The *Cerebral Cavernous Malformation* signaling pathway promotes vascular integrity via Rho GTPases

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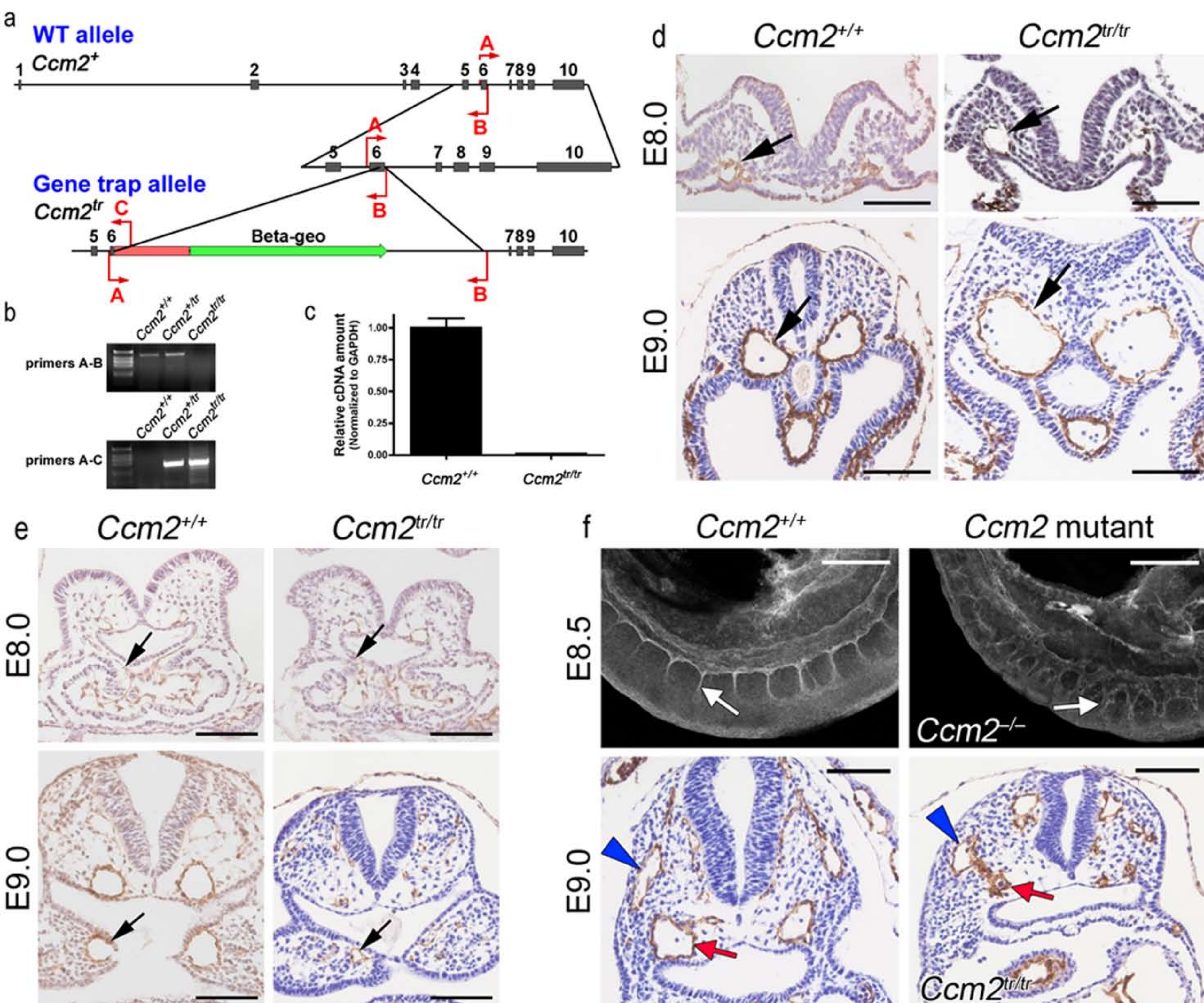
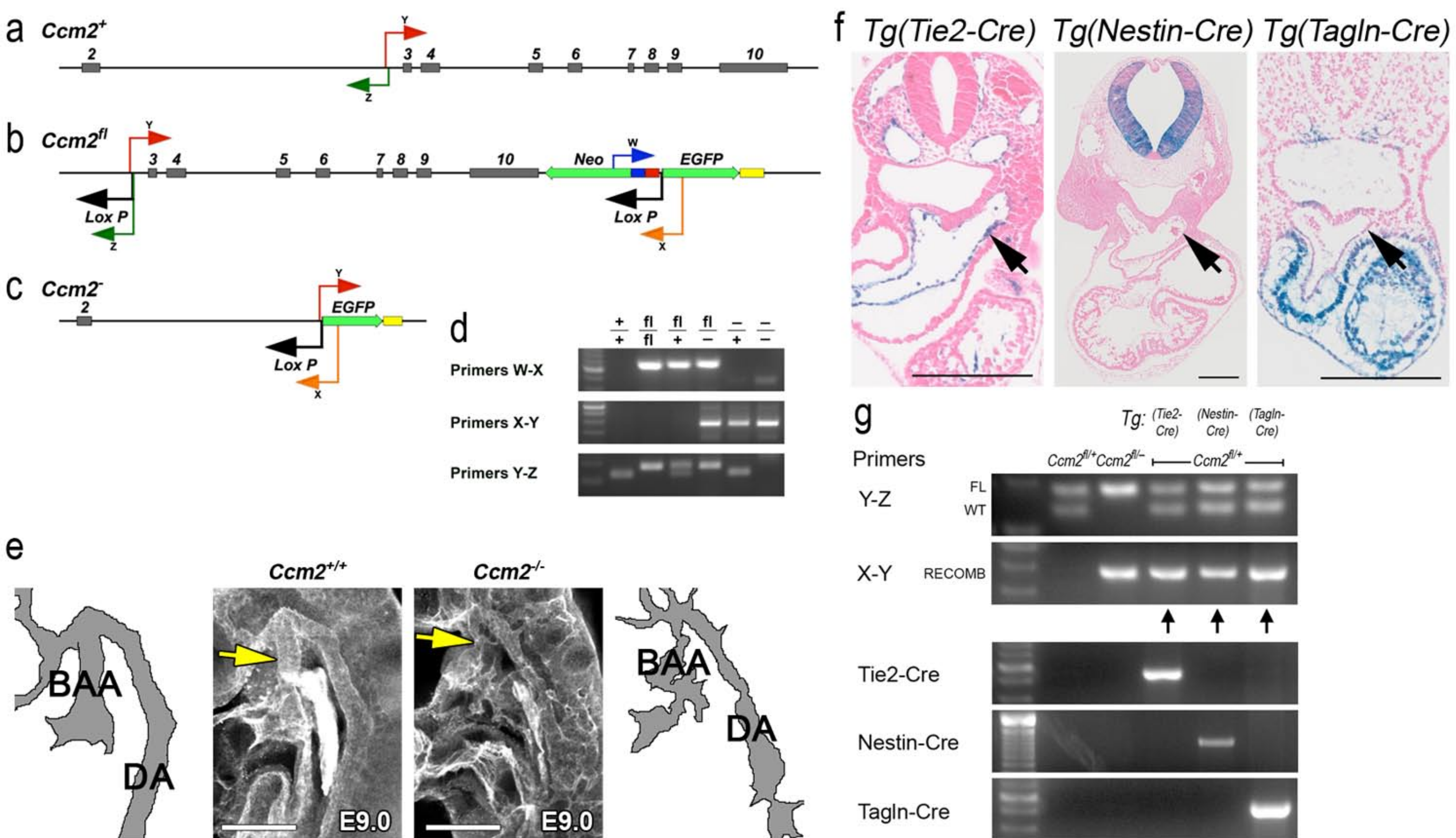


