

Supplementary Information

Binding of APC and dishevelled mediates Wnt5a-regulated focal adhesion dynamics in migrating cells

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Supplementary Methods

Materials and chemicals

Liver progenitor HPPL cells (Tanimizu *et al*, 2004) and pCS2-FLAG-mDvl2 were provided by Dr. A. Miyajima (Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo, Japan) and R. Habas (Department of Biochemistry, Robert Wood Johnson School of Medicine, Piscataway, New Jersey, USA), respectively. pQBI25-APC-GFP and anti-APC rabbit polyclonal and rat monoclonal antibodies were provided by Dr. Y. Mimori-Kiyosue (Cytoskeleton & Cell Motility Research Group, KAN Research Institute, Inc., Kobe, Japan). In this APC-GFP fusion construct, GFP is inserted between the 20 amino acid-repeat region and the basic region of APC (Mimori-Kiyosue *et al*, 2000). Anti-Dvl (DIX) antibody was prepared in rabbits by immunization with recombinant protein of Dvl1-(1-140) as described previously (Sakamoto *et al*, 2000). Conditioned media for Wnt3a and Wnt5a were prepared as described previously (Kishida *et al*, 2004; Kurayoshi *et al*, 2007). Wnt5a was purified to near homogeneity from Wnt5a conditioned medium (CM) (Kurayoshi *et al*, 2007). Rabbit polyclonal anti-Dvl2 and anti-Dvl3 antibodies and rabbit monoclonal anti-Wnt5a/b antibody were purchased from Cell Signaling Technology (Danvers, CA, USA). Mouse monoclonal anti-Dvl2 and anti-Dvl3 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-clathrin, anti-FAK, anti-paxillin, anti- β 1 integrin, anti- β 3 integrin, anti- α V integrin, anti- α 2 integrin, anti- α 3 integrin, and anti- α 5 integrin antibodies were from BD Biosciences (San Jose, CA, USA). Anti- β 1 integrin-adhesion blocking antibody was from Millipore (Billerica, MA, USA). Anti-vinculin, anti-talin, anti- β -tubulin, polyclonal anti-FLAG, and anti-FLAG (M2)

antibodies were from Sigma-Aldrich (St. Louis, MO, USA). Monoclonal anti-HA (16B12), polyclonal anti-HA, rat monoclonal anti-tubulin, and chicken anti-GFP antibodies were from Covance (Princeton, NJ, USA), MBL (Nagoya, Japan), Novus Biologicals (Littleton, CO, USA), and Aves (Tigard, OR, USA), respectively. Alexa Fluor 488-, 546-, 633- conjugated antibodies to rabbit, rat, chicken, and mouse immunoglobulin G and Alexa Fluor 633-, 546- and 488-phalloidin were purchased from Invitrogen (Carlsbad, CA, USA). Dithiobis (succinimidyl propionate) (DSP) from Pierce Biotechnology (Rockford, IL, USA). Other materials and chemicals were from commercial sources.

Plasmid constructions

pCGN/Dvl1-(full length), pCGN/Dvl1-(1-250), pCGN/Dvl1-(201-371), pCGN/Dvl1-(337-670), pCGN/Dvl1-(1-398), pMALc2/Dvl1-(full length), and pGEX4T1/Dvl1-(395-670) were constructed as described previously (Hino *et al*, 2001; Hino *et al*, 2003; Kishida *et al*, 2001; Kishida *et al*, 1999; Yamamoto *et al*, 1999). Standard recombinant DNA techniques were used to construct the following plasmids: pEGFP/Dvl2-(full length), pEGFP/Dvl2-(Δ DEP: Δ 423-509), pEGFP/Dvl2-(1-509), pEGFP/Dvl2-(506-736), pGEX4T1/APC-Arm+, pMALc2/APC-Arm+, pCDNA/FLAG-APC-Arm+, pEGFP/APC-Arm, pEGFP/APC-Arm4, pEGFP/APC-Arm1 and pCAG/Wnt5a.

Cell culture

HeLaS3, NIH 3T3, Vero, COS7, and MDCK cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HEK293T

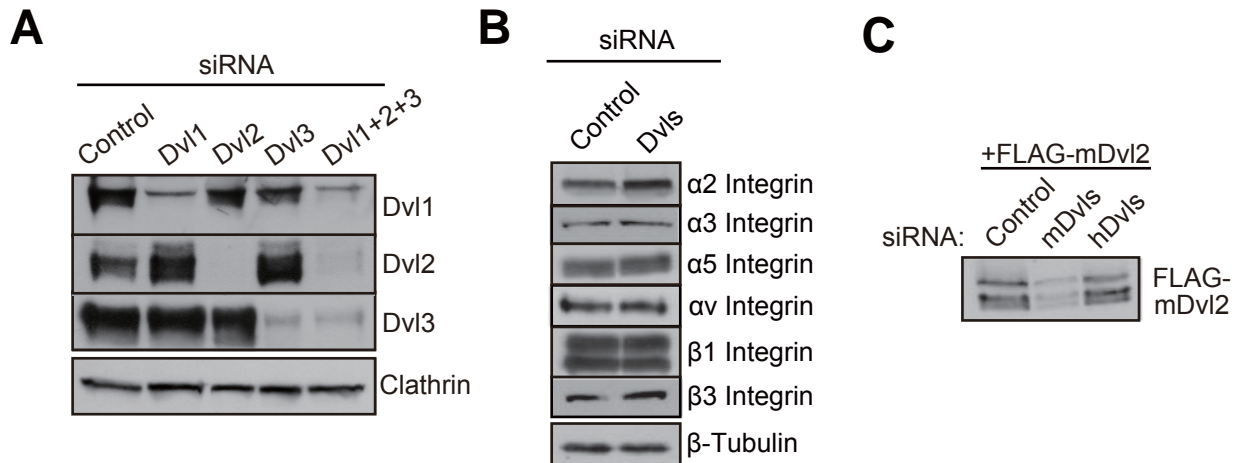
cells were grown in DH10 medium supplemented with 10% FBS. HPPL cells were grown in DH10 containing 10% FBS, $1 \times$ insulin/transferrin/selenium (Invitrogen, Carlsbad, CA, USA), 10 mM nicotinamide (Wako Pure Chemicals, Osaka, Japan), 0.1 μ M dexamethasone (Sigma-Aldrich), and 5 mM L-glutamine with 5 ng/ml hepatocyte growth factor (HGF) and epidermal growth factor (EGF).

siRNA experiment

Double-stranded RNA oligonucleotides were annealed *in vitro* before transfection. The RNA duplexes used in this study are shown in Supplementary Information, Table 1. HeLaS3, HEK293T, NIH 3T3, HPPL, and Vero cells were transfected with a mixture of siRNAs against Dvl1, Dvl2, and Dvl3 or Wnt5a-1 and 2 or APC-1 and 2 at 40 nM each using Oligofectamine (Invitrogen), Lipofectamine 2000 (Invitrogen), or Lipofectamine LTX (Invitrogen) and the cells were used for experiments at 72 h post-transfection.

