Congenic Fine-Mapping Identifies a Major Causal Locus for Variation in the Native Collateral Circulation and Ischemic Injury in Brain and Lower Extremity

Robert Sealock, Hua Zhang, Jennifer L. Lucitti, Scott M. Moore, James E. Faber

Rationale: Severity of tissue injury in occlusive disease is dependent on the extent (number and diameter) of collateral vessels, which varies widely among healthy mice and humans. However, the causative genetic elements are unknown. Recently, much of the variation among different mouse strains, including C57Bl/6J (B6, high extent) and BALB/cByJ (Bc, low extent), was linked to a quantitative trait locus on chromosome 7 (Candq1).

Objective: We used congenic mapping to refine Candq1 and its candidate genes to create an isogenic strain set with wide differences in collateral extent to assess their impact and the impact of Candq1, alone, on ischemic injury.

Methods and Results: Six congenic strains possessing portions of Candq1 introgressed from B6 into Bc were generated and phenotyped. Candq1 was refined from 27 to 0.737 Mb with full retention of effect, that is, return or rescue of phenotypes from the poor values in Bc to nearly those of wild-type B6 in the B6/B6 congenic mice as follows: 83% rescue of low pial collateral extent and 4.5-fold increase in blood flow and 85% reduction of infarct volume after middle cerebral artery occlusion; 54% rescue of low skeletal muscle collaterals and augmented recovery of perfusion (83%) and function after femoral artery ligation. Gene deletion and in silico analysis further delineated the candidate genes.

Conclusions: We have significantly refined Candq1 (now designated determinant of collateral extent 1; Dce1), demonstrated that genetic background–dependent variation in collateral vessels is a major factor underlying differences in ischemic tissue injury, and generated a congenic strain set with wide allele dose–dependent variation in collateral extent for use in investigations of the collateral circulation. (Circ Res. 2014;114:660-671.)

Key Words: collateral circulation • genetics • models, animal • peripheral vascular diseases

Cerebrovascular disease, stroke, coronary artery disease, peripheral artery disease, and other obstructive arterial diseases are the leading causes of morbidity and mortality worldwide.1 The extent (number and diameter) of native (preexisting) collaterals, a unique type of blood vessel within the microcirculation of most tissues, plays a major role in reducing the severity of ischemic tissue injury and cell death. Collaterals are arteriole-to-arteriole anastomoses that crossconnect a small percentage of the outer branches of adjacent arterial trees.2,3 When the trunk of one of the trees becomes obstructed, collateral flow from the adjacent tree provides retrograde perfusion of the occluded tree. Even a modest amount of collateral flow can significantly decrease tissue injury after acute obstruction. Moreover, chronic obstruction induces collaterals to increase their anatomic lumen diameter, that is, remodel by as much as 10-fold over days to weeks, depending on the tissue and species. Collateral remodeling (arteriogenesis), which is stimulated by fluid shear stress, is capable of greatly increasing collateral flow, depending on the extent of the native collaterals in the affected tissue and vigor of the molecular mechanisms that drive the remodeling process.

In This Issue, see p 587
Editorial, see p 591

It is well-known among stroke and neurovascular specialists, interventional cardiologists, and vascular surgeons that tissue ischemia and infarction vary greatly among patients, despite similar sites and severity of acute or chronic arterial obstruction. The mechanisms responsible for this variation remain ill-defined. Recent studies have reported wide variation among healthy individuals (ie, without obstructive disease in the tissue under examination) in the amount of blood flow provided by the native collateral circulation. In individuals without angiographically detectable coronary artery disease, that is, free of collateral remodeling that could differ and prevent...
interpretation of such measurements vis-à-vis native collateral extent, collateral flow index was distributed normally and varied by ≈40-fold, with 20% of individuals having low collateral flow indexes. Importantly, patients with coronary artery disease and poor collateral flows had 64% higher risk of mortality. Collateral flow index also varied significantly in the lower extremities of individuals without peripheral artery disease. And, similarly, in patients with sudden thromboembolic occlusion of the middle cerebral artery (MCAO), the most common cause of ischemic stroke, retrograde perfusion of the MCA tree—as assessed by neuroimaging during the hyperacute phase of stroke—varied widely, with 20% having poor pial collateral scores. Notably, such individuals sustain significantly larger infarct volumes, respond poorly to thrombolytic treatments, have increased risk for and severity of cerebral hemorrhage, and experience increased morbidity and mortality. Thus, collateral score is increasingly being viewed as an important diagnostic measurement to identify the optimal course of treatment and assess prognosis for recovery.

Nothing is known about the genetic mechanisms and little is known about the environmental mechanisms that are responsible for the wide variation in native collateral-dependent flow in humans. However, recent studies in mice have found that genetic background is a major factor. In the brain, pial collateral number and diameter varied by 35-fold and 4-fold, respectively, among 21 inbred strains. Collateral number and diameter varied by 35-fold and 4-fold, respectively, among 21 inbred strains. Moreover, the distribution of infarct volumes across 15 strains was closely predicted by the strain-specific values for collateral number, diameter, and length, plus hematocrit and MCA tree territory. Similarly, values for blood flow, hindlimb use, and ischemic appearance immediately after and 21 days after FAL of B6, Bc, and an F1 cross correlated with the high (B6), low (Bc), and intermediate (F1) number of collaterals in the adductor thigh region and cerebral cortex of these strains. This suggests that genetic variation in native collateral extent is the major physiological substrate responsible for the variation in blood flow and tissue injury in brain and hindlimb after arterial obstruction and for the 3 coincident QTLs on chromosome 7.

Taken together, these studies present a significant conundrum. They propose 3 different candidate gene lists for a single phenomenon. Although there is strong evidence that variation in the extent of the native collateral circulation is a major factor underlying variation in these ischemic injury traits, including the conclusion that Candq1 and Civq1 may be one and the same, differential metabolic sensitivity of cells to hypoxia/ischemia has recently been proposed to be a significant determinant of the variation in response to ischemia conferred by Candq1 and Lsq-1. This hypothesis would suggest that the 3 coincident QTLs cover multiple processes, the loci for which would presumably cease to overlap with dissection of Candq1 into smaller regions. To address these uncertainties, we constructed a series of congenic (CNG) mouse strains in which subregions of Candq1 from B6 were introgressed into the Bc genome and used them for the following: (1) to establish the most cogent gene list possible to guide future investigation; (2) to determine whether collateral extent, infarct volume after MCAO, and perfusion and tissue injury after FAL continue to segregate together as the introgressed region becomes smaller, because such a finding would strengthen the hypothesis that genetic-dependent variation in collateral extent underlies the aforementioned QTL and is the major physiological factor responsible for the differences in ischemic tissue injury in B6, Bc, and related mouse strains; and (3) to generate a congenic strain-set with wide differences in collateral extent for use in future investigations of the collateral circulation.

**Methods**

See Online Data Supplement for details.

**Animals**

Congenic strains were prepared by backcrossing B6-x-Bc recombinant inbred strains CXB3 and CBX4\* to Bc and then successively backcrossing the progeny to Bc (≈1500 mice bred and genotyped). Cln3\#x80;5 mice\#x26; Cln3\#x20;lox/lox (ex7/8) (on B6 backgrounds) were from Jackson Laboratories and Dr Beverly Davidson, University of Iowa, respectively. Mice engineered with floxed alleles or made globally hypomorphic at Jmjd5 (Jmjd5\#x20;lox; B6 background) were from Dr Takeshi Suzuki, Kanazawa University, Japan. Floxed mice were bred
with B6-Cre mice to produce mice null for Jmjd5 in endothelial cells (ECs; Jmjd5Δ/Δ) or globally haploinsufficient (Jmjd5−/+). Sex did not influence collateral extent in brain or hindlimb,18,21 and thus cohorts were half male and half female, and 2 to 4 months old. Approximately 2000 mice were bred, genotyped, and phenotyped for this study.

Collateral Number, Diameter, and Remodeling
The vasculature was maximally dilated and filled with Microfil with viscosity adjusted to minimize entrance into capillaries, followed by fixation and digital morphometry to obtain baseline collateral number and diameter and collateral remodeling 3 days after MCAO.16 Because collaterals of the neocortex in mouse (and human) reside in the pia mater, with none in the brain parenchyma,11,32 their extent can be fully quantified using these methods.

Cerebral Infarct Volume
The right MCA trunk below the midtemporals muscle was permanently occluded (MCAO).16 Brain slices were stained with 2,3,5-triphenyltetrazolium chloride (TTC) 24 hours later.

