

BASIC SCIENCES

RAB5C Increases Endothelial Release of VWF by Regulating Vesicle Trafficking

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BACKGROUND: Abnormal levels of VWF (von Willebrand Factor) are a risk factor for venous thromboembolism (VTE) and bleeding. Genome-wide association studies for VWF have identified novel candidate genes that may regulate VWF levels in humans, including *RAB5C* (RAS [rat sarcoma]-associated protein RAB5C). We hypothesized that RAB5C regulates VWF release from endothelial cells.

METHODS: We studied the effect of RAB5C on vesicle trafficking in human endothelial cells. We performed CRISPR (clustered regularly interspaced short palindromic repeats) interference targeting 2 genetic variants linked to altered VWF levels and evaluated RAB5C expression by reverse transcription–quantitative polymerase chain reaction. We silenced RAB5C or overexpressed RAB5C wild-type, constitutive active or dominant negative; and then, we measured VWF exocytosis from human umbilical vein endothelial cells to the media by ELISA. We performed proximity labeling and mass spectrometry to identify intracellular signaling pathways mediating the effects of RAB5C on VWF exocytosis.

RESULTS: We found that 2 genetic variants (rs9915255 and rs9912088 identified by genome-wide association studies for VWF levels) regulate *RAB5C* expression in stem cell–derived endothelial cells. We next silenced or overexpressed *RAB5C* in endothelial cells to assess its effect on VWF release. *RAB5C* silencing decreased VWF release after histamine stimulation, whereas overexpression of RAB5C or constitutively active RAB5C increased endothelial VWF release. To explore the intracellular signaling pathway mediating the effects of RAB5C on VWF exocytosis, we performed proximity labeling and mass spectrometry. We identified 147 proteins proximal to RAB5C, many of which are involved in vesicle trafficking. From this screen, we identified SNAP29 (synaptosome-associated protein 29), a SNARE (soluble NSF [N-ethylmaleimide-sensitive factor] attachment receptor)-associated protein that plays a crucial role in vesicle fusion, as a key RAB5C interactor regulating VWF exocytosis.

CONCLUSIONS: Taken together, our data demonstrate that RAB5C regulates VWF release in part through SNAP29 control of vesicle trafficking in endothelial cells. These findings validate genetic epidemiology data linking RAB5C to VWF levels in humans and provide new insights into the molecular mechanisms regulating VWF exocytosis.

GRAPHIC ABSTRACT: A [graphic abstract](#) is available for this article.

Key Words: endothelial cells ■ genome-wide association study ■ thrombosis ■ venous thromboembolism ■ von Willebrand Factor

Venous thromboembolism (VTE) includes deep vein thrombosis and pulmonary embolism.^{1,2} About 900 000 US adults develop VTE each year, and about 60 000 to 100 000 die from VTE each year.³ VTE can be caused by acquired conditions and inherited factors.⁴ Many genetic variants that cause VTE are still unknown.

Aberrant VWF (von Willebrand Factor) levels can lead to a spectrum of diseases extending from bleeding disorders to thrombotic diseases.^{5–9} Abnormally high levels

[See cover image](#)

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Nonstandard Abbreviations and Acronyms

Ang-2	angiopoietin-2
CA	constitutively active
CRISPRi	clustered regularly interspaced short palindromic repeats interference
DN	dominant-negative
ER	endoplasmic reticulum
GAP	GTPase protein
GEF	guanine nucleotide exchange factor
GST-VWF	VWF glutathione S-transferase
GWAS	genome-wide association studies
HUVEC	human umbilical vein endothelial cells
iPSC	induced pluripotent stem cells
PAR	protease-activated receptor
RAB5C	RAS-associated protein RAB5C
ROI	region of interest
siRNA	small interfering RNA
SNAP29	synaptosome-associated protein 29
SNARE	soluble NSF attachment receptor
VTE	venous thromboembolism
VWF	von Willebrand Factor
WPB	Weibel-Palade bodies
WT	wild-type

What Are the Clinical Implications?

Altered levels of VWF have important clinical consequences. High VWF (von Willebrand Factor) levels increase the risk of venous thromboembolism, ischemic stroke, coronary artery disease, and peripheral artery disease, whereas low levels lead to von Willebrand disease types 1 and 3. Because of this broad clinical impact, understanding how VWF is released from endothelial cells is essential for identifying new therapeutic approaches. Our study provides mechanistic insight into endothelial release of VWF by showing that *RAB5C*, a gene previously linked to VWF levels through human genome-wide association studies, directly regulates VWF secretion in vitro. Using proximity labeling, we identify SNAP29 (synaptosome-associated protein 29) as a protein that interacts with RAB5C and participates in the vesicle-trafficking steps required for VWF exocytosis. These findings help explain how genetic variation at *RAB5C* influences circulating VWF levels and reveal a new molecular pathway that controls endothelial VWF release. Clinically, defining this pathway could guide strategies aimed at reducing thrombotic risk in patients with high VWF levels or improving hemostasis in those with VWF deficiency. Overall, our work expands the mechanistic understanding of VWF biology and its relevance to both bleeding and thrombotic disease.

of VWF are a risk for thrombosis.^{10,11} Elevated VWF levels are associated with increased risk of VTE, ischemic stroke, coronary artery disease, and peripheral artery disease.^{12,13} Abnormally low levels of VWF increase the risk of bleeding. For example, von Willebrand disease types 1 and 3 are characterized by low or nonexistent levels of VWF, which increase the risk of bleeding.^{8,9,14} Understanding the biology of VWF expression and exocytosis (calcium-regulated secretion) is essential for developing novel targeted therapies for VWF-related thrombotic disorders.

VWF is a large, multimeric glycoprotein that mediates platelet adhesion to the vessel wall and mediates platelet aggregation to other platelets.¹⁵ VWF is released from endothelial cells after vascular injury.^{16,17} VWF is stored in endothelial granules called Weibel-Palade bodies (WPB) and then is transported to the endothelial wall and released into the blood in a process called exocytosis.^{18–22} Exocytosis involves several families of vesicle transport proteins including NSF (N-ethylmaleimide-sensitive factor), SNARE (soluble NSF attachment receptor), and Rab (Ras-related in brain) proteins.^{23–26}

Several groups have performed genome-wide association studies (GWAS) to identify genetic variants (single-nucleotide polymorphisms) that are associated with altered VWF levels.^{12,13,27–30} Recently, the CHARGE Consortium identified 11 novel candidate genes that might regulate VWF levels in humans.³¹ One

of these candidate genes is *RAB5C* (RAS [rat sarcoma]-associated protein RAB5C).

RAB5C is a GTPase that regulates vesicle trafficking in many cell types, including endothelial cells.^{32–35} Rab proteins are molecular switches that are activated when GEFs (guanine nucleotide exchange factors) catalyze GTP exchange onto the Rab protein.^{36,37} RAB proteins are inactivated by GAPs (GTPase proteins), which hydrolyze GTP to GDP on Rab proteins.^{38,39} In their GTP-bound active state, RAB interact with effectors and regulate different steps in vesicular trafficking.^{35,40–42}

Members of the RAB family regulate VWF trafficking from the endoplasmic reticulum (ER) to the Golgi to the granule to exocytosis. RAB10 (Ras-related protein Rab-10) and RAB8A (Ras-related protein Rab-8A) are implicated in WPB biogenesis,⁴³ RAB3 (Ras-related protein Rab-3) regulates WPB maturation and exocytosis,⁴⁴ RAB15 has been shown to positively regulate WPB exocytosis, cooperating with RAB27a.⁴⁵ RAB27a mediates the anchorage of WPB at the cortical actin cytoskeleton, allowing complete maturation of VWF before exocytosis, and supports WPB exocytosis by acting through the effector Slp4a (synaptotagmin-like protein 4a).^{41,45,46} RAB35 (Ras-related protein Rab-35) supports WPB exocytosis through a downstream effector cascade involving ACAP2 (ArfGAP with coiled-coil, ankyrin repeat and PH domains 2) and Arf6 (ADP-ribosylation factor 6).⁴⁰ The role of RAB5C in VWF release is not fully understood.

Here, we show that RAB5C regulates VWF release from endothelial cells in vitro. We use GWAS data to identify RAB5C as a potential regulator of VWF exocytosis. We use proximity labeling to identify proteins that are adjacent to RAB5C, and we identify SNAP29 (synaptosome-associated protein 29) as a protein that interacts with RAB5C and controls VWF exocytosis. Our results validate genetic associations between RAB5C and plasma VWF levels in humans and offer new mechanistic insight into the regulation of VWF exocytosis, advancing our understanding of pathways relevant to bleeding and thrombotic disease.

MATERIALS AND METHODS

Data Availability Statement

The authors will make their data, analytic methods, and study materials available to other researchers. The corresponding author is responsible for maintaining the availability of the data on request.

Genetics

Fine-mapping analysis was performed using a Bayesian model, SUSIE, which considers linkage disequilibrium (using the 1000 Genomes Phase 3 reference panel) and GWAS summary statistics³¹ to determine which of the variants in the locus are most likely to be causal for the observed association with VWF. Credible set variants are a group of single-nucleotide polymorphisms identified through Bayesian fine-mapping that collectively have a high probability (typically 95%) of containing the true causal variant. We then intersected the credible set variants identified by SUSIE with endothelial cell open chromatin regions based on Telo-HAEC ATAC-sequencing data (GSE126200). This analysis prioritized 2 variants as likely causal for the GWAS association.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Basel, Switzerland) and cultured in complete endothelial cell basal media according to the manufacturer's instructions (endothelial basal medium-2 [EBM-2] Basal Medium [CC-3156] and endothelial growth medium-2 [EGM-2] SingleQuots Supplements [CC-4176]). All experiments were performed using HUVEC grown for no >10 passages. HEK293 cells were purchased from ATCC (Manassas, Virginia) and cultured in DMEM media supplemented with 10%FBS and penicillin-streptomycin.

