

# Alterations in platelet secretion differentially affect thrombosis and hemostasis

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## Key Points

- VAMP isoforms regulate the kinetics and extent of platelet granule exocytosis.
- Manipulating platelet sensitive factor attachment protein receptors alters granule secretion, which affects the hemostatic balance.

We genetically manipulated the major platelet vesicle-associated membrane proteins (VAMP2, VAMP3, and VAMP8) to create mice with varying degrees of disrupted platelet secretion. As previously shown, loss of VAMP8 reduced granule secretion, and this defect was exacerbated by further deletion of VAMP2 and VAMP3. VAMP2<sup>Δ3Δ8</sup><sup>-/-</sup> platelets also had reduced VAMP7. Loss of VAMP2 and VAMP3 (VAMP2<sup>Δ3Δ</sup>) had a minimal impact on secretion when VAMP7 and VAMP8 were present. Integrin αIIbβ3 activation and aggregation were not affected, although spreading was reduced in VAMP2<sup>Δ3Δ8</sup><sup>-/-</sup> platelets. Using these mice as tools, we asked how much secretion is needed for proper thrombosis and hemostasis in vivo. VAMP2<sup>Δ3Δ</sup> mice showed no deficiency, whereas VAMP8<sup>-/-</sup> mice had attenuated formation of occlusive thrombi upon FeCl<sub>3</sub>-induced arterial injury but no excessive bleeding upon tail transection. VAMP2<sup>Δ3Δ8</sup><sup>-/-</sup> mice bled profusely and failed to form occlusive thrombi. Plasma-coagulation factors were normal in all of the strains, but phosphatidylserine exposure was reduced in VAMP2<sup>Δ3Δ</sup> and VAMP2<sup>Δ3Δ8</sup><sup>-/-</sup> platelets. From our data, an ~40% to 50% reduction in platelet secretion in vitro (dense and α granule) correlated with reduced occlusive thrombosis but no compromise in hemostasis. At a >50% reduction, thrombosis and hemostasis were defective in vivo. Our studies are the first systematic manipulation of platelet exocytic machinery to demonstrate a quantitative linkage between in vitro platelet secretion and hemostasis and thrombosis in vivo. The animals described will be invaluable tools for future investigations into how platelet secretion affects other vascular processes.

## Introduction

Platelets are anucleate cell fragments that contribute to a myriad of processes: thrombosis, hemostasis, angiogenesis, wound healing, and immunity.<sup>1,2</sup> Many of these functions are mediated by their releasate, which is generated through the exocytosis of cargo from the 3 types of granules: dense, α, and secretory lysosomes. The importance of these cargo is seen in dense and α-granulopathies, which cause a range of bleeding diatheses and other pathologies.<sup>3</sup> Given its importance, controlling releasate generation offers potential therapeutic usefulness. In this article, we develop a collection of genetically altered mice as tools to define how exocytosis is needed for proper hemostasis.

Platelet granule exocytosis is facilitated by a family of membrane proteins called soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNAREs). Based on localization and residues at the center of their SNARE domains, SNAREs are classified as target (t/Q, Glu) SNAREs and vesicle (v/R, Arg) SNAREs (also known as vesicle-associated membrane proteins [VAMPs]).<sup>4</sup> A complex of v- and t-SNAREs spans opposing bilayers to mediate membrane fusion and, thus,

vesicle/granule cargo release.<sup>5</sup> The t-SNAREs, SNAP23,<sup>6-8</sup> syntaxin-11,<sup>9</sup> and syntaxin-8,<sup>10</sup> are important for platelet secretion. Proteomic studies report 6 v-SNAREs (VAMP2, VAMP3, VAMP4, VAMP5, VAMP7, and VAMP8); however, 4 of these v-SNAREs (VAMP2, VAMP3, VAMP7, and VAMP8) are the most abundant in human and mouse platelets.<sup>11-14</sup> VAMP7 and VAMP8 play the most critical roles, whereas VAMP2 and VAMP3 have been considered secondary v-SNAREs.<sup>15,16</sup> The importance of VAMP8 is underscored by studies correlating single nucleotide polymorphisms in VAMP8 with early-onset myocardial infarction.<sup>17,18</sup> Additionally, a microRNA that regulates VAMP8 expression correlates with platelet hyperreactivity.<sup>19</sup> These data imply that the levels of VAMP8 play a dominant, but not exclusive, role in controlling platelet secretion.

To determine how much platelet secretion is necessary to attain a hemostatic balance, we generated mouse strains in which we genetically manipulated the major platelet VAMPs. We generated platelet-specific  $V2^{\Delta 3\Delta}$  and  $V2^{\Delta 3\Delta 8^{-/-}}$  animals and characterized secretion from their platelets. The hemostatic profiles of these animals were compared with wild-type (WT) and  $V8^{-/-}$  animals. We found that the loss of VAMP2 and VAMP3 had little effect on the kinetics or magnitude of secretion from platelet granules as long as VAMP7 and VAMP8 were present. However,  $V2^{\Delta 3\Delta 8^{-/-}}$  platelets showed significantly diminished granule secretion, and  $V2^{\Delta 3\Delta 8^{-/-}}$  animals bled profusely in tail-bleeding assays and failed to form occlusive thrombi. By comparing in vitro granule cargo release profiles and in vivo bleeding phenotypes, we distinguished the levels of platelet exocytosis required for normal hemostasis from those that are needed to sustain occlusive thrombus growth.

## Methods

The methods are described in detail in supplemental Methods.

### Mouse strains and genotyping

To delete VAMP2 and VAMP3, we crossed an RC::PFtoxin strain (generously provided by S. Dymecki, Harvard Medical School, Boston, MA<sup>20</sup>) with a PF4Cre strain (generously provided by R. Skoda, University Hospital, Basel, Switzerland<sup>21</sup>). The RC::PFtoxin strain has a genomic insertion encoding the catalytic subunit of the tetanus toxin endopeptidase, which can be expressed upon Cre-mediated excision of a STOP cassette. The STOP cassette consists of the 3' portion of the yeast His3 gene, an SV40 polyadenylation sequence, and a false translation initiation codon followed by a 5' splice donor site. The floxed STOP cassette is inserted between the promoter and tetanus toxin-coding sequences of a transgene, ensuring that few, if any, transcripts containing the coding region are generated. Tetanus toxin specifically cleaves only VAMP2 and VAMP3.<sup>22,23</sup> This strategy was needed because  $VAMP2^{-/-}$  mice are embryonically lethal,<sup>24</sup> as are  $VAMP3/8^{-/-}$  mice (S.W.W., unpublished observations). These RC::PFtoxin/PF4Cre mice ( $V2^{\Delta 3\Delta}$ ) were further crossed with a global  $VAMP8^{-/-}$  strain ( $V8^{-/-}$ ) to create an RC::PFtoxin/PF4Cre/ $VAMP8^{-/-}$  strain ( $V2^{\Delta 3\Delta 8^{-/-}}$ ). For genotyping RC::PFtoxin,<sup>20</sup> the primers were forward primer 5'-GCCGATCACCATCAACAACCTTC-3' and reverse primer 5'-GCAGAGCTTCACCAGCAACG-3' using the following conditions: 94°C for 4 minutes for 1 cycle, 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 1 minute for 35 cycles, and, finally, 72°C for 5 minutes. The genotyping of  $V8^{-/-}$  mice<sup>16</sup> and PF4Cre<sup>21</sup> was as described.

## Study design and controls

The breeding schemes produced 3 lines with normal VAMP levels: RC::PFtoxin<sup>+</sup>/PF4Cre<sup>-</sup>, RC::PFtoxin<sup>-</sup>/PF4Cre<sup>+</sup>, and RC::PFtoxin<sup>-</sup>/PF4Cre<sup>-</sup>. These strains were considered WT controls because their VAMP levels were unchanged (data not shown). They were evaluated alongside the VAMP-deletion strains in the tail bleeding time and FeCl<sub>3</sub>-induced arterial injury assays. Bleeding times and occlusion times showed no significant differences among these 3 WT strains (supplemental Figure 5). The in vitro experiments described in this article were performed using pooled blood from  $\geq 1$  of 3 WT control strains.

