

Supporting online material for
Conserved Eukaryotic Fusogens can Fuse Viral Envelopes to Cells

Ori Avinoam¹, Karen Fridman¹, Clari Valansi¹, Inbal Abutbul², Tzviya Zeev-Ben-Mordehai³,
Ulrike E. Maurer⁴, Amir Sapir^{1,*}, Dganit Danino², Kay Grunewald^{3,4}, Judith M. White⁵,
Benjamin Podbilewicz^{1,†}

¹Department of Biology, Technion - Israel Institute of Technology, Haifa 32000, Israel

²Department of Biotechnology and Food Engineering and The Russell Berrie Nanotechnology Institute, Technion - Israel Institute of Technology, Haifa 32000, Israel

³Oxford Particle Imaging Centre, Division of Structural Biology, Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, OX3 7BN, UK

⁴Department of Molecular Structural Biology, Max-Planck Institute of Biochemistry, D-82152 Martinsried, Germany

⁵Department of Cell Biology, University of Virginia, Charlottesville, VA 22908, USA

* Present address: Howard Hughes Medical Institute and Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA

†To whom correspondence should be addressed: E-mail: podbilew@technion.ac.il (BP)

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Materials and Methods

DNA constructs

For transient expression of FF proteins we inserted AFF-1::FLAG, EFF-1::V5, Tsp-FF-1::FLAG, and Bfl-FF-1::FLAG into the pCAGGS mammalian expression vector (1) (Tables S1 and S3). Unless otherwise indicated we used 5' KpnI and 3' NheI to clone into pCAGGS. To generate pOA20 (Table S3) the DNA encoded by pIZT-AFF-1 (2) was PCR amplified using primers OR55, OR56 (Table S4). To generate pOA19 the DNA encoded by pIZT-EFF-1A (3) was PCR amplified using primers OR54, OR55. To generate pOA35, DNA from a cDNA library (a kind gift from Isao Nagano) was PCR amplified using nested primers OR100-OR103. The PCR product was ligated into pGEMT-easy as recommended by Promega and then used as template for PCR amplification with primers OR111, OR112. To generate pOA60 the cDNA sequence corresponding to gi|210090015| with Flanking 5' KpnI 3' NheI was optimized for expression and synthesized (GeneScript). To label cytoplasm we used pRFPnes (4), kindly provided by Claudio Giraudo and James Rothman. To label the nucleus we used pCFPnls encoding CFP with two tandem repeats of the nuclear localization signal (nls) from simian virus large T-antigen. To generate pCFPnls primers OR147-148 were used with pCH44 (4) (kindly provided by Claudio Giraudo and James Rothman.) as template. The PCR product was cloned into the BamHI, EcoRI sites of pCDNA3.1 (+) (Invitrogen). To generate pOA6 *P. pacificus* genomic DNA (PS312) (5) was used as template with primers OR-19 and OR-22. The PCR product was ligated into pPD49.78 (6). To generate pRSETA-AFF1EC primers AM66 and AM 67 were used with AFF-1 cDNA as template (2). The PCR product was cloned into the BglII, KpnI sites of pRSET-A (Invitrogen). All sequences were verified by sequencing.

Nematode strains

Nematode strains were maintained according to standard protocols (5, 6). In addition to the wild-type strain N2, the following strains were used: LGII: BP347 *eff-1(ok1021)* (3). LGIV: SU93 *jcIs1[ajm-1::gfp, pRF4]* (7), BP421 *eff-1(ok1021)II; hyEx161[ajm-1::gfp, pOA6 (Cehsp::Ppa-ff-1)]*. To drive *Ppa-ff-1* ectopic expression in *C. elegans*, 10 ng/μl of pOA6 were co-injected with 10 ng/μl of the apical junction marker AJM-1::GFP (hyEx161).

Bioinformatics

Identification and characterization of new members of the FF family

FF proteins in nematodes were identified as described (8). For the Chordate, Ctenophore and Arthropod sequences the BLAST search provided by the National Center for Biotechnology Information (NCBI) was used. For annotation we used the Augustus gene prediction software (9) with the training set for *C. elegans*. In some cases we manually corrected the gene model based on the multiple sequence alignment (Fig. S1B). Accession numbers and databases are summarized in Table S1.

Phylogeny of FF proteins (Fig. 1A)

Phylogenetic analyses were conducted in MEGA4 (10). The evolutionary history was inferred using the Maximum Parsimony (MP) method (11). The MP tree was obtained using the Close-Neighbor-Interchange algorithm (12) with search level 3 (12, 13) in which the initial trees were obtained with the random addition of sequences (10 replicates). All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). The signal sequence was removed from the final dataset. Evolutionary relationships of 14 (right) and 25 (left) taxa is shown (Fig. 1A). For 14 taxa the full length sequence of the extracellular domain was used. Tree #1 out of 3 most parsimonious trees (length = 1165) is

shown. The consistency index is (0.926554), the retention index is (0.930667), and the composite index is 0.868356 (0.862313) for all sites and parsimony-informative sites (in parentheses). There were a total of 438 positions in the final dataset, out of which 344 were parsimony informative. For 25 taxa the amino acid sequences corresponding to the TGF β -RI like domains (2) AFF-1(84-192) were used; the sequence of the *N. gruberi* served as an outgroup. Tree #1 out of 9 most parsimonious trees (length = 469) is shown. The consistency index is (0.727876), the retention index is (0.717241), and the composite index is 0.529138 (0.522063) for all sites and parsimony-informative sites (in parentheses). There were a total of 75 positions in the final dataset, out of which 60 were parsimony informative.

Secondary structure prediction (Fig. S1C)

Predictions were performed using the JNET method (14, 15) available from the web services of the Jalview 2.5 software (16, 17).

Cells and reagents

All Baby Hamster Kidney cells (BHK) were BHK-21(ATCC) (18). BHK cells and their growth conditions were according to standard protocols. Dulbecco's modified Eagle's medium (DMEM), Penn/Strep, L-glutamine, and sodium pyruvate (Gibco), Fetal Bovine Serum (Biological Industries, Kibbutz Beit Haemek, Israel). Experiments with Sf9 cells and their growth conditions were as described (2, 3).

Cell-cell fusion assay

BHK cells at ~70% confluence were transfected using Fugene6 (Roche) with 2 μ g of pCAGGS DNA (FF or empty vector) and 0.5 μ g of pRFPnes DNA in 35 mm tissue culture dishes (Corning) containing a glass cover slip on the bottom (Knittel). At 14-24 hours post-transfection the cells were fixed with 4% paraformaldehyde in PBS and processed for

immunofluorescence. To assay multinucleated cells, we stained cell nuclei with Hoechst (1 $\mu\text{g/ml}$, H3570, Molecular Probes) or 1 $\mu\text{g/ml}$ DAPI for 10 min at room temperature (3). We counted the number of nuclei in expressing cells as marked by pRFPnes or antibody staining (see below), using either a Zeiss Axiovert 200M inverted or a Nikon Eclipse E800 upright fluorescence microscope. The fusion indexes (shown as percentage of fusion) were defined as the ratio between the number of nuclei in multinucleated cells and the total number of nuclei in fused cells and expressing cells that were in contact but did not fuse. The fusion indexes are presented as means \pm standard errors of at least eight independent experiments. Each experiment consisted of at least two replicates of the same transfection (2, 3). Transfection efficiency was evaluated as 40-60% based on pRFPnes and antibody staining.

Color mixing assay

Cytoplasmic content mixing assays were performed as described (4) with some modifications. The cytoplasm of cells that express AFF-1 was marked with a red fluorescent protein by expressing RFPnes. The nuclei of cells that express EFF-1 were marked with a CFPnls. Fused hybrid cells could be distinguished by their red cytoplasm surrounding multiple blue nuclei. The percentages of fused hybrid cells (red and cyan; purple) and multinucleated cells (red or cyan alone) were calculated by dividing the mean number of red, cyan and purple cells by the mean number of cells from four independent experiments. Experiments were repeated at least five times yielding similar results independent of whether the co-transfection fluorescent marker was RFPnes or CFPnls.

