Supplementary Information for

Super-resolution microscopy localizes endogenous Dvl2 to Wnt signaling-responsive biomolecular condensates

Antonia Schubert\textsuperscript{a,b,c}, Oksana Voloshenko\textsuperscript{a}, Franziska Ragaller\textsuperscript{a1}, Philipp Gmach\textsuperscript{a2}, Dominique Kranz\textsuperscript{a}, Christian Scheeder\textsuperscript{a}, Thilo Miersch\textsuperscript{a}, Matthias Schulz\textsuperscript{b}, Lorenz Trümper\textsuperscript{b}, Claudia Binder\textsuperscript{b}, Marko Lampe\textsuperscript{b}, Ulrike Engel\textsuperscript{b}, Michael Boutros\textsuperscript{a}

Antonia Schubert\textsuperscript{a,b,c}, Oksana Voloshenko\textsuperscript{a}, Franziska Ragaller\textsuperscript{a1}, Philipp Gmach\textsuperscript{a2}, Dominique Kranz\textsuperscript{a}, Christian Scheeder\textsuperscript{a}, Thilo Miersch\textsuperscript{a}, Matthias Schulz\textsuperscript{b}, Lorenz Trümper\textsuperscript{b}, Claudia Binder\textsuperscript{b}, Marko Lampe\textsuperscript{b}, Ulrike Engel\textsuperscript{b}, Michael Boutros\textsuperscript{a}

\textsuperscript{a} Division Signaling and Functional Genomics, German Cancer Research Center (DKFZ) and Department of Cell and Molecular Biology, Heidelberg University and BioQuant69120 Heidelberg, Germany

\textsuperscript{b} Department of Hematology and Medical Oncology, University Medical Center Göttingen, 37075 Göttingen, Germany

\textsuperscript{c} Department of Medical Oncology, National Center for Tumor Diseases (NCT), University Hospital Heidelberg, 69120 Heidelberg, Germany

\textsuperscript{d} Advanced Light Microscopy Facility (ALMF), European Molecular Biology Laboratory (EMBL), 69117 Heidelberg, Germany

\textsuperscript{e} Nikon Imaging Center (NIC), BioQuant and Centre for Organismal Studies (COS), Heidelberg University, 69120 Heidelberg, Germany

\textsuperscript{1} Present address: Science for Life Laboratory, Department of Women’s and Children’s Health, Karolinska Institutet, 17165 Solna, Sweden

\textsuperscript{2} Present address: Max Delbrück Center for Molecular Medicine (MDC) in the Helmholtz Association, 13125 Berlin, Germany

* Corresponding author:
Michael Boutros, Im Neuenheimer Feld 580, 69120 Heidelberg, Germany, Tel. +49 6221 42 1950, Fax +49 6221 42 1959
E-Mail: m.boutros@dkfz.de

This PDF file includes:
Supplementary Figures and Figure Legends S1 to S14
Supplementary Table S1
Legend for Supplementary Movie S1
Supplementary Materials and Methods
Supplementary References

Other supplementary materials for this manuscript include the following:
Movie S1
Supplementary Information

SI Figures

Fig. S1: Sequencing of Dvl2_mEos3.2 142_3 confirms correct integration of the fluorophore into the DVL2 locus. Sanger sequencing of the genomic DNA of HEK293T DVL2_mEos and alignment to the DVL2 sequence.
demonstrates correct integration of the mEos3.2 fluorophore into the DVL2 locus. Targeting sites of sgRNAs are indicated by arrows and silent mutations are indicated in gray.
Fig. S2: Knock-out of DVL1,2,3 confirms the specificity of the mEos3.2 signal to Dvl. (A) Dvl1 is not expressed in HEK293T cells. Cells were transfected with pLenti DVL1 for 48 h as expression control. Total cell lysates were used for Western blot analysis with the indicated antibodies. Beta-actin serves as a loading control. One of three independent experiments is shown. kDa = kilodaltons. (B) Knock-out efficiency of sgRNA targeting DVL1,2,3 as shown in Western blot after generation of HEK293T^{DVL2-mEos} DVL1,2,3\textsuperscript{KO} cells. Beta-actin serves as a loading control. One of three independent experiments is shown. kDa = kilodaltons. (C) The mEos3.2 signal is lost in the HEK293T^{DVL2-mEos} DVL1,2,3\textsuperscript{KO} pool. Correlative live-cell confocal microscopy. Cell membrane was visualized using CellMask Deep Red membrane dye (blue). Representative images of three replicates with imaging of ≥3 points-of-view. Scale bar 10 µm.
Fig. S3: Characterization of Dvl2_mEos3.2 condensates. (A) 20-30% of HEK293T^{DVL2_mEos} cells show endogenous Dvl2_mEos3.2 condensates. Manual quantification of Dvl2_mEos3.2 condensates performed on confocal stack or widefield images. Left: quantification of confocal mini stack images of fixed cells acquired on a Nikon Ti-E inverted microscope for colocalization experiments (60x magnification, fixation of cells 48 h after cell seeding). Analysis of 100 cells per replicate, four replicates. Right: quantification of Dvl2_mEos3.2 condensates performed on widefield fluorescent images of fixed cells taken with the InCell Analyzer 2200 for automated condensate quantification (20x magnification, fixation of cells 72 h after cell seeding). Manual counting of 100 cells in three different fields-of-view per replicate, four replicates. Graph represents the mean (±SEM) of four independent experiments that are shown by dots. (B) Confocal microscopy of Dvl2 in HEK293T and HEK293T^{DVL2_mEos} cells before and after DVL1,2,3^{KO}. Dvl2 was stained with the corresponding primary antibody and Alexa-647 (pink) secondary antibody. DNA was stained by Hoechst (blue). Maximum intensity projections HEK293T (z=26), HEK293T^{DVL1,2,3^{KO}} (z=17), HEK293T^{DVL2_mEos} (z=12), and HEK293T^{DVL2_mEos DVL1,2,3^{KO}} (z=25). Representative images of three replicates with imaging of ≥5 points-of-view. Scale bar 10 µm.
Fig. S4: Endogenous Dvl2_mEos3.2 supra-molecular condensates do not co-localize with common markers of cellular degradation machineries. (A) Cellular markers analyzed in this study for co-localization with Dvl2_mEos3.2 supra-molecular condensates: CellMask for the plasma membrane, Hoechst for DNA, Calnexin for ER and ER-associated degradation, Vimentin for aggresomes, proteasome, LAMP1 for lysosomes CEP164 and AAT for cilia, gamma-tubulin, PCM1, CEP164 for the centrosome. (B) Confocal microscopy demonstrates Dvl2_mEos3.2 supra-molecular condensates do not co-localize with common markers of cellular degradation machineries: aggresomes (Vimentin); proteasome, lysosome (LAMP1); ER and ER-associated degradation (Calnexin). HEK293T^Dvl2_mEos cells were fixed and stained with the indicated antibodies. Maximum intensity projections Vimentin (z=19), proteasome (z=18), LAMP1 (z=18), or Calnexin (z=12). Representative images of 3-6 replicates with imaging of ≥3 points-of-view. Scale bar 10 µm.
Fig. S5: Wnt-reporter activity in HEK293T^DVL2_mEos cells is concentration-dependent and independent of mEos3.2. (A) and (B) Concentration-dependent induction of TCF4-mediated Wnt activity in HEK293T^DVL2_mEos and HEK293T cells. HEK293T^DVL2_mEos and HEK293T cells were transfected with TCF4/Wnt-firefly luciferase and actin-Renilla-reporter plasmids (A) and increasing concentrations of the indicated plasmids (0.2/1/5/10 ng Wnt3 or Dvl2) (B). Recombinant Wnt3a was added 24 h after transfection (A). Luciferase activity was measured 16 h after treatment (A) or 52 h after transfection (B). Induced reporter activity was normalized to HEK293T cells stimulated with recombinant Wnt3a at a final concentration of 100 ng/ml (A) or HEK293T cells with 10 ng Wnt3 plasmid (B). Graphs represent the mean ±SEM of four independent experiments. (C) Knock-out efficiency of the sgRNA targeting mEos3.2 as shown in Western blot after generation of two HEK293T^DVL2_mEos mEos^KO2.1 pools (I+II). Beta-actin serves as a loading control. One of three independent experiments is shown. kDa = kilodaltons. (D) Knock-out of mEos3.2 has no influence on increased inducibility of the HEK293T^DVL2_mEos cell clone indicating a clonality effect independent of the mEos3.2 knock-in. Cells were transfected with pcDNA, or Wnt3 together with TCF4/Wnt-firefly luciferase and actin-Renilla-reporter plasmids. Luciferase activity was measured 48 h later. Induced reporter activity was determined by normalization to Wnt3 transfected HEK293T^DVL2_mEos cells. Graph represents the mean ±SEM of four independent experiments.
Fig. S6: Functionality of the endogenously tagged cell line HEK293T^DVL2_mEos. (A) TagRFP-tagged FZD7 rescues Wnt3a-induced reporter activation in HEK293T mFZD^KO (knock-out of FZD1,2,4,5,7,8; see (1)) as non-tagged FZD7. HEK293T mFZD^KO were transfected with the indicated plasmids (FZD7, C-terminally tagRFP-tagged FZD7, pcDNA, or Wnt3a) together with TCF4/Wnt-firefly luciferase and actin-Renilla-reporter plasmids. Luciferase activity was measured 52 h later. Induced reporter activity was determined by normalization to HEK293T mFZD^KO cells transfected with FZD7 and Wnt3a. Graph represents the mean ±SEM of four independent experiments. (B) ROR2 and ROR2_mScarlet induce invasiveness in the luminal A breast cancer cell line MCF7 using the modified Boyden Chamber assay. Stimulation with recombinant Wnt5a served as a positive control (2). Cells were transfected with the indicated plasmids (pcDNA, ROR2, c-terminally mScarlet-tagged ROR2). 96 h after seeding in the Boyden Chamber, invasive capacity was measured by counting the number of cells that had penetrated the ECM. Relative invasiveness was determined by normalization to cells transfected with pcDNA. Graph represents the mean ±SEM of three independent experiments, * p ≤ 0.05. (C) Dvl2_mEos3.2 is recruited to the plasma...
membrane after overexpression of FZD7_tagRFP and ROR2_mScarlet. Cells were transfected with the indicated plasmids for 48 h. Wnt activity was induced by Wnt3 overexpression for FZD7_tagRFP or Wnt5a overexpression for ROR2_mScarlet. The same laser settings were applied to all conditions for comparison of signal intensities. Cell membrane was visualized using CellMask Deep Red membrane dye (blue). Representative images of four replicates with imaging of ≥3 points-of-view. Scale bar 10 μm.
Fig. S7: No effect of Wnt stimulation on Dvl2_mEos3.2 condensate formation observed by confocal microscopy. (A-C) Overexpression of tagged and non-tagged Wnt3a and Wnt5a constructs in HEK293T<sub>DVL2_mEos</sub> has no effect on Dvl2_mEos3.2 condensate formation using confocal microscopy. HEK293T<sub>DVL2_mEos</sub> and HEK293T cells were transfected with the indicated siRNAs against DVL1/3 (B+C). 24 h later, cells were transfected with the indicated plasmids: pcDNA, Wnt3a_mCherry, or Wnt5a_mScarlet (A), pcDNA, or non-tagged Wnt3a (B), pcDNA, or Wnt3a_mCherry (C). C upper panel (HEK293T siCtl. pcDNA) represents control images of Fig. 4A. Images from Fig. 4A are shown again in the other panels for better comparison. Live-cell confocal microscopy was performed 48 h after transfection. Cell membrane was visualized using CellMask Deep Red membrane dye (blue). Green signal in HEK293T cells shows induced autofluorescence after siRNA/pcDNA transfection (B+C). Representative images of four replicates with imaging of ≥3 points-of-view. Scale bar 10 µm.
**Fig. S8:** Dvl2\_mEos3.2 condensate formation does not depend on Wnt transcriptional activity. 

