Constitutively active Notch4 receptor elicits brain arteriovenous malformations through enlargement of capillary-like vessels

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Arteriovenous (AV) malformation (AVM) is a devastating condition characterized by focal lesions of enlarged, tangled vessels that shunt blood from arteries directly to veins. AVMs can form anywhere in the body and can cause debilitating ischemia and life-threatening hemorrhagic stroke. The mechanisms that underlie AVM formation remain poorly understood. Here, we examined the cellular and hemodynamic changes at the earliest stages of brain AVM formation by time-lapse two-photon imaging through cranial windows of mice expressing constitutively active Notch4 (Notch4*). AVMs arose from enlargement of preexisting microvessels with capillary diameter and blood flow and no smooth muscle cell coverage. AV shunting began promptly after Notch4* expression in endothelial cells (ECs), accompanied by increased individual EC areas, rather than increased EC number or proliferation. Alterations in Notch signaling in ECs of all vessels, but not arteries alone, affected AVM formation, suggesting that Notch functions in the microvasculature and/or veins to induce AVM. Increased Notch signaling interfered with the normal biological control of hemodynamics, permitting a positive feedback loop of increasing blood flow and vessel diameter and driving focal AVM growth from AV connections with higher blood velocity at the expense of adjacent AV connections with lower velocity. Endothelial expression of constitutively active Notch1 also led to brain AVMs in mice. Our data shed light on cellular and hemodynamic mechanisms underlying AVM pathogenesis elicited by increased Notch signaling in the endothelium.

Significance

Brain arteriovenous malformations are focal lesions of enlarged, tangled vessels that shunt blood from arteries directly to veins. They can cause ischemia, hemorrhage, disability, and death, particularly in young people, accounting for 50% of childhood stroke. The molecular etiology of the disease remains poorly understood, hindering the development of therapeutic treatments. Here, we report that, in an animal model, the lesion arises from the enlargement of capillary-like vessels. Notch signaling in the endothelium of microvasculature and veins is critical for the disease initiation by increasing cell areas but not proliferation. Blood flow mediates disease progression by a positive feedback of increasing flow and vessel diameter. Our data shed light on the mechanism underlying the pathogenesis of this devastating disease.


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ECs results in spontaneous AVMs in mice (15–17, 21). Although this work has facilitated new research directions regarding the Notch pathway in AVMs, how aberrant Notch signaling leads to AVM remains unknown. Elucidating the initiating structural events that lead to AV shunt formation would provide insight into AVM pathogenesis.

A major obstacle to understanding AVM pathogenesis has been the inability to observe AVM formation and blood flow over time with high-resolution in vivo imaging. Here, we examined AVM formation in Tie2-Notch4* mice from the initial genetic event, illuminating disease progression using “5D” two-photon imaging, which allows high-resolution live imaging of vascular architecture (3D) and blood velocity (the fourth dimension) over time (the fifth dimension) (16, 22, 23). Our findings provide insights into the mechanism of brain AVM formation.

Results

Notch4*-Mediated AV Shunts Arise from the Enlargement of Capillary-Like Vessels. To determine the developmental origin of AVMs, we performed longitudinal live imaging in the brains of Tie2-Notch4* mice, in which Notch4* expression was repressed until birth by tetracycline treatment. We implanted cranial windows over the right parietal cortex of mice at postnatal day 7 (P7) and used in vivo two-photon microscopy to record vessel diameter and blood velocity over time. We measured lumen diameters of vessels connecting arteries and veins, or AV connections, at their narrowest point. We defined measurements >12.5 μm as AV shunts because AV connections of this diameter were not observed in control mice after P12 (SI Appendix, Fig. S1). We found that AV shunts developed from capillary-like vessels in Tie2-Notch4* mice beginning at ~P12 (Fig. 1 and SI Appendix, Figs. S2 and S3). Of 109 of the AV connections that we tracked, 41 (38%) grew to >12.5 μm between P14 and P25 in 12 mutants, and 4 of 109 (4%) grew to >30 μm (Fig. 1F and Movies S1 and S2). However, the initial diameter did not differ from that of controls (5.4 ± 1.6 vs. 5.3 ± 1.6 μm, Fig. 1B). We saw no correlation between the final and initial diameters of AV shunts (SI Appendix, Fig. S4). We conclude that AV shunts in Tie2-Notch4* mice enlarge from capillary-diameter AV connections, but that only a few continue to grow into large AVMs.

We examined the hemodynamics of AV shunts at their onset and as they enlarged. Using 5D two-photon imaging (16, 23), we simultaneously analyzed red blood cell velocity and AV connection diameter (Fig. 1C–E and SI Appendix, Fig. S2). For individual AV connections, we defined increased velocity and diameter by changes >2× the SD in control capillaries: 0.82 mm/s for velocity and 1.33 μm for diameter. (F and G) Whole-mount immunostaining of P12 surface cerebral cortex vasculature for α-smooth muscle actin (green) and VE-cadherin (red) in mutant (F) and control (G). Arrowheads indicate AV shunt (18 AV shunts in seven mice); corresponding lectin perfused vessels are shown (F’ and G’). (Scale bars, 50 μm.)

