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A novel cause of type 1 VWD: impaired exocytosis of Weibel-Palade bodies due to biallelic variants in MADD

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Abstract:
The regulated secretion of von Willebrand factor (VWF) from Weibel-Palade bodies (WPB) in endothelial cells is fundamental to hemostasis. This process relies on recruiting Rab GTPases and their effectors to the WPB membrane, with the guanine nucleotide exchange factor (GEF) MAP-kinase activating death domain (MADD) playing a central role. Biallelic variants in MADD lead to a pleiotropic neurological and developmental disorder that can include bleeding abnormalities. This study investigates the impact of pathogenic MADD variants on VWF secretion using patient-derived endothelial cells. We isolated endothelial colony forming cells (ECFCs) from three pediatric patients with biallelic MADD variants and unaffected heterozygous family members. All patients exhibited low VWF plasma levels (22-30 IU/dL). Proteomic analysis of patient-derived ECFCs revealed an absence of MADD peptides, reduced VWF, and downregulation of proteins involved in the exocytotic machinery, including Rab3D and the Rab3/27 effector Slp4-a. Functional assays demonstrated diminished Rab27A and Rab3D activity and their failure to localize to WPBs in patient cells. Biochemical and live-imaging studies showed that histamine-induced VWF and VWFpp secretion were significantly reduced in patient cells due to delayed and reduced degranulation of WPBs. Our findings demonstrate the critical role of MADD in maintaining the secretion competence of WPBs and the magnitude of VWF secretion by regulating the recruitment of the endothelial exocytotic machinery. This study highlights the in vivo significance of WPB exocytosis in maintaining plasma VWF levels and establishes MADD as the first causal gene for quantitative von Willebrand Disease (VWD) in patients without pathogenic VWF variants.

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A novel cause of type 1 Von Willebrand Disease: impaired exocytosis of Weibel-Palade bodies due to biallelic *MADD* variants

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Running title: Biallelic MADD variants can cause VWD

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- MADD regulates WPB secretion and VWF levels *in vivo*
- Defects in WPB exocytosis can cause type 1 VWD

Abstract

The regulated secretion of von Willebrand factor (VWF) from Weibel-Palade bodies (WPB) in endothelial cells is fundamental to hemostasis. This process relies on recruiting Rab GTPases and their effectors to the WPB membrane, with the guanine nucleotide exchange factor (GEF) MAP-kinase activating death domain (MADD) playing a central role. Biallelic variants in *MADD* lead to a pleiotropic neurological and developmental disorder that can include bleeding abnormalities. This study investigates the impact of pathogenic *MADD* variants on VWF secretion using patient-derived endothelial cells. We isolated endothelial colony forming cells (ECFCs) from three pediatric patients with biallelic *MADD* variants and unaffected heterozygous family members. All patients exhibited low VWF plasma levels (22-30 IU/dL). Proteomic analysis of patient-derived ECFCs revealed an absence of MADD peptides, reduced VWF, and downregulation of proteins involved in the exocytotic machinery, including Rab3D and the Rab3/27 effector Slp4-a. Functional assays demonstrated diminished Rab27A and Rab3D activity and their failure to localize to WPBs in patient cells. Biochemical and live-imaging studies showed that histamine-induced VWF and VWFpp secretion were significantly reduced in patient cells due to delayed and reduced degranulation of WPBs. Our findings demonstrate the critical role of MADD in maintaining the secretion competence of WPBs and the magnitude of VWF secretion by regulating the recruitment of the endothelial exocytotic machinery. This study highlights the *in vivo* significance of WPB exocytosis in maintaining plasma VWF levels and establishes *MADD* as the first causal gene for quantitative von Willebrand Disease (VWD) in patients without pathogenic *VWF* variants.

Introduction

The inherited bleeding disorder von Willebrand disease (VWD) is caused by quantitative and qualitative defects of the hemostatic plasma protein von Willebrand factor (VWF). VWD is a heterogeneous disease with three distinct subtypes, among which type 1 VWD is the most prevalent[1]. This subtype is characterized by reduced circulating levels of VWF (< 50 IU/dL) and a spectrum of mild to severe bleeding complications[2,3]. Interestingly, approximately 30% of individuals diagnosed with VWD type 1 do not have pathogenic variants in *VWF*[2]. This observation implies that additional genetic or regulatory elements outside the *VWF* locus may contribute to the deficiency in VWF levels. Genome-wide association studies (GWAS) have confirmed this, identifying other loci that influence VWF plasma concentrations. These involve both the clearance and secretion of VWF. Well-established loci that explain VWF level variability through clearance include the ABO blood group and the clearance receptors *CLEC4M*, *STAB2* and *SCARA5*[4–7]. On the other hand, the secretion of VWF into the vascular lumen is crucial for maintaining its plasma levels and ensuring an adequate response to vascular injury. In line with this, GWAS have identified secretory genes *STXBP5* and *STX2* to be associated with VWF levels[4]. Plasma VWF is mainly derived from endothelial cells. In these cells, VWF is synthesized and stored in specialized secretory organelles called Weibel-Palade bodies (WPBs)[8,9]. Before newly formed WPBs can be secreted, they must acquire soluble factors on their membrane, including Rab GTPases[10]. These Rab GTPases, the largest family of small GTPases, are vital regulators of vesicle trafficking. They exist in an inactive state bound to GDP in the cytosol and are activated by guanine nucleotide exchange factors (GEFs), which facilitate the exchange of GDP for GTP. The Rab3/Rab27 GEF MAP-kinase activating death domain (MADD) is an important regulator of WPB maturation and VWF secretion in cultured endothelial cells. The activation and recruitment of Rab27A and Rab3 isoforms to WPBs depend on MADD *in vitro*[11]. Biallelic variants in *MADD* have recently been associated with a rare multisystem disorder[12]. This severe disease frequently results in death during infancy[12–15].

The MADD protein contains several important domains that contribute to its function. The DENN domain binds Rabs and facilitates their activation, playing a key role in vesicle trafficking[16]. The death domain is involved in MAPK pathway signaling, which is crucial for various cellular processes including inflammation and apoptosis. The ubiquitous function of MADD is illustrated by the variety of cell types affected in its absence. The activation of Rab27A by MADD is required for the exocytosis of cytotoxic granules by lymphocytes[17,18], as well as in melanocytes and rat parotid acinar cells[19,20]. It also serves as a GEF that activates Rab3 isoforms in neuronal and neuroendocrine cells[16,21]. Moreover, abnormalities in MADD lead to hormonal imbalances[12,22,23] and impaired endocytosis of epidermal growth factor (EGF) by fibroblasts[12]. The common factor across the observations in these various cell types is defective vesicle trafficking, prompting us to investigate the endothelial secretory processes in these patients as a model for endothelial defects in context of VWD. To address this, our study investigates the impact of *MADD* variants on endothelial function using patient-derived endothelial colony-forming cells (ECFCs). Our primary aim is to find out whether biallelic MADD variants result in impaired VWF release *in vivo*, which will enhance our understanding of the regulatory processes involved in type 1 VWD. Our findings suggest that MADD plays a critical role in WPB maturation and VWF secretion by facilitating the acquisition of the necessary exocytotic machinery and that biallelic variants in *MADD* can result in VWD type 1.

Materials and Methods

Recruitment of patients

The patients studied in this research were initially reported as *MADD* patients by Schneeberger *et al. (*patients 2, 12 and 23 in their paper)[12]*.* Following the publication of their work, we obtained blood samples from these three unrelated patients and their unaffected heterozygous parents as part of the 2020-BOEC-MK study (NL72564.078.20), an ongoing study in our lab focused on isolating ECFCs from patients with VWD or defects in trafficking genes. The 2020-BOEC-MK study was approved by the Medical Ethical Committee of the Erasmus University Medical Center and conducted in accordance with the Declaration of Helsinki. Blood samples were collected with written informed consent from all participants and their parents.

