SUPPLEMENT MATERIAL

Online Figure I. Endothelial p120 conditional knockout is embryonic lethal. A p120-null allele was obtained by crossing mice harboring LoxP sites in the p120 gene with mice expressing Cre recombinase driven by the Tie2 promoter. Genotypes were confirmed using PCR (A). Breeding pairs of $p120^{flox/flox};Cre^{-}$ (x) $p120^{flox/wt};Cre^{+}$ were established to obtain mutant mice. Genotypes of neonatal mice revealed lower numbers of conditional mutant mice than the predicted 25% (B). Timed matings were conducted to determine the stage of development at which lethality occurred (C). Embryos were isolated at different time points and graded as viable or nonviable and then genotyped. Embryonic lethality among mutants first began to occur at E11.5, and continued to rise over three consecutive days compared to control littermates.

Online Figure II. Major vessels form normally in p120 endothelial conditional mutants. E9.5 embryos were cryosectioned transversely at heart level and stained for PECAM-1 and p120. p120 loss in the dorsal aortae (indicated by arrowheads) was verified by lack of colocalization between the two markers (C and F, inserts). Sections are shown with dorsal side upward. In all mutants analyzed, dorsal aortae were intact and showed no obvious defects. Scalebar in A is 20μM. To monitor intersomitic vessel formation, E9.5 embryos were whole-mounted and stained for PECAM-1. No defects were seen in mutant embryos (G) compared to control (H). Representative intersomitic vessels are indicated by blue arrowheads. Scalebar in G is 200μM.

Online Figure III. p120-null mutants exhibit decreased microvascular density and disorganized vascular networks. Placentas from E11.5 control and mutant embryos were stained with hematoxylin and eosin. Microvascular density in the labyrinthine layer was reduced in mutant tissue (C and D) compared to control (A and B). Boxes indicate areas enlarged in B and D, and arrowheads indicate representative microvessels. Scalebar in A is 100μM. Surviving E14.5 conditional mutant embryos exhibit reduced blood vessels in the head (G and H) compared to control littermates (E and F). PECAM-1-stained whole mount analysis revealed that microvascular networks in E13.5 brains are disorganized and exhibit decreased density in conditional mutant mice (J and L) compared to control littermates (I and K). Enlarged views of the eye show that the hyaloid vascular network (indicated by asterisks in I and J) is reduced in the mutant embryo (N, compared to M). Scalebar in I is 100μM.

Online Figure IV. Claudin 5 expression is not altered by endothelial p120 deletion. Cryosectioned E11.5 embryos were labeled for claudin 5, VE-cadherin, and p120 to identify blood vessels and verify the vascular ablation of p120 in mutant tissues. Representative fields from control (A-D) and mutant (E-H) mice are shown. No apparent changes in Claudin 5 expression or localization were observed in mutant animals. (compare B to F).

EXPANDED METHODS

Animals

Mice (Mus musculus) with LoxP sites inserted in introns 2 and 8 of the p120 gene were generated as described previously [1]. Tie2-Cre expressing C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) #004128[2]. All animal care and experimentation was performed in accordance with local and national regulations. For viability assessments, embryos that were discolored and partially or completely reabsorbed were scored as nonviable. All comparisons were made between mutant and wild-type littermates.

Tissue Processing and Staining

Tissue samples from adult and embryonic mice were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek U.S.A., Torrance, CA) and cut in 5μM sections using a Leica CM1850 cryostat (Leica Microsystems, Wetzlar, Germany). Sections were then mounted on glass Superfrost Plus microscope

slides (Fisher Scientific, Pittsburgh, PA), and fixed using methanol (Acros Organics, Geel, Belgium) or 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in phosphate-buffered saline with calcium and magnesium (PBS+) containing 2% bovine serum albumin (BSA) (Fisher Scientific), followed by permeabilization with 0.1% Triton (Roche Diagnostics Corporation, Indianapolis, IN) in PBS+, then subsequently stained. E9.5 embryos used for dorsal aorta analysis were incubated in 20% sucrose in PBS+ overnight following paraformaldehyde fixation, then embedded and cryosectioned. Whole-mounted embryos and yolk sacs were also fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton, and stained before mounting on slides. Paraffin-embedded sections from mouse embryos were also stained using hematoxylin and eosin.