## Results

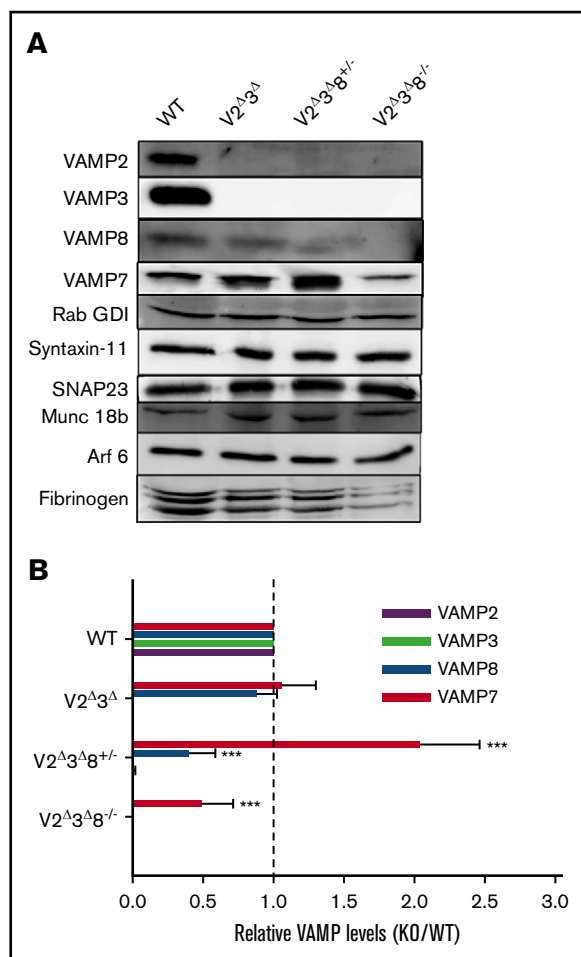
### Generation of platelet-specific $V2^{\Delta 3\Delta}$ and $V2^{\Delta 3\Delta 8^{-/-}}$ mouse models

To overcome embryonic lethality in  $V2^{-/-}$  animals, megakaryocyte-specific  $V2^{\Delta 3\Delta}$  animals were generated by crossing PF4Cre recombinase mice with a mouse harboring a tetanus toxin gene downstream of a floxed STOP cassette. Tetanus toxin light chain cleaves VAMP2 and VAMP3.<sup>23,24</sup> Megakaryocyte-specific  $V2^{\Delta 3\Delta}$  animals were crossed with global  $V8^{-/-}$  animals to generate  $V2^{\Delta 3\Delta 8^{-/-}}$  animals. Animals from both novel strains,  $V2^{\Delta 3\Delta}$  and  $V2^{\Delta 3\Delta 8^{-/-}}$ , were born at expected Mendelian ratios and were healthy, fertile, and had no gross developmental or anatomical abnormalities. The average weight of littermate controls at 12 weeks ( $24.41 \pm 2.96$  g) was comparable to  $V2^{\Delta 3\Delta}$  ( $23.48 \pm 2.97$  g) and  $V2^{\Delta 3\Delta 8^{-/-}}$  ( $23.58 \pm 1.90$  g) mice. Hematological parameters, including red blood cell count, white blood cell count, platelet number, and mean platelet volume, were not statistically different from littermate controls (supplemental Figure 2), indicating normal hematopoiesis. There were no significant defects in serotonin uptake (dense-granule cargo), PF4 ( $\alpha$ -granule cargo), or  $\beta$ -hexosaminidase (lysosomal cargo) levels in platelet lysates from these strains (data not shown). See supplemental Methods and supplemental Figure 5 for a discussion of WT controls.

Semiquantitative western blotting, using Rab GDI as a loading control,<sup>25</sup> confirmed the appropriate deletion or reduction of VAMP2, VAMP3, and VAMP8 in platelets from these strains (Figure 1). There was no change in expression of VAMP7 and VAMP8 in  $V2^{\Delta 3\Delta}$  platelets; consistent with our previous observations for VAMP2 and VAMP3.<sup>26</sup> However, manipulating VAMP8 levels on the  $V2^{\Delta 3\Delta}$  background surprisingly affected VAMP7 levels. A partial decrease in VAMP8 (eg,  $V2^{\Delta 3\Delta 8^{+/-}}$  platelets) correlated with increased VAMP7, whereas complete loss of VAMP8 resulted in decreased VAMP7. These data imply coordinated control of VAMP levels in platelets or megakaryocytes and demonstrate that the  $V2^{\Delta 3\Delta 8^{-/-}}$  platelets are hypomorphic for VAMP7. Expression of the t-SNAREs syntaxin-11 and SNAP23, as well as the t-SNARE regulator Munc18b, were unaffected. Expectedly, loss of VAMP3 reduced fibrinogen levels in platelets,<sup>27</sup> which was exacerbated in  $V2^{\Delta 3\Delta 8^{-/-}}$  platelets.

### Release from activated $V2^{\Delta 3\Delta 8^{-/-}}$ platelets was slower and less extensive

To measure how VAMP2, VAMP3, and VAMP8 influenced platelet granule secretion, we examined the agonist- and time-dependent release of cargo from all 3 platelet granules: dense,  $\alpha$ , and lysosomes. The release from  $V8^{-/-}$  platelets was reduced at lower



**Figure 1. Generation of V2 $\Delta$ 3 $\Delta$  and V2 $\Delta$ 3 $\Delta$ 8 $^{-/-}$  animals.** (A) A collage of representative western blots comparing protein levels among V2 $\Delta$ 3 $\Delta$ , V2 $\Delta$ 3 $\Delta$ 8 $^{+/-}$ , and V2 $\Delta$ 3 $\Delta$ 8 $^{-/-}$  platelets. Washed platelets ( $5 \times 10^7$  platelets per lane) were prepared from WT, V2 $\Delta$ 3 $\Delta$ , V2 $\Delta$ 3 $\Delta$ 8 $^{+/-}$ , and V2 $\Delta$ 3 $\Delta$ 8 $^{-/-}$  mice, and the indicated proteins were probed for. The blots for VAMP7, VAMP8, and Rab GDI were from the same membrane. (B) The VAMP isoforms in V2 $\Delta$ 3 $\Delta$ , V2 $\Delta$ 3 $\Delta$ 8 $^{+/-}$ , and V2 $\Delta$ 3 $\Delta$ 8 $^{-/-}$  platelets were quantified using Rab GDI as a loading control and ImageQuant TL software (v 7.0). The data were plotted as the ratio of knockout/WT. The vertical dashed line indicates a ratio of 1. Data are representative of platelets collected from individual mice from each strain and  $\geq 3$  independent experiments (see supplemental Figure 1 for additional blots for VAMP7 and VAMP8). A Student *t* test was used to analyze the data. \*\*\**P* < .001.

thrombin concentration but reached comparable levels for dense and  $\alpha$ -granule release from WT platelets as thrombin concentrations increased (Figure 2A-C). As previously seen,<sup>16</sup> loss of VAMP8 had more influence on  $\alpha$ -granule (~40%-50% reduction) and lysosome (~50% reduction) release than on dense granule release. This may be due, at least in part, to the defective release of secondary agonists from dense granules (adenosine triphosphate [ATP]/adenosine 5'-diphosphate [ADP], calcium, serotonin) that affects the release from  $\alpha$  granules and lysosomes.<sup>28</sup> Secretion of granule cargo from V2 $\Delta$ 3 $\Delta$  platelets, in response to increasing thrombin concentrations, was essentially equal to that from WT platelets (Figure 2A-C) and underlined the ancillary roles of VAMP2 and VAMP3 in platelet secretion. V2 $\Delta$ 3 $\Delta$ 8 $^{-/-}$  platelets showed an

