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SNARE-dependent Membrane Fusion Initiates α-Granule Matrix Decondensation in Mouse Platelets

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Running Title: Fusion-Dependent Granule Matrix Decondensation

Supplemental Materials and Methods

Antibodies and reagents - Antibodies against VAMP2/ synaptobrevin (NBP1-19332), anti-VAMP3/cellubrevin (NB300-510), anti-VAMP7 (NBP2-41183) were from Novusbio, Anti syntaxin-11 was from Synaptic System GmbH, anti-VAMP8/endobrevin*,* anti SNAP23 and anti Rab-GDI were as described previously.[1,2]

Measurement of platelet secretion - The secretion assay was carried out as described previously.[3] Briefly, washed platelets were labeled with [^{3}H]-serotonin (0.4 Ci/ml) (Perkin-Elmer Cetus Life Sciences, Boston, MA) for 30 min at 37°C. After washing with HEPES/ Tyrode buffer (10 mM HEPES/NaOH, pH 6.5; 5.56 mM glucose; 137 mM NaCl; 12 mM $NaHCO_3$; 2.7 mM KCl; 0.36 mM KH_2PO_4; and 1 mM $MgCl_2$) in the presence of 0.2 U/ml apyrase, the platelets were resuspended and adjusted to 2.5 × 10^8/ml and supplemented with 0.7 mM $CaCl_2$. For time course experiments, 0.1 U/ml thrombin (Chrono-Log, Havertown, PA) was added for the indicated times, and reactions were stopped with 2-fold excess of hirudin. Supernatants and pellets were recovered after centrifugation at 13,000 *g* for 2 min, and the pellets were lysed with an equal volume of lysis buffer (PBS, pH 7.4, and 1% Triton X-100) for 1 h on ice. Both supernatants and pellets were assayed for 3 granule cargo markers: [^{3}H]-serotonin for dense granules by scintillation counting, platelet factor 4 (PF4) for α granules by ELISA, and β-hexosaminidase for lysosomes by colorimetric assay using *p*-nitrophenyl-*N*-acetyl-β- *D*-glucosaminide, as previously described.[4] Secretion was then calculated as (supernatant/ [supernatant + pellet]) and expressed as a percentage.

Western blotting analysis - Mouse platelets were prepared from the indicated variants as described above. After adjusting to 1 x 10^9/ml, an equal volume of 2X SDS was added and the samples were incubated at 95°C for 5 min. An appropriate volume of this stock was loaded to achieve 5 x 10^7 platelets per well onto a 1.5 mm 12.5% SDS-PAGE gel and the separated proteins were transferred to Immobilon- P polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Bedford, MA) for 1 h at 100 V. The PVDF membrane containing the transferred proteins was first incubated with the blocking buffer (5% nonfat milk in TBST (25 mM Tris, 150 mM NaCl, 0.1% Tween-20, and pH 7.4) for 1 h at room temperature and then in the indicated dilution of primary antibody (in Blocking Buffer) overnight at 4°C. The membrane was washed with TBS-T and incubated with alkaline phosphatase-conjugated secondary antibody for 1 h at RT. After washing the membrane with TBS-T, Vista-ECF substrate (Amersham Biosciences) was incubated with the membrane for 5 min at RT. The proteins were visualized with a Typhoon 9400 scanner.

EM - High pressure freezing (HPF) and freeze substitution (FS) dehydration - Following fixation platelet preparations were washed 3 x 5 min with PBS and resuspended in PBS containing 2% ultra-low gel agarose (Sigma, St. Louis, MO). Platelets were then frozen using a Leica EMPACT2 high pressure freezer with rapid transfer system at high pressure (2100 bar) and further processed by freeze substitution (FS).[5] Samples were transferred under liquid nitrogen to cryovials containing 2% osmium tetroxide/0.1% glutaraldehyde/1% H_2O in acetone. Samples were freeze-substituted in a Leica AFS2 freeze substitution and low temperature embedding system under the following schedule: -90°C for 22 h, warm 3°C/h to -60°C, -60°C for 8 h, warm 3°C/h to -30°C, -30°C for 8 h, warm 3°C/h to 0°C. Following freeze substitution, samples washed 3 x 10 min in acetone followed by tannic acid at 4°C (1% tannic acid with 1% H_2O in acetone) for 1 h. Samples were washed 3 x 10 min in acetone followed by 1 h osmium wash (1% osmium tetroxide/1% H_2O solution in acetone) at 4°C. Samples were washed 3 x 10 min in acetone and dehydrated over a series of ethanol gradations (25%, 50%, 75%, 100%) using an automatic resin infiltration protocol for the PELCO Bio-Wave Pro laboratory microwave system. Resin polymerization was done at 60°C for 48 h.[5]

