Figures and figure supplements

The scaffolding protein flot2 promotes cytoneme-based transport of wnt3 in gastric cancer

Daniel Routledge *et al*

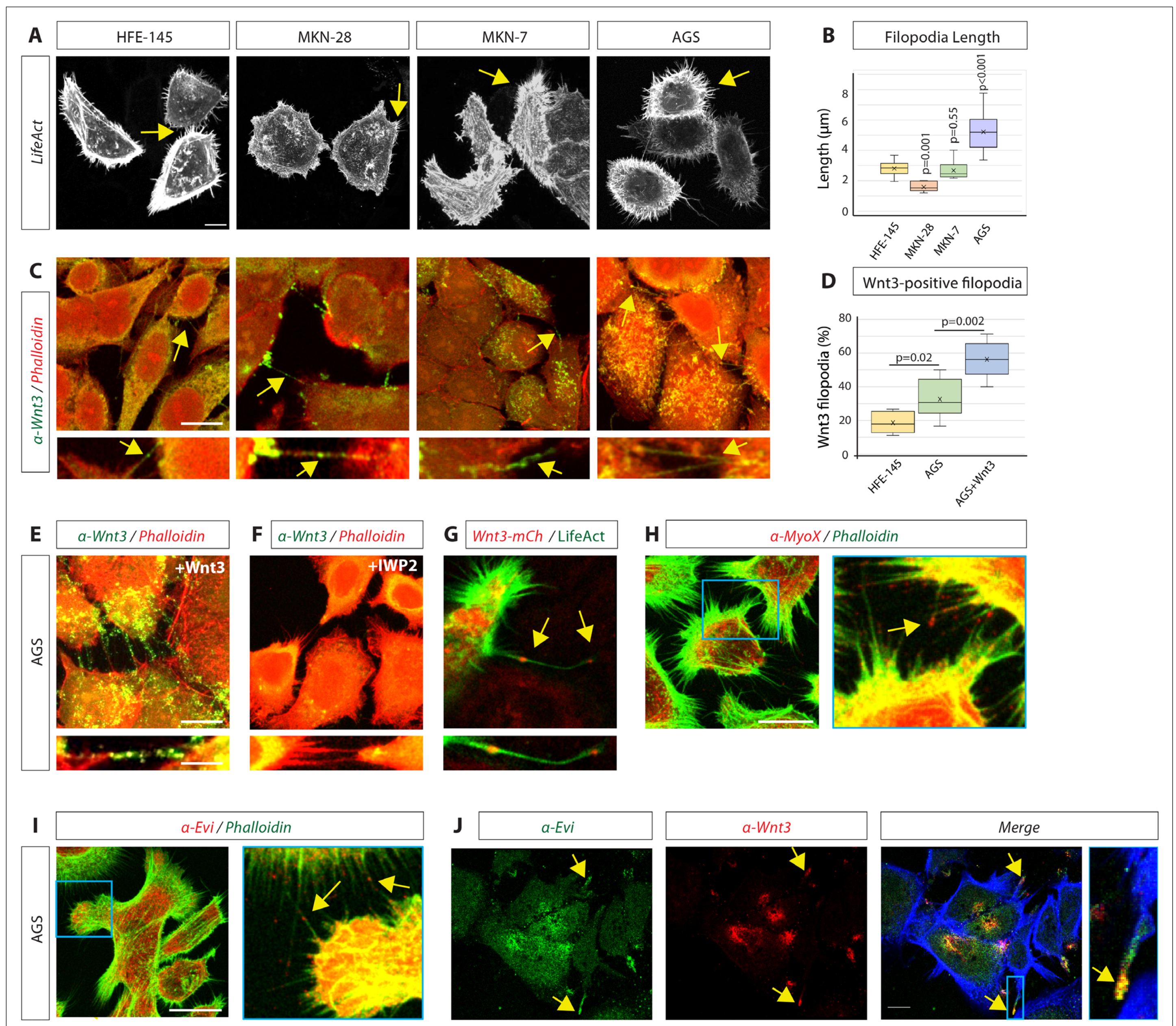

Figure 1. Gastric epithelial normal and cancer cell lines utilise cytonemes to transport Wnt3 intercellularly. (**A**) Confocal images of normal gastric epithelial cell line (HFE-145) and gastric cancer (GC) cell lines (MKN-28, MKN-7, and AGS) expressing LifeAct-GFP to visualise actin-based structures. Yellow arrows indicate examples of filopodia. (**B**) Quantification of filopodia length in GC cell lines MKN-28, MKN-7, and AGS (n=7, 8, 25; n=number of cells). Significance is calculated by Student's t-test. (**C**) Immunofluorescent images of HFE, MKN-28, MKN-7, and AGS, stained with antibodies against Wnt3 (green) and actin (Phalloidin-iFluor594, red). Scale bar 10 μm. High-magnification images indicate an example of a Wnt3-bearing cytonemes. Scale bar 2.5 μm. (**D**) Quantification of Wnt3-positive filopodia in gastric epithelial (HFE-145) and cancer (AGS) cells as a percentage of total filopodia (number of cells analysed = 6, 8, 6). Significance is calculated by Student's t-test. (**E**) Immunohistochemistry (IHC) images of AGS cells overexpressing Wnt3 and stained with an antibody against Wnt3 (green) and actin (iFluor594, red). Scale bar 10μm. High-magnification images highlight cytonemes. Scale bar 2.5 μm. (**F**) IHC images of AGS cells treated with the Porcupine inhibitor IWP2 (100 μM, 48 hr) and stained with an antibody against Wnt3 (green) and actin (iFluor594, red). (**G**) Live confocal cell imaging of AGS cells expressing Wnt3-mCherry and LifeAct-GFP. Cytoneme-localised Wnt3-mCherry highlighted by yellow arrows. (**H–J**) IHC images of AGS cells stained with antibodies against (**H**) Myosin-X (MyoX) and (**I**) Evi/Wntless (red) and Wnt3 (red) and (**J**) Evi/Wntless (green Scale bars 10 μm). Phalloidin labels actin (FITC-Phalloidin, green; Phalloidin-iFluor350, blue).

