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Supplementary Information for

Evolutionarily related small viral fusogens hijack distinct but modular actin nucleation pathways to drive cell-cell fusion

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Materials and Methods

Molecular Cloning

Aquareovirus fusion associated small transmembrane protein, p22 (Atlantic salmon reovirus Canada-2009, Accession number: C0L0N0), was synthesized and inserted into mammalian expression vector pcDNA3.1 with Kozak sequence and C-terminus tags (mCherry, eGFP, myc).

Point mutations and truncations were introduced with primers and verified with Sanger sequencing. mCherry was similarly inserted into pcDNA3.1. pcDNA3.1-p14-mCherry was used as previously described (23).

p14/p22 chimera with p14 ectodomain and transmembrane domain (1-57 AA) and p22 cytoplasmic tail (58-198 AA) was synthesized with Gibson assembly with C-terminus tags (mCherry, eGFP, myc). Other chimeras comprised all possible permutations of p14 ectodomain (1-35 AA), p14 transmembrane domain (36-57 AA), p14 cytoplasmic tail (58-125 AA), p22 ectodomain (1-34 AA), p22 transmembrane domain (35-57 AA), p22 cytoplasmic tail (58-198 AA) were synthesized with Gibson assembly with C-terminus mCherry tag.

GFP-EEA1 WT was a gift from Silvia Corvera (Addgene plasmid # 42307).

GFP-Intersectin Long, Intersectin-1 I SH3 A domain (human), Intersectin-1 I SH3 B domain (human), Intersectin-1 I SH3 C domain (human), Intersectin-1 I SH3 D domain (human), and Intersectin-1 I SH3 E domain (human), were gifts from Peter McPherson (Addgene plasmids #47395, #47413, #47414, #47415, #47416, #47417).

ΔGBD-mDia2 (258-1171 AA) was amplified from pCMV-eGFP-mDia2 (a kind gift from Scott Hansen) and inserted with a N-terminus FRB tag into pcDNA3.1.

Codon-swapped versions of SH3A domain from human Intersectin-1 (740-816 AA), where every third nucleotide is swapped so that it does not match siRNA targeting Intersectin-1 (EHU102031, Sigma Aldrich), were synthesized as a gblock (IDT). DH and PH domains from human Intersectin-1 (1226-1573 AA) was amplified and inserted downstream of SH3 A domain and eGFP into pcDNA3.1

Rab11a (human) was synthesized as a gblock from IDT and inserted with N-terminus mTagBFP2 tag.

GFP-caax was a gift from Alba Diz- Muñoz (EMBL-Heidelberg).

Cell culture, plasmid and siRNA transfection

Vero cells were obtained from UC-Berkeley Cell Culture Facility. Vero cells were grown in DMEM (Life Technologies) supplemented with 10% heat-inactivated FBS (Life Technologies), 10% non-essential amino acids (Life Technologies), and 1% Pen-Strep (Life Technologies), at 37 °C, 5% CO_2. N-WASP -/- and +/+ mouse embryonic fibroblasts cells were a kind gift from Scott Snapper (Harvard University, Cambridge, MA) and were grown in DMEM (Life Technologies) supplemented with 10% heat-inactivated FBS (Life Technologies) and 1% Pen-Strep (Life Technologies), at 37 °C, 5% CO_2.

Cells were negative for mycoplasma as verified with Mycoalert mycoplasma detection kit (Lonza).

Cells were transfected with plasmids with FuGENE HD (Promega) according to manufacturer's instructions. Cells were transfected with siRNA with Lipofectamine RNAimax (ThermoFisher) according to manufacturer's instructions.

Genome-edited human induced pluripotent stem cells (hiPSCs)

WTC10 hiPSC line was obtained from the Bruce Conklin Lab. hiPSCs were cultured on Matrigel (hESC-Qualified Matrix, Corning) in StemFlex medium (Thermo Fisher) with

Penicillin/Streptomycin in 37°C, 5% CO_2. Cultures were passaged with Gentle Cell Dissociation reagent (StemCell Technologies) twice every week.

The AP2M1 gene was edited in WTC10 hiPSCs as previously described using TALENs targeting exon 7 of AP2M1 gene (1). Both alleles of AP2M1 were tagged with tagRFP-T. Cas9-crRNA-tracrRNA complex electroporation method was used to edit ITSN1 gene in AP2M1-tagRFP-T genome edited hiPSCs. S. pyogenes NLS-Cas9 was purified in the University of California Berkeley QB3 MacroLab. TracrRNA and crRNA targeting AGGTGTTGGAAACTGAGCCA sequence in the immediate vicinity of the start codon of ITSN1 were purchased from IDT. Gibson assembly (New England Biolabs) was used to construct a donor plasmid containing the pCR8/GW/TOPO (Thermo Fisher) plasmid backbone, the mEGFP gene followed by a AAGTCCGGAGGTACTCAGATCTCGAGG linker sequence, and 450/447 base pair homology arm sequences. Three days after electroporation (Lonza, Cat#: VPH-5012) of Cas9-crRNA-tracrRNA complex and donor plasmid, the GFP positive cells were single cell sorted with a BD Bioscience Influx sorter (BD Bioscience) into Matrigel-coated 96-well plates. Clones were confirmed by PCR and sequencing of the genomic DNA locus around the mEGFP insertion site. Sequences were aligned using Benchling. Both alleles of ITSN1 were tagged with mEGFP in the hiPSC line used in this study.

hiPSCs sample preparation
2 days before fixation, hiPSCs were seeded on Matrigel-coated 4-well chambered cover glass (Cellvis). 20 hours before fixation, the p22 expressing plasmids were transfected using Lipofectamin Stem Transfection Reagent (Thermo Fisher). Halotag was labeled by JF635-HaloTag ligand (2). Cells were incubated in StemFlex medium with 100 mM JF635-HaloTag for 45min and the unbound ligands were washed away by three times of 5 min incubation in prewarmed StemFlex medium. Cells were fixed by 4% PFA in DPBS (Gibco) for 20 min and washed by DPBS three times.