Mouse anti-pp120 monoclonal antibody (mAb), rat anti-mouse VE-cadherin (CD144) mAb, and rat anti-mouse PECAM-1 (CD31) mAb were purchased from BD Biosciences (San Jose, CA). Rabbit polyclonal anti-NG2 chondroitin sulfate proteoglycan antibody was purchased from Millipore (Temecula, CA). Mouse anti-claudin 5 antibodies were obtained from Zymed (San Francisco, CA). Rat anti-mouse VE-cadherin mAb BV13 was a gift from Dr. E. Dejana (FIRC Institute of Molecular Oncology, Milan, Italy).

Microscopy

Whole unfixed embryos were photographed with a QIMAGING Retiga EXi-Fast camera and a Leica M2FLIII microscope (Leica Microsystems). Image manipulation was carried out using Adobe Photoshop CS software (Adobe Systems Incorporated, San Jose, CA). Stained tissue sections were analyzed using either a wide-field fluorescence microscope (model DMR-E; Leica, Wetzlar, Germany) equipped with narrow bandpass filters and a digital camera (model OrcaER; Hamamatsu Corporation, Sewickley, PA) or an inverted Leica DMI-6000B microscope equipped with an Infinity II confocal scanning module, 561- and 491-nm lasers, and a Hamamatsu CCD camera (C9100-12). Images were captured and processed with Simple PCI software (Hamamatsu).

For analysis of relative cadherin levels, line scan pixel intensity plots were generated from linear regions of interest (ROI) drawn perpendicular to vessels in Simple PCI. The peak fluorescence of the marker of interest (VE-cadherin, PECAM, or N-cadherin) was recorded along with the corresponding peak fluorescence of p120 from the image intensity 2D profile. Linear ROIs were repeated at 5 or 10 pixel increments along the entire vessel. A minimum of 150 paired data points were collected per field for 3 mosaic E10.5 embryos for VE-cadherin and PECAM comparison. Paired data points were sorted into p120 positive and negative groups based on a background threshold peak fluorescence measured from a non-vascular area. VE-cadherin or PECAM-1 fluorescence levels were plotted relative to p120 fluorescence. A minimum of 75 paired data points were collected from 2 control and 2 mutant E11.5 embryos and average N-cadherin fluorescence of p120-positive vessels and p120-negative vessels was compared.

Yolk sac vascular network analysis

Analysis of vessel branch point number and blind-ending vessels per field was performed by a blinded observer on paired mutant and control whole-mounted yolk sacs from five separate litters of E12.5 embryos. Branches and blind-ending vessels per field were averaged and a t-test was performed in SigmaPlot. For morphometric analysis of vascular networks, a magnification bar image was superimposed on images of PECAM-1 stained whole mount yolk sac samples in Adobe Photoshop CS to measure the diameter of a minimum of five vessels and adjacent avascular space per field for two mutant and two control E12.5 embryos. Vascular diameter (D_V) measurements were made from a straight line drawn perpendicularly across the vessel at a point equidistant from adjacent vessel branches. A contiguous line drawn to bisect the neighboring avascular space into approximately equal halves was measured as the paired avascular diameter (D_A) [3]. The paired measurements were plotted (D_V vs. D_A) and a linear regression was performed in SigmaPlot. The average ratio, D_V / D_A, was also compared between mutant and control groups.