![Fig. 1. Tie2-tTA;TRE-Notch4* mice developed AV shunts through enlargement of capillary-like vessels. (A and A’) Two-photon time-lapse imaging of FITC-dextran–labeled AV connections in the cerebral cortex through a cranial window. An AV shunt (red arrowheads and green arrows) developing from a capillary-diameter AV connection between P14 and P19. (B) Distribution of initial AV connection diameter (smallest lumen diameter) in AV connections in mutants (109 AV connections in 12 mice) and controls (36 AV connections in 5 mice, ages P8–P11). (C and D) Blood velocities (dotted lines) and lumen diameters (solid lines) measured over time in the capillary-like AV connections of (C1–C3) Notch4* mice and (D) controls. (E) Time of initial velocity increase vs. time of initial diameter increase for individual AV connections. Dotted red line represents simultaneous increase of blood velocity and vessel diameter. Points on the dotted vertical black line represent capillary-like vessels that enlarged but did not exhibit a significant increase in velocity. Increased velocity and diameter were defined by changes >2× the SD in control capillaries: 0.82 mm/s for velocity and 1.33 μm for diameter. (F and G) Whole-mount immunostaining of P12 surface cerebral cortex vasculature for α-smooth muscle actin (green) and VE-cadherin (red) in mutant (F) and control (G). Arrowheads indicate AV shunt (18 AV shunts in seven mice); corresponding lectin perfused vessels are shown (F’ and G’). (Scale bars, 50 μm.)](https://www.pnas.org/cgi/doi/10.1073/pnas.1415316111)
connection enlargement either coincided with or preceded the initial increase in velocity (Fig. 1C, 8/18 vessels in four mice). Some capillary-like vessels enlarged without a significant increase in velocity (Fig. 1C, 2/18 vessels in four mice), whereas others exhibited an increase in neither diameter nor velocity (Fig. 1C, 6/18 vessels in four mice). Neither velocity nor diameter increased in controls (Fig. 1D, 18 vessels in four mice). Data are summarized in Fig. 1E. Thus, our data suggest that blood velocity in AV connections that grew into AV shunts is initially capillary-like and becomes abnormally elevated only when connections enlarge.

As capillaries lack smooth muscle cell (SMC) coverage, we performed staining for α-smooth muscle actin (SMA) at P12, when AV shunts were first apparent. We identified 10 AV shunts from five mice that lacked SMA staining (Fig. 1F). In contrast, adjacent arteries were positive for SMA staining, similar to controls (Fig. 1G and SI Appendix, Fig. S5). We observed, as we have previously published (15–17), that advanced AVMs exhibited increased SMA staining. Thus, the absence of SMCs in the initial AV shunts is consistent with a capillary origin of AVMs in the Tie2-Notch4* mice.

We next examined the expression of Notch4* during the initiation of AV shunting. We previously demonstrated that Tie2-tTA effectively and specifically drives gene expression throughout the brain endothelium, through activation of the tetracycline repressor element (TRE) by the tetracycline transactivator (TTA) (17). Here we performed whole-mount staining of brain vasculature for the Notch4 intracellular domain (ICD), which also detects Notch4*, without the extracellular domain. We detected elevated Notch4-ICD throughout vasculature (reflecting Notch4* expression) in the mutants over the controls (SI Appendix, Fig. S6).

To examine the kinetics of Notch4* expression in real time during AV shunt formation, we analyzed TRE-H2b-eGFP, Tie2-tTA; TRE-Notch4* mice. TRE-H2b-eGFP reports TTA activity and thus TRE-Notch4* expression. As expected, there was a positive correlation between H2b-eGFP and Notch4* expression in fixed samples, validating the reporter assay (SI Appendix, Fig. S7).

We found that TTA activity preceded, by about 2 d, the enlargement in each segment (branch point to branch point) of the AV connections measured (Fig. 2A–C). Approximately 80% (159/198) of segments were positive for the TRE-H2b-eGFP reporter (Fig. 2D and SI Appendix, Fig. S8). About 50% (83/159) of the positive segments enlarged, and 40% (59/159) of these became part of AV shunts ≥12.5 μm in diameter. Only 15% (24/159) regressed (Fig. 2D and SI Appendix, Fig. S8). In contrast, only 10% of Notch4* negative segments enlarged (4/39); 80% (31/39) regressed. These data suggest that Notch4* expression promptly leads to AV shunting.

**EC Area, but Not Number or Proliferation, Increased in AV Shunt Formation.** To examine the cellular changes underlying the initiation of AV shunts, we used the R26R-confetti reporter (24) to track the position and number of labeled cells. We activated the reporter from P1 to P5 by tamoxifen (TAM) induction of Cdh5 (PAC)-CreERT2 in Tie2-tTA; TRE-Notch4*; Cdh5(PAC)-CreERT2; R26R-Confetti mice and littermate controls (with either TRE-Notch4* or Tie2-tTA, but not both), allowing the tracking of Notch4* expression during AV shunt formation (Fig. 3A). Surprisingly, we saw no detectable increase in cell number in the initial 48 h of AV shunt formation (Fig. 3A and B and SI Appendix, Fig. S9), despite a two- to threefold increase in AV shunt diameter (Fig. 3B).

Because our analysis was powered to detect as little as a twofold difference in cell number, these data suggest that AV shunt growth does not occur through a simple increase in EC number. To investigate whether cell proliferation is involved in the initiation of AV shunts, we performed BrdU analysis of tomato-lectin–labeled ECs. This assay showed no significant difference in the number of BrdU+ ECs (labeled for 48 h leading up to the analysis, Fig. 3C and D and SI Appendix, Fig. S10). Furthermore, we found no BrdU+ EC cells in 10/15 AV shunts with diameters ≥12.5 μm, again suggesting that the initiation of the AV shunt does not require increased EC proliferation.

