Supporting Online Material for

Wnt Induces LRP6 Signalosomes and Promotes Dishevelled-Dependent LRP6 Phosphorylation

Josipa Bilic, Ya-Lin Huang, Gary Davidson, Timo Zimmermann, Cristina-Maria Cruciat, Mariann Bienz, Christof Niehrs*

*To whom correspondence should be addressed. E-mail: Niehrs@DKFZ-Heidelberg.DE

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Materials and Methods

Constructs

All constructs used in this study have been described previously. Caveolin1-GFP construct was a gift from A. Helenius. Human Axin was subcloned into pECFP-N1 vector (Clontech) with and without intermitting stop codon, to produce wild type and ECFP-Axin fusion proteins, respectively.

Cell culture, transfections and *Dvl* knock down

All cell lines were cultured and Wnt3a conditioned medium was prepared as described (*1*). In all experiments involving LRP6 transfections, Mesd, mFz8, hAxin and GSK3β were co-transfected, to provide proportional amounts of key binding partners, except in Fig. 4A and Fig. S5. This proved to be essential for detection of phospho-LRP6 aggregates. Furthermore, in immunoflourescence experiments involving detection of endogenous LRP6 in P19 cells (Fig. S3A), 4 μM epoxomicine (Sigma) was added in the medium 2 hours during Wnt treatment.

For immunofluorescence cells were grown in 12 well plates on glass coverslips and transfected with the following amounts of DNA per well: 200 ng hLRP6-EYFP, 50 ng Mesd, 2 ng mFz8, 15 ng hAxin, 8 ng hGSK3β, 200 ng FLAG-hLRP6-ΔE1-4, 10 ng XDvl-GFP, 5 ng Caveolin-GFP, 5 ng Clathrin-L-chain-DsRed, 200 ng XCK1γ1^{K73R}, 200 ng M1-Dvl2, 200 ng M2-Dvl2. For sucrose gradient sedimentation assays MEF and HEK 293T cells were grown in 10 cm dishes. HEK 293T cells were transfected with: 2.5 μg hFLAG-LRP6, 640 ng Mesd, 18 ng mFz8, 260 ng hAxin, 100 ng GSK3β, 100 ng GFP, 2.5 μg FLAG-LRP6-ΔE1-4. All transfections were

performed with FuGene6 transfection reagent (Roche), and cells were harvested 24 hours post transfection.

For RNAi knock-down of *Drosphila* Dsh, S2R+ cells were transfected with 100ng pAc5.1-hLRP6 in 6-well format using effectene reagent (Qiagen) and plated in 96 wells with 1 μg dsRNA the next day, as previously described (*1*). After 5 days incubation with dsRNA, cells were treated for 1h with control or Wnt3a conditioned media and lysed in cholate buffer (1 % Na cholate, 50 mM Tris-HCl pH 7.0, 150 mM NaCl, 10 mM NaF, 5 mM Na_2VO_4, 1 mM PMSF and protease inhibitors). Lysates were analyzed by SDS-PAGE and immunoblot.

For siRNA knock-down of murine Dvl1,2,3 proteins, MEFs in 12-well plate were transfected with a 100 nM mixture of siRNA SmartPools (Dharmacon) targeting all 3 murine *dvl* transcripts using Dharmafect 1, according to manafacturer's protocol. 60 h after transfection, cells were treated for 15 min with control or Wnt3a medium, harvested in 0.5 ml low salt buffer (5 mM HEPES, pH 6.8, 1 mM $MgCl_2$, 10 mM sodium pyrophosphate, 10 mM NaF, 5 mM Na_2VO_4, 1 mM PMSF and protease inhibitors) and homogenized with 50 Dounce strokes. Nuclei were removed by centrifugation (5 min, 500g) and membranes pelleted (20 min, 13,000 rpm in a microfuge). Pellets were dissolved in cholate buffer and subjected to SDS-PAGE followed by immunoblot analysis.

