Variants of Rab GTPase–Effector Binding Protein-2 Cause Variation in the Collateral Circulation and Severity of Stroke

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Background and Purpose—The extent (number and diameter) of collateral vessels varies widely and is a major determinant, along with arteriogenesis (collateral remodeling), of variation in severity of tissue injury after large artery occlusion. Differences in genetic background underlie the majority of the variation in collateral extent in mice, through alterations in collaterogenesis (embryonic collateral formation). In brain and other tissues, ≈80% of the variation in collateral extent among different mouse strains has been linked to a region on chromosome 7. We recently used congenic (CNG) fine mapping of C57BL/6 (B6, high extent) and BALB/cByJ (BC, low extent) mice to narrow the region to a 737 Kb locus, Dce1. Herein, we report the causal gene.

Methods—We used additional CNG mapping and knockout mice to narrow the number of candidate genes. Subsequent inspection identified a nonsynonymous single nucleotide polymorphism between B6 and BC within Rabep2 (rs33080487). We then created B6 mice with the BC single nucleotide polymorphism at this locus plus 3 other lines for predicted alteration or knockout of Rabep2 using gene editing.

Results—The single amino acid change caused by rs33080487 accounted for the difference in collateral extent and infarct volume between B6 and BC mice attributable to Dce1. Mechanistically, variants of Rabep2 altered collaterogenesis during embryogenesis but had no effect on angiogenesis examined in vivo and in vitro. Rabep2 deficiency altered endosome trafficking known to be involved in VEGF-A→VEGFR2 signaling required for collaterogenesis.

Conclusions—Naturally occurring variants of Rabep2 are major determinants of variation in collateral extent and stroke severity in mice. (Stroke. 2016;47:3022-3031. DOI: 10.1161/STROKEAHA.116.014160.)

Key Words: amino acids ■ collateral circulation ■ endosomes ■ genetic background ■ stroke
increased cerebral blood flow to the area at risk and reduced infarct volume each by 85%.9

The Dce1 locus contains 28 protein-coding genes.2 This study sought to identify the causative gene(s). We first verified that genetic background at Dce1 determines pial collateral extent and infarct volume by deriving an additional congenic line. This was followed by phenotyping collateral extent in mutant mice deficient at 13 of the above 28 genes, allowing their provisional elimination as candidates genes. Among the remaining 15 genes, we previously identified Rabep2 (Rabaptin-5β, FRA) as the highest priority candidate.9 This was in part because it contains a SNP (rs33080487) that substitutes an adenine for a guanine in BC and almost all the other mouse strains with low collateral extent examined, resulting in an arginine-to-glutamine (R298Q) substitution predicted to be deleterious.9 We therefore hypothesized that variants of Rabep2 underlie the Dce1 locus and used gene editing to create B6 mice with either the BC SNP at rs33080487 or deletion of Rabep2 altogether. The results identify Rabep2 as the causative allele at Dce1.

Rabep2 is a novel gene. Our results show that variants of it alter collaterogenesis in the embryo and thus collateral extent and infarct volume in the adult. It is possible that the same or a related gene(s) may contribute to the wide collateral variation in humans, opening the way for the development of a biomarker for collateral extent, as well as a deeper understanding of why some patients with ischemic disease do better than others.

Methods

Mutant B6 mice were generated using CRISPR/Cas9 methodology: a guanine-to-adenine substitution (SNP), a 3-nucleotide deletion (Del3), a 1-guanine insertion (InsG), or a 1-guanine deletion (DelG) at rs33080487 (7:126440209) in Rabep2. Pial collaterals between the MCA and ACA trees were quantified in 3- to 4-month-old adults after maximal dilation and in embryos by immunohistochemistry/fluorescence. Infarct volume was determined 24 hours after pMCAO. Endosomes were measured with immunohistochemistry in embryonic pia. Neonatal retina, tumor, and aortic ring angiogenic assays were standard, as were quantitative polymerase chain reaction, immunoblot, immunoprecipitation, mass spectrometry, and genotyping. This study conformed to the recommendations of STAIR (2012; in the online-only Data Supplement). Data are mean±SEM with significance (P<0.05) determined by ANOVA, Student, and Bonferroni t tests. Methods are available in the online-only Data Supplement.

Results

A Refined Interval Containing Dce1 Narrows the Candidate Genes Underlying Collateral Variation

During backcrossing CNG5 to BC mice, we obtained a recombination that deleted 550 kb from CNG5 such that the introgressed region is 162 kb longer than the originally defined Dce1 (Figure 1 in the online-only Data Supplement). This CNG7B6 line has on average 13.0 pial collaterals (MCA–ACA) per individual, compared with 0.7 in CNG7BC littermates and 19 to 20 in B6 (Figure 1). Thus, the B6 allele of Dce1, when introgressed into BC, rescues 12 to 13 collaterals or ≈65% of the BC deficit in collateral number. Similarly, pial collaterals in CNG7B6 are almost 2-fold larger in diameter and infarct volumes after pMCAO than in CNG7BC littermates (Figure 1). These values are similar to those reported previously for lines CNG3, CNG5, and CNG6 and their CNGBC littermates (Figure 1).9 These data confirm our previous conclusion9 that a genetic element(s) in Dce1 is responsible for ≈80% of the variation in collateral extent and infarct volume between B6 and BC mice. However, it should be pointed out that in mice, multiple branching of the MCA can also be associated with small infarction volumes in suture models.

We did not examine infarct volume in the CNG6 line previously characterized9 and since extinguished. This prevented us from addressing a recent report that a variant lying 3′ to Dce1 does not affect collateral extent but is protective against infarct volume when of B6 genotype.10 We were unable to confirm this conclusion: infarct volume did not decrease but instead trended greater between CNG7B6 that lacks, and CNG5B6 and CNG3B6 that possess, B6 genome 3′ to Dce1 that includes the reported protective allele (Figure 1). One explanation is that the distal locus requires the presence of additional genetic material not present in our congenic lines.

To narrow the 28 protein-coding gene candidates in Dce1,9 we phenotyped collateral extent in available knockout mice. Collateral extent was unaffected in mice deficient in Nfatc2ip, Mapk3, Nupr1, or a 390 Kb region encompassing Slx1–Sept1b (Figures II and III in the online-only Data Supplement). These findings when combined with the absence of effect in Cln3+/− mice9 narrow the candidates to 13.

Variants of Rabep2 Confer Variation in Collateral Extent

Among the 28 genes in Dce1, a nonsynonymous coding SNP (rs33080487, 7:126440209 [GRCm38], c.1258G>A in BC) causes the alteration R298Q in the Rabex5-binding domain11 of Rabep2 (NP_085043.2), an alteration predicted to be deleterious by PolyPhen-2 (score=0.999). We thus edited this polymorphism into B6 mice (SNP line). Presumptive nonhomologous end-joining gave 3 additional lines (Table I and Figure IV in the online-only Data Supplement). Two lines, DelG and InsG, have a deletion and insertion, respectively, of one nucleotide leading to frameshift, stop codons, and predicted nonsense mediated decay. In the third line, Del3, the codon for R298 was deleted, leading to expression of full-length Rabep2 minus R298. Quantitative polymerase chain reaction of DelG confirmed that Rabep2 message was reduced to 15% of wild type (WT), with the residual presumably reflecting message that had not yet undergone nonsense mediated decay.

