



# JAM-A Acts via C/EBP- $\alpha$ to Promote Claudin-5 Expression and Enhance Endothelial Barrier Function

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**RATIONALE:** Intercellular tight junctions are crucial for correct regulation of the endothelial barrier. Their composition and integrity are affected in pathological contexts, such as inflammation and tumor growth. JAM-A (junctional adhesion molecule A) is a transmembrane component of tight junctions with a role in maintenance of endothelial barrier function, although how this is accomplished remains elusive.

**OBJECTIVE:** We aimed to understand the molecular mechanisms through which JAM-A expression regulates tight junction organization to control endothelial permeability, with potential implications under pathological conditions.

**METHODS AND RESULTS:** Genetic deletion of JAM-A in mice significantly increased vascular permeability. This was associated with significantly decreased expression of claudin-5 in the vasculature of various tissues, including brain and lung. We observed that C/EBP- $\alpha$  (CCAAT/enhancer-binding protein- $\alpha$ ) can act as a transcription factor to trigger the expression of claudin-5 downstream of JAM-A, to thus enhance vascular barrier function. Accordingly, gain-of-function for C/EBP- $\alpha$  increased claudin-5 expression and decreased endothelial permeability, as measured by the passage of fluorescein isothiocyanate (FITC)-dextran through endothelial monolayers. Conversely, C/EBP- $\alpha$  loss-of-function showed the opposite effects of decreased claudin-5 levels and increased endothelial permeability. Mechanistically, JAM-A promoted C/EBP- $\alpha$  expression through suppression of  $\beta$ -catenin transcriptional activity, and also through activation of EPAC (exchange protein directly activated by cAMP). C/EBP- $\alpha$  then directly binds the promoter of claudin-5 to thereby promote its transcription. Finally, JAM-A–C/EBP- $\alpha$ –mediated regulation of claudin-5 was lost in blood vessels from tissue biopsies from patients with glioblastoma and ovarian cancer.

**CONCLUSIONS:** We describe here a novel role for the transcription factor C/EBP- $\alpha$  that is positively modulated by JAM-A, a component of tight junctions that acts through EPAC to up-regulate the expression of claudin-5, to thus decrease endothelial permeability. Overall, these data unravel a regulatory molecular pathway through which tight junctions limit vascular permeability. This will help in the identification of further therapeutic targets for diseases associated with endothelial barrier dysfunction.

**GRAPHIC ABSTRACT:** A [graphic abstract](#) is available for this article.

**Key Words:** claudin-5 ■ endothelium ■ junctional adhesion molecule A ■ tight junctions ■ vascular permeability

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The Data Supplement is available with this article at <https://www.ahajournals.org/doi/suppl/10.1161/CIRCRESAHA.120.316742>.

For Sources of Funding and Disclosures, see page 1071.

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## Novelty and Significance

### What Is Known?

- Intercellular tight junctions are crucial for the regulation of endothelial barrier function and the control of vascular permeability. Their composition and integrity are affected in several pathological contexts, which include inflammation and cancers.
- JAM-A (junctional adhesion molecule A) is a transmembrane component of tight junctions with a role in maintenance of endothelial barrier function.
- Besides JAM-A, proteins of the claudin family are key components of tight junctions. Claudin-5 is an endothelial-specific protein that contributes to the selectivity of paracellular barriers.

### What New Information Does This Article Contribute?

- It defines the mechanism of action of JAM-A in the control of claudin-5 expression and, hence, in the regulation of the vascular barrier.
- It identifies the transcription factor C/EBP- $\alpha$  (CCAAT/enhancer-binding protein- $\alpha$ ) as a key player in controlling claudin-5 expression and endothelial permeability.

- It describes the dysregulation of the novel JAM-A-C/EBP- $\alpha$ -claudin-5 pathway in pathological conditions, such as cancer.

A detailed understanding of the molecular and cellular pathways that regulate vascular permeability is critical to the design of efficient and safe therapies for the targeted treatment of a wide range of human diseases. We describe a signaling circuit that involves JAM-A and claudin-5 and regulates endothelial permeability *in vivo* and *in vitro*. We show that the transcription factor C/EBP- $\alpha$  induces the expression of claudin-5 downstream of JAM-A, thus restricting endothelial permeability. This signaling cascade is negatively regulated by active  $\beta$ -catenin, which represses C/EBP- $\alpha$  expression in the absence of JAM-A. Finally, JAM-A-C/EBP- $\alpha$ -mediated regulation of claudin-5 is dysregulated in the ovarian cancer and glioblastoma vasculature, implicating this circuit in aberrant tumor vessels. The discovery of this pathway can help to identify new therapeutic targets for diseases associated with vascular barrier dysfunction.

## Nonstandard Abbreviations and Acronyms

<b>007</b>	8-(4-chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8-pCPT-2'-O-Me-cAMP)
<b>C/EBP-<math>\alpha</math></b>	CCAAT/enhancer-binding protein- $\alpha$
<b>CREB</b>	cAMP response-element binding protein
<b>DN</b>	dominant negative
<b>eGFP</b>	enhanced green fluorescent protein
<b>EPAC</b>	exchange protein directly activated by cAMP
<b>ERK</b>	extracellular signal-regulated kinase
<b>fLECs</b>	freshly isolated lung endothelial cells
<b>FoxO</b>	forkhead box protein O
<b>GBM</b>	glioblastoma multiforme
<b>iLECs</b>	immortalized lung endothelial cells
<b>JAM-A</b>	junctional adhesion molecule A
<b>PECAM-1</b>	platelet/endothelial cell adhesion molecule-1
<b>PKA</b>	protein kinase A
<b>TCF</b>	T-cell factor
<b>VE-cadherin</b>	vascular endothelial cadherin
<b>WT</b>	wild-type
<b>ZO</b>	zona occludens

The vascular barrier that separates the blood from the tissues is mainly composed of the endothelium, which is essential for maintenance of vascular hemostasis. Defective barrier function through increased endothelial permeability is a common feature of many pathological processes, such as inflammation and cancers.<sup>1</sup> The functional integrity of the endothelial barrier is determined by the fine-tuned organization and activity of the adherens and tight junction complexes localized at the intercellular contacts.<sup>2-4</sup>

Tight junctions are formed by different types of transmembrane adhesion proteins (eg, occludin, claudins, junctional adhesion molecules) and intracellular proteins (eg, cingulin, afadin, ZO-1-3 [zona occludens 1-3]). Collectively, these constitute a selective barrier to water, solutes, and larger molecules.<sup>5</sup> Among the transmembrane proteins, the immunoglobulin superfamily protein JAM-A (junctional adhesion molecule A) has a key role in maintenance of endothelial barrier function.<sup>6-10</sup> JAM-A is ubiquitously expressed and regulates several processes, including cell migration, angiogenesis, and stem cell and leukocyte transmigration.<sup>11-14</sup> Recent studies have shown that ZO-1 and JAM-A can form a cooperative unit that activates junctional actomyosin, and thus induces endothelial barrier formation.<sup>15</sup> Moreover, we have shown previously that JAM-A promotes activation and junctional localization of the small GTPase Rap-1 (Ras-related protein-1) through sustained expression of EPAC (exchange protein directly activated by cAMP)-1 and EPAC-2.<sup>13</sup> These proteins are potent activators of Rap-1, which, in

turn, enhances endothelial barrier function,<sup>16–18</sup> to maintain the correct organization of cell-cell junctions.

The main structural determinant of the paracellular endothelial barrier is claudin-5,<sup>19</sup> which belongs to the claudin family. Claudin-5 expression promotes the sealing of tight junctions, and as a consequence, decreased vessel permeability, and thus enhanced endothelial barrier function.<sup>20–22</sup> A previous report suggested a link between the expression of JAM-A and claudins. Indeed, in *in vitro* and *in vivo* epithelial models, the absence of JAM-A increased the levels of the permeability-enhancing claudin-10 and claudin-15, with no changes in claudin-2 or occludin, which suggested that JAM-A regulates the claudin composition of tight junctions.<sup>23</sup> However, the molecular basis of this regulation has received little attention and remains to be better defined.

Claudin-5 expression in endothelial cells is increased by cAMP, a known regulator of endothelial barrier function, through PKA (protein kinase A)-dependent and PKA-independent pathways.<sup>24,25</sup> However, the pathway that links JAM-A expression and cAMP-induced claudin-5 expression remains to be clarified.

In the present study, we define a novel signaling circuit that regulates endothelial permeability *in vivo* and *in vitro*. We demonstrate that JAM-A induces claudin-5 expression through an EPAC-dependent mechanism that is activated by cAMP. We identify C/EBP- $\alpha$  (CCAAT/enhancer-binding protein- $\alpha$ ) as the transcription factor that triggers claudin-5 expression downstream of JAM-A, which decreases endothelial permeability. We reveal that the cAMP-inducible C/EBP- $\alpha$  activity depends on EPAC, whereas C/EBP- $\alpha$  expression is suppressed by active  $\beta$ -catenin.<sup>26,27</sup> Indeed, we show that JAM-A deficiency promotes  $\beta$ -catenin signaling, which in turn inhibits C/EBP- $\alpha$  transcription and reduces claudin-5 expression.

It has been reported that the levels of JAM-A, C/EBP- $\alpha$ , and claudin-5 are decreased in different types of cancers.<sup>28–31</sup> Here, we observed that the expression of JAM-A, C/EBP- $\alpha$ , and claudin-5 are concomitantly downregulated in the fragile vasculature of glioblastoma multiforme (GBM) and ovarian cancer, which indicates the potential relevance of this signaling pathway for maintenance of vascular integrity in humans.

In summary, our data demonstrate a novel regulatory molecular pathway through which a component of the tight junctions, JAM-A, restricts vascular permeability by enhancing the expression of claudin-5, which is a crucial component of tight junctions. This will help to identify further therapeutic targets for diseases associated with endothelial barrier dysfunction.

## METHODS

Detailed Methods are available in the [Data Supplement](#). The data that support the findings of this study are available from the corresponding authors on reasonable request.

## RESULTS

JAM-A deficiency reduces the expression of claudin-5, which controls vascular permeability along with Rap-1.

Multiple studies have identified JAM-A as an important component of endothelial and epithelial tight junctions.<sup>9,32,33</sup> JAM-A was shown to be required for control of endothelial permeability to plasma solutes and for transmigration of inflammatory and immune cells.<sup>23,34</sup> However, the molecular mechanism through which JAM-A limits permeability and maintains the integrity of tight junctions has not been fully elucidated.

