CD36 regulates Factor VIII secretion from liver endothelial cells
Paula Reventun et al.

Methods

Single cell RNA-seq data processing

We re-analyzed a previous study of single cell RNA-seq data of human liver (GSE115469).[1] The counts matrices were loaded in R version 4.0.4, normalized based on sctransform and clustered with the k-nearest neighbor algorithm as implemented in Seurat.[2-6] Endothelial cell clusters were identified based on expression of known marker genes (CDH5, VWF, PECAM1).[7] The subpopulations of endothelial cells were identified based on gene expression of specific markers (SPARCL1, TM4SF1, CLEC14A, ID1, IGFBP7 and VWF for Cluster 12 (Sinusoidal Periportal HLEC), FCGR2B, S100A13, FCN3, LYVE1, STAB2, CLEC1B, FCN2, CRHBP, STAB1, F8 and CALCRL for Cluster 11 (Sinusoidal Central venous HLEC), ENG, PECAM1, RAMP3, INMT, DNASE1L3, LIFR, TIMP3, C7 and CTGF for Cluster 14 (portal endothelial cells) as previously described.[8,9]

Cell culture and sorting

Human umbilical vein endothelial cells (HUVEC) and human liver endothelial cells (HLEC) were from Lonza (Basel, Switzerland). To purify CD32+ HLEC, HLEC were incubated with CD32-FITC conjugated antibody (ab134384, Abcam, Cambridge, UK), washed, and sorted with a Beckman Coulter MoFlo XDP (California, US).[9] CD32+ HLEC passage 2 to 6 were grown on collagen-coated plates for three days, 30,000 cells were plated in 12 well plates and 10,000 in 96 well plates. hTERT- immortalized Human Aortic Endothelial Cells (TeloHAEC), EA.hy926 and HepG2 were from ATCC (Manassas,Virginia).

Quantitative real-time PCR

Total RNA was isolated from cells (RNeasy kit from Qiagen following the manufacturer's protocol). The A260/A280 ratio of all samples was between 1.9 and 2.1 as measured by spectrophotometry (NanoDrop; Thermo Scientific). 50 ng of RNA were used for a one step qPCR reaction performed with TaqMan probes (following the manufacturer´s instructions) on an CFX Connect thermal cycler (Bio-Rad). The qPCR probes Hs01634996_s1 FCGR2B, Hs03023943_g1 ACTB, Hs00252034_m1 F8, and Hs00354519_m1 CD36 were from Thermo Fisher Scientific. The Von Willebrand Factor (VWF) qPCR probe 450932996 was from Integrated DNA Technologies (Iowa, USA). Quantification was performed in triplicate for each sample. Expression results were calculated by the ΔΔCT method and were normalized to the reference gene ACTB. Statistical analysis was done by two-tailed unpaired Student's t-test and a $p < 0.05$ was considered significant.

Factor VIII Activity assay

CD32+HLEC were seeded in collagen-coated p12 well plates, 30000 cells were plated and three days after, media was replaced with fresh media (EGM-2 BulletKit (Lonza)), and then media was collected at 2, 4, 6, 8 and 24 h and FVIII activity was measured. Factor VIII activity levels in cell supernatants were detected with the commercially available plasma FVIII Chromogenic kit, Chromogenix Coatest® SP Factor VIII

(Diapharma). We followed the manufacturer's protocol, and we performed the assay in microplates with a 4 fold reduction of all volumes. The supernatants were analyzed without dilution, and we included a reference curve that was prepared by diluting a human plasma pool (sigma: P9523-1ml) in the cell culture medium (ECM complete). Absorbance was read at 405 nm and at 490 nm using a SpectraMax M5 Microplate Reader. Subtraction of measurements at 490 nm corrected for absorbance differences between wells.