For siRNA knock-down of human Dvl1,2,3, HEK 293T cells were transfected in 96-well plates in triplicates with a 50 nM mixture of siRNAs against *dvl1*, *dvl2* and *dvl3* and 50 nM siRNAs against *β-catenin* and non-targeting control siRNAs (Dharmacon) using Dharmafect 1 transfection reagent (Dharmacon). 24 h later, a luciferase reporter assay was carried out using

Fugene 6 transfection reagent (Roche). Per well a total of 100 ng DNA was transfected, including 20 ng pTOPFlASH, 8 ng pTK-Renilla, 10 ng mouse *Wnt1*, 7 ng *LRP6*, 2 ng mouse *frizzled8*, 20 ng *LRP6ΔE1-4*, 0.2 ng *X.tropicalis β-catenin*. 48 h after transfection, luciferase activity was determined using the Dual luciferase system (Promega). To test the efficiency of downregulation by siRNA, RNA levels of *Dvl1*, *Dvl2* and *Dvl3* were quantified by real time PCR using the LightCycler 480 with hydrolysis probes from Roche according to the manufacturer's protocol. Samples were normalized to beta actin.

Immunoflorescence, imaging and antibodies

Phosphorylated LRP6 was detected with Tp1479 antibody (*1*), total LRP6 was detected with T1479 antibody (*1*), Dvl2 was detected with antibody kindly provided by M. Semenov (*2*), and Axin antibody was kindly provided by R. Nusse. EEA1, TGN38, Calnexin and GSK3β antibodies, BD Bioscience; Cy3 conjugated anti-rabbit, FITC-conjugated anti-mouse, anti-GFP antibodies, Dianova; Anti-rabbit Alexa488, anti-rabbit Alexa546, Molecular Probes; monoclonal anti-FLAG M2, Sigma. Dextran Texas Red 10000 MW, lysine fixable was purchased form Invitrogen.

For HeLa and HEK 293T immunofluorescence staining, cells were treated 21h after transfection with Wnt3a or control conditioned medium for 3 hours before harvest, unless indicated otherwise. Cells were then processed as described (*3*). P19 cells were processed 24 hours after plating and treated for 2 hours with Wnt3a or control conditioned medium before harvest. For detection of endogenous LRP6 in P19 cells, an antigen retrieval method was used. Prior to antibody incubation, cells were microwave irradiated (90 W) for 3 min in 2.5mL 10mM sodium

citrate pH 6.0 and 0.05% Tween 20 (Sigma), left for 20 min to cool at RT and blocked with 1% BSA in PBS. For colocalization of endogenous LRP6 and Dvl2, or LRP6 and Axin proteins, a protocol was used that allows double antigen detection with two antibodies raised in rabbit (*4, 5*). P19 cells, after T1479 and anti-rabbit Alexa488 staining as described (*3*), samples were further blocked with 5% rabbit serum and unconjugated goat anti-rabbit IgG Fab fragments (1:25, Jackson ImmunoResearch Laboratories Inc.) for 1h. Explants were then post-fixed with 4% PFA and 0.2% glutaraldehyde for 10min. The second round of staining was performed with anti-Dvl2 or Axin antibody for 1h, followed by anti-rabbit Alexa546 for 1h at RT.

For detection of endogenous phosphorylated LRP6 proteins in *Xenopus* animal caps, explants were treated with Wnt3a or conditioned medium for 30 min at room temperature and then fixed in Dent's fixative overnight at -20°C. After rehydration, explants were blocked in 20% horse serum, 1% blocking reagent (Roche Molecular Biochemicals) in PBST. Tp1479 antibody was subtracted by incubating it overnight at 4°C with egg extract pre-bound to activated CH-Sepharose 4B (Sigma). Incubation with the primary antibodies Tp1479 overnight at 4°C was followed by anti-rabbit Alexa488 for 3 h at room temperature and Hoechst staining.

To permit double antigen detection with two antibodies raised in rabbit, after Tp1479 and anti-rabbit Alexa488 staining, explants were further blocked with 0.5% rabbit serum and unconjugated goat anti-rabbit IgG Fab fragments (1:20) for 1h respectively at room temperature. Explants were then post-fixed with 4% PFA for 15min at room temperature and microwave irradiated (630W) 6 times for 6.5min in 50mL 10mM sodium citrate (pH6.0) (with 1L tap water in the microwave tray) (*4, 5*). The second round of staining was performed on treated explants

with Dvl2 antibody (or H3 as control) at 4°C overnight, followed by anti-rabbit Alexa546 for 3 h at room temperature and Hoechst staining.

