

Genetic Inactivation of the $\beta 1$ adrenergic receptor prevents Cerebral Cavernous Malformations in zebrafish

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Abstract

Propranolol reduces experimental murine cerebral cavernous malformations (CCMs) and prevents embryonic caudal venous plexus (CVP) lesions in zebrafish that follow mosaic inactivation of *ccm2*. Because morpholino silencing of the $\beta 1$ adrenergic receptor (*adrb1*) prevents the embryonic CVP lesion, we proposed that *adrb1* plays a role in CCM pathogenesis. Here we report that *adrb1*^{-/-} zebrafish exhibited 86% fewer CVP lesions and 87% reduction of CCM lesion volume relative to wild type brood mates at 2dpf and 8-10 weeks stage, respectively. Treatment with metoprolol, a $\beta 1$ selective antagonist, yielded a similar reduction in CCM lesion volume. *Adrb1*^{-/-} zebrafish embryos exhibited reduced heart rate and contractility and reduced CVP blood flow. Similarly, slowing the heart and eliminating the blood flow in CVP by administration of 2,3-BDM suppressed the CVP lesion. In sum, our findings provide genetic and pharmacological evidence that the therapeutic effect of propranolol on CCM is achieved through $\beta 1$ receptor antagonism.

eLife Assessment

The authors follow up on a prior paper in which they showed that beta1 adrenergic receptors contributed to the pathogenesis of cavernous malformations. In the prior work, they used morpholinos and drugs to show this. In this new advance, they now extend this using a genetic knockout of the receptor. While both reviewers agree that this is **important** for the CV field, there are concerns about the labeling of figures and sample sizes used to make their claims, so the evidence is currently **incomplete**.

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Introduction

Cerebral cavernous malformations (CCMs), accounting for 5-15% of cerebrovascular abnormalities, are characterized by blood-filled endothelial-lined cavities, and can cause seizures, headaches, neurological deficits, and recurrent stroke risk¹. Familial CCMs are due to heterozygous loss of function mutations in the *KRIT1*, *CCM2*, or *PDCD10* genes with a second mutation inactivating the normal allele in random brain endothelial cells². We previously developed a zebrafish CCM model using CRISPR-Cas9 to inactivate *ccm2* in a manner that

replicates the mosaic genetic background of the human disease³. This zebrafish model exhibits two phenotypic phases: lethal embryonic caudal venous plexus (CVP) cavernomas at 2 days post-fertilization (dpf) in ~30% of embryos and histologically-typical CNS CCMs in ~100% of surviving 8 week old fish³. Both phases of this model, like their murine counterpart, depend on Krüppel-like factor 2 (KLF2), confirming shared transcriptional pathways. Furthermore, both phases of the zebrafish model exhibit similar pharmacological sensitivities (Supplementary Table 1) to murine and human lesions. Thus, this zebrafish model offers a powerful tool for genetic and pharmacological analysis of the mechanisms of CCM formation.

Anecdotal case reports^{4–6} and a recent phase 2 clinical trial have suggested that propranolol a non-selective β adrenergic receptor antagonist benefit patients with symptomatic CCMs⁷. In previous studies, we observed this effect of propranolol in both zebrafish and murine models of CCM⁸. Importantly, propranolol is a racemic mixture of R and S enantiomers and elegant work from the Bischoff lab has implicated the R enantiomer, which lacks β adrenergic antagonism, as the component that inhibits SOX18 thereby suppressing infantile hemangiomas⁹. We previously found that the anti-adrenergic S enantiomer rather than the R enantiomer inhibited development of embryonic CVP cavernomas in *ccm2* CRISPR zebrafish⁸. Furthermore, morpholino silencing of the gene that encodes the $\beta 1$ (*adrb1*) but not $\beta 2$ (*adrb2*) receptor also prevents CVP cavernomas⁸, suggesting that the $\beta 1$ adrenergic receptor ($\beta 1$ AR), which primarily impacts hemodynamics¹⁰, contributes to the pathogenesis of CCM. Here we have inactivated the *adrb1* gene to eliminate the $\beta 1$ AR and observed the expected reduction of heart rate and contractility which resulted in reduced blood flow through the CVP. These *adrb1*^{-/-} zebrafish were protected from embryonic CVP cavernomas and, importantly, also exhibited much reduced number and volume of adult brain CCM. Furthermore, a $\beta 1$ -selective antagonist, metoprolol, also inhibited adult CCM suggesting that $\beta 1$ AR-specific antagonists may be useful for CCM treatment and will carry less potential for $\beta 2$ AR-related side effects such as bronchospasm¹¹.

Results

$\beta 1$ AR is important in for development of CVP cavernomas

We previously reported that morpholino silencing of *adrb1* rescued CVP cavernomas in zebrafish embryos⁸. Because of morpholinos potential for off target effects^{12, 13}, we sought to confirm the $\beta 1$ AR's potential involvement in CCM pathogenesis by inactivating *adrb1*. We used CRISPR-Cas9 to generate an 8-bp deletion resulting in a premature stop codon at 57 bp (**Figure 1A**). To exclude potential off-target effects, the top 20 potential off-target sites predicted by Cas-OFFinder (www.rgenome.net/cas-offinder) (Supplementary Table 2) were sequenced and were not mutated (data not shown). We intercrossed *adrb1*^{+/-} F1 offspring resulting in *adrb1*^{-/-} zebrafish embryos that displayed reduced cardiac contractility (Supplementary Video 1) and decreased heart rate (Supplementary Figure 1A). Similar to *Adrb1*^{-/-} mice¹⁴, *adrb1*^{-/-} zebrafish exhibited a blunted chronotropic response to a $\beta 1$ AR agonist, isoprenaline hydrochloride (**Figure 1B**). *Adrb1*^{-/-} embryos had no obvious defects in vascular development and they survived to adulthood and were fertile.

