SUPPLEMENTARY MATERIALS

SUPPLEMENTARY FIGURE LEGEND

Supplemental Figure 1. VEC-GFP behaves as endogenous VE-cadherin. (A) Confluent HUVECs were transfected without (right column: Untransfected) or with the plasmid encoding GFP (left column) or VEC-GFP (middle column) as indicated at the top. The cells were starved in 0.5% BSA-containing medium 199 for 3 h, and stimulated with vehicle (Control) or 10 μM FSK for 30 min. The untransfected cells were stained with anti-VE-cadherin antibody and visualized with Alexa 488-conjugated secondary antibody. Both transfected and untransfected cells were stained with rhodamine-phalloidin to visualize F-actin. GFP, Alexa 488 and rhodamine images were obtained through a confocal microscope. GFP and Alexa 488 (VE-cadherin) images are shown in the upper panels. Rhodamine (F-actin) and the merged (Merge) images are shown in the middle and bottom panels, respectively. Scale bars, 20 μm. (B) 293T cells were transfected with the plasmid encoding GFP or VEC-GFP as indicated at the top. Cell lysates were immunoprecipitated with anti-GFP antibody. Immunoprecipitates (IP: GFP) and aliquots of cell lysates (Lysate) were subjected to Western blot analysis with the antibodies as indicated at the left.

Supplemental Figure 2. cAMP stabilizes VE-cadherin at cell-cell contacts through Rap1, but not PKA. (A) HUVECs starved in 0.1% BSA-containing medium 199 for 6 h were treated without (Control) or with 10 μM H89 (H89) for 10 min, and stimulated with vehicle (-) or 10 μM FSK (+) for 10 min. Cell lysates were subjected to Western blot analysis with anti-phospho-CREB and anti-CREB antibodies as indicated at the left. (B) FRAP analysis was performed to investigate the effect of H89 on FSK-induced stabilization of junctional VE-cadherin as described in the legend of Figure 1I. The recovery half time of VEC-GFP at cell-cell contacts was calculated similarly to the legend of Figure 1E. Data are expressed as mean ± s.e. of 6 independent experiments. (C) HUVECs were infected with adenoviruses encoding either LacZ (Ad-LacZ) or Rap1GAP (Ad-Rap1GAP) as indicated at the top. After 24 h, the cells were starved in 0.5% BSA-containing medium 199 for 6 h, and stimulated with vehicle (-) or 10 μM FSK (+) for 15 min. GTP-bound Rap1 was collected as described in the legend of Figure 1F, and subjected to Western blot analysis with anti-Rap1 antibody (GTP-Rap1). Aliquots of cell lysates were also subjected to Western blot analysis with anti-Rap1, anti-Rap1GAP and anti-β-tubulin antibodies as indicated at the left. (D) FRAP analysis

was performed to investigate the effect of Rap1GAP on FSK-induced stabilization of junctional VE-cadherin as described in the legend of Figure 1J. The recovery half time of VEC-GFP at cell-cell contacts was calculated similarly to the legend of Figure 1E. Data are expressed as mean ± s.e. of 4-8 independent experiments. Significant differences between two groups (B and D) are indicated as *, $p<0.05$. n.s. indicates no significance between two groups.

Supplemental Figure 3. H89 does not affect FSK-induced circumferential actin bundling and FSK-induced accumulation of VE-cadherin at cell-cell contacts. Monolayer-cultured HUVECs starved in 0.1% BSA-containing medium 199 for 3 h were pretreated without (first and second panels) or with 10 μM H89 (third and bottom panels) for 30 min, and subsequently stimulated with vehicle (first and third panels) or 10 μM FSK (second and bottom panels). The cells were then stained with anti-VE-cadherin antibody and visualized with Alexa 488-conjugated secondary antibody as described in the legend of Figure 1A. The cells were also stained with rhodamine-phalloidin to visualize F-actin. Alexa 488 (VE-cadherin: green), rhodamine (F-actin: red) and the merged (Merge) images are shown as indicated at the top of each column. The boxed areas marked by dotted line in the images are enlarged in the lower right corner of each image. Scale bars, 50 μm.

Supplemental Figure 4. Effect of 100 nM Lat.A on the organization of actin cytoskeleton and the localization of VEC-GFP. Confluent HUVECs transfected with the plasmid encoding VEC-GFP were starved in 0.5% BSA-containing medium 199 for 3 h, and incubated with vehicle (left column: (-)) or 100 nM Lat.A (right column: Lat.A) for 30 min. The cells were then stimulated with vehicle (Control) and 10 μM FSK (FSK) for 30 min as indicated at top, and stained with rhodamine-phalloidin as described in the legend of Figure 3A. GFP and rhodamine images were obtained through a confocal microscope. GFP (VEC-GFP), rhodamine (F-actin) and the merged (Merge) images are shown as indicated at the left. Scale bars, 20 μm. Note that 100 nM Lat.A less suppressed FSK-induced circumferential actin bundling and less decreased the junctional localization of VEC-GFP than 200 nM Lat.A.

Supplemental Figure 5. cAMP-induced circumferential actin bundling does not depend upon VE-cadherin-based cell-cell adhesions. Effect of depletion of either VE-cadherin (A), α-catenin (B), β-catenin (C) or p120-catenin (D) on FSK-induced circumferential actin bundling was examined as described in the legend of Figure 4.

Levels of F-actin at cell-cell contacts observed in Figure 4 were quantified as described in the legend of Figure 2B. Values are expressed as a percentage relative to that in the vehicle-treated cells transfected with control siRNA, and shown as mean ± s.e. of more than 100 contacts. Similar results were obtained in three independent experiments.

Supplemental Figure 6. Disruption of VE-cadherin based cell-cell adhesions by chelation of extracellular Ca^{2+} does not inhibit FSK-induced circumferential actin bundling. Monolayer-cultured HUVECs starved in 0.5% BSA-containing medium 199 for 3 h were treated without (first and second panels) or with (third and bottom panels) 5 mM EGTA for 30 min, and stimulated with vehicle (Control) or 10 μM FSK for 30 min as indicated at the left. The cells were then stained with anti-VE-cadherin antibody and visualized with Alexa 488-conjugated secondary antibody as described in Figure 2A. The cells were also stained with rhodamine-phalloidin to visualize F-actin. Alexa 488 and rhodamine images were obtained through a confocal microscope. Alexa 488 (VE-cadherin), rhodamine (F-actin) and the merged (Merge) images are shown as indicated at the top of each column. The boxed areas marked by dotted line in the images are enlarged in the lower right corner of each image. Scale bars, 50 μm.

