



# Endothelial Notch1 drives multicellular remodelling during hyaloid vessel regression

Shaymaa Khazaal<sup>1,2</sup> · Abdoul-Razak Sango<sup>1,2</sup> · Kawtar Zouine<sup>1,2</sup> · Alexa Silva Sosa<sup>1,2</sup>  · Ariane Vanessa Megne<sup>1,2</sup> · Rony Chidiac<sup>3</sup> · Kiran Bora<sup>4</sup> · Gaelle Mawambo<sup>1,2</sup> · Jing Chen<sup>4</sup> · Malika Oubaha<sup>1,2</sup>

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## Abstract

Hyaloid vessel regression is essential for vitreous transparency and normal vision, yet how this transient vascular network is dismantled remains unclear. Here we show that postnatal hyaloid regression in mice is not driven by a measurable increase in apoptosis, but instead by coordinated endothelial and mural cell delamination, extravascular redistribution and a transient plasticity program marked by *Snail1* and *Slug* induction. Notch1 signalling peaks during the regression window, and vascular endothelial-specific Notch1 deletion causes persistent hyaloid vessels, with excessive proliferation and failure of endothelial and mural cell disengagement. Mechanistically, loss of Notch1 preserves endothelial identity, suppresses an endothelial-mesenchymal transition-like transcriptional program and reduces expression of the Wnt co-receptors *Lrp5* and *Lrp6*. Wnt pathway mutant phenocopies the delamination defect, supporting functional convergence between Notch1 and Wnt signalling during vessel involution. Together, these findings identify Notch1 as a key driver of developmental vascular pruning. They also redefine hyaloid regression as an apoptosis-non-exclusive remodelling process, with broader implications for physiological and pathological vascular remodelling, and may guide therapeutic strategies to modulate vascular regression in ocular disorders.

**Keywords** Notch signaling · Hyaloid regression · Endothelial-to-mesenchymal transition · Vascular remodeling · Endothelial plasticity · Vision

## Introduction

Ocular vascular development is essential for normal eye growth and visual function [1]. During this process, the transient hyaloid vasculature supplies the developing lens and inner retina before undergoing regression as the retinal circulation matures. In mice, hyaloid vessels regress postnatally from approximately P4 to P21, whereas in humans, regression begins around 12–13 gestational weeks and is largely completed before birth [1–5]. This transition is crucial for vitreous transparency and retinal maturation, and its failure is associated with congenital disorders marked by persistent hyaloid vessels, aberrant retinal vascularization and vision loss, including persistent fetal vasculature, Norrie disease and retinopathy of prematurity [6–8].

Hyaloid regression is classically viewed as an apoptosis-driven process; however, accumulating evidence indicates that it is part of a broader multicellular remodelling program [9, 10]. The hyaloid vasculature is composed of endothelial cells (ECs), mural cells (MCs) which includes pericytes

Shaymaa Khazaal, Abdoul-Razak Sango, Kawtar Zouine and Alexa Silva Sosa have contributed equally to this work.

✉ Malika Oubaha  
oubaha.malika@uqam.ca

<sup>1</sup> Department of Biological Sciences, Faculty of Sciences, Université du Québec à Montréal (UQAM), Montreal, Canada

<sup>2</sup> Centre d'Excellence en Recherche sur les Maladies Orphelines-Fondation Courtois (CERMO-FC), Montreal, Canada

<sup>3</sup> Department of Pharmacology and Physiology, Université de Sherbrooke, Sherbrooke, QC, Canada

<sup>4</sup> Department of Ophthalmology, Boston Children's Hospital and Harvard Medical School, Boston, MA, USA

and vascular smooth muscle cells, and macrophages-lineage hyalocytes (HCs), which together coordinate vessel maintenance stabilization and clearance [3, 10–12]. Macrophage/HC-derived Wnt7b and canonical Wnt signalling through Norrin (Ndp)-Frizzled-4 and Lrp5 have been implicated in vessel involution, and genetic studies including those linked to Norrie disease have shown that defective regression is accompanied not only by reduced cell death but also by persistent immature vessels and altered endothelial behaviour [13–15]. Collectively, these observations suggest that hyaloid clearance depends on integrated changes in EC, MC association and coordinated multicellular remodelling rather than on apoptosis alone [3, 16].

Notch signalling is a central regulator of vascular development, where it controls endothelial quiescence, proliferation and vessel patterning [17–19]. In the retina, Dll4-Notch1 signalling promotes vascular pruning and limits excessive angiogenesis, yet its role in hyaloid regression remains undefined [20–22]. Here, we identify Notch1 as a previously unrecognized regulator of hyaloid vessel involution. We show that Notch1 controls hyaloid vascular cell proliferation, plasticity and vessel-wall disengagement, supporting a model in which hyaloid regression is driven by coordinated multicellular remodelling rather than apoptotic clearance alone. These findings refine the developmental logic of ocular vascular regression, position Notch1 as a key regulator of developmental vessel pruning and provide a framework for understanding pathological vessel persistence in the eye.

## Results

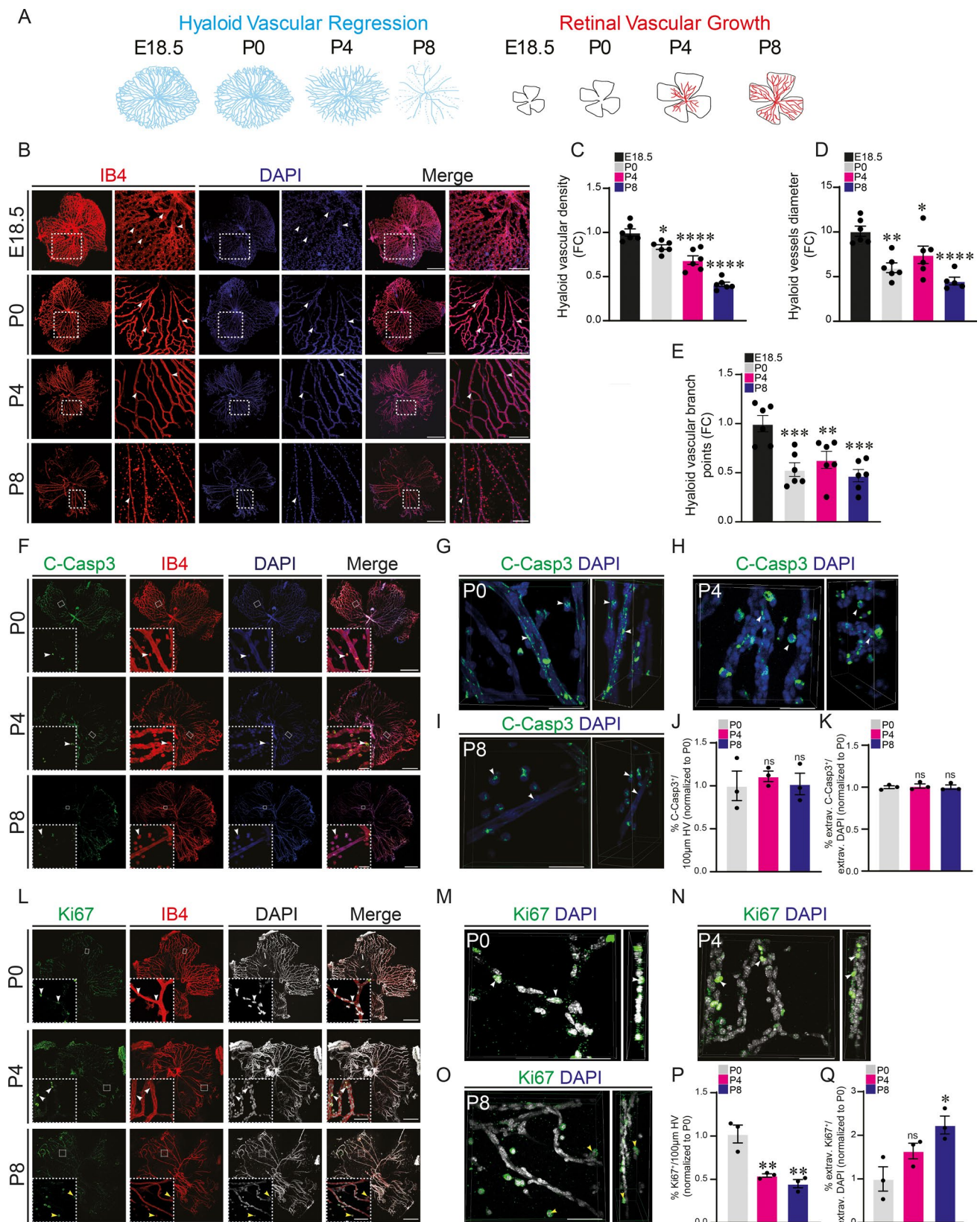
### Hyaloid vessel pruning involves cell relocation and extravascular proliferation without increased apoptosis

While apoptosis has been implicated in pruning redundant vasculature, its temporal and functional relevance to hyaloid vessel involution remains incompletely defined [23, 24]. In mice, the postnatal regression of hyaloid vessels occurs concomitantly with the growth of the retinal vasculature (Fig. 1A). To investigate the cellular basis of hyaloid regression, we delineated the developmental trajectory and early regression of the hyaloid vasculature in C57BL/6J mice, spanning late embryogenesis (E18.5) to early postnatal stages (P8). Isolectin B4 (IB4) labeling of flatmounted hyaloids revealed a progressive decrease in hyaloid vascular density over time (Fig. 1B). Quantification revealed a progressive decline, with ~ 15% reduction by P0, ~ 25% by P4, and ~ 64% by P8 compared to E18.5. (Fig. 1C). In parallel, we observed a marked narrowing of vessels diameter (Fig.

**Fig. 1** Cell relocation, apoptosis, and proliferation during postnatal hyaloid vessel pruning. **A** Schematic diagram of mouse hyaloid vessels (HV) regression and retinal vascular outgrowth at embryonic day 18.5 (E18.5) and postnatal days 0 (P0), 4 (P4), and 8 (P8). **B** Representative Isolectin B4 (IB4) and DAPI immunofluorescence staining of E18.5, P0, P4 and P8 HV flatmounts. **C–E** Quantification of HV density, vessel diameter and vascular branch points ( $n=5–6$ ). **F** Representative confocal micrographs of P0, P4 and P8 HV flatmounts labeled with cleaved caspase 3 (C-Casp3), IB4 and DAPI. **G–I** 3D reconstructions of HV at P0, P4, and P8 show the top and side views. Quantification of apoptotic cells (C-Casp3<sup>+</sup>) per 100  $\mu\text{m}$  of HV length (**J**), and extravascular (extrav.) apoptotic cells among total extravascular cells (**K**) ( $n=3$ ). **L** Representative images of Ki67, IB4 and DAPI immunofluorescence staining of P0, P4 and P8 HV flatmounts show proliferating cells within (white arrowheads) and outside (yellow arrowheads) the vascular compartment. **M–O** 3D reconstructions show proliferating cells in vascular and extravascular regions of HV at P0, P4 and P8. Quantification of proliferating cells (Ki67<sup>+</sup>) per 100  $\mu\text{m}$  of HV length (**P**), and extravascular proliferating cells among total extravascular cells (**Q**) ( $n=3$ ). Results are expressed as fold change (FC) relative to E18.5 (**C–E**) and as percentages (%) normalize to P0 (**J**, **K**, **P**, **Q**). Data are means  $\pm$  SEM. Represented  $p$  values are \* $\leq 0.05$ , \*\* $\leq 0.01$ , \*\*\* $\leq 0.001$ , \*\*\*\* $\leq 0.0001$  from ordinary one-way ANOVA test with Dunnett's multiple comparison. Non-significant (ns). Scale bars, 500  $\mu\text{m}$  (**B**, **F**, **L**) and 50  $\mu\text{m}$  [for higher magnification images in (**F–I**, **L–O**)]

