SUPPLEMENTARY MATERIALS

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Whole-mount immunofluorescence in embryos

Whole-mount staining was performed as previously described (Meadows et al., 2013). Fixed embryos were permeabilized in 0.1% Triton-X in PBS (PBST) for an hour then blocked in CAS block (Invitrogen) for an hour. Embryos were then incubated with Flk1-eGFP (1:500) or PECAM antibody (1:100) dissolved in CAS block overnight at 4^{o}C. The next day, the embryos were washed three times or an hour in PBS then incubated with secondary antibody (1:300, Invitrogen) dissolved in CAS block overnight at 4^{o}C. The next day, the embryos were washed in PBS, dehydrated into 100% methanol, then were visualized after clearing in BABB using a Zeiss AxioObserver epifluorescence microscope.

Whole-mount immunocytochemistry

PECAM staining was performed as previously described (Meadows et al., 2013). Embryos were fixed overnight at 4°C in 4% PFA in PBS. The following day, embryos were washed in PBS, transferred to 0.25% Trypsin (Hyclone) for 2 min, rinsed in PBS, and blocked in CAS-Block (Invitrogen) for 1 hr at room temperature. Following block, embryos were incubated overnight with PECAM antibody (BD Pharmingen; 1:300) in PBST (PBS + 0.1% Triton-X-100) at 4°C. The next day, embryos were washed with PBST and stained with DAB solution as per kit instructions (Vector labs). The staining reaction was stopped by rinsing in water and fixation in 4% PFA.

Immunofluorescence staining of embryonic tissues

Whole-mount staining was performed as previously described (Barry et al., 2015). Fixed tissues were washed (PBS), cryoprotected in 30% sucrose overnight, embedded in Tissue-Tek O.C.T. and sectioned. Sectioned tissues were soaked in PBS then heated in a 2100 antigen retriever in antigen retrieval buffer. Cytoplasmic proteins were stained using R-Buffer A (Electron Microscopy Sciences) and nuclear proteins were stained using R-Buffer B. Sections were washed (PBS) and blocked (1 h RT 5% serum). Primary antibody incubations were performed at 4°C overnight (for dilutions, see supplementary material Table S1). Slides were washed (PBS), incubated in secondary antibody (4 h, RT). Slides were washed (PBS) and mounted using Prolong Gold Mounting Medium with DAPI. Images were obtained using a LSM510 or LSM710 Meta Zeiss confocal. Phalloidin staining was performed without antigen retrieval. TSA immunofluorescent staining (PerkinElmer; individual fluorescein tyramide reagent pack, cat# SAT701) was used to stain pMLC.

Immunofluorescence staining of cultured ECs

Immunofluorescence staining of MS1cells was performed as previously described (Barry et al., 2015). MS1s (ATCC) were cultured on coverslips coated with dried gelatin were fixed with 4%PFA briefly then rinsed with PBSN (0.1%NP40/PBS). Cells were then permeabilized in PBSN for 15 minutes then blocked in CAS block (Invitrogen). Primary antibody dissolved in CAS block (1:60) was pipetted on parafilm then the glass coverslip was placed face down onto the droplet for an hour. Afterward, the coverslip was washed with PBSN then incubated with secondary antibody (1:200) in the same manner as the primary. After the secondary, the coverslip was washed with PBSN then mounted onto a slide with Prolong Gold anti-fade with DAPI (Invitrogen).Staining of HUVECs in 3D collagen matrices was performed as previously described (Norden et al., 2016).

Hematoxylin and Eosin staining

H&E staining was performed as previously described (Meadows et al., 2013). Embryos were dissected and fixed overnight at 4^{o}C in 4% PFA/PBS. The embryos were then paraffin embedded and sectioned. The sections were then washed in xylene, 100% ethanol, 95% ethanol, water, then Hematoxylin. The sections were then again washed in water, then for 5 seconds in 1% HCL/70%

ethanol. Next the sections were washed with water, Eosin solution, 95% ethanol, 100% ethanol, xylene, then were mounted with a coverslip and Permount solution (Fisher Scientific).

In situ hybridization

In situ hybridization staining on sections and whole mount were performed as previously described (Xu et al., 2009). Briefly, embryos stored in 75% ethanol at − 20 °C were rehydrated in stepwise fashion to PBST. Then, the embryos were treated with 10 μg/ml proteinase K, fixed in a 0.2% gluteraldehyde/4% paraformaldehyde (PFA) solution, and pre-hybridized at 65°C for 1 h. The samples were transferred into hybridization mix, containing 1 μg/ml Dig-labeled probes. Post-hybridization the tissue was washed and incubated with an anti-Dig antibody. Color development was carried out using BM purple solution (Roche). An Arhgap29 3' coding region fragment (1.2kb) and a Plexin D1 clone (MMM1013-66046 Open Biosystems) were used to generate Dig-labelled RNA probes.

siRNA transfection and recombinant protein expression

siGENOME siRNAs obtained from GE Dharmacon were transfected into cultured MS1 or HUVECs using standard protocols for transfection and western analysis blot, as previously described (Koh et al., 2008) (antibodies used for western blots are detailed in supplemental material Table S1 and siRNA sequences are detailed in supplemental material Table S2). Transfection of plasmids expressing Rasip1-GFP and GFP was performed 24 hours after siRNA transfection. 1μg of plasmid DNA was transfected onto cells cultured on a 12mm cover slip using 1μg Lipofectin (Invitrogen) dissolved in 300μl Optimem. Cells were fixed and stained 24 hours after transfection.

Whole embryo culture (WEC)

WEC protocol adapted from (Jones et al., 2005; Kalaskar and Lauderdale, 2014). Flk1-eGFP embryos were dissected with yolk sac intact at E8.0 in DMEM/8% FBS + 1% antibiotic antimycotic with HEPES. Embryos were cultured for 3hrs in 50% male rat serum and 50% DMEM with HEPES in Precision Incubator Unit (B.T.C. Engineering Milton Cambridge England). ROCK inhibitor (Y-27632, Millipore), Pak4 inhibitor (PF-03758309, Selleckchem), Rac1-3 inhibitor (EHT, Tocris), Rho inhibitor (Rho inhibitor I, Cytoskeleton), and the NMII inhibitor (blebbistatin, Sigma-Aldrich) were all added before culture at 10μM. After culture, embryos were imaged using a Zeiss AxioObserver epifluorescence microscope, then fixed at 4^{o}C with 4% PFA/PBS for 40min.