Rabep2 expression was assessed (Figure 2A). Whole brain and neocortex from WT and DelG mice probed with 2 specific rabbit sera (gifts of Dr M. Zerial) showed Rabep2 to be undetectable in DelG (Figure 2A, top). A commercial antibody (Proteintech 14625-AP) revealed several closely spaced bands in WT, BC, SNP, and Del3, although the lower band in DelG and InsG was absent. A pulldown from WT with this antibody yielded the lower band. Mass spectrometry (matrix-assisted laser desorption/ionization and LTQ-Velos-Orbitrap) confirmed that the product was Rabep2. We were unable to pull down enough of the upper 2 bands to get a definitive identification. However, mass spectrometry analysis showed that they are not Rabep2. Hence as predicted, both DelG and InsG mice are knockouts, although expression of Rabep2 in SNP and Del3 seems to be unaffected. Rabep1 levels by
quantitative polymerase chain reaction and immunoblot are not altered in the edited mice (Figure V A and VB in the online-only Data Supplement). Each line reproduced at Mendelian ratios (Table II in the online-only Data Supplement) and had normal growth. Founders were outcrossed to additional B6 for 2 to 5 generations, all WTs were littermates, and their values of collateral number and diameter were similar to our previous WT-B6,6–9 indicating no off-target effects.

Collateral number was reduced to 8.0 collaterals in homozygous DelG and InsG mice, a deficit of 11 to 12 collaterals compared with WT littermates and WT-B6 (Figure 2C). Thus, the reduction in collateral number because of the elimination of Rabep2 from B6 is virtually identical to the increase in collateral number caused by introduction of the B6 allele of Dce1 into BC, suggesting that variants of Rabep2 alone account for the entire effect of Dce1. Similarly, collateral diameter was reduced by 41% and 47% in DelG and InsG knockouts, representing 85% and 97% of the difference in diameter attributable to Dce1 (Figure 2B). This stronger effect on diameter than on number was previously noted for Dce1,9 where expression of the B6 allele in BC essentially restored diameter to the WT-B6 value.

Alterations at position 298 with retention of Rabep2 expression gave intermediate reductions in collateral number to 13.0 in Del3 (delR298) and 12.3 in SNP (R298Q). Thus, Rabep2 activity in these forms seems to be diminished, and variation at position 298 has less effect on the B6 background (reduction of 6–7 collaterals for R298Q) than on the BC background (rescue of 12–13 collaterals by R298Q). Diameter data yield similar conclusions (Figure 2B), although the difference between alteration and elimination of Rabep2 is less striking (Figure 2B). As expected, these changes in collateral extent have functional effects because infarct volumes after pMCAO were increased 2- to 3-fold in DelG and SNP mice (Figure 2D). Heterozygous mice had intermediate phenotypes (Figure VI in the online-only Data Supplement).

Rabep2 Favors Greater Collateral Extent and Smaller Infarcts in B6 Females

Infarct volume was 35% smaller in female B6-WT; this difference was abolished in Rabep2−/− mice in association with a trend toward fewer collaterals of smaller diameter (Table III in the online-only Data Supplement). Thus, on the B6 background, the B6 allele of Rabep2 favors greater collateral

Figure 1. The high collateral allele of the congenic Dce1 locus rescues low collateral number and diameter and large infarct volume. Collateral number in this and subsequent figures is the number of arteriole-to-arteriole anastomoses cross-connecting outer branches of the middle cerebral artery and anterior cerebral artery trees. Introgression of the congenic (CNG) Dce1 locus-7 of C57BL/6 (B6) mice into BALB/cByJ (BC) mice (CNG7BC, Figure I in the online-only Data Supplemental for genomic intervals of the 4 CNG lines shown) rescues 67% of the difference in number and 69% in average diameter of middle cerebral artery-to-anterior cerebral artery collaterals and 93% in infarct volume between B6 and CNG7BC (essentially BC wild type [WT]) mice. Lower right. Representative images of TTC staining 24 h after permanent middle cerebral artery occlusion [MCAO] and collaterals (stars). Historic values1 for B6, BC and CNG3, 5 and 6 provided for comparison. For this and subsequent figures, congenic BC lines are controls for congenic derivation, thus essentially wild-type BC; number of animals given in columns; *P<0.05, **P<0.01, ***P<0.001. nsd indicates no significant difference; and TTC, 2,3,5-triphenyltetrazolium chloride.
extent and smaller infarcts in females. On BC background Nfatc2ip−/− mice (discussed below), the B6 allele of Rabep2 does not confer protection against infarct volume.

Variants of Rabep2 Alter Collaterogenesis

Pial collateral extent in healthy adults is dependent on collateral formation in the embryo (ie, collaterogenesis).7 Consistent with this, collaterogenesis was inhibited at both E14.5 and E16.5 in Rabep2−/− mice, indicating that Rabep2 is required for robust collateral formation (Figure 3A). Likewise, CNG5B6 embryos formed more collaterals than CNG5BC, confirming that the B6 variant (of presumably Rabep2) increases collaterogenesis when on the BC background (Figure 3B). Consistent with diameter being larger and branch density lower for pial capillary plexus vessels of BC versus B6 embryonic neocortex,7 diameter was greater and branch number lower in Rabep2−/− (Figure 3C), indicating that Rabep2 also impacts vascular patterning. Previous studies have observed these same differences in the early embryonic capillary plexus of BC versus B6 and Vegfa and Flk1 mutants and shown that the accompanying differences in collaterogenesis are not secondary to the differences in the plexus, as reported in Lucitti et al7 and references therein.

Consistent with developmental and adult angiogenesis (sprouting of new capillary vessels from existing capillaries) being comparable in B6 and BC mice,7 angiogenesis in the newborn retina was similar in CNG5B6 and CNG5BC mice (Figure 4A). Likewise, orthotopic tumor growth and blood vessel infiltration (Figure 4B and 4C) and sprouting in aortic rings (Figure VII in the online-only Data Supplement) were not different in CNG mice. Thus, Rabep2 is critical in the pathway that drives collaterogenesis but is not required for developmental or tumor angiogenesis.

Rabep2 Deficiency Alters Endosome Trafficking

Little is known about the function of Rabep2 other than it binds activated Rabex5, Rab5, and Rab4 proteins during early endosome (EE) formation in cultured cells.12 Endosomes traffic receptors such as VEGFR2 to sites involved in downstream signaling, lysosomes, or back to the cell membrane.13–15 We thus examined EEs in pial membrane of Rabep2−/− mice at E14.5 when collaterogenesis is underway. Endosome diameter was larger in Rabep2−/−, although density was similar (Figure 5A through 5C). The requirement to examine EEs in situ precluded assessment of colocalization of VEGFR2 with EEA1. However, Vegfa expression, Vegfr2 expression, and Vegfr1 expression were similar between groups (Figure 5D), suggesting that aberrant levels of these proteins do not underlie the reduced collaterogenesis in Rabep2−/− mice.

Deficiency of Rabep2 had no effect on Rabex5 and Rab4 that Rabep2 is known to bind, nor on EEA1 or Rab7 which are present in late endosomes destined for recycling to the cell membrane (Figure 6A). These proteins are in the VEGFA→VEGFR2→endosome→VEGFR2 recycling and
signaling pathways (Discussion). These findings, which are consistent with lack of effect on expression of VEGFA, VEGFR2, and VEGFR1 (Figure 5D), strengthen the conclusion from Figure 5A through 5C that Rabep2 deficiency impairs endosome trafficking.

