

**BRIEF REPORT**

# Shear stress and platelet-induced tensile forces regulate ADAMTS13-localization within the platelet thrombus

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**Abstract**

**Background:** The multimeric glycoprotein von Willebrand factor (VWF) mediates platelet adhesion and aggregation at the site of vessel injury. The adhesive activity of VWF is influenced by its multimer length which is regulated by the metalloprotease ADAMTS13. The ability of ADAMTS13 to regulate platelet thrombus growth in a shear-dependent manner has been described, however, the mechanistic basis of this action has not been well characterized.

**Methods:** We developed an mCherry-tagged murine ADAMTS13 protein and utilized an ex vivo flow chamber system to visualize the localization of ADAMTS13 within the platelet thrombus under different conditions of shear. Using this system, we also assessed the influence of platelet-mediated tensile force on ADAMTS13 localization within the thrombus using gain-of-function GPIb binding and loss-of-function GPIIb/IIIa binding mutants in VWF/ADAMTS13 DKO mice.

**Results:** ADAMTS13 was visualized on the growing platelet thrombus under very high shear using ADAMTS13-mCherry. ADAMTS13-mCherry localized particularly at the top portion of the thrombus and reduced thrombus size as it grew to occlusion. At the pathological high shear of  $7500 \text{ s}^{-1}$ , platelet-mediated tensile force, involving GPIb but not GPIIb/IIIa receptors, influenced localization of ADAMTS13 to the thrombus under conditions of shear.

**Conclusions:** Tensile force applied on VWF produced by shear stress and platelet GPIb binding has a crucial role in ADAMTS13 activity at the site of thrombus formation. These results suggest that ADAMTS13 activity at the site of platelet thrombus formation is regulated by a shear stress and platelet-dependent feedback mechanism to prevent vessel occlusion and pathological thrombosis.

**KEYWORDS**

ADAMTS13, GPIb, platelet, shear stress, von Willebrand factor

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## Essentials

- The regulation of platelet thrombus growth by ADAMTS13 is not well characterized.
- Platelet thrombus formation in a flow chamber was assessed with mCherry-tagged ADAMTS13.
- ADAMTS13 colocalized with VWF and platelets to the middle and top portions of the thrombus.
- Platelet tethering to VWF by GPIb enhanced ADAMTS13 localization to the thrombus.

## 1 | INTRODUCTION

von Willebrand factor (VWF) is a multimeric glycoprotein that mediates platelet adhesion and aggregation at the site of blood vessel injury under. High molecular weight VWF multimers (HMWM) exhibit greater platelet-binding activities and have been associated with an increased risk for thrombosis. Shear stress applied to VWF causes a conformational change in VWF structure from its globular to an elongated form, exposing the platelet GPIb binding site in the VWF A1 domain<sup>1</sup> and resulting in platelet tethering to VWF. GPIb binding activates the GPIIb/IIIa receptor complex that mediates firm attachment of platelets through interactions with the Arg-Gly-Asp sequence in the VWF C4 domain, resulting in platelet thrombus formation.<sup>2,3</sup>

von Willebrand factor activity is regulated by the protease A disintegrin-like and metalloprotease with thrombospondin type 1 repeats-13 (ADAMTS13),<sup>4</sup> that cleaves HMWM to reduce the hemostatic efficacy of VWF. Exposure of VWF to shear stress or tensile stretching forces applied by captured platelets causes VWF to undergo a conformational change from a globular to an elongated form, thereby exposing cryptic ADAMTS13-binding exosites on VWF and facilitating ADAMTS13-mediated proteolysis.<sup>5,6</sup> Using an in vitro flow chamber model, we and others have previously demonstrated that ADAMTS13 can regulate platelet thrombus formation in a VWF-dependent manner under conditions of pathological shear.<sup>6-8</sup> However, the influence of platelet-applied tensile forces on ADAMTS13 activity within the platelet thrombus is unknown. Additionally, although we observed products of VWF cleavage by ADAMTS13 within the platelet thrombus, the localization of ADAMTS13 within the platelet thrombus has not been directly observed. In these studies, we generated a recombinant murine ADAMTS13 protein fused to the mCherry fluorophore to directly visualize the localization of ADAMTS13 within the platelet thrombus. We then characterized the contribution of tensile forces mediated by platelet-VWF tethering involving GPIb and GPIIb/IIIa receptors to ADAMTS13 localization and activity within a platelet thrombus using a flow chamber model.

