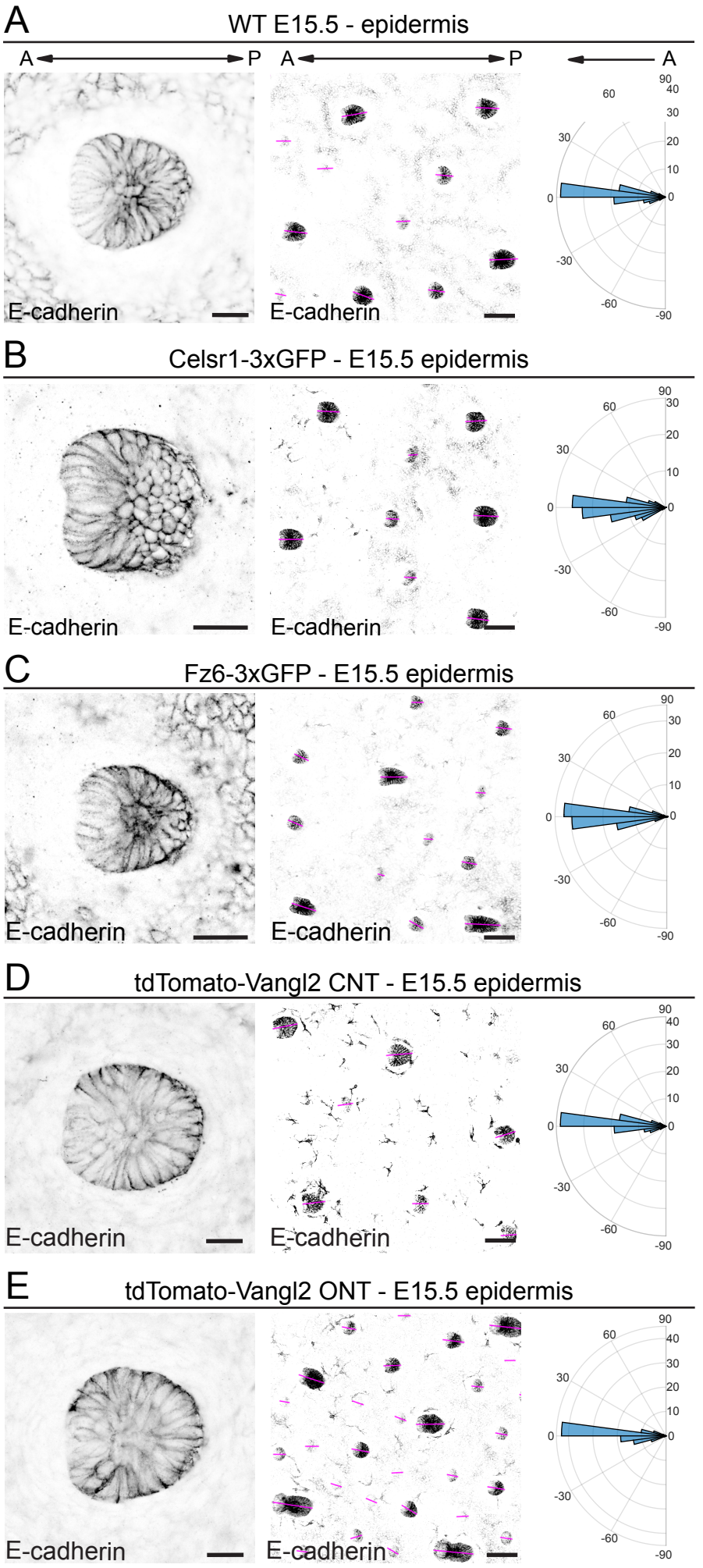


Fig. S1. Analysis of hair follicle orientation in Celsr1-3xGFP, Fz6-3xGFP, and tdTomato-Vangl2 homozygous embryos. (**A-E)** Hair follicle (HF) orientations at E15.5. Planar views of single follicles and larger areas of flat mount epidermis labeled with E-Cadherin are shown. Magenta lines overlaid developing HFs denote their orientation. Scale bar, 100 μm. Quantification of hair follicle alignment is shown in circular histograms. Anterior is to the left (at 0º). Vectors point toward the anterior, growing end of HFs. **(A)** wild type, n = 987 from 4 embryos. **(B)** Celsr1-3xGFP, n = 184 from 3 embryos. **(C)** Fz6-3xGFP homozygous, n = 361 from 3 embryos. **(D)** tdTomato-Vangl2 homozygotes with a curly tail and closed neural tube phenotype (CNT), n = 434 from 3 embryos. **(E)** tdTomato-Vangl2 homozygotes with an open neural tube (ONT) phenotype, n = 601 from 3 embryos. Note that in tdTomato-Vangl2 embryos of both phenotypes, hair follicles point anteriorly and align along the A-P axis similar to their orientations in wild type epidermis.