Imaging
All live cells were maintained at 37°C, 5% CO_2 with a stage top incubator (okolab) during imaging.

For confocal microscopy, cells were imaged with a spinning disk confocal microscope (Eclipse Ti, Nikon) with a spinning disk (Yokogawa CSU-X, Andor), CMOS camera (Zyla, Andor), and either a 4x objective (Plano Apo, 0.2NA, Nikon), 20x objective (Plano Fluor, 0.45NA, Nikon), 40x objective (Plano Fluor, 0.6NA ,Nikon) or a 60x objective (Apo TIRF, 1.49NA, oil, Nikon). Microscopes were controlled with Micro-Manager. Images were analyzed and prepared using ImageJ (National Institutes of Health).

Nuclei count
26,700 Vero cells were transfected with specified plasmids with FuGENE HD (Promega) and immediately plated onto fibronectin coated glass-bottomed dishes. 82,600 N-WASP -/- and +/+ mouse embryonic fibroblasts were transfected with p22 WT with Lipofectamine 3000 and immediately plated onto fibronectin coated glass bottomed dishes. For Vero cells expressing p22 WT and point mutations of p22 WT, cells were fixed 36 hours post transfection and imaged, while for p14/p22 chimera and N-WASP -/- and +/+, cells were fixed 22-24 hours post transfection. Vero cells co-expressing p14/p22 chimera-P149A-FKBP with either FRB-ΔGBD-mDia2 were transfected with FuGENE HD (Promega). 12-14 hours post transfection, 500 nM of rapalog (AP21967, Takara Bio) was added to media. Cells were fixed 24 hours post transfection. Twenty-five to thirty fields of view from each of three independent transfections were collected. Micrographs were processed in FIJI and cytoplasmic p22 fluorescence intensity was used to make a binary mask of cell bodies. Nuclei masks were made from cells stained with 5 μM Syto11 (Thermo Fisher), Hoechst 33342 (Thermo Fisher), or from the inverted mask of cytoplasm fluorescence. Nuclei per cell expressing fluorescently-tagged constructs were counted either manually or using the Speckle Inspector FIJI plugin (http://www.biovoxxel.de/). Cells expressing

the p14/p22 chimera were masked using pixel classification in Ilastik (https://www.ilastik.org/) and nuclei were counted as above. Cells with more than 2 nuclei were considered multinucleated.

splitYFP cell-cell fusion assay
As previously described (23), Vero cells were stably transduced with splitYFPa and splitYFPb lentivirus. Cells were passaged for a week prior to transfection with p22-WT-mCherry. 36 hours post transfection, cells were moved to 30°C, 5% CO_2 incubator to mature splitYFP fluorophore for 6 hours. Cells were imaged using confocal fluorescence microscopy.

Plasma membrane enrichment quantification
The plasma membrane of p22-mCherry and p14/p22-Chimera-mCherry expressing cells were labeled with CellMaskDeepRed (ThermoFisher). Cells were imaged with spinning disk confocal microscopy with a 60x objective. A linescan spanning the plasma membrane and 600-1000 nm proximal to the plasma membrane was analyzed in ImageJ. The plasma membrane location is determined by the maximum fluorescence intensity of CellMaskDeepRed, and the plasma membrane enrichment index is defined as the fluorescence intensity of mCherry-tagged proteins at the plasma membrane, normalized to the average fluorescence intensity of the protein in the cytosol.

Filopodia enrichment quantification
Vero cells co-expressing p14/p22 chimera-P149A-FKBP, FRB-ΔGBD-mDia2 and Lifeact-GFP were imaged with spinning disk confocal microscopy with a 60x objective at 37°C, 5% CO_2. 500 nM rapalog (AP21967, Takara Bio) was added to the media, and cells were imaged for 15 min. Linescans spanning 1.1 µm from the tip of filopodia were analyzed using ImageJ. The last 270 nm of filopodia is defined as the tip of the filopodia and the remaining 900 nm is defined as the length of the filopodia. Fluorescence intensity of p14/p22 chimera-P149A-mCherry-FKBP along the length of the filopodia is normalized to the average fluorescence intensity of the entire length of the filopodia. Approximately 30 filopodia at 0 min and 30 filopodia at 10 min after addition of rapalog, all selected at random, were analyzed.

Surface biotinylation
Cells were grown in 100 mm dish or T25 flask to 50% confluency and transfected using FuGENE reagent. After 24 hours cells were washed with PBS (pH 8) three times on ice and incubated with 1 mg Sulfo-NHS-Biotin in 0.5 mL PBS (pH 8) for 1 hour on ice. The biotinylation reaction was quenched with 100 mM glycine in PBS for 10 min at room temperature. Cells were washed three times with 100 mM glycine in PBS and lysed with 1 mL lysis buffer (1x RIPA buffer supplemented with 1x HALT protease inhibitor (Thermo Fisher Scientific)) for 30 minutes on ice. Cells were scraped into 1.5 mL microcentrifuge tube and sonicated on ice for 3 minutes. Cell debris was pelleted for 5 min at 17,900 rcf at 4°C. 30 µl of streptavidin magnetic beads (ThermoFisher) were washed with RIPA buffer at 4°C, the supernatant was added and incubated overnight. Beads were washed 5 times with RIPA buffer, suspended in Laemmli sample buffer (30 ul), denatured at 95°C for 5 min and separated on 4-20% acrylamide gradient gels by SDS-PAGE. Proteins were transferred onto nitrocellulose membrane with iBlot (Thermo Fisher Scientific). The membrane was blocked with 5% milk powder in TBST for 1 hour and probed with primary antibodies α-GFP (1:5000, g1544, Sigma Aldrich), α-myc (1:5000, 9e10, Sigma Aldrich), α-tubulin (1:5000, Clone YL1/2, Thermo) in 5% milk in TBST overnight at 4°C. The membrane was then probed with secondary antibodies, α-rabbit HRP (1:5000, 65-6120, Thermo Fisher), α-mouse HRP (1:5000, Jackson Labs), α-rat AlexaFluor 647 (1:5000, Life Technologies). Western blots were imaged on ChemiDoc (Bio-Rad). Densitometry of eluate and lysate lanes of western blots was used to quantify and normalize surface expression of p22 mutant to that of wild-type p22.

