

SUPPLEMENTAL MATERIALS

Gene-specific endothelial programs drive AVM pathogenesis in *SMAD4* and *ALK1* loss-of-function

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Figure S1.1

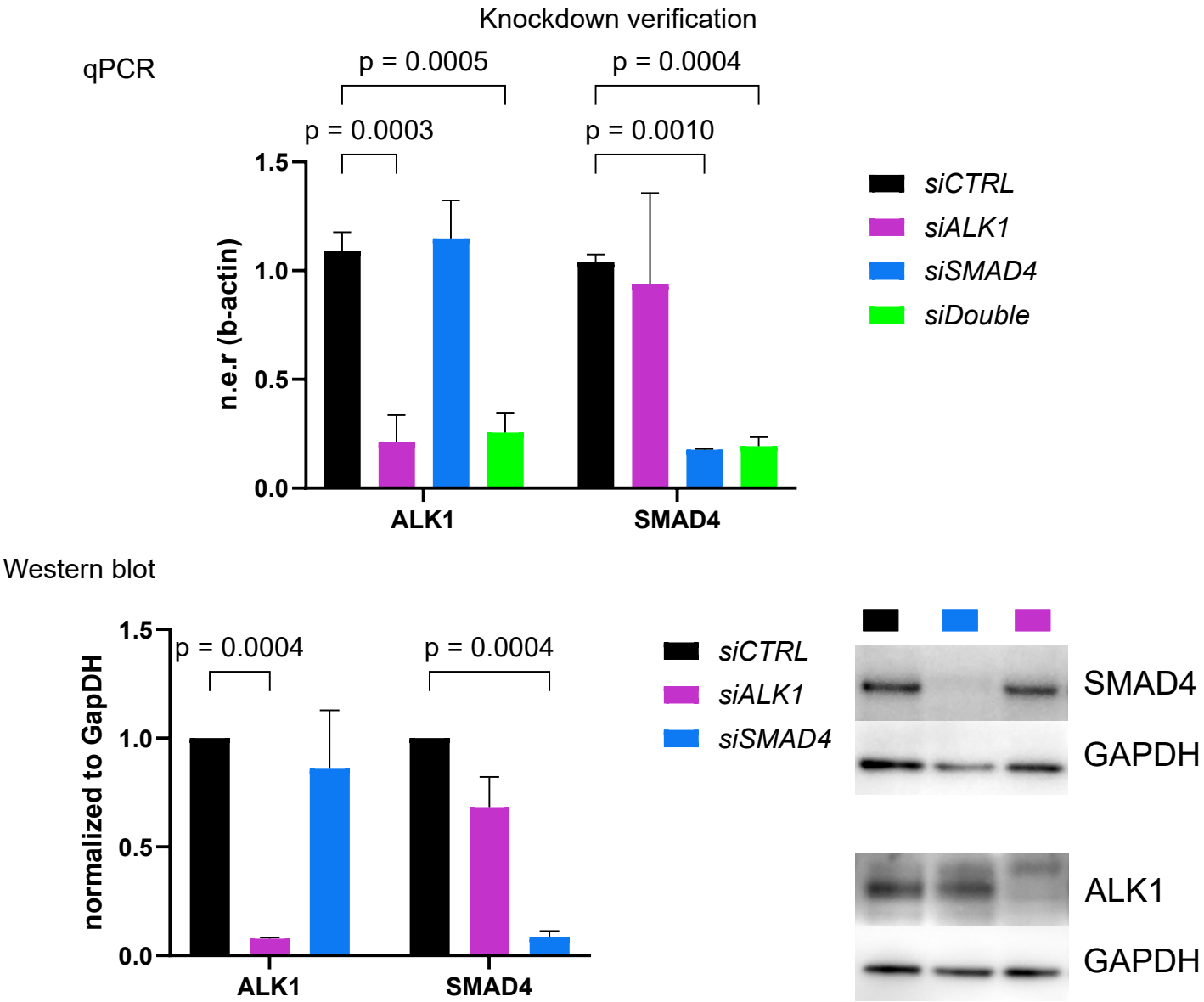


Figure S1.1:

Knockdown verification of *SMAD4*, *ALK1*. Top – qPCR analysis of *siCTRL* (black), *siSMAD4* (blue), *siALK1* (magenta), *siDouble* (lime), 48h post siRNA treatment. Log2FC values represent relative expression to b-actin. n=3 independent experiments. Bottom – Western blot analysis of *siCTRL*, *siSMAD4* and *siALK1* with representative images of blots, 48h post siRNA treatment. Bands normalized to GAPDH; Data presented as Mean $\pm$ SEM. n=3 independent experiments. Statistical analysis done using 2-way ANOVA with Dunnett's multiple comparisons test. Quantification data available in 01_statistics.xlsx. Raw blot images are included in WB.pdf