**A** Activity of murine rec. Wnt3a used in this study. HEK293^DVL2\_mEos^ cells were transfected with pcDNA, or Wnt3 together with TCF4/Wnt-firefly luciferase and actin-Renilla-reporter plasmids. Stimulation with 200 ng/ml rec. Wnt3a 16 h before reporter readout. Luciferase activity was measured 48h after transfection. Induced reporter activity was determined by normalization to HEK293^DVL2\_mEos^ cells stimulated with 10 ng Wnt3 plasmid. Graph represents the mean ±SEM of four independent experiments.

**B** CTNNB1 k.d. inhibits TCF4/Wnt reporter activation. For the Wnt-reporter assay, HEK293^DVL2\_mEos^ cells were reverse transfected with a pool of four siRNAs against CTNNB1, or siCtl. 24 h later, cells were transfected with pcDNA, or Wnt3 together with TCF4/Wnt-firefly luciferase and actin-Renilla-reporter plasmids. Luciferase activity was measured 48 h later. Induced reporter activity was determined by normalization to siCtl stimulated with Wnt3. Graph represents the mean ±SEM of four independent experiments.

**C** Automated condensate quantification after siRNA-mediated k.d. of CTNNB1 shows no effect on Dvl2\_mEos3.2 condensate formation in HEK293^DVL2\_mEos^ cells. Cells were seeded into 384-well plates and reverse transfected with the indicated siRNAs, or treated with RNAiMax only. The cells were fixed after 72 h and stained with Hoechst and Phalloidin. Wide-field images for condensate quantification were taken with an InCell Analyzer 2200 (20x magnification). Automated image analysis was performed as described in the SI Materials and Methods. The condensate count was normalized to the number of cells per image (condensates per 100 cells). The quantification via automated image analysis detects less condensates compared to manual counting. Box plot representing the average of relative, normalized condensate counts with dots representing the average relative, normalized condensate counts from three individual experiments.
Fig. S9: DVL1,2,3\textsuperscript{KO} in HEK293T cells reduces cell proliferation. Proliferation of HEK293T and HEK293T DVL1,2,3\textsuperscript{KO} cells was monitored by live-cell imaging using an Incucyte instrument. 2.5 x 10\textsuperscript{3} cells were seeded in clear Greiner flat-bottom polystyrene 96-well plates, and cultured under physiological conditions for 4 days. Images were acquired every 2 h to determine the cell count using the manufacturer’s software. Graph represents the mean (±SEM) of measurements in five wells per condition and timepoint. Representative of seven independent experiments.
Fig. S10: Full scans Figure 1. (A) Full scan PCR gel electrophoresis of Fig. 1B. (B) Full Western blot scans of Fig. 1C. The dashed lines indicate the selected areas shown in the respective main figure.
Fig. S11: Full scans Figure 3: Full Western blot scans of Fig. 3B. The dashed lines indicate the selected areas shown in the main figure.
Fig. S12: Full scans Figure 4: Full Western blot scans of Fig. 4B. The dashed lines indicate the selected areas shown in the main figure. Thick solid line indicates where the membrane was cut prior to antibody addition.
**Fig. S13: Full scans Figure S2:** Full Western blot scans of Fig. S2A (A) and S2B (B). The dashed lines indicate the selected areas shown in the respective supplemental figure. Thick solid lines indicate where the membrane was cut prior to antibody addition.
Fig. S14: Full scans Figure S5: Full Western blot scans of Fig. S5C. The dashed lines indicate the selected areas shown in the respective supplemental figure. Thick solid lines indicate where the membrane was cut prior to antibody addition.
## SI Table