We hypothesized that a change in the area covered by individual ECs might support the capillary enlargement in Tie2-Notch4* mice. To test this, we stained whole-mount cortical sections of mutants and controls for vascular endothelial cadherin (VE-cadherin, Cdh5) during the initial stages of AV shunt formation (P12). We found that the area covered by individual ECs increased more than threefold during the early stages of AV shunt formation (Fig. 3E and F). We asked where in the vascular tree Notch4* acts to elicit AV shunt formation. Our in vivo imaging suggested that a defect in the capillaries or microcirculation led to AV shunts. Because no capillary EC-specific TTA driver has been reported, we used Cdh5(PAC)-CreERT2 to induce Notch4* in all ECs and a recently developed arterial-specific line, BMMx(PAC)-CreERT2, to examine the effects of Notch4* specifically in the arterial ECs.

**Notch4* Expression in ECs of Arteries Was Not Sufficient to Induce AV Shunt Formation.** We asked where in the vascular tree Notch4* acts to elicit AV shunt formation. Our in vivo imaging suggested that a defect in the capillaries or microcirculation led to AV shunts. Because no capillary EC-specific TTA driver has been reported, we used Cdh5(PAC)-CreERT2 to induce Notch4* in all ECs and a recently developed arterial-specific line, BMMx(PAC)-CreERT2, to examine the effects of Notch4* specifically in the arterial ECs. We first confirmed the expression of the two inducibleCre lines in our system. Cdh5(PAC)-CreERT2 efficiently activated a mTmG Cre-reporter throughout the vascular endothelium, but not in circulating blood cells (SI Appendix, Figs. S11 and S12). The ROSA:LnL-ITA system effectively induced Notch4* expression, resulting in even higher protein levels than Tie2-tTA-driven expression (SI Appendix, Fig. S13 and Movie S3) (25). As demonstrated in other tissues (26), BMMx(PAC)-CreERT2 efficiently activated the same reporter throughout the arterial trunk.
Appendix, Table S1). In these mice, 82.8% of P7 mice by P18 after TAM injection at detested enlarged AV connections in mice have developed AV shunts (17). As expected, we readily detected Notch4* expression in the endothelium of BMX-CreERT2 positive arteries by injecting TAM at P7–P8, because we found that 62.7 ± 27.5% of total AV connections were enlarged by P18 (Fig. 4D and SI Appendix, Table S2). In contrast, in controls with one remaining Rbpj± allele, 22.3 ± 17.2% of total AV connections were enlarged (Fig. 4E and SI Appendix, Table S2). Thus, Notch4*-induced AV shunts require Notch signaling via Rbpj.

Because AV shunts were Rbpj-dependent, we tested whether blocking arterial Notchsignaling suppresses AV shunt formation. We deleted Rbpj by injecting TAM at P7–P8 in arterial ECs of Tie2-tTA;TRE-Notch4*; BMX(PAC)-CreERT2;Rbpj± mice. Excision of both Rbpj alleles using BMX(PAC)-CreERT2 did not suppress the AV shunt phenotype, because we found that 62.7 ± 27.5% of total AV connections were enlarged by P18 (Fig. 4F and SI Appendix, Table S2), which is no different from mice with excision of only one Rbpj allele [60.1 ± 36.4% of total AV connections enlarged (SI Appendix, Table S2)]. Taken together, these data suggest that the onset of AV shunts is mediated through Rbpj in the ECs of microvasculature and veins but not BMX(PAC)-CreERT2–positive arteries.

AV Shunts with Higher Velocity Increased in Velocity and Diameter, Whereas Adjacent Vessels with Lower Velocity Decreased in Velocity and Diameter. Despite Notch4* expression throughout the vascular endothelium, not all AV connections developed into AV shunts, and not all AV shunts continued to enlarge. We observed that AV connections were enlarged. By contrast, the BMX-CreERT2;ROSA:LNL:tTA;TRE-Notch4* mice resembled the negative littermate controls with only 2.1 ± 3.6% of total AV connections enlarged (Fig. 4B and C and SI Appendix, Table S1). Therefore, expression of Notch4* in the endothelium of BMX-CreERT2 positive arteries is not sufficient to induce AV shunts in the brain.

Fig. 3. Endothelial growth in the AV shunt accompanied by the expansion of endothelial cell surface area. (A) Two-photon time-lapse imaging showing Cdh5(PAC)-CreERT; Confetti-marked nuclear GFP+ and cytoplasmic YFP+ ECs in cerebral cortex of mutants and controls. Texas-Red-dextran labels plasma. (B) Quantification of total cell number and AV connection diameter over the same interval (22 AV connections in five mutants, 11 AV connections in three controls). (C) Whole-mount BrdU staining/lectin perfusion. P12 AV connection from mutant cerebral cortex is shown. Blue dots indicate Hoechst+cells, and the colored portion indicates the number of BrdU+cells. (D) Whole-mount staining of P12 cerebral cortex for VE-cadherin, counterstained by lectin perfusion. (E) Correlation of size of VE-cadherin-traced cells in P12 control (blue) and Notch4* (red) connections with AV connection diameter (24 AV connections in 11 mutants, 6 AV connections in three controls). (Scale bars: A, 100 μm; C, 25 μm.)

in the brain vasculature (SI Appendix, Fig. S11). Examination of mice with combined expression of the arterial marker ephrin-B2-H2b-eGFP and BMX(PAC)-CreERT2;Tie2-tTA;TRE-Notch4* revealed that BMX(PAC)-CreERT2 excision overlapped with ephrin-B2-H2b-eGFP in arteries but not in microvessels (SI Appendix, Fig. S14 and Movie S3). Therefore, BMX(PAC)-CreERT2 was uniformly active in arteries but not small arterioles. As such, the Cdh5(PAC)-CreERT2;ROSA:LNL:tTA driver induces Notch4* expression throughout the vascular endothelium, similar to the Tie2-tTA system, whereas the BMX(PAC)-CreERT2;ROSA:LNL:tTA driver induces Notch4* specifically in the endothelium of arteries.