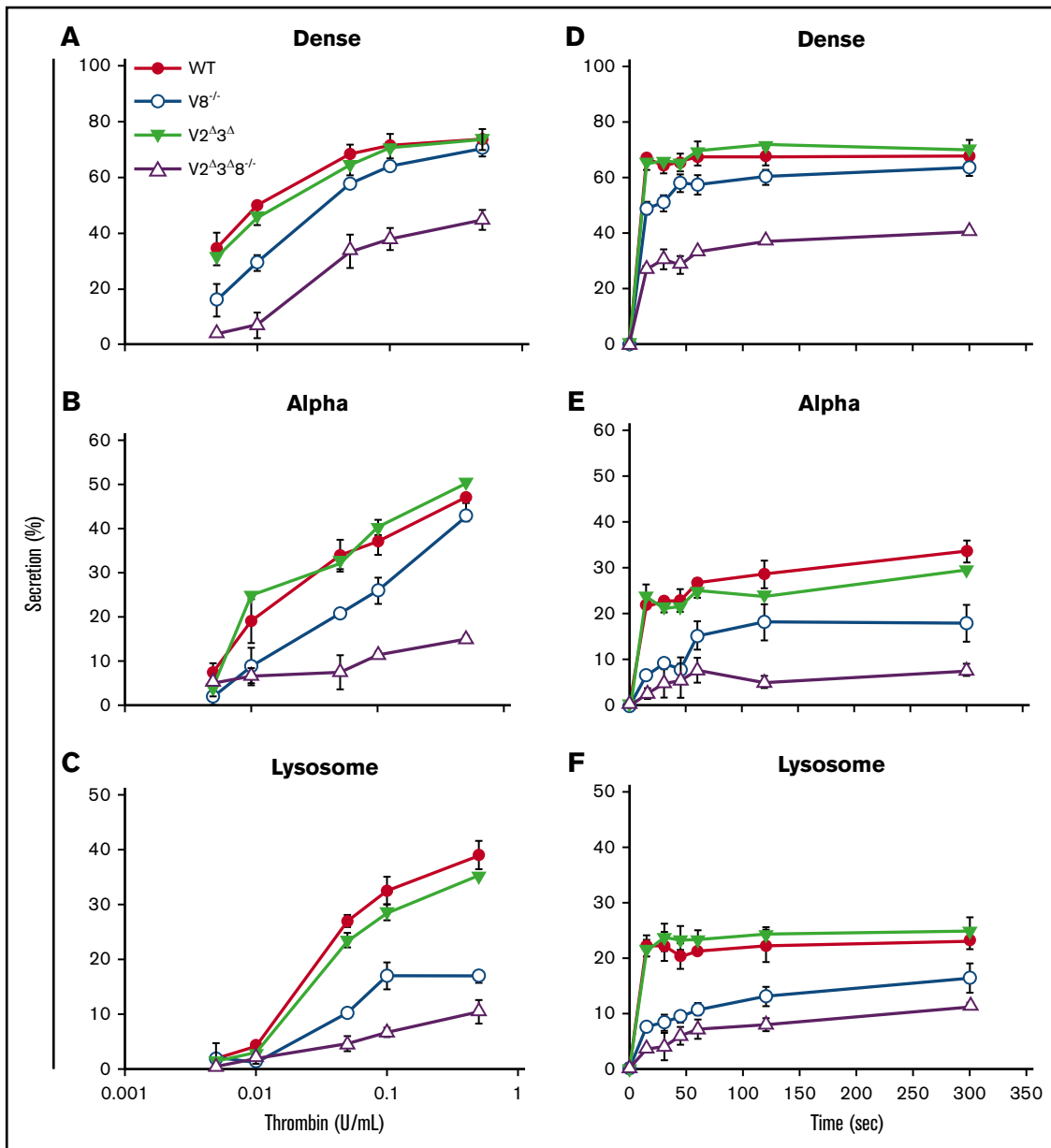
~50% decrease in dense granule secretion, an ~80% to 90% reduction in  $\alpha$ -granule secretion, and an ~70% reduction in lysosomal secretion upon stimulation with 0.5 U/mL thrombin (Figure 2A-C), indicating that loss of VAMP2, VAMP3, and VAMP8 had a synergistic effect on secretion that was greater than the deletion of VAMP8 alone, perhaps due to the additional loss of VAMP7.

Time-course analysis (using 0.05 U/mL thrombin) showed that the loss of VAMP8 affected the rates of early (first 30 seconds) dense granule release but not its maximal extent<sup>16</sup> (Figure 2D). Dense granule release rates in V2 $\Delta$ 3 $\Delta$  platelets were not affected, whereas V2 $\Delta$ 3 $\Delta$ 8 $^{-/-}$  platelets showed delayed dense granule release that reached only ~50% of WT at 5 minutes (Figure 2D). Similar patterns were observed in  $\alpha$  and lysosomal release (Figure 2E-F). These patterns are consistent with ancillary roles for VAMP2 and VAMP3<sup>16</sup> or, alternatively, the ~50% reduction in VAMP7 levels noted in Figure 1. These data demonstrate that manipulation of the VAMPs in platelets alters release kinetics and extents. These data imply differential roles for these 3 VAMPs in platelet exocytosis and perhaps in some steps of granule cargo sorting or biogenesis (eg, our report of a role for VAMP3 in fibrinogen trafficking<sup>27</sup>).

**V2 $\Delta$ 3 $\Delta$ 8 $^{-/-}$  platelets showed normal integrin activation and modest platelet aggregation defects.** To confirm the phenotypes indicated by the secretion assays, we used lumi-aggregometry to analyze ATP/ADP release. Release in response to 0.05, 0.1, and 0.5 U/mL thrombin was minimally affected in V2 $\Delta$ 3 $\Delta$  platelets but was less inhibited at the higher concentrations in V2 $\Delta$ 3 $\Delta$ 8 $^{-/-}$  platelets (Figure 3D-F). Aggregation was not affected at the higher agonist doses (Figure 3A-C). Under our in vitro conditions (0.05 U/mL thrombin), there was no ATP secretion from, and limited aggregation of, V2 $\Delta$ 3 $\Delta$ 8 $^{-/-}$  platelets. The ATP release at 0.1 and 0.5 U/mL thrombin was sufficient to support normal aggregation (Figure 3B-C).

Surface exposure of P-selectin and LAMP-1 ( $\alpha$ -granule and lysosome membrane markers, respectively) was analyzed by flow cytometry. Activation-dependent exposure of both granule membrane proteins was reduced in V2 $\Delta$ 3 $\Delta$ 8 $^{-/-}$  platelets compared with WT platelets (Figure 4A-B). Total surface staining for  $\alpha$ IIb $\beta$ 3 integrin decreased by 10% to 15% in V2 $\Delta$ 3 $\Delta$  platelets and by ~25% in V2 $\Delta$ 3 $\Delta$ 8 $^{-/-}$  platelets (Figure 4D). This may be due to an alteration of integrin trafficking to the plasma membrane (PM) and was not pursued further. In agreement with aggregometry data, there was no significant difference in activated integrin staining with phycoerythrin (PE)-conjugated JonA staining (Figure 4C). Thus, loss of VAMP2, VAMP3, and VAMP8 together, had minimal effects on  $\alpha$ IIb $\beta$ 3 integrin activation under the conditions tested.