Figure S1.2

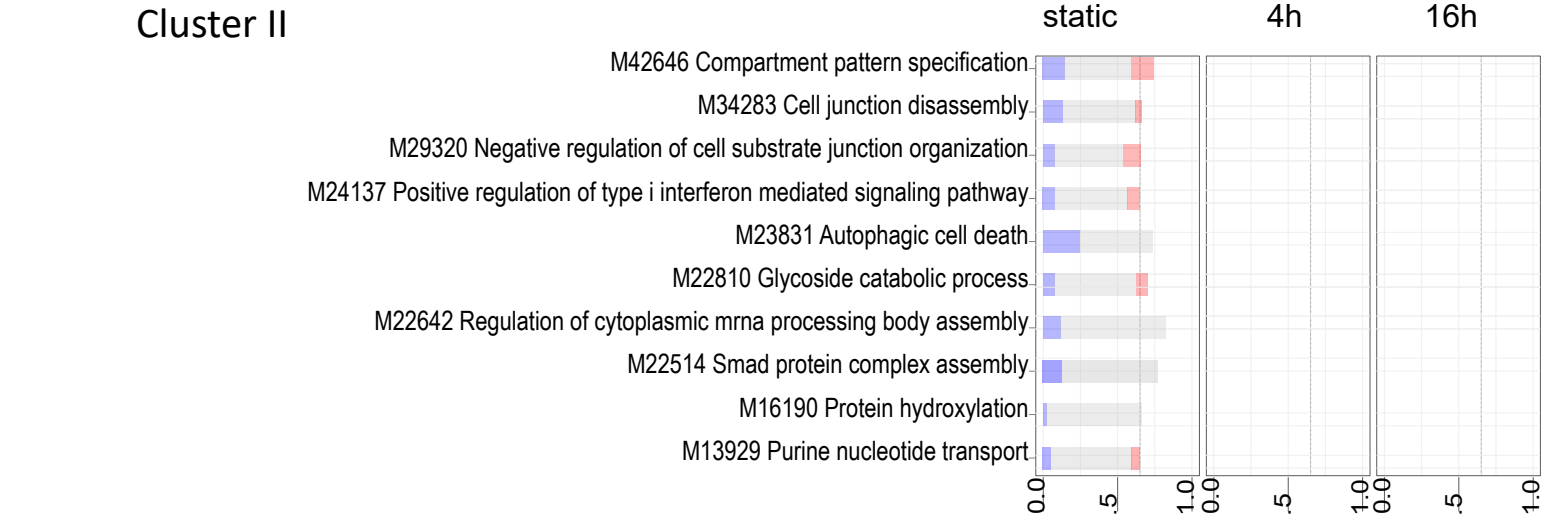


Figure S1.2 - continued

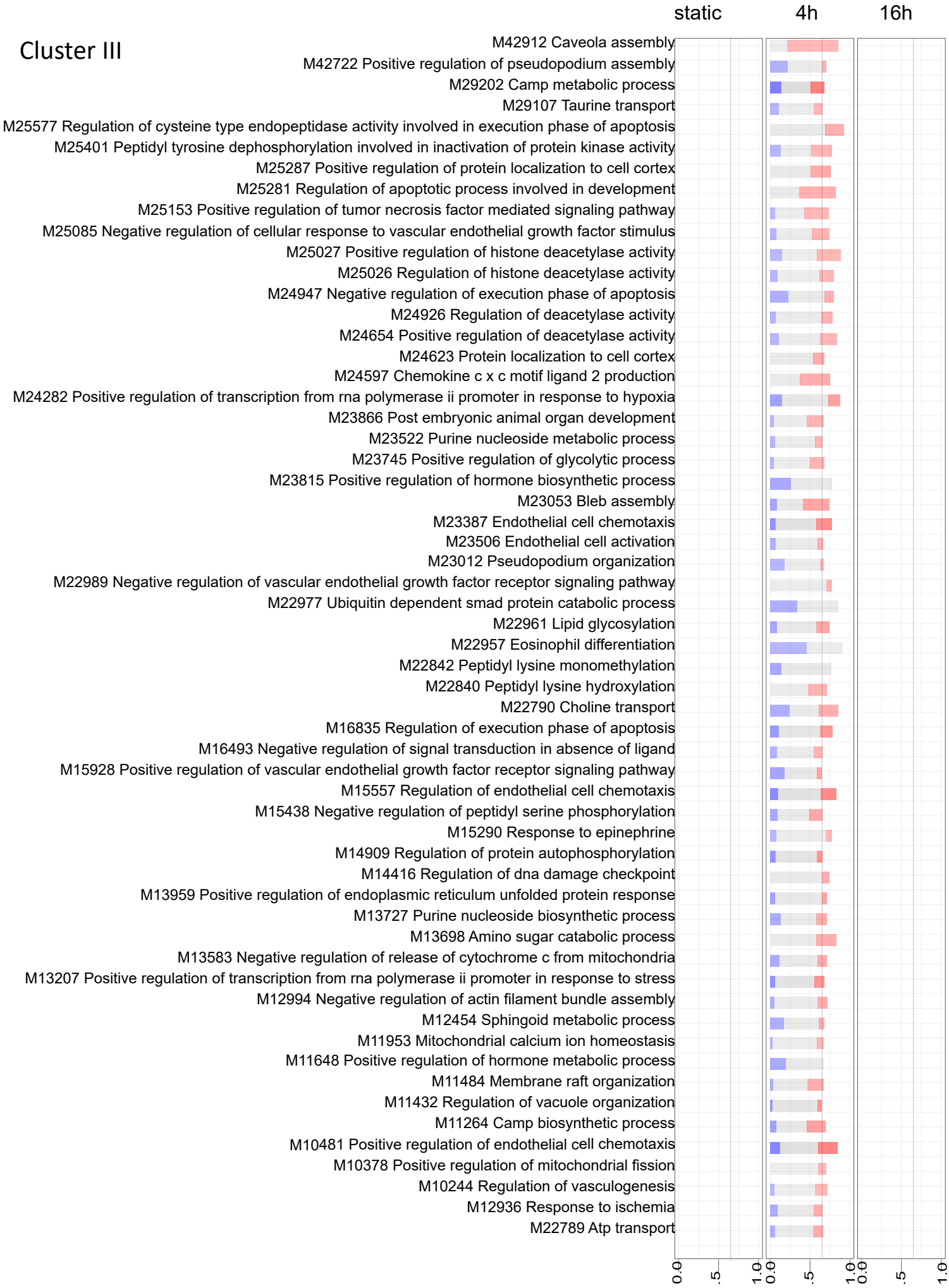
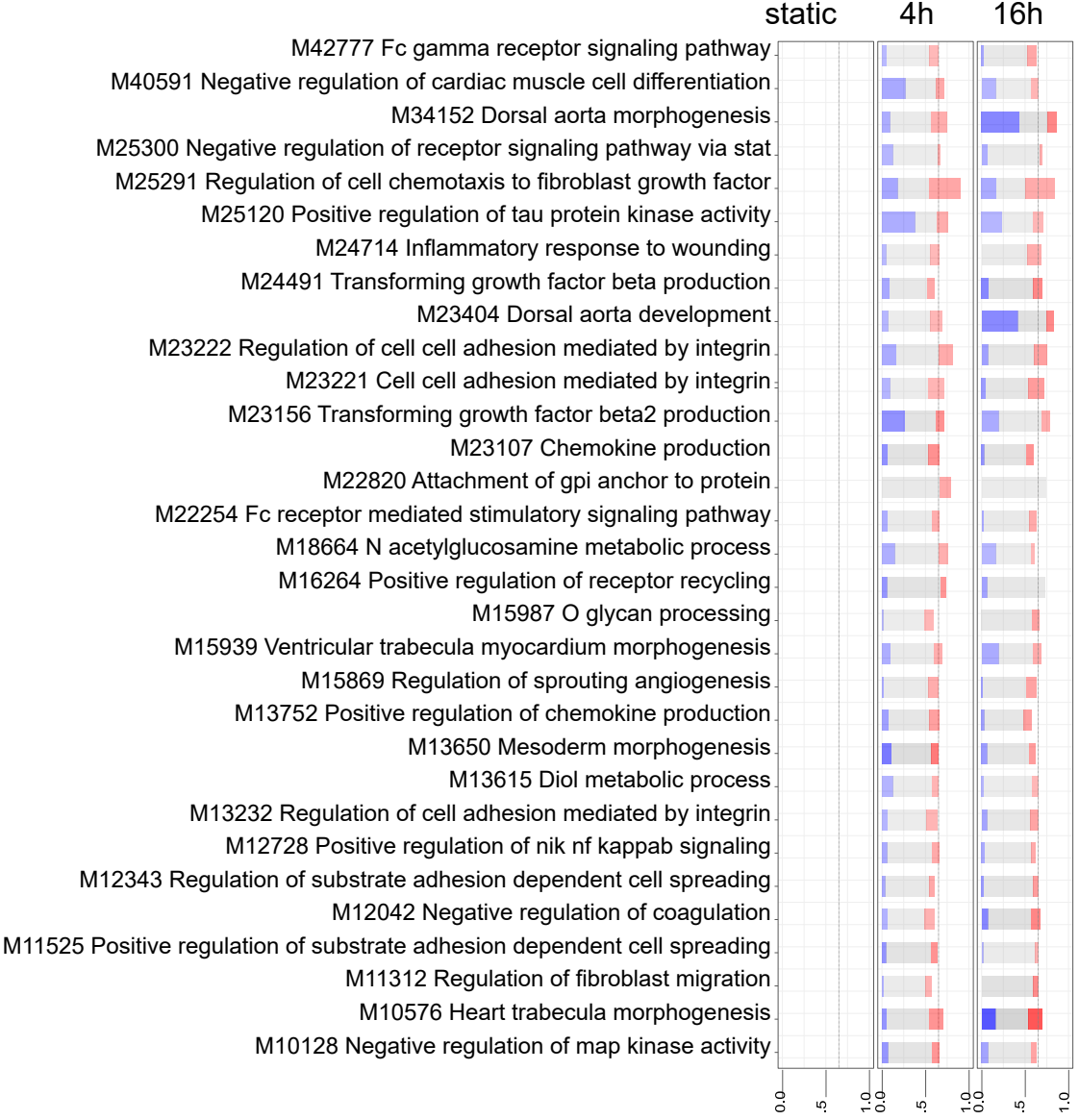