To evaluate the formation of AV shunts, we examined tamoxifen-perfused whole-mount preparations of Cdh5(PAC)-CreERT2;ROSA:LNL:tTA;TRE-Notch4* and BMX-CreERT2;ROSA:LNL:tTA;TRE-Notch4* at P18, when most Tie2-Notch4* mice have developed AV shunts (17). As expected, we readily detected enlarged AV connections in Cdh5(PAC)-CreERT2;ROSA:LNL:tTA;TRE-Notch4* mice by P18 after TAM injection at P7–P8 to induce Cre-mediated tTA expression (Fig. 4A and SI Appendix, Table S1). In these mice, 82.8 ± 21.8% of total AV connections were enlarged. By contrast, the BMX-CreERT2;ROSA:LNL:tTA;TRE-Notch4* mice resembled the negative littermate controls with only 2.1 ± 3.6% of total AV connections enlarged (Fig. 4B and C and SI Appendix, Table S1). Therefore, expression of Notch4* in the endothelium of BMX-CreERT2 positive arteries is not sufficient to induce AV shunts in the brain.

Fig. 4. Changes in Notch signaling in the artery alone did not affect AV shunt formation. (A–F) Lectin-perfused vasculature on P18 surface cerebral cortex. (A and B) ROSA:LNL:tTA;Notch4* mice in which Notch4* was expressed at P7 with temporally inducible Cre-recombinase. (D–F) Notch4* mutant or control mice with Cre-mediated Rbpj deletion at P7. (A) n = 83 AV connections in three mice. (B) n = 43 AV connections in three mice. (C) n = 103 AV connections in three mice. (D) n = 191 AV connections in five mice. (E) n = 186 AV connections in six mice. (F) n = 171 AV connections in seven mice. Blue arrowheads indicate AV connections. (Scale bars, 100 μm.)
investigated whether differences in blood flow velocity determine the growth of AV shunts. We identified arterial branch points in mutant mice in which one branch clearly led into an AV shunt (V1 in Fig. 5 A and B). We measured velocity and diameter in the higher-velocity AV shunt and the lower-velocity adjacent artery (V2 in Fig. 5 A and B) over time in Tie2-Notch4* mice and compared the changes in velocity and diameter to control arteries (V1 and V2 in Fig. 5 C and D). The velocities in control arteries were more stable, varying by an average of 16% between time points vs. 45% in the mutants. In five of seven cases where the velocity in the AV shunt increased, the velocity in the adjacent artery with lower velocity decreased (compare Fig. 5 A to B; see summary in Fig. 5 E “when AV shunt velocity increases” (see example in Movie S4)). In three cases, the velocity was reduced in both the AV shunt and its adjacent artery (Fig. 5 E, “when AV shunt velocity decreases”). The velocity in control arteries did not change (Fig. 5 E, “control”). Thus, increased velocity in AV shunts is often accompanied by decreased velocity in the adjacent arteries of Tie2-Notch4* mice.

We then investigated whether changes in velocity correlated with changes in diameter in Tie2-Notch4* mutants. We focused on the AV shunts in which velocity increased and adjacent arteries in which velocity decreased (represented by red dots in Fig. 5 E). We found that the adjacent downstream arteries indeed regressed, compared with either the AV shunt or arteries measured in control mice (Fig. 5 F, P < 0.02 by two-tailed Student t test). Therefore, changes in velocity correlated with changes in diameter, so that the highest velocity connections, which also tended to be the most proximal AV connections in the Tie2-Notch4* mice, exhibited increasing velocity and diameter, whereas the lower velocity and typically distal arterial branches exhibited reduced velocity and diameter.

For a broader perspective on AV shunt progression, we examined the whole brain by casting the vessels with radio-opaque microfil and imaging with microCT. Despite widespread AV shunting in mutant mice, AVM development appeared focal (Fig. 5 G). Thus, our data suggest that the unchecked growth of higher-velocity AV connections results in focal AVM development.

**Endothelial Expression of Activated Notch1 Induced AV Shunt Formation.**

To determine whether expression of constitutively active Notch1 in the endothelium also causes AV shunt formation, we combined a constitutively active, tTA-responsive Notch1* allele (16) with Tie2-tTA and activated Notch1* at birth. Five of five Tie2-tTA;TRE-Notch1* mice exhibited signs of ataxia or lethargy by P14, as well as large brain AVMs, whereas none of six controls were affected (Fig. 6). Thus, endothelial expression of constitutively active Notch1 induces brain AV shunts during neonatal development.

**Discussion**

Here, we provide novel mechanistic insights into AVM formation. Our data suggest that AV shunts arise from the enlargement of capillary-like vessels and that cell-autonomous Notch signaling in the endothelium of microvasculature and veins is sufficient and required for AVM formation. AV shunt initiation is accompanied by the enlargement of EC area but not increased proliferation. Blood flow mediates AV shunt progression by positive feedback of increasing flow and increasing diameter, leading to the selective growth of focal AVMs.

Our data shed light on the origin of AVMs. The vascular structure of brain AVMs has led to speculations that they arise from failed regression of primitive AV connections during development (3, 4). Extrapolation of histological analysis of skin AVMs led others to propose that brain AVMs arise from the dilatation of postcapillary venules (5). In contrast to the existing models, our results demonstrate that Notch4*-induced AV shunts grow from preexisting capillary-like connections with no detectable differences from normal capillaries in either their diameter or velocity. This suggests a new, potentially capillary-originated model of AVM initiation.

