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SHORT REPORT

Figures and figure supplements

R-propranolol is a small molecule inhibitor of the SOX18 transcription factor in a rare vascular syndrome and hemangioma

Jeroen Overman *et al*

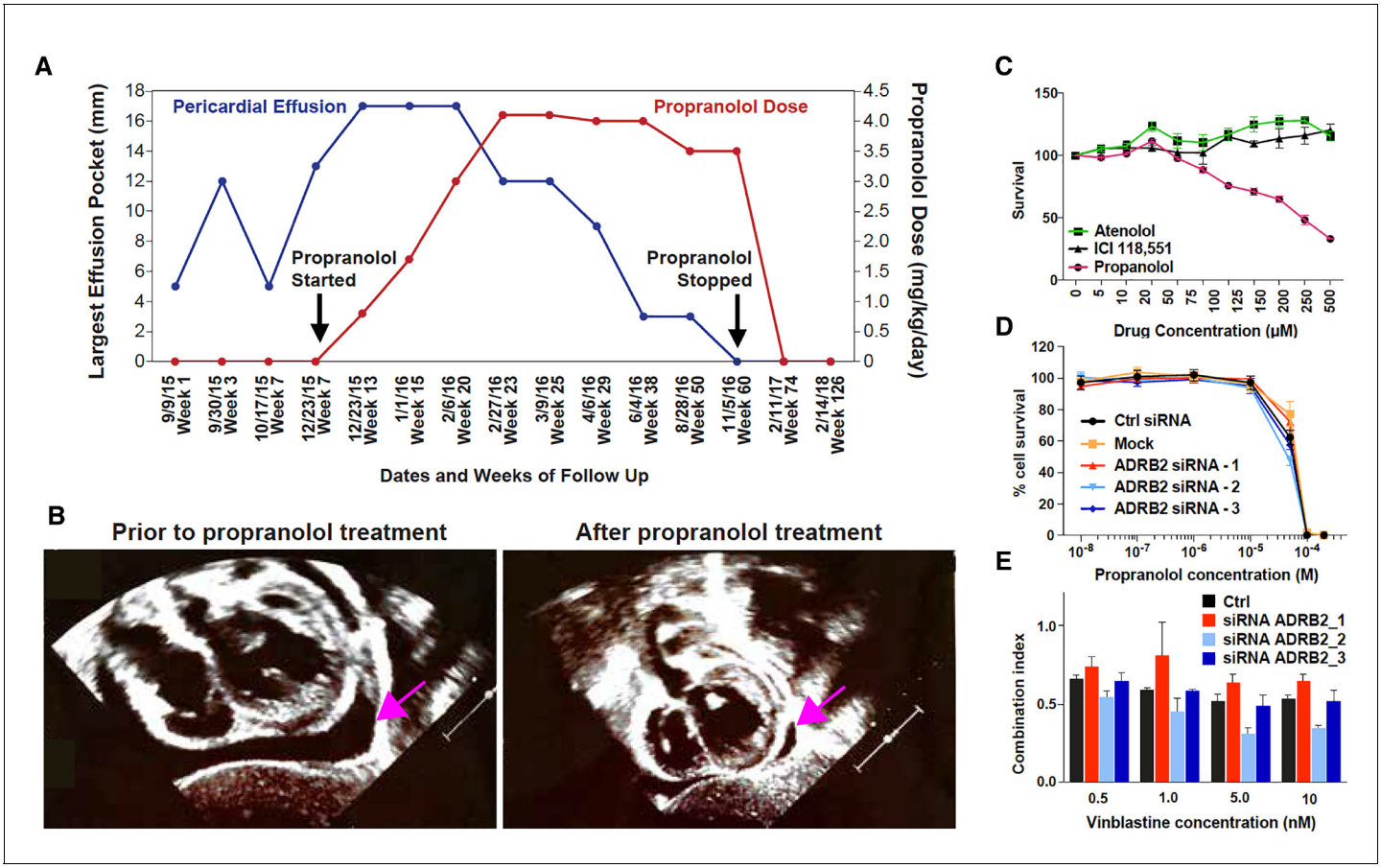

Figure 1. Propranolol treatment alleviates pericardial effusion severity in HLTRS patient and mediates β-adrenergic independent effects. (**A**) A 17 months old HLTRS patient was treated with propranolol, starting at 0.8 mg/kg/day in three divided doses and increasing gradually every 2–3 weeks to maximum of 4.1 mg/kg/day (red curve). In parallel the volume of ventricular peri-cardial effusion was measured at the end of the diastole (blue curve). (**B**) Echocardiography revealed that pericardiocentesis was not required anymore after propranolol treatment due to significant reduction in pericardial effusion (pink arrow) which did not recur as of June 2018 (time of the study). (**C**) Fetal endothelial colony forming cells (ECFC) were isolated from term placenta from healthy donors, expanded for three passages, and subjected to propranolol treatment followed by analysis of survival (percentage) as compared to vehicle control (DMSO). Propranolol affected the survival of ECFC at equivalent doses whereas Atenolol (specific β1 blocker) and ICI118,551 (specific β2) did not. (**D**) Cell survival assay performed on ISO-HAS angiosarcoma cells after transfection with three different siRNA sequences targeting *ADRB2* and following 72 hr incubation with propranolol (racemic mixture). Alamar Blue assay ; *Points*, mean of at least four independent experiments ; *Error bars*, standard error. (**E**) Combination indexes of propranolol and vinblastine in ISO-HAS angiosarcoma cells after transfection with three different siRNA sequences targeting *ADRB2* and following 72 hr drug incubation (50uM). Alamar Blue assay ; *Bars*, mean of at least four independent experiments ; *Error bars*, standard error. Statistical analysis for C was performed using Mann-Whitney non parametric t-test and for D-E using an unpaired two-tailed t test.

