig. 1) supports the hypothesis that ATP-bound torsinA is the form that binds to these proteins. To more generally define the role of nucleotide status in these interactions, we asked how torsinA

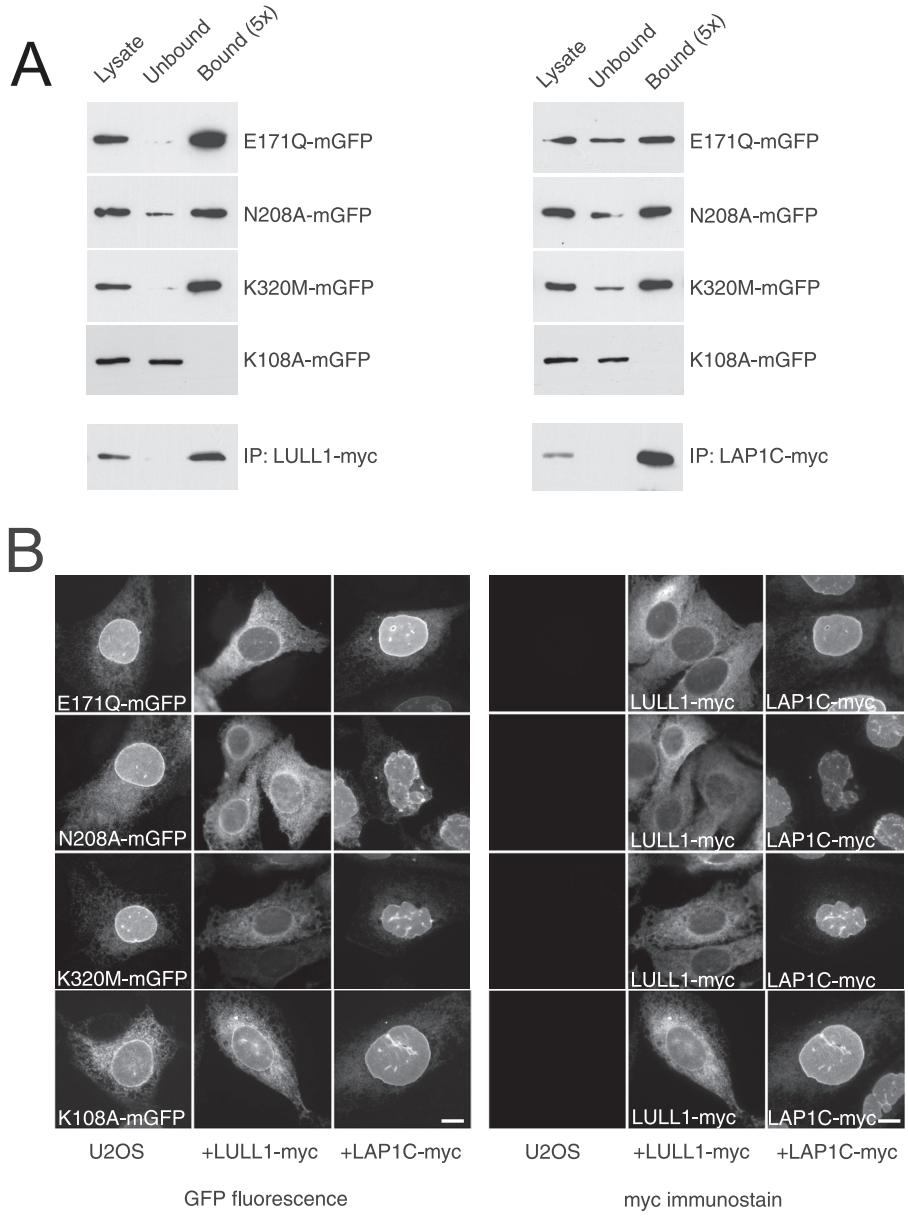

FIGURE 3. **Binding between torsinA and interacting proteins is stabilized by mutations in motifs implicated in ATP hydrolysis.** *A*, immunoblots monitoring co-immunoprecipitation of the indicated torsinA-mGFP mutants with LULL1-His_6myc (*left*) or LAP1C-His_6myc (*right*). For each experiment, the total lysate, unbound, and immunoprecipitated material are shown. Equal volumes of lysate and unbound samples are loaded, whereas the immunoprecipitated material is enriched 5-fold. GFP blots for each torsinA mutant show the extent to which torsinA co-immunoprecipitated with LULL1- or LAP1C-His_6myc. A representative blot of immunoprecipitated (*IP*) LULL1- or LAP1C-His_6myc is shown at the *bottom of each panel*. Each blot is representative of at least three experiments. *B*, localization of torsinA-mGFP mutants in U2OS cells or in U2OS cell induced to express LULL1-His_6myc or LAP1C-His_6myc. GFP fluorescence is shown in the *left panel*, and myc immunostaining is shown in the *right*. *Scale bars*, 10 μm.

with mutations in each of several independent nucleotide-interacting motifs, including an asparagine (Asn-208) in the sensor 1 motif that forms essential hydrogen bonds with the γ-phosphate of ATP, a lysine (Lys-320) in the sensor 2 motif also thought to interact with the γ-phosphate of ATP, and a lysine (Lys-108) in the Walker A motif that is an essential part of the nucleotide binding P-loop (11, 24, 25), interacts with LULL1 and LAP1C. We transiently transfected torsinA-mGFP with the indicated mutation into U2OS cells already expressing either LULL1- or LAP1C-His_6myc, immunoprecipitated myc-tagged proteins from lysates prepared in an optimized low-salt buffer containing CHAPS and Mg^{2+} ATP, and used immunoblots to determine how much torsinA was