Abortive Intussusceptive Angiogenesis Causes Multi-Cavernous Vascular Malformations

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Short title: Abortive Intussusceptive Angiogenesis and CCM

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Mosaic inactivation of *CCM2* in humans causes cerebral cavernous malformations (CCMs) containing adjacent dilated blood-filled multi-cavernous lesions. We used CRISPR-Cas9 mutagenesis to inactivate zebrafish *ccm2* resulting in novel lethal multi-cavernous lesions in the embryonic caudal venous plexus (CVP) caused by obstruction of blood flow by intraluminal pillars. These pillars mimic intussusceptive angiogenesis; however, the pillars failed to fuse to split the pre-existing vessel in two. Abortive intussusceptive angiogenesis stemmed from mosaic inactivation of *ccm2* leading to patchy *klf2a* over-expression and resulting aberrant flow signaling. Surviving adult fish manifested histologically-typical hemorrhagic CCM. Formation of mammalian CCM requires flow-regulated transcription factors, KLF2 and KLF4; fish CCM and the embryonic CVP lesion failed to form in *klf2a* null fish indicating a common pathogenesis with the mammalian lesion. These studies describe the first zebrafish CCM model and establish a mechanism that can explain the formation of characteristic multi-cavernous lesions.

Keywords: Cerebral Cavernous Malformations, intussusceptive angiogenesis, KLF2, Zebrafish

Abbreviations

CCM: Cerebral Cavernous Malformations

CRISPR: clustered regularly interspaced short palindromic repeats

dpf: days post fertilization

gRNA: guide RNA

hpf: hours post fertilization

NA: numerical aperture

CUBIC: clear, unobstructed brain imaging cocktails and computational analysis

CVP: caudal venous plexus

CNS: central nervous system

RBCs: red blood cells

H&E: Hematoxylin and eosin

Introduction

CCMs are central nervous system vascular anomalies that lead to significant morbidity and mortality [1]. CCMs affect ~1/200 humans and cause a lifelong risk of stroke and other neurological sequelae for which there is no pharmacologic therapy. Heterozygous loss of function mutations of three CCM genes (*KRIT1(CCM1), CCM2,* and *PDCD10(CCM3)*) are associated with development of venous capillary dysplasias with hemorrhage and increased vascular permeability [2] characteristic of CCM [1]. Heterozygous patients often exhibit a "second hit" on the normal *CCM* allele in CCM endothelial cells [3, 4] and loss of function of P53 or *Msh2*, genes that maintain genome stability, "sensitize" *Krit1*$^{+/-}$ or *Pdcd10*$^{+/-}$ mice for development of CCM. Thus, CCM are likely to arise following mosaic inactivation of both alleles of a given CCM gene. Neonatal endothelial-specific inactivation of murine *Krit1, Pdcd10 (Ccm3), or Ccm2* results in cerebellar and retinal vascular lesions that resemble CCM [5-7]. Thus, human CCM arise from venous capillaries as a consequence of mosaic inactivation of these genes in endothelial cells.

The most striking form of CCM are large complexes containing adjacent dilated blood-filled thin-walled vessels with surrounding hemosiderin deposition indicative of chronic bleeding. These are the lesions that are surgically resected either to relieve mass effects or recurrent bleeding and are therefore clinically relevant. The cellular mechanism whereby these hallmark multi-cavernous lesions form is unknown and we reasoned that the genetic and experimental accessibility of the zebrafish and the optical transparency of its embryos and larvae[8] could enable *in vivo* analysis of CCM development. In particular the fish is amenable to examination of genetic perturbations by study of mutant fish,

CRISPR/Cas9 mutagenesis, or morpholino silencing [9]. The Zebrafish “heart of glass” defect in cardiac development led to the discovery of HEG1 (a binding partner for KRIT1 [10]) and to the demonstration that loss of KRIT1 (*santa)* or its binding partner, CCM2 (*valentine*), produced an identical cardiac phenotype [11]. Importantly, neither mutation nor silencing of any of these genes have been reported to produce zebrafish CCM. Secondly, silencing of *pdcd10* does not produce either cardiac dilation or CCM in the fish [12]. Furthermore, there is compelling evidence that HEG1 mutations do not produce CCM in mice or humans[13]. These data indicate important differences between the consequences of deletion of these genes in the endocardium and in brain endothelial cells and underscore the need for a zebrafish model of authentic CCM.

Here we used CRISPR-Cas9 mutagenesis of *ccm2* to recapitulate the mosaic inactivation of a CCM gene. We observed a highly penetrant novel phenotype in embryos. By 2 days post fertilization (dpf) ~30% of embryos developed striking segmental dilatation of the caudal vein associated with slowed blood flow and formation of multiple dilated contiguous blood-filled chambers. We show that these lesions are caused by the intraluminal extension of pillars that lead to physical obstruction of blood flow. These pillars resemble the first steps of intussusceptive angiogenesis; however, in contrast to the physiological process, these pillars fail to organize and fuse together to split the pre-existing vessel in two. We show that this process of abortive intussusceptive angiogenesis is due to mosaic inactivation of *ccm2* leading to aberrant flow signaling and that, as in CCMs, KLF2 transcription factors play an important role in the formation of these lesions. In addition, typical CCM formed in the brains of all surviving adult and

juvenile fish. As in murine CCM models, KLF2 transcription factors played a key role in the pathogenesis of zebrafish CCMs. Thus, abortive intussusceptive angiogenesis, as a consequence of aberrant flow signaling, leads to formation of multi-cavernous venous lesions in zebrafish embryos that resemble human multi-cavernous CCM.

Methods

Zebrafish lines and husbandry

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: *ccm2*^{m201} [11], *klf2a*^{ig4} [14], *Tg(fli1:EGFP)*^{y1} [15], *Tg(gata1:dsred)*^{sd2} [16], *Tg(fli1:negfp)*^{y7} [17], *Tg(kdrl:mcherryras)*^{s896} [18], *Tg(klf2a:H2b-EGFP)* [19], *Tg(kdrl:mcherry)*^{is5} [20], and *casper* [21]. See Expanded Materials & Methods for morpholino injections.

Plasmids

pCS2-nls-zCas9-nls (47929) and pT7-gRNA (46759) were bought from Addgene. Crispr RNA (crRNA) sequences were listed in table of Expanded Materials & Methods. Target gRNA constructs were generated as described before [22]. PCS2-morangeccm2, pCS2-morangeccm2 mutant(L197R), pCS2-morangeklf2a, pCS2-morangeΔklf2a were cloned by infusion (Clontech) as follow: mOrange was cloned into ClaI, and linker sequence (5'-ggcagcgcgggcagcgcggcgggcagcggcgaattt-3') between ClaI and EcoRI. Then ccm2, L197R mutant, klf2a or Δklf2a sequence were cloned into EcoRI respectively. Primer sequences were listed in table of Expanded Materials & Methods.

RNA synthesis

For cas9 mRNA, pCS2-nls-zCas9-nls was digested by NotI, and then purified by column (Macherey-Nagel) as template. Capped nls-zCas9-nls RNA was synthesized using mMESSAGE mMACHINE SP6 Transcription Kit (ThermoFisher Scientific), and purified through lithium chloride precipitation described in the same kit. For gRNA synthesis, gRNA constructs were linearized by BamHI digestion and purified by column (Macherey-

Nagel). gRNA was synthesized by in vitro transcription using MEGAshortscript T7 Transcription kit (ThermoFisher Scientific), and purified by alcohol precipitation described in the same kit. The concentration of nls-zCas9-nls RNA and gRNA were measured by NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific), and their quality was confirmed by electrophoresis through a 1% (wt/vol) agarose gel. The final concentrations for RNA injection are as follow: cas9 750ng/μl, gRNA 120ng/μl, and injection volume is 0.5nl.

Whole mount in situ hybridization

Zebrafish embryos were collected at 48hpf, and fixed with 4% paraformaldehyde overnight. In situ hybridization was performed as described before [23]. The hybridization temperature is 68 ℃, and the probe concentration is 1ng/μl. For primers used to amplify the template DNA for probe synthesis, see Expanded Materials & Methods. The pictures for in situ hybridization was captured by Olympus MVX10, Macro-view.

Airyscan imaging and 3-D reconstruction

Embryos for imaging were anesthetized with egg water containing 0.016% tricaine (3-amino benzoic acid ethyl ester, Sigma-Aldrich), and then 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. Maximum projection was performed with ZEN (Zeiss). 3-D reconstruction was performed with Volocity (PerkinElmer).

Laser ablation of intravascular pillars

Laser ablation of intravascular pillars was performed using targeted ultra-fast laser pulses that were generated with a multi-pass Ti:Al2O3 amplifier of local construction that

followed a previously published design [24] and operated at a 5 kHz pulse-rate. The ablation beam and the imaging beam were combined with a polarizing beamsplitter [24] prior to the microscope objective. The two beams were focused in the same focal plane and the ablation beam was centered in the area that is raster-scanned by the imaging beam so that ablation occurred at the center of the TPLSM imaging field. The energy per pulse of the ablation beam was tuned with neutral density filters and the quantity of pulses was controlled by a mechanical shutter (Uniblitz LS3Z2 shutter and VMM-D1 driver; Vincent). The energy and number of pulses was adjusted based on damage evaluated from the real-time TPLSM images and ranged between 0.2-0.4μJ.