Pseudovirus preparation

Recombinant viruses were recovered as described (19) with some modifications (Fig. S3). BHK cells were grown to 70% confluence on 10 cm plates and then transfected with plasmids

encoding pCAGGS empty vector, pOA19 or pOA20 (Table S3). Following 24 hour incubation at 37°C in 5% CO₂, cells were infected with VSVG-complemented VSVΔG recombinant virus (VSVΔG-G) at a multiplicity of infection (MOI) of 2-5 for 1 hour at 37°C in a 5% CO₂ incubator in serum free culture medium (DMEM). Virus infected cells were washed at least 3 times with serum-free DMEM or PBS to remove unabsorbed VSVΔG-G virus. Following a 24 hour incubation period at 37°C the supernatant and cells containing the VSVΔG, VSVΔG-EFF-1, or VSVΔG-AFF-1 pseudoviruses were harvested and centrifuged at 600 g for 10 min at 4°C to clear cell debris. Virions were removed from the supernatant by pelleting at 100,000 g through a 20% sucrose cushion, and resuspended in 10% sucrose in Hepes/NaCl buffer (25 mM Hepes, 130 mM NaCl pH 7.4). For TEM, virions were additionally concentrated by centrifugation at 100,000 g.

Titering VSV pseudotype viruses on BHK cells

To determine the titer of each pseudovirus preparation, 3×10^4 BHK cells were plated into each well of a 96 well tissue culture plate (NUNC). To determine the titer of VSVΔG-AFF-1 or VSVΔG-EFF-1, BHK cells were initially transfected with 1 μg/ml *aff-1* or *eff-1*. Cells transfected with empty vector served as control. Six serial dilutions of the virus were performed and added to cells. After 18-24 hours of incubation, GFP expressing cells were counted in at least two dilutions using a Zeiss Axiovert 200M fluorescence microscope. Each experiment was repeated at least three times with duplicates. Inoculation was performed in the presence of anti-VSVG antibody mAb I1 (20) diluted 1:100 to inhibit infection due to residual presence of VSVG. Results were also confirmed by FACS analysis. For FACS analysis BHK cells were grown to 70% confluence and transfected with 1 μg/ml of plasmid encoding *aff-1* or *eff-1*. Following 24 hour incubation, cells were infected with VSVΔG-AFF-1 and incubated

for 24 hours. To measure the titer, cells were collected using EDTA and fixed in 4% paraformaldehyde. Samples were maintained on ice and examined for GFP expression using a BD FACS Calibur (N=20,000 cells, Fig. S5).

Biochemical analysis

To detect proteins by immunoblot, samples were treated with SDS-PAGE sample buffer containing 10% of β -mercaptoethanol, boiled for 5 minutes and examined on an 8%, 10% or 12% SDS polyacrylamide gel. For AFF-1 expressing cells (BHK-AFF-1) and viruses (VSV Δ G-AFF-1) bands were visualized using mouse anti-FLAG (M2, Sigma F3165) monoclonal antibody and mouse anti-M polyclonal antibody (Fig. S4). For EFF-1 expressing cells (BHK-EFF-1) and viruses (VSV Δ G-EFF-1) bands were visualized using mouse anti-V5 (Cat # 46-0705 Invitrogen) (data not shown). In controls rabbit anti-VSVG (Cat # V4888 Sigma-Aldrich) was used. Goat anti-mouse and anti-rabbit antibodies conjugated with HRP were used as secondary antibodies (Cat # 115-035-003, 111-035-003 Jackson). Bands were detected by chemoluminescence (ECL Plus Western Blotting Detection System, Amersham) using a FUJI LAS 3000 with the Image Gauge V3.12 software package. Data shown are representative of at least three independent experiments. InstantBlue (Expedeon) was used for Coomassie staining. Silver staining was performed using the ProteoSilver kit (Sigma) according to the manufacturer's instructions.

Mass spectrometry analysis

For proteolysis, each of the three viral like particles samples (VSV Δ G-AFF-1, VSV Δ G-G and VSV Δ G-bald) were incubated in 8 M Urea and 100 mM ammonium bicarbonate pH 8 and reduced with 2.8 mM DTT (60°C for 30 min). The samples were then alkylated with 8.8 mM iodoacetamide in 100 mM ammonium bicarbonate (in the dark at room temperature for 30

min) and digested in 2 M Urea, 25 mM ammonium bicarbonate with modified trypsin (Promega) at a 1:50 enzyme-to-substrate ratio, overnight at 37°C. The tryptic peptides were desalted using C18 stageTip tips, dried and re-suspended in 0.1 % formic acid. The peptides were resolved by reverse-phase chromatography on 0.075 X 200-mm fused silica capillaries (J&W) packed with Reprosil reversed phase material (Dr. Maisch GmbH, Germany). The peptides were eluted with linear 95 minutes gradients of 7 to 40% and 8 minutes at 95% acetonitrile with 0.1% formic acid in water at flow rates of 0.25 µl/min. Mass spectrometry was performed by an ion-trap mass spectrometer (Orbitrap, Thermo) in a positive mode using repetitively full MS scan followed by collision induced dissociation (CID) of the 7 most dominant ion selected peptides from the first MS scan.

The mass spectrometry data was analyzed using the Sequest 3.31 software (J. Eng and J.Yates, University of Washington and Finnigan, San Jose) searching against the UniProt database. Since a hamster database is not available cellular proteins listed in table S5 are representative homologs (21). The quantitation (peak area) was done using the PepQuant algorithm of the BioWorks software.

Production of mouse anti-AFF-1 polyclonal antibodies

The extracellular domain of AFF-1 (AFF-1EC) was sub-cloned into pRSET-A that introduced 6xHis at the N terminus (Table S3). The AFF-1EC::6xHis fusion protein was over-expressed in *E. coli* by adding 0.5mM IPTG and incubating the culture overnight at 16°C. Affinity purification with NiNTA beads (Qiagen Cat# 30210) was performed according to the QIAexpressionist manual (06/2003, QIAGEN). The protein was eluted by adding four 0.5 ml aliquots of elution buffer A (8M urea, 100mM NaH₂PO₄, 10mM Tris-HCl, pH 5.9) followed by another four aliquots of elution buffer B (8M urea, 100mM NaH₂PO₄, 10mM Tris-HCl, pH

4.5). Mouse polyclonal antibodies were prepared using AFF-1EC as antigen (Adar Biotech Inc., Israel). Sera obtained from mice, immunized with AFF-1EC, were tested by immunofluorescence using Sf9 cells expressing AFF-1::Flag. Sera #8 diluted 1:500 showed membrane and intracellular vesicular staining (Fig. S7A). Pre-immune sera (Fig. S7B) or secondary antibodies alone (Alexa Fluor 568 goat anti-mouse IgG (H+L) 1:500) gave no staining.

Immunofluorescence

BHK-21 cells were grown on tissue culture plates with glass cover slips on the bottom (Knittel). Cells were fixed with 4% paraformaldehyde in PBS, incubated in 40 mM NH₄Cl to block free aldehydes, washed in PBS, permeabilized in 0.1% tritonX-100 in PBS and blocked in 1% FBS in PBS. The cover slips were incubated 1 hour at 23°C with either anti-V5 1:500 (Invitrogen) or anti-FLAG 1:2000 (Sigma) mouse monoclonal antibodies. The secondary antibodies were goat anti-mouse and goat anti-rabbit coupled to Alexa488, 633 or 643 (Molecular Probes/Invitrogen). Transfected cells expressed cytoplasmic RFP from the pRFPnes vector and nuclei were visualized with DAPI or Hoechst staining as described above.

Transmission Electron Microscopy (TEM)

Negative staining-TEM

A 400-mesh carbon-coated grid was placed on a 20 µl sample drop for 2 min and blotted with a filter paper. The sample was chemically stained by placing the grid on a 20 µl drop of 2% uranyl acetate for 2 min followed by blotting with a filter paper and air-drying. Specimens were examined in a Tecnai T12 G² TEM (FEI) or in a Philips CM120 transmission electron microscopes operated at 120 kV. Images were recorded digitally on a Gatan UltraScan 1000

2k x 2k camera or the Gatan 791 wide-angle camera using the DigitalMicrograph software (Gatan, U.K.)