Capillary and microvessel densities in adult brain were not affected by Rabep2 deficiency (Figure 6B; Figure VIII in the online-only Data Supplement). This is consistent with data in Figure 4 and Figure VII in the online-only Data Supplement showing that angiogenesis was not altered in newborn retina, tumor growth, or aorta explants, reinforcing the conclusion that Rabep2 is important in the collaterogenic but not angiogenic pathways. Other genetic abnormalities have been associated with changes in capillary density, for example, Notch3 mutants with differences in pericyte coverage16,17 and aged mice harboring the human APP-Tg2576 transgene.18 However, expression of Notch3 was not different between CNG7BC and CNG7B6 mice (3 biological replicates done in triplicate and repeated 3×, P>0.8; data not shown).

The A-to-G SNP Present in 129 Background Mice Recapitulates the CNG Phenotype

Before deriving Rabep2 edited mice, we examined Nfatc2ip knockouts (a gift from Dr K. Mowen).19 Nfatc2ip−/− mice were made using 129/SvJae embryonic stem cells and backcrossed 6 to 12 generations to BALB/c. Genotyping identified that a 129Sv flanking region was retained that included Rabep2 with the B6 allele at rs33080487 (Figure III in the online-only Data Supplement). High-density SNP array analysis (GigaMUGA) confirmed the presence of ≈3 Mb of 129 derived sequence around targeted Nfatc2ip (≈7:125221537–128681374), extending well beyond Dce1 in both directions. Consistent with the presence of the B6 Rabep2 allele, Nfatc2ip−/− had 70% of collateral number and 93% of diameter of the difference attributable to the Dce1 locus evident in Figure 1. Likewise, they had the small infarct volume of B6-WT (Figures 1 and 2; infarct volumes were comparable, P=0.42; Figure IIIA through IIC in the online-only Data Supplement).
These values, which were reported previously and do not significantly differ from the CNG lines in Figure 1, confirm on a different background (BC) the major role of Rabep2 and SNP rs33080487 in collaterogenesis. These findings also show no contribution of Nfatc2ip.

Having identified Rabep2 as causal for Dce1, we combined new high-resolution SNP data (Sanger-1, Jackson Laboratories) with our own sequencing across Rabep2 to clarify uncertainties among 21 strains we previously phenotyped. Collateral phenotype now correlates almost exactly with genotype among 20 of 21 strains (Figure 6C; Table IV in the online-only Data Supplement); we also identified 3 new SNPs in the lone SJL/J outlier strain that could reconcile its phenotype to Dce1 (Table IV in the online-only Data Supplement footnotes).

**Discussion**

Collateral status has been proposed to be important in defining the course after treatment in acute ischemic stroke. However, collateral flow varies widely among patients for largely unknown reasons. Recent studies in mice have provided a potential clue. Strains with differences in genetic background exhibit wide variation in collateral extent, ≈80% of which depends on a polymorphic region on chromosome 7, Dce1. In this study, we demonstrate that a single gene, Rabep2, is responsible for this variation. However, its contribution varies depending on genetic background. When the BC allele of Rabep2 is on the B6 background, it accounts for 36% and 72% of the differences in collateral number and diameter attributable to Dce1—as determined in the SNP and genetically comparable Del3 mice—and accounts for 64% and 89% of number and diameter when it is deleted as in the Rabep2−/− mice. By comparison, when the B6 allele is present on the BC background (CNG7B6 and Nfatc2ip−/− mice), it rescues 70% and 93% of number and diameter in WT-B6 mice. These values are identical to those reported previously and make clear that other genetic elements interact with Rabep2 or contribute independently to collateral variation.

Rabep2 is a ubiquitously expressed gene about which little is known. Studies in cultured cells show that Rabep2 participates in vesicular trafficking wherein cell surface receptors are internalized into vesicles that fuse into EEs in a Rab4- and Rab5-dependent manner. Membrane cargo is then
returned to the plasma membrane via recycling endosomes (Rab4 dependent), concentrated in Golgi-associated stores, or transferred to lysosomal-bound late endosomes (Rab7 and Rab9 dependent).15,22–24 EE formation requires recruitment of Rab4, Rab5, and several Rab effectors. However, compared with the better known Rabep1 which when immunodepleted in HUVEC results in severe disruption of EE fusion, depletion of Rabep2 has only a minor effect which may explain why no functional studies have appeared since its original description.12

Rabep2 shares 42% sequence identity and 53% similarity with Rabep1. Although they complex with some of the same proteins, there is no evidence that they directly interact.12 Similar to Rabep1, Rabep2 is recruited to EEs by Rab4 and Rab5 GTPases and Rabex5 (RabGEF1), where it complexes with other endosomal proteins.12,13,15 Redundancy exists for Rabep1 because overexpression of other binding partners can compensate for dysfunctional trafficking caused by deleting its binding domains.19 The B6-BC amino acid change (rs33080487) is in the coiled-coil domain of Rabep2 that enables binding to Rabex5 (CC2-1, Figure 6C). Deleting the homologous domain of Rabep1 prevented its recruitment to EEs.15 Accordingly, it is possible that the R298Q change alters binding of Rabep2 to Rabex5 and hinders Rab4 or Rab5 activation and that deletion of Rabep2−/− further impairs activity. Consistent with this hypothesis, we observed larger endosomes in Rabep2−/− embryonic pia. This suggests that in Rabep2−/−, EEs initially form and fuse into larger vesicles (Rab5 function remains intact) but recycling is reduced because Rab4 action is hindered.

It is well established that VEGFR2 undergoes ligand-dependent and ligand-independent (constitutive) endocytosis,25–28 and that these signaling routes impact angiogenic sprouting and vascular development.29,30 Binding of VEGFA induces VEGFR2 trafficking through one of the several pathways, including the Rab4-mediated short-loop fast-recycling route back to the cell membrane via EEs. Whether endocytosis enhances or dampens VEGFR2 signaling seems to be context dependent. For example, downstream VEGFR2 effectors mitogen-activated protein kinase and AKT required for VEGFA angiogenic actions show sustained activation in endothelial cells when EE formation is impaired,15 suggesting that endocytosis is required for VEGFR2 signaling. In contrast, ligand binding causes ubiquitination of VEGFR2 thus targeting for lysosomal degradation.31 Further complicating matters, presumed newly synthesized VEGFR2 stored in the Golgi can be, after VEGF-VEGFR2 binding to vesicles, quickly mobilized/targeted to either the cell membrane or lysosomes,11 and
existing endosomes from plasma membrane can fuse with the Golgi. The signals that dictate where/when VEGFR2 is shuttled remain under investigation.

VEGFA→VEGFR2 signaling contributes importantly to embryonic collaterogenesis. Reduction of ligand or receptor during the narrow time window of collaterogenesis reduces collateral formation, thus collateral number and diameter in the adult. Herein, we found that the embryonic pial plexus of Rabep2−/− mice had larger vessel diameters and less branching. This phenocopies previous findings in Vegfa−/− and BC mice (compared with B6), the latter being consistent with reduced Vegfa expression in BC versus B6 mice reported in Lucitti et al and references therein. Because Vegfa, Vegfr2, and Vegfr1 transcripts were not decreased, and EEs were larger in E14.5 Rabep2−/− mice, we hypothesize that when Rabep2 is lacking, deficient EE recycling sequesters inactive VEGFR2, effectively reducing the number available for signaling, with the result that collaterogenesis is reduced. Significant perinuclear VEGFR2 is evident in pial endothelial cells of E14.5 embryos (data not shown); thus, transport from this location to the cell membrane may also be impaired.