Cell culture

Endothelial cells were obtained using methods previously described [4, 5]. Briefly, hearts and lungs were removed from mice of the $p120^{flox/flox}$;cre^{-} genotype between 8 and 10 days of age, or skins were removed from mice at 3 days of age. Tissues were finely minced and incubated with 2mg/mL collagenase type I (Worthington, Lakewood, NJ) at 37°C on a shaker for 30 minutes before trituration with a cannula. To purify endothelial cells, magnetic Dynal® Dynabeads (Invitrogen, Carlsbad, CA) were coated with PECAM-1 mAb (BD Biosciences) and added to the cell suspension. The beads and attached cells were washed to remove non-endothelial cells. Purified cells were plated on dishes coated with 0.1% gelatin or fibronectin (Sigma-Aldrich, St. Louis, MO) and re-purified during a later passage using magnetic beads coated with ICAM-2 mAb (BD Biosciences). The endothelial identity of the cells was verified by staining with antibodies to PECAM-1 and VE-cadherin (see above). Primary mouse endothelial cells were cultured in high-glucose DMEM (Mediatech, Herndon, VA) with 20% antibiotic/antimycotic solution (Mediatech), 100μg/mL heparin (Sigma-Aldrich), 100μg/mL endothelial cell growth supplement (ECGS) (Biomedical Technologies, Stoughton, MA), 1mM non-essential amino acids (Invitrogen), 1mM sodium pyruvate (Invitrogen), 2mM L-glutamine (Mediatech), and 25mM HEPES (Mediatech). To induce p120 knockout in cell culture, the cells were infected with an adenovirus expressing Cre (gift from Dr. L. Yang, Winship Cancer Institute, Emory University School of Medicine). Wild-type and mutant p120 adenoviruses were generated as previously described [6]. To ensure turnover of previously transcribed p120, a period of 72 hours was allowed before experiments. Control cells were infected with an empty adenoviral vector.

Endothelial barrier function and proliferation assays

Primary mouse endothelial cells were grown to confluency on Costar 3460 Transwell cell culture chambers (Corning Costar, Cambridge, MA). Following p120 ablation by adenoviral Cre (or infection with an empty adenoviral vector), Texas Red-labeled dextran (0.1 mg/mL) in growth media was added to the upper chamber and fluorescence readings were taken from the lower chamber at regular intervals (every 30 minutes) for two hours using a HTS 7000 Plus BioAssay Reader (Perkin Elmer, Waltham, MA) to measure the rate of dextran diffusion across the monolayer of cells, as described previously [7].
The proliferation potential of both p120-null cells and the control cells was evaluated using the in Situ Cell Proliferation Kit, FLUOS (Roche). Briefly, primary mouse endothelial cells were grown to 60-70% confluency on fibronectin-coated coverslips and infected with adenoviral Cre or empty virus. The cells were then incubated with BrdU labeling reagent for 1 hour at 37°. The incorporated BrdU was detected with anti-BrdU FLUOS and DAPI (Sigma-Aldrich) staining was performed to visualize nuclei. The results were analyzed by fluorescence microscopy and the percentages of BrdU positive cells were compared between the p120-null cells and control cells. Cells were also examined for p120 expression by immunofluorescence to verify successful Cre-mediated gene excision.

Western blot

Primary mouse microvascular endothelial cells were isolated from $p120^{flox/flox}$ mice and cultured in complete growth medium. Cells were infected with an adenoviral empty vector or adenoviral Cre recombinase and cultuted for 4 days to allow for p120 deletion and turnover. Cells were harvested in Laemmlin sample buffer (Bio Rad Laboratories, Hercules, CA) and samples were boiled for 5 minutes before loading on 7.5% SDS-PAGE gel for protein separation. Proteins were transferred to nitrocellulose membrane for immunoblotting and probed with antibodies against VE-cadherin: (eBioscience, San Diego, CA #16-1441-82 and BD Pharmingen #550548, diluted to 1:100 each), PECAM-1 (Santa Cruz, Santa Cruz, CA #SC-1506), p120 (Santa Cruz #SC-1101), or β-actin (Sigma-Aldrich #A5441). HRP-conjugated secondary antibodies (Bio-Rad Laboratories) were used at 1:3000 dilution and blots were developed with Amersham ECL Western Blotting Detection Reagents (GE Healthcare, Piscataway, NJ #RPN2106) or Amersham ECL Plus (GE Healthcare #RPN2132).
Growth curve

Primary mouse microvascular endothelial cells were isolated from $p120^{flox/flox}$ mice and cultured in complete growth medium. An equal number of cells were seeded into gelatin-coated 1.9cm^2 wells on day 0. Cells were allowed to attach for 4 hours and then infected with an empty adenoviral vector or adenoviral Cre recombinase. On day 1, cells were harvested by trypsinization and counted on a hemocytometer, with at least 3 wells per condition and 4 samples per well counted by two individual observers. Loss of p120 was confirmed in Cre-infected cells by immunofluorescence microscopy. Data are representative of four separate growth curve experiments.

