Von Hippel-Lindau (VHL) mutations disrupt vascular patterning and maturation via Notch

SI MATERIALS AND METHODS

Cell Culture, Immunostaining, and Vessel Analysis

The following methodologies have been previously described: *Vhl*$^{-/-}$, *Vhl*$^{2B/2B}$ mutant and WT ES cell derivation and maintenance[1], ES cell differentiation into primitive vascular networks[2], and fixation and staining of day 8 ES cell cultures[3]. The primary antibody used to label endothelial cells was rat anti-mouse PECAM-1 (BD Biosciences) at 1:1000, followed by incubation with the secondary antibody donkey anti-rat AlexaFluor488 (IgG; H+L) at 1:1000 (Invitrogen). Cultures were imaged using a confocal microscope (Leica TCS SP5) with a ×20 objective, and 5-8 z-axis confocal scans were generated and flattened. Vessel images were traced and processed using ImageJ plugins (BoneJ/Skeletonize 3D, Skeleton/AnalyzeSkeleton (2D/3D)) to generate branch point densities.

Endothelial Cell Enrichment and Real-Time Quantitative PCR Analysis

Magnet-assisted cell sorting (MACS) (Miltenyi Biotec) was used to isolate and enrich for endothelial cells from day 8 differentiated ES cell cultures as previously described[4]. Following TRIzol (Invitrogen) digestion of endothelial cell isolates, RNA was extracted with 1-Bromo-3-chloropropane (Sigma) and ethanol and purified with an RNeasy Plus Kit (Qiagen). Superscript II RT kit (Invitrogen) was used to retro-transcribe the RNA. Using the Applied Biosystems 7900HT Fast Real-Time PCR System and the TaqMan Universal Master Mix II (Applied Biosystems), real-time quantitative PCR was performed in triplicate using the following PCR primers: TATA binding protein (*Tbp*) (for normalization), *Vegf-A*, *Vegfr2/Flk-1*,

Dll4, *Jagged1*/*Jag1*, *Notch3*, and *Hey2* (Applied Biosystems). Expression changes were determined by the comparative CT method, and *Tbp* values facilitated normalization.

Mutant Mice and Inducible Genetic Recombination Experiments

All animal experiments were conducted with approval from the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. Our protocols are reviewed and approved by IACUC, and our NIH/PHS Animal Welfare Assurance Number is A3410-01 (Expires: 4/30/2021). Mice expressing Cre-recombinase ubiquitously [*Tg(UBC-cre/ERT2)*, JAX #007001] were bred with *R26R tdTomato* [*Gt(ROSA)26Sortm*$^{14(CAG-tdTomato)Hze}$, JAX #007914] to yield offspring homozygous for *R26R tdTomato* and cre/ERT2 positive or negative (*UBC*$^{CreER/+}$ or *UBC*$^{+/+}$, respectively)[5]. Pups were intraperitoneally (i.p.) injected daily with 1 mg/ml of tamoxifen (Fisher) from postnatal day 1 (P1) to 3, and retinal harvest occurred on P5. For Notch signaling gain-of-function experiments, this same Cre driver was crossed with R26R Notch1 Intracellular Domain (N1ICD) mice [*Gt(ROSA)26Sor*$^{tm1(Notch1)Dam}$/J, JAX #008159]. Tamoxifen was administered as described, and retinal harvest occurred on P7.

Retina Immunostaining, Imaging, and Analysis

Eyes were fixed by whole animal perfusion with 0.5% paraformaldehyde (PFA) followed by immersion in 2% PFA for 2 hours at room temperature. Following PBS rinse, retinas were dissected from the whole eye, immersed in 100% cold ethanol for 30 mins, rinsed with PBS containing 1% Triton X-100 (PBS-T) for 30 mins at room temperature, and then blocked in 3% donkey serum in PBS-T for 1 hour at room temperature. Primary antibody incubation was performed in PBS-T overnight at 4°C with isolectin GS-IB4 conjugated to AlexaFluor 488

(Molecular Probes, #I21411, 1:100). Retinas were washed 3×5 mins in PBS-T, flattened and mounted on slides in 50:50 PBS:glycerol with 1.5 coverslips. Images of retina whole mounts were acquired with a Zeiss LSM 880 confocal microscope with a ×40 objective, and 10-15 z-axis confocal scans were acquired and compressed. ImageJ was used to measure: 1) vessel branch point densities, as described above, 2) the percentage of the retina vessel area relative to total retina area, and 3) the number of vessel sprouts per length of the vascular front.