Confocal laser scanning of fixed cells was done on a Leica SP5 confocal microscope (Leica Microsystems, Germany). Live cell imaging was done on a Perkin Elmer Ultraview RS spinning disc confocal (Perkin Elmer Inc., CT, USA) connected to a Zeiss Axiovert 200 microscope. The live cell experiments were done with HeLa cells plated on glass bottom dishes (3.5 cm, MatTek, Ashland, MD, USA) at 37°C and 5% CO2 inside a microscope incubator box for environmental control (EMBLEM GmbH, Germany). The kymograph analysis was done using the Image J software available at http://rsb.info.nih.gov/ij/. *Xenopus* explants were examined on a confocal laser scanning microscope (Nikon C1Si).

Sucrose gradient sedimentation and coimmunoprecipitation

About 20 hours post transfection, HEK 293T cells were treated for 3 hours with Wnt3a or control conditioned medium. MEF cells were treated with 4 μM epoxomicine (Sigma) for 3 hours, and Wnt3a or control conditioned medium for 1 hour before harvest.

Cells were harvested in Hank's Balanced Salt Buffer on ice, pelleted, and lysed for 20 min in extraction buffer: 30 mM Tris (pH 7.3), 140 mM sodium chloride, 1% Triton X-100 (Sigma), 25 mM sodium fluoride, 3mM sodium ortho-vanadate, 2 mM PMSF, 100 nM okadaic acid and protease inhibitor cocktail tablete (Roche). Lysates were centrifuged, and supernatant was layered on top of a 15-40% sucrose gradient in 30 mM Tris (pH 7.3), 140 mM sodium chloride, 0.02% Triton X-100, 25 mM sodium fluoride, 3mM sodium ortho-vanadate and protease inhibitors.

Ultracentrifugation was done in a Beckman SW60 rotor at 43.000 rpm, 4 hours, 4°C. After centrifugation, fractions were collected from the bottom of the tube using a peristaltic pump and analyzed by SDS-PAGE and immunoblot.

For coimmunoprecipitation fractions were pooled, sucrose was diluted by adding 400 µl extraction buffer to 800 µl sample, and incubated for 4 h with FLAG M2 beads (Sigma) at 4°C. After washing with extraction buffer, proteins were eluted with Laemmli SDS-PAGE loading buffer at 50°C for 10 min.

References and notes

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3. C. M. Cruciat, C. Hassler, C. Niehrs, *J Biol Chem* **281**, 12986 (2006).
4. S. A. Lewis Carl, I. Gillete-Ferguson, D. G. Ferguson, *J Histochem Cytochem* **41**, 1273 (1993).
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SUPPLEMENTARY FIGURES:

