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Supplemental Information

Transcriptional Co-activator Functions of YAP and TAZ Are Inversely Regulated by Tyrosine Phosphorylation Status of Parafibromin

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Supplemental Information

Supplemental Figures

Figure S1

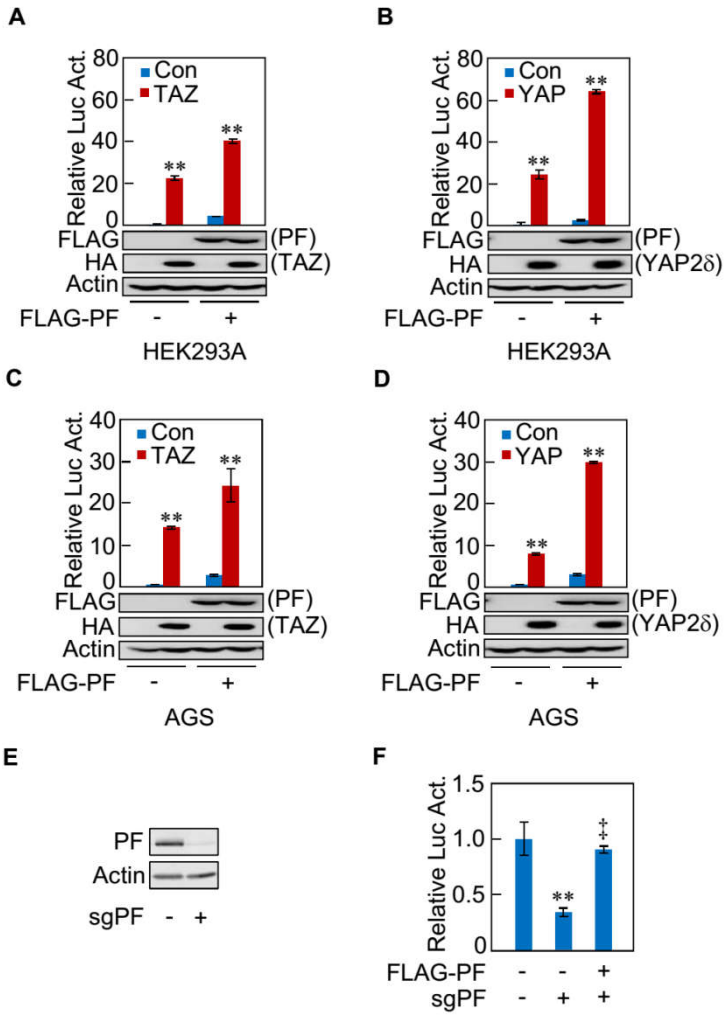

Figure S1. Related to Figure 1. Potentiation of the transcriptional co-activator activity of YAP and TAZ toward TEAD by Parafibromin

(A and B) HEK293A cells were transfected with indicated vectors together with reporter plasmids. Total cell lysates were subjected to immunoblotting with indicated antibodies.

(C and D) AGS cells were transfected with indicated vectors together with reporter plasmids. Total cell lysates were subjected to immunoblotting with indicated antibodies.

(E) HEK293T cells were transfected with indicated vectors. Total cell lysates were subjected to immunoblotting with indicated antibodies.

(F) HEK293T cells were transiently transfected with a TEAD luciferase reporter together with a sgPF vector together with or without PF vector.

Error bars, mean ± SD; n = 3; **p<0.01 versus control vector (A-D, F), or PF with control vector (A-D); ‡p<0.01 versus PF sgRNA with control vector (F).

Figure S2

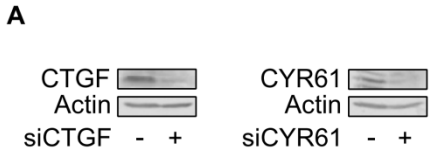

Figure S2. Related to Figure 2. Enhancement of YAP/TAZ-mediated biological actions by Parafibromin

(A) HEK293T cells were transiently transfected with *Ctgf*-specific siRNA, *Cyr61*-specific siRNA, or control siRNA. Total cell lysates were subjected to an immunoblotting with indicated antibodies.

