

Supplementary Materials for
**Cross-talk between TSC2 and the extracellular matrix controls pulmonary
vascular proliferation and pulmonary hypertension**

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Sci. Signal. **15**, eabn2743 (2022)
DOI: 10.1126/scisignal.abn2743

The PDF file includes:

Figs. S1 to S17
Tables S1 and S2

Other Supplementary Material for this manuscript includes the following:

MDAR Reproducibility Checklist

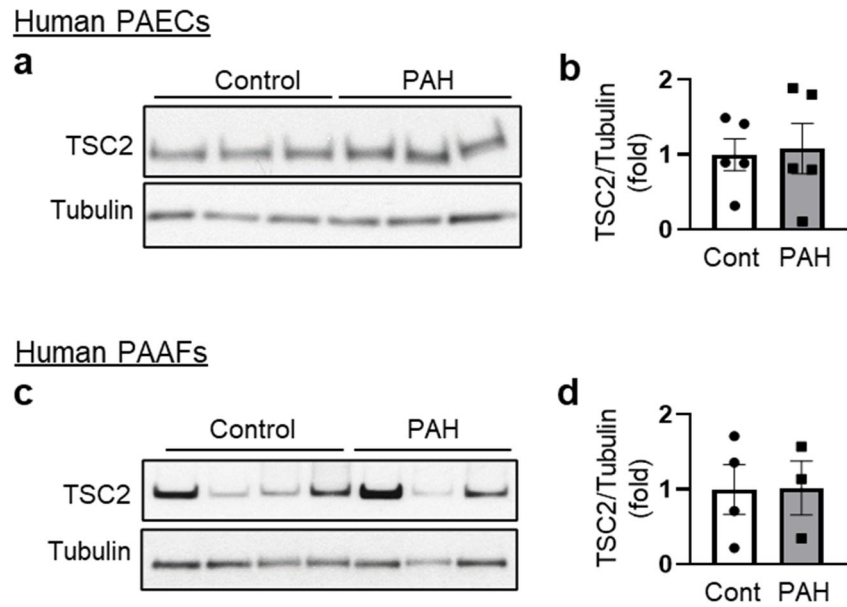


Figure S1. TSC2 protein levels are not reduced in PAAFs or PAECs from PAH lungs compared to those from control lungs

Immunoblot analysis of PAECs (**a,b**) and PAAFs (**c,d**) from non-diseased (control) and PAH subjects. Representative images (**a,c**) and analysis (**b,d**). $n=5$ subjects/group for PAECs, $n=4$ control and $n=3$ PAH subjects/group for PAAFs. Comparisons between PAH and control were determined to be not statistically significant by Mann Whitney U test.

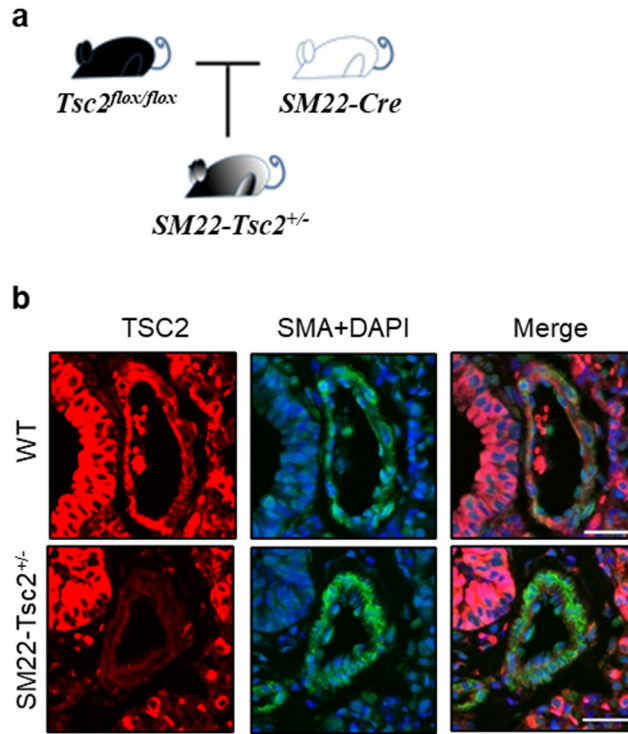


Figure S2. TSC2 protein reduction in SMA-positive areas in small PAs from *SM22-Tsc2^{+/-}* mice.

a: *SM22-Cre* mice were bred with *Tsc2^{flox/flox}* mice to generate *SM22-Tsc2^{+/-}* mice.

b: Immunohistochemical analysis of lung tissue sections of *SM22-Tsc2^{+/-}* mice to detect TSC2 (red), smooth muscle α -actin (SMA) (green), and DAPI (blue). Representative images from n=5 WT and 7 *SM22-Tsc2^{+/-}* mice and 5 PAs/mouse. Scale bar, 30 μ m.