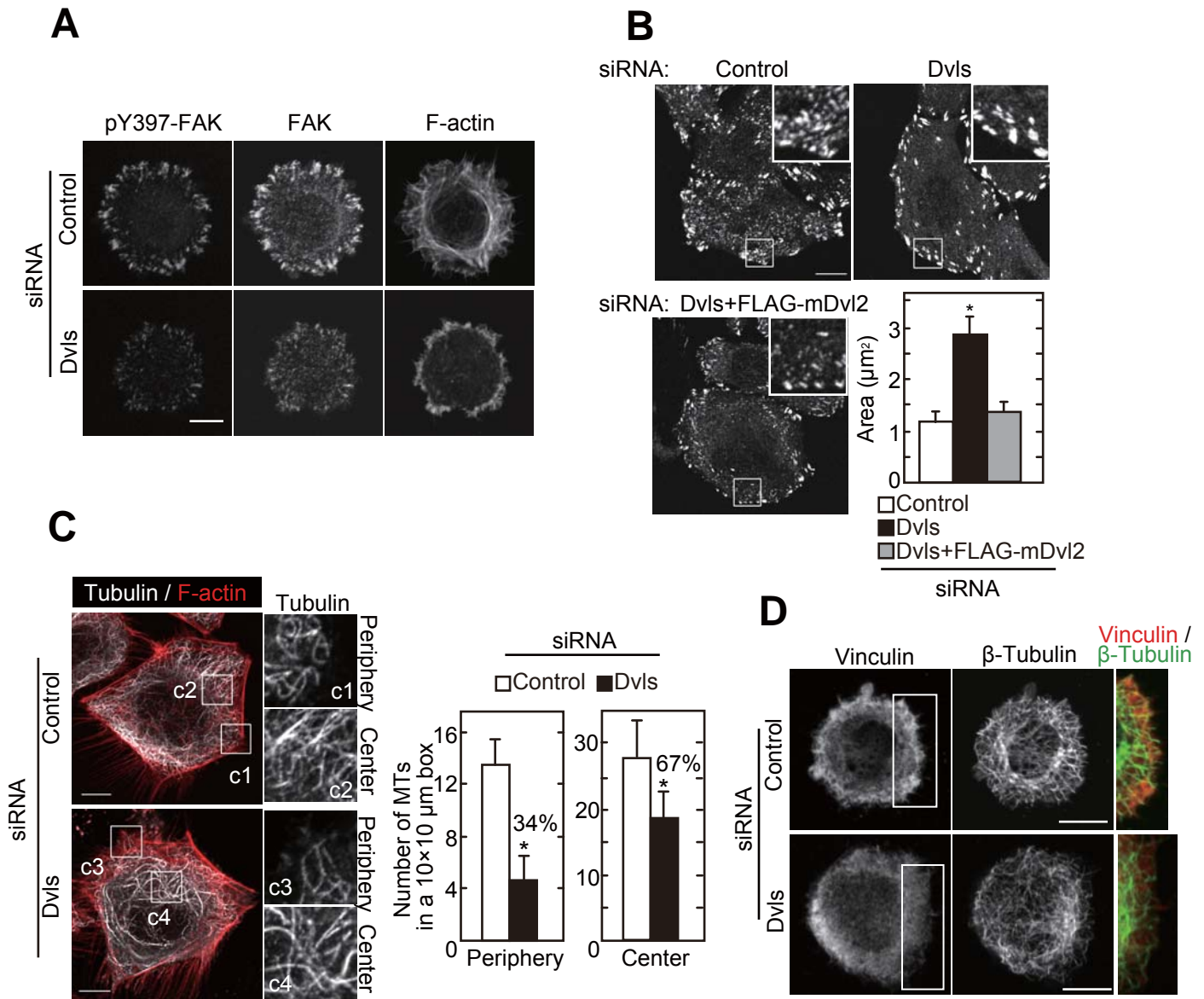
Supplementary References

- Hino S-I, Kishida S, Michiue T, Fukui A, Sakamoto I, Takada S, Asashima M, Kikuchi A (2001) Inhibition of the Wnt signaling pathway by Idax, a novel Dvl-binding protein. *Mol Cell Biol* 21: 330-342
- Hino S-I, Michiue T, Asashima M, Kikuchi A (2003) Casein kinase I ϵ enhances the binding of Dvl-1 to Frat-1 and is essential for Wnt-3a-induced accumulation of β -catenin. *J Biol Chem* 278: 14066-14073
- Kishida M, Hino S-I, Michiue T, Yamamoto H, Kishida S, Fukui A, Asashima M, Kikuchi A (2001) Synergistic activation of the Wnt signaling pathway by Dvl and casein kinase I ϵ . *J Biol Chem* 276: 33147-33155
- Kishida M, Koyama S, Kishida S, Matsubara K, Nakashima S, Higano K, Takada R, Takada S, Kikuchi A (1999) Axin prevents Wnt-3a-induced accumulation of β -catenin. *Oncogene* 18: 979-985
- Kishida S, Yamamoto H, Kikuchi A (2004) Wnt-3a and Dvl induce neurite retraction by activating Rho-associated kinase. *Mol Cell Biol* 24: 4487-4501
- Kurayoshi M, Yamamoto H, Izumi S, Kikuchi A (2007) Post-translational palmitoylation and glycosylation of Wnt-5a are necessary for its signalling. *Biochem J* 402: 515-523
- Mimori-Kiyosue Y, Shiina N, Tsukita S (2000) Adenomatous polyposis coli (APC) protein moves along microtubules and concentrates at their growing ends in epithelial cells. *J Cell Biol* 148: 505-518
- Sakamoto I, Kishida S, Fukui A, Kishida M, Yamamoto H, Hino S-I, Michiue T, Takada S, Asashima M, Kikuchi A (2000) A novel β -catenin-binding protein inhibits β -catenin-dependent Tcf activation and axis formation. *J Biol Chem* 275: 32871-32878
- Tanimizu N, Saito H, Mostov K, Miyajima A (2004) Long-term culture of hepatic progenitors derived from mouse Dlk+ hepatoblasts. *J Cell Sci* 117: 6425-6434
- Yamamoto H, Kishida S, Kishida M, Ikeda S, Takada S, Kikuchi A (1999) Phosphorylation of Axin, a Wnt signal negative regulator, by glycogen synthase kinase-3 β regulates its stability. *J Biol Chem* 274: 10681-10684



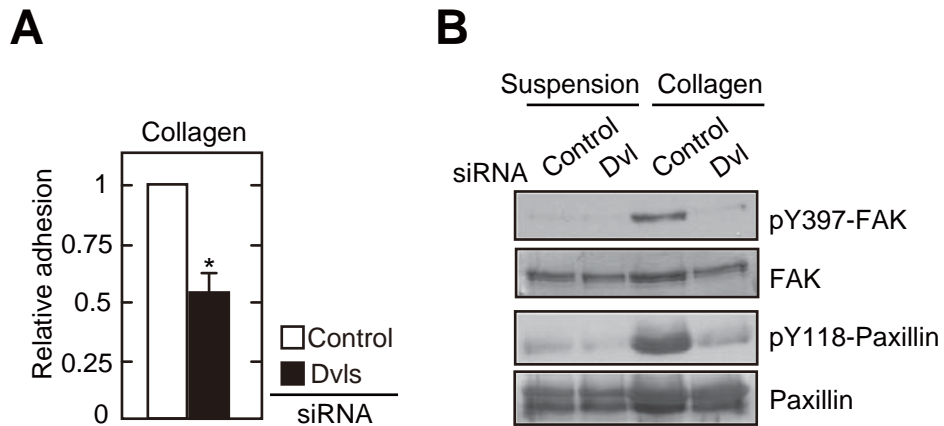
Supplementary Figure S1. Knockdown of Dvl

(A) Lysates of HeLaS3 cells transfected with the indicated siRNAs were probed with anti-Dvl1 (DIX), anti-Dvl2, or anti-Dvl3 antibody. Clathrin was used as a loading control. (B) Lysates of HeLaS3 cells transfected with control or Dvl siRNA were probed with antibodies against α 2, α 3, α 5, α v, β 1, and β 3 integrins. β -Tubulin was used as a loading control. (C) FLAG-mouse Dvl2 was expressed in HEK293T cells transfected with control, mouse Dvl1+2+3, or human Dvl1+2+3 siRNA, and then lysates were probed with anti-FLAG antibody.



Supplementary Figure S2. Knockdown of Dvl suppresses cell-substrate adhesion and the dynamics of microtubules at the cell periphery in HeLaS3 cells

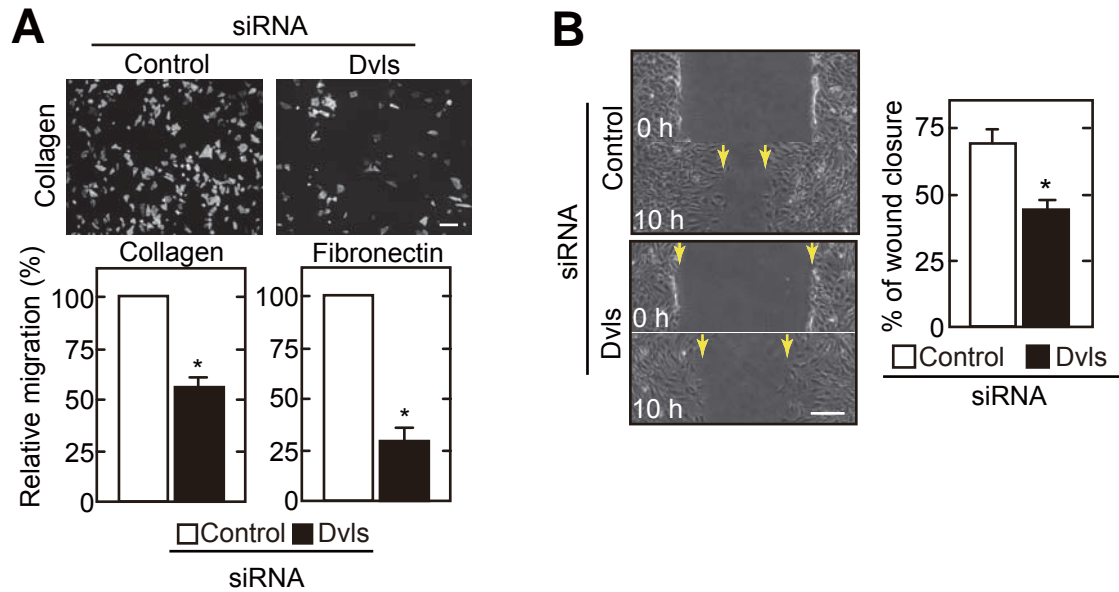
(A) After HeLaS3 cells transfected with control or Dvl siRNA were suspended in serum-free medium for 1 h, cells were plated on collagen for 15 min and stained with anti-pY397-FAK, anti-FAK antibodies and phalloidin. (B) FLAG-mouse Dvl2, which is resistant to siRNA for human Dvl1, Dvl2, and Dvl3, was expressed in HeLaS3 cells transfected with Dvl siRNA, and the cells were stained with anti-paxillin antibody. The lower right panel shows quantification of the individual areas of paxillin staining (focal adhesions). Five different focal adhesions in 20 cells were measured per each siRNA experiment. The region in the white box is shown enlarged. *, $P < 0.01$. (C) HeLaS3 cells transfected with control or Dvl siRNA were treated with 200 nM nocodazole for 10 min and stained with anti-tubulin antibody and phalloidin. The numbers of microtubules in a $10 \times 10 \mu\text{m}$ box located at the cell edge (periphery) and perinuclear region (center) were counted and the quantification is shown in the right-hand panel. Measurement was performed in 30 cells per each siRNA experiment. The regions in the white boxes (c1, c2, c3, and c4) are shown enlarged. *, $P < 0.01$. (D) After HeLaS3 cells transfected with control or Dvl siRNA were plated on collagen for 15 min and stained with anti-vinculin and anti- β -tubulin antibodies. The regions in the white boxes are shown enlarged. Scale bars, 10 μm .



