Molecular Medicine

VE-Cadherin–Mediated Epigenetic Regulation of Endothelial Gene Expression

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Rationale: The mechanistic foundation of vascular maturation is still largely unknown. Several human pathologies are characterized by deregulated angiogenesis and unstable blood vessels. Solid tumors, for instance, get their nourishment from newly formed structurally abnormal vessels which present wide and irregular interendothelial junctions. Expression and clustering of the main endothelial-specific adherens junction protein, VEC (vascular endothelial cadherin), upregulate genes with key roles in endothelial differentiation and stability.

Objective: We aim at understanding the molecular mechanisms through which VEC triggers the expression of a set of genes involved in endothelial differentiation and vascular stabilization.

Methods and Results: We compared a VEC-null cell line with the same line reconstituted with *VEC* wild-type cDNA. VEC expression and clustering upregulated endothelial-specific genes with key roles in vascular stabilization including *claudin-5*, vascular endothelial-protein tyrosine phosphatase (*VE-PTP*), and von Willebrand factor (*vWf*). Mechanistically, VEC exerts this effect by inhibiting polycomb protein activity on the specific gene promoters. This is achieved by preventing nuclear translocation of FoxO1 (Forkhead box protein O1) and β-catenin, which contribute to PRC2 (polycomb repressive complex-2) binding to promoter regions of *claudin-5*, *VE-PTP*, and *vWf*. VEC/β-catenin complex also sequesters a core subunit of PRC2 (Ezh2 [enhancer of zeste homolog 2]) at the cell membrane, preventing its nuclear translocation. Inhibition of Ezh2/VEC association increases Ezh2 recruitment to *claudin-5*, *VE-PTP*, and *vWf* promoters, causing gene downregulation. RNA sequencing comparison of VEC-null and VEC-positive cells suggested a more general role of VEC in activating endothelial genes and triggering a vascular stability-related gene expression program. In pathological angiogenesis of human ovarian carcinomas, reduced VEC expression paralleled decreased levels of claudin-5 and VE-PTP.

Conclusions: These data extend the knowledge of polycomb-mediated regulation of gene expression to endothelial cell differentiation and vessel maturation. The identified mechanism opens novel therapeutic opportunities to modulate endothelial gene expression and induce vascular normalization through pharmacological inhibition of the polycomb-mediated repression system. (*Circ Res*. 2018;122:231-245. DOI: 10.1161/CIRCRESAHA.117.312392.)

Key Words: blood vessels ■ cadherin ■ cell differentiation ■ endothelial cells ■ polycomb-group proteins

Several human pathological conditions are characterized by deregulated angiogenesis leading to the formation of unstable blood vessels.[1] Abnormal angiogenesis is also a hallmark of cancer. Solid tumors get their nourishment from newly formed vessels, which, however, present several structural abnormalities such as wide and irregular interendothelial junctions.[2]

Novelty and Significance

What Is Known?

- Pathological conditions such as inflammation, diabetic retinopathy, and age-related macular degeneration are characterized by deregulated angiogenesis and unstable blood vessels.
- Solid tumors receive nutrients and oxygen from newly formed vessels that display structural abnormalities such as lack of hierarchy, abnormal lumen, altered endothelial cell–cell junctions, and poor control of permeability.
- VEC (vascular endothelial cadherin) and its clustering at cell–cell adherens junctions upregulate claudin-5, key component of endothelial tight junctions regulating vessel permeability, via inhibition of FoxO1 (Forkhead box protein O1) and β-catenin nuclear translocation.

What New Information Does This Article Contribute?

- VEC expression and clustering upregulate a wide set of genes involved in endothelial differentiation and vascular stabilization, such as vascular endothelial-protein tyrosine phosphatase (*VE-PTP*) and von Willebrand factor (*vWf*) along with previously identified *claudin-5*.
- *Claudin-5*, *VE-PTP*, and *vWF* are expressed via inhibition of polycomb protein binding to their promoters by preventing the nuclear accumulation of FoxO1 and β-catenin, which associate with PRC2 (polycomb repressive complex-2) and promote its localization at target genes.
- VEC sequesters Ezh2 (enhancer of zeste homolog 2), a key component of PRC2, at the plasma membrane in a β-catenin–dependent and p120-catenin–dependent manner, further reducing polycomb nuclear activity.

Solid tumors and other human pathologies display abnormal, destabilized blood vessels, with wide and irregular endothelial cell–cell junctions. These leaky vessels favor metastatic dissemination of tumor cells, cause hemorrhages, and reduce delivery of therapeutic agents. We found that VEC is able to coordinate the expression of genes involved in endothelial differentiation and vascular stabilization. In particular, VEC expression and clustering upregulate stability-related genes *claudin-5*, *VE-PTP*, and *vWF* by preventing polycomb protein binding to their promoters via inhibition of FoxO1/β-catenin nuclear localization and sequestration of PRC2 component Ezh2 at the plasma membrane. This study shows that adherens junction establishment influences chromatin organization by modulating polycomb activity and that cadherin-mediated recruitment of a polycomb protein can influence gene expression. This mechanism depends on cadherin type, as no Ezh2/N-cadherin interaction is observed. Moreover, the work highlights a new role for FoxO1/β-catenin complex in localizing polycombs at target endothelial gene promoters. In vessels of human ovarian carcinomas, downregulation of VEC parallels reduced Claudin-5 and VE-PTP expression, pointing at a possible involvement of the identified mechanism. The data presented here suggest therapeutic opportunities to induce vascular normalization through inhibition of the polycomb-mediated repression system in pathological states characterized by vascular leakiness and fragility.

Nonstandard Abbreviations and Acronyms

Adm	adrenomedullin
AJs	adherens junctions
Ang	angiopoietin
ECs	endothelial cells
Eed	embryonic ectoderm development
Ezh2	enhancer of zeste homolog 2
H3K27me3	histone H3 trimethylated on lysine 27
Hey1	Hes-related family bHLH transcription factor with YRPW motif-1
HMEC-1	human dermal microvascular endothelial cells-1
IL	interleukin
Lmo2	LIM domain only-2
PcG	polycomb group
Pecam1	platelet/endothelial cell adhesion molecule-1
PI3K	phosphatidylinositol 3 kinase
PRC	polycomb repressive complex
Rbap48	retinoblastoma-binding protein 48
Sox18	SRY (sex determining region Y)-box-18
Stat6	signal transducer and activator of transcription-6
Suz12	suppressor of zeste 12
TAT	transactivator of transcription
Tcf	T-cell factor
TCF4-DN	dominant negative form of Tcf4
Tiam1	T-cell lymphoma invasion and metastasis-1
TJ	tight junction
TrxG	trithorax group

(Continued)

Nonstandard Abbreviations and Acronyms Continued

TSS	transcription start site
VEC	vascular endothelial cadherin
VEGFR2	vascular endothelial growth factor receptor-2
VE-PTP	vascular endothelial-protein tyrosine phosphatase
vWf	von Willebrand factor

VEC (vascular endothelial cadherin), the main component of endothelial adherens junctions (AJs), plays a major role in the process of vessel maturation and stability. This molecule, specifically expressed by endothelial cells (ECs), mediates homophilic adhesion at cell–cell contacts regulating vascular permeability.[3] VEC is indispensable for vascular maturation and inhibition of vascular regression.[3] VEC clustering at AJs triggers intracellular signals inducing contact inhibition of cell growth, protection from apoptosis, cell polarity, and inhibition of migration.[4]

In previous work, we found that VEC expression and clustering at cell–cell contacts relieve the inhibitory effect of the FoxO1 (Forkhead box protein O1)/β-catenin complex on the expression of claudin-5, an endothelial-specific TJ (tight junction) protein,[5] acting via the removal of a transcriptional repression mechanism.

The reversible nature of *claudin-5* gene repression suggested the involvement of PcG (polycomb group) proteins and epigenetic mechanisms in VEC-mediated regulation of *claudin-5* expression. Indeed, PcG proteins control the induction of reversible states of epigenetic silencing in most

Figure 1. Transcriptome profile determined by VEC (vascular endothelial cadherin) expression and clustering. A, Volcano plot showing the magnitude of differential expression between VEC-positive and VEC-null endothelial cells (ECs). Each dot represents 1 gene with detectable expression in both cell types. The horizontal dashed line (orange) together with the vertical lines (orange) mark thresholds used (*P* value ≤0.05 and |log2FC| ≥1) to define a gene as differentially regulated in VEC-positive (red). Genes that only passed threshold *P* value ≤0.05 are depicted in blue. Dots representing *claudin*-5, vascular endothelial-protein tyrosine phosphatase (*VE-PTP*), and von Willebrand factor (*vWF*) are labeled in the figure. **B**, Heat-map showing the expression pattern of significantly differentially expressed endothelial genes (*P* value ≤0.05 and |log2FC| ≥1) within and between biological replicates. Endothelial genes upregulated (red/orange) or downregulated (blue) in VEC-positive cells which were further investigated in this study are highlighted in the figure. Genes (*Continued*)

multicellular organisms, including humans, and regulate several developmental decisions by silencing genes involved in stem cell differentiation and specification of cellular identities.[6,7] Their activity is exerted through 2 multiprotein complexes called PRC (polycomb repressive complex)1 and PRC2.[6] PRC2 core subunits are Ezh (enhancer of zeste homolog)2, Suz (suppressor of zeste)12, Eed (embryonic ectoderm development), and Rbap (retinoblastoma-binding protein)48. Ezh2 is the catalytic subunit containing a SET [Su(var)3-9, enhancer of zeste and trithorax] domain, which harbors the active site for histone H3 trimethylation on lysine 27, although Suz12 and Eed association is needed for optimal enzymatic activity.[8] According to the canonical model, H3K27me3 (histone H3 trimethylated on lysine 27) is a silencing histone mark promoting the recruitment of PRC1 selectively at genes that are targeted for repression. PRC1 includes Ring1B, which mediates monoubiquitylation of histone H2A on lysine 119[9] blocking gene expression by multiple mechanisms such as chromatin compaction, inhibition of transcriptional initiation or elongation, recruitment of transcriptional inhibitors, and block of the binding of key activators.[6,10]

A fundamental step in PRC-mediated transcriptional repression is the ability of such complexes to be recruited specifically at target genes. In Drosophila, this is accomplished by polycomb response elements, clusters of DNA-binding sites for proteins that associate with PRC2 and PRC1.[11] Mammalian polycomb response elements have not been identified yet, and many aspects of polycomb recruitment still remain obscure.

Here, we report that VEC-mediated inhibition of FoxO1/β-catenin nuclear accumulation triggers a transcriptional program of endothelial differentiation and maturation. PcG proteins exert an essential role in such program by targeting the promoters of key endothelial stability genes *claudin-5*, vascular endothelial-protein tyrosine phosphatase (*VE-PTP*), and von Willebrand factor (*vWf*), causing their repression. VEC can inhibit such mechanism in multiple ways, by preventing polycomb binding to gene promoters through restriction of the nuclear localization of the FoxO1/β-catenin complex and by sequestering a fraction of Ezh2 at the plasma membrane.

These observations are supported by in vivo data in newborn mice treated with Ezh2 inhibitor UNC1999. Furthermore, dismantling of VEC clusters during pathological angiogenesis in human ovarian carcinomas is accompanied by the downregulation of Claudin-5 and VE-PTP.

These data highlight a key role of PcG proteins in the regulation of EC gene expression and open novel therapeutic opportunities to induce vascular normalization through pharmacological inhibition of the polycomb-mediated repression system.

Methods

Detailed Methods section is available in the Online Data Supplement.

The data that support the findings of this study are available from the corresponding author on reasonable request.

Results

VEC Clustering Triggers an Endothelial-Specific Transcription Program

To investigate whether VEC was able to upregulate other endothelial-specific genes besides *claudin-5*,[5] we performed an RNA sequencing comparative analysis of a mouse *VEC*-null cell line (VEC-null) and the same line reconstituted with *VEC* wild-type cDNA (VEC-positive). Several genes were upregulated by VEC expression and clustering (Figure 1A). Taking advantage of previously published data on endothelial gene expression,[12] we selected a list of endothelial genes whose expression was induced or repressed by VEC (Figure 1B; Online Tables I and II). Further validation of RNA sequencing data was performed by quantitative real-time polymerase chain reaction of *VE-PTP*,[13] *vWf*,[14] T-cell lymphoma invasion and metastasis-1 (*Tiam1*),[15] LIM domain only-2 (*Lmo2*),[16] signal transducer and activator of transcription-6 (*Stat6*)[17] and *Elk3* (ETS domain containing protein)[18,19] among VEC-induced genes, hes-related family bHLH transcription factor with YRPW motif-1 (*Hey1*)[20] and adrenomedullin (*Adm*)[21] among VEC-repressed genes, and platelet/endothelial cell adhesion molecule-1 (*Pecam1*)[22] and SRY (sex determining region Y)-box-18 (*Sox18*)[19] as genes not influenced by VEC expression (Online Figures IA and IIA through IIC). Similar transcriptional changes were induced by VEC clustering when comparing human dermal microvascular ECs (HMEC-1) in sparse and confluent conditions (Online Figure IID through IIG). Interestingly, VEC also seemed to coordinate the expression of several genes promoting vascular stability, according DAVID Functional Annotation Clustering tool.[23] For instance, we observed a general downregulation of genes involved in cell proliferation and sprouting angiogenesis, while extracellular matrix interaction and cell–cell adhesion were promoted (Figure 1C).

Within VEC-upregulated endothelial genes, we selected the tyrosine phosphatase *VE-PTP*[13] and the extracellular matrix protein *vWf*[14] for further studies. These 2 genes were selected by virtue of their endothelial specificity and their described role in the induction of vascular stability.[13,14] As reported for *claudin-5*,[5] both genes were strongly upregulated by VEC expression and clustering (Online Figure IA). A mutant version of VEC composed of the cadherin cytoplasmic tail fused to the transmembrane and extracellular domains of IL-2 (interleukin-2) receptor α-chain (IL2-VEC),[5] which is unable to cluster at cell–cell contacts, did not upregulate these genes, confirming the need of VEC clustering for this effect (Online Figure III). As for *claudin-5*,[5] FoxO1 and β-catenin activity inhibited *VE-PTP* and *vWf* expression. Infection of confluent VEC-positive cells with an adenovirus encoding a constitutively active form of FoxO1 (FKHR-TM [Forkhead transcription factor triple mutant])[24] significantly downregulated both *VE-PTP* and *vWf* expression (Online Figure IB). Similarly, increasing endogenous FoxO1 activity by LY294002-mediated inhibition of PI3K (phosphatidylinositol 3 kinase) reduced FoxO1-Ser256 (serine 256) phosphorylation

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Figure 1 Continued. are displayed in decreasing |log2FC| order (left to right). **C**, Heat-map showing normalized abundance of significantly changing genes across all samples. Genes belonging to selected functionally enriched terms are highlighted in green on the left of the plot. *Claudin-5*, *VE-PTP*, and *vWf* genes are highlighted in red. In (**B**) and (**C**), VEC-positive numbers 1/2/3 represent biological replicates in VEC-positive cells, whereas VEC-null numbers 1/2/3 represent biological replicates in VEC-null cells.

Figure 2. *Claudin-5*, vascular endothelial-protein tyrosine phosphatase (*VE-PTP*), and von Willebrand factor (*vWf*) are polycomb targets. A, Quantitative real-time polymerase chain reaction (qRT-PCR) for the transcription start site (TSS) of *claudin-5*, *VE-PTP*, and *vWf* performed on endogenous Ezh (enhancer of zeste homolog)2-, Suz (suppressor of zeste)12-, Bmi1 (B lymphoma Mo-MLV insertion region 1)-, and H3K27me3 (histone H3 trimethylated on lysine 27)-bound chromatin immunoprecipitated from confluent VEC (vascular endothelial cadherin)-null and VEC-positive endothelial cells (ECs). **B**, qRT-PCR for the TSS of *claudin-5*, *VE-PTP*, and *vWf* (*Continued*)

level (Online Figure IC, upper) and downregulated both genes under study (Online Figure IC). β-Catenin associates with FoxO1 and stabilizes its binding to *claudin-5* promoter.[5] The expression of a stabilized version of β-catenin (ΔN-β-catenin)[5] led to a marked downregulation of both *VE-PTP* and *vWf* genes (Online Figure ID), suggesting a regulatory mechanism similar to that of *claudin-5*. A promoter analysis spanning from 6000 bp upstream to 500 bp downstream of the transcription start site (TSS) of *VE-PTP* and *vWf* genes identified a series of paired Tcf (T-cell factor)/β-catenin/FoxO1-binding sites localized in 3 different regions on both promoters (Online Figure IE). Quantitative chromatin immunoprecipitation showed that FoxO1 binds all 3 regions in both promoters (Online Figure IF). Binding occurred only in confluent VEC-null and not in confluent VEC-positive cells, correlating with gene repression. Furthermore, β-catenin also bound all identified regions with different affinity (Online Figure IG), consistently with what previously reported.[5] Thus, VEC upregulates the endothelial-specific genes *vWf* and *VE-PTP* through a mechanism similar to *claudin-5* upregulation.

