Supplemental Material

Detailed methods

Mice

JAM-A–null mice were used for these studies (age-matched and sex-matched within each experiment)[57, 58]. The mice were grouped and randomised during the experiments using the online randomisation tool: http://www.randomizer.org. Moreover, both males and females (in equal proportions) within each experiment originated from different litters. All of the mice had a C57BL/6J background and were genotyped to verify the mutations[13]. None of the mice were excluded from the analysis. All of the animal procedures were in accordance with the Institutional Animal Care and Use Committee, and in compliance with the guidelines established in the Principles of Laboratory Animal Care (directive 86/609/EEC); they were also approved by the Italian Ministry of Health.

Patient material

All patients gave written informed consent to the surgical and clinical procedures (IRB1299), which followed the principles outlined in the Declaration of Helsinki. Tissues from a total of 11 gliomas and eight serous ovarian cancers were collected from patients while they underwent neurosurgical resection of their tumours. Part of each sample was immediately frozen and stored in a tumour bank. Another part of each sample was paraffin embedded and sent for routine histological and molecular analysis. Pathological and patient-matched healthy tissues were scored by a trained pathologist. Only patients with a histological diagnosis of glioblastoma multiforme (GBM) grade IV or high-grade serous ovarian cancer were included (Online Table I). Given that high-grade serous ovarian cancer is commonly diagnosed at a late

stage, with widespread invasion of the ovary and dissemination throughout the peritoneal cavity, it is very difficult to have access to patient-matched healthy tissue. Indeed, biopsies of normal ovary derived from eight independent patients operated on for non-ovarian cancer conditions were used as control tissues.

In-vivo extravasation assay

For extravasation assays with tracers of different molecular weights, the following protocols were performed.

EZ-link-sulpho-NHS-biotin

Adult mice (10-12 weeks old) were anaesthetised by intraperitoneal injection of Avertin (20 mg/kg; T48402; Sigma-Aldrich). The beating heart was perfused via the left ventricle with 50 mL EZ-link-sulpho-NHS-biotin (0.5 mg/mL; 443.43 Da; 21217; Thermo Fisher Scientific) in phosphate-buffered saline (PBS) with Ca^{2+} and Mg^{2+} (14040-091; Thermo Fisher Scientific) over the course of 5 min, followed by perfusion with 50 mL ice-cold 1% paraformaldehyde in PBS. The brains were then fixed in 4% paraformaldehyde for 1 h at room temperature, and transferred into PBS overnight at 4 °C. The next day, they were washed in PBS and processed for sectioning.

Lysine-fixable, Alexa Fluor-555 conjugated cadaverine and Alexa Fluor-488 conjugated dextran

Cadaverine (3.125 mg/mL in saline; 950 Da; A30677; Thermo Fisher Scientific) was injected intraperitoneally in adult mice (12 weeks old; 25 mg/kg). The circulation time was 2 h. The animals were anaesthesised by intraperitoneal injection of Avertin (20 mg/kg; T48402; Sigma-Aldrich) 20 min before sacrifice. Then, 200 µL dextran (5 mg/mL in saline; 10 kDa; D22910;

Thermo Scientific) was injected retro-orbitally in the venous sinus. After 15 min, the mice were perfused for 1 min to 2 min with Hank's balanced salt solution (14025-050; Thermo Fisher Scientific), followed by 5 min perfusion with 4% paraformaldehyde in PBS, pH 7.2. The brains were then removed and post-fixed in 4% paraformaldehyde at 4 °C for 5 h to 6 h.

For vibratome sections (1000 Plus; The Vibratome Company), the brains were embedded in 3% low-melting-point agarose, sectioned (100 μm) and immunostained. Sagittal vibratome sections were incubated in blocking/ permeabilisation solution (5% bovine serum albumin, 0.3% Triton X-100, in PBS), overnight at 4 °C. This was followed by incubation with streptavidin-Alexa Fluor 555 to detect sulpho-NHS-biotin leakage. Sections were mounted in Vectashield containing 4′,6-diamidino-2-phenylindole (DAPI; H-1200; Vector).

Cells and treatments

The cell lines used in this study included the following.

Immortalised lung endothelial cells (iLECs)

JAM-A–WT and *JAM-A*–null endothelial cells were derived from four lungs of adult WT and *JAM-A*–null mice, as previously described[57]. Briefly, the lungs (two lungs for each strain) were dissected out, pulled and dissociated with 1.5 mg/mL collagenase type A (11088793001; Roche) and 25 μg/mL DNase I (10104159001; Roche), for 1.5 h at 37 °C, and then passed through 70 μm and 40 μm cell strainers. After centrifugation, the red blood cells were lysed by incubation of the pellet with red blood cell lysis buffer (R7757; Sigma-Aldrich). The cells were then resuspended in the relevant buffer and incubated with anti-CD31–coated magnetic beads (Dynabeads; 11035; Thermo Fisher Scientific). After this incubation, the endothelial cells were separated using a magnet, and eluted and cultured overnight. The following day, the cells were immortalised using polyoma middle T oncogene, as previously described[59]. The endothelial

nature of the cells was confirmed by Western blotting and immunofluorescence with S-Endo-1/MUC18, VE-cadherin, PECAM-1, and other endothelial markers. The complete culture medium was Dulbecco's modified Eagle's medium plus GlutaMAX (10564011; Thermo Fisher Scientific), with 10% North American foetal bovine serum, penicillin/ streptomycin (100 U/mL, 0.1 mg/mL ECB3001D; Euroclone), heparin (100 μg/mL; from porcine intestinal mucosa; H3149; Sigma-Aldrich) and endothelial cell growth supplement (5 μg/mL; homemade, from calf brain). The starvation medium was MCDB131 (10372-019; Thermo Fisher Scientific) with 1% bovine serum albumin, inactivated for 1 h at 60 °C, plus penicillin/ streptomycin (100 U/mL, 0.1 mg/mL), with starvation for 16 h prior to treatments for 12 h with agents to activate PKA and EPAC (8-pCTP-cAMP, 250 μM; C3912; Sigma-Aldrich), and specifically to inactivate PKA (H89, 10 μM; B1427; Sigma-Aldrich) and to activate EPAC (007, 100 μM; C041; Biolog)[13, 24]. To determine whether β-catenin contributes to C/EBP-α and claudin-5 regulation, *JAM-A*–WT and *JAM-A*–null cells were exposed for 5 days to conditioned medium from L-cells producing Wnt-3a, as described previously[60], and then processed for RNA extraction.

Freshly isolated lung endothelial cells (fLECs)

JAM-A–WT and *JAM-A*–null endothelial cells were derived from 9-12 lungs of adult WT and *JAM-A*–null mice (7 weeks old), respectively. Briefly, the lungs were digested enzymatically in combination with gentle dissociation (MACS; Lung Dissociation kits; 130-095-927; Miltenyi Biotech). After dissociation, the endothelial cells were enriched by depletion of CD45-positive cells with CD45 MicroBeads (30-052-301; Miltenyi Biotech), followed by positive selection using CD31 MicroBeads (30-097-418; Miltenyi Biotech). The final cell pellets were washed with PBS and processed for protein extraction.

Freshly isolated brain endothelial cells (fBECs)

JAM-A–WT and *JAM-A*–null endothelial cells were derived from 9-12 brains of adult WT and *JAM-A*–null mice (7-8 weeks old), respectively. Briefly, the brains were digested enzymatically in combination with gentle dissociation (MACS; Adult Brain Dissociation kits; 130-107-677; Miltenyi Biotech). After dissociation, the myelin cell debris and erythrocytes were removed according to the manufacturer protocol. Endothelial cells were enriched by depletion of CD45-positive cells with CD45 MicroBeads (30-052-301; Miltenyi Biotech). After AN2 re-expression according to the manufacturer protocol, pericytes and astrocytes were depleted using AN2 Microbeads (130-097-171; Miltenyi Biotech) and ACSA-2 Microbeads (130-097-678; Miltenyi Biotech), respectively, followed by positive selection using CD31 MicroBeads (30-097-418; Miltenyi Biotech). The final cell pellets were washed with PBS and processed for protein extraction.

Alternatively endothelial cell isolation and cultured brain microvascular fragments (referred to as cultured BECs in the main text) were processed as previously described[61, 62]. Capillary fragments were seeded into collagen I (354236; BD Biosciences)-coated wells and cultured in MCDB131 with 20% foetal bovine serum (Hyclone) supplemented with 100 mg/mL heparin (H3149; Sigma-Aldrich) and 5 mg/mL Endothelial Cell Growth Supplement (E2759; Sigma-Aldrich). After 3 days of puromycin selection (4 mg/mL; AG-CN2-0078; Adipogen), the cells were cultured until subconfluence. The starvation medium was MCDB131 with 1% foetal bovine serum, plus penicillin/ streptomycin (100 U/mL, 0.1 mg/mL), with starvation for 20 h with agents to activate (007, 100 µM; C041; Biolog) or inactivate (3-[5-(*tert*-butyl)isoxazol- 3- yl]- 2-[2-(3-chlorophenyl)hydrazono]-3- oxopropanenitrile ESI-09, 10 µM; B133; Biolog) EPAC[63].

Plasmids and constructs

To overexpress C/EBP-α, three different constructs were produced (empty vector, C/EBP-α-WT, C/EBP-α-DN). The cDNAs coding for the C/EBP-α-WT and C/EBP-α-DN were subcloned into the pcDNA3 vector using restriction sites for *Bam*HI and *Xho*I, and the correct frame was verified by sequencing using the T7 and T3 primers. The *claudin-5* promoter constructs were obtained as described below in the dedicated section (*Claudin-5* promoter luciferase assay), and they were validated in the *JAM-A*–WT and *JAM-A*–null endothelial cells. TOP-FLASH and FOP-FLASH plasmids containing WT or mutant TCF/Lef binding sites upstream of a luciferase gene, respectively, and the pCMV plasmid for constitutive expression of β-galactosidase (pCMV-β-Gal), were kindly donated by Dr M.P. Cosma, Centre for Genomic Research, Barcelona, Spain[64]. These constructs were used in the TOP/FOP assay section, as described. Please see the Major Resources Table in the Supplemental Materials.

Lentiviral, retroviral and adenoviral preparations

The lentiviral vector used to stably express human claudin-5 and the empty vector (GFP, control) were obtained from Dr Fontijn (University of Amsterdam, The Netherlands)[65]. The lentiviruses were generated in HEK 293T cells, as described by Morini et al. (2018)[31]. The lentiviral vectors were produced as described by Dull et al. (1998)[66]. The packaging plasmids were kindly donated by L. Naldini (HSR-TIGET, San Raffaele Telethon Institute for Gene Therapy, Milan, Italy). Retroviral vectors coding for eGFP (control), human C/EBP-α-WT and human C/EBP-α-DN (encoding an N-terminal mutated C/EBP) were kindly provided by Dr Carol Stocking-Harbers (Leibniz Institute for Experimental Virology, Hamburg, Germany)[67]. Retroviruses were produced with the use of standard techniques[68]. The TCF4-DN adenovirus was donated by Dr S.J. George (Bristol Heart Institute, Bristol, UK). Infectious viruses were purified and titred using standard techniques. Two consecutive cycles of infections (5 h;

overnight) were performed with a multiplicity of infection of 300 in 1 mL complete medium. Please see the Major Resources Table in the Supplemental Materials.

