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Fusing a lasting relationship between ER tubules

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Abstract

Atlastin is an integral membrane GTPase localized to the endoplasmic reticulum (ER). *In vitro* and *in vivo* analyses indicate that atlastin is a membrane fusogen capable of driving membrane fusion, suggesting a role in ER structure and maintenance. Interestingly, mutations in the human atlastin-1 gene, SPG3A, cause a form of autosomal dominant hereditary spastic paraplegia (HSP). The etiology of HSP is unclear but two predominant forms of the disorder are caused by mutant proteins that affect ER structure, formation, and maintenance in motor neurons. In this review, we describe what is known about the molecular mechanism of atlastin function and its potential role in HSP. Greater understanding of the function of atlastin and associated proteins should lend significant insight into normal ER biogenesis and maintenance, as well as the pathology of disease.

Membrane fusion in organelle function

The biogenesis and maintenance of eukaryotic organelles is a complex and dynamic process that requires many protein and lipid components in order to generate the compartmentalization of function that is typical of the eukaryotic cell. In some cases, like the Golgi complex, proper organelle function depends upon communication between different compartments via vesicular transport. In other cases, such as mitochondria, organelle structure is highly dynamic with membranes undergoing regular fission and fusion events, a process that is required for normal mitochondrial function. Yet a third category of organelles, which includes the ER, lysosomes, and perhaps peroxisomes, employs both vesicular transport and fusion of parts of the organelle to carry out their normal biochemical functions. In all cases, proper organelle function requires membrane fusion events.

All known biological membrane fusion is driven by specific fusion proteins, or fusogens. The first membrane fusion proteins characterized in detail were viral fusion proteins from enveloped viruses[1–6]. Intracellular fusion in the secretory pathway mediated by a protein family collectively known as SNAREs [7,8] has received much attention over the last 15 years. Recently, the identification of fusogens responsible for organelle fusion, such as occurs in ER and mitochondria, have received closer attention. Mitochondria undergo constant fusion and fission events to form highly dynamic networks whose morphology results from a balance between these two processes[9–14]. Mitochondrial shape changes are important for proper function and inheritance[15] and loss of these dynamics can result in disease[13,16,17].

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Two large GTPases called Mitofusins (Fzo)[9,18–21] and OPA1 (MGM1)[22–26] are thought to fuse the outer and inner mitochondrial membrane, respectively.

The ER is also a highly dynamic organelle that exists as an interconnected network of tubes and sheets[27–29]. While ER sheets are mostly perinuclear and contiguous with the outer nuclear membrane, peripheral tubular extensions of the ER move along microtubule tracks, and frequently join together by membrane fusion. Regions of the peripheral ER maintain close contact with virtually all other cytoplasmic organelles, including mitochondria, peroxisomes, chloroplasts, and Golgi, as well as the plasma membrane. These diverse associations might allow non-vesicular transport of ER-synthesized lipids and sterols as well as interorganelle calcium homeostasis[29]. Membrane fusion allows the ER to maintain a dynamic network that can quickly change shape and preserve lumen continuity while adapting to the changing cytoplasmic environment.

Insight into how ER fusion occurs was recently revealed by the identification of a new membrane fusion protein called atlastin[30]. *In vitro* and *in vivo* evidence suggest that atlastin is likely responsible for generating and maintaining the dynamic nature of peripheral ER tubules [30,31]. In addition, atlastin dysfunction results in a form of Hereditary Spastic Paraplegia (HSP, also called familial spastic paraparesis or Strümpell-Lorrain disease), a group of inherited neurological disorders characterized by progressive lower extremity weakness and spasticity[32,33]. This intriguing evidence suggests that ongoing shape changes and lumen continuity are important for normal ER function, and that loss of these could be implicated in a disease state. In this review, we will focus on the mechanistic basis of ER membrane fusion driven by atlastin and discuss its potential role in the etiology of Hereditary Spastic Paraplegia.