We intercrossed *adrb*^{+/-} individuals and injected one-cell stage embryos with *ccm2* CRISPR and blindly scored the presence of CVP cavernomas at 48hpf. As expected we observed CVP cavernomas in 28% of *adrb1*^{+/-} embryos. In sharp contrast only 3% of *adrb1*^{-/-} embryos exhibited cavernomas (**Fig. 1C and D**) indicating that loss of $\beta 1$ AR prevents CVP cavernomas. these observations demonstrate that the $\beta 1$ AR is required for embryonic CVP cavernoma formation.

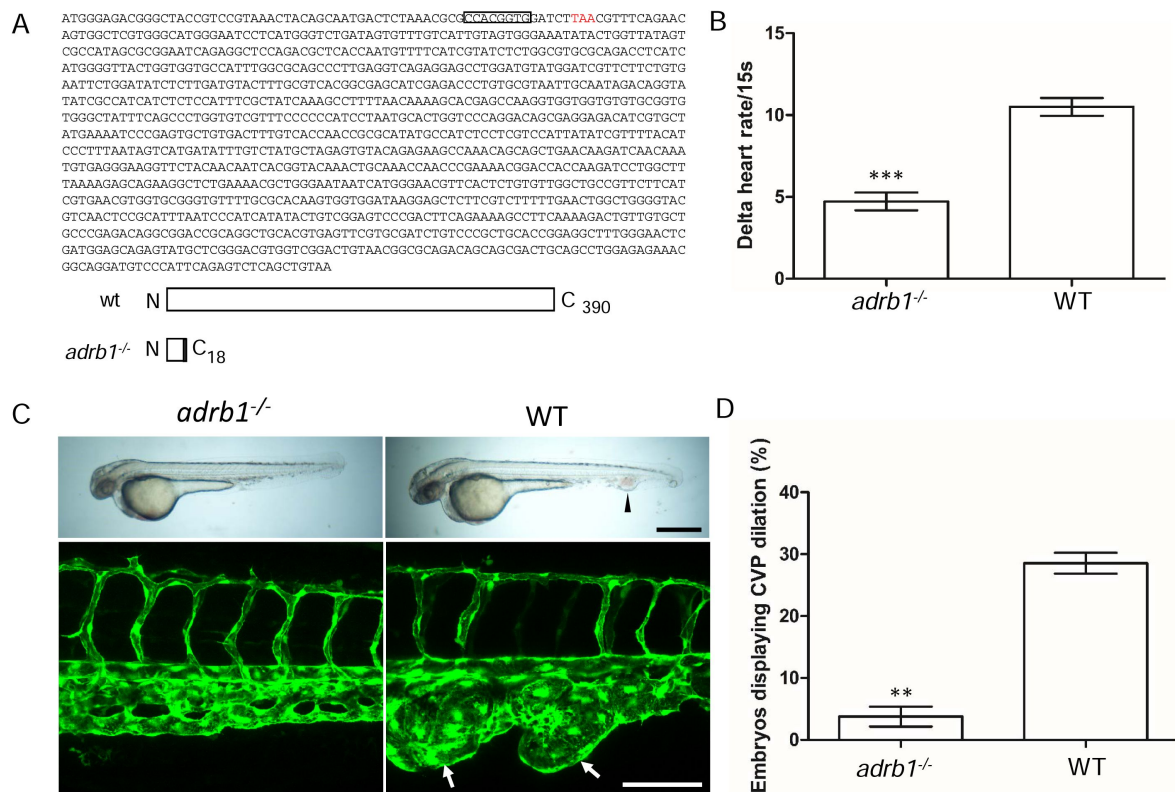


Figure 1.

Adrb1 signaling is essential for CVP dilation. (A) The targeted *adrb1* allele shows an 8-nucleotide deletion producing a pre-stop codon. *Adrb1* null cDNA is predicted to encode truncated *adrb1* protein. The wild type *adrb1* protein contains 390 amino acids, while the predicted *adrb1* null protein would contain 2 missense amino acids (gray bar) and would terminate after amino acid 18. (B) Isoprenaline hydrochloride (50 μ M) treatment at 72hpf lead to a heart rate increase in zebrafish, while the delta heart rate in *adrb1*^{-/-} is significantly smaller than that of wild type. Paired two-tailed t test, $P < 0.0001$. (C) After *ccm2* CRISPR injection, representative bright field and confocal images of 2dpf embryos show that wild type embryos display CVP dilation, while *adrb1*^{-/-} embryos were resistant to this defect. Arrowhead and arrows indicate the dilation in CVP. Scale bar: 500 μ m (bright field), 100 μ m (confocal). (D) Paired two-tailed t test shows that CVP dilation is significantly reduced in *adrb1*^{-/-} embryos compared to control. $P = 0.0012$.

β1AR mediates formation of adult CCM in the brain

Adult *ccm2* CRISPR zebrafish display highly penetrant CCMs throughout the central nervous system³. Brains from adult *ccm2* CRISPR fish on *adrb1*^{-/-} (12 brains) or wild type (13 brains) background were treated with CUBIC (clear, unobstructed brain/body imaging cocktails and computational analysis)¹⁵, and these transparent brains were then scanned with light-sheet microscopy and lesions were enumerated and volumes were estimated with NIH ImageJ. While the typical multi-cavern CCMs were observed in brains on wild type background appearing as blood filled dilated vessels (Figure 2A through C), *ccm2* CRISPR *adrb1*^{-/-} fish exhibited an 87% reduction in lesion volume (Figure 2D through G). Thus, genetic inactivation of β1AR prevented CCMs.

