Arteriosclerosis, Thrombosis, and Vascular Biology

BASIC SCIENCES

Desert Hedgehog-Driven Endothelium Integrity Is Enhanced by Gas1 (Growth Arrest-Specific 1) but Negatively Regulated by Cdon (Cell Adhesion Molecule-Related/Downregulated by Oncogenes)

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OBJECTIVE: Evidences accumulated within the past decades identified hedgehog signaling as a new regulator of endothelium integrity. More specifically, we recently identified Dhh (desert hedgehog) as a downstream effector of Klf2 (Kruppel-like factor 2) in endothelial cells (ECs). The purpose of this study is to investigate whether hedgehog coreceptors Gas1 (growth arrest-specific 1) and Cdon (cell adhesion molecule-related/downregulated by oncogenes) may be used as therapeutic targets to modulate Dhh signaling in ECs.

APPROACH AND RESULTS: We demonstrated that both Gas1 and Cdon are expressed in adult ECs and relied on either siRNAs- or EC-specific conditional knockout mice to investigate their role. We found that Gas1 deficiency mainly phenocopies Dhh deficiency especially by inducing VCAM-1 (vascular cell adhesion molecule 1) and ICAM-1 (intercellular adhesion molecule 1) overexpression while Cdon deficiency has opposite effects by promoting endothelial junction integrity. At a molecular level, Cdon prevents Dhh binding to Ptch1 (patched-1) and thus acts as a decoy receptor for Dhh, while Gas1 promotes Dhh binding to Smo (smoothened) and as a result potentiates Dhh effects. Since Cdon is upregulated in ECs treated by inflammatory cytokines, including TNF (tumor necrosis factor)-α and Il (interleukin)-1β, we then tested whether Cdon inhibition would promote endothelium integrity in acute inflammatory conditions and found that both fibrinogen and IgG extravasation were decreased in association with an increased Cdh5 (cadherin-5) expression in the brain cortex of EC-specific Cdon knockout mice administered locally with Il-1β.

CONCLUSIONS: Altogether, these results demonstrate that Gas1 is a positive regulator of Dhh in ECs while Cdon is a negative regulator. Interestingly, Cdon blocking molecules may then be used to promote endothelium integrity, at least in inflammatory conditions.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: cytokines ■ endothelial cells ■ endothelium ■ hedgehogs ■ therapeutics

Endothelium integrity, that is, preserved endothelial anatomic structure and function is essential to vascular homeostasis since a failure of this system represents a critical factor in cardiovascular and cerebrovascular disease pathogenesis. Indeed, the endothelium is involved in many physiological processes, such as regulation of vascular permeability, vascular tone, blood coagulation, as well as homing of immune cells to specific sites of the body. Conversely, endothelial dysfunction is associated with excessive vasoconstriction especially because of impaired endothelial nitric oxide production. Also, endothelial dysfunction is characterized by abnormal vascular leakage due to altered endothelial intercellular junctions. Finally, dysfunctional endothelial

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Nonstandard Abbreviations and Acronyms

BBB	blood-brain barrier
Cdh5	cadherin-5 or vascular endothelial cadherin
Cdon	cell adhesion molecule-related/down-regulated by oncogenes
Dhh	desert hedgehog
EBM-2	endothelial basal medium-2
EC	endothelial cell
FL-Dhh	full-length desert hedgehog
Gas1	growth arrest-specific 1
Gli	Gli family zinc finger
HBMEC	human brain microvascular EC
Hhip	hedgehog interacting protein
HUVEC	human umbilical vein EC
ICAM-1	intercellular adhesion molecule 1
Il-1β	interleukin-1β
Klf2	Kruppel-like factor 2
NF-κB	nuclear factor-κB
Ptch1	patched-1
Shh	sonic hedgehog
Smo	smoothened
TNF-α	tumor necrosis factor α
VCAM-1	vascular cell adhesion molecule 1

Highlights

- Gas1 (Growth arrest-specific 1) and Cdon (cell adhesion molecule-related/downregulated by oncogenes) are differentially regulated by inflammatory cytokines and modulate desert hedgehog effects in endothelial cells.
- Gas1 promotes desert hedgehog-induced endothelial immune quiescence by promoting desert hedgehog interaction with Smo (smoothened).
- Cdon prevents desert hedgehog binding to Ptch1 (patched-1) and by doing so, destabilizes endothelial adherens junctions and promotes angiogenesis.
- Cdon blocking molecules may be used to preserve endothelium integrity especially at the blood-brain barrier in the setting of neuroinflammation.

cells (ECs) acquire proinflammatory and prothrombotic phenotypes by expressing increased levels of adhesion and prothrombotic molecules, such as VCAM-1 (vascular cell adhesion molecule 1) and ICAM-1 (intercellular adhesion molecule 1).[1]

Evidences accumulated within the past decades identified hedgehog signaling as a new regulator of endothelium integrity.[2] For instance, hedgehog signaling was shown to promote blood-brain barrier (BBB) integrity and immune quiescence both in the setting of multiple sclerosis[3] and in the setting of stroke.[4] Additionally, we have shown that disruption of hedgehog signaling specifically in ECs induces blood-nerve barrier breakdown and peripheral nerve inflammation.[5] While investigating molecular mechanisms underlying hedgehog regulation of endothelium integrity, we found that Dhh (desert hedgehog) is the main hedgehog ligand express by ECs.[6,7] Of note, Indian hedgehog was reported to be expressed in some specific vascular bed including the one of the eye choroid[8,9] while Shh (sonic hedgehog) is not expressed by ECs.[6,7] Importantly, *Dhh* knockout in ECs leads to the disruption of Cdh5 (cadherin-5)/β-catenin interaction and spontaneous vascular leakage, an increased expression of adhesion molecules, including VCAM-1 and ICAM-1 and increased angiogenic capabilities.[10] Besides, Dhh, which is upregulated by blood flow and downregulated by inflammatory cytokines, appears to be a downstream effector of the master regulator of endothelial integrity Klf2 (Kruppel-like factor 2).[6]

The hedgehog family of morphogens, which includes Shh, Indian hedgehog, and Dhh, was identified nearly 4 decades ago in drosophila as crucial regulators of cell fate determination during embryogenesis.[11] The interaction of hedgehog proteins with their specific receptor Ptch1 (Patched-1) derepresses the transmembrane protein Smo (smoothened), which activates downstream pathways, including the hedgehog canonical pathway leading to the activation of Gli (Gli family zinc finger) transcription factors and so-called hedgehog noncanonical pathways, which are independent of Smo and Gli.[12]

The hedgehog ligand binding to Ptch1 is regulated by several coreceptors. Among these, Cdon (cell adhesion molecule-related/downregulated by oncogenes), Boc (Brother of Cdon), and Gas1 (growth arrest-specific 1) are suggested to promote hedgehog ligand interaction with Ptch1 while Hhip (Hedgehog interacting protein) inhibits it.[13]

Cdon and Boc proteins are cell surface glycoproteins belonging to a subgroup of the immunoglobulin superfamily of cell adhesion molecules, which also includes the Robo axon-guidance receptors. Their ectodomain, respectively, contains 5 and 4 Ig-like domains, followed by 3 FNIII (type III fibronectin) repeats (FNIII 1–3), a single transmembrane domain and a divergent intracellular region of variable length.[14] Cdon was shown to interact with all of the 3 N-terminal active hedgehog peptides through its third FNIII domain.

Gas1 was identified as one of 6 genes that were transcriptionally upregulated in NIH3T3 cells arrested in cell cycle upon serum starvation. *Gas1* encodes a 45 kDa GPI-anchored cell surface protein that binds N-terminal Shh with high affinity.[15]

The goal of the present study is to investigate whether Dhh-induced endothelial integrity depends on hedgehog coreceptors. This is essential to determine whether

such coreceptors could be used as therapeutic target to enhance Dhh-induced signaling in ECs under pathological conditions.

MATERIALS AND METHODS

The authors declare that all supporting data are available within the article and its Data Supplement.

Mice

Cdon floxed (*Cdon*^{Flox}) mice (Figure IA in the Data Supplement) were generated at the Institut Clinique de la Souris through the International Mouse Phenotyping Consortium from a vector generated by the European conditional mice mutagenesis program, EUCOMM. *Cdon* floxed mice were generated under a C57BL/6N background and backcrossed at least 6 times with C57BL/6J mice before they were used in any experiment. *Gas1*$^{tm3.1Fan}$ (*Gas1*^{Flox}) mice[16(p1)] were kindly given by C.M. Fan. Gas1 floxed mice were obtained under a 129 background and backcrossed at least 6 times with C57BL/6J mice before they were used in any experiment. Tg(Cdh5-cre/ERT2)1Rha (Cdh5-Cre/ERT2) mice,[17] which were a gift from RH. Adams were obtained and maintained under a C57BL/6J background.

Cdh5-Cre/ERT2 mice were genotyped using the following primers: 5′-TAAAGATATCTCACGTACTGACGGTG-3′ and 5′-TCTCTGACCAGAGTCATCCTTAGC-3′ that amplify 493 bp of the Cre recombinase sequence. Cdon floxed mice were genotyped using the following primers: 5′-CTTCCCAGAGGGTGTGAGAGCAATG-3′ and 5′-GAACCAG TAGCATGCATGATGCTGG-3′ which amplifies a 385 bp fragment of the WT allele or a 494 bp fragment if the allele is floxed. *Gas1* floxed mice were genotyped using the following primers: 5′-GAATCGAAGCGCCTGGACC-3′ and 5′-GGAA AACCGCACAGAAGAGGG-3′ which amplifies a 285 bp fragment of the WT allele or a 360 bp fragment if the allele is floxed.

