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Reducing Hypermuscularization of the Transitional Segment between Arterioles and Capillaries Protects Against Spontaneous Intracerebral Hemorrhage

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Julien Ratelade, Nicholas Klug, Damiano Lombardi, Monara Kaelle Servulo Cruz Angelim, Fabrice Dabertrand, et al.. Reducing Hypermuscularization of the Transitional Segment between Arterioles and Capillaries Protects Against Spontaneous Intracerebral Hemorrhage. *Circulation*, 2020, 141 (25), pp.2078-2094. 10.1161/CIRCULATIONAHA.119.040963 . inserm-02511921

HAL Id: inserm-02511921

<https://inserm.hal.science/inserm-02511921>

Submitted on 19 Mar 2020

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1 **Reducing hypermuscularization of the transitional segment between**
2 **arterioles and capillaries protects against spontaneous intracerebral**
3 **hemorrhage**

4 **Short title: A novel mechanism of intracerebral hemorrhage**

5

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20 Text word counts (does not include title, abstract, ref, table, legends): 5151

21

1 **ABSTRACT**

2

3 **Background:** Spontaneous deep intracerebral hemorrhage (ICH) is a devastating subtype of
4 stroke without specific treatments. It has been thought that smooth muscle cell (SMC)
5 degeneration at the site of arteriolar wall rupture may be sufficient to cause hemorrhage.
6 However, deep ICHs are rare in some aggressive small vessel diseases that are characterized
7 by significant arteriolar SMC degeneration. Here we hypothesized that a second cellular
8 defect may be required for the occurrence of ICH.

9 **Methods:** We studied a genetic model of spontaneous deep ICH using *Col4a1*^{+/*G498V*} and
10 *Col4a1*^{+/*G1064D*} mouse lines that are mutated for the alpha1 chain of Collagen type IV. We
11 performed high resolution imaging and molecular analyses of cerebroretinal microvessels,
12 genetic rescue experiments, vascular reactivity analysis and computational modeling. We also
13 examined post-mortem brain tissues from patients with sporadic deep ICH.

14 **Results:** We identified in the normal cerebroretinal vasculature a novel segment between
15 arterioles and capillaries, herein called the transitional segment (TS), that is covered by mural
16 cells distinct from SMCs and pericytes. In *Col4a1* mutant mice, this TS was
17 hypermuscularized, with a hyperplasia of mural cells expressing more contractile proteins,
18 whereas the upstream arteriole exhibited a loss of SMCs. Mechanistically, TS showed a
19 transient increase in proliferation of mural cells during post-natal maturation. Mutant brain
20 microvessels, unlike mutant arteries, displayed a significant upregulation of SM genes and
21 *Notch3* target genes, and genetic reduction of *Notch3* in *Col4a1*^{+/*G498V*} mice protected against
22 ICH. Retina analysis showed that hypermuscularization of the TS was attenuated but
23 arteriolar SMC loss unchanged in *Col4a1*^{+/*G498V*}, *Notch3*^{+/-} mice. Moreover,
24 hypermuscularization of the retinal TS increased its contractility and tone and raised the
25 intravascular pressure in the upstream feeding arteriole. Consistently, we similarly found

1 hypermuscularization of the TS and focal arteriolar SMC loss in brain tissues from patients
2 with sporadic deep ICH.

3 **Conclusions:** Our results suggest that hypermuscularization of the TS, via increased Notch3
4 activity, is involved in the occurrence of ICH in *Col4a1* mutant mice, by raising the
5 intravascular pressure in the upstream feeding arteriole and promoting its rupture at the site of
6 SMC loss. Our human data indicate that these 2 mutually reinforcing vascular defects may
7 represent a general mechanism of deep ICH.

8

9

1 **Clinical Perspective**

2 What is new ?

- 3 • *Col4a1* mutant mice, a genetic model of spontaneous deep intracerebral hemorrhage
4 (ICH), exhibit excessive muscularization of the transitional segment (TS) at the interface
5 between arterioles and capillaries, as a result of increased Notch3 activity, in addition to
6 focal loss of smooth muscle cells in arterioles, in the brain and the retina.
- 7 • Genetic reduction of *Notch3* in *Col4a1* mutant mice protects against ICH.
- 8 • Analysis of the retinal vasculature supports the concept of 2 mutually reinforcing vascular
9 defects as a mechanism of deep ICH, in which hypermuscularization of TS raises the
10 intravascular pressure in the upstream feeding arteriole crippled by smooth muscle cell
11 loss, and this ultimately leads to arteriolar rupture.

12 What are the clinical implications?

- 13 • Spontaneous ICH accounts for 10-20% of all strokes and is the most devastating subtype
14 of stroke, without specific treatments.
- 15 • Because these 2 mutually reinforcing vascular defects are present in the brain of patients
16 with sporadic deep ICH, this may represent a general mechanism of deep ICH and a
17 putative therapeutic target.

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1 INTRODUCTION

2 Spontaneous (non-traumatic) intracerebral hemorrhage (ICH) accounts for 9-27% of all
3 strokes¹, causing about 3 million cases worldwide per year². This stroke subtype is the most
4 devastating one because it is associated with high mortality rates (55% at one year) and major
5 permanent disabilities in patients who survive^{3,4}. Cerebral small vessel diseases underlie the
6 vast majority of ICHs⁵: so-called hypertension-related arteriopathy accounts for almost all
7 deep ICH (occurring in the basal ganglia, thalamus, cerebellum or brainstem), whereas just
8 over half of lobar ICH is associated with cerebral amyloid angiopathy. Importantly, as
9 population blood pressure control has improved, epidemiological studies have found a
10 decreasing prevalence of uncontrolled hypertension at the time of ICH diagnosis^{6,7}, yet ICH
11 incidence appears unchanged³.

12 Pathological studies on postmortem human brains have suggested that deep ICH results from
13 focal smooth muscle cell (SMC) degeneration in the media of cerebral arterioles and
14 replacement with collagen, which is structurally stiff and brittle⁸⁻¹⁰. It has been speculated that
15 the weakened arteriole dilates and eventually bursts at bifurcation sites and that this occurs
16 preferentially in deep brain regions where penetrating arterioles are exposed to high perfusion
17 pressure due to their proximity to the circle of Willis¹¹. However, SMC loss alone may not be
18 sufficient to cause ICH because deep ICHs are rare in some aggressive small vessel diseases
19 that are characterized by significant arteriolar SMC degeneration¹². Emerging evidence
20 indicates that mural cells exhibit a variety of cell phenotypes along the microvascular tree¹³,
21 raising the possibility that mural cells may respond in different ways to the factors underlying
22 the arteriopathy.

23 Animal models of spontaneous deep ICHs are very rare. Beside sporadic forms of deep ICH,
24 monogenic forms largely indistinguishable from sporadic forms have been characterized
25 offering excellent opportunities for mechanistic studies using genetic models. Heterozygous

1 mutations in the $\alpha 1$ (COL4A1) chain of collagen type IV, which is a major component of
2 vascular basement membranes (BM), are associated with spontaneous deep ICHs in humans
3 or mice in the absence of hypertension, although with incomplete penetrance^{14,15}.

4 Herein, we hypothesized that a second cellular defect may be required for the occurrence of
5 ICH and we used *Col4a1* mutant mice to test this hypothesis.

6

1 **METHODS**

2 The data that support the findings of this study are available from the corresponding author
3 upon reasonable request.

4 ***Human tissue samples***

5 Post-mortem frozen and paraffin embedded human brain samples were obtained from the UK
6 Medical Research Council Brain Bank of the University of Edinburgh, with informed consent
7 from patients or their families for their inclusion in the Lothian IntraCerebral Haemorrhage,
8 Pathology, Imaging and Neurological Outcome (LINCHPIN) study⁵. The use of postmortem
9 brain tissues for research was approved by the Institutional Review Board of INSERM
10 (IRB00003888, n°17-392).

11 ***Mice***

12 *Col4a1*^{+G498V} mice used in this study were generated on a mixed 50% C57BL/6 – 50%
13 129/Sv background as described previously^{14,16}. *Col4a1*^{+G1064D} mice on a C57BL/6
14 background were obtained from GlaxoSmithKline (Brentford, UK). *Notch3*^{+/-} mice were
15 described previously¹⁷ and maintained on a 129/Sv background. All animals were
16 randomized for their genotype information and were included in the study. Mice were
17 maintained in a specific pathogen-free environment. Experiments were conducted in
18 accordance with the guidelines of our local institutional Animal Care and Use Committee
19 (Lariboisiere-Villemin, CEA9).

20 All other methods are described in the supplementary material.

21 ***Statistical analysis***

22 Unless specified otherwise, data in figures and text are presented as means ± standard
23 deviation. Statistical significance was determined using either two-tailed Student's t-test, two-
24 tailed Mann-whitney test, one-way analysis of variance (ANOVA) followed by Tukey's post
25 hoc test, 2-way ANOVA followed by Bonferroni post hoc test or Kruskal-Wallis test followed

1 by Dunn's post hoc test, as specified in figure legends (GraphPad Prism 7 software). For large
2 samples ($n > 8$), data were analyzed using parametric or non-parametric tests depending on the
3 result of the D'Agostino-Pearson omnibus normality test. Chi-square test was used to
4 compare the penetrance of ICHs between different groups.
5

1 RESULTS

2 *An unbiased, marker-based mural cell characterization of the retinal vasculature*

3 We focused on the superficial retinal vascular network that exhibits many similarities with the
4 cerebral vasculature and offers the advantage of being nearly planar on flat-mounted retina
5 preparations and highly stereotyped (Fig 1A). Moreover, we recently established the validity
6 of the retinal vasculature as a study model in the COL4A1 disease¹⁴. Flat-mounted retinas of
7 adult mice were stained for various mural cell markers including smooth muscle contractile
8 proteins — α smooth muscle actin (α -SMA) or smooth muscle myosin heavy chain
9 (SMMHC)—, the intermediate filament protein desmin, the plasma membrane chondroitin
10 sulfate proteoglycan (NG2), Platelet-derived growth factor receptor-beta (PDGFR- β), or
11 aminopetidase N (CD13). Markers of endothelial cells (VE-cadherin), astrocytes (GFAP,
12 aquaporin 4), BM (perlecan, COL4A2) or elastic lamina (elastin or Alexa Fluor 633–
13 conjugated hydrazide¹⁸) were also used. Arterioles (average diameter 26 μ m) were surrounded
14 by α -SMA^{high}, SMMHC^{high}, desmin^{low}, CD13^{low}, NG2^{low} and PDGFR- β ^{low} SMCs. Here,
15 SMCs had a flat ring-shape and were circumferentially organized in a perpendicular manner
16 towards the vessel axis (Figs 1B, S1 and S2). Veins (average diameter 32 μ m) were
17 surrounded by stellate-shaped α -SMA^{low}, SMMHC^{low}, desmin^{high}, CD13^{low}, NG2^{low} and
18 PDGFR- β ^{high} SMCs (Figs 1B, S1 and S2). Capillaries (average diameter 4.1 μ m) were
19 covered by α -SMA^{negative}, SMMHC^{negative}, desmin^{high}, CD13^{high}, NG2^{high} and PDGFR- β ^{high}
20 mural cells, hereafter called pericytes, showing a protruding ovoid cell body (“bump on a
21 log”) embedded in the BM, and elongated thin processes (Figs 1B, S1 and S2). Interestingly
22 the vascular segment located between arterioles and capillaries (average diameter 10 μ m) was
23 covered by distinct mural cells, expressing both arteriolar SMC and pericyte markers. Namely
24 these cells were α -SMA^{high}, SMMHC^{high}, desmin^{high}, CD13^{high}, NG2^{high} and PDGFR- β ^{high}.
25 These mural cells had a pericyte-like protruding cell body, which contained the nucleus and

1 where NG2 staining was mostly concentrated (Fig S2B), but larger and irregular ensheathing
2 processes projecting orthogonally over the vessel, bright for α -SMA and SMMHC (Figs 1B,
3 S1 and S2). Thereafter, this vascular segment will be referred as “the transitional segment”
4 (TS) and its mural cells as “transitional cells”. TS were present at every arteriole/capillary
5 transition, usually at the 1st-3rd branch orders (0 being the proximal arteriole starting in the
6 center of the retina) (Fig S3). Interestingly, TS, unlike arterioles, were devoid of elastic fibers
7 (Fig. S4C-D). On the other hand, expression of VE-cadherin, perlecan, COL4A2, GFAP and
8 aquaporin 4 was almost comparable along the arteriovenous bed (Fig. S4 and data not shown).

