

The cerebral cavernous malformation proteins CCM2L and CCM2 prevent the activation of the MAP kinase MEKK3

Xavier Cullere^{a,1}, Eva Plovie^b, Paul M. Bennett^a, Calum A. MacRae^b, and Tanya N. Mayadas^a

^aCenter for Excellence in Vascular Biology, Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115; and ^bCardiovascular Division, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115

Edited by Igor B. Dawid, The Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, and approved October 9, 2015 (received for review May 29, 2015)

Three genes, *CCM1*, *CCM2*, and *CCM3*, interact genetically and biochemically and are mutated in cerebral cavernous malformations (CCM). A recently described member of this CCM family of proteins, CCM2-like (CCM2L), has high homology to CCM2. Here we show that its relative expression in different tissues differs from that of CCM2 and, unlike CCM2, the expression of CCM2L in endothelial cells is regulated by density, flow, and statins. In vitro, both CCM2L and CCM2 bind MEKK3 in a complex with CCM1. Both CCM2L and CCM2 interfere with MEKK3 activation and its ability to phosphorylate MEK5, a downstream target. The in vivo relevance of this regulation was investigated in zebrafish. A knockdown of *ccm2l* and *ccm2* in zebrafish leads to a more severe "big heart" and circulation defects compared with loss of function of *ccm2* alone, and also leads to substantial body axis abnormalities. Silencing of *mekk3* rescues the big heart and body axis phenotype, suggesting cross-talk between the CCM proteins and MEKK3 in vivo. In endothelial cells, CCM2 deletion leads to activation of ERK5 and a transcriptional program that are downstream of MEKK3. These findings suggest that CCM2L and CCM2 cooperate to regulate the activity of MEKK3.

cerebral cavernous malformation | signaling | MAP kinase | expression | endothelium

Cerebral cavernous malformations (CCMs) are characterized by endothelial cell channels in low-blood flow venous capillaries with poor coverage of pericytes and smooth cells and poorly developed tight and adherens junctions, resulting in increased permeability, hemorrhage, and subsequent neurologic deficits. Germline loss-of-function mutations in any one of three CCM genes—*CCM1* (*Krit1*), *CCM2* (*OSM*), or *CCM3* (*PDCD10*)—cause the familial and sporadic forms of CCMs. The proteins encoded by these genes interact in a cytosolic complex. Critical insights into their in vivo functions have been obtained from genetic manipulation in fish and mice.

In zebrafish, null mutations in either *ccm1* (*santa*, *san*) or *ccm2* (*valentine*, *vtn*) result in an enlarged heart ("big heart") and dilation of the subintestinal vessels and posterior cardinal vein, and morpholino (MO) studies indicate that *ccm1* and *ccm2* are in the same pathway during zebrafish cardiovascular development (1, 2). The contribution of *ccm3* or its downstream effectors in the *ccm1/2* pathway in zebrafish is less clear (3, 4). CCM proteins have been shown to regulate the signal strength of several pathways acting both as down-regulators and as activators. Reduced expression of CCM1, CCM2, or CCM3 increases RhoA activation (5). CCM2 and CCM3 siRNAs increase phosphorylation of AKT and the MAP kinases p38 and ERK1/2 (6). CCM1 promotes NOTCH activation (7) and deficiency in CCM proteins leads to endothelial-mesenchymal transition (EMT) by up-regulating TGF- β /BMP signaling (8). *ccm1* and *ccm2* deletion in zebrafish leads to the up-regulation of β 1-integrin signaling and *klf2* expression that results in a proangiogenic program (9). Ccm2-like (Ccm2l), a recently described paralog of Ccm2, was shown to have an antagonistic function to Ccm2 in cardiovascular development in mice and failed to rescue

the phenotype in *ccm2* null zebrafish (10). However, in another study, *ccm2l*, like *ccm2*, was required for cardiovascular development in zebrafish, suggesting redundant functions for these two proteins (11).

CCM2 has been shown to interact with the kinase MEKK3 (MAP3K3) (12), and MEKK3 is known to activate several signaling pathways via the formation of specific signaling modules. The MEKK3-MEK5-ERK5 kinase module promotes cellular functions from EMTs to cell proliferation (13) and, in vivo, represents an important signaling axis in early cardiovascular development in mice (14, 15).

Here we report differential regulation of CCM2 and CCM2L expression in endothelial cells, an interaction of these proteins with MEKK3's N-terminal regulatory domain that interferes with its activation in vitro. Furthermore, genetic studies in zebrafish and cultured endothelial cells demonstrate MEKK3 dysregulation in the absence of these proteins.

Results and Discussion

CCM2L Tissue Expression and Regulation and Its Interaction with CCM1. Several CCM2L isoforms exist (UniProt database). The canonical isoform 1 (long, 62 kD) and isoform 2 (short, 46 kD) retain a functional PTB domain (Fig. 1A), implicated in CCM2 interaction with CCM1 and other signaling molecules (2). Quantitative RT-PCR (qRT-PCR) primers that detect both isoforms 1 and 2 were used to evaluate their relative expression in vivo. mRNAs were detected in human umbilical vein endothelial cells (HUVEC)

Significance

The kinase MEKK3 (MAP3K) regulates cellular functions from proliferation to maintenance of cell identity, and plays an important role in cardiovascular development, yet little is known about how it is regulated. Loss-of-function mutations in *CCM1*, *CCM2*, or *CCM3* cause cerebral cavernous malformations characterized by dilated, leaky blood vessels. CCM proteins form a complex and regulate the signal strength of several pathways. We demonstrate that CCM2 and CCM2-like (CCM2L), a recently described paralog, strongly prevented the activation of MEKK3 in vitro. In zebrafish, *ccm2* and *ccm2l* genetically interacted to control cardiovascular development and body axis patterning during embryogenesis, and these were regulated by MEKK3. Therefore, two homologous CCM proteins regulate MEKK3 activity, and thus may modulate the strength of key signaling pathways.

