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Combinatorial interaction between CCM pathway genes precipitates hemorrhagic stroke

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SUMMARY

Intracranial hemorrhage (ICH) is a particularly severe form of stroke whose etiology remains poorly understood, with a highly variable appearance and onset of the disease (Felbor et al., 2006; Frizzell, 2005; Lucas et al., 2003). In humans, mutations in any one of three *CCM* genes causes an autosomal dominant genetic ICH disorder characterized by cerebral cavernous malformations (CCM). Recent evidence highlighting multiple interactions between the three *CCM* gene products and other proteins regulating endothelial junctional integrity suggests that minor deficits in these other proteins could potentially predispose to, or help to initiate, CCM, and that combinations of otherwise silent genetic deficits in both the CCM and interacting proteins might explain some of the variability in penetrance and expressivity of human ICH disorders. Here, we test this idea by combined knockdown of CCM pathway genes in zebrafish. Reducing the function of *rap1b*, which encodes a Ras GTPase effector protein for CCM1/Krit1, disrupts endothelial junctions in vivo and in vitro, showing it is a crucial player in the CCM pathway. Importantly, a minor reduction of Rap1b in combination with similar reductions in the products of other CCM pathway genes results in a high incidence of ICH. These findings support the idea that minor polygenic deficits in the CCM pathway can strongly synergize to initiate ICH.

INTRODUCTION

Endothelial cell-cell adhesion is important for proper blood vessel formation, maintaining the integrity of the vasculature, and regulating diffusion of molecules between the luminal and abluminal faces of blood vessels. Weakened endothelial junctions are implicated in intracranial hemorrhage (ICH), which are severe and debilitating forms of stroke in humans that include the cerebral cavernous malformation (CCM) disorders. CCM occur either sporadically or with an autosomal dominant inheritance pattern. So far, three *CCM* genes have been identified in the heritable disorders: *CCM1*/*KRIT1* (Laberge et al., 1999), *CCM2*/*malcavernin* (Denier et al., 2004) and *CCM3*/*programmed cell death 10* (*PDCD10*) (Bergametti et al., 2005), accounting for approximately 40%, 20% and 40% of the autosomal dominant disorders, respectively (Craig et al., 1998).

The *CCM1* locus codes for a protein called KRIT1 that contains four ankyrin repeat domains and a FERM (protein 4.1, ezrin, radixin, moesin) domain that mediates its interaction with Rap1, a small GTPase that facilitates KRIT1 localization to cell-cell junctions. In *Drosophila melanogaster*, Rap1 is not required for cell proliferation and cell fate specification, but is required for normal tissue morphogenesis and cell shape maintenance (Asha et al., 1999). Rap1 is localized to the adherens junctions and is actively required for their even distribution; loss of Rap1 leads to disrupted epithelial cell behavior (Knox and Brown, 2002). KRIT1/CCM1 also binds CCM2, CCM3, and a variety of additional interacting proteins, and it is thought that these proteins all function together in large multiprotein complexes regulating cell-cell junction formation (Hilder et al., 2007).

Despite the identification of the defective genes for the three *CCM* loci, little is known about the molecular mechanisms underlying CCM lesion formation. Furthermore, not all individuals harboring defective *CCM* genes develop ICH, reflecting incomplete penetrance of these mutations and/or involvement of additional genetic modifiers predisposing to lesion formation (Lucas et al., 2003). It has been suggested that multiple genetic factors play important roles in predisposition to hemorrhagic stroke and influence the likelihood of ICH events in both familial and sporadic disorders. Recent evidence that *CCM* genes act together in common intracellular complexes and/or signaling pathways (Dupre et al., 2003; Hilder et al., 2007; Voss et al., 2007; Zawistowski et al., 2005) suggested to us that minor functional perturbations of different genes in this pathway might act together to precipitate ICH. However, demonstrating multigene association is not possible in the small number of available human hemorrhagic stroke pedigrees, nor has functional evidence substantiating this idea been readily available from other model organisms.

We sought to explore the possibility that very minor, otherwise silent deficits in genes that function together to regulate endothelial junctional integrity might synergistically initiate hemorrhagic stroke. We turned to the zebrafish, a useful model organism for analysis of human disease genes and their pathophysiology (Lieschke and Currie, 2007). Zebrafish embryos are readily amenable to simultaneous functional knockdown of multiple genes (Pham et al., 2007), making them very useful for functional analysis of multigene families or interacting proteins. In this study, we show that a combined minor reduction in the expression of multiple CCM pathway genes can precipitate hemorrhagic stroke. Our results provide a rationale for understanding the variability in appearance and onset of this disease.

