Comparative phosphoproteomics analysis of VEGF and Angiopoietin-1 signaling reveals ZO-1 as a critical regulator of endothelial cell proliferation.

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Supplemental Figure S1: Correlations between phosphopeptide intensities of biological and technical replicates.

(A,B) Correlation scatter plots of peptide intensities between technical replicates (A) or biological replicates (B) in Control (CTL), VEGF and Ang-1 treated cells. Correlation between measurements is determined by Pearson coefficient (r). Log10 transformation of peptide intensities was used to visualize the correlation.

Supplemental Figure S2: Overview of the phosphoproteome profiling.

(A) A pie chart indicating the percentage of singly, doubly and multiply phosphorylated peptides identified in VEGF or Ang-1 treatment. (B) The relative abundance of non-redundant Serine, Threonine and Tyrosine phosphorylation sites identified in VEGF or Ang-1 treatment. (C) The distribution of the treated/non-treated ratios of all phosphopeptides shows a median distribution of 0.40 and 0.65 for VEGF and Ang-1 phosphopeptides, respectively. (D) The distribution of significant phosphopeptide abundance in VEGF or Ang-1 treated cells showed that 95% of the phosphopeptides are regulated. Vertical dashed lines indicate the cut-offs that defines the regulated peptides (-0.5>log2 fold change>0.5).

Supplemental Figure S3: Correlations between log2 fold-changes of phosphopeptides for the VEGF and Ang-1 treatment conditions.

(A,B) Log2 fold-changes for all quantified phosphopeptides between independent experiments of (A) VEGF or (B) Ang-1 treatment are plotted against each other and correlation between measurements is determined by Pearson coefficient (r). (C,D) Log2 fold-changes of significantly regulated phosphopeptides between independent experiments of (C) VEGF or (D) Ang-1 treatment are plotted against each other and correlation between measurements is determined by Pearson coefficient (r).

Supplemental Figure S4: Knockdown efficiency of siRNAs and effect of ZO-1, p120-catenin, β-catenin and VE-cadherin siRNA on endothelial cell proliferation.

(A) Quantification of the percentage of BrdU incorporation in BAECs transfected with two independent siRNAs against p120-catenin, ZO-1. Data are represented as mean ± SEM of three different experiments (*P<0.05 as compared to CT). The knockdown efficiency of p120-catenin and ZO-1 siRNAs was verified by immunoblot. β-actin was used as a loading control. Knockdown efficiency was measured by the densitometry of the protein expression levels in three independent experiments and the ratio is shown under the corresponding blot. (B) BAECs were transfected with siRNAs against p120-catenin, ZO-1, ZO-2, JUP, VE-cadherin, α-catenin or β-catenin for 48 hours and the corresponding protein expression was verified by immunoblot. β-actin was used as a loading control. Knockdown efficiency of the protein was measured by densitometry and the average ratio of three independent experiments is indicated. (C) Cyclin D1 mRNA expression increases significantly in ZO-1 downregulated cells. Real-time PCR analysis

of Cyclin D1 and ZO-1 mRNAs in BAECs transfected with a ZO-1 siRNA or control siRNA. Data was normalized to β-actin mRNA expression and represented as mean ± SEM. Statistical significance was determined by the standard two-tailed student's *t*-test (**P*<0.05 compared to control siRNA). (D) Downregulation of VE-cadherin or β-catenin does not affect proliferation in VEGF and Ang-1 treated cells. Quantification of the percentage of BrdU incorporation in BAECs transfected with VE-cadherin or β-catenin siRNA in response to VEGF (40 ng/ml) or Ang-1 (100 ng/ml) treatment. Data are represented as mean ± SEM of three different experiments. (**P*<0.05 compared to si-CT).

Supplemental Figure S5: The localization of p120-catenin, JUP or VE-cadherin was not affected in BAECs transfected with ZO-1 siRNA.

