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ORIGINAL RESEARCH

Dll4 Suppresses Transcytosis for Arterial Blood-Retinal Barrier Homeostasis

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RATIONALE: Central nervous system has low vascular permeability by organizing tight junction (TJ) and limiting endothelial transcytosis. While TJ has long been considered to be responsible for vascular barrier in central nervous system, suppressed transcytosis in endothelial cells is now emerging as a complementary mechanism. Whether transcytosis regulation is independent of TJ and its dysregulation dominantly causes diseases associated with edema remain elusive. Dll4 signaling is important for various vascular contexts, but its role in the maintenance of vascular barrier in central nervous system remains unknown.

OBJECTIVE: To find a TJ-independent regulatory mechanism selective for transcytosis and identify its dysregulation as a cause of pathological leakage.

METHODS AND RESULTS: We studied transcytosis in the adult mouse retina with low vascular permeability and employed a hypertension-induced retinal edema model for its pathological implication. Both antibody-based and genetic inactivation of Dll4 or Notch1 induce hyperpermeability by increasing transcytosis without junctional destabilization in arterial endothelial cells, leading to nonhemorrhagic leakage predominantly in the superficial retinal layer. Endothelial *Sox17* deletion represses Dll4 in retinal arteries, phenocopying Dll4 blocking-driven vascular leakage. Ang II (angiotensin II)–induced hypertension represses arterial Sox17 and Dll4, followed by transcytosis-driven retinal edema, which is rescued by a gain of Notch activity. Transcriptomic profiling of retinal endothelial cells suggests that Dll4 blocking activates SREBP1 (sterol regulatory element-binding protein 1)-mediated lipogenic transcription and enriches gene sets favorable for caveolae formation. Profiling also predicts the activation of VEGF (vascular endothelial growth factor) signaling by Dll4 blockade. Inhibition of SREBP1 or VEGF-VEGFR2 (VEGF receptor 2) signaling attenuates both Dll4 blockade–driven and hypertension-induced retinal leakage.

CONCLUSIONS: In the retina, Sox17-Dll4-SREBP1 signaling axis controls transcytosis independently of TJ in superficial arteries among heterogeneous regulations for the whole vessels. Uncontrolled transcytosis via dysregulated Dll4 underlies pathological leakage in hypertensive retina and could be a therapeutic target for treating hypertension-associated retinal edema.

VISUAL OVERVIEW: An online visual overview is available for this article.

Key Words: caveolae ■ edema ■ hypertension ■ retinal artery ■ tight junctions ■ transcytosis

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Hypertension associates with blood vessels intimately. Vascular narrowing promotes hypertension, and hypertension impairs vessels. Therefore, organs with hyper-dense vasculature, such as kidney and eye, are highly susceptible to hypertension.[1] In particular, the retina of the eye has the highest oxygen consumption rate under normal condition but its vessels become leaky in patients with severe hypertension, often leading to sight-threatening hypertensive retinopathy (HR).[2] Pathophysiology of vascular leakage associated with hypertension remains poorly understood, whereas that associated with metabolic stresses, such as diabetes mellitus and

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Novelty and Significance

What Is Known?

- Central nervous system tissues characterized by low vascular permeability display molecular diversity in endothelial cells.
- In addition to tight junction (TJ), suppression of transcytosis is emerging as a regulatory mechanism of low vascular permeability in central nervous system tissues including retina.

What New Information Does This Article Contribute?

- Dll4 preferentially expressed in retinal arteries maintains arterial blood-retinal barrier homeostasis by limiting transcytosis independently of TJ, revealing mechanistically diverse blood-retinal barrier regulation.
- Hypertension-driven uncontrolled transcytosis induces retinal edema by downregulating Dll4 expression.

Central nervous system tissues maintain low vascular permeability by organizing TJ and limiting endothelial transcytosis. Whether transcytosis regulation is independent of TJ and its dysregulation dominantly causes diseases associated with edema remain elusive. Here, endothelial Dll4-Notch signaling controls transcytosis independently of TJ in the retina and uncontrolled transcytosis via dysregulated Dll4 underlies pathological leakage in hypertensive mouse retina. While most of previous studies uncovered the permeability regulation in capillaries, inactivation of Dll4 or Notch1 induced hyperpermeability predominantly in arteries, identifying Dll4 signaling as a specialized mechanism for arterial blood-retinal barrier integrity in the retina with anatomic and vascular heterogeneities. Dll4 signaling may support the leakage-free delivery function inherent to retinal arteries by preventing material exchange en route to capillaries. Hypertensive stress downregulated Dll4 expression and induced transcytosis-driven periarterial leakage, which was rescued by gain of Notch activity and by inhibitors of VEGF (vascular endothelial growth factor) signaling. Regarding clinical relevance, patients with severe hypertension often experience vision-threatening leakage in the retinal arteries and are treated with blood pressure–lowering drugs. But, there is no established ocular therapeutics targeting hypertension-induced leakage. From clinical perspective, our findings suggest a potential utility of existing therapeutic inhibitors of VEGF-VEGFR2 (VEGF receptor 2) signaling for treating retinal edema caused by hypertension.

Nonstandard Abbreviations and Acronyms

Ang II	angiotensin II
BBB	blood-brain barrier
BP	blood pressure
BRB	blood-retinal barrier
CNS	central nervous system
EB	Evans blue
EC	endothelial cell
GO	gene ontology
HA	hemagglutinin
HR	hypertensive retinopathy
HREC	human retinal endothelial cell
MMA	mitomycin A
SRF	subretinal fluid
TJ	tight junction
VEGF	vascular endothelial growth factor
VEGFR2	VEGF receptor 2

hyperlipidemia, has been investigated well. Accordingly, current therapies for patients with hypertension-induced vascular leakage are limited to blood pressure (BP)–lowering medicine, and there is no established treatment for targeting vascular leakage.[1] Given that vascular leakage often persists to yield potentially blinding macular edema even after normalizing BP, the pattern and underlying mechanisms of hypertension-driven leakage of retinal vessels should be elucidated urgently.[3]

The central nervous system (CNS) consisting of brain and retina should maintain its unique tissue environment separated from blood. Endothelial cells (ECs) in CNS vessels employ (1) tight junction (TJ) and (2) inactive transcytosis to build blood-brain barrier (BBB) and blood-retinal barrier (BRB) with limited vascular permeability.[4] Disruption of BBB/BRB is involved in several brain and retina diseases with vascular edema such as Alzheimer disease, stroke, diabetic retinopathy, and age-related macular degeneration.[5,6] BBB/BRB disruption is also implicated in complications of hypertension including HR.[3] The pathophysiology of BBB/BRB disruption has been focused on junctional disintegration, leaving dysregulation in transcytosis poorly explored.

Signaling pathways, such as PDGFB-PDGFRβ, angiopoietin-Tie2, and YAP-TAZ (yes-associated protein-transcriptional coactivator with PDZ-binding motif), are fundamental for genesis and maturation of the blood-CNS barrier during development by regulating TJ alone and transcytosis both.[4,7] However, their inactivation has no effect on the barrier maintenance in adulthood, suggesting other molecular mechanisms for adult barrier maintenance. Recently, Mfsd2a (major facilitator super familydomain containing

2a), a lipid transporter, has been reported to regulate transcytosis independently of TJ in CNS capillaries.[8] Considering its high-order complexity, CNS tissues may have different mechanisms for transcytosis regulation in other types of vessels such as arteries. In addition, whether transcytosis contributes significantly to these barriers and whether uncontrolled transcytosis dominantly triggers pathological leakage remains to be determined.

Although Dll4 is a multifaceted player in vascular development,[9–11] its vascular role in adulthood just begins to be uncovered in lacteal and cardiac vessels.[12,13] In addition, the contribution of Dll4 to BBB/BRB integrity has not been revealed. In the present study, we found that Dll4 inhibition and hypertensive stress exhibited highly similar vascular phenotype with arterial leakage in the retina via increased transcytosis during adulthood. Therefore, we defined how Dll4 signaling contributes to arterial barrier homeostasis in steady state. Furthermore, we investigated the association between Dll4 signaling and hypertension and suggested a novel therapeutic approach to treat hypertension-induced pathological leakage in the retina.

METHODS

For details, please see the Expanded Methods and Major Resources Table in the Data Supplement. To minimize the possibility of unintentionally sharing information that can be used to reidentify private information, a subset of raw sequencing data generated for this study are available at NCBI GEO under the accession GSE142671.

Mice

Cdh5-CreER^{T2}[14] mice were interbred with *Dll4*$^{fl/fl}$, *Notch1*$^{fl/fl}$, and *Sox17*$^{fl/fl}$ (*SRY-related HMG-box 17*) mice to obtain mutant mice lacking *Dll4*, *Notch1*, and *Sox17* in ECs. *Cdh5*-CreER^{T2} mice were mated with *Gt(ROSA)26Sor*$^{tm1(Notch1)Dam/J}$ and *RPL22*$^{tm1.1Psam}$ mice to overexpress *N1ICD* and to express hemagglutinin-tagged ribosome in ECs. All mouse lines were C57BL/6J background. Cre-mediated deletion was induced as described previously.[15]

Hypertension Model

To induce hypertensive condition, Ang II (angiotensin II; Calbiochem) or PBS for controls was infused at a rate of 1 μg/(kg·min) for 4 weeks using an osmotic mini-pumps (Alzet) implanted subcutaneously with modifications (Figure 6A).[16,17] After 3 weeks, severe acute hypertension was generated by subcutaneously injecting Ang II (0.5 μg/g, twice per day) to reproduce hypertension-induced retinal edema by BRB disruption in patients with HR.[1] Systolic BP was measured using tail-cuff plethysmography (Kent Scientific).

Statistics

Statistical analyses were performed using the SPSS v.22 (IBM), R (v.3.6.0), and GraphPad Prism 7.0. Values are presented as mean±SD. No statistical methods were used to predetermine the sample size. The normality of distribution for variables were verified by Shapiro-Wilk test. Depending on normality, 2-tailed Student *t* test or Mann-Whitney *U* test was used to compare 2 groups and ANOVA test with the Tukey test or Kruskal-Wallis with Dunn's test was used to compare multiple groups. Pearson correlation test was used for correlation analyses. $P<0.05$ was considered statistically significant.

