**Research Report**

**Blood biomarkers in a mouse model of CADASIL**

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**A B S T R A C T**

Mutations in NOTCH 3 are the cause of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), a neurological disorder characterized by stroke, and vascular cognitive impairment and dementia. Loss of vascular smooth muscle cells (VSMC) and accumulation of granular osmiophilic material (GOM) deposits are hallmarks of CADASIL. There are no therapies for CADASIL and experimental endpoints to examine the preclinical efficacy of potential drugs are lacking. This study aims to use a mouse carrying the C455R mutation in Notch 3 to identify biomarkers associated with CADASIL.

Mass spectrometry and antibody arrays were used to explore the aorta and blood proteomes of CADASIL mice. ELISA assays were utilized for biomarker validation, a ligand-dependent assay was applied to examine the relationship between Notch signaling and biomarker expression, and retinal histology was performed for quantification of VSMC loss in arteries. Two-hundred day-old mice with the C455R CADASIL mutation in Notch 3 mice display robust VSMC loss in retinal arteries and had increased plasma levels of collagen18x1/endostatin (coll18x1) and high-temperature requirement A serine peptidase 1 (HTRA1) and reduced levels of Notch 3 extracellular domain (N3ECD), compared to control wild type mice.

Measurements of plasma endostatin, HTRA1 and N3ECD, along with VSMC quantification in retinal arteries, may serve as surrogate endpoints for assessing efficacy in preclinical therapeutic studies of CADASIL using mice.

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1. Introduction

Ischemic cerebral small-vessel disease (SVD) is a prevalent cause of stroke and a strong contributor to vascular cognitive impairment and dementia (Snyder et al., 2015). Multiple clinical studies show strong an association among SVD, aging, and cardiovascular risk factors (Kalra, 2012; Thompson and Hakim, 2009; Vermeer et al., 2007). Little is known about the cellular and molecular mechanisms underlying the development of SVD and therapies are lacking. Mendelian conditions resembling key aspects of SVD, in the absence of cardiovascular risk factors, have been described in adult patients (Gould et al., 2006; Hara et al., 2009; Joutel and Faraci, 2014). The study of these inherited SVDs may reveal key pathophysiological mechanisms, uncover biomarkers with diagnostic or prognostic potential, and define molecular targets for rational therapeutics. One such SVD is cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, (CADASIL), the most common form of inherited SVD (Chabriat et al., 2009).
Patients with CADASIL often suffer from migraines in childhood, have recurrent ischemic strokes in the third and fourth decade of life, and experience progressive cognitive impairment (Dichgans et al., 1998). CADASIL is caused by mutations in the NOTCH 3 gene, which encodes for one of four Notch receptor paralogues in mammals (Joutel et al., 1996). Most CADASIL mutations in Notch 3 affect cysteine residues located within the epidermal growth factor-like repeats in the extracellular domain (Brass et al., 2009; Dichgans et al., 2000; Joutel et al., 1997; Louvi et al., 2006). Postmortem studies of tissue from CADASIL patients reveal a non-amyloid, non-atherosclerotic, arteriopathy. The characteristics of this arteriopathy include VSMC death and the accumulation of deposits, detected by electron microscopy, known as granular osmiophilic material (GOM) (Baudrimont et al., 1993; Ruchoux et al., 1995; Ruchoux and Maurage, 1997; Tikka et al., 2009). GOM deposits are specific to CADASIL and their identification in skin biopsies or brain autopsies is diagnostic (Tikka et al., 2009). Biochemical studies of vessels of postmortem CADASIL cases and mouse models identified collagen 18 alpha 1(Col18α1)/endostatin, clusterin (Arboleda-Velasquez et al., 2011), Notch 3 ectodomain (N3ECD), and tissue inhibitor of metalloproteinases 3 (TIMP3) as GOM components (Ishiko et al., 2006; Monet-Lepretre et al., 2013).

For the current work, we studied published CADASIL mouse models carrying either the C455R or R1031C mutations in Notch 3. The C455R CADASIL mutation was described in a Colombian family in whom stroke occurs earlier and MRI abnormalities are more extensive than in patients carrying the R1031C, also described in a Colombian pedigree (Arboleda-Velasquez et al., 2002). Previous work showed that the C455R (located in the ligand-binding domain of the Notch 3 receptor) and the R1031C (located outside this region) mutations define different hypomorphic activity states of the Notch 3 receptor and that mice carrying these mutations develop GOM and other age-dependent phenotypes that resemble the human condition (Arboleda-Velasquez et al., 2011).

