

Cerebral cavernous malformation protein CCM1 inhibits sprouting angiogenesis by activating DELTA-NOTCH signaling

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Cerebral cavernous malformations (CCM) are frequent vascular abnormalities caused by mutations in one of the CCM genes. CCM1 (also known as KRIT1) stabilizes endothelial junctions and is essential for vascular morphogenesis in mouse embryos. However, cellular functions of CCM1 during the early steps of the CCM pathogenesis remain unknown. We show here that CCM1 represents an antiangiogenic protein to keep the human endothelium quiescent. CCM1 inhibits endothelial proliferation, apoptosis, migration, lumen formation, and sprouting angiogenesis in primary human endothelial cells. CCM1 strongly induces DLL4-NOTCH signaling, which promotes AKT phosphorylation but reduces phosphorylation of the mitogen-activated protein kinase ERK. Consistently, blocking of NOTCH activity alleviates CCM1 effects. ERK phosphorylation is increased in human CCM lesions. Transplantation of CCM1-silenced human endothelial cells into SCID mice recapitulates hallmarks of the CCM pathology and serves as a unique CCM model system. In this setting, the multikinase inhibitor Sorafenib can ameliorate loss of CCM1-induced excessive microvascular growth, reducing the microvessel density to levels of normal wild-type endothelial cells. Collectively, our data suggest that the origin of CCM lesions is caused by perturbed Notch signaling-induced excessive capillary sprouting, which can be therapeutically targeted.

endothelial cells | vascular malformations | CCM | KRIT1

Cerebral cavernous malformations (CCM) [OMIM: 116860] are frequent vascular abnormalities, predominately localized in the brain, affecting up to 0.5% of the human population (1). CCM lesions are characterized by grossly enlarged vascular channels, often lacking support of mural cells. The carriers can develop a symptomatic disease with headaches, seizures, focal neurological deficits, or hemorrhages. The disease is caused by mutations in one of the three known CCM genes, namely *KRIT1* (*CCM1*), *OSM* (*CCM2*), or *PDCD10* (*CCM3*), and can occur sporadically or familial with high penetrance (2). Recent data suggest a “two-hit mechanism” for the cause of localized lesions, where a germ-line loss of one allele is followed by a loss of the second allele of individual endothelial cells (3, 4).

CCM1 is expressed in astrocytes and endothelial cells, and can be associated to microtubules, membranes, and adherens junctions, but also the nucleus (5–8). The CCM1 protein is part of a large protein complex together with CCM2 and CCM3, components of the cytoskeleton and cell junctions, as well as components of signal transduction pathways and lipids (9). The interaction with RAP1 is essential for stabilizing endothelial cell-cell contacts (5). Additionally, the strong binding to the cytoplasmic $\beta 1$ -integrin binding protein-1 ICAP1 (ITGB1BP1) could affect $\beta 1$ -integrin conformation and endothelial-extracellular matrix interactions (10); however, detailed molecular and cellular functions remain elusive.

A *Ccm1* null mutation in mouse causes embryonic lethality. Many major blood vessels of mutant embryos are dilated, and others are narrowed and the expression of arterial marker genes is

strongly reduced, suggesting a defect in arterial/venous differentiation (11). In contrast, loss of *ccm1* in zebrafish does not alter arterial marker gene expression, but leads to dilation of major vessels because of excessive spreading of endothelial cells (12). The clinical observation that CCM lesions grow in response to VEGF (13) suggests that their formation could be driven by angiogenesis: the growth of new blood vessels from preexisting ones. We consequently hypothesized that CCM lesions may reflect a pathological angiogenesis. The experiments revealed a role of CCM1 as an activator of vascular DELTA-NOTCH signaling. Correspondingly, perturbed DELTA-NOTCH signaling was identified as a pathogenic cause of *CCM1* mutation-mediated vascular malformations.