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RESULTS

Rap1b function is required for vascular integrity

We began our analysis by studying *rap1b*, a gene implicated in the CCM pathway in endothelial cells (Cullere et al., 2005; Glading et al., 2007; Kehrer-Sawatzki et al., 2002; Laberge-le Couteulx et al., 1999) but not yet characterized in zebrafish. We obtained full-length clones for two *RAP1*-related zebrafish genes, *rap1a* and *rap1b*. The *rap1a* gene is expressed ubiquitously during early development (data not shown), but *rap1b* shows vascular-enriched expression by in situ hybridization (Fig. 1A). To knockdown *rap1b* function in zebrafish, we designed morpholino oligonucleotides (modified antisense oligonucleotides) (Nasevicius and Ekker, 2000), targeting either the ATG initiator codon or the second exon-intron boundary, to interfere with *rap1b* translation or splicing, respectively. Embryos injected with either one of these morpholinos appeared morphologically normal up to 4 days postfertilization (dpf) and were indistinguishable from control morpholino-injected embryos (data not shown and supplementary material Fig. S1A,B). However, by 48 hours postfertilization (hpf) more than 70% of embryos injected with either of the *rap1b* morpholinos (n=380) developed ICH (Fig. 1E,F). Most hemorrhage foci were localized in, and around, the hindbrain ventricle (Fig. 1E,F; supplementary material Fig. S1). There was also a left-side bias, with 62% (n=25/40) of hindbrain hemorrhages in *rap1b* morpholino-injected fish occurring on the left side. The efficacy of the *rap1b* splice morpholino was verified by reverse transcription (RT)-PCR (supplementary material Fig. S2). We also confirmed that *rap1b* morpholino-injected animals do not have defects in proper patterning of the nervous system (supplementary material Fig. S3A,B), endothelial specification, or arterial-venous differentiation (supplementary material Fig. S3C-J). The specificity of the observed phenotypic effects of *rap1b* knockdown was further verified by co-injection of synthetic human *RAP1B* mRNA with the zebrafish *rap1b* morpholino. Although most of the injected RNA would probably have been degraded by the time hemorrhage began to appear at 2 dpf, only 44% of the co-injected embryos (n=72) manifested cranial hemorrhages compared with 74% of the embryos injected with *rap1b* ATG morpholino alone (n=127) (Fig. 1G). These results indicate that the *rap1b* morpholinos target *rap1b* and specifically abrogate its function, and suggest that the human and zebrafish Rap1b proteins have a conserved function. To investigate whether Rap1b is specifically required in endothelial cells for maintenance of vascular integrity, we carried out a rescue experiment using the zebrafish *fli1* promoter (Villefranc et al., 2007) to drive endothelial expression of human Rap1b. When we co-injected a fli1:hRap1b DNA construct together with the zebrafish *rap1b* morpholino, cranial hemorrhage was reduced from 74% to only 30% of injected embryos, despite the mosaic expression of injected DNA (Fig. 1G). These results indicated that *rap1b* function is required in endothelial cells for vascular integrity.

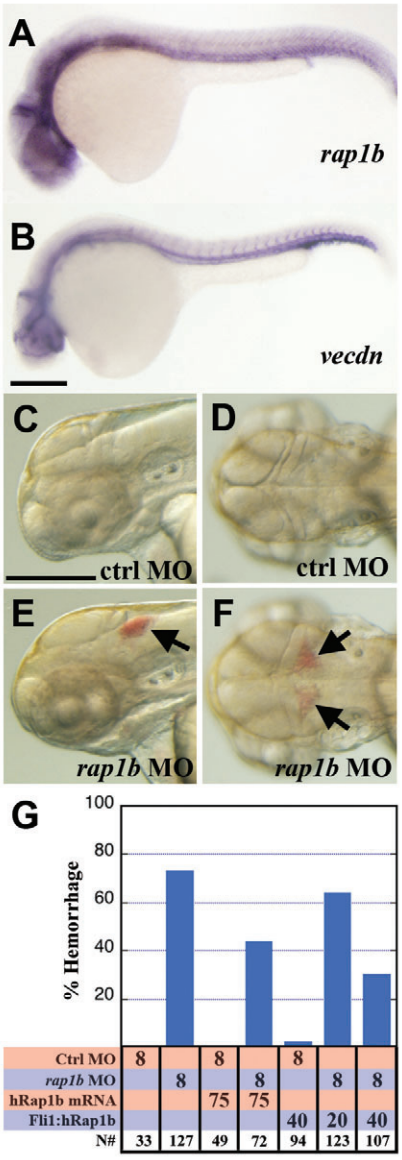

Fig. 1. Rap1b is required for vascular integrity in vivo. (A,B) Whole-mount in situ hybridization of 24 hpf zebrafish using probes for *rap1b* (A) and *vecdn* (VE-cadherin) (B). (C-F) Transmitted light images of the heads of animals injected with 8 nanograms of control (C,D) or *rap1b* (E,F) morpholino (MO). ICH in the *rap1b* MO-injected animals (black arrows) (E,F). (G) Quantitation of the percentage of animals developing ICH by 48 hpf after injection of: (1) control MO, (2) *rap1b* MO, (3) control MO + *hRap1b* mRNA, (4) *rap1b* MO + *hRap1b* mRNA, (5) control MO + fli1:hRap1b DNA, (6) *rap1b* MO + fli1:hRap1b DNA, (7) *rap1b* MO + fli1:hRap1b DNA. The amount of MO (in nanograms) and DNA and RNA constructs (in picograms) injected per animal, and the total number of embryos injected and scored (N #) are shown. Anterior is to the left, and dorsal is either above (A-C,E) or coming out of the plane of the page (D,F). Bars, 250 μM.

Rap1b function is required for proper endothelial cell-cell junction formation

We performed additional experiments to further explore the nature of the endothelial defects caused by Rap1b depletion. Ultrastructural examination of the cranial endothelium revealed normal well-formed endothelial junctions in control morpholino-injected animals (Fig. 2A). In contrast, most endothelial cell-cell junctions in *rap1b* morpholino-injected animals were either poorly formed or even absent, with open gaps observed between some endothelial cells (Fig. 2B-D), as also noted in human CCM patients (Clatterbuck et al., 2001). In order to further examine the role of Rap1 in endothelial junction formation, we used small interfering

