Supplemental Material

Propranolol Inhibits Cavernous Vascular Malformations by β1 Adrenergic Receptor Antagonism in Animal Models

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Supplemental Methods

Zebrafish embryo experiments

The following transgenic zebrafish lines were maintained under standard conditions: *Tg(fli1:EGFP)*^{y1} (1), and *Tg(gata1:dsred)*^{sd2} (2). CRISPR-Cas9 mutagenesis was used to generate mosaic *ccm2*-null zebrafish as previously described (3). Briefly, PCS2-nls-zCas9-nls (47929, Addgene) was linearized by NotI, and purified by column (Macherey-Nagel) as template for cas9 mRNA synthesis. Capped nls-zCas9-nls RNA was synthesized and purified with mMESSAGE mMACHINE SP6 Transcription Kit (ThermoFisher Scientific). DNA template for gRNA targeting zebrafish ccm2 was constructed as published (4). The crRNA sequences are as follow: gRNA1 5'-GGTGTTTCTGAAAGGGGAGA-3', gRNA2 5'-GGAGAAGGGTAGGGATAAGA-3', gRNA3 5'-GGGTAGGGATAAGAAGGCTC-3', gRNA4 5'-GGACAGCTGACCTCAGTTCC-3'. The gRNA constructs were linearized by BamHI and purified by column (Macherey-Nagel) as templates for gRNA synthesis. gRNAs were synthesized with MEGAshortscript T7 Transcription kit (ThermoFisher Scientific), and purified by alcohol precipitation described in the same kit. The concentration of above synthesized RNA was measured by NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific). The final concentrations for RNA injection are as follow: cas9 750ng/µl, gRNA 120ng/µl. The sequence and concentration of morpholinos used in this study are as follow: adrb1 MO 5'-acggtagcccgtctcccatgatttg-3' (4ng/µl), adrb2a MO 5'-gtattgaggaccttatgtttcccat-3' (2ng/µl). Microinjection volume is 0.5nl.

The 24hpf embryos were grouped into 24-well microplates, and each well contained a total volume of 1.5ml of egg water containing racemic propranolol (P0995, TCI), R-(+)-propranolol (0835100, R&D), S-(-)-propranolol (P8688, Sigma) or metoprolol (M1174, Spectrum), respectively. The concentration of the working solutions for racemic propranolol, R-(+)-propranolol, S-(-)-propranolol were 100µM and 400 µM for metoprolol. Egg water without drug was used as control. The allocation of different drugs to specific columns of the 24-well plate was randomized, as well as the allocation of individual zebrafish to individual wells.

The 48hpf embryos were anesthetized with 0.016% tricaine (E10521, Sigma), and embedded in 1% low melting point agarose (Invitrogen 16520050). Imaging was performed with Zeiss 880 Airyscan confocal under the standard Airyscan mode, and a 20x/NA 0.8 objective was used. Bright field pictures were captured by Olympus MVX10, Macro-view Statistical analysis was performed with GraphPad Prism. The mean and sample number are shown in the bar graphs.

Mouse models

The two CCM3 murine models used for this included 45 mice (43 males, 2 females) of the *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ model previously described (5). The Duke site maintains the animal line with the knockout allele of *Pdcd10* (6), obtained from Dr. Wang Min under a Material Transfer Agreement with Yale University, and the *Trp53* mice from the Jackson Laboratory. Mutant lines were maintained in the C57BL/6J inbred strain background (Jackson Laboratory). Both sexes were used in this model. A reduction of female *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ animals was observed previously (7). *Trp53*$^{-/-}$ females are more susceptible to neural tube defects during fetal development (8), resulting in fewer females born alive than males (9, 10) sometimes even to the point of complete loss of any females (11, 12). The skewed sex ratios that we observed with the *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ mice may be due to the loss of *Trp53* or possibly due to an interaction with *Pdcd10* heterozygosity that is making the skewing even worse.

The *Pdcd10*^{ECKO}model (*Pdcd10*$^{fl/fl}$*Pdgfb-iCreERT2*), in which a reduced dose (25 μg) of tamoxifen was injected at P6 (13), included 61 mice, utilizing nine mice for the isoproterenol dose response and 52 mice (31 males, 21 females) for the lesion burden and hemorrhage assessments.