Cryo electron microscopy

A 3- μ l drop of the sample was placed on a glow-discharged holey carbon coated copper electron microscopy grid (C-flat, Protochips). The drop was blotted, and the sample was vitrified by plunging into liquid ethane (-183°C). The specimen was then transferred to liquid nitrogen (-196°C) for storage. Vitrified specimens were examined on a Tecnai F30 Polara TEM (FEI) operated at 300 kV and equipped with a GIF2002 postcolumn energy filter (Gatan) operated in zero loss mode. 2K \times 2K images were acquired at a calibrated magnification of 27,500x, resulting in a pixel size of 0.5 nm at the specimen level. Projection images were recorded at defocus settings between -4 μm to -6 μm using SerialEM (22). In some experiments specimens were also examined in a Tecnai T12 G² TEM (FEI) operated at 120 kV (data not shown) and images were recorded digitally on a Gatan UltraScan 1000 2K x 2K camera using the DigitalMicrograph software (Gatan; (23, 24).

Cryo electron tomography

A 3- μ l aliquot of the pseudotyped virus preparations was pipetted onto a glow-discharged holey carbon coated copper electron microscopy grid (C-flat, Protochips). Colloidal 10 nm diameter gold particles coupled to bovine serum albumin (BSA) were added, excess liquid was absorbed using a filter paper and the grids were vitrified by plunge-freezing in liquid ethane. Vitrified grids were stored in liquid nitrogen until examined on a Tecnai Polara TEM (FEI) operated at 300 kV and equipped with a GIF2002 or Tridem postcolumn energy filter (Gatan) operated in zero loss mode and 2K \times 2K images were acquired at a calibrated pixel size of 0.5 nm or 0.47 nm at the specimen level, respectively. Tilt series were collected at a defocus of either -6 μm for the viral tomogram or -8 μm for the vesicle tomogram in two-

degree increments covering an angular range from -60° to 60° using SerialEM (22). The total electron dose was kept below 100 electrons/ \AA^2 . Tilt series were aligned using gold beads as fiducials. Three dimensional reconstructions were calculated from the tilt series in IMOD (25) using weighted back projection. Movies and slices for figures were prepared using Amira 5.2 (Visage Imaging).

Measurements

The width and length of the particles on the surface of viruses and vesicles were measured from images of negatively stained samples using ImageJ Software 1.410. The G glycoprotein was measured as control and the obtained size was compared to published dimensions (26). Unpaired *t* tests were performed ($P < 0.0001$).

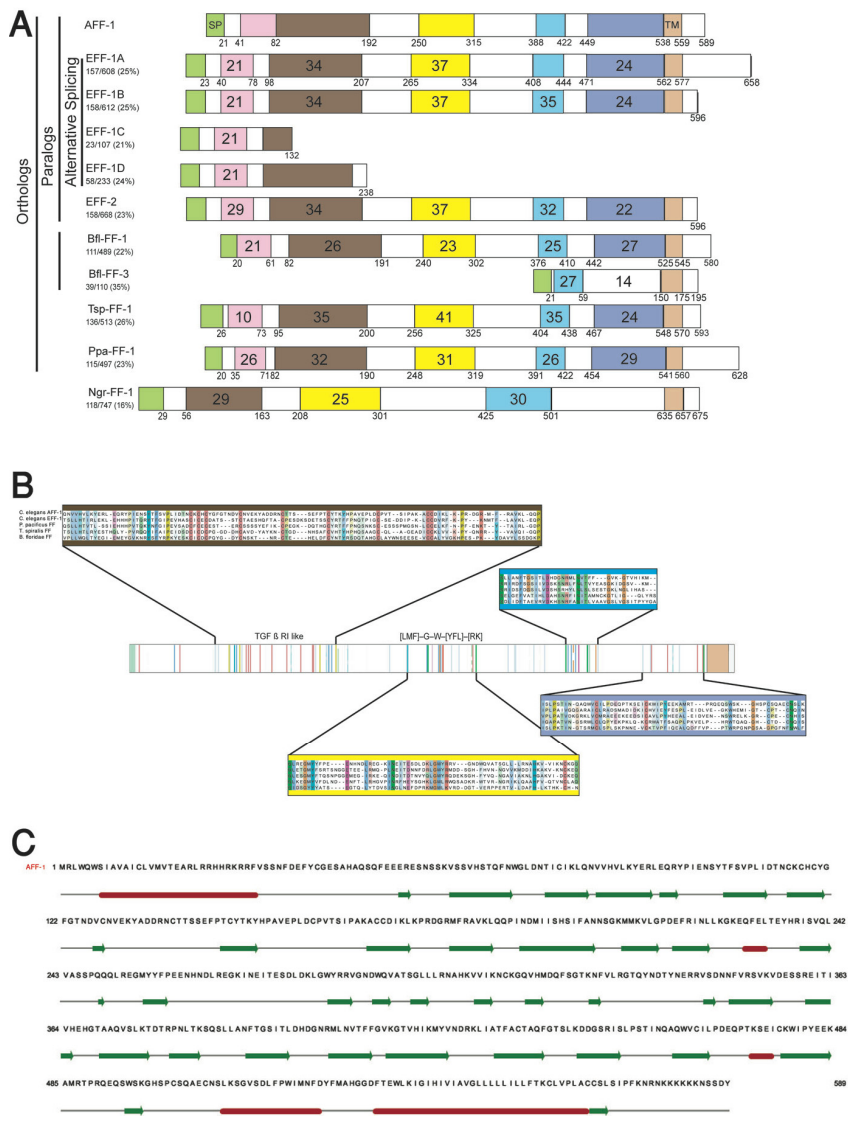
Immunogold labeling

Virus samples were pipetted onto carbon-coated grids and incubated for 5 min and then blocked with 1% BSA in PBS for 30 min at room temperature. The grids were then placed on a 50 μl drop of anti-AFF-1 (#8 serum; see Immunofluorescence section above) diluted 1:100 in PBS containing 1% BSA and incubated overnight at 4 $^\circ\text{C}$ in a sealed humidified chamber. Excess antibody was removed by placing grids sequentially onto three 50 μl drops of 0.1% BSA in PBS for 2 min each time. The grids were then placed on a 20 μl drop of goat anti-mouse IgG conjugated with 12-nm gold particles (Jackson lab, 1:20) for 1 hour at room temperature. Unbound gold conjugates were removed by three sequential 2-min washes with PBS. Samples were fixed by placing the grids on a 50 μl drop of 0.1% glutaraldehyde in PBS for 5 min. The grids were washed twice in PBS for 2 min and were then negatively stained by incubating the grids for 2 min on a 20 μl drop of 2% phosphotungstic acid in water (pH 7). Excess stain was removed and the grids were air-dried. Images were recorded digitally as described above.

Supplemental figures

Figure S1. Sequence analysis of FF proteins

(A) Distribution of conserved sequence motifs in FF paralogs and orthologs. Sequence motifs are color coded: Green – Signal peptide (SP), Pink – Pro-domain, Brown – TGF β -RI-like domain, Yellow- [LIMF]-G-W-[LYMF]-[RK] motif, Cyan – Putative protein-protein interaction domain, Purple – Membrane proximal stem, Ocher – Transmembrane domain (TM). *C. elegans* paralogs are listed by gene name AFF-1, EFF-1, EFF-2 (C26D10.7). EFF-1 Alternative splicing variants (EFF-1 A-D) are shown. Overall sequence identity to AFF-1 is indicated under the gene name. Local sequence identity to AFF-1 (%) is indicated within each domain. Sequence limits are indicated under the illustration unless it is identical to previously shown schematic.

