Supporting Information

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SI Materials and Methods

Constructs, Peptides, and Antibodies. Codon-optimized full-length *Drosophila melanogaster* atlastin (ATL) (residues 1–541) or the tailless mutant (residues 1–476) was cloned into pGEX-4T-3 or pGEX-6P-1. For the expression of human ATL1 in yeast at endogenous levels, the full coding region of ATL1 plus an N-terminal HA tag and 300-bp upstream sequences of SEY1 gene were amplified and cloned into YCplac111 (a LEU2/CEN plasmid). The insertion of the AAEEEEA sequence and the swapping of the *Drosophila* transmembrane (TM) region with that of human Sec61β (residues 61–97) or *Saccharomyces cerevisiae* Sac1p (residues 523–580) were achieved using PCR with overlapping primers. Point mutations were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). All constructs were confirmed by DNA sequencing. Peptides were synthesized by GL Biochem except for melittin, which was purchased from GenScript. The *Xenopus* ATL antibodies were generated in rabbits using the full-length protein as antigen.

Protein Expression and Purification. *Drosophila melanogaster* ATL was expressed in *Escherichia coli* as a GST fusion, as described (1). The cells were lysed in A100 buffer [25 mM Hepes (pH 7.5), 100 mM KCl, 1 mM EDTA, 2 mM β-mercaptoethanol, and 10% (vol/vol) glycerol]. The membranes were pelleted by centrifugation and solubilized in Triton X-100. The GST fusion proteins were isolated with glutathione Sepharose beads (GE Healthcare), washed, and eluted with 10 mM glutathione in A100 buffer with 0.1% Triton X-100. The GST tag was cleaved by thrombin or Prescission protease (GE Healthcare) and was removed with glutathione agarose.

Lipid-Mixing Assay. All in vitro lipid-mixing assays were performed as previously described (1). In brief, lipids [82:15:1.5:1.5 mole percent 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC):1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS): 7-nitrobenzoxadiazole (NBD)-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE):rhodamine-DPPE for donor vesicles or 83.5:15:1.5 mole percent POPC:DOPS:dansyl-DPPE for acceptor vesicles] were dried down to a film, hydrated with A100 buffer, and extruded through polycarbonate filters with a pore size of 100 nm as described previously (2). Proteins were reconstituted at a protein:lipid molar ratio of 1:2,000, except in Fig. 3*C*, where the ratio was 1:1,000. The final lipid concentration in the lipid mixing reaction was 0.6 mM, with donor and acceptor liposomes added at a 1:3 ratio. Peptides were used at a concentration of 15 μM in the lipid-mixing assay, unless otherwise indicated. The fluorescence intensity of NBD was monitored with an excitation of 460 nm and emission of 538 nm. The initial NBD fluorescence was set to zero, and the maximum fluorescence was determined after addition of dodecyl maltoside.

Dithionite-Quenching Assay. The dithionite quenching assays were performed using the same conditions as the lipid-mixing assays, except that 1 μL 100 mM dithionite in 25 mM Hepes (pH 10), 100 mM KCl, 10% (vol/vol) glycerol was added twice to 100 μL fusion reactions before peptide addition to selectively abolish NBD fluorescence on the outer leaflet of the bilayer.

Content-Mixing Assay. Content-mixing was assayed using an approach developed previously for SNARE-mediated membrane fusion (3). Donor liposomes (82:15:1.5:1.5 mole percent POPC:DOPS:Marina Blue-PE:NBD-DPPE) and acceptor liposomes (83.5:15:1.5 mole percent POPC:DOPS:dansyl-DPPE) were formed as for the lipid-mixing experiments. Reconstitution of ATL into proteoliposomes was done as described (2), except that 8 μM biotinylated R-phycoerythrin (RPE-biotin) (Invitrogen) was included during the reconstitution for donor vesicles and 8 μM Cy5-labeled streptavidin (SA-Cy5) (Invitrogen) was included for acceptor liposomes. Detergent removal by gradual addition of Bio-Beads SM-2 led to encapsulation of the dyes in the proteoliposomes. Unencapsulated content dyes were removed from the solution by incubation with NeutrAvidin resin (Pierce) for donor vesicles and biotin agarose (Sigma) for acceptor vesicles. The reaction was carried out as in the lipid-mixing assay, except that 20-μL volumes were used in 384-well plates. Content mixing and leakage was monitored by exciting RPE-biotin at 565 nm and detecting Cy5 fluorescence at 675 nm, and lipid mixing was monitored using dequenching of Marina Blue (excitation: 365 nm; emission: 460 nm). Biotin-dextran (1 μM) was included where indicated to block FRET resulting from contents that had been leaked from the vesicles during fusion. Maximum FRET caused by content mixing and leakage was determined by adding 1 μL of 10% (vol/vol) Thesit ($C_{12}E_9$; Affymetrix, Inc.) to reactions lacking biotin-dextran.