Endothelial but not hematopoietic Notch signaling is critical in the formation of AV shunts. Previous work has demonstrated a critical function for blood cells in separating the blood and lymphatic vascular systems (27). Our data, confirmed by two independent induction systems, demonstrate that increased Notch signaling specifically within the endothelium is sufficient to cause AV shunt formation without obvious involvement of the blood cells. Conversely, expression of Notch4* specifically in Scl-tTA-positive blood lineages did not lead to AV shunt formation (SI Appendix, Fig. S16). Furthermore, no increase in CD45+ cells was observed near AV shunts (SI Appendix, Fig. S17). Together, our data suggest that expression of Notch4* in the blood lineages is insufficient to induce AV shunts and that endothelial Notch4* elicits AVMs in a cell-autonomous fashion.

Initial enlargement of AV shunts correlates with area expansion of individual ECs, rather than increased proliferation. Consistent with this finding, we found that the converse is true: EC area is reduced during AV shunt regression upon turning off the
artery (16). Together, these findings suggest that sustained endothelial Notch signaling could not induce or suppress AV shunt formation, respectively. In the same genetic systems, global endothelial Cfl4(PAC)-CreERT2-mediated up- or down-regulation of Notch signaling could be performed on a single time point with lower resolution than we show here. We provide experimental evidence that endothelial Notch4* permits a “steal” and perpetuates a positive feedback loop, leading to selective growth of high-velocity connections at the expense of lower-velocity connections.

Our data also demonstrate that, in controls, blood velocity in adjacent two-branch arteries is maintained at a comparable rate over time. In mutants, the blood velocity in adjacent two-branch arteries varies greatly and with increasing disparity. Our earlier work shows that turning off Notch4 expression normalizes high-velocity AV shunts by rapidly reducing the flow and diameter of the AV shunt while increasing flow and diameter to the distal artery (16). Together, these findings suggest that sustained endothelial expression of Notch4* compromises biological checks that prevent AV connections from enlarging and “stealing” flow from adjacent vessels.

We believe that normal AV specification is critical in maintaining proper hemodynamics in the vasculature. Because Notch signaling is a critical determinant of AV specification, it is conceivable that either gain or loss of Notch activity could disrupt AV specification, compromising normal vascular control of hemodynamic forces and leading to AVMs. Indeed, both gain- and loss-of-function Notch mutants develop abnormal shunting in mouse embryos (13, 30). Our model is consistent with recent work hinting that reduced Notch signaling in HHT type 2 could contribute to AVMs (31). We propose that increased Notch signaling in the endothelium of nonarterial vessels disturbs bidirectional control of blood flow by allowing the persistent growth of high-flow AV connections at the expense of lower flow AV connections (SI Appendix, Fig. S18).

Materials and Methods

See SI Appendix for detailed information on materials and methods. Cranial windows, in vivo imaging, lectin-perfusion, Notch4-ICD, SMA, and VE-cadherin immunostaining was as described (16). BrdU immunostaining was as described (22). For peripheral blood isolation, blood was removed from right ventricles, and erythrocytes were lysed. Flow cytometry was performed following CD45 immunostaining and DAPI labeling. For MicroCT, images of Microfil-labeled vasculature were acquired using a μCT 40 system (Scanco Medical AG). All mouse lines used have been published. This study was carried out in strict accordance with National Institute of Health Regulations and the Institutional Animal Care and Use Committee at the University of California, San Francisco.

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Extended Materials and Methods:

In vivo Imaging. Cranial windows for long-term imaging were as we described (1). Images and line-scan data were obtained using a locally constructed two-photon laser scanning microscope through a long working distance 1.0 NA water-immersion objective (Zeiss) as we described (2). Low-energy femtosecond laser pulses were centered at 870nm to simultaneously excite Texas Red-dextran (2000kDa) and GFP, or at 800nm to excite fluorescein-dextran (2000kDa, Sigma).

Mice. This study was carried out in strict accordance with NIH regulations and the Institutional Animal Care and Use Committee at the University of California San Francisco. Tie2-tTA, TRE-Notch4* mice were generated as we described (3, 4). Tetracycline (Tet) sucrose solution (0.5-mg/ml Tet, 50-mg/ml sucrose, Sigma) was administered to pregnant mothers from plugging, and withdrawn from pups at birth. Notch4* expression was regulated in TRE-Notch4* mutants as we described (5). Rbpj$^{floxflox}$ (6), ROSA:LNL:tTA (7); TRE-H2b-eGFP (8), R26R-mT/mG (9), and R26R-confetti (10) mice have been published. Cdh5-CreERT$_2$ (11) and BMX-CreERT$_2$ (12) mice were kindly provided by the Adams lab. Tamoxifen (20mg/mL in corn oil) was given intragastrically (0.5mg at P7-P8) for induction of TRE-Notch4* by Cdh5(PAC)-CreERT$_2$; ROSA:LNL:tTA or BMX(PAC)-CreERT$_2$; ROSA:LNL:tTA or deletion of Rbpj by Cdh5(PAC)-CreERT$_2$ or BMX(PAC)-CreERT$_2$. For lineage tracing, Tamoxifen was given intragastrically (3x 0.1mg P1-P5) to Cdh5(PAC)-CreERT$_2$; R26R-confetti mice.

