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Supplementary Information

Endothelial YAP1 in regenerative lung growth through the angiopoietin-Tie2 pathway

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Materials and Methods

Materials. Angiopoietin1 (Ang1) was from R&D (Minneapolis, MN). Anti-CD31 and -TEAD1 antibodies were from Transduction Laboratories (Lexington, KY). Anti-aquaporin 5 (AQP5) antibody was from Abcam (Cambridge, MA). Anti-β-actin monoclonal antibody was from Sigma (St. Louis, MO). Anti-Tie2 monoclonal antibody was from Upstate (Lake Placid, NY). Anti-YAP1 and -Tie2 antibodies were from Santa Cruze Biotechnology (Dallas, TX). Anti-phospho-YAP1 (Ser 127) antibody was from Abcam. Human lung microvascular endothelial (L-HMVE) cells (Lonza) were cultured in EBM2 medium containing 5% FBS and growth factors (VEGF, bFGF and PDGF). Human adult lung fibroblasts (ATCC, Manassas, VA) were cultured in Eagle's Minimum Essential Medium (EMEM, ATCC). Human immortalized bronchial epithelial cells (HBEC3-KT, ATCC), which have been reported to differentiate into alveolar epithelial cells and to exhibit a bronchioalveolar phenotype (S1, S2), were cultured in Airway Epithelial Cell Basal Medium (AECBM, ATCC).

Plasmid construction and gene knockdown. The retroviral full length pQCXIH-

myc-YAP1 and pQCXIH-flag-YAP1-S127A were gifts from Kunliang Guan (Addgene plasmid # 33091 and # 33092) (S3). To generate the lentiviral pLenti-Tie2 construct, the full-length Tie2 cDNA from pSPORT-Tie2 (Open Biosystems (Huntsville, AL)) (S2) was inserted into pLenti vector (Addgene). As a control, plasmid with vector only was used. Generation of retroviral/lentiviral vectors was accomplished as reported (S2, S4). Viral supernatants were collected starting 48 h after transfection for four consecutive times every 12 h, pooled, and filtered through a 0.45 μm filter. Viral supernatants were then concentrated 100-fold by ultracentrifugation in a Beckman centrifuge for 1.5 h at 16,500 rpm. L-HMVE cells were incubated with viral stocks in the presence of 5 μg/ml polybrene (Sigma) and 90-100% infection was achieved 3 days later (S2, S4). Gene knockdown was performed using the RNA interference technique. siRNAs for human YAP1 #1 (5'- UCUCUGACCAGAAGAUGUC-3' and 5'-GACAUCUUCUGGUCAGAGA-3') and human YAP1 #2 (5'-AAGAAGUAUCUCUGACCAG-3' and 5'-CUGGUCAGAGAUACUUCUU-3') were purchased from Sigma Genosys (St. Louis, MO) and L-HMVE cells were transfected using silentfect (BioRad) (S2, S4). siRNA for human Tie2 was a smart pool siRNA from Dharmacon (S4). siRNA for human TEAD1 #1 was ON-TARGET plus TEAD1 siRNA (ORF J-012603-08, Thermo Scientific) and siRNA for human TEAD1 #2 was TEAD1_5 FlexiTube siRNA (SI04181261, QIAGEN) (S5). As a control, siRNA duplex with irrelevant sequence (QIAGEN) was used.

Molecular biological and biochemical methods. Quantitative reverse transcription (qRT)-PCR was performed with the iScript reverse transcription and iTaq SYBR Green qPCR kit (BioRad, Hercules, CA) using the BioRad real time PCR system (BioRad). β2 microglobulin and cyclophilin controlled for overall cDNA content. The

primers used for human Tie2 and β2 microglobulin and mouse Tie2 and cyclophilin were previously described (S2, S4). The primers used for human YAP1 forward; TAGCCCTGCGTAGCCAGTTA and reverse; TCATGCTTAGTCCACTGTCTGT, mouse Yap1 forward; ACCCTCGTTTTGCCATGAAC and reverse; TGTGCTGGGATTGATATTCCGTA, and human TEAD1 forward; ATGGAAAGGATGAGTGACTCTGC and reverse; TCCCACATGGTGGATAGATAGC (S5).