Dynamic Light Scattering. Fusion reactions using proteoliposomes containing tailless or wild-type ATL were carried out using the procedures and conditions used in the lipid-mixing experiments. Peptide (15 μM) was added 10 min before GTP addition in all reactions. Reactions, which included 5 mM $MgCl_2$, were diluted 1:2 into EDTA buffer (30 mM EDTA, 25 mM Hepes, 100 mM KCl, 10% (vol/vol) glycerol, 2 mM β-mercaptoethanol) to stop vesicle fusion. Effective hydrodynamic radii of proteoliposomes were determined using a DynaPro Nanostar instrument (Wyatt), which detects light scattered at 90° to the incident beam. Laser power and attenuation levels were set automatically by the instrument. The average of three measurements per sample was used to represent the average hydrodynamic radius of the vesicles.

Doxyl-Quenching Assay. Liposomes (84.5:15:0.5 mole percent POPC:DOPS:NBD-DPPE) either with or without 10 molar per cent doxyl PC (Avanti Polar Lipids) were formed as described previously (2). Peptide was mixed with liposomes at a peptide:lipid molar ratio of 1:40. Trp fluorescence was excited at 280 nm, and emission spectra were taken from 310–410 nm. Blank spectra containing only liposomes were subtracted from the data. Emission intensity at the peak maximum in the presence of liposomes with or without doxyl-PC was used to determine the extent of quenching. Experiments were performed at room temperature on a SpectraMax M5 Microplate Reader (Molecular Devices).

Circular Dichroism. Circular dichroism experiments were performed on a Jasco J-815 instrument. Each peptide was dissolved in 10 mM potassium phosphate (pH 7.5), 100 mM KCl and was analyzed in 1-mm path-length quartz cells at a concentration of 30 μM. Liposomes (85:15 mole percent POPC:DOPS), prepared as previously described (2), were included where indicated at a concentration of 1 mM. Spectra were collected at 25 °C from 200–260 nm. Each spectrum was the average of nine scans, performed at a bandwidth of 1 nm and a scan speed of 100 nm/min. Control spectra with buffer or liposomes only were subtracted from the corresponding peptide data.

Calcein-Leakage Assay. Liposomes (84.5:15:0.5 mole percent POPC:DOPS:Texas Red-DHPE) were prepared as described

previously (2), except that the dried lipid film was hydrated with 100 mM calcein in 25 mM Hepes (pH 7.5), 10% (vol/vol) glycerol, 1 mM EDTA, and the vesicles then were extruded through filters with a pore size of 200 nm. Unincorporated calcein was separated from the liposomes using a Sephadex G-50 column equilibrated in 25 mM Hepes (pH 7.5), 150 mM KCl, 10% (vol/vol) glycerol, 1 mM EDTA. Peptide was added to 0.1 mM lipids in a 96-well plate, and the fluorescence (excitation at 490 nm, emission at 520 nm) was monitored at room temperature using a SpectraMax M5 Microplate Reader (Molecular Devices). Control samples without peptide added were subtracted from the data.

Fluorescence Microscopy in Yeast. Yeast cells lacking *SEY1* and *YOP1* were cultured and visualized as previously described (4). In brief, cells were imaged in growth medium with an Olympus BX61 microscope, UPlanApo 1003/1.35 lens, QImaging Retiga EX camera, and IVision version 4.0.5 software.

Membrane-Association Assay. Proteoliposomes (10 μL) used for fusion were mixed with 300 μL of B100 buffer (A100 buffer without glycerol), 300 μL of 100 mM carbonate buffer (pH 11.0), or 300 μL 0.1% Triton X-100 in B100 buffer and were centrifuged in a Beckman TLA 100.3 rotor at 200,000 × *g* for 75 min. The supernatants and pellets were analyzed by SDS/PAGE and immunoblotting with anti-*Xenopus* ATL antibodies.

