Targeting and activity of the AAA+ ATPase torsinA within the endoplasmic reticulum

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TARGETING AND ACTIVITY OF THE AAA+ ATPASE TORSINA WITHIN THE ENDOPLASMIC RETICULUM

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Abstract

AAA+ ATPase enzymes couple ATP hydrolysis to the exertion of conformational change on a substrate. TorsinA is one of the few AAA+ ATPases that resides within the lumen of the mammalian endoplasmic reticulum (ER). A glutamic acid deletion (ΔE) in the catalytic domain of torsinA causes DYT1 dystonia, but torsinA’s function and the basis for disease remain unclear. I show that torsinA is dynamically targeted from the bulk ER to nuclear envelope (NE) upon coexpression with a cofactor protein, LULL1, and that torsinA’s movement into the NE is concomitant with displacement of select linker of the nucleoskeleton and cytoskeleton (LINC) complex proteins. These effects suggest a role for torsinA in modulating nuclear positioning via the LINC proteins. ΔE-torsinA also responds to LULL1, but only weakly displaces LINC proteins. These findings identify a possible mechanism for regulating torsinA localization and activity via LULL1, and suggest that activity at the NE is perturbed by the disease-causative ΔE mutation. An N-terminal hydrophobic domain (NTD) in torsinA is required for NE targeting. Surprisingly, deletion of this domain also abolishes retention of torsinA within the ER. I demonstrate that the NTD mediates stable monotopic association with the luminal leaflet of the ER, which in turn controls ER retention. Membrane domains from certain other ER resident proteins are thought to control targeting by selective partitioning within the membrane. I propose that this lipid-based sorting mechanism extends to monotopic membrane proteins, and identify a group of proteins that may share torsinA’s topology and ER retention mechanism. Returning to the interaction between torsinA and LULL1, I identify distinct regions of LULL1 that are responsible for torsinA binding and re-targeting to the NE. A conserved helix at the extreme C terminus of LULL1 mediates its interaction with torsinA, while the extreme N terminus of LULL1 must be present in order for torsinA to be targeted to the NE. Importantly, the N terminus of LULL1 is cytosolic, while the C-
terminal torsinA-binding domain is lumenal, raising the intriguing possibility that control of torsinA localization involves communication between the cytosol and the ER lumen. The work in this thesis provides new insight to torsinA’s cellular function and dysfunction in disease and defines novel mechanisms of controlling localization of membrane proteins.
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Chapter 1

Introduction
1.1 TorsinA is the causative gene in DYT1 dystonia

TorsinA was identified as the protein product of the human DYT1 locus, which is located on chromosome 9q34 and is linked to early onset (DYT1) torsion dystonia [1]. Dystonia is a dominantly inherited, rare movement disorder that manifests as uncontrollable twisting movements of the torso, neck, and limbs [2, 3]. Approximately 30% of carriers develop symptoms in adolescence. Understanding of the disease’s etiology has been limited by the fact that no defined neural pathology or degeneration correlates with disease symptoms. Both the absence of pathology and the developmental timing of onset have led to the proposal that the disease arises from abnormal development of neural circuits rather than the death of neurons. In carriers of the DYT1 mutation, brain imaging analyses find regions of increased metabolic activity [4] and evidence for abnormal neurotransmission in the basal ganglia [5] and cerebellum [6]. Recent functional MRI studies have identified specific differences amongst the brain activity patterns of carriers that correlate with clinical disease penetrance [7, 8]. It has been proposed that the sporadic appearance of secondary, presumably compensatory, alterations in brain circuitry underlies the low penetrance of dystonia [6, 7].

1.2 TorsinA is a member of the torsin subfamily of AAA+ ATPases

TorsinA is a member of the AAA+ ATPase superfamily of enzymes [1]. Deletion of a glutamic acid codon (ΔGAG) at position 302 or 303 within torsinA’s AAA domain is the most common mutation found in early onset torsion dystonia. TorsinA is an essential gene in mice, and the ΔGAG mutant cannot compensate for torsinA ablation, suggesting that the disease-causative mutation lacks the enzyme’s normal function [9]. TorsinA is expressed in most tissues, with particularly high expression in certain tissues and developmental timepoints [10, 11]. However, the disease that results from mutation to the enzyme is neurological, and over-expression of torsinA in mice can also cause
neuronal abnormalities [12, 13], suggesting a particular sensitivity of neurons to changes in torsinA function.

TorsinA is one of four AAA+ ATPases that are targeted to the mammalian secretory pathway, which comprise the torsin subfamily [1, 14]; the other family members are torsinB and the torsin-related proteins torp2A and torp3A. The torsinB gene is adjacent to torsinA on human chromosome 9q34, and is much more closely related to torsinA than are torp2A and torp3A. TorsinB is the only other family member that shares the pair of glutamic acids in the AAA domain that are mutated in dystonia [1], but no mutations in torsinB were found to be linked to dystonia [14]. Importantly, torsinB is highly expressed in most tissues of the body, but expression is relatively low in neurons [10] and during neural development [15]. Recent work suggests that torsinB protein may compensate for torsinA’s function in non-neuronal tissues [13, 16].

Since it was first identified as linked to dystonia, torsinA homologs have been identified in metazoans including mouse, rat, nematode, fruit fly, and zebrafish [14]. No torsin homologues were found in lower eukaryotes. Deletion of torsinA is lethal in mice [9]. Mutation to the C. elegans torsinA homolog OOC-5 causes a nuclear rotation defect early in embryonic development [17, 18]. Targeted down-regulation of the D. melanogaster homologue of torsina, torp4A, causes retinal degeneration [19].

1.3 The AAA+ superfamily of nucleotide hydrolases

AAA+ ATPase enzymes couple ATP hydrolysis to the exertion of conformational changes on substrates. This large enzyme family has members in all domains of life. AAA+ ATPases function as molecular chaperones, using their nucleotide hydrolase activity to exert changes on the folded state of their nucleic acid or protein substrates. This reaction may involve unwinding DNA, as in the case of helicases, or completely unfolding a protein into a polypeptide chain, such as in the case of substrate unfolding
by the 19S proteasome subunit. Other family members function to disassemble a stable protein complex into its individual folded protein components, as in the case of NSF disassembling SNARE complexes after vesicle fusion. In this latter case, the complex subunits remain functional and are recycled for further rounds of fusion [20].

AAA+ domains can occur in tandem (NSF, ClpB) or alone (spastin, torsinA) [21]. The AAA+ domain can be divided into two distinct subdomains: an N-terminal $\alpha/\beta$ Rossman fold and a C-terminal helical domain (Figure 1-1B). ATP binds in the interface between these subdomains, and key ATP-interacting motifs can be found in both regions. The relative positioning of these subdomains is dynamic through the ATP hydrolysis cycle [22]. In the $\alpha/\beta$ domain, Walker-A and Walker-B motifs fall in loops between secondary structure elements, and are features shared by P-loop NTPases and AAA+ enzymes [20, 23]. A conserved lysine residue in the Walker-A motif (GxxxxGK[T/S]) interacts with the $\beta$ and $\gamma$ phosphates of ATP, and the following threonine or serine coordinates Mg$^{2+}$ ions. The Walker-B motif (hhhhDE) consists of several hydrophobic residues followed by a conserved DE pair. The conserved glutamate residue participates in ATP hydrolysis by nucleophilic attack on the $\gamma$ phosphate of ATP. It has been shown for various AAA+s that mutations to the Walker-A motif abolish ATP binding [24, 25] while mutations to the Walker-B motif inhibit ATP hydrolysis but not binding [26, 27]. Since interaction with substrates and cofactors often occurs after ATP binding, mutation to either of these two motifs is a useful diagnostic tool in evaluating enzyme function.

Sensor I and II motifs are unique to AAA+ ATPases. These motifs are located in the C terminal portion of the $\alpha/\beta$ domain and in the helical domain, respectively, and interact with the $\gamma$-phosphate of ATP. Sensor I contains a polar residue that is closely apposed to the Walker-A and –B motifs; mutations to Sensor I impair ATP hydrolysis.
[28, 29]. Sensor II contains a conserved GAR sequence, with the arginine being key for ATP hydrolysis and in some cases, binding [30].

AAA+ ATPases generally assemble into hexamers with a flat ring structure [22, 31]. The oligomer is thought to be the catalytically active state, as ATP binding sites are positioned at subunit interfaces with ‘arginine finger’ residues from neighboring subunits contributing to binding and hydrolysis [22, 30]. AAA+ ATPases with two AAA+ domains in tandem, such as NSF, are often stable oligomers; those with only one AAA+ cassette tend to assemble transiently. The center of the oligomeric ring forms a pore through which substrates are translocated in many cases. Conserved aromatic, hydrophobic, and charged residues have been identified as key substrate contacts within the pore of various family members [21].

1.4 Features of the AAA+ domain in torsin subfamily members

The torsins contain a single AAA+ domain that is most homologous to the second AAA+ domain of the Clp/Hsp100 subfamily of AAA+ ATPases [1, 32, 33]. Torsin AAA+s, however, have some non-canonical features (Figure 1-2). The Walker-A motif diverges from the norm, having a conserved asparagine residue in place of the typical threonine or serine [32, 33]. Despite this difference, mutation to the Walker-A motif’s lysine (K108) affects torsinA behavior in cells, suggesting that this motif is functional [34]. Replacement of the canonical threonine in ClpB’s Walker-A with asparagine preserves nucleotide-stimulated oligomerization, but shifts nucleotide binding preference slightly towards ADP versus ATP, lowering baseline ATPase activity without affecting the ability of substrate to induce hydrolysis [35]. Similar behavior has been reported in the AAA+ family member and DNA helicase DnaC, which also contains an asparagine in its Walker-A motif. It requires cofactors in order to hydrolyze ATP effectively, and its ATP- and ADP- bound states each perform biologically important functions [36], leading to the
proposal that DnaC is an ATP/ADP switch. Catalysis by torsinA may have similar features.

Torsin Sensor IIs are also divergent, having the conserved sequence GCK instead of GAR [32] [33], yet mutation to this conserved lysine affects readouts of torsinA activity [32, 34]. The dystonia-causative ΔGAG deletion is predicted to fall in the helix preceding torsinA’s Sensor II loop, and could disrupt the helix and/or weaken ability to bind ATP [32, 33]. Torsins contain six conserved cysteines that could be involved in disulfide bonds in the oxidizing ER lumen; three of these fall in the AAA+ domain (Figure 1-1, 1-2) [33]. Interestingly, one of these cysteines directly precedes the conserved lysine in Sensor II. Analysis of the redox state of the nematode homolog OOC-5 indicates that there may be a conformational transition linked to the redox status of the Sensor II cysteine [33], making redox regulation of the torsins a possibility. A final distinctive characteristic of torsin AAA+s is the presence of two glycosylation sites in a loop between β2 and α2 [32]. Modification of both of these glycosylation sites has been confirmed [16, 37], and the presence of bulky sugar groups should be taken into account when considering the structure and conformational flexibility of these enzymes.

A structure of a torsin enzyme has not been solved, but weak ATP hydrolysis by a fragment of human torsinA has been observed [38], and the AAA+ domain of the nematode homolog OOC-5 is stabilized by nucleotide, suggesting that torsin proteins at least bind ATP [33]. Co-immunoprecipitation experiments [39, 40] support the idea that torsinA oligomerizes.

1.5 AAA+ ATPases use N terminal domains to engage adaptors and substrates

The combination of AAA+ domains with regulatory domains allows this superfamily of enzymes to function in diverse contexts. AAA+ cassettes have been reported joined to various protein-protein interaction domains, metal binding domains,
other catalytic domains, and DNA-binding motifs [23]. These domains may serve to recruit an AAA+ to a particular organelle or promote direct interaction with a substrate. The torsin enzymes have relatively minimal sequence outside of their AAA+ domain; human torsinA has a 67-residue N terminal domain preceding the AAA+ cassette (Figure 1-1A) [32]. This domain is mostly unstructured, but contains a few features of interest, including a hydrophobic region and three of torsinA’s six conserved cysteines. Cellular fractionation indicates that the hydrophobic region associates with membranes [41, 42]. TorsinA may thus be thought of as a membrane-targeted AAA+ ATPase.

1.6 TorsinA and the endoplasmic reticulum

TorsinA and its three homologs, torsinB, torp2A, and torp3A, are residents of the mammalian endoplasmic reticulum lumen [16, 37, 43]. The endoplasmic reticulum (ER) is the site of synthesis for the cell’s secretory and membrane proteins, as well as some of the cell’s lipids. The ER thus contains the machinery necessary for translocation of nascent polypeptides, assisted folding of polypeptides into functional proteins, degradation of misfolded proteins, and the sorting and packaging of secreted proteins for forward transport. In addition, the ER functions as an intracellular calcium store. This organelle has several distinct subdomains, including smooth ER (lipid synthesis, hormone synthesis, detoxification), rough ER (protein synthesis), transitional ER (anterograde protein transport), the nuclear envelope (see below), and contacts with other organelles, including the plasma membrane, mitochondria, peroxisomes, and lipid droplets. The ER membrane network comprises over 10% of the cell volume [44]. Amazingly, this network is disassembled, divided, and reassembled in daughter cells during each cell division.

The nuclear envelope (NE) is a double bilayered membrane that is contiguous with the bulk ER and defines the border of the nucleus. The NE appears to function as
an extension of the ER; ribosomes and COPII coat components have been observed on the outer nuclear membrane [45, 46]. In addition to the general ER functions defined above, the NE has some specialized features. For instance, the NE is the site where nuclear pore complexes (NPCs) regulate transport between the cytosol and nucleus. The inner surface of the nuclear membrane abuts the nuclear lamina, a cytoskeletal network that surrounds the nuclear volume. The lamina and inner nuclear membrane interact with and scaffold the cellular genome, influencing gene expression [47]. Proteins of the nuclear envelope connect the nuclear lamina to the cytoskeleton, allowing controlled positioning of the nucleus within the cell [48].

What essential function might torsinA perform in the ER? As an AAA+ ATPase, it is likely that torsinA modulates the folded state of ER resident protein substrate(s). TorsinA is unlikely to function as a general folding chaperone, as it is not upregulated when ER stress is induced [49]. Contradictory findings on torsinA’s ability to alter readouts of unfolded protein stress have been reported [40, 50, 51], but overexpression of torsinA does appear to promote the folding of some proteins [52-54]. It is possible that torsinA could exert a chaperone-like activity on a small number of substrates in a specific paradigm, rather than in the global cellular response to stress [55].

1.7 TorsinA binds to LAP1 and LULL1

Identification of torsinA binding partners has provided few clues to the enzyme’s cellular function. Lamina-associated polypeptide 1 (LAP1) was first identified as a torsinA binding protein in a microscopy-based screen for interacting proteins [56]. Lumenal domain-like LAP1 (LULL1) was then identified as an additional torsinA binding partner that shares the lumenal torsinA-binding domain first found in LAP1 [56]. Immunoprecipitation of torsinA and proteomic identification of co-precipitating bands [34] indicated that LAP1 and LULL1 are the major binding partners of torsinA in cultured
cells. Neither of these proteins has a well understood function other than binding to torsinA.

LAP1 and LULL1 are each type II single-pass transmembrane proteins of the ER/NE membrane system. The LAP1 and LULL1 genes are adjacent on human chromosome 1 [56], and based on the high homology of their lumenal domains, it is likely that a gene duplication event was involved in their genesis. The extraluminal domains of these proteins diverge from one another dramatically and may confer unique functions on the proteins. Despite low conservation of the extraluminal domains amongst LAP1 sequences or LULL1 sequences, one conserved feature of these domains is the basic pI in the case of LAP1 (pI ~9) and the acidic pI in the case of LULL1 (pI ~4) [34]. Perhaps because of its basic character, LAP1 is retained at the inner nuclear membrane by direct interaction of its extraluminal domain with the nuclear lamina [57]. LULL1’s extraluminal domain does not interact with the lamina, and LULL1 is localized to the bulk ER.

As is the case for the interaction of many AAA+ ATPases with their substrates and cofactors [20], torsinA’s binding to LAP1 and LULL1 appears to be linked to the nucleotide hydrolysis cycle. Stable interaction between torsinA and LAP1/LULL1 can be best detected when ATP hydrolysis is prevented by mutations to the catalytic domain [34, 56] or incubation with ATPγS. The ΔGAG mutation does not stabilize interaction with LAP1 and LULL1 [34]. Rather, ΔGAG weakens the interaction of ATP-trapped mutant torsinA with LAP1 and LULL1 [34], providing further evidence that this disease-causative mutation destabilizes the enzyme.

Conditions for interaction of torsinA with LAP1 can be screened by the ability of overexpressed LAP1 to shift some wild type torsinA or a large proportion of ATP-trapped torsinA into the NE [56]. Application of this analysis to other torsin family members indicated that only torsinB responded significantly to LAP1 overexpression [16]. These
data would suggest that torsinB but not torp2A or torp3A may share some or all of torsinA’s binding partners.

1.8 Evidence for a function for torsinA at the nuclear envelope

Several lines of evidence suggest that torsinA may function specifically at the nuclear envelope (NE), despite being localized throughout the ER. Firstly, neurons lacking torsinA or homozygous for the dystonia-causative ΔGAG mutation exhibited ultrastructural abnormalities at the nuclear envelope, consisting of vesicular structures in the perinuclear space [9]. The NE is the favored binding site for hydrolysis deficient “substrate trap” torsinA mutants [58, 59] as well as ΔGAG-torsinA [58, 59], suggesting that the enzyme may have substrate(s) there. Mutation of the C elegans homolog of torsinA, OOC-5, results in a nuclear rotation defect in the embryo [17]. The ooc-5 mutant shares some phenotypic similarities to nematodes lacking the NE-localized Sun or nesprin proteins [60, 61]. These proteins interact in the perinuclear space to form linker of nucleoskeleton and cytoskeleton (LINC) complexes, which tether the nucleus to the cytoskeleton and are important for processes involving nuclear movement [48]. Interestingly, nesprin-3 is abnormally distributed in fibroblasts from torsinA knockout mice, and these cells move slower than controls in a polarized cell migration assay [62]. Since nuclear movement is central to neuronal migration [63, 64], it is possible that torsinA’s dysfunction at the NE in dystonia could cause subtle changes to the establishment of neural circuits, leading in some cases to disease. Altogether, these data provide a compelling argument that torsinA may have an important function at the NE, but do not exclude the possibility of other function(s) in the bulk ER.
1.9 Summary of the thesis

When I first began my thesis work, I was intrigued by preliminary findings indicating that torsinA could be enriched in the nuclear envelope (NE) in the presence of a binding partner, LULL1. This was the first demonstration of the enzyme being targeted specifically to the NE, where other studies have suggested it may function.

I show that torsinA is dynamically targeted from ER to NE upon coexpression with LULL1, and that torsinA’s movement into the NE is concomitant with displacement of NE resident transmembrane proteins including Sun2, nesprin-2, and nesprin-3. Because Sun and nesprin proteins cooperate to anchor the nucleus to the cytoskeleton, these effects suggest a role for torsinA in modulating nuclear positioning. ΔGAG-torsinA can also be further enriched in the NE by LULL1, but is inefficient at displacing Sun2. Knockdown of LULL1 reduces the baseline level of ΔGAG-torsinA in the NE, suggesting a general role for LULL1 in controlling the distribution of torsinA within subdomains of the ER. These findings identify a possible mechanism for regulating torsinA localization and activity via LULL1, and suggest that activity at the NE is perturbed by the disease-causative ΔGAG mutation [65] (Chapter 2). We also determine that a rare second dystonia-causative mutation, R288Q [66], has a similar cellular phenotype to ΔGAG (Chapter 5), strengthening the argument that dysfunction of torsinA at the NE causes disease.

By analyzing the features of torsinA required for response to LULL1, we determined that an N-terminal hydrophobic domain (NTD) is required for NE targeting. Surprisingly, deletion of this domain also abolishes retention of torsinA within the ER. Although the NTD is physicochemically similar to a transmembrane domain (TMD), the NTD does not traverse the membrane, but mediates stable monotopic association with the lumenal leaflet of the ER. This association in turn controls ER retention. It has been
shown that TMDs from some ER resident proteins control targeting by selective partitioning among different domains of the membrane bilayer. I propose that this lipid-based sorting mechanism extends to monotopic membrane proteins, and identify a group of proteins that may share torsinA’s topology and ER retention mechanism. This group includes the pharmacologically important prostaglandin synthetic enzyme COX1 (Chapter 3). This study has several exciting implications. Firstly, it identifies a previously unknown way for lumenal membrane proteins to escape “bulk flow” secretion out of the ER without the intervention of recycling receptors or vesicular coat proteins. Secondly, many monotopic membrane domains have been shown to sense membrane curvature; torsinA’s NTD could preferentially partition into relatively flat membranes, and thus contribute to torsinA’s targeting to the NE by LULL1. Finally, homology models suggest that the NTD could place torsinA’s central catalytic pore proximal to the membrane, which could have consequences for engagement with transmembrane protein substrates.

Returning to the interaction between torsinA and LULL1, I identify non-overlapping regions of LULL1 that are responsible for (a) torsinA binding and (b) torsinA re-targeting to the NE. Specifically, a conserved helix at the extreme C terminus of LULL1 mediates its interaction with torsinA, while residues at the extreme N terminus of LULL1 must be present in order for torsinA to be effectively targeted to the NE. Importantly, the N terminus of LULL1 is cytosolic, while the C-terminal torsinA-binding domain is luminal, raising the intriguing possibility that control of torsinA localization involves communication between the cytosol and the ER lumen (Chapter 4). Separately, conserved cysteines in LULL1’s luminal domain must be present in order for LULL1 to re-target torsinA, which might suggest that regulation of torsinA by LULL1 involves a redox component.
The work in this thesis provides new insights into aspects of torsinA’s cellular function and dysfunction in disease, as well as the sorting mechanisms that lumenal membrane proteins, including torsinA, use to achieve ER localization.
References


Figure Legends

**Figure 1-1.** Domain organization of torsinA and AAA+ domain structure. (A) TorsinA consists of an N-terminal signal peptide (black), a hydrophobic domain (gray), an unstructured linker region (white), and a single AAA+ cassette (yellow). The position of the dystonia-causative glutamic acid deletion is indicated (E302/303); the positions of torsinA’s 6 conserved cysteines are indicated in red. (B) 3D model of the D2 AAA+ domain of *T. thermophilus* ClpB, a close homolog to torsinA. Predicted positions of relevant residues in torsinA are superimposed, including ATP-interacting sensor I and II motifs and the ΔGAG deletion. Adapted from [32].

**Figure 1-2.** Alignment of torsinA’s AAA+ domain to the D2 domain of *T. thermophilus* ClpB. Helices and sheets from ClpB are numbered and colored correspondingly to the 3D model in (1-1B). Key conserved motifs for AAA+ function are shaded. The two sequences are 21% identical and 40% similar. Adapted from [32].
Figure 1-1

A

GAG deletion

sensor 2

sensor 1

B

E302/303

21 43 70 332

AAA

*
Figure 1-2

TorsinA  (61) LQKDLDDNLFQGHLAKKIILNAVFGFINNPK-PKKPL-TLSLHGTWCTGK
ClpB D2  (552) LEEELHKRVVGQDEAIRAVADAAIRARRARAGLDPNRPIGSFLFLGPTGVGK

α0

TorsinA  (109) NFVSIIAENIYEYEGGLNSDYSVHFLFVATLHFPHASMITYKDQLQLWIRGN
ClpB D2  (602) TELAKTLAATLFDEEAMIRIDMTEYMEKHAVSRIGAPPGYVGYEEGQ

α1

ClpB D2 (702) TVIILSTNLGLSGPLILEGLQKGW----------------PYERIRDEVFKVLQQ

β2

ClpB D2 (786) LTEAAKDFLAERGYDPVFARPLRVIRQRELETplaQKILAGEVKEGDRV

TorsinA (159) VSACARY-------------SIFIFDEMKMTAGLIKPFLLDYDLVGF------VSYQK

β1

ClpB D2 (652) LTEAVRRPYSVIFDEIKAHPDVFNILLQILDGRLTDSHGRTVDFRN

α2

ClpB D2 (739) HFR-PEFLNR--LDEIVVFPLTKEQIRQIVEIQSLYLRALAEKRISLE

α3

**

ClpB D2 (836) QVDVG-------PAGLVFAVPARVEA--
Chapter 2

LULL1 retargets torsinA to the nuclear envelope revealing an activity that is impaired by the *DYT1* dystonia mutation
Acknowledgments

This work was performed in close collaboration with Teri Naismith, whose preliminary data initiated this project, and whose work appears in Figure 2-4. I would like to thank Seema Dalal for early work cloning LULL1 and creating stable cell lines, Soomin Shim for assistance with LULL1 shRNA cell lines, and Erik Snapp for performing fluorescence recovery after photobleaching experiments. LINC protein experiments were performed with advice, assistance, and reagents from Didier Hodzic and P.J. Stewart-Hutchison. Robert Mecham and members of his laboratory, specifically Jessica Wagenseil and Thomas Broekelmann, are gratefully acknowledged for assistance with time-lapse imaging. Many thanks also to all members of the Hanson lab for helpful discussions. This Chapter originally appeared as a publication of the same title by Vander Heyden AB, Naismith TV, Snapp EL, Hodzic D, and Hanson PI, in Molecular Biology of the Cell, Volume 20, Issue 11, June 2009, pg. 2661-72. It is reproduced here with the permission of the journal.
Abstract

TorsinA is an AAA+ ATPase in the endoplasmic reticulum (ER) lumen that is mutated in early onset DYT1 dystonia. TorsinA is an essential protein in mice, and is thought to function in the nuclear envelope (NE) despite localizing throughout the ER. Here, we report that transient interaction of torsinA with the ER membrane protein LULL1 targets torsinA to the NE. Fluorescence recovery after photobleaching and Blue Native PAGE indicate that torsinA is a stable, slowly diffusing oligomer in either the absence or presence of LULL1. Increasing LULL1 expression redistributes both wild-type and disease-mutant torsinA to the NE, while decreasing LULL1 with shRNAs eliminates intrinsic enrichment of disease-mutant torsinA in the NE. When concentrated in the NE, torsinA displaces the nuclear membrane proteins Sun2, nesprin-2G and nesprin-3 while leaving nuclear pores and Sun1 unchanged. Wild-type torsinA also induces changes in NE membrane structure. Since SUN proteins interact with nesprins to connect nucleus and cytoskeleton, these effects suggest a new role for torsinA in modulating complexes that traverse the NE. Importantly, once concentrated in the NE, disease-mutant torsinA displaces Sun2 with reduced efficiency and does not change NE membrane structure. Together, our data suggest that LULL1 regulates the distribution and activity of torsinA within the ER and NE lumen and reveal functional defects in the mutant protein responsible for DYT1 dystonia.
Introduction

Early-onset (DYT1) torsion dystonia is a neurological movement disorder characterized by twisting movements of the limbs and an absence of neuropathology or neurodegeneration [1, 2]. The disease is caused by autosomal dominant inheritance of a glutamic acid deletion in the protein torsinA, frequently referred to as the ΔGAG mutation because of the deleted codon [3]. Although torsinA is expressed ubiquitously [4], the only abnormalities described so far in animals lacking this essential protein are in neurons [5]. The failure of ΔGAG-mutant torsinA to rescue torsinA knock-out animals from perinatal lethality suggests that the ΔGAG mutant lacks whatever essential activity torsinA normally provides [5, 6].

The specific cellular functions ascribed to torsinA vary widely despite the fact that it has been a decade since the protein was first described and linked to dystonia [2]. TorsinA resides in the lumen of the endoplasmic reticulum (ER) [7-9]. Based on its membership in the AAA+ (ATPases associated with a variety of cellular activities) family of ATPases [3, 10], it is likely that torsinA disassembles or changes the conformation of a protein or protein complex within the ER or nuclear envelope (NE) lumen. While typically found throughout the ER, several things point to a function for torsinA in the NE. Reducing torsinA activity by gene knock-out in mice [5] or expressing a dominant negative form of the enzyme in cultured cells [11] selectively perturbs NE structure. The NE is the favored binding site for hydrolysis deficient “substrate trap” torsinA mutants [11-13]. Finally, the outer nuclear membrane protein nesprin-3 [14] is abnormally distributed in fibroblasts from torsinA knock-out mice, and these cells move slower than controls in a polarized cell migration assay [15]. Since nesprin-3 participates in linker of the nucleoskeleton and cytoskeleton (LINC) complexes [14, 16-19] these data suggest that torsinA activity may help regulate NE structure and connections between nucleus
and cytoskeleton.