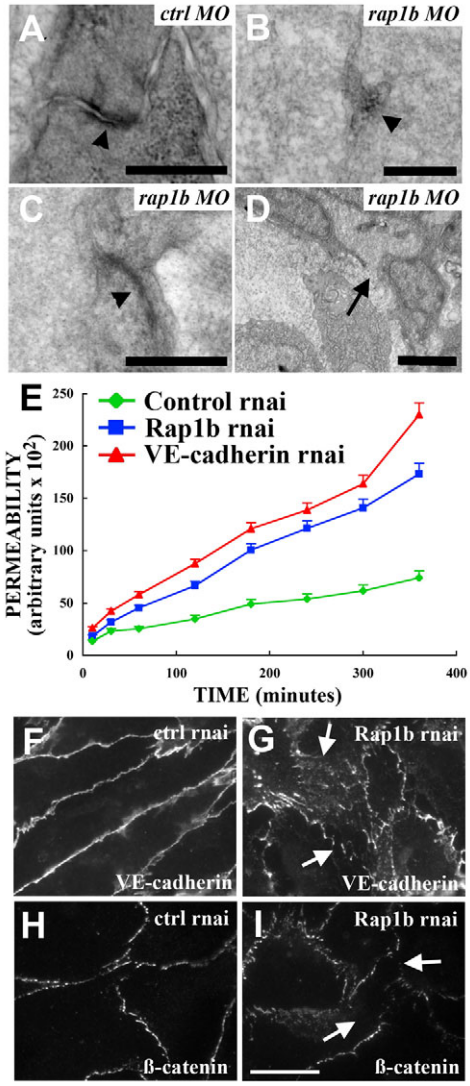

Fig. 2. Rap1b is required for proper endothelial junction formation in vivo and in vitro. (A-D) Transmission electron micrographs of endothelial cell-cell junctions in control (A) and *rap1b* morpholino-injected (B-D) animals. Normal junctions (arrowhead) between endothelial cells in control morphants (A). Poorly formed junctions (arrowheads) between endothelial cells in *rap1b* morphants (B,C). Gaps between endothelial cells (arrow) are also frequently observed in *rap1b* morphants (D). (E) Transwell permeability across a HUVEC endothelial monolayer. (F-I) Immunohistochemical staining of control (F,H) or Rap1b siRNA-treated (G,I) HUVEC monolayers, probed for VE-cadherin (F,G) or beta-catenin (H,I). Arrows in (G) and (I) indicate gaps and disruption of junctional organization of markers in Rap1b siRNA-treated monolayers. Bars, 500 nm (A-C), 2 µm (D) and 20 µM (F-I).

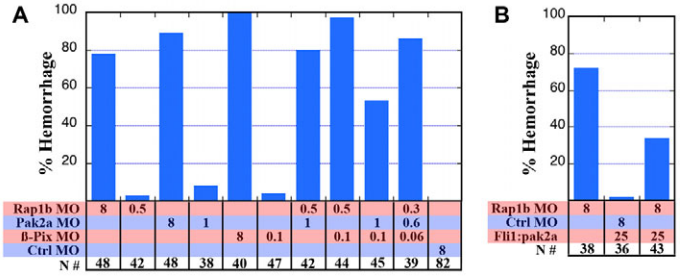

Fig. 3. Synergistic effects of defects in *rap1b* and downstream CCM pathway genes *pak2a* and *β*-pix. (A) Quantitation of the percentage of animals developing ICH by 48 hpf after injection of high or low doses of *rap1b*, *pak2a*, *β-pix* or control morpholinos (MO). (B) Quantitation of the percentage of animals developing ICH by 48 hpf following injection of *rap1b* MO, control MO + fli1:pak2a DNA or *rap1b* MO + fli1:pak2a DNA. The amount of MO and/or DNA construct injected per animal (in nanograms) and the number of embryos injected and scored (N #) are noted.

RNA (siRNA) to knockdown Rap1b in human umbilical vein endothelial cells (HUVECs) in vitro. We used Rap1b siRNA to reduce Rap1b and total Rap1 levels in HUVECs to 90% and 63% of normal, respectively (supplementary material Fig. S4A). Residual Rap1 failed to localize properly to junctions in Rap1b siRNA-treated HUVEC monolayers, similar to HUVECs treated with siRNA against vascular endothelial (VE)-cadherin or VE-cadherin null cells (supplementary material Fig. S4B-F). Transwell permeability tests showed increased permeability across Rap1b siRNA-treated endothelial monolayers, similar to endothelial monolayers treated with VE-cadherin siRNA (Fig. 2E). Furthermore, we observed loss or mislocalized staining of a variety of endothelial junction markers in Rap1b siRNA-treated monolayers, including the adherens junction markers VE-cadherin and β-catenin (Fig. 2F-I), and markers of proper tight junction formation (supplementary material Fig. S5A-F). These results indicate that Rap1 function is required for proper endothelial junction formation and maintenance both in vivo and in vitro.

CCM pathway molecules synergistically regulate vascular integrity

We took advantage of the ability to perform rapid combinatorial knockdown of gene function in the zebrafish (Pham et al., 2007) to examine the synergistic effects of combined partial loss-of-function for multiple members of the CCM/Rap1 signaling cascade. Previous reports have shown that cool1/β-pix, a guanine nucleotide exchange factor, and Pak2a, a downstream p21-activated kinase, control directed cell migration (Cau and Hall, 2005; Schwamborn and Puschel, 2004) and vascular permeability in zebrafish embryos (Buchner et al., 2007; Liu et al., 2007), and potentially function in a signaling pathway downstream from *ccm* gene products and Rap1b. Like the *rap1b* gene, *pak2a* is also preferentially expressed in the zebrafish vasculature, and mutants or morpholino-injected animals for either *pak2a* or *β-pix* display intracranial bleeding (Buchner et al., 2007; Liu et al., 2007) (supplementary material Fig. S6). We titrated doses of the respective morpholinos for each of these genes to levels that resulted in hemorrhage in less than 10% of injected animals, and then injected pairwise combinations of the morpholinos. At these very low doses of the splice-targeting morpholinos, we could not detect a significant reduction in correctly spliced mRNA levels by semi-quantitative RT-PCR (supplementary material Fig. S7). Nevertheless, injection of any two morpholinos resulted in strong synergistic effects, with dramatically elevated numbers of animals experiencing ICH (Fig. 3A). For example, co-injecting doses of *rap1b* and *pak2a* morpholinos, which, when given individually, caused hemorrhage in only 3% and 8% of animals, respectively,