Cerebral Blood Flow
In separate mice, the vasculature was maximally dilated and phosphate-buffered saline (PBS) containing 6-micron fluorescent microspheres and vasodilators was perfused retrograde via the thoracic aorta 24 hours after MCAO, followed by TTC staining of brain slices. Relative blood flow was quantified as the number of trapped microspheres in the volume of infarcted brain normalized to the number trapped in the corresponding region/volume on the nonligated side.33–35

Hindlimb Ischemia
FAL, laser Doppler measurement of plantar perfusion, which correlates closely with overall hindlimb blood flow,36 and measurement of hindlimb use and ischemic appearance were performed as described.20,21

Statistics
Values are mean±SE. Data were tested with Bonferroni post hoc tests (significance at P<0.05).

Results
Construction of Congenic Strains
Congenic strains were derived from 2 B6xBc (CXB) inbred lines in which homozygous blocks of B6 and Bc genotype are arranged in patchworks unique to each line.27 CXB3 and CXB4 were chosen because they contained potentially advantageous breakpoints within Candq1 near the EMMA region (Figure 1A). Congenic strains were developed by repeated backcrossing of CXB3 and CXB4 mice and progeny to Bc. At each round, congenic development was accelerated by screening progeny for loss of B6 genotype outside of Candq1. This resulted in 6 congenic strains; in 5 of these the introgressed B6 genotype successively converged on the center of the 95% confidence interval of Candq1 (Figure 1A). In the exception, CNG1, the block of B6 ends at least 1 Mb centromeric to EMMA (Figure 1A). Strains CNG1, CNG2, and CNG6 were derived from CXB3; CNG3, CNG4, and CNG5 were derived from CXB4.

Because the CXB input lines are 55% to 60% Bc and because we selected against B6 outside of Candq1 (see Methods section in Online Data Supplement), we began data collection at N5. To confirm that these mice were essentially congenic, 2 N5 mice each from CNG5 and CNG6 were subjected to whole-genome analysis using the megaMUGA chip (21 200 informative markers for B6 and Bc; FP de Villena, unpublished data). Small blocks of B6 genome outside Candq1 were variously found on chromosomes 5, 8, 11, and 14, but the percentages of B6 alleles among the markers, most of them heterozygous, were <0.1%, 0.3%, 0.7%, and 2.1%, respectively. The phenotyping results that were obtained with N5 and N6 mice (Figure 1C) were not different from preliminary phenotyping of N4 mice, confirming that this small degree of contamination is without effect on collateral extent. Subsequently, in 3 N8 CNG5-B6/Bc mice, B6 alleles were found at only 19, 34, and 34 SNPs of the 21 213 informative SNPs outside Dee1.

Collateral Number and Diameter Cosegregate and Identify a Single 737-kb Locus Responsible for the Large Variability in Collateral Extent Between B6 and Bc Mice
The collateral traits—number and diameter—for wild-type Bc, CNG1-B6/B6, and CNGx-Bc/Bc mice (x=2–6) were comparable (=1.0 collateral, ≈12 um in diameter; Figure 1C). Thus, other genetic elements outside of Candq1 that contribute to the more numerous and larger diameter collaterals that are present in wild-type B6 mice22 were eliminated from the CNGs by generation N5. CNG1 mice were not studied further. The heterozygous (CNG-B6/Bc) and homozgyous (CNGx-B6/B6) genotypes conferred a remarkably consistent allele dose–dependent increase in collateral number and diameter onto the Bc genome that is indistinguishable among the groups (P>0.7; Figure 1C). The amount of rescue of collateral number and diameter in CNG2–6 B6/B6 mice averaged 73±1% and 93±6%, respectively (83±4% for extent), of the wild-type B6 values. The residual differences in collateral number between CNG3/5 B6/B6 and wild-type B6 presumably reflect the impact of the 3 other small-effects QTLs identified for collateral number in B6-x-Bc F2 mice.22 By contrast, Candq1 was the only QTL identified for diameter in that study, which is consistent with the greater rescue of diameter in the CNGs. Importantly, both phenotypic traits among CNG2–6 remained the same as the introgressed regions converged on the overlap zone of CNG5 and CNG6, 133.229 to 133.966 Mb. Furthermore, they ceased to include the EMMA region19,22 (Figure 1A; shown in detail in Online Figure 1), indicating that this region (present in CNG2–4 but not in CNG5 and CNG6) has no detectable effect. These data suggest that the zone of overlap (0.737 Mb) contains the entire causal element of the originally defined22 27-Mb QTL, Candq1, for both number and diameter. We designated this region as determinant of collateral extent 1 (Dee1; detailed map in Online Figure 1B)

Differences in Collateral Extent Specified by Dee1 Account for the Differences in Severity of Tissue Injury in Mouse Models of Ischemic Stroke and Peripheral Artery Disease
Given their isogenic background outside of Dee1, the aforementioned congenic strains provide a unique opportunity to determine the impact of differences in collateral extent, alone, on tissue injury after arterial occlusion, free of the many other genetic-dependent hemodynamic and molecular differences
in B6 and Bc that could also cause differences in severity of ischemic tissue injury. In CNG3, CNG4, and CNG5 Bc/Bc mice, whose collateral extents do not differ from wild-type Bc (Figure 1C), infarct volume at 24 hours was not different from wild-type Bc (Figure 2). By comparison, in CNG3, CNG4, and CNG5-B6/B6 mice, whose collateral extents were rescued by 76%, 91%, and 82% (Figure 1C), infarct volume was dramatically returned toward the low value of wild-type B6 (rescue of 85%). Furthermore, like collateral extent (Figure 1), rescue of infarct volume was intermediate in the heterozygote condition. This congruency strongly strengthens the conclusion that the difference in pial collateral extent is the major factor that determines the large difference in infarct volume between Bc and B6 mice and, by inference, among 15 inbred mouse strains examined previously.25 As with collateral number, the small residual difference in infarct volumes between CNG3–5 B6/B6 and wild-type B6 may reflect the impact of the 3 other small-effects QTL identified for collateral number in B6-x-Bc F2 mice.22

To test the hypothesis that the protective mechanism provided by the B6 allele of Dce1 is collateral-dependent blood flow, we measured flow in the infarcted zone of CNG4-B6/B6 and CNG4-Bc/Bc 24 hours after MCAO using the microsphere method.33–35 Baseline blood flow (nonligated side) did not differ between strains (data not shown). Flow in the infarcted region was 4.5-fold higher in B6/B6 mice (or 73% lower in Bc/Bc; Figure 3). The higher flow reduced but did not prevent some infarction. The latter is expected, given that significant oxygen already diffuses from the anterior cerebral artery and posterior cerebral artery trees before traversing the collaterals and given that the estimated relative conductance of the native pial collateral network in aggregate (including PCA↔MCA collaterals that typically number ≈5 per hemisphere in wild-type B6 mice and are larger in diameter) is 15% to 20% of the conductance of the trunk of the MCA tree at the site of ligation in wild-type Bc (Faber et al, unpublished data).

We have shown that genetic strain-specific differences in collateral extent in brain are shared in other tissues of the same mouse strains, including skeletal muscle.20,21 To determine whether this extends to the Dce1 allele, we also examined skeletal muscle. Unlike pial collaterals that can be measured with high fidelity, quantification of native collaterals in the hindlimb that mostly reside deep in the adductor thigh where they cross-connect arterial trees that branch in 3 dimensions relies on the limited resolution of x-ray angiography20,21 or optical clearance of tissue followed by manual dissection.17 Therefore, we examined collaterals in the sheet-like abdominal wall musculature of the mouse where, like in the pia, the vascular trees are arranged in 2 dimensions. In agreement with these findings in brain, collateral number in CNG4-Bc/Bc did not differ from wild-type Bc, whereas the B6/B6 alleles of Dce1 rescued collateral number to 54% of the 6-fold greater number in wild-type B6 (Figure 4).
arterial occlusion. Consistent with this, the increase in shear stress stimulus that drives collateral remodeling after from its effect on baseline collateral diameter and, thus, the variation in collateral remodeling in B6 and Bc mice, apart erates. We previously found that adult and sets the baseline diameter on which remodeling op-

We next examined a model of peripheral artery disease to test the hypothesis that this finding extends to the hindlimb and has functional significance. In support of this, blood flow determined immediately after FAL and use and ischemic appearance scores measured 1 day later in CNG4-Bc/Bc and CNG4-B6/B6 mice—values that are predominantly determined by native collateral extent in the thigh—closely mimicked those previously reported in wild-type strains (Figure 5). That is, the B6/B6 allele of strongly rescued blood flow (perfusion was 73% greater than in CNG4-Bc/Bc immediately after FAL) and reduced/rescued the severity of hindlimb ischemia and use impairment on day 1 (88% and 100% reduction, respectively; Figure 5C).