Non-reducing SDS-PAGE, reduction and alkylation
Vero cells were transfected with p22 WT, C5S and C7S with myc tag. Vero cells were harvested 24 hours post transfection. Cells were washed with PBS, and lysed with lysis buffer (150 mM NaCl, 25 mM HEPES pH 7.4, 1 mM EDTA, 0.5% NP-40, 1x HALT protease inhibitor (Thermo Fisher Scientific) for 30 min at 4°C, and scraped and bath sonicated in ice for 3 min. Cell debris

was pelleted at 17,900 rcf for 10 min. To enrich for p22, supernatant was incubated with 5 µl of myc-Trap beads (Chromotek) overnight at 4°C. The beads were washed with lysis buffer five times. For p22 WT, C5S and C7S to be analyzed with non-reducing SDS-PAGE, samples were boiled in Laemmli sample buffer without reducing agents and separated on 4-20% acrylamide gradient gels by SDS-PAGE. For p22 WT reduced with DTT, samples were boiled in Laemmli sample buffer with 350 mM DTT. To cap cysteines in p22 WT, myc-Trap beads (Chromotek) were washed three times in alkylation buffer, 100 mM Tris, pH 8.0, 50 mM DTT before incubating at 85°C for 10 min. Reduced cysteines were capped immediately with 100 mM iodoacetamide (Sigma) for 15 min at room temperature in the dark. The eluted protein was boiled in Laemmli sample buffer and separated on 4-20% acrylamide gradient gels by SDS-PAGE. Proteins were transferred onto nitrocellulose membrane 16 hours at 4°C at 40 mA. The membrane was blocked in 5% milk in TBST for 1 hour and probed with primary antibody, α-myc (1:2500, 9E10, Sigma) in 5% milk in TBST overnight at 4°C. The membrane was then probed with secondary antibody, α-mouse HRP (1:2500, Jackson ImmunoResearch). Western blots were imaged on ChemiDoc (Bio-Rad).

Membrane fractionation

Vero cells were transfected with myc-tagged p22 WT, C5S and C7S. 36-48 hours post transfection, cells were lifted with 0.25% Trypsin, neutralized with media and then washed with PBS three times at 4°C. Cells were resuspended in lysis buffer (20 mM HEPES, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM TCEP, 1x HALT protease inhibitor (Thermo Fisher Scientific)), and lysed with 5 cycles of freeze/thaw. Nuclei were pelleted at 700 rcf for 5min, then mitochondria were pelleted at 10,000 rcf for 5 min. The resulting supernatant was centrifuged at 100,000 rcf for one hour at 4°C. The cytoplasmic supernatant was kept for analysis. The membrane pellet was washed with either high salt (20 mM HEPES, 500 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM TCEP, 1x HALT protease inhibitor (Thermo Fisher Scientific)) or high urea (20 mM HEPES, 2M urea, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM TCEP, 1x HALT protease inhibitor (Thermo Fisher Scientific). Membrane fraction was re-pelleted at 100,000 rcf for 1 hour at 4°C, and washed once. Membrane pellet was extracted by incubating in 2x Laemmli sample buffer for an hour at room temperature. Samples were boiled in Laemmli sample buffer and separated on 4-20% acrylamide gradient gels by SDS-PAGE. Proteins were transferred onto nitrocellulose membrane 1 hour at 4°C at 350 mA. The membrane was blocked in 5% milk in TBST for 1 hour and probed with primary antibody, α-myc (1:2500, 9E10, Sigma), α-tubulin (1:5000, Clone YL1/2, Thermo) in 5% milk in TBST overnight at 4°C. The membrane was then probed with secondary antibody, α-mouse HRP (1:2500, Jackson ImmunoResearch), α-rat AlexaFluor 647 (1:2500, Life Technologies). Western blots were imaged on ChemiDoc (Bio-Rad).

Drug treatment

To perturb downstream signaling of p22, 8-9 hours post transfection, Latrunculin A (abcam), Wiskostatin (Sigma Aldrich), CK666 (Sigma Aldrich), smifH2 (CalBioChem), ZCL278 (Cayman Chemical), ML141 (Cayman Chemical) were added to complete media at specified concentrations. DMSO was used as vehicle control, and 36 hours post transfection the cells were imaged to quantify the extent of cell-cell fusion.

siRNA knockdown endogenous ARPC3 and Intersectin-1 expression

26,700 Vero cells in fibronectin-coated imaging wells were transfected with 400 ng of either esiRNA targeting ARPC3 (EHU107121, Sigma-Aldrich), or esiRNA targeting ITSN-1 (EHU102031, Sigma-Aldrich), or control esiRNA (EHUEGFP, Sigma-Aldrich) with Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific), according to manufacturer's instructions. 8-12 hours post transfection, media was exchanged. 24 hours post transfection, media was exchanged and cells were transfected with p22-WT-mCherry alone or with rescue plasmids, codon-swapped SH3A, SH3A-DHPH, or GFP using FuGENE HD (Promega) according to manufacturer's instructions. The extent of cell-cell fusion was assayed 36 hours post plasmid transfection/66 hours post siRNA transfection. Expression of ARPC3 and Intersectin-1 is confirmed by lysing cells with RIPA buffer. Cell debris was pelleted at 17,900 rcf for 10 min and total protein is quantified with BCA assay. Samples were boiled in Laemmli sample buffer and separated on 4-

20% acrylamide gradient gels by SDS-PAGE. Proteins were transferred onto nitrocellulose membrane and probed with primary antibodies, α-ARPC3 (1:1000, Clone 26/p21-Arc, BD Biosciences), α-ITSN (1:1000, Clone 29, BD Biosciences), α-GFP (1:2500, gp1544, Life Technologies), α-tubulin (1:2500, Clone YL1/2, Thermo) and secondary antibodies, α-mouse HRP (1:2500, Jackson Labs), α-rabbit HRP (1:2500, 65-6120, Thermo Fisher), α-rat AlexaFluor 647(1:2500, Life Technologies).