FIGURE LEGENDS

Supplemental Figure 1. *Vhl* null and type 2B mutations have differential effects on ES cell-derived blood vessel dysmorphogenesis and gene expression. (A-C) Representative confocal images of Day 8 WT (A), $Vhl^{-/-}$ (B), and $Vhl^{2B/2B}$ Mutant (C) embryonic stem (ES) cell-derived blood vessels antibody labeled for platelet endothelial cell adhesion molecule-1 (PECAM-1)/CD31. Scale bar, 100 μm. (D) Branch points per vessel length measured from Day 8 vascular networks. n=3 experiments for each group. Values are averages ± SEM. *P≤0.05 vs. WT and $Vhl^{-/-}$ by one-way ANOVA followed by pair-wise comparisons with a 2-tailed Student t-Test. (E-J) Real-time quantitative polymerase chain reaction of vascular endothelial growth factor (VEGF) and Notch pathway components: VEGF-A (E), VEGF Receptor-2 (VEGFR2)/Flk-1 (F), Delta-like 4 (Dll4) (G), Jagged1 (H), Notch3 (I), and Hey2 (J) from WT, $Vhl^{-/-}$, $Vhl^{2B/2B}$ mutant endothelial cell-enriched preparations. (E) *P≤0.05 vs. WT and $Vhl^{-/-}$. (F) *P≤0.05 vs. WT. (G) *P≤0.05 vs. WT, **P≤0.05 vs. WT and $Vhl^{-/-}$. (H) *P≤0.05 vs. WT. (I) *P≤0.05 vs. WT. (J) *P≤0.05 vs. WT and $Vhl^{-/-}$. Values are averages ± SEM. n=3 experiments for each group. All statistical comparisons performed using one-way ANOVA followed by pair-wise comparisons with a 2-tailed Student t-Test.

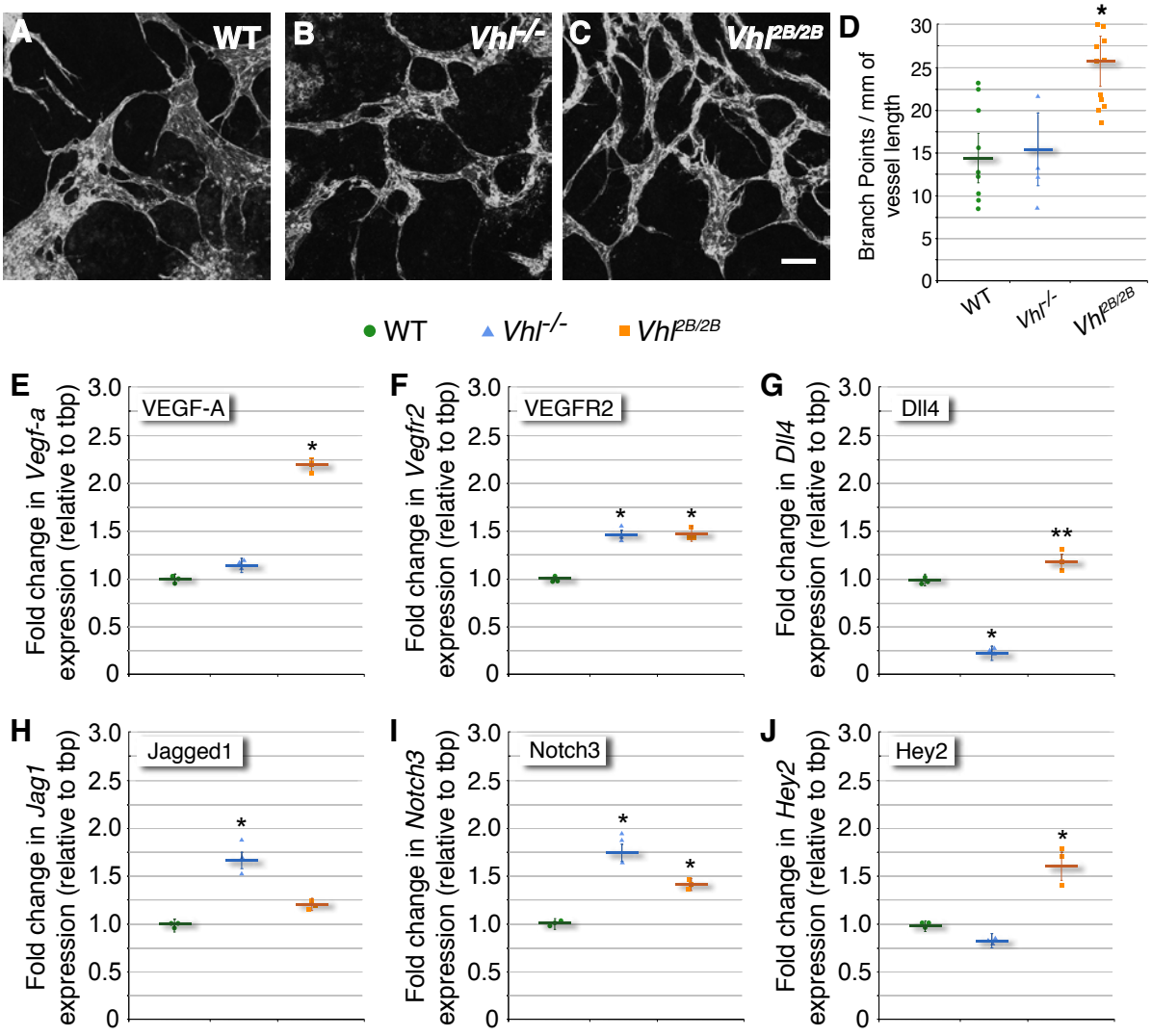
Supplemental Figure 2. *Vhl* 2B mutant and *floxed* conditional deletion constructs and *in vivo* experimental design. (A) Schematic of the genetic constructs designed to yield the *Vhl* 2B mutation and the *Vhl* floxed allele (Exon 1). Tamoxifen exposure and cre-recombinase activity yield a complete inactivation of the *Vhl* gene or presence of only the 2B *Vhl* mutant knock-in construct. (B) Timeline for postnatal tamoxifen injection, administration

