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GDP/GTP exchange factor MADD drives activation and recruitment of secretory Rab GTPases to Weibel-Palade bodies

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Abstract:
Von Willebrand factor (VWF) is an essential hemostatic protein that is synthesized and secreted by endothelial cells and stored in Weibel-Palade bodies (WPBs). The secretory Rab GTPases Rab27A, Rab3B and Rab3D have been linked with WPB trafficking and secretion. How these Rabs are activated and recruited to WPBs remains elusive. In this study, we identified MAP kinase-activating death domain (MADD) as the guanine nucleotide exchange factor (GEF) for Rab27A and both Rab3 isoforms in primary human endothelial cells. Rab activity assays revealed a reduction in Rab27A, Rab3D, and Rab3B activation upon MADD silencing. Rab activation, but not binding, was dependent on the DENN domain of MADD, indicating potential existence of two Rab interaction modules. Furthermore, immunofluorescent analysis showed that Rab27A, Rab3B, and Rab3D recruitment to WPBs was dramatically decreased upon MADD knockdown, revealing that MADD drives Rab membrane targeting. Artificial mistargeting of MADD using a TOMM70-tag abolished Rab27A localization to WPB membranes in a DENN domain-dependent manner, indicating that normal MADD localization in the cytosol is crucial. Activation of Rab3B and Rab3D was reduced upon Rab27A silencing, suggesting that activation of these Rabs is enhanced through prior activation of Rab27A by MADD. MADD silencing did not affect WPB morphology, but reduced VWF intracellular content. Furthermore, MADD-depleted cells exhibited decreased histamine-evoked VWF release, similar to Rab27A-depleted cells. In conclusion, MADD acts as a master regulator in VWF secretion by coordinating the activation and membrane targeting of secretory Rabs to WPBs.

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Key points

- MADD is a GDP/GTP exchange factor for secretory Rab GTPases and drives their recruitment to Weibel-Palade bodies
- MADD facilitates the regulated Von Willebrand factor secretion pathway

Abstract

Von Willebrand factor (VWF) is an essential hemostatic protein that is synthesized and secreted by endothelial cells and stored in Weibel-Palade bodies (WPBs). The secretory Rab GTPases Rab27A, Rab3B and Rab3D have been linked with WPB trafficking and secretion. How these Rabs are activated and recruited to WPBs remains elusive. In this study, we identified MAP kinase-activating death domain (MADD) as the guanine nucleotide exchange factor (GEF) for Rab27A and both Rab3 isoforms in primary human endothelial cells. Rab activity assays revealed a reduction in Rab27A, Rab3D, and Rab3B activation upon MADD silencing. Rab activation, but not binding, was dependent on the DENN domain of MADD, indicating potential existence of two Rab interaction modules. Furthermore, immunofluorescent analysis showed that Rab27A, Rab3B, and Rab3D recruitment to WPBs was dramatically decreased upon MADD knockdown, revealing that MADD drives Rab membrane targeting. Artificial mistargeting of MADD using a TOMM70-tag abolished Rab27A localization to WPB membranes in a DENN domain-dependent manner, indicating that normal MADD localization in the cytosol is crucial. Activation of Rab3B and Rab3D was reduced upon Rab27A silencing, suggesting that activation of these Rabs is enhanced through prior activation of Rab27A by MADD. MADD silencing did not affect WPB morphology, but reduced VWF intracellular content. Furthermore, MADD-depleted cells exhibited decreased histamine-evoked VWF release, similar to Rab27A-depleted cells. In conclusion, MADD acts as a master regulator in VWF secretion by coordinating the activation and membrane targeting of secretory Rabs to WPBs.

Introduction

Von Willebrand factor (VWF) is a multimeric glycoprotein that plays a crucial role in hemostasis.[1] Endothelial cells (ECs) synthesize VWF, which is processed by proteolytic cleavage of the VWF propeptide (VWFpp), glycosylation, and multimerization. Mature VWF multimers that emerge from the trans-Golgi network (TGN) are condensed into tubules and stored in specialized organelles called Weibel-Palade bodies (WPBs).[2] In steady-state, VWF from WPBs is continuously secreted into the vascular lumen through the basal pathway to maintain VWF plasma levels.[3,4] Upon vascular injury, rapid stimulus-induced WPB exocytosis occurs via the regulated pathway to enable formation of VWF strings under shear stress[5], which become a substrate for adhesion and subsequent activation and aggregation of platelets.[6]

The family of Rab GTPases, consisting of ~70 members in humans, plays a variety of roles in vesicle trafficking between intracellular compartments.[7] The secretory Rabs - Rab27A and Rab3 isoforms - have been previously implicated in WPB trafficking and secretion.[5,8] Rab27A, which is recruited during WPB maturation,[9] is able to both promote and inhibit VWF release in a secretagogue- and Rab effector-dependent manner.[10–12] Rab3B is present on WPBs independent of their maturation status, but its significance is unclear as Rab3B silencing had no effect on histamine-induced VWF secretion.[11,12] Rab3D also localizes to WPBs, and its overexpression resulted in reduced histamine-induced WPB exocytosis.[13] In contrast, Rab3D knockdown appeared to have either an inhibitory effect or no effect on VWF secretion.[11,12]

Rabs operate as molecular switches cycling between GDP-bound (inactive) and GTP-bound (active) states. Inactive Rabs are chaperoned by Rab-GDP dissociation inhibitor (Rab-GDI), which solubilizes hydrophobic regions to retain Rabs in the cytosol.[14,15] In their active state Rabs are targeted to specific organelle membranes, where they subsequently recruit effector proteins.[14,16]

In ECs several secretory Rab effectors are known, including Slp4-a, MyRIP, and Munc13 proteins. Each of these effectors is crucial for WPB trafficking and/or exocytosis by mediating interactions with the cytoskeleton (MyRIP),[10,11,17,18] as plasma membrane tethering factors (Munc13-2 and -4),[12,19–21] and through association with the SNARE complex that facilitates membrane fusion (Slp4-a).[11,22,23] Rab27A is able to bind all of these Rab effectors, whereas Rab3 isoforms only interact with Slp4-a and Munc13-2. Despite the extensive research on the function of Rabs and their associated Rab effectors in different stages of WPB release, the mechanism that controls the initial activation and recruitment of Rabs to WPBs is currently unclear.

Cycling of Rabs between GDP- and GTP-bound states is regulated by Rab-specific guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). The Differentially Expressed in Normal and Neoplastic cells (DENN) domain protein family is comprised of 18 members that each exhibit GEF activity towards specific Rabs.[24–26] MAP-kinase Activating Death Domain (MADD), alternatively referred to as DENN or Rab3 Guanine Exchange Protein (Rab3GEP), has been previously identified as a GEF for Rab3 isoforms in neuronal and (neuro)endocrine cells and for Rab27A in melanocytes and parotid acinar cells.[27–32] Besides the N-terminal DENN domain, MADD contains a C-terminal death domain (DD) that functions in MAP kinase signaling.[33] Physiological significance of MADD is underlined by a recent study from the Undiagnosed Disease Network, which reported that bi-allelic mutations in MADD are causative for an extremely rare multisystem disorder, delineated by neurological, endocrine, and exocrine dysfunction, which in some cases also manifests as hematological abnormalities.[34] This positions MADD as a crucial regulator within the neuroendocrine system that may also be involved in Rab27A-controlled release of lysosome-related organelles involved in hemostasis and immunity.[6]

In this study we investigated the role of MADD in regulation of WPB Rabs and VWF secretion. We found that MADD functions as a GEF for Rab27A, as well as Rab3 isoforms in ECs. Furthermore, MADD is required for the recruitment of Rab27A and Rab3D to WPBs. Finally, we show that, as a master regulator of WPB Rabs, MADD controls VWF secretion.

Methods

Antibodies

Antibodies used for immunoblotting and immunofluorescent stainings are listed in supplementary **Table S1**.

DNA constructs

GST-Slac2-b-SHD[35] and GST-RIM2-RBD[31] expression vectors for Rab activity assays were kind gifts of Prof. M. Fukuda and Prof. R. Regazzi, repectively. EGFP-tagged human MADD constructs (full length, ΔDENN, ΔDD, TOMM70-EGFP, and TOMM70-EGFP-MADD) have been described previously.[27] Lentiviral TOMM70-tagged fusions were made in the LVX-mEGFP-LIC vector[22] by swapping mEGFP for TOMM70-EGFP, TOMM70-EGFP-MADD or TOMM70-EGFP-MADD- ΔDENN. The pLKO.1-short hairpin (sh) RNA constructs from the TRC MISSION library (**Table S2**) were supplied by Dr. R. Beijersbergen and Dr. W. Van IJcken. shRNAs targeting MADD and Rabs were cloned into a pLKO.1-puro-CMV-mEGFP-U6-shRNA vector.[36] All constructs were verified by DNA sequence analysis.