9 *Col4a1^{+G498V} mice have hypermuscularized TS in addition to arteriolar SMC loss in the*
10 *retinal vasculature*

11 Next, we studied microvascular changes in the retina of adult *Col4a1^{+G498V}* mice (6-month-
12 old). Our previous work had shown segmental and focal loss and apoptosis of SMCs in the
13 retinal arterioles, starting from 3 months of age¹⁴. In keeping with this finding, we observed
14 that mural cell loss was strictly restricted to retinal arterioles and that remaining SMCs stained
15 negative for NG2 and low for desmin, suggesting that mutant SMCs have not undergone a
16 phenotypic switch towards a transitional cell or pericyte phenotype (Fig. S5). In striking
17 contrast, mutant TS exhibited a significantly higher number of mural cells with an increased
18 expression of contractile proteins including α -SMA or SMMHC (Fig. 2 A-C) that was already
19 present at one month of age (Fig S6), and we coined the term “hypermuscularization” to
20 designate these alterations. On the other hand, cell density and phenotype of pericytes were
21 unchanged in mutant capillaries although pericyte coverage was slightly increased (Fig. S7)
22 and no overt anomalies of SMCs were noticed in mutant veins.

23 To delineate the underlying cellular mechanisms of TS pathology, we examined whether there
24 was abnormal mural cell proliferation or apoptosis in mutant mice. The vascularization of the
25 murine retina occurs after birth and prior works suggest that mural cells are derived from a

1 common NG2+ precursor cell already present at birth^{19,20}. At post-natal day 10 (P10),
2 arterioles were already easily recognizable as α -SMA bright vessels, yet SMCs were still
3 NG2 bright and had a protruding cell body, whereas TS just started to become discernable
4 (Figs 2D and S8). At P10, TS consisted in small branches covered by rounded mural cells that
5 were NG2 bright and α -SMA low-to-very pale (white-boxed area in Fig. 2D). We found that
6 the length and α -SMA expression level of TS were comparable between wildtype and mutant
7 mice (Fig. 2F). Incorporation of 5-ethynyl-2'-deoxyuridine (EdU) revealed a low number of
8 proliferating mural cells in TS, but which was significantly higher in *Col4a1*^{+G498V} mice (Fig.
9 2D-F). Very few apoptotic mural cells were present in TS in wildtype and mutant mice (Fig
10 S8D). At P23, TS had further grown and almost reached the adult mature state described
11 above. The length of TS was comparable between wildtype and mutant mice but α -SMA
12 expression level was significantly increased in mutant mice. Proliferating mural cells were no
13 longer detected and almost no apoptotic mural cells were observed in *Col4a1*^{+/+} and
14 *Col4a1*^{+G498V} mice (Fig S8). SMC proliferation or apoptosis in arterioles was also very low at
15 P10 and P23, and notably comparable between wildtype and mutant mice (Fig. S8).

16 ***The brain vasculature of *Col4a1*^{+G498V} and *Col4a1*^{+G1064D} mutant mice display similar***
17 ***hypermuscularization of TS in addition to arteriolar SMC loss***

18 We next assessed whether the brain vasculature of *Col4a1* mutant mice displayed similar
19 changes to those of the retinal vasculature. Immunostaining of thick (100 μ m) brain slices
20 indicated that brain arterioles, like retinal arterioles, were surrounded by α -SMA^{high},
21 desmin^{low} and NG2^{low} SMCs, forming narrow circumferential bands, and brain TS were
22 surrounded by α -SMA^{high}, desmin^{high} and NG2^{high} mural cells having larger and irregular
23 ensheathing processes and a protruding cell body containing the nucleus wrapped by a bright
24 NG2 staining (Figs 3A-B and S9). Consistent with our previous work¹⁴, we detected in

1 *Col4a1*^{+/*G498V*} mice focal and segmental loss of SMCs in penetrating arterioles within regions
2 prone to ICH, like the thalamus (data not shown). Importantly, we found that α -SMA
3 expression in TS was significantly increased in *Col4a1*^{+/*G498V*} mice compared to *Col4a1*^{+/*+*}
4 mice, not only in the thalamus but also in other brain regions, such as the cortex and striatum,
5 that are spared by ICH (Fig 3C-D).

6 The combination of arteriolar SMC loss and hypermuscularization of TS was similarly
7 observed in *Col4a1*^{+/*G1064D*} mice, another independent *Col4a1* mutant line²¹⁻²³ in both the
8 retina and the brain, although vascular pathology was more severe than in *Col4a1*^{+/*G498V*} mice
9 (expanded results, Figs S10-S11).

10 To understand the reason behind the differences between TS and arterioles in mutant mice,
11 and because *Col4a1* mutations have been shown to decrease COL4 expression¹⁴, we asked
12 whether COL4 expression levels were different between mutant arterioles and TS. Brain
13 cryosections (20 μ m-thick) of *Col4a1*^{+/*+*} and *Col4a1*^{+/*G498V*} mice were immunostained with a
14 monoclonal antibody specific for COL4A2, which assembles with COL4A1 to form the
15 COL4A1A1A2 heterotrimer. Remarkably, COL4A2 immunoreactivity was significantly
16 decreased in mutant TS but unchanged in mutant arterioles (Fig. S12).

17 In the ensuing study, we focused on the role of the hypermuscularization of TS in ICH.

18

19 ***Expression of SMC genes and Notch3 target genes is upregulated in *Col4a1*^{+/*G498V*} brain***
20 ***microvessel preparations enriched in TS***

21 To investigate molecular changes occurring in TS, we purified brain microvessels from
22 *Col4a1*^{+/*+*} and *Col4a1*^{+/*G498V*} mice using sequential filtrations through nylon meshes of
23 decreasing pore sizes. Immunostaining of brain vessels retained on the 100 μ m-nylon mesh
24 mainly comprised arterioles and veins (Fig 4A, top panel) whereas the 40 μ m-retentate was

1 enriched in TS, and also in capillaries (Fig 4A, bottom panel). We found that genes coding for
2 the contractile proteins α -SMA and SMMHC were significantly upregulated in the TS
3 enriched fraction of *Col4a1*^{+G498V} mice, as well as genes coding for desmin, the actin-binding
4 proteins calponin and SM22 and the transcription factor myocardin, suggesting an increased
5 differentiation of mural cells (Fig 4B).

6 The Notch3 receptor is predominantly expressed in vascular SMCs and Notch3 signaling
7 pathway plays a pivotal role in the development and maturation of blood vessels²⁴. Moreover,
8 recent work suggests that activation of the Notch3 pathway promotes pulmonary arterial
9 hypertension by stimulating SMC proliferation and muscularization of small pulmonary
10 arteries²⁵. Given these observations, and the finding that Notch3 is also expressed in
11 transitional cells (Fig. S13), we suspected that hypermuscularization of the TS could be
12 mediated by a dysregulation of the Notch3 pathway, and thus we quantified the expression of
13 target genes regulated by Notch3^{26,27} (Supplementary table 1). Because our brain microvessel
14 preparations also contained endothelial cells, we selected Notch3 target genes that were
15 expressed exclusively in mural cells according to previous works^{26,27} or single cell RNAseq
16 analysis of mouse brain microvessels
17 (<http://betsholtzlab.org/VascularSingleCells/database.html>)¹³. We found that 7 out of 11 of
18 these Notch3 target genes were upregulated in brain microvessels of *Col4a1*^{+G498V} mice (Fig.
19 4C). In striking contrast, expression level of SM marker genes was essentially unchanged in
20 dissected brain arteries of *Col4a1*^{+G498V} mice and only 2 out of 11 of Notch3 target genes
21 were upregulated in mutant brain arteries (Fig. 4D,E). Altogether, our results suggest that the
22 increased muscularization of mutant TS arises from an increased activation of Notch3
23 pathway.

24 ***Genetic reduction of Notch3 in *Col4a1*^{+G498V} mice attenuates the hypermuscularization of***
25 ***TS in the retina and protects against ICH***

1 To confirm that activation of the Notch3 pathway contributed to the hypermuscularization of
2 TS and assess whether this hypermuscularization was involved in ICH in *Col4a1* mutant
3 mice, we adopted a genetic interaction approach. To avoid confounding effects induced by the
4 complete loss of Notch3, which causes prominent loss of SMCs^{17,28,29}, we generated and
5 analyzed *Col4a1*^{+/*G498V*} lacking 1 allele of Notch3, along with their control littermates, at 6
6 months of age. Loss of a single allele of Notch3 in wildtype mice (*Notch3*^{+/-}) has no
7 detectable morphological effect on the retinal microvasculature²⁹ but significantly reduces
8 Notch3 activity²⁶. Genetic reduction of Notch3 in *Col4a1* mutant mice (*Col4a1*^{+/*G498V*},
9 *Notch3*^{+/-}) had no significant effect on SMC loss in retinal arterioles (Fig. 5A,B), however, it
10 strikingly significantly attenuated α -SMA expression and mural cell number in TS (Fig
11 5C,D). Remarkably, the mean number of ICH was significantly reduced in *Col4a1*^{+/*G498V*},
12 *Notch3*^{+/-} mice compared to *Col4a1*^{+/*G498V*}, *Notch3*^{+/+} mice (Fig 5E,F). Moreover, the
13 penetrance of ICH was drastically reduced in *Col4a1*^{+/*G498V*}, *Notch3*^{+/-} mice, with ICH being
14 detected in only 20% of mice compared to near 60% in the *Col4a1*^{+/*G498V*}, *Notch3*^{+/+} group
15 ($P=0.024$ by the Chi-square test)..