Generation of Inducible Endothelial Cells Expressing dCas9-KRAB

Human induced pluripotent stem cells (iPSCs) expressing a catalytically inactive Cas9 fused to a Krüppel-associated box repressor (dCas9-KRAB; no. AICS-0090-391; Corriell) were differentiated into endothelial cells according to a previously published protocol.⁴⁷ iPSCs were seeded onto Geltrix (no. A1413201; Thermo) coated plates and maintained in Essential 8 Media (no. A2858501; Thermo) with Rock Inhibitor (Y-27632 dihydrochloride, no. 1254; Tocris). Mesoderm differentiation

was induced by culturing iPSCs in DMEM supplemented with B27 minus insulin and GSK3b (glycogen synthase kinase 3 beta) inhibitor (CHIR-99021) for 3 days. Endothelial differentiation was then achieved by culturing cells in EGM-2 media (no. CC-3162; Lonza) supplemented with VEGF (vascular endothelial growth factor) and FGF2b (basic fibroblast growth factor 2) for 5 days. Endothelial cells were isolated based on cell surface expression of CD31 (no. ab215912; Abcam) and CD144 (no. ab33168; Abcam) using cell sorting (fluorescence-activated cell sorter). All iPSC experiments received approval from the Johns Hopkins institutional stem cell research oversight committee under protocol no. 00000699.

Tube Formation Assay

Inducible endothelial cells (10 000 cells per well) were seeded in 96-well plates precoated with undiluted Geltrix. After overnight incubation to allow tube formation, cells were stained with Calcein AM dye. Fluorescent microscopy images were subsequently acquired.

Clustered Regularly Interspaced Short Palindromic Repeats Interference of Prioritized Variants

Transfection of dCas9-KRAB-expressing inducible endothelial cells with guide RNAs targeting the candidate variants prioritized in the *RAB5C* locus (17q21.2) was conducted using Lipofectamine RNAimax (no. 13778075; Thermo Fisher). The design of the single guide RNAs for rs9915255 and rs9912088 was performed using the CRISPR guide RNA design and analysis tool (CRISPOR),⁴⁸ and we chose a single guide RNA with fewer potential off-target effects. After a 6-hour transfection period, cells were refreshed with complete EGM-2 media and incubated for 48 hours at 37 °C. RNA was extracted and analyzed via reverse transcription-quantitative polymerase chain reaction to quantify RAB5C expression levels.

sgGuide rs9915255: TTGCTGGGAGAACTTGGATG TGG
sgGuide rs9912088: CTTCCAGGCTCCGATTAGT TGG

RNA Interference

HUVEC passages 2 to 6 were plated on gelatin-coated plates for 5 days and then transfected with small interfering RNA (siRNA). Cells were transfected with siRNA oligonucleotides targeting RAB5C or SNAP29 (Thermo Fisher Scientific), or with a siRNA control (Santa Cruz Biotechnologies, Thermo Fisher Scientific) using the transfection reagent Lipofectamine RNAimax (Thermo Fisher Scientific) and Opti-MEM (Gibco) at a concentration of 50 nmol/L for 6 hours. Two days after transfection, the media were replaced with fresh media (EGM-2 BulletKit, Lonza), the cells were cultured for 24 hours, then the cells were stimulated or not with histamine (10 µM for 1 hour), media were collected, and VWF was quantified by ELISA. Statistical analyses were performed using 1-way ANOVA.

Reverse Transcription-Quantitative Polymerase Chain Reaction

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 1-step quantitative polymerase chain reaction performed with TaqMan probes (following the manufacturer's instructions) on a CFX Connect thermal cycler (Bio-Rad). The quantitative polymerase chain reaction probes, Hs03023943_g1 ACTB, Hs00191150_m1 SNAP29, and Hs00904926_g1 RAB5C were from Thermo Fisher Scientific. The VWF quantitative polymerase chain reaction probe 450932996 was from Integrated DNA Technologies (Iowa). Quantification was performed in triplicate for each sample. Expression results were calculated by the $\Delta\Delta CT$ method and were normalized to the reference gene *ACTB*. Statistical analysis was done by 2-tailed unpaired Student *t* test, and a $P < 0.05$ was considered significant.

Enzyme-Linked Immunosorbent Assay

VWF antigen in media was measured using an ELISA, range 0.625 to 40 mIU/mL, sensitivity=0.38 mIU/mL (ab108918; Abcam) or a homemade ELISA using duo antibodies from Fitzgerald Industries (20R-VG001, 60R-VG002hrp). Angiopoietin-2 antigen in media was measured using an ELISA, range 46.9 to 3000 pg/mL, sensitivity=21.3 pg/mL (DANG20; R&D systems). Thrombospondin-1 antigen in media was measured using an ELISA, range 7.8 to 500 ng/mL, sensitivity=0.944 ng/mL (DTSP10; R&D systems). Protein S antigen in media was measured by a commercially available antibody duo ELISA, and the standard-curve was made by commercially available substrates performing a fold dilution starting at (200 ng/mL; Enzyme Research Labs Inc, South Bend, IN) according to the manufacturer's instructions.

GST-VWF Uptake Assay

A recombinant human GST-VWF (VWF glutathione S-transferase)-tagged protein was purchased from Proteintech (Ag2386). The VWF fragment contains amino acids 764 to 1035 of the human VWF, a region that includes the domains involved in the recycling of VWF. THP-1 monocytic cells (ATCC) were maintained in RPMI-1640 supplemented with 10% fetal FBS and differentiated into adherent macrophage-like cells by incubation with 100 nmol/L phorbol 12-myristate 13-acetate for 48 hours. HUVECs were cultured as previously described. For uptake experiments, HUVECs or THP-1 cells were incubated with 2 ng/mL GST-VWF for 30 minutes at 37°C in serum-free medium supplemented with 1 mmol/L $CaCl_2$.⁴⁹ After incubation, unbound ligand was removed by washing 3× with ice-cold PBS, and surface-bound protein was stripped by 2 washes with acid buffer (0.2 mol/L glycine, pH 2.5, 2 minutes each, 4°C), followed by neutralization with cold PBS. Cells were lysed, and Western blot was performed, and membranes were probed with anti-GST and for GAPDH.

RAB5C Constructs

The RAB5C wild-type (WT) construct was obtained from Origene (SC322014).

The V5-TurboID-NES_pCDNA3 was a gift from Alice Ting⁵⁰ (plasmid no. 107169; <http://n2t.net/addgene:107169>; RRID:Addgene_107169; Addgene). The fusion constructs were generated using In-Fusion Snap Assembly Master Mix, consisting of TurboID with a nuclear export signal and a V5 tag fused

to the N terminus of RAB5C. The constitutively active (CA) and dominant-negative (DN) RAB5C constructs were created using a kit (Q5 Site-Directed Mutagenesis Kit). All constructs were verified by Sanger sequencing before use in experiments. The primers used to construct the RAB5C mutated expression plasmids were as follows:

Mutation Q80L (CA) CAG (GLN)-CTG (LEU)
Forward CACAGCTGGACtGGAGCGGTATCACAGCCTGG
Reverse CAGGCTGTGATACCGCTCCaGTCCAGCTGTG
Mutation S35N (dominant negative) TCC (serine)-asparagine AAC
Forward GTAGGCAAAaCAGCCTCGTCCTCCGCTTTG
Reverse CGAGGCTGTTTtGCCTACCGCAGACTCC

Electroporation

HUVECs were electroporated using the Neon Electroporation system (Thermo Fisher) with the plasmid constructs at a concentration of 100 ng/ μ L (1400 V, 1 pulse, 20 ms). Cells were then seeded in a 6-well plate, allowed to recover overnight at 37°C, and the culture medium was replaced after 24 hours. Cells were harvested for analysis 72-hour postelectroporation. For measurements of endothelial release of VWF to the media by ELISA, cells were seeded in 96-well plates. Cells were allowed to recover overnight at 37°C, the culture medium was replaced after 24 hours, and cells were then grown for another 48 hours. Then cells were stimulated or not with histamine 10 μ M for 1 hour.

Lipofection

Cells were transfected with plasmid DNA using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, DNA-Lipofectamine complexes were prepared in Opti-MEM and added to cells at 80% confluence. After 6-hour incubation at 37°C, the medium was replaced with fresh complete medium. Confirmation of overexpression was performed by immunoblots or by reporter expression.

Rab5C Activation Assay

Rab5C activation was assessed using the Rab5C Pull-Down Activation Assay Kit (83501; NewEast Biosciences). Human embryonic kidney 293 cells (HEK293T) were transfected by lipofection with the TurboID-RAB5C constructs (TurboID-RAB5C-WT, TurboID-RAB5C-CA, and TurboID-RAB5C-DN). Cells were lysed in 1X Assay/Lysis Buffer supplemented with protease inhibitors on ice. Lysates were clarified by centrifugation at 12000×*g* for 10 minutes at 4°C. Lysates were incubated with GTP γ S or GDP in the presence of EDTA and $MgCl_2$. Active Rab5C was immunoprecipitated using an anti-Rab5-GTP monoclonal antibody and Protein A/G agarose beads, washed, and eluted in reducing SDS-PAGE sample buffer. Pull-down samples were resolved by SDS-PAGE, transferred to polyvinylidene fluoride membranes, and detected by Western blot using an anti-Rab5C rabbit polyclonal antibody, followed by secondary antibody detection and imaged on the Odyssey CLx imaging system.

Quantification of WPBs

The number of WPBs per cell was quantified following an image analysis approach originally described by Valentijn et al⁵¹ and

subsequently applied by Liu et al⁵² in endothelial cells. Both studies reported an average of 50 to 100 WPBs per cell under basal conditions, which served as a reference point for interpreting our results. Based on this, all VWF-positive structures detected by immunofluorescence were considered as WPBs if they had an area between 0.5 and 5 μm^2 and a circularity between 0.2 and 0.8. These thresholds were applied to include mature, elongated WPBs while excluding smaller vesicular structures or nonspecific aggregates. WPB counts were normalized per cell using DAPI (4',6-diamidino-2-phenylindole)-based nuclear segmentation.