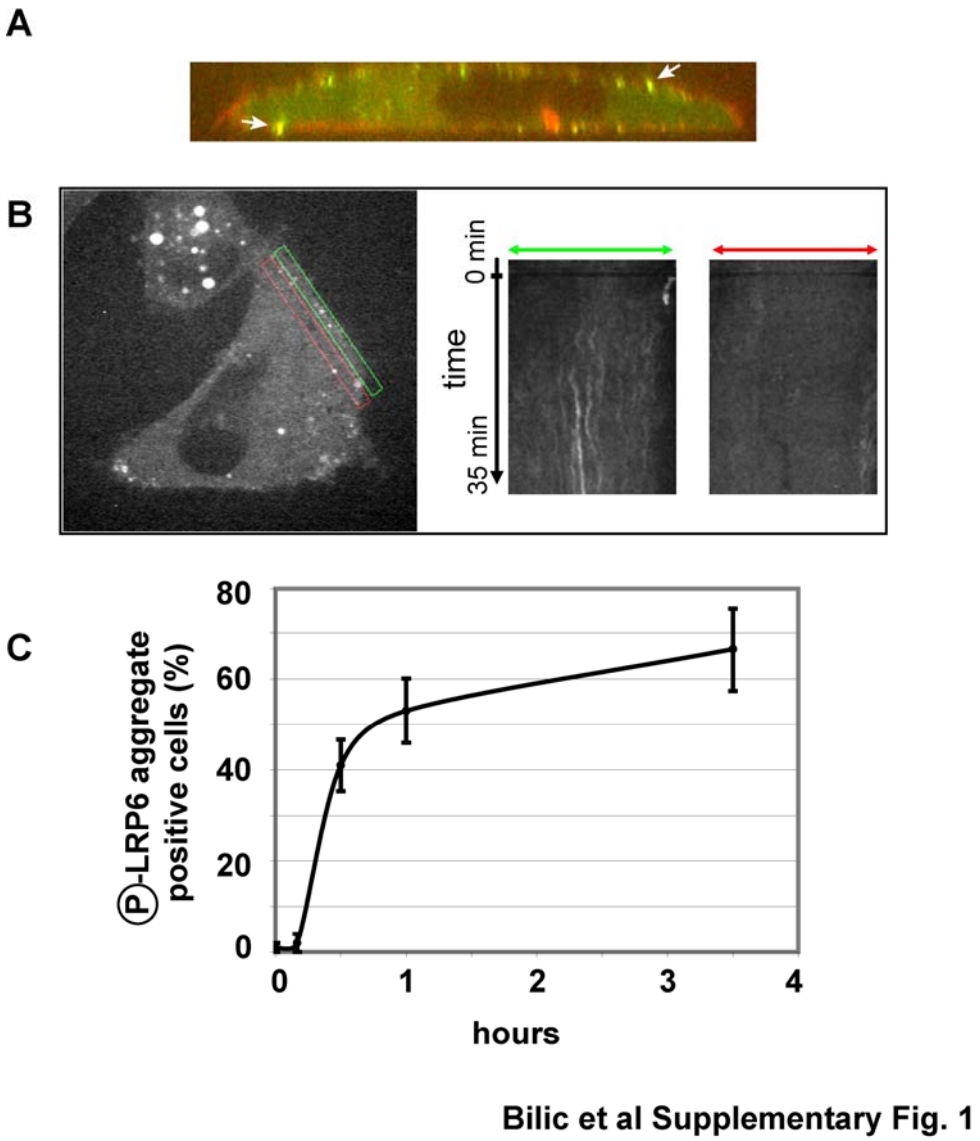


Fig. S1. (**A**) Confocal microscopy z- section of live HeLa cell expressing LRP6-EYFP (red) and ECFP-Axin (green), treated for 1 hour with Wnt. The two proteins colocalize (yellow, arrows) at the plasma membrane. (**B**) Kymograph (time/space plot) of ECFP-Axin expressing cell (left) life-imaged while treated with Wnt conditioned medium for 35 min. Two positions containing (green box) and not containing (red box) Axin aggregates are plotted. Note that the clusters show little change in size, or position following their formation during the analyzed interval. (**C**) Kinetics of phospho-LRP6 aggregate formation. HeLa cells were transfected with LRP6-EYFP and stimulated for 15 min, 30 min, 1 hour or 3.5 hours with Wnt conditioned medium. Cells were then fixed, stained with Tp1479 Ab and microscopically analyzed. LRP6-EYFP positive cells were scored for the presence of phospho-LRP6 aggregates.