Arteriogenesis, that is, anatomic enlargement of collateral lumen diameter that occurs after arterial obstruction (eg, pMCAO), is induced by shear stress. It is possible that the difference in arteriogenesis between these strains contributed to infarct volumes measured in this study. However, we previously showed that this difference maps to a quantitative trait locus on chromosome 11 different from Dce1/Rabep2 on chromosome 7. In agreement, introgression of Dce1 between the 2 strains had no effect on arteriogenesis of pial collaterals after pMCAO or hindlimb collaterals after femoral artery ligation. In addition, differences in infarct volume reported previously and in this study closely follow the genetically engineered differences in native collateral extent.

This study has several limitations. First, determining how altered Rabep2 disrupts collaterogenesis will be challenging because there is no in vitro model of collaterogenesis, mice with transgenic, marker, and conditional alleles of Rabep2 will have to be constructed, and little is known about the functions of Rabep2. Second, although effective in our immunoblot and pulldown assays, available Rabep2 antibodies showed significant nonspecific binding that precluded their use for
immunohistochemistry—findings we confirmed with mass spectrometry. Third, the importance of variants of Rabep2 in determining variation in collateral extent in mouse may not translate to human. However, because vascular developmental pathways are conserved among vertebrates, and mice and humans are 92% similar genetically, it is possible that polymorphisms at human RABEP2 are significant determinants. Other collateral genes are also likely important: 3 additional quantitative trait locus have been identified that account for almost all of the remaining variation in pial collaterals between B6 and BC mice beyond Rabep2, and several known angiogenic proteins significantly impact collaterogenesis, thus collateral extent, in pia and skeletal muscle (VEGFA, VEGFR2, Dil4, Notch, ADAM10, ADAM17, and Gja4, Gja5) and possibly others (Egln1 and Nfkb1). Last, we have previously reported in mice that aging, impaired eNOS activity, hypertension, metabolic syndrome, and other cardiovascular/stroke risk factors cause a reduction in pial collateral number and diameter, in association with increased infarct volume after pMCAO as reported in Moore et al and references therein. However, these environmental effects on collateral extent are smaller than those attributable to differences in genetic background.

Our findings regarding Dce1, Rabep2 and collaterals have recently been confirmed: P. Quax and coworkers (personal communication, 2016) examined a region on chromosome 7 in B6 versus BALB/c mice known to harbor variants of several genes resulting in differences in natural killer cell function. A similarly large reduction in pial collateral number was found in a B6 line in which the BALB/c variant of the natural killer cell locus had been introgressed during construction of a mutant line. While at first suggesting involvement of this locus in collateral variation, subsequent analysis identified that the flanking region of the natural killer cell locus contained the BALB/c variant of Dce1.

Summary/Conclusions
Collateral-dependent blood flow after stroke is primarily determined by collateral extent at baseline and the amount of collateral remodeling/arteriogenesis after large artery occlusion. Herein, we show that Rabep2 is the causal gene at Dce1 responsible for most of the difference in native collateral extent in B6 and BC mice and, by inference based on previous SNP association studies and new SNPs we identified herein, in 18 of the 19 other strains examined. Whether this extends to humans is under investigation in a prospective study, GENEDCSS (Genetic Determinants of Collateral Status in Stroke, J.E. Faber, PhD, personal communication, 2015). Confirmation that RABEP2 and other collateral genes link to variability in collateral status may lead to development of a test to identify individuals with poor collaterals before stroke or other occlusive disease occurs. In acute ischemic stroke patients, such a biomarker could also aid clinical decision-making and stratification into clinical studies; currently, there is no noninvasive method to assess collateral extent. In addition, the Rabep2 mutants described herein provide a set of mice that models individuals with poor, intermediate, and good collaterals on an otherwise isogenic background for study of the collateral circulation.

Acknowledgments
We thank Drs F. Pardo-Manuel de Villena, D. Cowley, K. Burridge, and D. Miller for tissue and GigaMUGA analysis, and the Animal Models Core.

Sources of Funding
This work was supported by National Institutes of Health(NIH)-National Heart, Lung and Blood Institute HL111070 and NIH-National Institute of Neurological Diseases and Stroke NS083633 (Dr Faber).

Disclosures
None.

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Stroke. 2016;47:3022-3031; originally published online November 3, 2016;
doi: 10.1161/STROKEAHA.116.014160

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SUPPLEMENTAL MATERIAL

Variants of Rab GTPase-effector binding protein-2 underlie variation in the collateral circulation and severity of stroke

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Supplemental Materials and Methods

Animals. Congenic strain 7-B6 (CNG7B6) resulted from a recombination while backcrossing CNG5B61 mice to BALB/cByJ (BC) to generation N12. PCR markers were used to identify blocks of B6 genotype and crossovers. Heterozygous mice were mated to produce homozygous CNG7B6 and CNG7BC congenic lines. CNG7B6 mice are backcrossed to BALB/cByJ > 11 generations. Four lines of CRISPR/Cas9 genetically modified B6 mice (see Supplemental Table I) were generated by UNC’s Animal Models Core. C57BL/6J (B6) mice with a guanine-to-adenine substitution (SNP), a 3-nucleotide deletion (Del3), a one guanine insertion (InsG) or a one guanine deletion (DelG) at rs33080487 (7:126,440,209) in the Rabep2 gene were generated. Mice were genotyped by sequencing PCR products. SNP mice were later genotyped by PCR using the primers F: 5’-GTCCTGGAACCTGCTTTGGTGTC, R-B6: 5’-TAGTCCTTTCCTGACTCATCTC, R-BC: 5’-TAGTCCTTTCCTGAGCTCATCTT. Nfatc2ip knockout mice (BC background) were a gift from Dr. Kerri Mowen at Scripps Research Institute (see Supplemental Table I). Mice possessing an engineered deletion of approximately 0.39Mb, including genes Slx1b through Fam57b at the 3’ end of Dce1, were purchased from Jackson Laboratories (# 013128, B6129S-Del (7Slx1b-Sept1) 4Am/J). Mapk3<sup>−/−</sup> mice were obtained from Jackson Labs ((B6.129(Cg)-Mapk3<tm1Gela>/J, stock# 019113). Nupr1<sup>−/−</sup> mice (B6 background) were a gift from Dr. Juan Iovana, INSERM, France. All experiments were performed per the University of North Carolina IACUC and NIH guidelines. Where possible, all experimental groups contained both sexes.

Pial collateral assessment in adult mice. After anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg), the thoracic aorta was cannulated retrograde with stretched PE50 tubing and the cortical pial circulation exposed via craniotomy. The vasculature was perfused with heparinized saline containing the vasodilators papaverine (40 μg/ml) and sodium nitroprusside (30 μg/ml), followed by a small bolus of Evans Blue (50 mg/ml in phosphate buffered saline). Yellow Microfil<sup>™</sup> (FlowTech Inc, Carver, MA) was then injected and allowed to cure. Brains were removed and fixed with 4% paraformaldehyde (PFA) overnight and imaged with a stereomicroscope. Pial collateral number was quantified as the number of arteriole-to-arteriole anastomoses cross-connecting outer branches of the MCA and ACA trees, identified after maximal dilation, fixation and filling of the cerebral arterial vasculature with Microfil. While viewing under a stereomicroscope, infusion of Microfil was stopped just before onset of filling of cerebral capillaries (~ 5 micron average diameter) and venules. The stopping point was aided by prior infusion of Evans blue PBS (Figure 1). Diameter measurements were made using ImageJ (NIH). Measurements were performed blindly.

