

Inhibition of the Plasma SCUBE1, a Novel Platelet Adhesive Protein, Protects Mice Against Thrombosis

Meng-Ying Wu, Yuh-Charn Lin, Wei-Ju Liao, Cheng-Fen Tu, Ming-Huei Chen, Steve R. Roffler, Ruey-Bing Yang

Objective—Signal peptide-CUB-EGF domain-containing protein 1 (SCUBE1), a secreted and surface-exposed glycoprotein on activated platelets, promotes platelet–platelet interaction and supports platelet–matrix adhesion. Its plasma level is a biomarker of platelet activation in acute thrombotic diseases. However, the exact roles of plasma SCUBE1 in vivo remain undefined.

Approach and Results—We generated new mutant (Δ) mice lacking the soluble but retaining the membrane-bound form of SCUBE1. Plasma SCUBE1-depleted Δ/Δ mice showed normal hematologic and coagulant features and expression of major platelet receptors, but Δ/Δ platelet-rich plasma showed impaired platelet aggregation in response to ADP and collagen treatment. The addition of purified recombinant SCUBE1 protein restored the aggregation of platelets in Δ/Δ platelet-rich plasma and further enhanced platelet aggregation in $+/+$ platelet-rich plasma. Plasma deficiency of SCUBE1 diminished arterial thrombosis in mice and protected against lethal thromboembolism induced by collagen-epinephrine treatment. Last, antibodies directed against the epidermal growth factor–like repeats of SCUBE1, which are involved in trans-homophilic protein–protein interactions, protected mice against fatal thromboembolism without causing bleeding in vivo.

Conclusions—We conclude that plasma SCUBE1 participates in platelet aggregation by bridging adjacent activated platelets in thrombosis. Blockade of soluble SCUBE1 might represent a novel antithrombotic strategy. (*Arterioscler Thromb Vasc Biol.* 2014;34:00-00.)

Key Words: blood platelets ■ thrombosis

SCUBE1 (signal peptide-CUB [complement protein C1r/SC1s, Uegf, and Bmp1]-EGF [epidermal growth factor] domain-containing protein 1) is the founding member of a secreted and membrane-associated SCUBE protein family.^{1,2} To date, 3 different members have been identified and are evolutionarily conserved in zebrafish, mice, and humans.^{1–12} These genes code for proteins of ≈ 1000 amino acids forming ≥ 5 domains: an NH₂-terminal signal peptide sequence, 9 tandem repeats of EGF-like motifs, a large N-glycosylated spacer region followed by 3 cysteine-rich (CR) repeats, and one CUB domain at the COOH terminus.^{2,5,10} When over-expressed in human embryonic kidney cells, recombinant SCUBE1 proteins form oligomers, which are either secreted into the conditioned medium or remain tethered to the cell surface where it behaves like a peripheral membrane (not membrane-spanning) protein.² Thus, SCUBE1 may have distinct functions depending on its distribution as a soluble or membrane-associated protein.

We and others have shown that *Scube1* is expressed during mouse development and in adult endothelial cells.^{1,2} However,

SCUBE1 protein is also expressed in platelets.¹³ SCUBE1 is stored within the α -granules of resting platelets, translocated to the platelet surface upon activation by thrombin, proteolytically released as smaller soluble fragments, and incorporated into thrombus.¹³ In addition, immunohistochemical analysis showed the deposition of SCUBE1 in the subendothelial matrix of human advanced atherosclerotic lesions.¹³ Similar to the known functions of the EGF-like domains in mediating homophilic protein–protein interactions as well as cell adhesion,^{14,15} the NH₂-terminal EGF-like domain repeats of SCUBE1 are also capable of promoting platelet–platelet interaction or supporting platelet–matrix adhesion in vitro.^{13,16}

Platelet activation and aggregation are primary reactions in arterial thrombosis and, accordingly, are responsible for the ischemic complications of acute thromboembolic diseases.^{17–19} Our recent clinical study showed that plasma SCUBE1 concentration is significantly elevated and is a potential biomarker of platelet activation in acute coronary syndrome and acute ischemic stroke.²⁰ In addition, plasma SCUBE1 levels are higher in hemodialysis or hypertensive patients.^{21,22}

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Nonstandard Abbreviations and Acronyms

CR	cysteine-rich
CUB	complement protein C1r/C1s, Uegf, and Bmp1
EGF	epidermal growth factor
PPP	platelet-poor plasma
PRP	platelet-rich plasma
SCUBE1	signal peptide-CUB (complement protein C1r/C1s, Uegf, and Bmp1)-EGF (epidermal growth factor) domain-containing protein 1

Furthermore, a genetic variant of *SCUBE1* gene is associated with enhanced risk of venous thromboembolism.²³ However, the precise functions of soluble SCUBE1 in thromboembolic vascular diseases remain largely unknown.

