## Supplementary Methods

### *Quantitative PCR*

RNA was isolated from HUVEC using the RNeasy kit (Qiagen) and cDNA synthesis was performed using Superscript III First-Strand Synthesis kit (ThermoFisher Scientific) according to the manufacturer's protocol. Gene expression was measured on a OneStepPlus Real Time PCR system (ThermoFisher Scientific) using SensiFast SYBR No-ROX kit (Bioline). Relative expression to *ACTIN* was calculated using the ΔCT method and normalized to the control. Primers are listed in **Table S4**.

### *Rab activity assay*

GST-Slac2-b-SHD and GST-RIM2-RBD fusions were produced using *E. coli* BL21 and coupled to glutathione magnetic beads (ThermoFisher Scientific, #78602). Rab activity pulldown assays were performed as described previously.[1] Briefly, GST-fusion-conjugated glutathione beads were washed 3x in Triton X-100 lysis buffer (20 mM Tris-HCl [pH7.5], 150 mM NaCl, 1% Triton X-100, 20 mM $MgCl_2$, 1% glycerol) and added to cleared cell lysates in the same lysis buffer with a protease inhibitor cocktail (Sigma, P8340) for 1 hour tumbling at 4°C. Input samples were saved for immunoblot analysis. Beads were washed 3x in Triton X-100 lysis buffer and resuspended in NuPAGE 4x LDS sample buffer (ThermoFisher Scientific, #NP0008) with 8% β-mercaptoethanol diluted 2x in Triton X-100 lysis buffer. Samples were boiled at 95°C for 5 minutes and stored at -20°C prior to Western blot analysis. Rab activity, assessed by densitometry, was calculated as the ratio of the active Rab fraction and total Rab levels normalized to the mean of the control.

### *TX-114 phase partitioning*

Cytosolic and membrane-bound protein fractions were separated using TX-114 (Sigma-Aldrich) detergent-based phase partitioning, performed as previously described.[2–4] Briefly, cells were lysed in TX-114 lysis buffer (PBS, 1% TX-114, 1 mM EDTA, protease inhibitor cocktail) and rotated at 4°C for 1 hour. After maximum speed centrifugation for 10 min at 4°C detergent and non-detergent phases in the supernatant were separated by raising the temperature from 0°C to 37°C for 1 minute and centrifuging for 1 minute at room temperature (RT) to obtain different layers. The lower detergent phase enriched for hydrophobic proteins was washed once with PBS. Proteins were precipitated using methanol-chloroform precipitation method using BSA as a carrier and analyzed by Western blot.[5]

### *Western blot*

Cells were lysed in NP-40 lysis buffer (50mM Tris-HCl [pH 7.4], 100mM NaCl, 10mM $MgCl_2$, 10% glycerol, 1% NP-40, protease inhibitor cocktail) or another lysis buffer as indicated and NuPAGE 4x LDS sample buffer (ThermoFisher Scientific) with 8% β-mercaptoethanol was added to obtain 1x final concentration. Proteins were separated on precast Novex NuPAGE 4-12% BisTris gels (ThermoFisher Scientific) and transferred on nitrocellulose membranes using the iBlot transfer system (ThermoFisher Scientific), program 3 for 7 minutes. Membranes were blocked for 30 minutes using 5% skim milk (ELK, Campina) in Tris-buffered saline-0.1% Tween-20 (TBST) and primary antibodies (**Table S1**) diluted in TBST were incubated overnight at 4°C. Membranes were washed three times for 10 minutes in TBST and incubated with species-specific secondary antibodies conjugated to IR680 or IR800 (LI-COR Biosciences) for 1 hour at RT. After washing membranes three times in TBST, they were imaged using the Odyssey imaging system (LI-COR Biosciences). Densitometry was measured using ImageJ software (NIH).

### *Immunofluorescence and image analysis*

Bovine fibronectin (Sigma) was diluted at 5 µg/ml final concentration in PBS. HUVEC were grown on fibronectin-coated 12 mm coverslips (ThermoFisher Scientific) for at least four days at full confluency. Cells were fixed with 4% PFA diluted in PBS for 10-15 minutes at RT, permeabilized with perm/quench (PBS, 0.2% (w/v) saponin, 50mM $NH_4$) for 10 minutes, and blocked using PGAS (PBS, 0.2% (w/v) gelatin, 0.02% (w/v) saponin) for ≥5 minutes. Coverslips were consecutively incubated with primary and secondary antibodies (**Table S1**) for 1 hour at RT with PBS washing steps in between. Coverslips were mounted in Mowiol and Z-stacks were imaged on a Leica TCS SP8 confocal fluorescence microscope using a 40x or 63x objective. Maximum projections were created in LasX software (Leica Microsystems) and processed using ImageJ software (NIH). Rab colocalization with VWF was analyzed in CellProfiler version 2.1.1 (Broad Institute) using a semi-automated pipeline that after manual identification of a GFP+ cell (IdentifyObjectsManually module) counts the total number of WPBs in each cell based on VWF staining (IdentifyPrimaryObjects module), and calculates overlap (MeasureImageOverlap module) between thresholded images of VWF and Rab27A or Rab3D stainings. Rab27A positivity of WPBs was scored as judged by overlap with VWF staining for TOMM70-EGFP, TOMM70-EGFP-MADD and TOMM70-EGFP-MADD-ΔDENN transduced cells that contained >5-100 WPBs total with a cut-off of 1 Rab27A+ WPB.

### *Transmission Electron Microscopy*

HUVEC were grown to full confluency in gelatin-coated 10 cm dishes. EGM-18 was replaced with Karnovsky fixative for 1 hour at RT and O/N at 4°C. Fixed samples were processed for TEM as previously described.[6] Images of WPBs in multiple cells were obtained using a FEI Tecnai T12 G2 transmission electron microscope (ThermoFisher Scientific). Mature and immature WPBs were scored across > 50 images for each condition.

