Supporting Information

Chen et al. 10.1073/pnas.1423026112

SI Materials and Methods

Antibodies. Anti-FLAG antibody (Clone M2) was obtained from Sigma-Aldrich. GAPDH antibody (5E10) was purchased from Gene Tex, and anti-BIP and anti-calnexin antibodies were a gift from Paul Kim (University of Cincinnati, Cincinnati, OH).

To express mLnp1 for antibody production, plasmids pGEX-6a-1-mLnp1 and pET-15b-mLnp1 were transformed into BL21 cells and expression was induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) during a 15-h incubation at 18 °C. Cells expressing GST-mLnp1 were harvested and resuspended in ice-cold PBS solution (25 mM NaPi, pH 7.4, 120 mM NaCl, 5 mM $MgCl_2$) containing 1× complete protease inhibitor (Roche), 1 mM PMSF and 1 mM DTT. Cells expressing His_6-mLnp1 were resuspended in Ni-NTA binding buffer (50 mM NaPi, pH 8.0, 300 mM NaCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 10 mM imidazole, 10% (vol/vol) glycerol, 1 mM PMSF, 1× Complete Protease Inhibitor). The resuspended cells were lysed by sonication, and Triton X-100 was added to a final concentration of 0.5%. The lysates were centrifuged at 25,000 × *g* at 4 °C for 30 min to remove cell debris, and the supernatants were treated as described later.

The supernatant containing GST-mLnp1 was incubated with glutathione-Sepharose 4B beads (1 mL of a 75% bead slurry/50 mL of supernatant) for 2 h at 4 °C before the beads were washed three times with PBS solution (25 mM NaPi, pH 7.4, 120 mM NaCl, 5 mM $MgCl_2$, 0.5% Triton X-100), and then heated at 100 °C in sample buffer for 10 min. The beads were pelleted and the supernatant was loaded onto a 10% SDS polyacrylamide gel. The gel was stained with 0.3 M $CuCl_2$ for 20 min, and the negatively stained GST-mLnp1 band (74.56 kDa) was excised and rinsed with Tris-EDTA buffer (250 mM Tris, pH 8.8, 250 mM EDTA) for 30 min to remove $CuCl_2$. Protein was eluted from excised gel strips by using Bio-Rad electro-eluter following the manufacturer's directions. The eluted protein was concentrated to 1 mg/mL in SDS buffer (0.1% SDS, 25 mM Tris, 192 mM glycine, pH 8.3), and sent to Cocalico Biologicals for antibody preparation.

The supernatant containing His_6-mLnp1 was incubated with Ni-NAT agarose beads (1 mL of a 50% bead slurry/50 mL of supernatant) for 2 h at 4 °C before the beads were washed three times with Ni-NTA wash buffer [50 mM NaPi, pH 8.0, 300 mM NaCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 20 mM imidazole, 10% (vol/vol) glycerol, 0.5% Triton X-100]. His_6-mLnp1 was eluted from the beads by using Ni-NTA elution buffer [50 mM NaPi, pH 8.0, 300 mM NaCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 250 mM imidazole, 10% (vol/vol) glycerol, 0.5% Triton X-100], and concentrated against the following buffer [20 mM Hepes, pH 7.4, 120 mM NaCl, 1 mM DTT, 30% (vol/vol) glycerol, 0.5% Triton X-100] using an Amicon-Ultra 10K filter.

Bio-Rad Affi-Gel 15 was used to affinity-purify the mLnp1 antibody. To make mLnp1-coupled Affi-Gel 15, a slurry of Affi-Gel 15 was equilibrated in Affi-Gel binding buffer [100 mM Hepes, pH 7.4, 150 mM NaCl, 5% (vol/vol) glycerol, 0.5 Triton X-100], and His_6-mLnp1 was added to a final concentration of 25 mg protein per milliliter of resin. The Affi-Gel 15/His_6-mLnp1 coupling reaction was then incubated for 4 h at 4 °C. Ethanolamine·HCl (pH 8.0) was added to the reaction (100 mM, final concentration), incubated for 1 h at 4 °C, and then washed twice with PBS solution (20 mM NaPi, pH 7.4, 120 mM NaCl), once with glycine buffer (100 mM glycine, pH 2.4, 150 mM NaCl), and then two more times with PBS solution. mLnp1 antibody serum (1.5–3 mL) was applied to the Affi-Gel, and incubated overnight at 4 °C. The Affi-Gel was transferred into an Econo-column (Bio-Rad) and washed with PBS solution until the OD_{280} of the flow through was less than 0.005. mLnp1 antibody was eluted with glycine buffer (100 mM glycine, pH 2.4, 150 mM NaCl), and immediately neutralized by using 5 M Tris·HCl (pH 8.0). The eluted antibody was concentrated using Amicon Ultra 30K filter and stored in PBS/glycerol buffer [20 mM NaPi, pH 7.4, 120 mM NaCl, 30% (vol/vol) glycerol].

DNA Constructs for the Liposome Fusion and GTPase Assay. Plasmid pJM693 (GST-Human Atlastin-1-His_8) containing human Atlastin-1 was generated by PCR by using oligos 581 (TAGTCGACATGGCCAAGAACC) and 589 (TTCTCGAGCATTTTTTTCTTTTC) and cDNA clone (ID no. 3869877; Open Biosystems) as template. The PCR fragment was cut with SalI and XhoI and ligated into pJM680 (1). Plasmid pJM992 [GST-DmLnp-His_8 (amino acids 99–387)] containing the DmLNP (CG8735) soluble domain was generated by PCR using oligos 878 (CCGGATCCCAGCGCAAGCTCAACAAAAATG) and 873 (CGCTCGAGGAGCTGGCAGCCACCTCAG) using a cDNA clone (GH24644) from the *Drosophila* Genomics Research Center as a template. The PCR product was digested with BamH1 and Xho1 and ligated into pJM680 (1) that was cut with the same enzymes.

