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## Elevated TGF $\beta$ Signaling Contributes to Cerebral Small Vessel Disease in Mouse Models of Gould Syndrome

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### Abstract

**Background:** Cerebral small vessel disease (CSVD) is a leading cause of stroke and vascular cognitive impairment and dementia. Studying monogenic CSVD can reveal pathways that are dysregulated in common sporadic forms of the disease and may represent therapeutic targets. Mutations in collagen type IV alpha 1 (*COL4A1*) and alpha 2 (*COL4A2*) cause highly penetrant CSVD as part of a multisystem disorder referred to as Gould syndrome. COL4A1 and COL4A2 form heterotrimers [ $\alpha 1\alpha 1\alpha 2(\text{IV})$ ] that are fundamental constituents of basement membranes. However, their functions are poorly understood and the mechanism(s) by which *COL4A1* and *COL4A2* mutations cause CSVD are unknown.

**Methods:** We used histological, molecular, genetic, pharmacological, and *in vivo* imaging approaches to characterize central nervous system (CNS) vascular pathologies in *Col4a1* mutant mouse models of monogenic CSVD to provide insight into underlying pathogenic mechanisms.

**Results:** We describe developmental CNS angiogenesis abnormalities characterized by impaired retinal vascular outgrowth and patterning, increased numbers of mural cells with abnormal morphologies, altered contractile protein expression in vascular smooth muscle cells (VSMCs) and age-related loss of arteriolar VSMCs in *Col4a1* mutant mice. Importantly, we identified elevated TGF $\beta$  signaling as a pathogenic consequence of *Col4a1* mutations and show that genetically suppressing TGF $\beta$  signaling ameliorated CNS vascular pathologies, including partial rescue of retinal vascular patterning defects, prevention of VSMC loss, and significant reduction of intracerebral hemorrhages in *Col4a1* mutant mice aged up to 8 months.

**Conclusions:** This study identifies a novel biological role for collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  as a regulator of TGF $\beta$  signaling and demonstrates that elevated TGF $\beta$  signaling contributes to CNS

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None

vascular pathologies caused by *Col4a1* mutations. Our findings suggest that pharmacologically suppressing TGF $\beta$  signaling could reduce the severity of CSVD, and potentially other manifestations associated with Gould syndrome and have important translational implications that could extend to idiopathic forms of CSVD.

### Keywords

TGF $\beta$ ; CSVD; type IV collagen; Gould syndrome; basement membrane

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### Introduction

Cerebral small vessel disease (CSVD) is a leading cause of stroke and vascular cognitive impairment and dementia (VCID)<sup>1–7</sup>. Radiological CSVD manifestations include white matter hyperintensities (WMH), dilated perivascular spaces, lacunar infarcts, microbleeds, and intracerebral hemorrhages (ICHs)<sup>8, 9</sup>. Although increasing age, hypertension, and cerebral amyloid angiopathy are the most common CSVD risk factors, family studies highlight the role of genetic susceptibility<sup>10–14</sup>. Importantly, familial and sporadic forms of CSVD share clinical and radiological features suggesting that the underlying pathogenic mechanisms may also be shared<sup>15–18</sup>. Therefore, studying monogenic forms of the disease could provide mechanistic insight and reveal potential therapeutic targets with broad relevance to CSVD.

Monogenic forms of CSVD have been attributed to rare and highly penetrant mutations in *NOTCH3*, *HTRA1*, *COL4A1*, *COL4A2*, *TREX1*, *FOXC1* and *GLA*<sup>16, 19–23</sup>. Notably, the pathological processes underlying monogenic forms of CSVD appear disparate. For instance, *NOTCH3* mutations that cause CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) lead to increased potassium current density in vascular smooth muscle cells (VSMCs) and impaired cerebral vasoreactivity<sup>24–26</sup>. In contrast, *HTRA1* mutations that cause CARASIL (cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy) appear to perturb TGF $\beta$  signaling<sup>27–29</sup>. *COL4A1/COL4A2*, *TREX1*, *GLA*, and *FOXC1* mutations cause impaired secretion of a core basement membrane component, mislocalization of a DNA exonuclease, loss of function of a glycoside hydrolase enzyme, and loss of function of a transcription factor, respectively. Thus, monogenic forms of CSVD appear to be a collection of diseases with overlapping clinical manifestations, for which a convergent molecular mechanism has not been identified. Moreover, whether the mechanisms underlying monogenic forms of CSVD also contribute to common sporadic forms of CSVD is unclear.

Among the genes responsible for monogenic forms of CSVD, *HTRA1* and *COL4A2* have emerged in highly powered genome wide associate studies (GWAS) with *COL4A2* being one of the genes most consistently associated with CSVD manifestations in the general population<sup>30–35</sup>. Independent large-scale genetic studies have also reported associations between *COL4A1* and/or *COL4A2* variants with intracranial aneurysms, myocardial infarction, arterial calcification, arterial stiffness, deep ICH, lacunar ischemic stroke, reduced white matter volume and vascular leukoencephalopathy<sup>33, 36–43</sup>. Furthermore, *COL4A2* is significantly associated both with ICH and WMH in stroke patients and

in community populations suggesting a generalizable role in cerebrovascular health<sup>35</sup>. Importantly, because of exclusion criteria for patients with syndromic findings in GWAS cohorts, the importance of *COL4A1* and *COL4A2* mutations in the etiology of CSVD may be underestimated<sup>44</sup>.

*COL4A1* and *COL4A2* form heterotrimers [ $\alpha 1\alpha 1\alpha 2(\text{IV})$ ] that assemble in the endoplasmic reticulum before being secreted and incorporated into basement membranes of nearly all tissues<sup>45–49</sup>. In general, mutations in *COL4A1* and *COL4A2* are thought to impair collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  assembly and secretion leading to intracellular accumulation and extracellular deficiency<sup>50–57</sup>. Accordingly, semi-dominant *COL4A1* and *COL4A2* mutations cause Gould syndrome – a multisystem disorder characterized primarily by variable cerebrovascular, ocular, renal, and skeletal muscle manifestations<sup>51, 58–62</sup>. Among these, cerebrovascular disease is the most highly penetrant feature and may manifest as porencephaly, stroke, WMH, subcortical microbleeds, enlarged perivascular spaces, and lacunar infarctions<sup>44, 59, 63–68</sup>. Notably, a recent study found *COL4A1* and *COL4A2* mutations in 19% of fetal ICH cases<sup>69</sup>. *Col4a1* and *Col4a2* mutant mice recapitulate the pathophysiological hallmarks of Gould syndrome, including cerebrovascular manifestations<sup>50, 51, 55, 58, 70–76</sup>. Using an allelic series of *Col4a1* and *Col4a2* mutant mice, we demonstrated that allelic heterogeneity has important implications for the penetrance and severity of Gould syndrome pathologies<sup>55, 73</sup>; and showed that allelic differences contribute to clinical variability, at least in part, via tissue-specific mechanistic heterogeneity<sup>73</sup>. Importantly, pharmacologically promoting collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  secretion using 4-phenylbutyrate (4PBA) reduced ICH severity in *Col4a1* mutant mice<sup>54, 73, 77–79</sup>; however, the mechanism(s) by which impaired collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  secretion causes CSVD remain unknown.

Despite being evolutionarily conserved core components of basement membranes throughout the animal kingdom, the biological functions of collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  are largely unknown<sup>80</sup>. Interestingly, several lines of *in vitro* and *in vivo* evidence support a link between collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  and TGF $\beta$  signaling<sup>81–86</sup>, which has well-established roles in vascular development and homeostasis. Notably, we recently demonstrated that elevated TGF $\beta$  signaling contributes to ocular dysgenesis in *Col4a1* mutant mice<sup>86</sup>. Importantly, altered TGF $\beta$  signaling contributes to multiple vascular diseases in humans, including hereditary hemorrhagic telangiectasia, Loeys–Dietz syndrome, and pulmonary arterial hypertension<sup>87–89</sup>. Here, we report that TGF $\beta$  signaling is elevated in two distinct *Col4a1* mutant mouse models of Gould syndrome. Importantly, we demonstrate that promoting collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  secretion using 4PBA reduced TGF $\beta$  signaling and that genetic suppression of TGF $\beta$  signaling significantly reduced clinically relevant CSVD manifestations in *Col4a1* mutant mice. Collectively, our findings have important implications with translational potential for individuals with Gould syndrome that may also be broadly applicable to idiopathic forms of CSVD and VCID.

## Results

### Pharmacologically promoting collagen $\alpha 1\alpha 1\alpha 2(\text{IV})$ secretion ameliorates developmental CNS vascular defects in *Col4a1* mutant mice

*Col4a1* mutations cause abnormal vascular development presumed to underlie pre- and perinatal ICH in *Col4a1* mutant mice<sup>78, 90</sup>. To better understand the role of collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  in developmental central nervous system (CNS) angiogenesis, we turned to the retina which is commonly used as a model to study CNS vascular development<sup>91</sup>. Notably, retinal vascular defects have been reported in *Col4a1* mutant mice and were shown to correlate with ICH<sup>58, 92, 93</sup>. Murine retinal angiogenesis begins as blood vessels enter from the optic nerve head shortly after birth to form a superficial primitive plexus that extends radially toward the peripheral retina<sup>91</sup>. Arteriovenous specification and remodeling of the primitive plexus follow a similar radial pattern to give rise to the mature hierarchical retinal vascular network<sup>94</sup>. To further characterize vascular development in *Col4a1* mutant mice, we used the *Col4a1*<sup>+/*G1344D*</sup> mutant mouse strain that carries a mutation causing severe  $\alpha 1\alpha 1\alpha 2(\text{IV})$  secretion impairment and severe ICH<sup>55, 78</sup>. We first immunolabeled retinal flat mounts with the vascular endothelial cell (VEC) marker CD31 and identified impaired vascular plexus outgrowth in postnatal day (P) 7 *Col4a1*<sup>+/*G1344D*</sup> mice compared to *Col4a1*<sup>+/*+*</sup> littermates (Figure 1A–B). We previously showed that pharmacologically promoting collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  secretion using 4PBA reduced ICH severity in *Col4a1* mutant mice<sup>54, 77, 78</sup>. To evaluate if promoting collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  secretion could also prevent retinal vascular defects in *Col4a1* mutant mice, 4PBA was provided in drinking water from P0. Postnatal 4PBA treatment did not prevent perinatal lethality in *Col4a1* mutant mice (Figure I in the Data Supplement)<sup>51, 71, 77</sup>. Interestingly, retinal vascular outgrowth tended to be decreased in *Col4a1*<sup>+/*+*</sup> mice and increased in *Col4a1*<sup>+/*G1344D*</sup> mice following 4PBA treatment. No statistical difference was observed between *Col4a1*<sup>+/*G1344D*</sup> and *Col4a1*<sup>+/*+*</sup> mice treated with 4PBA, suggesting there may be a relative improvement in mutant mice (Figure 1A–B). We next co-labeled P7 retinas for CD31 and the mural cell precursor marker NG2 (Ref.<sup>95</sup>) and found increased numbers of NG2<sup>+</sup> cells with abnormally protruding cell bodies on radial arterioles from *Col4a1*<sup>+/*G1344D*</sup> mice (Figure 1C–D and Figure II in the Data Supplement). Increased numbers of protruding NG2<sup>+</sup> cells were also observed on radial arterioles in a second *Col4a1* mutant mouse model (*Col4a1*<sup>+/*G394V*</sup>) which has milder collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  secretion impairment and ICH compared to *Col4a1*<sup>+/*G1344D*</sup> mice<sup>55, 78</sup>. Notably, 4PBA treatment reduced the number of NG2<sup>+</sup> cells treatment in both mutant strains (Figure 1C–F). Together, these data demonstrate retinal angiogenic defects characterized by impaired vascular plexus outgrowth and arteriolar mural cell abnormalities in *Col4a1* mutant mice that can be prevented, at least partially, by 4PBA.