A selective β1AR antagonist prevents CCMs

The non-selective β blocker propranolol reduces lesion volume in murine CCM models^{8, 16}. To ascertain whether propranolol had a similar effect in the zebrafish model, the chemical treatment was started from larval stage at concentration which allows the fish to develop to two months for CCM inspection. We added 12.5μM propranolol to or vehicle control to fish water of *ccm2* CRISPR zebrafish starting at 3 weeks of age. The water was refreshed daily with drug or vehicle until fish were sacrificed and brains were examined as described (Figure 3A). Quantification based on light-sheet scanning of zebrafish brains (Figure 3B) showed that compared to vehicle-treated controls (Figure 3C, D, and E), propranolol-treated groups displayed a 94% reduction in CCM lesion volume (Figure 3F, G, and H). Similarly, administration of 50μM racemic metoprolol a β1-selective antagonist produced a similar (98%) reduction in lesion volume (Figure 3B, I, J, and K). Importantly, neither drug at the doses administered reduced the growth of the fish or the volume of their brains. In sum, both genetic and pharmacological loss of β1 adrenergic receptor signaling markedly reduces the lesion burden in the zebrafish *ccm2* CRISPR model of CCM.

Loss of β1AR does not prevent increased

klf2a expression in *ccm2* null embryos

Inactivation of CCM genes leads to increased endothelial KLF2 expression^{17, 18}, a transcription factor important in cavernoma formation^{3, 19}. Nevertheless, silencing of *adrb1* did not prevent the expected increased endothelial *klf2a*¹⁷ expression in *ccm2* morphant *Tg(klf2a:H2b-EGFP)* fish in which the nuclear EGFP expression is driven by the *klf2a* promoter (Figure 4A and B). We previously reported that mosaic expression of KLF2a occurs in *ccm2* CRISPR fish and is sufficient to drive cavernoma formation when blood is flowing³. Nevertheless, the *adrb1* morphant (Figure 4C) and control morphant (Figure 4D) *ccm2* CRISPR *Tg(klf2a:H2b-EGFP)* embryos both displayed a mosaic increase of *klf2a* reporter (Figure 4E). Similarly, merely slowing the heart and reducing contractility with 2,3-butanedione monoxime (BDM)²⁰ also prevented CVP cavernomas (Fig 4F, and Supplementary Figure 2). Thus, silencing β1AR does not prevent the generalized increase in endothelial KLF2a in *ccm2* morphants nor does it prevent the mosaic KLF2a increase in *ccm2* CRISPR zebrafish.

Discussion

In this study, we provide genetic and pharmacological evidence to implicate the β1 adrenergic receptor (β1AR) in the pathogenesis of CCM. Our data suggest that inactivating β1AR via gene inactivation or pharmacological inhibition can significantly reduce the volume of CCM lesions in a zebrafish model.

Figure 2.

Genetic inhibition of *adrb1* signaling could rescue CCM in *ccm2* CRISPR zebrafish. (A through F) Representative light sheet microscopy scanning pictures of brains from *ccm2* CRISPR adult zebrafish of wild type (A through C) and of *adrb1*^{-/-} (D through F). Brains from *ccm2* CRISPR on wild type background show lesions indicated by arrows (A through C), while brains from *ccm2* CRISPR on *adrb1*^{-/-} do not show lesions (D through F). Scale bar: 1mm. (G) Statistical analysis of lesion volume by unpaired two-tailed t test. *P*=0.0005.