Expression and Purification of *Drosophila* and Human Atlastin-1. GST-dAtl-His_8 (pJM681) was produced as previously described (1). GST-HsAtl1-His_8 was produced in Rosetta (DE3) cells according to the manufacturer's instructions. Four liter cultures with a starting density of ~0.1–0.2 OD_{600} were grown at 25 °C to OD_{600} of 0.4–0.6 before the culture was moved to 16 °C, then induced with 0.2 mM IPTG and grown overnight (~16 h) at 16 °C. Cells were harvested, washed with 25 mM Hepes (pH 7.4) containing 200 mM KCl, and lysed in buffer A200 [25 mM Hepes, pH 7.4, 200 mM KCl, 10% (vol/vol) glycerol, 2 mM 2-mercaptoethanol] containing 4% (vol/vol) Triton X-100 2 mM EDTA and 1× complete protease inhibitor mixture (Roche). The extract was centrifuged for 40 min at 125,000 × *g* at 4 °C and the supernatant was incubated with 84 mg of swelled GSH-agarose beads (Sigma) for 1 h at 4 °C. Beads were washed with buffer A100 [25 mM Hepes, pH 7.4, 100 mM KCl, 10% (vol/vol) glycerol, 2 mM 2-mercaptoethanol] containing 1% Triton X-100 and 1 mM EDTA followed by a second wash in buffer A100 containing 0.1% Anapoe X-100 and 1 mM EDTA. Protein was eluted from GSH-agarose with buffer A100 containing 0.1% Anapoe X-100, 1 mM EDTA, and 10 mM glutathione. The frozen aliquots were stored at −80 °C.

Expression and Purification of soluble Human Lnp. His_6-SUMO-Human Lnp (99-428) was produced by expressing pJM967 in BL21(DE3) cells that were cultured as described earlier. Cells were harvested, washed with 25 mM Hepes (pH 7.4) and 200 mM KCl, and lysed in buffer A200 containing 1× complete protease inhibitor mixture (Roche). Cell debris was removed by centrifugation and filtered at 4 °C through a sterile 0.45-μM filter (Millipore). The clarified supernatant was then passed over a HiTrap HP Ni^{2+}-chelating column (GE Healthcare) on an ÄKTA Prime chromatography system (Amersham Biosciences). The column was washed with 10 column volumes of buffer A100 and eluted with a linear gradient of 20–500 mM imidazole in buffer A100 (10 column volumes). Peak fractions were pooled and the His_6-SUMO fragment removed by an overnight incubation with 0.15 mg of GST-SENP2 prepared as described

previously (2) at 4 °C. GST-SENP2 was removed by incubating the cleaved protein with GSH-agarose beads for 1 h at 4 °C, and then the supernatant was dialyzed overnight against 4 L of 25 mM Hepes·KOH, 50 mM KCl, and 10% (vol/vol) glycerol at 4 °C. The dialyzed protein was bound to a HiTrap SP-HP column and the protein was eluted with a linear salt gradient from 40 mM to 1 M KCl. Peak fractions were pooled and stored at −80 °C.

Expression and Purification of soluble *Drosophila* Lnp. GST-DmLnp-His_6 (99-387) (pJM992) was transformed into BL21(DE3) and grown as described earlier. Four-liter cultures with a starting density of ∼0.1–0.2 OD_{600} were grown at 37 °C until an OD_{600} of 0.4–0.6 was achieved, then induced with 0.2 mM IPTG and grown for 4 h at 37 °C. Cells were harvested, washed with 25 mM Hepes (pH 7.4) and 200 mM KCl, and lysed in buffer A200 containing 1× complete protease inhibitor mixture (Roche). Extracts were centrifuged, filtered, and purified by nickel chromatography as described earlier. Peak fractions were pooled and incubated with 84 mg of swelled GSH-agarose beads (Sigma) for 1 h at 4 °C. Beads were washed with buffer A100 and protein was eluted from GSH-agarose with buffer A100 containing 10 mM glutathione.

Proteoliposome Production, Reconstitution, and Fusion. Proteins purified in 0.1% Anapoe X-100 (Anatrace) were reconstituted into preformed POPC 100 nm liposomes as previously described (1, 2). Atlastin was reconstituted into labeled and unlabeled 100-nm liposomes by detergent-assisted insertion (3, 4). dAtl in 0.1% Anapoe X-100 was mixed with preformed liposomes at a 1:400 protein-to-lipid molar ratio and an effective detergent-to-lipid ratio of ∼0.8. Protein and lipid were allowed to mix for 1 h at 4 °C. Detergent was removed by Bio- Beads SM-2 Adsorbent (Bio-Rad) at 70 mg of Triton X-100 per 1 g of beads. Insoluble protein aggregates were pelleted by centrifugation of the samples in an Eppendorf microcentrifuge. Final lipid and protein concentrations were determined by liquid scintillation counting and amido black protein assay, respectively.

In vitro fusion assays were done as previously described (2) with the following modifications. Labeled and unlabeled populations of *Drosophila* Atlastin proteoliposomes (0.15 mM lipid each) were mixed with 0.5 mM GTP, 50 μM $ZnCl_2$, or buffer only in the presence of the indicated amounts of human or *Drosophila* Lnp and brought to total volume of 45 μL with buffer A100 in wells of 96-well FluoroNunc PolySorp plates (Nunc). The reactants in the plate were incubated at 37 °C in a fluorescent plate reader (Infinite M200; Tecan) for 5 min. To each well, 5 μL of 50 mM $MgCl_2$ was added, and nitrobenzoxadiazole (NBD) fluorescence was measured (excitation 460 nm, emission 538 nm) at 1-min intervals for 60 min. To determine the absolute NBD fluorescence, 10 μL of 2.5% (wt/vol) N-dodecylmaltoside was added at 60 min. Data were normalized to total fluorescence after detergent solubilization.

GTPase Measurements. GTPase activity was measured as previously described (2) by measuring the release of inorganic phosphate from GTP by using the EnzChek Phosphate Assay Kit (Molecular Probes). Recombinant human Atlastin-1 was mixed in a 100-μL reaction volume with 1 U/mL purine nucleoside phosphorylase, 200 μM 2-amino-6-mercapto-7-methylpurine riboside, 1 mM GTP, and 50 μM $ZnCl_2$ in the presence or absence of soluble Lnp at the indicated concentrations in a transparent 96-well plate. The plates were warmed to 37 °C in a microplate reader (Infinite M200; Tecan), and 5 mM $MgCl_2$ was added to start the reaction. Absorbance at 360 nm was measured in real time every 20 s for 20 min and normalized to phosphate standards, and the initial rates were calculated.