### Retinal vascular patterning defects in *Col4a1* mutant mice are partially rescued by 4PBA

During retinal vascular development, differentiation of NG2<sup>+</sup> mural cell precursors into mature VSMCs is marked by significant changes in the expression pattern of alpha smooth muscle actin ( $\alpha$ SMA) and the onset of the smooth muscle cell-specific proteins calponin and caldesmon that are critical for VSMC contraction. In the mature retina, dense concentric  $\alpha$ SMA labeling is detected throughout the arteriolar network while calponin expression is restricted to VSMCs along radial arterioles and is lost after the transition

to primary arterioles<sup>95</sup>. Notably, increased levels of arteriolar contractile proteins were reported in individuals with Gould syndrome and in *Col4a1* mutant mouse models<sup>68, 96</sup>, in which „hypermuscularization“ of the postarteriole transitional segment to the capillaries was proposed to contribute to ICH<sup>96</sup>. To evaluate contractile proteins in the retinal vasculature, we labeled retinal flat mounts from 1 month old (MO) mice for calponin and  $\alpha$ SMA (Figure 2). Similar to previous reports of altered contractile protein expression, the labeling intensities for both proteins were significantly increased in *Col4a1<sup>+/G1344D</sup>* mice compared to their *Col4a1<sup>+/+</sup>* littermates and we found that  $\alpha$ SMA levels were significantly decreased by 4PBA treatment irrespective of genotype (Figure 2A, C and F, H, respectively). Consistent with the retinal immunolabeling data, Western blot analyses revealed significant increases in  $\alpha$ SMA levels in brains from *Col4a1<sup>+/G1344D</sup>* mice compared to *Col4a1<sup>+/+</sup>* littermates (Figure III in the Data Supplement) that were reduced by 4PBA treatment (Figure IV in the Data Supplement).

In addition to elevated levels of contractile proteins, we found that the calponin labeling pattern was altered in *Col4a1<sup>+/G1344D</sup>* retinas (Figure 2A–E). Calponin labeling along radial arterioles extended further toward the retinal periphery (Figure 2D) and into the base of primary arterioles in *Col4a1<sup>+/G1344D</sup>* mice (Figure 2E). Moreover, ectopic calponin labeling in venules was also detected in *Col4a1<sup>+/G1344D</sup>* but not *Col4a1<sup>+/+</sup>* retinas (Figure VA–B in the Data Supplement). Of note, these calponin labeling parameters were not significantly altered in *Col4a1<sup>+/+</sup>* or *Col4a1<sup>+/G1344D</sup>* mice that received 4PBA (Figure 2D–E). Importantly, calponin and  $\alpha$ SMA immunolabeling also revealed retinal vascular patterning defects in *Col4a1<sup>+/G1344D</sup>* mice. Compared to their *Col4a1<sup>+/+</sup>* counterparts, *Col4a1<sup>+/G1344D</sup>* retinas had significantly fewer radial arterioles (marked by calponin labeling) with higher incidence of bifurcations (Figure V in the Data Supplement), increased numbers of primary arterioles (defined as  $\alpha$ SMA+ branches on radial arterioles) (Figure 2I), and reduced complexity of precapillary arborization characterized by decreased numbers of  $\alpha$ SMA+ secondary and tertiary arterioles (Figure 2J). Furthermore, while precapillary arborizations typically present as fan-shaped patterns in *Col4a1<sup>+/+</sup>* mice, spiked or herringbone morphologies were observed in *Col4a1<sup>+/G1344D</sup>* mice. Although no difference in the number of primary arterioles was detected following 4PBA treatment, precapillary arborization in *Col4a1<sup>+/G1344D</sup>* retinas tended to be more elaborate (Figure 2I and J). Notably, co-immunolabeling for calponin and  $\alpha$ SMA suggested the existence of „hybrid“ retinal arterioles in *Col4a1<sup>+/G1344D</sup>* mice that presented as large caliber vessels that resembled bifurcating radial arterioles but had faint or no calponin labeling (Figure VI in the Data Supplement).

### **Elevated TGF $\beta$ signaling in *Col4a1* mutant mice is inversely correlated with collagen $\alpha$ 1 $\alpha$ 2(IV) secretion**

TGF $\beta$  signaling plays important roles in vascular development<sup>97, 98</sup>, particularly in VSMC differentiation and regulation of contractile protein expression<sup>99</sup>. Notably, independent lines of evidence suggest that collagen  $\alpha$ 1 $\alpha$ 2(IV) modulates TGF $\beta$  superfamily signaling, raising the possibility that altered TGF $\beta$  signaling could contribute to the CNS vascular phenotypes in *Col4a1* mutant mice<sup>81, 84, 85</sup>. To test this hypothesis, we first evaluated the expression of TGF $\beta$  target genes in P7 brains and found that TGF $\beta$  signaling was

significantly elevated in *Col4a1<sup>+/G1344D</sup>* mice compared to their *Col4a1<sup>+/+</sup>* littermates (Figure 3A). To further test this hypothesis *in vivo* using an independent approach, we crossed *Col4a1<sup>+/G1344D</sup>* mice to a reporter line that expresses luciferase in response to SMAD2/3-mediated TGF $\beta$  signaling (*SBE-luc*)<sup>100</sup>. *In vivo* bioluminescence confirmed that *Col4a1<sup>+/G1344D</sup>* mice have elevated TGF $\beta$  signaling that was significantly suppressed by 4PBA (Figure 3B–C). Consistent with these observations, mRNA levels for several TGF $\beta$  target genes were also elevated in brains from 1MO *Col4a1<sup>+/G1344D</sup>* mice and were generally reduced by 4PBA (Figure 3D). Furthermore, we validated these findings in *Col4a1<sup>+/G394V</sup>* mice which showed significantly elevated SMAD2/3-mediated TGF $\beta$  signaling by *in vivo* bioluminescence imaging (Figure VIIA–B in the Data Supplement). The expression of TGF $\beta$  target genes was also elevated in VECs isolated from P7 *Col4a1<sup>+/G394V</sup>* brains and was suppressed by 4PBA (Figure VIIC–D in the Data Supplement).

### Genetically reducing TGF $\beta$ signaling ameliorates vascular defects in *Col4a1* mutant mice

To directly test whether excess TGF $\beta$  signaling contributes to vascular defects in *Col4a1* mutant mice, we sought to genetically reduce TGF $\beta$  signaling by crossing *Col4a1<sup>+/G1344D</sup>* mice to mice carrying a *Tgfb1* null allele<sup>101</sup> – an important TGF $\beta$  ligand isoform involved in vascular development and remodeling<sup>89, 102–104</sup>. We found that expression levels of TGF $\beta$  ligands are not increased in VECs isolated from P7 *Col4a1<sup>+/G1344D</sup>* brains and confirmed that *Tgfb1* heterozygosity reduced the mRNA levels of *Tgfb1*, but not *Tgfb2* or *Tgfb3*, in *Col4a1<sup>+/G1344D</sup>* mice and *Col4a1<sup>+/+</sup>* littermates (Figure VIII in the Data Supplement). Expression of TGF $\beta$  target genes in P7 and 1MO brains showed similar trends of being elevated in *Col4a1<sup>+/G1344D</sup>* mice and being reduced in both *Col4a1<sup>+/+</sup>* and *Col4a1<sup>+/G1344D</sup>* mice that were heterozygous for the *Tgfb1* null allele (Figure 4A–B). *Tgfb1* heterozygosity did not substantially improve viability of *Col4a1<sup>+/G1344D</sup>* mice (Figure IX in the Data Supplement) and did not affect retinal vascular plexus outgrowth (Figure 4C–D), but similar to 4PBA treatment, it prevented the increase in the number of radial arteriolar mural cells in *Col4a1<sup>+/G1344D</sup>* retinas (Figure 4E–F). Immunolabeling of 1MO retinas revealed that *Tgfb1* heterozygosity reduced the frequency of radial arteriole bifurcations in *Col4a1<sup>+/G1344D</sup>* mice but did not affect the number of radial arterioles or other calponin labeling parameters (Figure X in the Data Supplement and Figure 5A–E). Furthermore, while *Tgfb1* heterozygosity did not significantly alter  $\alpha$ SMA labeling intensity or the number of primary arterioles in *Col4a1<sup>+/+</sup>* or *Col4a1<sup>+/G1344D</sup>* mice, it significantly improved the complexity of precapillary arborization (Figure 5F–J).

### Genetically reducing TGF $\beta$ signaling significantly reduces ICH severity and prevents age-related VSMC loss in *Col4a1* mutant mice

Cerebrovascular disease, including ICH, is one of the most highly penetrant and clinically consequential manifestations in individuals with Gould syndrome<sup>105</sup>. To test if genetically reducing TGF $\beta$  signaling could reduce ICH severity in *Col4a1* mutant mice, we used Prussian blue staining to quantify hemosiderin in brains from P7, 1MO, and exercised-challenged 3MO mice. Consistent with previous observations<sup>51, 54, 58, 77, 78</sup>, ICH was detected in all *Col4a1<sup>+/G1344D</sup>* mice but never observed in *Col4a1<sup>+/+</sup>* littermates. Importantly, *Tgfb1* heterozygosity significantly reduced ICH severity in P7 and 1MO

*Col4a1<sup>+/G1344D</sup>* mice (Figure 6A–D) but did not prevent ICH in 3MO *Col4a1<sup>+/G1344D</sup>* mice that were challenged with exercise (Figure 6E–F).