**Fusion pore dynamics were affected by the loss of platelet VAMPs.** Because SNAREs mediate granule-PM fusion, we sought to determine how the loss of specific VAMPs affected fusion pore formation and/or dilation.<sup>29,30</sup> Based on Figure 2, we selected 2 time points, 90 seconds (partial cargo release) and 300 seconds (maximal cargo release), and used electron microscopy (EM) tomography to measure fusion pore size and prevalence in platelets stimulated with thrombin. We focused on  $\alpha$  granules because they are more abundant and readily identifiable. Fusion events were scored per platelet profile, and the widths of fusion pores were estimated across multiple z-steps in the EM tomographs (Figure 5). In the resting state, fusion pores were not observed (data not



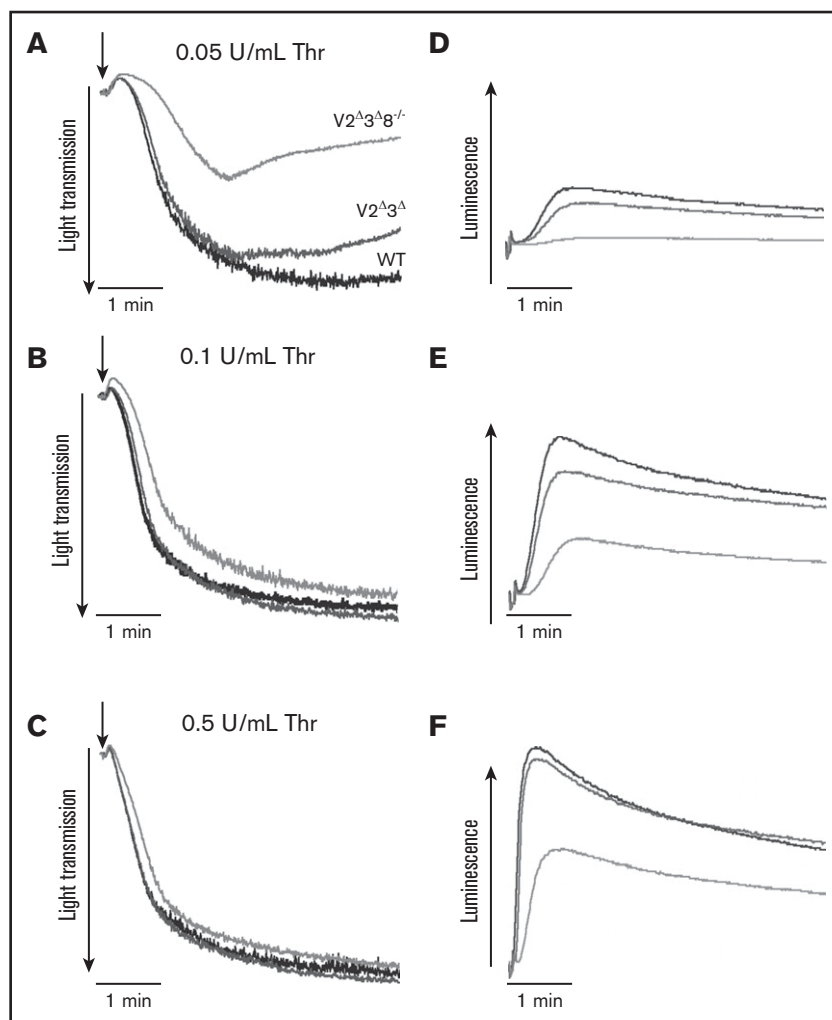
**Figure 2. Loss of VAMP2, VAMP3, and VAMP8 affects the kinetics and the extent of platelet secretion.** [ $^3\text{H}$ ]-5-[HT] (serotonin)-labeled platelets from WT,  $V8^{-/-}$ ,  $V2^{\Delta 3\Delta}$ , and  $V2^{\Delta 3\Delta 8^{-/-}}$  mice were prepared as described in supplemental Methods. The release of [ $^3\text{H}$ ]-5-[HT] from dense granules (A,D), PF4 from  $\alpha$  granules (B,E), and  $\beta$ -hexosaminidase from lysosomes (C,F) was measured, and percentage secretion was calculated as described in supplemental Methods. (A-C) For the thrombin dose-response experiment, platelets were stimulated for 2 minutes with the indicated concentrations of thrombin. (D-F) For the time-course experiments, platelets were stimulated with 0.05 U/mL thrombin for the indicated times. Data are mean  $\pm$  standard error of the mean of triplicate measurements and are representative of  $\geq 3$  independent experiments.

shown); however, upon stimulation, there was a time-dependent increase in prevalence and size, which culminated in granules becoming unidentifiable as they merged into the PM. For WT platelets, median pore diameter increased from 65 nm to 157 nm, whereas the mean was skewed slightly higher (90 seconds: 82.4 nm; 300 seconds: 166 nm; Figure 5A-B,E). This skew to larger pore size is further evidence that dilation is time dependent. In the  $V2^{\Delta 3\Delta 8^{-/-}}$  platelets, fusion pores were rare (2 per 35 profiles). Pores were only detected at the earliest time point, suggesting that

the pores produced were unsustainable. Interestingly, the fusion pore prevalence and size distribution in the absence of VAMP8 showed a delay relative to WT. In  $V8^{-/-}$  platelets, 1 fusion pore was identified in 24 profiles at 90 seconds, whereas 10 pores were identified in 18 profiles at 300 seconds. The median and mean values showed a similar skew to larger pores, as observed in WT platelets. The delayed pore formation in  $V8^{-/-}$  platelets and the lack of sustained pore formation in  $V2^{\Delta 3\Delta 8^{-/-}}$  platelets are consistent with the decreased  $\alpha$ -granule cargo release seen in Figure 2B,E.

**Figure 3. Depletion of VAMP2, VAMP3, and VAMP8 in platelets affects ATP/ADP release but not aggregation.**

Aggregation (A-C) and ATP/ADP release (D-F) were monitored simultaneously in a lumi-aggregometer. Washed platelets ( $4 \times 10^8$ /mL) from WT (black traces),  $V2^{\Delta 3\Delta}$  (dark gray traces), and  $V2^{\Delta 3\Delta 8^{-/-}}$  (light gray traces) platelets were stimulated with 0.05 U/mL (A), 0.1 U/mL (B), or 0.5 U/mL (C) thrombin (Thr), and ATP release and aggregation were measured for 5 minutes.



**Role of platelet secretion in spreading**

Previous studies reported a role for granule secretion in platelet spreading.<sup>15,31</sup> Using platelets from the mutant mice, we addressed the significance of granule secretion to spreading (supplemental Figure 4). When plated on fibrinogen-coated coverslips,  $V8^{-/-}$  platelets showed a slight reduction in spreading compared with WT platelets (supplemental Figure 4B).  $V2^{\Delta 3\Delta}$  platelets covered more area than WT platelets at 30 minutes, although the area covered was similar at later time points (supplemental Figure 4). This is most likely due to the loss of VAMP3, which we previously showed causes platelets to spread more rapidly.<sup>27</sup> Spreading of  $V2^{\Delta 3\Delta 8^{-/-}}$  platelets was markedly slower and the area covered was significantly reduced compared with WT platelets (supplemental Figure 4A).

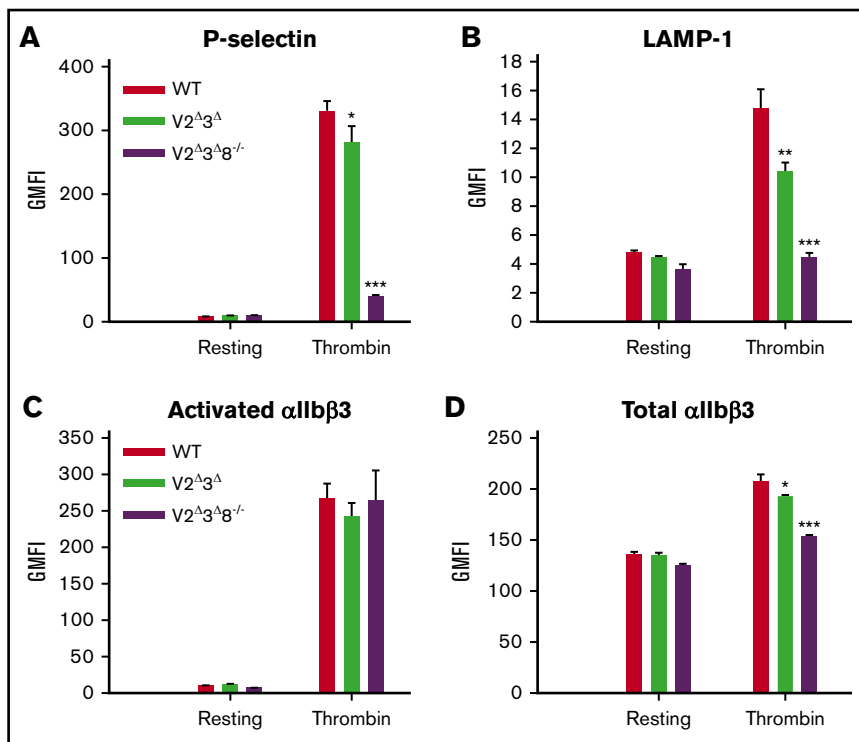
**Effect of platelet secretion on thrombosis and hemostasis**

