The atlastin membrane anchor forms an intramembrane hairpin that does not span the phospholipid bilayer

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Edited by Phyllis I. Hanson

The endoplasmic reticulum (ER) is composed of flattened sheets and interconnected tubules that extend throughout the cytosol and makes physical contact with all other cytoplasmic organelles. This cytoplasmic distribution requires continuous remodeling. These discrete ER morphologies require specialized proteins that drive and maintain membrane curvature. The GTPase atlastin is required for homotypic fusion of ER tubules. Atlastin homologs possess a conserved domain architecture consisting of a GTPase domain, a three-helix bundle middle domain, a hydrophobic membrane anchor, and a C-terminal cytosolic tail. Here, we examined several Drosophila-human atlastin chimeras to identify functional domains of human atlastin-1 in vitro. Although all chimeras could hydrolyze GTP, only chimeras containing the human C-terminal tail, hydrophobic segments, or both could fuse membranes in vitro. We also determined that co-reconstitution of atlastin with reticulon does not influence GTPase activity or membrane fusion. Finally, we found that both human and Drosophila atlastin hydrophobic membrane anchors do not span the membrane, but rather form two intramembrane hairpin loops. The topology of these hairpins remains static during membrane fusion and does not appear to play an active role in lipid mixing.

Organelle morphology is critical for correct localization and functionality. This is readily evident for the endoplasmic reticulum (ER), whose morphology consists of a dynamic network of sheetlike cisternae and tubular elongations that spread throughout the cytosol, all within a continuous membrane system. Disruption of ER morphology is often associated with lular dysfunction (1, 2). Specialized structural proteins form and maintain ER architecture, and mutation of genes encoding these structural proteins can lead to human disease (3, 4).

Membrane fusion is required to maintain ER luminal continuity and allow ER dynamics. ER homotypic membrane fusion is mediated in animals by the fusion GTPase atlastin (5–8). Atlastins have a conserved domain structure consisting of a large GTPase, a three-helix bundle middle domain, two tandem transmembrane domains (TMDs), and a short cytoplasmic C-terminal tail (reviewed by McNew et al. (6)). Membrane-shaping proteins called reticulons and REEPs are also required to form ER tubules and the edges of ER sheets (9, 10). Reticulons consist of a variable N-terminal domain followed by two short hairpin TMDs, separated by a long hydrophilic loop, and a C-terminal domain. The membrane domains of reticulons primarily reside in the outer leaflet of the ER membrane to introduce curvature and generate tubules (10–13). An additional ER resident called lunapark (14–17) works with the REEP/reticulon family and atlastin to maintain the exquisite morphology of the ER network.

Humans express three atlastin homologs: atlastin-1, atlastin-2, and atlastin-3. Mutations in human atlastin-1 (SPG3A) cause a disease called hereditary spastic paraplegia (HSP) (18, 19). Whereas mutations in many other loci can also cause HSP (18, 20), an emerging theme is that disruption of ER function may underlie many HSP phenotypes (21, 22). Atlastin-1 is found primarily in neurons, whereas atlastin-2 and atlastin-3 are more ubiquitously expressed. Mutations in atlastin-3 have recently been shown to cause a form of hereditary sensory and autonomic neuropathy (23).

Humans express four reticulon (24) and six REEP genes, each with multiple spliced isoforms. Mutations in REEP1 (SPG31) and reticulon-2 (SPG72) also cause HSP (18, 26, 27). In contrast to many species, Drosophila melanogaster has a single atlastin homolog (dAtl) (28) and one ubiquitously expressed reticulon (reticulon-like-1, Rtn1) (29).

Several pieces of evidence suggest that atlastin and REEP/reticulons physically and functionally interact in the ER membrane. Defects in synaptic transmission in Drosophila due to loss of dAtl can be partially rescued by the additional loss of Rtn1, strongly suggesting functional interaction in vivo (30). Co-immunoprecipitation experiments from rat brain extracts have also revealed an association between rat atlastin-1 and rat reticulon4a and reticulon3c. This work also suggested that the interaction between atlastin and reticulon was likely through the hydrophobic membrane-spanning segments (9).
Whereas the putative transmembrane domains of atlastin serve to anchor it in the ER membrane, experiments replacing the hydrophobic sequences with sequences from other ER-resident proteins led to loss of lipid-mixing activity (31), suggesting an additional role of this domain. Here, we show that the atlastin membrane anchor is an intramembrane hairpin rather than a bilayer-spanning transmembrane domain, and this topology is critical for membrane fusion.