Author contributions: X.C., E.P., C.A.M., and T.N.M. designed research; X.C., E.P., and P.M.B. performed research; X.C. contributed new reagents/analytic tools; X.C., E.P., P.M.B., and T.N.M. analyzed data; and X.C., E.P., and T.N.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

¹To whom correspondence should be addressed. Email: fcullere@rics.bwh.harvard.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1510495112/-DCSupplemental.

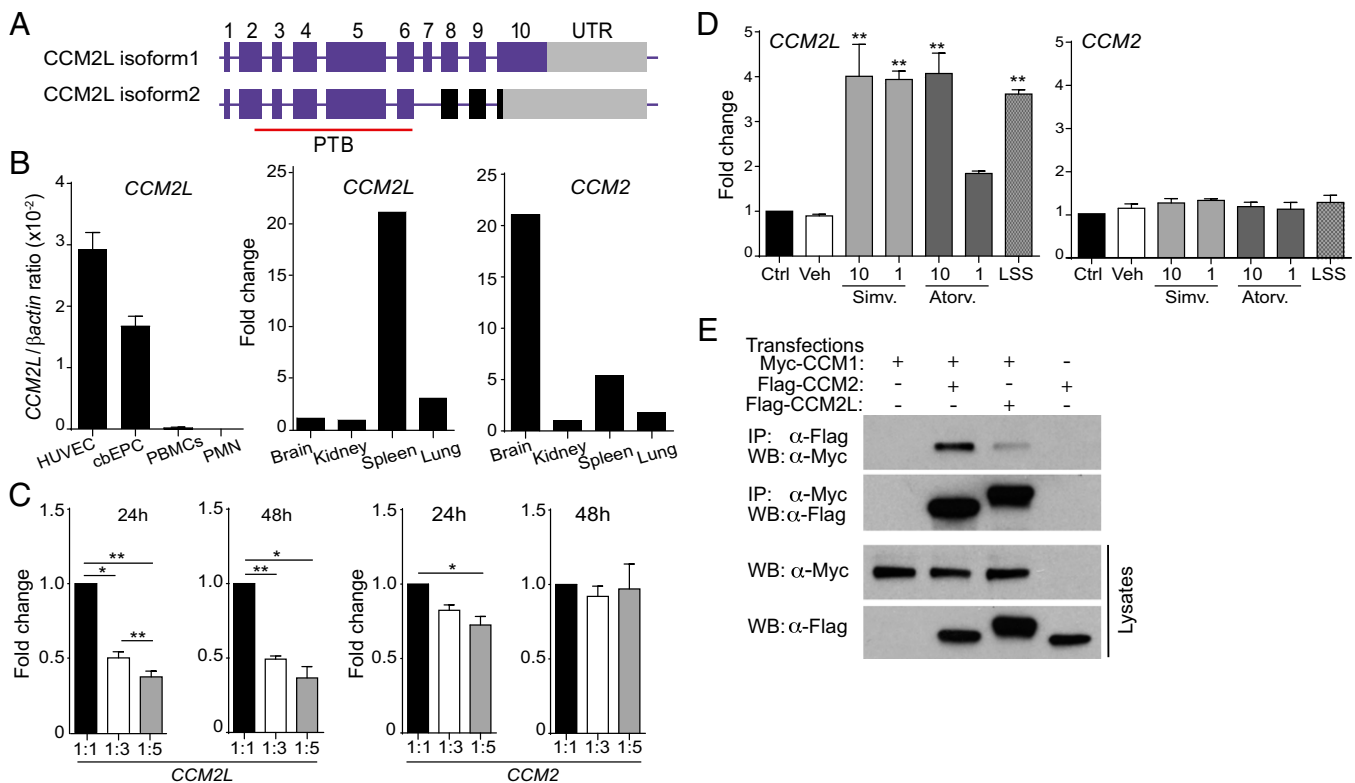


Fig. 1. CCM2L expression and interaction with CCM1. (A) Exons with identical amino acid sequences for canonical isoform 1 (62 kD) and shorter isoform 2 (46 kD) are in blue. Sequences only in isoform 2 are in black. The PTB domain is underlined, and the gray boxes represent the 3' UTR. (B) CCM2L and CCM2 mRNA analysis by qRT-PCR using primers for CCM2 and both CCM2L isoforms in endothelial cells (HUVEC), cord blood endothelial progenitor cells (cbEPC), peripheral blood mononuclear cells (PBMCs), neutrophils (PMN), or indicated samples of human tissue. (C) HUVEC were plated at confluency (1:1) or at indicated dilutions for 24 or 48 h. mRNA levels were evaluated by qRT-PCR. (D) HUVEC were treated with vehicle control, simvastatin, or atorvastatin at indicated μ M concentrations, or subjected to shear stress (LSS) for 24 h, followed by analysis of CCM2L and CCM2 mRNA levels by qRT-PCR. (E) HEK293 cells were transfected with Myc-tagged CCM1, Flag-tagged CCM2L, or Flag-tagged CCM2 as indicated. Cell lysates were subjected to IP with anti-tag antibodies, followed by Western blot analysis (WB) with indicated antibodies. CCM1 coimmunoprecipitated with CCM2L and CCM2. $n = 3$ independent experiments.

and endothelial progenitor cells (Fig. 1B). qRT-PCR primers that specifically detect only the long or short isoform indicated that the long isoform accounts for more than 90% of the mRNA for this gene product in all cells analyzed. The CCM2L isoforms were not detected in mononuclear cell fractions or neutrophils (Fig. 1B), suggesting selective expression in endothelial lineages versus hematopoietic cells.