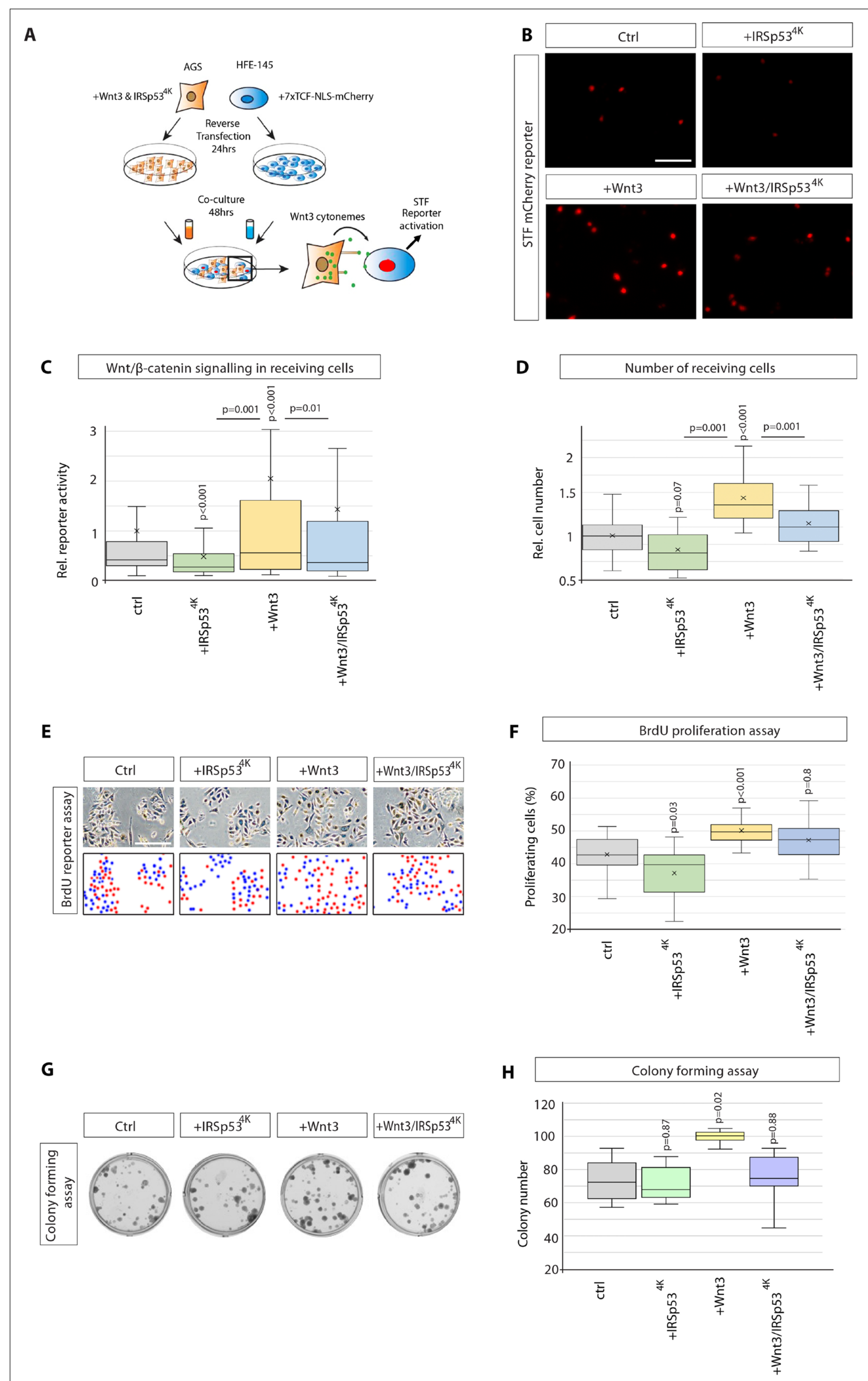

Figure 2. Wnt3 cytonemes regulate paracrine Wnt/β-catenin signalling and proliferation. (**A**) Experimental protocol for measuring paracrine Wnt signalling activation. HFE cells expressing the SuperTOPFlash (STF) reporter, 7×TCF-NLS-mCherry, were cocultivated with AGS cells expressing indicated constructs. Fluorescence of STF mCherry reporter was measured after 48 hr and compared to untransfected control cells. (**B**) Representative

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Figure 2 continued

images of STF reporter fluorescence for indicated conditions. Scale bar 100 µm. (**C**) Quantification of STF mCherry reporter fluorescence in HFE cells co-cultured with AGS (n per condition = 322, 394, 258, and 275). (**D**) Relative number of HFE cells per image after co-culture with AGS cells expressing indicated constructs. Significance calculated by Student's t-test with Bonferroni correction for multiple comparisons. (n per condition = 28, 26, 27, and 17; n=number of images). (**E**) Representative images of proliferating, BrdU-stained (red); co-cultured AGS and HFE-145 cells, as described in (**a**). Cells were counterstained with haematoxylin (blue dots). Scale bar 100 µm. Complementary images show $BrdU^+$ cells with red dots; blue dots mark $BrdU^-$ cells. (**F**) Quantification of BrdU-stained cells as a percentage of the population. Significance calculated by Student's t-test with Bonferroni correction for multiple comparisons. (n per condition = 20, 20, 20, and 20; n=number of images). (**G**), Colony-forming assay of AGS cells. AGS cells were transfected with the indicated constructs and co-cultured with AGS-RFP cells for 2 days. After sorting, AGS-RFP expressing cells were plated at clonal density for 10–12 days; (**H**), Quantification of spherical colonies. Significance is calculated by Student's t-test (n=9).

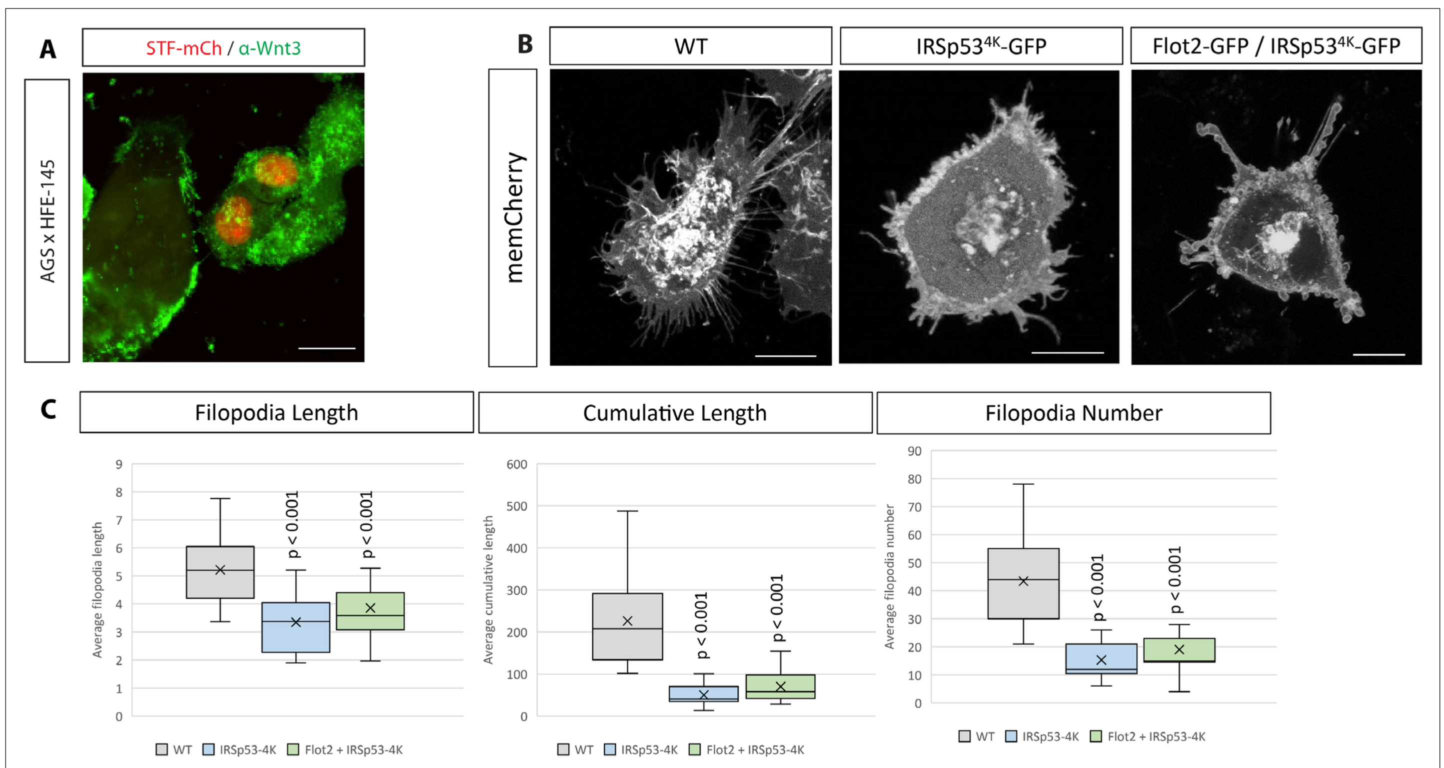

Figure 2—figure supplement 1. Wnt3 cytonemes depend on IRSp53 function. (**A**) Antibody staining against Wnt3 (green) after co-cultivation of SuperTOPFlash (STF)-mCh-expressing HFE-145 cells with AGS cells, showing Wnt3 staining in receiving HFE-145 cells. Scale bar 20 µm. (**B**) Confocal images of AGS cells expressing the dominant-negative IRSp53^{4K}-GFP mutant and memCherry, showing inhibition of filopodia formation (even in the presence of Flot2-GFP). Scale bar 10 µm. (**C**) Quantification of filopodia of cells from (**a**). (n per condition = 25, 13, 13; n=number of cells). Significance is calculated by Student's t-test.