For WPB size quantification, the same morphological criteria were applied, and the average area (μm^2) of all segmented WPBs was calculated per image as a measure of their relative maturation state. Each analyzed field covered a minimum area of 57 600 μm^2 , ensuring sufficient sampling for robust quantification. All data quantification was performed with ImageJ software.

The spatial distribution of WPBs relative to the nucleus was analyzed using Fiji (ImageJ). Multichannel images were first split into individual channels: CD31 (platelet endothelial cell adhesion molecule-1; membrane, red), VWF (WPBs, green), and DAPI (nucleus, blue). Individual cells were manually outlined in the CD31 channel using the freehand selection tool, and a binary mask was created to define the region of interest (ROI). Nuclei were segmented from the DAPI channel within the cell ROI by thresholding, converted to binary masks, and the centroid of each nucleus was measured and saved as an ROI. WPBs were segmented in the VWF channel by thresholding, converted to binary masks, and both centroid coordinates and area were measured and saved as ROIs. ROIs for nuclei and WPBs were imported altogether into the ROI Manager to extract x and y coordinates. Distances between WPB centroids (x_2, y_2) and the corresponding nuclear centroid (x_1, y_1) were calculated using the Euclidean formula:

$$d = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2}$$

This workflow allowed quantitative comparison of WPB positioning under different experimental conditions.

Immunofluorescence and Confocal Microscopy

HUVEC were seeded in gelatin-coated 15-mm coverslips, 25 000 cells per coverslip. Five days after seeding, cells reached confluence. 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 recognized: VWF (ab6994, Abcam Cambridge, United Kingdom), RAB5C (Santa Cruz), EEA1, Calreticulin, trans-Golgi network, (Abcam), SNAP29 (Cell Signaling) and CD31 to 647 conjugated (Abcam). Negative controls were incubated in PBS. The slides were washed with PBS and incubated with secondary antibodies for 1 hour at room temperature. Sections were washed twice with PBS and then stained with DAPI. 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), and 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 $\times 20$ and $\times 63$ magnification. Fluorescence images were acquired using a Leica TCS SP9 confocal microscope with identical acquisition settings for all samples. Colocalization

analysis was performed using Fiji (ImageJ). Pearson correlation coefficient was calculated using the Coloc 2 plugin. For each condition, Pearson correlation coefficients were calculated from 3 independent experiments, each including 3 fields containing ≈ 20 to 50 cells per field. Statistical significance was assessed using an unpaired 2-tailed Student t test (GraphPad Prism).

Proximity Labeling and Sample Preparation for Mass Spectrometry

Proximity labeling followed by proteomic mapping was performed, as previously described.⁵³ Briefly, a fusion construct was generated using In-Fusion Snap Assembly Master Mix, consisting of TurboID with a nuclear export signal and a V5 tag fused to the N terminus of RAB5C. HEK293T cells seeded in T75 flasks were transfected with the construct using Lipofectamine 3000, following the manufacturer's instructions. After 24 hours, cells were incubated with 50 μM biotin for 10 minutes. The labeling reaction was stopped by washing the cells 5 times with 1 mL of ice-cold DPBS. Cells were then lysed in 200 μL of RIPA buffer supplemented with protease inhibitors. Protein concentration was assessed using the BCA assay, and 1.5 mg of protein was incubated with 150 μL of streptavidin magnetic beads in 500 μL of RIPA buffer. The mixture was rotated for 1 hour at 4°C. Beads were washed as previously described. For on-bead digestion, the beads were incubated with 80 μL of 2 mol/L urea in 50 mmol/L Tris-HCl (pH 7.5), containing 1 mmol/L dithiothreitol and 0.4 μg trypsin, at 25°C for 1 hour with shaking at 1000 rpm. The supernatant was collected and transferred to fresh tubes. Beads were washed twice with 60 μL of 2 mol/L urea in 50 mmol/L Tris-HCl (pH 7.5), and the washes were combined with the initial digest supernatant. Peptides were reduced with 4 mmol/L dithiothreitol for 30 minutes and alkylated with 10 mmol/L iodoacetamide for 45 minutes in the dark. An additional 0.5 μg of trypsin was added to the sample, and digestion was allowed to proceed overnight at 25°C with shaking at 700 rpm. Alkylated peptides were acidified with 0.5% trifluoroacetic acid and applied to reversed-phase SepPak C18 cartridges (Waters). Peptides were eluted with 0.1% trifluoroacetic acid and 60% acetonitrile. The eluates were evaporated to dryness using a Vacufuge (Eppendorf) and redissolved in 50 mmol/L HEPES (pH 7.2), 50 mmol/L NaCl, and 10 mmol/L Na_2HPO_4 .

Liquid Chromatography Separation and Tandem Mass Spectrometry

Dried peptides were reconstituted in 2% acetonitrile and 0.1% formic acid and analyzed by nanoflow liquid chromatography-tandem mass spectrometry using an Easy-nano-liquid chromatography (nLC) interfaced with an Orbitrap Q Exactive Plus mass spectrometer (both instruments, Thermo Fisher Scientific). Peptide separation was performed with a 120-minute linear gradient (water/acetonitrile) at a flow rate of 300 nL/min using polyimide-coated, fused-silica, 25 $\text{cm} \times 360 \mu\text{m}$ outer diameter/75 μm inner diameter self-packed PicoFrit columns (New Objective) with built-in emitters (75 μm emitter inner diameter). Stationary phase in analytical columns consisted of ReproSil-Pur 120 C18-AQ, 2.4 μm particle size, 120 Å pore (Dr Maisch High Performance LC GmbH). Trap columns consisted of $\approx 1 \text{ cm} \times 360 \mu\text{m}$ outer diameter/75 μm inner diameter polyimide-coated, fused-silica tubing (New Objective), packed with 5 μm

particle size, 120 Å pore, C18 stationary phase (ReproSil-Pur), with a Kasil frit. Gradient conditions were as follows: 0%B at 0 minutes, step to 5%B at 0.1 minutes, 5%B to 20%B over 65 minutes, 20%B to 35%B over 30 minutes, 35 to 100%B over 10 minutes, hold at 100%B for 5 minutes, drop to 0%B and equilibrate until 120 minutes. Electrospray ionization was accomplished with 2.4 kV positive spray voltage and an ion transfer tube temperature of 250°C. Survey scans of precursor ions were acquired in the Orbitrap detector from 350 to 1800 m/z at 70 000 resolution at 200 m/z using an automatic gain control setting of 3×10⁶, maximum injection time of 60 ms, and an RF lens setting of 65. Precursor ions from each survey scan were isolated with a 1.6 m/z window and 0.5 m/z window offset, using the following filters: charge states 2 to 6, minimum intensity threshold 3.3×10⁴, and dynamic exclusion (duration 15 s, exclude isotopes). Precursors were fragmented using higher-energy collisional dissociation at 28% normalized collision energy and analyzed in the Orbitrap detector at a resolution of 35 000 at 200 m/z, using a maximum injection time of 150 ms and an automatic gain control target of 1×10⁵. Up to 15 tandem mass spectrometry (MS/ms) spectra were acquired between precursor survey scans.

Protein Identification

All database searches were done in Proteome Discoverer (v2.4, Thermo Fisher Scientific) with Mascot (v2.8, Matrix Science) and Byonic (v4.1, Protein Metrics). Data was searched by Mascot against a human RefSeq FASTA database (116 505 entries), using the following search criteria: tryptic cleavage (maximum 1 missed), 3 ppm precursor ion tolerance, 0.01 Da fragment ion mass tolerance, Met oxidation, Lys biotinylation, and Gln/Asn deamidation allowed as variable modifications, and Cys carbamidomethylation as a fixed modification. Peptide-spectrum matches from the Mascot search were validated by the target-decoy method, using a false-discovery rate cutoff of 1%. Filtering analyses identified a set of enriched proteins specifically associated with TurboID-fused RAB5C WT-transfected cells.

Network Analysis of TurboID-Enriched Proteins

Functional protein association/interaction networks were constructed by loading the gene identifiers of upregulated and downregulated proteins into stringApp 1.4.2 embedded in Cytoscape 3.7.1 and then searching the STRING v11 database. The default association/interaction threshold (STRING score >0.4) was used to map relationships between proteins. Network modularity was assessed with the Markov clustering function in the clusterMaker2 1.3.1 app using the STRING score (>0.6) for edge weighting. The granularity parameter (inflation value) was set empirically. The final networks are presented in an edge-weighted, spring-embedded layout using the Markov cluster (module) number for edge weighting. Intermodule associations were omitted, and modules were rearranged for clarity. Module annotation was conducted using STRING's multipathway enrichment analysis.

Immunoblot

Cells were harvested in RIPA buffer. Thereafter, 25 µg of total protein were separated in a 4% to 15% SDS-PAGE

and transferred to a Nitrocellulose membrane (Bio-Rad). Membranes were blocked for 1 hour at room temperature in Odyssey Blocking Buffer (LI-COR 927-50000). Primary antibodies were incubated overnight at 4°C. Antibodies to RAB5C and GAPDH were from Santa Cruz, antibody to V5 was purchased from Thermo Fisher, Streptavidin and anti-flag antibody were from Sigma. Washes were performed with TBS 0.1% Tween-20 before the addition of secondary antibody for 1h at room temperature. Primary antibodies were detected using IRDye 800CW Goat (polyclonal) anti-Mouse IgG (H+L) highly cross-adsorbed (No. 925-32210; LI-COR), IRDye 800CW Goat (polyclonal) anti-Rabbit IgG (H+L) highly cross-adsorbed (LI-COR no. 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.