16 ***Mutant TS are more contractile in response to thromboxane receptor agonist and increased***
17 ***intraluminal pressure***

18 To pinpoint the mechanism by which hypermuscularization of the TS contributed to the
19 occurrence of ICH, we next performed functional studies. To achieve this, we used the retina
20 where TS can be easily identified *ex vivo* under phase contrast microscopy (Fig S3). To study
21 vascular responses closest to physiological conditions, we developed an *ex vivo* pressurized
22 retina preparation (Fig 6A). We assessed the response of TS to two standard vasoconstrictors,
23 KCl and the thromboxane mimetic U-46619 on retina explants from *Col4a1*^{+/+} and
24 *Col4a1*^{+/*G498V*} mice aged 6 months (Fig. 6B and supplementary Table 2). Addition of 60 mM
25 KCl to the superfusion chamber elicited a robust and quick constriction of wildtype TS, with a

1 trend toward a stronger constriction in *Col4a1*^{+/*G498V*} vessels (Fig S14). U-46619 (10-100nM)
2 produced a concentration-dependent constriction of TS that was significantly increased in
3 *Col4a1*^{+/*G498V*} mice at 50 nM (36% diameter reduction in mutant mice versus 3% in control
4 mice) (Fig 6C,D). We also examined the contractile response of TS to increased intraluminal
5 pressure. Both *Col4a1*^{+/*+*} and *Col4a1*^{+/*G498V*} TS decreased luminal diameter in response to the
6 pressure change, however *Col4a1*^{+/*G498V*} TS exhibited a ~5.5-fold greater constriction than
7 *Col4a1*^{+/*+*} TS (Fig. 6E-F).

8 ***Hypermuscularization of the TS cripples the pressure drop in the microvasculature***

9 We then hypothesized that the higher tone and contractility of mutant TS could compromise
10 the pressure drop along the microvasculature. An abnormally higher intravascular pressure at
11 the level of arterioles combined with SMC loss might favor their rupture. Measuring
12 intravascular pressure *in vivo* in the microvasculature is extremely challenging and yet limited
13 to larger vessels. Moreover, assessing the pressure drop along the microvascular bed would
14 require several measurements at different locations which is not feasible *in vivo*. Therefore,
15 we used computational modeling to simulate the pressure distribution in different parts of the
16 microvasculature in the retina. We used an established mathematical model of an
17 artery/arteriolar human retinal network in which mural cells are implemented as fibers in the
18 vascular wall^{30,31} (Fig. 7A). In *Col4a1* mutant mice, density of mural cells was decreased in
19 arterioles to model SMC loss and increased in TS to simulate the hypermuscularization (Fig.
20 7B). Measurements of systolic and diastolic blood pressures by tail-cuff plethysmography
21 showed a 11% decrease in *Col4a1* mutant mice (Fig. S15). Hence, blood pressures at the
22 entry of the retinal vascular network were decreased proportionally in *Col4a1* mutant mice
23 and used as input parameters for the model (Fig. 7B). Intravascular pressure was then
24 simulated in control and mutant mice all along the arterioles and TS (Fig. 7C). Fig 7D shows
25 the values of systolic and diastolic intravascular pressures in 10 different locations during a

1 typical cardiac cycle. In control mice, pressure continuously dropped from 40 to 20 mmHg. In
2 *Col4a1* mutant mice, a lower pressure was observed in proximal arterioles (positions 1-3) due
3 to the slight systemic hypotension. However, pressure drop was reduced resulting in higher
4 intravascular pressure in middle-size arterioles (positions 4-8) and TS (positions 9-10)
5 compared to controls. Moreover, in this part of the vascular network the pulse pressure
6 (difference between the systolic and diastolic pressure) was larger in *Col4a1* mutant mice.
7 Thus, these data suggest that the hypermuscularization of TS leads to an abnormally elevated
8 mean and pulse pressure in the feeding upstream arteriole.

9 ***Diffuse mural cell loss is not associated with ICH***

10 Our findings above predict that, conversely, loss of transitional cells in TS in addition to SMC
11 loss in arterioles should not lead to ICH (Fig. 7E). Consistent with previous reports^{17,28,29},
12 cerebral and retinal arterioles of mice completely lacking *Notch3* exhibited prominent SMC
13 loss (Figs S16-S17). However, unlike *Col4a1* mutant mice, we found that TS in *Notch3*^{-/-}
14 mice also showed dramatic loss of transitional cells in both the retina and the brain (Figs S16-
15 S17). Computational modeling revealed that arterioles in *Notch3*^{-/-} mice were exposed to
16 lower intravascular pressure compared to controls (Fig. 7C,D). Remarkably, Perl's staining
17 revealed no ICH in *Notch3*^{-/-} mice aged 10-12 months (Fig. S17).

18 ***Cerebral TS are hypermuscularized in humans with sporadic deep ICH***

19 Finally, we tested if hypermuscularization of TS was also present in humans with sporadic
20 ICH. Similarly to murine cerebral vessels, human cerebral arteries were surrounded by flat
21 ring-shaped SMCs whereas α -SMA positive smaller vessels (diameter between 5 and 15 μ m)
22 were covered by mural cells having a protruding ovoid cell body (Fig. S18). These vessels are
23 most likely TS, although transitional cells could not be further characterized because none of
24 the antibodies against NG2, desmin or aminopeptidase N worked on human post-mortem

1 brain tissue in our experimental conditions. To assess vascular remodeling in human deep
2 ICH, we immunostained for α -SMA paraffin sections of postmortem brain tissues from adults
3 who suffered from spontaneous deep ICH (Fig. 8A) and age and sex-matched control subjects
4 (Supplementary table 3). To ensure that vascular lesions were not secondary to ICH, we used
5 tissue sampled in the hemisphere contralateral to the hemorrhage, and we analyzed sections
6 through the basal ganglia. In agreement to what has been previously reported⁸, we observed
7 that some, but not all, arteries in patients had a markedly reduced α -SMA staining in the
8 media with an uneven distribution of stained SMC, consistent with segmental and focal SMC
9 degeneration (Fig. S19). In contrast, TS in ICH patients often showed a marked increased
10 immunostaining for α -SMA compared to controls, suggesting an increased number or
11 hypertrophy of mural cells (Fig 8B). We imaged cross-sectional profiles of 525 randomly
12 selected TS/arterioles in patients and controls. Quantification of the ratio of the area stained
13 for α -SMA (media) over the area of the lumen confirmed that α -SMA staining was
14 significantly thicker in TS (lumen diameter between 5 and 15 μ m) from ICH patients whereas
15 α -SMA staining in arterioles (lumen diameter > 15 μ m) was comparable between ICH patients
16 and controls (Fig. 8C-D). Overall, our data suggest that hypermuscularization of TS is a
17 common pathological mechanism in spontaneous deep ICH.

1 **DISCUSSION**

2

3 In the present study, we investigated the microvascular pathology in mice mutant for
4 collagen type IV, a model of hereditary spontaneous deep ICH, and provided strong
5 experimental evidence for two mutually-reinforcing defects of the brain microvasculature as a
6 new mechanism for ICH. In our previous study, we had demonstrated that ICH originate from
7 deep brain arteries with focal and segmental loss of SMC¹⁴. In the present study, we
8 demonstrate that TS, the downstream microvessels at the interface between arteries and
9 capillaries, show an exaggerated muscularization with higher contractility and tone. Our data
10 support a mechanism in which hypermuscularization of mutant TS is driven by increased
11 Notch3 activity, cripples the pressure drop along the arterio-capillary axis, raising the
12 intravascular pressure in the upstream feeding arteriole and promoting its rupture at the site of
13 SMC loss (Fig. 7E). We found similar microvascular lesions in post mortem brain tissues
14 from patients with sporadic deep ICH, indicating that this may represent a general mechanism
15 of deep ICH.

16

17 **Zonation of microvascular pathology in Col4a1-related small vessel disease**

18 Several groups have highlighted the diversity of mural cells in the brain microvasculature
19 as arterioles branch into capillaries based on cellular morphology, α -SMA content or vascular
20 territory^{32,33}. Recently, Vanlandewijck, He and colleagues proposed the concept of mural cell
21 zonation, by which these cells can be grouped in distinct populations according to their gene
22 repertoire¹³. Herein, we took advantage of the flat mounted retina preparation, in which the
23 arteriovenous axis can be observed in a single plane, to precisely define the phenotype of
24 mural cells at the arteriole-capillary interface, by using a combination of several mural cell
25 markers and high-resolution imaging. Consistent with recent reports^{32,33}, we found that

1 microvessels at the interface between arterioles and capillaries (mean diameter 10 μm), herein
2 called TS, are covered by a population of mural cells that are distinct from the canonical ring-
3 shaped α -SMA positive SMCs located in arterioles and arteries, and the canonical α -SMA
4 negative pericytes, which have a protruding cell body and extend thin and long processes
5 along the capillaries. We further established that these mural cells robustly express markers of
6 both canonical SMCs and pericytes and extend large and short processes but have a
7 protruding cell body. We then demonstrated the presence of similar transitional cells in the
8 cerebral vessels. We believe that the name of transitional cell, rather than “contractile
9 pericyte”^{34,35}, “ensheathing pericyte”³² or “pre-capillary SMC”³³ better reflects the similarity
10 and the continuum between these mural cells and the adjacent canonical SMCs and pericytes.

11 Remarkably, the zonation of mural cells, as described above, coincides with the zonation
12 of microvascular pathology in *Col4a1* mutant mice. Notably, our time course, cellular,
13 molecular and genetic experiments strongly suggest that the exaggerated muscularization of
14 the TS and arteriolar SMC loss are driven by different mechanisms.

15

16 **Mechanism of excessive muscularization of TS in *Col4a1* mutant mice**

17 Increased α -SMA expression and mural cell numbers in TS include several possible
18 underlying mechanisms. Analysis of the retinal vasculature at P10, the earliest time point
19 when TS were discernable, revealed that proliferation of mural cells, although low, was
20 transiently increased in mutant TS. While the increased number of transitional cells may
21 largely contribute to the increased expression of SM contractile proteins, our finding of an
22 upregulation of several SM genes including myocardin, a transcription factor critically
23 involved in SMC differentiation, is consistent with an enhanced differentiation of transitional
24 cells in mutant TS— noting that we did not find evidence of a premature differentiation of
25 these cells.

1 Our findings that Notch3 target genes were upregulated in mutant brain microvessels
2 and that hypermuscularization of retinal TS in *Col4a1*^{+/*G498V*} mice was attenuated by genetic
3 reduction of Notch3, suggest that increased Notch3 activity is a driver of TS pathology. This
4 conclusion is consistent with a number of previous studies that clearly established a pivotal
5 role of Notch3 on early mural cell specification, differentiation and expression of SMC
6 genes^{17,28,29,36–38}. It is also worth mentioning that Notch3 has been identified as a mediator of
7 SMC proliferation and muscularization of small pulmonary arteries in the context of
8 pulmonary hypertension²⁵. Considering a recent report showing that COL4 can repress
9 Notch3 signaling³⁹, it is plausible that the reduction in COL4 expression in mutant TS could
10 be involved in this increased Notch3 activity. Nevertheless, further studies are warranted to
11 better understand the mechanisms by which *Col4a1* mutations increase Notch3 activity in
12 mutant TS and of Notch3-mediated hypermuscularization of mutant TS. In this regard, it will
13 be critical to better understand the molecular and physiological factors that underlie the
14 differentiation of mural cells in transitional or arteriolar vessels. However, one current
15 limitation is the absence of specific marker or genetically encoded reporter of transitional
16 cells.