Previously identified binding partners for torsinA include the transmembrane proteins LAP1 (also known as TOR1AIP1) in the NE and LULL1 (also known as TOR1AIP2 or NET9) in the ER, which control the distribution of hydrolysis deficient "substrate trap" torsinA between NE and ER in direct proportion to their relative abundance [20]. Here, we describe the surprising finding that increasing expression of LULL1 induces torsinA to redistribute from throughout the ER into the NE while decreasing it reverses concentration there. Once in the NE, torsinA displaces a subset of LINC complex components and gradually promotes structural changes in the NE. The disease-associated ΔGAG mutant is less efficient at enacting these changes. We conclude that LULL1 dynamically tunes the distribution of torsinA between the ER and NE and is thereby likely to regulate its function.
Methods

Plasmids

GFP in previously described torsinA-GFP plasmids [11] was changed to monomeric GFP (mGFP) by site-directed mutagenesis of L221K in GFP [22]. TorsinA-TagRFP was made by transferring torsinA mutants excised with XhoI and EcoRI from EGFP-N1 into a RFP-N1 vector containing TagRFP [44]. The torsinA Δ26-43 deletion was created by QuikChange mutagenesis (Stratagene). LULL1-myc was made by amplifying LULL1 (NM_145034, NP_659471) from human cDNA with primers containing HindIII and EcoRI restriction sites and ligating it into pcDNA4/TO/MycHis B (Invitrogen). LULL1-mCherry was made by excising LULL1 from EGFP-N1 using HindIII and EcoRI restriction sites and ligating into pmCherry-N1 (Clontech). ER-RFP was from [30]. Sun2-GFP is from [32]. Nesprin-3α-GFP is from [17]. The sequences of all constructs were verified by nucleotide sequencing.

Cell Culture and cell line generation

U2OS cells were grown in DMEM supplemented with 10% FBS and L-glutamine. Transient transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Clonal LULL1-myc tetracycline-inducible U2OS cell lines were made as described previously [45], with selection in hygromycin (50 µg/mL) and zeocin (100 µg/mL). LULL1-myc/Sun2-GFP dual-expressing cells were made by transfecting Sun2-GFP into LULL1-myc cells and selecting with 400 µg/mL G418. LULL1 was depleted by lentiviral transduction of shRNAs directed against human LULL1 (Sigma Mission shRNA, RefSeq # NM_145034). Lentiviruses were produced by cotransfecting hairpins in pLKO with the pCMV 8.2 ΔR packaging plasmid into 293T cells. U2OS cells were then transduced with viral particles according to manufacturer’s
instructions. Cells depleted of LULL1 were enriched for by selecting transduced cells with puromycin (10 µg/ml).

**Immunofluorescence**

Cells were fixed with 3% paraformaldehyde in PBS for 10 min, followed by permeabilization in 0.2% Triton-X-100 for 10 min. Coverslips were blocked in 2% goat serum in PBS for 1 hour before incubation with primary and Alexa Fluor-conjugated secondary antibodies. For epifluorescence imaging, coverslips were mounted in Mowiol (Calbiochem) and imaged with a Leica Diaplan microscope using a 63 x 1.4 N.A. objective and a Zeiss AxioCam MRm camera. Brightness and contrast were adjusted in Adobe Photoshop (Adobe Systems). For confocal imaging, coverslips were mounted in VectaShield (Vector Labs), sealed with nail polish, and imaged with a Bio-Rad Radiance 2000 microscope with a 63x/1.4 NA oil objective, using the 488- and 543-nm laser lines. Confocal z-series were acquired with a pinhole of 1.7 at 0.3 µm spacing. All confocal images were processed using the smooth function in Image J (v1.4, NIH), which replaces each pixel with the average in its 3 x 3 neighborhood. Maximum intensity projections of confocal z-series were made in ImageJ. Composite figures were prepared using Photoshop and Illustrator software (Adobe).

**NE/ER Ratio Analysis**

U2OS or LULL1 knock-down cells were transiently transfected with TorsinA-mGFP and ER-RFP as indicated. Confocal images were acquired as above. Fluorescence intensity was quantified using Metamorph 6.0 software (Universal Imaging). For each image, average intensities in the GFP and RFP channels were quantified in four regions each of the ER (~400 square pixels each) and NE (~200 square pixels each). Regions of interest
were delineated as boxes for ER and as hand-drawn outlines for NE and any overlapping perinuclear ER. These four values were averaged for each cell’s ER and NE and then used to compute the NE/ER ratio for each channel. The data shown represent the average of >20 cells per condition.

**Live imaging**

For experiments involving co-expression of LULL1 and torsinA, LULL1-myc expression was induced with tetracycline (1 µg/mL) 6 hrs prior to adding Lipofectamine 2000/torsinA-mGFP mixtures to initiate transfection. 1-2 hours later, coverslips were transferred to Bioptechs Delta T imaging dishes (Bioptechs Inc) and overlaid with phenol-red-free media containing 1% FBS. The dishes were placed on an enclosed stage (custom made by A. Czirok, Dept of Biological Physics, Eotvos University, Budapest, Hungary) attached to a CO₂ pump to maintain 5% CO₂ and to a temperature control device set to 37°C. Images were obtained with a 20 x 0.4 NA objective on an inverted epifluorescence/DIC microscope (Leica DMI6000B, Leica Microsystems). Images (696 by 520 pixels, 12-bit intensity resolution) were recorded with a QImaging Retiga Exi camera (QImaging) using 2 x 2 binning and pre-determined 1-2 sec exposures. Image acquisition and microscope settings were controlled by software based on [46]. Briefly, up to 16 pre-selected fields were visited in each scanning cycle, and epifluorescence images were taken in one or two channels. For each field and channel, 5 images were acquired in z positions centered around the chosen focal plane. Cells were imaged every 15 minutes for the duration of the experiment. Images were processed using software based on [46]. For each time point, the software chooses the most in-focus image of the “z-stack” for each 64 by 64 pixel block of the image. These best-focused pixel blocks are then collapsed into a single image. The software also
corrects for small shifts in x position. The resulting images were further processed using “Subtract Background” and “Enhance Contrast” tools in ImageJ. Time-lapse imaging (TiLa) code was created by the Computational Imaging Group at the University of Kansas Medical Center, under the direction of Drs. C. Little, B. Rongish, and A. Czirok. Dr. Czirok devised the original code for image acquisition and processing, which has been further developed and modified by Alan Petersen, Michael Filla, and Dr. Evan Zamir. A current version of this open source code is available from the Computational Imaging Group upon request (clittle@kumc.edu).

**FRAP analyses**

Cells were grown in 8-well Labtek chambers (Nunc) and imaged in phenol red-free RPMI supplemented with 10mM Hepes and 10% fetal bovine serum. Live cells were imaged on a 37°C environmentally controlled chamber of a confocal microscope system (Duoscan; Carl Zeiss MicroImaging, Inc.) with a 63X/1.4 NA oil objective and a 489-nm 100mW diode laser with a 500-550 nm bandpass filter for GFP. FRAP experiments were performed by photobleaching a region of interest at full laser power of the 489nm line and monitoring fluorescence recovery by scanning the whole cell at low laser power. No photobleaching of the cell or adjacent cells during fluorescence recovery was observed. Diffusion ($D$) measurements were calculated as described previously [23, 47]. Composite figures were prepared using Photoshop CS2 and Illustrator CS software (Adobe). Plotting of diffusion coefficients was performed with Prism 4.0c.

**Immunoblotting**

Western blots were developed as described [45]. For quantitative analysis using the Odyssey system (LiCOR Biosciences), samples were separated, blocked, and blotted according to manufacturer's instructions. Secondary antibodies conjugated to IRDye-680
or -800 were used.

**Antibodies**

The following antibodies were used: mouse anti-myc (9E10, Developmental Studies Hybridoma Bank, Univ. of Iowa), rabbit anti-Sun1 (Sigma), rabbit anti-Sun2 [32], chicken anti-nesprin2-Giant (Brian Burke, University of Florida, Gainesville), mouse anti-NPCs (mAb414, Babco), rabbit anti-calreticulin (Stressgen), mouse anti-torsinA DM2A8 [8], mouse anti-α-tubulin (Sigma), and rabbit anti-Sec61β (Joe Bass, Northwestern University). We generated an affinity-purified rabbit antibody against residues 1-217 of human LULL1 fused to GST. Protein purified from *Escherichia coli* was sent to Sigma Genosys for injection into rabbits. The resulting sera were depleted of GST-reactive antibodies by incubation with GST protein, and subsequently affinity purified on immobilized antigen. Secondary goat anti-mouse, goat anti-rabbit, and goat anti-chicken antibodies conjugated to Alexa 488, 555, or 595 were purchased from Molecular Probes. Goat anti-mouse and goat anti-rabbit antibodies conjugated to HRP were purchased from Bio-Rad.

**Triton X-114 phase separation**

Cells from a 6 cm plate of U2OS cells transfected with the indicated construct were collected and resuspended in 250 µl buffer containing 2% Triton X-114 and 200 mM NaCl. Samples were incubated at 4°C for 30 min and then centrifuged for 10 min at 10,000 x g to remove insoluble material. The soluble extract was incubated at 37°C for 10 min followed by centrifugation at 1,000 x g for 10 min at room temperature to separate phases. The top “aqueous” phase was transferred to a new tube, and the
bottom “detergent” phase brought to the same volume as the aqueous phase. Equal volumes were boiled and analyzed by SDS-PAGE and immunoblotting.

Blue native PAGE

10 cm plates of U2OS cells or U2OS cells with tetracycline-inducible LULL1-myc were induced with tetracycline and/or transfected with constructs as indicated. Cells were resuspended in sample buffer (50 mM imidazole, 50 mM NaCl, 2 mM EDTA, 2 mM ATP, 2 mM aminocaproic acid, 4 mM MgCl₂, PMSF, protease inhibitors, and dodecylmaltoside or digitonin detergent at indicated concentrations). The samples were incubated at 4°C for 15 minutes with agitation, then centrifuged for 30 minutes at 60,000 rpm at 4°C, followed by 15 minutes at 60,000 rpm at 4°C in fresh tubes. The protein concentration of the supernatant was determined by Bradford assay, and equivalent amounts of protein (~20 μg per sample) were supplemented with Coomassie G-250 at 0.125% w/v final concentration and glycerol to 5% final concentration. Samples were then loaded onto 7.5% BN-PAGE gels, run, and transferred to PVDF for Western blot detection. Samples were run alongside a native PAGE molecular weight marker (High Molecular Weight Calibration Kit for native electrophoresis, Amersham). BN-PAGE gels, buffer, and PAGE protocols were as previously described (Wittig et al., 2006).

FACS isolation of a LULL1, torsinA-mGFP positive cell population

15 cm plates of U2OS cells with tetracycline-inducible LULL1-myc were induced with tetracycline 6 hours before transfection of torsinA-mGFP. Either 12 or 22 hours later cells were trypsinized, washed in PBS, and resuspended in phenol red-free DMEM plus 1% FBS and 0.1 mM EDTA at 4°C. Cells were diluted to a concentration of 5-10 x 10⁶ cells/mL for sorting. Cells were collected by GFP fluorescence to obtain cells exclusively
expressing LULL1-myc- and torsinA-mGFP. FACS was performed at a WUSM core facility on a FACS Vantage Sorter (Becton Dickinson).

**Immunoprecipitation**

U2OS-LULL1 myc cells in 6 cm plates were induced to express LULL1-His6myc by adding 1 mg/ml tetracycline 6 hrs before adding Lipofectamine 2000 and the indicated torsinA-mGFP plasmid. On the following day, cells were collected and solubilized in 20 mM Hepes pH 7.2, 25 mM NaCl, 2 mM Mg2+ATP, 0.5% CHAPS, and complete protease inhibitor cocktail. LULL1-His6myc was then immunoprecipitated with 9E10 anti-myc monoclonal antibody and protein G Sepharose. Samples were resolved by SDS-PAGE and the distribution of mGFP tagged torsinA proteins monitored by immunoblotting with a GFP antibody.
Results

Dynamic regulation of torsinA distribution by LULL1

In an effort to understand how and when torsinA functions within the NE, we studied the subcellular localization of torsinA with monomeric GFP fused to its C-terminus in transfected U2OS cells. As expected, torsinA-mGFP is distributed evenly throughout the ER (Figure 2-1A, left). The dystonia-causative mutant, ΔGAG-torsinA, also localizes to the ER but is enriched in the contiguous NE as expected based on earlier studies (Figure 2-1A, middle) [11, 12, 21]. TorsinA’s binding partner LULL1-myc is also found throughout the ER (Figure 2-1A, right).

To our surprise, however, expressing torsinA-mGFP together with LULL1-myc shifted torsinA-mGFP almost entirely into the NE, where it displayed patterns ranging from concentration in a portion of the NE (Figure 2-1B, left hand cell) to coverage of the entire nucleus (Figure 2-1B, right hand cell). In the latter cells, the torsinA-mGFP containing NE was often distorted (Figure 2-1B”). A confocal slice from the z-stack used to generate the projected image in Figure 1B confirms that torsinA-mGFP concentrates at the nuclear periphery, and that the LULL1-myc containing ER appears generally normal (Figure 2-1B’). Overall, 65% of cells (n >150) expressing both proteins for 18 hours had torsinA-mGFP concentrated in the NE. A smaller, more variable proportion of LULL1-myc also relocalized to the NE (compare Figure 2-1A to 2-1B), leading us to hypothesize that LULL1 changes the subcellular targeting of torsinA rather than vice versa. ΔGAG-torsinA-mGFP similarly responded to coexpressed LULL1-myc by further concentrating in the NE (Figure 2-1C).

To assess the specificity of this phenomenon, we asked whether overexpressing LULL1 would cause another ER protein to redistribute, and conversely, whether introducing another ER protein would change the localization of torsinA. For this
analysis, we used Sec61\(\gamma\) tagged with GFP, which, similarly to LULL1, is a mobile single-pass transmembrane protein of the ER [22]. We found that expressing LULL1-myc did not change the distribution of mGFP-Sec61\(\gamma\) between bulk ER and NE (Figure 2-1D), and that introducing mGFP-Sec61\(\gamma\) did not cause torsinA to redistribute to the NE (Figure 2-1E). To ensure that the effect of LULL1 on torsinA is not tag-dependent, we examined different forms of the proteins and found that untagged torsinA and torsinA-TAG-RFP responded to LULL1-myc, and that LULL1-mGFP caused torsinA-myc to redistribute (Supplemental Figure 2-S1). To determine how much LULL1 is required to have this effect on torsinA, we induced LULL1 expression with 100-fold less (10 ng/ml) or 1000-fold less (1 ng/ml) tetracycline; 1 ng/ml tetracycline induced expression of LULL1 to \(~5\) times endogenous levels (Figure 2-S2D) and was also capable of moving torsinA-mGFP into the NE (Figure 2-S2A).

The variable distribution of wild-type torsinA in fixed LULL1- and torsinA-expressing cells suggested a dynamic process that we further explored in living cells. Shortly after transfecting torsinA-mGFP into LULL1-expressing cells, we transferred coverslips to an imaging chamber and collected pictures every 15 min for up to 24 hours. A typical field of cells is shown 2 hours after transfection of torsinA-mGFP and again 5 hours later (Figure 2-2A). These images demonstrate that as torsinA-mGFP expression begins, it is predominantly localized to the ER, recognizable at low magnification as a crescent of fluorescence surrounding a comparatively dark nucleus (Figure 2-2A, upper panel). Over time, torsinA-mGFP shifts to the NE in many of the expressing cells (Figure 2-2A, lower panel). A pair of cells viewed at higher magnification in Figure 2-2B show the typical manner in which torsinA redistributes. TorsinA starts to concentrate in the NE at one or a few points and proceeds to surround the whole nucleus in an average of 45 min (Figure 2-2B). This confirms that partially covered nuclei in fixed cells represent
intermediates in the redistribution of torsinA into the entire NE. In most cells, torsinA remained concentrated in the NE for the duration of the experiment (Figure 2-2B, right-hand cell). Rarely, it later returned to the ER demonstrating that the redistribution is reversible and implying that NE-localized torsinA is still responsive to changes in its environment (Figure 2-2B, left-hand cell). Mitosis was occasionally observed, demonstrating that NE breakdown and reformation remain possible in the presence of NE-localized torsinA (data not shown). Initiation of torsinA’s redistribution to the NE occurred randomly with respect to time of observation and position in the viewing field, suggesting that the redistribution is a cell-autonomous process initiated by factor(s) that could include a threshold amount of LULL1, torsinA, or something else. Imaging of ΔGAG-torsinA-mGFP in LULL1-myc-expressing cells showed that it too enriched in the NE with time, as expected based on the immunofluorescence of fixed cells (Supplemental Figure 2-S3A). However, the mutant enzyme never progressed with polarity into the NE (and we never saw partially covered nuclei in fixed samples). To be sure that LULL1 was responsible for the enhanced enrichment of ΔGAG-torsinA-mGFP in the NE, we also imaged it in U2OS cells with only endogenous levels of LULL1. In this case, its partitioning between NE and ER did not change over time (Supplemental Figure 2-S3B).

We next asked whether the LULL1-dependent shift of torsinA into the NE could be explained by selective immobilization there, analogous to the behavior of NE resident proteins such as the lamin B receptor (Ellenberg, 1997). To address this, we carried out quantitative FRAP (fluorescence recovery after photobleaching) experiments [23]. We found that expressed individually, torsinA-mGFP recovered slowly from photobleaching (Figure 2-2D, 2-2F) while LULL1-mGFP recovered an order of magnitude more quickly (Figure 2-2C, 2-2F). Coexpressing torsinA-mGFP with LULL1-mCherry did not change the slow mobility of torsinA in either the NE or ER (Figure 2-2E, 2-2F). Conversely,
coexpression with torsinA-mGFP did not change LULL1-mCherry diffusion (Figure 2-2E’, 2-2F). Notably, torsinA-mGFP is readily solubilized by mild detergents (data not shown, but see Figure 2-3 below), indicating that its slow diffusion and localization to the NE are not consequences of aggregation.

The diffusive behavior of torsinA suggested that it participates in a large protein complex in the ER and NE. Furthermore, AAA+ proteins typically function as higher order oligomers, typically either hexamers or dodecamers [10]. However, previous analyses of torsinA’s oligomeric state had not identified any such large assemblies [24], although torsinA can self-associate as judged by co-immunoprecipitation [21, 25]. To reconcile these two lines of data, we hypothesized that torsinA oligomers might be disrupted by the detergents used to prepare previous samples for analysis. We turned to Blue Native polyacrylamide gel electrophoresis (BN-PAGE), which separates native membrane proteins by size and shape in the presence of mild detergent and the protein binding dye Coomassie Brilliant Blue [26]. BN-PAGE of dodecylmaltoside (DDM)-solubilized torsinA reveals the presence of an oligomeric species of approximately hexameric size in samples containing 0.25% w/v DDM (Figure 2-3A, left lane). This oligomer is destabilized by higher concentrations of DDM (Figure 2-3A), indicating that it represents a complex of folded proteins rather than an aggregate. A comparable oligomer is detected in samples solubilized with 0.5% w/v digitonin (Figure 2-3B, upper panel). Further, the oligomeric species is completely dispersed on a second-dimension denaturing SDS-PAGE gel (Figure 2-3B, lower panel), where all immunoreactivity corresponds to the size of monomeric torsinA (~38 kDa). This suggests that torsinA forms a hexameric unit similar to other AAA+ ATPases [10], and together with earlier suggestions that torsinA is peripherally associated with the ER lumenal membrane (Liu et al., 2003; Callan et al., 2007) provides a likely explanation for its slow mobility in the FRAP experiments described above. Slow diffusion may explain why it takes an average
of 45 min (Figure 2-2B) for torsinA-mGFP to relocalize from ER to NE. Finally, torsinA is a stable oligomeric species of approximately hexameric size regardless of whether or not LULL1 is coexpressed (Figure 2-3C). The fact that LULL1 changes neither the oligomeric state nor the apparent mobility of torsinA suggests that LULL1 changes the enzyme’s targeting but not its fundamental organization.

*Features of TorsinA involved in its redistribution to the NE*

LULL1-promoted redistribution of torsinA to the NE was unexpected based on the earlier finding that hydrolysis deficient “substrate trap” mutant torsinA (containing a Walker B motif E171Q mutation) accumulates together with LULL1 in the peripheral ER and efficiently co-immunoprecipitates with it [20]. Because wild-type torsinA only inefficiently co-immunoprecipitates with LULL1 [20], we hypothesized that a transient, ATP-dependent interaction between active enzyme and LULL1 changes something, most likely in torsinA, that in turn targets it to the NE. To learn more about how torsinA responds to LULL1, we explored the effects of mutations in defined motifs within torsinA. Under conditions in which wild-type enzyme efficiently relocalizes to the NE, we found that, as expected (Goodchild and Dauer, 2005), torsinA(E171Q) remained distributed throughout the ER (Figure 2-4B). Interestingly, torsinA with a mutation in its Walker A ATP-binding motif (K108A) also did not redistribute into the NE (Figure 2-4B), establishing that torsinA needs to be able to engage ATP to move in response to LULL1.

We next wondered whether peripheral association of torsinA with the lumenal membrane is important for its targeting to the NE. We deleted the hydrophobic sequence (Δ26-43) implicated in membrane association [27, 28] and confirmed that torsinA no longer behaved as a hydrophobic protein in Triton X-114 phase partitioning experiments (Figure 2-4D). Interestingly, this hydrophilic torsinA did not move into the NE in cells expressing LULL1 (Figure 2-4B). Because deleting the hydrophobic
sequence does not affect binding of “substrate trap” E171Q mutant torsinA to LULL1 (Figure 2-4E), this mutant’s failure to redistribute suggests that the N-terminus of torsinA plays an essential role in LULL1-dependent NE targeting.

Removing LULL1 reduces enrichment of ΔGAG-TorsinA in the nuclear envelope

The above experiments indicate that overexpressing LULL1 shifts a large proportion of torsinA into the NE. This, together with the fact that ΔGAG-torsinA (and in some cell types, also wild-type torsinA) is known to be intrinsically enriched in the NE (Figure 2-1A) [11, 12, 29], led us to hypothesize that distribution of torsinA between ER and NE may normally be controlled by interaction with endogenous LULL1. To explore this possibility, we used RNAi to deplete LULL1 (Figure 2-4A) and compared the localization of ΔGAG-torsinA-mGFP to that of a cotransfected ER lumenal marker consisting of a prolactin signal sequence and a KDEL ER-retrieval sequence fused to mRFP (ER-RFP) [30]. Representative confocal sections show that ΔGAG-torsinA-mGFP is more concentrated around the nucleus than is ER-RFP in U2OS cells (Figure 2-4B), but not in LULL1 knock-down cells (Figure 4C). Wild type torsinA, meanwhile, is distributed similarly to ER-RFP in both cell types (images not shown, Figure 2-4D). Quantitative analysis (Figure 2-4D and Materials and Methods) confirms that wild type torsinA has no preference for the NE and/or perinuclear ER in U2OS cells. ΔGAG-torsinA-mGFP, in contrast, is enriched in the perinuclear region in U2OS cells but not in cells depleted of LULL1 (p = 0.003). The distribution of ΔGAG-torsinA-mGFP in the absence of LULL1 was indistinguishable from that of ER-RFP (p >0.05). Analysis of ΔGAG-torsinA-mGFP in the presence of another RNA hairpin directed against LULL1 showed the same effect (data not shown). This demonstrates that enrichment of ΔGAG-torsinA-mGFP in the NE requires LULL1. Together with the finding that high levels of
LULL1 shift torsinA into the NE, these experiments suggest that LULL1 has a general role in regulating localization of both wild-type and disease-mutant torsinA.

Redistributed torsinA displaces a subset of LINC complex components from the NE

To explore torsinA’s effects on the NE, we examined specific NE components by immunostaining. In cells containing NE-localized torsinA, the distribution and staining of nuclear pores did not change indicating that the overall integrity of the NE is maintained in these cells (Figure 2-6A). We next examined torsinA’s effects on the inner nuclear membrane proteins Sun1 and Sun2. While Sun1 was not affected by redistributed torsinA (Figure 2-6B), Sun2 immunostaining was notably diminished in regions of the NE where torsinA was concentrated suggesting that torsinA might displace Sun2 as it moves into the NE (Figure 2-6C). Orthogonal views of confocal z-series confirmed that Sun1 remains at the nuclear periphery in cells with torsinA in the NE (Figure 2-6B’) while Sun2 is decreased (Figure 2-6C’). The normal distribution of nuclear pores, known to colocalize with Sun1 but not Sun2 [31], could explain a selective effect of torsinA on Sun2. A survey of Sun2 immunoreactivity in cells containing LULL1-redistributed torsinA-mGFP indicated that changes in Sun2 were widespread; 60% of these cells lacked a clearly delineated nuclear rim of Sun2 compared to only 4% of untransfected cells (n > 110 for each). To rule out the alternative possibility that redistributed torsinA masked the lumenal Sun2 antibody epitope [32], we made a tetracycline inducible LULL1-myc cell line that also constitutively expressed Sun2-GFP and assessed the effect of introducing TorsinA fused to TagRFP (Merzlyak et al., 2007). Direct examination of protein fluorescence showed that Sun2-GFP was displaced by torsinA-TagRFP (Figure 2-6D), paralleling the effect of torsinA on endogenous Sun2. Importantly, despite being somewhat concentrated in the NE, E171Q-torsinA and ΔGAG-torsinA expressed individually in U2OS cells had little or no effect on Sun2 (Supplemental Figure 2-S5).
Further, expression of wild type torsinA on its own had no detectable effect. Sun2 in the NE thus appears to decrease specifically in response to LULL1-redistributed torsinA. The overall decrease in Sun2 intensity in cells with redistributed torsinA in the NE suggests that displaced Sun2 may be unstable and possibly degraded. Western blot analysis of Sun2 in such cells confirms a decrease in the overall level of Sun2 but not nucleoporins or a general ER marker (Figure 2-7). Increased degradation of INM proteins has been reported when lamin anchors are absent [33]; it is possible that torsinA could similarly destabilize Sun2 as a consequence of altering its association with the nuclear lamina.

SUN proteins participate in NE-spanning LINC complexes [34], which consist of SUN proteins in the inner nuclear membrane (INM) and nesprins in the outer nuclear membrane (ONM) [19]. Conserved domains of these proteins interact in the perinuclear space to physically connect the nucleus to the cytoskeleton [18, 19]. We therefore next asked whether redistributed torsinA affects the ONM-localized nesprins. Cells expressing LULL1-myc and torsinA-mGFP were stained with an antibody for nesprin2-Giant, revealing that it too was missing from the NE when torsinA was concentrated there (Figure 2-6E). This displacement occurred in 56% of cells expressing LULL1-myc and torsinA-mGFP compared to only 1% of untransfected cells (n > 35 for each). Similarly, transiently transfected nesprin3a-GFP was absent from regions of the NE containing torsinA-TagRFP (Figure 2-6F). These findings demonstrate that torsinA changes the behavior of a subset of LINC complex components, including Sun2 in the INM and nesprin2-Giant and nesprin-3 in the ONM. Indeed, some of the changes in NE membranes caused by redistributed wild-type torsinA (Figure 2-1B’, see also subsequent Figure 2-8A) are reminiscent of changes induced by manipulating components of LINC complexes [34]. A recent report of interaction between torsinA and nesprin-3 suggests that these effects could be direct [15], but further studies are needed.
to understand the molecular changes involved. Since live imaging analysis (Figure 2-2B) indicated that torsinA moves across the NE within an hour, the coincident patterns of Sun2 and nesprin-3 imply that their displacement is temporally related to the arrival of torsinA in the NE and that they could be its direct targets.