Coimmunoprecipitation

HEK293 cells were transfected with V5-tagged TurboID-RAB5C fusion protein, V5-tagged TurboID, and Flag-tagged SNAP29 using Lipofectamine 3000 following manufacture instructions. Two days after the transfection, cell lysates were extracted with IP lysis buffer supplemented with Protease Inhibitor Cocktail. Then, 500 µg of each sample was used for the assay. Immunoprecipitation was performed using magnetic beads pre-conjugated with anti-V5 or anti-Flag. Cell lysates were incubated with the antibody-conjugated beads for 1 hour at room temperature. Beads were then washed 3× with IP lysis buffer, and precipitants were eluted using 2× Laemmli sample buffer (Bio-Rad). Precipitants and input lysates were separated by 4% to 20% gradient SDS-PAGE (Bio-Rad) and transblotted to nitrocellulose. For the anti-FLAG IP, blots were immunoblotted with 1:1000 rabbit anti-V5 (Thermo Fisher) and then stripped (21059, Thermo Scientific) and reprobed with 1:1000 anti-Flag (Sigma). For the anti-V5 IP, blots were immunoblotted with 1:1000 primary mouse anti-Flag and then stripped and reprobed with 1:1000 mouse anti-V5. The FLAG-SNAP29 was a gift from Noboru Mizushima⁵⁴ (plasmid no. 45915; <http://n2t.net/addgene:45915>; RRID:Addgene_45915; Addgene). V5-TurboID-NES_pCDNA3 was a gift from Alice Ting⁵⁰ (plasmid no. 107169; <http://n2t.net/addgene:107169>; RRID:Addgene_107169; Addgene).

Statistical Analyses

Statistical analyses were carried out using Prism version 10. Differences between 2 groups were assessed using an unpaired 2-tailed Student *t* test, whereas differences among multiple groups were evaluated using 1-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons. Two-tailed *P* < 0.05 was considered statistically significant, with all error bars representing the SD. For the sample size of experiments, *n* denotes the number of independent experiments.

Data presented in bar graphs are shown as mean±SD. For box-and-whisker plots, the center line represents the median, the box shows the interquartile range (25th–75th percentiles), and the whiskers extend to the minimum and maximum values. All individual data points are displayed, and no outliers were removed.

RESULTS

Functional Genomics Screen Identifies a Role for RAB5C in Regulating Human VWF Levels

We previously performed a GWAS to identify new loci associated with VWF levels in human plasma.³¹ *RAB5C* was one of the candidate genes identified, with several genetic variants in the region significantly associated with VWF levels (Figure 1A). Fine-mapping of the genetic variants within this locus identified 25 variants that belong in the 95% credible set according to SUSIE (Figure S1). From that list, 2 variants, rs9915255 and rs9912088, are marked by endothelial ATAC-seq peaks in different introns of *RAB5C*, regions which are also enriched in activating histone modifications (H3K4me1, H3K4me2, and H3K27ac; Figure 1B). The minor allele frequency for rs9915255 is 26% and for rs9912088 is 26%. The colocalization of these variants with open chromatin regions in endothelial cells suggests that they may regulate *RAB5C* expression.

To validate our in silico findings at the *RAB5C* locus (17q21.2), we used clustered regularly interspaced short palindromic repeats interference (CRISPRi) to target the 2 prioritized variants (Figure 1C). We differentiated dCas9-KRAB⁺ expressing human iPSCs into endothelial cells using a previously described protocol and characterized their morphology and expression of endothelial markers (Figure S2).⁴⁷ We then performed CRISPRi by transfecting these cells with single guide RNAs that overlap our target variants, or a nontargeting single guide RNA as a control. *RAB5C* expression was significantly reduced after CRISPRi targeting of each variant region independently, supporting the hypothesis that the prioritized variants are found in enhancers that regulate the expression of *RAB5C* (Figure 1C). Notably, joint CRISPRi of both regions resulted in similar inhibition of *RAB5C* to that of individual CRISPRi of either region, possibly indicating a joint function between the 2 regulatory elements.

Taken together, our data suggest that genetic variants linked to altered VWF levels in humans also control *RAB5C* expression in endothelial cells.

RAB5C Regulates VWF Exocytosis From Endothelial Cells In Vitro

We investigated the role of *RAB5C* in regulating VWF release in endothelial cells. We used siRNA to silence *RAB5C* in HUVEC (Figure S3A and S3B). We then added media or histamine to HUVEC and measured the release of VWF into the cell media. *RAB5C* silencing did not affect basal release of VWF (Figure 2A). However, *RAB5C* silencing decreased histamine-induced VWF release (Figure 2A). *RAB5C* silencing did not change VWF mRNA (Figure S3C). However, *RAB5C* silencing increased intracellular VWF protein (Figure S3D). Conversely, *RAB5C* overexpression significantly increased VWF-stimulated release into the media (Figure 2B;

Figure S4A). These data suggest a key role for *RAB5C* in controlling VWF exocytosis in human endothelial cells.

RAB5C functions as a molecular switch, cycling between active and inactive conformations; the active form of *RAB5C* is bound to GTP. To determine whether the active form of *RAB5C* mediates VWF exocytosis, we overexpressed 2 *RAB5C* mutants in HUVECs: a CA form (*RAB5C*-CA, Q80L) and a DN form (*RAB5C*-DN, S35N; Figure S4B). We first validated their activity using an *RAB5C* GTP-binding assay: *RAB5C*-WT and *RAB5C*-CA bound GTP γ , whereas *RAB5C*-DN was unable to bind GTP γ , confirming that the mutants function as expected in transfected cells (Figure S5). Functionally, *RAB5C*-CA increased histamine-mediated VWF exocytosis, whereas *RAB5C*-DN decreased VWF release (Figure 2C), consistent with our silencing and overexpression experiments (Figure 2A and 2B). These findings suggest that *RAB5C* activation is essential for VWF exocytosis.

We observed that *RAB5C* mediates stimulated VWF release when the agonist is histamine. We next tested other secretagogues acting through different pathways. Histamine stimulates VWF exocytosis via H1/H2 receptor activation, leading to intracellular calcium mobilization and PKC (protein kinase C) activation.^{55,56} ATP induces VWF release through purinergic P2Y receptors, activating PLC (phospholipase C) and increasing intracellular calcium.⁵⁷ Calcium ionophore directly permeabilizes the plasma membrane, allowing calcium influx and bypassing receptor-mediated signaling.⁵⁸ Phorbol 12-myristate 13-acetate, a direct PKC activator, induces VWF exocytosis independently of calcium signaling.^{59,60} Thrombin, a physiological secretagogue of VWF, triggers release through PAR (protease-activated receptor)-1 and PAR-4, which couple to G-protein-mediated signaling cascades involving PLC, intracellular calcium mobilization, and PKC activation.⁶¹ *RAB5C* silencing decreases VWF exocytosis after stimulation with all agonists (Figure 2D; Figure S6). Our data suggest that *RAB5C* controls agonist-stimulated VWF exocytosis, independent of receptor signaling, suggesting a broader role for *RAB5C* in exocytosis.

Because *RAB5C* is known to facilitate endocytosis, we investigated whether released VWF is internalized and recycled by endothelial cells. We stimulated HUVEC with phorbol 12-myristate 13-acetate, and after stimulation, we added Dynasore to inhibit endocytosis. After 1 hour, we measured VWF levels in the cell media. No differences in extracellular VWF levels were observed compared with dimethyl sulfoxide-treated controls (Figure S7A). These data suggest that secreted VWF is not taken up by endothelial cells. To confirm this conclusion, we measured endothelial uptake of recombinant VWF. HUVEC incubated with recombinant VWF-GST for 30 minutes did not take up VWF, whereas activated THP-1 macrophages