The availability of multiple CADASIL animal models, including those used in this work, may allow for preclinical testing of experimental drugs (Arboleda-Velasquez et al., 2011; Joutel and Faraci, 2014). Such studies would be greatly facilitated by the identification of specific markers of disease progression that are measurable, sensitive, and robust. Here, we sought to identify blood biomarkers associated with CADASIL in a mouse model carrying non-synonymous mutations in Notch 3. We utilized blood plasma, from knock-in mice models carrying Notch 3 mutations identical to those found in Colombian families (Arboleda-Velasquez et al., 2008, 2011) for biomarker discovery and validation.

2. Results

2.1. Screening of CADASIL biomarkers in mouse plasma

Previous analyses of mice and humans carrying CADASIL mutations in Notch 3 identified col18α1/endostatin, a potent and pleiotropic anti-angiogenic factor, as a protein accumulating in CADASIL vessels and a GOM component (Arboleda-Velasquez et al., 2011; O’Reilly et al., 1997). We used an antibody array capable of detecting 53 angiogenesis-related proteins to determine whether endostatin, and/or other angiogenesis-related proteins, could be detected in plasma samples from our transgenic mice. We compared the plasma of knock-in mice expressing the human WT Notch 3 protein (N3KO; WT76+/−; SM22-Cre) to that of mice

![Fig. 1. Proteomic screen in CADASIL mice. Dot blots represent levels of specific proteins, in technical duplicate, found in plasma from two male CADASIL mice collected at 100 or 200 days of age (N3KO; C455R+/−; SM22-Cre) and a control (N3KO; WT76+/−; SM22-Cre). IGFBP-1, SDF-1, and collagen18α1/endostatin were found to be differentially expressed, as established by a variance analysis.](image-url)
expressing the C455R CADASIL mutant receptor (N3KO; C455R+/−; SM22-Cre) at 100 and 200 days old, all in a mouse endogenous notch 3 knockout background (N3KO) (Fig. 1 and Supplemental Fig. 1A). Furthermore, to examine whether any angiogenic-related protein candidates were regulated by Notch 3 function, we also compared their expression in plasma from 200-day old mice lacking notch 3, N3KO, and N3KO mice in which Notch 3 expression was restored in vessels using a conditional transgenic approach, N3KO; WT76+/−; SM22-Cre mice (Fig. 2 and Supplemental Fig. 1A).

We identified insulin-like growth factor-binding protein-1 (IGFBP-1), stromal cell-derived factor-1 (SDF-1), and col18α1/endostatin as potential biomarkers based on a variance analysis (see Methods). IGFBP-1 was increased in both 100- and 200-day old N3KO; C455R+/−; SM22-Cre mice (Fig. 1 and Supplemental Fig. 1A), with a fold change of 2.029 and 4.562, respectively. SDF-1 was more abundant in 100-day old N3KO; C455R+/−; SM22-Cre mice (1.752-fold change) and less abundant in the 200-day old N3KO; C455R+/−; SM22-Cre mice (0.344-fold change, Fig. 1 and Supplemental Fig. 1A). Col18α1/endostatin, was significantly less abundant (0.800 fold change) in the 100-day-old N3KO; C455R+/−; SM22-Cre mice, but, remained unchanged (1.025-fold change) in the 200-day old mice (Fig. 1 and Supplemental Fig. 1A). Because protein-protein interactions within complex samples have been reported when using antibody arrays (Kodadek, 2001), ELISA was utilized to validate the results. Analysis in 200-day old N3KO; C455R+/−; SM22-Cre mouse plasma using ELISA showed a significant increase in plasma circulating col18α1/endostatin (Fig. 3A, 1.76-fold change, P < 0.01).