(A) Representative confocal micrographs of immunofluorescence staining of BAECs transfected with control, ZO-1 or p120-catenin siRNA using antibodies against ZO-1 (red) and p120-catenin (green). (B) Representative confocal micrographs of immunofluorescence staining of BAECs transfected with control, ZO-1 or JUP siRNA using antibodies against ZO-1 (red) and JUP (green). The peri-nuclear ZO-1 staining is an artifact of the rabbit antibody used to visualize ZO-1. Scale bar represents 20 μm. (C) Representative confocal micrographs of immunofluorescence staining of BAECs transfected with control or ZO-1 siRNA using antibodies against ZO-1 (red) and VE-cadherin (green). Scale bar represents 20 μm.

Supplemental Figure S6: Representative FACS plot of ZO-1 intensity in BrdU positive or negative cells.

ZO-1 stained cell population was divided into BrdU negative cells (87.7%) and BrdU positive cells (11.8%). ZO-1 mean intensity was calculated in each population. Control for primary and secondary antibodies was performed and non-specific staining was removed from the two populations.

Supplemental Table S1: Complete list of phosphorylated peptides identified by mass spectrometry (Sheet 1). List of the regulated phosphopeptides quantified in VEGF or Ang-1 treatment (Sheet 2). Table headers are described in the legend (Sheet 3).

Supplemental Table S2: Complete list of gene ontology biological processes enrichments in VEGF or Ang-1 treatment (Sheet 1). Regulated phosphoproteins identified in VEGF or Ang-1 treatment were uploaded into STRING database and the biological processes annotations with a p-value less than 0.05 were retained. The complete list of pathways enriched in VEGF or Ang-1 treatment (Sheet 2). Regulated phosphoproteins identified in VEGF or Ang-1 treatment were used to characterize the pathway enrichment using IPA (ingenuity pathway analysis). Table headers are described in the legend (Sheet 3).

Supplemental Table S3: MCODE clustering results. The table contains the parameters used in M-code algorithm and the clusters identified in VEGF or Ang-1 interaction network.