In this study, we generated plasma SCUBE1-depleted mutant (Δ/Δ) mice to evaluate the pathophysiological roles of soluble SCUBE1 in vivo. Our results show that plasma SCUBE1 plays an important role in platelet aggregation and thrombus formation. In addition, genetic loss of soluble SCUBE1 or anti-SCUBE1 antibodies protected mice against fatal thromboembolism with a modest effect on bleeding. These data suggest that inhibition of plasma SCUBE1 might provide a novel therapeutic target/strategy to safely block thrombosis.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Deletion of a Part of the Spacer Region and the Entire CR Repeats of SCUBE1 Prevents Its Secretion

To explore the biological function of plasma SCUBE1 in vivo, we first designed and characterized a mutated recombinant protein with deficiency in producing the soluble form of SCUBE1. Because our previous studies indicated that the spacer region and CR repeats are involved in the secretion and membrane association of SCUBE1 and the COOH-terminal CUB domain is essential for embryonic brain development,¹⁶ we constructed a deletion mutant specifically lacking a part of the spacer region and the entire 3 CR repeats but preserving the CUB domain at the COOH-terminus (named SCUBE1- Δ ; see Figure 1A). To test its secretion and membrane-anchoring properties, we overexpressed the SCUBE1- Δ protein in human embryonic kidney-293T cells. Western blot analyses showed that SCUBE1- Δ indeed completely lost its secretory ability (Figure 1B), but retained its cell-surface expression (Figure 1C) at a lower level because SCUBE1- Δ still contains the NH₂-terminal part of the spacer region with a basic amino-acid stretch (amino acids 500~550) that is responsible for its membrane association through an electrostatic interaction with the acidic membrane phospholipids (unpublished data). Of note is that SCUBE1- Δ can form an oligomeric complex with itself and other SCUBE protein members when overexpressed in human embryonic kidney-293T cells and

determined by a pull-down assay (Figure I in the online-only Data Supplement).

Generation of a New Genetic Mutant Mouse Strain With Deficiency in Producing Plasma SCUBE1

Based on the in vitro deletion data, we next generated a targeting vector containing a *loxP*-flanked neomycin-resistant (Neo) cassette inserted in the *Scube1* intron 18 and an additional *loxP* site in the upstream intron 14 of the gene (Figure IIA in the online-only Data Supplement). Successful targeting of ES cells with this vector resulted in a conditional allele including the Neo cassette. Additional Cre-mediated recombination in ES cells deleted the Neo cassette and produced a conditional allele (*Scube1*^{Flox}) that introduced 2 *loxP* sites flanking exons 15 to 18 encoding a part of the spacer region and the complete 3 CR repeat motifs of *Scube1* (Figure IIA–IIC in the online-only Data Supplement). Heterozygous global *Scube1*^{Δ/+} mice were generated by crossing male protamine (Prm)-Cre; *Scube1*^{Flox/+} mice to wild-type female mice. Mice homozygous for the wild-type (+/+) allele or the targeted (Δ/Δ) allele were obtained by interbreeding the heterozygous ($\Delta/+$) offspring and were born with the expected Mendelian ratio (Figure IID in the online-only Data Supplement).

