**Supplementary Information**

# A combination of Wnt and growth factor signaling induces Arl4c expression to form epithelial tubular structures

Shinji Matsumoto[1], Shinsuke Fujii[1,5], Akira Sato[1], Souji Ibuka[1,2],
Yoshinori Kagawa[3,4,6], Masaru Ishii[4,6], and Akira Kikuchi[1,*]

## Supplementary Materials and Methods

### *Material and chemicals*

pCDNA-Arf6 and pCDNA-Arf6$^{T27N}$ were provided by Dr. K. Nakayama (Kyoto University, Kyoto, Japan). CSII-EF-MCS, CSII-CMV-MCS-IRES2-Bsd, and CS-RfA-EVBsd were provided by Dr. Miyoshi (RIKEN-BRC, Tsukuba, Japan) (Miyoshi et al, 1998). cDNA of mAG-hGem(1/110) and mKO2-hCdt1(30/120) were provided by Dr. Miyawaki, (RIKEN-BSI, Wako, Japan) and cloned into CSII-EF-MCS. B6N Mouse BAC clone (B6N01-089N24) was provided by RIKEN-BRC which is participating in the National Bio-Resources Project of the MEXT, Japan. pmt/Raf-CAAX, which is an active Raf kinase targeted to the plasma membranes by virtue of the addition of a C-terminal membrane localization signal from Ki-Ras, was provided by Dr. C. Marshall (Institute of Cancer Research, London) as described previously (Marais et al, 1997). Both Wnt3a and Wnt5a were purified to homogeneity as described previously (Kishida et al, 2004; Komekado et al, 2007; Kurayoshi et al, 2007). Anti-Dvl1 (DIX) antibody was prepared in rabbits by immunization with recombinant protein of Dvl1-(1-140) as described previously(Sakamoto et al, 2000). The primary antibodies used in this study are shown in Supplementary Table S2. Other materials and chemicals were obtained from commercial sources.

### *Cell culture*

X293T, HeLaS3, and MDCK type II (MDCK II) cells were maintained in Dulbecco modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. Parental IEC6 cells did not form branched tubular structures with continuous lumen efficiently under the treatment with Wnt3a/EGF, probably because of their heterogeneous characters. We established subclones of IEC6 cells which form branched tubular structures in response to Wnt3a/EGF, and used the subclones in this study. IEC6 cells were maintained in α minimum essential medium (αMEM)

supplemented with 10% FBS, penicillin-streptomycin, 4.5 mg/ml glucose, 10 μg/ml insulin, and 1×non-essential amino acid.

***Plasmid construction***

pEF-BOS-HA/hTcf4E Δ1-53 (DN-Tcf4), which lacks the N-terminal binding site for β-catenin, was constructed as described previously (Yamamoto et al, 2003). pCGN/β-catenin$^{SA}$, in which serine/threonine residues at the positions of 33, 37, 41, and 45 were changed to alanine, was constructed as described previously (Hino et al, 2005). Standard recombinant DNA techniques were used to construct the following plasmids: pEGFPN3-Arl4c, pEGFPN3-Arl4c$^{T27N}$, pEGFPC1-Rac1$^{G12V}$, and pEGFPC1-Rac1$^{T17N}$. To construct lentiviral vectors harboring a cDNA, EGFP, Arl4c$^{T27N}$-GFP, GFP-Rac1$^{G12V}$, GFP-Rac1$^{T17N}$, Arf6-HA, Arf6$^{T27N}$-HA, and FLAG-YAP$^{5SA}$ cDNAs were cloned into CSII-CMV-MCS-IRES2-Bsd. To construct a lentivirus vector harboring shRNA, a DNA fragment containing the H1 promoter and shRNA was cloned into CS-RfA-EVBsd using Gateway technology (Invitrogen). To generate reporter gene constructs, DNA fragments containing +2965 to +3690, +2965 to +3164, or +3195 to +3690 of the mouse Arl4c gene were cloned from BAC (B6Ng01-089N24) into the pGL4.27 vector.

***Microarray analyses***

IEC6 cells in 3D Matrigel culture remained untreated or were treated with 40 ng/ml Wnt3a, 5 ng/ml EGF-, or Wnt3a/EGF for 4 h or 24 h. The mRNA expression profile was produced by Bio Matrix Research (Chiba, Japan) using a gene microarray technology (GeneChip® Rat Genome 230 2.0 array, Affymetrix, Santa Clara, CA, USA). Data analyses were performed with GeneSpring GX 11 software (Agilent Technologies, Santa Clara, CA, USA), and the change in ratios between the hybridization intensities of Wnt3a and EGF treated and control samples were determined.