### *Secretion assay and VWF ELISA*

HUVEC were grown on gelatin-coated 6-well plates at full confluency for ≥4days and EGM-18 was replaced every other day. HUVEC were starved for 30 minutes in M199 serum-free medium (Gibco) supplemented with 2% bovine serum albumin (BSA, Serva) and stimulated with 100 µM histamine for 30 minutes. Media were collected and cells were lysed in PBS containing 1% Triton-X100 and protease inhibitor cocktail (Sigma, P8340). VWF content in media and lysates was assayed by sandwich ELISA as previously described.[6] Briefly, ELISA plates were coated overnight at 4°C with 0.5 µg VWF rabbit polyclonal anti-VWF antibody (DAKO, A0082) per well diluted in 1M $NaHCO_3$. The wells were blocked with TWEB (0.1% Tween-20, 1mM EDTA, 0.2% gelatin in PBS) for 1 hour and samples were incubated for 1 hour shaking at RT. Plates were washed 5 times with PBS-0.1% Tween-20 using the Skan microplate washer (Molecular Devices). HRP-conjugated anti-VWF antibody (DAKO, P0226) diluted in TWEB and 1 µg/well was incubated for 1 hour shaking at RT. Kinetic measurement using o-Phenylenediamine dihydrochloride (OPD) tablets (Sigma) was performed on a SpectraMAX microplate reader (Molecular Devices).

### *Statistical Analysis*

Statistical analyses were performed using Graphpad Prism 8 software. Datasets were tested for normal distribution (Shapiro-Wilk). For datasets following a normal distribution statistical significance was calculated using a parametric test. In case data did not fit a normal distribution a non-parametric test was used. Two-tailed unpaired t-test or Mann-Whitney-U test was used to compare two conditions. To compare more than two conditions, one-way analysis of variance (ANOVA) or Kruskall-Wallis test was used with Tukey's multiple comparisons test and Bonferroni correction method for multiple testing or with Dunnet's multiple comparisons test to compare each condition to a single control. For grouped data a two-way ANOVA was used with Sidak's multiple comparisons test. Results are shown as mean ± standard error of the

mean (SEM) or as indicated in figure legends and p-values are indicated with asterisks (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

**Supplementary tables**

**Table S1.** Antibodies

| Target | Species | Company/article | Clone/Cat# | IF | WB |
|---|---|---|---|---|---|
| Rab27A | Mouse mAb | Abnova | H00005873-M01 | - | 1:500 |
| Rab27A | Rabbit pAb | Bierings et al. (2012)[4] | Beavis B2324 | 1:50 | - |
| Rab3B | Mouse mAb | Abnova | 3F12 | 1:100 | 1:1000 |
| Rab3D | Rabbit pAb | Proteintech | 12320-1-AP | 1:50 | 1:1000 |
| MyRIP | Goat pAb | Abcam | ab10149 | 1:100 | - |
| VWF | Rabbit pAb | DAKO | A0085 | 1:5000 | 1:5000 |
| VWF | Mouse mAb | CLB | RAg20 | 1:5000 | - |
| VWF | Mouse mAb | CLB | RAg35 | 1:2000 | - |
| VE cadherin | Mouse mAb | BD Pharmingen | 55-7H1 | 1:200 | - |
| α-tubulin | Mouse mAb | Sigma | DM1A | - | 1:10000 |
| β-catenin | Rabbit pAb | SantaCruz | H102 | - | 1:10000 |
| Transferrin Receptor | Mouse mAb | Thermo Scientific | H68.4 | - | 1:2500 |
| GFP | Mouse mAb | Clontech | JL-8 | - | 1:2500 |
| Actistain-555 | - | Cytoskeleton Inc | PHDH-1-A | 1:400 | - |
| Hoechst-33342 | - | Invitrogen | H-1399 | 1:50000 | - |

*Secondary antibodies conjugated with fluorophores (Molecular Probes) were purchased from Invitrogen. Infrared dye-conjugated secondary antibodies were from LI-COR.

**Table S2.** Short hairpin RNAs.

| TRCN | Sequence |
|---|---|
| shCTRL-C002 * | CAACAAGATGAAGAGCACCAA |
| shMADD-37879 | CATCCTCAATCTGGAGAAAT |
| shMADD-37880 * | GCTCAACAAGTTCTATACTAA |
| shMADD-37881 | CCAGGAAATGATCGACAGGTA |
| shMADD-37882 | GCGAATCTATGACAATCCATA |
| shMADD-37883 | CCACAAGTACAAGACACCAAT |
| shRab27A-279985 * | CCAGTGTACTTTACCAATATA |
| shRab27A-380256 | CACAACAGTGGGCATTGATTT |
| shRab27A-297751 | CAGGAGAGGTTTCGTAGCTTA |
| shRab27A-380034 | GAAGGAGTGGTGCGATCAAAT |
| shRab27A-380306 | GATCTTCTCTATGATTGATAC |
| shRab3B-381145 | ACCATCACAACAGCCTATTAC |
| shRab3B-318484 | GCTACTCAGATCAAGACCTAC |
| shRab3B-349558 | GATGAGTTGCTGCTATTCTTT |
| shRab3B-318485 * | AGTGCAAAGGAGAACATCAGT |
| shRab3D-47778 | GCCTTCTAGCTTAGAACCATT |
| shRab3D-47779 | CATCAATGTGAAGCAGGTCTT |
| shRab3D-47780 | GACTATATGTTCAAACTGCTA |
| shRab3D-47781 * | CATCGCCAATCAGGAATCCTT |
| shRab3D-47782 | GACTTCCTTCCTGTTCCGATA |

*selected for further experiments.

**Table S3.** Small interference RNA oligo sequences.

| **Target** | **ON-TARGET*plus* SMARTpool** | **Oligo ID** | **sequence** |
|---|---|---|---|
| Human MADD | L004429 | J-004429-07 | ACACUGGAGUCACGCGAUA |
| | | J-004429-08 | GCAGUGAUAGUAUGGAUUA |
| | | J-004429-09 | GUACAUGCAGGGACAGAUA |
| | | J-004429-10 | UGAAUUGGGUGGCGAGUUC |

**Table S4.** Primers for qPCR.

| **Gene** | **Forward primer** | **Reverse primer** |
|---|---|---|
| *ACTIN* | CTTCCTTCCTGGGCATGGAGTC | CTCAGGAGGAGCAATGATCTTGATCTTC |
| *MADD* | AGTTAGCGCCGTGGTGAGTAATAG | CTGTGGGCTTTACCAAAGATGGTG |