Sucrose Gradient Centrifugation. Full-length or mutant *Drosophila* ATL was reconstituted at a protein:lipid molar ratio of 1:2,000. Proteoliposomes (30 μL) were treated with 1% of the indicated detergents for 1 h at 4 °C and then were loaded on top of a 250 μL 5–25% (wt/vol) sucrose gradient prepared in B100 buffer. Where indicated, 5 mM EDTA was added also. The samples were centrifuged in a Beckman TLS-55 rotor at 174,000 × *g* for 2 h, fractionated, and analyzed by SDS/PAGE and immunoblotting with antibodies to *Xenopus* ATL.

Mammalian Culture, Transfection, and Coimmunoprecipitation. COS-7 cells were maintained at 37°C with 5% CO_2 in DMEM containing 10% (vol/vol) FBS and were transfected using FuGENE HD (Roche). Coimmunoprecipitation was performed as described previously (4).

1. Bian X, et al. (2011) Structures of the atlastin GTPase provide insight into homotypic fusion of endoplasmic reticulum membranes. *Proc Natl Acad Sci USA* 108: 3976–3981.
2. Orso G, et al. (2009) Homotypic fusion of ER membranes requires the dynamin-like GTPase atlastin. *Nature* 460:978–983.
3. Zucchi PC, Zick M (2011) Membrane fusion catalyzed by a Rab, SNAREs, and SNARE chaperones is accompanied by enhanced permeability to small molecules and by lysis. *Mol Biol Cell* 22:4635–4646.
4. Hu J, et al. (2009) A class of dynamin-like GTPases involved in the generation of the tubular ER network. *Cell* 138:549–561.

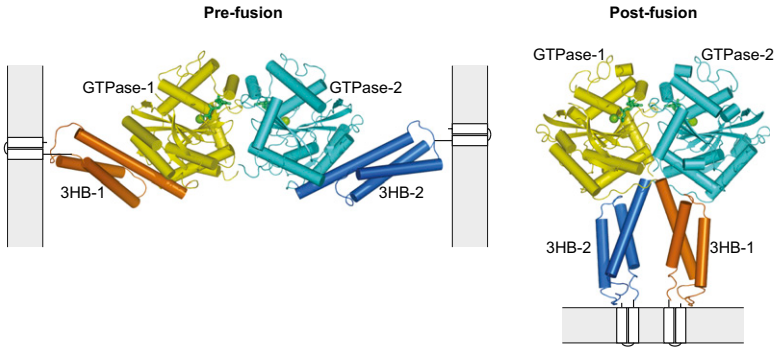

Fig. S1. Structures of human ATL1. Structures of the dimers of the N-terminal cytosolic domain of human ATL1, corresponding to the pre- and postfusion states (Protein Data Bank ID codes 3QOF and 3QNU, respectively), are shown in cartoon representation. The GTPase domains are colored in yellow and cyan, and the three-helix bundles are shown in orange and blue. GDP is shown in green stick representation, and an Mg^{2+} ion is shown as a lime-colored sphere. The positions of the TM segments following the N-terminal cytosolic domain and of the membranes are shown for reference.