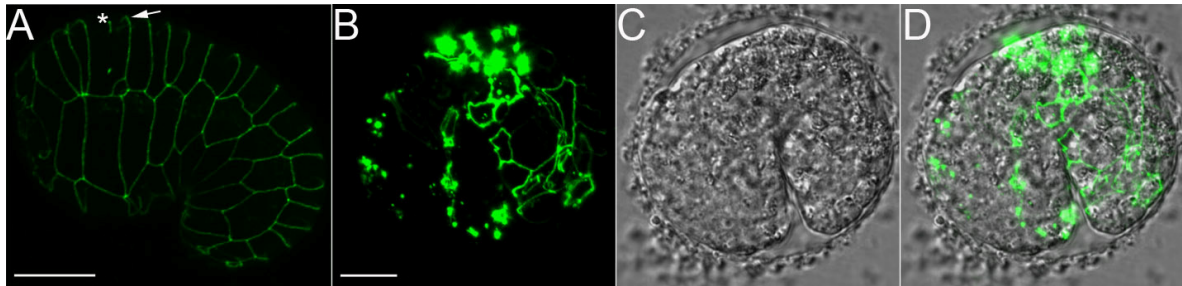


Sequences retrieved from *B. floridae* v2.0 assembly are listed as Bf- FF-1 and FF-3 which correspond to protein model id's 104514 and 104513 respectively (Table S1). *T. spiralis* (Tsp-FF-1) and *P. pacificus* (Ppa-FF-1) correspond to gi|162730680 and Contig235.2 of the PpaFreeze1.bases database. Sequences retrieved for *N. gruberi* (Ngr-FF-1) correspond to gi|284087402 (Table S1). Annotation was performed as previously described (2).

(B) Schematic representation of the multiple sequence alignment of FF proteins. Sequence alignment of conserved sequence motifs is shown. Alignment color code was according to the Clustal X color scheme with 40% conservation color increment in Jalview software (17). The background color of each domain is as in (A).

(C) Secondary structure prediction of AFF-1 based on the multiple sequence alignment of FF proteins. The consensus prediction is shown – alpha helices are marked as red tubes, and beta sheets as green arrows.

Figure S2. FF-1 from *P. pacificus* can fuse *C. elegans* cells



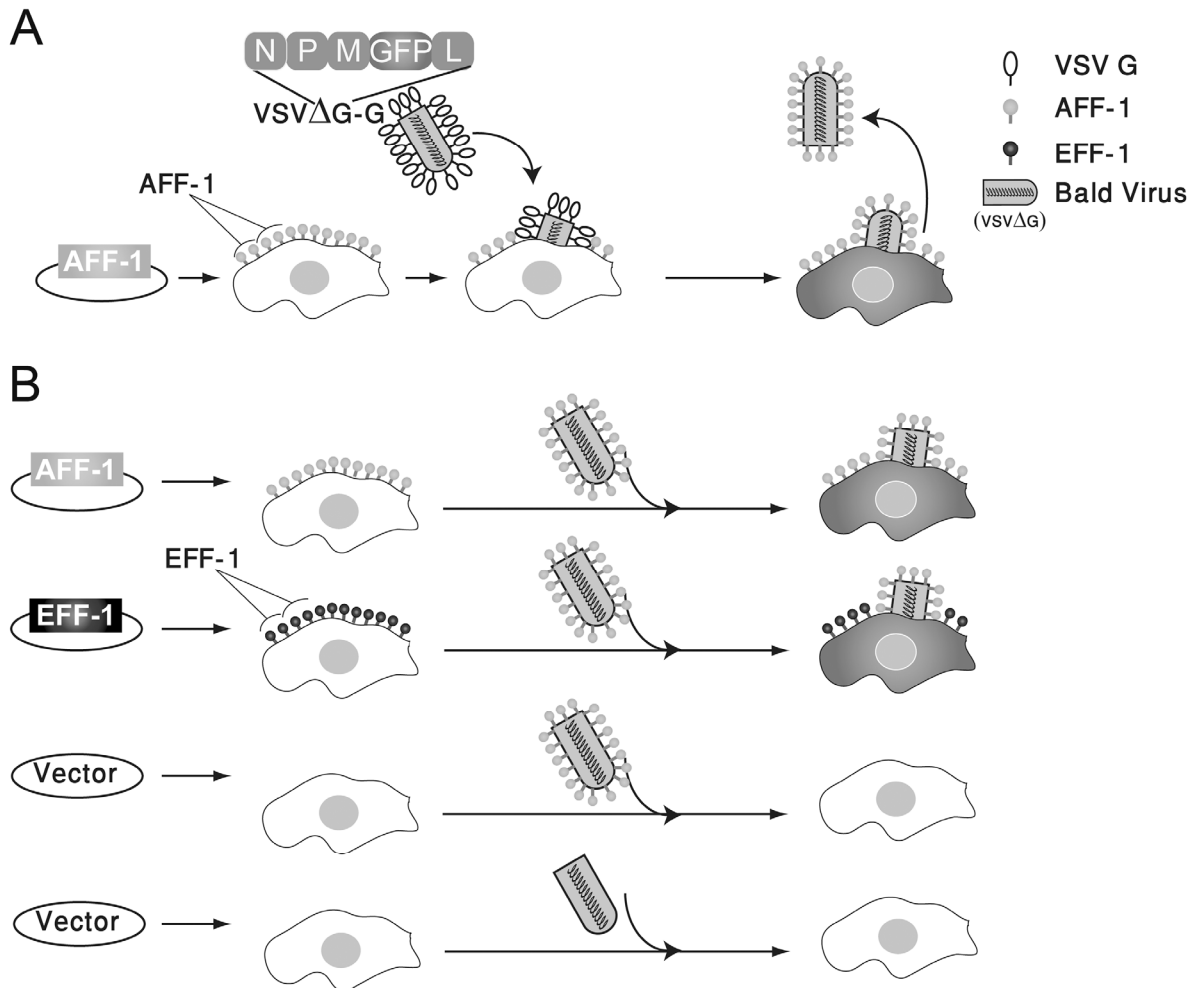
The gene *Ppa-ff-1* (Table S1) was PCR amplified from *P. pacificus* genomic DNA and cloned downstream to a heat shock promoter (*hsp16.2*) from *C. elegans*. Transgenic worms were generated using microinjection of the wild type strain (*N2*) and crossed to *eff-1(ok1021)* (27). Ectopic cell fusion was visualized by following the disappearance of an apical junction marker from fusing membranes (AJM-1::GFP) using confocal Z series reconstruction (2, 27) (n=9 embryos). (A and B) show fluorescence only, (C) is brightfield and (D) shows the merger between B and C.

(A-D) 1.5-fold stage embryos, anterior to the left and ventral down. Scale bars are 10 μ m.

(A) Wild-type embryo, two dorsal hypodermal cells undergo normal fusion (asterisk). Unfused junction (arrow).

(B-D) *eff-1* mutant embryo expressing *hsp::Ppa-ff-1* after heat shock. The disappearance of apical junctions between individual cells suggests that Ppa-FF-1 mediates fusion of the hypodermal cells in an *eff-1* independent manner.