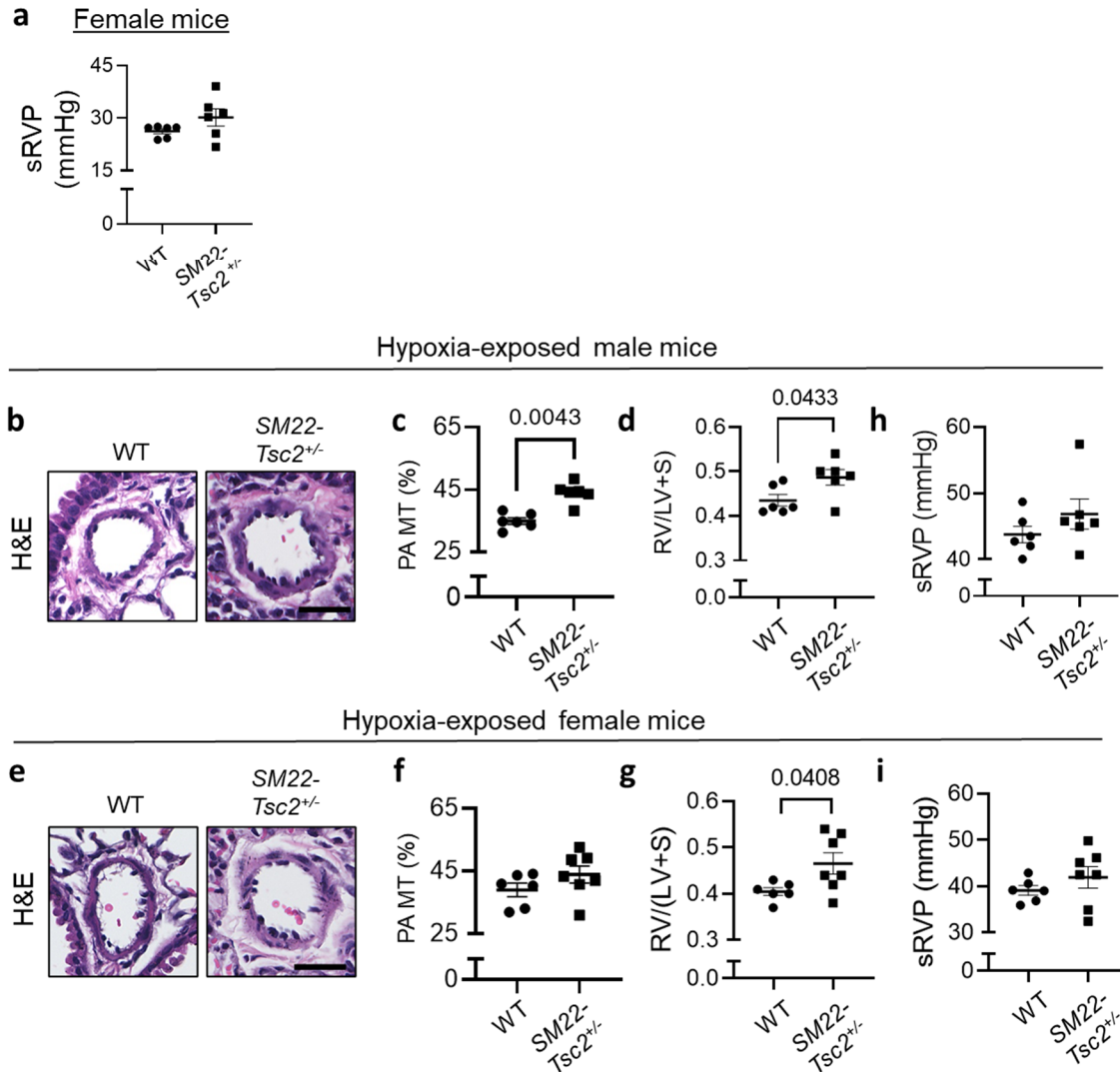


Figure S3. Chronic hypoxia exposure worsens pulmonary vascular remodeling and RV hypertrophy in *SM22-Tsc2*^{+/-} mice.

a: Systolic RV pressure (sRVP) of nine week old female *SM22-Tsc2*^{+/-} and wild type (WT) mice. Data are means±SE for n=6 WT mice and n=7 *SM22-Tsc2*^{+/-} mice.

b-i: Six week old male and female wild type (WT) and *SM22-Tsc2*^{+/-} mice were subjected to three weeks of chronic hypoxia exposure. PA medial thickness (PA MT) (**b, c, e, f**), right ventricular (RV) hypertrophy (RV/(LV+S) ratio) (**d, g**), and systolic right ventricular pressure (sRVP) (**h, i**) were analyzed. **b, e:** Images are representative from 6 mice/group, 10 PAs/mouse. Scale bar, 30 µm. **c, d, f, g, h, i:** Data are means±SE from n=6-7 mice/group, P values were determined by Mann Whitney U test.

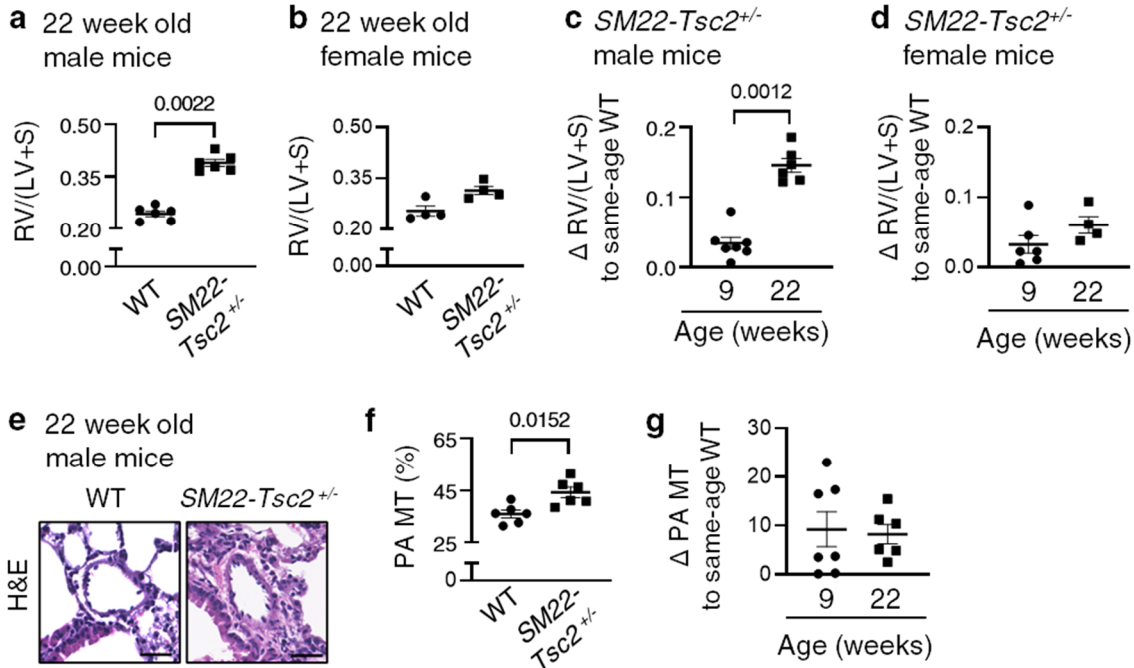


Figure S4. Male *SM22-Tsc2*^{+/-} mice develop severe RV hypertrophy with age.

a, b, Fulton index (RV/(LV + septum) weight ratio) was calculated in 22 week old wild type (WT) and *SM22-Tsc2*^{+/-} male (**a**) and female (**b**) mice. Data are means \pm SE from 6 (**a**) and 4 (**b**) mice/group. *P* values were determined by Mann Whitney U test.

c, d, RV/(LV + septum) weight ratios of *SM22-Tsc2*^{+/-} mice of indicated ages relative to same-age WT were determined. Data are means \pm SE from 6-7 (**c**) and 4-6 (**d**) mice/group. *P* values were determined by Mann Whitney U test.

e, f, Representative H&E images (**e**) and PA medial thickness (PA MT) analysis of 22 week old male mice from 6 mice/group, minimum of 10 PAs/mouse. Scale bar, 30 μ m. Data are means \pm SE. *P* values were determined by Mann Whitney U test.

g, PA MT of *SM22-Tsc2*^{+/-} mice of the indicated ages relative to same-age WT mice. Data are means \pm SE from 6 mice/group. *P* values were determined by Mann Whitney U test.