SUPPLEMENTAL REFERENCES

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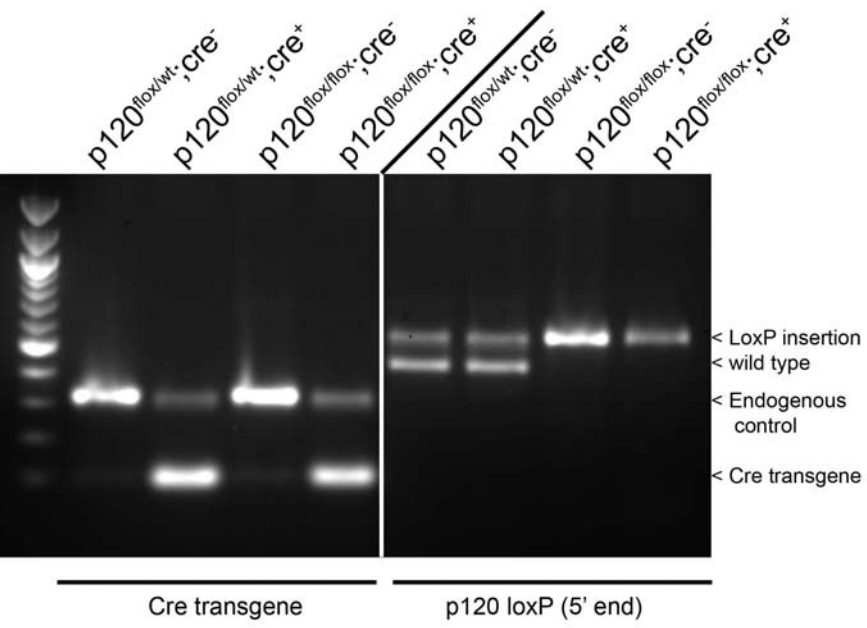

