Supplemental Material

Data Supplement Expanded Methods

Mice

Alk1$^{f/f}$ mice were kindly provided by Dr. S. Paul Oh. *Bmx Cre*^{ERT2} and *Esm1 Cre*^{ERT2} mice were kindly provided by Dr. Ralf Adams and *Mfsd2a Cre*^{ERT2} mice were kindly provided by Dr. Bin Zhou. Seven to eight weeks old *Alk1*$^{f/f}$ and *Bmx Cre*^{ERT2} *mTmG*, *Esm1 Cre*^{ERT2} *mTmG* mice or *Mfsd2a Cre*^{ERT2} *mTmG* mixed genetic background were intercrossed for experiments and *Alk1*$^{f/f}$ *Mfsd2a Cre*^{ERT2} *(mTmG)*, *Alk1*$^{f/f}$ *Esm1 Cre*^{ERT2} *(mTmG)* mice or *Alk1*$^{f/f}$ *Bmx Cre*^{ERT2} *(mTmG)* were used. Gene deletion was induced by intra-gastric injections with 100 µg Tx (Sigma, T5648; 2.5 mg ml^{-1}) into pups at P4 or P1-3. Tx-injected *Cre*^{ERT2} negative littermates were used as controls.

Latex dye injection

P6 pups were anaesthetized on ice, and abdominal and thoracic cavities were opened. The right atrium was cut, blood was washed out with 2 ml PBS and 1 ml of latex dye was slowly and steadily injected into the left ventricle with an insulin syringe. Retinas and GI tracts were washed in PBS and fixed with 4% paraformaldehyde (PFA) overnight. Brains and GI tracts were cleared in Benzyl Alcohol : Benzyl Benzonate (1:1) for 2-3 days before imaging.

Reagents and antibodies

For immunostaining: IB4 ([IsolectinB4] #I21412, 10 µg/mL; Life Technologies), GFP Polyclonal Antibody, Alexa Fluor 488 (#A-21311, 1:1000; Invitrogen), GOLPH4 (#ab28049, 1:400; abcam), YAP (#14074, 1:300; Cell Signaling), TAZ (#HPA007415, 1:300 Sigma), mouse ALK1 (#AF770, 1:300; R&D) human ALK1(#AF370, 1:300; R&D),

VE-Cadherin (#555289, 1:200; BD) GM-130 (#610822, 1:500; BD), cMYC (SC-40 1:200), DAPI (#D1306, 1:1000; Life Technologies), integrin β1 Alexa Fluor 647 (#303017,1:500; BioLegend), integrin α5 and αv [84].

For western blotting: ALK1 (7R-49334, 1:1000; Fitzgerald), integrin β1 (#34971, Cell Signaling), integrin α5 and αv, VEGFR2 (#9698, Cell Signaling), β-ACTIN (#A1978 1:3000; Sigma), YAP (#14074, 1:1000; Cell Signaling), TAZ (#HPA007415, 1:2000 Sigma), cMYC (#9402, 1:2000, Cell Signaling), Phospho-PI3K (#4228, 1:1000, Cell Signaling), PI3K (#06-195, 1:1000, Millipore), Phospho-AKT (#4060, 1:1000, Cell Signaling), AKT (#9272, 1:1000, Cell Signaling).

Appropriate secondary antibodies were fluorescently labeled (Alexa Fluor donkey anti-rabbit, Alexa Fluor donkey anti-goat) or conjugated to horseradish peroxidase (anti-rabbit and anti-mouse IgG [H+L], 1:8.000; Vector Laboratories).

ATN-161 (#S8454, Selleckchem), cilengitide trifluoroacetate (#S7077, Selleckchem), verteporfin (#S1786, Selleckchem), wortmannin (#S2758, Selleckchem). Full-length human *ALK1* was subcloned into pEGFP-N2 vector for fusing EGFP to the C-terminal of ALK1.

Cell culture and siRNA transfection

Human umbilical vein endothelial cells (HUVECs) were obtained from the Yale University Vascular Biology and Therapeutics Core Facility and cultured in EGM2-Bullet kit medium (CC-3156 & CC-4176, Lonza). Mouse primary brain microvascular endothelial cells were purchase from Cell Biologics (Cat # C57-6023) and cultured according to the manual. Depletion of *ALK1*, *SMAD4* or *ENG* was achieved by transfecting 20 pmol of small

interfering RNA (siRNA) against *ALK1* (Qiagen, mixture of 2 siRNAs: SI02659972 and SI02758392), *SMAD4* (Dharmacon, SMARTpool: ON-TARGETplus L-003902-00-0005) *ENG* (Dharmacon, ON-TARGETplus L-011026-00-0005), *VEGFR2* (Dharmacon, SMARTpool: ON-TARGETplus L-003148-00-0005) or ITGB1 (Dharmacon, SMARTpool: ON-TARGETplus L-004506-00-0005) using Lipofectamine RNAiMax (Invitrogen). Transfection efficiency was assessed by western blotting and quantitative qPCR. *VEGFR2* and/or *ITGB1* siRNAs were transfected 24 hours (h) after *ALK1* siRNA transfection. Experiments were performed 60-72 h post-transfection and results were compared with siRNA CTRL (ON-TARGETplus Non-Targeting Pool D-001810-10-05).