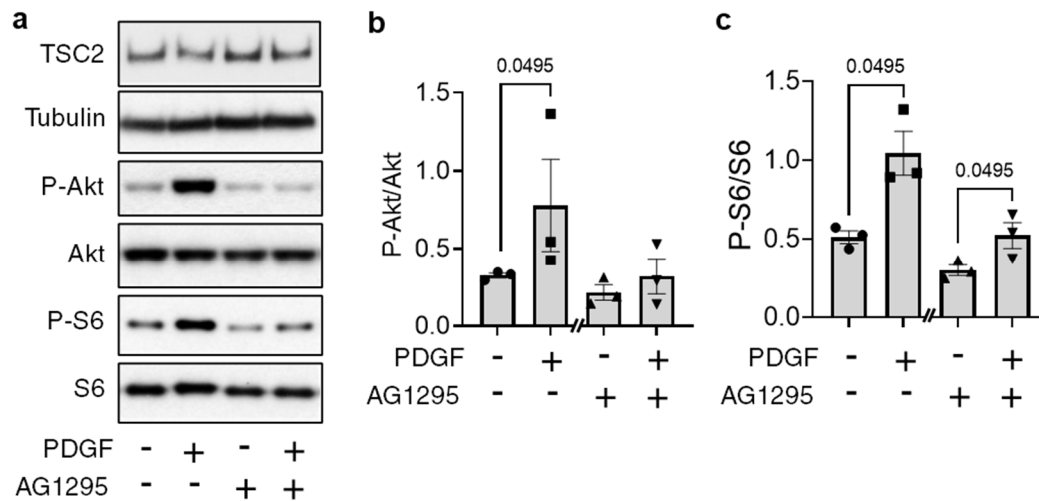


Figure S5. PDGF receptor inhibition decreases PDGF-induced Akt and S6 phosphorylation in human PAVSMCs.

Control human PAVSMCs were treated with 10 ng/ml PDGF-BB or diluent in the presence or absence of 10 μ M AG1295 for 48 hr and immunoblotted to detect the indicated proteins. Representative images (**a**) and analysis (**b, c**) of three independent experiments. Data are means \pm SE from n=3 subjects/group. Statistical analysis was performed using Mann Whitney U test.

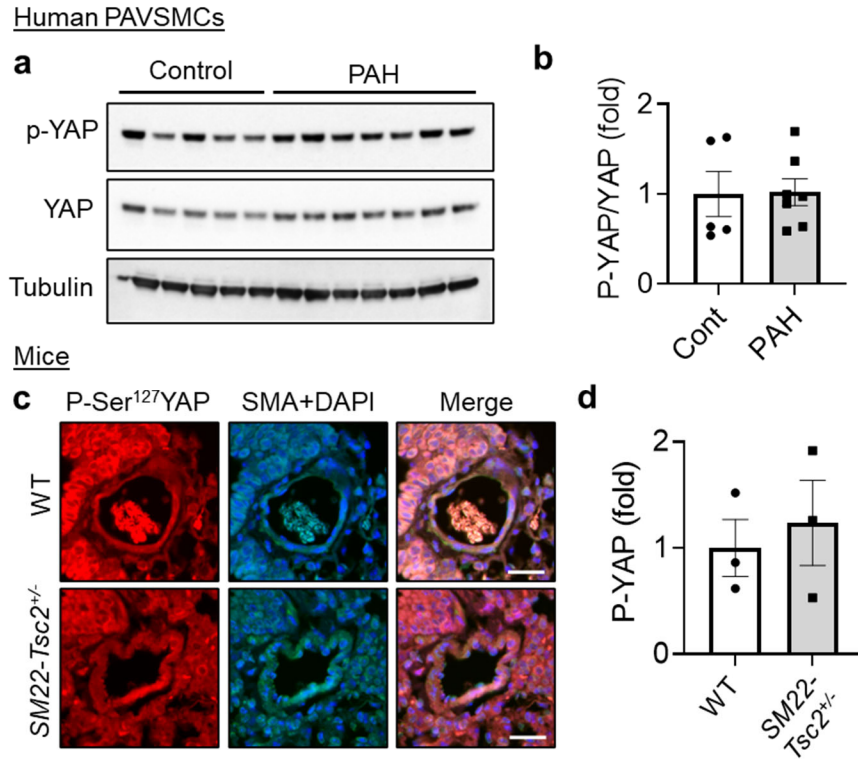


Figure S6. Phosphorylation state of Ser¹²⁷ in YAP in human PAVSMCs and small PAs from control and *SM22-Tsc2*^{+/-} mice.

a, b: Immunoblot analysis of human non-diseased (control) and PAH PAVSMCs. Data are means \pm SE from n=5 control subjects; n=7 PAH subjects.

c, d: Immunohistochemical analysis of lung tissue sections from nine weeks old male mice to detect phosphorylated Ser¹²⁷ in YAP (red), SMA (green), and DAPI (blue). **c:** Bar, 30 μ m. Images are representative from 3 mice/group, 10 PAs/mouse. **d:** Optical density (OD) measurement of Ser¹²⁷-YAP fluorescent signal in SMA-positive areas of small muscular PAs. Data are means \pm SE from 3 mice/group, 10 PAs/mouse. WT - wild type.