The improvement of blood flow that occurs over days to weeks after arterial occlusion primarily reflects anatomic lu-

Figure 2. Intrigression of Dce1 into the Bc genome rescues cerebral infarct volume. Cortical infarctions were produced in 2- to 3-month-old CNG3, CNG4, and wild-type (WT) mice by permanent middle cerebral artery occlusion. Twenty-four hours later, brains were sectioned (1 mm) and TTC-stained to reveal nonviable tissue (white). Sections from the same brain level are shown. Infarct volumes expressed as percentages of total brain volume were determined from the complete set of sections in each brain. Within each genotype, CNG3, CNG4, and CNG5 are not significantly different, although the introgressed regions in CNG3 and CNG4 extend beyond that in CNG5 and include the EMMA peak (Figure 1A). This supports the conclusion that the causal element for infarct volume, as cate (Figure II). In contrast to the previous candidate lists for Candq1 and coincident QTLs, in which no gene had clear ties to vascular biology, 7 genes in Dce1 can be at least provisionally tied to EC biology (Rabep2, Sh2b1, Cln3, Apobr, Il27, Mapk3, and Ppp4c; Figure 6). We focused on ECs because collaterogenesis occurs in the embryo by EC sprouting that involves vascular endothelial growth factor (VEGF)-A, Flk1 (VEGFR2), Clic4, and Notch signaling, because collateral extent is altered in adult mice with altered expression of endothelial nitric oxide synthase, DI4, connexin 40, and synectin, and because all of these genes are strongly expressed in ECs.

Two Dce1 genes listed contain nonsynonymous SNPs. The substitutions R298Q in Rabep2 and H120Y in Cln3 are predicted to be probably damaging for protein function (PolyPhen-2; scores >0.999–1.0). Both proteins are intriguing because they are involved in intracellular membrane trafficking, which is central to VEGF signaling. The amino acids at position 298 in Rabep2 and the corresponding position (K578) in Rabep1 are conserved among Rabep sequences. Replacement of K578 with R298Q in Rabep2 (Rabaptin-5beta) is a homolog of Rabep1 (Rabaptin-5), which in complex with Rabgef1 (Rabex-5) is an effector protein for Rab5 in the control of fusion of endocytic vesicles to form the early endosome and for Rab4 in subsequent control of rapid endocytic recycling. Rabep2 binds Rab5, Rab4, and Rabgef1, colocalizes with Rab5 on endosomal membranes, and is necessary for full endosome fusion activity in vitro. The amino acids at position 298 in Rabep2 and the corresponding position (K578) in Rabep1 are conserved among Rabep sequences. Replacement of K578 in Rabep1 by glutamine is predicted to be only possibly damaging (score 0.694 by PolyPhen-2), but there are no relevant experimental data on Rabep1 or Rabep2. Interestingly, Rabep2 contains the highest density of SNPs in Dce1 (Figure 6C). We are currently generating mice with disruption of Rabep2 because they have not been described.
Cln3, the gene responsible for juvenile Batten disease, codes an intracellular transmembrane protein thought to be involved in late endosomal trafficking and lysosome function. In mouse brain, Cln3 is expressed almost exclusively in ECs during the time of collaterogenesis (embryonic day [E] 14.5 to E18.5). We thus examined 2 different Cln3−/− mice but observed no effect on collateral extent (Online Figure III).

The only other nonsynonymous SNP in Dce1 is in the T-box transcription factor, Tbx6, giving the substitution Q331R, which is predicted to have no effect on protein function by Polyphen-2. This SNP and 3 others in Tbx6 also occur in ≥1 strains with high collateral extent (CAST/EiJ, NOD/ShiLtJ), suggesting that Tbx6 is an unlikely candidate gene (see SNP Analysis section). However, in a previous study, we determined relative expression levels of 106 genes, including all 28 in Dce1, in the pia mater dissected from the brains of B6 and Bc embryos at 3 time points spanning the time of collaterogenesis. Differential expression of Tbx6, Nfatc2ip, and Slx1b achieved significance (Bc>B6), with Tbx6 being the most strongly upregulated (2.9-fold). There are no reports of Tbx6 expression in ECs or interaction with VEGF. However, Tbx6 has been shown to cooperate with Notch intracellular domain in controlling the expression of Hes7 and Mesp2. Thus, Tbx6 could be involved in vessel patterning or collaterogenesis. We are currently generating mice with disruption of Tbx6 because they have not been described.

Nfatc2ip (Nip45) is a modulator of the type 2 T-helper cell immune response in mice, with the type 2 response being characteristic of Bc but not B6 mice. Nfatc2ip has a candidate SNP in its 3′UTR, which could explain the differential pial expression, although Nfatc2ip mRNA is not recognized as an experimentally supported miRNA target (TARbase version 12).

Figure 3. Introgression of Dce1 into the Bc genome rescues collateral number in skeletal muscle. Precapillary microcirculation in maximally dilated CNG4 and wild-type (WT) mice were filled with MicroFil. After fixation and optical clearing, collaterals between the epigastric and iliolumbar arteries, viewed from the peritoneal surface, were counted (red stars). Insets: Collateral zones at higher magnification. The homozygous B6 Dce1 allele restores 54% of the difference in collateral number between Bc and B6 WT mice.

Figure 4. Introgression of Dce1 into the Bc genome rescues collateral number in skeletal muscle. Precapillary microcirculation in maximally dilated CNG4 and wild-type (WT) mice were filled with MicroFil. After fixation and optical clearing, collaterals between the epigastric and iliolumbar arteries, viewed from the peritoneal surface, were counted (red stars). Insets: Collateral zones at higher magnification. The homozygous B6 Dce1 allele restores 54% of the difference in collateral number between Bc and B6 WT mice.
Slx1b is annotated as SLX1 structure–specific endonuclease subunit homolog B. There are no reports of either Nfatc2ip or Slx1b in ECs. We are currently generating mice with disruption of these genes, because they have not been described.

Jmjd5 (lysine-specific demethylase 8) was a particularly intriguing gene in the EMMA region because of possible roles in transcriptional and epigenetic regulation, and a strong argument was made for it as a candidate gene for Candq1. However, collateral extent in mice globally haploinsufficient, globally hypomorphic, or with EC-specific knockout of Jmjd5 were not different from wild-type littermates (Online Figure IV).

Evidence that Sh2b1, Apobr, Il27, and Ppp4c are potential candidate genes is summarized in the Online Data Supplement.

Figure 5. Introgression of Dce1 into the Bc genome rescues hindlimb perfusion after femoral artery ligation (FAL). A, Laser Doppler perfusion images of plantar region-of-interest (dotted line; quantified in B) before and after FAL. B, Seventy-three percent greater perfusion immediately after FAL in B6/B6 is in agreement with greater native collateral extent in skeletal muscle (Figure 4); larger deviations at day 3 and at day 10 are consistent with outward remodeling of a greater number of collaterals. Recovery of perfusion at day 10 was 83% greater in B6/B6. C, Severity of hindlimb use impairment and ischemic appearance were reduced/rescued on day 1 by 88% and 100%, respectively, and were less severe thereafter.

Figure 6. Haplotype, gene, and single nucleotide polymorphism (SNP) distribution in Dce1. A, Haplotype maps of B6 and Bc in Dce1 from the Mouse Phylogeny Viewer. Three haplotype blocks contain 11 of the 28 genes and 15 of the 27 candidate SNPs in Dce1 that distinguish Bc from B6. B, Genes within Dce1 that are involved in vascular biology, harbor candidate SNPs or occur in haplotype blocks. Rabep2, the only gene in all 3 categories, contains 7 candidate SNPs (5 intronic, 1 synonymous, 1 nonsynonymous) and lies in the small haplotype block number 2 (73 Kb), in which lie half of the candidate SNPs in Dce1. Numbers in parentheses indicate the number of candidate SNPs in each block. C, All SNPs (red bars) and candidate SNPs (black) are unevenly distributed in Dce1.
**Dce1** is partially syntenic with human 16p11.2, a locus strongly implicated in neurodegenerative disease, schizophrenia, autism, and severe early-onset obesity. However, we found no reports of vascular disease associated with 16p11.2 or hits in genome-wide association studies in 16p11 for vascular deficiencies.

**SNP Analysis**

SNPs in **Dce1** (Mouse Phenome Database; Mouse Genomes Project) were evaluated in the LookSeq pileups of deep sequencing data for Bc and B6 (see URLs in Online Table I). This gave 72 high-confidence SNPs. In a parsimonious model, a SNP responsible for low collateral extent would be present in Bc and AKR/J, a low-collateral strain (averaging 4.8 pial collaterals) that is phylogenically identical to Bc in **Dce1**, but would not be present in high-collateral strains. Accordingly, we examined each SNP location in high-collateral strains for which LookSeq data are available (LP/J, C3H/HeJ, CAST/EiJ, CBA/J, DBA/2J, FVB/NJ, NOD/ShiLtJ, and 129S1/SvImJ, plus A/J, a moderately low collateral strain; 7 collaterals). This yielded 27 candidate SNPs (Online Table III) present in Bc and AKR but not in high-collateral strains.