Live imaging of endothelial pillar ablation

Live images of the fish vessels were obtained with a two-photon laser scanning microscope of local design [24], which was adapted to include an ablation beam. Low-energy, 100-fs, 76-MHz pulses for TPLSM were generated by a Titanium:Sapphire laser oscillator (Mira F-900; Coherent Inc.) that was pumped by a continuous wave (CW) laser (Verdi V-10 Nd:YVO4 laser; Coherent Inc.). The imaging laser pulses were scanned in a raster pattern by galvanometric mirrors that are relay-imaged to the rear aperture of the objective. The two photon–excited fluorescence is reflected by a dichroic mirror and transmitted to a photomultiplier tube (PMT). To produce laser pulses for ablation while imaging, we employed a Pockels cell (QS-3 with NVP-525D driver and DD1 timing circuit; Quantum Technologies) to reroute 1 in 76,000 pulses from the oscillator pulse train to seed a multipass Titanium:Sapphire amplifier that is pumped by a Q-switched laser (Corona; Coherent). A half-wave plate (λ/2) rotates the polarization of the amplified pulses to lie perpendicular to that of the laser oscillator and thus permits both the ablation beam

and the imaging beam to be routed to the microscope objective with a polarizing beamsplitter. We used a 25x/NA 0.95, water-immersion objective (Olympus) for imaging and ablation.

Histology

Hematoxylin and eosin stain and Perl's Prussian blue stain were performed as described[25].

Zebrafish brain dissection, CUBIC treatment and Light sheet imaging

Zebrafish brain dissection was performed as previously described [26]. CUBIC was optimized on the basis of previous report [27]. The brains were fixed with PH7.5 4% PFA for 24 hours, and then washed with PBS for 24 hours. After PBS wash, CUBICR1 treatment was then performed at 37º C in water bath for 42 hours. Samples were imaged in CUBICR2 as medium with ZEISS Light sheet Z.1. Scanning was performed with 5X dual illumination optics and 5X objective.

Statistical analysis

Statistical analysis was performed with GraphPad Prism. P values were calculated by paired two- tailed Student's T-test unless otherwise specifically indicated. The mean and SD were shown in the bar graphs.

Results

ccm2 CRISPR zebrafish embryos display segmental dilation of the Caudal Venous Plexus (CVP)

As noted above, although null mutations of *krit1* and *ccm2* result in cardiac dilation and some vascular abnormalities, CCM have not been observed in zebrafish[11, 28]. We reasoned because humans with CCM are mosaic for homozygous inactivation of *CCM1* or *CCM2*, that induction of such mosaicism using a CRISPR-Cas9 system [29] could result in lesion formation. To create a mosaic animal, we co-injected Cas9 mRNA and gRNAs targeting the *ccm2* gene in zebrafish embryos (Supplementary Figure 1, Table1). As expected, both genomic DNA sequencing and whole mount in situ hybridization showed that *ccm2* was targeted in a variable mosaic pattern affecting all tissues (Supplementary Figure 1 B and C). About half of these mosaic embryos displayed lethal cardiovascular defect, and these embryonic phenotypes will be described below.

In ~30% of 2 dpf *ccm2* CRISPR embryos, we noted a new phenotype: localized dilatation of the Caudal Venous Plexus (CVP) associated with erythrocyte accumulation and sluggish blood flow (Figure 1A and B, Supplementary Video 1). This phenotype was clearly demonstrated in *Tg(fli1:EGFP)*^{y1} and *Tg(gata1:DsRed)*^{sd2} embryos in which erythrocytes are labelled with DsRed and endothelial cells with EGFP (Figure 1C and 1D). Examination of the dilated CVP revealed multiple large blood-filled chambers separated by thin-walled partitions that bore a striking resemblance to Stage 2 human multi-cavernous CCM (Figure 1C) in contrast to the normal architecture of control embryos (Figure 1D). In addition, ~5% of *ccm2* CRISPR embryos also displayed dilated cranial

vessels (CV) (Figure 1E). We also noted expected phenotypes previously reported in *ccm*2 morphants and mutants [11, 28], a small proportion (~10%) of *ccm2* CRISPR embryos exhibited both heart dilation at 2 dpf and increased branching of the subintestinal vein (SIV) at 3 dpf (Supplementary Figure 2). Co-administration of *ccm2* mRNA prevented both CVP dilation and heart dilation in *ccm2* CRISPR embryos (Figure 1G) confirming that both phenotypes are due to *ccm2* loss. Notably, the dilated heart and CVP dilation appeared to be mutually exclusive i.e. in over 200 embryos analyzed, we never observed both phenotypes in a single *ccm2* CRISPR embryo. Thus, the localized CVP dilation was the most prevalent phenotype observed in 2 dpf *ccm2* CRISPR embryos in comparison to the cardiac and subintestinal vein phenotypes that characterize *ccm2* null and *ccm2* morphant embryos (Figure 1H) [11, 28].

Abortive intussusceptive angiogenesis in the dilated CVP of ccm2 CRISPR embryos

We used confocal microscopy and 3-D reconstruction of the dilated area of the CVP in *Tg(fli1:EGFP;gata1:DsRed)* zebrafish to explore their underlying structural defect. We noted intraluminal endothelial pillars that partitioned the lumen (Figure 2A through C) of the dilated CVP. In contrast, as expected, a completely patent caudal and ventral vein lumen formed in control embryos (Figure 2D through F). Furthermore, the ventral vein, which normally forms by a combination of sprouting and intussusceptive angiogenesis[33], was lost in the dilated area of the CVP (Figure 2A through F). Importantly, these pillars were associated with pits on the external surface of the dilated CVP (Figure 2G arrows), a hallmark of the initial phase of intussusceptive angiogenesis [34]. In contrast to normal

intussusceptive angiogenesis, wherein transluminal pillars ultimately fuse to divide vessels longitudinally into new daughter vessels [34], the intussusceptions observed in *ccm2* CRISPR embryos were not coordinately formed and failed to fuse resulting in a honeycombed lumen. This honeycombing created a lumen with multiple chambers filled with red blood cells (RBCs) associated with sluggish blood flow (Figure 2I through K, Supplementary Video 2 and 3), whereas patent lumens and normal blood flow were observed in control embryos (Supplementary Video 4). Thus, in these mosaic *ccm2* null zebrafish, an expanded region of the CVP is formed by multiple dilated erythrocyte-filled chambers and is associated with evidence of incomplete intussusceptive angiogenesis.

Ablation of intravascular pillars reverts the dilated CVP phenotype

The multiple dilated compartments and sluggish blood flow suggested that this phenotype could be due to obstruction of free flow of erythrocytes by the meshwork of intravascular pillars. In support of this idea, we observed that spontaneous regression of an existing pillar was accompanied by reduced dilation of the CVP (Figure 3A through D). In addition, the trapped erythrocytes began to circulate freely. This rapid relief of both vessel dilation and blood stagnation suggested that the aberrant pillars may form a physical barrier thus resulting in accumulation of erythrocytes in dilated cavernous structures. To directly test the role of obstruction by intravascular pillars in dilation, we used targeted short pulses of near-infrared laser light to sever the pillars, a technique that generates negligible heat transfer and collateral damage to neighboring tissues [24]. There was near instantaneous reduction of the dilated vessel diameter (93.4µm) to near normal dimensions (69.4µm) in

the example shown (Figure 3E and F, Supplementary Video 5). Thus, the pillars are the underlying cause of the CVP dilation observed in *ccm2* CRISPR embryos.

Blood flow and red blood cells are required for CVP dilation

The importance of the pillars in CVP dilation suggested that obstruction of blood flow was responsible for the phenotype. Consistently, as noted above, *ccm2* CRISPR embryos displaying CVP dilation did not show heart dilation. Conversely, segmental CVP dilation was absent in *ccm2* null mutants or *ccm2* morphants that exhibit characteristic heart dilation (Supplementary Figure 3). These observations suggest that a normally pumping heart and thus normal blood flow is required for CVP dilation. To investigate the role of blood flow, we took advantage of the capacity of zebrafish embryos to obtain sufficient oxygen by diffusion to survive temporarily in the absence of circulating blood. We induced a silent heart phenotype by using a troponin T (*tnnt*) morpholino, resulting in ~65% reduction in the frequency of CVP dilation (Figure 4A). We also reasoned that the meshwork of pillars would not obstruct fluid flow but would present a barrier to free passage of erythrocytes. Reduction of erythrocytes using *gata1* or *tif1-gamma* morpholinos produced a similar dramatic reduction in the CVP dilation (Figure 4A). These data, indicate that the meshwork of pillars obstructs the passage of erythrocytes in flowing blood resulting in multiple erythrocyte-filled cavernous chambers that dilate the CVP .