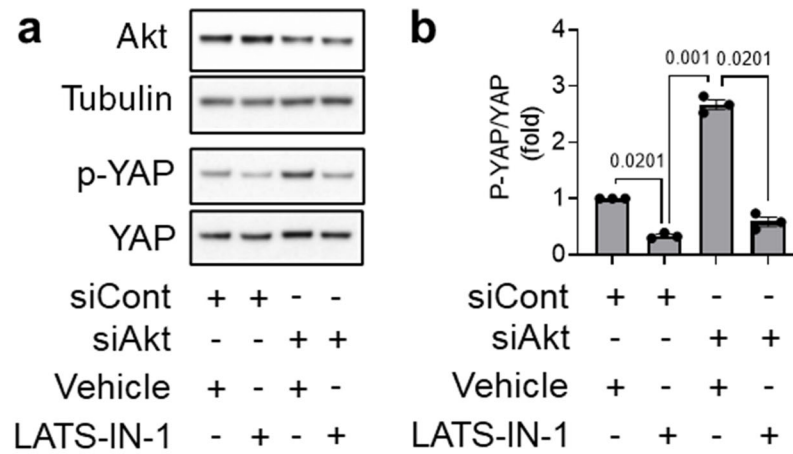


Figure S7. Effects of Akt and LATS on the Ser¹²⁷-YAP phosphorylation in human PAH PAVSMC.

Human PAH PAVSMCs were transfected with control scrambled siRNA (siCont) or siRNA directed against Akt1/2 (siAkt) or treated with 10 μ M LATS-IN-1 or vehicle separately or in combination for 48 hr and immunoblotted to detect the indicated proteins. **a**: Images are representative from three experiments, each performed on the cells from a different subject. **b**: Data are means \pm SE, n=3 subjects/group. *P* values were determined by Kruskal-Wallis rank test with Dunn's pairwise comparison.

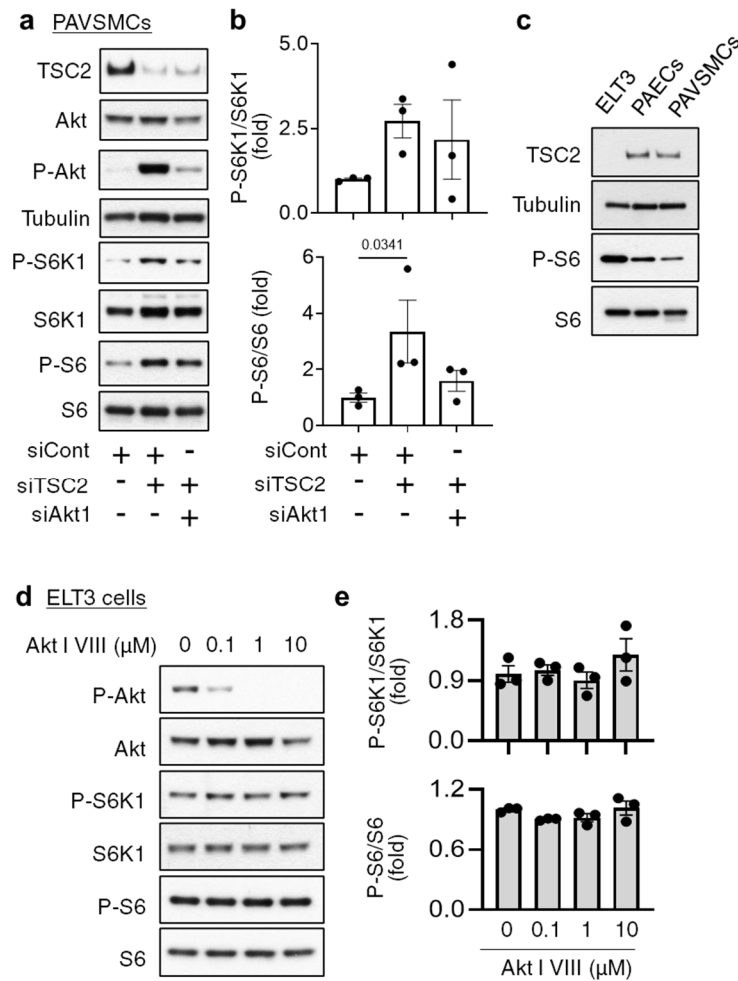


Figure S8. Loss of TSC2 attenuates mTORC1 activation by Akt.

a, b: Human control PAVSMCs were transfected with control siRNA GLO (siCont), siRNA directed against TSC2 (siTSC2), and siRNA directed against Akt1 (siAkt1) separately or in combination for 48 hr and immunoblotted to detect indicated proteins. **a:** Images are representative from three experiments, each performed on the cells from a different subject. **b:** Data are means \pm SE, $n=3$ subjects/group. P values were determined by Kruskal-Wallis rank test with Dunn's pairwise comparison.

c: Immunoblot analysis of ELT3 cells, human PA endothelial cells (PAECs), and human PAVSMCs to detect indicated proteins. Images are representative of three independent experiments.

d, e: ELT3 cells were treated with indicated concentrations of the Akt inhibitor VIII for 48 hr and immunoblotted to detect the indicated proteins. **d:** Images are representative from three experiments. **e:** Data are means \pm SE from 3 independent experiments. Statistical analysis was performed using Kruskal-Wallis rank test.

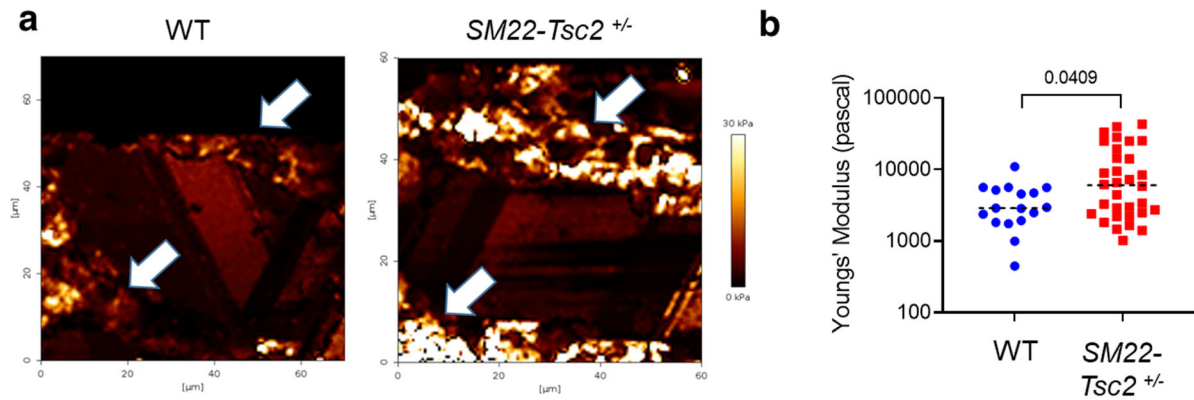


Figure S9. Stiffness of small PAs from male *SM22-Tsc2^{+/-}* and control (WT) mice.