Results

Biochemical analysis of human atlastin-1 and human Drosophila atlastin chimeras

Whereas Drosophila atlastin is an efficient fusogen in vitro (5, 31–35), the human atlastin proteins have been resistant to in vitro fusion analysis (36). We have confirmed that human atlastin-1 does not significantly promote membrane fusion in vitro (Fig. 1, black bars), although it maintains a robust GTPase activity (Fig. 1, blue bars). In an effort to identify potential differences between Atl-1 and dAtl, we constructed chimeras that subdivide the protein(s) into four primary domains: the GTPase domain, the three-helical bundle middle domain, the transmembrane domains, and the C-terminal tail. Whereas dAtl with the human Atl-1 tail (Fig. 1, chimera I) was functional, as previously reported (32), the only additional chimera that drove in vitro fusion was the inclusion of the human transmembrane domains (Fig. 1, chimera II). The remaining three chimeras tested (chimeras III, IV, and V) were incapable of membrane fusion while maintaining at least 50% of native Drosophila GTPase activity, suggesting that species-specific interactions between the GTPase domain and the middle domain are important for fusion.

Co-reconstitution of reticulon with atlastin does not influence the GTPase activity or membrane fusion propensity of atlastin

Atlastin has been shown to physically interact directly or indirectly with several proteins in the ER membrane, including reticulons, REEPs, spastin, and lunapark (6, 7, 17, 25, 37). Given the structural nature of the REEP/reticulon family (24, 38, 39) and the relatively large abundance of these proteins, the most likely interactions with atlastin that may regulate function are with the reticulons. To examine a potential regulatory role of reticulon, we analyzed the effects of Rtnl1 in dAtl-mediated in vitro fusion. Co-reconstitution of Rtnl1 and dAtl into preformed PC/PS liposomes was done by detergent-assisted insertion (5). Unincorporated protein was separated from proteoliposomes by flotation in a discontinuous Nycodenz gradient. We varied the amount of atlastin while keeping the reticulon concentration fixed to yield varying molar ratios of the two membrane proteins. Fig. 2D shows a Coomassie Blue–stained SDS-PAGE of proteoliposomes containing similar amounts of reticulon and decreasing concentrations of atlastin.

The influence of Rtnl1 on dAtl-mediated in vitro fusion was then assayed by lipid mixing (5, 32, 33). Three ratios of atlastin/reticulon were used, 1:1, 1:2, and 1:4, keeping Rtnl1 at a constant protein/lipid molar ratio of ~1:400. The reconstitution of atlastin was only modestly affected by the presence of reticulon (Fig. 2F). When membrane fusion was examined, both the rate (Fig. 2A) and extent (Fig. 2B) of lipid mixing were unaffected when normalized to atlastin reconstitution efficiency. These results suggest that reticulon does not influence atlastin’s ability to promote membrane fusion. Similarly, atlastin GTPase activity was also largely unaffected by reticulon (2.8 versus 2.5 μmol/min/μmol of protein) (Fig. 2C). Finally, we co-reconstituted Drosophila Rtnl1 with human atlastin-1 to determine whether the presence of reticulon influenced lipid mixing activity of the human protein. Whereas human Atl-1 was reconstituted with similar efficiency as dAtl when reticulon was included (Fig. S1), no improvement in lipid mixing activity was observed (data not shown).

Our results suggest that reticulons do not have a direct effect on the biochemical activities of atlastin under our conditions; however, this result does not preclude a role for atlastin–
reticulon interactions in localization to specific subdomains of the ER, such as within tubules or to the edges of ER sheets. Finally, the inability of human Atl-1 to promote fusion in vitro is unlikely to be due to the lack of a specific binding partner in the ER membrane, because chimera II, which contains the human transmembrane domains, is functional for fusion (Fig. 1), and inclusion of an abundant likely binding partner, reticulon, did not rescue activity.

The membrane anchor of atlastin forms two intramembrane hairpin loops

The hydrophobic membrane anchors of atlastin are similar in sequence across many species (Fig. S2), and our data show that their function in membrane fusion is also conserved between Drosophila and humans (Fig. 1). The putative transmembrane segments also have some unique physical characteristics that distinguish them from other, conventional transmembrane domains. In particular, whereas the N terminus of “TM1” and the C terminus of “TM2” both begin and end with a membrane-delimiting charge (mostly arginine, although lysine is also seen in the atlastin-2 isoforms), the presence of charge within the putative ER luminal loop that would define the end of “TM1” and start of “TM2” is highly variable across species (Fig. S2). Whereas atlastin-1 and atlastin-3 contain a positively charged aspartate or glutamate that could terminate TM1, dAtl and atlastin-2 isoforms do not possess any formal charge in this location (Fig. S2). These observations suggest that the hydrophobic segment could play an active role rearranging lipids during membrane fusion, rather than just acting as a passive membrane anchor.