Results

CCM1 Is a Negative Regulator of Sprouting Angiogenesis. Human umbilical vein endothelial cells (HUVEC) express *CCM1* mRNA as determined by quantitative real-time RT-PCR (qPCR). When cultured on Matrigel, HUVEC spontaneously organized into cords and formed a honeycomb-like network. Forced expression of CCM1 inhibited such network formation (Fig. 1*A* and *B*). HUVEC remained isolated or in clumps of cells and formed fewer tubes and branch points compared with GFP overexpression as control.

CCM1 expression caused a drastic reduction of VEGF- or FGF2-induced sprout formation in a 3D spheroidal system of endothelial differentiation and capillary formation (14) (Fig. 1*C*). The short sprouts failed to form a lumen. Because CCM lesions are typically caused by a loss of CCM1 function, we investigated whether down-regulation of this gene would lead to enhanced angiogenesis. Two independent CCM1 siRNAs, as well as a lentiviral shRNA vector, led to substantial silencing of *CCM1* mRNA expression (Fig. 1*D*). HUVEC with silenced CCM1 expression formed significantly more long capillary sprouts compared with nonsilencing siRNA-transfected control cells (Fig. 1*E*). When CCM1 shRNA transduced HUVEC (green fluorescent) were mixed with control cells (red fluorescent), the CCM1-depleted HUVEC formed more and larger sprouts (Fig. 1*F* and *Movie S1*). Cells overexpressing CCM1 formed only very few and short sprouts when mixed with control HUVEC (Fig. 1*G* and *Movie S2*).

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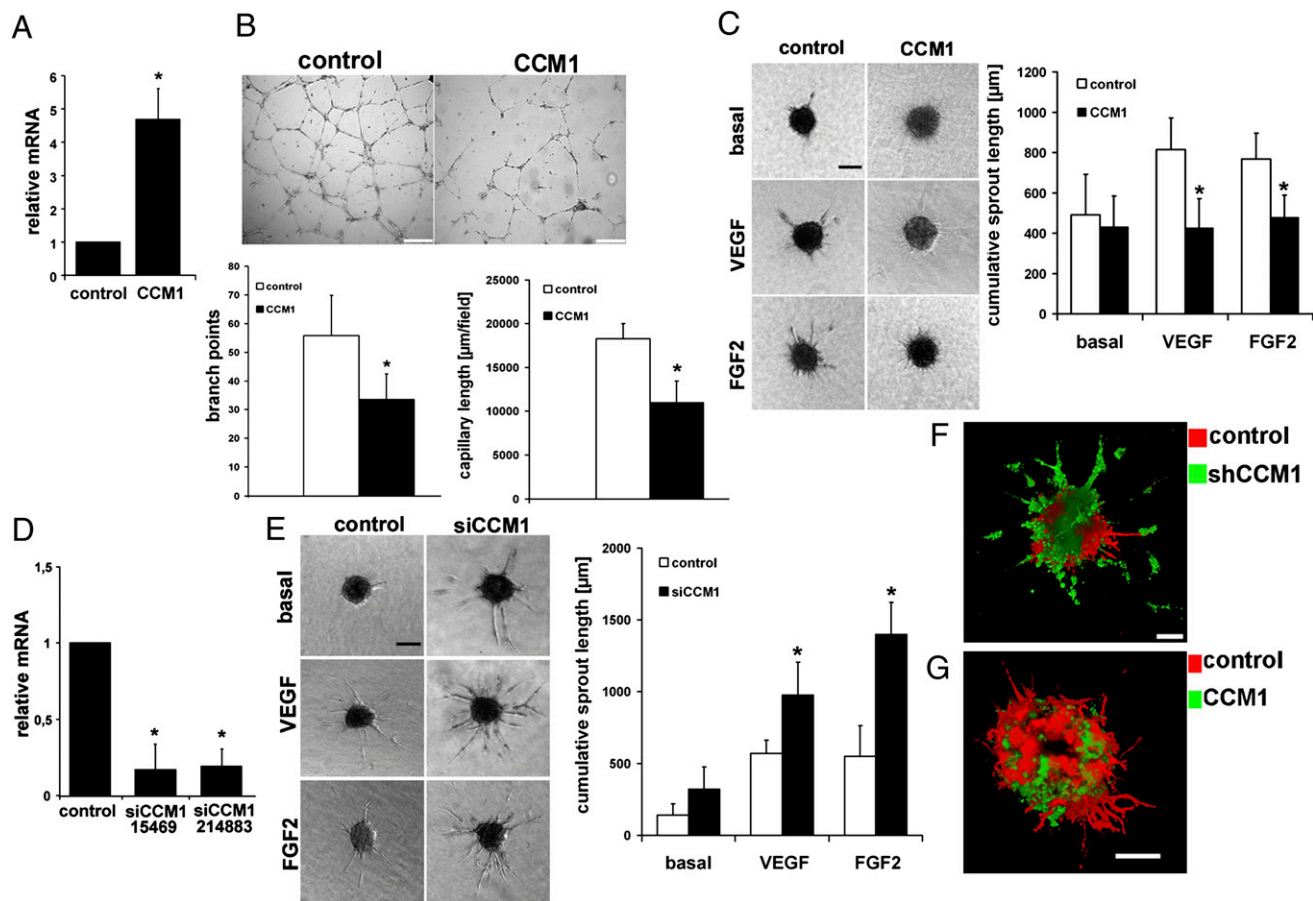