of DMSO with and without DAPT (if relevant), and tissue collection (TC), with associated Figures referenced.

Supplemental Figure 3. Conditional gain-of-function for Notch signaling reduces vessel formation in P7 mouse retinas similar to the induction of a *Vhl* null scenario. (A-B) Representative images of postnatal day 7 (P7) mouse retinal vasculature stained with isolectinB4. Regions are shown for *UBC*$^{+/+}$*; Rosa*^{N1IC} (A) and Notch1 Intracellular Domain (N1IC) gain-of-function (GOF) (i.e. *UBC*$^{CreER/+}$*; Rosa*^{N1IC}*)* (B) Scale bar, 100 μm. (C) Branch points per mm of vessel length measured from P7 vascular networks. *P≤0.05 vs. control *UBC*$^{+/+}$*; Rosa*^{N1IC}. (D) Percent of vascular area per total area within field of view. *P≤0.05 vs. control *UBC*$^{+/+}$*; Rosa*^{N1IC}. (E) Sprouting extensions per mm of vascular front. *P≤0.05 vs. *UBC*$^{+/+}$*; Rosa*^{N1IC}. Values are averages ± SEM. All statistical comparisons performed using one-way ANOVA followed by a 2-tailed Student t-Test.

Supplemental Figure 4. Summary of each *Vhl* genetic background and the associated changes in Notch signaling, retinal vessel branching, and arterial maturation, with and without Notch inhibition. (A-D) Arrow direction and number represent the nature of the observed changes for each category: Flat Arrow = No Changes/Normal, Up = Increase, Down = Decrease; 1 Arrow = Normal to Slight Change, 2 = Moderate, 3 = High, 4 = Severe. Each genetic background is represented by a different color: (A) *UBC*$^{+/+}$*; Vhl*$^{fl/fl}$: green, (B) *UBC*$^{+/+}$*; Vhl*$^{fl/2B}$: red, (C) *UBC*$^{CreER/+}$*; Vhl*$^{fl/fl}$: blue, and (D) *UBC*$^{CreER/+}$*; Vhl*$^{fl/2B}$: orange. Darker colors represent each genetic background in the context of Notch signaling inhibition.