Four additional strains support these SNP identifications. C57BLKS/J, 129X1/SvJ, and KK/HiJ are high-collateral strains that have the B6 allele at each candidate SNP. SWR, the strain having the lowest number of collaterals after Bc (2.6 collaterals), is phylogenically identical to Bc in **Dce1** and has the Bc allele at each candidate SNP. However, SJL (high, at 19.2 collaterals) and NZW (medium, at 8.3) are also identical to Bc in **Dce1**. The strong influence of **Dce1** for the determination of collateral extent can apparently be overridden by loci elsewhere in the genome. These may include the loci on chromosomes 1, 3, and 8 identified in B6-x-Bc F2 crosses, but a SJL-x-SWR-F2 failed to find any significant QTL. Recently, Chu et al. applied a similar strategy to B6 and C3H/HeJ, which are similar in collateral number but different in collateral diameter and cerebral infarct volume, and identified a locus linked to infarct volume on chromosome 8 (*Cigv4*) that is coincident with *Canq4*. Mapping of number and diameter did not identify a significant QTL on chromosome 8 in this cross (however, 50% fewer F2 mice were phenotyped for collateral traits and those traits had 5-fold smaller ranges than infarct volume). The authors concluded that *Cigv4* affects infarct volume in a collateral-independent manner. Such a finding could have important ramifications. However, the conclusion remains uncertain because, compared with B6 mice, C3H/HeJ have smaller collateral diameters, trend smaller in number, and have lower blood pressure and hemoglobin content compared with B6 (Mouse Phenome Database). These differences are relevant because oxygen delivery across a collateral network after occlusion is proportional to (collateral number × diameter × arterial pressure × arterial oxygen content). Thus, even a small reduction in collateral extent in the C3H/HeJ strain would result in a significantly greater infarct volume after MCAO.

**Haplotype Analysis**

The genomes of classical inbred mouse strains are assemblies of blocks of DNA (haplotypes) identical by descent from ancestral populations and recognizable by their numbers and distributions of SNPs. Therefore, an alternative model is that the causal element in **Dce1** lies in a haplotype, possibly enclosing collateral-influencing elements that are linked or have interacting effects. **Dce1** contains 3 haplotype blocks between B6 and Bc (Figure 6A). These blocks contain 11 genes and 15 of the 27 candidate SNPs (Figure 6B). Of the 11 genes, only 2 (*Rabep2* and *Sh2b1*) can be linked, through the literature, to possible roles in vascular biology.

The genes identified for relevance to vascular biology, proximity to candidate SNPs, and location in Bc haplotype blocks are listed in Figure 6B. The only gene present in all 3 lists is *Rabep2*, in haplotype block 2, which contains 14 of the 27 candidate SNPs, 7 of which are in *Rabep2* (Figure 6C). These analyses thus converge on haplotype block 2 and suggest *Rabep2*, in particular, as a possible causal element in **Dce1**.

**Other Genetic Elements in **Dce1****

No conventional miRNAs were found in **Dce1**, but miRBase lists two 5p-tailed miRNAs or miRNAs located in intronic sequences at splice junctions. Nothing is known of their possible functions. NONCODE version 4 lists 51 long noncoding RNAs (≥2 exons, >200 base pairs), including 24 long intervening RNAs (ie, between coding genes) and 27 that overlap protein coding genes (reviewed in Ulitsky and Bartel). None of these seems to be annotated regarding regulatory or small peptide coding functions or strain differences in expression. The longest of these transcribes exons 3 to 11 of *Rabep2* and terminates at exons 14 and 15 of *Atp2a1* (transcribed from the opposite strand), adding to the intrigue of *Rabep2*. We also examined indels and structural and copy number variations in Bc versus B6 in the Mouse Phenome Database. All 60 indels (Sanger2 data set in the Mouse Phenome Database) were intergenic or intronic and usually located in highly repetitive sequences. Three structural variations were small (240, 225, and 3 bases; Sanger3 data set) and located intergenically or near the center of large introns. No copy number variations were listed (Ampen1 data set). None of these elements were studied further.

**Discussion**

The possibility that variation in the extent of native collateral circulation is an important determinant of variation in ischemic injury when acute arterial occlusion occurs or disease becomes manifest has historically been overlooked or minimized, with the exception of those who study or treat acute ischemic stroke (and references therein), some coronary investigators (and references therein), and among vascular surgeons who frequently encounter or perform arterial occlusions. This presumably extends, in part, from the small diameter typical of native collaterals in most healthy individuals that is beyond the resolution of digital angiography (>0.2 mm), from the misconception that such minute vessels cannot mediate significant flow in the acute setting until remodeling has occurred, and from the inability to experimentally change native collateral extent to test its importance. The latter restriction has begun to yield in recent studies in which collateral extent was found to vary widely in mice with differences in genetic background. This variation was strongly correlated with...
differences in blood flow, tissue injury and functional impairment after acute occlusion, and in chronic recovery of flow and function during collateral remodeling. However, whether these associations reflect a causal relationship could not be determined because it has not been possible to change native collateral extent while keeping genetic background constant. Even in inbred mice, including Bc and B6 strains, differences in genetic background have significant effects on arterial pressure, microvascular regulation, blood rheology, sensitivity of tissue to ischemia, immune response, inflammation, expression of many vascular-acting genes, and additional mechanisms that can each affect collateral flow and tissue injury after acute and chronic arterial occlusion independent of differences in native collateral extent.

The magnitude of the residual trait differences between Bc and B6 strains, including Bc and B6 congenic strains (Bc × B6 hosts for congenic number or diameter). Thus, congenic substitution background have significant effects on arterial pressure, microvascular regulation, blood rheology, sensitivity of tissue to ischemia, immune response, inflammation, expression of many vascular-acting genes, and additional mechanisms that can each affect collateral flow and tissue injury after acute and chronic arterial occlusion independent of differences in native collateral extent (see also Mouse Phenome Database).

In the present study, we addressed this uncertainty by generating a congenic set (CNG5, currently backcrossed to effectively N10; see Results section) with Dce1 allele dose–dependent differences in collateral extent that are essentially isogenic elsewhere in their genomes. We showed that congenic introgression of the Dce1 locus (737 Kb) of high-collateral B6 mice into the genome of low-collateral Bc mice confers near-B6 levels of native collateral number and diameter in the brain, as well as cerebral blood flow and infarct volume after MCAO. Furthermore, we obtained similar effects for native collaterals in skeletal muscle, as well as hindlimb blood flow, ischemic tissue appearance and use impairment, and recovery of these parameters immediately after and on subsequent days after FAL. This confirms previous studies showing that genetic effects on collateral extent apply to multiple tissues for a given genetic background. The magnitude of the residual trait differences between congenic B6 strains and B6 wild-type mice agree with previous estimates of the effect size of 3 additional minor QTLs for collateral number or diameter. Thus, congenic substitution of a discrete locus strongly reduced the tissue injury in models of stroke and peripheral artery disease that occurs in a strain with poor collateral extent. These findings demonstrate the importance of native collateral circulation in determining the severity of ischemic injury and capacity to recover blood flow and tissue function after arterial occlusion. They also validate this same conclusion suggested in previous studies examining different mouse strains. Thus, at least in healthy young adult strains examined thus far, genetic-dependent variation in native collateral extent strongly dominates the aforementioned collateral-independent factors in determining tissue injury.

Congenic introgression of the B6 allele of Dce1 into the Bc background rescued (restored) collateral number to 85% of wild-type B6 in brain, but only to 55% in skeletal muscle (Figures 1 and 4). This difference may reflect that muscle types differ greatly in arterial branch patterning and metabolic fiber type composition; that is, the external and internal oblique muscles and rectus abdominus, which compose the abdominal area that we studied, may not be representative of all skeletal muscles viewed as a whole, with regard to collateral extent. As stated in the Results section, we phenotyped this muscle area because, unlike other muscles but much like the pial circulation, its arterial architecture is arranged in 2 dimensions and thus can be imaged with high fidelity. This difference could also reflect differences in the timing of collaterogenesis that may exist among different tissue types during development and tissue growth or differences in contribution of Dce1 to the extent of native collateral circulation. Regarding the latter, 3 additional small-effect QTLs for collateral number were identified in our earlier study. However, despite the difference in rescue shown herein, these findings confirm previous work showing the dominance of the chromosomal 7 locus, now refined to Dce1, in determining the majority of the differences in collateral abundance in multiple tissues of the mouse.