Figure S3

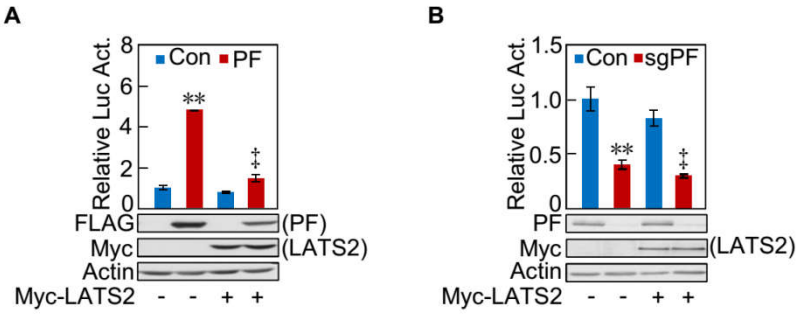

Figure S3. Related to Figure 3. Effect of Hippo signaling on Parafibromin-mediated TEAD activation

(A) HEK293T cells were transiently transfected with a TEAD luciferase reporter together with Parafibromin vector or a control empty vector in the presence or absence of a Myc-LATS2 vector. Total cell lysates were subjected to a luciferase assay or an immunoblotting with indicated antibodies.

(B) HEK293T cells were transiently transfected with a TEAD luciferase reporter together with Parafibromin-specific sgRNA vector or a control empty vector in the presence or absence of a Myc-LATS2 vector. Total cell lysates were subjected to a luciferase assay or an immunoblotting with indicated antibodies.

Error bars, mean ± SD; n = 3; **p<0.01 versus control vector (A and B); ‡p<0.01 versus PF with control vector (A), or PF sgRNA with control vector (B).

Figure S4

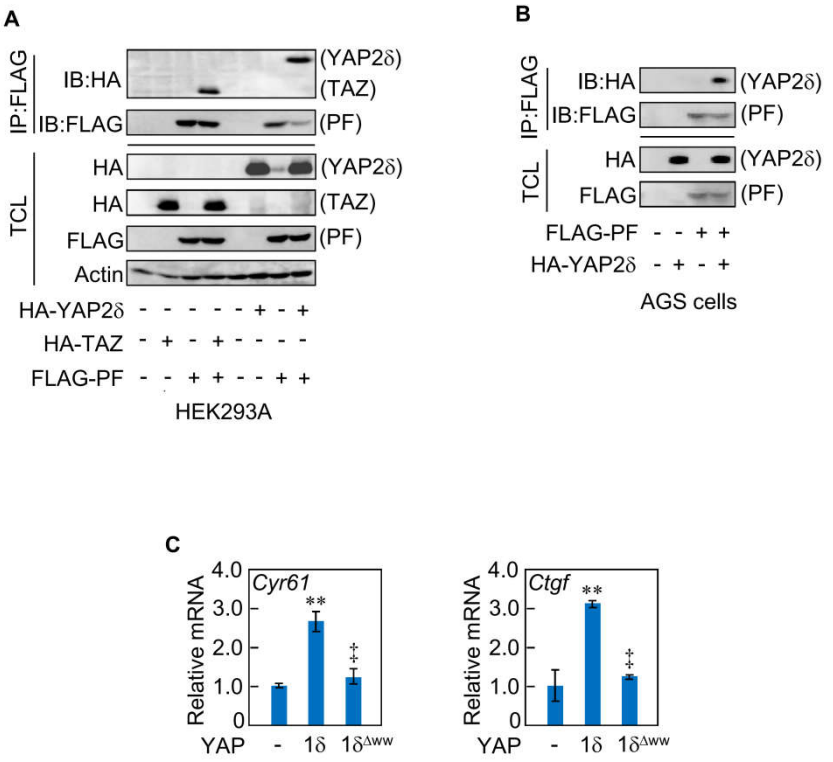

Figure S4. Related to Figure 4. Physical interaction between Parafibromin and YAP/TAZ

(A) HEK293A cells were transfected with expression vectors of either HA-YAP2δ or HA-TAZ, together with that of FLAG-Parafibromin. Anti-FLAG IP from cell lysates and TCL were analyzed by immunoblotting with anti-FLAG and anti-HA antibodies.

(B) AGS cells were transfected with expression vectors of HA-YAP2δ and FLAG-Parafibromin. Anti-Flag IP from cell lysates and TCL were analyzed by immunoblotting with anti-FLAG and anti-HA antibodies.

(C) qRT-PCR analysis of Cyr61 and Ctgf mRNA expression in HEK293T cells transiently transfected with a HA-YAP1δ, HA-YAP1$\delta^{\Delta ww}$ or control vector.

Error bars, mean ± SD; n = 3; **p<0.01 versus control vector; ‡p<0.01 versus YAP1δ.