RT-polymerase chain reaction analyses of platelet mRNA followed by direct sequencing of the PCR product confirmed that the coding sequences for a portion of the spacer region and the 3 CR repeats (exons 15–18) were successfully deleted, yet the COOH-terminal CUB domain was transcribed via in-frame coding in Δ/Δ mouse platelets (Figure 1D, left). In addition, the mRNA expression of other *Scube* genes (*Scube2* and *Scube3*) remained unchanged between +/+ and Δ/Δ mice (Figure 1D, left). Because anti-SCUBE1 antibodies were ineffective in detecting endogenous mouse SCUBE1 protein expression by immunoblotting, we first used confocal immunofluorescent staining with anti-CUB specific antibody to verify the expression of SCUBE1-wild-type or Δ mutant protein in α -granules under high magnification (Figure III in the online-only Data Supplement). In addition, immunostaining using anti-CR specific antibody confirmed a complete absence of these 3 CR repeats but conservation of the CUB domain (anti-CUB immunostaining) in Δ/Δ platelets (Figure 1D, right). Consistent with previous in vitro studies (Figure 1B), in +/+ mice the plasma concentration of SCUBE1 was \approx 150 ng/mL, whereas soluble SCUBE1 was undetectable in Δ/Δ mice, and both wild-type and Δ protein could be released from α -granules and tether on the platelet surface upon activation that could be suppressed by inhibiting platelet secretion by aspirin (Figure IV in the online-only Data Supplement).

Because platelet endothelial aggregation receptor-1 (PEAR1), a novel EGF-like repeat-containing transmembrane receptor, is expressed on the platelet surface and involved in platelet contact-induced activation.²⁴ We then performed further analysis of the interaction between PEAR1 and SCUBE1 by using a variety of SCUBE1 deletion constructs containing the EGF-like repeats, the spacer, the CR, or the CUB domain, respectively (Figure V in the online-only Data Supplement). Our pull-down assay showed