Figure S1.2 - continued



Figure S1.2 - continued

Cluster V

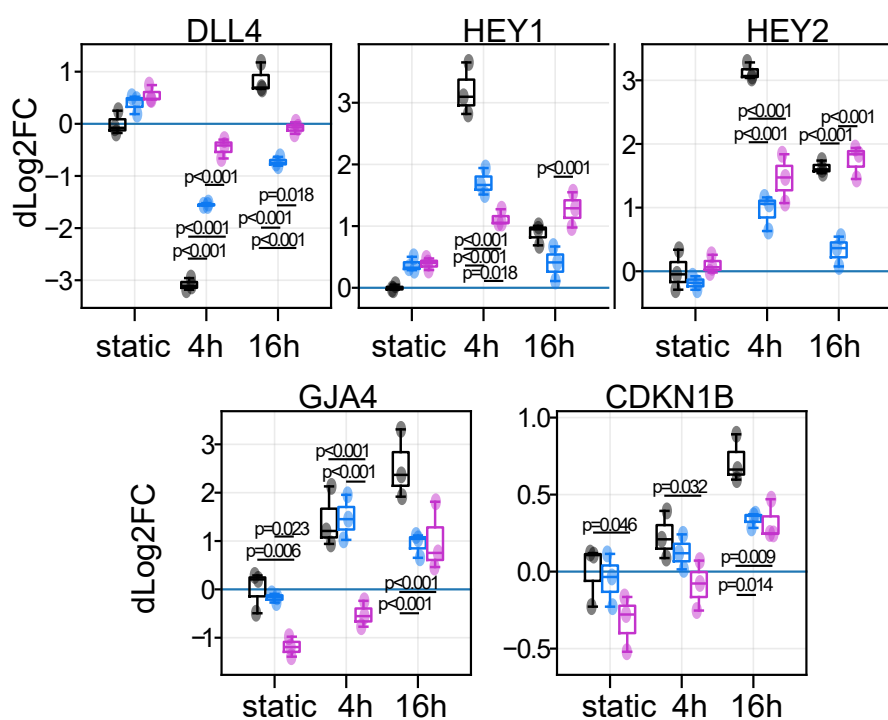
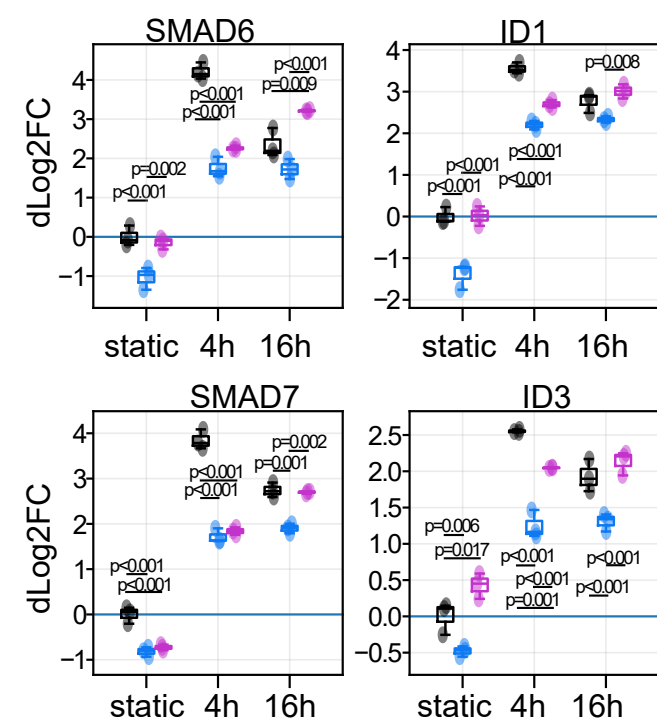
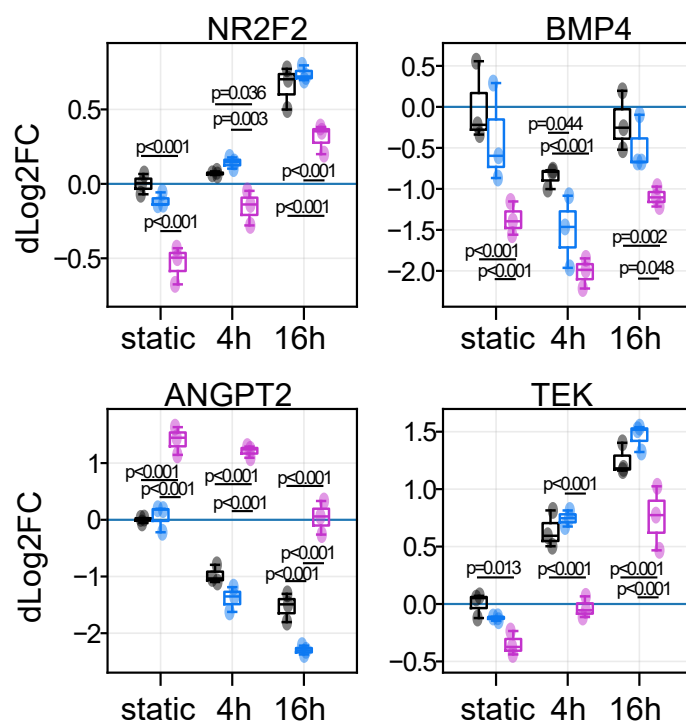
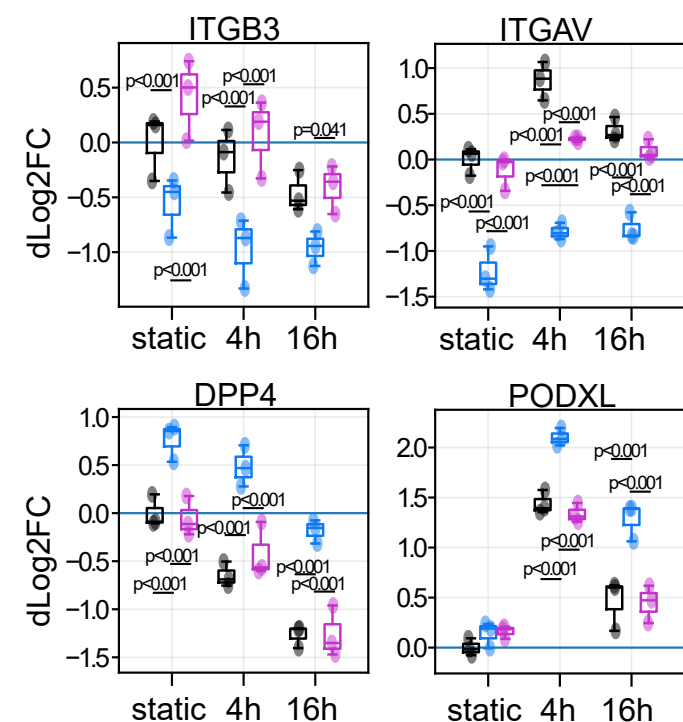


Cluster VI



Figure S1.2

Full panel plot of GO_BP of *siSMAD4* vs. *siALK1* in static, 4h and 16h of LSS. GO_BPs are clustered according to their enrichment in the different flow conditions: Cluster I – enrichment in all flow conditions; Cluster II – enrichment in static only; Cluster III – enrichment in 4h LSS only; Cluster IV – enrichment in 16h LSS only; Cluster V – enrichment in both 4h and 16h LSS; Cluster VI – enrichment in static and 4h LSS **OR** 16h LSS.