Immunostaining

For angiogenesis studies the eyes of P6/P8 pups were prefixed in 4% PFA for 8 min at room temperature. Retinas were dissected, blocked for 30 min at room temperature in blocking buffer (1% fetal bovine serum, 3% BSA, 0.5% Triton X-100, 0.01% Na deoxycholate, 0.02% Sodium Azide in PBS at pH 7.4) and then incubated with specific antibodies in blocking buffer overnight at 4°C. The next day, retinas were washed and incubated with IB4 together with the corresponding secondary antibody overnight at 4°C, then washed and post-fixed with 0.1% PFA and mounted in fluorescent mounting medium (DAKO, USA). High-resolution pictures were acquired using ZEISS LSM800 and Leica SP8 confocal microscope with a Leica spectral detection system (Leica TCS SP8 detector), and the Leica application suite advanced fluorescence software. Quantification of retinal vasculature was done using ImageJ and then Prism 7 software for statistical analysis.

For cell immunostaining, cells were plated on gelatin coated dishes. Growing cells were fixed for 10 min with 4% paraformaldehyde (PFA) and permeabilized with 0.1% Triton X-100 for 10 min prior to overnight incubation with primary antibody and then secondary antibody conjugated with fluorophore.

Shear stress experiments.

HUVECs were re-plated on glass slides coated with the indicated proteins for 6 h and wound scratch was carried out on the slides before application of flow. The slides were loaded into parallel plate flow chambers. Laminar shear at 15 dynes/cm^2 was used to mimic high flow in retinal veins.

For real-time imaging, PH-AKT-mClover3 was modified from PH-AKT-GFP (addgene #51465). Plasma membrane targeting sequence of LCK tagged with mRuby3 (LCK-mRuby3, modified from addgene #98822) was co-expressed in the same vector by IRES sequence as a plasma membrane marker. HUVECs were transfected with siRNAs followed by lentiviral transduction coding PH-AKT-mClover3 and LCK-mRuby3. The infected cells were mixed with uninfected cells in 1:2 ratio, then seeded on microfluidic chamber (IBIDI u-slide 0.4 luer, 1x10^5 total cell/slide) and cultured additional 24-48 h more for imaging. Imaging was performed on an Eclipse Ti microscope equipped with an Ultraview Vox spinning disk confocal imaging system, with 20X objective (Plan Apo, Nikon). Each pixel intensity was plotted as y with corresponding distance from upstream of the cell as x, which was normalized to have length 1 to the direction of flow (0 to 1). Then slope of the plot at each time frame was measured as a representative value of cell polarity.

Western blotting

Cells were lysed with Laemmli sample buffer including phosphatase and protease inhibitors (Thermo Scientific, 78420, 1862209). 20 µg of proteins were separated on 4% to 15% Criterion precast gels (567–1084, Biorad) and transferred on 0.23 um nitrocellulose membranes (Biorad). Western blots were developed with chemiluminescence horseradish peroxidase substrate (Millipore, WBKLS0500) on a Luminescent image Analyzer, ChemiDoc XRS+ imaging system (Biorad). Bands were quantified using ImageJ.

Immunoprecipitation

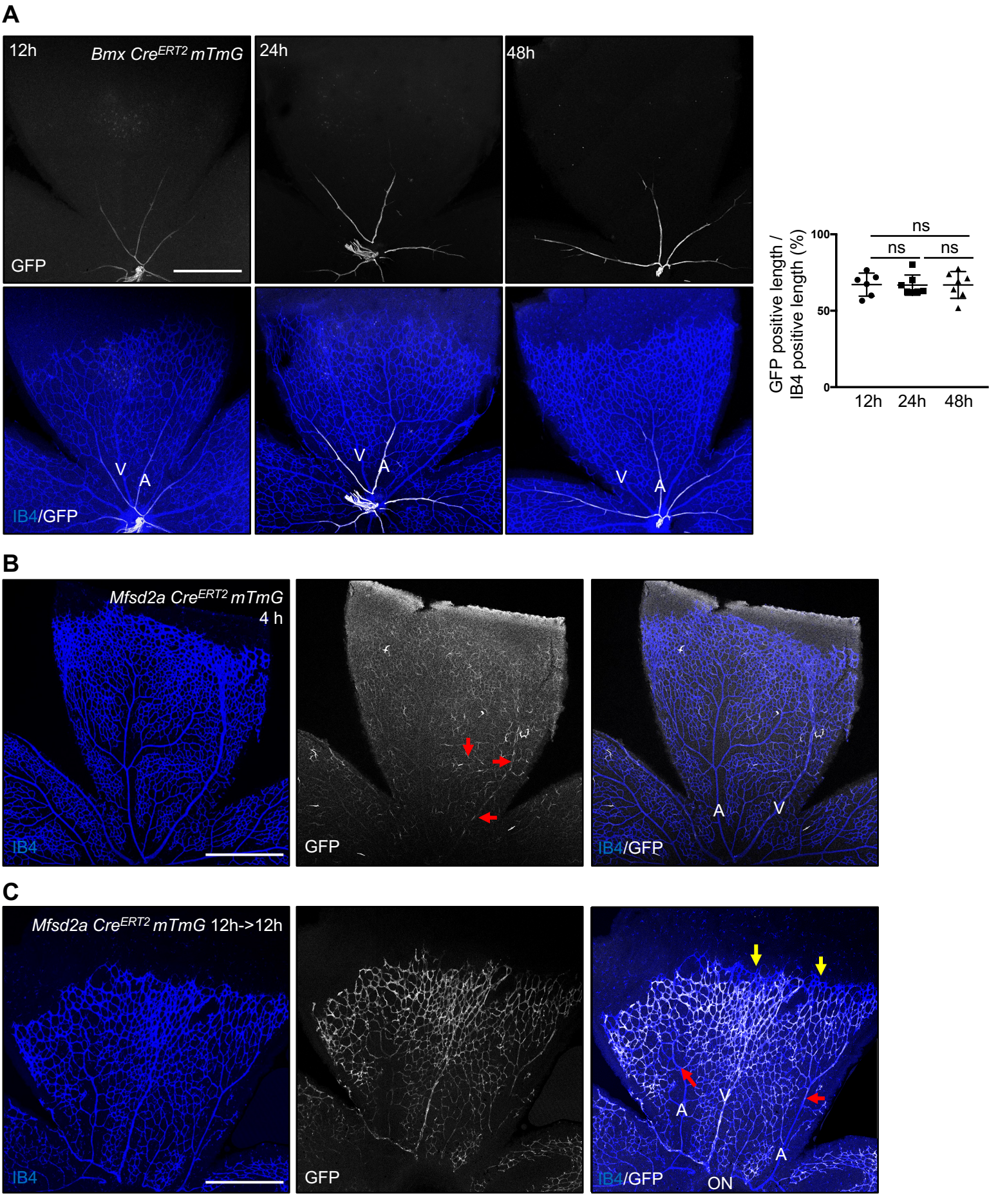
Cell lysates were prepared in 50 mM Tris-HCl at pH 7.4, 50 mM NaCl, 0.5% Triton X-100, phosphatase and protease inhibitors, centrifuged at 16,000 × *g* for 20 min. Protein concentration was quantified using BCA assay (Pierce). In total, 500 µg of protein from cell lysate were incubated overnight at 4 °C with 10 µg/ml of anti-VEGFR2, and finally incubated with protein A/G magnetic beads (88802, Thermo Scientific) for 2 h at 4 °C. The immunocomplexes were washed three times in lysis buffer and resuspended in 1X Laemmli sample buffer. For western-blot analysis, 50 µg of protein was loaded for each condition.