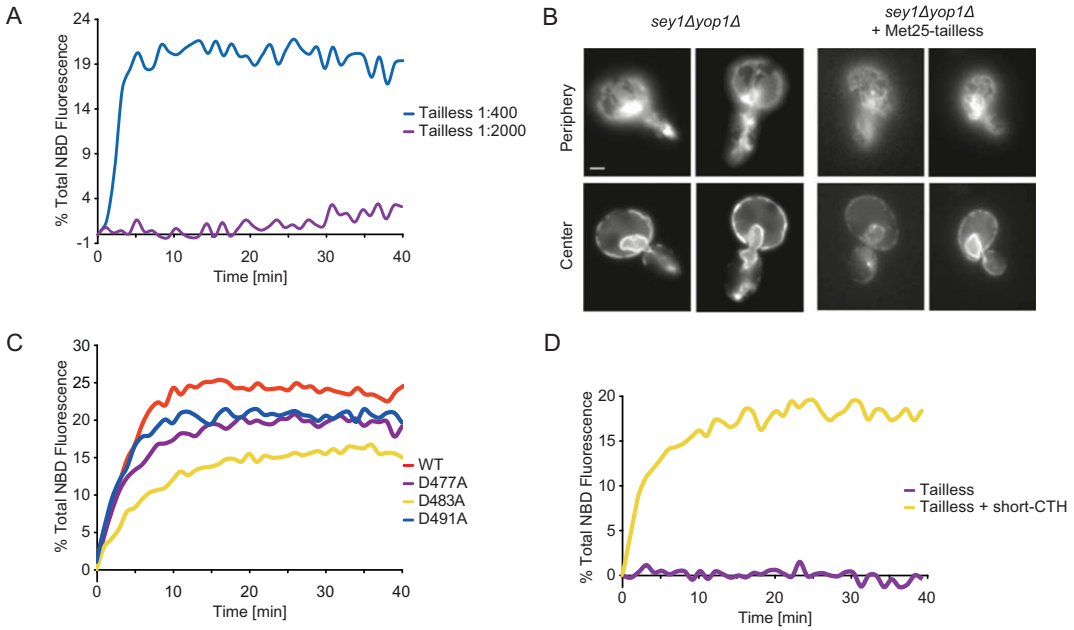

Fig. S2. Membrane fusion with C-terminal tail (CT) mutants of ATL. (*A*) The purified tailless ATL mutant (residues 1–476) was reconstituted into liposomes at a protein:lipid molar ratio of 1:400 or 1:2,000 and was tested in the fusion assay. Note that the tailless ATL mutant promotes membrane fusion at higher concentrations. (*B*) The endoplasmic reticulum (ER) morphology was analyzed in *S. cerevisiae* cells lacking Sey1p and Yop1p (*sey1Δ yop1Δ* cells) in the presence or absence of tailless human ATL1. The tailless mutant was expressed under the MET25 promoter. The ER was visualized by expressing Sec63-GFP, focusing the microscope on either the periphery or the center of the cells. (Scale bar, 1 μm.) (*C*) ATL with point mutants in the CT region was reconstituted at a protein:lipid molar ratio of 1:1,000 and was tested in the fusion assay. (*D*) The fusion of vesicles containing tailless ATL was determined in the absence or presence of a synthetic peptide corresponding to the N-terminal part of the amphipathic helix (short-CTH; residues 479–494 of *Drosophila* ATL) at 45 μM.

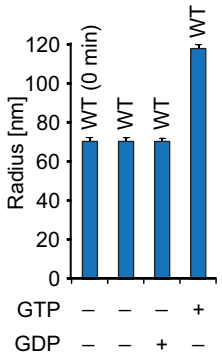

Fig. S3. Fusion with wild-type ATL measured by dynamic light scattering. Vesicles containing wild-type ATL at a protein:lipid ratio of 1:400 were incubated with GTP or GDP or without nucleotide for 10 min. The reactions were terminated after 10 min by the addition of EDTA, followed by analysis by dynamic light scattering. One representative experiment is shown, with the radii given as the average of three instrument readings. Error bars indicate SE.

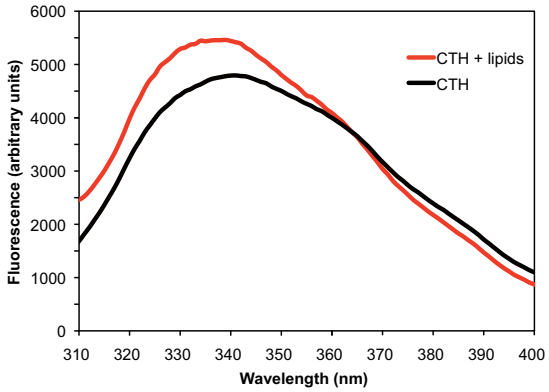

Fig. S4. Fluorescence spectrum of the CTH peptide. The fluorescence of the single Trp490 residue in the CTH peptide was monitored in the presence and absence of liposomes (84.5:15:0.5 molar percent POPC:DOPS:NBD-DPPE). Controls with only buffer or liposomes were subtracted from the data. A correction for the slight fluorescence attenuation caused by light scattering by liposomes was determined by measuring the fluorescence of the amino acid Trp in the presence and absence of liposomes.

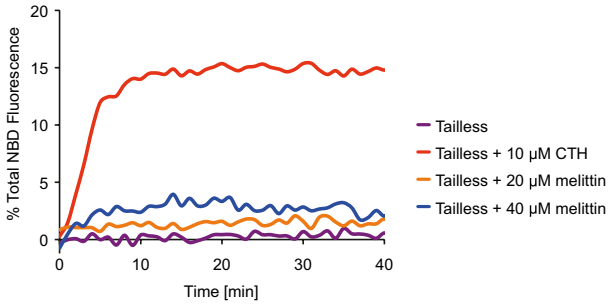

Fig. S5. Melittin has only a weak ability to stimulate fusion of tailless ATL. The fusion activity of tailless ATL (reconstituted at a protein:lipid ratio of 1:1,000) was determined in the presence of different concentrations of melittin. For comparison, the curve for tailless ATL in the presence of CTH is shown.