Isolation and culture of ECFCs

ECFCs were isolated from peripheral blood of study participants and healthy control donors following established protocols[24]. Cells were cultured on gelatin-coated plates in endothelial cell growth medium (EGM-2; Promocell), supplemented with 18% fetal calf serum (Sigma-Aldrich), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco) (EGM-18) at 37°C and 5% CO_2. ECFCs were used up to passage 7, and every experiment was performed on cells of equal passage.

NGS and qPCR analysis

Genomic DNA was isolated from ECFCs using the DNeasy Blood & Tissue kit (QIAGEN). An in-house targeted gene panel for Next Generation Sequencing (NGS; Illumina) was used to assess the genotypes of patients and their parents, with a target depth of 300x. Sequencing reads were aligned to the human genome Hg38. Variants were identified using STRELKA[25] for variant calling and DELLY[26] for detecting structural variants and copy number changes. ECFCs were kept at confluence in 6-wells plates for 5 to 7 days before RNA isolation, which was performed using the RNeasy mini kit (QIAGEN) and analyzed as described previously[27]. For

imaging and secretion experiments ECFC clones from healthy controls and patients were matched based on RNA profile[27].

Mass spectrometric analysis

Proteomics analysis was performed as described previously[28]. Details on cell work-up and initial data processing can be found in the **Supplemental Methods.** Proteins were filtered for proteotypic, ≥2 unique peptides per protein and should be quantified in at least 4 samples. LFQ values were transformed in log2 scale and missing values were imputed by normal distribution (width = 0.3, shift = 2.5). Label free statistical analyses were preformed using LIMMA[29] and moderated t-test were used to determine significant proteins (BH adjusted $p < 0.05$ and log2 fold change > 0.5).

Rab activity assay

The active fractions of Rab3D and Rab27A in ECFCs were determined using pulldown assays for the active form of both GTPases, followed by western blot analysis, as previously described[11]. Details of the antibodies used can be found in **Supplemental Table 1**.

Lentiviral transfection and transduction

Lentivirus encoding mEGFP-Rab3D, mEGFP-Rab27A or mEGFP-Slp4-a was produced in human embryonic kidney 293T (HEK293T) cells as described previously[30], using calcium phosphate transfection of third-generation lentiviral packaging plasmids pMD2.G, pRSV- REV, and pMDLg/pRRE (Addgene). Virus containing medium was collected over three days, concentrated, pooled, and frozen at -20°C. Endothelial cells were transduced by an overnight incubation with the lentivirus.

Immunocytochemistry, confocal microscopy and image analysis

ECFCs grown on gelatin-coated coverslips (Marienfeld) were fixed and stained as described previously[31]. The antibodies used are detailed in **Supplemental Table 1.** Images were taken

on a Leica SP8 confocal microscope. For quantitative analysis, tilescans were acquired on a Leica Stellaris 5 X LIA scanning microscope using a 63x oil immersion objective. Maximum intensity projections were used for subsequent analysis, which was carried out in CellProfiler[32] using a customized version of OrganelleProfiler[33] (**Supplemental File 1**).

Secretion assay

The secretion of VWF and VWF propeptide (VWFpp) from ECFCs after 30 minutes stimulation with 100 µM histamine was assessed as previously described[11]. Protein levels were quantified by enzyme-linked immunosorbent assay (ELISA). The percentage of secretion was calculated by dividing the concentration of VWF or VWFpp in the conditioned medium by the total amount, which is the sum of VWF or VWFpp concentrations in both the medium and the cell lysates. VWF and VWFpp levels were measured by ELISA as described previously[34], details are stated in the **Supplemental Methods**.

Live cell imaging of WPB exocytosis

Epifluorescence imaging of WPB secretion in two patients and two healthy controls was performed as previously descibed[35] but using VWFpp fused to mStayGold[36], a brighter and more photostable green fluorescent protein[37] (**Supplemental Figure 1; Supplemental Methods**).

Results

Reduced VWF plasma levels in patients with biallelic MADD variants

ECFCs were successfully isolated from three unrelated pediatric patients with biallelic variants in *MADD* and from one heterozygous parent of each patient (**Figure 1A,B**). The plasma VWF levels of the heterozygous parents were within the normal range (103-126 IU/dL). In contrast, all 3 patients with biallelic *MADD* variants exhibited significantly reduced VWF:Ag (22-37 IU/dL) and VWFpp:Ag (27-41 IU/dL) levels (**Figure 1B**). Notably, Patient 3, aged 12 years,

experienced recurrent nosebleeds, requiring frequent emergency hospital visits and blood transfusions. No bleedings were reported for Patients 1 (aged 4 years) and 2 (aged 14 years). The historically lowest VWF level <30 IU/dL of Patient 1 and 3 classifies them as VWD type 1, whereas Patient 2 is right at the cut-off of 30 IU/dL[3]. Genomic analysis confirmed the *MADD* genotypes of all study participants as reported by Schneeberger *et al.*[12] (**Figure 1C**). Using NGS covering the entire *VWF* locus we did not find pathogenic *VWF* variants in any of the three patients that could directly explain the low plasma VWF levels.

An unbiased proteomics approach shows loss of MADD protein and altered VWF exocytosis proteins in patient ECFCs

To assess the effect of biallelic variants on protein levels of MADD and to discern if other proteins are affected by MADD defects, we performed an unbiased proteomic analysis (**Figure 2A**). Samples included ECFCs isolated from the three patients, from one heterozygous family member per patient and from 11 healthy donors. Varying number of clones were isolated per patient and donor. Employing an unbiased proteomics workflow using mass spectrometry data independent acquisition, we quantified 7300–7894 proteins per sample (**Supplemental Figure 2A**). To assess protein levels of MADD, we plotted peptide coverage of the MADD sequence per sample. In total we detected 26 unique peptides with a mean coverage of 17% +/- 2.2 of the MADD protein in the controls (**Figure 2B**). Strikingly, no peptides were identified for any patients. To identify differentially abundant proteins between controls and patients, we performed t-tests, grouping all patients, versus all controls. Ten proteins were significantly downregulated in patients while four were upregulated ($p < 0.05$ & $LFC > 0.5$) (**Figure 2C and Figure 2D**). Besides MADD, downregulated proteins included those involved in the VWF exocytosis machinery such as RAB3D, SYTL4 (Slp4-a) and VWF. Other downregulated proteins were NPDC1, DPP7, CST3, CAPN5, IFIT5 and THEM4. Of those, NPDC1[38], DPP7[39] and IFIT5[40] have been linked to WPBs by proteomic screens of WPB composition and interactors in human umbilical vein endothelial cells (HUVECs). Strikingly, among the upregulated proteins, GSTM1 was not only upregulated in patients, but also in patients' family

members compared to healthy controls (**Supplemental Figure 2B**). Three proteins were uniquely upregulated in patients: SYS1, LCP1 and UBL5. Next, to assess coregulated proteins to MADD and VWF we determined protein level correlations of all proteins with MADD and VWF and filtered for highly correlating proteins (Pearson correlation > 0.75) (**Figure 2E**). Consistent with our statistical analysis, RAB3D and NPDC1 had a high correlation with MADD (both 0.87) and VWF (0.85 and 0.77 respectively). Overall, differentially regulated and correlating proteins contained integral proteins for WPB membrane tethering and fusion such as SYTL4 (Slp4-a)[41], MYRIP[42] and RAB3D[43,44] (**Figure 2F**). Interestingly, NPDC1 was (1) significantly downregulated in patient ECFCs and (2) had a high correlation with both VWF and MADD, suggesting a direct role in the regulation of both proteins. In summary, our findings show that *MADD* variants alter the proteome of patient ECFCs, particularly affecting proteins involved in VWF exocytosis and WPB function.