Supplementary Figure S3. Knockdown of Dvl suppresses cell-substrate adhesion in Vero cells

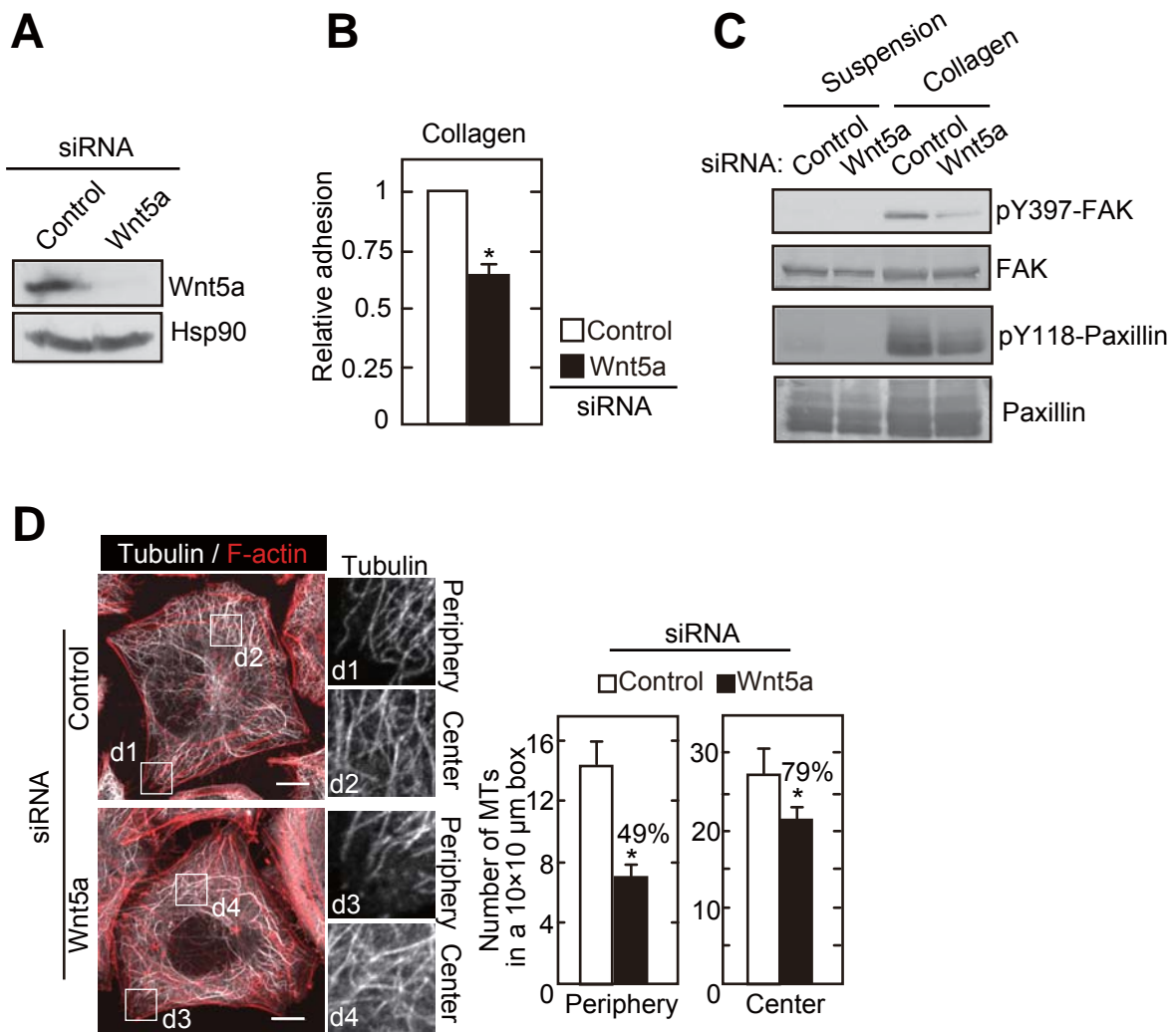
(A) Vero cells transfected with control or Dvl siRNA were plated on collagen (Col) for 15 min and subjected to the adhesion assay. The results are expressed as the ratio of adhesion activity of cells treated with control siRNA and indicate means \pm SE from three independent experiments. *, $P < 0.01$.

(B) After Vero cells transfected with control or Dvl siRNA were suspended in serum-free medium for 1 h, they were kept in suspension or plated onto collagen-coated dishes for 1 h. Lysates were probed with anti-pY397-FAK and anti-pY118-paxillin antibodies. FAK and paxillin were used as loading controls.



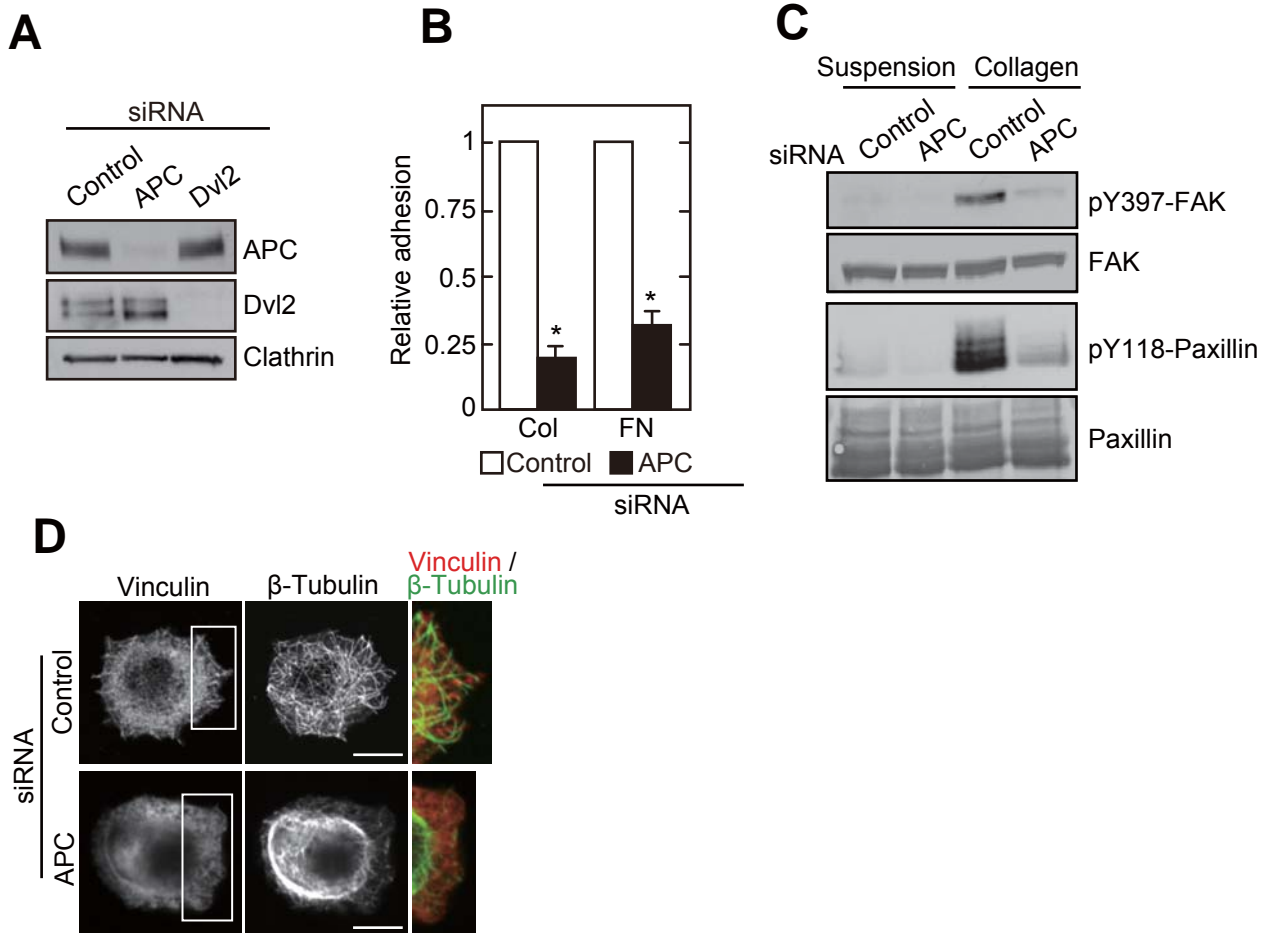
Supplementary Figure S4. Knockdown of Dvl suppresses cell migration