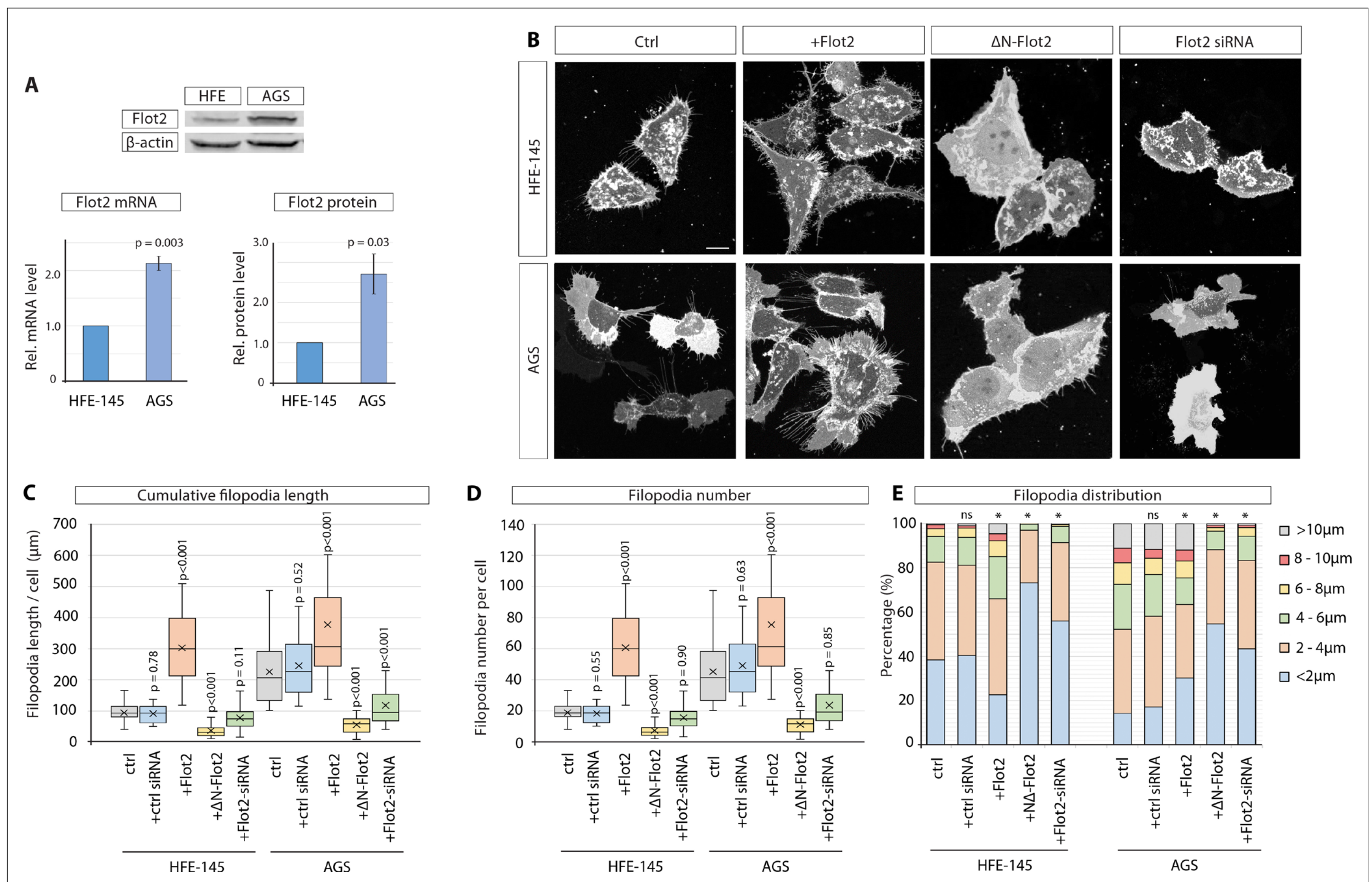

Figure 3. Flotillin-2 is over-expressed and promotes filopodia formation and elongation in gastric cancer cells. (**A**) Flot2 protein levels in HFE-145 and AGS cells as quantified by Western blot after normalising to beta-actin levels (n=3) and by RT-qPCR after normalising to Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) as a housekeeping gene (n=4). Relative protein and mRNA levels are compared to HFE-145. Error bars represent SEM. Significance is calculated by Student's t-test. (**B**) Representative images of HFE and AGS cells expressing membrane-mCherry and indicated Flotillin-2 (Flot2) constructs or siRNA after 48 hr. Scale bars 10 µm. (**C–D**) Filopodia quantifications of HFE and AGS cells transfected with indicated Flot2 plasmids or siRNA. Significance calculated by Student's t-test with Bonferroni correction for multiple comparisons. Average cumulative filopodia length (**C**), average filopodia number per cell (**D**). (n per condition [HFE]=22, 19, 25, 23, 24). (n per condition [AGS]=25, 21, 25, 25, 25; n=number of cells measured). (**E**) Distribution of filopodia, categorised by length as a percentage of total filopodia per HFE or AGS cell 48 hr post-transfection with indicated Flot2 plasmids or siRNA. A Pearson's $\chi 2$ test was performed to test for significance between control (ctrl) group (expected) and experimental groups (observed) with 5 degrees of freedom (df) and a p-value <0.05. The specific $\chi 2$ values are as follows, HFE: ctrl siRNA 0.86, Flot2 0.001, dnFlot2 <0.001, Flot2 siRNA <0.001, and for AGS: ctrl siRNA 0.65, Flot2 0.007, dnFlot2 <0.001, and Flot2 siRNA <0.001. Asterisks mark significant differences.

