Supplemental Figure 1.

Reduced *RASA1* mRNA leads to excessive angiogenesis in mouse retina.

(**A-F**) Representative c

onfocal fluorescence micrographs showing projected images of vessels stained with Alexa-594-isolectin in whole-mount retina of a control siRNA (**A**-**C**) or RASA1 siRNA (**D**-**F**) transfected eyes. Control or RASA1 siRNA was intravitreally injected into the right or left eye, respectively, at p6 (C57BL/6). Higher magnification of peripheral region (white boxes) and middle region (yellow boxes) are also shown in (**B** and **E**) or (**C** and **F**), respectively. Scale bars, 600 μm (**A** and **D**), 150 μm (**B**-**F**). (**G**) RT-PCR indicate mouse *RASA1* mRNA levels in each retina (normalized to mRNA levels in retina injected with control siRNA; n=6 per condition, mean ± SEM; *p<0.05). (**H**) Graph showing percentage of vascularized area in the middle or peripheral regions of retinae (n=5 per condition, mean ± SEM; **p<0.01).

Supplemental Figure 2.

RASA1 is structurally and functionally conserved between zebrafish and human. (**A**) Alignment of predicted amino acid sequences for human RASA1 and zebrafish RASA1a and RASA1b (82% and 76% identical to human RASA1, respectively) were conducted using ClustalW and Boxshade programs. Residues in black are identical, residues in gray indicate conserved amino acids. RASA1,

human RASA1; rasa1a and rasa1b, zebrafish RASA1. (**B**) Whole mount *in situ* staining shows the diffused, and non-vascular-specific expression of *RASA1a* and *RASA1b* at 18 somite, 24 hpf and 48 hpf. *VEGFR2* staining shows the vascular-specific staining. (**C**) RT-PCR analysis showed efficient knockdown of *RASA1a* and *RASA1b* transcripts by splice morpholinos. wt: control sample; MO: splice morpholino injected sample.

Supplemental Figure 3.

Efficient knockdown of *EphB4a* and *ephrin-B2a* by morpholinos.

Validation of EphB4a and ephrin-B2a (efnb2a) morpholinos (MOs). (**A** and **B**) EphB4a morphant at 48 hpf generated by a splice MO (250 µM; **A**), confirmed phenotype from AUG-targeted MO (higher magnification of boxed region shown in **B**). Scale bars, 500 µm (**A**), 100 µm (**B**). (**C**) Western blot analysis for EphB4a AUG MO knockdown efficiency (compare lanes 1 and 2). (**D**) RT-PCR analysis showed efficient knockdown by the EphB4a splice MO. wt: control sample; MO: splice MO injected sample. (**E**) Western blot analysis for ephrin-B2a AUG MO knockdown efficiency (compare lanes 1 and 2). Non-radioactive in vitro transcription-translation system was used for Western blots (**C** and **E**; also see **Methods**). PCR product with or without MO target site (w/ MO Target or No MO Target, respectively), was incubated with or without MO (MO or control, respectively).

S**upplemental Figure 4.**

Predicted RASA1 binding sites on EphB4a are necessary for efficient rescue of EphB4a morphants.

EphB4a morphant rescue experiment with EphB4a wildtype or mutant mRNA. Usage of a double transgenic line (32, 33) facilitated visualization of endothelial (green; **A**, **C**, **E**) and blood cells (red; **B**, **D**, **F**) in the same embryo at 48 hpf. All mRNAs were injected at 30 ng/µl. (**A** and **B**) Co-injection of EphB4a MO with wildtype *EphB4a* mRNA rescued caudal vessel structure and function. (**C**-**F**) Co-injection with each mutant EphB4a receptor (EphB4a mRNA lacking two potential RASA1 binding sites ($EphB4a_{FF}$; **C** and **D**), or EphB4a mRNA lacking three potential RASA1 binding sites ($EphB4a_{EE+Grb2}$; **E** and **F**)) was unable to rescue the EphB4a morphant caudal vascular malformation.

Supplemental Figure 5.

Schematic diagram of arterial or venous intersegmental vessel formation.

(**A** and **B**) The formation of arterial or venous intersegmental vessels (IS Artery or IS Vein, respectively) occurs by interaction of secondary venous sprout (2°sprout) with the primary intersegmental vessel (1°segment) (**A**): no connection (IS Artery in red) or connection (IS Vein in blue). However, under reduced EphB4 or RASA1 function, more veins were formed (**B**).

Supplemental Figure 6.

mTORC1 inhibitor restores caudal blood flow in EphB4a and RASA1a morphants.