**Supplementary Figures with legends**

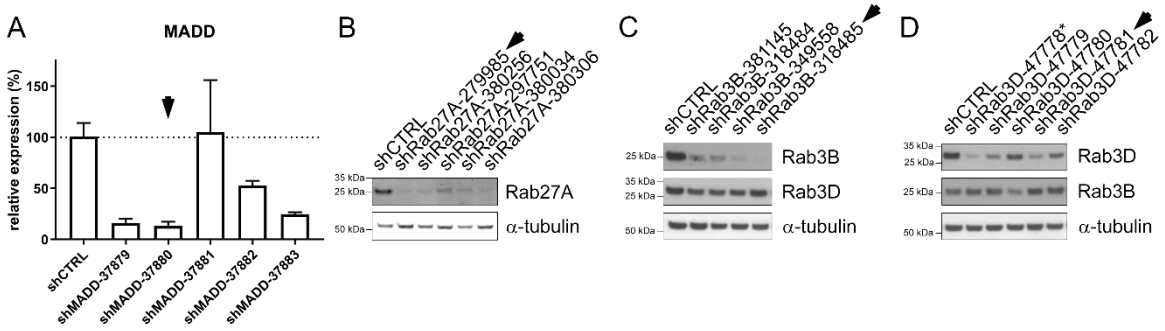


**Figure S1. Validation of short hairpin RNAs targeting MADD and secretory Rabs in HUVEC.** (**A**) Quantitative PCR (qPCR) showing *MADD* expression normalized to *ACTIN* using the ΔCT method (mean±SEM) in HUVEC transduced with different short hairpin (sh)RNAs. Western blot analysis of Rab27A (**B**), Rab3B (**C**), and Rab3D (**D**) knockdown in HUVEC transduced with target-specific shRNAs. Selected shRNAs are indicated with arrows. The shRNAs that induced the largest reduction of expression, indicated with arrows, were selected for further experiments. The shRNA that inferred most efficient knockdown of Rab3D (indicated with an asterisk) had lethal effects in long-term culture.

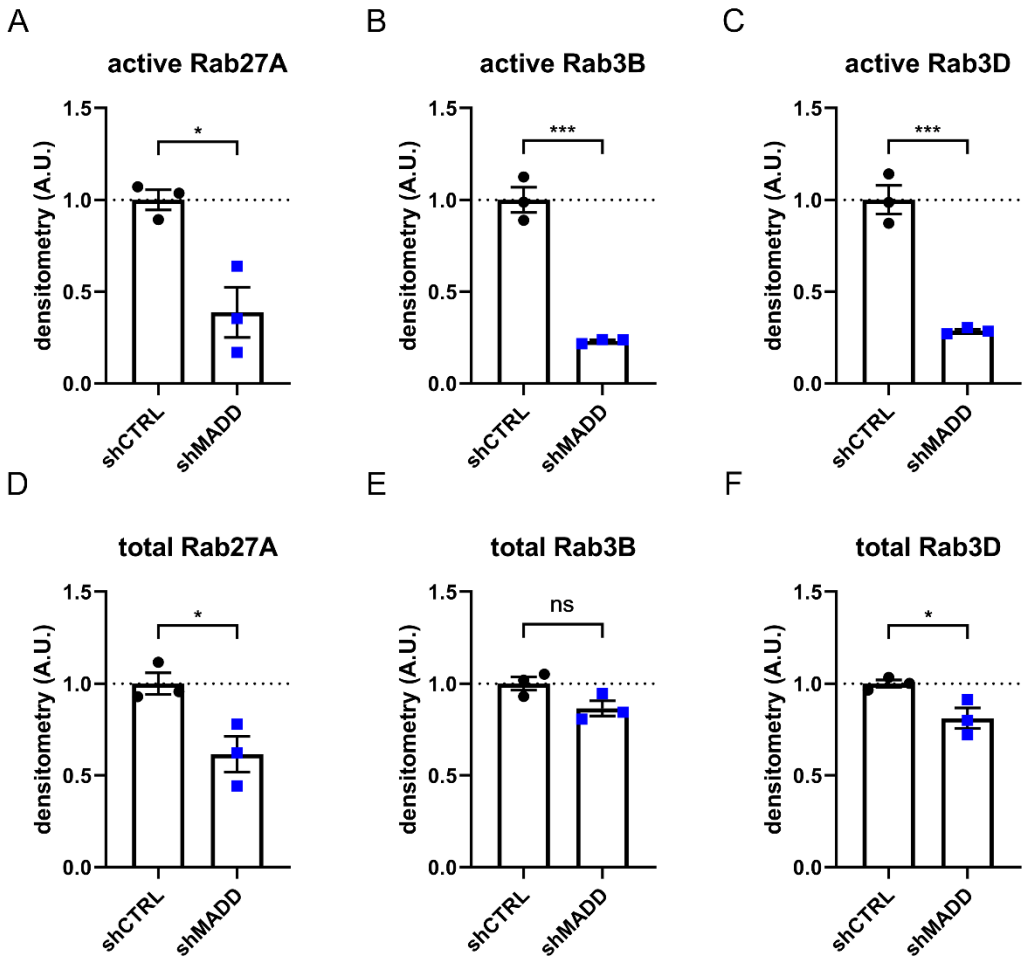


**Figure S2. Active Rab fraction and total input.** Active and total levels of Rab27A, Rab3B, and Rab3D determined in Slac2-b and RIM2 pulldown assays with shCTRL and shMADD-transduced HUVEC lysates (n=3 biological replicates). Densitometry (arbitrary units: A.U.) was determined using ImageJ and normalized to the mean of the control. (mean±SEM, unpaired two-tailed t-test, ns: not significant, *P<0.05, **P<0.01, ***P<0.001).

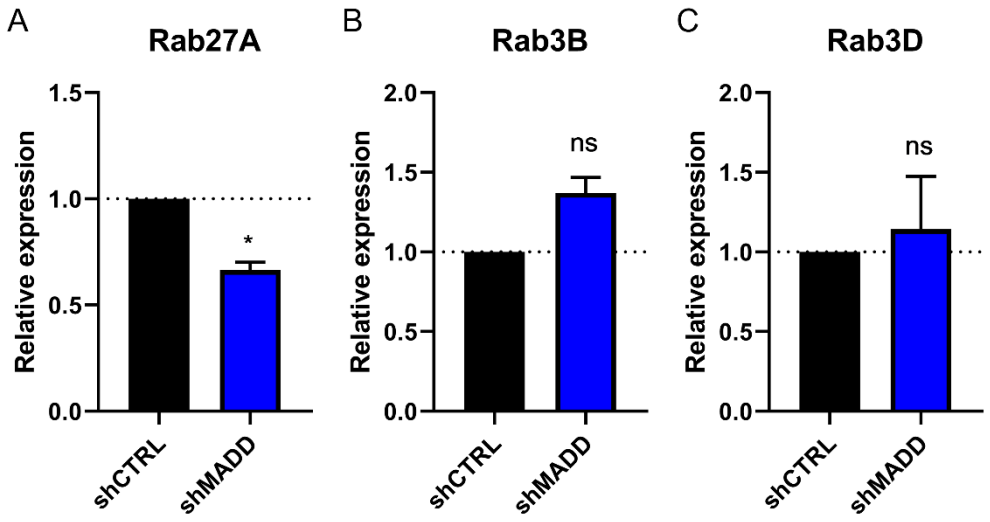


**Figure S3. Rab27A mRNA levels are decreased upon MADD silencing.** Rab27A (**A**), Rab3B (**B**), and Rab3D (**C**) mRNA expression was analyzed by qPCR in shCTRL- and shMADD-transduced HUVEC (mean±SEM, n=3 biological replicates, unpaired two-tailed t-test, ns: not significant, *P<0.05).