Fig. 1. CCM1 inhibits sprouting angiogenesis. (A–C) HUVEC were adenovirally transduced with CCM1 or GFP as control leading to an about 5-fold over-expression of CCM1 mRNA as determined by qPCR (A). (B) CCM1 inhibited tube formation and branching on a Matrigel matrix. (Scale bars, 2,000 μm .) (C) CCM1 inhibited VEGF (25 ng/mL) or FGF2 (25 ng/mL) stimulated sprouting angiogenesis in a collagen matrix. (Scale bar, 200 μm .) (D) Down-regulation of CCM1 mRNA expression by two siRNAs (Ambion) led to about 80% reduction of mRNA levels. (E) Small interfering RNA-mediated CCM1 silencing strongly enhanced endothelial sprouting in collagen gels under basal conditions and after VEGF or FGF2 stimulation. (Scale bar, 200 μm .) (F and G) Control HUVEC were labeled with the red fluorescent membrane dye PKH26 and mixed with CCM1 shRNA (F) or cDNA (G) expressing HUVEC stained with the green membrane dye PKH67. After sprouting in collagen beds (25 ng/mL VEGF), spheroids were imaged with confocal laser microscopy and 3D images were calculated. (F) CCM1 depletion caused more and larger sprouts. (Scale bar, 100 μm .) (G) CCM1-expressing HUVEC formed few regular sprouts compared with control. * $P < 0.05$. (Scale bar, 100 μm .) Error bars are means \pm SD of $n = 10$ in C and E.

Control cells exhibited normal sprouting behavior, suggesting a cell-autonomous function of CCM1. These experiments established CCM1 as a negative regulator of angiogenesis.

CCM1 Impairs Endothelial Migration, Proliferation, and Apoptosis. Angiogenesis involves multiple cellular processes, including endothelial cell migration and proliferation. Migration of CCM1-expressing HUVEC was significantly delayed compared with control. This delay was detected in a scratch wound assay (Fig. 2A) and in the Boyden chamber using VEGF or FGF2 as chemoattractants (Fig. 2B). Conversely, CCM1 silencing enhanced endothelial migration (Fig. 2A and B), which was associated with an increased number of polarized focal adhesions at cellular protrusions (Fig. 2C).