The caudal functional assay was performed on embryos co-injected with EphB4a and ephrin-B2 morpholinos (dMO) or on RASA1a morphants at 72 hpf, after 48 h treatment with rapamycin (Rapa; 400 nM). The double transgenic line (32, 33), was used to facilitate visualization of endothelial (green; **A**, **C**, **E**, **G**, **I**) and blood cells (red; **B**, **D**, **F**, **H**, **J**). (**A** and **B**) Control embryo shows proper vascular architecture and blood flow. (**C**-**J**) Treatment with Rapamycin restored caudal blood flow by 72 hpf in dMO (**C**-**F**) or in RASA1a MO (**G**-**J**). See also **Supplemental Figure 7**.

Supplemental Figure 7.

PI3K-mTORC1 pathway inhibitors significantly restore the proportion of EphB4a and RASA1a morphants with normal caudal blood flow.

(**A**-**D**) The caudal functional assay was performed on embryos co-injected with EphB4a and ephrin-B2 morpholinos (dMO; **A** and **B**) or on RASA1a morphants (**C** and **D**) at 72 hpf, after 48 h treatment with rapamycin (Rapa; 400 nM), GDC0941 (GDC; 250 nM), or BEZ235 (BEZ; 250 nM). dMO or RASA1a morphant phenotypes at 48 hpf (severe, mild, or normal) converted into percentages (**A** and **C**) or percentages were normalized against dMO or RASA1a morphant to show the percentage increase in embryos with normal blood flow in comparison with dMO or RASA1a morphant (**B** and **D**). Data are shown as mean ± SEM from three independent experiments (n=100 per condition; *p<0.05, **p<0.01, ***p<0.001).

Supplemental Figure 8.

Generation of the *Tg(fli1:RhebS16H)* transgenic line to study the consequence of active mTORC1 signaling in endothelial cells.

(**A** and **B**) Arteriovenous connection assay was performed at 48 hpf on embryos injected with *RhebS16H* mRNA. A counting region containing eight ISVs (from mid-trunk to end of yolk extension) was used for each embryo at 48 hpf (n=20 per condition). *RhebS16H* mRNA injection generated more IS veins (**A**), similar to the phenotype observed in EphB4a and RASA1 morphants. Arteriovenous intersegmental vessel proportion in *RhebS16H* mRNA injected embryo was normalized by rapamycin treatment (400 nM; **B**), suggesting the mTORC1 role for proper arteriovenous connection. (**C**-**F**) Embryos were examined by incrossing F_1 *Tg(fli1:RhebS16H)* line. Those lacking EGFP in their hearts are wildtype siblings of the *Tg(fli1:RhebS16H)* line, showing normal vasculature (**C**; higher magnification of boxed region shown in **D**). However, those with positive *cmlc2:EGFP* (which indicates that these embryos also carry the *RhebS16H* transgene) showed a mild vascular phenotype in the caudal plexus (**E**; higher magnification of boxed region shown in **F**). Experiments for this paper were conducted using F_3 and F_4 embryos. (**G**) *Tg(fli1:RhebS16H)* line was generated using Tol2 kit. Briefly, transposase mRNA was injected with a transposon donor plasmid containing Tol2 construct with a *fli1* promoter and the gene encoding *RhebS16H* into a 1-cell stage zebrafish embryo. The Tol2 construct was excised from the donor plasmid, before integration into the genomic DNA.

Supplemental Figure 9.

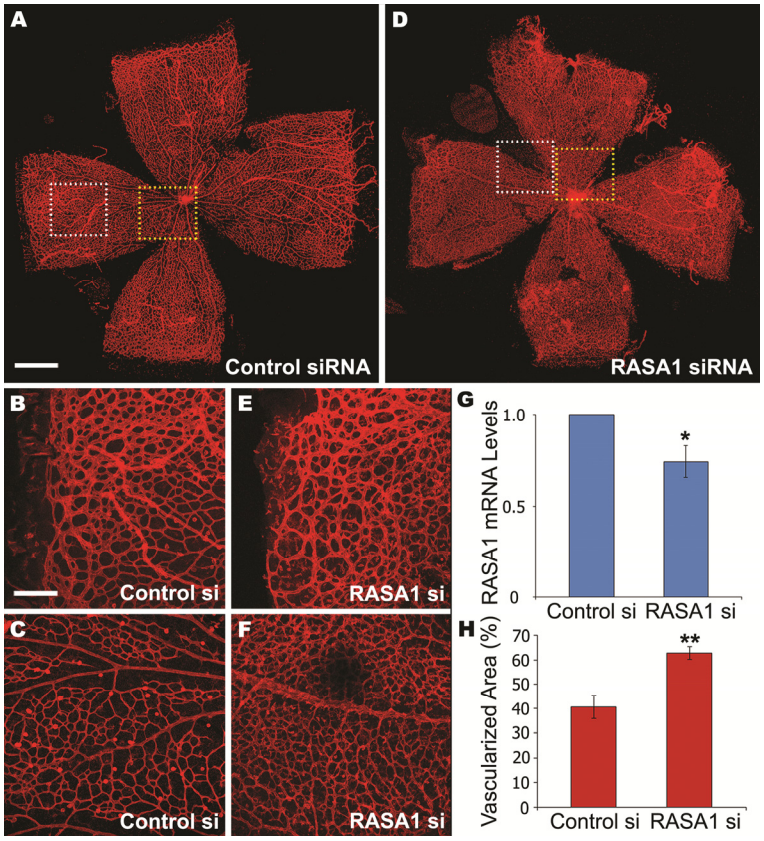
Endothelial mTORC1 activation is unique in vascular anomalies. Representative images from immunohistochemical staining for CD31 (endothelial cells), p-S6 (S235/236, indicates increased mTORC1 activity) and p-ERK1/2 (indicates increased RAF-MEK activity) in resected vascular anomalies. All experiments were performed on serial sections. (**A** and **B**) Resected tissues from patients (#1 and #2) with Parkes Weber Syndrome (PWS). (**C**-**E**) Resected tissues from three patients (#1, #2 and #3) with AVMs. These AVMs were not tested for *RASA1* mutations. Black arrowheads indicate positive endothelial staining (overlapped staining with CD31); red arrowheads indicate no endothelial staining; black arrow indicates positive staining in additional cell types. Scale bars, 50 μm (**A** and **B**), 100 μm (**C**-**E**).