Figure S1.3**a. Canonical BMP/TGF-beta associated****b. Notch, Arterial identity associated****c. Endothelial adhesion associated****d. Angiogenic, venous identity associated**

siCTRL siSMAD4 siALK1

Figure S1.3

Expression patterns of individual genes over time (RNA-seq data). Y axis is Log2FC scale normalized to *siCTRL* static, X axis are flow conditions. Expression patterns of (a) Canonical BMP/TGF-beta associated genes; (b) Notch and Arterial identity associated genes; (c) Endothelial adhesion associated genes; and (d) Angiogenic and venous identity associated genes. *siCTRL* – black; *siSMAD4* – blue; *siALK1* – magenta. Adjusted p-values under 0.05 are indicated.

Figure S2

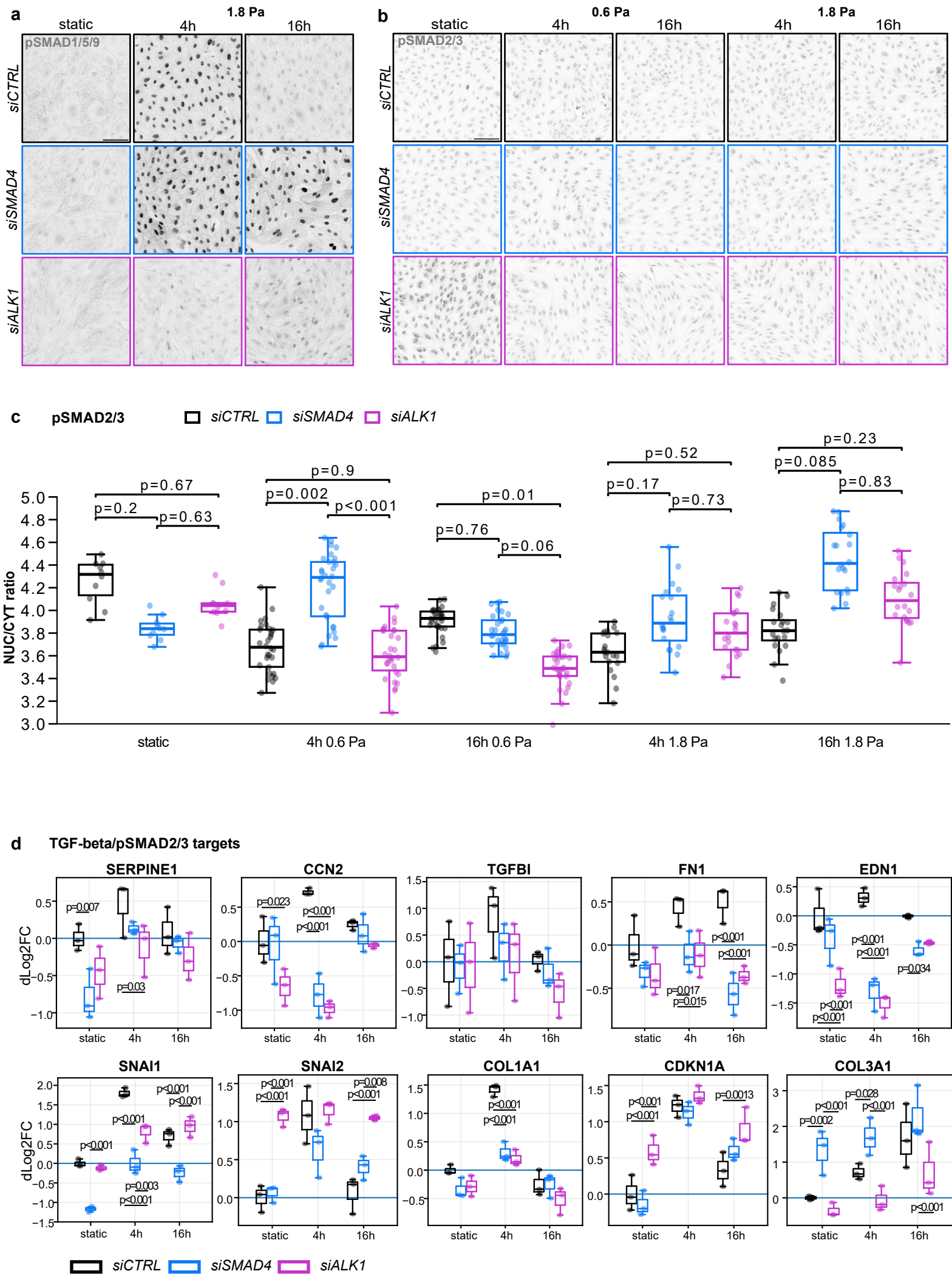


Figure S2

- a. Representative pSMAD1/5/9 immunofluorescence images of siRNA-treated HUVECs after exposure to HSS (1.8 Pa). Scale bar 100 μ m.
- b. Representative pSMAD2/3 immunofluorescence images of HUVECs after exposure to static, 4h and 16h, 0.6 and 1.8 Pa conditions. Scale bar 100 μ m.
- c. Quantification of nuclear pSMAD2/3 signal at all conditions, normalized to cytosolic signal. Box- and scatter plots display the distribution of raw image-level measurements (≤ 10 images per experiment per condition). Statistical analysis was done on experimental means using 2-way ANOVA with Tukey's multiple comparison correction. Sample size: n_static=1; n_0.6_Pa=3 (both time points); n_1.8_Pa=2 (both time points). Full statistical analysis outputs are available in 02_statistics.xlsx
- d. Individual expression patterns of TGF-beta/pSMAD2/3 targets under 0.6 Pa (LSS) flow (RNA-seq data). Y axis is Log2FC scale normalized to *siCTRL* static. Expression patterns of a. *siCTRL* – black; *siSMAD4* – blue; *siALK1* – magenta. Adjusted p-values under 0.05 are indicated.