Figure S1

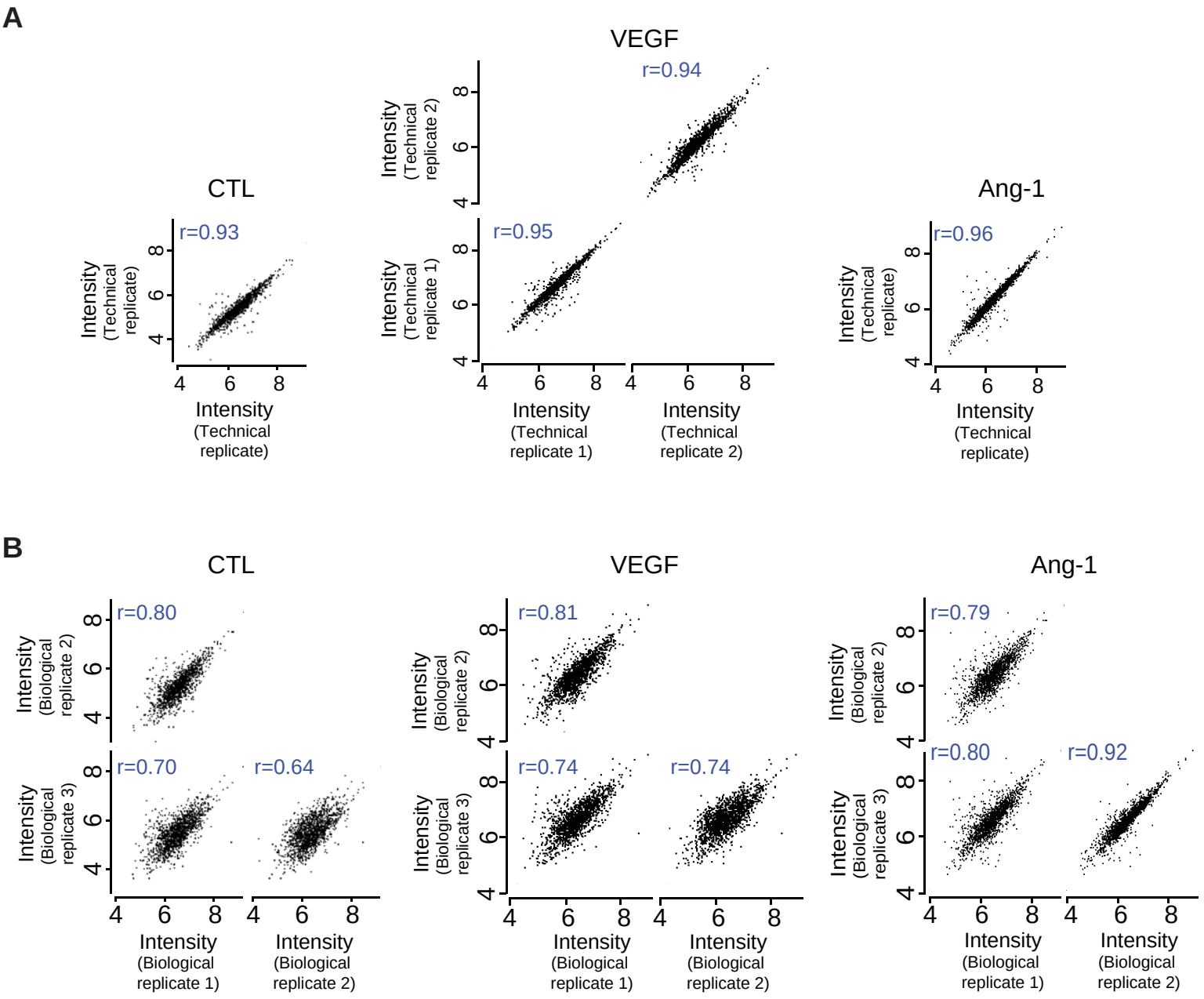

A
CTL
r=0.93
Intensity (Technical replicate)
Intensity (Technical replicate)
VEGF
r=0.94
r=0.95
Intensity (Technical replicate 2)
Intensity (Technical replicate 1)
Intensity (Technical replicate 1)
Intensity (Technical replicate 2)
Ang-1
r=0.96
Intensity (Technical replicate)
Intensity (Technical replicate)
B
CTL
r=0.80
r=0.70
r=0.64
Intensity (Biological replicate 2)
Intensity (Biological replicate 3)
Intensity (Biological replicate 1)
Intensity (Biological replicate 2)
VEGF
r=0.81
r=0.74
r=0.74
Intensity (Biological replicate 2)
Intensity (Biological replicate 3)
Intensity (Biological replicate 1)
Intensity (Biological replicate 2)
Ang-1
r=0.79
r=0.80
r=0.92
Intensity (Biological replicate 2)
Intensity (Biological replicate 3)
Intensity (Biological replicate 1)
Intensity (Biological replicate 2)