(A) HeLaS3 cells transfected with control or Dvl siRNA were placed in a Transwell chamber for the migration assay. Migration activities were expressed as percentages of control cells. The results shown are means \pm SE from four independent experiments. *, $P < 0.01$. (B) Confluent NIH3T3 cells transfected with control or Dvl siRNA were placed on a collagen coated coverslip for the wound-healing assay. Wound monolayers were allowed to heal for 10 h. Images were captured shortly after wounding and at 10 h after wounding. Arrows indicate the cell front. The percentage of wound closure was measured and is shown in the right-hand panel. *, $P < 0.01$. Scale bars in A, 100 μm ; in B, 30 μm .



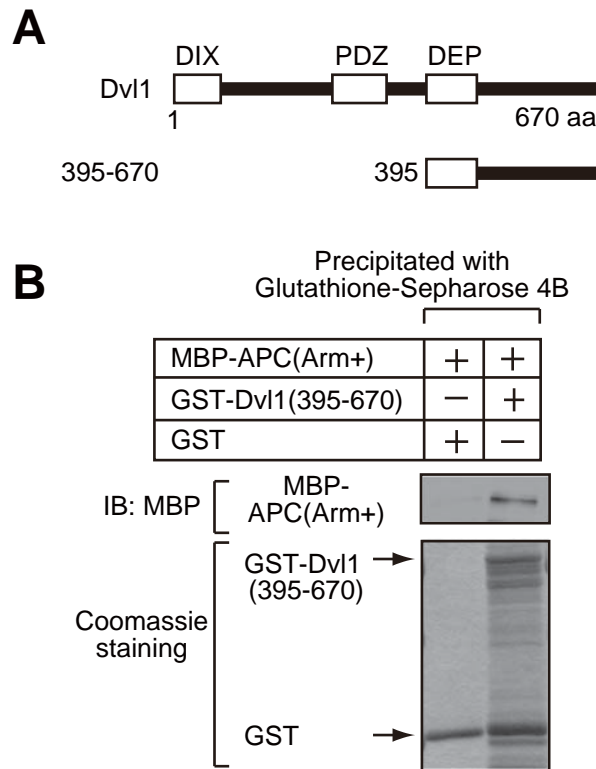
Supplementary Figure S5. Knockdown of Wnt5a suppresses cell-substrate adhesion and the stability of microtubules at the cell periphery

(A) Lysates of HeLaS3 cells transfected with control or Wnt5a siRNA were probed with anti-Wnt5a antibody. Hsp90 was used as a loading control. (B) HeLaS3 cells transfected with control or Wnt5a siRNA were plated on collagen for 15 min and subjected to the adhesion assay. The results are expressed as the ratio of adhesion activity of cells treated with control siRNA and indicate means \pm SE from three independent experiments. *, $P < 0.01$. (C) After HeLaS3 cells transfected with control or Wnt5a siRNA were suspended in serum-free medium for 1 h, they were kept in suspension or plated onto collagen-coated dishes for 1 h. Lysates were probed with anti-pY397-FAK and anti-pY118-paxillin antibodies. FAK and paxillin were used as loading controls. (D) HeLaS3 cells transfected with control or Wnt5a siRNA were treated with 200 nM nocodazole for 10 min and stained with anti-tubulin antibody and phalloidin. The numbers of microtubules in a $10 \times 10 \mu\text{m}$ box located at the cell edge (periphery) and perinuclear region (center) were counted and the quantification is shown in the right-hand panel. Measurement was performed in 30 cells per each siRNA experiment. The regions in the white boxes (d1, d2, d3, and d4) are shown enlarged. *, $P < 0.01$.



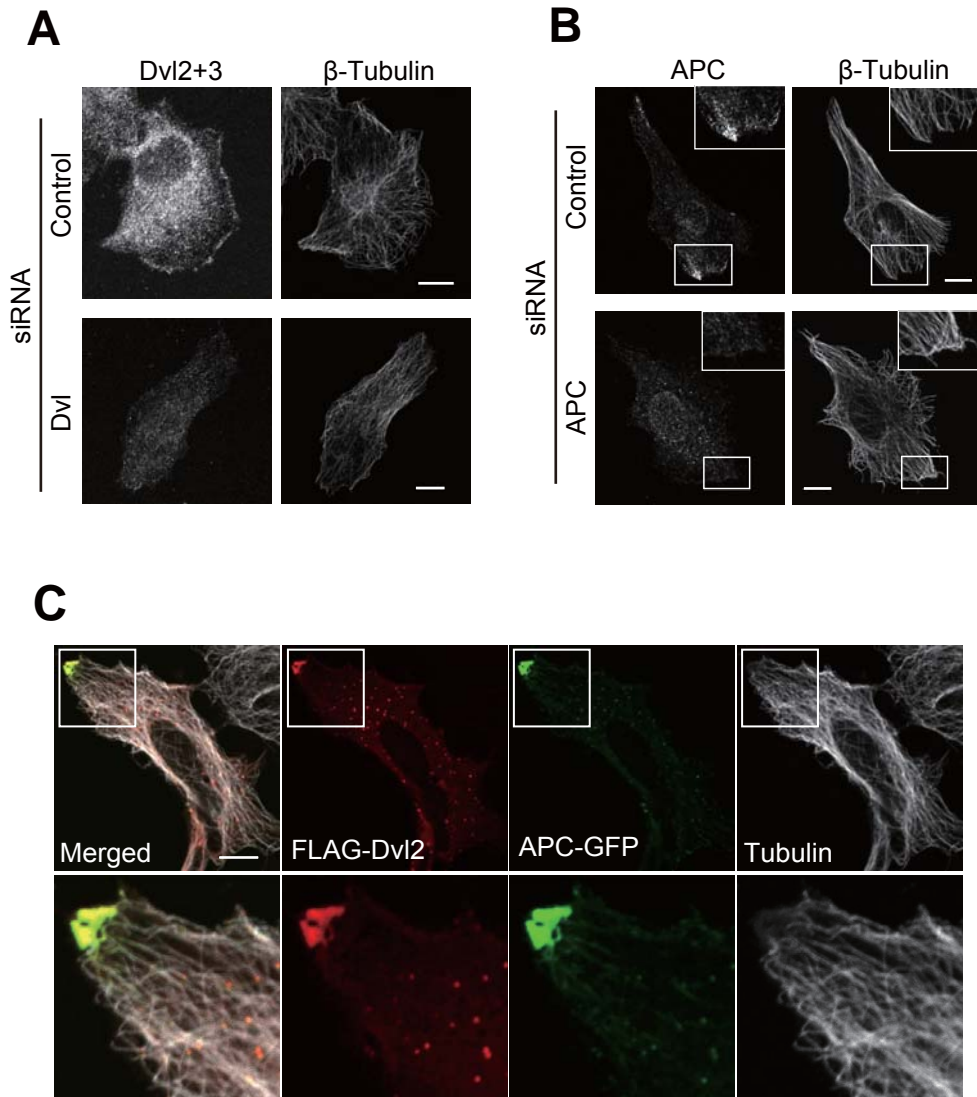
Supplementary Figure S6. Knockdown of APC suppresses cell-substrate adhesion

(A) Lysates of HeLaS3 cells transfected with the indicated siRNAs were probed with anti-APC or anti-Dvl2 antibody. Clathrin was used as a loading control. (B) HeLaS3 cells transfected with control or APC siRNA were plated on collagen (Col) or fibronectin (FN) for 15 min and subjected to the adhesion assay. The results are expressed as the ratio of adhesion activity of cells treated with control siRNA and indicate means \pm SE from three independent experiments. *, $P < 0.01$. (C) After HeLaS3 cells transfected with control or APC siRNA were suspended in serum-free medium for 1 h, they were kept in suspension or plated onto collagen-coated dishes for 1 h. Lysates were probed with anti-pY397-FAK and anti-pY118-paxillin antibodies. FAK and paxillin were used as loading controls. (D) HeLaS3 cells transfected with control or APC siRNA were plated on collagen for 15 min and stained with anti-vinculin and anti- β -tubulin antibodies. The regions in the white boxes are shown enlarged. Scale bars, 10 μ m.