1D) and a significant decrease in branching complexity relative to E18.5 as reflected by reduced vascular branch points (Fig. 1E). Although apoptosis has been reported in embryonic hyaloid vessels at mid-gestation, before the onset of postnatal regression, its contribution to postnatal involution remains unclear [9, 11]. We therefore quantified cleaved caspase-3-positive (C-Casp3<sup>+</sup>) cells across the regression timeline (Fig. 1F). 3D reconstructions at P0, P4 and P8 validated the presence of C-Casp3<sup>+</sup> cells both along and outside the hyaloid vasculature throughout this period (Fig. 1G–I). Quantitative analyses showed that neither total apoptotic cells nor extravascular apoptotic cells increased between P0 and P8 (Fig. 1J, K). Given the lack of a measurable increase in apoptosis, we next asked whether proliferation accompanies postnatal hyaloid remodelling. We therefore quantified Ki67<sup>+</sup> cells across the regression timeline (Fig. 1L). 3D reconstructions at P0, P4 and P8 revealed Ki67<sup>+</sup> cells both along and outside the hyaloid vasculature throughout this period (Fig. 1L–O). Quantitative analysis showed that the number of proliferating cells per 100  $\mu\text{m}$  of hyaloid vessel declined significantly from P0 to P4 and P8 (Fig. 1P). Interestingly, the proportion of extravascular proliferating cells increased over time and reached significance at P8 (Fig. 1Q).

These data indicate that postnatal hyaloid vessel regression is not accompanied by an increase in apoptosis. To assess the specificity of Ki67 and C-Casp3 antibodies in the immunofluorescence staining, P0, P8 control samples processed in parallel with omission of the primary antibody were included. The absence of detectable signal in these negative controls supported staining specificity (Fig. S1A).



Taken together, these findings show that postnatal hyaloid vessel regression in mice occurs without a measurable increase in apoptosis. Instead, regression is characterized by reduced vascular cell proliferation and a spatial shift in proliferative activity to the extravascular compartment. These observations support a model in which hyaloid vessel involution is coupled to dynamic extravascular remodelling, rather than being driven primarily by apoptotic clearance as previously proposed.

### Cell-type-specific extravascular proliferation accompanies hyaloid vessel regression

Because postnatal hyaloid vessel involution occurred without increased apoptosis, but with a shift in proliferative activity from the intravascular to the extravascular compartment (Fig. 1L–Q), we next analysed the dynamics of ECs, MCs and HCs to determine how each population contributes to vascular regression.

To assess whether extravascular cells arise from the endothelial lineage during postnatal hyaloid regression, we performed tamoxifen-inducible endothelial lineage tracing in *Rosa26-mT/mG; Cdh5-CreERT2* mice. In the absence of tamoxifen, hyaloid vessels remained tdTomato<sup>+</sup> with virtually no GFP signal, indicating negligible background recombination. Following tamoxifen administration, GFP<sup>+</sup> cells were detected not only within the vessel wall but also in the extravascular compartment (Fig. 2A).

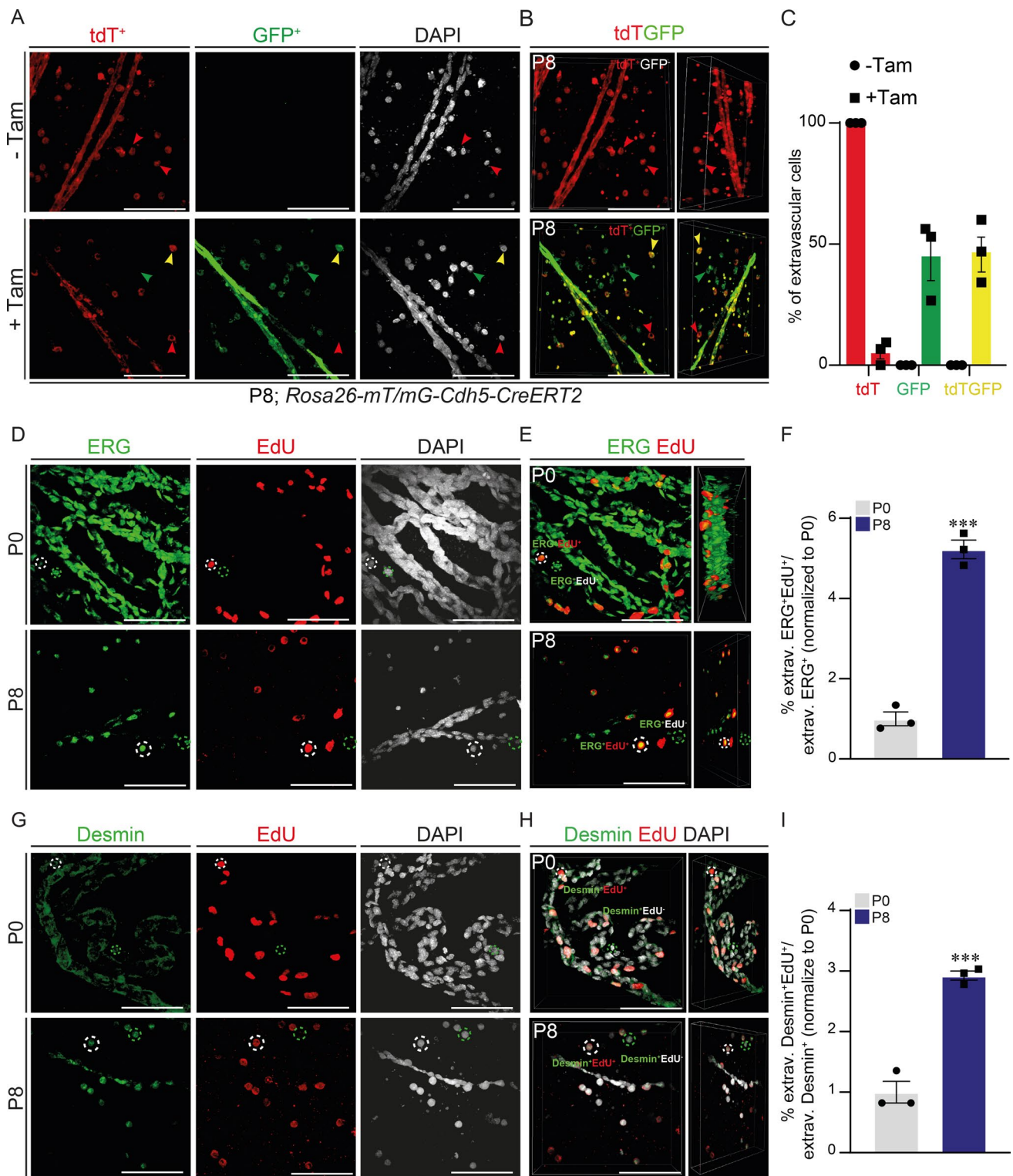
Extravascular cells included GFP<sup>+</sup> cells, tdTomato<sup>+</sup> cells and tdTomato<sup>+</sup>GFP<sup>+</sup> cells. The tdTomato<sup>+</sup>GFP<sup>+</sup> population is consistent with ongoing reporter conversion in endothelial-lineage cells, although we cannot exclude that some cells represent non-recombined hyalocytes/macrophages that have engulfed GFP<sup>+</sup> endothelial material [9, 10]. 3D reconstruction confirmed the extravascular localization of GFP<sup>+</sup> and tdTomato<sup>+</sup>GFP<sup>+</sup> cells (Fig. 2B). Quantification showed that extravascular cells in control samples were almost exclusively tdTomato<sup>+</sup>, whereas tamoxifen-treated samples contained a marked increase in GFP<sup>+</sup> and tdTomato<sup>+</sup>GFP<sup>+</sup> extravascular cells, with only a small residual tdTomato-only population (Fig. 2C). Together, these results identify endothelial-lineage-labelled cells within the extravascular population that accumulates during hyaloid vessel involution, while also suggesting that this compartment remains heterogeneous and may include non-recombined cells and phagocytosed vascular material.

To independently validate the identity and localization of these extravascular endothelial-derived cells, we next combined endothelial and basement membrane markers. CD31 and Collagen IV co-staining identified Collagen IV<sup>+</sup>CD31<sup>−</sup> empty vascular sleeves, consistent with regressing vessels, and further revealed expansion of the

**Fig. 2** Proliferative endothelial and mural cells delamination accompanies hyaloid vessels regression. **A** Representative confocal micrographs of P8 HV flatmounts from *Rosa-mT/mG; Cdh5-CreERT2* mice in the absence (-Tam) or following tamoxifen induction (+Tam), showing tdTomato (tdT), GFP, and DAPI labeling. **B** 3D reconstructions of HV illustrating tdT<sup>+</sup>, GFP<sup>+</sup>, and tdT<sup>+</sup>/GFP<sup>+</sup> and their extravascular localization. Arrowheads indicate tdT<sup>+</sup> cells (red), GFP<sup>+</sup> cells (green), and tdT<sup>+</sup>/GFP<sup>+</sup> cells (yellow). **C** Quantification of extravascular tdT<sup>+</sup>, GFP<sup>+</sup>, and tdT<sup>+</sup>/GFP<sup>+</sup> cells, expressed as a percentage of total extravascular cells in -Tam and +Tam conditions ( $n=3$ ). **D** Representative confocal images of P0 and P8 HV flatmounts showing EdU incorporation in ECs (ERG1/2/3<sup>+</sup>). Nuclei are labeled with DAPI. **E** 3D reconstructions showing proliferating ECs (white circle) and non-proliferating ECs (green circle) in vascular and extravascular regions of HV at P0 and P8. **F** Quantification of EdU<sup>+</sup> ECs relative to total number of extravascular ERG1/2/3<sup>+</sup> cells in HV ( $n=3$ ). **G** Representative confocal images of P0 and P8 HV flatmounts showing EdU incorporation in mural cells (MCs; Desmin<sup>+</sup>). Nuclei are labeled with DAPI. **H** 3D reconstruction of HV at P0 and P8 showing proliferating MCs (white circle) and non-proliferating MCs (green circle). **I** Quantification of extravascular proliferating MCs among total extravascular MCs ( $n=3-4$ ). Results are expressed as percentages (%) from total cells (C) or as % normalized to P0 stage (F, I). Data are shown as means  $\pm$  SEM. Represented  $p$ -values are  $***\leq 0.001$  from two-tailed parametric unpaired  $t$ -test. Scale bars, 50  $\mu$ m

extravascular compartment at P8 relative to P0 (Fig. S1B). Notably, CD31<sup>+</sup> ECs were frequently detected outside these sleeves at P8, supporting their displacement from the regressing vessel wall. Quantification further showed that the number of empty sleeves increased significantly at P8 relative to P0 (Fig. S1C).