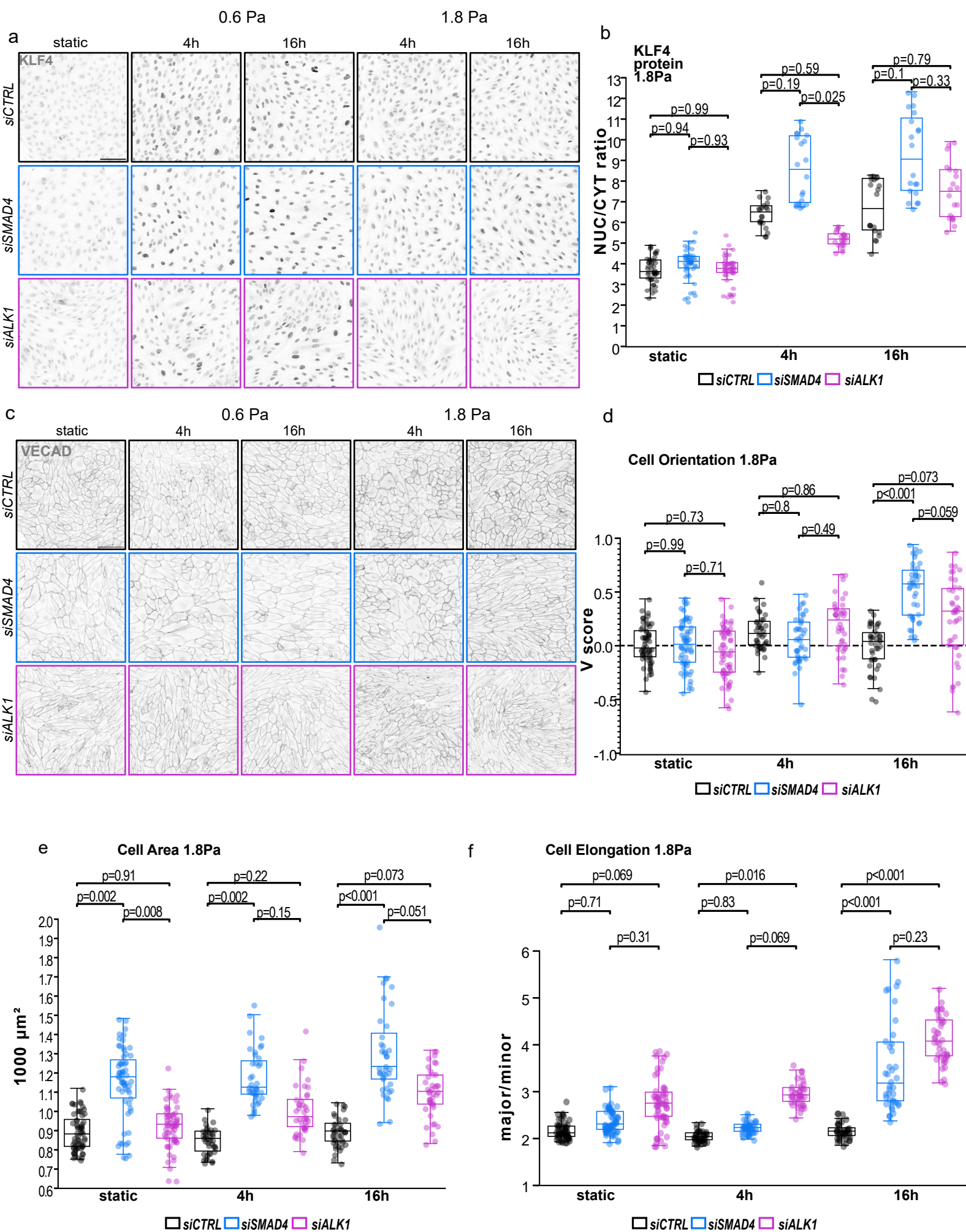
Figure S3

Figure S3

- a. Representative *KLF4* immunofluorescence images of HUVECs after exposure to static, 4h and 16h, 0.6 and 1.8 Pa conditions. Scale bar 100 μ m.
- b. Quantification of nuclear *KLF4* signal at 1.8 Pa, normalized to cytosolic signal. Box- and scatter plots display the distribution of raw image-level measurements (≤ 10 images per experiment per condition). n=2 independent experiments. Statistical analysis was done on experimental means using 2-way ANOVA with Tukey's multiple comparison correction.
- c. Representative VEcad immunofluorescence images of HUVECs after exposure to static, 4h and 16h, 0.6 and 1.8 Pa conditions. Scale bar 100 μ m.
- d. – f. Statistical analysis of cell orientation (d), cell area (e) and cell elongation (f) of *siCTRL*-, *siSMAD4*- and *siALK1*-treated HUVECs at static, 4h and 16h at 1.8Pa (HSS).). Box- and scatter plots display the distribution of raw image-level measurements (≤ 10 images per experiment per condition). Statistical analysis was done on experimental means using 2-way ANOVA with Tukey's multiple comparison correction. Full statistical analysis outputs are available in 03_statistics.xlsx

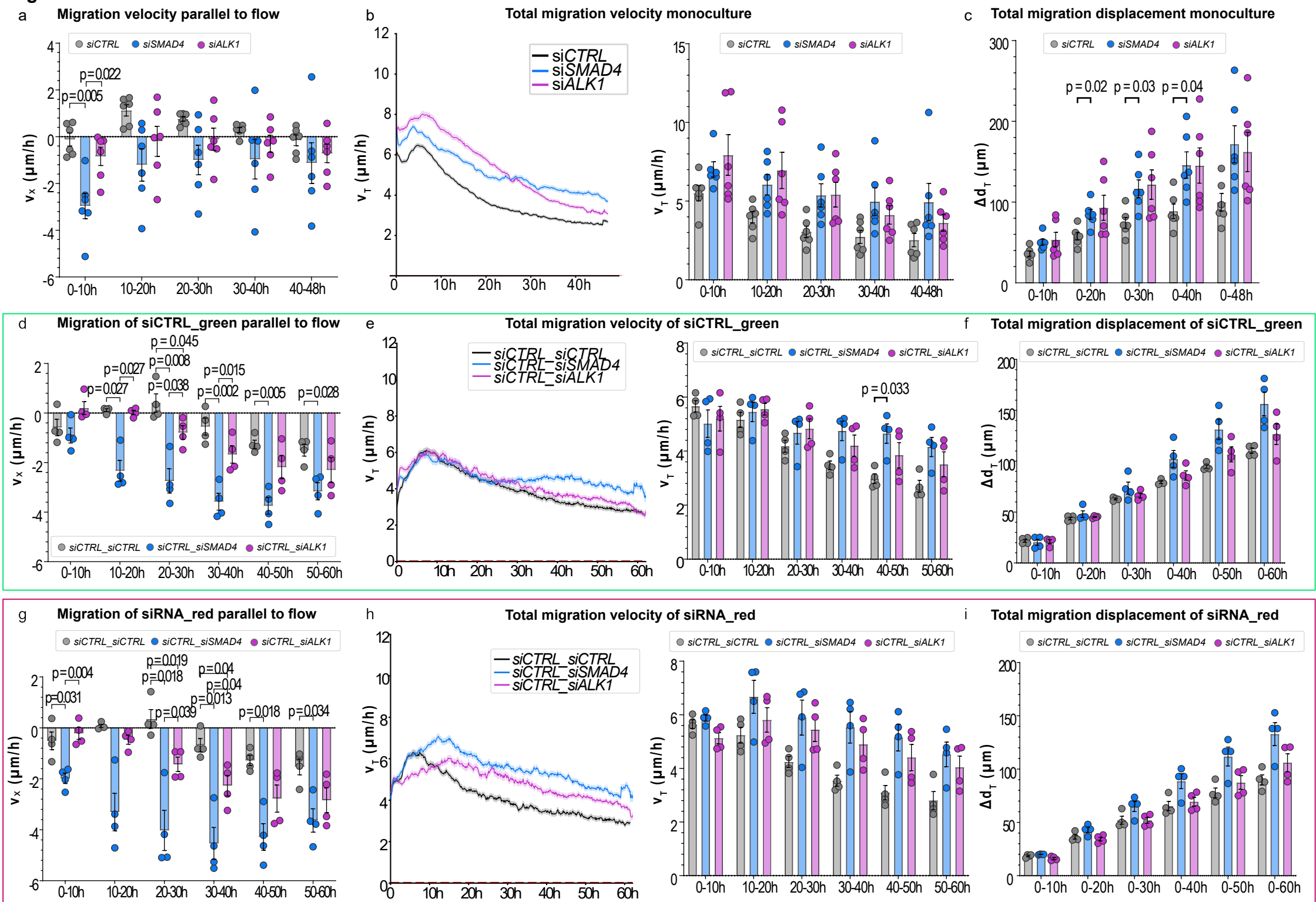
Figure S4

Figure S4

a-c. Migration analysis of cells in monoculture. (a) Quantification of mean migration velocity parallel to flow direction of *siCTRL*-, *siSMAD4*- or *siALK1*-treated HUVECs. (b) Total migration velocity plot and Quantification of *siCTRL*-, *siSMAD4*- or *siALK1*-treated HUVECs. (c) Total migration displacement analysis of *siCTRL*-, *siSMAD4*- or *siALK1*-treated HUVECs.