Supplementary figure 1 – A gene trap mutation results in loss of *Ccm2* expression and angiogenesis defects. (**a**) The genomic structure of wild type *Ccm2* is disrupted by insertion of the gene trap vector within exon 6. The location of genotype primers is demonstrated. (**b**) The results of PCR genotyping for the three possible genotypes is illustrated. (**c**) Real-time quantitative RT PCR with primers in exons 8 and 9 for *Ccm2* message in total RNA derived from *Ccm2*$^{tr/tr}$ embryos. The quantity of *Ccm2* cDNA was normalized to *Gapdh* (values +/- s.d.). (**d**) The aorta of the embryo (arrows) caudal to the heart and venous inflow in wild type and mutant embryos. The aortae of the mutant enlarge by E9.0 (lower right). (**e**) Development of the first branchial arch artery in mice lacking *Ccm2*. A cord of endothelial cells is present at E8.0 in both wild type and mutant embryos (arrows, upper panels). The mutant has endothelial cells without proper lumen at E9.0 (arrows, lower panels). (**f**) Angiogenesis defects involve the intersomitic arteries in mice lacking *Ccm2*. Intersomitic artery sprouts (white arrows) are broad and irregular in mutant embryos. An abnormal, direct connection between the cardinal vein (blue arrowheads) and dorsal aorta (red arrows) is seen in a mutant E9.0 embryo (lower right). Scale bars: 100 µm.