In vitro fibrin gel angiogenesis assay. Fibrin gel angiogenesis assays were performed as previously described (S2, S5, S6). Briefly, $1X10^5$ L-HMVE cells were incubated with 3000 Cytodex 3 microcarrier beads (GE Healthcare Life Sciences, Pittsburgh, PA) in 1 ml EGM2 in a glass tube for 4 hours with gentle agitation. The beads coated with the cells were transferred to 25 cm^2 tissue culture flask and incubated with or without siRNA or virus treatment. As a control, cells were treated with control siRNA with irrelevant sequence or control virus (vector alone). After 16 h incubation, 250 beads coated with L-HMVE cells were suspended in 500 µl of 2.5 mg/ml fibrinogen solution (Sigma) and mixed with 500 µl of thrombin solution (0.5 U) in a 24-well plate. After fibrin gels were solidified, 1 ml of EGM2 containing $2X10^4$ human lung fibroblasts was seeded on top of each fibrin gel in a 24-well plate. Ang1 (20 ng/ml total protein) was added to the medium at day 1 and the medium was changed every other day. After incubation of beads in the fibrin gels for 5 days, the area and length of the sprout from the beads were quantified using ImageJ software.

Co-culture of HBEC3-KT and HUVE cells. Co-culture of HBEC3-KT and HUVE cells was performed as previously described with slight modifications (S1, S2). Briefly,

human adult lung fibroblasts were seeded as a feeder layer at a density of $1X10^5$ cells in a 24-well plate. After 2 days, the medium was aspirated and 300 µl of growth factor reduced Matrigel (BD Biosciences) was overlaid on top of the fibroblasts. The gels were solidified at 37°C for 15 min in the incubator. HUVE cells ($5X10^4$ cells) treated with targeted siRNA or virus and HBEC3-KT ($1X10^5$ cells) were suspended in 1 ml of medium (mixture of bronchial epithelium medium and EGM2) and applied on top of the solidified Matrigel. Ang1 (20, 100 ng/ml total protein) was added into the medium and the medium was changed every other day. Bronchio-alveolar phenotypes (i.e., epithelial budding) were evaluated after 5 days in culture.

Mouse lung EC isolation. *Yap1$^{fl/fl}$* mice were obtained from Dr. Fernando Camargo (Harvard Medical School) (S7) and crossed with Cdh5(PAC)-CreERT2 mice (obtained from Dr. Ralf Adams, Max Planck Institute) (S8), an inducible cre deleter under the control of VE-cadherin promoter, to create VE-cadherin-specific Yap1 conditional knockout (*Yap1$^{fl/fl}$-Cdh5(PAC)-Cre^{ERT2}*) mice, in which cre recombination is induced in ECs by administration of tamoxifen. Mouse lung ECs were isolated from *Yap1$^{fl/fl}$* and *Yap1$^{fl/fl}$-Cdh5(PAC)-Cre^{ERT2}* mouse lungs using anti-CD31 conjugated magnetic beads (S9). Isolated ECs were validated by FACS and more than 95% of the isolated cells expressed EC markers (not shown). Yap1 protein and mRNA expression decreased by 82% and 79%, respectively in lung ECs isolated from *Yap1$^{fl/fl}$-Cdh5(PAC)-Cre^{ERT2}* mice compared with those from control *Yap1$^{fl/fl}$* mice 48 hours after 4-hydroxytamoxifen (4-OHT, 1 μM) treatment (Fig. 4A, B).

Fibrin gel implantation on the mouse lung in vivo. The *in vivo* animal study was carried out in strict accordance with the recommendations in the Guide for the Care

