Atlastin GTPases are required for Golgi apparatus and ER morphogenesis

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Received December 18, 2007; Revised January 16, 2008; Accepted February 7, 2008

The hereditary spastic paraplegias (SPG1-33) comprise a cluster of inherited neurological disorders characterized principally by lower extremity spasticity and weakness due to a length-dependent, retrograde axonopathy of corticospinal motor neurons. Mutations in the gene encoding the large oligomeric GTPase atlastin-1 are responsible for SPG3A, a common autosomal dominant hereditary spastic paraplegia. Here we describe a family of human GTPases, atlastin-2 and -3 that are closely related to atlastin-1. Interestingly, while atlastin-1 is predominantly localized to vesicular tubular complexes and cis-Golgi cisternae, mostly in brain, atlastin-2 and -3 are localized to the endoplasmic reticulum (ER) and are most enriched in other tissues. Knockdown of atlastin-2 and -3 levels in HeLa cells using siRNA (small interfering RNA) causes disruption of Golgi morphology, and these Golgi structures remain sensitive to brefeldin A treatment. Interestingly, expression of SPG3A mutant or dominant-negative atlastin proteins lacking GTPase activity causes prominent inhibition of ER reticularization, suggesting a role for atlastin GTPases in the formation of three-way junctions in the ER. However, secretory pathway trafficking as assessed using vesicular stomatitis virus G protein fused to green fluorescent protein (VSVG-GFP) as a reporter was essentially normal in both knockdown and dominant-negative overexpression conditions for all atlastins. Thus, the atlastin family of GTPases functions prominently in both ER and Golgi morphogenesis, but they do not appear to be required generally for anterograde ER-to-Golgi trafficking. Abnormal morphogenesis of the ER and Golgi resulting from mutations in atlastin-1 may ultimately underlie SPG3A by interfering with proper membrane distribution or polarity of the long corticospinal motor neurons.

INTRODUCTION

The hereditary spastic paraplegias (HSPs) are a clinically and genetically diverse cluster of inherited neurological disorders in which the primary manifestations are progressive spasticity and weakness of the lower limbs due to a length-dependent retrograde axonopathy of the corticospinal upper motor neurons (1–5). The autosomal dominant HSP SPG3A is the second most common HSP overall, and most common young-onset form (6–11). It is caused by well over 30 different mutations, predominantly missense mutations, in the atlastin-1 gene. Based on its similarity to proteins in the dynamin superfamily of large GTPases (4,6,12–14) and on recent studies in heterologous cell culture systems (15), atlastin-1 has been implicated in intracellular membrane trafficking, particular at the ER (endoplasmic reticulum)-to-Golgi interface (12,15). However, atlastin-1 is predominantly localized to the central nervous system, and its expression levels are much lower in peripheral tissues (6,12), indicating that it may have specific functions in the central nervous system. To gain further insight into the cellular functions of atlastin-1, we have investigated two closely related human proteins, atlastin-2 and atlastin-3 (12,14). Although these proteins are highly similar structurally to atlastin-1, their
distributions among tissues vary substantially. We have found that knockdown of atlastin-2 and -3 in cells causes abnormalities in Golgi morphology, most commonly fragmentation. Interestingly, expression of SPG3A mutant and dominant-negative forms of atlastin-1, -2 or -3 that lack GTPase activity resulted in prominent changes in ER morphology, with loss of typical reticularization. However, both dominant-negative and atlastin siRNA knockdown studies revealed essentially normal ER-to-Golgi trafficking. Thus, the atlastins constitute a family of large GTPases that function in morphogenesis of the ER and Golgi apparatus. Since a number of SPG3A mutations in atlastin-1 impair its GTPase activity (13), abnormal morphogenesis of the ER and Golgi may underlie this form of HSP.

RESULTS

Atlastin family of GTPases

We investigated several large GTPases highly homologous to the SPG3A protein atlastin-1. In humans there are three atlastin family members, which we have named atlastin-1, -2 and -3 (12,14). Indeed, a ClustalW phylogenetic tree of these proteins indicates that this division is conserved in a variety of rodents and higher mammals (Fig. 1A). However, some species such as Drosophila melanogaster (D. melanogaster) (16), Caenorhabditis elegans (C. elegans), and the sea urchin Strongylocentrotus purpuratus (S. purpuratus) express only one atlastin, indicating that the three atlastins in higher...
species may have at least partially overlapping functions. Indeed, in humans and rodents, atlastin-1 is expressed at very low levels outside of the central nervous system (6,12).