ICH severity in *Col4a1* mutant mice increases with age, however, the nature of ICH in perinatal and adult mice is qualitatively, and potentially mechanistically, different<sup>77, 78, 96, 106</sup>. We previously reported that hemosiderin puncta were evenly distributed throughout the brains of P7 *Col4a1<sup>+/G1344D</sup>* mice<sup>78</sup>. Between P7 and 1MO, cortical hemorrhages begin to resolve and by 8MO, hemosiderin staining is predominantly subcortical<sup>77, 78</sup> (Figure 7A). The change in the ICH distribution is accompanied by a shift from numerous microhemorrhages to fewer macrohemorrhages<sup>78, 106</sup> and a recent study implicated progressive loss of arteriolar VSMCs as an important factor contributing to age-related macrohemorrhages in *Col4a1* mice<sup>106</sup>. To test whether elevated TGFβ signaling also contributes to age-related vascular pathologies, we quantified retinal radial arteriolar VSMC coverage in *Col4a1<sup>+/+</sup>* and *Col4a1<sup>+/G1344D</sup>* littermates in the context of *Tgfb1* heterozygosity. In agreement with previously published work<sup>96, 106</sup>, we found that VSMC coverage was comparable between *Col4a1<sup>+/+</sup>* and *Col4a1<sup>+/G1344D</sup>* mice at 1MO but there was a significant loss of arteriolar VSMCs between 1 and 3 months in *Col4a1<sup>+/G1344D</sup>* mice. Notably, *Tgfb1* heterozygosity protected *Col4a1<sup>+/G1344D</sup>* mice from age-related arteriolar VSMC loss even when aged up to 8MO (Figure 7B–C). In addition, an increase in arteriolar diameter was detected as early as 1MO in *Col4a1<sup>+/G1344D</sup>* mice (Figure 7B and D) and while *Tgfb1* heterozygosity reduced arteriolar diameter in 1MO mice, it did not affect arteriolar diameter at 3 and 8MO (Figure 7B–D). Importantly, genetically reducing TGFβ signaling also significantly decreased ICH severity in 8MO *Col4a1<sup>+/G1344D</sup>* mice (Figure 7E–F). Collectively, these findings demonstrate that excess TGFβ signaling contributes to developmental and progressive CNS vascular manifestations in *Col4a1* mutant mice.

## Discussion

CSVD accounts for up to 30% of strokes and is a leading cause of VCID<sup>9, 107–109</sup>; however, limited understanding of the pathogenic mechanisms represents a major obstacle in developing targeted therapeutic strategies. Importantly, inherited and idiopathic forms of CSVD share clinical manifestations suggesting that studying monogenic forms of disease might inform broader disease mechanisms. *COL4A1* and *COL4A2* mutations have emerged as an important cause of CSVD in humans and *Col4a1* mutant mice have ICH - a pathological hallmark of CSVD. The severity of ICH was previously shown to correlate with that of retinal vascular defects in *Col4a1* mutant mice<sup>106</sup>. In the present study, we have used complementary approaches in the retina and brain of *Col4a1* mutant mice to further characterize developmental CNS vascular defects and show, for the first time, that elevated TGFβ signaling contributes to CSVD in this model. Collectively, our findings suggest that regulation of TGFβ signaling is a critical biological function of collagen α1α1α2(IV) and that the TGFβ signaling pathway may represent a potential therapeutic target for individuals with Gould syndrome that could also be of broad relevance to idiopathic CSVD.

Semi-dominant coding *COL4A1* and *COL4A2* mutations cause Gould syndrome – a highly variable multisystem disorder that includes ocular dysgenesis, myopathy, and cerebrovascular disease ranging from germinal matrix hemorrhage and porencephaly to



cerebral microbleeds and age-related diffuse WMH<sup>59, 60, 79</sup>. COL4A1 and COL4A2 are extracellular matrix proteins that together constitute a major component of nearly all basement membranes. Importantly, the two genes are tightly linked on Chromosome 13 and the locus is reproducibly associated with CSVD manifestations in large scale GWAS<sup>30-35</sup>. Notably, *COL4A1* mutations were found in 16% of patients with porencephaly<sup>110</sup> and 19% of fetal ICH cases<sup>69</sup> and a retrospective study of 52 individuals with *COL4A1* mutations found stroke occurred in 17% of subjects and MRI showed WMH (63%), subcortical microbleeds (52%), porencephaly (46%), enlarged perivascular spaces, (19%), and lacunar infarctions (13%)<sup>64</sup>. Together, these findings clearly establish a role for *COL4A1* and *COL4A2* mutations in the etiology of CSVD. *Col4a1* and *Col4a2* mutant mouse models faithfully recapitulate the variable pathological features observed in individuals with Gould syndrome, including CSVD manifestations<sup>50, 51, 53-55, 58, 60, 61, 70-74, 76-78, 93, 96, 106, 111, 112</sup>. The primary consequence of *COL4A1* and *COL4A2* mutations is impaired collagen  $\alpha 1(\text{I})\alpha 2(\text{IV})$  biosynthesis leading to intracellular accumulation of mutant collagen  $\alpha 1(\text{I})\alpha 2(\text{IV})$  heterotrimers at the expense of their secretion into basement membranes<sup>50, 51, 55, 59, 77, 78, 113, 114</sup>. However, biological functions of collagen  $\alpha 1(\text{I})\alpha 2(\text{IV})$  and the mechanism(s) by which impaired  $\alpha 1(\text{I})\alpha 2(\text{IV})$  secretion cause CSVD are unknown.

We have previously reported defects in CNS vascular angiogenesis in *Col4a1* mutant mice including increased density and tortuosity of the vascular plexus in the embryonic hindbrain and altered organization of the retinal vascular network<sup>58, 78, 92</sup>. Consistent with these findings, we show delayed retinal vascular plexus outgrowth and altered patterning of the retinal arterial network in *Col4a1* mutant mice. Furthermore, we detected increased numbers of NG2<sup>+</sup> mural cell progenitors with abnormally protruding cell bodies in P7 retinas and elevated levels of contractile proteins along radial arterioles and localization beyond their normal spatial distribution pattern in 1MO *Col4a1* mutant retinas. Interestingly, a recent study described a small but significant increase in the number of proliferating NG2<sup>+</sup> mural cell progenitors in P10 retinas, and an increase in the number of NG2<sup>+</sup>; $\alpha$ SMA<sup>+</sup> mural cells and elevated contractile protein levels in retinas from adult *Col4a1* mutant mice. However, the increase in mural cell number and contractile protein expression was restricted to the postarteriole transitional segment of the retinal vascular network which led the authors to postulate that „hypermuscularization“ at the postarterial transition might be a key pathogenic feature. Supporting a potentially important role for this region of the vascular network, we detected retinal vascular patterning defects in *Col4a1*<sup>+/G1344D</sup> mice that include reduced complexity and abnormal morphology of precapillary arborization. We also identified „hybrid“ arterioles in *Col4a1*<sup>+/G1344D</sup> retina that presented as relatively large caliber vessels resembling bifurcating radial arterioles with high  $\alpha$ SMA<sup>+</sup> but faint or no calponin labeling. In agreement with previous reports and in addition to developmental vascular defects, we observed age-related loss of arteriolar VSMCs. Despite subtle differences between our findings and published work that may be attributable to variability in approach, models, or genetic background, our observations corroborate and extend findings from previous studies by validating the proposed importance of developmental mural cell defects and progressive VSMC loss in *Col4a1* mutant mouse models of CSVD.

Accumulating evidence supports the integration of distinct insults that may conspire to cause ICH in *Col4a1* mutant mice – developmental precapillary hypermuscularization and progressive arterial VSMC loss<sup>96</sup>. These elements are proposed to collectively impinge on blood pressure gradients at critical locations of the vascular network causing rupture of penetrating arterioles. Age-related VSMC loss is well documented in idiopathic CSVD<sup>9</sup> and its occurrence in *Col4a1* mutant mice highlights how *Col4a1* mutant mouse models may relate more broadly to CSVD. However, VSMC loss does not always lead to ICH and systemic hypotension has been reported in multiple *Col4a1* mutant mouse models<sup>58, 96, 112</sup>, which emphasizes the importance of precapillary hypermuscularization in this model. Increased  $\alpha$ SMA has been reported in postmortem brains from individuals with idiopathic CSVD, including those with *COL4A1* mutations<sup>68</sup>, and we found that *Col4a1* mutant mice have elevated cerebral  $\alpha$ SMA levels. Supporting the idea that precapillary constrictions may be a critical feature underlying the pathology, a recent study described elevated NOTCH3 signaling in *Col4a1* mutant mice, and genetically reducing *Notch3* prevented hypermuscularization of the postarteriole transitional segment and ICH but not VSMC degeneration<sup>78, 96, 106</sup>.

Notably, this model underscores a critical role for VSMC loss in addition to precapillary hypermuscularization in the etiology of deep, spontaneous macrohemorrhages but does not address the mechanisms contributing to developmental vascular defects underlying porencephaly and widespread perinatal microhemorrhages that are observed before the onset of VSMC loss. Furthermore, whether reduced *Notch3* protects against developmental vascular defects remains to be determined. It is notable that *Tgfb1* heterozygosity prevented VSMC loss, but *Notch3* heterozygosity did not, suggesting that TGF $\beta$  signaling may be the more proximate insult<sup>115</sup>. It is important to consider that there are distinct temporal phases to the cerebrovascular pathology and that developmental and progressive defects (or other sub-phenotypes within each category) may reflect different pathogenic mechanisms rather than a disease continuum. For example, in early life, ICHs are observed as microhemorrhages evenly distributed throughout the brain but, with age, most cortical hemorrhages resolve, and subcortical hemorrhages become predominant<sup>78, 106</sup>. Early postnatal microhemorrhages occur at the level of capillaries and are associated with a transient increase in blood brain barrier permeability, whereas age-related macrohemorrhages affect arteries in deep brain regions and are associated with progressive focal loss of VSMC<sup>106</sup>. The progressive nature of age-related CNS vascular defects in *Col4a1* mutant mice is an important consideration for long term management of patients with Gould syndrome and may present valuable interventional opportunities.

