Desert Hedgehog-driven endothelium integrity is enhanced by Gas1 but negatively regulated by Cdon

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SUPPLEMENTAL MATERIALS

Supplemental Methods

Chemical/recombinant proteins

Recombinant TNF α was from Shenandoah Biotechnology Inc and used at 1 to 100 nmol/L to treat HUVECs. We used BAY 11-7085 (Sigma-Aldrich) at 2 µmol/L to inhibit NF- κ B. Recombinant II-1 β was from Shenandoah Biotechnology Inc and used at 10 nmol/L to treat HUVECs and HBMECs. SAG was from SelleckChem and used at 100 nmol/L to activate Smo.

siRNA/Transfection

HUVECs were transfected with human Gas1 siRNA: rArGrCrArCrArUrUrCrUrUrArGrGrArUrUrArArGrGrGdTdC, human Cdon siRNA: rGrCrUrArUrArGrUrGrArCrArGrCrArGrCrArGrCrUrCrCdTdC

human Dhh siRNA: rArCrUrCrCrUrUrArArArGgArGrGrArCrUrArUrUrUrArGdCdC, human Ptch1 siRNA: rArGrArArArUrArCrCrCrArCrArGrCrArUrArGrUrGrArCdCdT: or universal scrambled negative control siRNA duplex (Origen) using JetPRIME[™] transfection reagent (Polyplus Transfection), according to the manufacturer's instructions.

Plasmids/Transfection

The human Gas1 encoding vector, pcDNA3-Gas1 was kindly given by C.M. Fan ¹, the GFP tagged-mouse Cdon encoding vector, pCA-mCdonEGFP, was a gift from A. Okada ², the myc-tagged human Ptch1, Ptch1-1B-myc was kindly given by R. Toftgard ³ and the human full length Dhh was previously described ⁴. A myc tag was added by PCR at the N-terminal of human full length Dhh to generate the myc-tagged Dhh encoding vector.

HeLa cells were transfected using JetPRIME[™] transfection reagent (Polyplus Transfection), according to the manufacturer's instructions.

Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Following manufacturer's instructions, RNAs were isolated and homogenized, from 3 x 10⁵ cells or from tissues previously snap-frozen in liquid nitrogen, using Tri Reagent® (Molecular Research Center Inc). For quantitative RT-PCR analyzes, total RNA was reverse transcribed with M-MLV reverse transcriptase (Promega) and amplification was performed on a DNA Engine Opticon®2 (MJ Research Inc) using B-R SYBER® Green SuperMix (Quanta Biosciences). Primer sequences are reported in Supplemental table I. The relative expression of each mRNA was calculated by the comparative threshold cycle method and normalized to Actb mRNA expression.

Immunoprecipitation/Western blot analysis

Prior to western blot analysis, Dhh, Ptch1 ou Smo were immunoprecipitated with mouse anti-Dhh antibodies (Santa Cruz Biotechnology, Cat# sc-271168), anti myc-tag antibodies (Millipore, Cat# 05-724) or mouse anti-Smo antibodies (Santa Cruz Biotechnology, Cat# sc-166685).

Expression of Cdon, Gas1, Dhh, Ptch1 and Smo were evaluated by SDS PAGE using goat anti-mouse Cdon antibodies (R&D systems, Cat#AF2429), goat anti-human Gas1 antibodies (R&D systems, Cat# AF2636), mouse anti-Dhh antibodies (Santa Cruz Biotechnology, Inc, Cat#sc-271168), rabbit anti-Ptch1 antibodies (Abcam, Cat#ab53715) and mouse anti-Smo antibodies (Santa Cruz Biotechnology, Cat# sc-166685) respectively.

Expression of human ICAM-1 and VCAM-1 expression were evaluated by SDS PAGE using mouse anti-human ICAM1 antibodies (Santa Cruz Biotechnology, Cat# sc-8439) and rabbit anti-VCAM-1 (Abcam, Cat# ab134047) respectively.

Protein loading quantity was controlled using a monoclonal anti-α-tubulin antibody (Sigma, Cat# T5168). Secondary antibodies were from Invitrogen, Cat#A-21039, A-21084, A-21036). The signal was then revealed by using an Odyssey Infrared imager (LI-COR).

In vitro permeability assay

100 000 cells were seeded in Transwell® inserts. The day after, 0.5 mg/mL 70 kDa FITC-Dextran (Sigma) was added to the upper chamber. FITC fluorescence in the lower chamber was measured 1 hour later.