17

18 **Why TS respond to *Col4a1* mutations with hypermuscularization whereas arteries do** 19 **not?**

20 We found that arteries differed from TS in several respects. Notably, *Col4a1* mutation did not
21 affect expression of COL4 and marginally affected expression of Notch3 target genes in
22 mutant arteries. This different response of arterial SMCs and transitional cells to *Col4a1*
23 mutation may originate from their distinct intrinsic molecular identity highlighted in this
24 study. Furthermore, the distinct environment of SMCs in arterioles compared to mural cells in
25 transitional segments offers an additional possible explanation. Indeed, communication

1 between endothelial cells and SMCs in arteries is hampered by the presence of an internal
2 elastic lamina which restricts cell-cell communication to myoendothelial projections⁴⁰. In
3 contrast, endothelial cells are in direct contact with transitional cells in TS, which are devoid
4 of elastic lamina. Thus, the distinct pathology of arteriolar SMCs and transitional cells in
5 *Col4a1* mutant mice may arise from cell autonomous effects versus endothelial cell-mediated,
6 i.e. non-cell autonomous, effects respectively⁴¹. Future work is needed to investigate this
7 hypothesis.

8

9 **Two mutually-reinforcing defects of the brain microvasculature as a key mechanism for** 10 **spontaneous deep ICH**

11 We recently reported that *Col4a1* mutant mice exhibited arteriolar SMC loss in the brain
12 and the retina, and that ICH originated from arterioles with severe loss of SMC¹⁴. In this
13 study, we demonstrated that mutant TS in the brain and the retina were hypermuscularized.
14 Our Notch3 genetic reduction experiment in *Col4a1* mutant mice further suggests that
15 hypermuscularization of the TS plays a role in the occurrence of ICH. By analyzing the retina,
16 which is a tractable central nervous system preparation where vessels of the superficial layer
17 are at similar depths and can be easily fully imaged, we demonstrated that
18 hypermuscularization of TS was attenuated and arteriolar SMC loss unchanged in *Col4a1*
19 mutant mice haploinsufficient for *Notch3*. Remarkably, these mice were protected against
20 hemorrhage. Additional studies would be desirable to confirm that hypermuscularization of
21 TS is similarly attenuated and arteriolar SMC loss similarly unchanged in the brain of *Col4a1*
22 compound heterozygotes, although such quantifications are still extremely challenging
23 because the cerebral vasculature spans several spatial scales and forms a highly complex
24 network. Moreover, our demonstration in *Notch3* null mice that a severe loss of mural cells in
25 both arterioles and TS does not necessarily lead to ICHs, lends further support to the concept

1 that the hypermuscularization of TS in addition to arteriolar SMC loss is involved in ICH
2 occurrence.

3 Hypermuscularization of TS is predicted to locally decrease vessel diameter, increase
4 vascular resistance and raise the intravascular pressure in the upstream proximate arteriole,
5 hence favoring its rupture at the site of SMC loss. Both our experimental data and our
6 computational modeling support these predictions. First, using our pressurized retina
7 preparation, we found that pressure increase caused a significantly higher reduction in the
8 diameter of TS in *Col4a1* mutant mice compared to control mice. Second, our computational
9 modeling revealed that the pressure drop along the arterio-TS axis was crippled in *Col4a1*
10 mutant mice, ie with an abnormally higher intravascular pressure in the proximate feeding
11 arteriole, despite the fact that *Col4a1* mutant mice were slightly hypotensive. Notably, our
12 computational modeling approach showed that arterioles in *Notch3* null mice were instead
13 exposed to lower intravascular pressure, which likely protected them against rupture and
14 hemorrhage as observed experimentally.

15 ICHs in *Col4a1* mutant mice preferentially occur in the deep parts of the brain. This
16 anatomical specificity of ICH is likely attributed in part to the following factors. First, in a
17 previous study, we reported that arteriolar SMC loss predominated in the deep penetrating
18 arteries¹⁴. Secondly, although hypermuscularization of TS is not restricted to ICH prone
19 regions, the mechanism by which it favors arteriolar rupture, namely by raising the
20 intravascular pressure at the site of SMC loss, suggests that it could contribute to the regional
21 specificity of ICH. Indeed, recent computational modeling in human suggests that
22 intravascular pressure at the level of perforating arteries in the deep parts of the brain is higher
23 than the one at the level of penetrating arteries in the cortex, owing to their anatomical
24 proximity to the circle of Willis⁴². Therefore, it is anticipated that it is in those deep

1 penetrating arteries that hypermuscularization of TS should produce the highest local
2 elevation of intravascular pressure.

3

4 **Excessive muscularization of the TS in sporadic deep ICH**

5 In patients with sporadic deep ICH, SMC degeneration has been documented at the
6 presumed site of arterial wall rupture for many years^{10,11}. So far, the assumption has been
7 made that vessel wall degeneration was uniquely responsible for bleeding. Our findings in
8 *Col4a1* mutant mice, a genetic model of spontaneous deep ICH, prompted us to test the
9 possibility that sporadic deep ICH in humans may also arise from a similar zonation of
10 microvascular pathology. Remarkably, analysis of human post-mortem brain tissues from
11 patients with spontaneous deep ICH revealed, in addition to segmental SMC degeneration in
12 arteries, a thickening of α -SMA staining in smaller vessels, consistent with a hyperplasia or
13 hypertrophy of mural cells. Although, we could not unequivocally identify these vessels as TS
14 because most of the mural cell markers used in the mouse did not work on human tissues in
15 our experimental conditions, the presence of protruding cell nuclei and the internal diameter
16 of the affected vessels, comprised between 5-15 μ m, strongly suggest that these are TS.
17 Hence our data suggest that the mechanism of ICH identified in *Col4a1* mutant mice also
18 operates in human sporadic deep ICH. Yet, whether Notch3 activity is similarly increased in
19 TS in patients needs to be further investigated.

20 Our findings open new opportunities towards the development of preventive therapies
21 for ICH. Although several studies have established the benefit of blood pressure lowering in
22 preventing the recurrence of ICH (secondary prevention)⁴³⁻⁴⁵, our data suggest that
23 pharmacological compounds targeting hypermuscularization of the TS may be more effective
24 for both primary and secondary prevention of ICH. Notably, hypermuscularization of the TS
25 may prove to be more easily druggable than arteriolar SMC loss.

1 **Conclusion**

2 Collectively our work is consistent with the new concept of two mutually-reinforcing
3 defects of the brain microvasculature, with arteriolar SMC loss and exaggerated
4 muscularization of the TS, as a new mechanism underlying hereditary and sporadic deep ICH.
5 Hypermuscularization of this segment may represent a novel therapeutic target for ICH
6 prevention.

7

1 **ACKNOWLEDGEMENTS**

2 We thank the adults included in the LINCHPIN study, and their relatives and carers. Confocal
3 images were acquired at the ImagoSeine (Jacques Monod Institute) and Neurimag (Institute of
4 Psychiatry and Neuroscience of Paris) facilities. The SP8 confocal microscope (Institute of
5 Psychiatry and Neuroscience of Paris) was funded by a grant from Fondation Leducq.

6

7 **SOURCES OF FUNDING**

8 This work was supported by Fondation Leducq (Transatlantic Network of Excellence on the
9 Pathogenesis of Small Vessel Disease of the Brain) to AJ and MTN, the European Union
10 (Horizon 2020 Research and Innovation Programme SVDs@target under the grant agreement
11 n° 666881), the French National Agency of Research (ANR I-Can) and the French Fondation
12 for Rare Diseases to AJ, the National Institute of Neurological Disorders and Stroke (NINDS)
13 and National Institute of Aging (NIA) (R01NS110656), the National Institutes of Health
14 under Award Number R35HL140027 and the Henry M. Jackson Foundation for the
15 Advancement of Military Medicine (HU0001-18-2-0016) to MTN. The LINCHPIN study was
16 funded by UK Medical Research Council (MRC) and The Stroke Association. The Edinburgh
17 Brain Bank, part of the MRC UK Brain Bank Network, curates the brain tissue from
18 LINCHPIN study tissue donors and controls who died suddenly from non-neurological
19 conditions. The Edinburgh Brain Bank is funded by both MRC and The Stroke Association.

20

21 **DISCLOSURES**

22 None.

23

24 **SUPPLEMENTAL MATERIALS**

25 Expanded Material & Methods

1 Online Figures 1-19

2 Online Tables I-III

3 References 46-51

4

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11 Cardiovascular Sciences, University of Manchester, Manchester, UK (MTN); DHU
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14