Pial collateral assessment in embryonic mice. Mice were paired, and noon on the day that a vaginal plug was detected was considered embryonic day (E) 0.5. On a specified day, embryos were harvested and the brains were fixed in situ in 2% paraformaldehyde in PBS for 2-10 hours. Whole brains were rinsed, permeablisized with 0.1 % Triton-X for 12 hours, blocked with 5% donkey serum in PBS, and exposed to anti-CD31 antibodies (BD Biosciences, #550274) (1:300) diluted in PBS overnight. Brains were then rinsed and exposed to HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, #111-065-144), horseradish peroxidase (Vectastain RTU #PK-7100, Vector Laboratories), DAB (SK-4100, Vector), and DAB
Enhancing solution (H-2200) following the manufacturer instructions. Pial collaterals interconnecting the middle cerebral artery (MCA) and anterior cerebral artery (ACA) trees were identified and counted in each hemisphere using a fluorescent stereomicroscope (MZ16FA, Leica). Measurements were performed blindly.

Embryonic pial morphometrics. Morphometrics were measured with Image J and conducted blindly. Four to 5 high-magnification (150X) images of the plexus between the MCA and ACA (the “collateral zone”) were taken for each hemisphere. An ROI was created and the average capillary diameter was determined for ~10 capillary segments per ROI and in approximately 6 ROIs per embryo. Branch number was determined by counting the number of branch points within the ROI within the collateral zone of each hemisphere. Measurements within an embryo were averaged to create one value per embryo.

Middle cerebral artery occlusion. A 4mm incision was made just caudal to the right eye and the temporal muscle was retracted. A 2mm² craniotomy was drilled (18000-17, FST, Foster City, CA) over the MCA trunk, which was cauterized (18010-00, FST, modified). The temporal muscle was reseated and the incision closed. The brain was removed 24h later and sliced into 1mm coronal sections that were incubated in a 2% 2,3,5-triphenyltetrazolium chloride (TTC) in PBS at 37°C. Sections were coded to blind the investigator to genotype and imaged with a stereomicroscope. Cortical area and area devoid of TTC staining (infarct area) were measured (ImageJ) for each section, summed, and percent infarct volume was calculated.

Endosome diameter measurements. Brains of 6 WT and 6 Delc E14.5 embryos were fixed in 2% PFA at 4°C overnight. Brains were permeabilized with 0.01% Triton (4°C overnight), blocked and exposed to anti-VEGFR2 (1:300, R&D AF644) and anti-EEA1 (Thermo Scientific MA5-14794) at 4°C overnight. The vessels were secondarily stained using Alexafluor-conjugated antibodies (Life Technologies) and the pia mater was gently separated from the cortex and mounted on slides. Three to 5 fields per hemisphere per mouse were imaged with a Zeiss 510 or Zeiss 880 confocal microscope. The acquired z-stacks were loaded into Imaris and the ImarisCell module (Bitplane.com) EEA1-positive vesicles in the plane of the capillary plexus were detected, segmented, counted and measured for diameter. The average values per hemisphere were averaged to create one set of values per embryo. All measurements were conducted blindly and de-coded for statistical analysis.

Retinal Assay. Postnatal day-7 mice were euthanized by isofluorane overdose, eyes were removed and fixed in 2% paraformaldehyde for 2 hours. One eye per mouse was secured by micropins optic nerve-side down to a silastic-coated petri dish. Using vannas scissors, an incision was made on the cornea and using fine forceps, the outer sclera and pigmented retina layers are separated. The lens, vitreous humor, and hyaloid vessels were then removed. Four deep radial cuts were made in the retinal cup, and the cup was transferred to a 96-well plate containing PBS. On an orbital rotator, retinas were incubated with 3% donkey serum for 30’, followed by 0.5% Triton-X in PBS for 30’, followed by rat anti-mouse CD31 antibody (BD Pharmingen #550274) in PBS for 2 hours at RT or overnight at 4°C, and then AlexaFluor donkey anti-rat secondary (1:500) for 60’. After thorough rinsing in PBS, retinas were transferred to a charged slide, excess PBS was removed, mounting media was added and a coverslip was placed
over the retina. Retinas were imaged on a Zeiss 510META confocal microscopy and measurements were performed using ImageJ.

**Tumor injections.** Mouse mammary tumor cell line 4T1 was purchased from ATCC (CRL-2539) and maintained in 10% FBS DMEM. To prepare for injection, Matrigel (Corning, 356234) was diluted to 40% in HBBS. 4T1 cells were then harvested and suspended at a density of $1.0 \times 10^7$ cells/mL in 40% Matrigel. One million cells (100 μL cell-Matrigel suspension) were injected orthotopically into the third right mammary fat pad of each BALB/cByJ mouse. To determine tumor volumes, an external caliper was used to measure daily the greatest longitudinal diameter (length) and the greatest transverse diameter (width) of the tumors. Tumor volumes were then calculated with the modified ellipsoidal formula: $\text{Tumor volume} = \frac{1}{2} (\text{length} \times \text{width}^2)$. Mice were sacrificed when tumors reached 1 cm$^3$ in size. Excised tumors were weighed, cut in half, fixed in 4% PFA, infiltrated with 10%, then 20%, then 30% sucrose in PBS, flash frozen in liquid nitrogen, and cryosectioned for histology.

**Aortic ring angiogenesis assay.** Six 0.5mm thoracic aortic rings from each 4-6 week-old mouse were serum-starved in Opti-MEM (Life Technologies, #31985-070) for 24h and plated in 75μl collagen type 1 (Millipore #08-115) (modified from 2). On day-6, rings were fixed and stained with anti-VE-cadherin (1:300, BD, #55048) and an AlexaFluor-conjugated secondary. Sprout counts were obtained live on blinded samples using a dissecting microscope, and sprout length was determined using Image J.

**Quantitative RT-PCR.** Cerebral cortical tissues were collected and stored in RNAlater® (Sigma #R0901). Total RNA was isolated using the Qiagen RNeasy Kit. Reverse transcription was performed with SuperScript™ First-Strand Synthesis System (Invitrogen, #11904-018) following manufacturer instructions. Amplification was achieved with SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma, #S4438) using StepOnePlus (AB Biosciences) and analyzed using the delta-delta CT method and StepOnePlus software. Three to 4 samples per group were analyzed in triplicate.