Isoproterenol dose response

In vivo isoproterenol dose response hemodynamics was performed as previously described (14). Briefly, *Pdcd10*^{ECKO}mice were anesthetized with ketamine (100 mg/kg) and xylazine (2.5 mg/kg), intubated and connected to a rodent ventilator. After bilateral vagotomy, a 1.4 French (0.46 mm) high fidelity micromanometer catheter (ADInstruments, Dunedin, New Zealand) was inserted into the right carotid artery and advanced retrograde across the aortic valve to left ventricular (LV) pressure. Continuous high fidelity LV pressure was recorded at baseline and 45–60 sec after injection of incremental doses of isoproterenol (50 – 5000 pg) in a cannulated jugular vein using LabChart 8 software (ADInstruments). Parameters measured were heart rate, LV systolic and end diastolic pressure, and the maximal and minimal first derivative of LV pressure (LV dP/dtmax per min). Ten sequential beats were averaged for each measurement.

Experimental design

Guidelines from the National Institute of Neurological Disorders and Stroke, implemented for objectivity in preclinical research, were followed, including randomization, blinding of outcome assessment, appropriate sample size estimation based on the primary outcome, and prespecified data analyses (15). Mice receiving treatment and placebo controls were raised contemporaneously. The *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ model received 50 mg/kg/day of propranolol in the drinking water for 90 days starting at P21 (n=23) or placebo (n=22), while the *Pdcd10*^{ECKO}model received 50 mg/kg/day of propranolol (n=27) in the drinking water for 35 days starting at P21 or placebo (n=25). New water bottles with fresh drug were changed once per week, and they were topped off periodically (when levels were low). The concentration of propranolol in the drinking water was calculated based on the average amount of water consumed per mouse per day (averaged each month since weekly weight gain was low). This dosing scheme was endorsed by our veterinarians, in view of the short half-life of oral propranolol, making oral gavage impractical. We verified that the drug is at a therapeutic level using isoproterenol challenge showing that the propranolol was exhibiting the expected physiologic effect at that dose.

Mice were euthanized if they exhibited signs of suffering, such as large skin abscesses or tumors. This propranolol treatment did not affect the body weights for the *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ model or the *Pdcd10*^{ECKO} model compared to the placebo controls (Supplemental Table).

In the present study, doses other than 50 mg/kg were not assessed in mice. This dose was chosen based on effecting physiologic β1 receptor inhibition. The human equivalent dose for a drug is estimated from United States Food and Drug Administration guidelines (16) by multiplying the no observed adverse effect level of the drug in a mouse by 0.081 (17), the human equivalent dose for propranolol is 4.05 mg/kg/day for an equivalent no observed adverse effect level of 50 mg/kg/day in a mouse. For a 70 kg human adult, an equivalent dose of propranolol is calculated at approximately 280 mg/day. In fact, propranolol doses between 20 and 320 mg per day are widely used clinically. In the ongoing randomized controlled pilot trial, Treat_CCM, a dose of propranolol between 40 and 80 mg per day is being used to treat subjects with CCM disease (18).

Lesion burden and hemorrhage analyses

Following the final drug treatment, the brains were removed from the mice. CCM lesion and total brain volumes were determined by micro-computed tomography, as described previously (19). For each mouse, lesion burden was calculated as the percentage of total lesional volume per total brain volume. For the *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ model, lesions were identified and selected as either Stage 1 single cavern or Stage 2 mature multi-cavernous as previously described (19) by three observers (T.M., R.G. and N.H.), who were blinded to the treatment regimen. Volumes for each of the two stages were separately added to a segmented group volume (i.e. material) and total volumes were determined for each material by multiplying the number of voxels segmented by the voxel size.

The brains were soaked in formalin after imaging and cut into 1-mm thick coronal slices. Slices from the *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ model were photographed if determined microscopically to have putative CCM lesions that anatomically correlated with the micro-computed tomography. From the *Pdcd10*^{ECKO} model, which had numerous lesions throughout the brain, one slice was randomly selected from the forebrain and another randomly selected from the hindbrain.