To study the biochemical properties, distributions, and functions of the atlastin family of GTPases, we generated antibody and peptide antibodies specific for atlastin-2 and -3; we had previously generated specific antibodies to atlastin-1 (12,13). As shown in Figure 1B, these antibodies were subtype-specific in their detection of recombinant proteins overexpressed in COS7 cells. Atlastin-2 exists in two splice forms with different C-termini (isoforms 2a and 2b; Supplementary Material, Fig. S1), and one antibody against atlastin-2 was specific for the 2b isof orm (Fig. 1B). Since the atlastin-2b antibody had much higher affinity than the anti-atlastin-2ab antibody, we used this antibody preparation for subsequent experiments investigating atlastin-2. An antibody generated against a peptide sequence identical in all three atlastins detected all subtypes equally, confirming that expression levels of the recombinant proteins were similar (Fig. 1B). Antibodies against atlastin-1 and atlastin-2b detected endogenous proteins of identical size to the recombinant proteins overexpressed in 293 cells (Fig. 1C). However, two different start sites for translation initiation have been suggested for human atlastin-3 (Supplementary Material, Fig. S1). Overexpressing short and long forms using these different start sites and comparing their migrations on SDS–PAGE to that of the endogenous atlastin-3 protein in 293 cell extracts indicated that the shorter form resulting from use of the more 3′ initiator ATG codon corresponds most closely in size to the endogenous protein. Thus, numbering for the human atlastin-3 amino acid sequence commences at this latter initiator methionine (Supplementary Material, Fig. S1).

We examined the distributions of atlastin proteins in human tissues and cell lines. In human tissues, we found that while atlastin-1 is largely localized to brain, atlastin-2 and -3 were expressed at higher levels in peripheral tissues, and much less so in brain. Similarly, in multiple cell lines tested the atlastin proteins had mostly complementary distributions, with some overlap (Fig. 1D and E).

**Homo-oligomerization of atlastins**

Previously, we suggested that the atlastin-1 protein is an integral membrane protein with both N- and C-termini facing the cytoplasm using protease protection assays. Also, using Triton X-114 phase partitioning and membrane association assays, we found that the C-terminal hydrophobic domains are necessary and sufficient for atlastin-1 membrane localization (12). We similarly found that both atlastin-2 and -3 are integral membrane proteins (Fig. 2A and B) with N- and C-termini facing the cytoplasm, using the same approaches (Fig. 2C and D).

Atlastin-1 exists as an oligomer, most likely a tetramer based on chemical cross-linking and gel-exclusion fast protein liquid chromatography (FPLC) studies (12). We found similar chemical cross-linking patterns for atlastin-2 and -3 and similar elution patterns on gel-exclusion FPLC (Fig. 3A and B). Importantly, co-immunoprecipitation studies of atlastin-1, -2 and -3, all of which are present in human 293 cells (Fig. 1E), demonstrated that anti-atlastin-1 antibodies only co-immunoprecipitate atlastin-1, whereas atlastin-2 antibodies only co-precipitate atlastin-2 (Fig. 3C). Thus, endogenously these proteins occur as homomeric complexes. On the other hand, upon overexpression these proteins form heterologous complexes, and in yeast two-hybrid assays, each atlastin showed the capacity to interact with the others as well (Supplementary Material, Fig. S2).

**Atlastin-2 and -3 localize to the ER along microtubules**

To determine whether the fact that atlastins form homomers in vivo reflects distinct subcellular localizations, we examined the distributions of atlastin-2 and -3 in HeLa cells. In contrast to the atlastin-1, atlastin-2 and -3 exhibited very little co-localization with the cis medial–Golgi marker GM130 (Fig. 4A). However, in HeLa cells transfected with the temperature-sensitive ts045 vesicular stomatitis virus G protein fused to green fluorescent protein (VSVG-GFP) construct and held at 40°C to label the ER, both atlastin-2 and atlastin-3 showed prominent localization to the ER (Fig. 4B); similar results were seen using anti-calnexin antibodies to label the ER (unpublished data). Even so, the atlastin-2 staining pattern consistently appeared more reticular, whereas atlastin-3 staining was more punctate.