| HEK293T<sup>DVL2</sup>_mEoS | Cell name | Gene | % of sequences with mutations |
|-------------------------------|-----------|------|------------------------------|
| EVI<sup>KO1</sup>_1          | EVI/WLS   |      | 95.48                        |
| EVI<sup>KO1</sup>_3          | EVI/WLS   |      | 91.9                         |
| mFZD<sup>KO</sup>            | FZD1      |      | 86.64                        |
|                               | FZD2      |      | 91.43                        |
|                               | FZD4      |      | 87.73                        |
|                               | FZD5      |      | 84.54                        |
|                               | FZD7      |      | 87.36                        |
|                               | FZD8      |      | 85                           |

Table S1: Summary of MiSeq-Analysis showing the percent of sequences with mutations in the respective CRISPR knock-out pools.
SI Movie legend

**Movie S1: Cell cycle-dependent formation of Dvl2_mEos3.2 condensates in HEK293T cells.** Live-cell imaging revealed that Dvl2_mEos3.2 condensates dissolve before mitosis. Time-lapse movie of two cells (marked by arrows) that lose condensates before and regain condensates after mitosis. Cells were seeded into ibidi chamber slides 24 h before images were taken on an A1 Nikon microscope every 15 minutes for 15 h under physiological conditions. Maximum projections of four slices spanning a total of 3 µm are shown.
SI Material and Methods

Cell culture

HEK293T cells were cultured in Dulbecco's Modified Eagle's Medium from Thermo Fisher Scientific (Gibco DMEM, 41965062, Waltham, USA) and supplemented with 10% fetal bovine serum (FCS, F7524-500 ML, Sigma Aldrich, St. Louis, USA). HEK293T cells were obtained from the ATCC (CRL-11268, Manassas, USA) and authenticated by SNP profiling (Multiplexion, Heidelberg, Germany). MCF-7 cells were obtained from DSMZ (ACC115, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), cultured in RPMI-1640 (C10046, Thermo Fisher Scientific, Waltham, USA) supplemented with 10% FCS. Mycoplasma contamination was ruled out regularly.

Transfections

Plasmids used in this study are listed below. Plasmid transfection was performed using TransIT-LT1 Transfection Reagent (731-0027, Mirus Bio, Madison, USA) according to the manufacturer's instructions. For siRNA transfection Lipofectamine RNAiMAX (13778150, Thermo Fisher Scientific, Waltham, USA) was used according to the manufacturer's instructions.

Cloning of tagged Wnt pathway expression constructs

Gateway cloning was used to generate the C-terminally tagged human Fzd7 expression construct. Gateway clones were provided by the Genomics and Proteomics Core Facility (DKFZ) and are listed below. The plasmid pEXP-FZD7_tagRFP was obtained by recombining pENTR223 hFZD7 (BC015915) and pDEST26-RFP-C (tagRFP) using the Gateway LR Clonase II Enzyme Mix (11791020, Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's protocol.

C-terminally tagged ROR2, ROR2_mScarlet and DVL2_mEos3.2 expression constructs were generated by In-Fusion cloning (In-Fusion HD Cloning Plus, 638909, Takara Bio, Kusatsu, Japan). ROR2 was cloned from the genomic locus of HeLa cells using PCR amplification. mScarlet as reported by Bindels and colleagues (3) was cloned from a designed gBlock (Linker: Ala-Ser). ROR2 alone, or ROR2 and mScarlet were inserted into pcDNA3.2/V5-DEST (Invitrogen, through DKFZ-vector and clone repository) with insertion of a stop-codon before the V5. For DVL2_mEos3.2, ROR2_mScarlet was removed using the restriction enzymes Ascl and Xhol. DVL2 was cloned from pDONR DVL2 (BC014844); mEos3.2 and the linker from a custom DVL2_mEos3.2 gBlock designed for the endogenous tagging (Integrated DNA Technologies (IDT), Leuven, Belgium). Oligonucleotides are listed below. For the In-Fusion reaction, 80 ng of backbone, 100 ng of each insert and 2 μl 5x In-Fusion Master Mix were mixed with water to reach a total volume of 10 μl following an incubation at 50 °C for 1 h. 2 μl of the In-Fusion reaction were transformed into competent bacteria.

Endogenous tagging of DVL2 in HEK293T cells

Four different sgRNAs targeting human DVL2 at its C-terminus or the beginning of the 3'-UTR were designed using the E-CRISP web service and cloned into the PX459 vector (4). Sequences are listed below. A gBlock (synthesized by IDT, Leuven, Belgium) served as a homology-directed repair template for the CRISPR/Cas9-induced double-strand breaks. It was designed to contain 400-nt long 5′- and 3′- homology arms flanking a flexible (GGGGS); linker sequence and the coding sequence of mEos3.2 with introduced mutations (mammalian codon-optimized) as reported in (5) (see Fig. 1 for schematic representation and Fig. S1 for sequence alignment with the DVL2 locus). For the transcription of the linker and mEos3.2, the stop codon at the C-terminus of DVL2 was depleted and re-introduced after the mEos3.2 sequence. Silent point mutations were inserted into the gBlock to interfere with the base pairing of the DVL2 sgRNAs and avoid Cas9 targeting the homology arms. For the generation of the HEK293T_DVL2_mEos142_3 clone, 3-4x10⁵ HEK293T cells were transfected in 6-well plates using 500 ng
of the PX459 sgDVL2_142 construct and either 250 ng or 500 ng of the DVL2_mEos3.2 gBlock. After 48 hours, cells were selected by 4 μg/ml puromycin (P9620, Sigma Aldrich, St. Louis, USA) treatment for 24 hours. Fluorescent single-cell clones were picked from 10 cm dishes using sterile 8 mm x 8 mm cloning cylinders (C1059-1EA, Sigma Aldrich, St. Louis, USA). After an additional serial dilution to assure single-cell clonality, clones were further characterized.

Validation of HEK293T DVL2_mEos single-cell clones

Single-cell clones were validated using polymerase chain reaction (PCR) and Sanger sequencing, fluorescence microscopy, and immunoblot analysis. PCR was conducted using the primers listed below and Q5 Hot Start High-fidelity DNA Polymerase (M0493S, New England Biolabs, Ipswich, USA). PCR products were loaded on a 1% agarose gel followed by gel extraction using a NucleoSpin Gel and PCR clean-up kit (740609.250, Machery-Nagel, Düren, Germany). Eurofins Genomics (Ebersberg, Germany) performed Sanger sequencing of PCR fragments.