Claudin-5, VE-PTP, and *vWf* Are Polycomb Target Genes

We then aimed at clarifying how the FoxO1/β-catenin complex might act on *claudin-5*, *VE-PTP*, and *vWf* genes to induce their repression. Given the endothelial specificity of the identified genes, we hypothesized the possible involvement of PcG proteins, a group of transcriptional regulators involved in cell specification, which mediate reversible inhibition of transcription.[6] We performed quantitative chromatin immunoprecipitation for PcG proteins on the TSS of *claudin-5*, *VE-PTP*, and *vWf* genes. Gene TSSs displayed higher enrichment in the components of both PRC2 (Ezh2 and Suz12) and PRC1 (Bmi1; B lymphoma Mo-MLV insertion region 1) in confluent VEC-null compared with VEC-positive cells (Figure 2A, top and lower left). The mark of Ezh2 enzymatic activity, H3K27me3, also showed a similar enrichment pattern (Figure 2A, lower right), and quantitative chromatin immunoprecipitation for total histone H3 showed comparable enrichments at the analyzed regions in VEC-null and VEC-positive cells (Online Figure IV), ruling out the possibility that the higher H3K27me3 signal in VEC-null cells could be because of widespread higher histone density. PcG-mediated repression is counteracted by the activity of TrxG (trithorax group) proteins.[6] As expected, *claudin-5*, *VE-PTP*, and *vWf* TSSs showed a higher enrichment in H3K4me3, marker of TrxG activity and gene activation, in confluent VEC-positive cells than in VEC-null cells (Figure 2B, upper). RNA polymerase II phosphorylated on Ser5, a modification needed for the enzyme to escape the promoter and transcribe the gene,

was also increased at the TSSs of genes in confluent VEC-positive cells (Figure 2B, lower).

When we overexpressed the PRC2 member Suz12 in confluent VEC-positive cells using lentiviral-mediated gene delivery (Figure 2C), *claudin-5*, *VE-PTP*, and *vWf* expression was reduced (Figure 2E). PRC2 proteins are known to promote each other's stability by physical interaction.[8] Suz12 overexpression led to an almost 2-fold increase in Ezh2 protein level (Figure 2D). The promoter region of *claudin*-5 (3169 bp upstream of the coding sequence; Online Figure VA), comprising all 3 regions of paired Tcf/β-catenin/FoxO1-binding sites[5] was cloned upstream of the firefly luciferase gene. As expected, transfection of this reporter in confluent VEC-null and VEC-positive cells showed higher luciferase activity in the presence of VEC expression and clustering (Online Figure VB). Suz12 overexpression in VEC-positive ECs significantly decreased luciferase expression (Online Figure VC). *Lmo2* and *Stat6* were also downregulated by Suz12 overexpression (Online Figure VIA), suggesting that the identified mechanism might regulate a wider set of endothelial genes. *Hey1* expression was induced by Suz12 (Online Figure VIB), likely as a result of polycomb-mediated downregulation of a *Hey1* gene repressor, while *Pecam1* and *Sox18* levels were not altered (Online Figure VIC). Conversely, Suz12 knockdown led to a marked decrease in Ezh2 and to the abrogation of detectable H3K27me3 levels (Figure 2F). FoxO1 protein levels were ≈30% lower on Suz12 knockdown (Figure 2F), although FoxO1 mRNA expression was unchanged (Online Figure VII). Suz12 knockdown abolished Suz12 and H3K27me3 signal at the TSSs of *claudin-5*, *VE-PTP*, and *vWf* genes (Figure 2G) causing a partial reactivation of their expression (Figure 2H). The incomplete rescue of expression might be because of the lack of specific gene activators missing in a VEC-null context or to PRC1 activity keeping genes partially repressed in the absence of PRC2.[25] Furthermore, Suz12 knockdown induced an increase in *claudin-5* promoter activity as assessed by luciferase reporter assay (Online Figure VD).

FoxO1/β-Catenin Enhance PcG Protein Binding to *Claudin-5, VE-PTP,* and *vWf* Promoters

We then hypothesized that the FoxO1/β-catenin complex could positively modulate polycomb interaction with the promoters of the identified genes. By coimmunoprecipitation, we found that constitutively active FKHR-TM or endogenous FoxO1 interact with Ezh2 (Figure 3A). Another member of PRC2, Suz12, also coimmunoprecipitated with FKHR-TM (Figure 3B). FKHR-TM overexpression in VEC-positive confluent cells increased PcG protein binding to the

Figure 2 Continued. performed on endogenous H3K4me3-bound and RNA polymerase II (p-PolII) Ser5–bound chromatin immunoprecipitated from confluent VEC-null and VEC-positive ECs. **C**, Western blot (WB) analysis of indicated proteins in extracts of confluent VEC-null and VEC-positive ECs upon Suz12 overexpression. **D**, Quantification of WB in (**C**). Suz12 and Ezh2 levels were normalized to tubulin. Columns are means±SEM of 3 independent experiments. **E**, qRT-PCR analysis of *claudin-5*, *VE-PTP*, and *vWf* expression in confluent VEC-null and VEC-positive ECs upon Suz12 overexpression. **F**, WB analysis of indicated proteins in extracts of confluent VEC-null ECs upon Suz12 knockdown (sh-Suz12). **G**, qRT-PCR for the TSS of *claudin-5*, *VE-PTP*, and *vWf* performed on endogenous Suz12- and H3K27me3-bound chromatin immunoprecipitated from confluent VEC-positive, VEC-null-sh-Empty, and VEC-null-sh-Suz12 ECs. **H**, qRT-PCR analysis of *claudin-5*, *VE-PTP*, and *vWf* expression in confluent VEC-null-sh-Empty and VEC-null-sh-Suz12 ECs. **A**, **B**, **G**, Levels of DNA are normalized to input, columns are means±SD of triplicates from a representative experiment. **C**, **F**, Tubulin and vinculin are the loading controls. **E**, **H**, Levels of mRNA are normalized to 18S; columns are means±SEM of triplicates from a representative experiment. In (**A**) and (**B**), *P<0.05; **P<0.01, *t* test VEC-null vs VEC-positive. In (**G**), *P<0.05; **P<0.01, *t* test VEC-null Sh-Empty vs VEC-null Sh-Suz12. In (**D**), (**E**), and (**H**), *P<0.05; **P<0.01, *t* test. nd indicates not detectable.

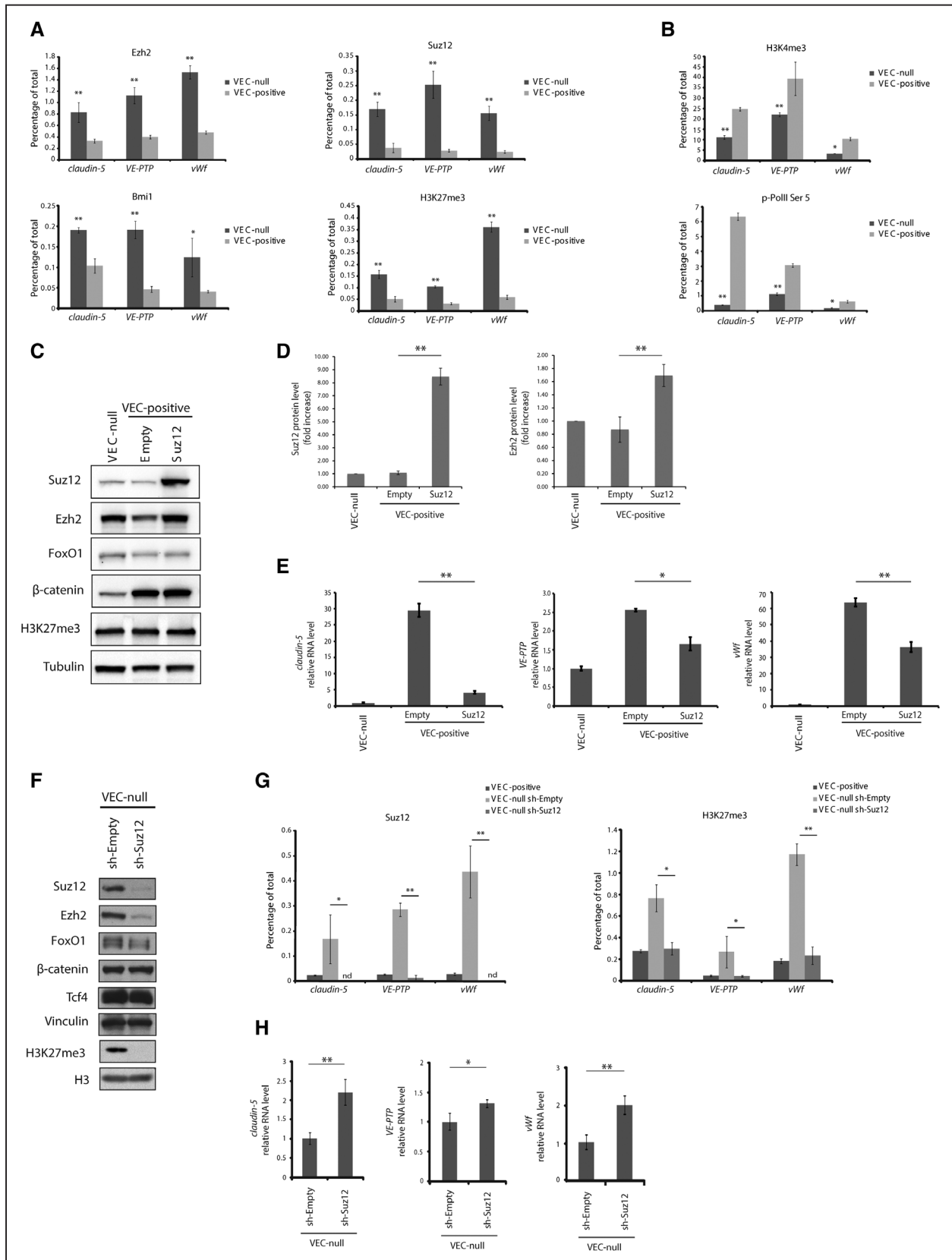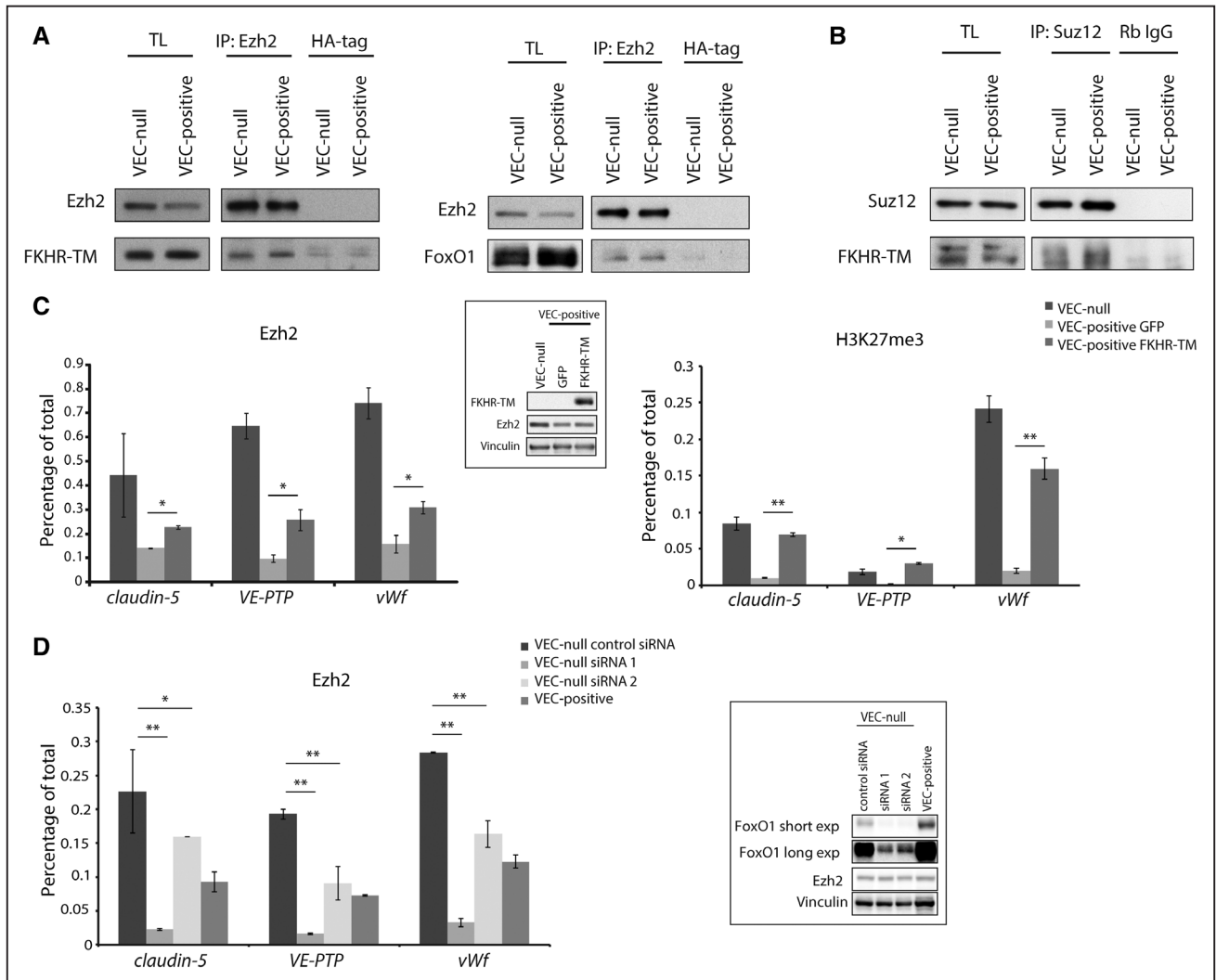**Figure 3. FoxO1 (Forkhead box protein O1) enhances PcG (polycomb group) protein association to *claudin-5,* vascular endothelial-protein tyrosine phosphatase (*VE-PTP*), and von Willebrand factor (*vWf*) promoters. A**, **B**, Coimmunoprecipitation and Western blot (WB) of endogenous Ezh (enhancer of zeste homolog)2 or Suz (suppressor of zeste)12 and endogenous FoxO1 or FKHR-TM (Forkhead transcription factor triple mutant) from extracts of confluent VEC (vascular endothelial cadherin)-null and VEC-positive endothelial cells (ECs) or the same cells types expressing FKHR-TM (myc-tagged). **C**, Quantitative real-time polymerase chain reaction (qRT-PCR) for the transcription start site (TSS) of *claudin-5*, *VE-PTP*, and *vWf* performed on endogenous Ezh2- and H3K27me3 (histone H3 trimethylated on lysine 27)-bound chromatin immunoprecipitated from confluent VEC-null and VEC-positive ECs expressing either FKHR-TM or GFP (green fluorescent protein; negative control). Inset: WB analysis of FKHR-TM and Ezh2. **D**, qRT-PCR for the TSS of *claudin-5*, *VE-PTP*, and *vWf* performed on endogenous Ezh2-bound chromatin immunoprecipitated from confluent VEC-positive or VEC-null ECs transfected with control siRNA or with 2 siRNAs targeting FoxO1 mRNA. Inset: WB analysis of FoxO1 and Ezh2. Two different film exposure timings are shown for FoxO1. **C**, **D**, Vinculin is the loading control. Levels of DNA are normalized to input, columns are means±SD of triplicates from a representative experiment. In (**C**), *$P<0.05$; **$P<0.01$, t test VEC-positive GFP vs VEC-positive FKHR-TM. In (**D**), *$P<0.05$; **$P<0.01$, t test VEC-null control siRNA vs VEC-null siRNA1 or VEC-null siRNA2. IP indicates immunoprecipitation; and TL, total cell lysate.

TSS of *claudin-5*, *VE-PTP*, and *vWf* genes, as shown by Ezh2 quantitative chromatin immunoprecipitation (Figure 3C, left). Consequently, H3K27me3 repressive histone mark was increased (Figure 3C, right). This is consistent with gene downregulation observed on FKHR-TM overexpression (Online Figure IB). No increase in Ezh2 protein levels was induced by FKHR-TM (Figure 3C inset), proving that the augmented Ezh2 enrichment was independent of protein up-regulation. Conversely, FoxO1 knockdown strongly reduced Ezh2 recruitment at target sites in VEC-null cells to levels comparable to those detected in VEC-positive cells, or even lower (Figure 3D), in the absence of any Ezh2 downregulation

(Figure 3D inset). Interestingly, this effect was dose dependent. SiRNA 2 was less efficient than siRNA 1 in knocking down FoxO1 (see FoxO1 long exp, Figure 3D inset), and this resulted in a weaker reduction of Ezh2 binding to gene TSSs.

Coexpression of Suz12 and FKHR-TM in VEC-positive confluent cells induced a stronger repression of *claudin-5* than the expression of FKHR-TM alone (75.3% versus 53.1%; Online Figure VIIIA and VIIIB), further supporting the hypothesis that FoxO1 acts in concert with polycomb activity.