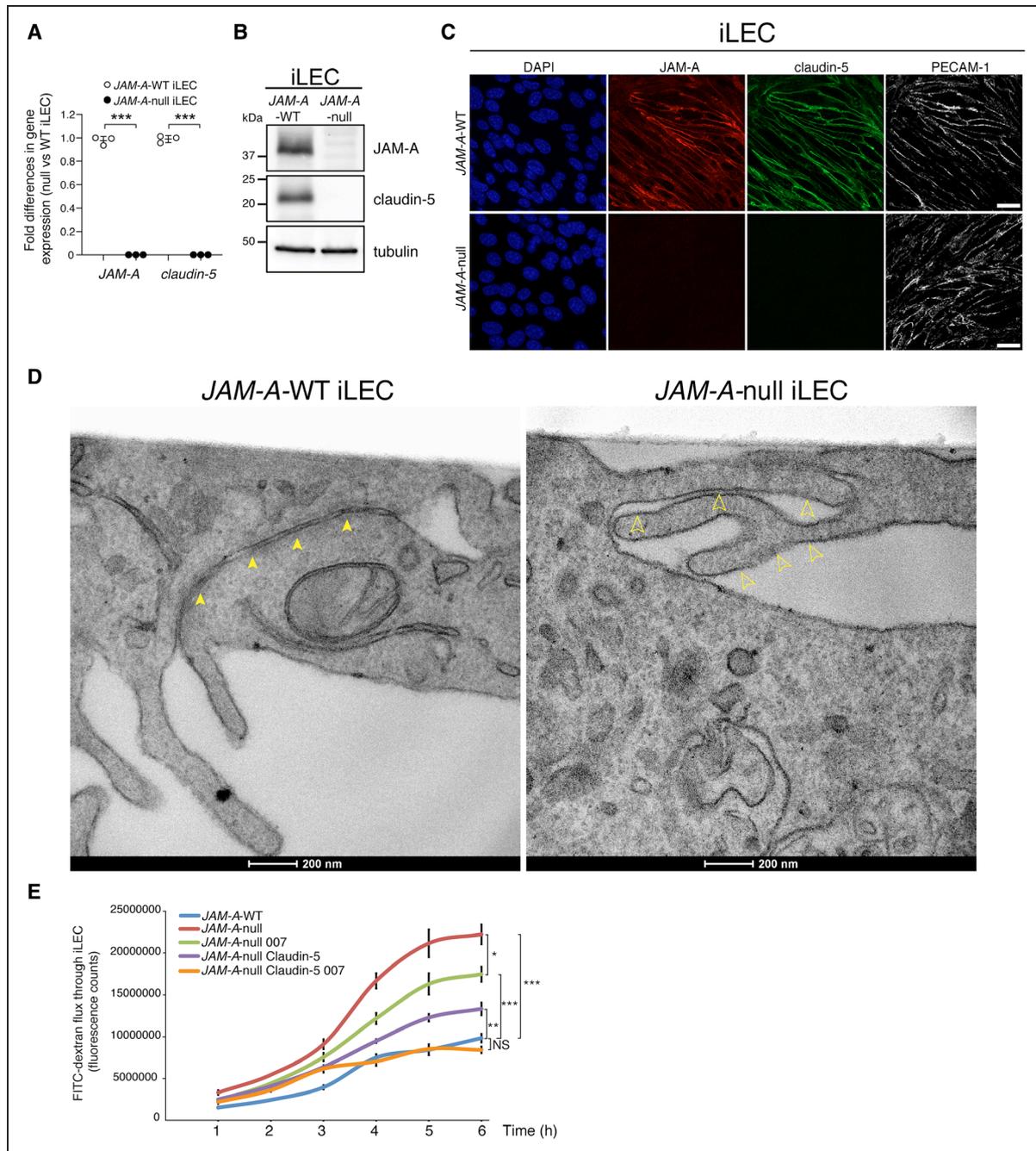
To identify transcripts regulated by JAM-A expression, we carried out Affymetrix analysis of gene expression through a comparison of immortalized lung endothelial cells (iLECs) of wild-type (WT) mice and *JAM-A*-null mice. We examined the endothelial components described in the literature as key molecules for the organization of both tight and adherens junctions. Among the prominently affected genes of the tight junctions, *claudin-5* showed greater significant downregulation in the absence of JAM-A ( $P < 0.05$ , local-pooled error test; fold change  $> 2$ ), whereas adherens junctions molecules were not significantly affected (Figure 1A in the [Data Supplement](#)).

The differences in the expression levels of claudin-5 were confirmed by real-time quantitative polymerase chain reaction analysis and also at the protein level by immunoblotting and immunofluorescence staining (Figure 1A–1C). Downregulation of claudin-5 expression in iLECs was also observed upon acute depletion of JAM-A with small-interfering RNAs (Figure 1B through 1D in the [Data Supplement](#)).

Overall, these data demonstrate a positive role for JAM-A in triggering claudin-5 expression.

To accurately examine tight junction morphology, we used transmission electron microscopy to compare *JAM-A*-WT and *JAM-A*-null iLECs. As shown in Figure 1D, in comparison with the control *JAM-A*-WT, the absence of JAM-A led to discontinuous tight junction strands, with widening of the paracellular spaces, which suggested that these junctions were not formed correctly.

As claudin-5 is the major determinant in the selectivity of paracellular barriers,<sup>20–22</sup> the overall data prompted us to postulate that its downregulation in the absence of JAM-A is responsible for increased vascular permeability. To address this point, *in vitro* permeability was determined through measurement of the passage of fluorescein isothiocyanate (FITC)-labeled dextran across monolayers of *JAM-A*-WT and *JAM-A*-null iLECs and after restoring claudin-5 expression in *JAM-A*-null iLECs. The expression and junctional localization of claudin-5 were evaluated using immunofluorescence and Western blotting, respectively (Figure 1E and 1F in the [Data Supplement](#)). As shown in Figure 1E, endothelial permeability was increased in the absence of JAM-A compared with WT cells. Moreover, reestablishment



**Figure 1. JAM-A (junctional adhesion molecule A) controls endothelial permeability through regulation of claudin-5 expression and Rap-1 (Ras-related protein-1) activation.**

**A**, Quantification of fold-differences in *JAM-A* and *claudin-5* gene expression in immortalized lung endothelial cells (iLECs) from *JAM-A*-wild-type (WT) and *JAM-A*-null mice following real-time quantitative polymerase chain reaction analysis. Data are means $\pm$ SD from 3 independent experiments. *P* value determined by 2-sided unpaired Welch *t* test. *JAM-A*-WT vs *JAM-A*-null: *JAM-A* \*\*\* $P=9.54\times 10^{-7}$ ; *claudin-5* \*\*\* $P=4.17\times 10^{-7}$ . **B**, Representative immunoblotting for *JAM-A* and *claudin-5* *JAM-A*-WT and *JAM-A*-null iLECs. Tubulin is shown as the loading control. Data are representative of 3 independent experiments. **C**, Representative confocal microscopy of *JAM-A* (red) and *claudin-5* (green) expression in *JAM-A*-WT and *JAM-A*-null iLECs. PECAM-1 (platelet/endothelial cell adhesion molecule-1; white) is an endothelial junctional marker; DAPI (4',6-diamidino-2-phenylindole; blue) stains nuclei. Data are representative of 3 independent experiments. Scale bars: 20  $\mu$ m. **D**, Representative transmission electron microscopy of *JAM-A*-WT and *JAM-A*-null iLECs. Arrows indicate the tight junction strands of the endothelial cells. Scale bars: 200 nm. **E**, Tracer flux assay. Permeability to fluorescein isothiocyanate (FITC)-dextran of *JAM-A*-WT and *JAM-A*-null iLECs and *JAM-A*-null stably expressing GFP (green fluorescent protein; *JAM-A*-WT and *JAM-A*-null) or *claudin-5* (*JAM-A*-null *claudin-5*) with treatment with vehicle or 8-(4-chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8-pCPT-2'-O-Me-cAMP; 007; 100  $\mu$ mol/L), to activate Rap-1. Data are means $\pm$ SEM of triplicates from a single experiment, as representative of 3 independent experiments. *P* values determined by Brown-Forsythe ANOVA followed by Dunnett T3 test for multiple comparisons. Comparison at 6 h: (overall \*\*\*\* $P=5.3\times 10^{-7}$ ), *JAM-A*-WT vs *JAM-A*-null \*\*\* $P=3\times 10^{-4}$ ; *JAM-A*-WT vs *JAM-A*-null 007 \*\*\* $P=5\times 10^{-4}$ ; *JAM-A*-WT vs *JAM-A*-null *claudin-5* \*\* $P=4.3\times 10^{-3}$ ; *JAM-A*-WT vs *JAM-A*-null *claudin-5* 007,  $P=0.25$ , not statistically significant [NS]; *JAM-A*-null vs *JAM-A*-null 007 \* $P=0.0117$ .

of claudin-5 expression in these *JAM-A*-null iLECs showed 72% recovery of the permeability phenotype, which indicated that claudin-5 controls endothelial barrier function downstream of JAM-A (Figure 1E; Figure IE through IG in the [Data Supplement](#)).

Our previous study showed that JAM-A promotes activation and junction localization of the small GTPase Rap-1 through sustained expression of EPAC-1 and EPAC-2.<sup>13</sup> These last 2 proteins are potent activators of Rap-1, which maintains the correct organization of cell-cell junctions and enhances endothelial barrier function.<sup>16–18</sup> Thus, we hypothesized that JAM-A regulates both claudin-5-dependent and Rap-1-dependent control of endothelial permeability. Indeed, complete rescue of endothelial function was obtained by restoring both claudin-5 expression and activating Rap-1 using the EPAC-specific agonist 8-(4-chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8-pCPT-2'-O-Me-cAMP; known as 007) in *JAM-A*-null iLECs (Figure 1E).

Taken together, these data show that JAM-A is an important regulator of vascular permeability that acts through the control of both claudin-5 expression and Rap-1 activation.

### Cyclic AMP Increases Claudin-5 Expression Through EPAC Signaling, Which Is Impaired in the Absence of JAM-A

To unravel the molecular mechanisms that underlie reduced expression of claudin-5 in the absence of JAM-A, we analyzed the different factors that might be involved in the regulation of claudin-5 expression. cAMP has been reported to enhance barrier function of tight junctions in brain endothelial cells through both PKA-dependent and PKA-independent induction of claudin-5 expression.<sup>24,25,35</sup> Moreover, the main cAMP targets are PKA and EPAC-1/2, which act in concert to tighten the endothelial barrier. Based on this evidence, we hypothesized that PKA- and EPAC-dependent pathways sustain claudin-5 expression in endothelial cells.

Therefore, we investigated different membrane-permeable cAMP analogs for their impact on claudin-5 expression. Treatment of *JAM-A*-WT iLECs with 8-chlorophenylthio-cAMP, which activates PKA and EPAC-1/2, increased claudin-5 levels by 4-fold, whereas in the absence of JAM-A, this was lower (1.5-fold; Figure 2A). Although pretreatment with the PKA inhibitor H89 did not affect the basal levels of claudin-5, this reduced 8-chlorophenylthio-cAMP-mediated claudin-5 induction by 50%, which confirmed that cAMP increases claudin-5 expression via PKA-dependent and PKA-independent (potentially EPAC-mediated) pathways in iLECs (Figure 2A). Moreover, in the absence of JAM-A, claudin-5 expression induced by 8-chlorophenylthio-cAMP was

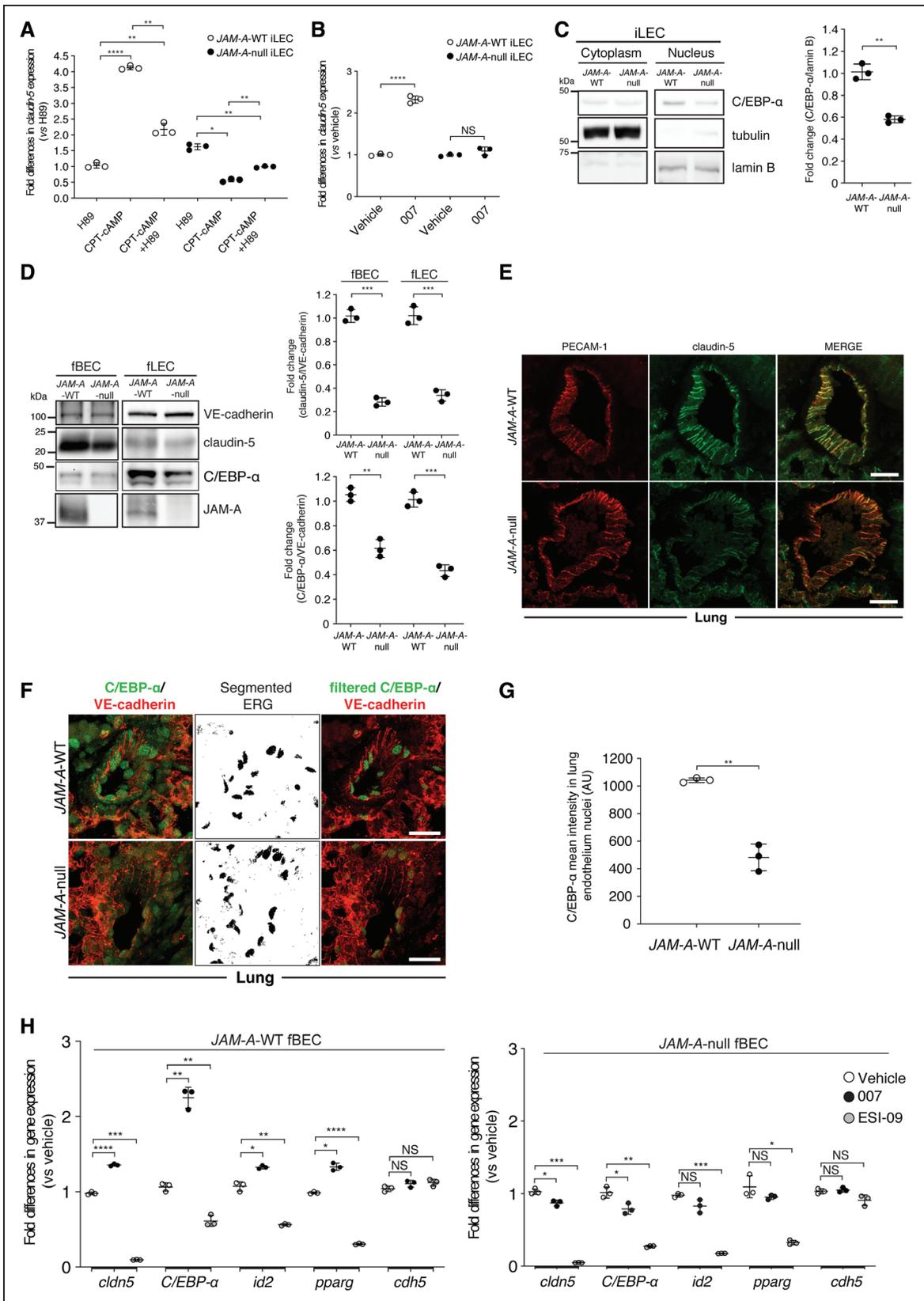
completely abrogated by H89, which suggests that the claudin-5 expression is controlled by PKA alone. Thus, we hypothesized that in *JAM-A*-null iLECs, EPAC-dependent claudin-5 expression is affected by the low expression of EPAC-1/2.<sup>13</sup> Indeed, treatment with 007 increased the claudin-5 mRNA levels only in the presence of JAM-A (by 2-fold), whereas it failed to do so in the absence of JAM-A, which confirmed that JAM-A regulates claudin-5 expression through EPAC (Figure 2B). Finally, to rule out a role for JAM-A in PKA-dependent regulation of claudin-5, we analyzed the activation of CREB (cAMP response-element binding protein), a downstream effector of PKA.<sup>36</sup> Here, comparison of *JAM-A*-WT and *JAM-A*-null iLECs showed no differences in CREB phosphorylation at serine residue 133<sup>36</sup> (Figure IIA and IIB in the [Data Supplement](#)).