Generation of CRISPR knock-out cells

Multitargeting sgRNAs for the DVL genes were generated as previously reported for mFZDKO (1). In short, the gene sequences of DVL1, DVL2, and DVL3 were aligned using Clustal Omega (6). We determined highly conserved regions in the PDZ-domain. Here, all NGG protospacer-adjacent motif (PAM) sequences were identified. When the preceding 18- to 20-nt were almost identical in the three DVL genes, we predicted the specificity of the identified sgRNAs for the DVL genes. Assisting in this, the sgRNA design tool E-CRISP was used (4). All identified sgRNAs are specific for the DVL genes and did not show predicted off-target binding sites. For EVIKO two sgRNAs were designed targeting Exon 1 of the EVI/WLS locus (Ex1_1 and Ex1_3). For multiple knock-outs of the predominant Frizzled receptors in HEK293T cells (Fzd 1, 2, 4, 5, 7, 8) the construct published in (1) was used. Eurofins Genomics (Ebersberg, Germany) synthesized oligonucleotides. They were annealed and cloned into the PX459 vector as previously described (1). Respective HEK293T cells were transfected with the indicated plasmids containing the sgRNAs and Cas9. After 48 h, cells were selected with 1-2 μg/ml puromycin (P9620, Sigma Aldrich, St. Louis, USA) for 48-72 h. Cell pools were grown for at least 7 d before the continuation of experiments. We performed MiSeq-analysis to further characterize the mutations in the HEK293T DVL2_mEos mFZDKO and EVIKO pools.

Mutation analysis by indel-nested PCR and Illumina MiSeq sequencing

To characterize the induced mutations in CRISPR/Cas9 knock-out pools, we used nested PCR and MiSeq Next Generation Sequencing as previously reported (1) (Table S1). In brief, we designed primer pairs for FZD1, FZD2, FZD5, FZD7, FZD8, and EVI/wntless with the help of Primer3 Web (7) to amplify 100-150 bp up and downstream of the sgRNA target regions. Specific adapters for the second step of the indel-nested PCR were added (1). For the analysis, genomic DNA was extracted from the CRISPR-k.o. pools using the DNeasy Blood and Tissue Kit (14398, Qiagen, Germantown, MD, USA) according to the manufacturer’s protocol. The first 20 cycle PCR was performed with the gene-specific primers. After PCR purification using the NucleoSpin Gel and PCR Clean-up from Machery-Nagel (740609.10, Düren, Germany), the second step indel-nested PCR was performed with the standard set of primers. After the 2-step indel-nested PCR, we conducted MiSeq sequencing (Illumina, San Diego, CA, USA). For detection of mutations in amplified regions, multiple sequence alignment was carried out using ClustalOmega (https://www.ebi.ac.uk/Tools/msa/clustalo) and CRISPResso2 (8). The primer sequences are listed below.

TCF4/Wnt-reporter assay

We quantified Wnt signaling activity by using TCF4/Wnt-luciferase assays with the indicated HEK293T cell lines as previously described (1). In 384-well white, flat-bottom polystyrene plates (14239, Greiner,
Frickenhausen, Germany) 7,500 cells per well were seeded in DMEM (41965062, Thermo Fisher Scientific, Waltham, USA) supplemented with 10% FCS (F7524-500 ML, Sigma Aldrich, St. Louis, USA). After 24 h, cells were transfected with 20 ng of TCF4/Wnt firefly luciferase-reporter, 10 ng of actin–Renilla luciferase-reporter, and 20 ng of DVL, Wnt, beta-catenin or control (pcDNA3.2/V5-DEST) plasmids using the TransIT-LT1 Transfection Reagent (731-0027, Mirus Bio, Madison, USA). If indicated, the total amount of DNA transfected was adjusted by adding a control plasmid (pcDNA3.2/V5-DEST). Unless otherwise indicated, paracrine induction of Wnt signaling was achieved by treating cells with 100 ng/ml recombinant mouse Wnt3a (315-20-10, PeproTech, Hamburg, Germany). Recombinant Wnt3a was added 16 h before the luciferase activity readout. 48 h after transfection, luminescence was measured using the plate reader Mithras LB940 Multimode Microplate Reader (Berthold Technologies, Bad Wildbad, Germany). For data analysis, the TCF4/Wnt-luciferase signal was normalized to the actin-Renilla signal.

**Blue Sepharose**

To validate the loss of Evi in HEK293TDVL2_mEos EVIKO, secreted Wnts in the supernatant from cell culture supernatants were analyzed using Blue Sepharose 6 Fast Flow (17-0948-01, GE Healthcare, Chicago, USA) pulldown for enrichment followed by Western blotting as previously reported (9). 3-4x10⁵ HEK293TDVL2_mEos EVIKO cells per well were seeded in 6 well plates. 24 h after a medium change, 2 ml of cell culture supernatant from nearly confluent cells were collected. After centrifugation for 10 min at 8000 g to remove dead cells and debris, the supernatant was transferred into new tubes. Triton X-100 (T8787-250ml, Sigma Aldrich, St. Louis, USA) was added to a final volume of 1%. After washing the beads three times in washing buffer (50 mM Tris-HCl, pH 7.5 (75746-250G, Sigma Aldrich, St. Louis, USA); 150 mM KCl (P-9541, Sigma Aldrich, St. Louis, USA); 1% Triton X-100 in H₂O) and centrifugation (3 min, 2700 g), 40 µl of Blue Sepharose beads were added to each sample. The beads were incubated on a tube rotator overnight at 4 °C. Subsequently, the beads were washed again three times in the washing buffer. After the last centrifugation, the pellet was resuspended in 200 µl 2x Laemmli-buffer and incubated at 95 °C for 5 min. 30 µl of the supernatant was loaded on a gel for SDS-PAGE.

**Immunoblot analysis**

Protein extraction was performed using a Triton-containing lysis buffer (as described in (1)) or the commercially available RIPA lysis buffer (89900, Thermo Fisher Scientific, Waltham, USA) supplemented with Complete Mini protease inhibitors (11836153001, Roche, Basel, Switzerland) and a phosphatase inhibitor cocktail (78420, Thermo Fisher Scientific, Waltham, USA). Protein concentrations were measured using the Pierce BCA Protein Assay Kit (23225, Thermo Fisher Scientific, Waltham, USA). 10-30 µg of protein and PageRuler Plus Prestained Protein Ladder (26619, Thermo Fisher Scientific, Waltham, USA) were loaded onto pre-cast Bolt 4-12 % Bis-Tris Plus gels (NW04120BOX, NW04122BOX, or NW04125BOX, Thermo Fisher Scientific, Waltham, USA) in MOPS-buffer (20X: 1 M MOPS (A1076, AppliChem, Darmstadt, Germany), 1 M Tris-Base; 20 mM EDTA; 69.3 mM SDS in H₂O). Protein was transferred to Amersham Protran 0.45 µm Western blot nitrocellulose membranes (GE10600002; GE Healthcare, Waukesha, USA) in 10% methanol (32213, Sigma Aldrich, St. Louis, USA) containing transfer buffer (20X: 500 mM Bicine (sc-216087A, Santa Cruz Biotechnology, Dallas, USA), 20 mM Bis-Tris (sc-216088A, Santa Cruz Biotechnology, Dallas, USA) 20 mM EDTA). After the transfer, membranes were blocked using 5% skim milk (70166-500G, Sigma Aldrich, St. Louis, USA) / TBS-T at room temperature for 60 min. After the incubation with primary antibodies at 4 °C overnight, the membranes were washed three times for 5 min in TBS-T. Incubation with a horseradish peroxidase (HRP)-coupled secondary antibody was performed at room temperature for 45-60 min. After three additional washing steps in TBS-T for 5 min, enhanced chemiluminescence (ECL) substrate was added (Immobilon WBKLS0100, Merck Millipore, Burlington, USA, or SuperSignal West Femto Maximum Sensitivity Substrate 34095, Thermo Fisher Scientific, Waltham, USA).
Amersham Hyperfilm ECL (GE28-9068-36, Cytiva, Marlborough, USA) and the COMPACT 2 NDT developer machine (PROTEC, Oberstenfeld, Germany) were used for visualization of HRP-induced light signals. Antibodies and complete Western blot scans can be found below.