Rab3D and Rab27a are not activated and therefore not recruited to WPB in MADD patient ECFCs

To investigate the impact of MADD loss, we assessed the activation and recruitment of Rab3D and Rab27A in patient ECFCs. Due to limited availability of cells for P1 not all experiments could be performed for this patient. Pull down GTPase activity assays for P2 and P3 showed a significant reduction in the active, GTP-bound forms of Rab3D (83-89% decrease, $p< 0.01$) and Rab27A (77-88% decrease, $p< 0.01$) in patient cells compared to healthy controls (**Figure 3A,B**). Immunofluorescence analysis revealed that this decrease in activity was accompanied by complete absence of endogenous Rab3D and Rab27A from the WPB membrane (**Supplemental Figure 3**). To confirm that the observed effect was due to a lack of Rab activation in the absence of MADD, rather than low available Rab levels, we overexpressed mEGFP-tagged Rab3D and Rab27A. Representative images show that mEGFP-Rab3D was completely absent from WPBs in P1 and P3, and strongly reduced in P2 ECFCs (**Figure 3C**). Quantification of the mean intensity of the Rab3D signal on WPB in a large number of cells for P2 and P3 confirms this observation (**Figure 3D)**. Similarly, mEGFP-Rab27A was absent from

WPBs in P1 and P3, and reduced in P2 (**Figure 3E**). This reduction was also confirmed by image quantification (**Figure 3F**). Additionally, we investigated the recruitment of Slp4-a, a known effector of Rab3 and Rab27, to WPBs in P2 and P3. Overexpression of Slp4-a revealed that it is recruited to WPBs in healthy and heterozygous family member ECFCs. However, there is only a faint signal of mEGFP-Slp4-a on WPBs in P2 cells, and it is absent in P3 cells, indicating that the entire Rab3/Rab27-Slp4-a axis is disrupted in patient cells (**Figure 4**). These results indicate that the loss of MADD impairs the activation and recruitment of Rab3D and Rab27A to WPBs, severely disrupting the exocytotic machinery required for WPB function.

The loss of effector complex formation results in the loss of VWF secretion capacity

To investigate the impact of loss of MADD on secretion of WPB cargo, we evaluated histamine (100 µM; 30 minutes) evoked secretion of VWF from patient-derived ECFCs. Our analysis revealed a significant reduction in stimulus induced VWF secretion in ECFCs from patients P1 and P3 compared to controls, with an 81% reduction in P1 ($p<0.01$) and a 76% reduction in P3 ($p<0.01$) (**Figure 5A**). Similarly, the levels of secreted VWFpp were reduced by 66% in P1 ($p<0.01$) and 86% in P3 ($p<0.05$) (**Figure 5B**). In contrast, VWF and VWFpp secretion from P2 cells was not reduced compared to control ECFCs. Since these *in vitro* release assays can lack in sensitivity due to their extended stimulation times and do not provide detail on timing and extent of release of individual organelles, we used live-cell imaging to observe release of WPBs that were fluorescently labeled with the VWFpp-mStayGold probe (**Figure 6A,B**). Due to limited availability of cells for P1, these experiments were performed for P2 and P3 only. The mean WPB release per cell was lower in both P2 and P3, with a significant proportion of P3 ECFCs showing no WPB release at all (**Figure 6C**). Histograms of the times of WPB exocytotic events (bin width 2 seconds) for 40 cells per individual showed a reduced number of events (**Figure 6D**). Moreover, the delay to the first fusion events following the rise in intracellular calcium was notably prolonged in patient cells. In P2, the delay was 12±2 seconds, and in P3, it was 26±9 seconds, compared to 3±1 second in control cells (**Figure 6E**). Interestingly, while ELISA results for P2 did not show a significant reduction in VWF and

VWFpp secretion, live-cell imaging revealed a reduced mean WPB release per cell and a prolonged delay in the first fusion events. This discrepancy suggests that while the overall secretion levels measured by ELISA might appear unaffected, the dynamics and efficiency of WPB exocytosis are impaired in P2 cells. Cumulative plots of WPB fusion times scaled to the mean fraction of fluorescent WPB exocytosed clearly distinguished patient ECFCs from control ECFCs (**Figure 6F**). MADD patient cells showed significantly reduced histamine-induced degranulation, with only 17% of fluorescent WPB undergoing exocytosis in P2 and 5% fraction in P3, compared to 36-39% in control ECFCs (**Figure 6F**). Taken together, these results highlight the impaired WPB fusion process in MADD-deficient ECFCs.

Discussion

This is the first study to demonstrate that a defect in the endothelial cell secretory pathway can result in VWD type 1. This is illustrated by three patients with historically lowest circulating VWF:Ag levels between 22-30 IU/dL that all have distinct, biallelic variants within one gene that lead to (1) deficiency of MADD protein expression in endothelial cells, (2) reduced Rab3/Rab27A activation and (3) loss of key regulators of exocytosis from WPBs. MADD has been recognized as the GEF for Rab27A, Rab3B and Rab3D across various cell types[11,16,18–20] and our findings align with previous research on the role of MADD in vesicle trafficking, but provide new insights into its specific involvement in VWF secretion. We show through real-time observation of WPB fusion that the absence of MADD impairs VWF secretion by reducing the secretion competence of WPBs. Since endothelial cells are the main source of circulating VWF, this ultimately translates in the lower VWF plasma levels we observed in patients with deleterious *MADD* variants. Plasma VWF propeptide levels are also low in these patients (Figure 1), consistent with reduced synthesis or secretion of VWF rather than increased clearance as an underlying mechanism. Our study thus further highlights the contribution of secretion of VWF from WPBs to maintenance of adequate levels of circulating VWF and positions MADD as an important regulator of VWF levels *in vivo*.

Despite the absence of MADD, some VWF secretion still occurs, which is also evident from the low but persistent number of WPB exocytosis events in patient ECFCs. Residual WPB release could be supported by alternative routes for recruitment of exocytotic machinery, such as via the MADD-independent Rab15 - Munc13-4 complex[44] or by SNAREs and exocyst subunits arriving from the endolysosomal pathway[45–47]. Whole proteome analysis revealed total absence of MADD peptides in patient ECFCs, regardless of the specific variant. Loss of expression as a result of nonsense-mediated decay was to be expected for patients with variants that result in premature termination codons (Patient 1 and 3), but Patient 2 harbors one allele that encodes a missense variant (G305V). Although we were also unable to detect

MADD-derived peptides in this patient, we cannot rule out that minimal levels of MADD protein, below the detection limit of mass spectrometry, remain. This could explain why there still was some residual Rab3/Rab27A and Slp4-a targeting to WPBs in ECFCs of this particular patient. Loss of MADD was associated with decreased expression of Slp4-a and RAB3D, suggesting that MADD may influence the stability of these exocytotic components, which could further define the phenotype. Next to RAB3D, NPDC1 was the only protein both significantly downregulated in patients and highly correlating to both MADD and VWF. NPDC1 has been shown to regulate (neuronal) cell proliferation and although it was also identified in a proteomic screen on purified WPB fractions in HUVECs, it has not been described as a VWF interactor[39]. However, it has been shown to colocalize with MADD homologue Rab3 GEP in synaptic vesicles in rats[48] and to bind to MADD homologue AEX-3, regulate RAB27A and potentially regulate secretion of dense-core vesicles (DCVs) in *C. Elegans* (where it is termed CAB-1)[49–51]. If NPDC1 has similar functions in WPB secretion or trafficking in humans remains to be further investigated, but provides an interesting research outlook.

While it is generally accepted that circulating VWF primarily originates from WPBs, the pathways through which it is released remain unresolved. It has been argued that VWF in plasma primarily originates from basal (i.e. unstimulated) secretion of VWF from WPBs[52,53], a concept that still requires confirmation *in vivo*. On the other hand, there is also evidence for a significant contribution of regulated release of VWF by exocytosis of WPBs through stimulus-induced activation of endothelial cells. This is illustrated by (1) elevations in plasma VWF following activation by the WPB secretagogue epinephrine[54,55], (2) the link between arginine vasopressin 2 receptor (V2R) variants and circulating VWF levels[56], but also by (3) reductions in plasma VWF levels in *TLR2* knockout mice, a Toll-like receptor found on endothelial cells that activates release of WPBs[57,58]. All these pathways converge on the release mechanism of WPBs and can therefore be expected to depend on crucial components in their exocytotic machinery, such as MADD. Our data show that defects in this machinery reduce the ability for WPBs to undergo exocytosis, but do not affect basal release of VWF (Supplemental Figure 4),

which suggests that the reduction in VWF in plasma in these patients is the result of diminished regulated secretion.