Having established that endothelial-lineage-labelled cells accumulate outside the regressing vessel wall, we next asked whether proliferating ECs are redistributed to the extravascular compartment during postnatal hyaloid involution. EdU incorporation combined with ERG immunostaining identified proliferating ECs both within and outside the vasculature and revealed a marked increase in extravascular EdU<sup>+</sup>ERG<sup>+</sup> cells at P8 relative to P0, when EdU<sup>+</sup>ERG<sup>+</sup> cells were largely confined to the vessel wall (Fig. 2D–F). We independently confirmed this result using a second set of complementary EC and proliferation markers (Fig. S1D, E). ERG marks EC nuclei, whereas CD31 labels the EC membrane, allowing more precise positional assignment of ECs relative to the vessel wall (Fig. S1D). In parallel, EdU labels S-phase cells, whereas Ki67 identifies cycling cells more broadly (Fig. S1E). Although these markers are not expected to fully colocalize, their complementary distributions provide independent support for the same conclusion and validate the increase in extravascular proliferating ECs at P8 compared to P0. Consistent with the results obtained using EdU and ERG (Fig. 2D–F), Ki-67 and CD31 co-immunostaining revealed increased displacement of proliferating ECs to the extravascular compartment at P8 compared to P0 (Fig. S2A–C). Together, these data indicate that hyaloid regression is accompanied not only by



EC displacement, but also by proliferative EC redistribution beyond the vascular scaffold.

We next asked whether this remodelling program is restricted to ECs or also involves MCs. Desmin immunostaining combined with EdU incorporation identified proliferating MCs both along the vascular scaffold and outside the

vessel wall (Fig. 2G). 3D reconstruction validated the presence of extravascular EdU<sup>+</sup>Desmin<sup>+</sup> cells at P8 (Fig. 2H). Quantification revealed a significant increase in extravascular proliferating MCs at P8 relative to P0 (Fig. 2I). To exclude marker-dependent bias, we repeated the analysis using Ki67 co-immunostaining with Desmin and a second

MCs marker, CD13. Desmin and CD13 yielded the same overall pattern, identifying proliferating MCs both along the vessel wall and within the extravascular compartment (Fig. S2D–I). 3D and quantification likewise showed a significant increase in extravascular Ki67<sup>+</sup>Desmin<sup>+</sup> (Fig. S2E, F) and Ki67<sup>+</sup>CD13<sup>+</sup> cells at P8 compared with P0 (Fig. S2H, I). These findings indicate that MCs, like ECs, participate in the extravascular proliferative remodelling that accompanies vessel involution.

We next examined whether HCs also contribute to the expanding extravascular proliferative compartment. F4/80 and Ki67 co-immunostaining identified proliferating HCs outside the vascular wall at both P0 and P8, and 3D reconstruction validated the extravascular localization of Ki67<sup>+</sup>F4/80<sup>+</sup> cells (Fig. S2J, K). However, in contrast to ECs and MCs, the number of proliferating HCs remained unchanged over this period (Fig. S2L). Thus, unlike ECs and MCs, HCs are already extravascular at early postnatal stages and do not show evidence of progressive redistribution during hyaloid regression.

Together, these data define a cell-type-specific proliferative remodelling program during postnatal hyaloid vessel involution. ECs and MCs undergo extravascular proliferative shift but HCs remain largely unchanged. These findings demonstrate that ECs and MCs contribute to the expanding extravascular proliferative compartment during hyaloid vessel regression.

### Hyaloid vessel regression proceeds through multicellular remodelling rather than increased apoptosis

To determine whether hyaloid vessel involution is driven by apoptosis of specific vascular cell types, we profiled dissociated hyaloid vessels by flow cytometry using CD31, Annexin V and 7-AAD (Fig. 3A). The total number of recovered 7-AAD<sup>−</sup>CD31<sup>+</sup> ECs did not significantly change from P0 to P8, and CD31 median fluorescence intensity within this population was also unchanged (Fig. 3B, C). Among recovered 7-AAD<sup>−</sup>CD31<sup>+</sup> ECs, the proportion of Annexin V<sup>+</sup> apoptotic cells remained stable over this period (Fig. 3D), indicating that hyaloid regression is not accompanied by a detectable increase in apoptosis within this population. Similarly, apoptotic cells within the 7-AAD<sup>−</sup>CD31<sup>−</sup> compartment, which includes MCs and HCs, did not vary detectably across this period (Fig. 3A, D).

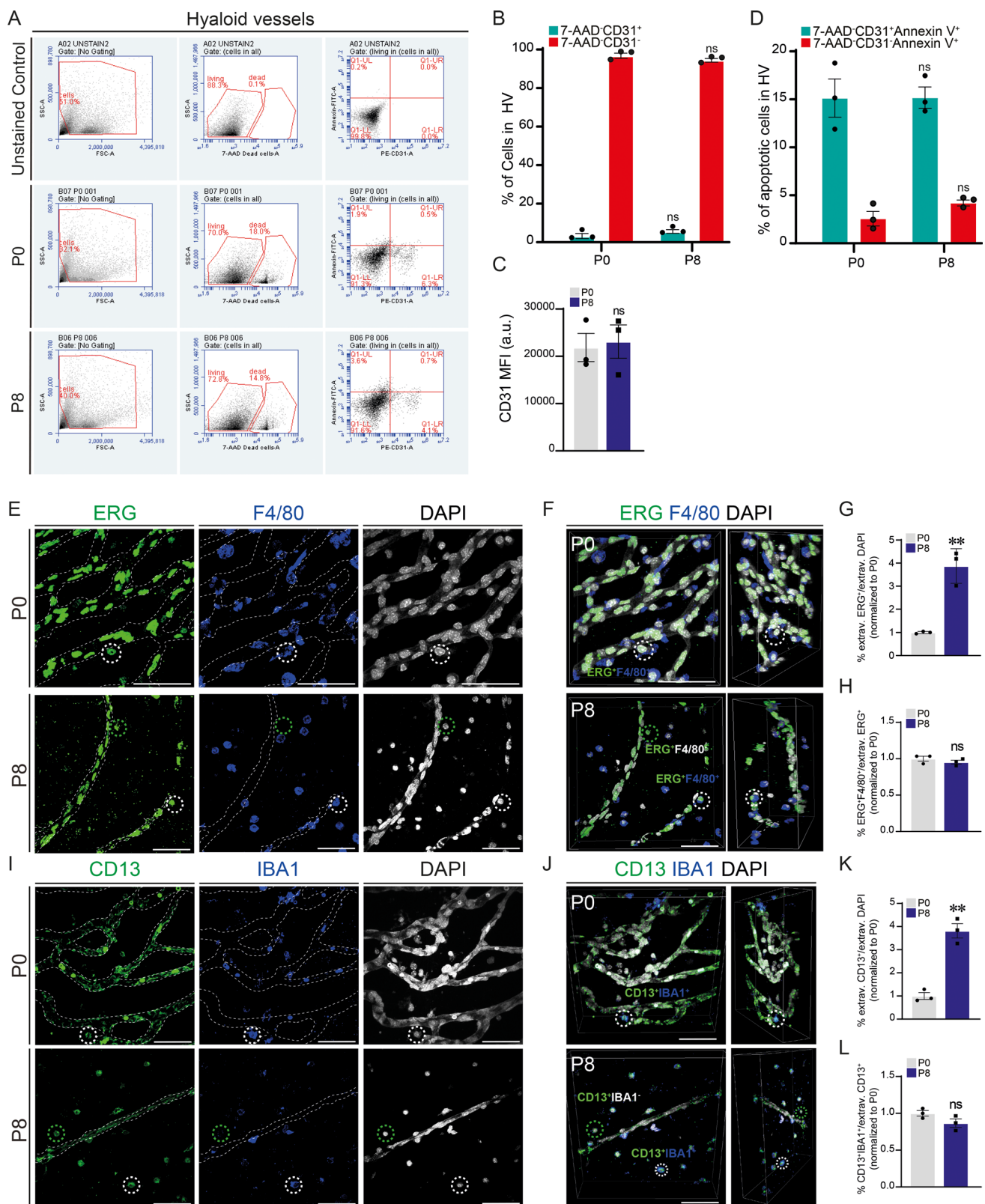
To directly assess apoptosis in defined vascular cell types in situ, we performed hyaloid flatmount co-immunostaining for CD31 and C-Casp3 to identify apoptotic ECs (Fig. S3A). Three-dimensional reconstruction and quantification showed a decrease in C-Casp3<sup>+</sup> ECs in the extravascular compartment (Fig. S3B, C). Consistent with the

**Fig. 3** Phagocytic uptake of ECs and MCs by hyalocytes and steady state apoptosis during hyaloid regression. **A** Flow cytometry (FACS) analysis of ECs and non-ECs apoptosis from HV at P0 and P8. Representative FACS plots show the gating strategy followed. Numerals shown in the plots indicate the percentages of the indicated cell population within each plot. **B** Quantification of total ECs (7-AAD<sup>−</sup>PE-CD31<sup>+</sup>) and non-ECs (7-AAD<sup>−</sup>PE-CD31<sup>−</sup>). **C** CD31 median fluorescence intensity (MFI) and **D** apoptotic (FITC-AnnexinV<sup>+</sup>) cells within each population at P0 and P8 ( $n=3$ ). **E** Representative confocal micrographs and **F** 3D reconstructions of ERG1/2/3, F4/80, and DAPI immunofluorescence in HV at P0 and P8 reveal two EC populations: ERG1/2/3<sup>+</sup> cells positive for the hyalocyte (HC) marker F4/80 (white circles; ERG1/2/3<sup>+</sup>F4/80<sup>+</sup>) and ERG1/2/3<sup>+</sup> cells negative for F4/80 (green circles; ERG1/2/3<sup>+</sup>F4/80<sup>−</sup>). **G** Quantification of extravascular ERG1/2/3<sup>+</sup> cells among total extravascular cells, and **H** extravascular ERG1/2/3<sup>+</sup>F4/80<sup>+</sup> cells, expressed relative to the total number of extravascular ERG1/2/3<sup>+</sup> cells ( $n=3$ ). **I** Representative confocal micrographs and **J** 3D reconstructions of P0 and P8 HV flatmounts labeled for CD13, IBA1, and DAPI reveal two MCs (CD13<sup>+</sup>) populations: MCs co-expressing the HC marker IBA1 (white circles; CD13<sup>+</sup>IBA1<sup>+</sup>) and MCs lacking IBA1 expression (green circles; CD13<sup>+</sup>IBA1<sup>−</sup>). **K** Quantification of extravascular CD13<sup>+</sup> cells among total extravascular cells, and **L** extravascular CD13<sup>+</sup>IBA1<sup>+</sup> cells, expressed relative to the total number of extravascular CD13<sup>+</sup> cells ( $n=3$ ). Results are expressed as percentages (%) (**B**, **D**) and normalized to P0 stage (**G**, **H**, **K**, **L**) or as arbitrary units (a.u.) (**C**). Data are shown as means $\pm$ SEM. Represented  $p$ -values are  $^{**}\leq 0.01$  from two-tailed parametric unpaired  $t$ -test. Non-significant (ns). Scale bars, 50  $\mu$ m

flow cytometry analysis, whole-mount CD31/C-Casp3 co-immunostaining showed that the total number of C-Casp3<sup>+</sup> ECs did not change significantly between P0 and P8 (Fig. S3D). In contrast, immunostaining of the hyaloid flatmount revealed a significant decrease in total CD31<sup>+</sup> cells over the same period (Fig. S3E), in agreement with western blot analysis of whole isolated hyaloid vessel lysates showing reduced CD31 protein abundance from P0 to P8 (Fig. S3F). Together, these findings indicate that hyaloid regression is accompanied by a tissue-level reduction in CD31<sup>+</sup> cells and CD31 protein abundance, without a detectable decrease in CD31 expression or increase in apoptosis within the recovered CD31<sup>+</sup> EC population by flow cytometry.