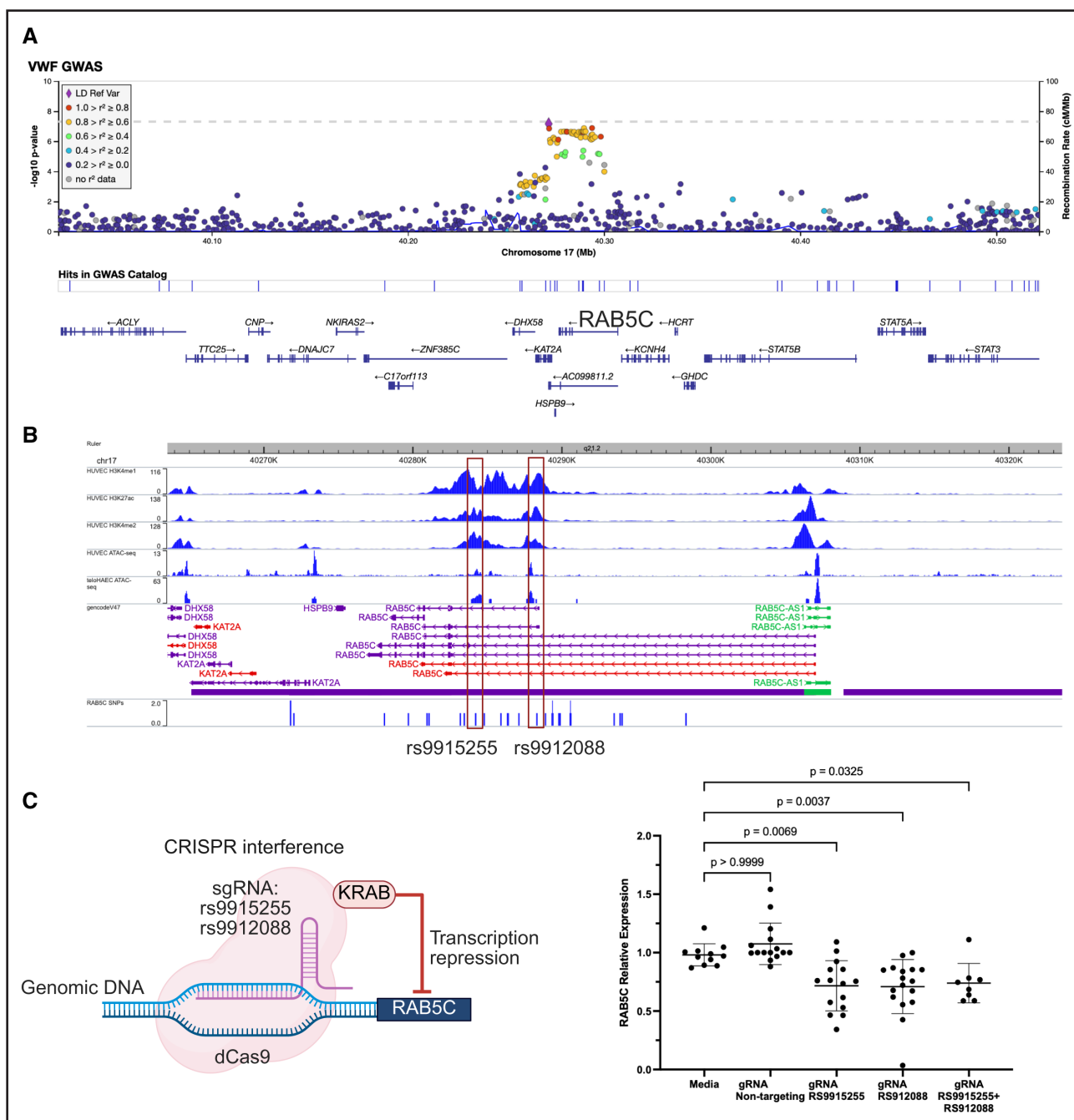


Figure 1. Functional genomics screen identifies a role for *RAB5C* (RAS-associated protein RAB5C) in human VWF (von Willebrand Factor) levels.

A, Fine-mapping of genetic variants in the *RAB5C* locus that are linked to VWF levels in humans. **B**, Epigenetic fine-mapping of credible set variants in the *RAB5C* locus (17q21.2) identifies 2 conserved variants in the *RAB5C* locus as candidate regulators of *RAB5C* and drivers of the VWF genome-wide association studies (GWAS) signal. In red are the variants that are localized in open chromatin regions of endothelial cells according to ATAC-seq (assay for transposase-accessible chromatin using sequencing) of human umbilical vein endothelial cell (HUVEC) and telomerase-immortalized human aortic endothelial cells (Telo-HAEC) analysis. **C**, CRISPR (clustered regularly interspaced short palindromic repeats) interference in inducible endothelial cells targeting 2 candidate fine-mapped variants indicates that the region surrounding rs9915255 and rs9912088 regulates *RAB5C* expression. Relative expression was measured by reverse transcription–quantitative polymerase chain reaction using the $\Delta\Delta C_t$ method (comparative threshold cycle method) with ACTB (β -actin) as the housekeeping control ($n=8-15$, mean $\pm$ SD). AS1 indicates antisense RNA 1; Chr, chromosome; dCas9, catalytically inactive Cas9; DHX, DEAH-box helicase; gRNA, guide RNA; H3K4me1, histone H3 lysine 4 monomethylation; H3K4me2, histone H3 lysine 4 dimethylation; H3K27ac, histone H3 lysine 27 acetylation; HSPB9, heat shock protein beta-9; KAT, lysine acetyltransferase; KRAB, Krüppel-associated box; sgRNA, single-guide RNA; and SNP, single-nucleotide polymorphism.

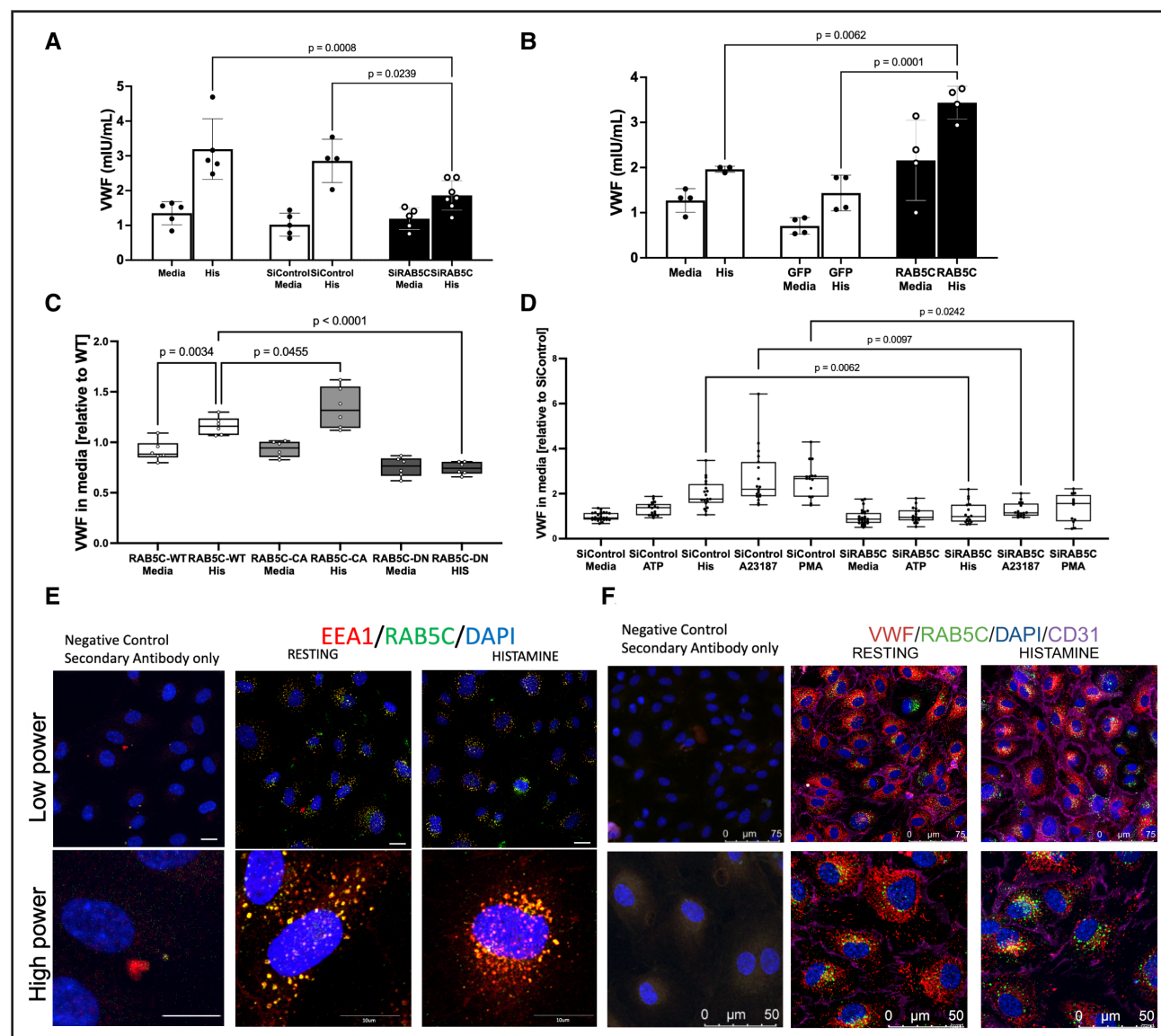


Figure 2. RAB5C (RAS-associated protein RAB5C) increases VWF (von Willebrand Factor) release.

VWF levels in the cell media were measured after human umbilical vein endothelial cells (HUVECs) were stimulated with histamine (His) or media after transfection with (A) small interfering RNA (siRNA) against RAB5C (siRAB5C) or siRNA control (SiControl), (B) vectors expressing GFP (green fluorescent protein) or RAB5C, (C) vectors expressing RAB5C–wild-type (RAB5C–WT), RAB5C–constitutively active (RAB5C–CA) and RAB5C–dominant negative (RAB5C–DN; $n=3-7$, median, interquartile range (IQR; [25th–75th percentiles], whiskers=minimum–maximum, all points shown). D, VWF levels in the cell media were measured after HUVEC were transfected with siRNA scramble or against RAB5C and stimulated with His, ATP, calcium ionophore, and phorbol 12-myristate 13-acetate ($n=6-20$, median, IQR [25th–75th percentiles], whiskers=minimum–maximum, all points shown). Overexpression of RAB5C increases VWF release; overexpression of constitutively active RAB5C increases VWF release, and expression of RAB5C–DN decreases VWF release. E and F, Subcellular localization of RAB5C. E, HUVECs were treated with vehicle or His for 1 hour, fixed, and immunostained with antibodies against RAB5C and EEA1 (early endosome antigen 1), then imaged by confocal microscopy. RAB5C and EEA1 colocalized, with Pearson correlation coefficients of 0.80 ± 0.02 under resting conditions and 0.75 ± 0.031 after His stimulation (green, RAB5C; red, EEA1; blue, DNA; yellow, colocalization of RAB5C/EEA1). F, Cells were fixed and immunostained with antibodies against RAB5C and VWF, and imaged by confocal microscopy (green, RAB5C; red, VWF; purple, CD31 [platelet endothelial cell adhesion molecule-1]; blue, DNA). RAB5C and VWF did not colocalize, with Pearson correlation coefficients of 0.43 ± 0.014 under resting conditions and 0.46 ± 0.034 after His stimulation. Similarly, RAB5C and CD31 did not colocalize, with Pearson correlation coefficients of 0.09 ± 0.016 under resting conditions and 0.09 ± 0.038 after His stimulation. Representative images are shown using the 20 $\times$ objective (top) and higher magnification views (bottom). Scale bars: 10 μ m, 50 μ m, and 75 μ m. Negative control images were acquired using secondary antibodies only. DAPI indicates 4',6-diamidino-2-phenylindole.

efficiently internalized VWF–GST (Figure S7B). We also confirmed that inhibition of endocytosis before stimulation with histamine does inhibit VWF release

(Figure S7C). We conclude that HUVEC do not take up VWF, which suggests that RAB5C does not affect VWF recycling.