Protease Protection Assay. Protease protection assays were performed as previously described (5, 6) with minor modifications. COS-7 cells were plated in a 12-well culture dish and grown to 90% confluence in DMEM. Cells were washed with PBS solution, and cell monolayers were left untreated or permeabilized with 200 μL digitonin (100 μg/mL) in proteinase K buffer (20 mM Tris·HCl, pH 7.4, 120 mM NaCl, 250 mM sucrose, 1 mM EDTA) at room temperature for 1 min. The cells were then treated with proteinase K (100 μg/mL) in proteinase K buffer for 3 min at 20 °C in the absence or presence of 1% Triton X-100. At the end of the incubation, the reaction was stopped by the addition of complete protease inhibitor mixture (Roche) and PMSF. Cells were lysed in RIPA buffer, and protein concentration was determined by using the Bradford assay reagent. mLnp1, BIP, and calnexin were detected by Western blot analysis.

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2. Moss TJ, Andreazza C, Verma A, Daga A, McNew JA (2011) Membrane fusion by the GTPase atlastin requires a conserved C-terminal cytoplasmic tail and dimerization through the middle domain. *Proc Natl Acad Sci USA* 108(27): 11133–11138.
3. Rigaud JL, Lévy D (2003) Reconstitution of membrane proteins into liposomes. *Methods Enzymol* 372:65–86.
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5. Kalbfleisch T, Cambon A, Wattenberg BW (2007) A bioinformatics approach to identifying tail-anchored proteins in the human genome. *Traffic* 8(12):1687–1694.
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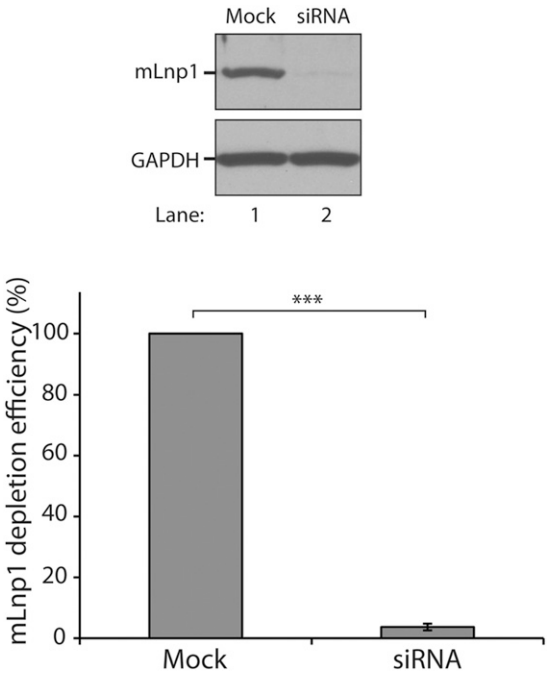


Fig. S1. mLnp1 depletion efficiency by siRNA. (*Top*) COS-7 cells were transfected with siRNA against mLnp1 mRNA. Cell lysates were immunoblotted with anti-mLnp1 antibody to demonstrate efficient depletion of mLnp1. GAPDH was used as a loading control. (*Bottom*) Quantification of the mLnp1 depletion. Error bars are SEM from three separate experiments (***$P < 0.001$, Student t test).

