JAM-C induces endothelial cell permeability through its association and regulation of {beta}3 integrins

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Supplement Material

Additional Details for Figures

Figure 1. Regulation of JAM-C levels regulates EC permeability

a**.** Permeability of HUVECs infected with EV or JAM-C (J-C). This is a representative of 4 experiments *$p < 0.01$ compared to EV.

b. Confluent HUVECs were immunostained with an anti-JAM-C antibody. Without treatment (A)**,** treated with 0.1 U/ml thrombin for 30 seconds (B), pre-treated with angiopoietin-1 at 0.1 μg/ml and then treated with thrombin for 30 seconds (C).

c. Permeability of control (A, B) or JAM-C (C, D) siRNA transfected cells untreated (A,C) or treated with 1U/ml thrombin (B,D). This is a pool of 2 experiments. D compared to B *$p<0.05$

Figure 2 JAM-C regulates integrin function

a**.** EV (A,C) or JAM-C (B,D) adenovirus infected cells were immunostained for β_3 integrin (top) and β_1 integrins (bottom).

b**.** HUVECs were untreated (A) or treated with thrombin (0.1U/ml) for 30 seconds (B) and then immunostained for β_3 integrin.

c. The percentage of attached EV (□) or JAM-C (■)infected cells on fibronectin(Fn), vitronectin(Vn), laminin(Ln) or Matrigel after 60 minutes. JAM-C compared to EV *$p < 0.05$.

d. The percentage of attached control (□) or JAM-C siRNA (■) transfected cells on fibronectin or vitronectin after 60 minutes. JAM-C siRNA compared to control * $p < 0.05$

Figure 5. JAM-C regulates Rap1 activity

a. Lysates from EV or JAM-C infected cells or b. control or JAM-C siRNA transfected cells were assessed for active Rap1 (top) and total Rap1(bottom panel).

c-d. HUVECs were transfected with a control siRNA(Csi) or siRNA targeted to human Rap1b(Rsi). c. shows percentage of cells attached to fibronectin and d. permeability

*$p < 0.01$ compared to control cells.

e. Permeability of HUVECs transfected with control siRNA(Csi), siRNA to JAM-C(J-Csi), Rap1b(Rsi) or JAM-C and Rap1b together (J+R). This is pooled data of 2 experiments * $p < 0.05$ compared to control. NS=not significant

Supplementary Figure Legends

FigureI.

a. HUVECs were transfected with a non-silencing control siRNA or siRNA targeted to human JAM-C. After 48 hours, cells were lysed and the lysates subjected to SDS-PAGE and immunoblotted for JAM-C.

b. HUVECs were transfected with control siRNA(siC) or siRNA targeted to JAM-C(siJ-C) cultured inTranswells and then assessed for permeability given as the FITC-dextran passaged (ng/ml) after 30 minutes. This is a representative of 3 experiments * $p < 0.01$ compared to control siRNA.

c. HUVECs were infected with JAM-C adenovirus then plated into LabTek slides for a further 24 hours. Cells were fixed and stained with anti-JAM-C antibody.

d**.** Cytosolic (C) and membrane (M) fractions of extracts from HUVECs (above) were probed for JAM-C.

e. HUVECs that had been grown to confluence were either unstimulated (A), stimulated for 30 seconds (B) or 2 minutes (C) with 0.1U/ml thrombin and stained for JAM C localization.

Figure II

a**.** HUVECs were infected with either empty vector (EV) or JAM-C (JAM-C) adenovirus for 24 hours and cultured in LabTek slides for a further 24 hours. Cells were then fixed and immunostained for VE cadherin (top panel) or PECAM (bottom panel).

b. HUVECs were infected with either empty vector (EV) or JAM-C (JAM-C) adenovirus for 48 hours. The cell lysates were subjected to SDS-PAGE and immunoblotting with anti VE cadherin or anti PECAM (upper panel) or anti phosphotyrosine PY 100 (lower panel) antibody.

Figure III.

