Supporting Information

Li et al. 10.1073/pnas.1013170108

SI Methods

Zebrafish Strains and Husbandry. Zebrafish were maintained in an Emory University facility under standardized conditions in compliance with Institutional Animal Care and Use Committee regulations. Wild-type and transgenic zebrafish embryos were maintained at 28.5 °C and staged as previously described (1). The *Tg* (*TOP:GFP*)$^{w25/+}$ transgenic line contains four Lef binding sites and a minimal promoter upstream of GFP (2) and was used as the reporter line for the canonical Wnt signaling pathway. For in vivo canonical Wnt assays, embryos obtained by in-crossing parents homozygous for the transgene were used. *Tg*(*hsp70l:Δtcf3-GFP*)^{w26} (*Δtcf*) transgenic zebrafish was kindly provided by Richard Dorsky (Salt Lake City). The *Δtcf* line contains Tcf3a with GFP in place of the N terminus under the control of a heat-shock promoter (3). Transgene activation was achieved by heat-shocking at 37 °C for 1 h according to previous protocols (3). Embryos were sorted according to GFP expression and raised at 28.5 °C until fixation. Where appropriate, wild-type embryos were heat-shocked under the same conditions to serve as controls.

Yeast Two-Hybrid Analysis. Vangl2 cDNA encoding its C-terminal 238–521 amino acids was cloned into the plasmid pGBKT7 (Clontech) and used as a bait to screen an embryonic day 15 mouse cochlear epithelial cDNA library built in pGADT7-RecAB. Positive clones expressing reporters His3, Ade2, α-galactosidase (α-gal), and β-gal were selected and rescreened. Plasmid DNAs were prepared from confirmed positive clones, digested diagnostically to preliminarily classify clones, and sequenced.

Coimmunoprecipitation, Western Blot Analysis, and Immunohistochemistry. Coimmunoprecipitation assays of Rack1 and Vangl2-GFP were performed with brain and/or cochlear extracts prepared from embryonic day 16.5 mouse embryos expressing Vangl2-GFP fusion protein. The mice expressing Vangl2-GFP were generated previously by using BAC-mediated transgenesis and carried two copies of Vangl2-GFP under the control of Vangl2 gene regulatory elements. Vangl2-GFP shows asymmetric subcellular localization identical to that of the endogenous Vangl2 protein. For mapping Vangl2-interacting domains in Rack1 protein, plasmids encoding Rack1 WD40 1–4 or Rack1 WD40 5–7 motifs tagged with HA (gifts from Pablo Lopez Bergami, Mount Sinai School of Medicine, New York, NY) were cotransfected into HEK293 cells with a plasmid encoding Vangl2-GFP by using FuGENE 6 (Roche). Protein extracts were prepared in RIPA buffer (Upstate Biotechnology) containing a protease inhibitor mixture (Roche).

For coimmunoprecipitation, 500 μL (1 μg/μL) of protein extracts was incubated with a designated primary antibody at 4 °C overnight, followed by incubation with protein A beads (Invitrogen) pretreated with 5% BSA for 1 h at 4 °C. After three washes, samples were eluted in 50 μL of 2× SDS sample loading buffer (100 mM Tris·HCl, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) and heat-treated before being subjected to standard SDS/PAGE. The primary antibodies used for coimmunoprecipitation were: rabbit (Santa Cruz Biotechnology) or mouse anti-Rack1 (BD Biosciences), rabbit (Santa Cruz Biotechnology) or mouse anti-GFP (Chemicon), and rabbit (Santa Cruz Biotechnology) and mouse anti-HA (Cell Signaling).

For Western blot analysis, the following primary antibodies were used: mouse anti-Rack1 (1:1,000; BD Biosciences), mouse anti-GFP (1:500; Chemicon), rabbit anti-aPKCζ (1:1,500; Santa Cruz Biotechnology), mouse anti-GADPH (1:1,000; Proteus BioSciences), mouse anti-HA (1:1,000; Cell Signaling), and mouse anti–α-tubulin (1:10,000; Sigma).