Polarity index calculation

Briefly, after segmenting each channel corresponding to the Golgi and nuclear staining, the centroid of each organelle was determined and a vector connecting the center of the nucleus to the center of its corresponding Golgi apparatus was drawn. The Golgi-nucleus

assignment was done automatically minimizing the distance between all the possible couples. The polarity of each cell was defined as the angle between the vector and the scratch line. An angular histogram showing the angle distribution was then generated. Circular statistic was performed using the Circular Statistic Toolbox. To test for circular uniformity, we applied the polarity index (PI), calculated as the length of mean resultant vector for a given angular distribution[29]. (N = number of cells, α = angle)

$$\text{Polarity Index} = \sqrt{\left(\frac{1}{N}\sum_{1}^{N}\cos\alpha\right)^2 + \left(\frac{1}{N}\sum_{1}^{N}\sin\alpha\right)^2}$$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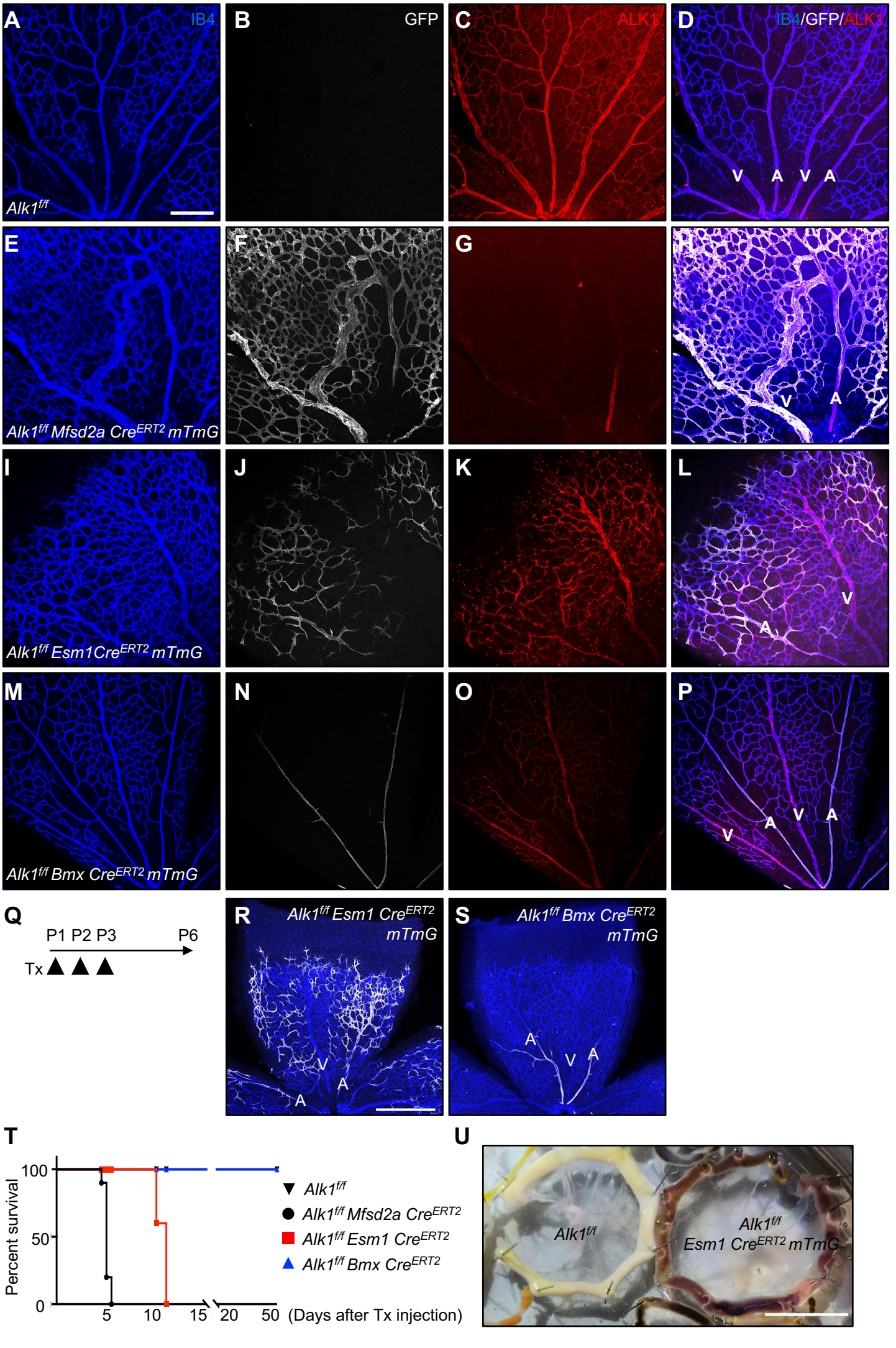