HUVECs were transfected with a non-silencing control siRNA or siRNA targeted to human β_3 integrin.

a. After 48 hours, cells were lysed and the lysates subjected to SDS-PAGE and immunoblotted for β_3 integrin (upper panel) and actin (lower panel).

b. After 24 hours the cells were plated onto Transwells and 24 hours later the permeability was measured. The FITC-dextran passaged (ng/ml) after 30 minutes is shown. This is pooled data of 2 experiments * $p < 0.01$ compared to control siRNA.

c. EV (□) or JAM-C (■) infected cells were plated onto the wells coated with fibronectin or with mouse anti-human integrin $\alpha v\beta_3$ or mouse anti-human integrin β_1 antibody. The percentage of cells attached relative to the EV-infected cells plated on fibronectin is shown. *$p < 0.001$ compared to EV.

d. The cells were also stained with HUTS21 antibody and assessed for level of fluorescence(EV, grey line or JAM-C, black line). This is a representative of 2 experiments performed.

Figure IV.

a. normal HUVECs immunostained for JAM-C (green) or β_1 (red), with areas of colocalization seen as yellow.

b. HUVECs were transfected with a non-silencing control siRNA or siRNA targeted to human Rap1b. After 48 hours, cells were lysed and the lysates subjected to SDS-PAGE and immunoblotted for Rap1 (upper panel) and actin (lower panel).

c. HUVECs, transfected with a non-silencing control siRNA (siC), siRNA targeted to JAM-C (siJ-C), siRNA targeted to Rap1b (siR) or siRNA to both Rap1b and JAM-C (siJ-C+R) were plated onto fibronectin-coated plates and allowed to attach for 60 minutes. The percentage of

cells attached relative to the control siRNA transfected cells is shown. *p < 0.01 compared to control cells. NS= not significantly different from control

Figure V.

a HUVECs were infected with empty vector (□) or JAM-C (■) adenovirus for 48 hours.Cells were plated onto the wells coated with mouse anti-human VE cadherin or mouse anti-human PECAM antibody. 30 minutes later wells were washed. MTS assay was used to assess number of attached cells. The percentage of cells attached relative to the EV-infected is shown. *p < 0.05 compared to EV.

b. HUVECs were transfected with a non-silencing control siRNA (□) or siRNA targeted to human JAM-C (■). Cells were plated onto the wells coated with mouse anti-human VE cadherin or mouse anti-human PECAM antibody. 30 minutes later, wells were washed. MTS assay was used to assess number of attached cells. The percentage of cells attached relative to the control is shown. *p < 0.01 compared to control.

Figure VI

a. HUVECs were transfected with control siRNA or siRNA directed to AF6. After 48 hours , cells were lysed and the lysates subjected to SDS PAGE and immunoblotted for AF6.

b. Cells were grown to confluence in LabTek slides then fixed and immunostained with anti-JAM-C (A) or anti-AF-6 (B) antibodies. Colocalization (C) is shown in yellow and arrowed.

c. Thrombin regulates AF6 localisation. HUVECs were gown to confluence and then either unstimulated (A,B,C) or stimulated (D,E,F) with thrombin for 2 minutes. Cells were fixed and stained for JAM C (green) or AF6 (red). Areas of colocalisation are seen in insert

Materials and Methods

Reagents

Angiopoietin-1 was obtained from R & D Systems (Minneapolis, MN), human thrombin and FITC-conjugated dextran (MW 40,000) were from Sigma-Aldrich (St. Louis, MO). Fibronectin was from Boehringer Mannheim Biochemica (Roche), vitronectin from Promega (Madison, WI), laminin from Roche (Penzberg, Germany) and Matrigel from Becton Dickinson (Bedford, MA).

JAM-C and recombinant adenoviral constructs.

Recombinant adenoviruses were made by subcloning human JAM-C fragments from pcDNA3–JAM-C constructs into the pAdEasy-1 vector (Qbiogene Inc, Carlsbad, CA). Virus was amplified in HEK293 cells and purified by CsCl gradient ultracentrifugation. Virus titers were determined using the $TCID_{50}$ method, as recommended by Qbiogene.