Cell culture, transfection and transduction

Pooled, cryo-preserved human umbilical cord endothelial cells (HUVEC, Promocell) were cultured on gelatin-coated surfaces in endothelial cell growth medium (Promocell, EGM-2) supplemented with endothelial growth factor mix (Promocell), 18% fetal calf serum (FCS, Bodinco), 100 U/mL penicillin, and 100 µg/mL streptomycin (P/S, Gibco). Gelatin from porcine skin (Sigma) was dissolved at final concentration of 1% in phosphate-buffered saline (PBS, Fresenius Kabi). HUVEC were kept at 37°C, 5% CO_2 and used in experiments up to passage 5-6. Human embryonic kidney 293T (HEK293T) cells were cultured in Dulbecco's modified eagle medium (DMEM) containing D-glucose and L-glutamine supplemented with 10% FCS and P/S.

Lentivirus was produced in HEK293T cells using the 3rd generation lentiviral packaging plasmids pMD2.G, pRSV-REV, and pMDLg/pRRE (Addgene) using TransIT-LT-1 (Mirus Bio) as described previously.[37] HUVEC were transduced with two batches of fresh virus and selected for 3 days using 0.5 µg/ml puromycin. All other constructs were transfected in HEK293T using TransIT-LT-1 or in HUVEC using the Neon transfection system (Invitrogen) delivering 1 pulse of 1300 Volt, and a pulse width of 30 ms. A pool of small interference RNA (siRNA) oligo duplexes targeting human MADD (ON-TARGET plus SMARTpool, #L004429, Dharmacon) and non-targeting siRNA control (ON-TARGET plus non-targeting pool control, #D001810) were transfected in HUVEC by Nucleofection (Lonza) as described previously.[11] Oligo sequences are in supplementary **Table S3**.

Additional methods can be found in the Supplemental material.

Results

MADD activates secretory Rabs in a DENN-dependent manner

Since MADD has been shown to exhibit GEF activity towards Rab3 isoforms and Rab27A in different cell types,[27–32,38] we hypothesized that MADD functions as a secretory RabGEF in ECs. To investigate this, we silenced MADD in HUVEC using stable expression of shRNAs targeting MADD (shMADD) or a non-targeting control (shCTRL) (**Fig. 1A, Fig. S1A**). We then performed pulldown assays in shCTRL- and shMADD-transduced HUVEC to capture the active, GTP-bound fractions of endogenous Rab27A and Rab3B/D. GST-tagged Rab effector domains Slp-homologue lacking C2 domains B - Slp Homology Domain (GST-Slac2-b-SHD) and Rab3-Interacting Molecule 2 - Rab Binding Domain (GST-RIM2-RBD), which specifically bind to GTP-Rab27A and GTP-Rab3 isoforms, respectively, were used as bait. Upon MADD knockdown, both the amount and the proportion of active GTP-bound Rab27A, Rab3B, and Rab3D were substantially reduced, indicating that MADD is indeed required for their activation in ECs (**Fig. 1B-G, Fig. S2A-C**). Interestingly, Rab27A and Rab3D total protein levels were also significantly decreased upon MADD knockdown (**Fig. S2D-F**), which corresponds to previous observations that MADD knockout melanocytes have lower Rab27A expression levels.[27] This may be attributed to an increased degradation of inactive Rabs,[39] decreased mRNA expression in case of Rab27A (**Fig. S3A-C**) or both.

MADD contains DD and DENN domains that are important for its GEF function.[27,31] It is assumed that a Rab binding site is located within the DENN domain, as this is also the case for other DENN domain proteins.[24] To delineate if MADD activates all three Rabs using its DENN domain, we compared the effect of full length MADD and a mutant lacking the DENN domain (ΔDENN) on Rab activity in HEK293T cells. Full length MADD markedly increased the active GFP-Rab27A, GFP-Rab3B, and GFP-Rab3D fractions, whereas the ΔDENN mutant did not (**Fig. 2A-F**). However, Rab activation was not completely abolished by ΔDENN, but a reduction

to the basal activation level was observed, indicating that the truncation mutant is not dominant negative. Notably, we found that full-length MADD as well as ΔDENN co-precipitated with GTP-bound Rabs (**Fig. S4A-C**), implying that the DENN domain is not crucial for the Rab-GEF interaction. Thus, MADD has the capacity to activate Rab27A, Rab3B, and Rab3D and its exchange activity depends on the DENN moiety.

MADD drives recruitment of secretory Rabs to WPBs

To investigate if MADD is involved in targeting Rab27A and Rab3 to WPBs we evaluated their localization after MADD depletion in HUVEC. For comparison we also silenced expression of these Rabs directly with specific shRNAs targeting Rab27A, Rab3B, or Rab3D (**Fig. 3A-C, Fig. S1B-D**). As expected, WPB-localized Rab27A was significantly reduced in shRab27A-transduced ECs when compared to shCTRL (**Fig. 3D,E**). Strikingly, in MADD-depleted cells Rab27A staining no longer colocalized with VWF, indicating that MADD is involved in Rab27A targeting to WPBs. As also previously documented, Rab27A immunoreactivity on WPBs appeared less intense in cells with a high number of WPBs, which is explained by a limited pool of Rab27A that distributes over an increasing number of WPBs, thereby diluting the Rab27A signal.[11] Indeed, quantification revealed higher WPB numbers clearly decreased the proportion of Rab27A+ WPBs in shCTRL cells, a pattern that was not observed after Rab27A or MADD depletion (**Fig. 3E**).

Since MADD also exhibits GEF activity on Rab3B/D, we tested whether their targeting to WPBs was also dependent on MADD. Rab3D staining was visible on WPBs in shCTRL cells, but not in shRab3D cells, and significantly decreased upon MADD knockdown (**Fig. 3F,G**). Silencing MADD using siRNAs (siMADD) and comparing to a non-targeting siRNA control (siCTRL) also showed a clear depletion of Rab27A, Rab3B, as well as Rab27A-effector MyRIP from WPBs

(**Fig. S5A-C**), complementary to our observations in shRNA-transduced ECs. Moreover, phase partitioning showed that upon MADD knockdown Rab27A, Rab3B and Rab3D levels were decreased in the detergent phase (i.e. membrane-bound), although not significant in case of Rab27A (**Fig. S6A,B**), suggesting that Rab membrane-association was reduced. Taken together, we conclude that impaired targeting of Rabs to WPBs upon MADD silencing consists of a reduction of (active) Rab levels (**Fig. 1, Fig. S2**) as well as decreased Rab recruitment, pointing towards a role for MADD in directing secretory Rabs to WPBs.

MADD has been observed in the cytosol of different cell types, while its target Rab27A localized to secretory granules.[27,28] In lack of an antibody that detects endogenous MADD, we determined the intracellular localization in HUVEC by ectopic expression of EGFP-MADD or EGFP-MADD-ΔDENN. Both constructs exhibited primarily cytosolic distribution and were not enriched on WPBs, suggesting that another mechanism is responsible for the recruitment of secretory Rabs to WPBs (**Fig. S7A,B**). Although we cannot rule out that during WPB Rab activation, presence of MADD at the WPB membrane is so transient that this precludes detection, our result suggest that MADD does not activate Rabs exclusively in proximity of WPBs.

To further substantiate its role in membrane targeting of Rabs, we used EGFP-MADD fused with a TOMM70-tag, which directs MADD to mitochondria by virtue of a mitochondrial targeting motif[27] and tested the contribution of MADD localization to correct targeting of one of its substrates, Rab27A. The TOMM70-EGFP control localized primarily to (aggregated) mitochondria (**Fig. 4A**) as was seen previously[27], whilst endogenous Rab27A was localized to WPBs as shown by scoring cells exhibiting Rab27A co-staining with VWF (**Fig. 4A,D**). However, in cells expressing TOMM70-EGFP-MADD WPBs were no longer positive for Rab27A (**Fig. 4B,D**). Intriguingly, TOMM70-EGFP-MADD-ΔDENN did not affect Rab27A localization, suggesting that endogenous MADD function remains unrestricted by this truncation mutant and that the DENN moiety is required for the dislocation of Rab27A (**Fig. 4C,D**). These data reveal

that aberrant targeting of MADD to mitochondria prevents DENN-domain dependent recruitment of Rab27A to WPBs, indicating that correct localization of MADD is essential.