Figure S2

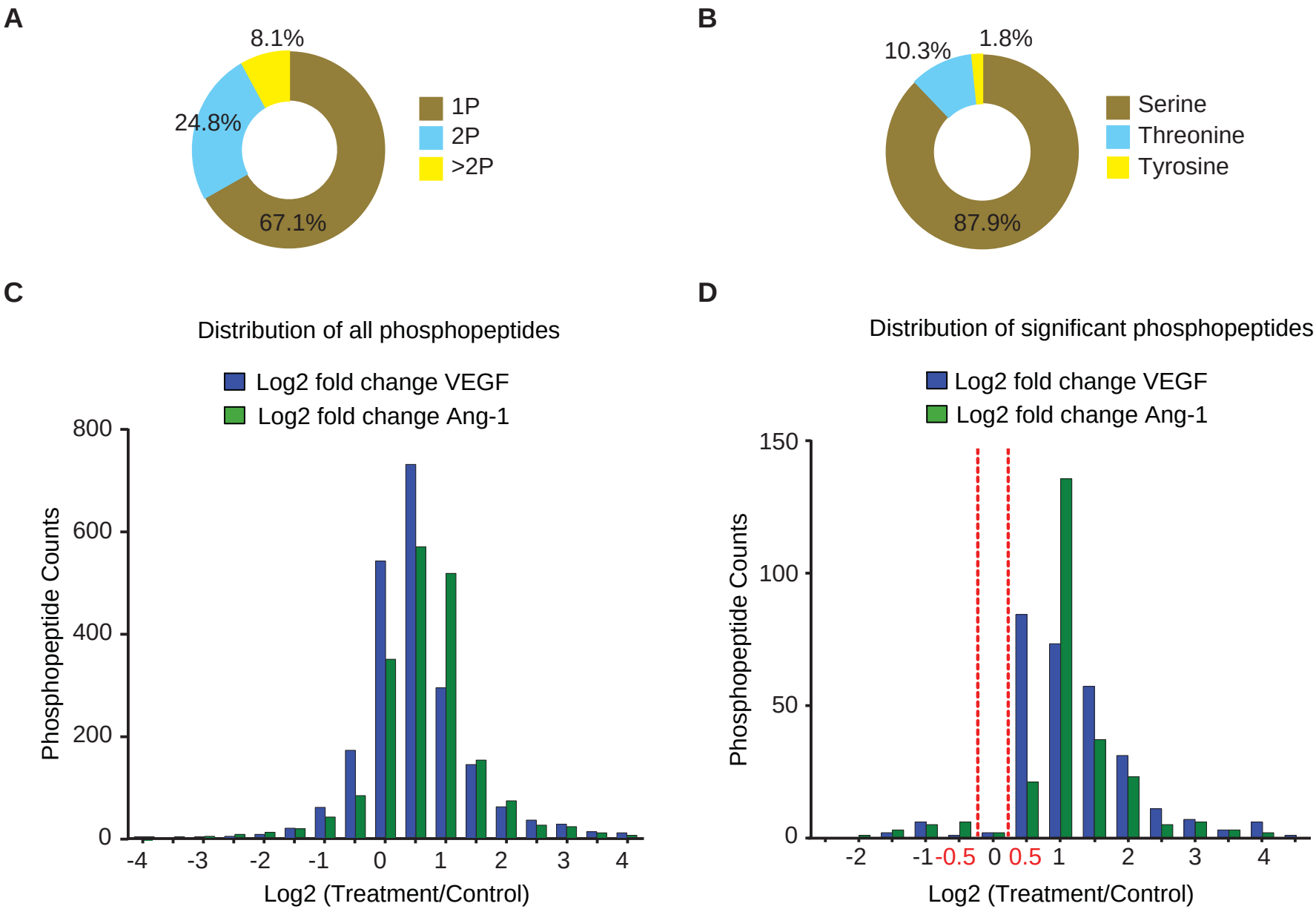

A
8.1%
24.8%
67.1%
1P
2P
>2P
B
10.3%
1.8%
87.9%
Serine
Threonine
Tyrosine
C
Distribution of all phosphopeptides
Log2 fold change VEGF
Log2 fold change Ang-1
Phosphopeptide Counts
800
600
400
200
0
-4
-3
-2
-1
0
1
2
3
4
Log2 (Treatment/Control)
D
Distribution of significant phosphopeptides
Log2 fold change VEGF
Log2 fold change Ang-1
Phosphopeptide Counts
150
100
50
0
-2
-1
-0.5
0
0.5
1
2
3
4
Log2 (Treatment/Control)