Supplemental Figure 10.

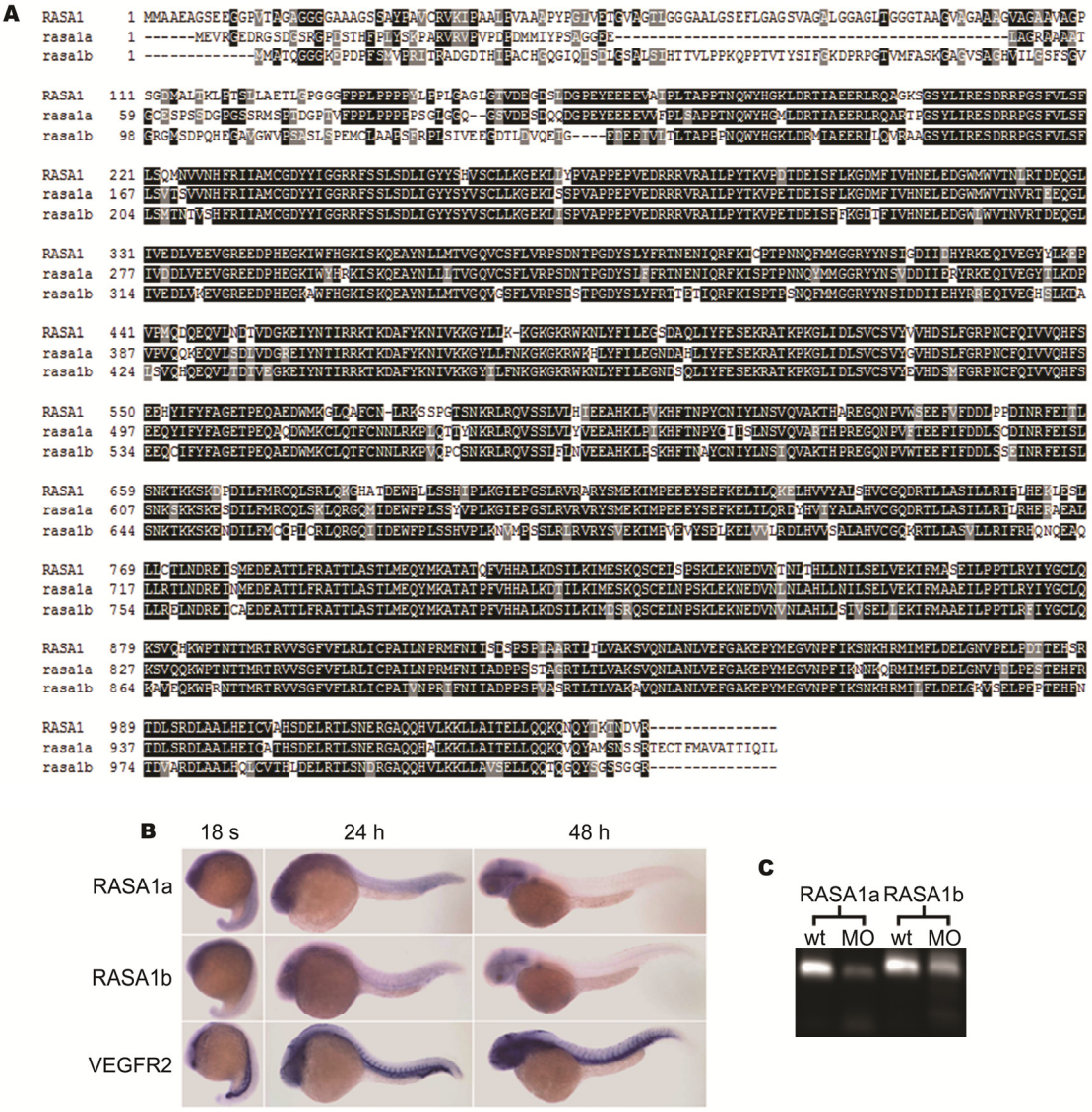
Proposed model describing the molecular mechanisms that leads to normal and abnormal vessel connections. (**A**) Under normal conditions, cell contact dependent ephrin-B2 stimulation of EphB4 recruits RASA1 to the receptor at the membrane. This model proposes that EphB4-RASA1 interaction is critical for the suppression of mTORC1 pathway activity. Attenuation of the mTORC1 pathway generates the signaling cascade necessary for proper vessel hierarchy and capillary formation. (**B**) Partial loss of function of RASA1 hinders normal mTORC1 suppression leading to overactive mTORC1 signaling, therefore,