We examined the localization of atlastin-2 and -3 by immunogold electron microscopy and found that gold particles not only decorated ER tubules and vesicular tubular complexes (VTCs) in the vicinity of the Golgi apparatus, but also localized to presumptive ER membranes extending along the length of microtubules (Fig. 5). Although both atlastins were associated to the same membrane compartments ultrastructurally, overall differences were observed in the density of gold particles. For instance, the atlastin-2 staining pattern revealed a particularly high proportion of gold particles associated with ER membranes along microtubules as compared with atlastin-3 staining (Fig. 5). Neither atlastin-2 nor atlastin-3 was visualized on the nuclear envelope (unpublished data).

**Knockdown of atlastin-2 and -3 disrupts Golgi morphology**

To assess the functional roles of the atlastins in intracellular membrane trafficking and morphology, we examined the effects of depleting atlastin-2 or -3 in HeLa cells using siRNAs. HeLa cells express atlastin-2 and -3 abundantly, but have extremely low levels of atlastin-1 (Fig. 1E). We were able to achieve significant knockdown of atlastin-2 and -3 at 72 h after transfection and found that upon the specific knockdown of either of these isoforms, there was no up-regulation of the other atlastin within these cells (Fig. 6A). We also achieved significant double knockdown of both atlastin-2 and -3 at 72 h after transfection (Fig. 6B). We then examined the cells for morphological changes, and we found a substantial change in Golgi morphology upon depletion of atlastin-2, -3, or both (Fig. 6C and D, and unpublished data). On the other hand, ER morphology was normal except in a small subset of double knockdown cells where the ER clearly was more tubular, with loss of typical reticularization (unpublished data).

Two main Golgi phenotypes as assessed by GM130 staining were detected with both the atlastin-2 and atlastin-3 specific siRNAs. One was a widespread distribution of fragmented...
GM130-positive structures (Fig. 6D, middle panels), whereas the other was characterized by a more elongated, tubular pattern (Fig. 6D, lower panels). The proportion of cells showing each pattern differed among the knockdown groups, with the atlastin-2 siRNA group producing a higher percentage of cells with the tubular phenotype and the atlastin-3 siRNA and the double knockdown groups having a majority of cells with a more dispersed, ‘mini-stack’ distribution pattern. Mini-stacks are small, organized stacked structures with Golgi proteins adjacent to ER exit sites. After microtubule disassembly using drugs such as nocodazole, electron microscopy studies have shown that VTCs form initially, but over time, a stacked morphology more typical of the Golgi complex appears (17). The Golgi apparatus was considered ‘disrupted’ in those cells exhibiting either a fragmented or tubular Golgi morphology. Importantly, disruption of Golgi morphology was present in ~80% of atlastin-2 and -3 siRNA cells, significantly higher than the percentage in control siRNA cells ($P < 0.001$; Fig. 6C).

Abnormal Golgi structures in atlastin-2 or -3 depleted cells are brefeldin A sensitive

To determine whether the GM130-positive structures are within the ER or else membrane compartments formed after exiting the ER, we used brefeldin A (BFA), a drug commonly used to block transport of secretory cargoes from the ER to the Golgi apparatus by inhibiting the Arf1 GTPase. HeLa cells transfected with control, atlastin-2, or atlastin-3 siRNAs were re-transfected with an YFP-Golgi construct and then treated with BFA a day later. Live images of the cells were acquired every 30 s during the BFA treatment to assess any
redistribution of cargo protein from the abnormal membrane compartments present in the atlastin-2 and -3 siRNA cells (Fig. 7). Surprisingly, both the punctate and the tubular structures identified in the atlastin-2 and -3 siRNA groups were BFA-sensitive, confirming that upon loss of either atlastin, proteins are still able to exit the ER. Redistribution of YFP-Golgi can be followed over 25 min following BFA treatment in control siRNA cells (Supplementary Material, Video 1), and a similar redistribution can be clearly seen for the same duration following BFA treatment in atlastin-2 and atlastin-3 siRNA cells (Supplementary Material, Videos 2 and 3).