12h
Bmx CreERT2 mTmG
24h
48h
GFP
IB4/GFP
V
A
GFP positive length / IB4 positive length (%)
100
50
0
ns
ns
ns
12h
24h
48h
B
Mfsd2a CreERT2 mTmG
4 h
IB4
GFP
IB4/GFP
C
Mfsd2a CreERT2 mTmG 12h->12h
ON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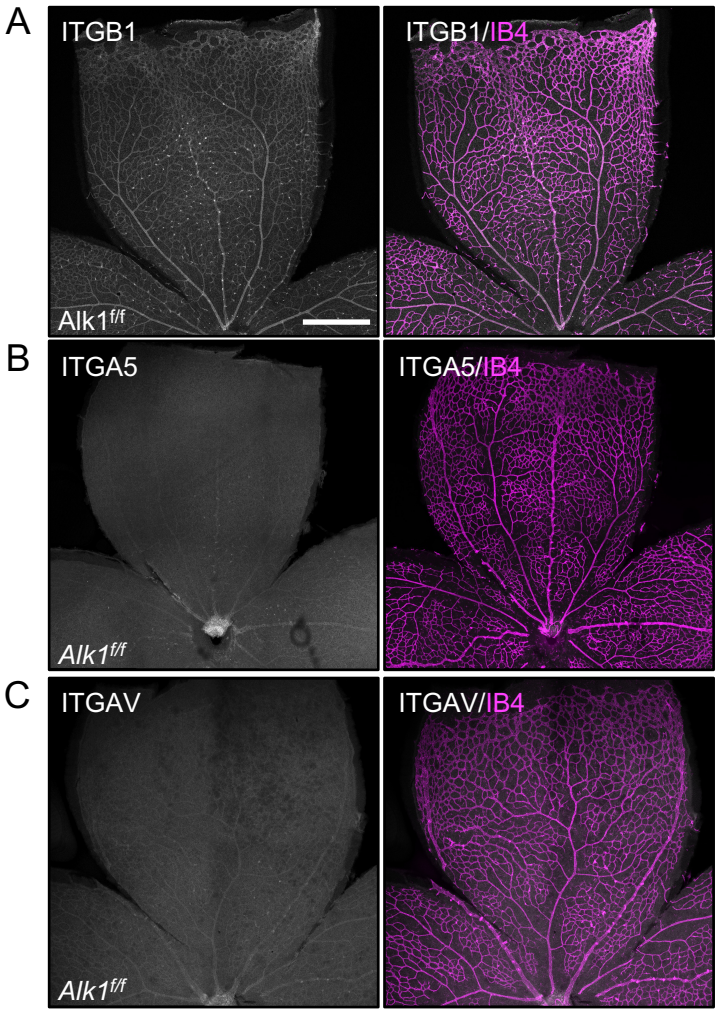
IB4
Alk1f/f
B
GFP
C
ALK1
D
IB4/GFP/ALK1
V A V A
E
Alk1f/f Mfsd2a CreERT2 mTmG
F
G
H
V A
I
Alk1f/f Esm1CreERT2 mTmG
J
K
L
V
A
M
Alk1f/f Bmx CreERT2 mTmG
N
O
P
A V A
V
Q
P1 P2 P3
P6
Tx
R
Alk1f/f Esm1 CreERT2