We next performed co-immunostaining for CD13 and C-Casp3 to identify apoptotic MCs (Fig. S3G). Three-dimensional reconstructions and quantification likewise confirmed the presence of MCs in the extravascular compartment (Fig. S3H, I) and the frequency of C-Casp3<sup>+</sup> MCs remained unchanged between P0 and P8 (Fig. S3J). Thus, during hyaloid regression, ECs and MCs accumulate in the extravascular compartment without a detectable increase of apoptosis; overall apoptotic extravascular ECs and MCs remain low at the analysed stages.

We next asked whether these displaced vascular cells are subsequently cleared by HCs. To distinguish extravascular ECs and MCs from HCs and quantify their spatial association, we performed whole-mount immunostaining using ERG or CD13 together with the HC markers F4/80



(Fig. 3E–H) or IBA1 (Fig. 3I–L) respectively. Consistent with our earlier results, the fraction of extravascular ERG<sup>+</sup> cells increased significantly by P8 (Fig. 3G). However, the

proportion of extravascular ERG<sup>+</sup> cells associated with F4/80<sup>+</sup> HCs did not change (Fig. 3H). Similarly, extravascular CD13<sup>+</sup> cells accumulated during involution (Fig. 3K),

whereas their association with IBA1<sup>+</sup> HCs remained unchanged (Fig. 3L). Together, these findings indicate that postnatal hyaloid regression is not accompanied by a detectable increase in apoptosis or HC-associated clearance. Instead, it is characterized by the progressive displacement of ECs and MCs to the extravascular compartment during the regression process.

### Notch1 promotes an EndoMT-like transcriptional program during hyaloid regression

The accumulation of proliferative ECs and MCs outside the regressing vessel wall at later stages of hyaloid involution suggested that vascular regression may involve not only cell displacement but also changes in endothelial cell state. We therefore asked whether this remodeling process is associated with an endothelial-to-mesenchymal transition (EndoMT)-like program.

This transition could facilitate EC displacement by coupling loss of endothelial identity to acquisition of mesenchymal features. qRT-PCR analysis of whole hyaloid lysates revealed marked downregulation of the endothelial markers *Vegfr2*, *Pecam1*, *Vwf* and *Tie2* between P0 and P8 (Fig. 4A). This was accompanied by induction of the mesenchymal regulators *Snail1*, *Slug* and *Acta2/α-Sma*, whereas *Twist1* remained unchanged (Fig. 4B).

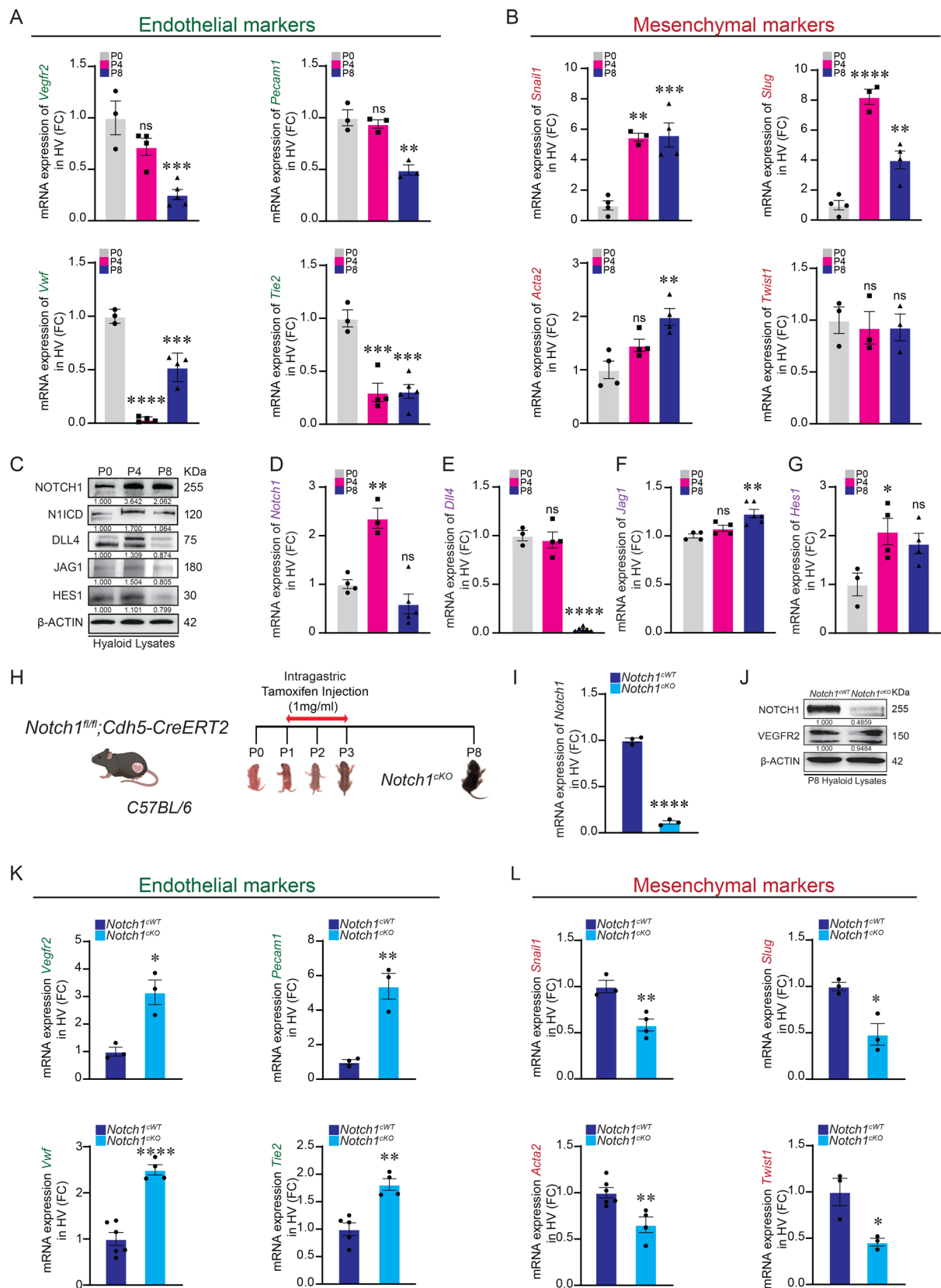
Although these measurements were obtained from whole hyaloid lysates and therefore do not assign these transcriptional changes to a specific cell type, the overall expression profile is consistent with selective engagement of an EndoMT-like remodeling program during hyaloid regression. The induction of *Snail1* and *Slug* in the absence of *Twist1* upregulation further supports a plastic, early transition state rather than a fully stabilized mesenchymal program [25–28].

Prompted by these observations, we next investigated upstream pathways that might regulate endothelial remodeling during hyaloid regression. Notch1 was of particular interest given its established roles in vascular stabilization and EndoMT [29–31]. However, whether Notch1 contributes to hyaloid vessel regression has not, to our knowledge, been previously addressed. We therefore examined the temporal expression of Notch1, its ligands and canonical downstream targets across the regression window from P0 to P8. Western blot analysis of isolated hyaloid vessels revealed a progressive increase in total NOTCH1 protein from P0 to P8, with a peak at P4. The cleaved active form, NICD, was likewise maximal at P4 (Fig. 4C). DLL4 and JAG1 displayed similar temporal dynamics at the protein level, with transient upregulation at P4 before returning towards baseline by P8. HES1 was also elevated at P4, indicating a transient increase in Notch signalling activity at this stage

**Fig. 4** Notch1 drives endothelial identity loss and plasticity-associated gene expression during hyaloid regression. **A** Quantitative reverse transcription polymerase chain reaction (qRT-PCR) shows downregulation of endothelial cell (EC) identity markers *Vegfr2*, *Pecam1*, *Vwf* and *Tie2* during hyaloid regression. **B** qRT-PCR analysis shows induction of endothelial mesenchymal transition (EndoMT)-associated transcription factors: *Snail1*, *Slug* and *Acta2* in hyaloid from P0 to P8. *Twist* expression remains unchanged. **C** Western blot analysis of hyaloid cell lysates at P0, P4 and P8 showing expression of Notch1, its active form (Notch1 intracellular domain, NICD), the ligands DLL4 and JAG-1 and the downstream target gene HES1 ( $n=2-3$ ). Densitometric quantification of each protein is shown relative to the loading control,  $\beta$ -ACTIN. **D** qRT-PCR analysis shows the levels of *Notch1*, *EDil4*, *FJag1* and *GHes1* transcripts in HV at P0, P4, and P8. **H** Schematic diagram of tamoxifen-induced Notch1 deletion in ECs in *Notch1<sup>fl/fl</sup>; Cdh5-CreERT2 C57BL/6* transgenic mice. **I** Validation of *Notch1* deletion by qRT-PCR in *Notch1<sup>ckO</sup>* versus *Notch1<sup>cWT</sup>* P8 HV. **J** Western blot analysis of P8 hyaloid cell lysates from *Notch1<sup>cWT</sup>* and *Notch1<sup>ckO</sup>* shows the protein expression of Notch1 and VEGFR2 ( $n=2-3$ ). Densitometric quantification of each protein is shown relative to the loading control,  $\beta$ -ACTIN. **K** qRT-PCR shows upregulation of EC identity markers *Vegfr2*, *Pecam1*, *Vwf* and *Tie2* in *Notch1<sup>ckO</sup>* versus *Notch1<sup>cWT</sup>* P8 HV. **L** qRT-PCR analysis shows reduction of EndoMT-associated transcription factors *Snail1*, *Slug*, *Acta2* and *Twist* in *Notch1<sup>ckO</sup>* P8 HV versus *Notch1<sup>cWT</sup>*.  $\beta$ -actin was used as a reference gene. Results are presented as fold change (FC) normalize to P0 stage (A, B, D–G) or P8 *Notch1<sup>cWT</sup>* (I, K, L). Data are shown as means  $\pm$  SEM. Represented  $p$  values are \* $\leq 0.05$ , \*\* $\leq 0.01$ , \*\*\* $\leq 0.001$ , \*\*\*\* $\leq 0.0001$  from ordinary one-way ANOVA test with Dunnett's multiple comparison (A, B, D, E, G), Kruskal-Wallis test with Dunn's multiple comparisons (F) or two-tailed parametric unpaired t-test (I, K, L).  $n=3-5$  for *Vegfr2* and *Tie2*;  $n=3$  for *Pecam1* and *Twist*;  $n=3-4$  for *Vwf*, *Snail1* and *Slug*;  $n=4$  for *Acta2* (A, B);  $n=3-4$  for *Notch1* D and *Hes1* (G);  $n=3-6$  for *Dil4* (E);  $n=4-6$  *Jag1* (F);  $n=3$  for *Notch1* (I);  $n=3$  for *Vegfr2* and *Pecam1*;  $n=4-6$  for *Vwf*;  $n=4-5$  for *Tie2*;  $n=3-4$  for *Snail1*;  $n=3$  for *Slug* and *Twist1*;  $n=4-6$  for *Acta2* (K, L). Non-significant (ns)

(Fig. 4C). Consistent with these protein changes, qRT-PCR analysis showed a transient increase in *Notch1* mRNA at P4 that declined to baseline by P8 (Fig. 4D). *Dil4* transcripts also decreased by P8, whereas *Jag1* expression was significantly increased at this stage, indicating divergent ligand regulation during hyaloid regression (Fig. 4E, F). Transcriptional profiling of canonical Notch1 targets further revealed upregulation of *Hes1* at P4 (Fig. 4G), consistent with ongoing Notch1 pathway activity during involution.

