Supporting Information

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SI Methods

Reagents. Protein A/G-conjugated agarose beads and antibodies and specific siRNAs of netrin-1, MST1, MST2, UNC5B, UNC5C, DCC, and control siRNA were from Santa Cruz. Smart pool siRNA oligonucleotides toward human Lats1 or Last2 were purchased from Dharmacon. Recombinant human netrin-1 was from R&D Systems. Glutathione Sepharose 4B was supplied by Amersham Pharmacia. All of the chemicals not included above were from Sigma. Antibodies of YAP, TAZ, phosphor-YAP (S127), phosphor-YAP (S397), phosphor-Akt (S473), phosphor-MST1/2 (T183/180), phosphor-Lats1 (S909), phosphor-Lats1 (S1079), Lats1, PARP, and Caveolin-1 were obtained from Cell Signaling. Anti–β-actin, anti-Flag, and anti-HA antibodies were purchased from Sigma. Anti-CTFG and anti-Ub antibodies were from Santa Cruz Biotechnology. The antibody against PP1A was purchased from Abcam.

Transfection of Plasmids/siRNAs. Cells were transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. siRNAs were delivered into cells using RNAiMAX (Invitrogen) according to the manufacturer's instructions.

In Vitro Dephosphorylation Assay. In vitro dephosphorylation reactions were carried out by incubating immunprecipitated PP1A from netrin-1–treated cells with immunoprecipitated phospho-YAP (Ser-127)/phospho-YAP (Ser-397) from confluent cells for 1 h at 37 °C in buffer containing 50 mM Hepes, 100 mM NaCl, 1 mM $MnCl_2$, 2 mM DTT, 0.1 mM EGTA, and 0.025% Tween-20 followed by SDS/PAGE gel and immunoblotting with the indicated antibodies.

Immunofluorescence Staining. Cells were seeded on coverslips and fixed with 4% (vol/vol) paraformaldehyde (prepared in PBS) for 30 min and permeabilized with 0.1% Triton X-100 for 30 min. Following blocking in 3% (vol/vol) FBS in PBS for 1 h, cells were incubated with an antibody at 4 °C overnight. After washing with PBS three times, cells were incubated with FITC-conjugated secondary antibodies (1:1,000) for 2 h at room temperature. Then the cells were counterstained with 4′,6-diamidino-2-phenylindole and examined under a fluorescence microscope.

Cell Proliferation and Migration Assay. Cells were seeded in 96-well plates at a density of 3,000 cells per well. The next day, the medium was replaced with fresh medium containing drugs or vehicle controls. Cells were incubated at 37 °C for the indicated times. The cell proliferation was monitored by MTT and BrdU incorporation (Cell Signaling) assays according to the manufacturer's protocols. Cell migration assay was performed using BD Falcon cell culture inserts for 24-well plates with a 8.0 μm pore filter according to the manufacturer's instructions. Treated cells (equal number of viable cells counted by Trypan blue staining) were seeded into the upper chamber of the insert in serum-free media, and the lower chamber was filled with media containing 5% (vol/vol) FBS. After 24 h, cells were fixed using 100% (vol/vol) methanol and stained using 0.05% crystal violet. Cells in the upper chamber were carefully removed, and cells that migrated through the filter were determined by counting the cells attached on the filters.

Western Blotting and Immunoprecipitation. Western blotting was performed using the standard protocol. Cell were lysed in lysis buffer A (50 mm Tris, pH 7.4, 40 mm NaCl, 1 mm EDTA, 0.5% Triton X-100, 1.5 mm Na_3VO_4, 50 mm NaF, 10 mm sodium pyrophosphate, 10 mm sodium β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 5 mg/mL aprotinin, 1 mg/mL leupeptin, and 1 mg/mL pepstatin A). After centrifugation for 10 min at 14,000 × *g* at 4 °C, the cell lysate was quantified using Brandford assay. An equal amount of protein (20–50 μg) was loaded for blotting with the indicated specific antibodies. For immunoprecipitation, after centrifugation, the supernatants were collected, quantified, and the antibody was mixed with the supernatant for 1 h, and then protein A/D agarose beads were added in overnight. Immunoprecipitates were washed four times with lysis buffer, and proteins were eluted with SDS/PAGE sample buffer.