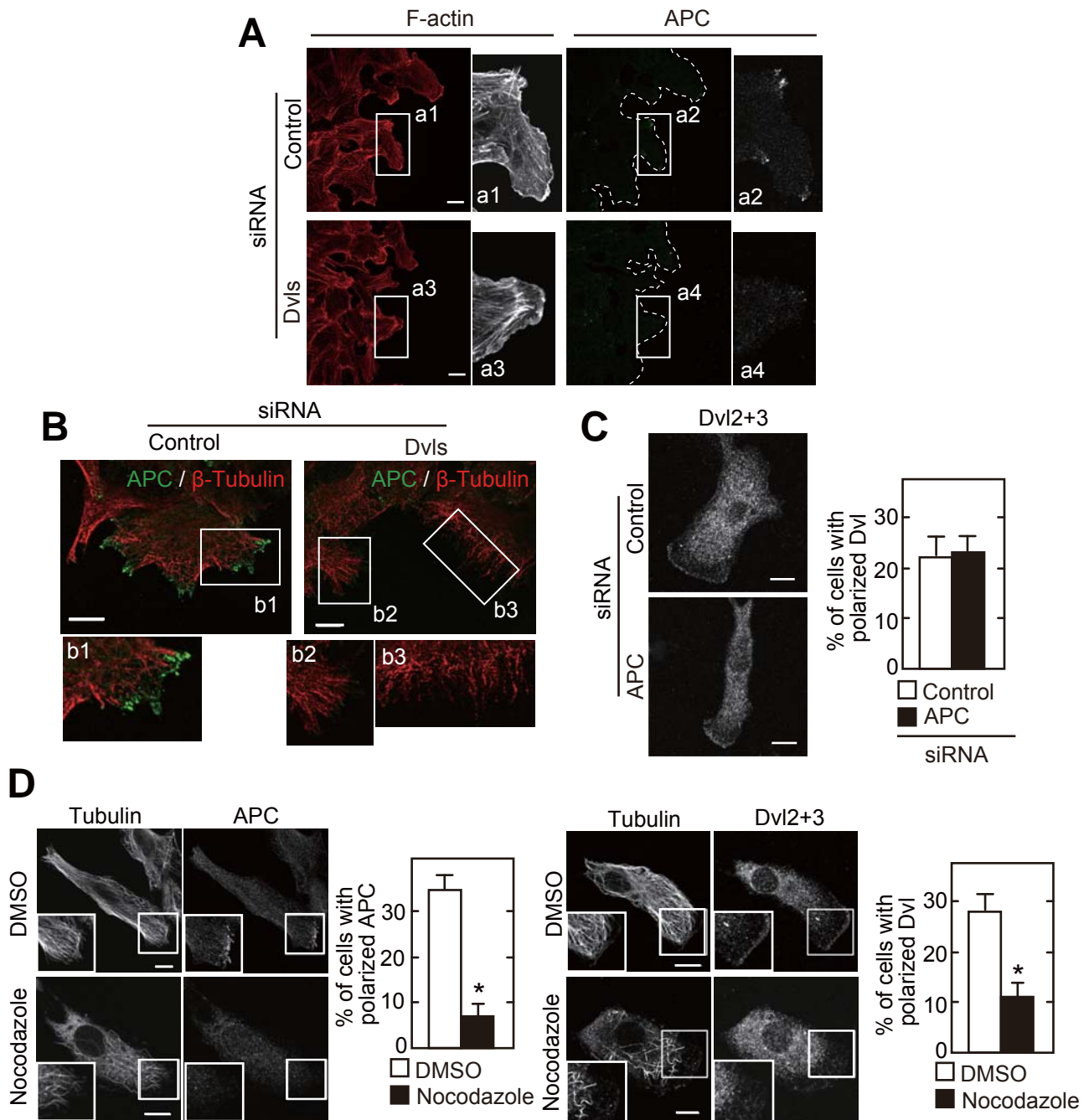
Supplementary Figure S7. Direct binding of Dvl and APC

(A) Schematic representation of the deletion mutants of Dvl1 used in this study. Amino acid numbers are indicated. (B) After MBP-APC(Arm+) was incubated with GST-Dvl1(395-670) or GST, GST-Dvl1(395-670) and GST were precipitated with glutathione-Sepharose and then the precipitates were probed with anti-MBP antibody (top panel). Recombinant GST-Dvl1(395-670) and GST were stained with Coomassie Brilliant Blue (bottom panel).

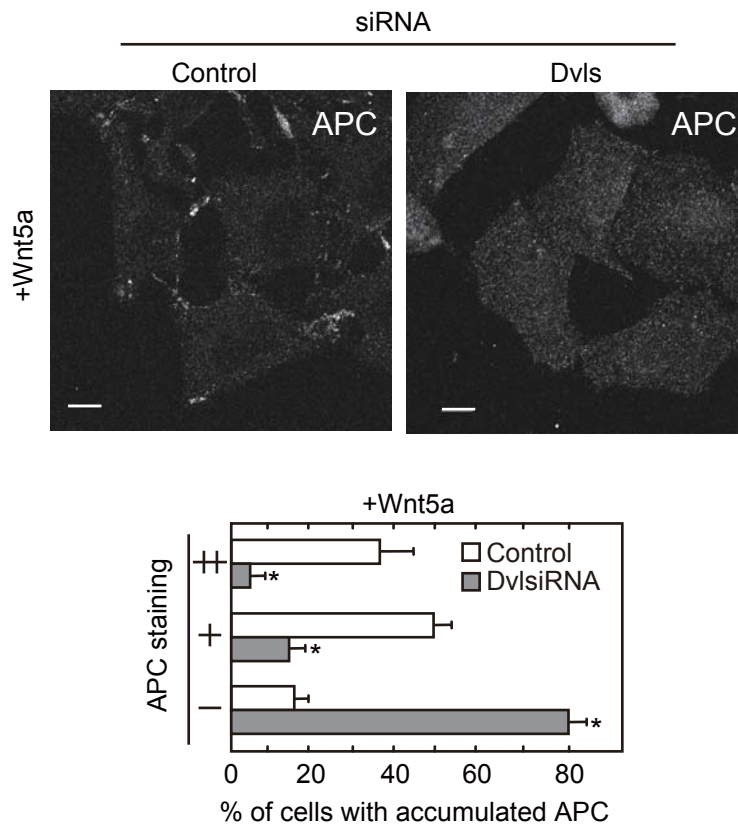


Supplementary Figure S8. Polarized localization of Dvl and APC to the leading edge

(A) Vero cells transfected with control or Dvl siRNA were fixed with methanol and stained with anti-β-tubulin and anti-Dvl2+3 (a mixture of anti-Dvl2 and anti-Dvl3 antibodies) antibodies. (B) Vero cells transfected with control or APC siRNA were fixed with methanol and stained with anti-β-tubulin and anti-APC antibodies. The regions in the white box are shown enlarged. (C) HPPL cells coexpressing FLAG-Dvl2 and APC-GFP were stained with anti-FLAG (red), anti-GFP (green), and anti-tubulin (gray) antibodies. The regions in the white box are shown enlarged in the bottom panels. Scale bars, 10 μm.