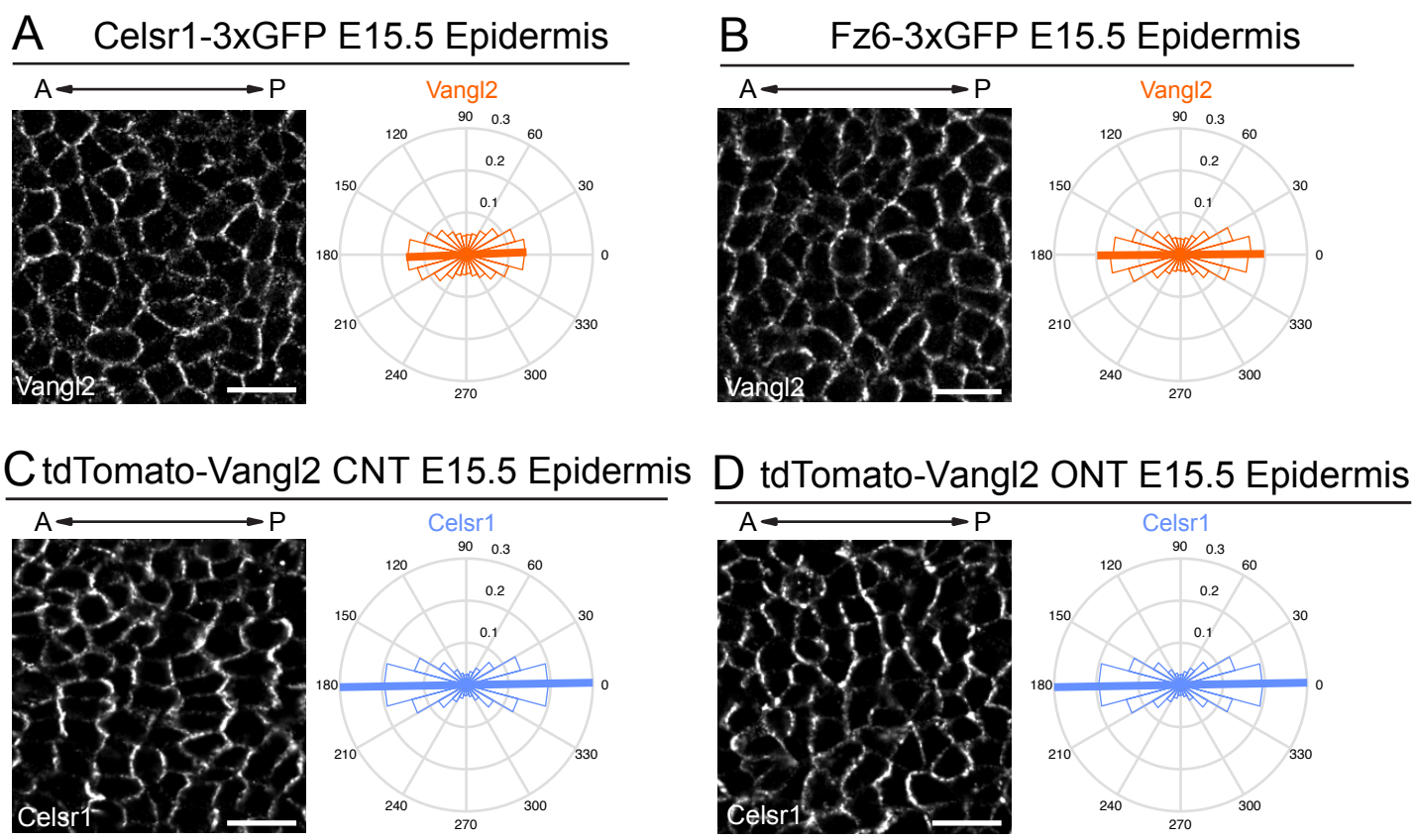


Fig. S2. Comparison of PCP protein asymmetry in Celsr1-3xGFP, Fz6-3xGFP, and tdTomato-Vangl2. **(A-B)** Representative planar views of Vangl2 immunofluorescence in the basal layer of the IFE from Celsr1-3xGFP and Fz6-3xGFP homozygous embryos at E15.5. Quantification of Vangl2 polarity is shown below on circular histograms. **(A)** Vangl2 in Celsr1-3xGFP epidermis, n = 13,605 basal cells from 3 embryos. **(B)** Vangl2 in Fz6-3xGFP epidermis, n = 9,778 from 3 embryos. **(C-D)** Representative planar views of Celsr1 immunofluorescence in the basal layer of the IFE from tdTomato-Vangl2 homozygous embryos at E15.5. Circular histograms below display quantification of Celsr1 polarity **(C)** Celsr1 localization in tdTomato-Vangl2 homozygotes with the curly tail and closed neural tube phenotype (CNT), n = 10,775 basal cells from 3 embryos. **(D)** Celsr1 localization in tdTomato-Vangl2 homozygotes with open neural tube (ONT) phenotypes, n = 9,884 basal cells from 3 embryos. Scale bars, 20 μm.

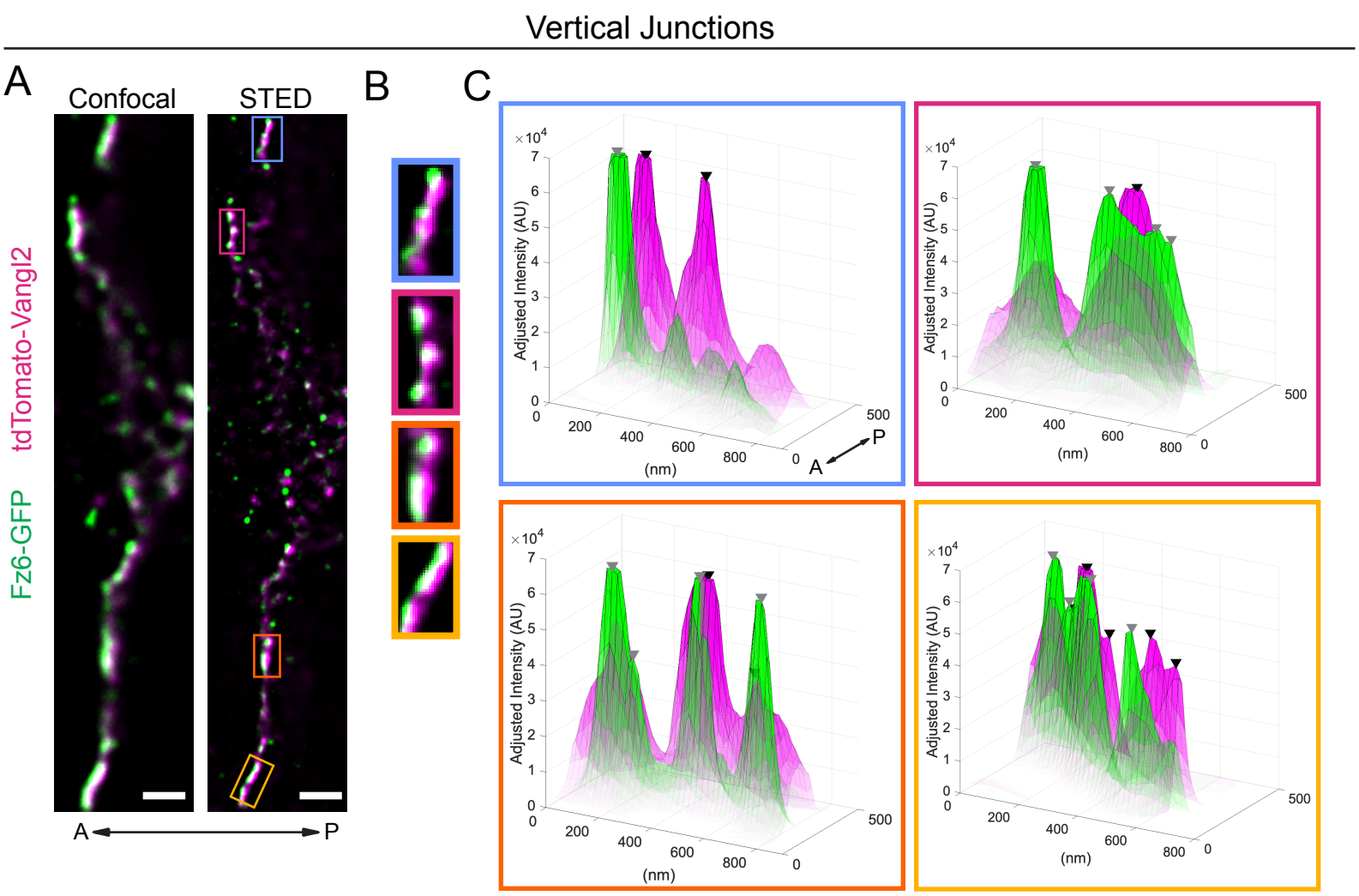


Fig. S3. Super resolution (STED) imaging of Fz6-3xGFP and tdTomato-Vangl2 resolves their localization at vertical and horizontal junctions. (A) Additional representative examples of Fz6-3xGFP and tdTomato-Vangl2 localization across two vertical junctions imaged using standard confocal imaging and STED. Scale bar, 1 μm. **(B)** Magnified regions of the junction corresponding to the boxed areas in (A). **(C)** Corresponding surface plots of GFP and tdTomato fluorescence intensity along the junction of the magnified regions in (B). Arrowheads mark peaks of fluorescence intensity. Opacity of the plot corresponds to pixel intensity, with low opacity indicating low pixel intensity and high opacity indicating high pixel intensity. **(D, G)** Additional representative examples of Fz6-3xGFP and tdTomato-Vangl2 localization across two horizontal junctions imaged using STED. Scale bar, 500 nm. **(E, H)** Magnified regions of the junction corresponding to the boxed areas in (D, G). **(F, I)** Corresponding surface plots of GFP and tdTomato fluorescence intensity along the junction of the magnified region outline in magenta in (D, E) and the region outlined in orange in (G, H). Arrowheads mark peaks of fluorescence intensity. Opacity of the plot corresponds to pixel intensity, with low opacity indicating low pixel intensity and high opacity indicating high pixel intensity.

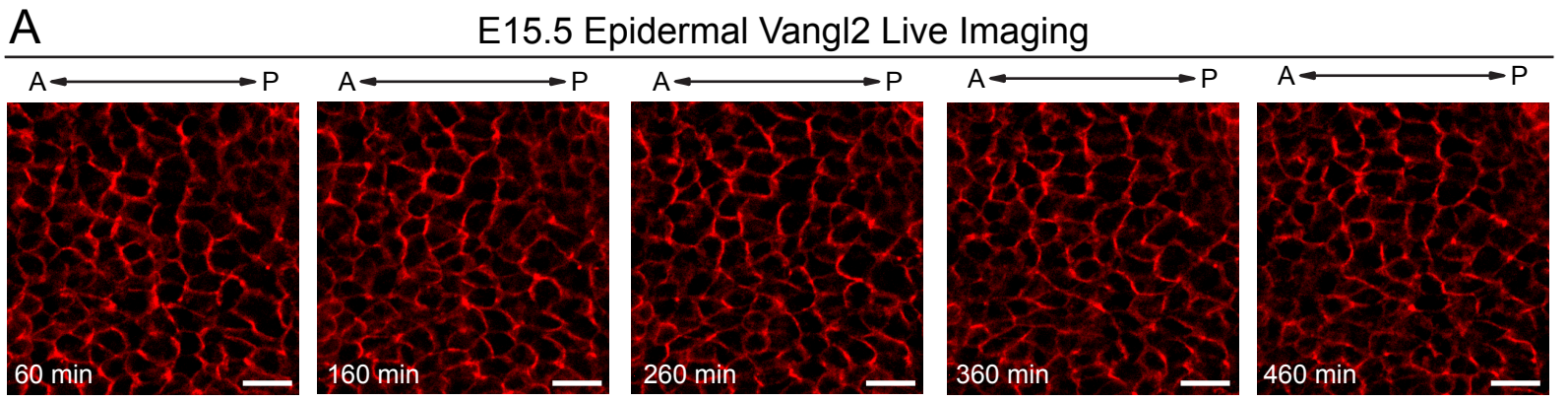


Fig. S4. tdTomato-Vangl2 live imaging. Still frames from live imaging of tdTomato-Vangl2 homozygous E15.5 skin explants at indicated time points. Scale bar, 40 μm.