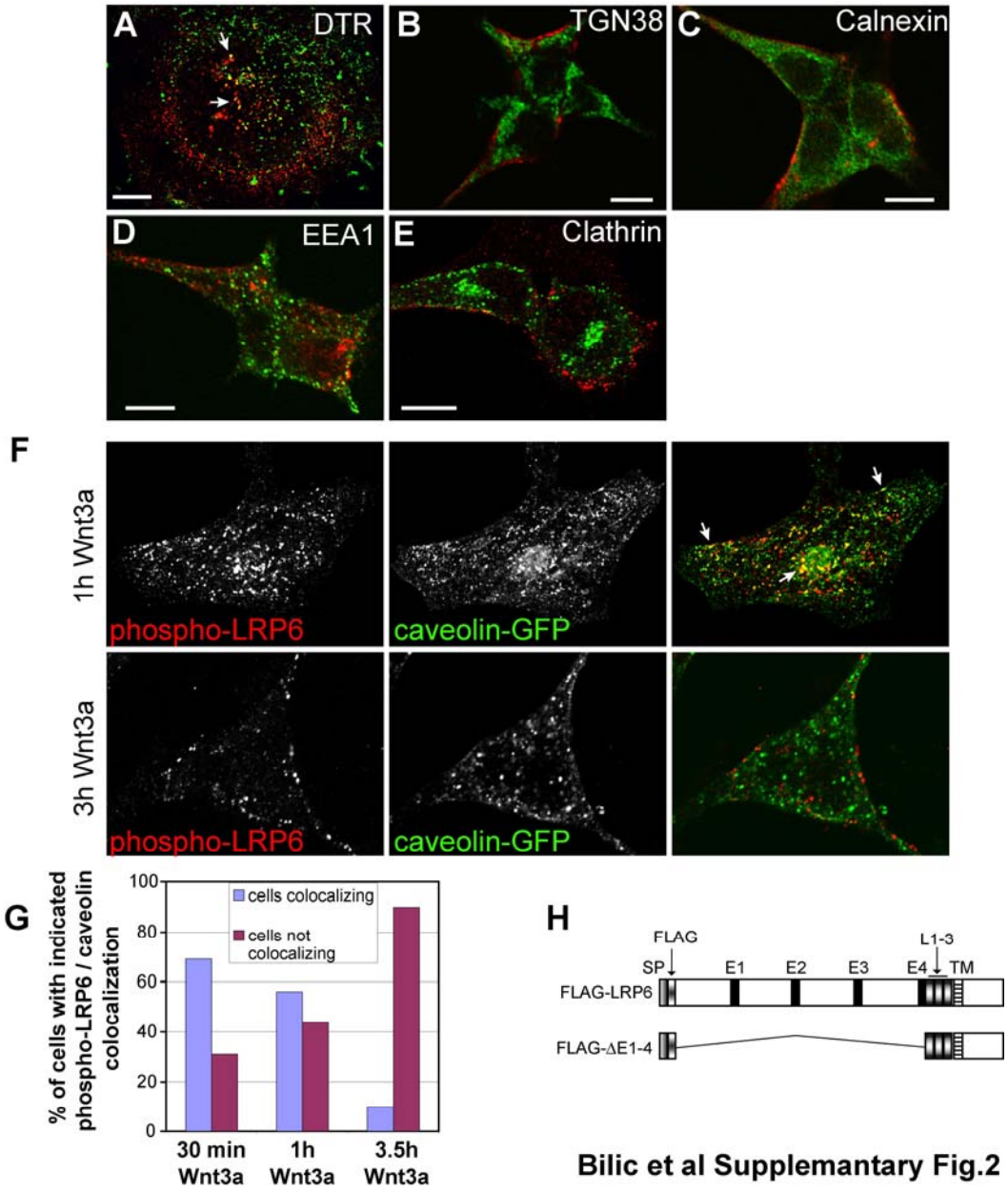


Fig. S2. (**A** to **E**) Confocal microscopy of transfected HeLa (A and E) or HEK 293T (B-D) cells, treated for 1h (A) or 3 hours (B and C) with Wnt, fixed and stained for phospho-LRP6 (red). Bar is 10μm. (A) Cells were simultaneously treated with Wnt and the fluid phase marker Texas Red Dextran (DTR, green). Arrows indicate occasional colocalization (yellow). (B) Phospho-LRP6 aggregates do not significantly colocalize with TGN38, (C) Calnexin, (D) EEA1 or (E) cotransfected DsRed-Clathrin light chain. (F) Confocal images showing phospho-LRP6 colocalization with Caveolin-GFP. (**G**) Cells as in (F) were analyzed for phospho-LRP6/Caveolin-GFP colocalization and scored positive if 30% or more of phospho-LRP6 aggregates were also Caveolin positive. (**H**) Schematic drawing of full length FLAG-tagged LRP6 receptor, and its constitutively active form ΔE1-4. SP, signal peptide; E1-4, EGF repeats; L1-3, LDL repeats; TM, transmembrane domain.