Figure S5

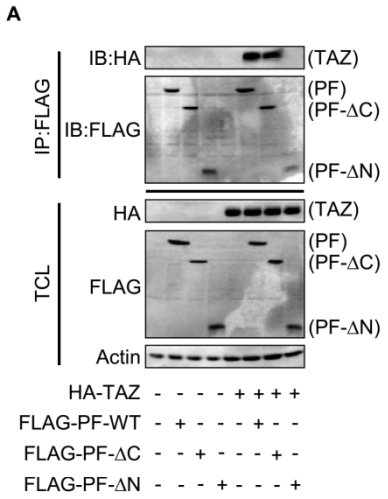

Figure S5. Related to Figure 5. Potentiation of transcriptional co-activator TAZ by tyrosine dephosphorylation of Parafibromin

(A) HEK293T cells were transfected with expression vectors of FLAG-Parafibromin, FLAG-Parafibromin-ΔC or FLAG-Parafibromin-ΔN, together with that of HA-TAZ. Anti-Flag IP from cell lysates and TCL were analyzed by immunoblotting with anti-FLAG and anti-HA antibodies.

Figure S6

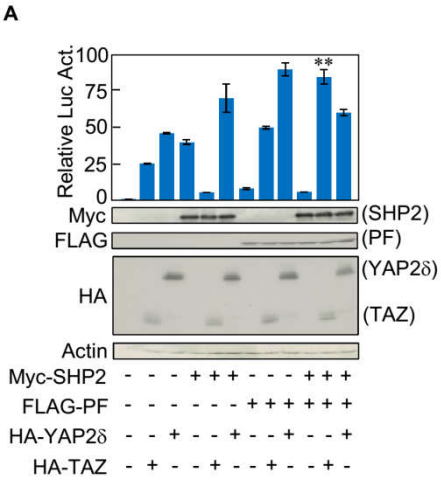

Figure S6. Related to Figure 6. Differential regulation of YAP by tyrosine dephosphorylation of Parafibromin

(A) HEK293T cells were transfected with indicated vectors together with a TEAD reporter plasmid. Total cell lysates were subjected to immunoblotting with indicated antibodies.

Error bars, mean ± SD; n = 3; **p<0.01 versus PF with TAZ.