d-f. Migration analysis of *siCTRL*_green cells in mosaic co-cultures. (d) Quantification of mean migration velocity parallel to flow direction of *siCTRL*-treated HUVECs when co-cultured either with themselves (*siCTRL*_siCTRL, gray), or with *siSMAD4*- or *siALK1*-treated HUVECs (*siCTRL*_siSMAD4, blue and *siCTRL*_siALK1, magenta). (e) Total migration velocity plot and Quantification of *siCTRL*-treated HUVECs when co-cultured either with themselves (*siCTRL*_siCTRL, gray), or with *siSMAD4*- or *siALK1*-treated HUVECs (*siCTRL*_siSMAD4, blue and *siCTRL*_siALK1, magenta). (f) Total migration displacement analysis of *siCTRL*-treated HUVECs when co-cultured either with themselves (*siCTRL*_siCTRL, gray), or with *siSMAD4*- or *siALK1*-treated HUVECs (*siCTRL*_siSMAD4, blue and *siCTRL*_siALK1, magenta).

g-i. Migration analysis of *siRNA*_red cells in mosaic co-cultures. (g) Quantification of mean migration velocity parallel to flow direction of red fraction of *siRNA*-treated HUVECs when co-cultured with *siCTRL*_green (*siCTRL*_siCTRL, gray; *siCTRL*_siSMAD4, blue; or *siCTRL*_siALK1, magenta). (h) Total migration velocity plot and Quantification of red fraction of *siRNA*-treated HUVECs when co-cultured with *siCTRL*_green (*siCTRL*_siCTRL, gray; *siCTRL*_siSMAD4, blue; or *siCTRL*_siALK1, magenta). (i) Total migration displacement analysis of *siRNA*-treated HUVECs when co-cultured with *siCTRL*_green (*siCTRL*_siCTRL, gray; *siCTRL*_siSMAD4, blue; or *siCTRL*_siALK1, magenta).

Statistical analysis was done using 2-way ANOVA with Holm-Šídák multiple comparison correction. Full statistical outputs available in 04_statistics.xlsx.

Figure S5

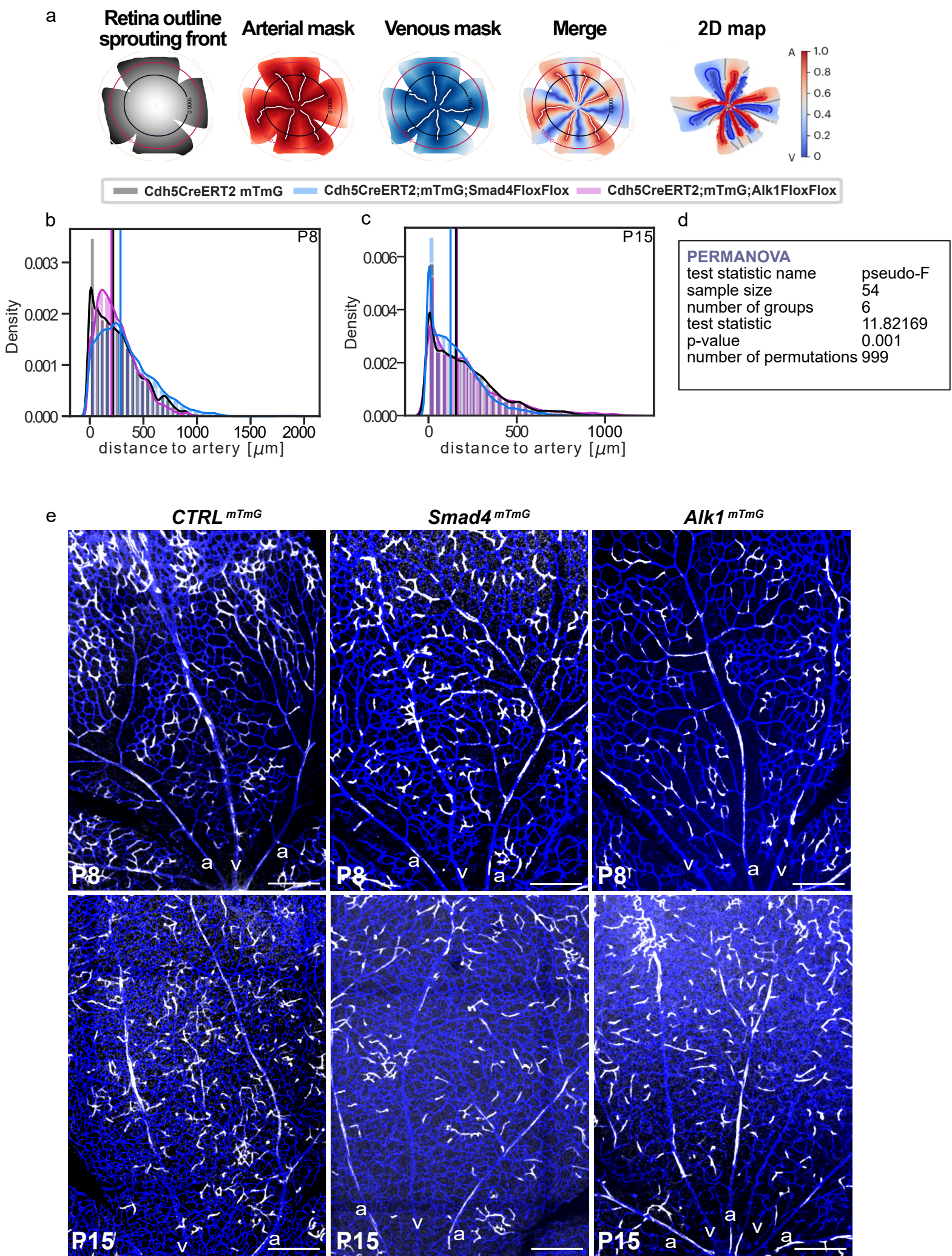


Figure S5

- a. Analysis overview: two-dimensional masks of retina outline, arteries and veins in flat-mounted retinae, result in a 2D map. Each GFP⁺ EC's coordinates are based on its location relative to the vein (0.0) and artery (1.0) and the radial distance from the optic nerve to the sprouting front. Kernel Density Estimate (KDE) plots represent the cumulative location of all GFP⁺ EC across all retinae for each genotype.
- b. – c. Density histogram of GFP⁺ pixel distribution relative to the arteries at P8 (b) and P15 (c), respectively. Vertical lines represent means of each genotype (gray – CTRL^{mTmG}; blue – Smad4^{mTmG}; magenta – Alk1^{mTmG}).
- d. PERMANOVA analysis output of PC1 and PC2.
- e. Higher magnification images of retina sections shown in Figure 5. Scale bar 250µm. a-artery; v-vein.