Animal experiments were performed in accordance with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and approved by the local Animal Care and Use Committee of Bordeaux University.

The Cre recombinase in Cdh5-Cre/ERT2 mice was activated by intraperitoneal injection of 1 mg tamoxifen for 5 consecutive days at 8 weeks of age. Mice were phenotyped 2 weeks later. Successful and specific activation of the Cre recombinase is shown in Figure IB and IC in the Data Supplement.[6] Notably, only female were used in both the corneal angiogenesis and Miles assays because male fight and are more likely to have their cornea and back skin injured. Only males were used in the lipopolysaccharide and Ad-Il (interleukin)-1β protocols. At the end of experiments animal was euthanized via cervical dislocation.

Mouse Corneal Angiogenesis Assay

Pellets were prepared as previously described.[18] Briefly, 5 µg of VEGFA (vascular endothelial growth factor A; Shenandoah biotechnology diluted in 10 µL sterile PBS was mixed with 2.5 mg sucrose octasulfate-aluminum complex (Sigma-Aldrich Co, St Louis, MO), then 10 µL of 12% hydron in ethanol was added. The suspension was deposited on a 400-µm nylon mesh (Sefar America Inc, Depew, NY), then both sides of the mesh were covered with a thin layer of hydron and allowed to dry.

Female mice were anesthetized with an intraperitoneal injection of ketamine 100 mg/kg and xylazine 10 mg/kg. The eyes of the mice eyes were topically anesthetized with 0.5% proparacaine. The globe of the eye was proptosed with jeweler's forceps taking care not to damage the limbus vessel surrounding the base of the globe. Sterile saline was also applied directly to each eye as needed during the procedure to prevent excessive drying of the cornea and to facilitate insertion of the pellet into the lamellar pocket of the eyes. Using an operating microscope, a central, intrasomal linear keratotomy was performed with a surgical blade parallel to the insertion of the lateral rectus muscle. Using a modified von Graefe knife, a lamellar micro pocket was made toward the temporal limbus by rocking the von Graefe knife back and forth.

VEGFA containing pellet was placed on the cornea surface with jeweler's forceps at the opening of the lamellar pocket. A drop of saline was applied directly to the pellet and using the modified von Graefe knife, the pellet was gently advanced to the temporal end of the pocket. Buprenorphine was given at a dose of 0.05 mg/kg subcutaneously on the day of surgery.

Nine days after pellet implantation, mice were euthanized, and then eyes were harvested and fixed with 2% paraformaldehyde. Capillaries were stained with rat anti-mouse CD31 antibodies (BMA Biomedicals, catalog no. T-2001), primary antibodies were visualized with Alexa 568-conjugated anti-rat antibodies (Invitrogen). Pictures were taken under ×50 magnification. Angiogenesis was quantified as the CD31$^+$ surface area using Image J software. Notably, such quantification is a rough quantification of angiogenesis, which does not discriminate increased vessel density from increased vessel length. The corneas were given a number at the time of collection so that pictures were taken blinded and quantifications were done blinded.

In Vivo Permeability Assessment (Miles Assay)

The back of female mice was shaved. Seventy-two hours, later mice were administered with 100 µL 1% Evans blue via retro-orbital injection. Subsequently, they were administered with 50 µL NaCl 0.9% containing or not 20 ng VEGFA (Shenandoah biotechnology) subcutaneously at 6 spots on their back. Buprenorphine was given at a dose of 0.05 mg/kg subcutaneously on the day of surgery.

Thirty minutes later, mice were euthanized, skin biopsy around each injection point were then harvested to quantify Evans blue extravasation. Evans blue dye was extracted from the skin by incubation at 65 °C with formamide. The concentration of Evans blue dye extracted was determined spectrophotometrically at 620 nm with a reference at 740 nm. The genotype of the mice was not known by the experimenter during the entire procedure.

Lipopolysaccharide-Induced Systemic Inflammation

Mice were administered with 0.05 mg/kg buprenorphine subcutaneously to avoid any pain. Thirty minutes later, they were administered with 10 mg/kg lipopolysaccharide (Sigma) intraperitoneally. Finally, mice were euthanized by cervical

dislocation 6 hours after lipopolysaccharide administration, the left lung was harvest, fixed in methanol, paraffin embedded, and cut into 7 µm thick sections.

GR1$^+$ cells were quantified after GR1 staining of lung cross sections, using Image J software in 10 pictures taken under ×20 magnification. One section per lung was quantified for each mouse. Lungs were given a number at the time of collection, so that pictures were taken blinded and quantifications were done blinded.

Ad-Il-1β Stereotaxic Injections

Mice were anesthetized using isoflurane and placed into a stereotactic frame (Stoelting). An ophthalmic ointment was applied at the ocular surface to maintain eye hydration during the time of surgery. The skull was shaved and the skin incised on 1 cm to expose the skull cap. Then, a hole was drilled into the cerebral cortex and 3 µL of an Ad-Il-1β[19] or AdDL70 control (AdCtrl), (10^7 pfu) solution was microinjected at y=1 mm caudal to bregma, x=2 mm, z=1.5 mm using a Hamilton syringe, into the cerebral cortex and infused for 3 minutes before removing the needle from the skull hole.[20] Mice received a subcutaneous injection of buprenorphine (0.05 mg/kg) 30 minutes before surgery and again 8 hours postsurgery to assure a constant analgesia during the procedure and postoperatively. Mice were euthanized 7 days postsurgery. For histological assessment, brains were harvested and fixed in formalin for 3 hours before being incubated in 30% sucrose overnight and optimal cutting temperature embedded. Then, for each brain, the lesion area identified by the puncture site was cut into 7 µm thick sections.

BBB permeability was evaluated by measuring tight junction integrity and plasmatic protein extravasation. For each brain, Cdh5$^+$, Fibrinogen$^+$, and IgG$^+$ areas were quantified, using Image J software, in 20 pictures taken at the margins of the lesion area under ×40 magnification. CD45$^+$ leukocytes were counted in 20 pictures randomly taken under ×40 magnification. CD11b$^+$, Gfap$^+$ (glial fibrillary acidic protein), and NeuN$^+$ areas were quantified in 10 pictures taken in and around the lesion area under ×20 magnification. One section, localized in the Il-1β-induced inflammatory lesion area, per brain was quantified for each mouse. Brains were given a number at the time of collection, so that pictures were taken blinded and quantifications were done blinded.

Immunostaining

Before staining, whole-mount corneas were fixed with 2.5% formaline for 10 minutes, brain was fixed with 10% formaline for 3 hours, heart and lungs were fixed with methanol for 24 hours, and cultured cells were fixed with 10% formaline for 10 minutes.

Capillaries were identified using rat anti-mouse CD31 antibodies (BMA Biomedicals, catalog no. T-2001). Neutrophils were stained with rat anti-Ly6G (GR1) antibodies (BD Pharmingen Inc, catalog no. 551459). Human Cdh5 was stained using mouse anti-human Cdh5 antibodies (Santa Cruz Biotechnology, Inc, catalog no. sc-9989). Mouse Cdh5 was stained using goat anti-mouse Cdh5 antibodies (R&D systems, catalog no. AF1002). Albumin and fibrinogen were stained using sheep anti-albumin antibodies (Abcam, catalog no. ab8940) and rabbit anti-fibrinogen antibodies (Dako, catalog no. A0080), respectively. Mouse IgGs were stained with Alexa Fluor 568-conjugated donkey anti-mouse IgG (Invitrogen, catalog no. A-10037). Pan-leucocytes were identified using rat anti-mouse CD45 antibodies (BD Pharmingen Inc, catalog no. 550539). CD11b$^+$ microglia and macrophages were identified using rat anti-CD11b antibodies (Invitrogen, catalog no. 14-0112-82). GFAP was stained using rabbit anti-GFAP antibodies (Invitrogen, catalog no. OPA1-06100). Neurons were identified using anti-NeuN antibodies (Millipore, catalog no. ABN78). Cdon was stained using goat anti-mouse Cdon antibodies (R&D Systems, catalog no. AF2429). Gas1 was stained using goat anti-human Gas1 antibodies (R&D Systems, catalog no. AF2636). Dhh was stained using mouse anti-Dhh antibodies (Santa Cruz Biotechnology, Inc, catalog no. sc-271168). Ptch1 was stained using rabbit anti-Ptch1 antibodies (Abcam, catalog no. ab53715). For immunofluorescence analyzes, primary antibodies were resolved with Alexa Fluor-conjugated secondary polyclonal antibodies (Invitrogen, catalog no. A-21206, A-21208, A-11077, A-11057, A-31573, and A-10037), and nuclei were counterstained with DAPI (1/5000). For both immunohistochemical and immunofluorescence analyses, negative controls using secondary antibodies only were done to check for antibody specificity.

Cell Culture

In vitro experiments were performed using human umbilical vein ECs (HUVECs; Lonza), human dermal microvascular ECs (Lonza), or human brain microvascular ECs (HBMECs; Alphabioregen). HUVECs and HBMECs were cultured in EBM-2 (endothelial basal medium-2) supplemented with EGM-2 BulletKits (Lonza). Human dermal microvascular ECs were cultured in EBM-2 supplemented with EGM-2 MV BulletKits (Lonza). Cells from passage 3 to passage 6 were used. Before any treatment cells were serum starved in 0.5% fetal bovine serum medium for 24 hours. HUVECs were cultured in Opti-MEM to perform assays in serum-free conditions. HeLa ATCCCCL-2 cells were cultured in Roswell Park Memorial Institute medium supplemented with 10% fetal bovine serum.