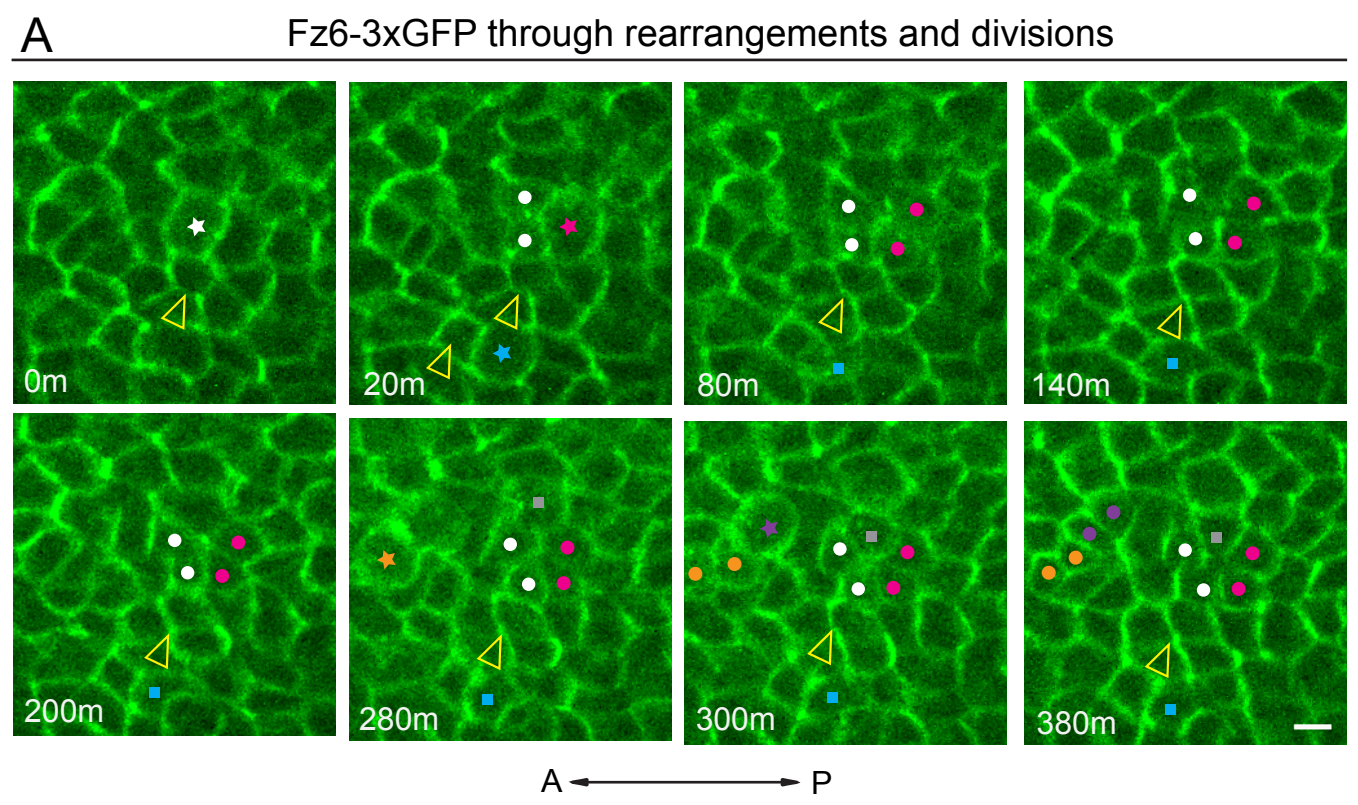


Fig. S5. Live imaging of Fz6-3xGFP through cell rearrangements and divisions. Additional examples of Fz6-3xGFP dynamics through time. **(A)** Still frames of Fz6-3xGFP live images during cell divisions and rearrangements. Each division is indicated by a colored star in mitosis and a pair of dots after cytokinesis. Yellow arrowhead marks a junctional remodeling event. Note the horizontal junction at 0m shrinks to a 4-cell vertex at 140m. **(B-C)** Representative examples of Fz6-3xGFP during cell division (dashed outlines). Neighboring cells are darkened to emphasize Fz6-3xGFP in the dividing cell. Note in these examples, the polarity of daughter cells does not align identically with the polarity of the mother cell prior to mitosis.

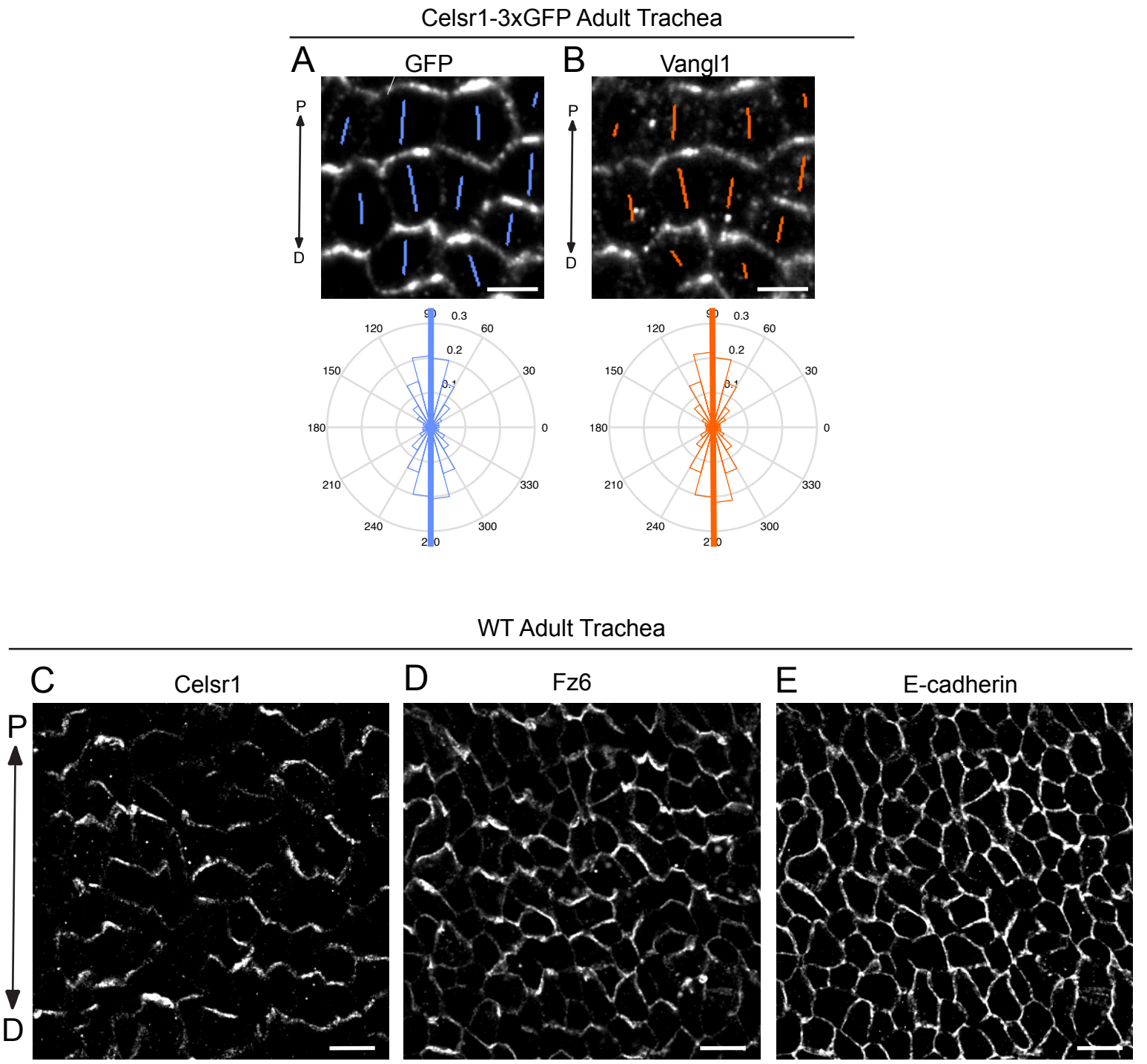


Fig. S6. Celsr1-3xGFP polarity quantification in adult trachea and comparison in wild-type trachea. Representative immunofluorescence images of Celsr1-3xGFP **(A)** and Vangl1 **(B)** in adult trachea. Lines represent the magnitude (line length) and axis (line angle) of polarity. Circular histograms below show the distribution of Celsr1-3xGFP polarity (A) and Vangl1 (B) in the adult trachea, n = 3,228 cells from 3 adult tracheas. Scale bars, 5 μm. **(C-E)** Representative immunofluorescence images of Celsr1 (C), Fz6 (D) and E-cadherin (E) in wild-type adult trachea. Scale bars, 10 μm.