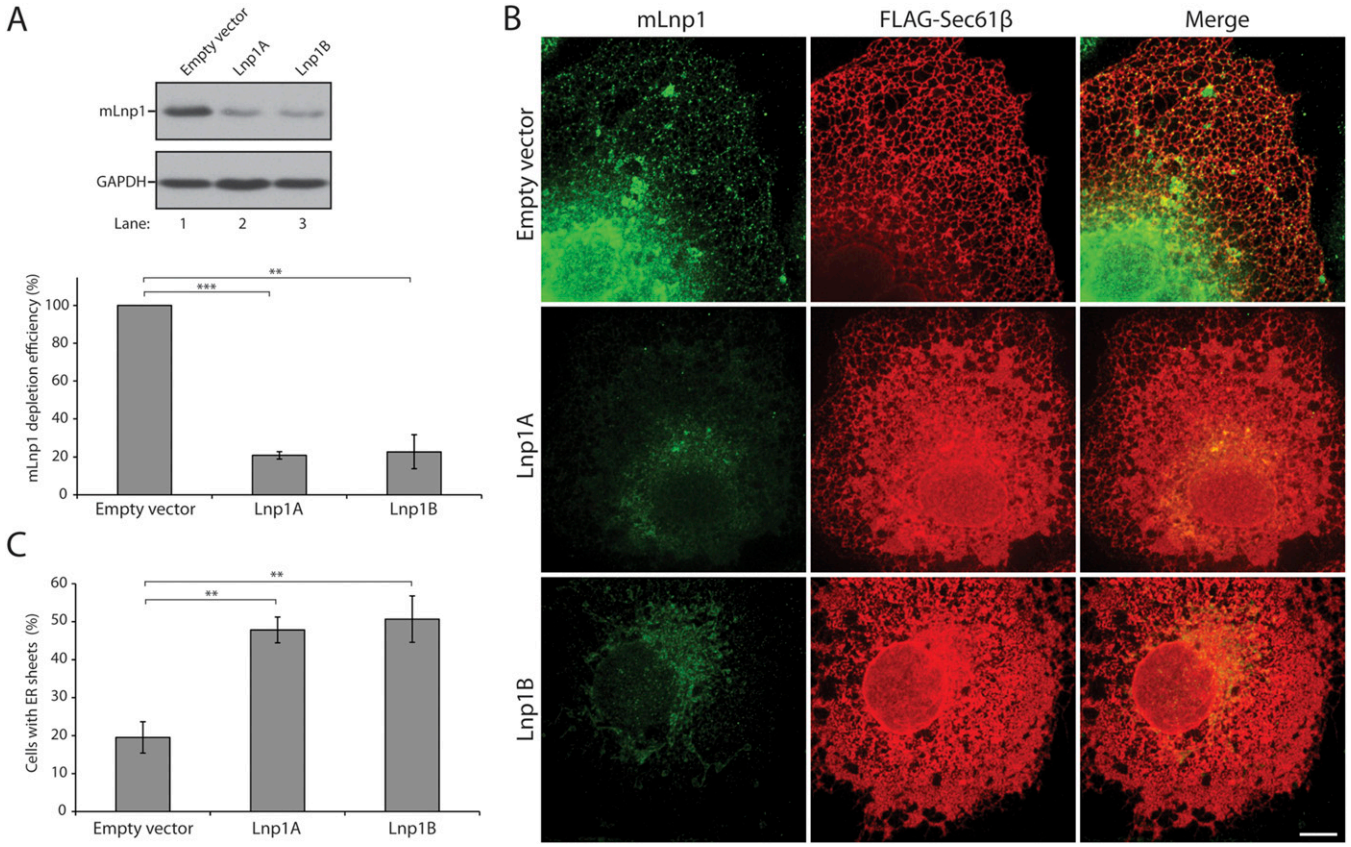


Fig. S2. Depletion of mLnp1 reduces the tubular ER network. (*A*) (*Top*) Two different shRNAs, Lnp1A and Lnp1B, directed against mLnp1 mRNA were transfected into COS-7 cells, and lysates were immunoblotted with antibody to Lunapark. GAPDH was used as a loading control. (*Bottom*) Quantification of the mLnp1 depletion. Error bars are SEM from three separate experiments (***$P < 0.001$ and **$P < 0.005$, Student *t* test). (*B*) COS-7 cells were cotransfected with FLAG-Sec61β and mLnp1 shRNA vectors and immunostained for mLnp1 and FLAG-Sec61β. (*C*) The percentage of cells with sheet-like ER morphology was quantified. Error bars are SEM for three separate experiments; $n = 340$ cells for empty vector, $n = 274$ cells for Lnp1A, and $n = 338$ cells for Lnp1B (**$P < 0.01$, Student *t* test).

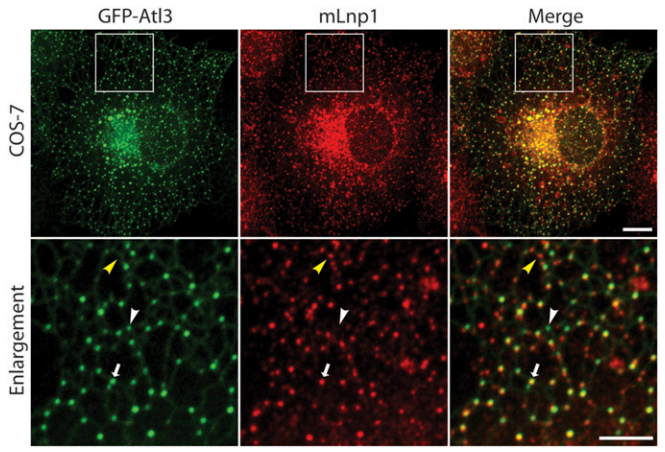


Fig. S3. mLnp1 partially colocalizes with GFP-Atl3. COS-7 cells were transfected with GFP-Atl3 and immunostained with anti-mLnp1 antibody. Boxed area (*Top*) is enlarged below. The arrow points to mLnp1 and GFP-Atl3 that colocalize; the white arrowhead points to a GFP-Atl3 punctum that is not colocalized with mLnp1; the yellow arrowhead points to an mLnp1 punctum that does not colocalize with GFP-Atl3. (Scale bars: *Top*, 10 μm; *Bottom*, 5 μm.)

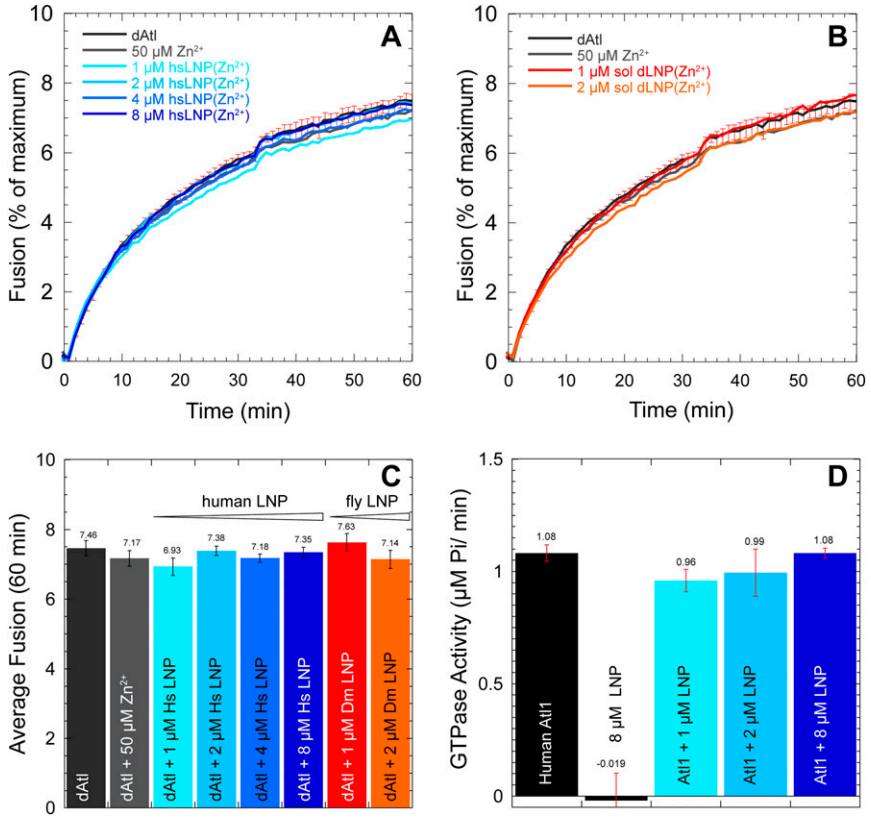


Fig. S4. Lunapark does not affect fusion or GTPase activity of Atlastin. (*A*) Kinetic fusion graph of fluorescently labeled dAtl (*Drosophila* atlastin) donor proteoliposomes fused with equimolar amounts of unlabeled dAtl acceptor proteoliposomes (~0.66 μM total dAtl) with increasing amounts of soluble human LNP (Zn^{2+}) and (*B*) soluble *Drosophila* LNP (Zn^{2+}). (*C*) Average fusion at 60 min. Fusion of dAtl is unaffected by increasing amounts of hsLNP and dLNP. (*D*) GTPase activity of human Atlastin-1. Increasing amounts of the soluble domain of human LNP does not alter GTPase activity. Histograms are an average of three experiments. Error bars represent SEM.