It has been largely assumed that the dAtl hydrophobic domains traverse the ER membrane twice and have an approximately 5-residue luminal loop (see Fig. 4A (a)) (10, 31, 33, 40). Our interest in understanding whether the atlastin hydrophobic domains actively move lipids during membrane fusion led us to determine the topology of the hydrophobic segments. The
conventional membrane topology model (Fig. 4A (a)) places a short loop inside the ER lumen or inside the proteoliposome in our reconstituted system. This topology predicts that the loop residues are protected from modification by reagents added to the outside of the liposome. We targeted the accessibility of phenylalanine 448, which resides between hydrophobic segments in the center of the ~5-amino acid loop (Fig. 4A and Fig. S2) by expressing a mutation at this location that allows for covalent modification by copper-free click chemistry (strain-promoted azide-alkyne cycloaddition). To this end, we expressed dAtl in bacteria that contains an amber stop codon (TAG) in place of the codon for phenylalanine at position 448 and a suppressor tRNA that codes for the nonnative amino acid p-azidophenylalanine (AzF) (41). The ability of AzF-448 to be labeled by reagents added to the outside of intact proteoliposome will determine whether this residue is exposed to the outside of the membrane or inside the lumen. Atlastin containing this modified amino acid was used to probe the location of phenylalanine 448 relative to the phospholipid bilayer. The location of this residue was determined by coupling the azido functionality of AzF with the membrane-impermeant strained alkyne, dibenzocyclooctyl (DBCO), conjugated to a fluorescent carbocyanine dye, Cy5.5, utilizing copper-free click chemistry. This covalent attachment was performed with dAtl (F448AzF) reconstituted into proteoliposomes, whereas maximum labeling was achieved by protein labeling in detergent micelles. Unreacted AzF was quenched by the addition of NaN₃, and the fluorescence intensity of atlastin-coupled Cy5.5 was determined in gel using fluorescence imaging (Fujifilm LAS-4000).

Fig. 3 examines the rate and extent of labeling of F448AzF followed by quenching unreacted DBCO with excess NaN₃. Extensive labeling (~40% for dAtl (F448AzF) and ~70% for chimera II (I450AzF)) is seen at the earliest time point (2 min), which increases to ~55 and 90%, respectively, over the next 2 h.

Fig. 4 shows a summary of AzF-448 labeling in liposomes over several experiments relative to labeling in detergent. Reconstituted dAtl(F448AzF) labeled to ~54%, suggesting very strongly the Phe-448 is exposed to the outside of the proteoliposome rather than residing inside the proteoliposome lumen (Fig. 4B, blue circle). A similar extent of labeling was seen when a residue known to reside in a cytoplasmic loop (10, 11) of Rtn1 (Phe-106) was interrogated in a similar manner (Fig. 4A, c and B (red squares)). The inability of DBCO-Cy5.5 to label residues inside proteoliposomes was confirmed using AzF labels in two locations within the Rtn1 reticulon homology domain, specifically Val-69 and Gly-170 (Fig. 4A (c)). Whereas proteins containing V69AzF and G170AzF were efficiently labeled in detergent micelles (Fig. 4C, + TX-100), they were not significantly modified when reconstituted into proteoliposomes (Fig. 4, B (V69AzF (blue squares) and G170AzF (green squares)) and C), suggesting the DBCO-Cy5.5 dye does not have access to the interior of the proteoliposomes. Additionally, the speed of labeling shown in Fig. 3 and the inability of the much smaller molecule dithionite to leak into liposomes (Fig. S3) strongly argue against the possibility that internally labeling occurs. Overall, these data suggest an alternative domain topology in which the presumed luminal loop is facing the cytosol and the putative transmembrane domains form two intramembrane hairpins that do not fully traverse the lipid bilayer (Fig. 4A (b)).