Co-immunoprecipitation

Vero cells were transfected with specified plasmids. Vero cells were harvested 24-36 hours post transfection. Cells were washed with PBS, and lysed with lysis buffer (150 mM NaCl, 25 mM HEPES pH 7.4, 1 mM EDTA, 0.5% NP-40, 1x HALT protease inhibitor (Thermo Fisher Scientific) for 30 min at 4°C, and scraped and bath sonicated in ice for 3 min. Cell debris was pelleted at 17,900 rcf for 10 min. Supernatant was incubated with 7.5 µl of washed GFP-Trap beads (Chromotek) overnight at 4°C. The beads were washed with lysis buffer five times, before boiled in Laemmli sample buffer and separated on 4-20% acrylamide gradient gels by SDS-PAGE. Proteins were transferred onto nitrocellulose membrane and probed with primary antibodies, α-ITSN (1:1000, Clone 29, BD Biosciences), α-GFP (1:5000, gp1544, Life Technologies), and secondary antibodies, α-mouse HRP (1:5000, Jackson Labs), α-rabbit HRP (1:5000, 65-6120, Thermo Fisher), α-rat AlexaFluor 647(1:5000, Life Technologies). Western blots were imaged on ChemiDoc (Bio-Rad).

Protein purification

N-terminus GST-tagged SH3A, SH3B, SH3C, SH3D, SH3E domains from human Intersectin-1 was expressed in Rosetta for 4 hours at 37°C. Cells were resuspended in lysis buffer (25 mM HEPES pH 7.2, 150 mM NaCl, 1 mM DTT, 1x PMSF) and lysed by sonication. Cell debris was pelleted and the supernatant was bound onto glutathione resin (GBiosciences). Resin was washed with 10 column volumes of wash buffer (25 mM HEPES pH 7.2, 150 mM NaCl, 1 mM DTT) and protein was eluted with elution buffer (25 mM HEPES pH 7.2, 150 mM NaCl, 1 mM DTT, 30mM glutathione). Protein was desalted into storage buffer (25 mM HEPES pH 7.2, 150 mM NaCl, 1 mM DTT, 10% glycerol) and flash frozen.

SH3 binding assay

HEK293T cells were transfected with pcDNA3.1-p22-myc with TransIT-293 according to manufacturer's instructions. 24 hours post transfection, the cells were washed with PBS and lysed by incubating in lysis buffer (150 mM NaCl, 25 mM HEPES pH 7.4, 1 mM EDTA, 0.5% NP-40, 1x HALT protease inhibitor (Thermo Fisher Scientific) for 30 min in 4°C and bath sonicated for 3 min. Cell debris was pelleted at 17,900 rcf for 15 min and the supernatant was bound to α-myc beads (Chromotek) for 4 hours at 4°C. Beads were washed twice with wash buffer and (150 mM NaCl, 25 mM HEPES pH 7.2, 1 mM EDTA, 0.5% NP-40) and incubated with 30 µM of GST-SH3A, GST-SH3B, GST-SH3C, GST-SH3D, GST-SH3E for an hour at 4C. Beads were washed twice with (1 M NaCl, 25 mM HEPES pH 7.4, 0.1% NP-40, 1 mM EGTA, 5 mM $MgCl_2$) and three times with (100 mM NaCl, 25 mM HEPES pH 7.4, 0.1% NP-40, 1 mM EGTA, 5 mM $MgCl_2$). The beads were washed with lysis buffer five times, boiled in Laemmli sample buffer, and separated on 4-20% acrylamide gradient gels by SDS-PAGE. Proteins were transferred onto nitrocellulose membrane and probed with primary antibodies, α-GST (1:5000, abcam) as described above.

Flow cytometry

Cells were transfected with GFP-tagged p14, p22, and chimera. 24 hours post transfection, the cells were lifted with short treatment of 0.25% trypsin, neutralized with full media and the GFP intensity was analyzed with Attune (Thermo Fisher). GFP-expressing population was identified by comparing with non-transfected cells, and the average GFP intensity of the GFP-expressing population was calculated using FlowJo.

To quantify expression levels, cells were transfected with mCherry-tagged p22 WT and p22 mutants. 36 hours post transfection, the cells were lifted as above and the mCherry intensity was analyzed with Attune (Thermo Fisher). mCherry-expressing population was identified by

comparing with non-transfected cells, and the average mCherry intensity of the mCherry-expressing population was calculated using FlowJo.

Statistical Analysis

Two-way student's t-test was used to compare between two conditions, one-way-ANOVA with Dunnett's test when we are comparing across more than two conditions to a control. Kolmogorov-Smirnov test was used compare distributions of the number of nuclei in p22-expressing cells. Kolmogorov-Smirnov test was calculated using SciPy and other statistics were calculated using Prism 8 (Graphpad).

Fig. S1. Amino acid sequence of FAST proteins. Amino acid sequence of p14 (Reptilian orthoreovirus, Q80FJ1), p10 (Avian reovirus-176, Q77ND6), p15 (Baboon orthoreovirus, Q918V6), p10 (Nelson Bay orthoreovirus, Q9J1B2), p13 (Broome virus, D6MM29), p16 (Grass Carp reovirus, Q8JU66) and p22 (Atlantic salmon reovirus-Canada 2009, C0L0N0) with known Grb2 binding motifs labeled in cyan and predicted topology and domains notated. The number of cytoplasmic tyrosines and virus genus, species/strain is shown.