Pathogenicity of *Col4a1* and *Col4a2* mutations is generally attributed to impaired collagen  $\alpha 1\alpha 1\alpha 2$ (IV) secretion and pharmacologically promoting  $\alpha 1\alpha 1\alpha 2$ (IV) secretion using 4PBA can reduce severity of ocular dysgenesis, skeletal myopathy, and ICH in *Col4a1* mutant mice<sup>50, 51, 55, 59, 77, 78, 113, 114</sup>. Here we show that postnatal 4PBA treatment also normalized the numbers of mural cell precursors in P7 retinas and significantly reduced  $\alpha$ SMA levels in retinas and brains from *Col4a1*<sup>+/G134D</sup> mice. Importantly, we demonstrate that altered TGF $\beta$  signaling contributes to CNS vascular pathologies in *Col4a1* mutant mice. Using a combination of molecular, genetic, and *in vivo* imaging approaches, we showed that TGF $\beta$  signaling was significantly elevated in *Col4a1* mutant mice and correlated

with collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  secretion impairment. Notably, 4PBA reduced TGF $\beta$  signaling in *Col4a1* mutant mice suggesting a role for  $\alpha 1\alpha 1\alpha 2(\text{IV})$  as a regulator of the TGF $\beta$  pathway and that reducing TGF $\beta$  signaling might prevent pathology in *Col4a1* mutant mice. Genetically reducing TGF $\beta$  signaling using heterozygosity for a null allele of *Tgfb1* reduced developmental arterial mural cell defects, prevented age-related VSMC loss, and significantly reduced ICH severity in *Col4a1* mutant mice aged up to 8MO. Western blots using whole brain lysates from 1MO mice suggested an increase in of pSMAD2/3 in *Col4a1<sup>+/-</sup>G1344D* compared to *Col4a1<sup>+/-</sup>* samples, however the effect was not statistically significant (data not shown) and additional biochemical studies on samples enriched for the vasculature may be required. Collectively, these data demonstrate the functional relevance of this pathway in the pathogenesis of CSVD and its translational potential as a therapeutic target.

However, it is important to note that *Tgfb1* heterozygosity did not completely rescue CSVD manifestations in *Col4a1* mutant mice. For instance, we have shown previously that exercise exacerbates ICH severity in *Col4a1* mutant mice<sup>77, 78</sup> and while *Tgfb1* heterozygosity prevented arteriolar hypermuscularization and VSMC loss, it was insufficient to reduce ICH severity in 3MO *Col4a1* mutant mice subjected to an exercise challenge. While other possibilities exist, including the need for more precise control of TGF $\beta$  signaling than is achieved by systemic *Tgfb1* heterozygosity, other TGF $\beta$  ligands<sup>86, 116–118</sup> could contribute to CNS vascular dysfunction in *Col4a1* mutant mice. Of note, our recent findings suggest that altered TGF $\beta$ 2-mediated signaling partially contribute to ocular pathology in *Col4a1* mutant mice<sup>86</sup>. Alternatively, additional pathways could be involved which would be consistent with the concept that collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  is a multifunctional signaling platform that regulates various cellular processes.

TGF $\beta$  signaling plays pivotal roles during vascular developmental and disease. While details of TGF $\beta$  regulation and signaling are well established, the consequences of decreased or increased signaling can be confounding and are context dependent. The role of TGF $\beta$  signaling in human disease is clearly illustrated by Loeys–Dietz syndrome which result from loss of function mutations for factors involved at various steps along the pathway, including ligands (*TGFB2/3*), receptors (*TGFBR1/2*) and signal transducers (*SMAD2/3*). Hereditary hemorrhagic telangiectasia is also caused by loss of function mutations in a receptor (*ACVRL1*), co-receptor (*ENG*) or signal transducer (*SMAD4*) of TGF $\beta$  signaling. Our data support consideration for inclusion of Gould syndrome as part of the family of “TGF $\beta$  signalopathies”<sup>119</sup>.

The discovery that TGF $\beta$  signaling is elevated in *Col4a1<sup>+/-</sup>Mut* mice strengthens an argument for the existence of convergent mechanisms underlying monogenic and idiopathic forms of CSVD. Elevated TGF $\beta$  signaling is an intuitive candidate that may explain matrix accumulation and has been proposed previously to contribute to CSVD<sup>120, 121</sup>. There is significant support for the role of VSMCs in age-related CSVD and VCID and excessive TGF $\beta$  signaling in mice is sufficient to induce arteriolar VSMC loss and alter vessel caliber<sup>120</sup>. Importantly, perturbation of TGF $\beta$  signaling is also proposed to underlie CARASIL caused by *HTRA1* mutations, however, a consensus for the pathogenic mechanism is still unclear. *HTRA1* is a serine protease that has been proposed to suppress

NOTCH3 and TGF $\beta$  signaling intracellularly and extracellularly<sup>27, 122, 123</sup>. Mutations in this gene consequently increases both NOTCH3 and TGF $\beta$  signaling pathways. Because the crosstalk between NOTCH3 and TGF $\beta$  signaling is not fully understood, it remains unclear whether HTRA1 regulates NOTCH3 and TGF $\beta$  independently or co-regulates downstream pathways<sup>124, 125</sup>.

The potential mechanistic convergence between *COL4A1*, *COL4A2*, *HTRA1* and *NOTCH3* is intriguing as is the implication that elevated TGF $\beta$  signaling is broadly relevant for idiopathic CSVD, sporadic ICH, and VCID. However, recent data from CADASIL mouse models suggest the involvement of a seemingly unrelated channelopathy in the etiology of CSVD that is mediated by increased voltage-gated potassium channels (Kv1) in pial and parenchymal VSMCs and impair cerebral autoregulation<sup>26</sup>. Moreover, although *COL4A1* and *COL4A2* are consistently associated with idiopathic CSVD, large scale GWAS studies detect common variants that are often non-coding, and it is far from certain that regulatory variants would have the same consequences as highly penetrant and rare coding mutations that impair collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  secretion.

Despite being conserved throughout the animal kingdom, the biological functions of collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  are largely unknown and regulation of TGF $\beta$  signaling could occur at multiple levels. Supporting a role for collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  as a suppressor of TGF $\beta$  signaling, the magnitude of TGF $\beta$  signaling elevation in *Col4a1* mutant mice inversely correlates with impaired collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  secretion, with greater TGF $\beta$  signaling elevation in *Col4a1*<sup>G1344D</sup> compared to *Col4a1*<sup>G394V</sup> mice and pharmacologically promoting  $\alpha 1\alpha 1\alpha 2(\text{IV})$  secretion using 4PBA reduced TGF $\beta$  signaling in *Col4a1* mutant mice.

Moreover, TGF $\beta$  signaling regulation occurs at multiple levels, from ligand activation to receptor engagement to signal amplitude or duration and thus, the molecular mechanism(s) by which collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  regulates TGF $\beta$  signaling may be complex and remain unknown. Collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  has been shown to directly bind TGF $\beta$  superfamily ligands<sup>81, 84, 85</sup> and may regulate their bioavailability, or signaling domain. Alternatively, collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  could also act downstream of TGF $\beta$  ligands. For example, collagen binding integrins have been shown to recruit the TCPTP phosphatase to dephosphorylate TGFBR2, suppressing TGF $\beta$  signaling<sup>82, 126</sup>. In this scenario, extracellular collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  deficiency would de-repress TGF $\beta$  signaling. This may represent a feedback mechanism whereby cells sense collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  deficiency and respond with compensatory upregulation of ECM production programs, including the TGF $\beta$  signaling pathway.

This study has important translational implications for individuals with Gould syndrome. Pharmacologically promoting  $\alpha 1\alpha 1\alpha 2(\text{IV})$  secretion is an attractive therapeutic approach because it is agnostic to the number and nature of biological functions for collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  and can simultaneously restore multiple downstream pathways. However, we have demonstrated that depending on the mutation, promoting secretion of mutant heterotrimers can exacerbate some pathologies<sup>73</sup>. Further understanding of collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  roles in the basement membrane and the location of functional subdomains

responsible for their execution might allow genetic stratification of patients for personalized therapy. For individuals with mutations in specific functional subdomains, it may be undesirable to promote mutant collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  secretion and preferable to target only the distal mechanisms. Moreover, the opportunity for the greatest impact from promoting  $\alpha 1\alpha 1\alpha 2(\text{IV})$  secretion is during embryogenesis<sup>77</sup> and the ability to „reconstruct“ a defective basement membrane may decrease significantly with age if collagen  $\alpha 1\alpha 1\alpha 2(\text{IV})$  is long lived with low levels of turnover. Thus, directly targeting the downstream pathways, such as TGF $\beta$  signaling, represent an important alternative. While additional pathogenic mechanisms might be involved, elevated TGF $\beta$  signaling appears to be a fundamental pathway contributing to Gould syndrome pathology. We propose that interventions aimed at targeting TGF $\beta$  signaling could have an important impact for this devastating syndrome.

## Experimental Procedures

### Animals

All experiments were conducted in compliance with protocols approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco (Protocols AN159737 and AN182181). The *Col4a1* mutant mouse lines *Col4a1*<sup>G394V</sup> and *Col4a1*<sup>G1344D</sup> were described previously<sup>55, 71</sup>. All *Col4a1* mutant mice were heterozygous for a given mutation that was iteratively crossed to C57BL/6J mice (N 20). *Tgfb1*<sup>+/-</sup> mice<sup>101</sup> and the TGF $\beta$  reporter line expressing luciferase in response to Smad2/3-dependent TGF $\beta$  signaling activation (*SBE-luc*)<sup>100</sup> were maintained on a C57BL/6J background (N 5) and bred to *Col4a1*<sup>+/-Mut</sup> mice to generate *Col4a1*<sup>+/-Mut</sup> and *Col4a1*<sup>+/+</sup> littermates heterozygous for the *Tgfb1* null mutation or carrying the *SBE-luc* reporter transgene. Both male and female mice were used for all experiments and no differences were observed between sexes.

### 4PBA Treatment

Mice were provided 9.3g/L 4-phenylbutyrate (4PBA, Scandinavian Formulas Inc) from birth in drinking water refreshed weekly as described previously<sup>77</sup>.

### Exercise challenge

Mice were exercise challenged on a treadmill in a single session 24 h prior to harvesting for the 3MO time-point as described previously<sup>77</sup>. Each exercise session included a 2-min acclimation period, followed by a 30-min exercise challenge with a 15° downhill grade on a treadmill equipped with a shock plate (Exer 3/6, Columbus Instruments, Columbus, OH, USA). Animals were started at 7 m/min and increased by 3 m/min every 2 min until a maximum speed of 12 m/min was reached.