In ECs Rab27A is present on mature WPBs, whereas Rab3B is already detected at an earlier stage.[11] This raises the question whether sequential or cooperative activation of Rab3 and Rab27A underpins their recruitment during WPB maturation. To explore the possibility that secretory Rabs facilitate each other's activation, we determined Rab3B and Rab3D activation after Rab27A silencing (**Fig. 5A-C**). Interestingly, levels of GTP-Rab3B and GTP-Rab3D were both significantly decreased upon Rab27A knockdown, implying that Rab27A augments activation of Rab3 isoforms (**Fig. 5D, Fig. S8A,B**). In the reverse setup, Rab27A activity remained unchanged upon Rab3B silencing, whilst Rab3D depletion resulted in slightly lower GTP-Rab27A levels, suggesting Rab3D might also enhance Rab27A GDP/GTP exchange (**Fig. 5E-G, Fig. S8C,D**). Analysis of Rab localization in IF images showed no difference in Rab3D presence on WPBs upon Rab27A knockdown, and also Rab27A localization remained unchanged in Rab3B and Rab3D depleted cells, suggesting that normal recruitment of active Rabs still occurs (**Figure S9A-D**). Taken together, the levels of active Rab3B and Rab3D appear to be dependent on the presence of (active) Rab27A, which suggests that activation of Rab27A precedes or promotes Rab3B and Rab3D activation.

MADD is crucial for histamine-evoked WPB exocytosis

Rab3 and Rab27 have been shown to coordinate secretory organelle maturation, transport, and exocytosis through interactions with a variety of effectors.[40] Since MADD is necessary for their activation and recruitment to WPBs (**Fig. 1-4**), we investigated MADD's role in the endothelial secretory pathway. MADD or individual Rab depletion did not have an effect on WPB morphology as the classical cigar-shaped organelles were observed, suggesting that

individually they are dispensable for normal WPB formation (**Fig. 6A, Fig. S10**). Nonetheless, we noted a decrease in the number of WPBs per cell upon MADD knockdown, pointing towards a VWF storage defect (**Fig. 6B**). Although differences in total VWF levels between shCTRL and shMADD were not as clear on Western blot (**Fig. 6C**).

To address if MADD is involved in WPB maturation, we examined WPB maturation status by electron microscopy. In both shCTRL and shMADD ECs classical cigar-shaped organelles could be distinguished throughout the cytoplasm, including immature WPBs in close proximity to the Golgi and WPBs in different stages of maturation as judged by the condensation of VWF bundles (**Fig. S11A,B**), with no difference in their proportions between control and MADD depleted cells (**Fig. S11C**). Collectively, these results indicate that MADD does not play an indispensable role in biogenesis and maturation of WPBs.

Since secretory Rabs and their effectors facilitate WPB exocytosis,[11,12] we performed secretion assays to test if MADD, as an upstream regulator of Rab function, is involved in VWF secretion. MADD silencing significantly reduced intracellular VWF content in unstimulated cells, as well as histamine-stimulated VWF secretion, both in absolute levels and as a percentage of the (unstimulated) intracellular content (**Fig. 6D-F**). These data indicate that besides facilitating regulated WPB exocytosis, MADD could play an additional role in VWF storage/turnover of WPBs. Similar to previous reports that used siRNA-based knockdown,[11,12] Rab27A knockdown using shRNAs resulted in a significant reduction of the absolute histamine-evoked VWF secretion, and a reduction, although not significant, in the secreted VWF as a percentage of intracellular content (**Fig. S12A-D**). Rab3B and Rab3D had no significant effect on absolute levels intracellular VWF, nor on histamine-stimulated VWF secretion, however, the % VWF secreted by shRab3B cells was significantly increased, opposite to MADD knockdown. The latter result may be explained by a secondary effect of long term Rab3B knockdown, which appears to upregulate Rab27A expression (**Fig. S12D**), perhaps in order to compensate for the

depletion of Rab3B. In conclusion, based on our data and previous reports,[11,12] we speculate that the effect of MADD on regulated WPB exocytosis is mainly attributed to its crucial role in Rab27A activation and recruitment.

Discussion

Thus far, it was unknown how secretory Rabs in ECs were activated and targeted to WPBs. In this study, we identified MADD as the GEF for three WPB-localized Rabs: Rab27A, Rab3B, and Rab3D. We have shown that MADD is responsible for the activation and targeting of these Rabs to WPBs, thereby promoting VWF secretion. Our findings are in line with reports showing that MADD exhibits GDP/GTP exchange activity on Rab27A,[27,28,32] and all four Rab3 isoforms in other cell types.[30,31] MADD controls Rab27A activation and recruitment to melanosomes in melanocytes and amylase-containing granules in parotid acinar cells.[27,28,32] Through regulation of Rab3, MADD mediates neurotransmitter and hormone release from (neuro)endocrine cells.[41–43] MADD KO mice exhibited impaired synaptic vesicle transport and reduced neurotransmitter and insulin release.[42,44,45] Overall, MADD functions in the transport and/or exocytosis of (secretory) organelles through regulation of secretory Rab function, which is consistent with our data in ECs.

Although GEFs typically localize to intracellular compartment(s) where Rabs are active[26,46], we observed that EGFP-MADD was not enriched on WPBs, but instead showed localization throughout the cytosol. Microscopy data from melanocytes[27] and subcellular fractionation experiments in parotid acinar cells also showed the vast majority of MADD in the cytosolic fraction.[28] Although presence of MADD on WPBs does not appear to be required for correct localization of secretory Rabs, artificial sequestering of MADD to the mitochondrial membrane severely impacted Rab27A localization, hinting that mobility of MADD in the cytosol may be crucial. Thus, despite the predominantly cytosolic localization of EGFP-MADD the possibility

remains that transient association of MADD with WPBs is needed for delivery of the secretory Rabs to these organelles.

Apart from MADD, Rab-GAPs may also control the localization of these secretory Rabs on WPBs.[14,47] A RabGAP screen performed in ECs found that TBC1D10A inhibits VWF secretion via inactivating and presumably removing Rab35 from WPBs, indicating that RabGAPs can also influence Rab localization.[48] Recently, the RabGAP TBC1D22A was detected by proximity-labeling in a proteomics study investigating the Rab27A/Rab3B interactomes in ECs, suggesting that this RabGAP could be a candidate for inactivating these Rabs, thereby destabilizing association with the WPB membrane.[19]

A number of reports have identified MADD as a specific GEF for Rab3 and Rab27A by screening the GDP release or GTP loading of different purified Rab proteins[25,30,32] or via isolating the activated Rab fraction from cell lysates using effector pulldown assays[27,28,31,32] similar to our approach. However, many of these studies focused solely on its role in activation of either Rab3[30,31,43,45] or Rab27A[27,28,32,49], with the exception of one study in *Caenorhabditis elegans* neurons[50], showing that MADD homolog AEX-3 activates RAB-3 and RAB-27, which in turn both regulate synaptic transmission. Since multiple secretory Rabs are concurrently expressed in ECs, our system offers the opportunity to study the intricate balance between them in mammalian cells, with Rab isoforms adding an additional layer of complexity.

Previous evidence for a sequential Rab27A-Rab3A activation cascade was found in sperm cells, in which Rab27A and effector Rabphilin3-a, both present on dense core granules, recruit the GEF for Rab3A (GRAB) that in turn recruits and activates Rab3A.[38,51] We show that in ECs Rab27A and Rab3 activity levels are interrelated judging from attenuation of Rab3B/D activation when Rab27A is silenced, which suggests a positive feedback mechanism is normally present. Furthermore, *in vitro* GDP dissociation assays showed that MADD by itself cannot fully activate Rab3D and is completely unable to activate Rab3B[30], whilst Rab activation assays in cellular

context presented here and also by others[31] revealed that Rab3B/D activation was unequivocally dependent on MADD. This points to requirement for additional cellular factors for allosteric regulation of MADD activity, for instance by activated Rab27A. However, further studies are needed to elucidate the complete mechanism.