RNA interference

siRNAs against mouse C/EBP-α (s63854: AAAGCUGAGUUGUGAGUUAtt; s63855: ACUCAAAACUCGCUCCUUUtt; Thermo Fisher Scientific) were used to knock-down C/EBP-α. siRNAs against JAM-A ON-TARGET plus a set of four Upgrade siRNAs (J-054103-09:UAUUCUAGCUAGCGUAUAA;J-054103-10:CAUCAGAGCUGUUCCGUAG; J-054103:CCAUCCAAGCCGACGAUCA;J-054103-12: TCAGCATTGTGTAACCCCAGT; Thermo Fisher Scientific) were used to knock-down JAM-A. Transfection was performed using Lipofectamine 2000 (LS11668019; Thermo Fischer Scientific), according to the manufacturer instructions. Please see the Major Resources Table in the Supplemental Materials.

Tracer flux analysis

Endothelial cells were seeded on 6.5-mm Transwell permeable supports (pore size, 0.4 μm; 3413; Corning), cultured for 72 h in complete culture medium, and assayed for permeability to fluorescein isothiocyanate (FITC)-dextran (40 kDa; FD40S; Sigma-Aldrich). For the treatments, the cells were starved for 16 h, then pretreated for 1 h with 007, which remained with the cells through the time courses. Next, 1 mg/mL FITC-dextran was added to the medium of the transwell apical compartment. After different times of incubation, 50 μL aliquots of the medium were collected from the basal compartment, and the paracellular tracer flux was measured as the amount of FITC-dextran in this basal medium, determined using a fluorometer (Wallac Victor3 1420 multi-label counter; Perkin Elmer). At the end of the permeability assay, the endothelial monolayer on the permeable supports were fixed with 4% paraformaldehyde

for 10 min at room temperature. Once fixed, the cells were stained with Gram's crystal violet solution (1092180500; Merk) for 5 min, and then washed three times with PBS with Ca^{2+} and Mg^{2+}. The monolayers were monitored using a widefield microscope (IM-5FLD; Optika, Italy).

Nuclear cytoplasm fractionation

For cytosol extracts

Cells were washed with ice-cold PBS with Ca^{2+} and Mg^{2+}, scraped from the plates after the addition of hypotonic buffer (20 mM HEPES, pH 7.5, 1 mM EDTA, pH 8.0, protease/ phosphatase inhibitors), and incubated for 20 min on ice. The cells were then passed through a 22-gauge syringe needle and centrifuged at 16000× *g* for 5 min at 4 °C, to separate the cytoplasm (supernatant) from the nuclei (pellet).

For nuclear extracts

The pellets obtained were washed three times with hypotonic buffer, to eliminate cytosol contamination, and centrifuged at 16,000× *g* for 30 s at 4 °C. The pellets were then resuspended in hypertonic buffer (14 mM HEPES, pH 7.5, 0.7 mM EDTA, pH 8.0, 450 mM NaCl, 10% glycerol, protease/ phosphatase inhibitors) and ultracentrifuged at 50,000× *g* for 30 min at 4 °C (Optima Max Ultracentrifuge), to sediment the membranes. The resulting supernatant represented the nuclear protein extract.

Transcription factor binding site analysis and chromatin immunoprecipitation assays

To identify C/EBP-α consensus sequences on the putative *claudin-5* promoter region, the Genomatix software was used. This programme identifies transcription-factor binding sites in nucleotide sequences using a large library of weight matrices. Here, 6000 base pairs upstream

of the *claudin-5* coding sequence were analysed, and a prediction of the potential combination of transcription-factor binding sites was obtained with reasonable accuracy.

For the chromatin immunoprecipitation assays, the cells were starved overnight and crosslinked with 1% paraformaldehyde for 10 min at room temperature. The paraformaldehyde was then inactivated by addition of 125 mM glycine. The cells were washed and resuspended in lysis buffer. The samples were sonicated (Bioruptor 200) under the following conditions: H power, 30 s ON – 60 s OFF, for 20 min. Chromatin extracts containing 200 µg DNA fragments with a mean size of 500 bp were immunoprecipitated with 5 µg anti-C/EBP-α antibody (14AA X; sc-61). The DNA was recovered and amplified by RT-qPCR, using the oligonucleotides that flanked the assayed promoter regions. The primer sequences used are given in Online Table II. For RT-qPCR analyses, the DNA was diluted with specific primers (10 mM each) to a final volume of 25 µL, in SYBR Green Reaction Mix (4385617; Thermo Fisher Scientific).

Claudin-5 *promoter luciferase assay*

Functional assays were performed in HEK-293T cells plated in six-well plates and transfected for 5 h in Optimem, using Lipofectamine 2000 (LS11668019; Thermo Fisher Scientific). The reporter plasmids (PGL3-basic vector; Promega) expressed luciferase cDNA under the control of the *claudin-5* promoter portions[20]. pCDNA3 expression vectors containing C/EBP-α-WT or C/EBP-α-DN were transfected where indicated. A renilla-expressing plasmid was used as the internal control, to normalise for transfection efficiency. The Dual Luciferase Reporter Assay system (E1980; Promega) was used to measure the firefly and renilla luciferase activities 30 h after transfection (GloMax luminometer; Promega). The claudin-5 promoter was generated by cloning 6000 bp upstream of the claudin-5 coding sequence, as follows. The 6000-bp promoter was first amplified by PCR from genomic DNA from immortalised murine endothelial cells, using the following primers:

0FW 5’GTCGACTTTATAATTCATGTA3’,

0RW 5’TAAAAAGAATTCCAAGATAGT3’,

1FW 5’ACTATCTTGGAATTCTTTTTA3’,

1brev 5’ATCCGTTGACCTTCCATGG3’,

2FW 5’CCATGGAAGGTCAACGGAT3’,

2brev 5’ATATATTAACTGCTGCCTTCT3’,

34FW 5’TTACATCTACTGAGCAGGGA3’,

34RW 5’AAGCTTGGGTTCTGAAGTAACCT3’,

These primers were used to generate four shorter fragments of the promoter (named 0, 1, 2, 34). Fragment “0” ranging from -6000 to -4420; fragment “1” from -4440 to -2782; fragment “2” from -2801 to -1344; and fragment “34” from -1420 to 0. The amplified fragments were then ligated into pGEM-T easy (Promega) and fully sequenced. Then, the individual fragments were sequentially excised from the pGEM-T easy, and assembled to generate the longer fragments used in the experiments. The assembly procedure was carried out as follows. First, fragments “34” and “2” were digested with *Xba*/*Hind*III and *Eco*RI/*Xba*, respectively, and simultaneously ligated into the pSP72 vector (Promega), to generate fragment “234”. Then fragment “1” was digested with *Eco*RI/*Nco*I from pGEM-T, and ligated upstream of fragment “234” in pSP72 digested with *Eco*RI/*Nco*I, to generate fragment “1234” in pSP72. Fragment “0” was digested with *Sal*I, blunted with Klenow, and subsequently digested with *Eco*RI before ligation into fragment “1234” in pSP72 digested with *Cla*1 (blunted with Klenow)/ *Eco*RI, to generate the full-length promoter in pSP72. All of the assembly steps were verified by DNA sequencing. Finally, the full-length promoter and fragment “234” were excised from pSP72 by Klenow blunted digestion with *Sal*I/*Hind*III and *Nco*I/*Hind*III, respectively, and subcloned into pGL3-basic vector (Promega), digested with *Sma*I, and dephosphorylated to avoid self-ligation

of the empty vector. Fragment "34" was excised from pGEM-T easy by Klenow blunted digestion with *Xba*/*Hind*III, and subcloned in PGL3-basic vector, as above.

TOP/FOP assay

TOP/FOP assays were performed using a previously described standard technique[20]. Briefly, *JAM-A*–WT and *JAM-A*–null cells were plated in six-well plates for 80% confluent cultures at the time of transfection. The cells were transfected overnight in Optimem, using Lipofectamine 2000 (LS11668019; Thermo Fischer Scientific), according to the manufacturer instructions. To normalise for transfection efficiency, 1 μg pCMV-β-Gal plasmid was co-transfected with 3 μg of either TOP-FLASH or FOP-FLASH plasmids. Luciferase activity was assayed 48 h after transfection, using the dual light luciferase and β-galactosidase reporter gene assay system (T1003; Thermo Fisher Scientific). TCF/Lef β-catenin–mediated gene transcription was defined by the ratio of the TOP-FLASH or FOP-FLASH luciferase activities, where the β-Gal activity of the internal control reporter pCMV–β-Gal was used to correct for differences in transfection efficiency.

Quantitative RT-PCR analysis

Total RNA from the cells was isolated using RNeasy mini kits (74104; Qiagen) or Maxwell RSC simplyRNA cell and tissue kits (AS1390; Promega), and reverse transcribed with random hexamers (High-Capacity cDNA Archive kits; Applied Biosystems), following the manufacturer instructions. For gene expression analysis, 5 ng cDNA was amplified (in triplicate) in a reaction volume of 10 μL that contained the following reagents: 5 μL "TaqMan Fast Advanced Master Mix (4444557; Applied Biosystems), 0.5 μL TaqMan Gene expression assay 20× (Thermo Fisher Scientific). Real-time PCR was carried out on a real-time PCR system (7500; Thermo Fisher Scientific) using a pre-PCR step of 20 s at 95 °C, followed by 40

cycles of 1 s at 95 °C and 20 s at 60 °C. A specific TaqMan assay (Thermo Fisher Scientific) was used for each gene. Please see Major Resources Table in the Supplemental Materials. Raw data (Ct) were analysed using the Biogazelle qbase plus software, and the fold-changes are expressed as calibrated normalized relative quantities (CNRQs) with standard error (SE). The GeNorm Software chose GAPDH and 18s as the best housekeeping genes, and the geometric mean of GAPDH and 18s was used to normalise the data.

Vessels from pathological and (patient-matched) healthy paraffin-embedded tissues were scored by a trained pathologist and micro-dissected to be further processed for RNA isolation using Maxwell RSC RNA FFPE kits (AS1440; Promega), according to the manufacturer protocol. RNA samples were quantified using a fluorometer (Qubit) with RNA kits (Thermo Fisher Scientific), and monitored for size and quality (Agilent Bioanalyser) with Pico RNA kits (Agilent, Santa Clara, CA, USA). After cDNA synthesis, the samples were preamplified using TaqMan PreAmp Master Mix (Thermo Fisher Scientific), then diluted 1:4 with TE 1× buffer, and 2 uL/well were used for RT-PCR analysis.

Western blotting

Total proteins were extracted by solubilising cells in boiling sample buffer (2×; 2.5% sodium dodecyl sulphate, 20% glycerol, 0.125 M Tris-HCl, pH 6.8) enriched in protease inhibitor R cocktail set III (EDTA free; 539134; Calbiochem) and phosphatase inhibitors (cocktail 2; P5726; and cocktail 3; P0044; Sigma-Aldrich). Lysates were incubated for 5 min at 100 °C, to promote protein denaturation, and then centrifuged at 16,000× *g* for 5 min, to pellet the cell debris. For adult mouse brains, the tissue was digested enzymatically in combination with gentle dissociation (MACS; Adult Brain Dissociation kits; 130-107-677; Miltenyi Biotech) After dissociation, the myelin cell debris and erythrocytes were removed according to the manufacturer protocol.

Endothelial cells were enriched by depletion of CD45-positive cells with CD45 MicroBeads (30-052-301; Miltenyi Biotech), followed by positive selection using CD31 MicroBeads (30-097-418; Miltenyi Biotech). The final cell pellets were washed with PBS and processed for protein extraction. For the isolation of endothelial cells of adult mouse lung, the lungs were dissected out and dissociated with 1.5 mg/mL collagenase type A (11088793001; Roche) and 25 μg/mL DNase I (10104159001; Roche), for 1.5 h at 37 °C, and then passed through 70 μm and 40 μm cell strainers.