In vitro lumen formation assay

HUVEC lumen and tube formation in 3D collagen matrices were performed as previously described (Koh et al., 2008). HUVECs (Lonza) were then suspended at 2 x 10^6 cells/mL in 2.5 mg/mL collagen type I matrices. SCF, IL-3, SDF-1α, and FGF-2 were added at 200 ng/mL into collagen type I. Cultures were fed with media containing reduced serum supplement (RSII), ascorbic acid, and FGF-2 at 40 ng/mL. Cultures were allowed to assemble into capillary networks over a period of 0–120 hr when cultures were fixed or collected for further processing. Samples were fixed in 2% paraformaldehyde or 3% glutaraldehyde in PBS. Cultures fixed in paraformaldehyde were then stained for fluorescent microscopy imaging, whereas cultures fixed in glutaraldehyde were stained in 0.1% toluidine blue in 30% methanol.

Cell culture image analysis and statistics

Object Identification and features extraction were performed using CellProfiler (Carpenter et al., 2006). DAPI channel was used to identify nuclear objects. EnhanceEdges module (Sobel was used as an edge-finding method) was applied to VE-cadherin images prior to identifying cell boundaries. Then 3 features were extracted for each cell – 2 for Adhesion and the other one for Stress Fiber/Actin. The detail of each feature is followed.

A. Adhesion
   1. Junction discontinuity (**Online Figure VII A1**)

First, each set of 'Edge-enhanced' VE-cadherin images were manually threshold to obtain positive/foreground VE-cadherin pixels. A junction discontinuity is defined by adding all the foreground pixels within a cell edge and divided by a cell edge area. A cell edge is identified by a 10 pixel ring within a cell boundary (dark gray area left panel). If the junction is perfectly smooth, the ratio would be close to one. (By visual observation, the width of the VE-cadherin stain on cell boundary is on average about 10 pixels) as shown in Example 1. On the other hand, if the junction is disrupted, the value would be much lesser than 1 as shown in Example 2.

2. Junction width (**Online Figure VII A2**)

   Each cell is equally divided into 10 bins radially from its center. Then we obtained a ratio of total intensity of the VE-cadherin stain in the 9^{th} bin (dark gray ring) to the total intensity of the VE-cadherin stain in the whole cell (light gray left panel). If the junction is smooth and continuous, there wouldn't be any VE-cadherin stain in the 9^{th} bin; hence the junction width would be close to zero (Example 1). In the case that junction is 'jagged', the value of the junction width would be higher as shown in Example 2.

B. Stress Fiber/Actin

1. Ratio of Phalloidin area per cell area (**Online Figure VII B**)

First, EnhanceOrSuppressFeatures module was applied to Phalloidin images to enhance the stress fibers (Feature type: Neurites, Enhancement method: Line Structures, Feature size: 20). Then the images were manually threshold to obtain positive/foreground Actin pixels. Actin area is obtained by adding all the foreground pixels within a cell. Then the ratio of Actin area (sum all black pixels) to cell area (sum all light gray pixels) is obtained. For a treatment with reduced stress fiber, the ratio would be lesser than the normal/control condition.

ONLINE FIGURE LEGENDS

Online Figure I. Angioblasts form adhesion complexes with each other, polarize the adhesions laterally, then open a blood vessel lumen in an anterior to posterior manner. (**A**) Lateral view of a Flk1-eGFP embryo, stained for GFP, showing that the dorsal aortae open in an anterior to posterior manner. Dotted line arrow, progression of lumen opening. (**B-D**) Developing angioblasts come into contact and establish adhesion foci at contacting interface (pre-apical membranes). (**E-E'''**) Adhesion complexes are remodeled (redistributed) between the apical and basal membrane once the angioblasts polarize and become ECs. (**F**) Transfection of Rasip1-GFP into HUVECs shows Rasip1 at cell-cell junctions. Arrowheads, EC cell-cell junction. (**G**) Schematic showing clearance of junction 'ribbons' from the pre-apical luminal surface, as lumen opens. (**H-I''**) HUVECs expressing AcGFP-Rasip1 and PODXL-mCherry protein during lumen formation in a 3D lumen formation assay. Rasip1 tethers the apical membrane during and after lumen formation. Arrows, apical Rasip1; L, lumen. (**J-L**) Antibody localization of Rasip1 and endosomal proteins Rab7, Rab8 and Rab5 in MS1 cells. (**M-M''**) MS1 cells transfected with constitutively active Rab5(Q79L)-mCherry produce enlarged early endosomes that display Rasip1 enrichment. Arrow heads, enlarged endosomes. Scale bars: A 30μm, B-D 5μm, E-E''' 2μm, F 10μm, G-H'' 10μm, I-I''5μm, J-L 10μm, M 7μm.

Online Figure II. Endocytosis is not necessary for vascular cord adhesion remodeling. (**A-D'''**) WEC was performed in the presence of Pitstop2 or the Pitstop2 negative control. Treatment of either drug did not prevent vascular cord adhesion remodeling (marked by VEcad or ZO-1) to the periphery of the apical membrane (marked by Moesin) (n=3 control and treated, 16 FOV, quantified in E). ns = not significant. EC, endothelial cell. (**F-G''**) pMLC localizes to VEcad -positive adhesion complexes as they remodel from the center of vascular cords to the periphery (n=3, 15 FOV). (**H-I'**) F-actin localizes to adhesion complexes as they remodel away from the center of vascular cords (n=12, 60 FOV). EC, endothelial cell; L, lumen; arrow heads, adhesion complexes; arrows, direction of adhesion relocalization. Scale bars: A-D''' 2μm, F-I' 2μm.