Together, these data demonstrate that VAMP-deficient mice have distinct platelet exocytosis profiles. These mice were used to assess how exocytosis affects platelet function in vivo, using 2 assays: tail bleeding time and  $\text{FeCl}_3$ -induced carotid injury (Figure 6A-B). Tail bleeding times were comparable for WT,  $V3^{-/-}$ ,  $V2^{\Delta 3\Delta}$ , and  $V8^{-/-}$

mice. Even after reducing VAMP8, the tail bleeding times did not increase in  $V2^{\Delta 3\Delta 8^{+/-}}$  mice. However, tail bleeding was significantly increased in  $V2^{\Delta 3\Delta 8^{-/-}}$  mice (mean time,  $571.74 \pm 72.61$  seconds) compared with WT mice (mean time,  $250.07 \pm 86.82$  seconds). For most  $V2^{\Delta 3\Delta 8^{-/-}}$  mice, application of pressure was required to stop the bleeding. In the  $\text{FeCl}_3$ -induced injury model, the strains responded differently. The average times to occlusion were comparable for WT (~6.5 minutes),  $V8^{+/-}$  (~7 minutes),  $V2^{\Delta 3\Delta}$  (~7 minutes), and  $V2^{\Delta 3\Delta 8^{+/-}}$  (~6.5 minutes) mice. Occlusion times were more variable in  $V8^{-/-}$  mice, and the average was significantly increased (~21 minutes). This was even more true in  $V2^{\Delta 3\Delta 8^{-/-}}$  mice (~28 minutes), which generally failed to form occlusive thrombi during our observation period of 30 minutes. Males and females showed no differences in bleeding or occlusion times (supplemental Figure 6). These data demonstrate that platelet exocytosis contributes differentially to thrombosis vs hemostasis, because normal thrombosis appears to require more platelet secretion.

Because several factors control hemostasis, we examined the coagulation cascades and the degree of phosphatidylserine (PS) exposure in our strains. Prothrombin time (PT) and activated partial thromboplastin time (APTT) assays of plasma showed no differences among the strains, suggesting no defects in the coagulation





**Figure 4. V2<sup>Δ3Δ8</sup><sup>-/-</sup> platelets have lower P-selectin and LAMP-1 exposure and reduced integrin αIIbβ3 surface levels but normal activation.** Washed platelets ( $5 \times 10^7$ ) from WT, V2<sup>Δ3Δ</sup>, and V2<sup>Δ3Δ8</sup><sup>-/-</sup> mice were held resting or stimulated with thrombin (0.1 U/mL) for 2 minutes and then incubated with fluorescein isothiocyanate-conjugated anti-P-selectin (A), PE-conjugated LAMP-1 (B), PE-conjugated JonA (C), or fluorescein isothiocyanate-conjugated CD41/61 (D) antibodies for 20 minutes at room temperature. Fluorescence intensities were measured by flow cytometry. Shown are representative data and geometric mean fluorescence intensity (GMFI) (mean  $\pm$  standard error of the mean) of  $\geq 2$  independent experiments. A Student *t* test was used to analyze the data. \**P* < .05, \*\**P* < .01, and \*\*\**P* < .001.

system of these animals (Figure 6C). Although the loss of VAMP8 did not affect PS exposure, loss of VAMP2/3 did ( $\sim 30\%$  reduction), as measured by lactadherin binding. Further deletion of VAMP8 (ie, V2<sup>Δ3Δ8</sup><sup>-/-</sup>) exacerbated this defect to an  $\sim 50\%$  decrease in PS exposure in response to thrombin (Figure 6D). Although not significant in cases of V2<sup>Δ3Δ</sup> and V2<sup>Δ3Δ8</sup><sup>+/-</sup>, the *P* values (.15 and .14, respectively) indicate a trend toward significance. These data reveal a connection between VAMP isoforms and PS exposure, perhaps through platelet granule secretion and/or intracellular trafficking in platelets. To gain insight into this effect, we probed for transmembrane protein 16F (TMEM16F), a phospholipid scramblase important for PS exposure.<sup>32-34</sup> Its levels did not differ significantly in any of the strains tested (Figure 6E).

## Discussion

In this article, we describe a collection of genetically engineered mice in which the platelet-secretory machinery is altered to yield differential effects on platelet exocytosis, thrombosis, and hemostasis (Figure 7A). We have measured platelet secretion and defined how the 4 major platelet VAMPs mediate the release of cargo from the 3 classes of granules. Using these mice, we have estimated the level of secretion needed for hemostasis in tail bleeding and occlusive thrombosis in a FeCl<sub>3</sub>-injury model. Our studies represent the first attempt at defining the extent to which platelet secretion is needed for thrombosis and hemostasis in vivo.

### Role of VAMPs in platelet secretion

In previous reports,<sup>15,16</sup> single deletion of VAMP7 or VAMP8 blocks cargo release, but not completely, suggesting that compensatory effects or alternative membrane-fusion pathways could occur. Using recombinant VAMPs as standards and quantitative western blotting, Graham et al showed that VAMP2 and VAMP8 are most