Supplemental Figure 7. α- and β-catenins are essential for cAMP-induced accumulation of VE-cadherin at cell-cell contacts. (A, B) HUVECs were transfected with control siRNA (upper panels of each figure) or with different siRNAs targeting against α-catenin (A) and β-catenin (B) from those used in Figure 5A and 5B (lower panels of each figure). The cells were then stimulated with vehicle (Control) or FSK as described in the legend of Figure 4. After fixation, the cells were immunostained with either anti-α-catenin (A) or anti-β-catenin (B) antibody and with anti-VE-cadherin antibody, and visualized with Alexa 488- and Alexa 546-conjugated secondary antibodies, respectively. Phase contrast, Alexa 488 and Alexa 546 images were obtained using Olympus IX81 inverted microscope. Phase contrast (Bright field), Alexa 488 (α-catenin in A and β-catenin in B), Alexa 546 (VE-cadherin) and the merged (Merge) images are shown as indicated at the top of each column. The boxed areas marked by dotted line in the images are enlarged in the lower right corner of each image. Scale bars, 30 μm.

Supplemental Figure 8. Depletion of p120-catenin results in the loss of VE-cadherin expression at cell-cell contacts. HUVECs were transfected with control siRNA (upper panels) or with two independent siRNAs targeting against p120-catenin (middle and

lower panels), starved in medium 199 containing 0.5% BSA for 3 h, and stimulated with vehicle (Control) or FSK as described in the legend of Figure 4. The cells were fixed and immunostained with anti-p120-catenin and anti-VE-cadherin antibodies, and visualized with Alexa 488- and Alexa 546-conjugated secondary antibodies, respectively. Phase contrast, Alexa 488 and Alexa 546 images were obtained using Olympus IX81 inverted microscope. Phase contrast (bright field), Alexa 488 (p120-catenin), Alexa 546 (VE-cadherin) and the merged (Merge) images are shown as indicated at the top of each column. The boxed areas marked by dotted line in the images are enlarged in the lower right corner of each image. Scale bars, 30 μm.

Supplemental Figure 9. VEC-GFP, PECAM1-GFP and their mutants. (A) Structures of VEC-GFP, PECAM1-GFP and its mutants. VEC-GFP: VE-cadherin carboxy-terminally tagged with GFP, VEC-GFPΔβ-GFP: a VEC-GFP mutant lacking the β-catenin binding domain of VE-cadherin, VECΔC-GFP: a VEC-GFP mutant lacking the cytoplasmic region of VE-cadherin, VECΔC-α-GFP: a VEC-GFP mutant in which the cytoplasmic region of VE-cadherin is replaced with α-catenin, VECΔC-αΔN-GFP: a VEC-GFP mutant in which the cytoplasmic region of VE-cadherin is replaced with α-catenin lacking N-terminal β-catenin binding domain, PECAM1-GFP: PECAM1 carboxy-terminally tagged with GFP, PECAM1ΔC-GFP: a PECAM1-GFP mutant lacking the cytoplasmic region of PECAM1, PECAM1ΔC-α-GFP: a PECAM1-GFP mutant in which the cytoplasmic region of PECAM1 is replaced with α-catenin, PECAM1ΔC-VEC/C-GFP: a PECAM1-GFP mutant in which the cytoplasmic region of PECAM1 is replaced with that of VE-cadherin. (B) 293T cells were transfected with the plasmids as indicated at the top. After 24 h, the cell lysates were subjected to Western blot analysis with anti-GFP and anti-β-actin antibodies as indicated at the left. (C) Confluent HUVECs were transfected with the plasmids as indicated at the top of each panel and cultured for 24 h. After fixation, GFP fluorescence images were obtained through a confocal microscope. Scale bars, 20 μm. (D) HUVECs were transfected with the plasmids as indicated at the top of each panel and cultured for 24 h. The cells were then subjected to FACS analysis as described in the Materials and Methods. The X- and Y-axes indicate GFP fluorescence intensity and cell count, respectively. Note that expression levels of VEC-GFP, PECAM1-GFP and their mutants are similar. (E) 293T cells were transfected with the plasmids as indicated at the top. Cell lysates were subjected to immunoprecipitation (IP) with anti-β-catenin antibody followed by Western blot analysis with anti-GFP antibody. Aliquots of total cellular lysates (Lysate) were also subjected to Western blot analysis

with anti-GFP, anti-β-catenin and anti-β-actin antibodies as indicated at the left of each panel.