Atlastin genetics, domain architecture, and structure

Atlastin and HSP

Atlastin is the product of the SPG3A (Spastic Paraplegia Gene 3A) locus[34] and mutation of this gene is responsible for a form of HSP. The neuropathological basis for compromised motor function in HSP is likely length-dependent axonopathy of the corticospinal tract[33]. Genetic analysis has identified more than 40 different loci involved in HSP (*SPG1-45*)[32,33,35,36] and 20 HSP loci have been molecularly identified[35]. Over half of all autosomal dominant HSP (ADHSP) cases occur due to mutation in one of three proteins: Spastin (*SPAST*, *SPG4*,), a microtubule severing protein; Atlastin-1 (*ATL1*, *SPG3A*), a GTPase; and receptor expression enhancing protein -1 (*REEP1, SPG31*[37]), first identified for its role in trafficking receptors to the plasma membrane. Spastin mutations occur in 40–45% of ADHSP[38], while Atl1 and REEP1 account for 10%[39] and 3%[40], respectively. Molecular analysis of patients with HSP has identified 44 mutations in the *ATL1* gene. All of these lesions are dominant alleles and mutations have been identified in all domains of the protein.

Domain architecture

Human atlastin-1 (Atl1) is a 558 amino acid (63 kDa) multidomain protein (Figure 1). It has a short N-terminal domain, followed by a well-conserved GTPase domain, a middle domain with undefined function, two tandem transmembrane domains and a C-terminal cytoplasmic domain[41]. The original atlastin gene was renamed atlastin-1 following the identification of two additional paralogs (atlastin-2 and atlastin-3)[41].

Atlastin homologs in other species share a very similar domain architecture, typified by *Drosophila melanogaster* atlastin (Datl). The *Drosophila* genome produces a single atlastin protein that is 541 amino acids in length (Figure 1). The Datl sequence is highly

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homologous with all three human isoforms, ranging between 44–49% identical (61–68% similar) over the entire length of the protein. Functional homologs of atlastin are also found in yeast (Sey1p) and plants (RHD3)[42,43]. These proteins share limited sequence similarity with human atlastin, yet they are GTPases and possess a similar domain structure[31].

The atlastins are closest in sequence to the human guanylate binding proteins (GBPs). Together, they have been grouped with the dynamin family of GTPases and loosely termed "dynamin-like" family members[44]. The inclusion of atlastins and GBPs in this group is based entirely on homology within the N-terminal GTPase domain. The other conserved regions that define dynamins and are now known to provide membrane fission activity, including the GED, PH and PRD domains[45], are absent in atlastin. Additionally, atlastin contains two tandem membrane spanning regions that are necessary for membrane fusion activity and are absent in dynamins. Distinct differences are even present within the conserved GTPase domain. Atlastins and GBPs share a unique RD motif in the G4 GTP-binding domain that interacts with the nucleotide base that is different from what is found in dynamins, dynamin-like proteins and even mitofusins[44,46]. Based on recent functional analysis of atlastin[30] and the strong *in vivo* evidence that mitofusins are involved in mitochondrial membrane fusion[47], we conclude that atlastins and mitofusins act as fusion GTPases, while dynamins and related fission molecules such as DRP1 act as fission GTPases.

All atlastins contain two hydrophobic regions that are predicted to span the membrane. Biochemical fractionation revealed that Atl1 is an integral membrane protein with both N- and C-termini exposed to the cytoplasm[41]. Most large GTPases form higher order oligomeric structures and Atl1 has been shown to self-associate. *In vitro* analysis of the N-terminal cytoplasmic domain of Atl1 revealed that it is a monomer that shifts to a size consistent with a dimer in the presence of nonhydrolyzable GTP[48]. However, immunoprecipitated full-length Atl1 migrated as an apparent homotetramer by gel filtration[41], as did Atl2 and Atl3[49]. Either the transmembrane segments and the C-terminal tail influence oligomerization, or the immunoprecipitated material from detergent extracts contained additional mass not attributable to atlastin. Attempts to identify heteroligomeric complexes has met with mixed results. Endogenous Atl1 and Atl2 do not coprecipitate other atlastins; however, mixed heteroligomeric complexes can be coprecipitated when atlastins are overexpressed[49].