**Immunoblots.** Whole brain tissue (~25 mg) was homogenized in 400 μl of 100mM NaCl-50mM Tris-5mM EDTA-1% Triton X-100-0.5% IGEPAL CA-630 (immunoprecipitation (IP) buffer,) plus 1/100 volume HALT protease inhibitor cocktail (ThermoFisher 87786) and 1mM dithiothreitol (IP buffer HALT-DTT). Antibodies were rabbit anti-Rabep2, 1:1000 (Proteintech14625-1-AP), a rabbit serum directed against Rabep2, 1:500 (a generous gift of Dr. Marino Zerial), rabbit anti-Rabex5, 1:1000 (Novus Biologicals, NBP1-49938), rabbit anti-Rab4, 1:500 (Abcam, ab13252), rabbit anti-EEA1, 1:1000 (ThermoFisher, MA5-14794) and rabbit anti-Rab7, 1:1000 (Cell Signaling Technology, 9367S). For labeling with the Proteintech anti–Rabep2 antibody, lysates were denatured in SDS sample buffer at 70°C and size-separated by gel electrophoresis on a 7% tris-acetate gel (Invitrogen, EA03585BOX ) for all other antibodies, 4-15% Mini-Protean® TGX Stain-Free™ Precast Gel (Bio-Rad, 456-8086) were used. Proteins were transferred to a PVDF-membrane in a Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked by incubation in Tris-buffered Saline containing 0.1% Triton X-100 and 5% powdered milk (TBST-milk) or 5% bovine serum albumin (BSA) (Sigma-Aldrich, A2153) for 1 hour and incubated overnight with the primary antibody and mouse anti-actin 1:10,000 (Abcam ab6276) in TBST-milk or TBST-BSA. Membranes were washed several times.
with TBST and incubated for 1 hour with IRDye® 680RD donkey anti-rabbit IgG (H + L), 1:5,000 (Li-Cor 925-68073) and IRDye® 800CW goat anti-mouse IgG (H + L) 1:5,000 (Li-Cor 926-32212). Blots were read on a LiCor CLx. For rabbit anti-EEA1, details were the same except use of HRP-conjugated goat anti-rabbit IgG (Vector Laboratories, Inc., PI-1000), detection by SuperSignal® West Pico Substrate (Thermo Scientific, 34080) and imaging on a FluorChem™ M Imaging system (ProteinSimple, 3102257).

**Immunoprecipitation.** Mice were euthanized by cervical dislocation after isofluorane anesthesia, and brains were removed, frozen on dry ice, and stored at -80°C. Lysates were prepared by homogenizing 3g whole brain in 50 mL IP buffer-HALT-DTT using Omni Tips disposable rotor stator generator probes (Omni International) and an Omni International homogenizer (30-seconds). Homogenates were cleared of particulate material at 12100 x g rcf. Cyanogen bromide-activated sepharose 4B (Sigma C9142, 80 mg dry mass) was washed, swelled and coupled to antibody (60 µg IgG protein) according to the manufacturer’s instructions, pre-treated with 1 ml packed volume sepharose under gentle agitation at 4°C for 30 minutes and recovered by passage through a 5-ml mini-column (Pierce 89897). This was repeated with 1 ml of fresh sepharose. The homogenates were then incubated for 4 hours at 4°C with activated sepharose coupled to Proteintech anti-Rabep2 antibody (60 µg) according to the manufacturer’s instructions. The sepharose was collected in a fresh column and the treated homogenate saved for immunoblot analysis. The sepharose was washed with 5 column-volumes of IP buffer-HALT-DTT followed by 5 column volumes of IP buffer containing no detergent. Proteins bound to the beads were eluted with 0.1 M glycine (pH 1.7) in four 0.5 mL fractions. Each fraction was precipitated with 125 µL trichloroacetic acid (TCA) with incubation on ice for 10 minutes followed by a 10 minute centrifugation at 16000 rcf. Pellets were combined, washed in cold acetone, centrifuged again, dried and dissolved in gel electrophoresis sample buffer. The resuspended pellets were resolved on 7% Tris-acetate acrylamide gels (Invitrogen, EA03585BOX) either for immunoblot analysis as above or mass spectrometry analysis.

**Mass spectrometry.** Mass spectrometry was performed by the UNC Proteomics core. Three separate WT and DelG samples were first in-gel digested with trypsin for matrix-assisted laser desorption/ionization (MALDI) Orbitrap. The sample was then run on a nanoAcquity-Orbitrap Velos LC-MS/MS instrument. Raw data was searched against the Uniprot mouse database. Only peptides with false discovery rate (FDR) < 5% were considered.

**Genomic DNA from Nfatc2ip<sup>-/+</sup>.** To determine the background strain of the Nfatc2ip KO and WT mice and the extent of the 129 DNA remaining in the KO genome, RNA from each group was run on the third generation of the Mouse Universal Genotyping Array (MUGA), GigaMUGA. The array compared 143,259 known SNPs from the Nfatc2ip<sup>-/-</sup> and Nfatc2ip<sup>++/+</sup> mice, and samples from BALBc/J, BALB/cByJ, BALB/cAnNHsd, 129T2/SvEmsJ, 129S5/SvEvBrd, 129X1/SvJ, 129S4/SvJaeJ, 129S1/SvImJ, and C57BL/6J strains to the reference C57BL/6J genome. The Nfatc2ip KO and WT mice shared genetic material from each of the BALB/c strains on the array and most closely matched BALB/cAnNHsd. However, the match was not robust and it is possible that the mice contain genetic material from a BALB/c substrain not yet genotyped by GigaMUGA or that the mice represent a unique BALB/c substrain via genetic drift. Approximately 3Mb of 129-derived sequence remains (approximately
Capillary and microvascular density and area were obtained from 5 and 6 month-old male and female DelG, SNP and wildtype mice.\textsuperscript{5,6} Mice were perfused for 5 minutes with 100 μM sodium nitroprusside in PBS (Corning, 46-013-CM) for maximal dilation, followed by 1% PFA. Brains were post-fixed for 24 hours in 1% PFA at 4°C, cryoprotected overnight in 30% sucrose, then embedded in OCT tissue-freezing medium. Ten micron coronal sections between 1.0 mm and 0.6 mm relative to bregma were taken at 100-um intervals starting where the corpus callosum becomes continuous. Sections were stained for Glut-1 (Glucose transporter 1). Briefly, sections were labeled with 1:200 rabbit anti mouse Glut-1 (Abcam, AB652) at 4°C overnight, then probed with 1:200 Alexa-488 Goat anti rabbit (Life Technologies, A10042) for 1 hour at room temperature. Images were collected on a Zeiss 880 confocal laser scanning microscope. Capillary and microvessel density and area were measured within the M1 motor cortex (gray matter) and underlying corpus callosum (white matter). Regions of interest for gray matter excluded pial membrane and for white matter excluded the edges of the corpus callosum. Binary images were analyzed in ImageJ using the Watershed algorithm and the Analyze Particles function\textsuperscript{7} with a fixed setting of 1 pixel = 0.417 um. Capillaries were defined as structures between 7.53 and 188.4 pixels and microvessels as structures between 7.53 and 3014.4 pixels.

Statistics. Statistics were performed with SPSS (PASW Statistics 18). Values are expressed as mean ± SE. Unless stated otherwise, independent t-test or one-way ANOVA (with Bonferroni post-hoc analysis) were used to determine differences between groups. A probability value of p<0.05 was considered significant.

STAIR criteria. The following recommendations of STAIR (2012)\textsuperscript{8} were adhered to in this study: investigators were blinded to genotype, gene dose-response was evaluated by studying wildtype, heterozygous and homozygous mutant mice, both sexes were studied, pMCAO was used to permanently recruit pial collaterals, no data point was excluded, n-sizes of ≥ 10 were used for most groups, and all negative results were reported.