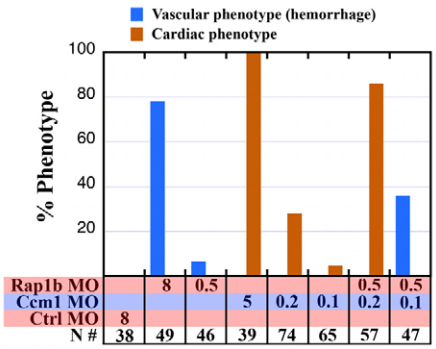

Fig. 4. Synergistic effects of defects in *rap1b* and upstream *CCM* genes. Quantitation of the percentage of animals developing enlarged hearts and loss of circulation (orange bars) or ICH (blue bars) by 48 hpf after injection of high or low doses of *rap1b*, *ccm1* or control morpholinos (MO). The amount of MO injected per animal (in nanograms) and the number of embryos injected and scored (N #) are noted.

resulted in 80% of animals developing ICH. To investigate whether functional interaction between *rap1b* and *pak2a* is specifically required in endothelial cells for maintenance of vascular integrity, we carried out another endothelial cell-specific rescue experiment. The zebrafish *fli1* promoter was used to drive mosaic endothelial expression of *pak2a*. When we co-injected this fli1:pak2a DNA construct together with the zebrafish *rap1b* morpholino, ICH was reduced from 75% with morpholino alone to only 35% (Fig. 3B). These results indicate that excess Pak2a in endothelium can complement the defect in *rap1b* morphants. Co-injection of morpholinos against all three genes (*rap1b*, *pak2a*, *β-pix*), at doses of two-thirds of the amount used for the double morpholino injections, resulted in 86% of animals developing ICH (Fig. 3A). These results indicate that very minor deficits in multiple members of the Rap1b/Pak2a/β-pix pathway lead to unexpectedly dramatic hemorrhagic outcomes.

To examine whether combined partial functional reduction in a *ccm* gene product and Rap1b could precipitate hemorrhagic stroke, we used morpholinos to partially knockdown zebrafish *ccm1* together with *rap1b*. Loss of CCM1 function in either mice (Whitehead et al., 2004) or zebrafish (Mably et al., 2006) has been previously shown to cause a defect in cardiac function, precluding analysis of potential vascular integrity defects. Co-injection of low doses of *ccm1* and *rap1b* morpholinos resulted in synergistic enhancement of the cardiac phenotype, similar to previously reported combined partial knockdown of *ccm1* and *ccm2* (Mably et al., 2006). Interestingly, however, co-injection of even lower doses of *ccm1* and *rap1b* morpholinos resulted in 36% of animals developing ICH, a phenotype not previously reported for knockdown of *ccm1* in zebrafish (Fig. 4). To rule out the possibility that the observed effects were because of genetic cross-regulation rather than functional synergy between gene products, we carried out semi-quantitative RT-PCR for *ccm1*, *pak2a*, *rap1b*, *β-pix* and *mapk1* on embryos injected with high doses of either *ccm1* or *rap1b* morpholinos. We did not detect any changes in transcript levels of any of the genes, other than *ccm1* or *rap1b* that were being directly targeted by their respective morpholinos (supplementary material Fig. S8).

DISCUSSION

Rap1b in vascular homeostasis

Formation of seamless endothelial tubes is crucial for the development and function of blood vessels, and requires proper assembly of endothelial cell-cell junctions. Defects in the maintenance of endothelial junctions in cranial vessels lead to ICH, with devastating consequences. The *RAP1* gene has been shown to be important for cadherin-mediated cell adhesion in other epithelial cell types, including Drosophila epithelial cells (Knox and Brown, 2002). Our findings demonstrate that Rap1b is essential for junctional integrity in endothelium. Depletion of Rap1b in cultured endothelial cells leads to defective endothelial cell-cell junctions and loss of barrier function. Rap1-depleted cultured endothelial monolayers display defective localization of markers of both adherens junctions and tight junctions, similar to defects in the distribution of adherence junctions that have been observed in Rap1-deficient Drosophila epithelial cells (Knox and Brown, 2002). Rap1b knockdown in zebrafish embryos leads to defective formation of endothelial junctions and ICH, with lesions appearing in similar anatomical locations to those commonly recognized for ICH lesions. Generally, in humans, stroke occurs more frequently on the left side of the brain than on the right side (Foerch et al., 2005), and we found that zebrafish embryos injected with *rap1b* morpholino also display slightly more hindbrain hemorrhages on the left side. Together, our results suggest that Rap1 has a conserved role in regulating cell-cell junction formation from invertebrates to higher vertebrates including humans.

Active Rap1 localizes to cell-cell contacts and physically interacts with Krit1/CCM1, and it has been proposed that Rap1 is important for recruiting Krit1 to junctions (Voss et al., 2007). In addition to showing that other junctional markers failed to localize properly, our results also demonstrate that residual Rap1 (from Rap1a) does not localize properly to cell-cell junctions in Rap1b siRNA-treated endothelial monolayers in culture, indicating that junctional targeting of Rap1 is dependent on its own function.