## 2 | METHODS

### 2.1 | Plasmid construction and recombinant protein production

The mCherry cDNA (Clontech, Mountainview, CA) was cloned into the full-length murine ADAMTS13 cDNA pcDNA3.1 following a 12-amino acid linker (GGGSGGGSGGG). The gain-of-function platelet GPIb binding mutation (V1316M)<sup>9</sup> and loss-of-function platelet GPIIb/IIIa

binding mutation (RGG)<sup>2</sup> were introduced by mutagenesis into the full-length murine VWF cDNA in pCIneo<sup>10</sup> using the Quikchange Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA). Recombinant mADAMTS13-mCherry and murine VWF were produced in HEK 293 T cells by calcium phosphate transfection.<sup>11,12</sup> VWF:Ag was measured by enzyme-linked immunosorbent assay (ELISA) using rabbit anti-human VWF antibodies A0082 and P0226 (Dako, Agilent, Santa Clara, CA, USA). ADAMTS13 activity was measured by cleavage of the VWF 73mer.<sup>13</sup>

### 2.2 | Flow chamber experiments and real time visualization of rADAMTS13 cherry

A flow chamber slide was coated with collagen I/III (MP Biomedicals, Solon, OH, USA) at 300 µg/mL in sodium carbonate/bicarbonate buffer at room temperature overnight, washed with phosphate-buffered saline (PBS), blocked with 5% bovine serum albumin for 1 hour and washed again prior to the experiment. Whole blood was obtained from ADAMTS13 knockout (KO) or VWF/ADAMTS13 double knockout (DKO) mice by cardiac puncture and anticoagulated with 200 µmol/L argatroban (Enzo Life Science, Farmingdale, NY). Platelets were labeled with 3,3',9'-dihexyloxycarbocyanine iodide (DiOC<sub>6</sub>, 1 µmol/L; Invitrogen). Two U/mL of mADAMTS13-mCherry diluted in PBS or mock transfected media diluted in PBS were added to the whole blood and were perfused into the prepared flow chamber at the indicated shear rates for 9 minutes controlled by a syringe pump (New Era Pump Systems Inc., Farmingdale, NY, USA; NE-1600). Platelet accumulation and ADAMTS13-cherry association was imaged using a Quorum WaveFX-X1 spinning disk confocal system (Quorum Technologies, Inc., Guelph, ON, Canada) with a Hamamatsu Orca high resolution camera (Hamamatsu, Japan). In some experiments, whole blood was obtained from VWF/ADAMTS13 DKO mice and 2 U/mL recombinant mADAMTS13 and 2 U/mL mVWF (WT, V1316M, RGG) were added and used for the experiments.

For the direct visualization of ADAMTS13-cherry association with accumulating platelets, rabbit anti-mCherry antibody (BioVision, Milpitas, CA, USA) and an anti-rabbit IgG-Alexa568 (Molecular Probes, Eugene, OR, USA) were incubated for 30 minutes and mixed with mADAMTS13-mCherry for 15 minutes and added to anticoagulated blood prior to the experiments. All mouse experiments were reviewed and approved by the Queen's University Animal Care Committee.

### 2.3 | Immunostaining of thrombi post-flow chamber experiments

Thrombi were fixed with Cytoperm/Cytofix (Becton Dickinson, Franklin Lakes, NJ, USA) and immunofluorescent staining was performed to further analyze the distribution of platelets, VWF and ADAMTS13. The

thrombus was permeabilized with PBS/1% Triton X-100 for 10 minutes at room temperature, blocked with serum free protein block (Dako) for 20 minutes and incubated with a rabbit anti-mCherry antibody overnight at 4°C, followed by goat anti-rabbit IgG-Alexa568 for 8 hours at room temperature. Stained thrombi were fixed with Cytoperm/Cytofix for 20 minutes at room temperature, followed by incubation with phalloidin-Alexa488 (Molecular Probes) and anti-VWF antibody (Dako) coupled with Alexa647 (ThermoFisher Scientific, Waltham, MA) overnight at 4°C. Thrombi were mounted in Dako fluorescence mounting medium (Dako) prior to imaging. Both isotype IgG control antibodies for ADAMTS13 and VWF staining, and staining for VWF and ADAMTS13 with thrombi formed from blood collected from VWF/ADAMTS13 DKO mice were used to confirm staining experiments.