We then investigated whether β-catenin could interact with PcG protein complex. Coimmunoprecipitation experiments showed an interaction between β-catenin and both Ezh2
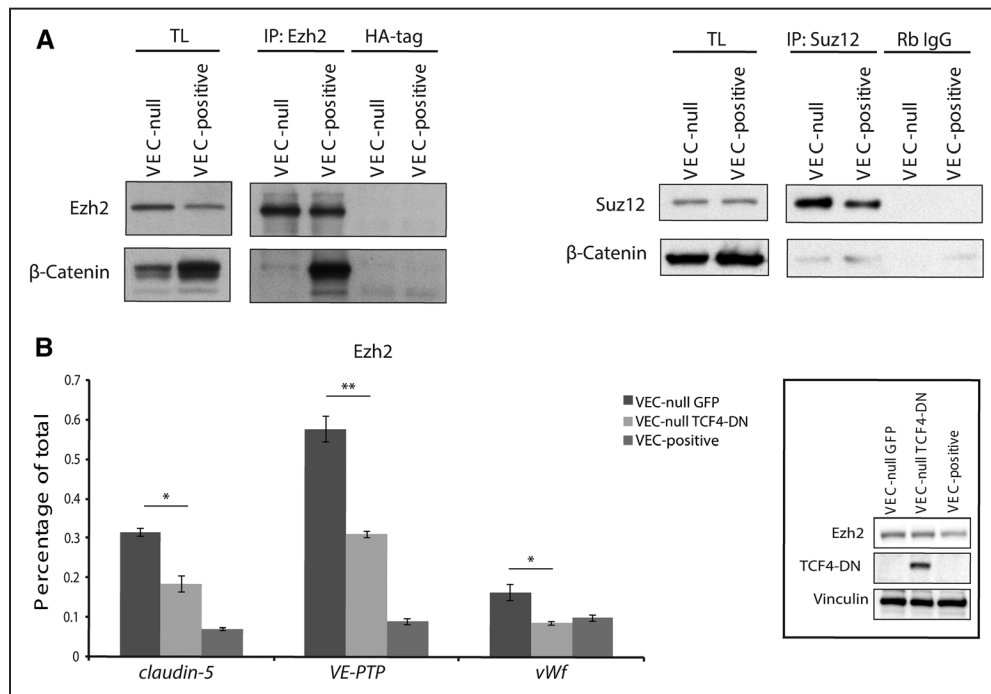
Figure 4. β-Catenin stabilizes polycomb/DNA interaction on *claudin-5*, vascular endothelial-protein tyrosine phosphatase (*VE-PTP*), and von Willebrand factor (*vWf*) promoters. A, Coimmunoprecipitation and Western blot (WB) analysis of endogenous Ezh (enhancer of zeste homolog)2 or Suz (suppressor of zeste)12 and β-catenin from extracts of confluent VEC (vascular endothelial cadherin)-null and VEC-positive endothelial cells (ECs). **B**, Quantitative real-time polymerase chain reaction (qRT-PCR) for the TSS of *claudin-5*, *VE-PTP*, and *vWf* performed on endogenous Ezh2-bound chromatin immunoprecipitated from confluent VEC-positive and VEC-null ECs expressing dominant negative form of Tcf4 (TCF4-DN) or GFP (green fluorescent protein; negative control). Inset: WB analysis of TCF4-DN and Ezh2 in extracts from confluent VEC-positive and VEC-null ECs expressing TCF4-DN or control GFP. Vinculin is the loading control. *$P<0.05$; **$P<0.01$, t test VEC-null GFP vs VEC-null TCF4-DN. IP indicates immunoprecipitation; and TL, total cell lysate.

and Suz12 (Figure 4A). Surprisingly, we detected a strong Ezh2/β-catenin association in VEC-positive confluent cells that was unlikely to be connected to the analyzed recruitment mechanism (Figure 4A, left). The meaning of this interaction will be further explored in the next section.

β-Catenin/FoxO1 association is known to stabilize FoxO1 binding to *claudin-5* promoter. When TCF4-DN (dominant negative form of Tcf4), lacking the β-catenin–interacting region, was overexpressed in VEC-null cells, β-catenin/DNA interaction was abrogated,[5] and Ezh2 binding to *claudin-5*, *VE-PTP*, and *vWf* promoters was strongly weakened (Figure 4B), suggesting a stabilizing role for β-catenin on PcG protein association to target sites.

VEC Associates With Ezh2 and Sequesters It at the Plasma Membrane

The strong Ezh2/β-catenin association detected in confluent VEC-positive cells (Figure 4A, left) suggested an alternative role for such interaction in this cell type. Indeed, Ezh2 coimmunoprecipitated with endogenous full-length VEC (Figure 5A, left), whereas no interaction was detected between Ezh2 and N-cadherin (Figure 5A, right). Ezh2–VEC interaction was confirmed in vivo in adult mice-derived whole lung extracts (Figure 5B). Biotinylation of cell surface proteins further proved that Ezh2 associates with VEC exposed on the cell membrane (Figure 5C). Moreover, junctional staining of Ezh2 was detected in confluent VEC-positive but not in VEC-null ECs (Figure 5D, arrow). Taken together these data

demonstrate that the observed VEC–Ezh2 interaction sequesters the polycomb protein at the cell surface.

To define whether Ezh2 interacts directly with VEC cytoplasmic tail and which Ezh2 domain is involved in such interaction, we designed a peptide array displaying 20-mers covering the entire amino acid sequence of mouse Ezh2 (746 amino acids). Each peptide spotted on the membrane overlapped the next one by 19 amino acids, resulting in a change of only a single amino acid per peptide and providing a high resolution in determining the interaction sites. Such peptide array was probed with glutathione *S*-transferase (GST)–tagged VEC cytoplasmic tail[26] and free GST as control for nonspecific interactions (Online Figure IXA and IXB). Peptides corresponding to the spots displaying high signal in VEC cytoplasmic tail–probed array and no or low signal in GST-probed control (Online Figure IXB, red dots) were synthesized as biotinylated and used in streptavidin pull-down experiments to confirm protein interactions (Figure 6A). Peptides M6 and M10 corresponding to amino acids 450 to 469 and 454 to 473, respectively, were identified as the sites of Ezh2/VEC direct interaction (Figure 6A and 6B).

We then investigated the role of VEC cytoplasmic partner β-catenin in such interaction. Ezh2/VEC association was reduced in a β-catenin–null EC line (β-catenin knockout) compared with its wild-type counterpart (β-catenin wild type; Figure 6C and 6D). When VEC-null cells were reconstituted with a truncated mutant of VEC lacking the β-catenin–binding domain (Δβcat),[5] thus unable to sequester β-catenin at the membrane, VEC displayed a marked reduction of Ezh2
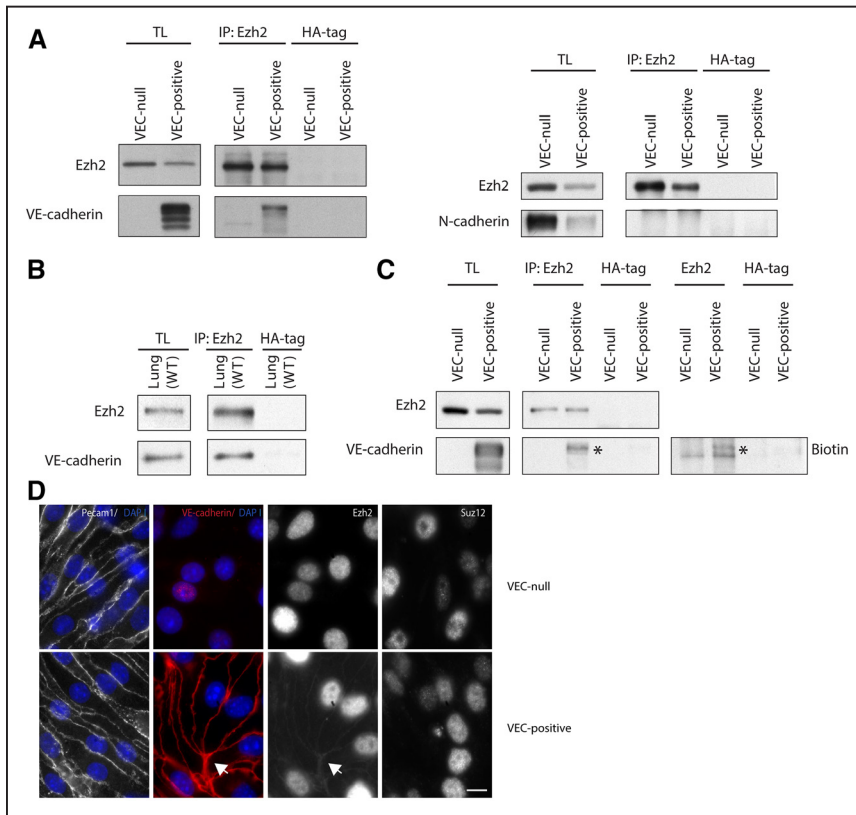
Figure 5. VEC (vascular endothelial cadherin) sequesters Ezh2 (enhancer of zeste homolog 2) at the plasma membrane. A, Coimmunoprecipitation and WB of endogenous Ezh2 and VEC or N-cadherin from extracts of confluent VEC-null and VEC-positive endothelial cells (ECs). **B**, Coimmunoprecipitation and Western blot (WB) of endogenous Ezh2 and VEC from wild-type (WT) murine whole lung extracts. **C**, Coimmunoprecipitation and WB of endogenous Ezh2 and VEC from extracts of confluent VEC-null and VEC-positive ECs after biotinylation of cell surface proteins. Asterisk highlights Ezh2-associated total and surface VEC bands. **D**, Immunofluorescence analysis of Ezh2 junctional localization (arrow) in confluent VEC-null and VEC-positive ECs. Junctional Suz (suppressor of zeste)12 was not detected. Platelet/endothelial cell adhesion molecule-1 (Pecam1) and VEC were used as junctional markers. Scale bar: 10 μm. IP indicates immunoprecipitation; and TL, total cell lysate.

binding (Figure 6G and 6H). These results strongly suggested that β-catenin association to VEC cytoplasmic tail is required for optimal Ezh2 junctional recruitment. Ezh2 peptide array was probed with GST-tagged β-catenin (Online Figure IXC). Biotinylated peptide pull-down with peptides corresponding to the spots displaying high signal in β-catenin–probed array and no or low signal in GST-probed control (Online Figure IXC, red dots) confirmed Ezh2 interaction with β-catenin at amino acids 522 to 541 and 585 to 604, corresponding to peptides O4 and P30 (Figure 6E and 6F).

Ezh2 also associated with another constituent of VEC cytoplasmic junctional complex, p120-catenin (Online Figure X). VEC-null cells reconstituted with a mutant version of VEC lacking the juxtamembrane p120-catenin binding region showed a marked reduction of VEC/Ezh2 interaction (Figure 6G and 6H), suggesting a role for p120-catenin in such association. Ezh2 peptide array was probed with GST-tagged p120-catenin, and the identified putative interacting peptides (Online Figure IXD, red dots) were selected for validation by biotinylated peptide pull-down (Figure 6I). Amino acids 652 to 671, corresponding to peptide R23, were identified as Ezh2 interaction site with p120-catenin (Figure 6I and 6J).

Overall, these data prove that Ezh2 interacts with VEC junctional complex at multiple sites. Whether the identified interactions correspond to different junctional pools of Ezh2 or a single complex is formed is a matter of future investigation.

Inhibition of Ezh2/VEC Interaction Downregulates Claudin-5, VE-PTP, and vWf Expression

To understand whether Ezh2 sequestration at AJs impacts the expression of *claudin-5*, *VE-PTP*, and *vWf*, we aimed at inhibiting Ezh2/VEC and Ezh2/β-catenin interactions. Selected Ezh2

peptides M6-M10 (VEC-binding sites) and O4-P30 (β-catenin–binding sites) were synthesized in tandem with transactivator of transcription (TAT) of human immunodeficiency virus, to allow peptide entry into the cell. Such TAT-conjugated peptides are expected to bind VEC or β-catenin engaging the domains needed for Ezh2 interaction, thus acting in a dominant negative fashion to inhibit Ezh2 junctional recruitment. VEC-positive cells were treated from subconfluent state throughout the period of time needed to establish AJs to saturate Ezh2-binding sites before the polycomb protein could localize at cell–cell contacts. Treatment with peptides TAT-P30 and TAT-M10 reduced VEC/Ezh2 association by 28% and 45.3%, respectively (Figure 7A). This caused an increase in Ezh2 recruitment to the TSS of *claudin-5*, *VE-PTP*, and *vWf* (Figure 7B), which, in the case of TAT-M10 treatment, corresponded to a significant repression of gene expression in comparison to treatment with a nonbinding TAT-control peptide (Figure 7C). The lack of gene downregulation after TAT-P30 treatment might be because of interference of this peptide with the correct assembly of the FoxO1/β-catenin/PRC2 complex at gene promoters.

Polycomb Activity Correlates With Claudin-5, VE-PTP, and vWf Repression In Vivo

To verify whether the inhibition of PRC2 activity could enhance the expression of the identified endothelial genes in vivo, we analyzed the effect of Ezh2/Ezh1 inhibition in mouse pups by pharmacological treatment with UNC1999. As reported in Figure 8A, drug administration increased *VE-PTP* and *vWf* expression in lung ECs, but was unable to increase *claudin-5* expression over physiological levels.

EZH2 overexpression is frequent in tumor cells and in vessels of human epithelial ovarian cancers and is associated
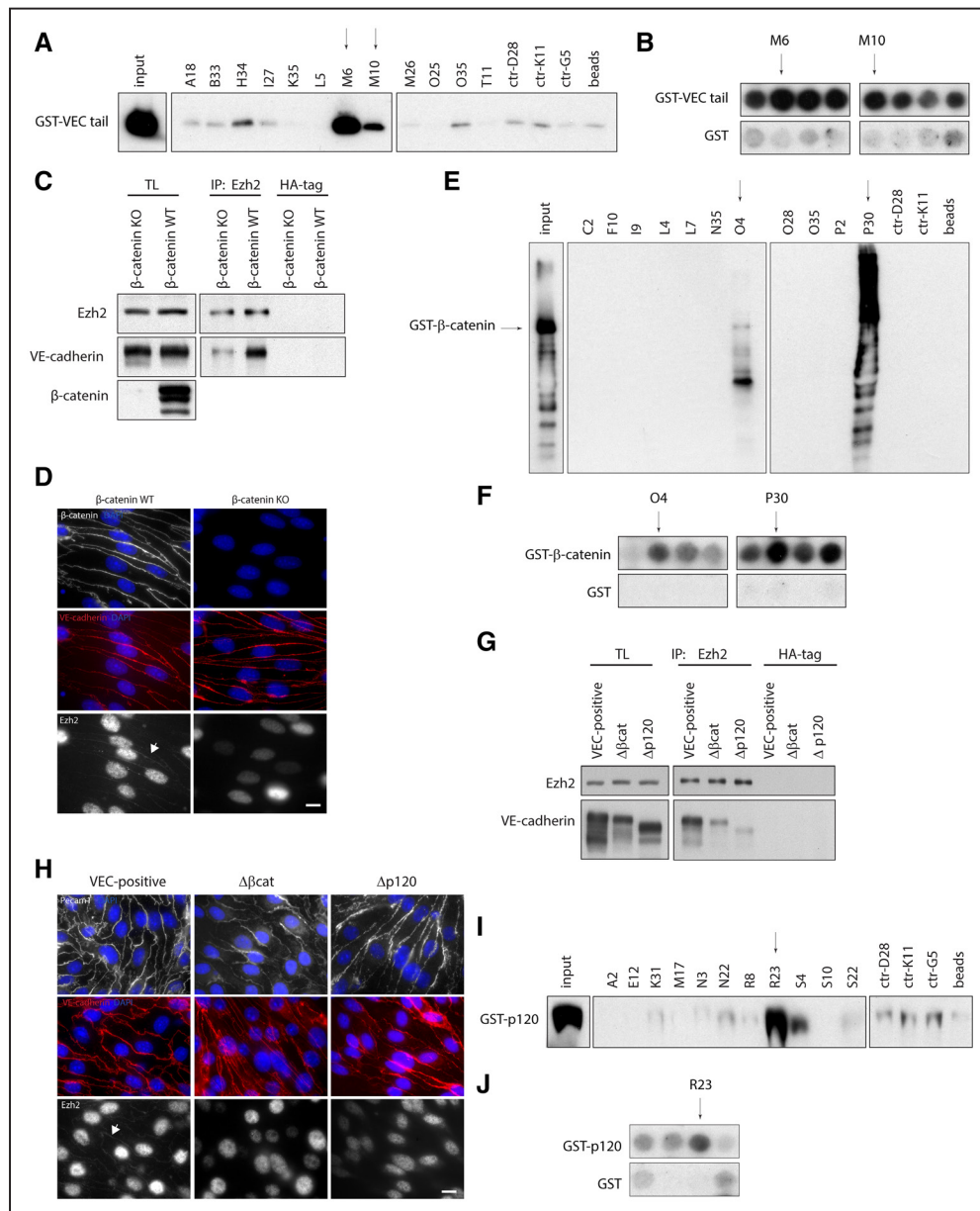
Figure 6. Analysis of Ezh2 (enhancer of zeste homolog 2) interaction with VEC (vascular endothelial cadherin) junctional complex.
A, Streptavidin pull-down of selected biotinylated Ezh2 peptides and GST-tagged VEC cytoplasmic tail. GST-VEC cytoplasmic tail (400 ng) was loaded as input. Peptides displaying no interaction in peptide array were used as controls. Arrows indicate peptides showing positive signal. **B**, Regions of peptide array in the Online Figure IXA and IXB corresponding to selected peptides in (**A**). **C**, Coimmunoprecipitation and Western blot (WB) of Ezh2 and VEC from extracts of confluent β-catenin knockout (KO) and β-catenin wild-type (WT) endothelial cells (ECs). **D**, Immunofluorescence analysis of Ezh2 (arrow) and β-catenin junctional localization in confluent β-catenin KO and β-catenin WT ECs. VE-cadherin (red) was used as junctional marker. **E**, Streptavidin pull-down of selected biotinylated Ezh2 peptides and GST-tagged β-catenin. GST-β-catenin (400 ng) was loaded as input. Peptides displaying no interaction in peptide array were used as controls. Arrows indicate peptides showing positive signal. **F**, Regions of peptide array in the Online Figure IXA and IXC corresponding to peptides in (**E**). **G**, Coimmunoprecipitation and WB of Ezh2 and VEC from extracts of confluent VEC-positive, Δβcat, and Δp120 ECs. **H**, Immunofluorescence analysis of Ezh2 junctional localization (arrow) in confluent VEC-positive, Δβcat, and Δp120 ECs. VEC and platelet/endothelial cell adhesion molecule-1 (Pecam1) were used as junctional markers. **I**, Streptavidin pull-down of selected biotinylated Ezh2 peptides and GST-tagged p120-catenin. GST-p120-catenin (300 ng) was loaded as input. Peptides displaying no interaction in peptide array were used as controls. Arrow indicates peptide showing positive signal. **J**, Regions of peptide array in the Online Figure IXA and IXD corresponding to selected peptides in (**I**). In (**D**) and (**H**), scale bar: 10 μm. GST indicates glutathione *S*-transferase; HA, human influenza hemagglutinin; IP, immunoprecipitation; and TL, total cell lysate.