deregulation of EphB4-RASA1-mTORC1 promoting abnormal vessel connections. This model provides a possible mechanism for *RASA1*-dependent vascular malformation.

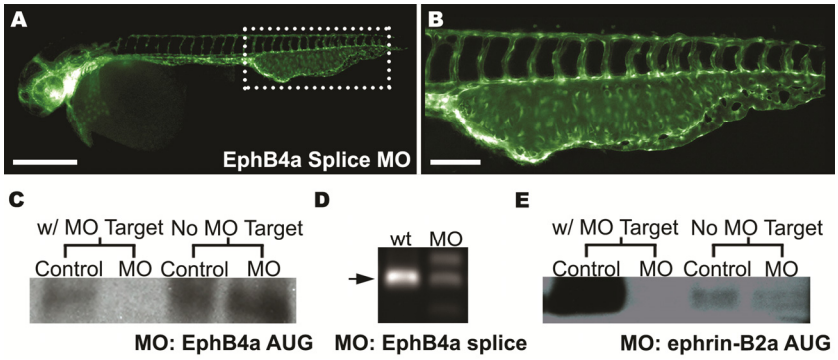
Supp Fig 1


A
Control siRNA
D
RASA1 siRNA
B
Control si
E
RASA1 si
C
Control si
F
RASA1 si
G
RASA1 mRNA Levels
1.0
0.5
0
*
Control si
RASA1 si
H
Vascularized Area (%)
70
60
50
40
30
20
10
0
**
Control si
RASA1 si

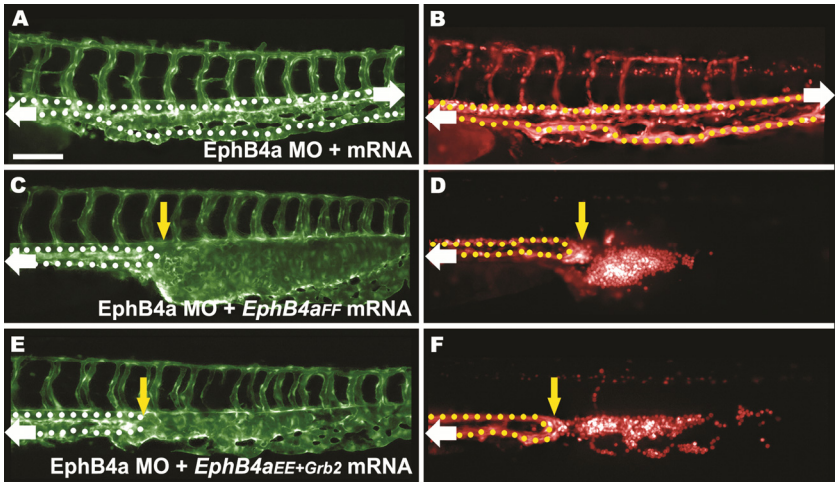
Supp Fig 2


A
RASA1
rasa1a
rasa1b
B
18 s
24 h
48 h
RASA1a
RASA1b
VEGFR2
C
RASA1a RASA1b
wt MO wt MO