and Use of Laboratory Animals of the National Institutes of Health. The protocol was reviewed and approved by the Animal Care and Use Committee of Medical College of Wisconsin. CD1 mice (Charles River Laboratory) and *Yap1*$^{fl/fl}$ and *Yap1*$^{fl/fl}$*-Cdh5(PAC)-Cre*^{ERT2} mice were used for the study. Fibrin gel was fabricated as described (S6, S9). Briefly, we added thrombin (2.5 U/ml) with angiogenic factors (VEGF and bFGF at 100 ng/ml) to the fibrinogen solution (12.5 mg/ml), mixed well, and incubated drops of the mixture at 37 °C for 30 min until they solidified (S6, S9). For gel implantation on the mouse lungs (S6, S9), mice were mechanically ventilated and thoracotomy was performed in the fifth left intercostal space. After thoracotomy, a small area of the left visceral pleura (0.5 mm^2) was scraped using forceps and the fabricated fibrin gel was implanted on the mouse lung surface using a fibrin glue. We pretreated *Yap1*$^{fl/fl}$*-Cdh5(PAC)-Cre*^{ERT2} or control *Yap1*$^{fl/fl}$ mice with tamoxifen (5 mg, oral gavage, every other day) for four days, and implanted the gel on these mouse lungs for 7 days. Angiogenesis and alveolar epithelial cell recruitment are evaluated by counting the number of blood vessels and alveolar epithelial cells that are stained positive for EC marker (CD31) and alveolar type-I epithelial cell marker (AQP5), respectively, from five different areas of the gel (S6, S9). This system is important to study the vascular morphogenesis in the lung, because blood vessel structures in the gel are significantly different in the gel implanted on the mouse lung compared to those in the gel implanted under the skin; vascular density was 67% lower and vessel diameter was 4.1-times larger in the subcutaneously implanted gel, suggesting that blood vessels are formed in the gel in an organ-specific manner (Supplementary Fig. S4A). Fluorescent images are taken on a Leica TCS SP5 confocal laser scanning microscope and morphometric

analysis is performed using ImageJ software as we reported (S2, S4-S6, S9).

Unilateral PNX. Unilateral PNX was performed as described (S2). In brief, mice (CD1, *Yap1*$^{fl/fl}$, *Yap1*$^{fl/fl}$*-Cdh5(PAC)-Cre*^{ERT2}, 8-12 week old, approximately 25 g) were anesthetized with Ketamine/Xylazine (IP), and intubated with a 21-gauge cannula and mechanically ventilated at 120 cycle/min with a tidal volume of 10 ml/kg using a rodent ventilator (MiniVent, Harvard Apparatus, Holliston, MA). After ensuring adequate anesthesia, a 1 cm incision was made through the skin, muscle above the left lung along the intercostal space between the fourth and fifth ribs were cut, and thoracotomy was performed. A small retractor was placed to provide access to the thoracic cavity. The left lung was gently lifted through the incision and a 5-0 silk suture was passed around the hilum and tied. The hilum was then transected distal to the tie. The remaining portions of the hilum and tie were returned back to the thoracic cavity. The mouse was extubated and observed for return of spontaneous respirations. Sham operated mice underwent thoracotomy without PNX. Since the cardiac lobe is routinely evaluated for compensatory lung growth (S10), the weight of the cardiac lobe was measured and normalized to body weight (BW) after the experiments. Histological samples were prepared as previously described with modifications (S4). Briefly, lungs were fixed with 4% paraformaldehyde solution through the trachea under a constant pressure of 15 cm H_2O. The trachea was then ligated, and the lungs were immersed in fixiative overnight at 4°C. Lungs were then processed and embedded in OCT compound. Serial step sections, 5 μm in thickness were taken along the longitudinal axis of the lobe. The fixed distance between the sections was calculated so as to allow systematic sampling of random sections across the whole lobe. Morphological analysis

of mean linear intercept (MLI) and alveolar numbers was performed on the hematoxylin and eosin (H&E) stained lung sections as described (S4). Vascular structure was characterized using the microfil vascular casting system (S11). After heparinization, mice were euthanized and the cardiac apex was cut. Microfil (0.5-1 ml, Flow Tech) was injected into the pulmonary arteries through right ventricle. After solidification of Microfil, the lungs were fixed with 4% paraformaldehyde, dehydrated with ethanol, cleared with methyl salicylate, and imaged. Quantification of vasculatures was performed using the AngioTool software (NCI/NIH).

Statistical Analysis. All phenotypic analyses including image selection and computational image analysis for EC sprouting assay, epithelial budding assay, mouse lung gel implantation assay, and lung tissues after PNX were performed by masked observers unaware of the identity of experimental groups. Error bars (SEM) and *p* values were determined from the results of three or more independent experiments. The F test (for two samples) or the Levene test (for more than two samples) was performed to confirm that the variances are homogeneous. Student's t-test was used for statistical significance for two groups. For more than two groups, one-way ANOVA with a post-hoc analysis using the Bonferroni test was conducted.