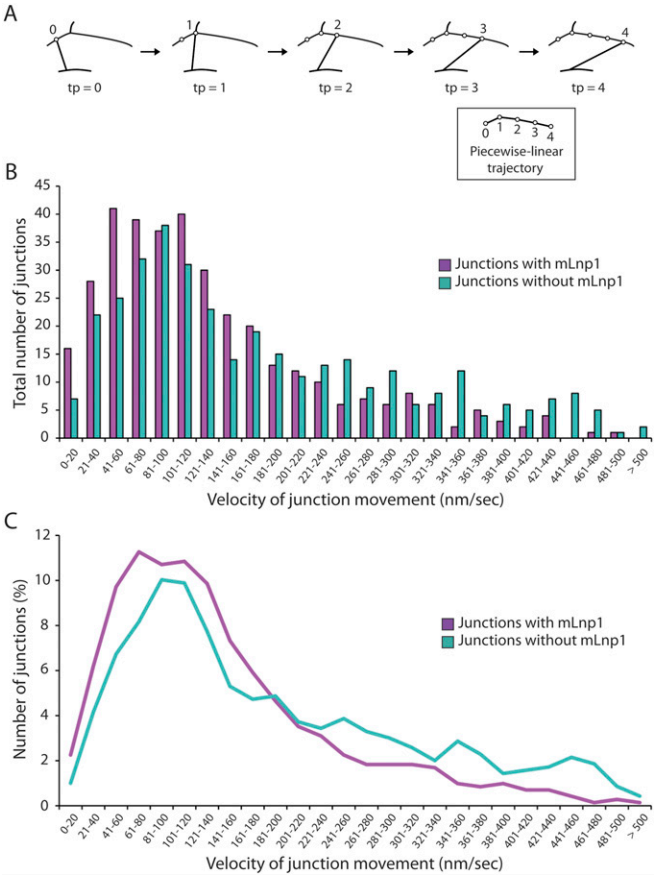


Fig. S5. Preexisting junctions with mLnp1 move at lower velocities than those lacking mLnp1. (*A*) A diagram showing how junction velocity was measured. The positions of a tracked junction within image frames are marked at each time point (tp). The piecewise-linear trajectory and the total distance that a junction travels along an ER tubule are obtained by linking subsequent positions. The average velocities are calculated by dividing the total distance by the total elapsed time. (*B*) Quantification of the velocities of preexisting junctions; $n = 355$ for junctions with mLnp1 (magenta), $n = 349$ for junctions without mLnp1 (cyan). (*C*) A trendline of the velocities of preexisting junctions based on the data in *B*.

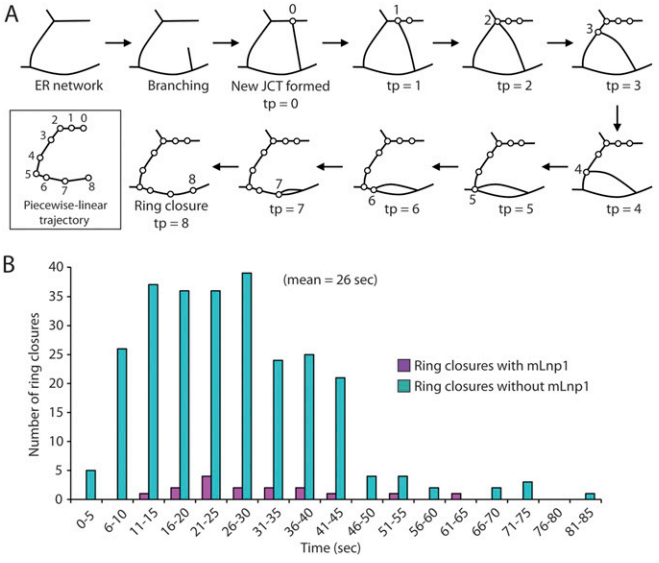


Fig. S6. Duration of junctions before ring closure. (*A*) A diagram showing a newly formed junction that undergoes ring closure, and how junction velocity is measured as described in the legend of Fig. S5*A*. Time is defined as "0" when a branching tubule fuses to a preexisting tubule, generating a new junction. (*B*) According to the diagram in *A*, the duration is from tp 0 until the time at which ring closure is completed. $n = 281$ for junctions undergoing ring closure.

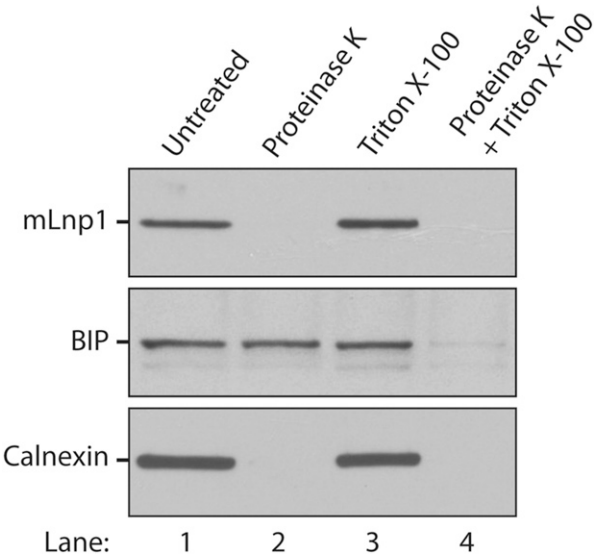
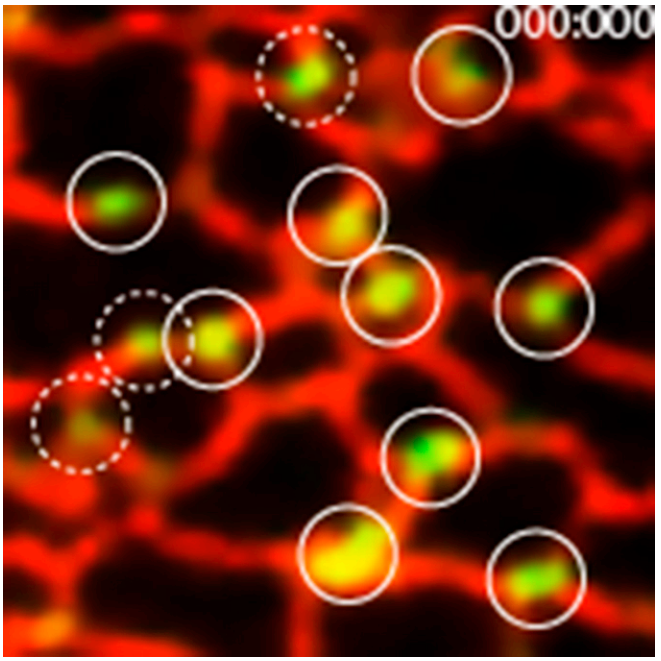
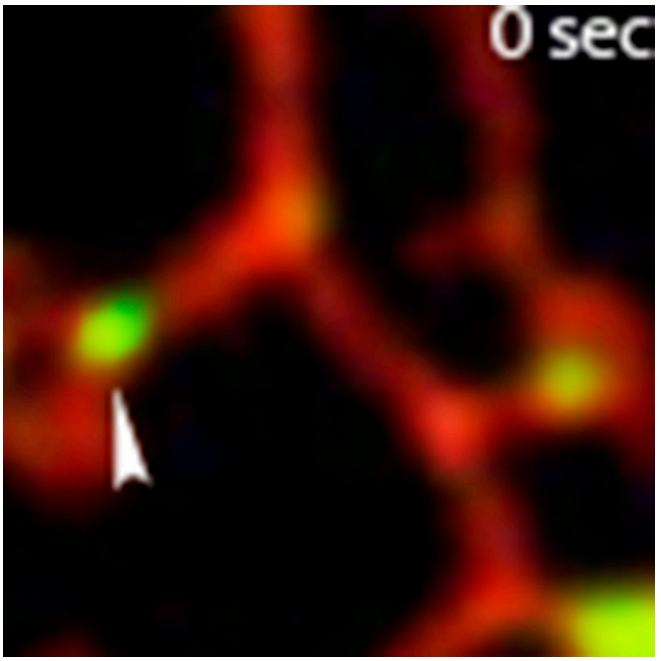