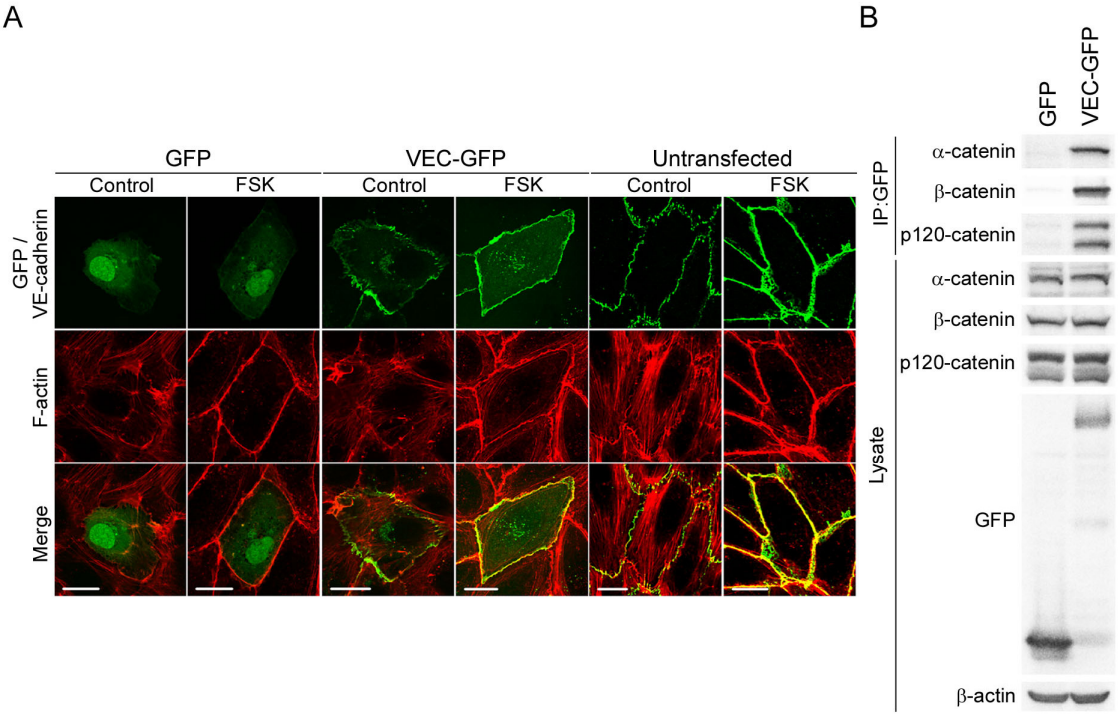
Supplemental Figure 10. VECΔC-α-GFP, but not VEC-GFP, accumulates on the bundled actin filaments in the absence of α- and β-catenins. HUVECs in 6-cm collagen-coated dishes were transfected with control siRNA and siRNA targeting against either α-catenin (A, C) or β-catenin (B, D) as indicated at the left of each panels. After 48 h, the cells were re-plated on collagen-coated glass-base dish, and transfected with the plasmid encoding either VEC-GFP (A, B) or VECΔC-α-GFP (C, D). After 24 h, the cells were starved in medium 199 containing 0.5% BSA for 3 h, and stimulated with vehicle (Control) or 10 μM FSK (FSK) for 30 min. Then, the cells were immunostained with either anti-α-catenin (A, C) or anti-β-catenin (B, D) antibody, and visualized with Alexa 633-conjugated secondary antibody as described in the legend of Figure 2A. The cells were also stained with rhodamine-phalloidin to visualize F-actin. GFP, rhodamine and Alexa 633 images were obtained through a confocal microscope. GFP (VEC-GFP in A and B, VECΔC-α-GFP in C and D), rhodamine (F-actin), Alexa 633 (α-catenin in A and C, β-catenin in B and D) and the merged (Merge) images are shown as indicated at the top. The border between the untransfected cell and the cell-expressing GFP tagged-VE-cadhein is shown. Scale bars, 5 μm.

Supplemental Figure 11. Circumferential actin bundle formation is responsible for cAMP-induced stabilization of VECΔC-α-GFP at cell-cell contacts. Confluent HUVECs expressing VECΔC-α-GFP were starved in medium 199 containing 1% BSA for 3 h, treated with or without 100 nM Lat.A for 30 min, and stimulated with vehicle (Control) or 10 μM FSK for 30 min as indicated at the bottom of each graph. The cells were then subjected to FRAP analysis as described in the legend of Figure 1B. The mobile fraction of VECΔC-α-GFP (A) and its recovery half time (B) were calculated as described in the legend of Figure 1D and 1E, respectively. Data are expressed as mean ± s.e. of 4 independent experiments. Significant differences between two groups are indicated as *, $p<0.05$. n.s. indicates no significance between two groups.

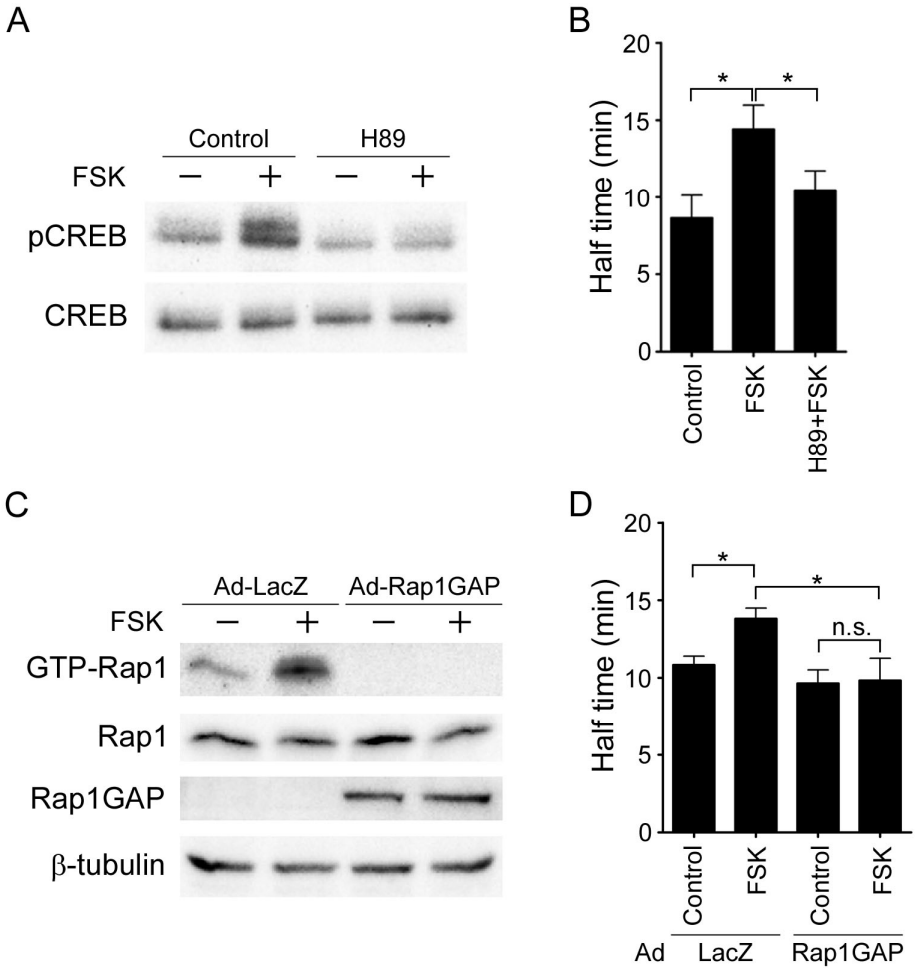
Supplemental Figure 12. PECAM1ΔC-α-GFP accumulates on the bundled actin filaments in the absence of β-catenin. HUVECs in 6-cm collagen-coated dishes were transfected with control siRNA or siRNA targeting against β-catenin as indicated at the left of each panels. After 48 h, the cells were re-plated on collagen-coated glass-base dish, and transfected with the plasmid encoding PECAM1ΔC-α-GFP. After 24 h, the

cells were starved in medium 199 containing 0.5% BSA for 3 h, and stimulated with vehicle (Control) or 10 μM FSK (FSK) for 30 min. Then, the cells were immunostained with anti-β-catenin antibody, and visualized with Alexa 633-conjugated secondary antibody as described in the legend of Figure 2A. The cells were also stained with rhodamine-phalloidin to visualize F-actin. GFP, rhodamine and Alexa 633 images were obtained through a confocal microscope. GFP (PECAM1ΔC-α-GFP), rhodamine (F-actin), Alexa 633 (β-catenin) and the merged (Merge) images are shown as indicated at the top. The border between the untransfected cell and the cell-expressing GFP tagged-PECAM1 is shown. Scale bars, 5 μm.