**In vitro invasiveness (modified Boyden chamber assay)**

To test the functionality of ROR2 after labeling with the mScarlet-fluorophore, we performed an *in vitro* invasiveness assay (modified Boyden chamber assay) as previously published (2). MCF7 cells were transfected with the constructs pcDNA, ROR2, or ROR2_mScarlet. After 24 h, 1 × 10⁵ cells were seeded onto a polycarbonate membrane with a pore diameter of 10 µm (PC99CP81030, Pieper Filter, Bad Zwischenahn, Germany) coated with ECM (3432-005-01, Cultrex PathClear Basement Membrane Extract, Bio-Techne, Minneapolis, USA). As a positive control, recombinant Wnt5a (645-WN-010/CF, Bio-Techne, Minneapolis, USA) was added to one well containing control cells (MCF7 pcDNA) (2). Cells were grown for a total of 96 h. Tumor cell invasive capacity was measured by counting the number of cells penetrating the ECM in the lower wells and relating the number of cells to the MCF7 pcDNA control cells. Three biologically independent experiments were performed.

**Double thymidine block and quantification of mitotic condensate formation**

Before quantifying the Dvl2_mEos3.2 condensate-positive mitotic cells, cells were synchronized using a double thymidine block (10) to enrich the mitotic cells. In brief, HEK293T and HEK293T<sub>Dvl2</sub> mEos were seeded in flat-bottom black polystyrene 96-well plates with µClear bottom (655090, CELLSTAR Greiner Bio-one, Frickenhausen, Germany). 24 h later, thymidine (T1895, Sigma Aldrich, St. Louis, USA) was added at a final concentration of 2 mM. After overnight incubation, the thymidine-containing medium was removed, and the cells were carefully washed three times with pre-warmed PBS, followed by incubation with thymidine-free medium for 9 h. Subsequently, the cells were again incubated with 2 mM thymidine-containing medium for 18 h to arrest cells in G1/S. The cells were washed again and the medium was replaced with thymidine-free medium. Following the release into the cell cycle, the cells were imaged after 0, 6, 8, 10, and 12 h. For evaluation of mitotic cells, nuclear staining was performed using Hoechst 30 min before imaging. The highest fraction of mitotic cells was observed 8-10 h after the double thymidine block. Wide-field images for condensate quantification were taken with an InCell Analyzer 6000 (GE Healthcare, Buckinghamshire, United Kingdom). Manual counting of mitotic cells with and without Dvl2_mEos3.2 condensates was performed in four fields-of-view per experiment (20x magnification).

**Live-cell imaging**

24-48 hours before live-cell imaging, 1x10⁵ cells were transferred into 8-well ibidi µ-Slides (80826, ibidi, Gräfelfing, Germany). For membrane visualization, cells were carefully washed once with PBS. CellMask Deep Red membrane stain (1:1000 in FCS-free RPMI; C10046 Thermo Fisher Scientific, Waltham, USA) was added for 5-10 min at 37 °C. Cells were again carefully washed with PBS (10010056, Thermo Fisher Scientific, Waltham, USA). Live-cell imaging was performed with either of the following set-ups A) phenolred-free Leibovitz medium (10 % FCS; 21083027 Thermo Fisher Scientific, Waltham, USA) or B) phenolred-free DMEM (10 % FCS; 31053028, Thermo Fisher Scientific, Waltham, USA) using a point-scanning A1 or AX Nikon confocal microscope and a Nikon 60x water immersion objective (NA 1.27) (both Nikon Europe BV, Amsterdam, Netherlands), see description in live-cell time-lapse imaging. In all confocal images, mEos3.2 is shown after excitation with 488 nm wavelength.

**Live-cell time-lapse imaging**
Images were acquired under physiological conditions using the point-scanning A1 Nikon confocal microscope with a stage top incubation unit (TokaiHit, Fujinomiya, Japan). Images were acquired every 15 minutes for 15 hours with a 40x objective (NA 0.95). The NIS-Elements software 5.2 (Nikon Europe BV, Amsterdam, Netherlands) was used for image processing. For time lapse data, denoising was performed with the inbuilt Denoise AI function. Maximum projections of four slices spanning a total of 3 µm are shown.

**Incucyte live-cell proliferation analysis**

Proliferation curves were generated using an Incucyte live-cell microscope. 2.5 x10³ cells were seeded in clear Greiner flat-bottom polystyrene 96-well plates with µClear bottom (655087, CELLSTAR Greiner Bio-one, Frickenhausen, Germany) and cultured under physiological conditions for the indicated time. Proliferation curves were generated using the manufacturer’s software.

**Immunofluorescence staining**

24-48 hours before fixation, cells were seeded on 10 mm coverslips in 24-well plates or ibidi μ-8-well slides. Cells were fixed with 2-4 % PFA (28908, Thermo Fisher Scientific, Waltham, USA) for 7-20 min. For methanol fixation (gamma-tubulin), cells were incubated with ice-cold methanol (32213, Sigma Aldrich, St. Louis, USA) at -20 °C for 15 minutes. Then cells were washed twice with PBS and permeabilized with 0.2 % Triton-X100 in PBS for 10 min. After blocking with 5 % normal goat serum (5425S, Cell Signaling, Danvers, USA) in PBS for 1 h, the indicated primary antibodies (1:50-500; see Appendix) were incubated for 2 h at room temperature or overnight at 4 °C followed by secondary antibodies (1:500) and Hoechst nuclear dye (H1399, Thermo Fisher Scientific, Waltham, USA; 1:1000) in the dark at room temperature for 1 h. The cells were washed three times with PBS before imaging in ibidi slides or mounting the coverslips onto a microscopy slide with Prolong Gold/Diamond (P36934/P36961, Thermo Fisher Scientific, Waltham, MA, USA). Fixed samples were imaged with a spinning disk confocal microscope based on the Yokogawa head X1 on a Nikon Ti-E inverted microscope operated with Volocity software (PerkinElmer UltraVIEW VoX). Confocal stacks with 400 nm distance were acquired with a Nikon 60x water immersion objective (NA 1.27) and an EM-CCD camera (Hamamatsu C9100-02, Hamamatsu, Japan). Alternatively, the SP8 confocal microscope, a 40X oil-immersion objective, and the LasX software (Leica Microsystems, Wetzlar, Germany) were used. If indicated, maximum intensity projections for better localization of the Dvl2_mEos3.2 condensates in relation to other cellular structures were generated using Fiji (11).