As shown in Figure 2, the sprouts that form the ventral vein are lost in the dilated region of the CVP. Because CVP development in *tnnt* morphants is nearly normal [35], we inspected the regions of the CVP displaying loss of ventral sprouting in *tnnt* morphant

ccm2 CRISPR embryos. In 11 such embryos, in spite of the defective ventral sprouting and ventral vein formation, we observed no intravascular pillars. This result suggests that blood flow, in addition to causing the CVP dilation, is required for intussusceptive pillar formation (Figure 4B and C) as it is for normal CVP arborization[33]. Inactivation of either *ccm1* or *ccm2* markedly upregulates expression of KLF2 a flow-regulated transcription factor required for normal cardiovascular development and for CCM formation [28, 32, 36]. *In situ* hybridization revealed that *klf2a* was also upregulated in a patchy pattern in the CVP of *ccm2* CRISPR embryos (Figure 4D and E). Thus, dilation was associated with the patchy upregulation of a flow-sensitive transcription factor, KLF2, in the *ccm2* CRISPR CVP. Taken together, these results suggest that patchy KLF2 expression in combination with blood flow leads to formation of these dilated red blood cell filled multi-cavernous lesions in the CVP.

Mosaic expression of ccm2 is necessary for cavernoma formation in the CVP

ccm2 CRISPR caused mosaic inactivation of *ccm2* and the dilated CVP phenotype, whereas global inactivation of *ccm2* in *ccm2* null mutants or *ccm2* morphants does not. We therefore questioned whether mosaicism, *per se,* played a role in the CVP dilation. To test this idea, we globally reduced *ccm2* expression by co-injecting a sub lethal dose of *ccm2* morpholino with the *ccm2* gRNA CRISPR mixture. The chosen morpholino dose did not increase the frequency of observable heart defects; however, the percentage of embryos displaying CVP dilation decreased dramatically (Figure 5A). We then reasoned that because *ccm2* acts as a scaffold connecting *krit1* to *ccm3* [37], the overexpression of *ccm2* might have a dominant negative effect. Indeed, when we injected linearized DNA

containing *ccm2* fused to m-Orange, *ccm2* mosaic over expression led to CVP dilation and aberrant intussusceptions similar with those observed in *ccm2* CRISPR embryos (Figure 5B and B'). In sharp contrast, injection of a plasmid encoding a loss of KRIT1 binding function *ccm2(L197R)* mutant [38] resulted in embryos displaying a normal vascular development (Figure 5C and C'). Thus, mosaicism for *ccm2* expression causes the formation of multi-cavernous erythrocyte-filled structures in the CVP.

Mosaic upregulation of KLF2a is sufficient for cavernoma formation in CVP

Inactivation of *ccm2* leads to upregulation of KLF2 and, as shown above, *ccm2* mosaicism results in the dilated CVP phenotype. Thus, we hypothesized that the resultant mosaic KLF2 upregulation could initiate the abortive intussusceptive angiogenesis. To test this idea, we used a *klf2a* reporter line, *Tg(klf2a:H2AEGFP)*, together with an endothelial cell-specific marker line (*Tg(kdrl:mcherry)*^{is5}) to observe the activity of the *klf2a* promoter. *Ccm2* morphants displayed a generalized increase in *klf2a* reporter expression (Supplementary Figure 4 A and A'), whereas the loss of blood flow in the *tnnt* morphant caused much reduced reporter expression in endothelial cells (Supplementary Figure 4 B through C'). Consistent with previous reports [28, 39], these opposing changes confirm that *ccm2* and flow can regulate expression of KLF2a. In 23 hpf *ccm2* CRISPR embryos, examined prior to onset of blood flow, a patchy increase in *klf2a* reporter expression was observed in *ccm2* CRISPR CVP endothelial cells; whereas reporter expression was uniformly low in control embryos at the same stage (Figure 6A, B). Furthermore, in *tnnt* morphant 2dpf *ccm*2 CRISPR embryos, there was also a striking mosaic increase in endothelial *klf2a* reporter expression (Figure 6C).

The variable increase in KLF2a expression in the CVP endothelial cells of *ccm2* CRISPR embryos and requirement for blood flow suggested the possibility that these two factors led to the formation of cavernomas in the CVP. To address the role of KLF2, we injected *klf2a* and *klf2b* morpholinos and observed reversal of both CVP dilation and heart dilation in the *ccm2* CRISPR embryos (Figure 6D). Furthermore, *ccm2* CRISPR treatment of *klf2a*$^{-/-}$ embryos caused no CVP dilation (Supplementary Figure 5). Thus, *klf2a* is required for the CVP dilation phenotype.

In *ccm2* CRISPR embryos, KLF2a was both upregulated in a mosaic fashion and required for CVP dilation; we therefore asked whether mosaic upregulation of KLF2a expression *per se* causes cavernoma formation. Mosaic overexpression was accomplished by injecting a plasmid encoding KLF2a *into Tg(fli1:EGFP)*^{y1} embryos; ~6% of such embryos displayed CVP dilation compared to control embryos injected with ΔKLF2a plasmid expressing KLF2a with a deleted DNA binding domain [40] (Figure 6E and F). Affected embryos exhibited intussusceptions within the CVP lumen accompanied by dilation (Figure 6G). These observations show that mosaic upregulation of KLF2a expression is sufficient for cavernoma formation when blood is flowing. Combined with the capacity of mosaic deletion or overexpression of *ccm2* to cause CVP dilation, these results show that uncoordinated flow sensing caused by mosaic KLF2a expression leads to the abortive intussusceptive angiogenesis that obstructs the lumen to form these cavernoma-like lesions.

Cerebral cavernous malformations in adult zebrafish

The foregoing data indicated that mosaic inactivation of *ccm2* resulted in a multi-cavernous lesion in the embryonic CVP that resembles mammalian CCM in gross architecture and dependence on KLF2. We then asked if authentic CCM would develop in the ~50% of *ccm2* CRISPR embryos that developed with a normal gross morphology and survived to adulthood. Brain vascular lesions were observed in virtually all of these adult *ccm2* CRISPR zebrafish (Figure 7A and C) and not in control fish (Figure 7E and G). In order to image the lesions at the whole brain level, clear, unobstructed brain imaging cocktails and computational analysis (CUBIC) was applied to these brains, and the transparent brains were scanned by light sheet microscopy (Figure 7B, D, F, and H). The distribution of lesions included cerebrum, cerebellum, brain stem and in some fish also the spinal cord (Figure 7I). This distribution pattern is similar to that found in patients[30]. Hematoxylin and eosin stained sections showed dilated multi-cavernous vascular channels filled with nucleated erythrocytes and lacking mature vessel wall angioarchitecture (Figure 7J). Perl's Prussian blue staining indicated prior hemorrhage adjacent to the lesions (Figure 7K). These histological findings were absent in control fish (Figure 7L and M) and resemble those in CCM patients (Figure 7N and O) [31]. CCM were not seen in *ccm2* CRISPR in *klf2a*^{ig4} null zebrafish [14] (Figure 7P) consistent with previous murine studies in which inactivation of *Klf2* prevented CCM formation[32]. In addition, similar to humans, these adult zebrafish also developed extracranial lesions (Supplementary Figure 6).

Discussion

Familial CCM lesions form as a consequence of mosaic complete inactivation of *CCM1, 2,* or *3.* Here we have used Cas9-CRISPR mutagenesis to create such a mosaicism for *ccm2* in zebrafish and show that surviving adult *ccm2* CRISPR animals develop brain and extracranial lesions that closely resemble those observed in humans with CCM. In ~30% of embryos, we observed a novel phenotype, the formation of segmental dilatation of the caudal vein associated with slowed blood flow and formation of multiple markedly dilated blood-filled chambers, resembling a multi-cavernous CCM. These lesions are caused by intussusceptive intraluminal pillars that obstruct the passage or erythrocytes resulting in the development of multiple dilated blood filled chambers. These pillars form as a consequence of a combination of blood flow and mosaic over expression of a flow-dependent transcription factor, KLF2a, leading to aberrant flow sensing in the developing CVP. In sum, our studies describe the first zebrafish model for CCM and provide a new mechanism that can explain the formation of the characteristic multi-cavernous lesions seen in humans.

CVP dilation is due to blood flow obstruction by intravascular pillars.

The segmental dilation of the CVP is due to intussusceptive pillars that fail to fuse normally, thus honeycombing the vein lumen and obstructing the free flow of blood. Evidence for the role of obstruction includes the marked slowing of blood flow within the lesions, dependence of dilation on blood flow and erythrocytes (Fig. 5A), and the relief of dilation by spontaneous or induced regression of the pillars (Fig 4A through F).