Figure S3. Generation and infection of recombinant single round VSV Δ G-AFF-1

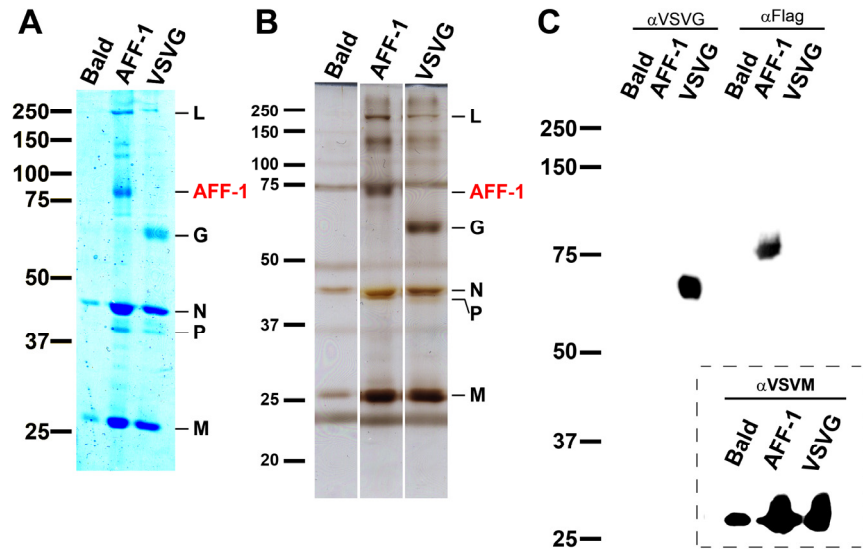


(A) BHK cells were transfected with a plasmid encoding *aff-1* and expressed AFF-1 protein on the surface. Cells were then infected with VSV Δ G-G. The viral genome encodes GFP in place of the VSVG. Infection results in viral induced expression of GFP by target cells (gray cytoplasm). VSV Δ G-AFF-1 pseudoviruses were harvested from the supernatant. Incorporation of AFF-1 was confirmed in purified virions by Western blot (Fig. S4)

(B) Cells were transfected with *aff-1*, *eff-1* or vector plasmid and infected with VSV Δ G-AFF-1. Cells transfected with empty vector and infected with un-complemented VSV Δ G (bald), served as negative controls.

Figure S4. Biochemical analysis of purified VSVΔG-AFF-1 pseudoviruses shows AFF-1 is efficiently incorporated into pseudotypes.

VSVΔG - Bald, AFF-1 and VSVG virions were collected from the supernatant by pelleting through a 20% sucrose cushion. Virions were purified by ultracentrifugation on a 20% to 60% continuous sucrose gradient, re-pelleted and examined by coomassie dye, silver staining and western blot.



(A) Coomassie staining of VSVΔG- Bald, AFF-1, VSVG showing an AFF-1 band at an apparent molecular weight (MW) of 75 kDa and VSVG at an apparent MW of 69 kDa. Bald virions served as negative control.

(B) Silver staining of VSVΔG- Bald, AFF-1, VSVG showing an AFF-1 band at an apparent MW of 75 kDa and VSVG at an apparent MW of 69 kDa. Bald virions served as negative control.

(C) Western blot analysis VSVΔG- Bald, AFF-1 and VSVG virions. A sample of each pseudotyped virus was split in three and each third was loaded in a separate lane of a 10% SDS – polyacrylamide gel followed by immunoblot. Rabbit anti-VSVG antibody recognized VSVG at an apparent MW of 69 kDa. Mouse anti-FLAG antibody recognized AFF-1-FLAG at an apparent MW of 75 kDa. The apparent MW of AFF-1 in Sf9 insect cells was also 75 kDa (2). The theoretical MW of AFF-1 is 67 kDa (28). The presence of viral proteins was confirmed by mouse anti-VSVM that identified a band at an apparent MW of 26 kDa in all three samples. Viral proteins L, G, N, P, M and AFF-1 are indicated.

Figure S5. VSV Δ G-G infection of cells expressing CeFFs and in the presence of anti-VSVG

BHK cells expressing AFF-1, EFF-1 or transfected with empty vector were infected with VSV Δ G-G pseudo typed virus (1.5×10^7 IU). Infection was performed in the presence (+) or absence (-) of anti-G antibodies (α G) (1:100). Cells were examined by FACS after 24 hours (total number of cells counted 20,000 cells). Transfection/Expression of FF proteins in the target cells did not significantly affect infection efficiency by VSV Δ G-G pseudo typed virus (The two-tailed P value equals 0.49-0.89). α G efficiently blocked infection of VSV Δ G-G. Results are presented as mean of three independent experiments \pm standard error.

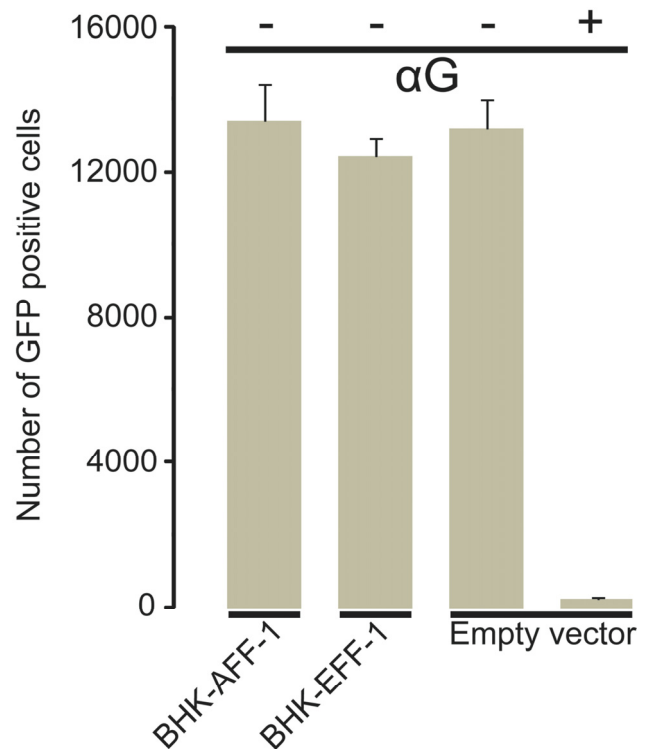
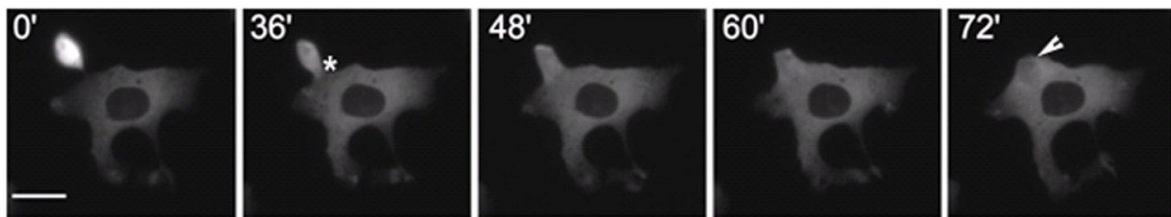
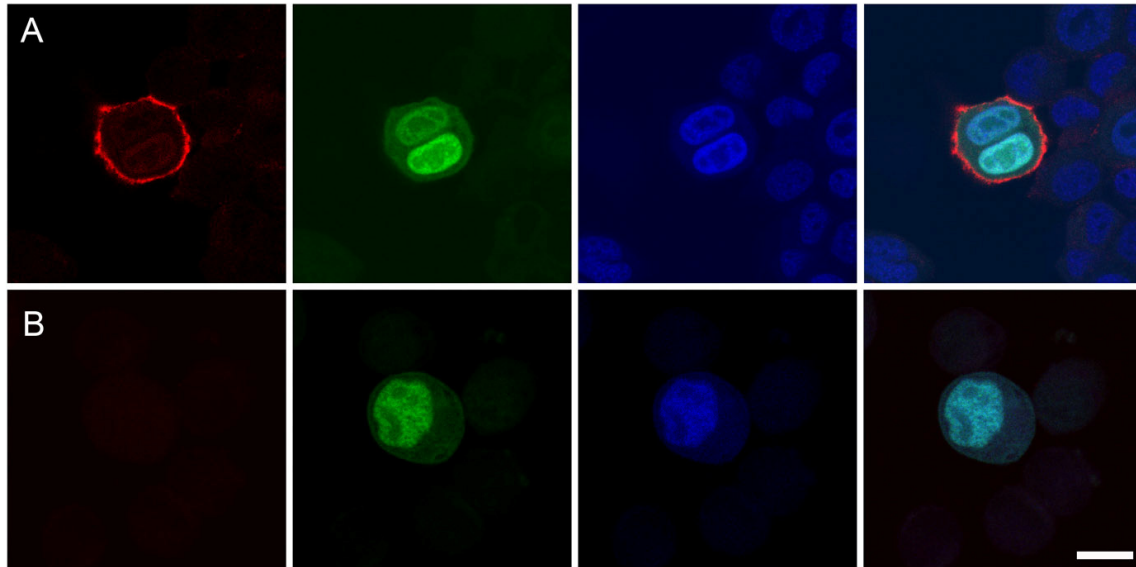