RNA Extraction and Reverse Transcription. RNA samples were prepared using RNeasy Plus mini kit (QIAGEN). Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem) according to the manufacturer's instruction.

PCR and Real-Time PCR. PCR was performed using the PCR Master Mix (Thermo Scientific) according to the manufacturer's instruction. Complementary DNA was used for quantification (with GAPDH gene as a control) by real-time PCR, which was performed using SYBR qPCR master mix (Applied Biosystems) and the 7300 real-time PCR system (Applied Biosystems). Primer pairs used in this study are as follows: UNC5A, CAACATGACCTATGGGACCTT/CCTCGCACGACTGCTTTT; UNC5B, ACCACCCAGCTGGGACCTTAT/AGGGTCGACCTGCCCTCCCGAGGCGTCC; UNC5C, CAAGTGTAATAGTGAATGGGTT/GGCCTGCTTTATGATGAG; UNC5D, CTTTGTACTCGGGCTTGG/AATGATTCTTGCCGTGGT; DCC, ACCCGACGCTCTTCA/CCTCTCCAGAGTGGACATAGGCC; netrin-1, GACAAGGCGGGGGACTGG/GGCCTTCTTGCACTTGCCC; GAPDH, TTGCCATCAATGACCCCTTCA/CGCCCCACTTGATTTTGGA; YAP, CGCTCTTCAACGCCGTCA/AGTACTGGCCTGTCGGGAGT; TAZ, CCAGTGCCTCAGAGGTCCA/ATCTGCTGCTGGTGTTGGTG; and CTGF, CCAATGACAACGCCTCCTG/TGGTGCAGCCAGAAAGCTC. These primers were synthesized by Eurofins MWG.