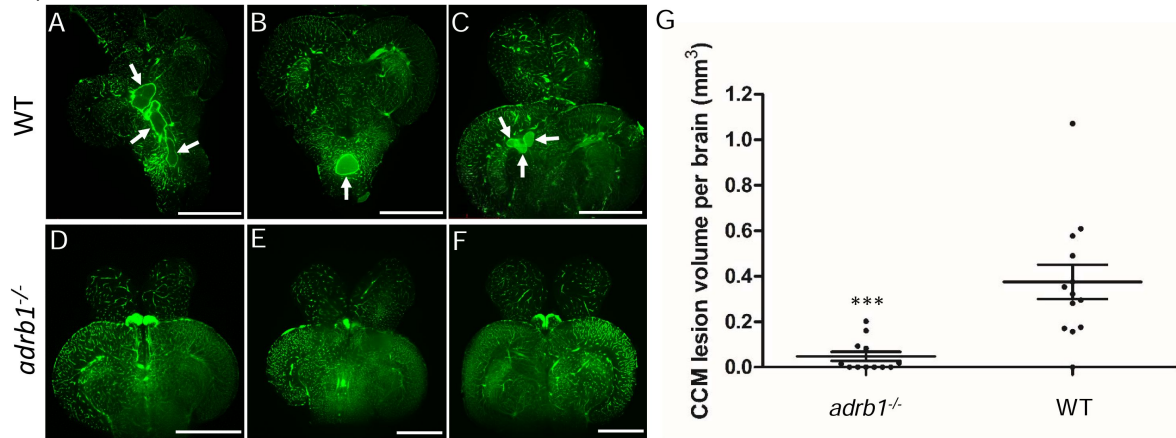
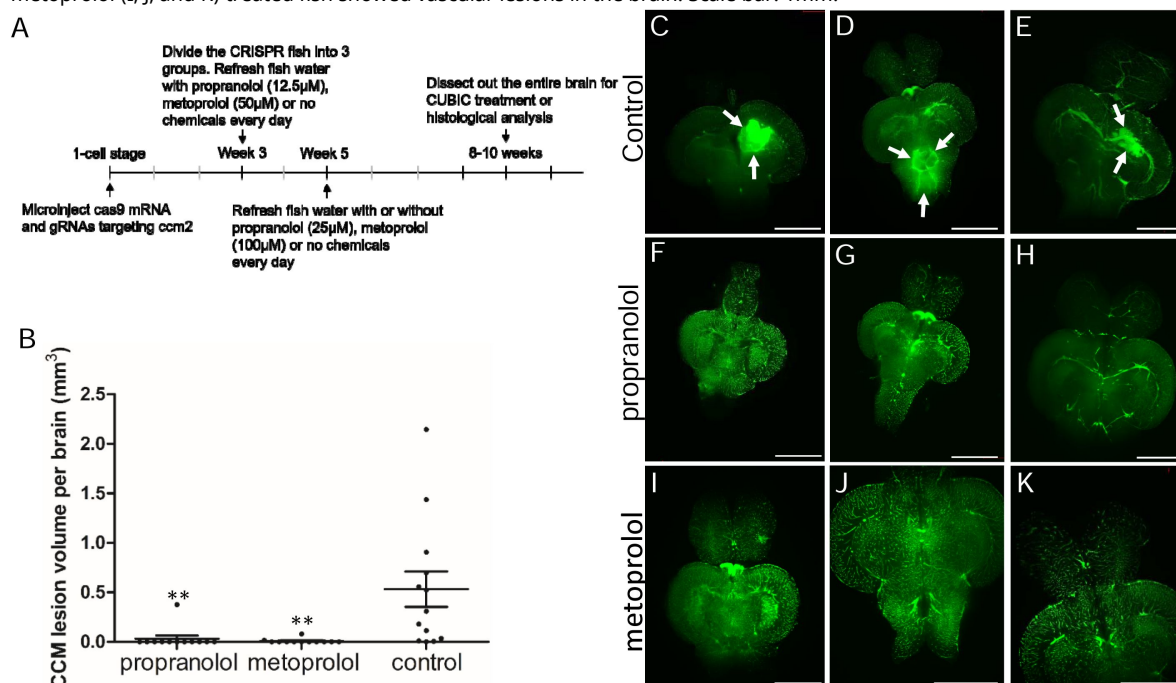


Figure 3.

Both propranolol and metoprolol could rescue CCM in *ccm2* CRISPR zebrafish. (A) A diagram outlines the drug treatment experiment, CUBIC treatment and following recording of CCMs in adult zebrafish brain. The chemical treatment was started from week 3 with 12.5 μ M propranolol or 50 μ M metoprolol, and increased to 25 μ M propranolol and 100 μ M metoprolol, respectively from week 5. The fish water with chemicals or vehicle control are refreshed on a daily basis. (B) Statistical analysis of lesion volume by one-way ANOVA followed by Tukey's multiple comparison test. *P*<0.01. (C through K) Representative light sheet microscopy scanning pictures of brains from *ccm2* CRISPR adult zebrafish. In controls without chemical treatment (C, D, and E) there were vascular anomalies indicated by arrows. Neither propranolol (F, G, and H) nor metoprolol (I, J, and K) treated fish showed vascular lesions in the brain. Scale bar: 1mm.



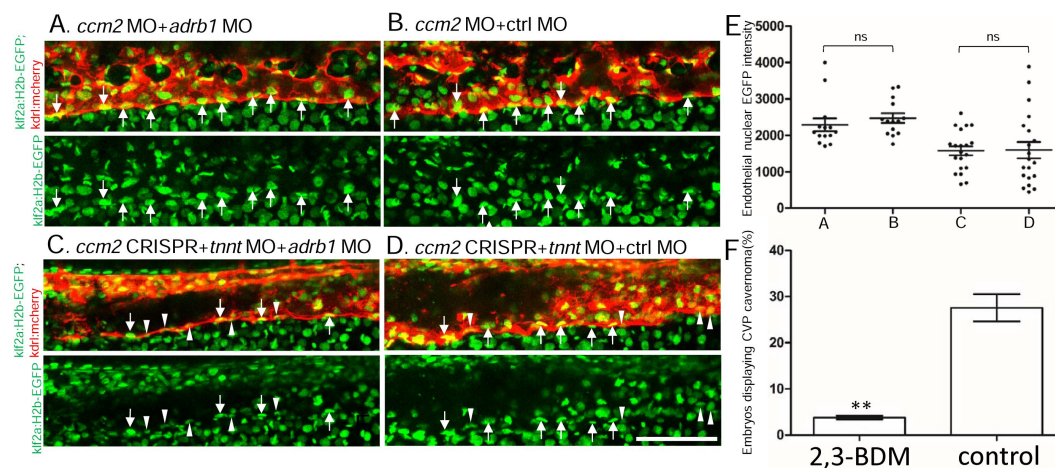


Figure 4.