Confocal microscopy

30,000 CD32+HLEC were seeded in collagen-coated coverslips 15 mm. Three days after seeding cells reached confluency. Cells were fixed with paraformaldehyde, permeabilized with Triton 0.15%, blocked with normal goat serum, and then incubated with primary antibodies overnight at 4°C. Primary Antibodies: Mouse monoclonal FVIII (GMA-8015 Green Mountain), Rabbit Polyclonal COPII (17913-1-AP Proteintech), Rabbit Polyclonal VWF and Rabbit monoclonal CD63 (ab6994, ab252919 Abcam Cambridge, UK), Rabbit Monoclonal RAB27 and Goat Polyclonal Angiopoietin (MAB7245 , AF623 R&D) Rabbit Polyclonal Antibody CD32 (A12553 Abclonal). (Negative controls without the primary antibody but with the secondary antibody were incubated in PBS.) The slides were washed with PBS and incubated with secondary antibodies for 1 h at room temperature. Sections were washed twice with PBS and then stained with DAPI. Images were taken for data quantification using a Leica TCS SP9 confocal microscope. The fluorescence-conjugated secondary antibodies were Alexa 488 (excitation wavelength, 543 nm; emission wavelength, 586–590 nm), Alexa-568 (excitation wavelength, 578 nm; emission wavelength 603 nm), Alexa-647 (excitation wavelength, 650 nm; emission wavelength, 665 nm). Nuclei were stained with DAPI (excitation wavelength, 405 nm; emission wavelength, 424 nm). Fluorescent images were captured at ×20 and x63 magnification.

RNA interference

CD32+ HLEC passage 2 to 6 were plated on collagen-coated plates for three days and transfected with small interfering RNA (siRNA). Cells were transfected with siRNA oligonucleotides targeting CD36, or with a siRNA control (Santa Cruz Biotechnologies) using the transfection reagent Lipofectamine RNAimax (Thermo Fisher Scientific) and Opti-MEM (Gibco) at a concentration of 50 nM for 6 hours. Two days after transfection, the media was replaced with fresh media (EGM-2 BulletKit (Lonza), the cells were cultured for 24 h, then media was collected and FVIII concentration was measured by ELISA. Statistical analysis was performed using one-way ANOVA.

Immunoblot

Cells were harvested in RIPA buffer with Complete mini and phosfo stop reagents were from Roche (Switzerland). 25 µg of total protein were separated in a 4-15% SDS-polyacrylamide gel electrophoresis and transferred to Nitrocellulose membrane (Biorad, Hercules, California). Membranes were blocked for one hour at room temperature in Odyssey Blocking Buffer (TBS) (LI-COR 927-50000). Primary antibodies were incubated overnight at 4 °C. Antibodies to P38, phospho-P38, HSP27 and phospho-HSP27 were from Cell Signaling (Massachusetts, USA), antibody tdTomato was purchased from Origene (Maryland, USA).Washes were performed with TBS 0.1%

Tween-20 (TBST) before the addition of secondary antibody for one hour at room temperature. Primary antibodies were detected using IRDye 800CW Goat (poly-clonal) anti-Mouse IgG (H + L) highly cross-adsorbed (LI-COR#925- 32210), IRDye 800CW Goat (polyclonal) anti-Rabbit IgG (H + L) highly cross-adsorbed (LI-COR# 926-32211). All blots were imaged on the Odyssey® CLx imaging system using 680 nm and 780 nm channels. Protein detection was performed using Image Studio Ver 5.2.

OxLDL uptake

OxLDL uptake was quantified by adding 10 µg/mL of fluorescent (DiI) labeled oxLDL (DiI-oxLDL, Thermo Scientific) into basal media for 1 hour. Cells were washed with PBS twice, once with 0.5 M NaCl 0.2M acetic acid, and twice with PBS again. Cells were imaged using a Leica TCS SP9 confocal microscope Average cellular fluorescence was assessed in ImageJ with at least 3 images per condition for each experiment.

CD36 variant construct and transfection

mCherry-CD36 was a gift from Michael Davidson (Addgene plasmid # 55011 ; http://n2t.net/addgene:55011 ; RRID:Addgene_55011). We used this plasmid encoding mCherry-CD36(WT) to construct a plasmid encoding mCherry-CD36(Y325X). Q5 Site-Directed Mutagenesis kit (NEB, Massachusetts, US) was used to perform the cDNA mutation NM_001001548.3(CD36):c.975 T to G (p.Tyr325Ter) according to the manufacturer's instructions using the following primers:
Forward: GTACATCATAgGGTGTGCTAG and Reverse:
AATTTTTTGAGATAATTTTTTCTGTG. Constructs were sequenced by Sanger

Telo-HAEC cells were lifted, and Electroporation was performed using the Neon Electroporator system (ThermoFisher) and the constructs were added at a concentration of 100 ng/µL (1400 V, 1 pulse, 20 ms). Cells were seeded in coverslips for Immunofluorescence and in p6 well plate for Western blot as previously describe. Cells were allowed to recover overnight at 37 °C. Media was replaced after 24 hours and cells were used after 72 hours.