Figure S3

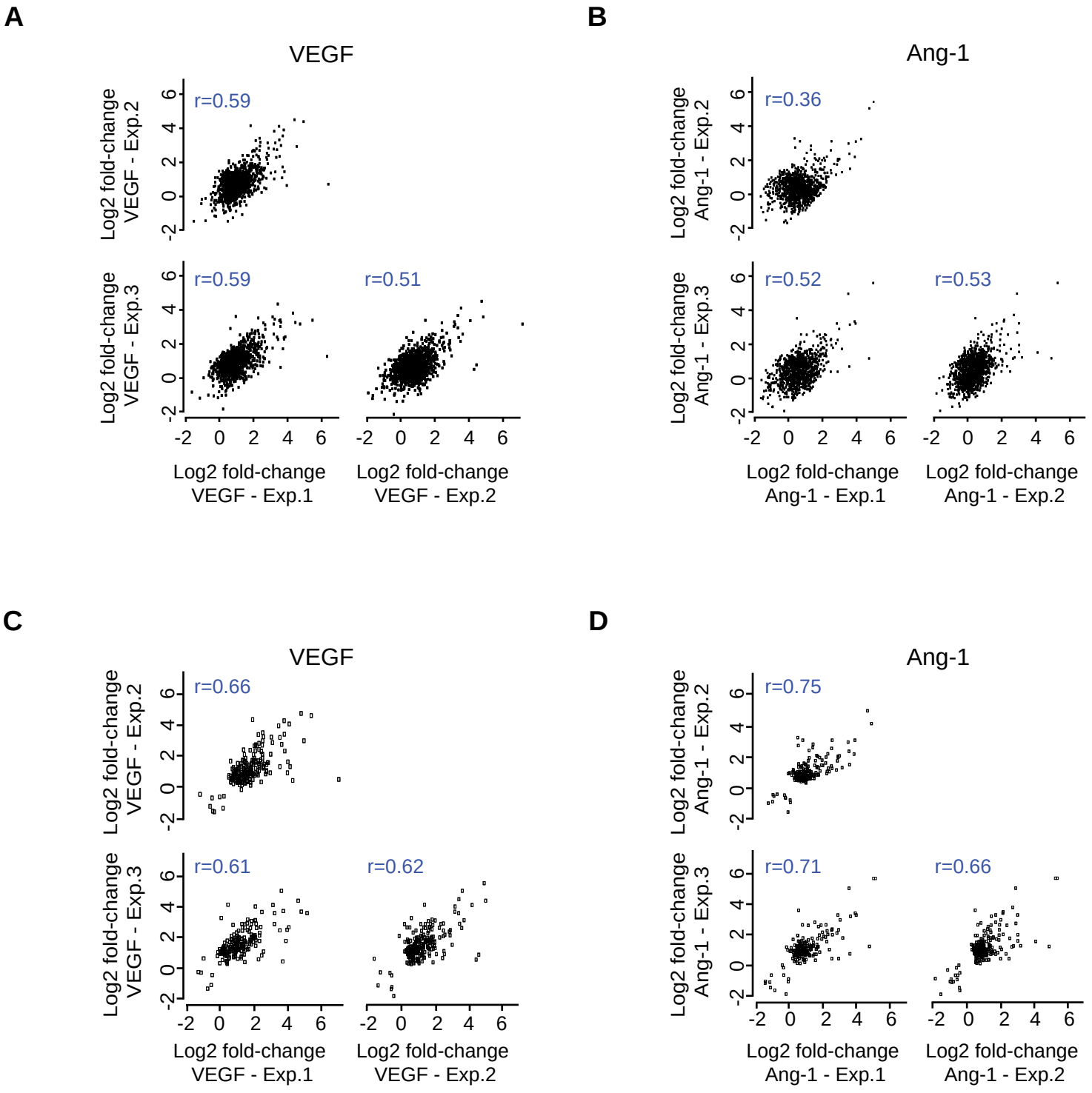

A
VEGF
r=0.59
r=0.59
r=0.51
Log2 fold-change VEGF - Exp.2
Log2 fold-change VEGF - Exp.3
Log2 fold-change VEGF - Exp.1
Log2 fold-change VEGF - Exp.2
B
Ang-1
r=0.36
r=0.52
r=0.53
Log2 fold-change Ang-1 - Exp.2
Log2 fold-change Ang-1 - Exp.3
Log2 fold-change Ang-1 - Exp.1
Log2 fold-change Ang-1 - Exp.2
C
VEGF
r=0.66
r=0.61
r=0.62
Log2 fold-change VEGF - Exp.2
Log2 fold-change VEGF - Exp.3
Log2 fold-change VEGF - Exp.1
Log2 fold-change VEGF - Exp.2
D
Ang-1
r=0.75
r=0.71
r=0.66
Log2 fold-change Ang-1 - Exp.2
Log2 fold-change Ang-1 - Exp.3
Log2 fold-change Ang-1 - Exp.1
Log2 fold-change Ang-1 - Exp.2