PAs < 200 μm from nine weeks old control (WT) and *SM22-Tsc2^{+/-}* male mice were mechanically characterized using AFM microindention. Representative images (a) and analysis (b) of $n=3$ WT mice, $n=4$ *SM22-Tsc2^{+/-}* mice, 5-10 PAs/mouse are shown. **b**: Horizontal lines represent the mean Young's modulus of each group; each symbol corresponds to one PA. *P* value was determined by a mixed model with genotype as a fixed effect and individual mice as a random effect. White arrows indicate PA walls.

Control PAVSMCs on the matrices produced by control or PAH PAVSMCs

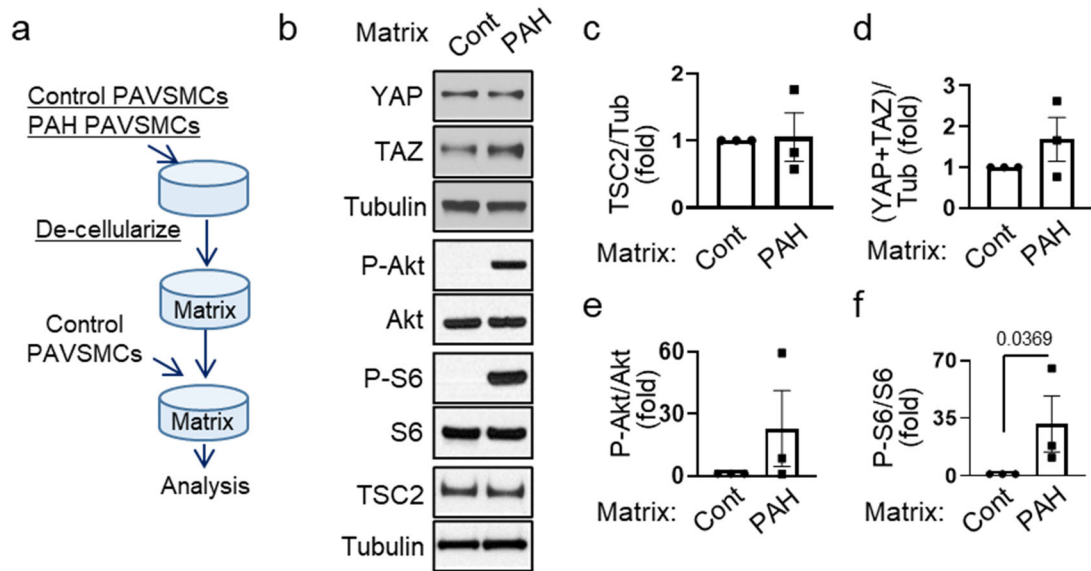


Figure S10. ECM produced by human PAH PAVSMCs promotes YAP/TAZ accumulation and increases Akt and S6 phosphorylation in control PAVSMCs

a: Preconfluent human PAH or control PAVSMCs were grown for 7 days. After cell removal, equal numbers of non-diseased (control) PAVSMCs were plated on remaining matrices. 4 days post-plating, immunoblot analyses were performed.

b. Immunoblots are representative of three independent experiments, each performed on the cells from a different subject.

c-f. Data are means \pm SE from n=3 subjects/group. PAH was normalized to control, which was set at 1. Comparisons between PAH and control were determined to be not statistically significant by Mann Whitney U test, except for P-S6/S6.

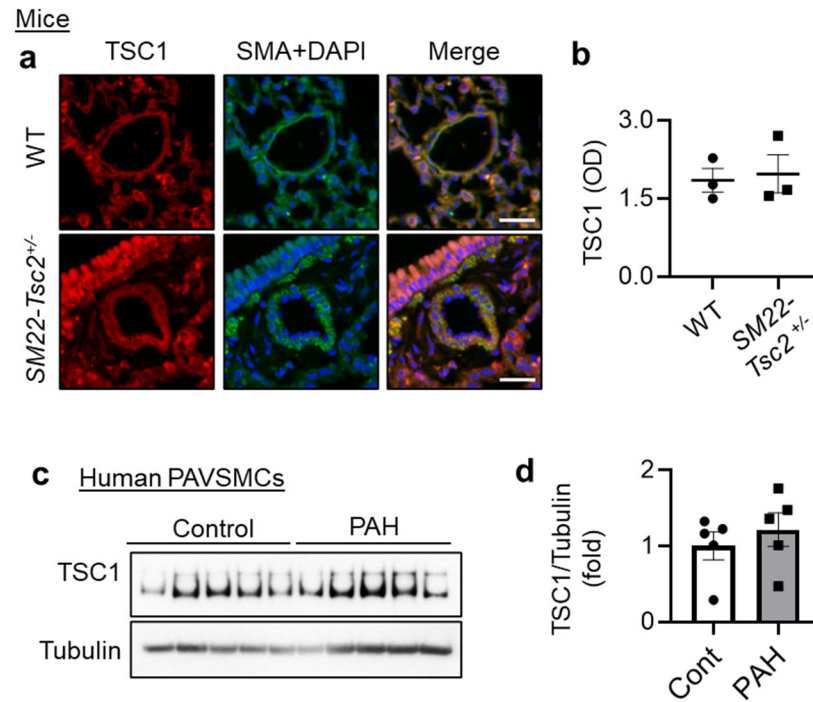


Figure S11. PAVSMC TSC1 protein levels are not reduced in in PH-related conditions.

a, b: Immunohistochemical analysis of lung tissue sections from nine weeks old male mice to detect TSC1 (red), SMA (green), and DAPI (blue). **a:** Bar, 30 μ m. Images are representative from 3 mice/group, 10 PAs/mouse. **b:** Optical density (OD) measurement of TSC1 fluorescent signal in SMA-positive regions of small muscular PAs. Data are means \pm SE from 3 mice/group, 10 PAs/mouse. WT - wild type.

c, d: Immunoblot analysis of human non-diseased (control) and PAH PAVSMCs. Data are means \pm SE. PAH was normalized to control which was set to 1. N=5 subjects/group.