Overall, these data demonstrate that JAM-A regulates claudin-5 expression through an EPAC-dependent mechanism.

### JAM-A Deficiency Reduces Expression of C/EBP- $\alpha$

Evidence suggested that cAMP can also regulate gene expression through nonclassical routes that are independent of CREB and PKA.<sup>37</sup> In this context, a group of transcription factors known as C/EBPs has been shown to have cAMP-inducible activities that are dependent on EPAC.<sup>38,39</sup> To date, 6 C/EBP genes have been identified ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ,  $\zeta$ ). These all function as master regulators of cellular processes, including cell proliferation and differentiation, and inflammatory responses.<sup>40</sup> As we showed that EPAC is involved in the regulation of claudin-5 expression, we investigated whether the expression of specific C/EBP isoforms is affected by JAM-A. First, we compared mRNA and protein expression levels of the different C/EBP isoforms in *JAM-A*-WT and *JAM-A*-null iLECs. Real-time polymerase chain reaction and immunoblotting revealed that C/EBP- $\gamma$  and C/EBP- $\delta$  levels remained unchanged, whereas the C/EBP- $\beta$  isoforms, known as LIP and LAP, were upregulated in *JAM-A*-null iLECs (Figure IIC and IID in the [Data Supplement](#)). Conversely, C/EBP- $\alpha$  was significantly downregulated in the absence of JAM-A, which suggests that it is involved in the regulation of claudin-5 downstream of EPAC (Figure IIC and IID in the [Data Supplement](#)).

To further strengthen this evidence, we compared *JAM-A*-WT and *JAM-A*-null iLECs in terms of C/EBP- $\alpha$  nuclear localization and transcriptional activity. Nuclear C/EBP- $\alpha$  levels in the *JAM-A*-null cells were significantly reduced compared with the *JAM-A*-WT counterpart (Figure 2C; Figure IIE and IIF in the [Data Supplement](#)). Moreover, significant reduction in C/EBP- $\alpha$  expression was detected upon small-interfering RNA-mediated acute depletion of JAM-A in iLECs (Figure IIG



**Figure 2. JAM-A (junctional adhesion molecule A) sustains claudin-5 expression through an EPAC (exchange protein directly activated by cAMP)-dependent mechanism and C/EBP- $\alpha$  (CCAAT/enhancer-binding protein- $\alpha$ ) regulation.**

**A** and **B**, Quantification of fold-differences in *claudin-5* gene expression in *JAM-A*-wild-type (WT) and *JAM-A*-null immortalized lung endothelial cells (iLECs) following real-time quantitative polymerase chain reaction (RT-qPCR) analysis. Cells were treated with vehicle (**A** and **B**; fold-difference, 1.0) and the specific PKA (protein kinase A) inhibitor H89 (10  $\mu$ mol/L), and the PKA and EPAC activator (*Continued*)

in the [Data Supplement](#)). Consistent with this, when C/EBP- $\alpha$  transcriptional activity was analyzed by monitoring mRNA levels of its known target genes (ie, *csf1f1*; *csf1f3*, *id2*, *pparg*),<sup>41</sup> these were significantly lower in *JAM-A*-null iLECs compared with the WT (Figure IIH in the [Data Supplement](#)).

To determine the physiological relevance of these in vitro findings, we analyzed claudin-5 and C/EBP- $\alpha$  protein levels in freshly isolated endothelial cells from different organs (ie, freshly isolated lung endothelial cells [fLECs] and freshly isolated brain endothelial cells) obtained from the *JAM-A*-WT and *JAM-A*-null mice. We first tested the preparation purity using real-time polymerase chain reaction for cell-specific markers. The endothelial cell enrichment in comparison to other cell types from the brain and lungs was measured. As reported in Figure IIIA in the [Data Supplement](#), both the fLEC and freshly isolated brain endothelial cells preparations showed strong enrichment of endothelial cell-specific markers, with barely detectable contamination of other cell types, such as pericytes, smooth muscle cells, epithelial cells, fibroblasts, and astrocytes. Consistent with the previous in vitro data, the absence of *JAM-A* led to significant decreases in claudin-5 and C/EBP- $\alpha$  protein levels compared with the WT controls (Figure 2D). Densitometric analysis showed 30%

downregulation of claudin-5 expression in both lungs and brain (Figure 2D), whereas C/EBP- $\alpha$  protein levels were reduced by 40% in fLECs and 60% in freshly isolated brain endothelial cells (Figure 2D). In vivo immunofluorescence analysis of the lungs confirmed significant downregulation of claudin-5 and C/EBP- $\alpha$  expression in blood vessels that lacked *JAM-A*, with no changes in the levels of PECAM-1 (platelet/endothelial cell adhesion molecule-1) and VE-cadherin (vascular endothelial cadherin; Figure 2E through 2G). Furthermore, in both fLECs and iLECs, *JAM-A* expression did not affect the levels of additional tight junction and adherens junction proteins, which suggests that the iLECs are a suitable cellular model for further studies (Figure IIIB in the [Data Supplement](#)).

Finally, to confirm EPAC activity in the brain vessels, we examined the impact of its activation and inhibition in cultured endothelial cells from brain isolated from *JAM-A*-WT and *JAM-A*-null mice. Treatment with the EPAC-specific agonist 007 led to a significant increase in the mRNA and protein levels of *claudin-5* and C/EBP- $\alpha$  and its targets only in the presence of *JAM-A*, whereas it failed to do so in the absence of *JAM-A* (Figure 2H; Figure IIIC in the [Data Supplement](#)). Conversely, the EPAC inhibitor ESI-09 reduced mRNA and protein levels of EPAC downstream effectors in both

**Figure 2 Continued.** CPT-cAMP (8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate sodium salt; 250  $\mu$ mol/L) alone and in combination with H89 (**A**), or selective EPAC activator 8-(4-chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8-pCPT-2'-O-Me-cAMP; 007; 100  $\mu$ mol/L; **B**). Data are means $\pm$ SD from 3 independent experiments. *P* values determined by Brown-Forsythe ANOVA followed by Dunnett T3 test for multiple comparisons. *JAM-A*-WT: (overall \*\*\**P*=10<sup>-4</sup>), H89 vs CPT-cAMP \*\*\*\**P*=2 $\times$ 10<sup>-5</sup>; H89 vs CPT-cAMP+H89 \*\**P*=5.6 $\times$ 10<sup>-3</sup>; CPT-cAMP vs CPT-cAMP+H89 \*\**P*=7 $\times$ 10<sup>-3</sup>; *JAM-A*-null: (overall \*\*\**P*=2.2 $\times$ 10<sup>-4</sup>), H89 vs CPT-cAMP \**P*=1.67 $\times$ 10<sup>-2</sup>; H89 vs CPT-cAMP+H89 \*\**P*=2.4 $\times$ 10<sup>-3</sup>; CPT-cAMP vs CPT-cAMP+H89 \*\**P*=10<sup>-3</sup>. **B**, *P* values determined by 2-sided unpaired Welch *t* test. *JAM-A*-WT: vehicle vs 007 \*\*\**P*=6 $\times$ 10<sup>-4</sup>; *JAM-A*-null: vehicle vs 007 *P*=0.23, not statistically significant (NS). **C, left**, Representative immunoblotting for nuclear/cytoplasm distribution of C/EBP- $\alpha$  in *JAM-A*-WT and *JAM-A*-null iLECs. Tubulin and lamin B are shown as cytoplasmic and nuclear markers, respectively. **Right**, C/EBP- $\alpha$ /lamin B ratio quantified by densitometry scan and expressed as fold changes. Data are means $\pm$ SD from 3 independent experiments. *P* value determined by 2-sided unpaired Welch *t* test. C/EBP- $\alpha$ : *JAM-A*-WT vs *JAM-A*-null \*\**P*=3.2 $\times$ 10<sup>-3</sup>. **D, left**, Representative immunoblotting for *JAM-A*, claudin-5, and C/EBP- $\alpha$  in freshly isolated endothelial cells from brains (fBECs) and lungs (fLECs) of *JAM-A*-WT and *JAM-A*-null mice. VE-cadherin (vascular endothelial cadherin) is shown as loading control for endothelial cell numbers. **Right**, Claudin-5/VE-cadherin and C/EBP- $\alpha$ /VE-cadherin ratios quantified by densitometry scan and expressed as fold changes. Data are means $\pm$ SD from 3 independent experiments. *P* values determined by 2-sided unpaired Welch *t* test. Claudin-5: fBEC, *JAM-A*-WT vs *JAM-A*-null \*\*\**P*=1.2 $\times$ 10<sup>-4</sup>; fLEC, *JAM-A*-WT vs *JAM-A*-null \*\*\**P*=5.4 $\times$ 10<sup>-4</sup>; C/EBP- $\alpha$ : fBEC, *JAM-A*-WT vs *JAM-A*-null \*\**P*=1.5 $\times$ 10<sup>-3</sup>; fLEC, *JAM-A*-WT vs *JAM-A*-null \*\*\**P*=2.9 $\times$ 10<sup>-4</sup>. **E**, Representative confocal microscopy for PECAM-1 (platelet/endothelial cell adhesion molecule-1; red) and claudin-5 (green) in paraffin-embedded lung sections from *JAM-A*-WT and *JAM-A*-null mice (n=3, *JAM-A*-WT; n=3, *JAM-A*-null). Sections were 4  $\mu$ m thick. Scale bars: 20  $\mu$ m. **F**, Representative confocal microscopy for C/EBP- $\alpha$  (green) and VE-cadherin (red) in paraffin-embedded lung sections from *JAM-A*-WT and *JAM-A*-null mice. As C/EBP- $\alpha$  was detected in the cytoplasm of the perivascular cells in the lung, ERG (ETS-related gene) staining (nuclear marker) was segmented with threshold 350-4.096 to isolate nuclear C/EBP- $\alpha$  (**middle**), for merged images of filtered C/EBP- $\alpha$  and VE-cadherin (**right**). Sections were 4  $\mu$ m thick. Scale bars: 20  $\mu$ m (n=3, *JAM-A*-WT; n=3, *JAM-A*-null). **G**, Quantification of nuclear C/EBP- $\alpha$  signal in lung vessels sections from *JAM-A*-WT and *JAM-A*-null mice as nuclear C/EBP- $\alpha$  mean fluorescence intensity in 4 sections containing at least 10 nuclei, expressed as arbitrary units (AU); n=3, *JAM-A*-WT; n=3, *JAM-A*-null, means $\pm$ SD. *P* value determined by 2-sided unpaired Welch *t* test. *JAM-A*-WT vs *JAM-A*-null \*\**P*=8.3 $\times$ 10<sup>-3</sup>. **H**, Quantification of fold-differences for *cldn-5*, C/EBP- $\alpha$ , *id2*, *pparg*, *cdh5* expression in *JAM-A*-WT (**left**) and *JAM-A*-null (**right**) fBECs following RT-qPCR analysis. Cells were treated with vehicle (fold-difference, 1.0), the selective EPAC activator 007 (100  $\mu$ mol/L), and the specific EPAC inhibitor ESI-09 (10  $\mu$ mol/L). Data are means $\pm$ 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 T3 test for multiple comparisons. *JAM-A*-WT fBECs: *cldn-5*: (overall \*\*\*\**P*=6.3 $\times$ 10<sup>-6</sup>), vehicle vs 007 \*\*\*\**P*=2.4 $\times$ 10<sup>-5</sup>; vehicle vs ESI-09 \*\*\**P*=2.6 $\times$ 10<sup>-4</sup>; C/EBP- $\alpha$ : (overall \*\*\**P*=1.1 $\times$ 10<sup>-4</sup>), vehicle vs 007 \*\**P*=1.4 $\times$ 10<sup>-3</sup>; vehicle vs ESI-09 \*\**P*=1.9 $\times$ 10<sup>-3</sup>; *id2*: (overall \*\*\**P*=6.4 $\times$ 10<sup>-4</sup>), vehicle vs 007 \**P*=1.1 $\times$ 10<sup>-2</sup>; vehicle vs ESI-09 \*\**P*=8.4 $\times$ 10<sup>-3</sup>; *pparg*: (overall \*\*\**P*=3.4 $\times$ 10<sup>-4</sup>), vehicle vs 007 \**P*=1.1 $\times$ 10<sup>-2</sup>; vehicle vs ESI-09 \*\*\*\**P*=2.9 $\times$ 10<sup>-7</sup>; *cdh5*: (overall *P*=0.12, NS), vehicle vs 007 *P*=0.21, NS; vehicle vs ESI-09 *P*=0.13, NS. *JAM-A*-null fBECs: *cldn-5*: (overall \*\*\*\**P*=5.7 $\times$ 10<sup>-6</sup>), vehicle vs 007 \**P*=1.4 $\times$ 10<sup>-2</sup>; vehicle vs ESI-09 \*\*\**P*=8.4 $\times$ 10<sup>-4</sup>; C/EBP- $\alpha$ : (overall \*\*\**P*=2.5 $\times$ 10<sup>-4</sup>), vehicle vs 007 \**P*=3.7 $\times$ 10<sup>-2</sup>; vehicle vs ESI-09 \*\**P*=5.8 $\times$ 10<sup>-3</sup>; *id2*: (overall \*\**P*=2.6 $\times$ 10<sup>-3</sup>), vehicle vs 007 *P*=0.17, NS; vehicle vs ESI-09 \*\*\**P*=6 $\times$ 10<sup>-4</sup>; *pparg*: (overall \*\**P*=9.2 $\times$ 10<sup>-3</sup>), vehicle vs 007 *P*=0.39, NS; vehicle vs ESI-09 \**P*=2.1 $\times$ 10<sup>-2</sup>; *cdh5*: (overall *P*=0.058, NS), vehicle vs 007 *P*=0.74, NS; vehicle vs ESI-09 *P*=0.14, NS.