Cell culture assays are described in the Data Supplement.

Statistics

Results are reported as mean±SEM. Comparisons between groups were analyzed for significance with the nonparametric Mann-Whitney test or the Kruskal-Wallis test followed by Dunn multiple comparisons test using GraphPad Prism v8.0.2 (GraphPad Inc, San Diego, CA). Differences between groups were considered significant when $P \leq 0.05$ (*$P \leq 0.05$; **$P \leq 0.01$; and ***$P \leq 0.001$).

RESULTS

ECs Express Cdon, Gas1, and Hhip but Not Boc

First, we searched for *Cdon*, *Boc*, *Gas1*, and *Hhip* expression in human ECs from different origin, including HUVECs, human dermal microvascular ECs, and HBMECs via real-time polymerase chain reaction. As shown in Figure 1A, human ECs express *Hhip*, *Cdon*, and *Gas1* mRNA while *Boc* mRNA is express at a lower level.

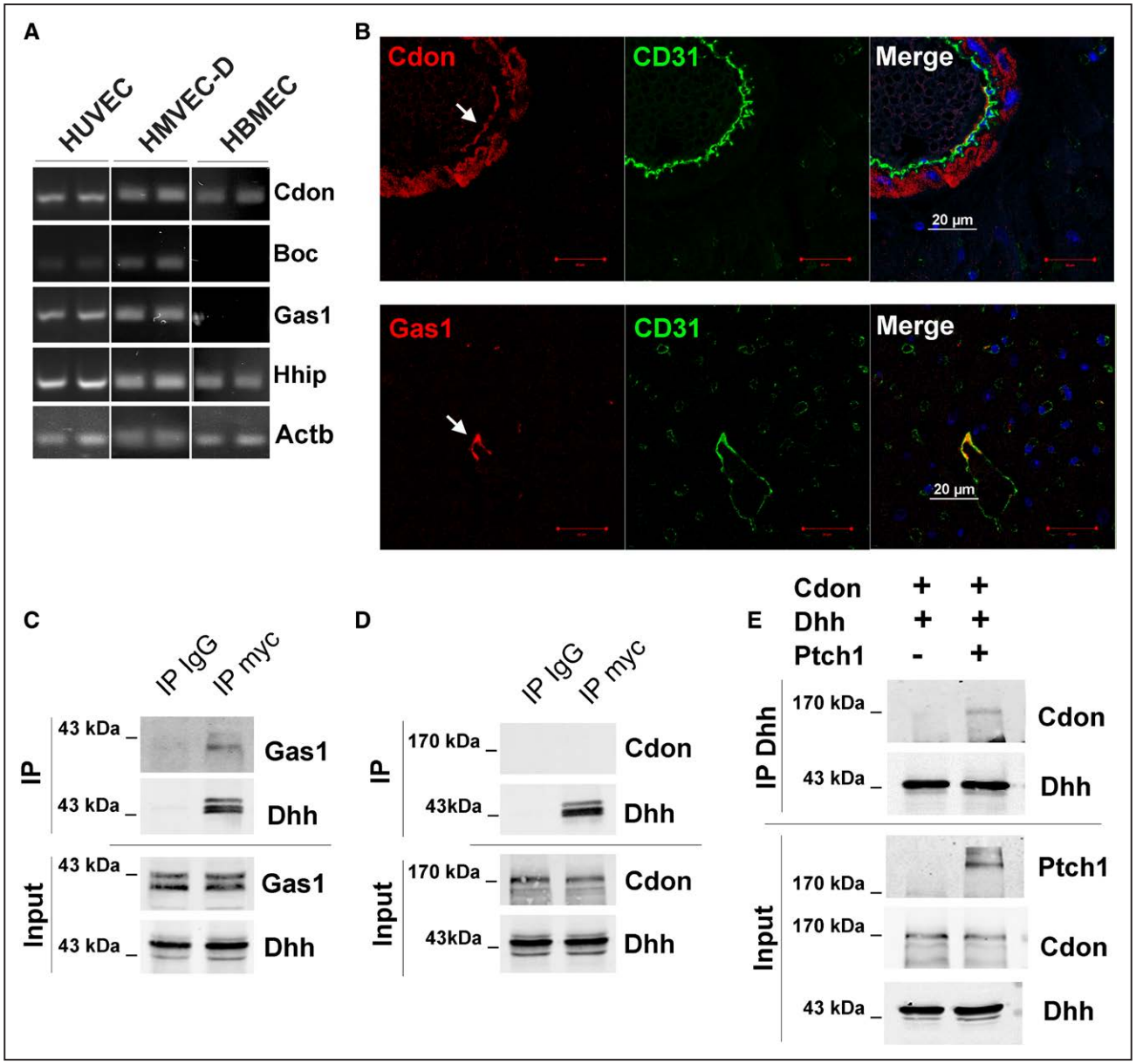

Figure 1. Endothelial cells (ECs) express Cdon (cell adhesion molecule-related/downregulated by oncogenes), Gas1 (growth arrest-specific 1), and Hhip (hedgehog interacting protein).
A, *Cdon*, *Boc*, *Gas1*, *Hhip*, and *Actb* mRNA expression were evaluated via real-time polymerase chain reaction (RT-PCR) in human umbilical vein ECs (HUVECs), human dermal microvascular ECs (HMVECs-D), and human brain microvascular ECs (HBMECs). **B**, Heart cross section from wild-type mice were coimmunostained with anti-Cdon (in red) or anti-Gas1 (in red) antibodies together with anti-CD31 (in green) antibodies to identify Cdon or Gas1 expression in ECs, respectively. **C**, HeLa were cotransfected with Gas1 and myc-tagged desert hedgehog (Dhh) encoding vectors. Gas1 interaction with Dhh was evaluated by coimmunoprecipitation assay. **D**, HeLa were cotransfected with Cdon and myc-tagged Dhh encoding vectors. Cdon interaction with Dhh was evaluated by coimmunoprecipitation assay. **E**, HeLa were cotransfected with Cdon, Ptch1 (patched-1), and Dhh encoding vectors. Cdon interaction with Dhh was evaluated by coimmunoprecipitation assay.

Notably, *Gas1* is not detected in HBMECs. Since the role of endothelial Hhip has already been reported in several articles,[21–23] we focused our investigations on Gas1 and Cdon and confirmed their endothelial expression via immunostaining (Figure 1B). Interestingly, while TNF (tumor necrosis factor)-α inhibits Ga*s1* mRNA expression in HUVECs (Figure IIA in the Data Supplement), it increases *Cdon* mRNA expression (Figure IIB in the Data Supplement). Notably, TNF-α-induced *Cdon* mRNA expression depends on NF-κB (nuclear factor-κB) activity (Figure IIC in the Data Supplement).

Next, we performed coimmunoprecipitation assays to verify that Dhh is able to bind these receptors. Indeed, Dhh is the main hedgehog ligand expressed by ECs, especially HUVECs, human dermal microvascular ECs, and HBMECs (Figure IID in the Data Supplement). While we found that Dhh binds Gas1 directly (Figure 1C), Cdon alone cannot bind Dhh (Figure 1D).

However, Dhh can bind Cdon in the presence of Ptch1 (Figure 1E). Consistently, Cdon colocalizes with Dhh only in the presence of Ptch1 while Gas1 colocalizes with Dhh both in the presence and absence of Ptch1. Both Gas1 and Cdon are internalized once they bind Dhh (Figure III in the Data Supplement).

With the aim to investigate the role of Gas1 and Cdon in ECs, we performed a series of in vitro and in vivo assays using siRNAs and EC-specific conditional knockout mice, respectively.

Cdon Promotes EC Proliferation, Migration, and Angiogenesis

The role of Gas1 and Cdon in angiogenesis was investigated using the mouse corneal angiogenesis assay. Mice deficient for Gas1 or Cdon expression in EC together with their respective control littermates were implanted with VEGFA containing pellets. While VEGFA-induced angiogenesis was not different in *Gas1*^{ECKO} mice from their control littermates (Figure 2A and 2B), VEGFA-induced angiogenesis was significantly inhibited in *Cdon*^{ECKO} mice compared with their control littermates (Figure 2C and 2D). Consistently, in vitro experiments performed in HUVECs showed that both EC proliferation (Figure 2E) and VEGFA-induced EC migration (Figure 2F) were decreased after *Cdon* knockdown (Figure IVA in the Data Supplement). *Gas1* knockdown (Figure IVB in the Data Supplement) did not modify EC proliferation or VEGFA-induced migration (Figure 2E and 2F). However, *Gas1* knockdown did promote EC migration in the absence of VEGFA (Figure 2F).

This set of data demonstrates that Cdon is proangiogenic. Notably, this effect works in the opposite direction to Dhh anti-angiogenic effect.[10]

Gas1 Prevent EC Activation and Lipopolysaccharide-Induced Neutrophil Recruitment

To investigate the role of Gas1 and Cdon in regulating EC immune quiescence, HUVECs were transfected with *Gas1*, *Cdon*, or control siRNAs. VCAM-1 and ICAM-1 expression was measured via both real-time quantitative polymerase chain reaction and Western blot analyses. While *Gas1* knockdown significantly increased both VCAM-1 and ICAM-1 expression, *Cdon* knockdown did not (Figure 3A and 3C). These results were confirmed in TNF-α-treated cells (Figure 3D and 3E). Finally, to assess the functional consequences of EC activation in vivo, we quantified neutrophils recruitment in the lungs of mice that were administered with lipopolysaccharide. As expected, neutrophil density in the lung of *Gas1*^{ECKO} mice was significantly increased (Figure 3F and 3G) compared with their control littermates while we found no difference between *Cdon*^{ECKO} mice and control littermates (Figure 3H and 3I).