Supplementary figure 2. Conditional targeting of *Ccm2*. The three alleles of *Ccm2* that result from our targeting strategy are shown. (**a**) Wild type *Ccm2* has 10 exons, the final 9 of which are shown. (**b**) The conditional (floxed) allele includes LoxP sites that flank exons 3 through 10 of *Ccm2*. The floxed allele can be detected with primers W and X, or can be recognized by the upward shift in band size with primers Y and Z relative to wild type due to LoxP insertion. (**c**) Cre recombinase deletes exons 3–10 of *Ccm2*. The mutant allele can be detected with primers X and Y. (**d**) PCR genotyping results are shown for all 6 possible combinations. (**e**) Confocal immunofluorescence (CD31 antibody) of branchial arch arteries (yellow arrows) and aorta in an embryo homozygous for a germline recombined allele of *Ccm2* compared to a wild type littermate. Scale bars: 100 μm. (**f**) X-gal staining of embryos containing LacZ reporter allele and tissue specific Cre drivers as specified. The branchial arch artery endothelium is indicated (arrows). Scale bars: 500 μm. (**g**) PCR for the recombined allele in embryos (primers X-Y, “RECOMB”). Primers Y-Z also define the status of the wild type (“WT”) and floxed (“FL”) alleles. The appearance of PCR product for the recombined allele in *Ccm2*$^{fl/+}$ embryos (arrows) indicates Cre-mediated recombination for each of the tissue specific drivers.

Supplementary table 1. *Ccm2*$^{tr/tr}$ mutant phenotype: normal appearing embryos.

	Ccm2$^{+/+}$	*Ccm2*$^{+/tr}$	*Ccm2*$^{tr/tr}$
<E8.5	72	161	79
E8.5	217	362	177
E9.0	48	80	2
>E9.0	79	121	0

Supplementary Methods

Mouse strains.

Mice with gene trap mutations of *Ccm2* were derived from an embryonic stem cell clone (Bay Genomics). A construct for the conditional allele of *Ccm2* was derived from genomic sequence obtained from a BAC clone (RP22 library, Invitrogen). The construct extended from a *Sal*I site 5' of exon 3 through a *Bam*HI site 3' of exon 10. The construct contained inserts as outlined in Fig. S2. All mice were backcrossed into the C57BL6/J strain. Experiments performed prior to the 5th cross were performed with littermate controls. LacZ reporter mice (*R26R1*) and *Tie2-Cre* mice were generously provided by Phil Soriano and Masashi Yanagisawa respectively. *HPRT-Cre*, *Nestin-Cre* and *Tagln-Cre* mice were obtained from The Jackson Laboratory. Genotypes were determined by PCR analysis of genomic DNA isolated from either ear biopsies or yolk sac tissues using primers outlined in **Supplementary Fig. 1** and **2** (sequences available upon request).

Confocal immunofluorescence of embryos.

Embryos were prepared for confocal immunofluorescent detection of PECAM antigen as previously described[39] with the following exception. For improved signal detection in thick specimens, following the final wash steps after applying secondary antibody, embryos were processed through graded methanols before mounting in benzyl alcohol – benzyl benzoate (BABB) based mounting medium. Images were aquired with an Olympus FV300 confocal microscope, and stacks were chosen to visualize only one of the paired dorsal aortae. Multiple images were required to visualize the entire embryo. Photoshop was used to assemble source images into a final composite (image junctions shown in final assembly).

Digital Subtraction.

Ultrasound images were selected for digital subtraction by choosing frames unaffected by motion from maternal respiration. Sequential images were applied to each other in Photoshop (Adobe Systems, Inc.) using a subtractive filter to remove static portions of the image. Several resulting images of dynamic pixels were merged together using an additive filter. This composite of dynamic pixels was colorized using a gradient overlay. To provide some anatomic perspective, the colorized image was given 75% opacity and projected over an unfiltered source image.

Histology.

Embryos were studied with antibodies to PECAM (1:250 dilution, clone MEC13.3, BD Biosciences). Improved visualization on paraffin sections was obtained using a biotinylated tyramide signal amplification (TSA) kit (PerkinElmer) according to the manufacturer's instructions. Cardiac smooth muscle was demonstrated with antibodies to alpha smooth muscle actin (1:500 dilution, clone 1A4, Sigma) without signal amplification. To demonstrate tissue specificity of transgenic Cre

lines, embryos with appropriate LacZ reporter alleles were stained with X-gal as previously described[42].

Cell culture.
Human umbilical vein endothelial cells (HUVEC) and human dermal microvascular endothelial cells (HMVEC) were obtained from Lonza and grown according to the manufacturer's instructions in EGM-2 media (HUVEC) or EGM-2MV media (HMVEC). Human embryonic kidney (HEK 293T) cells (from ATCC) were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco) with 10% fetal bovine serum (Hyclone) supplemented with antibiotics.