*JAM-A*-WT and *JAM-A*-null endothelial cells from brain. As expected, neither of the 007 and ESI-09 treatments affected the mRNA levels of *cdh5* (Figure 2H; Figure IIIC in the [Data Supplement](#)).

Thus, these data demonstrate that *JAM-A* sustains C/EBP- $\alpha$  expression, which suggests a role for C/EBP- $\alpha$  in EPAC-mediated claudin-5 induction in endothelial cells.

### JAM-A Triggers Claudin-5 Expression Through C/EBP- $\alpha$ and Regulates Endothelial Permeability

To investigate the specific role of C/EBP- $\alpha$  in the regulation of claudin-5 expression, we used several approaches to modulate C/EBP- $\alpha$  expression and its activity as a transcription factor. First, we induced acute C/EBP- $\alpha$  downregulation using small-interfering RNAs. This markedly reduced the C/EBP- $\alpha$  mRNA and protein expression, by  $\approx$ 75% to 80% compared with the control (Figure 3A and 3B). This reduction of C/EBP- $\alpha$  led to significant decreases in claudin-5 expression at both the mRNA and protein levels, compared with control cells (Figure 3A and 3B). Second, we stably overexpressed full-length C/EBP- $\alpha$  and a 30-kDa C/EBP- $\alpha$  dominant-negative (C/EBP- $\alpha$ -DN)<sup>42</sup> in both *JAM-A*-WT and *JAM-A*-null iLECs. As shown in Figure 3C and 3D, C/EBP- $\alpha$ -DN decreased claudin-5 mRNA and protein expression, whereas these were upregulated by full-length-C/EBP- $\alpha$  (C/EBP- $\alpha$ -WT). The fold-differences in claudin-5 expression induced by C/EBP- $\alpha$  and C/EBP- $\alpha$ -DN were similar in *JAM-A*-WT and *JAM-A*-null cells (Figure 3C). When C/EBP- $\alpha$ -WT was expressed, the absolute level of claudin-5 in *JAM-A*-null iLECs was  $\approx$ 4% (ie, 0.0417-fold) that of the WT counterpart, although the levels of C/EBP- $\alpha$ -WT were comparable in both cell types (Figure 3C, p42 isoform, highlighted in blue). This suggests an inhibitory mechanism in *JAM-A*-null cells, where claudin-5 expression is maintained low even in the presence of C/EBP- $\alpha$ . This inhibitory mechanism is explored further below. Consistent with this, the mRNA levels of the selected C/EBP- $\alpha$  target genes *id2* and *pparg* showed similar trends to that of *claudin-5* (Figure 3D).

Similar data on claudin-5 protein expression and regulation of C/EBP- $\alpha$  target genes at the mRNA level were obtained using transient overexpression of C/EBP- $\alpha$ -WT and C/EBP- $\alpha$ -DN (Figure IIID and IIIE in the [Data Supplement](#)). Taken together these data demonstrate that C/EBP- $\alpha$  is a novel regulator of claudin-5 expression.

Finally, we sought to determine whether the increase in claudin-5 expression induced by C/EBP- $\alpha$  can reverse the higher endothelial permeability caused by the absence of *JAM-A*. For this purpose, we performed

in vitro permeability assays to compare *JAM-A*-WT and *JAM-A*-null iLECs infected with vectors to stably express full-length C/EBP- $\alpha$ -WT or C/EBP- $\alpha$ -DN, or eGFP (enhanced green fluorescent protein) as the control. As shown in Figure 3E, the expression of C/EBP- $\alpha$ -WT in *JAM-A*-null cells (*JAM-A*-null C/EBP- $\alpha$ -WT) decreased the permeability compared with the control cells (*JAM-A*-null eGFP). This suggests that the increase in claudin-5 expression that is mediated by the transcription factor C/EBP- $\alpha$  induces the tightening of the junctions. As expected, in *JAM-A*-null C/EBP- $\alpha$ -WT-expressing cells, the permeability defect was not completely rescued, as C/EBP- $\alpha$  failed to re-establish claudin-5 expression to the level of the *JAM-A*-WT control cells, as previously shown (Figure 3C; Figure IIIF in the [Data Supplement](#)).

Conversely, the expression of C/EBP- $\alpha$ -DN in *JAM-A*-WT iLECs increased the permeability compared with the control cells (*JAM-A*-WT eGFP), due to the downregulation of claudin-5 (see Figure 3C; Figure IIIF in the [Data Supplement](#)). This set of data confirms the novel role of the transcriptional activity of C/EBP- $\alpha$  in the control of endothelial barrier permeability (Figure 3E).

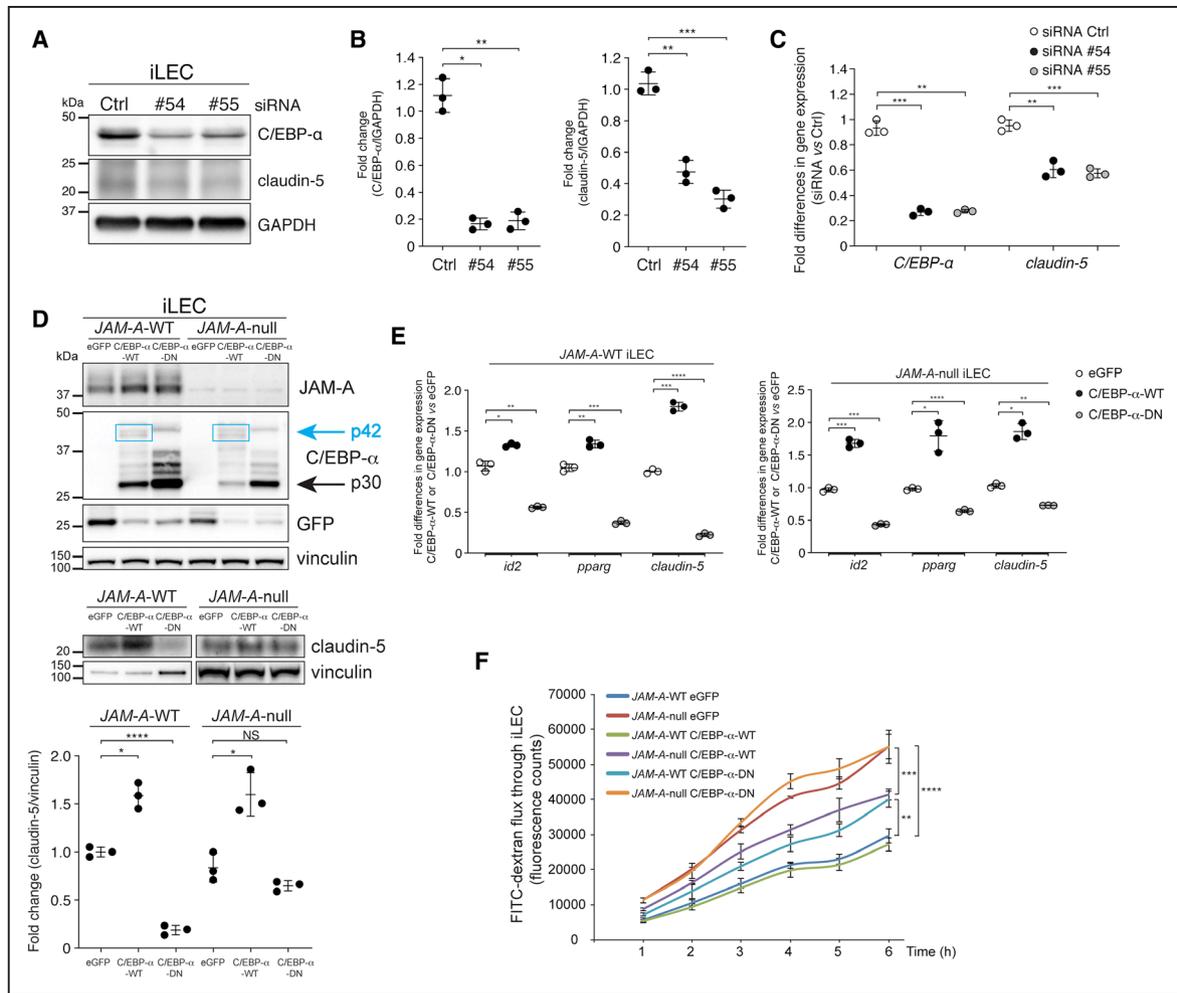
### JAM-A Promotes C/EBP- $\alpha$ Binding to the Claudin-5 Promoter and Induces Gene Transcription

We then wanted to determine how C/EBP- $\alpha$  acts on the *claudin-5* gene to induce its expression. As 3 putative C/EBP- $\alpha$  binding sites were identified within the *claudin-5* promoter region within  $-6.0$  kb of the transcription start site, we hypothesized that the *claudin-5* gene is a direct transcriptional target of C/EBP- $\alpha$  (Figure IVA in the [Data Supplement](#)).

In chromatin immunoprecipitation assays, C/EBP- $\alpha$  binding was enriched at the selected sites on the *claudin-5* promoter in the *JAM-A*-WT cells, compared with the *JAM-A*-null iLECs (Figure 4A). This suggested that C/EBP- $\alpha$  sustains claudin-5 transcription by directly binding to its promoter and that this mechanism is compromised in the absence of *JAM-A*, where C/EBP- $\alpha$  is downregulated (Figure 4A) and less active.