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Supplementary Figure Legends

Supplementary Fig. S1. YAP1 controls Tie2 expression in L-HMVE cells. A) Graph showing YAP1 and Tie2 mRNA levels in L-HMVE cells treated with YAP1 siRNA #2 or control siRNA with irrelevant sequences (n=3, *, $p<0.05$). **B)** Immunoblots showing YAP1, Tie2, and β-actin protein levels in L-HMVE cells treated with YAP1 siRNA #2 or control siRNA with irrelevant sequences (*left*). Graph showing YAP1 and Tie2 protein levels in L-HMVE cells treated with YAP1 siRNA #2 or control siRNA with irrelevant sequences (*right*, n=3, *, $p<0.05$). **C)** Graph showing TEAD1 and Tie2 mRNA levels in L-HMVE cells treated with TEAD1 siRNA #2 or control siRNA with irrelevant sequences (n=3, *, $p<0.05$). Error bars represent s.e.m.

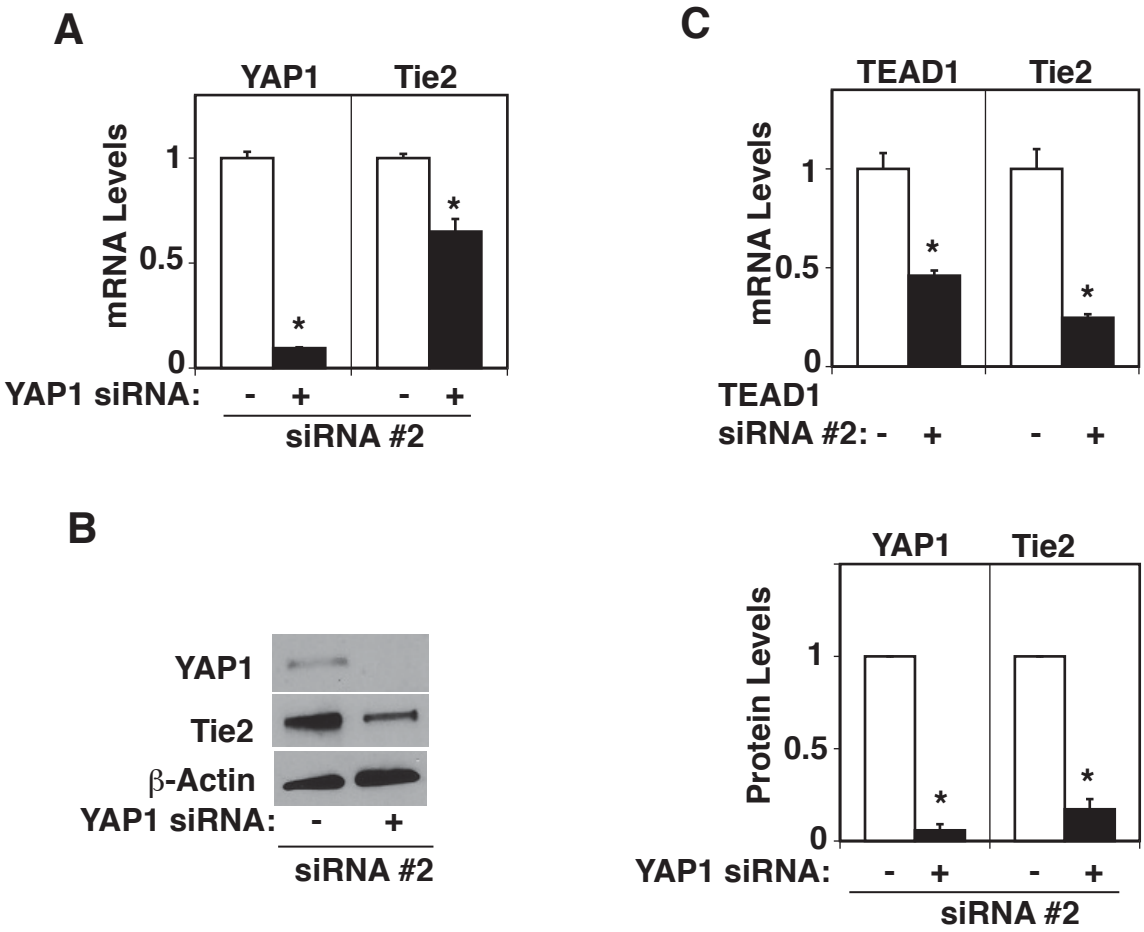
Supplementary Fig. S2. YAP1 stimulates EC sprouting in L-HMVE cells. A) Graph showing the Tie2 mRNA levels in L-HMVE cells treated with Tie2 siRNA, Tie2 lentivirus, control siRNA with irrelevant sequences, or control virus (n=3, *, $p<0.05$). **B)** Phase contrast images showing EC sprouting from each bead in L-HMVE cells treated with Ang1 or in combination with virus encoding full-length YAP1 or YAP1S127A mutant construct, siRNA targeting Tie2, control siRNA with irrelevant sequences, or control virus (vector alone). Scale bar, 150 μm. **C)** Graphs showing the changes in sprout area and length of the sprout in L-HMVE cells treated with Ang1 or in combination with lentivirus encoding full-length YAP1 or YAP1S127A mutant construct, siRNA targeting Tie2, control siRNA with irrelevant sequences, or control virus (vector alone) (n=3, *, $p<0.05$). Error bars represent s.e.m.

