Supporting Information

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SI Materials and Methods

In Silico Analysis of the LegC Effectors. Coiled-coil (CC) motifs were predicted using the protein sequences of the *Legionella* effectors LegC2 (YP_095901.1), LegC3 (AAU27781.1), and LegC7 (AAU28360.1) using COILS (48) (window = 28 and cutoff = 0.9). Sequences of the LegC effectors and mammalian Qa SNAREs like Stx6, Stx7, and Stx16 were aligned using MUSCLE (49) and conservation scores were calculated using Jalview (50) based on the AMAS method (51). Details about the Zappo color scheme can be found at: www.jalview.org/help/html/colourSchemes/clustal.html. Transmembrane helices (TMHs) were predicted by the SMART 7 (52). CC motifs and TMDs shown in Fig. 1*B* are as follows: LegC2, TMD1 (79–101), TMD2 (105–127), CC1 (169–289), and CC2 (330–361); LegC3, CC1 (59–96), CC2 (232–259), CC3 (310–357), TMD1 (373–395), TMD2 (402–424), and CC4 (472–508); and LegC7, TMD (97–119), CC1 (155–272), and CC2 (325–371).

***Legionella* Strains and Plasmids.** The desired genes were amplified by PCR reactions using chromosomal DNA from *Legionella* strain KS79 with the following combinations of primers. Gene *legC2* was amplified using primers: ATCGCATATGACAGACACTCCAAAAGC and ATCGCTCGAGCTAACCTGTGAGAGTTTG. Gene *legC3* was amplified using primers: ATCGCATATGATTATGTTTTTGGCCAACTGC and ATCGCTCGAGTTACGCTATCTCATTAACTG. Gene *legC7* was amplified with primers: ATCGCATATGGCTACTAATGAAACAGAGC and ATCGCTCGAGTTAATTGACTAAAGCAATAG. The PCR products were individually cloned into NdeI and XhoI sites of pET15b and correct clones were confirmed by sequencing. These plasmids were used to express 6×-His–LegC2, 6×-His–LegC3, and 6×-His–LegC7. All of the experiment were performed with *L. pneumophila* JR32 (53) a Philadelphia-1–derived strain, with *dotA* (a JR32 *dotA*::Tn903dIIlacZ strain LELA3118) or with KS79 (20) a hypercompetent *comR* mutant of JR32. *Legionella* strains carrying a plasmid expressing β-lactamase effector protein fusions were constructed as previously described (20). *Legionella* strains harboring a plasmid pXDC50 to express mCherry protein for *Legionella* growth curve were constructed in a previous study (27).

Cell Culture. THP-1 cells were obtained from ATCC and grown in Advanced RPMI 1640 (Invitrogen) supplemented with 10% (vol/vol) FBS and 2 mM glutamine at 37 °C in a CO_2 incubator. THP-1 cells were differentiated into macrophage-like cells by resuspending them into RPMI + 2 mM glutamine + 10% (vol/vol) FBS + 30 μM PMA. Following 72 h of treatment with PMA, the differentiated THP-1 cells were washed and resuspended in RPMI + 2 mM glutamine + 10% (vol/vol) FBS for infection (20).

Infection of THP-1 Cells by *Legionella* and Coimmunoprecipitation of LegC Proteins and VAMP4. Coimmunoprecipitation experiments were carried out following the manual of the Pierce Crosslink IP Kit (Thermo Fisher Scientific). *Legionella* strains overexpressing either TEM or N-terminal TEM-tagged versions of the effector proteins LegC2, LegC3, or LegC7, were used to infect differentiated THP-1 macrophages. After 6 h of infection, 4×10^7 infected THP-1 cells were harvested and lysed on ice with lysis buffer [25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 5% (vol/vol) glycerol, pH 7.2] in the presence of protease inhibitors (Halt Protease Inhibitor Mixture; Thermo Fisher Scientific). The supernatant was collected and precleared with 50% (wt/vol) Protein A/G agarose. The precleared supernatant was incubated overnight with Protein A/G agarose charged with 500 μg affinity-purified, polyclonal, rabbit anti-TEM antibodies (prepared at Pocono Farms). The beads were washed three times and eluted with elution buffer at pH 2. The precleared supernatants and elutions were analyzed by immunoblot analysis with anti-TEM antibody and different human R-SNARE antibodies (Fig. 1*C* and Fig. S1). TEM (30 kDa), TEM-LegC2 (76 kDa), TEM-LegC3 (93 kDa), and TEM-LegC7 (78 kDa) are present in the precleared supernatant as well as in eluted samples at the expected sizes.