Figure S6. Time lapse images of AFF-1-mediated fusion of mammalian cells



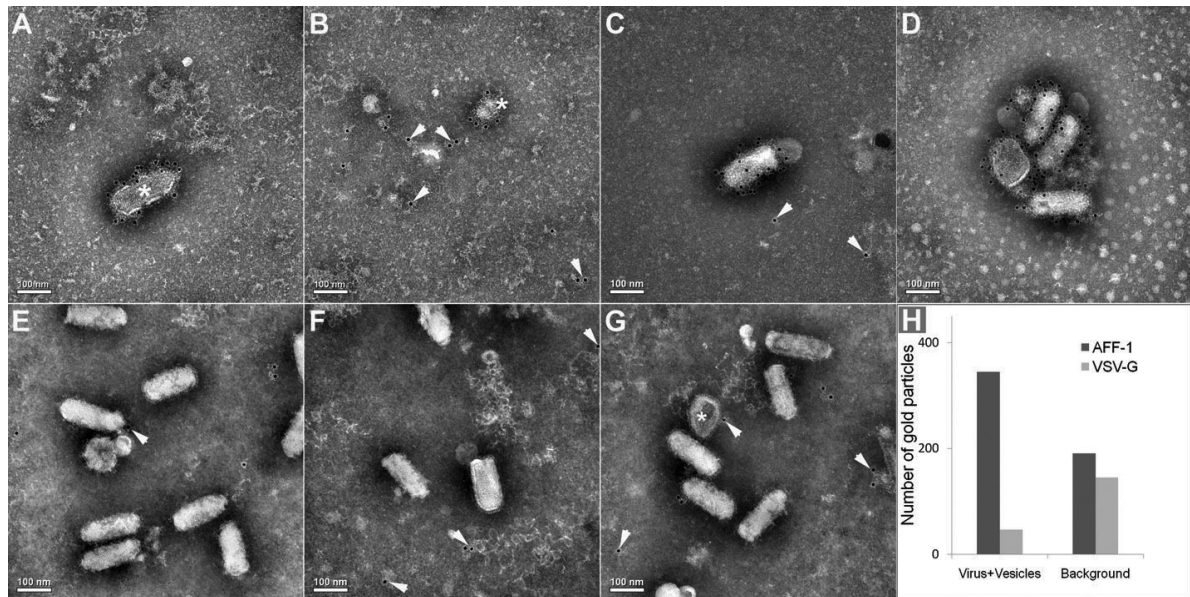
BHK cells co-transfected with AFF-1 and pRFPnes (white). The cells fuse as indicated by the diffusion of the cytoplasmic marker from the brighter cell (36 min, asterisk) to the larger cell. After 72 minutes the marker is homogeneously distributed and excluded from the second nucleus (arrowhead). Scale bar 20 μ m. $n > 3$ experiments (Movie S1).

Figure S7. Mouse polyclonal anti-AFF-1 antibody recognizes AFF-1 proteins on the plasma membrane of Sf9 cells



Immunofluorescence in Sf9 cells expressing AFF-1-FLAG (2) (transfected with 3 $\mu\text{g/ml}$ *aff-1* plasmid) with either (A) #8 mouse polyclonal antibodies against AFF-1 diluted 1:500 in TBST or (B) pre-immune serum. Secondary antibody - Alexa Fluor 568 goat anti-mouse IgG (H+L, Invitrogen Cat#A11004) diluted 1:500 in TBST. AFF-1 (red); Transfection marker - (nuclear/cytoplasmic GFP (green); DAPI, nuclei (blue). Scale bar is 10 μm .

Figure S8. Quantification of immunogold labeling shows specificity of anti-AFF-1 antibodies



In order to quantify the specificity of the immunogold labeling, we counted the number of gold particles on VSVΔG-AFF-1 (A-D) or VSVΔG-G (E-G) in negative stained preparations.

(A) Immunogold labeled VSVΔG-AFF-1 (asterisk).

(B) Immunogold labeled vesicle isolated from VSVΔG-AFF-1 prep (asterisk) with some background staining (arrowheads).

(C) Immunogold labeled VSVΔG-AFF-1 (center) with some background staining (arrowheads).

(D) Immunogold labeled vesicles and VSVΔG-AFF-1 viruses.

(E) VSVΔG-G stained with anti-AFF-1 (negative control) antibody showing some non-specific immunogold labeling (arrowheads).

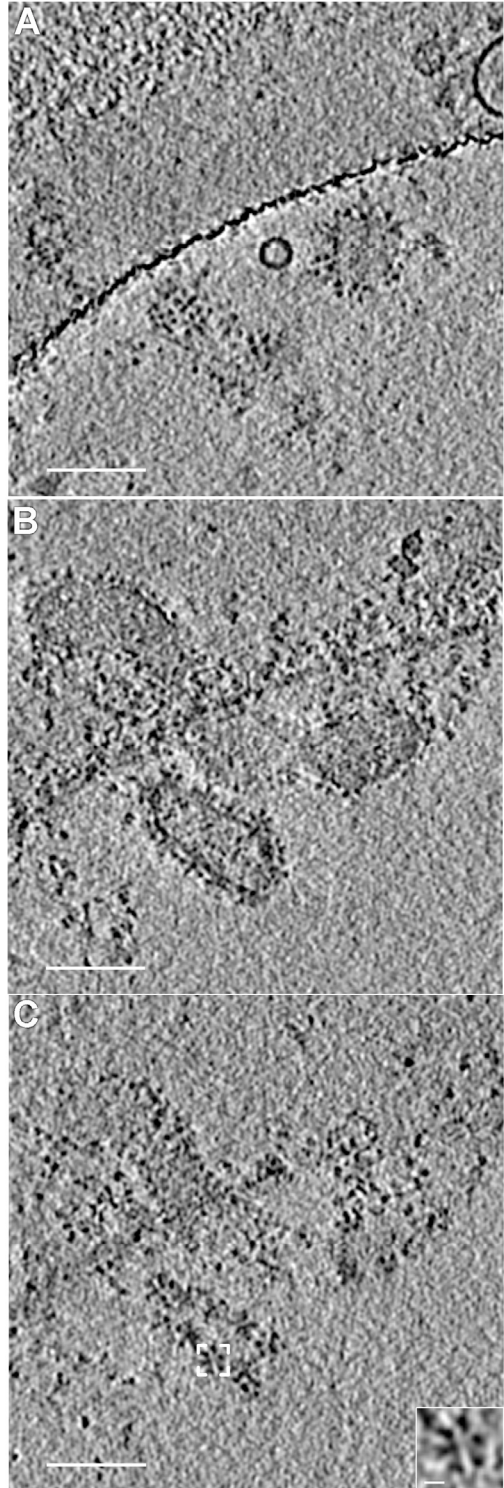
(F) VSVΔG-G (center) with some background staining (arrowheads).

(G) Immunogold labeled vesicles isolated from VSVΔG-G prep (asterisk) with some background staining (arrowheads).

(H) Number of gold particles (Y axis) recognizing viruses and vesicles versus background in VSVΔG-AFF-1 or VSVΔG-G samples stained with anti-AFF-1. Anti-AFF-1 showed specific virus/vesicle recognition of VSVΔG-AFF-1 compared to control VSVΔG-G. The difference in background staining between VSVΔG-AFF-1 and VSVΔG-G grids was not statistically significant ($P=0.4588$; $n=30$ quantified images per pseudovirus type).

Figure S9. Slices from cryoET of vesicles that co-purified with VSV Δ G-AFF-1

(A-C) Top, center and bottom slices from cryoET of vesicle preparations displaying penta- or hexa-meric “flower” shaped assemblies (see Movie S5). Scale bars are 100 nm and 10 nm for the inset; white box: magnified area shown in inset.



