SUPPLEMENTAL MATERIAL
Supplemental Material and Methods

Cell culture

For the direct isolation of human umbilical vein endothelial cells (HUVECs), the cells were obtained by collagenase treatment of term umbilical cord veins as described[1], and then incubated with red blood cell (RBC) lysis buffer (BioLegend, San Diego, CA) for 15 min at room temperature (RT). These freshly isolated quiescent HUVECs were centrifuged at 1400 rpm for 5 min and resuspended in 1 mL of 0.05% BSA PBS with FcR blocking reagent (1:200) for 30 min. HUVECs were incubated with APC anti-human CD45, FITC anti-human CD31 antibodies (BioLegend), and PE anti-human CD105 (BioLegend) for 45 min, washed twice with cold PBS and analyzed on a FACSort flow cytometer (BD Biosciences, San Jose, CA). All $CD31^+$ $CD45^-$ ECs were also $CD105^-$. A portion of these cells was collected directly in Trizol and RNA was used for miR-17~92 expression analysis (freshly isolated quiescent HUVECs). The remainder of cells was directly plated on 0.1% gelatin-coated flasks in M199 10% FBS supplemented with VEGF-165 (50 ng/mL) (BD Biosciences) for different time points (stimulated freshly isolated HUVECs) to determine the effect of VEGF on miR-17~92 expression.

Regular HUVECs were obtained from the tissue culture core laboratory of the Vascular Biology and Therapeutics program (Yale University, New Haven, CT) and were cultured as previously described [2-6]. Human aortic endothelial cells (HAECs) (Lonza, Allendale, NJ) were cultured in EGM™ BulletKit™.

To determine the effect of VEGF or bFGF (Sigma, St Louis, MO) stimulation on miR-17~92 expression, HUVECs or HAECs were starved in M199 or EGM™ 0.1% BSA respectively for 12 hours prior to stimulation with VEGF-165 (50 ng/mL) or bFGF (25 ng/mL).

miR-17~92 cluster Inhibition *in vitro*

HUVECs were transfected with 70nM mix of miR-17~92 inhibitors [10 nM each, including Inh-miR-17, Inh-miR-17*, Inh-miR-18, Inh-miR-19a, Inh-miR19b, Inh-miR-20 and Inh-miR-92a (Dharmacon, Lafayette, CO)] using Oligofectamine (Life Technologies, Carlsbad, CA) as previously described[2, 3]. Control samples were treated with an equivalent concentration (70nM) of an inhibitor negative control sequence (CI).

siRNA Knockdown of Elk-1 and PTEN

HUVECs were transfected with 30 nM Elk-1 siRNA (5'GCAAGGCAAUGGCCACAUC 3'), 30 nM PTEN siRNA SMARTpool or 30 nM non-silencing siRNA (NS) (5'AATTCTCCGAACGTGTCACGT3') (Dharmacon) using Oligofectamine (Life Technologies) as previously described[2-4].

Vector construction

The promoter region of 1353 bp (pro1353) of the human miR-17~92 cluster was kindly provided by Dr. Hammond[7]. Deletion constructs were generated with the following restriction endonucleases: EcoRI 975 bp construct, PstI 805 bp construct and SmaI 335 bp construct. Deletion of Elk-1 binding site within miR-17~92 promoter region (944-937) was generated using Multisite-QuikChange (Stratagene, La Jolla, CA) according to the manufacturer's protocol. For Elk-1 overexpression, Elk-1 expression vector was purchase from Life Technologies. For PTEN overexpression, human PTEN clone MGC11227 was amplified and cloned with 5' KnpI and 3' MfeI sites, ligated to a 5' EcoRI and 3' NotI T2A-EGFP-bGHpoly(A) obtained from the pSpCas9(BB)-2A-GFP plasmid (Addgene Plasmid #48138), inserted into a pENTR1A no ccDB (Addgene Plasmid #17398), then into pEZY3 (Addgene Plasmid #18672) with Clonase (Invitrogen), this vector is referred as PTEN OE and produce PTEN-T2A fusion protein. A pEZY3-EGFP plasmid was used as an empty control, referred as CT OE.

Transient transfection and reporter gene assay

miR-17~92 promoter and deletion constructs transfection

HUVECs ($10x10^4$ cells/well) plated in 12-well plate were co-transfected with 0.5 µg of the indicated miR-17~92 promoter reporter constructs (see above) and 0.01 µg of *Renilla* luciferase control reporter vector using Lipofectamine LTX (Life Technologies) for 6 hours. Then, HUVECs were washed with PBS and incubated with 20% FBS M199 for 8 hours. Finally, cells were starved overnight (O/N) and then stimulated with VEGF (50 ng/mL). Luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega, Madison, WI). Firefly luciferase activity was normalized to the corresponding *Renilla* luciferase activity.

miR-17~92 promoter and Elk-1 construct transfection

HUVECs were co-transfected as described above with 0.25 µg of the miR-17~92 full length promoter construct (pro1353), 0.25 µg of Elk-1 overexpression vector (Life Technologies) and 0.01 µg of *Renilla* luciferase control reporter vector.

miR-17~92 inhibition and PTEN construct transfection

For PTEN overexpression HUVECs were transfected with a mix of miR-17~92 inhibitors or CI and 0.25 µg of PTEN-T2A or control overexpression vector (CT OE) using lipofectamine LTX as described above.

RNA isolation and qRT-PCR

Total RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's protocol. cDNA was synthesized using iScript RT supermix (Bio-Rad, Richmond, CA), following the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed in triplicate using iQ SYBR green supermix (Bio-Rad) on an iCycler real-time detection system (Bio-Rad). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) or 18S rRNA was used for normalization.

Pri-miRNA levels were detected using the TaqMan RT kit (Life Technologies) according to manufacturer's protocol. The members of the miR-17∼92 cluster are tightly grouped within an 800-bp region of human chromosome 13[8]. Pri-miR-17~92 was detected using two different primers, pri-miR-17~92 (1) and (2) that recognize the miR-17-miR-18a-miR-19a stem loops and miR-20a-miR-19b-miR-92a stem loops, respectively. Mature miRNA levels of the different miR-17∼92 cluster members were detected using TaqMan miRNA Assay kit (Life Technologies) according to manufacturer's protocol. Quantitative real-time PCR was performed using TaqMan Universal Master Mix (Life Technologies)[2-6]. 18S rRNA or small RNA U6 was used for normalization, respectively.