Altogether these data demonstrate that Gas1 prevents EC activation similarly to Dhh.[6] On the contrary, Cdon does not seem to participate in the regulation of EC activation.

Cdon Disrupts Adherens Junction Integrity

The role of Gas1 and Cdon in controlling endothelial intercellular junction integrity was first investigated in vitro. HUVECs were transfected with *Gas1*, *Cdon*, or control siRNAs. Adherens junction integrity was quantified after Cdh5 immunostaining (Figure 4A) and endothelium permeability using Transwells. We found that *Gas1* knockdown disrupts Cdh5-dependent junction integrity (Cdh5 junctions acquire a thicker, nonlinear phenotype; Figure 4B) while *Cdon* knockdown prevents EC permeability (Figure 4C). *Gas1* knockdown did not show any effects in the permeability assay test. Indeed, endothelial Cdh5 junction may have a nonlinear phenotype but still be continuous.[24] Consistently, in the Miles assay in vivo, VEGFA-induced vascular permeability was not different between *Gas1*^{ECKO} and control mice (Figure 4D) while it was decreased in the absence of endothelial Cdon (Figure 4E). This last set of data demonstrate that Cdon strongly increases vascular permeability unlike Dhh[6,10] and that Gas1 may slightly modify Cdh5 junction organization without functional consequences.

To conclude on this first set of results, Gas1 may promote hedgehog signaling in ECs since Gas1 knockdown mostly recapitulates the effects of *Dhh* knockdown. On the contrary, Cdon most likely inhibits hedgehog signaling since *Cdon* knockdown induces opposite effect to those of *Dhh* knockdown. Notably, Cdon has been previously identified as a hedgehog decoy receptor in the zebrafish optic vesicle.[25]

Therefore, in the second part of this study, we chose to perform a series of experiments aiming to investigate whether and how Gas1 and Cdon modulate hedgehog signaling in ECs.

Gas1 Promotes Dhh Interaction With Smo While Cdon Prevents Dhh Interaction With Ptch1

First, we tested whether Gas1 or Cdon modulates Dhh interaction with Ptch1 and Smo. Notably, Smo has been recently shown to be a receptor for hedgehog ligands especially in the case of cell autonomous signaling.[26] Interestingly, we found that Gas1 prevents Dhh interaction with Ptch1 but promotes Dhh interaction with Smo. On the contrary, Cdon prevents Dhh interaction with Ptch1 but does not modify Dhh interaction with Smo (Figure 5A and 5B).

Since *Gas1* knockdown phenocopies most features of *Dhh* deficiency, we tested whether Gas1 effects on

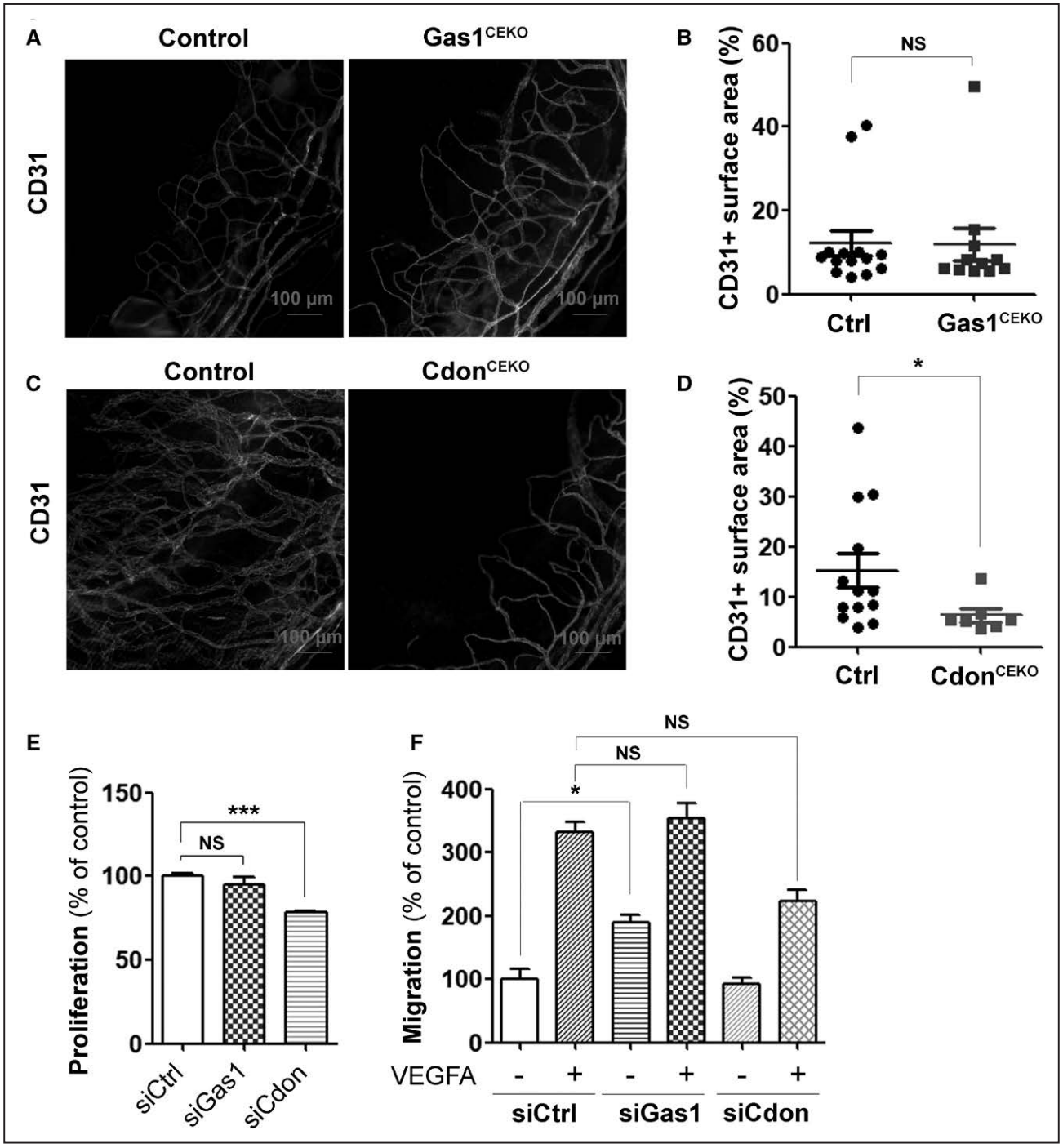

Figure 2. Cdon (cell adhesion molecule-related/downregulated by oncogenes) promotes endothelial cell (EC) migration, proliferation, and angiogenesis.

A and **B**, VEGFA (vascular endothelial growth factor A) containing pellets were implanted in the corneas of Cdh5-Cre/ERT2 *Gas1*$^{Flox/Flox}$ (*Gas1*^{ECKO}) and *Gas1*$^{Flox/Flox}$ (control) mice 2 wk after they were administered with tamoxifen (n=14 and 11 corneas, respectively). **A**, Whole-mount corneas were immunostained with anti-CD31 antibodies to identify blood vessels. Representative pictures are shown. **B**, Angiogenesis was quantified as the percentage of CD31$^+$ surface area. **C** and **D**, VEGFA containing pellets were implanted in the corneas of Cdh5-Cre/ERT2; *Cdon*$^{Flox/Flox}$ (*Cdon*^{ECKO}) and *Cdon*$^{Flox/Flox}$ (control) mice 2 wk after they were administered with tamoxifen (n=13 and 7 corneas, respectively). **C**, Whole-mount corneas were immunostained with anti-CD31 antibodies to identify blood vessels. Representative pictures are shown. **D**, Angiogenesis was quantified as the percentage of CD31$^+$ surface area. **E** and **F**, Human umbilical vein ECs (HUVECs) were transfected with growth arrest-specific 1 (*Gas1*), *Cdon*, or control siRNAs. **E**, Cells proliferation was assessed using methyl thiazolyl tetrazolium (MTT). The experiment was repeated 3 times, each experiment included n=8 wells/condition. **F**, Cell migration was assessed in a chemotaxis chamber in the presence or not of 50 ng/mL VEGFA. The experiment was repeated 3 times, each experiment included n=4 wells/condition. Mann-Whitney test or Kruskal-Wallis test followed by Dunn multiple comparisons test. NS indicates not significant. *P≤0.05; **P≤0.01; ***P≤0.001.

