Supporting Information

Arboleda-Velasquez et al. 10.1073/pnas.1101964108

SI Materials and Methods

IHC and Immunofluorescence. Paraffin-embedded sections were deparaffinized with xylene, hydrated with descending series of ethanol, and washed with PBS solution. The sections were blocked with 10% normal serum and 1% BSA in PBS solution for 1 h. Sections for IHC were further incubated with 0.5% H_2O_2 in methanol to reduce endogenous peroxidase activity. After washing with PBS solution, sections were incubated overnight with primary antibodies against smooth muscle cell α-actin (A5228; Sigma), clusterin (AF2937; R&D Systems), and endostatin (AF1098; R&D Systems) diluted in antibody diluent (Dako). After washing with PBS solution, sections were incubated for 30 min at room temperature with secondary antibodies coupled to biotin or fluorochrome in antibody diluent. Sections were washed with PBS solution and mounted with ProLong mounting solution (Invitrogen) for immunofluorescence. For IHC, sections were developed with DAB according to the manufacturer's instructions, counterstained with hematoxylin, and dehydrated with ascending series of ethanol and xylene before mounting.

EM. Following intracardiac perfusion of deeply anesthetized animals with 2.5% glutaraldehyde and 2% paraformaldehyde (PFA) in 0.1 M sodium cacodylate buffer (pH 7.4), brain tissue was dissected, rinsed, dehydrated in a series of ethanol dilutions (50–100%), and embedded in epoxy resin (Embed 812; Electron Microscopy Sciences). Ultrathin sections (60 nm) were cut on a Reichert ultramicrotome and collected on Formvar- and carbon-coated grids. The samples were stained with 2% uranyl acetate and lead citrate and examined on a Philips Tecnai 12 BioTWIN electron microscope (FEI). Images were captured digitally using a CCD camera (Morada; Soft Imaging Systems).

Immuno-EM. Human brains were stored in formalin since postmortem extraction (>5 y). To allow for simultaneous visualization of GOMs and detection of gold particles upon immuno-EM, small blocks of tissue were postfixed in 2.5% glutaraldehyde and 2% PFA in 0.25 M Hepes buffer (pH 7.4), embedded in epoxy resin, and sectioned as described earlier. Epon sections were etched in 1% H_2O_2 and processed as described (1). Grids then were immunolabeled with antibodies against clusterin (1:20; AF2937; R&D Systems) or endostatin (1:20; AF1098; R&D Systems) for 30 min at room temperature, washed in PBS solution, labeled with secondary antibodies, washed in PBS solution, labeled with Protein A Gold (Universitair Medisch Centrum Utrecht) for 30 min at room temperature, washed in PBS solution, postfixed in 1% glutaraldehyde for 5 min, washed in distilled water, stained with 2% neutral uranyl acetate for 10 min, washed briefly in water, and rinsed with 1.8% methylcellulose/0.5% uranyl acetate. Samples were examined on a Philips Tecnai 12 BioTWIN electron microscope. Given the fixation conditions and epon embedding, signal intensity (reflected by numbers of gold particles) was expected to be low. Images were captured digitally by using a CCD camera (Morada; Soft Imaging Systems).

In one case (Fig. 5G), brain tissue was stored frozen since postmortem extraction (10 y), thawed and postfixed in 4% PFA, rinsed in PBS solution, resuspended in 10% gelatin, trimmed in smaller blocks and placed in 2.3 M sucrose overnight at 4 °C. Blocks were frozen rapidly on aluminum pins in liquid nitrogen, trimmed on a Leica Cryo-EMUC6UltraCut, and 75-nm-thick sections were collected as previously described (1). The frozen sections were collected, thawed, and placed on a nickel formvar/carbon-coated grid floated in a dish of PBS solution. For immunolabeling, grids were placed section-side-down on drops of 0.1 M ammonium chloride for 10 min to quench untreated aldehyde groups, then blocked for nonspecific binding on 1% fish skin gelatin in PBS solution for 20 min. Grids were incubated on goat anti-endostatin antibody (1:10) for 30 min, rinsed, incubated with a rabbit anti-goat bridge, and placed on protein A gold for 30 min. All grids were rinsed in PBS solution, fixed using 1% glutaraldehyde for 5 min, and then rinsed in a uranyl acetate/methylcellulose drop for 10 min.