After centrifugation, the red blood cells were removed by incubation of the pellet with red blood cell lysis buffer (R7757; Sigma-Aldrich). The cells were then resuspended in the relevant buffer and incubated with anti-CD31-coated magnetic beads (Dynabeads; 11035; Thermo Fisher Scientific). After this incubation, the endothelial cells were separated using a magnet, eluted in sample buffer (2×), and then used for Western blotting.

Protein concentrations were determined using BCA Protein Assay kits (23225; Pierce), according to the manufacturer instructions. Equal amounts of protein were loaded onto gels and separated by SDS-PAGE, then transferred to membranes (Protran nitrocellulose hybridisation transfer membrane; pore size, 0.2 μm; 10600001; GE Healthcare) and blocked for 1 h at room temperature in 5% milk or 5% bovine serum albumin, in 0.05% Tween-20 in Tris-buffered saline (TBS). The membranes were incubated overnight at 4 °C or for 1 h at room temperature with the primary antibodies diluted in blocking solution. Next, the membranes were rinsed three times with washing solution (0.05% Tween-20 in TBS) for 10 min each, and incubated for 1 h at room temperature, with horseradish-peroxidase-linked secondary antibodies (diluted in blocking solution). The membranes were rinsed three times with washing solution for 5 min each, and the specific binding was detected by the enhanced chemiluminescence (ECL) technique (RPN2106; GE Healthcare), or using Super Signal West Femto Maximum Sensitivity Substrate (34096; Thermo Fisher Scientific) or Super Signal West

Dura Extended Duration Substrate (34076; Thermo Fisher Scientific), using a ChemiDoc MP system (BioRad). The molecular masses of the proteins were estimated relative to the electrophoretic mobility of a co-transferred prestained protein marker (Precision Plus Protein Standards Dual Colour; 161-0374; Bio-Rad). Please see the Major Resources Table in the Supplemental Materials.

Densitometry analysis of the bands was carried out used the Fiji software (open source; htpp://fiji.sc/)[71] and with the band analysis tools of the ImageLab software, version 4.1 (Bio-Rad). GAPDH, tubulin or VE-cadherin (loading control) were used to normalize the quantified bands, as specified in the Figures. The densitometry data obtained for the control was set to 1, and the ratios for *JAM-A*-null cells *versus* WT are shown for each protein.

Immunofluorescence microscopy

Immunofluorescence microscopy staining was performed using a standard technique described previously[70]. Briefly, to enhance endothelial cell adhesion, the slides were coated with glutaraldehyde cross-linked gelatin, as follows. The culture supports were incubated for 1 h at room temperature with 1% gelatin, followed by cross-linking with 2% glutaraldehyde for 15 min at room temperature. The glutaraldehyde was replaced by 70% ethanol. After 1 h, five washes were performed with PBS, followed by an overnight incubation with PBS containing 2 mM glycine (3908.1; Roth). Before cell seeding, the slides were washed five times with PBS. The cells were cultured in 8-well l-Slides (80826; Ibidi). For C/EBP-α staining, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Once fixed, the cells were washed three times with PBS with Ca^{2+} and Mg^{2+}, and were subsequently permeabilised with 0.1% Triton X-100 in PBS with Ca^{2+} and Mg^{2+} for 5 min. For all of the other staining, the cells were fixed and permeabilised in ice-cold methanol at 20 °C for 5 min. Fixed cells were incubated for 1 h in blocking solution: 2% bovine serum albumin, 5% normal donkey serum

(017-000-1210; Jackson ImmunoResearch) and 0.05% Tween-20 in TBS. The cells and slices were then incubated overnight at 4 °C or for 2 h at room temperature, with the primary antibodies diluted in blocking buffer. The appropriate secondary antibodies (Alexa Fluor 488, 555; 647; 1:400; Jackson ImmunoResearch) were applied to the cells for 45 min at room temperature. Before mounting the cells, the cell nuclei were stained with DAPI (1:5000; D9542; Sigma-Aldrich) for 5 min at room temperature.

For paraffin-embebbed tissue sections (i.e., human GBM, ovarian tissue, lungs), the samples were de-paraffinised and hydrated following standard protocols, and subjected to antigen unmasking in sodium citrate buffer, pH 6, for 45 min at 95 °C (for C/EBP-α), or in EDTA (for claudin-5, JAM-A, PECAM-1, ERG, VE-cadherin). For the immunofluorescence analysis, negative controls using isotype matched normal IgG were carried out to check for antibody specificity. Please see Major Resources Table in the Supplemental Materials.

The samples were observed under confocal microscopy (TCS SP5; Leica), equipped with yellow (Argon, 488 nm), red (561 nm, solid state laser) and blue (633 nm, HeNe laser) excitation laser lines. Image acquisition was performed using 40× and 63×/1.4 NA oil immersion objectives (HCX PL APO 63 9; Lbd Bl, Leica), with spectral detection bands and scanning modalities optimised for the removal of channel cross-talk.

Image processing

Image analysis was carried out used the Fiji software (open source; htpp://fiji.sc/)[71]. The Figures were assembled and processed using Adobe Photoshop and Adobe Illustrator. The only adjustments used in the preparation of the Figures were for brightness and contrast. For comparison purposes, different sample images of the same antigen were acquired under constant acquisition settings.

To asses sulpho-NHS-biotin leakage, a custom Fiji plugin was developed. The leakage was measured on the z-maximum projection as the ratio between the leakage (streptavidin) area and the total area of the brain (PECAM-1 or podocalixyn). The leakage area was defined using the Yen thresholding[72] method after filtering the image with a median and a Gaussian filter. The brain area was defined using the Li thresholding method[73] after filtering the image with a median filter. Quantification of the leakage was carried out using two distinct operators. The first operator acquired the confocal stacks, while the second operator performed the quantification in a blind test.

To quantify endothelial nuclear β-catenin and C/EBP-α in cell cultures, a Fiji plugin was developed. The endothelial area was identified on the VE-cadherin channel using the Triangle thresholding method[73], after use of a Gaussian filter. Within the endothelial area identified, the nuclei were defined on the DAPI channel using the Otsu thresholding method[74], after rolling ball background (https://zenodo.org/record/845874) subtraction and use of a Gaussian filter. Then the endothelial nuclear β-catenin and C/EBP-α mean intensities were measured after the background subtraction. Quantification was carried out using two distinct operators. The first operator acquired the confocal stacks, while the second operator performed the quantification in a blind test.

To quantify lung and ovarian cancer tissue, a customised Image[75] macro was developed to analyse the images. As the first step, the type of staining was selected. This included: DAPI for nuclear staining; ERG for specific endothelial nuclear staining; and VE-cadherin for endothelial junction staining in lung and in ovarian cancer samples. As the second step, all of the vessels were drawn by hand. On the staining channel, a grey level threshold was set manually. As the third step, after background subtraction in the channel of interest, the resulting mask image was used to measure the mean intensity of nuclear C/EBP-α in the lung and ovarian

cancer vessels, or the mean intensity of JAM-A and claudin-5 at the endothelial junctions in ovarian cancer vessels.

For the ovarian cancer samples, the mean intensity of each nucleus in the selected vessel was measured. For the lung samples, the mean intensity of all of the nuclei in the selected vessel was measured. For the endothelial junctions, the mean intensity of all of the junctional area in the selected vessel was measured. Quantification was carried out by two distinct operators. The first operator acquired the confocal stacking, while the second operator performed the quantification in a blind test.

Electron microscopy

Basic electron microscopic examination and transmission electron microscopy tomography were performed as described previously[76]. The procedure for the tissue block preparation for electron microscopy analysis was as described previously[77].

Statistical analysis

The normality of the datasets was assessed using Shapiro-Wilk normality tests. For datasets with normal distributions, two-sided unpaired Welch's t-tests (for pairwise comparison) or one-way Brown-Forsythe ANOVA followed by Dunnett's T3 tests (for *post-hoc* multiple comparison) were used. Non-parametric (Mann-Whitney) tests were applied to datasets that did not show normal distributions. Wherever applicable, the details about the statistical test applied are specified in the Figure legend. The standard software package GraphPad Prism (version 8.4.2) was used.

Antibodies

The antibodies used in this study were: anti-C/EBP-α rabbit monoclonal (8178S; 1:500 for Western blotting [WB]; 1:200 for immunofluorescence [IF]); anti-C/EBP-α rabbit polyclonal (ab140479; 1:100 for IF on paraffin sections); anti-C/EBP-α rabbit polyclonal (14AA X; sc-61, 5 µg for 200 µg of total protein, for chromatin immunoprecipitation); anti-C/EBP-β rabbit polyclonal (3082S; 1:500 for WB); anti-C/EBP-γ rabbit polyclonal (sc25769; 1:250 for WB); anti-C/EBP-δ rabbit polyclonal (sc-636; 1:250 for WB); anti-ERG mouse monoclonal (ab136152; 1:5 for IF on paraffin sections); anti-GAPDH mouse monoclonal (sc-32233; 1:1000 for WB); anti-JAM-A rat polyclonal BV12 (homemade; 1:600 for IF); anti-JAM-A goat polyclonal (AF1077; 1:2500 for WB); anti-JAM-A rabbit monoclonal (ab52647; 1:100 for IF on paraffin sections), anti-claudin-5 rabbit polyclonal (ab53765; discontinued; 1:100 for IF on paraffin sections), anti-claudin-5 mouse monoclonal Alexa Fluor 488 (352588; 1:200 for IF on paraffin sections); anti-claudin-5 rabbit polyclonal (ab15106; 1:50 for IF on paraffin sections); anti-claudin-5 mouse monoclonal (352500; 1:250 for WB); anti-PECAM-1 goat polyclonal (AF3628; 1:40 for IF on paraffin sections); anti-PECAM-1 hamster monoclonal (MAB1398Z; 1:400 for IF); anti-lamin B goat polyclonal (sc-6217; 1:2000 for WB); anti-cingulin rabbit polyclonal (PA5-55673; 1:400 for WB); anti-afadin mouse monoclonal (610732; 1:500 for WB); anti-occludin mouse monoclonal (33-1500; 1:500 for WB); anti-zo-1 rabbit polyclonal (ab216880; 1:1000 for WB); anti-tubulin mouse monoclonal (T9026 1:2000 for WB and IF); anti-CREB rabbit polyclonal (D76D11; 1:1000 for WB), anti-phospho-CREB Ser133 rabbit monoclonal (87G3; 1:500 for WB), anti-β-catenin (BD610154; 1:2000 for WB), anti-phospho-β-catenin Ser552 (5651; 1:1000 for WB); anti-VE-cadherin goat (sc-6458; since discontinued; 1:500 for WB; 1:50 for IF on paraffin sections); anti-podocalyxin (AF1556; 1:400 for IF); anti-GFP (sc-9996; 1:8000 for WB); anti-4',6-diamidino-2-phenylindole (D9542; 1:5000 for IF); phalloidin-TRITC (P1951; 1:200 for IF), anti-vinculin mouse monoclonal (9131; 1:5000 for WB), streptavidin, Alexa Fluor 555 (S32355, 1:400 for IF). The anti-mouse, anti-rat and anti-

rabbit secondary antibodies conjugated to horseradish peroxidase were from Cell Signaling (1:2000); the anti-goat secondary antibodies conjugated to horseradish peroxidase were from R&D Systems (1:1000); and the Alexa Fluor 488, 555 and 647 donkey secondary antibodies were from Jackson ImmunoResearch (1:400). Please see the Major Resources Table in the Supplemental Materials.