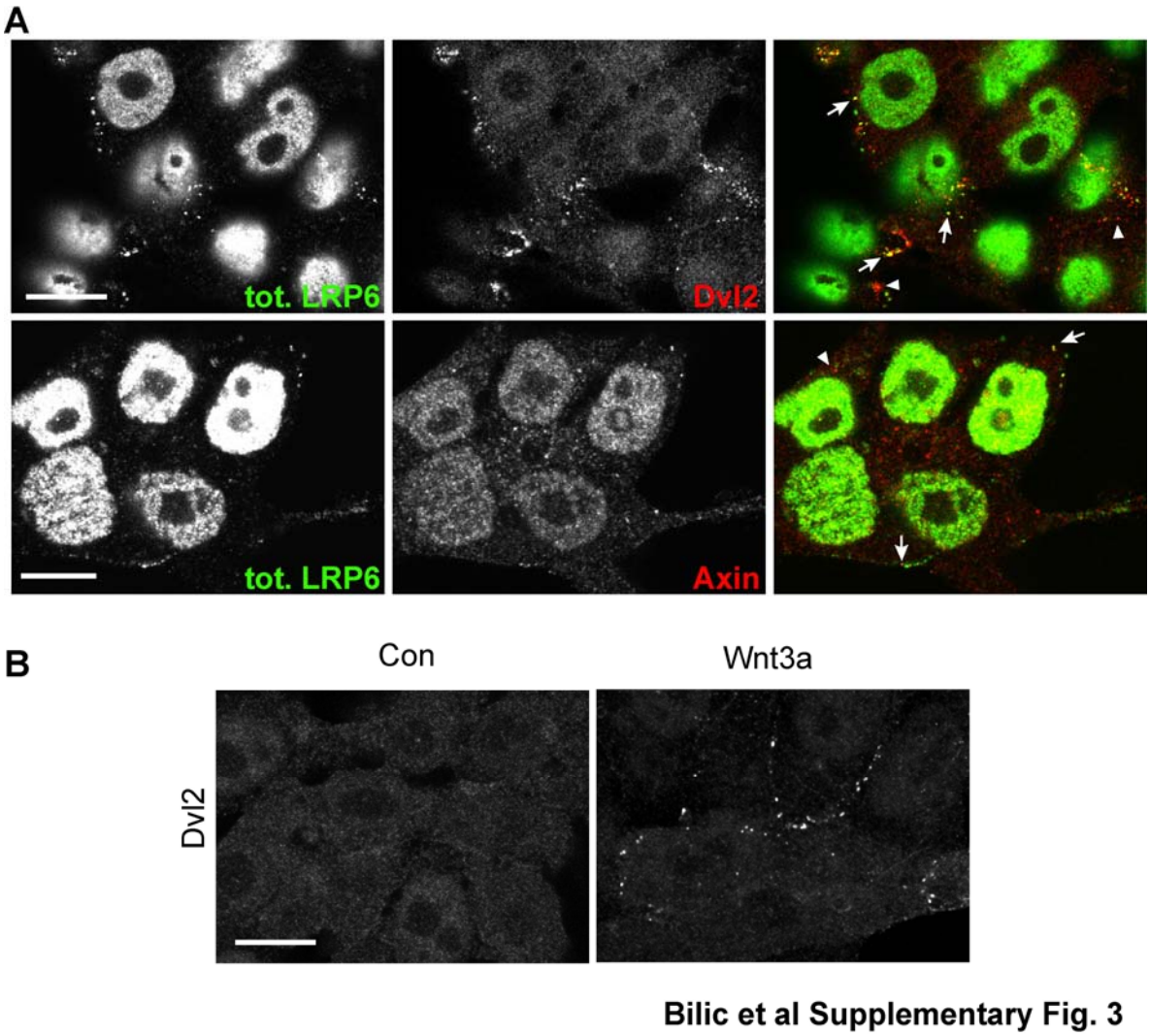