RESULTS

Dll4 Blocking Induces Transient Periarterial Leakage

Dll4 is a key regulator of angiogenesis and arterial differentiation in developing retina; however, its role in mature vessels with BRB integrity has not been revealed.[9–11] Accordingly, Dll4 expression was robust in angiogenic front and arteries in the growing mouse retina. Unexpectedly, we found persistent arterial Dll4 expression in adult retina (Figure IA and IB in the Data Supplement). To gain insight into the role of Dll4 in BRB maintenance, we injected anti-Dll4 antibody (α-Dll4 Ab) or IgG (as a control) intravitreally into adult mice only once (to exclude unwanted systemic and long-term secondary effects) and examined the extravasation of intravenously infused tracers in the retina 7 days later (Figure 1A). Extravasation of Evans blue (EB) and dextran (4 and 10 kDa) was prominent in the superficial vascular plexus compared with the deep vascular plexus of the α-Dll4 Ab-treated retina (Figure 1B through 1D). Following intravitreal α-Dll4 Ab treatment, EB leakage from arteries was observed after 3 days, increased up to the capillaries and veins after 7 days, and was restricted to periarterial areas with a decline after 10 days, indicating reversible leakage predominant in retinal arteries and suggesting a correlation between Dll4 levels and the amount and duration of leakage in each vessel type (Figure 1E and 1F). These findings indicate that Dll4 is indispensable for arterial BRB homeostasis.

However, intravitreal α-Dll4 Ab treatment yielded no changes in the vascular pattern and density and the coverage by NG2-positive pericytes and SMA (smooth muscle actin)-positive smooth muscle cells (Figure IC through IF in the Data Supplement). Dll4 blocking resulted in a loss of collagen type IV and tortuosity in retinal arteries as shown by intravital imaging, suggesting that it may facilitate the diffusion of leaked fluid by weakening the extracellular matrix (Figure IG through IJ in the Data Supplement).

Endothelial Dll4-Notch1 Signaling Maintains Barrier Integrity Via Transcytosis Suppression

As BRB integrity is maintained mainly by specialized TJ and limited transcytosis in ECs,[18] we studied whether α-Dll4 Ab treatment impairs TJ and enhances transcytosis in the adult retina. Dll4 blocking did not alter the level and distribution pattern of VE-cadherin, claudin-5, occludin,

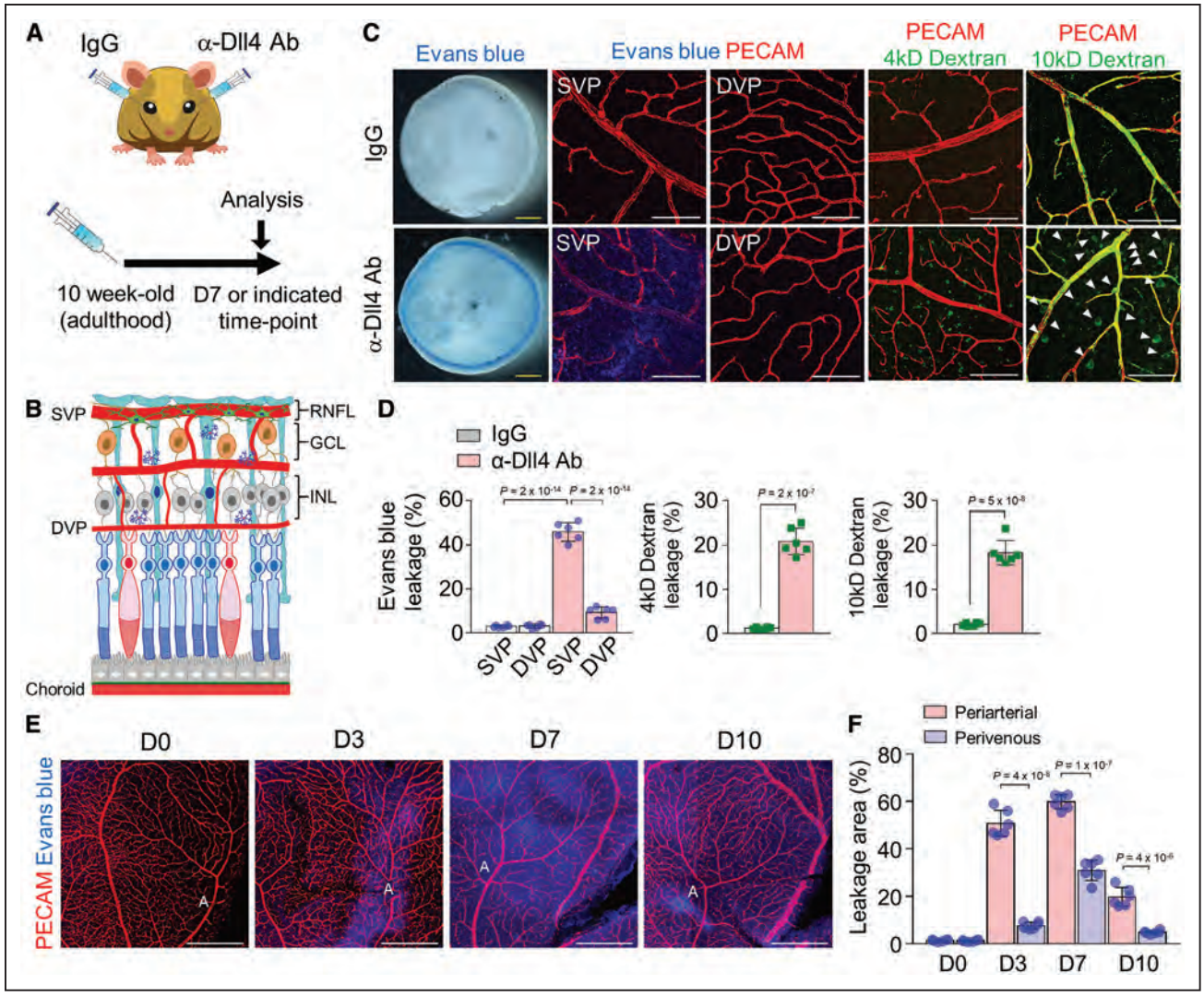

Figure 1. Dll4 blocking leads to abundant and reversible periarterial leakage in the adult mouse retina.
A, Schematic of transient and local inhibition of retinal Dll4 by single intravitreal injection of anti-Dll4 (α-Dll4 Ab) or control (IgG) antibodies into adult mice. **B**, Schematic of the retina with laminated tissue and vessel organization. **C**, Gross images of the retinal cup and PECAM (platelet endothelial cell adhesion molecule)-immunostaining of whole-mounted retina showing vascular leakages of tracers in the superficial, but not deep, vascular layer 7 days after α-Dll4 Ab treatment. Arrowheads indicate extravasated 10 kD dextran. **D**, Quantification of leakage (n=6 retinas per group). **E**, Serial images of whole-mounted retina after α-Dll4 Ab treatment, showing reversible leakage prevalent in arteries over time. **F**, Quantification of Evans blue leakage in the periarterial and perivenous area (n=6 retinas per group). Data are presented as mean±SD. Two-tailed Student *t* test. Scale bars, 500 μm (**C**, yellow; **E**), 100 μm (**C**, white). A indicates artery; DVP, deep vascular plexus; GCL, ganglion cell layer; INL, inner nuclear layer; RNFL, retinal nerve fiber layer; and SVP, superficial vascular plexus.

and ZO-1 immunostaining (Figure IIA and IIB in the Data Supplement), but it increased the immunostaining of PLVAP (plasmalemma vesicle-associated protein) and caveolin-1, the markers for caveolar-mediated transcytosis,[19] in retinal arteries and arterioles (Figure 2A through 2C), and their protein levels in retinal lysates (Figure IK in the Data Supplement). Using transmission electron microscopy, we verified the maintenance of TJ and the increased number of transcellular vesicles in ECs treated with α-Dll4 Ab (Figure 2D and 2E). A flask-shaped invaginations of plasmalemma in the luminal side as well as docking and fusion on the abluminal side were increased.

We also inhibited retinal Notch1, a primary receptor for Dll4,[9] by intravitreal α-Notch1 Ab injection into adult mice. Similar to Dll4 blocking, α-Notch1 Ab treatment induced extravasation of tracers and arterial PLVAP and caveolin-1 expressions in ECs without alterations of VE-cadherin and claudin-5 expressions (Figure IIIA through IIIG in the Data Supplement). Coverage of mural cells was unaltered, but loss of collagen type IV with arterial tortuosity was observed following α-Notch1 Ab treatment (Figure IIIH through IIIK in the Data Supplement).

We further studied the role of Dll4 and Notch1 by genetically inactivating these genes (Figure IVA in the Data Supplement). Consistent with the data of intravitreal