We have shown that in ECs MADD promotes histamine-stimulated VWF secretion to a similar extent as Rab27A in absolute levels, suggesting that MADD acts as an upstream regulator of WPB exocytosis. Also other Rabs have been shown to influence WPB exocytosis, including Rab15, Rab35, and Rab46,[12,48,52] which could be mobilized by other GEFs, such as DENND1 for Rab35,[25] functioning alongside MADD in regulation of Rabs on WPBs. There is no consensus in literature regarding the role of Rab3 isoforms in stimulated VWF release[11–13] and also here our data did not give a conclusive answer to the question if Rab3B and Rab3D independently play a role in stimulated VWF secretion as long term Rab knockdown appears to influence expression of other (redundant) Rabs, which in turn may (partially) compensate for the depletion. The question remains whether Rab3B and Rab3D are redundant in ECs, like in neurons,[53] as they are highly similar with >80% amino acid sequence identity and recruit the same effector Slp4-a.[11]

Low circulating levels of VWF, such as in Von Willebrand disease (VWD; VWF <30 IU/dL) or in case of “low VWF” (30-50 IU/dL), give rise to an increased risk of bleeding.[54,55] VWF deficiency can be caused by mutations in VWF that affect its biosynthesis and/or clearance. Yet, in ~25% of VWD type 1 patients and in ~60% of low VWF patients no causative mutation in VWF can be detected and the pathogenic mechanisms that underpin their reduced VWF levels often remain unresolved. In the normal population plasma VWF levels are distributed over a wide range and they have been shown to be largely genetically determined.[56,57] The heritability of VWF plasma levels consists predominantly of quantitative trait loci outside the VWF gene[58] and it is to be expected that some will overlap with non-VWF loci responsible for unexplained quantitative

VWF deficiencies in VWD1 and low VWF patients. Genome-wide association studies for modifiers of VWF levels have identified among others links with genetic variants in SNARE proteins,[59–61] indicating that alterations in secretory processes are responsible for some of the variability in VWF plasma levels. However, a significant portion of the heritable component of VWF levels is still unaccounted for[58] and we speculate that additional (pathogenic) mechanisms exist that impair the endothelial cells' capacity to secrete VWF. Possibly, genetic variants in (regulators of) MADD may exist that affect its efficacy in recruiting Rab GTPases and their downstream effectors (including SNAREs) to the exocytic machinery of WPBs, thereby reducing basal and/or vascular injury-induced release of VWF by endothelial cells.

In a recent report Schneeberger et al. describe a cohort of pediatric patients with biallelic mutations in MADD who suffer from a severe pleiotropic disorder with a spectrum of developmental, neurological and hematological anomalies and poor life expectancy.[34] Cellular studies with patient fibroblasts indicate that a subgroup of these patients show a phenotype consistent with disturbed vesicular trafficking. Some of these patients have mutations in the DENN domain, which we and others have shown to be essential for Rab27A activation and recruitment to WPBs. Interestingly a number of patients with biallelic MADD mutations present with clinical symptoms and laboratory phenotypes that are consistent with an increased risk of bleeding, such as frequent nosebleeds as well as prolonged Ivy bleeding time and abnormal platelet degranulation (Unpublished observations, Prof. K. Freson). In platelets, formation as well as degranulation of alpha- and dense granules involves Rab27A and Rab27B,[62–64] which is also a substrate for MADD exchange activity.[25] If degranulation of WPBs is also affected in these patients is currently unknown, but in future studies we will focus on establishing whether an endothelial (secretory) component also exists in the pathology of this multisystem disorder.

In conclusion, the discovery of MADD as a WPB-Rab GEF adds a new component to the VWF/WPB secretory machinery in secretory organelle exocytosis. MADD depletion results in

decreased levels of activated secretory Rabs on the WPB membrane, ultimately leading to reduced VWF secretion. Therefore, we propose a model in which MADD coordinates activation and recruitment of Rab27A, Rab3B, and Rab3D to WPBs as an upstream master regulator in the endothelial VWF secretory pathway.

Data Sharing Statement

For data sharing, please contact the corresponding author at r.bierings@erasmusmc.nl.

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Authorship Contributions

MK, PB, HJ, IDC and IC performed research and analyzed data; ANH and TC contributed vital reagents and expertise; MK, JV, CM and RB designed the research and wrote the paper.

Disclosure of Conflicts of Interest

The authors report no conflicts of interest.

References

1. Sadler JE. Biochemistry and genetics of Von Willebrand Factor. *Annu. Rev. Biochem.* 1998;67(1):395–424.

2. Springer TA. von Willebrand factor, Jedi knight of the bloodstream. *Blood*. 2014;124(9):1412–1425.

3. Giblin JP, Hewlett LJ, Hannah MJ. Basal secretion of von willebrand factor from human endothelial cells. *Blood*. 2008;112(4):957–964.

4. Lopes da Silva M, Cutler DF. von Willebrand factor multimerization and the polarity of secretory pathways in endothelial cells. *Blood*. 2016;128(2):277–285.

5. Schillemans M, Karampini E, Kat M, Bierings R. Exocytosis of Weibel–Palade bodies: how to unpack a vascular emergency kit. *J. Thromb. Haemost.* 2019;17(1):6–18.

6. Karampini E, Bierings R, Voorberg J. Orchestration of Primary Hemostasis by Platelet and Endothelial Lysosome-Related Organelles. *Arterioscler. Thromb. Vasc. Biol.* 2020;40(6):1441–1453.

7. Zhen Y, Stenmark H. Cellular functions of Rab GTPases at a glance. *J. Cell Sci.* 2015;128(17):3171–3176.

8. Nightingale T, Cutler D. The secretion of von Willebrand factor from endothelial cells; an increasingly complicated story. *J. Thromb. Haemost.* 2013;11(Suppl1):192–201.

9. Hannah MJ, Hume AN, Arribas M, et al. Weibel-Palade bodies recruit Rab27 by a content-driven, maturation-dependent mechanism that is independent of cell type. *J. Cell Sci.* 2003;116(19):3939–3948.

10. Nightingale TD, Pattni K, Hume AN, Seabra MC, Cutler DF. Rab27a and MyRIP regulate

the amount and multimeric state of VWF released from endothelial cells. *Blood*. 2011;117(12):3476–3476.

11. Bierings R, Hellen N, Kiskin N, et al. The interplay between the Rab27A effectors Slp4-a and MyRIP controls hormone-evoked Weibel-Palade body exocytosis. *Blood*. 2012;120(13):2757–2767.

12. Zografou S, Basagiannis D, Papafotika A, et al. A complete rab screening reveals novel insights in Weibel-Palade body exocytosis. *J. Cell Sci.* 2012;125(20):4780–4790.

13. Knop M, Aareskjold E, Bode G, Gerke V. Rab3D and annexin A2 play a role in regulated of vWF, but not tPA, from endothelial cells. *EMBO J.* 2004;23(15):2982–2992.

14. Müller MP, Goody RS. Molecular control of Rab activity by GEFs, GAPs and GDI. *Small GTPases*. 2018;9(1–2):5–21.

15. Pfeffer SR, Dirac-Svejstrup AB, Soldati T. Rab GDP dissociation inhibitor: Putting Rab GTPases in the right place. *J. Biol. Chem.* 1995;270(29):17057–17059.

16. Oesterlin LK, Pylypenko O, Goud B. Effectors of Rab GTPases: Rab binding specificity and their role in coordination of rab function and localization. *Ras Superfamily Small G Proteins Biol. Mech. 2 Transp.* 2014;39–66.

17. Conte IL, Hellen N, Bierings R, et al. Interaction between MyRIP and the actin cytoskeleton regulates Weibel-Palade body trafficking and exocytosis. *J. Cell Sci.* 2016;129(3):592–603.

18. Rojo Pulido I, Nightingale TD, Darchen F, et al. Myosin Va acts in concert with Rab27a and MyRIP to regulate acute von-Willebrand factor release from endothelial cells. *Traffic*. 2011;12(10):1371–1382.

19. Holthenrich A, Drexler HCAA, Chehab T, Naß J, Gerke V. Proximity proteomics of

endothelial Weibel-Palade bodies identifies novel regulator of von Willebrand factor secretion. *Blood*. 2019;134(12):979–982.

20. Chehab T, Santos NC, Holthenrich A, et al. A novel Munc13-4/S100A10/annexin A2 complex promotes Weibel-Palade body exocytosis in endothelial cells. *Mol. Biol. Cell*. 2017;28(12):1688–1700.

21. Criado Santos N, Chehab T, Holthenrich A, Gerke V. Analysis of Ca 2+ -dependent Weibel–Palade body tethering by live cell TIRF Microscopy: Involvement of a Munc13-4/S100A10/Annexin A2 complex. *Methods Mol. Biol.* 2019;437–445.

22. van Breevoort D, Snijders AP, Hellen N, et al. STXBP1 promotes Weibel-Palade body exocytosis through its interaction with the Rab27A effector Slp4-a. *Blood*. 2014;123(20):3185–3194.

23. Schillemans M, Karampini E, Van Den Eshof BL, et al. Weibel-palade body localized syntaxin-3 modulates von willebrand factor secretion from endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* 2018;38(7):1549–1561.

24. Marat AL, Dokainish H, McPherson PS. DENN domain proteins: Regulators of Rab GTPases. *J. Biol. Chem.* 2011;286(16):13791–13800.

25. Yoshimura SI, Gerondopoulos A, Linford A, Rigden DJ, Barr FA. Family-wide characterization of the DENN domain Rab GDP-GTP exchange factors. *J. Cell Biol.* 2010;191(2):367–381.