Online Table I. Clinical parameters of patients

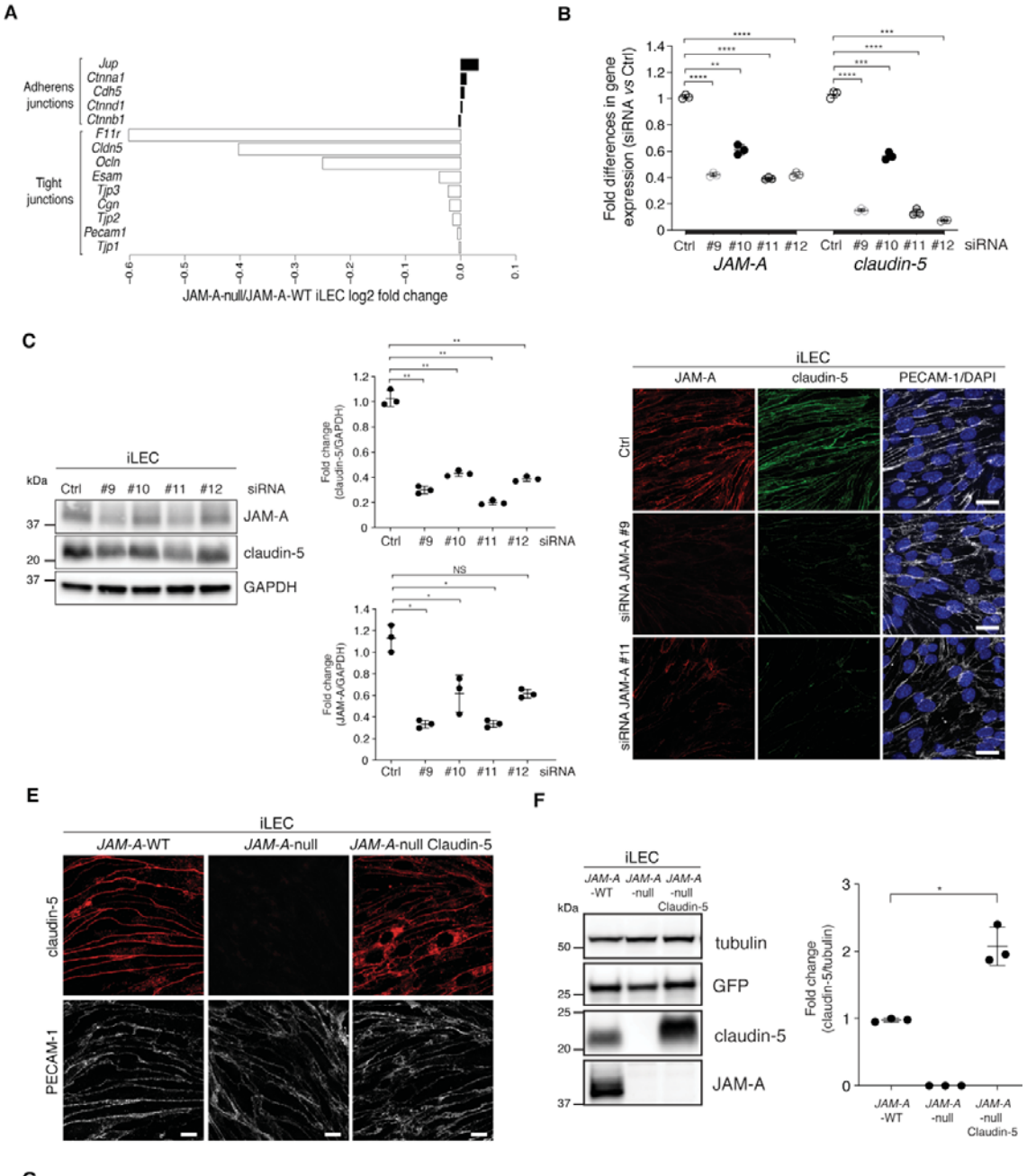
Ovarian cancer patients				
TYPE	**DIAGNOSIS**	**GRADE**	**AGE**	**GENDER**
NP	Endometrioid endometrium carcinoma		66	Female
NP	Squamous cell carcinoma of uterine cervix		47	Female
NP	Adenocarcinoma of uterine cervix		50	Female
NP	Ovarian fibroma		56	Female
NP	Ovarian cystadenoma		67	Female
NP	Endometrioid endometrium carcinoma		63	Female
NP	Leyomioma of uterus		49	Female
NP	Endometrioid uterus carcinoma		65	Female
P	Serous surface papillary carcinoma	G3	78	Female
P	Serous cystadenocarcinoma	G3	62	Female
P	Serous cystadenocarcinoma	G3	46	Female
P	Serous surface papillary carcinoma	G3	50	Female
P	Serous cystadenocarcinoma	G3	45	Female
P	Serous cystadenocarcinoma	G3	78	Female
P	Serous surface papillary carcinoma	G3	49	Female
P	Serous surface papillary carcinoma	G3	66	Female

Glioblastoma patients				
TYPE	**DIAGNOSIS**	**GRADE**	**AGE**	**GENDER**
NP/P	Glioblastoma	IV	64	Female
NP/P	Glioblastoma	IV	51	Female
NP/P	Glioblastoma	IV	56	Female
NP/P	Glioblastoma	IV	47	Male
NP/P	Glioblastoma	IV	48	Male
NP/P	Glioblastoma	IV	33	Male
NP/P	Glioblastoma	IV	30	Female
NP/P	Glioblastoma	IV	32	Male
NP/P	Glioblastoma	IV	59	Male
NP/P	Glioblastoma	IV	41	Male
NP/P	Glioblastoma	IV	43	Female

Online Table II. Primers used for the chromatin immunoprecipitation assays.

Region	Direction	Primer
#1	FW	GACCAGCATAATCCTTAACAACAA
	R	CCACCAAGAAACTTTGCTGAG
#2	FW	GTGCCTCAGTACCTTCGAGC
	R	TCAGCATTGTGTAACCCCAGT
#3	FW	AGATAGAAGTAAGGCTGGGGT
	R	GGTTCCTGGGGCTTGGATTT

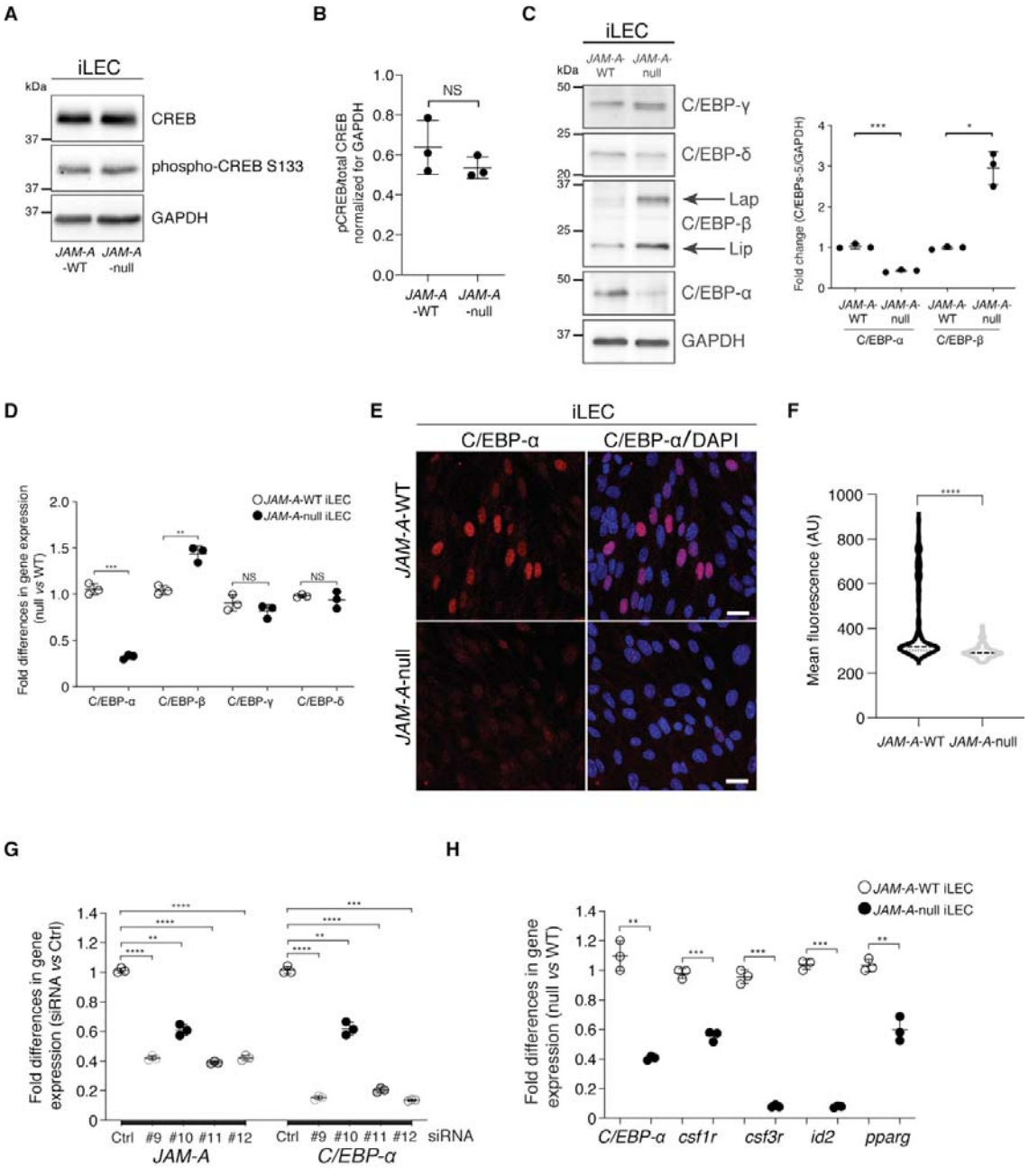
Online Figures



Online Figure I. JAM-A positively regulates claudin-5 expression.

A, Bar chart showing mean log2 (fold-changes) from JAM-A-null/JAM-A-WT immortalised lung-derived endothelial cells (iLECs) for genes involved in formation of adherens (black) and tight (white) junctions (two mice per condition). **B**, Quantification of fold differences in JAM-A and claudin-5 gene expression in iLECs following RT-qPCR analysis. Cells were treated with non-targeting siRNA (Ctrl; fold-difference, 1.0) and four siRNAs against *JAM-A* (#9, #10, #11, #12), as indicated. Data are means ±SD from 3 independent experiments. *P* values determined by Brown-Forsythe ANOVA followed by Dunnett's T3 test for multiple comparisons. *JAM-A*: (overall ****, $P = 2.6 \times 10^{-6}$), siRNA Ctrl *vs* siRNA #9 ****, $P = 3.9 \times 10^{-6}$; siRNA Ctrl *vs* siRNA #10 **, $P = 1.2 \times 10^{-3}$; siRNA Ctrl *vs* siRNA #11 ****, $P = 3.2 \times 10^{-5}$; siRNA Ctrl *vs* siRNA #12 ****, $P = 5.3 \times 10^{-6}$; *Claudin-5*: (overall ****, $P = 1.7 \times 10^{-9}$), siRNA Ctrl *vs* siRNA #9 ****, $P = 5 \times 10^{-5}$; siRNA Ctrl *vs* siRNA #10 ***, $P = 10^{-4}$; siRNA Ctrl *vs* siRNA #11 ****, $P = 5.8 \times 10^{-6}$; siRNA Ctrl *vs* siRNA #12 ***, $P = 7.4 \times 10^{-4}$. **C**, Left: Representative immunoblotting for JAM-A and claudin-5 in iLECs transfected with non-targeting siRNA (Ctrl) or four siRNAs against JAM-A (#9, #10, #11, #12), as shown in (**A**). GAPDH is shown as loading control. Right: JAM-A/GAPDH and claudin-5/GAPDH ratios quantified by densitometry scanning and expressed as fold-changes. Data are means ±SD from 3 independent experiments. *P* values determined by Brown-Forsythe ANOVA followed by Dunnett's T3 test for multiple comparisons. *Claudin-5*: (overall ****, $P = 2.8 \times 10^{-5}$), siRNA Ctrl *vs* siRNA #9 **, $P = 1.1 \times 10^{-3}$; siRNA Ctrl *vs* siRNA #10 **, $P = 1.9 \times 10^{-3}$; siRNA Ctrl *vs* siRNA #11 **, $P = 5.3 \times 10^{-3}$; siRNA Ctrl *vs* siRNA #12 **, $P = 9.1 \times 10^{-3}$; *JAM-A*: (overall **, $P = 1.8 \times 10^{-3}$),siRNA Ctrl *vs* siRNA #9 *, $P = 2.1 \times 10^{-3}$; siRNA Ctrl *vs* siRNA #10 *, $P = 4.2 \times 10^{-3}$; siRNA Ctrl *vs* siRNA #11 *, $P = 2.1 \times 10^{-3}$; siRNA Ctrl *vs* siRNA #12 $P = 0.051$, NS. Representative confocal microscopy of JAM-A (red) and claudin-5 (green) expression in