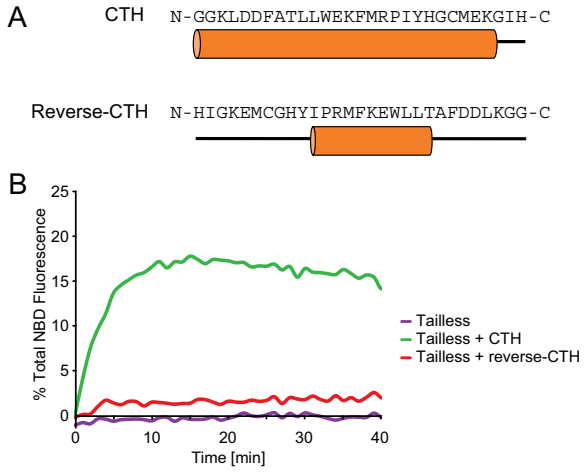

Fig. S6. A CTH peptide with the reversed sequence has a reduced ability to stimulate fusion of tailless ATL. (*A*) Prediction of helices for the wild-type peptide (CTH) and a peptide with the reverse sequence (reverse-CTH). (*B*) Fusion activity of the tailless ATL in the presence of 15 μM CTH or reverse-CTH peptide.

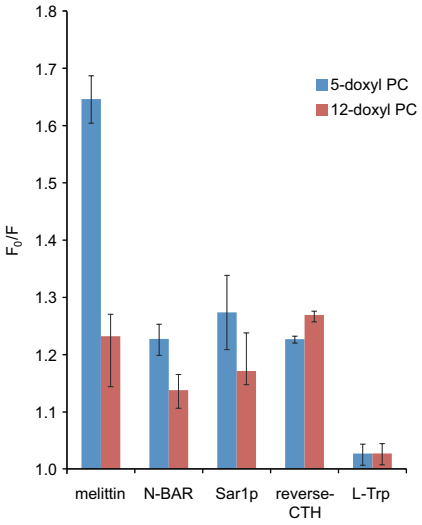

Fig. S7. Lipid interaction of amphipathic peptides used in Fig. 3 *C* and *D* and Fig. S6. The following peptides were added to liposomes containing or lacking phosphatidylcholine with doxyl groups at position 5 or 12 of the hydrocarbon chain: melittin, N-BAR helix (residues 1–24 of Rvs161p), Sar1p helix (residues 1–23 of Sar1p), and reverse CTH (a peptide with the reverse sequence of CTH; see Fig. S6). The quenching of the fluorescence of Trp residues in each of the peptides was measured and is expressed as F_0/F (maximal fluorescence with doxyl-free liposomes divided by maximal fluorescence with doxyl-containing liposomes). A control using the amino acid Trp (L-Trp) instead of a peptide is shown for comparison. Results shown are the mean of three experiments. Error bars indicate SE.