Online Figure III. Gene deletion before blood vessel lumen formation using Tie2-Cre occurs in the yolk sac vasculature, but not in the dorsal aorta.
Schematic of gene deletion using CAG-CreERT2 or Tie2-Cre in the dorsal aorta or the yolk sac vasculature. (**A-B**) Rosa26Reporter-LacZ expression shows that angioblasts express Tie2-Cre in early angioblasts in the aorta and yolk sac vasculature (n=4). Arrow heads, angioblasts. (**C-D**) Rosa26Reporter-LacZ expression shows that CAG-CreERT2 can induce recombination in all cells of the embryo, including endothelial cells, after a low dose of tamoxifen (n=6). NT, neural tube; M, mesoderm; End, endoderm; A + red dotted line, aorta. (**E-F**) Staining of RhoA in the dorsal aorta, showing that deletion of RhoA using Tie2-Cre does not occur before lumen formation (n=5 control and mutant, >36 FOV). EC, endothelial cell; L, lumen. (**G-H'**) Staining of RhoA in the yolk sac, showing that deletion of RhoA using Tie2-Cre during lumen formation (n=5 control and mutant, >36 FOV). (**I-J'**) Staining of Cdc42 in the dorsal aorta, showing that deletion of Cdc42 using Tie2-Cre does not occur before lumen formation (n=6 control and mutant, 30 FOV, quantified in I). ns = not significant. Arrowheads, Cdc42 staining. (**K-L'**) Staining of Cdc42 in yolk sac endothelial cells, showing that deletion of Cdc42 using Tie2-Cre does occur before lumen formation in the yolk sac (n=6 control and mutant, 30 FOV, quantified in L). ****P<0.0001. Scale bars: A 100μm, B 50μm, C 250μm, D 20μm, E-F 10μm, G-H' 10μm, I-J' 5μm, K-L' 14μm.

Online Figure IV. Cdc42 is necessary for angioblast migration, adhesion remodeling, and EC proliferation.
(**A-A'**) Flk1-LacZ staining shows that Cdc42 deletion using Tie2-Cre causes aberrant angioblast migration and cord formation in the yolk sac vasculature (n=5 control and n=3 mutant). Red arrow, vascular cord. (**B-B'**) Rosa26Reporter-YFP expression shows that lumen formation fails in the yolk sac vasculature after deletion of Cdc42 using Tie2-Cre. EC, endothelial cell; L, lumen (n=6 control and mutant). (**C-C'**) Flk1-eGFP expression shows that deletion of Cdc42 using CAG-CreERT2 causes failed angioblast migration in the posterior aortae (n>23 control and n=23 mutant). (**D-D'**) Cross sections show that angioblasts fail to develop into vascular cords after deletion of Cdc42 using CAG-CreERT2 (n>6 control and mutant). (**E-H'**) Cross sections of yolk sac ECs showing that ECs fail to remodel cell-cell adhesions and form patent lumens in the yolk sac after deletion of Cdc42 using Tie2-Cre (n=6 control and mutant). Arrow heads, adhesion complexes. (**I-M**) Cdc42 deletion causes less pHH3 positive ECs to develop after deletion using CAG-CreERT2 or Tie2-cre (n=3 control and mutant, 15 FOV, quantified in K and N). *P<0.05. Arrow heads, pHH3 positive cells. (**O-S**) Cdc42 deletion using CAG-CreERT2 or Tie2-cre does not influence apoptosis as assessed by Cleaved Caspase 3 staining (n=3 control and mutant, 15 FOV, quantified in Q and T). ns = not significant. Scale bars: A-A' 40μm, B-B' 7μm, C-C' 100μm, D-D' 7μm, E-H' 5μm, I-J 10μm, L-M 18μm, O-P 10μm, R-S 18μm.

Online Figure V. Rac1 is not necessary for vascular lumen formation but is necessary for vascular remodeling.
(**A-A'**) $Rac1^{Tie2KO}$ embryos display hypoplasia by E11.5. (**B-B'**) Whole-mount PECAM immunocytochemistry stain showing failed vascular plexus remodeling in E9.5 $Rac1^{Tie2KO}$ embryos. (**C-C'**) H&E stain of E8.5 $Rac1^{Tie2Het}$ and $Rac1^{Tie2KO}$ embryos show that lumen formation occurs normally in the yolk sac during vasculogenesis. L, lumen. (**D-D'**) Whole-mount PECAM staining of E8.0 (3s) $Rac1^{CAGHet}$ and $Rac1^{CAGKO}$ embryos show normal dorsal aortae after Rac1 deletion (n=3 control and mutant). (**E-F'**) Staining of F-actin and PECAM/endomucin on $Rac1^{Tie2Het}$ and $Rac1^{Tie2KO}$ embryos showing that Rac1 is not necessary for blood vessel lumen formation or vascular cord adhesion remodeling (n=3 control and mutant, 15 FOV, quantified in G). n= not significant. Scale bars: A-A' 1.25mm, B-B' 125μm, C-C' 50μm, D-D' 50μm, E-F' 7μm.
Online Figure VI. RhoA suppresses blood vessel lumen expansion by suppressing EC proliferation and cell spreading.
(**A-A'**) Bright field images showing RhoA deletion using Tie2-Cre causes embryonic lethality and hypoplasia at E9.25 from vascular defects (n>4 control and mutant). (**B-B'**) Blood vessels visualized by PlexinD1 *in situ* become expanded and fail to remodel after deletion of RhoA using Tie2-Cre. (**C-C'**) Cross sections of $RhoA^{Tie2Het}$ and $RhoA^{Tie2KO}$ embryos expressing Rosa26-YFP reporter. $RhoA^{Tie2KO}$ embryos have larger blood vessel lumens (n=3 control and mutants, 15 FOV, quantified in D). ****P<0.0001. L, lumen. (**E-F**) Graph showing that nuclei become more distant between ECs suggesting increased cell spreading after deletion of RhoA (graph E) or inhibition of ROCK (graph F). (n=3 control and mutant, 15 FOV). *P<0.05. (**G-I'**) RhoA deletion causes more pHH3 positive ECs to develop after deletion using CAG-CreERT2 or Tie2-cre (n=3 control and mutant, 15 FOV, quantified in H and J). *P<0.05, **P<0.01. EC, endothelial cells; End, endoderm; arrow heads, pHH3 positive cells. (**K-M'**) RhoA deletion using CAG-CreERT2 or Tie2-cre slightly increases apoptosis as assessed by Cleaved Caspase 3 staining (n=3 control and mutant, 15 FOV, quantified in L

and N). *P<0.05, **P<0.01. B, blood. Scale bars: A-A' 250μm, B-B' 100μm, C-C' 18μm, G-G' 10μm, I-I' 25μm, K-K' 10μm, M-M' 7μm.