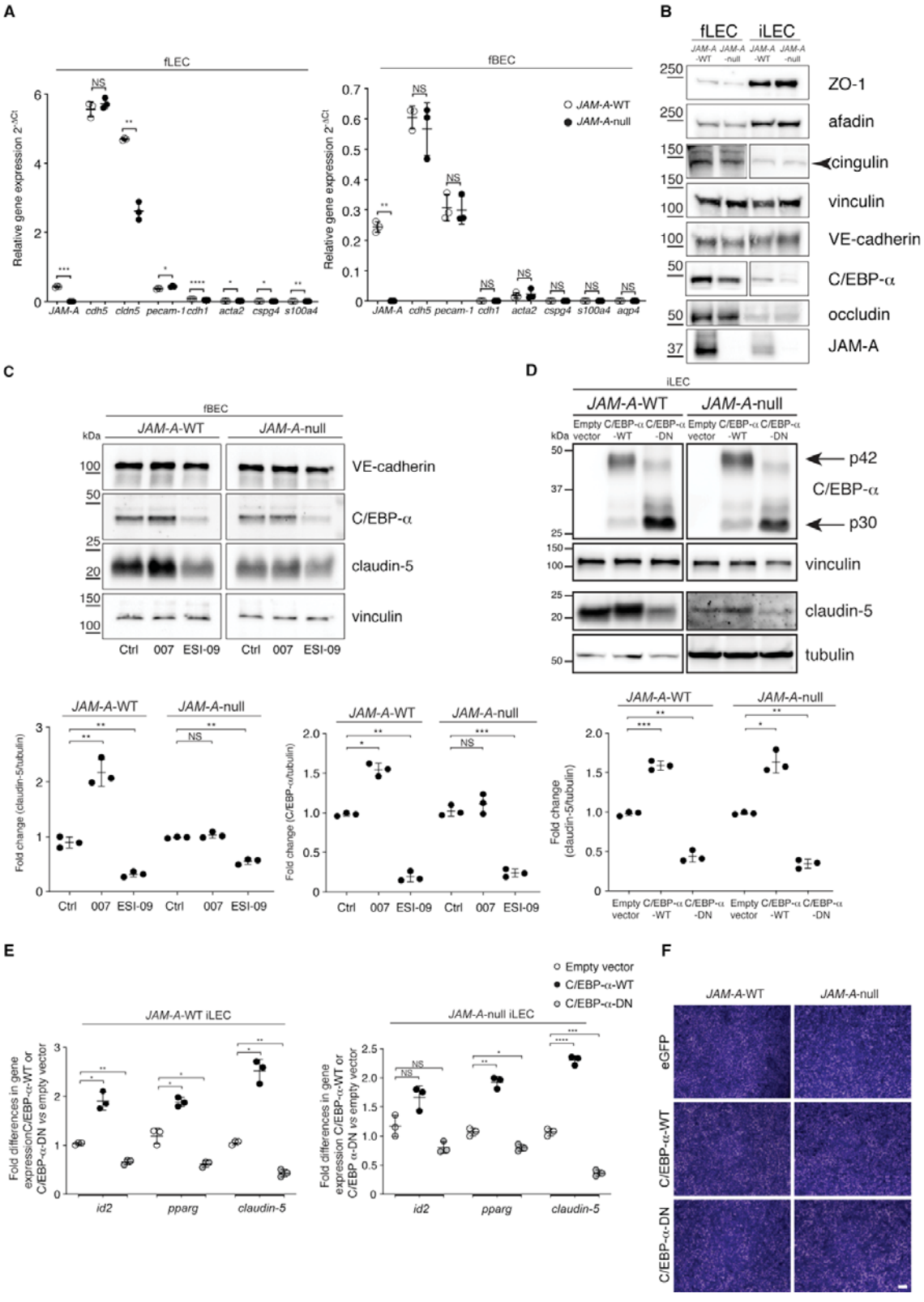
iLECs treated as in (**A**). PECAM-1(white) is shown as an endothelial junctional marker. DAPI (blue) stains nuclei. Data are representative of 3 independent experiments. Scale bars: 20 μm. **E**, Representative confocal microscopy of claudin-5 (red) and PECAM-1 (white) in iLECs stably expressing GFP (JAM-A–WT, JAM-A–null, Ctrl) or claudin-5 (JAM-A–null claudin-5). Data are representative of 3 independent experiments. Scale bars: 10 μm. **F**, Left: Representative immunoblotting for JAM-A, claudin-5 and GFP in lysates from cells treated as in (**E**). Tubulin is shown as loading control. Right: claudin-5/tubulin ratios quantified by densitometry scanning and expressed as fold-changes. Data are means ±SD from 3 independent experiments. *P* value determined by two-sided unpaired Welch's t-test. *JAM-A*–WT *vs JAM-A*–null Claudin-5 *, $P = 2.2 \times 10^{-2}$. **G**, Representative crystal violet staining of filters from *JAM-A*–WT and *JAM-A*–null iLECs and *JAM-A*–null stably expressing GFP (*JAM-A*–WT, *JAM-A*–null, Ctrl) or claudin-5 (*JAM-A*–null claudin-5) with treatments with vehicle or 007 (100 μM), as shown in Figure 1**E**. Monolayers were stained at the end of the permeability assay (6 h). Scale bar: 50 μm (triplicate filters/condition; n = 3).


A
iLEC
kDa
CREB
phospho-CREB S133
GAPDH
37
37
37
JAM-A -WT
JAM-A -null
B
pCREB/total CREB normalized for GAPDH
NS
JAM-A -WT
JAM-A -null
C
iLEC
JAM-A- WT
JAM-A- null
kDa
C/EBP-γ
C/EBP-δ
Lap
C/EBP-β
Lip
C/EBP-α
GAPDH
Fold change (C/EBPs-5/GAPDH)
C/EBP-α
C/EBP-β
D
JAM-A-WT iLEC
JAM-A-null iLEC
Fold differences in gene expression (null vs WT)
C/EBP-α
C/EBP-β
C/EBP-γ
C/EBP-δ
E
iLEC
C/EBP-α
C/EBP-α/DAPI
JAM-A-WT
JAM-A-null
F
Mean fluorescence (AU)
JAM-A-WT
JAM-A-null
G
Fold differences in gene expression (siRNA vs Ctrl)
Ctrl
#9
#10
#11
#12
siRNA
JAM-A
C/EBP-α
H
JAM-A-WT iLEC
JAM-A-null iLEC
Fold differences in gene expression (null vs WT)
C/EBP-α
csf1r
csf3r
id2
pparg

Online Figure II. JAM-A deficiency reduces expression and transcriptional activity of C/EBP-α independent of PKA signalling.

A, Representative immunoblotting for total CREB and its phosphorylation (phospho-CREB S133) in *JAM-A*–WT and *JAM-A*–null immortalised lung-derived endothelial cells (iLECs). GAPDH is shown as loading control. Data are representative of 3 independent experiments. **B**, Quantification of data illustrated in (**A**). Data are means ±SD. *P* value determined by two-sided unpaired Welch's t-test. *JAM-A*–WT *vs JAM-A*–null $P = 0.32$, NS. **C**, Representative immunoblotting for C/EBP proteins in *JAM-A*–WT and *JAM-A*–null iLECs. GAPDH is shown as loading control. Right: C/EBP-α/GAPDH and C/EBP-β/GAPDH ratios quantified by densitometry scanning and expressed as fold-changes. Data are means ±SD from 3 independent experiments. *P* value determined by two-sided unpaired Welch's t-test. *JAM-A*–WT *vs JAM-A*–null: C/EBP-α ***, $P = 5.7 \times 10^{-4}$; C/EBP-β *, $P = 1.3 \times 10^{-2}$. **D**, Quantification of fold-differences in *C/EBP* genes expression in *JAM-A*–WT and *JAM-A*–null iLECs following RT-qPCR analysis. Data are means ±SD from 3 independent experiments. *P* value determined by two-sided unpaired Welch's t-test. *JAM-A*–WT *vs JAM-A*–null: C/EBP-α ***, $P = 4.9 \times 10^{-4}$; C/EBP-β **, $P = 4.5 \times 10^{-3}$; C/EBP-γ $P = 0.24$, NS; C/EBP-δ $P = 0.50$, NS. **E**, Representative confocal microscopy of C/EBP-α (red) and DAPI (blue; nuclear marker) in *JAM-A*–WT and *JAM-A*–null iLECs. Data are representative of 3 independent experiments. Scale bars: 20 μm. **F**, Quantification of mean fluorescence (arbitrary units; AU) for C/EBP-α expression in nuclei of *JAM-A*–WT and *JAM-A*–null iLECs. Data are means ±SD from 3 independent experiments. *P* value determined by two-sided Mann–Whitney test. *JAM-A*–WT *vs JAM-A*–null, ****, $P < 10^{-15}$. **G**, Quantification of fold-differences for *JAM-A* and *C/EBP*-α gene expression in iLECs following RT-qPCR analysis. Cells were treated with non-targeting siRNA (Ctrl; fold-difference, 1.0) or different siRNAs against JAM-A (#9, #10, #11, #12). Data are means ±SD from 3 independent experiments. *P* values determined by Brown-Forsythe ANOVA followed