Figure S4

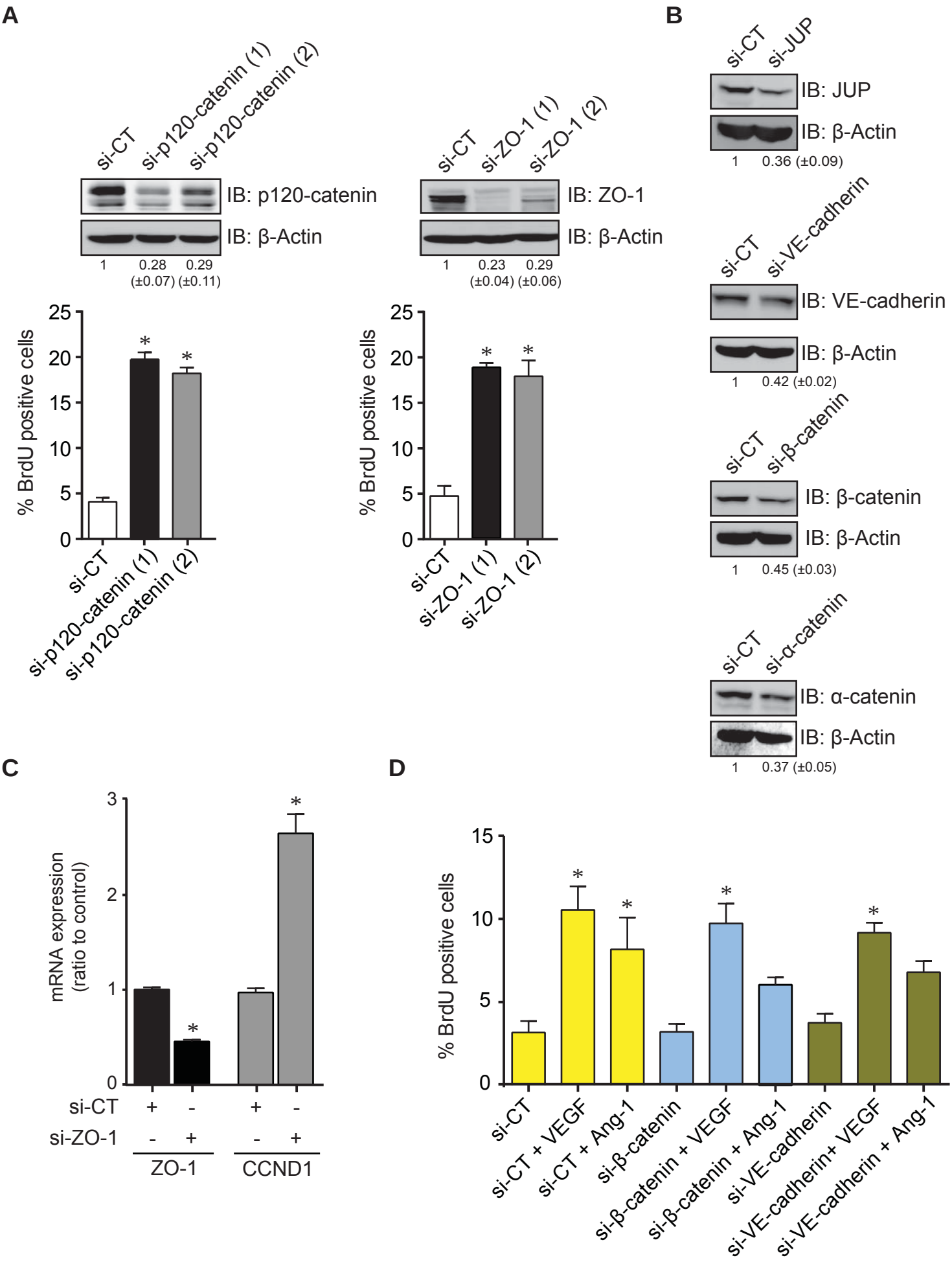

A
si-CT
si-p120-catenin (1)
si-p120-catenin (2)
IB: p120-catenin
IB: β-Actin
1 0.28 (±0.07) 0.29 (±0.11)
si-CT
si-ZO-1 (1)
si-ZO-1 (2)
IB: ZO-1
IB: β-Actin
1 0.23 (±0.04) 0.29 (±0.06)
% BrdU positive cells
si-CT
si-p120-catenin (1)
si-p120-catenin (2)
si-ZO-1 (1)
si-ZO-1 (2)
B
si-CT
si-JUP
IB: JUP
IB: β-Actin
1 0.36 (±0.09)
si-VE-cadherin
IB: VE-cadherin
1 0.42 (±0.02)
si-β-catenin
IB: β-catenin
1 0.45 (±0.03)
si-α-catenin
IB: α-catenin
1 0.37 (±0.05)
C
mRNA expression (ratio to control)
si-CT
si-ZO-1
ZO-1
CCND1
D
% BrdU positive cells
si-CT
si-CT + VEGF
si-CT + Ang-1
si-β-catenin
si-β-catenin + VEGF
si-β-catenin + Ang-1
si-VE-cadherin
si-VE-cadherin+ VEGF
si-VE-cadherin + Ang-1