IGFBP-1 and SDF-1 levels were not changed in the plasma of mice from functional rescue comparison (N3KO vs. N3KO; WT76+/−; SM22-Cre), whereas col18α1/endostatin was more abundant in the N3KO; WT76+/−; SM22-Cre mouse when compared to the N3KO mouse, suggesting that col18α1/endostatin expression may be regulated by Notch 3 signaling (Fig. 2 and Supplemental Fig. 1A). Using a ligand-dependent Notch signaling assay (Arboleda-Velasquez et al., 2011) we found that coculture of MEFs expressing either the WT or C455R mutant NOTCH 3 with

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**Fig. 2.** Proteomic screen in N3KO mice. Dot blots show levels of specific proteins found in plasma from two male notch 3 knockout (N3KO) mice (200 d) or two rescued/control (N3KO; WT76+/−; SM22-Cre) in which a wild type human Notch 3 is expressed in VSMC. Col18α1/endostatin was found to be differentially expressed, as established by a variance analysis.

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**Fig. 3.** Endostatin is misregulated in CADASIL. (A) ELISA detection of endostatin in plasma from mice (200 d) expressing the human Notch 3 CADASIL mutation (N3KO; C455R+/−; SM22-Cre), or control (N3KO; WT76+/−; SM22-Cre) showing that there is significantly more endostatin protein circulating in the blood of CADASIL mice. Number of biological replicates is indicated in parenthesis. (B) Primary cultures of MEFs from Cre-inducible CADASIL (N3KO; C455R+/+) or control mice (N3KO; WT76+/+) were placed in monocultures or cocultures with cells expressing DLL1 (ROSA26DLL1/+) a Notch ligand. Monocultures had no significant change in col18α1 mRNA expression, whereas cocultures with DLL1 significantly increased col18α1 mRNA expression. Expression was significantly more robust, however, in N3KO; WT76+/+ cocultures compared to N3KO; C455R+/+ cocultures. Asterisks (*) indicate P-value < 0.05 (unpaired t-test).
DLL1-expressing cells led to the expression of col18α1 mRNA, (Fig. 3B), though induction was significantly less pronounced in the MEFs expressing the C455R mutant Notch 3 receptor, compared to the WT (Fig. 3B). These data are in line with the hypomorphic nature of the C455R allele (Arboleda-Velasquez et al., 2011) and suggests that col18α1 expression is dependent on Notch 3 functionality.

2.2. Biomarkers in degenerating vessels

We have previously shown that expression of the R1031C CADASIL mutant Notch 3 receptor triggered mural cell degeneration in the aortas of one-year old mice (Arboleda-Velasquez et al., 2011). To search for candidate biomarkers, we compared the proteomes of aortae of R1031C+/−; SM22-Cre mice with that of mice expressing the human WT Notch 3 protein (WT76+/−; SM22-Cre). Using quantitative MS (See Methods), we identified 10 proteins that were more abundant and 32 proteins that were less abundant in aortas from R1031C+/−; SM22-Cre mice compared to control WT76+/−; SM22-Cre mice (Supplemental Fig. 1B). From this list, we focused on high-temperature requirement A serine protease (HTRA1), which was upregulated by about 2-fold in CADASIL aortas, because mutations in HTRA1 have previously been identified as the cause of a similar SVD called cerebral autosomal recessive with subcortical infarcts and leukoencephalopathy (CARASIL) (Hara et al., 2009). To further support the notion that HTRA1 is misregulated in NOTCH 3 mutant mice, we used ELISA to measure the plasma levels of HTRA1 in the C455R model (the model used in the antibody arrays above). ELISA analysis of plasma from the CADASIL N3KO; C455R+−; SM22-Cre mice showed a significant increase in HTRA1, compared to N3KO; WT76+/−; SM22-Cre controls (Fig. 4, fold-change of 1.83, P < 0.05).

2.3. Notch 3 ectodomain as a CADASIL biomarker

We investigated whether N3ECD could be detected in plasma and whether its levels were changed in the presence of Notch 3 mutations. As a commercial ELISA is not available, we designed a custom assay (see Methods). We detected no signal above background in WT C57BL/6 mice or in the Notch 3 knockout mouse (N3KO; WT76+/−) whereas signal was copious in mice expressing the human Notch 3 protein (N3KO; WT76+/−; SM22-Cre), indicating that our N3ECD ELISA was specific for the human N3ECD (Data not shown and Fig. 5). The maximum linear range of the ELISA was 2 μg/ml (Fig. 5A). This assay was also capable of detecting N3ECD in both human plasma and serum samples, with higher levels found in the serum (Fig. 5B, 14 ng/ul versus 51 ng/ul, P < 0.01). There was a significant decrease in circulating N3ECD in the plasma of CADASIL N3KO; C455R+/−; SM22-Cre mice when compared to N3KO; WT76+/−; SM22-Cre controls (Figs. 5C, 0.6-fold change, P < 0.01). RNA transgene expression was similar for CADASIL and control mice (Fig. 5D).