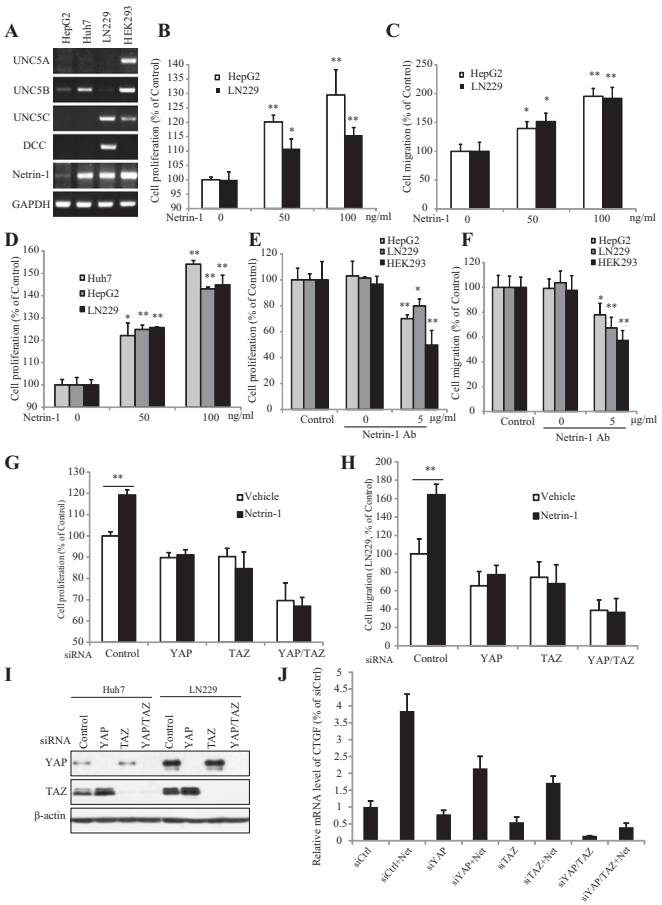

Fig. S1. YAP/TAZ is required for netrin-1 to promote cell proliferation and migration (related to Fig. 1). (*A*) The expression spectrum of canonical netrin receptors in various cell lines. Several indicated gene expressions in HepG2, Huh7, and LN229 and HEK293 cells were analyzed by RT-PCR as described in *Methods*. HepG2 cells weakly express UNC5B, and Huh7 cells strongly express UNC5B. LN229 cells contain both DCC and UNC5C receptors. (*B* and *C*) Netrin-1 enhances cell proliferation and migration in HepG2 and LN229 cells. Netrin-1 treatment enhances cell proliferation in HepG2 and LN229 cells. Following treatment with netrin-1 (100 ng/mL) for 48 h, cell proliferation was examined by MTT assay (*B*). Netrin-1 promotes migration of HepG2 and LN229 cells. Following treatment with netrin-1 (100 ng/mL), cell migration was determined by transwell migration assay (*C*). Data are presented as mean ± SEM (*$P < 0.05$, **$P < 0.01$, one-way ANOVA; $n = 3$). (*D*) Effect of netrin-1 on cell proliferation examined by BrdU incorporation assay. Cells were treated with different concentrations of netrin-1 for 48 h, and the effect of netrn-1 on cell proliferation was determined by BrdU incorporation assay, as described in *Methods*. Data are represented as mean ± SEM (*$P < 0.05$, **$P < 0.01$, one-way ANOVA; $n = 3$). (*E* and *F*) Antibody against netrin-1 decreases cell proliferation and migration. Cells were treated with the antibody of netrin-1 (5 μg/mL) for 48 h. Cell proliferation and migration were examined by MTT assay (*D*) and transwell cell migration assay (*E*). Data are presented as mean ± SEM (*$P < 0.05$, **$P < 0.01$, one-way ANOVA; $n = 3$). (*G* and *H*) YAP/TAZ are required for netrin-1 to stimulate LN229 cell proliferation and migration. YAP/TAZ were knocked down in LN229 cells and treated with or without 100 ng/mL netrin-1 for 48 h. Netrin-1 was replenished every day. Cell proliferation was determined by MTT assay (*G*). Knockdown of YAP/TAZ blocks netrin-1–induced cell migration. Migration of LN229 cells transfected with control siRNA or YAP/TAZ siRNA was assessed by transwell cell migration assays (*H*). Data are presented as mean ± SEM (**$P < 0.01$, two-tailed Student's t test; $n = 3$). (*I*) Validation of siRNA knockdown on YAP/TAZ expression in Huh7 and LN229 cells. Cells were transfected with the indicated siRNA, and the knockdown efficiency was determined by Western blotting. (*J*) YAP/TAZ are required for netrin-1 to induce their responsive CTGF gene expression. Following transfection with the indicated siRNA, LN229 cells were treated by netrin-1 (100 ng/mL) for 2 h. The mRNA levels of the CTGF were measured by real-time PCR. Data are presented as mean ± SEM.