26. Ishida M, Oguchi ME, Fukuda M. Multiple types of guanine nucleotide exchange factors (GEFS) for rab small GTpases. *Cell Struct. Funct.* 2016;41(2):61–79.

27. Sanzà P, Evans RD, Briggs DA, et al. Nucleotide exchange factor Rab3GEP requires DENN and non-DENN elements for activation and targeting of Rab27a. *J. Cell Sci.*

2019;132(9):jcs212035.

28. Imai A, Ishida M, Fukuda M, Nashida T, Shimomura H. MADD/DENN/Rab3GEP functions as a guanine nucleotide exchange factor for Rab27 during granule exocytosis of rat parotid acinar cells. *Arch. Biochem. Biophys.* 2013;536(1):31–37.

29. Niwa S, Tanaka Y, Hirokawa N. KIF1Bβ- and KIF1A-mediated axonal transport of presynaptic regulator Rab3 occurs in a GTP-dependent manner through DENN/MADD. *Nat. Cell Biol.* 2008;10(11):1269–1279.

30. Wada M, Nakanishi H, Satoh A, et al. Isolation and characterization of a GTP/GTP exchange protein specific for the Rab3 subfamily of small G proteins. *J. Biol. Chem.* 1997;272(7):3875–3878.

31. Coppola T, Perret-Menoud V, Gattesco S, et al. The death domain of rab3 guanine nucleotide exchange protein in GDP/GTP exchange activity in living cells. *Biochem. J.* 2002;362(2):273–279.

32. Figueiredo AC, Wasmeier C, Tarafder AK, et al. Rab3GEP is the non-redundant guanine nucleotide exchange factor for Rab27a in melanocytes. *J. Biol. Chem.* 2008;283(34):23209–23216.

33. Schievella AR, Chen JH, Graham JR, Lin L-L. MADD, a Novel Death Domain Protein That Interacts with the Type 1 Tumor Necrosis Factor Receptor and Activates Mitogen-activated Protein Kinase. *J. Biol. Chem.* 1997;272(18):12069–12075.

34. Schneeberger PE, Kortüm F, Korenke GC, et al. Biallelic MADD variants cause a phenotypic spectrum ranging from developmental delay to a multisystem disorder. *Brain*. 2020;

35. Kuroda TS, Fukuda M, Ariga H, Mikoshiba K. The Slp homology domain of

synaptotagmin-like proteins 1-4 and Slac2 functions as a novel Rab27A binding domain. *J. Biol. Chem.* 2002;277(11):9212–9218.

36. Lenzi C, Stevens J, Osborn D, et al. Synaptotagmin 5 regulates Ca 2+ -dependent Weibel–Palade body exocytosis in human endothelial cells. *J. Cell Sci.* 2019;132(5):jcs221952.

37. Karampini E, Schillemans M, Hofman M, et al. Defective AP-3-dependent VAMP8 trafficking impairs Weibel-Palade body exocytosis in Hermansky-Pudlak Syndrome type 2 blood outgrowth endothelial cells. *Haematologica*. 2019;104(10):2091–2099.

38. Bustos MA, Lucchesi O, Ruete MC, Mayorga LS, Tomes CN. Rab27 and Rab3 sequentially regulate human sperm dense-core granule exocytosis. *Proc. Natl. Acad. Sci. U. S. A.* 2012;109(30):E2057-66.

39. Takahashi T, Minami S, Tsuchiya Y, et al. Cytoplasmic control of Rab family small GTP ases through BAG 6. *EMBO Rep.* 2019;20(4):e46794.

40. Fukuda M. Regulation of secretory vesicle traffic by Rab small GTPases. *Cell. Mol. Life Sci.* 2008;65(18):2801–2813.

41. Miyoshi J, Takai Y. Dual role of DENN/MADD (Rab3GEP) in neurotransmission and neuroprotection. *Trends Mol. Med.* 2004;10(10):476–480.

42. Li LC, Wang Y, Carr R, et al. IG20/MADD plays a critical role in glucose-induced insulin secretion. *Diabetes*. 2014;63(5):1612–1623.

43. Oishi H, Sasaki T, Nagano F, et al. Localization of the Rab3 small G protein regulators in nerve terminals and their involvement in Ca2+-dependent exocytosis. *J. Biol. Chem.* 1998;273(51):34580–34585.

44. Yamaguchi K, Tanaka M, Mizoguchi A, et al. A GDP/GTP exchange protein for the Rab3

small G protein family up-regulates a postdocking step of synaptic exocytosis in central synapses. *Proc. Natl. Acad. Sci. U. S. A.* 2002;99(22):14536–14541.

45. Tanaka M, Miyoshi J, Ishizaki H, et al. Role of Rab3 GDP/GTP exchange protein in synaptic vesicle trafficking at the mouse neuromuscular junction. *Mol. Biol. Cell*. 2001;12(5):1421–1430.

46. Pfeffer SR. Rab GTPase regulation of membrane identity. *Curr. Opin. Cell Biol.* 2013;25(4):414–419.

47. Seabra MC, Wasmeier C. Controlling the location and activation of Rab GTPases. *Curr. Opin. Cell Biol.* 2004;16(4):451–457.

48. Biesemann A, Gorontzi A, Barr F, Gerke V. Rab35 protein regulates evoked exocytosis of endothelial Weibel–Palade bodies. *J. Biol. Chem.* 2017;292(28):11631–11640.

49. Tarafder AK, Wasmeier C, Figueiredo AC, et al. Rab27a targeting to melanosomes requires nucleotide exchange but not effector binding. *Traffic*. 2011;12(8):1056–1066.

50. Mahoney TR, Lin Q, Itoh T, et al. Regulation of synaptic transmission by RAB-3 and RAB-27 in Caenorhabditis elegans. *Mol. Biol. Cell*. 2006;17(6):2617–2625.

51. Quevedo MF, Bustos MA, Masone D, et al. Grab recruitment by Rab27A-Rabphilin3a triggers Rab3A activation in human sperm exocytosis. *Biochim. Biophys. Acta - Mol. Cell Res.* 2019;1866(4):612–622.

52. Miteva KT, Pedicini L, Wilson LA, et al. Rab46 integrates Ca2+ and histamine signaling to regulate selective cargo release from Weibel-Palade bodies. *J. Cell Biol.* 2019;218(7):2232–2246.

53. Schluter OM. A Complete Genetic Analysis of Neuronal Rab3 Function. *J. Neurosci.* 2004;24(29):6629–6637.

54. Leebeek FWG, Eikenboom JCJ. Von Willebrand's Disease. *N. Engl. J. Med.* 2016;375(21):2067–2080.

55. Lavin M, Aguila S, Schneppenheim S, et al. Novel insights into the clinical phenotype and pathophysiology underlying low VWF levels. *Blood*. 2017;130(21):2344–2353.

56. SADLER JE. von Willebrand factor: two sides of a coin. *J. Thromb. Haemost.* 2005;3(8):1702–1709.

57. Desch KC, Ozel AB, Siemieniak D, et al. Linkage analysis identifies a locus for plasma von Willebrand factor undetected by genome-wide association. *Proc. Natl. Acad. Sci.* 2013;110(2):588–593.

58. Swystun LL, Lillicrap D. Genetic regulation of plasma von Willebrand factor levels in health and disease. *J. Thromb. Haemost.* 2018;16(12):2375–2390.

59. Smith NL, Chen M-H, Dehghan A, et al. Novel Associations of Multiple Genetic Loci With Plasma Levels of Factor VII, Factor VIII, and von Willebrand Factor. *Circulation*. 2010;121(12):1382–1392.

60. van Loon J, Dehghan A, Weihong T, et al. Genome-wide association studies identify genetic loci for low von Willebrand factor levels. *Eur. J. Hum. Genet.* 2016;24(7):1035–1040.

61. Sabater-Lleal M, Huffman JE, de Vries PS, et al. Genome-Wide Association Transethnic Meta-Analyses Identifies Novel Associations Regulating Coagulation Factor VIII and von Willebrand Factor Plasma Levels. *Circulation*. 2019;139(5):620–635.

62. Barral DC, Ramalho JS, Anders R, et al. Functional redundancy of Rab27 proteins and the pathogenesis of Griscelli syndrome. *J. Clin. Invest.* 2002;110(2):247–257.

63. Tiwari S, Italiano JE, Barral DC, et al. A role for Rab27b in NF-E2-dependent pathways of

platelet formation. *Blood*. 2003;102(12):3970–3979.

64. Tolmachova T, Abrink M, Futter CE, Authi KS, Seabra MC. Rab27b regulates number and secretion of platelet dense granules. *Proc. Natl. Acad. Sci.* 2007;104(14):5872–5877.