Atlastin structure

The structure of the N-terminal cytoplasmic domain (residues 1–446) of human Atl1 was solved by X-ray crystallography in two recent studies[48,50] (Figure 2C). The globular GTPase domain bears a strong resemblance to the equivalent region of guanylate binding protein1 and dynamin. The middle domain folds into a three-helix bundle that connects to the GTPase domain by a flexible linker. Interestingly, two crystal forms were identified that differed in the relative arrangement of the three-helix bundle and the GTPase domain resulting from alterations in the linker (Figure 2C). Additionally, crystal packing contacts between adjacent monomers in the crystal suggested that dimerization could occur between GTPase domains, similar to GBPs[48]. Dimer models of both crystal forms suggest the intriguing possibility that form 2 is a dimer that could bridge membranes and that form 1 represents a post-fusion structure (Figure 2B and C) [51]. While these dimer models are suggestive, they must be interpreted cautiously. Additionally, analysis of Atl1 monomers and dimers in solution by small angle X-ray scattering (SAXS) showed that the solution structure differs significantly from the static X-ray structures of the monomer and the hypothetical dimers[48].

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Atlastin function in cultured cells and model organisms

Localization

The subcellular distribution of atlastin has been examined in many cell types by a variety of techniques. Atl1 has been found in the ER[52,53], Golgi[41], and vesicular structures around the ER[43]. The Atl1 paralogs Atl2 and Atl3 have also both been localized to the ER[49], as has Drosophila atlastin[30]. Recent analysis of Atl1 localization in zebrafish primary neurons found that Atl1 co-localized with endosomal markers[54]. All atlastins that have been examined contain an ER retention signal at their extreme C-terminus, are most commonly found in the ER, and therefore likely function in this compartment; however, additional sites of action cannot be excluded due to the observed localization to other compartments.

Phenotypic effects of up- and down-regulation of atlastin expression

HeLa cells normally express low levels of endogenous Atl1 and abundant amounts of Atl2 and Atl3[49]. siRNA-mediated knockdown of Atl2 or Atl3 results in a normal ER morphology by fluorescence microscopy with a small subset of double knockdown cells showing more tubular, less reticular ER. The most pronounced effect of atlastin reduction is on Golgi morphology[49]. In this study, the Golgi in Atl2 or Atl3 knockdown cells was fragmented resembling “mini-stacks” or elongated tubules, however, protein trafficking was normal. The reason for the dramatic change in Golgi morphology and relatively minor effect on ER structure remains obscure. These morphological changes may be an indirect result of altered communication between the ER and subsequent secretory compartments that is not reflected in bulk protein traffic.

In this same study, overexpression of wildtype myc-Atl1 -2 or -3 did not noticeably affect ER morphology by light microscopy, but produced a fragmented Golgi[49]. However, overexpression of GTPase-deficient mutants of atlastin resulted in more elongated and tubular ER with less branching, as well as a fragmented Golgi. These effects may be due to a dominant negative effect of the overexpressed protein. Overexpression of either wildtype or mutant atlastins did not significantly affect protein trafficking. The effects of atlastin reduction were also examined in neurons, the primary site of action for HSP. Atl1 knockdown in primary rat cerebral cortical neurons showed reduced axonal length, an increased number of neurons without axons and a reduced number of dendrites per cell[55]. It is possible that specific ER functions such as appropriate calcium homeostasis are required for proper neurite outgrowth or axonal pathfinding and these functions are disrupted when atlastin-mediated fusion is lost.

Atlastin interactions—Atl1 has been shown to associate with spastin, a microtubule severing protein[52,5657], and recent work has also demonstrated that atlastin interacts with all of the ER tubule forming proteins in the Reticulon and REEP/Yop/DP1 family[31,57]. An interaction between Atl1 and spastin has been reported by several groups[49,52,56,57], but there is conflicting evidence with regard to the interacting regions. In one case, the N-terminal 80 amino acids of spastin (which includes a putative membrane spanning domain) were required for an interaction with the N-terminal cytoplasmic domain of Atl1, a construct that lacks transmembrane segments[52]. In another series of experiments, the C-terminus of Atl1 (residues 408–558, which contains the transmembrane domains and the C-terminal tail) was required for an interaction with full-length spastin, suggesting a mutually exclusive region of Atl1 required for spastin interaction [56]. Most recently, an association between three HSP-related proteins, atlastin, spastin and REEP1, was reported[57]. This work showed that the interaction between atlastin and REEP1 likely occurs through hydrophobic membrane spanning domains of each protein[57], similar to atlastin s interaction with other reticulons[31]. This recent study also reported that the interaction between spastin and atlastin required the

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N-terminal transmembrane segment of the M1 isoform[57] of spastin and the two tandem TMDs of atlastin. The disagreement in the literature on which portion of atlastin is responsible for the interaction with spastin suggests that this interaction should be interpreted cautiously. Additionally, spastin interaction with atlastin is limited to atlastin-1, since atlastin-2 and atlastin-3 do not appear to interact with spastin[49]. While an association between the ER tube forming proteins and atlastin may spatially restrict the location of atlastin within the ER tube where it is functionally required for fusion, a functional link between the microtubule severing protein spastin and atlastin could be utilized to organize ER tubules and the microtubule cytoskeleton into a regulated network.