For immunostaining in siRNA experiments, the monoclonal anti-RACK1 antibody was from Transduction Laboratories. Texas Red goat anti-mouse IgG, nucleic acid stain DAPI, and ProLong Gold antifade reagent were purchased from Molecular Probes and Invitrogen.

Morpholino Oligonucleotides (MOs), cDNA Constructs, and in Situ Hybridization. The translation-blocking MO against *rack1* was as follows: 5′-CCC TTA CTG TCA TCT GCT CGG TCAT-3′. The *vangl2* MO has been previously described (4). For single-morpholino injections, 2 ng of MO was injected into embryos at the one- or two-cell stage. For double-morpholino injections, ∼1 ng of each MO was injected. The control morpholino sequence used was 5′-CCT CTT ACC TCA GTT ACA ATT TATA-3′. All of the MOs were synthesized by Gene Tools.

For RNA synthesis, cDNA sequences encoding zebrafish or mouse Rack1 were cloned into pCS2+ vectors to prepare RNA for in situ hybridization or mRNA rescue experiments, respectively. DNA sequences encoding human Rack1$^{WD1–4}$ or Rack1$^{WD5–7}$ motifs were cloned into pCS2+ to generate RNA for overexpression analysis. Approximately 100–200 pg of mouse *rack1* or *vangl2* mRNA was injected into embryos at the one- or two-cell stage together with *rack1* or *vangl2* MO for rescue experiments. For overexpression of Rack1 WD40 motifs, 500–2,000 pg of mRNA was used.

Standard procedures were used to carry out in situ hybridization for *chd*, *dlx3b*, and *myoD1*.

Monitoring Gastrulation Cellular Movements, Mitotic Spindle Orientation, and Notochord Cellular Morphology. Convergent extension (CE) movements during gastrulation were analyzed as previously described (5), with minor modifications. Fluorescein dextran (10,000 M_r) was injected into a single blastomere of a 32-cell morphant embryo. Only embryos with fluorescent clones located within the dorsal region of embryos at the onset of gastrulation were included for imaging. Images shown are representative of at least 10 embryos containing dorsal fluorescent clones of each experimental group. Live images were taken at 30% epiboly, shield, and 75% epiboly stages of the same embryos.

Mitotic spindle orientation in dorsal epiblast cells during early gastrulation was recorded and analyzed as described (6). Briefly, control or *rack1* MOs were injected along with RNAs encoding Histone 2B-RFP and membrane GFP (gifts from Dr. Scott Fraser, Pasadena, CA) into embryos at the one- or two-cell stage. Live *Z* images were taken from the dorsal side around the shield stage to record mitotic spindle orientation through the depth of the dorsal epiblast. The angles formed between each mitotic spindle and the animal–vegetal axis of the embryo was measured and plotted for each experimental group. Student's *t* test was carried out to evaluate the significance of difference among the three experimental groups.

To assay notochord cell morphology, embryos were raised in 10 μM BODIPY ceramide (Invitrogen) as previously described (7). Optical scanning images in the most anterior part of notochord were taken at the entire depth from carefully stage-matched morphant embryos. Optical scanning images at compatible regions were compared. The geometric long axis for each cell (irregular polygon) was calculated and plotted by using a program we developed. The angle formed between the long axis of each cell

and the mediolateral axis of each embryo was measured by using the program we developed and plotted with the Oriana 3 program.

Lef/Tcf Canonical Wnt Reporter Assays. TOPFlash luciferase reporter construct (Randall T. Moon, University of Washington, Seattle, provided by Xing Dai, University of California, Irvine) and a pSV-β-gal expression vector (a gift from Harish Joshi, Emory University) were cotransfected with the plasmid expressing Rack1 or with the control plasmid into HEK293 cells by using FuGENE 6. Transfected cells were treated with Wnt3a conditioned medium (isolated from Wnt3A-expressing cell line CRL-2647; ATCC) or control medium (isolated from L2648; CRL-2648 ATCC) for 4, 8, or 12 h and harvested for standard luciferase assays to detect the canonical Wnt activity, all of which were normalized with β-gal activity. The difference between samples of control plasmid and Rack1-expressing plasmid was subjected to two-tailed unpaired type 2 Student's *t* test.

1. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the zebrafish. *Dev Dyn* 203:253–310.
2. Dorsky RI, Sheldahl LC, Moon RT (2002) A transgenic Lef1/β-catenin-dependent reporter is expressed in spatially restricted domains throughout zebrafish development. *Dev Biol* 241:229–237.
3. Lewis JL, et al. (2004) Reiterated Wnt signaling during zebrafish neural crest development. *Development* 131:1299–1308.
4. Park M, Moon RT (2002) The planar cell-polarity gene *stbm* regulates cell behaviour and cell fate in vertebrate embryos. *Nat Cell Biol* 4:20–25.
5. Gerdes JM, et al. (2007) Disruption of the basal body compromises proteasomal function and perturbs intracellular Wnt response. *Nat Genet* 39:1350–1360.
6. Gong Y, Mo C, Fraser SE (2004) Planar cell polarity signalling controls cell division orientation during zebrafish gastrulation. *Nature* 430:689–693.
7. Jessen JR, et al. (2002) Zebrafish *trilobite* identifies new roles for Strabismus in gastrulation and neuronal movements. *Nat Cell Biol* 4:610–615.

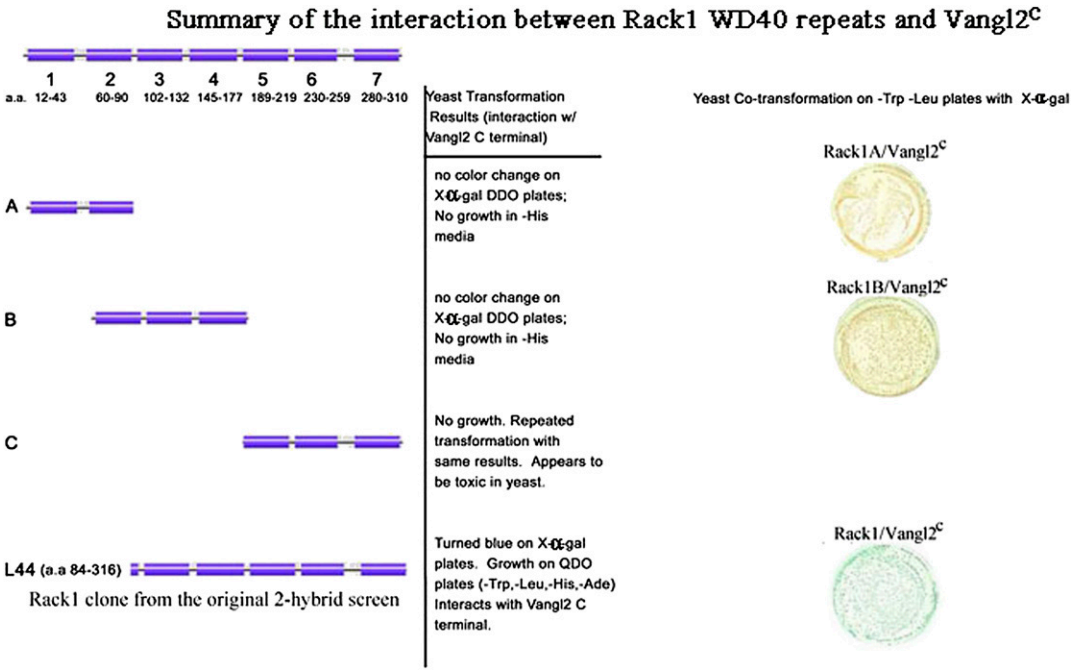