Silencing of CCM1 caused a moderate, nonsignificant enhancement of HUVEC proliferation as determined by BrdU incorporation and cell-number counting. The apoptosis rate under basal conditions and in response to the apoptosis-inducing agent staurosporin was also not significantly altered (Fig. S1). Conversely, forced CCM1 expression led to reduced cell proliferation (Fig. 2D). This finding was associated with a reduced fraction of S-phase cells, an increased G1-phase population (Fig. 2E), and elevated mRNA levels of the cell cycle inhibitors *p21* (*Waf1* or *CIP1*) and *p27* (*KIP1*) (Fig. 2F). CCM1 expression significantly reduced ERK phosphorylation, whereas higher phospho-ERK levels could

be detected after CCM1 silencing (Fig. 2G). These findings were corroborated by analysis of protein lysates derived from surgical material of human CCM lesions. Compared with control brain tissue, lesions from six CCM patients (15) had significantly higher levels of phosphorylated ERK protein (Fig. 2H).

CCM1 expression did not induce apoptosis under normal culture conditions and even protected HUVEC from staurosporine-induced apoptosis (Fig. 2I). In line with the potent antiapoptotic and prosurvival functions of active AKT (16, 17), CCM1 expression significantly elevated the amount of active AKT protein phosphorylated at serine 473 (Fig. 2J), whereas knockdown of CCM1 decreased AKT phosphorylation (Fig. 2K). Collectively, the data establish a role of CCM1 in shifting the balance from ERK-mediated proliferation and migration to AKT-mediated cell survival and endothelial quiescence.

CCM1 Regulates Sprouting Angiogenesis in Vivo. Human endothelial cells during angiogenesis can functionally be studied in vivo by grafting them in immunocompromized mice, where they will form a network of perfused capillaries that anastomoses with the mouse vasculature (18, 19). We sought to advance this assay to mimic a genetic human vascular disease in mice (Fig. 3A). Lentivirally CCM1-silenced HUVEC and control shRNA transduced cells were comparatively studied for their ability to form vascular net-