To assess whether RAB5C controls the release of other proteins in WPB, we silenced RAB5C and measured exocytosis of Ang-2 (angiopoietin-2). Silencing RAB5C decreased stimulated Ang-2 release (Figure S8A). We also evaluated the role of RAB5C in the basal or constitutive secretion of other proteins not contained in WPB, such as thrombospondin-1 and protein S. RAB5C also regulated endothelial basal release of thrombospondin and protein S (Figure S8B and S8C). These data suggest that RAB5C regulates endothelial exocytosis of proteins contained within WPB (such as VWF and Ang-2) as well as proteins not contained in vesicles (such as thrombospondin and protein S).

Subcellular Localization of RAB5C and VWF

To better understand the role of RAB5C in regulated VWF exocytosis, we analyzed its subcellular localization in HUVECs using confocal microscopy. RAB5C appeared as a punctate signal in the cytoplasm (Figure 2E). Colocalization studies revealed that RAB5C localizes in the cytoplasm, colocalizing with EEA1 (early endosome antigen 1), a marker of early endosomes (Figure 2E). RAB5C does not localize with the plasma membrane or ER (Figure 2F), and RAB5C only partially colocalizes with the trans-Golgi network (Figure S9A and S9B). Furthermore, no colocalization between RAB5C and VWF was detected (Figure 2F), and coimmunoprecipitation assays confirmed no direct interaction between RAB5C and VWF (Figure S9C).

We next examined whether RAB5C changes WPB size or location within the cell. Colocalization studies of VWF with the plasma membrane, trans-Golgi network, and ER were performed in HUVECs with or without RAB5C silencing. RAB5C silencing did not affect WPB location (Figure S10A) or VWF distance from the nucleus (Figure S10B). RAB5C did not affect the size of WPB (Figure S10C). Silencing RAB5C had a small effect on the number of WPB (Figure S10D). These findings suggest that RAB5C regulates VWF exocytosis indirectly, likely through interactions with other molecular partners.

RAB5C Proximity Labeling

To investigate how RAB5C regulates VWF exocytosis, we identified protein partners adjacent to RAB5C using proximity labeling followed by mass spectrometry. We generated a fusion protein containing the TurboID enzyme fused to RAB5C.⁵³ This construct included a nuclear export sequence to ensure cytosolic localization, and a V5 tag for detection (Figure 3A). HEK293T cells were transfected with this construct, biotin was added for 10 minutes, and the cells were lysed. Biotinylated proteins near RAB5C were captured using streptavidin-coated magnetic beads (Figure 3A; Figure S11A and S11B) and analyzed by mass spectrometry.

To define the RAB5C-proximal neighborhood (≈ 10 nm), we filtered the protein list to consider proteins that were identified in the RAB5C-Turbo WT samples but absent from TurboID and GFP (green fluorescent protein) control groups (Figure S11C). This filter identified 147 proteins adjacent to RAB5C. These proteins were subjected to pathway analysis (Figure S11D). STRING functional association network analysis identified 98 proteins, yielding about 20 clustered modules (Figure 3B). Ontologies related to the endosome, vesicle transport, and endosome recycling constitute a major theme of the network. A comparison with WPB proteins identified by Holthenrich et al,⁶² revealed 26 shared proteins (Figure S11E).

Among these, SNAP29 emerged as a strong potential candidate, as we found it was in close proximity to RAB5C in our proximity labeling, and SNAP29 is a component of WPB, and SNAP29 is involved in vesicle trafficking.

To test for an interaction between RAB5C and SNAP29, we performed coimmunoprecipitation. HEK293T cells were transfected with the RAB5C-TurboID plasmid along with a FLAG-tagged SNAP29 construct. Immunoprecipitation of V5-tagged RAB5C coprecipitated FLAG-SNAP29 (Figure 3C).

Taken together, our data show that RAB5C and SNAP29 are in close proximity to each other and also interact with each other.

SNAP29 Regulates VWF Trafficking

We investigated the role of SNAP29 in VWF release using HUVEC. SNAP29 has been found in WPBs in endothelial cells.^{62,63} We silenced SNAP29 in HUVEC using siRNA (Figure S12A), and stimulated VWF exocytosis with histamine. Silencing SNAP29 decreased histamine-stimulated VWF exocytosis (Figure 4A) without changing VWF mRNA levels (Figure S12B). The effect on VWF exocytosis of silencing SNAP29 was similar to the effect of silencing RAB5C (Figure 4A).

We next examined whether SNAP29 changes VWF localization within the cell. Colocalization analysis by confocal microscopy revealed partial colocalization between SNAP29 and VWF with a Pearson correlation coefficient of $P=0.6\pm0.05$ (Figure 4B). Colocalization studies of VWF with the plasma membrane, trans-Golgi network, and ER showed that SNAP29 knockdown did not alter the subcellular distribution of VWF (Figure S12C and S12D). However SNAP29 knockdown decreased WPB number and size (Figure S12E and S12F).

To explore how RAB5C affects SNAP29, we performed confocal microscopy of endothelial cells with or without RAB5C silencing. Silencing RAB5C decreased the presence of SNAP29 in the Golgi (trans-Golgi network, Figure 4C) and significantly reduced the colocalization between SNAP29 and VWF (Figure 4B). Our results show that RAB5C silencing disrupts SNAP29 localization.

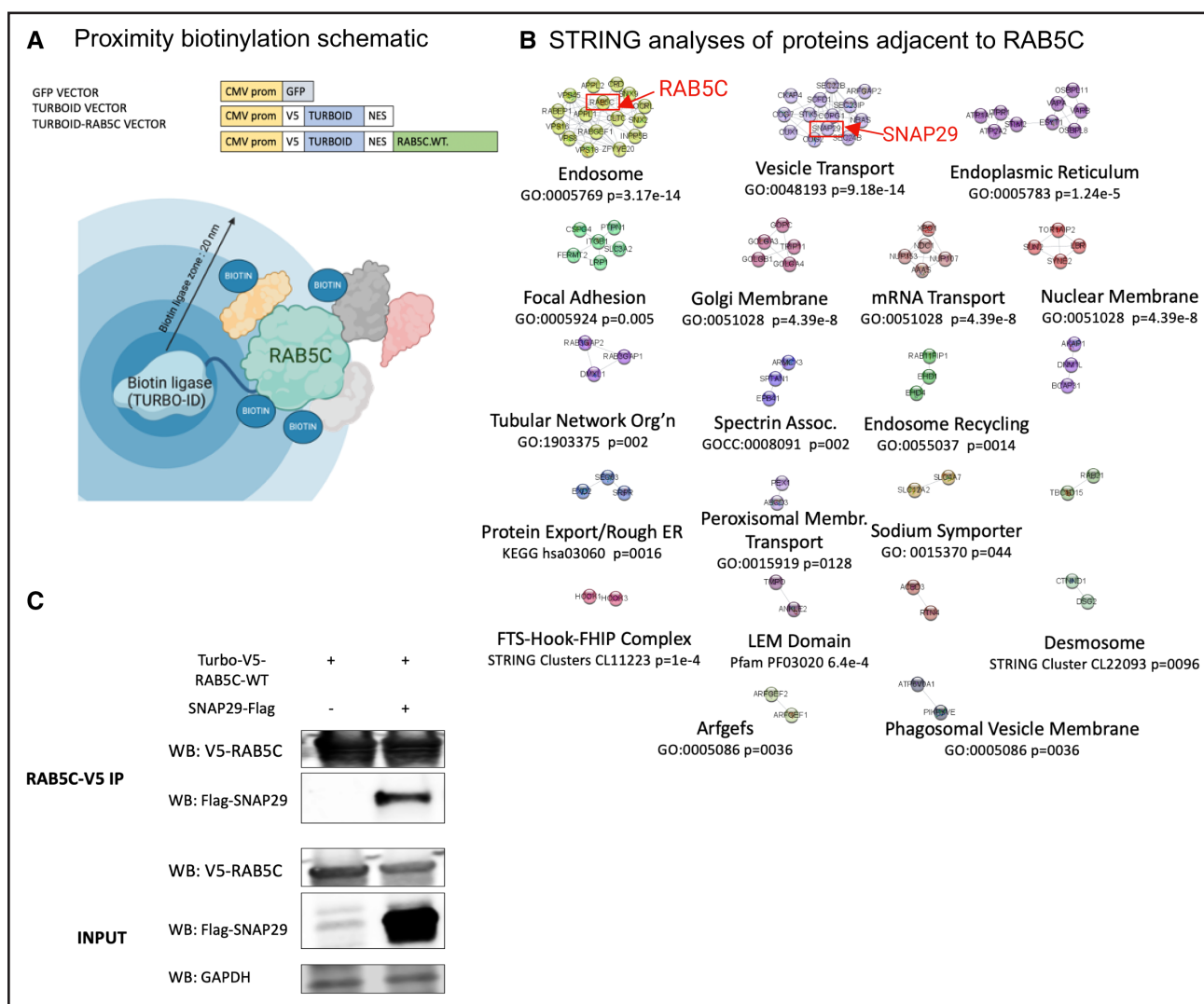


Figure 3. Proximity labeling identifies proteins adjacent to RAB5C (RAS-associated protein RAB5C).

A, top, Schematic of the constructs used for proximity biotinylation. A GFP (green fluorescent protein) vector was used as a control, the TurboID vector containing V5 tag was used as another control, and as an experimental vector, we used a vector of TurboID fused with RAB5C.