Supplementary Fig. S3. Endothelial YAP1 controls alveolar epithelial

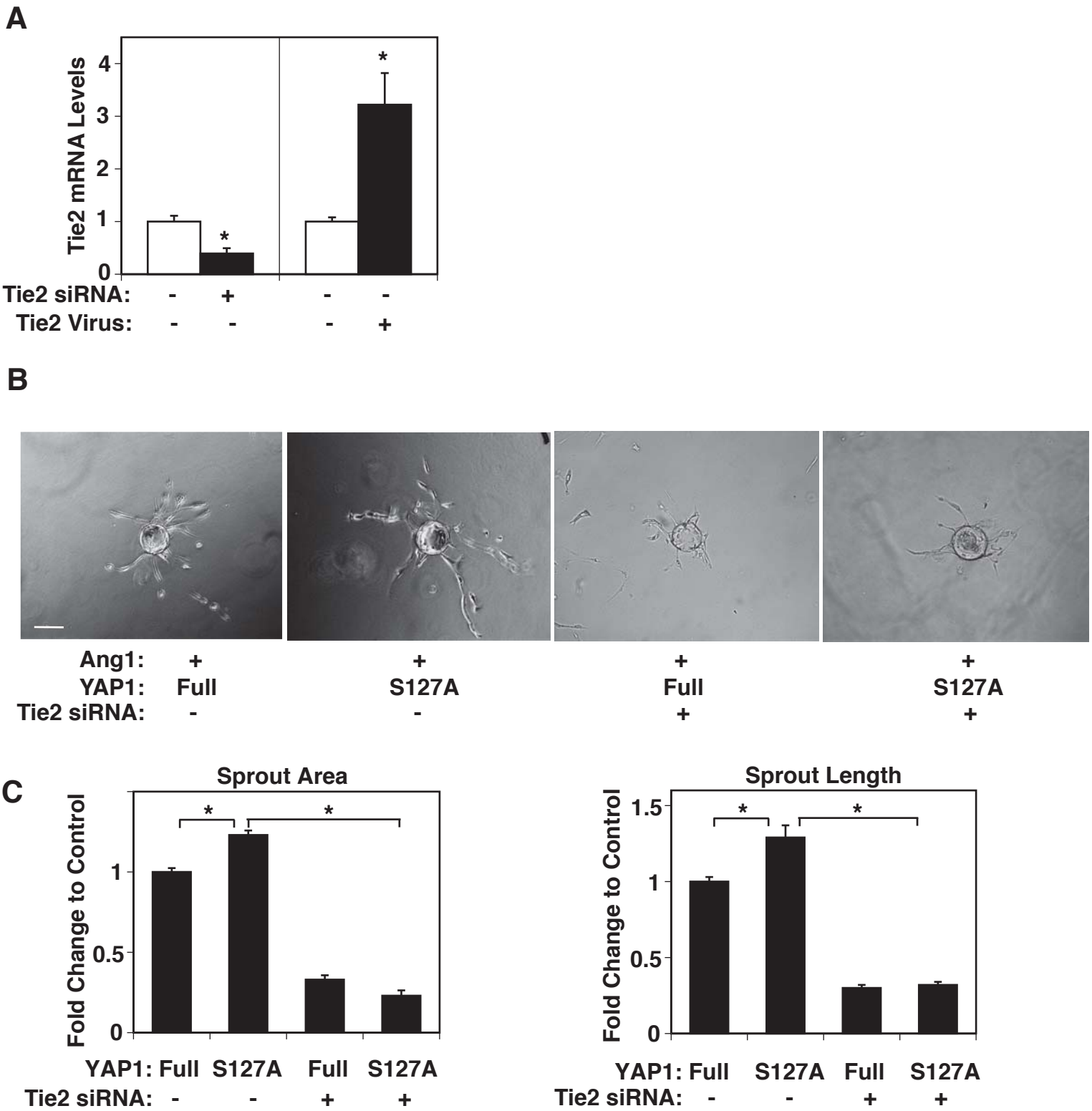
morphogenesis in vitro. A) Phase contrast images showing epithelial cell budding (scale bar, 50 μm) in co-culture of HBEC3-KT and HUVE cells treated with Ang1 (0, 20, 100 ng/ml) or in combination with virus encoding full-length YAP1, YAP1S127A mutant construct, Tie2 siRNA, control siRNA or control virus (vector alone) for 5 days. **B)** Graph showing the changes in the number of buds in co-culture of HBEC3-KT and HUVE cells treated with Ang1 (0, 20, 100 ng/ml) or in combination with virus encoding full-length YAP1, YAP1S127A mutant construct, Tie2 siRNA, control siRNA or control virus (vector alone) for 5 days (n=3, *, $p<0.05$). Error bars represent s.e.m.