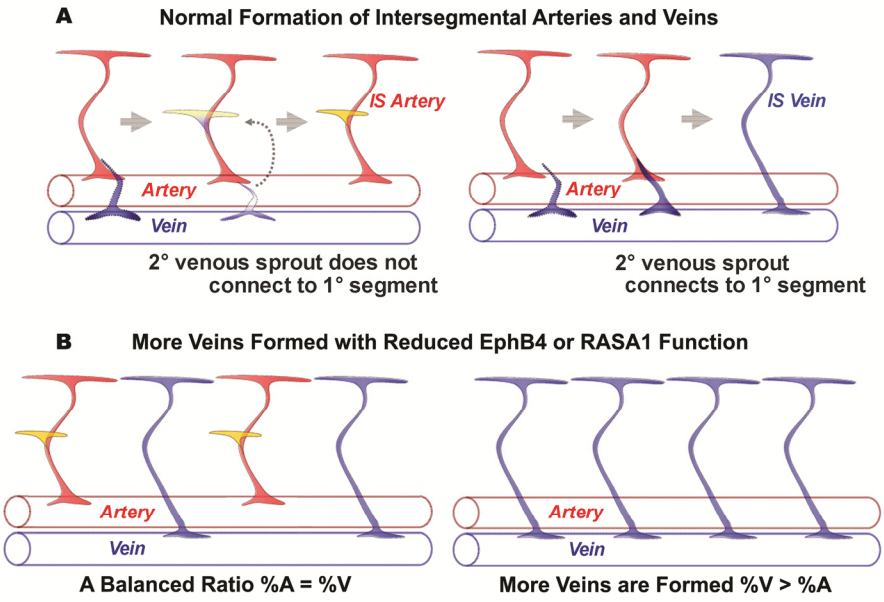
Supp Fig 3


A
EphB4a Splice MO
B
C
w/ MO Target
No MO Target
Control
MO
Control
MO
MO: EphB4a AUG
D
wt
MO
MO: EphB4a splice
E
w/ MO Target
No MO Target
Control
MO
Control
MO
MO: ephrin-B2a AUG

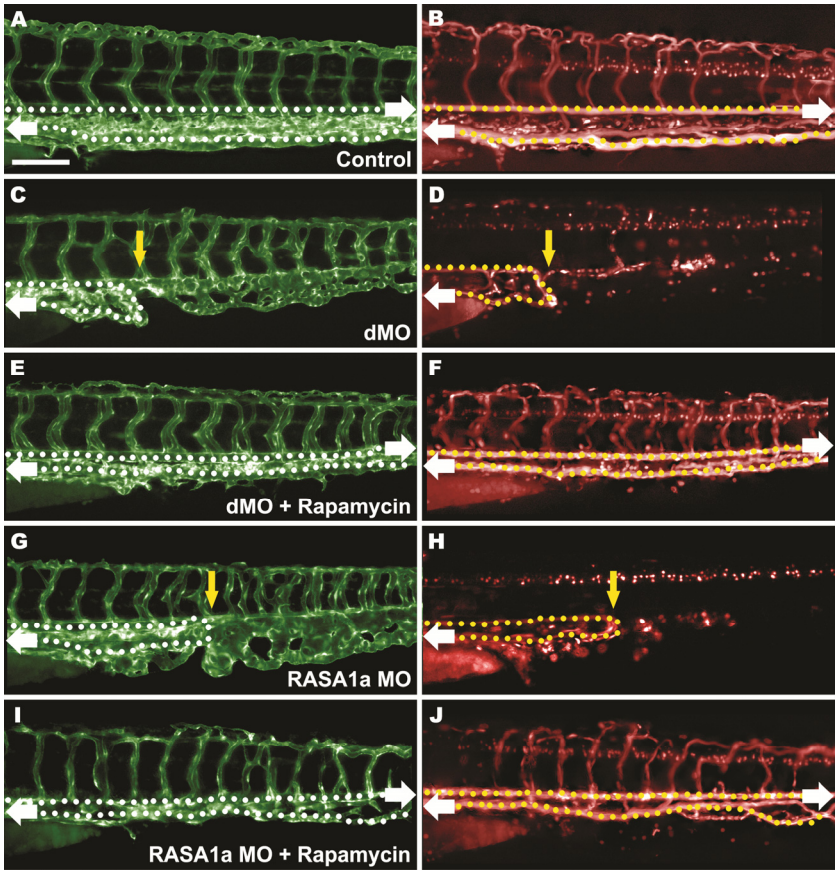
Supp Fig 4


A
B
EphB4a MO + mRNA
C
D
EphB4a MO + EphB4aFF mRNA
E
F
EphB4a MO + EphB4aEE+Grb2 mRNA