Figure S6

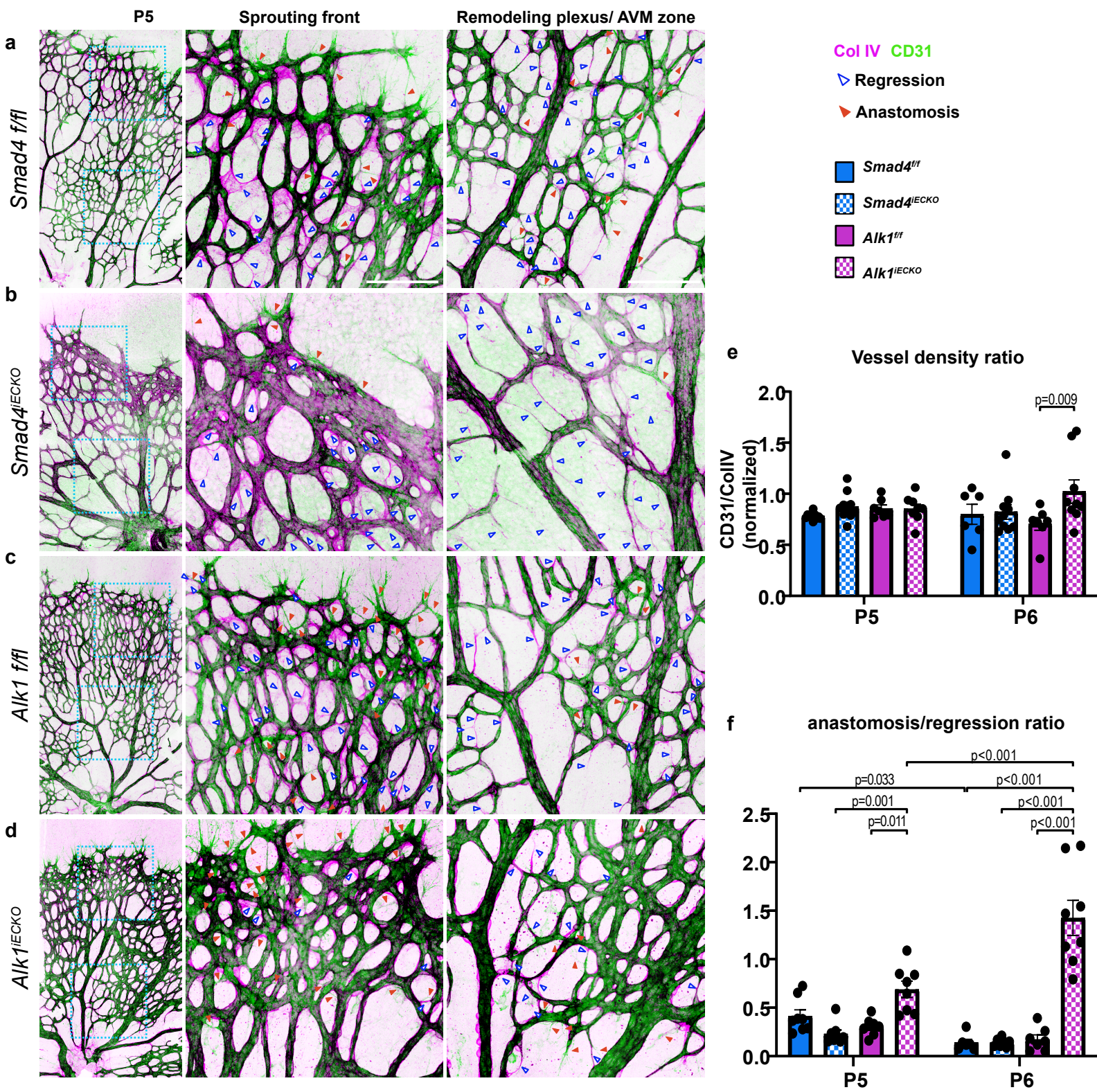


Figure S6

a–d. Microscopy images of P5 retinæ of *Smad4*^{fl/fl} (a), *Smad4*^{iECKO} (b), *Alk1*^{fl/fl} (c), *Alk1*^{iECKO} (d). Light blue dashed rectangles mark areas of sprouting front (SF) and remodeling plexus/AVM zone (RP) on the right of each image. Scale bar 100µm. Anastomosis events marked in orange triangles; Regression events marked in dark blue unfilled triangles.

e–f. Vessel density (vessel volume/ROI volume x100%) (e); and anastomosis/regression events ratios (f) in littermate controls (full-color bars) and KO retinæ (dotted bars). Collection time points (P5 or P6) are annotated under each group. 2-way ANOVA test was used to determine statistical significance with Tukey's multiple correction. Data are represented as mean ± SEM. Full statistical outputs available in 06_statistics.xlsx.

Supplementary movies:

Live imaging of endothelial migration under flow.

S1-3. Representative time lapse imaging of HUVECs labeled with SPY505 and exposed to LSS for 48h. 1. *siCTRL*; 2. *siSMAD4*; 3. *siALK1*. Frame interval – 7.5 minutes.

S4-6. Representative time lapse imaging of HUVECs labeled with CellTracker Green (*siCTRL*) and CellTracker Red (*siCTRL/siSMAD4/siALK1*) and exposed to LSS for 62h. 4. *siCTRL* mosaic (green + red); 5. *siCTRL* green+*siSMAD4* red; 6. *siCTRL* green+*siALK1* red.

Major Resources Table

Mouse Lines

Strain	Vendor or Source	Background Strain	Sex	Diet, housing&husbandry conditions	Persistent ID / URL
<i>Alk1^{ff}</i>	Dr. S.Paul Oh, Barrow Neurological Institute, Phoenix, Arizona	129/C57BL/6J	M &F	Mice were fed a standard laboratory diet using Inotiv Teklad 2018S Sterilizable Rodent diet. Mice were housed in the Yale Animal Resources Center (YARC) facilities under a standard 12-hour light/12-hour dark cycle (typically lights on at 7:00 AM and off at 7:00 PM, or reversed depending on the room) to support circadian rhythms. Housing conditions were maintained at 65–75°F with 40–60% humidity.	https://pubmed.ncbi.nlm.nih.gov/19805914/
<i>Smad4^{ff}</i>	JAX (Strain# 017462)	C57BL/6J	M &F		https://www.jax.org/strain/017462
<i>mTmG</i>	JAX (Strain# 007676)	C57BL/6J	M &F		https://www.jax.org/strain/007676#
<i>Cdh5 Cre^{ERT2}</i>	Dr. Ralf Adams, Max Planck Institute for Molecular Biomedicine, Münster, Germany	C57BL/6J	M &F		https://pubmed.ncbi.nlm.nih.gov/20445537/ Taconic Biosciences, Model #: 13073

Mouse Models Used in This Study

Strain	Background Strain	Sex	Breeding scheme
<i>Alk1^{ff} Cdh5 Cre^{ERT2} mTmG</i>	C57BL/6J	M&F	Generated with <i>Alk1^{ff}, Cdh5 Cre^{ERT2}, mTmG</i> F1 : <i>Cdh5 Cre^{ERT2} X mTmG</i> F2 : <i>Alk1^{ff} X Cdh5 Cre^{ERT2} mTmG</i> F3 : <i>Alk1^{ff} Cdh5 Cre^{ERT2} mTmG</i>
<i>Smad4^{ff} Cdh5 Cre^{ERT2} mTmG</i>	C57BL/6J	M&F	Generated with <i>Smad4^{ff}, Cdh5 Cre^{ERT2}, mTmG</i> F1 : <i>Cdh5 Cre^{ERT2} X mTmG</i> F2 : <i>Smad4^{ff} X Cdh5 Cre^{ERT2} mTmG</i> F3 : <i>Smad4^{ff} Cdh5 Cre^{ERT2} mTmG</i>
<i>Alk1^{ff} Cdh5 Cre^{ERT2}</i>	C57BL/6J	M&F	Generated with <i>Alk1^{ff}, Cdh5 Cre^{ERT2}</i> F1 : <i>Alk1^{ff} X Cdh5 Cre^{ERT2}</i> F2 : <i>Alk1^{ff} Cdh5 Cre^{ERT2}</i>
<i>Smad4^{ff} Cdh5 Cre^{ERT2}</i>	C57BL/6J	M&F	Generated with <i>Smad4^{ff}, Cdh5 Cre^{ERT2}</i> F1 : <i>Smad4^{ff} X Cdh5 Cre^{ERT2}</i> F2 : <i>Smad4^{ff} Cdh5 Cre^{ERT2}</i>
<i>Cdh5 Cre^{ERT2} mTmG</i>	C57BL/6J	M&F	Generated with <i>Cdh5 Cre^{ERT2}, mTmG</i> F1 : <i>Cdh5 Cre^{ERT2} X mTmG</i>