Whole mount staining and fluorescence quantification. Immunostaining was performed as we described (1). Fluorescence quantification was performed using ImageJ and MATLAB (MathWorks). Notch4-ICD fluorescence staining intensity in mutants was normalized to the average staining intensity in littermate controls, and nuclear TRE-H2b-GFP intensities were normalized to background levels. Areas of VE-cadherin outlined ECs were quantified by tracing the outline of clearly visible individual ECs. For quantification of AV shunts in fixed samples, P18 mouse brains were sliced and flat-mounted to image surface vessels of the same region of the cortical surface. Images of tomato lectin-perfused vasculature were acquired (3-7 random fields of view per sample). AV connections within individual fields of view were identified and scored as enlarged if the minimum diameter was $\geq 12.5\mu$m. The ratio of enlarged/total AV connections was computed for each animal, and the mean ratio (n = # of mice) was compared between groups.
**BrdU incorporation and immunostaining.** BrdU (5-bromo-2′-deoxyuridine)(100 mg/kg body weight in 0.9% saline)(Fisher) was injected IP into pups on P10, P11, P12. Four hours post-injection on P12, 50ug biotinylated-Lycopersicon esculentum (tomato) lectin (Vector Labs)/45 ug Alexa647-streptavidin (Jackson ImmunoResearch)/PBS was perfused via IVC. Whole mount immunostaining was followed according to Pitulescu et al. (2010), using anti-BrdU (1:50)(BD Biosciences) and Cy3-conjugated secondary (Jackson ImmunoResearch). Tissue was incubated in 2ug/mL Hoechst 33342 (Molecular Probes) 30 min prior to imaging. Z stacks (1um steps) were captured with Yokogawa Spinning Disk confocal microscopy and ImageJ software at the UCSF Biological Imaging Development Center.

**Isolation of peripheral blood and flow cytometry.** Blood (200-300uL) was removed from right ventricle of anesthetized mouse using 20G needle/syringe, dispensed into 5mM EDTA, and erythrocytes were lysed (155mM NH₄Cl, 12mM NaHCO₃, 0.1mM EDTA, pH 7.5). Cells were pelleted, washed in PBS, fixed with 2% PFA, immunostained against CD45-Alexa647 (1:200)(BioLegend), and incubated in 2ug/mL DAPI (Sigma). The UCSF Liver Center Core performed flow cytometry using BD Biosciences LSRII cytometer.

**MicroCT.** Mice were exsanguinated by cannulating the left ventricle and perfusing saline from the heart through the vasculature using an IV drip bag and butterfly needle. An incision was made in the right atrium allowing an outflow track for blood. The descending aorta was ligated with 6.0 suture after exsanguination to maximize brain perfusion. Without removing the butterfly needle from the left ventricle, a syringe containing a mixture of yellow Microfil MV122-yellow (1:10 catalyst to MV compound, Flow Tech Inc) was used to gently perfuse the vasculature until internal organs had a yellow appearance. Brains were harvested after 90 minutes allowing the Microfil to cure. Specimens were stored in 10% formalin until imaging. Brain specimens were scanned with a micro-computed tomography system (μCT 40, Scanco Medical AG, Basserdorf, Switzerland) with a 12-μm isotropic nominal resolution. Images were acquired by scanning in the transaxial plane over a ~1.5cm section extending from the rostral to caudal edges of each brain. Data visualization was conducted at the microCT workstation. Segmentation was performed by applying a predetermined threshold radio-attenuation value on all scans to eliminate signal voxels generated outside of the brain.
Statistics. Comparisons were made using a two-tailed Student’s T-Test. Comparison of multiple groups was made using ANOVA with post-hoc Tukey’s HSD analysis.

References:
Supplemental Figure Legends

Supplemental Figure 1. AV diameter increases over time in the cerebral vasculature of Notch4* expressing mutants.
A) Mean AV diameter measured at the narrowest point of each of the indicated timepoints in Tie2-tTA (control) and Tie2-tTA; TRE-Notch4* (mutant) mice. Red dots indicate the diameter of all AV connections ≥12.5µm. B) The percentage of AV connections ≥12.5µm diameter at each timepoint.

Supplemental Figure 2. Relationship between lumen diameter and blood velocity in capillary-like vessels studied over time.
A) Plots of capillary-like vessels with paired increase of both lumen diameter (solid line) and blood velocity (dotted line) during the imaging period. B) Plots of capillary-like vessels with increase in diameter but minimal or no increase in blood velocity over the imaging period. C) Plots of capillary-like vessels with minimal or no increase in either diameter or blood velocity over the imaging period. D) Plots of capillaries in control mice demonstrating variation of lumen diameter and blood velocity over imaging period. Velocity and diameter analysis in all vessels that remained through the imaging interval (N=18 AV connection in 4 Notch4* mutant and N=18 AV connections in 4 control mice). Additional examples from these mice are shown in Figure 1.

Supplemental Figure 3. Control mice did not develop AV shunts. Two-photon time-lapse imaging of FITC-dextran labeled AV connections on cerebral cortex through a cranial window. An AV connection (red arrowheads) in a control mouse remains capillary diameter between P14 to P19. Scale bar = 50µm.

Supplemental Figure 4. No correlation between the initial diameter of the AV connection (smallest lumen diameter) and the final AV shunt diameter (largest lumen diameter).

Supplemental Figure 5. Vascular smooth muscle cell coverage is grossly normal in Notch4* expressing mice at P12.
Whole mount immunofluorescent staining of cortex from Notch4* mutant and control mice. Anti-α-smooth muscle actin in red and lectin perfusion in green. Scale bar = 200µm.

Supplemental Figure 6. Strong expression of Notch4* transgene throughout endothelium of the microvasculature.
Fluorescence immunostaining for Notch4 intracellular domain in cerebral cortex whole mount shows increased levels Notch4-ICD in the Notch4* expressing mice than in their littermate controls. Note that expression is elevated not only in the AV shunt (arrowhead), but also in the artery, vein and capillaries not obviously enlarged (arrows, magnified view in lower panels). Scale bars = 100µm in upper panels and 25µm in lower panels.