The DYT1 ΔGAG mutation impairs the effects of torsinA on the NE

ΔGAG-torsinA has consistently been found to be more enriched in the NE than is wild-type torsinA [11, 12, 21, 29], leading to the hypothesis that mislocalization to the NE might contribute to the development of dystonia [12]. At the same time, ΔGAG-torsinA is thought to be less functional and less stable than wild-type torsinA such that loss of normal enzyme function could underlie the disease [5, 21, 35]. As shown in Figure 2-1 and Supplemental Figure 2-S3, coexpressing LULL1 with ΔGAG-torsinA-mGFP caused it to enrich further in the NE, albeit without the distinctive polarity that characterized the shift of wild type protein. We therefore compared the behavior of ΔGAG- and wild-type torsinA in more detail. Maximum intensity projections of nuclei in cells in which these proteins were concentrated for up to 18 hours revealed that wild-type torsinA distorted NE membranes into both the nucleus and the cytoplasm (Figure 2-8A, 2-8A', left) in 68% of cells with NE-localized torsinA (n =136). In contrast, ΔGAG-torsinA-mGFP left the NE largely unchanged (Figure 2-8A, 2-8A', right), causing NE distortions in only 7% of cells containing NE-localized protein (n = 74). These differences suggest that ΔGAG-torsinA interacts differently than wild-type enzyme with components of the NE.

To determine how redistributed ΔGAG-torsinA affects specific NE components, we looked for changes in Sun2. In cells expressing LULL1-myc and ΔGAG-torsinA-mGFP Sun2 was diminished (Figure 2-8D, 2-8D'). However, a difference between mutant and wild-type enzyme became apparent when we used time-lapse imaging to
compare the effects of wild-type or ΔGAG torsinA-TagRFP on Sun2-GFP.

Representative sequential images show that transiently transfected wild-type torsinA-TagRFP displaces Sun2-GFP as it appears in the NE (Figure 2-8E). In the cells shown, Sun2-GFP decreases at one end of the nucleus while torsinA-TagRFP is too dim to see, but as torsinA-TagRFP becomes visible it is apparent that the boundary between increasing torsinA-TagRFP and decreasing Sun2-GFP remains closely apposed (see especially 90 min and 120 min frames of Figure 2-8E). In contrast, time lapse imaging of ΔGAG-torsinA-TagRFP revealed a much slower effect of ΔGAG-torsinA on Sun2-GFP (Figure 2-8F). Whereas Sun2-GFP started to decrease within 15 min of when wild-type torsinA began to concentrate in the NE, it took ~7 hrs for this to happen in cells expressing ΔGAG-torsinA. Together with the lack of structural abnormalities in the NE of cells expressing ΔGAG-torsinA, this suggests that ΔGAG-torsinA is less efficient than wild-type torsinA at changing NE components.
Discussion

Controlling the localization of enzymes within the cell is an important general mechanism for regulating their activity. Post-translational modifications and conformational changes are known to retarget many cytoplasmic enzymes from one place to another, but dynamic changes in the distribution of enzymes within the ER lumen are less well characterized. We find that the torsinA binding partner LULL1 – an ER transmembrane protein – drives redistribution of this lumenal AAA+ ATPase from throughout the ER network specifically into the NE (Figure 2-9), where previous in vitro and in vivo studies have suggested a function[5, 11, 12]. The idea that regulated distribution between the ER and NE might control torsinA’s activity toward spatially restricted substrate(s) arose initially from studies showing that so-called "substrate trapped" torsinA mutants accumulated in the NE [11, 12, 36]. The wild-type enzyme, in contrast, is diffusely distributed throughout the ER except in a few cell types [21] and after certain pharmacological manipulations [8, 15, 37]. We hypothesized that additional factor(s), which perhaps are more abundant in cell types where torsinA exhibits NE preference [21], were therefore likely to control the targeting and activity of wild-type torsinA. Extrapolating between the extremes of LULL1 overexpression (Figures 2-1, 2-2, & 2-6) and LULL1 silencing (Figure 2-5) leads us to propose that transient interaction with LULL1 positively regulates the targeting and activity of torsinA in the NE. The abundance of LULL1 thus emerges as a critical regulator of torsinA activity. Future studies will address whether variations in endogenous LULL1 levels explain previously described cell-type specific differences in torsinA distribution [21] and whether there are peaks in the expression or stabilization of LULL1 that correlate with important events in neuronal development or plasticity.

LULL1 is a ~70 kDa single-pass transmembrane protein with no defined functional motifs and no known binding partners other than torsinA [20]. Several features
suggest that LULL1 may be regulated at both the transcriptional and post-translational levels, making it an attractive potential regulatory protein. There are significant variations in the level of LULL1 message in different cells and tissues [20, 38] and over the course of \textit{in vitro} muscle cell differentiation [38]. Further, the extralumenal domain of LULL1 has a preponderance of charged residues and a dearth of hydrophobic residues, both of which are likely to predispose it to rapid and potentially regulated turnover [39]. Consistently, secondary structure prediction algorithms indicate a lack of stably folded structures in this region, and the PESTfind algorithm (http://www.at.embnet.org/toolbox/pestfind) finds two high scoring sequences that may correlate with rapid protein turnover [40]. Finally, as a transmembrane protein, LULL1 provides a way to directly or indirectly transmit signals across the ER membrane to torsinA.

Using LULL1 overexpression to concentrate torsinA in the NE, we were able uncover molecular and structural changes that are likely to represent torsinA’s normal activity, including alterations in a subset of NE proteins that participate in NE-bridging LINC complexes (Figures 2-6 & 2-7). LINC complexes are formed when INM-localized Sun proteins and ONM-localized nesprin proteins interact within the NE lumen, and have recently attracted attention as important connectors between the cytoskeleton, NE membranes, and elements within the nucleus including the lamina and telomeres [19]. While much remains to be learned about the cell biology of LINC complexes, it is clear that they have important roles in such diverse processes as nuclear anchorage, cell migration, and regulating gene expression [16, 41]. Our data showing that some LINC complex proteins – Sun2, nesprin-2G, and nesprin-3 – are destabilized by torsinA as it accumulates in the NE suggest possible roles for torsinA in these same processes. In support of this, a recent study showed that fibroblasts from torsinA knock-out mice migrate more slowly than controls in a polarized cell migration assay [15].
Although LINC complex proteins may help recruit torsinA to the NE, the subset that torsinA displaces (i.e. at least Sun2, nesprin-2G, and nesprin-3) seem unlikely to be directly responsible for retaining it there since torsinA remains in the NE even after these proteins are gone. Future work will need to explore possible roles for other NE proteins in this process, perhaps especially remaining LINC complex proteins such as Sun1 and the known torsinA binding partner LAP1.

The mechanism by which LULL1 induces torsinA retargeting is an area of ongoing interest. Here, we present several observations about torsinA that constrain the possible explanations. Using BN-PAGE (Figure 2-3) and FRAP (Figure 2-2), we established that torsinA assembles into a large, membrane associated oligomer – probably a hexamer – within the ER lumen. The oligomeric state of torsinA did not change following interaction with LULL1, and LULL1 remained highly mobile whether or not it was transiently engaging and affecting torsinA. These results are the first demonstration that torsinA indeed assembles into the kind of oligomer expected of an AAA+ protein, and establish that LULL1 changes torsinA targeting without affecting its fundamental structure. Our mutagenesis experiments indicate that an N-terminal hydrophobic sequence distinct from torsinA’s core AAA+ domain [13, 42] is required for this retargeting (Figure 2-4). It is therefore attractive to hypothesize that interaction with LULL1 causes a conformational change involving this N-terminal domain, enhancing torsinA’s affinity for something within the NE. Future work will focus on defining these states and further delineating the mechanism(s) responsible for controlling the transition between the ER-distributed and NE-enriched forms of torsinA.

Importantly, we found that DYT1-associated ΔGAG-torsinA is also redirected to the NE by LULL1 (Figures 2-1 & 2-8), but once there it is less effective at enacting changes in NE structure and protein composition (Figure 2-8). These results suggest a molecular loss-of-function that may correlate with the previously described inability of
ΔGAG-torsinA to rescue the lethality of torsinA knock-out in the mouse [5, 6]. These functional deficiencies could ultimately contribute to the development of DYT1 dystonia. The fact that torsinA is now established to be an oligomeric enzyme (Figure 2-3) supports the possibility that mixed oligomers containing wild-type and mutant subunits could turn a loss-of-function mutation into a dominantly inherited trait. Separately, data from other groups have shown that overexpressing ΔGAG-torsinA can have toxic effects on the function of the secretory pathway [43], raising the possibility that a combination of the loss-of-function shown here and gain-of-function shown elsewhere might explain the dominant inheritance of DYT1 dystonia. The discovery that LULL1 regulates the distribution and activity of torsinA within the ER and the NE paves the way for future exploration of how changes in its activity correlate with the development of disease.
References


Figure Legends

**Figure 2-1.** LULL1 promotes relocalization of TorsinA to the NE.

(A) Distribution of torsinA-mGFP, ΔGAG-torsinA-mGFP and LULL1-myc expressed individually in U2OS cells. (B) Distribution of co-expressed torsinA-mGFP and LULL1-myc, shown in maximum intensity projections (B, B'') and a confocal slice (B'). (C) Distribution of co-expressed ΔGAG-torsinA-mGFP and LULL1-myc. (D) Distribution of co-expressed Sec61γ-mGFP and LULL1-myc. (E) Distribution of co-expressed Sec61γ-mGFP and torsinA-myc. (A-C, E) are all maximum intensity projections of confocal z-series. (D) is an epifluorescence image. Scale bars, 10 μm.

**Figure 2-2.** Live imaging and FRAP analysis of LULL1-directed torsinA relocalization to the NE. (A) Selected images from time-lapse observation of torsinA-mGFP in cells expressing unlabelled LULL1-myc. (B) Selected images from zoomed-in portion of a field similar to (A). TorsinA-mGFP fills the NE over an average time of 45 minutes (+/- 26 min, n = 52 cells). (C) Time series showing FRAP of LULL1-mGFP in U2OS cells. (D) Comparable time series showing FRAP of torsinA-mGFP in U2OS cells. (E) Time series showing FRAP of torsinA-mGFP in a cell coexpressing LULL1-mCherry. (E') Corresponding time series showing FRAP of LULL1-mCherry in a cell expressing torsinA-mGFP. (F) Diffusion coefficient values for LULL1 and torsinA-mGFP, determined as described in [23, 47] from recovery curves such as those shown in Supplemental Figure S3. LULL1’s diffusion coefficient averages ~0.4 μm²/s in U2OS cells and in cells coexpressing torsinA-mGFP, while torsinA’s diffusion coefficient ranges between 0.09 and 0.13 μm²/s in both U2OS and LULL1-mCherry-expressing cells. For comparison, luminal ER-GFP has a $D_{eff}$ of ~10 um²/s [23], an ER-localized transmembrane protein has a $D_{eff}$ of ~0.4 um²/s [23], and a polysome-associated
translocon has a $D_{\text{eff}}$ of \(\sim 0.05 \text{ um}^2/\text{s}\) [48].

Scale bars, 10 \(\mu\text{m}\).

**Figure 2-3.** Blue Native PAGE separation of torsinA oligomers. (A) BN-PAGE separation of untagged TorsinA expressed in U2OS cells. TorsinA is detectable as a species of approximately hexameric size in 0.25% w/v DDM; this species is decreased in abundance in the presence of higher concentrations of DDM. (B) 2D PAGE of untagged torsinA expressed in U2OS cells. TorsinA was solubilized in 0.5% w/v digitonin, run on 1st dimension Blue Native PAGE, then separated in a second dimension by SDS-PAGE. (C) Blue Native PAGE separation of untagged torsinA expressed alone or with LULL1-myc, solubilized in 1% w/v digitonin.

**Figure 2-4.** Structural requirements for LULL1-directed torsinA redistribution. (A) Schematic of torsinA structure. (B) Representative epifluorescence images of torsinA-mGFP containing indicated mutation in LULL1-myc (not shown) expressing U2OS cells. Scale bars, 10 \(\mu\text{m}\). (C) Quantitation of redistribution. \(n>150\) cells for each mutant. (D) Immunoblots of equal fractions of aqueous and hydrophobic phases from a Triton-X114 phase partitioning assay show that wild type torsinA partitions with the hydrophobic phase, but shifts to the aqueous phase after deleting the protein's N-terminal hydrophobic domain (amino acids 26-43). (E) E171Q-torsinA-mGFP immunoprecipitates efficiently with LULL1-myc, and deletion of the N-terminal hydrophobic domain (amino acids 26-43) does not abolish binding.

**Figure 2-5.** Removing LULL1 by RNAi reverses enrichment of $\Delta$GAG-torsinA in NE. (A) Immunoblot of lysates from cells transduced with the indicated shRNA probed for
LULL1. (B and C) Representative confocal images of ΔGAG-torsinA-mGFP and ER-RFP coexpressed in control U2OS cells (B) vs. in U2OS cells transduced with shRNA #2088 (C). (D) Relative fluorescence intensity of ER-RFP, wt-torsinA-mGFP, and ΔGAG-torsinA-mGFP in NE vs. ER. ΔGAG-torsinA-mGFP has a significantly higher NE/ER ratio in U2OS cells than in LULL1 knockdown cells (p = 0.003). The NE/ER ratio of ΔGAG-torsinA-mGFP is not significantly different from that of ER-RFP in LULL1 knockdown cells (p = 0.19). n > 21 cells for each condition. Scale bars, 10 µm.

**Figure 2-6.** Redistributed torsinA displaces LINC complex components from the NE. (A) Nuclear pore components stained with mAb414 in cells expressing LULL1-myc (not shown) and torsinA-mGFP, epifluorescence image. (B) Emerin in cells expressing LULL1-myc (not shown) and torsinA-mGFP, epifluorescence image. (C) Sun1 in cells expressing LULL1-myc (not shown) and torsinA-mGFP. Shown are maximum intensity projections of a confocal z-series. (C’) XZ orthogonal view: top, torsinA-mGFP; bottom, Sun1. Approximate position of xz slice is marked by asterisk in (C). (D) Sun2 immunostaining in the same cells. 60% of cells containing torsinA-mGFP in the NE lack a nuclear rim of Sun2, compared to 4% of untransfected cells (n > 110 for each). (D’) XZ orthogonal view: top, torsinA-mGFP; bottom, Sun2. Approximate position of xz slice is marked by asterisk in (D). (E) Sun2-GFP and torsinA-TagRFP in cells also expressing LULL1-myc, projected z-series. (F) Nesprin2-Giant and torsinA-mGFP in cells also expressing LULL1-myc (not shown), imaged by epifluorescence microscopy. 56% of cells containing torsinA-mGFP in the NE lack NE-localized nesprin2-Giant, compared to 1% of untransfected cells (n > 35 for each). (G) Transfected nesprin3α-GFP in cells also expressing torsinA-TagRFP and LULL1-myc (not shown), projected z-series. Scale bars, 10 µm.
Figure 2-7. Sun2 protein levels are decreased as a consequence of LULL1-directed NE localization of torsinA. Western blot analysis of cells coexpressing LULL1-myc and torsinA-mGFP for 12 or 22 hours and sorted by GFP fluorescence indicates that Sun2 protein levels decrease, while NPCs (mAb 414) and a representative ER protein (Sec61β) are not affected.

Figure 2-8. Comparison of wild-type and ΔGAG-torsinA-mGFP effects on the NE. (A) Comparison of NE in cells expressing LULL1-myc (not shown) with redistributed wild-type torsinA-mGFP (left) and ΔGAG-torsinA-mGFP (right). NE membrane distortions occur in 68% of cells with NE-localized wild-type torsinA (n = 136) and in 7% of cells with NE-localized ΔGAG-torsinA (n = 74). Shown are maximum intensity projections of confocal z-series, with orthogonal xz views in (A'). Approximate position of xz orthogonal slice marked by asterisk in (A). (B) Effect of LULL1-redistributed ΔGAG-torsinA-mGFP on Sun2. Projected z-series, with orthogonal views of ΔGAG-torsinA (top) and Sun2 (bottom) in (B'). Approximate position of xz slice is marked by asterisk in (B). (C) Time-lapse imaging of Sun2-GFP and torsinA-TagRFP in LULL1-myc expressing cells. (D) Time-lapse imaging of Sun2-GFP and ΔGAG-torsinA-TagRFP in LULL1-myc expressing cells. The median delay between the onset of torsinA-TagRFP redistribution and initiation of Sun2-GFP loss was <15 min (between consecutive frames) for wild type (+/-20 min, n = 32 cells) and 420 min for ΔGAG-TorsinA (+/-378 min, n = 33 cells). Scale bars, 10 µm.

Figure 2-9. Model of LULL1-dependent torsinA function at the NE. LULL1 interacts with the torsinA hexamer in the peripheral ER, which promotes an activating change in
torsinA that requires catalytic residues and association with the membrane. TorsinA then moves into the NE, where it displaces components of LINC complexes, including Sun2, nesprin2-Giant, and nesprin3. This gives torsinA the ability to alter contacts between the nuclear lamina (or nuclear contents), the nuclear envelope membranes, and the cytoskeleton.

**Supplemental Figure Legends**

**Figure 2-S1.** Differently tagged torsinAs can redistribute to the NE, and differently tagged LULL1s can target TorsinA to the NE. (A) Untagged, myc-tagged, and TagRFP-tagged versions of torsinA all redistribute to the NE when coexpressed with LULL1-myc. (B) LULL1-mGFP also sends torsinA-myc to the NE. Scale bars, 10 µm.

**Figure 2-S2.** A wide expression range of LULL1 is capable of inducing redistribution of transiently transfected torsinA. (A-C) LULL1-myc is expressed in a tetracycline-responsive manner over a wide range of tetracycline dosage. Low (A, 1 ng/mL tet), intermediate (B, 10 ng/mL tet) and high (C, 1 µg/mL tet) levels of LULL1 expression are all capable of causing redistribution of coexpressed torsinA-mGFO to the NE. (D) Western blot comparison of overexpressed myc-tagged LULL1 (upper band) to endogenous LULL1 (lower band) expression in U2OS cells. LULL1 is expressed at low levels in U2OS cells; induction of LULL1-myc to ~5 times endogenous expression levels (with 1 ng/mL tet) is capable of inducing torsinA redistribution to the NE.

**Figure 2-S3.** ΔGAG-torsinA-mGFP enriches in the NE over time when coexpressed with LULL1-myc, but not when expressed alone in U2OS cells. (A) Time lapse live imaging of ΔGAG-torsinA-mGFP coexpressed with LULL1-myc and (B) imaging of ΔGAG-torsinA-
mGFP expressed alone. In both, fluorescent protein signal increases over the imaging period, but partitioning into the NE increases over time in (A) but not in (B). Thus, ΔGAG-torsinA-mGFP responds to the presence of LULL1 with a time-dependent enrichment in the NE.

**Figure 2-S4.** FRAP parameters for LULL1-mGFP and torsinA-mGFP. (A)
Representative fluorescence recovery curves for LULL1-mGFP in U2OS cells (“LULL”), torsinA-mGFP in U2OS cells (“torsinA”), and torsinA-mGFP in LULL1-mCherry-expressing cells (“torsinA+LULL”). TorsinA recovers to a much lesser extent than LULL1.

**Figure 2-S5.** Sun2 is only displaced from the NE by coexpressed LULL1 and torsinA. (A-C) Sun2’s NE localization is not changed by torsinA-myc expression (A), E171Q-torsinA-myc expression (B), or ΔGAG-torsinA-myc expression (C). Compare to displacement of Sun2 caused by LULL1-myc and torsinA-mGFP in Figure 5.
Figure 2-1
Figure 2-2

A. 2 hours post transfection

B. 7 hours post transfection

C. LULL1-mGFP

D. TorA-mGFP

E. TorA-mGFP + LULL1-mCherry

F. Diffusion coefficients (D) for different treatments
Figure 2-4

A.

Δ26-43  K108A  E171Q  Δ302/303

1  21  70  332

- signal sequence
- hydrophobic domain
- AAA+ domain

B.

wtA  E171Q  K108A  Δ26-43

C.

% cells with TorA in NE

D.

Aqueous  Hydrophobic

TorA mGFP  Δ26-43 TorA mGFP

E.

lysate  unbound  IP x 5

IB: E171Q-TorA-mGFP
IB: E171Q/Δ26-43-TorA-mGFP
IP: LULL1-myc
Figure 2-5

A. LULL1 shRNAs

B. ΔGAG-mGFP  ER-RFP  merge

C. U2OS

D. ER-RFP  WTA-mGFP  ΔGAG-mGFP

NE/ER Intensity Ratio

U2OS 2088 U2OS 2088

ER-RFP  wtA-mGFP  ΔGAG-mGFP
Figure 2-6

A. TorA-mGFP  mAb414  merge

B. TorA-mGFP  Sun1  merge

C. TorA-mGFP  Sun2  merge

D. Sun2-GFP  TorA-RFP  merge

E. TorA-mGFP  nesprin2G  merge

F. nesprin3α-GFP  TorA-RFP  merge
Figure 2-7

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- Sun2
- p153
- p116
- p62
- Sec61β
- myc
- GFP
Figure 2-8

A. TorA-mGFP  ΔGAG-mGFP

B. ΔGAG-mGFP  Sun2  merge

C. Sun2-GFP

D. ΔGAG-TorA-RFP

Time points: 0 min, 15 min, 45 min, 60 min, 90 min, 120 min, 15 min, 45 min, 115 min, 205 min, 385 min, 415 min
Figure 2-9

LULL1

TorA

intact LINC

destabilized LINC

ER

ONM

INM

lamina

cytoskeleton

69
Figure 2-S1

A. In U2OS + LULL1-myc

B. In U2OS

Figure 2-S2

A. B. C. D.

1 ug/mL tet 10 ng/mL tet 1 ng/mL tet

1 ug/mL 10 ng/mL 1 ng/mL

U2OS
tetracycline

LULL1-myc
endogenous
LULL1
Figure 2-S3

A. $\Delta$GAG-TorA in U2OS + LULL1-myc

B. $\Delta$GAG-TorA in U2OS
Figure 2-S4

Figure 2-S5
Chapter 3

Static retention of the lumenal monotopic membrane protein torsinA in the endoplasmic reticulum
Acknowledgments

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Abstract
TorsinA is a membrane-associated enzyme in the endoplasmic reticulum (ER) lumen that is mutated in DYT1 dystonia. How it remains in the ER has been unclear. We report that a hydrophobic N terminal domain (NTD) directs static retention of torsinA within the ER by excluding it from ER exit sites, as has been previously reported for short transmembrane domains (TMDs). We show that despite the NTD’s physicochemical similarity to TMDs, it does not traverse the membrane, defining torsinA as a luminal monotopic membrane protein and requiring a new paradigm to explain retention. ER retention and membrane association are perturbed by a subset of non-conservative mutations to the NTD, suggesting that a helical structure with defined orientation in the membrane is required. TorsinA preferentially enriches in ER sheets, as might be expected for a luminal monotopic membrane protein. We propose that the principle of membrane-based protein sorting extends to monotopic membrane proteins, and identify other proteins including the monotopic luminal enzyme cyclooxygenase 1 (prostaglandin H synthase 1) that share this mechanism of retention with torsinA.
Introduction

Early-onset (DYT1) torsion dystonia is a neurological movement disorder [1] caused by a glutamic acid deletion (ΔE) in the catalytic domain of torsinA [2]. TorsinA is an AAA+ ATPase of the endoplasmic reticulum (ER) and contiguous nuclear envelope (NE). The specific cellular functions ascribed to torsinA vary widely despite the fact that it has been a decade since the protein was first described and linked to dystonia [3]. Based on its membership in the AAA+ family of ATPases [2, 4], it is likely that torsinA disassembles or changes the conformation of a protein or protein complex in the ER or NE. The ΔE mutation is thought to compromise this function [5, 6].

TorsinA is targeted to the ER lumen by an N-terminal signal peptide. Analyses of torsinA’s subcellular localization, processing, and glycosylation show that the signal peptide is cleaved and the mature protein resides in the lumen of the ER [7-9] where it is a stable protein [10] [11]. TorsinA’s binding partners include the transmembrane proteins LULL1 in the ER and LAP1 in the NE [12] [13]. Abnormalities in NE structure [6, 14] and effects on NE-localized LINC complex proteins [15] when torsinA levels are perturbed suggest an important function for this enzyme specifically at the NE. Other studies point to additional functions elsewhere in the ER [16].

The steady state localization of torsinA in the ER and NE demands that it escapes forward flux out of the ER into the secretory pathway. This is a significant issue for all ER resident proteins, and the underlying mechanisms are what define the composition of the ER. Proteins intended for efficient secretion are concentrated in nascent COPII vesicles at ER exit sites (ERES) by specific interactions with subunits of the COPII coat or, in the case of luminal proteins, with transmembrane receptors that in turn interact with COPII subunits [17]. However, proteins without specific export signals also leave the ER in COPII vesicles at a rate referred to as bulk flow. For soluble
proteins in the ER lumen, recent quantitative measurements indicate that bulk flow empties the equivalent of half of the lumenal volume every 40 minutes [18]. Proteins that leave the ER by bulk flow may return to the ER in COPI vesicles if they are recognized by a recycling receptor. The prototype for this is the KDEL receptor that recycles lumenal proteins with the tetrapeptide KDEL at their C-terminus [19]. For membrane proteins there is less quantitative information about the rate of bulk flow. There are some membrane proteins that never leave the ER and are statically retained by their TMD [20-25]. This retention is attributed to preferential partitioning of short TMDs into the thinner and less ordered membrane of the ER, and the best characterized of these TMDs has been shown to partition differentially among subdomains of the ER [24]. However, that there is a bulk flow of membrane proteins is clear from the need for the TMD-specific recycling receptor, Rer1 [26], and from studies showing that small increases in the hydrophobicity of ER-retained TMDs allow their escape from the ER [22-25].

How does torsinA achieve its localization to the ER? An early study indicated that a hydrophobic domain at the N terminus of the protein was required for ER localization of human torsinA in heterologous cells [8]. However, the mechanism underlying localization was unclear as neither the N-terminal domain nor other regions of the torsinA sequence contain any canonical targeting motifs. A subsequent proposal was that torsinA remains in the ER lumen because of protein-protein interactions with other resident proteins [27]. The facts that the N terminus is not involved in interactions with known abundant binding partners including LULL1 in the ER and LAP1 in the NE [28], and that even highly overexpressed torsinA remains in the ER [7], suggest that this is unlikely. These discrepancies raise the question of whether a previously unknown mechanism might be responsible for keeping torsinA and similar proteins in the ER. In this study, we provide evidence that torsinA’s N-terminal domain is a monotopic membrane associating domain that is directly responsible for static retention in the ER.
lumen. Further, we identify other membrane proteins that appear to behave similarly, providing new insight into protein sorting in the early secretory pathway.
Methods

Plasmids

TorsinA-mGFP and Δ26-43-torsinA-mGFP are as described previously [28]. (1-67), (1-43), and (1-25) torsinA-mGFP were made by PCR amplification of the indicated sequences with primers containing XhoI and EcoRI sites, followed by ligation into the mGFP-containing pEGFP-N1 vector. All NTD substitution and insertion mutations were introduced by QuikChange mutagenesis. MBP-(21-43) and MBP-(21-67) were made by PCR amplification of the corresponding sequence with primers containing EcoRI and BamH1 restriction sites, followed by ligation into pMAL-c (NEB). The Lep reporter vector, Lep 13A/6L, and 18A/1L constructs were generously provided by Gunnar von Heijne (Stockholm University). The NTD was inserted into the Lep reporter vector as described [29]: oligonucleotides encoding residues 21-43 of human torsinA in forward (N_lum-C_cyto orientation) or reverse (N_cyto-C_lum orientation) sequence were annealed together and ligated into Lep vector that had been digested with Spel and KpnI. The COX-1 expression vector was provided by Robert J. Kulmacz (University of Texas, Houston). VSVG-(ts045)-GFP [30] and NPY-GFP [31] were as described.

Reagents

A peptide consisting of residues 21-43 of human torsinA, an aminohexanoic acid linker and a biotin tag was synthesized and verified by mass spectrometry by the W.M. Keck Foundation Biotechnology Resource Laboratory (Yale University). 7-methyl diguanosine triphosphate cap structure analog and amylose resin were from NEB (Ipswich, MA). Rabbit reticulocyte lysate, canine rough microsomes, and NTPs were from Promega (Madison, WI). Complete protease inhibitor cocktail was from Boehringer (Ridgefield, CT). Protein G Sepharose was from GE/Amersham (Piscataway, NJ). Antibodies used include: mouse monoclonal anti-bCOP (clone maD, Sigma), mouse monoclonal anti-
Sec31A (BD Biosciences, San Jose, CA), mouse monoclonal anti-GFP (clone B-2, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-GFP [32], rabbit polyclonal anti-His (Cell Signaling, Beverley, MA), mouse monoclonal ERGIC-53 (Alexis Biochemicals, San Diego, CA), and rabbit polyclonal anti-COX-1 (Cayman Chemical, Ann Arbor, MI). All other chemicals were from Sigma (St. Louis, MO).