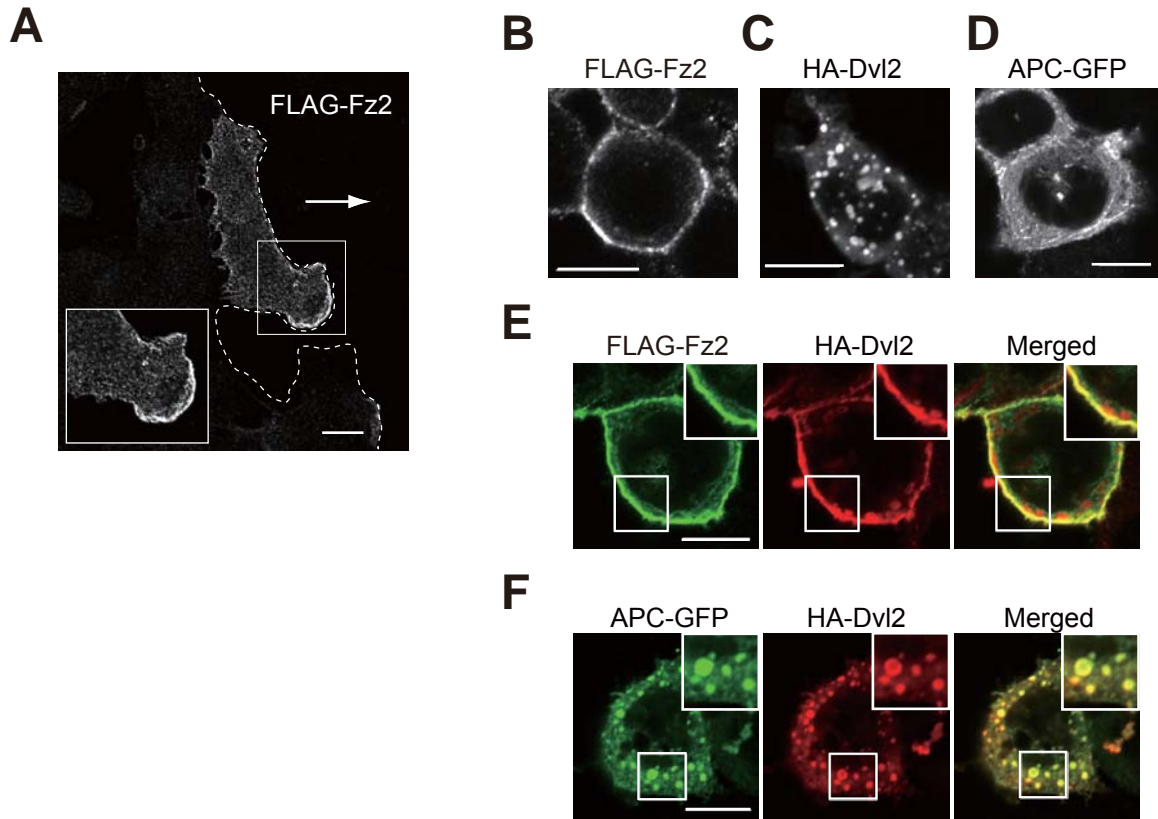


Supplementary Figure S9. APC is not required for the localization of Dvl to the leading edge (A) Confluent Vero cells transfected with control or Dvl siRNA were wounded. After 4 h, the cells were stained with anti-APC antibody (green) and phalloidin (red). The regions in white boxes (a1, a2, a3, and a4) are shown enlarged. (B) Confluent HPPL cells transfected control or Dvl siRNA were wounded. After 5 h, the cells were stained with anti-APC and anti- β -tubulin antibodies. The regions in white boxes (b1, b2, and b3) are shown enlarged in the bottom panel. (C) Vero cells transfected control or APC siRNA were stained with anti-Dvl2+3 antibodies. The numbers of cells with polarized Dvl at the cell cortex were counted and the quantification is shown in the right-hand panel. Measurement was performed in at least 50 cells per siRNA experiment. (D) Vero cells were treated with or without 10 μ M nocodazole for 10 min and stained with β -tubulin and anti-Dvl2+3 or anti-APC antibodies. Numbers of cells with polarized APC or Dvl at the cell cortex was counted and the quantification is shown in the right-hand panel. Measurement was performed in at least 50 cells per treatment. *, $P < 0.01$. Scale bars, 10 μ m.



Supplementary Figure S10. Localization of APC to the cell periphery in response to Wnt5a

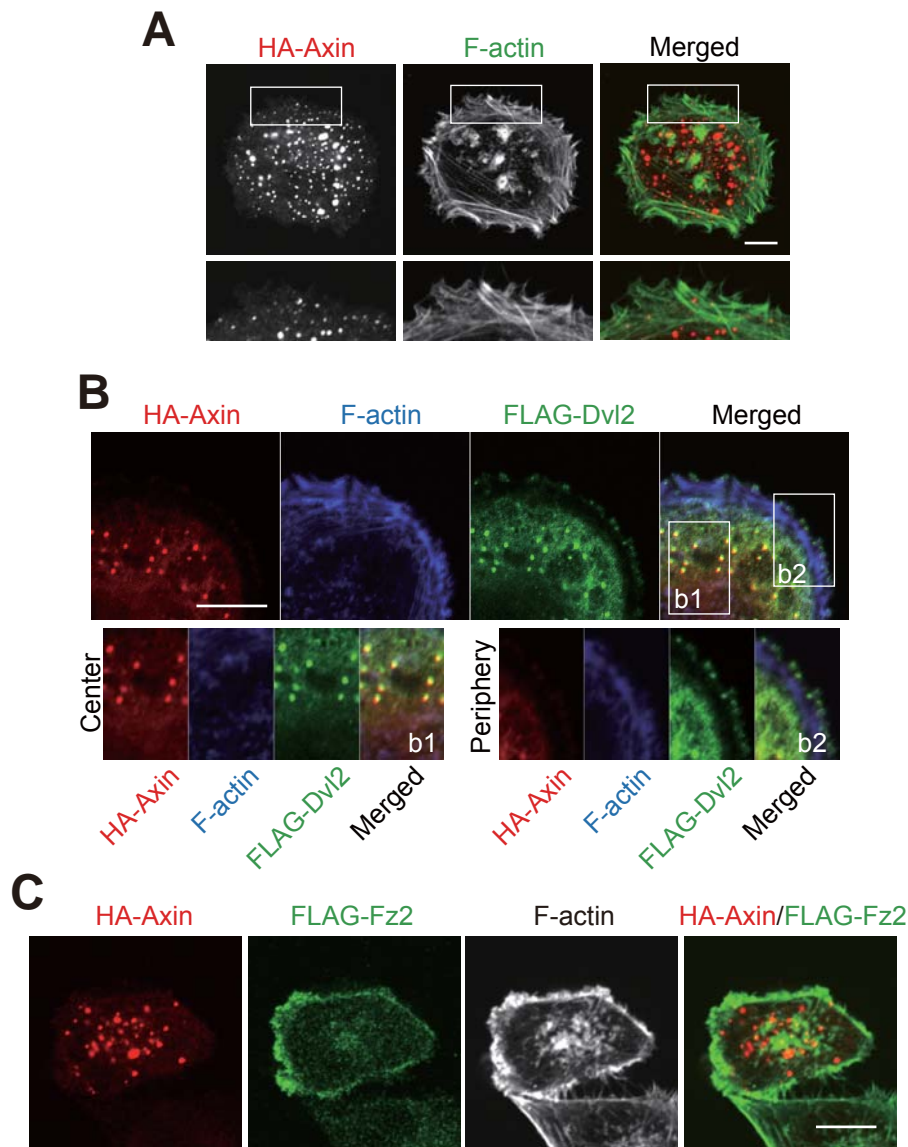
After HeLaS3 cells transfected with control or Dvl1 siRNA were serum-starved for 36 h and stimulated with or without 400 ng/ml purified Wnt5a for 60 min, the cells were stained with anti-APC antibody. Cells with accumulated APC (-, +, ++) at the cell periphery was counted in at least 50 cells per each treatment. Scale bar, 10 μ m. *, $P < 0.01$.



Supplementary Figure S11. Colocalization of FLAG-Fz2, HA-Dvl2, and APC-GFP

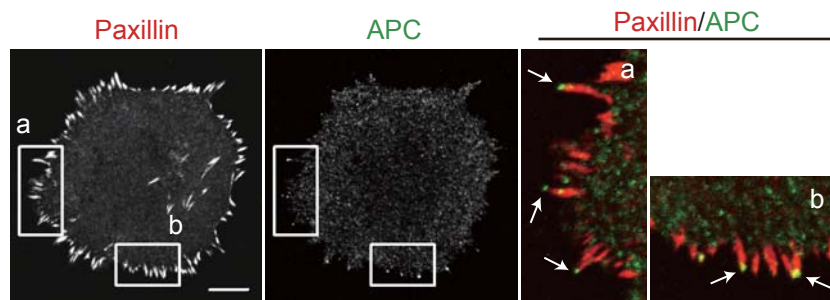
(A) Confluent Vero cells expressing FLAG-Fz2 were wounded. After 4 h the cells were fixed and stained with polyclonal anti-FLAG antibody without permeabilization. White arrow, migration direction. Dashed lines indicate a front line of scratched cells. The region in the white box is shown enlarged. Polarized localization of FLAG-Fz2 was observed in 40% of 30 cells expressing FLAG-Fz2 in the front line of scratched cells.

(B) HEK293T cells expressing FLAG-Fz2 were stained with polyclonal anti-FLAG antibody without permeabilization. (C, D) HEK293T cells expressing HA-Dvl2 (C) or APC-GFP (D) were stained with anti-HA antibody or anti-GFP antibody. (E) HEK293T cells coexpressing FLAG-Fz2 and HA-Dvl2, were stained with polyclonal anti-FLAG antibody before permeabilization. Thereafter, intracellular HA-Dvl2 was stained with anti-HA antibody. The region in the white box is shown enlarged. (F) HEK293T cells coexpressing HA-Dvl2 and APC-GFP were stained with anti-HA and anti-GFP antibodies. The region in the white box is shown enlarged. Scale bars, 10 μm.