Migration assay

Cell migration was evaluated with a chemotaxis chamber (Neuro Probe, Inc., Gaithersburg, MD, USA). Briefly, a polycarbonate filter (8-µm pore size) (GE Infrastructure, Fairfield, CN, USA) was coated with a solution containing 0.2% gelatin (Sigma-Aldrich Co.) and inserted between the chambers, then 5×10^4 cells per well were seeded in the upper chamber, and the lower chamber was filled with EBM-2 medium containing 0.5% FBS. Cells were incubated for 8 hours at 37°C then viewed under 20× magnification, and the number of cells that had migrated to the lower chamber were counted in 3 HPFs per well; migration was reported as the mean number of migrated cells per HPF. Each condition was assayed in triplicate and each experiment was performed at least three times.

Methyl thiazolyl tetrazolium (MTT) cell proliferation Assay

 $5x10^3$ cells per well were seeded in a 96-well plate. At the indicated time points, 10 µL of 5 mg/mL MTT were added to each wells. Cells were incubated 3-4 hours at 37°C then culture medium was replaced by 100 µL DMSO. OD was read at 590 nm with a reference at 620 nm. Each condition included eight wells in each experiment and each experiment was performed at least three times.

Supplemental figures and figure legends



Supplemental Figure I: (A) Schema representing the Cdon Floxed allele. (**B**) Cdh5-Cre/ERT2 *Cdon^{Flox/Flox}* and *Cdon^{Flox/Flox}* mice were administered with tamoxifen and sacrificed 2 weeks later. Heart cross section were co-immunostained with anti-Cdon (in red) and anti-CD31 (in green) antibodies to identify Cdon expression in ECs. (C) Cdh5-Cre/ERT2 *Gas1^{Flox/Flox}* and *Gas1^{Flox/Flox}* mice were administered with tamoxifen and sacrificed 2 weeks later. Heart cross section were co-immunostained with anti-Gas1 (in red) and anti-CD31 (in green) antibodies to identify Cdon expression in ECs. (C) Cdh5-Cre/ERT2 *Gas1^{Flox/Flox}* mice were administered with tamoxifen and sacrificed 2 weeks later. Heart cross section were co-immunostained with anti-Gas1 (in red) and anti-CD31 (in green) antibodies to identify Gas1 expression in ECs.



Supplemental Figure II: Cdon and Gas1 are regulated by TNF α in an opposite manner. HUVECs were treated with 1, 10 and 100 ng/mL TNF α for 6 hours. *Cdon* (**A**) and *Gas1* (**B**) mRNA expression was quantified via RT-qPCR. The experiment was repeated 3 times, each experiment included triplicates. (**C**) HUVECs were treated with 10 ng/mL TNF α in the presence or not of 2 µM BAY 11-7085 for 6 hours. Cdon mRNA expression was quantified via RT-qPCR. The experiment included triplicates. (**D**) *Dhh*, *Shh*, *Ihh Actb* mRNA expression was evaluated via RT-PCR in HUVECs, HMVECs-D and HBMECs. *: p≤0.05; ***: p≤0.001. Kruskal-Wallis test followed by Dunn's multiple comparisons test.



Supplemental Figure III: (A) HeLa were co-transfected with Cdon, Dhh, and/or Ptch1 encoding plasmids. Cdon (in green), Dhh (in red) and Ptch1 (in purple) localization was assessed by immunostaining. (B) HeLa were co-transfected with Gas1, Dhh, and/or Ptch1 encoding plasmids. Gas1 (in green), Dhh (in red) and Ptch1 (in purple) localization was assessed by immunostaining.



Supplemental Figure IV: (A) HUVECs were transfected with Cdon or control siRNAs. Cdon mRNA expression was quantified via RT-qPCR. The experiment was repeated 3 times, each experiment included triplicates. (B) HUVECs were transfected with Gas1 or control siRNAs. Gas1 mRNA expression was quantified via RT-qPCR. The experiment was repeated 3 times, each experiment included triplicates. (C-G) HUVECs were transfected with Gas1 or control siRNAs, then treated or not with 100 nM SAG. (C) Cdh5 localization was evaluated by immunofluorescent staining (in red) of a confluent cell monolayer and (D) quantified as the mean junction thickness using Image J software. The experiment was repeated at least 4 times. (E) Cell migration was assessed in a chemotaxis chamber. The experiment was repeated 3 times, each experiment included n=4 wells/condition. VCAM-1 (F) and ICAM-1 (G) mRNA expression was quantified via RT-qPCR. The experiment was repeated 3 times, each experiment included triplicates. (H-J) HUVECs were transfected with Ptch1 or control siRNAs. Ptch1 (H), VCAM-1 (I) and ICAM-1 (J) mRNA expression was quantified via RTgPCR. The experiment was repeated 3 times, each experiment included triplicates. *: p≤0.05; ***: p≤0.001. NS: not significant. Mann Whitney test or Kruskal-Wallis test followed by Dunn's multiple comparisons test.