Small interfering RNA (siRNA) transfection

siRNA targeted to human JAM-C (AUGUAGUUAACUCCAUCUGGUUUCC) and (GGAAACCAGAUGGAGUUAACUACAU), human Rap1b (GCGUGAGUAUAAGCUAGUCGUUCUU) and (AAGAACGACUAGCUUAUACUCACGC), human β_3 intergrin (CCACUGUAUAAAGAGGCCACGUCUA) and (UAGACGUGGCCUCUUUAUACAGUGG), siRNA targeted to AF6 (AUCCAUGGUAAGCAAAGUCGUUGCC) and (GGCAACGACUUUGCUUACCAUGGAU) and control non-silencing siRNA were synthesized by Invitrogen (Carlsbad, CA).

Immunofluorescence

Detection of JAM-C, β_1 integrin, β_3 integrin, VE cadherin, PECAM and AF-6 was by the use of appropriate primary antibodies and Alexa Fluor 594 goat anti-mouse IgG, or Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR).Cells were imaged by epifluorescence microscopy on an Olympus BX-51 microscope (Olympus, Hamburg, Germany) equipped

with excitation filters for fluorescein and acquired to a Photometrics Cool Snap FX charge-coupled device camera (Roper Scientific GmbH, Germany). Images were adjusted for brightness and contrast using V^{++} software (Digital Optics Ltd., Auckland, New Zealand). Orthogonal images were aquired using a Leica SP5 confocal system (Leica Microsystems Gmbh, Wetzler, Germany) with Volocity v5 software from Improvision (Coventry, England).

Immunoblotting and immunoprecipitation

Cells were lysed in ice-cold lysis buffer and Equal amounts of protein were separated by SDS–PAGE, transferred to PVDF membrane, blocked with 5% skim milk powder and 0.1% Triton X100 in phosphate buffered saline (PBS), and probed with polyclonal rabbit anti JAM-C antibody, monoclonal anti β_3 integrin antibody (Cell Signaling, Danvers, MA), monoclonal anti AF6 antibody (BD Transduction, Franklin Lakes, NJ), polyclonal anti Rab1 (Upstate, Lake Placid, NY) or monoclonal anti VE cadherin and anti PECAM antibody. After washing, membranes were incubated with HRP-conjugated secondary antibody and reactive bands were detected by chemiluminescence. Membranes were stripped using stripping buffer (Re-Blot Plus Western Blot Recycling Kit, Chemicon, Temecula CA) and reprobed with anti actin (Chemicon) or anti PY100 (Cell Signaling) antibodies.

For immunoprecipitation, 1 mg of protein was used for each precipitation. Lysates were pre-incubated with protein G-Sepharose beads (Zymed, San Francisco, CA) for 30 minutes at 4 °C. After brief centifugation, the lysates were treated with anti JAM-C, anti β_3 integrin or anti AF6 antibody and protein G-Sepharose beads for 2 hours at 4 °C. Immunocomplex-captured beads were washed with lysis buffer and then boiled in 25 μl of Laemmli sample buffer. Proteins were separated by SDS–PAGE and Western blotting performed.

Cell fractionation

HUVECs were infected with adenovirus and grown for 48 hours. Cells were washed with PBS and harvested by scraping into 1 ml of homogenization buffer. Cells were mechanically homogenized and centrifuged for 15 min at 1200 × *g* at 4 °C. The supernatant was further centrifuged for 1 hour at 125 000 × *g* at 4 °C. The resultant supernatant, containing the cytosolic components, was removed and the pellet, containing the membrane components, was resuspended in 0.5 ml of homogenization buffer supplemented with 0.5% Triton X100 and 100 mM NaCl. Proteins in 20 μl of each fraction were separated by SDS–PAGE, transferred to PVDF membranes and probed with the anti-JAM-C antibody

Attachment Assays

Adenovirus infected cells were detached with trypsin. Cells were plated into fibronectin, vitronectin, laminin or Matrigel-coated 96-well microtiter trays at 4×10^4 cells per well for 60 minutes and washed three times with serum-free medium. MTS (3-(4,5 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl-2-(4-sulfophenyl)-2H-tetrazolium; Promega) assay was used to assess cell attachment. Absorbance at 490 nm was measured.