There is emerging evidence of an endothelial autonomous function for the CCM genes (5, 16, 17). Here we analyzed CCM2L expression in endothelial cells under various biological settings. Several genes, such as claudin-5 (18), are regulated by cell density. Confluent HUVEC monolayers replated at lower densities exhibited reduced CCM2L levels, suggesting that cell-cell contacts may be required to maintain optimal CCM2L levels (Fig. 1C), whereas CCM2 levels were largely unaffected, indicating distinct regulation of these two genes. Hemodynamic forces (shear stress) are strong modulators of the endothelial phenotype, and patterns of gene expression induced by flow can be recapitulated by statin treatment (19). HUVEC monolayers subjected to shear stress or treated with statins showed up-regulation of CCM2L, but not CCM2, mRNA (Fig. 1D). Thus, whereas CCM2L levels are dynamically regulated, CCM2 levels are not similarly responsive to cues that modulate the endothelial phenotype. All subsequent analyses of CCM2L were conducted in heterologous cells owing to the observed variability of expression in endothelial cells.

Mutations that impair CCM1-CCM2 interactions result in vascular malformations. The ability of CCM2L to interact with CCM1 was examined in transient transfection assays. As expected, CCM2 interacted with CCM1. Similarly, both CCM2L isoforms

were found in complex with CCM1 (Fig. 1E), suggesting regulation of CCM2L by canonical CCM pathways.

CCM2 and CCM2L Interact with the MEKK3/MEK5 Complex and Regulate MEKK3 Activity.

CCM2 has been identified as a scaffold protein for hypertonicity-induced MEKK3-p38 activation (12). Here we show an interaction of CCM2 and CCM2L with MEKK3 (Fig. 2A) that did not involve the catalytic domain of the kinase, as they bound MEKK3 lacking this region and failed to interact with the kinase domain alone (Fig. 2B). The binding of MEKK3 with CCM2L was consistently more robust than that with CCM2 (Fig. 2A). Similarly, transfected CCM2L interacted with endogenous MEKK3 in HUVEC as assessed in immunoprecipitation (IP) assays (Fig. S1A). The region of CCM2L mediating interaction with MEKK3 mapped to the C terminus (amino acids 357-571) of the canonical CCM2L, as isoform 2, which lacks these sequences, failed to interact with MEKK3 while retaining binding to CCM1 (Fig. 2C). A recent study localized the region of interaction of CCM2 with MEKK3 to the harmonin homology domain of the CCM2L C terminus (20). Based on these data, we mutated four hydrophobic amino acids in the predicted second α -helix of this domain in CCM2L, because in CCM2, the $\alpha 1$ and $\alpha 2$ helices form a hydrophobic cleft that accommodates the N-terminal α -helix of MEKK3 (20). We found that this mutant did not bind MEKK3 (Fig. 2D). CCM2L and CCM2 were also present in a ternary complex with CCM1 and MEKK3, because CCM1 immunoprecipitates contained MEKK3 only in the presence of CCM2L or CCM2 (Fig. 2E).