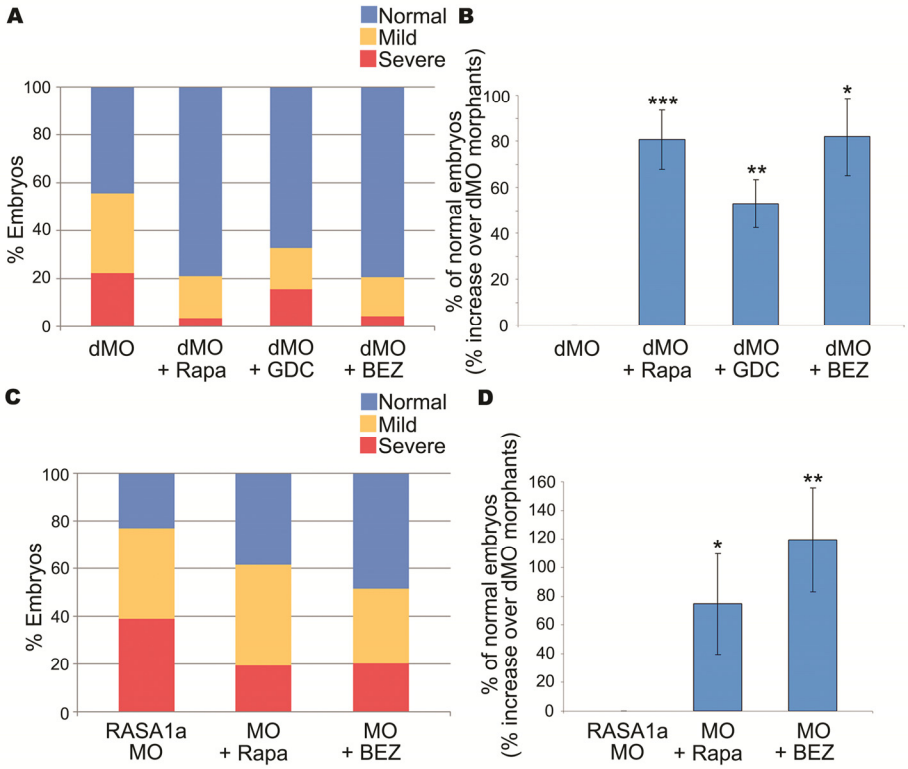
Supp Fig 5


A
Normal Formation of Intersegmental Arteries and Veins
IS Artery
Artery
Vein
IS Vein
Artery
Vein
2° venous sprout does not connect to 1° segment
2° venous sprout connects to 1° segment
B
More Veins Formed with Reduced EphB4 or RASA1 Function
Artery
Vein
Artery
Vein
A Balanced Ratio %A = %V
More Veins are Formed %V > %A