## 2.4 | Imaging analysis

Image analysis was performed on fixed and permeabilized immunostained thrombi obtained at the end of the flow chamber experiments. The surface coverage and thrombus volume were measured with

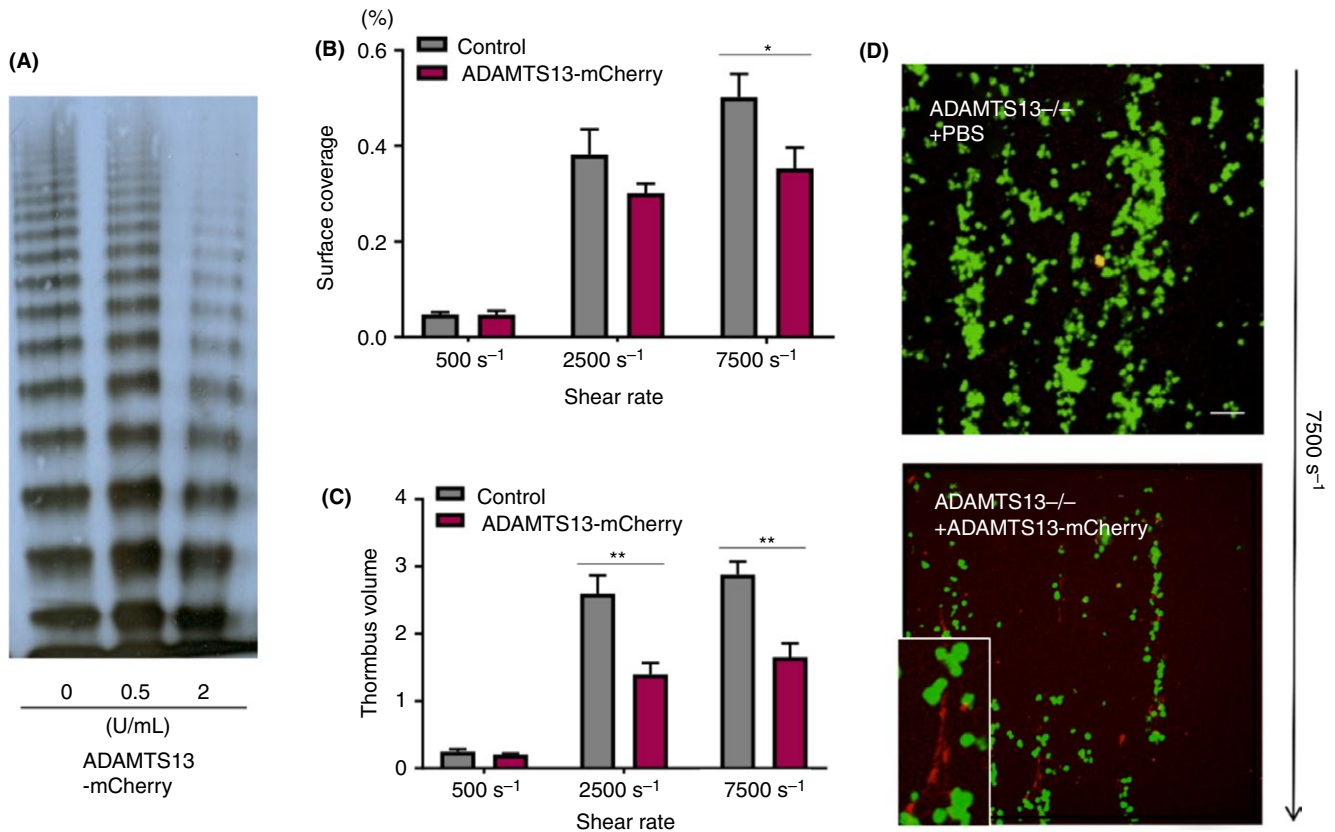
ImagePro 6.0.<sup>11</sup> The intensity of platelet, VWF, and ADAMTS13 accumulation was measured by the Quorum WaveFX-X1 spinning disk confocal system. To analyze the distribution of VWF and ADAMTS13, the thrombus height was divided into three layers (top, middle, and bottom) and intensity of accumulation was measured respectively. The overlap coefficient for ADAMTS13 and VWF were measured by Coloc-2 in Fiji Is Just ImageJ (National Institute of Health, Bethesda, MD, USA).