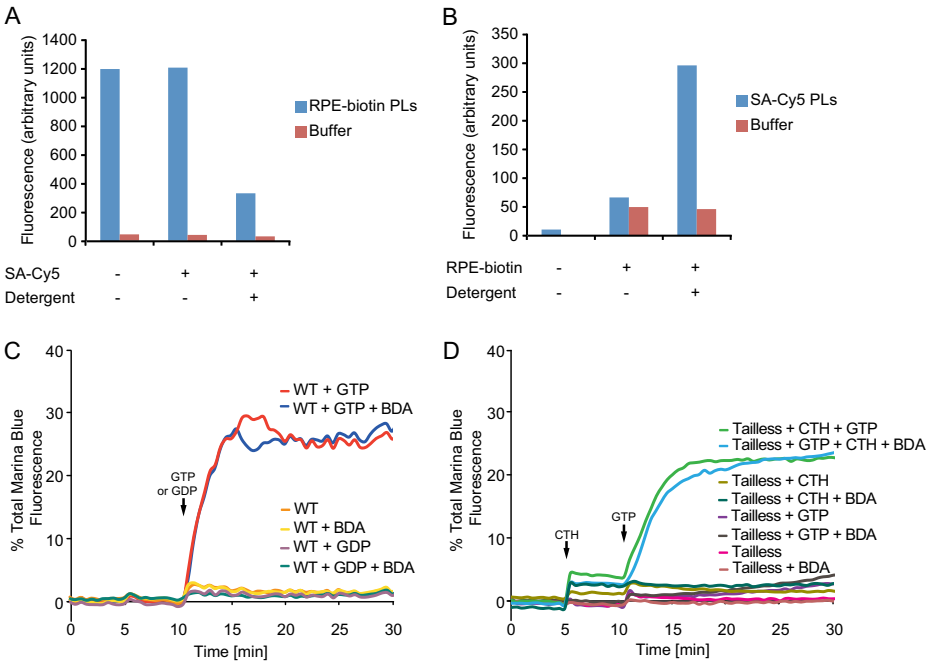

Fig. S8. Encapsulation of content-mixing dyes into proteoliposomes reconstituted with wild-type ATL. (*A*) SA-Cy5 was added to proteoliposomes containing RPE-biotin in the absence or presence of detergent (1% Thesit). RPE-biotin fluorescence was measured in a SpectraMax M5 plate reader with an excitation wavelength of 540 nm and an emission wavelength of 575 nm. Quenching of RPE-biotin by SA-Cy5 occurred only when detergent was added to lyse the liposomes. (*B*) As in *A*, but RPE-biotin was added to proteoliposomes containing SA-Cy5 or buffer. FRET was measured by exciting RPE-biotin at a wavelength of 565 nm and detecting the fluorescence emission of SA-Cy5 at 675 nm. (*C*) Lipid mixing with wild-type ATL was measured in parallel with content mixing shown in Fig. 4*A*. The dequenching of the fluorescence of Marina Blue was monitored. (*D*) As in *C*, but with the content-mixing experiments shown in Fig. 4*B*.

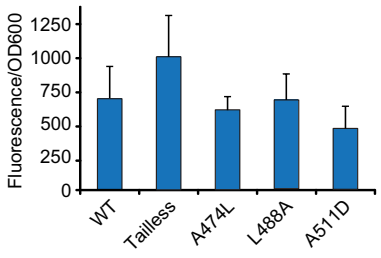

Fig. S9. Protein expression levels in the yeast complementation assay shown in Fig. 5. Wild-type human ATL1 or the indicated mutants were expressed under the SEY1 promoter in *S. cerevisiae* cells lacking Sey1p and Yop1p (*sey1Δ yop1Δ* cells). The expression levels were determined by immunoblotting with anti-ATL1 antibodies. Data shown are the mean of three experiments. Error bars indicate SE.