Atlastin function in model organisms—The first phenotypic characterization of atlastin function in animals was done in *Drosophila* where a P-element insertion in the fly atlastin gene (atl^1) was identified during a large scale behavioral screen[58]. atl^1 mutants are temporarily paralyzed by mechanical shock and classified as “bang-sensitive”. More recently, another atlastin mutant (atl^2) was generated by imprecise excision of the original atl^1 P-element[59]. This ~1,600 bp deletion mutant is likely a null allele and is pupal lethal. Immunolocalization experiments reveal that Datl is broadly expressed in the larval CNS as well as body wall muscle, but is not enriched within synapses. Analysis of the atl^2 mutant indicated that loss of atlastin produces subtle effects on the neuromuscular junctions such as slightly increased synaptic bouton number and marginally reduced body wall muscle size. Examination of microtubule organization in muscle suggests that Datl may regulate microtubule organization and dynamics, as has been suggested by association with spastin. Furthermore, these authors suggest that Datl may also functionally and physically interact with spastin.

Datl function has been examined *in vivo* by RNA interference. Reduction of Datl results in minor changes in ER structure when examined by fluorescence microscopy; however, transmission electron microscopy reveals substantial ER fragmentation[30]. Fluorescence loss in photobleaching (FLIP) experiments demonstrate that Datl depletion and ER fragmentation results in pervasive ER lumen discontinuity. This disorganization of the ER likely has profound effects on normal ER function. Moreover, overexpression of Datl in flies produces a grossly aberrant ER that also contains Golgi markers. When the ER was examined by electron microscopy, abnormally enlarged cisternae were also observed[30]. The cellular phenotypes of Datl reduction and overexpression strongly suggest that Datl plays an important role in ER maintenance and biogenesis. In the absence of Datl function, the ER fragments and continuity between ER subdomains is lost.

A recent study examined the consequences of altering Atl1 levels in the zebrafish *Danio rerio* by morpholino-mediated knockdown and overexpression[54]. Decreased levels of Atl1 resulted in aberrant spinal motor axons, which caused a reduction of larval movement. Molecular analysis indicated that bone morphogenic protein (BMP) signaling was upregulated in the knockdown and inhibited by Atl1 overexpression, suggesting a role in receptor trafficking. These authors also showed that Atl1 localizes to endosomes and that Atl1 deletion did not affect ER structure when examined by fluorescence microscopy. While it is possible that Atl1 functions differently in teleosts, the abnormalities seen in motor axons could also be explained by ER morphological changes not seen by light microscopy, although this remains to be tested. The presence of Atl1 in endosomes is a bit more difficult to reconcile with work from other species.

Atlastin is a fusion protein—The atlastin phenotypes observed *in vivo* in *Drosophila* are consistent with a role for Datl in the fusion of ER membranes. The protein(s) responsible for the fusion of ER membranes have been unclear until the recent functional characterization of Datl[30]. The role of atlastin as a membrane fusion protein was explored by reconstitution

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studies and enzymatic analysis[30]. Bacterially expressed Datl reconstituted into synthetic liposomes [60] was analyzed for membrane fusion activity utilizing a lipid mixing assay[61] that has been used extensively for the analysis of membrane fusion by the SNARE proteins[8,62–72]. Atlastin promotes robust lipid mixing that is completely dependent on divalent metals and GTP. No fusion occurs when non-hydrolyzable GTP analogs are included in the reaction. Additionally, a mutant atlastin (K51A) that prevents nucleotide binding does not support membrane fusion. In fact, this mutant is a dominant inhibitor of membrane fusion when liposomes that contain wildtype atlastin are mixed with the liposomes containing the K51A mutant atlastin.

Additional support for the role of atlastin as an ER fusogen was recently provided by antibody inhibition studies of ER tubule formation *en route* to nuclear envelope regeneration in *Xenopus* egg extracts[31]. Reformation of ER tubules from fragmented ER vesicles is known to be a GTP-dependent process[73], and antibodies against *Xenopus* atlastin inhibits tubule reformation similar to GTPγS[31,57]. This result suggests that atlastin plays a role in ER tubule formation in *Xenopus*.