After appropriate brain slices were processed and embedded in paraffin, the blocks were sectioned at 5-μm thicknesses. Sections from all mice from both models were stained with H&E and analyzed for acute hemorrhage by two observers (R.S. and T.M.), who were blinded to the treatment regimen. Acute bleeding is identified on the respective histologic images, measuring the area with the aid of a software polygon tool for a digital camera (DP22) mounted on a CX41 microscope (Olympus, Waltham, MA) of extra-lesional bleeding (not confined by endothelium lining) and the lesional area (including any blood confined within endothelium lined caverns), with the following formula expressing

$$\textit{\% extra-lesional bleeding} = 100 \text{ X } \frac{\sum \text{extra-lesional bleed areas } (\mu m^2)}{\sum \text{lesional areas} + \sum \text{extra-lesional bleed areas } (\mu m^2)}.$$

Additional sections from the *Pdcd10*^{ECKO}model were stained with Perls stain, which stains hemosiderin and other non-heme iron, indicating chronic bleeding, and for CD3 positive T cells and CD20 positive B cells, both indicating inflammation, by methods previously described (7). Slides were examined microscopically to determine which mice had detectable chronic hemorrhage and/or inflammation.

The H&E-stained sections from the *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ model, were examined by two observers (R.S. and T.M.), who had no knowledge of treatment assignment during examination, to determine lesion maturity. Stage 1 lesions are single caverns at least 100 mm in diameter, while Stage 2 lesions are mature, multi-cavernous lesions, as previously described (5). After anatomically correlating with micro-computed tomography, the total volumes of the Stage 1 and Stage 2 lesions were determined. Adjacent sections containing Stage 2 lesions were stained with Perls stain, scanned and relative amounts of Perls stain for each Stage 2 lesion were determined, as described previously (5). The ratio of integrated Perl staining per lesional area was determined for each Stage 2 lesion.

Statistical analysis

For comparisons of cardiovascular effects in zebrafish, the two-tailed Fisher exact test was used.

For the isoproterenol dose response, statistical significance was determined with 2-way repeated-measures ANOVA with Sidak correction for multiple comparison in GraphPad Prism, with post hoc independent samples t-test (test statistic t) for contractility and post hoc Tukey's (test statistic q) honestly significant difference for heart rate comparisons.

For primary outcome assessment, we hypothesized that the CCM lesion burden determined by micro-computed tomography at the conclusion of treatment will be significantly decreased in each of

the propranolol-treated groups as compared to placebo control mice receiving the same drug-free diet and drinking water. Power calculations assumed an effect size of 1.3, determined from similar studies in the *Pdcd10*$^{+/-}$ model comparing CCM lesion burden in placebos with mice depleted of B cells (5). Based on an effect size of 1.27, sample size was calculated to be 15 per group using the two-sample t test for mean difference (test statistic t) to test the significance between the propranolol-treated and control groups (α=0.05, 1-β=0.92, 2-tailed).

Because of the even greater rate of mice produced with the *Pdcd10*^{ECKO} model than the *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ model, at least 21 of these mice were included per group. Based on the same effect size of 1.27, sample size was calculated to be 21 per group using the two-sample t test for mean difference (test statistic t) to test the significance between the propranolol-treated and placebo groups (α=0.01, 1-β=0.92, 2-tailed).

Statistical outliers were defined as >2 standard deviations above or below the mean of their respective group (Z-score > 2). After the statistical outliers were removed, data were tested for normality by the D'Agostino & Pearson omnibus normality test (test statistic K^2). Unlike the normally distributed lesion burden data in the B cell depletion studies on *Pdcd10*$^{+/-}$ mice previously reported (5), upon which the sample size calculations for the present studies were based, the lesion burden data in all of the present studies were not normally distributed. Hence, non-parametric tests were used to test for statistical significance. In situations when most of data per group was non-zero, the 2-sided Conover 2-sample test (test statistic t) was used to test for significant differences per group – a test in which sensitivity was improved because the individual data points are weighted (20). The Conover test is an enhanced version of the Mann-Whitney U test by weighing the ranks of the original values. It provides an improved use of the non-zero data. If one of the groups contained more zero values than non-zero values, the Mann-Whitney U test (test statistic U) was used to test for significant differences per group to avoid possible statistical errors due to over emphasis of weighted outlying data points when analyzed by the 2-sided Conover 2-sample test. Similar non-parametric statistical analyses were conducted with the acute hemorrhage and non-heme iron deposition data, in which these data were not normally distributed as well.

Other prespecified secondary outcome analyses also used appropriate statistical tests. Since the body weights of mice were normally distributed, parametric tests were used to test for statistical significance. The F test (test statistic F) was used to evaluate the variances between two unpaired groups. Since the body weights had equal variances between propranolol treated mice and placebo controls, the differences between the two groups were compared using Student's t-test (test statistic t). The log-rank (Mantel-Cox) test (test statistic Z) was used to compare the survival of animals between treatment groups.