Supplemental Figure 5. TdTomato expression within retina endothelial cells confirming tamoxifen-induced genetic recombination. (A-C) Representative images of postnatal day 5 (P5) mouse retinal vasculature labeled with isolectinB4 (A) and expressing the red fluorescent reporter TdTomato (C) following 3 day exposure to tamoxifen.



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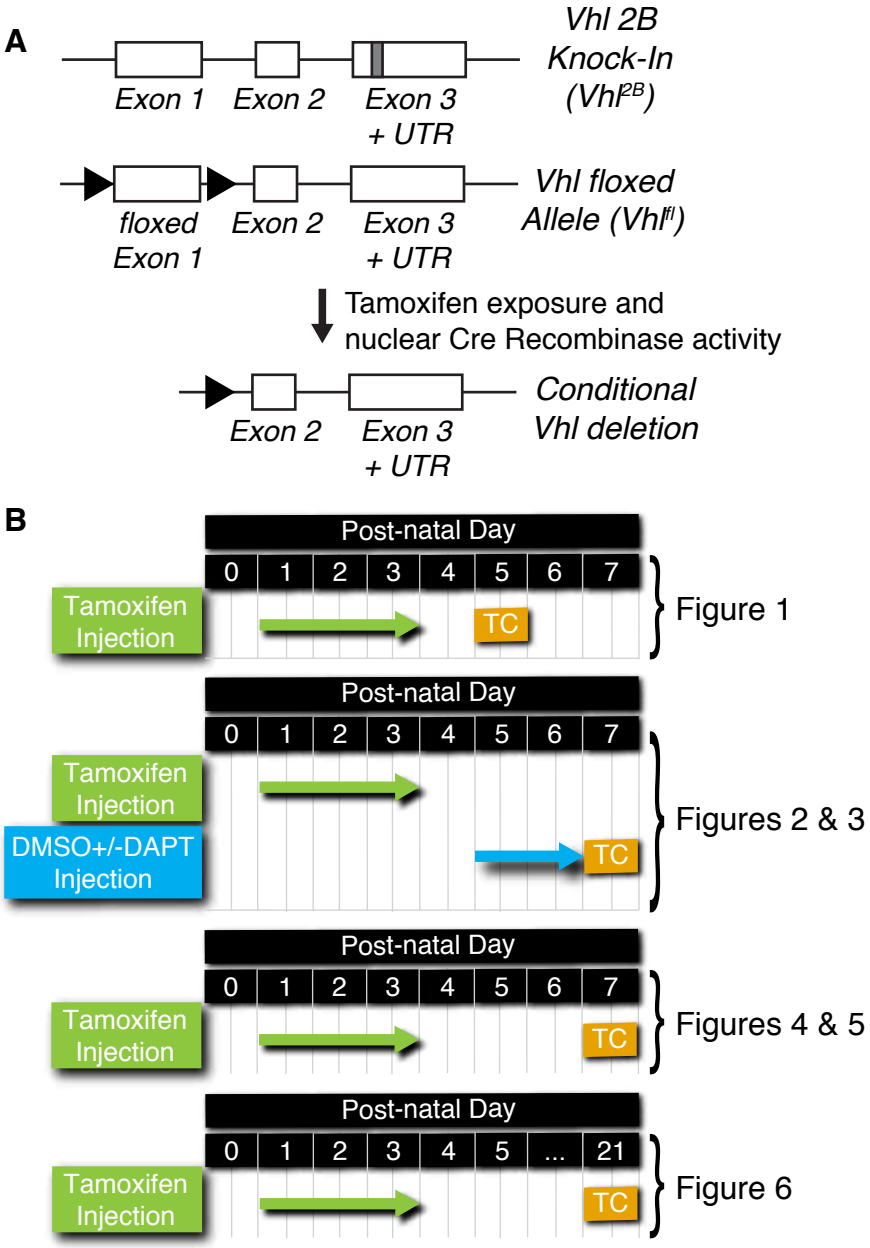