Cell culture

U2OS cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and L-glutamine. Transient transfections were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

Immunofluorescence

For temperature block experiments, coverslips were transferred to HEPES-buffered media supplemented with 10% FBS and L-glutamine, and incubated for 2 h in a water bath set to 10°C or 15°C. For BFA treatment, cells were transfected approximately 16 h before adding 1 mM BFA to the medium for 4 h. Cells were washed in PBS and then fixed with 4% paraformaldehyde in PBS for 10 min, followed by permeabilization in 0.2% Triton-X-100 for 10 min. Coverslips were blocked in 2% goat serum in PBS for 1 h before incubation with primary and Alexa Fluor-conjugated secondary antibodies. For selective permeabilization with digitonin, cells were washed once in PBS before transfer to 0.0025% digitonin in PBS for 5 min at 4°C. Cells were then fixed, blocked, and stained as described above. Coverslips were mounted in Mowiol (Calbiochem, San Diego, CA). Epifluorescence imaging was performed with a Diaplan microscope (Leica Microsystems, Bannockburn, IL) using a 63 x 1.4 NA objective and a Zeiss MRm camera (Thornwood, NY). Confocal imaging was performed on an Olympus FV500 microscope.
using a 60 x 1.4 NA objective. Brightness and contrast were adjusted with Adobe Photoshop (Adobe Systems, San Jose, CA) and composite figures were prepared in Adobe Illustrator.

**Quantifying colocalization**

To monitor localization of candidate cargo proteins to ER exit sites, cells expressing the indicated candidate proteins were costained for Sec31A and viewed by confocal microscopy. Colocalization analysis was restricted to ERES as described [33]. Briefly, a binary mask of the Sec31A signal was created, manually thresholded, and added as an additional channel in the RGB image. Colocalization was then quantified in the regions defined by the mask, and expressed as Pearson’s correlation coefficient. Colocalization was analyzed in >20 cells for each condition reported. Statistical comparison of data sets was performed by t-test.

**Immunoprecipitation from culture medium**

60-mm plates of U2OS cells were transiently transfected with the indicated plasmids and incubated for ~16 h. For experiments involving BFA treatment, fresh media was added with or without 1 mM BFA and cells were incubated for an additional 6 h. The media was then collected, placed on ice, and supplemented with 1 mM PMSF, 1x complete protease inhibitors, and 5 mM EDTA. The media was centrifuged at 4000 rpm for 10 min at 4°C to remove cell debris. Protein-G-sepharose beads covalently conjugated to affinity purified rabbit anti-GFP antibody were then added to the supernatant and incubated 2h at 4°C. The beads were then pelleted and washed 3 x in PBS/0.5% Triton-X-100 with PMSF and protease inhibitors, then boiled in 50 uL SDS-PAGE sample buffer for analysis. The cells, meanwhile, were scraped and pelleted in PBS and solubilized in
~300 ul PBS/0.5% Triton-X-100 with PMSF and protease inhibitors for 30 min at 4°C. The insoluble material was pelleted at 15,000 x g for 10 min at 4°C. An aliquot of the supernatant was boiled in SDS-PAGE sample buffer. 15 ul each of the cell lysate and immunoprecipitate were separated on SDS-PAGE and analyzed by immunoblotting.

**Far UV circular dichroism**

Far UV CD spectra were recorded using a 0.2 mm pathlength cuvette in a Jasco J715 spectropolarimeter at ambient temperature, scanning from 260 to 190 nm in 0.2 nm steps at 100 nm/min, averaging 5 spectra per condition. Peptide was diluted to 1 mg/ml in 10 mM sodium phosphate buffer, pH 7.0 supplemented with trifluoroethanol (Sigma) or SDS as indicated. Solvent spectra gave negligible signal and were subtracted from sample spectra. Data shown are expressed as mean residue ellipticity (deg cm²/dmol). The percentage of α-helix was estimated from the molar ellipticity at 222 nm ($\theta_{222}$) using the equation $f_h = (\theta_{222}/\theta_{222a}) + (i\kappa/N)$ where $f_h$ is the fraction in the α-helical form, $\theta_{222}$ is the mean residue ellipticity at 222 nm, $\theta_{222a}$ is the molar ellipticity at 222 nm for an infinitely long α-helix (-39,500 deg cm²/dmol), $i$ is the number of helices (assumed to be one), $\kappa$ is a wavelength specific constant (2.6 at 222 nm), and $N$ is the number of peptide bonds in the peptide (defined as 26 for the 23 residues of torsinA plus aminohexanoic acid linker and biotin) [34].

**Purification of MBP fusion proteins**

BL21(DE3) *Escherichia coli* transformed with MBP, MBP-21-43, or MBP-21-67 were grown in 0.5 liters of Terrific Broth and induced to express protein by addition of isopropyl 1-thio-β-D-galactopyranoside and shaking for 3 hours at room temperature. After pelleting, bacteria were lysed in 20 mM Tris pH 7.4, 200 mM NaCl, 1 mM EDTA,
5% glycerol, 1 mM PMSF by sonication. N-octyl glucoside was added to 1% w/v and samples were incubated for 30 min at 4°C, followed by centrifugation at 9000 x g for 20 min at 4°C. Amylose resin was added to the supernatant and incubated overnight at 4°C. Unbound material was removed by washing in lysis buffer, and proteins were eluted in lysis buffer supplemented with 10 mM maltose. MBP-21-67 was incubated sequentially with 10 mM DTT (30 min at room temperature) and 50 mM NEM (30 min at room temperature) to reduce and alkylate cysteines. For use in proteoliposome preparations, proteins were dialyzed into 10 mM sodium phosphate pH 7.0, 150 mM NaCl, 30 mM n-octyl glucoside overnight at 4°C. Proteins were clarified by centrifugation at 200,000 x g for 1 hr at 4°C before use in proteoliposome preparations. Protein concentrations were quantitated by Bradford assay with BSA as standard and snap frozen.

**Proteoliposome preparation and characterization**

1,2-dioleoyl-sn-glycerol-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids (Alabaster, AL). 60 µg of DOPC in chloroform was dried under a stream of nitrogen and then under vacuum for several hours. The lipid film was resuspended in 1 mL of 10 mM sodium phosphate, pH 7.0, 150 mM NaCl, and 30 mM n-octyl glucoside, followed by addition of MBP fusion proteins for a final protein:lipid ratio of 2:1 by weight. Liposomes were generated by removing detergent by dialysis at 37°C in 2000 Da molecular weight cutoff dialysis cassettes (Pierce) against 3 x 1 L changes of buffer lacking detergent for 48 hours. To determine how much protein was associated with liposomes, samples were loaded on the bottom of a sucrose cushion and centrifuged to separate liposomes from unincorporated protein by flotation. A 150 µL aliquot of the proteoliposome preparation was mixed with 100 µL 2.2 M sucrose/10 mM sodium phosphate pH 7.0/150 mM NaCl in a polycarbonate centrifuge tube. This mixture was
overlaid with a 200 µL cushion of 0.75 M sucrose/10 mM sodium phosphate pH 7.0/150 mM NaCl, then with a 50 µL layer of buffer lacking sucrose. The step gradients were centrifuged for 80 min at 240,000 g (55,000 rpm) in a Beckman TLS 55 rotor. Three fractions were collected from the bottom using a syringe (250 µL, 150 µL, and 100 µL). Aliquots of each fraction were analyzed by SDS-PAGE and Coomassie stain.

**In vitro translation**

Template was generated by amplifying the coding region of interest using a 5’ primer that contains the T7 promoter sequence and a 3’ primer that anneals just beyond the stop codon. The resulting product was purified using the Qiagen PCR purification kit and eluted in 50 µL water. 2 µL of the product was then mixed with 0.2 µL T7 RNA Polymerase (Promega), 0.2 µL water, and 7.6 µL T1 mix (final concentration 40 mM HEPES, pH 7.6, 6 mM MgCl2, 2 mM spermidine, 10 mM DTT, 500 µM ATP, 500 µM UTP, 500 µM CTP, 100 µM GTP, and 500 µM 7-methyl diguanosine triphosphate). This transcription mix was incubated for 1 h at 37°C, then RNA was precipitated by adding 1.5 µL 5 M NaCl and 24 µL of 100% ethanol and incubating at -20°C overnight. The RNA was pelleted at 15,000 x g for 30 min at 4°C and resuspended in 10 µL of nuclease-free water. In vitro translation reactions were then set up as follows: 12.5 µL rabbit reticulocyte lysate, 0.5 µL TnT reaction buffer (Promega), 0.5 µL amino acids – Met (Promega), 1 µL 35S-methionine (Perkin Elmer), 5 µL of the freshly prepared transcript, 2 µl canine rough microsomes, and water to a 25 µL final reaction volume. Translation reactions were incubated for 1 h at 30°C. The reaction was stopped by boiling in SDS-PAGE sample buffer; samples were then separated by SDSPAGE and detected by autoradiography.
Sorting proteomics data

[35] identified 1430 proteins of the secretory pathway proteome by mass spectrometry analysis of rat liver rough microsome (RM), smooth microsome (SM), Golgi, and COPI vesicle fractions. To identify ER resident proteins similar to torsinA, these 1430 proteins were sorted using the following criteria: (1) presence of a predicted cleavable signal peptide (Gilchrist et al Table S1C); (2) similar biochemical behavior to torsinA in salt extraction and Triton X-114 partitioning experiments (Gilchrist et al Table S3B); and (3) enrichment in rough microsomal and smooth microsomal fractions over Golgi and COPI fractions (Gilchrist et al Table S3D). Approximately 150 proteins including torsinA matched these criteria. Literature and database searches were then used to eliminate proteins known to be localized to different compartments and identify proteins for which the cell biological data are consistent with a lumenal orientation in the ER, and for which other established ER retention or retrieval mechanisms either do not exist or do not fully explain sorting behavior. Selected proteins of interest from this set are highlighted in Table 1.

Transmembrane segment prediction

To explore the potential for hydrophobic sequences in torsinA to adopt a TMD configuration, the full sequence of human torsinA was analyzed in the MPEx program (http://blanco.biomol.uci.edu/mpex/) [36] in biological hydrophobicity scale mode (“translocon TM analysis”). The predicted ΔG for membrane insertion (ΔGpred) was calculated for the MPEx-identified protein sequence and mutants to that region using the dGpred prediction algorithm (http://dgpred.cbr.su.se/) [37]; these values are reported in Figure 5A.
Triton-X-114 phase separation and Fluorescence recovery after photobleaching (FRAP) were performed as described in [28]. SDS-PAGE and Immunoblotting were performed as described [32].

Linescan analysis

To analyze ER subdomain preference, cells expressing the indicated proteins were fixed and costained for CRT as a bulk ER marker. The distribution of red and green fluorescence was analyzed by linescan in Metamorph. Two lines per cell were drawn from the nuclear periphery to the cell edge, and a line in a cell-free region of the coverslip was drawn for background measurement. The average background intensities were subtracted and the intensities normalized to 1 before replotting the data in Microsoft Excel.
Results

TorsinA is a life-long ER resident

While all transmembrane and secreted proteins are transiently found in the ER, proteins that reside in the ER either never leave and are said to be statically retained or are retrieved from the ER-to-Golgi intermediate compartment (ERGIC) by COPI-mediated retrograde transport. Both are commonly referred to as ER retention mechanisms. To determine how torsinA remains in the ER, we incubated cells expressing mGFP-tagged torsinA or cargo proteins at temperatures that selectively block different trafficking steps. At 15°C, COPII-mediated transport from the ER proceeds but recycling and forward transport from the ERGIC do not, causing all proteins that leave the ER to accumulate in the ERGIC [38] while statically retained ER proteins are unchanged. At 10°C, COPII components assemble together with cargo at ER exit sites (ERES) but do not generate free vesicles [39-41]. VSVG-(ts045)-mGFP, a plasma membrane-targeted transmembrane protein that directly interacts with COPII, accumulated dramatically with the COPI component βCOP in the ERGIC (Figure 3-1A). TorsinA-mGFP, in contrast, remained distributed throughout the ER and was absent from the ERGIC after incubation at 15°C (Figure 3-1B). After incubation at 10°C, GFP fusions of VSVG-(ts045), the secreted neuropeptide Y (NPY), and ER-GFP appeared at ERES marked by the COPII component Sec31A, but torsinA-mGFP did not (Figure 3-2A-D). Quantitating colocalization confirmed that VSVG-(ts045), NPY, and ER-GFP are present in ERES at 10°C, while torsinA is not (Figure 3-2E). Notably, the large increase in colocalization of VSVG-(ts045) with Sec31A at 10°C vs. 39.5°C is because VSVG-(ts045) is a temperature sensitive mutant protein that misfolds at 39.5°C and interacts with folding chaperones that keep it away from ERES; once folded at a permissive temperature its diacidic COPII interacting motif promotes rapid accumulation at ERES [40]. The fact that
torsinA avoids ERES to an extent comparable to misfolded VSVG, while ER-GFP is readily detectable in ERES despite the lack of a forward transport signal, suggests specific exclusion of torsinA from ERES. This exclusion is similar to that previously described for short TMD-containing proteins (Ronchi et al., 2008). Finally, overexpressed torsinA-mGFP is efficiently retained in the ER at physiological temperature (Figure 3-1B) and is completely EndoH sensitive [7], implying that torsinA rarely leaves the ER. Altogether, these data indicate that torsinA is statically retained in the ER.

An N-terminal domain (NTD) directs ER retention

Mature torsinA consists of a hydrophobic N-terminal domain (NTD), a short linker region, and the AAA+ domain (Figure 3-3A). Building on an earlier report showing that deleting the NTD led to secretion of human torsinA from Drosophila S2 cells [8], we deleted residues 26-43 from human torsinA and found that the mutant protein appeared in the Golgi (Figure 3-3B) and in the cell medium (Figure 3-3C) when expressed in human U2OS cells. Both changes were blocked by brefeldin A (BFA), indicating that without its NTD, torsinA traffics through the classical secretory pathway. Deletion of the N terminus allows torsinA to access ERES to an extent comparable to the lumenal marker ER-GFP (Figure 3-4), consistent with truncated torsinA exiting the ER by bulk flow transport.

While the deletion led to secretion of all torsinA variants analyzed (Figure 3-3C), changes in intracellular distribution were most apparent in cells expressing the Δ26-43 deletion in combination with an E171Q mutation in the AAA+ domain. A likely explanation for this difference is that the E171Q mutant is trapped in its ATP-bound state [14] and may therefore be more conformationally stable than the wild-type enzyme, which can cycle through different nucleotide states. In support of this, OOC-5, the C. elegans ortholog of torsinA, is more thermostable in the presence of nucleotide [42] and
protein stability often correlates with the efficiency of secretion [43].

To determine whether the NTD is sufficient for ER retention or whether the AAA+ domain is also required, we attached torsinA’s signal sequence (1-25), the signal sequence plus the NTD (1-43), or the entire pre-AAA sequence (1-67) to mGFP. (1-43)-mGFP and (1-67)-mGFP localized to the ER while (1-25)-mGFP was present in the Golgi (Figure 3-3D). Furthermore, only (1-25)-mGFP was detectable in the cell medium (Figure 3-3E). The NTD is thus both necessary and sufficient for ER retention, and determining why it stays in the ER should delineate the mechanism controlling torsinA localization.

**Physical properties of the NTD**

The NTD contains a pattern of hydrophobic and nonpolar residues that is well conserved among torsinA orthologs (Figure 3-5A), suggestive of a membrane-associating domain. As previously shown, torsinA partitions almost completely into the hydrophobic phase in Triton X-114 phase separation experiments; this is reversed by deleting the NTD (Figure 3-5B) [8, 28]. The NTD alone controls this partitioning (Figure 3-5B) providing hydrophobic behavior typical of a transmembrane domain (TMD) [44]. To determine whether cellular factors or modifications contribute to this hydrophobicity, we purified the NTD fused to maltose binding protein from *E. coli*. Residues 21-43 or 21-67 of torsinA shifted a fraction of this 45 kDa soluble protein into the hydrophobic phase (Figure 3-5C), confirming that hydrophobicity is intrinsic to the NTD and is not the result of post-translational modification or binding to another protein.

Since α-helices are the predominant secondary structure found in membrane-associating domains [45], we analyzed the secondary structure of torsinA’s NTD. Far UV circular dichroism shows that a synthetic peptide corresponding to residues 21-43 of torsinA is unstructured in aqueous buffer, but becomes partially helical as
trifluoroethanol (TFE) is added (Figure 3-5E). To test whether helicity also increases in a membranous environment, we added detergent. In the presence of 20 mM SDS, the NTD peptide became as helical as in 50% TFE (Figure 3-5E). This stabilization of NTD structure in detergent micelles implies that the peptide may also be helical in the lipid bilayer [46].

The NTD associates directly and stably with membranes

To study the behavior of the NTD in cellular membranes, we analyzed the diffusion of an NTD-mGFP fusion protein by fluorescence recovery after photobleaching (FRAP) [47]. While free GFP in the ER lumen has a diffusion coefficient ($D_{\text{eff}}$) of 7.67 $\pm$ 0.44 $\mu$m$^2$/sec, (1-67)-mGFP diffuses at least an order of magnitude slower (0.69 $\pm$ 0.07 $\mu$m$^2$/sec) (Figure 3-5D). Single-pass TMD proteins of the ER have a similar $D_{\text{eff}}$ [47]. The NTD’s behavior in cellular membranes is thus most comparable to that of a membrane protein.

To determine whether the NTD associates directly with membranes, we asked whether residues 21-67 of torsinA fused to MBP could be co-reconstituted into proteoliposomes with phosphatidylcholine, which is a major component of the ER membrane [48]. We combined protein with 1,2-dioleoyl-sn-glycerol-3-phosphocholine (DOPC) lipid in the presence of n-octyl glucoside, then generated liposomes by dialyzing away the detergent. Liposomes were separated from soluble protein by flotation through a sucrose step gradient. The MBP-NTD fusion protein floated to the top of a sucrose gradient with the lipids, while the MBP protein remained at the bottom (Figure 3-5F). This experiment shows that the NTD associates directly with membranes in the absence of other proteins or modifications, and thus has the ability to mediate stable and direct association with the ER membrane.
The NTD is a monotopic membrane interacting domain

Proteins known to be statically retained in the ER appear to rely on preferential partitioning of short and/or polar TMDs into the thinner and less ordered ER bilayer [23-25]. We have established that torsinA’s NTD is necessary and sufficient for ER retention and associates directly with membranes. We do not, however, know how the NTD is positioned in the membrane. While originally thought to be a transmembrane protein [8, 49], torsinA has more recently been termed a peripheral membrane protein based on partial extraction from membranes by alkaline wash and modification of a glycosylation site inserted into the NTD [27]. However, bona fide TMD proteins can be sensitive to alkaline extraction [50] and inserting an Asn residue could itself perturb handling of a TMD by the translocon [37]. Indeed, based on a biologically based TMD prediction algorithm [36], torsinA’s NTD contains a 23-residue sequence (Ile24 - Phe46) that might traverse the bilayer (Figure 3-7A below). Because both torsinA’s ER retention (Figure 3-3) and LULL1-dependent NE targeting [28] rely on the NTD, we set out to definitively assess its membrane topology.

The bulk of torsinA’s sequence is in the lumen of the ER, as demonstrated by its protection from protease digestion [7, 9]. Importantly, if the NTD were to function as a stop-transfer sequence, the protein’s C terminus would be in the cytosol, the reverse of what is seen in cells. However, the unusually close apposition of torsinA’s signal peptide and NTD could influence topogenesis in more complex ways [51]. In addition, there are examples of post-translational insertion of hydrophobic domains across the membrane, particularly when that domain falls at a protein terminus [52]. We therefore wanted to directly test whether torsinA’s N terminus is exposed to the cytosol. To do this, we asked whether a polyhistidine tag inserted between the signal peptide and the NTD (Figure 3-6A) is accessible from the cytosol. We verified that the epitope tag did not disrupt
lumenal targeting (Figure 3-6B) and confirmed that selective permeabilization with
digitonin allows detection of the cytosolic Sec31A epitope (Figure 3-6C) but not of the
lumenal PDI epitope (Figure 3-6D). We found that the His tag was accessible to antibody
when membranes were fully permeabilized with Triton-X-100 (Figure 3-6E), but was
inaccessible when only the plasma membrane was permeabilized with digitonin (Figure
3-6F). To test whether the NTD is read as a TMD when not adjacent to torsinA’s signal
peptide, we inserted residues 21-43 of torsinA into a TMD reporter construct [53] and
assessed whether the NTD integrates into the membrane or translocates into the lumen
by monitoring glycosylation after \textit{in vitro} translation (Figure 3-S1). Comparing the
behavior of the NTD to test segments that do (Lep 13A/6L) or do not (Lep 18A/1L) insert
into the ER membrane [29, 37] shows that the NTD is most similar to the latter segment
(Figure 3-S1). These results suggest that in any context, torsinA’s NTD does not
traverse the bilayer, but rather associates with the lumenal side of the ER membrane.
Together with its direct membrane association demonstrated above, these experiments
define the NTD as a monotopic membrane interacting domain.

\textit{Mutations to the NTD have distinct effects on topology and ER retention}

Given that the NTD is not behaving as a TMD, we wondered what distinguishes it from a
TMD and as a TMD, how it would be sorted within the secretory pathway. Energetic
predictions suggest that small changes to the sequence will decrease the energy barrier
to membrane insertion (Figure 3-7A); we increased hydrophobicity either by inserting the
sequence LALALA between Ala 31 and Gly 32, or by replacing polar residues in the
NTD itself with Leu. Both inserting the LALALA sequence and replacing two or more
polar residues with Leu caused the domain to be read as a stop-transfer sequence,
monitored by cytosolic exposure of the C-terminal GFP tag to antibody (Figure 3-7B-F).
This shows that the NTD is close to the edge of recognition as a type I TMD.
Significantly, there were noticeable differences in the steady state subcellular distribution of these hydrophobic mutant proteins. The LALALA and 5 x Leu mutants clearly accumulated in a perinuclear region suggestive of the Golgi in many cells (Figure 3-7E, 6F). Upon closer inspection, (1-67)-LALALA-GFP, (1-67)-5 x Leu-GFP, and (1-67)-3 x Leu-GFP all partially colocalize with the intermediate compartment marker ERGIC-53 (Figure 3-S2). In contrast, the 2 x Leu construct did not colocalize with ERGIC-53 (Figure 3-S2) despite its transmembrane orientation (Figure 3-7C). Thus, the small increase in hydrophobicity created by two leucine substitutions promotes integration as a TMD optimized for retention in the ER, while further hydrophobic substitution or extension of the sequence allows the same TMD to escape to post-ER compartments. These data indicate that both topology and sorting of torsinA is affected by changes in NTD amino acid composition. The changes in sorting once the NTD becomes a TMD parallel those previously reported when the hydrophobicity of other ER transmembrane domains was increased [22-25].

**A map of the orientation of the NTD in the ER bilayer**

We next performed scanning mutagenesis along the NTD to explore its orientation with respect to the membrane. Ala or Gly substitutions had no effect on targeting (Figure 3-S4A) or topology (not shown) of torsinA. We therefore introduced Arg residues, which are typically tolerated on the surface and in interfacial regions of the bilayer but not in the hydrophobic core [37]. We analyzed the effects of these Arg substitutions on E171Q-torsinA’s steady state localization, secretion, and diffusion in cellular membranes. Arg substitutions at positions 24, 28, 29, 31, and 38 had little to no effect on ER localization (Figure 3-8B and Figure 3-S3B), retention (Figure 3-8C and Figure 3-S5), or diffusive behavior in cells (Figure 3-8F and not shown). In contrast, Arg substitutions at positions 26, 30, and 34 caused partial relocalization to an ERGIC-53-labeled compartment.
(Figure 3-8A and Figure 3-S4) and some secretion into the cell medium (Figure 3-8C and Figure 3-S5). These mutations also caused an increase in diffusion as measured by FRAP (Figure 3-8F and not shown), although not to the extent observed when the NTD is deleted (Figure 3-8E). Altogether, these data indicate that selected mutations perturb torsinA’s association with the membrane and retention in the ER. Importantly, the residues most affected by single Arg substitutions would fall along one face of a helix formed by the NTD (Figure 3-8D). This less tolerant helical face where Leu 26, 30, and 34 lie is therefore likely to be buried in the hydrophobic core of the lipid bilayer, while the more tolerant helical surface where Ile 24, Leu 28, Ala 29 and 31, and Ile 38 lie may be positioned in the interfacial region or on the membrane surface [37]. While the NTD is not a strikingly amphipathic sequence, the asymmetry in distribution of its small nonpolar residues vs. leucine residues results in a low hydrophobic moment ($\mu_H = 0.22$) that may define the orientation of the domain in the membrane. Hydrophobic residues along one surface of this domain are necessary for retention of torsinA in the ER.

*Other lumenal membrane proteins are also excluded from ER exit sites*

To identify proteins that might share torsinA’s topology and sorting mechanism, we took advantage of the proteomic analysis of secretory pathway components published a few years ago by Bergeron and colleagues [35] (see Methods). From a group of hydrophobic ER-enriched proteins, we selected those for which the bulk of the protein is known to be in the ER lumen and which were either known to be monotopic lumenal membrane proteins or had less well characterized hydrophobic domains that could be either monotopic membrane associating domains or TMDs (Table 3-I). The mechanism(s) underlying the ER localization of these proteins are unknown, although data in each case pointed to a role for their association with the membrane in ER retention.

The best studied of these proteins is COX-1, a key enzyme in prostaglandin
synthesis [54]. Structures and simulations show that it uses segments of four amphipathic helices to associate monotopically with the lumenal bilayer leaflet [54, 55]. COX-1 was originally proposed to reside in the ER because of a KDEL-like S/P-TEL sequence at its C terminus [56]. However, mutation or deletion of this sequence had no effect on ER localization [57, 58]. Rather, deletion studies suggest that the membrane-associating region of COX-1 is independently retained in the ER [59], although a mechanistic explanation for this result has been lacking. A similar situation applies to the unrelated ER protein 11-β-hydroxysteroid dehydrogenase, which produces cortisol and uses an amphipathic helix to associate monotopically with the lumenal bilayer leaflet [55, 60]. To test whether one of these known lumenal monotopic membrane proteins exhibits torsinA-like ER retention, we analyzed the effects of low temperature blocks on the localization of transfected COX-1 (Figure 3-9). Immunostained COX-1 is distributed throughout the ER at physiological temperature. Like torsinA, COX-1 is absent from the ERGIC after incubation at 15°C and is efficiently excluded from ERES after incubation at 10°C (Figure 3-9B,C). These data support the idea that COX-1 is statically retained in the ER by exclusion from ERES.

We also identified a number of less well studied ER membrane proteins that may share a similar retention mechanism, although detailed studies of their hydrophobic membrane interacting domains are lacking (Table 3-I, Groups II and III). Mutagenesis analyses on Group II proteins point to a role for their hydrophobic domains in ER localization. This group includes arylacetamide deacetylase, whose N terminal hydrophobic domain is responsible for localization in the ER and bears similarity to the monotopic hydrophobic domain of 11-β-hydroxysteroid dehydrogenase [61]. The UDP-glucuronosyltransferases (UDP-GTs) contain a lumenal hydrophobic domain that is responsible for ER localization independently of a C-terminal TMD and dilysine motif.
[62]. Proteins in Group III are minimally studied, having confirmed ER localization and proposed membrane-associating domains based on hydrophobicity. These include malectin, a recently identified lectin involved in protein N-glycosylation in the ER, and the peptidyl-prolyl cis-trans isomerase FKBP19; each has a proposed N-terminal signal peptide and C-terminal hydrophobic domain [63, 64]. Our analysis of COX-1 indicates that this enzyme shares features with torsinA; whether the other proteins of this group are similarly excluded from ER exit sites will be the focus of a future study.