Figure legends

Figure 1. MADD is a guanine nucleotide exchange factor for secretory Rabs. (**A**) *MADD* expression was analyzed by quantitative PCR seven days after transduction of HUVEC with shCTRL or shMADD (mean±SEM, n=3 biological replicates). (**B**) Pulldown (PD) of active, GTP-bound Rab27A using GST-Slac2-b-SHD (Slac2-b)-coupled beads in shCTRL- and shMADD-transduced HUVEC lysates. GTP-bound Rab3B (**C**), and Rab3D (**D**) were extracted using GST-RIM2-RBD (RIM2)-coupled beads. PD experiments were performed in biological triplicate and representative Western blots are shown. Bar graphs represent normalized active fractions of Rab27A (**E**), Rab3B (**F**), and Rab3D (**G**) relative to total input levels (mean±SEM, unpaired two-tailed t-test, *P<0.05, **P<0.01).

Figure 2. MADD activates secretory Rabs in a DENN domain-dependent manner. HEK293T cells were transfected with GFP-Rab27A (**A**), GFP-Rab3B (**B**), or GFP-Rab3B (**C**) alone or co-transfected with GFP-MADD-full-length or GFP-MADD-ΔDENN. GTP-bound Rabs were extracted from lysates using Slac2-b or RIM2 PD as indicated. GFP-tagged proteins were visualized using an anti-GFP antibody. Experiments were performed in technical duplicates and representative Western blots are shown. Bar graphs represent mean normalized active fractions of Rab27A (**D**), Rab3B (**E**), and Rab3D (**F**) relative to input levels.

Figure 3. Rab27/3 recruitment to WPBs is decreased upon MADD silencing. HUVEC were transduced with pLKO-shRNAs targeting Rab27A (**A**), Rab3B (**B**), and Rab3D (**C**) or non-targeting control (shCTRL) and representative Western blots and quantifications n=3 to 5 biological replicates are shown confirming knockdown after seven days. (mean±SEM, unpaired t-test, ***P<0.001, ****P<0.0001). HUVEC were transduced with a pLKO-GFP-shRNA co-

expression construct containing shCTRL, shRab27A, shRab3D, or shMADD (all shown in green) and immunostained for VWF (blue), and Rab27A or Rab3D (red) as indicated (**D,F**). Individual channels are shown in gray scale below. Boxed areas are magnified on the right. Yellow and cyan arrowheads indicate Rab+ and Rab- WPBs, respectively. Scale bars represent 10 µm. The Rab+ proportion of Weibel-Palade bodies (WPBs) per cell were quantified (**E,G**). In (**E**) the proportion of Rab27A+ WPBs and divided over three bins based on the amount of WPBs in shCTRL (n=56) is compared to shRab27A (n=55) and shMADD (n=54) (mean±SD, two-way ANOVA, Tukey's multiple comparisons test, ns: not significant, ****P<0.0001). In (**G**) the proportion of Rab3D+ WPBs in shCTRL (n=42) is compared to shRab3D (n=31) and shMADD (n=33) without binning (mean±SD, one-way ANOVA, Tukey's multiple comparisons test, ns: not significant, ****P<0.0001).

Figure 4. Targeting MADD to mitochondria displaces Rab27A from WPBs. HUVEC were transduced with TOMM70-EGFP (**A**) or TOMM70-EGFP-MADD (**B**) or TOMM70-EGFP-MADD-ΔDENN (**C**) (all shown in green) and immunostained for VWF (blue), Rab27A (red), and VE cadherin (not shown). Boxed areas are magnified on the right. Yellow and cyan arrowheads indicate Rab27A+ and Rab27A- WPBs, respectively. Scale bars represent 10 µm. (**D**) Quantification of the percentage of transduced (GFP+) HUVEC containing Rab27A+ WPBs in TOMM70-EGFP (175 cells analyzed), or TOMM70-EGFP-MADD (96 cells analyzed), and TOMM70-EGFP-MADD-ΔDENN (38 cells analyzed) (mean, one-way ANOVA, Dunnet's multiple comparisons test, ns: not significant, *P<0.05).

Figure 5. Rab27A augments Rab3B/D activation. GTP-bound Rab3B (**A**) and Rab3D (**B**) fractions were extracted by RIM2 PD in shCTRL- and shRab27A-transduced HUVEC lysates.

(**C**) Western blot analysis of Rab27A expression in shCTRL and shRab27A. In (**D**) normalized Rab3B/D activity levels are plotted relative to total input levels. (**E**) GTP-bound Rab27A fractions were extracted by Slac2-b PD in shCTRL-, shRab3B, and shRab3D-transduced HUVEC lysates. (**F**) Western blot analysis of Rab3B and Rab3D expression in shCTRL-, shRab3B, and shRab3D. In (**G**) normalized Rab27A activity levels are plotted relative to total input levels. Representative Western blots are shown of n=3 (Slac2-b PD) and n=5 (RIM2 PD) biological replicates. Statistical analysis was an unpaired t-test comparing each condition to shCTRL (mean±SEM, unpaired t-test, ns: not significant, *P<0.05, **P<0.01).

Figure 6. MADD promotes VWF secretion in endothelial cells. (**A**) HUVEC were transduced with shCTRL or shMADD and immunostained for VWF (green), vascular endothelial (VE) cadherin (red) and nuclei (blue). Boxed areas are magnified on the right (1) and below (2). Scale bars represent 10 µm. (**B**) The number of WPBs was quantified per cell (small dots) and averaged per image (large dots) in shCTRL (n=15) and shMADD (n=6) images (mean±SEM, unpaired t-test, ****P<0.0001). (**C**) Western blot analysis for VWF expression in shCTRL- and shMADD-transduced HUVEC. ELISA data showing intracellular VWF content in unstimulated cells (**D**), 30-minute histamine-stimulated VWF secretion in absolute amounts (expressed in picomole (pmol)) (**E**), and as a percentage of intracellular content of resting/non-stimulated cells (**F**) (mean±SEM, n=5 biological replicates, unpaired t-test, *P<0.05, **P<0.01).