Western Blot. Cells were harvested 48 h after infection with adeno-Cre using RIPA buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% DOC, 0.1% SDS, and 5 mM EDTA) for the analysis described in Fig. 1*A*. For the analysis described in Fig. 6*C*, aortas were dissected from euthanized mice and homogenized in protein extraction buffer containing 5 M urea, 50 mM NaCl, and 75 mM Tris, pH 8. All samples were reduced in loading buffer containing 2% SDS and DTT 100 mM before SDS-PAGE. The M-20 primary antibody was used for detection of Notch 3 (sc-7424; Santa Cruz). Clusterin was detected using the same antibody used for tissue expression studies, whereas a monoclonal anti-endostatin antibody [hES (1D1)] against a recombinant human polypeptide corresponding to the mouse endostatin domain (provided by B. Olsen, Harvard Medical School, Boston) was used for detection of COL18A1/endostatin (2). Similarly to the antibody we used for immunostaining, this antibody recognizes endostatin whether free (cleaved from COL18A1) or part of the COL18A1 molecule.

Characterization of Granular Osmiophilic Deposits. Proteins were extracted from LCM caps by using Hot SDS sample buffer (0.125 M Tris, pH 6.8, 10% glycerol, 2% SDS, 0.1 M DTT; no protease inhibitors added). Cold sample buffer (50 μL) containing DTT was placed on the bottom of a 0.5-mL tube and sealed using the LCM caps as lids. The tube was inverted and incubated for 15 min at 65 °C in an oven. The buffer was used again to extract proteins sequentially from a total of five caps. Individual aliquots were then mixed to obtain a total volume of approximately 250 μL, homogenized by sonication for 10 s, and frozen at −80 °C. Before gel electrophoresis, samples were thawed and supplemented with NuPAGE LDS Sample Buffer (NP0007; Invitrogen) and NuPAGE Sample Reducing Agent (NP0004) according to the manufacturer's instructions. Samples were denatured by incubation at 70 °C for 15 min. Running buffer (Mops SDS, NP0001) was supplemented with NuPAGE Antioxidant (NP0005). Novex NuPAGE gels (NP0335) were used as indicated by the manufacturer and run at 150 V. Gels were recovered in 2% acetic acid/40% methanol for 1 h, rinsed, stained with colloidal Coomassie (B-2025; Sigma), followed by brief rinses with 5% acetic acid/25% methanol, and finally washed in 25% methanol (two to three changes) for 1 to 2 h at room temperature.

Sample Preparation and MS. Each gel lane was divided into four parts based on size, excised, and prepared for trypsin digestion as described previously (3). Briefly, gel pieces were cut into 1-mm cubes, destained using 50% acetonitrile/50 mM NH_4HCO_3 at 37 °C, dehydrated with 100% acetonitrile, and dried for 10 min under vacuum. Dry gel pieces were rehydrated with 10 ng/mL trypsin (in 50 mM NH_4HCO_3, pH 8.2) on ice for 1 h, and then digested at 37 °C overnight, extracted twice using a solution of 50% acetonitrile/5% formic acid, dried completely under vac-

uum, and stored at −80 °C. All samples were analyzed by liquid chromatography/tandem MS (MS/MS) in duplicate on a hybrid linear ion trap–Orbitrap mass spectrometer (LTQ Orbitrap; ThermoFisher). Peptide mixtures were loaded onto a 125-μm inner diameter fused-silica microcapillary column packed in-house to 18 cm length with C18 resin (Magic C18AQ, 5 μm, 200 Å; Michrom Bioresources) and separated by using a 55-min gradient from 5% to 27% solvent B (0.15% formic acid in acetonitrile). The mass spectrometer was operated in a data-dependent mode with dynamic exclusion of previously fragmented ions for 30 s. A full-scan MS was followed by 10 MS/MS experiments on the 10 most abundant ions detected in the full-scan MS. The AGC target values were set to 1×10^6 ions for MS full scans at a resolution of 60,000 whereas the AGC target was set at 5,000 ions for MS/MS experiments, and the maximum ion accumulation times were 1,000 ms for the MS and 120 ms for the MS/MS mode. Isolation widths were ±2.0 *m/z* for MS/MS precursors, and ions were selected for MS/MS when their intensities exceeded a minimum threshold of 3,000 counts. Singly charged peptides and peptides with unassigned charge states were excluded from MS/MS fragmentation. The normalized collision energy was set to 29%, and one microscan was acquired per spectrum. All data sets were converted from the .RAW format to the .mzXML format (4) and imported into an in-house data management system. MS/MS data files were searched using the Sequest algorithm (5) against a composite target-decoy protein sequence database (6) in which the target component was comprised of protein sequences derived from the IPI Human database (www.ebi.ac.uk/IPI/) and sequences of known contaminant proteins, such as porcine trypsin and human keratins. Searches were performed with full tryptic specificity; oxidized Met (+15.9949) and carboxyamidomethylated Cys (+57.0215) were set as dynamic and static modifications, respectively. The precursor mass tolerance was set at 50 ppm and monoisotopic precursor and fragment masses were used in all calculations. The fragment ion tolerance was set at 1.0 Da, and a maximum of two missed cleavages were allowed. Search results were filtered by using a target-decoy strategy to obtain a peptide identification false positive rate of approximately 1% while maximizing sensitivity. For each of the four samples, the total number of redundant peptides [peptide spectral matches (PSMs)] for each protein were calculated for each duplicate separately and then averaged. These averaged values for the experimental samples were then subtracted from their control counterparts. The variation in each sample was estimated as follows:

$$Var = \sqrt{\frac{(Control_1 - Control_2)^2 + (Experimental_1 - Experimental_2)^2}{2}}$$

Proteins were considered to be differentially expressed if the absolute value of the difference between the average peptide counts (i.e., PSM) in the experimental sample minus the average PSM in the control sample exceeded three times the variation and a value of four.

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Table S1. Gene Ontology analyses of proteins identified in control human brain vessel samples by MS

Biological process	Reference proteins (n = 25,431)	Detected proteins (n = 448)	Expected	+/–	P value
Biological process unclassified	11,321	50	194.98	–	1.14^{-49}
Cell structure	687	91	11.83	+	1.69^{-49}
Cell structure and motility	1,148	106	19.77	+	1.58^{-44}
Intracellular protein traffic	1,008	62	17.36	+	2.48^{-16}
Glycolysis	46	14	0.79	+	2.53^{-11}
Transport	1,306	57	22.49	+	5.76^{-9}
Protein folding	186	20	3.2	+	2.7^{-8}
Carbohydrate metabolism	592	33	10.2	+	1.97^{-7}
Muscle contraction	198	18	3.41	+	5.99^{-7}
Endocytosis	277	22	4.77	+	8.38^{-7}
Protein complex assembly	68	11	1.17	+	6.41^{-6}
Neuronal activities	569	29	9.8	+	1.04^{-5}
Ectoderm development	692	34	11.92	+	1.09^{-5}
Stress response	200	17	3.44	+	1.78^{-5}
General vesicle transport	251	19	4.32	+	0.000019
Cell adhesion	622	30	10.71	+	2.02^{-5}
Synaptic transmission	279	20	4.81	+	0.000021
Receptor mediated endocytosis	111	13	1.91	+	2.17^{-5}
Tricarboxylic acid pathway	36	8	0.62	+	4.29^{-5}
Calcium ion homeostasis	38	8	0.65	+	6.42^{-5}
Amino acid metabolism	230	16	3.96	+	0.000115
Other protein targeting and localization	18	6	0.31	+	0.000133
Cell motility	352	21	6.06	+	0.000193
Ion transport	616	29	10.61	+	0.000234
mRNA transcription regulation	1,459	6	25.13	–	0.000703
Exocytosis	163	13	2.81	+	0.00105
Signal transduction	3,406	89	58.66	+	0.00119
Homeostasis	196	13	3.38	+	0.00149
Developmental processes	2,152	62	37.06	+	0.00158
Immunity and defense	1,318	43	22.7	+	0.00177
Extracellular matrix protein-mediated signaling	62	8	1.07	+	0.00308
Cation transport	482	23	8.3	+	0.00311
Neurotransmitter release	107	10	1.84	+	0.00436
Cell communication	1,213	41	20.89	+	0.00576
mRNA transcription	1,914	13	32.96	–	0.00596
Protein metabolism and modification	3,040	78	52.36	+	0.00703
Nucleoside, nucleotide and nucleic acid metabolism	3,343	34	57.58	–	0.00802
Antioxidation and free radical removal	38	6	0.65	+	0.00882
Cell adhesion-mediated signaling	379	19	6.53	+	0.00889
Muscle development	143	10	2.46	+	0.0461

Reference proteins indicates the number of proteins from the complete National Center for Biotechnology Information *H. sapiens* database belonging to a specific category. Detected proteins indicates the number of proteins from that category detected in the MS study. Expected indicates the number of proteins stochastically expected to appear in the list of detected proteins, based on the reference list. +/– indicates whether the number of proteins from a given category are over- (+) or under–represented (–).