The intramembrane hairpin topology or “W” conformation of atlastin hydrophobic segments is conserved in human atlastin-1. When the human-Drasophila chimera II protein (Fig. 1) was engineered to code for a modifiable amino acid in a similar position (I450AzF), DBCO-Cy5.5 labeling was even stronger than with the endogenous Drosophila membrane anchor (Figs. 3 and 4B (green circle)). In this case, labeling in proteoliposomes was as efficient as in detergent micelles (98%). This difference in labeling between WT dAtl and human-Drosophila chimera II may be attributed to differences in dye access to the modifiable residue. Additionally, differential association states of the recombinant proteins in the plane of the bilayer may alter labeling. Regardless, our data support a model that the atlastin mem-
brane domain can adopt a dual intramembrane hairpin loop topology.

Atlastin-mediated fusion does not require conformational alterations of the membrane anchor for lipid mixing

The physical mechanism of lipid movement during membrane fusion is still a poorly understood process. Viral fusion proteins utilize a bilayer-penetrating fusion peptide for membrane perturbation (42–46), whereas SNARE proteins are thought to move lipids as rigid transmembrane helices are pulled together during zippering of the SNARE four-helix bundle (47–51). We have previously shown that one way that atlastin drives membrane destabilization during fusion is by using a C-terminal amphipathic α-helix that embeds in the membrane (32). Given the unconventional properties of the atlastin transmembrane segment, we considered the possibility that this
region of the protein may also aid membrane destabilization and lipid mixing by changing conformations during fusion. The relatively few charges in the loop between hydrophobic segments (Fig. S2) raised the possibility that the topology of the membrane anchor could switch from the conventional “V” to the newly identified “W” topology (Fig. 4A(a) versus Fig. 3A(b)). To test for the possibility that movement of the small loop across the bilayer is part of the mechanism of atlastin-mediated fusion, we designed mutations that would stabilize the C-terminal end of the first hydrophobic segment (N450K), the N-terminal end of the second hydrophobic segment (Y466K), or both (N450K/Y466K). These mutations should prevent transbilayer movement of the intervening loop sequence, regardless of the initial topology (“W” or “V”). When proteins containing these mutations were expressed and reconstituted into proteoliposomes, no significant difference in lipid-mixing activity was observed (Fig. 5A), suggesting that the location of the loop remains unchanged during fusion. Additionally, we performed lipid-mixing assays with dAtl(F448AzF) labeled in proteoliposomes prior to the fusion reaction. As shown in Fig. 4, the method results in atlastin that contains a bulky, charged moiety on the outside of the proteoliposomes. DBCO-Cy5.5–conjugated atlastin (F488AzF) fused as efficiently as the AzF-containing protein that remained unlabeled (Fig. 5B). The ability of this covalently labeled atlastin to continue to drive membrane fusion further suggests that the “W” topology does not change during the fusion cycle.

The intramembrane hairpin topology of atlastin is important for function

Previous studies have shown that replacing the hydrophobic anchor of atlastin with a single transmembrane domain or the two transmembrane domains of the resident ER phosphatase SacI resulted in a nonfunctional protein, suggesting that the atlastin hydrophobic anchor has unique properties (31). Next, we chose to examine another hydrophobic membrane anchor that is known to reside in a similar ER subdomain of atlastin, namely reticulon. We expressed an atlastin–reticulon chimera in which the dAtl hydrophobic anchor was replaced with the first hydrophobic segment of the reticulon homology domain (amino acids 48–88) of Rtnl1 (Fig. 5C(b)). Although this chimera was expressed and reconstituted similarly to WT atlastin, it was unable to promote membrane fusion (Fig. 5D). Taken together, these results suggest that the unique “W” topology of the atlastin membrane anchor is functionally important for membrane fusion driven by atlastin.

Atlastin can form ER-like tubular networks in vitro

The observation that the atlastin membrane anchor likely occupies the outer leaflet of the ER membrane is consistent
Atlastin membrane anchors do not span the bilayer

with the studies localizing atlastin primarily to ER tubules or the edges of ER sheet (i.e. areas of high membrane curvature) (16, 52, 53). Additionally, the “W” topology suggests that atlastin may also play a structural role in shaping ER membranes, similar to the defined role of the REEP/reticulon family (10–13). Recent work has demonstrated that ER-like networks can be formed in vitro using Xenopus egg extracts in an atlastin- and GTP-dependent manner (14). Additionally, proteoliposomes containing Drosophila atlastin can generate ER-like networks (54). We have been able to recapitulate an ER-like tubular network with atlastin proteoliposomes attached to coverslips with polylysine (Fig. 6). These results demonstrate that atlastins have the capacity to generate membrane curvature and produce stable tubular structures. The intramembrane hairpin topology described in this work is consistent with the capacity to wedge bilayer structure to generate curvature.