VAMP4 Knockdown. *Escherichia coli* strains carrying pCMV or pMD helper plasmids were grown in LB medium with 100 μg/mL carbenicillin. *E. coli* strains carrying plasmids pGIPZ (vector control) or pGIPZ shRNA for knockdown of VAMP4 (V3LHS_364980, V3LHS_364977, and V3LHS_364975), obtained from Thermo Fisher Scientific and were grown in low-salt LB medium with 100 μg/mL ampicillin and 25 μg/mL zeocin. pCMV or pMD helper plasmids were prepared with Qiagen Plasmid Maxi Kit and pGIPZ shRNA plasmids were extracted with Qiagen Plasmid Midi Kit following supplier's protocols. Phoenix cells (a gift from Jose Silva, Columbia University, New York) were cultivated in DMEM with 10% (vol/vol) FBS and transfected with jetPEI transfection reagent (Polyplus) following supplier's protocols. Briefly, 2×10^5 cells were seeded in a six-well plate 24 h before transfection and media were replaced by fresh DMEM with 10% (vol/vol) FBS 1 h before transfection. One microgram of pMD, 1 μg of pCMV, and 2 μg of pGIPZ shRNA or pGIPZ plasmid alone were diluted into 150 mM NaCl to a final volume of 100 μL, and 8 μL of jetPEI transfection reagent was diluted with 150 mM NaCl to a final volume of 100 μL. The plasmid solution was mixed with jetPEI transfection reagent solution briefly and further incubated at room temperature for 20 min. The mixture was added to Phoenix cells and incubated at 37 °C in a CO_2 incubator. After 20 h of transfection, the medium was replaced with 2 mL fresh DMEM + 10% (vol/vol) FBS. After 48 h of transfection, 2 mL DMEM supernatant containing viral particles was collected and filtered with Millex-HA filter. To construct stable VAMP4 knockdown cell lines in THP-1 cells, 1×10^6 THP-1 cells in 1.25 mL Advanced RPMI + 2 mM glutamine + 10% (vol/vol) FBS, were seeded in a six-well plate shortly before infection. A mixture of 0.75 mL viral suspension produced in Phoenix cells as described above and 8 μg Polybrene were added to the THP-1 cells. These THP-1 cells were centrifuged at 1,000 × *g* for 1 h at room temperature and further incubated at 37 °C in a CO_2 incubator. After 4 h of infection, THP-1 cells were spun down, washed once with Advanced RPMI + 2 mM glutamine + 10% FBS, resuspended into 3 mL Advanced RPMI + 2 mM glutamine + 10% (vol/vol) FBS, and returned to the CO_2 incubator at 37 °C. After 48–60 h of infection, infected THP-1 cells were sorted using a MoFlo-HTS cell sorter (Dako Cytomation) and GFP^+ THP-1 cells were collected. The GFP^+ cells were grown for several generations before further experiments.