Online Figure VII. Arhgap29 null embryos die embryonically from failure to attach the allantois to the ectoplacental cone.
(**A-B''**) Bright field and Flk1-eGFP images showing that Arhgap29 mutant embryos are not able to fuse the allantois to the chorion (n=3 control and mutant). (**C-F'**) In situ hybridization of Arhgap29 in whole-mount and in section showing that Arhgap29 is expressed in the aorta, yolk sac vasculature, and the allantois (n=3 control and mutant). Red arrows and dotted lines, Arhgap29 positive vessels. NT, neural tube. (**G-H'''**) Arhgap29 mutants do no display defects in EC polarity as assessed by junctional ZO-1, apical PODXL, and basal pPaxillin staining in the aorta (n=3 control and mutant, 15 FOV). Arrow heads, EC-EC adhesions; EC, endothelial cell; L, lumen; M, mesoderm. (**I-K''**) NMII and Rasip1 are transiently colocalized to the apical membrane during vascular lumen expansion (n=3 control and mutant, 15 FOV). Scale bars: A-B'' 250μm, C-E 50μm, F 500μm, G-H''' 7μm, I-K'' 7μm.

Online Figure VIII. RhoA deletion or ROCK inhibition does not influence EC cell-cell adhesion morphology.
(**A-C**) MS1 cells treated with siRNA targeted to RhoA or the ROCK inhibitor Y-27632 did not change EC cell-cell adhesion morphology as marked by VEcad and phalloidin staining (n=3 control and treated, 15 FOV, quantified in D). (**E**) siRNA immunoblot confirmation after reduction of Rasip1, Cdc42, NMHCIIA, or RhoA. (**F**) Cell culture image analysis. For cell culture image analysis see supplementary methods. Scale bars: A-C 5μm.

SUPPLEMENTARY VIDEOS

Movie S1. Live imaging of Flk1-eGFP embryo yolk sacs treated with DMSO.
Movie S2. Live imaging of Rasip1-/- Flk1-eGFP embryo yolk sacs.
Movie S3. Live imaging of Flk1-eGFP embryo yolk sacs treated with 10μm Cytochalasin D.
Movie S4. Live imaging of Flk1-eGFP embryo yolk sacs treated with 10μM blebbistatin.
Movie S5. Live imaging of $Cdc42^{CAGKO}$ Flk1-eGFP embryo yolk sacs.
Movie S6. Live imaging of Flk1-eGFP embryo yolk sacs treated with 10μM PF-03758309.
Movie S7. Live imaging of $RhoA^{CAGKO}$ Flk1-eGFP embryo yolk sacs.
Movie S8. Live imaging of Flk1-eGFP embryo yolk sacs treated with 10μM Y-27632.
Movie S9. Live imaging of Flk1-eGFP embryo yolk sacs with open vessels treated with DMSO.
Movie S10. Live imaging of Flk1-eGFP embryo yolk sacs with open vessels treated with 10μM blebbistatin.
Movie S11. Live imaging of Flk1-eGFP embryo yolk sacs with open vessels treated with 10μM Y-27632.

Table S1. Antibodies and Dilutions

Antibody	Raised in	Staining Dilution	Western Dilution	Source
PECAM	Rat	1:100		BD Biosciences
Endomucin	Rat	1:100		Santa cruz
GFP	Chicken	1:500		Aves
Rasip1	Goat	1:100		Novus Biologicals
pH3	Rabbit	1:100	1:1000	Millipore
C.Caspase3	Rabbit	1:100	1:1000	Cell Signaling
VE-cadherin	Goat	1:100		Santa cruz
pPaxillin	Rabbit	1:100	1:1000	Cell Signaling
RhoA	Rabbit		1:1000	Cell Signaling
Podocalyxin	Rabbit	1:100		Millipore
β1 integrin	Goat	1:100		Santa cruz
ZO-1	Mouse	1:100		Cell Signaling
pMLC (Ser19)	Mouse	1:100	1:1000	Cell Signaling
MLC	Rabbit		1:1000	Cell Signaling
NMHCIIA		1:100		BioLegend
GAPDH	Mouse		1:1000	Thermo Scientific
β-actin	Mouse		1:2500	Cell Signaling
Pak2	Rabbit		1:1000	Cell Signaling
pPak2 (Ser20)	Rabbit		1:1000	Cell Signaling
Pak4	Rabbit		1:1000	Cell Signaling
pPak4 (Ser141)	Rabbit		1:1000	Cell Signaling
RFP	Rabbit	1:1000		Invitrogen
Rab5	Rabbit	1:60		Cell Signaling
Rab7	Rabbit	1:60		Cell Signaling
Rab8A	Rabbit	1:60		Protein Tech

Table S1. siRNAs and Source

siRNA	Company	Catalogue #
siControl	GE Dharmacon	D-001206-13-50
siCDC42	Ambion	s2765
siCdc42	GE Dharmacon	M-043087-01-0050
siPAK2	Ambion	M-040615-01-0020
siPAK4	Invitrogen	NM_001014834_stealth_749
siNMIIA	GE Dharmacon	M-040013-01-0050
siMRCKβ	Invitrogen	NM_006035_stealth 691
siRasip1	GE Dharmacon	M-065864-00-0050
siRac1	GE Dharmacon	M-041170-01-0050
siROCK1	Ambion	s12097
siRhoA	GE Dharmacon	M-042634-01-0050