Polygenic regulation of CCM lesions

In addition to demonstrating that Rap1b is a key component in stabilizing cell-cell junctions in vivo, our results also confirm that it acts in a common molecular pathway with the *ccm* genes. In humans, hereditary CCM disorders are associated with mutations at the *CCM1*/*KRIT1* (Laberge et al., 1999), *CCM2*/*malcavernin* (Denier et al., 2004) and *CCM3*/*PDCD10* (Bergametti et al., 2005) loci. The familial CCM disorders all show dominant inheritance patterns with variable penetrance and expressivity, and virtually all *CCM* alleles are loss-of-function mutations (Felbor et al., 2006). However, the underlying basis for lesion formation in these disorders is still unclear. Haploinsufficiency has been documented in other human genetic vascular disorders including collagen 3A1 deficiency (Schwarze et al., 2001; Wenstrup et al., 2000), and it may be similarly important in CCM (Cave-Riant et al., 2002). One report documented a loss of heterozygosity by second mutation in the same *CCM* locus that was associated with a CCM lesion (Gault et al., 2005). However, it has been difficult to establish the factors that initiate most lesion formation in CCM and why the appearance of these lesions is so variable.

Second-site genetic mutations have also been suggested as a possible mechanism for initiating lesion formation in CCM (Gunel et al., 1996). Second-site genetic modifier(s) have been well documented for many other human genetic diseases including

Huntington's disease (Wexler et al., 2004) and sickle cell anemia (Sebastiani et al., 2005). In sickle cell anemia, 6-8% of affected individuals also show overt stroke, a phenotype caused by a genetic modifier from the TGF-β signaling pathway (Sebastiani et al., 2005). In the case of CCM, the proteins encoded by the three known disease loci have been shown to physically interact with one another (Voss et al., 2007; Zawistowski et al., 2005), and with additional proteins including Rap1 (Beraud-Dufour et al., 2007; Hilder et al., 2007; Serebriiskii et al., 1997). As noted above, Rap1 itself has been implicated in junctional localization of CCM1/KRIT1 (Voss et al., 2007). Based on these results it seemed possible that the phenotypic consequences of reduced levels of CCM proteins in human heterozygotes could be readily influenced by mutations in, or reduced levels of, some of these interacting proteins, and that these second-site defects could either trigger lesion formation or influence the penetrance, onset, or severity of the disease.

As a first test of this idea, we used the zebrafish to examine whether minimal deficits in some of these genes, which are individually phenotypically silent, could combine to yield defects in junction formation and hemorrhage. Highly conserved *ccm1* and *ccm2* orthologs are expressed in the zebrafish vasculature, and animals with homozygous null mutations in either of the genes (the *santa* and *valentine* mutants, respectively) develop enlarged non-functional hearts and lack blood circulation, as do animals with full-dose knockdowns in either gene (Mably et al., 2006), precluding analysis of potential vascular integrity defects. Similar cardiac defects have been reported in mice with homozygous targeted deletion of *Ccm1*/*Krit1* (Whitehead et al., 2004). Combined partial knockdown of *ccm1* and *ccm2* by morpholinos also 'phenocopies' the cardiac phenotypes (Mably et al., 2006). However, our results show that very low-level depletion of *ccm1* and *rap1b* synergistically enhances the cranial hemorrhage phenotype seen in *rap1b* morphants. Strongly synergistic hemorrhage defects are also seen when zebrafish embryos are injected with combinations of very low doses of morpholinos against other genes implicated in the CCM pathway, including *rap1*, *ccm1*, *β-pix* and *pak2a*.

It remains to be seen whether similar second-site defects in interacting genes influence lesion formation, or disease manifestation, in human patients heterozygous for *CCM* mutations. This is likely to be difficult to determine given the small patient population sizes and large number of potential interacting genes. Furthermore, the extraordinary level of synergy that we have documented in our zebrafish studies suggests that maintenance of junctional integrity is extremely sensitive to even minimal combined functional disruption. This may make it difficult to determine whether a second site polymorphism detected in human CCM patients is only a harmless polymorphism, or a significant genetic modifier of the disease. However, evidence that these genes interact closely, both physically and functionally, in junction formation and vascular integrity leads to the hope that approaches designed to modulate the downstream activity or function of the CCM pathway might have positive benefits for a large number of affected individuals.

METHODS

Zebrafish lines and husbandry

Zebrafish lines used in the study are wild-type EK, *Tg*(*fli1:EGFP*)^{y1} and *Tg*(*flk:GFP*); *Tg*(*gata:DsRed*). All lines were maintained as previously described (Westerfield, 2000).

Cloning, expression and immunocytochemistry

Zebrafish *rap1a* and *rap1b* orthologs were identified based on identity searches to human genes. A *rap1a* cDNA fragment was amplified using primers rap1aF: AAAAACACACGCCTTCCCGCCTTG and rap1aR: TGGAGTAAACCAGAGCAAAACCC, and cloned into pTOPO (Clontech). The zebrafish *rap1b* in situ clone was obtained from ZIRC (cb1026). In situ hybridization was carried out as previously described (Pham et al., 2007). A complete cDNA clone for human Rap1b was obtained from Open Biosystems and used for synthetic mRNA synthesis and for generation of an endothelial specific pTolfli1ep:human Rap1b construct (Villefranc et al., 2007). To detect axonal projections (pan-neuronal), a monoclonal antibody generated against acetylated tubulin (Sigma T6793) was used.