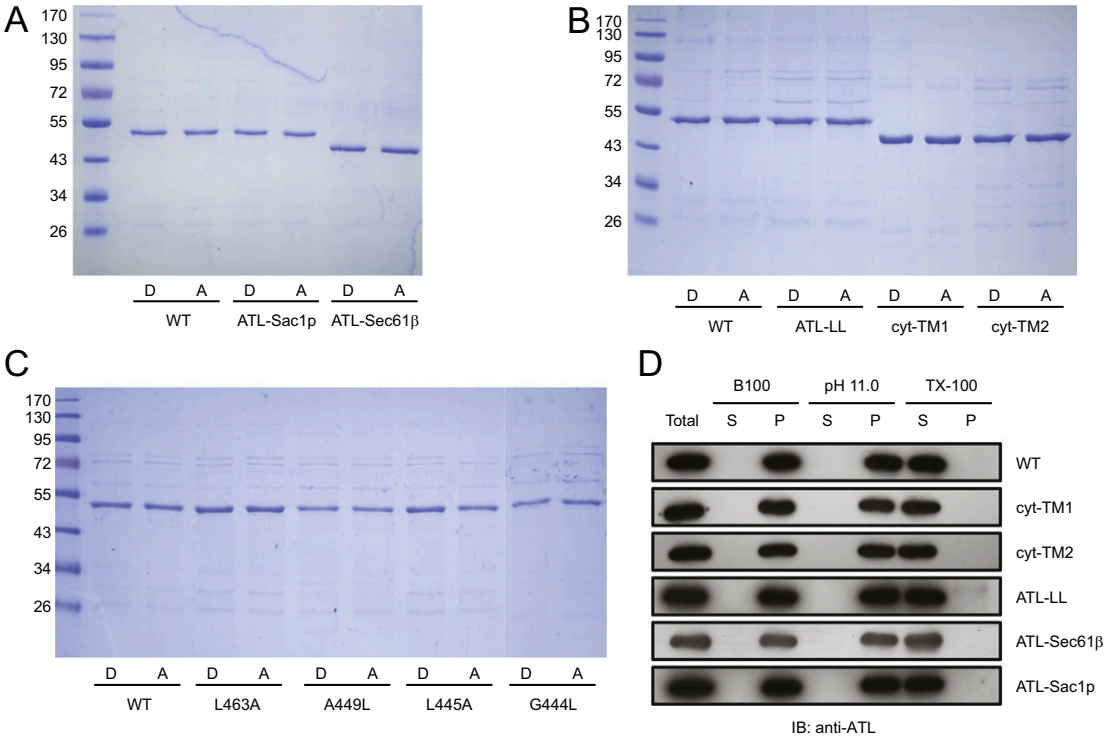

Fig. S10. Reconstitution of TM mutants of ATL into liposomes. (*A*) Donor (D) and acceptor (A) vesicles, which were used for the fusion assays shown in Fig. 6*B*, were analyzed by SDS/PAGE and Coomassie blue staining. The vesicles contained wild-type or TM-replacement mutants of ATL. (*B*) As in *A*, but with TM insertion or deletion mutants shown in Fig. 6*C*. (*C*) As in *A*, but with TM point mutants shown in Fig. 6*D*. (*D*) Proteoliposomes containing WT or mutant ATL were incubated with a physiological buffer (B100), carbonate (pH 11.0), or 0.1% Triton X-100 (TX-100). The samples were centrifuged, and supernatants (S) and pellets (P) were analyzed by SDS/PAGE and immunoblotting with antibodies to *Xenopus* ATL, which cross-react with *Drosophila* ATL.