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Persistent ID / URL
<i>In vitro IF</i>				
VEcadherin	R&D Systems	AF938	1:1000	https://www.rndsystems.com/products/human-ve-cadherin-antibody_af938
GM130	BD Bioscience	610822	1:500	https://www.bdbiosciences.com/en-de/products/reagents/microscopy-imaging-reagents/immunofluorescence-reagents/purified-mouse-anti-gm130.610822?tab=product_details
pSMA D159	Cell Signaling	13820S	1:500	https://www.cellsignal.com/products/primary-antibodies/phospho-smad1-ser463-465-smad5-ser463-465-smad9-ser465-467-d5b10-rabbit-monoclonal-antibody/13820?srsId=AfmBOoo5FnI_TsfF7sJLPxdwfZukovTt954A_MldzwSKdSj-a84KTiy9
pSMA D23	Proteintech	80427-2-RR	1:500	https://www.ptglab.com/products/Phospho-SMAD2-Ser465-467-Antibody-80427-2-RR.htm
KLF4	Sigma Aldrich	HOA002926	1:500	https://www.sigmaaldrich.com/DE/en/product/sigma/hpa002926?srsId=AfmBOorL51iyJLpnhSoihzJTP1bfVGzBwqt0PIL98Ik519kZfO_ORoI
anti-goat IgG 568	Thermo fisher	A11057	1:400	https://www.thermofisher.com/antibody/product/Donkey-anti-Goat-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11057
anti-mouse IgG 488	Thermo fisher	A21202	1:400	https://www.thermofisher.com/antibody/product/Donkey-anti-Mouse-IgG-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-21202
anti-rabbit IgG 647	Thermo fisher	A31573	1:400	https://www.thermofisher.com/antibody/product/Donkey-anti-Rabbit-IgG-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-31573
<i>In vivo IF</i>				
Isolectin B4	Thermo fisher	132450	1:400	https://www.thermofisher.com/order/catalog/product/de/en/132450
GFP polyclonal antibody Alexa Fluor 488	Thermo fisher	A-21311	1:1000	https://www.thermofisher.com/antibody/product/GFP-Antibody-Polyclonal/A-21311
CD31	BD Bioscience	553370	1:200	https://www.bdbiosciences.com/en-dk/products/reagents/microscopy-imaging-reagents/immunohistochemistry-reagents/purified-rat-anti-mouse-cd31.553370?tab=product_details
Col IV	Bio-Rad	2150-1470	1:400	
Anti-rat Alexa Fluor 488	Thermo fisher	A21208	1:500	https://www.thermofisher.com/antibody/product/Donkey-anti-Rat-IgG-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-21208
Anti-rabbit Alexa Fluor 568	Donkey, Thermo fisher	A10042	1:500	https://www.thermofisher.com/antibody/product/Donkey-anti-Rabbit-IgG-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A10042

DOI [to be added]

WB				
SMAD 4	Cell Signaling	38454S	1:1000	https://www.cellsignal.com/products/primary-antibodies/smad4-d3m6u-rabbit-monoclonal-antibody/38454?site-search-type=Products&N=4294956287&Ntt=38454t&fromPage=plp&_requestid=186987
ALK1	Biosynth	70R-49334	1:500	https://www.biosynth.com/p/70R-49334/alk1-antibody
GAPDH	Cell Signaling	971669	1:1000	https://www.cellsignal.com/products/primary-antibodies/gapdh-d4c6r-mouse-mab/97166
ECL anti-mouse IgG HRP	GE Healthcare	NA931V	1:5000	https://www.cytivalifesciences.com/en/us/shop/protein-analysis/blotting-and-detection/blotting-standards-and-reagents/amersham-ecl-hrp-conjugated-antibodies-p-06260
anti-rabbit IgG HRP	Cell Signaling	7074S	1:5000	https://www.cellsignal.de/products/secondary-antibodies/anti-rabbit-igg-hrp-linked-antibody/7074

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)	Persistent ID / URL
HUVECs	Promocell	Mixed donor (M+F)	Cat.No. C-12203

Data & Code Availability

Description	Source / Repository	Persistent ID / URL
Raw RNAseq reads	GEO database	GSE282952
Python scripts	Github	https://github.com/gerhardt-lab/

Analysis and statistics files

Description	Persistent ID / URL
GO_BP: biological process for siSMAD4/vs_siALK1 in static, 4h and 16h if flow exposure The analysis was filtered out GO terms that had an AUC below 0.65, and padj values above 0.05	Data Set 1.xlsx
DEG tables filtered through Log2FC of either above 0.5 or below -0.5, and padj of 0.05	Data Set 2.xlsx
Statistical outputs for Fig. S1.1	01_statistics.xlsx
Statistical outputs for Fig. 2	02_statistics.xlsx
Statistical outputs for Fig. 3	03_statistics.xlsx
Statistical outputs for Fig. 4	04_statistics.xlsx
Statistical outputs for Fig. 5	05_statistics.xlsx
Statistical outputs for Fig. 6	06_statistics.xlsx

ARRIVE GUIDELINES

The ARRIVE guidelines (<https://arriveguidelines.org/>) are a checklist of recommendations to improve the reporting of research involving animals. Key elements of the study design should be included below to better enable readers to scrutinize the research adequately, evaluate its methodological rigor, and reproduce the methods or findings.

Study Design

Groups	Sex	Age	Number (prior to experiment)	Number (after termination)	Littermates (Yes/No)	Other description
Figure 5						
<i>Alk1^{f/f} Cdh5 Cre^{ERT2} mTmG</i>	Neonatal mice of both sexes were included without distinction	P8	5	5	No	Two retinas from each pup were used for analysis.
<i>Smad4^{f/f} Cdh5 Cre^{ERT2} mTmG</i>		P8	4	4	No	
<i>Cdh5 Cre^{ERT2} mTmG</i>		P8	4	4	No	
<i>Alk1^{f/f} Cdh5 Cre^{ERT2} mTmG</i>		P15	3	3	No	
<i>Smad4^{f/f} Cdh5 Cre^{ERT2} mTmG</i>		P15	7	7	No	
<i>Cdh5 Cre^{ERT2} mTmG</i>		P15	4	4	No	
Figure 6						
<i>Alk1^{f/f}</i>	Neonatal mice of both sexes were included without distinction	P5	4	4	Yes	Both retinas from each pup were used for analysis ; however, some were not used due to insufficient quality for reliable quantification
<i>Alk1^{f/f} Cdh5 Cre^{ERT2}</i>		P5	4	4	Yes	
<i>Smad4^{f/f}</i>		P5	5	5	Yes	
<i>Smad4^{f/f} Cdh5 Cre^{ERT2}</i>		P5	5	5	Yes	
<i>Alk1^{f/f}</i>		P6	4	4	Yes	
<i>Alk1^{f/f} Cdh5 Cre^{ERT2}</i>		P6	4	4	Yes	
<i>Smad4^{f/f}</i>		P6	3	3	Yes	
<i>Smad4^{f/f} Cdh5 Cre^{ERT2}</i>		P6	5	5	Yes	

Study Design:

Species, age, sex, strain, and sources of animals are described:

Yes

Sample Size:

Exact number of experimental units allocated to each group, the total number in each experiment, the total number of animals used described:

Yes

Inclusion/exclusion Criteria

For mosaic experiments several obtained images were not used for analysis due to insufficient quality for reliable quantification.

Randomization

Pups of several litters were obtained for each group.

Statistical Methods: details of the statistical methods used for each analysis, including software used are described:

Yes