**Online Table I.** Sequence alterations to create mouse lines generated by CRISPR/Cas9

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Operation*</th>
<th>Consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNP</td>
<td>126440209G&gt;A</td>
<td>rs33080487 into B6; R298Q in NP_085043.2</td>
</tr>
<tr>
<td>DelG</td>
<td>126440209DelG</td>
<td>Alters reading frame and truncates protein</td>
</tr>
<tr>
<td>InsG</td>
<td>126440209_126440210InsG</td>
<td>Alters reading frame and truncates protein</td>
</tr>
<tr>
<td>Del3</td>
<td>126440208_126440210Del</td>
<td>Removes codon (CGG) for R298 in NP_085043.2 with retention of reading frame</td>
</tr>
</tbody>
</table>

*Positions refer to B6 reference sequence of chromosome 7.
**Online Table II.** Birth number ratios from heterozygote-heterozygote breedings by genetic line.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wildtype (%)</th>
<th>Heterozygous (%)</th>
<th>Homozygous (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabep2 DelC</td>
<td>23 (27)</td>
<td>44 (52)</td>
<td>17 (20)</td>
</tr>
<tr>
<td>Rabep2 InsC</td>
<td>36 (23)</td>
<td>83 (53)</td>
<td>39 (25)</td>
</tr>
<tr>
<td>Rabep2 Del3</td>
<td>20 (23)</td>
<td>42 (49)</td>
<td>24 (28)</td>
</tr>
<tr>
<td>Rabep2 SNP</td>
<td>30 (25)</td>
<td>61 (51)</td>
<td>29 (24)</td>
</tr>
<tr>
<td>Nfatc2ip</td>
<td>31 (29)</td>
<td>55 (51)</td>
<td>22 (20)</td>
</tr>
</tbody>
</table>
Table III. Interaction of sex, genetic background and *Rabep2* alleles on pial collateral number, diameter and infarct volume.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Sex</th>
<th>Number</th>
<th>Diameter</th>
<th>Group</th>
<th>n</th>
<th>Infarct volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRISPR WT</td>
<td>12</td>
<td>F</td>
<td>19.3 ± 0.8</td>
<td>23.6 ± 0.9</td>
<td>CRISPR WT</td>
<td>10</td>
<td>3.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>M</td>
<td>20.0 ± 0.9</td>
<td>24.7 ± 0.7</td>
<td></td>
<td>10</td>
<td>5.1 ± 0.8</td>
</tr>
<tr>
<td>DelG &amp; InsG</td>
<td>9</td>
<td>F</td>
<td>6.8 ± 0.9</td>
<td>11.8 ± 0.4</td>
<td>DelG</td>
<td>3</td>
<td>11.2 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>M</td>
<td>8.9 ± 1.0</td>
<td>13.5 ± 0.9</td>
<td></td>
<td>8</td>
<td>11.0 ± 1.0</td>
</tr>
<tr>
<td>Del3 &amp; SNP</td>
<td>13</td>
<td>F</td>
<td>12.4 ± 0.8</td>
<td>16.4 ± 0.7</td>
<td>SNP</td>
<td>7</td>
<td>10.8 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>M</td>
<td>11.6 ± 1.4</td>
<td>15.9 ± 0.4</td>
<td></td>
<td>3</td>
<td>8.4 ± 0.8</td>
</tr>
<tr>
<td>Nfatc2ip WT</td>
<td>6</td>
<td>F</td>
<td>1.3 ± 0.4</td>
<td>8.4 ± 0.8</td>
<td></td>
<td>6</td>
<td>11.7 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>M</td>
<td>2.0</td>
<td>9.6</td>
<td></td>
<td>2</td>
<td>10.9 ± 0.7</td>
</tr>
<tr>
<td>Nfatc2ip KO</td>
<td>9</td>
<td>F</td>
<td>14.5 ± 0.5</td>
<td>21.5 ± 0.7</td>
<td></td>
<td>3</td>
<td>6.5 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>M</td>
<td>13.3 ± 0.8</td>
<td>21.4 ± 0.8</td>
<td></td>
<td>6</td>
<td>6.0 ± 1.0</td>
</tr>
</tbody>
</table>

Mice were pooled by genetic manipulation [CRISPR WT founders, KO (DelG & InsG), amino acid deletion (Del3 and SNP)] to increase n-size. Infarct volume is smaller in female WT. This is absent in mice with deletion of *Rabep2* in association with a trend toward fewer collaterals of smaller diameter. Thus on the C57BL/6 (B6) background and compared to male sex, the B6 allele of *Rabep2* favors maintenance of collateral abundance and smaller infarction in females. On the BALB/c background of the *Nip45* mice, the B6 allele of *Rabep2* (present in the Nfatc2ip KO mouse) does not confer this protection per infarct volume. Additional studies are required to determine the basis for the collateral extent dependence on sex with the B6 allele of *Rabep2*. Values are mean ± SEM for n number of animals; 1-tailed t-tests per sex.
Table IV. Correlation of collateral number and genotype at Rabep2 for 21 previously phenotyped mouse strains (see also next page)

<table>
<thead>
<tr>
<th>Strain</th>
<th># collaterals</th>
<th>Genotype at Rabep2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>High collateral number strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBA</td>
<td>21.4</td>
<td>B6</td>
</tr>
<tr>
<td>C57BLKS/J</td>
<td>20.8</td>
<td>B6, by haplotype shared with LP</td>
</tr>
<tr>
<td>129X1/J</td>
<td>20.6</td>
<td>B6, by haplotype shared with LP</td>
</tr>
<tr>
<td>SJL/J</td>
<td>19.0</td>
<td>BC; R298Q</td>
</tr>
<tr>
<td>B6</td>
<td>18.8</td>
<td>B6</td>
</tr>
<tr>
<td>NZO</td>
<td>18.8</td>
<td>B6</td>
</tr>
<tr>
<td>129S1</td>
<td>18.3</td>
<td>B6</td>
</tr>
<tr>
<td>NOD</td>
<td>18.3</td>
<td>B6</td>
</tr>
<tr>
<td>NON</td>
<td>17.5</td>
<td>B6, by haplotype shared with LP</td>
</tr>
<tr>
<td>DBA</td>
<td>17.5</td>
<td>B6</td>
</tr>
<tr>
<td>CAST/EiJ</td>
<td>17.1</td>
<td>B6</td>
</tr>
<tr>
<td>C3H</td>
<td>16.3</td>
<td>B6, by haplotype shared with B6</td>
</tr>
<tr>
<td>FVB</td>
<td>16.2</td>
<td>B6</td>
</tr>
<tr>
<td>KK</td>
<td>13.1</td>
<td>B6, by haplotype shared with LP</td>
</tr>
<tr>
<td>LP</td>
<td>11.9</td>
<td>B6</td>
</tr>
<tr>
<td><strong>Low collateral number strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZW</td>
<td>8.2</td>
<td>BC; R298Q</td>
</tr>
<tr>
<td>A/J</td>
<td>6.9</td>
<td>B6 at rs33080487 but polymorphic at rs31196589; R426H</td>
</tr>
<tr>
<td>LEWES/EiJ</td>
<td>5.9</td>
<td>B6 at rs33080487 but G to C polymorphism at 7:126438655</td>
</tr>
<tr>
<td>AKR</td>
<td>3.3</td>
<td>BC; R298Q</td>
</tr>
<tr>
<td>SWR/J</td>
<td>2.5</td>
<td>BC; R298Q</td>
</tr>
<tr>
<td>BC</td>
<td>0.8</td>
<td>BC; R298Q</td>
</tr>
</tbody>
</table>

SNPs were from Sanger 1 or CGD-MDA1 databases at http://phenome.jax.org/db/q?rtn=snp/ret01&refine=g6jcBQcY3

SNP in LEWES from http://www.sanger.ac.uk/sanger/Mouse_SnpViewer/rel-1505

Haplotype information from http://msub.csbio.unc.edu/

Genotypes at rs33080487 were confirmed (A/J) or found (SJL/J, LEWES/EiJ, SWR/J) by PCR / sequencing, as was the SNP at 7:126438655 in LEWES/EiJ.