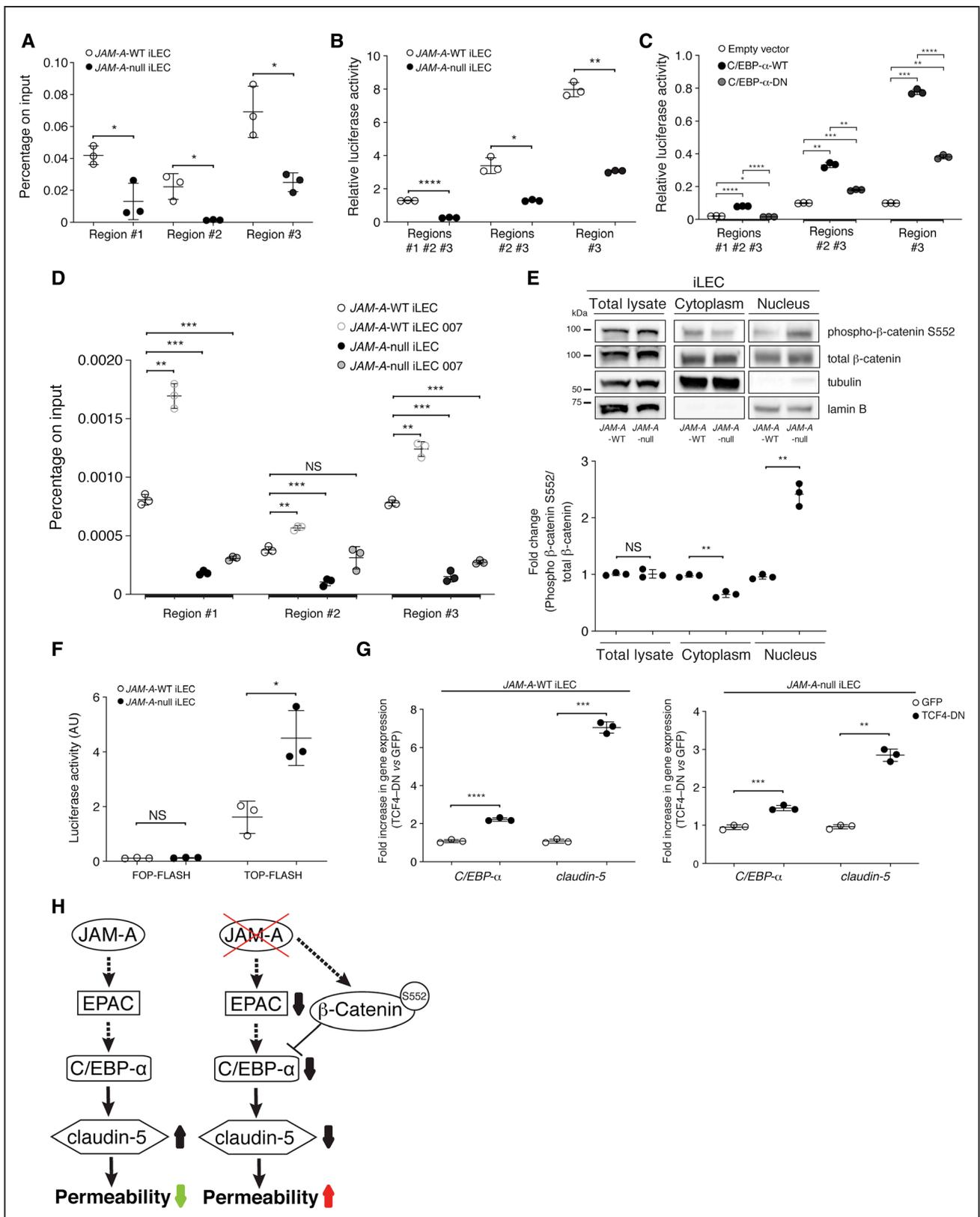
To confirm these data, the sequences of the *claudin-5* promoter were cloned upstream of the firefly luciferase gene; these comprised all 3 of the C/EBP- $\alpha$  binding site regions (ie, no. 1, no. 2, no. 3) or regions no. 1 and no. 2, or only region no. 3 (Figure IVB in the [Data Supplement](#)). As expected, the transfection of these reporters into iLECs resulted in higher luciferase activities in the presence of *JAM-A* (Figure 4B).

To demonstrate that the binding of C/EBP- $\alpha$  to the *claudin-5* promoter is functionally active, a transcriptional reporter assay was performed in HEK-293T cells, where claudin-5 reporter constructs were cotransfected with



**Figure 3. C/EBP- $\alpha$  (CCAAT/enhancer-binding protein- $\alpha$ ) controls the endothelial barrier through claudin-5 expression.**

**A, left**, Representative immunoblotting for C/EBP- $\alpha$  and claudin-5 in immortalized lung endothelial cells (iLECs) transfected with nontargeting small-interfering RNA (siRNA; Ctrl [control]) or with siRNAs against C/EBP- $\alpha$  (no. 54, no. 55). GAPDH is shown as loading control. **Right**, C/EBP- $\alpha$ /GAPDH and claudin-5/GAPDH ratios quantified by densitometry scanning and expressed as fold changes. Data are means $\pm$ SD from 3 independent experiments. *P* values determined by Brown-Forsythe ANOVA followed by Dunnett T3 test for multiple comparisons. *C/EBP- $\alpha$* : (overall \*\*\**P*=5.8 $\times$ 10<sup>-4</sup>), siRNA Ctrl vs siRNA no. 54 \**P*=1 $\times$ 10<sup>-2</sup>, siRNA Ctrl vs siRNA no. 55 \*\*\**P*=2.5 $\times$ 10<sup>-3</sup>. *Claudin-5*: (overall \*\*\*\**P*=3.9 $\times$ 10<sup>-5</sup>), siRNA Ctrl vs siRNA no. 54 \*\**P*=1.3 $\times$ 10<sup>-3</sup>; siRNA Ctrl vs siRNA no. 55 \*\*\**P*=2.9 $\times$ 10<sup>-4</sup>. **B**, Quantification of fold-differences for *C/EBP- $\alpha$*  and *claudin-5* gene expression in iLECs following real-time quantitative polymerase chain reaction analysis, as shown in **A**. Cells were treated with nontargeting siRNA (siRNA Ctrl; fold-difference, 1.0) and siRNAs against C/EBP- $\alpha$  (no. 54, no. 55). Data are means $\pm$ SD from 3 independent experiments. *P* values determined by Brown-Forsythe ANOVA followed by Dunnett T3 test for multiple comparisons. *C/EBP- $\alpha$* : (overall \*\*\**P*=2.7 $\times$ 10<sup>-4</sup>), siRNA Ctrl vs siRNA no. 54 \*\*\**P*=6.5 $\times$ 10<sup>-4</sup>; siRNA Ctrl vs siRNA no. 55 \*\**P*=4.4 $\times$ 10<sup>-3</sup>. *Claudin-5*: (overall \*\*\**P*=5.1 $\times$ 10<sup>-4</sup>), siRNA Ctrl vs siRNA no. 54 \*\**P*=2.9 $\times$ 10<sup>-3</sup>; siRNA Ctrl vs siRNA no. 55 \*\*\**P*=6.3 $\times$ 10<sup>-4</sup>. **C**, Representative immunoblotting for JAM-A (junctional adhesion molecule A), C/EBP- $\alpha$  (upper) and claudin-5 (lower) in JAM-A-wild-type (WT) and JAM-A-null iLECs stably expressing full-length C/EBP- $\alpha$ -WT (p42), C/EBP- $\alpha$ -dominant-negative (DN; p30), and eGFP (enhanced green fluorescent protein) as control. Vinculin is shown as loading control. **Upper**, Blue boxes highlight bands corresponding to full-length C/EBP- $\alpha$  (p42 isoform). **Bottom**, 10  $\mu$ M JAM-A-WT and 80  $\mu$ M JAM-A-null were loaded. Claudin-5/vinculin ratios quantified by densitometry scanning and expressed as fold changes. Data are means $\pm$ SD from 3 independent experiments. *P* values determined by Brown-Forsythe ANOVA followed by Dunnett T3 test for multiple comparisons. *JAM-A*-WT: (overall \*\*\**P*=5.5 $\times$ 10<sup>-4</sup>), eGFP vs C/EBP- $\alpha$ -WT \**P*=10<sup>-2</sup>; eGFP vs C/EBP- $\alpha$ -DN \*\*\*\**P*=6.7 $\times$ 10<sup>-5</sup>; *JAM-A*-null: (overall \*\**P*=5.2 $\times$ 10<sup>-3</sup>), eGFP vs C/EBP- $\alpha$ -WT \**P*=2.8 $\times$ 10<sup>-2</sup>; eGFP vs C/EBP- $\alpha$ -DN *P*=0.27, not statistically significant (NS). **D**, Quantification of fold-differences for *id2*, *pparg*, and *claudin-5* gene expression in *JAM-A*-WT (left) and *JAM-A*-null (right) iLECs, under stable expression of eGFP (control; fold-difference 1.0) or C/EBP- $\alpha$ -WT or C/EBP- $\alpha$ -DN. Data are means $\pm$ SD from 3 independent experiments. *P* values determined by Brown-Forsythe ANOVA followed by Dunnett T3 test for multiple comparisons. *JAM-A*-WT iLECs: *id2* (overall \*\*\**P*=6.4 $\times$ 10<sup>-4</sup>), eGFP vs C/EBP- $\alpha$ -WT \**P*=1.1 $\times$ 10<sup>-2</sup>; eGFP vs C/EBP- $\alpha$ -DN \*\**P*=8.4 $\times$ 10<sup>-3</sup>; *pparg*: (overall \*\*\*\**P*=4.1 $\times$ 10<sup>-6</sup>), eGFP vs C/EBP- $\alpha$ -WT \*\**P*=2.6 $\times$ 10<sup>-3</sup>; eGFP vs C/EBP- $\alpha$ -DN \*\*\**P*=3 $\times$ 10<sup>-4</sup>; *cln-5*: (overall \*\*\*\**P*=2.8 $\times$ 10<sup>-6</sup>), eGFP vs C/EBP- $\alpha$ -WT \*\*\**P*=3 $\times$ 10<sup>-4</sup>; eGFP vs C/EBP- $\alpha$ -DN \*\*\*\**P*=4.6 $\times$ 10<sup>-6</sup>. *JAM-A*-null iLECs: *id2*: (overall \*\*\*\**P*=3.9 $\times$ 10<sup>-5</sup>), eGFP vs C/EBP- $\alpha$ -WT \*\*\**P*=6.6 $\times$ 10<sup>-4</sup>; eGFP vs C/EBP- $\alpha$ -DN \*\*\*\**P*=2.1 $\times$ 10<sup>-4</sup>; *pparg*: (overall \**P*=1.5 $\times$ 10<sup>-2</sup>), eGFP vs C/EBP- $\alpha$ -WT \**P*=4.3 $\times$ 10<sup>-2</sup>; eGFP vs C/EBP- $\alpha$ -DN \*\*\*\**P*=5.9 $\times$ 10<sup>-5</sup>; *cln-5*: (overall \*\**P*=2.9 $\times$ 10<sup>-3</sup>), eGFP vs C/EBP- $\alpha$ -WT \**P*=1.2 $\times$ 10<sup>-2</sup>; eGFP vs C/EBP- $\alpha$ -DN \*\**P*=5.9 $\times$ 10<sup>-3</sup>. **E**, Quantification of permeability to fluorescein isothiocyanate (FITC)-dextran (40 kDa) in *JAM-A*-WT and *JAM-A*-null iLECs stably expressing full-length C/EBP- $\alpha$ -WT (p42), C/EBP- $\alpha$ -DN (p30), and eGFP as control. Data are means $\pm$ SEM of triplicates from a single experiment, representative of 3 independent experiments. *P* values determined by Brown-Forsythe ANOVA followed by Dunnett T3 test for multiple comparisons. Comparison at 6 h: (overall \*\*\*\**P*=7.8 $\times$ 10<sup>-7</sup>), *JAM-A*-WT eGFP vs *JAM-A*-null eGFP \*\*\*\**P*=1.3 $\times$ 10<sup>-5</sup>; *JAM-A*-WT eGFP vs *JAM-A*-WT C/EBP- $\alpha$ -DN \*\**P*=1.7 $\times$ 10<sup>-3</sup>; *JAM-A*-null eGFP vs *JAM-A*-null C/EBP- $\alpha$ -DN null \*\*\**P*=6.9 $\times$ 10<sup>-4</sup>.