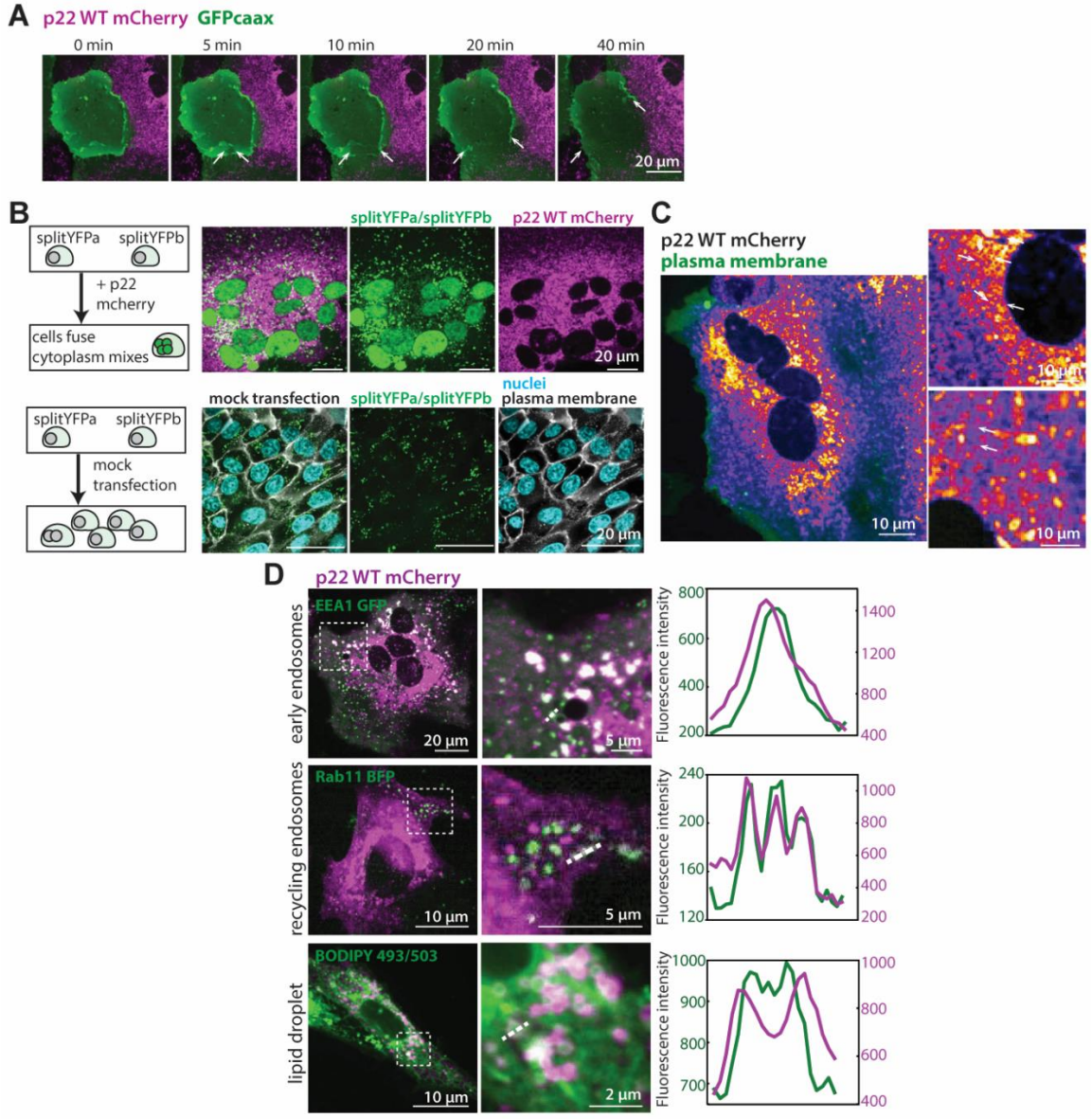

Fig. S2. Cell-cell fusion mediated by p22 and intracellular localization of p22. (A) Confocal image timeseries of p22-mCherry (magenta) expressing cell fusing with a neighboring naïve cell. Plasma membrane is visualized with GFP-caax (green) and pore expansion is denoted with white arrows. (B) Multinucleated Vero cell expressing splitYFPa and splitYFPb (green) and mCherry-tagged p22-WT (magenta). Mock transfected Vero cells expressing splitYFPa and splitYFPb (green), with nuclei stained with Hoechst 33342 (cyan) and plasma membrane stained with CellMaskDeepRed (white). (C) Figure 1D false-colored with fire lookup table and plasma membrane is labeled with CellMaskDeepRed (green). Regions are magnified and vesicles are denoted with white arrows. (D) Representative confocal images of p22-mCherry (magenta) co-expressed with EEA1-GFP (green) and Rab11-BFP (green) to label early and recycling endosomes. Lipid droplets were labeled with BODIPY 493/503 (green). Regions boxed are magnified and fluorescence intensity of line scan of dotted line is shown.