Supplementary Figure 1



Supplementary Figure 2



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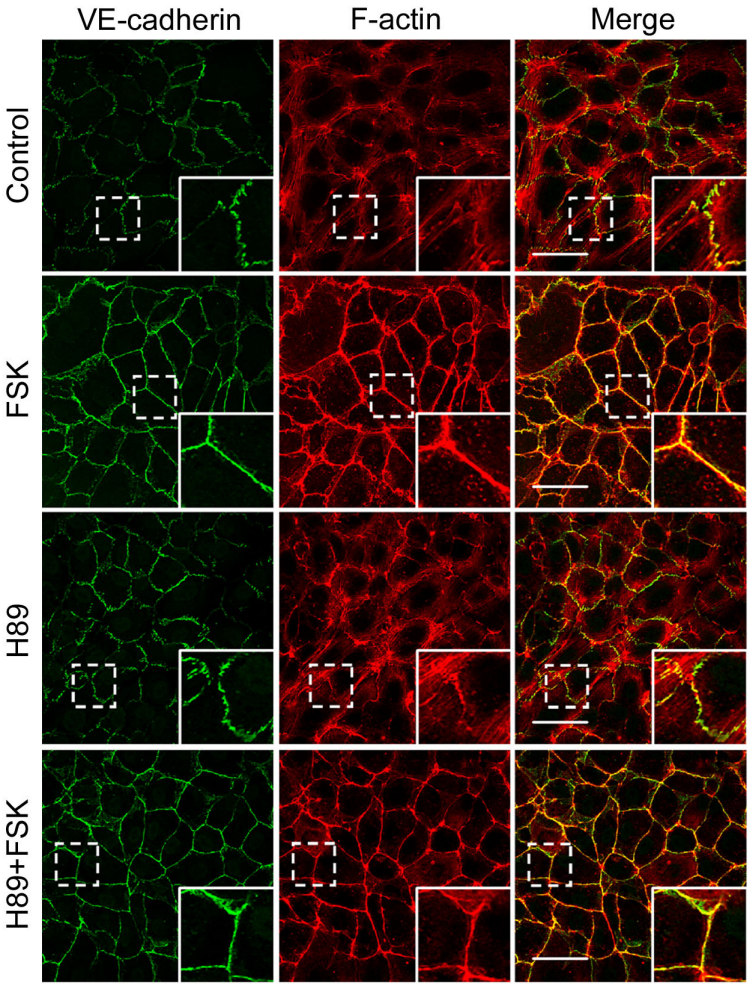

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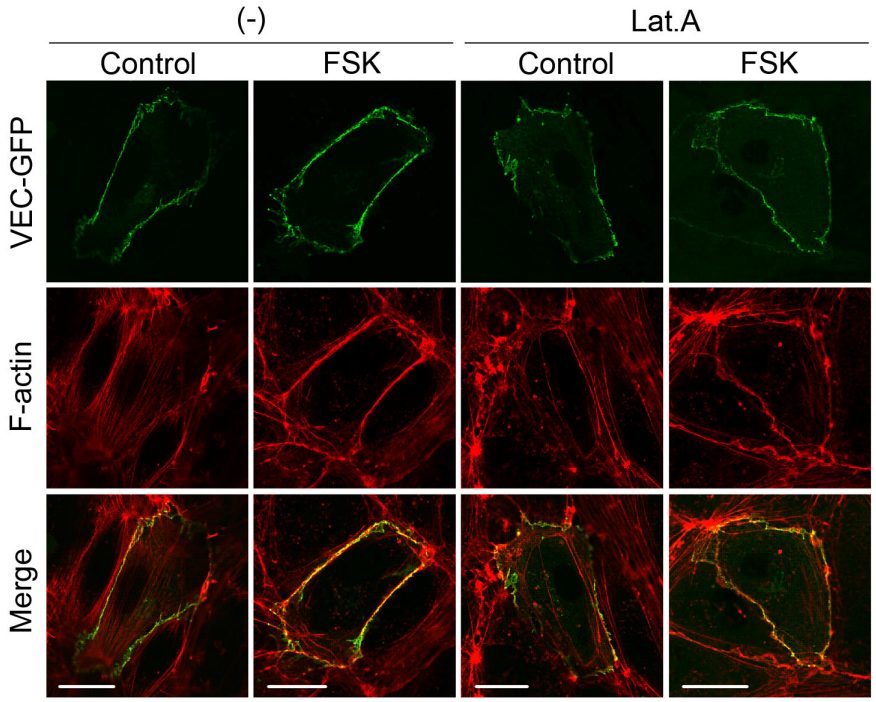

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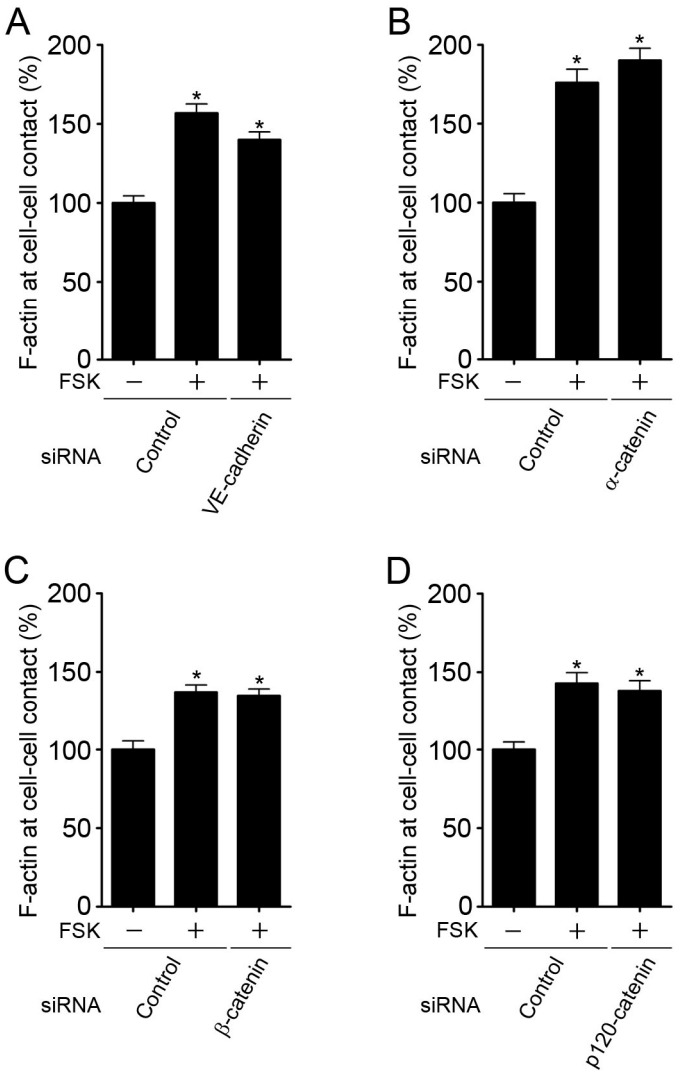