**Condensate quantification**

For automated condensate quantification, 2500 cells (HEK293T, HEK293T_Dvl2_mEos, HEK293T_Dvl2_mEos EVI[KOEX1_1] and EVI[KOEX1_3] and HEK293T_Dvl2_mEos mFZDKO) per well were seeded in DMEM supplemented with 10% FCS into 384-well black, flat-bottom µClear plates (781092, Greiner, Frickenhausen, Germany). If indicated, a reverse siRNA transfection was performed when seeding the cells. If indicated, plasmid transfection was done 24 h after cell seeding. Treatment with murine rec. Wnt3a was performed at the recorded timepoints before cell fixation. 72 hours after seeding, cells were fixed with PFA. Staining was done with DyLight 650 Phalloidin (1:500) (12956S, Cell Signaling, Danvers, USA) and Hoechst (1:3000). Cells were imaged with 20x magnification (Nikon SAC 20x objective, NA = 0.45) using a InCell Analyzer 2200 (GE Healthcare, Buckinghamshire, United Kingdom). Per image and channel, a 16-bit grey-scale image (2048 x 2048 pixels) was acquired. Per cell line, at least three individual experiments were performed. For each experiment comprising Figure 4F, four fields of view per well in 240 wells on multiple plates were imaged. For each plate, 16 representative fields of view were chosen for automated condensate quantification (in total 112 fields of view from seven plates per cell line). For each experiment in Figure 4G, 15 wells with four fields of view per well were analyzed per cell line, treatment and timepoint (in total 2880 fields of view from four
plates). For each experiment in Figure S8C, 42 wells with four fields of view per well were analyzed per condition (in total 1512 fields of view from three plates). An automated image analysis using R/EBImage was used to quantify condensates (12). In a first step, single cells were detected based on the selective staining of DNA with Hoechst and F-Actin with Phalloidin. In brief, intensity-based thresholding and region growing was used to identify individual nuclei and cell bodies based on the DNA and F-actin staining. In a second step, condensates were identified per image using intensity-based thresholding on the FITC:mEos signal inside regions covered by cells. For downstream analysis, the number of cells and the condensate count per image were saved as an R data frame. To compare the condensate counts between cell lines the number of detected condensates was normalized per 100 cells in each image and further averaged per plate. For plotting and statistical testing, the R packages ggplot and ggbubr were used. Statistical significance was calculated using the Wilcoxon signed rank test.

We manually quantified condensates on confocal mini stack and widefield images. The quantification of confocal mini stack images was performed on pictures of fixed cells acquired on a Nikon Ti-E inverted microscope for colocalization experiments (60x magnification, fixation of cells 48 h after cell seeding). Here, we analyzed 100 cells per replicate in four replicates. The quantification of Dvl2_mEos3.2 condensates was performed on widefield fluorescent images of fixed cells taken with the InCell Analyzer 2200 for automated condensate quantification (20x magnification, fixation of cells 72 h after cell seeding). Manual counting of 100 cells in three different fields-of-view per replicate, four replicates.

Structured illumination microscopy (SIM)

For SIM, immunofluorescence stainings were performed as described above. Samples were imaged on a Nikon N-SIM system on a Ti-E equipped with TIRF Apochromat, 100x/1.49 NA oil immersion objective (both Nikon Europe BV, Amsterdam, Netherlands), and an EM-CCD camera (Andor iXon DU-897, Oxford Instruments, Abingdon, United Kingdom). The system is equipped with 405, 488, 561 and 640 nm laser. Acquisition and reconstruction of 3D-SIM was run with NIS-Elements 4.6 software (Nikon Europe BV, Amsterdam, Netherlands).

Single-molecule localization microscopy using PALM and DNA-PAINT

For single-molecule localization microscopy, cells were seeded on round 24 mm #1.5HP coverslips (PK26.1, Carl Roth, Karlsruhe, Germany) in 6-well plates. For DNA-PAINT, cells were fixed with 4 % PFA for 10 minutes, washed, and permeabilized with 0.2 % Triton X-100. The CEP164 primary antibody was incubated for 60 min at a dilution of 1:300. Oligo-nucleotide labeled secondary anti-mouse antibodies and the complementary DNA-PAINT imager strands labeled with Atto655 were used according to the manufacturer's instructions (Massive-AB 1-Plex DNA-PAINT kit anti-mouse atto655, Massive Photonics, Gräfeling, Germany). In brief, after washing the cells three times with PBS after primary antibody incubation, slides were washed once with the washing buffer. Secondary DNA-PAINT antibodies were diluted 1:100 in the antibody incubation buffer. After incubation for 1 h at room temperature, slides were washed three times and stored until imaging in the washing buffer. Right before imaging, slides were washed once in the imaging buffer and then mounted in imaging buffer containing 1 nM imager strands on slides with a central, single depression well (5916600, Carl Roth, Karlsruhe Germany). Coverslips were sealed with two-component silicone glue (13001000, Picodent Twinsil, Wipperfürth, Germany). For two-color imaging, mEos3.2 was sequentially imaged after the DNA-PAINT acquisition without buffer exchange.

For PALM-imaging of mEos3.2 only, cells were fixed with 4 % PFA for 10 minutes, washed, and the coverslips were mounted in PBS prepared with (90 %) D2O as described above.

Super-resolution microscopy was performed on a Leica SR GSD microscope (Leica Microsystems, Wetzlar, Germany) and using a HC PL APO 160x / NA 1.43 oil objective and an Andor iXon 897 EMCCD
camera (Oxford Instruments, Abingdon, United Kingdom) controlled by LasX software (Version 1.9.0.13747). Fluorophores were excited by 488 nm (mEos3.2 - green form), 532 nm (mEos3.2 - red form), and 640 nm (Atto655) lasers (300, 500, and 500 mW maximum power output, respectively). Photo-conversion of mEos3.2 was conducted with a 405 nm laser (50 mW maximum power output). Image data analysis was performed using LasX and ImageJ/Fiji in part using the ThunderSTORM plugin (13). High-density data was processed with the HAWK-plugin prior to ThunderSTORM processing (14).

**Statistical Analysis**

If not stated otherwise, a two-tailed Student t-test was used to calculate the statistical significance.

### siRNAs

| Name                                           | Supplier                  | Catalogue Number       |
|------------------------------------------------|---------------------------|------------------------|
| Silencer Select Negative Control #2 siRNA      | Ambion; Thermo Fisher Scientific | 4390846                |
| Human CTNNB1 siGENOME siRNA, set of 4          | Dharmaco; Horizon Discovery | MQ-003482-00-0002      |
| Human DVL1 siGENOME siRNA #6                   | Dharmaco; Horizon Discovery | D-004068-06            |
| Human DVL2 siGENOME siRNA #2 (in Fig. 1C #1)   | Dharmaco; Horizon Discovery | D-004069-02            |
| Human DVL2 siGENOME siRNA #3 (in Fig. 1C #2)   | Dharmaco; Horizon Discovery | D-004069-03            |
| Human DVL2 siGENOME siRNA #4 (in Fig. 1C #3)   | Dharmaco; Horizon Discovery | D-004069-04            |
| Human DVL3 siGENOME siRNA #1                   | Dharmaco; Horizon Discovery | D-004070-01            |
| Human UBC siGENOME siRNA #1                    | Dharmaco; Horizon Discovery | D-019408-01-0002       |
| Human UBC siGENOME siRNA #2                    | Dharmaco; Horizon Discovery | D-019408-02-0002       |
| Human UBC siGENOME siRNA #3                    | Dharmaco; Horizon Discovery | D-019408-03-0002       |
| Human UBC siGENOME siRNA #4                    | Dharmaco; Horizon Discovery | D-019408-04-0002       |

### Oligonucleotides

| Name                                           | Sequence 5’ to 3’ | Description |
|------------------------------------------------|-------------------|-------------|

27
D2mEosF1.FOR  
```
agcaggctcgcggccCTCGAGatggcgggtagc
gacacttg
```
InFusion cloning pcDNA DVL2_mEos3.2, Insert 1 Dvl2, forward primer

D2mEosF1.REV  
```
TCCGCCGGAGCCCCCTCCGCcataacat
ccacaaagaactgctgggat
```
InFusion cloning pcDNA Dvl2_mEos3.2, Insert 1 Dvl2, reverse primer

D2mEosF2.FOR  
```
gttctttgtgatgttagGGCGGAGGG
```
InFusion cloning pcDNA DVL2_mEos3.2, Insert 2 mEos3.2, forward primer

D2mEosF2.REV  
```
agaaagctgggtcggcgcgccTCATCTTCTGGC
ATTGTCAGGCAATCCAG
```
InFusion cloning pcDNA Dvl2_mEos3.2, Insert 2 mEos3.2, reverse primer