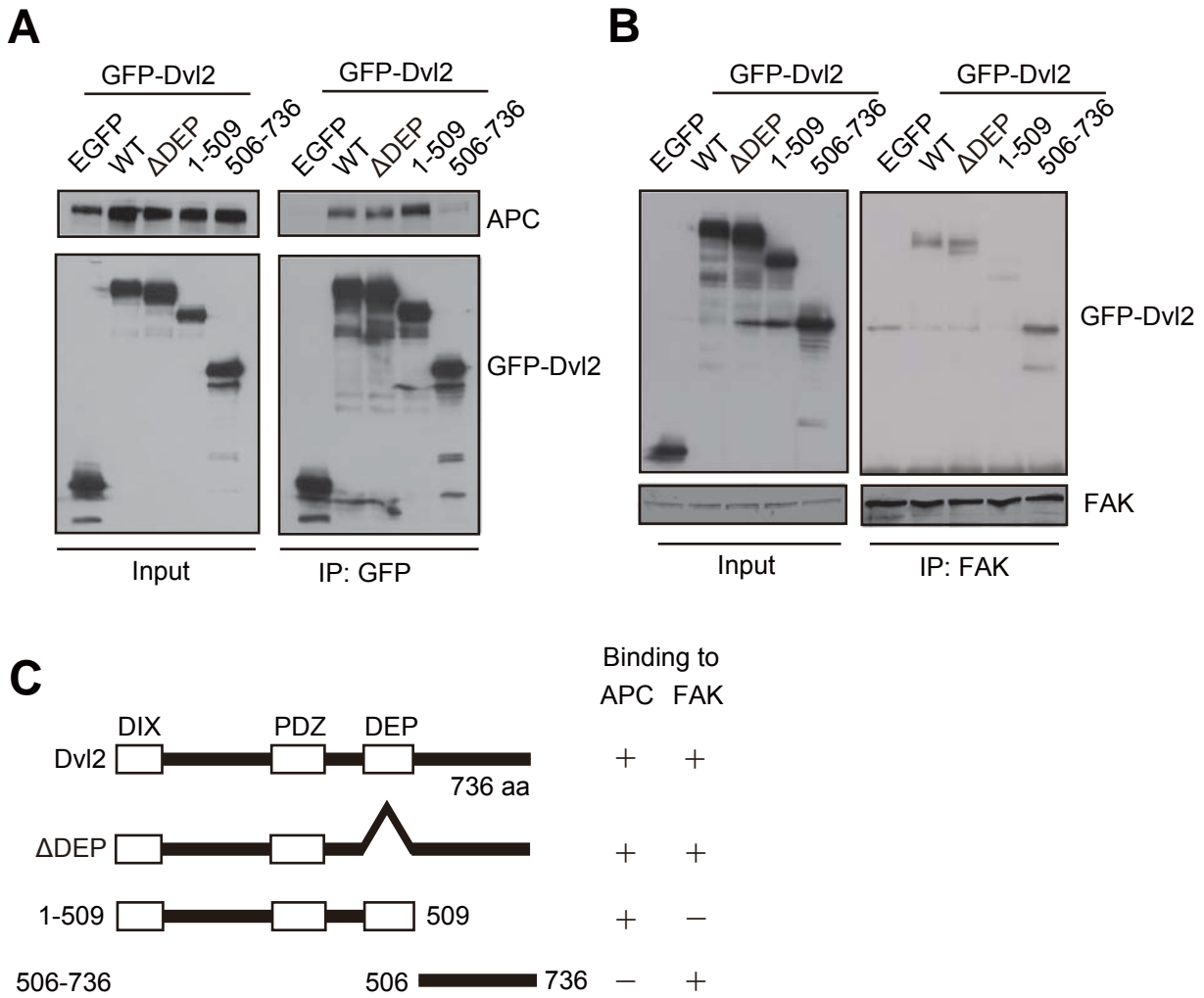
Supplementary Figure S12. Axin does not localize to the cell periphery with Fz2 or Dvl

(A) Vero cells expressing HA-Axin were plated on collagen-coated dishes for 1 h and stained with anti-HA antibody and phalloidin. The region in the white box is shown enlarged in the bottom panels. (B) Vero cells coexpressing FLAG-Dvl2 and HA-Axin were plated on collagen-coated dishes for 1 h and stained with anti-FLAG and anti-HA antibodies and phalloidin. The regions in the white boxes (b1 and b2) are shown enlarged in the bottom panels. (C) Vero cells coexpressing HA-Axin and FLAG-Fz2 were fixed and stained with polyclonal anti-FLAG antibody before permeabilization. Thereafter, Axin and F-actin were stained with anti-HA antibody and phalloidin. Scale bars, 10 μ m.



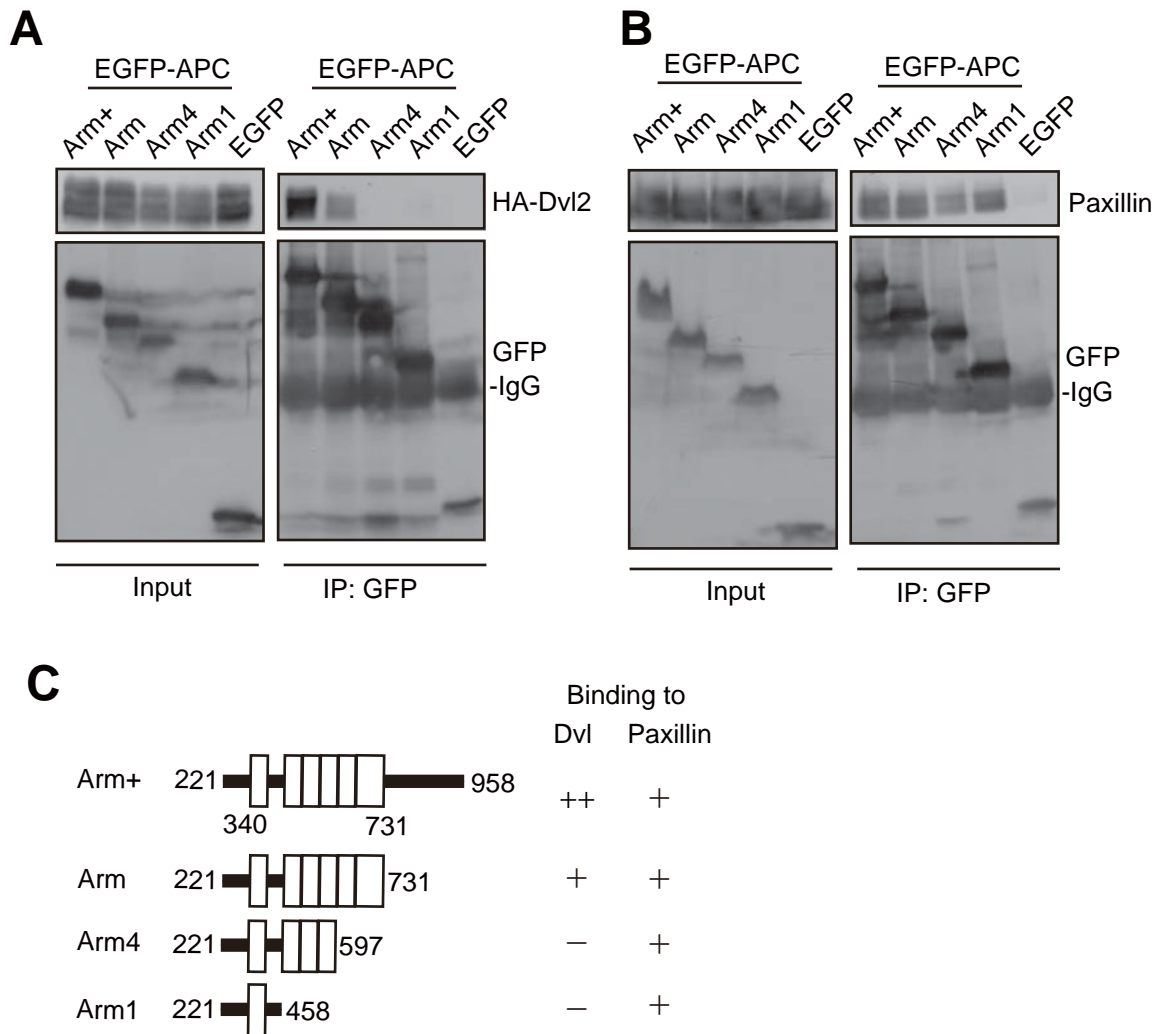
Supplementary Figure S13. APC localizes adjacent to paxillin

HPPL cells were plated on collagen for 1 h and stained with anti-APC and anti-paxillin antibodies. The regions in the white boxes (a and b) are shown enlarged in the right-hand panels. Localization of APC at the tip of paxillin staining was observed in 77% of 90 cortical APC staining. Scale bar, 10 μ m.