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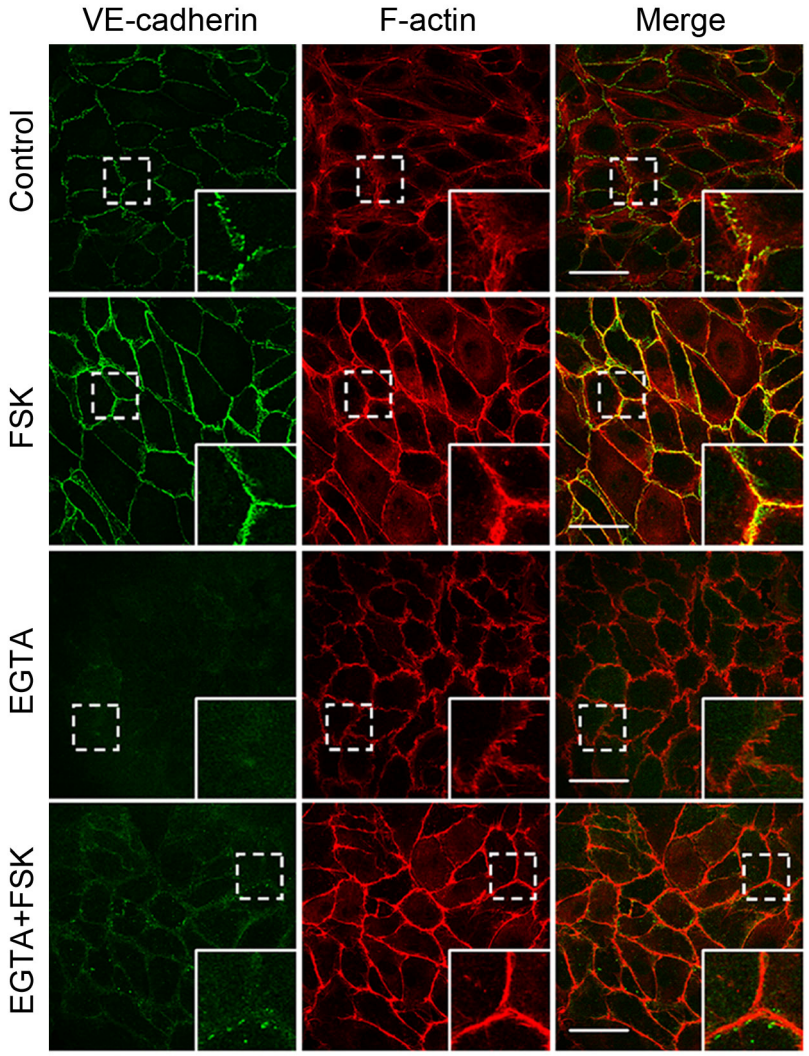

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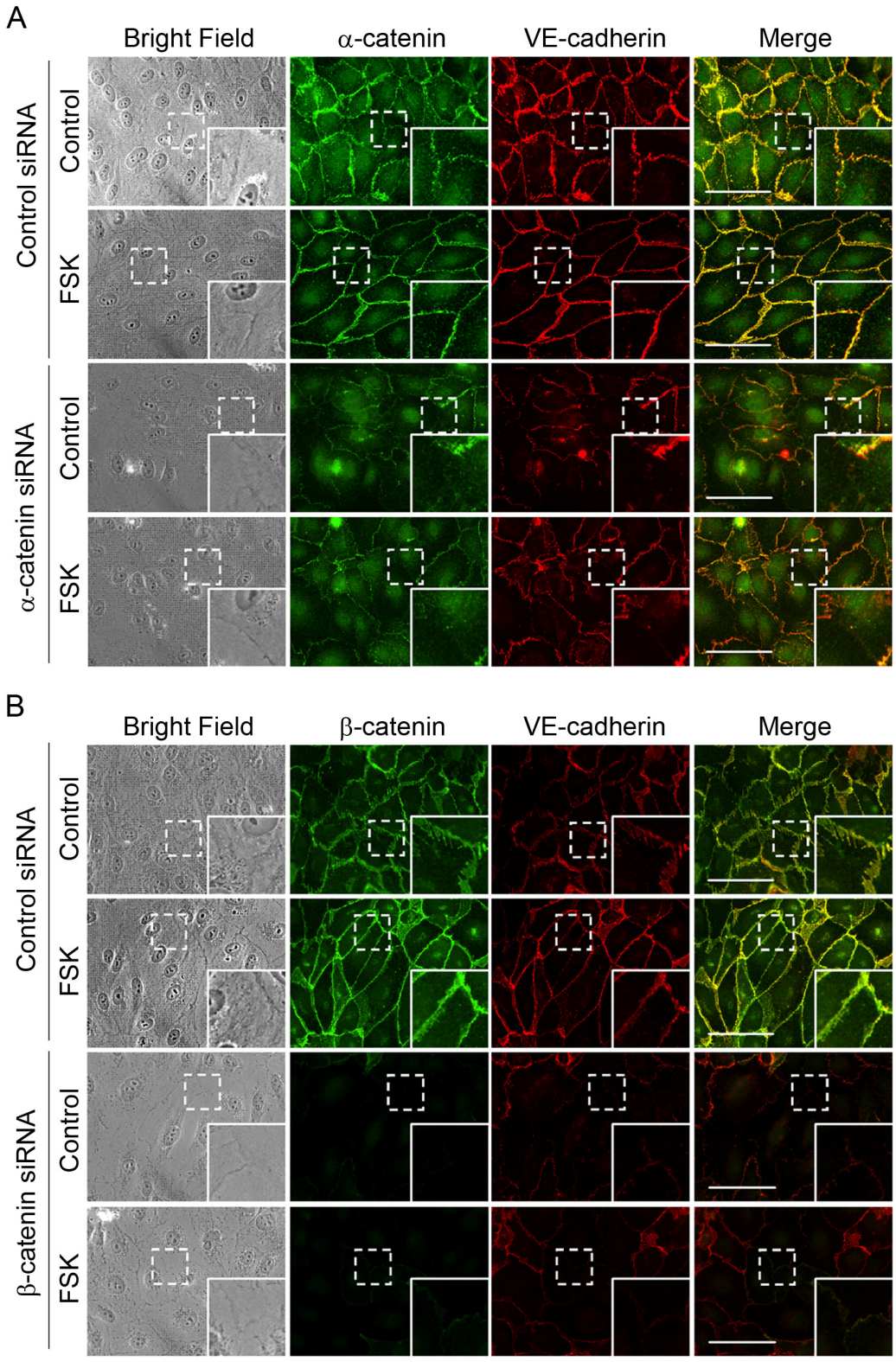