Dvl2_Cterm_fw  
```
AGGGTGGATGAGAGGGGAG
```
Sequencing primer: verification of an endogenous insertion of the DVL2_mEos3.2 gBlock

Dvl2_3-UTR_rev  
```
CAGGCTGCTGTCTAGGATGC
```
Sequencing primer: verification of an endogenous insertion of the DVL2_mEos3.2 gBlock at DVL2 locus, binds 3'-UTR of Dvl2 behind homology arm of the gBlock in reverse direction

DVL2-gBlock_fwd  
```
GAGGCCCGAGGAGCGGGC
```
Sequencing primer: verification of endogenous insertion of the DVL2_mEos3.2 gBlock, primer binds the very beginning of the gBlock DVL2_mEos3.2

DVL2-gBlock_rev  
```
TACCGCTGACCACCCCCAACC
```
Sequencing primer: verification of endogenous insertion of mEos3.2 gBlock, primer binds the end of the gBlock DVL2_mEos3.2

pROR2_F1.FOR  
```
aaaaagcaggctcgcggccCTCGAGATGGCC
CGGGGCTCGGCG
```
InFusion cloning pcDNA ROR2 and ROR2_mScarlet, Insert 1 ROR2, forward primer

pROR2_1_F1.REV  
```
gccgcgcacccctcagaatTCAAGCTTCCAGC
TGGACTTGGGCTCCTG
```
InFusion cloning pcDNA ROR2, reverse primer, overhangs for integration into pcDNA3.2

pROR2_1ms_F1.REV  
```
TGCCCTCGCCCTTGTCTCAATCgtagcAG
CTTCCAGCTTGGACTTGCGGCTCCTG
```
InFusion cloning pcDNA ROR2_mScarlet, Insert 1 ROR2, reverse primer,
overhangs to anneal with Insert 2 (mScarlet)

pROR2_1mS_F2.FOR

CCCAAGTCCAGCTGGAAGCTgctagcatgg
tgcaagggcgaggg

InFusion cloning pcDNA ROR2_mScarlet, Insert 2 mScarlet, forward primer

pROR2_mS_F2.REV

GCGCGCcaccctctcagaattTCAacttacagctc
gtcatgccGC

InFusion cloning pcDNA ROR2_mScarlet, Insert 2 mScarlet, reverse primer

FZD7_fwd

tctacacctacttggttgacatgc

Sequencing primer FZD7, forward primer

DVL2_SNPy_fwd

agaggacgtcatctctcagcag

Sequencing primer DVL2, forward primer

mEos3.2_rev

tgtgcctcgaatatggatactgtgg

Sequencing primer mEos3.2, reverse primer

NP_Evi_ex1_If

TCCCTACAGACGctcttcgatctATGGCTG
GGGCAATT ATAGA

Sequencing primers for indel-nested PCR 1, EVI Exon 1

NP_Evi_ex1_Ir

AGTTCAACGACGTGctcttcgatctTCATAG
AAGCAAGG CAGTGA

Sequencing primers for indel-nested PCR 1, EVI Exon 1

NP_Evi_ex1_2f

TCCCTACAGACGctcttcgatctAATGGCT
GGGGCAAT TATAG

Sequencing primers for indel-nested PCR 1, EVI Exon 1

NP_Evi_ex1_2r

AGTTCAACGACGTGctcttcgatctGATCAT
AGAAGCAGG CAGTGA

Sequencing primers for indel-nested PCR 1, EVI Exon 1

Plasmids

Expression constructs

| Name             | Source                        | Catalogue Number/reference |
|------------------|-------------------------------|----------------------------|
| beta-catenin     | pcDNA3 beta-catenin           | M.B. laboratory             |
| DVL2_mEos3.2     | pcDNA Dvl2_mEos3.2            | -                           |
| DVL2             | pDONOR DVL2 (BC014844)        | DKFZ-vector and clone       |
|                  |                               | repository                  |
| FZD7_tagRFP      | pEXP-Fzd7_tagRFP              | -                           |
| pcDNA            | pcDNA3.2/V5-DEST              | 12489019                    |
| Name                        | sgRNA sequence (5'→3')                  | Source/reference                        |
|-----------------------------|----------------------------------------|----------------------------------------|
| pDEST26-RFP-C               | pDEST26-RFP-C (tagRFP), TagRFP-C (6xHis-NT) | DKFZ-vector and clone repository       |
| pENTR223 hFZD7              | hFzd7 (BC015915)                       | DKFZ-vector and clone repository       |
| Renilla luciferase          | pAct-RL, Renilla luciferase             | D. Nickles, M.B. laboratory            |
| ROR2                        | pcDNA ROR2.1                            | -                                      |
| ROR2_mScarlet               | pcDNA ROR2.1_mScarlet                   | -                                      |
| s-luc                       | Secreted luciferase                     |                                        |
| TCF4/Wnt firefly luciferase | 6xKD; pGL4.26 6xTcF-Firefly luciferase  | K. Demir, M.B. laboratory              |
| Wnt3                        | pcDNA Wnt3                              | Addgene #35909                         |
| Wnt3a                       | pcDNA Wnt3a                             | Addgene #35908                         |
| Wnt3a-mCherry               | pCS2+ mCherry-mWnt3a                    | Takada laboratory                      |
| Wnt5a                       | pcDNA Wnt5a                             | Addgene #35911                         |
| Wnt5a mScarlet              | pcDNA Wnt5a-mScarlet                    | A. Schubert                             |

**CRISPR/Cas9 constructs**

| Name                        | sgRNA sequence (5'→3')                  | Source/reference                        |
|-----------------------------|----------------------------------------|----------------------------------------|
| PX459 pSpCas9(BB)-2A-Puro   | -                                      | Addgene 48139 (20)                      |
| PX459 sgDVL1,2,3_II         | GTACATTGGCTCCATCATGAA                   | This publication: sgRNA sequence for DVL1,2,3<sup>Δ</sup> |
| PX459 sgDVL2_142            | gCCATGGCCATGTGGAAGCTT                   | This publication: sgRNA for mEos3.2 knock-in |
| PX459 sgDVL2_225            | GGCCTCCTGTTGTTGTACT                     | This publication: sgRNA for mEos3.2 knock-out |
| PX459 sgEos2.1              | gCGTATGGAAAGGCAAAGTAAA                  | This publication: sgRNA for mEos3.2 knock-out |
| PX459 sghEvi_ex1_1_70       | GCAAATCATCGCCTTTCTGGT                   | This publication: sgRNA sequence for Evi<sup>Δ</sup> |
| PX459 sghEvi_ex1_3_70       | GAATCAAGCCTCACCAGAA                    | This publication:                           |
### sgRNA sequence for EVI\(^{KOE1,3}\)