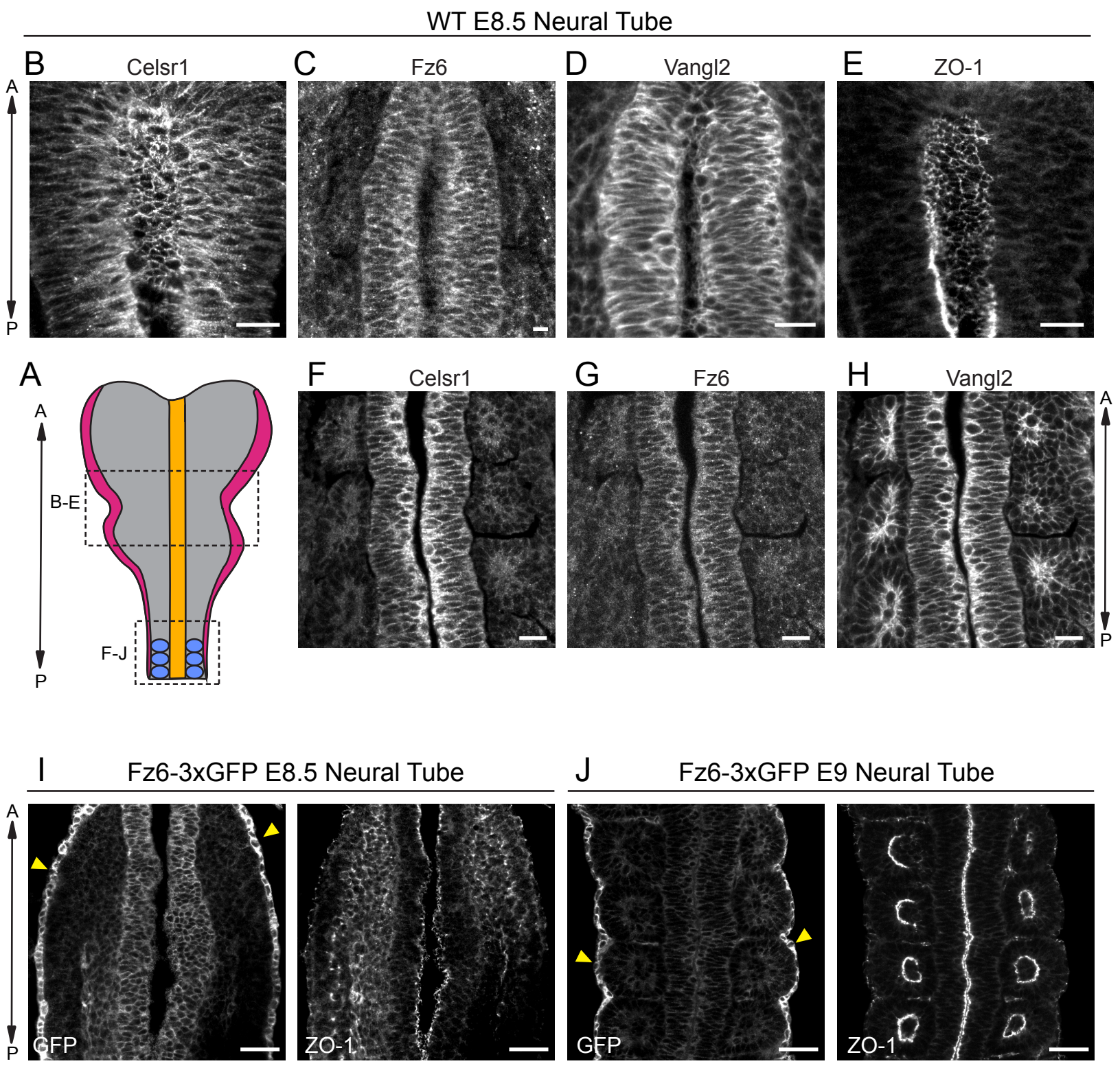


Fig. S7. Expression of PCP proteins in wild-type E8.5 embryos and Fz6-3xGFP expression in the E8.5 and E9.5 surface ectoderm and neural tube. (A) Schematic of dorsal view of E8.5 embryo showing neural folds and somites. Schematics adapted from (Brooks et al., 2020). **(B-E)** Representative images of PCP protein localization in the rostral neural tube of E8.5 wild-type embryos as detected by immunofluorescence of endogenous proteins. Maximum intensity projections of 3-7 μm are shown. Scale bars, 20 μm. **(B)** Celsr1 **(C)** Fz6 **(D)** Vangl2 **(E)** ZO-1 to mark the apical positions of neural epithelial cells. **(F-G)** Representative images showing PCP protein localization in the neural tube and somites at more caudal positions of E8.5 wild-type embryos. Scale bars, 20 μm. **(F)** Celsr1 **(G)** Fz6 **(H)** Vangl2. **(I)** Representative image of Fz6-3xGFP and ZO-1 expression in the midline and surface ectoderm of Fz6-3xGFP homozygous E8.5 embryos. Scale bars, 10 μm. **(J)** Representative image of Fz6-3xGFP and ZO-1 in the midline and somites of Fz6-3xGFP homozygous E9 embryos in which the neural tube is fully closed. Note high expression of Fz6-3xGFP in the surface ectoderm (yellow arrowheads) compared to the neural epithelium and somites. Scale bars, 40 μm.