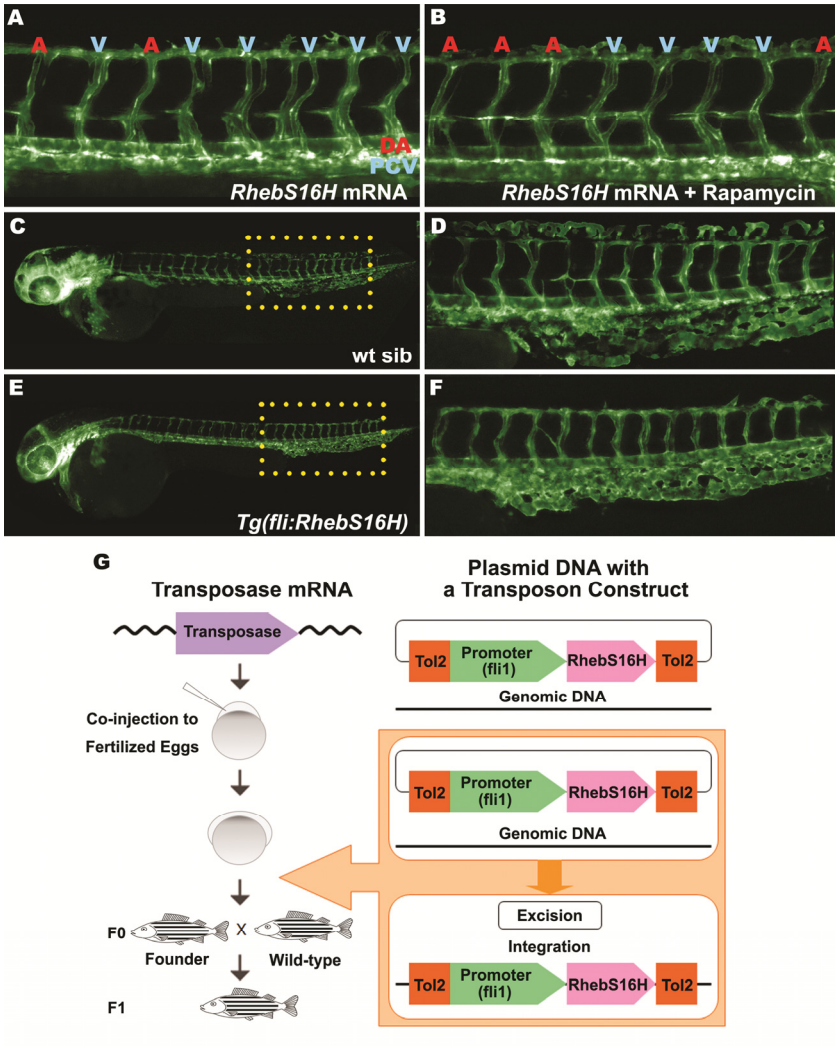
Supp Fig 6


A
B
Control
C
D
dMO
E
F
dMO + Rapamycin
G
H
RASA1a MO
I
J
RASA1a MO + Rapamycin

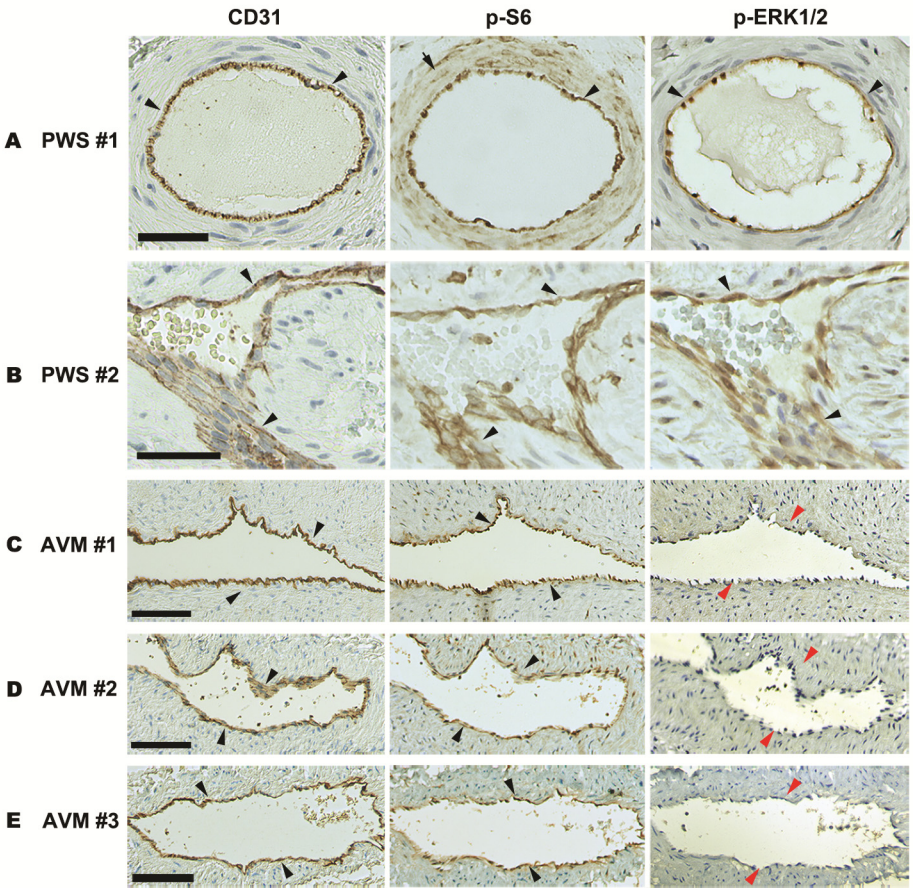
Supp Fig 7


A
Normal
Mild
Severe
% Embryos
100
80
60
40
20
0
dMO
dMO + Rapa
dMO + GDC
dMO + BEZ
B
% of normal embryos (% increase over dMO morphants)
100
80
60
40
20
0
***
**
*
dMO
dMO + Rapa
dMO + GDC
dMO + BEZ
C
Normal
Mild
Severe
% Embryos
100
80
60
40
20
0
RASA1a MO
MO + Rapa
MO + BEZ
D
% of normal embryos (% increase over dMO morphants)
160
140
120
100
80
60
40
20
0
*
**
RASA1a MO
MO + Rapa
MO + BEZ