Protein Purification. N-terminal 6× His-tagged, full-length versions of LegC2, LegC3, and LegC7 were cloned into pET15b (Novagen) vector for overexpression in the *E. coli* strain BL21 (DE3) and affinity purified using Ni^{2+}-nitrilotriacetic acid (NTA) agarose (Qiagen) resin followed by thrombin cleavage to remove the 6×-His tags. The proteins were further purified by ion-exchange chromatography using the ÄKTA system (GE Healthcare) as described earlier for purification of SNAREs (54). Purity of the proteins was analyzed by SDS/PAGE and Coomassie blue staining (Fig. S4*A*). N-terminal 6× His-tagged, full-length VAMP4 (1–220) and α-SNAP (1–298) were overexpressed and purified as described

earlier (37, 39). Recombinant NSF from Chinese hamsters was expressed and purified as described earlier (39). A cytoplasmic fragment of a cysteine mutant (S28C) of VAMP2 (Syb2, 1–96) was expressed using a pET28a vector and purified as described earlier (40) and then labeled with Oregon-Green 488 Iodoacetamide (Molecular Probes) as per manufacturer's instructions. Soluble fragments of R-SNAREs were expressed and purified as described earlier (55). As positive control for disassembly, a truncated, ternary neuronal SNARE complex was formed by mixing Syntaxin 1A (183–288), SNAP-25 (1–206), and Oregon-Green–labeled (at position S28C) cytoplasmic domain (1–96) of Syb (VAMP2*), in the molar ratio = 1:1:1.5 and purified as described earlier (41). Purified proteins were snap frozen with liquid nitrogen and stored at −80 °C until use.

Characterization of LegC/SNARE Hybrid Complexes. For the formation of SDS-resistant complexes, purified, full-length versions of LegC2, LegC3, and LegC7 were mixed in equal stoichiometric ratio in the presence or absence of VAMP4 and incubated overnight at 4 °C on a rotary mixer. Both samples were resolved by SDS/PAGE without prior heat denaturation, followed by Coomassie blue staining (Fig. 4*B*). See Fig. 4*A* for the individual components used in the experiment. The band of lower mobility (larger arrowhead), visible only in the presence of VAMP-4, was cut out and analyzed by nano LC/MS-MS as described earlier (56). In short, proteins were separated by 1D SDS/PAGE [4–12% (wt/vol) NuPAGE Bis·Tris gel; Invitrogen] followed by Coomassie staining. The desired band was sliced out as a gel piece, reduced with 10 mM DTT for 55 min at 56 °C, alkylated with 55 mM IAA (iodoacetaide) for 20 min at 26 °C, and digested with modified trypsin (Serva) overnight at 37 °C. Tryptic peptides were injected into a C18 precolumn [2.5 cm, 360 μm o.d. (outer diameter), 150 μm i.d. (inner diameter), Reprosil-Pur 120 Å, 5 μm, C18-AQ; Dr. Maisch] at a flow rate of 10 μL/min. Bound peptides were eluted and separated on a C18 capillary column (15 cm, 360 μm o.d., 75 μm i.d., Reprosil-Pur 120 Å, 3 μm, C18-AQ; Dr. Maisch) at a flow rate of 300 nL/min, with a gradient from 7.5% to 37.5% (vol/vol) ACN (acetonitrile) in 0.1% formic acid for 50 min using an Agilent 1100 nano-flow LC system (Agilent Technologies) coupled to an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Electron). The mass spectrometer was operated in the data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey MS spectra were acquired in the Orbitrap (m/z 350–1,600) with the resolution set to 30,000 at m/z 400 and automatic gain control target at 5 × 105. The eight most intense ions were sequentially isolated for CID MS/MS fragmentation and detection in the linear ion trap. Ions with single and unrecognized charge states were excluded. The peptide hits generated were searched against a local database comprising the proteins LegC2, LegC3, LegC7, and VAMP4 using Mascot (Matrix Science) and results were viewed using the Scaffold Viewer (Proteome Software).