*Preferred partitioning of luminal monotopic membrane proteins into ER sheets*

The fact that both torsinA and COX-1 are monotopically associated with the ER membrane via short helical domains predicts preferential positioning of their membrane interacting domains in the luminal leaflet of the ER membrane. The ER consists of an anastamosing network of flat sheets and curved tubules [65]. Because of the inherent curvature of the tubules, we would expect proteins in the luminal leaflet to distribute preferentially into the sheets rather than the tubules. To test this prediction, we analyzed the distribution the known sheet-preferring protein CLIMP63 or torsinA-mGFP in the sheets and tubules of COS-7 cells (Figure 3-10) [65]. Both proteins were compared to the distribution of calreticulin, which is a soluble luminal protein that is present throughout the ER. We found a striking segregation of both torsinA and CLIMP63 to the perinuclear ER with its predominant population of sheets and away from the more dispersed tubules of the peripheral ER, which were stained well with calreticulin (Figure 3-10A-B). Linescan analysis demonstrated preferential accumulation of torsinA-mGFP in sheet structures (Figure 3-S4). At higher levels of expression, torsinA could in some cases be observed in tubules while in other cases, highly expressed torsinA appeared to promote formation of additional sheet structures, similarly to what has been described with CLIMP-63 overexpression [65]. Importantly, sheet preference of torsinA was
abolished when the NTD was deleted (Figure 3-10C); the Δ26-43-torsinA-mGFP signal was superimposable on calreticulin. We also examined cells expressing COX-1 and saw robust colocalization of it with endogenous CLIMP-63 in ER sheets (Figure 3-10D). In many cases, COX1 overexpression also appeared to cause sheet proliferation. Overall, this analysis indicates that the monotopic lumenal proteins torsinA and COX-1 segregate into ER sheets, and at least in the case of torsinA, that this segregation correlates with the presence of ER retention determinants. Notably, in a previous study of TMD-based ER retention, a plasma membrane-targeted TMD partitioned into ER tubules before it exited the ER, while a shorter ER-retained TMD preferred ER sheets [24]. Thus, it appears that preference for less curved membranes decreases the likelihood of diffusing into an ER exit site.
Discussion

Protein flux into and out of the ER is governed by the cell’s need to create, fold, and secrete proteins. Direct or indirect interaction of secreted proteins with the COP II coat results in concentration into transport vesicles and efficient secretion; alternatively, proteins may passively enter COP II vesicles and exit the ER by bulk flow. Retention and retrieval mechanisms counter the forward flux of the secretory pathway to keep resident proteins in the ER. Although many sorting signals have been defined, how the unique protein and lipid composition of the ER is established and maintained remains a topic of study and debate [18]. Here, we identify a monotopic membrane interacting domain in the lumenal enzyme torsinA that allows it to escape bulk flow out of the ER, and is both necessary and sufficient for static retention in the ER. Importantly, this sorting behavior is attributable to the domain’s direct association with the membrane, and results in exclusion of the protein from ER exit sites (ERES). We find evidence for similar behavior in other lumenal monotopic proteins.

TorsinA has over the years been called either a transmembrane [49] [8] or a peripheral membrane-associated protein [27]. Our data show that its NTD is hydrophobic (Figure 3-5B,C) and interacts directly with membranes in the absence of any other proteins (Figure 3-5F) but in a monotopic and not transmembrane configuration (Figure 3-6). Several observations indicate that the NTD controls ER retention independently of other proteins. Firstly, retention of torsinA or the NTD alone in the ER is not readily saturated by overexpression. Secondly, torsinA achieves ER localization by static retention rather than by retrieval from post-ER compartments (Figure 3-1, 3-2). As has been previously noted [24], a receptor-mediated mechanism for excluding a protein from ER exit sites is currently considered unlikely and would both require enough statically retained receptor protein to interact with torsinA over its entire lifetime and move the problem of defining a retention mechanism from torsinA to the receptor. In addition,
although the NTD controls ER retention it is not involved in interaction with the predominant torsinA binding partners LAP1 and LULL1 [13, 28] (not shown), ruling these out as potential retention factors. Finally, conservative substitutions along the length of the NTD (Figure 3-S3A, Figure 3-S4B) have no effect on ER retention, which would be unexpected if the NTD were to harbor a protein-protein interacting motif. We therefore conclude that ER retention is mediated by physical features of the NTD.

Membrane-based sorting of proteins along the secretory pathway is a well characterized phenomenon for transmembrane proteins, generally explained by a matching of TMD length and composition to bilayer thickness, which in turn depends on lipid composition [66, 67]. The ER bilayer is generally thinner, less ordered, and less charged than post-ER membranes [48]. Based on our analysis of torsinA and COX1, we suggest that the underlying principles of membrane organization that result in sorting of TMDs [67] extend to monotopic membrane interacting domains (Figure 3-11). We propose that there are two potentially complementary effects arising from association of monotopic domains with bilayers that could contribute to ER retention. First, the energetic cost of inserting a monotopic domain into the lumenal bilayer leaflet is likely to be lower in the loosely packed membrane of the ER relative to the more ordered membranes of the Golgi and later secretory pathway (Figure 3-11, box i). Second, monotopic domains may preferentially partition into less curved bilayers because the inner leaflet of curved ER domains, such as tubules and budding vesicles, will be relatively contracted (Figure 3-11, box ii). Crowding of coat-associated cargo in the membrane may amplify these effects in COPII vesicles in vivo. We propose that these and potentially other membrane-based sorting effects may explain the retention of torsinA and similar proteins in the ER.

While proteins that associate with the lumenal leaflet of the ER membrane have not been extensively studied, we identified a few that share both torsinA's topology and
its exclusion from ER exit sites (Table 3-I, Figure 3-11). Two of these – COX1 and 11-β-hydroxysteroid dehydrogenase – have been structurally characterized as lumenal monotopic proteins. Subsequent molecular modeling of one of these proteins, COX-1, in membranes indicates that its monotopic membrane association disorders the lumenal leaflet [68] and affects membrane curvature [69]. Both of these effects could influence partitioning of the domain within the lipid bilayer (as schematized in Figure 3-11) and ultimately protein sorting. Although a structure of torsinA’s NTD in a membrane will be important to confirm a similar relationship between it and the ER membrane, our finding that it tolerates non-conservative mutations on only one face of the helix involved in membrane association suggest that it adopts a defined position in the membrane, perhaps as an in-plane helix.

The exclusion of torsinA from the ERES microenvironment and its preferential concentration in ER sheets raises questions about the composition of ERES and the transitional ER. This membrane domain is characterized by a lack of ribosomes and an abundance of COPII components and cargo [70] [71]. Phosphatidylinositol 4-phosphate is found on the cytosolic surface of ERES [72] and long-chain phosphatidylserine is concentrated in COPII vesicles relative to the bulk ER [73], suggesting that the transitional ER may also be a distinct lipid environment. Notably, the transitional ER is a stable ER subdomain that is marked throughout the cell cycle by Sec16 [74]. A recent study formalized the idea that rough and smooth ER correspond approximately to sheets and tubules, respectively, in mammalian cells [65]. Further studies will be needed to delineate the relationships between ER subdomains with differing composition, membrane curvature, and membrane protein sorting.

As is typical for AAA+ family members [4], torsinA is an oligomeric enzyme [28] held together by interactions among its AAA+ domains. It is possible that the NTD helices on such an oligomer will function together to perturb lipid structure and thereby
amplify the effects of the NTD on protein distribution among different subdomains of the ER. The topologically similar dimeric COX1 enzyme contributes four helices per subunit to anchor itself in the membrane [54]. Thus, cooperative effects of membrane interacting domains may amplify the partitioning of monotopic membrane proteins between subdomains of the ER.
References


**Figure Legends**

**Figure 3-1.** TorsinA is a static ER resident. (A, B, C) Epifluorescence microscopy of U2OS cells expressing VSVG-(ts045)-GFP (at 39.5°C), ER-GFP, or torsinA-mGFP (at 37°C). (A', B', C') Costaining with βCOP (A', C') or ERGIC53 (B') after 2 hr incubation at 15°C.

**Figure 3-2.** TorsinA is excluded from ER exit sites. (A-D) Confocal microscopy of cells expressing VSVG-(ts045)-GFP (at 39.5°C), NPY-GFP, ER-GFP, or torsinA-mGFP (at 37°C). (A'-D') Costaining with Sec31A after 2 hr incubation at 10°C. Scale bars, 10 μm.

(E) Quantification of colocalization of the indicated GFP-tagged proteins with Sec31A. N > 20 cells for each condition. Bars indicate standard error of the mean. * Indicates significant difference between conditions (p <0.05).

**Figure 3-3.** Residues 26-43 of torsinA’s NTD are necessary and sufficient for ER retention. (A) Schematic view of torsinA sequence: signal peptide (1-20, black), NTD (21-43, gray), linker region (44-70), AAA domain (71-332), ΔE mutation (*, 302/303), and C-terminal mGFP tag. (B) Confocal microscopy of torsinA-mGFP, Δ26-43-torsinA-mGFP, E171Q/Δ26-43-torsinA-mGFP, and E171Q/Δ26-43-torsinA-mGFP in the presence of BFA. (C) Immunoblot of the indicated GFP fusion proteins in cell lysates or media immunoprecipitates. (D) Confocal microscopy of torsinA’s signal sequence (1-25), NTD (1-43), or NTD plus linker region (1-67) fused to mGFP. Scale bars, 10 μm. (E) Immunoblot of the indicated GFP fusion proteins in cell lysates or media immunoprecipitates.
**Figure 3-4.** N-terminally deleted torsinA accesses ER exit sites. Confocal microscopy of cells expressing Δ26-43-torsinA-mGFP or E171Q/Δ26-43-torsinA-mGFP at 37°C. (F’-G’)

Costaining with Sec31A after 2 hr incubation at 10°C. Scale bars, 10 µm. (H)

Quantification of colocalization of the indicated GFP-tagged proteins with Sec31A. N > 20 cells for each condition. Bars indicate standard error of the mean.

**Figure 3-5.** TorsinA’s NTD is intrinsically hydrophobic, helical, and stably associates with membranes. (A) Alignment of torsinA’s NTD (residues 21-43 of human torsinA). Completely conserved residues are indicated in green, conserved residues in yellow, conservative substitutions in cyan, and nonconservative substitutions in white. (B) Anti-GFP Immunoblot of equal proportions of aqueous and Triton-X-114 detergent phases of the indicated constructs. (C) Coomassie stain of equal proportions of aqueous and Triton-X-114 detergent phases of recombinant MBP chimeras. (D) Diffusion coefficients determined by FRAP (ER-GFP, 7.67 +/- 0.44 µm²/s, n = 10; 1-67-mGFP, 0.69 +/- 0.07 µm²/s, n = 8). (E) Far UV circular dichroism of a synthetic peptide of residues 21-43 of human torsinA in aqueous buffer, in increasing concentrations of trifluoroethanol, and in 20 mM SDS. The 50% TFE and 20 mM SDS conditions induced 28.5% and 29.3% helicity, respectively (see Methods for calculation). (F) Coomassie stain showing the distribution of MBP chimeras following sucrose flotation of DOPC liposomes generated by detergent dialysis in the presence of the indicated proteins.

**Figure 3-6.** TorsinA’s N terminus is not accessible from the cytosol. (A) Schematic diagram of His-torsinA-mGFP construct. (B) His-torsinA-mGFP migrates at the same apparent molecular weight as wild type torsinA-mGFP and is similarly sensitive to EndoH. (C-D) Selective permeabilization with digitonin allows detection of the cytosolic
Sec31A epitope (C) but not the lumenal PDI epitope (D). (E-F) Cells expressing His-torsinA-mGFP were treated with Triton-X-100 (E) or with digitonin under conditions that permeabilize only the plasma membrane (F). The N-terminal His tag was detected using anti-His antibody and gave specific signal in Triton-X-100 but not in digitonin treated samples. Transfected cells are indicated with asterisks (*); note that background nuclear staining by the anti-His antibody is unrelated to His-TorA-GFP expression. Scale bars, 10 \( \mu \text{m} \).

**Figure 3-7.** TorsinA’s NTD can be converted into a transmembrane domain. (A) MPEx predicted TM segment in torsinA and \( \Delta G_{\text{pred}} \) for membrane insertion of wild type and mutated NTD sequences. (B-F) Epifluorescence microscopy of cells expressing the indicated GFP fusion proteins, stained with anti-GFP antibody after either selective permeabilization in digitonin (left panels) or complete permeabilization in Tx100 (right panels). Scale bars, 10 \( \mu \text{m} \).

**Figure 3-8.** ER retention correlates with membrane association. (A-B) Epifluorescence microscopy of E171Q-torsinA-mGFP with the indicated mutations, costained with ERGIC-53. Scale bars, 10 \( \mu \text{m} \). (C) Immunoblot of cell lysates or media immunoprecipitates from cells expressing the indicated torsinA-mGFP constructs. (D) Helical wheel plot summarizing results of nonconservative mutations placed throughout the NTD. Sensitive residues marked in red, insensitive residues marked in gray, and untested residues marked in white. See Figure S3 for supporting data. Arrowhead indicates direction of hydrophobic moment of wild type sequence (\( \mu H = 0.22 \)). (E-F) Diffusion coefficients of the indicated mutations in E171Q-torsinA-mGFP determined by FRAP (E: E171Q, 0.09 +/- 0.01 \( \mu \text{m}^2/\text{s} \), n = 9; E171Q/\( \Delta \text{26-43} \), 1.08 +/- 0.12 \( \mu \text{m}^2/\text{s} \), n =
(F: L28R, 0.17 +/- 0.02 µm²/s, n = 12; L30R, 0.27 +/- 0.02 µm²/s, n = 12; A31R, 0.20 +/- 0.02 µm²/s, n = 12; L34R, 0.33 +/- 0.03 µm²/s, n = 12.) Means and SEMs are indicated by lines and brackets. * indicates p <0.05; L28R vs A30R, A31R vs L34R, and L28R vs L34R are significantly different, as determined by ANOVA followed by Tukey's post test.

**Table 3-I.** Identification of proteins with similar characteristics to torsinA from Gilchrist et al., 2006. Hydrophobic ER-enriched proteins with experimentally confirmed ER localization and lumenal orientation are listed. See text for details.

**Figure 3-9.** COX-1 is a static ER resident and is excluded from ER exit sites. (A) Epifluorescence microscopy of cells expressing untagged COX-1 at 37°C. (A') Costaining with ERGIC-53 after 2 hr at 15°C. (B) Confocal microscopy of cells expressing untagged COX-1 at 37°C. (B') Costaining with Sec31A after 2 hr at 10°C. Scale bars, 10 µm. (C) Quantification of colocalization of the indicated proteins with Sec31A. N > 50 cells for each condition. Bars indicate standard error of the mean. * Indicates significant difference between conditions (p<0.05).

**Figure 3-10.** Preference of torsinA and COX1 for ER sheets. (A-C) Confocal microscopy of COS-7 cells stained for or expressing the indicated GFP-tagged proteins, zoomed in to show a section of the perinuclear and peripheral ER. ‘N’ indicates the nucleus. (D) Confocal microscopy of a COS-7 cell expressing untagged COX1, costained for CLIMP-63. Scale bars, 10 µm.
Figure 3-11. Model of retention of monotopic lumenal proteins within the ER. Monotopic lumenal proteins associate with the lumenal leaflet of the ER. This membrane association favors partitioning away from sites of ER-to-Golgi transport. This effect on protein sorting may be achieved by monotopic domains selectively partitioning into less ordered bilayers (box i) or less curved bilayers (box ii).

Figure 3-S1. TorsinA’s NTD does not traverse the ER bilayer in an in vitro system. (A) Diagram of the Lep membrane insertion reporter construct with test domain in gray and bracketing glycosylation sites in red. If the test domain is inserted as a TMD, only one site is glycosylated (left); if it is not, both are glycosylated (right). (B) In vitro translations of Lep reporter constructs in the absence or presence of canine rough microsomes (RMs) with the following test domains: the model TM segments Lep13A/6L, Lep18A/1L (Hessa et al., 2007); torsinA residues 21-43 (NTD) in Nlum-Ccyto (type I) or Ncyto-Clum (type II) orientation. Unglycosylated, singly glycosylated, and doubly glycosylated bands are designated 0g, 1g, and 2g, respectively. While unglycosylated bands remain prominent, the membrane-inserted Lep13A/6L segment is also singly glycosylated, while the translocated Lep18A/1L segment is doubly glycosylated. The torsinA segment in either orientation was doubly glycosylated, similarly to the latter model segment, indicating that it is translocated into the ER lumen.

Figure 3-S2. Increasing hydrophobicity of torsinA’s NTD allows ER exit. Colocalization of the indicated TorsinA (1-67)-mGFP constructs with the intermediate compartment marker ERGIC-53. (1-67)-GFP (A) and (1-67) 2 x Leu- GFP (B) do not appreciably accumulate in the ERGIC-53 stained compartment. (1-67) 5 x Leu-GFP (D), (1-67)-LALALA-GFP (E), and to a lesser extent (1-67) 3 x Leu-GFP (C) do colocalize partially with ERGIC-53, indicating that these mutants are able to escape the ER. (1-67) 2 x Leu-
GFP, 3 x Leu- GFP, and LALALA-GFP traverse the ER bilayer as transmembrane domains (Figure 5).

**Figure 3-S3.** Effects of scanning mutagenesis on torsinA’s ER localization I. (A) The intracellular localization of torsinA is unaffected by conservative substitutions throughout the NTD. (B) Nonconservative replacement of residues 24, 29, 31, or 38 with Arg has no effect on torsinA’s ER localization. All mutations were made in ATP-stabilized E171Q-torsinA-mGFP; similar results were seen with wild type torsinA-mGFP.

**Figure 3-S4.** Effects of scanning mutagenesis on torsinA’s ER localization II. Epifluorescence microscopy of cells expressing the indicated mutations to E171Q-torsinA-mGFP, costained for ERGIC-53. (A) Nonconservative replacement of residues 26, 30, or 34 with Arg cause partial mislocalization to the ERGIC-53 stained intermediate compartment. (B) A conservative Ala substitution at these positions does not have this effect. All mutations were made in ATP-stabilized E171Q-torsinA-mGFP; similar results were seen with wild type torsinA-mGFP.

**Figure 3-S5.** A subset of mutations to torsinA’s NTD causes secretion. Immunoprecipitation of NTD mutants from cell media. Mutation of residues 26, 30, or 34 to Arg leads to secretion of torsinA-mGFP into the cell medium. Conservative Ala substitutions at these positions did not cause secretion. Mutation of residues 24, 28, 29, 31, or 38 to Arg also did not cause secretion.

**Figure 3-S6.** Linescan analysis of ER subdomain preference. (A-C) Representative confocal images of COS-7 cells costained for (A) endogenous CLIMP-63 and calreticulin (CRT); (B) torsinA-mGFP and CRT; (C) ∆26-43-torsinA-mGFP and CRT. Linescans of
The relative intensities of the two signals are shown to the right; two linescans per image were performed, a background linescan was subtracted, and the intensities were normalized before plotting.
Figure 3-1

A 39.5°C
VSVG-GFP

A' 15°C
VSVG-GFP βCOP merge

B 37°C
TorA-mGFP

B' TorA-mGFP βCOP merge
Figure 3-2

[A] VSVG-GFP
[B] NPY-GFP
[C] ER-GFP
[D] TorA-mGFP

10°C

[E] Pearson's correlation coefficient
Figure 3-4

A  Δ26-43-mGFP

A'  Δ26-43-mGFP  Sec31A  merge

B  E171Q/Δ26-43-mGFP

B'  E171Q/Δ26-43-mGFP  Sec31A  merge

C

Pearson's correlation coefficient

37°C  10°C  37°C  10°C

Δ26-43-mGFP  E171Q/Δ26-43-mGFP
Figure 3-5

A

Homo sapiens
Mus musculus
Rattus norvegicus
Sus scrofa
Ornithorhynchus anatinus
Drosophila melanogaster

B

Aqueous Hydrophobic

TorA mGFP
Δ26-43 mGFP
1-67 mGFP
1-43 mGFP
1-25 mGFP

C

Aqueous Hydrophobic

MBP
21-43 MBP
21-67 MBP

D

D_{eff} (μm^2/s)

1-67 ER-mGFP GFP

E

mean residue ellipticity (deg cm^2/dmol)

190 200 210 220 230 240 250 260

wavelength (nm)

10 mM sodium phosphate pH 7.0
20% TFE
40% TFE
50% TFE
20 mM SDS

F

load 30% bottom 0% top sucrose

21-67 MBP
MBP

122
Figure 3-6

A

B

triton-X-100

anti-His  GFP

Digitonin  Triton-X-100

anti-His  GFP

E302/303

PDI

6xHIS

NTD

AAA

GFP

-  -  +  +

Endo H

WT  His  WT  His

-  -  +  +

1

21  70

Endo H
A torsinA mutant sequence $\Delta G_{\text{pred}}$

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Figure 3-8

A

L30R/E171Q  ERGIC-53  merge

B

L28R/E171Q  ERGIC-53  merge

C

cells  media

E171Q  L30R/E171Q  ERGIC-53  merge

L28R/E171Q  L30R/E171Q  ERGIC-53  merge

DEFF (µm²/s)

E

L30R/E171Q  L28R/E171Q

D  23-PISLGLALAGVLTGYIYP-39

F

L28R  L30R  A31R  L34R

* * *

Deff (µm²/s)

**

**

**

**

125
Table 3-I. Identification of proteins with similar characteristics to TorsinA from Gilchrist et al., 2006.

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| Abbreviations used: HD, hydrophobic domain; TMD, transmembrane domain; UDP-GT, UDP-glucuronosyltransferase; FKBP19, FK506-binding protein of 19 kDa.

Group I: structurally characterized as monotopic lumenal membrane proteins with defined UDP-glucuronosyltransferase; FKBP19, FK506-binding protein of 19 kDa.

Group II: deletion mutagenesis analyses indicate hydrophobic domains that are necessary and/or sufficient for ER localization.

Group III: confirmed ER localization and proposed membrane-associated domains based on hydrophobicity.

Figure 3-9

A 37°C

A' 15°C

B 37°C

B' Sec31A

39.5°C 10°C 37°C 10°C 37°C 10°C

VSVG -GFP

TorA -mGFP

COX1

Pearson's correlation coefficient

0.35

0.30

0.25

0.20

0.15

0.10

0.05

39.5°C 10°C 37°C 10°C 37°C 10°C

VSVG -GFP

TorA -mGFP

COX1

*
Figure 3-10
Figure 3-11

**i. lipid order**
- low sterol
- some sterols, sphingolipids

**ii. membrane curvature**
- bulk ER
- ERES
Figure 3-S1

A

inserted
translocated

G1
G2

N

C

lumen
cytoplasm

B

18A/1L 13A/6L N_{NTD} N_{NTD} N_{NTD}
- + - + - + - +

NTD
N_{Lum-Cyto}

NTD
N_{Cyto-Lum}

RMs
- 2g
- 1g
- 0g
Figure 3-S3

A

P23G/E171Q  S25A/E171Q  V33A/E171Q  T35A/E171Q

Y39A/E171Q  P40G/E171Q  R41D/E171Q  R41A/E171Q

B

I24R/E171Q  A29R/E171Q

A31R/E171Q  I38R/E171Q
Figure 3-S4

A

L26R/E171Q  ERGIC53  merge

L30R/E171Q  ERGIC53  merge

L34R/E171Q  ERGIC53  merge

B

L26A/E171Q  ERGIC53  merge

L30A/E171Q  ERGIC53  merge

L34A/E171Q  ERGIC53  merge
Figure 3-S5

- TorA
- Δ26-43

- L26R
- L30R
- L34R

- L26A
- L30A
- L34A

- L28R
- A29R
- A31R
- I24R
- I38R
Figure 3-S6

A  CLIMP63 vs CRT

B  TorsinA-mGFP vs CRT

C  Δ26-43-mGFP vs CRT
Chapter 4

Molecular characterization of torsinA’s interaction with its cofactor LULL1
Acknowledgments

I would like to thank Seema Dalal, who initially made the LULL1 241-470 deletion mutant, Teri Naismith for helping me with cloning difficulties, and an undergraduate student, Neha Yakhmi, who worked with me for a summer and assisted with analyzing various LULL1 cysteine mutant phenotypes. Her work appears in Figures 4-8C and 4-9H. I would also like to thank other members of the Hanson lab for helpful discussions.
Abstract

TorsinA binds robustly to two cofactor proteins, LAP1 and LULL1. These are partially homologous, but have distinct effects on torsinA behavior (Chapter 2): LULL1 activates torsinA to displace NE resident proteins, while LAP1 does not. The goal of this chapter is to identify conserved torsinA-binding regions and unique torsinA-activating regions.

LAP1 and LULL1 are most homologous at their C-terminus, and I determine that a C-terminal helix in LULL1 (which is conserved in LAP1) is required for binding to torsinA.

LULL1 and torsinA are each oligomeric proteins, and I find that ATP-trapped torsinA and LULL1 are associated as a stable hetero-oligomer; this may indicate that hetero-oligomers assemble at some point during the cycle of interaction. Additional features in LULL1 that are required to target torsinA to the NE include a large proportion of LULL1’s cytosolic domain and conserved cysteines within the lumenal domain. These findings are suggestive of (a) signaling being transduced from the cytosol to the ER lumen via LULL1, and (b) a redox component to regulation of torsinA by LULL1. Future experiments will determine whether either or both of these factors are mechanistically important for LULL1 activation of torsinA.
Introduction

TorsinA has two known binding partners, lamina-associated polypeptide 1 (LAP1) and lumenal domain like LAP1 (LULL1). These proteins were identified as potential torsinA binding partners in a microscopy-based screen for proteins that modulate torsinA localization [1]. Immunoprecipitation of torsinA and proteomic analysis of co-precipitating bands indicated that LULL1 and LAP1 are the preeminent torsinA binding partners in U2OS cells [2].

LAP1 and LULL1 are each type II single-pass transmembrane proteins of the ER/NE membrane system. The LAP1 and LULL1 genes are adjacent on human chromosome 1 [1], and based on the high homology of their lumenal domains, it is likely that a gene duplication event was involved in their genesis. The extralumenal domains of these proteins diverge from one another dramatically (Figure 4-1) and may confer unique function on the proteins. LAP1 is specifically retained in the inner nuclear membrane (INM) by association of its extralumenal domain with the nuclear lamina [3], while LULL1 is present throughout the bulk ER.

TorsinA’s binding to LAP1 and LULL1 appears to be linked to torsinA’s ATP hydrolysis cycle, as a stable interaction can be best detected when ATP hydrolysis is prevented by various mutations to the catalytic domain [1, 2]. This transient interaction with LAP1 and LULL1 is important for torsinA function; LULL1 levels control the amount of torsinA present and active in the NE (Chapter 2), and LAP1 knockdown has similarities to torsinA knockdown in mice [4]. These proteins have distinct roles in modulating torsinA activity, however, as only LULL1 can dynamically re-target torsinA from the bulk ER to the NE (Chapter 2). LAP1, on the other hand, can enrich torsinA in the NE to a lesser extent, and does not induce torsinA to displace the NE resident LINC complex proteins. Because LAP1 and LULL1 are homologous, yet LULL1 has unique effects on torsinA, comparison of LULL1 and LAP1 sequence will allow identification of
conserved torsinA-binding regions (in LAP1 and LULL1) and unique torsinA re-targeting regions (in LULL1). These analyses will provide insight into the mechanism(s) used to restrict torsinA’s activity to the NE.
Methods

Plasmids

TorsinA-mGFP, E171Q-torsinA-mGFP, LULL1-myc, and LAP1-myc were as described (Vander Heyden et al 2009, Naismith et al 2009). LULL1-GFP and RFP-LULL1 were made by amplifying the coding sequence with primers containing HindIII and EcoRI restriction sites and ligating into eGFP-N1 and TagRFP-C1 [5], respectively. Point mutations to LULL1 were made by Quikchange mutagenesis (Stratagene). Deletion mutations to LAP1 and LULL1 were made by PCR amplification of the desired fragments with primers containing HindIII and EcoRI restriction sites and ligating into pcDNA4/TO/MycHis B (Invitrogen). The sequences of all constructs were verified by nucleotide sequencing.

Non-reducing SDS-PAGE

35 mm dishes of U2OS cells, U2OS-derived stable cell lines, or transiently transfected U2OS cells were grown to confluency. Cells were washed in PBS, then lysed by incubation in buffer (PBS, 0.5% Tx100, 20 mM NEM, 1 mM PMSF, and protease inhibitors) for 30’ at 4°C with gentle agitation. SDS-PAGE sample buffer and benzonase enzyme were added to the lysates, and the lysates were incubated for 1 hr at room temperature before separation on 6-8% SDS-PAGE gels. Gels were then transferred to PVDF and immunoblotted as described.