recovered together with LULL1- or LAP1C-His_6myc. Note that because we transfected torsinA mutants into clonal cell lines expressing LULL1- or LAP1C-His_6myc, all torsinA has access to the proteins being immunoprecipitated. For comparison, we included torsinA with the previously characterized Walker B (E171Q) mutation. TorsinA with mutations all three of the motifs predicted to impair ATP hydrolysis (Walker B (E171Q), sensor 1 (N208A), and sensor 2 (K320M)) co-immunoprecipitated efficiently with both LULL1 and LAP1C (Fig. 3*A*). In contrast, torsinA with a K108A mutation in its Walker A motif was not significantly recovered, suggesting that ATP binding is required for stable interaction of torsinA with these two proteins (Fig. 3*A*). As will be discussed further in the next section, recovery of the wild-type enzyme was intermediate between that of the ATP-locked and ATP-free mutants (data not shown, see Fig. 4). The fact that we were able to significantly deplete extracts of overexpressed ATP-bound torsinA mutants by immunoprecipitating overexpressed LULL1 or LAP1C-His_6myc suggests that interactions between these proteins are likely to be direct.

To probe interactions with torsinA in their native environment, we asked how increases in LULL1 or LAP1C affect the distribution of torsinA mutants within the ER and NE. Inspection of GFP fluorescence revealed shifts in the distribution of ATP hydrolysis mutants (Walker B E171Q, sensor 1 N208A, and sensor 2 K320M) in response to increased expression of LULL1 or LAP1C (Fig. 3*B*). Each of the mutants was enriched in the NE in U2OS cells (Fig. 3*B*, *left panel*). Increased LULL1-myc in the ER abolished this (Fig. 3*B*, *middle panel*), whereas increased LAP1C-myc in the NE further concentrated the torsinA mutants in the NE (Fig. 3*B*, *right panel*). These changes in localization were less pronounced for the Walker A K108A mutant, although it too shifted somewhat into the NE in cells overexpressing LAP1, indicating that in the milieu of the intact