Bottom, Diagram of proximity biotinylation showing how the TurboID of the fused protein attaches biotin to any protein near the bait protein (RAB5C) within a radius of 20 nm. **B**, Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) analysis of the RAB5C adjacent proteins identified by mass spectrometry, after filtering out proteins adjacent to control vectors. **C**, Coimmunoprecipitation of V5-RAB5C and Flag-SNAP29 (synaptosome-associated protein 29) proteins overexpressed in human embryonic kidney 293T cells (HEK293T). AAAS indicates aladin WD repeat nucleoporin; ABCD3, ATP-binding cassette subfamily D member 3; ACBD3, acyl-CoA-binding domain-containing 3; AKAP1, A-kinase-anchoring protein 1; ANKLE2, ankyrin repeat and LEM domain-containing 2; APPL1, adaptor protein phosphotyrosine interacting with PH domain and leucine zipper 1; APPL2, adaptor protein phosphotyrosine interacting with PH domain and leucine zipper 2; ARFGAP2, ADP ribosylation factor GTPase-activating protein 2; ARFGEF1, ADP ribosylation factor guanine nucleotide exchange factor 1; ARFGEF2, ADP ribosylation factor guanine nucleotide exchange factor 2; ARFIP1, ADP ribosylation factor-interacting protein 1; ARM CX3, armadillo repeat containing X-linked 3; ATP1A1, ATPase Na⁺/K⁺ transporting subunit alpha 1; ATP2A2, ATPase sarcoplasmic/endoplasmic reticulum Ca²⁺ transporting 2; ATP6V0A1, ATPase H⁺ transporting V0 subunit a1; ATP7A, ATPase copper transporting alpha; BCAP31, B-cell receptor-associated protein 31; CKAP4, cytoskeleton-associated protein 4; CLCC1, chloride channel CLIC-like 1; CLTC, clathrin heavy chain; CMV prom, cytomegalovirus promoter; COG2, component of oligomeric golgi complex 2; COG7, component of oligomeric golgi complex 7; COPG1, coatamer protein complex subunit gamma 1; CTNND1, catenin delta 1; CUX1, cut-like homeobox 1; DNM1L, dynamin 1-like; DSG2, desmoglein 2; ECE1, endothelin-converting enzyme 1; EHD1, EH domain-containing 1; EHD4, EH domain-containing 4; ESYT1, extended synaptotagmin 1; EXD2, exonuclease 3'-5' domain-containing 2; FNDC3A, fibronectin type III domain-containing 3A; GFP, green fluorescent protein; GOLGA3, golgin A3; GOLGA4, golgin A4; GOLGB1, golgin B1; GOPC, golgi-associated PDZ and coiled-coil motif containing; HK1, hexokinase 1; HOOK1, hook microtubule-tethering protein 1; HOOK3, hook microtubule-tethering protein 3; INPP5B, inositol polyphosphate-5-phosphatase B; IP, immunoprecipitation; ITPR1, inositol 1,4,5-trisphosphate receptor type 1; KIF16B, kinesin family member 16B; LAMTOR1, late endosomal/lysosomal adaptor MAPK and MTOR activator 1; LBR, lamin B receptor; LNPEP, leucyl and cystinyl aminopeptidase; LRP1, LDL receptor-related protein 1; MTDH, metadherin; NBAS, neuroblastoma amplified sequence; NDC1, nuclear division cycle 1; NDRG1, N-myc downstream-regulated gene 1; NES, nuclear export signal; NUP107, nucleoporin 107; NUP153, nucleoporin 153; OCRL, oculocerebrorenal syndrome of Lowe; OSBPL8, oxysterol-binding protein-like 8; OSBPL11, oxysterol-binding (Continued)

Taken together, our results suggest that RAB5C controls the location of SNAP29, SNAP29 colocalizes with VWF, and SNAP29 regulates stimulated endothelial exocytosis of VWF (Figure 5). However, RAB5C does not affect basal secretion.

DISCUSSION

The major finding of our study is that RAB5C is a novel regulator of VWF exocytosis in endothelial cells. A set of proteins is adjacent to RAB5C, including SNAP29. We show that this regulation occurs via SNAP29, which interacts with RAB5C, colocalizes with VWF, and regulates VWF exocytosis.

Prior work has established RAB5 as a regulator of vesicle trafficking.^{26,64} The Rab family of proteins regulates vesicle trafficking in diverse cell types, including endothelial cells.³⁷ RAB5C was first identified as a regulator of endocytosis, including recycling of membrane proteins, formation of endosomes, and autophagosomes.^{33,42} (Subsequent work showed that RAB5C plays a role in cell adhesion, neuronal migration, and axonal transport.^{34,35,65}) Our current work and previous studies have reported that endocytosis contributes to VWF exocytosis. However, our work shows that endothelial cells do not take up VWF. This raises the possibility that RAB5C's role in endocytosis could indirectly influence VWF release, consistent with our observed effects of RAB5C in HUVEC. The RAB5 isoform RAB5A has been shown to regulate compound exocytosis, but the role of RAB5C in exocytosis has not previously been shown.

We show that RAB5C controls stimulated release of VWF but not constitutive (basal) release. The pathway of stimulated exocytosis involves discrete stages, including: agonist interaction with its cell surface receptor, internalization of surface receptors, intracellular signaling cascades, intracellular calcium transients, granule trafficking to the plasma membrane, SNARE interactions with calcium sensors, membrane fusion, and recycling of vesicle components.^{59,66} The precise stage at which RAB5C acts in this pathway is unclear. Based on our results, one possible secretory role for RAB5C is that RAB5C controls trafficking of

key proteins, such as SNAP29, from Golgi to granule that are necessary for exocytosis. Taken together, our results show that RAB5C is necessary for the process of exocytosis.

We discovered that within the cell, RAB5C is spatially adjacent to a set of 147 proteins, including proteins that regulate phagosome maturation, inositol phosphate metabolism, endocytosis, calcium transport, and autophagy (Figure 3; Figure S11). This suggests that RAB5C might affect VWF granule exocytosis at several steps. For example, RAB5C might affect the maturation of the Weibel-Palade granules that contain VWF in endothelial cells. Also, RAB5C might control phosphatidyl inositol phosphate signaling that regulates vesicle trafficking between the ER and Golgi and granule. RAB5C might also control the location of proteins that mediate calcium signals that trigger exocytosis.

Of the 147 proteins that we identified as adjacent to RAB5C, we selected SNAP29 for further study for several reasons. First, the literature shows that SNAP29 regulates ER to Golgi trafficking.⁶⁷ Second, the literature shows that SNAP29 is a component of VWF granules in endothelial cells.⁶² SNAP29 is a member of the SNARE family. SNAP29 regulates vesicle trafficking in several cellular pathways, including autophagy, ER to Golgi trafficking, and vesicle fusion with the plasma membrane.

Our data support the concept that SNAP29 mediates some of the effects of RAB5C on endothelial exocytosis of VWF. First, we found that SNAP29 is adjacent to RAB5C (Figure 3). Second, we found that SNAP29 physically interacts with RAB5C (Figure 3). Third, we have shown that SNAP29 partially colocalizes with VWF granules (Figure 4). Fourth, we show that silencing SNAP29 decreased endothelial release of VWF (Figure 4). Silencing RAB5C or its partner SNAP29 has the same effect on VWF release. Finally, we show that RAB5C regulates the location of SNAP29, maintaining its location in the Golgi and on WPB. Taken together, our data suggest that RAB5C decreases exocytosis in part by acting on a pathway that includes SNAP29.

There are several possible pathways through which SNAP29 might affect endothelial release of VWF. One possibility is that SNAP29 might be necessary

Figure 3 Continued. protein-like 11; PEX1, peroxisomal biogenesis factor 1; PHB2, prohibitin 2; PIKFYVE, phosphoinositide kinase FYVE-type zinc finger containing; PTPN1, protein tyrosine phosphatase nonreceptor type 1; RAB3GAP1, RAB3 GTPase-activating protein subunit 1; RAB3GAP2, RAB3 GTPase-activating protein subunit 2; RAB11FIP1, RAB11 family interacting protein 1; RAB21, RAB21 member RAS oncogene family; RABEP1, RAB GTPase-binding effector protein 1; RABGEF1, RAB guanine nucleotide exchange factor 1; RPN1, ribophorin I; RTN4, reticulum 4; SCAMP1, secretory carrier membrane protein 1; SCFD1, sec1 family domain containing 1; SEC22B, SEC22 homolog B vesicle-trafficking protein; SEC23IP, SEC23-interacting protein; SEC24B, SEC24 homolog B; SEC63, SEC63 homolog protein translocation regulator; SLC3A2, solute carrier family 3 member 2; SLC12A2, solute carrier family 12 member 2; SNAP29, synaptosome-associated protein 29; SNX1, sorting nexin 1; SNX2, sorting nexin 2; SNX3, sorting nexin 3; SNX9, sorting nexin 9; SRPR, signal recognition particle receptor subunit alpha; STIM2, stromal interaction molecule 2; STX5, syntaxin 5; SUN2, Sad1 and UNC84 domain containing 2; SYNE2, spectrin repeat containing nuclear envelope protein 2; TMPO, thymopoietin; TOR1AIP1, torsin A-interacting protein 1; TOR1AIP2, torsin A-interacting protein 2; TRIP11, thyroid hormone receptor interactor 11; VAPA, VAMP-associated protein A; VAPB, VAMP-associated protein B; VPS13C, VPS13 homolog C; VPS16, VPS16 core subunit of CORVET/HOPS complexes; VPS18, VPS18 core subunit of CORVET/HOPS complexes; VPS45, VPS45 homolog; WB, Western blot; XPO1, exportin 1; and ZFYVE20, zinc finger FYVE-type containing 20.