Figure S7

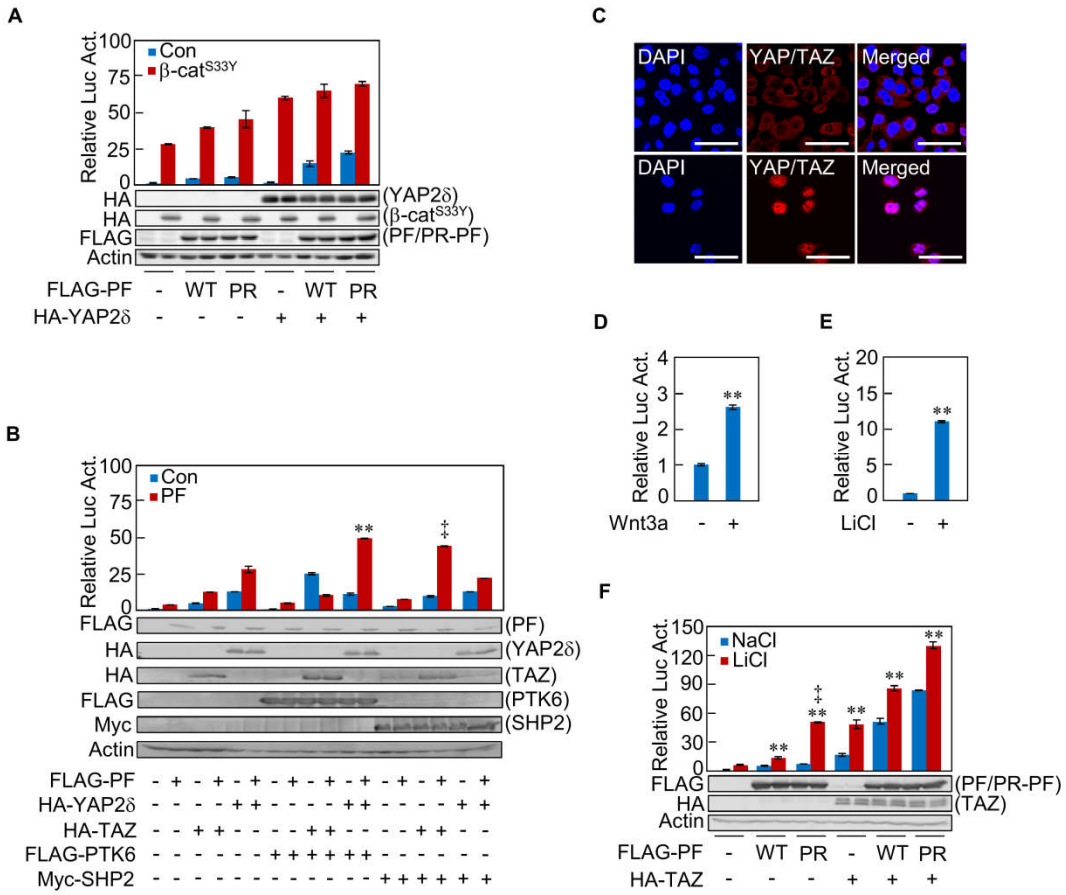

Figure S7. Related to Figure 7. Synergism between nuclear TAZ and Wnt signaling pathways by Parafibromin

(A) HEK293T cells were transfected with a TOP-*tk* luciferase reporter together with a FLAG-PF or FLAG-PR-PF vector with an HA-β-catenin^{S33Y} vector and/or an HA-YAP2δ vector. TCLs were subjected to luciferase assay and immunoblotting analysis with indicated antibodies.

(B) HEK293T cells were transfected with a FLAG-PF vector together with an HA-YAP2δ vector or an HA-TAZ vector with a FLAG-PTK6 vector or a Myc-SHP2 vector. TCLs were subjected to luciferase assay and immunoblotting analysis with indicated antibodies.

(C) AGS cells were seeded at high (upper) or low (lower) cell density, and cultured for 24 h. Immunnostaining was performed with indicated antibody and DAPI. Scale bars, 50 μm.

(D and E) HEK293T cells were transfected with a TOP-*tk* luciferase reporter. Cells were then treated with Wnt3a-conditioned medium or control medium(C), or treated with LiCl or NaCl (D).

(F) HEK293T cells were transfected with a TOP-*tk* luciferase reporter together with a FLAG-PF or FLAG-PR-PF and/or an HA-TAZ vector. Cells were then treated with LiCl or NaCl. TCLs were subjected to luciferase assay and immunoblotting with the indicated antibodies.

Error bars, mean ± SD; n = 3; **p <0.01 versus PF with YAP2δ (B), control medium (D), NaCl (E), PF (F), PR-PF (F), TAZ (F), TAZ with PF (F) or TAZ with PR-PF (F); ‡p <0.01 versus PF with TAZ (B) or PF with LiCl (F).

Transparent Methods

Cell culture and transfections

HEK293T, HEK293A, NIH3T3 and *Hrpt2*$^{flox/flox}$*;CAG-CreER* MEF cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). AGS cells were cultured in RPMI 1640 medium with 10% FBS. To activate endogenous Wnt signaling, HEK293T cells were treated with 25 mM LiCl, which inhibits GSK-3β and prevents β-catenin degradation. Transient transfection was carried out by using Lipofectamine reagent (Invitrogen) with Plus reagent (Invitrogen) for HEK293T, HEK293A and NIH3T3 cells, and using Lipofectamine 2000 reagent (Invitrogen) for AGS cells. *Hrpt2*$^{flox/flox}$*;CAG-CreER* MEFs were treated with 500 nM 4-OHT (Sigma) for 72 h to conditionally delete *Hrpt2* alleles.