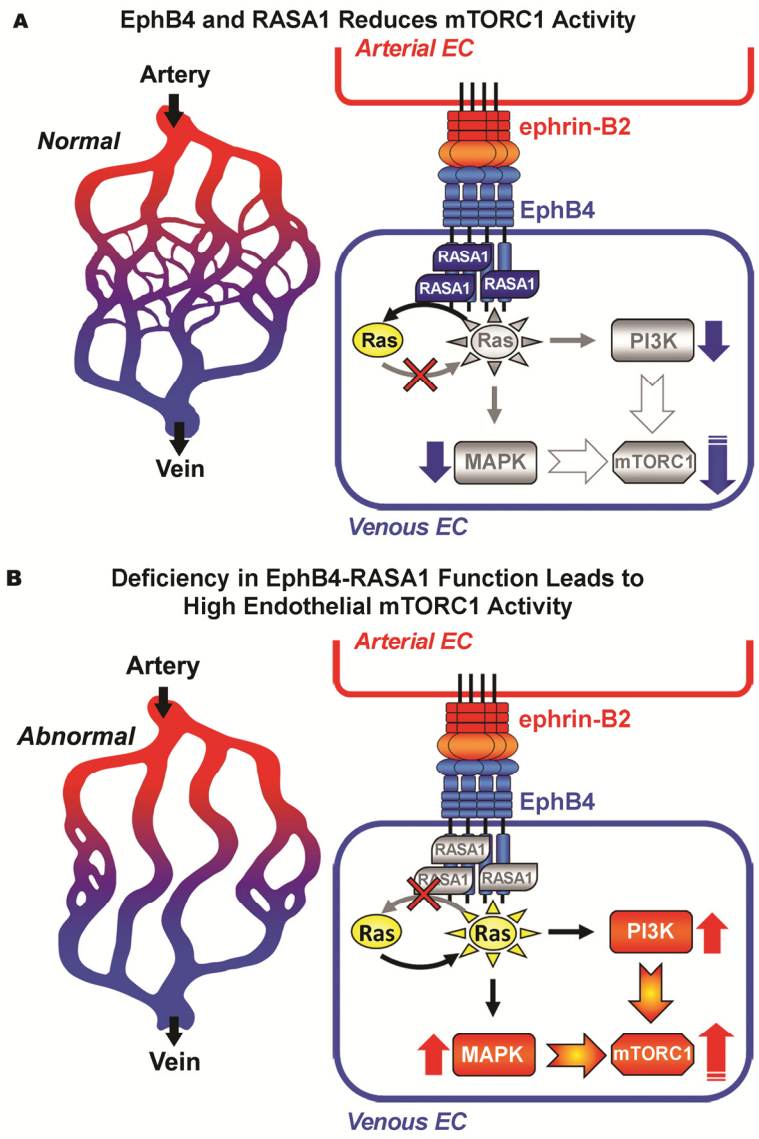
Supp Fig 8


A
A V A V V V V V
DA
PCV
RhebS16H mRNA
B
A A A V V V V A
RhebS16H mRNA + Rapamycin
C
wt sib
D
E
Tg(fli:RhebS16H)
F
G
Transposase mRNA
Transposase
Co-injection to
Fertilized Eggs
F0
X
Founder
Wild-type
F1
Plasmid DNA with
a Transposon Construct
Tol2
Promoter
(fli1)
RhebS16H
Tol2
Genomic DNA
Tol2
Promoter
(fli1)
RhebS16H
Tol2
Genomic DNA
Excision
Integration
Tol2
Promoter
(fli1)
RhebS16H
Tol2

Supp Fig 9



Supp Fig 10


A
EphB4 and RASA1 Reduces mTORC1 Activity
Artery
Normal
Vein
Arterial EC
ephrin-B2
EphB4
RASA1
RASA1
RASA1
Ras
Ras
PI3K
MAPK
mTORC1
Venous EC
B
Deficiency in EphB4-RASA1 Function Leads to
High Endothelial mTORC1 Activity
Artery
Abnormal
Vein
Arterial EC
ephrin-B2
EphB4
RASA1
RASA1
RASA1
Ras
Ras
PI3K
MAPK
mTORC1
Venous EC

Supplemental Table 1.

siRNA	siRNA Sequence
RASA1 #1 (mouse)	5'-UGUCCAACACCUAACAACCAGUUUA-3'

Supplemental Table 2.

Primer	qRT-PCR Primer Sequence (5'-3')
mouse RASA1 sense	5'-TGTGGTGATTACTACATTGGTGG-3'
mouse RASA1 antisense	5'-CGCCTTCTATCTTCTACTGGCTC-3'

Supplemental Table 3.

Morpholino	Morpholino Sequence
EphB4a (AUG)	5'- GCGGAATCACGAGTGTTTTACTTGT -3'
EphB4a (splice)	5'-CTGGAAAACACACACGAGAGATAGA-3'
ephrin-B2a	5'-AATATCTCCACAAAGAGTCGCCCAT-3'
RASA1a	5'-TAATCTCCACACATCGCAATGATCC-3'
RASA1b	5'-TGCGATGATCCTGCAAACGATCATT-3'

Supplemental Table 4.

Primer	RT-PCR Primer Sequence (5'-3')
EphB4a sense	5'-ATGGAGCTCTTCTCCAGGAATGTG-3'
EphB4a antisense	5'-ATAGGTCCGCACACTGTTGTTCTC-3'
RASA1a sense	5'-CCGAACCATTGCTGAAGAGCGATT-3'
RASA1a antisense	5'-ACCAATGAGGTCTGAAAGGGACGA-3'
RASA1b sense	5'-ATGATCGCAGAGGAGCGTTTGCTT-3'
RASA1b antisense	5'-GGCAGGACACGTAGCTGTAATAACCA-3'