Based on differential responses of skeletal muscle cells from B6 and Bc mice to hypoxia/ischemia in vitro, it was recently proposed that, in addition to controlling collaterogenesis, Candq1 also harbors a nonvascular, muscle–autonomous gene(s)—involved in plasticity and survival of muscle precursor cells and expression of vascular factors and their cognate receptors—that is important in the response to ischemia independent of differences in collateral extent. This proposal, together with the fact that Civq1 for infarct volume after MCAO is coincident with Candq1 and Lsq-1, thus suggests that the 3 QTLs cover multiple processes. If correct, the loci for these different processes should cease to overlap during congenic dissection of Candq1. This should result in a retention of the trait values for (ie, rescue of) collateral extent and collateral-dependent perfusion but in a reduction in the rescue of cerebral infarct volume after MCAO and hindlimb ischemic injury and use after FAL, or a separation of the 2 processes in certain congenic strains. However, our findings that congenic strains successively narrowing Candq1 from 27 to 737 kb retain the same values for all of these traits as the original interval does not support this proposal. Notwithstanding the possibility that this nonvascular process remains within this narrow locus, the current findings strengthen the conclusion that Candq1, Civq1, and Lsq-1 are QTLs for a single phenomenon, collateral extent, and that the common causal element, that is, one of the genetic elements presented in the Results section and in Figure 6, lies in Dce1.

The identification of Dce1 adds a fourth list of candidate genes in the search for the genetic basis of variation in collateral extent. The EMMA analysis of 21 strains, which confirmed our F2 cross, came very close to pinpointing Dce1. It may have missed because of this limited number of strains and possibly because of effects of strains A/J (very different from Bc at Dce1 but with low collateral number) and SJL/J (identical to Bc at Dce1 but with high collateral number). Similarly, the efforts to identify causal elements and candidate gene lists in Civq1 and Lsq-1 were deflected from Dce1 because they depended on the reasonable assumption that causal elements would be identical between A/J and Bc, and distinct from B6. This assumption was based on the finding that chromosome substitution strain 7, in which B6 chromosome 7 is replaced by A/J chromosome 7, nearly phenocopies A/J. However, the haplotype structure of A/J in Dce1 is quite distinct from those of Bc and B6 (1513 and 1526 SNPs, respectively; Mouse Phenome Database), and none of the candidate SNPs in Figure 6 is present in A/J. These findings, when combined with our previous observation that collateral number and diameter were reduced by 75% in B6 mice whose chromosome 7 had been substituted with that of the A/J strain, suggest that a different locus or one that interacts with Dce1 is present on chromosome 7.
of A/J mice. An obvious model that would reconcile these results would suppose an element on chromosome 7, outside of Dce1 and identical in Bc and A/J, that favors high collateral number if activated by a signal from an element in Dce1 having the B6 genotype. The sequences of neither Bc nor A/J would be capable of such activation.

In conclusion, the results of this study provide strong evidence that genetic-dependent variation in collateral extent underlies the 3 previously identified QTLs, Candrq1, Civq1, and Lsq-1, and is the major physiological factor responsible for differences in ischemic tissue injury in brain and hindlimb after arterial occlusion in B6, Bc, and related19 mouse strains. Our findings have also greatly narrowed the locus, denoted Dce1 (737 kb), and provided a gene list refined by in silico analysis and use of knockout mice to guide future investigation. Although the causative genetic element at Dce1 has not yet been identified, we speculate that it is a major driver gene or critical link in the pathway that governs collaterogenesis, a process that occurs late during gestation and determines collateral extent in the adult. Because the pathways governing angiogenesis during development are highly conserved, polymorphisms of this same genetic element or a related one in the signaling pathway controlling collaterogenesis may be important in the wide variation in collateral status in humans,4,11 that is, may constitute a risk allele for collateral insufficiency in brain and other tissues. This hypothesis, which is currently undergoing investigation in patients with acute ischemic stroke,23 is aided by the large effect size of Dce122,25 and the fact that the locus is contiguous on human chromosome 16. Finally, we have generated a congenic strain set (CNG5) with wide allele dose–dependent differences in collateral extent. Because these mice are isogenic elsewhere in the genome, they provide a unique strain set for future investigations of the collateral circulation, including modeling patients with poor, intermediate, and good collateral status.

Acknowledgments

We are grateful to Dr Kun Gao for phenotyping Cln3+/- Davidson mice; Drs Kirk Wilhelmsen, Fernando Pardo-Manuel de Villena, and Tim Wiltshire for helpful discussion; Drs Darla Miller and Fernando Pardo-Manuel de Villena for megaMUGA genotyping; and Drs Beverly Davidson and Takeshi Suzuki for generous gifts of Cln3+/- mice and Jmjd5-engineered mice, respectively.

Sources of Funding

Supported by National Institutes of Health National Heart, Lung, and Blood Institute (HL090655, HL111073) and by National Institute of Neurological Disorders and Stroke (NS083633) to J.E.F.

Disclosures

None.

References


Sealock et al

Genetics of Collateral Circulation and Stroke 669


42. Hutagaluh AH, Novick PJ. Role of Rab GTPases in membrane traffic and cell physiology. Physiol Rev. 2011;91:119–149.


Novelty and Significance

What Is Known?

- In most tissues, adjacent arterial trees are connected by a small number of arteriole-to-arteriole anastomoses, termed collateral vessels, that in the event of occlusion of an artery supplying one of the trees (e.g., in stroke, myocardial infarction, or peripheral artery disease) can have a significant protective effect.
- Collateral-dependent blood flow varies widely among healthy humans, that is, those without obstructive disease; however, the mechanisms responsible for this variation are unknown.
- The number and diameter of collaterals vary widely among strains of inbred mice, indicating that collateral extent is strongly influenced by genetic background; a quantitative trait locus (QTL) on chromosome 7 has been linked to the majority of this variation, but proof that this locus also causes the variation in tissue injury among the strains has not been obtained.

What New Information Does This Article Contribute?

- Congenic mice have been generated in which successively smaller regions of chromosome 7 of C57Bl/6J mice (B6; high collateral number and diameter) are introgressed into the BALB/cByJ strain (Bc; very low collateral number and diameter).
- The main genetic element determining variation in collateral extent in the cerebral pial and skeletal muscle circulation in these congenic strains has been localized to 737 Kb (Dce1) on chromosome 7.
- Substitution of the B6 allele of Dce1 for the Bc allele imparts to Bc mice high collateral extent and correspondingly greater tissue perfusion and protection in models of stroke and peripheral artery disease, proving that genetic variation in native collateral extent is the major determinant of variation in ischemic damage.

Although no studies have been performed, understanding the genetic determinants of the extent of the native collateral circulation in humans could have significant impact in assessing the risk for severe ischemic outcomes and suggesting lifestyle alterations before ischemic events, assessing suitability and choice of recanalization treatment after an event, and stratifying patients by genetically assessed risk in clinical trials. Previous work in mice identified a major 27-Mb-wide QTL for variation in collateral extent on chromosome 7. Others identified apparently identical QTLs for infarct volume in a stroke model and tissue loss in a peripheral artery disease model. Efforts to identify the causal elements in these QTLs relied on indirect analyses and inference to identify several candidate genes, none of which is a known angiogenic gene. The direct congenic approach used here has sharply localized the causal region (Dce1) and suggested new candidate genes, several of which are known to have vascular actions. The causal relationship between collateral extent and tissue response in congenic strains strongly suggests that the 3 QTLs all arise from a single phenomenon, collateral extent. This study provides a new basis for identifying collateral genes and, potentially, for assessing genetic influences on collateral extent in humans.
Congenic Fine-Mapping Identifies a Major Causal Locus for Variation in the Native Collateral Circulation and Ischemic Injury in Brain and Lower Extremity
Robert Sealock, Hua Zhang, Jennifer L. Lucitti, Scott M. Moore and James E. Faber

Circ Res. 2014;114:660-671; originally published online December 3, 2013; doi: 10.1161/CIRCRESAHA.114.302931

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/114/4/660

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2013/12/03/CIRCRESAHA.114.302931.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
Online supplemental material

Congenic fine-mapping identifies a major causal locus for variation in the native collateral circulation and ischemic injury in brain and lower extremity

Robert Sealock PhD¹, Hua Zhang MD¹,³, Jennifer L. Lucitti PhD¹,³, Scott M. Moore MD² and James E. Faber PhD¹,³

¹Department of Cell Biology and Physiology, ²Department of Surgery and ³The McAllister Heart Institute, School of Medicine, University of North Carolina at Chapel Hill

Detailed materials and methods
Online Table I: Principle websites used
Online Table II: Genes in Dce1
Online Table III: Candidate SNPs in Dce1
Online Figure I: Detailed map of Dce1
Online Figure II: Pial collateral remodeling and hindlimb perfusion
Online Figure III: Cln3⁻/⁻ mice
Online Figure IV: Jmjd5-engineered mice
Additional information on genes in Dce1 having putative vascular roles not discussed in Discussion.
Online supplemental references
Detailed materials and methods