To study the role of Notch1 in EndoMT and regression we used a tamoxifen-inducible, EC-specific Notch1 knockout mouse model (*Notch1<sup>fl/fl</sup>; Cdh5-CreERT2*) identified as *Notch1<sup>ckO</sup>*. This mouse model allows for temporally controlled deletion of Notch1 specifically in vascular ECs upon administration of tamoxifen. To target the period of active hyaloid vessels remodeling, tamoxifen was administered via intragastric injection to neonatal pups from P1 to P3, and hyaloid vessels were collected at P8 as demonstrated in the model (Fig. 4H). Efficient deletion was confirmed by the near-complete loss of *Notch1* mRNA in *Notch1<sup>ckO</sup>* hyaloids relative to tamoxifen-treated Cre-negative littermate controls (*Notch1<sup>cWT</sup>*) (Fig. 4I), accompanied by NOTCH1



protein levels at P8 (Fig. 4J). To assess whether the loss of endothelial identity was similarly evident at the protein level, we next analysed VEGFR2. Although *Vegfr2* mRNA was significantly increased in *Notch1<sup>ckO</sup>* hyaloids, VEGFR2 protein was not detectably upregulated at P8 (Fig. 4J, K), indicating that the effect of endothelial Notch1 deletion on endothelial marker VEGFR2 recovery is more evident at the transcriptional than the protein level at this stage. This model provides a robust platform to dissect the endothelial-specific function of Notch1 in postnatal vascular regression without confounding developmental lethality.

Consistent with this, mRNA profiling of P8 hyaloid lysates showed that endothelial Notch1 deletion increased the expression of the endothelial markers *Vegfr2*, *Pecam1*, *Vwf* and *Tie2* in *Notch1<sup>ckO</sup>* hyaloids relative to *Notch1<sup>cWT</sup>* controls (Fig. 4K). In parallel, the mesenchymal regulators *Snail1*, *Slug*, *Acta2* and *Twist1* were significantly reduced (Fig. 4L). Together, these data are consistent with a role for endothelial Notch1 in promoting the EndoMT-like transcriptional changes associated with physiological hyaloid regression, including the attenuation of endothelial identity marker expression during involution.

### Endothelial Notch1 is required for hyaloid regression and postnatal retinal vascular development

Having established that endothelial Notch1 promotes an EndoMT-like transcriptional program during hyaloid regression (Fig. 4), we next asked whether Notch1 is functionally required for hyaloid vessel involution in vivo. In striking contrast to the regressed hyaloid network observed in P8 *Notch1<sup>cWT</sup>* controls, IB4 staining of flatmounted hyaloids revealed marked vascular persistence in *Notch1<sup>ckO</sup>* littermates (Fig. 5A). Quantitative morphometric analysis confirmed a significant increase in vessel density, branching complexity and vessel diameter in *Notch1<sup>ckO</sup>* hyaloids relative to controls (Fig. 5B–D). These findings identify endothelial Notch1 as a key driver of physiological hyaloid vessel regression.

Because endothelial Notch1 is already known to control postnatal retinal vascular development, we analyzed the retinal vasculature in parallel with the hyaloid phenotype [32–34]. IB4 staining of flatmounted P8 retinas revealed a marked delay in formation of the superficial vascular layer in *Notch1<sup>ckO</sup>* mice relative to *Notch1<sup>cWT</sup>* controls (Fig. 5E). Quantitative morphometric analysis confirmed a marked reduction in retinal vascular outgrowth in *Notch1<sup>ckO</sup>* retinas, with an approximately 70% decrease in vascular area and a 50% decrease in branching relative to controls (Fig. 5F, G). Consistent with this early defect, 3D reconstruction of IB4-stained retinas showed that *Notch1<sup>ckO</sup>* mice failed

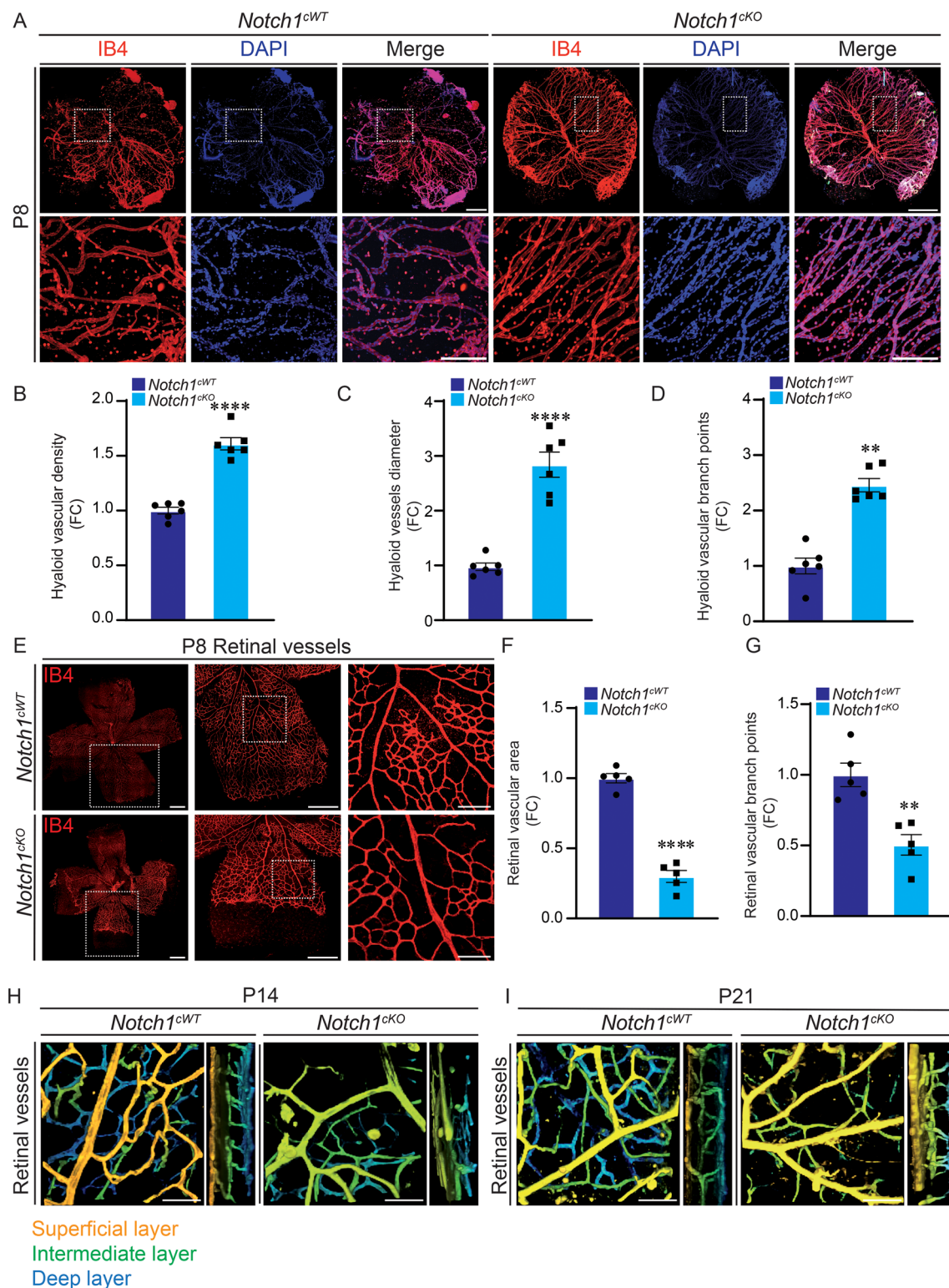
**Fig. 5** Endothelial-specific Notch1 deletion in vivo disrupts hyaloid vascular regression and retards retinal vascular superficial layer formation **A** Representative confocal micrographs of P8 HV from *Notch1<sup>ckO</sup>* and *Notch1<sup>cWT</sup>* mice immunostained with IB4 and DAPI. **B** Quantification of hyaloid vascular density, **C** vessels diameter, and **D** hyaloid vascular branch points of confocal microscopy data in **(A)** ( $n=6$ ). **E** Representative IB4 staining and **F** quantification of retinal vascular area, and **G** vascular branch points in P8 flatmounted retinal vasculature in *Notch1<sup>ckO</sup>* versus *Notch1<sup>cWT</sup>* ( $n=5$ ). **H**, **I** 3D reconstructions of P14 and P21 retinal vasculature labeled with IB4 show three vascular layers [first superficial layer (yellow), second deep layer (blue) and third intermediate layer (green)] in *Notch1<sup>ckO</sup>* versus *Notch1<sup>cWT</sup>* ( $n=3$ ). Results are presented as fold change (FC) relative to *Notch1<sup>cWT</sup>* control HV. Data are shown as means  $\pm$  SEM. Represented  $p$ -values are  $***\leq 0.01$ ,  $****\leq 0.0001$  from two-tailed parametric unpaired t-test (**B**, **C**, **F**, **G**) or Mann-Whitney test (**D**). Scale bar, 500  $\mu$ m (**A**, **E**) and 200  $\mu$ m [for higher magnification images in (**A**)] and 100  $\mu$ m [for higher magnification images in (**E**)], and 50  $\mu$ m (**H**, **I**),

to fully establish the deep vascular plexus at P14 and P21, in contrast to *Notch1<sup>cWT</sup>* littermates (Fig. 5H, I). Together, these data validate the requirement for endothelial Notch1 in postnatal retinal vascular development and identify a previously unrecognized role for Notch1 in fetal hyaloid clearance, with endothelial Notch1 loss leading to persistent fetal vasculature.

### Loss of endothelial Notch1 impairs endothelial and mural cell redistribution during hyaloid regression

Having established a requirement for endothelial Notch1 in hyaloid vessel regression, we next sought the cellular basis of the persistence phenotype in *Notch1<sup>ckO</sup>* mice. To determine the identity and localization of proliferating cells, we analysed ERG and EdU whole mounts labeling by 3D reconstruction. *Notch1<sup>cWT</sup>* hyaloids displayed a clear population of extravascular ERG<sup>+</sup> ECs, whereas in *Notch1<sup>ckO</sup>* hyaloids ERG<sup>+</sup> cells remained largely restricted to the vascular scaffold (Fig. 6A, B). Quantification confirmed a near-complete loss of extravascular ERG<sup>+</sup> cells in *Notch1<sup>ckO</sup>* hyaloids (Fig. 6C). In parallel, the proportion of total proliferating ECs (EdU<sup>+</sup>ERG<sup>+</sup>) was significantly increased in *Notch1<sup>ckO</sup>* hyaloids relative to controls (Fig. 6D). Together, these findings indicate that endothelial Notch1 deletion prevents EC displacement from the regressing vessel wall while retaining proliferating ECs within the persistent vasculature.