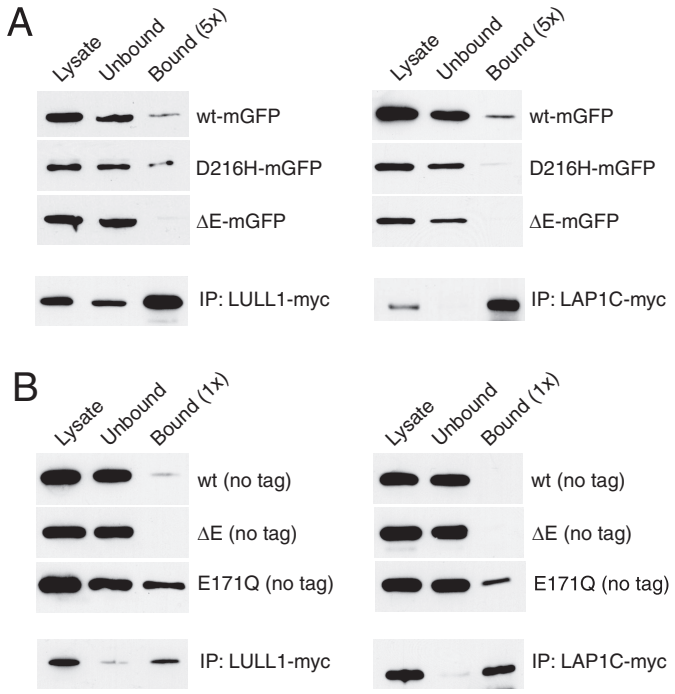

FIGURE 4. **Dystonia-associated torsinAΔE does not interact stably with LULL1 or LAP1C.** *A*, immunoblots monitoring co-immunoprecipitation of the indicated torsinA-mGFP mutants with LULL1-His_6myc (*left*) or LAP1C-His_6myc (*right*). For each experiment, the total lysate, unbound, and immunoprecipitated (*IP*) material are shown. Equal volumes of lysate and unbound samples are loaded, whereas the immunoprecipitated material is enriched 5-fold. GFP blots for each torsinA mutant show the extent to which torsinA co-immunoprecipitated with LULL1- or LAP1C-His_6myc. A representative blot of immunoprecipitated LULL1- or LAP1C-His_6myc is shown at the *bottom of each panel*. *B*, immunoblots monitoring co-immunoprecipitation of the indicated untagged torsinA mutants with LULL1-His_6myc (*left*) or LAP1C-His_6myc (*right*). In this case, equal proportions of lysate, unbound, and bound samples are shown. Each blot is representative of at least three experiments. *wt*, wild type.

cell, it remains able to associate to some degree with these binding partners. Altogether, the immunoprecipitation and immunofluorescence experiments establish that mutations in three distinct motifs expected to impair ATP hydrolysis stabilize the interaction of torsinA with its binding partners. In terms of localization, the effects of increased expression of LAP1C and LULL1 confirm that ATP-bound torsinA distributes within the ER/NE system according to the relative abundance of binding partners in different places. The fact that LAP1 is an abundant NE protein in many cells likely explains the tendency of hydrolysis-deficient mutants to concentrate in the NE (14, 24, 26).

Disease-associated ΔE Mutation Does Not Stabilize Interaction with LULL1 and LAP1—Studies of the disease-associated torsinAΔE protein transfected into non-neuronal or neuronal cells show that it enriches in the NE at low expression levels and in membranous inclusions at high expression levels (14, 26, 35, 36). Endogenous torsinAΔE has also been reported to concentrate in the NE of *DYT1* patient fibroblasts (26). Because several distinct ATPase-deficient torsinA mutants accumulate in the NE (14, 24, 26, and see above), one attractive hypothesis has been that the ΔE deletion might partially impair ATP hydrolysis (perhaps by perturbing the positioning of the adjacent sensor 2 motif (24, 25)), causing the mutant protein to behave as a weak substrate trap (15). If this is the case, the prediction is that interaction of torsinAΔE with its binding partners should be stabilized in a manner similar to that of the three ATP-hydrolysis mutants studied above.