**Figure 4. JAM-A (junctional adhesion molecule A) promotes upregulation of claudin-5 expression via C/EBP- $\alpha$  (CCAAT/enhancer-binding protein- $\alpha$ ), which is inhibited by active  $\beta$ -catenin.**

**A**, Quantification of chromatin immunoprecipitation analysis of C/EBP- $\alpha$  binding to the *claudin-5* promoter in JAM-A-wild-type (WT) and JAM-A-null immortalized lung endothelial cells (iLECs; for details see Figure IVA in the Data Supplement). DNA levels were normalized to the input. Data are means $\pm$ SD of triplicates from a single experiment, as representative of 3 experiments. *P* values determined by 2-sided unpaired Welch *t* test. JAM-A-WT vs JAM-A-null: region no. 1  $P=3\times 10^{-2}$ ; region no. 2  $P=4.5\times 10^{-2}$ ; region no. 3  $P=2.9\times 10^{-2}$ . (Continued)

plasmids that drive the overexpression of C/EBP- $\alpha$ -WT or C/EBP- $\alpha$ -DN, respectively (Figure IVC in the [Data Supplement](#)). Here, C/EBP- $\alpha$ -WT induced *claudin-5* promoter activity, and this effect was reduced when the C/EBP- $\alpha$  transcriptional activity was compromised (C/EBP- $\alpha$ -DN; Figure 4C).

Finally, to determine whether EPAC activation regulates the binding of C/EBP- $\alpha$  to the *claudin-5* promoter, chromatin immunoprecipitation assays were performed in the presence of 007. Indeed, this treatment strengthened C/EBP- $\alpha$  binding to the *claudin-5* promoter only in the presence of JAM-A, with no effects seen in the absence of JAM-A. These last data support the concept that EPAC is required for C/EBP- $\alpha$  binding to the *claudin-5* promoter through JAM-A (Figure 4D).

Overall, our data demonstrate that C/EBP- $\alpha$  increases *claudin-5* expression in response to EPAC activation. This signaling pathway is positively regulated by JAM-A.

### JAM-A Deficiency Increases $\beta$ -Catenin Signaling, Which In Turn Reduces Claudin-5 Expression Through C/EBP- $\alpha$ Inhibition

Previous studies have demonstrated that activation of Wnt/ $\beta$ -catenin signaling suppresses the expression of C/EBP- $\alpha$  mRNA.<sup>26,27</sup> JAM-A negatively regulates  $\beta$ -catenin signaling in epithelial cells.<sup>11</sup> Based on

this evidence, we hypothesized that JAM-A depletion enhances  $\beta$ -catenin signaling, to thus inhibit C/EBP- $\alpha$  and claudin-5 expression. Therefore, we compared JAM-A-WT and JAM-A-null cells in terms of the nuclear localization and transcriptional signaling of  $\beta$ -catenin. Here, the total amount of active  $\beta$ -catenin that was phosphorylated at Ser552 was similar in the JAM-A-WT and JAM-A-null iLECs and fLECs (Figure 4E; Figure IVD through IVF in the [Data Supplement](#)). Conversely, in the absence of JAM-A, there was marked increase in the levels of active nuclear  $\beta$ -catenin, compared with the WT counterpart (Figure 4E; Figure IVE and IVF in the [Data Supplement](#)). Consistent with this,  $\beta$ -catenin transcriptional activity was detected by measurement of the expression of its target genes *axin2* and *ccnd1* and using a TCF (T-cell-factor) luciferase reporter, which showed that it was significantly higher in the absence of JAM-A (Figure 4F; Figure IVG in the [Data Supplement](#)). Thus, we can conclude here that JAM-A regulates  $\beta$ -catenin/TCF-mediated gene transcription.

To determine whether  $\beta$ -catenin contributes to C/EBP- $\alpha$  and claudin-5 regulation, JAM-A-WT and JAM-A-null iLECs were treated with Wnt-3a (wingless-type MMTV integration site family, member 3a), which stabilizes  $\beta$ -catenin,<sup>43</sup> to trigger the expression of its target genes. Indeed, Wnt-3a upregulated *axin2*, while it inhibited C/EBP- $\alpha$  and *claudin-5* at the mRNA level, compared with

**Figure 4 Continued. B**, Quantification of transcriptional reporter assays in JAM-A-WT and JAM-A-null iLECs under overexpression of luciferase reporters for all 3 C/EBP- $\alpha$  binding site regions of the *claudin-5* promoter (region no. 1, no. 2, no. 3), or both region no. 1 and no. 2 (region no. 1 and no. 2), or only region no. 3 (region no. 3; for details see Figure IVB in the [Data Supplement](#)). Data are means $\pm$ SD of triplicates from a single experiment, representative of 3 experiments. *P* values determined by 2-sided unpaired Welch *t* test. JAM-A-WT vs JAM-A-null: region no. 1, no. 2, no. 3 \*\*\*\**P*=4.9 $\times$ 10<sup>-7</sup>; region no. 2 and no. 3 \**P*=1.6 $\times$ 10<sup>-2</sup>; region no. 3 \*\**P*=1.9 $\times$ 10<sup>-3</sup>. **C**, Quantification of transcriptional reporter assays in HEK-293T cells overexpressing *claudin-5* promoter-reporter plasmids (as in **B**) together with empty vector as control, and full-length C/EBP- $\alpha$ -WT, or C/EBP- $\alpha$ -dominant-negative (DN), as indicated. Data are means $\pm$ SD of triplicates from a single experiment, as representative of 3 experiments. *P* values determined by Brown-Forsythe ANOVA followed by Dunnett T3 test for multiple comparisons. Region no. 1, no. 2, and no. 3: (overall \*\*\*\**P*=1.8 $\times$ 10<sup>-7</sup>), empty vector vs C/EBP- $\alpha$ -WT \*\*\*\**P*=3.2 $\times$ 10<sup>-6</sup>; empty vector vs C/EBP- $\alpha$ -DN \**P*=3.2 $\times$ 10<sup>-2</sup>; C/EBP- $\alpha$ -WT vs C/EBP- $\alpha$ -DN \*\*\*\**P*=2.2 $\times$ 10<sup>-5</sup>; region no. 2 and no. 3: (overall \*\*\**P*=7 $\times$ 10<sup>-4</sup>), empty vector vs C/EBP- $\alpha$ -WT \*\**P*=3.3 $\times$ 10<sup>-3</sup>; empty vector vs C/EBP- $\alpha$ -DN \*\*\**P*=3 $\times$ 10<sup>-4</sup>; C/EBP- $\alpha$ -WT vs C/EBP- $\alpha$ -DN \*\**P*=8.1 $\times$ 10<sup>-3</sup>; region no. 3: (overall \*\*\*\**P*=9.2 $\times$ 10<sup>-7</sup>), empty vector vs C/EBP- $\alpha$ -WT \*\*\**P*=3.7 $\times$ 10<sup>-4</sup>; empty vector vs C/EBP- $\alpha$ -DN \*\**P*=1.3 $\times$ 10<sup>-3</sup>; C/EBP- $\alpha$ -WT vs C/EBP- $\alpha$ -DN \*\*\*\**P*=10<sup>-5</sup>. **D**, Quantification of chromatin immunoprecipitation analysis of C/EBP- $\alpha$  binding to the *claudin-5* promoter in JAM-A-WT and JAM-A-null iLECs treated with vehicle as control, or with 8-(4-chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8-pCPT-2'-O-Me-cAMP; 007). DNA levels were normalized to the input. Data are means $\pm$ SD of triplicates from a single experiment, representative of 3 experiments. *P* values determined by Brown-Forsythe ANOVA followed by Dunnett T3 test for multiple comparisons. Region no. 1: (overall \*\*\**P*=2.1 $\times$ 10<sup>-4</sup>), JAM-A-WT vs JAM-A-WT 007 \*\**P*=2.1 $\times$ 10<sup>-3</sup>; JAM-A-WT vs JAM-A-null \*\*\*\**P*=4.8 $\times$ 10<sup>-4</sup>; JAM-A-WT vs JAM-A-null 007 \*\*\**P*=9.3 $\times$ 10<sup>-4</sup>; region no. 2: (overall \*\**P*=7.4 $\times$ 10<sup>-3</sup>), JAM-A-WT vs JAM-A-WT 007 \*\**P*=1.5 $\times$ 10<sup>-3</sup>; JAM-A-WT vs JAM-A-null \*\*\**P*=8.7 $\times$ 10<sup>-3</sup>; JAM-A-WT vs JAM-A-null 007 *P*=0.64, not statistically significant (NS); region no. 3: (overall \*\*\*\**P*=4 $\times$ 10<sup>-6</sup>), JAM-A-WT vs JAM-A-WT 007 \*\**P*=3.2 $\times$ 10<sup>-3</sup>; JAM-A-WT vs JAM-A-null \*\*\**P*=5.8 $\times$ 10<sup>-4</sup>; JAM-A-WT vs JAM-A-null 007 \*\*\**P*=2.1 $\times$ 10<sup>-4</sup>. **E**, Representative immunoblotting for total lysate, cytoplasm and nuclear distribution of total and active  $\beta$ -catenin S552 (phosphor- $\beta$ -catenin) in JAM-A-WT and JAM-A-null iLECs. Tubulin and lamin B are shown as cytoplasmic and nuclear markers, respectively. **Bottom**, phospho- $\beta$ -catenin S552/total- $\beta$ -catenin ratios quantified by densitometry scan and expressed as fold changes. Data are means $\pm$ SD from 3 independent experiments. *P* values determined by 2-sided unpaired Welch *t* test. Total lysate: JAM-A-WT and JAM-A-null, *P*=0.94, NS; cytoplasm: JAM-A-WT and JAM-A-null \*\**P*=3 $\times$ 10<sup>-3</sup>; nucleus: JAM-A-WT and JAM-A-null \*\**P*=4.9 $\times$ 10<sup>-3</sup>. **F**, Quantification of transcriptional activity of TCF (T-cell-factor)- $\beta$ -catenin in JAM-A-WT and JAM-A-null iLECs underexpression of TOP-FLASH or FOP-FLASH constructs. Data are means $\pm$ SD from 3 independent experiments. *P* values determined by 2-sided unpaired Welch *t* test. JAM-A-WT and JAM-A-null: FOP-FLASH *P*=0.38, NS; TOP-FLASH, \**P*=1.9 $\times$ 10<sup>-2</sup>. **G**, Quantification of fold-differences of C/EBP- $\alpha$  and *claudin-5* gene expression in JAM-A-WT (**left**) and JAM-A-null (**right**) iLECs, underexpression of GFP (green fluorescent protein) or TCF4-DN. Data are means $\pm$ SD from 3 independent experiments. *P* values determined by 2-sided unpaired Welch *t* test. C/EBP- $\alpha$ : JAM-A-WT: GFP vs TCF4-DN \*\*\*\**P*=8.6 $\times$ 10<sup>-6</sup>; *claudin-5*: JAM-A-WT: GFP vs TCF4-DN \*\*\**P*=2.3 $\times$ 10<sup>-4</sup>; JAM-A-null: GFP vs TCF4-DN \*\*\**P*=7.1 $\times$ 10<sup>-4</sup>; *claudin-5*: JAM-A-null: GFP vs TCF4-DN \*\**P*=10<sup>-3</sup>. **H**, Schematic model of JAM-A signaling in the regulation of vascular permeability. Full arrows, direct effects on downstream targets; dashed arrows, regulation of downstream targets (eg, mRNA, protein expression or activity). The molecular mechanisms of this regulatory pathway remain to be defined. AU indicates arbitrary units; and EPAC, exchange protein directly activated by cAMP.

vehicle-treated cells (Figure IVH in the [Data Supplement](#)). In contrast, inhibition of endogenous  $\beta$ -catenin signaling using a dominant-negative TCF4 construct in *JAM-A*-WT and *JAM-A*-null iLECs increased the mRNA expression of *C/EBP- $\alpha$*  and *claudin-5* compared with the control cells (Figure 4G). Taken together, these data demonstrate that *JAM-A* positively regulates *C/EBP- $\alpha$*  expression by suppressing  $\beta$ -catenin transcriptional activity. *C/EBP- $\alpha$* , in turn, sustains *claudin-5* expression, which controls the vascular permeability (Figure 4H).