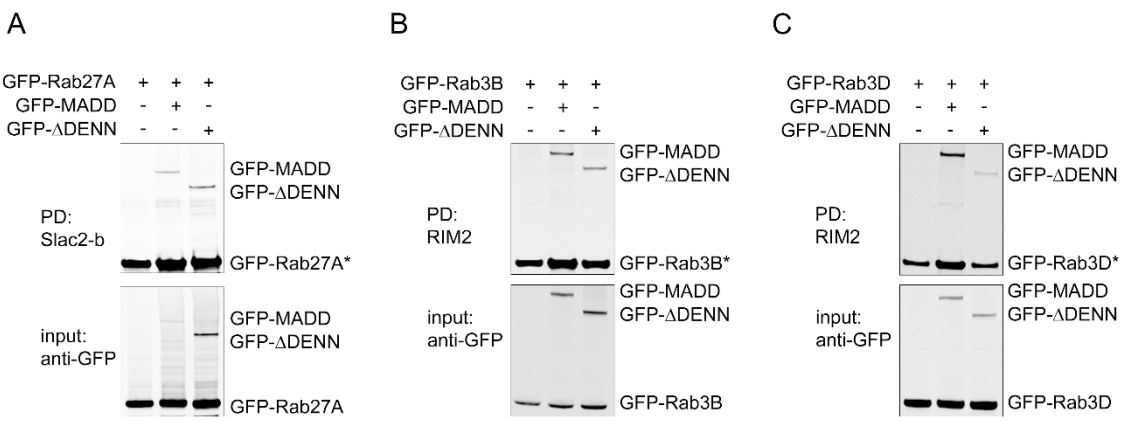


**Figure S4. MADD-ΔDENN binds to GTP-bound Rab27A and Rab3.** HEK293T cells were transfected with GFP-Rab27A (**A**), GFP-Rab3B (**B**), or GFP-Rab3B (**C**) alone or co-transfected with GFP-MADD-full-length or GFP-MADD-ΔDENN. GTP-bound Rabs were extracted from lysates using Slac2-b or RIM2 pulldown (PD) as indicated. In Slac2-b and RIM2 PD assays for active Rab27A (**A**), Rab3B (**B**) and Rab3D (**C**) GFP-MADD and –ΔDENN coprecipitate.

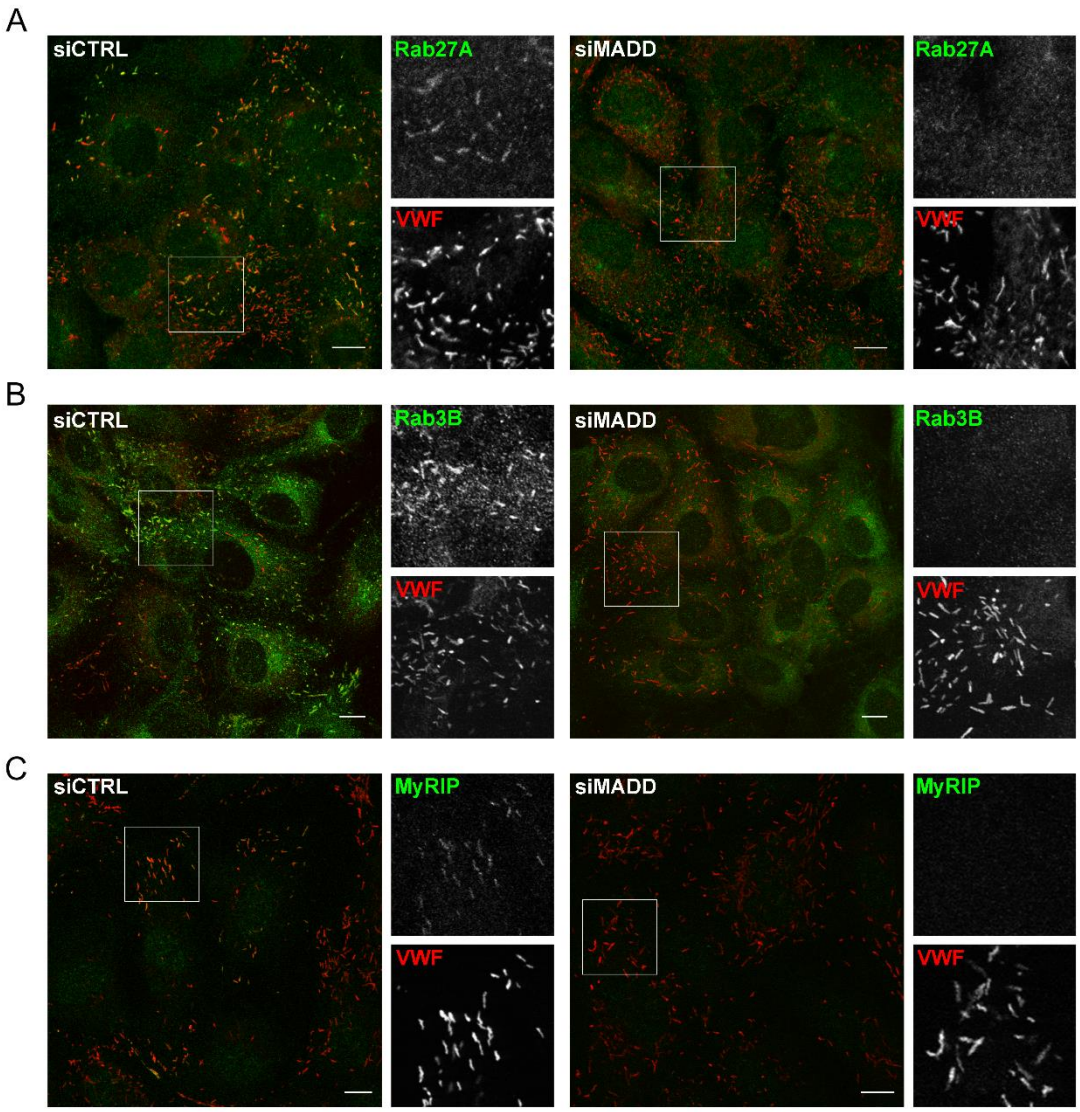


**Figure S5. siMADD diminishes Rab27A, Rab3B, and MyRIP localization to WPBs.** HUVEC were transfected with siCTRL or siMADD immunostained for VWF (red) and Rab27A (**A**), Rab3B (**B**), or MyRIP (**C**) in green as indicated. Individual channels are shown in gray scale. Boxed areas are magnified on the right. Scale bars represent 10 µm.