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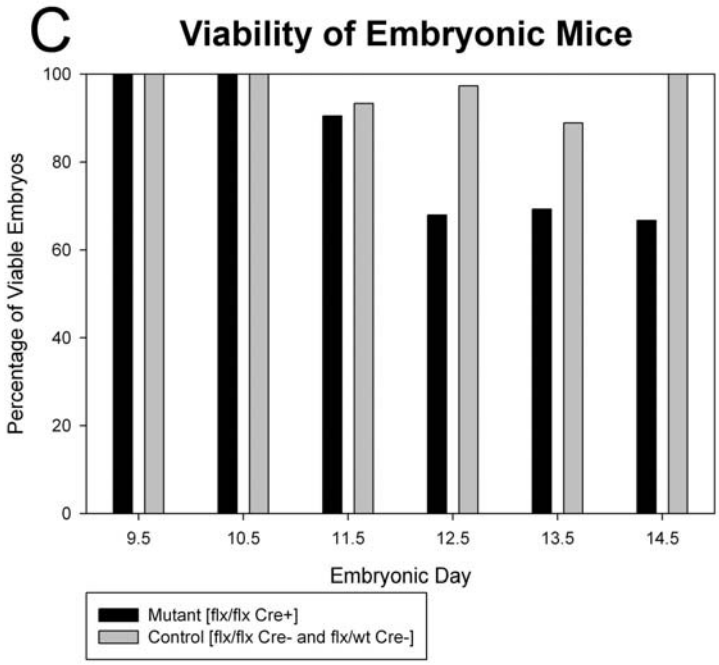
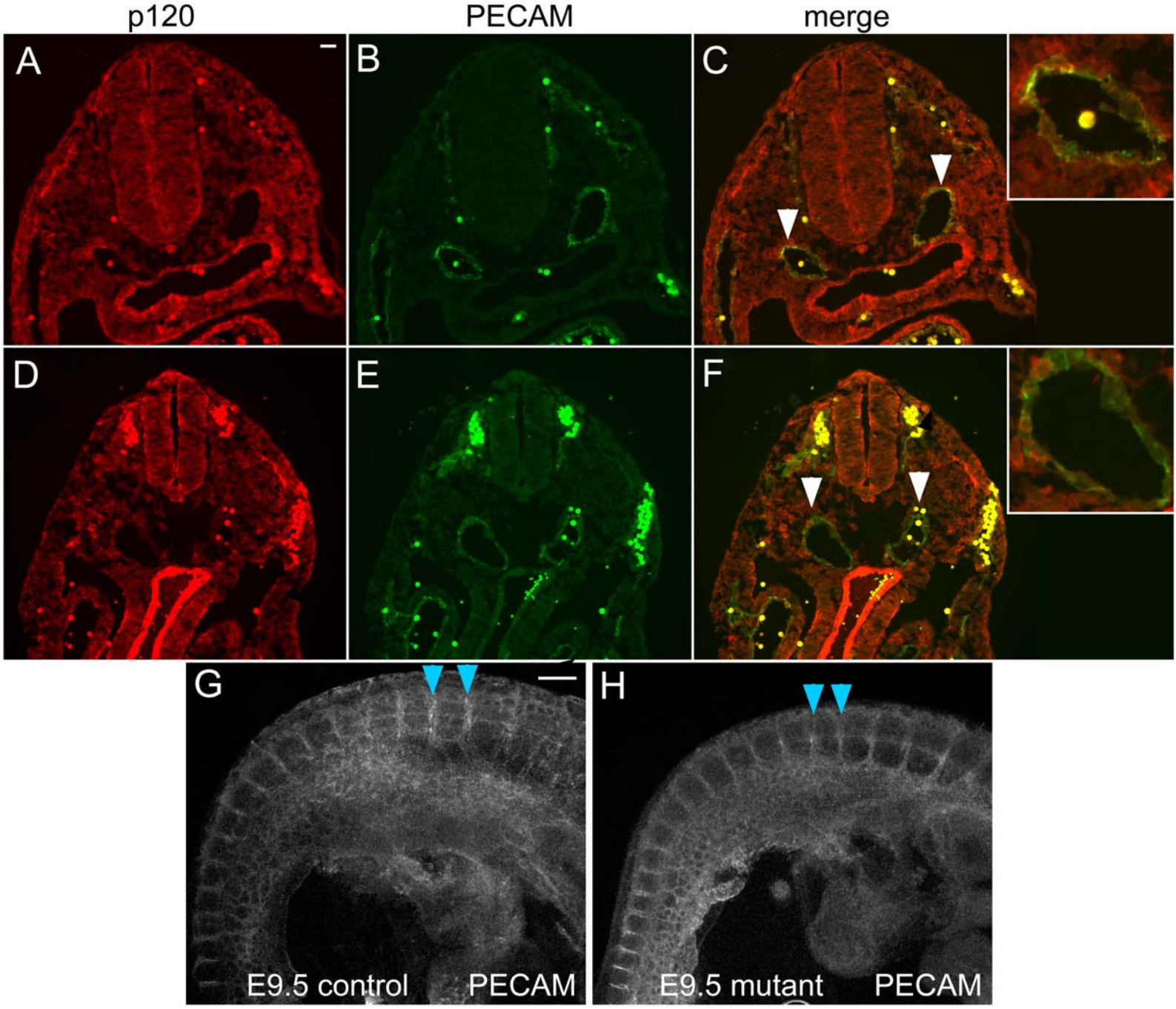


B

Viability of Neonatal Mice

Genotype	Number	% Actual	% Expected
$p120^{flox/wt};cre^{-}$	95	35.2	25.0
$p120^{flox/wt};cre^{+}$	55	20.4	25.0
$p120^{flox/flox};cre^{-}$	76	28.1	25.0
$p120^{flox/flox};cre^{+}$	44	16.3	25.0