SUPPLEMENTARY MATERIALS REFERENCES

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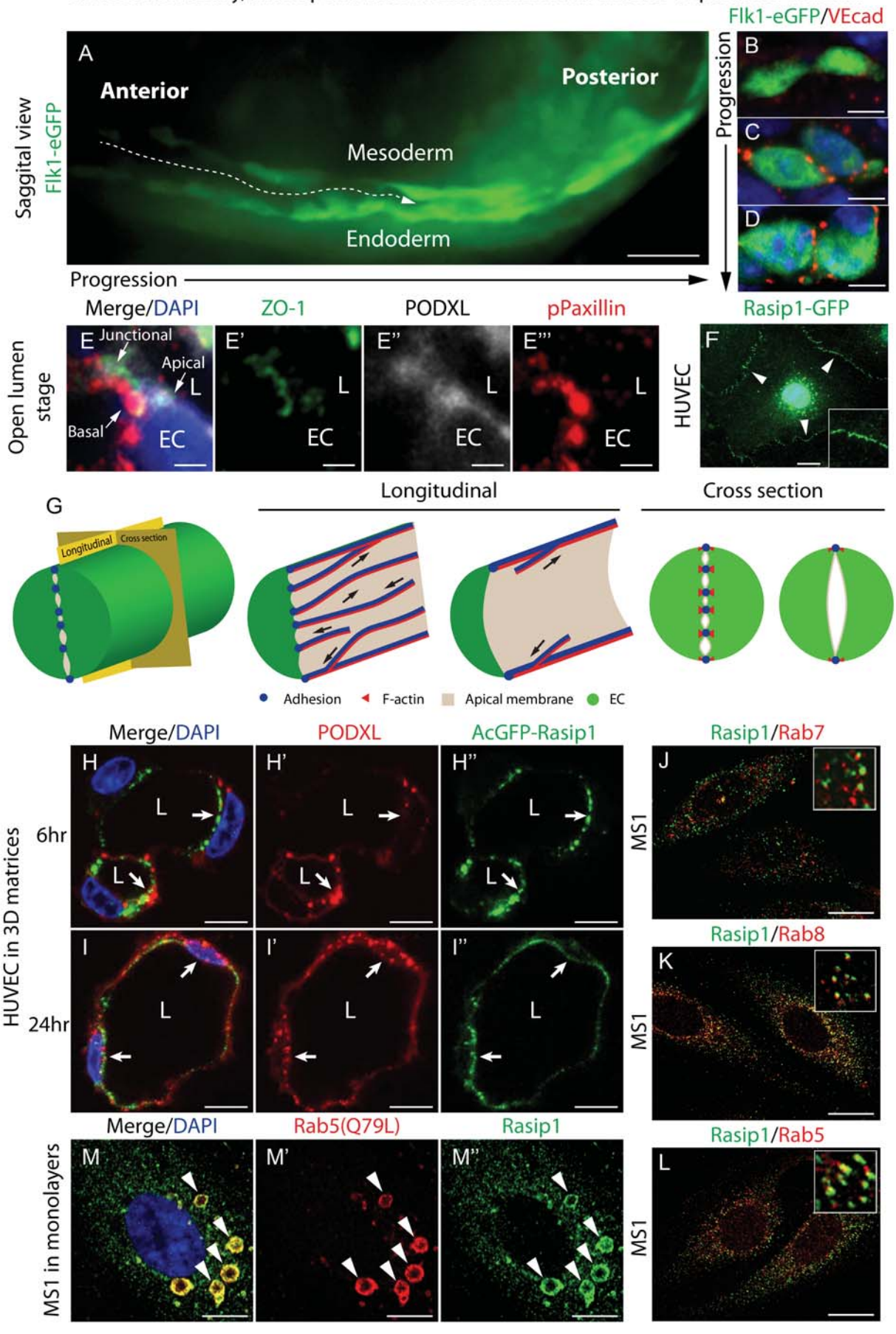
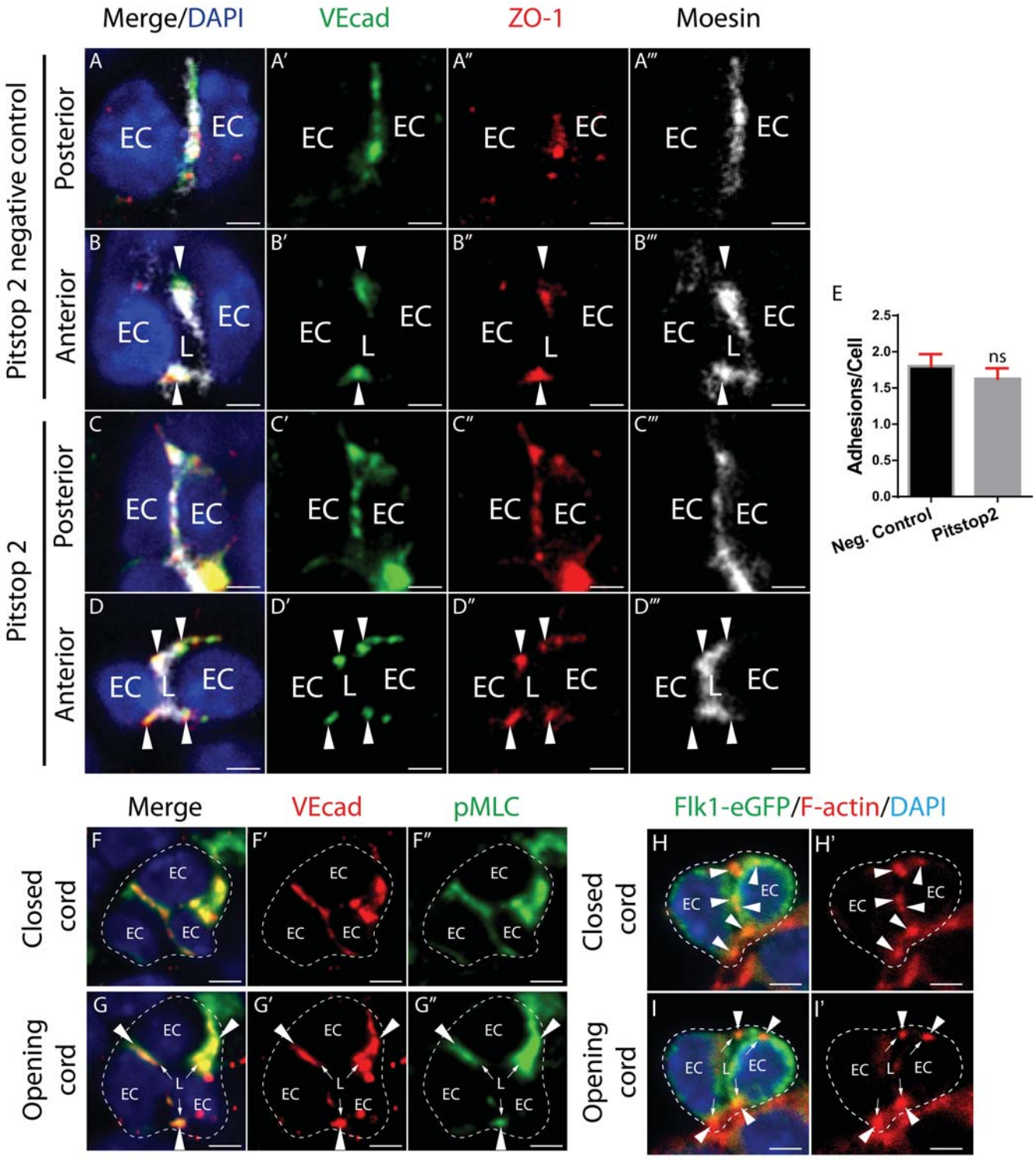
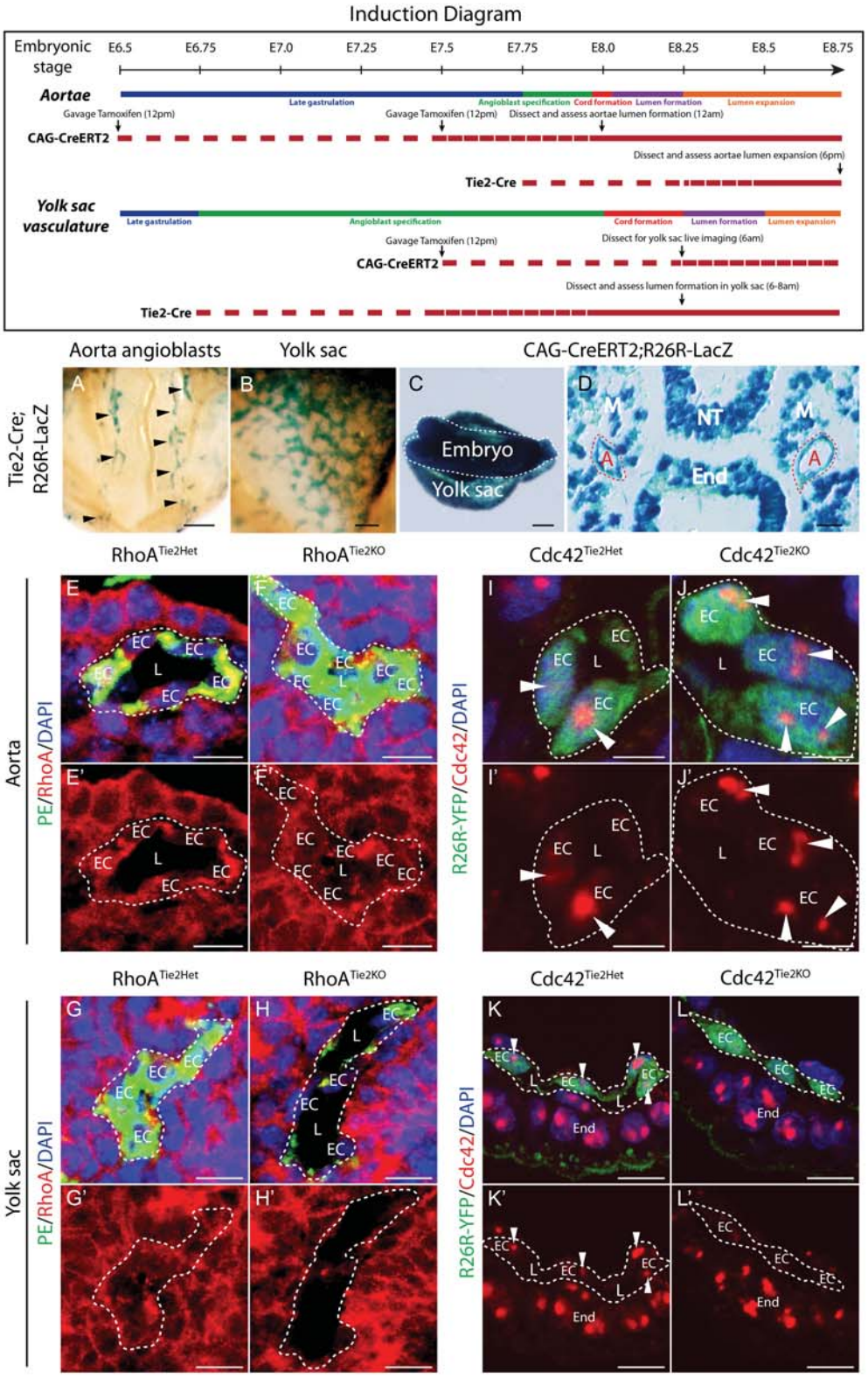
Online Figure I. Angioblasts form adhesion complexes with each other, polarize the adhesions laterally, then open a blood vessel lumen in an anterior to posterior manner.