Microinjection

Morpholinos were injected at described doses into 1-2 cell stage zebrafish embryos. Morpholinos used in this analysis are listed below:
rap1b translation start site (start site underlined) MO 5′ GGACTACTAACTTGTATTCACGCAT 3′
rap1b exon 2 splice donor site MO 5′ AAATGATGCAGAACTTGCCTTTCTG 3′
rap1b mismatch (mismatches underlined) MO 5′ GGAGTAGTAACTTCTATTGACGGAT 3′
pak2a exon 8 splice donor site MO 5′ AATAGAGTACAACATACCTCTTTGG 3′
β-pix exon 6 splice donor site MO 5′ GCGCATCTCTCTTACCACATTATAG 3′
ccm1 exon 1 splice donor site MO 5′GCTTTATTTCACCTCACCTCATAGG 3′

PCR analysis

Total cellular RNA from morpholino-injected embryos was extracted using Trizol reagent and treated with DNAase I. cDNA was synthesized using the ThermoScript kit (Invitrogen). Semi-quantitative PCR was performed using cDNA generated from equal amounts of RNA from different samples. The linear range of amplification cycles was determined for each target gene. Target and reference (β-actin) genes were amplified to the same cycle number for each sample. PCR primers used to analyze zebrafish genes are listed in the supplementary material.

Transmission electron microscopy

Zebrafish embryos were fixed at room temperature in 2.5% glutaraldehyde (pH 7.3) made in 0.13 M sodium cacodylate buffer. Embryos were embedded in Epoxy resin and semi-thin 1 µm plastic sections were cut and stained with Toluidine Blue O stain. Thin sections were prepared on a Reichert-Jung Ultracut-E ultramicrotome. The grids were post-stained with uranyl acetate and lead citrate.

Cell culture and RNA interference

Human endothelial cells from umbilical cord vein (HUVEC) were seeded on gelatin-coated tissue culture vessels. They were cultured for 24 hours in MCDB 131 with 20% FCS, ECGS and heparin (as described in Lampugnani et al., 2006) and were transfected with siRNA (supplementary material).

TRANSLATIONAL IMPACT

Clinical issue

Stroke is the third leading cause of death worldwide and the most common cause of disability in developed nations. Intracranial hemorrhage (ICH) accounts for 10 percent of stroke and is a particularly severe form of the disease, associated with high rates of death and long-term disability. A substantial number of individuals have inherited ICH disorders caused by mutations in one of three *CCM* (cerebral cavernous malformation) genes. The incidence of stroke is highly variable within affected families, and the factors that trigger ICH in either inherited or sporadic forms of the disease are unknown. Limited understanding of the genetic contributors to this disease has hampered treatment and there is currently no effective prophylactic drug therapy for ICH.

Results

This study is the first definitive demonstration that ICH can be triggered by a combination of very minor defects in CCM proteins or in their effectors. By injecting specialized antisense oligonucleotides into zebrafish, the authors simultaneously inhibited the expression of multiple CCM pathway genes in various combinations. They show that a subtle decrease in each of these genes alone caused little or no effect independently, but when combined resulted in very high frequencies of ICH.

Implications and future directions

These findings have important implications for the diagnosis and treatment of hemorrhagic stroke. Single mutations in CCM proteins may not be enough to induce stroke, but accompanying subtle secondary mutations may have significant consequences. In addition, mutations to effectors in the CCM pathway may initiate lesions, contributing to the highly variable penetrance of familial CCM disorders. In humans, subtle genetic second hits in individuals that are outwardly normal, but genetically 'sensitized' by minor deficits in CCM pathway genes, could lead to sporadic forms of hemorrhagic stroke. Besides the potential for improved diagnostics for stroke predisposition, these results also suggest that downstream manipulation of CCM signaling could be useful for future development of effective pharmacologic therapies for treatment and prevention of ICH.

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Antibodies

Antibodies used were: Rap1 for immunofluorescence and western blot (rabbit polyclonal, code sc-65, Santa Cruz); Rap1b for western blot (rabbit, code 36E1, Cell Signaling); VE-cadherin for immunofluorescence and western blot (goat polyclonal, code sc-6458, Santa Cruz); KRIT1 for immunofluorescence and western blot (goat polyclonal, code sc-23997, Santa Cruz); beta-catenin (mouse monoclonal, clone 14, BD transduction laboratories); claudin 5 (rabbit polyclonal, kindly donated by Dr H. Wolburg, Institute of Pathology, University of Tubingen, Tubingen, Germany); cingulin (rabbit polyclonal, code 36-4401, Zymed); ZO-1 (rabbit polyclonal, code 61-7300, Zymed); vinculin (mouse monoclonal, clone h-VIN1, Sigma); and tubulin for western blot (mouse monoclonal, clone B-5-1-2, Sigma).

Immunocytochemistry, transwell permeability assays and western blots were carried out as previously described (Lampugnani et al., 2006). Details of the protocols can be found in the supplementary material.

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COMPETING INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

A.V.G., M.G.L., E.D. and B.M.W. conceived and designed the experiments; A.V.G., M.G.L. and L.D. performed the experiments; A.V.G., M.G.L., E.D. and B.M.W. analyzed the data; A.V.G., M.G.L., E.D. and B.M.W. wrote the paper.

SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at http://dmm.biologists.org/content/1/4-5/275/suppl/DC1

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REFERENCES

Asha, H., de Ruiter, N. D., Wang, M. G. and Hariharan, I. K. (1999). The Rap1 GTPase functions as a regulator of morphogenesis *in vivo*. *EMBO J.* **18**, 605-615.