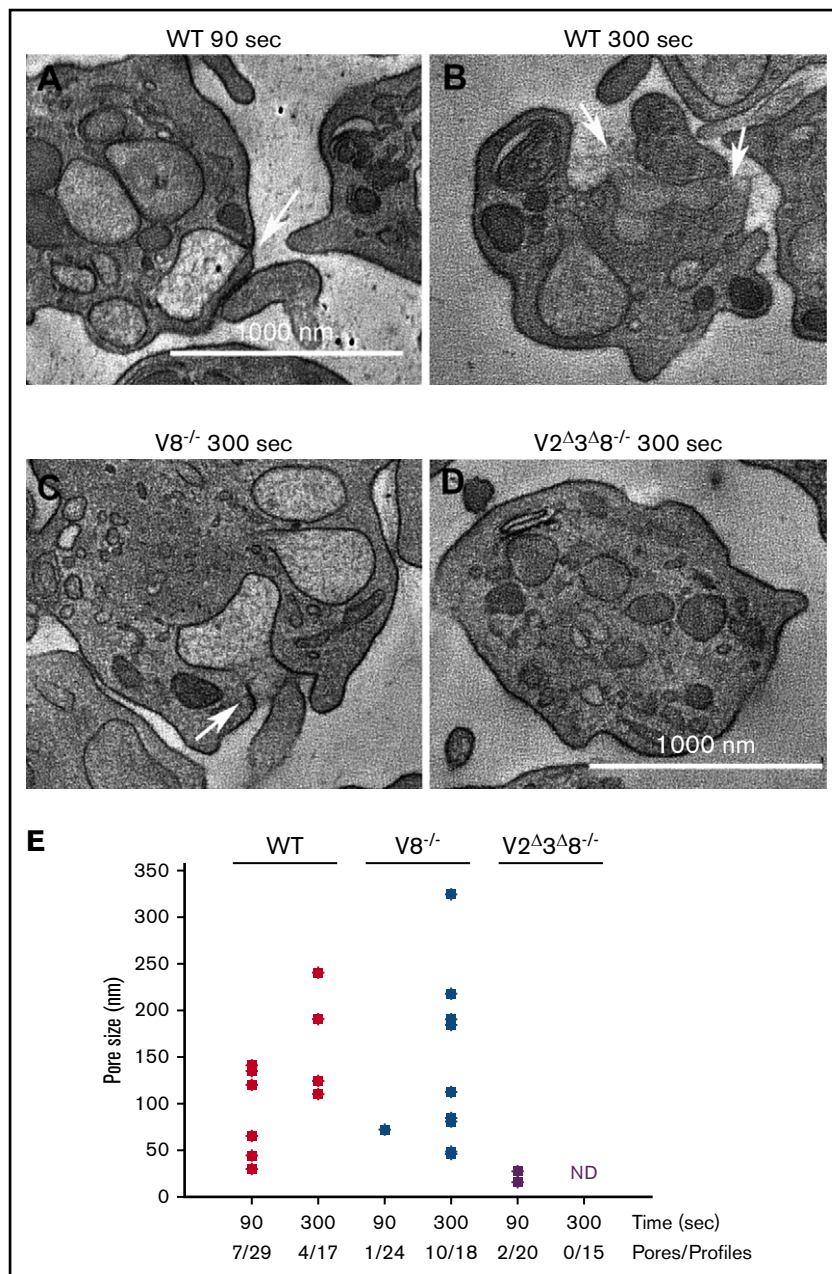
abundant in mouse platelets, whereas VAMP3, VAMP7, and VAMP8 are abundant in human platelets.<sup>12</sup> This was validated by mass spectrometry.<sup>11,13</sup> Using the semiquantitative western blotting data in Figure 1, we can estimate the remaining relative VAMP levels in our mice (Figure 7A) and, thus, correlate their levels to platelet secretion. Discordance of the curves in Figure 7B demonstrate that total VAMP levels, in aggregate, do not correlate with platelet secretion. This rebuts promiscuous VAMP functions, which might be predicted based on the heterogeneity of VAMP interactions observed in some systems.<sup>35-37</sup> Instead, our data confirm major contributions for VAMP7 and VAMP8.<sup>15,16</sup>

Although the loss of VAMP2/3 had minimal effect on its own, it exacerbated the effects of VAMP8 loss, perhaps due to effects on VAMP7. V2<sup>Δ3Δ8</sup><sup>-/-</sup> platelets had significant secretion defects at all agonist levels, and V2<sup>Δ3Δ8</sup><sup>-/-</sup> mice had profound bleeding and occlusion defects in vivo. Although this suggests some contributions from VAMP2 and VAMP3, the nature of their role is unclear. VAMP3 mediates integrin trafficking and endocytosis of granule cargo in platelets.<sup>27</sup> VAMP8 is also needed for endocytic trafficking in some cells.<sup>38</sup> Possibly, VAMP3 and VAMP8 function in overlapping pathways; thus, the double deletion of VAMP3 and VAMP8 affects the trafficking and/or localization of VAMP7, accounting for its reduction in V2<sup>Δ3Δ8</sup><sup>-/-</sup> platelets (Figure 1; supplemental Figure 1A). Consistently, we have been unable to create a global VAMP3/8 deletion strain due to embryonic lethality (S.W.W., unpublished observations). VAMP2's role is unclear because its levels in human platelets are low.<sup>11,12,14,16</sup> In practical terms, our V2<sup>Δ3Δ8</sup><sup>-/-</sup> mice are hypomorphic for VAMP7 (Figure 1), which, given the importance of VAMP7 and VAMP8, explains the severity of the secretion and hemostatic defects measured (Figure 6). Fortunately, we have created a strain with a greater reduction in secretion than either of the single VAMP deletions.

**Figure 5. The loss of VAMP8 delays fusion pore formation and dilation.**

Platelets from the indicated strains were isolated and fixed in resting or thrombin-stimulated states (0.1 U/mL thrombin for 90 or 300 seconds). EM tomographs were analyzed for fusion pore structures, and the diameters were measured in z-series. Representative EM images of WT platelets at 90 seconds (A) and at 300 seconds (B),  $V8^{-/-}$  platelets at 300 seconds (C), and  $V2^{\Delta 3\Delta 8^{-/-}}$  platelets at 90 seconds (D). The arrows indicate fusion pores. (E) The distribution of fusion pore size in platelets at early (90 seconds) and late (300 seconds) times poststimulation for the indicated strains is graphed as a scatter plot.

Mean pore sizes are as follows: (WT: 90 seconds, 82.4 nm; WT: 300 seconds, 166 nm;  $V8^{-/-}$ : 90 seconds, 72 nm;  $V8^{-/-}$ : 300 seconds, 160.8 nm;  $V2^{\Delta 3\Delta 8^{-/-}}$ : 90 seconds, 21 nm,  $V2^{\Delta 3\Delta 8^{-/-}}$ : 300 seconds, pores could not be detected). Pores detected per platelet profile are indicated. ND, not detected.

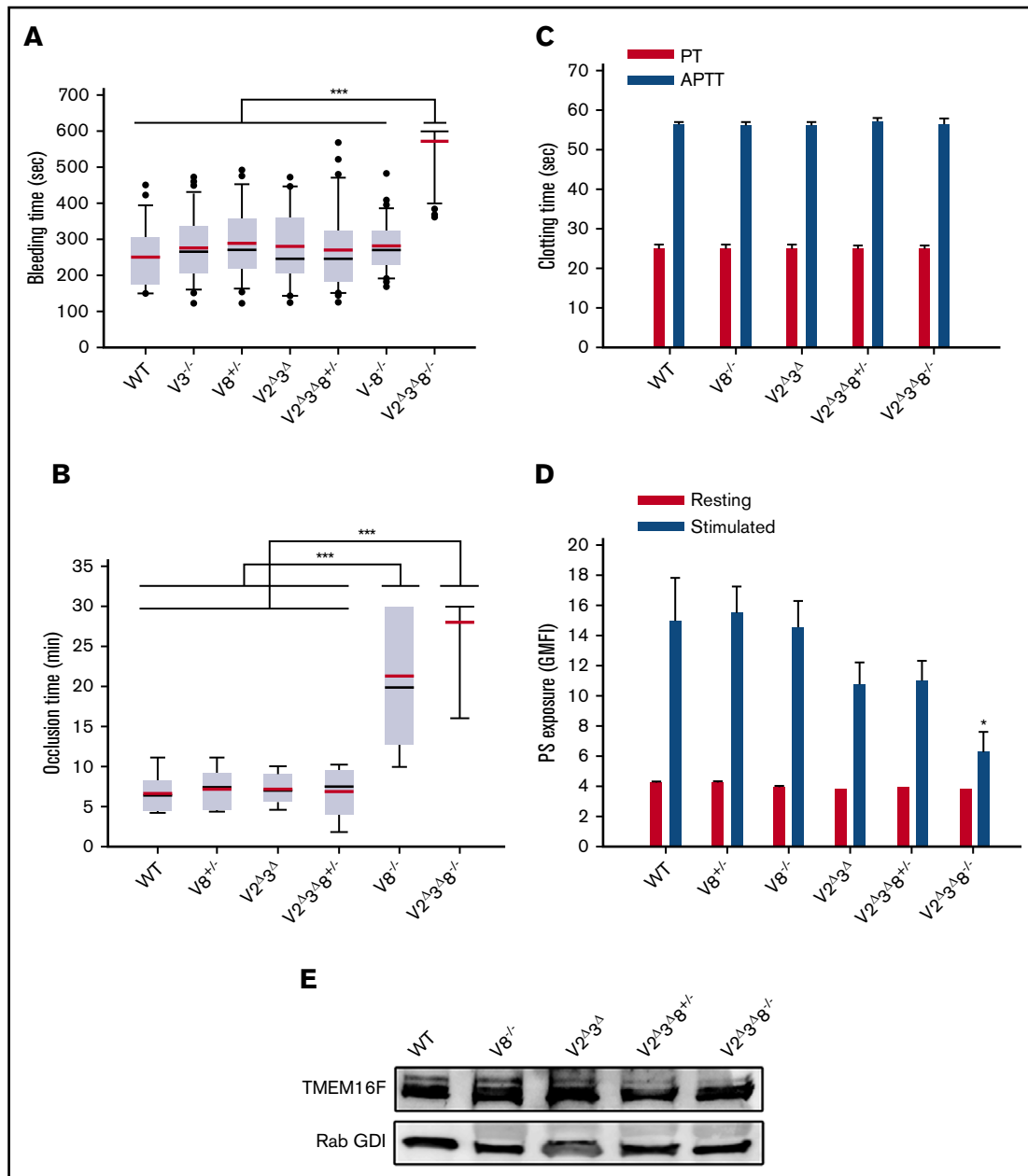


Future attempts to create  $VAMP7/8^{-/-}$  mice are underway to fully address this point.