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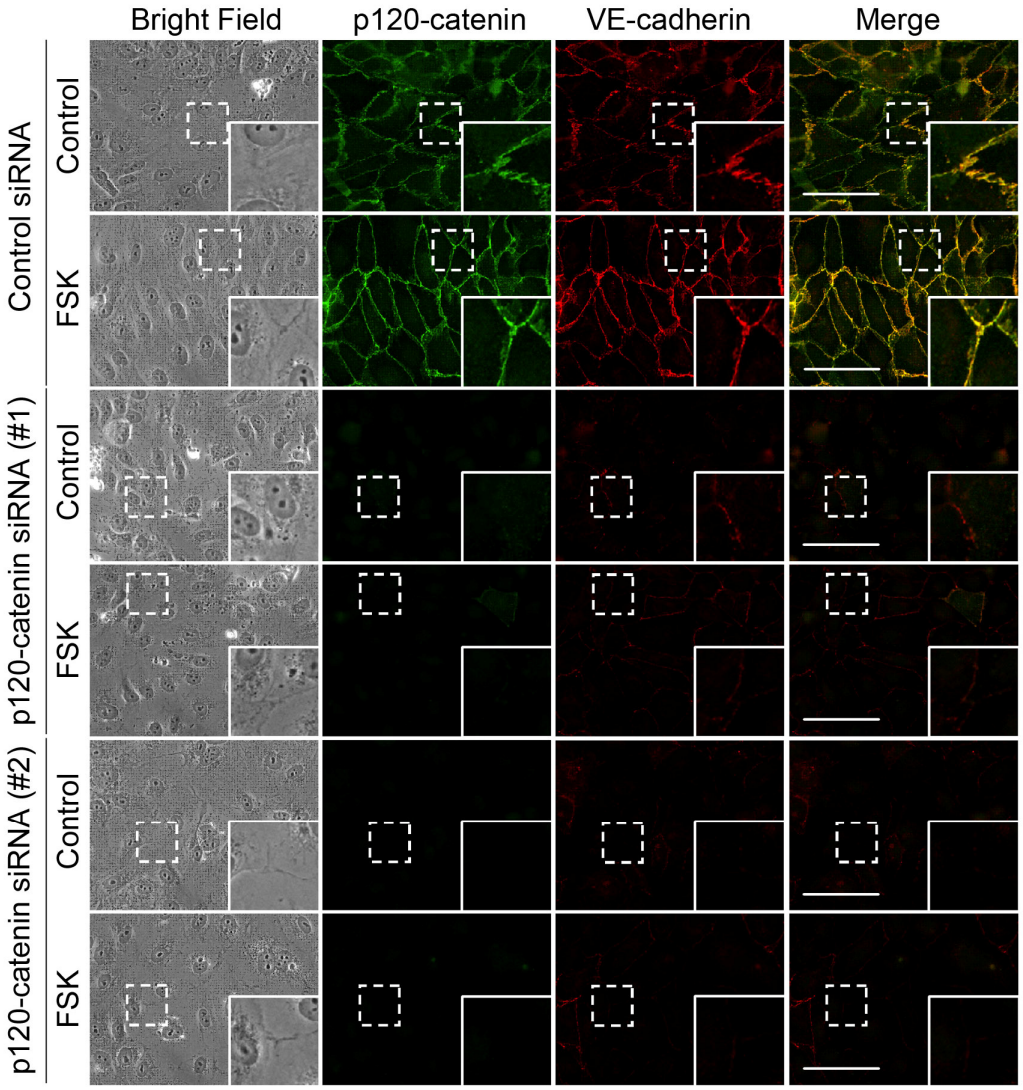