Congenic mice
The input strains to the congenic strains were BALB/cByJ (Bc; Jackson Laboratories stock no. 001026), CXB3/ByJ (CXB3; #000353) and CXB4/ByJ (CXB4; #000354). CXB3 and CXB4 are recombinant inbred lines derived from BALB/cByJ and C57Bl/6ByJ (B6; #001139). The very different distributions of blocks of B6 and Bc genotypes in CXB3 and CXB4 have been mapped in detail. Of the 1078 genome-wide markers used in the mapping, 59.6% (CXB3) and 55.5% (CXB4) are the Bc allele. Congenic lines were generated using a speed congenics approach by backcrossing CXB3 and CXB4 to Bc mice to give F1 mice. Starting at the second backcross (F1 to Bc; N2 generation), PCR markers (SNPs, simple sequence length polymorphisms, and restriction fragment length polymorphisms) in the blocks of B6 genotype were used to select for mice that lost the B6 genotype outside of Candq1 but retained it within Candq1. Congenic lines from CXB3 and CXB4 were developed and maintained separately. Control B6 mice (C57Bl/6J; #000664) were obtained from Jackson Laboratories as new stocks and phenotyped contemporaneously with the congenic lines. The Mouse Phenome Database indicates no known genetic differences between BALB/c and BALB/cByJ strains in the region of interest in this study. Phenotyping of congenics and control B6 and Bc was done by a single observer (HZ). Cln3Δex7/8 mice and Cln3LacZ/LacZ (∆ex1-8) (both on C57Bl/6J background) were from Jackson Laboratories (#017895) and Dr. Beverly Davidson, respectively. Mice genetically engineered at exon 4 of Jmjd5 (B6 background) were from Dr. Takeshi Suzuki. They were bred with B6-Cre mice to produce mice null for Jmjd5 in endothelial cells (Jmjd5ECΔ), globally haploinsufficient (Jmjd5Δ/+) or globally hypomorphic (Jmjd5Neo/+). Gender does not influence collateral parameters, thus cohorts were roughly half male and half female, and 2-3 months old. All animal procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Cerebral collateral visualization and morphometry
Filling of the pre-capillary pial circulation for counting and analysis of collateral diameters was as described previously in detail, except that yellow Microfil was used instead of polyurethane. Briefly, under deep anesthesia [ketamine (100mg/kg ip) and xylazine (15 mg/kg ip)], the circulation was flushed with PBS-heparin via retrograde cannulation of the abdominal artery, then maximally dilated with adenosine and papaverine. The dorsal calvarium and adherent dura mater were removed to allow visual control. The thoracic aorta was cannulated retrograde and yellow MicrofilR (FlowTech, Inc, Carver, MA) with viscosity adjusted to minimize entrance into capillaries was infused under a stereo microscope. The tissue was then fixed with topical 4% paraformaldehyde. On digital images (Leica MZ16FA, Bannockburn, IL) of the pial circulation, collaterals connecting the anterior cerebral (ACA) and middle cerebral (MCA) artery trees in both hemispheres were counted and measured for lumen diameter (ImageJ, NIH, Bethesda, MD), defined as the collateral diameter at its point halfway between the distal arterioles of the MCA and ACA tree that it cross-connects.

Middle cerebral artery occlusion and infarct volume
Permanent occlusion of the right MCA trunk by micro-cautery midway between the zygomatic arch and the pinna of the ear has been described in detail. Briefly, mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg ip). A 4-5mm incision was made between the
right eye and ear, the temporal muscle was separated at its midpoint, and a ~2mm burr-hole over the trunk of the MCA in the right hemisphere was drilled with a handheld drill. The MCA was cauterized and transected, and the incision was closed. Mice received cephazolin and buprenorphine and were allowed to recover for 24 hours, then killed with an overdose of ketamine (100mg/kg ip) and xylazine (15 mg/kg ip). The brain was removed, cooled on dry ice until the tissue became stiff, and coronal slices (1mm) were incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC) in PBS at 37 degrees for 20 min, fixed with 1% PFA overnight. Infarct volume was calculated as the sum of the unstained volumes and expressed as a percent of total right cortical volume.9

Cerebral blood flow
24 hours after MCAO, mice were anesthetized (ketamine, 100 mg/kg) and retrogradely perfused via the thoracic aorta at 100 mmHg with PBS containing nitroprusside (0.1 mM) and papaverine (40 µg/ml) for maximal dilation for 2-3 minutes. 3 x 10^6 6 um diameter red-fluorescent 580/605 polystyrene FluoSpheres (Life Technologies) were sonicated vigorously and bolus-injected into the perfusion line just prior to the point of aortic cannulation and perfusion was continued for one minute. The brains were then removed, cooled on dry ice until the tissue became stiff, sliced (1 mm) and prepared for assessment of infarct volume as above and visualization of trapped microspheres by fluorescence microscopy. For each brain, microspheres in the infarct regions of the slices were counted in reverse-contrast images and summed. Density was calculated as the ratio of that count to the count in the corresponding zone on the contralateral, un-operated side.

Skeletal muscle collateral morphometry
Mice were anesthetized with ketamine (100mg/kg) and xylazine (10mg/kg), and heparinized. The heart was externalized through a partial sternotomy, with care not to damage the internal mammary arteries, aorta, and great vessels, as adequate abdominal wall filling requires patency of these vessels. A cardiotomy was then created in the left ventricle and a PE50 catheter with a tapered and flanged tip was inserted, with the tip positioned in the ascending aorta and secured with 6-0 suture placed around the aortic root. The heart was gently returned to its normal anatomic position prior to perfusion to prevent kinking of the aorta and its branches. Exsanguination and maximal dilation of the vasculature was accomplished with PBS containing sodium nitroprusside (40µg/ml) and papaverine (40µg/ml). Abdominal wall vasculature was then infused with Microfil (Flowtech, Inc, Carver, MA), with a composition of 8:1:1 (compound:diluent:curing agent) to optimize filling of the arterial and native collateral circulations while minimizing entrance into capillaries. Adequate and equivalent filling of each animal was ensured by hand-filling until Microfil first became visible within the femoral vein, which in pilot studies was shown to reliably reflect complete filling within the abdominal wall. Immediately after filling, 4% paraformaldehyde was instilled into the peritoneal cavity in order to fix the abdominal wall vasculature in the maximally dilated state. Following overnight fixation in 4% PFA, the abdominal wall musculature was dissected free from the viscera and overlying skin, and then underwent ethanol-based dehydration followed by methyl salicylate clearance to fully visualize filled abdominal wall vessels. Native collaterals were counted between the epigastric and iliolumbar arteries, as viewed from the peritoneal surface. Because slight differences in the completeness of filling was sometimes seen between the right and left sides, both sides were counted and the side with the most collaterals was used for data analysis. All vessel counting and data analysis was conducted in a blinded fashion.
**Femoral artery ligation (FAL) and blood flow**

Femoral artery ligation, laser Doppler flowmetry, and assessment of hindlimb use and ischemia appearance were assessed as described in detail.\(^\text{10,11}\) Briefly, animals were anesthetized with ketamine (100 mg/kg im) and xylazine (15 mg/kg im). Hair was removed from the hindquarters with a depilating cream, with care taken to avoid erythema. Body temperature was maintained at 37.0 ± 0.5°C. The femoral artery was exposed aseptically through a 2-mm incision and isolated from the femoral vein and nerve, with care taken to avoid damage to vessels or nerve or retracting the incision. The femoral artery was ligated just proximal to the trifurcation of the genu, saphenous and popliteal arteries. The superficial epigastric artery was also ligated, but venous structures were left intact. The wound was irrigated with sterile saline, the incision was closed, and cefazolin (50 mg/kg im) and buprenorphine were administered. Blood flow was measured with a scanning laser-Doppler perfusion imager (model LDI2-IR, Moor Instruments, Wilmington, DE) that was modified for high resolution and depth of penetration (2 mm) with an 830-nm-wavelength infrared 2.5-mW laser diode, 100-\_m beam diameter, and 15-kHz bandwidth. Animals were anesthetized with 1.125% isoflurane supplemented with 2:3 oxygen-air, and rectal temperature was closely maintained at 37.0 ± 0.5°C.