with poor prognosis.[31] Vessels of human healthy ovarian tissue presented very low EZH2 expression, while VEC, Claudin-5, and PECAM1 were clearly detectable (Figure 8B, upper). In contrast, in tumor vasculature, high nuclear EZH2 staining paralleled a significant reduction in VEC and Claudin-5,

whereas PECAM1 expression was unaltered (Figure 8B, lower, and 8C). EC-associated vWf staining was highly variable in both healthy ovarian tissue and tumor samples, preventing a quantitative evaluation of its expression. VE-PTP staining quantification also presented problems of antibody specificity.
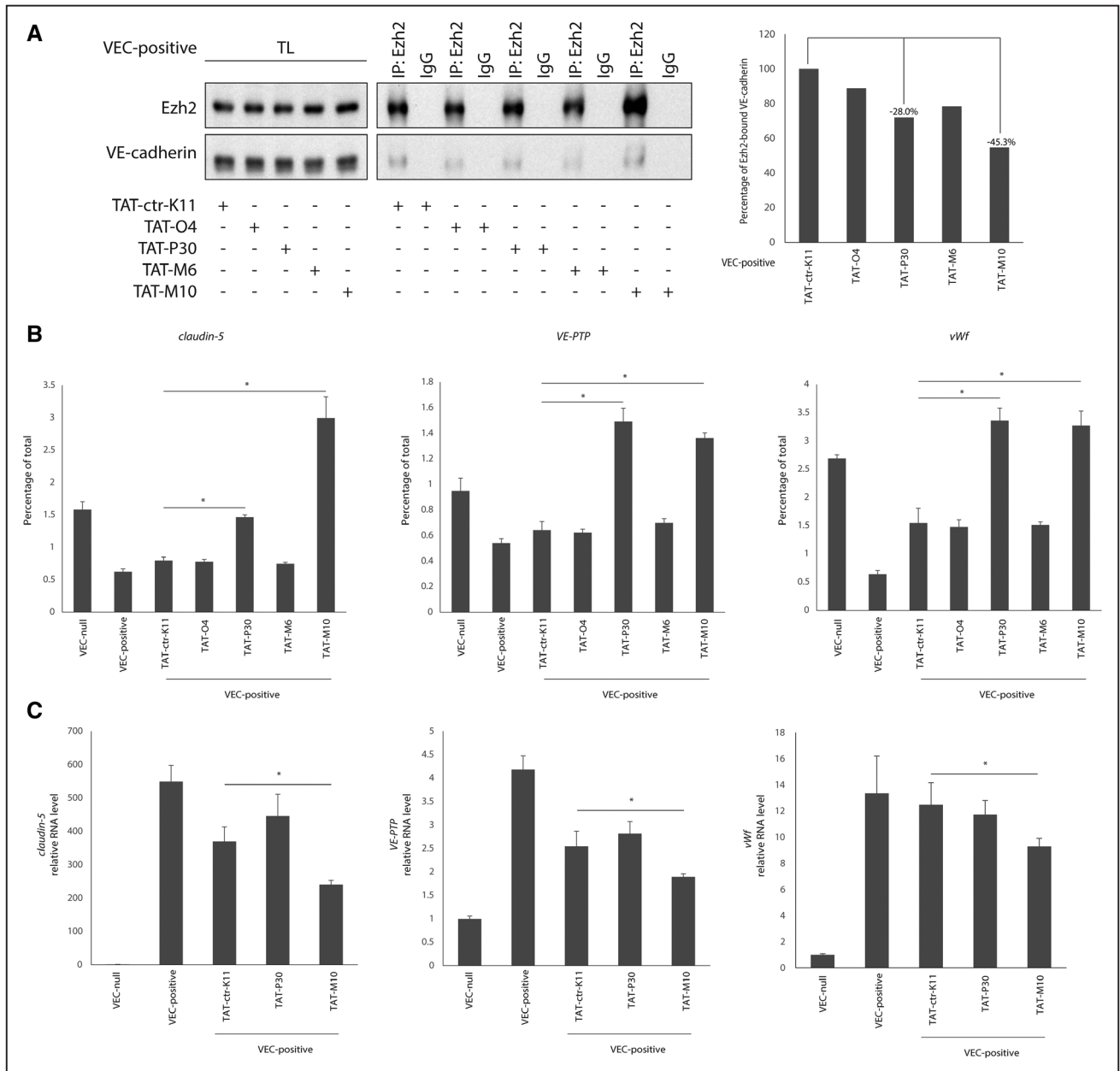
Figure 7. Inhibition of Ezh2 (enhancer of zeste homolog 2)/VEC (vascular endothelial cadherin) interaction causes *claudin-5*, **vascular endothelial-protein tyrosine phosphatase (***VE-PTP***), and von Willebrand factor (***vWf***) downregulation. A,** Coimmunoprecipitation and Western blot (WB) of endogenous Ezh2 and VEC from extracts of VEC-positive endothelial cells (ECs) treated with VEC-binding transactivator of transcription (TAT)-M6 and TAT-M10 peptides, β-catenin–binding TAT-O4 and TAT-P30 peptides or nonbinding TAT-ctr-K11 peptide as control (**left**). Quantification of coprecipitated VEC protein normalized on precipitated Ezh2 level (**right**). **B**, Quantitative real-time polymerase chain reaction (qRT-PCR) for the transcription start site (TSS) of *claudin-5*, *VE-PTP*, and *vWf* performed on endogenous Ezh2-bound chromatin immunoprecipitated from VEC-positive ECs treated with VEC-binding TAT-M6 and TAT-M10 peptides, β-catenin–binding TAT-O4 and TAT-P30 peptides or nonbinding TAT-ctr-K11 peptide as control. Levels of DNA are normalized to input; columns are means±SD of triplicates from a representative experiment. *$P<0.01$, *t* test TAT-ctr-K11 vs TAT-P30 or TAT-M10 treatment. **C**, qRT-PCR analysis of *claudin-5*, *VE-PTP*, and *vWf* expression in VEC-positive ECs treated with VEC-binding TAT-M10 peptide, β-catenin–binding TAT-P30 peptide or nonbinding TAT-ctr-K11 peptide as control. Levels of mRNA are normalized to GAPDH; columns are means±SEM of triplicates from a representative experiment. *$P<0.01$, *t* test TAT-ctr-K11 vs. TAT-M10 treatment. IP indicates immunoprecipitation; and TL, total cell lysate.

Nonetheless, a reduction of VE-PTP signal could be appreciated in tumor vessels (Online Figure XI).

Discussion

In this study, we describe a novel mechanism through which VEC expression and clustering upregulate endothelial-specific genes and contribute to endothelial differentiation and stability. We found that VEC engagement at cell–cell contacts acts by inhibiting PcG protein–mediated epigenetic modifications of EC chromatin. PcG proteins have been implicated in several developmental processes,[7,8] and Ezh2 activity was claimed to be involved in the regulation of genes important in tumor
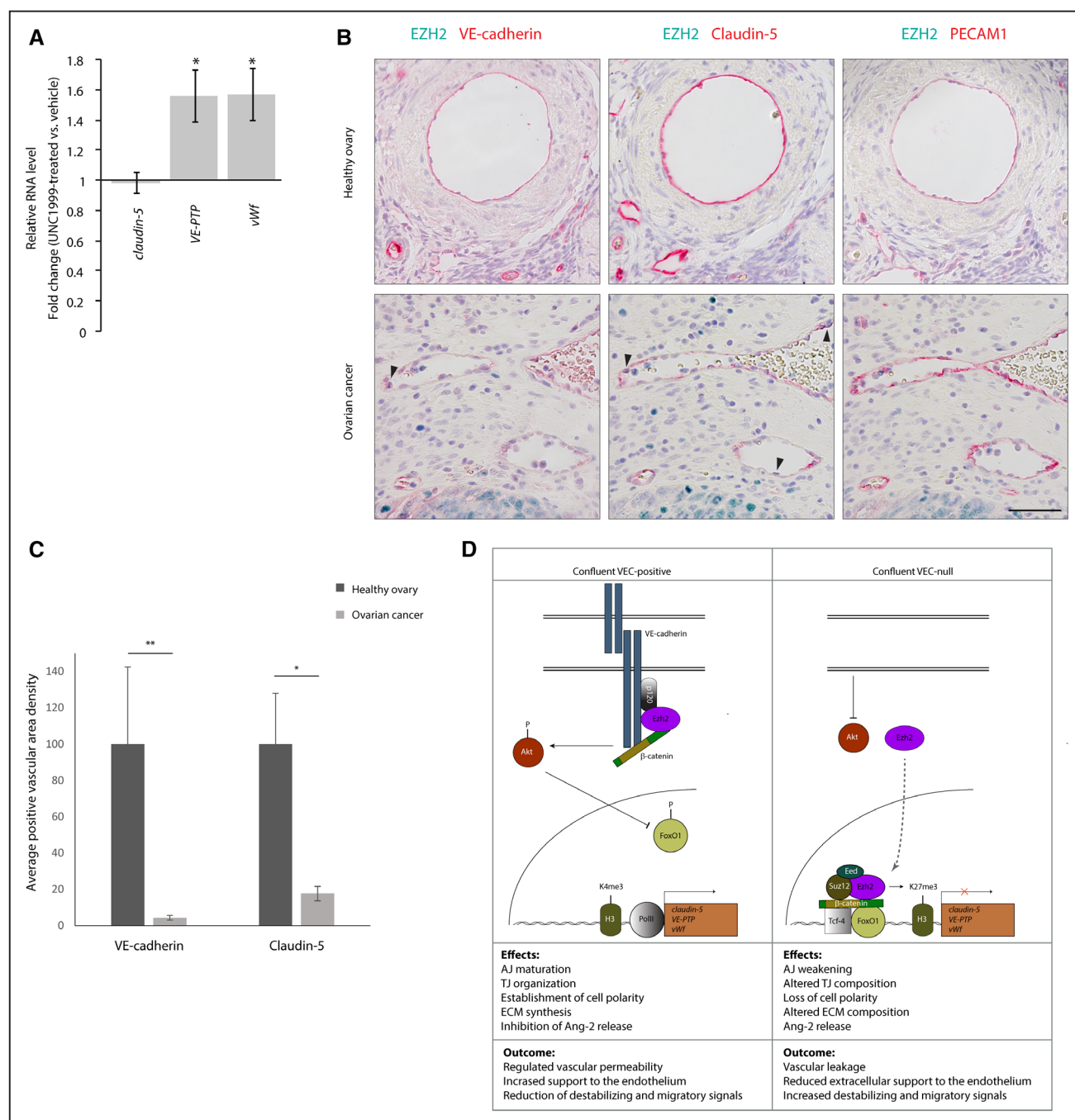
Figure 8. Ezh2 (enhancer of zeste homolog 2) activity correlates with *claudin-5*, vascular endothelial-protein tyrosine phosphatase (*VE-PTP*), and von Willebrand factor (*vWf*) repression in vivo. A, Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of *claudin-5*, *VE-PTP*, and *vWf* expression in lungs of vehicle- or UNC1999-treated pups (P6). Data are represented as fold change of UNC1999 treated vs vehicle and are means±SD from at least 4 mice per group. Gene expression was normalized to *VEC* (vascular endothelial cadherin) expression. **B**, Immunohistochemistry (IHC) staining of VEC, Claudin-5, platelet/endothelial cell adhesion molecule-1 (PECAM1; red) and EZH2 (green) expression in serial sections of human healthy ovary (**upper**) or serous surface papillary ovarian carcinoma (**lower**). Black arrowheads point to tumor vessel endothelial cells (ECs) expressing high levels of EZH2. Scale bar: 50 μm. **C**, Quantification of IHC stainings in (**B**). For VEC and claudin-5, areas of specific signal, divided by the total measured area, were normalized to the corresponding values of PECAM1 staining. Columns are means±SEM (n=3 healthy ovaries; 4 ovarian carcinomas; at least 3 fields per sample). **D**, Suggested model for the regulation of *claudin-5*, *VE-PTP*, and *vWf* genes. Clustered VEC recruits β-catenin and activates Akt leading to FoxO1 (Forkhead box protein O1) phosphorylation and inhibition.[5] Furthermore, Ezh2 is sequestered at the cell membrane by association with VEC cytoplasmic tail (**left**). These mechanisms allow gene activation by impeding the recruitment of PcG (polycomb group) proteins to gene promoters. Claudin-5 expression allows the correct organization of tight junctions (TJs) and regulation of vessel permeability.[5,27] VE-PTP regulates adherens junction (AJ) maturation[28] and VEGFR2 (vascular endothelial growth factor receptor-2) activity,[13,29] whereas vWf contributes to extracellular matrix (ECM) formation and inhibits Ang (angiopoietin)-2 release.[14,30] These effects are likely to contribute to vessel stabilization and prevent vascular leakage. In (**A**) and (**C**), *P<0.05; **P<0.01, t test.

angiogenesis and Kaposi Sarcoma.[31] Furthermore, Ezh2 was reported to play a role in maintaining vascular integrity during embryonic development.[32]

However, these studies mainly focused on the effects of Ezh2 inhibition without investigating in detail the mechanisms of polycomb regulation in ECs. We observed that VEC expression and clustering were able to trigger a gene expression program contributing to endothelial differentiation. Some of these endothelial genes seemed to be regulated by polycomb activity, thus pointing to a possible central role of VEC/polycomb crosstalk in orchestrating endothelial specification.

Within the set of genes upregulated by VEC, we selected endothelial-specific *claudin-5*, *VE-PTP*, and *vWf*. Claudin-5 is a major component of endothelial TJs and determines size and charge selectivity of endothelial paracellular permeability.[4,5,27,33]

VE-PTP exerts different context-specific activities including associating with VEC and reducing its tyrosine phosphorylation.[34] Absence of VE-PTP impairs AJ maturation, inducing vessel destabilization and increased permeability.[28,35] VE-PTP associates with Tie-2 receptor modulating Ang (angiopoietin) signaling[36] and interacts with VEGFR2 (vascular endothelial growth factor receptor-2) limiting its signaling activity.[13,29]

vWf is a crucial component of EC extracellular matrix that provides mechanical support to the EC monolayer[30] and limits the release of vessel-destabilizing Ang-2 from endothelial Weibel–Palade bodies.[14] Consistent with our data, a larger pool of vWf is present in confluent ECs compared with subconfluent condition.[37]

Although our analysis was mainly focused on these 3 genes, other genes regulated by VEC are involved in inhibition of cell proliferation, decrease of sprouting angiogenesis, promotion of cell adhesion, and production of extracellular matrix, suggesting a broader role of VEC expression and clustering in inducing vessel stabilization.

We previously found[5] that VEC expression derepresses the endothelial-specific gene *claudin-5* by restraining the activity of FoxO1 and β-catenin.[33] Here, we show that nuclear FoxO1 and β-catenin increase polycomb targeting to the promoter regions of a set of endothelial genes. We observed that induction of *claudin-5*,[5] *VE-PTP*, and *vWf* relies on VEC capacity to activate the PI3K/AKT pathway, leading to FoxO1 inactivation,[4] and to sequester β-catenin at the cell membrane. The absence of VEC clustering prevented the expression of this set of genes. The work presented here confirms and extends what previously observed for *claudin-5*[5] and further underlines the role of FoxO1 as a key transcription factor in endothelial differentiation and homeostasis.[38] This is in agreement with a recent report highlighting a key role of Akt1 activation and FoxO inhibition in stabilizing the endothelial barrier and preventing vascular leakage.[39]

Little is known about the molecular mechanisms of FoxO1-mediated gene repression. We found that FoxO1 physically associates with PcG proteins and, through still unknown mechanisms, contributes to their association to a selected set of endothelial gene promoters inducing chromatin conformational changes and gene inhibition. β-Catenin takes part in such multiprotein complex and stabilizes PcG protein binding

to DNA, as TCF4-DN–mediated abrogation of β-catenin/DNA binding destabilizes Ezh2/promoter interaction.

PcG proteins have been previously reported to have atypical extranuclear localizations.[40,41] We show here that VEC also reduces polycomb nuclear activity by sequestering Ezh2 at the plasma membrane in a β-catenin–dependent and p-120-catenin–dependent manner. Ezh2 interaction with VEC was confirmed in vivo in mouse lung extracts in the absence of any crosslinking reaction, whereas no association was detected between Ezh2 and the other major classical endothelial cadherin, N-cadherin, likely as a result of the reduced binding of this adhesion molecule to p120-catenin.[42] Recently, EZH2 has been shown to interact with β-catenin in liver cancer stem cells[43] through its N-terminal domain (amino acids 1–334). This was defined by domain mapping assays using EZH2 truncation mutants. Our analysis identified the Ezh2 sites of direct interaction with β-catenin in the domain comprised between amino acids 522 and 604. Deletion of domain 1 to 334 might alter the protein structural integrity, affecting EZH2/β-catenin interaction at downstream residues.

Ezh2/VEC association was shown to play a functional role in regulating gene expression. Inhibition of such interaction, and likely the consequent release of Ezh2 protein in the cytoplasm, increased its recruitment to *claudin-5*, *VE-PTP*, and *vWf* gene promoters causing their downregulation. The strong increase of Ezh2 recruitment at gene promoters, however, did not parallel a similarly strong repression of gene expression.

This was likely because of reduced FoxO1 and β-catenin nuclear abundancy in the confluent VEC-positive condition, impeding the correct assembly of the nuclear repressive complex.