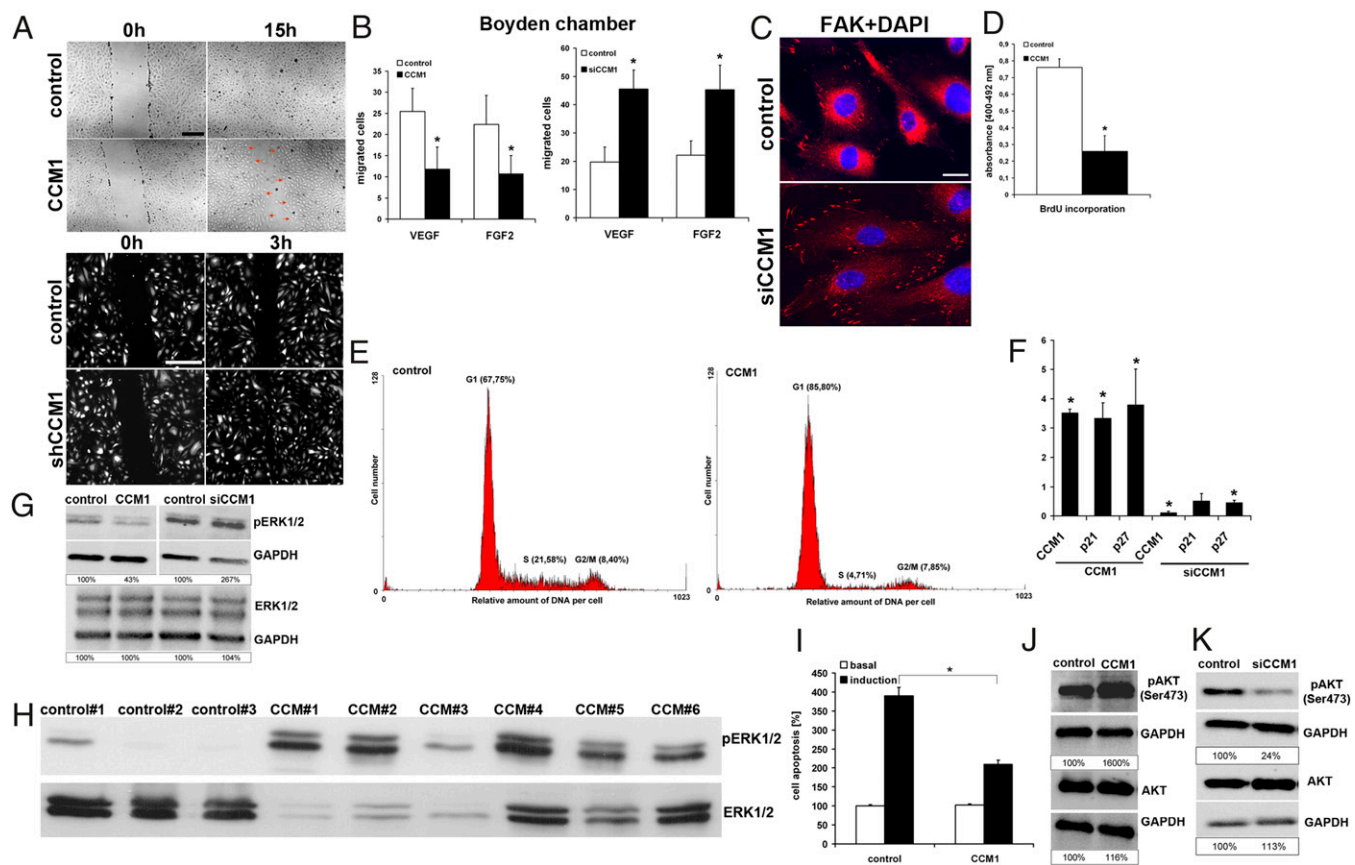


Fig. 2. CCM1 inhibits endothelial migration and proliferation. (A) Confluent HUVEC were wounded and migration was assayed. Short hairpin RNA against CCM1 enhanced closure of the gap, whereas lentiviral CCM1 expression inhibited cell migration (arrows). Because shRNA vectors also expressed GFP, fluorescence pictures are shown, whereas CCM1 over expression is shown as bright field image. (Scale bars, 200 μm .) (B) Transmigration through a collagen coated filter (8- μm pore size) toward VEGF (25 ng/mL) or FGF2 (25 ng/mL) was inhibited by CCM1 and enhanced after CCM1 knockdown. (C) Antibody staining against focal adhesion kinase (FAK) revealed a higher number and more polarized focal adhesion contacts at the cell periphery after CCM1 knockdown. (Scale bar, 50 μm .) (D–G) CCM1 inhibited endothelial proliferation as shown by reduced BrdU incorporation (D), decreased fraction of cells in S-phase as determined by FACS (E), and up-regulation of the cell cycle inhibitors *p21* and *p27* mRNA (F). (G) Western blot analysis 48 h after CCM1 adenovirus transduction showed that CCM1 reduced phosphorylation of ERK1/2 proteins, whereas siRNA treatment elevated phospho-ERK1/2 levels. (H) Protein lysates of human CCM1 lesion exhibit high phospho-ERK1/2 amounts. (I) Apoptosis of HUVEC under normal culture conditions and 2 h after addition of 250 nmol/L staurosporine was measured by detection of caspase-3 and -7 activities with a luminescent substrate. CCM1 expression reduced the rate of staurosporine-induced cell death. (J and K) CCM1 expression increased the amount of phosphorylated AKT protein at serine 473. * $P < 0.05$. Error bars are means \pm SD of $n = 5$.

works following xenotransplantation. Twenty-eight days after transplantation, CCM1-silenced HUVEC had formed a significantly denser vessel network in vivo (Fig. 3C). These vessels also had more protrusions and had larger diameters than control HUVEC vasculatures (Fig. 3B and D and Movie S3 and Movie S4), thus representing hallmarks of the CCM disease.