### Retinal whole mount and immunolabeling

Mice were anesthetized and transcardially perfused with phosphate buffered saline [pH 7.4] (PBS). Eucleated eyes were immersion fixed in 2% paraformaldehyde (PFA) overnight at 4°C and incubated 48 hours in PBS at 4°C. Retinas were isolated, cut into quadrants and incubated in blocking buffer (5% BSA, 5% normal serum and 0.5% Triton X-100 in PBS) (T8787, Sigma Aldrich) overnight at 4°C. Retinas were incubated in primary

antibodies against CD31 (1:200; 553370, BD Pharmagen), NG2 (1:200; AB5320, Millipore Sigma), Calponin (1:200; ab46794, Abcam) and/or  $\alpha$ -smooth muscle actin (SMA) (1:100; 48938S, Cell Signaling Technology) overnight at 4°C. Retinas were washed in PBS containing 0.5% Triton X-100 (PBST) and incubated with AlexaFluor-conjugated secondary antibodies (raised in donkey, 1:500, Invitrogen-Molecular Probes) overnight at 4°C in the dark. Immunolabeled retinas were washed in PBST and mounted using ProLong Gold Antifade Mountant (P36934, Thermo-Fisher Scientific). Images were captured using a Zeiss AxioImager M.1 microscope equipped with AxioCam MRm camera and AxioVision software or a Zeiss LSM700 confocal microscope equipped with plan-Apochromat objectives (63x/1.4 oil immersion or 20x/0.8) and ZEN software (Carl Zeiss Microscopy).

### Retinal analyses

Retinal vascular outgrowth was quantified by normalizing the distance between the optic nerve head (ONH) and the edge of the vascular front to the distance between the ONH and the edge of the retina in all quadrants of CD31 immunolabeled retinas and averaged. Retinal radial arteriolar mural cell numbers were quantified from 220×165 $\mu$ m field of view images from retinas co-labeled for CD31 and NG2. Images of radial arterioles were captured after the first branch point from the ONH unless otherwise stated and mural cells were counted over 200 $\mu$ m of vessel length. Mural cell numbers represent averages from three independent masked observers. The number of radial arterioles, average distance of calponin labeling along radial arterioles, and the number of calponin immunolabeled primary arterioles averaged from three radial arterioles were quantified from whole retina images. The number of primary arterioles, complexity of the precapillary arteriolar arborization, and the intensity of  $\alpha$ SMA and calponin immunolabeling were quantified from 1280×1280 $\mu$ m field of view images captured at a central location of the retina. Mean gray value intensities of  $\alpha$ SMA and calponin immunolabeling were quantified and averaged from three radial arterioles using ImageJ (National Institutes of Health). The number of bifurcated radial arterioles was evaluated and normalized to the total number of radial arterioles immunolabeled for calponin.  $\alpha$ SMA immunolabeling was used to evaluate the number of primary arterioles and precapillary arteriolar arborization.

Radial arteriolar VSMC coverage and diameter were quantified using CellProfiler software from 220×165 $\mu$ m field of view images of radial arterioles captured after the first branch from the ONH. VSMC coverage was measured as the percentage of  $\alpha$ SMA and CD31 co-labeled area per field of view. Arteriolar diameters were averaged at three equally spaced locations along the vessel for each image.

### Bioluminescence imaging

P7 *Col4a1<sup>+/-Mut</sup>* mice and *Col4a1<sup>+/+</sup>* littermates carrying the TGF $\beta$  reporter transgene (*SBE-luc*) were injected with RediJect D-Luciferin Bioluminescent Substrate (770504, PerkinElmer) (150mg/kg, i.p.) and anesthetized with isoflurane. Bioluminescence imaging was performed using the Spectrum In Vivo Imaging System (IVIS) (124262, PerkinElmer) 10 minutes after injection using 5 seconds exposure.

### Isolation of brain vascular endothelial cells

Microvascular endothelial cells were isolated as previously described<sup>127</sup>. Briefly, P7 mouse brains were harvested, and brain stems, cerebella and thalami were removed. The remaining brain regions were minced using a scalpel and homogenized by pipetting. Cells were dissociated in digestion buffer [Hanks' Balanced Salt solution (HBSS) containing 2mg/mL collagenase type 2 (LS004176, Worthington biochemical), 2mg/mL collagenase/dispase (10269638001, Sigma Aldrich), and 0.1mg/mL DNase I (AK3778-0100, Akron Biotech)] and incubated on a thermo-shaker set to 800 rpm for 1 hour at 37 °C. Cell suspensions were mixed with 2mL bovine serum albumin (BSA)/HBSS (22% w/v) and centrifuged at 1000xg for 20 minutes at 4°C. Cell pellets were washed in HBSS and collected by centrifugation (1000xg) for qRT-PCR analyses.

### Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using Qiagen RNeasy Plus Mini Kit (74134, Qiagen) and reverse transcribed using iScript cDNA Synthesis Kit (1708841, Bio-Rad). qRT-PCR was performed on a C1000 Touch Thermal cycler and CFX96 or CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories) using SsoAdvanced™ SYBR Green® Supermix (1725271, Bio-Rad) and primers listed in supplemental table 1. Briefly, 25ng of cDNA and 0.5µM primers were used per 10 µl reaction. Each cycle consisted of denaturation at 95°C for 5s, followed by annealing and extension at 60°C for 20s. Each reaction was run as technical duplicates and a minimum of 4 biological replicates was used per group. The relative expression level of each gene was normalized to that of *Gapdh* or *b-actin* and analyzed using the  $2^{-CT}$  method<sup>128</sup>.

### Perl's Prussian blue staining and intracerebral hemorrhage quantification

Anesthetized mice were transcardially perfused with PBS and brains fixed by immersion in 4% PFA overnight at 4°C, cryoprotected in 30% sucrose in PBS for 48 hours and embedded in Tissue-Tek OCT compound (4583, Sakura Finetek). For ICH quantification, four sets of eight alternating 40µm coronal cryosections centered to bregma per brain were collected. One set was stained with Perl's Prussian blue as previously described<sup>58, 77, 78</sup>. Images were acquired using a SteREO Discovery.V8 microscope and AxioCam ICc3 camera and AxioVision 4.6 software (Carl Zeiss Microscopy). Hemorrhage areas were normalized to total brain area in percentage computationally using CellProfiler software<sup>77, 129</sup>.

### Protein extraction and western blot analysis

Brains were harvested from mice transcardially perfused with PBS and homogenized in tissue protein extraction reagent (T-PER) buffer supplemented with phosphatase and protease inhibitor cocktail (78440, ThermoFisher). Protein samples were separated on 4%–12% Bis-Tris gels (NW04125BOX, Invitrogen) using Bolt MOPS SDS Running Buffer (B0001, Invitrogen) and transferred to polyvinylidene fluoride (PVDF) membranes (1620177, Bio-Rad). Membranes were incubated in blocking buffer (5% non-fat milk in tris-buffered saline supplemented with 0.1% Tween-20 (TBST)) for 1 hour at room temperature and incubated with αSMA antibody (1:400; ab5694, Abcam) in blocking buffer at 4°C overnight. GAPDH antibody (1:10,000; MAB374, Millipore-Sigma) was used as a loading

control. After washing in TBST, membranes were incubated in horseradish peroxidase-conjugated secondary antibodies (1:10,000; Jackson ImmunoResearch Laboratories) in blocking buffer for 2 hours at room temperature. The membranes were visualized by chemiluminescence (Immobilon forte WBLUF0100, Millipore or Pierce™ ECL Western Blotting Substrate, 32106). Densitometric analysis was performed using ImageJ.

### Statistical analyses

Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, Inc., La Jolla, CA, USA) and data are presented as means ± standard deviation. Two-group comparisons were performed using Student t-test. Multiple-group comparisons to test for difference between genotypes and effect of treatment or *Tgfb1* heterozygosity in *Col4a1<sup>+/+</sup>* and *Col4a1<sup>+/-Mut</sup>* mice were performed using 2-way ANOVA followed by Tukey's post hoc test as specified in figure legends.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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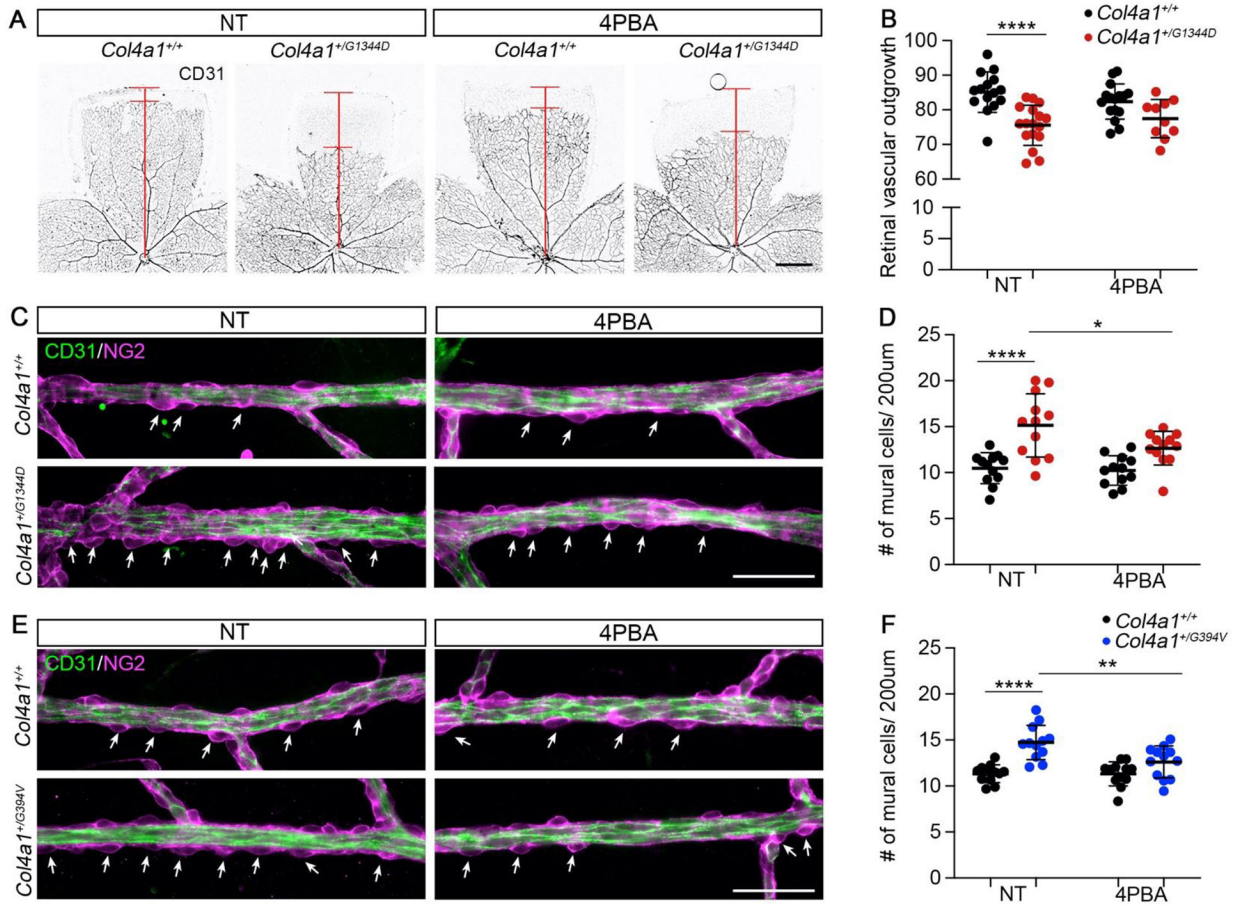


