Supplementary information 1

**Plasmids and antibodies** Plasmids encoding full-length cDNAs of mouse EphB2 and human ephrin-B1, and the Fc-fusion protein expression construct of ephrin-B1 were described previously (Tanaka *et al.*, 2004; Wang *et al.*, 2002). The mutant of ephrin-B1 containing the extracellular domain (ephrin-B1 ECD) was generated using a PCR-based technique and tagged with GST by cloning into pGEX4T2 (Amersham Pharmacia). Ephrin-B1 mutant containing the transmembrane and cytoplasmic domain (ephrin-B1 cyto) was also cloned into pGEX4T2. Ephrin-B1 with mutations of four tyrosine residues in the cytoplasmic domain (Y313, 317, 324, 329), ephrin-B1 4YF, was generated using the Altered sites Mutagenesis system (Promega). The plasmids encoding mouse claudin1 and claudin4 were donated by M. Furuse and S. Tsukita. Mutants of claudin1 shown in Fig. 2A were also generated using the PCR-based technique, and cloned into pEBB with the addition of a flag epitope tag at the carboxyl-terminus. To generate GST-tagged claudin1 mutants shown in Fig. 2B, ECD1 (aa 1-103), ECD2 (aa 115-188), C1 (aa 81-145) and C2 (aa 160-211) were cloned into pGEX4T2. To generate the recombinant retrovirus, cDNA of wild type claudin4 was subcloned into the vector pDON-AI (Takara). For making claudin1-Fc fusion protein, the first and the second extracellular domain of claudin1 (claudin1 and claudin1, respectively) were connected with flag tag. The cDNA fragment encoding the signal peptide of secreted alkaline phosphatase (pSEAP2, Clontech) was ligated to the amino-terminus, and the Fc-region of mouse IgG was ligated to the carboxyl-terminus. The Fc-fusion proteins of Eph, ephrin and claudin1 were purified from a culture medium of COS1 cells transfected with plasmids encoding those Fc-fusion proteins by passing them through a Protein A sepharose column as described previously (Wang *et al.*, 2002). pCSMT was from D. Turner and R. Rupp (Hutchinson Cancer Research Center, Seattle, WA). The monoclonal antibodies for the flag tag and the myc tag were from Sigma and Santa Cruz, respectively. The rabbit polyclonal antibodies for ephrin-B1 and EphB2, which react with the extracellular domain of each protein, were purchased from R&D systems. The monoclonal antibody for phosphotyrosine (4G10) was from Upstate Biotechnology. The Alexa Fluor-labeled second
antibodies of anti-goat IgG, anti-rabbit IgG and anti-mouse IgG were from Molecular Probes. Polyclonal antibody against tyrosine-phosphorylated ephrin-B1 (ephrin-B1 pY317, amino acid 314-321) was raised in rabbits and affinity-purified.

**Screening of cDNA library** The cDNA was synthesized from poly(A)^+ RNA of stage 10-12 *Xenopus laevis* embryos using oligodeoxythymidylate primers and Moloney murine leukemia virus reverse transcriptase (BRL). Staging of embryos was according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). The synthesized cDNAs were directionally cloned into pCSMT expression vector using EcoRI and XhoI sites to construct the proteins tagged with 6 copies of myc-epitope at amino-terminus. The cDNA library was divided into small pools, initially containing around 500 cDNAs for each. Ephrin-B1 together with a pool of *Xenopus* cDNA library were transiently expressed in HEK293T cells. The cDNA pools containing ephrin-B1 binding proteins were identified through immunoprecipitation of the cell lysates with anti-ephrin-B1 antibody and subject to immunoblotting with the antibody against myc. A single clone was isolated through four or five rounds of sequential enrichment.

**Cell culture and transfection**

HT29 colon carcinoma cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum, while MDCK, L and Cos1 cells were cultured in DMEM with 10% fetal bovine serum. For transient expression assays, Cos1 cells were transfected with plasmid DNA using FuGene6 reagent (Roche). In some experiments, transfected cells were treated with PP2 or PP3 (Calbiochem) at a final concentration of 10 µM for 30 min before making cell lysates. L cells stably expressing ephrin-B1 and/or claudins were established through selection in DMEM containing hygromycin B at a concentration of 400 µg/ml or G418 (Gibco BRL) at 0.6 mg/ml for 2 to 3 weeks. Well-isolated colonies were characterized further. To establish MDCK ephrin-B1 Tet-ON cells, we used the Rev Tet-ON gene expression system (Clontech) to introduce the tetracycline-regulating element, tTA, and subsequently, the Rev-TRE carried human ephrin-B1 cDNA into the MDCK cells. In some experiments, plates were coated with claudin1-Fc fusion protein as described previously (Tanaka et al., 2004). Briefly, plates were filled with 2 µg/ml fusion protein plus 20 µg/ml of goat anti-IgG Fc (ICN) in PBS (-). Plates were washed, then incubated in PBS (-)
containing 2 % BSA (w/v) to block the remaining protein binding sites. Cells were plated on the coated surface until cells attached and spread on each plate almost to the same degree.

**Immunoprecipitation and Affinity Precipitation**

Transfected cells were harvested 48 hrs after transfection, and cell lysates were prepared with protease inhibitors in a PLC buffer [50 mM Hepes (PH 7.5), 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 100 mM NaF, 1 mM Na₃VO₄, and 1% Triton X-100]. To purify the proteins, 1 µg of monoclonal or affinity purified polyclonal antibodies was incubated with 500 µg of cell lysate for 2 hrs at 4°C. They were precipitated with protein A agarose for 1 hr at 4°C. Immunoprecipitates were extensively washed with PLC buffer, separated by SDS-PAGE, and immunoblotted.

**Cell staining**

MDCK or L cells were plated on glass cover slips. The cells were fixed for 5 min at room temperature with 4% PFA in PBS and then permeabilized for 10 min with 0.2% Triton X-100. The cells were preincubated in 2% BSA (bovine serum albumin) with 5% normal serum for 0.5 hr and then incubated with the specific antibodies for 1 hr at room temperature. After washing, the cells were incubated with Alexa-conjugated secondary antibodies with or without TOTO-3 iodide (Molecular Probes) for 0.5 hr at room temperature. Photos were taken using a Radiance 2100 confocal microscope (BioRad).