### JAM-A Deficiency Increases Endothelial Permeability in the Brain Microvasculature

Multiple studies have demonstrated that *JAM-A* is involved in positive regulation of epithelial barrier function.<sup>10,23,44</sup> Moreover, *claudin-5* is a key component of the tight junction strands, and it is highly expressed in brain endothelial cells, where it decreases permeability to small molecules (<800 Da).<sup>19</sup> Given that *claudin-5* regulates the size-dependent paracellular pathway in the blood-brain barrier, we investigated whether loss of *JAM-A* leads to increased endothelial permeability to the low molecular weight (443.43 Da) lysine-reactive biotinylation reagent sulfo-N-hydroxysuccinimide-biotin, from the intravascular space into the brain parenchyma. Compared with *JAM-A*-WT littermates, *JAM-A*-null adult mice showed significantly increased leakage of sulfo-N-hydroxysuccinimide-biotin in the cerebral cortex, striatum, hypothalamus, and midbrain (Figure 5A and 5B). Interestingly, the brain areas with widespread leakage also showed downregulation of *claudin-5* protein expression, which is consistent with loss of barrier function (Figure 5C; Figure VA in the [Data Supplement](#)). To determine the size selectivity of the endothelial barrier dysfunction, we examined extravasation of larger molecular weight tracers in the brain, using cadaverine (950 Da) and dextran (10 kDa). Interestingly, while no leakage of the high molecular weight dextran was detected, in the absence of *JAM-A* the permeability to cadaverine was slightly, but significantly, increased in the cortex and the hypothalamus (Figure 5D; Figure VB and VC in the [Data Supplement](#)). This suggested that *JAM-A* signaling can regulate the paracellular passage of molecules into the brain in a *claudin-5*-like size-dependent manner.

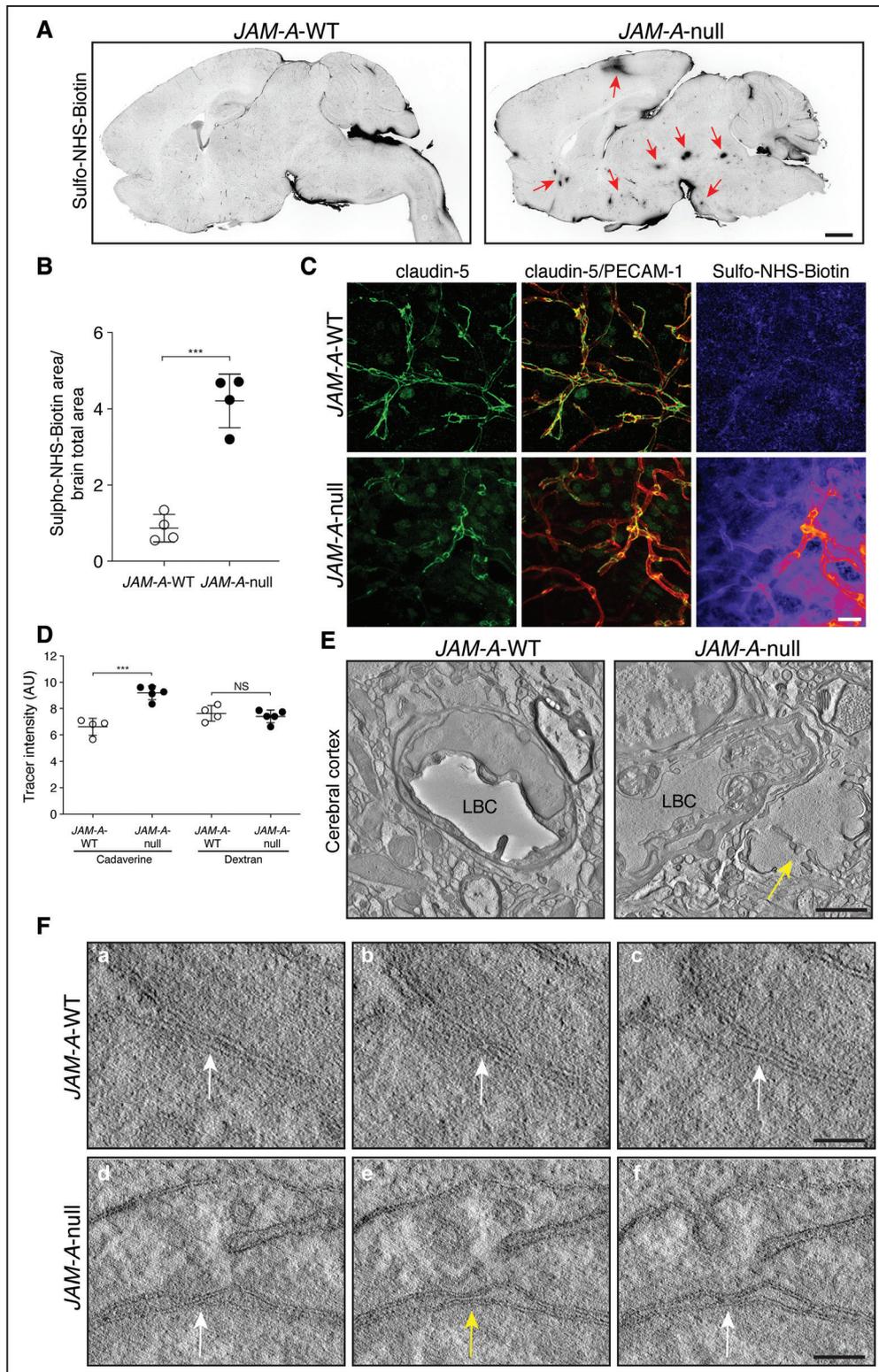
Furthermore, in the cortex of *JAM-A*-null mice, fluid leakage from blood capillaries and edema were confirmed using 2-step transmission electron microscopy tomography. We sectioned the samples, using serial sections with interleaved thicknesses. Initially, the first section (60 nm) was examined under electron microscopy, and an area where edema was evident in the absence of *JAM-A* was selected (Figure 5E). Then the first 200-nm section was examined for the cross-section of the blood capillary located near the edematous area (Figure VIA in the [Data Supplement](#)). Next, 2 serial tomography boxes were obtained at higher

magnification. In the control littermate, the capillaries on consecutive cross-sections of the electron microscopy tomography box were examined. Here, a tight junction appeared as a translucent area (osmiophobic channel) between 2 plasma membranes, where they were attached to each other (external side, relative to the cells), in the dark layers of the plasma membrane (Figure 5F, a through c; Figure VIA in the [Data Supplement](#)). This pale channel was observed in all of the consecutive serial tomography sections without any break or interruption, and the strands of the tight junctions were observed in close vicinity to each other (Figure 5F, a through c; Figure VIA in the [Data Supplement](#)). In *JAM-A*-null capillaries located near an edematous locus, the strands were significantly more distant from each other (Figure VIA in the [Data Supplement](#)), and in the consecutive sections, there was an interruption along the pale strand channel (Figure 5E, d through f; Figure VIA in the [Data Supplement](#)). This pattern typically indicated that the barrier function of these tight junctions was impaired and that this break was responsible for the leakage observed. Thus, the absence of *JAM-A* can impair the organization of the tight junctions in correspondence with areas of edema, which supports the central role of *JAM-A* in the control of endothelial barrier function in vivo.

### JAM-A Positively Regulates Claudin-5 and C/EBP- $\alpha$ Expression in Cancer Vasculature

The data presented above describe a novel molecular mechanism that links the expression of *JAM-A* and *claudin-5* and that is important for the maintenance of the integrity of the endothelial barrier (Figure 4H).

One of the characteristics of brain tumors is significant peritumoral edema, which can be explained as the result of alterations to the tight junction organization and *claudin-5*.<sup>45</sup> Given the reduction in *claudin-5* expression in the GBM vasculature,<sup>46,47</sup> we hypothesized that this is related to *JAM-A* expression. Indeed, downregulation of *JAM-A* has been described for several solid tumors, such as endometrial, pancreatic, and kidney tumors, and has been shown to correlate with tumor size and progression, as well as with poor prognostic outcome.<sup>48-50</sup> Of note, as well as mediating *JAM-A*-positive regulation of *claudin-5* transcription, *C/EBP- $\alpha$*  has a tumor suppressor role in different solid tumors.<sup>28</sup> Hence, we evaluated the expression of *JAM-A*, *claudin-5*, and *C/EBP- $\alpha$*  mRNAs in blood vessels from tissue biopsies of 11 patients with grade IV GBM. As illustrated in Figure 6A, *JAM-A*, *claudin-5*, and *C/EBP- $\alpha$*  mRNA levels were significantly lower in GBM, compared with patient-matched healthy tissues. Moreover, in the healthy tissue, *JAM-A* and *claudin-5* showed intense staining patterns at the endothelial cell-to-cell contacts, whereas in the GBM vessels, neither *JAM-A* nor *claudin-5* were detectable (Figure 6B). In parallel, nuclear *C/EBP- $\alpha$*  staining in the tumor vasculature was lower compared with the vasculature of the healthy tissue (Figure 6C).



**Figure 5. Endothelial permeability is increased in vivo by JAM-A (junctional adhesion molecule A) deficiency in areas of decreased claudin-5.**

**A**, Representative confocal microscopy for streptavidin to detect sulpho-N-hydroxysuccinimide-biotin (sulpho-NHS-biotin) leakage in vibratome brain sections (100  $\mu$ m) from *JAM-A*-wild-type (WT) and *JAM-A*-null mice. Red arrows (**right**) indicate foci of leakage in different brain regions. Scale bar: 1 mm ( $n=3$ , *JAM-A*-WT;  $n=3$ , *JAM-A*-null). **B**, Quantification of sulpho-NHS-biotin leakage in *JAM-A*-WT and *JAM-A*-null mice brain sections, as shown in **A**. Data are means $\pm$ SD, as ratio of sulpho-NHS-biotin area/ brain total area ( $n=4$ , *JAM-A*-WT;  $n=4$ , *JAM-A*-null mice). *P* value determined by 2-sided unpaired Welch *t* test. *JAM-A*-WT vs *JAM-A*-null \*\*\**P*= $6.4 \times 10^{-4}$ . **C**, Representative confocal microscopy for claudin-5 (green), PECAM-1 (platelet/endothelial cell adhesion molecule-1; red), and streptavidin, for sulpho-NHS-biotin leakage (pseudocolor) in brain sections from *JAM-A*-WT and *JAM-A*-null mice. Scale bar: 20  $\mu$ m ( $n=3$ , *JAM-A*-WT;  $n=3$ , *JAM-A*-null). (Continued)

Finally, we extended this approach to high-grade serous ovarian cancer, where the expression of JAM-A, claudin-5, and C/EBP- $\alpha$  in blood vessels was evaluated in patient-derived tissue biopsies by immunofluorescence. The endothelial levels of JAM-A, claudin-5, and C/EBP- $\alpha$  were significantly lower in the ovarian cancer tissues, compared with the healthy ovarian controls (Figure VIIA through VII E in the [Data Supplement](#)).

Taken together, these data suggest that the dysregulation of these molecular mechanisms that involve the tight junction proteins JAM-A and claudin-5 has pathological implications.

## DISCUSSION

One of the hallmarks of deteriorating vascular integrity is increased vessel permeability, which occurs in pathological contexts such as inflammation and tumor growth. The vascular barrier is mainly maintained through the stability of the endothelial cell-to-cell junctions.<sup>51</sup> Claudin-5 is the major constituent of endothelial cell-to-cell tight junctions, and it has a critical role in the maintenance of the vascular barrier.<sup>52</sup> We describe here a novel role for the transcription factor C/EBP- $\alpha$  that acts downstream of JAM-A (a component of cell-to-cell tight junctions) and EPAC to promote the transcription of claudin-5, thus strengthening the endothelial barrier *in vivo* and *in vitro*. Therefore, as a component of tight junctions, JAM-A contributes to the organization and function of tight junctions through enhanced expression of claudin-5.

Claudin-5 transcription can be controlled by various signaling pathways, and its activity can be regulated post-translationally.<sup>53</sup> Here, we have shown that C/EBP- $\alpha$  binds directly to the claudin-5 promoter to induce transcription of the *claudin-5* gene, thus enhancing claudin-5 expression.

The mechanism by which JAM-A activates C/EBP- $\alpha$  requires EPAC, which in turn activates C/EBP- $\alpha$ . In this context, in contrast,  $\beta$ -catenin acts as a negative regulator of claudin-5 expression. Indeed, JAM-A deficiency increases  $\beta$ -catenin transcriptional activity, which, in turn, inhibits the expression of C/EBP- $\alpha$ , thus reducing claudin-5 transcription. A role for active  $\beta$ -catenin in suppression of the expression of C/EBP- $\alpha$  is in agreement with previous reports in other cell types.<sup>26,27,38,39</sup> Indeed, our data indicate that JAM-A deficiency increases  $\beta$ -catenin nuclear localization and signaling, which in turn reduces claudin-5 expression through inhibition of C/EBP- $\alpha$ .