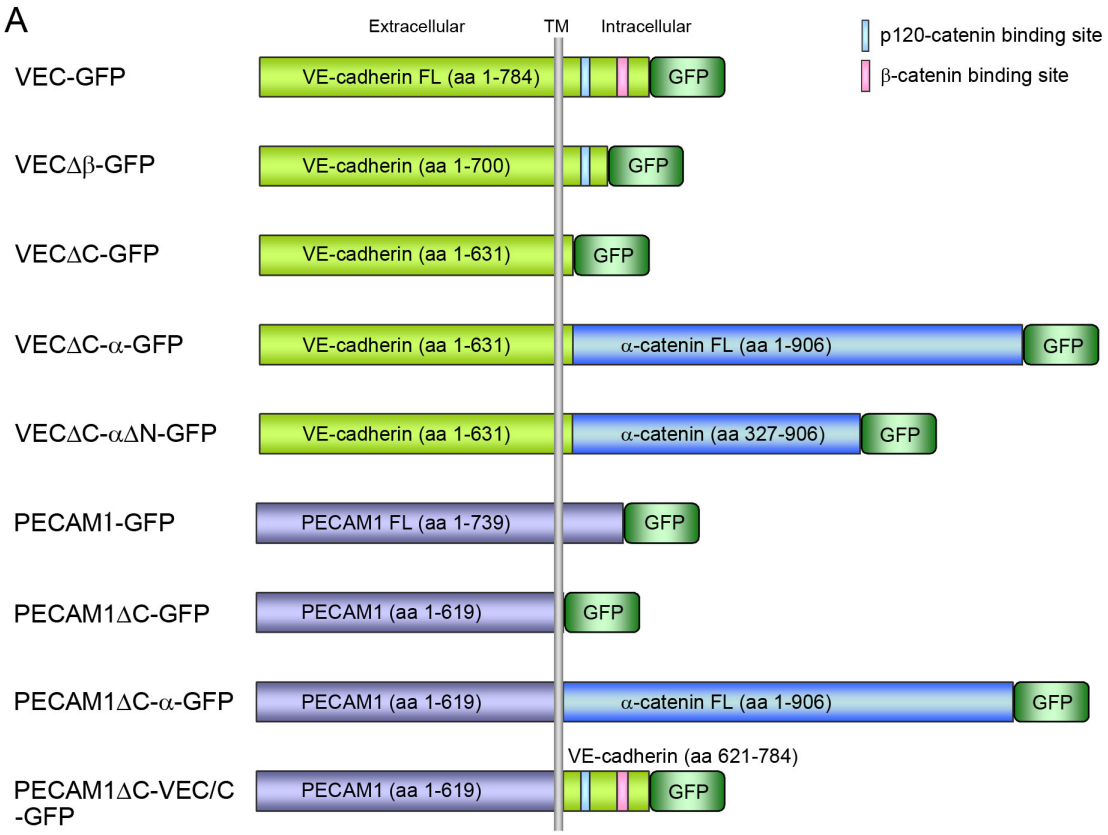
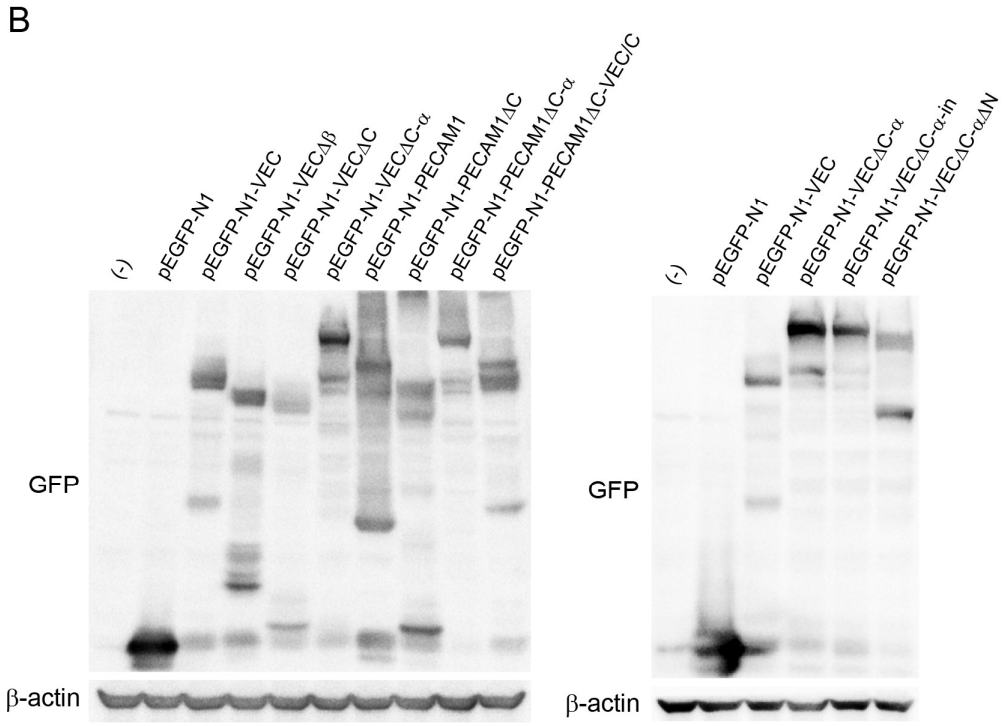
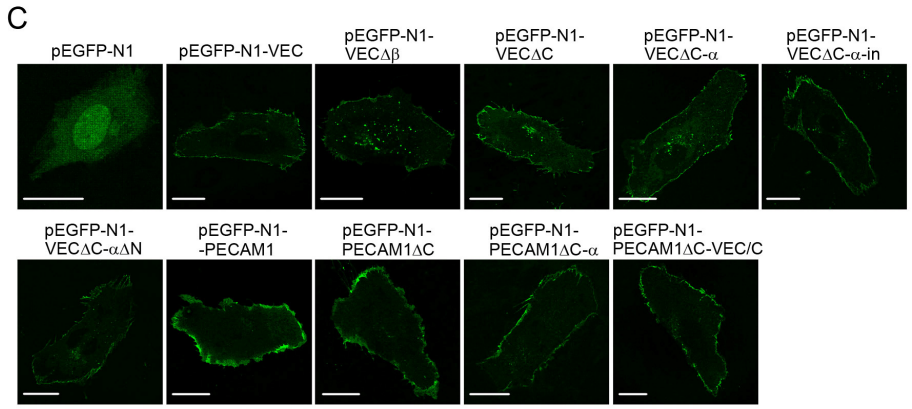