For attachment to antibody coated wells, 96-well microtiter trays were pre-coated with mouse IgG 50μg/ml for 18 hours at 4°C followed by a block with 1% BSA at 37°C for 30 minutes. The plates were washed once with PBS then coated with mouse anti-human integrin $\alpha v\beta_3$, mouse anti-human integrin β_1, mouse anti-human VE cadherin or mouse anti-human PECAM antibody (50μg/ml) 60 minutes at 37°C, washed again with PBS and blocked with 1% BSA at 37°C for another 30 minutes. The wells were then washed twice with PBS, and cells at 10^4 cells per well were added for 30 minutes and washed three times with serum-free medium. MTS assay was used to assess cell attachment.

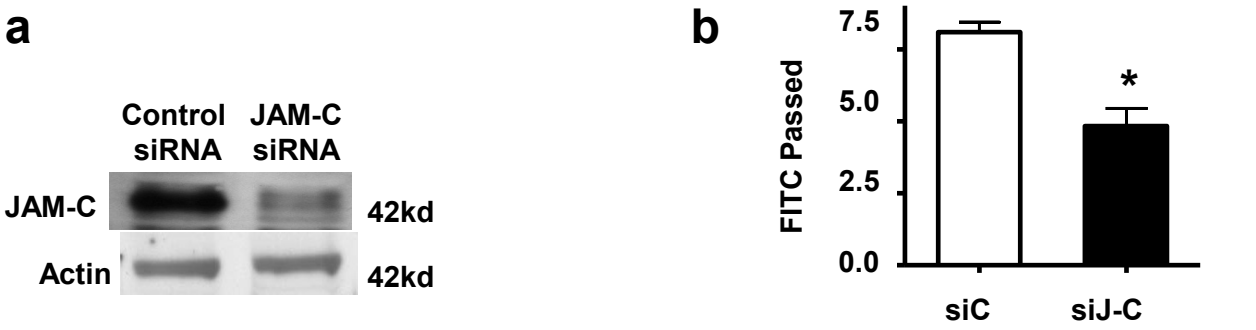
Endothelial Permeability Assays

Adenovirus-infected HUVECs were plated onto Transwells (3μm, Corning Costar Corp, Cambridge, MA) 24 hours after the infection procedure and then cultured for 24 hours in complete medium and then in 2% FCS medium for a further 24 hours. siRNA transfected cells were plated onto Transwells 24 hours after transfection then incubated in complete medium for another 24 hours before the assay. FITC-conjugated dextran was added to the upper chamber of all wells. The amount of FITC–dextran in the lower chambers of the Transwells was determined using a LS 50B Luminescence Spectrometer (Perkin Elmer, Beaconsfield, Buckinghamshire, UK; excitation wavelength, 485 nm; emission wavelength 530 nm). Permeability is given as the amount of FITC–dextran passing from the upper chamber to the lower chamber.

Rap1 Activity assays

Cell lysates were incubated with agarose beads conjugated with Ral GDS-Rap-binding domain for 60 minutes at 4°C. The beads were washed three times in lysis buffer, resuspended in 2 × reducing SDS sample buffer and boiled. The samples were separated by SDS–PAGE and active Rap1 was detected by immunoblotting.)

Figure I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Control siRNA
JAM-C siRNA
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Actin
42kd
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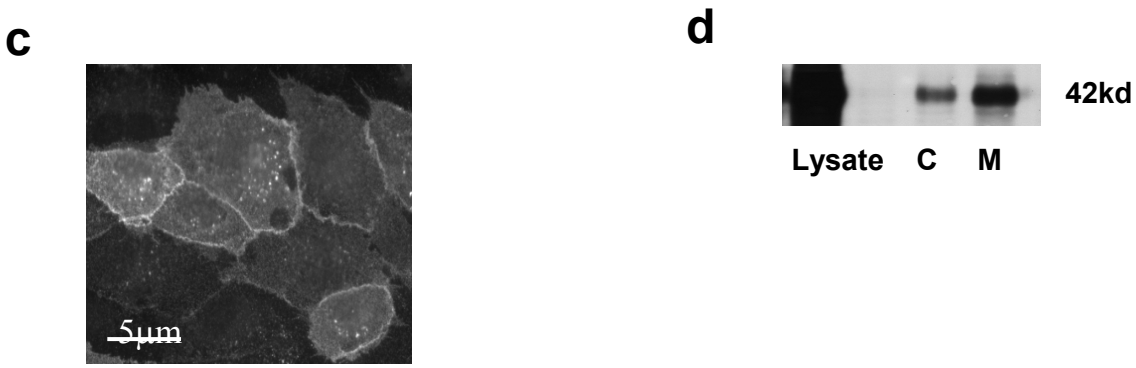
2.5
0.0
*
siC
siJ-C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5μm
d
42kd
Lysate
C
M