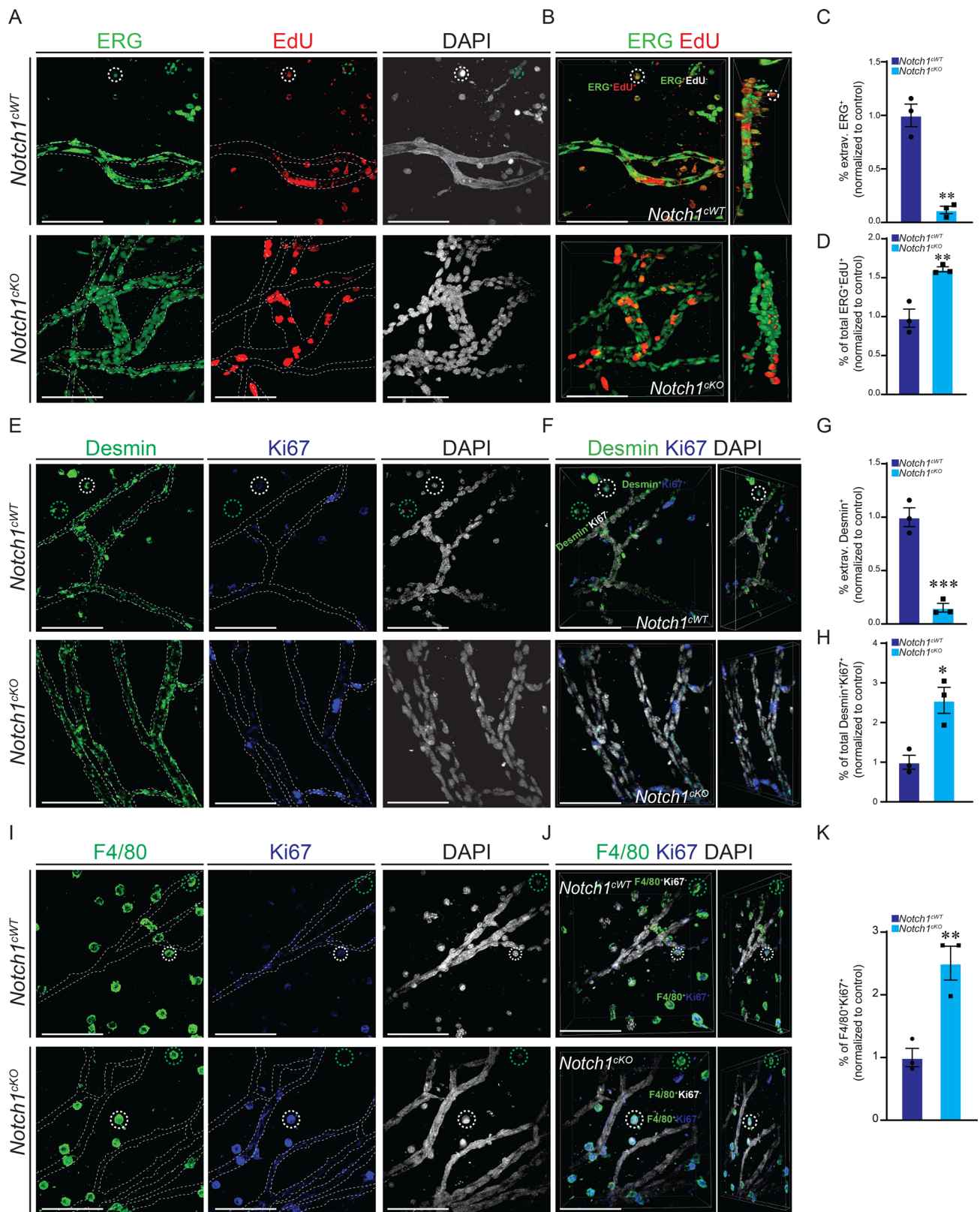
We then examined whether this defect extends to MCs. Desmin and Ki67 co-staining showed that, in *Notch1<sup>cWT</sup>* hyaloids, proliferating MCs were distributed both along the vessel wall and within the extravascular compartment. By contrast, in *Notch1<sup>ckO</sup>* hyaloids, most Ki67<sup>+</sup>Desmin<sup>+</sup> MCs remained closely associated with the vascular scaffold (Fig. 6E). 3D reconstruction and quantification revealed a significant increase in total MC number together with a reduction in extravascular MC redistribution in the absence of Notch1 (Fig. 6G, H). These data indicate that endothelial



Notch1 deletion disrupts not only EC displacement, but also the normal redistribution of MCs during hyaloid regression.

Co-immunostaining for F4/80 and Ki67 revealed increased HC proliferation in *Notch1<sup>cKO</sup>* hyaloids compared with *Notch1<sup>cWT</sup>* controls at P8, as confirmed by 3D

reconstruction and quantification of F4/80<sup>+</sup>Ki67<sup>+</sup> cells (Fig. 6I–K). Notably, these HCs remained extravascular but closely apposed to the persistent vessels, resembling the early postnatal configuration observed before the onset of regression. Together, these data suggest that, in the absence



**Fig. 6** Loss of endothelial Notch1 alters hyaloid endothelial and mural cells positioning and proliferation. **A** Representative confocal micrographs of P8 HV flatmounts from *Notch1<sup>cWT</sup>* and *Notch1<sup>cKO</sup>* mice showing EdU incorporation in ERG1/2/3<sup>+</sup> cells. Nuclei are labeled with DAPI. **B** 3D reconstructions show ERG1/2/3<sup>+</sup>EdU<sup>+</sup> cells (white circle) and ERG1/2/3<sup>+</sup>EdU<sup>-</sup> cells (green circle) in P8 HV from *Notch1<sup>cWT</sup>* and *Notch1<sup>cKO</sup>* mice ( $n=3$ ). **C** Quantification of extravascular ERG1/2/3<sup>+</sup> among total extravascular cells and **D** ERG1/2/3<sup>+</sup>EdU<sup>+</sup> cells, expressed relative to total ERG1/2/3<sup>+</sup> in P8 HV in *Notch1<sup>cKO</sup>* versus *Notch1<sup>cWT</sup>* ( $n=3$ ). **E** Representative confocal micrographs and **F** 3D reconstructions of Desmin, Ki67 and DAPI immunofluorescence in P8 from *Notch1<sup>cWT</sup>* and *Notch1<sup>cKO</sup>* mice, showing distinction between proliferating MCs (Desmin<sup>+</sup>Ki67<sup>+</sup>) (white circle) and non-proliferating MCs (Desmin<sup>+</sup>Ki67<sup>-</sup>) (green circle). **G** Quantification of extravascular Desmin<sup>+</sup> among total extravascular cells and **H** Desmin<sup>+</sup>Ki67<sup>+</sup>, expressed relative to total Desmin<sup>+</sup> of confocal microscopy data in **(E)** ( $n=3$ ). **I** Representative confocal micrographs and **J** 3D reconstructions of F4/80, Ki67 and DAPI immunofluorescence in P8 from *Notch1<sup>cWT</sup>* and *Notch1<sup>cKO</sup>* mice, showing distinction between proliferating HCs (F4/80<sup>+</sup>Ki67<sup>+</sup>) (white circle) and non-proliferating HCs (F4/80<sup>+</sup>Ki67<sup>-</sup>) (green circle). **K** Quantification of extravascular F4/80<sup>+</sup>Ki67<sup>+</sup> of confocal microscopy data in **(I)** ( $n=3$ ). Results are expressed as percentages normalized to *Notch1<sup>cWT</sup>* control HV. Data are shown as means  $\pm$  SEM. Represented  $p$ -values are \* $\leq 0.05$ , \*\* $\leq 0.01$  and \*\*\* $\leq 0.001$  from two-tailed parametric unpaired T-test. Scale bars, 50  $\mu$ m

of endothelial Notch1, the hyaloid vasculature fails to progress into the regression program and instead retains immature pre-regression features.

We further asked whether the persistent hyaloid vasculature in *Notch1<sup>cKO</sup>* mice results from altered apoptosis. Cleaved caspase-3 staining revealed comparable numbers of apoptotic cells in *Notch1<sup>cKO</sup>* and *Notch1<sup>cWT</sup>* hyaloids at P8 (Fig. S4A, B), excluding defective apoptotic cell loss as the primary cause of persistence. Instead, Collagen IV and CD31 co-staining showed that, whereas control hyaloids contained Collagen IV<sup>+</sup>CD31<sup>-</sup> empty sleeves and extravascular CD31<sup>+</sup> ECs, *Notch1<sup>cKO</sup>* hyaloids retained CD31<sup>+</sup> ECs within Collagen IV<sup>+</sup> vascular tracks and formed fewer empty sleeves (Fig. S4C, D), consistent with impaired vascular disengagement.

Together, these findings show that endothelial Notch1 is required for the remodelling events that drive hyaloid regression. In its absence, ECs and MCs fail to disengage from the vessel wall, collagen sleeve formation is reduced without a change in apoptosis, and the hyaloid network persists in an immature, pre-regression configuration.

### Notch1 and Wnt pathway mutants converge on defective endothelial and mural cell delamination during hyaloid regression

Given the established role of Wnt signaling in hyaloid vessel regression [13, 14, 35, 36], we next asked whether endothelial Notch1 loss alters the expression of core components of this pathway. As essential co-receptors for canonical Wnt signaling, LRP5 and LRP6 are key determinants of cellular

responsiveness to Wnt ligands. qRT-PCR analysis revealed a marked reduction in both *Lrp5* and *Lrp6* expression in *Notch1<sup>cKO</sup>* hyaloid lysates relative to littermate controls (Fig. 7A, B), suggesting that Notch1 activity helps sustain Wnt signaling competence during regression. To further examine this relationship in an independent genetic context, we re-analysed publicly available single-cell RNA-sequencing datasets from P3 *Fz5<sup>-/-</sup>* mice, which exhibit persistent hyaloid vasculature [37]. Loss of *Fz5* was associated with suppression of Notch1-related signaling, supporting reciprocal coupling between Wnt and Notch pathway activity during hyaloid regression (Fig. 7C, D). To determine whether this interdependence extends more broadly across the Norrin/Wnt axis, we next examined *Ndp*-deficient mice, a well-established model of failed hyaloid regression [15, 35, 36]. As expected, *Ndp<sup>-/-</sup>* mice retained a dense hyaloid network at P8, which we confirmed by whole-mount imaging and quantification of vascular density (Fig. 7E, F).