The patients with biallelic *MADD* variants in our study had historically lowest circulating VWF levels between 22-30 IU/dL, and according to the current ASH-ISTH-NHF-WFH guidelines[3] levels <30 IU/dL are defined as type 1 VWD regardless of bleeding phenotype. Patients with this VWD type are at increased risk of bleeding, but even in cases with confirmed pathogenic *VWF* variants symptom severity may vary[59] or excessive bleeding may even be absent, especially in children who did not yet encounter hemostatic challenges[60,61]. Indeed, only one of the three patients in our study has a history of abnormal bleeding. The diagnostic workup of this patient (Patient 3) was performed before inclusion in our study and also showed prolonged closure time in platelet function analysis (PFA). A prolonged PFA has, independent from our study, also been reported for Patient 2[18], although she did not experience excessive bleeding.

It is currently unclear if our results are generalizable to all patients with *MADD* variants. So far, in literature a total of 42 patients have been reported with homozygous or compound heterozygous *MADD* variants[12–15,22,62,63]. The phenotypic spectrum is broad and does not seem to correlate with the type of variant, or whether it was located in the GEF activity containing DENN domain or in the MAP kinase activity containing DEATH domain of MADD[12]. While our study primarily attributes the effects of biallelic *MADD* variants on VWF secretion to the loss of GEF activity, we did not explore the impact of MADD deficiency on MAP kinase signaling or a role for that pathway in regulation of VWF. Detailed phenotypic descriptions and mechanisms by other investigators include a splice site variant (c.4377+2T>G) that does not affect GEF activity but is linked to an endocrine phenotype, with normal MADD protein levels observed in these patients[22]. Another splice site variant (c.963+1G>A) results in an in-frame deletion of 30 base pairs that causes loss of MADD protein expression, and is associated with impaired platelet function due to reduced ATP secretion from platelet dense granules and a cytotoxic defect due to impaired cytotoxic granule secretion in NK and CD8 T cells, characteristic of

hemophagocytic lymphohistiocytosis (HLH)[12,18]. Features of HLH, such as anemia and thrombocytopenia, are prevalent in most of the patient population. Reports on VWF levels or bleeding abnormalities are lacking from the small population of patients available. It should however be noted that these patients are young, have a low life expectancy and often suffer from physical impairments, which may limit the number of hemostatic challenges that they experience. Furthermore, due to the severe symptoms of the other manifestations of their disease, these patients are often not directly seen by hematologists. This is relevant because VWD type 1 is notoriously under-diagnosed, partly due to insufficient disease awareness in non-specialist health care professionals[1]. Our findings highlight that defects in the WPB exocytosis pathway –of which MADD is one example– can underlie reduced plasma VWF levels and a type 1 VWD phenotype. However, we show that heterozygous *MADD* variants do not affect WPB exocytosis and that heterozygous family members do not have reduced VWF plasma levels. Considering the rarity and severe condition of patients with biallelic *MADD* variants, it is unlikely that these account for a substantial number of patients within the broader type 1 VWD patient population. Probably for that same reason, *MADD* has not emerged as a significant locus in previous GWAS of VWF plasma levels. GWAS are designed to detect associations with common variants of modest effect and are less effective at identifying rare variants, such as the *MADD* variants we describe here.

Patients with type 1 VWD or mild to moderate Hemophilia A are often treated with the synthetic vasopressin analogue DDAVP[64], which raises VWF and FVIII levels by eliciting WPB exocytosis. However, not all patients show a (complete) response to DDAVP[65] and the reasons for this lack of response are currently not fully understood. None of the patients in our study have been treated with DDAVP in the past. Performing a DDAVP trial in neurologically and developmentally affected patients for research purposes is ethically problematic. Even more so because the impaired secretion of WPBs observed in our *ex vivo* ECFC secretion assays, which indirectly address the mechanism of action of DDAVP, would predict that these patients will not benefit from DDAVP treatment.

Our combined results serve as an example that defects in the WPB exocytosis pathway can result in type 1 VWD and warrant further exploration of genetic variants in this pathway in the pathogenesis of type 1 VWD and the mechanisms of DDAVP non-response.

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Authorship contributions

S.H. and S.A.G. performed experiments, analyzed data and drafted the manuscript; P.E.B. performed experiments; S.N.J.L. performed experiments and analyzed data; G.C.K., T.H., and D.B. included patients and provided clinical input; F.W.G.L provided clinical input. M.v.d.B. supervised research and drafted the manuscript; P.A.S. made and provided vital reagents used in this study; T.C. performed experiments, analyzed data, supervised research and drafted the manuscript; R.B. designed the study, supervised research and drafted the manuscript. All authors have read and approved the final version of the manuscript.

Disclosure of Conflicts of Interest

F.W.G.L. received research support from CSL Behring and Shire/Takeda for performing the Willebrand in the Netherlands (WiN) study and is a consultant for uniQure, Takeda, CSL Behring, and BioMarin, of which the fees go to the institution. The other authors declare no conflicts of interest.

Data availability

The mass spectrometry proteomics data and DIANN analysis files have been deposited to the ProteomeXchange Consortium via the PRIDE[66] partner repository with dataset identifier PXD058221.

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Figure Legends

Figure 1. Patient characteristics. (A) Pedigrees of the three families harboring pathogenic *MADD* variants. Affected individuals are indicated by filled symbols. Different colored rectangles represent the variants found in each individual as indicated above the pedigree. (B) Venous blood plasma measurements and clinical features of patients and healthy family members at inclusion in the 2020-BOEC-MK study. For Patient 3, additional plasma measurements were previously performed at the University Hospital of Antwerp due to bleeding problems. (C) Schematic of the MADD protein (NP_003673.3) showing the DENN and DD domains, alongside the *MADD* variants confirmed by NGS.

Figure 2. Absence of MADD peptides and reduced abundance of secretory machinery in patient ECFCs (A) Schematic overview of proteomics workflow. (B) MADD peptide coverage plot per sample, color gradient indicates log10(intensity). Percentages are means of control, patient or patient family ECFC clones. (C) Volcano plot of patient versus control ECFCs. Dotted lines indicate p-value (-log10(0.05)) and log fold change (LFC > 0.5) thresholds, colors indicate down- (blue) and upregulated proteins (orange). Proteins of interest are labeled. (D) Relative protein abundance (LFQ) values. Colors indicate grouping: controls (CTL; grey circle), patient (P; red square) and patient family member (PF; blue diamond). (E) Proteins with Pearson correlation > 0.7 to MADD (green) and VWF (purple), connecting line indicates protein correlation to both. (F) Schematic overview of WPB tethering proteins which were differently regulated (brown) or correlated to VWF (purple), MADD (purple) or both (red).

Figure 3. Loss of Rab targeting to WPBs in MADD patient ECFCs. (A,B) Normalized levels of active Rab3D (A) and Rab27a (B) measured by pulldown assays, relative to input levels. (C) ECFCs transduced with LVX-mEGFP-Rab3D (green) and immunostained for VWF (red) to visualize Rab recruitment to WPBs. Representative confocal images are shown. Scale bar is 10 µm. (D) Intensity (a.u.) of Rab3D fluorescence on the WPB membrane. Each dot represents the mean of a cell. (E) Confocal images of ECFCs transduced with LVX-mEGFP-Rab27a

(green) and immunostained for VWF (red). Scale bar is 10 μm. (F) Quantification of Rab27a fluorescence intensity (a.u.) on WPBs. Each dot represents the mean of a cell.