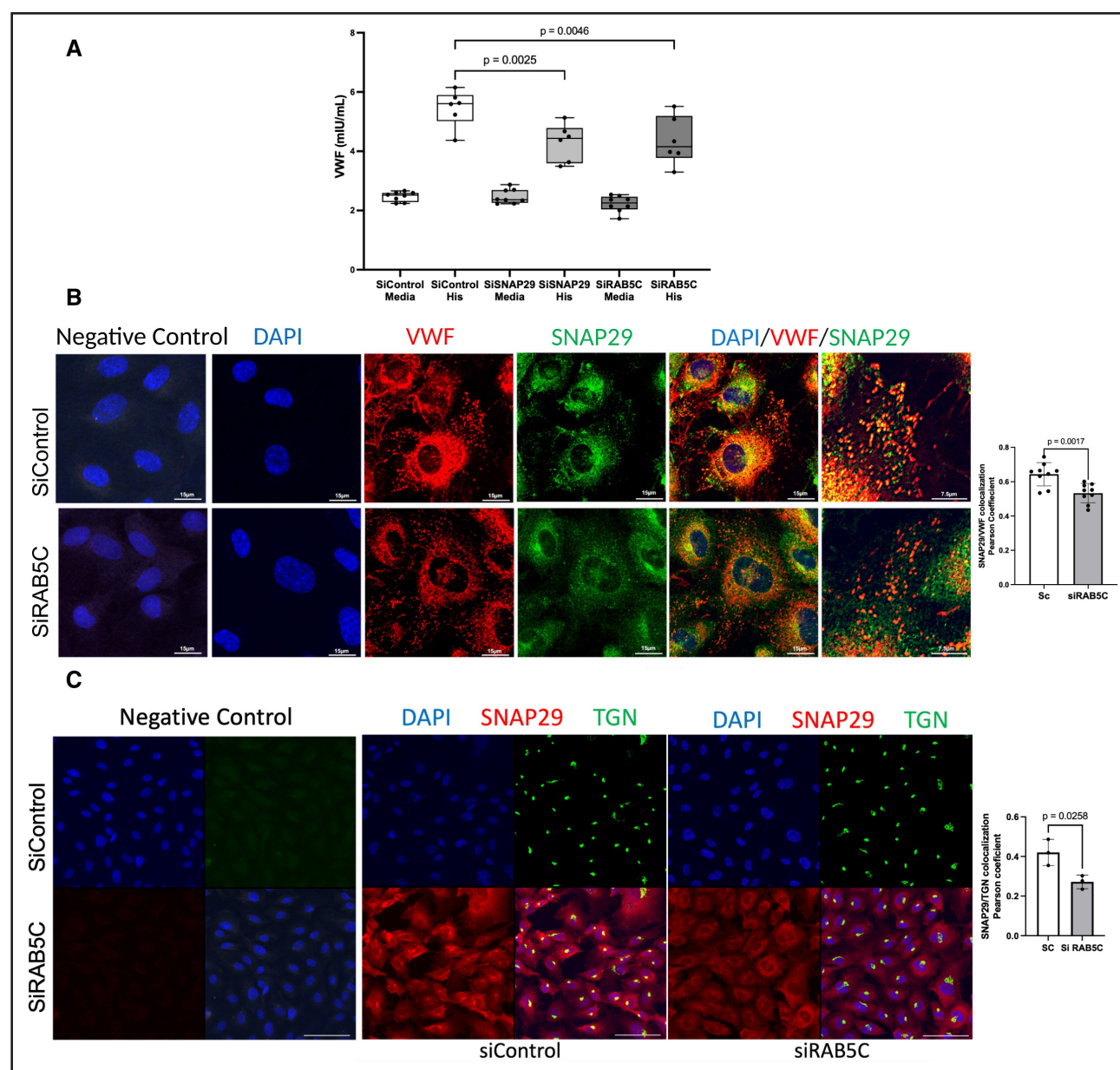


Figure 4. SNAP29 (synaptosome-associated protein 29) regulates VWF (von Willebrand Factor) trafficking.

RAB5C (RAS-associated protein RAB5C) regulates VWF release and SNAP29 localization in endothelial cells. **A**, VWF levels in the culture media were measured after human umbilical vein endothelial cells (HUVECs) were transfected with siControl or siRAB5C and stimulated with or without histamine for 1 hour ($n=6-8$; median, interquartile range [IQR; 25th–75th percentiles], whiskers=minimum–maximum, all points shown). **B**, VWF partially colocalizes with SNAP29. HUVECs were transfected with small interfering RNA (siRNA) control (siControl; **top**) or siRNA against RAB5C (siRAB5C; **bottom**), immunostained with antibodies against SNAP29 and VWF, and imaged by confocal microscopy using a $\times 20$ objective and magnified region (scale bars: 15 and 7.5 μm). VWF and SNAP29 partially colocalize, and colocalization decreases in RAB5C-silenced cells (green, SNAP29; red, VWF; blue, DNA; yellow, SNAP29/VWF colocalization). Colocalization was quantified using the Pearson correlation coefficient ($N=9$, mean $\pm$ SD). **C**, RAB5C controls SNAP29 localization in the trans-Golgi network (TGN). HUVECs were transfected with siRNA control (siControl; **left**) or siRNA against RAB5C (siRAB5C; **right**), immunostained with antibodies against SNAP29 (red) and the Golgi marker TGN (green), and imaged by confocal microscopy using a $\times 20$ objective (scale bar: 75 μm). Colocalization of TGN and SNAP29 was quantified by Pearson correlation coefficient. Silencing RAB5C reduces SNAP29 presence in the Golgi (blue, DNA; red, SNAP29; green, TGN; yellow, SNAP29/TGN colocalization; $n=3$, mean $\pm$ SD).

to regulate the transport of VWF or other WPB components from the ER to the Golgi or from the Golgi to the WPB. We found SNAP29 is localized in the Golgi and on WPB. Indeed, SNAP29 controls the maturation of lamellar granules necessary for exocytosis from

keratinocytes.⁶⁸ SNAP29 might also play a role in priming of VWF granules before fusion, as SNAP29 has been shown to interact with RAB3 and promotes glial cell exocytosis of myelin.⁶⁹ Another possibility is that SNAP29 is necessary for endocytosis and recycling of proteins

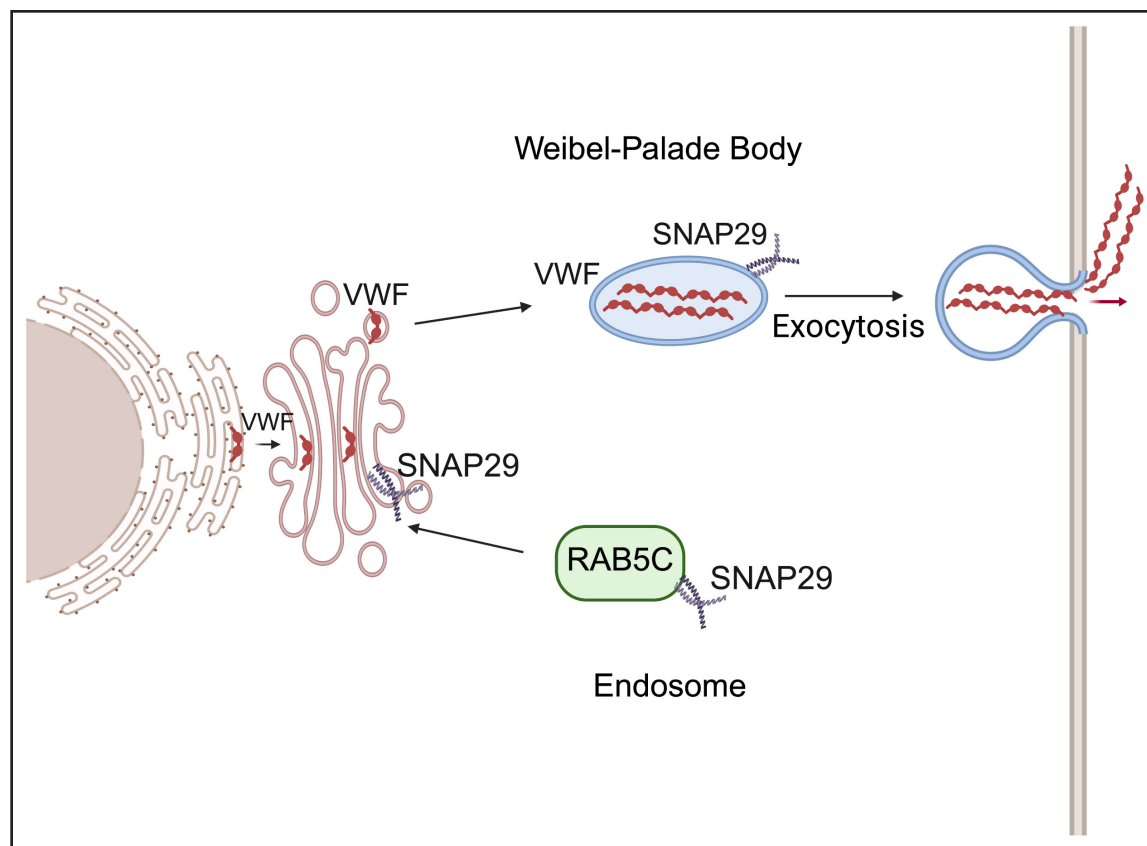


Figure 5. Scheme for RAB5C (RAS-associated protein RAB5C) regulation of VWF (von Willebrand Factor) exocytosis.

RAB5C interacts with SNAP29 (synaptosome-associated protein 29). RAB5C directs SNAP29 to the Golgi. SNAP29 localizes with Weibel-Palade bodies and facilitates exocytosis. Created in BioRender. Lowenstein, C (2025) <https://BioRender.com/1y0yb32>.

that are necessary for exocytosis.⁷⁰ A final possibility is that SNAP29 regulates autophagocytosis, a process that others have shown to be important in VWF release.⁷¹ Further work is needed to identify the precise stage at which SNAP29 controls exocytosis.

Our study has potential limitations. There are many interactors of RAB5C in addition to SNAP29, and some of these other partners probably play additional roles in mediating the effects of RAB5C on VWF exocytosis. Although we show that RAB5C controls the location of SNAP29 in the Golgi, the precise stage at which SNAP29 affects exocytosis is not fully understood. Our studies rely on human genetic data and cellular studies, and future studies are needed to explore the role of RAB5C control of VWF release in vivo.

In summary, we used GWAS data to identify genetic variants in humans that are associated with VWF exocytosis. We tested the role of 1 candidate gene, RAB5C, and found that it regulates VWF exocytosis from endothelial cells. These studies identify new components of the VWF secretory pathway.

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Disclosures

None.

Supplemental Material

Figures S1–S12

Major Resources Table

Unedited Western Blots

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