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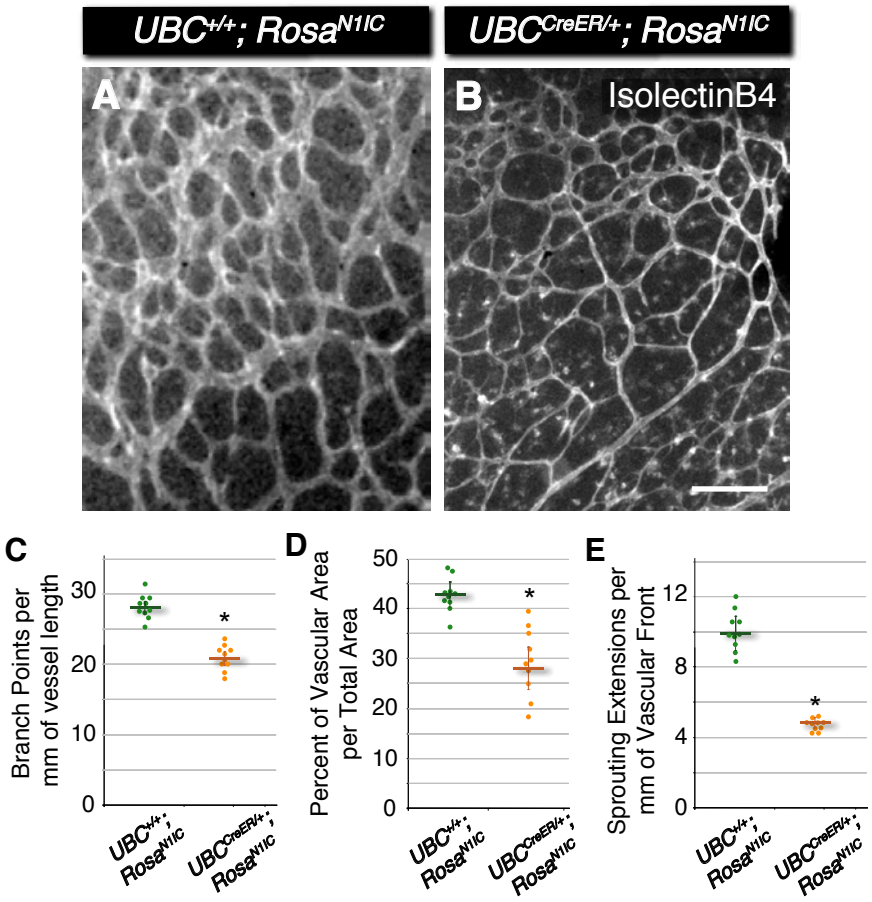

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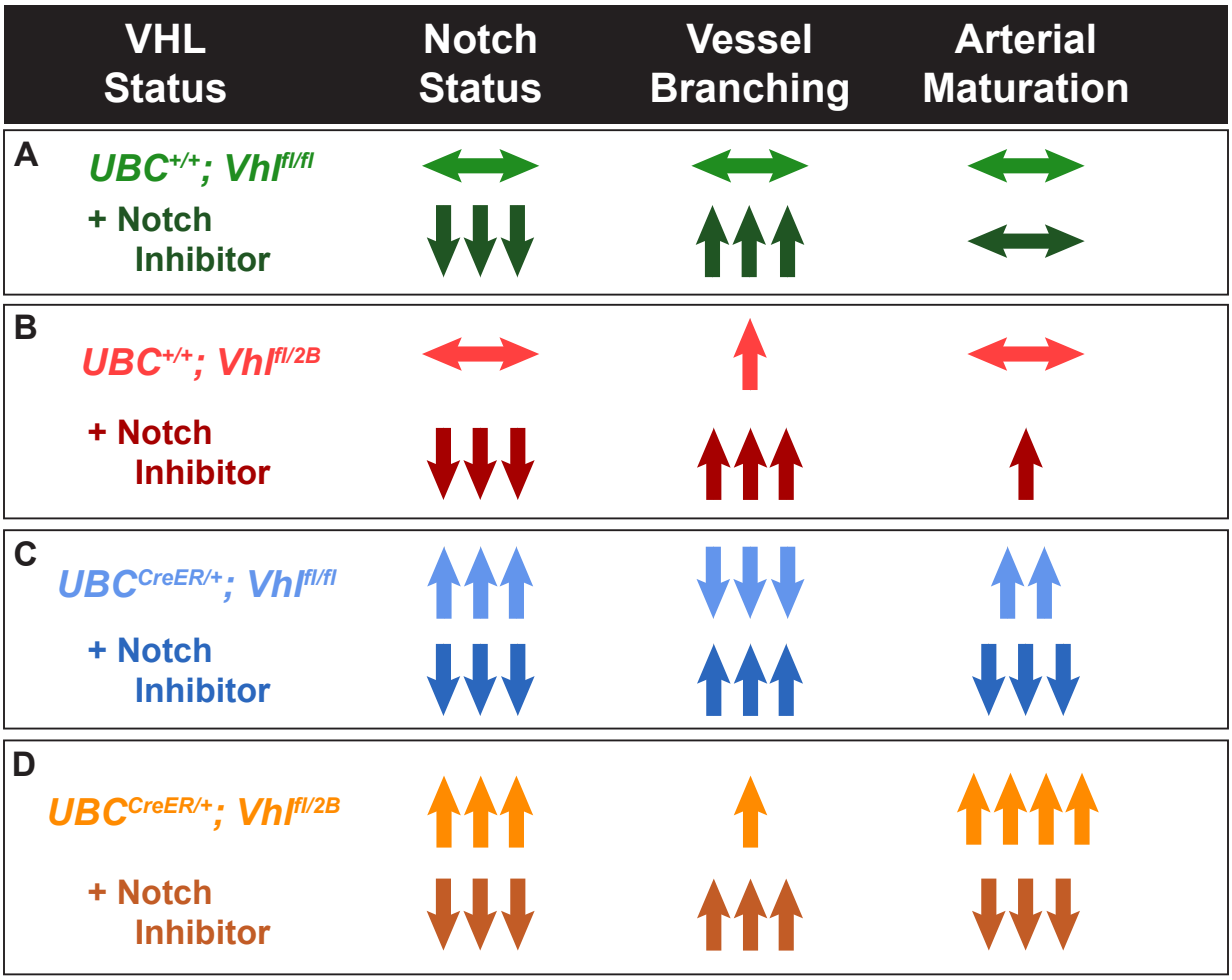