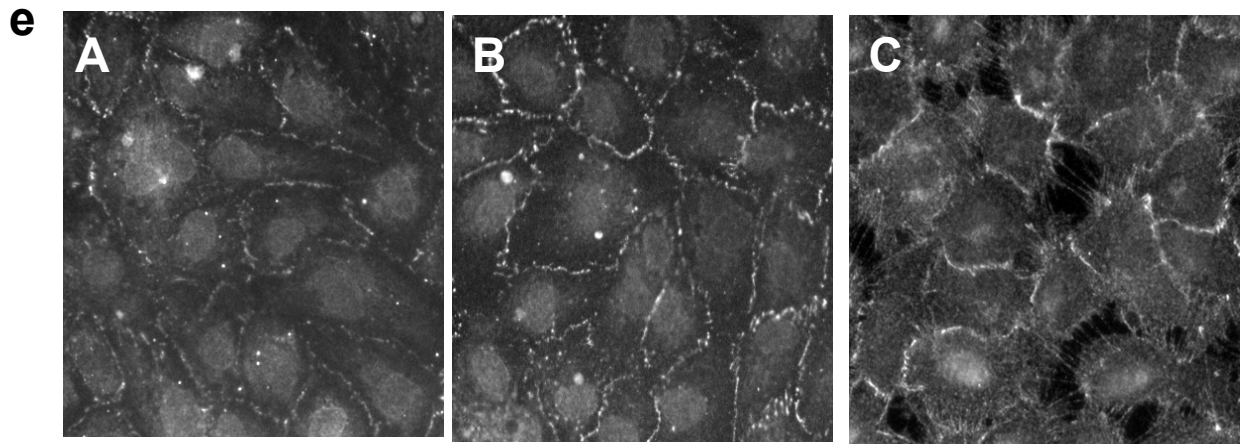
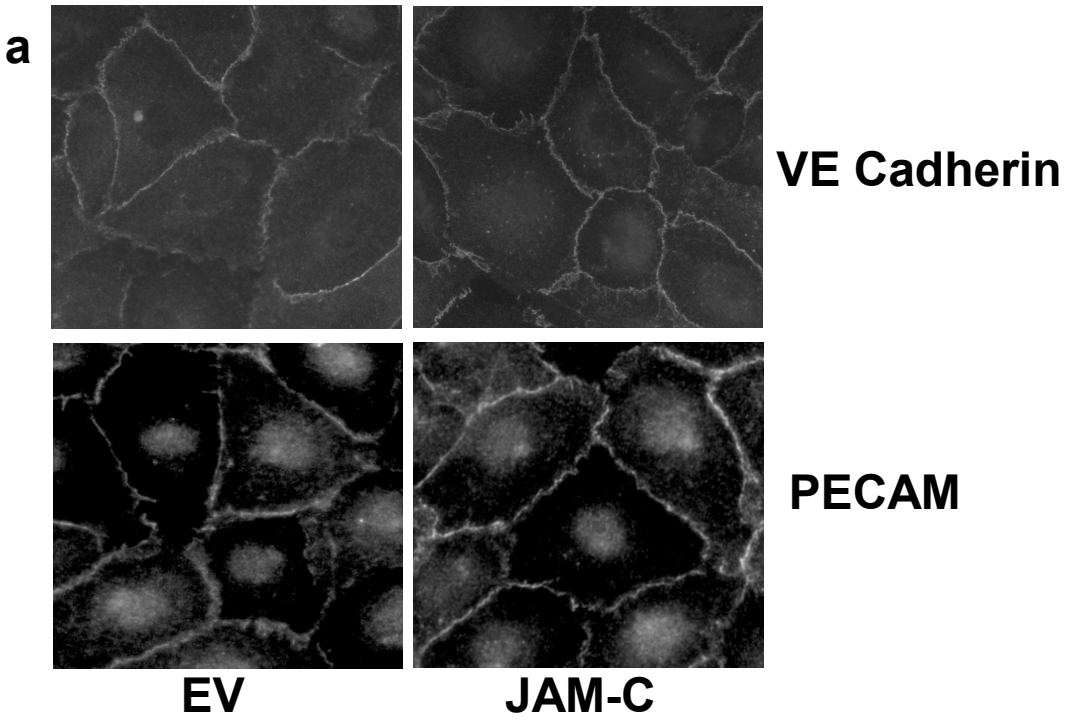