Thus, we propose that an event happening at cell–cell contacts, that is, VEC clustering, triggers and orchestrates a network of pathways and epigenetic events, regulating several aspects of endothelial homeostasis and vascular stability (Figure 8D).

Pharmacological inhibition of Ezh2/Ezh1 led to the upregulation of *VE-PTP* and *vWf* in mouse pups, confirming that these genes are dependent on polycomb activity in vivo. C*laudin-5* was not significantly upregulated by such treatment, likely because its expression is unable to increase over a physiological threshold. Additional studies are needed for understanding the kinetics of expression of this gene in the presence or absence of polycombs in different vascular regions.

Deregulated angiogenesis is a characteristic of several human pathologies and is a hallmark of cancer. Tumor vessels present altered endothelial cell–cell junctions, are hyperpermeable, have low pericyte coverage, and altered basement membrane composition and stiffness. Tumor vessel stabilization by increased VEC has been shown to reduce tumor metastasis.[44,45] Ezh2 expression is increased in tumor-associated ECs, and this has been linked to increased vascular density and reduced vessel maturation.[31] Our results show that reduced VEC and increased EZH2 nuclear staining in human ovarian carcinoma vessels parallel the downregulation of Claudin-5 and VE-PTP, suggesting a possible alteration of the identified signaling mechanism.

In conclusion, this work introduces a novel role for VEC in the regulation of polycomb activity and, consequently, EC

differentiation and vascular maturation. The available genetic and pharmacological approaches to inhibit polycomb function will be instrumental to identify the role of this mechanism in different pathological conditions of the vascular system and to investigate new therapeutic opportunities to induce vessel normalization in cancer.[4]

Disclosures

None.

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Circulation Research

VE-Cadherin–Mediated Epigenetic Regulation of Endothelial Gene Expression

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SUPPLEMENTAL MATERIAL

VE-cadherin-mediated Epigenetic Regulation of Endothelial Gene Expression

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DETAILED METHODS

Data Disclosure

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Cell culture

The cell lines used in this study were:

- ECs derived from murine embryonic stem cells with homozygous null mutation of the *VEC* gene (VEC-null)[1]. The wild type form of *VEC* was introduced in these cells (VEC-positive) using retrovirus-mediated transfer as described in detail by Lampugnani et al.[2];

- Δβcat cells, ECs expressing a truncated mutant of VEC (lacking residues 703-784 of human VEC)[3], which correspond to the β-catenin-binding region[2];

- Δp120 cells, ECs expressing a truncated mutant of VEC (lacking residues 621-702 of human VEC), which correspond to the p120-catenin-binding region[2];

- IL2-VEC cells, ECs expressing a mutated version of VEC made up of the VEC cytoplasmic domain (amino acids 621-784) fused to the extracellular and transmembrane domains of IL-2 receptor α-chain (from Andrew Kowalczyk, Emory University, Atlanta, GA)[4, 5];

- β-Catenin WT and β-catenin KO ECs were derived from lungs of adult β-catenin[flox/flox] mice[6], immortalized as previously described[1, 7] and infected with an adenovirus encoding GFP (control) or CRE recombinase to obtain *β-catenin* gene recombination.

For all ECs of murine origin, culture medium was DMEM (GIBCO) with 20% North American (NA) fetal bovine serum (FBS) (HyClone), glutamine (2 mM; Sigma), penicillin/streptomycin (100 units/l; Sigma), sodium pyruvate (1 mM; Sigma), heparin (100 μg/ml, from porcine intestinal mucosa; Sigma), and EC growth supplement (ECGS) (5 μg/ml, made in our lab from calf brain) (complete culture medium). Starving medium was MCDB 131 (GIBCO) with 1% bovine serum albumin (BSA) (EuroClone), glutamine (2 mM), penicillin/streptomycin (100 units/l) and sodium pyruvate (1mM).

- Human dermal microvascular endothelial cells-1 (HMEC-1) (from STP Cell Services, The Francis Crick Institute, London, UK) were cultured in MCDB 131 (Thermo Fisher) with 10% North American (NA) fetal bovine serum (FBS) (HyClone), glutamine (10 nM; Sigma), penicillin/streptomycin (100 units/l; Sigma), epidermal growth factor (EGF) (30ng/ml; Sigma) and hydrocortisone (1 μg/ml; Sigma);

- 293T-Phoenix-Ecotropic packaging cells were provided by IFOM Cell Culture facility and cultured in DMEM medium supplemented with 10 % South American (SA) FBS (Hyclone), glutamine (2 mM) and sodium pyruvate (1 mM);

- Low passage AD-HEK293 cell line (human embryonic kidney, American Type Culture Collection, Manassas, VA), used for adenoviral production, were provided by IFOM Cell Culture facility and grown in DMEM medium supplemented with 10% FBS NA, glutamine (4 mM), penicillin/streptomycin (100 units/l), and sodium pyruvate (1 mM).

All cells were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Lentiviral and adenoviral preparations

ΔN-βcatenin construct was obtained from C. Brancolini (University of Udine, Udine, Italy). Lentiviral vectors used to stably express a short hairpin RNA against Suz12 (sh-Suz12), to overexpress Suz12 and the respective controls were a kind gift of D. Pasini (IEO, Milan, Italy). Packaging plasmids were kindly donated by L. Naldini (HSR-TIGET, San Raffaele Telethon Institute for Gene Therapy, Milan, Italy).

Lentiviral vectors were produced as described by Dull et al.[8]. Briefly, on day 1, 293T-Phoenix-Ecotropic packaging cells were transfected with the viral genome using calcium phosphate and incubated overnight with the transfection mix. On day 2 the medium containing the transfection mix was removed and 293T-Phoenix-Ecotropic cells were grown in as little medium as possible to concentrate the virus. On day 3 the medium containing the virus was removed, passed through a 0.45 μm diameter filter, supplemented with Polybrene (8 μg/ml, from IFOM Cell Culture facility) and placed on cells to be infected. The same procedure was repeated on day 4. Sh-Suz12- and sh-Empty-infected cells were selected with hygromycin 300 μg/ml. Cells were kept under selection until control non-infected cells died. Suz12-overexpressing cells and their Empty control were selected with Puromycin 3 μg/ml. Cells were kept under selection until control non-infected cells died.

The FKHR-TM adenovirus has been previously described[9]. The TCF4-DN adenovirus was kindly donated by S. J. George (Bristol Heart Institute, Bristol, UK)[10]. Infectious viruses were purified and titered using standard techniques. Briefly, for adenovirus production AD-293T cells were infected with 2 pfu/cell in DMEM without serum for 1 h at 37°C. Then, the infection medium was removed and cells were grown in an appropriate volume of DMEM + 5% horse serum until complete cell lysis was obtained (usually 72h later). The medium containing the viruses was then subjected to 3 freeze-and-thaw cycles in order to destroy all the cells and to free as many virions as possible. The resulting supernatant was then centrifuged at 3000 rpm for 30 min at 4°C to eliminate the cellular debris, aliquoted and stored at -80°C. For the infection of ECs two consecutive cycles of infection [5 h and overnight (O/N)] were performed with MOI of 300 in 1 ml of complete culture medium.

Immunofluorescence microscopy
Immunofluorescence microscopy staining was performed using standard technique, as previously described[11]. Briefly, cells were seeded on 0.5% cross-linked gelatin. Cells were fixed and permeabilized in ice-cold methanol at -20°C for 5 min. Fixed cells were incubated for 30 min in a blocking solution [phosphate buffer saline (PBS) containing Ca^{2+} and Mg^{2+}, 2.5% skim-milk, 0.3% TritonX-100]. Cells were then incubated for 1 h at RT with primary antibodies diluted in blocking buffer. Alternatively, cells were fixed with 1% PAF in triethanolamine, pH 7.5, containing 0.1% Triton X-100 and 0.1% NP-40 for 20 min. Fixed cells were incubated for 30 min in a blocking solution [Tris-buffered saline (TBS) containing 5% BSA, 0.3%TritonX-100]. Cells were then incubated overnight at 4°C with primary antibodies diluted in blocking buffer. Appropriate secondary antibodies were applied on cells for 45 min at RT and cells were then mounted with VECTASHIELD containing DAPI (Vector Biolabs).

Samples were observed under an epifluorescence microscope (DMR; Leica) using a 63X objective. Images were captured using a charge-coupled camera and processed with Adobe Photoshop. Only adjustments of brightness and contrast were used in the preparation of the figures. For comparison purposes, different sample images of the same antigen were acquired under constant acquisition settings.

Western blot analysis
Total proteins were extracted by solubilising cells in boiling Laemmli buffer [2.5% SDS and 0.125 M Tris-HCl (pH 6.8)]. Lysates were incubated for 5 min at 100°C to allow protein denaturation and then spinned for 5 min at 13200 rpm to discard cell debris. The supernatants were collected and the concentration of protein was determined using a BCA™ Protein Assay Kit (Pierce) according to manufacturer's instructions. Equal amounts of proteins were loaded on gel and separated by SDS-PAGE, transferred to a Protran Nitrocellulose Hybridization Transfer Membrane 0.2 μm pore size (Whatman) and blocked for 1 h at RT in 1X TBS Tween (TBST) [150 mM NaCl, 10 mM Tris-HCl (pH 7.4), and 0.05% Tween] containing 5% (w/v) skim-milk. The membranes were incubated overnight at 4°C or 1 h at RT with primary antibodies diluted in 1X TBST-5% BSA. Next, membranes were rinsed 3 times with 1X TBST for 5 min each and incubated for 1 h at RT with HRP-linked secondary antibodies (diluted in 1X TBST-5% BSA). Membranes were rinsed 3 times with TBST for 5 min each and specific binding was detected by the enhanced chemiluminescence (ECL) system (Amersham Biosciences) using HyperfilmTM (Amersham Biosciences) or the ChemiDoc gel imaging system (BIORAD). The molecular masses of proteins were estimated relatively to the electrophoretic mobility of co-transferred prestained protein marker, Broad Range (Cell Signalling Technology).

Co-immunoprecipitation
Cells were grown until confluent and starved overnight. Cells were then washed once with DMEM without serum and incubated with 0.4 mg/ml of dithiobis(succinimidyl)propionate (DSP) (Pierce) for 30 min at 37°C. After several washes with ice-cold PBS, cells were lysed in ice-cold modified RadioImmunoPrecipitation Assay (RIPA) buffer (Tris HCl pH 7.5 100 mM, NaCl 150 mM, Deoxycholic acid 1%, SDS 0.1%, $CaCl_2$ 2

mM). The protein lysate was precleared with an appropriate volume of Protein G Sepharose 4B (Zymed) for 3 h at 4°C. Protein concentration was determined with BCA™ Protein Assay Kit and an equal amount of protein was incubated with either immune antibodies or species-matched control antibodies overnight at 4°C. On the following day immunocomplexes were collected using Protein G Sepharose 4B for 3 h at +4°C. Beads were then washed several times with modified RIPA buffer and boiled in an appropriate volume of Laemmli buffer. Samples were analysed by standard Western blot analysis as described above[11].

CoIP following biotinylation of membrane proteins was performed using the same protocol as above. Before cell lysis with modified RIPA buffer cells were incubated with Sulfo-NHS-LC-Biotin (Pierce ThermoScientific) 0.55 mg/ml in PBS containing Ca^{2+} and Mg^{2+} pH 8.0 for 30 min at 4°C. After biotinylation cells were washed with PBS containing Ca^{2+} and Mg^{2+}pH 8.0 + Glycine 100 mM to quench the reaction.

CoIP from lung tissue: lungs from wild type adult age-matched mice were lysed in ice-cold modified JS buffer [Hepes pH 7.5 72 mM, NaCl 210 mM, glycerol 0.5 %, Triton X-100 1%, $MgCl_2$ 2 mM, EGTA 7.2 mM, SDS 0.1 %, Sodium Orthovanadate 300 mM, Pefabloc SC 1 mM (Sigma) and Sodium Fluoride 1 mM] using Tissue Lyser II (QIAGEN) (two 30-sec pulses at maximum frequency). Samples were precleared for 4 h at 4°C with an appropriate volume of Protein G Sepharose 4B. Protein concentration was determined with BCA™ Protein Assay Kit and an equal amount of protein was incubated with Protein G Sepharose 4B pre-coupled with either immune antibodies or species-matched control antibodies overnight at 4°C. The day after, beads were then washed with modified JS buffer and boiled in an appropriate volume of Laemmli buffer. Samples were analysed by standard western blot analysis[11]. All the buffers contained freshly added protease inhibitor cocktail (IFOM Kitchen Facility).

RNA-seq library preparation and sequencing
1 μg total RNA per sample (in triplicate) was used to prepare RNA sequencing libraries of VEC-null and VEC-positive cells with SMARTer Stranded Total RNA Sample Prep kit - High input Mammalian kit (Clontech), according to the manufacturer's instructions. This kit includes depletion of ribosomal RNA prior to library synthesis, barcoding and amplification. For validation of the amplified RNA-seq library, 1 μl of the library was used with the Agilent High Sensitivity DNA kit (Agilent). The samples were sequenced on a HiSeq2500 sequencing system with v4 chemistry from Illumina at the Swedish National Genomics Infrastructure, Uppsala node, SNP&SEQ technology platform (Science for Life Laboratory, Uppsala). The samples were run in pools of 6 samples with equimolar amounts of dsDNA from each of the six samples. Each pool was run on a single lane (paired-end sequencing, 125bp reads).

Bioinformatics analysis of RNA-seq data
Each sample received ~24 to 46 million of paired-end reads. Trim Galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) was used for quality/adapter trimming of the raw reads. In addition, 5 nucleotides from both 5' and 3' end of the reads were clipped to remove unwanted bias. Then trimmed reads were mapped to the mm10 mouse genome with > 70% overall mapping rate using TopHat v2.1.1[12] and annotated with GO terms using R package Biomart[13]. Differential gene expression (DEG) analysis was performed using DESeq2[14]. Genes were considered significantly differentially expressed if the $|log2FC| \geq 1$ and the adjusted p-value ≤ 0.05.

Identification of enriched functional annotation categories
Genes significantly up and downregulated in confluent VEC-positive vs. VEC-null cells were used to query DAVID's[15] "Functional Annotation Clustering" tool to look for enrichment of annotation terms from GOTERM_BP_DIRECT, KEGG, BIOCARTA and UP_KEYWORDS using the *Mus musculus* genome as background. Annotation terms with overlapping gene sets were grouped (see Online Table III for details) and used to annotate an expression heat-map of the same genes across all samples. For the purposes of visualisation abundance is presented as log2 (normalised read count + 1).

Quantitative Real Time (qRT)-PCR analysis
Total RNA was isolated by RNeasy kit (QIAGEN) and 1 μg was reverse transcribed with random hexamers (High Capacity cDNA Archive Kit, Applied Biosystems) according to the manufacturer's instructions. cDNA (5 ng) was amplified in triplicate in a reaction volume of 15 μl using TaqMan Gene Expression Assay (Applied Biosystems) and an ABI/Prism 7900 HT thermocycler, using a pre-PCR step of 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 60 sec at 60°C. Preparations of RNA template without reverse transcriptase were used as negative controls. For any sample the expression level, normalized to the housekeeping gene

18S, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β2-microglobulin, was determined using the comparative threshold cycle (CT) method as previously described[16].

Transcription factor binding site analysis
In order to identify FoxO1 and Tcf/β-catenin consensus sequences on the putative *VE-PTP* and *vWf* promoter regions we used the program MatInspector[17], which identifies transcription factor binding sites (TFBS) in nucleotide sequences using a large library of weight matrices. We analyzed the sequence spanning from 6000 bp upstream and 500 bp downstream the transcription start site (TSS) of the genes and obtained a prediction of a potential combination of TFBS. The TFBS sequences considered in the analysis were [AG][GA][TG][AC]AACAA[AC] for FoxO1 binding and [TAG][GT][AG][CT][AT]x(2)CAAAG[GCT][GAC][AC][GCA] for Tcf/β-catenin binding.

Claudin-5 **promoter luciferase assay**
Claudin-5 putative promoter region including all three regions of paired Tcf/β-catenin-FoxO1 binding sites[18] (3169 bp upstream of the CDS) was cloned into the XhoI-HindIII site of the multiple cloning region of pGL3-Basic Vector (Promega) using the following primers: 5'-AAAAACTCGAGAAATGGCTCTGGGCAAGAAG-3' and 5'-AGGGAAAGCTTGGCTAAAGACTGAATGCTCA-3'. XhoI and HindIII restriction enzymes were from New England BioLabs. Cloning was confirmed by sequencing, performed by STP Genomics Equipment Park (The Francis Crick Institute, London, UK). VEC-positive cells, wild type or infected with the indicated constructs, were seeded at 30.000 cells/cm²; VEC-null cells, wild type or infected with the indicated constructs, were seeded at 55.000 cells/cm² in 35 mm diameter plates (Corning) in complete culture medium without penicillin/streptomycin. The day after, cells were transiently transfected with 2 μg/well of *Claudin-5*-pGL3-luciferase vector and 1 μg/well of pRL-TK vector (Promega) using 8 μl/well of LipofectAMINE 2000 (Invitrogen) in OptiMEM (Invitrogen) according to the manufacturer's instructions. After 8.5 h of incubation, cells were incubated in fresh complete culture medium and 72 h after transfection, firefly and Renilla luminescence were detected using Dual-Glo Luciferase Assay System (Promega), according to the manufacturer's instructions, with EnVision 2102 MultiLabel Reader (PerkinElmer). Renilla luminescence signal was used as normalizer for transfection efficiency.