Enzyme-linked immunosorbent assay (ELISA)

CD32+ HLEC were seeded in collagen-coated 12 well plates, 30,000 cells were plated, and after three days the media was replaced with fresh media (EGM-2 BulletKit (Lonza)), and then media was collected at the specific times. FVIII antigen in the media was measured using an ELISA following the manufacturer's instructions (F5055 LSBIO ELISA with a listed sensitivity of 31 pg/ml) (LSBio, Seattle, WA).

To evaluate the release of VWF to the media of HUVEC and CD32+HLEC, 10,000 cells of HUVEC and CD32+ HLEC were seeded in p96-collagen coated plates, cultured for three days, then the media was replaced, and cells were treated with or without histamine for 1 h. We collected media and measured VWF levels by an ELISA. Supernatants were collected after treatment with media or 10 µM histamine for 1 h, and the concentration of VWF released into the media was measured by ELISA as previously described. The levels of VWF antigen in media were measured by an ELISA using antibodies from Fitzgerald Industries (20R-VG001, 60R-VG002hrp).

Statistical Analyses

The sample size N is the number of biological repeat experiments. Statistical analysis for qRT-PCR was done by two-tailed unpaired Student's t-test and a $P < 0.05$ was considered significant. Statistical analyses for the enzyme-linked immunosorbent assay (ELISA) were done by one-way ANOVA, using the Bonferroni Test for multiple comparisons, and a $P < 0.05$/number of tests was considered significant.