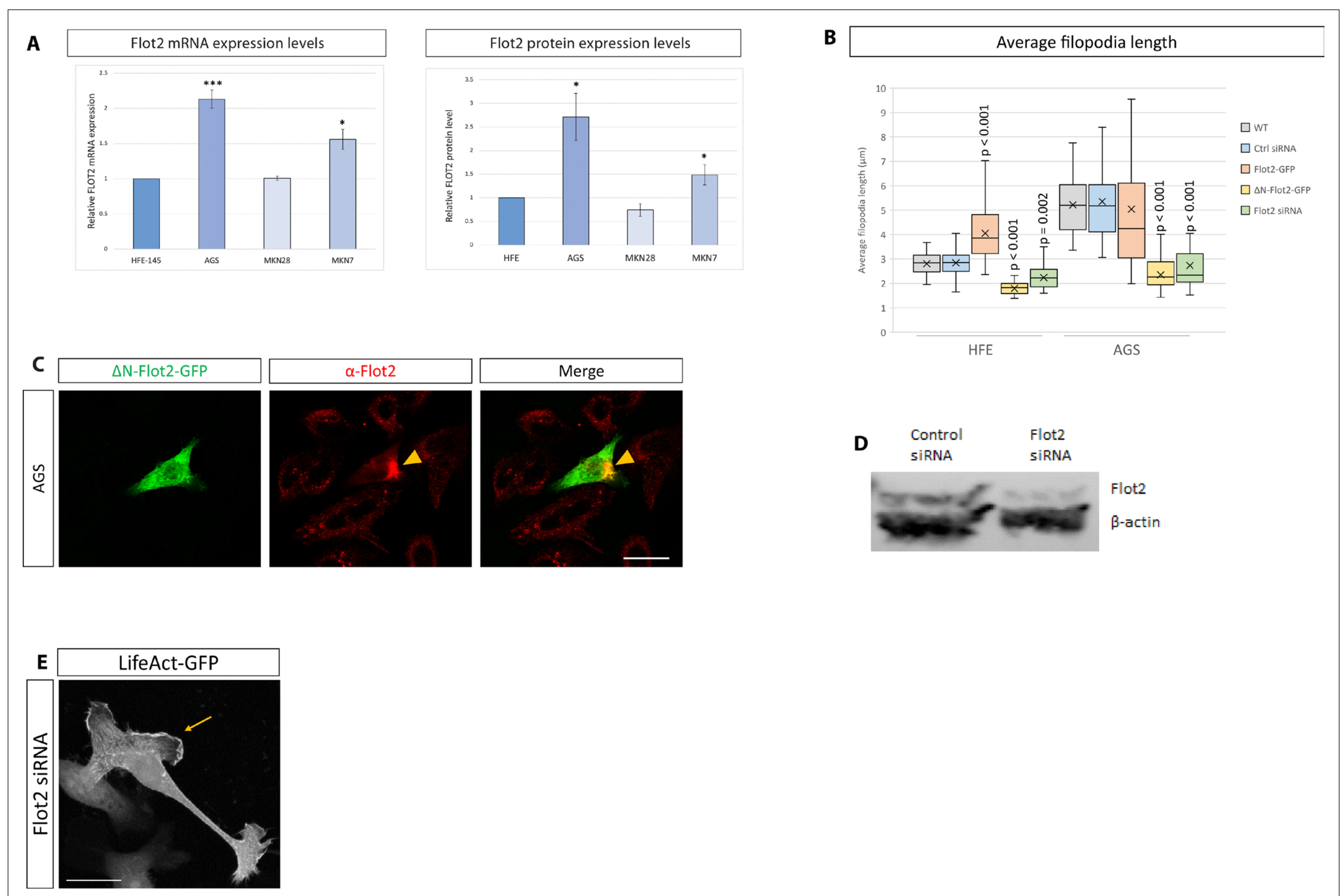

Figure 3—figure supplement 1. Effects of Flot2 on filopodia and Wnt3. (**A**) Quantification of Flot2 expression levels (mRNA and protein) in HFE-145, AGS, MKN-28, and MKN-7 cells. Significance is calculated by Student's t-test. (**B**) Quantification of filopodia length in HFE and AGS cells expressing indicated constructs. Significance is calculated by Student's t-test with Bonferroni correction for multiple comparisons. (**C**) Antibody staining of AGS cells expressing ΔN-Flot2-GFP and stained for endogenous WT Flot2 (red), showing the effect of the dominant-negative mutant on Flot2 localisation. Scale bar 20 µm. (**D**) Analysis of efficiency of siRNA-mediated knock-down of Flot2 in AGS cells by Western blot. (**E**) Confocal image of an AGS cell with depleted Flot2 (siRNA-mediated) and expressing LifeAct-GFP to visualise actin. Yellow arrow highlights an example of lamellipodia frequently seen in Flot2-depleted cells. Scale bars 10 µm.

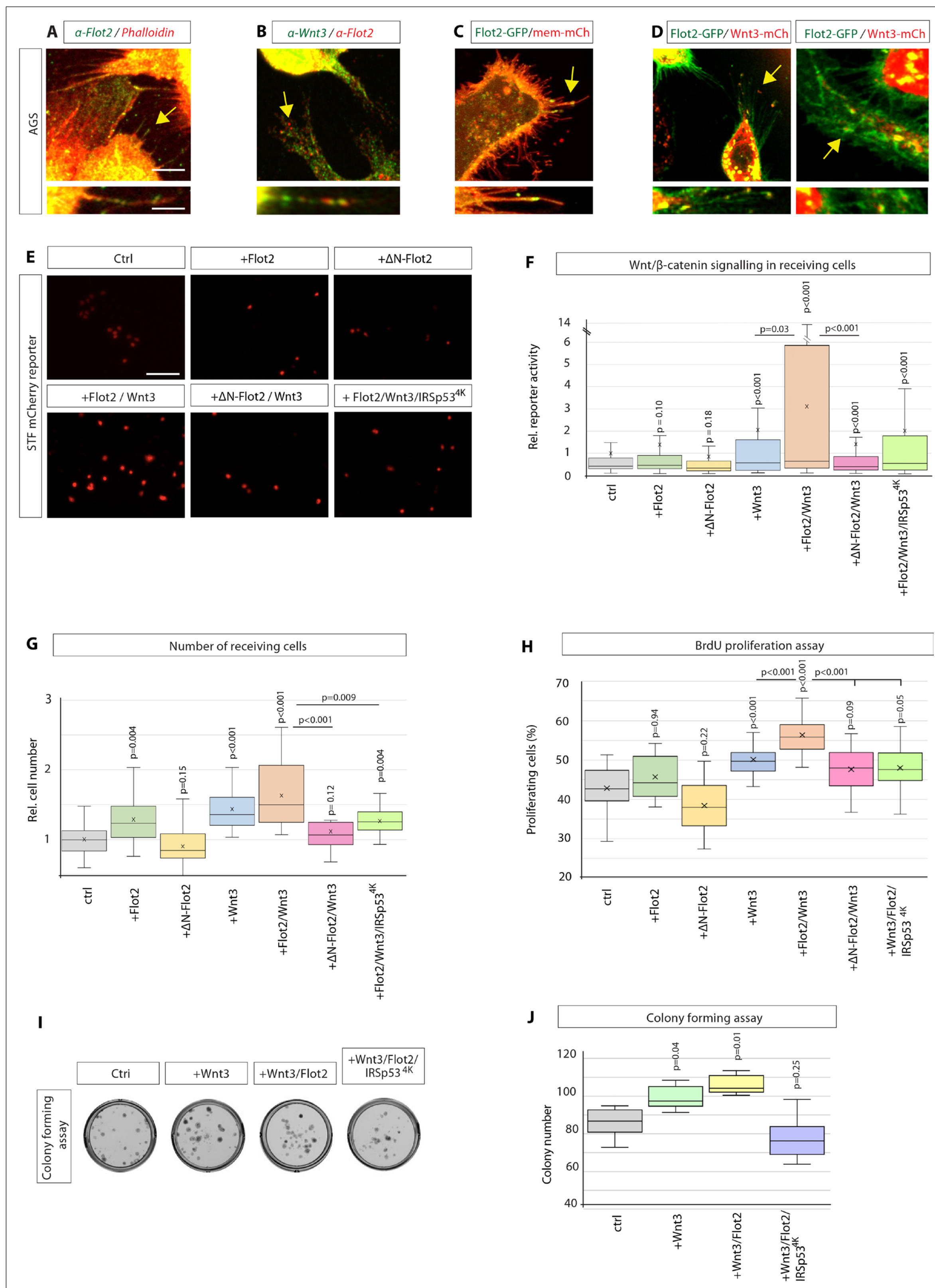

Figure 4. Flotillin-2 marks Wnt3 cytonemes and influences paracrine Wnt/β-catenin signalling and proliferation. (**A**) Immunohistochemistry (IHC) analysis showing endogenous localisation of Flot2 (green) in AGS cells. TRITC phalloidin was used to visualise actin. Arrows indicate the localisation of Flot2 to filopodia. Scale bars 5 μm. High-magnification images indicate an example of a Flot2-bearing cytonemes. Scale bars 2.5 μm. (**B**), IHC analysis shows that Flot2 co-localises with Wnt3 on cytonemes. (**C**) Confocal images showing the subcellular localisation of Flot2-GFP in AGS cells. Arrows indicate