Proteoliposome Preparation and Fusion Assay. For the fusion assays, SUVs (average diameter of 50 nm) were prepared by detergent removal as described earlier (57). In brief, purified, full-length LegC2, LegC3, and LegC7 in 1% CHAPS were mixed (molar ratio 1:1:1) overnight at 4 °C and then added to the lipid mixture (DOPC:DOPE:DOPS:cholesterol:Oregon-Green-DHPE (1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine) mol% ratio 55:22:11:11:1 in 5% cholate), with final protein:lipid molar ratio of 1:1,000. The mixture was passed through a Sephadex G-50 column using the HP150 (20 mM Hepes, 150 mM KCl, pH 7.4) buffer to form small unilamellar proteoliposomes by detergent removal. All lipids were bought from Avanti Polar Lipids. Using the same procedure, proteoliposomes containing purified, full-length VAMP4 or VAMP2 (with 1 mol% Texas-Red-DHPE label) were prepared (final protein:lipid molar ratio 1:1,000). Liposome concentrations were quantified as total lipid content based on phospholipid concentrations measured by Fiske's reagent (58, 59) to ensure using them in equal stoichiometric ratios for the fusion assay. Proteoliposome fusion was monitored by a lipid mixing assay based on FRET using a spectrofluorimeter (FluoroMax-2; Jobin Yvon) as described earlier (29). In short, 25 μL of Oregon-Green–labeled LegC SUVs in 1.2 mL HP150 buffer (20 mM Hepes pH 7.4, 150 mM KCl), were excited at 496 nm in a quartz cuvette; the emission was monitored at 515 nm. The emission was allowed to stabilize upon the initial drop. To start the fusion reaction, equal amounts of Texas-Red–labeled R-SNARE SUVs were added and fusion was monitored via FRET, λ_{ex} = 496 nm and λ_{em} = 605 nm. Acceptor fluorescence (F_t) was normalized to the initial value (F_0) (at the instant (t_0) of addition) as F_t/F_0 and plotted with respect to time (t). For competitive inhibition, the LegC SUVs were incubated with excess soluble SNARE domains of the R-SNAREs for 30 min, before being used for the assay. Raw data were analyzed and plotted using Origin Pro-9.0 (OriginLabs).

NSF-Mediated Disassembly of SNARE Complexes Monitored by Fluorescence Anisotropy. Because both VAMP4 and VAMP2 proteoliposomes were able to fuse with LegC proteoliposomes (Fig. 3*A*) due to in vitro "promiscuity" (typical for many SNAREs), we decided to use an Oregon-Green–labeled cytoplasmic fragment of Synaptobrevin (VAMP2*) as the R-SNARE partner with neuronal Q-SNAREs or LegCs to monitor NSF-mediated disassembly of such complexes using fluorescence anisotropy. To check whether the soluble VAMP2 formed complexes with the neuronal Q-SNAREs and LegC effectors to be reconstituted on the respective SUVs, the proteoliposomes were subjected to coflotation assay by density gradient centrifugation as described earlier (60) followed by detection of the fluorescently labeled VAMP2*. Full-length LegC2, LegC3, LegC7, and Oregon-Green–labeled (at residue 28) cytoplasmic domain of Synaptobrevin (VAMP2*) were mixed overnight at 4 °C to generate LegC–VAMP2* hybrid complex. The complex was reconstituted into SUVs by detergent removal (57) using a protein:lipid ratio of 1:1,000. As control, truncated, ternary neuronal SNARE complexes (with the same VAMP2* fragment) were also reconstituted into SUVs with a protein:lipid ratio of 1:1,000. For the anisotropy experiments, we used 10 μL of proteoliposomes in 0.6 mL "disassembly buffer" (20 mM Hepes-KOH pH = 7.4, 20 mM KCH_3COO, 120 mM KGlu) (30). Anisotropy of VAMP2* was measured using a FluoroLog 3 spectrometer in T configuration equipped for polarizers (model FL322; Jobin Yvon). All experiments were done at 37 °C. Disassembly was performed adding 60 nM NSF, 500 nM α-SNAP, 2 mM ATP, and 5 mM Mg^{+2} (39). The G factor was calculated according to $G = I_{HV}/I_{HH}$, where I is the fluorescence intensity, the first subscript letter indicates the direction of the exciting light, and the second subscript letter the direction of emitted light. The intensity of the vertically (V) and horizontally (H) polarized emission light after excitation by vertically polarized light was measured. The anisotropy (r) was determined according to $r = (I_{VV} - G \times I_{VH})/(I_{VV} + 2G \times I_{VH})$.