The functional results of ischemia were assessed by use and appearance scores at days 1, 3, and 10 after femoral artery ligation. Animals were individually inspected for a foot appearance score or index of ischemia: 0, normal; 1–5, cyanosis or loss of nail(s), where the score is dependent on the number of nails affected; 6–10, partial or complete atrophy of digit(s), where the score reflects number of digits affected; 11, partial atrophy of forefoot. Hindlimb use scores (index of muscle function) were obtained by observation: 0, normal; 1, no toe flexion; 2, no plantar flexion; 3, dragging foot.\(^\text{12}\)

**Jmjd5-deficient mice**

Mice harboring the floxed Jmjd5 allele (Jmjd5\(^{fl/fl}\)), mice constitutively deficient in Jmjd5 (Jmjd5\(^{Δ*/+}\)), and mice hypomorphic for Jmjd5 (Jmjd5\(^{neo/+}\)) were generous gifts from Takeshi Suzuki.\(^\text{5}\) To create a constitutive endothelial cell-specific Jmjd5 knockout, Jmjd5\(^{fl/fl}\) mice were crossed with a Cdh5-Cre mouse (Jackson Laboratory, #006137) and backcrossed to Jmjd5\(^{fl/fl}\) to produce Cdh5-Cre\(^{+/-}\);Jmjd5\(^{fl/+}\) (Jmjd5\(^{ECD}+)\) mice.
Online Table I. Principle web sites used.

miRNA search: miRBase
  http://www.mirbase.org/search.shtml
miRNA targets: TarBase 6.0
SNPs & Indels:
  Jackson Laboratories Mouse Phenome database
  http://phenome.jax.org/db/q?rtn=snp/ret1&uproj=CGD-IMP2&usampreg=1
  Sanger Mouse Genomes Project
  http://www.sanger.ac.uk/cgi-bin/modelorgs/mousegenomes/snps.pl
Sequence pileups for mouse strains: Sanger Mouse Genomes Project
  http://www.sanger.ac.uk/cgi-bin/modelorgs/mousegenomes/lookseq/index.pl?show=1:10000000-10000000,paired_pileup&win=100&lane=129S1.bam&width=700
Non-synonymous SNP effect predictions:
  Polyphen-2
  http://genetics.bwh.harvard.edu/pph2/
  Provean
  http://provean.jcvi.org/seq_submit.php
Mouse phylogeny viewer
  http://msub.csbio.unc.edu/
Gene and transcript lists:
  NCBI map viewer
  UCSC Genome Browser
  http://genome.ucsc.edu/cgi-bin/hgGateway?clade=vertebrate&org=Mouse&db=0&hgsid=76761400
Synteny: NCBI homology maps
Remapping between Builds 37 and 38 (remaps between mm9 and mm10)
Regulatory features search: Ensembl
  http://useast.ensembl.org/Mus_musculus/Info/Index
Human GWAS studies of 16p11.2
  http://www.genome.gov/gwastudies/
Long noncoding RNA database (mouse): NONCODE
  http://159.226.118.44/NONCODEv3/index.htm
Relative expression levels: BioGPS
  http://biogps.org/#goto=welcome
**Online Table II**: Genes in *Dce1*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Start location</th>
<th>Build 37</th>
<th>Build 38</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xpo6</td>
<td>7:133245232</td>
<td>7:126101718</td>
<td></td>
</tr>
<tr>
<td>Sbk1</td>
<td>7:133416133</td>
<td>7:126272619</td>
<td></td>
</tr>
<tr>
<td>Lat</td>
<td>7:133507341</td>
<td>7:126363827</td>
<td></td>
</tr>
<tr>
<td>Spns1</td>
<td>7:133513574</td>
<td>7:126370060</td>
<td></td>
</tr>
<tr>
<td>Nfatc2ip</td>
<td>7:133526368</td>
<td>7:126382854</td>
<td></td>
</tr>
<tr>
<td>Cd19</td>
<td>7:133551962</td>
<td>7:126408448</td>
<td></td>
</tr>
<tr>
<td>Rabep2</td>
<td>7:133572281</td>
<td>7:126428767</td>
<td></td>
</tr>
<tr>
<td>Atp2a1</td>
<td>7:133589374</td>
<td>7:126445860</td>
<td></td>
</tr>
<tr>
<td>Sh2b1</td>
<td>7:133610508</td>
<td>7:126466994</td>
<td></td>
</tr>
<tr>
<td>Tufm</td>
<td>7:133630869</td>
<td>7:126487355</td>
<td></td>
</tr>
<tr>
<td>Atxn2l</td>
<td>7:133635222</td>
<td>7:126491708</td>
<td></td>
</tr>
<tr>
<td>Eif3c</td>
<td>7:133690425</td>
<td>7:126546911</td>
<td></td>
</tr>
<tr>
<td>Cln3</td>
<td>7:133714914</td>
<td>7:126571400</td>
<td></td>
</tr>
<tr>
<td>Apobr</td>
<td>7:133728522</td>
<td>7:126585008</td>
<td></td>
</tr>
<tr>
<td>Il27</td>
<td>7:133732809</td>
<td>7:126589295</td>
<td></td>
</tr>
<tr>
<td>Nupr1</td>
<td>7:133766760</td>
<td>7:126623246</td>
<td></td>
</tr>
<tr>
<td>Ccdc101</td>
<td>7:133792823</td>
<td>7:126649309</td>
<td></td>
</tr>
<tr>
<td>Sult1a1</td>
<td>7:133816384</td>
<td>7:126672870</td>
<td></td>
</tr>
<tr>
<td>Slx1b</td>
<td>7:133832441</td>
<td>7:126688927</td>
<td></td>
</tr>
<tr>
<td>Bola2</td>
<td>7:133839514</td>
<td>7:126696000</td>
<td></td>
</tr>
<tr>
<td>Coro1a</td>
<td>7:133843288</td>
<td>7:126699774</td>
<td></td>
</tr>
<tr>
<td>Mapk3</td>
<td>7:133903140</td>
<td>7:126759626</td>
<td></td>
</tr>
<tr>
<td>Gdpd3</td>
<td>7:133909928</td>
<td>7:126766414</td>
<td></td>
</tr>
<tr>
<td>Ypel3</td>
<td>7:133920489</td>
<td>7:126776975</td>
<td></td>
</tr>
<tr>
<td>Tbx6</td>
<td>7:133924997</td>
<td>7:126781483</td>
<td></td>
</tr>
<tr>
<td>Ppp4c</td>
<td>7:133929382</td>
<td>7:126785868</td>
<td></td>
</tr>
<tr>
<td>Aldoa</td>
<td>7:133938748</td>
<td>7:126795234</td>
<td></td>
</tr>
<tr>
<td>Fam57b</td>
<td>7:133960399</td>
<td>7:126816885</td>
<td></td>
</tr>
</tbody>
</table>
Online Table III: Candidate SNPs in *Dce1*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Identifier</th>
<th>Build 37</th>
<th>Build 38</th>
<th>B6</th>
<th>Bc</th>
<th>Consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xpo6</td>
<td>rs33063624</td>
<td>7:133295562</td>
<td>7:126152048</td>
<td>C</td>
<td>T</td>
<td>Intron</td>
</tr>
<tr>
<td>Xpo6</td>
<td>rs25093239</td>
<td>7:133307606</td>
<td>7:126164092</td>
<td>C</td>
<td>T</td>
<td>Intron</td>
</tr>
<tr>
<td>Xpo6</td>
<td>rs227712042</td>
<td>7:133387775</td>
<td>7:126195261</td>
<td>C</td>
<td>T</td>
<td>Intron</td>
</tr>
<tr>
<td></td>
<td>rs33068721</td>
<td>7:13372819</td>
<td>7:126229305</td>
<td>A</td>
<td>G</td>
<td>Intergenic</td>
</tr>
<tr>
<td></td>
<td>rs33072147</td>
<td>7:13380810</td>
<td>7:126237296</td>
<td>G</td>
<td>C</td>
<td>Intergenic</td>
</tr>
<tr>
<td></td>
<td>rs33073821</td>
<td>7:133513295</td>
<td>7:126369781</td>
<td>C</td>
<td>G</td>
<td>Intergenic</td>
</tr>
<tr>
<td>Spns1</td>
<td>rs33077365</td>
<td>7:133516160</td>
<td>7:126372646</td>
<td>G</td>
<td>A</td>
<td>Intron</td>
</tr>
<tr>
<td>Nfatc2ip</td>
<td>rs33079501</td>
<td>7:133526623</td>
<td>7:126383109</td>
<td>G</td>
<td>A</td>
<td>3' UTR</td>
</tr>
<tr>
<td></td>
<td>rs33078730</td>
<td>7:133561029</td>
<td>7:126417515</td>
<td>G</td>
<td>A</td>
<td>Intergenic</td>
</tr>
<tr>
<td></td>
<td>rs243867849</td>
<td>7:133568397</td>
<td>7:126424883</td>
<td>G</td>
<td>A</td>
<td>Intergenic</td>
</tr>
<tr>
<td></td>
<td>rs227824226</td>
<td>7:133569845</td>
<td>7:126426331</td>
<td>A</td>
<td>G</td>
<td>Intergenic</td>
</tr>
<tr>
<td></td>
<td>rs246194872</td>
<td>7:133570124</td>
<td>7:126426610</td>
<td>C</td>
<td>A</td>
<td>Intergenic</td>
</tr>
<tr>
<td></td>
<td>rs215526206</td>
<td>7:133570533</td>
<td>7:126427019</td>
<td>G</td>
<td>T</td>
<td>Intergenic</td>
</tr>
<tr>
<td></td>
<td>rs259462571</td>
<td>7:133571315</td>
<td>7:126427801</td>
<td>C</td>
<td>T</td>
<td>Intergenic</td>
</tr>
<tr>
<td>Rabep2</td>
<td>rs236423818</td>
<td>7:133573990</td>
<td>7:126430476</td>
<td>C</td>
<td>A</td>
<td>Intron</td>
</tr>
<tr>
<td>Rabep2</td>
<td>rs258037007</td>
<td>7:133574019</td>
<td>7:126430505</td>
<td>A</td>
<td>G</td>
<td>Intron</td>
</tr>
<tr>
<td>Rabep2</td>
<td>rs261416329</td>
<td>7:133577552</td>
<td>7:126434038</td>
<td>C</td>
<td>T</td>
<td>Intron</td>
</tr>
<tr>
<td>Rabep2</td>
<td>rs33083620</td>
<td>7:133581943</td>
<td>7:126438429</td>
<td>C</td>
<td>T</td>
<td>Cs</td>
</tr>
<tr>
<td>Rabep2</td>
<td>rs33080487</td>
<td>7:133583723</td>
<td>7:126440209</td>
<td>G</td>
<td>A</td>
<td>Cn R298Q</td>
</tr>
<tr>
<td>Rabep2</td>
<td>rs254286371</td>
<td>7:133584920</td>
<td>7:126441406</td>
<td>A</td>
<td>G</td>
<td>Intron</td>
</tr>
<tr>
<td>Rabep2</td>
<td>rs213531450</td>
<td>7:133586656</td>
<td>7:126443142</td>
<td>T</td>
<td>C</td>
<td>Intron</td>
</tr>
<tr>
<td>Atp2a1</td>
<td>rs233753984</td>
<td>7:133591983</td>
<td>7:126448469</td>
<td>A</td>
<td>C</td>
<td>Intron</td>
</tr>
<tr>
<td></td>
<td>rs227071387</td>
<td>7:133656437</td>
<td>7:126512923</td>
<td>G</td>
<td>T</td>
<td>Intergenic</td>
</tr>
<tr>
<td></td>
<td>rs234324273</td>
<td>7:133687493</td>
<td>7:126543979</td>
<td>C</td>
<td>G</td>
<td>Intergenic</td>
</tr>
<tr>
<td>Cln3</td>
<td>rs33089967</td>
<td>7:133723863</td>
<td>7:126580349</td>
<td>G</td>
<td>A</td>
<td>Cn H120Y</td>
</tr>
<tr>
<td></td>
<td>rs33092841</td>
<td>7:133747498</td>
<td>7:126603984</td>
<td>A</td>
<td>G</td>
<td>Intergenic</td>
</tr>
<tr>
<td>Nupr1</td>
<td>rs233244480</td>
<td>7:133769153</td>
<td>7:126625639</td>
<td>C</td>
<td>T</td>
<td>5' UTR</td>
</tr>
</tbody>
</table>