Primer sequences for quantitative real time PCR:

Elk-1 forward primer 5' CAGCCAGAGGTGTCTGTTACC 3' and Elk-1 reverse primer 5' GAGCGCATGTACTCGTTCC' 3.

GAPDH forward primer 5' GAAGGTGAAGGTCGGAGTC 3' and GAPDH reverse primer 5' GAAGATGGTGATGGGATTTC 3'.

18S forward primer 5' GCTTAATTTGACTCAACACGGGA 3' and 18S reverse primer 5' AGCTATCAATCTGTCAATCCTGTC 3'.

NR4A2 forward primer 5' GGTTGCAATGCGTTCGTGGC 3' and NR4A2 reverse primer 5'AGCCCGTGTCTCTCTGTGACC 3'.

EGR3 forward primer 5' TGCTATGACCGGCAAACTCGC 3' and EGR3 reverse primer 5' AGGGGAGTCGAAGGCGAACT 3'.

NR4A2 and EGR3 gene expression was used as a positive control for VEGF stimulation[9] (Data not shown).

Human PTEN forward primer 5' TGGATTCGACTTAGACTTGACC 3' and human PTEN reverse primer 5' TGTCTTTCAGCACAAAGATTGTAT 3'.

Mouse PTEN forward primer 5' AGCCATCATCAAAGAGATCGT 3' and mouse PTEN reverse primer 5' GCTTTGAATCCAAAAACCTTACTAC 3'.

Western blot analysis

Nuclear and cytoplasmic fractions were isolated using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Life Technologies) according to the manufacturer's protocol. Whole cell lysate isolation and western blot were performed as previously described[2-6]. Rabbit polyclonal antibody against Phospho-Erk (Thr202/Tyr204) (1:1000), rabbit polyclonal antibody against Phospho-Elk-1 (Ser383) (1:500), rabbit polyclonal antibody against PTEN (1:1000), and mouse monoclonal antibody against Erk (1:1000) were obtained from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal antibodies against Elk-1 (1:500) and Lamin B1 (1:1000) were purchased from Abcam (Cambridge, MA). Mouse monoclonal Hsp90 (1:3000) antibody was purchased from BD Biosciences. Secondary antibodies were fluorophore-conjugated antibodies (LI-COR Biotechnology, Lincoln, NE). Bands were visualized by using the Odyssey Infrared Imaging System (LI-COR Biotechnology).

Bioinformatics analysis

Promoter analysis, transcription factor prediction

Sequence promoter analysis to identify potential transcription factors was performed using Softberry (http://www.softberry.com), TF bind (http://tfbind.hgc.jp) and TF search (http://www.cbrc.jp/research/db/ TFSEARCH.html) software.

miRNA target prediction analysis

Mouse miRNA target predictions were obtained from miRwalk[10] Only targets predicted by at least 3 algorithms were chosen for further analysis. Human miRNA targets were obtained from miRanda (The microRNA.org resource: targets and expression [11]) and TargetScan[12]. miRNAs targets from the same seed were combined and entered as a single entry in Venny 2.0.2 [Oliveros, J.C. (2007-2015) http://bioinfogp.cnb.csic.es/tools/venny/index.html] Venny is an interactive tool for comparing lists with Venn's diagrams where the targets common for all four seeds were identified. Venny 2.0.2 was also used to find targets that were common for all four seeds in mouse and human. The targets in bold are genes that are expressed in ECs (with at least 10 fpkm in HUVEC [13]).

Gene expression array analysis

HUVECs were transfected with a mix of miR-17~92 inhibitors or CI as described above for 36 hours and incubated in the presence of VEGF (stimulated conditions). PCR array gene expression of human genes related to angiogenesis (Qiagen) was performed according to the manufacturer's protocol and as described previously[4]. Genes upregulated >1.3 or downregulated < -1.3 in Inhibitor miR-17~92 over CI and with *p*-value <0.1 were analyzed with the Disease and Functions feature in Ingenuity Pathway Analysis software (Qiagen) to find predicted activated or deactivated pathways. Only pathways relevant to endothelial cells with significant p-value were considered. Blue lines connecting the genes and pathways indicate predicted inhibitory effect. Green genes are downregulated, while red genes are upregulated in Inhibitor miR-17~92 compared to CI.

Chromatin Immunoprecipitation Assay

HUVECs starved for 12 hours and then treated with VEGF (50 ng/mL) were cross-linked in 1% formaldehyde for 15 min at RT. DNA from fixed chromatin cells were then subjected to immunoprecipitation using a ChIP assay kit (Millipore, Billerica, MA), and antibodies against Elk-1 (Abcam) or anti-rabbit IgG (Millipore). Purified DNA was analyzed by quantitative real time PCR which produced different fragments of the miR-17~92 promoter containing the Elk-1 binding site.

Primer 1 forward 5' CTGAGAATTCCGGAATTTCCT 3' and primer 1 reverse 5' CAGCTGCATTTAGTAAGAACTC 3'
Primer 2 forward 5' CAGTGATATGTGCTTTGCAG 3' and primer 2 reverse 5' CTCCAGTAGAAATAGCATAGCTC 3'
Primer 3' forward 5' GAATTTCCTGAACCACAATGTG 3' and primer 3 reverse 5' GCATTTAGTAAGAACTCTGGGT 3'
MCL1 primer forward 5' AGTCCCCAACTATGCCCTCT 3' and MCL1 primer reverse 5' CTCTGTGCTTCCCTGAGACC 3'
GNGT1 primer forward 5' ATTGCAAAGAGGGCAGAAGA 3' and GNGT1 primer reverse 5' TTGTGAGCGTTTTTCCAACA 3'
MCL1 and GNGT1 promoters are used as positive and negative controls respectively[14].

Cell number assessment

HUVECs were transfected with a mix of miR-17~92 inhibitors or CI as described above. 24 or 48 hours post-transfection, cells were collected and cell number was assessed by using a hemocytometer as previously described[2, 3]. Viability was determined by Trypan blue dye exclusion.