Global Gene Expression Analysis to Uncover Molecular CCM1 Effects. Comparative microarray analyses of CCM1 expressing and control GFP-transduced HUVEC identified 262 transcripts with at least 2-fold changed expression (Fig. S2 and Table S1). The transcriptional changes of 12 candidates were verified by qPCR Table S2. The majority of the regulated transcripts were involved in cell-cycle progression, proliferation, and cell migration (Fig. 4B) and these genes were also highly functional connected with each other (Fig. S3 and Fig. S4).

Among the regulated genes, we identified a considerable number of transcripts associated with angiogenesis (Fig. S2C). Angiogenic factors (*EGRI*, *ESM1*, *ROBO4*) and genes involved in cell migration (*CDC42EP2*, *ETV5*, *HMMR*, *IQGAP3*) were down-regulated. In contrast, extracellular matrix molecules (*COL1A2*, *FIBULIN2*) and BMP/TGF β related/regulated genes (*ANGPTL4*, *BAMBI*, *COL1A2*, *ESM1*, *GDF3*, *TGFA*) were up-regulated. No-

tably, CCM1 induced *HEY1*, a direct target of NOTCH signaling (20), which acts as a strong inhibitor of sprouting angiogenesis (21).

CCM1 Acts Upstream of the DELTA-NOTCH Pathway. In addition to the up-regulation of *HEY1*, we identified a significant induction of the NOTCH ligand *DLL4* and the NOTCH target gene *HEY2* (Fig. 4A), which was accompanied by higher levels of cleaved, and thus activated, NOTCH protein (Fig. 4B). Active NOTCH signaling inhibits endothelial proliferation, migration, sprouting, and branching, whereas loss-of-function causes the opposite effects with excessive sprouting angiogenesis (22).

Silencing of CCM1 expression significantly diminished *DLL4*, *HEY1*, and *HEY2* mRNA levels (Fig. 4A). The down-regulation of the NOTCH ligand *DLL4* was between 50 and 80%, a sufficient reduction known to cause excessive angiogenesis (23–25). NOTCH activity is tightly linked to CCM1 because the down-regulation of *CCM1* mRNA after 8 h was followed by rapid *DLL4*, *HEY1*, and *HEY2* mRNA reduction. Conversely, *CCM1* mRNA expression, observable after 18 h of transduction, was accompanied by rapid *DLL4*, *HEY1*, and *HEY2* induction (Fig. S5).

DLL4-NOTCH Signaling Regulates AKT and ERK Phosphorylation. CCM1 increased AKT phosphorylation (Figs. 2 and 4C). AKT is