Discussion

Membrane fusion relies on protein machines to exert force on the membrane to allow for lipid and content mixing. SNARE-mediated fusion as well as viral fusion utilize the energy from protein folding to accomplish the task of membrane merger (7, 42–47, 49–51). The third class of membrane fusion proteins typified by atlastin and mitofusin are fusion GTPases (2, 5, 6) that use the chemical energy of GTP hydrolysis to drive conformational changes necessary to fuse membranes. Recent work has provided mechanistic insights into the conformational changes that occur during GTP hydrolysis (31, 34, 55–58), yet the role of the required hydrophobic membrane anchor remains relatively unexplored. Additionally, several proteins have been shown to interact with atlastin, primarily through the hydrophobic membrane anchor, including reticulin/REEP proteins, lunapark, and spastin (6, 7, 17, 25, 37).

Whereas membrane fusion by Drosophila atlastin has been characterized extensively in vitro (5, 31–35), other species of atlastins, including human, have been difficult to characterize in vitro (36). We generated protein chimeras between dAtl and HA1-1 to identify potential functional regions of the human homolog. We found that the hydrophobic membrane anchor and cytoplasmic tail from HA1-1 were functional when grafted into the Drosophila atlastin protein. The remaining chimeras were capable of cleaving GTP to at least 50% of WT, but none were competent for membrane fusion (Fig. 1).

One potential reason for the inability of human atlastin to promote membrane fusion is the absence of a required binding partner. We addressed the role of the most likely binding partner, reticulin, by co-reconstituting with atlastin. The presence of reticulin in the membrane in stoichiometric proportions did not influence the ability of dAtl (Fig. 2) or Atl-1 (Fig. S1) (data not shown) to drive membrane fusion or cleave GTP.

Our interest in probing an active role for the atlastin membrane anchor in the fusion process prompted us to examine the topology of the atlastin hydrophobic segments. Using copper-free click chemistry, we found that the predicted luminal loops between presumptive membrane spans one and two were, in fact, exposed to the outside of proteoliposomes (Fig. 4). This observation strongly suggests that the atlastin membrane anchor is not transmembrane, but rather two intramembranous hairpins that primarily occupy the outer leaflet of the membrane, forming a “W” topology rather than a conventional “V” (Fig. 4A, a versus b). This property is also conserved in Atl-1, suggesting that this topology may be a general feature of this class of proteins.

Regardless of the ultimate topology model, we wanted to explore the possibility that the transmembrane anchor was mobile, changing its conformation during the fusion cycle. To test this possibility, we expressed mutants that contained membrane-delimiting charges to define the boundaries of the hydrophobic segments. These mutations should prevent bilayer passage of lesser hydrophilic loops in their absence. However, we found that these mutants were virtually WT for membrane fusion (Fig. 5). Similarly, covalent attachment of the large, charged DBCO-Cy5.5 moiety to the loop between hydrophobic segments on the outside of proteoliposomes did not affect membrane fusion propensity, confirming that the “W” topology does not need to convert to a “V” topology as part of the fusion mechanism. The newly identified “W” topology of the atlastin membrane anchor appears to be a required part of the molecule. Attachment of atlastin to the membrane with a reticulin-spanning domain results in a nonfunctional protein (Fig. 5).

Finally, the “W” topology may also serve to shape membrane, similar to REEP/reticulin proteins. We have confirmed a published report (54) that Drosophila atlastin alone can produce an ER-like tubular network in vitro under conditions that drive membrane fusion (Fig. 6).

In summary, we report that both human and Drosophila atlastin contain a novel membrane anchor that forms two intramembranous hairpins that primarily occupy the outer leaflet of the membrane, forming a stable “W” topology that is a requirement for atlastin to function as a membrane fusion protein.
Experimental procedures

Molecular biology

The dAtl construct, pJM681, previously described (5), with an N-terminal GST tag and C-terminal eight-histidine tag in a modified pGEX-4-T3 bacterial expression vector was used for making recombinant dAtl. All dAtl point mutants or chimera used this plasmid as a backbone. All point mutants of dAtl were made by PCR using a QuikChange II site-directed mutagenesis kit (Agilent Technologies). The mutant dAtl (Y446K), pJM1141, was made using the primer (CTTTGGATTGTGGTTCACGAGGTCTCCAATCTGCTTCCG) and its complementary primer. In the same manner, dAtl (N450K), pJM1142, was made with (GTCTCTATACGTGGGCAGTATCCATCTGCAATCTGATTATG) and its corresponding complementary primer; additionally, the double mutant dAtl (Y446K/N450K), pJM1143, was made by introducing this same mutation in pJM1142. The mutant dAtl (F448AzF), pJM1139, was made with a primer coding for an amber stop codon (TAG) (GGTTGGTCTATACGTGGGCAGTATCCATCTGCAATCTGCTTCCG) and its corresponding complementary primer.