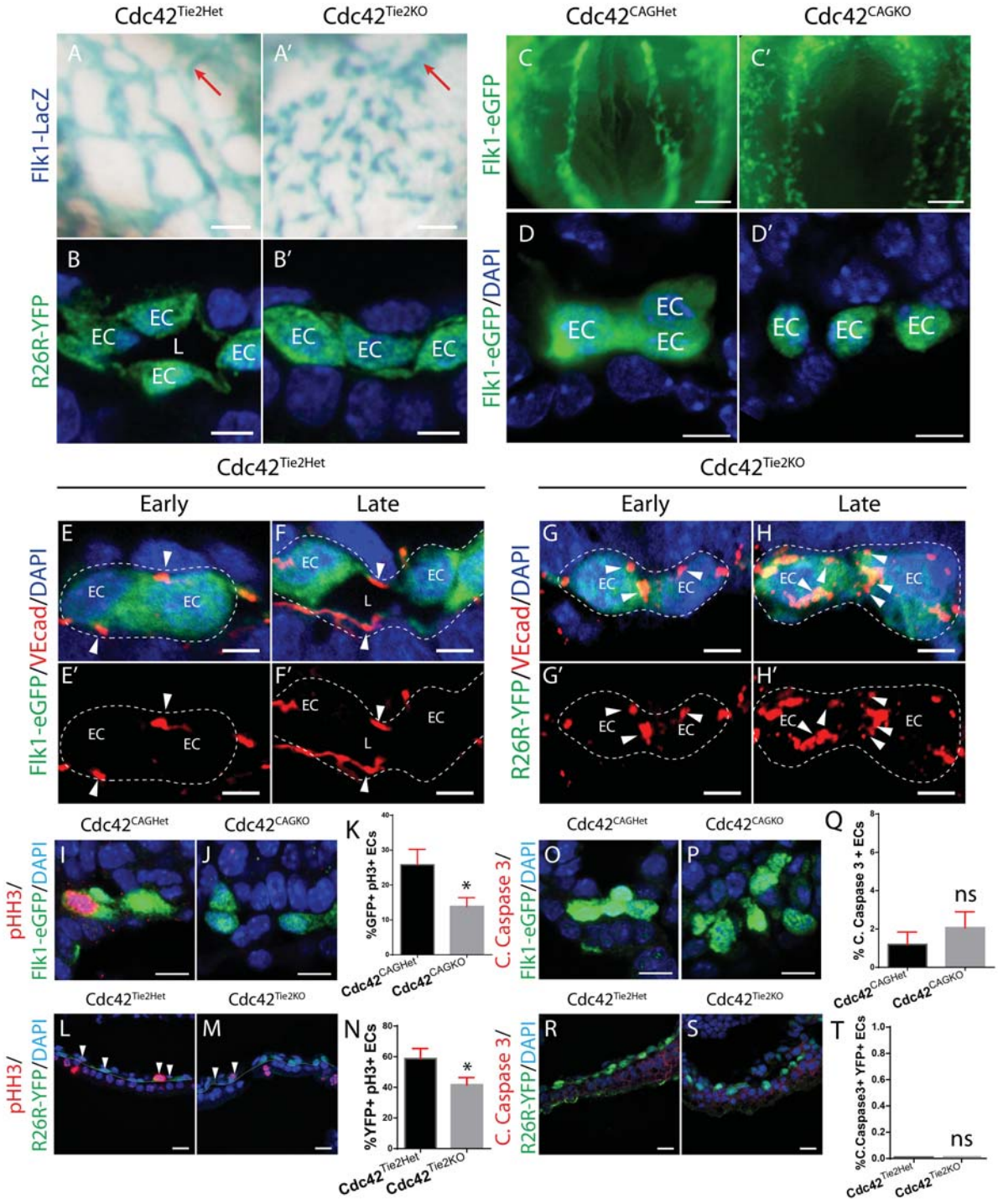
Online Figure II. Endocytosis is not necessary for vascular cord adhesion remodeling.