To test this hypothesis, we transfected wild-type or torsinAΔE-mGFP into cells expressing LULL1- or LAP1C-His_6myc and assessed their interaction by co-immunoprecipitation. In contrast to mutants with changes in motifs required for nucleotide hydrolysis, neither wild-type torsinA (with either Asp or His at the polymorphic residue 216 (24)) nor torsinAΔE-mGFP efficiently co-immunoprecipitated with either LULL1 or LAP1C (Fig. 4*A*). This is the expected result for enzymes able to hydrolyze ATP and thereby release their interacting partners. Because the ΔE glutamic acid deletion falls within the C-terminal α-helical domain of torsinA (24, 25), we repeated these experiments with torsinA variants lacking C-terminal mGFP tags. Untagged torsinA with a Walker B mutation (E171Q) again co-immunoprecipitated with LULL1 and LAP1C, whereas wild-type and ΔE mutant torsinA did not (Fig. 4*B*). We note that immunoprecipitating LULL1- and LAP1C-myc depleted a smaller proportion of untagged compared with mGFP-tagged E171Q torsinA from cell lysates because of different expression levels, with untagged torsinA expressed at much higher levels than mGFP-tagged torsinA (data not shown). These results establish that the disease-associated ΔE deletion does not stabilize the interaction of torsinA with either LULL1 or LAP1C and thus affects torsinA differently than do mutations in defined ATP-interacting motifs.

Disease-associated ΔE Deletion Impairs Binding of ATP-bound torsinA to LULL1 and LAP1C—If the loss of function associated with torsinAΔE is not attributable to slowed ATP hydrolysis and stabilized binding to LULL1 and LAP1, we wondered whether the deletion might instead impair the interaction of torsinA with these proteins. To discover whether this is the case, we deleted the glutamic acid from each of the three ATP-hydrolysis mutants that bound tightly to LULL1 and LAP1 above. We again coexpressed these torsinA mutants (E171Q-ΔE, N208A-ΔE, and K320M-ΔE) with LULL1- or LAP1C-myc and asked whether the expected complexes were present (Fig. 5*A*). Strikingly, the ΔE mutation greatly reduced binding of each of these mutants to LULL1 and LAP1C. Based on our previous finding that the polymorphic replacement of Asp-216 with His-216 partially normalizes the subcellular distribution of the ΔE mutant protein (24), we also asked whether changing the typical Asp to the polymorphic His might restore binding of torsinA(E171Q-ΔE) to LULL1 or LAP1C. As shown, it did not.

Because co-immunoprecipitation is a stringent test of protein-protein interactions that might not detect transient low affinity interactions, we also asked how the ΔE mutation affects the redistribution of hydrolysis-deficient torsinA mutants when they are coexpressed with LAP1C or LULL1. As shown in Fig. 5*B*, some of the double mutant proteins shifted detectably when coexpressed with LULL1 or LAP1C, but the changes were less pronounced than seen for the same mutants without the ΔE deletion (Fig. 3*B*). Among the three double mutants, the sensor 1 N208A-ΔE mutant was the least responsive to the presence of LULL1 and LAP1C, indicating that this mutant's interaction with these binding partners is more sensitive to the ΔE deletion than that of the others.