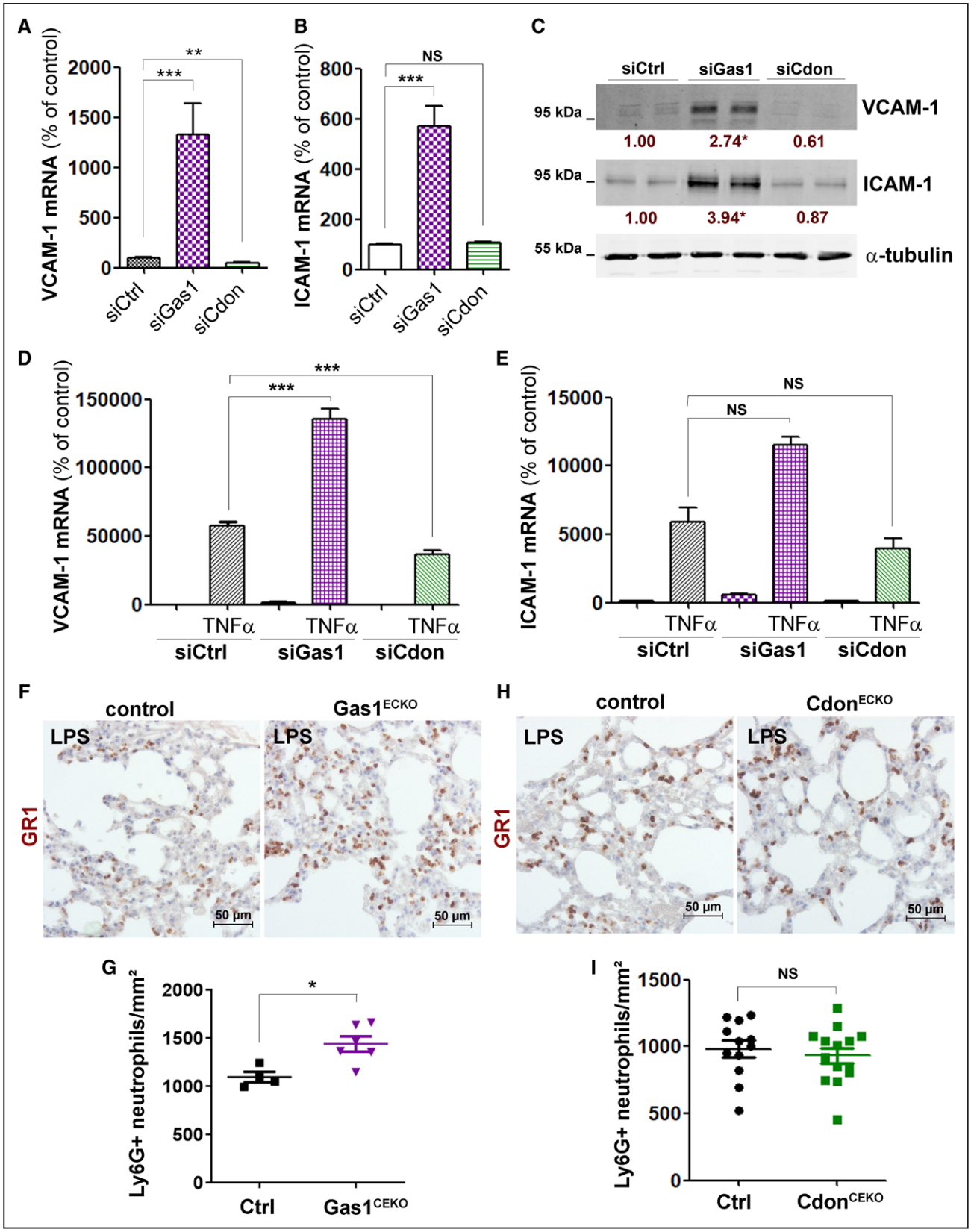

Figure 3. Gas1 (Growth arrest-specific 1) prevents endothelial cell (EC) activation and neutrophil recruitment.
A–**C**, Human umbilical vein ECs (HUVECs) were transfected with *Gas1*, *Cdon*, or control siRNAs. *VCAM-1* (vascular cell adhesion molecule 1; **A**) and *ICAM-1* (intercellular adhesion molecule 1; **B**) mRNA expression was quantified via real-time quantitative polymerase chain reaction (RT-qPCR). The experiment was repeated 3 times, each experiment included triplicates. **C**, VCAM-1 and ICAM-1 protein expression was quantified by Western blot analysis the experiment was repeated at least 3 times, each experiment included duplicates. (*Continued*)

endothelial adherens junction's integrity and migration depend on hedgehog signaling. To do so, we performed rescue experiments. HUVECs were either transfected with *Gas1* or control siRNAs and then treated or not with the Smo agonist SAG. As shown in Figure IVC and IVD in the Data Supplement, *Gas1* knockdown-induced Cdh5 junction thickening was prevented in the presence of SAG. Similarly, *Gas1* knockdown failed to induce EC migration in the presence of SAG (Figure IVE in the Data Supplement). However, SAG had no effect on *Gas1* knockdown-induced VCAM-1 and ICAM-1 (Figure IVF and IVG in the Data Supplement). Notably, VCAM-1 and ICAM-1 seem to be downstream of Ptch1 rather than Smo since *Ptch1* knockdown is sufficient to increase their expression (Figure IVH and IVJ in the Data Supplement).

Because Cdon has opposite effects to Dhh ones, we hypothesized that Cdon is a decoy receptor for Dhh at the surface of EC and thus tested whether si*Cdon*-induced effects are prevented in the absence of Dhh. HUVECs were transfected with *Cdon* siRNAs alone or in combination with *Dhh* siRNAs. While si*Cdon* alone decreased adherens junction thickness and endothelium permeability, in the si*Cdon* + si*Dhh* condition, effects were no longer significant (Figure 5D and 5F) confirming our hypothesis. Beside, we verified whether si*Cdon* still decrease endothelium permeability in the absence of serum (Figure 5G), confirming that Cdon does improve endothelial junction integrity not by interfering with any circulating hedgehog ligands but rather by interfering with EC-derived Dhh.

Cdon Deficiency at the Endothelium Prevents BBB Opening in the Setting of Acute Inflammation

Finally, since Cdon appears to act as a negative regulator of Dhh-induced signaling in ECs, we hypothesized that blocking Cdon may promote Dhh-induced signaling in ECs and subsequently promote maintenance of endothelium integrity in pathological conditions.

To test such hypothesis, we administered adenoviruses encoding Il-1β locally in the cortex of both $Cdon^{ECKO}$ mice and their control littermate to induce acute brain inflammation and BBB breakdown.

Notably, Cdon expression is significantly increased upon Il-1β treatment in both HUVECs and HBMECs (Figure 6A and 6B). In accordance with our hypothesis, endothelial adherens junctions were preserved in the absence of Cdon, as attested by an increased Cdh5 expression in the cortical lesion area of $Cdon^{ECKO}$ mice injected with Il-1β, compared with control littermates (Figure 6C and 6D). Consistently, both fibrinogen and IgG extravasation were decreased (Figure 6C, 6E, and 6F). BBB tightness in $Cdon^{ECKO}$ mice was associated with a decreased leucocyte infiltration, a decreased microglia and astrocyte activation and finally with an increased neuron survival (Figure VA through VE in the Data Supplement).

These last results demonstrate that blocking Cdon might indeed be a working therapeutic strategy to preserve endothelium integrity in pathological setting such as acute neuroinflammation.

Cdon Blocking Antibodies May be Used as a Therapeutic Tool to Maintain Endothelial Junctions in the Setting of Inflammation

We then tested whether Cdon antibodies may be used as a therapeutic tool to block Dhh binding to Cdon and improve endothelial integrity. To do so, HUVECs were treated or not with TNF-α, in the presence or not of Cdon blocking antibodies. As shown in Figure 6G and 6H, TNF-α-induced Cdh5 junction thickening is prevented in the presence of Cdon blocking antibodies.

DISCUSSION

Hedgehog signaling has been described to be regulated by several coreceptors including Hhip, Boc, Cdon, and Gas1 especially in the setting of embryogenesis.[27] The purpose of the present study was to investigate the role of Gas1 and Cdon in ECs in adults. Importantly, hedgehog signaling in ECs is original by several aspects. First, it exclusively involves noncanonical signaling[28,29]; second, it is activated by full-length unprocessed Dhh (FL-Dhh)[10]; and third, it occurs cell autonomously.[6] It is important to have in mind that full-length unprocessed hedgehog ligands may not only bind Ptch1 but also Smo directly.[26] In this particular setting, the present study demonstrates that Cdon prevents FL-Dhh binding to Ptch1. Gas1 also prevents FL-Dhh binding to Ptch1 but promotes FL-Dhh binding to Smo. By doing so, Cdon mainly acts as a negative regulator of FL-Dhh and destabilizes EC junctions to promote angiogenesis while

Figure 3 Continued. **D** and **E**, HUVECs were transfected with *Gas1*, *Cdon*, or control siRNAs and then treated or not with 10 ng/mL TNF (tumor necrosis factor)-α for 6 h. *VCAM-1* (**D**) and *ICAM-1* (**E**) mRNA expression was quantified via RT-qPCR. The experiment was repeated 3 times, each experiment included triplicates. **F** and **G**, Cdh5-Cre/ERT2 $Gas1^{Flox/Flox}$ ($Gas1^{ECKO}$) and $Gas1^{Flox/Flox}$ (control) mice were treated with 10 mg/kg lipopolysaccharide (LPS) and euthanized 6 h later. **F**, Lung sections were immunostained with anti-Ly6G (GR1) antibodies to identify neutrophils. **G**, Neutrophil infiltration was quantified as the number of $Ly6G^+$ cells/mm^2 (n=5 and 6, respectively). **H–I**, Cdh5-Cre/ERT2 $Cdon^{Flox/Flox}$ ($Cdon^{ECKO}$) and $Cdon^{Flox/Flox}$ (control) mice were treated with 10 mg/kg LPS and euthanized 6 h later. **H**, Lung sections were immunostained with anti-Ly6G (GR1) antibodies to identify neutrophils. **I**, Neutrophil infiltration was quantified as the number of $Ly6G^+$ cells/mm^2 (n=14 and 12, respectively). Mann-Whitney test or Kruskal-Wallis test followed by Dunn multiple comparisons test. NS indicates not significant. *$P \leq 0.05$ and ***$P \leq 0.001$.