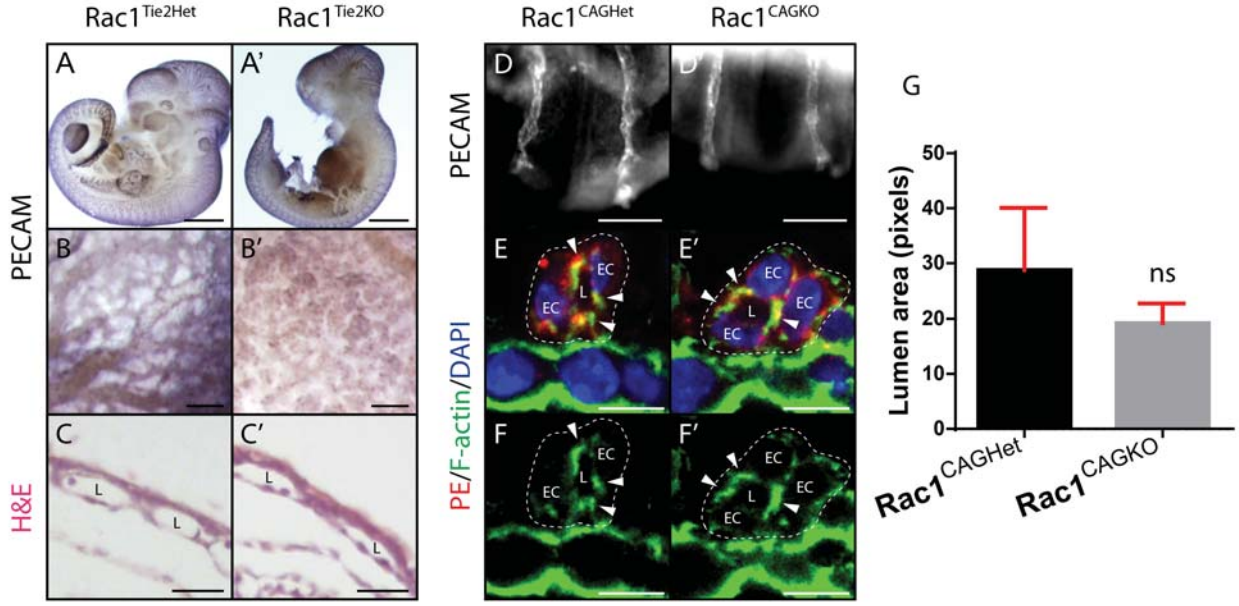
Online Figure III. Gene deletion before blood vessel lumen formation using Tie2-Cre occurs in the yolk sac vasculature but does not occur in the dorsal aorta.