2.4. CADASIL mice have a robust vascular smooth muscle cell loss phenotype

Our analyses indicate that CADASIL mutations are associated with changes in plasma levels of endostatin, HTRA1 and N3ECD in mice. We examined the level of VSMC degeneration in the retina of CADASIL N3KO; C455R+−; SM22-Cre mice. We focused on the retina because it is part of the central nervous system, has a blood barrier similar to that of the brain, and it has a highly stereotypic vascular bed allowing for systematic quantification of VSMC loss. Using smooth muscle actin (SMA), a marker for VSMC, we observed that mice carrying the C455R mutation have large gaps in the vasculature (Supplementary Fig. 2). This loss appeared to occur uniformly throughout the retina (Fig. 6, a representative example is shown). We confirmed this observation by using anti-col18α1/endostatin and HTRA1, which are markers of VSMC loss. Immunohistology revealed strong N3ECD staining throughout the retina vasculature of the CADASIL mice, N3KO; C455R+/−; SM22-Cre, most prominently in large caliber arteries, whereas expression in the control N3KO; WT76+/−; SM22-Cre mice was less robust (Supplementary Fig. 3). Col18α1/endostatin staining was also localized to retinal vessels (Supplementary Fig. 4), but in contrast to the N3ECD staining was detected predominantly in capillaries at the periphery of the retina in CADASIL N3KO; C455R+−; SM22-Cre mice.

3. Discussion

In the present work we used mouse models of CADASIL, carrying either the C455R or the R1031C mutations in Notch 3, to identify increased plasma levels of col18α1/endostatin and HTRA1, decreased levels of N3ECD, and loss of VSMC in retinal vessels as candidate biomarkers associated with CADASIL mutations in mice. Mice expressing the C455R mutant Notch 3 receptor displayed robust VSMC loss in the retinal vasculature by six-months of age. We are currently characterizing the cerebrovasculature of these mice carrying the C455R mutation in Notch 3, however, analyses of the retina offer significant advantages including clear visualization of coverage along the length of individual vessels and their branches in a tissue containing a blood barrier similar to that of the brain. Previous analyses of the retinal vasculature in Notch 3 mutant mouse models have also been helpful in identifying pericyte loss further validating the use of the retinal vasculature as informative for CADASIL phenotypes (Henshall et al., 2015; Koller et al., 2015).

Antibody arrays for angiogenesis-related proteins were used to determine whether proteins in this category could be dysregulated in CADASIL mice. Antibody arrays are not without limitations.

![Fig. 4. HTRA1 protein is more abundant in the plasma of CADASIL mice. ELISA detection of HTRA1 in the plasma of mice (200 d) expressing the human Notch 3 CADASIL mutation (N3KO; C455R+−; SM22-Cre), or control (N3KO; WT76+/−; SM22-Cre) showing that there is significantly more HTRA1 protein circulating in the blood of CADASIL mice. Number of biological replicates is indicated in parenthesis. Asterisks (*) indicate P-value < 0.05 (unpaired t-test).](image-url)
because the results could be affected by protein-protein interactions within the sample mixture (Kodadek, 2001), which may explain the discrepancy in the results for endostatin between antibody arrays and ELISA. Notwithstanding these limitations, the endostatin finding may be functionally relevant to the pathobiology of CADASIL as we identified possible differences in the vascular distribution of col18α1/endostatin in the retina and differences in circulating levels of endostatin have been shown to be prognostic for ischemic stroke (Navarro-Sobrino et al., 2011). Furthermore, our analyses indicated that the increased plasma levels of col18α1/endostatin in mice carrying the C455R mutation was not directly related to the effects of the CADASIL mutation on Notch 3 signaling; col18α1/endostatin mRNA expression levels were increased in cells expressing WT human Notch 3 and reduced in cells expressing the C455R mutant.