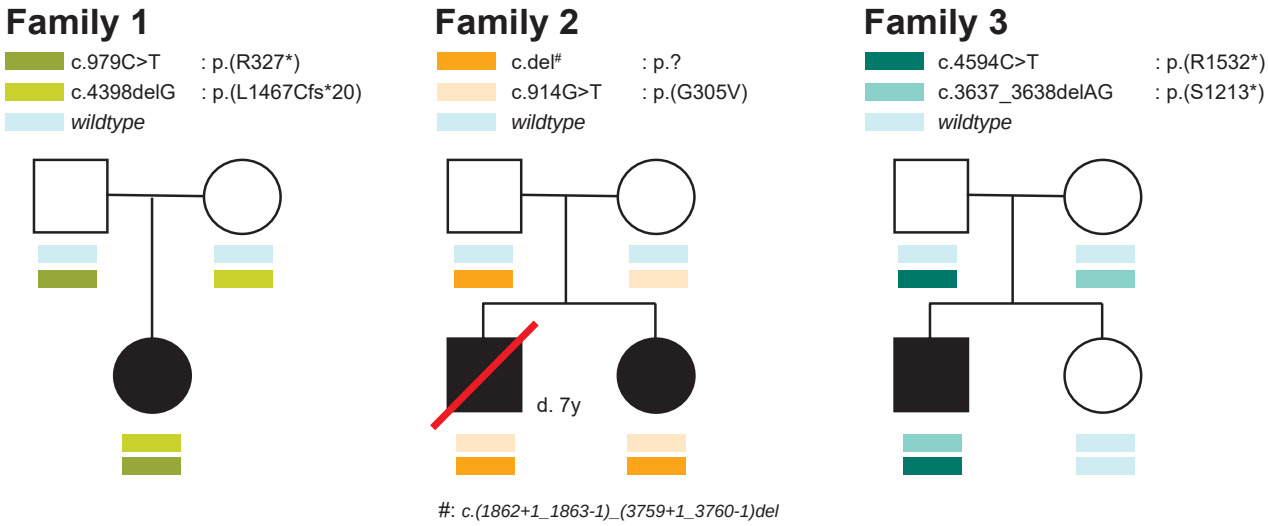
Figure 4. The Rab effector Slp4-a is not recruited to WPB in absence of MADD. ECFCs were transduced with LVX-mEGFP-Slp4-a (green) and immunostained for VWF (red). Representative confocal images are shown. Magnified views of the areas indicated by the white square are presented on the right. Scale bar is 20 μm.

Figure 5. Reduced VWF and VWFpp secretion from MADD patient ECFCs. (A) VWF secretion measured by ELISA in medium of ECFCs stimulated with 100 μM histamine for 30 minutes. Release is normalized to control. (B) VWFpp secretion measured by ELISA in medium of ECFCs stimulated with 100 μM histamine for 30 minutes. Release is normalized to control. Results are presented as mean ± SD from three independent experiments, each performed in triplicate. Statistical significance is indicated as *P ≤ 0.05, **P ≤ 0.01 (t-test).

Figure 6. MADD deficiency impairs WPB exocytosis. (A) Schematic of the live cell secretion workflow. ECFCs are nucleofected with VWFpp-mSG to label WPBs. After 24 hours, cells are stimulated with 100 μM histamine, and individual fusion events are recorded over time. (B) Example of experiment output data in a healthy control ECFC. T=0 is set at the time of intracellular calcium rise. The left side shows three subsequent still images from the video, with timepoints indicated above each image, where WPB release is observed over time. Scale bar is 10 μm. In the histogram on the right side the WPB fusion events of this single cell are counted (bin width 2 seconds). (C) Percentage of WPBs released, plotted for each cell that was imaged (n=39). (D) Delay from calcium rise (measured by a rise in 355 nm/380nm Fura-2 ratio) to the first exocytosis event, plotted per cell (n=39). (E) Histograms (bin width: 2 seconds) of WPB secretion events from MADD P2 ECFCs (top, yellow, 344 events) and P3 ECFCs (bottom, green, 107 events) ECFCs compared to healthy controls (1151 and 1012 events respectively). (F) Cumulative plot of histamine-evoked WPB fusion times, scaled to the fraction of WPB that underwent exocytosis.

Figure 1

A



#: *c.(1862+1_1863-1)_(3759+1_3760-1)del*

B

	Sex	Blood group	VWF:Ag [IU/dL]	VWFpp:Ag [IU/dL]	Bleeding diathesis	Schneeberger *et al.* [12]
Patient 1	F	non-O	22	30	Not reported	Patient 12
Mother Patient 1	F	O	108	72	Not reported	-
Patient 2	F	O	30	27	Not reported	Patient 2
Mother Patient 2	F	non-O	126	63	Not reported	-
Patient 3	M	O	24-37*	32-41**	Yes	Patient 23
Father Patient 3	M	O	103	89	Not reported	-

** Range of 3 independent measurements* *** Range of 2 independent measurements*

C

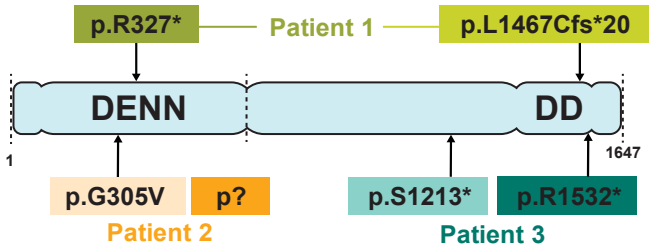
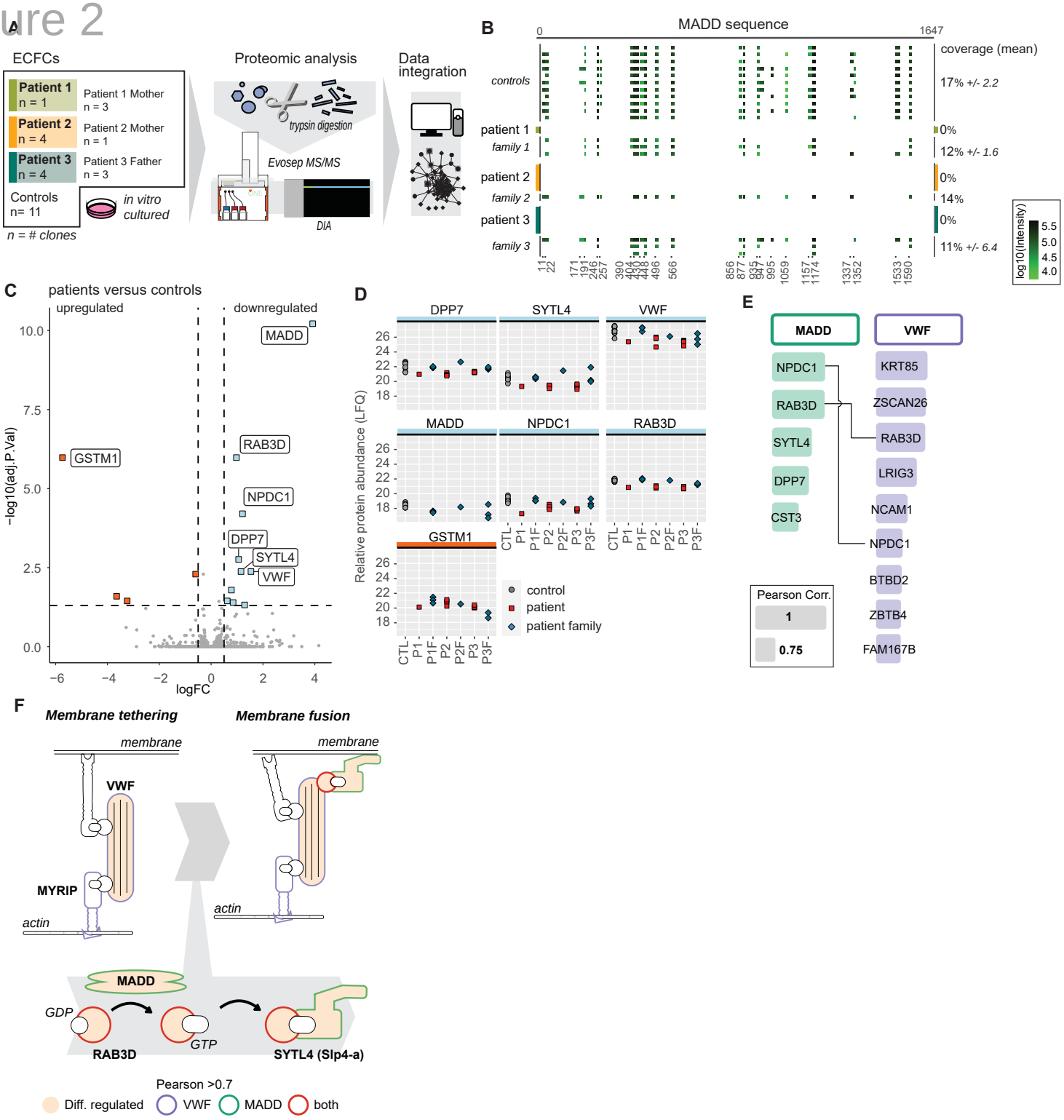