VSVG trafficking is not impaired in cells lacking atlastin-2 and -3

Because depletion of atlastin-2 or -3 altered the Golgi morphology, we next assessed whether knockdown of atlastin-2, -3, or both impaired protein trafficking through the Golgi apparatus, using ts045 VSVG-GFP as a reporter. Two days after transfection of HeLa cells with control, atlastin-2, atlastin-3, or both atlastin-2 and -3 siRNAs, all groups were transfected with VSVG-GFP and placed overnight at a temperature (40°C) that is non-permissive for VSVG exiting the ER. Cells were then transferred to a permissive temperature (32°C) and examined at various time points. In control cells, VSVG was observed at the plasma membrane as early as 90 min after the temperature change, and the majority of VSVG was present at the plasma membrane by 180 min, consistent with previous reports (18). VSVG trafficked normally to the plasma membrane in all atlastin knockdown groups examined (Fig. 8, and unpublished data), suggesting that atlastins are not required for general secretory pathway trafficking from the ER to the plasma membrane.

Dominant-negative atlastin proteins disrupt ER morphology

We found that upon expression of Myc-atlastin-1, Myc-atlastin-2, or Myc-atlastin-3 a normal, reticular ER morphology was identified (Fig. 9, top panels). However, when an SPG3A mutant form of atlastin-1 containing a mutation in a critical residue in the RD loop of the GTP-binding domain (R217Q) as well as the corresponding mutant forms of atlastin-2 and -3 (Supplementary Material, Fig. S1) were expressed in HeLa cells, the appearance of the ER changed to a more tubular, less reticular morphology (Fig. 9, middle panels). We also created dominant-negative forms of these proteins with point mutations in a key lysine residue required for GTP binding in other dynamin-related GTPases (12,19), which resulted in a very similar, and even slightly more pronounced, ER phenotype (Fig. 9, bottom panels).

Dominant-negative atlastin proteins disrupt Golgi morphology but not protein trafficking

We found that overexpression of either wild-type or SPG3A mutant forms (20) of all three atlastin proteins can result in a fragmented Golgi phenotype; however, these results were variable. Fortunately, we were able to identify a very consistent trend using the dominant-negative mutants. Overexpression of any of the dominant negative constructs resulted in significant Golgi fragmentation (Fig. 10A) in 60–65% of transfected cells (Fig. 10B). Importantly, we did not detect any impairment of VSVG trafficking to the Golgi mini-stacks and cell surface upon overexpression of any of the wild-type or mutant atlastin proteins (Fig. 10C, and unpublished data).

Interaction of atlastin proteins with spastin

Atlastin-1 has been shown to interact with microtubule-severing AAA ATPase spastin that is mutated in the most common form of autosomal dominant hereditary spastic
paraplegia, SPG4 (21,22). Since atlastin-2 and -3 localize prominently to ER membranes along microtubules (Fig. 5), we investigated whether atlastin-2 and -3 interact with spastin as well. Using yeast two-hybrid tests, we found a robust interaction of atlastin-1 with spastin, as published previously (21,22). However, neither atlastin-2 nor -3 interacted with spastin (Fig. 11A). The C-terminal 150 residues of atlastin-1 were sufficient for the interaction, but we were unable to narrow down the interaction domain further using the yeast two-hybrid system (Fig. 11B). Lauring and co-workers (22) were able to identify the C-terminal cytoplasmic domain of atlastin-1 as the region mediating the spastin interaction using fusion protein pull down assays, and this region is divergent among atlastin-1, -2 and -3 (Supplementary Material, Fig. S1).