References for Methods

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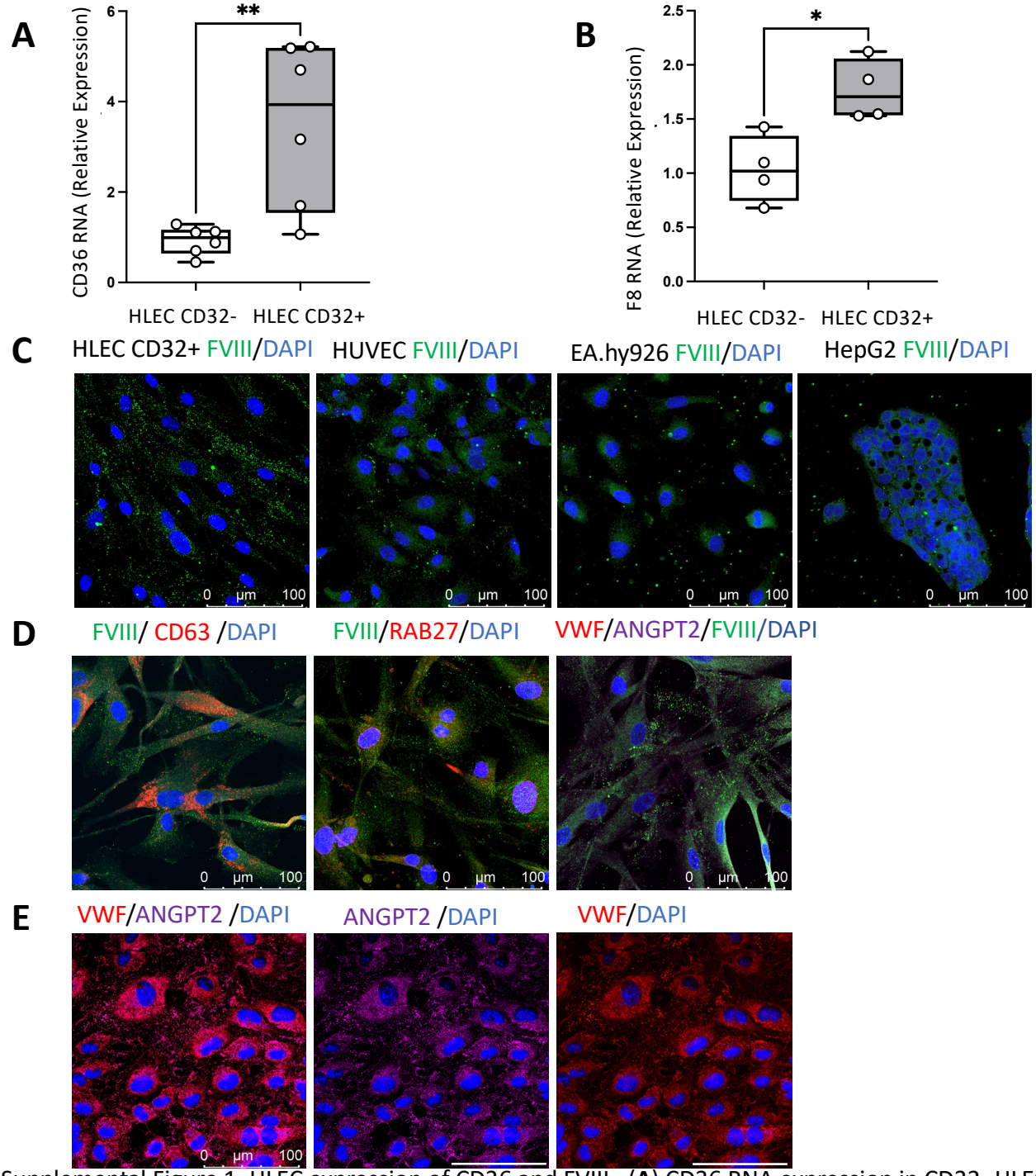