Fig. S1. Summary of the interaction between Rack1 WD40 repeats and Vangl2 C-terminal cytoplasmic domain. Rack1 WD40 repeats 1–2, 2–4, and 5–7 were tested for their interaction with Vangl2 C-terminal domain (Vangl2^{C}). A positive interaction leads to the expression of reporter genes, and the cells turn blue. In contrast to clone L44, which encodes Rack1 WD40 repeats 3–7 and gives a positive reading for its interaction with Vangl2^{C}, Rack1 WD40 repeats 1–2 and 2–4 do not show a detectable interaction with Vangl2^{C}. Rack1 WD40 repeats 5–7 are toxic to yeast cells.

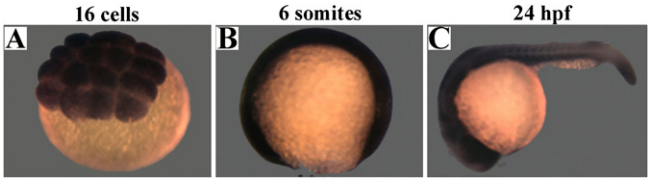

Fig. S2. Rack1 is expressed ubiquitously during early zebrafish embryogenesis. Embryos from 16-cell (*A*), six-somite (*B*), and 24-h postfertilization (hpf) (*C*) stages were subjected to in situ hybridization analysis with *rack1* RNA probe.

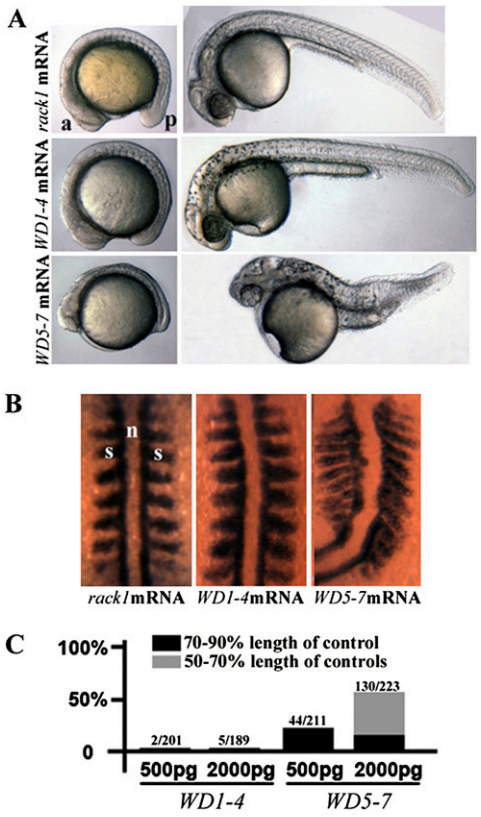

Fig. S3. Dominant-negative effect of Rack1$^{WD5–7}$ on gastrulation. (*A* and *B*) Live and *myoD1* in situ hybridization images of embryos injected with full-length mouse *rack1* mRNA, mRNA encoding Rack1$^{WD1–4}$, or mRNA encoding Rack1$^{WD5–7}$. (*C*) Gastrulation defect was quantified. a, anterior; p, posterior; s, somite; n, notochord.