**DISCUSSION**

We have investigated the functions of the atlastin family of GTPases, emphasizing studies of atlastin-2 and -3. We had previously reported that the SPG3A protein atlastin-1 co-localizes prominently with p115, a protein present at VTCs and cis-Golgi (12,13,23), mostly in brain, and also...
Consistent with this notion, species such as Drosophila and C elegans have only one atlastin protein. Because our experiments were carried out in HeLa cells, and levels of atlastin-1 are extremely low in these cells, we focused on knocking down atlastin-2 and -3 to assess for any intracellular changes. With the specific depletion of either atlastin-2 or -3 we saw two different types of changes in Golgi morphology, but no consistent ER phenotype. In cells where atlastin-2 and -3 double knockdowns were performed, we saw a similar degree of Golgi disruption, but also saw a more tubular ER morphology in a small subset of cells (unpublished data). In complementary experiments, we examined the structural and functional changes that occurred upon overexpression of either wild-type, SPG3A-type mutant (20), or dominant-negative forms of all three atlastins. In all cases, overexpression of the mutant, but not wild-type, atlastin proteins resulted in a change in ER morphology to a more tubular phenotype with very few interconnections by three-way junctions, which are points of homotypic fusion events in smooth ER that result in a polygon-like structure (26–28). The fact that all six atlastin mutants defective in GTPase activity produced the same morphological change, while the wild-type atlastins did not, may reflect the fact that each overexpressed mutant atlastin protein can oligomerize with all three endogenous atlastins (Supplementary Material, Fig. S2), thus impairing GTPase activity of heteromeric complexes comprising all of the atlastins. In assessing Golgi morphology, we found that overexpression of both the wild-type and SPG3A-type constructs caused fragmentation of the Golgi into what appeared to be ‘mini-stacks,’ but this phenotype varied among the mutant forms. These differences may reflect functional specificity of the different protein or variations in expression levels within the cells. Expression of dominant-negative atlastin proteins produced a more consistent effect on Golgi morphology. However, on examining effects on protein trafficking in these overexpression paradigms, we found that there did not seem to be inhibition of VSVG trafficking to the plasma membrane, even in cells with significantly altered ER morphology.

One explanation for the differences between dominant-negative studies and siRNA depletion studies, particularly with respect to changes in ER morphology, is that the loss of a single isoform may have more modest effects because the other isoforms can compensate. Furthermore, even in the atlastin-2 and -3 double knockdown cells the degree of knockdown of each isoform may not be sufficient in a majority of the cells to elicit a phenotype, and there is also a low level of atlastin-1 in HeLa cells. On the other hand, the changes in Golgi morphology upon knockdown of a single atlastin may reflect more specialized functions of each isoform at the level of VTCs or Golgi apparatus. This notion is consistent with the fact that although both Golgi phenotypes were present upon knockdown of atlastin-2 or -3, there was a clear difference in proportion of cells displaying each phenotype in each atlastin siRNA condition. Thus, the majority of atlastin-2 siRNA cells exhibited more elongated Golgi tubules, whereas the majority of atlastin-3 siRNA cells had a more fragmented ‘mini-stack’ Golgi morphology.

We considered the possibility that, in our siRNA treatment conditions, the fragmented GM130- and YFP-Golgi-positive membranes may reflect Golgi proteins retained at ER exit sites, and consequently that the more elongated, tubular structures might represent Golgi proteins trapped within the ER. However, these GM130- and YFP-Golgi-labeled compartments were BFA sensitive, clearly demonstrating that they are}

**Figure 5.** Atlastin-2 and -3 localize to ER membranes and along microtubules by immunogold electron microscopy. Electron microscopic analysis of HeLa cells immunostained for endogenous atlastin-2 (top and middle panels) or atlastin-3 (bottom panel) reveals gold particles not only at ER and VTC/cis-Golgi (top and bottom panels), but also decorating membranes along microtubules (middle panel) (G, Golgi apparatus; MT, microtubules). Bars, 100 nm.
formed after proteins have trafficked from the ER in the form of VTCs. It is likely that once VTCs have formed from the ER, atlastins are important in the movement of these vesicles along microtubules towards the MTOC (microtubule organizing center), the fusion of vesicular tubular complex (VTC) membranes, or perhaps both. The fact that these structures are BFA-sensitive is also consistent with our results showing that all atlastin siRNA cells examined were still able to traffic VSVG to the plasma membrane, with only a very small subset of cells showing a delay in trafficking. Indeed,