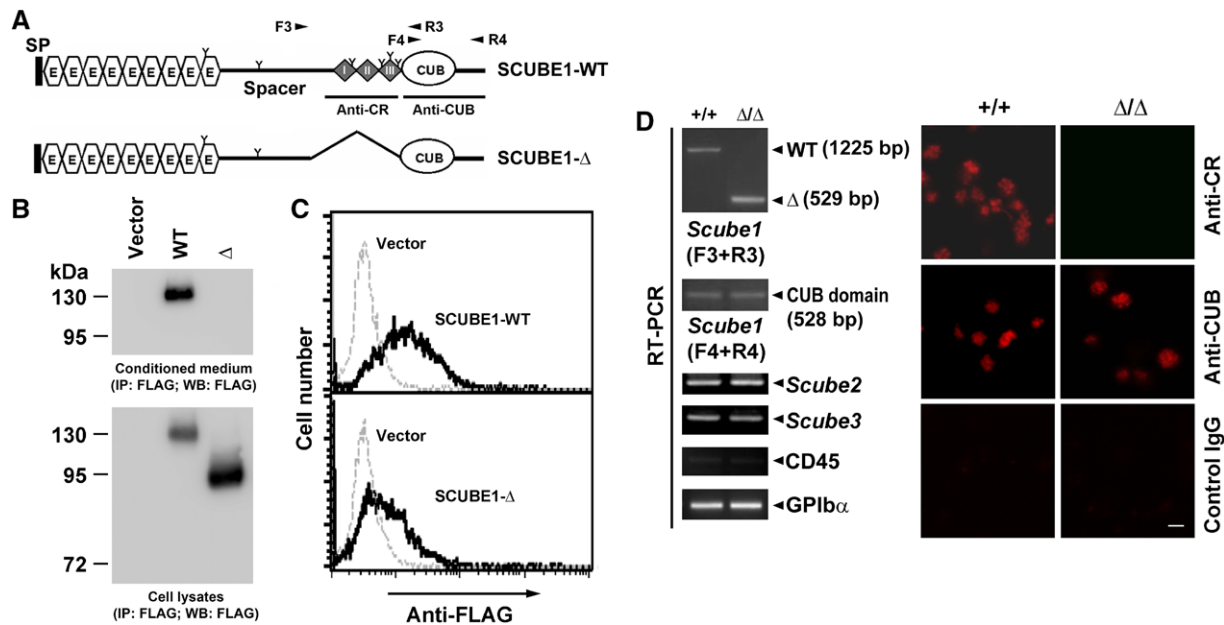


Figure 1. Deletion of a part of the spacer region and entire cysteine-rich (CR) repeats prevent its secretion. **A**, Domain structure of signal peptide-CUB-EGF domain-containing protein 1 (SCUBE1) wild-type (WT) and Δ mutant protein. A FLAG epitope tag was added immediately after the signal peptide sequence at the NH₂ terminus for easy detection. Locations of the polymerase chain reaction (PCR) primers and the corresponding immunogen for anti-CR or anti-CUB antibodies are marked. **B**, SCUBE1- Δ mutant is a secretion-deficient protein. HEK-293T cells were transfected with the indicated expression plasmid DNA. Two days after transfection, protein samples from the conditioned medium or cell lysates were immunoprecipitated with anti-FLAG antibody, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. Recombinant SCUBE1 proteins were detected by western blotting using anti-FLAG antibody. **C**, Cell surface expression of SCUBE1- Δ mutant. HEK-293T cells were transiently transfected with an empty vector (Vector) or the expression plasmid encoding FLAG epitope-tagged SCUBE1 WT or Δ mutant (Δ). Two days after transfection, cells transfected as above were stained with anti-FLAG antibody to determine cell surface expression. **D**, Correct targeting of the Δ allele at the mRNA and protein level. RT-PCR analysis verified the mRNA expression of SCUBE1- Δ in Δ/Δ platelets and mRNA expression of *Scube2* and *Scube3* remained unaltered. In addition, the expression of CD45 or glycoprotein Iba was verified and used as marker genes for leukocytes or platelets, respectively (left panels). Likewise, expression of SCUBE1- Δ protein lacking the entire 3 CR repeats but retaining the CUB domain in Δ/Δ platelets was confirmed by immunofluorescent staining using the domain-specific antibody as indicated (right panels) in isolated platelets adhered on fibrinogen-coated glass slides. Note that +/+ platelets were positive for both anti-CR and anti-CUB staining, and Δ/Δ platelets were only positive for anti-CUB staining. Control IgG was used as a negative staining control. Bar, 2 μ m. CR indicates cysteine-rich repeats; CUB, CUB domain; E, epidermal growth factor-like domain; IP, immunoprecipitation; SP, signal peptide; and WB, Western blot analysis.

the adhesive module of the EGF-like repeats and the CUB domain of SCUBE1 can interact with PEAR1.

These Δ/Δ mutant mice showed normal development, survival, and reproduction and no spontaneous bleeding or thrombosis. In addition, blood cell counts (including platelets) and hematologic and coagulation parameters, as well as protein or mRNA levels of major platelet surface receptors, including glycoprotein VI, glycoprotein IX, glycoprotein Ia (α 2), β 3 integrin, and ADP receptors (P2Y₁ and P2Y₁₂), were similar between +/+ and Δ/Δ mice (Table II and Figure VI in the online-only Data Supplement). This indicates that SCUBE1 is not required for the development of hematopoietic lineages, including the formation and maturation of platelets.

Platelet Aggregation Was Impaired in Plasma SCUBE1-Deficient Δ/Δ Platelet-Rich Plasma (PRP) Compared With +/+ PRP

ADP, collagen, and thrombin are the major platelet activators at the sites of vascular injury.²⁵ To determine the role of plasma SCUBE1 in platelet function, we first isolated PRP and measured platelet aggregation in response to ADP, collagen, and the thrombin agonist PAR-4 peptide (Figure 2). With PAR-4 peptide (50–250 μ mol/L), platelet aggregation

was indistinguishable in PRP samples from +/+ and Δ/Δ mice (Figure 2; Figure VII in the online-only Data Supplement). However, in response to ADP at 5 and 10 μ mol/L or collagen at a low concentration, 2.5 μ g/mL, platelets in PRP from Δ/Δ mice reproducibly aggregated less than that from +/+ mice. All agonists induced a comparable activation-dependent change from discoid to spherical shape in +/+ and Δ/Δ platelets, which can be seen as a transient upward deflection in the aggregation traces with the addition of agonists (Figure 2). Therefore, plasma SCUBE1 is critical for a maximal response to ADP or a low concentration of collagen but not for the initial shape change after agonist stimulation.