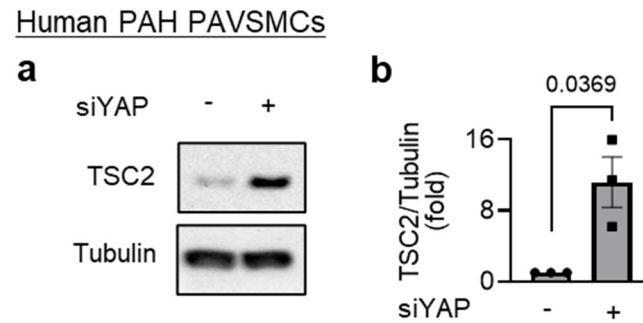


Figure S12. YAP depletion in human PAH PAVSMC results in TSC2 accumulation

Immunoblot analysis of human PAH PAVSMCs transfected with siRNA YAP (+) or control siRNA GLO (-) for 48hr. (a) Representative immunoblots. (b) Data are means \pm SE from n=3 subjects/group. siYAP was normalized to control, which was set at 1. *P* value for siYAP compared to siRNA GLO was determined by Mann-Whitney U test.

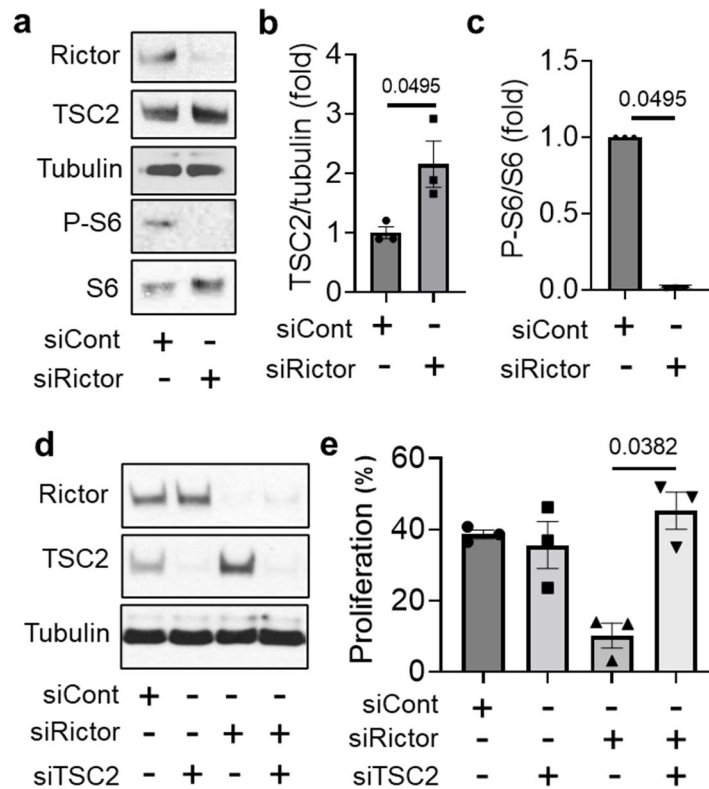


Figure S13. Rictor-dependent reduction of TSC2 protein levels contributes to PAH PAVSMC proliferation.

a-c: Human PAH PAVSMCs were transfected with siRNA directed against Rictor (siRictor) or control siRNA GLO (siCont) for 48 hr and immunoblotted to detect indicated proteins. **a:** images are representative from three experiments, each performed on the cells from a different subject. **b:** Data are means \pm SE, $n=3$ subjects/group. P values for siRictor compared to siCont were determined by Mann-Whitney U test.

d, e: Human PAH PAVSMCs were transfected with siRNA directed against Rictor (siRictor) and TSC2 (siTSC2) separately or in combination and control siRNA GLO (siCont) for 48 hr and immunoblotted for the indicated proteins (**d**) or proliferation analyzed as measured by BrdU incorporation (**e**). Data are means \pm SE from $n=3$ subjects/group. P values were determined by Kruskal-Wallis rank test with Dunn's pairwise comparison.

Human PAH PAVSMCs

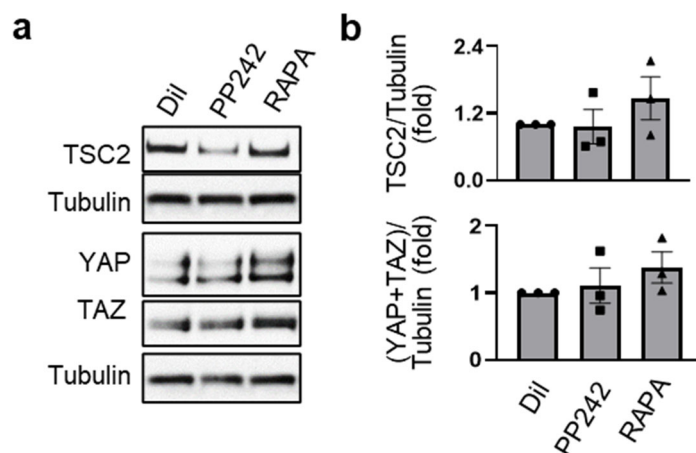


Figure S14. Pharmacological inhibition of mTOR does not affect TSC2 or YAP/TAZ protein levels in human PAH PAVSMCs

Human PAH PAVSMCs treated with 10 μ M PP242, 10 nM rapamycin (RAPA), or diluent (Dil) were immunoblotted for TSC2, YAP, and TAZ. (a) Representative immunoblots. (b) Data are means \pm SE from n=3 subjects/group. PP242 and rapamycin were normalized to diluent, which was set at 1. Comparisons between PP242 or rapamycin and diluent were determined to be not statistically significant by Kruskal-Wallis rank test with Dunn's pairwise comparison.