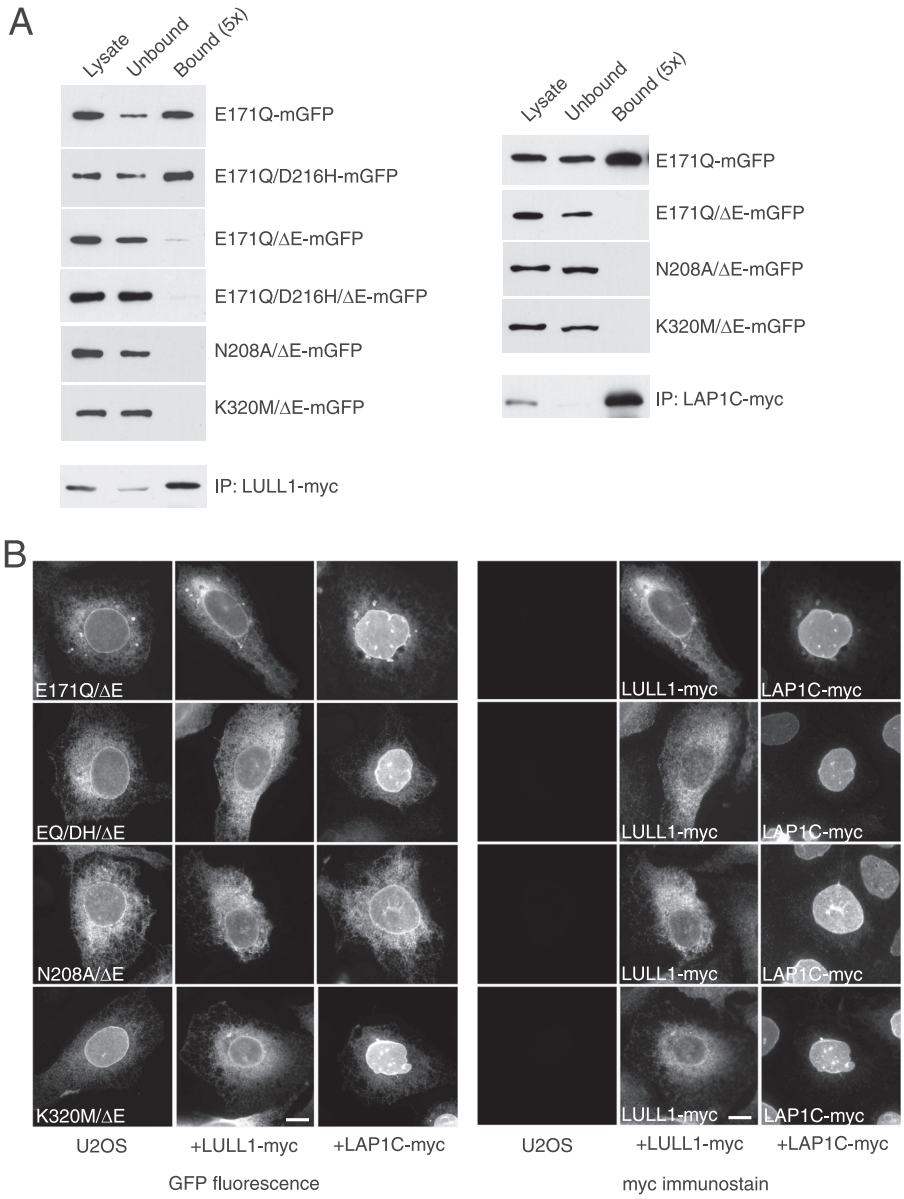

FIGURE 5. **Dystonia-associated ΔE mutation impairs interaction of ATP-hydrolysis-deficient mutant proteins with LULL1 and LAP1C.** *A*, immunoblots monitoring co-immunoprecipitation of the indicated torsinA-mGFP mutants with LULL1-His_6myc (*left*) or LAP1C-His_6myc (*right*). For each experiment, total lysate, unbound, and immunoprecipitated (*IP*) material are shown. Equal volumes of lysate and unbound samples are loaded, whereas the immunoprecipitated material is enriched 5-fold. GFP blots for each torsinA mutant show the extent to which torsinA co-immunoprecipitated with LULL1- or LAP1C-His_6myc. A representative blot of immunoprecipitated LULL1- or LAP1C-His_6myc is shown at the *bottom of each panel*. Each blot is representative of at least three experiments. *B*, localization of torsinA-mGFP mutants in U2OS cells or in U2OS cell induced to express LULL1-His_6myc or LAP1C-His6myc. GFP fluorescence is shown in the *left panel*, and myc immunostaining is shown in the *right panel*. *Scale bars*, 10 μm.

Altogether, our results indicate that the disease-linked ΔE mutation does not enhance association between torsinA and LAP1C or LULL1. Unexpectedly, this mutation instead destabilizes interaction of ATP-bound torsinA with these proteins, suggesting that the loss of function associated with the ΔE deletion may reflect a reduced affinity for these major binding partners caused by a ΔE-induced structural perturbation of torsinA.

DISCUSSION

Our primary goal in this study was to determine how the *DYT1*-causative ΔE mutation changes interactions between torsinA and proteins that it interacts with in the ER and NE. After establishing that the major binding partners for torsinA in our cell culture model include two transmembrane proteins, LULL1 and LAP1C, already known to bind to torsinA (Fig. 1), we used a panel of defined mutations to show that torsinA preferentially interacts with them in its ATP-bound state (Figs. 3 and 4). Parallel experiments with the *DYT1*-causative ΔE mutant protein showed that this mutation did not stabilize interaction with LULL1 and LAP1 (Fig. 4). In fact, introducing the ΔE deletion into three independent ATP-hydrolysis defective torsinA mutants decreased their otherwise robust interaction with LULL1 and LAP1C (Fig. 5). Defects in the cellular function of torsinAΔE may therefore be caused by impaired interactions with LULL1 and/or LAP1. Restoring these could provide a promising avenue for therapeutic development.