by Dunnett's T3 test for multiple comparisons. *JAM-A*: (overall ****, $P = 2.6 \times 10^{-6}$), siRNA Ctrl *vs* siRNA #9 ****, $P = 3.9 \times 10^{-6}$; siRNA Ctrl *vs* siRNA #10 **, $P = 1.2 \times 10^{-3}$; siRNA Ctrl *vs* siRNA #11 ****, $P = 3.2 \times 10^{-5}$; siRNA Ctrl *vs* siRNA #12 ****, $P = 5.3 \times 10^{-6}$; *C/EBP*-α: (overall ****, $P = 4.7 \times 10^{-6}$), siRNA Ctrl *vs* siRNA #9 ****, $P = 4 \times 10^{-5}$; siRNA Ctrl *vs* siRNA #10 **, $P = 2.6 \times 10^{-3}$; siRNA Ctrl *vs* siRNA #11 ****, $P = 5.5 \times 10^{-5}$; siRNA Ctrl *vs* siRNA #12 ***, $P = 6.8 \times 10^{-4}$. **H**, Quantification of fold-differences for *C/EBP*-α and its target gene (*csf1r*, *csf3r*, *id2*, *pparg*) expression in *JAM-A*–WT (fold-difference, 1.0) and *JAM-A*–null iLECs following RT-qPCR analysis. Data are means ±SD from 3 independent experiments. *P* value determined by two-sided unpaired Welch's t-test. *JAM-A*–WT *vs JAM-A*–null iLECs: *C/EBP-α* **, $P = 6.2 \times 10^{-3}$; *csf1r* ***, $P = 1.1 \times 10^{-4}$; *csf3r* ***, $P = 6.6 \times 10^{-4}$; *id2* ***, $P = 2.7 \times 10^{-4}$; *pparg* **, $P = 4.1 \times 10^{-3}$.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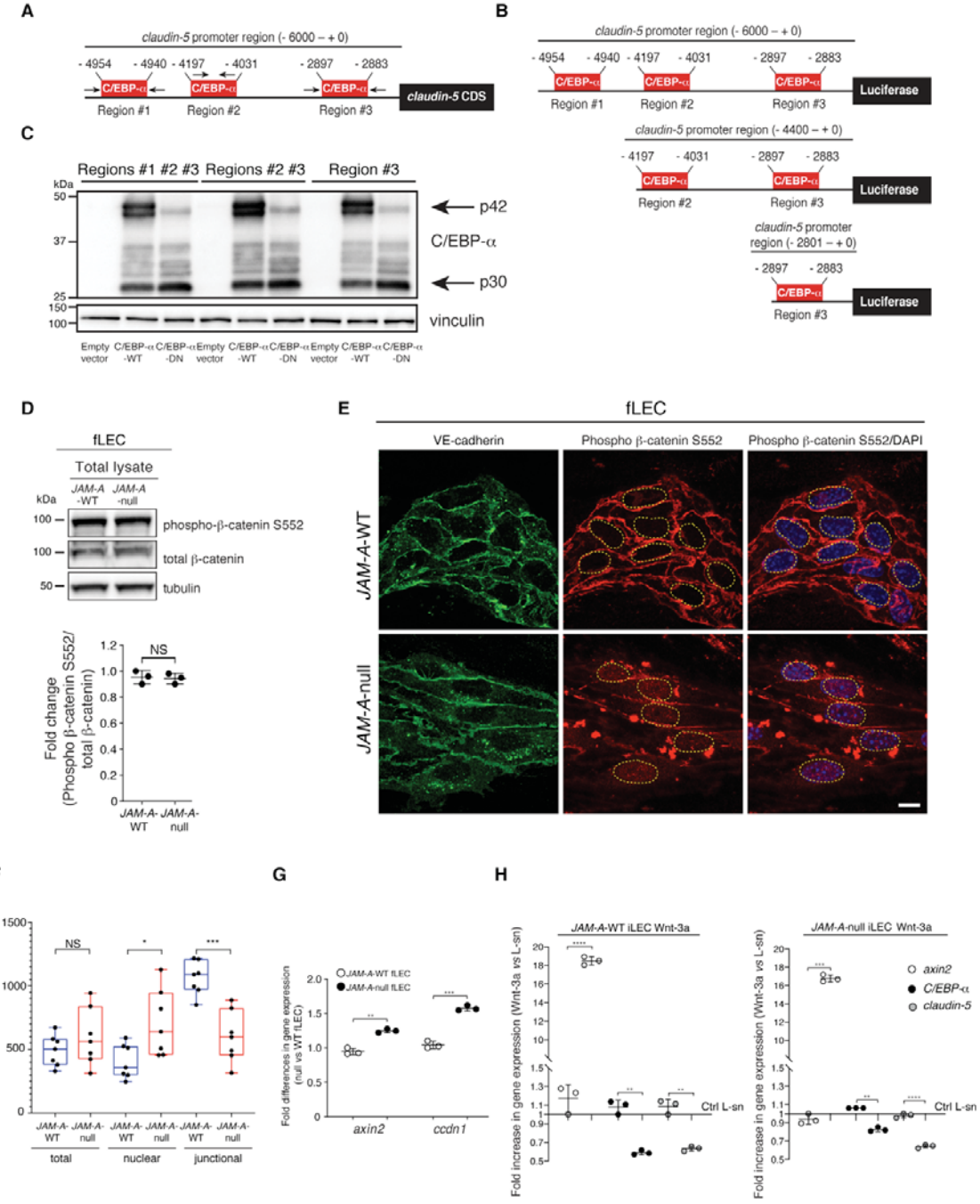
fLEC
fBEC
Relative gene expression 2-ΔCt
JAM-A-WT
JAM-A-null
JAM-A
cdh5
cldn5
pecam-1
cdh1
acta2
cspg4
s100a4
aqp4
B
fLEC
iLEC
JAM-A -WT
JAM-A -null
ZO-1
afadin
cingulin
vinculin
VE-cadherin
C/EBP-α
occludin
JAM-A
C
fBEC
JAM-A-WT
JAM-A-null
kDa
VE-cadherin
C/EBP-α
claudin-5
vinculin
Ctrl
007
ESI-09
Fold change (claudin-5/tubulin)
Fold change (C/EBP-α/tubulin)
D
iLEC
Empty vector
C/EBP-α -WT
C/EBP-α -DN
p42
C/EBP-α
p30
vinculin
claudin-5
tubulin
Fold change (claudin-5/tubulin)
E
JAM-A-WT iLEC
JAM-A-null iLEC
Empty vector
C/EBP-α-WT
C/EBP-α-DN
Fold differences in gene expression C/EBP-α-WT or C/EBP-α-DN vs empty vector
id2
pparg
claudin-5
F
JAM-A-WT
JAM-A-null
eGFP
C/EBP-α-WT
C/EBP-α-DN

Online Figure III. JAM-A acts through EPAC-C/EBP-α regulation to up-regulate expression of claudin-5 in immortalised and freshly isolated endothelial cells.

A, Quantification of genes expressed by endothelial cells (*JAM-A, cdh5, cldn5, pecam-1*) and by other contaminant cells (*cdh1, acta2 cspg4, s100a4, aqp4*) in freshly isolated lung endothelial cells (fLECs) and freshly isolated brain endothelial cells (fBECs) derived from *JAM-A*–WT and *JAM-A*–null mice following RT-qPCR analysis. Data are means ±SD from 3 independent experiments (n = 12, *JAM-A*–WT; n = 12, *JAM-A*–null mice; for each experiment). *P* value determined by two-sided unpaired Welch's t-test. *JAM-A*–WT *vs JAM-A*–null fLECs: *JAM-A* ***, $P = 7 \times 10^{-4}$; *cdh5* $P = 0.33$, NS; *cldn5* **, $P = 4 \times 10^{-3}$; *pecam-1* *, $P = 10^{-2}$; *cdh1* ****, $P = 4.8 \times 10^{-6}$; *acta2* *, $P = 1.8 \times 10^{-2}$; *cspg4* *, $P = 4.7 \times 10^{-2}$; *s100a4* *, $P = 7.7 \times 10^{-3}$; *JAM-A*–WT *vs JAM-A*–null fBECs: *JAM-A* **, $P = 1.6 \times 10^{-3}$; *cdh5* $P = 0.54$, NS; *pecam-1* $P = 0.84$, NS; *cdh1* $P = 0.42$, NS; *acta2* $P = 0.72$, NS; *cspg4* $P = 0.49$, NS; *s100a4* $P = 0.95$, NS; *aqp4* $P = 0.48$, NS. **B**, Representative immunoblotting for Afadin, ZO-1, cingulin, occludin and VE-cadherin in immortalised lung-derived endothelial cells (iLECs) and fLECs derived from lungs of WT and JAM-A-null mice. Vinculin is shown as loading control. Data are representative of 3 independent experiments (for fLECs: n = 4, *JAM-A*–WT; n = 4, *JAM-A*–null mice; for each experiment). **C**, Top: Representative immunoblotting for C/EBP-α, claudin-5 and VE-cadherin in fBECs. Cells were treated with vehicle (fold-difference, 1.0), and the selective EPAC activator 007 (100 µM) and specific EPAC inhibitor ESI-09 (10 µM). Data are means ±SD from 3 independent experiments Bottom: Claudin-5/vinculin and C/EBP-α/vinculin ratios quantified by densitometry scan and expressed as fold-changes. Data are means ±SD from 3 independent experiments. (n = 12, *JAM-A*–WT; n = 12, *JAM-A*–null mice; for each experiment). *P* values determined by Brown-Forsythe ANOVA followed by Dunnett's T3 test for multiple comparisons. Claudin-5: *JAM-A*–WT (overall **, $P = 2 \times 10^{-3}$), Ctrl *vs* 007 **, $P = 6.4 \times 10^{-3}$; Ctrl *vs* ESI-09 **, $P = 5 \times 10^{-3}$; *JAM-A*–null (overall ***, $P = 2 \times 10^{-4}$), Ctrl

vs 007 $P = 0.47$, NS; Ctrl *vs* ESI-09 **, $P = 8.7 \times 10^{-3}$; C/EBP-α: *JAM-A*–WT (overall ****, $P = 2.2 \times 10^{-5}$), Ctrl *vs* 007 *, $P = 1.1 \times 10^{-2}$; Ctrl *vs* ESI-09 **, $P = 3.8 \times 10^{-3}$; *JAM-A*–null (overall ***, $P = 5.8 \times 10^{-4}$), Ctrl *vs* 007 $P = 0.53$, NS; Ctrl *vs* ESI-09 ***, $P = 1.6 \times 10^{-4}$. **D**, Representative immunoblotting for C/EBP-α (upper panel) and claudin-5 (three lower panels) in *JAM-A*–WT and *JAM-A*–null iLECs overexpressing empty vector as control, or full-length C/EBP-α-WT (p42) or C/EBP-α-DN (p30). Upper panel: vinculin is shown as loading control. Bottom panel: 10 µg JAM-A-WT; 80 µg JAM-A-null were loaded. Tubulin was used as loading control. Claudin-5/tubulin ratios quantified by densitometry scanning and expressed as fold-changes. Data are means ±SD from 3 independent experiments. *P* values determined by Brown-Forsythe ANOVA followed by Dunnett's T3 test for multiple comparisons. *JAM-A*–WT: (overall ****, $P = 1.5 \times 10^{-5}$), empty vector *vs* C/EBP-α-WT ***, $P = 8.5 \times 10^{-4}$, empty vector *vs* C/EBP-α-DN **, $P = 2 \times 10^{-3}$; *JAM-A*–null (overall **, $P = 1.5 \times 10^{-3}$): empty vector *vs* C/EBP-α-WT *, $P = 2.4 \times 10^{-2}$; empty vector *vs* C/EBP-α-DN **, $P = 4.4 \times 10^{-3}$. **E**, Quantification of fold-differences of *id2*, *pparg* and *claudin-5* gene expression in *JAM-A*–WT (left) and *JAM-A*–null (right) iLECs, for overexpression of empty vector (control; fold-difference 1.0) and C/EBP-α-WT or C/EBP-α-DN. Data are means ±SD from 3 independent experiments. *P* values determined by Brown-Forsythe ANOVA followed by Dunnett's T3 test for multiple comparisons. *JAM-A*–WT iLECs: *id2*: (overall **, $P = 4.7 \times 10^{-3}$), empty vector *vs* C/EBP-α-WT *, $P = 2.4 \times 10^{-2}$; empty vector *vs* C/EBP-α-DN **, $P = 2.9 \times 10^{-3}$; *pparg*: (overall ***, $P = 5.9 \times 10^{-4}$), empty vector *vs* C/EBP-α-WT *, $P = 1.3 \times 10^{-2}$; empty vector *vs* C/EBP-α-DN *, $P = 1.9 \times 10^{-2}$; *cldn5*: (overall **, $P = 2 \times 10^{-3}$), empty vector *vs* C/EBP-α-WT *, $P = 1.4 \times 10^{-2}$; empty vector *vs* C/EBP-α-DN **, $P = 2 \times 10^{-3}$. *JAM-A*–null iLECs: *id2*: (overall **, $P = 4.5 \times 10^{-3}$), empty vector *vs* C/EBP-α-WT $P = 6.4 \times 10^{-2}$, NS; empty vector *vs*