Rtnl1 (PB isoform) and any corresponding point mutants were expressed from a bacterial expression pGEX-4T-3 vector with an N-terminal GST-SUMO tag, pJM1115. All point mutations were made by PCR using a QuikChange II site-directed mutagenesis kit. Rtnl1 (V69AzF), pJM1158, was made with the primer (CATCTCCAGCTTCTGATAGCTCAGGTGCCTTGC) and its complementary primer. In the same manner, Rtnl1 (G106AzF), pJM1163, was made with CGAGGGTGCTCCCTAGAGAGCTGCTGATGCTGTCGC and Rtnl1 (G170AzF), pJM1159, was made with GTGCCGTGTCCTCAATTGATGACTCTGCTGTCG and their respective complementary primers.

The dAtl-Rtnl1 (TM1), pJM1160, chimera was constructed using a manufactured gene fragment G-block (IDT) containing the sequence for dAtl (354–421)–Rtnl1 (48–88)–dAtl (477). Our dAtl in pGEX was digested with SacI and Afill, and this fragment was then ligated to the G-block by Gibson assembly.

Chimera I, pJM866, was constructed with Hs-Atl1 C-terminal tail generated by PCR from pJM693, GST-Hs-Atl1-H8 in pGEX4T3, using primers BspEI-Hs-Atl1-c-tail (ATTCGGAGAAATCCCGAGCTGCTG) and Hs-Atl1-Xhol (TTTCTGACGCACTTTTTTTTTTTC). The PCR product was digested with BspEI and Xhol ligated into pJM681 cut with the same enzymes.

Chimera II, pJM1007, was constructed by generating Hs-Atl1 middle domain with C-terminal domain by PCR from pJM693 using primers Hs-Atl1-3HB-SphI (GAGGATCCCATGTTACAGG) and Hs-Atl1-TMD-SacI reverse (CGCGAGCTCCCGGATATATGCGCCCAAGTG). The PCR product was digested with Ncol and SacI and ligated into pJM681 cut with the same enzymes.

Chimera IV, pJM917, was constructed by generating Hs-Atl1 middle domain with C-terminal domain by PCR from pJM693 using primers 3HB-SphI-forward (GAGGATCCCATGTTACAGG) and Hs-Atl1-TMD-SacI reverse (TTTCTGACGCAACTTTTTTTTTTTC). The PCR product was digested with SphI and Xhol and ligated into pJM681 cut with the same enzymes.

Chimera V, pJM908, was constructed by generating the Hs-Atl1 GTase domain by PCR from pJM693 (GST-Hs-Atl1-H8 in pGEX4T-3) using primers Ncol-Hs-Atl1-forward (ACACATGGCAAGAACCACAGG) and SphI-Hs-Atl1-reverse (CGCGAGCTCCCGGATATATGCGCCCAAGTG). The PCR product was digested with SphI and Xhol and ligated into pJM681 cut with the same enzymes.

Protein expression and purification

Protein expression—Recombinant proteins were purified with an N-terminal GST tag and an eight-histidine tag, as described previously (5), or a GST-SUMO tag, as described (33), with the following modifications. A 200-ml Luria-Bertani medium with 100 μg/ml ampicillin preculture was inoculated with 5 ml of transformed BL21 (DE3) Escherichia coli (Agilent Technologies) and incubated with shaking overnight at 25 °C. The culture was then pelleted at 2000 rcf for 10 min, using an Allegra tabletop centrifuge at 25 °C. The supernatant was removed, and the pellet was resuspended and inoculated to a 4-liter Luria-Bertani medium with 100 μg/ml ampicillin. The culture was allowed to reach an A600 of 0.4–0.5 at 25 °C before inducing with 0.2 mM isopropyl 1-thio-β-D-galactopyranoside, followed by an overnight shaking incubation at 16 °C.