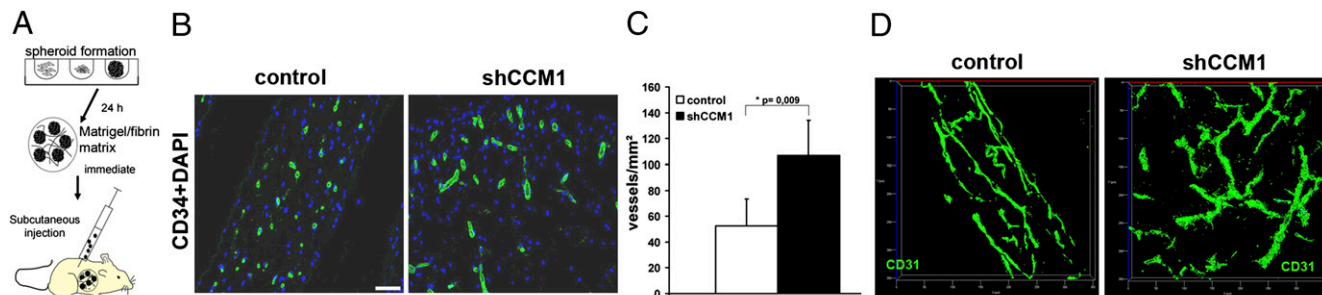


Fig. 3. CCM1 inhibits angiogenesis in transplanted endothelial cells. (A) Scheme of the spheroid-based angiogenesis assay. (B) Representative sections through plugs stained against human CD34 (green) for endothelial cells. HUVEC expressing control shRNA formed a regular capillary vascular network. CCM1 silenced HUVEC formed a denser network with larger vessels. (Scale bar, 50 μ m.) (C) Quantification of microvessel density determined by the number of vessels per square millimeter. $n = 7$ plugs of control and CCM1 shRNA. (D) Thick sections (50 μ m) were stained with human specific anti-CD31 to stain the endothelial network and assessed by confocal laser microscopy (LSM710; Zeiss). shRNA against CCM1 led to formation of a denser network with larger vessels compared with control.

phosphorylated after interaction with phosphatidylinositol trisphosphates (PIP3) at the cell membrane. PIP3 is generated from PIP2 by phosphatidylinositol-3-kinase (PI3K), and CCM proteins can interact with PIP2 or PIP3 molecules (9, 26). The PI3K inhibitors Wortmannin and LY294002 almost completely abolished CCM1-induced AKT phosphorylation (Fig. 4), implicating that CCM1 functions are transmitted in a PIP3-dependent manner.

Active NOTCH1 could strongly induce phosphorylation of AKT (Fig. 4D). However, we ruled out the possibility that CCM1-induced AKT phosphorylation was only a secondary effect because of induction of NOTCH signaling. The blockage of NOTCH with the γ -secretase inhibitor DAPT did not prevent phosphorylation of AKT1 after CCM1 expression (Fig. 4C).

Furthermore, CCM1 inhibited ERK phosphorylation (Figs. 2 and 4E). However, this inhibition was abolished when HUVEC were treated with DAPT, suggesting that this CCM1 effect is fully mediated through NOTCH signaling. Our assumption was supported by the finding that constitutive active NOTCH1 strongly inhibited ERK1/2 phosphorylation (Fig. 4F). Taken together, these data imply that CCM1 promotes AKT phosphorylation in a NOTCH-dependent and independent manner, and it inhibits ERK1/2 phosphorylation indirectly through activation of the DELTA-NOTCH cascade (Fig. 4G).

Rescue of the CCM1 Phenotype. To rescue the endothelial defects caused by CCM1 gain-of-function, cells overexpressing CCM1

were treated with the γ -secretase inhibitor DAPT to prevent NOTCH activation. This treatment was sufficient to restore the responsiveness to VEGF and caused an almost normal sprouting behavior of HUVEC (Fig. 5A and B).

Finally, we aimed at developing a unique therapeutic strategy to improve the treatment of CCM. Our data showed that loss of CCM1 leads to enhanced ERK signaling and angiogenesis. The multiple kinase inhibitor Sorafenib (Nexavar) is a clinically approved anti-angiogenic drug which inhibits the Raf/ERK pathway, as well as VEGF receptor signaling, besides some other tyrosine kinases (27). Although doses smaller than 5 μ M were not effective, the addition of 10 μ M Sorafenib could almost completely block VEGF-induced sprouting after CCM1-silencing in HUVEC (Fig. 5C and D). Thus, antiangiogenic therapy might be beneficial to prevent disease progression. To test this theory, we grafted CCM1-depleted HUVEC s.c. into the flanks of SCID mice. Two groups were formed after 28 d. One group received 40 mg/kg per day Sorafenib per oral gavage, whereas the control group was mock-treated with the solvent. The plugs were analyzed after treatment for 7 d. The mock-treated mice showed again the formation of a hyper-dense and irregular vasculature with large vessel diameters. Treatment with Sorafenib was able to strongly ameliorate the excessive vasculature. Sorafenib decreased the microvessel density to rates comparable with untreated endothelial cells (Fig. 5E–G). Taking these data together, we conclude that the elucidation of CCM1-regulated signaling pathways