C/EBP-α-DN $P = 9.2 \times 10^{-2}$, NS; *pparg*: (overall ****, $P = 3.4 \times 10^{-5}$), empty vector *vs* C/EBP-α-WT **, $P = 2 \times 10^{-3}$; empty vector *vs* C/EBP-α-DN *, $P = 10^{-2}$; *cldn5*: (overall ****, $P = 2.6 \times 10^{-7}$), empty vector *vs* C/EBP-α-WT ****, $P = 5 \times 10^{-5}$; empty vector *vs* C/EBP-α-DN ***, $P = 2.1 \times 10^{-4}$. **F,** Representative crystal violet staining of filters from JAM-A–WT and JAM-A–null iLECs stably expressing full-length C/EBP-α-WT (p42), C/EBP-α-DN (p30), and eGFP as control, as shown in Figure 3**E**. The monolayers were stained at the end of the permeability assay (6 h). Scale bar: 50 μm (triplicate filters/condition; n = 3)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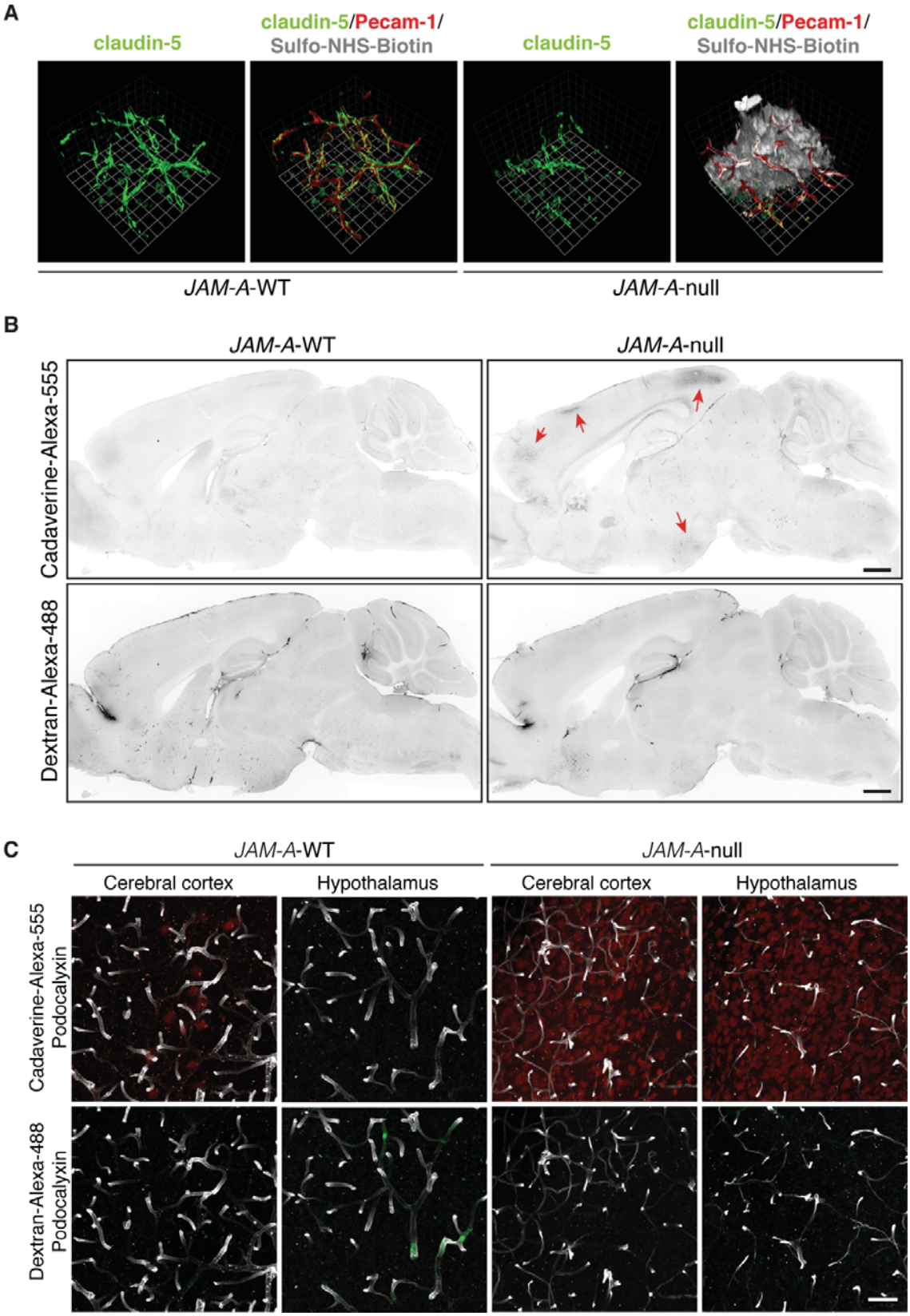
- 4954
- 4940
- 4197
- 4031
- 2897
- 2883
C/EBP-α
claudin-5 CDS
Region #1
Region #2
Region #3
B
claudin-5 promoter region (- 6000 – + 0)
Luciferase
claudin-5 promoter region (- 4400 – + 0)
claudin-5 promoter region (- 2801 – + 0)
C
Regions #1 #2 #3
Regions #2 #3
Region #3
kDa
50
37
25
150
100
p42
C/EBP-α
p30
vinculin
Empty vector
C/EBP-α -WT
C/EBP-α -DN
D
fLEC
Total lysate
JAM-A -WT
JAM-A -null
phospho-β-catenin S552
total β-catenin
tubulin
Fold change (Phospho β-catenin S552/ total β-catenin)
NS
JAM-A- WT
JAM-A- null
E
fLEC
VE-cadherin
Phospho β-catenin S552
Phospho β-catenin S552/DAPI
JAM-A-WT
JAM-A-null
F
Phospho-β-catenin S552 mean intensity (AU)
total
nuclear
junctional
G
JAM-A-WT fLEC
JAM-A-null fLEC
Fold differences in gene expression (null vs WT fLEC)
axin2
ccdn1
H
JAM-A-WT iLEC Wnt-3a
JAM-A-null iLEC Wnt-3a
Fold increase in gene expression (Wnt-3a vs L-sn)
Ctrl L-sn
axin2
C/EBP-α
claudin-5

Online Figure IV. C/EBP-α increases claudin-5 expression and is inhibited by active β-catenin.

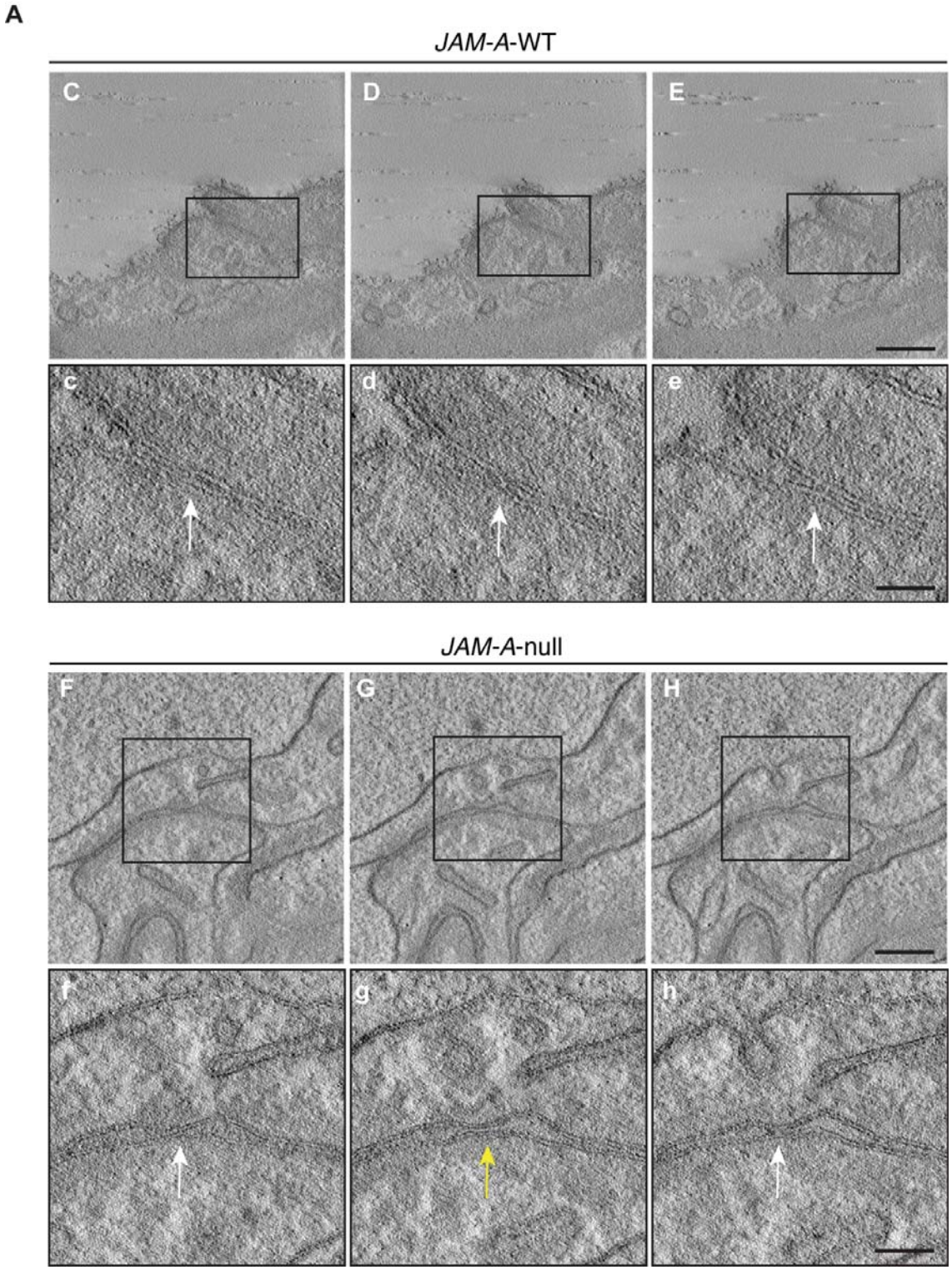
A, Schematic representation of the putative C/EBP-α binding sites (red boxes) in the promoter region of the *claudin-5* gene. Arrows indicate primers used for quantitative chromatin immunoprecipitation experiments shown in Figure 4. **B,** Schematic representation of the regions of *claudin-5* putative promoter cloned upstream of the firefly luciferase gene. C/EBP-α binding sites are depicted as red boxes. These constructs were used in the luciferase reporter assays shown in Figure 4. **C**, Representative immunoblotting for C/EBP-α in HEK-293T cells overexpressing *claudin-5* promoter reporter plasmids (indicated in **B**) together with empty vector as control, and full-length C/EBP-α-WT (p42) or C/EBP-α-DN (p30). Vinculin is shown as loading control. Data are representative of 3 independent experiments. **D**, Top: Representative immunoblotting for total and active β-catenin S552 (phospho-β-catenin) in *JAM-A*–WT and *JAM–A*-null freshly isolated lung endothelial cells (fLECs). Tubulin is shown as loading control. Bottom: Phospho-β-catenin S552/total-β-catenin ratios quantified by densitometry scanning and expressed as fold-changes. Data are means ±SD from 3 independent experiments (n = 3, *JAM-A*–WT; n = 3, *JAM-A*–null mice; for each experiment). *P* value determined by two-sided unpaired Welch's t-test. *JAM-A*–WT *vs JAM–A*-null $P = 0.80$, NS. **E**, Representative confocal microscopy of VE-cadherin (green) and phospho-β-catenin S552 (red) expression in *JAM-A*–WT and *JAM-A*–null fLECs. Data are representative of 3 independent experiments (n = 4, *JAM-A*–WT; n = 4, *JAM-A*–null mice; for each experiment). Scale bar: 10 μm. **F**, Quantification of total, nuclear and junctional phospho-β-catenin S552 (active-β-catenin) signals in *JAM-A*–WT and *JAM–A*-null fLECs as mean fluorescence intensities, expressed as arbitrary units (AU). Data are means ±SD; *P* value determined by two-sided unpaired Welch's t-test. *JAM-A*–WT *vs JAM–A*-null:, total $P = 0.31$, NS; nuclear *, $P = 2.4 \times 10^{-2}$; junctional ***, $P = 4.7 \times 10^{-4}$. **G**, Quantification of fold-differences in β-catenin