Azidophenylalanine mutant proteins were expressed in a similar manner as the other tagged proteins as described (59) with the following modifications. BL21 DE3 cells were transformed with the corresponding mutant protein plasmid and pEVOL-AzF (Addgene entry 31186) (41). Bacterial cultures were grown with 100 μg/ml ampicillin and 35 μg/ml chloramphenicol in M9 minimal medium supplemented with 0.2% glucose and 0.1% casamino acids. A 2-liter culture was allowed to reach an A600 of 0.4–0.5 before inducing with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside, 0.06% arabinose, and 1.5 mM p-azidophenylalanine (Bachem); after induction, the culture was incubated overnight with shaking at 16 °C.

Lysate preparation—Cells were harvested by pelleting for 15 min at 7500 rcf at 4 °C on an Avanti JHC centrifuge with a JS-5.0 rotor. The supernatant was discarded, and cells were resuspended in A200 (25 mM HEPES (pH 7.4) and 200 mM KCl). Cells were then centrifuged for 5 min at 4 °C at 10,950 rcf in a JA-10 rotor. Cells were then resuspended in 40 ml of breaking buffer (A200 plus 10% glycerol, 2 mM 2-mercaptoethanol, 4% Triton X-100, 40 mM imidazole, and one Complete protease inhibitor mixture tablet (Roche Applied Science)). GST-SUMO–tagged proteins omitted imidazole. Cells were then passed three times through an Emulsiflex C3 high-pressure homogenizer (Avastin) at 15,000–20,000 p.s.i. Cell extracts were then cleared by centrifugation at 125,000 rcf at 4 °C using a Type 45-Ti rotor in an Optima LE-80K ultracentrifuge. Cleared lysate was then fil-

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tered through a 0.45-μm pore cellulose nitrate sterile membrane filter (Whatman).

**GST-H8–tagged proteins**—dAtl proteins were tagged with an N-terminal GST tag and an eight-histidine tag. Lysates were passed through an equilibrated (A100 (25 mM HEPES (pH 7.4) and 100 mM KCl) + 10% glycerol, 2 mM 2-mercaptoethanol, 1% Triton X-100, 40 mM imidazole) HiTrap Chelating HP column (GE Healthcare) in an Akta prime LC system (Amersham Biosciences). The column was then washed with 25 ml of A100 plus 10% glycerol, 2 mM 2-mercaptoethanol, 0.1% Anapoe X-100, 40 mM imidazole. Protein was then eluted in a 30-ml linear gradient of imidazole from 40 mM to 1M KCl. Peak fractions were pooled and stored on an Akta prime LC system. Using its cation-exchange column, protein was then passed through a SP-Sepharose column (GE Healthcare) in an Akta prime LC system (Amersham Biosciences). The elution was then diluted with 25 mM HEPES (pH 7.4) supplemented with 10 mM reduced GSH. Protein was then aliquoted, plunged into liquid nitrogen, and stored at −80 °C. Purity was analyzed by SDS-PAGE and Coomassie staining and quantified by Amido black (60).

**GST-SUMO–tagged proteins**—Reticulon and its mutants were purified with a GST-SUMO tag as described (33). Lysates were incubated for 3 h at 4 °C with 70 mg of equilibrated swollen GSH-agarose beads. Beads were pelleted, and unbound protein was removed by aspiration. Beads were then transferred to 10-ml Polyprep columns (Bio-Rad) and washed with 25 ml of A100 with 10% glycerol, 2 mM 2-mercaptoethanol, 0.1% Anapoe X-100, and 1 mM EDTA. Protein was then eluted with 1.5 ml of wash buffer (pH 7.4) supplemented with 10 mM reduced GSH. Protein was then aliquoted, plunged into liquid nitrogen, and stored at −80 °C. Purity was also analyzed by SDS-PAGE and Coomassie staining and quantified by Amido black (60).