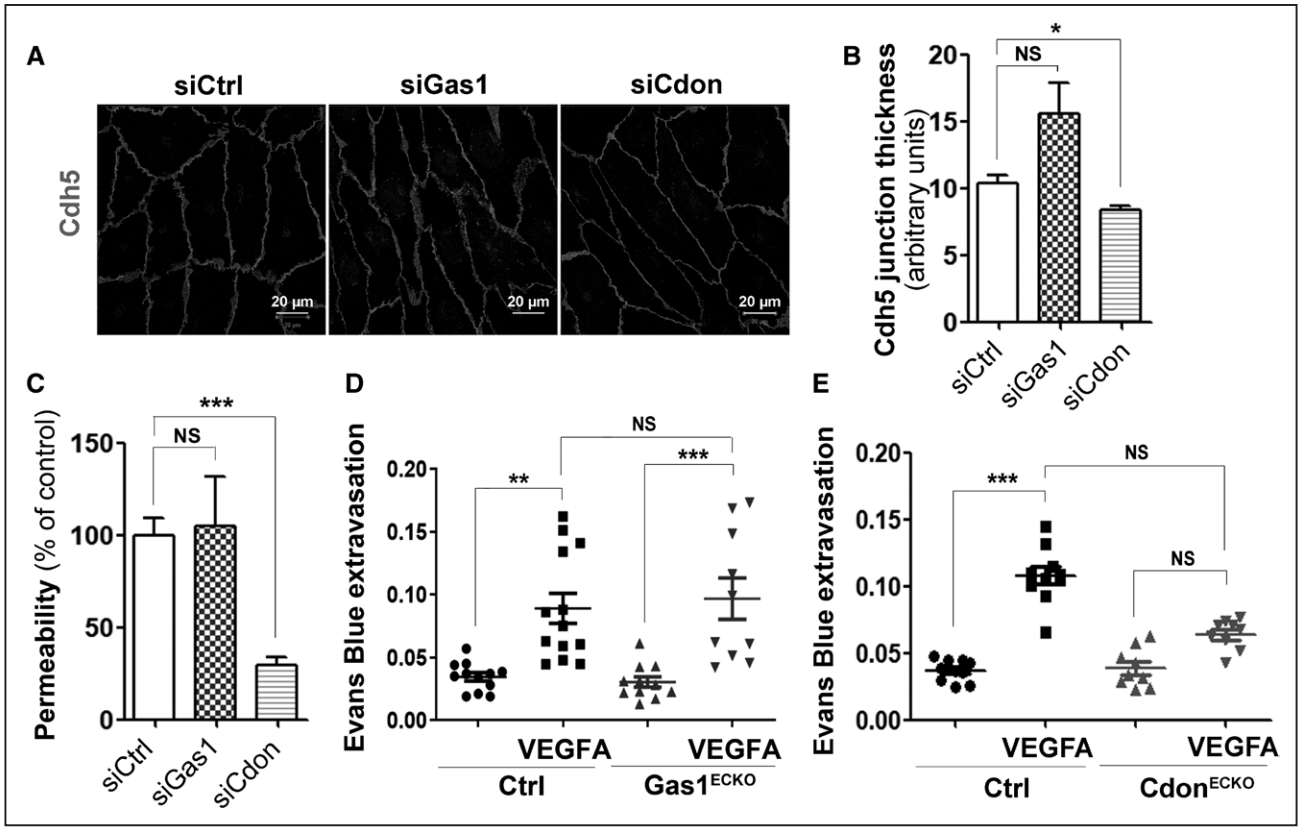

Figure 4. Gas1 (Growth arrest-specific 1) promotes adherens junction integrity while Cdon (cell adhesion molecule-related/downregulated by oncogenes) disrupts it.

A–**C**, Human umbilical vein endothelial cells (HUVECs) were transfected with *Gas1*, *Cdon*, or control siRNAs. **A**, Cdh5 localization was evaluated by immunofluorescent staining (in white) of a confluent cell monolayer and (**B**) quantified as the mean junction thickness using Image J software. The experiment was repeated at least 4 times. **C**, Endothelial monolayer permeability to 70 kDa FITC-Dextran was assessed using Transwells. The experiment was repeated 3 times, each experiment included duplicates. **D**, VEGFA (vascular endothelial growth factor A)-induced permeability was assessed in both Cdh5-Cre/ERT2 $Gas1^{Flox/Flox}$ ($Gas1^{ECKO}$) and $Gas1^{Flox/Flox}$ (control) mice using the Miles assay (n=10 and 14 mice, respectively). **E**, VEGFA-induced permeability was assessed in both Cdh5-Cre/ERT2; $Cdon^{Flox/Flox}$ ($Cdon^{ECKO}$) and $Cdon^{Flox/Flox}$ (control) mice using the Miles assay (n=9 and 10 mice, respectively). *P≤0.05; **P≤0.01; and ***P≤0.001. Kruskal-Wallis test followed by Dunn multiple comparisons test. NS: not significant.

Gas 1 is a positive regulator of FL-Dhh which prevents EC activation (Figure VI in the Data Supplement).

Cdon, Gas1, and Boc are typically believed to be positive regulators of hedgehog signaling[13] in line with the fact that Gas1, Cdon, and Boc were shown to be equally capable of promoting Shh signaling during neural patterning since overexpression of any individual component results in ectopic ventral cell fate specification.[27] Additionally, while genetic removal of Gas1, Cdon, or Boc individually has only modest effects on Shh signaling, removal of any 2 components results in significantly reduced Shh-dependent ventral neural patterning.[27] However, conflicting results have been published: Gas1 was first shown to bind Shh in 2001. In this study, it was suggested to reduce the availability of active Shh in the somite based on ectopic expression studies.[15] In 2007, experiments using *Gas1* deficient mice revealed, on the contrary, that Gas1 is a positive regulator of Shh signaling and facilitates Shh low-level effects.[30] Similarly, Cdon was shown to positively regulate Shh-induced signaling especially in the developing brain[31,32] while it was more recently shown to act as a hedgehog decoy receptor during proximal-distal patterning of the optic vesicle.[25] Whether Gas1 and Cdon are positive or negative regulators of hedgehog signaling may then most likely depend on the type of ligand and cell type involved.

Hedgehog signaling in ECs is still far from being fully understood.[2] We have previously shown that Dhh prevents EC activation by downregulating VCAM-1 and ICAM-1 and protects adherens junction integrity by promoting Cdh5 interaction with β-catenin.[6] The present study suggests that Dhh regulates EC activation and EC junction integrity via distinct pathways. Indeed, while Cdon mainly affects Dhh regulation of endothelial junctions, Gas1 mainly regulates Dhh regulation of EC immune quiescence. In both cases, a dialog between Ptch1 and Smo seems to be involved, since Cdon modulates Dhh interaction with Ptch1 to regulate EC junctions, while we previously found that Cdh5 junction integrity depends on Smo.[10] Similarly, Gas1 promotes Dhh binding