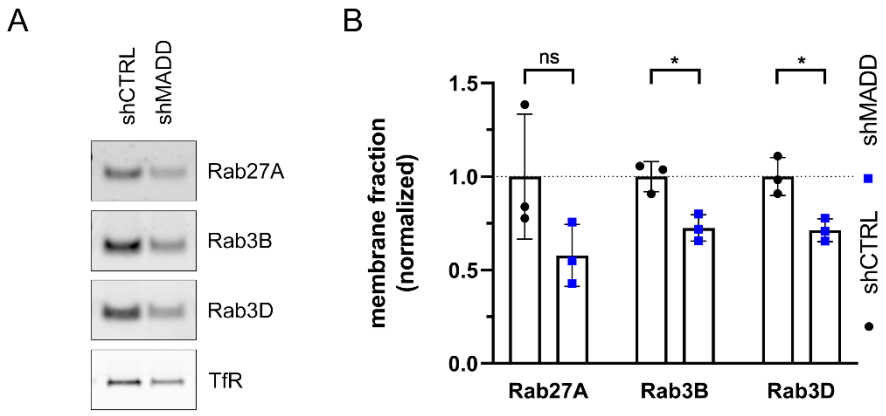


**Figure S6. MADD knockdown reduces Rab membrane-association.** (**A**) TX-114-based phase partitioning and Western blot analysis of Rab27A, Rab3B, and Rab3D and Transferrin Receptor (TfR) in the detergent phase (i.e. membrane-bound) of shCTRL- and shMADD-transduced HUVEC. (**B**) Densitometry was determined using ImageJ and normalized to TfR and the mean of each respective control (mean±SEM, n=3 biological replicates, unpaired two-tailed t-test, ns: not significant, *P<0.05).

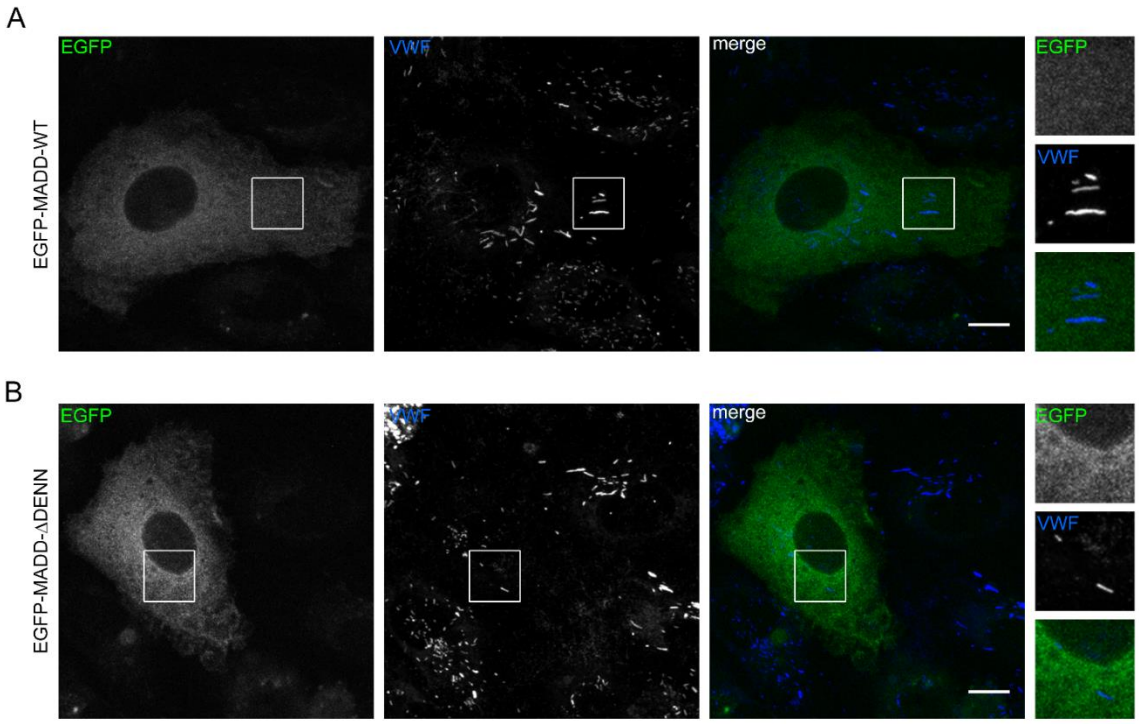


**Figure S7. GFP-MADD is not enriched on WPBs in endothelial cells.** HUVEC were transfected with a EGFP-MADD-WT (**A**) or EGFP-MADD-ΔDENN (**B**) construct (both in green) and immunostained for VWF (blue). Individual channels are shown in gray scale. Boxed areas are magnified on the right. Scale bars represent 10 µm.