Flow cytometry analyses of proliferation and apoptosis

DNA synthesis–based cell proliferation assay, BrdU incorporation, was performed described[3]. Briefly, HUVECs were transfected with a mix of miR-17~92 inhibitors or CI as described above. At 44 hours post-transfection, HUVECs were incubated with 100 μM 5-bromo-2'-deoxyuridine (BrdU) (Sigma) for 4 hours at 37°C and fixed in 70% cold ethanol. Then, cells were incubated with 2M HCl for 20 min, washed with PBS and incubated with PBS, 0.5% Tween 20 and 1% normal goat serum. Finally, cells were centrifuged and labeled with FITC anti-BrDU (BD Biosciences) for 1 hour at RT. Ten thousand events per sample were acquired in a FACScalibur flow cytometer (Becton Dickinson) for data analysis using CellQuest software by selective gating to exclude doublet cells.

Cell cycle analysis, apoptotic measurement (SubG1), was ascertained as previously described [4]. Briefly, at 48 hours post-transfection, cells (floating and attached) were collected and fixed in 70% cold ethanol for 1 hour, washed with PBS, and stained with PI as described[15] and analyzed by flow cytometry as described above. Apoptotic cells were determined by their hypochromic, subdiploid staining profiles (subG1 population) using WinMDI 2.9 sofware (J. Trotter 1993-1998). Incubation with 5 ng/mL of TNF (R&D Systems, MN) plus 5μg/mL Cyclohgeximide (Sigma) was used as positive control of apoptosis in ECs and incubation with pan-caspase inhibitor 50μM zVAD (R&D) was use to inhibit apoptosis[16].

Fibrin gel bead assay

HUVECs were transfected with a mix of miR-17~92 inhibitors or CI as described above. Fibrin gel bead assay was performed as described[17, 18]. Briefly, $1x10^6$ HUVECs were mixed with 2500 beads in EGM™ BulletKit™ for 4 hours at 37°C. Coated beads were transferred to T25 flask in 5mL of EGM™ BulletKit™ and left O/N. The following day, the coated beads were resuspended in fibrinogen solution (2.0 mg/mL fibrinogen, 0.15 Units/mL of aprotinin) at a concentration of ~200 beads/mL. 0.625 Units/mL of thrombin was added to each well of a 24-well plate and then 0.5 mL of the fibrinogen/bead suspension was added to each well. Finally, the plate was placed at 37°C for 10-15 min to generate a clot and 1 mL of EGM™ BulletKit™ was added to each well. HUVECs were allowed to undergo morphogenesis for 2-3 days. Angiogenic sprouting was quantified by measuring cumulative sprout length, branches, sprouts, and number of detached cells using NIH ImageJ software. Sprouting was defined as a vessel with length greater than or equal to the diameter of the bead. Branching was defined as a segment of a vessel that has branched off from the major vessel sprout[17, 18]. For each

experiment, at least 10 spheroids per triplicate well and condition were analyzed in each experiment.

Mice

All mouse experiments were approved by the Institutional Animal Care Use Committee at the Yale School of Medicine. To generate inducible vascular endothelial cell-specific miR-17~92 iEC-KO mice, we crossed miR-17–92$^{flox/flox}$ mice[19] with a Tamoxifen (TMX)-inducible expressed Cre-recombinase (Cre-ERT2) under the regulation of vascular endothelial cadherin promoter (Cdh5)[20, 21] to achieve specific inactivation of miR-17~92 cluster in ECs. For analysis at postnatal day (P) 6 neonates, 50 µg of TMX was injected via i.p. on three consecutive days, P1-P3[21, 22]. Percentage of reduction of the levels of miR-17~92 cluster members in miR-17~92 iEC-KO mice compared to control littermates ranged from 33% to 77% (Supplemental Figure 5C).

Mouse Lung and Retina Endothelial Cell Isolation

Mouse lung endothelial cells (MLECs) were isolated, as previously described[5, 22], from three pairs of lungs dissected from 3 weeks-old wild-type (WT) mice or P6 miR-17~92 iEC-KO mice. Briefly, freshly isolated lung tissue was minced with scissors and allowed to digest at 37 °C with 2 mg/mL ≈ 175 u/mg Type I collagenase (Sigma) for 1 hour. Lung tissue was further subjected to mechanical disruption by passage ≈ 12 times through a 14-gauge needle and filtration through 70 µm steel mesh. Cells were washed once with DMEM 10% FBS, centrifuged at 1300 rpm for 8 min, and resuspended in 2 mL of cold 0.1% BSA PBS. ECs were immuno- isolated using sheep anti-rat IgG–coated magnetic beads precomplexed with 12.5 µg of rat α-platelet/endothelial cell adhesion molecule 1 (PECAM-1) antibody (Pharmingen, San Diego, CA). A portion of ECs were collected for miRNA expression analysis, the remainder plated on 0.1% gelatin-coated flasks in EGM™ BulletKit™ supplemented with VEGF-164 (50 ng/mL) (BD Biosciences).

Retinal ECs were isolated from retina dissected from WT mice at P2, P5, P12, P17, and adult mice as described above.

Mouse retina vascular system analysis

For retina staining, procedures were followed as described[22, 23]. Briefly, retinas were dissected out and stained with Alexa-594-conjugated isolectin B4 (1:200) (Life Technologies), mouse anti-phospho-histone H3 (1:200) (Cell Signaling) and rabbit anti-COL IV (1:200) (AbD Serotec, Richmond, CA) and rabbit anti-cleaved caspase 3 (1:200) (Cell Signaling). Slides were analyzed using a Leica TCS SP5 microscope. Branch point numbers were quantified as described[22, 23], and the retinal area and vascular density were quantified with ImageJ.