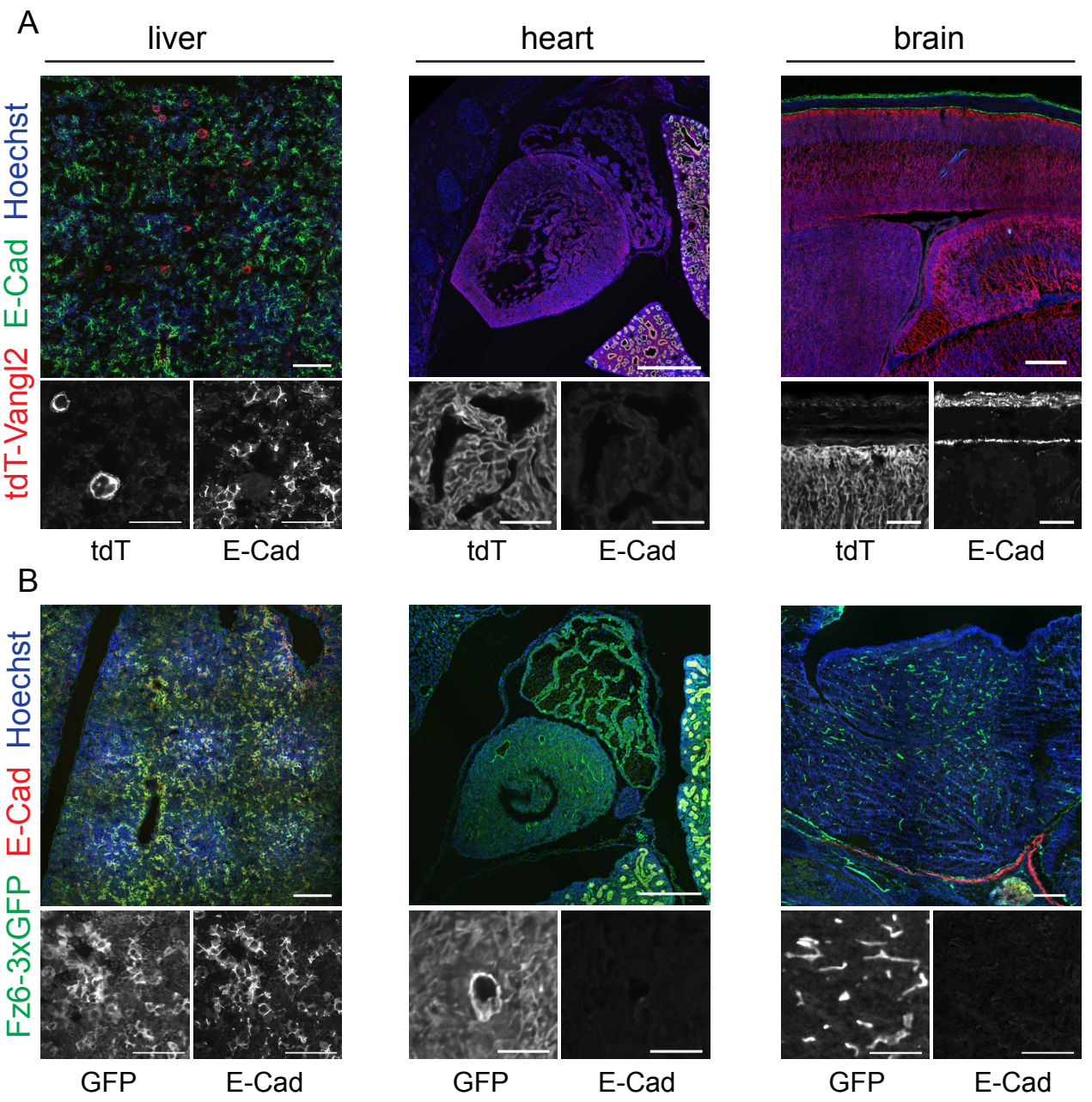
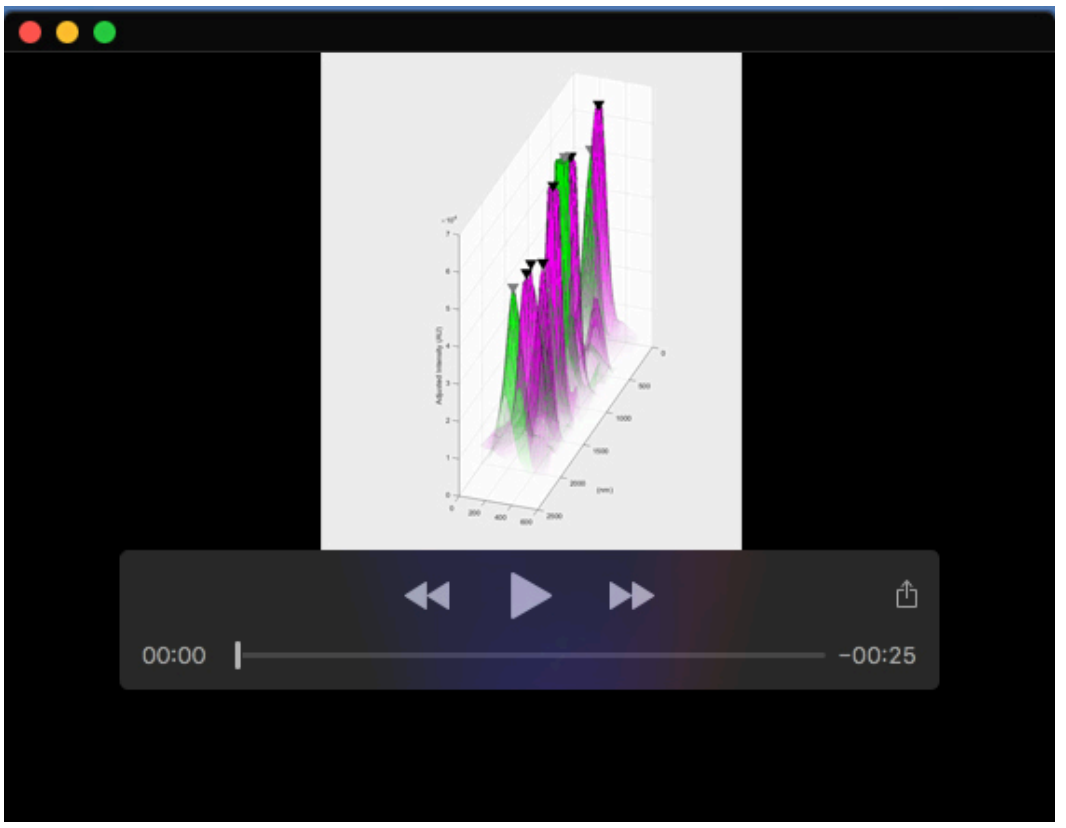
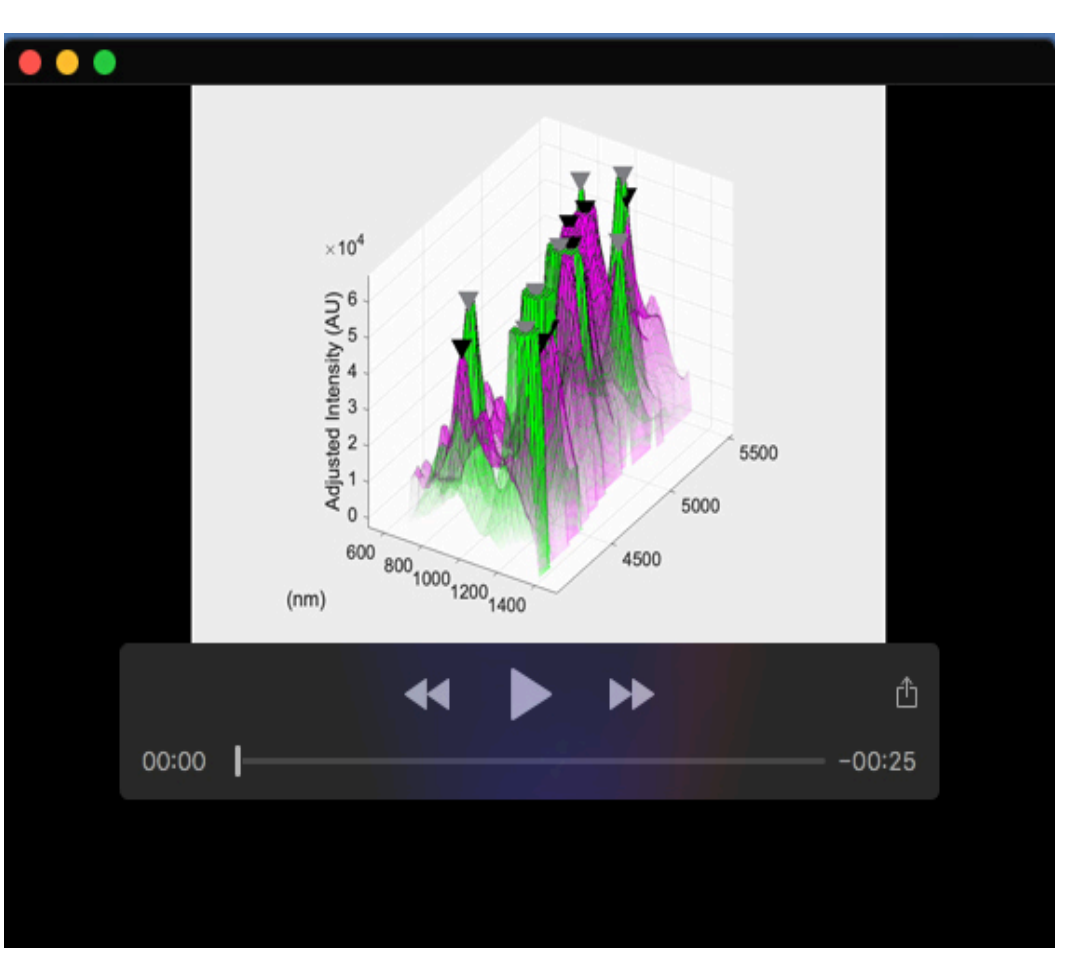