Supplemental Figure 7. TRE-eGFP is a reporter of Notch4* expression.
A) Notch4-ICD staining in sagittal brain sections of Tie2-tTA; TRE-Notch4*; TRE-H2B-GFP mice at P10 (a1), P12 (a2), and P18 (a3). (a4) Notch4-ICD staining in littermate control. (a5) Negative control of Notch4 staining using non-specific IgG on adjacent section in mutant specimen. B) Notch4-ICD staining intensity in lectin-perfused vessel segments of Notch4* mice, normalized to age-matched genetic controls. C) Correlation between Notch4 staining intensity and GFP intensity in lectin-perfused vessel segments of Notch4* mice over time.

Supplemental Figure 8. In vivo assessment of the correlation between TRE-eGFP expression and diameter.
Time history of number of GFP+ cells and vessel segment diameter of Notch4* mice. Change in vessel segment diameter was scored as increase, constant, decrease, or regress. Each line trajectory corresponds to an individual vessel segment. Color transitions of line trajectory indicate time progression. Circle indicates vessel segment was part of an AV shunt. Cross indicates vessel segment regressed. Number of GFP+ cells was normalized to the initial vessel segment diameter and expressed as per unit length. Bold line trajectory corresponds to mean number of GFP+ cells and mean vessel segment diameter.

Supplemental Figure 9. No significant difference in the number of Confetti labeled ECs in AV connections as they grow into AV shunts. Correlation plot showing the relationship between the change in diameter and the change in cell number over the 48hr interval. Triangle indicates mean change in diameter and cell number. Marker size represents number of observations.

Supplemental Figure 10. No significant difference in BrdU labeled endothelium in Notch4* mutant mice. A) Quantification of BrdU labeled cells as a percentage of all DAPI+ lectin+ ECs in the indicated vessels (-Shunt indicates that the artery or vein is not directly connected to an AV shunt) and B) in the AV connections or AV shunts of the indicated mice. C) Quantification of minimal AV diameter in the measured connections. Error bars reflect standard deviation among individual animals (N=4 for AV shunt group and N=5 for all other groups).

Supplemental Figure 11. Cdh5(PAC)-CreERT2 and BMX(PAC)-CreERT2 mediate endothelial- and arterial endothelial-specific gene excision, respectively. (A) Cdh5(PAC)-CreERT2 and (B) BMX(PAC)-CreERT2 activity was visualized in vivo using a membrane-targeted eGFP Cre-reporter. Plasma in perfused vessels is labeled by Texas-red dextran. Higher magnification image shows differential labeling of the artery, capillary and vein (A’ & B’). Arrow indicates capillaries. Scale bars = (B) 150μm and (B’) 50μm.

Supplemental Figure 12. Cdh5(PAC)-CreERT2 is active in vascular endothelium and AV shunts but not blood cells. (A) FACs showing the level of Cre-activated mGFP in Cdh5(PAC)-CreERT2 mice (with and without ROSA:LNL:tTA; TRE-Notch4*) and positive and negative controls. (B&C) Whole mount imaging showing strong mGFP expression throughout the vasculature of Notch4* expressing mice (B) and the control (C).

Supplemental Figure 13. Cdh5(PAC)-CreERT2; ROSA:LNL:tTA is a strong driver of TRE-Notch4* expression. Quantification of Notch4 immunofluorescence staining intensity in 10 fields per brain, normalized against littermate controls. (N=4 Cdh5(PAC)-CreERT2; ROSA:LNL:tTA; TRE-Notch4* mice and N=6 Tie2-tTA; TRE-Notch4* mice.)

Supplemental Figure 14. Variable BMX(PAC)-CreERT2-mediated excision in the distal ends of the arterioles. Two-photon imaging of the cerebellum of BMX(PAC)-CreERT2; mT/mG; ephrinB2-H2b-eGFP mice with counter stain by tomato lectin perfusion. Note extension of the nuclear ephrinB2-H2b-eGFP signal beyond the end of BMX(PAC)-CreERT2; mT/mG+ (cytoplasmic) arteries. Scale bar = 50μm.

Supplemental Figure 15. Growth of an AV shunt and regression of a lower resistance distal vessel. 3D rendering of in vivo time-lapse data of an AV shunt and distal artery (arrowhead).

Supplemental Figure 16. Scl-tTA; TRE-Notch4* does not result in AVM.
Vasculature of (A) Scl-tTA; TRE-Notch4* and (B) littermate Tie2-tTA; TRE-Notch4* mutants, revealed by fluorescent tomato lectin perfusion at P28 after tetracycline removal at birth. (A’, B’) Magnified view of boxed regions in (A, B). Scale bar = 100μm (A, B) and 50μm (A’, B’).

Supplemental Figure 17. No increase in CD45+ hematopoetic cells in the AV shunts of Notch4* expressing mice.
Fluorescence immunostaining for CD45 in cerebral cortex whole mount shows no detectable increase in hematopoetic cells in the AV shunts of Notch4* expressing mouse, and very few hematopoetic cells in general. Scale bars = 100μm.

Supplemental Figure 18. Model for the flow-mediated selection of AV shunts
Schematic depicting a normal cortical brain vascular network (A) and the remodeling of the vasculature induced by Notch4* expression (B-D). In the earliest stage, AV shunts throughout the brain are enlarged (arrowheads in B). Following initial enlargement, a few AV shunts in which flow is highest continue to enlarge (arrowheads in C). After sequential flow-mediated selections steps, AVM-like lesions develop, composed of dilated and tortuous arterial and venous vessels which shunt large amounts of blood from distal capillaries (arrowhead in D).