Plasmids

The expression vectors for N-terminally FLAG-tagged wild-type Parafibromin, phospho-resistant Y290/293/315F Parafibromin (PR-Parafibromin), Y290F Parafibromin, Y293F Parafibromin, Y315F Parafibromin and Myc-tagged human SHP2 were described previously (Takahashi et al., 2011). The expression vector of a PxxY motif deficient mutant of FLAG-tagged Parafibromin, FLAG-tagged Parafibromin (P287A), was generated from the FLAG-tagged wild-type Parafibromin expression vector by site-directed mutagenesis. The cDNA encoding human PTK6 was cloned into the pRc/CMV vector (Invitrogen). The cDNA encoding hemagglutinin (HA)-tagged human TAZ was amplified from AGS cells with an HA-tag sequence in 5' and cloned into the pcDNA3 vector as previously described (Tsutsumi et al., 2013). The cDNA encoding HA-tagged human YAP isoforms were amplified from AGS cells with an HA-tag sequence in 5' and cloned into the pSP65-SRα vector. The WW domain-deleted mutant was generated from the expression vector YAP1δ isoform. Expression vectors of a WW domain-deficient mutant of TAZ, HA-TAZ WW domain-mutant (W152A/P155A), and a phosphorylation-resistant mutant of TAZ (TAZ-S89A) were generated from the HA-TAZ expression vector by site-directed mutagenesis. Expression vectors of either WW domain-deficient mutant of YAP2δ, HA-YAP2δ WW1 domain-mutant (W199A/P202A) and WW2 domain-mutant (W258A/P261A), and a double WW domains-deficient mutant of YAP2δ, HA-YAP2δ (W199A/P202A, W258A/P261A) were generated from the HA-YAP2δ expression vector by site-directed mutagenesis. The expression vector for HA-tagged β-catenin (S33Y) was generated from the expression vector for HA-β-catenin by introducing a point mutation (S33Y) by site-directed mutagenesis according to the method described previously (Takahashi et al., 2011). Wnt-responsive Top-*tk* and YAP/TAZ-responsive 8×GT TEAD-luciferase reporter plasmids have been described previously (Tsutsumi et al., 2013). Expression vectors of shRNA for human TAZ and human YAP were designed as pSUPER-based plasmids that target sequences of 5'-ACGTTGACTTAGGAACTTT-3', and 5'-GACATCTTCTGGTCAGAGA-3', respectively, as described previously (Tsutsumi et al., 2013).

CRISPR/Cas9 to Parafibromin/CDC73 construction and transfection

Expression vectors of sgRNA for human Parafibromin/CDC73 was designed as pX330-based plasmids that target sequences of 5'-CACCGAAGAAGGAGATTGTGGTGA-3'(#1), 5'-CACCGGGAGACGAAGTGATCTTCG-3'(#2), and 5'-CACCGAAGACCAACTATGTTGTTT-3'(#3), respectively. Sequences were designed using the CRISPR DESIGN tool (http://crispr.mit.edu/). All specific target sequences were amplified and cloned,

and verified by DNA sequencing. After the transient transfection of pX330-sgParafibromin plasmids together with a puromycin-resistant plasmid into cells by using Lipofectamine reagent (Invitrogen), puromycin (2 μg/ml) (Invitrogen) treatment for 7 d was employed for selection and then cells were expanded in the regular culture medium.

Antibodies

Anti-FLAG (M2, SIGMA), anti-HA (3F10, Roche and 16B12, COVANCE), anti-YAP/TAZ (D24E4, Cell Signaling), anti-Parafibromin (A300-170A and A300-171A, Bethyl), anti-β-catenin (H102, Santa Cruz), anti-c-Myc (9E10, Santa Cruz), anti-Cyclin D1 (H295, Santa Cruz) and anti-Actin (C-11, Santa Cruz) antibodies were used as primary antibodies for immunoblotting.

Luciferase assay

Luciferase activities were measured by using the dual luciferase reporter assay (Promega) according to the manufacturer's protocol. pRL/TK-luciferase reporter plasmid was used as a second reporter. The data were obtained by analyzing triplicated samples. The pSP65-SRα, pcDNA3, pSUPER or pX330 empty vector was used for a control plasmid.

Immunoprecipitation and immunoblotting

Cells were harvested and lysed in lysis buffer containing 100 mM NaCl, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1% Brij35, 2 mM Na_3VO_4, 10 mM NaF, 2 mM β-glycerophosphate and 2 mM PMSF. For immunoprecipitation, lysates were incubated with respective antibodies and protein G-beads (GE Healthcare). The beads were then washed five times with the lysis buffer, and the immune complex was eluted with SDS–PAGE sample buffer. Lysates and immunoprecipitates were subjected to SDS-PAGE, followed by immunoblotting. Proteins were visualized using western blot chemiluminescence reagent (Perkin-Elmer Life Sciences). The obtained chemiluminescence was exposed to X-ray film (Fuji Film).

Immunostaining

AGS cells were washed with PBS and fixed with 4% paraformaldehyde. Cells were then permeabilized with 0.1% TritonX-100 and incubated in 1% BSA/PBS, followed by first antibody treatment. Fluorescent images were obtained using FV1200 (OLYMPUS) confocal microscope systems.