Washed Δ/Δ Platelets Show Normal Morphological Features, Aggregation, and Signaling

To determine whether impaired PRP aggregation was due to an intrinsic morphological or signaling abnormality in platelets, we next prepared washed platelets from +/+ and Δ/Δ mice for ultrastructural and aggregation assays. Electron microscopy revealed no apparent differences in ultrastructure or the numbers of α - or dense granules between +/+ and Δ/Δ platelets (Figure VIIIA in the online-only Data Supplement). In addition, ADP or collagen-induced aggregations of washed platelets were

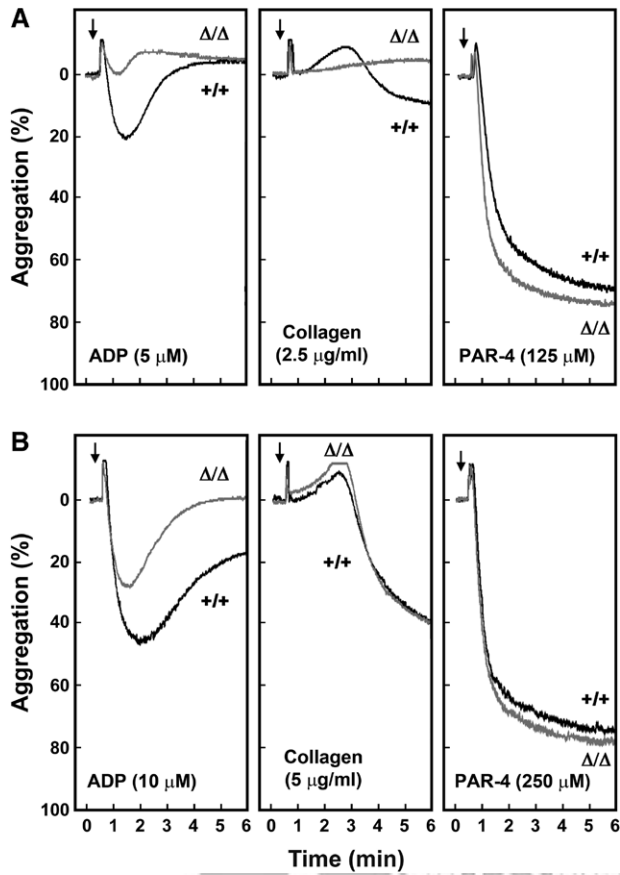


Figure 2. Impaired ADP- and collagen-induced platelet-rich plasma (PRP) aggregation in Δ/Δ mice compared with $+/+$ mice. Blood was collected from $+/+$ and Δ/Δ mice. PRP was obtained by pooling blood from animals with the same genotype. The platelet number in each sample was adjusted to $2.5 \times 10^8/\text{mL}$ with platelet-poor plasma. Low (A) or high (B) concentrations of ADP, collagen, or PAR-4 peptide were added to stirred PRP from $+/+$ or Δ/Δ mice. Aggregation was measured and expressed as the percentage change in light transmission, with the value for the blank (platelet-poor plasma) set at 100%. Traces are representative of data obtained from ≥ 3 independent experiments. Arrows in all panels indicate application of platelet agonists.

comparable for $+/+$ and Δ/Δ mice (Figure IXA in the online-only Data Supplement) as was downstream activation of Akt kinase induced by ADP,²⁶ which was measured by its phosphorylation status (Figure IXB in the online-only Data Supplement) or total platelet tyrosine phosphorylation profiles during collagen-induced aggregation (Figure X in the online-only Data Supplement). In agreement with this finding, flow cytometry showed that the active conformation of $\alpha_{\text{IIb}}\beta_3$ integrin induced by inside-out activation and the surface exposure of fibrinogen, von Willebrand factor, or P-selectin caused by α -granule secretion in response to agonist stimulation was virtually identical between $+/+$ and Δ/Δ platelets (Figure VIIIB in the online-only Data Supplement). Likewise, the secretion of platelet dense granules as determined by measuring ATP release after activation was comparable between $+/+$ and Δ/Δ platelets (data not shown).

Plasma SCUBE1 Is Involved in Platelet Aggregation

Because intrinsic platelet structure and signaling seemed normal in Δ/Δ mice (Figures VIII and X in the online-only Data