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Figure 4 continued

the localisation of Flot2-GFP on cytonemes. (**D**) Confocal images highlighting co-localisation of Flot2-GFP and Wnt3-mCh on cytonemes in AGS cells (arrows). Flot2-GFP and Wnt3-mCherry also cluster and co-localising at a cytoneme contact point (arrow). (**E**) Representative images of SuperTOPFlash (STF) reporter fluorescence for indicated conditions. Scale bar 100 uM. (**F**) Relative quantification of STF mCherry reporter fluorescence in HFE cells co-cultivated with AGS cells expressing indicated constructs. Quantifications are relative to AGS control. (n per condition = 322, 443, 403, 258, 336, 306, and 297; n=number of nuclei measured). (**G**) Relative number of HFE cells per image after co-cultivation with AGS cells expressing indicated constructs. Significance calculated by Student's t-test with Bonferroni correction for multiple comparisons. (n per condition = 28, 27, 26, 27, 22, 24, and 15; n=number of images). (**H**) Quantification of BrdU-stained cells as a percentage of the population, after co-cultivation of AGS and HFE cells, as described in ***Figure 2a***. significance calculated by Student's t-test with Bonferroni correction for multiple comparisons. (n per condition = 20; n=number of images). (**I**), Colony-forming assay of AGS cells. AGS cells were transfected with the indicated constructs and co-cultivated with AGS-RFP cells for 2 days. After sorting, AGS-RFP expressing cells were plated at clonal density; (**J**), Quantification of colonies after 10–12 days. Significance is calculated by Student's t-test (n=9).

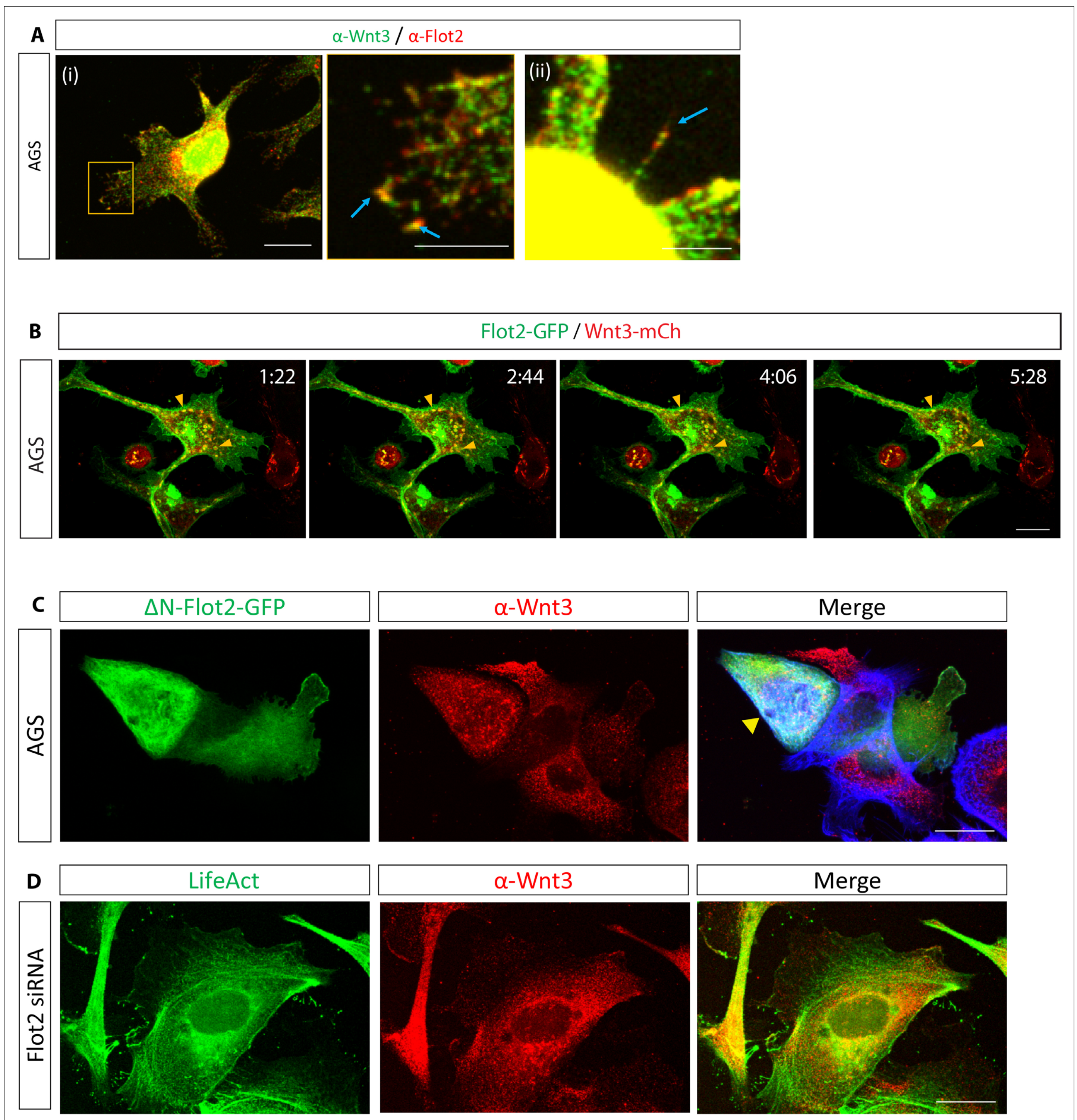

Figure 4—figure supplement 1. Co-localisation study of Flot2 and Wnt3. (**A**) Immunofluorescent images of AGS cells stained for Wnt3 (green) and Flot2 (red). Co-localisation of Flot2 and Wnt3 highlighted by blue arrows. Scale bars represent (**i**) 10 µm (left) and 5 µm (right), (ii) 5 µm. (**B**) Confocal time-lapse images of AGS cells expressing Flot2-GFP and Wnt3-mCh. Yellow arrows highlight the co-localisation of Flot2 and Wnt3 intracellularly. Scale bar 10 µm. (**C**) AGS cells have been transfected with indicated constructs to compare Wnt3 localisation in AGS depleted for Flot2 function.