Tumor-induced neovascularization

LLC cells (10^6) were injected s.c. in the dorsal flank of 6-week-old as described[2, 5], of previously treated TMX, miR-17~92 iEC-KO or CT mice . After 14 days, animals were euthanized and tumor tissues were collected and frozen in Tissue-Tek OCT (Sakura Finetek, Torrance, CA). Frozen sections of LLC tumors were stained with anti-mouse PECAM-1-PE labeled antibody (BD Biosciences) and DAPI. Stained slides were imaged with a Zeiss Axiovert 200M inverted microscope and a Hamamatsu camera. For quantification, 3 sections per mouse sample were analyzed and from each sample 2 images were captured from random areas of each tissue section. Microvessel density was quantified by measuring the PECAM-1 per sample area. ImageJ was used to determine the number of positive structures or pixels per sample area.

Ear angiogenesis

Adenoviruses encoding murine VEGF-A164 (Ad5CMV VEGF164) (Gene Transfer Vector Core, University of Iowa) (2 x 10^8 viral particles) were injected intradermally as described [5] into the right ears of 6 week-old, of previously treated TMX, miR-17~92 iEC-KO or CT mice. The left ears were injected with a control virus expressing eGFP (Ad5CMV eGFP)

(Gene Transfer Vector Core, University of Iowa). After 3 days, animals were euthanized and the ears were removed and finally embedded and frozen in OCT compound (Tissue-Tek; Sakura) for staining with anti-mouse PECAM-1-PE labeled antibody (BD Biosciences) and DAPI. Stained slides were imaged using a Zeiss Axiovert 200M inverted microscope and quantification of PECAM-1 positive structures was performed as above indicated.

Statistics

Statistical analyses were performed with GraphPad Prism 6 software (GraphPad, San Diego, CA), using the two-tailed, unpaired Student's *t*-test or one-way ANOVA, when appropriate. Data are expressed as mean ± S.E.M. *P* values $P \leq 0.05$ were considered statistically significant.

Supplemental References

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Online Figure Legends

Online Figure I. VEGF stimulates the expression of the miR-17~92 cluster in ECs.
qRT-PCR analysis of pri-miRNA-17~92 levels in HUVECs starved for 12 hours and then treated with VEGF (50 ng/mL) for the indicated times. Data correspond to the mean ± S.E.M. of three independent experiments. * P≤0.05

Online Figure II. Efficiency of siRNA mediated knockdown of Elk-1 in ECs.
qRT-PCR and Western blot analysis (insert) of Elk-1 mRNA and protein levels respectively, of HUVECs transfected with Elk-1 siRNA or non-silencing (NS) control siRNA for 48 hours. Data correspond to the mean ± S.E.M. of three independent experiments, * P≤0.05.

Online Figure III. FGF stimulates the expression of the miR-17~92 cluster in ECs.
(A) qRT-PCR analysis of pri-miRNA-17~92 levels in HUVECs starved for 12 hours and then treated with FGF (25 ng/mL) for 3 hours. (B) qRT-PCR analysis of pri-miRNA-17~92 levels human aortic endothelial cells (HAECs) starved for 12 hours and then treated with VEGF (50 ng/mL) for 3 hours. Data correspond to the mean ± S.E.M. of three independent experiments. For (B) each experiment was performed with different batch of HAECs ,* P≤0.05.

Online Figure IV. miR-17~92 cluster inhibition does not induce apoptosis in ECs.
(A) HUVECs were transfected with 70nM mix of miR-17~92 inhibitors (Inh-miR-17~92) or control inhibitor (CI) for 24 hours. Cells were harvested and treated with anti-BrdU antibody and analyzed by flow cytometry as described in online Material and Methods. One representative experiment out of three with similar results is shown. HUVECs were transfected as indicated in A. After 12 hours post-transfection, HUVECs were treated with zVAD (50μM) or vehicle control (DMSO) and harvested at different time points. (B) Total number of cells. Data are expressed as mean of triplicates ± S.D. of one experiment out of three with similar results. (C) Flow cytometry analysis of DNA content at 48 hours. Percentage of apoptotic cells correspond to Sub G0/G1 population is shown. One representative experiment out of three is shown. Last row of plots shows the controls for the induction of apoptosis and ZVAD action. HUVECs were treated with TNF (5ng/mL) plus Cycloheximide (5μg/mL) ± zVAD (50μM) for 12 hours and collected for analysis of apoptosis as described above.

Online Figure V. Expression of miR-17~92 cluster in mouse ECs.
(A) qRT-PCR analysis of individual miRNAs from the miR-17~92 cluster in freshly isolated mouse lung endothelial cells (MLECs) upon VEGF (50 ng/mL) stimulation. Data are expressed as relative miRNA levels vs. freshly isolated control ECs and correspond to one representative experiment out of three independent experiments with similar results. (B) qRT-PCR analysis of individual miRNAs from the miR-17~92 cluster retinal ECs isolated from wild-type (WT) mice. Data are expressed as relative miRNA levels vs. P2 retinal ECs. (C) qRT-PCR analysis of individual miRNAs from the miR-17~92 cluster in freshly MLECs from miR-17~92 iEC-KO mice (TMX P1-P3) at P6. Data are expressed as relative miRNA levels vs. control littermates.

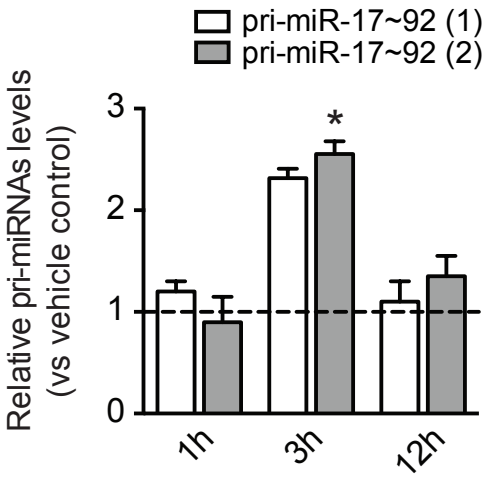
Online Figure VI. Endothelial-specific 17~92 deletion reduces developmental retinal angiogenesis. Mice were injected with TMX P1-P3 and assessed at P6 [CT, n = 6; miR-17~92 iEC-KO, n = 7]. (A) Retinal whole mount, (B) Vascular front and (C) Vascular plexus Isob4 immunostaining representative images. Magnification: 20X.