Flotation Assay to Separate Soluble VAMP2* Released from NSF-Mediated Disassembly of Complexes. The same proteoliposomes as above were incubated with α-SNAP (500 nM), NSF (60 nM), Mg^{+2} (5 mM), and ATP (2 mM) for 10 min at room temperature in disassembly buffer (20 mM Hepes-KOH pH = 7.4, 20 mM KCH_3COO, 120 mM KGluconate). To distinguish proteins attached to liposomes from those solubilized upon disassembly, samples were subjected to flotation assay based on density gradient centrifugation using a Nycodenz gradient as described earlier (60). Fractions from the gradient were resolved by SDS/PAGE and distribution of the fluorescent VAMP2* among the fractions (in the gel) was detected (Fig. 5 *B–D*) using the FLA-7000 Imager (Fujifilm).

Immunoprecipitation of VAMP4 Confirms Interaction with LegC2, LegC3, and LegC7 Using Anti-VAMP4 Antibody. Similar to the experiment shown in Fig. 1*C*, we used lysates of THP-1 cells that were infected with *Legionella* strains overexpressing either TEM or TEM-tagged versions of LegC2, LegC3, or LegC7, respectively. Precleared supernatants of the corresponding cell lysates were then incubated with anti-VAMP4 antibody immobilized on Protein A/G agarose, followed by washing and elution of the bound antigen complex as described in *Materials and Methods*. Starting supernatants and eluted antigen complexes were analyzed by immunoblotting using anti-VAMP4 and anti-TEM antibodies, respectively (Fig. S1). For details see *Materials and Methods* above and the legend to Fig. 1*C*.

Measurement of Cell Viability of Infected Cells After Knockdown of VAMP4. To test whether VAMP4 knockdown has a negative impact on cell viability after infection with *Legionella*, we infected control cells and three VAMP4-knockdown cell lines (*Materials and Methods*) with wild-type *Legionella* and measured cell viability using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) as reporter (61). In brief, 1×10^5 cells were plated in 96-well plates, followed by infection with 10-fold serial dilutions from 1×10^7 bacteria per well to 10 bacteria per well of *L. pneumophila* as described in *Materials and Methods*. After 120 h of infection, 20 μL of 5 mg/mL MTT was added to each well and the plate was further incubated for 4 h at 37 °C in a CO_2 incubator. Afterward, the supernatant was removed by aspiration and 100 μL of isopropanol containing 40 mM HCl was added to each well to solubilize the Formazan dye. A total of 20 μL of 10% (wt/vol) SDS was added and the samples were mixed by incubating 10 min at room temperature on a shaker. A_{570} was measured using a plate reader (TECAN Infinite 200 PRO). The results (Fig. S2) showed no significant differences between the mock transfected and the three VAMP4 knockdown cell lines (KD_A, KD_B, and KD_C). Hence cytotoxicity can be excluded as a cause for the retarded intracellular growth of *Legionella* in the THP-1 macrophage cell lines with VAMP4 knockdown.

Assay of *Legionella* Uptake. *Legionella* wild-type or *dotA* mutant strains overexpressing mCherry (27) were used to infect THP-1 cell lines transfected with VAMP4 knockdown or mock constructs. As a negative control, cells were treated with Cytochalasin D (an inhibitor for actin elongation, which inhibits the entry of *Legionella*) 30 min before infection. As a positive control, cells were incubated with anti-MOMP (major outer membrane protein) antibodies 30 min before infection. After 40 min of infection, cells were fixed and further stained with rabbit anti-MOMP antibodies and anti-rabbit Alexa Fluor 647 for staining external bacteria. The percent of entry (number of intracellular bacteria/total bacteria) was calculated and plotted (Fig. S3). There was no significant difference in the levels of uptake of *Legionella* into the cell lines, between the VAMP4 knockdown and mock constructs.