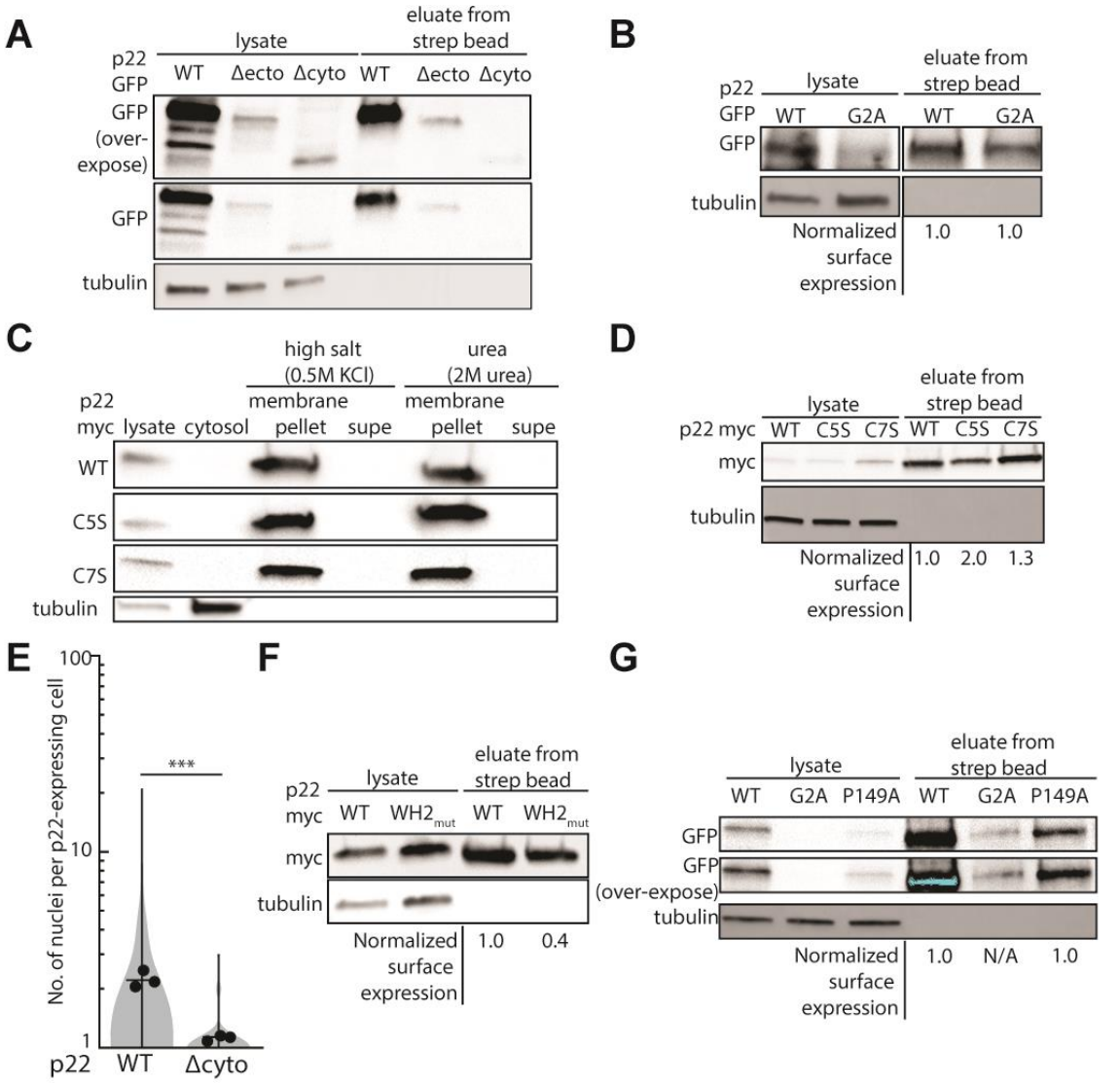

Fig. S3. Quantification of surface expression and membrane localization of wild-type and mutant p22. (A) Western blot from surface biotinylation of GFP-tagged p22-WT, p22-Δecto and p22-Δcyto expressing cells. (B) Western blot from surface biotinylation of GFP-tagged p22-WT and p22-G2A expressing cells. p22-G2A surface expression normalized to that of p22-WT. (C) Membrane fractionation and high-salt and high urea wash of myc-tagged p22-WT, p22-C5S and p22-C7S. (D) Western blot from surface biotinylation of myc tagged p22-WT, p22-C5S and p22-C7S expressing cells. p22- C5S and p22-C7S surface expression normalized to that of p22-WT. (E) Distribution of number of nuclei in p22-WT and p22-Δcyto expressing cells from three independent transfections, with mean number of each replicate, average from three independent transfections shown. *** represent $p<0.001$ using two-tailed Student's t-test. (F) Western blot from surface biotinylation of myc-tagged p22-WT, p22-$WH2_{mut}$ expressing cells. p22 $WH2_{mut}$ surface expression normalized to that of p22-WT. (G) Western blot from surface biotinylation of GFP-tagged p22-WT, p22-G2A and p22 P149A expressing cells. p22 P149A surface expression normalized to that of p22-WT. Overexposed regions of GFP blot is highlighted in cyan.