e
A
B
C

Figure II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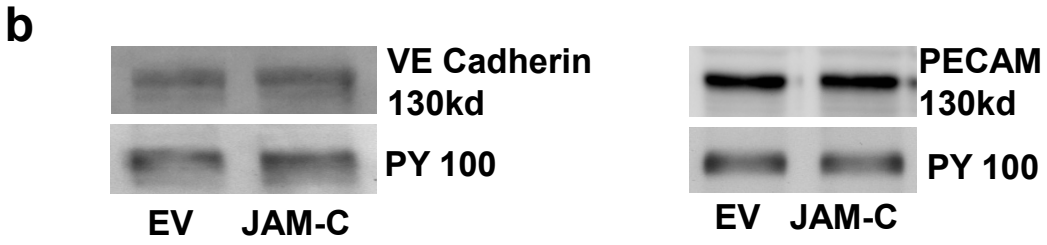
PECAM
EV
JAM-C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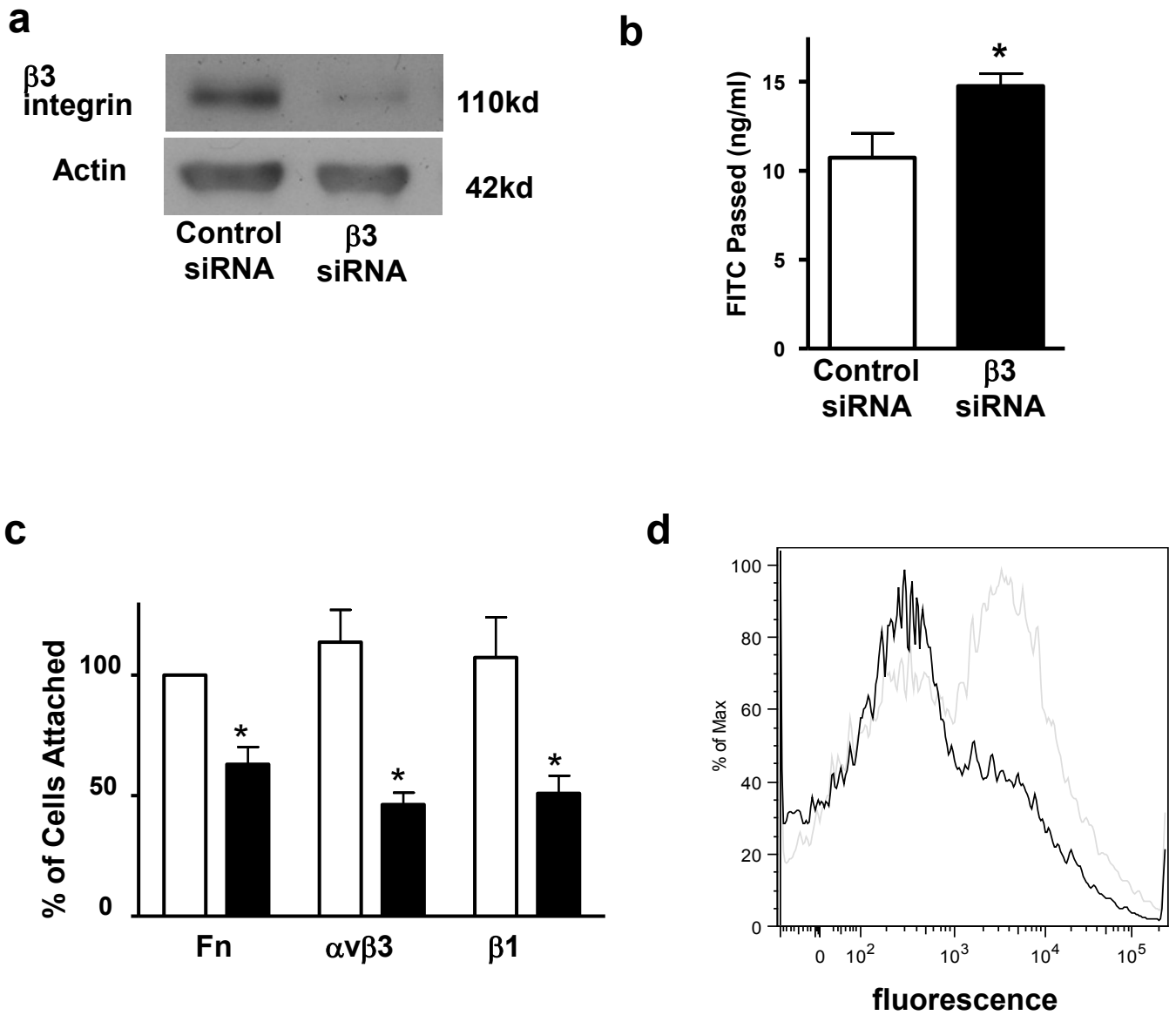
PY 100
EV
JAM-C
PECAM
130kd
PY 100
EV
JAM-C