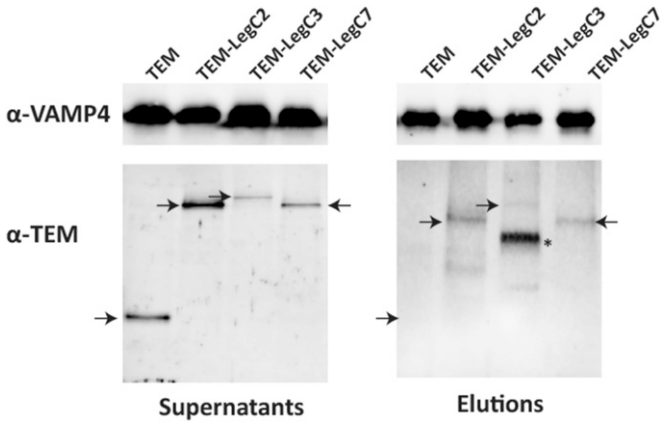

Fig. S1. *LegC* effectors interact with VAMP4. Immunoprecipitation of VAMP4 reveals coprecipitation of TEM-tagged LegC2, LegC3 (rather weak only), and LegC7. Both the starting supernatants and the eluted antigen complexes were analyzed for VAMP4 and TEM by immunoblotting, the position of the LegC proteins is marked with arrows. The band marked with an asterisk was not identified; it may represent a breakdown product.