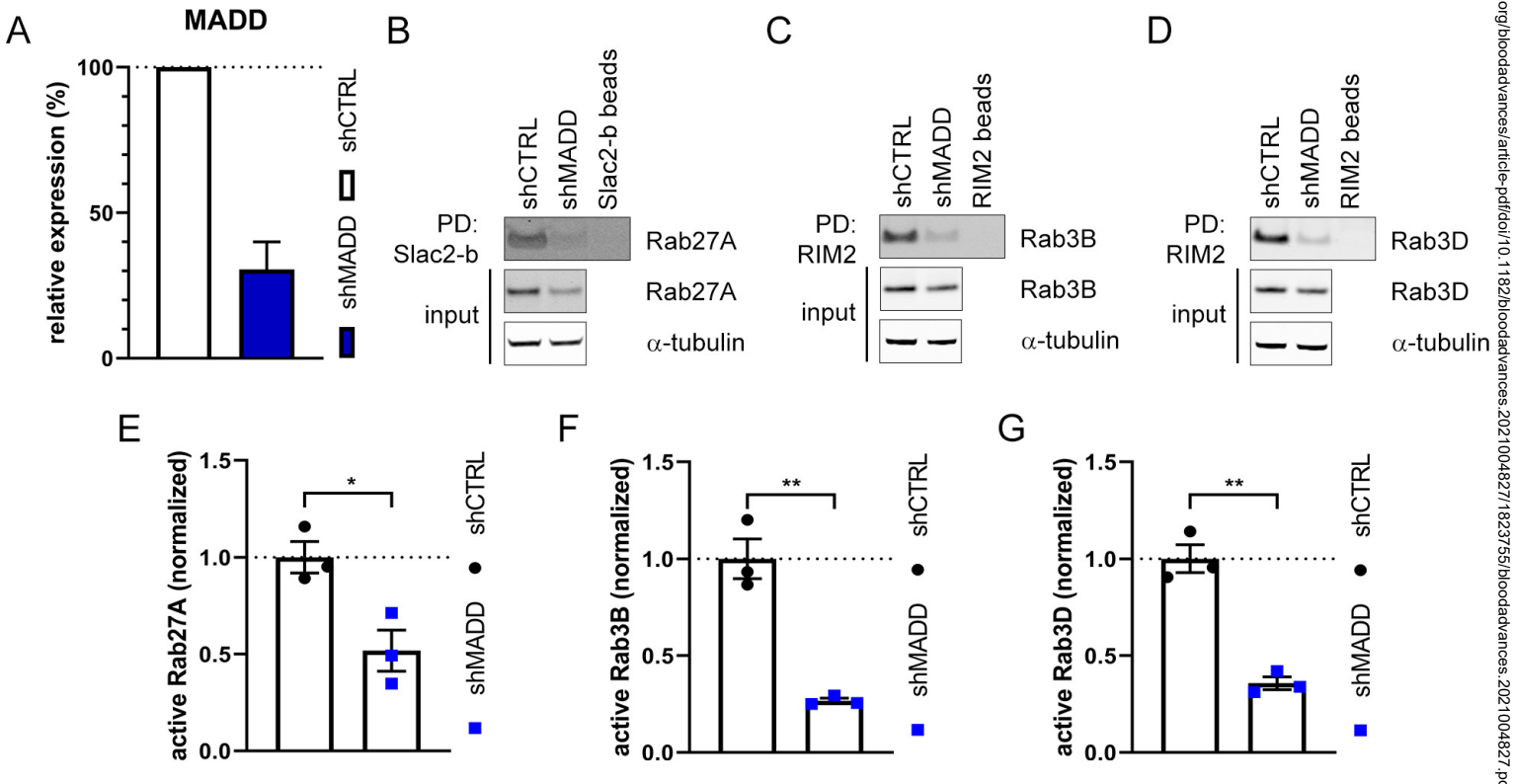


Figure 2

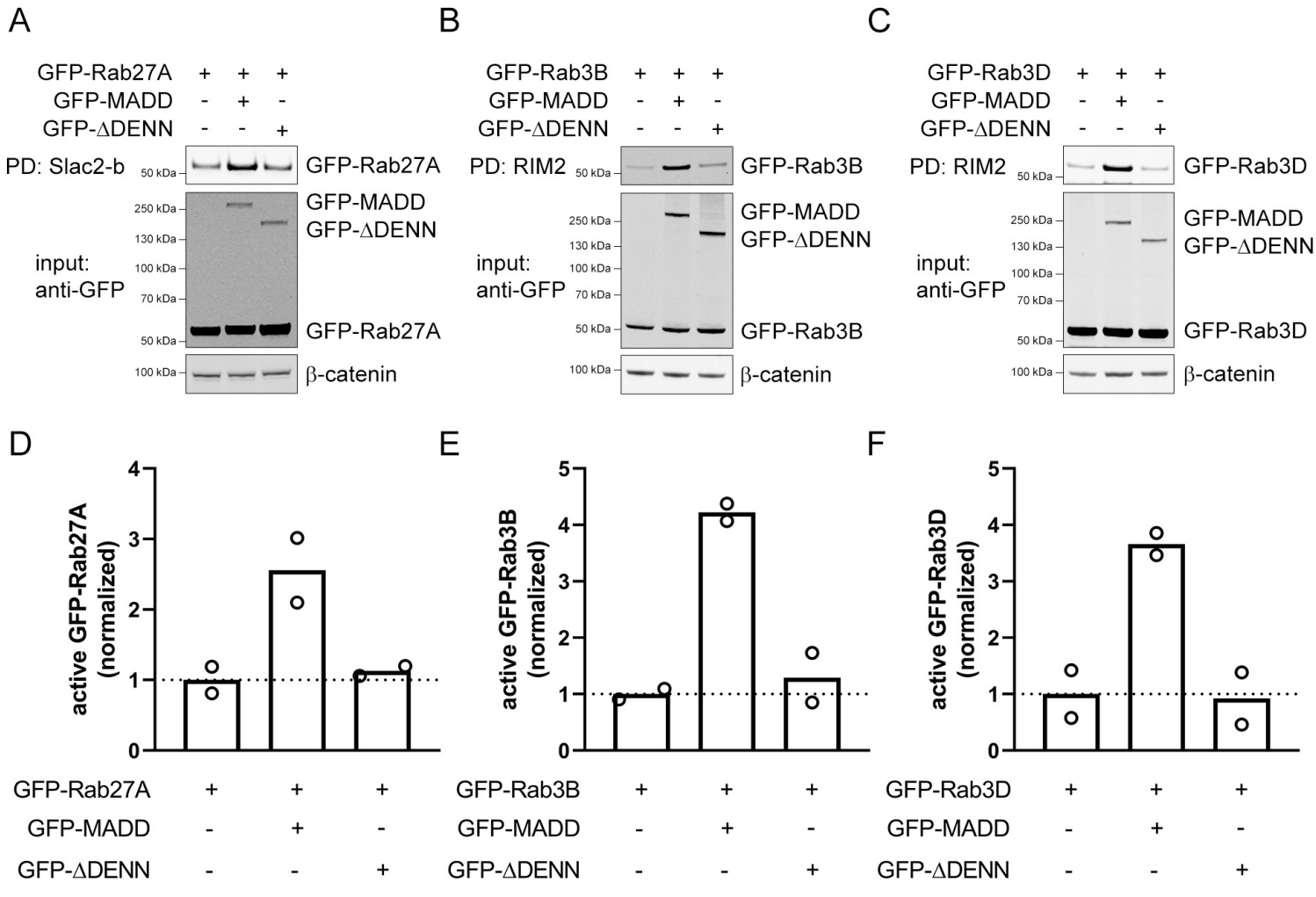


Figure 3

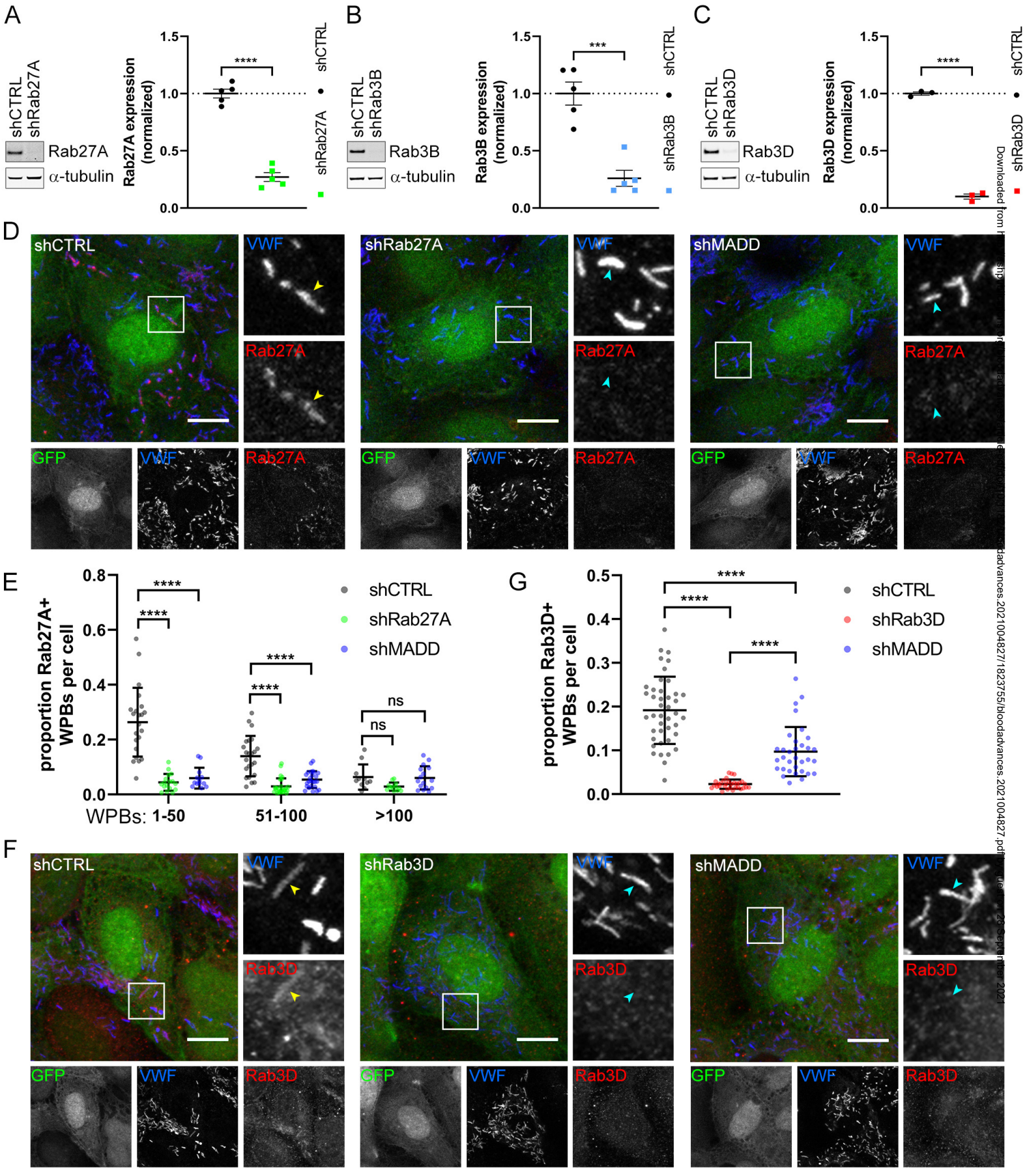