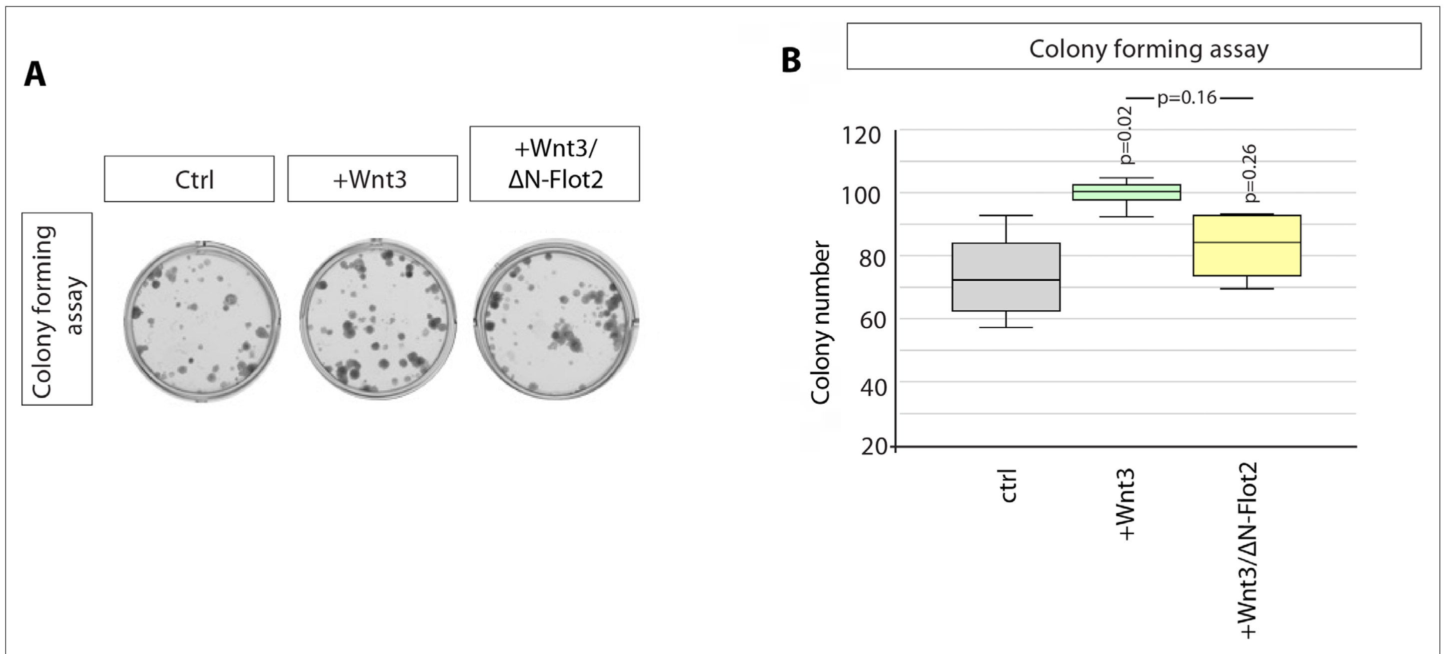

Figure 4—figure supplement 2. Colony formation assay. (**A**) Quantification of spherical colonies after 8–10 days. Results for AGS-RFP cells co-cultured with Ctrl and Wnt3 expressing AGS cells are the same displayed in ***Figure 2G and (B***), Significance calculated by Student's t-test (n=9).

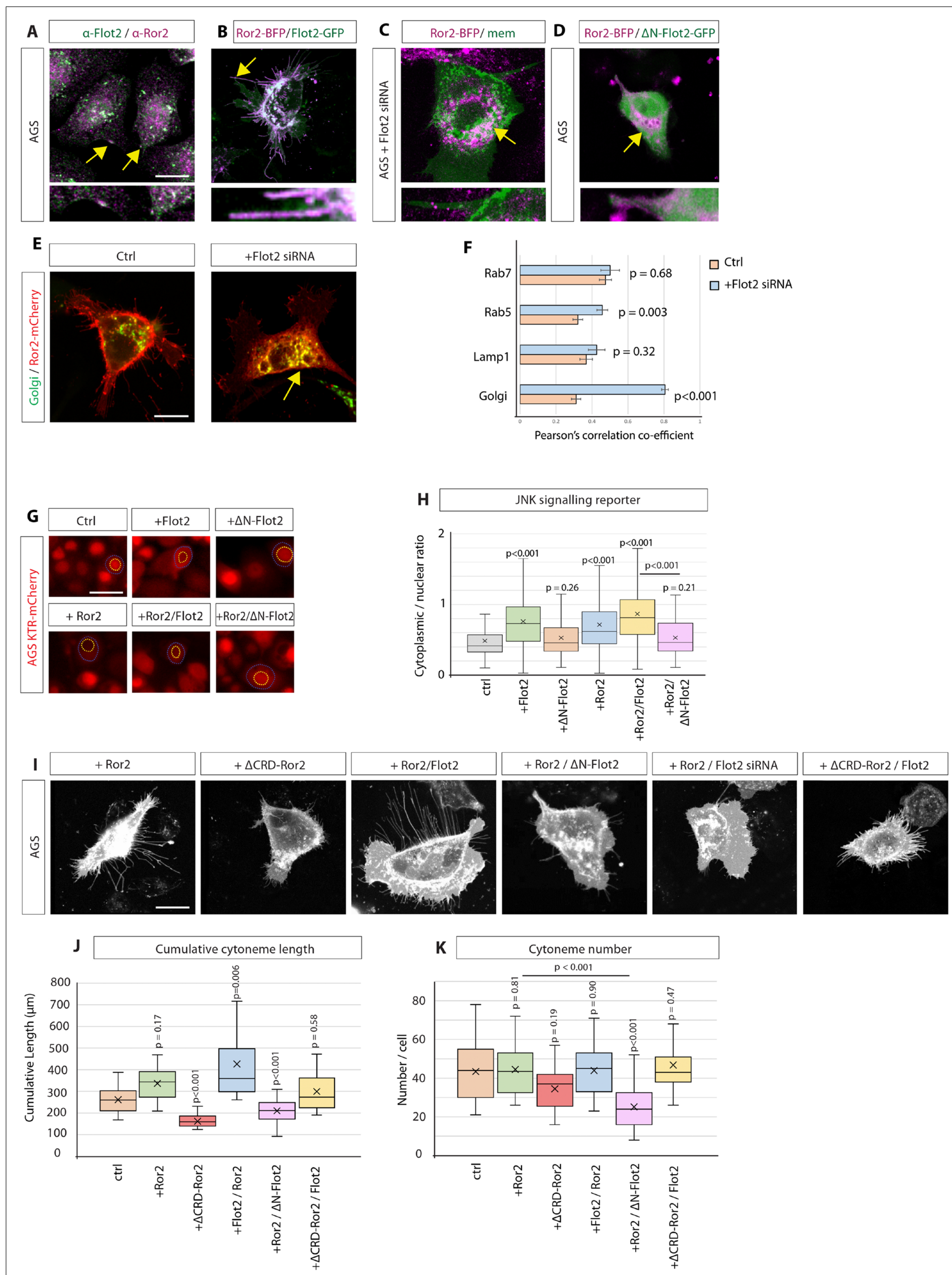

Figure 5. Flotillin-2 is required for Ror2 membrane localisation, Ror2/PCP signalling and Ror2-mediated cytoneme formation. (**A**) Immunohistochemistry (IHC) analysis of AGS cells stained for Ror2 (red) and Flot2 (green). Flot2 and Ror2 show co-localisation with a Pearson's correlation coefficient (PCC) of 0.65 (n=10), highlighted at the membrane by arrows. Scale bars represent 10 µm, and in high-magnification images, 2.5 µm (right). (**B–D**), Confocal live-cell imaging of AGS cells expressing Ror2-BFP with Flot2-GFP (**B**), Flot2 siRNA (**c**) or ΔN-Flot2-GFP (**D**) and memCherry. Arrows highlight subcellular

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regions of co-localisations. (**E**) Live confocal images of AGS cells expressing Ror2-mCherry and indicated organelle markers +/- Flot2 siRNA. Arrows highlight the co-localisation of Ror2-mCherry and mTurq2-Golgi. Scale bar 10 µm F, Quantification of co-localisation of Ror2-mCherry with indicated markers, assessed by PCC. Significance is calculated by Student’s t-test. (n per condition [WT]=7, 10, 8, and 10) (n per condition [Flot2 siRNA]=7, 6, 7, 8). (**G**) Representative images of AGS cells stably expressing the JNK-KTR-mCherry reporter and indicated constructs after 48 hr. Blue dotted line encircles the cytoplasm and yellow dotted line the nucleus of a representative cell. Scale bar 20 µm. (**H**) Quantification of the JNK-KTR-mCherry reporter. Nuclear and cytoplasmic fluorescence of cells were measured, and the cytoplasmic: nuclear ratio was calculated. Significance is calculated by one-way ANOVA with Bonferroni correction for multiple comparisons. (n per condition = 136, 109, 74, 109, 82, and 79). (**I**) Representative confocal images of AGS cells expressing memCherry and indicated constructs for 48 hr. Scale bars 10 µm. (**J, K**) Quantification of cytoneme length and number of AGS cells transfected with constructs indicated in (**i**). Significance calculated by Student’s t-test with Bonferroni correction for multiple comparisons. (n per condition = 25, 22, 21, 23, 25, and 21; n=number of cells).