Adrb1 signaling does not alter *klf2a* expression in *ccm2* CRISPR embryos. *Tg(klf2a:H2b-EGFP; kdrl:mcherry)* embryos were injected and nuclear EGFP signal in mcherry labeled vascular endothelial cells is recorded by confocal. Representative images from each group are shown. (A and B) Neither embryos injected with *ccm2* MO together with *adrb1* MO (A) nor control MO (B) displayed mosaic pattern of endothelial nuclear EGFP intensity. (C through E) All the embryos are absent of blood flow due to *tnnt* morpholino injection. Compared to that of control (C), *ccm2* CRISPR embryos co-injected with *adrb1* MO (D) or control MO (E) both displayed a mosaic increase of nuclear EGFP intensity of vascular endothelial cells. Scale bar:100 μ m. (F) EGFP intensity of endothelial nuclei from 2 embryos in each group are quantified with Image J. Statistical analysis is performed by one-way ANOVA followed by Tukey's multiple comparison test. There is no significant difference between *ccm2* morphants coinjected with *adrb1* MO (A) and control MO (B). There is also no significant difference between *ccm2* CRISPR embryos coinjected with *adrb1* MO (D) and control MO (E). $P > 0.05$. (F) At 2dpf, 2,3-BDM prevented the CVP cavernoma dramatically. 164 embryos in 2,3-BDM treated group and 177 in control group were used for Two-tailed paired t-test. $P = 0.0013$.

Case reports^{4,6,21} and our previous study⁸ revealed the potential benefit of the non-selective β -adrenergic receptor blocker propranolol in reducing CCMs in patients and mouse models, respectively. However, propranolol is a racemic mixture, and its R enantiomer which lacks β -adrenergic antagonism was reported to show therapeutic effect for infantile hemangiomas in an animal study⁹. Notably, our study demonstrates that *adrb1*^{-/-} zebrafish are significantly protected against the formation of CCMs, suggesting that propranolol's therapeutic effect on CCM is through β 1AR antagonism. Together with the observed significant reduction of CCM lesion volume upon metoprolol treatment, a selective β 1AR antagonist, supports the therapeutic potential of β 1AR antagonism in CCMs. β 1 selective blockers offer the advantage of causing fewer mechanism-based side effects compared to propranolol, such as bronchospasm²², and are already in clinical use (including agents like atenolol, metoprolol, nebivolol, and bisoprolol). Thus, further studies are warranted to evaluate the potential value of β 1 selective antagonists in this disease.

Consistent with the *Adrb1KO* mice¹⁴, the *adrb1*^{-/-} zebrafish embryos displayed the decreased chronotropic response to a beta-adrenergic agonist, isoprenaline, and decreased basal heart rate compared to that of *adrb1*^{+/+} brood mates; however, CVP morphology of *adrb1*^{-/-} embryo was not perturbed (Supplementary Figure 3). *adrb1*^{-/-} embryo also displayed a marked decrease of blood flow in CVP (Supplementary Video 2, and Supplementary Figure 1B). A similar reduction of heart rate and contractility by 2,3-BDM, a cardiac myosin ATPase inhibitor, prevented CVP dilation (Figure 4F). We previously found that arresting blood flow prevents aberrant intussusceptive angiogenesis and the resulted CVP cavernomas in *ccm2* CRISPR embryos³. Taken together, these data suggest that reduced blood flow secondary to reduced cardiac function accounts for the protective effect of loss of β 1AR signaling on CVP lesions and on CCM. Importantly, although β 1AR are highly expressed in cardiomyocytes and contribute to increased cardiac output²³, β 1ARs are also expressed in other tissues^{24,25} including endothelial cells of vascular anomalies in patients²⁶. Thus, future studies will be required to define the tissue specificity of the role of the β 1AR in CCM pathogenesis.

Experimental procedures

Zebrafish lines and handling

Zebrafish were maintained and with approval of Institutional Animal Care and Use Committee of the University of California, San Diego. The following mutant and transgenic lines were maintained under standard conditions: *Tg(fli1:EGFP)*^{1,27}, *Tg(klf2a:H2b-EGFP)*²⁸, and *Tg(kdrl:mcherry)*^{is5,29}. *adrb1*^{-/-} zebrafish was obtained by coinjection of Cas9 protein (EnGen Spy Cas9 NLS, M0646, NEB) with gRNA targeting *adrb1*. Genotyping of *adrb1*^{-/-} was performed with forward primer (5'-AGAGCAGAGCGCGGATGGAA-3') and reverse primer (5'-GATCCATACATCCAGGCT-3').

Plasmids and morpholino

pCS2-nls-zCas9-nls (47929) and pT7-gRNA (46759) were bought from Addgene. The CRISPR RNA (crRNA) sequences used in this study are as follow: *ccm2*-1 5'-GGTGTCTTCTGAAAGGGGAGA-3', *ccm2*-2 5'-GGAGAAGGGTAGGGATAAGA-3', *ccm2*-3 5'-GGGTAGGGATAAGAAGGCTC-3', *ccm2*-4 5'-GGACAGCTGACCTCAGTTCC-3', *adrb1* 5'-GACTCTAAACGCGCCACGG-3'. Target gRNA constructs were generated as described before³⁰. Morpholino sequence used in this study are as follow: *adrb1* (5'-ACGGTAGCCCGTCTCCCATGATTG-3'), *ccm2* (5'-GAAGCTGAGTAATACCTTAACCTT CC-3'), *tnnt2a* (5'-CATGTTTGCTCTGATCTGACACGCA-3'), *control* (5'-CCTTTACCTC AGTTACAATTTATA-3').