mTmG
V
A
A
S
Alk1f/f Bmx CreERT2
mTmG
A
V A
T
100
50
0
Percent survival
5
10
15
20
50
(Days after Tx injection)
Alk1f/f
Alk1f/f Mfsd2a CreERT2
Alk1f/f Esm1 CreERT2
Alk1f/f Bmx CreERT2
U
Alk1f/f
Alk1f/f
Esm1 CreERT2 mTmG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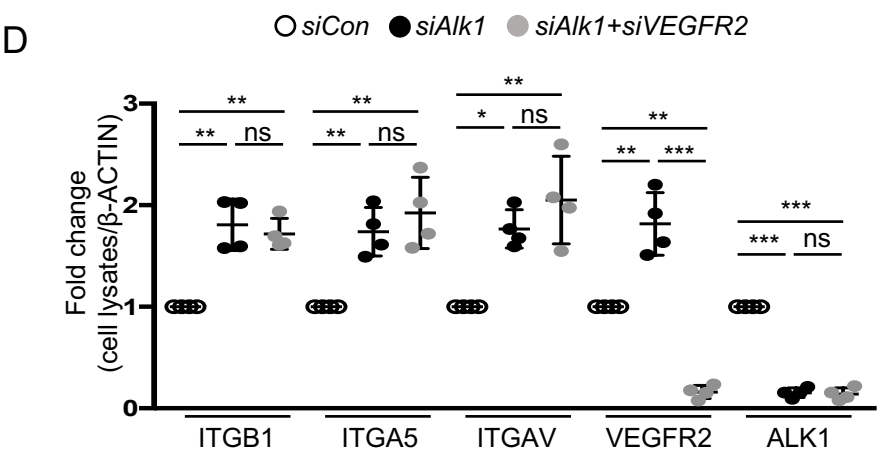
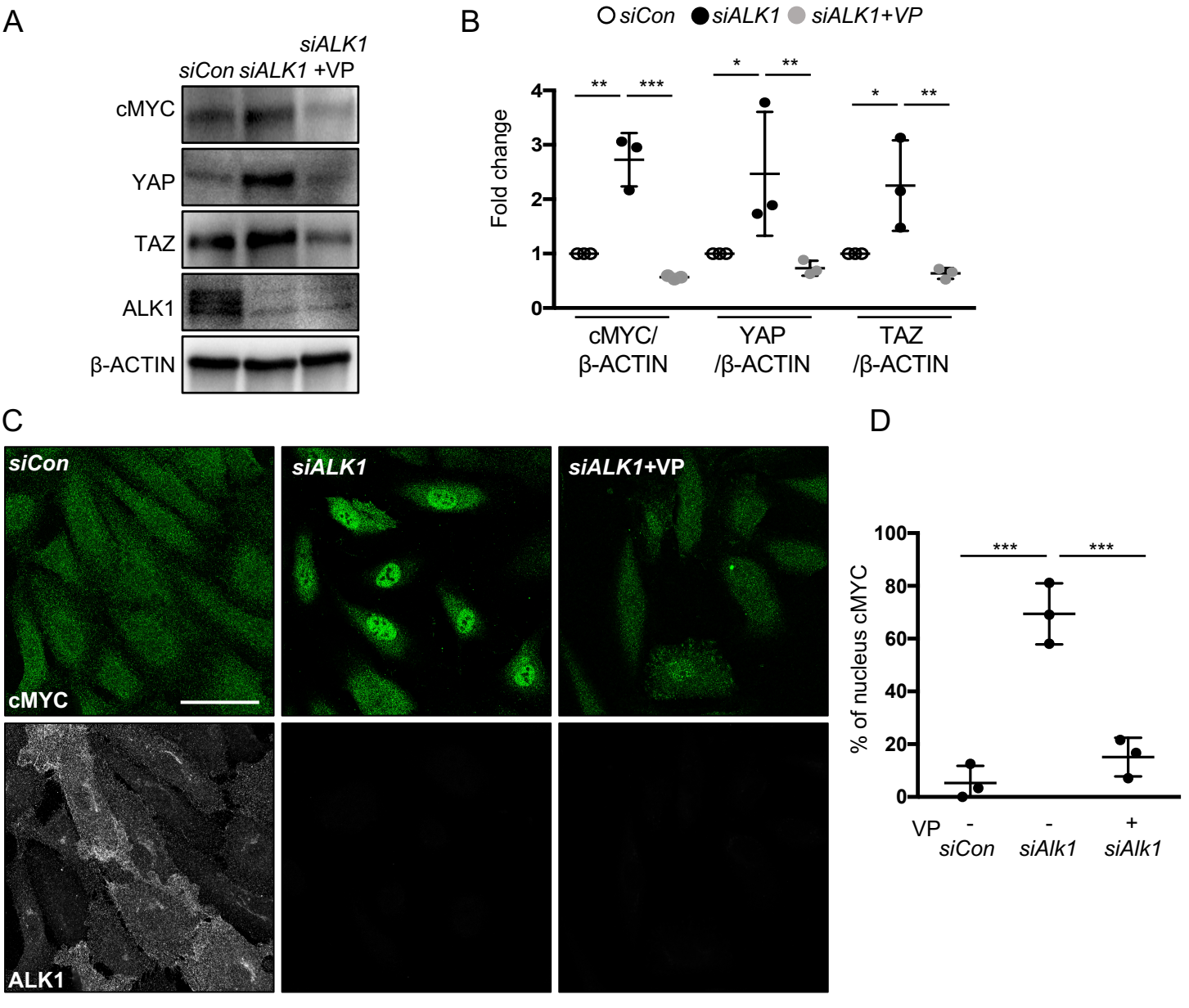
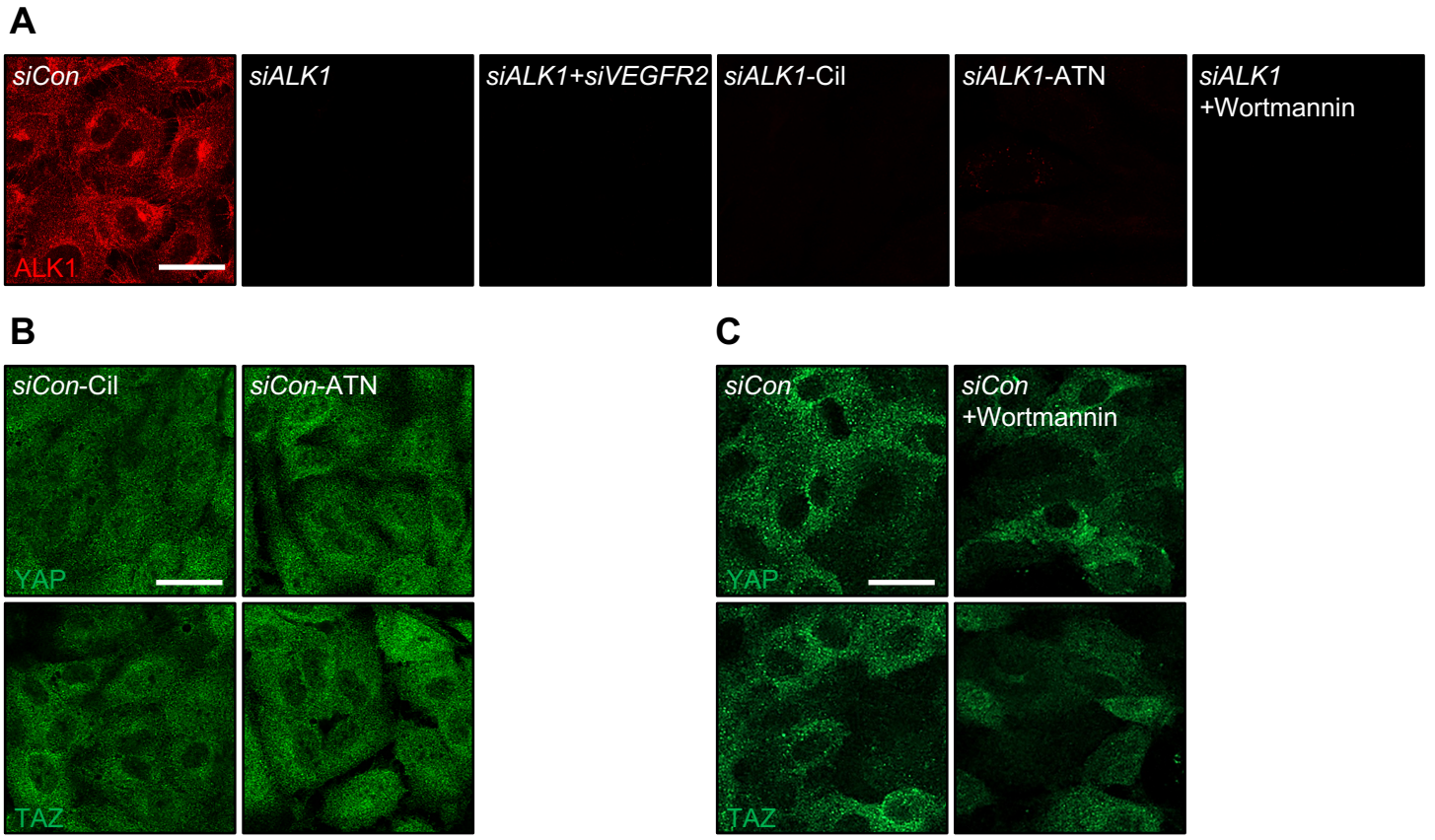
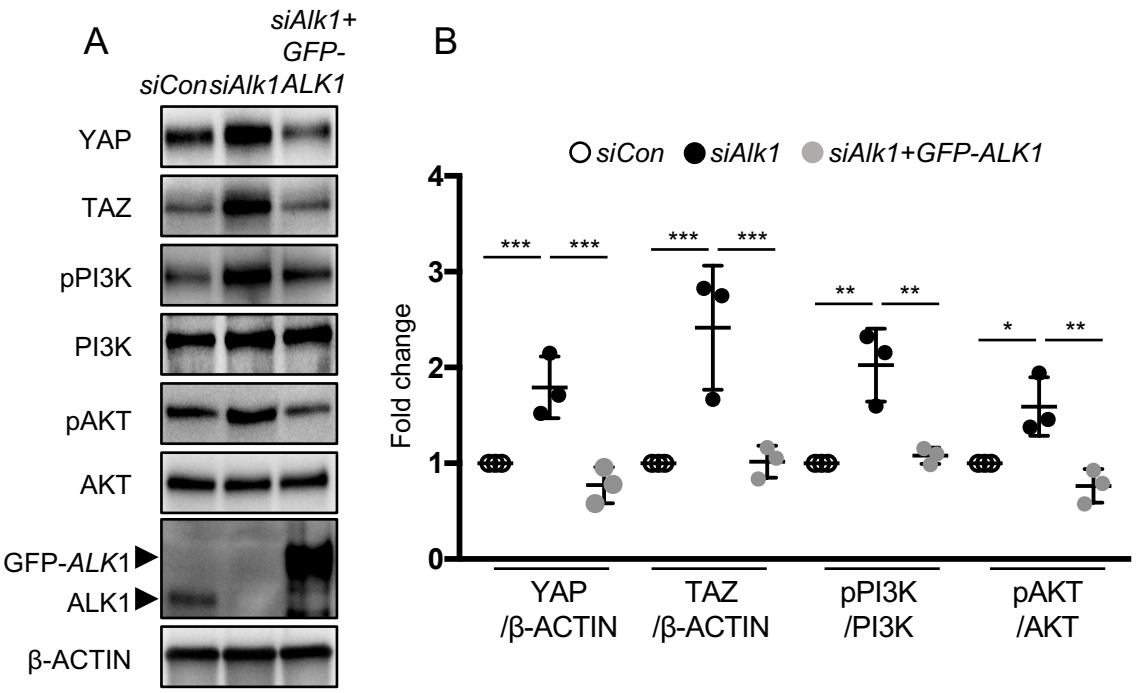
ITGB1
ITGB1/IB4
Alk1f/f
B
ITGA5
ITGA5/IB4
Alk1f/f
C
ITGAV
ITGAV/IB4
Alk1f/f