15

16

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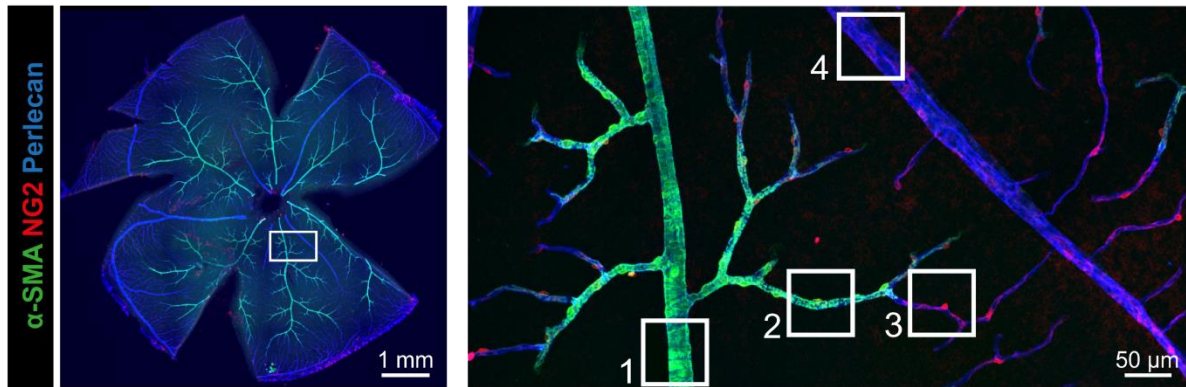
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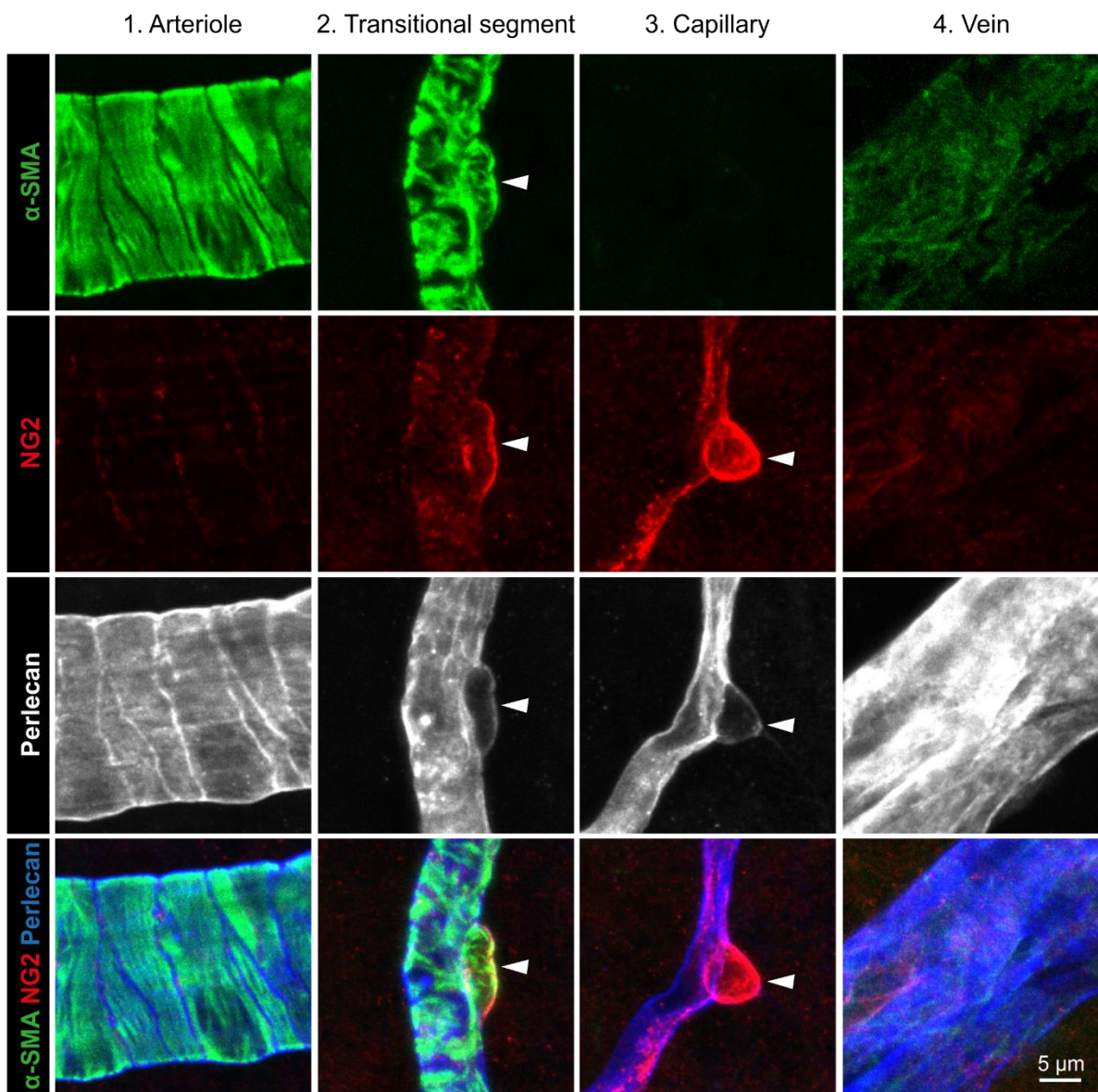
18

1 FIGURES and LEGENDS

A



B

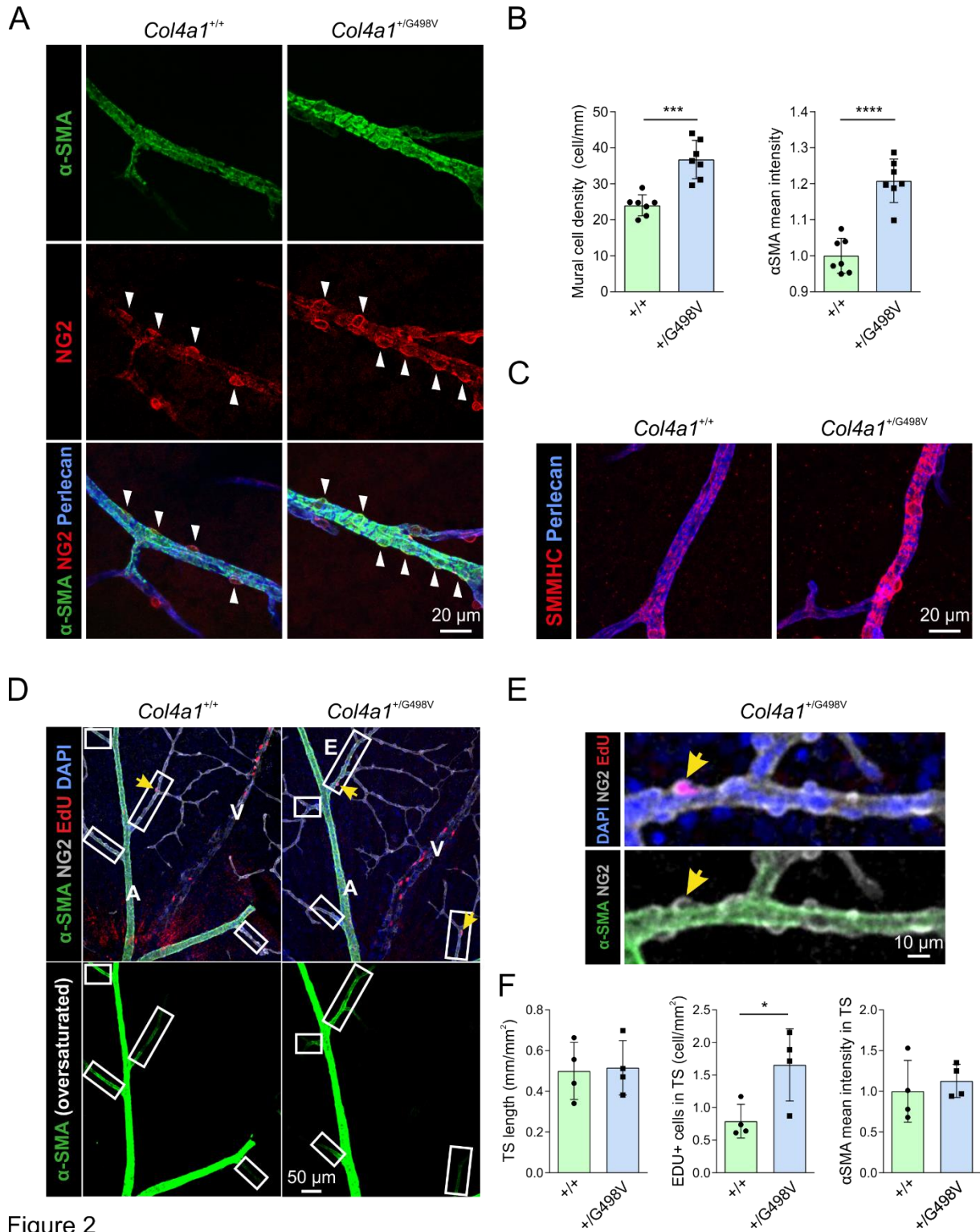


2 Figure 1

1 **Figure 1. Heterogeneity of mural cells in retinal vessels.** Confocal pictures of adult mouse
2 retina immunostained for α -SMA, NG2 and perlecan. **A.** Whole flat mounted retina (left
3 panel). Higher magnification of the boxed region (right panel). **B.** Higher magnification
4 pictures of the boxed regions numbered in A, showing SMCs in arterioles (1), transitional
5 cells in transitional segments (2), pericytes in capillaries (3) and SMCs in veins (4).
6 Arrowheads show protruding cell body in transitional segments and capillaries. Scale bars: 1
7 mm (A, left), 50 μ m (A, right) and 5 μ m (B).

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1 Figure 2

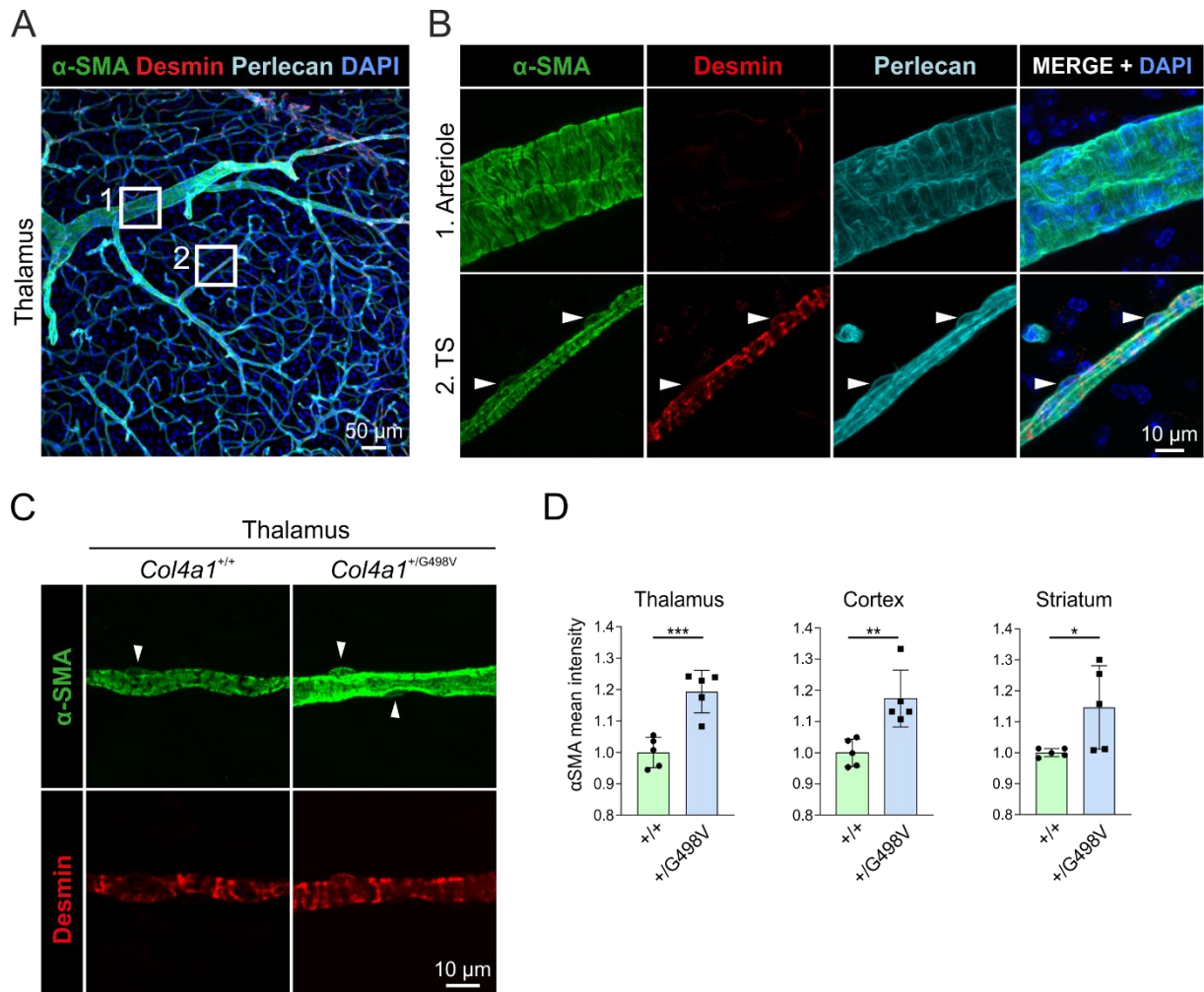
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3 **Figure 2. TS in *Col4a1*^{+/G498V} retina are hypermuscularized in adult mice and exhibit**