Supplementary Fig. S4. Endothelial YAP1 is required for TEAD1 and Tie2 expression in the lung after PNX. A) Fluorescence micrographs showing formation of vascular networks (CD31-positive) in the fibrin gel supplemented with VEGF and bFGF (100 ng/ml) 7 days after implantation on the mouse lung (*left*) or under the skin (*right*). Scale bar, 25 μm. Graphs showing quantification of vessel density (*left*) and diameter (*right*) in the gel (n=5, mean±s.e.m., * $p<0.05$). **B)** Graphs showing quantification of TEAD1 and Tie2 expression in the *Yap1*$^{fl/fl}$*-Cdh5(PAC)-Cre*^{ERT2} or *Yap1*$^{fl/fl}$ mouse lungs 7 days after PNX (n=7, mean ± s.e.m., *, $p<0.05$). **C)** Graph showing the quantification of the immunoblots of YAP1, Tie2, and TEAD1 in ECs isolated from *Yap1*$^{fl/fl}$*-Cdh5(PAC)-Cre*^{ERT2} or control *Yap1*$^{fl/fl}$ mouse lungs 7 days after PNX (n=4, mean±s.e.m., *, $p<0.05$).

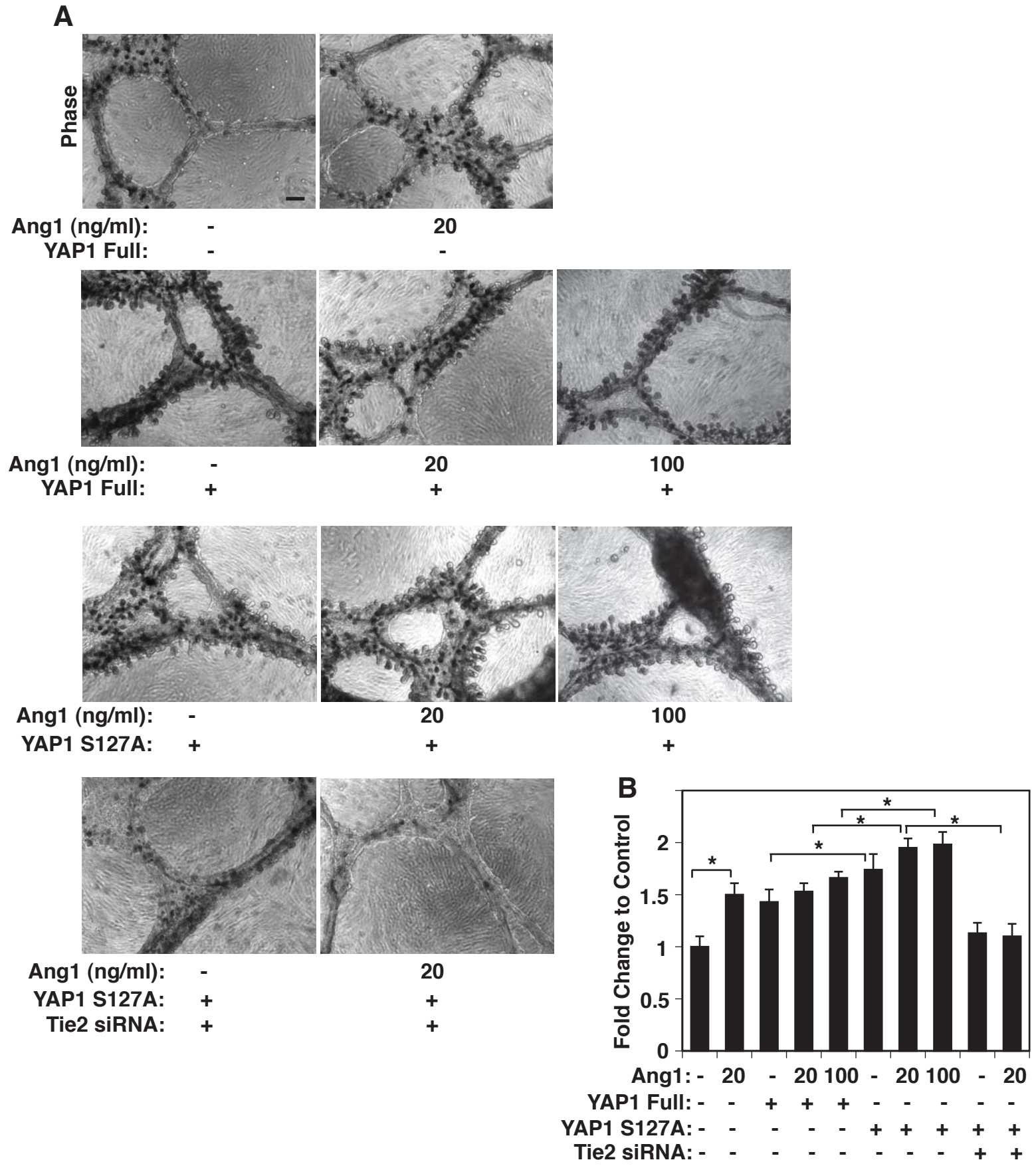
Supplementary Fig. S5. Knockdown of endothelial YAP1 inhibits Tie2 expression in the mouse lung. Original immunoblots for the images in Fig. 4A showing YAP1, Tie2, and β-actin protein levels in ECs isolated from *Yap1*$^{fl/fl}$*-Cdh5(PAC)-Cre*^{ERT2} or control *Yap1*$^{fl/fl}$ mouse lungs treated with 4-OHT for 48 hours.