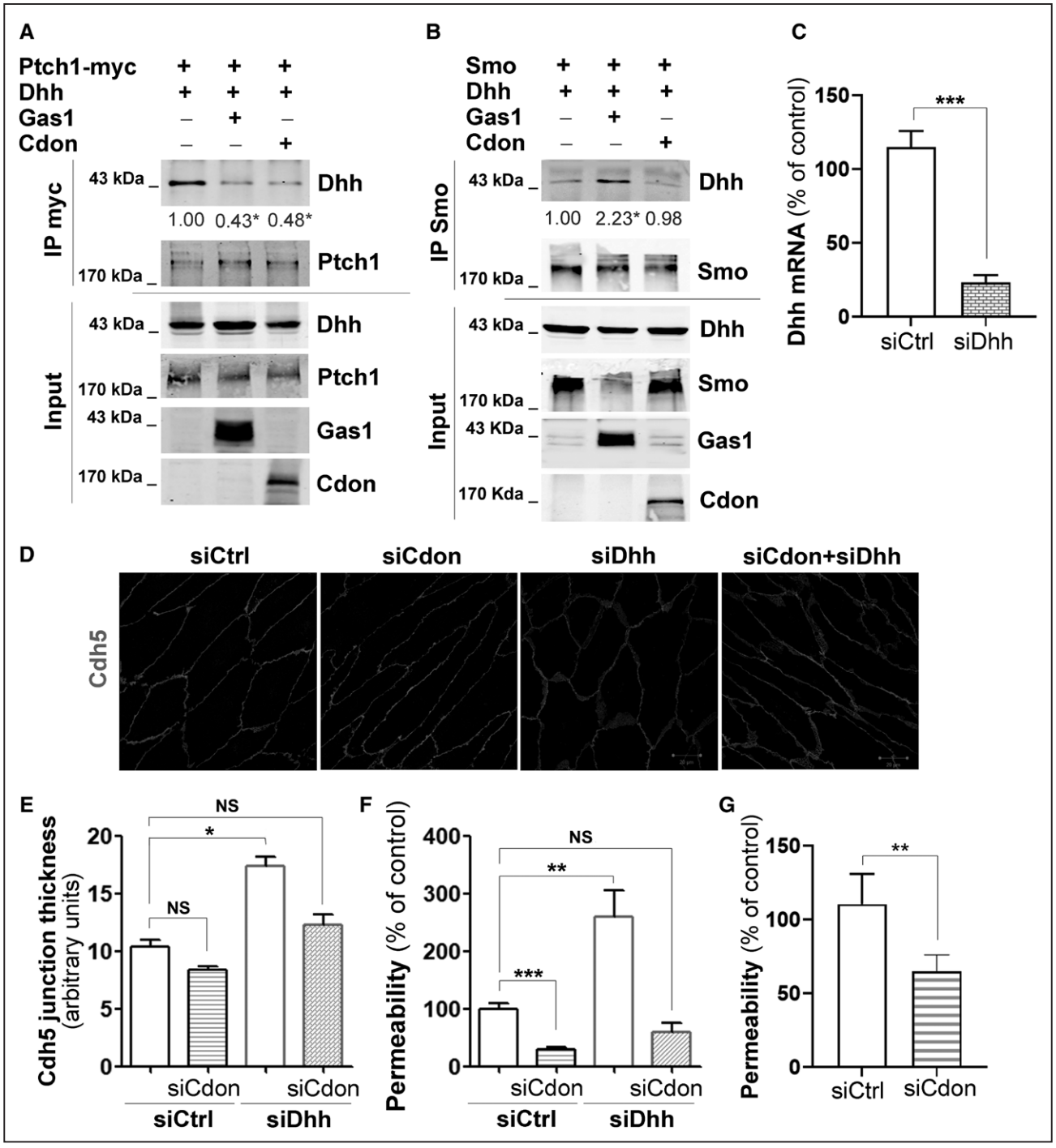

Figure 5. Gas1 (Growth arrest-specific 1) promotes desert hedgehog (Dhh) binding to Smo (smoothened) while Cdon (cell adhesion molecule-related/downregulated by oncogenes) prevents Dhh binding to Ptch1 (patched-1).
A, HeLa were cotransfected by Ptch1-myc and Dhh encoding plasmids together with or without Gas1 or Cdon encoding plasmids. Dhh interaction with Ptch1 was assessed by coimmunoprecipitation assay. The experiment was repeated 4 times. **B**, HeLa were cotransfected Smo and Dhh encoding plasmids together with or without Gas1 or Cdon encoding plasmids. Dhh interaction with Smo was assessed by coimmunoprecipitation assay. The experiment was repeated 4 times. **C**, Human umbilical vein endothelial cells (HUVECs) were transfected with *Dhh* or control siRNAs. Dhh mRNA expression was measured via real-time quantitative polymerase chain reaction (RT-qPCR). The experiment was repeated 3 times, each experiment included triplicates. **D–F**, HUVECs were cotransfected with *Cdon* or control siRNAs together with or without *Dhh* siRNAs. **D**, Cdh5 localization was evaluated by immunofluorescent staining (in white) of a confluent cell monolayer and (**E**) quantified as the mean junction thickness using Image J software. The experiment was repeated at least 4 times. **F**, Endothelial monolayer permeability to 70 kDa FITC-Dextran was assessed using Transwells. The experiment was repeated 3 times, each experiment included duplicates. **G**, HUVECs were transfected with *Dhh* or control siRNAs. Endothelial monolayer permeability to 70 kDa FITC-Dextran was assessed using Transwells in serum-free conditions. The experiment was repeated 3 times, each experiment included duplicates. Kruskal-Wallis test followed by Dunn multiple comparisons test. NS indicates not significant. *$P \leq 0.05$; **$P \leq 0.01$; and ***$P \leq 0.001$.

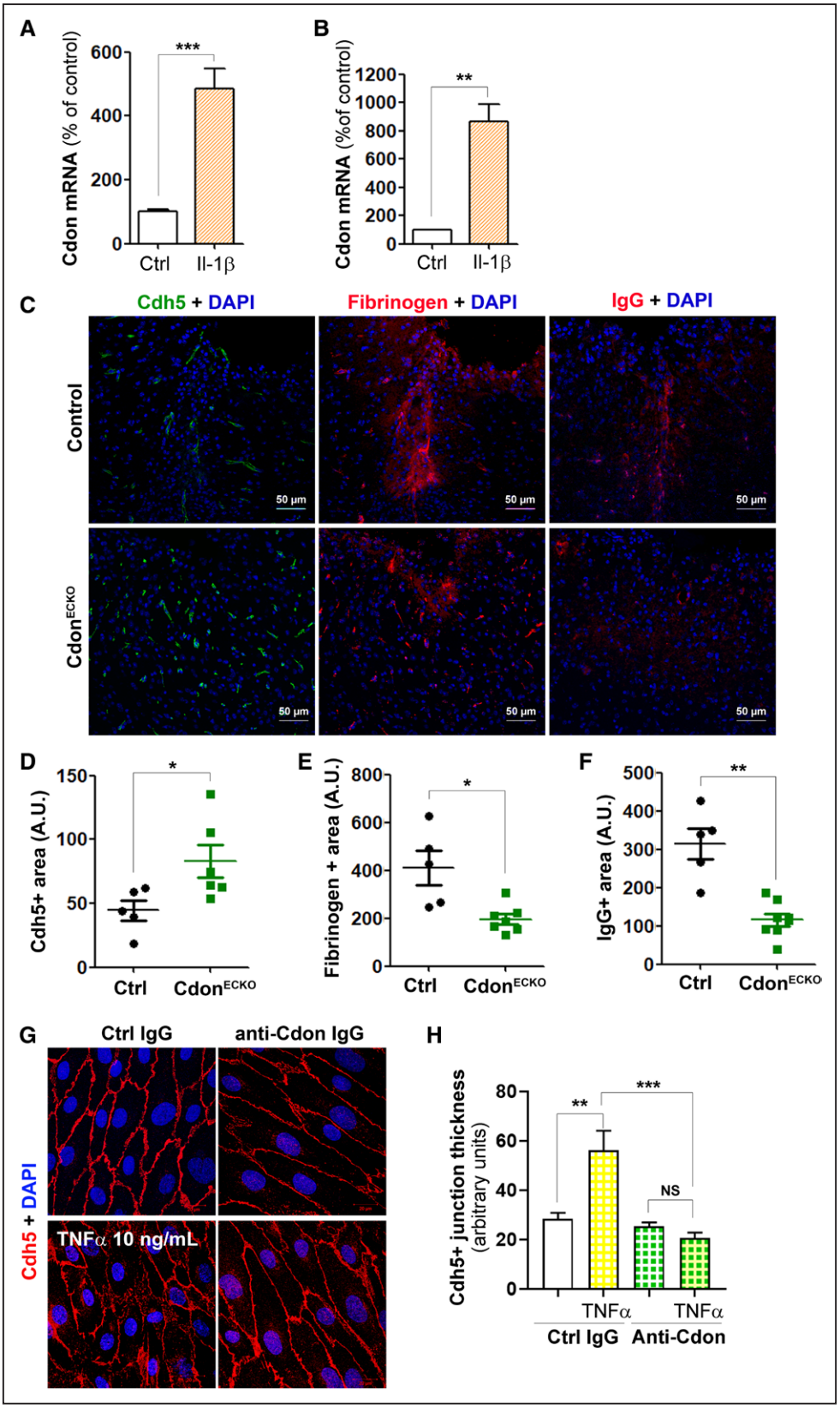

Figure 6. Cdon (cell adhesion molecule-related/downregulated by oncogenes) deficiency in endothelial cells (ECs) prevents Il-1β-induced blood-brain barrier (BBB) disruption.

Human umbilical vein endothelial cells (HUVECs; **A**) and human brain microvascular ECs (HBMECs; **B**) were treated or not with 10 ng/mL Il-1β for 6 h. Cdon mRNA expression was quantified via real-time quantitative polymerase chain reaction (RT-qPCR). Experiments were repeated 3 times, each experiment included triplicates. **C–F**, Both Cdh5-Cre/ERT2; *Cdon*$^{Flox/Flox}$ (*Cdon*^{ECKO}) and *Cdon*$^{Flox/Flox}$ (*Continued*)

Figure 6 Continued. (control) mice were administered in the cerebral cortex with adenoviruses encoding Il-1β (n=7 and 5 mice, respectively). Mice were euthanized 7 days later. **C**, Brain sagittal sections were immunostained with anti-Cdh5 (in green), anti-fibrinogen (in red), or anti-IgG (in red) antibodies. Representative confocal images are shown. **D**, Cdh5 expression was quantified as the $Cdh5^+$ surface area. **E**, Fibrinogen extravasation was quantified as the fibrinogen+surface area. **F**, IgG extravasation was quantified as the IgG^+ surface area. **G–H**, HUVECs were treated or not with 10 ng/mL TNF (tumor necrosis factor)-α in the presence of 1.5 µg/mL Cdon blocking antibodies or 1.5 µg/mL unspecific IgGs for 2 h. **G**, Cdh5 localization was evaluated by immunofluorescent staining (in red) of a confluent cell monolayer and (**B**) quantified as the mean junction thickness using Image J software. The experiment was repeated at least 4 times. Mann-Whitney test or 1 way ANOVA followed by Bonferroni multiple comparisons test. NS indicates not significant. *$P \leq 0.05$; **$P \leq 0.01$; and ***$P \leq 0.001$.

with Smo to prevent EC activation, while we found that *Ptch1* knockdown is sufficient to increase VCAM-1 and ICAM-1 expression in ECs. We then hypothesized that both dialogs going from Ptch1 to Smo and Smo to Ptch1 exist (Figure VI in the Data Supplement) based on the reciprocal regulation of Ptch1 and Smo by Smurf family of E3 ubiquitin ligases.[33]

Finally, the main goal of this study was to investigate whether hedgehog coreceptors may be used to modulate hedgehog signaling in ECs for therapeutical purposes. By identifying Cdon as a negative regulator of Dhh in ECs and by demonstrating that Cdon knockout prevents BBB opening in the setting of brain inflammation, the present study offers the possibility of using Cdon blocking molecules including blocking antibodies (Figure 6G and 6H) as therapeutic tools to preserve endothelial integrity at least in the setting of inflammation. Notably, inflammatory cytokines, including TNF-α and Il-1β, increase Cdon expression in ECs.

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Disclosures

None.