***Infection using lentivirus harboring a cDNA or shRNA***

Lentiviral vector CSII-CMV-MCS-IRES2-Bsd harboring a cDNA or CS-RfA-EVBsd harboring a shRNA was transfected with the packaging vectors pCAG-HIV-gp and pCMV-VSV-G-RSV-Rev into X293T cells using FuGENE HD transfection reagent (Roche Applied Science, Basel, Switzerland).

To generate IEC6 cells stably expressing Arl4c-GFP, Arl4c-GFP$^{T27N}$, GFP-Rac1$^{G12V}$, GFP-Rac1$^{T17N}$, Arf6-HA, Arf6$^{T27N}$-HA, FLAG-YAP$^{5SA}$, mAG-hGem (1-110), or mKO2-hCdt1 (30-120), 5 x $10^4$ parental cells/well in a 12-well plate were treated with lentiviruses and 10 μg/ml polybrene, centrifuged at 1200 x *g* for 1 h, and incubated for 24 h. The cells were selected and maintained in the medium containing 2.5 μg/ml Blastcidin S. In analyses with shRNAs, the following target sequences were used. Control shRNA targeting luciferase, 5'-GTGCGTTGCTAGTACCAAC-3'; mouse Arl4c, 5'-GCTCTATGAGATGATCCTG-3'. Viral supernatant was concentrated by ultracentrifugation at 50000 x *g* for 120 min. Isolated ureteric buds were treated with lentiviruses and 10 μg/ml polybrene, centrifuged at 1200 x *g* for 30 min, and cultured for 7 days.

***Immunofluorescence staining***

Cells grown on glass coverslips were fixed for 10 min at room temperature in PBS containing 4% (w/v) paraformaldehyde and permeabilized in PBS containing 0.2% (w/v) Triton X-100 and 2 mg/ml BSA for 10 min. IEC6 or MDCK II cells grown in 3D culture were fixed for 30 min at room temperature in PBS containing 4% (w/v) paraformaldehyde and permeabilized and blocked in PBS containing 0.5% (w/v) Triton X-100 and 40 mg/ml BSA for 30 min.

Kidney rudiments cultured for 48 h *in vitro* were fixed for 10 min in ice-cold MeOH and permeabilized and blocked in PBS containing 0.5% (w/v) Triton X-100 and 40 mg/ml BSA for 30 min. Organoids of ureteric buds (UBs) grown in 3D culture were removed from Matrigel using cell recovery solution (BD Biosciences) and fixed for 30 min at room temperature in PBS containing 4% (w/v) paraformaldehyde. Organoids were permeabilized and blocked in PBS containing 0.5% (w/v)

Triton X-100 and 40 mg/ml BSA for 30 min.

The cells or organoids were incubated with primary antibodies for 3 h at room temperature or overnight at 4˚C and with secondary antibodies in accordance with the manufacturer's protocol (Molecular Probes, Carlsbad, CA, USA). The samples were viewed and analysed with LSM510 and LS710 laser scanning microscope (Carl-Zeiss, Jana Germany). LSM image browser (Carl-Zeiss) was used to create stacking images in the experiments with MDCK II cells and kidney organoids.

***Reporter gene assay***

HeLaS3 cells were transfected with pEGFPC1, pCGN-SA-β-catenin, and/or Pmt-Raf-CAAX, *Arl4c-Luc*, and pCMV-LacZ. At 24 h after transfection, the cells were lysed, and the luciferase activity was measured with PicaGene reagent (Toyo Ink, Tokyo, Japan) as described previously (Yamamoto et al, 2003). β-Galactosidase activities were determined to normalize the transfection efficiency.

***Chromatin immunoprecipitation (ChIP) assay***

IEC6 Cells ($1\times10^7$) were stimulated with5 μM CHIR99021 and/or 5 ng/ml EGF for 3 h or 6 h and then the cells were cross-linked with 1% formaldehyde for 10 min at room temperature. The cell pellets were lysed with sodium dodecyl sulfate (SDS) lysis buffer (50 mM Tris/HCl [pH 8.0], 10 mM EDTA, and 0.5% SDS) and sonicated to shear DNA to a size range between 200 and 1000 bp. Sheared chromatin samples were diluted in ChIP dilution buffer (16.7 mM Tris/HCl [pH 8.0], 167 mM NaCl, 1.2 mM EDTA, and 1.1% Triton X-100) supplemented with protease inhibitors, and precleared with salmon sperm DNA/protein A-agarose (Millipore, Billerica, MA, USA) and incubated for 12 h at 4˚C with 5 μg of anti-histone H4 (acethyl K8) (Abcam, Cambridge, United Kingdom), anti-Tcf4 (6H5-3) (Millipore), anti-Ets1 (C-20) (Santa Cruz Biotechnology, CA, USA) antibodies, or negative control IgG (Diagenode, Liége, Belgium). Immunocomplexes were absorbed with salmon sperm DNA/protein A-agarose beads, and washed once with high salt buffer (20 mM Tris/HCl [pH 8.1], 500 mM NaCl, 0.1% SDS, 1% TritonX-100, and 2 mM EDTA), once with LiCl