With identification of Rabep2 as the causal gene in Dce1, it is possible to nearly exactly correlate phenotype and genotype for the 21 inbred strains we previously phenotyped and thus clarify some previous uncertainties. ref 6 of manuscript No additional SNPs were found in Rabep2 of LEWES/EiJ by all-exon PCR / sequencing. In SJL/J, the same procedure confirmed rs33083620
(a synonymous SNP in exon 4) and found rs32027070 (a SNP in the 5’ untranslated region not previously noted in this strain).

**Analysis**

Eight of the 15 strains with high collateral number ($\geq 11.9$ collaterals) are B6 at rs33080487. Five others are predicted B6 because they share haplotype with strains B6 or LP (known to be B6 at rs33080487). The single outlier among the high collateral-number-strains is SJL/J, which shares haplotype, rs33080487, rs33083620 and rs32027070 with BC. This outlier status points to the existence of other locations in the SJL/J genome able to strongly influence collateral extent. Among the 6 low-number strains, 4 are BC at rs33080487. A fifth strain, A/J, shares haplotype with LP and its high-collateral-number relatives but has a non-synonymous variant not previously recognized (rs31196589) causing alteration R426H (Figure 6C), predicted to be deleterious by Polyphen-2 (score 0.999). This mutation appears to resolve the confusion caused by the fact that substitution of A/J chromosome 7 into B6 gives a low-collateral-number strain. In LEWES/EiJ, the sixth low-collateral-number strain, the single variant causes alteration P200R (Figure 6C), predicted possibly deleterious by Polyphen-2 (score 0.54). These results strengthen the concept that naturally occurring variants of *Rabep2* can be major determinants of collateral variation.
Figure I. **Introgressed intervals of congenic (CNG) mice.** Map showing SNP references and genomic positions on chromosome 7 used to define CNG5, CNG6 and CNG7 mouse lines and the Dce1 locus as defined previously. Gray text and bars depict BC genome, black text and bars depict B6, and white blank intervals denote regions of uncertainty. Genomic positions are from GRCm38 (mm10).
Figure II. Deletion of other genes in Dce1 does not affect collateral extent. A, Adult collateral number and diameter, as well as collaterogenesis (B), were not different in mice heterozygous for a 0.39 Mb deletion stretching from Slx1b to Sept1 (COD interval) from B6 (WT). Homozygous COD KO mice are embryonic lethal thus were not examined. C,D, Adult collateral number and diameter were not affected in Nupr1−/− or Mapk3−/− mice. The reason for larger diameter in Nupr1−/− mice is not apparent although the measurements for the 2 groups were made by different investigators.
Figure III. See next page for legend.
Figure III. Flanking genome of the 129Sv strain, which has the B6 SNP at rs34404087, confers the B6 collateral phenotype onto Nfatc2ip^+/− mice that are on the BC background.  A, Dose-dependent loss of Nfatc2ip mRNA shown by qRT-PCR in adult neocortex.  B, The complimentary DNA strand shows that BC.Nfatc2ip^−/− mice have the A→G SNP in the Rabep2 gene (rs33080487). This SNP recapitulates the CNGB6 phenotype.  C, BC.Nfatc2ip^−/− have increased collateral number and diameter that are identical to CNGB6 mice. Infarct volume after pMCAO was reduced by almost 50% in BC.Nfatc2ip^−/− mice and was similar to CNGB6 mice.  D, Comparison of SNP differences in Nfatc2ip^{+/−}, Nfatc2ip^−/− and representative mouse strains to the C57BL/6J mouse genome as determined by GigaMUGA high density array (http://www.csbio.unc.edu/CCstatus/index.py?run=AvailableLines.info). Black lines represent a homozygous nucleotide difference from the reference strain (C57BL/6J), gray lines represent a heterozygous difference, white lines represent homozygous similarity. Approximately 3Mb of 129-derived sequence remains (approximately 7:125,221,537 - 128,681,374) around the targeted Nfatc2ip gene (7: 126,382,854-126,396,737, yellow line).
Figure IV. Description of CRISPR-derived Rabep2 mutant mice. A, Guide and template sequences used to target the nucleotide at 7:126,440,209 of mouse Rabep2 gene. Representative traces from the complimentary DNA strand used to genotype the CRISPR/Cas9 mice. The B6 WT nucleotide sequence has a cytosine (complimentary to guanine) at rs33080487. “Del3” mice have an in-frame 3-cytosine/guanine deletion (immediately before red arrow) and are predicted to produce a full-length protein lacking one amino acid. “DelG” mice lack the cytosine/guanine at rs33080487 (immediately before red arrow), which causes a frameshift mutation that produces an immediate stop codon. This is predicted to lead to nonsense-mediated decay (NMD) of the transcript and a functional knockout mouse. “InsG” mice have an inserted cytosine at rs33080487 (red arrow) that causes a frameshift mutation, forming a stop codon that initiates NMD. The “SNP” mice have the BC adenine at rs33080487 (the complimentary thymidine is shown, red arrow) exchanged for the B6 guanine, resulting in the translational substitution R298Q characteristic of the BC strain. Rabep2 expression is reduced in homozygous adult (B) and embryonic (C) DelG cortex.
Figure V. Expression of Rabep1 is not altered in Rabep2 CRISPR/Cas9 edited mice. A, Lysates of 22-26 mg whole brain were prepared and immunoblotted in equal loadings as in Methods. Blots were probed with Proteintech rabbit anti-Rabep1 antibody (14350-1-AP) at a dilution of 1:500 and prepared for analysis on the LiCor Odyssey CLx (see Methods). Each sample is from a different mouse. The calculated molecular weight for Rabep1 (NP_062273.2; http://www.bioinformatics.org/sms/prot_mw.html) is shown. SeeBlue plus 2 standards (St; ThermoFisher) were myosin and bovine serum albumin which migrate on NuPage 7% Tris-acetate gels with the apparent molecular weights shown (from package insert). Other labels are as in Figure 2. B, RNA was extracted from adult cerebral cortexes and cDNA was prepared as described in Methods. Rabep1 expression is compared between WT and DelG mice (n=3 in each group).

210 kDa
99.6 kDa
71 kDa

WT WT WT InsG InsG WT InsG InsG DelG DelG WT WT St

Rabep1 expression, fold Δ
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4

WT DelG
3 3
Figure VI. Collateral phenotype in adult mice heterozygous at CRISPR-edited alleles.
Figure VII. *Dce1* does not affect sprout outgrowth in aortic ring angiogenesis assay. Sprout number and length in aortic explants from congenic (CNG) BC mice (ie, BC-WT) and CNGB6 mice (BC with B6 *Dce1* allele introgressed in place of BC allele).
Figure VIII. Rabep2 deletion does not change expression of early endosome-associated proteins. Immunoblots in WT and Rabep2−/− (DelG) E16.6 mouse brain (3 of each). Beta-actin serves as loading controls. qPCR showed no change in expression of Rabex5 and Rab4; other above were not examined.