Fig. S7. Lunapark is exposed to the cytosolic side of the ER membrane. The membrane topology of Lnp1 was determined by using a protease protection assay as described in *SI Materials and Methods*. Protease K was added to permeabilized COS-7 cells in the presence or absence of Triton X-100, and cell lysates were immunoblotted with anti-Lnp1, anti-BIP, and anti-calnexin antibodies.



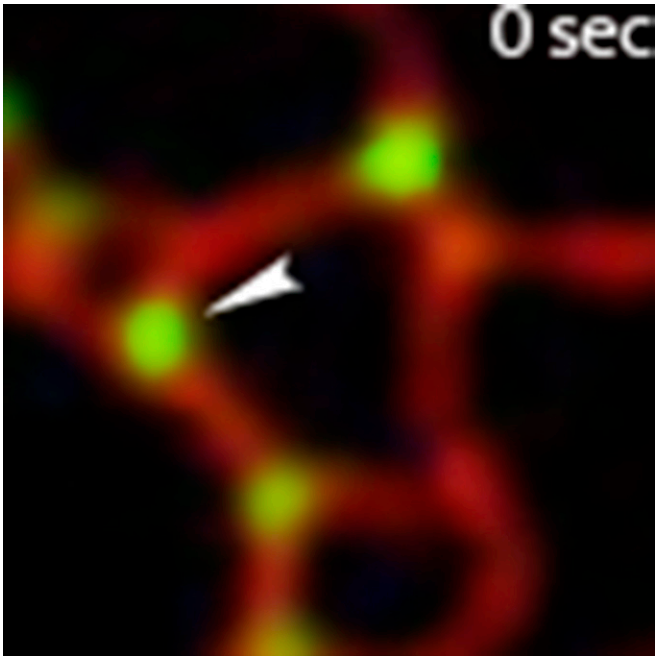
Movie S1. mLnp1 at ER junctions is not very mobile. Solid circles (diameter, 1.5 μm) mark the original position of stationary mLnp1 puncta. Dashed circles (diameter, 1.5 μm) mark the original position of mobile mLnp1 puncta.

Movie S1



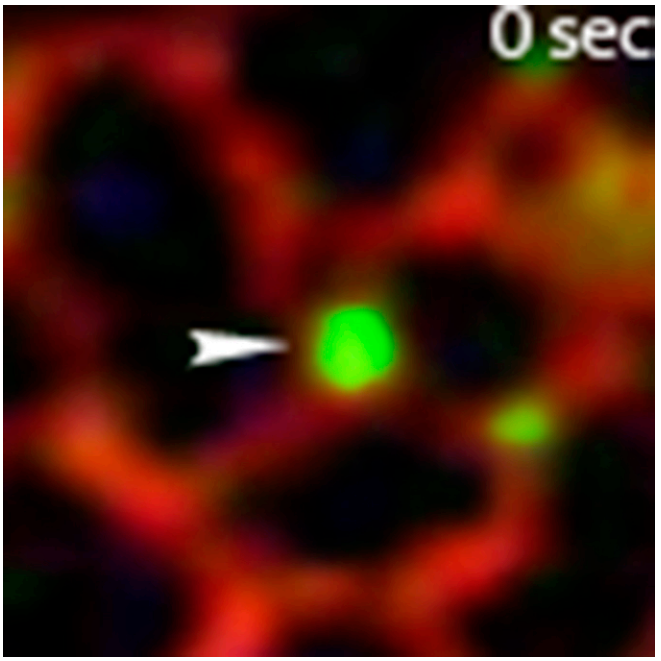
Movie S2. mLnp1 travels from one junction to the next junction. The arrowhead points to an mLnp1 punctum in motion.