The structure-function studies of Datl[30] and the recent crystal structure of Atl1[48] have led us to develop the following working model of atlastin function in membrane fusion (Figure 2). ER tubules often form three-way junctions when the tip of a mobile tubule fuses with the side of an existing tubule (Figure 2A and B). Atlastin may localize along the length and tips of the tube, perhaps directly though an association with the reticulon/REEP tube forming proteins. We suggest that atlastin exists in multiple conformations, both monomer and cis-dimers (i.e. dimers within the same membrane). We also make the assumption that atlastin undergoes a monomer to dimer transition that is dependent on bound nucleotide. This assumption is supported by gel filtration data with Atl1[48] and our unpublished data. The observation that Atl1 crystalizes in two distinct forms suggests that conformational rearrangement of the middle domain three-helix relative to the GTPase domain may also be important for the fusion mechanism. Crystal form 2 (Figure 2C) is oriented in a manner that would allow dimerization between the GTPase domains and connect adjacent bilayers (Figure 2B and C). We model this interaction as a first point of contact. An interaction between catalytic surfaces of the GTPase domain is plausible based on crystal packing contacts and homology with GBP1; however, we favor the idea that additional associations between juxtamembrane regions are required for fusion, based on the mechanism of other known fusion proteins such as SNAREs and viral envelope protein. We suggest that GTP binding permits association between the GTPase domains, although this interaction is unlikely to be the major contacts that drive dimerization. Figure 2C (left) depicts the crystal form 2 structures interacting between the membrane of an approaching tubule tip and the side of an established tubule, promoted by GTP binding. Nucleotide hydrolysis would then drive a structural rearrangement of the linker region that repositions the middle domain three-helix bundle. The transition from crystal form 2 to crystal form 1 could be accomplished by rotating the three-helix bundle about the axis indicated in Figure 2D. Since the C-terminus of the mobile three-helix bundle is firmly anchored in the membrane by two transmembrane domains, the movement shown in the schematic would induce close proximity between the two opposing bilayers, perhaps close enough to exclude water in the hydration shell. Additionally, the mechanical motion generated by the three-helix bundle transition could distort and sufficiently perturb the phospholipid bilayer structure to induce lipid mixing and membrane fusion.

Concluding remarks

The ER provides a plethora of functions required for normal cell function ranging from calcium homoestasis, glycosylation, and lipid biosynthesis, to acting as the initial way

station for all secreted proteins, as well as most transmembrane proteins. Given the fundamental importance of such ER functions, it's not difficult to envision how disrupting ER function could cause dire cellular consequences. Less obvious is the need to maintain specific ER morphologies. Why does the ER need to be constructed as sheet and tubes? Perhaps form does follow function and the diversity of ER biochemistry requires its elaborate reticular structure. Analysis of the ER fusion protein atlastin further suggests that the dynamic relationship between tube and sheet structures, and the ability to generate new connections, is also paramount.

Considering our limited understanding of the functional consequences of ER morphological changes, it is difficult to precisely interpret all of the phenotypes of atlastin misexpression. Regardless, cogent arguments can be made to explain many of them in the context of ER dysfunction. The pathology of HSP suggests that long motor neurons may be one of the most susceptible tissues to alterations in ER structure. Perhaps ER tubules that provide luminal connections to the cell body are needed in the synapses of these very long axons. The inability of motor neurons with mutations in Atl1 to generate ER that spans the physical distance between synaptic boutons and the cell body may lead to inappropriate neuronal activity and eventually neurodegeneration.

The identification of atlastin as a novel GTP-dependent membrane fusion protein also expands our understanding of the ways biological membranes merge. Traditional membrane fusion proteins such as viral fusion proteins and SNAREs use energy derived from metastable protein folding intermediates to drive fusion[74,75]. The use of chemical energy in the form of nucleotide hydrolysis at the point of membrane fusion is unique to a new class of membrane fusion protein exemplified by atlastin and perhaps mitofusin. Detailed analysis of this type of fusion mechanism has yet to be explored. Future mechanistic analysis will determine how atlastin uses GTP hydrolysis to generate force and move lipids. The knowledge that atlastin controls ER fusion opens an exciting new area of possibilities for understanding ER morphogenesis and function in general, as well as a providing a potential mechanistic basis of the pathophysiology of Hereditary Spastic Paraplegia.