Quantitative RT-PCR

Quantitative RT-PCR was performed as previously described (Takahashi et al., 2011). Briefly, RNA extracted from cells using TRIzol reagent (Invitrogen) were subjected to reverse transcription by SuperScriptII (Invitrogen). cDNAs were analyzed by Step-One-Plus Real-Time PCR System (Applied Biosystems) using SYBR Premix Ex Taq (TaKaRa, Japan). *GAPDH* was used to normalize input. Following primers were used:

human *GAPDH*, 5'-CCTCAACTACATGGTTTACATGTTCC-3' and
5'-GAAGATGGTGATGGGATTTCCATTG-3';
mouse *Ctgf*, 5'-GGACACCTAAAATCGCCAAGC-3' and
5'-ACTTAGCCCTGTATGTCTTCACA-3';
mouse *Cyr61*, 5'-ATCTGCAGAGCTCAGTCAGAAGG-3' and
5'-AGACAGTTCTTGGGGACACAGAGG-3'.

Wound healing assay

Wound healing assay was performed as previously described (Tang et al., 2015). Briefly, AGS cells (4×10^5 cells) were cultured in six-well plate and were transfected with indicated expression vectors. 24 h after transfection, cells were subjected to serum starvation for 12 h. After rinsed with medium to remove unattached cells, the confluent layer of cells was scratched with a sterile tip to create an artificial wound. Cell migration to the wounded gap was then monitored by microscopy after 9 h and the distance between the edges of the wound was analyzed using ImageJ software.

Colony formation assay

Colony formation assay was performed as previously described (Tang et al., 2015). Briefly, NIH3T3 cells were transfected with indicated expression vectors. At 24 h after transfection, the cells were seeded in 6-well plates at a density of 1000 cells/well in 2 ml medium. After culturing for two weeks, colonies were stained with 0.5% crystal violet in 2% ethanol, and colonies were counted and photographed.

Cell proliferation assay

Cell proliferation assays were carried out in 96-well plates, and living cells were counted at each time point using MTS assays with a Cell Counting Kit (Promega) according to the manufacturer's instructions. Absorbance was measured at 450 nm.

Mice

Hrpt2$^{flox/flox}$ mice (Wang et al., 2008) were crossed with *CAG-CreER* transgenic mice to generate *Hrpt2*$^{flox/flox}$*;CAG-CreER* mice. At ages of 4 to 6 weeks, *Hrpt2*$^{flox/flox}$*;CAG-CreER* mice and the control littermates were daily injected intraperitoneally with tamoxifen dissolved in corn oil (4 mg/40 g body weight). Mice were euthanized and examined at 5 days after exposure to tamoxifen. All animals were treated and maintained in accordance with the protocol approved by the Ethics Committees for Animal Experiment at the Graduate School of Medicine, the University of Tokyo.

Immunohistochemistry

Freshly sampled mouse tissues were flushed with ice-cold PBS and fixed by incubation in 4% paraformaldehyde in PBS overnight at 4ºC. Fixed tissues were dehydrated, embedded in paraffin, and sectioned. The sections were de-waxed and rehydrated. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide in methanol for 30 min at room temperature. Antigen retrieval was performed by boiling for 15 min in Citrate buffer pH 6.0 or Tris-EDTA pH 9.0. Tissues were incubated overnight at 4ºC with the following primary antibodies: anti-CTGF (ab6992, Abcam, 1:400), anti-CYR61 (ab24448, Abcam, 1:200) and anti-Parafibromin (A300-170A, Bethyl, 1:500). Staining was performed using the Vectastain ABC-Elite kit according to the manufacturer's instructions.

Statistics

All the numerous data were expressed as mean±SD, and were analyzed by two-tailed unpaired Student's t test. Statistical significance was assessed at $p<0.05$ and $p<0.01$. Representative experiments are shown.

Supplemental References

Tang, C., Mei, L., Pan, L.Y., Xiong, W.Y., Zhu, H.B., Ruan, H.F., Zou, C.C., Tang, L.F., Iguchi, T., and Wu, X.M. (2015). Hedgehog signaling through GLI1 and GLI2 is required for epithelial–mesenchymal transition in human trophoblasts. BBA-General Subjects 1850, 1438-1448.

Wang, P., Bowl, M.R., Bender, S., Peng, J., Farber, L., Chen, J., Ali, A., Zhang, Z., Alberts, A.S., Thakker, R.V., et al. (2008). Parafibromin, a component of the human PAF complex, regulates growth factors and is required for embryonic development and survival in adult mice. Mol. Cell. Biol. 28, 2930-2940.