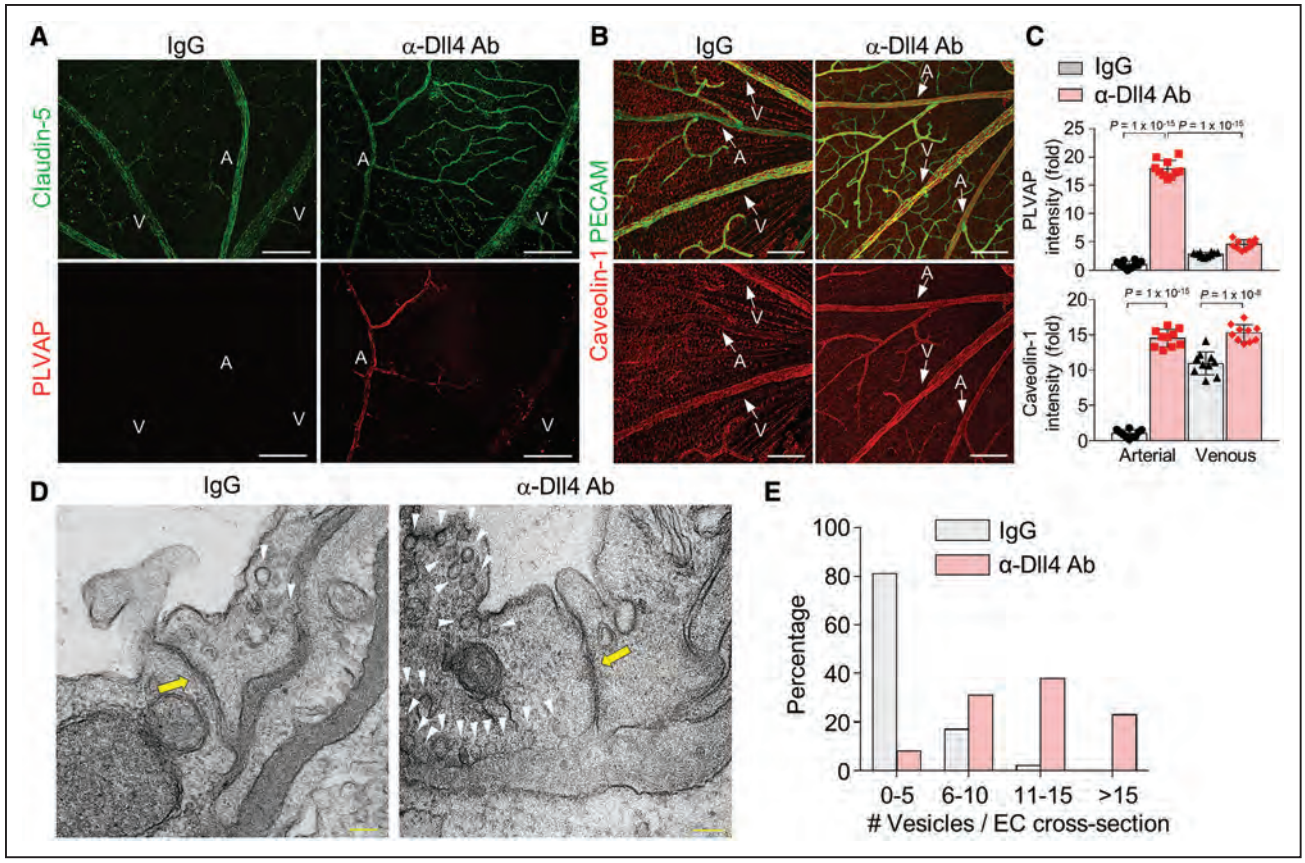

Figure 2. Dll4 blocking increases endothelial transcytosis but not tight junction instability in retinal arteries.
A and **B**, Immunostaining of whole-mounted retina 7 days after intravitreal α-Dll4 Ab or control IgG injection shows transcytosis markers PLVAP (plasmalemma vesicle-associated protein; **A** and caveolin-1 (**B**) in arteries and arterioles after α-Dll4 Ab treatment. No change in tight junction (TJ) marker claudin-5. **C**, Quantification of immunostaining intensities compared with control arteries (n=10 retinas per group). Data are presented as mean±SD. Two-tailed Student *t* test. **D**, Transmission electron microscopy images showing intact TJ and increased vesicle number (arrowheads) in retinal endothelial cells (ECs) 7 days after α-Dll4 Ab treatment. **E**, Quantification of vesicles per EC cross-section (n=100 sections per group). Scale bars, 100 μm (**A** and **B**), 200 nm (**D**). A indicates artery; PECAM, platelet endothelial cell adhesion molecule; and V, vein.

antibody injection, adult mice lacking *Dll4* and *Notch1* in ECs (*Dll4*$^{i\Delta EC}$ and *Notch1*$^{i\Delta EC}$, respectively) showed no remarkable changes in mural cell coverage (pericytes and smooth muscle cells; Figure IVB and IVC in the Data Supplement). In addition, *Dll4*$^{i\Delta EC}$ and *Notch1*$^{i\Delta EC}$ mice phenocopied arterial BRB disruption by α-Dll4 Ab treatment, indicating ECs as a major cell type for Dll4-Notch1 signaling in adult BRB maintenance (Figure IVD through IVH in the Data Supplement). The *Slco1c1* CreER^{T2} line, which was generated originally for gene deletion in CNS ECs,[20] had *Dll4* excised from arterial ECs specifically in adult mouse retina (*Dll4*$^{i\Delta RAEC}$), which is different from pan-endothelial *Dll4* deletion in *Dll4*$^{i\Delta EC}$ mice (Figure VA through VB in the Data Supplement). *Dll4*$^{i\Delta RAEC}$ mice also exhibited PLVAP expression and tracer leakage predominantly in retinal arteries (Figure VC through VH in the Data Supplement), underscoring the importance of Dll4 in arterial BRB homeostasis. Nonetheless, we do not exclude the possibility that Dll4 may play a role in BRB maintenance in capillaries and veins.

In human umbilical vein endothelial cells, α-Notch1 Ab treatment increased albumin uptake, which was reversed by Notch1 activation (Dll4 coating) and dynasore (transcytosis inhibitor) but not by adrenomedullin (paracellular transport inhibitor; Figure VIA through VID in the Data Supplement), verifying transcytosis as a major route of leakage when blocking Dll4-Notch1 signaling. However, α-Notch1 Ab treatment and Dll4 coating did not affect the transepithelial electrical resistances (Figure VIE and VIF in the Data Supplement), suggesting that paracellular transport is independent of Notch regulation. In human retinal EC (HREC) culture, α-Notch1 Ab, but not Notch activation by Dll4 coating, upregulated several structural components of caveolar vesicles, such as PLVAP, caveolin-1, phospho-caveolin-1, and cavin-1 at the protein level (Figure VII in the Data Supplement). These findings point to the cell-autonomous Dll4-Notch regulation of caveolae-mediated transcytosis. Although Mfsd2a has been suggested to regulate transcytosis for postnatal BRB maturation,[8,21] it was expressed mainly in the capillaries of the adult retina with minimal overlap

with Dll4 expression (Figure VIIIA through VIIIB in the Data Supplement). In addition, α-Dll4 Ab treatment resulted in no discernable change in Mfsd2a expression in the retina, suggesting that Dll4 functions in an Mfsd2a-independent manner (Figure VIIIC in the Data Supplement). Taken together, these results indicate that EC-autonomous Dll4-Notch1 interaction keeps the permeability of retinal arteries low by selectively limiting transcytosis.

Sox17 Deletion Phenocopies Transcytosis-Mediated Periarterial Leakage by Dll4 Blocking

Because transcription factor Sox17 promotes Dll4-mediated arterial development in the retina, we studied whether Sox17 is involved in Dll4-mediated arterial BRB regulation in adulthood. Adult mice lacking endothelial *Sox17* (*Sox17*$^{i\Delta EC}$) had repressed Dll4 immunostaining, whereas endothelial *Dll4* deletion did not affect arterial Sox17 immunostaining in the retina (Figure 3A, 3B, 3G, and 3H), indicating that Sox17 is an upstream regulator of arterial Dll4 expression. Consistent with phenotypes of Dll4 inhibition, *Sox17* deletion resulted in extravasation of tracers, upregulated PLVAP and caveolin-1 expression, and increased the number of transcellular vesicles in arterial ECs without claudin-5 and TJ changes (Figure 3C through 3K). Immunoblot analysis of retinal lysates also confirmed repressed Dll4 levels and increased levels of PLVAP and caveolin-1 in the retinas of *Sox17*$^{i\Delta EC}$ mice (Figure 3L and 3M). In addition, a gain in Notch activity via overexpression of N1ICD in ECs (*N1ICD*^{iOE})[13] attenuated EB leakage and arterial PLVAP expression in the retinas of *Sox17*$^{i\Delta EC}$ mice (Figure IX in the Data Supplement). These findings suggest that Sox17 is upstream of Dll4-regulated arterial BRB maintenance.

Lipogenic Transcription Factor SREBP1 Mediates Dll4 Blockade-Induced Transcytosis

To understand the underlying molecular mechanisms, we generated EC-specific RiboTag mice (*Cdh5-Cre*ERT2;*RPL22*$^{m1.1Psam}$)[22] and injected IgG or α-Dll4 Ab intravitreally, followed by isolation of highly EC-enriched transcripts 3 days later for RNA sequencing (Figure XA through XC in the Data Supplement). Principal component analysis and hierarchical clustering of RNA-sequencing results revealed clear discrimination of the gene expression profiles between IgG and α-Dll4 Ab-treated retinal ECs (Figure XD and XE in the Data Supplement). Among 12 756 differentially expressed genes, 801 genes were identified as significant differentially expressed genes, composed of 544 upregulated and 257 downregulated genes. Gene ontology (GO) enrichment analysis identified regulation of transport as one of the top gene ontology terms (biological process), and Ingenuity Pathway Analysis predicted activation of transport and export of molecules in Dll4-inhibited retinal ECs (Figure XF and XG in the Data Supplement). Interestingly, gene set enrichment analysis revealed positive enrichment of gene sets related to raft formation, vesicle endocytosis, and vesicle transport in α-Dll4 Ab-treated retinal ECs, pinpointing Dll4-regulated steps in the whole process of transcytosis (Figure 4A). In line with this, RNA-sequencing data exhibited upregulated structural components of caveolar vesicles in Dll4-inhibited retinal ECs, which was verified by quantitative RT-PCR (Figure XH and XI in the Data Supplement). However, the gene set related to cell-cell junction was not significantly enriched (Figure XJ in the Data Supplement).

Ingenuity Pathway Analysis upstream analysis of differentially expressed genes suggested that Dll4 inhibition activates EGR1 and SREBP1 (sterol regulatory element-binding protein 1; a master lipogenic transcription factor[23]), which are involved in caveolae formation (Figure 4B).[24,25] Moreover, several upregulated SREBP1 target genes, such as *STAT6*, *RAB10*, *CORO1A*, and *PLCB3*, were associated with caveolae or transcytosis (Figure 4C).[26,27] Compared with the plasma membrane, caveolae are composed of a higher ratio of cholesterol and sphingomyelin but a lower ratio of phospholipids.[28] Dll4-inhibited retinal ECs revealed positive enrichment of gene sets related to cholesterol homeostasis and bile acid transport essential for cholesterol maintenance but negative enrichment of gene sets related to phospholipids (Figure 4D). Taken together, these transcription profiles suggest that Dll4 inhibition initiates EC transcytosis by creating a caveolae-favorable lipid environment. Next, we verified the role of EGR1 and SREBP1 in vivo by systemically administering mitomycin A (MMA, EGR1 inhibitor) or fatostatin (SREBP1 inhibitor) after intravitreal α-Dll4 Ab injection (Figure 4E). Fatostatin attenuated arterial leakage, PLVAP expression (Figure 4F and 4G), and the number of transcellular vesicles (Figure XI in the Data Supplement) induced by α-Dll4 Ab, suggesting that Dll4 signaling limits transcytosis by suppressing SREBP1 in retinal ECs. In contrast, MMA exacerbated arterial hyperpermeability, implying inhibitory regulation by EGR1 activation.