| PX459 sgFZD1,2,5,7,8,(4) | gATGGCCAGCTCCATCTGGT |

### Antibodies

| Target                  | Species | Supplier                     | Catalogue Number | Usage | Dilution |
|-------------------------|---------|------------------------------|------------------|-------|----------|
| **primary antibodies**  |         |                              |                  |       |          |
| AAT                     | mouse   | Sigma-Aldrich                | T7451            | IF    | 1:500    |
| beta-actin -HRP         | mouse   | Santa Cruz Biotechnology     | sc47778-HRP      | WB    | 1:5000   |
| APC (C-term)            | rabbit  | Abcam                        | ab15270          | IF    | 1:300    |
| Axin1                   | rabbit  | Cell Signaling Technology    | 2087S            | IF    | 1:300    |
| Calnexin                | mouse   | Santa Cruz Biotechnology     | sc11397/23954    | IF    | 1:100    |
| CD107a (LAMP1)          | mouse   | BioLegend                    | 328601           | IF    | 1:50     |
| CEP164                  | rabbit  | Novus Biologicals           | NBP1-81445       | IF    | 1:300    |
| Dvl1 (3F12)             | mouse   | Santa Cruz Biotechnology     | sc8025           | WB    | 1:1000   |
| Dvl2                    | rabbit  | Cell Signaling Technology    | 3216S            | WB    | 1:1000   |
| Dvl2 (30D2)             | rabbit  | Cell Signaling Technology    | 3224S            | IF    | 1:50     |
| Dvl3 (4D3)              | mouse   | Santa Cruz Biotechnology     | sc8027           | WB    | 1:1000   |
| Dvl pan (B-4)           | mouse   | Santa Cruz Biotechnology     | sc166303         | WB    | 1:1000   |
| Evi / Wls/ GPR177 YJ5   | mouse   | BioLegend                    | 655902           | WB    | 1:1000   |
| GM130                   | mouse   | eBiosciences                 | 610823           | IF    | 1:50     |
| HSC70                   | mouse   | Santa Cruz                   | 7298             | WB    | 1:1000   |
| LRP6 (C5C7)             | rabbit  | Cell Signaling               | 2560             | IF    | 1:50     |
### Protein Antibodies

| Name                  | Species    | Supplier                          | Catalog # | Dilution          |
|-----------------------|------------|-----------------------------------|-----------|-------------------|
| PCM1                  | rabbit     | Sigma-Aldrich                     | HPA023370 | IF 1:300          |
| Proteasome            | mouse      | Abcam                             | 55628-100 | IF 1:300          |
| RFP                   | rabbit     | Invitrogen; Thermo Fisher Scientific | R10367    | WB 1:1000 - 1:3000 |
| ROR2 (6FD10)          | mouse      | ProMab                            | 30623     | WB 1:1000         |
| alpha-tubulin         | mouse      | Sigma-Aldrich                     | T9026     | IF 1:50           |
| gamma-tubulin         | mouse      | Sigma-Aldrich                     | T5326     | IF 1:300          |
| Vimentin (D21H3)      | rabbit     | New England Biolabs              | 5741S     | IF 1:300          |
| Wnt3(a)               | rabbit     | GeneTex                           | GTX128101 | WB 1:1000         |
| Wnt5a/b (C27E8)       | rabbit     | Cell Signaling Technology         | 2530      | WB 1:1000         |

#### Secondary Antibodies

| Antibody              | Species    | Supplier                          | Catalog #   | Dilution |
|-----------------------|------------|-----------------------------------|-------------|----------|
| mouse IgG (HRP)       | goat       | Jackson ImmunoResearch            | 115-035-003 | WB 1:2000 |
| rabbit IgG (HRP)      | goat       | Jackson ImmunoResearch            | 111-035-003 | WB 1:2000 |
| mouse IgG (AF568)     | goat       | Invitrogen; Thermo Fisher Scientific | A11004   | IF 1:500   |
| mouse IgG (AF647)     | goat       | Invitrogen; Thermo Fisher Scientific | A21240   | IF 1:500   |
| rabbit IgG (AF647)    | goat       | Invitrogen; Thermo Fisher Scientific | A21245   | IF 1:500   |
| mouse IgG (AF633)     | goat       | Invitrogen; Thermo Fisher Scientific | A21126   | IF 1:500   |
| rabbit IgG (AF633)    | goat       | Invitrogen; Thermo Fisher Scientific | A21070   | IF 1:500   |

**Massive-AB 1-Plex DNA-PAINT kit anti-mouse atto655** (Massive Photonics GmbH, Gräfelfing, Germany)

### Dyes

| Name                      | Supplier                          | Dilution |
|---------------------------|-----------------------------------|----------|
| CellMask Deep Red         | Thermo Fisher Scientific, C10046   | 1:1000   |
| Stain                        | Supplier                          | Dilution          |
|------------------------------|-----------------------------------|-------------------|
| Hoechst                      | Thermo Fisher Scientific, H1399   | 1:1000            |
| DyLight 650 Phalloidin       | Cell Signaling, 12956S            | 1:500-1000        |
SI References

1. O. Voloshenko, P. Gmach, J. Winter, D. Kranz, M. Boutros, Mapping of Wnt-Frizzled interactions by multiplex CRISPR targeting of receptor gene families. The FASEB Journal 31, 4832–4844 (2017).

2. T. Pukrop, et al., Wnt 5a signaling is critical for macrophage-induced invasion of breast cancer cell lines. Proc. Natl. Acad. Sci. U. S. A. 103, 5454–5459 (2006).

3. D. S. Bindels, et al., mScarlet: a bright monomeric red fluorescent protein for cellular imaging. Nat. Methods 14, 53–56 (2017).

4. F. Heigwer, G. Kerr, M. Boutros, E-CRISP: fast CRISPR target site identification. Nat. Methods 11, 122–123 (2014).

5. M. Zhang, et al., Rational design of true monomeric and bright photoactivatable fluorescent proteins. Nat. Methods 9, 727–729 (2012).

6. F. Sievers, et al., Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol. 7, 539 (2011).

7. A. Untergasser, et al., Primer3--new capabilities and interfaces. Nucleic Acids Res. 40, e115 (2012).

8. K. Clement, et al., CRISPResso2 provides accurate and rapid genome editing sequence analysis. Nat. Biotechnol. 37, 224–226 (2019).

9. K. Glaeser, M. Boutros, J. C. Gross, "Biochemical Methods to Analyze Wnt Protein Secretion" in Wnt Signaling: Methods and Protocols, Methods in Molecular Biology., Q. Barrett, L. Lum, Eds. (Springer, 2016), pp. 17–28.

10. G. Chen, X. Deng, Cell Synchronization by Double Thymidine Block. Bio Protoc 8 (2018).

11. J. Schindelin, et al., Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682 (2012).

12. G. Pau, F. Fuchs, O. Sklyar, M. Boutros, W. Huber, EBlImage--an R package for image processing with applications to cellular phenotypes. Bioinformatics 26, 979–981 (2010).

13. M. Ovesný, P. Křižek, J. Borkovec, Z. Svindrych, G. M. Hagen, ThunderSTORM: a comprehensive ImageJ plug-in for PALM and STORM data analysis and super-resolution imaging. Bioinformatics 30, 2389–2390 (2014).

14. R. J. Marsh, et al., Artifact-free high-density localization microscopy analysis. Nat. Methods 15, 689–692 (2018).

15. D. Nickles, C. Falschlehner, M. Metzig, M. Boutros, A genome-wide RNA interference screen identifies caspase 4 as a factor required for tumor necrosis factor alpha signaling. Mol. Cell. Biol. 32, 3372–3381 (2012).

16. V. L. Katanaev, et al., Reggie-1/flotillin-2 promotes secretion of the long-range signalling forms of Wingless and Hedgehog in Drosophila. EMBO J. 27, 509–521 (2008).
17. K. Demir, et al., RAB8B is required for activity and caveolar endocytosis of LRP6. *Cell Rep.* **4**, 1224–1234 (2013).

18. R. Najdi, et al., A uniform human Wnt expression library reveals a shared secretory pathway and unique signaling activities. *Differentiation* **84**, 203–213 (2012).

19. R. Takada, et al., Assembly of protein complexes restricts diffusion of Wnt3a proteins. *Commun Biol* **1**, 165 (2018).

20. F. A. Ran, et al., Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* **8**, 2281–2308 (2013).