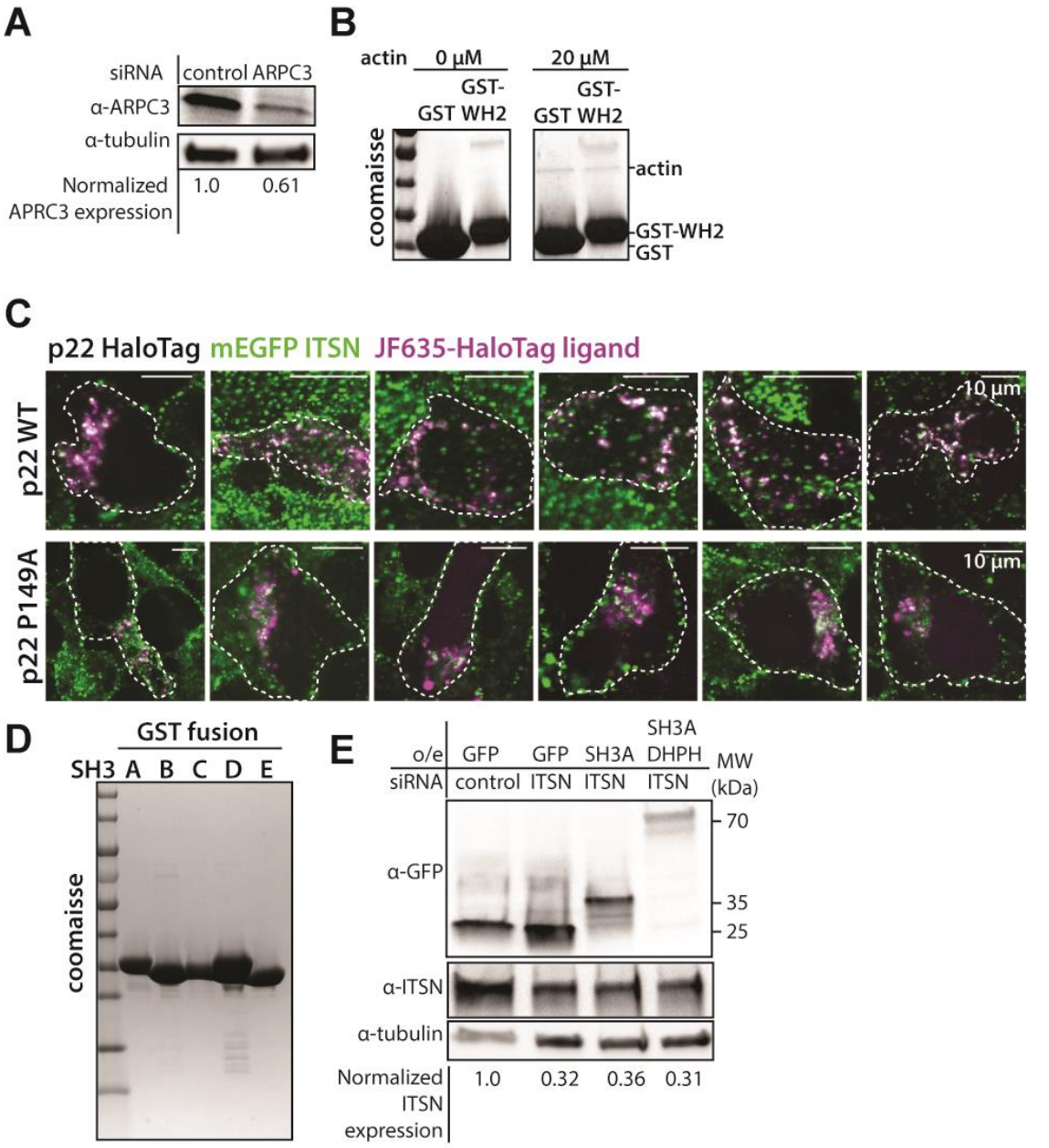

Fig. S4. Quantification of p22 mutants and cytoskeletal drug treated p22-expressing cells. (A) Western blot of ARPC3 expression in cells treated with control siRNA and siRNA targeting ARPC3. (B) Coomassie stained SDS-PAGE gel of *in vitro* binding assay of GST and GST-tagged WH2 motif with actin monomers. (C) Confocal images of endogenously mEGFP-tagged Intersectin-1 (green) cells expressing p22-WT-HaloTag (magenta) or p22-P149A-HaloTag (magenta) conjugated with JF635. The periphery of cells is outlined with a white dotted line. (D) Coomassie stained SDS-PAGE gel of purified GST-tagged SH3 domains of Intersectin-1. (E) Western blot of ITSN-1 expression in cells treated with control siRNA and siRNA targeting ITSN-1 and/or over-expressing GFP-tagged SH3A, and SH3A-DHPH.

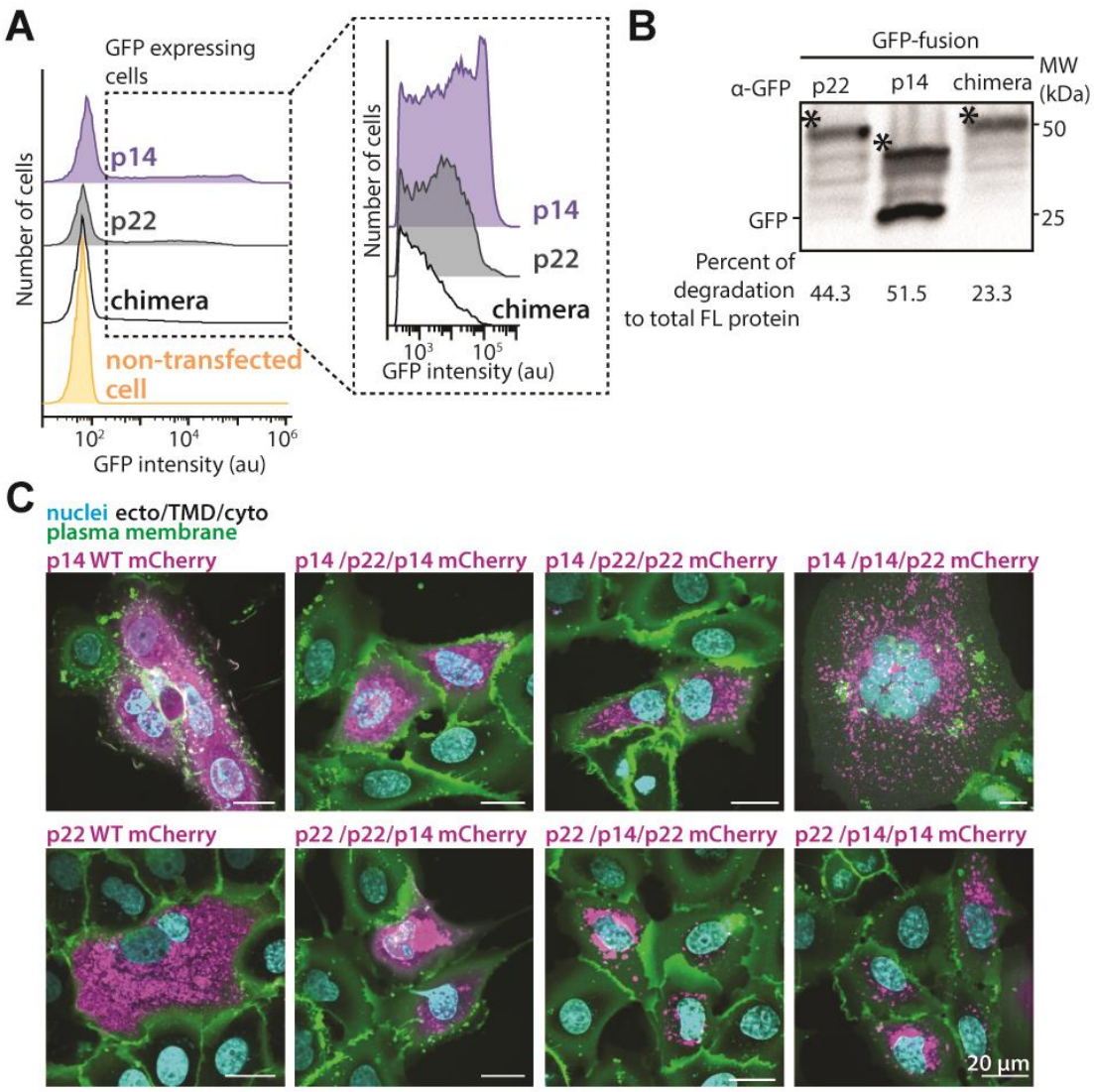

Fig. S5. p14/p22 chimeric fusogen expression and distribution of number of nuclei in chimera-expressing cells. (A) Distribution of GFP intensity of cells expressing GFP-tagged p14, p22 and p14/p22 chimera and non-transfected cells by flow cytometry. Boxed regions are magnified. (B) Western blot of GFP-tagged p22, p14 and p14/p22 chimera. Full-length p22, p14, and p14/p22 chimera is annotated with *. Ratio of degradation products to that of full-length protein is quantified. (C) Representative fluorescent confocal images of cells expressing mCherry-tagged p14, p22, and all possible permutations of p14 and p22 chimeras (magenta). Nuclei stained with Hoechst 33342 (cyan) and plasma membrane stained with CellMaskDeepRed (green).