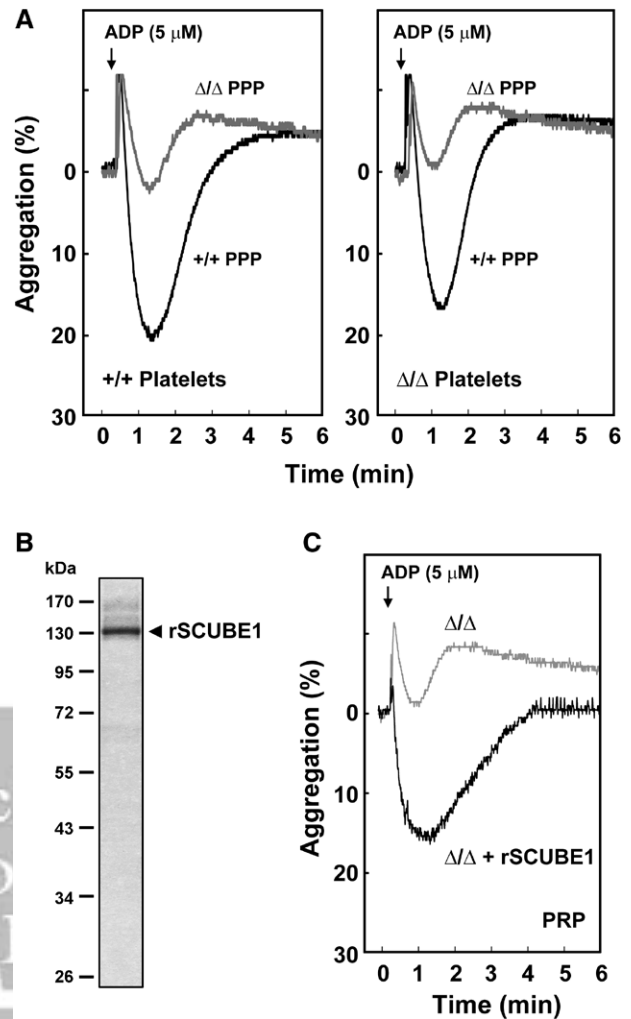


Figure 3. Mixing study with plasma and platelets from mice of different genotypes or by the addition of recombinant murine signal peptide-CUB-EGF domain-containing protein 1 (rSCUBE1). **A**, Plasma containing SCUBE1 is essential for normal ADP-induced platelet aggregation. Isolated platelets from $+/+$ and Δ/Δ mice were suspended in platelet-poor plasma (PPP) from $+/+$ or Δ/Δ mice to a platelet concentration of $2.5 \times 10^8/\text{mL}$ and activated with ADP ($5 \mu\text{mol/L}$). **B**, Production of rSCUBE1 purified from the conditioned medium of HEK-293T cells overexpressing the murine SCUBE1 carrying a His tag at the COOH terminus. One microgram of rSCUBE1 was analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue. **C**, Restoration of the Δ/Δ phenotype by rSCUBE1. The addition of rSCUBE1 (150 ng/mL) restored impaired aggregation of Δ/Δ PRP in response to ADP ($5 \mu\text{mol/L}$) to comparable levels as in $+/+$ PRP. Each panel shows representative tracing from 3 independent experiments. Arrows indicate the addition of ADP agonist.

Supplement), we further evaluated whether plasma SCUBE1 is involved in agonist-stimulated aggregation. We performed platelet and plasma exchange studies with washed platelets from $+/+$ or Δ/Δ mice resuspended in platelet-poor plasma (PPP) from either genotype. Interestingly, $+/+$ platelets aggregated less in SCUBE1-depleted (Δ/Δ) PPP than in SCUBE1-positive ($+/+$) PPP (Figure 3A, left). Conversely, Δ/Δ platelets aggregated more in $+/+$ PPP than in Δ/Δ PPP (Figure 3A, right). These data indicate that plasma SCUBE1 is involved in platelet aggregation.

To further confirm that the impaired aggregation in Δ/Δ mice was directly attributable to plasma deficiency of SCUBE1, we evaluated the effect of recombinant murine SCUBE1 (rSCUBE1) on platelet aggregation by adding rSCUBE1 protein in vitro. Although rSCUBE1 at concentrations ≤ 5 $\mu\text{g}/\text{mL}$ had no effect on shape change, aggregation and agonist-induced signaling of $+/+$ platelets (Figure XI in the online-only Data Supplement), the addition of rSCUBE1 at normal plasma level (150 ng/mL) was sufficient to restore the defective aggregation of Δ/Δ PRP in response to ADP (Figure 3C). Of note, the magnitude of platelet aggregation in Δ/Δ PRP samples with rSCUBE1 was rescued to the level in $+/+$ PRP samples (Figure 2A) or the level in Δ/Δ platelets mixed with $+/+$ PPP (Figure 3A). In addition, rSCUBE1 further enhanced ADP-induced aggregation in $+/+$ PRP but not when $\alpha_{\text{IIb}}\beta_3$ function was inhibited (Figure XII in the online-only Data Supplement).