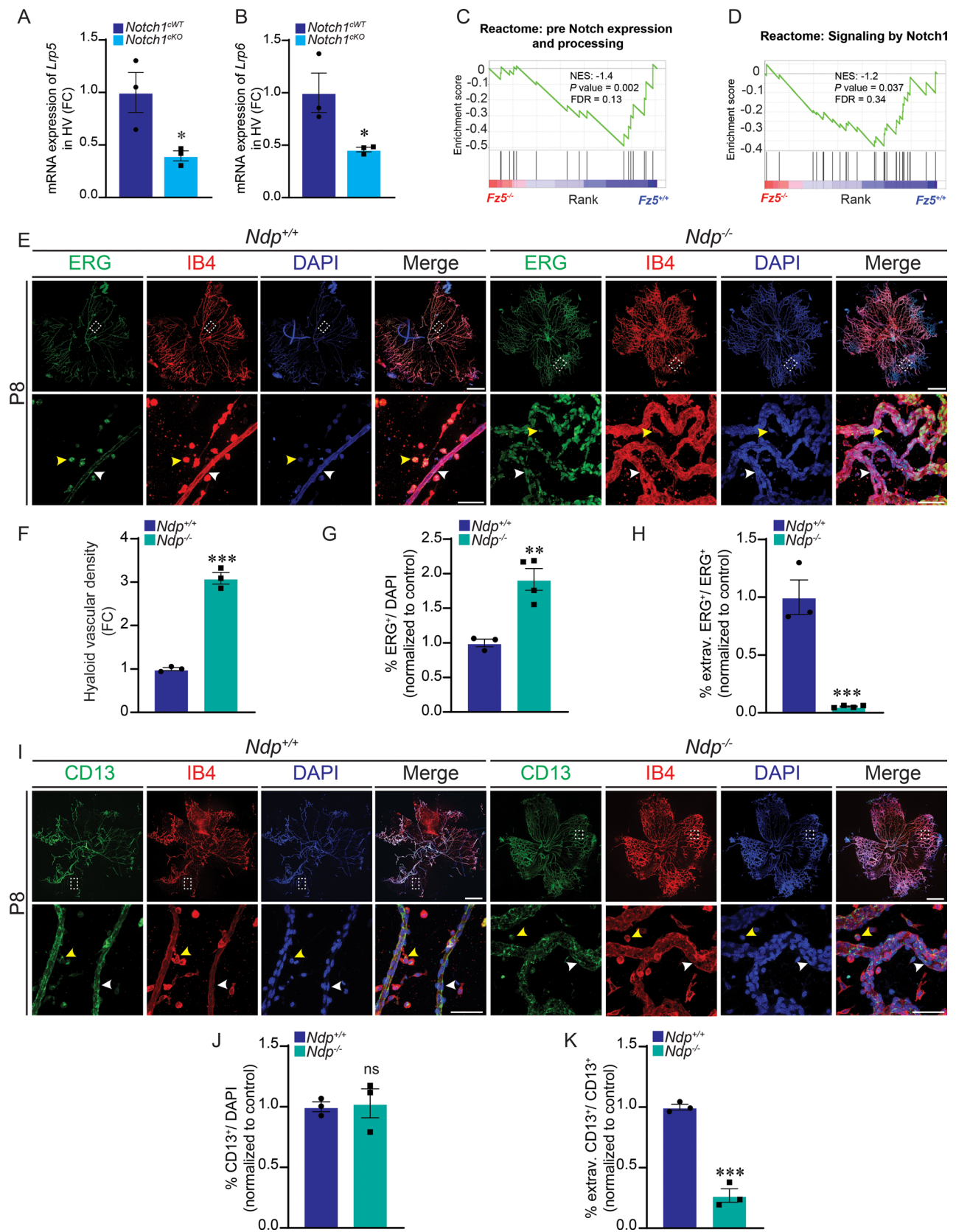
We then examined EC localization by ERG and IB4 co-staining. Strikingly, in *Ndp<sup>-/-</sup>* hyaloids, ERG<sup>+</sup> cells remained largely confined to IB4<sup>+</sup> vascular structures, with an almost complete loss of extravascular ECs, closely phenocopying the defect observed in *Notch1<sup>cKO</sup>* hyaloids (Fig. 7E). Quantification showed an increase in total ERG<sup>+</sup> cell number together with a marked reduction in extravascular ERG<sup>+</sup> cells, consistent with impaired EC delamination (Fig. 7G, H).

Similarly, CD13 and IB4 co-staining showed that, whereas extravascular CD13<sup>+</sup> cells were readily detected in controls, CD13<sup>+</sup> cells in *Ndp<sup>-/-</sup>* hyaloids remained closely associated with the persistent vascular scaffold (Fig. 7I–K). Although total CD13<sup>+</sup> cell number was unchanged, extravascular CD13<sup>+</sup> cells were markedly reduced (Fig. 7J, K), indicating that impaired EC and MC redistribution is a shared feature of persistent hyaloid vessels in both *Notch1*- and *Ndp*-deficient mice.

Together, these findings identify impaired EC and MC displacement from the regressing vessel wall as a shared feature of Notch- and Wnt-pathway mutants. Although the molecular hierarchy between these pathways remains unresolved, the data support a model in which coordinated and functional interdependence of Wnt-Notch signalling drives EC and MC delamination during physiological hyaloid vessel regression.

## Discussion

The prevailing view of hyaloid vessel regression has emphasized apoptosis and HC-driven clearance as the dominant mechanisms of involution [10, 13, 38]. Our data refine that model. Across the early postnatal regression window, we find no measurable increase in total or cell-type-specific apoptosis, nor evidence for increased association



**Fig. 7** Notch1-Wnt transcriptional coupling orchestrates endothelial and mural plasticity during hyaloid vessel regression. **A** qRT-PCR analysis shows transcripts expression of *Lrp5* and *Blrp6* in P8 *Notch1<sup>CKO</sup>* versus *Notch1<sup>WT</sup>* HV. **C, D** Gene Set Enrichment Analysis (GSEA) plots showing normalized enrichment scores (NES), False Discovery Rate (FDR) and *P*-value for Notch 1-associated Reactome pathway analysis of EC cluster scRNA-seq data. Genes were ranked based on differential expression between postnatal day (P3) *Fz5<sup>-/-</sup>* and *Fz5<sup>+/+</sup>* control. In *Fz5<sup>-/-</sup>*, pathways associated with pre-Notch expression and processing, and Notch1 signaling are downregulated relative to *Fz5<sup>+/+</sup>* controls. **E** Representative confocal micrographs showing ERG1/2/3, IB4 and DAPI immunofluorescence of P8 HV from *Ndp<sup>-/-</sup>* and *Ndp<sup>+/+</sup>* mice. **F** Quantification of HV density, **G** total and **H** extravascular ERG1/2/3<sup>+</sup> cells of confocal microscopy data in (**E**) (*n*=3–4). **I** Representative confocal micrographs of P8 HV flatmounts from *Ndp<sup>-/-</sup>* and *Ndp<sup>+/+</sup>* mice immunostained with CD13, IB4 and DAPI. **J** Quantification of total and **K** extravascular CD13<sup>+</sup> cells of confocal microscopy data in (**I**) (*n*=3). White arrowheads indicate ERG1/2/3<sup>+</sup> or CD13<sup>+</sup> cells within the vasculature, and yellow arrowheads indicate extravascular ERG1/2/3<sup>+</sup> or CD13<sup>+</sup> cells. Results are expressed as fold change (FC) relative to *Notch1<sup>WT</sup>* (**A, B, F**) and as percentages (%) normalized to *Ndp<sup>+/+</sup>* (**G, H, J, K**) control HV. Data are means ± SEM. Represented *p*-values are \*≤ 0.05, \*\*≤ 0.01 and \*\*\*≤ 0.001 from two-tailed parametric unpaired t-test. Non-significant (ns). Scale bars, 500 μm and 50 μm [for higher-magnification images in (**E, I**)]

of displaced vascular cells with HCs. Instead, regression is accompanied by the progressive accumulation of viable extravascular ECs and MCs, together with reduced vessel density, narrowing of vessel caliber and loss of branching complexity. These findings suggest that postnatal hyaloid regression is not driven solely by apoptotic elimination but may also involve multicellular remodeling associated with the redistribution of vascular cells away from the regressing scaffold. This interpretation is consistent with broader concepts of vessel pruning, in which vascular cells rearrangement, displacement and selective destabilization can contribute alongside cell death [39].

This distinction is important because it places the postnatal hyaloid system in a different mechanistic frame from the classical HC-WNT7b-apoptosis axis. HCs are required for ocular vascular remodeling, and WNT7b signaling through FZD4/LRP5/LRP6 has been firmly linked to hyaloid regression and endothelial cell apoptosis [16, 36]. Likewise, BIM-dependent apoptosis contributes to physiological hyaloid involution [40]. Our findings do not contradict those studies; rather, they suggest that apoptosis is not the dominant readout during the postnatal phase examined here. One plausible interpretation is that HCs and Wnt signals act permissively or instructively upstream of vascular destabilization, whereas the immediate cellular execution of postnatal regression includes EC and MC disengagement from the vessel wall. In that sense, HC function during regression may be more paracrine than phagocytic over the P0-P8 interval analysed here.

A central advance of this study is the identification of a cell-type-specific extravascular remodeling. Endothelial

lineage tracing, complementary EC markers, and the appearance of collagen IV-positive empty sleeves together indicate that ECs leave the regressing vascular scaffold rather than simply disappearing in situ. MCs show a parallel redistribution, whereas HCs do not exhibit the same progressive proliferative shift. This hierarchy argues that ECs expand in the extravascular compartment, with MCs following the destabilizing vascular scaffold. More broadly, these observations align hyaloid involution with emerging models of vascular regression in which cell rearrangement and physical disengagement can precede or substitute for overt apoptotic pruning.

Our data further suggest that this endothelial redistribution is linked to an EndoMT-like state. During regression, hyaloid lysates lose endothelial markers while inducing *Snail1*, *Slug* and *Acta2*, yet *Twist1* remains unchanged. In the EndoMT literature, this type of transcriptional profile is generally associated with a more plastic and potentially reversible endothelial transition rather than a fully stabilized mesenchymal conversion [41–43]. That interpretation fits well with our imaging data: ECs become extravascular, but the system remains developmental and transient rather than fibrotic or pathological. At the same time, our RNA measurements were performed on whole hyaloid lysates, so the present data support an EndoMT-like transcriptional program at the tissue level, rather than proving that every displaced EC undergoes a full lineage transition. Resolving the precise extent and reversibility of this state will require single-cell or spatial approaches.

Within this framework, our results identify endothelial Notch1 as a key regulator of physiological hyaloid involution. Notch signaling is well established as a context-dependent controller of endothelial quiescence, branching behavior and vascular maturation, including in the postnatal retina [17, 44, 45]. Here, however, Notch1 may have an additional role in a transient fetal vascular bed, where its loss is associated with altered transcriptional and cellular changes during EC and MC disengagement from the regressing vessel wall. Endothelial Notch1 deletion preserves endothelial marker expression, suppresses mesenchymal regulators, abolishes the extravascular EC and MC pool, reduces collagen sleeve formation and causes marked hyaloid persistence without altering basal apoptosis. Thus, in the hyaloid vascular bed, Notch1 does not simply restrain angiogenesis; it appears to enable the transition from a stable vascular tube to a remodeling, regression-competent state.

The phenotype of the endothelial *Notch1<sup>CKO</sup>* mice also highlights a broader developmental principle: hyaloid regression and retinal vascular growth are coordinated, but mechanistically distinct, arms of postnatal ocular vascular remodeling. The delayed superficial plexus expansion and defective deep plexus formation in mutant retinas are

consistent with established roles for endothelial Notch1 in retinal vascular development [17]. What our study adds is that the same endothelial pathway is also required to clear the transient fetal vasculature. In this sense, Notch1 couples two complementary developmental tasks: promoting orderly retinal vascularization while enabling removal of the fetal hyaloid network. Failure of this coordination offers a conceptual bridge between persistent hyaloid phenotypes and broader congenital vitreoretinopathies in which fetal vessel clearance and retinal vascular maturation are both disrupted.