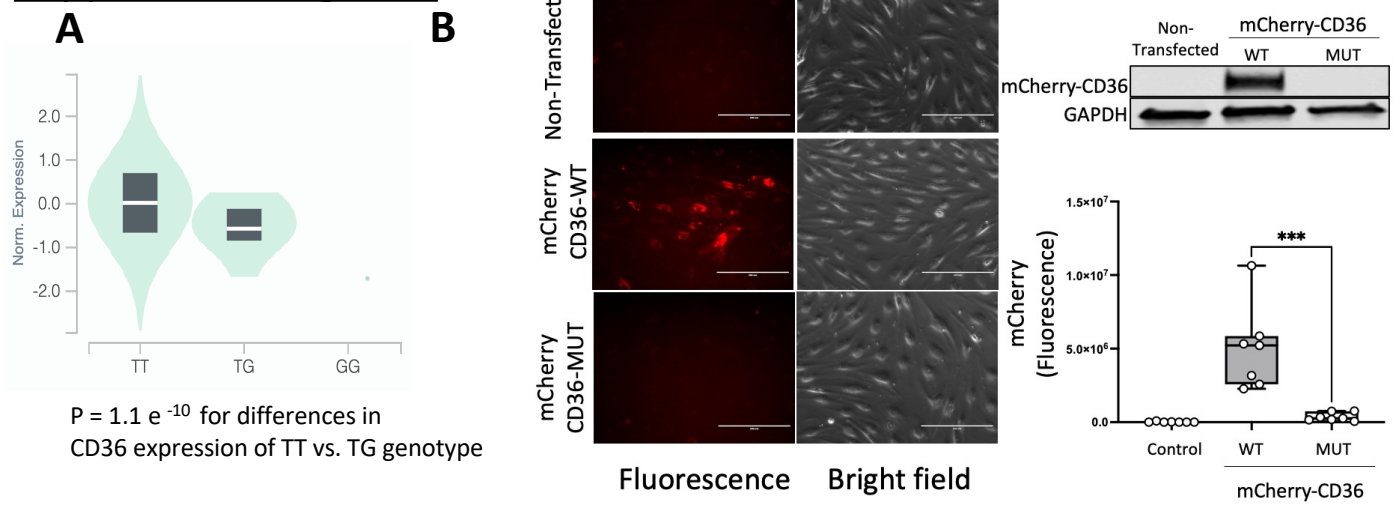
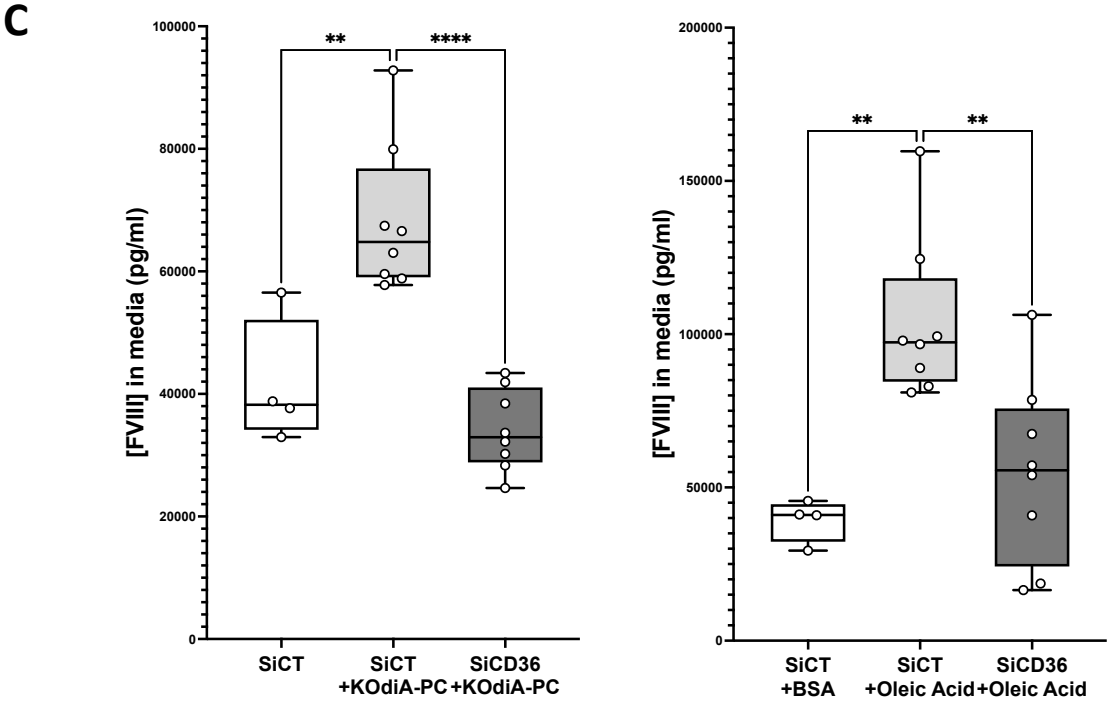
Supplemental Figure 1