To examine whether the EGF-like repeats are participated in promoting homophilic cell-cell aggregation, we then evaluated the effects of a GST fusion protein containing the EGF-like repeats (GST-S1-E4-9) on the aggregating property of a stable cell line with surface overexpression of the EGF-like repeats of SCUBE1 (S1-E4-9) by cell aggregation assay. Monolayer cultures of the parental (control) or the transfected stable cell line (S1-E4-9) were dissociated into single cells and allowed to aggregate in suspension culture together with GST or GST-S1-E4-9 protein, respectively. When the suspensions were gently shaken, cells expression S1-E4-9 tended to aggregate in the presence of GST-S1-E4-9 ($\approx 40\%$) but not GST alone ($\approx 5\%$), whereas the parental control cells aggregated poorly in suspension ($< 3\%$) (Figure XIII in the online-only Data Supplement). These data suggested that the EGF-like repeats of SCUBE1 is capable of promoting the cell aggregation, possibly through trans-homophilic interactions

and cross-linking the surface-expressed SCUBE1 proteins. However, soluble SCUBE1 could not compete with fibrinogen binding to activated platelets, suggesting that SCUBE1 may not bind activated $\alpha_{\text{IIb}}\beta_3$ integrin (Figure XIV in the online-only Data Supplement). Overall, these findings are in agreement with our previous studies in humans^{13,16} and indicate that soluble SCUBE1 is an adhesive molecule, which cross-links adjacent activated platelets, thus promoting aggregation and stabilizing aggregates.

Δ/Δ Mice Are Protected Against Thrombosis and Have a Slightly Prolonged Bleeding Time

We then investigated the role of soluble SCUBE1 in platelet function by assessing thrombus formation in vitro. A whole-blood perfusion assay was performed over collagen-coated surfaces at a high shear rate of 1000 s^{-1} (Figure 4). After 3 minutes of perfusion, the area covered by thrombi was $27.6 \pm 4.1\%$ for $+/+$ mice and $14.5 \pm 2.2\%$ for Δ/Δ mice (47% lower than $+/+$; $P < 0.01$). Similarly, the thrombus volume obtained from Δ/Δ mice was 56% smaller than that for $+/+$ mice (1.97 ± 0.17 versus $4.47 \pm 0.8 \mu\text{m}^3 \times 10^6$; $P < 0.01$). Interestingly, transfer of $+/+$ plasma can restore impaired thrombus formation in Δ/Δ mice (Figure 4). It is noteworthy that the addition of tirofiban (an $\alpha_{\text{IIb}}\beta_3$ inhibitor) markedly suppressed in vitro thrombus formation by $+/+$ or Δ/Δ platelets (data not shown). These data suggest that plasma SCUBE1 is important for platelet cross-linking (thrombus formation) in vitro under arterial shear conditions. Furthermore, we used 2 thrombosis models to determine the in vivo effects of plasma SCUBE1 deficiency. In the first model, we induced platelet- and fibrin-rich thrombus formation by ferric chloride injury and denudation of the carotid artery.²⁷ The size of arterial thrombus in Δ/Δ mice was 35% less on average than in $+/+$ mice ($P < 0.05$; Figure 5A–5C). Next, we used a model of platelet-dependent intravascular