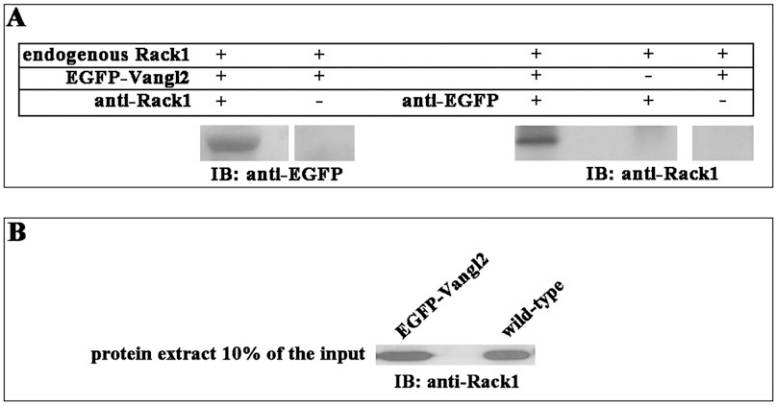

Fig. S4. Rack1 is associated with Vangl2 in zebrafish embryo extracts. RNA encoding EGFP-Vangl2 (200 pg) was injected into zebrafish embryos at the one- or two-cell stage. The embryos were collected from the bud stage (10 hpf) to 10-somite stage (14 hpf) and processed for yolk removal and protein extraction from the dissected EGFP-Vangl2–expressing embryos. Similarly, extracts were made from control embryos. (*A*) Coimmunoprecipitation experiments were performed as described. Note that EGFP-Vangl2 was pulled down only in the presence of Rack1 antibody and that Rack1 was coimmunoprecipitated only in the presence of EGFP-Vangl2 and EGFP antibody. (*B*) The protein extracts from the control and EGFP-Vangl2–injected embryos were loaded directly and blotted with an antibody against Rack1 as input controls.

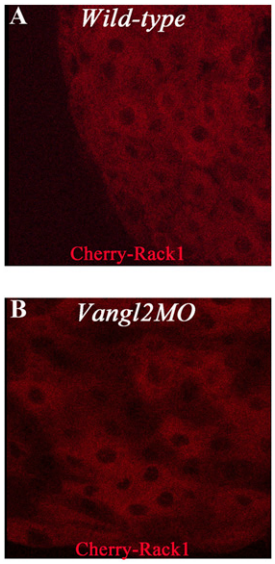

Fig. S5. Rack1 localization. RNA encoding Cherry-Rack1 was injected to control (*A*) and *vangl2* (*B*) morphants. Views are of enveloping layer cells of shield-stage embryos.