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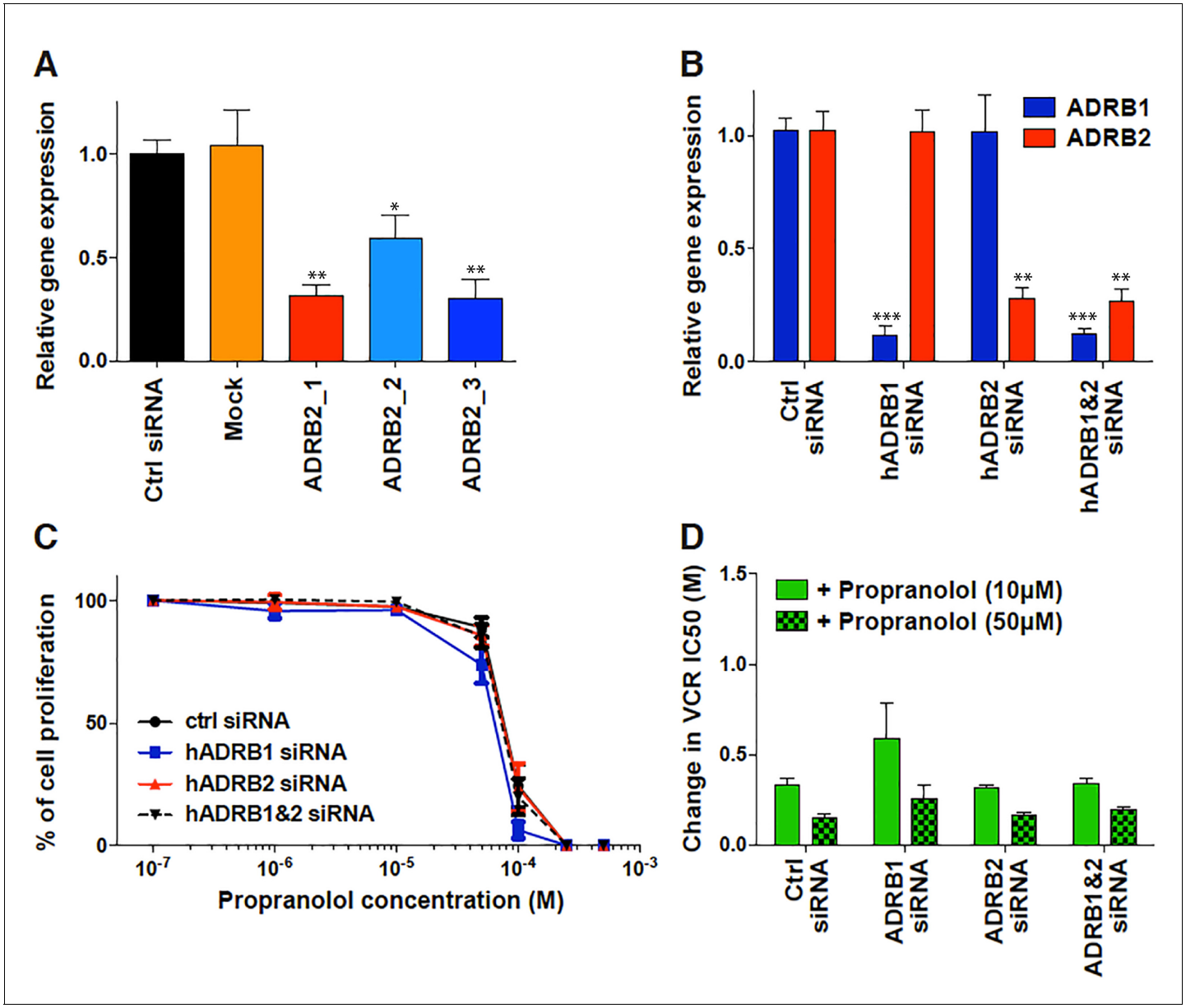

Figure 1—figure supplement 1. β-adrenergic independent effects of propranolol. (**A and B**) β-adrenergic receptor gene expression following 72 hr transfection with siRNA in ISO-HAS angiosarcoma cells (**A**) and SHEP neuroblastoma cells (**B**), as determined by qRT-PCR using specific primers for *ADRB1* and *ADRB2*, and compared with *YWHAZ* as housekeeping gene. (**C**) Cell viability assay performed by Alamar Blue on SHEP neuroblastoma cells following siRNA transfection and 72 hr drug incubation; *Data points*, mean of at least four independent experiments; *Error bars*, standard error. (**D**) Change in vincristine EC50 (i.e. concentration causing a 50% reduction in cell viability after 72 hr drug incubation) in the presence of 10 or 50 µM propranolol as compared with vincristine alone following transfection of SHEP neuroblastoma cells with either *ADRB1*, *ADRB2* or both siRNA. *Bars*, mean of at least four independent experiments ; *Error bars*, standard error. Classic unpaired Student's t test with the following legend: *p<0.05; **p<0,01; ***p<0001.