Blue Native PAGE was performed as described in Chapter 2, with the caveat that ATP and MgCl2 were omitted from buffers when samples were intended for analysis of LULL1’s oligomeric state alone.
Clear Native PAGE

Clear Native PAGE is a variant of Blue Native PAGE that uses gentle non-ionic detergents to confer a charge shift on membrane protein complexes instead of Coomassie dye [6]; it is often more gentle and gives improved band resolution over Blue Native PAGE. 60 mm plates of U2OS cells, U2OS-derived stable cell lines, or transiently transfected U2OS cells were grown to confluency. Cells were resuspended in sample buffer (50 mM imidazole, 50 mM NaCl, 2 mM EDTA, 2 mM aminocaproic acid, 1 mM PMSF, protease inhibitors, and dodecylmaltoside or digitonin detergent at indicated concentrations). The samples were incubated at 4°C for 15 minutes with agitation, then centrifuged for 30 minutes at 60,000 rpm at 4°C, followed by 15 minutes at 60,000 rpm at 4°C in fresh tubes. 1 μL of benzonase was added to each sample, and the samples were then incubated at 4°C overnight. Samples were supplemented with 5x CNPAGE sample buffer (Ponceau red, 50% glycerol), then loaded onto a 7.5% Native PAGE gel. Gels were run in buffer containing 0.01% dodecylmaltoside and 0.05% deoxycholate (ref) and transferred to PVDF for Western blot detection. The gel and buffer formulations were as previously described [7].

Pulse-chase analysis of protein turnover

35 mm dishes of U2OS cells inducibly expressing LULL1-myc were grown to ~90% confluency. Expression of LULL1-myc was induced by addition of 1 μg/ml tetracycline; cells were incubated for 4 hours. Cells were deprived of methionine and cysteine for 1 hour in medium lacking these amino acids and supplemented with 10% FBS, L-glutamine, and 1 μg/ml tetracycline. Cells were labeled in medium supplemented with 112.5 μCi of 35S-methionine/cysteine (NEN) for 10 minutes, and chased with medium containing cold methionine and cysteine for the indicated time periods. At each time
point, cells were collected in cold lysis buffer (150 mM NaCl, 25 mM HEPES pH 7.4, 1% 
Tx100, 2 mM EDTA, 1 mM PMSF, protease inhibitors) and flash frozen in liquid N₂. After 
collection of all samples, LULL1-myc was immunoprecipitated with myc antibody. 
Immunoprecipitates were separated on SDS-PAGE, Coomassie stained, dried, and 
exposed to phosphor screen to detect radioactivity.

**Immunoprecipitation, Immunoblotting, and Immunofluorescence** were performed as 
described in Chapter 2.
Results

*TorsinA binds to the homologous lumenal domains of LAP1 and LULL1*

LAP1 and LULL1 bind to torsinA via their homologous lumenal domain, which is approximately 250 residues long in both proteins. Comparison of the sequences of human LAP1 and LULL1 (Figure 4-1) indicates that the C-terminal ~100 residues are the most well conserved between the two proteins in humans (86% homology, 77% identity) and in other species (not shown); the membrane-proximal ~130 residues and transmembrane segments are less well conserved (70% homology, 50% identity). The extralumenal domains of LAP1 and LULL1 are least well conserved between the two proteins (15% identity) or amongst orthologs in different species (not shown).

The higher conservation in sequence and predicted secondary structure at the C-terminal ends of LAP1 and LULL1 suggest that a conserved torsinA-binding domain could lie within this region. To test this prediction, the highly homologous ~100 amino acids were deleted from LAP1 and LULL1. The resulting LAP1 1-367 and LULL 1-373 truncated mutants localized to the NE and bulk ER, respectively, as expected (Figure 4-2B,E). Full length LAP1 and LULL1 co-immunoprecipitate with E171Q-torsinA-mGFP (Figure 4-2C,G), although a significant proportion of LAP1 remains insoluble because of association of its extralumenal domain with the nuclear lamina. LAP1 1-367 and LULL1 1-373, in contrast, do not co-immunoprecipitate with E171Q-torsinA-mGFP (Figure 4-2C,G). When compared to overexpressed full length LAP1 and LULL1, it is apparent that the truncated mutants do not affect the intracellular localization of wild type torsinA-mGFP (Figure 4-2B,E). Overall, these data are consistent with the torsinA-binding domain falling within the region at the C termini of LAP1 and LULL1 that is best conserved between the two proteins. Because of LULL1’s more tractable solubility, and because of its dramatic effects on torsinA localization and activity (Chapter 2), I chose to further characterize torsinA’s interaction with LULL1 alone.
LULL’s C terminal helix binds to torsinA

To more finely resolve where the torsinA-interacting domain lies in LULL1, I made smaller deletions starting from the C-terminus. Deletion of a predicted C-terminal helix from LULL1 (residues 440-470) abolished both torsinA-binding and torsinA-targeting behavior (Figure 4-2F,G). The extreme C-terminus of LULL1, and probably the same region of LAP1, is therefore important for interaction with torsinA. In LULL1, this helix has pronounced charged and hydrophobic faces (Figure 4-3H). To determine what surface(s) of this helix interact with torsinA, selected charged residues were replaced with apolar residues and vice versa. Substitution of aspartate 441 or serine 444 with alanine was tolerated; these mutants were comparable to wild type LULL1 in binding to E171Q-torsinA-mGFP (Figure 4-4B) and redistributing wild type torsinA-mGFP from the ER to the NE (Figure 4-3B, C, Figure 4-4A). Replacing leucine 446 with arginine had an intermediate effect, weakening binding to E171Q-torsinA-mGFP (Figure 4-4B) and decreasing the frequency of wild type torsinA’s movement into the NE (Figure 4-3D, Figure 4-4A). Mutating arginine 449 to alanine, isoleucine 450 to arginine, or leucine 453 to arginine completely abolished both binding to E171Q-torsinA-mGFP (Figure 4-4B) and NE targeting of wild type torsinA (Figure 4-3E-G, Figure 4-4A). The sensitivity of the LULL1-torsinA interaction to these point mutations supports the proposal that LULL1’s (and LAP1’s) extreme C terminus comprises the torsinA binding region. Attachment of small (myc) or large (GFP) epitope tags (Figure 4-2 and not shown) to the C terminus of LULL1 or LAP1 does not appear to affect the interaction with torsinA, suggesting that the C termini are not buried when binding to torsinA.
**LULL1’s cytosolic domain is required for targeting torsinA to the NE**

I determined that the extreme C terminal helix of LULL1 is required for binding to torsinA. This helix falls within the region of LAP1 and LULL1 homology, corresponding in LULL1 to residues 373-470, which independently immunoprecipitates with E171Q-torsinA-mGFP (Figure 4-5B). However, this region alone cannot affect torsinA’s intracellular localization (Figure 4-6B,G), suggesting that additional domain(s) are involved in re-targeting torsinA from the ER to the NE. To identify these domain(s), I generated mutants containing incrementally more of the LULL1 sequence N-terminal to the region of homology (Figure 4-5A). The complete luminal domain of LULL1 (residues 241-470) and a construct lacking the cytosolic N-terminal 100 residues (101-470) also associate with E171Q-torsinA-mGFP (Figure 4-5B) but do not affect wild type torsinA’s localization (Figure 4-6C,D,G). Attaching an RFP tag to the N terminus of LULL1 did not affect targeting of torsinA (Figure 4-6F,G), indicating that a free N terminus is not required. Surprisingly, a construct lacking just the N-terminal 50 residues (51-470) was capable of targeting torsinA to the NE to an extent comparable to full length LULL1 (Figure 4-6E, G), indicating that the region containing residues 50-100 of LULL1’s cytosolic domain is important for this function.

How might this region of LULL1 control torsinA’s localization? The cytosolic domain of LULL1 is predicted to be unstructured; it is possible that LULL1’s cytosolic domain could confer a short half-life on the protein [8] and thereby regulate torsinA’s localization. However, LULL1-myc appears to be a stable protein (Figure 4-S1). One notable feature of this region of LULL1 sequence is two conserved serine residues that could be phosphorylated; indeed, phosphopeptides corresponding to this sequence have been isolated in a broad-scale proteomic study [9]. However, mutating these residues to either an aspartate residue (to simulate constitutive phosphorylation) or to an alanine
residue (constitutively de-phosphorylated) had no effect on the ability of LULL1 to target torsinA to the NE (not shown).

The fact that LULL1’s cytosolic domain is required for targeting torsinA to the NE raises the intriguing possibility that LULL1 transduces a signal from the cytosol to torsinA in the lumen of the ER. I was unable to identify any cytosolic proteins that stably interact with LULL1’s cytosolic domain (not shown), but it remains possible that transient interaction with a cytosolic factor could lead to modulation of LULL1 and in turn, torsinA. A future goal will be to elucidate the precise role of LULL1’s cytosolic domain in torsinA activation.

**LULL1 and ATP-trapped torsinA coassemble**

TorsinA is a hexameric enzyme (Chapter 2); to understand whether LULL1 can co-assemble with torsinA, I performed native PAGE analysis on cell extracts expressing exogenous untagged torsinA and LULL1-myc. This analysis demonstrated that torsinA assembles into hexamer-sized species in the absence or presence of co-expressed LULL1-myc (Figure 2-3C; Figure 4-7A). However, if ATP-trapped E171Q-torsinA and LULL1-myc are co-expressed and subjected to native PAGE separation, E171Q-torsinA and LULL1 co-assemble into a stable high molecular weight complex (Figure 4-7B). This finding illustrates the nucleotide state dependence of the torsinA-LULL1 interaction and suggests that a heterooligomeric complex may be an intermediate in the cyclical interaction of hydrolysis-competent torsinA with LULL1. Separately, this analysis indicates that LULL1-myc assembles into high molecular weight species when expressed alone in U2OS cells (Figure 4-7B). We therefore analyzed the oligomeric state of LULL1 in more detail.
**LULL1 multimerizes**

LULL1 has three luminal cysteines that are likely to be involved in intra- and/or inter-molecular disulfides in the oxidizing environment of the ER lumen (Figure 4-1). When transiently transfected into U2OS cells, LULL1-myc assembles into disulfide-bonded dimers and perhaps tetramers as detected on nonreducing SDS-PAGE (Figure 4-8A). These high molecular weight species originate from disulfide bonds, as they are completely absent when LULL1’s three luminal cysteines are mutated (Figure 4-8A). Under native conditions, LULL1-myc is an obligate dimer and is also detected in hexameric and even larger species (Figure 4-8B). LULL1-myc and LULL1-GFP co-immunoprecipitate, and this interaction persists when the luminal cysteines are mutated (Figure 4-8C). Taken together, these analyses suggest that noncovalent interactions and disulfide bonds cooperate to make LULL1 oligomers.

Interestingly, LULL1-myc reproducibly separated into at least three distinct bands in the dimeric size range on non-reducing SDS-PAGE (Figure 4-8A). It is likely that these three bands represent distinct dimeric species that have varying levels of compactness. One explanation would be that the fastest-migrating band would have the most disulfide bonds, while the slowest would have the least disulfide bonds. A trivial explanation would be that combined with endogenous LULL1, LULL1-myc forms oligomers of slightly differing molecular weights. To better understand how LULL1 assembles, I mutated LULL1’s luminal cysteines, expressed these mutant proteins in cells depleted of endogenous LULL1, and compared their migration on non-reducing SDS-PAGE (Figure 4-8D). LULL1-myc’s patterning on non-reducing SDS-PAGE was unchanged by depletion of endogenous LULL1. LULL1 C310S was present in the two more compact dimer states, while LULL1 C382S appeared to sample all three; LULL1 C468S was detectable only in the slowest-migrating dimer state. If these distinct disulfide-bonded states are relevant to LULL1’s function, one may expect that their perturbation would
impair LULL1’s effect on torsinA localization. This is indeed the case; the C310S, C468S, and triple C/S mutations each completely abrogate the ability of LULL1 to retarget torsinA to the nuclear envelope (Figure 4-9A, B, D-F), while the C382S mutation, which did not detectably alter LULL1 assembly as assessed by non-reducing SDS-PAGE (Figure 4-8D), was still active (Figure 4-9C, F). Importantly, each of these mutations had no effect on binding to torsinA (Figure 4-9G,H). Rather, it appears that the ability of LULL1 to sample different conformational states correlates with its ability to affect torsinA localization.

*Endogenous LULL1 is predominantly monomeric*

LULL1 is expressed in U2OS cells, yet endogenous LULL1 levels are not sufficient to redistribute torsinA from the bulk ER to the NE. I previously determined that complete removal of LULL1 abrogated the enrichment of ∆GAG-torsinA in the NE (Chapter 2), implying that the amount of torsinA in the NE is a direct consequence of the expression level of LULL1. To understand how different levels of LULL1 cause a graded effect on torsinA’s localization, I analyzed the oligomeric state of endogenous LULL1. Surprisingly, endogenous LULL1 is predominantly monomeric on nonreducing SDS-PAGE (Figure 4-10A), indicating that it is not engaged in intermolecular disulfides. Rather, the protein migrates as a stable but non-covalent dimer under native conditions (Figure 4-10B). This is somewhat surprising since LULL1 contains 3 luminal cysteines that cannot all be paired intramolecularly. It is tempting to speculate that LULL1’s oligomerization is inducible, and that transient induction of oligomerization would correlate with the ability of LULL1 to target torsinA to the NE. An important caveat in analyzing these experiments, however, is that overexpression of thiol-containing proteins in the ER (as in Figure 4-8) could in turn alter the redox equilibrium of the organelle, and lead to generation of nonphysiological disulfides. Further experiments may be needed to
determine which of LULL1’s cysteines are paired and unpaired in vivo, and whether those pairings change in response to torsinA levels or other environmental stimuli.

**A putative TxxC motif in LULL1**

LULL1’s lumenal cysteines are key to LULL1’s effects on torsinA, yet these cysteines are conserved in LAP1 (Figure 4-1). Two of these 3 cysteines (382 and 468) fall within LAP1 and LULL1’s region of homology, but the region surrounding cysteine 310 is not as well conserved between the two proteins. We noticed a threonine at position 307 that is conserved among LULL1 orthologs: a TxxC motif (Figure 4-11A). TxxC motifs are a variant of the CxxC motif found in oxidoreductase proteins in the thioredoxin family, including protein disulfide isomerase (PDI). TxxC motifs occur in the peroxiredoxins, which are thioredoxin-related proteins that become disulfide-bonded in presence of peroxide and transfer that disulfide to another protein [10].

Since this position contains an alanine or valine in LAP1 sequences (Figure 4-11A), I mutated LULL1’s threonine to alanine and assessed its affect on LULL1 activity. Strikingly, torsinA was completely insensitive to LULL1 T307A (Figure 4-11C,D). While this finding indicates functional importance for this threonine in LULL1, additional work will be needed to address whether this putative TxxC motif is involved in an oxidoreductase-like function. Importantly, torsinA’s sequence contains 6 conserved cysteines, and certain combinations of mutations to these cysteines do abolish torsinA’s response to LULL1 (T. Naismith, data not shown). Intriguingly, changes to torsinA molecular weight and localization in response to oxidative stress have been reported [11]. An interesting model would be that under some conditions, LULL1 would become disulfide-bonded, and would act as an oxidoreductase on torsinA, forming or isomerizing disulfides in torsinA. This alteration in torsinA conformation could then allow torsinA to bind specifically to elements of the NE.
Discussion

The data presented here indicate that the C-terminal region of homology in LAP1 and LULL1 interacts with torsinA; further analysis in LULL1 more finely maps the binding region to a C-terminal helix. LAP1 and LULL1 are large proteins: 462 and 470 amino acids in length, respectively. The lumenal domains alone are ~230 residues long, yet it appears that a single helix at the C termini may be responsible for binding to torsinA.

What function(s) might the other regions of these proteins perform? We have determined that a large portion of LULL1’s 470 amino acids is required for torsinA targeting activity. While many AAA+ enzymes use accessory domains to recruit substrate, torsinA’s sequence is relatively minimal outside of its AAA domain. One interesting possibility could be that other portions of LAP1 and LULL1’s lumenal domains could function as accessory domains to torsinA, facilitating interaction with substrate(s). In the case of LULL1, these substrates may be the LINC complex proteins in the NE (Chapter 2).

AAA+ ATPase enzymes function as oligomeric ring structures [12]; I have determined that torsinA assembles into a hexamer (Chapter 2). Work presented here demonstrates that LULL1 is also capable of homo-oligomerization; it appears to be an obligate dimer, yet can assemble into hexameric and larger species. When ATP hydrolysis in torsinA is blocked, isolation of a stable hetero-oligomeric complex of torsinA and LULL1 becomes possible. Altogether, these data point to hetero-oligomeric assemblies of torsinA and LULL1 working together to achieve function.

What region of torsinA interacts with LAP1 and LULL1? While a structure of torsinA has not been solved yet, homology models to other AAA+ ATPase family members can provide some insight into possible structural features [13, 14]. One interesting characteristic of the torsinA AAA+ domain is the presence of a helix-loop-helix motif that is inserted between canonical structural elements of the AAA+ domain. ClpAB subfamily members also use this motif to interact with each other [15], but
comparatively, this region is extended in torsinA. This helix-loop-helix motif may be surface-exposed in the holoenzyme [13] and could interact with LULL1’s C-terminal helix, perhaps by lateral packing of the helices. Data on the implicated regions in LULL1 and torsinA could support this model. Mutations to LULL1’s C terminal helix that affect binding cluster on one side of the helix. Separately, preliminary data suggest a role for torsinA's helix-loop-helix motif in binding to LULL1; deletion of the loop between the helices does abolish binding to LULL1 (T. Naismith, data not shown). However, it is possible that such a deletion may locally or broadly destabilize the protein fold, and identification of point mutations in the vicinity of this loop that abolish binding will be a next step.

**Could LULL1 be a redox regulator of torsinA?**

Another distinguishing feature of torsinA is the presence of six conserved cysteines in its sequence. As a resident of the ER lumen, these cysteines would be susceptible to oxidation. One of these cysteines falls within the Sensor II motif; the oxidation state of this cysteine could affect the functioning of Sensor II, and thus impact how the enzyme binds and/or hydrolyzes ATP [14]. Three of the cysteines are adjacent to the N-terminal membrane domain (Chapter 3), and their disulfide-bonded state could influence torsinA’s disposition in the membrane. An intriguing possibility is that LULL1 (and perhaps LAP1) could modulate torsinA’s conformational state by a redox mechanism.

LULL1 has a TxxC motif that must be intact for the protein to target torsinA to the NE (Figure 4-9). In peroxiredoxin TxxC motifs, the alcohol sidechain of the threonine residue is thought to stabilize the nearby cysteine side chain as a thiolate anion, lowering the pKa of the cysteine and increasing its reactivity [10]. This reactive cysteine, termed the peroxidatic cysteine, then attacks a peroxide substrate to form a cysteine-
sulfenic acid moiety. This species is resolved by attack from another cysteine on a neighboring subunit, the resolving cysteine, to form an intersubunit disulfide. The peroxiredoxin may then interact with a redox partner to transfer the disulfide, analogously to disulfide bond formation by PDI.

Several features of LULL1 are comparable to peroxiredoxins. The peroxidatic cysteine (in the TxxC motif) would be cysteine 310, and the resolving cysteine could be the C-terminal cysteine 468. Both of these cysteines are required for torsinA-targeting activity (Figure 4-7). Because the reaction cycle involves formation of an intersubunit disulfide, peroxiredoxins are monomeric and oligomeric at different stages of their reaction cycle. LULL1 can oligomerize into large assemblies (Figure 4-6) and it is possible that this oligomerization could be inducible (Figure 4-7). Peroxiredoxin reactions are involved in various cellular processes, including Ero1-independent protein folding in the ER [16]; transcriptional activation [17]; and even the circadian cycle [18]. In the case of LULL1 and torsinA, perhaps an oxidoreductase cycle involving the two proteins could shift torsinA into a form that is targeted to the NE.
References


Figure Legends

Figure 4-1. LAP1 and LULL1 are homologous in their lumenal domains. (A) Alignment of *Homo sapiens* LAP1 and LULL1 protein sequences. Approximate region of transmembrane domain is boxed. Conserved lumenal cysteines marked with asterisks. (B) Schematic representation of LAP1 and LULL1 conservation. The C-terminal ~100 residues are best conserved in sequence and secondary structure.

Figure 4-2. LAP1 and LULL’s C terminal region of homology binds to torsinA. (A) LAP1-myc overexpression causes partial enrichment of torsinA-mGFP in the NE. (B) LAP1 1-367-myc localizes to the NE but has no effect on the localization of torsinA-mGFP. (C) LAP1-myc co-immunoprecipitates with E171Q-torsinA-mGFP but LAP1 1-367-myc does not. (D) LULL1-myc overexpression causes torsinA-mGFP to move into the NE. (E-F) LULL1 1-373-myc and 1-439-myc localize to the bulk ER and do not affect the localization of torsinA-mGFP. (G) LULL1-myc co-immunoprecipitates with E171Q-torsinA-mGFP but LULL1 1-373-myc and LULL 1-439-myc do not. Scale bars, 10 µm.

Figure 4-3. Point mutations to LULL1’s C terminal helix abolish torsinA targeting. (A-G) Epifluorescence microscopy of cells coexpressing the indicated LULL1-myc constructs and torsinA-mGFP. Scale bars, 10 µm. (H) Helical wheel diagram of LULL1’s C terminal helix with residues that have been mutated indicated by asterisks.

Figure 4-4. Point mutations to LULL1’s C terminal helix abolish torsinA binding. (A) Quantification of the fraction of cells expressing LULL1 and torsinA that have torsinA in the NE (representative images in Figure 4-3). N>100 cells per condition. (B) Co-immunoprecipitation of the indicated LULL1-myc mutants with E171Q-torsinA-mGFP.
**Figure 4-5.** Truncated LULL1 mutants lacking N-terminal sequences bind to torsinA. (A) Schematic of LULL1-myc deletion mutants. (B) Co-immunoprecipitation of the indicated LULL1-myc constructs with E171Q-torsinA-mGFP.

**Figure 4-6.** Residues 51-100 of LULL1 are required for targeting torsinA to the NE. (A-F) Epifluorescence microscopy of cells expressing the indicated LULL1-myc mutants with torsinA-mGFP. Scale bars, 10 µm. (G) Quantification of the fraction of cells expressing the indicated LULL1-myc mutants and torsinA-mGFP having torsinA in the NE. N>50 cells per condition.

**Figure 4-7.** ATP-trapped torsinA and LULL1 form a stable oligomeric complex. (A) Untagged torsinA migrates as a stable hexamer on Blue Native PAGE in 0.25% dodecylmaltoside in the absence or presence of coexpressed LULL1-myc. (B) Untagged E171Q-torsinA also migrates partially as a hexamer; coexpression of LULL1-myc induces the formation of a distinctly migrating complex that is reactive both for torsinA and myc. Overexpressed LULL1-myc on its own migrates as a dimer and higher-order species.

**Figure 4-8.** Analysis of LULL1-myc oligomerization. (A) Nonreducing SDS-PAGE of exogenous LULL1-myc or LULL1 C/S-myc transiently transfected into U2OS cells. (B) Blue Native PAGE of exogenous LULL1-myc transiently transfected into U2OS cells, in the presence of 0.5% or 1% dodecylmaltoside. (C) Co-immunoprecipitation of LULL1-myc or LULL1 with the three luminal cysteines substituted with serines (LULL1 C/S-myc) with LULL1-GFP. (D) Nonreducing SDS-PAGE of the indicated LULL1-myc mutants transiently transfected into cells stably depleted of endogenous LULL1.
Distinctly migrating bands in the dimeric size range are numbered, and two distinctly migrating monomeric species are indicated by arrows.

**Figure 4-9.** A subset of LULL1’s lumenal cysteines are required for re-targeting torsinA to the NE. (A-E) Epifluorescence microscopy of the indicated LULL1-myc mutants coexpressed with torsinA-mGFP. (F) Quantification of the fraction of doubly expressing cells having torsinA in the NE; n>50 cells per condition. Scale bars, 10 μm. (G,H) Co-immunoprecipitation of the indicated LULL1-myc mutants with E171Q-torsinA-mGFP.

**Figure 4-10.** Analysis of endogenous LULL1 oligomerization. (A) Nonreducing SDS-PAGE of endogenous LULL1 in U2OS cells. (B) Clear Native PAGE of endogenous LULL1 in U2OS cells in the presence of 0.25% dodecylmaltoside (DDM).

**Figure 4-11.** A putative TxxC motif in LULL1 is required for re-targeting torsinA to the NE. (A) Alignment of the region surrounding cysteine 310 in LAP1 and LULL1. Predicted helical secondary structure (h) (PsiPred) is indicated above the alignment. (B-C) Epifluorescence microscopy of the indicated LULL1-myc mutants coexpressed with torsinA-mGFP. Scale bars, 10 μm. (D) Quantification of the fraction of doubly expressing cells having torsinA in the NE; n>100 cells per condition.

**Figure 4-S1.** LULL1 is a stable protein. Cells inducibly expressing LULL1-myc under control of a tetracycline-inducible promoter were labeled for 10 minutes with 150 μCi/mL $^{35}$S-methionine/cysteine, then chased in medium for the indicated times. LULL1-myc was immunoprecipitated with myc antibody, and $^{35}$S labeled immunoprecipitates (top) or total cell lysate (bottom) were detected by exposure to phosphor screen.
Figure 4-1

A

LAP1  MKTRRTRLQQGHSEPFPSPVMTRRGLRDSHSEEEVAS 42
LULL1  --MADSGLREPQEDSKADENFSVNSOAQETTIASNAEA 40

consensus  mk---t---Q---Q-L---P-m---g---s-----E---

LAP1  SQTDLSQTISKTQSVSELVRLRFP---LRYPRYEALSVQKVNT 93
LULL1  EILHSACGLSDHQVEVETGPEALGTDSSESEDANVKHKPDKFEDNQSF 94

consensus  -----1SK---D----r---F-eanl-----e-T---qK---F

LAP1  SEKETEEEDDLLSSSSTVQARSQDSTTRSEEQYIESFQSQ--- 144
LULL1  LDQGKHHILPSNLGKEQPLDPSPHPKSDKVRADAHLSSTVAPKEADGTG 148

consensus  -e-G-----d----------SD--G------S-i-----S-gtg

LAP1  SQNFTAHEKPSVLSGYQKTOPQWAPQTAIRTRMASONSLKESLGNQSPST 197
LULL1  ASQEPPTTSQ-EAQSPGHSAGQQEDETLKRRLLAPEASHPQQTEQLEIKE 201

consensus  aSQ-----D-Qp-----S-G-----Qe-----R---

LAP1  SRRQVTGQPNAFVKRNRWLLPILIAALASFP-WFFSEPEVTT---AVQQF 247
LULL1  NAQDTMQGINKGWSYGPVILVNLVLVALVAVASVNSYSSPAQQVPKNPALEA 255

consensus  ---Q-q---F-FlL-L1-A1-----S-n---fStP---kmpAv---

LAP1  QNEMNLLKNVQGDEKLKSTQTFLEXHLNSHFRSQPAILLFAANDAEAL 301
LULL1  LAPSLEDKFGQSSFLQGGRKLFQLHNAEP-TESATLFAAERETL 308

consensus  --Q-G-ky-GL-FLA-KHLN-S-PaS-FA-ll-TAARDa-e-L

*  

LAP1  RCLSEQIADAYSFSRSQRAIRIGDKMATQDSTVKEVDQVLNSKRNQNA 355
LULL1  KCLSHKVAAYSQKSPIQDQAGRTQWQDSTYLVEDLSEYPQKNA 362

consensus  rclS-----iADAYsS---V-i-IDG--k---QDSSTVLK-VD-ELS-GF-NQ-QA

*  

LAP1  VVRFESFPAGSTLFYKYCDHENAASFKDVALVLTLVLEETIGTSGLLKEVEE 409
LULL1  VVRFESFPAGSTLFYKYCDHENAASFKDVALVLTLVLEETLEASVGPRTTE 416

consensus  VVH-FESFPAGSTLFYKYCDHENAASFKDVALVLTLVLEETL---SIG-k-EE

*  

LAP1  KVRDFKVTFTSNPPSNYHMDPKLGLWSRISHLVLVPQFENALKRGICF 462
LULL1  KVRDFKVTFTSNPPSNYHMDPKLGLWSRISHLVLVPQFSTBEQGCF 470

consensus  KVRD-L--KFTNS-TP-SyNHMD-DKL-GLWSRISHLVLVPQ---l---CLf

B

extraluminal

TM

luminal

15% identity

61% identity, 77% homology

50% identity, 70% homology

77% identity, 86% homology

α

β

α

β

COOH
Figure 4-2

A
TorsinA-mGFP
LAP1-myc
merge

B
TorsinA-mGFP
LAP1 1-367-myc
merge

C
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<th>bound</th>
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<tr>
<td>LAP1 1-367</td>
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<tr>
<td>E171Q-torsinA (IP)</td>
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</table>

D
TorsinA-mGFP
LULL1-myc
merge

E
TorsinA-mGFP
LULL 1-373-myc
merge

F
TorsinA-mGFP
LULL 1-439-myc
merge

G
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<th>lysate</th>
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<tr>
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<td>LULL 1-439</td>
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<td>E171Q-torsinA (IP)</td>
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</table>
Figure 4-3

[Images of fluorescence microscopy results showing different mutations of TorsinA-mGFP and myc channel with corresponding merge images for each mutation.]