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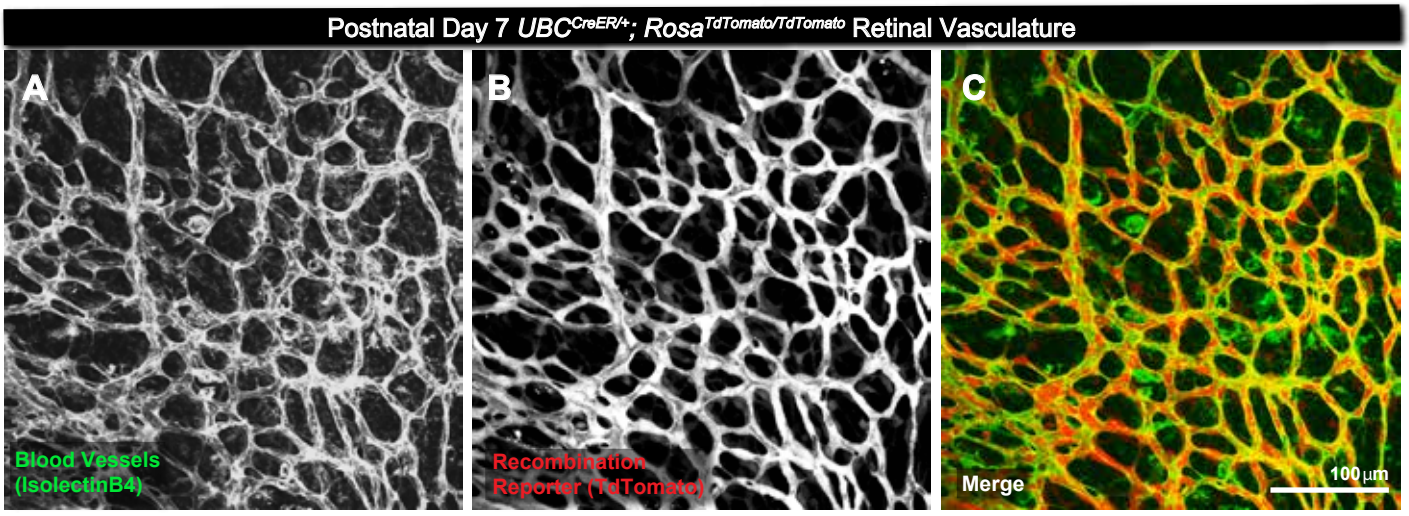

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