Beraud-Dufour, S., Gautier, R., Albiges-Rizo, C., Chardin, P. and Faurobert, E. (2007). Krit 1 interactions with microtubules and membranes are regulated by Rap1 and integrin cytoplasmic domain associated protein-1. *FEBS J.* **274**, 5518-5532.

Bergametti, F., Denier, C., Labauge, P., Arnoult, M., Boetto, S., Clanet, M., Coubes, P., Echenne, B., Ibrahim, R., Irthum, B. et al. (2005). Mutations within the programmed cell death 10 gene cause cerebral cavernous malformations. *Am. J. Hum. Genet.* **76**, 42-51.

Buchner, D. A., Su, F., Yamaoka, J. S., Kamei, M., Shavit, J. A., Barthel, L. K., McGee, B., Amigo, J. D., Kim, S., Hanosh, A. W. et al. (2007). pak2a mutations cause cerebral hemorrhage in redhead zebrafish. *Proc. Natl. Acad. Sci. USA* **104**, 13996-14001.

Cau, J. and Hall, A. (2005). Cdc42 controls the polarity of the actin and microtubule cytoskeletons through two distinct signal transduction pathways. *J. Cell Sci.* **118**, 2579-2587.

Cave-Riant, F., Denier, C., Labauge, P., Cecillon, M., Maciazek, J., Joutel, A., Laberge-Le Couteulx, S. and Tournier-Lasserve, E. (2002). Spectrum and expression analysis of KRIT1 mutations in 121 consecutive and unrelated patients with Cerebral Cavernous Malformations. *Eur. J. Hum. Genet.* **10**, 733-740.

Clatterbuck, R. E., Eberhart, C. G., Crain, B. J. and Rigamonti, D. (2001). Ultrastructural and immunocytochemical evidence that an incompetent blood-brain barrier is related to the pathophysiology of cavernous malformations. *J. Neurol. Neurosurg. Psychiatr.* **71**, 188-192.

Craig, H. D., Gunel, M., Cepeda, O., Johnson, E. W., Ptacek, L., Steinberg, G. K., Ogilvy, C. S., Berg, M. J., Crawford, S. C., Scott, R. M. et al. (1998). Multilocus linkage identifies two new loci for a mendelian form of stroke, cerebral cavernous malformation, at 7p15-13 and 3q25.2-27. *Hum. Mol. Genet.* **7**, 1851-1858.

Cullere, X., Shaw, S. K., Andersson, L., Hirahashi, J., Luscinskas, F. W. and Mayadas, T. N. (2005). Regulation of vascular endothelial barrier function by Epac, a cAMP-activated exchange factor for Rap GTPase. *Blood* **105**, 1950-1955.

Denier, C., Goutagny, S., Labauge, P., Krivosic, V., Arnoult, M., Cousin, A., Benabid, A. L., Comoy, J., Frerebeau, P., Gilbert, B. et al. (2004). Mutations within the MGC4607 gene cause cerebral cavernous malformations. *Am. J. Hum. Genet.* **74**, 326-337.

Dupre, N., Verlaan, D. J., Hand, C. K., Laurent, S. B., Turecki, G., Davenport, W. J., Acciarri, N., Dichgans, J., Ohkuma, A., Siegel, A. M. et al. (2003). Linkage to the CCM2 locus and genetic heterogeneity in familial cerebral cavernous malformation. *Can. J. Neurol. Sci.* **30**, 122-128.

Felbor, U., Sure, U., Grimm, T. and Bertalanffy, H. (2006). Genetics of cerebral cavernous angioma. *Zentralbl. Neurochir.* **67**, 110-116.

Foerch, C., Misselwitz, B., Sitzer, M., Berger, K., Steinmetz, H. and Neumann-Haefelin, T. (2005). Difference in recognition of right and left hemispheric stroke. *Lancet* **366**, 392-393.

Frizzell, J. P. (2005). Acute stroke: pathophysiology, diagnosis, and treatment. *AACN Clin. Issues* **16**, 421-440; quiz 597-598.

Gault, J., Shenkar, R., Recksiek, P. and Awad, I. A. (2005). Biallelic somatic and germ line CCM1 truncating mutations in a cerebral cavernous malformation lesion. *Stroke* **36**, 872-874.

Glading, A., Han, J., Stockton, R. A. and Ginsberg, M. H. (2007). KRIT-1/CCM1 is a Rap1 effector that regulates endothelial cell cell junctions. *J. Cell Biol.* **179**, 247-254.

Gunel, M., Awad, I. A., Finberg, K., Steinberg, G. K., Craig, H. D., Cepeda, O., Nelson-Williams, C. and Lifton, R. P. (1996). Genetic heterogeneity of inherited cerebral cavernous malformation. *Neurosurgery* **38**, 1265-1271.

Hilder, T. L., Malone, M. H., Bencharit, S., Colicelli, J., Haystead, T. A., Johnson, G. L. and Wu, C. C. (2007). Proteomic identification of the cerebral cavernous malformation signaling complex. *J. Proteome Res.* **6**, 4343-4355.

Kehrer-Sawatzki, H., Wilda, M., Braun, V. M., Richter, H. P. and Hameister, H. (2002). Mutation and expression analysis of the KRIT1 gene associated with cerebral cavernous malformations (CCM1). *Acta Neuropathol.* **104**, 231-240.

Knox, A. L. and Brown, N. H. (2002). Rap1 GTPase regulation of adherens junction positioning and cell adhesion. *Science* **295**, 1285-1288.