Supplemental Figure V: Both Cdh5-Cre/ERT2; *Cdon^{Flox/Flox}* (*Cdon^{ECKO}*) and *Cdon^{Flox/Flox}* (control) mice were administered in the cerebral cortex with adenoviruses encoding II-1 β (n=7 and 5 mice respectively). Mice were sacrificed 7 days later. (**A**) Brain sagittal sections were immunostained with anti-CD45 (in green), anti-CD11b (in green), anti-GFAP (in green) or anti-NeuN (in red) antibodies. Representative confocal images are shown. (**B**) Leucocyte infiltration was quantified as the number of CD45+ cells/mm². (**C**) Microglia activation was quantified as the CD11b+ surface area. (**D**) Reactive astrocytes were quantified as the GFAP+ surface area. (**E**) Neuronal survival was quantified as the NeuN+ surface area. *: p≤0.05; **: p≤0.01. Mann Withney test.



Supplemental Figure VI: Schema recapitulating Gas1 and Cdon effects in ECs. In the presence of Cdon, Dhh interaction with Ptch1 is compromised and Smo is inactive. This induces Cdh5-dependent junction opening. In the presence of Gas1, Dhh binds Smo while Ptch1 inhibits ICAM-1 and VCAM-1 expression suggesting a dialogue going from Smo to Ptch1.

Supplemental Table

hActb		5'-GGAGGAGCTGGAAGCAGCC-3'
	R	5'-GCTGTGCTACGTCGCCCTG-3'
hCdon	F	5'-TGGCCAGTTGCCGGAGGAGA-3'
	R	5'-ACAGCCCTCGGGGACAGGTG-3'
hBoc	F	5'-GACCCTCACCAGACTTGACC-3'
	R	5'-GGTCGTCTCTCTGGATCTGC-3'
hGas1	F	5'-GCTAGCTGCAGTGTTCAGGA-3'
	R	5'-ATCCTCACTGGCCACAATCTG-3'
hHhip	F	5'-CTCGGCACTAGTGGGTCCTG-3'
	R	5'-TGCAGGTTGTACCGTGGCTC-3'
hDhh	F	5'-CTTGGACATCACCACGTCTG-3'
	R	5'-ATGTAGTTCCCTCAGCCCCT-3'
hShh	F	5'-GTCGAGCAGTGGACATCACC-3'
	R	5'-GAAACAGCCGCCGGATTTGG-3'
hlhh	F	5'-TGTCAAGTCTGAGCATTCGG-3'
	R	5'-GTCTCGATGACCTGGAAAGC-3'
hPtch1	F	5'-GGCAGCGGTAGTAGTGGTGT-3'
	R	5'-GTCGTGTGTGTCGGTGTAGG-3'
hVCAM-1	F	5'-GGCCCAGTTGAAGGATGCGGG-3'
	R	5'-AGAGCACGAGAAGCTCAGGAGAA-3'
hICAM-1	F	5'-ACGCCGGAGGACAGGGCATT-3'
	R	5'-GGGGCTATGTCTCCCCCACCA-3'

F forward, R reverse

 β -actin was used as the household gene

Supplemental Table I: List of primers used for reverse transcription (RT) quantitative polymer chain reaction (qPCR)

Major Resources Table

Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex	Persistent ID / URL

Genetically Modified Animals

	Species	Vendor or	Background	Other	Persistent ID / URL
		Source	Strain	Information	
Parent	mus	R. Adams	C57BL/6J-		http://www.informatics.ja
- Male	musculus	Laboratory	Tg(Cdh5-		x.org/allele/MGI:384898
			cre/ERT2)1Rha		2
Parent	mus	C.M. Fan	129.C57BL/6J-		http://www.informatics.ja
-	musculus	laboratory	Gas1 ^{tm3.1Fan}		x.org/allele/key/871662
Female					

	Species	Vendor or	Background	Other	Persistent ID / URL
		Source	Strain	Information	
Parent	mus	R. Adams	C57BL/6J-		http://www.informatics.ja
- Male	musculus	Laboratory	Tg(Cdh5-		x.org/allele/MGI:384898
			cre/ERT2)1Rha		2
Parent	mus	Institut	C57BL/6N.C57B		N/A
-	musculus	Clinique	L/6J-		
Female		de la	Cdon Floxed		
		Souris			