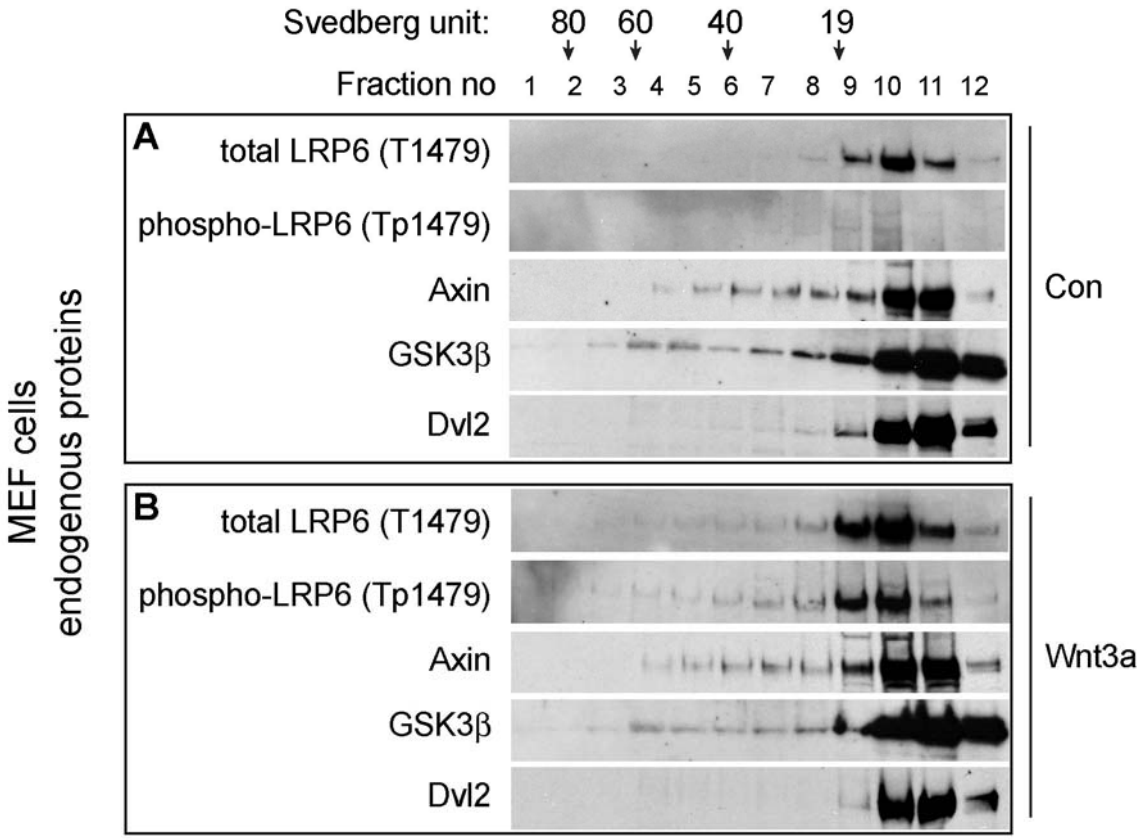