Laberge, S., Labauge, P., Marechal, E., Maciazek, J. and Tournier-Lasserve, E. (1999). Genetic heterogeneity and absence of founder effect in a series of 36 French cerebral cavernous angiomas families. *Eur. J. Hum. Genet.* **7**, 499-504.

Laberge-le Couteulx, S., Jung, H. H., Labauge, P., Houtteville, J. P., Lescoat, C., Cecillon, M., Marechal, E., Joutel, A., Bach, J. F. and Tournier-Lasserve, E. (1999). Truncating mutations in CCM1, encoding KRIT1, cause hereditary cavernous angiomas. *Nat. Genet.* **23**, 189-193.

Lampugnani, M. G., Orsenigo, F., Gagliani, M. C., Tacchetti, C. and Dejana, E. (2006). Vascular endothelial cadherin controls VEGFR-2 internalization and signaling from intracellular compartments. *J. Cell Biol.* **174**, 593-604.

Lieschke, G. J. and Currie, P. D. (2007). Animal models of human disease: zebrafish swim into view. *Nat. Rev. Genet.* **8**, 353-367.

Liu, J., Fraser, S. D., Faloon, P. W., Rollins, E. L., Vom Berg, J., Starovic-Subota, O., Laliberte, A. L., Chen, J. N., Serluca, F. C. and Childs, S. J. (2007). A betaPix Pak2a signaling pathway regulates cerebral vascular stability in zebrafish. *Proc. Natl. Acad. Sci. USA* **104**, 13990-13995.

Lucas, M., Costa, A. F., Garcia-Moreno, J. M., Solano, F., Gamero, M. A. and Izquierdo, G. (2003). Variable expression of cerebral cavernous malformations in carriers of a premature termination codon in exon 17 of the Krit1 gene. *BMC Neurol.* **3**, 5.

Mably, J. D., Chuang, L. P., Serluca, F. C., Mohideen, M. A., Chen, J. N. and Fishman, M. C. (2006). santa and valentine pattern concentric growth of cardiac myocardium in the zebrafish. *Development* **133**, 3139-3146.

Nasevicius, A. and Ekker, S. C. (2000). Effective targeted gene 'knockdown' in zebrafish. *Nat. Genet.* **26**, 216-220.

Pham, V. N., Lawson, N. D., Mugford, J. W., Dye, L., Castranova, D., Lo, B. and Weinstein, B. M. (2007). Combinatorial function of ETS transcription factors in the developing vasculature. *Dev. Biol.* **303**, 772-783.

Schwamborn, J. C. and Puschel, A. W. (2004). The sequential activity of the GTPases Rap1B and Cdc42 determines neuronal polarity. *Nat. Neurosci.* **7**, 923-929.

Schwarze, U., Schievink, W. I., Petty, E., Jaff, M. R., Babovic-Vuksanovic, D., Cherry, K. J., Pepin, M. and Byers, P. H. (2001). Haploinsufficiency for one COL3A1 allele of type III procollagen results in a phenotype similar to the vascular form of Ehlers-Danlos syndrome, Ehlers-Danlos syndrome type IV. *Am. J. Hum. Genet.* **69**, 989-1001.

Sebastiani, P., Ramoni, M. F., Nolan, V., Baldwin, C. T. and Steinberg, M. H. (2005). Genetic dissection and prognostic modeling of overt stroke in sickle cell anemia. *Nat. Genet.* **37**, 435-440.

Serebriiskii, I., Estojak, J., Sonoda, G., Testa, J. R. and Golemis, E. A. (1997). Association of Krev-1/rap1a with Krit1, a novel ankyrin repeat-containing protein encoded by a gene mapping to 7q21-22. *Oncogene* **15**, 1043-1049.

Villefranc, J. A., Amigo, J. and Lawson, N. D. (2007). Gateway compatible vectors for analysis of gene function in the zebrafish. *Dev. Dyn.* **236**, 3077-3087.

Voss, K., Stahl, S., Schleider, E., Ullrich, S., Nickel, J., Mueller, T. D. and Felbor, U. (2007). CCM3 interacts with CCM2 indicating common pathogenesis for cerebral cavernous malformations. *Neurogenetics* **8**, 249-256.

Wenstrup, R. J., Florer, J. B., Willing, M. C., Giunta, C., Steinmann, B., Young, F., Susic, M. and Cole, W. G. (2000). COL5A1 haploinsufficiency is a common molecular mechanism underlying the classical form of EDS. *Am. J. Hum. Genet.* **66**, 1766-1776.

Westerfield, M. (2000). *The Zebrafish Book: A Guide for Laboratory Use of Zebrafish (Danio rerio)*. Eugene: University of Oregon Press.

Wexler, N. S., Lorimer, J., Porter, J., Gomez, F., Moskowitz, C., Shackell, E., Marder, K., Penchaszadeh, G., Roberts, S. A., Gayan, J. et al. (2004). Venezuelan kindreds reveal that genetic and environmental factors modulate Huntington's disease age of onset. *Proc. Natl. Acad. Sci. USA* **101**, 3498-3503.

Whitehead, K. J., Plummer, N. W., Adams, J. A., Marchuk, D. A. and Li, D. Y. (2004). Ccm1 is required for arterial morphogenesis: implications for the etiology of human cavernous malformations. *Development* **131**, 1437-1448.

Zawistowski, J. S., Stalheim, L., Uhlik, M. T., Abell, A. N., Ancrile, B. B., Johnson, G. L. and Marchuk, D. A. (2005). CCM1 and CCM2 protein interactions in cell signaling: implications for cerebral cavernous malformations pathogenesis. *Hum. Mol. Genet.* **14**, 2521-2531.