Statistical analyses were performed using SAS9.4 (SAS Institute Inc., Cary, NC), R v3.4.4 (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism 7.00 (GraphPad Software Inc., La Jolla, CA). All differences were considered to be statistically significant at $P< 0.05$.

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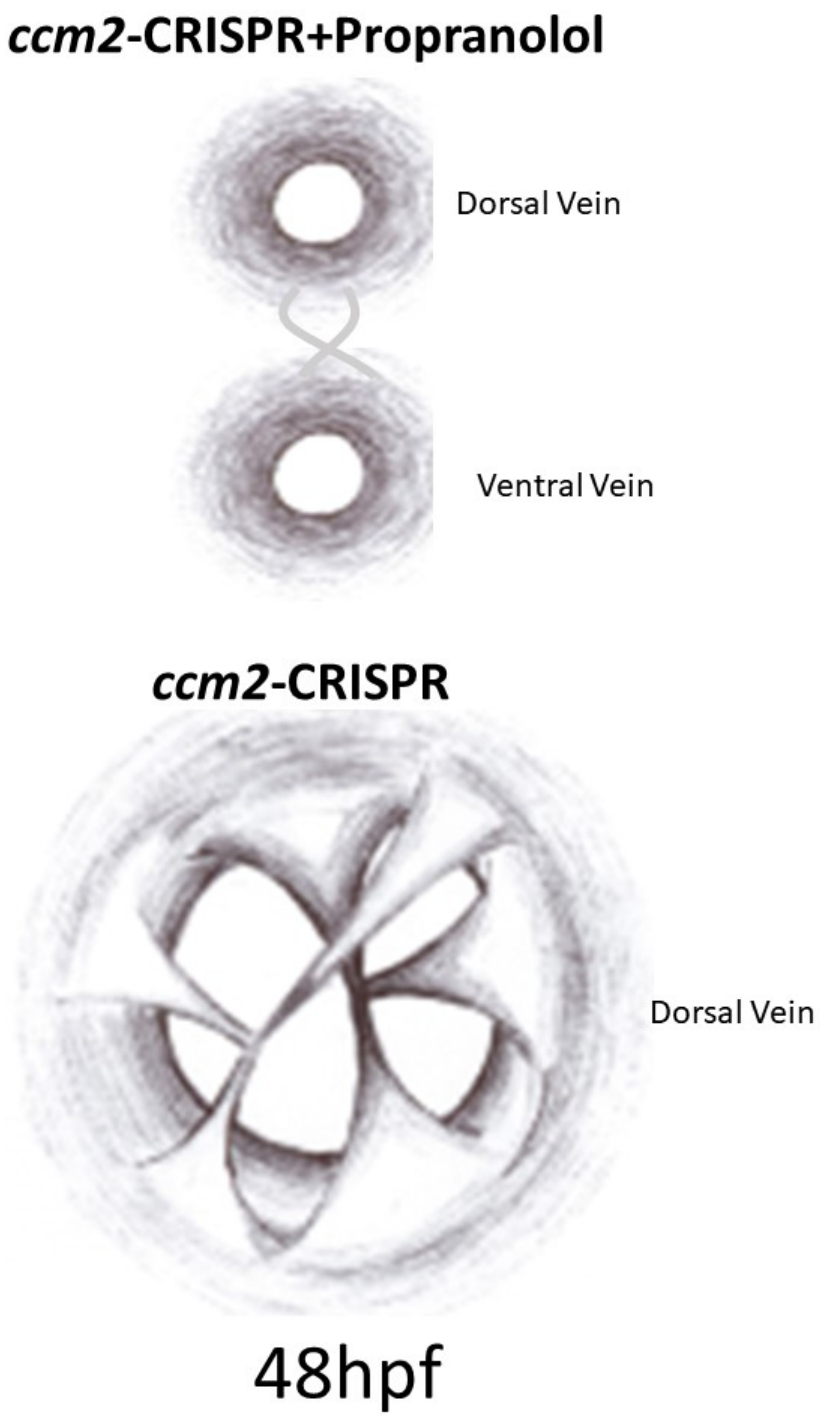
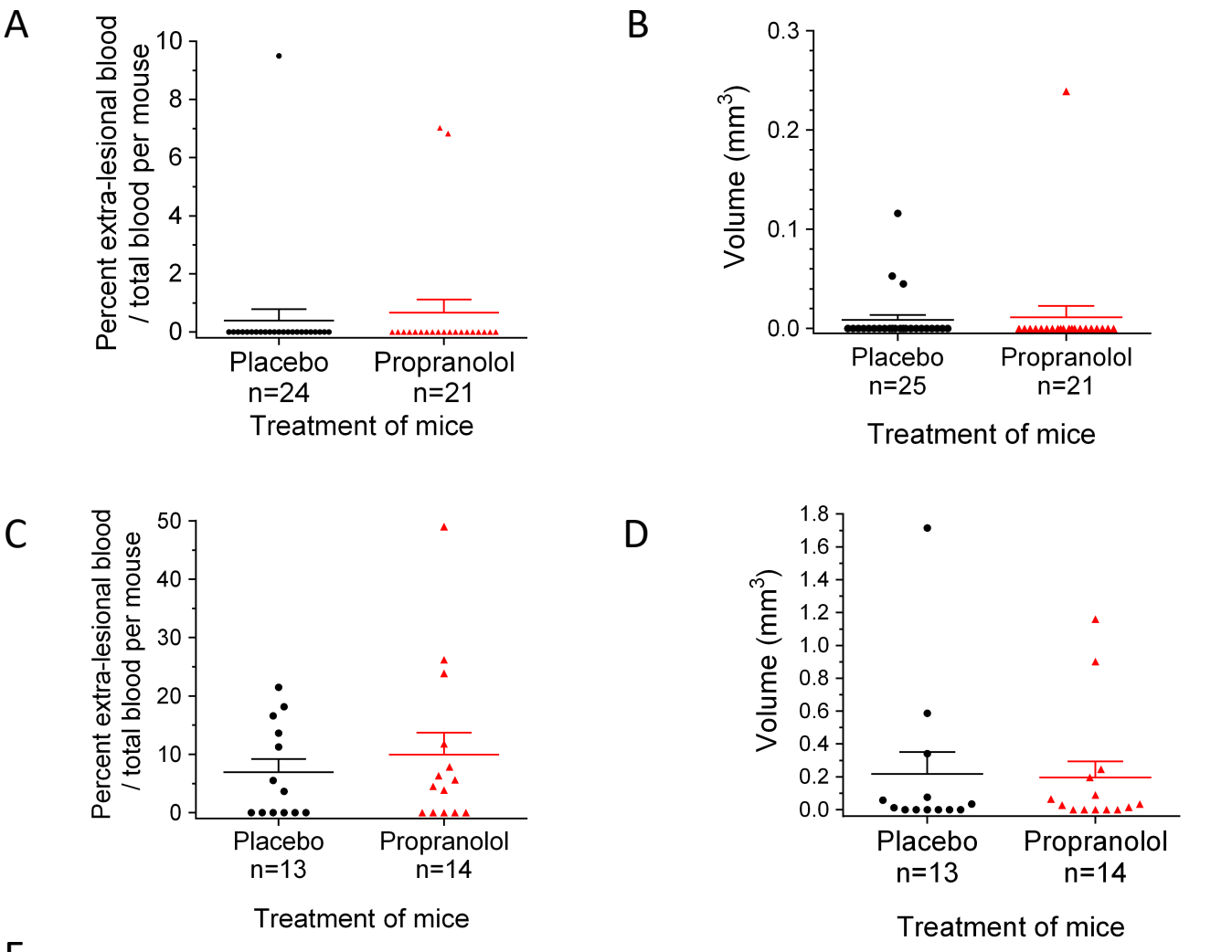
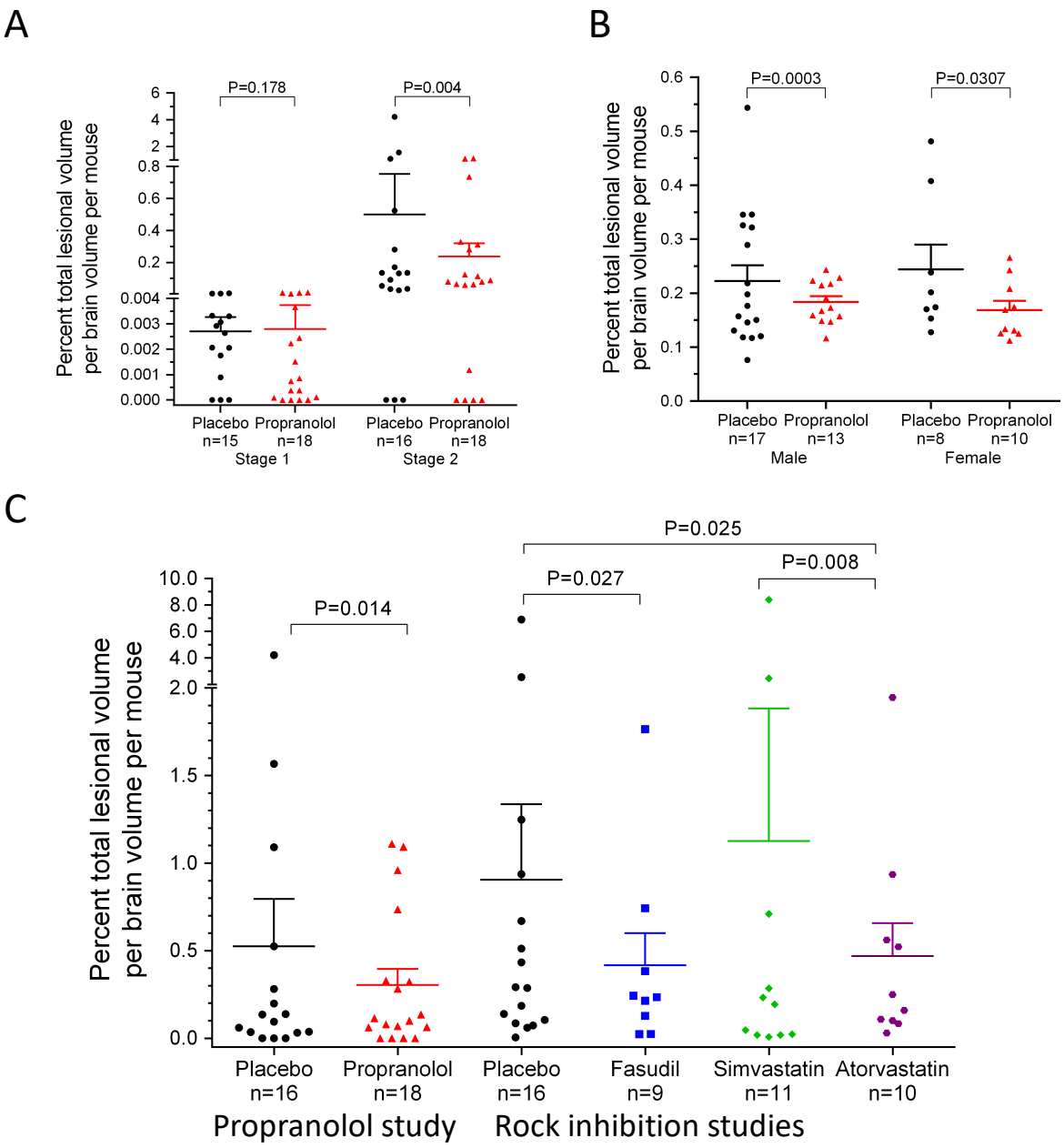