buffer (10 mM Tris/HCl [pH 8.1], 0.25 M LiCl, 1 mM EDTA, 1% deoxycholic acid, and 1% Nonidet P-40), and three times with TE buffer (10 mM Tris/HCl (pH 8.1), and 1 mM EDTA). Immune complexes extracted in elution buffer (1% SDS and 100 mM $NaHCO_3$ ) were incubated for 4 h at 65°C to revert DNA-protein cross-links. Then the DNA was extracted by incubation in proteinase K (final concentration of 50 μg/ml) buffer for 1 h at 45 °C. The purified DNA was used in PCR to assess the presence of target sequences. Forward and reverse primers were as follows: fragment containing Ets-binding sites, 5'-ACCAGTCAGTTGGAGGGCTA-3' and 5'-GCTGTGAGTGAGCACGGAGT-3.

***Complex formation and immunoprecipitation***

HeLaS3 cells or HeLaS3 cells expressing HA-Tcf4 or HA-DN-Tcf4 (100-mm diameter dish) were treated with 5 μM CHIR99021 and/or 5 ng/ml EGF for 3 h and then lyzed in 500 μl of lysis buffer (10 mM Tris/HCl [pH 7.4], 140 mM NaCl, 5 mM EDTA, 1% NP40, 25 mM NaF, 20 μg/ml leupeptin, 20 μg/ml aprotinin, and 10 mM PMSF). The lysates were immunoprecipitated with 4 μg of anti-Ets1 antibody (C-20) (Santa Cruz) or negative control IgG (R&D), and the immunoprecipitates were probed with the indicated antibodies.

***Arf6 assay***

Activation of Arf6 was assayed using glutathione-S-transferase (GST)-GGA3 PBD (Santy & Casanova, 2001). IEC6 cells were lysed in 400 μl of 50 mM Tris/HCl [pH 7.5], 100 mM NaCl, 2 mM $MgCl_2$, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol with protease inhibitors (1 mM phenylmethylsulfonyl-fluoride, 1 mg/ml leupeptin, and 1 mg/ml aprotinin) containing 20 μg of GST-GGA3 PBD. After the lysates were centrifuged at 20000 x *g* for 10 min, the supernatants were incubated with glutathione-Sepharose (20 μl each) for 2 h at 4°C. Glutathione-Sepharose was precipitated by the centrifugation, and the bound proteins were probed with the anti-Arf6 antibody.

***Quantitative PCR***

Total RNA was isolated from IEC6 cells, HeLaS3 cells, or mouse tissues, and quantitative RT-PCR was performed (Hino et al, 2005). The results are expressed as fold increases compared with mRNA levels of the indicated genes in control cells. Forward and reverse primers were as follows: *rat GAPDH*, 5'-ATCAACGACCCCTTCA-3' and 5'-TTTGGCCCCACCCTTC-3'; *human, rat and mouse Axin2*, 5'-CTGGCTCCAGAAGATCACAAAG-3' and 5'-CATCCTCCCAGATCTCCTCAAA-3'; *rat Arl4c*, 5'-CTGCTGGTCATCGCCAACAA-3' and 5'-CCTGAAACGCAGGAAGTCTC-3'; *rat LRP5*, 5'-ACAGGGGAGAAAAGGAAGGA-3' and 5'-GCCATTGTCCTGCAACTGTA-3'; *rat LRP6* 5'-GGAACAAATCGCATTGAGGT-3' and 5'-CTCGACCCACATTTGGAACT-3'; *rat β-catenin*, 5'-GCCAGTGGATTCCGTACTGT-3' and 5'-GAGCTTGCTTTCCTGATTGC-3'; *rat Dvl1*, 5'-GGGAGTCAGCAGAGTGAAGG-3' and 5'-CCTGACTTCGAGGGCTACTG-3'; *rat Dvl2*, 5'-TGTGGCTCAAGATCACCATC-3' and 5'-CTGAGGTCCCCGAAAACATA-3'; *rat Dvl3*, 5'-GGCCTATGGCTTTCCCTTAC-3' and 5'-TGACTTTGAGTCCCCAGCTT-3'; *rat Arf6*, 5'-CTATGAGGGGCTCACATGGT-3' and 5'-AACAAAGAAAACCCCCAACC-3'; *rat ARNO*, 5'-CTGAACCTGTCTGTGCTCCA-3' and 5'-CACGTAGCAGGTGTCTGTGG-3'; *rat CTGF*, 5'-TAGCAAGAGCTGGGTGTGTG-3' and 5'-TTCACTTGCCACAAGCTGTC-3'; *rat ANKRD1*, 5'-AGCGGAGCAACCAGCTATAA-3' and 5'-AAGTCTTGCTCCCCCAAAAT-3'; *mouse* and *human GAPDH*, 5'-AGCCCAGAACATCATCCCTG-3' and 5'-CACCACCTTCTTGATGTCATC-3'; *mouse Arl4c*, 5'- CTCCTGGTTATCGCCAACA-3' and 5'- GAGACTTCCTGCGTTTCAGG-3', *human Arl4c*, 5'-GTGCTCTACCGGCTCAAGTT-3' and 5'-ACCGAGTCCACCACGTAGAT-3', *dog Arl4c*, 5'-GTGCTCTACCGGCTCAAGTT-3' and 5'-ACCGAGTCCACCACGTAGAT-3'.