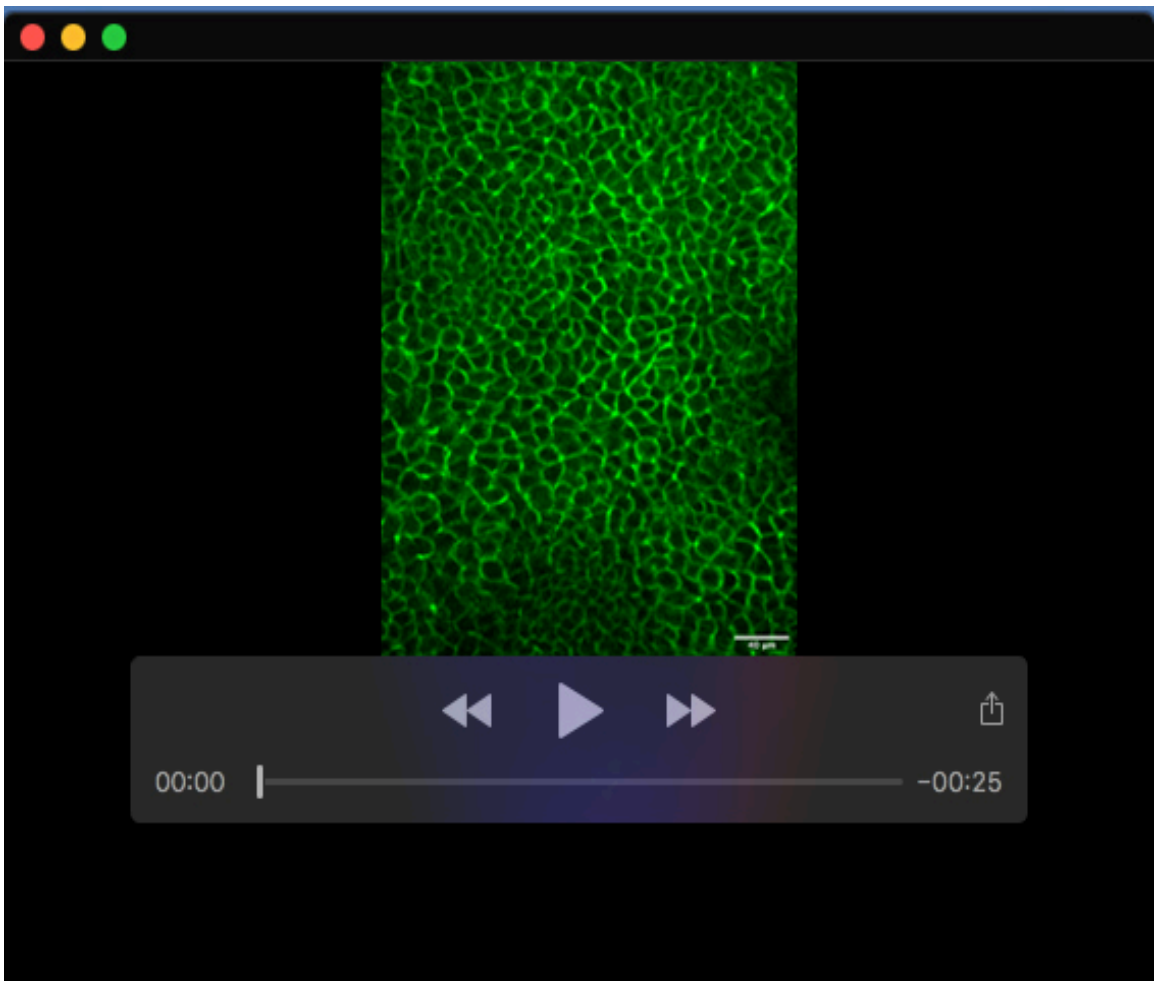
Fig. S8. Fz6-3xGFP and tdTomato-Vangl2 expression in the liver, heart and brain. Sagittal sections from tdTomato-Vangl2 and Fz6-3xGFP embryos at E16.5 labeled with E-Cadherin and Hoechst. **(A-B)** Composite images of E16.5 sagittal sections showing tdTomato-Vangl2 (A, red) and Fz6-3xGFP (B, green) expression in the liver, heart, and brain as indicated. Below each merged image, zoomed-in regions of individual channels in greyscale are shown. **(A)** tdTomato-Vangl2 (red); E-Cadherin (green), Hoechst (blue). tdTomato-Vangl2 is expressed is sparsely labeled cells scattered throughout the liver, broadly throughout the heart including cardiac muscle, and in neurons in the brain. **(B)** Fz6-3xGFP (green), E-Cadherin (red), Hoechst (blue). Note Fz6-3xGFP is expressed in liver epithelium as well as the vasculature in all three tissues. Scale bars, 100 μm for merged images, 500 μm for composite images of the heart, and 50 μm for greyscale images.



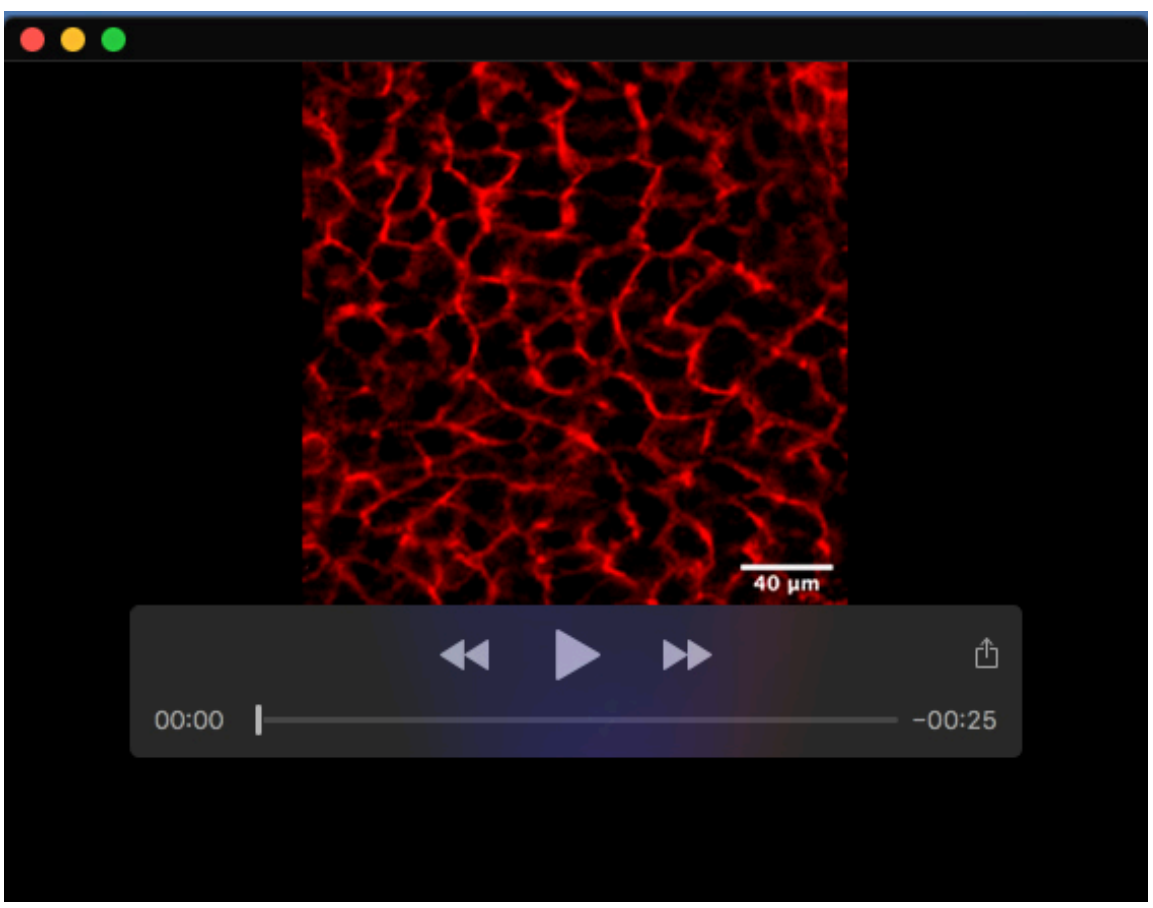
Movie 1. Surface plot of Fz6-3xGFP and tdTomato-Vangl2 peaks in a vertical junction. Rotating surface plot obtained from STED imaging of Fz6-3xGFP and tdTomato-Vangl2 peaks corresponding to the junction in Figure 4C, D. Fluorescence intensity peaks marked by arrowheads.