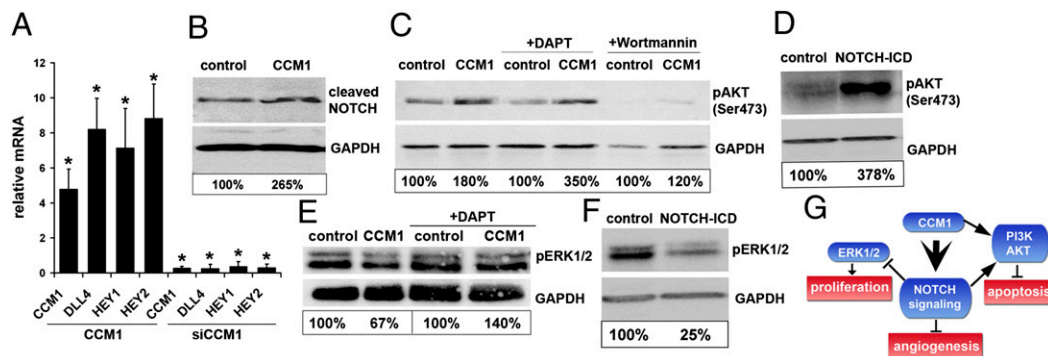


Fig. 4. CCM1 activates DELTA-NOTCH signaling. (A) Quantitative RT-PCR showing significantly elevated mRNA levels of the NOTCH ligand *DLL4* and the target genes *HEY1* and *HEY2* 48 h after adenoviral CCM1 transduction. Small interfering RNA treatment against CCM1 down-regulated expression of *DLL4* and NOTCH target genes. (B) CCM1 expression in HUVEC increased the amounts of cleaved NOTCH receptor proteins as detected by a cleavage-specific antibody. (C) Western blotting revealed strongly increased levels of phospho-AKT after 36 h of adenoviral CCM1 transduction. The NOTCH inhibitor DAPT (25 μ M) could not prevent AKT phosphorylation. Inhibition of PI3K activity by Wortmannin (2 μ M) prevented AKT phosphorylation independent of CCM1 expression. (D) Adenoviral expression of constitutive active NOTCH1 (intracellular domain, ICD) increased phospho-AKT levels. (E) Adenoviral CCM1 expression decreased ERK1/2 phosphorylation and this could fully be prevented with the NOTCH inhibitor DAPT (25 μ M). (F) Active NOTCH1 strongly inhibited ERK1/2 phosphorylation. (G) Proposed scheme of CCM1-mediated regulation of endothelial functions. * $P < 0.05$.

is in line with a recent report showing that AKT1 can activate NOTCH1 in melanoma cells (32). Thus, we propose that CCM1 activates NOTCH by stimulation of AKT phosphorylation and cleaved NOTCH1 further stimulates AKT phosphorylation in a positive feedback loop. Additionally, we found that active NOTCH1 could strongly diminish ERK phosphorylation. Thus, CCM1 leads to potent reduction of ERK-mediated cell proliferation but to an increase of AKT-driven endothelial survival via NOTCH signaling.

We envisage that antiangiogenic therapy could be beneficial to prevent progression of CCM lesions. Sorafenib, an approved antiangiogenic drug, targets VEGF receptors and the Raf/ERK pathway (27). Our data suggest that ERK signaling is enhanced in the angiogenic endothelium of CCM lesions, making Sorafenib an ideal candidate for initial drug testing. Indeed, Sorafenib efficiently blocked angiogenic sprouting of HUVEC silenced for CCM1 expression. In vivo, Sorafenib led to a drastic regression of CCM1-deficient blood vessels. Thus, antiangiogenic therapy might be beneficial for the treatment of cerebral cavernous malformations in the future.

Materials and Methods

Plasmids, RNAi. CCM1 cDNA was cloned into pENTR3c-IRES2-EGFP. Control siRNA-1 and siRNAs against CCM1 (15469, 214883) (5) were from Ambion. CCM1 shRNAs (RH54430-98913140, -98820292, RMM4431-98978703) were from Open Biosystems. RH54430-98820292 achieved excellent knockdown.

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