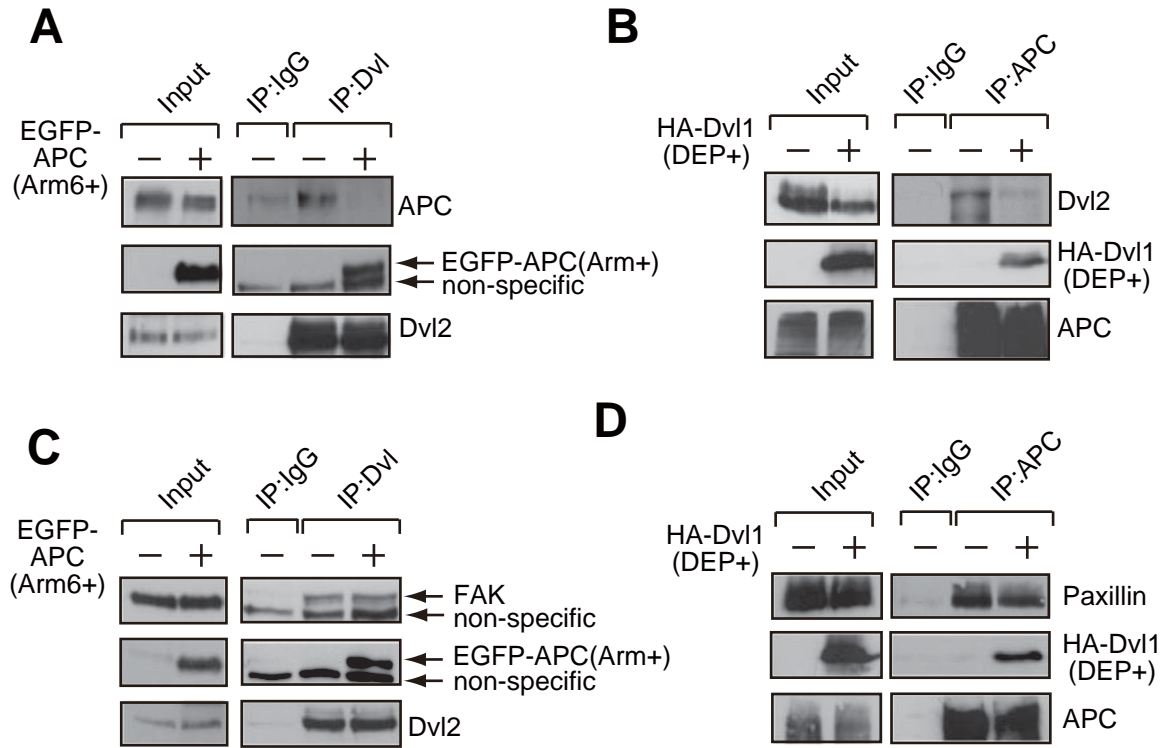
Supplementary Figure S14. Binding regions of Dvl to APC and FAK

(A) Lysates of COS7 cells expressing GFP-Dvl2 deletion mutants were immunoprecipitated with anti-GFP antibody and the immunoprecipitates were probed with anti-GFP and anti-APC antibodies. WT, wild type. (B) Lysates of COS7 cells expressing GFP-Dvl2 deletion mutants were immunoprecipitated with anti-FAK antibody and the immunoprecipitates were probed with anti-GFP and anti-FAK antibodies. (C) Schematic representation of the deletion mutants of GFP-Dvl2 used in this study. Amino acid numbers are indicated.



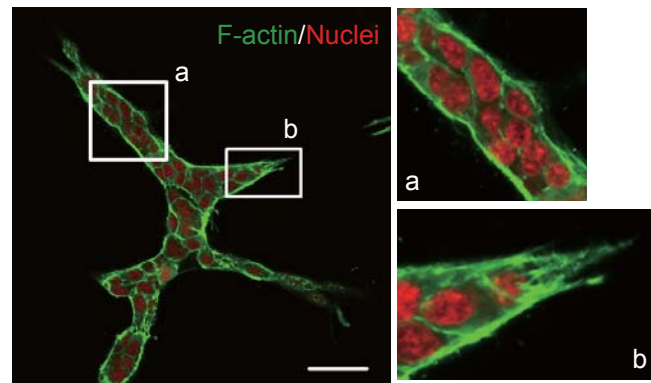
Supplementary Figure S15. Binding regions of APC to Dvl and paxillin

(A) Lysates of COS7 cells coexpressing HA-Dvl2 and EGFP-APC deletion mutants were immunoprecipitated with anti-GFP antibody and the immunoprecipitates were probed with anti-HA and anti-GFP antibodies. (B) Lysates of COS7 cells expressing EGFP-APC deletion mutants were immunoprecipitated with anti-GFP antibody and the immunoprecipitates were probed with anti-paxillin and anti-GFP antibodies. (C) Schematic representation of the deletion mutants of EGFP-APC used in this study. Amino acid numbers are indicated.

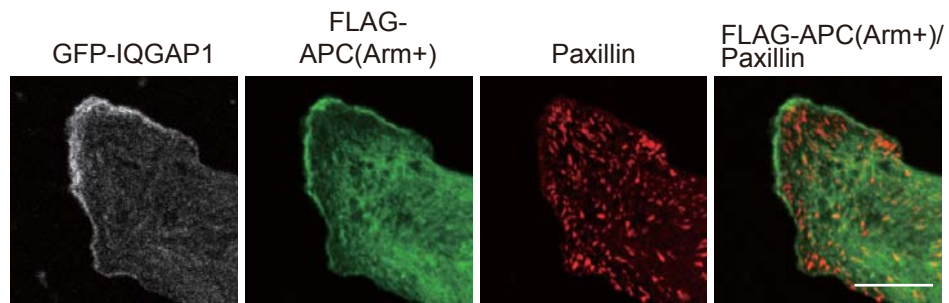


Supplementary Figure S16. Complex formation of Dvl, APC, and paxillin or FAK

(A) Lysates of HEK293T cells transfected with or without EGFP-APC(Arm6+) were immunoprecipitated with anti-Dvl(DIX) antibody and the immunoprecipitates were probed with anti-APC, anti-GFP, and anti-Dvl2 antibodies. (B) Lysates of HEK293T cells transfected with or without HA-Dvl1(DEP+) were immunoprecipitated with anti-APC antibody and the immunoprecipitates were probed with anti-Dvl2, anti-HA, and anti-APC antibodies. (C) Lysates of HEK293T cells transfected with or without EGFP-APC(Arm6+) were immunoprecipitated with anti-Dvl(DIX) antibody and the immunoprecipitates were probed with anti-FAK, anti-GFP, and anti-Dvl2 antibodies. (D) Lysates of HEK293T cells transfected with or without HA-Dvl1(DEP+) were immunoprecipitated with anti-APC antibody and the immunoprecipitates were probed with anti-paxillin, anti-HA, and anti-APC antibodies.



Supplementary Figure S17. HPPL cells form multicellular epithelial tube-like structures in 3D collagen gel. HPPL cells were cultured in collagen gel for 5 days and were stained with phalloidin and PI. The regions in the white boxes (a and b) are shown enlarged. Scale bar, 50 μm .



Supplementary Figure S18. IQGAP1 does not recruit the APC-paxillin complex to the cell cortex
Vero cells coexpressing FLAG-APC(Arm+) and GFP-IQGAP1 were stained with anti-paxillin (red), anti-FLAG (green), and anti-GFP (gray) antibodies. Scale bar, 10 μ m.

Table S1. siRNAs probes used in this study

siRNA Probe	Sequence
human Dvl1	5' -GGAGGAGAUCUUUGAUGACTT-3'
human Dvl2	5' -GGAAGAAAUUUCAGAUGACTT-3'
human Dvl3	5' -GGAGGAGAUCUCGGAUGACTT-3'
mouse Dvl1	5' -GGAGGAGAUCUUCGAUGACTT-3'
mouse Dvl2	5' -GGAAGAGAUCUCCGAUGACTT-3'
mouse Dvl3	5' -GGAAGAGAUCUCGGACGACTT-3'
Wnt5a-1	5' -GUUCAGAUGUCAGAAGUAUTT-3'
Wnt5a-2	5' -GUGGAUAACACCUCUGUUUTT-3'
APC-1	5' -GAGCGGCAGAAUGAAGGUCAA-3'
APC-2	5' -GAGUGGCAGGAAGCUCAUGAA-3'
non-specific	5' -CAGUCGCGUUUGCGACUGGTT-3'