Thin section (50 nm) transmission electron microscopy - Thin sections were cut at a thickness of 50 nm and post-stained with uranyl acetate and lead citrate (EMS). Samples were imaged using a FEI Tecnai TF20 intermediate-voltage electron microscope operated at 80 keV (FEI). Images were acquired with a FEI Eagle 4k digital camera controlled with FEI software.

Semi-thick section (300 nm) transmission electron tomography - Samples were sectioned at 300 nm and post-stained with uranyl acetate and lead citrate, followed by carbon coating. 15 nm gold (EMS) was applied to each side of the grids. Samples were imaged using a FEI Tecnai TF20, 200 keV field-emission gun, using SerialEM software (University of Colorado, Boulder, CO). [6]. Dual axis tilt series were collected ± 60° with a tilt increment of 1°. Images were taken at 7800x, XY pixel size 1.42 nm.

Tomogram reconstructions - Reconstructions were computed using weighted back projection (IMOD software, University of Colorado). STEM tomograms were binned by 2 and post-processed with an anisotropic diffusion filter.[7]

Surface rendering and quantitative analysis of platelet features – Platelet features in the 3D image volumes were segmented manually in the Amira Software 6.5.0 environment (FEI SAS, part of Thermo Fisher Scientific). Polygons were then generated to create surface models of the corresponding features. Fractional weights, automatically generated during segmentation, were used to produce smooth boundary interfaces. From known voxel dimensions, distances, areas, and volumes were quantified within the software. Results were then exported to Excel spreadsheets for further analysis, *e.g.,* the generation of means and standard errors. A full user guide for image processing and analysis using Amira software is available as a free download (no registration required) at https://www.fei.com/software/amira-user-guide/.