## 2.5 | Data presentation and statistics

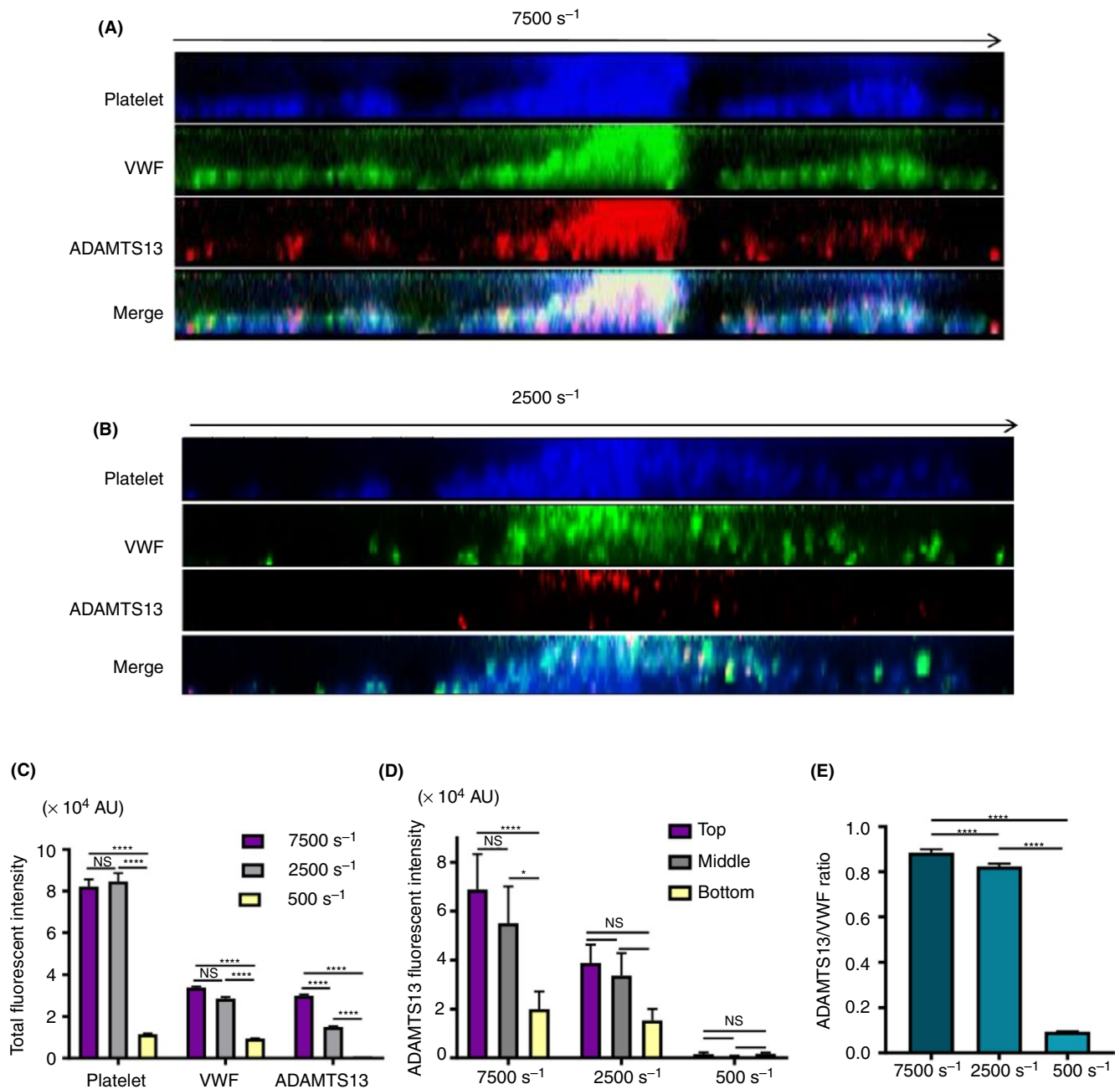
All data and statistical analysis was performed using Graphpad Prism 6.0 (Graphpad software). The results are shown as means  $\pm$  SEM. Statistical significance was assessed using Student's *t* tests or two-way Analysis of variance.