Online Figure VII. Loss of endothelial miR-17~92 does not affect apoptosis *in vivo.* Mice were injected with TMX P1-P3 and assessed at P6 [CT, n = 6; miR-17~92 iEC-KO, n = 7]. Isolectin B4/Cleaved Caspase3 immunostaining representative images. Magnification: 40X

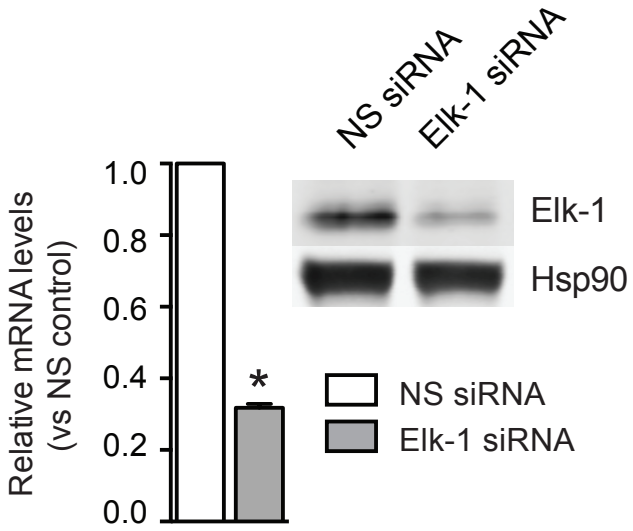
Online Figure VIII. Inhibition of miR-17~92 in endothelial angiogenic program.

(A) Gene expression array analysis in HUVECs transfected with 70nM mix of Inh-miR-17~92 or CI and incubated in the presence of VEGF (stimulated conditions). Samples were collected 36 post-transfection. The positive regulators of angiogenesis are indicated in red, negative regulators in blue, positive/negative regulators in grey. Data are expressed as fold change compared to CI (dashed line) *, $P \leq 0.05$. (B) Genes upregulated >1.3 or downregulated < -1.3 in Inh-miR-17~92 over CI and with *P*-value <0.1 were analyzed with the Disease and Functions feature in Ingenuity Pathway Analysis software (Qiagen) to find predicted activated or deactivated pathways. Only pathways relevant to endothelial cells with significant P-value are depicted. Blue lines connecting the genes and pathways indicate predicted inhibitory effect. Green genes are downregulated, while red genes are upregulated Inh-miR-17~92 compared to CI.