Our final set of experiments places Notch1 within a larger signaling architecture that includes the Wnt pathway, long known to regulate hyaloid regression [13, 15, 37]. Endothelial Notch1 loss reduced expression of *Lrp5* and *Lrp6*, whereas re-analysis of *Fz5*-mutant single-cell datasets indicated suppression of Notch-related signaling. Moreover, *Ndp*-deficient hyaloids phenocopied the Notch1 mutant with respect to the near-complete loss of extravascular ECs and MCs. Together, these findings argue that defective EC and MC displacement in the extravascular compartment is a shared endpoint of both Notch and Wnt pathway disruption. Whether this reflects direct transcriptional cross-regulation, parallel convergence on a common EC and MC states, or a staged signaling hierarchy remains unresolved. Nonetheless, interaction between Wnt/ $\beta$ -catenin and Notch signaling has been described in other vascular contexts, including regulation of *Dll4* and endothelial remodeling, making such coupling mechanistically plausible in the hyaloid vascular bed as well [46].

**Limitations of the study.** First, the EndoMT-like signature was inferred from bulk hyaloid lysates and therefore cannot yet assign transcriptional changes to specific cell types with single-cell resolution. Second, our study focuses on the early postnatal window and does not exclude a temporally restricted contribution of apoptosis at earlier embryonic stages or later phases of involution. Third, the stable association of HCs with persistent vessels in Notch1-deficient eyes suggests that HC-lineage cells may still shape regression indirectly, even if increased HC-associated clearance is not the dominant fate of displaced vascular cells during the window examined here. Future live imaging, lineage-resolved transcriptional profiling and cell type-specific perturbation of Wnt-Notch nodes should define the order of these events more precisely.

In summary, our study supports an apoptosis-non-exclusive model of hyaloid vessel regression in which endothelial Notch1 drives a multicellular remodeling centered on EC displacement, partial EndoMT-like plasticity and coordinated disengagement of ECs and MCs from the regressing scaffold. By linking Notch1 to both hyaloid clearance and Wnt-associated regression pathways, these findings

expand the conceptual framework of developmental vessel pruning and suggest that pathological persistence of fetal ocular vessels may arise not only from failed cell death, but also from failed endothelial state transitions. This revised view may be relevant to persistent fetal vasculature and related pediatric vitreoretinopathies in which regression and retinal vascular maturation fail in parallel.

## Materials and methods

### Murine model

The manipulations carried out on mice as part of this project were approved by the Institutional Animal Protection Committee (CIPA) of the University of Quebec in Montreal (UQAM) accredited by the Canadian Council on Animal Care (CCAC). For inducible endothelial lineage-tracing model, *Rosa26-mT/mG* and *Cdh5-CreERT2* mice were crossed to obtain (*Rosa26-mT/mG*; *Cdh5-CreERT2*). For inducible endothelial deletion of *Notch1*, *Notch1<sup>fl/fl</sup>* and *Cdh5-CreERT2* mice were crossed to obtain (*Notch1<sup>fl/fl</sup>*; *Cdh5-CreERT2*) mice.

*Notch1<sup>fl/fl</sup>* mice were kindly provided by Przemyslaw Sapieha and Dr Nathalie Labrecque while *Cdh5-CreERT2* and *Rosa26-mT/mG* mice by Bruno Larrivée. Mice strains were of *C57BL/6J* genetic background. Genotyping was performed by polymerase chain reaction (PCR) analysis of genomic DNA extracted from ear punches. Mice with the correct genotype were selected for subsequent experiments.

*C57BL/6J* wild-type (*WT*) and *Ndp* knock-out (*Ndp<sup>-/-</sup>*) (stock # 012287) mice were from Jackson Laboratory.

### Tamoxifen injection of mice

To activate Cre recombinase, tamoxifen (4-hydroxy-tamoxifen; Sigma-Aldrich, T5648) was dissolved in corn oil (Sigma, C8267) at 1 mg/ml. *Rosa26-mT/mG*; *Cdh5-CreERT2* and *Notch1<sup>fl/fl</sup>*; *Cdh5-CreERT2* mice received 50  $\mu$ l tamoxifen by intragastric injection at the milk spot from postnatal day 1 (P1) to postnatal day 3 (P3). Endothelial-specific Notch1-deficient mice are referred to as *Notch1<sup>cKO</sup>*, and tamoxifen-treated Cre-negative littermates were used as controls (*Notch1<sup>cWT</sup>*).

### Gene set enrichment analyses (GSEA)

Gene set enrichment analyses (GSEA) were conducted using GSEA v4.0.1 software provided by Broad Institute of Massachusetts Institute of Technology and Harvard

University. We used GSEA to validate correlation between molecular signatures in phenotypes of interest. Publicly available single-cell RNA-seq data from Chen et al., IOVS 2023 [37] were reanalyzed following the pipeline described in the Methods. Differential gene expression analysis was conducted using the FindMarkers function in Seurat to compare P3 *Fz5*<sup>-/-</sup> mutant versus P3 *Fz5*<sup>+/+</sup> control mice. The resulting ranked gene list was used as input for subsequent GSEA. Pathway-based gene sets were obtained from Reactome for enrichment analysis.

### Flow cytometry

Hyaloid vessels were isolated from postnatal mice at P0 and P8, dissociated using 1× trypsin (MULTICELL, Cat. 325-542-CL) and washed with PBS (1×). For surface marker detection, cells were resuspended in PBS and incubated with PECAM-1 (CD31)- PE (Santa Cruz Biotechnology; sc-376764 PE) for 30 min on ice, followed by washing with PBS. Apoptotic cells were labeled with Annexin V-FITC (BioLegend; 640906) by incubating samples for 15 min at room temperature. Dead cells were labeled with 7-AAD (eBioscience, 00-6993-50). All staining steps were performed protected from light using aluminum foil. Flow cytometric acquisition was performed on a BD Accuri C6 Plus (2-laser, 4-color), and data were analyzed using BD Accuri C6 Plus Analysis software.

### Collection of pre and postnatal eyes for immunofluorescence

Pregnant females were euthanized at embryonic day 18.5 (E18.5). Following a midline abdominal incision, uterine horns were exposed, and fetuses were removed from the amniotic sacs. Embryos at E18.5 and pups at postnatal day 0 (P0), P4, and P8 were sacrificed by decapitation. Eyes were enucleated, and a small puncture was made through the cornea using a 26G needle. Samples were fixed in 4% paraformaldehyde (PFA) for 1 h at room temperature, followed by three washes in PBS. Eyes were either processed immediately at room temperature or stored in PBS at 4 °C until further use.

### Immunofluorescence

For visualization of hyaloid and retinal vasculature in embryonic and postnatal mice, eyes were enucleated and fixed in 4% PFA at room temperature, followed by three washes in cold PBS. Hyaloid tissues were permeabilized and blocked in PBS containing 10% FBS, 1% Triton

X-100, and 0.01 M glycine. Retinas were treated with a permeabilization-blocking solution composed of 3% BSA, 3% FBS, 0.01% sodium deoxycholate, and 0.5% Triton X-100 in PBS.

For blood vessels staining, hyaloids and retinas were incubated with isolectin B4 (IB4, 1:200; DL-1208-0.5, CA, Newark) in the corresponding blocking solution. For protein localization, flat-mounted hyaloids and retinas were incubated with primary antibodies (see Table S1), followed by secondary antibodies: donkey anti-rabbit Alexa Fluor 488 IgG (1:500, A21206; Invitrogen) or donkey anti-rabbit Alexa Fluor 594 IgG (1:500, A21207; Invitrogen), donkey anti-rat Alexa Fluor 488 IgG (1:400, A48269TR; Invitrogen) or donkey anti-goat DyLight 488 IgG (1:500; SA5-10086; Invitrogen). After staining, tissues were washed in PBS and counterstained with DAPI (1 µg/ml, D9542; Sigma).

Retinas were flat-mounted directly, whereas hyaloids were embedded in 2% ultra-low-melting-point agarose (800-016-TC) and incubated at 37 °C in a humidified chamber to allow gradual gel diffusion. Hyaloids were then transferred to 4 °C and subsequently flat-mounted. All samples were mounted in glycerol (Sigma-Aldrich) and imaged using a confocal microscope (Olympus FV1000) with 10× and 60× oil-immersion objectives. Images were processed using Photoshop CS4 (Adobe Systems). Immunofluorescence quantification was performed on 3–5 Z-Stack images acquired at 60× of hyaloid vessels using ImageJ software.

### EdU labeling and imaging

To label proliferating cells in vivo, the thymidine analog EdU (5-Ethynyl-2'-deoxyuridine, 50 µg/g body weight, in PBS) was injected into postnatal 8 (P8) mice. After 6 h, the eyes were enucleated and fixed with 4% PFA for 1 h at room temperature. EdU labeling was detected using the Click-iT™ EdU Alexa Fluor 647 Imaging kit (Invitrogen, Cat: C10086).

### Quantification of hyaloid vascular parameters

Hyaloid vascular density, vessels diameter and vascular junctions in flatmounted hyaloids vessels were analyzed using ImageJ software as described before [47].

### RNA extraction and real-time quantitative PCR

Total RNA was extracted from total hyaloid vessels and suspended in commercial RNase-free water with a RNeasy Minikit (Qiagen, Germany). After DNase I treatment, cDNA was synthesized from 1 µg total RNA using

the SuperScript II Reverse Transcriptase kit for RT-PCR (Thermo Fisher Scientific) according to the manufacturer's instructions. cDNA (5 ng) was amplified in triplicate using SYBR Select Master Mix or Power Up SYBR Master Mix (Thermo Fisher Scientific).

Gene expression analysis was performed using the comparative cycle threshold ( $\Delta$ CT) method, normalized with the expression of reference gene  $\beta$ -actin and presented as the mean fold change ( $\pm$  SEM) compared with control samples. The specific mouse primers pairs (Forward/Reverse) from the Invitrogen company are listed in Table S2.

## Western blot

Hyaloid vessels were lysed in NP40 and protein content extracted using RIPA lysis buffer with mechanical help. After quantification, protein probes were loaded in polyacrylamide gels and separated using an SDS-page electrophoresis. Proteins were then transferred to a Nitrocellulose membrane and blocked in 3% skim milk in TBS-T for 1 h at room temperature. Membranes were incubated with diverse antibodies (Table S3) overnight at 4 C, followed by a 1 h incubation with species-appropriate HRP-conjugated secondary antibodies. Proteins were detected with ECL clarity method (Bio-Rad) in a chemiluminescence imaging system.

## Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA). Data are presented as mean  $\pm$  SEM or as individual values, as indicated. Prior to statistical testing, normal data distribution was assessed using the Shapiro–Wilk normality test with a significance level of 0.05. For comparisons between two groups, two-tailed parametric unpaired *t*-tests were used in a normal distributed data; otherwise, non-parametric Mann–Whitney *U* tests were applied. For comparisons involving more than two groups, ordinary one-way ANOVA followed by Dunnett's multiple-comparison test was used for normally distributed data. When data distribution did not follow, the non-parametric Kruskal–Wallis test followed by Dunn's multiple-comparison correction was applied. It was considered statistically significant if the *p* values are  $\leq 0.05$ ,  $\leq 0.01$ ,  $\leq 0.001$ ,  $\leq 0.0001$ . N is indicated pertinently for each experiment.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s10456-026-10062-8>.

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**Author contributions** Author Contributions: SAR, KS, ZK, SSA, designed and performed the experiments, analyzed the data, prepared figures, KS, SSA, ZK performed, analyzed experiments and wrote the figures legends; GM analyzed experiments. CR performed bioinformatic analysis; CJ, BK provided resources; MO obtained financing, designed, and supervised the experiments, analyzed the data, prepared figures, and wrote the manuscript. All authors reviewed and edited the manuscript.

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**Data availability** No datasets were generated or analysed during the current study.

## Declarations

**Conflict of interest** The authors declare no competing interests.

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