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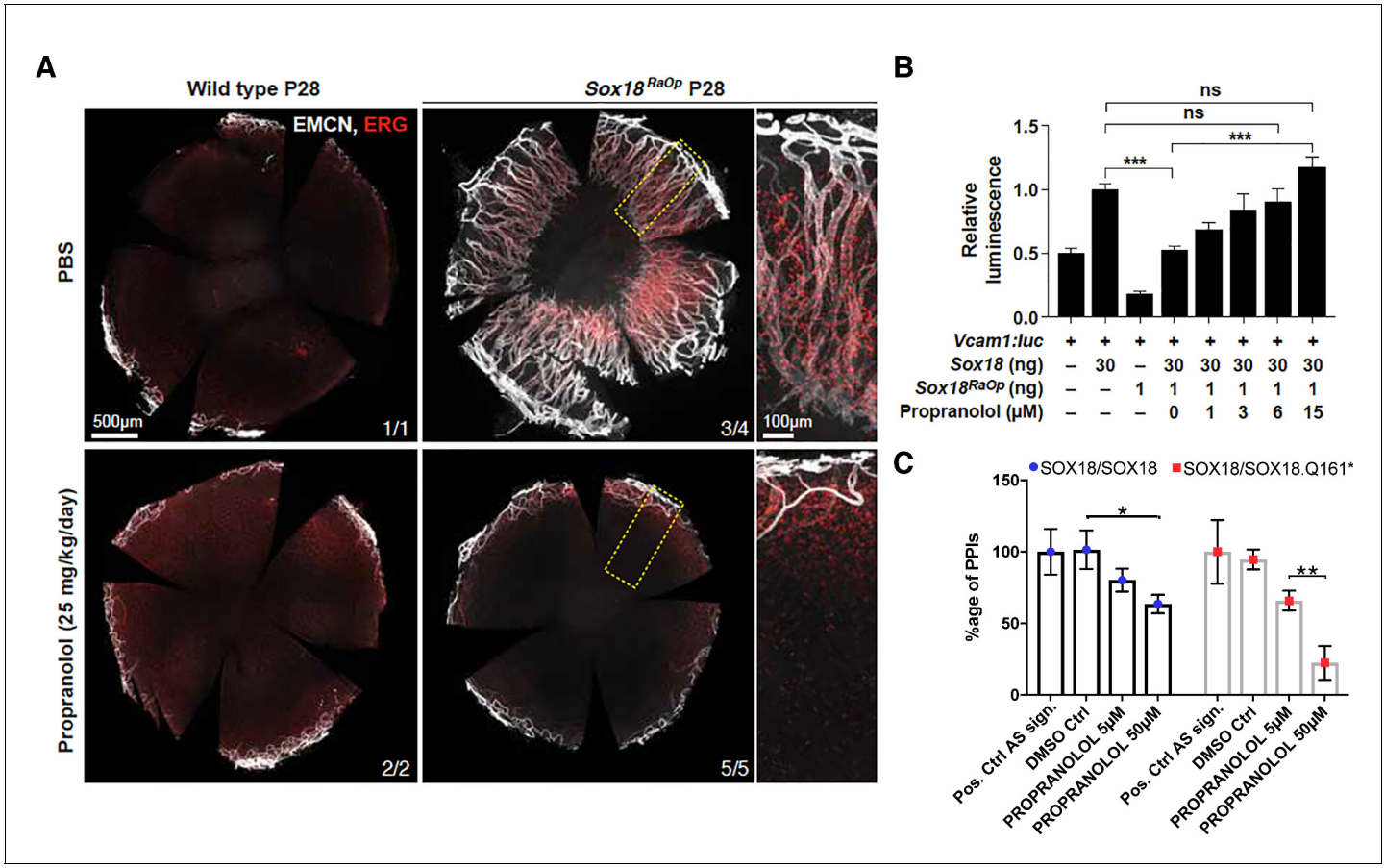

Figure 2. Propranolol rescues corneal neo-vascularization phenotype in a mouse pre-clinical model of HLTRS and SOX18 dominant-negative transcriptional repression via protein-protein interaction disruption. (**A**) Fluorescent images of corneal flat mounts, showing blood vessel penetration into the cornea at P28 stage using endothelial cell markers ERG and endomucin (EMCN). *Sox18* WT and *RaOp* mice were treated from P8 to P28 with either vehicle PBS or propranolol. Propranolol has no obvious effect on WT cornea, but prevents CNV in *RaOp* pups. Number of predominant phenotype shown in bottom right. Scale bar left 500 µm, right 100 µm. (**B**) COS-7 cells were transfected with SOX18 responsive Vcam1:*luciferase* construct and a combination of *Sox18* wild type plasmid DNA and RaOp plasmid DNA. *RaOp* behaves in a dominant negative fashion and is capable to inhibit SOX18 WT function even at low 30:1 (w/w DNA) allelic ratios. Addition of propranolol to the media rescues SOX18 dependent activity of the Vcam1 promoter in presence of *RaOp*. Effect is concentration dependent and normal SOX18 activity on this construct is achieved at 15 µM propranolol. *Sox18*. *** p-value≤0.001, Kruskal-Wallis multiple comparison test. Data shown is mean ± SD of n ≥ 8. (**C**) The bar graph shows ALPHAScreen signal as a measure of the level of protein-protein interaction between SOX18 and its mutant counterpart RaOp (red square) and SOX18 homodimer formation (blue dot) in absence or presence of propranolol treatment. Propranolol is a small compound with the ability to disrupt SOX18 self-recruitment. Statistical analysis in 2B one-way ANOVA with Bonferroni post-hoc test and in 2C ANOVA Sidak's multiple comparison test. Analysis of the protein pair by ALPHAScreen assay was performed in three different biological experiment with three technical replicates.