If the functional difference between B6 and Bc at *Dce1* should be attributable to one or more SNPs, the listed SNPs were judged to be candidates because they are present in Bc and AKR/J vs B6, but are not found in any of the high collateral number strains for which LookSeq pileup data is available (LP/J, C3H/HeJ, CAST/EiJ, CBA/J, DBA/J, FVB/NJ, NOD/ShiLtJ, and 129S1/SvImJ). An additional 42 SNPs found in Bc and AKR/J were judged not to be candidates because they are also present in one or more of the high collateral number strains.
Online Figure I. Maps of Dce1. A. EMMA and Dce1 compared. The EMMA algorithm\textsuperscript{13} was applied to Cangd1 and 21 inbred mouse stains, yielding a highly significant peak at 132.356 to 132.817 Mb.\textsuperscript{8,14} Dce1 lies on the right shoulder of the EMMA peak, beginning at 133.229, or only 0.412 Mb further 3'. B. Detailed map showing the important markers (black text) that define Dce1, which is the zone of overlap between the introgressed regions of CNG5 and CNG6. B6 genotype is in black, Bc background genotype is gray, and regions of uncertain genotype between limiting markers are in white. Markers that delimit the introgressed regions outside of the overlap are in gray text. Locations in megabases beneath each marker are from MGSCv37 (mm9) (upper) and GRCm38 (mm 10) (lower).
Online Figure II. Collateral remodeling (A) and recovery from FAL (B) in congenic and wild-type strains do not differ. A, Pial collateral remodeling. Hatched bars: baseline diameter prior to MCAO. Open bars: Remodeled diameter 24 hrs after middle cerebral artery occlusion (MCAO), which is maximal by day-3, is comparable between CNG5 mice and the corresponding wild-types. This is expected since amount of remodeling is primarily determined by baseline collateral diameter (solid bars) before MCAO. Wildtype data are from Zhang et al.7 B, Laser-Doppler perfusion imaging values (perfusion units) after femoral artery ligation do not differ between CNG5- and CNG4-B6/B6 strains, and both are comparable to wild type C57BL/6 mice.11 Values are mean ± SEM for n number of animals. B6, C57BL/6. Bc, BALB/c. Data for CNG4-B6/B6 are from Figure 5.
Online Figure III. Collateral number and diameter are not affected by absence of Cln3. Collateral number and diameter were measured in homozygous Cln3ΔEx7/8 mice (JAX, stock number 017895, Jackson Laboratories) and homozygous Cln3ΔEx1-8 mice, obtained from Dr. Beverly Davidson, University of Iowa, both stated by suppliers to be on the B6 background. Neither parameter was significantly different from B6 control mice (p > 0.1 in all cases). Values in this and subsequent figures are mean ± SEM. Numbers of mice in this and subsequent figures are number of animals. JAX: 5 female and 5 male, 2-3 mos-age, 20-29 grams. IOWA: 4 male and 3 female, 2-7 mos-age, 23-35 grams.
Online Figure IV. Differences in expression of *Jmjd5* do not impact collateral extent. *Jmjd5*^fl/fl*, *Jmjd5*^neo/+* and *Jmjd5*Δ/+ were obtained from Dr. Takeshi Suzuki,5 while *Jmjd5*ECΔ mice were made from *Jmjd5*^fl/fl* mice at UNC (see Online Data Supplement – Detailed materials and methods). The cerebral vasculature of adult (3-4 month old) *Jmjd5*-engineered mice was filled with yellow Microfil™ and analyzed as in “Cerebral collateral visualization and morphometry” above. Collateral number and diameter in both hemispheres were quantified in endothelial cell-specific knockout (*Jmjd5*ECΔ), global hypomorphic (*Jmjd5*neo/+), and global haploinsufficient (*Jmjd5*Δ/+) mice and compared with wild-type littermates. Collateral number was similar in all groups. Although moderate *Jmjd5* knockdown (*Jmjd5*neo/+) slightly increased collateral diameter compared to the wild-type littermate (*, p<0.05 by 2-tailed t-test), diameter was unchanged in the other groups. These data demonstrate that *Jmjd5* has negligible control over collaterogenesis, consistent with its physical location outside of *Dce1* (0.6 Mb 5’ of *Dce1*).
Additional information on putative candidate genes in *Dce1* not discussed in the main text.

**SH2B1** (src-homology-2 adapter protein 1) is an SH2 domain-containing adapter protein for SRC and JAK2 kinases. SRC transduces signals from VEGFR2 leading to modulation of focal adhesions and VECadherin. Jak2 activation promotes endothelial cell migration and angiogenesis.\(^{15}\) *SH2B1* is expressed at modest levels in most tissues, including endoglin-positive endothelial cells (BioGPS). Polymorphisms in an *SH2B1* obesity locus have been associated with reduced insulin-stimulated NO synthase activity in endothelial cells.\(^{16}\)

**Ppp4c** is a serine, threonine phosphatase expressed in vasculature and other cells. Morpholino knockdown in zebrafish leads to intersegmental endothelial cell tracks that look normal but are not tubulated (ie, they don’t accept blood from the dorsal aorta).\(^{17}\)

**APOBR** is a receptor that binds to the apolipoprotein B48 of dietary triglyceride (TG)-rich lipoproteins. It is expressed in a variety of immune cells (neutrophils, macrophages, etc.) and at low levels in endothelial cells. The ApoB protein particle in ApoB-containing lipoproteins negatively regulates angiogenesis via downregulation of VEGFR1.\(^{18}\) Multiple additional papers attest to the negative effects of ApoB on endothelial cells, but the receptor(s) is unknown.

**IL-27** is a broadly active cytokine from immune cells and vascular smooth muscle cells. Numerous papers and reviews\(^{19,20}\) attest to its roles in anti-angiogenesis, promotion of a proadhesive state for endothelial cells, “global changes in HUVEC gene expression in response to IL-27”,\(^{21}\) induction of anti-angiogenic CXCL9 and CXCL10 expression in endothelial cells, and pro-inflammatory actions.
Online References