REFERENCES

1. Peng Z, Shu B, Zhang Y, Wang M. Endothelial response to pathophysiological stress. *Arterioscler Thromb Vasc Biol*. 2019;39:e233–e243. doi: 10.1161/ATVBAHA.119.312580
2. Chapouly C, Guimbal S, Hollier P-L, Renault M-A. Role of Hedgehog signaling in vasculature development, differentiation, and maintenance. *Int J Mol Sci*. 2019;20:3076. doi: 10.3390/ijms20123076
3. Alvarez JI, Dodelet-Devillers A, Kebir H, Ifergan I, Fabre PJ, Terouz S, Sabbagh M, Wosik K, Bourbonnière L, Bernard M, et al. The Hedgehog pathway promotes blood-brain barrier integrity and CNS immune quiescence. *Science*. 2011;334:1727–1731. doi: 10.1126/science.1206936
4. Xia YP, He QW, Li YN, Chen SC, Huang M, Wang Y, Gao Y, Huang Y, Wang MD, Mao L, et al. Recombinant human sonic hedgehog protein regulates the expression of ZO-1 and occludin by activating angiopoietin-1 in stroke damage. *PLoS One*. 2013;8:e68891. doi: 10.1371/journal.pone.0068891
5. Chapouly C, Yao Q, Vandierdonck S, Larrieu-Lahargue F, Mariani JN, Gadeau AP, Renault MA. Impaired Hedgehog signalling-induced endothelial dysfunction is sufficient to induce neuropathy: implication in diabetes. *Cardiovasc Res*. 2016;109:217–227. doi: 10.1093/cvr/cvv263
6. Caradu C, Couffinhal T, Chapouly C, Guimbal S, Hollier PL, Ducasse E, Bura-Rivière A, Dubois M, Gadeau AP, Renault MA. Restoring endothelial function by targeting desert Hedgehog downstream of Klf2 improves critical limb ischemia in adults. *Circ Res*. 2018;123:1053–1065. doi: 10.1161/CIRCRESAHA.118.313177
7. Hollier P-L, Guimbal S, Mora P, Diop A, Cornuault L, Couffinhal T, Gadeau A-P, Renault M-A, Chapouly C. Genetic disruption of the blood brain barrier leads to protective barrier formation at the glia limitans. *bioRxiv*. Preprint posted online March 15, 2020. doi:10.1101/2020.03.13.990762
8. Lehmann GL, Hanke-Gogokhia C, Hu Y, Bareja R, Salfati Z, Ginsberg M, Nolan DJ, Mendez-Huergo SP, Dalotto-Moreno T, Wojcinski A, et al. Single-cell profiling reveals an endothelium-mediated immunomodulatory pathway in the eye choroid. *J Exp Med*. 2020:217. doi: 10.1084/jem.20190730
9. Dakubo GD, Mazerolle C, Furimsky M, Yu C, St-Jacques B, McMahon AP, Wallace VA. Indian Hedgehog signaling from endothelial cells is required for sclera and retinal pigment epithelium development in the mouse eye. *Dev Biol*. 2008;320:242–255. doi: 10.1016/j.ydbio.2008.05.528
10. Hollier P-L, Chapouly C, Diop A, Guimbal S, Cornuault L, Gadeau A-P, Renault M-A. Endothelial cell response to Hedgehog ligands depends on their processing. *bioRxiv*. doi:10.1101/2020.03.03.974444
11. Nüsslein-Volhard C, Wieschaus E. Mutations affecting segment number and polarity in Drosophila. *Nature*. 1980;287:795–801. doi: 10.1038/287795a0
12. Robbins DJ, Fei DL, Riobo NA. The Hedgehog signal transduction network. *Sci Signal*. 2012;5:re6. doi: 10.1126/scisignal.2002906
13. Ramsbottom SA, Pownall ME. Regulation of Hedgehog signalling inside and outside the cell. *J Dev Biol*. 2016;4:23. doi: 10.3390/jdb4030023
14. Sanchez-Arrones L, Cardozo M, Nieto-Lopez F, Bovolenta P. Cdon and Boc: two transmembrane proteins implicated in cell-cell communication. *Int J Biochem Cell Biol*. 2012;44:698–702. doi: 10.1016/j.biocel.2012.01.019
15. Lee CS, Buttitta L, Fan CM. Evidence that the WNT-inducible growth arrest-specific gene 1 encodes an antagonist of sonic hedgehog signaling in the somite. *Proc Natl Acad Sci U S A*. 2001;98:11347–11352. doi: 10.1073/pnas.201418298
16. Jin S, Martinelli DC, Zheng X, Tessier-Lavigne M, Fan CM. Gas1 is a receptor for sonic hedgehog to repel enteric axons. *Proc Natl Acad Sci U S A*. 2015;112:E73–E80. doi: 10.1073/pnas.1418629112
17. Azzoni E, Conti V, Campana L, Dellavalle A, Adams RH, Cossu G, Brunelli S. Hemogenic endothelium generates mesoangioblasts that contribute to several mesodermal lineages in vivo. *Development*. 2014;141:1821–1834. doi: 10.1242/dev.103242
18. Kenyon BM, Voest EE, Chen CC, Flynn E, Folkman J, D'Amato RJ. A model of angiogenesis in the mouse cornea. *Invest Ophthalmol Vis Sci*. 1996;37:1625–1632.
19. Horng S, Therattil A, Moyon S, Gordon A, Kim K, Argaw AT, Hara Y, Mariani JN, Sawai S, Flodby P, et al. Astrocytic tight junctions control inflammatory CNS lesion pathogenesis. *J Clin Invest*. 2017;127:3136–3151. doi: 10.1172/JCI91301
20. Argaw AT, Gurfein BT, Zhang Y, Zameer A, John GR. VEGF-mediated disruption of endothelial CLN-5 promotes blood-brain barrier breakdown. *Proc Natl Acad Sci U S A*. 2009;106:1977–1982. doi: 10.1073/pnas.0808698106
21. Agrawal V, Kim DY, Kwon YG. Hhip regulates tumor-stroma-mediated upregulation of tumor angiogenesis. *Exp Mol Med*. 2017;49:e289. doi: 10.1038/emm.2016.139

22. Sekiguchi H, Ii M, Jujo K, Renault MA, Thorne T, Clarke T, Ito A, Tanaka T, Klyachko E, Tabata Y, et al. Estradiol triggers sonic-hedgehog-induced angiogenesis during peripheral nerve regeneration by downregulating hedgehog-interacting protein. *Lab Invest.* 2012;92:532–542. doi: 10.1038/labinvest.2012.6
23. Nie DM, Wu QL, Zheng P, Chen P, Zhang R, Li BB, Fang J, Xia LH, Hong M. Endothelial microparticles carrying hedgehog-interacting protein induce continuous endothelial damage in the pathogenesis of acute graft-versus-host disease. *Am J Physiol Cell Physiol.* 2016;310:C821–C835. doi: 10.1152/ajpcell.00372.2015
24. Wang Y, Alexander JS. Analysis of endothelial barrier function *in vitro*. *Methods Mol Biol.* 2011;763:253–264. doi: 10.1007/978-1-61779-191-8_17
25. Cardozo MJ, Sánchez-Arrones L, Sandonis A, Sánchez-Camacho C, Gestri G, Wilson SW, Guerrero I, Bovolenta P. Cdon acts as a Hedgehog decoy receptor during proximal-distal patterning of the optic vesicle. *Nat Commun.* 2014;5:4272. doi: 10.1038/ncomms5272
26. Casillas C, Roelink H. Gain-of-function Shh mutants activate Smo cell-autonomously independent of Ptch1/2 function. *Mech Dev.* 2018;153:30–41. doi: 10.1016/j.mod.2018.08.009
27. Allen BL, Song JY, Izzi L, Althaus IW, Kang JS, Charron F, Krauss RS, McMahon AP. Overlapping roles and collective requirement for the coreceptors GAS1, CDO, and BOC in SHH pathway function. *Dev Cell.* 2011;20:775–787. doi: 10.1016/j.devcel.2011.04.018
28. Renault MA, Roncalli J, Tongers J, Thorne T, Klyachko E, Misener S, Volpert OV, Mehta S, Burg A, Luedemann C, et al. Sonic hedgehog induces angiogenesis via Rho kinase-dependent signaling in endothelial cells. *J Mol Cell Cardiol.* 2010;49:490–498. doi: 10.1016/j.yjmcc.2010.05.003
29. Chinchilla P, Xiao L, Kazanietz MG, Riobo NA. Hedgehog proteins activate pro-angiogenic responses in endothelial cells through non-canonical signaling pathways. *Cell Cycle.* 2010;9:570–579. doi: 10.4161/cc.9.3.10591
30. Martinelli DC, Fan CM. Gas1 extends the range of Hedgehog action by facilitating its signaling. *Genes Dev.* 2007;21:1231–1243. doi: 10.1101/gad.1546307
31. Tenzen T, Allen BL, Cole F, Kang JS, Krauss RS, McMahon AP. The cell surface membrane proteins Cdo and Boc are components and targets of the Hedgehog signaling pathway and feedback network in mice. *Dev Cell.* 2006;10:647–656. doi: 10.1016/j.devcel.2006.04.004
32. Zhang W, Kang JS, Cole F, Yi MJ, Krauss RS. Cdo functions at multiple points in the Sonic Hedgehog pathway, and Cdo-deficient mice accurately model human holoprosencephaly. *Dev Cell.* 2006;10:657–665. doi: 10.1016/j.devcel.2006.04.005
33. Li S, Li S, Wang B, Jiang J. Hedgehog reciprocally controls trafficking of Smo and Ptc through the Smurf family of E3 ubiquitin ligases. *Sci Signal.* 2018;11:eaan8660. doi: 10.1126/scisignal.aan8660