Protein expression and purification
For GST-tagged VEC-cytoplasmic tail production, bacterial expression vector[19] was transformed into BL21 strain of *E. coli*. For growth, a single colony was picked from freshly transformed plate and allowed to grow overnight at 37°C in 20 ml LB medium. This culture was used to inoculate 2 x 1 liter of LB and TB media. Cultures were allowed to grow until the OD600nm reached 0.6 at which point protein expression was induced by the addition of 1 mM IPTG. Induction was allowed to occur at 37°C for 4 h. The cells were then harvested by centrifugation and stored at -80°C until required. Cells obtained from 1 liter of LB or TB media were treated in the same way. Frozen pellets were thawed and resuspended in 20 ml ice cold lysis buffer containing 50 mM HEPES (pH 7.5), 2 mM EDTA, 1 mM DTT and protease inhibitors (Sigma) (2 protease inhibitor tablets were added to 50 ml total lysis buffer). To ensure complete lysis the suspension was sonicated for 5 x 30 sec, 20% amplitude. The insoluble fraction was removed by centrifugation (30,000 rpm, 20 mins, 4°C) and the soluble fraction incubated with 200 μl glutathione sepharose for 1 h at 4°C with constant rolling. The resin was then washed extensively with lysis buffer and bound protein eluted using lysis buffer containing 20 mM reduced glutathione. Elutions were concentrated to a volume of 270 μl to which 30 μl of glycerol was added. The protein concentration was determined by Bradford reagent, snap frozen in liquid nitrogen and stored at -80 °C until required.
For GST-tagged p120-catenin production, competent *E.coli* DH10 Bac cells (Invitrogen) were transformed with GST-Ctnnd1-pFastBAC1 plasmid (NovoPro).   White colonies were picked from freshly transformed plates and grown in liquid culture in order to purify bacmid DNA. This DNA was then used to generate virus by transfecting Sf21 insect cells (Invitrogen). Two further rounds of amplification generated high titre virus stocks suitable for infecting large scale cultures for expression of the protein. Sf21 insect cells were infected at a cell density of 1.2 x 10⁶ cells/ml and at a MOI of 1. Infected cultures were allowed to grow for 3 days with shaking (140 rpm) at 27ºC. Cells were then harvested and cell pellets stored at -80 ºC until required. 2L of cell pellet was thawed and resuspended in 30 ml lysis buffer (50 mM HEPES [pH 7.5], 250 mM NaCl, 10% glycerol, 1 % Triton X-100, 1 mM NaF, 10 mM benzamidine, 0.5 mM EDTA, 10 mM B-glycerophosphate, 1 mM Na₂VO₄, 1mM DTT) and lysed by sonication on ice (3 min total time, 20 sec on 5 sec off, 20%

amplitude). Insoluble material was removed by centrifugation at 30.000 rpm for 20 mins. The soluble fraction was incubated with 300 µl bed volume of glutathione sepharose resin for 2 h (with rolling at 4ºC) after which time the resin was washed extensively in buffer containing 50 mM HEPES (pH7.5), 250 mM NaCl, 1 mM DTT. Protein was eluted in the same wash buffer supplemented with 15 mM reduced glutathione and 5% glycerol.

Peptide arrays
Peptide arrays were synthesised on an Intavis Multipep Peptide Synthesiser (Intavis Bioanalytical Instruments AG), using N-Fmoc amino acids, onto a cellulose sheet derivitised to have 8-10 ethylene glycol units between the sheet and an amino group for synthesis. Each amino acid was coupled as a chlorohydroxybenzatriazole active ester, automatically formed immediately prior to use. Once the required number of cycles of coupling and deprotection and washing were completed, the membranes were treated with a solution of 20 mls containing 95% trifluoroacetic acid, 3% triisopropylsilane and 2% water for 4 h. Following this treatment, membranes were washed 4 times with dichloromethane, 4 times with ethanol, twice with water, once with ethanol.
Membranes were activated with 100% methanol for 10 seconds and washed twice with 1X TBST (0.1% Tween) for 5 minutes each wash. Membranes were then blocked for 1 h at RT in 1X TBST (0.1% Tween) containing 5% (w/v) skim-milk, and incubated 2 h at RT with free GST (kind gift of S. Tooze, The Francis Crick Institute, London, UK) or GST-tagged VEC-cytoplasmic tail[19], β-catenin (Sino Biological Inc.) or p120-catenin, diluted to 1 µg/ml in 1X TBST-5% BSA. Next, membranes were rinsed 5 times with 1X TBST (0.1% Tween) and incubated overnight at 4°C with HRP-linked anti-GST antibody (diluted in 1X TBST-5% skim-milk). Membranes were rinsed 5 times with 1X TBST (0.1% Tween) for 5 min each time and specific binding was detected by the enhanced chemiluminescence (ECL) system (Amersham Biosciences) using HyperfilmTM (Amersham Biosciences).

Peptide pull-down assays
Solid phase synthesis of the peptides was carried out on an Intavis Multipep Peptide Synthesizer (Intavis Bioanalytical Instruments AG) on a Rink amide LL resin, using N-Fmoc amino acids and HCTU as the coupling reagent. In the final steps of chain assembly biotin was incorporated at the N-terminal after an aminohexanoic acid spacer. Following this, the peptidylresin was added to 10 ml of 92.5% trifluoroacetic acid, 2.5% water, 2.5% ethanedithiol and 2.5% triisopropyl silane. After 2 h reaction, the resin was removed by filtration and the peptide was precipitated from the acid solution with diethyl ether on ice. The peptide was isolated by centrifugation, then dissolved in water and freeze dried overnight. After dissolving in water portions of the peptides were purified on a C8 reverse phase HPLC column (Agilent PrepHT Zorbax 300SB-C8, 21.2x250 mm, 7 m). Buffer A was 1% acetonitrile, 0.08% trifluoroacetic acid in water, buffer B was 90% acetonitrile, 0.08% trifluoroacetic acid in water. The elution gradient was from 10% to 50% B over 40 minutes at a flow rate of 8 ml/minute. The peak fraction was analysed by LC–MS on an Agilent 1100 LC-MSD. The calculated molecular weights of the peptide were in agreement with the mass found. For the complete list of peptides used in this study see Online Table IV.
NeutravidinTM biotin binding beads (Perbio) were washed 3 times in 1 ml of cold Buffer Y [Tris-HCl pH 7.5 50 mM, NaCl 150 mM, EDTA 1 mM, NP-40 1 % v/v, DTT 1mM, EDTA-free Protease Inhibitor Cocktail (Sigma)] and incubated with the desired biotin-conjugated peptide (1.5 mg/ml in water) at 4°C for 1 h with rotation. Peptide-conjugated beads were washed 3 times in cold Buffer Y and incubated with 2 µg of purified protein in a volume of 500 µl of Buffer Y overnight at 4°C with rotation. Beads were then washed 3 times in Buffer Y and boiled in an appropriate volume of Laemmli buffer. Samples were analysed by standard Western blot analysis as described above.

TAT-peptide treatment
VEC-positive cells were plated at a concentration of 55.000 cells/cm^2 in complete culture medium with 30 µg/ml of TAT-conjugated peptide. Treatment was repeated the following day at the same concentration, followed by 2 other treatments at 10 µg/ml on day 3 and 5. On day 7, after cells had been at confluence for 48 h, cells were starved overnight in starving medium containing 10 µg/ml of TAT-conjugated peptide, then processed for co-immunoprecipitation, ChIP and qRT-PCR.

Chromatin immunoprecipitation (ChIP)

ChIP assays were carried out as previously described[20]. Briefly, cells were starved overnight and cross-linked with 1% PFA for 10 min at RT. PFA was then inactivated by the addition of 125 mM glycine. Cells were then washed and resuspended in lysis buffer. Samples were sonicated with a BIORUPTOR™ 200 using the following conditions: H power, 30 sec ON - 60 sec OFF for 20 min. Chromatin extracts containing from 200 μg to 1000 μg of DNA fragments with an average size of 500 bp were incubated overnight with using 5 μg of either immune antibody or matched non-immune antibodies and Dynabeads protein G (Invitrogen) or Ultralink resin protein A/G (Pierce ThermoScientific). On the following day, beads were recovered and washed twice with Mixed Micelle Wash Buffer (NaCl 150 mM, TrisHCl pH 8.1 20 mM, EDTA 5 mM, Sucrose 5.2 % w/v, NaN$_3$ 0,02%, Triton X-100 1%, 0.2 % SDS), Buffer 500 (Deoxycholic acid 0,1% w/v, NaCl 500 mM, HEPES pH 7.5 25 mM, EDTA 1 mM, NaN$_3$ 0.02%, Triton X-100 1%), LiCl Detergent wash (Deoxycholic acid 0,5% w/v, LiCl 250 mM, EDTA 1 mM, NP-40 0.5% v/v, NaN$_3$ 0.02%, Tris HCl pH 8.0 10 mM). Proteins/DNA complexes were detached from beads by heating the samples at 65°C for 10 min. De-crosslinking was done at 65°C overnight. DNA was purified using phenol/chloroform and precipitated in 70% ethanol according to standard protocol. DNA was amplified by qRT-PCR techniques using oligonucleotides flanking the assayed promoter regions.

Primers used in this experiments were: 5'-GTCGGGTGAGCATTCAGTCT-3' and 5'-ATCAAGCCCACCCATCCTAC-3' (Claudin-5 TSS); 5'-TGCAGAAGGAGAAAACAATGC-3' and 5'-GCAGCAACGTGTGTCAGTGT-3' (VE-PTP Region 1); 5'-TGGATCCTGTAGCCATATTTGA-3' and 5'-CATCATATAACTGCAACAAAGCAC-3' (VE-PTP Region 2); 5'-GACATAAGTAGCCAAGAACAGGTTT-3' and 5'-TCAAATCACTAGGAGGAATAAGACA-3' (VE-PTP Region 3); 5'-GCTCAACAAGTGGTACCCAGA-3' and 5'-TGCACGACGCTCAGTGTTAT-3' (VE-PTP TSS); 5'-GTTTGTTTGAGCCAGGGTCT-3' and 5'-CAGGAGGTCGAAGCAAGATG-3' (vWf Region 1); 5'-GCAGGTCTTGGGTTCTATGC-3' and 5'-GGGGTGGAAATGATGGTTC-3' (vWf Region 2); 5'-TGGTGGCAACTTGGAGCTAT-3' and 5'-AGGGGCTTCAAAGTCCTCAG-3' (vWf Region 3/TSS).

For qRT-PCR analyses DNA was diluted in the presence of specific primers (0.4 μM each) to a final volume of 25 μl in SYBR Green Reaction Mix (Perkin Elmer).

RNA interference

Stealth RNAi Duplexes (Life Technologies) and the correspondent Low GC Stealth RNAi Control Duplexes (Life Technologies) were used to knockdown FoxO1. The sequences of the two siRNAs used were the following: 5'-CCAAGUGACUUGGAUGGCAUGUUUA-3' and 5'-CAGACACUUCAGGACAGCAAAUCAA-3'. Transfection was performed using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions.

***In vivo* pharmacological treatment**

All animal procedures were performed in accordance with the Institutional Animal Care and Use Committee (IACUC), in compliance with the guidelines established in the Principles of Laboratory Animal Care (directive 86/609/EEC) and approved by the Italian Ministry of Health.

Ezh2 and Ezh1 were inhibited in C57Bl/6J background pups by intraperitoneal injection with 50 mg/kg UNC1999[21] in 10% DMSO and 90% corn oil or vehicle only, at P3, P4 and P5. Total RNA from lungs of UNC1999-treated pups (P6) was isolated and processed as described in details previously[22].

Immunohistochemistry (IHC)

Paraffin-embedded human ovarian tissue samples were scored as pathologic or non-pathologic by a trained pathologist. Samples were de-paraffinized and hydrated following standard protocol and subjected to antigen unmasking in Sodium Citrate Buffer pH 6 for 45 min at 95°C. Samples were stained using MATCH 2 Double Stain 2 Kit (Biocare). Haematoxylin/eosin staining was performed according to standard protocol and samples were mounted in Eukitt (Bio-Optica).

For image quantification we used NIS-Elements AR software (version 4.51.01; Nikon). A hue saturation intensity (HSI) threshold was defined by selecting pixels of specific signal around the vessel perimeter. This threshold was applied to every image, followed by morphometrics steps of image smoothing, cleaning and filling. An area of interest was drawn on images to exclude regions of non-specific staining. Areas of detected specific signal were divided by the total measured surface, and values obtained for Claudin-5 and VE-cadherin were then normalized on the measurements obtained for PECAM1 in the corresponding regions.

Antibodies and chemicals
Antibodies used in this study were: anti-claudin-5 mouse monoclonal clone 4C3C2, anti-V5 tag mouse monoclonal R960-25 (Life Technologies); anti-claudin-5 rabbit polyclonal ab53765, anti-claudin-5 rabbit monoclonal ab131259, anti-CD31 rabbit polyclonal ab28364, anti-total histone H3 ab1791, anti-H3K4me3 ab8580, and anti-RNA pol II phospho Ser 5 (ABCAM); anti-β-catenin mouse monoclonal 610154, anti-Ezh2 mouse monoclonal 612666, anti-N-cadherin mouse monoclonal 610921, anti-Pecam1 rat monoclonal 550274 (BD Biosciences); anti-β-catenin rabbit polyclonal 06-734, anti-Ezh2 rabbit polyclonal 07-689, anti-H3K27me3 rabbit polyclonal 07-449, anti-TCF4 mouse monoclonal clone 6H5-3 05-511 (Millipore); anti-FKHR (H-130X) rabbit polyclonal sc-67140, anti-VE-cadherin goat polyclonal sc-6458, anti-vWf clone H-300 rabbit polyclonal sc-14014, anti-Suz12 goat polyclonal sc-46264 (Santa Cruz Biotechnology); anti-vWf rabbit polyclonal AB7356 (Chemicon); anti-FoxO1 rabbit monoclonal clone C29H4 2880, anti-phospho-FoxO1 (Ser 256) rabbit polyclonal 9461, anti-Myc-tag rabbit polyclonal 2272, anti-Suz12 rabbit monoclonal D39F6 3737, anti-H3K27me3 clone C36B11 rabbit monoclonal BK9733BFS, anti-β-catenin rabbit monoclonal 8480S (Cell Signalling), anti-Ezh2 mouse monoclonal NCL-L-EZH2 (Novocastra-Leica), peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories), anti-HA-tag mouse monoclonal clone 12CA5, anti-Bmi1 mouse monoclonal clone AF27 [from K. Helin, Biotech research and Innovation Centre (BRIC), University of Copenhagen], anti-Ezh2 clone AC22 and AE25-13, anti-α-tubulin mouse monoclonal, anti-vinculin mouse monoclonal (from internal service); anti-human VE-cadherin mouse monoclonal (BV9)[23], anti-VE-PTP Rabbit polyclonal (produced in our laboratory), anti-human VE-PTP Rabbit Polyclonal hPTP1-8 from D. Vestweber[24]; HRP-linked anti-GST RPN1236 (GE/Amersham); anti-VE-cadherin rabbit polyclonal 36-1900 (Thermofisher); anti-Ezh2 rabbit polyclonal 21800-1-AP (Proteintech).
The following reagents were used in this study: pan-caspase inhibitor Z-VAD-FMK (Promega); PI3K inhibitor LY294002 (Cell Signalling Technology). To inhibit PI3K activity, cells were starved for 24h in starving medium + 1% BSA and then treated overnight with LY294002 10 µM or Dimethyl sufoxide (DMSO) as a control. To avoid apoptotic cell death during FKHR-TM overexpression experiments, 24h after the beginning of the infection, cells were treated with Z-VAD-FMK 50 µM.

Statistical analysis
Student's two-tailed unpaired t-test was used to determine statistical significance. The significance level was set at $P < 0.05$.

ONLINE FIGURE LEGENDS

Online Figure I. VEC clustering induces *VE-PTP* and *vWf* expression through FoxO1/β-catenin inhibition.
(A) qRT-PCR and WB analysis of *VE-PTP* and *vWf* expression in sparse (sp) and confluent (con) VEC-null and VEC-positive ECs (upper panel). Immunofluorescence analysis of VEC (green) and *VE-PTP* or *vWf* expression (red) in confluent VEC-null and VEC-positive ECs. Scale bar: 10 µm (lower panel).
(B) qRT-PCR analysis of *VE-PTP* and *vWf* expression in VEC-null and VEC-positive ECs expressing FKHR-TM or GFP as a negative control. To limit the pro-apoptotic effect of FKHR-TM, after adenoviral infection the pan-caspase inhibitor Z-VAD-FMK (50 µM) was added to culture medium. qRT-PCR was performed 72 h after infection. The levels of mRNA are normalized to Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*), columns are means ± s.e.m. of triplicates from a typical experiment. nd, not detectable.
(C) qRT-PCR analysis of *VE-PTP* and *vWf* expression in confluent VEC-null and VEC-positive ECs treated with the PI3K inhibitor LY294002 (10 µM, overnight). Upper panel: WB analysis of p-FoxO1 Ser 256 (arrow) and total FoxO1 protein.
(D) qRT-PCR analysis of *VE-PTP* and *vWf* expression in confluent VEC-null and VEC-positive cells expressing ΔN-β-catenin or GFP as negative control.
(E) Schematic representation of the putative promoter regions of *VE-PTP* and *vWf*. FoxO1 (F) binding sites are depicted as white boxes and TCF/β catenin (T) binding sites as black boxes. Arrows identify primers used for qChIP shown in panels F and G.
(F) qRT-PCR for Region 1, 2 and 3 of *VE-PTP* and *vWf* promoter regions performed on endogenous FoxO1-bound chromatin immunoprecipitated from confluent VEC-null and VEC-positive ECs.

(G) qRT-PCR for Region 1, 2 and 3 of *VE-PTP* and *vWf* promoter regions performed on endogenous β-catenin-bound chromatin immunoprecipitated from confluent VEC-null and VEC-positive ECs.

In (A) and (C) vinculin was used as loading control.