Movie S2



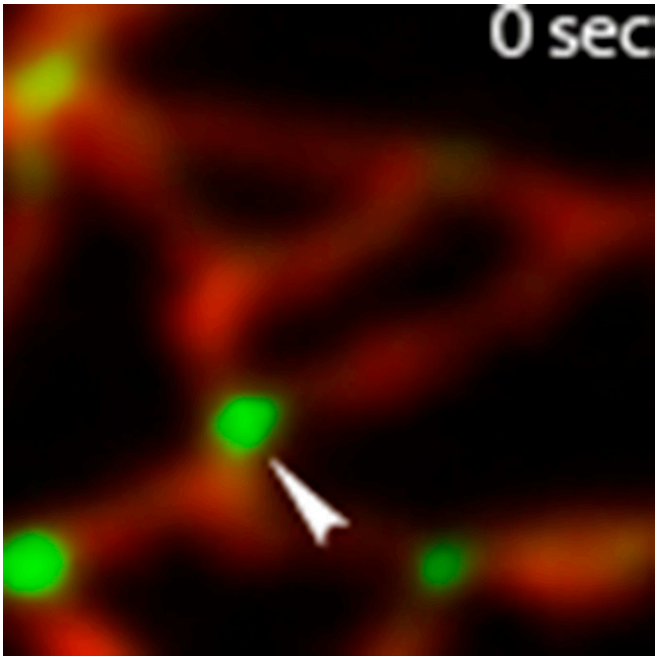
Movie S3. An mLnp1 punctum travels and merges with another mLnp1 punctum. The arrowhead points to a traveling mLnp1 punctum.

Movie S3



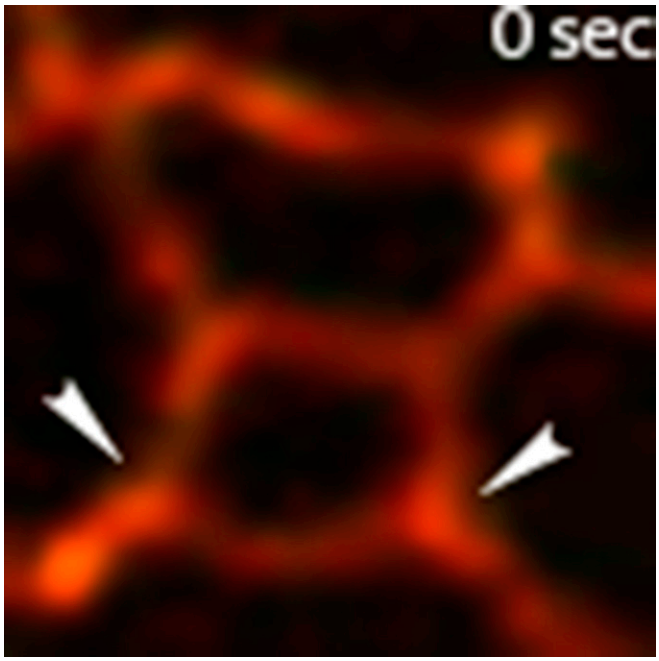
Movie S4. An mLnp1 punctum splits into two puncta. The arrowheads point to an mLnp1 punctum that splits.

Movie S4



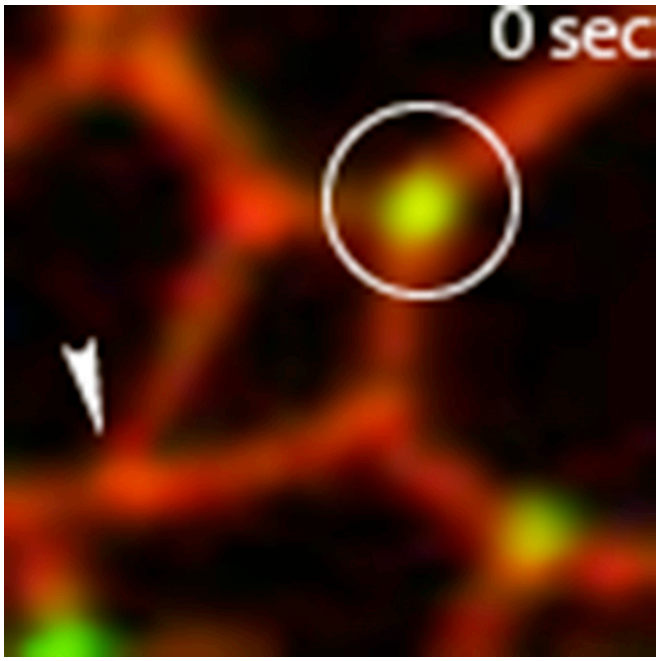
Movie S5. mLnp1 travels with the junction. The arrowhead points to a traveling mLnp1 punctum and junction.

Movie S5



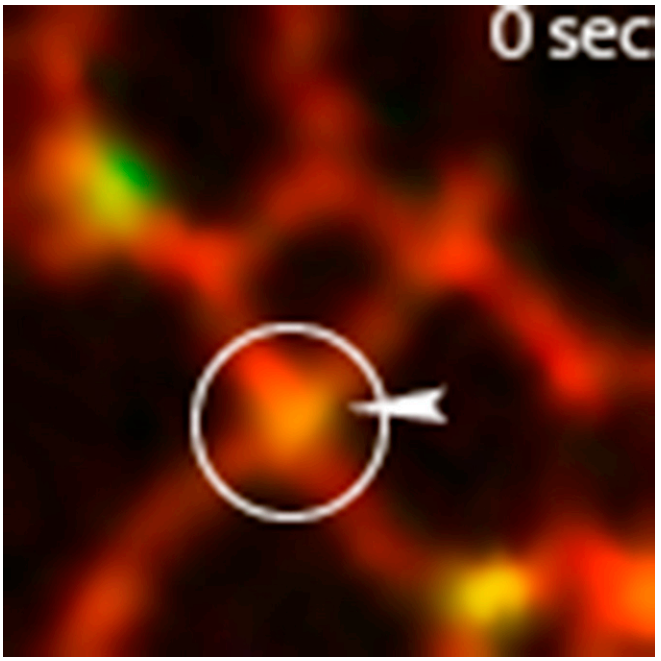
Movie S6. An mLnp1-free junction moves to an mLnp1-free junction to complete a ring closure. The arrowheads point to mLnp1-free junctions.