Online Figure I



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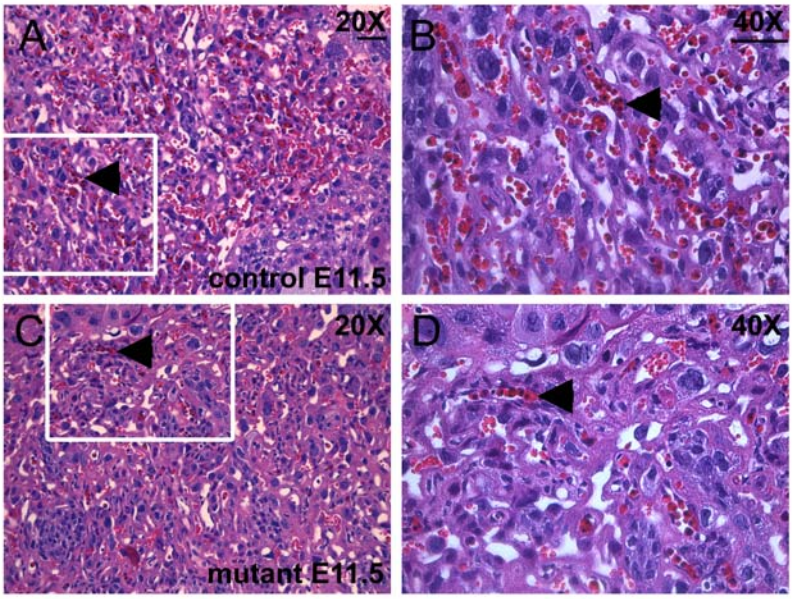