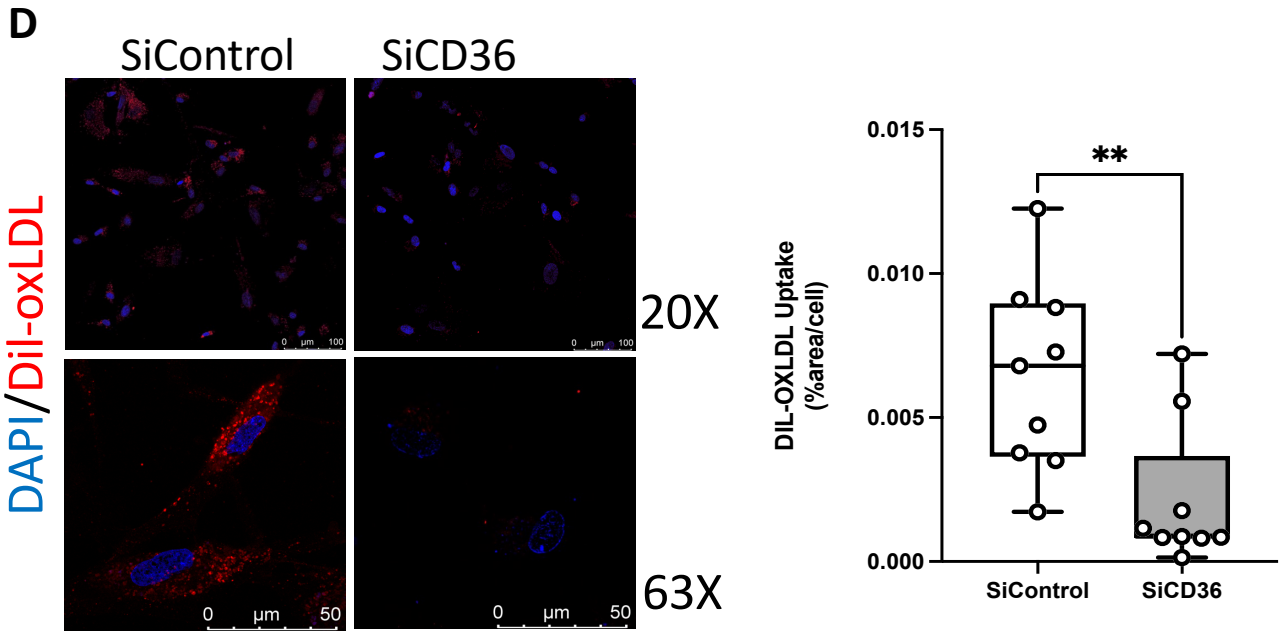


Supplemental Figure 1. HLEC expression of CD36 and FVIII. (**A**) CD36 RNA expression in CD32- HLEC and CD32+ HLEC as measured by qRT-PCR relative to beta-actin. (N = 4-6, mean ± SD. * = $p < 0.05$) (**B**) F8 mRNA expression in CD32- HLEC and CD32+ HLEC as measured by qRT-PCR (N = 4-6, mean ± SD. * = $p < 0.05$) (**C**) FVIII antibody staining of CD32+ HLEC cells and other endothelial cells and hepatocytes. HLEC CD32+, HUVEC, EA.hy926 and HepG2 were stained with FVIII monoclonal primary antibody (green) and with DAPI for nuclei (blue). **(D)** CD32+ HLEC and Weibel-Palade body markers. In CD32+ HLEC, FVIII does not co-localize with Weibel Palade Body markers including (left) CD63 and (middle) RAB27. CD32+ HLEC were hybridized with antibodies to FVIII (green) and to (left) CD63 (red), (middle) RAB27 (red), and (right) VWF (red), and Angiopoietin-2 (purple) and imaged by confocal microscopy. **(E)** HUVEC express VWF and ANGPT2 in granules. HUVEC hybridized with antibodies to FVIII (green), VWF (red), and Angiopoietin-2 (purple) and imaged by confocal microscopy. Representative images at 20X (scale bar=100um). Pearson's correlation coefficient of CD63 and FVIII = 0.143 ± 0.06 and of RAB27 and FVIII = 0.190 ± 0.1 (N = 3). Pearson's correlation coefficient of VWF and ANGPT2 in HUVEC = 0.559± 0.04 (N =3).

Supplemental figure 2







Supplemental Figure 2

Supplemental Figure 2. CD36 regulates FVIII release.
(A) Genetic variant of CD36 is associated with lower expression levels of CD36. GTEX portal expression quantitative trait loci (eQTL) analysis of rs3211938 variant in humans. Violin plot of the normalized expression of CD36 mRNA in Artery-Tibial Tissue from the major allele (TT) of the mutation and heterozygous carriers of the mutation (TG) showing carriers of the mutation have a decrease in CD36 expression ($p = 1.1 \times 10^{-10}$, NES: -0.93).

(B) Genetic variant of CD36 does not express CD36 protein in endothelial cells. A wild-type or mutant mCherry-CD36 construct was transfected into Telo-HAEC endothelial cells. (Left Panel) Cells were imaged by fluorescence (left) and bright field imaging (right). The mCherry-CD36-mutant CD36(Tyr325X) does not produce a fluorescent signal. Representative image at 20X objective (scale bar= 200 μM). (Right lower panel) Quantification of fluorescent images (N = 7,mean ± S.D. ***p < 0.0001). (Right upper panel) Immunoblot of cell lysates after transfection with wild-type or mutant CD36. The mCherry-CD36-mutant CD36(Tyr325X) does not express mCherry-CD36

(C) CD36 agonists increase CD32+ HLEC release of FVIII. CD32+ HLEC were transfected with siCD36 or siControl oligonucleotides for 48 h, and then treated with the CD36 agonists (left) KOdiA-PC 10 μg/ml and (right) oleic acid 40 μM for 16 h, and FVIII release into the media was measured by ELISA. Both KOdiA-PC and oleic acid increase FVIII release, and CD36 mediates the effect of these compounds (N = 4, mean ± S.D. * p < 0.005).