Intussusceptive angiogenesis differs from sprouting angiogenesis by splitting the existing vessel intraluminally as a response to increased blood flow [34]. Recent elegant studies have shown that localized reduction in fluid shear stress occurs at the site of intussusceptive pillars and is contiguous around those pillars that are fusing to divide a vessel[33]. Similar to physiological intussusceptive angiogenesis, the hallmark pits and intraluminal pillars were also observed in the CVP of *ccm2* CRISPR embryos (Figure 3A, B, C and G). However, these pillars failed to undergo orderly fusion to split the vessel due to mosaic over-expression of *klf2a*, a flow-sensitive transcription factor, thus disrupting the orderly flow signaling required for intussusceptive CVP arborization[33]. The meshwork formed by these intraluminal endothelial pillars partitioned the patent lumen into multiple blood-filled chambers (Figure 3I and J, Supplementary Videos 2 and 3). As more and more red blood cells accumulated, the CVP became dilated (Supplementary Video 1). In contrast to the necessity of blood flow for formation of the multi-cavernous CVP lesions, blood flow suppresses endothelial proliferation and simple vessel dilation in *krit1* global null zebrafish [42] in which this CCM gene is deleted throughout the vasculature. As shown here, vessels mosaic for expression of a CCM gene require blood flow to form the intravascular pillars that obstruct blood flow and cause multi-cavernous lesions. Previous studies termed dilated capillaries Stage 1 CCM and multi-cavernous lesions stage 2 CCM [25]. The differential flow requirements for formation of dilated vessels and multi-cavernous lesions in zebrafish suggest that the multi-cavernous form of CCM may employ a distinct pathogenetic mechanism.

Ccm2 **mosaicism causes multi-cavernous malformations**

Initially we ascribed the absence of segmental CVP dilation in *ccm2* null fish [11, 28] solely to the reduced blood flow caused by the dilated heart. This explanation is insufficient because rescue of the heart phenotype in *krit1 (ccm1)* null fish was not reported to cause segmental CVP dilation or CCMs [42]. Normal intussusceptive angiogenesis requires an orderly patterning of high and low flow signaling[33]. *Ccm2* mosaicism causes a random upregulation of *klf2a*, a key effector of flow signaling, thereby disrupting this orderly patterning of flow signaling. In contrast, the global knockout uniformly upregulates klf2a so that the patterning of other flow sensitive signals can guide the completion of the intussusceptive arborization.

The dilated multi-cavernous CVP lesions described here resemble human multi-cavernous CCM [3] and their formation required mosaicism. Recent elegant studies found that multi-cavernous murine CCM are mosaic for inactivation of *Ccm3* [45, 46]. The mouse studies emphasized that simply dilated vessels are not mosaic and contained only *Ccm3* null endothelial cells [45, 46] . Mosaicism in multi-cavernous murine CCM was ascribed to recruitment of wild type cells to the clonal CCM[45, 46]. Importantly, the mouse studies did not address the mechanism by which multi-cavernous lesions form. Here we, have shown that mosaicism is a pre-requisite for formation of these multi-cavernous lesions because it disorganizes the flow signaling required for orderly sprouting and intussusceptive angiogenesis that remodel the CVP.

***Ccm2* CRISPR zebrafish are an authentic CCM model**

As in humans [3, 4], the fish lesions arise as a consequence of mosaic inactivation of CCM genes. Secondly, as in humans, chronic bleeding leads to iron deposition; this finding contrasts with the lack of iron deposition seen in acute mouse CCM models [25]. Thirdly, similar to humans, histologically typical lesions are distributed throughout the CNS in contrast to the hindbrain-restricted lesions in acute mouse models[25]. Fourth, as is true in mouse models [13, 41], the development of CCM depends on *klf2a*, the orthologue of murine *Klf2* and paralogue of *Klf4*, indicating that they form by the same pathogenetic mechanism. There are chronic mouse models which do exhibit hemosiderin deposits and lesions throughout the CNS [3]; however, these models require cumbersome breeding schemes and mice of more than three or more months of age. That said, a recent report that postnatal induction of brain-endothelial-cell-specific ablation of the *Ccm2* gene using the inducible *Slco1c1*-CreER^{T2} mouse results in iron deposits around CCM throughout the murine brain at three months of age has great promise [43]. In contrast to existing chronic mouse models, the present model uses CRISPR-Cas9 to generate highly penetrant typical lesions throughout the CNS, requires about two months, and can be induced in mutant strains without additional breeding. Thus, this model should be useful tool in future studies to assess the effect of the many genetic manipulations possible in zebrafish [8] on the pathogenesis of CCM and to provide a complement to pharmacological screens directed at the dilated heart phenotype of *ccm1* or *ccm2* mutants [44].

In sum, the present work reveals a new embryonic vascular malformation, a multi-cavernous dilation of the CVP that resembles multi-cavernous Stage 2 CCM. The CVP malformation requires blood flow and mosaic inactivation of *ccm2* and is caused by

abortive intussusceptive angiogenesis as a consequence of imbalanced flow signaling. The high penetrance and resemblance of the embryonic CVP malformation to multi-cavernous CCM suggest that it will be a useful phenotype for pharmacologic or morpholino-based screens. In addition, we report a tractable zebrafish model of CCM that mimics the mammalian disease in mosaicism, lesion histology, and dependence on KLF2 transcription factors. A particularly appealing feature of the two models is that disease pathogenesis can be studied on mutant backgrounds without the need for additional breeding. Hits in the embryonic screen can then readily be tested for effects on the formation of brain CCM that occur in the 50% of *ccm2* CRISPR embryos that survive to adulthood.

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Supplemental Materials

Expanded Materials & Methods

Supplementary Figures 1-6

Supplementary Videos 1-5

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Figure Legends

Figure 1. *ccm2* CRISPR zebrafish embryo display novel vascular phenotypes. Endothelial cells and red blood cells were labeled by EGFP and DsRed respectively in double transgenic *Tg(fli1:EGFP)*^{y1}*;Tg(gata1:DsRed)*^{sd2}*.* embryos (A) Red blood cells accumulate in dilated segments of the caudal vein of *ccm2* CRISPR fish at 2dpf. (B) cas9 mRNA injected Control embryo. (C) *ccm2* CRISPR embryos showed accumulation of red blood cells and intraluminal endothelial cells in a dilated segment of caudal vein in contrast to a control embryo (D). (E) *ccm2* CRISPR embryos occasionally showed dilations of cerebral veins, whereas control embryos (F) showed normal development of cerebral veins (F). MCeV: mid cerebral vein, PMBC: primordial midbrain channel, PHBC: primordial hindbrain channel. (G) The dilated CVP and heart of *ccm2* CRISPR embryos were rescued by ccm2 mRNA injection. p Values were calculated using an unpaired two-tailed Student's t test. (H) Phenotypic distribution of dilated heart, CVP, and Cerebral Veins (CV) in *ccm2* CRISPR embryos at 2dpf in the . *$p < 0.05$; **$p < 0.01$. Error bars indicate SD. Scale bar: 1mm in A and B, and 100µm in C through F.

Figure 2. **Intravascular pillars honeycomb the lumen of the caudal vein in *ccm2* CRISPR embryos.** (A-F) XZ planes and 3-D projection along Y axis of Airyscan images revealed intraluminal endothelial pillars at 2dpf (A-C), whereas Cas9 injected control embryos displayed a normal patent lumen in both a dorsal and ventral caudal vein (D-F). Endothelial cells were labeled by EGFP in *Tg(fli1:EGFP)* embryos. Arrow, arrowhead, and asterisk indicated the dorsal aorta, dorsal vein and ventral vein, respectively. (G and H) Ventral view of 3-D reconstruction show the irregular surface of the dramatically dilated caudal vein segment in *ccm2* CRISPR embryo (G) and normal ventral vein (H). Arrows in

G indicate small pits where the endothelial pillars originate. (I-K) Intraluminal view of 3-D reconstruction of *ccm2* CRISPR embryo reveal the intraluminal pillars honeycombing the lumen and the accumulated red blood cells (I). Erythrocytes were not imaged in J to reveal pillars and the area within the box in (J) was magnified in (K), and arrowhead indicates the intravascular pillar. Endothelial cells and red blood cells were labeled by EGFP or DsRed respectively in *Tg(fli1:EGFP)*^{y1}*;Tg(gata1:DsRed)*^{sd2} embryos. Scale bar: 20µm.

Figure 3. Intravascular pillars obstruct blood flow leading to vessel dilation in *ccm2* CRISPR embryos. (A through D) Time lapse images reveal spontaneous retraction of an intravascular pillar leading to re-entry of blood cells into circulation and reduced dilation of the caudal vein. Endothelial cells were labeled by mCherry, and their nucleus and some red blood cells were labeled by EGFP in the *Tg(fli1:nEGFP)*^{y7}*;Tg(kdrl:mcherryras)*^{s896} embryos. The retracted pillar is outlined by dotted lines for emphasis. Note that pillar retraction and vessel dilation were temporally correlated. (E and F) Laser ablation of pillar reduced CVP diameter. The diameter of the dilated vein (E) was reduced after ablation (F). Note the pillars indicated by arrows in (E) are gone after ablation in (F). Dashed line indicates the diameter of the vein before and after ablation. Scale bar: 50µm.

Figure 4. Blood flow is required for pillar formation and vessel dilation. Morpholinos targeting *tnnt*, *gata1*, *tif1gamma*, or a control morpholino were co-injected with *ccm2* guide and Cas9 RNA. (A) Reduction of blood flow in *tnnt* morphants (A, B, C) resulted in reduced CVP dilation (A) and intravascular honeycombing (B, C) in 2dpf *ccm2* CRISPR *Tg(fli1:EGFP)* embryos. Arrows indicate intussusceptions. Scale bar: 100µm. (A) Loss of erythrocytes in *gata1 or tif1gamma* morphant *ccm2* CRISPR embryos also reduced the incidence of CVP dilation. P Values were calculated using one-way ANOVA. **$p < 0.01$.