Using aortas from mice carrying the R1031C mutation, our MS analysis identified HTRA1 as a misregulated protein. In humans, CADASIL affects primarily small caliber vessels in the brain (Louvi et al., 2006), although possible effects in large vessels have not been systematically analyzed. In CADASIL mice, however, the phenotype reported was overt, with aortas losing about half their width by one year of age (Arboleda-Velasquez et al., 2011), providing a rationale for using this tissue in our study. The finding of HTRA1 changes in affected vessels from a CADASIL mouse model is of particular interest because mutations in this protein are associated with a similar SVD known as CARASIL (Hara et al., 2009). The similarities between CADASIL and CARASIL have been reviewed in detail (Tikka et al., 2014), however, notable similarities include cerebral small vessel disease associated with a loss of VSMC coverage, stroke and cognitive impairment. It is unclear how the Notch 3 CADASIL mutations lead to HTRA1 accumulation in vessels and in plasma and whether HTRA1 may contribute to
Cadasil pathobiology. Mutations in Carasil have been proposed to cause a loss of function in Htrai activity, and, by effect, a gain of function in Tgfb signaling, as the mutant Htrai fails to repress Tgfb signaling (Hara et al., 2009). However, another hypothesis postulates that Carasil pathobiology is associated with diminished Tgfb signaling caused by defective Htrai-mediated cleavage of latent Tgfb binding protein 1 (Ltbp-1), a known Tgfb facilitator protein (Baudouin et al., 2014).

The C455R Cadasil mouse model had less detectable N3ecd in plasma when compared to controls and this difference was not explained by differences in levels of mRNA expression. The decreased levels of N3ecd may be related to impaired Notch 3 signaling as it is known that upon proteolytic cleavage of the Notch receptors the Notch ectodomain is released from the receptor expressing cell and transcytosed into the ligand-expressing cell (Klug and Muskavitch, 1999), a process that may be impaired by deficient signaling. Alternatively, it is possible that mutant N3ecd accumulates in vessels as goms, as it has been suggested occurs in humans and mouse models (Monet-Lepretre et al., 2013; Joutel et al., 2010), resulting in less detectable N3ecd in plasma of the Cadasil mice (Fig. 5C). In support of this concept, N3ecd staining was found to be more prominent in vessels from Cadasil (C455R) mice compared to controls. The notion that the extracellular domain of the Notch 3 receptor circulates in blood and that its levels may change in Cadasil is novel and it remains to be established if they are functional in this compartment. The use of N3ecd as a plasma biomarker may have some practical advantages over recently reported “Notch 3 score”, which represents a measure for the Notch 3 accumulation based upon immunohistochemistry analysis of postmortem brain tissue from mice (Rutten et al., 2015), as plasma N3ecd could be measured over time from live animals allowing prospective analyses.

Monitoring the level of these biomarkers at the beginning and end of a trial may offer a means to quantify the efficacy of a treatment that is both inexpensive and technically feasible. Possible therapies include modulation of Notch 3 signaling to test the Notch 3 signaling defect hypothesis in Cadasil (Arboleda-Velasquez et al., 2011). Other approaches include therapeutic Notch 3 cysteine correction in Cadasil using exon skipping or allele specific regulation of Notch 3 expression, which will constitute a direct test of the Notch 3(ECD) toxic accumulation cascade hypothesis (Rutten et al., 2016).

In conclusion, we have identified three circulating proteins in a C455R mouse model of Cadasil: endostatin, Htrai, and Notch 3, which we propose as biomarkers. Changes in these proteins occurred in mice by 200-days of age, coincident with a significant loss of VSMC in the retina. We speculate that quantification of these three proteins, along with analyses of VSMC coverage in the retina, may be used as a surrogate endpoints in future preclinical trials of Cadasil.

4. Experimental procedure

4.1. Animal models

All mouse models used in this study were previously described (Arboleda-Velasquez et al., 2008, 2011). Briefly, mice express either a wild type human notch3 transgene (WT76) or a mutated human notch3 transgene (C455R or R1031C), or a notch ligand (DLL1); transgenes are inserted into the ROSA26 locus (Soriano, 1999) and expression occurs through Cre-mediated recombination. This study was approved by the Schepens Eye Research Institute Institutional Animal Care and Use Committee (IACUC). All procedures followed were in accordance with institutional guidelines. Both male and female animals were included in the study.