***Knockdown of protein expression by siRNA***

In analyses with siRNAs, the following target sequences were used. Randomized control,

5’-CAGTCGCGTTTGCGACTGG-3’; *rat LRP5,* 5’-GCAGTACCCGGAAGATCAT-3’; *rat LRP6,* 5’-GCTGGCATGTGATTGGCTT-3’; *rat Dvl1,* 5’-CCAGTAGCCGAGATGGAAT-3’; *rat Dvl2,* 5’-GCGAGACGAAGGTGATTTA-3’; *rat Dvl3,* 5’-GCATCACAGACTCCACTAT-3’; *rat β-catenin,* 5’-CCATGGAGCCAGACAGAAA-3’; *rat Arl4c,* 5’-GCTGTGGGAACTGAGTAAT-3’; *rat YAP,* 5’-CCGGGATGACTCAGGAATT-3’; *rat TAZ,* 5’-CCTCGTACTCACACTCCTT-3’; *rat Elk1,* 5’-CCTTCTTCTCAGTCAGCTT-3’; *rat Ets1,* 5’-GCCACATTCCATTGGCCAT-3’; *rat Ets2,* 5’-CCATTTCAGAAGTCAGTTT-3’; *rat Arf6,* 5’-CCAAGATCTTCGGGAACAA-3’; *rat ARNO,* 5’-CCTGACTCCTTATTTGTAA-3’. IEC6 cells were transfected with a mixture of siRNAs against genes of interest at 2.5-20 nM each using RNAiMAX (Invitrogen Carlsbad, CA, USA) and the cells were used for experiments at 36-48 h post-transfection.

***Others***

Western blotting data were representative of at least four independent experiments. Rac and Rho activities were measured using glutathione-S-transferase (GST)-CRIB and GST-Rhotekin as described previously (Sato et al, 2010).

## Supplementary References


Hino S, Tanji C, Nakayama KI, Kikuchi A (2005) Phosphorylation of β-catenin by cyclic AMP-dependent protein kinase stabilizes beta-catenin through inhibition of its ubiquitination. *Mol Cell Biol* 25: 9063-9072

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

Komekado H, Yamamoto H, Chiba T, Kikuchi A (2007) Glycosylation and palmitoylation of Wnt-3a are coupled to produce an active form of Wnt-3a. *Genes Cells* 12: 521-534

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

Marais R, Light Y, Paterson HF, Mason CS, Marshall CJ (1997) Differential regulation of Raf-1, A-Raf, and B-Raf by oncogenic ras and tyrosine kinases. *J Biol Chem* 272: 4378-4383

Miyoshi H, Blomer U, Takahashi M, Gage FH, Verma IM (1998) Development of a self-inactivating lentivirus vector. *J Virol* 72: 8150-8157

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

Santy LC, Casanova JE (2001) Activation of ARF6 by ARNO stimulates epithelial cell migration through downstream activation of both Rac1 and phospholipase D. *J Cell Biol* 154: 599-610

Sato A, Yamamoto H, Sakane H, Koyama H, Kikuchi A (2010) Wnt5a regulates distinct signalling pathways by binding to Frizzled2. *EMBO J* 29: 41-54

Yamamoto H, Ihara M, Matsuura Y, Kikuchi A (2003) Sumoylation is involved in β-catenin-dependent activation of Tcf-4. *EMBO J* 22: 2047-2059