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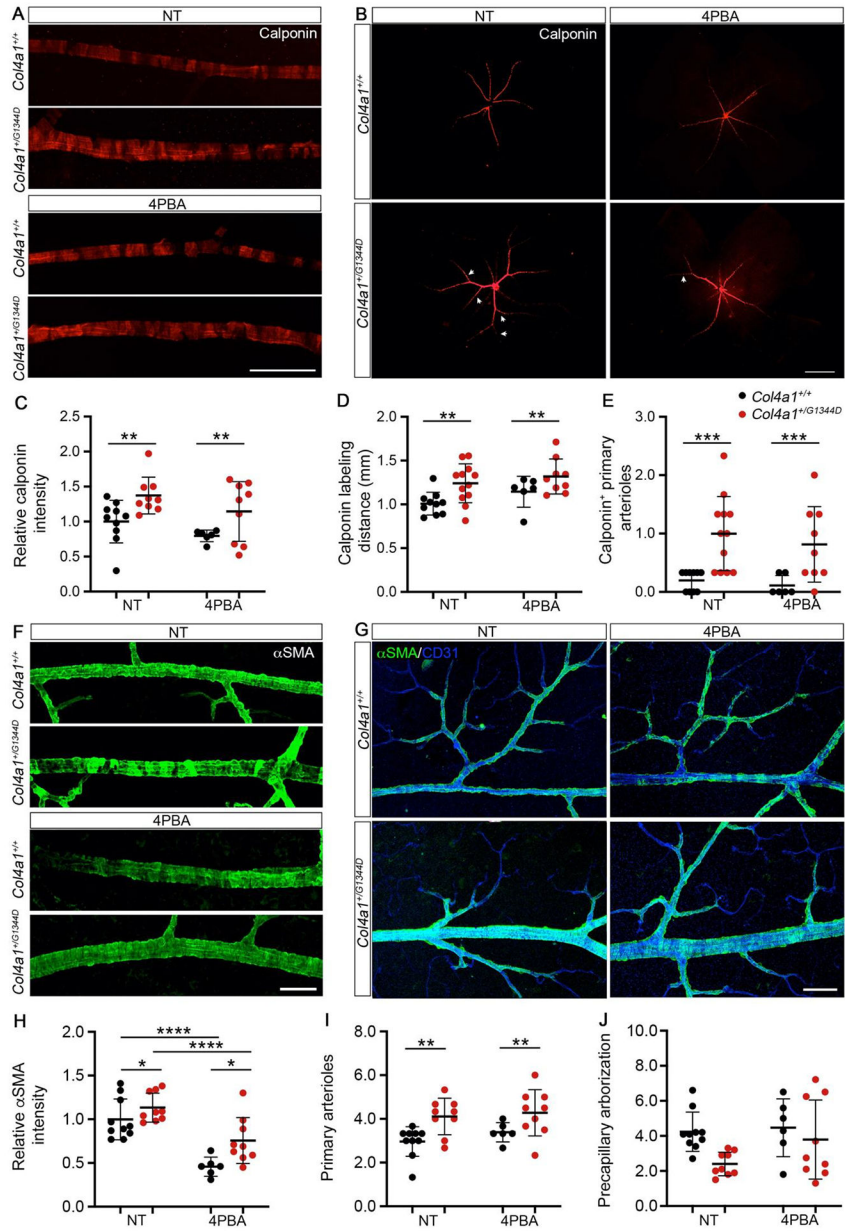
**Highlights**

- *Col4a1* mutant mice model Gould syndrome and cerebral small vessel disease
- Elevated TGF $\beta$  signaling causes cerebral small vessel disease in *Col4a1* mutant mice
- Promoting collagen  $\alpha 1(I) \alpha 2(IV)$  secretion reduces TGF $\beta$  signaling in *Col4a1* mutant mice
- Genetically reducing TGF $\beta$  decreases intracerebral hemorrhage severity in mutant mice



**Figure 1. 4PBA partially ameliorates developmental retinal vascular defects in *Col4a1* mutant mice.**

(A) P7 retinas immunolabeled for CD31 and (B) quantification of vascular outgrowth showing significantly impaired retinal angiogenesis in *Col4a1*<sup>+/G1344D</sup> mice but not their 4PBA treated counterparts when compared to their respective controls. From left to right, n= 15, 17, 16, and 10 retinas. (C-F) P7 radial arterioles immunolabeled for CD31 (green) and NG2 (magenta) and quantification showing increased numbers of arteriolar mural cells (white arrows in C and E) in *Col4a1*<sup>+/G1344D</sup> (C-D) and *Col4a1*<sup>+/G394V</sup> (E-F) mice that are reduced by 4PBA. n= 12 mice per group. Data are presented as mean±SD, \*p<0.05; \*\*p<0.01; \*\*\*\*p<0.0001, two-way ANOVA. Scale bars: 500µm (A), and 100µm (C and E). NT, no treatment.



**Figure 2. *Col4a1* mutant mice have abnormal retinal vascular patterning.** (A) Retinal radial arterioles and (B) whole retinas immunolabeled for calponin and (C) quantification showing increased intensity of arteriolar calponin labeling, (D) peripheral extension of calponin labeling along radial arterioles and (E) increased number of calponin<sup>+</sup> primary arterioles (white arrows in B) in 1MO *Col4a1*<sup>+/G1344D</sup> mice that are not prevented by 4PBA treatment. From left to right, n= 10, 12, 6, and 9 retinas. (F) Retinal radial arterioles immunolabeled for  $\alpha$ SMA and (G) retinas immunolabeled for CD31 and  $\alpha$ SMA and (H) quantification showing increased arteriolar  $\alpha$ SMA labeling intensity, (I) increased numbers of primary arterioles and (J) reduced complexity of precapillary arteriolar arborization in 1MO *Col4a1*<sup>+/G1344D</sup> mice. 4PBA reduced  $\alpha$ SMA labeling intensity in *Col4a1*<sup>+/+</sup> and *Col4a1*<sup>+/G1344D</sup> retinas and improved precapillary arteriolar arborization in

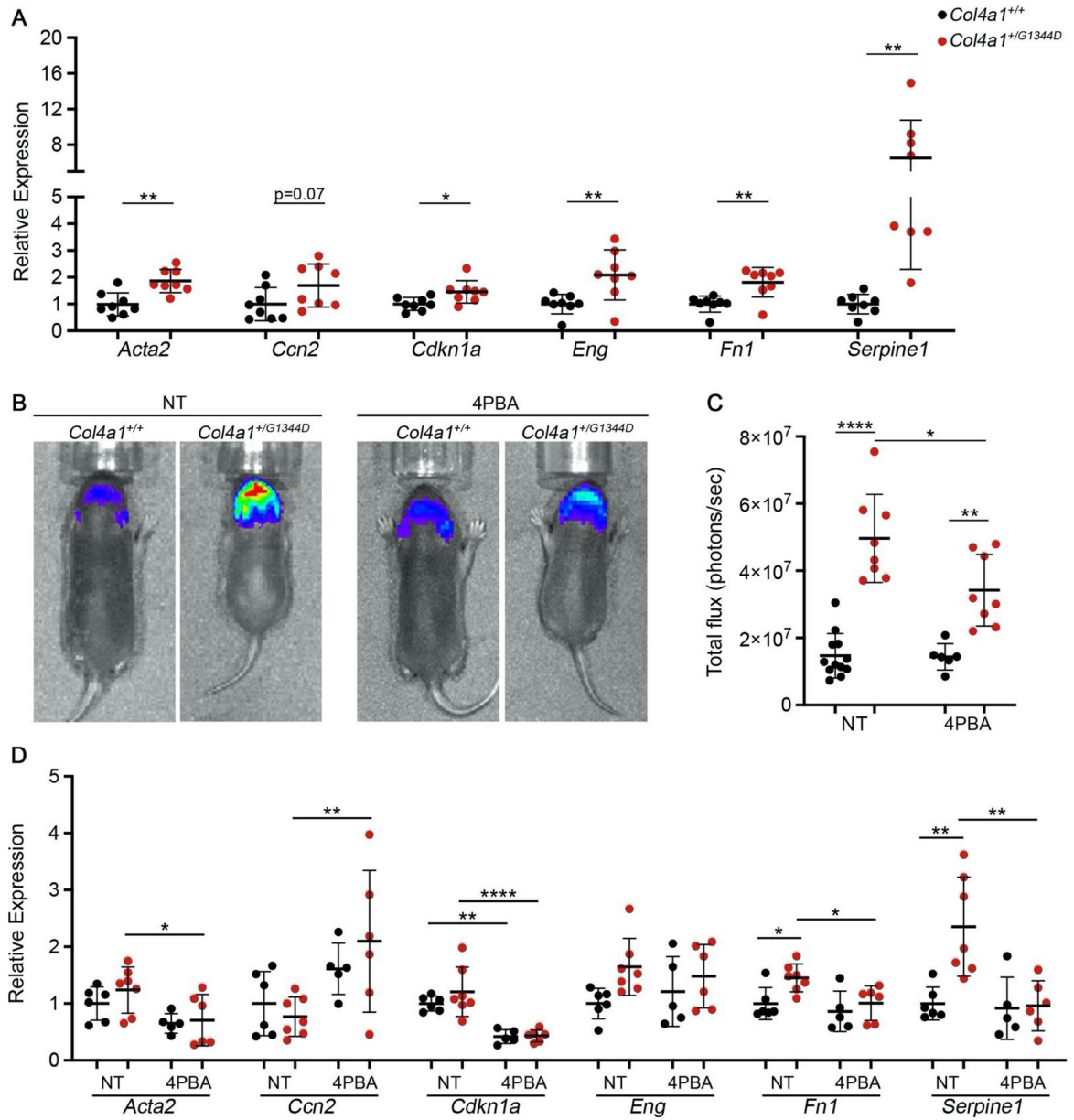
*Col4a1*<sup>+/*G1344D*</sup> retinas. From left to right, n= 10, 9, 6, and 9 retinas. Data are presented as mean±SD, \*p<0.05; \*\*p<0.01; \*\*\*p<0.001; \*\*\*\*p<0.0001, two-way ANOVA. Scale bars: 100µm (**A and B**) and 50µm (**F and G**).