Human PAH PAVSMCs

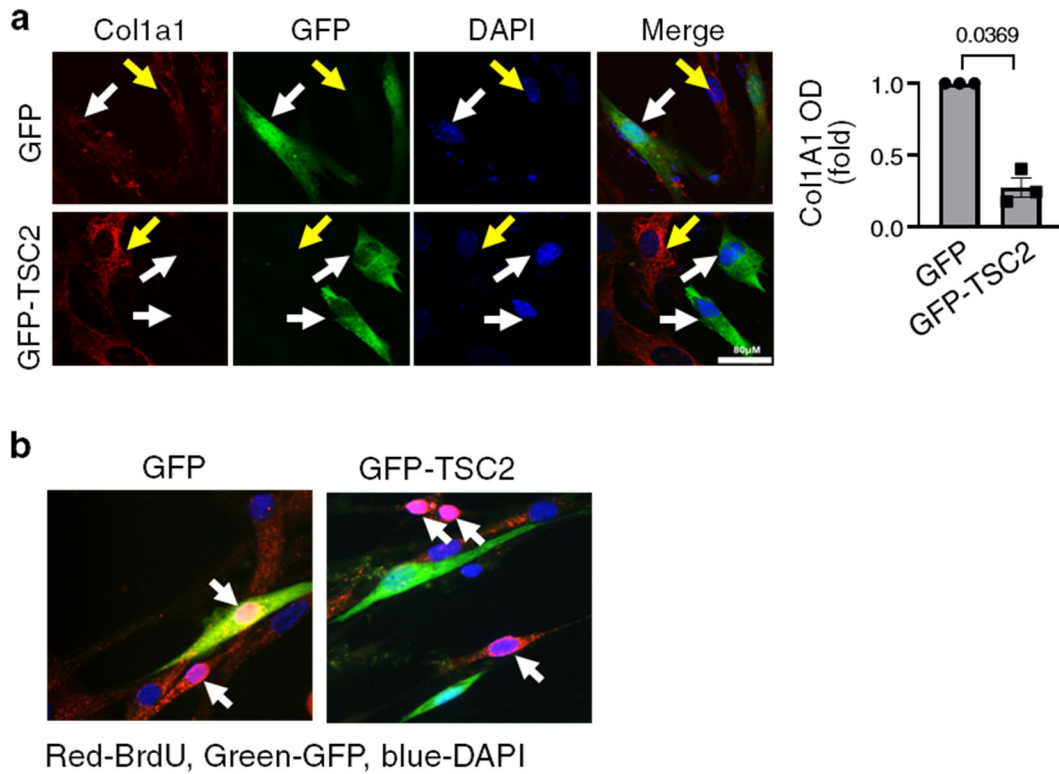


Figure S15. TSC2 reduces Col1A1 levels in human PAH PAVSMCs

a. Human PAH PAVSMCs were transfected with GFP or GFP-TSC2. Immunocytochemical analysis was performed 48hr post-transfection to detect Col1A1 (red), GFP (green) and DAPI (blue). White and yellow arrows indicate cells that were transfected or not transfected with plasmids, respectively. Scale bar, 80µm. Optical density of Col1A1 in GFP⁺ cells were measured by ImageJ. Data are means±SE from n=3 subjects/group, 12 cells/subject. GFP-TSC2 was normalized to GFP, which was set at 1. *P* value for GFP-TSC2 compared to GFP was determined by Mann-Whitney U test.

b. Representative images of human PAH PAVSMCs transfected with GFP (left, green) or GFP-tagged human TSC2 (right, green) were subjected to the BrdU incorporation assay (BrdU, red; DAPI, blue). Yellow – overlap between GFP (green) and BrdU (red).

Human PAH PAVSMCs

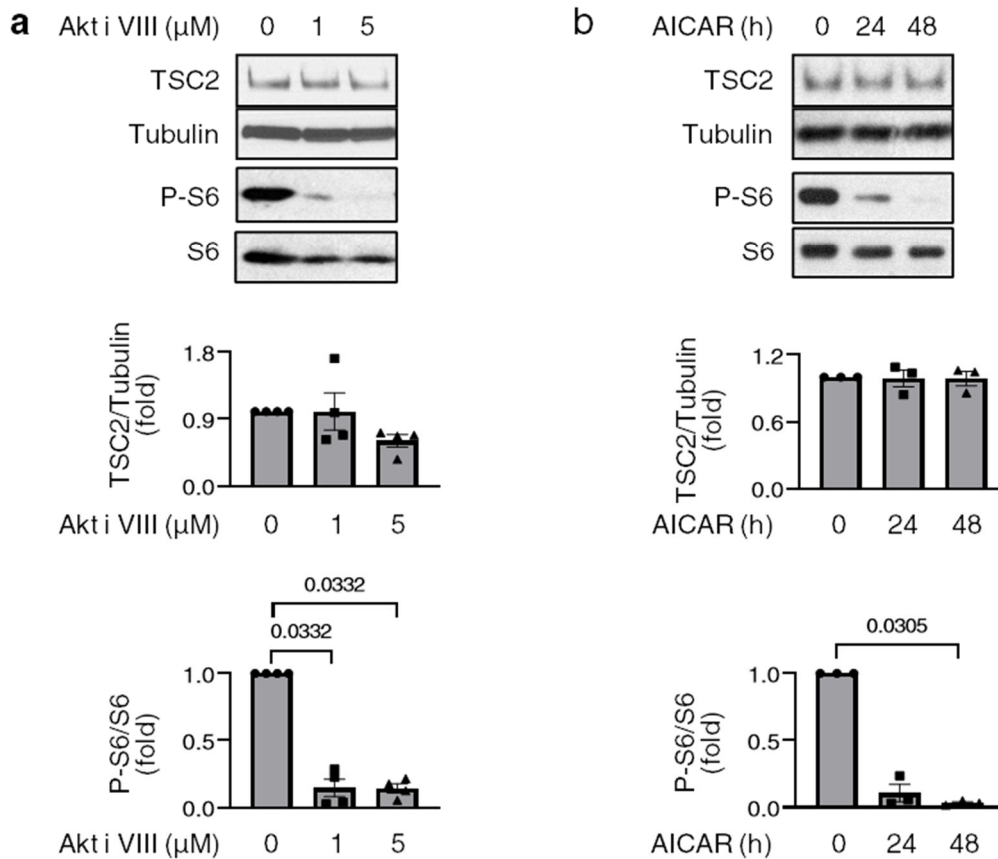
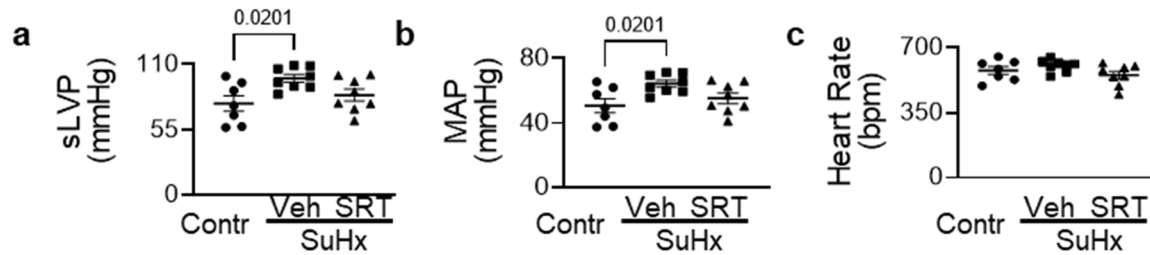