RNA synthesis

The pCS2-nls-zCas9-nls plasmid containing Cas9 mRNA was digested with NotI enzyme, followed by purification using a Macherey-Nagel column, serving as the template. The capped nls-zCas9-nls RNA was synthesized using the mMESSAGE mMACHINE SP6 Transcription Kit from ThermoFisher Scientific. The resulting RNA was purified through lithium chloride precipitation, as per the instructions provided in the kit. For gRNA synthesis, the gRNA constructs were linearized using BamHI enzyme and purified using a Macherey-Nagel column. The gRNA was synthesized via in vitro transcription using the MEGashortscript T7 Transcription Kit from ThermoFisher Scientific. After synthesis, the gRNA was purified by alcohol precipitation, as instructed in the same kit. The concentration of the nls-zCas9-nls RNA and gRNA was measured using a NanoDrop 1000 Spectrophotometer from ThermoFisher Scientific, and their quality was confirmed through electrophoresis on a 1% (wt/vol) agarose gel.

Microinjection

All injections were performed at 1-cell stage with a volume of 0.5nl. The final injection concentrations are as follow: Cas9 protein (10μM), Cas9 mRNA (750ng/μl), gRNA 120ng/μl, *adrb1* MO (4ng/μl), *ccm2* MO (4ng/μl), *tnnt2a* MO (5.3 ng/μl), control MO (4ng/μl).

Chemical treatment

Propranolol (P0995, TCI) (12.5μM) and metoprolol (M1174, Spectrum) (50μM) were used to treat the zebrafish larva from Day 21. Beginning from Day 35, adjusted concentration of propranolol (25μM) or metoprolol (100μM) were used to treat the juvenile fish. The chemicals were dissolved in fish water, and fish water containing chemicals were refreshed daily. Egg water without above chemicals was refreshed daily for fish used as negative control. 2,3-butanedione monoxime (BDM) (6mM) was added to egg water of the *ccm2* CRISPR embryos at 25hpf, and CVP cavernoma was observed at 2dpf.

Airyscan imaging and fluorescence intensity analysis

To prepare the embryos for imaging, they were first anesthetized using egg water containing 0.016% tricaine (3-amino benzoic acid ethyl ester) from Sigma-Aldrich. Subsequently, the anesthetized embryos were embedded in 1% low melting point agarose obtained from Invitrogen (product number 16520050). The imaging process was carried out using a Zeiss 880 Airyscan confocal microscope, utilizing the standard Airyscan mode, with a 20x/NA 0.8 objective. The intensity of nuclear EGFP (enhanced green fluorescent protein) was quantified using ImageJ software.

Heartbeat and blood flow recording

Heartbeat and blood flow were recorded using Olympus MVX10. The embryos were treated with 0.004% tricaine which does not have effect on heartbeat^{31, 32}.

Zebrafish brain dissection, CUBIC treatment and lightsheet imaging

The dissection of zebrafish brains followed the methodology described in a previous study by Gupta et al.³³. The CUBIC method was optimized based on the findings from a previous report by Susaki et al.¹⁵. The brains were fixed in 4% paraformaldehyde (PFA) with a pH of 7.5 for 24 hours and subsequently washed with PBS (phosphate-buffered saline) for an additional 24 hours. Following the PBS wash, the brains underwent CUBICR1 treatment at 37°C in a water bath for 42

hours. For imaging, the samples were placed in CUBICR2 as the imaging medium and imaged using a ZEISS Lightsheet Z.1 microscope. Scanning was carried out utilizing 5X dual illumination optics in combination with a 5X objective.

Statistical analysis

The statistical analysis was conducted using GraphPad Prism software. P-values were calculated using an unpaired two-tailed Student's t-test, unless otherwise specified. The bar graphs display the mean values along with their corresponding standard deviations (SD).

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References

1. Leblanc GG, Golanov E, Awad IA (2009) **Young WL and Biology of Vascular Malformations of the Brain NWC. Biology of vascular malformations of the brain** *Stroke* **40**:e694–702
2. Labauge P, Denier C, Bergametti F, Tournier-Lasserre E (2007) **Genetics of cavernous angiomas** *Lancet Neurol* **6**:237–44
3. Li W, Tran V, Shaked I, Xue B, Moore T, Lightle R, Kleinfeld D, Awad IA, Ginsberg MH (2021) **Abortive intussusceptive angiogenesis causes multi-cavernous vascular malformations** *Elife* **10**
4. Goldberg J, Jaeggi C, Schoeni D, Mordasini P, Raabe A, Bervini D (2018) **Bleeding risk of cerebral cavernous malformations in patients on beta-blocker medication: a cohort study** *J Neurosurg* :1–6
5. Moschovi M, Alexiou GA, Stefanaki K, Tourkantoni N, Prodromou N (2010) **Propranolol treatment for a giant infantile brain cavernoma** *J Child Neurol* **25**:653–5
6. Reinhard M, Schuchardt F, Meckel S, Heinz J, Felbor U, Sure U, Geisen U (2016) **Propranolol stops progressive multiple cerebral cavernoma in an adult patient** *J Neurol Sci* **367**:15–7
7. Lanfranconi S, Scola E, Meessen J, Pallini R, Bertani GA, Al-Shahi Salman R, Dejana E, Latini R, Treat CCM (2022) **Safety and efficacy of propranolol for treatment of familial cerebral cavernous malformations (Treat_CCM): a randomised, open-label, blinded-endpoint, phase 2 pilot trial** *Lancet Neurol*
8. Li W *et al.* (2021) **Propranolol inhibits cavernous vascular malformations by beta1 adrenergic receptor antagonism in animal models** *J Clin Invest* **131**
9. Overman J *et al.* (2019) **R-propranolol is a small molecule inhibitor of the SOX18 transcription factor in a rare vascular syndrome and hemangioma** *Elife* **8**
10. van den Meiracker AH, Man in't Veld AJ, Boomsma F, Fischberg DJ, Molinoff PB, Schalekamp MA (1989) **Hemodynamic and beta-adrenergic receptor adaptations during long-term beta-adrenoceptor blockade. Studies with acebutolol, atenolol, pindolol, and propranolol in hypertensive patients** *Circulation* **80**:903–14
11. MacLagan J, Ney UM (1979) **Investigation of the mechanism of propranolol-induced bronchoconstriction** *Br J Pharmacol* **66**:409–18
12. Robu ME, Larson JD, Nasevicius A, Beiraghi S, Brenner C, Farber SA, Ekker SC (2007) **p53 activation by knockdown technologies** *PLoS Genet* **3**
13. Eisen JS, Smith JC (2008) **Controlling morpholino experiments: don't stop making antisense** *Development* **135**:1735–43
14. Rohrer DK, Desai KH, Jasper JR, Stevens ME, Regula DP, Barsh GS, Bernstein D, Kobilka BK (1996) **Targeted disruption of the mouse beta1-adrenergic receptor gene: developmental and cardiovascular effects** *Proc Natl Acad Sci U S A* **93**:7375–80