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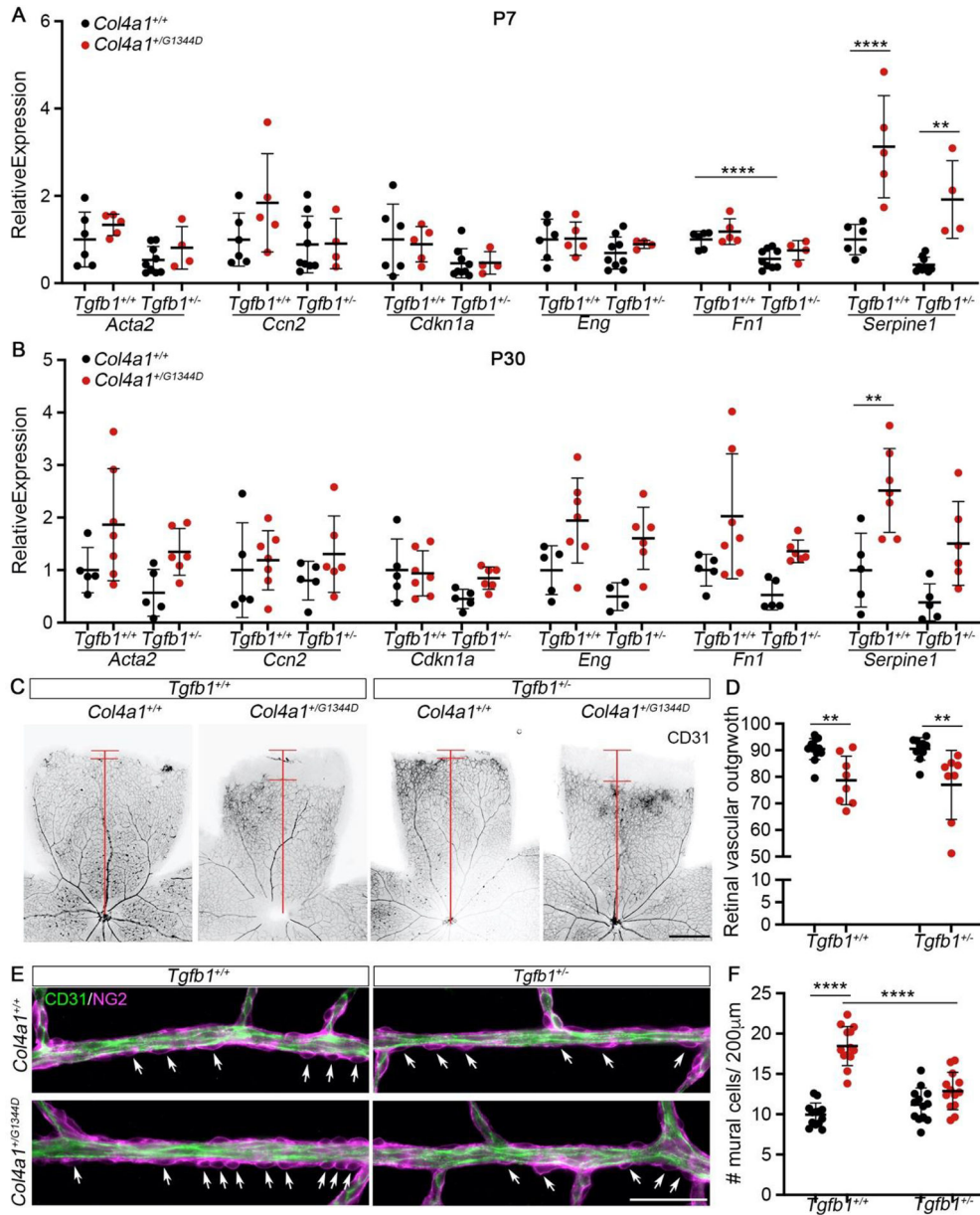
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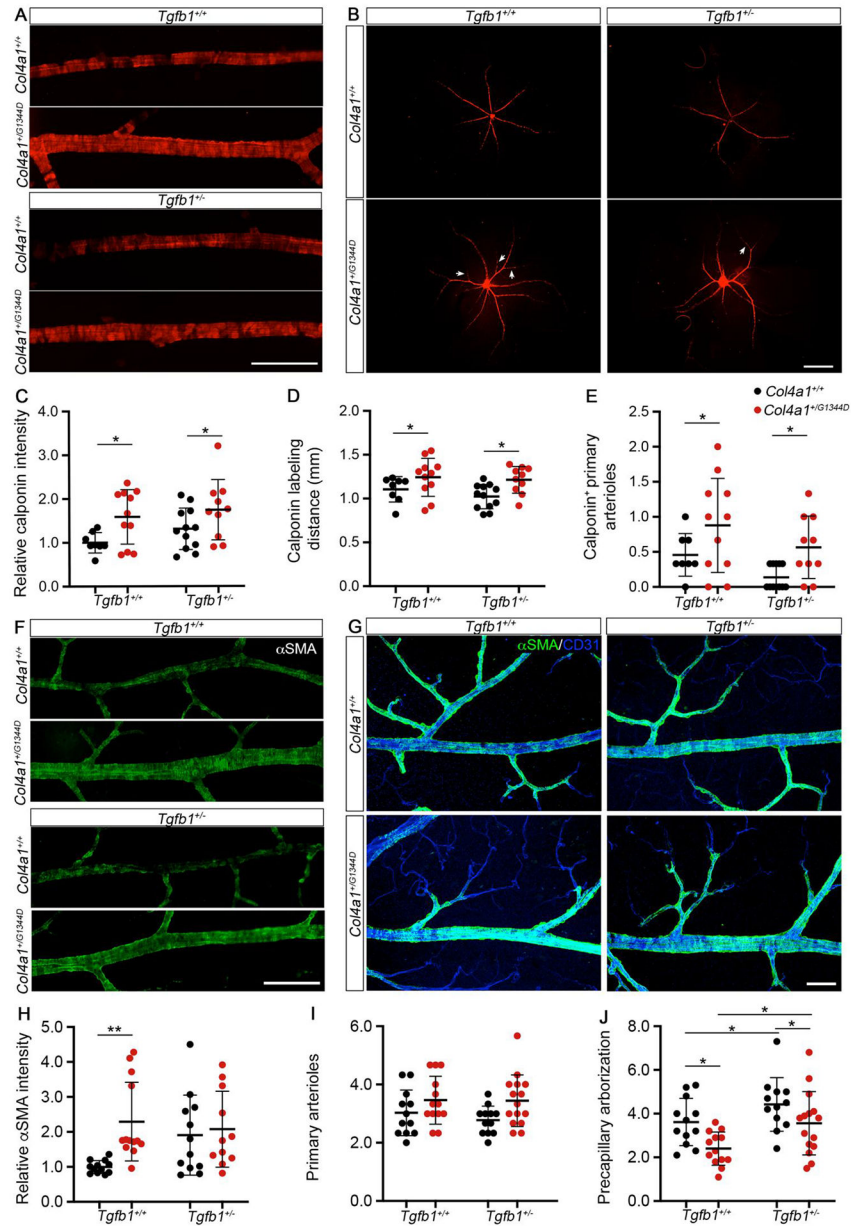
**Figure 3. *Col4a1* mutant mice have elevated TGFβ signaling that is reduced by 4PBA.** (A) qPCR analyses showing increased expression of TGFβ target genes in P7 *Col4a1*<sup>+/G1344D</sup> brains. n= 8 brains per group. (B) Representative images and (C) quantification of *in vivo* bioluminescence in P7 mice carrying the SBE-luciferase reporter gene showing elevated TGFβ signaling in *Col4a1*<sup>+/G1344D</sup> mice that is reduced by 4PBA. From left to right n= 12, 8, 6, and 8 mice. (D) qPCR analyses showing increased expression of TGFβ target genes in 1MO *Col4a1*<sup>+/G1344D</sup> brains that is generally reduced by 4PBA. From left to right, n= 6, 7, 5, and 6 brains. Data are presented as mean±SD, \*p<0.05; \*\*p<0.01; \*\*\*\*p<0.0001, two-way ANOVA.



**Figure 4. Genetically reducing TGFβ signaling partially ameliorates developmental retinal vascular defects in *Col4a1* mutant mice.**

(A) qPCR analyses of P7 and (B) P30 brains showing that *Tgfb1* heterozygosity decreased TGFβ target gene expression in brains from *Col4a1*<sup>+/+</sup> and *Col4a1*<sup>+/*G1344D*</sup> mice. From left to right, n= 6, 5, 9, and 4 mice (A), and n= 5, 7, 5, and 6 mice (B). (C) P7 retinas immunolabeled for CD31 and (D) quantification of retinal vascular outgrowth showing that *Tgfb1* heterozygosity does not prevent retinal angiogenesis defects in *Col4a1*<sup>+/*G1344D*</sup> mice. From left to right n= 14, 8, 10, and 8 retinas. (E) P7 retinal arterioles immunolabeled for CD31 and NG2 and (F) quantification showing that *Tgfb1* heterozygosity normalizes the number of arteriolar mural cells (white arrows in C) in *Col4a1*<sup>+/*G1344D*</sup> retinas. n= 13 retinas per group. Data are presented as mean±SD, \*\*p<0.01; \*\*\*\*p<0.0001, two-way ANOVA. Scale bars: 500µm (C) and 100µm (E).