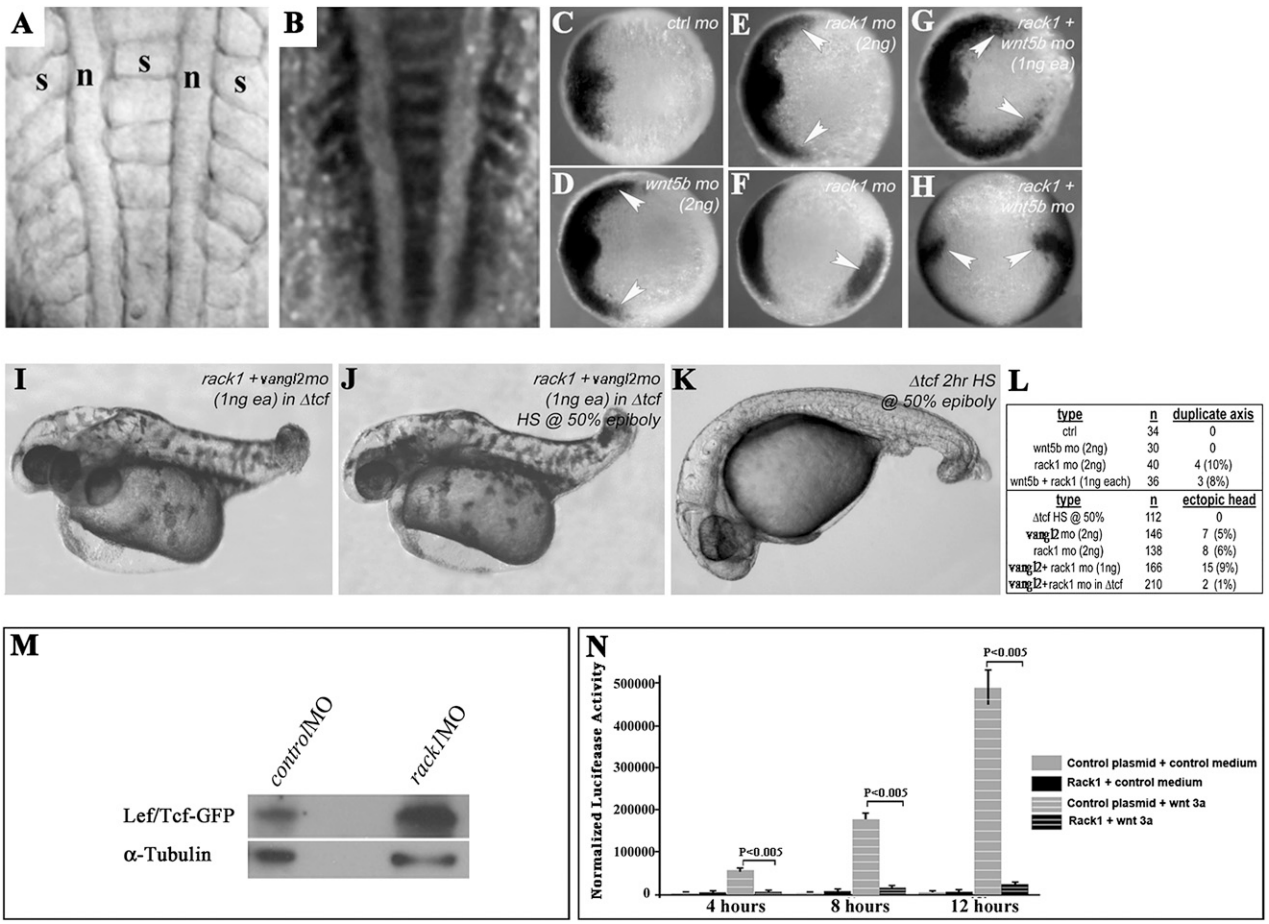

Fig. S6. Rack1 antagonizes canonical Wnt activity. (*A–L*) Knockdown of Rack1 or Vangl2 led to the formation of duplicated body axes (*A* and *B*) as shown in live imaging (*A*) and with *myoD1* expression (*B*). The expression of a canonical Wnt target gene, *chd*, in control (*C*), *wnt5b* (*D*), *rack1* (*E* and *F*), and *rack1* and *wnt5b* (*G* and *H*) morphants. Embryos injected with *rack1* and *vangl2* MO s without (*I*) or with (*J*) heat-shock treatment that inactivates canonical Wnt activity. Control embryos with heat-shock treatment (*K*) show a typical canonical Wnt loss-of-function phenotype. The quantification of the duplicated body axes was summarized in *L*. (*M*) Western blot with an antibody against GFP or α-tubulin using protein extracts from control- or *rack1* MO–injected 24-h postfertilization embryos carrying canonical Wnt reporter GFP. (*N*) The graph summarizing normalized luciferase activities detected in HEK293 cells cotransfected with the canonical Wnt reporter construct and the control or Rack1-expressing plasmid with or without Wnt3a stimulation. The error bars represent the SDs among the normalized reporter activities from at least three independently transfected samples. a, anterior; p, posterior; n, notochord; s, somite.

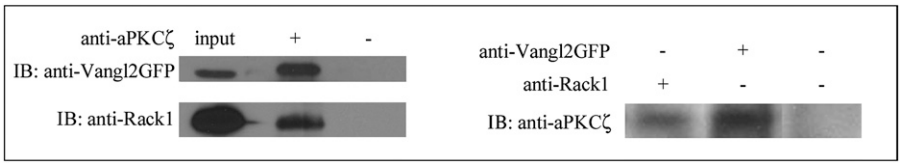

Fig. S7. Rack1 and Vangl2 both form complexes with aPKCζ. An antibody against aPKCζ specifically pulled down both Vangl2GFP and Rack1 (*Left*), and antibodies against either Vangl2GFP or Rack1 coimmunoprecipitated specifically aPKCζ (*Right*) from embryonic day 16 mouse brain extracts.