4 **increased mural cell proliferation during the post-natal maturation of the retinal**

5 **vasculature. A. Representative confocal pictures of retinal TS from *Col4a1*^{+/+} and**

1 *Col4a1*^{+/*G498V*} mice aged 6 months stained for α -SMA, NG2 and perlecan. TS in mutant mice
2 exhibit greater α -SMA fluorescence intensity and an increased number of transitional cells
3 (arrowheads). **B.** Quantification of mural cell density and α -SMA mean fluorescence intensity
4 in retinal TS of *Col4a1*^{+/*+*} and *Col4a1*^{+/*G498V*} mice at 6 months (n=7 mice per group). ***
5 $P=0.0001$ **** $P<0.0001$ by Student's t-test. **C.** Representative confocal pictures of TS of
6 *Col4a1*^{+/*+*} and *Col4a1*^{+/*G498V*} mice at 6 months stained for SMMHC and perlecan showing
7 higher SMMHC expression in mutant vessels. **D.** Representative confocal images of retinal
8 arterioles (A) and TS (white boxes) of *Col4a1*^{+/*+*} and *Col4a1*^{+/*G498V*} mice aged 10 days,
9 stained for EdU incorporation (4 hours) together with α -SMA, NG2, and DAPI— Shown are
10 oversaturated images of α -SMA staining to better visualize TS (bottom panel). Yellow arrows
11 depict proliferating mural cells in TS. Note the sustained cell proliferation in wildtype and
12 mutant veins (V). **E.** Higher magnification views of the boxed area in D showing a
13 proliferating mural cell in the TS of a *Col4a1*^{+/*G498V*} mouse (yellow arrow) **F.** Quantification
14 of the total length of TS ($P=0.8746$) stained by α -SMA, mural cell proliferation in TS (*
15 $P=0.0298$) and α -SMA mean fluorescence intensity in TS ($P=0.5753$). Data were analyzed by
16 Student's t-test (n=4 mice per group). Scale bars: 20 μ m (A, C), 50 μ m (D) and 10 μ m (E).
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1 Figure 3

2 **Figure 3. TS are present in the brain vasculature and are hypermuscularized in**

3 **$Col4a1^{+/G498V}$ mice. A, B** Representative confocal pictures of an adult wildtype mouse brain

4 immunostained for α -SMA, desmin, perlecan and Dapi. **A.** Low magnification view of a

5 vascular tree. **B.** Higher magnification pictures of the boxed regions numbered in A, showing

6 SMCs in arterioles (1), and transitional cells in TS (2). Arrowheads point to the protruding

7 cell body of transitional cells in the TS, containing the nucleus. **C.** Representative confocal

8 pictures of TS in the thalamus from $Col4a1^{+/+}$ and $Col4a1^{+/G498V}$ mice aged 6 months stained

9 for α -SMA and desmin, showing increased α -SMA immunoreactivity in mutant TS.

10 Arrowheads point to cell nucleus of transitional cells. **D.** Quantification of α -SMA mean

11 fluorescence intensity in TS of $Col4a1^{+/+}$ and $Col4a1^{+/G498V}$ mice aged 6 months in the

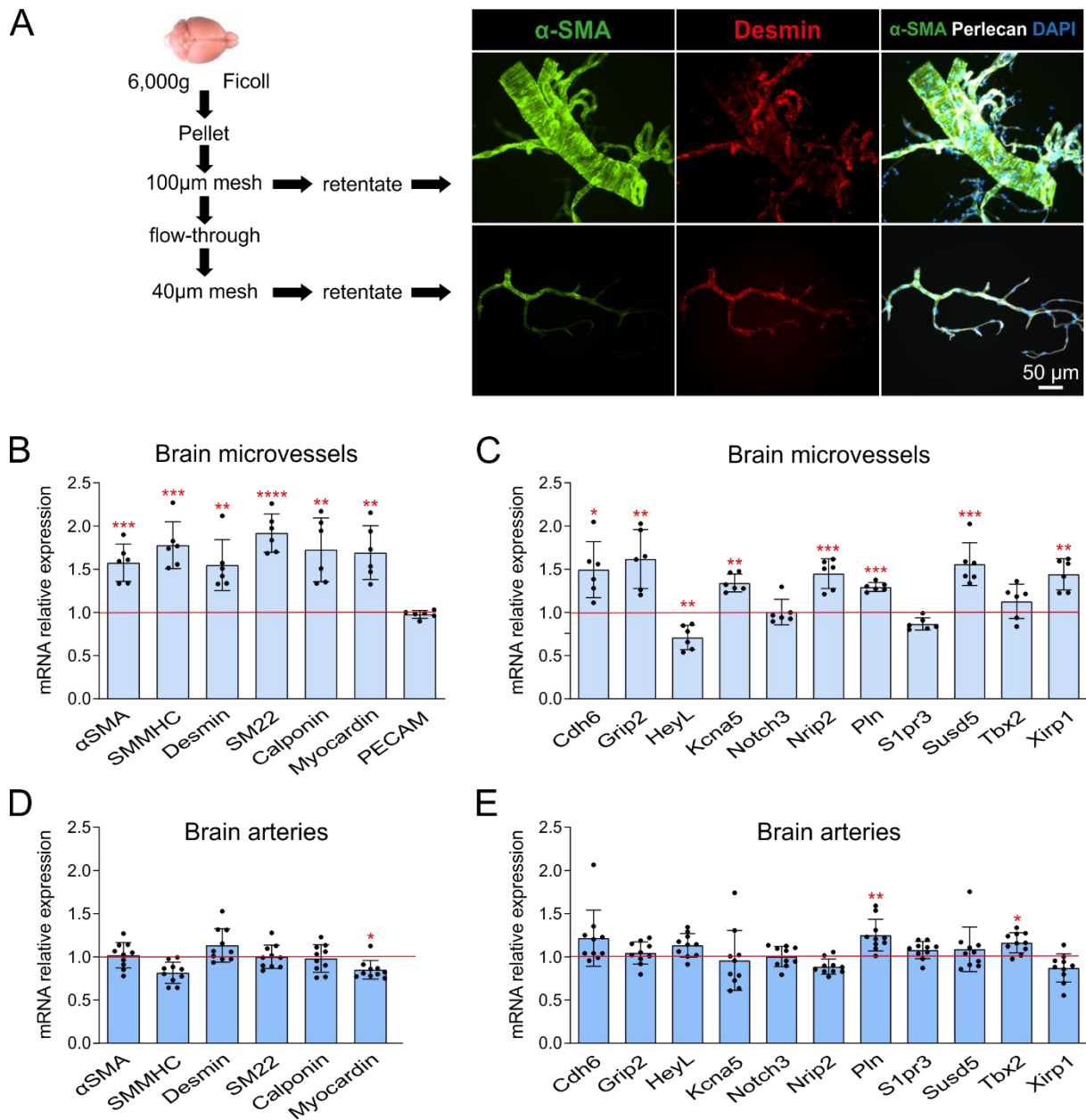
1 thalamus (** $P=0.0008$), cortex (** $P=0.005$) and striatum (* $P=0.0422$). Data were
2 analyzed by Student's t-test ($n=5$ mice per group). Scale bars: 50 μm (A) and 10 μm (B,C).

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1 Figure 4
 2 **Figure 4. Gene expression level of SMC genes and Notch3 target genes in brain**
 3 **microvessels and brain arteries of *Col4a1*^{+/+} and *Col4a1*^{+/G498V} mice.** A. Microvessels were
 4 prepared from mouse brain by homogenization and centrifugation into Ficol solution as
 5 described in the supplementary method section. Vessel preparations were then fractionated
 6 successively through 100 and 40 µm nylon meshes. Pictures show vessels retained on each
 7 nylon mesh (100 and 40 µm-fractions) stained for α-SMA, desmin, perlecan and DAPI.
 8 Arterioles (bright for α-SMA and low for desmin) were retained in the 100 µm-fraction and

1 40 μm -fractions were enriched in TS (bright for α -SMA and desmin) and capillaries. **B, C.**
2 Relative mRNA levels of smooth muscle genes (**B**) and Notch3 target genes (**C**) measured by
3 quantitative RT-PCR on 40 μm -fractions prepared from *Col4a1*^{+/+} and *Col4a1*^{+/*G498V*} adult
4 mice (n=6 mice per group). Data are reported as a fold change over control mice and analyzed
5 by Student's t-test. α -SMA (***P*=0.0007), SMMHC (***P*=0.0002), Desmin (**
6 *P*=0.0018), SM22 (*****P*<0.0001), Calponin (** *P*=0.0041), Myocardin (** *P*=0.0013),
7 PECAM (*P*=0.4934), Cdh6 (* *P*=0.0117), Grip2 (** *P*=0.0048), HeyL (** *P*=0.0035), Kcna5
8 (** *P*=0.0060), Notch3 (*P*=0.9697), Nrip2 (***P*=0.0002), Pln (***P*=0.0007), S1pr3
9 (*P*=0.0530), Susd5 (***P*=0.001), Tbx2 (*P*=0.1972), and Xirp1 (** *P*=0.0085). **D, E.**
10 Relative mRNA levels of smooth muscle genes (**D**) and Notch3 target genes (**E**) measured by
11 quantitative RT-PCR in dissected brain arteries from *Col4a1*^{+/+} and *Col4a1*^{+/*G498V*} adult mice
12 (n=10 mice per group). Data are reported as a fold change over control mice and analyzed by
13 Student's t-test. α -SMA (*P*=0.7811), SMMHC (*P*=0.0506), Desmin (*P*=0.1680), SM22
14 (*P*=0.9903), Calponin (*P*=0.8291), Myocardin (* *P*=0.0407), Cdh6 (*P*=0.0609), Grip2
15 (*P*=0.5982), HeyL (*P*=0.1397), Kcna5 (*P*=0.8060), Notch3 (*P*=0.9669), Nrip2 (*P*=0.1677),
16 Pln (** *P*=0.0062), S1pr3 (*P*=0.4643), Susd5 (*P*=0.4996), Tbx2 (* *P*=0.0309), and Xirp1
17 (*P*=0.2664). Scale bar: 50 μm (A).