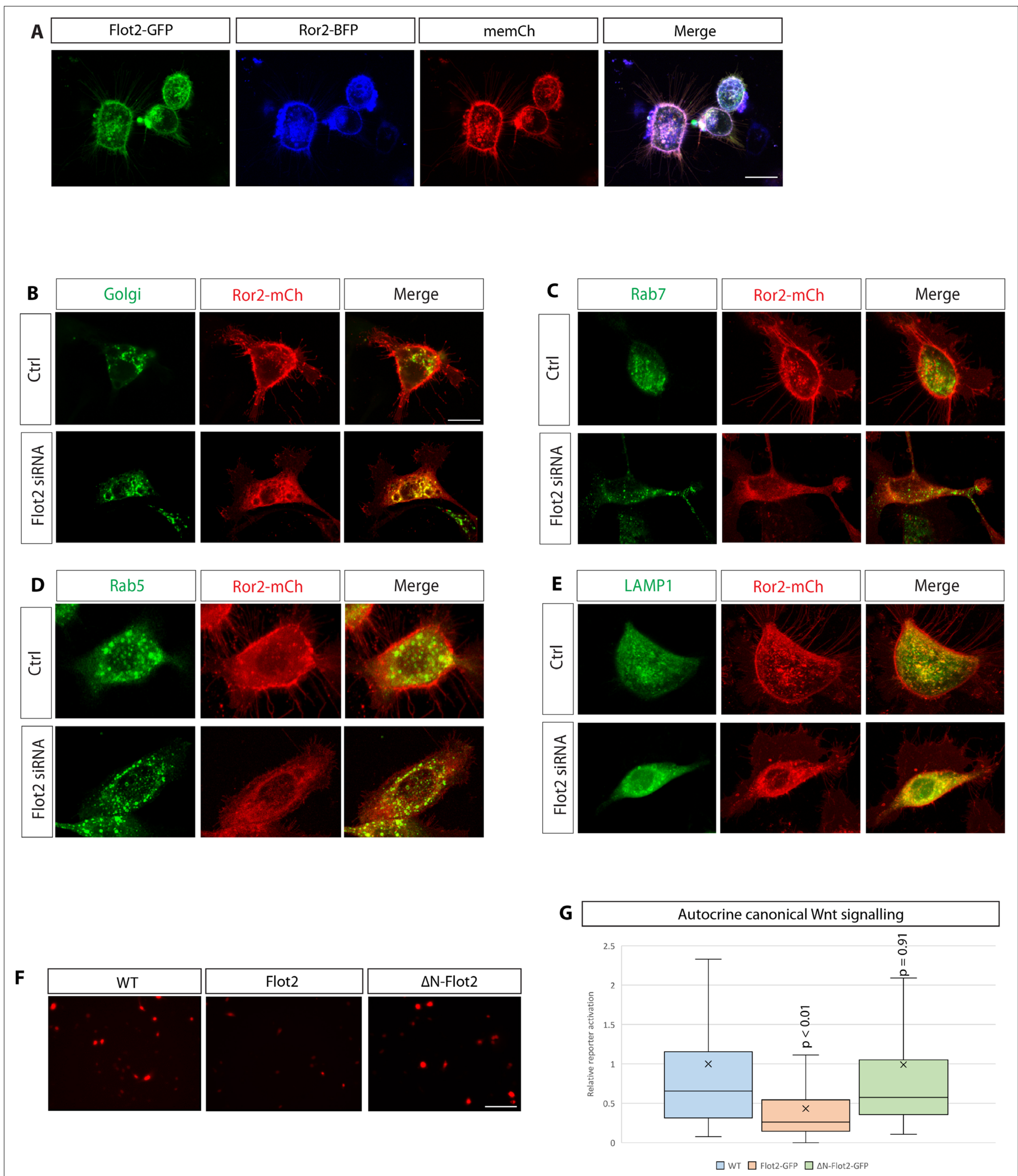

Figure 5—figure supplement 1. Flotillin-2 and Ror2 – localisation and signalling. (**A**) Confocal images of AGS cells expressing Flot2-GFP, Ror2-BFP and membrane-mCherry to highlight membrane localisation of Flot2 and Ror2. Scale bar 20 µm. (**B – E**) Confocal images of AGS cells expressing Ror2-mCherry and indicated cell organelle markers. Scale bars represent 20 µm. (**F**) Representative images of AGS cells transfected with the 7×TCF-NLS-mCherry reporter and indicated constructs for 48 hr. Scale bar 20 µm G, Relative quantification of 7×TCF-NLS-mCherry fluorescence compared to untransfected control. Significance is calculated by Student's t-test. (n per condition = 406, 212, and 469; n=number of nuclei measured).