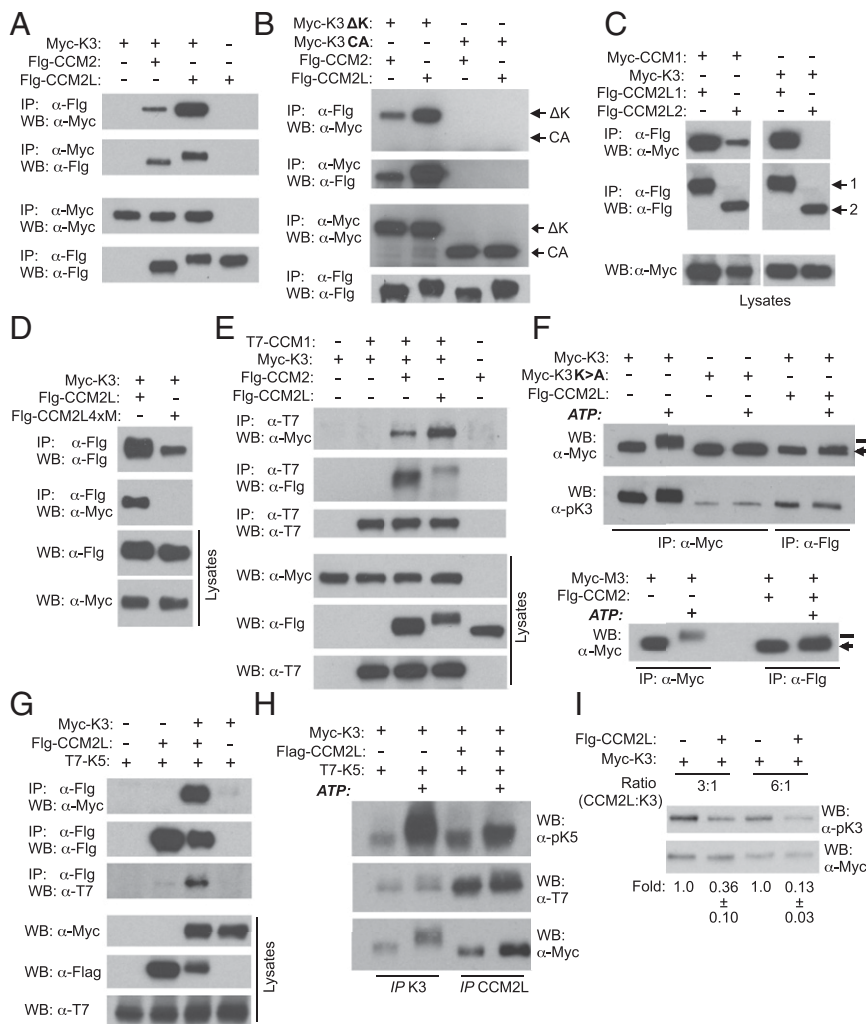


Fig. 2. CCM2L and CCM2 interact with MEKK3 and prevent its autophosphorylation. HEK293 cells were transfected with the indicated Myc-, Flag (Flag)-, or T7-tagged constructs alone or in combination. Anti-tag immunoprecipitated proteins and cell lysates were analyzed by Western blot (WB). (A) CCM2L and CCM2 interact with MEKK3 (K3). (B) CCM2L and CCM2 interact with only the N-terminal domain of MEKK3 (K3 Δ K, lacking the kinase domain), and not with the kinase domain (K3CA, catalytically active kinase domain). (C) CCM2L isoform 1 (-1) and isoform 2 (-2) interact with CCM1, but only isoform 1 (-1) binds MEKK3. (D) CCM2L, but not CCM2L4xM (with mutations in four hydrophobic residues), interacts with MEKK3. (E) MEKK3 is in a complex with CCM1 only when CCM2L or CCM2 are present. (F) CCM2L (Upper) and CCM2 (Lower) prevent MEKK3 autophosphorylation. Immunoprecipitated MEKK3 and kinase dead MEKK3 (K3K > A) alone or with CCM2L or CCM2 were subjected to an in vitro kinase assay (+ATP). MEKK3 p-Ser526 status was analyzed by Western blot analysis. MEKK3, but not MEKK3K > A, shows a slower migrating band, indicating autophosphorylated MEKK3 (bar). MEKK3 present in CCM2L or CCM2 immunoprecipitates is unable to autophosphorylate (arrow), and MEKK3 complexed with CCM2L has less p-Ser526. (G) MEKK3 (K3), MEK5 (K5), and CCM2L are present in a complex. Anti-CCM2L immunoprecipitates contain MEK5 only when MEKK3 is also cotransfected. (H) MEK5 phosphorylation by MEKK3 is reduced in the presence of CCM2L. In vitro kinase assays of indicated immunoprecipitates were followed by WB using anti-tag antibodies and p-MEK5-specific antibody (Ser311/Thr315). (I) Cells were transfected with MEKK3 with and without CCM2L at the indicated plasmid ratios. Aliquots of cell lysates containing similar amounts of MEKK3 (Lower) were evaluated for MEKK3 p-Ser526 (Upper). Densitometric analyses of WBs are reported as fold reduction in CCM2L-containing samples. $n = 3$ –4 independent experiments for each panel.

We next evaluated whether CCM2/CCM2L interacting with MEKK3 altered its function. Unlike other MAPKs, MEKK3 undergoes spontaneous activation in overexpression studies owing to its ability to autophosphorylate critical residues necessary for its kinase activity (21). Endogenous MEKK3 is not constitutively active in the cell without the appropriate stimulus, indicating that self-activation is tightly regulated to avoid excessive kinase activation. Cotransfections of MEKK3 with CCM2L or CCM2 were performed, followed by MEKK3 pull-down and an in vitro kinase assay (Fig. 2F). In this assay, on the addition of ATP, MEKK3 autophosphorylation results in reduced mobility of the kinase in SDS/PAGE gels (22). Interestingly, the ability of CCM2L-bound MEKK3 to undergo autophosphorylation was substantially impaired; that is, the significant shift in the wild-type

(WT) kinase did not occur in the presence of CCM2L and indeed was similar to that of an MEKK3 kinase inactive mutant (Fig. 2F). Phosphorylation of Ser526 (p-Ser526) in the activation loop of MEKK3 plays a critical role in its activation (21). MEKK3 complexed to CCM2L exhibited a significant reduction in p-Ser526, and the signal did not increase after the addition of ATP (Fig. 2F). Like CCM2L, CCM2-bound MEKK3 showed diminished autophosphorylation (Fig. 2F). Dimerization of MEKK2 is important for its activation by transphosphorylation (23). We propose that the interaction with CCM2L or CCM2 prevents MEKK3 dimerization and thus its spontaneous activation. This may restrict MEKK3's activity and/or enable its activation in a stimulus-specific manner. This hypothesis predicts that the lack of this controlled