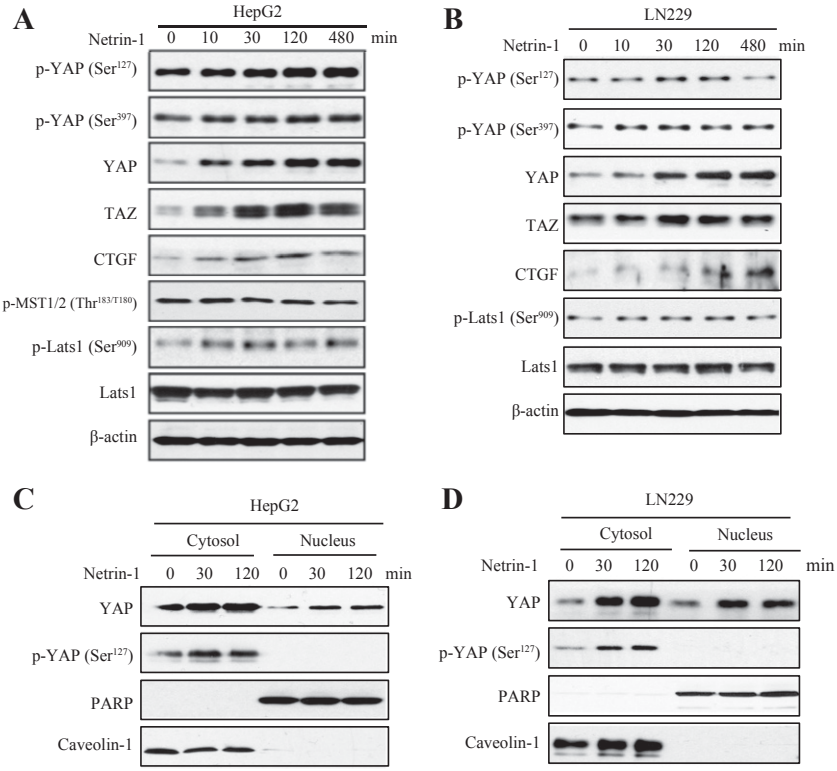

Fig. S2. Netrin-1 up-regulates YAP expression and accumulation in the nucleus of HepG2 and LN229 cells (related to Fig. 2). (*A*) Netrin-1 enhances YAP expression in HepG2 cells. Cells were treated with netrin-1 (100 ng/mL) for the indicated times. Cell lysates were subjected to Western blotting with the indicated antibodies. (*B*) Netrin-1 enhances YAP expression in LN229 cells. Cells were treated with netrin-1 (100 ng/mL) for the indicated times. Cell lysates were subjected to Western blotting with the indicated antibodies. (*C*) Netrin-1 induced YAP accumulation in the nucleus in HepG2 cells. Cells were treated with netrin-1 for 30 or 120 min and subjected to subcellular fractionation. Equal amounts of proteins from the nuclear and cytosolic fractions were subjected to immunoblotting with antibodies of PARP and Caveolin-1. (*D*) Netrin-1 induces YAP accumulation in the nucleus in LN229 cells. Cells were treated with netrin-1 for 30 or 120 min and subjected to subcellular fractionation. Equal amounts of proteins from the nuclear and cytosolic fractions were subjected to immunoblotting with antibodies of PARP and Caveolin-1.

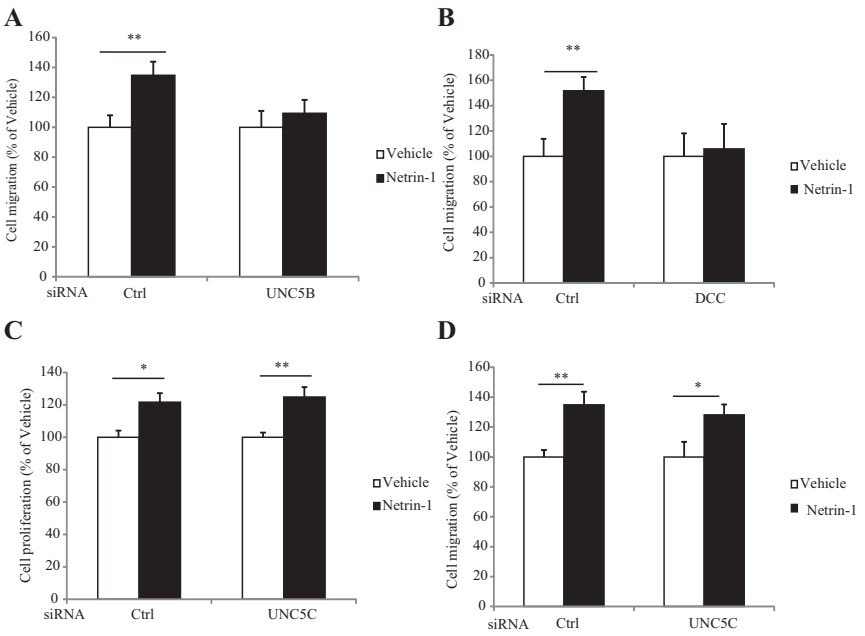

Fig. S3. Involvement of netrin receptors in netrin-1 induced cell proliferation and migration (related to Fig. 3). (*A*) Knockdown of UNC5B abolishes netrin-1–induced cell migration in Huh7 cells. Cells were transfected with control siRNA or UNC5B siRNA and then subjected to transwell migration assay in the presence of netrin-1 (100 ng/mL). (*B*) Knockdown of DCC abolished netrin-1–induced cell migration in LN229 cells. Cells were transfected with control siRNA or UNC5B siRNA and then subjected to transwell migration assay in the presence of netrin-1 (100 ng/mL). (*C* and *D*) UNC5C is not required for the regulation of cell proliferation and migration by netriin-1. Cell proliferation (*C*) and migration (*D*) were determined by MTT and transwell migration assays, respectively. Data are presented as mean ± SEM (*$P < 0.05$, **$P < 0.01$, two-tailed Student's *t* test; $n = 3$).