In (A), (C) and (D) the levels of mRNA are normalized to 18S; columns are means ± s.e.m. of triplicates from a representative experiment.

In (B), (C) and (D) **$P < 0.01$, *t*-test.

In (F) and (G) the levels of DNA are normalized to input, columns are means ± st.dev. of triplicates from a representative experiment. nd, not detectable. *$P < 0.05$; **$P < 0.01$, *t*-test VEC-null vs. VEC-positive.

Online Figure II. Validation of RNA-seq results.
(A) qRT-PCR analysis of *Tiam1*, *Lmo2*, *Stat6* and *Elk3* expression in confluent VEC-null and VEC-positive ECs.
(B) qRT-PCR analysis of *Hey1* and *Adm* expression in confluent VEC-null and VEC-positive ECs.
(C) qRT-PCR analysis of *Pecam1* and *Sox18* expression in confluent VEC-null and VEC-positive ECs.
(D) qRT-PCR analysis of *Claudin-5*, *VE-PTP* and *VWF* expression in sparse (sp) and confluent (con) HMEC1.
(E) qRT-PCR analysis of *TIAM1*, *LMO2*, *STAT6* and *ELK3* expression in sparse (sp) and confluent (con) HMEC1.
(F) qRT-PCR analysis of *HEY1* and *ADM* expression in sparse (sp) and confluent (con) HMEC1.
(G) qRT-PCR analysis of *PECAM1* and *SOX18* expression in sparse (sp) and confluent (con) HMEC1.
In the figure, the levels of mRNA are normalized to *GAPDH*; columns are means ± s.e.m. of triplicates from a representative experiment. *$P < 0.05$; **$P < 0.01$, *t*-test.

Online Figure III. *VE-PTP* and *vWf* genes are repressed in IL2-VEC ECs.
qRT-PCR analysis of *VE-PTP* and *vWf* expression in confluent VEC-null, VEC-positive and IL2-VEC ECs. The levels of mRNA are normalized to 18S; columns are means ± s.e.m. of triplicates from a representative experiment. *$P < 0.01$, *t*-test.

Online Figure IV. Histone density on the TSS of *claudin-5*, *VE-PTP* and *vWf* is not affected by VEC expression.
qRT-PCR for the TSS of *claudin-5*, *VE-PTP* and *vWf* performed on endogenous histone H3-bound chromatin immunoprecipitated from confluent VEC-null and VEC-positive ECs. The levels of DNA are normalized to input, columns are means ± st.dev. of triplicates from a representative experiment.

Online Figure V. *Claudin-5* luciferase reporter assays.
(A) Schematic representation of the putative promoter region of *claudin-5* cloned upstream of firefly luciferase gene. FoxO1 (F) binding sites are depicted as white boxes and TCF/β catenin (T) binding sites as black boxes.
(B) *Claudin-5* luciferase reporter assay performed on confluent VEC-null and VEC-positive cells.
(C) *Claudin-5* luciferase reporter assay performed on confluent VEC-positive ECs upon Suz12 overexpression.
(D) *Claudin-5* luciferase reporter assay performed on confluent VEC-null ECs upon Suz12 knockdown (sh-Suz12).
In (B), (C) and (D) *$P < 0.05$; **$P < 0.01$, *t*-test.

Online Figure VI. Effect of Suz12 overexpression on endothelial genes.
(A) qRT-PCR analysis of *Lmo2* and *Stat6* expression in confluent VEC-positive ECs upon Suz12 overexpression.
(B) qRT-PCR analysis of *Hey1* expression in confluent VEC-positive ECs upon Suz12 overexpression.
(C) qRT-PCR analysis of *Pecam1* and *Sox18* expression in confluent VEC-positive ECs upon Suz12 overexpression.
In the figure, the levels of mRNA are normalized to *GAPDH*; columns are means ± s.e.m. of triplicates from a representative experiment. *$P < 0.05$; **$P < 0.01$, *t*-test.

Online Figure VII. FoxO1 mRNA levels are unaltered upon stable PcG protein knockdown.
qRT-PCR analysis of *FoxO1* expression in confluent VEC-null sh-Empty and VEC-null sh-Suz12 ECs. The levels of mRNA are normalized to 18S; columns are means ± s.e.m. of triplicates from a representative experiment.

Online Figure VIII. FoxO1 and Suz12 cooperate for repressing *claudin-5* expression.
(A) WB analysis of FKHR-TM, Suz12, H3K27me3 and claudin-5 in extracts of confluent VEC-null, VEC-positive-Empty and VEC-positive-Suz12 ECs expressing FKHR-TM or GFP (negative control). Tubulin is the loading control.
(B) qRT-PCR analysis of *claudin-5* expression in VEC-null, VEC-positive-Empty and VEC-positive-Suz12 ECs expressing FKHR-TM or GFP (negative control). Levels of mRNA are normalized to GAPDH. Columns are means ± s.e.m. of triplicates from a representative experiment. *$P<0.01$, t-test.

Online Figure IX. Ezh2 peptide arrays.
Peptide arrays with overlapping 20-mers spanning the whole sequence of murine Ezh2 spotted on membrane, probed with indicated GST-tagged proteins in (B), (C) and (D) or free GST as control (A), and analysed by WB. Red dots identify peptides selected for streptavidin pull-down experiments shown in Figure 6. Green boxes define the areas shown in Figure 6B, 6F and 6L.

Online Figure X. Ezh2 interacts with p120-catenin.
Co-immunoprecipitation and WB of endogenous Ezh2 and p120-catenin from extracts of confluent VEC-null and VEC-positive ECs.

Online Figure XI. VE-PTP downregulation in vessels of serous surface papillary ovarian carcinoma.
IHC staining of VE-PTP expression in sections of human healthy ovary (left panel) or serous surface papillary ovarian carcinoma (right panel). Black arrowheads point to tumor vessel ECs expressing high levels of EZH2. Scale bar: 40 μm.

ONLINE TABLES

Online Table I. Significantly upregulated endothelial genes in VEC-positive vs VEC-null ECs.
Complete list of endothelial genes significantly upregulated in VEC-positive cells according to the threshold
|log2FC| ≥ 1 and adjusted p-value (padj) ≤ 0.05. List refers to Figure 1B.

Ensembl_gene_id	External_gene_name	log2FoldChange	padj
ENSMUSG00000021458	2010111I01Rik	1.030144555	0.000155219
ENSMUSG00000020681	Ace	1.080455751	2.39E-08
ENSMUSG00000054808	Actn4	1.571394864	5.72E-85
ENSMUSG00000000530	Acvrl1	3.011281082	3.07E-116
ENSMUSG00000022893	Adamts1	1.207992156	3.01E-34
ENSMUSG00000069833	Ahnak	1.002778723	4.12E-19
ENSMUSG00000019256	Ahr	2.306899238	8.88E-74
ENSMUSG00000066406	Akap13	1.147082083	3.31E-30
ENSMUSG00000021895	Arhgef3	1.557740572	2.19E-62
ENSMUSG00000055116	Arntl	1.63486021	1.38E-11
ENSMUSG00000051669	AU021092	2.631170051	4.87E-77
ENSMUSG00000037458	Azin1	1.694521168	9.20E-37
ENSMUSG00000049792	Bag5	1.037512828	5.20E-14
ENSMUSG00000059588	Calcrl	2.353845544	1.21E-91
ENSMUSG00000007655	Cav1	1.227512235	2.91E-32
ENSMUSG00000000058	Cav2	1.463840099	1.96E-19
ENSMUSG00000022661	Cd200	1.565319838	2.62E-12
ENSMUSG00000033502	Cdc14a	1.780455692	9.75E-33
ENSMUSG00000036533	Cdc42ep3	2.127347302	4.40E-35
ENSMUSG00000023067	Cdkn1a	1.582084046	2.75E-12
ENSMUSG00000071637	Cebpd	1.007602909	0.011257482
ENSMUSG00000041378	Cldn5	5.617650065	5.83E-76
ENSMUSG00000023959	Clic5	1.947174651	8.18E-105
ENSMUSG00000003617	Cp	3.425553469	3.73E-39
ENSMUSG00000006360	Crip1	4.100612414	8.93E-20
ENSMUSG00000061353	Cxcl12	1.61906941	4.58E-11
ENSMUSG00000028195	Cyr61	1.614984821	1.58E-10
ENSMUSG00000022150	Dab2	1.108024746	5.42E-21
ENSMUSG00000002257	Def6	2.77107749	1.67E-11
ENSMUSG00000040631	Dok4	1.004119184	1.66E-14
ENSMUSG00000003518	Dusp3	1.335031683	3.23E-57
ENSMUSG00000028108	Ecm1	1.087583892	2.06E-12
ENSMUSG00000008398	Elk3	1.080804982	2.57E-37
ENSMUSG00000022505	Emp2	1.577772246	8.46E-12
ENSMUSG00000024140	Epas1	1.005688736	1.04E-33
ENSMUSG00000038776	Ephx1	1.351199263	1.93E-14
ENSMUSG00000024778	Fas	4.169362351	4.39E-36
ENSMUSG00000003420	Fcgrt	1.978980212	6.33E-51
ENSMUSG00000040170	Fmo2	2.04526583	0.000500035

ENSMUSG00000009687	Fxyd5	1.339637934	8.36E-24
ENSMUSG00000015312	Gadd45b	1.483447133	0.000612635
ENSMUSG00000034201	Gas2l1	1.006587513	1.30E-09
ENSMUSG00000021360	Gcnt2	1.904024924	2.17E-15
ENSMUSG00000055737	Ghr	1.411347191	3.38E-16
ENSMUSG00000054435	Gimap4	2.783636744	4.28E-105
ENSMUSG00000050105	Grrp1	1.129108898	0.025674785
ENSMUSG00000040562	Gstm2	2.083053527	1.45E-12
ENSMUSG00000067212	H2-T23	1.48958506	3.67E-20
ENSMUSG00000071379	Hpcal1	1.160288402	4.98E-09
ENSMUSG00000003541	Ier3	2.349737755	2.52E-10
ENSMUSG00000074896	Ifit3	1.814510316	3.07E-15
ENSMUSG00000005533	Igf1r	1.840922626	0.000836188
ENSMUSG00000022969	Il10rb	1.029032008	4.36E-10
ENSMUSG00000017057	Il13ra1	1.234914814	0.036664413
ENSMUSG00000018899	Irf1	1.203207384	6.74E-14
ENSMUSG00000027276	Jag1	1.101653799	6.97E-17
ENSMUSG00000021294	Kif26a	1.432481827	1.15E-15
ENSMUSG00000031788	Kifc3	1.976925202	9.04E-28
ENSMUSG00000055148	Klf2	1.488630726	1.41E-18
ENSMUSG00000003032	Klf4	2.801834854	6.83E-94
ENSMUSG00000033863	Klf9	2.562506604	2.76E-36
ENSMUSG00000021959	Lats2	1.193141326	5.64E-25
ENSMUSG00000054263	Lifr	1.084566843	2.40E-06
ENSMUSG00000032698	Lmo2	1.511260593	5.22E-37
ENSMUSG00000016520	Lnx2	1.290227959	1.83E-20
ENSMUSG00000068015	Lrch1	1.419034427	7.97E-37
ENSMUSG00000040488	Ltbp4	1.12334367	8.90E-07
ENSMUSG00000001089	Luzp1	1.028630163	5.55E-19
ENSMUSG00000075602	Ly6a	1.812932107	1.17E-16
ENSMUSG00000022587	Ly6e	1.028516047	2.27E-11
ENSMUSG00000042622	Maff	1.194577554	0.017765841
ENSMUSG00000028862	Map3k6	1.348510986	0.038153326
ENSMUSG00000001493	Meox1	4.746823504	1.38E-22
ENSMUSG00000022353	Mtss1	1.425709869	1.67E-56
ENSMUSG00000037235	Mxd4	1.105213479	3.24E-35
ENSMUSG00000021365	Nedd9	1.012790987	7.52E-23
ENSMUSG00000003847	Nfat5	1.085110516	2.67E-30
ENSMUSG00000021025	Nfkbia	1.26736757	5.21E-09
ENSMUSG00000021806	Nid2	2.24487386	1.32E-126
ENSMUSG00000020889	Nr1d1	2.192153131	2.56E-11
ENSMUSG00000020019	Ntn4	1.060251028	2.58E-13
ENSMUSG00000022146	Osmr	2.214586778	5.94E-05
ENSMUSG00000024725	Ostf1	1.483312676	2.43E-32
ENSMUSG00000024805	Pcgf5	1.427740658	0.000138545
ENSMUSG00000051177	Plcb1	2.625979803	3.78E-89

ENSMUSG00000032377	Plscr4	2.938641363	1.61E-09
ENSMUSG00000017754	Pltp	3.603782243	4.70E-44
ENSMUSG00000047714	Ppp1r2	1.133018396	2.00E-41
ENSMUSG00000053198	Prx	1.312060881	4.93E-07
ENSMUSG00000027864	Ptgfrn	1.760648665	0.001114651
ENSMUSG00000059895	Ptp4a3	1.076091502	4.20E-14
ENSMUSG00000020154	Ptprb	1.870491603	2.59E-102
ENSMUSG00000020151	Ptprr	3.906271548	1.03E-14
ENSMUSG00000009291	Pttg1ip	1.494446887	6.28E-54
ENSMUSG00000062232	Rapgef2	1.384227356	1.15E-36
ENSMUSG00000054364	Rhob	1.181079153	3.54E-19
ENSMUSG00000033107	Rnf125	1.575761287	1.32E-12
ENSMUSG00000021067	Sav1	1.563868928	3.56E-41
ENSMUSG00000028780	Sema3c	1.166899613	0.006795405
ENSMUSG00000071178	Serpina1b	4.035515589	4.23E-19
ENSMUSG00000071177	Serpina1d	3.738797408	3.27E-13
ENSMUSG00000072849	Serpina1e	3.335872183	3.95E-10
ENSMUSG00000017756	Slc12a7	2.252879677	5.02E-69
ENSMUSG00000037434	Slc30a1	1.178611424	2.16E-21
ENSMUSG00000025993	Slc40a1	1.018321315	5.11E-10
ENSMUSG00000030096	Slc6a6	1.770344183	2.31E-17
ENSMUSG00000002504	Slc9a3r2	1.286876353	7.09E-34
ENSMUSG00000032548	Slco2a1	2.471737479	6.58E-10
ENSMUSG00000025006	Sorbs1	1.153251032	5.20E-14
ENSMUSG00000031626	Sorbs2	1.075504026	3.75E-14
ENSMUSG00000002147	Stat6	1.511251856	4.96E-28
ENSMUSG00000014813	Stc1	1.078916739	8.98E-40
ENSMUSG00000039156	Stim2	1.240683389	1.07E-45
ENSMUSG00000030711	Sult1a1	1.055824496	0.014286389
ENSMUSG00000096054	Syne1	1.043358879	1.65E-14
ENSMUSG00000038213	Tapbpl	1.210269439	3.14E-06
ENSMUSG00000006642	Tcf23	2.402352937	1.27E-08
ENSMUSG00000020034	Tcp11l2	1.137117364	1.82E-08
ENSMUSG00000002489	Tiam1	2.185948789	4.27E-102
ENSMUSG00000020044	Timp3	1.710305929	9.82E-89
ENSMUSG00000034640	Tiparp	3.307295131	3.95E-127
ENSMUSG00000020023	Tmcc3	2.227468759	9.42E-09
ENSMUSG00000037573	Tob1	1.334672963	6.87E-13
ENSMUSG00000020601	Trib2	1.368183264	4.98E-15
ENSMUSG00000047821	Trim16	1.05040397	5.32E-08
ENSMUSG00000020773	Trim47	1.808934868	3.01E-18
ENSMUSG00000042116	Vwa1	4.388395603	7.03E-88
ENSMUSG00000001930	Vwf	2.10472827	3.69E-88
ENSMUSG00000044786	Zfp36	1.277531768	0.007208359
ENSMUSG00000041703	Zic5	1.557135924	0.011888894

Online Table II. Significantly downregulated endothelial genes in VEC-positive vs VEC-null ECs.
Complete list of endothelial genes significantly downregulated in VEC-positive cells according to the threshold |log2FC| ≥ 1 and adjusted p-value (padj) ≤ 0.05. List refers to Figure 1B.