The fact that association of torsinA with these two membrane proteins is sensitive to mutations expected to affect nucleotide hydrolysis or nucleotide binding extends earlier observations suggesting that torsinA adopts distinct conformational states in response to nucleotide, as expected of a functional AAA^+ protein (11). The high efficiency with which we co-immunoprecipitated ATP-bound torsinA with LULL1 or LAP1C argues that their interaction is likely to be direct, although definitive proof of this will require reconstitution with purified proteins. Binding of other AAA^+ proteins to their substrates is known to be controlled by nucleotide status, often in exactly this way, but the same is true of interactions between AAA^+ proteins and proteins that function as cofactors to recruit *bona fide* substrates. An example of the latter is the ATP-dependent interaction between the AAA^+ protein *N*-ethylmaleimide-sensitive factor and its cofactor α-SNAP, which in turn binds to SNARE complexes that *N*-ethylmaleimide-sensitive factor ultimately disassembles (37). Whether LULL1 and LAP1C are substrates or cofactors of torsinA is therefore not yet clear. It will be important to learn more about what LULL1 and LAP1 interact with, particularly in the ER lumen, to further define their relationship to torsinA.

An attractive hypothesis to explain some of the cellular properties of the torsinAΔE mutant was that it might be a substrate

trap displaying stabilized interactions with NE-localized factors, similar to what is seen with ATP-hydrolysis defective mutants, including the well studied Walker B (E171Q) mutant (14, 26). Our results (Figs. 3–5) demonstrate that this is not the case and imply that other changes must underlie the tendency of low concentrations of torsinAΔE to accumulate in the NE (26, 36, 38). Supporting a significant difference between ΔE and hydrolysis-impaired torsinA mutants is the fact that they behave differently when highly overexpressed, with the ΔE mutant accumulating in membranous inclusions or "whorls" structurally reminiscent of ER- or NE-derived karmellae (7, 35), whereas the ATP-bound mutants spill into the peripheral ER as would be expected following saturation of NE binding sites (14 and data not shown). Our recent study of the dynamic control of torsinA distribution between peripheral ER and NE suggests that the residual interaction between torsinAΔE and LULL1 may play a part in targeting this mutant to the NE (42).

An important question is raised by these results: what precisely is wrong with torsinAΔE? Our data, showing that this deletion not only does not trap the enzyme in its ATP-bound state (Fig. 4) but also overrides the effect of other mutations that do (Fig. 5), point to a structural effect of the ΔE deletion that does not parallel in any simple way the more clearly defined effects of point mutations in known nucleotide-interacting motifs. Several recent observations indicate that this single glutamic acid deletion may, among other changes, destabilize the protein. The overall half-life of the mutant enzyme is shorter than that of the wild-type protein (36, 39), and expression of the mutant enzyme in mouse brain leads to lower total levels of torsinA (5). Underlying this may be the fact that wild-type and ΔE torsinA access different pathways for their degradation, with the ΔE mutant being handled at least in part by the proteasome presumably via the ER-associated degradation pathway typically reserved for misfolded proteins (36, 39). In addition, once in the NE, torsinAΔE has less effect on NE structure and composition than does the wild-type enzyme (42). Whereas detailed structural information on how the ΔE mutation changes torsinA will clearly be required, our finding that ATP-bound torsinAΔE is less able to efficiently engage LULL1 and LAP1C demonstrates that an additional effect of what appears to be a structurally destabilizing deletion (24, 25) is to perturb the otherwise robust interactions of torsinA with its major binding partners. Future efforts to restore a normal interaction between torsinAΔE and LULL1 and LAP1 may therefore be promising avenues for therapeutic exploration.

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