H

440-SDKLSGLWSRISHLVLPV-457

[Diagram showing amino acid sequence with highlighted residues for mutation study.]
Figure 4-4

A

![Bar graph showing fraction of cells with NE torsinA](image)

B

![Western blot images](image)

- LULL
- LULL D441A
- LULL S444A
- LULL L446R
- LULL R449A
- LULL I450R
- LULL L453R
- E171Q-TorA (IP)
Figure 4-5

A

extraluminal  TM  luminal

-1-470

373-470

241-470

101-470

51-470

B

lysate  unbound  bound

LULL FL

373-470

LULL 241-470

LULL 101-470

LULL 51-470

E171QA-torsinA (IP)
Figure 4-6

A  
TorsinA-mGFP  LULL1-myc  merge

B  
TorsinA-mGFP  LULL1 373-470 -myc  merge

C  
TorsinA-mGFP  LULL1 241-470 -myc  merge

D  
TorsinA-mGFP  LULL1-101-470 -myc  merge

E  
TorsinA-mGFP  LULL1 51-470 -myc  merge

F  
TorsinA-mGFP  RFP-LULL1  merge

G  
fraction cells with TorA in NE

1.00
0.75
0.50
0.25
0.00

LULL  373-470  241-470  101-470  51-470  RFP-LULL
Figure 4-7

A

+       +             +         +                -           E171Q-TorsinA
-        +            -          +                +          LULL1-myc

440 kDa
232 kDa
140 kDa
67 kDa

BN-PAGE

B

+       +             +         +                -           E171Q-TorsinA
-        +            -          +                +          LULL1-myc

440 kDa
232 kDa
140 kDa
67 kDa

WB: TorA
WB: AP-LULL
**Figure 4-8**

A. Immunoblot (WB) of LULL1 and LULL1 C/S proteins. The blot shows bands at various molecular weights.

B. Densitometry analysis showing the relative intensity of bands at different DDM concentrations (0.5 and 1%)

C. Summary of gel bands for untransfected, LULL1, LULL1 C/S, and LULL1-GFP (IP)

D. Gel analysis of untransfected and transfected samples showing bands at molecular weights 12, 250, 150, 100, 75, and 50 kDa.

1. Band at 125 kDa
2. Band at 232 kDa
3. Band at 440 kDa
Figure 4-10

A

B

WB: LULL1

WB: LULL1

10 sec 1 min

0.25 0.25 % DDM
Figure 4-11

A  

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<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
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<td>TAARBGEETKCLS</td>
</tr>
<tr>
<td>Mus musculus LULL1</td>
<td>TAARBGEETKCLS</td>
</tr>
<tr>
<td>Rattus norvegicus LULL1</td>
<td>TAARBGEETKCLS</td>
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<td>TAARBGEETKCLS</td>
</tr>
<tr>
<td>Homo sapiens LAP1</td>
<td>TARAABEETKCLS</td>
</tr>
<tr>
<td>Canis familiaris LAP1</td>
<td>TARAABEETKCLS</td>
</tr>
<tr>
<td>Mus musculus LAP1</td>
<td>TAAQDAAEETKCLS</td>
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<tr>
<td>Rattus norvegicus LAP1</td>
<td>TAAQDAAEETKCLS</td>
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</table>

B  

C  

D  

**Fraction of cells with TorA in NE**

<table>
<thead>
<tr>
<th></th>
<th>LULL</th>
<th>T307A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.00</td>
<td>0.75</td>
</tr>
</tbody>
</table>

* *
Figure 4-S1
Chapter 5

Preliminary Data and Future Directions
Acknowledgments

I would like to thank Teri Naismith for making the R288Q mutation to torsinA and analyzing its behavior in cells; her work appears in Figure 5-5. I would also like to thank Soomin Shim for assistance in generating LULL1 shRNA cell lines, and for passing on her painstakingly thorough RNAi protocols to me. I would like to thank Joshlean Fair, who worked with me for a summer as an undergraduate, and performed preliminary immunofluorescence experiments in torsinA knockdown cell lines. Finally, thanks to other members of the Hanson lab for helpful discussions.
Abstract

This chapter contains preliminary data of interest to various aspects of torsinA biology. We identify a conserved arginine residue that is required for torsinA’s activity at the NE, mutation to which has been observed in a case of dystonia. This mutant is phenotypically similar to the more common ΔGAG mutation, strengthening the argument that dysfunction of torsinA at the NE causes dystonia. Cells depleted of LULL1, torsinA, and LAP1 were separately generated, and the status of NE resident proteins in these cells evaluated. LULL1 depletion reproducibly caused a decrease in the amount of Sun2 at the NE. Loss of either LULL1 or torsinA caused imbalances in nuclear pore complex organization and number. LAP1 depletion had little to no effect on other NE residents or on torsinA’s dynamic localization to the NE. ΔGAG-torsinA was confirmed to migrate as a hexamer on Blue Native PAGE. Finally, the oligomeric state of torsin family members was determined by Blue Native PAGE alone or in combination with torsinA. TorsinA, torsin2A, and torsin3A form stable oligomers, while torsinB does not. However, torsinA is able to coassemble into a hetero-oligomeric species with torsinB only.
Methods

Generation of cell lines stably expressing short hairpin RNAs

To make cell lines stably lacking LULL1 expression, U2OS cells were transduced with Sigma Mission shRNAs directed against human LULL1 (Sigma Aldrich, RefSeq # NM_145034). Lentiviruses for transduction were produced by cotransfecting hairpins in pLKO with the pCMV 8.2 ΔR packaging plasmid into 293T cells. U2OS cells were then transduced with viral particles according to manufacturer’s instructions. Cells depleted of LULL1 were enriched for by selecting transduced cells with puromycin (10 µg/ml).

To generate cell lines depleted of torsinA, U2OS cells were transfected with shRNAs in the pLKO vector, then subjected to selection with puromycin (10 µg/ml). Individual colonies were isolated and grown to generate clonal lines. Two cell lines were generated with no detectable LULL1 expression (hairpins 1507 and 2088), and one cell line was generated with ~10% of endogenous LULL1 expression (hairpin 1269). See Figure 2-5 for a representative Western blot. Hairpins 1269 and 1507 fall within the coding sequence of LULL1, while hairpin 2088 falls in the 3’ untranslated region of LULL1.

Transient transfection of short interfering RNAs

To knock down LAP1, we selected a siRNA that is directed to the 3’ UTR of the LAP1 gene, which should target all LAP1 isoforms (A, B, and C). Coverslips in 6-well or 12-well plates were seeded at a density of 3 x 10^5 or 5 x 10^4 cells per well, respectively, the day before transfection with siRNA. Cells were transfected with 10-50 nM siRNA in transfection mixtures prepared according to the manufacturer’s instructions (Thermo Scientific). Dharmafect formulation #1 was used as the transfection reagent for U2OS
cells. Culture medium was replaced with fresh medium ~18 hr after transfection. Cells were collected for analysis after ~90 hr of siRNA transfection.

**Analysis of NPC intensity in synchronized cells**

This assay was performed essentially as described in (Doucet et al. 2010). Briefly, U2OS cells were grown to confluency in 10-cm dishes. Cells were synchronized in mitosis by treatment with 0.6 ug/ml nocodazole for 18 hr. Mitotic cells were harvested by shake-off, washed twice in warm PBS, and plated on coverslips in fresh culture medium. Coverslips were fixed in cold 4% PFA in PBS 5 hr (G1 time point) and 19 hr (G2 time point) after replating, stained with mAb414 to visualize NPCs, and imaged by epifluorescence microscopy. Images were obtained at the same exposure time for 5 hr and 19 hr time points, taking care not to allow pixel saturation. NPC number is a function of total fluorescent intensity; this value was quantified for individual nuclei in Image J. For presentation, NPC intensity values were normalized to U2OS cells at the 5 hr timepoint.

**Blue Native PAGE, immunoprecipitations, and immunofluorescence** were performed as described in Chapter 2.
Results and Discussion

Our data are consistent with torsinA exerting an enzymatic activity at the NE (Chapter 2). Mutagenesis indicates that movement of torsinA into the NE depends on the ability of the AAA domain to bind and hydrolyze ATP. Specifically, mutations that prevent ATP binding (the Walker A motif mutant, K108A) or ATP hydrolysis (the Walker B mutant, E171Q; the sensor II mutant, K320M) prevent LULL1-directed movement into the NE. These mutations alter binding to LAP1 and LULL1. If LAP1 and LULL1 are cofactors to torsinA, and NE resident LINC proteins are its substrates, it might be possible to identify mutations that do not affect binding to LAP1 and LULL1, but alter either targeting to the NE or activity on NE resident proteins. Identification of such mutants would deepen our understanding of the molecular changes that torsinA undergoes as it binds to LULL1, translocates to the NE, and displaces LINC complex proteins. With this in mind I revisited the alignment of torsinA’s AAA domain (Figure 5-1).

Mutation to a possible pore loop blocks NE localization of torsinA

AAA+s use pore loops that protrude into the central pore of the holoenzyme to engage substrates. In various cases, pore loops have been identified that use hydrophobic, aromatic, or charged residues to contact substrate [1]. Mutations to these pore loops will often hinder activity on substrates without affecting oligomerization or ATPase activity of the enzyme; these mutations can thus be thought of as uncoupling the nucleotide hydrolase activity from its effect on a substrate. The ClpB D2 domain, a close homolog to torsinA, has a pore loop with a conserved GYVG sequence [2]. The structure-based alignment of torsinA to ClpB’s D2 domain (see Figure 1-2) [7] indicates a region in torsinA that could correspond to this pore loop. While torsinA does not contain the typical GYVG sequence, it does contain a sequence with conserved residues including a tyrosine (Y147) that could function similarly to the key tyrosine in ClpB.
(residues 147-155, boxed, Figure 5-1). I made triple alanine mutations across this sequence (which were termed pore loop mutants 1a, 1b, and 1c, see Figure 5-2A) and assessed their affect on torsinA behavior.

The three pore loop mutants all moved to the NE with lower frequency in response to LULL1 (Figure 5-2B-F) and were ineffectual at displacing Sun2 (Figure 5-3A-E). These mutations did not, however, abolish binding to LULL1 (Figure 5-3F). It thus appears that these mutations specifically affect torsinA’s targeting to and activity in the NE, without affecting interaction with cofactor proteins. Individual point mutations in the pore loop region will clarify which residue(s) are required for normal function. If this sequence does in fact contain a pore loop, it might be possible to crosslink the pore loop to NE-localized substrates using a modified amino acid, as was previously done with ClpB [2].

Mutation to a conserved arginine in renders torsinA inactive at the NE

An additional feature that I noticed in torsinA’s AAA+ domain was the presence of an absolutely conserved arginine residue in helix 5 at residue 288; this arginine and the following glycine are conserved from *H sapiens* torsinA to the *C elegans* OOC-5 homolog (Figure 5-1, arrow). Arginine residues in AAA domains have been implicated in cross-subunit interactions with nucleotide, which stabilizes oligomers [3], as well as interacting with substrate [4]. I mutated this residue to an alanine and assessed the effect of this mutation on torsinA. I find that this mutant redistributes to the NE with approximately normal frequency in LULL1-myc expressing cells (Figure 5-4B’, C). However, torsinA-R288A does not displace the NE resident protein Sun2 from the NE (Figure 5-4E,F). This loss of activity at the NE occurs without an effect on torsinA’s ability to bind to LULL1 (Figure 5-4G) or hexamerize (Figure 5-4H). These findings make
a role for R288 in ATP binding or oligomerization unlikely, and may be most consistent with R288 being a substrate-interacting residue.

Intriguingly, a recent case study described a mutation of this arginine to a glutamine (R288Q) in a case of hereditary dystonia [5]. We also made this substitution and assessed its effects. It appears that the R288Q substitution also allows torsinA to move into the NE (Figure 5-5B',C), but is unable to displace Sun2 from the NE (Figure 5-5E,F), as seen with the alanine substitution. This phenotype is similar to what we previously observed for the more common dystonia-causing \( \Delta \text{GAG} \) mutation: \( \Delta \text{GAG} \)-torsinA can move into the NE in response to LULL1, but is much less efficient at displacing Sun2 once there (Chapter 2). Thus, we have identified a similar cellular phenotype arising from two disease-related mutations. These data further strengthen the model that torsinA-related disease arises from loss of function or dysfunction of torsinA at the NE.

While these two disease-associated mutants similarly affect torsinA’s function in cellular assays, there may be important distinctions between them. The \( \Delta \text{GAG} \) mutation has been shown to weaken the interaction of torsinA with LAP1 and LULL1 [6], whereas the R288Q mutation probably does not have this effect (not shown, but see IP of R288A with LULL1 in Figure 5-4G). The available data on the \( \Delta \text{GAG} \) mutation are suggestive of this mutation destabilizing the protein. The deleted residue is predicted to fall within an alpha helix directly preceding the ATP-interacting Sensor II motif [7, 8]. A single alanine or glutamine substitution in helix 5 (in the case of the R288A/R288Q mutations), on the other hand, is less likely to perturb secondary structure. It is attractive to speculate that this highly conserved arginine may be involved in substrate interaction. Since mutations to R288 allow movement to the NE but not displacement of Sun2, one possibility is that these mutations could stabilize interaction with Sun2 and/or other NE substrates.
Characterization of cells individually depleted of LULL1, LAP1, and torsinA

As described in Chapter 2, I found that depletion of LULL1 prevented enrichment of ΔGAG-torsinA-mGFP in the NE. This finding, combined with the fact that LULL1 over-expression causes dramatic relocalization of torsinA to the NE, suggests that LULL1 levels directly regulate the extent of torsinA enrichment at and activity in the NE. NE resident proteins including Sun2, nesprin3, and nesprin2-Giant are displaced in cells co-expressing exogenous torsinA and LULL1. I therefore further analyzed the localization of these and other NE resident proteins in LULL1 knockdown cell lines. U2OS or LULL1 shRNA 2088 cells were stained for antibodies to Sun1, Sun2, nesprin2-Giant, nesprin3, and LAP1 (Figure 5-6). Sun1 and nesprin3 levels appeared subtly increased at the NE in LULL1 2088 cells compared to U2OS cells at the same exposure time (Figure 5-6A, D). Occasional nuclei were observed in LULL1 2088 cells that were completely lacking in Sun2 signal or lower in LAP1 signal (Figure 5-6B, E). Nesprin2-Giant appeared unaffected (Figure 5-6C). The loss of Sun2 staining in some cells expressing LULL1 shRNA 2088 was quite dramatic, so I assessed the effect of LULL shRNAs 1269 and 1507 on Sun2 protein localization. Cells expressing these shRNAs also had diminished Sun2 at the NE (Figure 5-7B,C). Additional controls will be required to confirm that these alterations in LINC protein levels are directly related to LULL1 protein depletion.

Since NE-localized torsinA can alter the stability of NE resident proteins, I analyzed NE resident proteins in cells depleted of torsinA. A representative Western blot showing knockdown of torsinA in two clonal cell lines expressing hairpin 376 is shown in Figure 5-8A. Somewhat surprisingly, Sun2 appeared grossly normal in most cells. In cells stained with mAb414, I noted frequent nuclear pore complex (NPC) disorganization. Normally, NPCs are abundant and relatively evenly distributed across the NE surface. In torsinA knockdown cells, I noted many cells with blotches of NPC-free NE, as well as occasional cells with very low levels of mAb414 staining, or having a pole
of the nucleus completely lacking in NPC signal (Figure 5-8C, D, arrows). Since these 
poles also lacked Sun2 staining (Figure 5-8C,D, arrows), it is possible that these are 
regions where the NE and/or underlying lamina are perturbed.

I also obtained short interfering RNA (siRNA) against LAP1. A representative 
Western blot of the level of LAP1 knockdown achieved is shown in Figure 5-10A. I 
analyzed the effect of LAP1 knockdown on other NE resident proteins. Cells lacking 
LAP1 had grossly normal levels and localization of Sun1, Sun2, nesprin2-Giant, and 
nesprin3 (Figure 5-9B-E). Occasionally, cells lacking Sun2 staining at the NE could be 
found, as was also noted with LULL1 knockdown (Figure 5-9C, asterisks). Endogenous 
LULL1 also appeared grossly normal (Figure 5-9F).

TorsinA moves into the NE when LULL1 is coexpressed, but it is unclear what 
anchors torsinA in that compartment. A likely candidate would be LAP1, as this protein 
binds to torsinA [6] and is a stable [9] and abundant component of the NE. To test 
whether LAP1 is required for anchoring torsinA at the NE, I transfected LAP1 siRNAs 
into a cell line inducibly expressing LULL1-myc. Cells were transfected with siRNA, 
icubated for 60 hours, and expression of LULL1-myc was induced with tetracycline. 
TorsinA-mGFP was transiently transfected into the cells 6-8 hours later. Cells were fixed 
and stained for microscopy after ~18 hours of LULL1-myc and torsinA-mGFP co-
expression. Despite the efficient knockdown of LAP1 protein at the NE, torsinA-mGFP 
remained targeted to the NE (Figure 5-10C-E). Treatment with the control RNAi (which 
targets firefly luciferase) caused a partial but significant decrease in frequency of NE 
localization (Figure 5-10B,F). This is somewhat surprising since other groups have 
reported that LAP1 is required for localization of the "substrate trapped" E171Q-torsinA 
mutant to the NE [10]. These data suggest that LAP1 and LULL1 independently 
influence torsinA localization. The question of what retains wild type torsinA in the NE 
thus remains unanswered.
**TorsinA and LULL1 are required for interphase assembly of nuclear pore complexes**

In my initial characterization of cell lines stably expressing torsinA or LULL1 shRNAs, I noticed that many cells had disorganized or diminished nuclear pore complex (NPC) populations (Figure 5-8 and not shown). Our laboratory previously described the accumulation of E171Q-torsinA-mGFP in pore-depleted islands of the NE [11]. Since NPCs are normally evenly distributed across the NE, this could indicate that membranes containing mutant torsinA are not competent for NPC insertion. We decided to analyze the relationship between the levels of torsinA and LULL1 and NPC homeostasis in more detail.

In metazoan cells, NPCs are assembled by two distinct mechanisms at different stages of the cell cycle [12]. Post-mitotic NPC assembly involves the recruitment of soluble and membrane-bound NPC components to naked chromatin, and is coupled to the reassembly of the NE. NPC insertion into the NE continues after the NE has reformed during interphase. This latter mode of insertion requires the controlled fusion of the inner and outer nuclear membranes and is less well understood. A recent study describes a simple assay, based on the observation that total NPC numbers roughly double through the cell cycle [13], to assess the relative efficiency of postmitotic versus interphase NPC assembly [14]. I used this assay in U2OS cells to determine whether changes to torsinA or LULL1 protein levels have any effect on NPC number.

**U2OS cells or cells stably expressing shRNAs to torsinA or LULL1 were synchronized in mitosis by nocodazole treatment and shake-off of the rounded mitotic cells. Nocodazole was washed out and the cells were replated on coverslips to allow the cell cycle to resume.** Samples were collected after 5 hours and 19 hours. The former timepoint is predominated by cells in early G1; these cells have just completed division and NE reassembly and have a number of NPCs that were inserted concomitantly with NE assembly. The latter timepoint is populated by cells in late G2; these nuclei have
grown in surface area, DNA content, and NPC number through S phase. Coverslips from each timepoint were stained with mAb414 to detect NPCs, and the total fluorescence intensity of NPCs was quantified. When U2OS cells are subjected to this analysis, the intensity and thus overall number of NPCs roughly doubles between early and late stages of the cell cycle (Figure 5-11A,D). In cells depleted of torsinA or LULL1, however, NPC doubling is impaired (Figure 5-11B-D). These data suggest that torsinA and LULL1 may be involved in insertion of new NPCs into the NE.

It was previously noted that overexpressed E171Q-torsinA anti-localizes with NPCs in the NE [11]. I also assessed cells overexpressing wild type or E171Q-torsinA-mGFP and found that overexpression of either form of torsinA had a dominant effect on NPC insertion; no increase in NPC intensity was observed through the cell cycle in cells expressing wild type or E171Q-torsinA-mGFP (Figure 5-11E-H). It could be that if too much of torsinA’s enzymatic activity is present at the NE, it could have a deleterious effect on the process of NPC insertion. Alternatively, it may be that overexpression of any protein that localizes partially or specifically to the NE inhibits NPC insertion. Important controls to assess the specificity of this phenomenon will include knockdown and overexpression of a NE and/or ER resident protein that is not expected to have a function in NPC insertion.

**\(\Delta GAG\) torsinA can oligomerize**

Using native gel electrophoresis, I determined that torsinA can assemble into an oligomer that migrates at an approximately hexameric size (Chapter 2, Figure 2-3). Inheritance of one allele carrying the \(\Delta GAG\) mutation can lead to dystonia. One explanation for the dominant inheritance of dystonia could be that disease-mutant and wild-type torsinA can coassemble. I find that \(\Delta GAG\)-torsinA is also detectable as a hexamer on BN-PAGE (Figure 5-12A). Blue Native PAGE separation of samples
expressing differently tagged &Delta;GAG and wild type torsinA isoforms could be performed to determine whether coassembly of mixed oligomers occurs. If coassembly is detected, this could suggest that the &Delta;GAG mutation contributes to disease pathogenesis by “poisoning” oligomers of wild type torsinA. If coassembly is not detected, this outcome could also contribute to our understanding of dystonia. Because oligomeric species of each form of torsinA are clearly detectable on Blue Native PAGE (Figure 2-3 and Figure 5-12A), a negative result would indicate that hetero-oligomers do not form or are significantly less stable than homo-oligomers.

TorsinA and TorsinB can hetero-oligomerize

TorsinA has three homologs in humans; it has been suggested that these homologs could compensate for loss of torsinA function in disease, perhaps explaining why mutation to a ubiquitously expressed enzyme could lead to an exclusively neuronal phenotype. It is thus desirable to understand whether the other torsin homologs could be expected to be active AAA ATPases. To that end, I used Blue Native PAGE to determine whether these homologs are also stable oligomers. Myc-tagged torsinA, torsinB, torsin2A, or torsin3A were expressed in U2OS cells and subjected to Blue Native PAGE in the presence of 0.5% digitonin. Oligomeric species were detected for torsinA, torsin2A, and torsin3A, while torsinB was detectable only as a monomer (Figure 5-12B).

A related question of interest is whether torsin family members can hetero-oligomerize together to create mixed oligomers. To answer this question, I transiently transfected cells stably expressing torsinA-mGFP with myc-tagged torsinB, torsin2A, or torsin3A. These samples were subjected to Blue Native PAGE in the presence of 0.5% digitonin, and the torsinA-mGFP signal was examined for unique bands when homologs were co-expressed. Only torsinB-myc expression caused the migration of a GFP-reactive band of unique size (~140 kDa) (other combinations not shown) (Figure 5-12D).
This band was also detected by myc antibody, indicating that the band is composed of torsinA-mGFP and torsinB-myc molecules (Figure 5-12E). These data suggest that torsinA and torsinB could coassemble in cells. TorsinA and torsinB are the most closely related members of the torsin subfamily, with torsin2A and torsin3A being more distantly related. Interestingly, torsinB is the only other torsin family member that shares the double Glu residues, which are commonly mutated in DYT1 dystonia, with torsinA [15]. Further, recent work in mice indicates that torsinB is highly expressed along with torsinA in all tissues except brain [16, 17]; if torsinB can compensate for and/or cooperate with torsinA, this could explain how mutation to torsinA causes exclusively neuronal dysfunction.
References


Figure Legends

Figure 5-1. Multiple sequence alignment of torsin AAA+ domains, aligned to residues 71-332 of human torsinA. Completely conserved residues are highlighted in green, conserved residues in yellow; conservative substitutions are highlighted in cyan, and nonconservative substitutions in white. Predicted secondary structure elements, based on the alignment to ClpB in [7], are indicated below the alignment. Key regions of the sequence are boxed in red. An absolutely conserved arginine residue at position 288 is indicated (arrow).

Figure 5-2. Mutations to a possible pore loop affect torsinA’s targeting to the NE. (A) Alignment to residues 145-156 of human torsinA where a pore loop may lie (see red boxed region in Figure 5-1). Three mutants were generated: pore loop 1a (147YKD149 → AAA), pore loop 1b (150QLQ152 → AAA), pore loop 1c (153LWI155 → AAA). (B-E) Epifluorescence microscopy of torsinA-mGFP (B-B’), torsinA-PL1a-mGFP (C-C’), torsinA-PL1b-mGFP (D-D’), torsinA-PL1c-mGFP (E-E’) alone (B,C,D,E) or in cells coexpressing LULL1-myc (B’,C’,D’,E’). (F) Quantification of the fraction of torsinA-mGFP and LULL1-myc coexpressing cells having torsinA in the NE. N>100 cells per condition. * indicates significant difference from WT by unpaired t-test (p<0.05).

Figure 5-3. Mutations to a possible pore loop affect torsinA’s activity in the NE. (A-D) Epifluorescence microscopy of cells coexpressing torsinA-mGFP (A), torsinA-PL1a-mGFP (B), torsinA-PL1b-mGFP (C), or torsinA-PL1c-mGFP (D) and LULL1-myc (not pictured), costained for endogenous Sun2. Scale bars, 10 µm. (E) Quantification of the fraction of GFP-expressing cells lacking detectable Sun2 in the NE. N>100 cells per condition. ER-GFP was used as a control for effect of transfection on Sun2 protein
levels. * indicates significant difference from WT by unpaired t-test (p<0.05). (F) Co-immunoprecipitation of the indicated myc-tagged torsinA constructs with endogenous LULL1.

**Figure 5-4.** Effect of R288A mutation on torsinA function. (A-B’) Epifluorescence microscopy of torsinA-mGFP (A-A’) or torsinA-R288A-mGFP (B-B’) alone (A,B) or in cells coexpressing LULL1-myc (A’, B’). (C) Quantification of the fraction of torsinA-mGFP and LULL1-myc coexpressing cells having torsinA in the NE. N>100 cells per condition. (D-E) Epifluorescence microscopy of cells coexpressing torsinA-mGFP (D) or torsinA-R288A-mGFP (E) and LULL1-myc (not pictured), costained for endogenous Sun2. Scale bars, 10 µm. (F) Quantification of the fraction of GFP-expressing cells lacking detectable Sun2 in the NE. N>100 cells per condition. ER-GFP was used as a control for effect of transfection on Sun2 protein levels. * indicates significant difference from WT by unpaired t-test (p<0.05). (G) Co-immunoprecipitation of the indicated myc-tagged torsinA constructs with endogenous LULL1. (H) Clear Native PAGE of GFP-tagged torsinA in the presence of 0.25% dodecylmaltoside detergent. (I) SDS-PAGE separation of the samples shown in (H).

**Figure 5-5.** Effect of R288Q mutation on torsinA function. (A-B’) Epifluorescence microscopy of torsinA-mGFP (A-A’) or torsinA-R288Q-mGFP (B-B’) alone (A,B) or in cells coexpressing LULL1-myc (A’, B’). (C) Quantification of the fraction of torsinA-mGFP and LULL1-myc coexpressing cells having torsinA in the NE. N>100 cells per condition. (D-E) Epifluorescence microscopy of cells coexpressing torsinA-mGFP (D) or torsinA-R288Q-mGFP (E) and LULL1-myc (not pictured), costained for endogenous Sun2. Scale bars, 10 µm. (F) Quantification of the fraction of GFP-expressing cells lacking detectable
Sun2 in the NE. N>100 cells per condition. ER-GFP was used as a control for effect of transfection on Sun2 protein levels. * indicates significant difference from WT by unpaired t-test (p<0.05).