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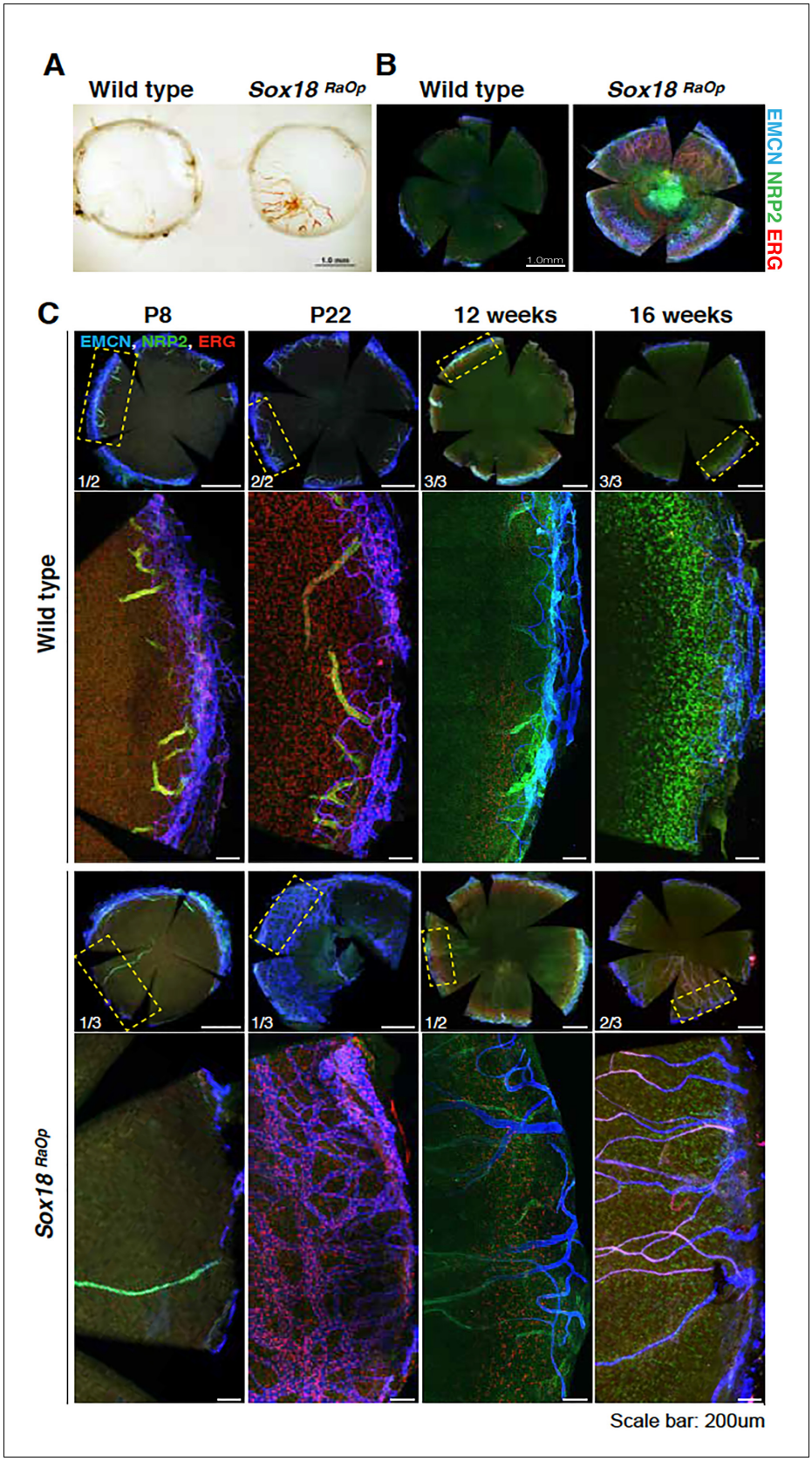

Figure 2—figure supplement 1. Time course of the corneal phenotype. (**A**) Corneal flat mount from adults (8 weeks old mice) wild type control and RaOp ±animals showing blood vessel invasion. (**B**) Immunofluorescence

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Figure 2—figure supplement 1 continued

further confirms blood (Endomucin, EMCN, blue, ERG, red) and lymphatic (Neuropillin-2, NRP2, green) vessels outgrowth in corneal tissues (8 weeks old mice). (**C**) Corneal tissues were harvested from mice at different stages, ranging from postnatal day 8 to 16 weeks of age. Penetrance of corneal vessels was analysed by immunofluorescence for vascular endothelial cells markers EMCN, NRP2 and ERG. At early stages (**P8–P22**), wild type pups have NRP2-positive, EMCN-negative vessels projecting from the sclera into the cornea. This is not observed at later stages. Onset of corneal neo-vessel formation in *RaOp* mice appears between P22 and 12 weeks. Scale bar whole corneas: 0.5 mm, detail: 0.2 mm.

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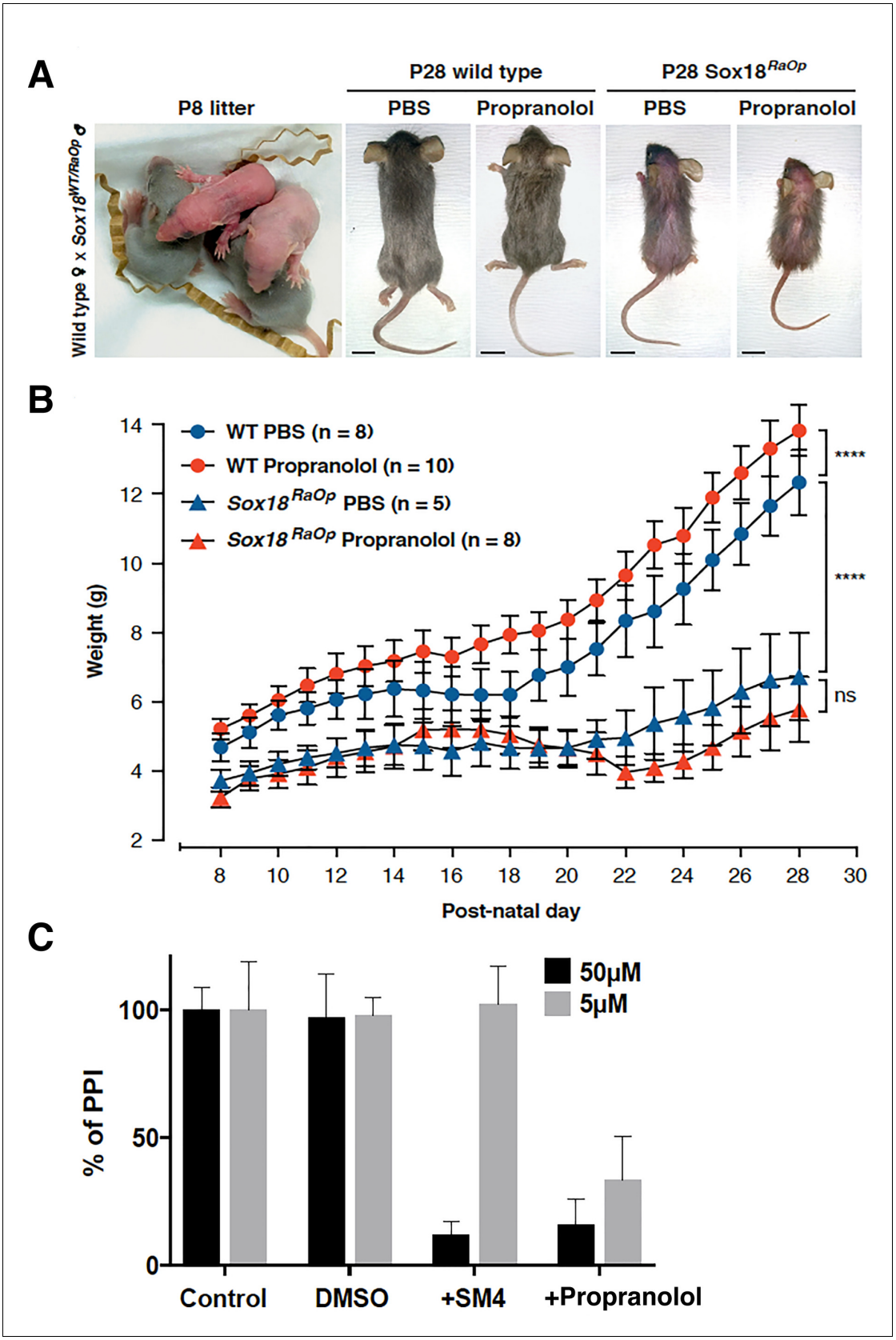