## 3 | RESULTS AND DISCUSSION

The activity of ADAMTS13-mCherry was first measured by a functional ELISA using the VWF73mer substrate. This proved



**FIGURE 1** Murine A Disintegrin And Metalloproteinase with Thrombospondin type 1 motif, 13 (ADAMTS13)-mCherry reduces murine von Willebrand factor (VWF) multimer size and decreases platelet thrombus formation in a flow chamber model. (A) ADAMTS13-mCherry reduces the high molecular weight content of VWF multimers. Recombinant mouse VWF was treated with ADAMTS13-mCherry (0, 0.5, 2 U/mL) in the presence of 0.75 mol/L urea and the reactions were terminated by the addition of Ethylenediaminetetraacetic acid (EDTA, 10 mmol/L). The multimer profiles of these treated proteins were confirmed on the 1% sodium dodecyl sulfate agarose gel electrophoresis. ADAMTS13-mCherry was added to whole blood from ADAMTS13 KO mice and perfused across the collagen-coated flow chamber. Platelets were labeled with 3,3'-dihexyloxycarbocyanine iodide (DiOC<sub>6</sub>, green). Influence of ADAMTS13-mCherry on (B) surface area coverage and (C) platelet thrombus volume after 9 min under 500 s<sup>-1</sup>, 2500 s<sup>-1</sup>, or 7500 s<sup>-1</sup> shear conditions. (D) Visualization of ADAMTS13-mCherry at the sites of platelet accumulation. Platelets were labeled with DiOC<sub>6</sub> (green). The signal from ADAMTS13-mCherry was enhanced with an anti-cherry antibody (red). Scale bar = 25  $\mu$ m. \**P* < 0.05, \*\**P* < 0.01



**FIGURE 2** Association between von Willebrand factor (VWF), A Disintegrin And Metalloproteinase with Thrombospondin type 1 motif, 13 (ADAMTS13), and platelets under high and very high shear stress. 2 U/mL of ADAMTS13-mCherry was added to the whole blood obtained from ADAMTS13 KO mice and perfused into the collagen coated flow chamber at 7500 s<sup>-1</sup>. After 9 min of perfusion, the thrombus was fixed and immunostaining was performed. Platelet, VWF and ADAMTS13 are shown in blue, green and red pseudo-color, respectively. Merged images are also shown and white pseudo-color indicates the three color co-localization. Representative images after 9 min of perfusion from seven separate experiments are shown. (A) Longitudinal view of a representative thrombus formed under 7500 s<sup>-1</sup>. (B) Longitudinal view of a representative thrombus formed under 2500 s<sup>-1</sup>. (C) Total fluorescent intensity of platelet, VWF, and ADAMTS13 under 7500 s<sup>-1</sup>, 2500 s<sup>-1</sup>, and 500 s<sup>-1</sup> are shown. (D) The difference of ADAMTS13 fluorescent intensity in the top, middle, and bottom portion of thrombus under 7500 s<sup>-1</sup>, 2500 s<sup>-1</sup>, and 500 s<sup>-1</sup> are shown. Each data point represents the means of 21 areas examined (three areas randomly selected in seven independent perfusions). Data was expressed in arbitrary units (AU). (E) Ratio of ADAMTS13 to VWF staining in incorporated thrombus. \**P* < 0.05, \*\*\*\**P* < 0.0001

to be comparable to wild type ADAMTS13 (data not shown) and the concentration was normalized to normal pooled plasma (defined as 1 U/mL).<sup>12</sup> The ability of ADAMTS13-mCherry to cleave

urea-treated murine rVWF was confirmed by multimeric analysis (Figure 1A). We next evaluated the ability of ADAMTS13-mCherry to cleave VWF in a flow chamber model. In this model, the rate of

shear within the flow chamber is initially applied at a controlled rate by the syringe pump. As the platelet thrombus grows in size, it generates a partial occlusion within the chamber that increases the localized shear stress overlying the platelet thrombus, that in turn promotes the transformation of VWF from its globular to its elongated form,<sup>14</sup> thereby exposing the cryptic ADAMTS13 cleavage site on thrombus-anchored VWF. The ability of ADAMTS13-mCherry to reduce the platelet thrombus surface area coverage and thrombus volume in a shear-dependent manner was confirmed after a 9-minute perfusion (Figure 1B,C). We also directly visualized the association of ADAMTS13-mCherry with platelet-VWF structures using a fluorescently tagged anti-mCherry antibody under conditions of very high shear ( $7500\text{ s}^{-1}$ ) (Figure 1D and Figure S1). The localization of ADAMTS13 was partially associated with platelets, and resembled VWF string-like structures. ADAMTS13 staining was not detected at  $2500\text{ s}^{-1}$  and  $500\text{ s}^{-1}$  (data not shown).