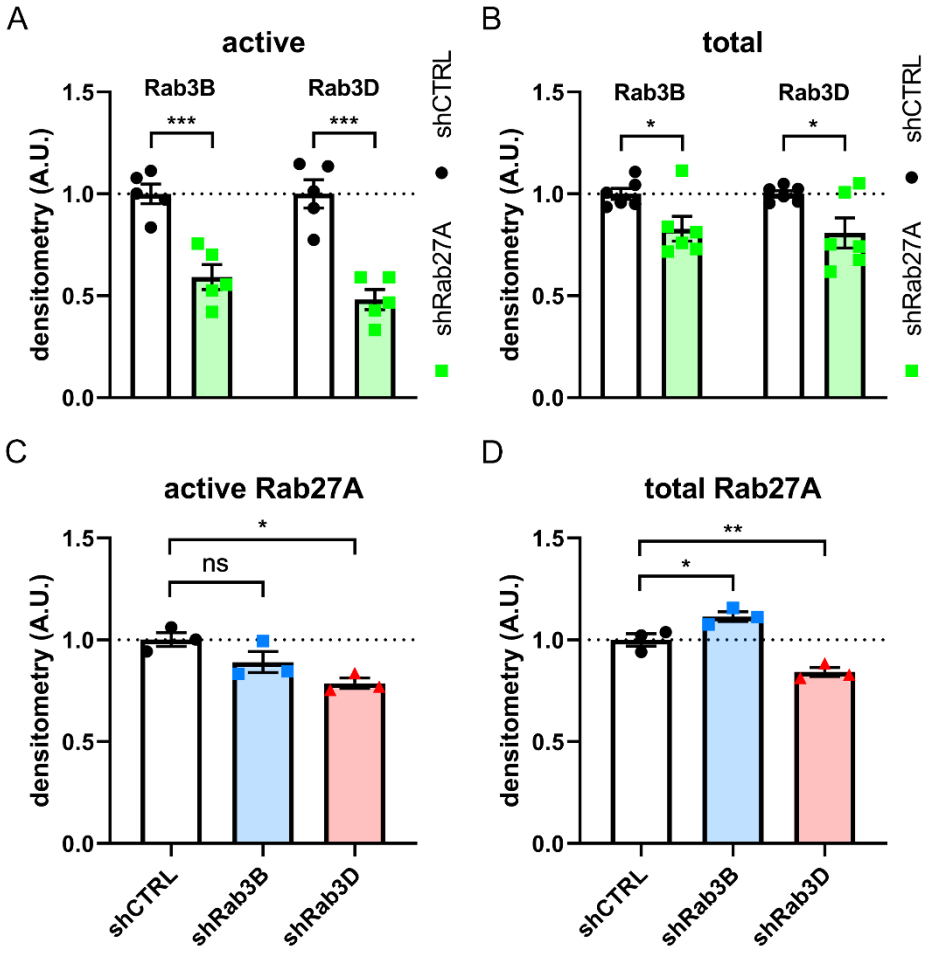


**Figure S8. Active Rab- and total input levels.** Active (**A**) and total levels (**B**) of Rab3B and Rab3D determined in a RIM2 pulldown assay with shCTRL and shRab27A-transduced HUVEC lysates (n=5 biological replicates). Active (**C**) and total levels (**D**) of Rab27A determined in a Slac2-b pulldown assay with shCTRL, shRab3B, and shRab3D-transduced HUVEC lysates (n=3 biological replicates). Densitometry data (arbitrary units: A.U.) was determined using ImageJ and normalized to the mean of the control. (mean±SEM, unpaired two-tailed t-test, ns: not significant, *P<0.05, **P<0.01, ***P<0.001).

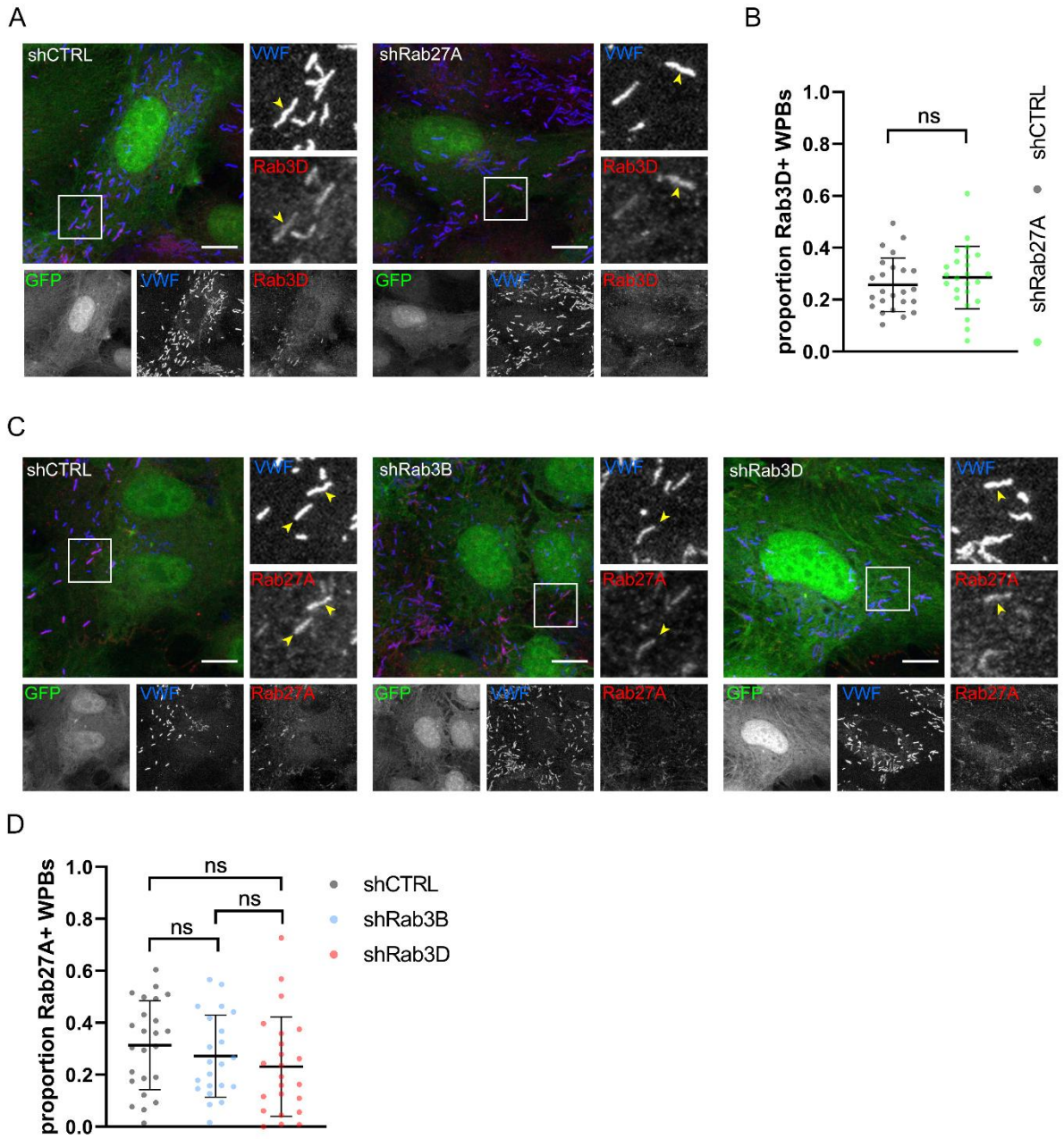


**Figure S9. Rab3D and Rab27A localization is not affected by knockdown of other WPB Rabs.** HUVEC were transduced with a pLKO-GFP-shRNA co-expression construct containing shCTRL, shRab27A, shRab3B, or shRab3D (all shown in green) and immunostained for VWF (blue), and Rab27A or Rab3D (red) as indicated (**A,C**). Individual channels are shown in gray scale below. Boxed areas are magnified on the right. Yellow arrowheads indicate Rab+ WPBs. Scale bars represent 10 µm. In (**B**) the proportion of Rab3D+ WPBs per cell in shCTRL (n=24) is compared to shRab27A (n=24) (mean±SD, t-test, ns: not significant) and in (**D**) the proportion of Rab27A+ WPBs in shCTRL (n=24) is compared to shRab3B (n=22) and

shRab3D (n=23) (mean±SD, one-way ANOVA, Tukey's multiple comparisons test, ns: not significant). For the proportion of Rab27A+ WPBs cells with >50 WPBs were excluded.