**Figure 5-6.** Immunofluorescence of NE resident proteins in LULL1-depleted cells. U2OS or LULL1 shRNA line 2088 cells were stained for endogenous Sun1 (A), Sun2 (B), nesprin2-Giant (C), nesprin3 (D), and LAP1 (E) and imaged by epifluorescence microscopy. Images were acquired at similar exposure times to compare relative levels of these proteins between U2OS and LULL1-depleted cells. Nuclei lacking Sun2 staining are indicated with asterisks. Scale bars, 10 µm.

**Figure 5-7.** Sun2 is decreased in a survey of LULL1-depleted cell lines. U2OS (A), LULL1 shRNA line 1269 (B), LULL1 shRNA line 1507 (C), and LULL1 shRNA line 2088 (D) cells were stained for endogenous Sun2. Images were acquired at similar exposure times to compare relative levels of Sun2 amongst the different cell lines. Sun2 protein levels are reproducibly decreased in the three LULL1-depleted cell lines tested. Nuclei lacking detectable Sun2 are indicated with asterisks. Scale bars, 10 µm.

**Figure 5-8.** Stable knockdown of torsinA causes NE and NPC disorganization. (A) Representative Western blot of four puromycin-selected clonal lines stably expressing either hairpin 376 or hairpin 1911. The two lines expressing hairpin 376 show efficient knockdown of torsinA. (B-D) Epifluorescence microscopy of U2OS (B), torsinA shRNA line 376 clone 8 (C), or torsinA shRNA line 376 clone 21 (D), stained with mAb414 to visualize NPCs and Sun2. Instances of partial or complete NPC displacement are indicated by arrows. Scale bars, 10 µm.
**Figure 5-9.** Immunofluorescence of NE resident proteins in LAP1-depleted cells. (A) Representative Western blot of U2OS cell lysates that had been transfected with the indicated concentrations of LAP1 or control (to firefly luciferase) siRNA for ~90 hours. (B-F) LAP1-depleted cells were stained for Sun1 (B), Sun2 (C), nesprin2-Giant (D), nesprin3 (E), or LULL1 (F). Images were acquired at similar exposure times to compare relative levels of the indicated proteins between treatment conditions. All proteins tested appeared grossly normal, with rare displacement of Sun2 from the NE (asterisks). Scale bars, 10 µm.

**Figure 5-10.** LAP1 depletion has no effect on the ability of torsinA to move into the NE in response to LULL1. Cells stably expressing LULL1-myc were transfected with the indicated concentrations of control (to firefly luciferase) or LAP1 siRNA. After 60 hours of siRNA incubation, tetracycline was added to induce LULL1 expression; torsinA-mGFP was transfected into the cells 8 hours later. Coverslips were collected and processed by immunofluorescence after ~18 hours of torsinA expression. (A-E) Cells transfected with the indicated concentrations of siRNA and expressing torsinA-mGFP were stained for endogenous LAP1 and myc to detect LULL1 (not shown). (F) The fraction of doubly expressing cells with torsinA in the NE was quantified for each condition (N>100 cells per condition). * indicates significant difference from no RNAi and Sham transfection controls by unpaired t-test (p<0.05).

**Figure 5-11.** Altering LULL1 and torsinA expression levels inhibits the insertion of NPCs into the NE. (A-C) Epifluorescence microscopy of U2OS, torsinA shRNA line 376-8, or LULL1 shRNA line 2088 were stained with mAb414 antibody 5 hours and 19 hours after being released from cell cycle block. (D) Quantification of total mAb414 fluorescence
intensity. N>50 cells per condition. (E-G) U2OS, torsinA-mGFP, or E171Q-torsinA-mGFP stably expressing cells were processed as in A-C. (H) Quantification of total mAb414 fluorescence intensity. N>50 cells per condition.

**Figure 5-12.** Blue Native PAGE separation of torsin family members. (A) Separation of overexpressed untagged wild type or ∆GAG-torsinA on BN-PAGE in the presence of 0.25% dodecylmaltoside. (B) Separation of the indicated myc-tagged torsin family members on Blue Native PAGE in the presence of 0.5% digitonin. TorsinA, torsin2A, and torsin3A are detectable in various oligomeric species, but no oligomers of torsinB were observed. (C) SDS-PAGE separation of the samples from (A). (D) The indicated myc-tagged constructs were transfected into cells stably expressing torsinA-mGFP and the lysates were separated on Blue Native PAGE in the presence of digitonin. A unique ~140 kDa band is detected by GFP (D) and myc (E) antibodies in samples coexpressing both torsinA-mGFP and torsinB-myc, suggesting that torsinA and torsinB can coassemble. No coassembly was observed with torsinA and torsin2A or torsin3A (not shown).
Figure 5-2

A

H sapiens Torsin A TLKDOQLQLMER
M musculus Torsin A TQLKDQLOLWR
X tropicalis Torsin A DKKDQLOLWR
H sapiens Torsin B KLKDQLOQKRR
C elegans OOC-5 BBQDOMRR

Pore loop 1a (PL1a) ...LAAAQL...
Pore loop 1b (PL1b) ...DAALW...
Pore loop 1c (PL1c) ...QAAAIR...

B

B’
torsinA-mGFP
torsinA-mGFP
LULL1-myc
merge

C

C’
PL1a-mGFP
PL1a-mGFP
LULL1-myc
merge

D

D’
PL1b-mGFP
PL1b-mGFP
LULL1-myc
merge

E

E’
PL1c-mGFP
PL1c-mGFP
LULL1-myc
merge

F

Fraction of cells with TorA in NE

WT PL1a PL1b PL1c

*
Figure 5-3

(A) torsinA-mGFP, Sun2, merge

(B) PL1a-mGFP, Sun2, merge

(C) PL1b-mGFP, Sun2, merge

(D) PL1c-mGFP, Sun2, merge

(E) Bar graph showing the fraction of GFP-expressing cells lacking Sun2.

(F) Western blot analysis with bands for TorA-myc and LULL1 (IP).
Figure 5-4

(A, A') torsinA-mGFP
(B, B') R288A-mGFP
(LULL1-myc) 
merge

(C) fraction cells with TorA in NE

(D) torsinA-mGFP
(Sun2) 
merge

(E) R288A-mGFP
(Sun2) 
merge

(F) fraction GFP-positive cells lacking Sun2

(G) E171Q, E171Q-R288A, wild type
(torsinA-myc) 
LULL1 (IP)

(H) wild type, R288A

(I) 

669 kDa
440 kDa
232 kDa
140 kDa
Figure 5-7

(A) U2OS

(B) hairpin 1269

(C) hairpin 1507

(D) hairpin 2088
Figure 5-8

A

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<th>376-21</th>
<th>1911-2</th>
<th>1911-8</th>
</tr>
</thead>
</table>
| ![TorsinA](image)
| ![α-tubulin](image) |

B

U2OS

mAb414

Sun2

C

mAb414

Sun2

DAPI

merge

D

hairpin 376 clone 8

![Hairpin 376 clone 8](image)

D

hairpin 376 clone 21

![Hairpin 376 clone 21](image)
Figure 5-9

A

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<th>50 nM LAP1</th>
<th>20 nM ctrl</th>
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</tr>
<tr>
<td>25 kDa</td>
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</tr>
</tbody>
</table>

α-tubulin

20 nM control siRNA  | 20 nM LAP1 siRNA

B
Sun1

C
Sun2

D
Nesprin2G

E
Nesprin3

F
LULL1
Figure 5-10

A

TorA-mGFP

no siRNA

20 nM control siRNA

B

20 nM LAP1 siRNA

10 nM LAP1 siRNA

C

D

E

F

fraction cells with TorA in NE

no RNAi

SHAM

20 nM ctrl

10 nM RNAi

20 nM RNAi

50 nM RNAi

*
Figure 5-11
Figure 5-12

A. TorA-myc TorB-myc Tor2-myc Tor3-myc

B. TorA-myc TorB-myc Tor2-myc Tor3-myc

C.

D. WB: GFP

E. WB: myc
Chapter 6

Summary and Discussion
Summary of the thesis

AAA+ ATPase enzymes couple ATP hydrolysis to the exertion of conformational change on a substrate. TorsinA is one of the few AAA+ ATPases that reside within the lumen of the mammalian endoplasmic reticulum (ER), where it performs an essential function. As an AAA+ ATPase, torsinA is expected to modulate the folded state of protein substrate(s). Determining the cellular function of this enzyme is the broad goal of this thesis.

The work presented here demonstrates that torsinA may be targeted specifically to the NE, where other studies have suggested it may function. TorsinA is dynamically targeted from ER to NE upon coexpression with LULL1; this is concomitant with displacement of NE resident transmembrane proteins including Sun2, nesprin-2, and nesprin-3. Because Sun and nesprin proteins cooperate to anchor the nucleus to the cytoskeleton, these effects suggest a role for torsinA in modulating nuclear positioning. The dystonia-causative ΔGAG mutant can also be enriched in the NE by LULL1, but is extremely inefficient at displacing Sun2. These findings identify a possible activity of torsinA at the NE as well as a mechanism for regulating this activity via LULL1, and suggest that activity at the NE is perturbed by the disease-causative ΔGAG mutation (Chapter 2). We also find that a rare second dystonia-causative mutation, R288Q, has a similar cellular phenotype to ΔGAG (Chapter 5), strengthening the argument that dysfunction of torsinA at the NE causes disease.

By analyzing the features of torsinA required for response to LULL1, we determined that an N-terminal hydrophobic domain (NTD) is required for NE targeting (Chapter 2). Surprisingly, deletion of this domain also abolishes retention of torsinA within the ER. I demonstrate that the NTD mediates stable monotopic association with the lumenal leaflet of the ER, which in turn controls ER retention (Chapter 3). It has been
shown that TMDs from some ER resident proteins control targeting by selective partitioning among different domains of the membrane bilayer. I propose that this lipid-based sorting mechanism extends to monotopic membrane proteins, and identify a group of proteins that may share torsinA’s topology and ER retention mechanism. This group includes the pharmacologically important prostaglandin synthetic enzyme COX1 (Chapter 3). This study has several exciting implications. Firstly, it identifies a previously unknown way for lumenal membrane proteins to escape “bulk flow” secretion out of the ER without the intervention of recycling receptors or vesicular coat proteins. Secondly, many monotopic membrane domains have been shown to sense membrane curvature; torsinA’s NTD could preferentially partition into relatively flat membranes, and thus contribute to torsinA’s targeting to the NE by LULL1. Finally, homology models suggest that the NTD could place torsinA’s central catalytic pore proximal to the membrane, which could have consequences for engagement with transmembrane protein substrates.

Returning to the interaction between torsinA and LULL1, I identify non-overlapping regions of LULL1 that are responsible for torsinA binding and torsinA re-targeting to the NE. Specifically, a conserved helix at the extreme C terminus of LULL1 mediates its interaction with torsinA, while residues at the extreme N terminus of LULL1 must be present in order for torsinA to be effectively targeted to the NE. Importantly, the N terminus of LULL1 is cytosolic, while the C-terminal torsinA-binding domain is lumenal, raising the intriguing possibility that control of torsinA localization involves communication between the cytosol and the ER lumen (Chapter 4). Separately, conserved cysteines in LULL1’s lumenal domain must be present in order for LULL1 to re-target torsinA, which might suggest that regulation of torsinA by LULL1 involves a redox component.
The work in this thesis provides new insights into aspects of torsinA's cellular function and dysfunction in disease, as well as the sorting mechanisms that lumenal membrane proteins, including torsinA, use to achieve ER localization.
Discussion

*Understanding torsinA’s dysfunction in dystonia*

Deletion of one of a pair of glutamic acids (E302/303) is the most common causative mutation in early onset torsion dystonia [1]. The ∆GAG mutation cannot rescue the lethality of torsinA knockout [2], lacks the ability to displace NE resident proteins in a cellular assay [3] (Chapter 2), and weakens binding to LAP1 and LULL1 [4], supporting the hypothesis that the mutant lacks the enzyme’s normal function. However, the ∆GAG mutation causes mislocalization to the NE [5, 6], overexpressed ∆GAG-torsinA inhibits flux through the secretory pathway [7], and ∆GAG-torsinA can also assemble into a hexamer (Chapter 5), suggesting a means for a toxic gain of function. Unfortunately, mice heterozygous (∆GAG/+) for the DYT1 mutation, which should model the human disease state, do not exhibit outward signs of disease or inward signs of pathology [2], which has proven a roadblock to studying the disease. Brain imaging studies of human subjects have made some headway in identifying changes to brain circuitry in the disease state [8, 9], and these differences were recently confirmed to also exist in the mouse model [10]. A key consideration in understanding torsinA function may be the developmental window in which torsinA is required. ∆GAG/∆GAG mice gestate normally, but die shortly after birth; nuclear envelope membrane deformations become more pronounced as neurons mature [2]. One possibility may be that torsinA functions in the NE during or at the conclusion of neuronal migration. Continued analysis of the physiological, cell biological, and biochemical effects of this mutation will be needed to determine how and when it affects normal enzyme function and causes disease.
Is torsinA a functioning AAA+ ATPase?

Work in this thesis and contemporaneous work by other groups supports the possibility of torsinA being an active AAA+ ATPase enzyme. We determined that in cellular extracts, torsinA assembles into oligomers of approximately hexameric size (Chapter 2), which is typical of Class II AAA+s. Purified OOC-5 (the C elegans homolog) becomes more thermostable in the presence of nucleotide, which likely means that torsinA at least binds to ATP [11]. Weak hydrolysis of ATP has been reported with the purified AAA+ domain of human torsinA, although this fragment was monomeric [12]. Further, various mutations to the catalytic domain have been shown by our laboratory and others to affect torsinA’s behavior in vivo [4-6, 13]. We have determined that torsinA associates with membranes via a hydrophobic N terminal domain (Chapter 3). Deletion of this domain affects cellular readouts of torsinA activity (Chapter 2), and it may also be that this domain (and an associated membrane) must be present for optimal ATPase activity of torsinA. A future goal should be to determine conditions for isolation of an active oligomer with ATPase activity that can be reproducibly observed; it would then be possible to determine whether this activity is impaired by dystonia-causative mutations or modulated by the NTD, LAP1, LULL1, or putative substrate proteins such as the Suns and nesprins.

Functional significance of torsinA’s effects on LINC complex proteins

We have established that torsinA moves into the NE and displaces select NE resident proteins when induced to move there by LULL1. These proteins are components of the linker of nucleoskeleton and cytoskeleton (LINC) complex, which functions in nuclear positioning by tethering the nuclear lamina to the cytoskeleton [14, 15]. Correct LINC complex function is required for establishment of cell polarity and subsequent cellular migration; these proteins are thus important for proper development.
of the brain [16] and other tissues [17, 18]. Data in model organisms [19] and in cellular assays [20] support the idea that torsinA has a role in nuclear movement. However, antibody limitations prevent us from determining whether endogenous torsinA enriches in the NE in response to increased LULL1 expression. Depletion of endogenous torsinA by RNAi did not seem to affect LINC complex protein levels or localization in our hands (Chapter 5) although nesprin-3 displacement has been reported in torsinA -/- fibroblasts [20] and depletion of the cofactor LULL1 did cause mislocalization of Sun2 (Chapter 5).

The displacement of LINC complex proteins from the NE is admittedly an indirect readout of torsinA’s putative activity. We know that torsinA causes the displacement of Sun2, nesprin2, and nesprin3 from the NE when it is concentrated there. However, we know little about the molecular changes taking place to these proteins and their binding partners at the NE. It may be that these three proteins show this change in localization because torsinA has dissolved the interaction(s) responsible for retaining them at the NE. If torsinA is disassembling intact LINC complexes, this could then allow the nucleus to move or rotate within the cell. It is important to note that similar conformational changes could be happening to other proteins that could affect their oligomeric state or interaction with partners without causing their displacement from the NE. We do not yet have a full picture of torsinA’s activity on NE structure and components.

Preliminary data (Chapter 5) indicate that torsinA may play a role in interphase NPC insertion, a process that every cycling cell participates in [21]. Our laboratory previously reported that overexpressed E171Q-torsinA concentrates in pore-depleted regions of the NE, inducing ultrastructural deformations including NE thinning and bubbling [6]. The idea of a molecular machine such as an ATPase participating in membrane deformation to allow NPC insertion is intuitively attractive. Intriguingly, very recent (unpublished) work indicates that Sun1, which is known to associate more intimately with NPCs than Sun2 [22], is involved in this process as well (Talamas et al, in
press). We found that torsinA does not displace Sun1 from the NE (Chapter 2), but it is possible that torsinA could modulate Sun1 in other ways. For instance, it is known that Sun1 and Sun2 hetero-oligomerize via their lumenal coiled-coil domains [23], although it is not clear how formation of these oligomers relates to participation in LINC complexes.

To gain greater resolution of torsinA’s activity on the LINC proteins, it will be necessary to reconstitute the interaction of torsinA with individual LINC proteins and intact LINC complexes.

The significance of torsinA’s interactions with LAP1 and LULL1

LAP1 and LULL1 bind to torsinA in an ATP-dependent fashion [4, 24], and LULL1 co-assembles into an oligomer with ATP-trapped E171Q-torsinA (Chapter 4). Similarly to other AAA+ ATPases with divergent Walker-A motifs [25, 26], it could be that interaction with cofactors stimulates torsinA’s ATPase activity. This interaction would then be a means of regulating torsinA’s enzymatic activity on substrate protein(s).

The consequences of torsinA’s interaction with LULL1 have been a major focus of the work presented here. LULL1’s ability to promote accumulation of torsinA in the NE has parallels to the ability of other AAA+ cofactors to direct enzymes to their targets [27]. The activating signal could involve the transmission of a conformational change from LULL1 to torsinA; alternatively, other domains of LULL1 could serve as a platform for interaction of torsinA with NE resident binding partners. Control of this signal could be achieved by controlling LULL1 expression level or addition of activating/inhibitory modifications to LULL1, to name a few possibilities. Analysis of the features of the LULL1-torsinA interaction and identification of any additional cellular factors that promote torsinA activation will continue to further our understanding of regulation of torsinA by LULL1.
Less is understood about the importance of LAP1 to torsinA function. LAP1 is an abundant component of the inner nuclear membrane that directly interacts with the nuclear lamina [28]. Mutations that trap torsinA on ATP (so-called ‘substrate traps’) also cause torsinA to accumulate in the NE [6], and this relies on the presence of LAP1 in the NE [29]. However, interaction of torsinA with LAP1 at the NE does not promote displacement of LINC proteins as seen with LULL1 [3](Chapter 2). Depletion of LAP1 in mice is perinatally lethal, and causes ultrastructural abnormalities at the NE that bear similarity to the phenotype observed in neurons lacking torsinA [30]. Depletion of LAP1 in cells did not significantly affect LINC complex protein localization or LULL1-dependent torsinA targeting (Chapter 5). It may be that LULL1 and LAP1 promote distinct functions of torsinA at the NE. Separately, it is possible that LAP1 has torsinA-independent functions the nuclear periphery.

LAP1 function has not been studied extensively; however, it was noted that LAP1 is found in membrane vesicles associated with the mitotic spindle during mitosis [31]. More recently, it was demonstrated in an RNAi screen that ablation of LAP1 results in a mitotic delay [32]. This delay was determined to arise from failures in mitotic spindle establishment. Somewhat surprisingly, torsinA did not exhibit a related phenotype in this screen. Such a finding could indicate an independent function for LAP1, or could suggest that a redundant enzyme compensates when torsinA is knocked down. Regardless, a potential role of LAP1 in mitotic spindle formation could be an exciting area for future exploration.

*Do torsinA and torsinB have overlapping functions?*

TorsinA and TorsinB are the most closely related torsin family members, having ~70% homology between the human sequences [33]. TorsinB can interact with LAP1 to some extent [29, 30], so it is possible that LAP1 and/or LULL1 might also influence
torsinB activity. TorsinA-null mice have distorted NEs exclusively in neurons; knockout of torsinB in fibroblasts from these mice caused the emergence of these ultrastructural defects [30]. Combined with the fact that torsinA and B are coexpressed in most tissues, but only torsinA is expressed in neurons, these data suggest that torsinB may compensate for torsinA loss in non-neuronal tissues [30]. If this is the case, this may help to explain the contradiction between the relatively mild effects of depleting torsinA in most cells and the basic cellular functions ascribed to the enzyme. Dual depletion of torsinA and torsinB may reveal more dramatic effects on NE structure and function.
References


Appendix I

Generation of affinity-purified LAP1 and LULL1 antibodies
In order to study the roles of LAP1 and LULL1 proteins in regulating torsinA function, it is desirable to have a method of detecting the endogenous proteins. We chose to generate antibodies to human LAP1 and LULL1, using the divergent extraluminal domains of these two proteins as antigens.

A glutathione-S-transferase (GST) epitope tag was affixed to the N terminus of the protein domains for affinity purification. Plasmids encoding GST-tagged LAP1 or LULL1 residues 1-217 were transformed into *E. coli* BL21-CodonPlus(DE3)-RIL cells (Stratagene) and grown on agar plates. The resulting colonies were used to inoculate 2 x 500 mL flasks of TB media. Cultures were grown at 37°C until density reached an OD$_{600}$ of ~0.8. Cultures were then cooled to room temperature, induced with 0.5 mM IPTG, and grown an additional 3 hours at room temperature. The cultures were collected by centrifugation and lysed in buffer containing 100 mM HEPES pH 7.4, 300 mM NaCl, 5% glycerol, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF by 3 x 30-second pulses of sonication on ice. Triton-X-100 detergent was then added to 1% final concentration and samples were solubilized for 30 minutes at 4°C. Soluble material was harvested by centrifugation at 12,000 rpm for 10 minutes at 4°C. Glutathione-conjugated Sepharose beads (GE Healthcare) were equilibrated in lysis buffer, then incubated with the supernatant for 1 hour at 4°C. The mixture was then centrifuged at 1000 rpm for 5 minutes at 4°C to separate unbound material. The resin was washed with lysis buffer supplemented with Triton-X-100 detergent, then incubated in lysis buffer supplemented with 50 mM glutathione to elute protein. Elutions were collected by centrifugation at 1000 rpm. Analysis of the eluates by SDS-PAGE and Coomassie stain demonstrates that the GST fusion proteins were purified to homogeneity (Figure A-1). GST-LAP1 1-217 did not elute efficiently from the glutathione resin; protein was released by boiling the resin in SDS-PAGE sample buffer. Several milligrams of purified protein were then run on a
preparative SDS-PAGE gel, and the fixed and Coomassie-stained gel slices were sent to Sigma Genosys for rabbit immunization and serum production.

The crude sera were reactive to overexpressed and endogenous protein (Figure A-2) as well as to GST (not shown). The sera were depleted of GST-reactive antibodies by incubation with GST protein, and subsequently affinity purified on immobilized GST-LAP 1-217 or LULL 1-217. Affinity purification increased antibody specificity and ability to detect endogenous protein. For an example of detection of LULL1 by Western blot with affinity-purified serum, see Figure 2-3. See Figure A-3 for an example of specific detection of LAP1 with affinity-purified serum. LAP1 is spliced into up to three distinct proteins- LAP1A, LAP1B, and LAP1C; the variable sequences are found in the nucleoplasmic domain, which was used as the antigen for antibody production. The affinity-purified LAP1 serum variably recognizes one to three bands of distinct molecular weight in U2OS cells.

The ability of the affinity purified sera to detect LAP1 and LULL1 by immunofluorescence was also tested. U2OS cells expressing LULL1-myc or LAP1-mGFP were fixed for 10 minutes in 4% paraformaldehyde, followed by permeabilization for 10 minutes in 0.2% Triton-X-100, blocking for 30 minutes in 2% goat serum, and incubation with the indicated antibodies. The affinity purified antibody signals were comparable to the epitope tag signals (Figure A-4A, A-4B). In U2OS cells, which express sufficient endogenous LAP1 and LULL1 to be detected reliably on a Western blot (see above), immunofluorescence signals in the expected cellular compartments are observed. LULL1 is detected in the NE and ER; the signal appears better preserved in cells that were permeabilized with 0.1% saponin (Figure A-4C) than in cells permeabilized with 0.2% Triton-X-100 (Figure A-4D). LAP1, conversely, is detected clearly at the NE in cells permeabilized with 0.2% Triton-X-100 (Figure A-4F), and less so in cells permeabilized with 0.1% saponin (Figure A-4E). This detection only after
incubation with more stringent detergent may reflect the fact that the LAP1 epitope lies within the domain known to associate with the nuclear lamina.

Finally, the ability of the sera to immunoprecipitate endogenous LAP1 and LULL1 was assessed. U2OS cell lysates from a 35 mm culture dish were incubated with two microliters of affinity-purified anti-LAP1 or anti-LULL1 serum, followed by immunoprecipitation of the protein:antibody complexes with Protein G-conjugated Sepharose. As demonstrated in Figure A-5, the affinity purified sera also immunoprecipitate endogenous LAP1 and LULL1 well.
Figure Legends

Figure A-1. GST affinity purification of LAP1 and LULL1 extralumenal domains from *E. coli*. (A) GST-LULL1 1-217 or (B) GST-LAP1 1-217 were expressed and purified from *E. coli* by glutathione resin affinity purification. Aliquots of unbound material, glutathione-eluted material, and material remaining on glutathione resin were analyzed by SDS-PAGE and Coomassie stain.

Figure A-2. Western blots with sera generated against LAP1 and LULL1. (A) Cell lysates from cells expressing LULL1-myc were subjected to Western blot with preimmune serum at 1:1000 dilution or anti-GST-LULL1 1-217 serum at 1:1000 dilution. LULL1-myc is detected by serum raised to the LULL1 1-217 antigen but not by preimmune serum. (B) Cell lysates from cells transfected (+) or not (-) with LAP1-myc were subjected to Western blot with anti-GST-LAP1 1-217 serum at 1:500 dilution. LAP1-myc and a slightly smaller band that could be endogenous LAP1 are detected by the serum. Nonspecific lower molecular weight bands are also apparent.

Figure A-3. Affinity purified serum to human LAP1 specifically detects endogenous LAP1 in U2OS cells. A specific ~60 kDa band corresponds to human LAP1 and is detected in untreated or control siRNA-treated cells, but is absent in cells treated with 10-50 nM of LAP1 siRNA.

Figure A-4. Immunofluorescent detection of LAP1 and LULL1 with affinity purified sera. (A) Cells stably expressing LULL1-myc were costained with myc and AP-LULL1 antibodies at 1:500 dilution; myc and AP-LULL1 signals coincide. (B) Cells transiently expressing LAP1-mGFP were stained with AP-LAP1 antibody at 1:100 dilution. AP-LAP1 recognizes LAP1-mGFP and more dimly, endogenous LAP1 in nearby cells. (C-F) After
fixing, U2OS cells were permeabilized with the indicated detergents for 10 minutes, followed by blocking in goat serum and incubation with (C-D) AP-LULL at 1:100 dilution or (E-F) AP-LAP1 at 1:100 dilution.

Figure A-5. Affinity purified antibodies immunoprecipitate endogenous LULL1 and LAP1. (A-B) U2OS cell lysates from 35 mm dishes were incubated with two microliters of the indicated antibodies, followed by immunoprecipitation of the protein:antibody complexes with Protein G-conjugated Sepharose beads. Immunoprecipitated material was detected by Western blot with the same antibodies. The relative positions of the immunoprecipitated protein and the cross-reacting antibody chains (IgG) are indicated
Figure A-2

A

preimmune

LULL serum

150 kDa
100 kDa
75 kDa
50 kDa

LULL1-myc

B

- + LAP1-myc

75 kDa
50 kDa

LAP1-myc

LAP1
Figure A-3

[Image: Western blot analysis showing LAP1 and α-tubulin.]

- LAP1 bands at 25 kDa, 37 kDa, 50 kDa, and 75 kDa.
- α-tubulin bands at 25 kDa.

Conditions:
- 10 nM LAP1
- 20 nM LAP1
- 50 nM LAP1
- 20 nM ctrl
- Negative control
Figure A-4

A. LULL1-myc  AP-LULL1 1:500  merge

B. LAP1-mGFP  AP-LAP1 1:100  merge

C. AP-LULL1 1:100  0.1% saponin

D. AP-LULL1 1:100  0.2% Tx100

E. AP-LAP1 1:100  0.1% saponin

F. AP-LAP1 1:100  0.2% Tx100
Figure A-5

A

-  + AP-LULL1

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LULL1

IgG

B

-  + AP-LAP1

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LAP1

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