4.2. Plasma collection

Plasma was collected from mice with the following genotypes: a mouse endogenous notch 3 knockout (N3ko), and a N3ko mouse heterozygous for the WT (WT76) human or mutant (C455R) human notch3 transgene, both driven by Sm22-Cre (N3ko; WT76+/−; Sm22-Cre and N3ko; C455R+/−; Sm22-Cre, respectively). Mice were anesthetized using a mixture of ketamine (120 mg/kg) and xylazine (20 mg/kg) using a 0.5%, 27 G needle via intraperitoneal injection. Under anesthesia, the anterior chest wall was removed and the left ventricle was pierced with a 0.5", 27 G needle. Blood was aspirated and dispensed, into a BD Vacutainer...
K2 EDTA, lavender top, blood collection tube (BD 367841). This tube was inverted several times to ensure the blood made contact with the entire surface of the EDTA-coated tube. The tube was then placed on ice and immediately processed for plasma following the manufacturer’s protocol. Plasma was aliquoted into 100 μl volumes in Eppendorf LoBind Protein Microcentrifuge Tubes (Eppendorf, 022431102) then stored at −80 °C until use.

4.3. Human plasma collection

Donors provided their informed consent before study entry under guidelines approved by the institutional review board at the University of Antioquia, Medellin, Colombia. Plasma was prepared from blood obtained through venipuncture using BD Vacutainer K2 EDTA tubes then stored at −80 °C until use.

4.4. Candidate biomarker screening using an angiogenesis antibody array

Plasma (200 μl) from control mice (N3KO; WT76+/−; SM22-Cre), CADASIL mutant mice (N3KO; C455R+/−; SM22-Cre), and endogenous notch 3 knockout mice (N3KO) was processed on the Proteome Profiler Angiogenesis Array Kit (R&D Systems, ARYO1S), according to the manufacturer’s protocol. For all antibody arrays, two control and two experimental mice were used. Dot blot analysis was performed via Image J by measuring integrated density. Background was accounted for by subtracting the integrated density of the dot’s surrounding area. A variance analysis was conducted on each array (Figs. 1 and 2) to identify potential candidate proteins, as previously described (Arboleda-Velasquez et al., 2011). Proteins of interest were considered to be differentially abundant in plasma if the absolute value of the difference between the two control (N3KO; WT76+/−; SM22-Cre) and two experimental (N3KO; C455R+/−; SM22-Cre) mice exceeded 1.5 times the variation (equation below).

\[
\text{Var} = \sqrt{\frac{(\text{Control}_1 - \text{Control}_2)^2 + (\text{Experimental}_1 - \text{Experimental}_2)^2}{2}}
\]

Candidate proteins that were differentially abundant in at least two of the three arrays were selected for validation via ELISA.

4.5. Candidate biomarker identification utilizing a MS proteomic screen

The material used for MS was obtained from a cohort of previously published mice and analyzed for different parameters (Arboleda-Velasquez et al., 2011). The genotypes of the mice, all wild type for the endogenous mouse notch 3, were heterozygous for the WT (WT76) human or mutant (R1031C human NOTCH3 transgene, driven by SM22-Cre (WT76+/−; SM22-Cre and R1031C+/−; SM22-Cre), respectively).

Aortas were isolated from one-year old mice and homogenized in 8 M urea and 50 mmol/L Tris–HCl (pH 8). Protein from each sample was collected, alkylated and digested using 1 μg of Lys-C at 37 °C overnight. Peptides from proteolytic digestion were desalted and labeled using reductive dimethylation (Boersema et al., 2008; Hsu et al., 2003). Samples were labeled with light (CH2O-NaBH3SCN) and heavy (CD2O-NaBD3SCN) reagents, respectively. The samples were washed with 0.1% formic acid and eluted from the column using 80% ACN and 0.5% acetic acid. Samples were then dried down using a vacuum centrifuge. Peptides from light or heavy aorta samples were separated by strong cation-exchange chromatography into 20 fractions. Each fraction was dried down by vacuum centrifugation and desalted using self-packed C18 STAGE-tips (Rappsilber et al., 2003). All liquid chromatography-tandem mass spectrometry (LC-MS/MS) data were obtained using an LTQ-Orbitrap Discovery hybrid mass spectrometer (Thermo Fisher, San Jose, CA). Each sample was loaded onto a reverse phase column and separated using 95 min LC gradient of 5–27% buffer B at a flow of 0.5–1 μl/min. MS analysis was performed using a top 10 method where the MS1 scan was acquired in the Orbitrap, followed by 10 data dependent MS/MS scans on the 10 most intense ions in the LTQ with CID for fragmentation. MS/MS spectra assignments were made with the Sequest algorithm (Eng et al., 1994) using the entire mouse IPI database (version 3.6).