Error bars indicate SD. (D and E) In situ hybridization showed a patchy increase of endothelial *klf2a* expression in 30hpf *ccm2* CRISPR embryos (D) compared to control (E).

Figure 5. Mosaic *ccm2* expression caused CVP dilation. (A) Low dose *ccm2* morpholino reduced the incidence of CVP dilation but did not significantly increase heart dilation in *ccm2* CRISPR embryos. (B and C) Mosaic *ccm2* but not inactive *ccm2(L197E)* over expression caused CVP dilation. Arrows indicate pillars in the CVP. (B' and C') Mosaic expression of mOrange-tagged *ccm2* or *ccm2(L197E)*. Scale bar: 100µm. Error bars are ±S.D.

Figure 6. Mosaic KLF2a expression caused CVP dilation. (A and B) At 23hpf, *ccm2* CRISPR *Tg(klf2a:H2b-EGFP)* embryos displayed a mosaic increase of EGFP expression in endothelial cells in the CVP (A), compared with cas9 mRNA control embryos (B). Scale bar: 25µm. (C) *ccm2* CRISPR and *tnnt* morpholino injected *Tg(klf2a:H2b:EGFP* 2dpf embryos displayed a mosaic increase of endothelial nuclear EGFP expression in dorsal vein. Scale bar: 50µm. In A through C, EGFP expression was driven by *klf2a* promoter in *Tg(klf2a:H2b:EGFP)* embryo, and endothelial cells were labelled by mcherry in *Tg(kdrl:mcherry)* transgenic line. Arrows indicated the endothelial nuclei with increased EGFP, and arrowheads indicated the other endothelial nuclei along the ventral wall of dorsal vein. (D) Both the CVP dilation and heart dilation were rescued by injection of *klf2* morpholinos in 2dpf *ccm2* CRISPR embryos. **$p < 0.01$. Error bars indicate SD. (E) *pCS2-*KLF2a plasmid-injected 2.5 dpf embryos displayed CVP dilation, whereas injection of a PCS2 plasmid encoding a DNA binding domain deleted ΔKLF2a mutant showed normal development. Arrow indicates the CVP dilation and retained erythrocytes. Scale bar: 1mm. (F) Quantification of the prevalence of CVP dilation following KLF2a or ΔKLF2a

overexpression. The mean and SD are shown. (G and H) Representative images show the honeycombed lumen and dilated CVP in 1.5dpf KLF2a injected embryo (G) and normal CVP of ΔKLF2a injected embryo. Arrow indicates honeycombing. Scale bar: 100µm.

Figure 7. Adult *ccm2* CRISPR zebrafish develop typical CCM lesions. The ~50%of *ccm2* CRISPR fish that survived developed highly penetrant CCMs. (A and C). Arrows indicate superficial lesions on dorsal (A) and ventral (C) surface of the brain. Note hemorrhage into the ventricles. Lesions are absent in control embryos (E and G). CUBIC clearing (B,D,F,H) enables visualization of CCM burden by light sheet microscopy. Arrows indicate the lesions that corresponded to those seen in bright field, and arrowhead indicates a deeper lesion. L: left, R: right. Scale bar: 1mm. (I) Cavernomas were dispersed throughout the central nervous system including cerebrum, cerebellum, brain stem and spinal cord. (J) Hematoxylin and eosin (H&E) stained brain section reveals nucleated erythrocytes filling a dilated vessel with adjacent Prussian Blue stained iron deposition (K) in *ccm2* CRISPR fish and the absence of lesions or iron deposition in control fish (L,M). (N,O) a CCM from a patient stained with H&E (N) or Prussian Blue (O). Note similar appearance to the zebrafish lesion shown in (J,K). Arrow indicates dilated vessel. Scale bar: 50µm. (P) CCM were prevented in *klf2a*^{ig4} null *ccm2* CRISPR adults. Error bars in panel I and P are ±S.D.

Fig.1

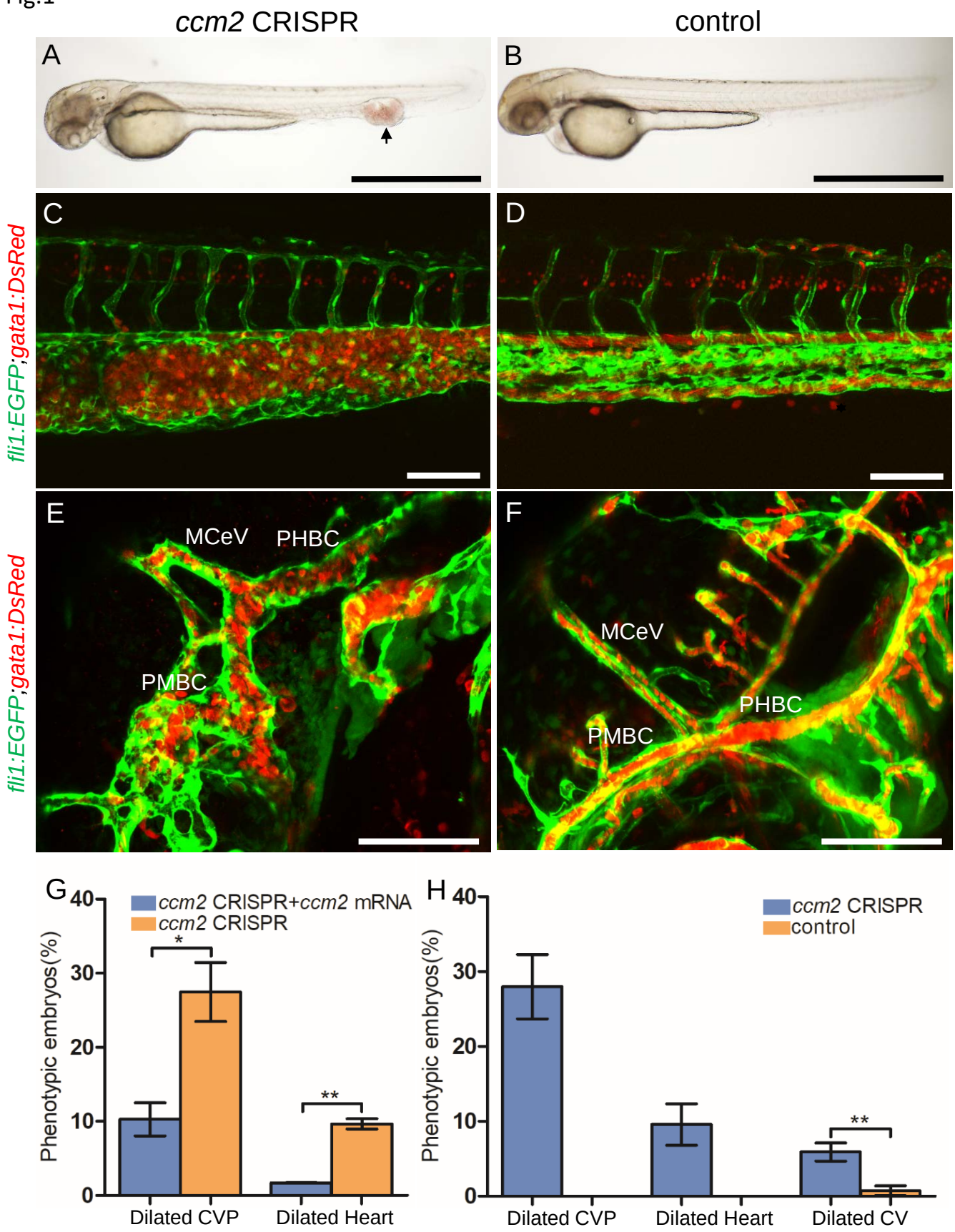
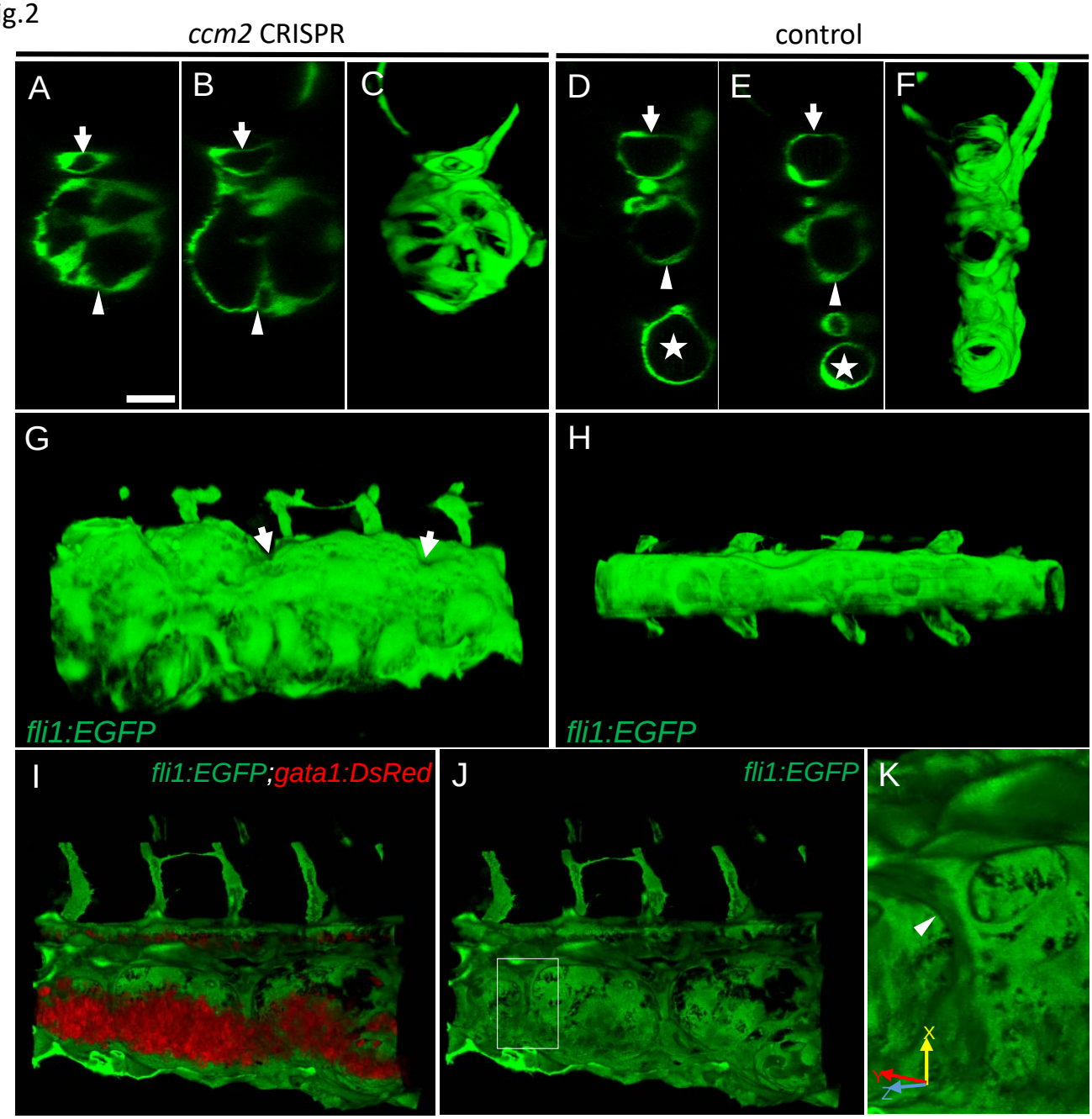