To understand how VAMPs affect the kinetics of platelet granule secretion, we conducted detailed EM tomographic studies and examined fusion pore formation and subsequent dilation. Such pores are the most likely route of granule cargo release, and our data demonstrate that VAMPs are required for fusion pore formation and expansion (Figure 5). Few pores were detected in  $V2^{\Delta 3\Delta 8^{-/-}}$  platelets. The delayed pore formation in  $V8^{-/-}$  platelets confirms the importance of VAMP8 for initial membrane fusion and parallels the delayed secretion noted in Figure 2. The remaining VAMP7 is not sufficient for rapid fusion pore formation and/or expansion. These data imply that VAMP8 and VAMP7 contribute to

normal fusion pore formation/dilation. Consistently, reconstituted in vitro assays show that alterations in VAMP levels affect fusion pore dynamics and stability.<sup>39</sup> Such alterations clearly affect granule cargo release (Figure 2).

A previous analysis<sup>40</sup> suggests that VAMP8 is preferred for compound granule-granule fusion, whereas VAMP7 mediates PM-granule (primary) fusion. However, these discrete roles should not be considered mutually exclusive nor absolute. Loss of VAMP8 ( $V8^{-/-}$ ) delayed primary fusion of  $\alpha$  granules, and loss of VAMP8 and reduction of VAMP7 ( $V2^{\Delta 3\Delta 8^{-/-}}$ ) almost eliminated it (Figure 5). The partial release defects seen in platelets from the single-deletion mouse strains<sup>15,16</sup> (Figure 2) argue that VAMP7 and VAMP8 are used for primary fusion, which is required for



**Figure 6. Hemostasis and thrombus formation are impaired in  $V2^{\Delta 3\Delta}8^{-/-}$  animals.** (A) Tail bleeding times were measured after tail transection of WT ( $n = 28$ ),  $V3^{-/-}$  ( $n = 38$ ),  $V2^{\Delta 3\Delta}$  ( $n = 20$ ),  $V8^{+/-}$  ( $n = 26$ ),  $V8^{-/-}$  ( $n = 31$ ),  $V2^{\Delta 3\Delta}8^{+/-}$  ( $n = 41$ ), and  $V2^{\Delta 3\Delta}8^{-/-}$  ( $n = 34$ ) mice. (B) Thrombus formation in the carotid artery was induced by topical application of 5%  $FeCl_3$ , and blood flow was monitored in WT ( $n = 6$ ),  $V8^{+/-}$  ( $n = 6$ ),  $V2^{\Delta 3\Delta}$  ( $n = 7$ ),  $V2^{\Delta 3\Delta}8^{+/-}$  ( $n = 9$ ),  $V8^{-/-}$  ( $n = 7$ ), and  $V2^{\Delta 3\Delta}8^{-/-}$  ( $n = 7$ ) mice. For panels A and B, data are mean  $\pm$  standard error of the mean. The  $P$  values (log-rank test) are indicated and \*\*\* $P < .001$ . The red line indicates the mean, and the black line indicates the median. The box represents 25th-75th percentiles. Whiskers/error bars above and below the box indicate the 90th and 10th percentiles, respectively. Outliers are as shown in tail bleeding figure. (C) APTT and PT were measured in platelet-poor plasma prepared from WT ( $n = 7$ ),  $V8^{-/-}$  ( $n = 5$ ),  $V2^{\Delta 3\Delta}$  ( $n = 8$ ),  $V2^{\Delta 3\Delta}8^{+/-}$  ( $n = 3$ ), and  $V2^{\Delta 3\Delta}8^{-/-}$  ( $n = 5$ ) mice. (D) PS exposure on the surface of thrombin-stimulated platelets from WT,  $V8^{+/-}$ ,  $V8^{-/-}$ ,  $V2^{\Delta 3\Delta}$ ,  $V2^{\Delta 3\Delta}8^{+/-}$ , and  $V2^{\Delta 3\Delta}8^{-/-}$  mice was measured by fluorescein isothiocyanate-lactadherin binding and flow cytometry. The data are representative of 2 independent assays. \* $P < .05$ , Student  $t$  test. (E) A representative western blot showing the levels of TMEM16F in the indicated strains. Rab GDI was used as a loading control.

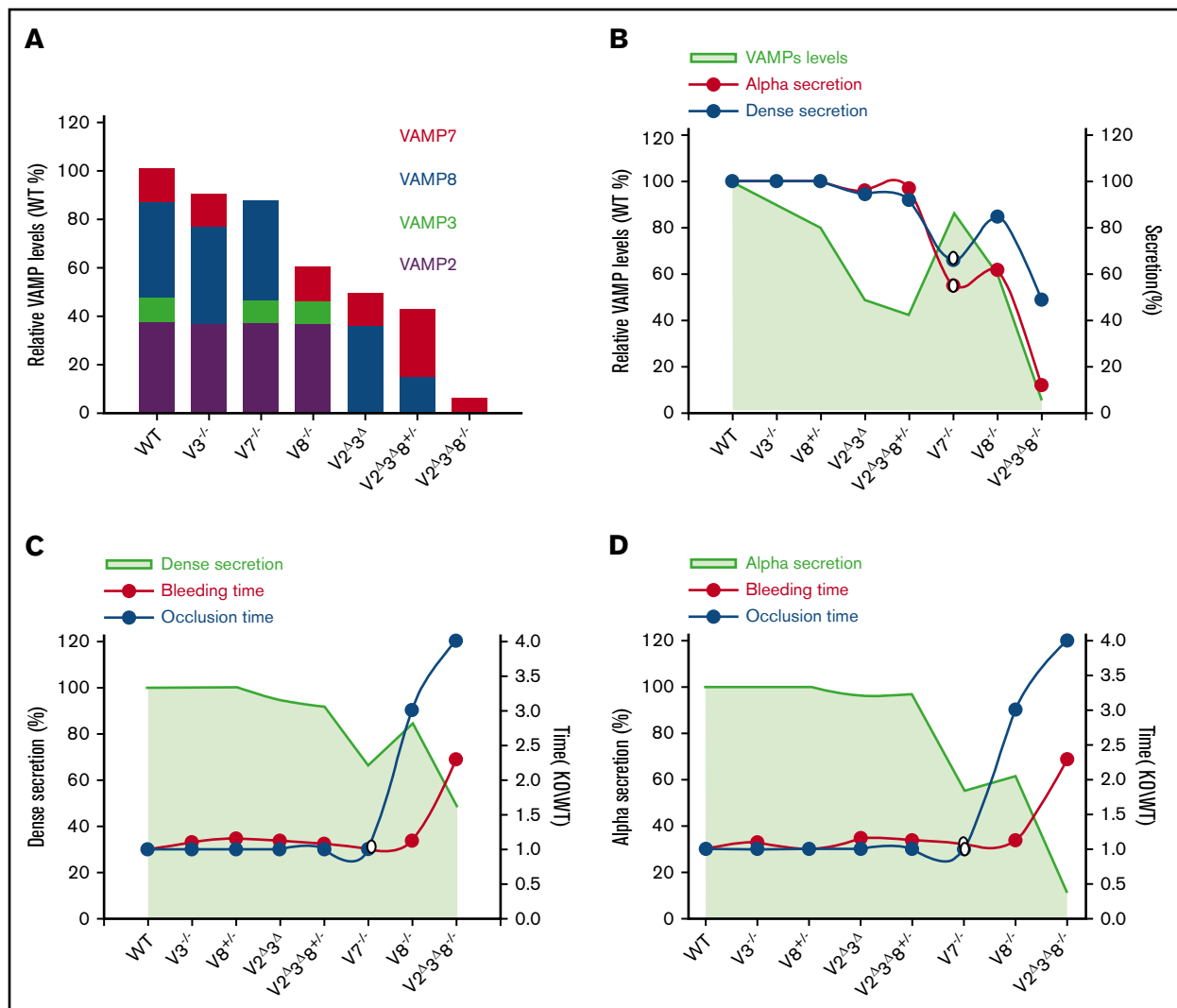
measurable cargo release. VAMP8's abundance relative to that of VAMP7 makes it a logical choice to mediate compound fusion, because more of those events are likely to be required; however, further analysis of  $V7^{-/-}$  platelets will be needed to directly address this point. It should be noted that using primary and compound fusion would differentially affect cargo release rates and extents and