Fig. S3. (**A**) Colocalization of total LRP6 (T1479 Ab, green) and Dvl2 or Axin (red) in untransfected P19 cells. Cells were stimulated for 2h with Wnt. Colocalizing (yellow) aggregates are indicated by arrows. Dvl2 or Axin aggregates not colocalizing with LRP6 are indicated by arrowheads. Note that nuclear staining is LRP6-unrelated cross-reactivity, not detected in nuclear lysates by immunoblot. (**B**) Immunofluorescence of endogenous Dvl2 aggregates in P19 cells treated for 2h with control (Con) or Wnt3a conditioned medium. Bar is 10 μm.



Bilic et al Supplementary Figure 4.

Fig. S4. Sucrose gradient sedimentation of Triton X-100 lysates of mouse embryonic fibroblasts (MEF) treated for 1h with control (**A**) or Wnt3a conditioned medium (**B**). Total and phospho-LRP6, Axin, GSK3β and Dvl2 were analyzed by immunoblot. Note Wnt dependent LRP6 aggregates in fractions 3-8. Axin and GSK3β aggregates are Wnt independent.

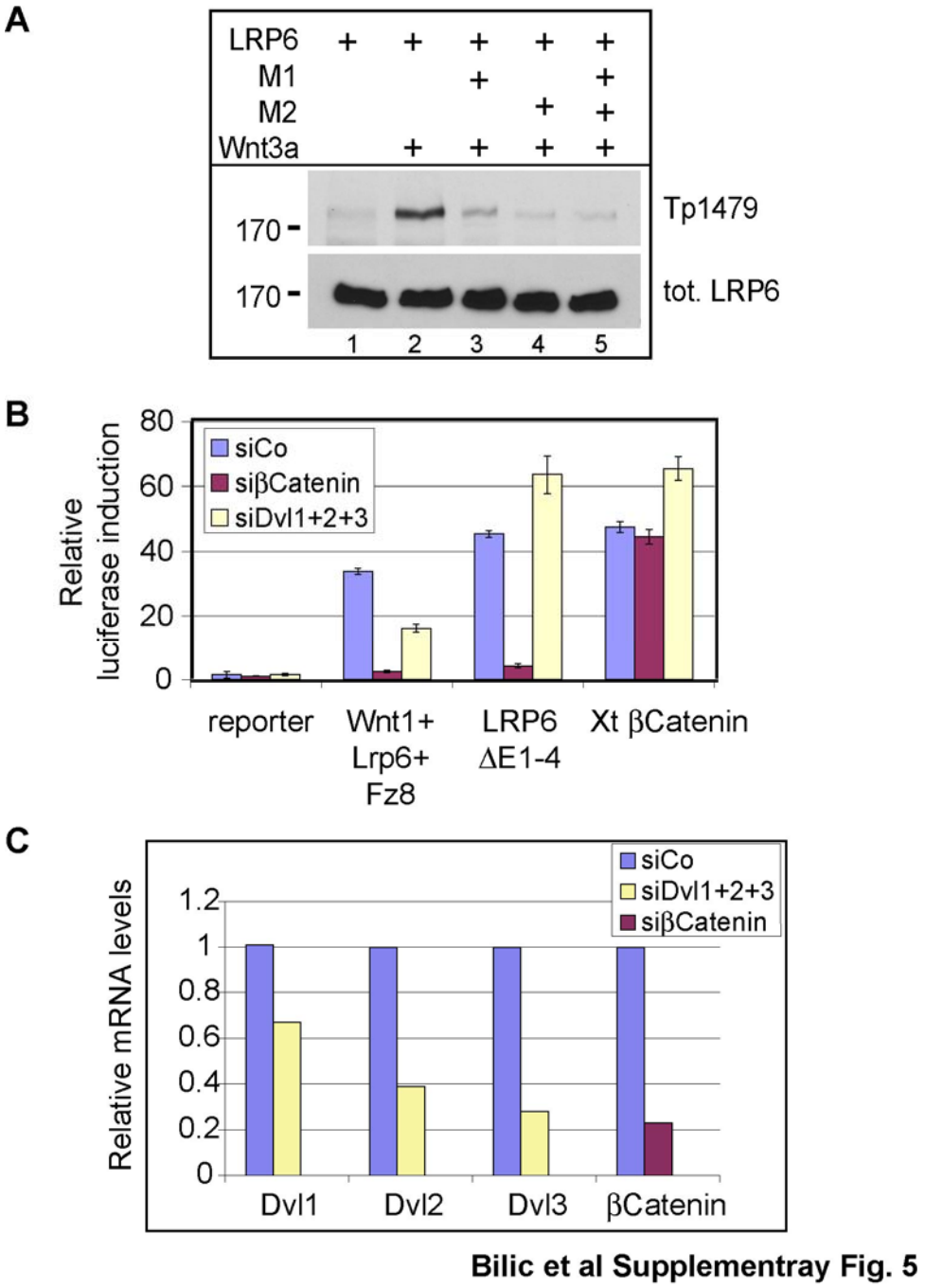


Fig. S5. (**A**) Immunoblot of HEK 293T cells transfected as indicated and treated with control or Wnt3a conditioned medium for 1h. M1 and M2 are dominant negative *dvl2* mutants that show impaired self-polymerization. (**B**) Wnt luciferase reporter assay in HEK 293T cells stimulated by transfection with the indicated constructs, and in the presence of the indicated siRNAs. (**C**) Efficiency of siRNA knock-down in (B) was monitored by real time RT-PCR.

Movie 1

Time lapse confocal life cell imaging of HeLa cells transfected with ECFP-Axin (green) and LRP6-EYFP (red). Recording was for 50 min and Wnt3a conditioned medium was added after 5 min. Note appearance of yellow punctate structures at the plasma membrane after Wnt medium addition.