Therefore, our data and previous findings<sup>20</sup> indicate that  $\beta$ -catenin can repress the transcription of claudin-5 through different parallel mechanisms: indirectly through inhibition of C/EBP- $\alpha$  expression; and directly through interactions with FoxO1 (forkhead box O1) and TCF.<sup>20</sup>

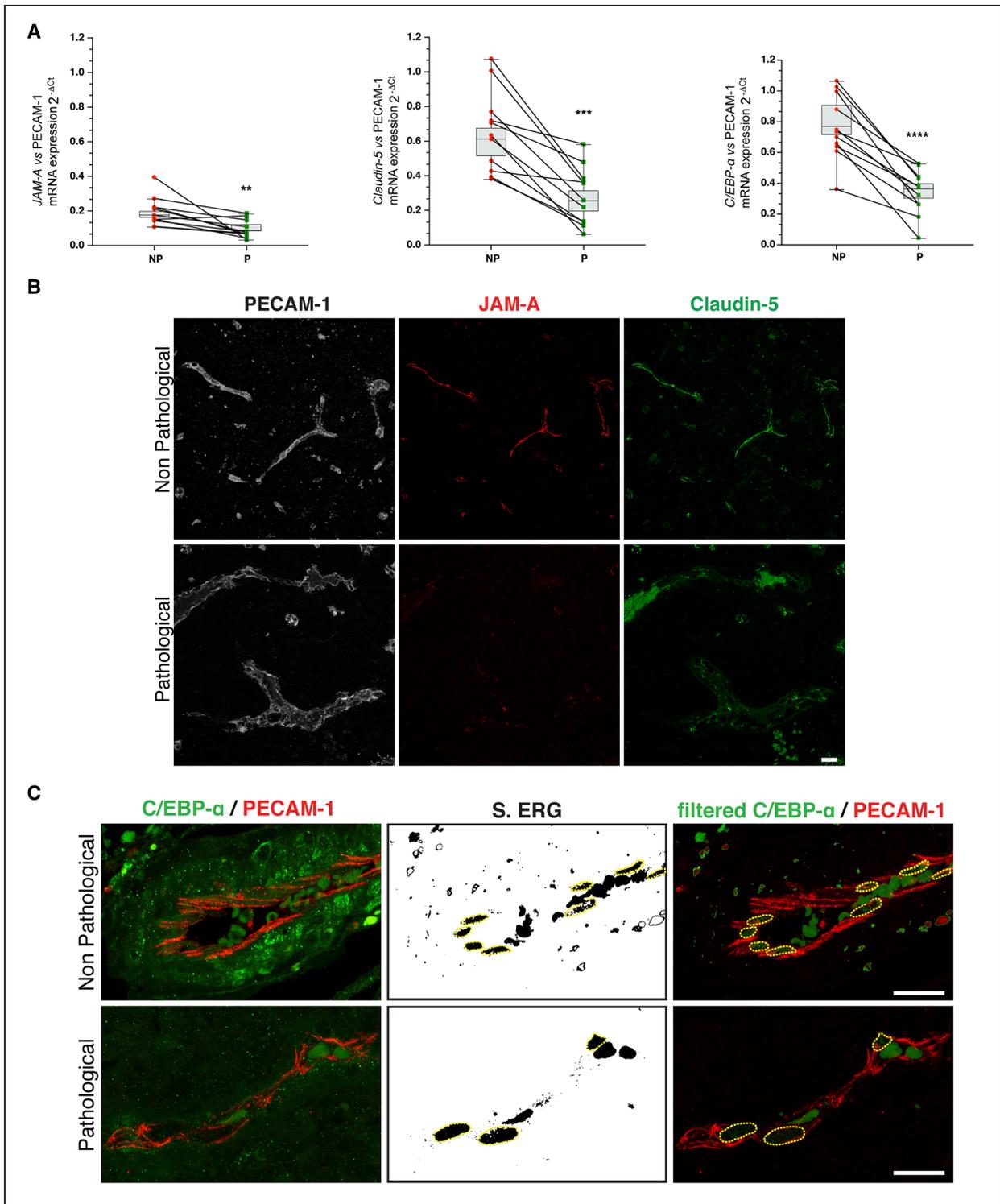
We have shown here that EPAC activates C/EBP- $\alpha$  downstream of JAM-A in endothelial cells. However, the molecular mechanisms of this regulation remain to be explored further. Covalent modification of C/EBP- $\alpha$  by the intermediate EPAC-activated protein kinases might occur. In this respect, it has been demonstrated that certain C/EBP isoforms are substrates for ERK (extracellular signal-regulated kinase), ribosomal S6 kinase, and protein kinase C protein kinases. The resulting phosphorylation enhances the activity of C/EBPs, although the different C/EBP proteins are regulated by specific mechanisms without functional redundancy.<sup>39,40</sup>

We have previously demonstrated that the absence of JAM-A impairs EPAC-mediated activation of Rap-1, which is required to restrict transmigration of leukocytes and mesoangioblasts.<sup>13</sup> Here, we now shown that JAM-A regulates vascular permeability via the control of the strength of tight junctions through claudin-5 expression. This highlights a dual role for JAM-A, as an important regulator of the vascular barrier properties to both solutes and circulating cells.

Interestingly, although increasing evidence has suggested that the mechanisms underlying vascular leakage might vary in different organ-specialized vasculatures,<sup>54</sup> the signaling pathway of JAM-A–EPAC–C/EBP- $\alpha$  that we have identified here is conserved in diverse tissues, such as brain and lung. This underlines the possible broad-spectrum relevance of this signaling in the maintenance of vascular integrity in physiology and its possible alterations in pathologies. Indeed, it has been reported that the levels of JAM-A, C/EBP- $\alpha$ , and claudin-5 are decreased in several diseases<sup>55,56</sup> and different cancers,<sup>28–30</sup> which also implies a role for these molecules as tumor suppressors. Our findings show that reduced JAM-A in the human GBM and ovarian carcinoma vasculature parallels the downregulation of claudin-5 and C/EBP- $\alpha$ , which suggests a correlation between these players in pathological situations in humans.

In conclusion, our data demonstrate a novel regulatory loop through which a component of the tight junctions, JAM-A, restricts vascular permeability by enhancing the expression of claudin-5, a crucial functional component of the tight junctions themselves. This pathway should help to identify novel therapeutic targets for diseases associated with vascular barrier dysfunction.

**Figure 5 Continued. D**, Quantification of cadaverine and dextran leakage in *JAM-A*-WT and *JAM-A*-null mice brain sections. Data are means $\pm$ SD, measured as relative intensities (arbitrary units; AU; n=4, *JAM-A*-WT; n=5, *JAM-A*-null mice). *P* values determined by 2-sided unpaired Welch *t* test. *JAM-A*-WT vs *JAM-A*-null: cadaverine \*\*\**P*=7.9 $\times$ 10<sup>-4</sup>; dextran *P*=0.56, not statistically significant (NS). **E**, Representative transmission electron microscopy of cerebral cortex from *JAM-A*-WT and *JAM-A*-null mice. Arrow denotes locus of edema. Scale bar: 1200 nm (n=3, *JAM-A*-WT; n=3, *JAM-A*-null). **F**, Representative 2-step transmission electron microscopy tomography of cerebral cortex from *JAM-A*-WT (a–c) and *JAM-A*-null mice (d–f). Parts (a–c) and (d–f) are 3 consecutive serial tomography sections showing a tight junction strand. White arrows, tight junction that appears as the translucent area (osmiophobic channel) between 2 plasma membranes; yellow arrow; interruption along the pale strand channel. Scale bars (a–c): 80 nm; (d–f) 90 nm (n=3, *JAM-A*-WT; n=3, *JAM-A*-null). LBC indicates lumen of brain capillary.



**Figure 6. JAM-A (junctional adhesion molecule A) positively regulates claudin-5 and C/EBP- $\alpha$  (CCAAT/enhancer-binding protein- $\alpha$ ) expression in glioblastoma multiforme.**

**A**, Quantification of *JAM-A*, *claudin-5*, and *C/EBP- $\alpha$*  expression in tissue biopsies from 11 patients with grade IV glioblastoma multiforme (GBM) following real-time quantitative polymerase chain reaction analysis. mRNA expression for each gene was normalized to PECAM-1 (platelet/endothelial cell adhesion molecule-1). The lines link values between GBM and patient-matched healthy tissues. *P* values determined by 2-sided paired *t* test. Non-pathological (NP) vs pathological (P): *JAM-A*  $**P=6.8\times 10^{-3}$ ; *claudin-5*  $***P=1.3\times 10^{-4}$ ; *C/EBP- $\alpha$*   $****P=2.1\times 10^{-5}$ . **B**, Representative confocal microscopy for PECAM-1 (white), JAM-A (red), and claudin-5 (green) in paraffin-embedded tissue sections (4  $\mu$ m) from GBM and patient-matched healthy tissue. Images are representative of 11 patients. Scale bars: 20  $\mu$ m. **C**, Representative confocal microscopy for C/EBP- $\alpha$  (green) and PECAM-1 (red) in paraffin-embedded tissue sections (4  $\mu$ m) from GBM and patient-matched healthy tissue. ERG (ETS-related gene) staining was segmented with threshold 350-4.096 (S. ERG) for merged images of filtered C/EBP- $\alpha$  and PECAM-1 (filtered C/EBP- $\alpha$  PECAM-1). Yellow dashed circles outline ERG-positive nuclei to exclude signals belonging to circulating cells. Scale bars: 20  $\mu$ m.

## ARTICLE INFORMATION

Received February 10, 2020; revision received July 10, 2020; accepted July 13, 2020.

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## Acknowledgments

We thank Amanda Oldani for image processing support and Fabrizio Orsenigo for sharing luciferase claudin-5 promoter constructs. We thank Christopher P. Berrie for editorial assistance, Lorenzo Bello for collecting patient informed consent according to the clinical procedure (IRB1299) and Giorgio Binelli for statistical support. We are indebted to Ruud D. Fontijn for providing the lentiviral human claudin-5 constructs, and with Daniel Tenen for providing the retroviral C/EBP- $\alpha$  constructs. M. Giannotta and E. Dejana conceived and designed the study; L. Ferrari, M. Giannotta, and A.A. Scalise performed the in vitro experiments and analyzed the results with C. Giampietro; C. Maderna, M. Ravà and N. Kakociannos performed the in vivo experiments and analyzed the results with M. Giannotta; F. Pisati performed paraffin-embedded tissue sectioning; E. Martini and I. Costa performed imaging analysis. M.G. Lampugnani, C. Giampietro, N. Kakociannos, M. Malinverno, and A. Taddei contributed to scientific discussions. M. Lupia and U. Cavallaro provided tissue biopsies from healthy and ovarian cancer patients and analyzed the data with M. Giannotta; B. Fernandes and N. Rudini provided tissue biopsies from healthy patients and patients with glioblastoma multiforme and analyzed the data with M. Giannotta; G.V. Beznoussenko, and A.A. Mironov performed the electron microscopy experiments; M. Giannotta and M.G. Lampugnani wrote the article, which was revised by E. Dejana, C. Giampietro, and A. Taddei. The final form of the article has been approved by all of the authors.

## Sources of Funding

This study was supported by *Associazione Italiana per la Ricerca sul Cancro* (AIRC; Investigator Grants 18683 and 21320), the AIRC 5x1000 call Metastatic disease: the key unmet need in oncology of the MYNERVA project, no. 21267 (Myeloid Neoplasms Research Venture AIRC), the European Research Council (project EC-ERC-V-EPC, contract 742922), Initial Training Networks BtRAIN grant 675619, the CARIPO (Cassa di Risparmio delle Provincie Lombarde) Foundation (2014-1038, 2016-0461), the Italian Ministry of Health (Ricerca Finalizzata PE-2016-02362551), the Telethon Foundation (GGP19202), the IEO-CCM (Istituto Europeo di Oncologia-Centro Cardiologico Monzino) Foundation, the Umberto Veronesi Foundation, the Swedish Science Council, and the Knut and Alice Wallenberg Foundation.

## Disclosures

None.

## Supplemental Materials

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