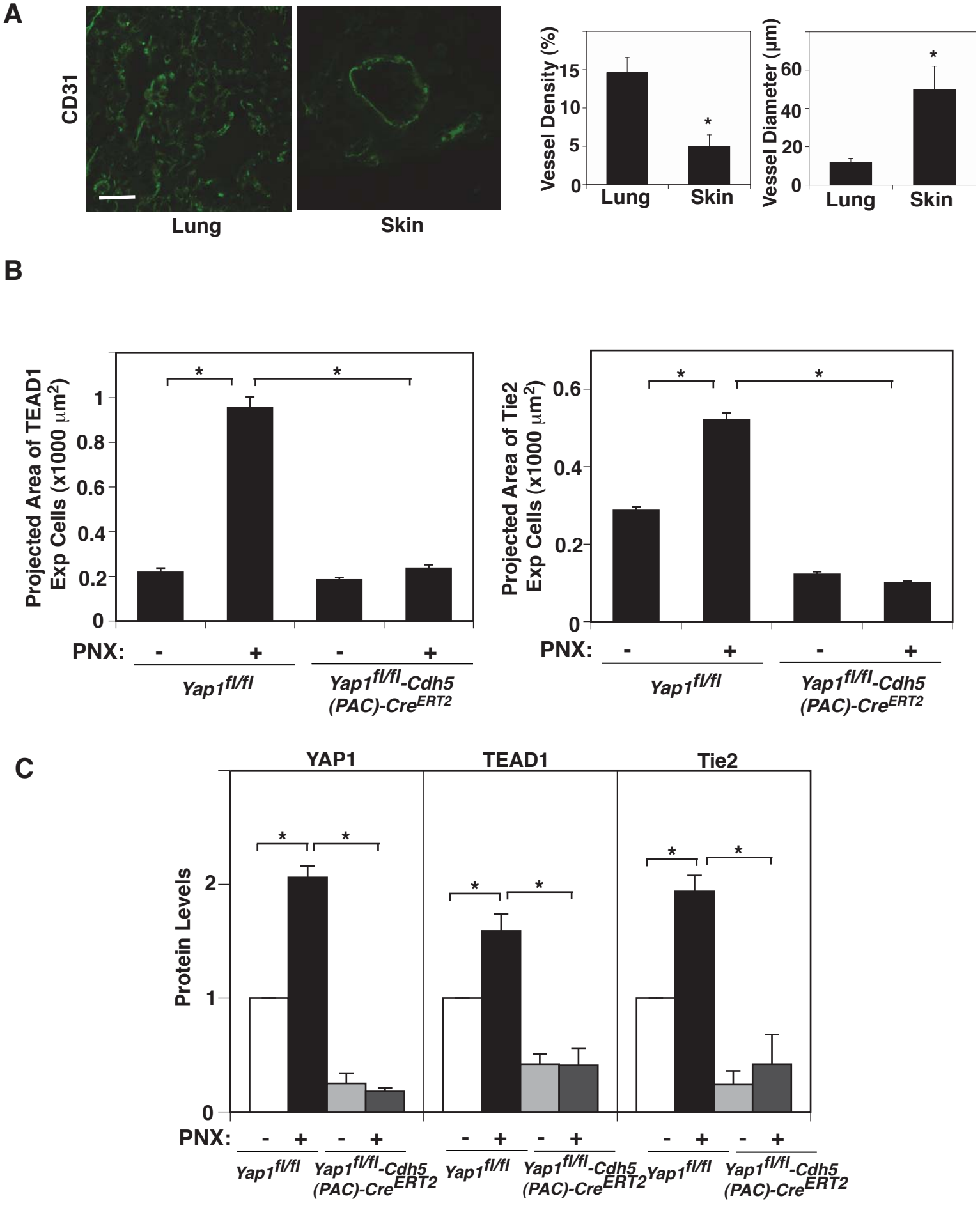
Supplementary Fig. S1



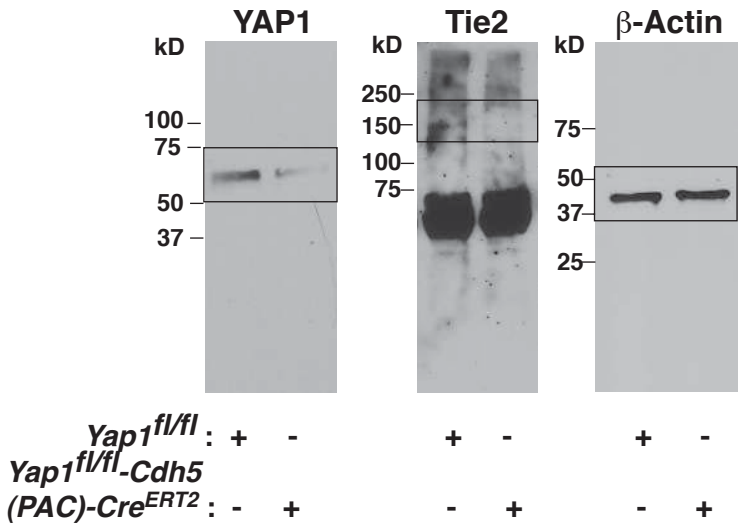
Supplementary Fig. S2



Supplementary Fig. S3



Supplementary Fig. S4



Original uncut gel images for Fig 4A

Supplementary Fig. S5