Figure 2


A
ECFCs
Patient 1 n = 1
Patient 1 Mother n = 3
Patient 2 n = 4
Patient 2 Mother n = 1
Patient 3 n = 4
Patient 3 Father n = 3
Controls n= 11
in vitro cultured
n = # clones
Proteomic analysis
trypsin digestion
Evosep MS/MS
DIA
Data integration
B
MADD sequence
0
1647
coverage (mean)
controls
17% +/- 2.2
patient 1
family 1
0%
12% +/- 1.6
patient 2
family 2
0%
14%
patient 3
family 3
0%
11% +/- 6.4
log10(Intensity)
5.5
5.0
4.5
4.0
C
patients versus controls
upregulated
downregulated
MADD
GSTM1
RAB3D
NPDC1
DPP7
SYTL4
VWF
−log10(adj.P.Val)
logFC
D
DPP7
SYTL4
VWF
MADD
NPDC1
RAB3D
GSTM1
Relative protein abundance (LFQ)
CTL
P1
P1F
P2
P2F
P3
P3F
control
patient
patient family
E
MADD
VWF
NPDC1
RAB3D
SYTL4
DPP7
CST3
KRT85
ZSCAN26
RAB3D
LRIG3
NCAM1
NPDC1
BTBD2
ZBTB4
FAM167B
Pearson Corr.
1
0.75
F
Membrane tethering
Membrane fusion
membrane
VWF
MYRIP
actin
MADD
GDP
GTP
RAB3D
SYTL4 (Slp4-a)
Pearson >0.7
Diff. regulated
VWF
MADD
both

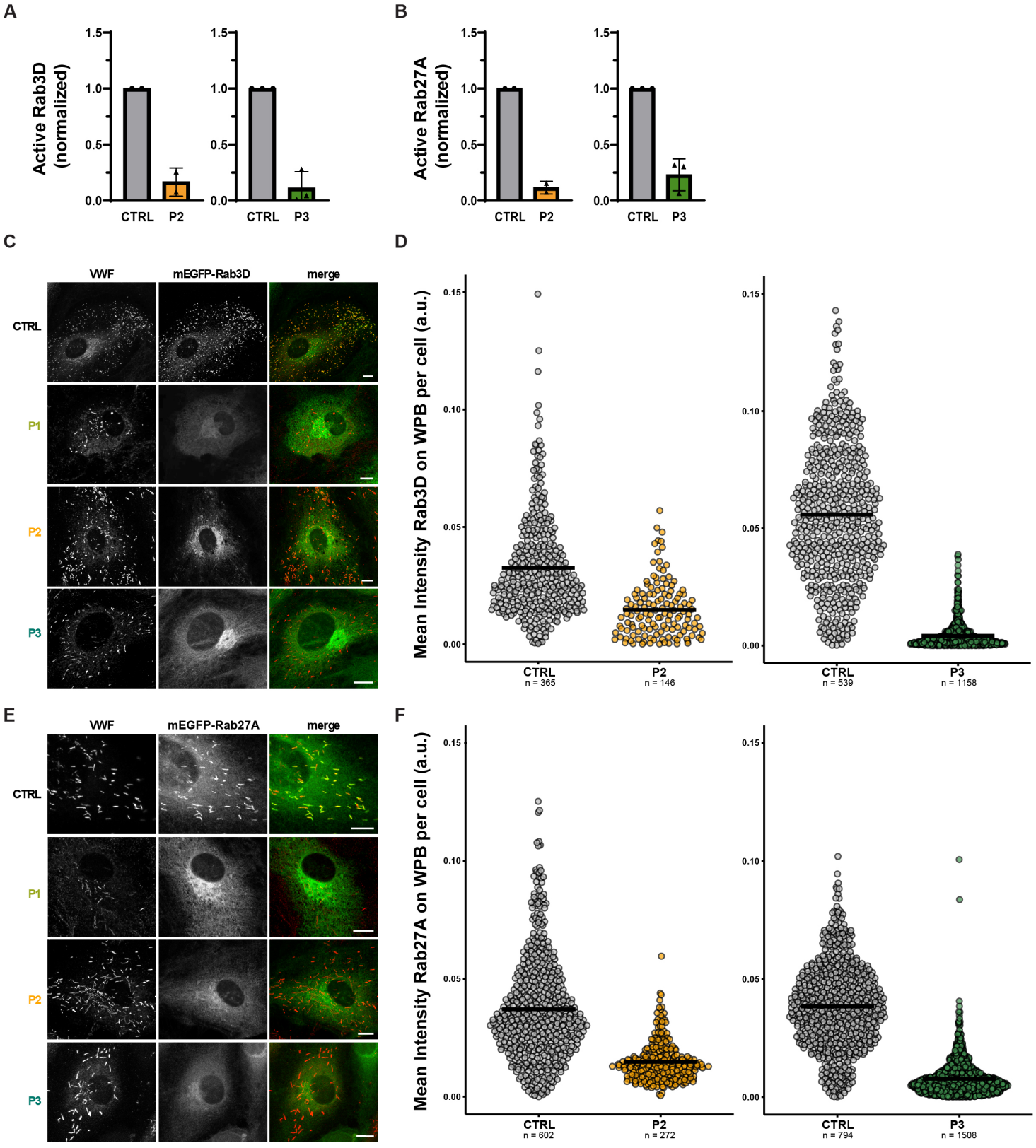

A
Active Rab3D (normalized)
1.5
1.0
0.5
0.0
CTRL
P2
CTRL
P3
B
Active Rab27A (normalized)
CTRL
P2
CTRL
P3
C
VWF
mEGFP-Rab3D
merge
CTRL
P1
P2
P3
D
Mean Intensity Rab3D on WPB per cell (a.u.)
0.15
0.10
0.05
0.00
CTRL
n = 365
P2
n = 146
CTRL
n = 539
P3
n = 1158
E
VWF
mEGFP-Rab27A
merge
CTRL
P1
P2
P3
F
Mean Intensity Rab27A on WPB per cell (a.u.)
CTRL
n = 602
P2
n = 272
CTRL
n = 794
P3
n = 1508