ccm2 CRISPR
control
A
B
C
D
E
F
fli1:EGFP;gata1:DsRed
fli1:EGFP;gata1:DsRed
MCeV
PHBC
PMBC
MCeV
PHBC
PMBC
G
ccm2 CRISPR+ccm2 mRNA
ccm2 CRISPR
Phenotypic embryos(%)
40
30
20
10
0
*
**
Dilated CVP
Dilated Heart
H
ccm2 CRISPR
control
Phenotypic embryos(%)
40
30
20
10
0
**
Dilated CVP
Dilated Heart
Dilated CV

Fig.2


ccm2 CRISPR
control
A
B
C
D
E
F
G
H
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I
fli1:EGFP;*gata1:DsRed*
J
fli1:EGFP
K
X
Y
Z

Fig.3

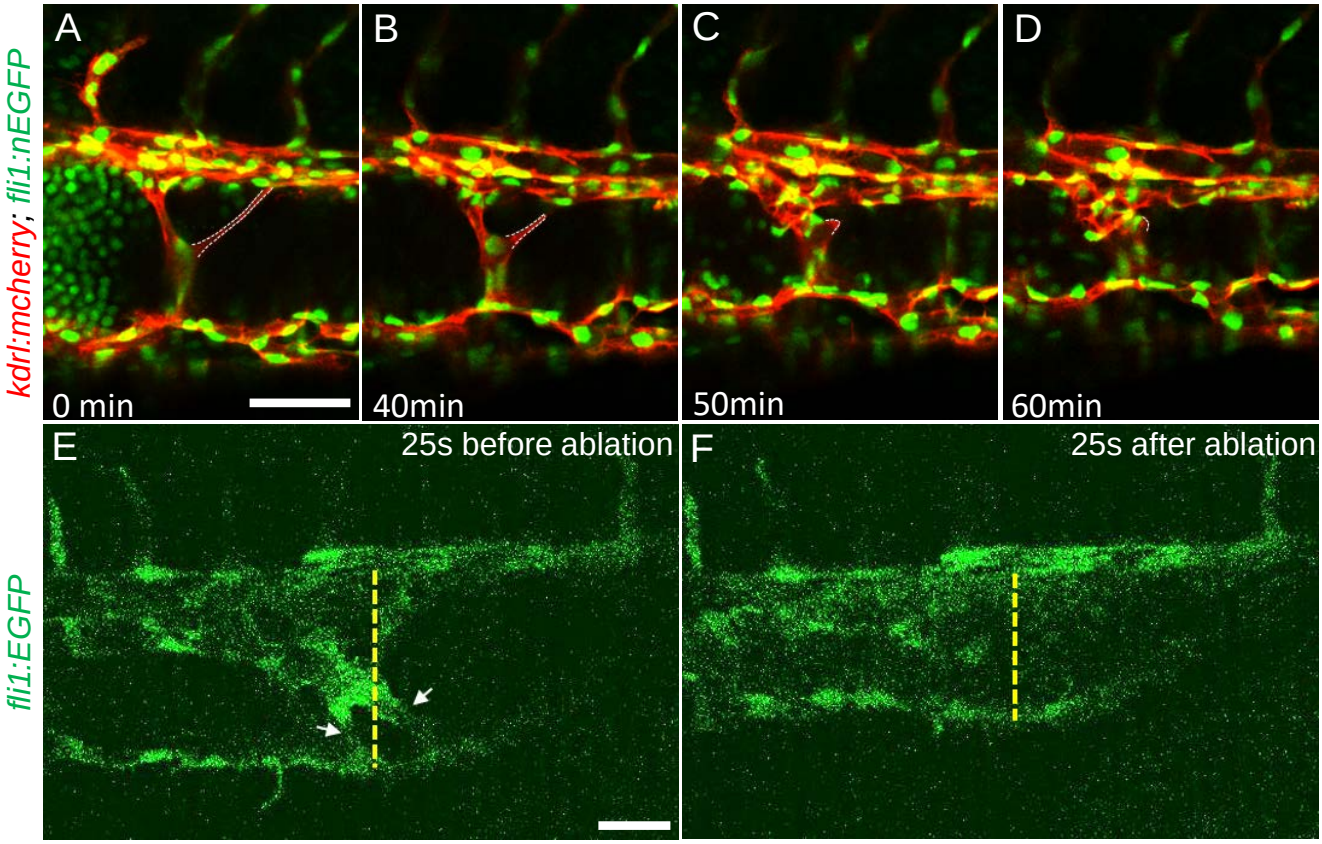

A
B
C
D
kdrl:mcherry; fli1:nEGFP
0 min
40min
50min
60min
E
25s before ablation
F
25s after ablation
fli1:EGFP