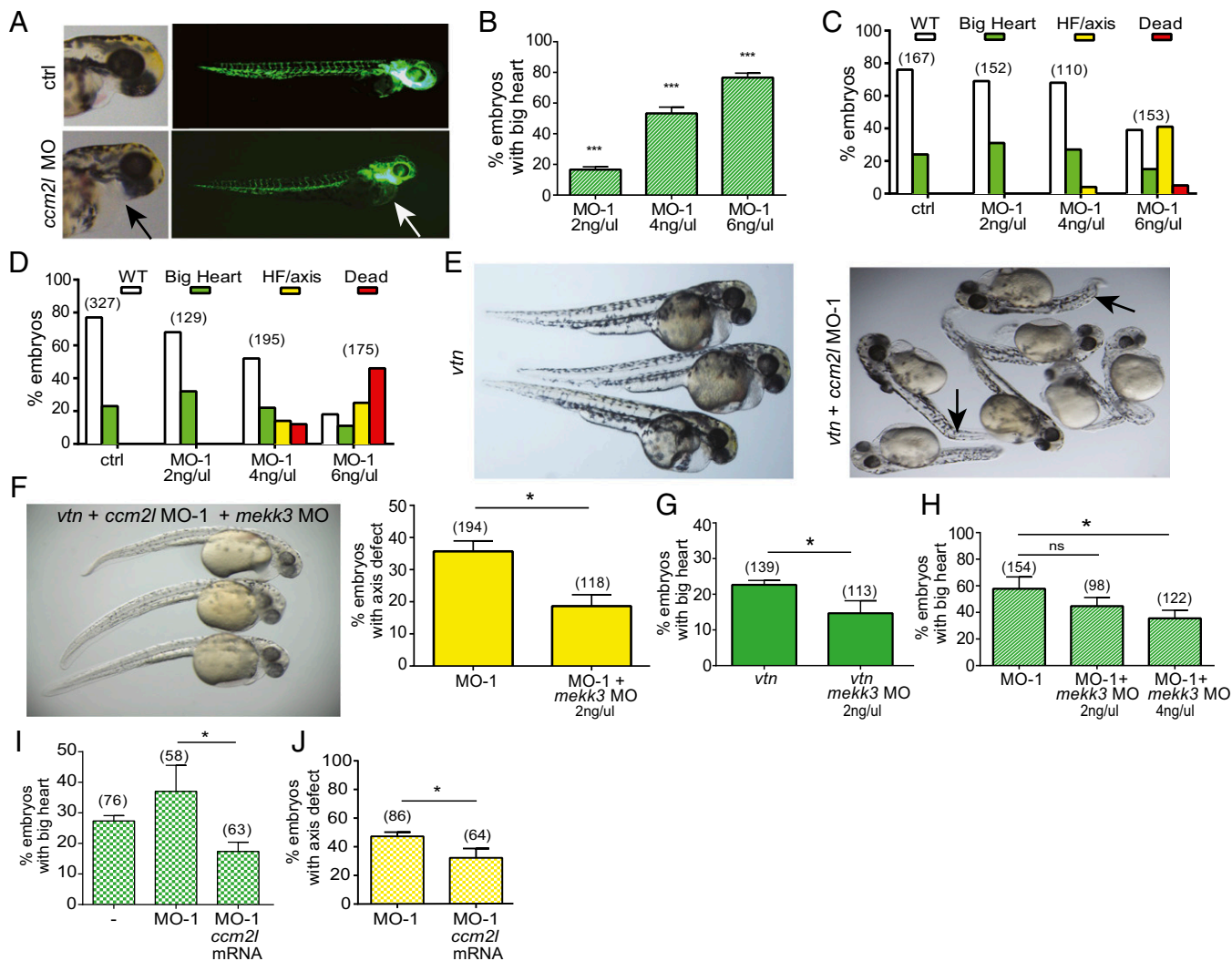


Fig. 3. Genetic interaction of *ccm2l* with *ccm1* and *ccm2* and modulation of *mekk3* activity in zebrafish. (A) Single-cell *flk-GFP* embryos were injected with control (ctrl) or *ccm2l* MO (MO-1), and images were obtained at 48 h postfertilization. The arrow indicates cardiac enlargement/big heart. (B) The big heart phenotype was evaluated with increasing doses of *ccm2l* MO-1. (C–E) Different amounts of *ccm2l* MO-1 or ctrl MO were injected into embryos from *san/ccm1* (C) and *vtn/ccm2* (D) heterozygous crosses. The *vtn/ccm2* zebrafish with *ccm2l* knockdown show further enlargement of the heart in combination with heart failure (HF) and tail/axis defects (arrows). Phenotypes were classified as WT (no phenotype), big heart, HF/axis (HF and axis/tail defects), or dead (lack of survival). (E) Representative pictures of *vtn/ccm2* mutants without (Left) or with *ccm2l* MO-1 injected (Right). (F) Rescue of the axis phenotype in *ccm2/ccm2l*-deficient fish with 4 ng/ μ L *mekk3* MO. Embryos from *vtn/ccm2* heterozygous crosses were injected with *ccm2l* alone or in combination with ctrl or *mekk3* MO, and the axis defect phenotype was scored. (G) Rescue of the big heart phenotype in *vtn/ccm2* mutant fish (*vtn*) with ctrl or 2 ng/ μ L *mekk3* MO. (H) Rescue of big heart in WT plus 4 ng/ μ L *ccm2l* MO-1 injection with ctrl MO or indicated concentrations of *mekk3* MO. (I and J) Rescue of big heart (I) and tail axis defects (J) in *vtn + ccm2l* MO-1 zebrafish, with human CCM2L mRNA. The big heart phenotype in *vtn* alone (I, –) is shown for comparison. * $P < 0.05$, unpaired t test. Graphs represent data pooled from three independent experiments. Error bars represent SEM.

activity will result in an unregulated population of MEKK3 under homeostatic conditions.

MEK5, a kinase that interacts with MEKK3 and an important downstream effector, was also present in CCM2L immunoprecipitates, but only when MEKK3 was coexpressed (Fig. 2G). We found that CCM2L in the MEKK3/MEK5 complex significantly attenuated MEKK3-mediated MEK5 phosphorylation (MEK5 p-Ser311/p-Thr315) in its activation loop (Fig. 2H). The strong interaction of MEKK3 and CCM2L would predict a greater amount of inactive MEKK3 in the presence of CCM2L in total cell lysates. In agreement with this, cells containing MEKK3 and CCM2L showed a significant reduction in p-Ser526 compared with cells with MEKK3 alone (Fig. 2I).

In summary, CCM2L and CCM2 interact with MEKK3, with CCM2L demonstrating greater binding, and both proteins prevent

MEKK3 activity. Other MEKK3-interacting proteins also reportedly regulate kinase activation in *in vitro* assays. The interaction of TAK1 kinase with MEKK3 prevents its basal activation, and dissociation of the complex increases MEKK3 activity (22). Moreover, the interaction of MEKK3 and MEKK2 with 14-3-3 proteins limits their ability to dimerize and autophosphorylate in the activation loop (24, 25). The interaction of CCM2L with MEKK3 may prevent a feed-forward loop of MEKK3 activation by transphosphorylation, but might not in itself interfere with MEKK3's ability to phosphorylate all of its downstream targets. In this case, CCM2L may serve as a scaffold, as has been shown for CCM2. When CCM2L interacts with inactive MEKK3, it forms a stable complex (Fig. S1A) that keeps MEKK3 inactive and prevents its activation by autophosphorylation (Fig. 2F), whereas the interaction with active MEKK3 results in CCM2L phosphorylation,

as demonstrated by its reduced mobility in SDS gels (Fig. S14). Whether phosphorylated CCM2L dissociates from this complex and allows dimerization and further kinase activation is an area of interest for future investigation. Thus, the regulation of MEKK3 likely is related to local amounts of CCM2L, whose dynamic expression may serve this purpose. The finding that the total pool of active MEKK3 in cell lysates is markedly reduced in the presence of CCM2L highlights the significance of this regulation.