Transfection of ECs with siRNAs.
Human *CCM2* siRNA was obtained from Dharmacon. Luciferase GL2 duplex or scramble siRNA (Dharmacon) were used as a control. EC transfection with siRNAs was carried out in growth media with 1% serum. Details of the siRNA transfection protocol have been described previously[43].

Reverse transcription-polymerase chain reaction (RT-PCR).
Total RNA was extracted from EC vasculogenesis assay at indicated time points or from siRNA-treated (Luciferase or *CCM2*) ECs using the Totally RNA Isolation kit (Ambion) according to the manufacturer's instructions. RNA (1 μg) was reverse transcribed using AccuScript High Fidelity 1st strand cDNA synthesis kit (Stratagene). RT-PCR amplification primer sequences are available upon request. For quantitative real-time PCR, total RNA was extracted from cultured endothelial cells or from embryos using the NucleoSpin RNA II kit (Clontech) according to the manufacturer's instructions. Reverse transcription was performed with random primers using the RetroScript kit (Ambion). Quantitative PCR was performed with TaqMan assays (Applied Biosystems) for human *CCM2* and *GAPDH*, or mouse *Ccm2* and *Gapdh*. Quantification was performed by standard curve method, and *CCM2* transcripts were normalized to *GAPDH* for comparisons.

Immunofluorescent Cell Staining.
Glass chamber slides were coated with human fibronectin (Biomedical Technologies, Inc.), and transfected cells were seeded at 50,000 cells per well. For the RHOA and ROCK experiment, cells were treated 4 d after seeding with 40 μg mL^{-1} of cell-permeable C3 transferase (Cytoskeleton, Inc.) or 20 μM Y-27632 (Santa Cruz Biotechnology) for 4 h. For the simvastatin experiment, cells were treated 3 d after seeding with 40 μM simvastatin or equivalent amounts of ethanol in growth media for 24 h. Cells were fixed in 4% formaldehyde and incubated with an antibody against β-catenin (BD Biosciences). Fluorescent secondary antibody (Molecular Probes) was used to visualize β-catenin staining. Actin cytoskeleton was visualized using fluorescently-conjugated phalloidin (Molecular Probes). Images were obtained with an Olympus FV300 confocal microscope.

GTPase Activation Assays.
Activity of RHOA, RAC1, and CDC42 were measured using activation assay kits (Upstate) according to manufacturer's instructions. Briefly, transfected cells were scraped into Mg^{2+} lysis buffer supplemented with protease inhibitors (Roche) and phosphatase inhibitors (Sigma). A small portion of the lysate was retained as total cell lysate and the rest was incubated with the assay reagent. GTP-bound forms were eluted from the assay reagent using Laemmli sample buffer and analyzed by western blotting. The total cell lysate was analyzed by western blotting for total GTPase input.

Immunoprecipitation.
An EST for *CCM2* (IMAGE: 2924210) was obtained from ATCC and cloned into a pcDNA3.1 Hygro+ plasmid modified to encode a C-terminal V5 tag. Constructs for myc-tagged RHOA, RAC1, and CDC42 were obtained from Addgene[44] (Addgene plasmid 15899, Addgene plasmid 15902, and Addgene plasmid 15905 respectively). Plasmids were transfected into HEK 293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. 3 d post transfection, cells were scraped into lysis buffer (50 mM Tris-HCl at pH 7.5, 100 mM NaCl, 0.5% Triton X-100) supplemented with protease and phosphatase inhibitors. A portion of cell lysate was retained as whole cell lysate, and the rest incubated with antibodies against RHOA (Santa Cruz Biotechnology), RAC1, or CDC42 (Upstate) as indicated at 4°C for 2 h, followed by incubation with Protein A/G beads (Santa Cruz Biotechnology). The beads were washed three times with lysis buffer and bound proteins were eluted using Laemmli sample buffer. Presence of CCM2-V5 was detected using an anti-V5 antibody (Invitrogen). Presence of myc-tagged RHOA, RAC1, and CDC42 were detected using an anti-myc antibody (Santa Cruz Biotechnology).

MAPK Profiling.
siRNA transfected cells were lysed in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40) supplemented with protease and phosphatase inhibitors. Lysates were then analyzed by western blotting. Antibodies to phospho-JNK and total JNK were from Santa Cruz Biotechnology. Antibodies to phospho-ERK, phospho-p38, phospho-MKK4, phospho-MKK7, total ERK, total p38, total MKK4, and total MKK7 were from Cell Signaling Technology. The effect of ROCK inhibitor on JNK was tested by treating cells with 10 μM Y-27632 for 30 min prior to cell lysis. The effect of simvastatin on JNK was determined by treating cells with 10 μM simvastatin for 24 h prior to cell lysis.