Fig. 4

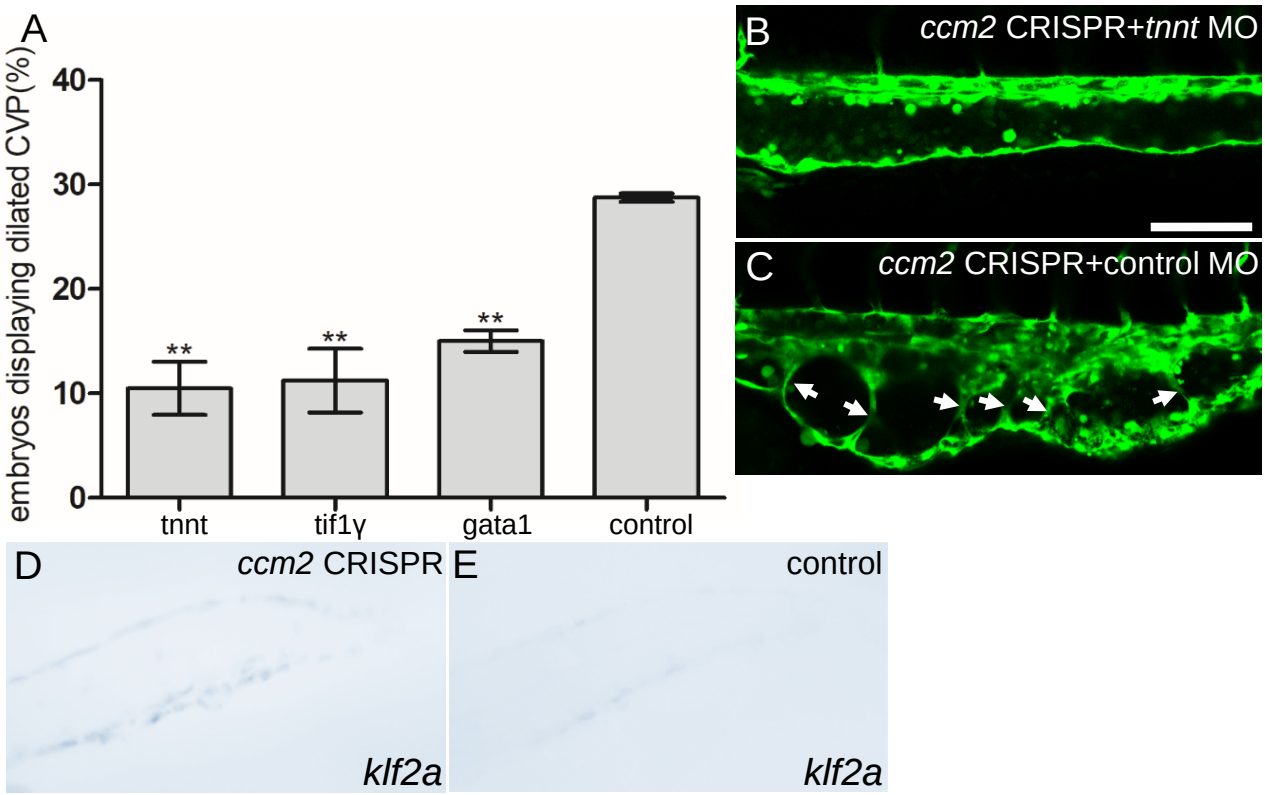
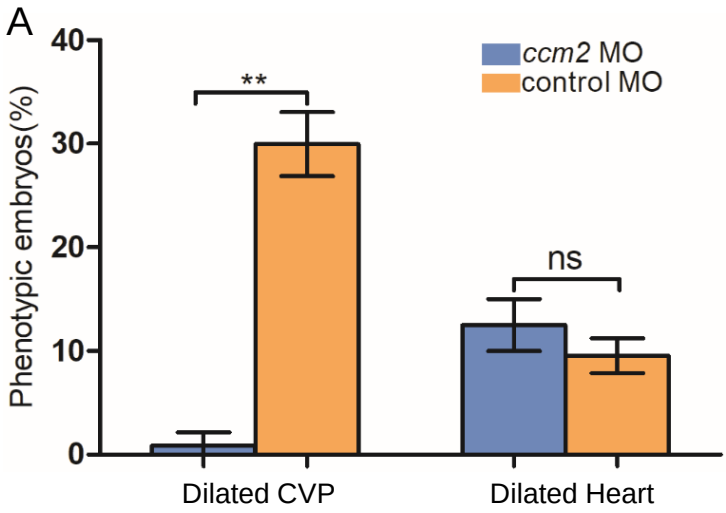

A
embryos displaying dilated CVP(%)
40
30
20
10
0
**
**
**
tnnt
tif1γ
gata1
control
B
ccm2 CRISPR+tnnt MO
C
ccm2 CRISPR+control MO
D
ccm2 CRISPR
klf2a
E
control
klf2a

Fig.5


A
Phenotypic embryos(%)
40
30
20
10
0
**
ns
ccm2 MO
control MO
Dilated CVP
Dilated Heart