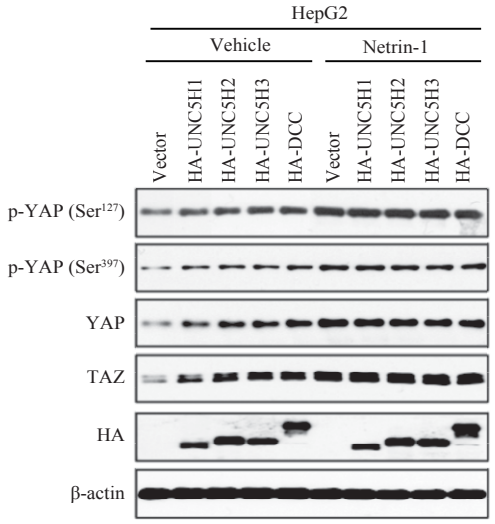

Fig. S4. Overexpression of the canonical receptors of netrin-1 up-regulates YAP phosphorylation and expression (related to Fig. 3). Overexpression of the netrin receptor induces YAP phosphorylation and its expression increases. HepG2 cells were transfected with the indicated netrin-1 receptors, followed by treatment with vehicle or netrin-1 (100 ng/mL) for 2 h, and cell lysates were subjected to Western blotting with the indicated antibodies.

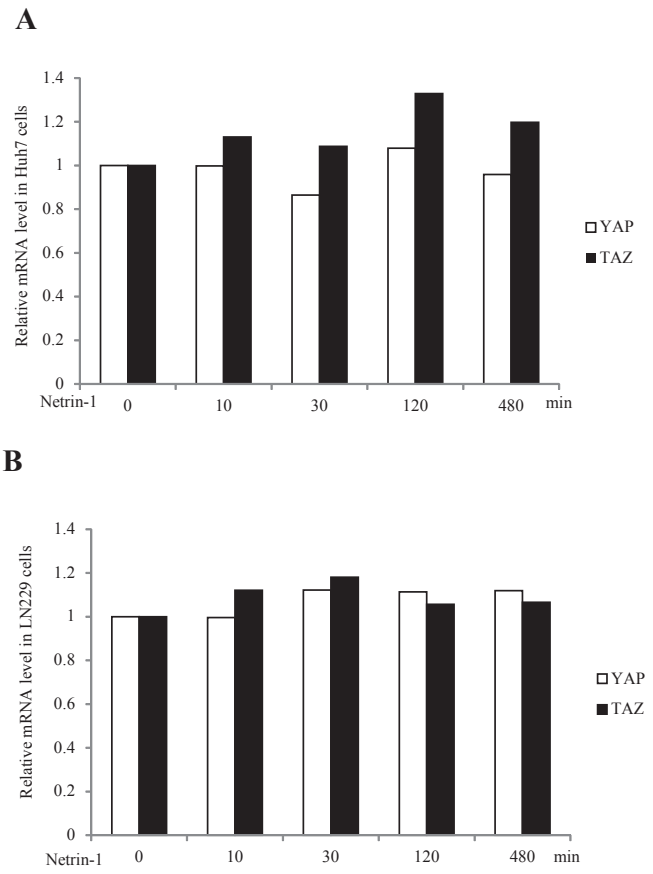

Fig. S5. The mRNA levels of YAP/TAZ cannot be regulated by netrin-1 in Huh7 and LN229 cells (related to Fig. 5). Following treatment with netrin-1 for the indicated times, Huh7 (*A*) or LN229 (*B*) cells were collected and mRNAs were extracted. The mRNA levels of the YAP/TAZ were measured by real-time PCR as described in *Methods*.