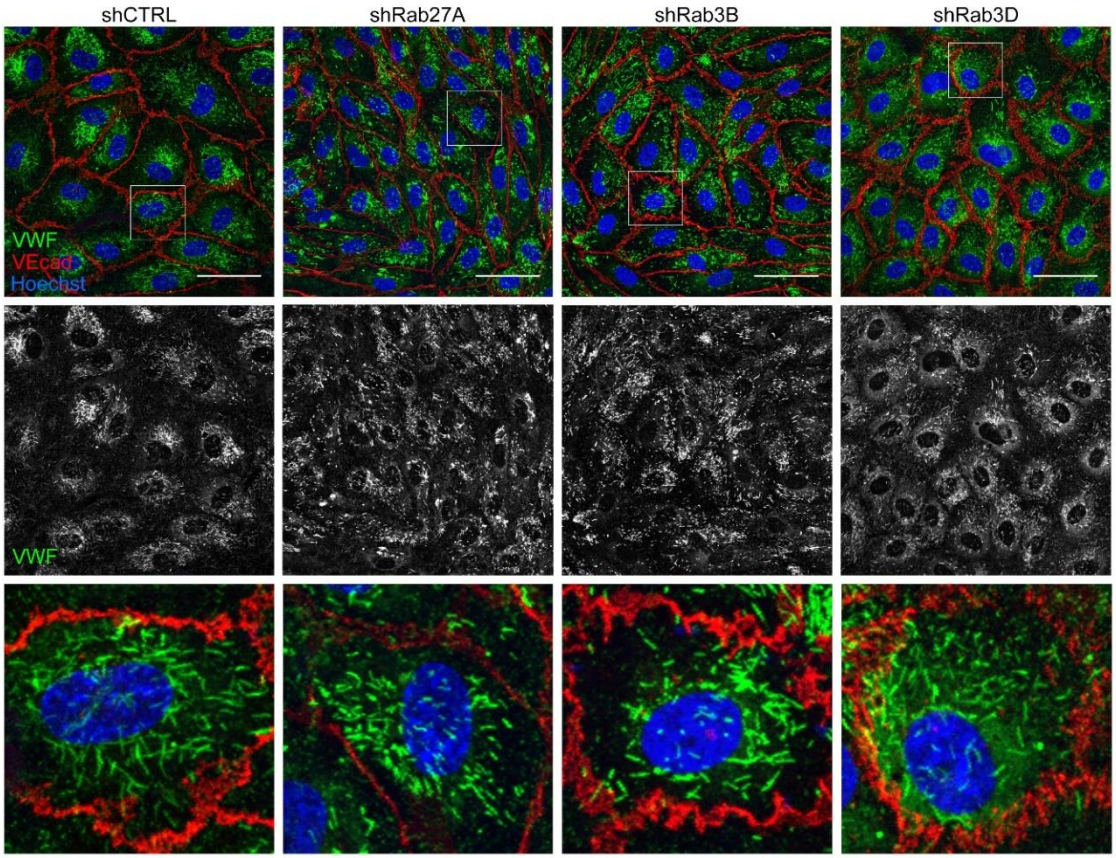


**Figure S10. WPB formation in secretory Rab-silenced monolayers.** Immunofluorescent staining of Von Willebrand factor (VWF, green), vascular endothelial (VE)-cadherin (red), and nuclei (blue) in shRNA-transduced HUVEC. The individual green channel (VWF) and the merged channels of one magnified cell are shown in gray scale below. Scale bars represent 40 µm.