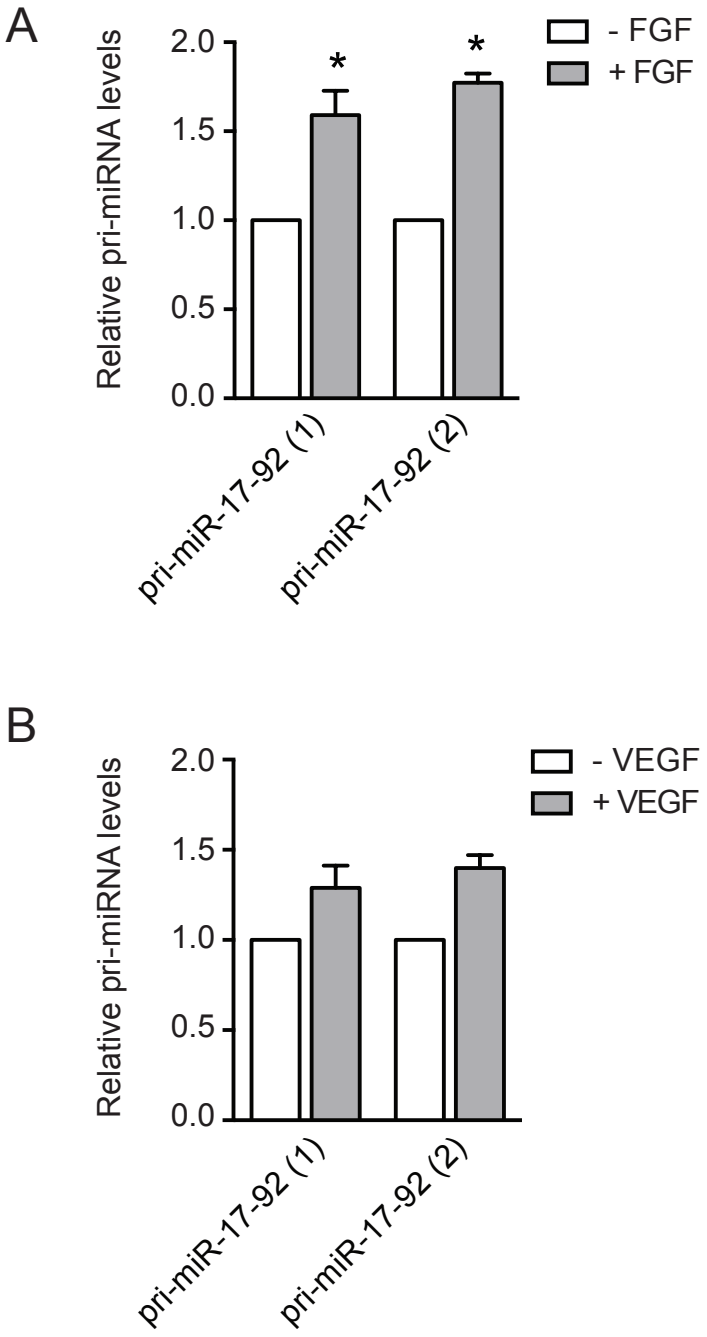
Online Figure I



Online Figure II

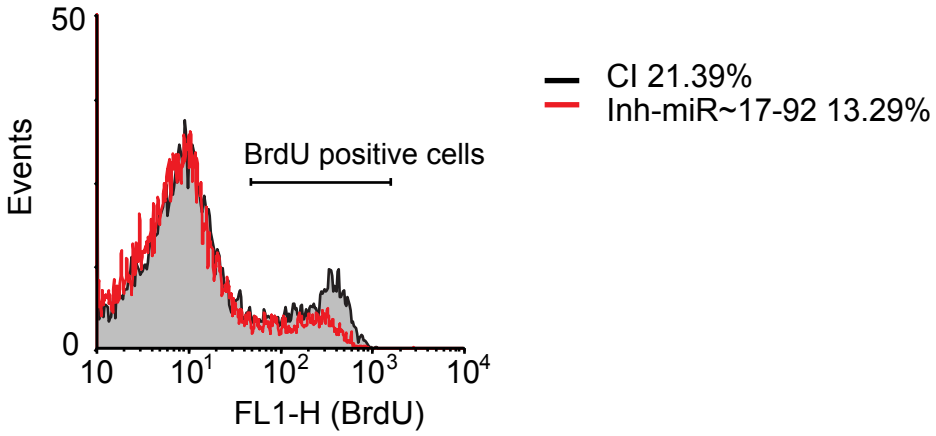


Online Figure III

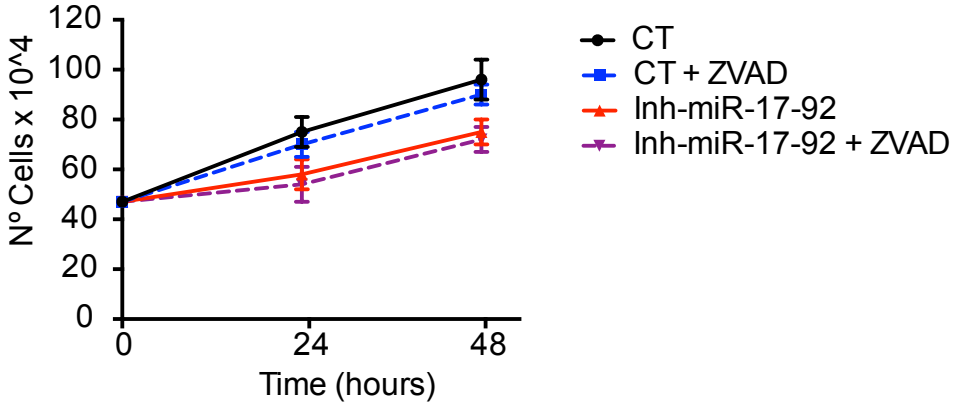