Genetic Interaction of *ccm2l* with *ccm2* and *ccm1* in Zebrafish. We exploited the zebrafish model to address whether *ccm2* and *ccm2l* are in the same pathway and regulate *mekk3* function in vivo. Mutations in the zebrafish orthologs of CCM proteins result in distinct phenotypes that include a “big heart,” characterized by an abnormally enlarged heart cavity, lack of circulation, and disruption of intersomitic vessels (26). A MO designed to knock down *ccm2l* was injected into one-cell stage embryos of zebrafish expressing GFP in endothelial cells (*flk-GFP*) to visualize the vasculature. At 48 h postfertilization, *ccm2l* MO-injected fish displayed an enlarged atrium, ventricle, and pericardial cavity (big heart) (Fig. 3A), associated with mild reductions in heart rate [mean, 156 ± 8 bpm in controls ($n = 6$) vs. 140 ± 9 bpm in morphants ($n = 11$); $P < 0.005$] and cardiac output [44 ± 7 nL/min in controls ($n = 6$) vs. 32 ± 10 nL/min in morphants ($n = 11$); $P < 0.05$]. Vasculogenesis appeared grossly unaffected. This phenotype was dose-dependent; increasing amounts of *ccm2l* MO-1 resulted in more embryos with the heart enlargement/big heart phenotype (Fig. 3B), which is consistent with a previous report (11).

Injections of *ccm2l* MO into *santa* (*san/ccm1*) (Fig. 3C) and *valentine* (*vtn/ccm2*) (Fig. 3D) heterozygous pair crosses demonstrated a strong genetic interaction between *ccm2l* and *ccm1* or *ccm2*, which was more prominent with *vtn/ccm2*, as evidenced by a decrease in survival rate, a complete heart failure (HF) phenotype, and appearance of a markedly distorted body axis (Fig. 3D and E). Here 14% of *vtn/ccm2* mutants sensitized with the *ccm2l* MO-1 (4 ng/ μ L) had a severe HF/axis phenotype, and 12% died. This was even more pronounced with the higher-dose injections (6 ng/ μ L), where 25% of *vtn/ccm2* mutants showed HF/axis defects and 46% died. In similarly sensitized *san/ccm1* mutants, 4% had the HF/axis defect and 0% died at 4 ng/ μ L *ccm2l* MO, and 41% had the HF/axis defect and 5% died at higher MO concentrations.

To further confirm the specificity of this phenotype and the genetic interaction with the CCM pathway, we injected a second MO (MO-2) against *ccm2l* in WT embryos and embryos generated by *vtn/ccm2* intercrosses. We observed the same big heart phenotype in the injected WT embryos (Fig. S2A and B). Moreover, MO-2 injection in *vtn/ccm2* embryos (Fig. S2C and D) again resulted in a decrease in survival rates, and embryos with the HF/axis phenotype. These experiments were performed

with lower doses of the MO-2, because the higher doses produced toxicity and poor survival.

Individual, differently classified zebrafish embryos from experiments were genotyped. The embryos with the axis defects correlated with embryos containing a mutant *vtn* allele in the presence of *ccm2l* MO, whereas no WT fish were detected in this group (Fig. S2E). Control injections using a cardiac-specific MO (troponinT; *tnnt2*) in *vtn/ccm2* mutants and the injection of *ccm2l* MO-1 into the silent heart mutants (*sih/tnnt2*) resulted in the expected big heart and silent phenotype, respectively, but with no additional tail axis defects (Fig. S2F and G). This indicates that the distorted axis/tail phenotype is specific to the *ccm2l/ccm2* interaction, and that a defect in heart function is not responsible for the observed phenotype. These results suggest perturbed body axis patterning (27), a phenotype not previously reported in *ccm* mutant lines (11, 26, 28) or in WT fish injected with *ccm2l* MO alone (11).

Role for MEKK3 in a CCM Genetic Pathway in Zebrafish. Our biochemical results prompted us to investigate the role of MEKK3 in the body axis phenotype observed in the *vtn/ccm2l* morphants. Overexpression of MEKK3 in the *vtn/ccm2l* morphants by injection of in vitro translated MEKK3 mRNA resulted in more severe tail axis defects and cardiac failure (Fig. S2H); however, MOs directed to *mekk3* efficiently rescued the tail axis defect (Fig. 3F). Because rescue experiments usually require careful dosing of MOs, we chose to use the milder *ccm2l* MO 1 (MO-1) to rescue the CCM signaling loss-of-function-specific phenotypes. In this experiment, 33% of *vtn/ccm2* embryos receiving only the *ccm2l* MO-1 showed a pronounced axis defect, compared with 19% receiving coinjection with the *mekk3* MO (Fig. 3F). Thus, *mekk3* knockdown decreased the severity of the axial phenotypes, indicating that *ccm2* and *ccm2l* modulation of *mekk3* activity may be an important regulator of body axis patterning. Ccm2 and Ccm2l play antagonistic functions in cardiovascular development in mice (10), whereas in zebrafish, *ccm2l* deficiency enhances *ccm1* and *ccm2* mutant phenotypes in heart development, as shown here by our experiments as well as in a previous study (11).

Injection of the *mekk3* MO into embryos generated by *vtn/ccm2* intercrosses also reduced the prevalence of the big heart phenotype from 23% to 15% in *mekk3* MO-injected embryos (Fig. 3G), consistent with a recent report (29). Similarly, the *mekk3* MO reduced the big heart phenotype in WT fish injected with *ccm2l* MO (Fig. 3H). The rescue of the body axis patterning phenotype in *ccm2/ccm2l*-deficient zebrafish and the big heart phenotype in single gene deficiency with *mekk3* MOs demonstrates the importance of MEKK3 regulation by these two CCM-related proteins. Notably, *ccm2l* mRNA is located in the tail of embryos both in the cell mass ventral to the notochord and in the presumptive notochord (11), an important structure in vertebral column development. *ccm2* is also present in the ventral