References

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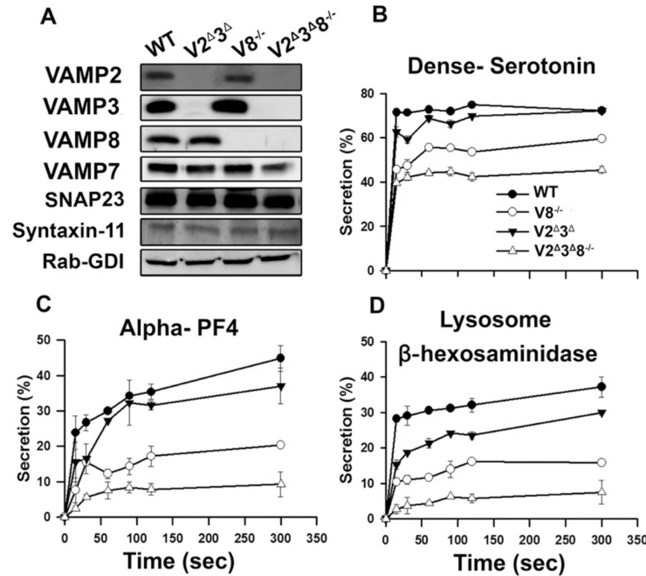
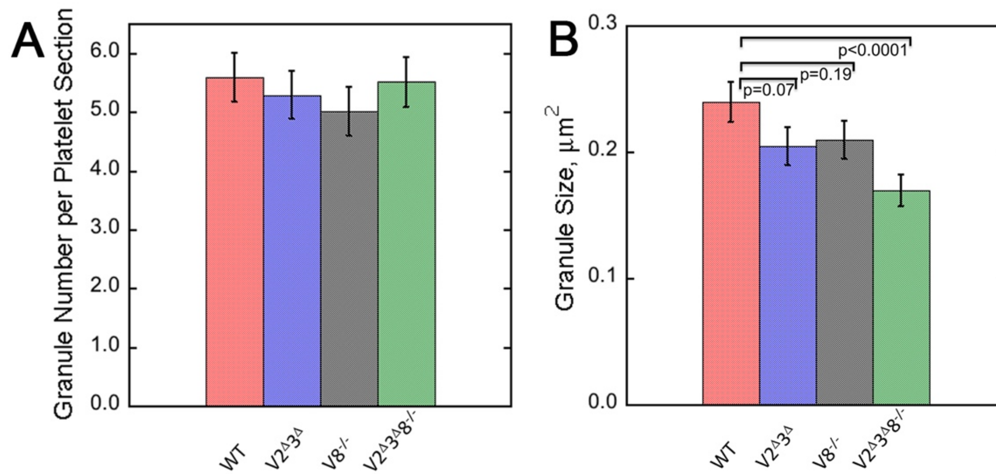
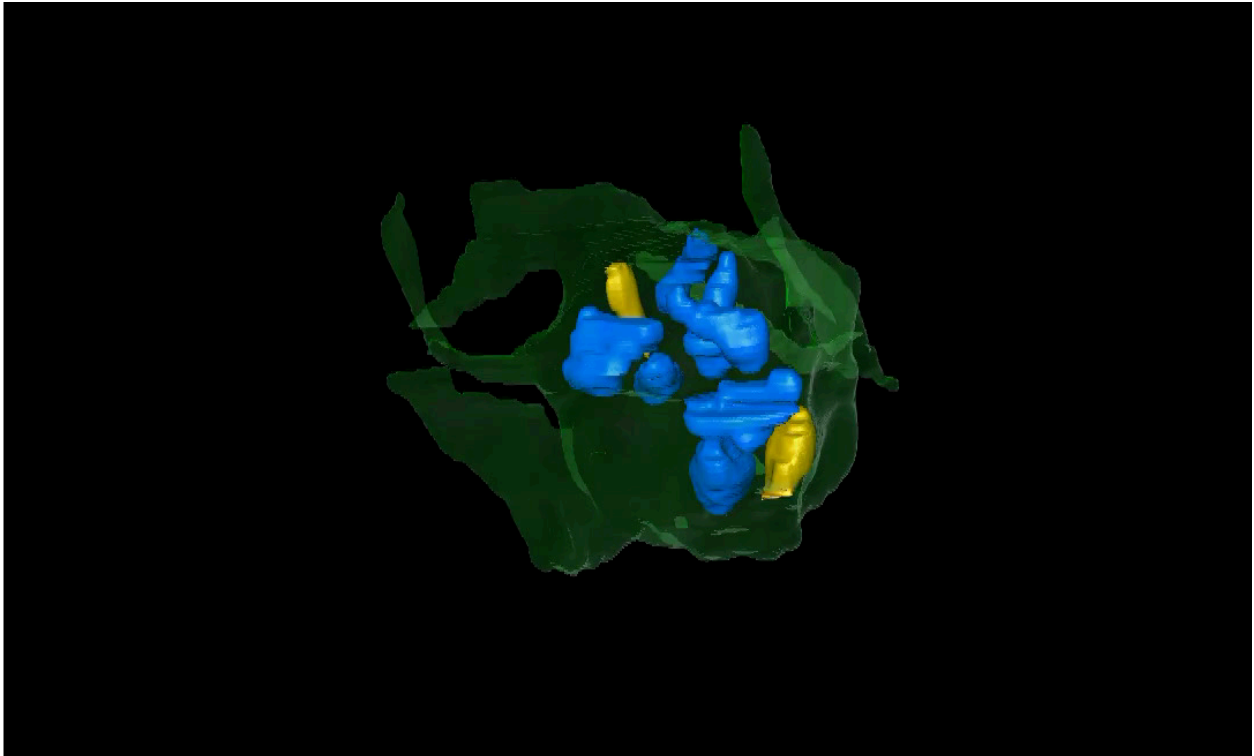