Movie S6



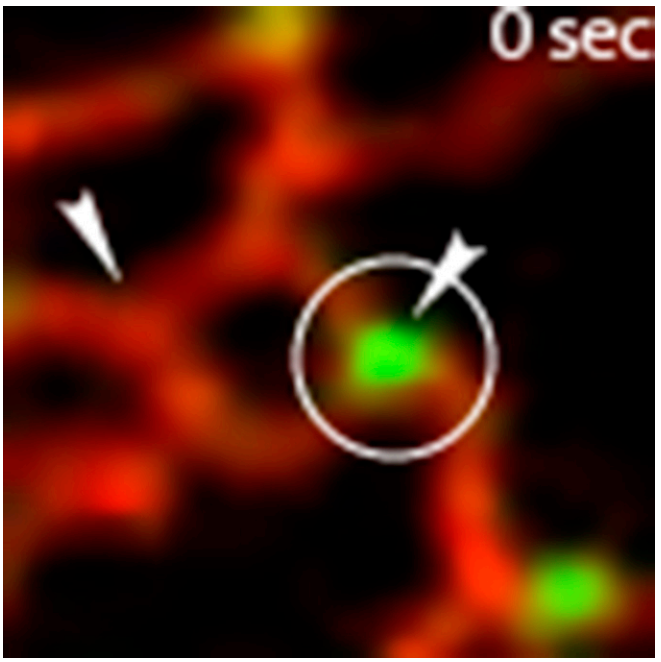
Movie S7. An mLnp1-free junction moves to a stationary junction with mLnp1 to complete a ring closure. The arrowhead points to mLnp1-free junctions. The solid circle marks a stationary mLnp1 junction.

Movie S7



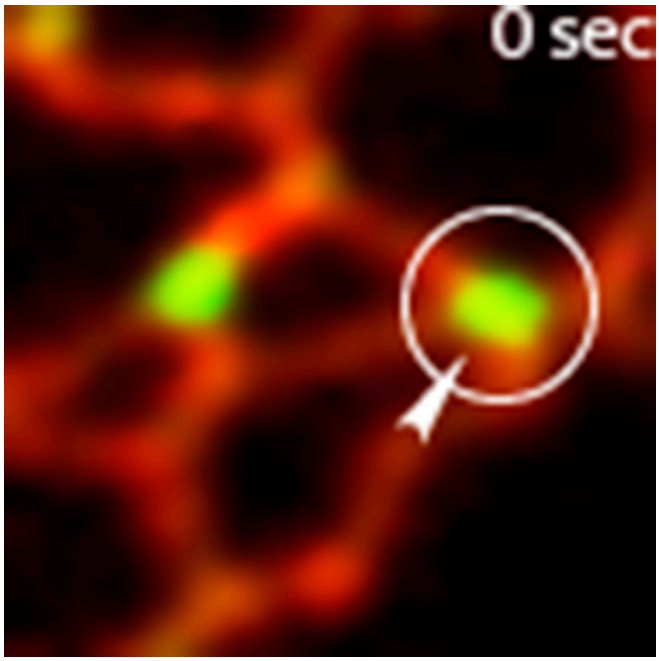
Movie S8. A junction leaves a stationary mLnp1 punctum and moves toward another junction to complete a ring closure. The arrowhead points to a junction without mLnp1. The solid circle marks a stationary junction with mLnp1.

Movie S8



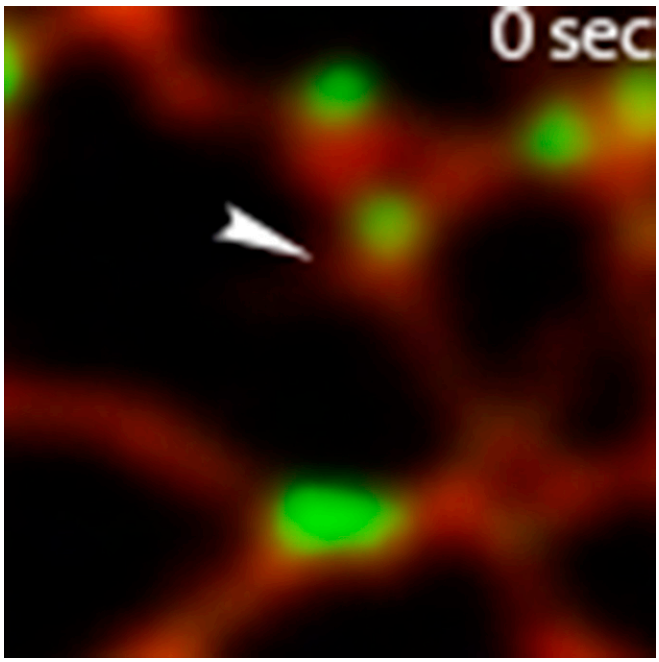
Movie S9. A junction containing mLnp1 and an mLnp1-free junction move toward each other to complete a ring closure. The arrowheads point to an mLnp1-free junction or an mLnp1 containing junction that move toward each other. The solid circle marks the original position of a junction with mLnp1.

Movie S9



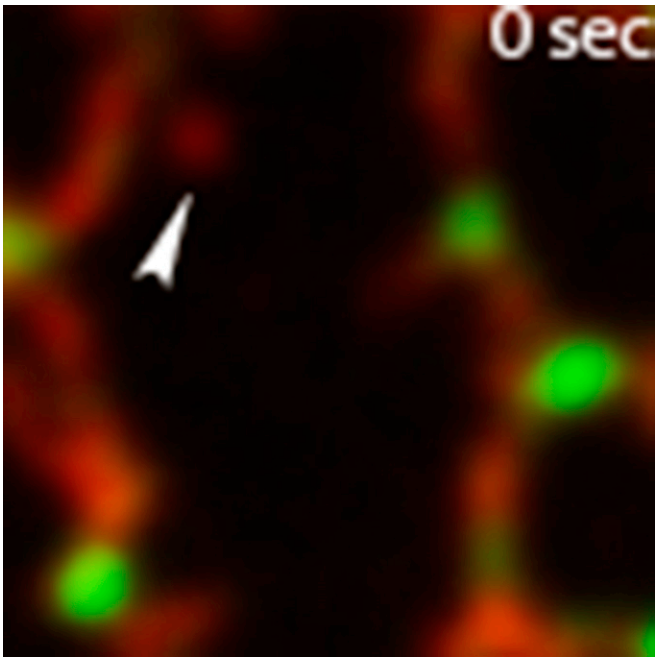
Movie S10. A junction containing mLnp1 moves to a stationary junction to complete a ring closure. The arrowhead points to a junction containing mLnp1. The solid circle marks the original position of a junction with mLnp1.

Movie S10



Movie S11. A newly formed junction that does not acquire mLnp1 undergoes ring closure and ultimately disappears. The arrowhead points to a new junction that undergoes ring closure.

Movie S11



Movie S12. A newly formed junction that acquires mLnp1 remains stable. The arrowhead points to a new junction that acquires mLnp1 and remains stable.

Movie S12