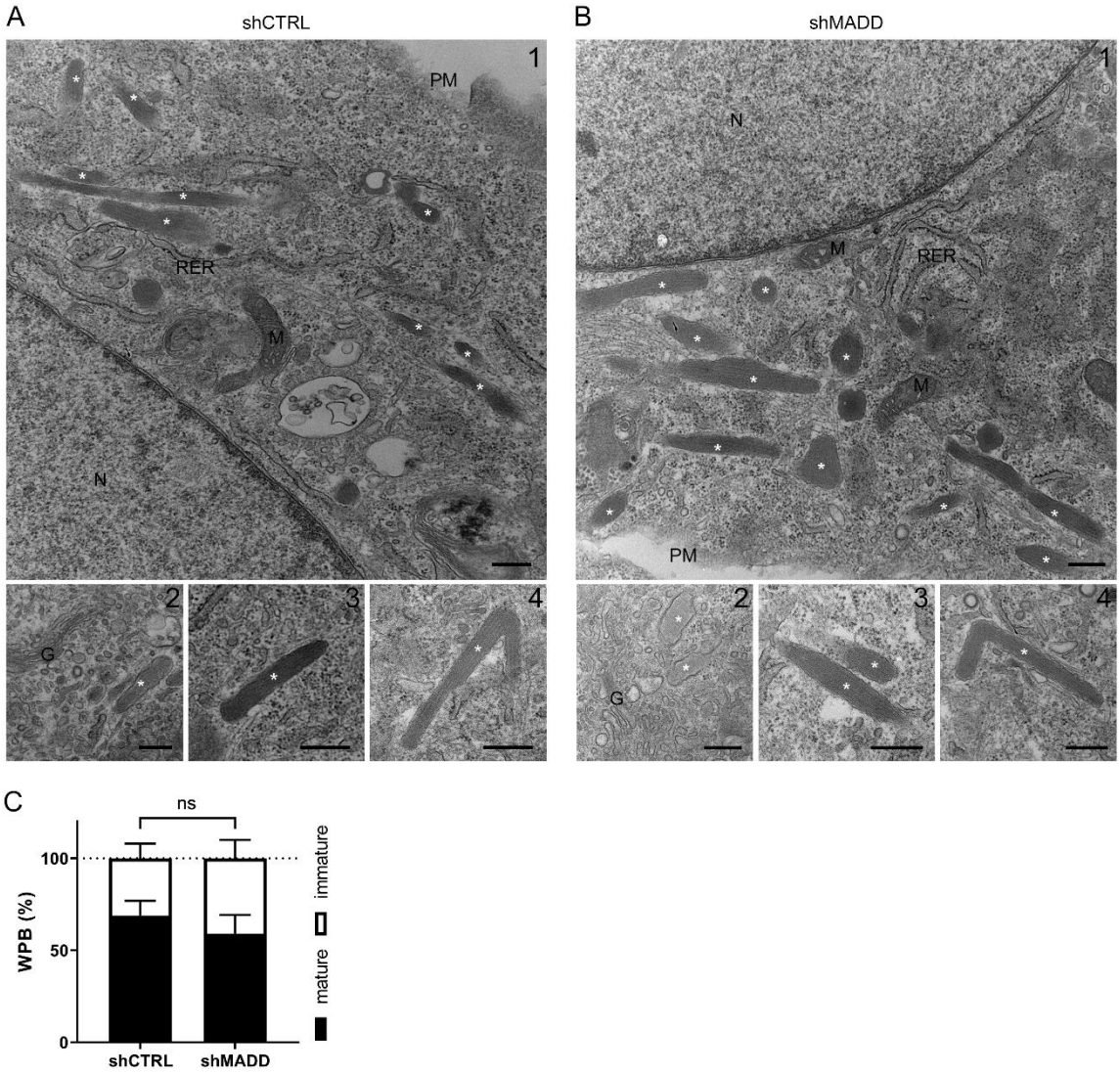


**Figure S11. WPB biogenesis and maturation remains unchanged upon MADD silencing.** HUVEC transduced with shCTRL (**A**) or shMADD (**B**) were analyzed using transmission electron microscopy (TEM). Representative TEM images per condition showing an overview of one single cell containing WPBs (1) indicated with white asterisks (*), and detail images below show immature (2), mature (3), and hinged (4) WPBs. N=nucleus, PM=plasma membrane, RER=rough endoplasmic reticulum, M=mitochondrion, G=Golgi. Scale bars represent 400 nm. In (**C**) mature and immature WPBs were scored in shCTRL-, and shMADD-transduced HUVEC and percentages are shown (mean±SEM, n=3 biological replicates, two-way-ANOVA with Sidak's multiple comparisons test, ns: not significant).

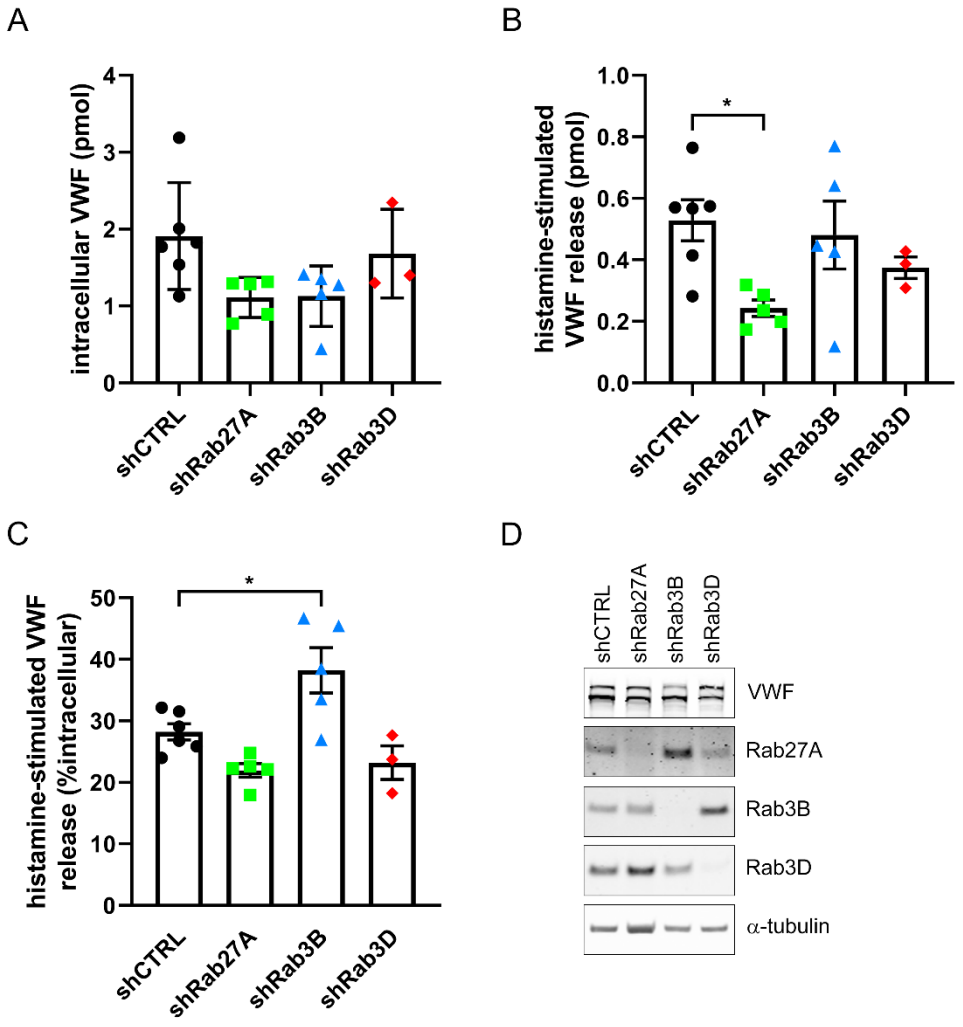


**Figure S12. Rab27A silencing recapitulates effects of MADD knockdown on histamine-stimulated VWF secretion.** ELISA data of VWF secretion assay with shCTRL- and shRab-transduced HUVEC showing intracellular VWF content (**A**), 30-minute histamine-stimulated VWF secretion in picomole (pmol) (**B**), and as a percentage of intracellular content (**C**) (mean±SEM, n=3-5 biological replicates, one-way ANOVA with Dunett's multiple comparisons test, *P<0.05, **P<0.01). (**D**) shows a representative Western blot analysis of Rab27A, Rab3B, Rab3D, and VWF expression in shCTRL- and different shRab-transduced HUVEC.

## Supplementary References


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