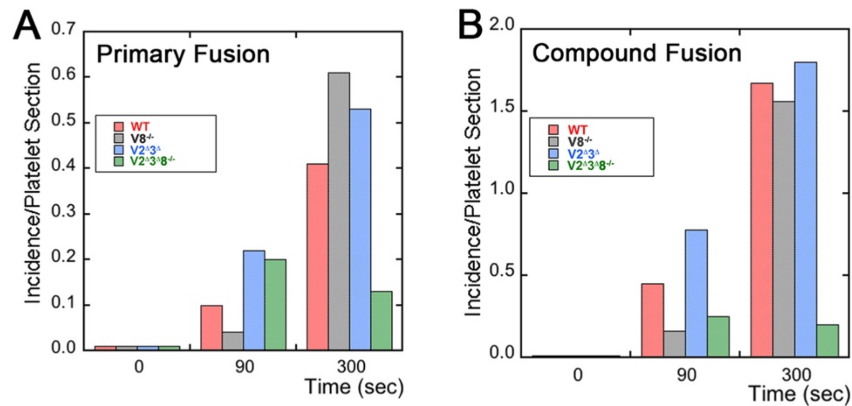
Supplemental Figure 1. VAMPs are important for platelet secretion. A) Shown is a collage of representative western blots comparing the expression of the manipulated VAMPs and other key elements of the secretory machinery among $V2^{\Delta}3^{\Delta}$, $V8^{-/-}$ and $V2^{\Delta}3^{\Delta}8^{-/-}$ platelet extracts (n>3). Rab-GDI was generally used as a loading control to compare expression levels. In the figure shown, GDI is the loading control for VAMP7. For additional information and examples, see Joshi *et al,* 2018 (main paper reference [33]). **B-D**) Time course of exocytosis from the three types of platelet granules. Platelets from the indicated strains were stimulated with 0.1 U/ml thrombin for increasing times. Releasate and platelet pellets were separated by centrifugation and three cargo markers were measured: platelet factor 4 (PF4) for α-granules (**B**), [^{3}H]-serotonin for dense granules (**C**), and β-hexosaminidase activity for lysosomes (**D**). Percent release was calculated using the equations ((releasate/ [pellet + releasate]) x 100). Data are the averages of three replicates with standard deviations indicated.



Supplemental Figure 2. VAMP deletion slightly affected α-granule size but not number per platelet. Thin section, electron microscopy images of immediately fixed platelets from the indicated mouse strains were analyzed for α-granule number per platelet profile (**A**) and cross-sectional granule area (**B**). In (**A**), 50 randomly chosen platelet profiles were scored for each mouse strain and the averages and standard error of the means are presented. In (**B**), 50 randomly chosen α-granule profile areas were measured for each mouse strain and averages and standard error of the means are presented as above. Student's t-test was used to establish statistical significance as indicated.



Supplemental Figure 3 Movie. Condensed and decondensed α-granule distribution remains predominantly central during shape-change in stimulated platelets. In this movie a rendered platelet that has been stimulated for 90 sec is shown. The decondensed granules are in tan/yellow and condensed granules are in blue. The PM is shown in green and rendered to be transparent. Movie can be downloaded as a separate supplemental file.



Supplemental Figure 4. Effect of SNARE variants on primary versus compound granule-granule fusion. Samples were fixed at various stimulation times and embedded for electron tomography of 300 nm thick sections. Platelet profiles were scored at 90 and 300 sec for primary and compound fusion. For 90 sec, profiles scored were: wild type (n = 29), $V8^{-/-}$ (n = 24), $V2^{\Delta}3^{\Delta}$ (n = 18), $V2^{\Delta}3^{\Delta}8^{-/-}$ (n = 20). For 300 sec, profiles scored were: wild type (n = 17), $V8^{-/-}$ (n = 18), $V2^{\Delta}3^{\Delta}$ (n = 15), $V2^{\Delta}3^{\Delta}8^{-/-}$ (n = 15).