D
siCon
siAlk1
siAlk1+siVEGFR2
Fold change (cell lysates/β-ACTIN)
0
1
2
3
**
ns
*
***
ITGB1
ITGA5
ITGAV
VEGFR2
ALK1


A
siCon siALK1 siALK1 +VP
cMYC
YAP
TAZ
ALK1
β-ACTIN
B
siCon siALK1 siALK1+VP
Fold change
cMYC/ β-ACTIN
YAP /β-ACTIN
TAZ /β-ACTIN
C
siCon
siALK1
siALK1+VP
cMYC
ALK1
D
% of nucleus cMYC
VP
siCon
siAlk1
siAlk1


A
siCon
ALK1
siALK1
siALK1+siVEGFR2
siALK1-Cil
siALK1-ATN
siALK1
+Wortmannin
B
siCon-Cil
siCon-ATN
YAP
TAZ
C
siCon
siCon
+Wortmannin
YAP
TAZ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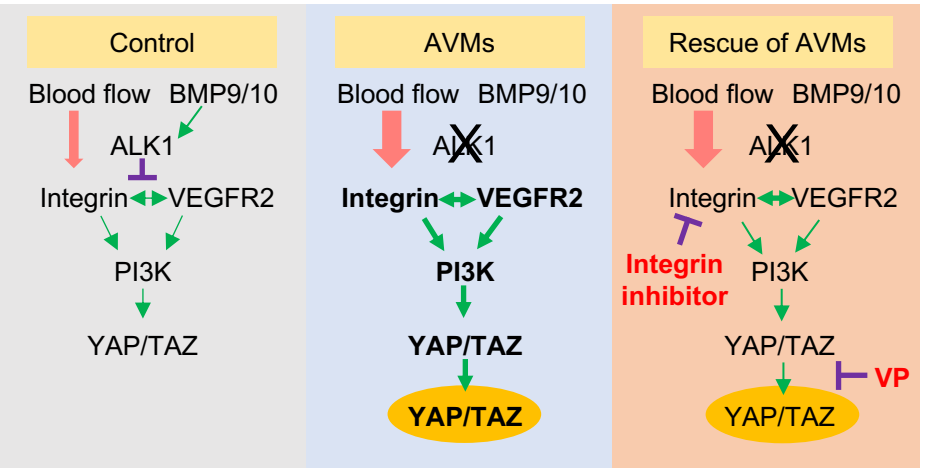
siCon
siAlk1
siAlk1+ GFP-ALK1
YAP
TAZ
pPI3K
PI3K
pAKT
AKT
GFP-ALK1
ALK1
β-ACTIN
B
siCon
siAlk1
siAlk1+GFP-ALK1
Fold change
0
1
2
3
4
***
***
***
***
**
**
*
**
YAP /β-ACTIN
TAZ /β-ACTIN
pPI3K /PI3K
pAKT /AKT


Control
Blood flow
BMP9/10
ALK1
Integrin
VEGFR2
PI3K
YAP/TAZ
AVMs
Blood flow
BMP9/10
ALK1
Integrin
VEGFR2
PI3K
YAP/TAZ
YAP/TAZ
Rescue of AVMs
Blood flow
BMP9/10
ALK1
Integrin
VEGFR2
Integrin inhibitor
PI3K
YAP/TAZ
VP
YAP/TAZ

Data Supplement Figure legends I – VIII

Supplemental Figure I. *Mfsd2a* positive cells migrate against the direction of blood flow.

(A) *Bmx Cre^{ERT2}mTmG* mice injected with 100μg Tx at P5.5 (for 12h), P5 (for 24 h) and P4 (for 48 h) and dissected at P6. GFP expressing ECs are located in proximal arteries. Quantification of *Bmx Cre^{ERT2} mTmG* GFP expressing vessel length over IB4 positive vessel length from optic nerve. n = 6-8 retinas per time point. ns: nonsignificant, One-way ANOVA with Sidak's multiple comparisons test. (B) *Mfsd2a Cre^{ERT2} mTmG* mice injected with 100 μg Tx at P6 and dissected after 4 h. GFP expressing ECs are located in capillaries and veins (red arrows) but not in arteries. (C) P6 retina flat mount images labeled with IB4 (blue) and GFP (white) from *Mfsd2a Cre^{ERT2} mTmG* mice injected with 100 μg Tx at P5, dissected after 12h (P5.5) and cultured for an additional 12 h in vitro (P6). Yellow arrows indicate tip cells and red arrows indicate location of GFP-expressing ECs in arteries. ON: optic nerve, V: vein, A: artery, Scale bar: 500 μm

Supplemental Figure II. Genetic deletion of *Alk1* in *Mfsd2a*, *Esm1* and *Bmx Cre^{ERT2} mTmG* retinas.