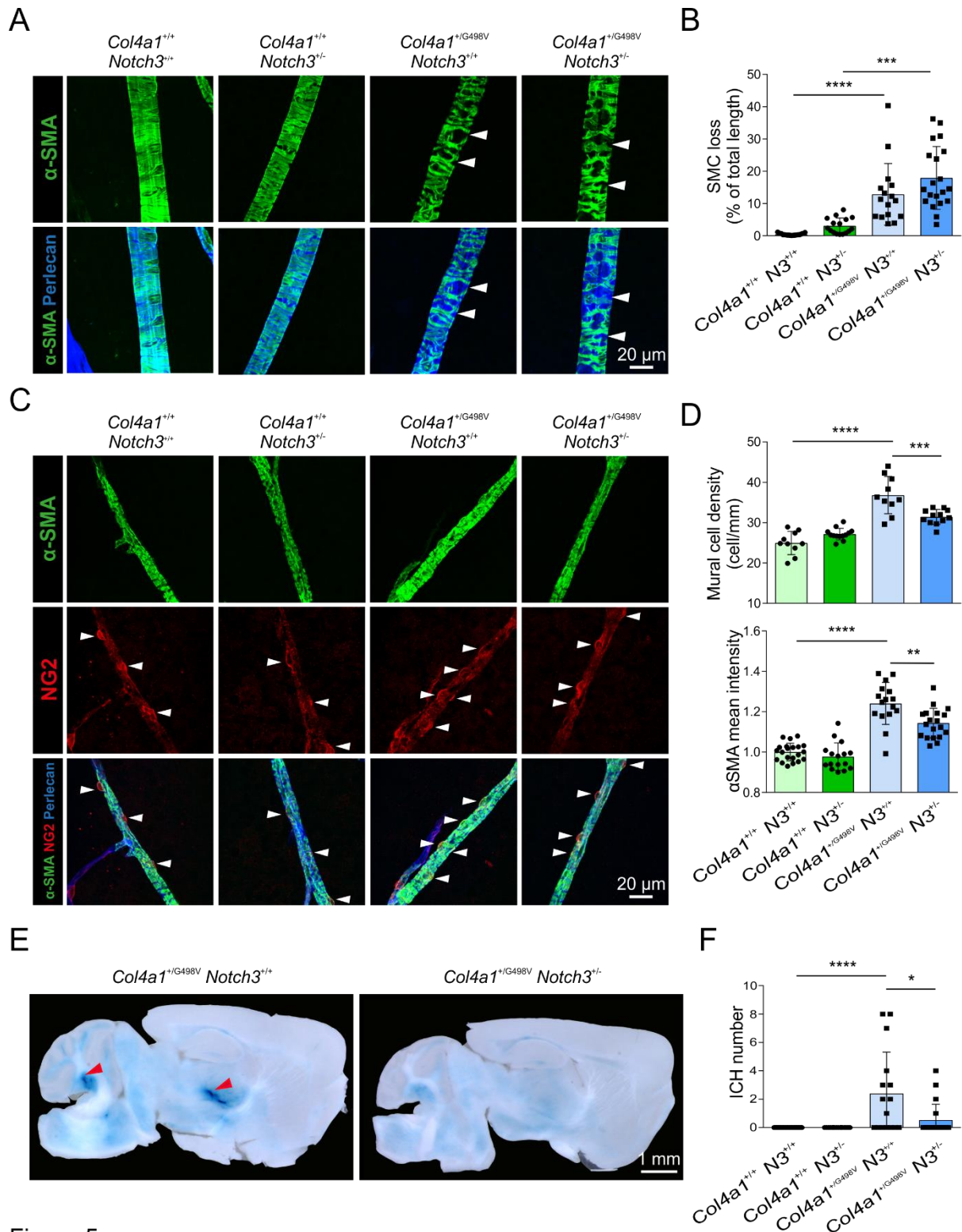


Figure 5

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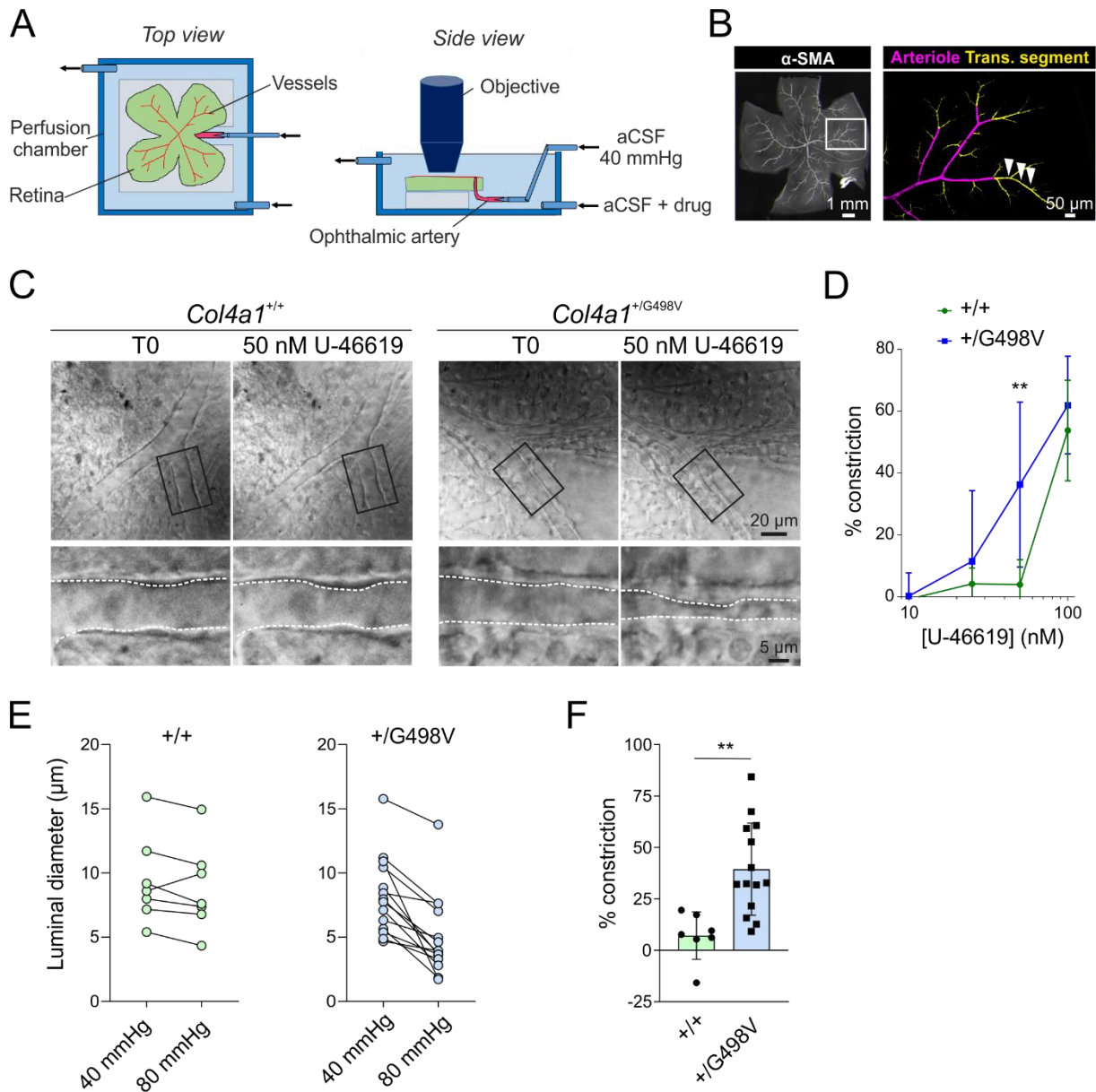
2 **Figure 5. Genetic reduction of Notch3 attenuates the hypermuscularization of TS in the**

3 **retina and protects $Col4a1^{+/G498V}$ mice against ICH. $Col4a1^{+/G498V}$ mice were crossed**

1 with *Notch3*^{+/-} mice and issued mice of the four following genotypes were screened for
2 microvascular lesions and ICHs at 6 months of age: *Col4a1*^{+/+}, *Notch3*^{+/+}; *Col4a1*^{+/+},
3 *Notch3*^{+/-}; *Col4a1*^{+/*G498V*}, *Notch3*^{+/+} and *Col4a1*^{+/*G498V*}, *Notch3*^{+/-}. **A.** Representative confocal
4 pictures of retinal arterioles stained for α -SMA and perlecan, showing SMC loss
5 (arrowheads). **B.** Quantification of SMC loss in retinal arterioles showing no significant
6 difference between *Col4a1*^{+/*G498V*}, *Notch3*^{+/+} and *Col4a1*^{+/*G498V*}, *Notch3*^{+/-} mice (from left to
7 right n=21, 16, 16 and 20 mice). Data were analyzed with Kruskal-Wallis and Dunn's post-
8 hoc test. *** $P=0.0005$ **** $P < 0.0001$. **C.** Representative confocal pictures of retinal TS
9 stained for α -SMA, NG2 and perlecan. Arrowheads show cell bodies of transitional cells. **D.**
10 Quantification of mural cell density (from left to right n=10, 13,10 and 12 mice) and α -SMA
11 mean fluorescence intensity (from left to right n=21, 16,16 and 20 mice) in TS showing a
12 significant reduction in mural cell density and α -SMA intensity in *Col4a1*^{+/*G498V*} *Notch3*^{+/-}
13 mice compared to *Col4a1*^{+/*G498V*} *Notch3*^{+/+} mice. Data were analyzed by one-way ANOVA
14 and Tukey's post-hoc test. Mural cell density *** $P=0.0004$, **** $P<0.0001$. α -SMA
15 intensity **** $P<0.0001$. **E.** Representative images of sagittal brain sections stained for
16 hemosiderin. Arrowheads depict deep ICHs. **F.** Quantification of the number of ICH showing
17 a significant decrease in ICH number in *Col4a1*^{+/*G498V*}, *Notch3*^{+/-} mice compared to
18 *Col4a1*^{+/*G498V*}, *Notch3*^{+/+} mice (from left to right n=21, 16,16 and 20 mice). Data were
19 analyzed by Kruskal-Wallis and Dunn's post-hoc test. * $P=0.020$, **** $P<0.0001$. Scale bars:
20 20 μ m (A, C) and 1 mm (E).