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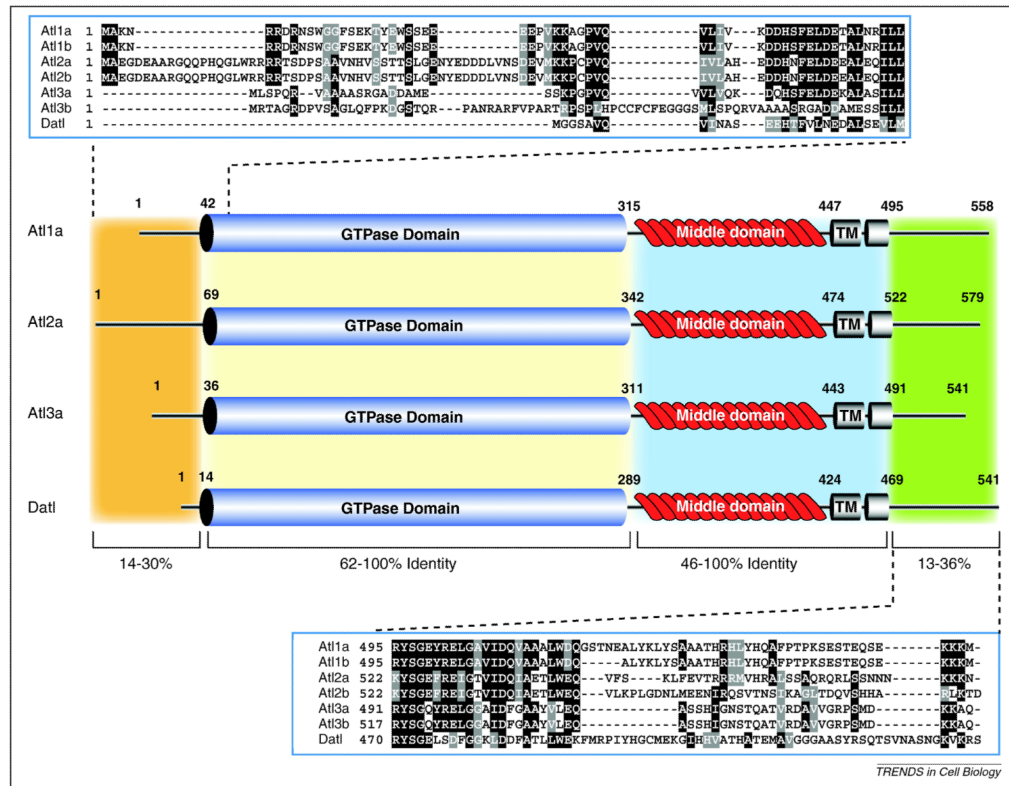

Figure 1. Domain architecture and protein similarity between Human and *Drosophila* atlastins
Human Atl1a (NP_056999.2), Atl2a (NP_071769.2), Atl3a (NP_056274.3) and Datl (AAF56318.1) are shown. The number of amino acid residues and location of identified domains are indicated on the protein schematic. The three human paralogs and *Drosophila* ortholog are subdivided into four regions indicated by the colored boxes. The variable length N-terminus is colored orange, the highly conserved GTPase domain depicted as a blue cylinder, the INTERPRO domain IPR015894 (GBP-N-term)[76] is colored tan, the “middle domain” containing the 2 tandem transmembrane domains is colored light blue and the variable length C-terminus is colored light green. Multiple sequence alignments (ClustalW2) of the extreme N- and C-terminus of all 6 human atlastin isoforms and Datl are shown (top and bottom) to highlight sequence divergence.