Figure 6. siRNA-mediated knockdown of atlastin-2 and -3 disrupts the Golgi apparatus. (A) Lysates from HeLa cells transfected with three different siRNAs specific for atlastin-2 (AT2) or else control siRNA were immunoblotted for endogenous atlastin-2 and -3 at 72 h post-transfection (left panels). Equal protein loading was monitored by immunoblotting for PLCγ. There is no upregulation of atlastin-3 protein expression upon knockdown of atlastin-2. Lysates from HeLa cells transfected with three different siRNAs specific for atlastin-3 (AT3) or else control siRNA were immunoblotted for atlastin-2 and -3, with PLCγ levels monitored as a loading control (right panels). Atlastin-2 expression is not upregulated upon knockdown of atlastin-3. An asterisk in atlastin-3 immunoblots identifies a cross-reactive band. (B) Lysates from HeLa cells transfected with both AT2 siRNA #1 and AT3 siRNA #3 or else control siRNA were immunoblotted for atlastin-2 and -3, with PLCγ levels monitored as a control for protein loading. (C) Graphical representation of percentage of cells with disrupted Golgi morphology in atlastin-2, -3, or double knockdown (DKD) conditions as compared with control siRNA-transfected cells (n = 3; 100 cells per condition). *P < 0.01; **P < 0.001. (D) HeLa cells transfected with control siRNA or atlastin-2 siRNA #1 were co-immunostained for atlastin-2 and GM130 (left panels) 72 h after transfection. HeLa cells were also transfected with control siRNA or atlastin-3 siRNA #3 and co-immunostained for atlastin-3 and GM130 (right panels). Boxed areas are enlarged in the insets to show fragmentation or tubular Golgi extending beyond the perinuclear region. Bars, 10 μm.
secretory protein trafficking does not require a stacked Golgi morphology, nor does there need to be a centralized location of the Golgi apparatus (29). This trafficking, however, may not be as efficient as in control cells, potentially accounting for the delay in VSVG trafficking seen in a small subset of atlastin-2 and -3 siRNA cells.

Thus, our results suggest that while depletion of atlastins by siRNAs and overexpression of dominant-negative, GTPase-deficient atlastin proteins have substantial effects on the morphology of the Golgi apparatus and the reticular ER structure, respectively, protein trafficking in the secretory pathway does not seem to be greatly affected. This is particularly interesting in light of a recent study that found that mutations in proteins critical for ER-to-Golgi transport in the secretory pathway affected development of dendrites far more than axons in *Drosophila* (30). Conversely, since SPG3A is a long axonopathy, and loss of atlastin-1 causes effects predominantly on the development of axons in cultured cortical neurons (13), atlastin-1, and possibly atlastin-2 and -3, may be important in other intracellular trafficking pathways, perhaps those involved in the establishment and maintenance of specific polarized membranes or membrane processes. Indeed, Sannerud *et al.* (31) have provided evidence for such a ‘Golgi-bypass’ pathway defined by Rab1 that is linked to the dynamics of the smooth ER as well as pre-Golgi intermediate compartments and functions in the delivery of membranes to developing neurites.

Mechanistically, the atlastin GTPases may serve an important role in fusion of ER membranes to form the more widely-spread ER reticulum by way of three-way junctions or else by influencing the association of ER with microtubules. Alternatively, atlastins may also play a role in VTC or Golgi ‘mini-stack’ movement along microtubules towards the MTOC as well as the formation of the normal stacked Golgi structure. In this regard, the interaction of atlastin-1, but not atlastin-2 or -3, with the microtubule-severing AAA ATPase spastin that is mutated in the most common HSP, SPG4, is particularly intriguing. It will be interesting to determine whether atlastin-2 and -3 interact with other microtubule-severing proteins, or whether this interaction with microtubule-severing enzymes is specific to atlastin-1 and represents an important functional specialization with relevance for the pathogenesis of the HSPs.

Importantly, spastin is also present at the ER, and this distribution is increased upon overexpression of atlastin-1. Furthermore, overexpression of *SPG4* mutant spastin disrupts ER morphology in conjunction with microtubule abnormalities.
and overexpression of wild-type spastin results in increased lateral mobility of the translocon complexes that are part of membrane-bound polysomes (32). Thus, proper ER morphology may be an important determinant of axon development and maintenance. Lastly, at least one additional HSP protein, the SPG17 protein seipin, localizes prominently to ER membranes (33). Thus, in addition to proteins implicated in endocytosis that are mutated in a number of HSPs (4), regulation of ER and Golgi morphology and possibly novel trafficking pathways important for membrane addition or dynamics at axons may also be a major theme in HSP disease pathogenesis.