Although previous studies have reported the elevated presence of proteolyzed VWF within the platelet thrombus in the presence of ADAMTS13,<sup>6</sup> the localization of ADAMTS13 accumulation within the platelet thrombus has not been directly imaged. We performed multicolored immunostaining of thrombi generated under conditions of moderate ( $500\text{ s}^{-1}$ ), high ( $2500\text{ s}^{-1}$ ) and very high ( $7500\text{ s}^{-1}$ ) shear to visualize the association between VWF, platelets, and ADAMTS13 within these thrombi (Figure 2).

The staining intensity of ADAMTS13 was measured throughout the thrombus. The amount of ADAMTS13 within the thrombus increased in a shear dependent manner, with very high shear conditions ( $7500\text{ s}^{-1}$ ) associated with the greatest ADAMTS13 intensity (Figure 2C). Moreover, this increase in ADAMTS13 localization was associated with the top and middle layers of the thrombus (Figure 2D). ADAMTS13 binding under conventional physiological high shear ( $2500\text{ s}^{-1}$ ) was observed predominantly on the top layer of thrombus where shear stress increases as the thrombus grows and the lumen of the chamber becomes increasingly stenotic with the actual shear rate being much higher than  $2500\text{ s}^{-1}$ . This pattern of ADAMTS13 accumulation did not achieve statistical significance by image analysis. In contrast, at the low shear condition ( $500\text{ s}^{-1}$ ), ADAMTS13 staining was very low. No significant differences in VWF or platelet staining was observed between the top, middle, and bottom portions of the thrombi under these low shear conditions (data not shown). We observed that the ratio of ADAMTS13/VWF staining was essentially the same for thrombi formed under  $7500\text{ s}^{-1}$  and  $2500\text{ s}^{-1}$  shear rates, but lower at  $500\text{ s}^{-1}$ , suggesting that incorporation of ADAMTS13 into the thrombus is not just dependent on the amount of VWF:Ag present, but also on the amount of localized shear (Figure 2E).

Platelet-VWF complexes are the preferred substrate for ADAMTS13 cleavage in fluid phase, where the tensile forces applied by platelets under shear transforms VWF from its globular to an elongated conformation.<sup>5</sup> We observed a strong association between ADAMTS13, VWF, and platelets suggesting involvement