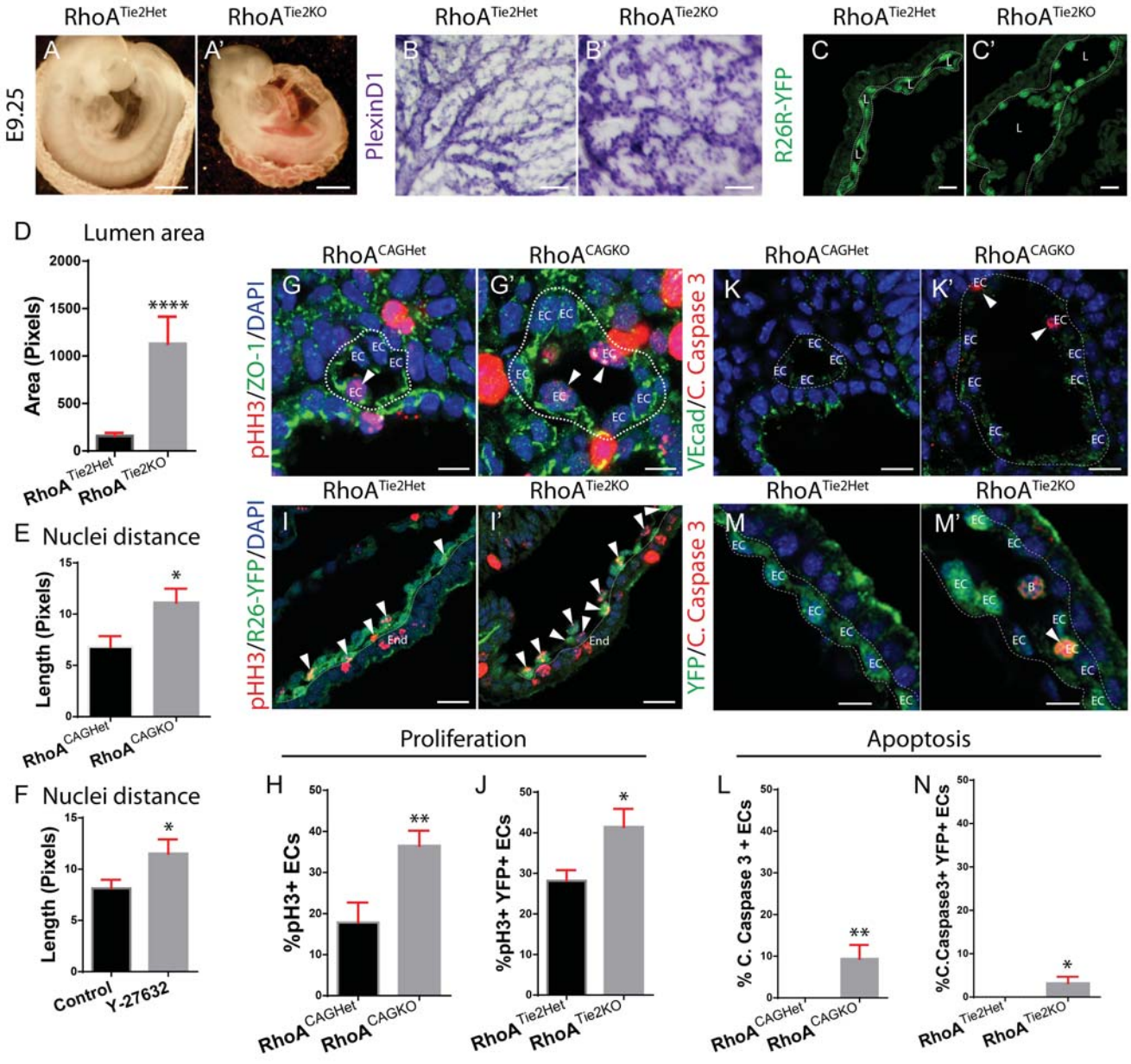
Online Figure IV. Cdc42 is necessary for angioblast migration, adhesion remodeling, and EC proliferation.



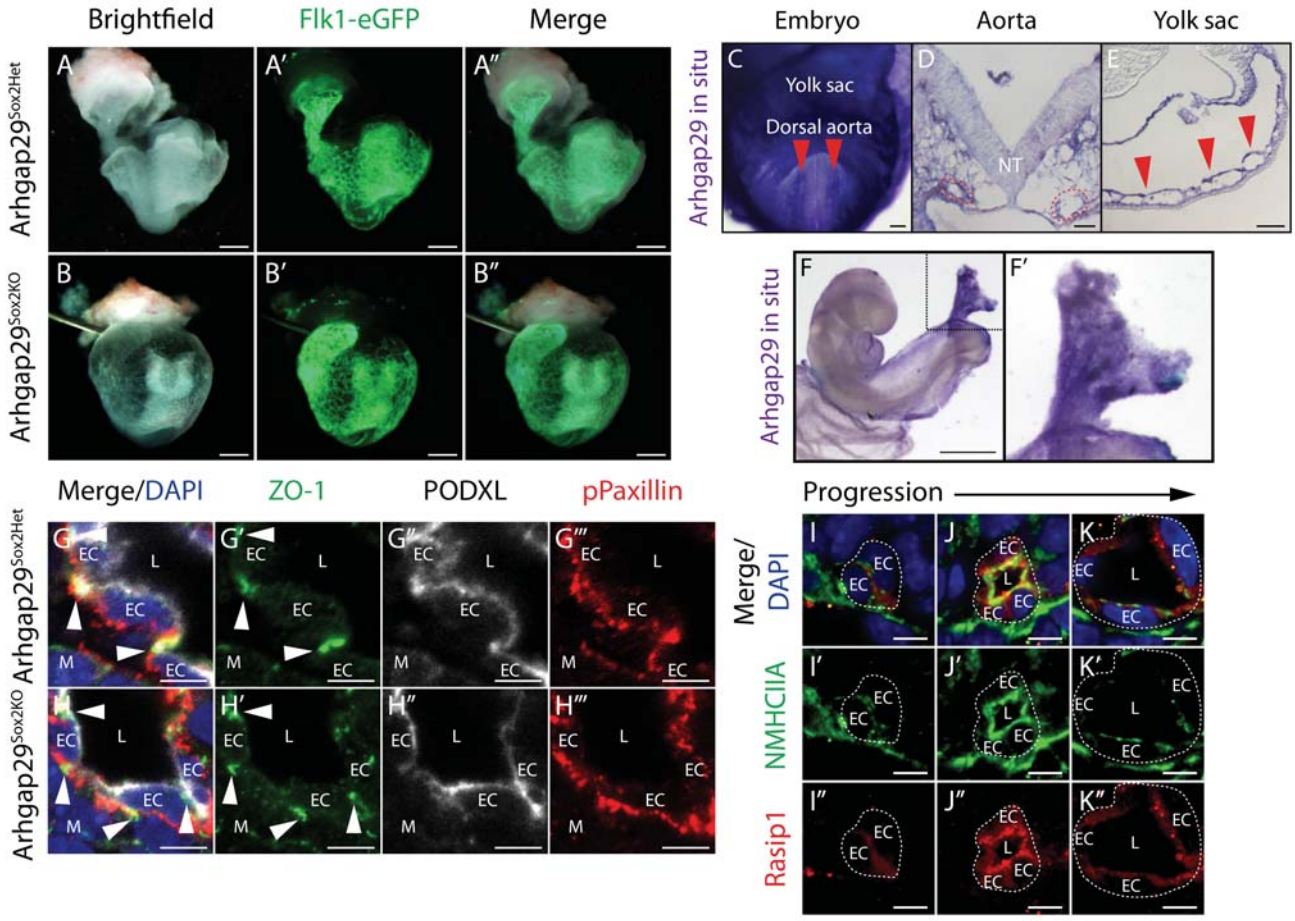
Online Figure V. Rac1 is not necessary for vascular lumen formation but is necessary for vascular remodeling.



Online Figure VI. RhoA suppresses blood vessel lumen expansion by suppressing EC proliferation and cell spreading.



Online Figure VII. Arhgap29 null embryos die embryonically from failure to attach the allantois to the ectoplacental cone.



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