Transcriptomic Profiling Predicts the Association Between Dll4 and Hypertension in ECs

Unexpectedly, the top enriched gene sets (C5 category) were related to arterial BP regulation, and one of the top-ranked pathways in Dll4-inhibited retinal ECs was EDN1 (endothelin-1) signaling, which is crucial for BP elevation (Figure 5A through 5C). Consistently, Dll4 inhibition upregulated BP-elevating genes (*EDN1*, *EDN2*, *F2R*, *EMG*, and *ECE*) but downregulated BP-lowering *ADORA1* (adenosine receptor A; Figure 5D and 5E). These data suggest that Dll4 inhibition is associated

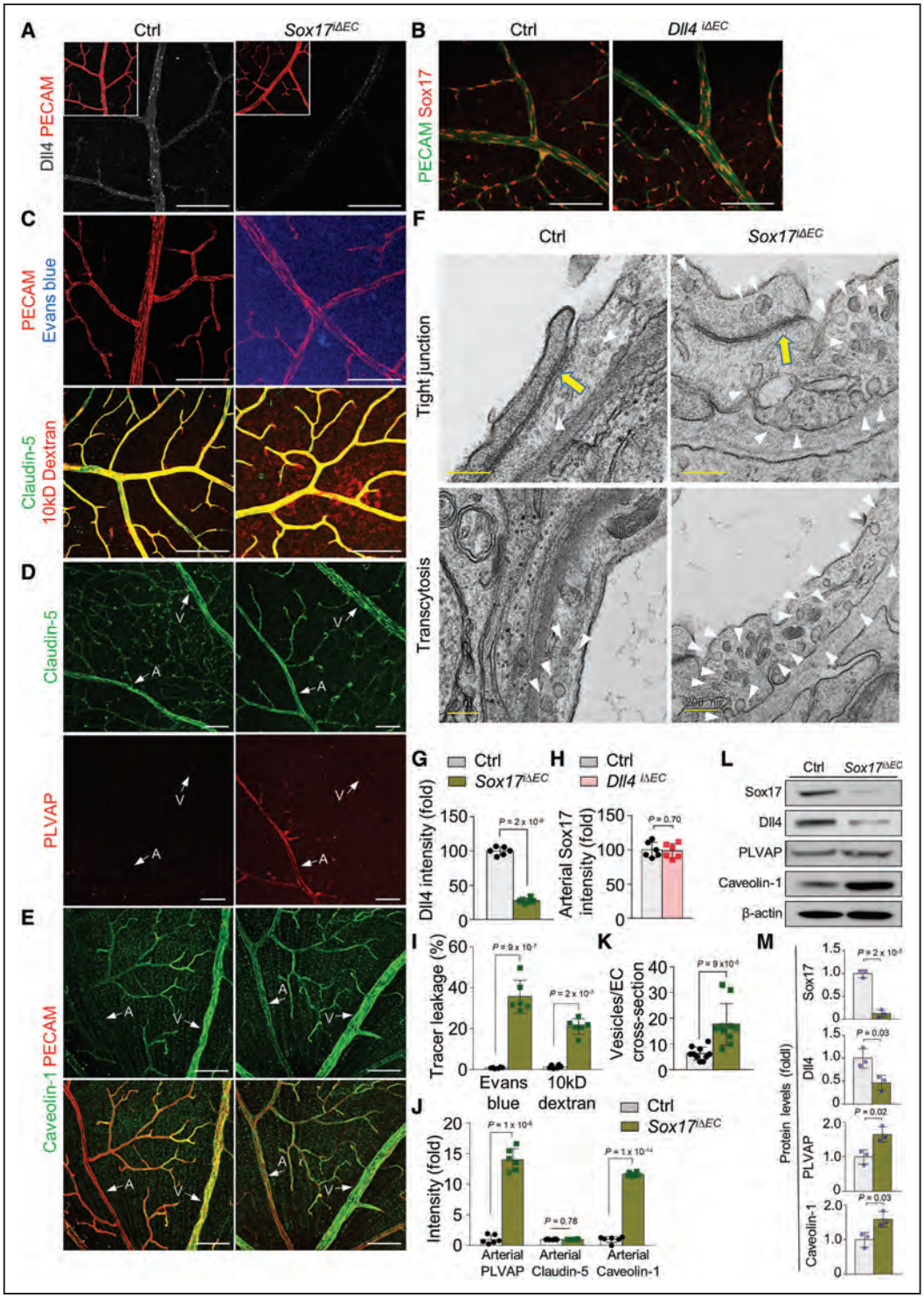

Figure 3. Endothelial *Sox17* deletion leads to enhanced transcytosis by downregulating Dll4 in retinal arteries.
A–**E**, Immunostaining of whole-mounted retina from adult control (Ctrl) and *Sox17*$^{i\Delta EC}$ mice 2 wk after tamoxifen treatment. **A**, Decreased Dll4 immunostaining in retinal arteries by endothelial cell (EC) *Sox17* deletion (**G**, quantification, n=6 retinas per group). **B**, No discernable change in Sox17 immunostaining in adult retinal arteries by EC *Dll4* deletion (*Dll4*$^{i\Delta EC}$; **H**, quantification, n=6 retinas per group). **C**, Extravasated Evans blue (EB; **top**) and 10 kD dextran (**bottom**) by EC *Sox17* deletion (**I**, quantification, n=6 retinas per group). **D** and **E**, Appearance of PLVAP (plasmalemma vesicle-associated protein) and caveolin-1 (**E**) in *Sox17*-deficient retinal arteries without changes of claudin-5 (**D**; **J**, quantification of arterial PLVAP, caveolin-1, and claudin-5, n=6 retinas per group). **F**, Transmission electron microscopy (TEM) images showing increased vesicle number (arrowheads) and intact TJ (arrows) in *Sox17*-deficient retinal ECs (**K**, quantification of vesicle number per EC cross-section, n=10 sections per group). **L**, Immunoblot of Sox17, Dll4, PLVAP, and caveolin-1 in retinal lysates from adult control and *Sox17*$^{i\Delta EC}$ mice (**M**, quantification, n=3 per experiment). Data are presented as mean±SD. Mann-Whitney *U* test (**K**), 2-tailed Student *t* test (**G**, **H**, **I**, **J**, and **M**). Scale bars, 200 μm (**D** and **E**), 100 μm (**A**–**C**), 200 nm (**F**). A indicates artery; PECAM, platelet endothelial cell adhesion molecule; and V, vein.

with hypertension in the retinal ECs, which is in line with hypertension development in a subset of cancer patients treated with α-DII4 Ab.[29] However, ocular treatment with α-DII4 Ab had no effect on systemic BP (Figure 6C).

Hypertension Induces Nonhemorrhagic Leakage in the Retina by Downregulating Sox17 and DII4

Interestingly, similar to DII4 blocking in mice, severe hypertension in humans induces periarterial leakage in the retina.[1] To elucidate the potential relationship, we generated a hypertension-induced retinal edema model by infusing Ang II into adult mice chronically, followed by acute Ang II stresses (Figure 6A through 6F). Notably, Ang II–induced hypertension, *Sox17* deletion, and α-DII4 Ab treatment resulted in nonhemorrhagic leakage in common. In contrast, copious hemorrhagic leakage due to severe junction disruption was caused by α-PDGFRβ Ab treatment in diabetic retinopathy models[30] (Figure 6G and 6H; Figure XII in the Data Supplement). Importantly, some portions of vision-threatening retinal edema (including macular edema), which is occasionally found in patients with severe hypertension, is characterized by nonhemorrhagic BRB disruption with exudations.[1] The common leakage phenotype suggests that hypertension-induced leakage is mechanistically associated with Sox17 and DII4.

Ang II–induced hypertension repressed endothelial Sox17 and DII4 in the retinal arteries (Figure 7A through 7C). Ang II treatment also repressed Sox17 and DII4 and upregulated SREBP1 and VEGFR2 (vascular endothelial growth factor 2) in HRECs (Figures XIII, XIVM through XIVO in the Data Supplement). As Ang II generated reactive oxygen species in ECs,[31] we treated HRECs with the antioxidant N-acetyl cysteine. N-acetyl cysteine decreased intracellular reactive oxygen species and significantly restored levels of Sox17, DII4, SREBP1, and VEGFR2 in Ang II-treated HRECs toward control levels (Figure XIII in the Data Supplement). These results suggest that hypertensive insults may decrease Sox17 via reactive oxygen species and subsequently elicit changes in the expression of downstream molecules. We validated the effect of reactive oxygen species in the retina. PEG-bilirubin, which has an improved anti-oxidative capacity,[32] recovered Sox17 and DII4 levels and repressed PLVAP and caveolin-1 expression in retinal arteries from Ang II–infused mice (Figures XIVA through XIVF, XIVI, and XIVK in the Data Supplement). PEG-bilirubin also attenuated extravasation of tracers and retinal edema (Figure XIVF through XIVH, XIVJ, and XIVL in the Data Supplement). Furthermore, long-term intake of N-acetyl cysteine decreased arterial PLVAP expression and EB leakage in hypertensive retinas (Figure XV in the Data Supplement).

The magnitude of EB leakage showed an inverse relationship with DII4 immunoreactivity, suggesting DII4 downregulation as one of the mechanisms of hypertension-induced BRB disruption (Figure 7D). A gain in Notch activity in ECs reduced hypertension-induced EB leakage, PLVAP expression, and transcellular vesicles in retinal arteries (Figure 7E through 7H). Furthermore, SREBP1 inhibition by fatostatin injection decreased EB leakage, retina edema, and PLVAP expression in the retina from Ang II–infused mice similar to treatment with α-DII4 Ab (Figure XVI in the Data Supplement). Ang II infusion and related *Sox17* deletion and DII4 blockade did not result in detectable immunostaining of cleaved caspase-3, Ki67, and Edu in retinal ECs (Figure XVII in the Data Supplement); thus, apoptotic damage to ECs followed by reactive proliferation is unlikely to be involved in Ang II–induced retinal leakage. These findings suggest downregulation of Sox17 and DII4, followed by SREBP1-mediated transcytosis, as one of the mechanisms underlying hypertension-induced pathological leakage in the retina.

Periarterial Superficial Leakage in Patients With HR

For a clinical relevance, we evaluated retinal angiography and optical coherence tomography of patients with HR to know the leakage pattern in the retina. While patients with diabetic retinopathy showed smeared capillary leakage and deep vascular layer edema,[33] a significant proportion of patients with HR (Table I in the Data Supplement) presented periarterial leakage and edema specific to the superficial vascular layer containing the ganglion cell complex, resembling the α-DII4 Ab-treated or hypertensive mouse retina (Figure XVIII in the Data Supplement). The distinct leakage site suggests substantial pathophysiological difference of vascular leakage between HR and diabetic retinopathy. In line with this, hypertensive, but not diabetic, stress decreased DII4 expression in superficial arteries of the mouse retina, whereas DII4 was not detectable in deep vessels (Figure XIX in the Data Supplement).