Sequest searches were performed using a target-decoy strategy (Elias and Gygi, 2010) with the mouse IPI database in correct orientation (forward database) and the same database but with all sequences in reverse orientation (reverse database). Sequest searching was performed with a precursor ion tolerance of 50 ppm with LysC specificity. For dimethyl labels, a static modification of 28.0313 Da was used on the N-terminus and lysine residues. In addition, a differential modification of 8.044336 Da was used for heavy proteins, respectively, on the N-terminus and lysine residues. A protein level false discovery rate of 1% was used as a threshold for protein identifications using the target decoy strategy. Quantification of each protein was determined using the peak heights for light and heavy forms for that protein. The criterion for protein quantification was a signal-to-noise ratio of 5 for at least one of the protein species (light or heavy). Quantification of protein level was performed by calculating the median value of the ratios of light to heavy. Using this method of quantification, the contribution of single peptide protein species in the data set was low (5%), presumably reflecting the high accuracy and sensitivity of the MS methods and the high signal-to-noise cutoff used. Z scores were given to the proteins of the R1031C mice based on the standard deviation of the control mice. A median score of plus or minus two was used as a cut off to generate a list of candidate proteins (Supplemental Fig. 1B). Proteins identified as candidates passed this cut off criteria in two independent MS experiments, each utilizing one aorta per genotype.

4.6. Candidate validation using ELISA

Endostatin and HTRA1 were measured using commercially available ELISA kits (MyBioSource, MBS266186 and MBS90656, respectively), according to the provider’s protocol. The genotype of the samples tested was masked to the investigator conducting the assays and samples were randomized in the plates to prevent position effects. No samples were excluded from the analyses on the basis of being outliers.

4.7. Notch 3 extracellular domain ELISA

The N3ECD sandwich enzyme-linked immunosorbent assay was designed using commercially available antibodies. A monoclonal capture antibody (R&D systems MAB 1559) was coated on a high affinity binding ELISA plate (Fisher Scientific 3590) at 625 ng/ml in 100 μl of PBS, overnight, on a rotator, at 4 °C. The plate was then blocked using 300 μl of a 10% BSA solution in PBS at room temperature on a rotator for 2–3 h and washed three times with wash buffer diluted in distilled water from a 25X stock (R&D systems WA126). Recombinant human Notch 3 extracellular domain region (N3ECD), which was originally used to raise the antibodies (R&D systems 1559-NT-050), was used as a positive control and standard. Recombinant N3ECD serially diluted from 2 ng/ml to 0.125 ng/ml, along with experimental serum or plasma samples diluted 1:10 in PBS, were applied to the plate and left to incubate on a shaker overnight at 4 °C.

Biotinylated polyclonal antibody raised against the N3ECD (R&D Systems BAF1559) was used for detection. This antibody was
diluted in solution of PBS containing 2.5% BSA (Sigma) to a final concentration of 0.001 mg/ml. One hundred μl was applied to each well and left at room temperature on a rotator for 2 h. The plate was washed three times and a Horseradish Peroxidase-streptavidin (HRP-Strep) complex was added (R&D Systems DY998). The HRP-Strep complex was diluted using Reagent Calibrator Diluent 2 (R&D Systems DY008) at a 1X concentration. For each well 5 μl of HRP-Strep was added to 95 μl of the 1X calibrator diluent. One hundred μl of the diluted HRP-Strep complex was applied to the plate and left on a rotator at room temperature for 40 min. The plate was then washed five times to ensure complete removal of the HRP-Strep. After this, 100 μl of tetrathymelbenzidine (TMB) (R&D Systems DY008) was added to the plate, initiating the detection phase of the reaction. Termination of this reaction took place after 20 min using 50 μl of 3-IRES-eGFP) was added (R&D Systems DY998). The plate was then read using a SPECTRAmax plus 384 from Molecular Devices using a 450 nm wavelength.

To test the ability of our ELISA to detect circulating N3ECD in human samples, serially diluted plasma and serum samples, from patients with no CADASIL mutations, were used.