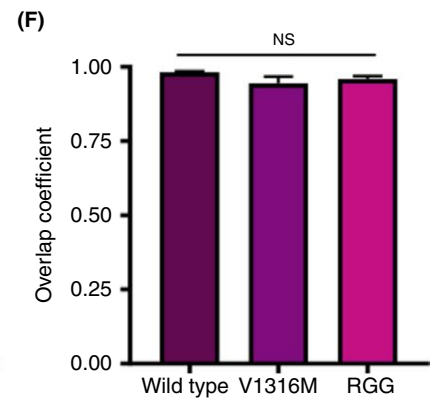
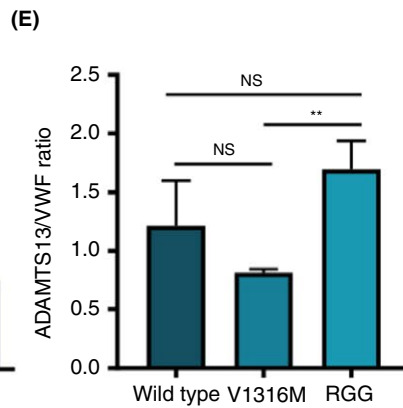
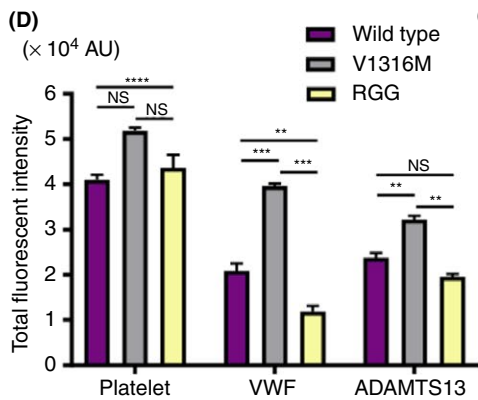
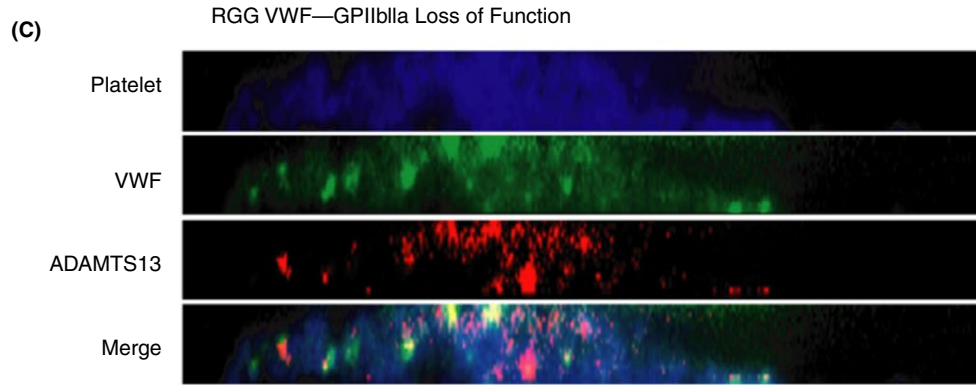
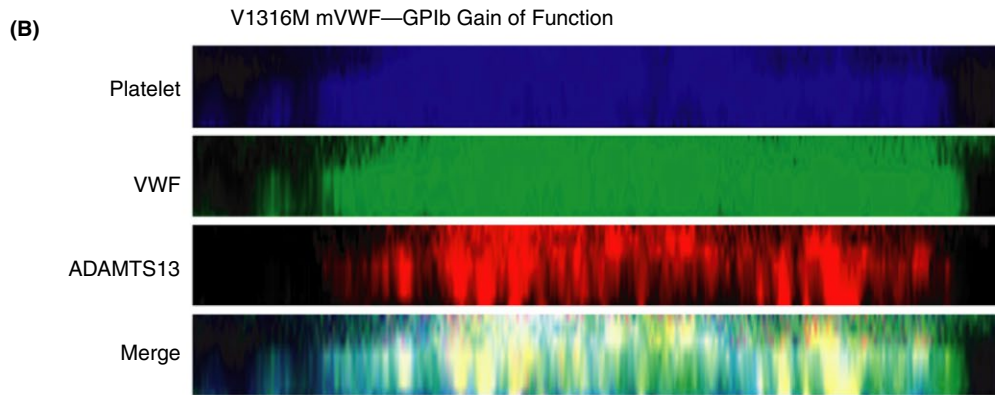
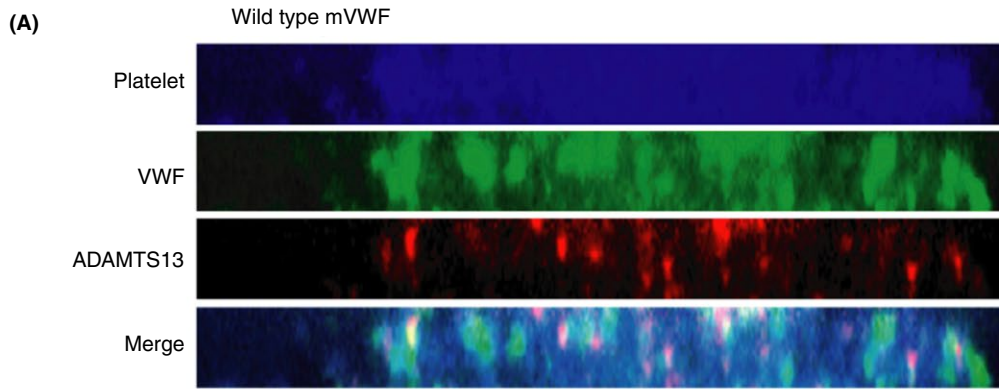
of platelet GPIb and GPIIb/IIIa receptors in facilitating these interactions. We then modified the platelet-binding ability of our recombinant murine VWF using the gain-of-function GPIb binding mutation (V1316M) seen in type 2B von Willebrand disease (VWD), and the loss-of-function GPIIb/IIIa binding mutation (RGG). Whole blood was obtained from VWF/ADAMTS13 DKO mice, supplemented with  $2\text{ U/mL}$  of wild-type, V1316M or RGG VWF in the presence of  $2\text{ U/mL}$  of ADAMTS13-mCherry, and blood was perfused in a flow chamber at a shear rate of  $7500\text{ s}^{-1}$ .