Figure 2—figure supplement 2. Effects of propranolol on the overall morphology of treated *RaOp* mice and disruption of SOX18/RBPJ protein-protein interaction. (**A**) DBA/2JArc wild type females were crossed with a heterozygous *RaOp* male (B6D2-RaOp/J) to generate *Sox18* mutant pups

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Figure 2—figure supplement 2 continued

heterozygous for the Ra^{Op} allele. *RaOp* mice have a distinct sparse coat, and are smaller than wild type littermates. Pups were treated daily through oral gavage with vehicle PBS or 25 mg/kg/day propranolol, from P8 until P28. No obvious gross morphological defects were observed in propranolol treated mice compared to vehicle PBS. Scale bar 1 cm. (**B**) Weight of the mice was recorded daily throughout the time course of the treatment. *RaOp* weighted less than wild type littermates. Propranolol increased the mean weight of the wild type mice, but not of *RaOp* mice. (**C**) The bar graph represents ALPHAScreen signals as a measure of the protein-protein interaction between SOX18 and RBPJ (NOTCH signaling effector) transcription factors. Control conditions (ctrl and DMSO ctrl) show the reference signal for SOX18/RBPJ interaction, results are shown as a fold response compared to control. Upon addition of SOX18 small molecule inhibitor Sm4, PPI is disrupted at 50 µM, whereas propranolol showed efficacy from 5 µM. Experiments were performed in three independent replicates. **** *p*adj ≤1e-4, 2way ANOVA Tukey multiple comparisons. Data shown is mean ± SEM, n-number is indicated in graph.

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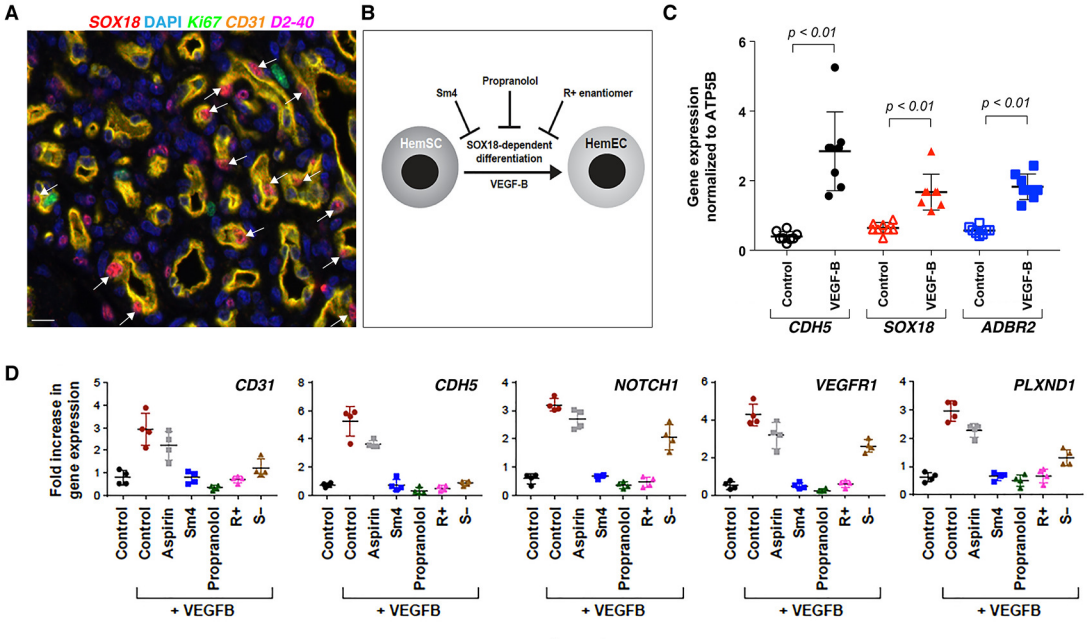