4.8. Ligand-dependent Notch signaling assay

Ligand-dependent Notch signaling assay was performed as described previously (Arboleda-Velasquez et al., 2011). Briefly, mouse embryonic fibroblasts (MEFs) were isolated from N3KO; WT76+/+ or N3KO; C455R+/+ embryos for signal-receiving cells, and from ROSA26DLL1+/+ embryos for signal-sending cells. Induction of the different transgenes at the ROSA26 locus was achieved by in vitro infection with an adenovirus encoding the Cre recombinase (adeno-Cre, 12.5 d.p.c.). This inducible transgene system was chosen to avoid creating an early selection pressure in the generation of the MEFs used in these experiments. MEFs were first generated in non-induced cells and then induction was performed prior to coculture.

Signal-sending and signal-receiving cells were plated together at a ratio of 2:1 respectively to ensure every signal-receiving cell was exposed to the DLL1 ligand, cultured together for 48 h, before signal-receiving cells were sorted by FACS based upon their eGFP expression (the transgene is bicistronic including the NOTCH 3-ires-eGFP) and directly lysed into RLT lysis buffer for RNA extraction with the RNeasy kit (Qiagen). Following retro-transcription of 600 ng total RNA (High Capacity Archive RNA-to-cDNA Kit, Applied Biosystems), col18a1/endostatin cDNA levels were assessed by TaqMan qPCR (Gene Expression Assay Mm00487131_m1) following manufacturer’s guidelines (Applied Biosystems). Differential expression was calculated using the 2^-ΔΔCT method with mouse Tbp (Mm00446973_m1) used to normalize cDNA input. Relative expression was calculated by normalizing on control samples where signal-receiving cells were cocultured with ROSA26DLL1+/+ cells that were not induced with Cre and therefore did not over-express the DLL1 ligand.

4.9. Quantification of smooth muscle cell loss in the vasculature

VSMC loss in the retinal vasculature was quantified in 10 animals: five control N3KO; WT76+/+; SM22-Cre mice and five CADASIL N3KO; C455R+/−; SM22-Cre mice. Animals were sacrificed and whole eyes were removed and immediately fixed overnight in 4% paraformaldehyde at 4 °C. Following dissection and removal of the fixed retina from the eyeball, retina was washed three times in 2 ml of phosphate buffered saline (PBS) at room temperature for three minutes. Blocking buffer was then applied for a minimum of 5 h at room temperature. Blocking buffer consists of 1 μmol/L CaCl2, 1 μmol/L MgCl2, 1 μmol/L MnCl2, 3.9 mmol/L sodium citrate, 1% Triton, and 0.3% goat serum, all diluted in PBS.

The retinas were then incubated with 5 μg/ml of Alexa 488-conjugated isolectin B4 glycoprotein (IB4) [1 in 200] (Invitrogen I21411) and 10 μg/ml of Cy3-conjugated smooth muscle actin antibody (SMA) [1 in 100] (Sigma C6198), in a final volume of 300 μl of blocking buffer overnight at 4 °C on a rotator before the retina was then washed with PBS and then mounted for imaging. Whole retina images were taken under 5x (1x2.5) using the Zeiss Axioskpe 2 mot plus microscope, using the red and green channels. The SMA coverage was quantified using ImageJ macros as follows. The program utilizes the signal of the IB4 positive staining to “outline” the area of the vessels to obtain a measurement of the total vascular area. Then the red channel is assessed for positive signal and the area for the regions of positive signal are recorded. The macro then produces an analyzed image, outlining the vascularized area in blue and the SMA positive area in red, and a table of results of vascular area and SMA positive area to be utilized for calculating percent coverage from the ratio of SMA positive area to total vascularized area.

4.10. Notch 3 and endostatin retina staining

Staining was performed in retinas of 6-month old N3KO; C455R+/−; SM22-Cre, and N3KO; WT76+/−;SM22-Cre mice with antibodies against either Notch 3 extracellular domain (1 in 200) (EMD Millipore MABC594) or endostatin (1 in 120) (R&D Systems BAF570) with Col IV, a vessel marker, [1:200] (Abcam ab6586) in a total volume of 300 μl per retina. Retinas were prepared as described above and then placed in 300 μl of blocking solution with one of the two pairs of antibodies and imaged as described above.

4.11. Statistical analysis

An unpaired students t-test function assuming a two tailed distribution and equal variance as statistical comparison of the ELISA results and VSMC loss quantification. P values < 0.05 were considered significant and are indicated with an asterisk (*).

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Appendix A. Supplementary material

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