Supplemental Figure 1. A schematic diagram depicting a coronal section through the normal caudal venous plexus and a cavernous malformation in the *ccm2* CRISPR zebrafish at 48hpf. The normal caudal venous plexus is composed of dorsal vein, ventral vein and the plexus connecting them. In 30% of *ccm2* CRISPR embryos the dorsal vein does not form the ventral sprouts that lead to creation of the ventral vein and intervening plexus. Instead, uncoordinated intussusceptive growth partitions the lumen into small caverns which leads to dilation due to accumulation of trapped red blood cells.



Supplemental Figure 2. Effect of propranolol (50 mg/kg/day) on hemorrhage in murine models. In the *Pdcd10*^{ECKO} model, propranolol did not affect the **(A)** ratio of extra-lesional blood / total blood (P=0.592) or **(B)** volume of extra-lesional blood (P=0.614) compared to placebos. The Mann-Whitney U test was used (non-normal data distribution, majority of zero values in treated cohort). In **(A)** four outliers were excluded (2 placebo, 2 propranolol). In **(B)** three outliers were excluded (1 placebo, 2 propranolol). Means (longer lines) and SEM (shorter lines above the mean lines) are indicated. In the *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ model**, (C)** propranolol did not affect the ratio of extra-lesional blood / total blood compared to placebos (P=0.291), and **(D)** there was a trend for decrease volume of extra-lesional blood (P=0.083) in propranolol treated mice compared to placebos. The Two-Sample Conover Test was used (non-normal data distribution). Seven mice lacking Stage 2 lesions were excluded (3 placebos and 3 propranolol with no lesions, and 1 propranolol with only a stage 1 lesion). Two outliers were excluded (1 placebo, 1 propranolol). The mean (longer lines) and SEM (shorter lines above the mean lines) are indicated for each group. **(E)** Effect of propranolol on chronic hemorrhage in Stage 2 lesions of the *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ model. Propranolol did not affect the ratio of integrated iron / lesional area compared to placebos (P=0.32). The Two-Sample Conover Test was used (non-normal data distribution). Six outliers were excluded (3 placebo, 3 propranolol). The mean (longer lines) and SEM (shorter lines above the mean lines) are indicated for each group.



Supplemental Figure 3. Lesion burden effect of propranolol (50 mg/kg/day) analyzed by lesion stage and sex. (A) Effect of propranolol on Stage 1 and Stage 2 lesion burden in the *Ccm3*$^{+/-}$*Trp53*$^{-/-}$ model. Propranolol did not affect the burden of Stage 1 single cavern lesions (left, P=0.178). Three outliers were excluded (2 placebo, 1 propranolol). Propranolol significantly decreased the burden of Stage 2 mature, multicavernous lesions (right, P=0.004) compared to placebo controls. Two outliers were excluded (1 placebo, 1 propranolol). The Two-Sample Conover Test was used (non-normal data distribution). The mean (longer lines) and SEM (shorter lines above the mean lines) are indicated for each group. **(B)** Effect of sex on lesion burden in the propranolol-treated *Pdcd10*^{ECKO} model. (Left) Propranolol significantly decreased lesion burden in male models (P=0.0003). One outlier was excluded (placebo). (Right) Similarly, propranolol significantly decreased lesion burden in female models (P=0.0307). The Two-Sample Conover Test was used (non-normal data distribution). The mean (longer lines) and SEM (shorter lines above the mean lines) are indicated for each group. **(C)** Comparative effect of various pharmacotherapies in the *Ccm3*$^{+/-}$*Trp53*$^{-/-}$ model. Propranolol (50 mg/kg) for 90 days (P=0.014), as well as Rock inhibitors fasudil (100 mg/kg) for 100 days (P=0.027) and atorvastatin (80 mg/kg) for 100 days (P=0.025), but not simvastatin (40 mg/kg) for 100 days, decreased lesion burden compared to placebo, as measured by micro-computed tomography, when treatment started at P21. The Two-Sample Conover Test was used (non-normal data distribution). Rock inhibition studies are based on data from Shenkar R, Peiper A, Pardo H, Moore T, Lightle R, Girard R, et al. Rho Kinase Inhibition Blunts Lesion Development and Hemorrhage in Murine Models of Aggressive Pdcd10/Ccm3 Disease. Stroke. 2019;50:738-744. The mean (longer lines) and SEM (shorter lines above the mean lines) are indicated for each group.

Supplemental Table. **Weights of Murine Models**

Murine Model	Time after treatment	Weights (placebo group) n	g (mean ± SD)	Weights (propranolol group) n	g (mean ± SD)	p-value F test	t test
Pdcd10$^{+/-}$*Trp53*$^{-/-}$	0 months	17	9.5 ± 2.1	19	9.5 ± 3.4	0.07	0.99
	1 month	17	21.7 ± 2.6	19	21.6 ± 2.6	0.93	0.92
	2 months	17	25.5 ± 2.9	19	26.0 ± 2.4	0.47	0.61
	3 months	17	27.0 ± 4.0	19	27.2 ± 2.9	0.19	0.83
Pdcd10^{ECKO}	0 weeks	26	8.6 ± 1.5	23	9.3 ± 1.5	0.96	0.13
	1 week	26	13.3 ± 1.8	23	13.7 ± 2.2	0.40	0.45
	2 weeks	26	17.3 ± 2.0	23	17.7 ± 2.3	0.55	0.45
	3 weeks	26	18.9 ± 2.2	23	19.2 ± 2.1	0.84	0.64
	4 weeks	26	20.4 ± 2.4	23	20.7 ± 2.1	0.56	0.68
	5 weeks	26	20.8 ± 2.4	23	21.2 ± 2.5	0.76	0.57