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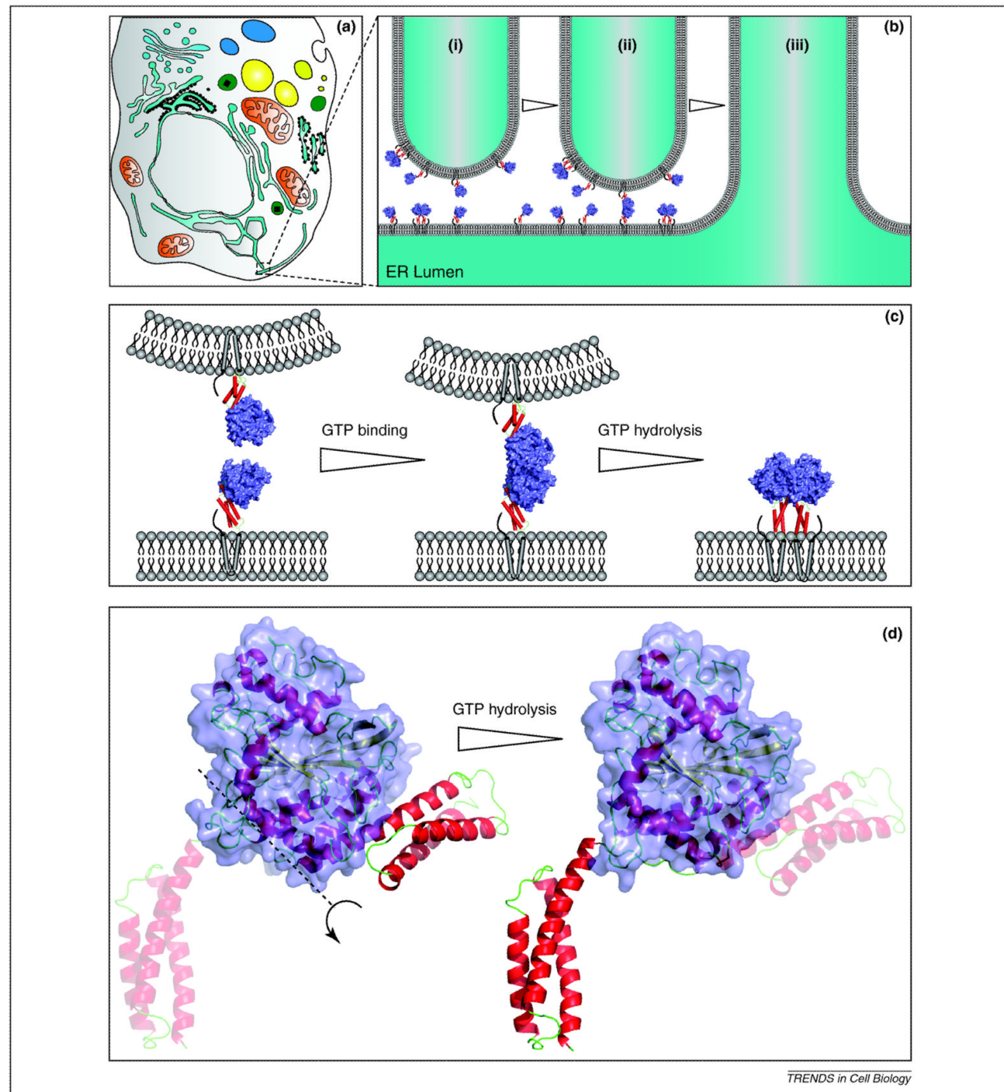

Figure 2. Proposed model for atlastin function in ER membrane fusion

(A) A stylized eukaryotic cell depicting cytoplasmic organelles and the phycial relationship to the ER. A growing ER tubule approaching a peripheral ER tubule is highlighted in the dashed box. (B) A 50 nm diameter ER tubule is shown in proximity to a perpendicular ER tubule (i). Various atlastin molecules are shown to scale in both membranes. As the growing tube makes a close approach, form 2 atlastin monomers signal GTP binding and dimerization is favored (ii). GTP hydrolysis triggers the transition from form 2 to form 1 driving membrane fusion (iii). (C) Larger scale representations of the relevant interactions shown in panel B. (D) Structural models of Atl1crystal form 2 (left) and crystal form 1 (right). The proposed conformational changes that rotates the three-helix bundle out of the plane of the page about the axis marked by a dashed line results in form 2. The transparent three helix bundle on the left is the future location following GTP hydrolysis while the transparent three helix bundle on the right is the previous location after the conformational change. The GTPase domain is shown as a surface representation (blue) in all panels while the three-helix bundle segments are shown as red cylinders (B and C) or red helices (D). The location of the transmembrane domains is indicated by grey cylinders and the C-terminal tail

as a thick black line in B and C. Atl1 crystal form 2 (3Q5E) and crystal form 1 (3Q5D) were rendered in pymol.

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