Abundant leakage can lead to subretinal fluid (SRF) accumulation.[34] SRF accumulation was prominent in a subset of patients with superficial edema (group 1 and 2) compared with patients lacking an edematous appearance (group 3; Figure XXA and XXB in the Data Supplement). In addition, the thicknesses of the superficial, but not deep, vascular layer significantly positively correlated with SRF height in HR (Figure XXC in the Data Supplement). SRF in α-DII4 Ab-treated and hypertensive mouse retina was likely to accumulate by leakage in retinal vessels without choroidal leakage (Figure XXD in the Data Supplement). This leakage pattern suggests that DII4 downregulation and subsequent superficial vascular leakage may contribute to hypertension-induced retinal edema, including macular edema, in which the origin of the leakage is controversial and poorly defined yet.

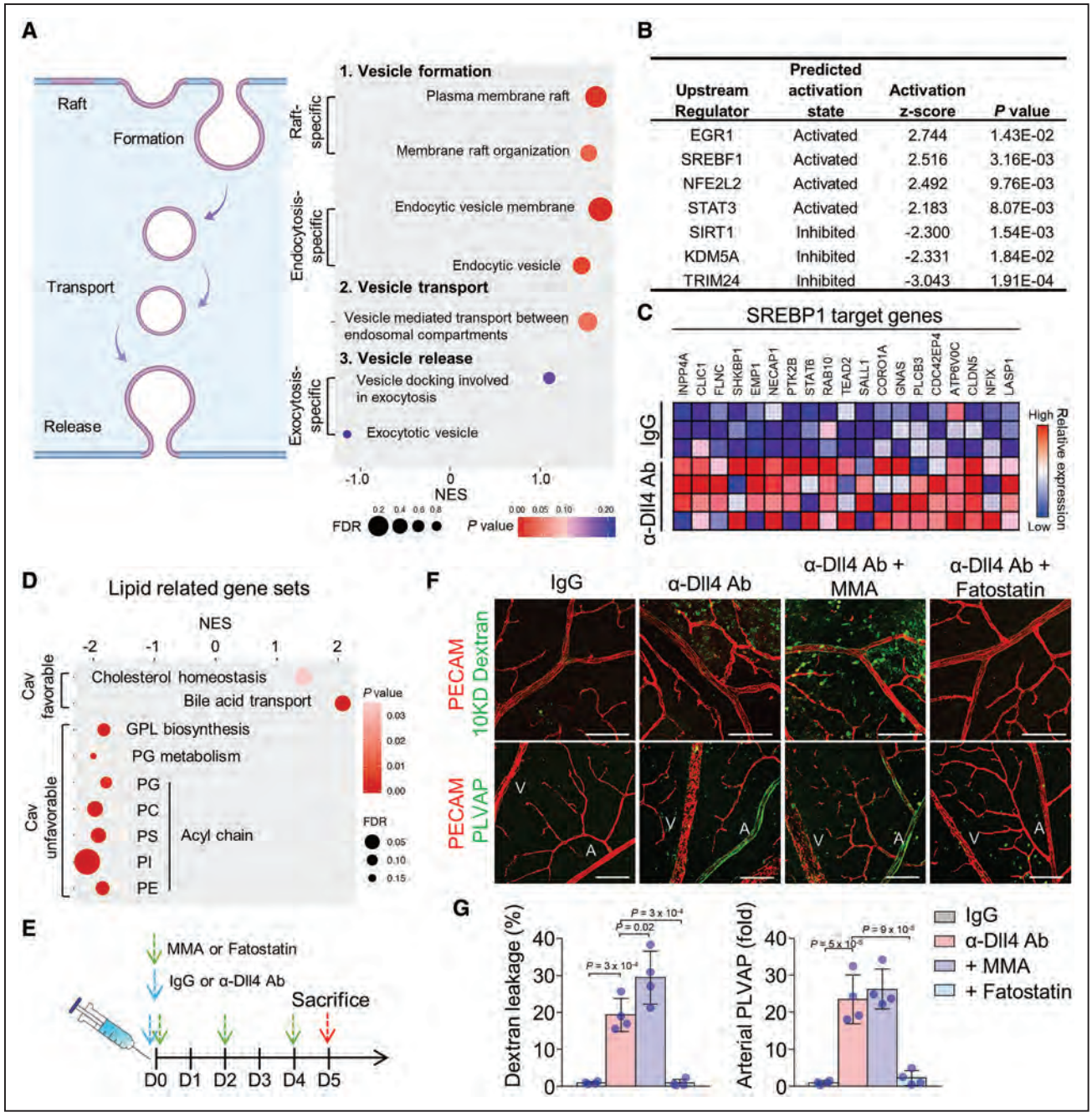

Figure 4. The Sox17-Dll4 axis limits retina vascular permeability by restricting SREBP1 (sterol regulatory element-binding protein 1)-mediated caveolae formation.

A, Schematic of the processes of transcytosis (**left**) and related dot plot of gene set enrichment analysis (GSEA) results in retinal endothelial cells (ECs) treated with α-Dll4 Ab compared to IgG (**right**). Schematic created with BioRender. **B,** Top transcription factors after α-Dll4 Ab treatment predicted by the ingenuity pathway analysis (IPA) upstream analysis. *P* values by Fisher exact test. **C**, Heatmap showing upregulation of SREBP1 target genes (SREBP1_Q6 gene set in MSigDB) in retinal ECs treated with α-Dll4 Ab. **D**, Dot plot of GSEA results related to lipid compositions favorable or unfavorable for caveolae (Cav) formation. *P* values are presented by color gradients and FDR values by circle size (**A** and **D**). n=3 samples for IgG-treated retinal ECs and n=4 samples for α-Dll4 Ab-treated retinal ECs (**A**–**D**). **E**–**G**, Validation of the role of EGR1 and SREBP1 in Dll4-blocking–induced retinal leakage. **E**, Experimental schedule showing single intravitreal α-Dll4 Ab or IgG injection followed by systemic injection of mitomycin A (MMA, EGR1 inhibitor) and fatostatin (SREBP1 inhibitor). **F**, Extravasation of 10 kD dextran (**top**) and PLVAP (plasmalemma vesicle-associated protein) immunostaining (**bottom**) in adult retina. **G**, Quantification of dextran leakage and arterial PLVAP intensity (n=4 retinas per group). Data are presented as mean±SD. One-way ANOVA followed by Tukey post hoc analysis. Scale bars, 100 μm (**F**). A indicates artery; FDR, false discovery rate; GPL, glycerophospholipid; NES, normalized enrichment score; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserin; and V, vein.

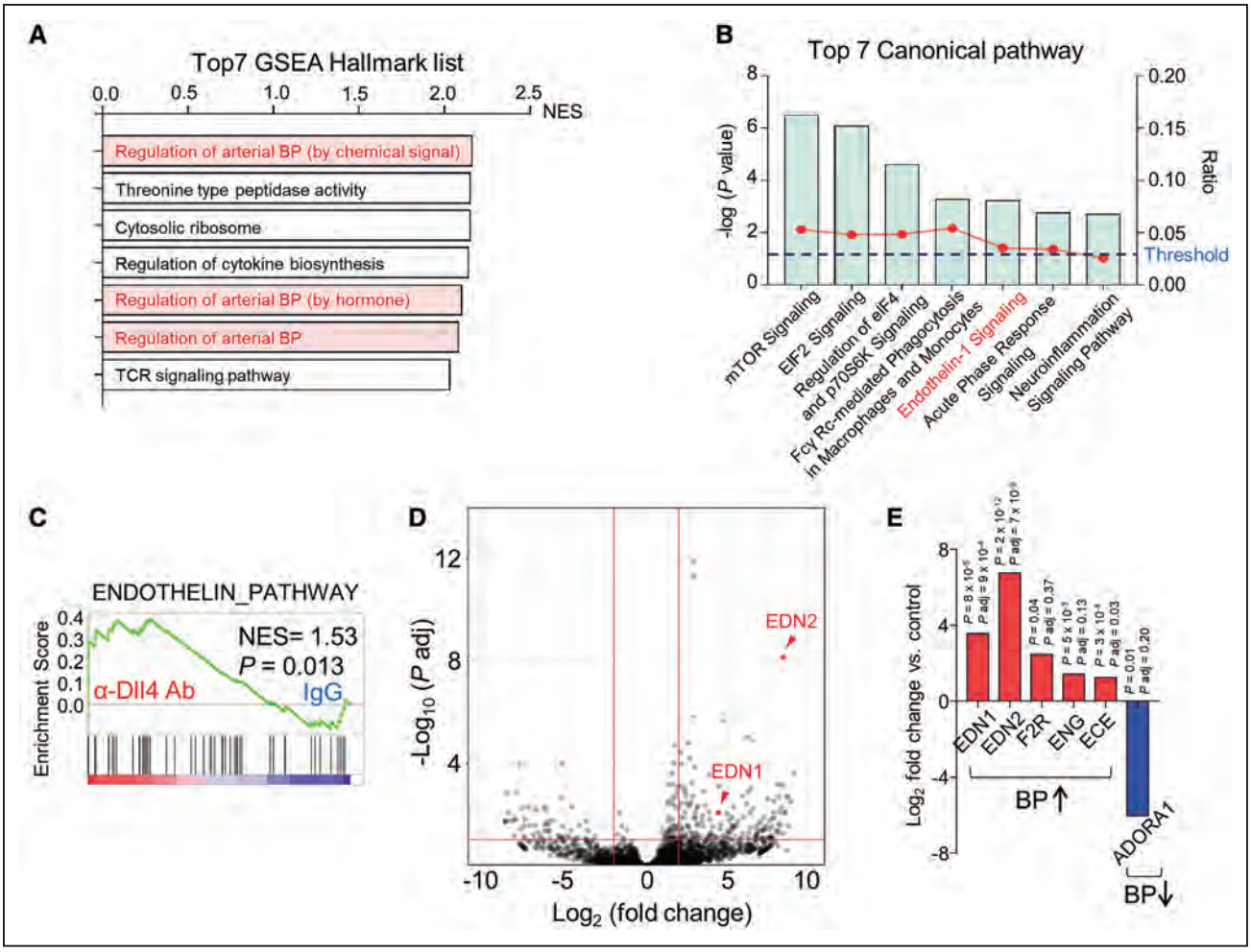

Figure 5. Transcriptomic profiling suggests the association of Dll4 inhibition with hypertension regulation in retinal endothelial cells (ECs).