MATERIALS AND METHODS

Eukaryotic DNA expression constructs

The pGW1 construct expressing Myc-tagged human atlastin-1 and the pRK5 construct expressing the untagged protein have been described previously (12). The full coding sequences of human atlastin-2a (GenBank accession number NM_022374) and atlastin-2b (GenBank accession number AAM97342) cDNAs were cloned into the EcoRI sites of the pGW1-Myc expression vector for production of N-terminal Myc-tagged proteins as well as the pRK5 expression vector for production of untagged proteins. The full coding sequence for human atlastin-3 (GenBank accession number AK097588) preceded by a Kozak consensus sequence was cloned into the XmaI sites of pGW1-Myc and pRK5. Where indicated for atlastin-3, another candidate initiator Met residue (as in GenBank accession number NM_015459; Supplementary Material, Fig. S1) was used. Atlastin deletion constructs were generated by PCR using Pfu Turbo, and site-directed mutagenesis was performed using the QuikChange method (Stratagene). The YFP-Golgi expression construct was purchased from BD Biosciences Clontech and the ts045 VSVG-GFP construct was provided by Dr J. Lippincott-Schwartz (34).

Antibodies

Polyclonal antibodies raised in rabbits and guinea pigs against atlastin-1 (No. 5409; residues 1–18) have been described previously (12,13). Affinity-purified polyclonal antibodies

Figure 9. Dominant-negative atlastin proteins prominently disrupt ER morphology. HeLa cells expressing Myc-tagged atlastin-1, -2 or -3 (upper panels) exhibit a typical reticular ER morphology, as revealed by co-labeling with VSVG-GFP at 4°C. However, expression of Myc-tagged SPG3A atlastin-1 mutant R217Q and the equivalent mutations atlastin-2R244Q or atlastin-3R187Q result in a more tubular, elongated ER morphology with much less branching (middle panels). This profound effect on ER morphology was also seen to the same, if not greater, extent upon expression of Myc-tagged dominant-negative mutations atlastin-1K80A, atlastin-2K107A, or atlastin-3K47A (bottom panels). Bars, 10 μm.
were generated commercially (Quality Controlled Biochemicals) in rabbits and goats against atlastin-2b (No. 6955; residues 561–578; acetyl-CIKAGLTDQVSHHARLKTD-OH) and atlastin-3 (No. 7053; residues 550–567; acetyl-CATVRDAVVGRPSMDKKAQ-OH) as well as to a region common to atlastin-2a and -2b (No. 6327; residues 1–17; MAEGDEAARGQQPHQGLC-OH) and a region identical in atlastin-1, -2 and -3 (No. 6944; residues 341–358 in atlastin-1; acetyl-LPHPKSMLQATAEANNLAC-OH), with terminal cysteine residues added to facilitate coupling to keyhole limpet hemocyanin. Other antibodies used include mouse monoclonal anti-GM130 (IgG1, clone 35; BD Transduction Laboratories), anti-Myc-epitope (IgG1, clone 9E10; Santa Cruz Biotechnology), PLCγ-1 (mixed IgGs, #05-163, Millipore), and anti-BiP/Grp78 (IgG2a, clone 40; BD Transduction Laboratories) as well as rabbit polyclonal anti-calnexin (H-70; Santa Cruz Biotechnology).

**Tissue preparation, fractionation and protease digestion**

Human tissue homogenates were obtained from BD Biosciences Clontech. Membrane association, Triton X-114
phase partitioning, and protease digestion assays were performed as described previously (12).

Protein interaction studies

Chemical cross-linking studies were performed as described previously (12). Co-immunoprecipitation studies were performed using affinity-purified goat anti-atlastin-2b (5 μg) and guinea pig anti-atlastin-1 (5 μg) antibodies. Immune complexes were collected using protein A-Sepharose, washed, resolved by SDS–PAGE, and immunoblotted with rabbit polyclonal antibodies against atlastin-1, -2b, and -3. FPLC Gel-exclusion FPLC was performed as described previously (12). Yeast two-hybrid assays were conducted in the yeast strain AH109 containing a pGBKT7-spastin bait construct (22) and atlastin-1, -2a, -2b, -3 prey in pGAD10 or pGADT7 vectors. Strength of interaction was assessed by nutritional selection on histidine-lacking media as described previously (35,36).