15. Susaki EA, Tainaka K, Perrin D, Yukinaga H, Kuno A, Ueda HR (2015) **Advanced CUBIC protocols for whole-brain and whole-body clearing and imaging** *Nat Protoc* **10**:1709–27
16. Oldenburg J *et al.* (2021) **Propranolol Reduces the Development of Lesions and Rescues Barrier Function in Cerebral Cavernous Malformations: A Preclinical Study** *Stroke* **52**:1418–1427
17. Renz M *et al.* (2015) **Regulation of beta1 integrin-Klf2-mediated angiogenesis by CCM proteins** *Dev Cell* **32**:181–90
18. Zhou Z *et al.* (2015) **The cerebral cavernous malformation pathway controls cardiac development via regulation of endocardial MEKK3 signaling and KLF expression** *Dev Cell* **32**:168–80
19. Zhou Z *et al.* (2016) **Cerebral cavernous malformations arise from endothelial gain of MEKK3-KLF2/4 signalling** *Nature* **532**:122–6
20. Bartman T, Walsh EC, Wen KK, McKane M, Ren J, Alexander J, Rubenstein PA, Stainier DY (2004) **Early myocardial function affects endocardial cushion development in zebrafish** *PLoS Biol* **2**
21. Miquel J, Bruneau B, Dupuy A (2014) **Successful treatment of multifocal intracerebral and spinal hemangiomas with propranolol** *J Am Acad Dermatol* **70**:e83–e84
22. Ji Y, Chen S, Wang Q, Xiang B, Xu Z, Zhong L, Yang K, Lu G, Qiu L (2018) **Intolerable side effects during propranolol therapy for infantile hemangioma: frequency, risk factors and management** *Sci Rep* **8**
23. Rohrer DK, Chruscinski A, Schauble EH, Bernstein D, Kobilka BK (1999) **Cardiovascular and metabolic alterations in mice lacking both beta1- and beta2-adrenergic receptors** *J Biol Chem* **274**:16701–8
24. Guimaraes S, Moura D (2001) **Vascular adrenoceptors: an update** *Pharmacol Rev* **53**:319–56
25. Osswald W, Guimaraes S (1983) **Adrenergic mechanisms in blood vessels: morphological and pharmacological aspects** *Rev Physiol Biochem Pharmacol* **96**:53–122
26. Stanculescu MC, Popoiu MC, Cimpean AM, David VL, Heredea R, Cerbu S, Boia ES (2021) **Expression of beta1 adrenergic receptor in vascular anomalies in children** *J Int Med Res* **49**
27. Lawson ND, Weinstein BM (2002) **In vivo imaging of embryonic vascular development using transgenic zebrafish** *Dev Biol* **248**:307–18
28. Heckel E, Boselli F, Roth S, Krudewig A, Belting HG, Charvin G, Vermot J (2015) **Oscillatory Flow Modulates Mechanosensitive klf2a Expression through trpv4 and trpp2 during Heart Valve Development** *Curr Biol* **25**:1354–61
29. Jin SW, Beis D, Mitchell T, Chen JN, Stainier DY (2005) **Cellular and molecular analyses of vascular tube and lumen formation in zebrafish** *Development* **132**:5199–209
30. Jao LE, Wente SR, Chen W (2013) **Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system** *Proc Natl Acad Sci U S A* **110**:13904–9

31. Schwerte T, Uberbacher D, Pelster B (2003) **Non-invasive imaging of blood cell concentration and blood distribution in zebrafish *Danio rerio* incubated in hypoxic conditions in vivo** *J Exp Biol* **206**:1299–307
32. Langheinrich U, Vacun G, Wagner T (2003) **Zebrafish embryos express an orthologue of HERG and are sensitive toward a range of QT-prolonging drugs inducing severe arrhythmia** *Toxicol Appl Pharmacol* **193**:370–82
33. Gupta T, Mullins MC (2010) **Dissection of organs from the adult zebrafish** *J Vis Exp*

Editors

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Reviewer #1 (Public review):

Summary:

This work seeks to provide genetic evidence for a role for beta-adrenergic receptors that regulate heart rate and blood flow on cavernous malformation development using a zebrafish model, and to extend information regarding beta-adrenergic drug blockade in cavernous malformation development, with the idea that these drugs may be useful therapeutically.