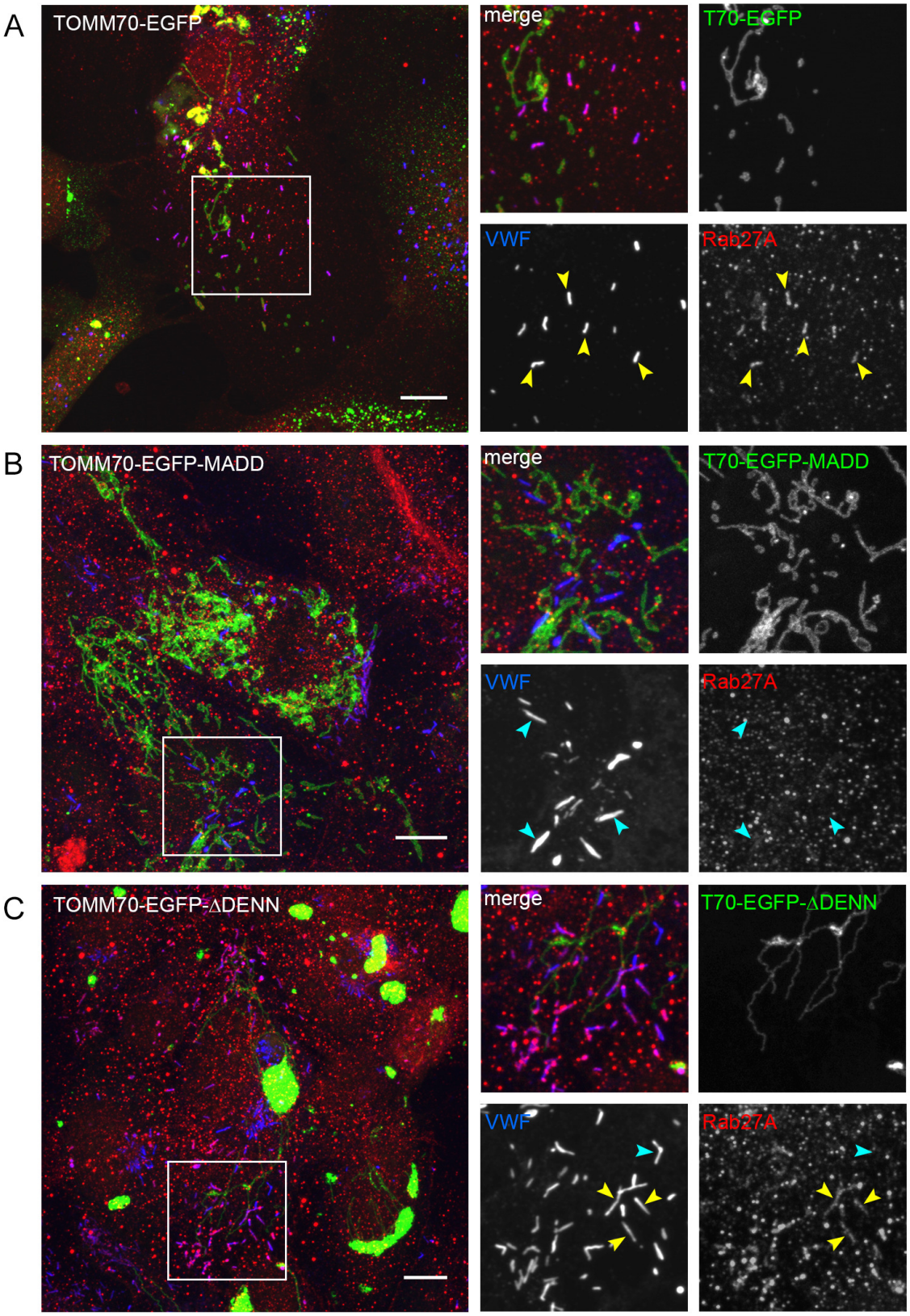


Figure 4



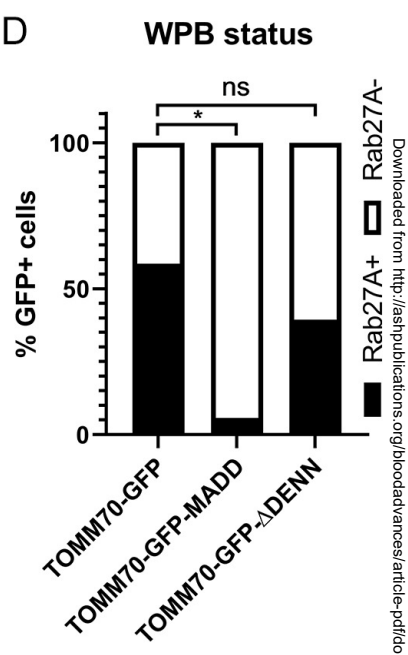


Figure 5

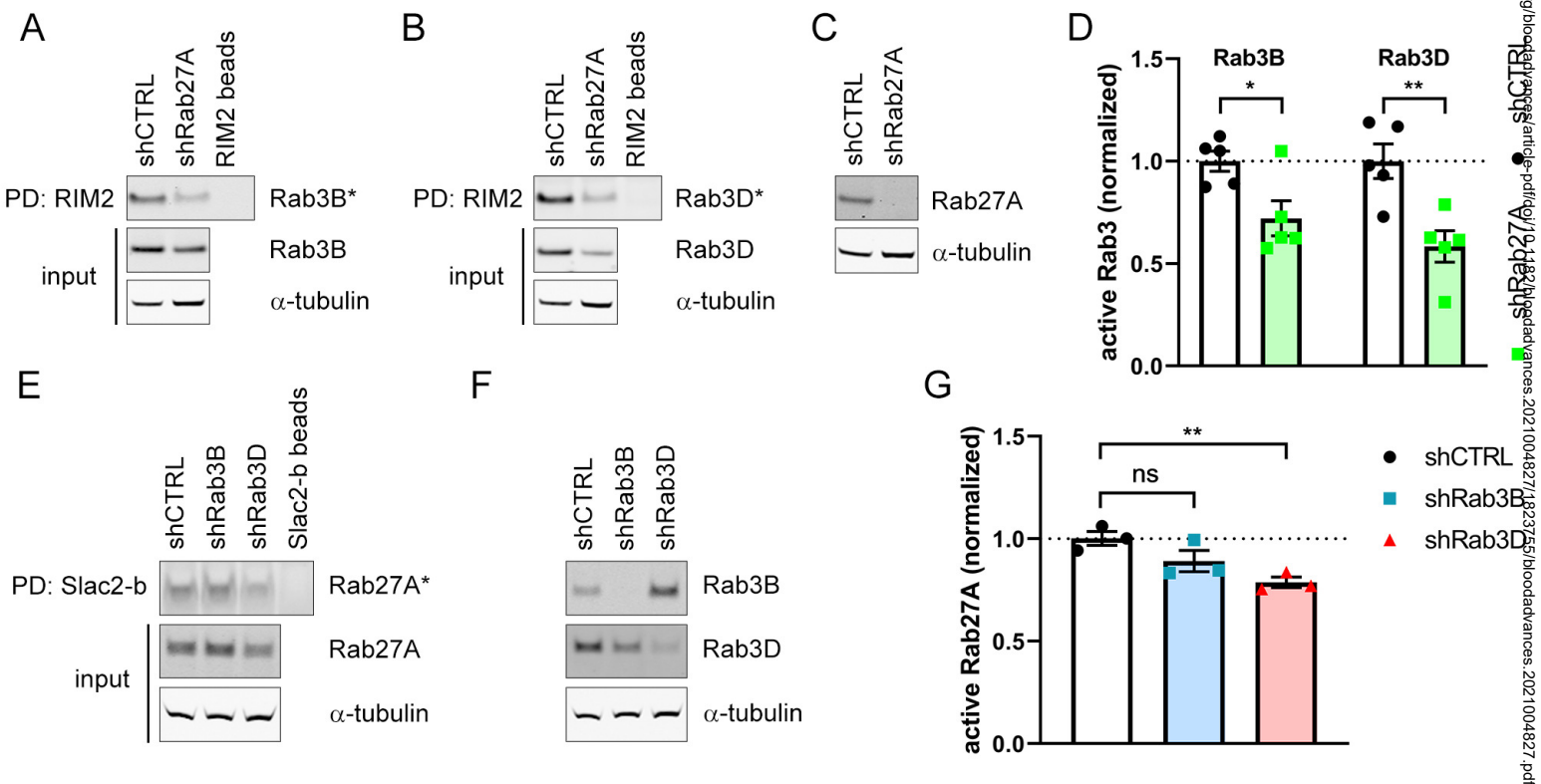


Figure 6