Antibodies

Target antigen	Vendor or Source	Catalo g #	Working concentration	Lot # (preferred but not required)	Persistent ID / URL
Mouse CD31	BMA Biomedicals	T-2001	2 µg/mL		http://www.bma.ch/en/produ cts/t-2001
Mouse Ly6G	BD Pharmingen Inc	551459	5 μg/mL		https://www.bdbiosciences.c om/us/reagents/research/ant ibodies-buffers/immunology- reagents/anti-mouse- antibodies/cell-surface- antigens/purified-rat-anti- mouse-ly-6g-1a8/p/551459
human Cdh5	Santa Cruz Biotechnology , Inc	sc- 9989	1 µg/mL		https://www.scbt.com/fr/p/ve- cadherin-antibody-f-8
Mouse Cdh5	R&D systems	AF1002	2 µg/mL		https://www.rndsystems.com /products/mouse-ve- cadherin-antibody_af1002
Albumin	Abcam	ab8940	40 µg/mL		https://www.abcam.com/hum an-serum-albumin-antibody- ab8940.html
fibrinogen	Dako	A0080	1.2 µg/mL		https://www.agilent.com/cs/li brary/packageinsert/public/1 02273002.PDF

mouse CD45	BD Pharmingen Inc	550539	0,625 µg/mL	https://www.bdbiosciences.c om/us/applications/research/ stem-cell-research/cancer- research/human/purified-rat- anti-mouse-cd45-30- f11/p/550539
CD11b	Invitrogen	14- 0112- 82	5 μg/mL	https://www.thermofisher.co m/antibody/product/CD11b- Antibody-clone-M1-70- Monoclonal/14-0112-82
GFAP	Invitrogen	OPA1- 06100	? (1/200)	https://www.thermofisher.co m/antibody/product/GFAP- Antibody-Polyclonal/OPA1- 06100
NeuN	Millipore	ABN78	1 μg/mL	https://www.merckmillipore.c om/FR/fr/product/Anti-NeuN- Antibody-rabbit,MM_NF- ABN78
Cdon	R&D systems	AF2429	2 μg/mL (IF) 0,4 μg/mL (WB)	https://www.rndsystems.com /products/mouse-cdo- antibody_af2429
Gas1	R&D systems	AF2636	2 μg/mL (IF) 0,4 μg/mL (WB)	https://www.rndsystems.com /products/human-gas1- antibody_af2636
Dhh	Santa Cruz Biotechnology , Inc,	sc- 271168	1 μg/mL (IF) 0,4 μg/mL (WB)	https://www.scbt.com/fr/p/dh h-antibody-f-9?Ntt=sc- 271168
Ptch1	Abcam	ab5371 5	5 μg/mL (IF) 1 μg/mL (WB)	https://www.abcam.com/patc hed-ptch1-antibody- ab53715.html
Smo	Santa Cruz Biotechnology , Inc	sc- 166685	0,4 μg/mL (WB)	https://www.scbt.com/fr/p/sm o-antibody-e-5
ICAM1	Santa Cruz Biotechnology , Inc	sc- 8439	0,4 μg/mL (WB)	https://www.scbt.com/p/icam -1-antibody-g- 5?requestFrom=search
VCAM-1	Abcam	ab1340 47	0,47 μg/mL (WB)	https://www.abcam.com/vca m1-antibody-epr5047- ab134047.html
Alpha- tubuline	Sigma	T5168	? (1/2000)	https://www.sigmaaldrich.co m/catalog/product/sigma/t51 68?lang=fr®ion=FR

DNA/cDNA Clones

Clone Name	Sequence	Source / Repository	Persistent ID / URL

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)	Persistent ID / URL
	Course	amaiomi	

HUVEC	Lonza	F	https://bioscience.lonza.com/lonza_bs/C H/en/Primary-and-Stem- Cells/p/00000000000184665/HUVEC- %E2%80%93-Human-Umbilical-Vein- Endothelial-Cells%2C-Pooled%2C-in- EGM-2
HMVEC-D	Lonza	unknown	https://bioscience.lonza.com/lonza_bs/C H/en/Primary-and-Stem- Cells/p/00000000000185014/HMVEC- dAd-%E2%80%93-Human-Dermal- Microvascular-Endothelial-Cells- %E2%80%93-Adult
HBMEC	Alphabioregen	unknown	https://alphabioregen.com/products/alhe 02- frozen?_pos=1&_sid=0c8f7a447&_ss=r
HeLa	ATCC	F	https://www.lgcstandards- atcc.org/products/all/CCL- 2.aspx?geo_country=fr#

Data & Code Availability

Description	Source / Repository	Persistent ID / URL

Other

Description	Source / Repository	Persistent ID / URL
Smoothened Agonist (SAG) HCI	SelleckChem	https://www.selleckchem.com/products/smoothened-agonist- sag-hcl.html
Human TNF-alpha, E.coli derived	Shenendoah Biotechnology Inc.	https://www.clinisciences.com/autres-produits-186/human-tnf- alpha-e-coli-derived-38000075.html
Human IL-1	Shenendoah	https://www.clinisciences.com/autres-produits-186/human-il-
beta, E.coli	Biotechnology	1-beta-e-coli-derived-38000235.html
derived	Inc.	
LPS	Sigma-Aldrich	
Bay 11-7085	Sigma-Aldrich	https://www.sigmaaldrich.com/catalog/product/sigma/b5681?l ang=fr®ion=FR

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