Figure S16. TSC2 protein levels in human PAH PAVSMCs treated with an Akt inhibitor and AMPK activator

Human PAH PAVSMCs were treated with the indicated concentrations of the Akt inhibitor VIII for 24hr (**a**) or the AMPK activator AICAR (100mM) for the indicated periods of time (**b**). PAVSMCs were immunoblotted for the indicated proteins. Data are means \pm SE from 3 subjects/group. *P* values for Akt inhibitor VIII or AICAR treatment compared to diluent (0) or 0hr treatment were determined by Kruskal-Wallis test with Dunn's pairwise comparison.

C57/BL6 mice



Sprague-Dawley Rats

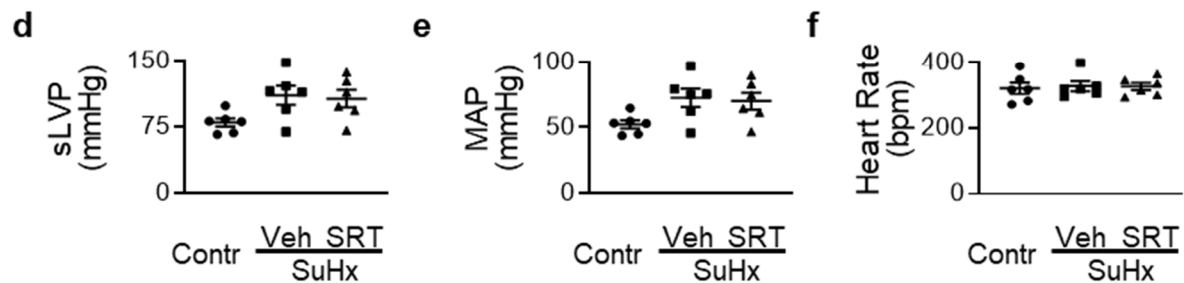


Figure S17. SRT2104 does not affect systemic blood pressure or heart rate of mice and rats with SuHx-induced PH

a-c: Systolic left ventricular pressure (sLVP) (a), mean arterial pressure (MAP) (b), and heart rate (c) of mice without PH or with SuHx-induced PH and treated with vehicle or SRT2104. Data are means±SE from n=7 control mice (3 male and 4 female), n=8 PH+Veh mice (4 male and 4 female), and n=8 PH+SRT mice (4 male and 4 female). *P* values were determined by one-way ANOVA with Holm-Sidak's posthoc test.

d-f: sLVP (d), MAP (e), and heart rate (f) of control rats or rats with SuHx-induced PH treated with vehicle or SRT2104. Data are means±SE from 6 rats/group. *P* values between SuHx and Contr were determined to be not statistically significant by one-way ANOVA.

Table S1. Characteristics of human subjects.

Diagnosis	Sex	Age, years
Non-diseased	F	28
Non-diseased	F	29
Non-diseased	F	33
Non-diseased	F	34
Non-diseased	F	36
Non-diseased	F	37
Non-diseased	F	38
Non-diseased	F	40
Non-diseased	F	41
Non-diseased	F	43
Non-diseased	F	44
Non-diseased	F	46
Non-diseased	F	48
Non-diseased	F	50
Non-diseased	F	50
Non-diseased	F	53
Non-diseased	F	56
Non-diseased	F	57
Non-diseased	F	64
Non-diseased	M	24
Non-diseased	M	25
Non-diseased	M	35
Non-diseased	M	35
Non-diseased	M	36
Non-diseased	M	40
Non-diseased	M	41
Non-diseased	M	44
Non-diseased	M	47
Non-diseased	M	53
Non-diseased	M	70
IPAH	F	16
IPAH	F	16
IPAH	F	29
IPAH	F	32
IPAH	F	39

IPAH	F	40
PAH	F	49
IPAH	F	50
IPAH	F	53
IPAH	F	58
IPAH	F	62
IPAH	M	21
IPAH	M	31
PAH	M	45
IPAH	M	51
IPAH	M	53

IPAH: Idiopathic pulmonary arterial hypertension; PAH: pulmonary arterial hypertension

Table S2. Chronic hypoxia promotes PH and RV hypertrophy in mice

Group/exposure	Normoxia	Hypoxia	
Male WT sRVP (mmHg)	28.285±0.581	43.778±1.239*	<i>P</i> =0.0022
Male <i>SM22-Tsc2</i> ^{+/-} sRVP (mmHg)	32.406±1.264	46.843±2.283*	<i>P</i> =0.0012
Female WT sRVP (mmHg)	26.200±0.696	39.075±1.027*	<i>P</i> =0.0252
Female <i>SM22-Tsc2</i> ^{+/-} sRVP (mmHg)	30.158±2.460	41.924±2.343*	<i>P</i> =0.0082
Male WT RV/(LV+S)	0.288±0.007	0.435±0.013*	<i>P</i> =0.0022
Male <i>SM22-Tsc2</i> ^{+/-} RV/(LV+S)	0.324±0.008	0.487±0.017*	<i>P</i> =0.0012
Female WT RV/(LV+S)	0.278±0.015	0.405±0.008*	<i>P</i> =0.022
Female <i>SM22-Tsc2</i> ^{+/-} RV/(LV+S)	0.310±0.013	0.466±0.023*	<i>P</i> =0.0012

Systolic right ventricular pressure (sRVP) and Fulton index (RV/(LV+S)) of control (WT) and *SM22-Tsc2*^{+/-} mice exposed to normobaric hypoxia (10% O₂) or normoxia for three weeks. Data are means±SE, 6-7 mice/group. *P* values for hypoxia compared to normoxia were determined by Mann-Whitney U test.