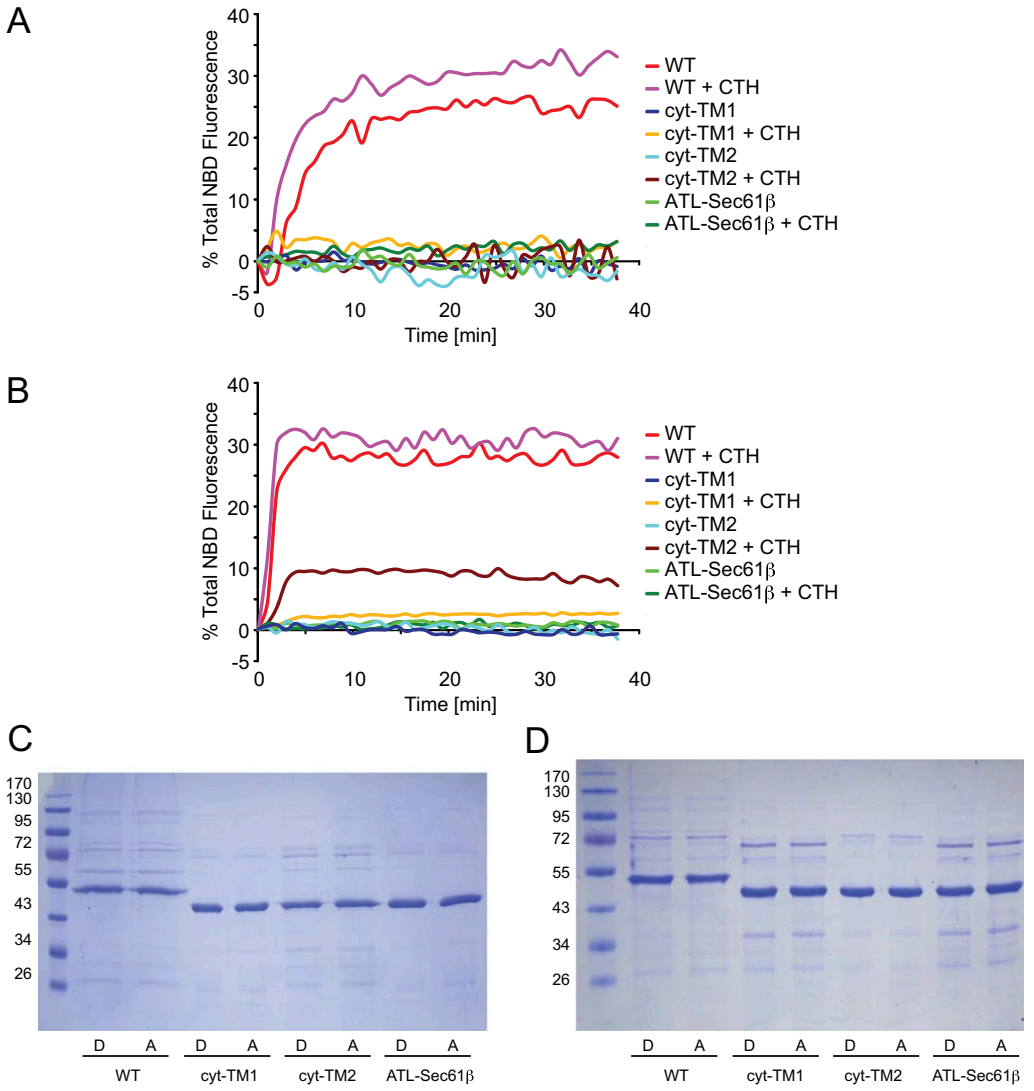

Fig. S11. Membrane fusion by wild-type ATL and TM mutants in the presence of CTH peptide. (*A*) Wild-type ATL or TM mutants lacking CT (cyt-TM1, cyt-TM2, and ATL-Sec61β) were reconstituted into liposomes at a protein:lipid molar ratio of 1:2,000. The membrane fusion assays were performed in the absence or presence of CTH peptide. (*B*) As in *A*, but the proteins were reconstituted at a protein:lipid molar ratio of 1:400. Note that the cyt-TM2 mutant showed some low fusion activity in the presence of CTH peptide. (*C*) The donor (D) and acceptor (A) vesicles used in *A* were analyzed by SDS/PAGE and Coomassie blue staining. (*D*) As in *C*, but with vesicles used in *B*.

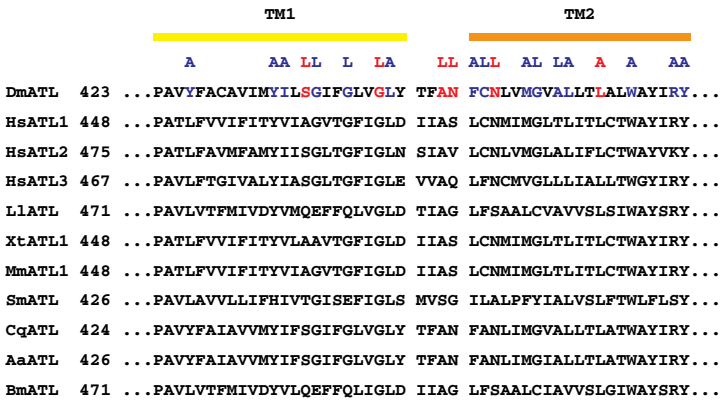

Fig. S12. Sequence alignment of TMs of ATLs from various species and fusion activity of tested TM mutants: *Drosophila melanogaster* ATL (DmATL), *Homo sapiens* ATL1 (HsATL1), *Homo sapiens* ATL2 (HsATL2), *Homo sapiens* ATL3 (HsATL3), *Loa loa* ATL (LlATL), *Xenopus tropicalis* ATL1 (XtATL1), *Mus musculus* ATL1 (MmATL1), *Schistosoma mansoni* ATL (SmATL), *Culex quinquefasciatus* ATL (CqATL), *Aedes aegypti* ATL (AaATL), *Brugia malayi* ATL (BmATL). The indicated DmATL residues were changed to the residues shown above the DmATL sequence. The fusion activity of the purified mutants was determined and classified into wild type-like (blue) and reduced activity (red).

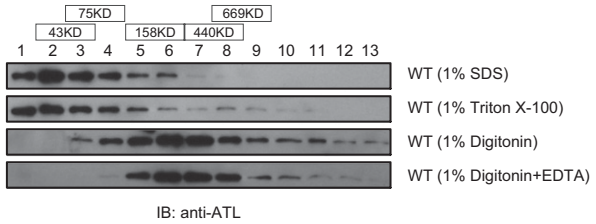

Fig. S13. ATL forms nucleotide-independent oligomers. Proteoliposomes containing wild-type ATL were solubilized in 1% SDS, 1% Triton X-100, 1% digitonin, or 1% digitonin and EDTA, and the extracts were subjected to sucrose gradient centrifugation. Fractions were analyzed by SDS/PAGE and immunoblotting with antibodies to *Xenopus* ATL. Note that ATL oligomers are dissociated by both SDS and Triton X-100. Molecular mass standards in the sucrose gradient are indicated.