**Figure 5. Genetically reducing TGF $\beta$  signaling partially restores vascular patterning in *Col4a1* mutant retinas.**

(A) Retinal radial arterioles and (B) whole retina immunolabeled for calponin and (C-E) quantification showing that *Tgfb1* heterozygosity does not change the intensity (C) or pattern of calponin labeling (D-E) in 1MO *Col4a1*<sup>+/-</sup>*G1344D* mice. White arrows in B indicate calponin<sup>+</sup> primary arterioles. From left to right, n= 8, 11, 12, and 10 retinas. (F) Radial arterioles immunolabeled for  $\alpha$ SMA and (G) radial arterioles immunolabeled for CD31 and  $\alpha$ SMA and (H-J) quantification showing that *Tgfb1* heterozygosity improves precapillary arteriolar arborization (J) but not arteriolar  $\alpha$ SMA labeling intensity (H) or number of primary arterioles (I) in 1MO *Col4a1*<sup>+/-</sup>*G1344D* mice. From left to right, n= 12, 13, 12, and

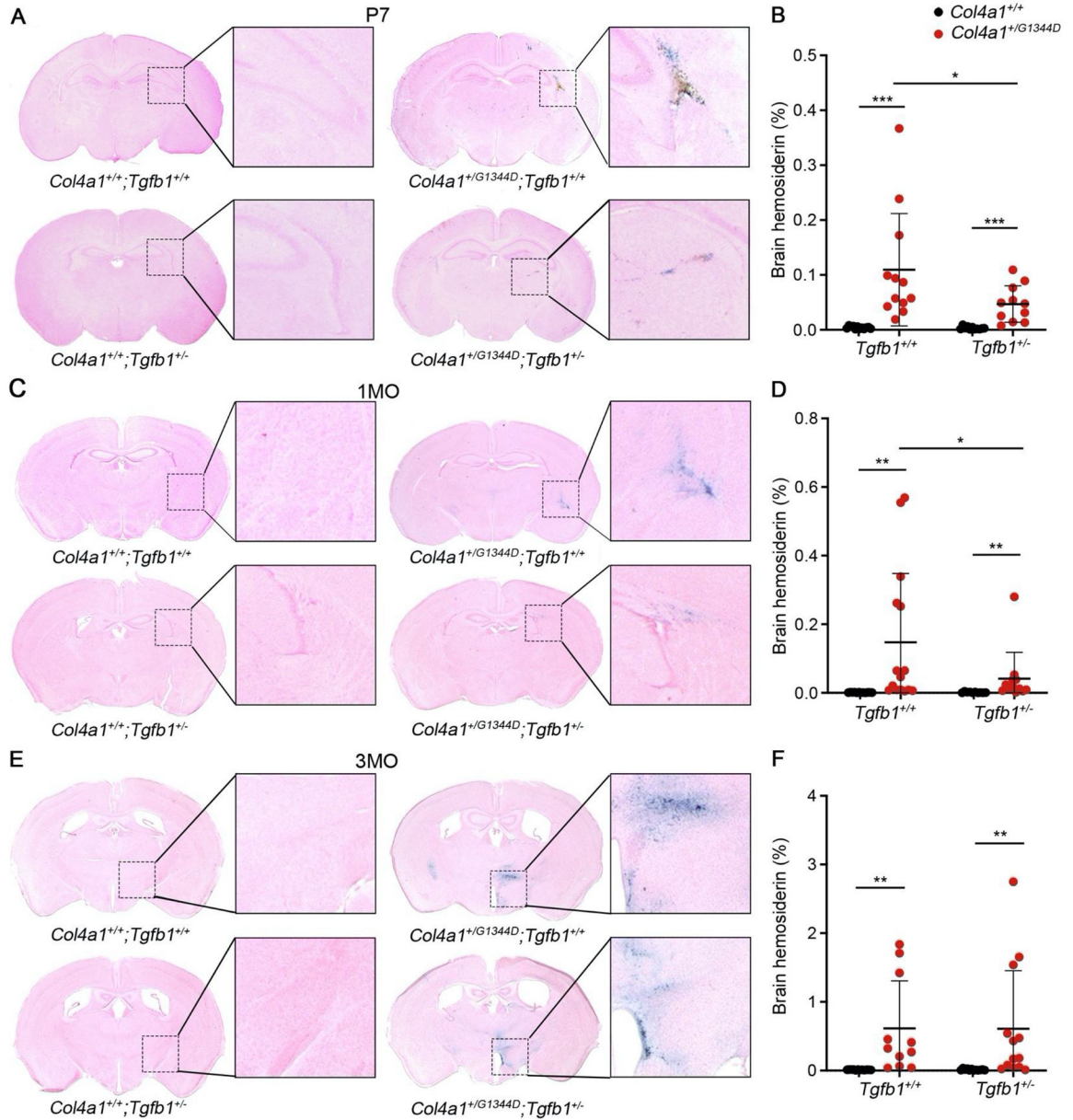
11 retinas (**H**) and n= 12, 13, 12, and 15 retinas (**I-J**). Data are presented as mean±SD, \*p<0.05; \*\*p<0.01, two-way ANOVA. Scale bars: 100µm (**A, B, F**) and 50µm (**G**).

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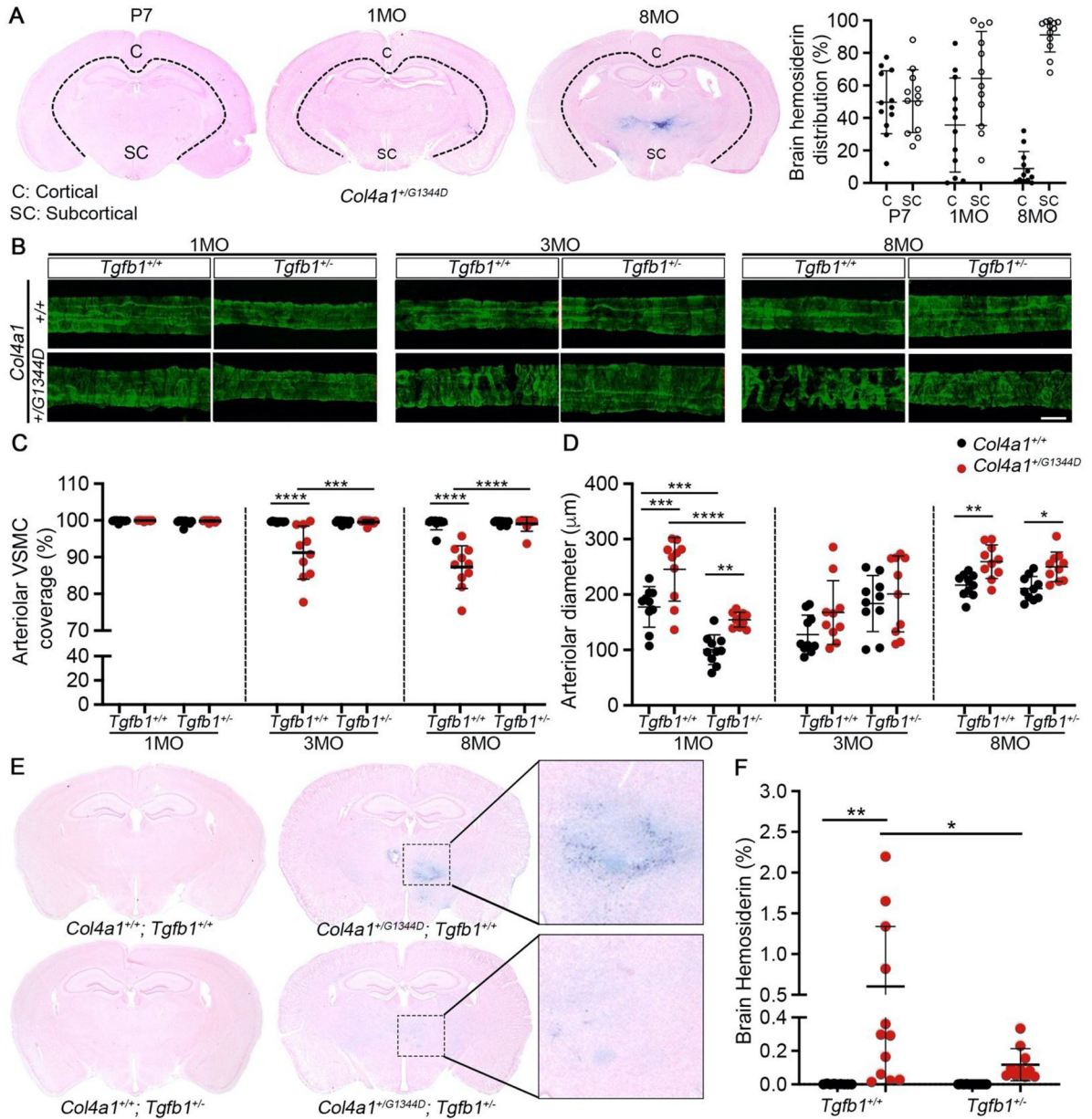
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**Figure 6. *Tgfb1* heterozygosity reduces ICH severity in *Col4a1* mutant mice.** (A-F) Prussian blue-stained brain sections and quantification of brain hemosiderin showing that *Tgfb1* heterozygosity reduces ICH severity in P7 (A-B) and 1MO (C-D) *Col4a1<sup>+/G1344D</sup>* mice but not in exercised 3MO *Col4a1<sup>+/G1344D</sup>* mice (E-F). From left to right, n= 12, 12, 11, and 11 mice (B), n= 12, 15, 10, and 12 mice (D), and n=12, 11, 12, 13 mice (F). Data are presented as mean±SD, \*p<0.05; \*\*p<0.01; \*\*\*p<0.001, two-way ANOVA.



**Figure 7. *Tgfb1* heterozygosity prevents age-related vascular defects in *Col4a1* mutant mice.** (A) Prussian blue-stained brain sections from P7, 1MO and 8MO *Col4a1*<sup>+/*G1344D*</sup> mice and quantification of hemosiderin showing an age-dependent shift in ICH distribution. C, cortical; and SC, subcortical. n= 12 mice per group. (B) 1MO, 3MO and 8MO radial arterioles immunolabeled for αSMA and quantification showing (C) a progressive loss of VSMC coverage and (D) increased arteriolar diameter in *Col4a1*<sup>+/*G1344D*</sup> mice. Notably, *Tgfb1* heterozygosity prevented VSMC loss in *Col4a1*<sup>+/*G1344D*</sup> mice and reduced arteriolar diameter in both *Col4a1*<sup>+/*+*</sup> and *Col4a1*<sup>+/*G1344D*</sup> mice at 1MO. n=10 mice per group. (E) Prussian blue-stained brain sections from 8MO mice and (F) quantification of hemosiderin showing that ICH severity in *Col4a1*<sup>+/*G1344D*</sup> is significantly reduced by *Tgfb1* heterozygosity. From left to right, n= 9, 12, 11, and 10 mice. Data are presented as

mean±SD, \*p<0.05; \*\*p<0.01, \*\*\*p<0.001; \*\*\*\*p<0.0001, two-way ANOVA. Scale bar: 100µm.

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