Figure III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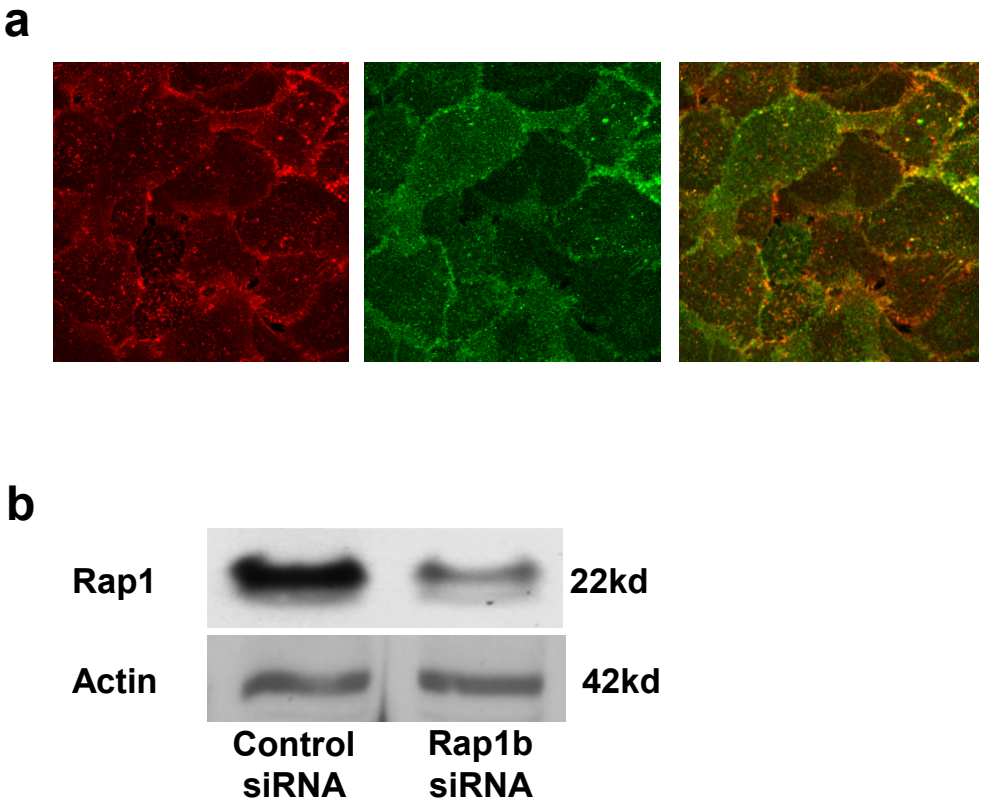
Actin
110kd
42kd
Control siRNA
β3 siRNA
b
FITC Passed (ng/ml)
15
10
5
0
*
Control siRNA
β3 siRNA
c
% of Cells Attached
100
50
0
*
*
*
Fn
αvβ3
β1
d
% of Max
100
80
60
40
20
0
0
10^2
10^3
10^4
10^5
fluorescence