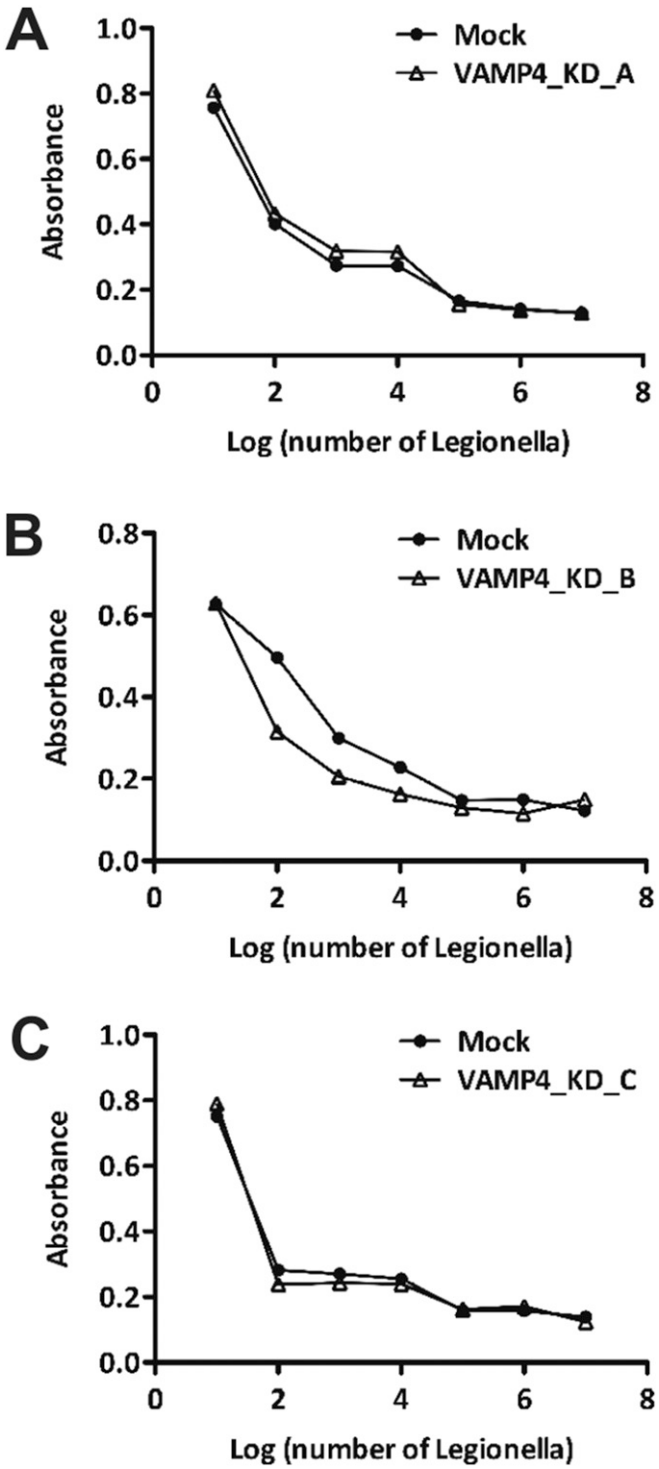

Fig. S2. Knockdown of VAMP4 does not cause cytotoxicity. (*A*–*C*) Cell viability, assayed by the MTT reaction, upon infection with increasing concentrations of *Legionella* shows no significant differences between the mock-transfected and the VAMP4 knockdown cell lines.

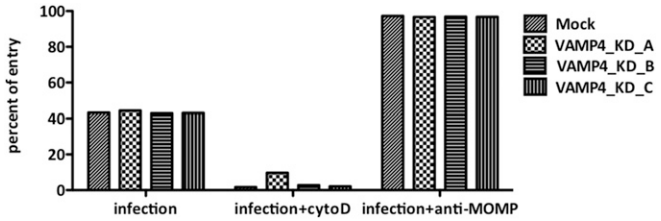

Fig. S3. VAMP4 knockdown does not affect *Legionella* uptake. Percent of entry of the *Legionella* showing no significant difference between VAMP4 knockdown and mock constructs. Percentage of entry for negative control with Cytochalasin D and positive control with anti-MOMP antibody as comparison.

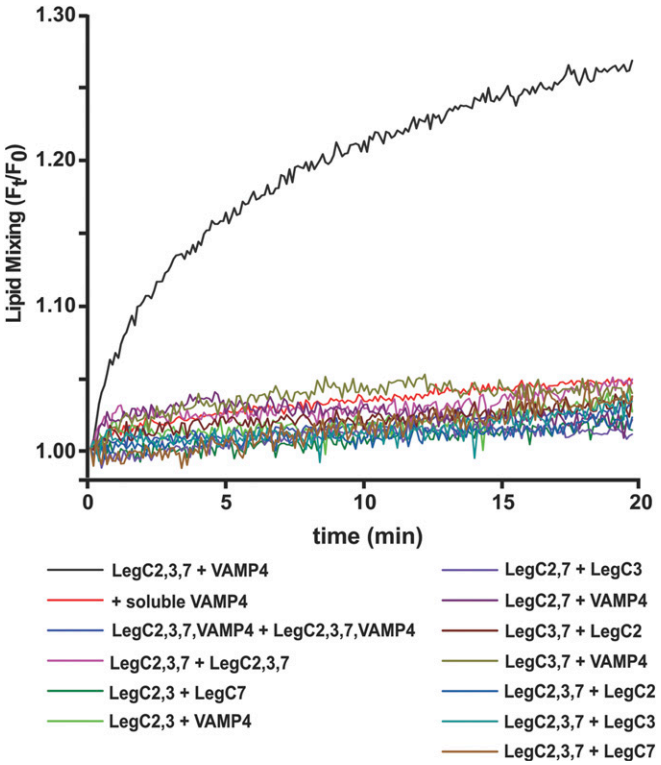

Fig. S4. LegC-reconstituted liposomes fuse with VAMP4 liposomes in only one topology. Twelve different pairs of liposomes were prepared containing LegC2, LegC3, LegC7, and VAMP4, in various combinations as indicated. Each pair was then tested for fusion as in Fig. 3. LegC effectors were found to drive in vitro membrane fusion together with VAMP4 only in the topology LegC2:LegC3:LegC7 + VAMP4.