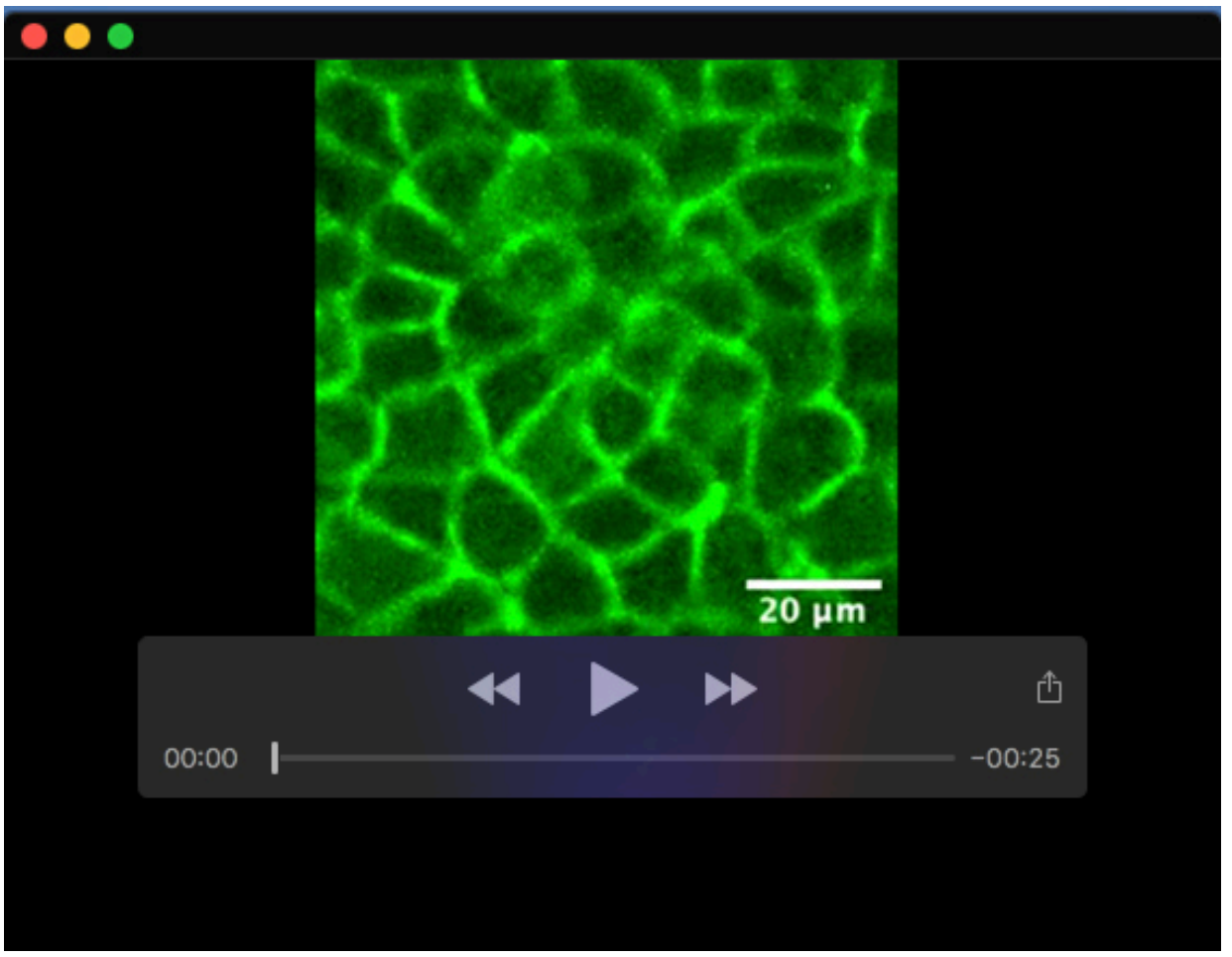
Movie 2. Surface plot of Fz6-3xGFP and tdTomato-Vangl2 peaks in a horizontal junction. Rotating surface plot obtained from STED imaging of Fz6-3xGFP and tdTomato-Vangl2 peaks corresponding to the junction in Figure 4G, H. Fluorescence intensity peaks marked by arrowheads.



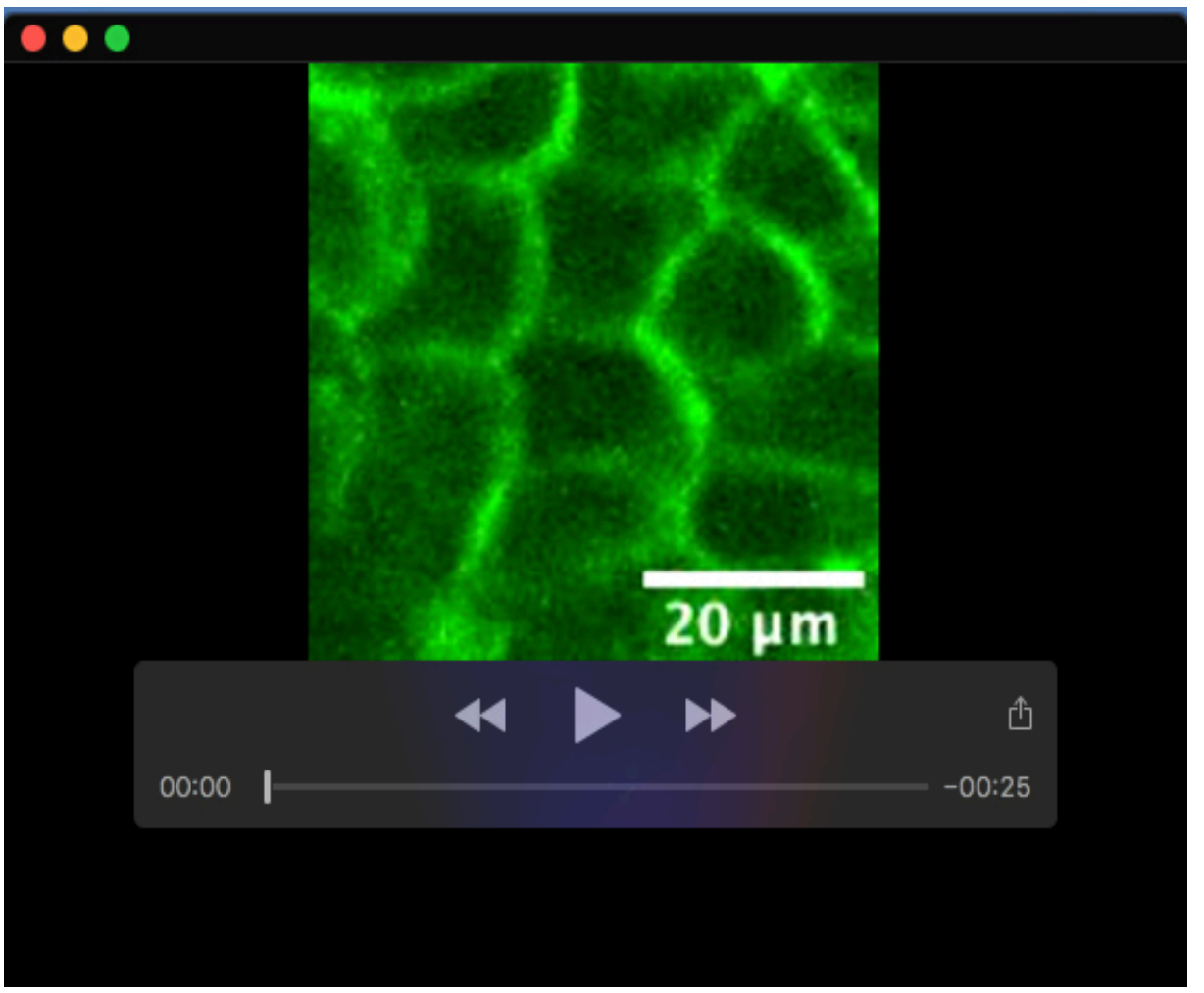
Movie 3. Live imaging of Fz6-3xGFP E15.5 epidermis. Time-lapse imaging of E15.5 Fz6-3xGFP homozygous explants. Imaging was acquired over the course of 7.3 hours with 20 minute intervals. Anterior is to the left. Scale bar, 40 µm.