Figure IV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b
Rap1
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Actin
42kd
Control siRNA
Rap1b siRNA
c
% of Cells Attached
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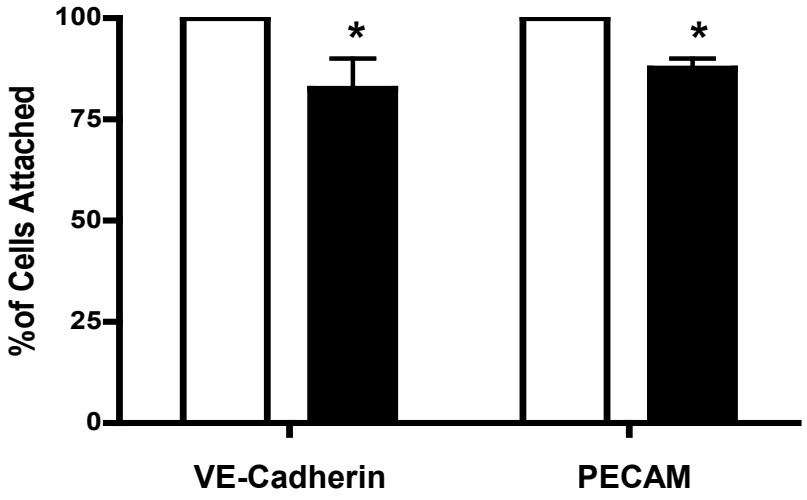
100
50
0
*
*
NS
siC
siJ-C
siR
siJ-C+R

Figure V

a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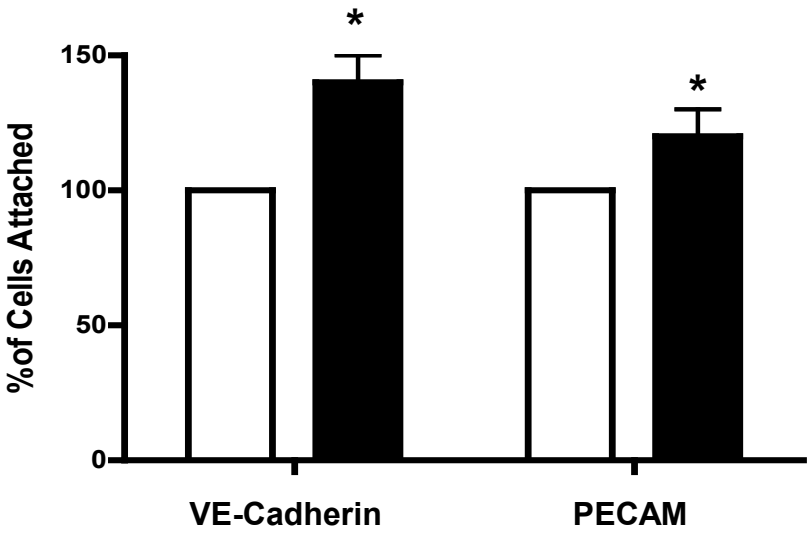
50
25
0
%of Cells Attached
*
*
VE-Cadherin
PECAM

b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100
50
0
%of Cells Attached
*
*
VE-Cadherin
PECAM

Figure VI