Figure S5

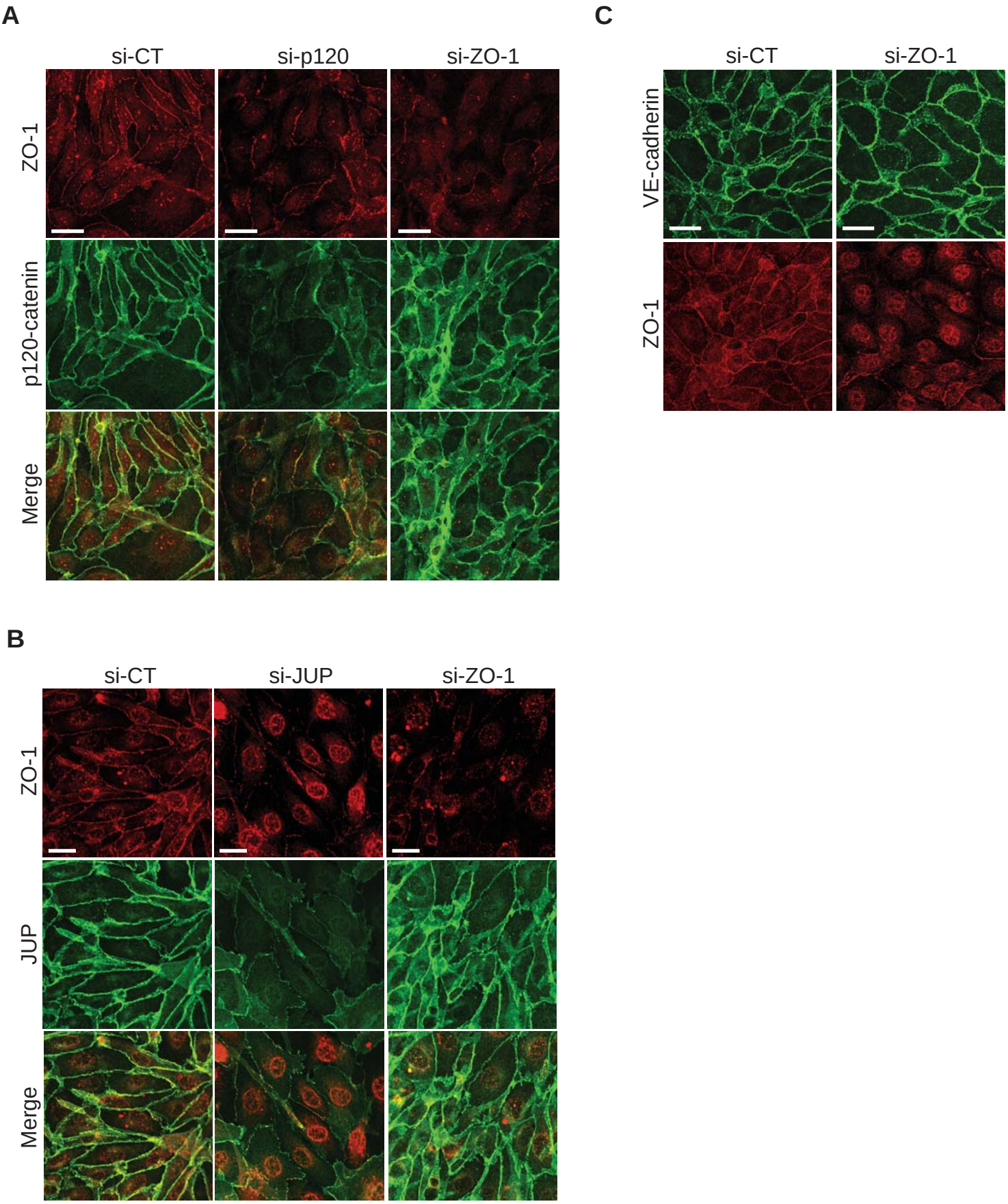
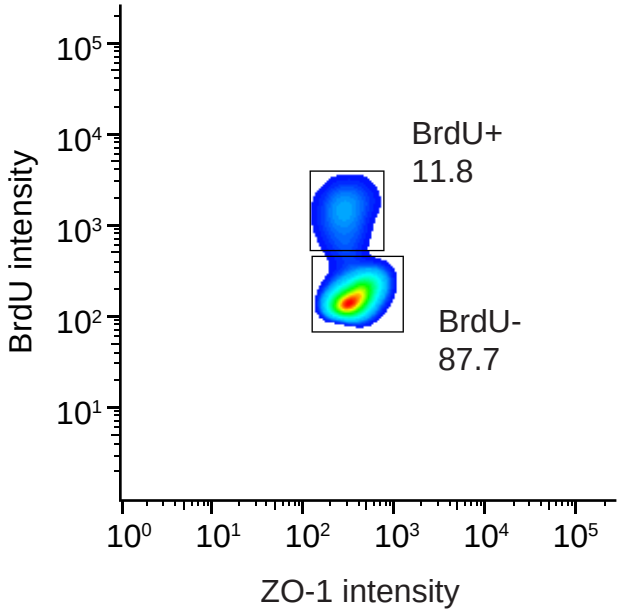

A
si-CT
si-p120
si-ZO-1
ZO-1
p120-catenin
Merge
C
si-CT
si-ZO-1
VE-cadherin
ZO-1
B
si-CT
si-JUP
si-ZO-1
ZO-1
JUP
Merge

Figure S6


BrdU intensity
10^5
10^4
10^3
10^2
10^1
BrdU+
11.8
BrdU-
87.7
10^0
10^1
10^2
10^3
10^4
10^5
ZO-1 intensity