Movie 4. Live imaging of tdTomato-Vangl2 E15.5 epidermis. Time-lapse imaging of E15.5 tdTomato-Vangl2 homozygous explants. Imaging was acquired over the course of 8 hours with 20 minute intervals. Anterior is to the left. Scale bar, 40 µm.



Movie 5. Live imaging of Fz6-3xGFP through cell rearrangements and divisions. Time-lapse imaging of cell rearrangements and divisions in E15.5 Fz6-3xGFP homozygous explants seen in Figure 5B. Imaging was acquired over the course of 7.3 hours with 20 minute intervals. Scale bar, 20 µm.



Movie 6. Live imaging of Fz6-3xGFP through cell division. Time-lapse imaging of cell division in E15.5 Fz6-3xGFP homozygous explants seen in Figure 5C. Imaging was acquired over the course of 7.3 hours with 20 minute intervals. Scale bar, 20 µm.

Table S1. sgRNA target sequences

Targeted allele	Construct design	Targeted genomic sequence with sgRNA
Celsr1-3xGFP	C-terminal fusion	TGAGGCACAGTCAACC(C→A)CACAG(A→G)CTGCCGGTCAAGCCCTCAGACCTT
Celsr1-3xGFP	C-terminal fusion	CCATCAGGAAACCATGAGGCACAGTCAACCCCACAGACTGCCGGTCAAGC
Fz6-3xGFP	C-terminal fusion	GGGTGCTGGCAGCCATTCCGACGCTTGAAGAAAACTGTCTCGTTCCCCCA
Fz6-3xGFP	C-terminal fusion	TGGCAGCCATTCCGACGCTTGAAGAAAACTGTCTCGTTCCCCCAGAAGCA
tdTomato-Vangl2	N-terminal fusion	ACACACGGCGTTCGGACGCCATGGACACCGAGTCCCAGTACTCGGGC
tdTomato-Vangl2	N-terminal fusion	ATGGA…TATTC(C→A)TACAAGTC(G→C)GGCCACTCCCGC

Underline: sgRNA sequence, Red: stop codon, Green underline: start codon, Blue: PAM site, Dashed line: silent mutations introduced

Table S2. Primer sequences

PCR	Name of genotyping primer	Genotyping Primer Sequences	Where the primer binds	Product Size WT animals	Product size knockin animals
Celsr1-3xGFP insertion PCR	Celsr1.FOR	GGGTGGCCATGAATGTACGC	Upstream of 3xGFP	544 bp	2753 bp
	Celsr1.REV	CGGAGCTATGCCAGCCTTAAT	Downstream of 3xGFP		
GFP PCR	Celsr1.FOR	GGGTGGCCATGAATGTACGC	Upstream of 3xGFP	N/A	551 bp
	3xGFP.REV	CTTCATGTGGTCGGGGTAGC	Within GFP		
Fz6-3xGFP insertion PCR	Fz6.FOR	GCCTCAGTAATTGTGTCCGC	Upstream of 3xGFP	942 bp	3153 bp
	Fz6.REV	CCCTTCCCATCCCCACATTT	Downstream of 3xGFP		
GFP PCR	Fz6.FOR	GCCTCAGTAATTGTGTCCGC	Upstream of 3xGFP	N/A	651 bp
	3xGFP.REV	CTTCATGTGGTCGGGGTAGC	Within GFP		
tdTomato-Vangl2 insertion PCR	Vangl2.FOR	GGTATTCTCATGGAGCGCCT	Upstream of tdTomato	665 bp	2090 bp
	Vangl2.REV	TCTTTGATGACCTCCTCGCC	Downstream of tdTomato		
tdTomato PCR	Vangl2.FOR	GGTATTCTCATGGAGCGCCT	Upstream of tdTomato	N/A	331 bp
	tdTomato.REV	AAACCACTTTAGCCCCAGTGC	Within tdTomato		

Table S3. Antibodies and reagents

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional Information
Antibody	Anti-Celsr1 (Guinea pig polyclonal)	Devenport and Fuchs, 2008		1:1000
Antibody	Anti-E-cadherin, clone DECMA-1 (rat monoclonal)	Thermo Fisher Scientific	Cat #MA1-25160	1:1000 (IF, whole mount) 1:2000 (IF, sections)
Antibody	Anti-E-cadherin (rabbit monoclonal)	Cell Signaling Technology	Cat #3195	1:250
Antibody	Anti-Vangl1 rabbit polyclonal)	Sigma Aldrich	Cat #HPA025235-10	1:500
Antibody	Anti-Vangl2 (rat monoclonal)	Millipore	Cat #MABN750	1:100
Antibody	Anti-Frizzled6 (Goat polyclonal)	R&D Biosystems	Cat #AF1526	1:400
Antibody	Anti-GFP (chicken polyclonal)	Abcam	Cat #ab6556	1:2000
Antibody	Anti-RFP (rabbit	Rockland Inc	Cat #600-4010379	1:200 (IF, whole mount)

	polyclonal)			1:500 (IF, sections)
Antibody	Anti-ZO-1 (rat monoclonal)	DSHB	Cat # R26.4C	1:100
Antibody	Anti-Guinea Pig, Alexa Fluor 488 (Goat)	Invitrogen	Cat #A11073	1:2000
Antibody	Anti-Guinea Pig, Alexa Fluor 647 (Donkey)	Invitrogen	Cat #A21450	1:2000
Antibody	Anti-Chicken, Alexa Fluor 488 (Goat)	Invitrogen	Cat #A-11039	1:2000
Antibody	Anti-Chicken, Alexa Fluor 488 (Donkey)	Jackson ImmunoResearch	Cat #703-545-155	1:2000
Antibody	Anti-Rabbit, Alexa Fluor 555 (Donkey)	Invitrogen	Cat #A-31572	1:2000
Antibody	Anti-Rabbit, Alexa Fluor 488 (Donkey)	Jackson ImmunoResearch	Cat #711-545-152	1:2000
Antibody	Anti-Rat, Alexa Fluor 647 (Donkey)	Jackson ImmunoResearch	Cat #712-605-153	1:2000
Antibody	Anti-Rat, Alexa Fluor 555 (Goat)	Invitrogen	Cat #A-21434	1:2000
Antibody	Anti-Rat, Alexa Fluor 488 (Donkey)	Invitrogen	Cat #A-21208	1:2000

Antibody	Anti-Goat, Alexa Fluor 647 (Donkey)	Jackson ImmunoResearch	Cat #705-605-147	1:2000
Antibody	Anti-chicken STAR ORANGE (goat)	Abberior	STORANGE-1005	1:100
Antibody	Anti-rabbit STAR RED (goat)	Abberior	STRED-1002	1:100
Recombinant DNA reagent	pAAV RhoB YFP Ms2	(Wilson et al., 2017)	Addgene #131777	
Recombinant DNA reagent	fat2-3xGFP floxDsRed	(Barlan et al., 2017)		
Recombinant DNA reagent	pUC vector	(Ravindran et al., 2020)		
Recombinant DNA reagent	pCAG-TAG	(Trichas et al., 2008)	Addgene #26771	
Recombinant DNA reagent	pAAV Celsr1-3xGFP	This paper		
Recombinant DNA reagent	pAAV Fz6-3xGFP	This paper		
Recombinant DNA reagent	pUC tdTomato-Vangl2	This paper		
Software, algorithm	Matlab	MathWorks	https://www.mathworks.com/products/matlab.html	
Software, algorithm	Tissue Analyzer	(Aigouy et al., 2010)	https://grr.gred-clermont.fr/labmirouse/software/WebPA/index.html	
Software, algorithm	R	R project	https://www.R-project.org/	
Software, algorithm	ggplot2	(Hadley Wickham and Thomas Lin, 2016)	https://ggplot2.tidyverse.org	
Software, algorithm	CaptureFigVid	MathWorks	https://www.mathworks.com/matlabcentral/fileexchange/41093-create-video-of-rotating-3d-plot	