Cell culture and transfection

HeLa cells were maintained in Dulbecco’s MEM supplemented with 10% fetal bovine serum (Gibco). For immunostaining experiments, HeLa cells plated on coverslips in 6-well plates were transfected with 1 μg of plasmid DNA using Lipofectamine (Invitrogen). Twenty-four hours later, cells were washed in phosphate-buffered saline [PBS(phosphate-buffered saline); pH 7.4] and fixed using 4% formaldehyde. Cells to be used for immunoblot analysis were washed with PBS and lysed with 0.1% Triton X-100 in PBS.

For siRNA transfections, HeLa cells were plated at 50% confluence and transfected the next morning with 100 nM siRNA oligonucleotides using Oligofectamine (Invitrogen) for 4 h. Cells in the double knockdown conditions were treated with 150 nM of each siRNA oligonucleotide duplex. Cells were then scraped for immunoblot analysis or fixed for immunocytochemical analysis 48 and 72 h after transfection. Specific siRNA oligonucleotides (Invitrogen) for atlastin-2 were targeted against the following sequences: #1, GGGCUACCUCAUGAACAUUCAUA; #2, UCCUGUCUCUAAGUUGCAACUAUAU; and #3, GAGAGCUUCGAAACUGGUUCCAUU. Atlastin-3-specific siRNAs (Invitrogen) were as follows: #1, GCCGCACAUUGAAGGGAAAUUAAA; #2, GGCGUAUCAGGGUAUCUGGUCAA; #3, GGUUAGAGAUGGGAGUUCCCUAU. The control siRNA oligonucleotide was obtained from Ambion.

Confocal microscopy

All cells were imaged using a Zeiss LSM510 confocal microscope with a 63 × 1.4 NA Plan-APoCHROMAT lens. Acquisition was performed using LSM 510 version 3.2 SP2 software (Carl Zeiss Microimaging), and data were processed using Adobe Photoshop 7.0 and Adobe Illustrator CS2 software. For quantification of cell biology studies, at least 100 cells were counted per experimental group, with experiments conducted at least three times. Cells with fragmented or else tubular Golgi not restricted to tight cisternae within the perinuclear region were considered ‘disrupted’.

BFA treatment and VSVG-GFP trafficking

HeLa cells plated in two-well glass bottom chambers were transfected with 0.5 μg of YFP-Golgi DNA, and the following day they were placed in growth media containing 5 μg/ml BFA (Epicentre). An image was acquired before addition of the BFA and subsequent images were taken every 30 s after addition of BFA.

For VSVG-GFP trafficking studies, HeLa cells grown on coverslips were transfected with the ts045 VSVG-GFP construct and immediately placed in a 40°C incubator. Sixteen hours after transfection, HeLa cells were moved to 32°C to allow for VSVG trafficking from ER to Golgi, and then fixed and processed for immunocytochemical analysis 0, 30, 60, 90, 120 and 180 min after the temperature change.

Immunogold electron microscopy

For atlastin-2 immunogold electron microscopy, HeLa cells were fixed in 2% paraformaldehyde and 0.1% glutaraldehyde in PBS for 1 h. For atlastin-3 immunogold staining, HeLa cells were fixed in 4% paraformaldehyde in PBS for 1 h. Cells were then processed for electron microscopic analysis and imaged as described previously (12,13).
Statistical analysis
Statistical significance was assessed using two-tailed, unpaired Student’s t-tests, assuming unequal variance.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS
We thank J. Nagle and D. Kaufmann (National Institute of Neurological Disorders and Stroke DNA Sequencing Facility) for DNA sequencing, Dr J.-H. Tao-Cheng and V. Tanner-Crocker (National Institute of Neurological Disorders and Stroke EM Facility) for assistance with electron microscopy, Drs B. Lauring and J. Lippincott-Schwartz for providing materials, and Dr C.-R. Chang for experimental assistance.

Conflict of Interest statement
None declared.

FUNDING
This work was supported by the Intramural Research Program of the National Institute of Neurological Disorders and Stroke, National Institutes of Health.

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