Online Figure IV

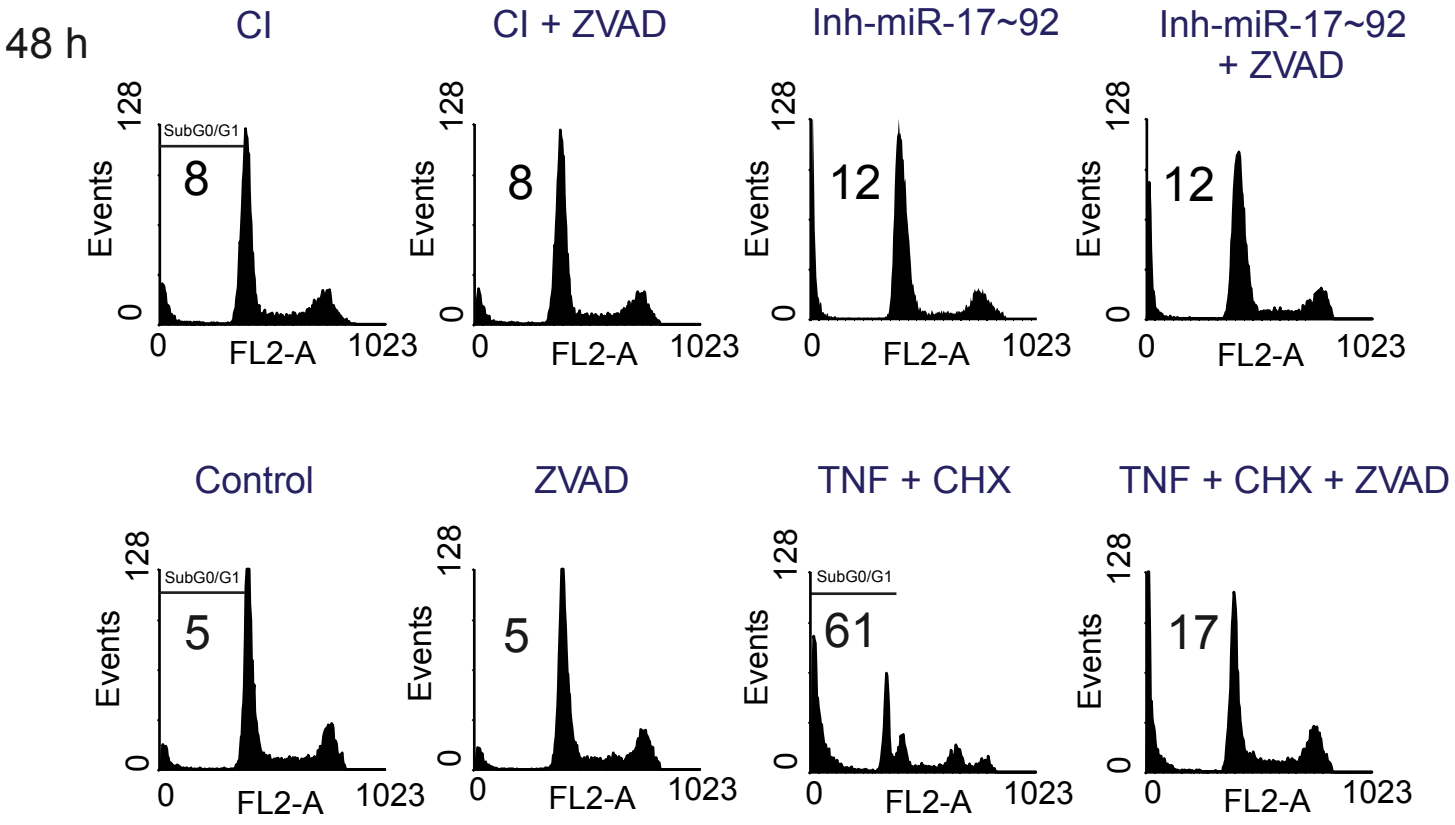
A



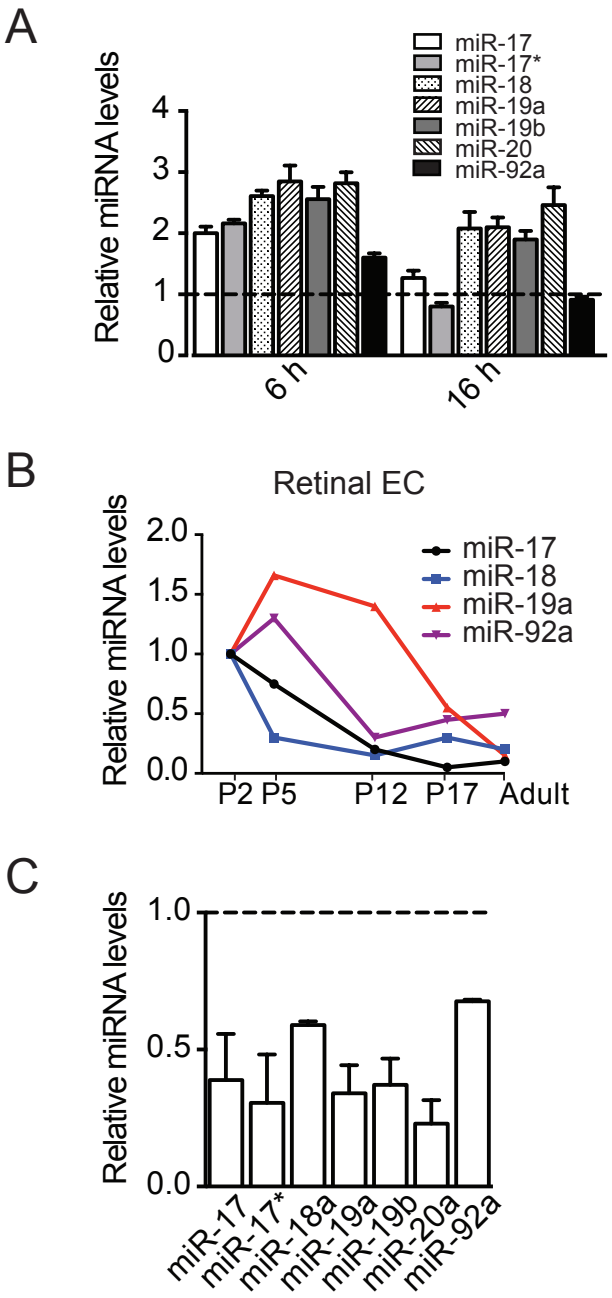
B



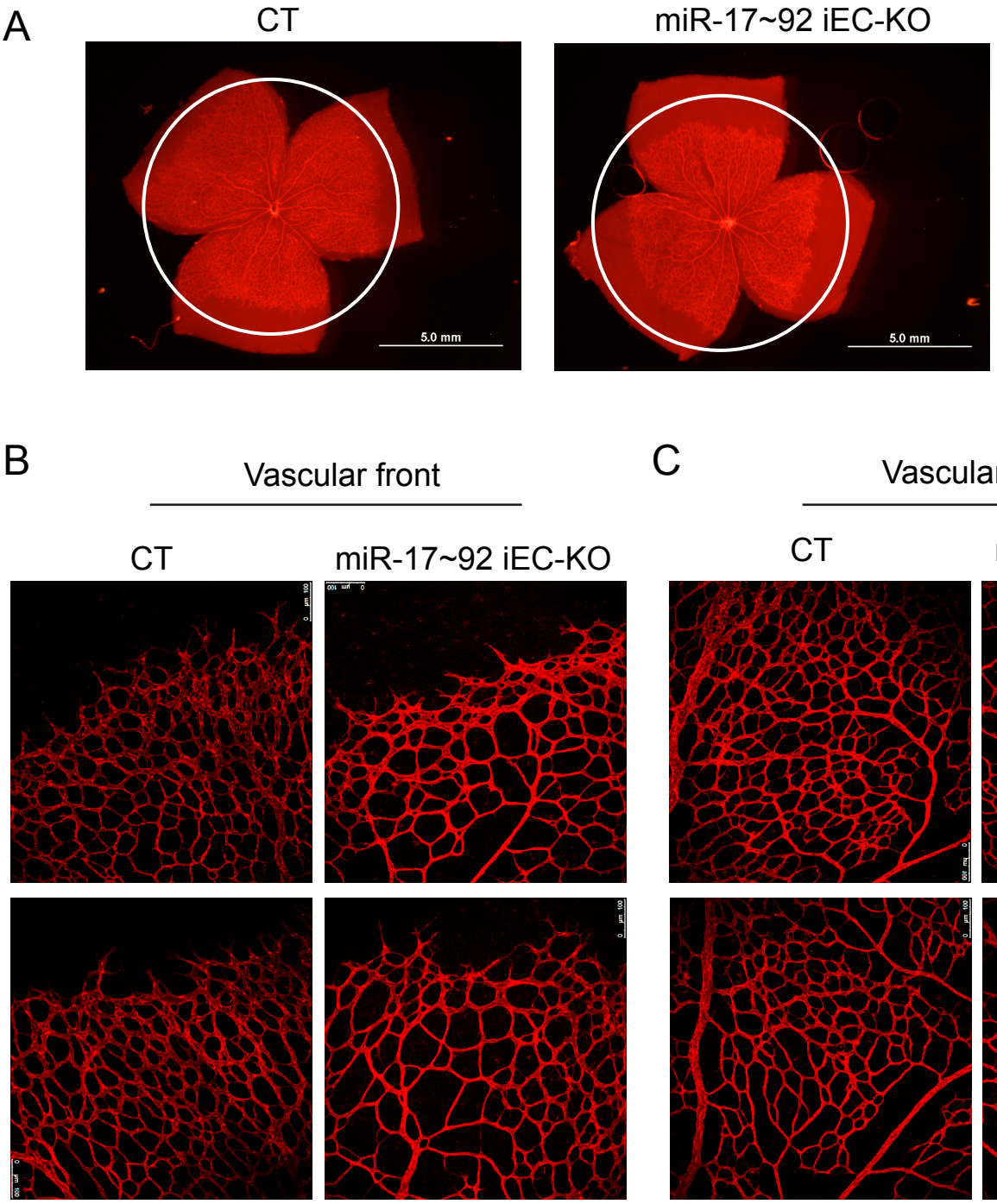
C



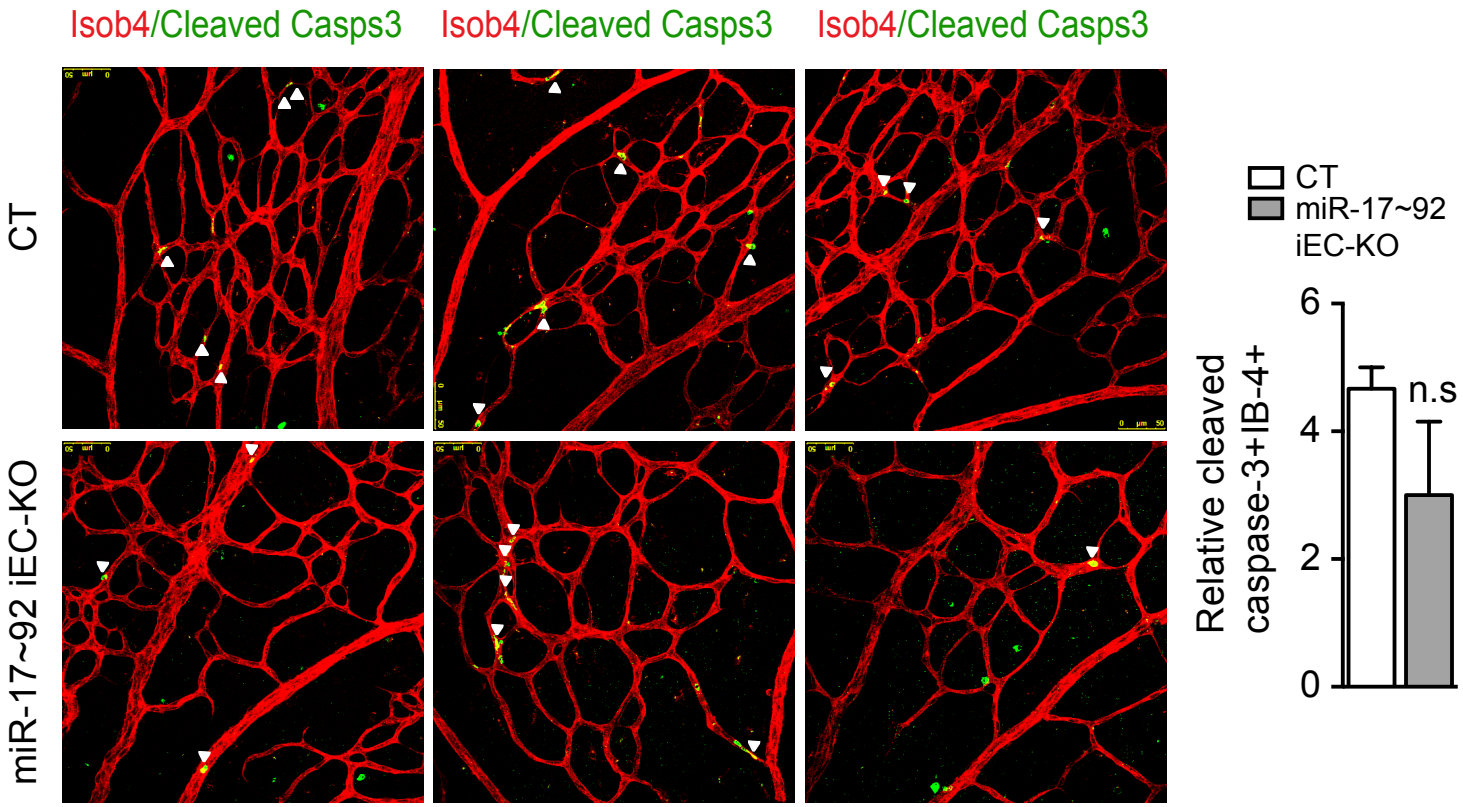