Supplemental tables

Table S1. Sequence identifiers

Species	Sequence Identifier
<i>Caenorhabditis elegans</i>	CeAFF-1
	CeEFF-1
	C26D10.7 (CeEFF-2)
<i>Caenorhabditis briggsae</i>	Cbr-aff-1
	Cbr-eff-1
<i>Caenorhabditis japonica</i>	CJA05978
	CJA03218
<i>Caenorhabditis ramanei</i>	Cre-aff-1
	Cre-eff-1
<i>Caenorhabditis brenneri</i>	Database: Caenorhabditis_PB2801-4.0-contigs (Contig1645.2)
	CBN17896
<i>Caenorhabditis sp5,7,9,11</i>	Database: Caeno_sp5_DRD-2008_JU800_1.fna [contig_262626]
	Database: Caeno_sp7_ju1286_454scaffolds_1.fna [scaffold00005]
	Database: Caeno_sp9_ju1422_454scaffolds_1.fna [scaffold00235, scaffold00002]
	Database: Caeno_sp11_JU1373_454scaffolds_1.fna [scaffold01488]
<i>Pristionchus pacificus</i>	Database: PpaFreeze1.bases Contig235.2 (Ppa FF-1)
	Database: PpaFreeze1.bases Contig162.29 (Ppa FF-2)
	Database: PpaFreeze1.bases Contig735.1 + Contig735.2 (Ppa FF-3)
<i>Pristionchus entomophagus</i>	Database: Pristionchus_entomophagus-3.0.bases Contig1225.3
<i>Pristionchus maupasi</i>	Database: p.maup genome Contig3990.1
<i>Trichinella spiralis</i>	gi 162730680 (<i>Tsp-ff-1</i>)
	gi 162732036 (<i>Tsp-ff-2</i>)
<i>Trichinella pseudospiralis</i>	gi 149208398 gb EF601568.1
<i>Trichinella papuae</i>	gi 149208399 gb EF601569.1
<i>Meloidogyne incognita</i>	gi 19265127
<i>Meloidogyne arenaria</i>	gi 15768755
<i>Meloidogyne hapla</i>	gi 207096946
<i>Globodera pallida</i>	gi 54548408
<i>Ancylostoma caninum</i>	gi 157990577
<i>Brugia Malayi</i>	Bm1_09970 + Bm1_09975
	Bm1_24045
<i>Haemonchus contortus</i>	gb CA869252.1
	gi 27320801
<i>Ascaris suum</i>	gi 113050648

<i>Oscheius tipulae</i>	Database: Oscheius_tipulae_clc3_1.fna [Contig 4684, contig 5292]
<i>Dirofilaria immitis</i>	Database: Dirofilaria_immitis_clc_1.fna
<i>Howardula aoronymphium</i>	Database: Howardula_aoronymphium_clc_1.fna
<i>Litomosoides sigmodontis</i>	Database: Litomosoides_sigmodontis_abyss_1.fna
<i>Heterodera glycines</i>	GI:170569983
<i>Romanomermis culicivorax</i>	contig05859, contig06497 (Kindly provided by W. Kelley Thomas)
<i>Trichuris muris</i>	Database: T_muris_contigs.fasta
<i>Strongyloides ratti</i>	Database: S.ratti.reads
<i>Onchocerca volvulus</i>	Database: O_volvulus_all454_contigs.fna
<i>Teladorsagia circumcincta</i>	Database: T_circumcincta_reads.fasta
<i>Wuchereria bancrofti</i>	GI:285822425, GI:285835743
<i>Loa loa</i>	GI:285852521, GI:285851695
<i>Branchiostoma floridae</i>	jgi Brafl1 104514 fgenesh2_pg.scaffold_465000022 (Bfl-ff-1)
	jgi Brafl1 104513 fgenesh2_pg.scaffold_465000021
<i>Pleurobrachia pileus</i>	gi 167791107
<i>Calanus finmarchicus</i>	gi 190134016
<i>Lepeophtheirus salmonis</i>	gi 293020530
<i>Naegleria gruberi</i>	gi 284087402; gi 284087338 ; gi 284083966 ; gi 284083965 est's GI:168534442; GI:168542950

Table S2. Measured size of AFF-1 and VSVG

Mean Size	VSVG			AFF-1		
	Length (nm)	Width (nm)	N	Length (nm)	Width (nm)	N
Negative Staining	14.5±0.5	8.7±0.4	20	10.9±0.5	6.0±0.3	32
Crystal Structure (26)	12.5	6.0				

* Measurements are shown as mean size ± Standard Error, N – number of measurements

Table S3. Plasmids

Plasmid Name	Description	Reference
pOA6	<i>P. pacificus</i> <i>eff-1</i> genomic sequence in pPD49.78 (hsp 16.2)	This work
pOA19	<i>C. elegans</i> <i>eff-1</i> fused to a C-terminal V5 tag (EFF-1::V5) in pCAGGS	This work
pOA20	<i>C. elegans</i> <i>aff-1</i> fused to a C-terminal FLAG tag (AFF-1::FLAG) in pCAGGS	This work
pOA35	<i>T. spiralis</i> <i>ff-1</i> with a kozak sequence fused to a C-terminal FLAG tag (Tsp-FF-1::FLAG)	This work
pOA60	<i>B. floridae</i> <i>ff-1</i> with a kozak sequence fused to a C-terminal FLAG tag (Bfl-FF-1::FLAG)	This work
pCFPnls	CFP with two tandem nuclear localization signals	This work
pRSETA -AFF1EC	The extracellular domain of AFF-1 (residues 60-536)	This work
pCAGGS	A chicken beta-actin/rabbit beta-globin hybrid promoter with a human cytomegalovirus immediate early promoter (CMV-IE) enhancer	(1)
pIZT-AFF-1::FLAG	<i>C. elegans</i> <i>aff-1</i> fused to a C-terminal FLAG tag (AFF-1::FLAG) in pIZT	(2)
pRFPnes	DsRed2 with a nuclear export signal (pCH19)	(4)
pCAGGS-Gind	VSV G Indiana strain	(29)

Table S4. Primers

Name	Sequence
OR-19	ATGATCTTCTCTCTCTCTCTACTGTATAC
OR-22	TCATACATAATCTCCAGGTAGAACATC
OR-54	TTAATTGGTACCACTATGGAACCGCCGTTGAGTGG
OR-55	AATTAAGCTAGCTCAACCGGTACGCGTAGAATCGAGACC
OR-56	TTAATTGGTACCACTATGGTACTGTGGCAATGGTCAATAGCC
OR-100	ATGTTCTCACCACCTTTTTGTCTTCTTCTTCTG
OR-101	AACTGCCTCGCCCAAGAATATGCC
OR-102	ATATTCTTGGGCGAGGCAGTTGACC
OR-103	TCACAATTTGTTAGCATTTCGTTCTG
OR-111	TTAATTGGTACCATGTTCTCACCACCTTTTTGTCTTCTTCTTCTG
OR-112	AATTAAGCTAGCTCATTTGTCATCGTCCTTGTAGTCCAATTTGTTAGCATTCGTTCTGCCATTTCC
OR-147F	AATTAAGGATCCATGGTGAGCAAGGGCGAGGAGCTG
OR-148R	AATTAAGAATTCTTATACCTTTCTTCTTTTTGGATCTACC
AM-66	TATGTCTTAGATCTCCAAAGTCTCATCAGTACACAGTACT
AM-67	TGTATCATGGTACCCTCTGTGAAATCCCCACCATGAGC

Table S5. Proteins identified in purified pseudovirus-preparations by mass spectrometry