Immunofluorescent staining of wild-type recombinant VWF was mostly associated with the middle and top layers of the platelet thrombus (Figure 3A). The V1316M VWF GPIb binding gain-of-function mutation demonstrated dramatically enhanced ADAMTS13 and VWF deposition throughout, indicating that GPIb binding mediates a significant tensile force on the VWF molecule and facilitates ADAMTS13 binding to VWF. Interestingly, type 2B VWD patients demonstrate loss of HMWMs and a bleeding phenotype related in part to increased ADAMTS13-mediated VWF proteolysis secondary to enhanced GPIb tensile forces applied to the VWF molecule.<sup>15,16</sup>

In contrast, the VWF RGG variant, with loss of GPIIb/IIIa binding, had minimal influence on ADAMTS13 localization (Figure 3C). There are several potential reasons for this observation. First, the GPIIb/IIIa-binding site on VWF is located in the C-terminal C4 domain distal to the ADAMTS13-binding shear-sensitive A2 domain, and thus may have a minimal impact on A2 domain unfolding. Second, the interaction between VWF and GPIIb/IIIa occurs after the initial process of VWF binding to GPIb, an event that decelerates or arrests circulating platelets so that the GPIIb/IIIa-VWF interaction may not exert as much tensile force on the VWF molecule.

The ratio of ADAMTS13/VWF staining for the V1316M VWF GPIb binding gain-of-function mutation is much lower when compared with the RGG variant, confirming that the amount of VWF incorporated into the thrombus is not the only factor that modifies localization of ADAMTS13. In this case, the decreased ADAMTS13/VWF ratio associated with the V1316M variant may be limited to the amount of ADAMTS13 available in this system for incorporation into the thrombus (Figure 3E). Importantly, the overlap coefficient between ADAMTS13 and VWF staining for all conditions is 1.0, suggesting that all ADAMTS13 incorporation in these thrombi is VWF-dependent (Figure 3F).

These results suggest that ADAMTS13 function within the platelet thrombus is localized predominantly to sites of pathological shear stress. Exposure of VWF to pathological shear stress results in unfolding of the molecule, and enhances platelet GPIb-mediated platelet adhesion, that in turn applies an additional tensile force on the VWF molecule. These shear and tensile forces unfold the ADAMTS13-cleavage site on VWF, resulting in the reduction of HMW VWF multimers and limits further platelet thrombus growth. Under conditions of high shear, ADAMTS13 activity is exquisitely regulated so as to maintain effective hemostatic thrombus formation but preventing pathologic occlusive thrombosis.



**FIGURE 3** Influence of Glycoprotein Ib (GPIb), and Glycoprotein IIb/IIIa (GPIIb/IIIa), on the association between von Willebrand factor (VWF), A Disintegrin And Metalloproteinase with Thrombospondin type 1 motif, 13 (ADAMTS13), and platelets. (A) Wild type murine recombinant (r) VWF, (B) murine V1316M rVWF, (C) murine RGG rVWF were added to whole blood from VWF/ADAMTS13 double knockout (DKO) mice and perfused into the flow chamber, respectively. Longitudinal views of a representative thrombus after 9 min of perfusion from four separate experiments are shown. Platelet, VWF, ADAMTS13, and merged images are shown. (D) The total fluorescent intensity of platelets, VWF and ADAMTS13 are shown. Data was expressed in arbitrary units (AU). Each data point represents the means of 8 areas examined (two to three areas randomly selected in three to four independent perfusions). (E) Ratio of ADAMTS13 to VWF staining in incorporated thrombus. (F) Overlap coefficient between ADAMTS13 and VWF as determined by Coloc 2 analysis. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$

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## RELATIONSHIP DISCLOSURE

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## AUTHOR CONTRIBUTIONS

YS designed and performed experiments, analyzed, and interpreted the data and wrote the manuscript. LLS analyzed and interpreted the data and wrote the manuscript. JM, CB, KN, and OD performed experiments. JR and CH designed experiments and interpreted the data. DL designed experiments, interpreted the data and edited the manuscript.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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