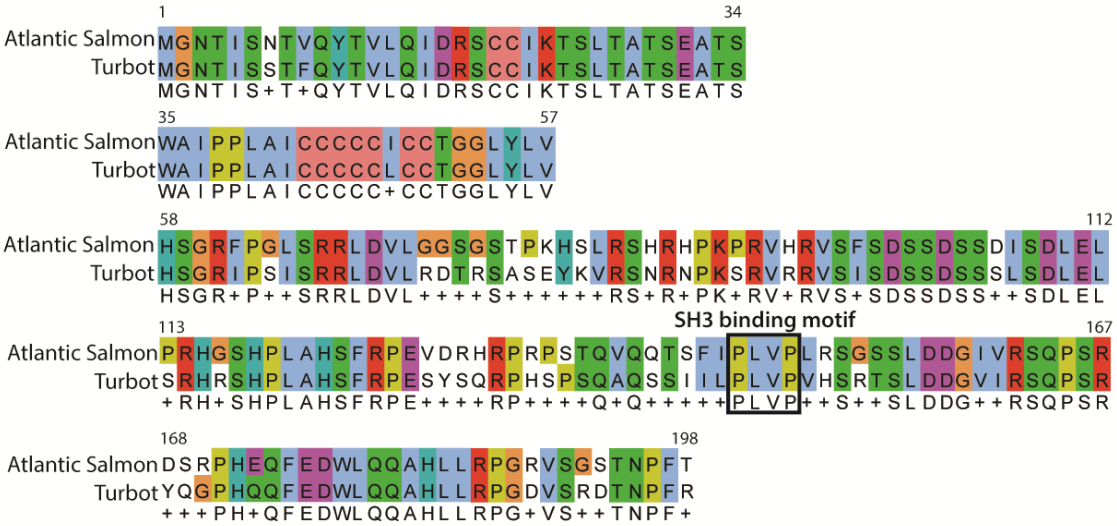

Fig. S6. Sequence alignment of FAST proteins from Turbot reovirus and Atlantic salmon reovirus using ClustalW. Sequence alignment of FAST proteins from Turbot reovirus (ADZ31982.1) and Atlantic salmon reovirus (C0L0N0) using ClustalW.

		ks-test p-value
Figure 1C	WT	1
	mCherry	5.21E-54
Figure 1F	WT	1
	delecto	8.92E-06
Figure 1G	WT	1
	G2A	2.97E-44
Figure 1J	WT	1
	C5S	3.55E-14
	C7S	1.14E-16
Figure 2A	DMSO	1
	LatA 50nM	3.90E-04
	LatA 100nM	9.60E-03
	LatA 250nM	1.79E-04
	CK666 50uM	3.21E-05
	CK666 100uM	1.20E-04
	CK666 200uM	5.00E-05
	smifH2 50uM	0.09
Figure 2B	cont	1
	ARPC3 siRNA	0.06
Figure 2D	WT	1
	WH2mut	1.00E-04
	P149A	6.33E-25

		ks-test p-value
Figure 3C	cont	1
	ITSN siRNA	1.38E-04
	ITSN siRNA SH3A	1.59E-11
	ITSN siRNA SH3ADHPH	0.64
Figure 3D	DMSO	1
	ZCL278 1uM	0.015
	ZCL278 10uM	0.002
	ZCL278 100uM	0.00179
	ML141 0.2uM	0.0029
	ML141 2uM	0.0029
	ML141 200uM	2.03E-07
	Wiskostatin 5uM	5.44E-09
	Wiskostatin 7.5uM	1.02E-07
	Wiskostatin 10uM	2.50E-04
Figure 3E	N-WASP +/+	1
	N-WASP -/-	0.02
Figure 4D	p22	1
	p14	0.99
	chimera	3.07E-15
Figure 5D	0nM rapalog	1
	500nM rapalog	5.36E-07
Supp. Figure 3E	WT	1
	delcyto	2.17E-07

Fig. S7. p-values from ks-test on all distribution of number of nuclei compared in previous figures.

Movie S1 (separate file). Confocal timelapse of a Vero cell expressing p22-mCherry (magenta) and GFPcaax (green) fusing with a Vero cell expressing only GFPcaax (green). Scale bar is 20 μm.

Movie S2 (separate file). Confocal timelapse of a Vero cell expressing p22-mCherry (magenta) and GFPcaax (green) fusing with a Vero cell expressing only GFPcaax (green). Scale bar is 20 μm.

Movie S3 (separate file). Confocal timelapse of a Vero cell expressing p22-mCherry (magenta) and GFPcaax (green) fusing with a Vero cell expressing only GFPcaax (green). Scale bar is 20 μm.

Movie S4 (separate file). Confocal timelapse of a Vero cell expressing chimera-P149A-mCherry-FKBP (magenta), FRB- ΔGBD-mDia2 and Lifeact-GFP (green) upon addition of 500 nM rapalog. Scale bar is 10 μm.

Movie S5 (separate file). Confocal timelapse of a magnified region of Video 2. Vero cell expressing chimera-P149A-mCherry-FKBP (magenta), FRB- ΔGBD-mDia2 and Lifeact-GFP (green) upon addition of 500 nM rapalog. Scale bar is 10 μm.

Dataset S1. Aligned sequencing results of genome-edited human induced pluripotent stem cells (hiPSCs) with eGFP-tagged Intersectin-1.

SI References

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