Table S2. Gene Ontology analyses of proteins identified in human CADASIL brain vessel samples by MS

Biological process	Reference proteins (n = 25,431)	Detected proteins (n = 448)	Expected	+/–	P value
Biological process unclassified	11,321	49	197.21	–	1.7^{-51}
Cell structure	687	87	11.97	+	3.2^{-45}
Cell structure and motility	1,148	103	20	+	1.4^{-41}
Intracellular protein traffic	1,008	57	17.56	+	3.5^{-13}
Glycolysis	46	14	0.8	+	3^{-11}
Protein folding	186	20	3.24	+	3.3^{-8}
Transport	1,306	55	22.75	+	6.6^{-8}
Carbohydrate metabolism	592	34	10.31	+	7.4^{-8}
ECM protein-mediated signaling	62	12	1.08	+	3.4^{-7}
Muscle contraction	198	18	3.45	+	7.1^{-7}
Cell adhesion-mediated signaling	379	26	6.6	+	1.2^{-6}
Cell communication	1,213	51	21.13	+	1.3^{-6}
Cell adhesion	622	32	10.84	+	2.7^{-6}
Stress response	200	18	3.48	+	3.9^{-6}
Endocytosis	277	21	4.83	+	4.9^{-6}
Developmental processes	2,152	70	37.49	+	1.1^{-5}
Signal transduction	3,406	97	59.33	+	2^{-5}
mRNA transcription regulation	1,459	4	25.42	–	2.2^{-5}
Amino acid metabolism	230	17	4.01	+	3^{-5}
Protein complex assembly	68	10	1.18	+	6.9^{-5}
Calcium ion homeostasis	38	8	0.66	+	7^{-5}
General vesicle transport	251	18	4.37	+	0.0001
Neuronal activities	569	27	9.91	+	0.00012
Other protein targeting and localization	18	6	0.31	+	0.00014
Cell motility	352	21	6.13	+	0.00023
Immunity and defense	1,318	46	22.96	+	0.00025
Synaptic transmission	279	18	4.86	+	0.00045
mRNA transcription	1,914	11	33.34	–	0.00054
Antioxidation and free radical removal	38	7	0.66	+	0.00087
Ectoderm development	692	30	12.05	+	0.00097
Receptor mediated endocytosis	111	11	1.93	+	0.00111
Mesoderm development	551	25	9.6		0.00273
Pentose-phosphate shunt	10	4	0.17		0.00478
Protein metabolism and modification	3,040	79	52.96	+	0.00616
Homeostasis	196	12	3.41	+	0.00665
Tricarboxylic acid pathway	36	6	0.63	+	0.00699
Nucleoside, nucleotide and nucleic acid metabolism	3,343	35	58.23	–	0.0107
Skeletal development	123	10	2.14		0.0152
Exocytosis	163	11	2.84	+	0.0249
Neurotransmitter release	107	9	1.86	+	0.0267
Blood circulation and gas exchange	89	7	1.55		0.0337
Ion transport	616	24	10.73	+	0.0391
Muscle development	143	10	2.49	+	0.0504

Reference proteins indicates the number of proteins from the complete National Center for Biotechnology Information *H. sapiens* database belonging to a specific category. Detected proteins indicates the number of proteins from that category detected in the MS study. Expected indicates the number of proteins stochastically expected to appear in the list of detected proteins, based on the reference list. +/– indicates whether the number of proteins from a given category are over- (+) or under–represented (–).

Table S3. Comparative proteomic analysis of human brain vessels from control and CADASIL samples

Reference	Name	Control	CADASIL
ANXA2P2	Annexin A2 pseudogene 2	11	18
CLU	Clusterin	5	24
COL12A1	Collagen 12 α 1	0	8
COL14A1	Collagen 14 α 1	0	6
COL18A1	Collagen 18 α 1 (endostatin included)	1	12
COL1A2	Collagen 1 α 2	1	8
HBA1, HBA2	Hemoglobin, α	77	28
HSPG2	Perlecan	8	34
INA	Internexin, α	24	10
LAMA5	Laminin, α 5	4	15
LAMC1	Laminin, γ 1	14	21
MFGE8	Lactadherin (medin included)	0	7
MYH11	Smooth muscle myosin heavy chain	14	60
NEFL	Neurofilament protein, light polypeptide	21	8
NEFM	Neurofilament 3	29	8
NFASC	Neurofascin	8	2
PRELP	Leucine rich repeat proteoglycans	0	9
SLC4A1	Solute carrier family 4	21	4
VCL	Vinculin	7	19

Proteins found to be either more or less prevalent in both CADASIL samples compared with both controls. This analysis considered the number of total peptides detected for each protein and the variability of the measurements between replicates (*Methods*). Average number of total peptides found is listed for each protein.