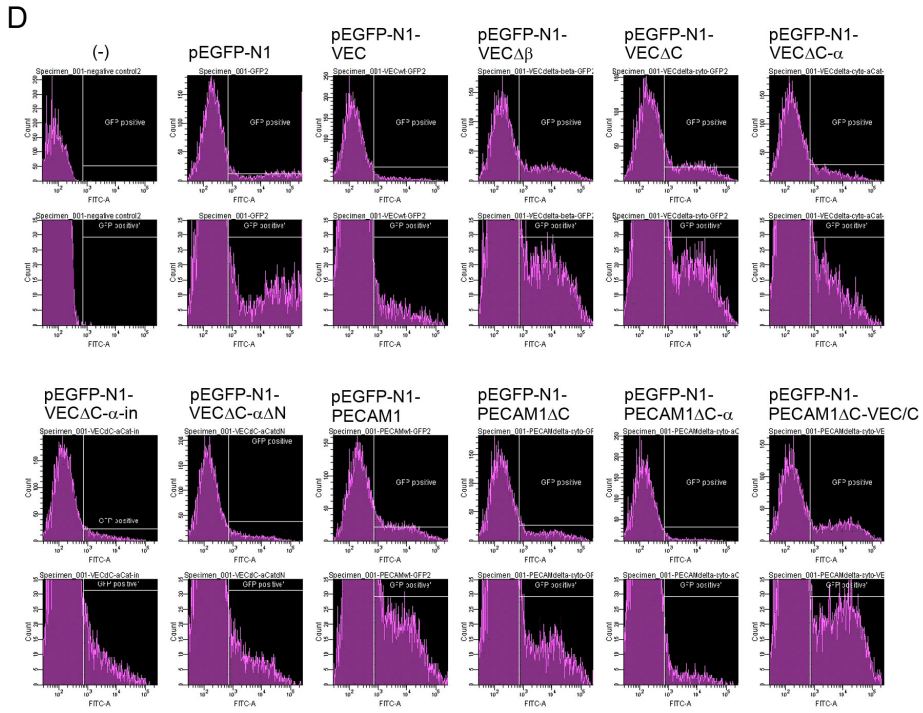
Supplementary Figure 9 A-B

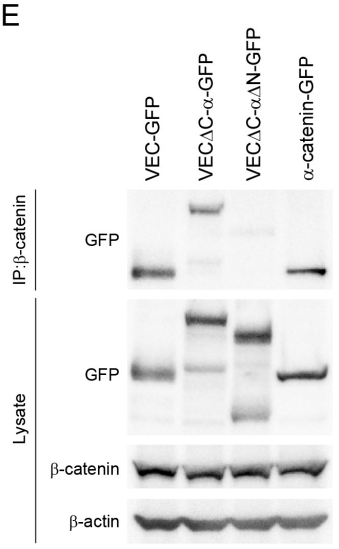




Supplementary Figure 9 C-E







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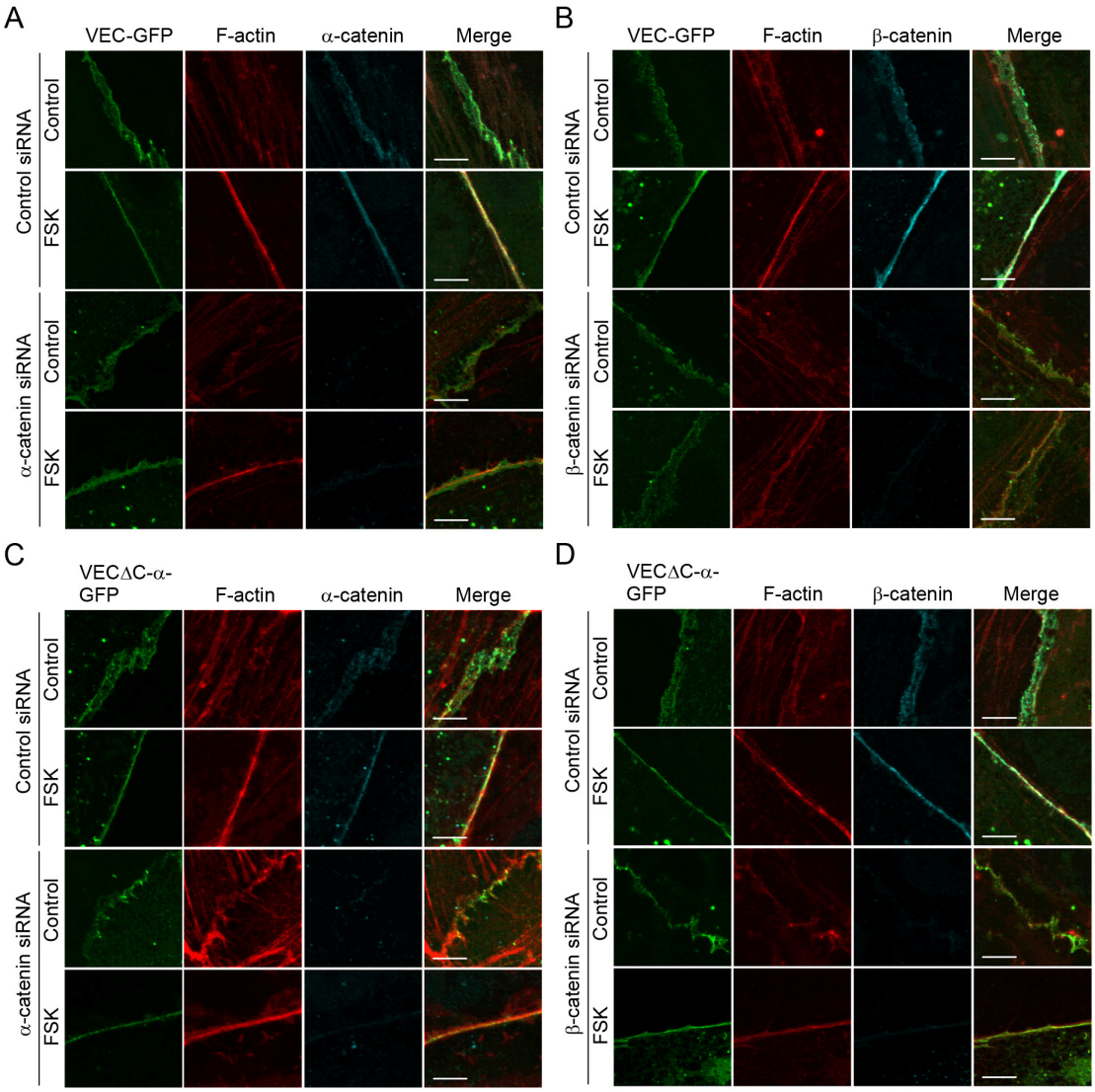

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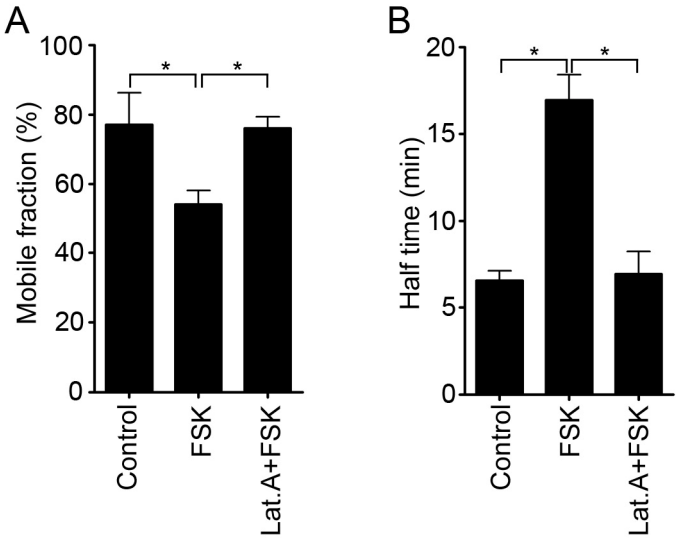

Supplementary Figure 12