Ensembl_gene_id	External_gene_name	log2FoldChange	padj
ENSMUSG00000025085	Ablim1	-1.945664238	5.37E-24
ENSMUSG00000026003	Acadl	-1.086609504	2.58E-10
ENSMUSG00000030790	Adm	-1.608017282	0.010413999
ENSMUSG00000038587	Akap12	-1.726967454	2.61E-34
ENSMUSG00000041688	Amot	-1.758695706	2.43E-32
ENSMUSG00000071847	Apcdd1	-1.149995659	0.001802338
ENSMUSG00000027792	Bche	-1.514352417	0.013541964
ENSMUSG00000026278	Bok	-1.289180293	0.000255985
ENSMUSG00000026029	Casp8	-1.067999624	2.80E-19
ENSMUSG00000041598	Cdc42ep4	-1.048941056	0.008913035
ENSMUSG00000037664	Cdkn1c	-1.557952614	4.96E-06
ENSMUSG00000052560	Cpne8	-2.080693021	6.82E-28
ENSMUSG00000044258	Ctla2a	-1.245822534	7.41E-19
ENSMUSG00000074874	Ctla2b	-1.670778565	6.31E-25
ENSMUSG00000019891	Dcbld1	-1.138077823	0.004201829
ENSMUSG00000057098	Ebf1	-2.111413582	9.99E-12
ENSMUSG00000027954	Efna1	-1.200342638	1.03E-18
ENSMUSG00000041773	Enc1	-1.79080039	1.66E-23
ENSMUSG00000043556	Fbxl7	-3.775920835	5.86E-14
ENSMUSG00000038372	Gmds	-1.004691345	0.004715295
ENSMUSG00000056870	Gulp1	-1.419817989	0.020827657
ENSMUSG00000040289	Hey1	-2.020566228	6.67E-08
ENSMUSG00000007872	Id3	-1.394713645	2.94E-07
ENSMUSG00000026896	Ifih1	-1.325927571	0.010695402
ENSMUSG00000078853	Igtp	-1.198266255	0.020467277
ENSMUSG00000031304	Il2rg	-3.162676661	2.01E-14
ENSMUSG00000031734	Irx3	-2.38750018	6.26E-20
ENSMUSG00000031239	Itm2a	-1.759963614	4.14E-06
ENSMUSG00000098557	Kctd12	-1.851946008	1.84E-06
ENSMUSG00000057722	Lepr	-1.490989244	0.005647257
ENSMUSG00000018169	Mfng	-2.644030792	9.44E-15
ENSMUSG00000023094	Msrb2	-1.333390874	0.000634309
ENSMUSG00000020900	Myh10	-1.492692468	1.59E-35
ENSMUSG00000018417	Myo1b	-3.305487303	1.90E-133
ENSMUSG00000046949	Nqo2	-1.085224089	1.64E-09
ENSMUSG00000033377	Palmd	-1.624208436	0.004043486
ENSMUSG00000031379	Pir	-4.724267197	1.95E-22
ENSMUSG00000042842	Serpinb6b	-1.942757029	1.63E-06
ENSMUSG00000045629	Sh3tc2	-6.052872043	1.87E-43
ENSMUSG00000040710	St8sia4	-2.233886401	7.77E-46
ENSMUSG00000037820	Tgm2	-5.967240498	4.34E-191

ENSMUSG00000060548	Tnfrsf19	-1.869105773	2.51E-11
ENSMUSG00000020577	Tspan13	-5.490186323	1.60E-39
ENSMUSG00000001473	Tubb6	-1.290729983	4.37E-30

Online Table III. Grouping of functional annotation categories.
Explanation of how different annotation terms with overlapping gene sets were grouped in Figure 1C graphical representation.

Direction	Group	Group in Figure 1C
down	UP_KEYWORDS: Cell cycle	cell cycle
down	GOTERM_BP_DIRECT: GO:0007049~cell cycle	cell cycle
down	UP_KEYWORDS: Cytoskeleton	cytoskeleton
down	UP_KEYWORDS: Phosphoprotein	phosphoprotein / signal
down	GOTERM_BP_DIRECT: GO:0007052~mitotic spindle organization	mitotic spindle organization
down	UP_KEYWORDS: Cell projection	cell projection
down	UP_KEYWORDS: Cyclin	cell proliferation / cyclin
down	GOTERM_BP_DIRECT: GO:0008283~cell proliferation	cell proliferation / cyclin
down	GOTERM_BP_DIRECT: GO:0002040~sprouting angiogenesis	sprouting angiogenesis
up	UP_KEYWORDS: Glycoprotein	glycoprotein
up	UP_KEYWORDS: Disulfide bond	disulfide bond
up	UP_KEYWORDS: Signal	phosphoprotein / signal
up	KEGG_PATHWAY: mmu04510:Focal adhesion	ECM-receptor interaction / focal adhesion
up	KEGG_PATHWAY: mmu04151:PI3K-Akt signaling pathway	PI3K-AKT signaling pathway
up	KEGG_PATHWAY: mmu04512:ECM-receptor interaction	ECM-receptor interaction / focal adhesion
up	UP_KEYWORDS: Developmental protein	developmental protein / differentiation
up	UP_KEYWORDS: Differentiation	developmental protein / differentiation
up	UP_KEYWORDS: Membrane	membrane / transmembrane
up	UP_KEYWORDS: Transmembrane	membrane / transmembrane
up	UP_KEYWORDS: Protein phosphatase	protein phosphatase / transferase
up	UP_KEYWORDS: Kinase	kinase
up	UP_KEYWORDS: Transferase	protein phosphatase / transferase
up	UP_KEYWORDS: Serine/threonine-protein kinase	kinase
up	BIOCARTA: m_raccycdPathway:Influence of Ras and Rho proteins on G1 to S Transition	influence of Ras and Rho proteins on G1 to S transition
up	UP_KEYWORDS: Cell cycle	cell cycle

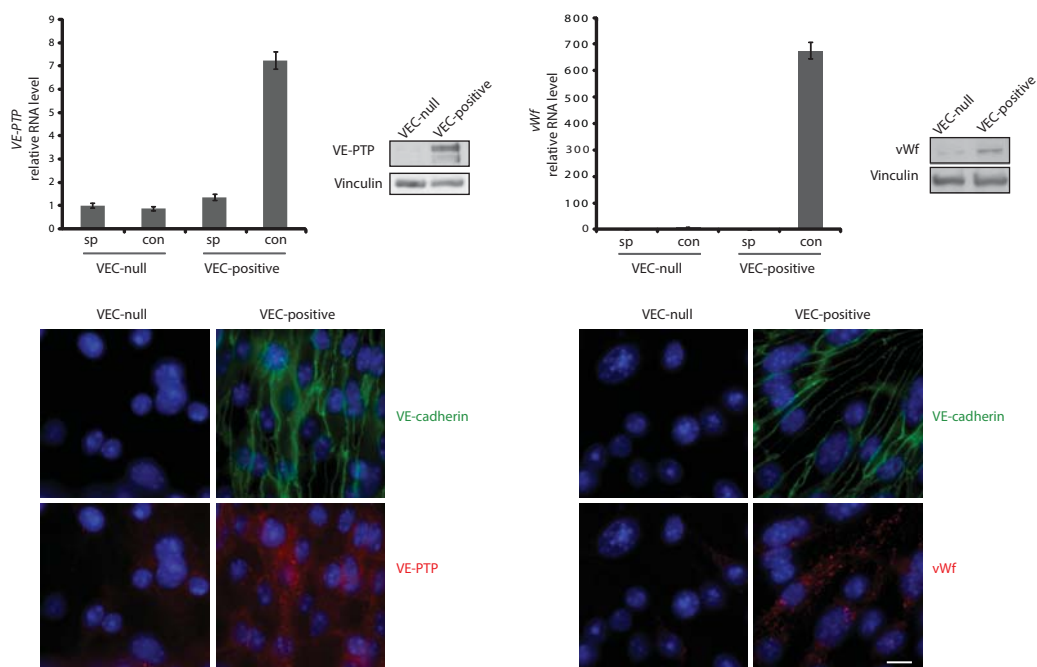**Online Table IV. Complete list of peptides used in this study.**

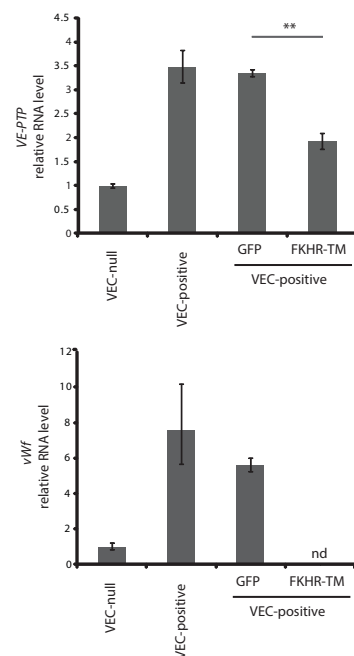|	
Biotinylated peptides used in pull-down assays	
A2	Biotin-X-G-Q-T-G-K-K-S-E-K-G-P-V-C-W-R-K-R-V-K-S-CONH2
A18	Biotin-X-R-V-K-S-E-Y-M-R-L-R-Q-L-K-R-F-R-R-A-D-E-CONH2
B33	Biotin-X-I-M-T-S-V-S-S-L-R-G-T-R-E-C-S-V-T-S-D-L-CONH2
C2	Biotin-X-S-L-R-G-T-R-E-C-S-V-T-S-D-L-D-F-P-A-Q-V-CONH2
E12	Biotin-X-D-R-E-C-G-F-I-N-D-E-I-F-V-E-L-V-N-A-L-G-CONH2
F10	Biotin-X-E-R-E-E-K-Q-K-D-L-E-D-N-R-D-D-K-E-T-C-P-CONH2
H34	Biotin-X-D-C-F-L-H-P-F-H-A-T-P-N-T-Y-K-R-K-N-T-E-CONH2
I9	Biotin-X-T-Y-K-R-K-N-T-E-T-A-L-D-N-K-P-C-G-P-Q-C-CONH2
I27	Biotin-X-Q-C-Y-Q-H-L-E-G-A-K-E-F-A-A-A-L-T-A-E-R-CONH2
K31	Biotin-X-K-D-E-T-S-S-S-S-E-A-N-S-R-C-Q-T-P-I-K-M-CONH2
K35	Biotin-X-S-S-S-S-E-A-N-S-R-C-Q-T-P-I-K-M-K-P-N-I-CONH2
L4	Biotin-X-N-S-R-C-Q-T-P-I-K-M-K-P-N-I-E-P-P-E-N-V-CONH2
L5	Biotin-X-S-R-C-Q-T-P-I-K-M-K-P-N-I-E-P-P-E-N-V-E-CONH2
L7	Biotin-X-C-Q-T-P-I-K-M-K-P-N-I-E-P-P-E-N-V-E-W-S-CONH2
M6	Biotin-X-N-F-C-A-I-A-R-L-I-G-T-K-T-C-R-Q-V-Y-E-F-CONH2
M10	Biotin-X-I-A-R-L-I-G-T-K-T-C-R-Q-V-Y-E-F-R-V-K-E-CONH2
M17	Biotin-X-K-T-C-R-Q-V-Y-E-F-R-V-K-E-S-S-I-I-A-P-V-CONH2
M26	Biotin-X-R-V-K-E-S-S-I-I-A-P-V-P-T-E-D-V-D-T-P-P-CONH2
N3	Biotin-X-D-V-D-T-P-P-R-K-K-K-R-K-H-R-L-W-A-A-H-C-CONH2
N22	Biotin-X-C-R-K-I-Q-L-K-K-D-G-S-S-N-H-V-Y-N-Y-Q-P-CONH2
N35	Biotin-X-H-V-Y-N-Y-Q-P-C-D-H-P-R-Q-P-C-D-S-S-C-P-CONH2
O4	Biotin-X-P-C-D-H-P-R-Q-P-C-D-S-S-C-P-C-V-I-A-Q-N-CONH2
O25	Biotin-X-C-E-K-F-C-Q-C-S-S-E-C-Q-N-R-F-P-G-C-R-C-CONH2
O28	Biotin-X-F-C-Q-C-S-S-E-C-Q-N-R-F-P-G-C-R-C-K-A-Q-CONH2
O35	Biotin-X-C-Q-N-R-F-P-G-C-R-C-K-A-Q-C-N-T-K-Q-C-P-CONH2
P2	Biotin-X-F-P-G-C-R-C-K-A-Q-C-N-T-K-Q-C-P-C-Y-L-A-CONH2
P30	Biotin-X-C-L-T-C-G-A-A-D-H-W-D-S-K-N-V-S-C-K-N-C-CONH2
R8	Biotin-X-F-I-S-E-Y-C-G-E-I-I-S-Q-D-E-A-D-R-R-G-K-CONH2
R23	Biotin-X-D-R-R-G-K-V-Y-D-K-Y-M-C-S-F-L-F-N-L-N-N-CONH2
S4	Biotin-X-N-N-D-F-V-V-D-A-T-R-K-G-N-K-I-R-F-A-N-H-CONH2
S10	Biotin-X-D-A-T-R-K-G-N-K-I-R-F-A-N-H-S-V-N-P-N-C-CONH2
S22	Biotin-X-N-H-S-V-N-P-N-C-Y-A-K-V-M-M-V-N-G-D-H-R-CONH2
T11	Biotin-X-R-A-I-Q-T-G-E-E-L-F-F-D-Y-R-Y-S-Q-A-D-A-CONH2
ctr-D28	Biotin-X-L-D-Q-D-G-T-F-I-E-E-L-I-K-N-Y-D-G-K-V-H-CONH2
ctr-G5	Biotin-X-I-S-S-M-F-P-D-K-G-T-A-E-E-L-K-E-K-Y-K-E-CONH2
ctr-K11	Biotin-X-D-R-E-A-G-T-E-T-G-G-E-N-N-D-K-E-E-E-E-K-CONH2
TAT-peptides used in VEC-positive EC treatment	
TAT-M6	Biotin-X-N-F-C-A-I-A-R-L-I-G-T-K-T-C-R-Q-V-Y-E-F -YGRKKRRQRRR-CONH2
TAT-M10	Biotin-X-I-A-R-L-I-G-T-K-T-C-R-Q-V-Y-E-F-R-V-K-E-YGRKKRRQRRR-CONH2
TAT-O4	Biotin-X-P-C-D-H-P-R-Q-P-C-D-S-S-C-P-C-V-I-A-Q-N-YGRKKRRQRRR-CONH2
TAT-P30	Biotin-X-C-L-T-C-G-A-A-D-H-W-D-S-K-N-V-S-C-K-N-C-YGRKKRRQRRR-CONH2
TAT-ctr-K11	Biotin-X-D-R-E-A-G-T-E-T-G-G-E-N-N-D-K-E-E-E-E-K-YGRKKRRQRRR-CONH2

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Online Figure I
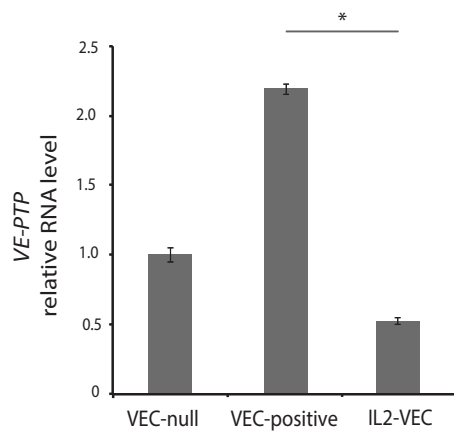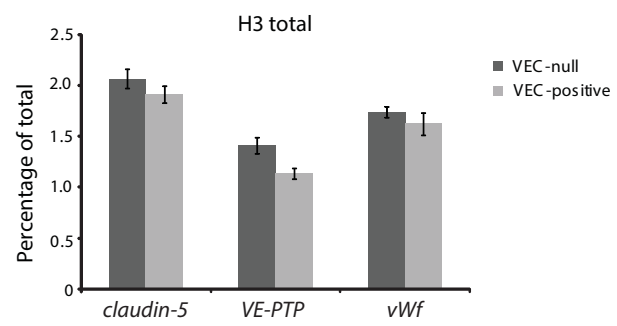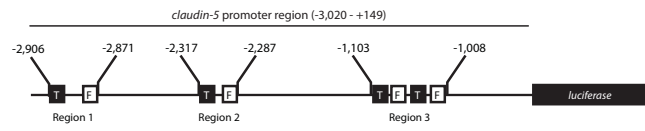
Online Figure II

Online Figure III

H3 total

VEC-null
VEC-positive

Online Figure IV

A

claudin-5 promoter region (-3,020 - +149)

-2,906   -2,871   -2,317   -2,287   -1,103   -1,008

T F   T F   T F T F   luciferase

Region 1   Region 2   Region 3

B

**

Relative luciferase activity

700
600
500
400
300
200
100
0

VEC-null   VEC-positive

C

*

Relative luciferase activity

120
100
80
60
40
20
0

Empty   Suz12

VEC-positive

D

**

Relative luciferase activity

110
108
106
104
102
100
98
96
94
92
90

sh-Empty   sh-Suz12

VEC-null

Online Figure V

A

Lmo2 relative RNA level

Stat6 relative RNA level
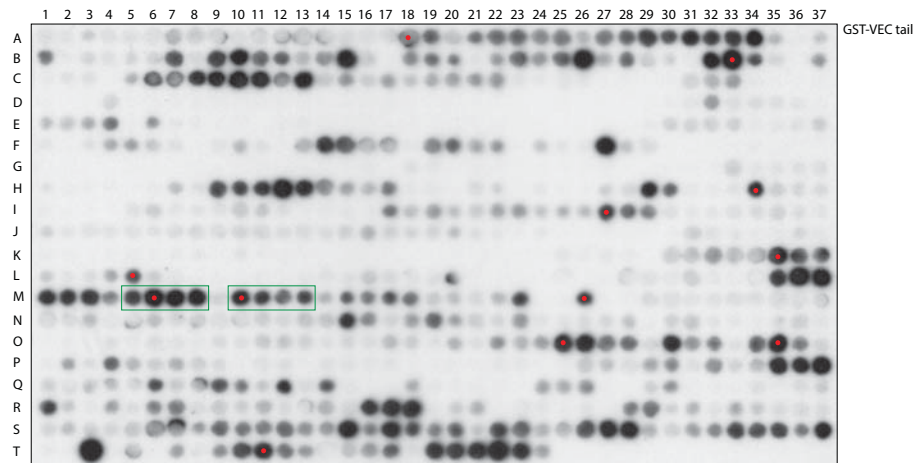
B

Hey1 relative RNA level

C

Pecam1 relative RNA level

Sox18 relative RNA level

Online Figure VI

Online Figure VII

A

VEC-positive

	VEC-null	Empty-GFP	Empty-FKHR-TM	Suz12-GFP	Suz12-FKHR-TM

FKHR-TM

Suz12

H3K27me3

Claudin-5

Tubulin

B

	VEC-null	VEC-positive		
GFP		+	+	
FKHR-TM			+	+
Empty		+	+	
Suz12				+	+

Online Figure VIII

Online Figure IX

Online Figure X

Healthy ovary　　　　　　　Ovarian cancer

EZH2　VE-PTP

Online Figure XI