A, Top 7 enriched gene sets of the C5 category in α-Dll4 Ab-treated retinal ECs. Note the 3 gene sets related to arterial blood pressure (BP) regulation highlighted in red. **B**, Top 7 IPA-based canonical pathways altered in α-Dll4 Ab-treated retinal ECs. Note the endothelin-1 signaling crucial for BP elevation. Red dots, proportion of the genes found in each pathway out of the total genes in the analysis. Light green bars, *P* value by Fisher exact test. The dotted line represents the threshold for significance (*P*=0.05). **C**, Gene set enrichment analysis (GSEA) plot showing positive enrichment of the endothelin pathway in α-Dll4 Ab-treated retinal ECs. **D**, Volcano plot of differentially expressed genes in α-Dll4 Ab-treated retinal ECs with BP-regulating genes highlighted. *P* adj, adjusted *P* value calculated with Benjamini-Hochberg correction. **E**, RNA-seq data showing upregulation of BP-elevating genes (red) and downregulation of BP-lowering gene (blue) in α-Dll4 Ab-treated retinal ECs. n=3 samples for IgG-treated retinal ECs and n=4 samples for α-Dll4 Ab-treated retinal ECs. Data are presented as mean±SD. ADORA1 indicates adenosine receptor A; ECE, endothelin converting enzyme; EDN, endothelin; ENG, endoglin; mTOR, mammalian target of rapamycin; and NES, normalized enrichment score.

Blocking VEGF-VEGFR2 Pathway Rescues BRB Integrity in Dll4-Inhibited or in Hypertensive Retina

Activated VEGF signaling is a major cause of vascular leakage in endothelial disorders, including several retinal diseases.[35–37] Not surprisingly, gene set enrichment analysis revealed enrichment of the VEGFR signaling pathway in Dll4-inhibited retinal ECs (Figure 8A). VEGFR2 immunostaining was robust in arterial ECs from α-Dll4 Ab-treated retinas but was undetectable in IgG-treated retinas, and immunoblot analysis of retinal lysates also confirmed VEGFR2 upregulation (Figure 8B; Figure XXIA and XXIB in the Data Supplement). *VEGFR2* levels were increased subsequent to the downregulation of Notch target genes in retinal ECs after α-Dll4 Ab treatment (Figure XXIC in the Data Supplement), consistent with Notch control of VEGFR2 expression at the transcriptional level as reported previously.[38] Consistent with previous reports,[35] VEGF, but not IgG, injected into the eye induced EB extravasation and PLVAP upregulation in the retinal vessels (Figure XXII in the Data Supplement), implicating VEGF signaling in transcytosis-mediated retinal leakage. Intravitreal injection of anti-VEGFR2 antibody (DC101) or VEGF-trap (aflibercept) reduced Dll4 blockade-induced arterial EB leakage, transcellular vesicle numbers in retinal

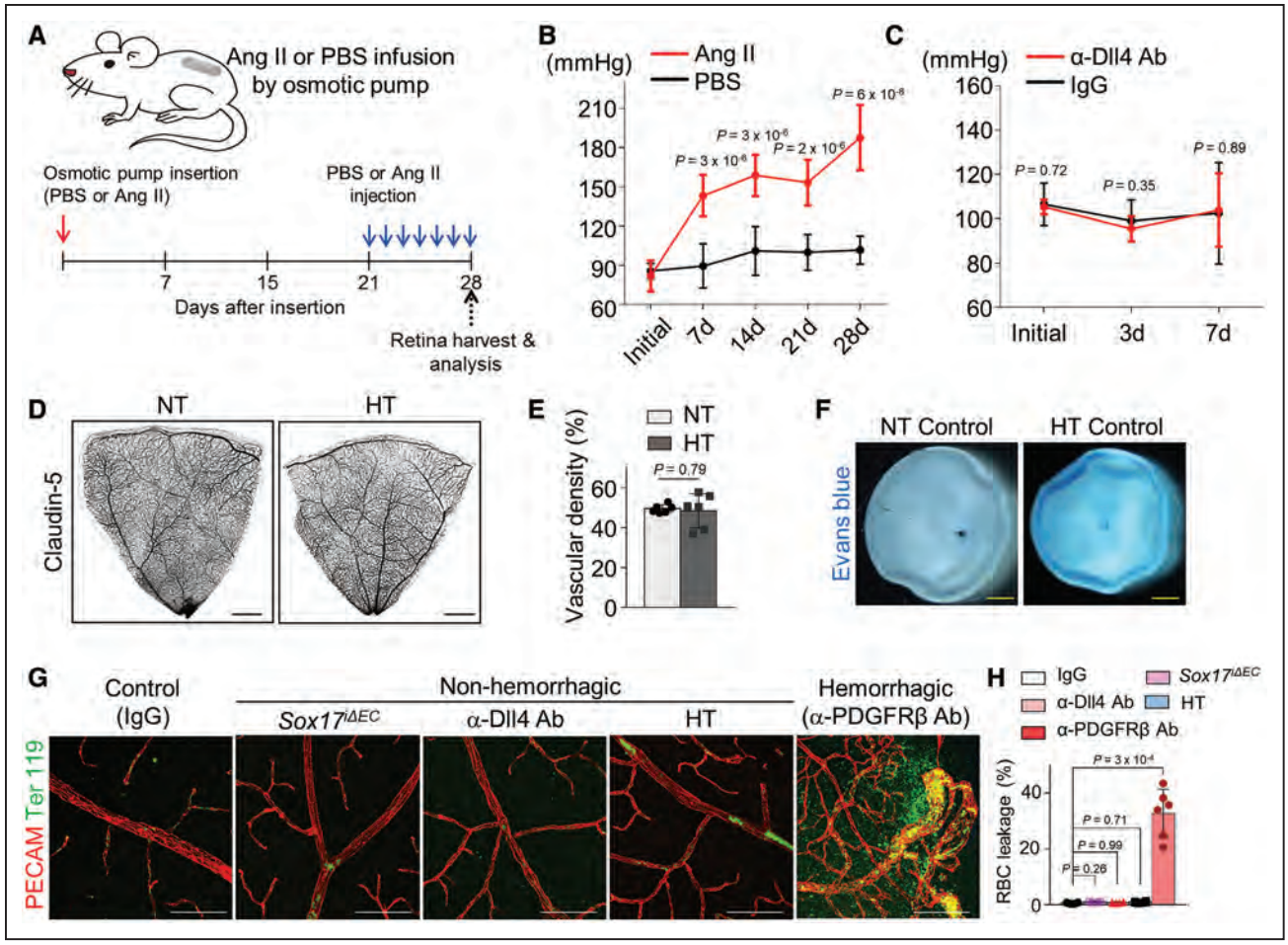

Figure 6. Dll4 inhibition, endothelial *Sox17* deletion, and hypertensive stress induce nonhemorrhagic leakage in adult retinal vessels. **A**–**F**, Mouse model of severe hypertension (HT)-induced blood-retinal barrier (BRB) disruption. **A**, Experimental setting for the HT-induced retinal edema model. **B**, Blood pressure elevation after Ang II (angiotensin II) treatment (n=10 mice per group) and intravitreal α-Dll4 Ab or control IgG injection (**C**, n=9 mice per group). **D**, Immunostaining of claudin-5 in whole-mounted adult retina. Note no apparent change in vascular morphology with HT. **E**, Quantification of vessel density (n=6 retina per group). **F**, Gross images of retinal cups showing abundant EB leakage. (**G** and **H**) Undetectable micro-hemorrhage by endothelial *Sox17* deletion, α-Dll4 Ab treatment, and HT. **G**, Ter119 immunostaining of whole-mounted retina. Note the abnormal vascular morphology and severe hemorrhage only with α-PDGFRβ Ab treatment, mimicking diabetic retinopathy. **H**, Quantification of the Ter119-positive extravascular hemorrhagic area (n=6 retinas per group). Data are presented as mean±SD. Two-tailed Student *t* test (**B**, **C**, and **E**), Kruskal-Wallis test followed by Dunn post hoc analysis (**H**). Scale bars, 500 μm (**D** and **F**), 100 μm (**G**). NT indicates normotension; and PECAM, platelet endothelial cell adhesion molecule.

ECs, and arterial PLVAP expression (Figure 8H through 8I), suggesting VEGFR2 induction as one of the mechanisms underlying transcytosis-mediated retinal leakage in Dll4 inhibition.

Interestingly, arterial VEGFR2 expression was also upregulated in the hypertensive retina (Figure XXIA and XXIB in the Data Supplement). α-VEGFR2 Ab significantly decreased hypertension-induced EB leakage, the thickness of the superficial vascular layer, and SRF height (Figure XXIII in the Data Supplement). Gain of endothelial Notch activity further suppressed these pathological characteristics, implying the existence of VEGFR2-independent downstream mechanisms. Although VEGF signaling was activated under Dll4-inhibited and hypertensive conditions, angiogenic phenotype was not observed. Taken together, these findings indicate that Dll4 signaling maintains BRB homeostasis by repressing VEGFR2 in the steady state and suggest that blockade of VEGF-VEGFR2 signaling could be a readily applicable therapeutic means for treating hypertension-associated retinal edema, which has no therapeutic consensus now.

As SREBP1 and VEGF signaling are critically involved in transcytosis-mediated BRB disruption, we explored how they work together in this pathophysiology. Intravitreal treatment with α-Dll4 Ab or VEGF upregulated SREBP1 in retinal arteries in vivo (Figure XXIVA and XXIVE in the Data Supplement). SREBP1 is produced initially in precursor form and then cleaved into a mature form that translocates into the nucleus to exert its functions.[26] Immunoblotting showed that α-Dll4 Ab treatment increased both the precursor and mature forms of SREBP1 in retinal lysates (Figure XXIVB through XXIVD in the Data Supplement).