Strengths:

The work shows that genetic loss of a specific beta-adrenergic receptor in zebrafish, *adrb1*, prevents embryonic venous malformations and CCM in adult zebrafish brains. Two drugs, propranolol and metoprolol, also blunt CCM in the adult fish brain. These findings are predicted to potentially impact the treatment of human CCM, and they increase understanding of the factors leading to CCM.

Weaknesses:

There are minor weaknesses that detract slightly from enthusiasm, including poor annotation of the Figure panels and lack of a baseline control for the study of *Klf2* expression (Figure 4).

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Reviewer #2 (Public review):

Summary:

Previously, the authors developed a zebrafish model for cerebral cavernous malformations (CCMs) via CRISPR/Cas9-based mosaic inactivation of the *ccm2* gene. This model yields CCM-like lesions in the caudal venous plexus of 2 days post-fertilization embryos and classical CNS

cavernomas in 8-week fish that depend, like the mouse model, on the upregulation of the KLF2 transcription factor. Remarkably, the morpholino-based knockdown of the gene encoding the Beta1 adrenergic receptor or B1AR (*adrb1*; a hemodynamic regulator) in fish and treatment with the anti-adrenergic S enantiomer of propranolol in both fish and mice reduce the frequency and size of CMM lesions.

In the present study, the authors aim to test the model that *adrb1* is required for CCM lesion development using *adrb1* mutant fish (rather than morpholino-mediated knockdown and pharmacological treatments with the anti-adrenergic S enantiomer of propranolol or a racemic mix of metoprolol (a selective B1AR antagonist).

Strengths:

The goal of the work is important, and the findings are potentially highly relevant to cardiovascular medicine.

Weaknesses:

(1) The following figures do not report sample sizes, making it difficult to assess the validity of the findings: Figures 1B and D (the number of scored embryos is missing), Figures 2G and 3B (should report both the number of fish and lesions scored, with color-coding to label the lesions corresponding to individual fish in which they were found).

(2) Figure 4 has a few caveats. First, the use of *adrb1* morphants (rather than mutants) is at odds with the authors' goal of using genetic validation to test the involvement of *adrb1* in CCM2-induced lesion development.

Second, the authors should clarify if they have validated that the *tnnt* (*tnnt2a*) morpholino phenocopies *tnnt2a* mutants in the context in which they are using it (this reviewer found that the *tnnt2a* morpholino blocks the heartbeat just like the mutant, but induces additional phenotypes not observed in the mutants).

Third, the data in Figure 4E is from just two embryos per treatment, a tiny sample size. Furthermore, judging from the number of points in the graph, only a few endothelial PCV cells appear to have been sampled per embryo. Also, judging from the photos and white arrowheads and arrows (Figure 4A-D), only the cells at the ventral side of the vessel were scored (if so, the rationale behind this choice requires clarification).

Fourth, it is unclear whether and how the *Tg(kdrl:mcherry)*^{is5} endothelial reporter was used to mask the signals from the *klf2a* reporter. The reviewer knows by experience that accuracy suffers if a cytosolic or cell membrane signal is used to mask a nuclear green signal.

Finally, the text and legend related to Figure 4 could be more explicit. What do the authors mean by a mosaic pattern of endothelial nuclear EGFP intensity, and how is that observation reflected in graph 4E? When I look at the graph, I understand that *klf2a* is decreased in C-D compared to A-B. Are some controls missing? Suppose the point is to show mosaicism of *Klf2a* levels upon *ccm2* CRISPR. Don't you need embryos without *ccm2* CRISPR to show that *Klf2a* levels in those backgrounds have average levels that vary within a defined range and that in the presence of *ccm2* mosaicism, some cells have values significantly outside that range? Also, in 4A-D, what are the white arrowheads and arrows? The legend does not mention them.

Given the practical relevance of the findings to cardiovascular medicine, increasing the strength of the evidence would greatly enhance the value of this work.

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Author response:

Thank you for your thoughtful and constructive feedback on our manuscript. We greatly appreciate your recognition of the strengths in our work, particularly regarding the genetic evidence demonstrating the role of beta-adrenergic receptors in cavernous malformation (CCM) development and the therapeutic potential of beta-blocker drugs.

We acknowledge your concerns and have addressing them to improve the clarity and rigor of our study. Specifically:

For Reviewer 1:

(1) Figure Annotation: We will enhance the annotation of the figure panels to provide clearer and more detailed descriptions, ensuring that each panel is easily interpretable and contributes effectively to the overall narrative of the study.

(2) Baseline Control for *Klf2* Expression: We will include this control (*klf2* expression in control Morpholino-injected embryos) in our revised figures and text to provide a more complete context for the changes in *klf2* expression observed in response to genetic loss of *adrb1* in *ccm2* morphants and *ccm2*-CRISPR embryos.

For Reviewer 2:

(3) Sample Sizes in Figures 1, 2, and 3: We agree that reporting sample sizes is crucial for the transparency and reproducibility of our findings. For Figures 1B and D, as well as Figures 2G and 3B, we will update the figure legends to include the number of embryos and adult fish in which lesions were scored.

(4) Figure 4 Concerns: Use of *adrb1* Morphants: We will note that the *adrb1* morphant shows similar hemodynamic changes to the *adrb1* mutant and that the morphants did not prevent the mosaic *klf2* expression in *ccm2* CRISPR embryos. Thus supporting our conclusion that protection from CVP cavernomas in the *adrb1* mutant are not due to altered hemodynamics blocking mosaic *klf2* expression.

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