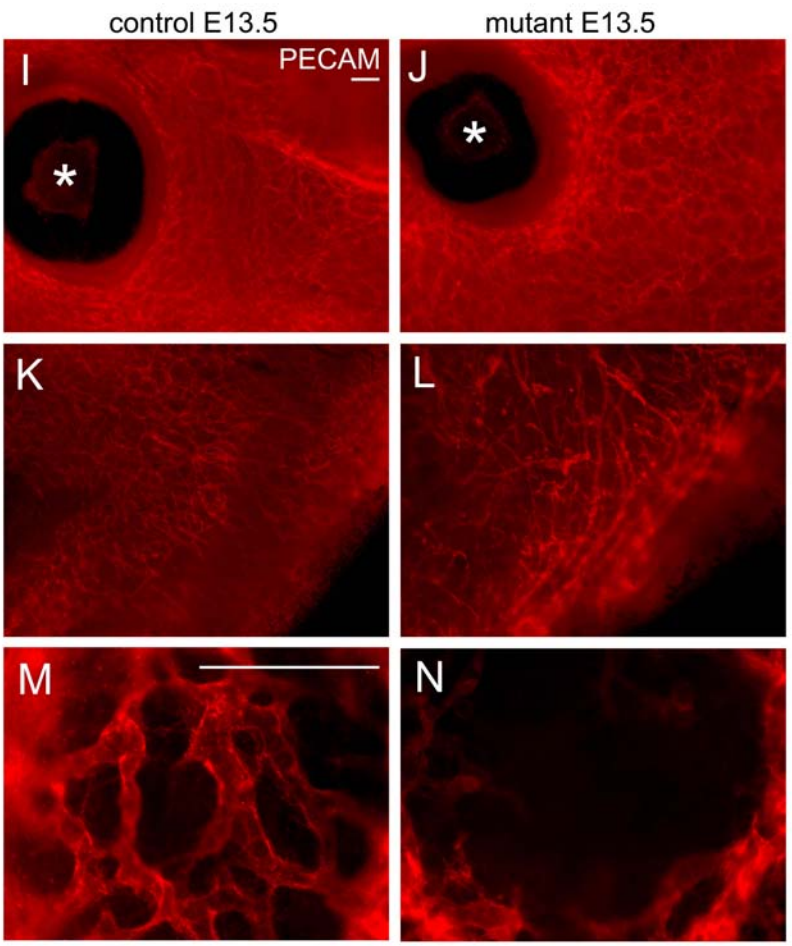

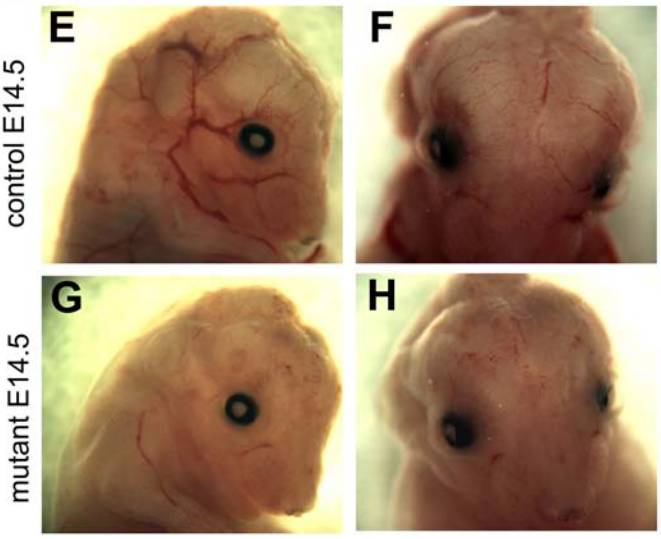

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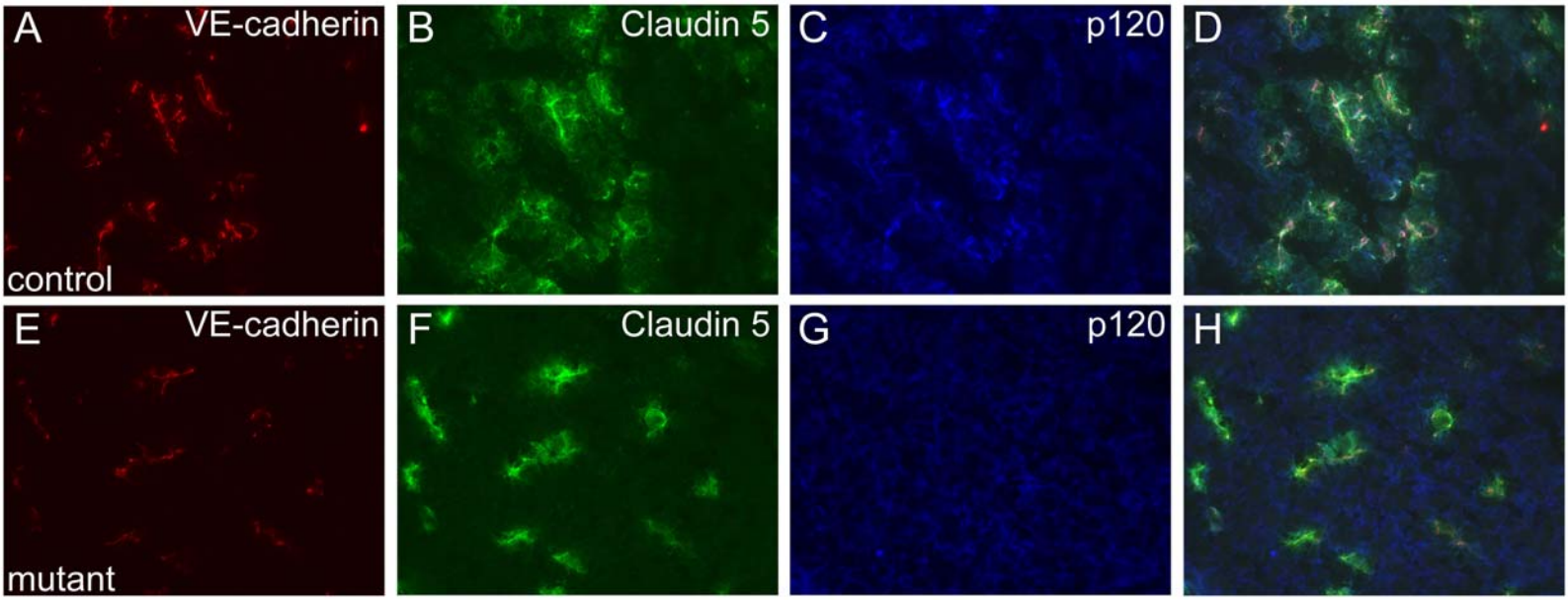

Online Figure IV