Online Figure V



Online Figure VI



Online Figure VII



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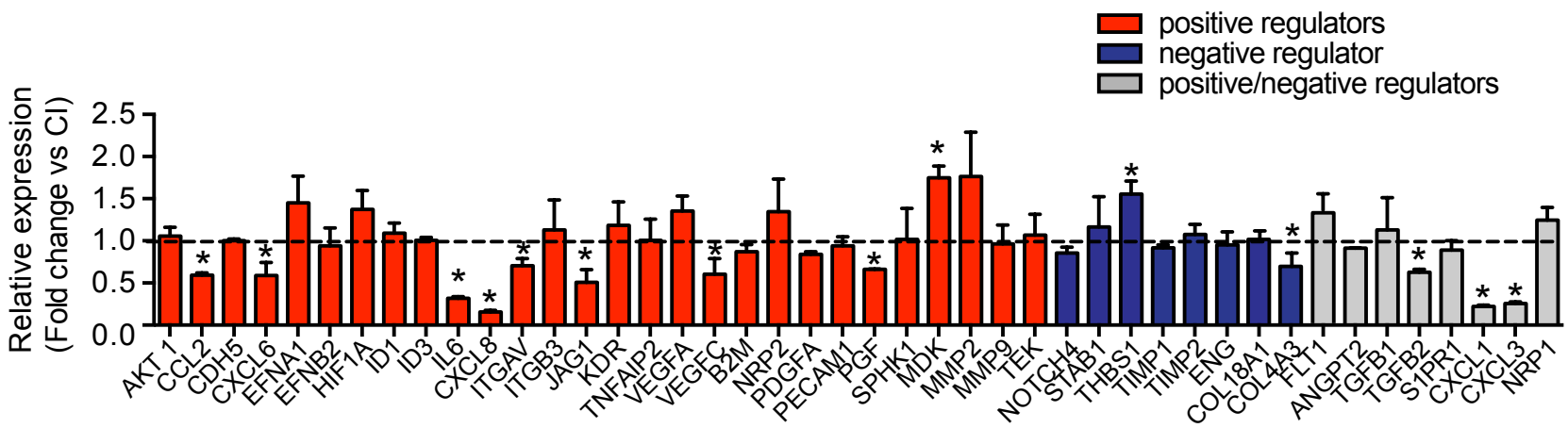
Online Figure VIII

A



B