perhaps account for the heterogeneity of  $\alpha$ -granule cargo release noted previously.<sup>41</sup>

### Platelet secretion and thrombosis

Thrombi appear stratified with highly activated platelets forming a central core and less activated platelets at the periphery (ie, the





**Figure 7. Correlating VAMP-mediated exocytosis to platelet function.** VAMP levels, secretion, and function are graphically correlated using our data (closed circles) and that from Koseoglu et al.<sup>15</sup> (open circles). (A) Calculated VAMP levels remaining in each of the strains tested, relative to WT, were based on the data in Figure 1B and from Graham et al.<sup>12</sup> (B) The VAMP levels in each strain are represented by green shading. The red line indicates  $\alpha$ -granule secretion, and the blue line indicates dense granule secretion. Secretion levels are from Figure 2 (0.05 U/mL thrombin for 2 minutes). Secretion from dense and  $\alpha$  granules of V7<sup>-/-</sup> platelets (open symbols) were estimated based on ATP release and P-selectin exposure, respectively.<sup>15</sup> All values are normalized to WT. Dense granule release (C) and  $\alpha$ -granule release (D) are as in panel B and are plotted as green shading. The red line indicates tail bleeding times, and the blue line indicates the occlusive thrombosis times in the FeCl<sub>3</sub>-injury model. Data are normalized to WT. These data were taken from other figures in this article in which variations were indicated; therefore, error indicators were omitted from these graphs to improve figure clarity.

shell).<sup>42-45</sup> We posit that the low-agonist doses in our assays resemble platelet secretion in the shell, whereas the high-agonist doses resemble what occurs in the core of a thrombus, proximal to the tissue damage (Figure 2A-C). At either low- or high-agonist doses, loss of VAMP2/3 had no significant effect on secretion and no effect on our in vivo assays of thrombosis and hemostasis. Loss of VAMP8 alone had more effect on  $\alpha$ -granule release than on dense granule release. V8<sup>-/-</sup> animals showed delayed thrombosis, with smaller thrombi in the laser-injury model.<sup>12</sup> Consistently, there was also an effect on FeCl<sub>3</sub>-induced occlusion, but there was no overt defect on tail bleeding (Figure 6A-B). Thus, that level of secretion is sufficient to stop bleeding but is insufficient for extensive thrombus growth. Single deletion of VAMP7 caused no

thrombosis or bleeding defects.<sup>15</sup> Deletion of VAMP2, VAMP3, and VAMP8 together caused a profound effect on release from dense and  $\alpha$  granules at all agonist levels. The defect was sufficient for a bleeding diathesis and an FeCl<sub>3</sub>-induced occlusion defect (Figure 6A-B). It should be noted that release from these platelets was not completely abolished; however, what little is released (presumably via VAMP7-mediated fusion) was insufficient for normal hemostasis or thrombosis.

Analysis of our data (Figure 7) demonstrates several aspects of how secretion may affect hemostasis and thrombosis. First, lysosomal release did not appear to be required. It was profoundly defective in several of our strains (Figure 2), but that, on its own, failed to correlate with any hemostatic defects. Second, defects in dense

granule release had a greater impact on bleeding than did defects in  $\alpha$ -granule release (Figure 7C-D). This is consistent with the phenotypes of patients (and mouse models) with dense and  $\alpha$  granulopathies. Patients with Hermansky-Pudlak syndrome or Chédiak-Higashi syndrome, in general, have more severe bleeding than do patients with gray platelet syndrome.<sup>46</sup> Third, bleeding was most profound when the platelets were less able to secrete at the highest agonist doses. Milder release defects with low-dose thrombin did not correlate with bleeding but did affect thrombus growth<sup>16</sup> (Figure 7C-D). Taken together, our data underline the importance of rapid and maximal release from dense granules and indicate a threshold for effective hemostasis. Our data demonstrate that thrombosis is more sensitive to manipulations of platelet secretion than is hemostasis.

### VAMP deficiency and coagulation

Given the bleeding phenotypes (Figure 6A-B) and the fact that coagulation factors are secreted proteins, it was important to measure coagulation in our mice. There were no differences in PT or APTT, indicating no defects in extrinsic or intrinsic pathways, respectively (Figure 6C). Exposure of PS on platelet surfaces drives the formation and localization of prothrombinase complexes.<sup>47</sup> Surprisingly, platelets from 3 of our mouse strains showed some defect in lactadherin binding.  $V2^{\Delta 3\Delta 8^{-/-}}$  platelets had the most robust impairment (~50% decrease), which could explain their hemostasis defects (Figure 6A-B). However, platelets from  $V2^{\Delta 3\Delta}$  and  $V2^{\Delta 3\Delta 8^{+/+}}$  mice had lower lactadherin binding (~30% decrease); however, neither had secretion (Figure 2; supplemental Figure 3) or hemostatic (Figures 6 and 7) defects. This surprising finding yields insights into another facet of how platelet secretion could affect hemostasis and suggests a threshold for PS exposure requirements.  $Nbeal2^{-/-}$  animals also have a PS exposure defect consistent with  $\alpha$  granules being important for PS exposure.<sup>48</sup> It is unclear whether the loss of dense granule release also contributes to this deficit, because the secondary agonists in dense granules (ie, ADP) could be needed to induce PS exposure.<sup>28</sup> Alternatively, given VAMPs' roles in intracellular trafficking (ie, proposed effect on VAMP7), sorting and/or targeting of the enzymes needed for PS exposure could have been influenced during platelet biogenesis. However, TMEM16F, which is a  $Ca^{2+}$ -dependent phospholipid scramblase important for PS exposure,<sup>32-34</sup> was unchanged in our strains (Figure 6E). Thus, further investigations are necessary to address these various questions.

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## Significance

This is the first study that defines the contributions of platelet granule secretion to hemostasis and thrombosis. By altering the types and amounts of VAMPs, we created a set of mouse strains with distinct platelet-secretion profiles that were used to define the threshold of secretion needed for occlusive thrombosis without bleeding. About 50% to 60% of dense and  $\alpha$ -granule secretion is needed for this crucial balance. This insight is invaluable in developing novel antithrombotic strategies and better managing current antithrombotic regimens. Furthermore, the animals described in this study will be vital tools to address how platelet secretion affects other physiological and pathophysiological processes.

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## Authorship

Contribution: S.J. and S.W.W. designed and performed the experiments, analyzed data, and wrote the manuscript; M.B., A.K., and J.Z. assisted with some of the platelet-secretion and spreading experiments and the biochemical analyses; and I.D.P. and B.S. performed the electron microscopy and image analysis.

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