Figure 3. The R(+) enantiomer of propranolol and SOX18 small molecule inhibitor halt infantile hemangioma stem cell differentiation. (**A**) Infantile hemangioma tissue section stained for *SOX18* (red), *Ki67* (green), *CD31* (orange), *D2-40* (pink) and DAPI (blue) reveals the presence of *SOX18* expression in a large subset of hemangioma endothelial cells (arrows). (**B**) Schematic representation of infantile hemangioma stem cell (HemSC) endothelial differentiation assay. VEGF-B stimulates HemSC to differentiate into hemangioma endothelial cells (HemEC). This differentiation process is inhibited by propranolol, the R(+) enantiomer of propranolol, and by *SOX18* small molecule inhibitor Sm4 (all at 5 uM). (**C**) VEGF-B treatment of HemSC from four different infantile hemangiomas resulted in increased *CDH5* (an endothelial cell marker), SOX18 and ADBR2 (β2 adrenergic receptor) mRNA. Means and standard deviations are shown. (**D**) The effects of SOX18 inhibitor Sm4, its scaffold aspirin as a negative control, propranolol and its purified R(+) and S(-) enantiomers on HemSC-to-HemEC differentiation from two infantile hemangioma patients. Endothelial differentiation markers, *CD31* and *CDH5* and hemangioma endothelial markers *NOTCH1*, *PLXND1* and *VEGFR1* under each treatment condition in four biological replicates, determined by qPCR, were standardized as described (***Willems et al., 2008***). Means and standard deviations are shown. Statistical analysis in 3C and 3D was done using one-way ANOVA, Fisher Tests, and two-tailed two independent sample T-Tests.

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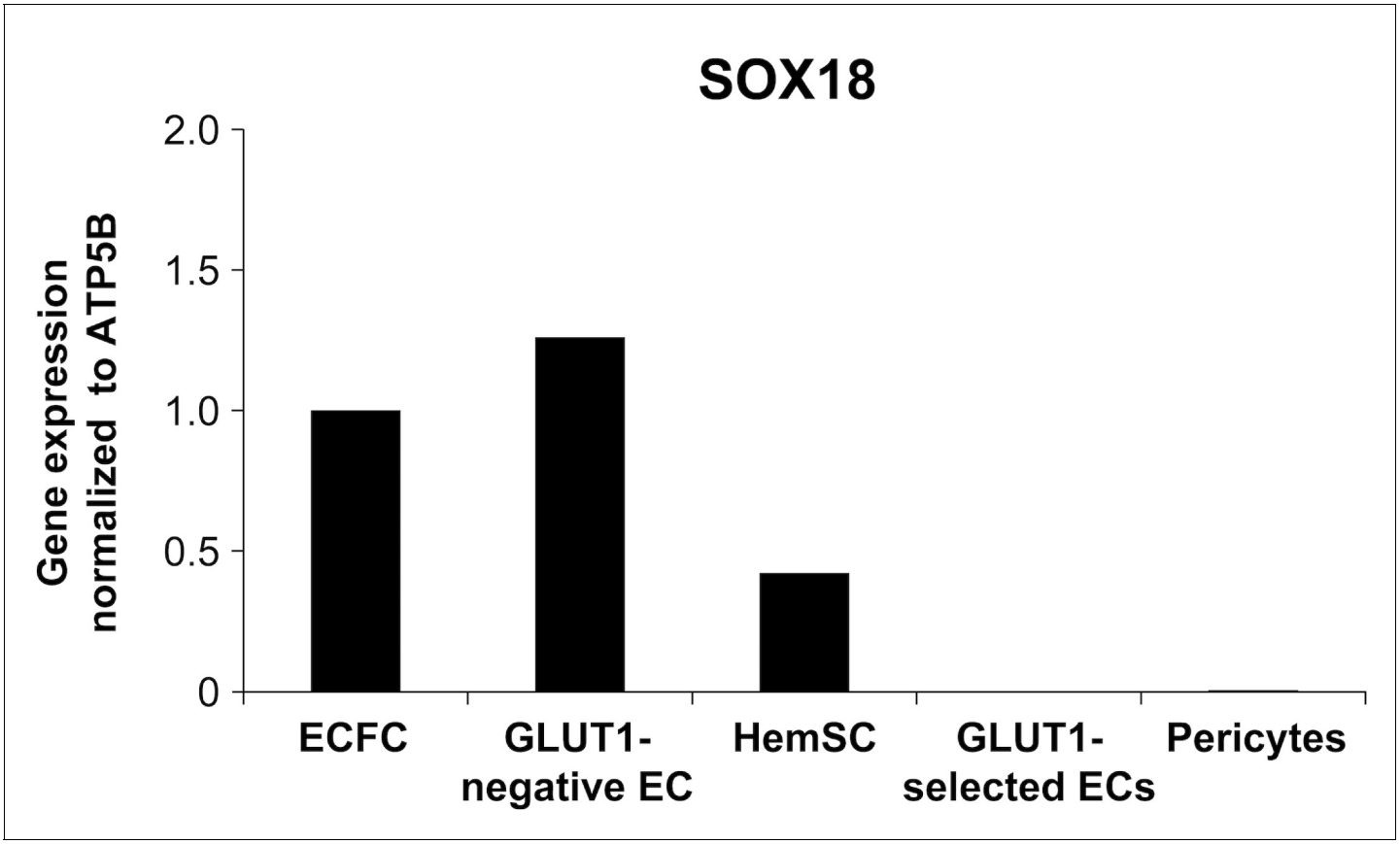

Figure 3—figure supplement 1. SOX18 expression in infantile hemangioma-derived cells. Human ECFC served as a positive control for SOX18. Hemangioma-derived GLUT1-negative ECs, HemSC, GLUT1-positively selected ECs, and pericytes were analysed for SOX18 mRNA expression by qPCR analysis.