Accession	Protein	MW (kDa)	AFF-1			VSV-G			Bald			Subcellular location
			Peptide hits	Coverage (%)	Peak Area (%)	Peptide hits	Coverage (%)	Peak Area (%)	Peptide hits	Coverage (%)	Peak Area (%)	
P03523	VSV-L	241	96	48.50	6.32	85	37.65	5.34	43	23.95	4.95	Virion; Host cytoplasm
P03521	VSV-N	47	49	87.20	36.23	49	85.55	39.41	36	78.91	43.03	Virion; Host cytoplasm
Q18592	AFF-1	67	40	60.40	9.79	0	NA	NA	0	NA	NA	Membrane; Single-pass type I membrane protein
P03522	VSV-G	57	0	NA	NA	32	36.40	21.21	0	NA	NA	Virion membrane; Single-pass type I membrane protein
P03519	VSV-M	26	24	56.30	27.45	22	47.60	14.34	18	47.60	33.41	Virion membrane; Peripheral membrane protein
P03520	VSV-P	30	14	48.70	10.14	13	47.17	9.25	11	26.42	4.71	Virion; Host cytoplasm
P17852	Integrin alpha-3	118	22	23.20	1.65	14	15.29	0.69	14	13.70	1.88	Membrane; Single-pass type I membrane protein
Q9WV91	Prostaglandin F2 receptor negative regulator	98	19	19.00	0.58	9	9.56	0.13	7	8.30	0.56	ER membrane; Single-pass type I membrane protein
P09055	Integrin beta-1	88	15	21.70	1.36	11	16.67	0.65	9	14.04	0.62	Membrane; Single-pass type I membrane protein
P54289	Voltage-dependent calcium channel subunit alpha-2/delta-1	124	6	5.30	0.03	0	NA	NA	0	NA	NA	Membrane; Single-pass type I membrane protein
P62998	Ras-related C3 botulinum toxin substrate 1	21	6	31.30	0.23	4	24.48	0.25	0	NA	NA	Membrane; Lipid-anchor; Cytoplasmic side
Q10741	Disintegrin and metalloproteinase domain-containing protein	84	6	6.80	0.11	3	5.48	0.02	0	NA	NA	Membrane; Single-pass type I membrane protein
Q8R366	Immunoglobulin superfamily member 8	65	5	8.80	0.15	2	3.93	0.05	0	NA	NA	Membrane; Single-pass membrane protein
P11688	Integrin alpha-5	115	4	4.10	0.03	0	NA	NA	0	NA	NA	Membrane; Single-pass type I membrane protein
Q08992	Syntenin-1	32	4	10.70	0.14	0	NA	NA	0	NA	NA	Membrane; Peripheral membrane protein
Q2HJ49	Moesin	68	4	6.60	0.05	13	15.60	0.16	0	NA	NA	Membrane; Peripheral membrane protein; Cytoplasmic side
Q8WUM4	Programmed cell death 6-interacting protein	96	14	17.70	1.76	15	16.92	0.21	2	2.76	0.03	Cytoplasm
Q07891	Transferrin receptor protein 1	85	4	2.90	0.06	6	9.38	0.11	3	3.90	0.6	Membrane; Single-pass type II membrane protein
P62833	Ras-related protein Rap-1A	21	4	19.60	0.08	3	13.59	0.04	0	NA	NA	Membrane; Lipid-anchor
P21956	Lactadherin	51	4	8.00	0.14	3	6.48	0.08	2	4.75	0.15	Membrane; Peripheral membrane protein
P19120	Heat shock cognate 71 kDa protein	71	8	23.20	0.09	11	14.40	0.25	4	8.74	0.24	Cytoplasm
Q5E9R3	EH domain-containing protein 1	61	3	5.80	0.01	0	NA	NA	0	NA	NA	Membrane; Peripheral membrane protein; Cytoplasmic side
P04272	Annexin A2	39	10	31.30	0.11	10	31.27	0.40	0	NA	NA	Basement membrane
Q9H223	EH domain-containing protein 4	61	3	6.30	0.03	5	9.24	0.08	0	NA	NA	Cell membrane; Peripheral membrane protein
Q95JC7	Neutral amino acid transporter B	56	3	6.30	0.07	2	4.45	0.09	0	NA	NA	Membrane; Multi-pass membrane protein
P18246	Gap junction alpha-1	43	3	10.20	0.03	2	6.27	0.01	0	NA	NA	Cell membrane; Multi-pass membrane protein
Q8NG11	Tetraspanin-14	31	3	14.10	0.06	2	10.37	0.02	3	14.07	0.1	Membrane; Multi-pass membrane protein
P13549	Elongation factor 1-alpha	50	4	9.70	0.09	5	14.94	0.22	0	NA	NA	Cytoplasm
Q92030	Sodium/potassium-transporting ATPase subunit alpha-1	113	2	8.30	0.02	7	2.15	0.03	0	NA	NA	Cell membrane; Multi-pass membrane protein
P31976	Ezrin	69	2	3.30	0.06	5	8.26	0.24	0	NA	NA	Peripheral membrane protein; Cytoplasmic side
Q9Z0G9	Claudin-3	23	2	7.30	0.01	4	14.16	0.09	5	14.16	0.42	Cell membrane; Multi-pass membrane protein
Q62419	Endophilin-A2	41	2	6.50	0.02	3	9.78	0.03	0	NA	NA	Early endosome membrane; Peripheral membrane protein
Q90474	Heat shock protein HSP 90	83	0	NA	NA	3	4.55	0.04	0	NA	NA	Cytoplasm
P15924	Desmoplakin	332	0	NA	NA	0	NA	NA	10	3.34	1.17	Desmosome
P30932	CD9 antigen	25	0	NA	NA	0	NA	NA	3	15.04	0.09	Membrane; Multi-pass membrane protein
Q8SPJ1	Junction plakoglobin	82	0	NA	NA	0	NA	NA	3	4.97	0.11	Membrane; Peripheral membrane protein
P98157	Prolow-density lipoprotein receptor-related protein 1	507	0	NA	NA	10	1.45	0.09	0	NA	NA	Membrane; Single-pass type I membrane protein
P20020	Plasma membrane calcium-transporting ATPase 1	139	0	NA	NA	6	7.23	0.04	0	NA	NA	Membrane; Multi-pass membrane protein
P29317	Ephrin type-A receptor 2	108	0	NA	NA	4	4.71	0.05	0	NA	NA	Membrane; Single-pass type I membrane protein
P35950	Low-density lipoprotein receptor	94	0	NA	NA	3	10.98	0.06	0	NA	NA	Membrane; Single-pass type I membrane protein

Virial, membrane, membrane-associated and previously-identified cytosolic proteins (21) are shown. Serum proteins (e.g. transferrins and albumins), cytosolic proteins (e.g. actin, tubulin, histones and enzymes) and proteins identified by fewer than 3 peptides are not shown. Peptide hits - total number of identified peptides. Peak area - calculated integrated height of the reconstructed ion chromatogram peaks. Coverage - Displays the percentage of the protein sequence covered by the identified peptides.

Supplemental movie legends

Movie S1. Fusion of BHK cells mediated by AFF-1 (Fig. S6). Timer presents hh::mm. Merger of cytoplasms is revealed by the transfer of bright fluorescence (white) from the small cell to the bigger cell (arrowhead). Scale bar is 20 μm .

Movie S2. Multinucleated cells expressing AFF-1 fuse (phase contrast). Scale bar is 20 μm . Timer presents hh::mm.

Movie S3. CryoET reconstruction (series of computational slices through the tomogram) of VSV Δ G-AFF-1 (See Fig. 4 I to K).

Movie S4 and S5 CryoET reconstruction (series of computational slices through the tomogram) of vesicles co-purified in VSV Δ G-AFF-1 preparations displaying penta- or hexameric “flower” shaped assemblies (See Figs. 4 L, M and S9).

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30. O.A. performed the experiments in tissue culture, molecular biology work, inoculations and bioinformatics. O.A. and K.F prepared the pseudotyped viruses. K.F., I.A., B.P. and D.D. performed the negative staining and cryo-TEM experiments. U.M., T.B. and K.G. performed the cryo-ET and cryo-TEM experiments. K.F. performed the immunogold labeling. A.S. prepared the construct for expression of AFF-1EC in bacteria. C.V. screened the anti AFF-1 antibodies. O.A., B.P. and J.W. designed and analyzed the pseudotyped viruses. O.A. and B.P. analyzed the experiments and wrote the manuscript. All authors discussed the results and commented on the manuscript.