target gene (*axin2*, *ccdn1*) expression in *JAM-A*–WT (fold-difference, 1.0) and *JAM-A*–null fLECs following RT-qPCR analysis. Data are means ±SD from 3 independent experiments (n = 4, *JAM-A*–WT; n = 4, *JAM-A*–null mice; for each experiment). *P* value determined by two-sided unpaired Welch's t-test. *JAM-A*–WT *vs JAM–A*-null fLECs: *axin2* **, $P = 1.6 \times 10^{-3}$; *ccdn1* ***, $P = 2.4 \times 10^{-4}$. **H**, Quantification of fold-differences of *C/EBP*-α, *claudin-5* and *axin-2* gene expression in cells from *JAM-A*–WT (left) and *JAM-A*–null (right) iLECs, upon treatment with L-cell–conditioned medium (Ctrl L-sn; fold-difference 1.0) or Wnt-3a. Data are means ±SD of triplicates from a single experiment, as representative of 3 experiments. *P* value determined by two-sided unpaired Welch's t-test. *JAM-A*–WT: Ctrl L-sn *vs* Wnt-3a: *axin2* ****, $P = 5 \times 10^{-5}$; *C/EBP*-α **, $P = 3.6 \times 10^{-3}$; *claudin-5* **, $P = 2.4 \times 10^{-4}$; *JAM-A*–null: Ctrl L-sn *vs* Wnt-3a: *axin2* ***, $P = 1.4 \times 10^{-4}$; *C/EBP*-α **, $P = 3.3 \times 10^{-3}$; *claudin-5* ****, $P = 5.4 \times 10^{-5}$.


A
claudin-5
claudin-5/Pecam-1/
Sulfo-NHS-Biotin
claudin-5
claudin-5/Pecam-1/
Sulfo-NHS-Biotin
JAM-A-WT
JAM-A-null
B
JAM-A-WT
JAM-A-null
Cadaverine-Alexa-555
Dextran-Alexa-488
C
JAM-A-WT
JAM-A-null
Cerebral cortex
Hypothalamus
Cerebral cortex
Hypothalamus
Cadaverine-Alexa-555
Podocalyxin
Dextran-Alexa-488
Podocalyxin

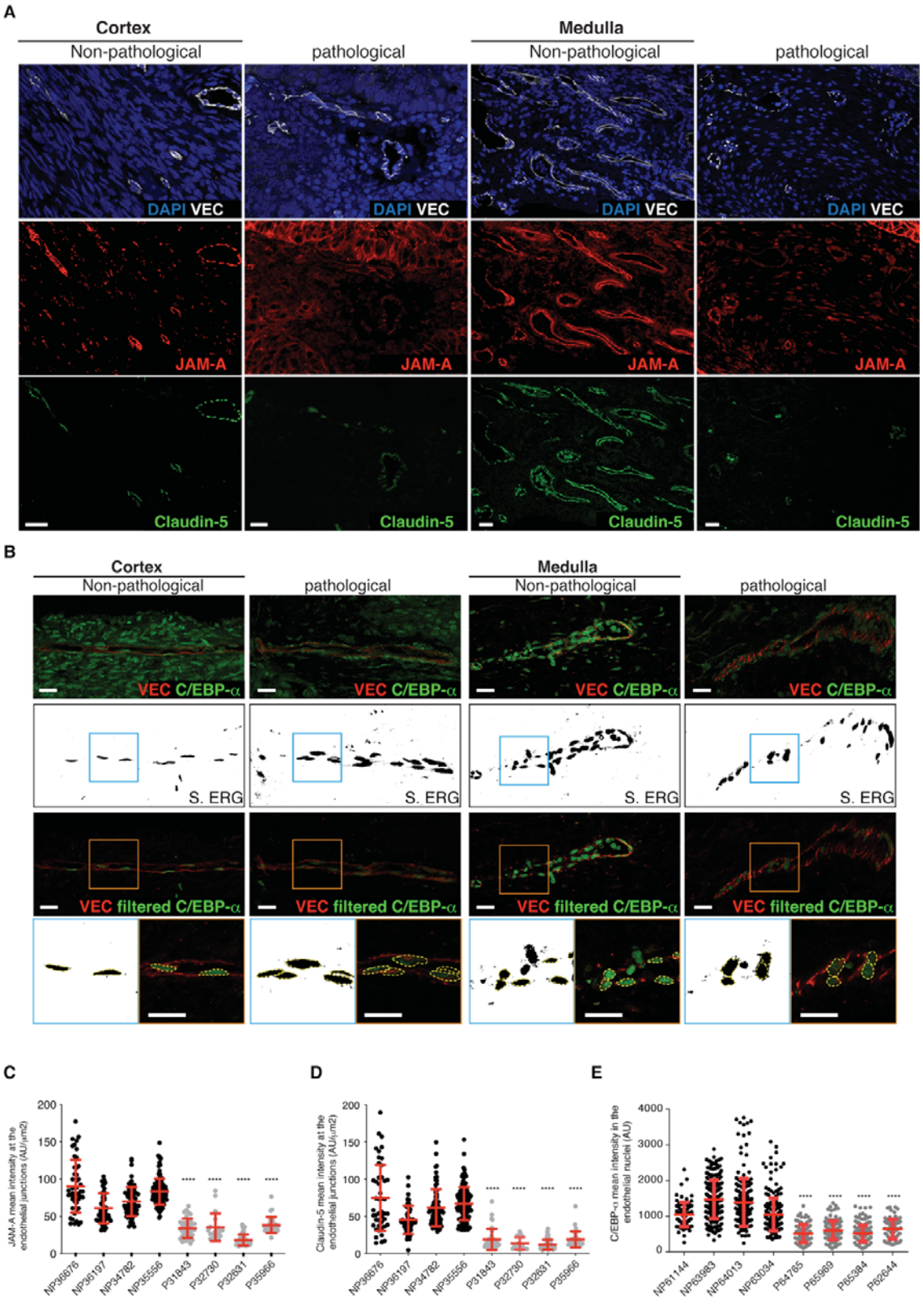
Online Figure V. JAM-A deficiency increases endothelial permeability in brain microvasculature in a size-dependent manner.

A, Representative three-dimensional reconstructions of confocal microscopy for claudin-5 (green), PECAM-1 (red) and streptavidin for detection of sulpho-NHS-biotin leakage (grey), in brain sections from *JAM-A*–WT and *JAM-A*–null mice (n = 3, *JAM-A*–WT; n = 3, *JAM-A*–null). **B**, Representative confocal microscopy to detect cadaverine-Alexa-555 and dextran-Alexa-488 in vibratome brain sections (100 μm) from *JAM-A*–WT and *JAM-A*–null mice. Red arrows (right): foci of cadaverine leakage in the cerebral cortex and in the hypothalamus. Scale bar: 1 mm (n = 4, *JAM-A*–WT; n = 5, *JAM-A*–null). **C,** Representative confocal microscopy for podocalyxin (white), cadaverine-Alexa-555 (red) and dextran- Alexa-488 (green) of cerebral cortex and hypothalamus brain sections from *JAM-A*–WT and *JAM-A*–null mice. Scale bar: 40 μm (n = 4, *JAM-A*–WT; n = 5, *JAM-A*–null).


A
JAM-A-WT
C
D
E
c
d
e
JAM-A-null
F
G
H
f
g
h

Online Figure VI. Tight junction strands are impaired in the absence of JAM-A

A, Representative two-step transmission electron microscopy tomography of the cerebral cortex from *JAM-A*–WT (C-E) and *JAM-A*–null mice (F-H). Upper panels (C-E) and (F-H) show three consecutive serial tomography sections. Panels (c-e) and (f-h) show the enlargement of the boxed areas in the corresponding upper panels. Panels (c-e) and (f-h) are reported also in Figure 5F as panels (a-c) and (d-f) respectively. White arrows indicate a tight junction that appears as a translucent area (osmiophobic channel) between two plasma membranes. Yellow arrows indicate an interruption along the pale strand channel. Scale bars: 400 nm (C-E); 270 nm (F-H); 80 nm (c-e); 90 nm (f-h) (n = 3, *JAM-A*–WT; n = 3, *JAM-A*–null).


A
Cortex
Medulla
Non-pathological
pathological
Non-pathological
pathological
DAPI VEC
JAM-A
Claudin-5
B
Cortex
Medulla
Non-pathological
pathological
Non-pathological
pathological
VEC C/EBP-α
S. ERG
VEC filtered C/EBP-α
C
D
E
JAM-A mean intensity at the endothelial junctions (AU/um2)
Claudin-5 mean intensity at the endothelial junctions (AU/um2)
C/EBP-α mean intensity in the endothelial nuclei (AU)
NP36676
NP36197
NP34782
NP35556
P31843
P32730
P32631
P35966
NP61144
NP63983
NP64013
NP63034
P64765
P65969
P65384
P62644

Online Figure VII. JAM-A positively regulates claudin-5 and C/EBP-α expression in ovarian cancer vasculature.

A, Representative confocal microscopy for VE-cadherin (VEC, white), DAPI (blue), JAM-A (red) and claudin-5 (green) in paraffin-embedded cortex and medulla sections (4 μm) from healthy human ovary (non-pathological) or ovarian carcinoma (pathological). Images are representative of 4 healthy and ovarian cancer patients. Scale bars: 20 μm. **B**, Top three rows: Representative confocal microscopy for C/EBP-α (green) and VE-cadherin (VEC, red) in paraffin-embedded cortex and medulla sections (4 μm) from healthy human ovary (non-pathological) or ovarian carcinoma (pathological). ERG staining was segmented with threshold 350-4,096 (S. ERG) for merged images of filtered C/EBP-α and VE-cadherin (VEC filtered C/EBP-α). Bottom row: Magnification of boxed regions indicated in relevant upper rows. Yellow dashed circles outline ERG-positive nuclei to exclude signals belonging to circulating cells. Images are representative of 4 healthy and ovarian cancer patients. Scale bars: 20 μm. **C-E**, Quantification of junctional JAM-A (**C**), Claudin-5 (**D**) and nuclear C/EBP-α (**E**) signals in the vasculature of tissue biopsies from healthy (coded NP) and ovarian cancer (coded P) patients, as shown in (**A**), (**B**), as mean fluorescence intensities, expressed as arbitrary units (AU). Data are means ±SD. *P* value determined by two-sided Mann–Whitney test. NP *vs* P ****, $P < 10^{-15}$.