Figure 4

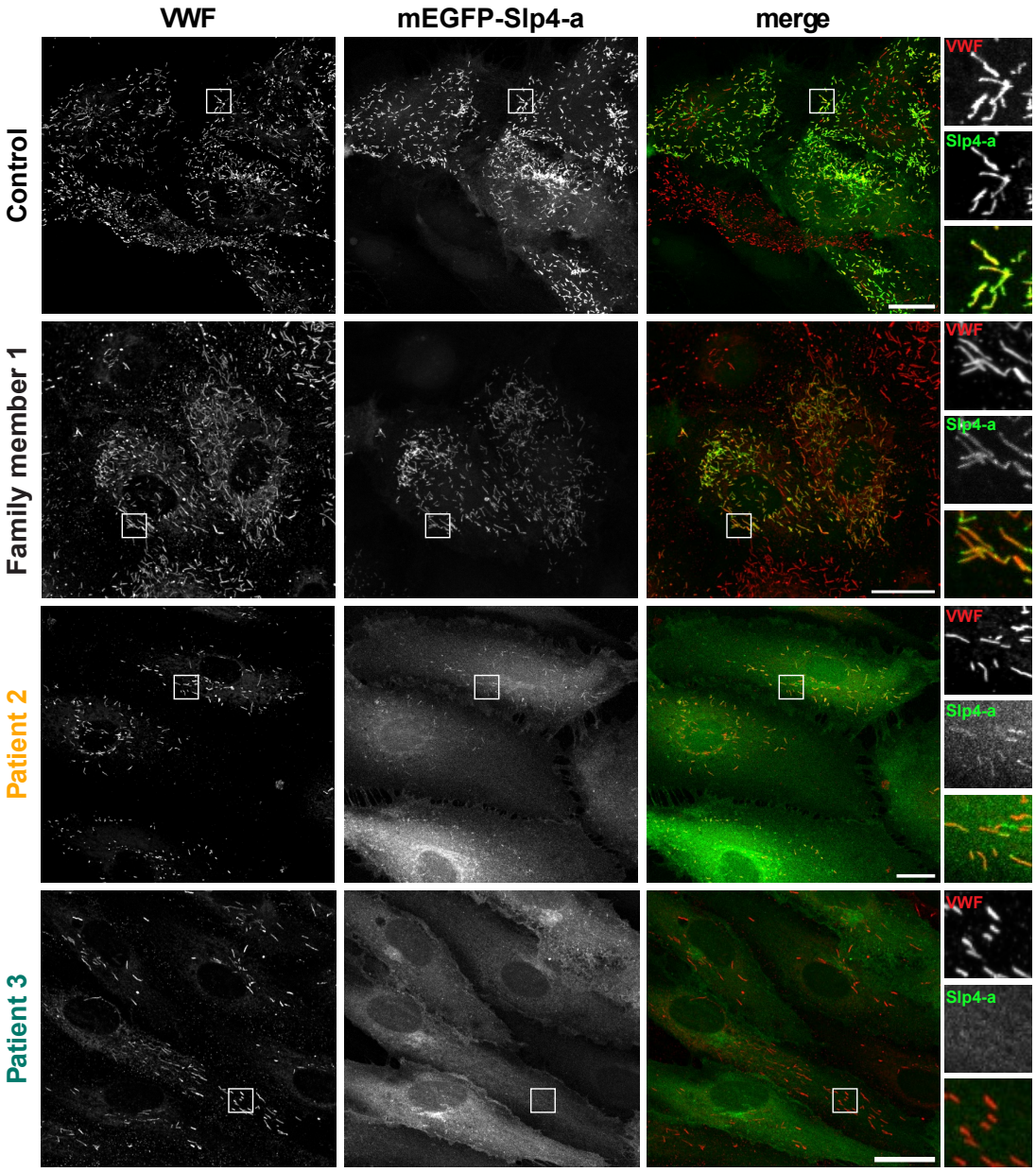

VWF
mEGFP-Slp4-a
merge
Control
Family member 1
Patient 2
Patient 3
VWF
Slp4-a

Figure 5

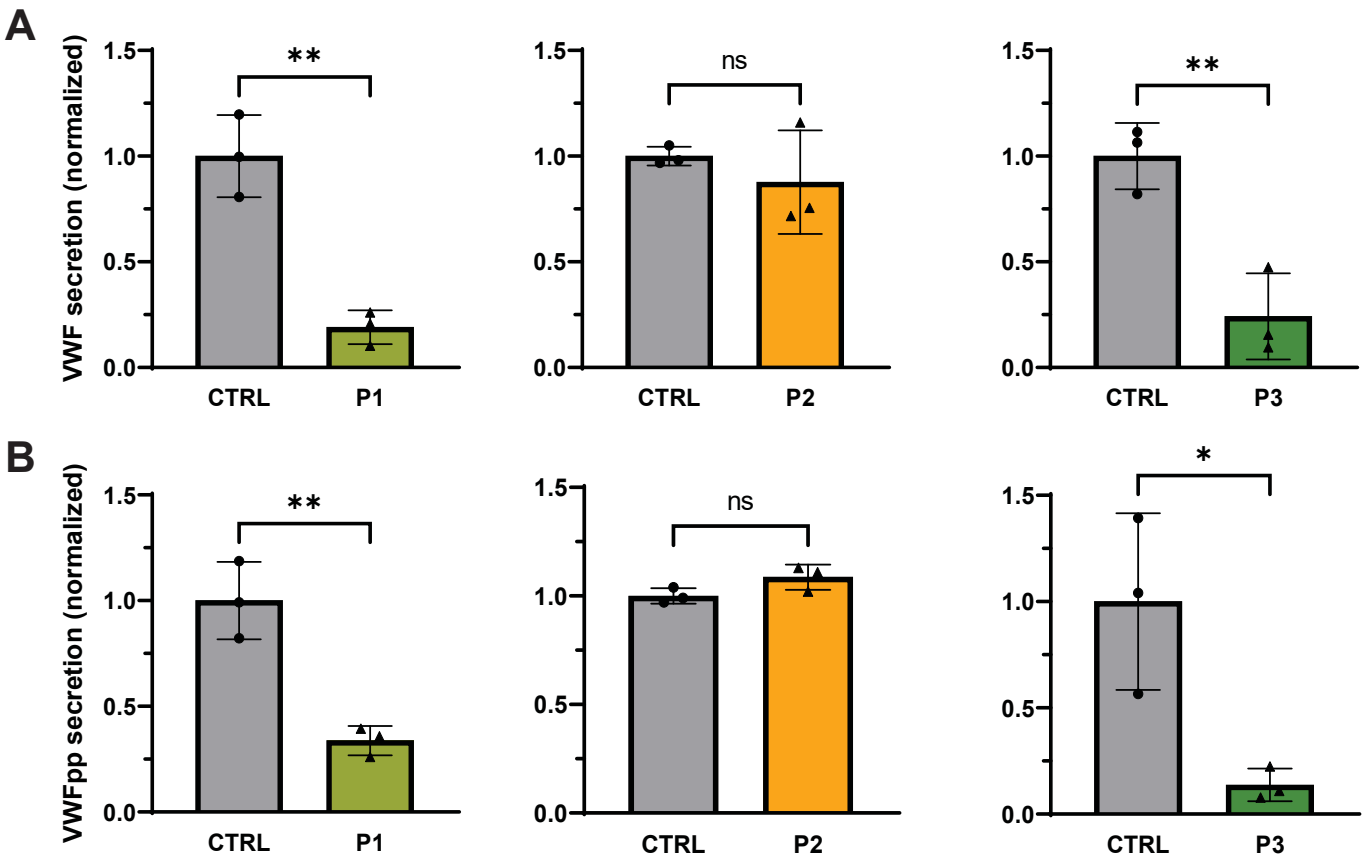
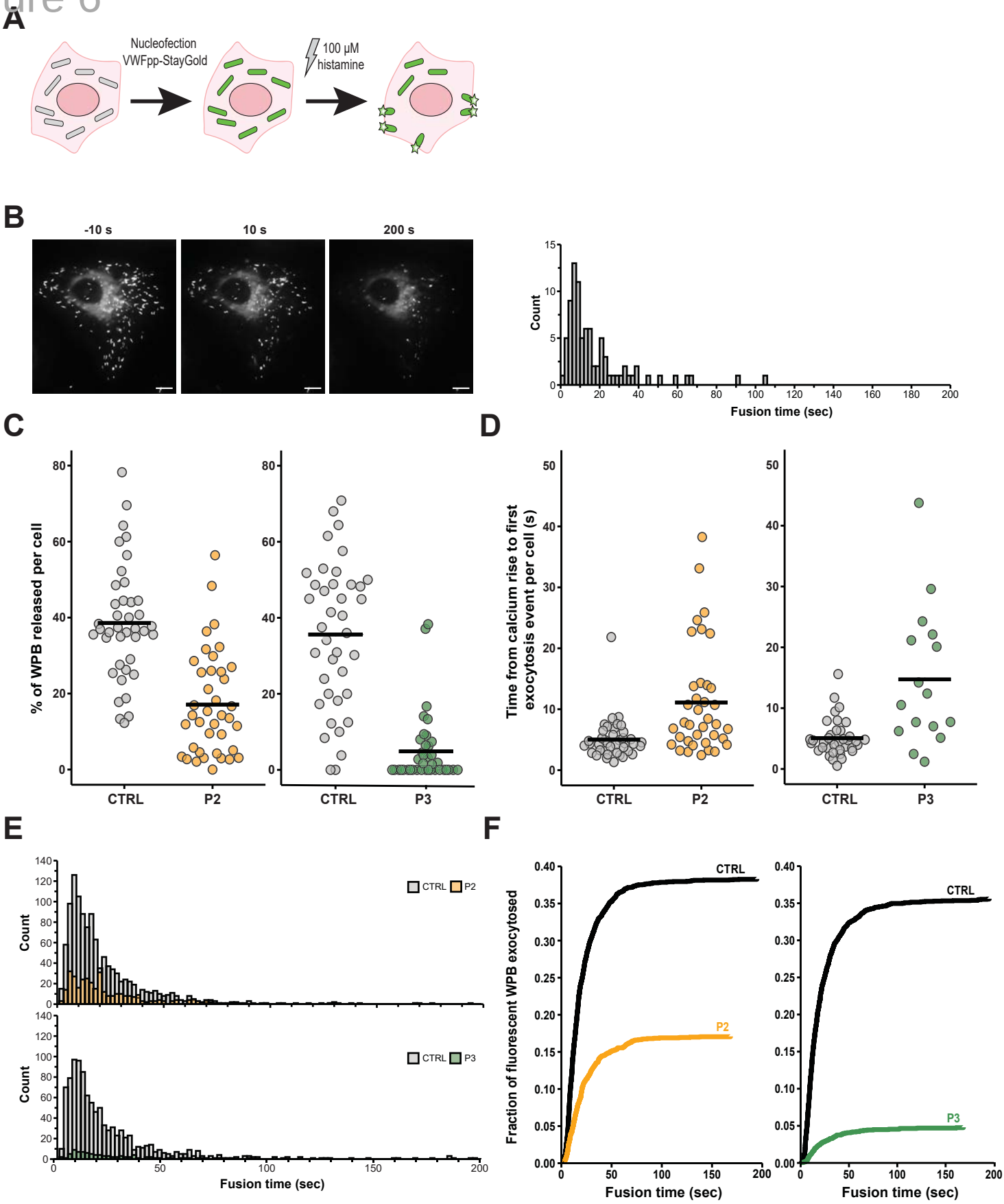