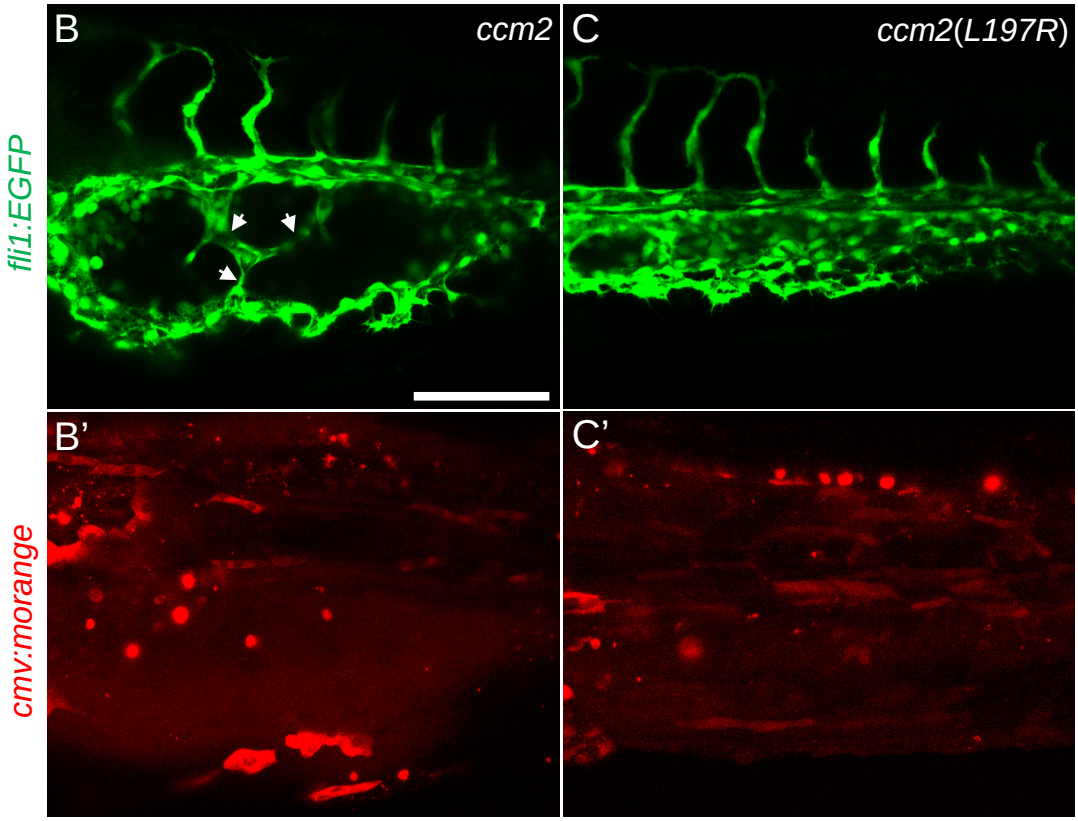

B
ccm2
C
ccm2(L197R)
fli1:EGFP
B'
C'
cmv:morange

Fig.6

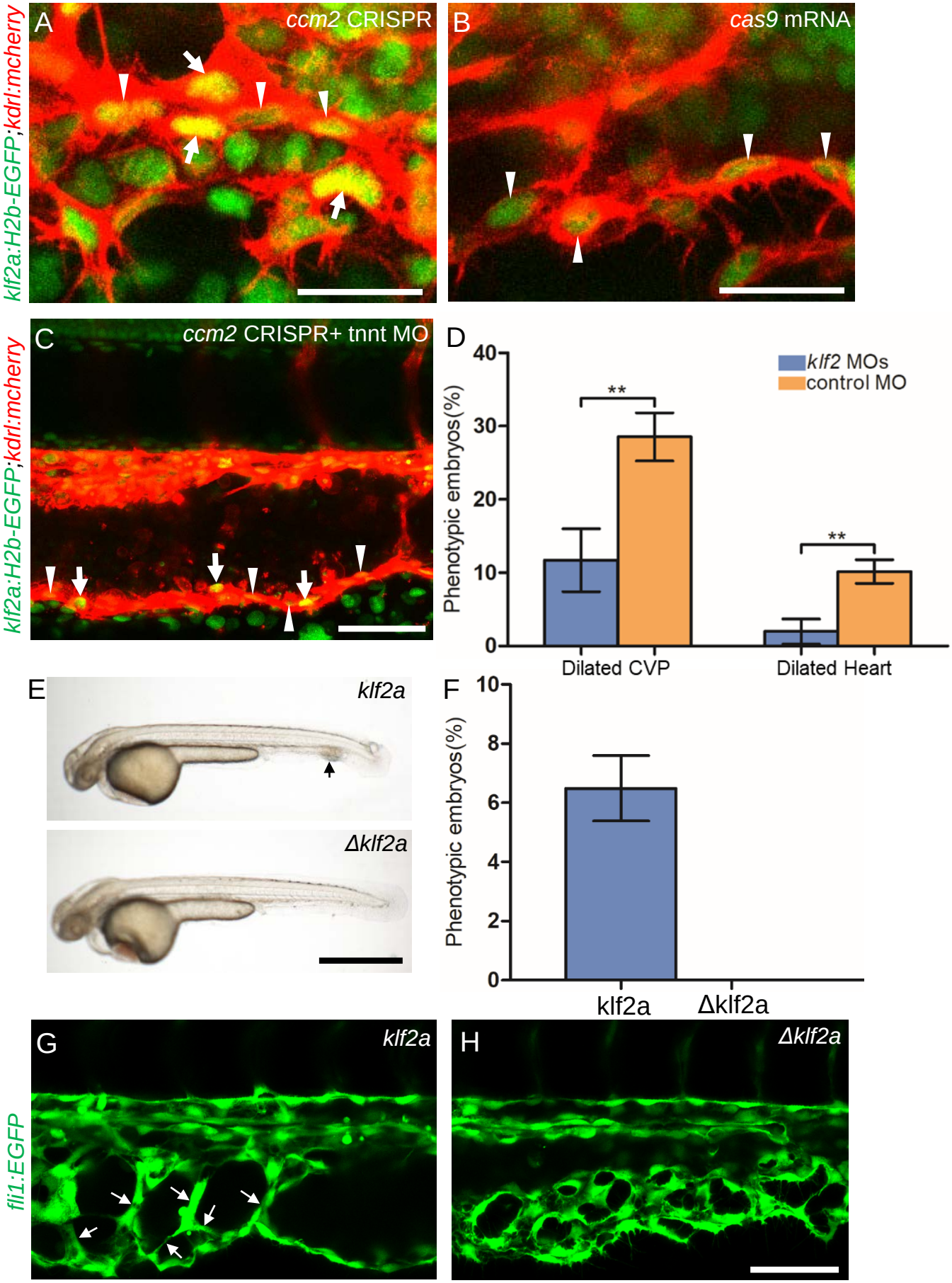
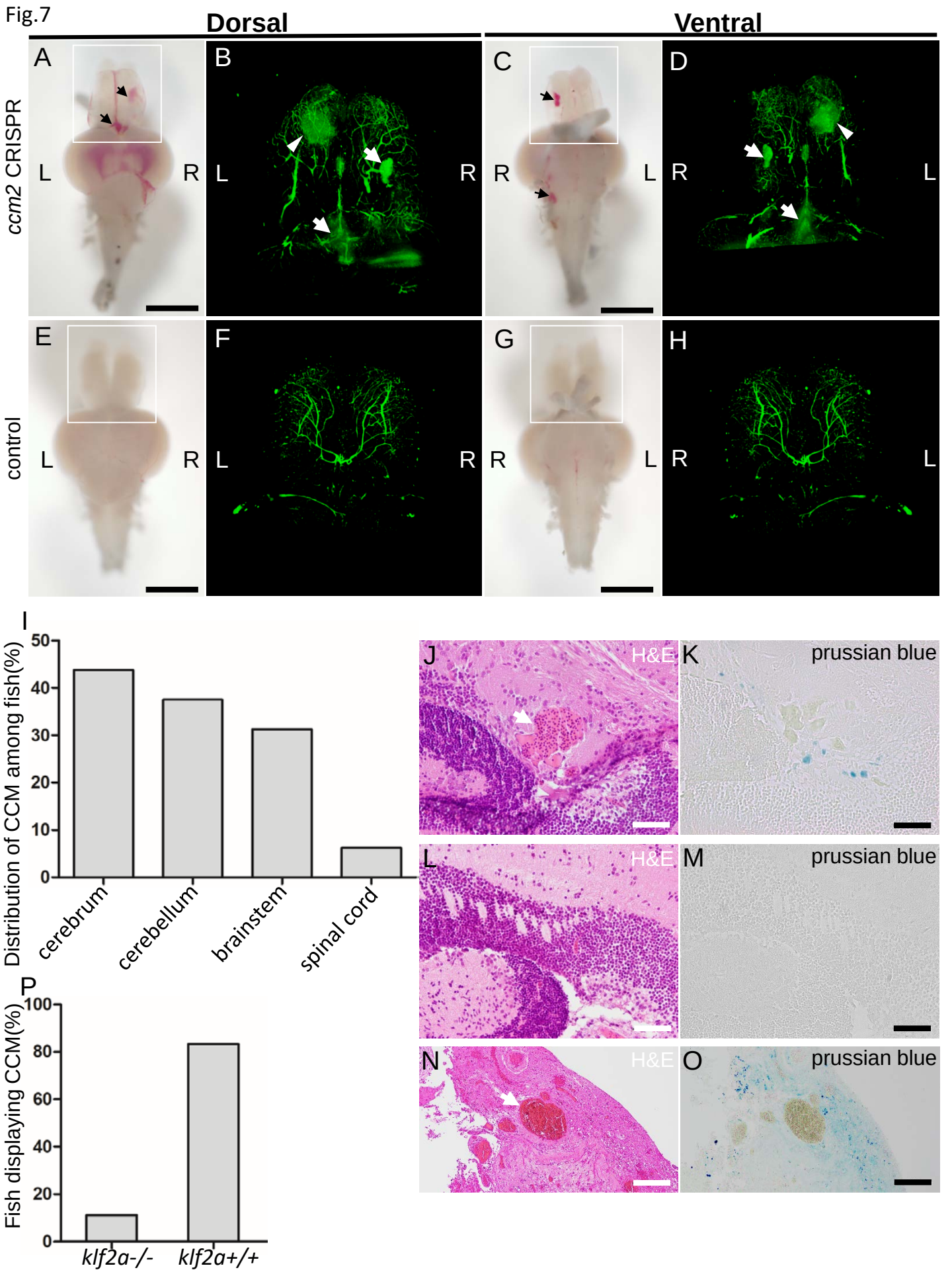

A
ccm2 CRISPR
B
cas9 mRNA
klf2a:H2b-EGFP;kdrl:mcherry
C
ccm2 CRISPR+ tnnt MO
klf2a:H2b-EGFP;kdrl:mcherry
D
klf2 MOs
control MO
Phenotypic embryos(%)
40
30
20
10
0
**
**
Dilated CVP
Dilated Heart
E
klf2a
Δklf2a
F
Phenotypic embryos(%)
10
8
6
4
2
0
klf2a
Δklf2a
G
klf2a
H
Δklf2a
fli1:EGFP

Fig.7

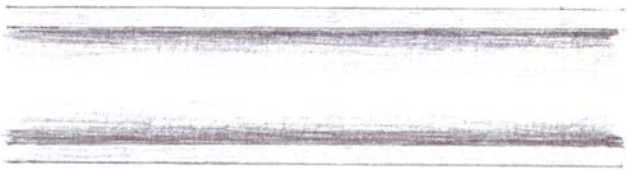

Dorsal
Ventral
ccm2 CRISPR
control
A
B
C
D
E
F
G
H
L
R
I
Distribution of CCM among fish(%)
50
40
30
20
10
0
cerebrum
cerebellum
brainstem
spinal cord
J
K
L
M
N
O
H&E
prussian blue
P
Fish displaying CCM(%)
100
80
60
40
20
0
klf2a-/-
klf2a+/+

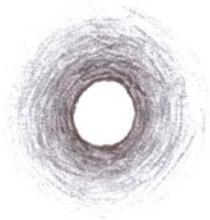
diagram

Sagittal view

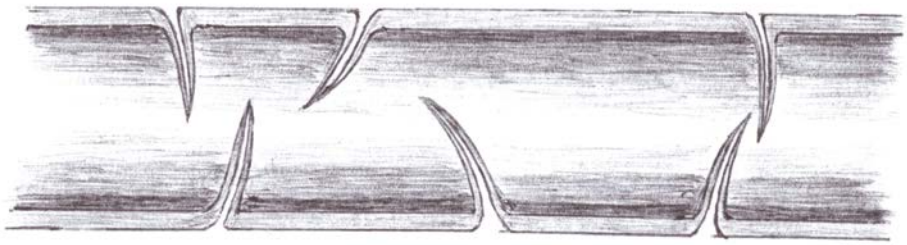
Transverse view

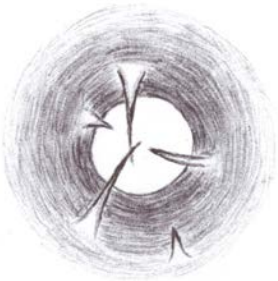
24hpf





30hpf





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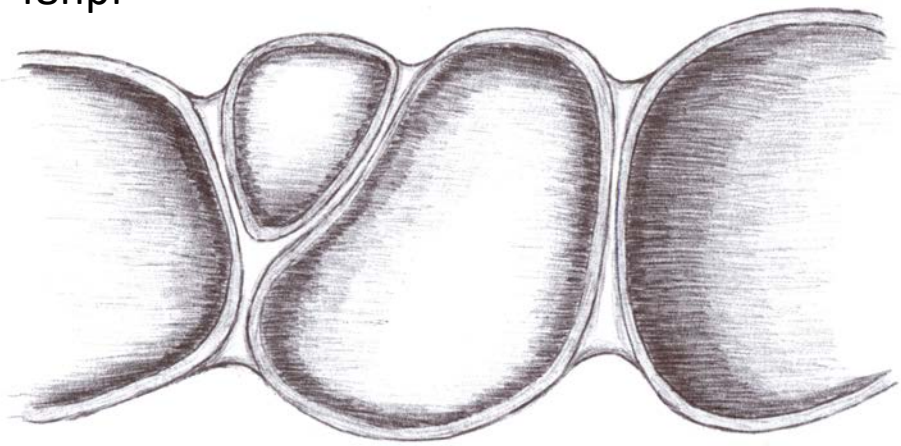

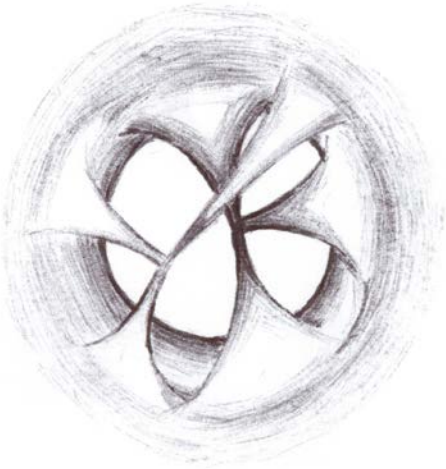

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