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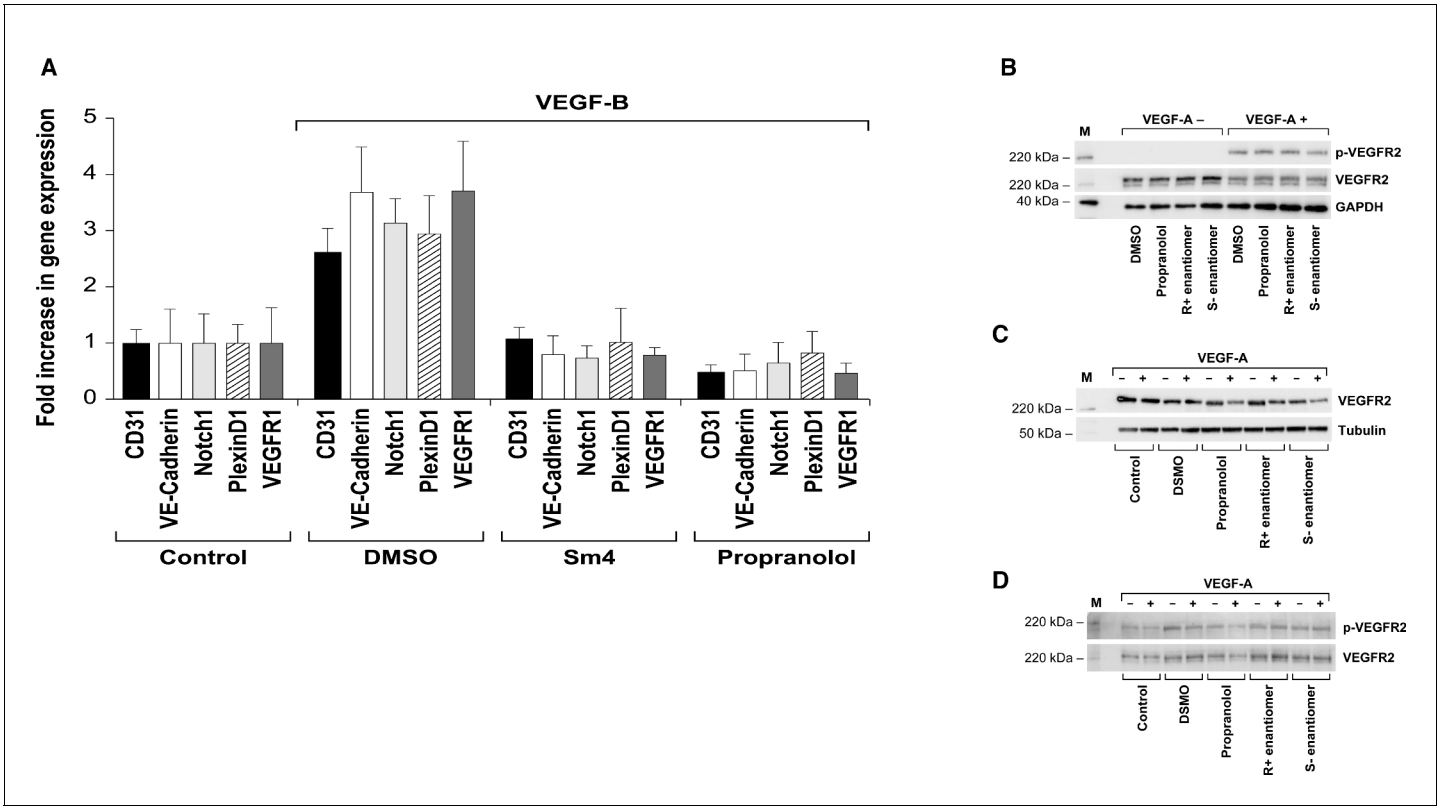

Figure 3—figure supplement 2. SOX18 inhibitor (Sm4) and propranolol block HemSC to EC differentiation. (**A**) VEGF-B treatment of HemSC from four different infantile hemangiomas resulted in increased *CD31* and *CDH5* (endothelial cell markers), and hemangioma endothelial markers NOTCH1, *PLXND1* and *VEGFR1*, determined by qPCR. Inclusion of SOX18 inhibitor (Sm4) or propranolol blocked the VEGF-B induced increases in these markers. Results from eight biological replicates were standardized as described (***Willems et al., 2008***). Means and standard deviations are shown. All P values were < 0.001 for Control versus DMSO (VEGF-B treated); DMSO versus Sm4 and DMSO versus propranolol. (**B–D**) VEGFR2 protein and phosphorylation were not affected by pre-treatment with propranolol, R + enantiomer or S(-) enantiomer (each tested at 5 uM). (**B**) Human endothelial colony forming cells (ECFC) were pre-treated for one hour with drug as indicated, stimulated with 25 ng/ml VEGF-A for 5 min, lysed and analysed by Western blotting. (**C**) HemSC were induced to differentiate for 5 days with VEGF-B; control indicates cells in differentiation media without VEGF-B. Differentiated cells were pre-treated for one hour with drugs as indicated, stimulated with 25 ng/ml VEGF-A for 5 min, and lysed for VEGFR2 Western blotting. (**D**) HemSCs were treated as in B, cell lysates were immunoprecipitated with anti-VEGFR2, followed by Western blotting for phosphorylated VEGFR2 and total VEGFR2. B-D, M indicates lane with molecular weight standards.