A
VWF secretion (normalized)
1.5
1.0
0.5
0.0
**
CTRL
P1
ns
CTRL
P2
**
CTRL
P3
B
VWFpp secretion (normalized)
**
CTRL
P1
ns
CTRL
P2
*
CTRL
P3

Figure 6


A
Nucleofection
VWFpp-StayGold
100 µM
histamine
B
-10 s
10 s
200 s
Count
Fusion time (sec)
C
% of WPB released per cell
CTRL
P2
CTRL
P3
D
Time from calcium rise to first exocytosis event per cell (s)
CTRL
P2
CTRL
P3
E
Count
CTRL
P2
Count
CTRL
P3
Fusion time (sec)
F
Fraction of fluorescent WPB exocytosed
CTRL
P2
Fusion time (sec)
CTRL
P3
Fusion time (sec)

Biallelic *MADD* variants and von Willebrand factor secretion

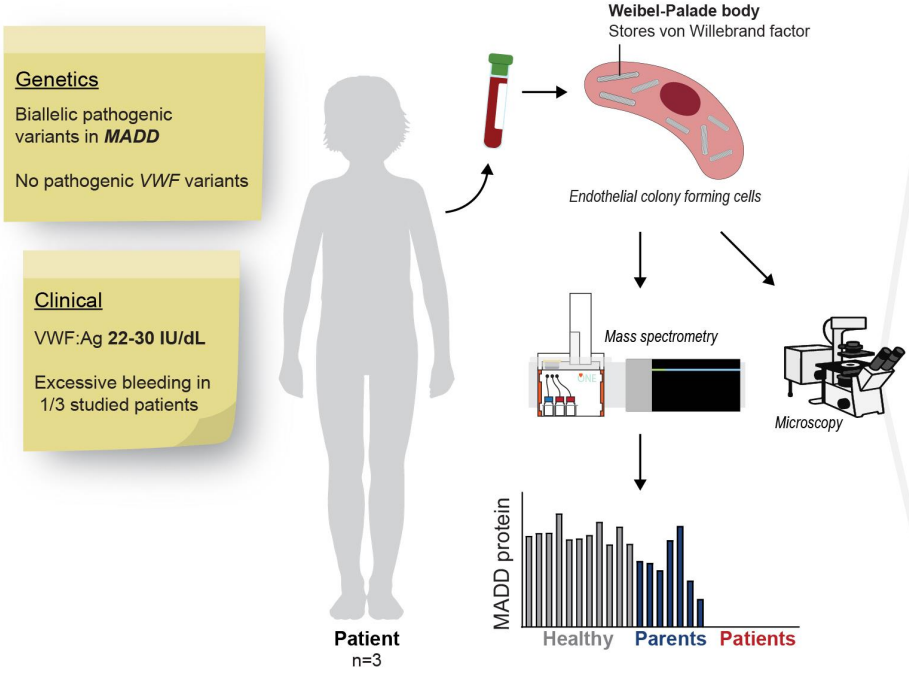
Context of Research

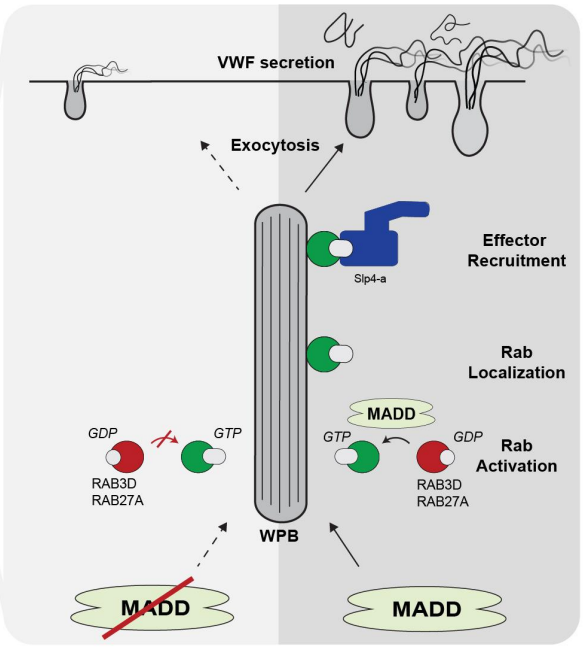
30-50% of patients with type 1 von Willebrand disease (VWD) do not have a pathogenic variant in von Willebrand factor (*VWF)*

Aim of This Study

To find out whether biallelic *MADD* variants result in defective VWF release *in vivo*

Findings





Conclusions: MADD regulates WPB secretion and VWF levels *in vivo.* Defects in WPB exocytosis can cause type 1 VWD.

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