Supplemental Table 1. Quantification of enlarged AV connections in mice with endothelial- or arterial endothelial-specific Notch4* expression.
Cdh5(PAC)-CreERT2 and BMX(PAC)-CreERT2 were used to turn on Notch4* in all vessels and in arteries, respectively. Lectin-perfused AV connections on the surface of the cerebral cortex of Notch4* mice (see Fig. 4) were scored as enlarged if the minimum diameter was ≥12.5μm. The ratio of enlarged/total AV connections was computed for each animal (see Supplemental Table 1 raw data), and the mean ratio (n = # of mice) was compared between groups. See Supplemental Methods for detailed methodology. (Data are mean±SD)

Supplemental Table 2. Quantification of Notch4*-mediated enlarged AV connections after endothelial- or arterial endothelial-specific deletion of Rbpj.
Cdh5(PAC)-CreERT2 and BMX(PAC)-CreERT2 were used to delete floxed-Rbpj in all vessels and in arteries, respectively. Either both or a single Rbpj allele was excised. Lectin-perfused AV connections on the surface of the cerebral cortex of Notch4* and control mice (see Fig. 4) were scored as enlarged if the minimum diameter was ≥12.5μm. The ratio of enlarged/total AV connections was computed for each animal (see Supplemental Table 2 raw data), and the mean ratio (n = # of mice) was compared between groups. See Supplemental Methods for detailed methodology. (Data are mean±SD).
Supplemental Figure 2A-C.

A

B

C
Supplemental Figure 2D.
Supplemental Figure 4.

Correlation of Smallest vs. Largest Lumen Diameters for Same Vessels Within Imaging Period

- Mutant
- Control

Largest Lumen Diameter Measured Across All Timepoints vs. Smallest Lumen Diameter Measured Across All Timepoints
Supplemental Figure 5.

*Tie2-tTA; TRE-Notch4*  
*Tie2-tTA*
Supplemental Figure 6.

*Tie2-tTA; TRE-Notch4*

*Tie2-tTA*

Art.

Vein

anti-Notch4+/lectin perfusion

anti-Notch4+/isolated channel

anti-Notch4+/lectin perfusion
Supplemental Figure 7.

(A) 

Tie2-tTA; TRE-Notch4*; TRE-H2B-GFP

(a1) Notch4-ICD/GFP
P10

(a2) Notch4-ICD/GFP
P12

(a3) Notch4-ICD/GFP
P18

Tie2-tTA;
TRE-H2B-GFP

(a4) Notch4-ICD/GFP
P18

(a5) Non-specific IgG/GFP
P18

(B) Notch4 staining in vessel segments

Relative intensity vs. P10, P12, P18

(C) Scatter plot

Relative intensity
TRE-H2B-GFP vs. Notch4-ICD immunofluorescence

r=0.78
Supplemental Figure 8.
Supplemental Figure 11.
Supplemental Figure 13

Notch4 staining in vessel segments

Relative intensity (AU)

Cdhr5(PAC)-CreERT2; ROSA:LNLT7A; TRE-Notch4*
Tie2-tTA; TRE-Notch4*
Supplemental Figure 17

Anti-CD45 lectin perfusion

Tie2-tTA; TRE-Notch4*

Tie2-tTA

# CD45+ cells (mm⁻²)

Tie2-tTA; TRE-Notch4*  |  Tie2-tTA

0  |  20  |  40  |  60  |  80
Supplemental Figure 18

A. Normal vasculature

B. Notch4* mutant: Initial Dilation of AV Shunts

C. Notch4* mutant: Flow mediated selection

D. Notch4* mutant: Progression to AVM
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Supplemental Table 1 raw data

**Cdh5(PAC)-CreERT2; ROSA:LNL:tTA; TRE-Notch4**

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**ROSA:LNL:tTA; TRE-Notch4**

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**BMX(PAC)-CreERT2; ROSA:LNL:tTA; TRE-Notch4**

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**ROSA:LNL:tTA; TRE-Notch4**

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Supplemental Table 2 raw data

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### BMX(PAC)-CreERT2; RBPJ\(^{fl/fl}\); Tie2-tTA

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Supporting Information

Murphy et al. 10.1073/pnas.1415316111

Movie S1. Vascular network in Notch4* mutant before AV shunt formation. Three-dimensional rendering of a two-photon imaging stack from the P14 Notch4* mutant shown in Fig. 1A.

Movie S1
Movie S2. Vascular network in Notch4* mutant after AV shunt formation. Three-dimensional rendering of an imaging stack from P19 Notch4* mutant shown in Fig. 1A and Movie S1, 5 d later. An AV shunt can be observed in the center of the field, where only capillary connections had been seen previously.

Movie S2

Movie S3. Overlapping expression of ephrinB2-H2b-eGFP and BMX(PAC)-CreERT2 induced mT/mG. Optical z-stack from two-photon imaging of the cortical surface of the brain of a BMX(PAC)-CreERT2; mTmG; ephrin-B2-H2b-eGFP mutant mouse following perfusion with Alexa-647–labeled tomato lectin. Green channel: ephrin-B2-H2b-eGFP and BMX(PAC)-CreERT2 activated mTmG reporter; red channel: lectin perfusion. Note that the BMX(PAC)-CreERT2 reporter ends in the large arterioles before ephrin-B2-H2b-eGFP capillaries.

Movie S3
Movie S4. Dramatic reduction in distal blood flow with proximal steal effect. Single-plane movie from a two-photon imaging stack taken at a late time point in AV shunt progression in a Tie2-tTA; TRE-Notch4* mutant mouse. Movement of dark-red blood cells can be observed by contrast Texas-Red–dextran labeled plasma. Slowly moving red blood cells can be observed as distinct spheres, and quickly moving blood cells appear as streaks. Blood flow and vessel diameter was reduced in this distal branch over time as the proximal connection grew.

Other Supporting Information Files

SI Appendix (PDF)