(D) CD36 mediates cell uptake of Dil-OxLDL in HLEC CD32+ cells. Left: CD32+ HLEC were transfected with oligonucleotides directed against CD36 or a control sequence, and 48 h later cells were incubated with Dil-oxLDL 10 μg/mL for 1h and then washed and imaged by confocal microscopy. Silencing CD36 (siCD36) decreases the uptake of Dil-OxLDL. Representative images at 20X and 63X immersion objective (scale bar= 100 μM and 50 μM). Right: quantification of the fluorescence intensity as % area per cell (N =9, mean ± SD. ** = p < 0.005)

Appendices

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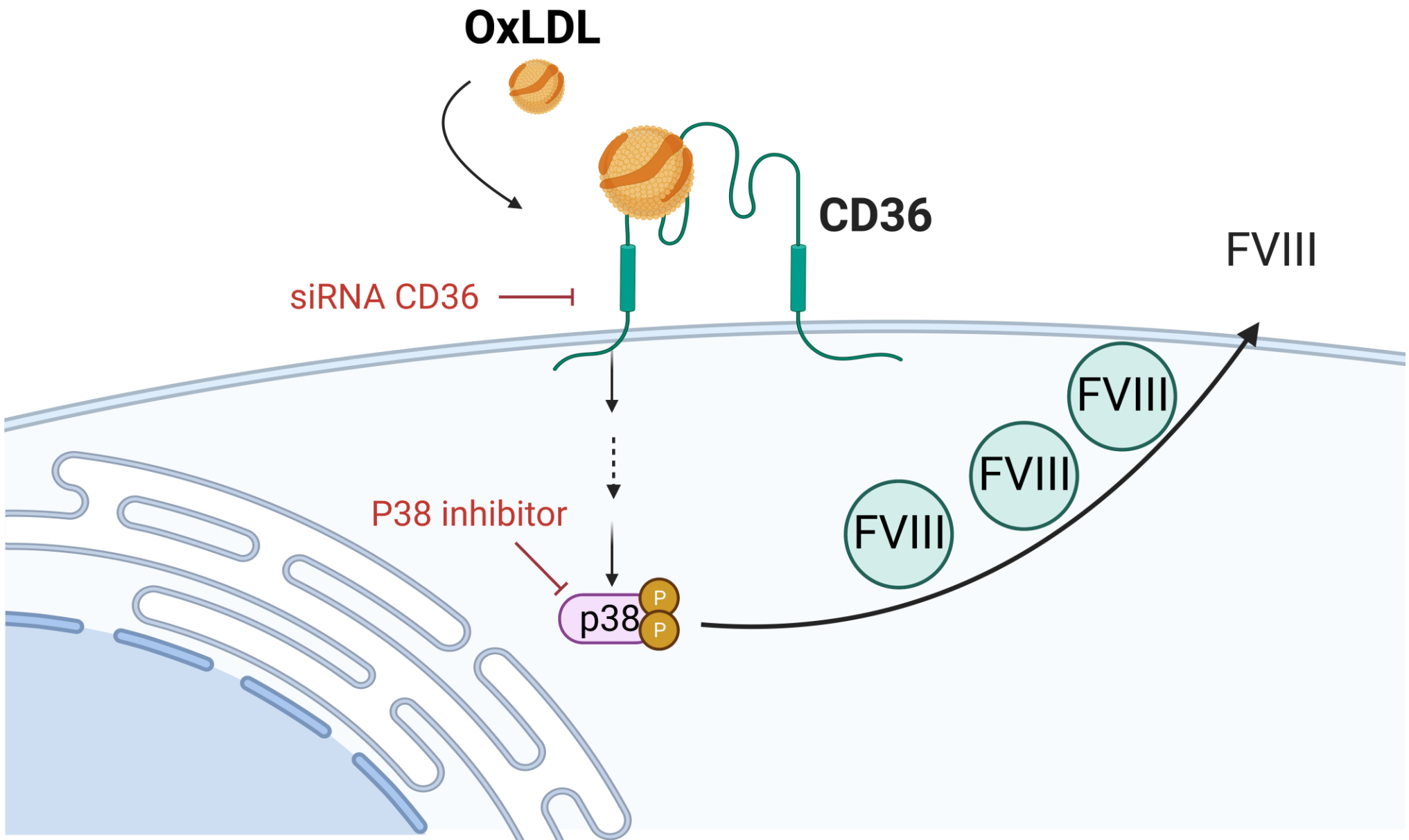
OxLDL
CD36
FVIII
siRNA CD36
FVIII
FVIII
FVIII
P38 inhibitor
p38
P
P