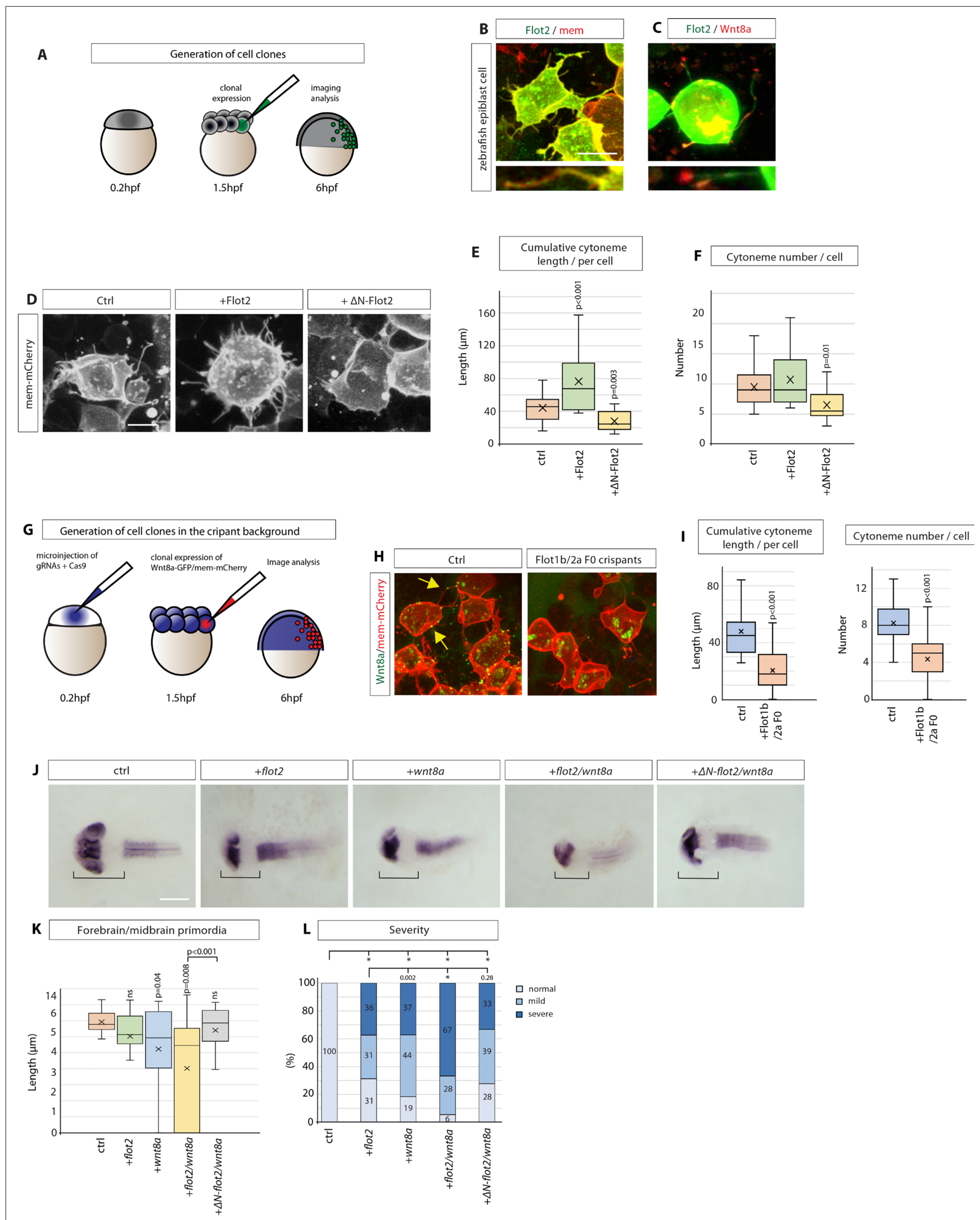

Figure 6. Flotillin-2 promotes cytoneme formation and Wnt8a signalling in zebrafish development. (**A**) Experimental setting to generate small clones expressing indicated constructs in the zebrafish embryo. (**B, C**) Confocal images of zebrafish epiblast cells injected with Flot2-GFP / memCherry and Flot2-GFP / Wnt8a-mCherry and imaged at 8 hpf. Scale bars represent 10 µm (**D**) Representative images of zebrafish epiblast cells injected with memCherry indicated constructs. Scale bar 10 µm. (**E**), (**F**), Quantification of filopodia from epiblast cells injected. Significance is calculated by

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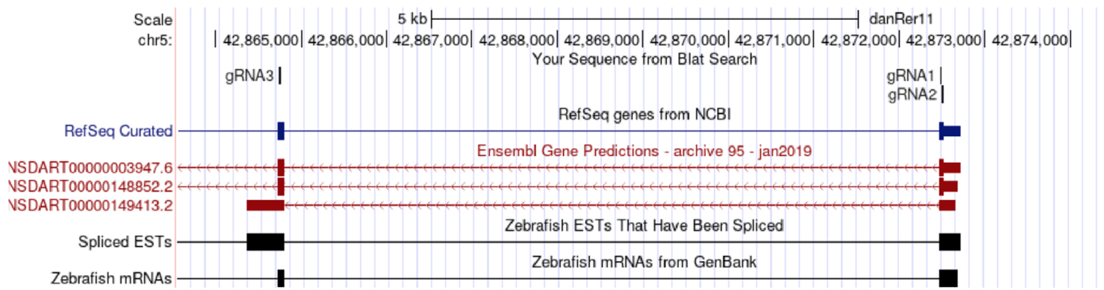
Student's t-test. (n per condition = 17, 20, and 14). (**G**), Experimental strategy to generate Wnt8a-GFP/mCherry cell clones in F0 Flot1b/2 a Crispants background. (**H**), Confocal images of zebrafish epiblast cells expressing indicated constructs. (**I**) Quantification of cytoneme length and number in injected epiblast cells. Significance is calculated by Student's t-test. (n per condition = 18, 15) (**J**) In situ hybridisation against *pax6a* in zebrafish embryos at 30hpf after microinjection of 100 ng/µl of indicated DNA constructs and imaged. Scale bar represents 100 µm. (**K**) Quantification of forebrain and midbrain primordia length in zebrafish embryos injected as in (**A**). Significance is calculated by Student's t-test. (n per condition = 23, 16, 27, 18, and 18; n=number of embryos). (**L**) Qualitative analysis of phenotype severity in zebrafish embryos injected as indicated in (**A**). Phenotypes are classified into the categories normal, mild and severe. Numbers in bars represent percentages of total embryos. A Pearson's $\chi 2$ test revealed a significant difference between the ctrl group (expected) and experimental groups (observed) with 2 degrees of freedom (df) and a p-value < 0.05 of $\chi 2$ <0.001. Distribution comparison of *flot2* injected embryos revealed a significant difference to the *wnt8a* group ($\chi 2$=0.002) and the *flot2 + wnt8* a group ($\chi 2$<0.001), but not to *wnt8a+ΔN-flot2* ($\chi 2$=0.28). Asterisks mark significant differences.

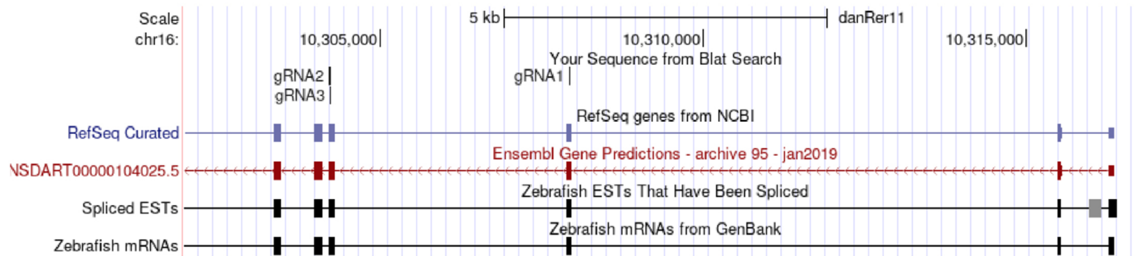
A

gRNA design for Flot2a and Flot1b

flot2a



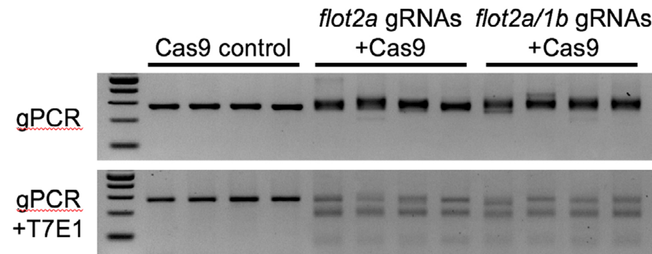
flot1b



B

Heteroduplex assay

flot2a (exon 1)



flot1b (exon 4)

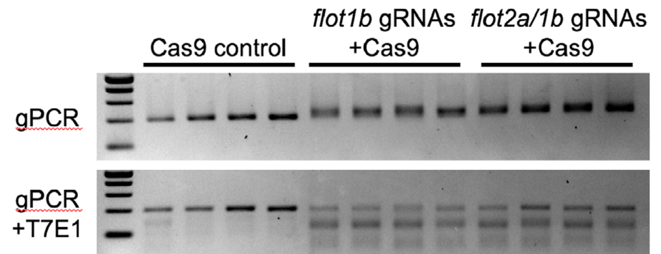


Figure 6—figure supplement 1. Site-specific mutagenesis of zebrafish *flot2a* and *flot1b* genes. (**A**) Location of guide RNA (gRNA) target sites. Three individual gRNAs were designed to target coding exons of *flot2a* and *flot1b* genes (annotated with blue and red boxes). Two gRNAs and one gRNA target exon 1 and exon 2 of *flot2a*, respectively. For *flot1b*, one gRNA and two gRNAs were designed to target exon 3 and exon 4, respectively. (**B**) Site-specific mutagenesis was detected by T7 endonuclease I (T7E1) assay. Genomic sequences including gRNA target sites in *flot2a* exon 1 or *flot1b* exon 4 were amplified by PCR from genomic DNA extracted from single embryo injected with Cas9 (control) or gRNAs/Cas9 ribonucleoprotein complex (n=4 per condition). Heteroduplex DNA in the PCR products was cleaved by T7E1.