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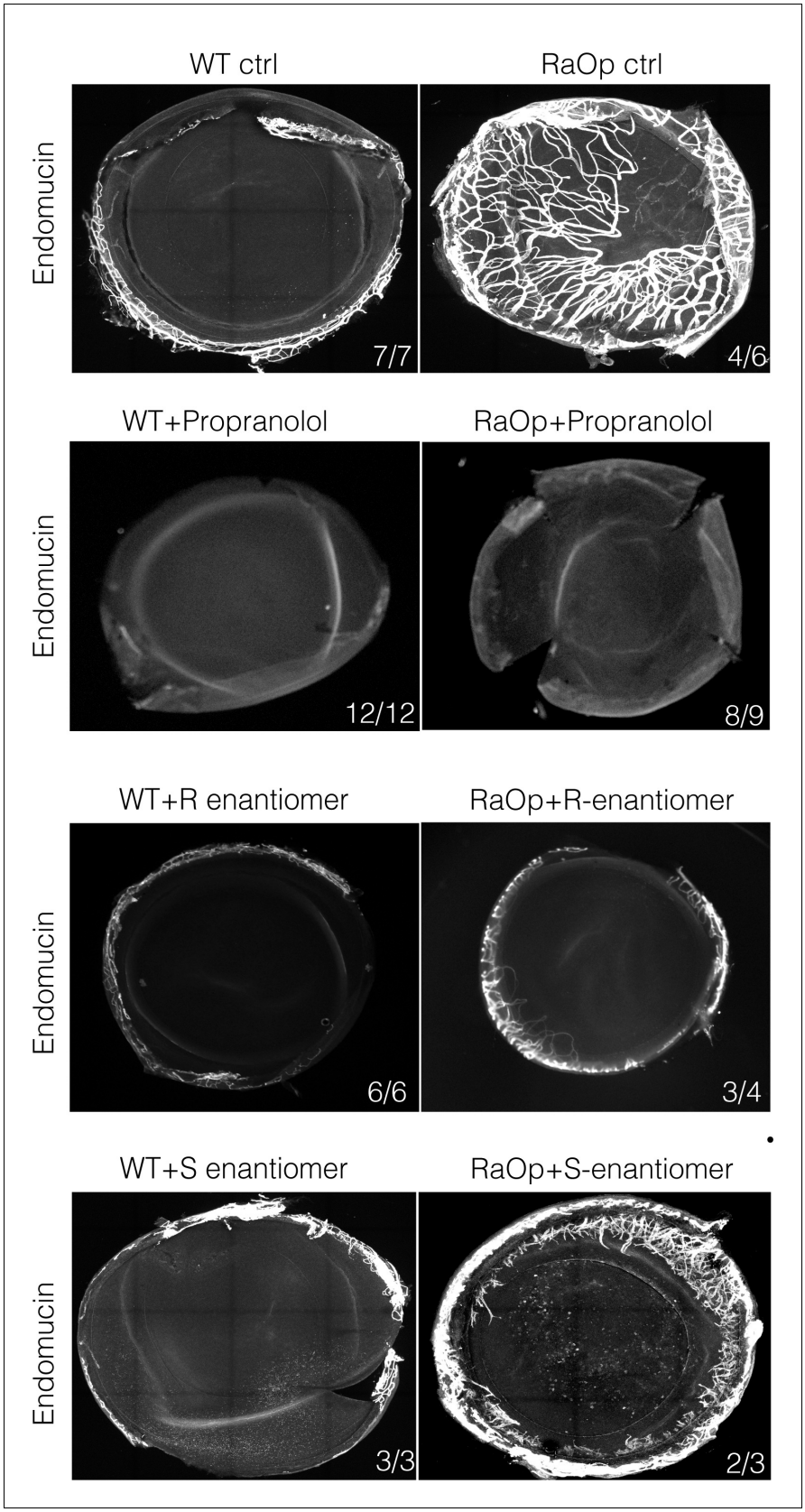

Figure 3—figure supplement 3. The racemic mixture Propranolol and its enantiomer (R(+) and S(-)) rescues corneal neo-vascularization phenotype in a mouse pre-clinical model of HLTRS. Fluorescent images of corneal flat

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Figure 3—figure supplement 3 continued

mounts, showing blood vessel penetration into the cornea at P28 stage using endothelial cell markers endomucin (EMCN). Sox18 WT and RaOp mice were treated from P8 to P28 with either vehicle PBS or propranolol or R-enantiomer or the S-enantiomer at 25 mg/Kg/day. Propranolol has no obvious effect on WT cornea, but prevents CNV in RaOp pups. Number of predominant phenotype shown in bottom right.

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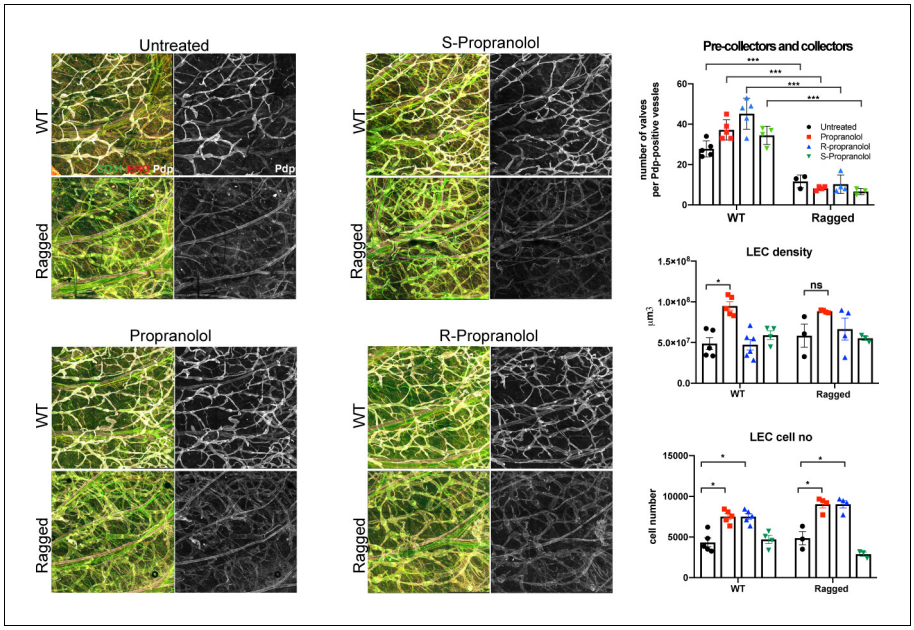

Figure 3—figure supplement 4. Propranolol and its R(+) and S(-) enantiomers do not rescue the dermal lymphatic vascular phenotype of the *Ragged Opossum* mutant mouse model. Ear whole mount immunofluorescence for Podoplanin (white), ERG (red) and CD31 (green) was performed after wild type and RaOp mutant animals were treated by propranolol, S(-) and R (+)enantiomer (P1-P28 at 25 mg/kg/day). Quantification of the number of valves in pre-collector and collector vessels, LEC vessel density and LEC cell number showed that none of the drug treatment was efficient to mitigate the RaOp phenotype. n = 3–6 animal per genotypes and drug treatment.

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