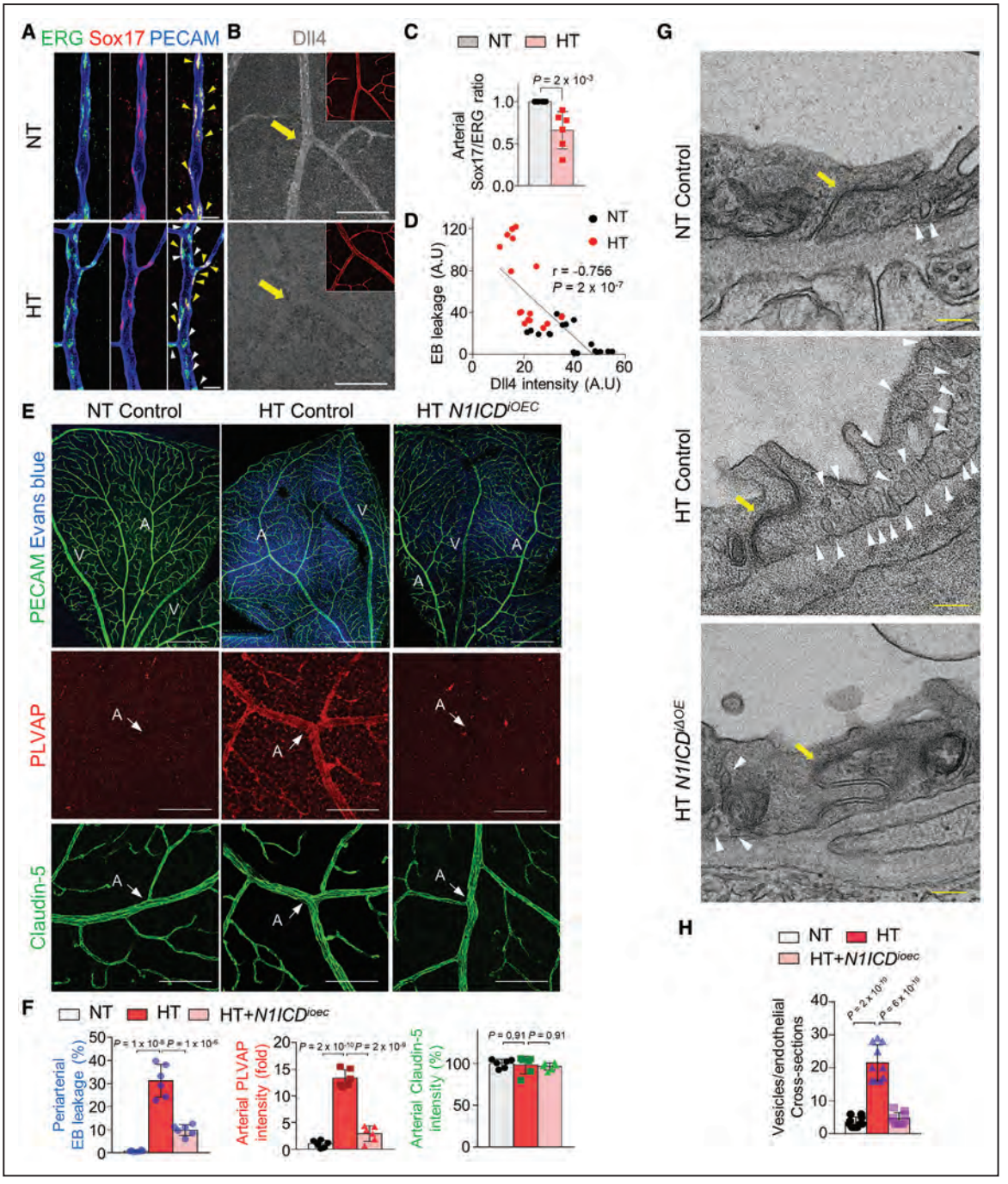

Figure 7. Hypertensive stress induces transcytosis-mediated hyperpermeability in retinal arteries by downregulating Sox17 and DII4.

A and **B**, Repressed Sox17 (**A**) and DII4 (**B**) immunostaining in retinal arteries by Ang II (angiotensin II)–induced hypertension (HT) compared to normotension (NT). **A**, ERG (ETS-related gene), marker of endothelial cell (EC) nuclei. White and yellow arrowheads indicate EC nuclei without and with Sox17 expression, respectively. **B**, Insets, PECAM (platelet endothelial cell adhesion molecule) images. Yellow arrow, artery. **C**, Sox17/ERG ratio in retinal arteries (n=6 retinas per group). Reference value is the ratio of Sox17/ERG in NT condition. Two-tailed Student *t* test. **D**, Scatter plot and linear regression showing an inverse relationship between arterial DII4 expression and periarterial Evans blue (EB) leakage. *r*, Pearson correlation coefficient. **E**–**H**, Rescued blood-retinal barrier (BRB) from HT with gain of EC Notch activity. **E**, Extravasated EB (**upper**) and immunostaining of PLVAP (plasmalemma vesicle-associated protein; **middle**) and claudin-5 (**bottom**) in adult retinas from control mice with NT (NT Control), control mice with HT (HT Control), and mutant mice overexpressing Notch1 intracellular domain in ECs with HT (HT *N1ICD*^{iOE}). **F**, Quantification of periarterial EB leakage and arterial PLVAP (plasmalemma vesicle-associated protein) and claudin-5 intensity (n=6 retinas per group). **G**, Transmission electron microscopy images showing vesicles (white arrowheads) and TJ (yellow arrows) in retinal ECs. No apparent change in TJ. **H**, Quantification of number of vesicles per EC section (n=10 sections per group). Data are presented as mean±SD. One-way ANOVA followed by Tukey post hoc analysis (**F** and **H**). Scale bars, 500 μm (**E**, **upper**), 100 μm (**A**, **B**, and **E**, **middle** and **bottom**), 200 nm (**G**). A indicates artery; and V, vein.

Although VEGF treatment also upregulated both forms, it increased mature SREBP1 higher than precursor SREBP1 and promoted nuclear localization of SREBP1 (Figure XXIVF and XXIVG in the Data Supplement). Notch inhibition using α-Notch1 Ab also increased SREBP1 expression and nuclear localization in HRECs, which were attenuated by co-treatment with α-VEGFR2 Ab (Figure XXIVG through XXIVI in the Data Supplement). These findings suggest that disrupted Dll4-Notch signaling increases SREBP1 expression and promote the nuclear translocation of SREBP1 via VEGF signaling.

DISCUSSION

Here we revealed a novel role of the Dll4-Notch pathway, supporting heterogeneous regulation of CNS vascular permeability in several aspects. First, different molecular players regulate the BRB at different time points. The PDGFB-PDGFRβ, angiopoietin-Tie2, and YAP-TAZ signaling pathways are indispensable for BRB formation and maturation in development but dispensable for adult BRB maintenance, meaning that the mechanisms of adult BRB homeostasis are unknown.[7,39] In the present study, inhibition of retinal Dll4 disrupted adult BRB integrity. Second, Dll4 blocking triggered vascular leakage in the superficial vascular layer specifically in the retina consisting of multiple cell type–specific layers. Dll4 may be specialized to protect ganglion cells located in the superficial layer. Third, regarding vessel type, Dll4 blocking induced rapid and predominant leakage from arteries compared with capillaries and veins. Given that arteries deliver oxygen and nutrients to capillaries instead of a material exchange, Dll4 may support leakage-free delivery inherent to retinal arteries. Regarding clinical relevance, Dll4 expression was repressed in the retinal arteries by hypertensive stress, which induced periarterial leakage. Gain of Notch activity rescued arterial BRB from hypertensive stress. Downregulation of Dll4 may be one of major pathogenic factors of hypertension-induced vascular leakage.

We also demonstrate that TJ and transcytosis can be regulated independently. Though previous studies discovered several players that regulate TJ alone or both TJ and transcytosis, no regulators specific for transcytosis have been identified except Mfsd2a.[18] Dll4 inhibition increased caveolae-mediated transcytosis without TJ disruption in ECs. In addition, partial loss of extracellular matrix may facilitate the diffusion of leaked fluid. Though hemorrhage has been considered a key characteristic of retinal vascular leakage, Dll4 inhibition induced nonhemorrhagic leakage. Considering that hemorrhage occurs due to severe junctional disruption, it could be difficult to find regulatory mechanisms specific for transcytosis in previous studies.

Our study also reveals mechanistically diverse BRB regulation. Pericytes extrinsically control endothelial TJ and transcytosis in growing retinal vessels,[4,40] whereas the endothelial Dll4-Notch pathway regulates transcytosis in a cell-autonomous manner. Antibody-based blockade of retinal Dll4 resulted in transient vascular leakage, followed by BRB recovery, suggesting that it may activate feedback mechanisms for BRB homeostasis. The Notch signaling pathway equipped with feedback mechanisms is useful for maintaining homeostasis in several biological contexts.[13] In line with this, transcriptomic profiling predicted that Dll4 blocking can activate the EGR1 pathway, which is known to inhibit the formation of caveolar vesicles,[24] suggesting a potential feedback mechanism in response to Dll4 blocking. In contrast, inhibition of retinal PDGFRβ by a single injection of blocking antibody resulted in long-lasting BRB disruption.

Our findings indicate that Dll4 preferentially expressed in superficial retinal arteries plays a role in maintaining the arterial BRB in the superficial layer by keeping EC transcytosis in check. On the contrary, our study raises new questions. What mechanisms inhibit the transcytosis of capillaries and veins in the retina? For example, Mfsd2a was reported to regulate transcytosis in retinal capillaries.[18] Dll4 and Mfsd2a may regulate transcytosis complementarily in arteries and capillaries, respectively. How is vascular permeability suppressed in the deep vascular layer of the retina, where Dll4 is rarely expressed? As diabetic retinopathy frequently accompanies vascular leakage in the deep layer, studies of diabetic retinal edema will elucidate the mechanisms involved.[33]

EC-specific transcription factor Sox17 promotes arterial differentiation by driving Dll4 expression in primitive vessels of the growing retina.[41] Sox17 was continuously expressed in arterial ECs in the stabilized adult retina. Specific deletion of *Sox17* from adult ECs repressed Dll4 expression and phenocopied vascular defects in the retina with Dll4 inhibition. These findings establish the conserved role of the Sox17-Dll4 axis in arterial contexts. Sox17 belongs to the SoxF subgroup, with Sox7 and Sox18 based on the high similarity of their nucleic acid sequences.[42] Triple deletion of these genes, but not single deletion of *Sox17*, from ECs, results in dermal vascular leakage.[43] However, *Sox17* deletion alone from ECs disrupts the BRB, suggesting heterogeneous ways for maintaining vascular integrity depending on the tissue type.