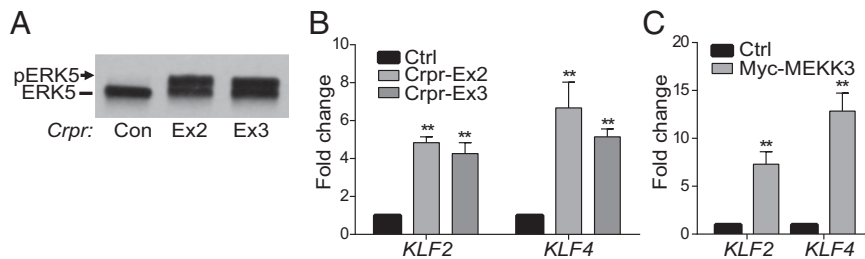


Fig. 4. ERK5 activation in CCM2 deleted endothelial cells. (A and B) HUVEC transduced with control or CCM2 exons 2 or 3 CRISPR/Cas9 (Crpr) lentivirus were analyzed for ERK activation by Western blot (A) or for *KLF2* or *KLF4* mRNA by qRT-PCR (B). Activation of ERK5 is detected by the appearance of a slower migrating form indicating phosphorylation (arrow). (C) HUVEC were transduced with lentiviral particles to express Myc-tagged MEKK3 and harvested for qRT-PCR after 48 h, and *KLF2* and *KLF4* mRNA levels were evaluated. *** $P < 0.05$. $n = 4$ experiments. Error bars represent SEM.

cell mass, whereas *ccm1* mRNA is present in the notochord (26). Moreover, MAPK pathways play key roles in notochord differentiation, with interference in this pathway leading to distortion in the tail axis (30). Finally, using in vitro transcribed human CCM2L, we found that CCM2L isoform 1 significantly rescued the greater heart and tail axis defects observed in zebrafish mutants (Fig. 3 I and J). This suggests that the defects observed in *ccm2l* MO-injected zebrafish are specific and not due to off-target effects.

CCM2 Deletion Leads to Activation of an MEKK3-Dependent Pathway.

In endothelial cells, activation of ERK5, a downstream effector of MEKK3, up-regulates Kruppel-like transcription factors (KLFs), which contribute to endothelial cell homeostasis. To examine whether CCM2 in endothelial cells regulates this pathway, we genetically deleted *CCM2* using CRISPR/Cas9 lentiviral approaches in HUVEC. Knockdown of CCM2 using two independent guide RNAs led to ERK5 hyperactivation and up-regulation of *KLF2* and *KLF4*. In agreement with the role of MEKK3 in this pathway, overexpression of this kinase in endothelial cells led to significant increases in *KLF2* and *KLF4* expression (Fig. 4).

In conclusion, hyperactivation of MEKK3 in the absence of the CCM proteins CCM2 and/or CCM2L interferes with heart development and body axis patterning in zebrafish. This correlates in vitro with interference of MEKK3 hyperactivation by these CCM proteins. Although loss-of-function mutations in the newly identified CCM2L in human CCM lesions have not yet been reported, we predict that CCM2L regulation of MEKK3 can

modify the severity of CCM disease. Because CCM2L expression is regulated by flow, low-flow conditions in CCM lesions may result in a reduction in CCM2L expression and subsequent exacerbation of MEKK3 activation, the tight regulation of which is required for formation of a normal vasculature. Genetic inactivation of members of the MEKK3 pathway results in embryonic lethality, characterized by defects in angiogenesis and early cardiovascular development (14, 15). Our data in zebrafish are informative of the molecular mechanism of action of CCM2L; however, the link with the CCM disease in humans remains unclear, given that the phenotypes observed with a deficiency of CCM2L or other CCM proteins are not recapitulated in CCM patients. Nevertheless, features of the phenotypes reported in zebrafish, such as dilated vessels and defective tight junctions, are observed in CCM lesions.

Materials and Methods

All experiments were performed in Tuebingen AB zebrafish or *Danio* fish obtained from EkkWill Waterlife Resources. Zebrafish were bred and maintained at 28.5 °C with 14-h light and 10-h dark exposure (day/night cycle), in accordance with the protocol approved by the Harvard Subcommittee for Animal Research. Detailed information on cDNA constructs and experimental procedures is provided in [SI Materials and Methods](#).

ACKNOWLEDGMENTS. We thank Dr. Dan Milner (Brigham and Women's Hospital) for providing human tissue samples, Dr. Joyce Bischoff (Boston Children's Hospital) for providing cord blood endothelial precursor cells, and Kay Case (Brigham and Women's Hospital) for providing cultured HUVEC. This work was supported by National Institutes of Health Grants P01 HL036028 (to T.N.M. and X.C.) and R01 HL109264 (to C.A.M.).