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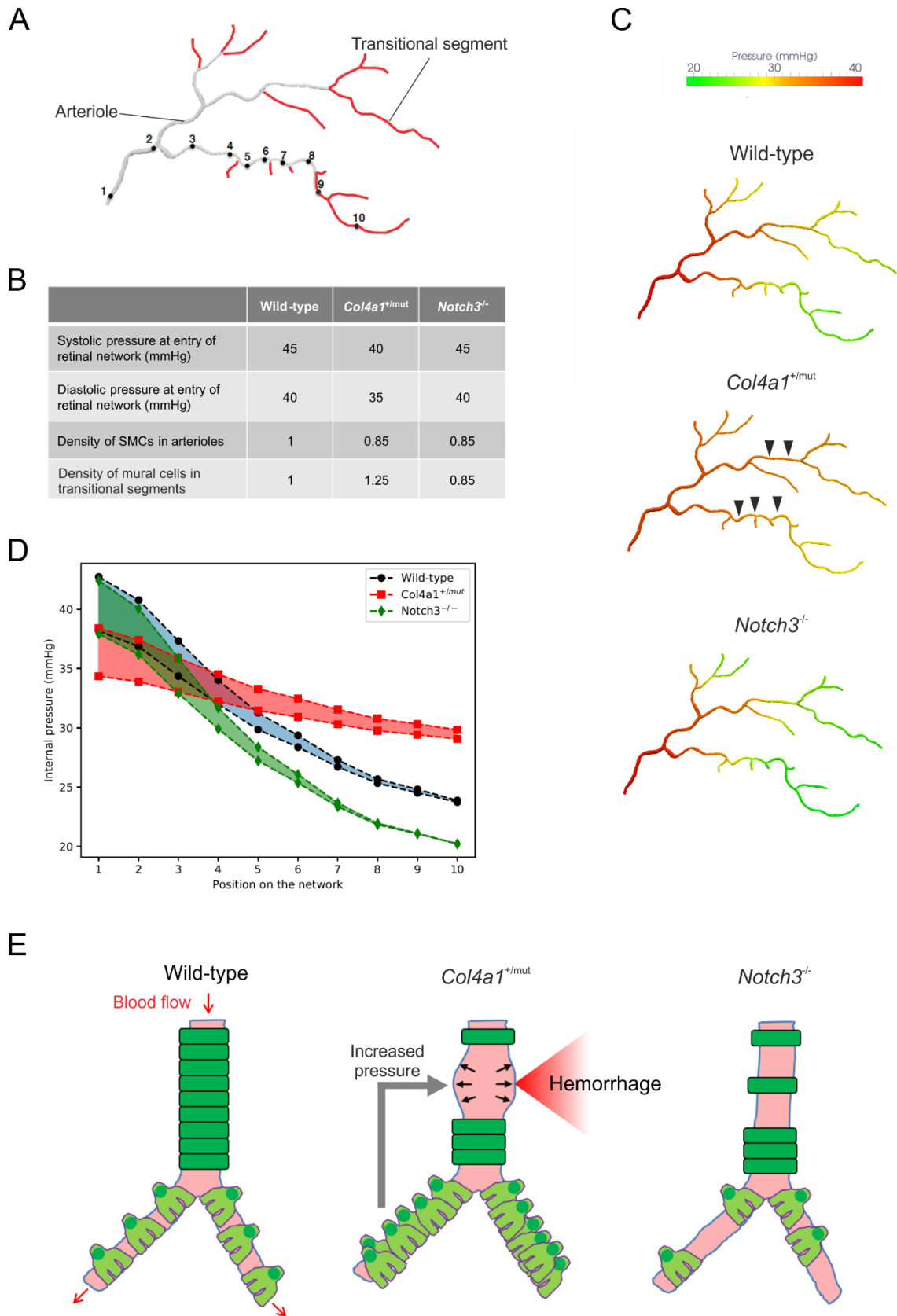
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1 Figure 6
 2 **Figure 6. Agonist and pressure-induced contractile responses are greater in TS of**
 3 ***Col4a1*^{+/G498V} mice.** **A.** Experimental setup to analyze contractility and tone of the TS in retina
 4 explants. The retina was dissected with the ophthalmic artery and pinned on a silicon platform
 5 with the vessels facing upwards and immersed in a perfusion chamber filled with circulating
 6 aCSF at 37°C. To pressurize retinal vessels, ophthalmic artery was cannulated on a
 7 micropipette fed with a gravity column of CSF at 40 mmHg. Changes in vessel diameters
 8 were observed using a water immersion 40X objective. **B.** Whole retina (left panel).

1 Schematic depicting TS (yellow, arrowheads) and arterioles (purple) analyzed in these
2 experiments (right panel)— noting that the capillaries are not depicted in this schematic. **C.**
3 Differential interference contrast images of TS from *Col4a1*^{+/+} and *Col4a1*^{+/G498V} mice aged 6
4 months before (T0) and after addition of 50 nM thromboxane agonist U-46619 to the
5 perfusion chamber. Bottom pictures show higher magnification view of boxed regions.
6 Dashed white lines delineate the vessel wall. **D.** Dose response curve showing increased
7 constriction of *Col4a1*^{+/G498V} TS in response to U-46619 (n=5 *Col4a1*^{+/+} and 9 *Col4a1*^{+/G498V}
8 mice). **E.** Decreasing diameters of individual TS following 40 mmHg to 80 mmHg pressure
9 increase at the ophthalmic artery **F.** Increased pressure-induced constriction in *Col4a1*^{+/G498V}
10 TS, shown as percent constriction (relative to 40 mmHg diameter, n=7 vessels from 3
11 *Col4a1*^{+/+} mice and n=14 vessels from 5 *Col4a1*^{+/G498V} mice). Data were analyzed by repeated
12 measure 2-way ANOVA and Bonferroni's multiple comparison test (panel D, ** $P=0.0051$),
13 or Student's t-test (panel F, ** $P=0.0021$). Scale bars: 1mm (B, left), 50 μm (B, right), 20 μm
14 (C, top) and 5 μm (C, bottom).

15



1 Figure 7

1 **Figure 7. Computational modeling predicts that excessive muscularization of the TS**
2 **increases intravascular pressure in the upstream arteriolar segment. A.** Geometry of the
3 human retinal vascular tree used for mathematical modeling. Arterioles are depicted in grey
4 and transitional segments in red. **B.** Input parameters used for mathematical modeling. **C.**
5 Color-coded representation of systolic intravascular pressures along the retinal vascular tree
6 simulated with the model in wild-type (top), *Col4a1* mutant (middle) and *Notch3*^{-/-} (bottom)
7 mice, showing that pressure is elevated in terminal arterioles and TS of *Col4a1* mutant mice
8 (arrowheads) but reduced in *Notch3*^{-/-} mice. **D.** Graph showing simulated systolic and
9 diastolic pressures at 10 different points on the vascular tree represented in A. **E.** Proposed
10 working model of the mechanism of arteriolar wall rupture in *Col4a1* mutant mice.
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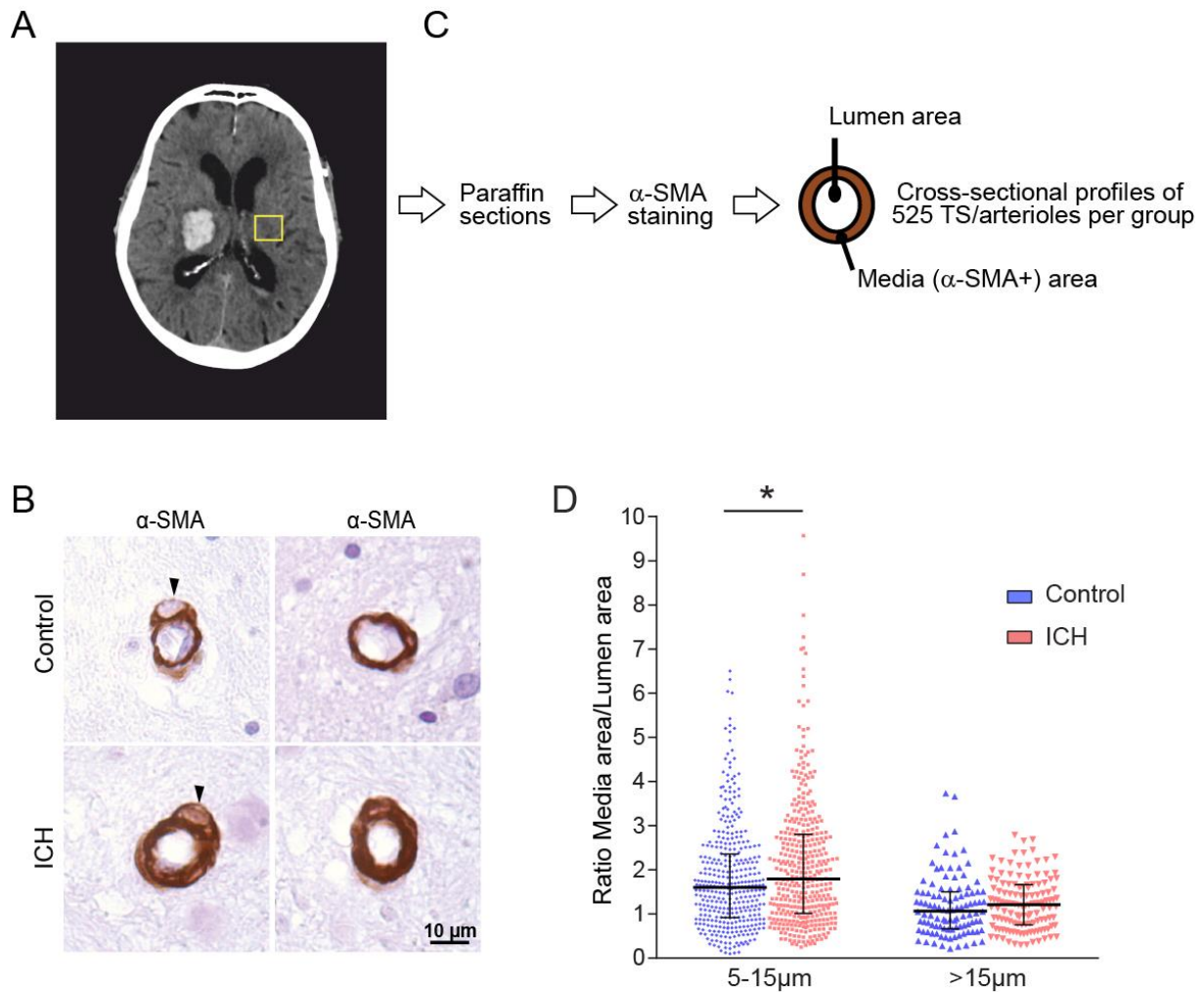


Figure 8

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2 **Figure 8. Hypermuscularization of the TS in humans with deep ICH.** A. Representative
3 image of a CT-scan showing a deep ICH in the left thalamus. Brain tissue was sampled after
4 death in the contralateral basal ganglia for immuno-histochemistry (boxed region) and
5 embedded in paraffin. B. Representative images of cerebral TS (basal ganglia) stained for α -
6 SMA showing an hypermuscularization in ICH patients. Arrowheads point to transitional cell
7 bodies. C. Schematic of the methodology used to quantify vessel wall remodeling. Cross-
8 sectional profiles of 525 randomly selected arterioles/TS were analyzed in each group. D.
9 Ratio of the vessel area stained for α -SMA (media) over the area of the lumen in cerebral

1 arterioles/TS from control and ICH patients. Vessels were stratified into two categories
2 according to their lumen diameter (5 to 15 μm , TS and $> 15 \mu\text{m}$, arterioles). From left to right
3 $n = 368$ and 382 TS (5-15 μm), 106 and 128 arterioles ($>15\mu\text{m}$) analyzed. Data are presented
4 as scatter plots with median value and first/third quartiles. Data were analyzed by Kruskal-
5 Wallis and Dunn's post-hoc test. * $P=0.016$ Scale bar: $10 \mu\text{m}$ (B).
6