ECs exhibited a range of transcriptional changes that respond to Dll4 inhibition. It may include bases with unrecognized changes in our analyses. Nevertheless, transcriptional profiling in the present study predicted significant changes in gene sets associated with vesicles and transport after Dll4 blocking, confirming the changes in gene expression involved in several steps of transcytosis induction. Notably, SREBP1, the master lipogenic transcription factor, is one of the pathways predicted to govern this transcriptional change.[23] SREBP1 activated by blocking retinal Dll4 may shift the lipid composition of the plasma membrane from unfavorable to favorable for caveolae formation in retinal

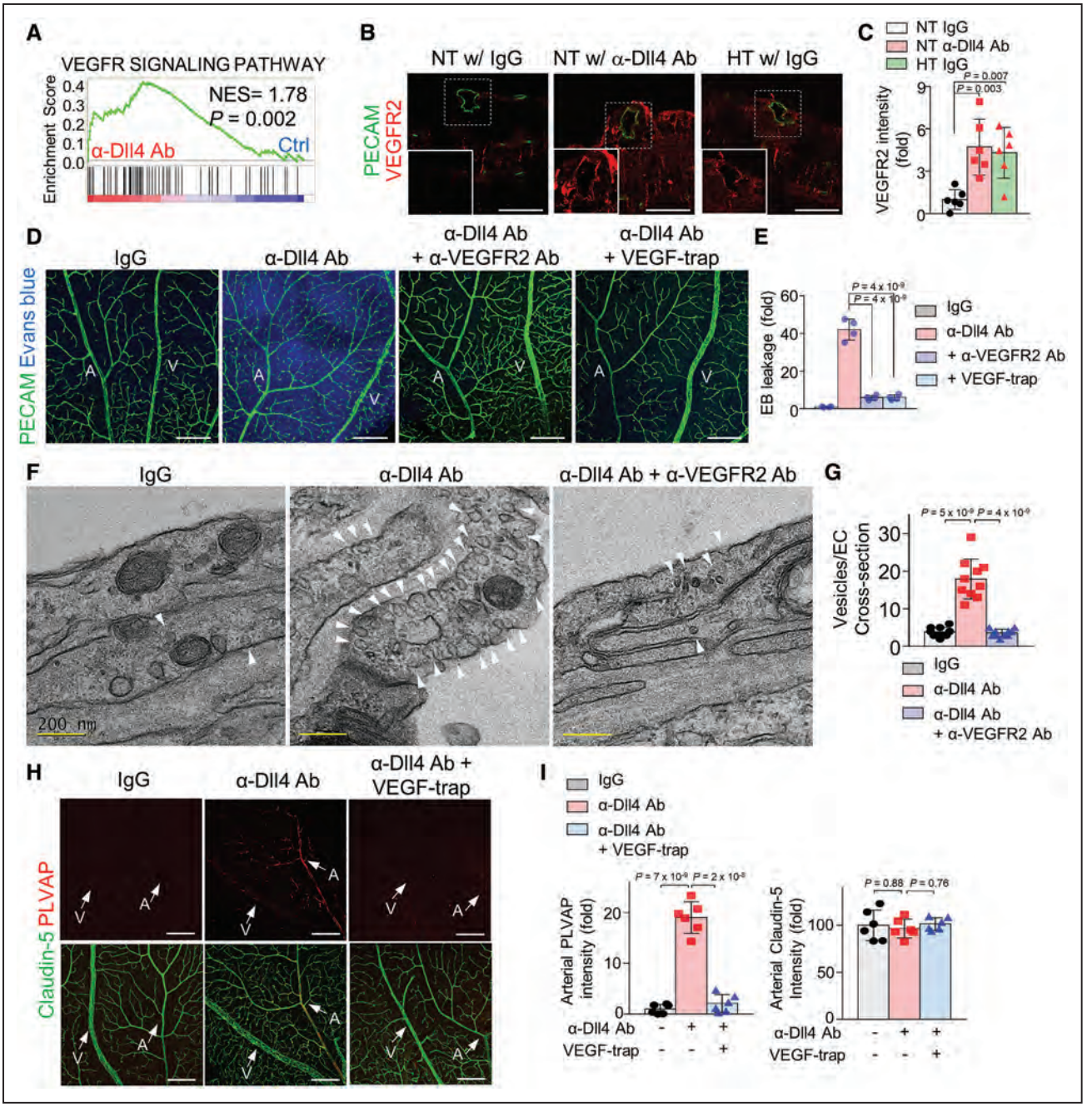

Figure 8. Blocking the VEGF (vascular endothelial growth factor)-VEGFR2 (VEGF receptor 2) pathway attenuates DII4 inhibition–induced retinal leakage by suppressing endothelial cell (EC) transcytosis.

A, Gene set enrichment analysis (GSEA) plots showing upregulation of gene sets related to VEGFR (vascular endothelial cell growth factor receptor) signaling pathway in α-DII4 Ab-treated retinal endothelial cells (ECs). **B**, Cryosectioned images of adult retina showing increased VEGFR2 immunostaining in arterial ECs with α-DII4 Ab treatment and Ang II–induced hypertension (HT). Insets are magnified images of the outlined area. **C**, Quantification of VEGFR2 intensity in arterial ECs (n=6 retinas per group). **D**, Attenuation of DII4 inhibition induced Evans blue (EB) leakage by blocking VEGF signaling (**E**, quantification of extravasated EB, n=4 retinas per group). VEGF-trap, aflibercept. **F**, Transmission electron microscopy image showing a decreased number of transcytotic vesicles (arrowheads) in α-DII4 Ab-treated retinal ECs by α-VEGFR2 Ab treatment. **G**, Quantification of vesicle number per EC cross-section (n=10 sections per group). **H**, DII4 inhibition–induced arterial PLVAP (plasmalemma vesicle-associated protein) immunostaining by VEGF trap. No changes in claudin-5 immunostaining. **I**, Quantification of arterial PLVAP and claudin-5 intensity (n=6 retinas per group). Data are presented as mean±SD. One-way ANOVA followed by Tukey post hoc analysis. Scale bars: 200 μm (**D** and **H**), 50 μm (**B**), 200 nm (**H**). A indicates artery; NES, normalized enrichment score; NT, normotension; PECAM, platelet endothelial cell adhesion molecule; and V, vein.

ECs, leading to the formation of caveolar vesicles. In line with this, a previous study reported that SREBP1 promotes transcription of *caveolin-1* in ECs by binding to sterol regulatory element sites.[44] Importantly, SREBP1 inhibition rescued BRB integrity under DII4 inhibition and hypertensive stress. These findings suggest the

molecular axis of Sox17-Dll4-SREBP1 as a key regulatory system of transcytosis in arterial ECs.

In this work, retinal ECs exhibited hypertension-related transcriptional changes responding to Dll4 blocking. Similarly, hypertension was one of the most frequent adverse events in cancer patients treated with α-Dll4 antibodies.[29] Thus, Dll4 blocking may be associated with hypertension. Hypertensive stress induces nonhemorrhagic leakage from arteries in the superficial layer of the mouse retina, similar to periarterial leakage and macular edema, a representative symptom resulting from nonhemorrhagic leakage in patients with severe hypertension. Importantly, a gain in Notch activity in ECs rescued the BRB from hypertensive stress. Mechanistically, hypertensive stress repressed the expression of both Sox17 and Dll4, which are crucial for arterial BRB maintenance, partially via oxidative stress, and subsequently induced transcytosis-mediated leakage. This result suggests that dysregulation of specific regulatory mechanisms in disease contexts may cause selective, rather than random, leakage regarding region and process.

Existing retinopathy models accompany bleeding due to severe vascular damage, hindering the validation of whether transcytosis is involved in pathological leakage.[45] In contrast, hypertensive mouse models have minimal morphological and structural changes in retinal vessels and are useful for identifying transcytosis as a major cause of pathological leakage in the retina. Importantly, Dll4 blocking and hypertensive stress led to SRF accumulation, suggesting that transcytosis can induce copious leakage.

The leakage pattern of retinal vessels in hypertensive mouse models was very similar to that of patients with severe hypertension. Hypertensive stress resulted in superficial vascular edema specifically in the mouse retina and caused SRF accumulation. Similarly, in retinal image analysis, a fraction of patients with severe hypertension exhibited both thickening of the superficial layers and SRF accumulation. Considering that vascular leakage is predominant in the deep vascular layers in diabetic retinopathy,[33] our findings suggest that hypertensive stress and diabetic stress may induce retinal edema mediated by a regionally different type of vascular leakage.

Blockade of VEGF is an effective therapy for curing retinal edema.[35,46] Retinal ECs after Dll4 blocking had a gene expression signature of activated VEGF signaling, although they did not show an angiogenic phenotype. Dll4 blocking upregulated VEGFR2 expression in arterial ECs of the retina, and VEGFR2 neutralizing antibody effectively attenuated the retinal vascular leakage induced by Dll4 inhibition and hypertensive stress. No therapeutic consensus has been reached for hypertension-associated retinal edema,[3,47] but our results suggest blockade of VEGF signaling as a readily applicable therapeutic means. Mechanistically, blockade of VEGF signaling may suppress transcytosis, as shown by repressed PLVAP expression. This suggests that the activated VEGF pathway is contextually able to increase vascular permeability via transcellular transport in retinal vessels, though it also triggers hyperpermeability by junctional transport in general. In accordance, recent studies reported that VEGF signaling increases transcellular transport in lymphatics.[48,49] How VEGF signaling promotes transcytosis has not been well-elucidated, but our findings suggest that activated VEGF signaling stimulates the expression and nuclear localization of SREBP1, which is important for caveolar vesicle formation.

We revealed the association between VEGF signaling and transcytosis in HR and suggest that transcytosis is implicated in other retinal diseases with leakage. We also found that transcytosis can induce pathological leakage without hemorrhage. In clinic, retinal leakage often persists even after hemorrhage is absorbed,[34] pointing to different underlying pathophysiology between hemorrhagic and nonhemorrhagic leakage. Nonhemorrhagic leakage appears to underlie macular edema or hard exudate in a spectrum of diseases, including HR, diabetic retinopathy, and Coats disease.[1,50,51] Moreover, additional complications of hypertension such as hypertensive encephalopathy and hypertensive nephropathy involve vascular leakage as a major pathology.[52] Future studies will uncover whether transcytosis is responsible for leakage in these hypertensive complications. Our findings suggest that transcytosis may be a target to develop therapeutic strategies in vascular disorders with vascular leakage.

In conclusion, we defined Dll4 signaling as a specialized mechanism for arterial BRB homeostasis in the retina with anatomic and vascular heterogeneities (Figure XXV in the Data Supplement). Dll4 signaling protects retinal ganglion cells located in the superficial retinal layers from the risk of edema and supports the delivery function inherent to retinal arteries by preventing material exchange en route to capillaries. Hypertension induces selective, not global, leakage in the retinal arteries via Dll4 downregulation, underscoring the strategic advantage of architecturally divided BRB regulation in disease. Our findings identify the molecular axis of Sox17-Dll4-SREBP1 as a key regulatory pathway of transcytosis in arterial ECs independent of TJ integrity and elucidate that uncontrolled transcytosis is associated with pathological leakage in retinal diseases.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Expanded Methods
Online Figures I–XXV
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