(A-P) 100 μg Tx was injected intragastrically at P4 in *Alk1$^{f/f}$*, *Alk1$^{f/f}$ Mfsd2a Cre^{ERT2}*, *Alk1$^{f/f}$ Esm1 Cre^{ERT2}* and *Alk1$^{f/f}$ Bmx Cre^{ERT2} mTmG* pups, and retinas were dissected at P6. IB4 (blue), GFP (white) and ALK1 (red) staining of retinal flat mounts. GFP and ALK1 staining shows non-overlapping expression. V: vein, A: artery, (Q) Schematic representation of the experimental strategy used to delete *Alk1* in mice. Arrowheads indicate injection of 100 μg Tx at P1, P2 and P3 in *Alk1$^{f/f}$ Esm1* and *Bmx Cre^{ERT2} mTmG*

pups. (R and S) IB4 (blue) and GFP (white) staining of retinal flat mount from *Alk1$^{f/f}$ Esm1 Cre^{ERT2} mTmG* (R) and *Alk1$^{f/f}$ Bmx Cre^{ERT2} mTmG* (S). (T) Survival curves for *Alk1$^{f/f}$ Mfsd2aCre^{ERT2}*, *Alk1$^{f/f}$ Esm1Cre^{ERT2}*, *Alk1$^{f/f}$ BmxCre^{ERT2}* and *Alk1$^{f/f}$* mice injected with 100 μg Tx at P4. n = 8-10 mice/group. ****P<0.001, Log-rank (Mantel-Cox) test. (U) Freshly dissected small intestines from P14 mice with the indicated genotypes after 100 μg Tx injection at P4. *Alk1$^{f/f}$ Esm1 Cre^{ERT2} mTmG* mice displayed intestinal hemorrhage. Scale bars : 200 μm (A-P), 500 μm (R-S), 1 cm (U)

Supplemental Figure III. Integrin staining of *Alk1$^{f/f}$* retinas.

(A-C) IB4 (Magenta) and ITGB1(A, white), ITGA5 (B, white) or ITGAV (C, white) staining of retinal flat mounts from P8 *Alk1$^{f/f}$* pups. A scale bar : 500 μm (A-C) (D) Quantification of ITGB1, ITGA5, ITGAV, VEGFR2 or ALK1 levels normalized to β-ACTIN. *P<0.05, **P<0.01, ***P<0.001,ns : nonsignificant, One-way ANOVA with Sidak's multiple comparison test.

Supplemental Figure IV. VP blocks YAP/TAZ activity in *ALK1* deleted HUVECs.

(A) Western blot analysis of HUVECs transfected with control or *ALK1* siRNAs with DMSO or VP (1 μM) for 24 h. (B) Quantification of cMYC, YAP or TAZ levels normalized to β-ACTIN. *P<0.05, **P<0.01, ***P<0.001, One-way ANOVA with Sidak's multiple comparisons test. (C) cMYC staining for control, *ALK1* siRNAs transfected HUVECs treated with DMSO or VP (1 μM) for 24 h. Nuclear cMYC localization in *siALK1* ECs is blocked by VP treatment. (D) Quantification of nuclear cMYC. ***P<0.001, Two-way ANOVA with Tukey's multiple comparisons test. A scale bar : 50 μm

Supplemental Figure V. PI3K acts upstream of YAP/TAZ.

(A) ALK1 staining for *siCon*, *siALK1*, si ALK1+siVEGFR2, siALK1+Cil, siALK1+ATN and siALK1+Wortmannin treated HUVECs. (B) YAP and TAZ staining for control siRNAs transfected HUVECs treated with Cilengitide (Cil, 5 μM) or ATN161 (ATN, 5 μM) for 12 h. (C) YAP and TAZ staining for control siRNAs transfected HUVECs treated with PBS, Wortmannin (100 nM) for 12 h. Scale bars: 50 μm

Supplemental Figure VI. ALK1 regulates PI3K/AKT-YAP/TAZ signaling.

(A) Western blot analysis of mouse brain ECs transfected with control or *Alk1* siRNAs with GFP-*ALK1*. (B) Quantification of YAP/TAZ levels normalized to β-ACTIN and pPI3K/PI3K and pAKT/AKT. *P<0.05, **P<0.01, ***P<0.001, One-way ANOVA with Sidak's multiple comparisons test.

Supplemental Figure VII. Model for ALK1-integrin-VEGFR2 signaling.

A model for ALK1-integrin-YAP/TAZ signaling in maintenance of vascular quiescence. In quiescence, ALK1 signaling represses PI3K activation downstream of integrin-VEGFR2 signaling, thereby inhibiting YAP/TAZ nuclear localization. ALK1 deletion results in increased integrin-VEGFR2-PI3K signaling, and excessive YAP/TAZ expression and localization to the nucleus, thereby inducing vascular defects. Blocking integrin-ECM interaction with integrin inhibitors or YAP/TAZ localization with YAP/TAZ inhibitor rescues vascular malformations in *Alk1* deficient mice.

Data supplement Movies

Supplemental Movie I. ALK1 regulates EC polarization against the direction of blood flow.

Movies of *siCon* or *siALK1* HUVECs stably transduced with PH-AKT-mClover3 and plasma membrane targeting sequence of LCK-mRuby3. HUVEC monolayers in microfluidic chambers were exposed to 12 dynes/cm^2 LSS under microscope. 0 – 5 min is static condition and flow starts after 5 min. Color intensity indicates AKT-PH sensor.