- Fischer A, Zalvide J, Faurobert E, Albiges-Rizo C, Tournier-Lasserre E (2013) Cerebral cavernous malformations: From CCM genes to endothelial cell homeostasis. *Trends Mol Med* 19(5):302–308.
- Fisher OS, Boggon TJ (2014) Signaling pathways and the cerebral cavernous malformation proteins: Lessons from structural biology. *Cell Mol Life Sci* 71(10):1881–1892.
- Zheng X, et al. (2010) CCM3 signaling through sterile 20-like kinases plays an essential role during zebrafish cardiovascular development and cerebral cavernous malformations. *J Clin Invest* 120(8):2795–2804.
- Yoruk B, Gillers BS, Chi NC, Scott IC (2012) Ccm3 functions in a manner distinct from Ccm1 and Ccm2 in a zebrafish model of CCM vascular disease. *Dev Biol* 362(2):121–131.
- Richardson BT, Dibble CF, Borikova AL, Johnson GL (2013) Cerebral cavernous malformation is a vascular disease associated with activated RhoA signaling. *Biol Chem* 394(1):35–42.
- Zhu Y, et al. (2010) Differential angiogenesis function of CCM2 and CCM3 in cerebral cavernous malformations. *Neurosurg Focus* 29(3):E1.
- Wüsthube J, et al. (2010) Cerebral cavernous malformation protein CCM1 inhibits sprouting angiogenesis by activating DELTA-NOTCH signaling. *Proc Natl Acad Sci USA* 107(28):12640–12645.
- Maddaluno L, et al. (2013) EndMT contributes to the onset and progression of cerebral cavernous malformations. *Nature* 498(7455):492–496.
- Renz M, et al. (2015) Regulation of β 1 integrin-Klf2-mediated angiogenesis by CCM proteins. *Dev Cell* 32(2):181–190.
- Zheng X, et al. (2012) Dynamic regulation of the cerebral cavernous malformation pathway controls vascular stability and growth. *Dev Cell* 23(2):342–355.
- Rosen JN, Sogah VM, Ye LY, Mably JD (2013) *ccm2*-like is required for cardiovascular development as a novel component of the Heg-CCM pathway. *Dev Biol* 376(1):74–85.
- Uhlik MT, et al. (2003) Rac-MEKK3-MKK3 scaffolding for p38 MAPK activation during hyperosmotic shock. *Nat Cell Biol* 5(12):1104–1110.
- Drew BA, Burrow ME, Beckman BS (2012) MEK5/ERK5 pathway: The first fifteen years. *Biochim Biophys Acta* 1825(1):37–48.
- Yang J, et al. (2000) Mekk3 is essential for early embryonic cardiovascular development. *Nat Genet* 24(3):309–313.
- Rose BA, Force T, Wang Y (2010) Mitogen-activated protein kinase signaling in the heart: Angels versus demons in a heart-breaking tale. *Physiol Rev* 90(4):1507–1546.
- Boulday G, et al. (2009) Tissue-specific conditional CCM2 knockout mice establish the essential role of endothelial CCM2 in angiogenesis: Implications for human cerebral cavernous malformations. *Dis Model Mech* 2(3-4):168–177.
- Boulday G, et al. (2011) Developmental timing of CCM2 loss influences cerebral cavernous malformations in mice. *J Exp Med* 208(9):1835–1847.
- Taddei A, et al. (2008) Endothelial adherens junctions control tight junctions by VE-cadherin-mediated up-regulation of claudin-5. *Nat Cell Biol* 10(8):923–934.
- Gimbrone MA, Jr, Garcia-Cardena G (2013) Vascular endothelium, hemodynamics, and the pathobiology of atherosclerosis. *Cardiovasc Pathol* 22(1):9–15.
- Wang X, et al. (2015) Structural insights into the molecular recognition between cerebral cavernous malformation 2 and mitogen-activated protein kinase kinase kinase 3. *Structure* 23(6):1087–1096.
- Fritz A, et al. (2006) Phosphorylation of serine 526 is required for MEKK3 activity, and association with 14-3-3 blocks dephosphorylation. *J Biol Chem* 281(10):6236–6245.
- Di Y, Li S, Wang L, Zhang Y, Dorf ME (2008) Homeostatic interactions between MEKK3 and TAK1 involved in NF- κ B signaling. *Cell Signal* 20(4):705–713.
- Cheng J, et al. (2005) Dimerization through the catalytic domain is essential for MEKK2 activation. *J Biol Chem* 280(14):13477–13482.
- Matitau AE, Gabor TV, Gill RM, Scheid MP (2013) MEKK2 kinase association with 14-3-3 protein regulates activation of c-Jun N-terminal kinase. *J Biol Chem* 288(39):28293–28302.
- Matitau AE, Scheid MP (2008) Phosphorylation of MEKK3 at threonine 294 promotes 14-3-3 association to inhibit nuclear factor κ B activation. *J Biol Chem* 283(19):13261–13268.
- Mably JD, et al. (2006) *santa* and *valentine* pattern concentric growth of cardiac myocardium in the zebrafish. *Development* 133(16):3139–3146.
- Hammerichmidt M, Mullins MC (2002) Dorsoroventral patterning in the zebrafish: Bone morphogenetic proteins and beyond. *Pattern Formation in Zebrafish (Results and Problems in Cell Differentiation)*, ed Solnica-Krezel L (Springer, Berlin, Germany).
- Mably JD, Mohideen MA, Burns CG, Chen JN, Fishman MC (2003) *heart of glass* regulates the concentric growth of the heart in zebrafish. *Curr Biol* 13(24):2138–2147.
- 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(2):168–180.
- Hawkins TA, Cavodeassi F, Erdélyi F, Szabó G, Lele Z (2008) The small molecule Mek1/2 inhibitor U0126 disrupts the chordamesoderm-to-notochord transition in zebrafish. *BMC Dev Biol* 8:42.
- Wu X, et al. (2004) Tissue-engineered microvessels on three-dimensional biodegradable scaffolds using human endothelial progenitor cells. *Am J Physiol Heart Circ Physiol* 287(2):H480–H487.
- Bevilacqua MP, Pober JS, Wheeler ME, Cotran RS, Gimbrone MA, Jr (1985) Interleukin 1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes, and related leukocyte cell lines. *J Clin Invest* 76(5):2003–2011.
- Dardik A, et al. (2005) Differential effects of orbital and laminar shear stress on endothelial cells. *J Vasc Surg* 41(5):869–880.
- dela Paz NG, Walshe TE, Leach LL, Saint-Geniez M, D'Amore PA (2012) Role of shear-stress-induced VEGF expression in endothelial cell survival. *J Cell Sci* 125(Pt 4):831–843.
- Ellinger-Ziegelbauer H, Brown K, Kelly K, Siebenlist U (1997) Direct activation of the stress-activated protein kinase (SAPK) and extracellular signal-regulated protein kinase (ERK) pathways by an inducible mitogen-activated protein kinase/ERK kinase 3 (MEKK) derivative. *J Biol Chem* 272(5):2668–2674.
- Asimaki A, et al. (2014) Identification of a new modulator of the intercalated disc in a zebrafish model of arrhythmic cardiomyopathy. *Sci Transl Med* 6(240):240ra74.