**Click chemistry and proteoliposome flotation**

Labeling of azidophenylalanine mutants with DBCO-Cy5.5 was done to proteoliposomes at a concentration of 150 μM at 4 °C for 2.5 h. A sample of the proteoliposomes was solubilized by adding 1% Triton X-100; this was used as the positive control. This was followed by a quenching reaction with 500 μM NaN₃ for 2.5 h at 4 °C. Proteoliposomes were then floated in a Nycodenz gradient to separate free protein and unclicked dye using a three-layer discontinuous gradient as described previously (63) with the following modifications. A stock solution of 80% Nycodenz in A100 was diluted with the proteoliposomes in A100 with 10% glycerol, 2 mM 2-mercaptoethanol, and 1 mM EDTA, to 40% Nycodenz, to a final volume of 300 μl. This was added to a 5 × 41-mm Ultra-Clear tube (Beckman). A 30% Nycodenz solution was layered on top. Finally, 50 μl of the same buffer with no glycerol was layered at the top. The gradient was then centrifuged in an SW55-Ti rotor for 4 h at 48,000 rpm at 4 °C. The resulting layers were harvested and analyzed by SDS-PAGE.

Gel imaging was done on a LAS4000 luminescent imager (Fuji), using red light and Cy5 filter for Cy5.5 fluorescence, and the gel was then stained with Coomassie Blue and imaged in the same instrument with the diascopic illumination settings. Densitometry of the gel bands was done using Multi Gauge version 3.X analysis software.

To determine the kinetics of azide click in proteoliposomes, we clicked proteoliposomes with 150 μM DBCO-Cy5.5 and quenched the reaction at different time points with 5 mM sodium azide. To determine a zero time point, the dye was mixed with sodium azide before mixing with the proteoliposomes. At the final time point, 150 min, 1% Triton X-100 was added to solubilize the lipids and the proteoliposomes. The samples were then analyzed by SDS-PAGE and Coomassie staining.
added and incubated for another 30 min, to determine its maximum labeling. Additionally, we had solubilized proteoliposomes in 1% Triton X-100 clicked and quenched in the same time points (only 0 and 180 min are shown). Samples were then analyzed by SDS-PAGE and quantified in the same manner as described.

**In vitro lipid mixing assays**

Fusion was measured by lipid-mixing assays based on the method described previously (5). Labeled and unlabeled proteoliposomes were brought to a final concentration of 0.15 mM in A100 with 10% glycerol, 2 mM 2-mercaptoethanol, and 1 mM EDTA supplemented with 5 mM MgCl₂. After a 5-min incubation and mixing at 37 °C, fusion was induced with 5 mM GTP. NBD fluorescence was then monitored for 1 h using a TECAN M200 plate reader. After 1 h, 2.5% (w/v) n-dodecyl β-D-maltoside was added to solubilize the proteoliposomes and measure maximum NBD fluorescence; this was measured every minute for 15 min.

**Pᵢ release assays**

GTPase activity was analyzed by Pᵢ release as described previously (5). Purified protein in detergent and proteoliposomes solubilized in A100 with 10% glycerol, 2 mM 2-mercaptoethanol, 0.1% Anapoe X-100, 1 mM EDTA, and 0.5 mM MgCl₂ were assayed for GTPase activity using the EnzChek phosphate release assay kit (Molecular Probes). Protein was incubated at 37 °C for 5 min before adding 0.5 mM GTP. Absorbance at 360 nm was recorded every 30 s for 30 min.

**In vitro networks on polylysine-coated coverslips**

Glass coverslips were spotted with 1 mg/ml poly-L-lysine hydrobromide (Sigma-Aldrich) for 1 h at room temperature in a biological hood. The polylysine solution was then removed, and the coverslips were washed twice with deionized water. The coverslips were dried overnight in the biological hood and stored. Approximately 150 μM proteoliposomes were incubated at 37 °C for 30 min in fusogenic conditions (50 mM GTP and 50 mM MgCl₂) or nonfusogenic conditions (no GTP or 50 mM GTP/50 mM MgCl₂). The reaction was then diluted 1:10 in A100 buffer and spotted into a polylysine-coated coverslip, placed in a glass slide, and sealed with nail polish. The liposome networks were then imaged in a Nikon A1-rs1 confocal microscope using the settings to image rhodamine.

**Author contributions**—M. A. B.-S. and J. A. M. conceptualization; M. A. B.-S. formal analysis; M. A. B.-S. and T. D. investigation; M. A. B.-S. and T. D. methodology; M. A. B.-S. and J. A. M. writing—original draft; M. A. B.-S. and J. A. M. writing—review and editing; J. A. M. funding acquisition; J. A. M. project administration.

**Acknowledgments**—We thank Dr. Mike Stern and Dr. Joseph Faust for comments and ideas for this project, Dr. Jun Ohata and Dr. Emily Thomas for guidance in noncanonical residue mutagenesis and click chemistry, and Alexandra Rangel and Calvin Su for help with the molecular biology.

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