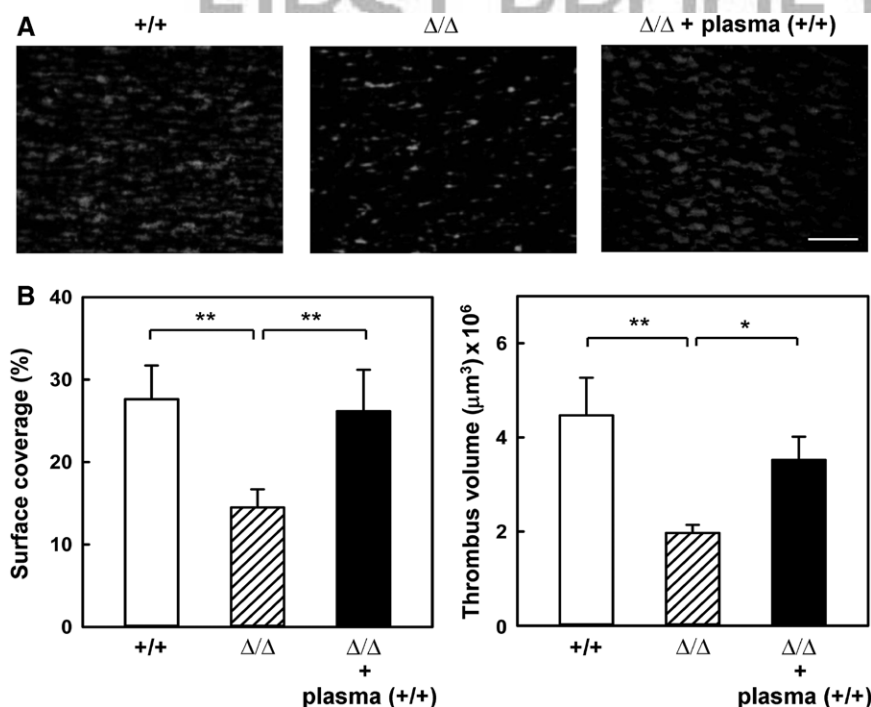


Figure 4. Flow chamber assay showed impaired thrombus formation by the Δ/Δ platelets that can be restored by $+/+$ plasma transfer. Fluorescence (DiOC₆) labeled platelets in whole blood from $+/+$, Δ/Δ , or Δ/Δ mixed with $+/+$ plasma were perfused over a collagen-coated (0.2 mg/mL) surface at a well shear rate of 1000 s^{-1} . **A**, Representative images of thrombus formation after perfusion for 3 minutes. Bar, 100 μm . **B**, Quantitative data of surface coverage (left) and thrombus volume (right) measured by the sum of the detected surface areas of all images of the Z-stack by fluorescent confocal microscope scanning (means \pm SD, $n = 5$ mice). * $P < 0.05$; ** $P < 0.01$.

thrombosis and fatal pulmonary embolism induced by systemic intravenous injection of a mixture of collagen (0.3 mg/kg) and epinephrine (60 μ g/kg). All +/+ mice died within 8 minutes after injection, whereas the survival rate for Δ/Δ mice was $\approx 60\%$ ($P < 0.05$ compared with +/+ mice; Figure 5D). Thus, thrombus formation in Δ/Δ mice did not develop as rapidly or as extensively as in +/+ mice. Indeed, macroscopic and histological analysis revealed widespread pulmonary thromboembolism in +/+ mice (Figure 5E) but not in surviving Δ/Δ mice (Figure 5F). Thus, loss of plasma SCUBE1 significantly protected mice against arterial thrombosis.

Hemostasis involves both platelet aggregation and blood coagulation. To test whether the defect in plasma SCUBE1 affected hemostasis, we measured bleeding times in mice after tail-tip amputation (Figure 5G). The mean bleeding time in +/+ mice was 65 ± 5.6 seconds ($n=24$), whereas bleeding was highly variable and mildly prolonged in Δ/Δ mice (140 ± 17.5 seconds, $n=24$, $P < 0.05$). To determine whether a defect in coagulation contributed to this increase in bleeding, we assessed activated partial thromboplastin time and prothrombin time as indices of intrinsic and extrinsic coagulation pathways, respectively. Neither activated partial thromboplastin time nor prothrombin time was altered in Δ/Δ mice (Table II in the online-only Data Supplement). Thus, plasma SCUBE1 has an important function in hemostasis mediated by platelet aggregation, not coagulation.

Infusion of SCUBE1 Antibodies Protects Mice Against Fatal Thromboembolism

To examine whether blocking plasma SCUBE1 might be useful to prevent thrombosis, we studied the effect of specific antibodies against SCUBE1 on platelet aggregation *in vitro* and on thromboembolism *in vivo*. We used monoclonal antibodies raised specifically against the EGF-like repeats of SCUBE1 (Figure XVb in the online-only Data Supplement), the domain which is involved in reciprocal and lateral interactions between SCUBE1 proteins in homophilic adhesions.¹⁶ In contrast to the isotype-matched control antibody, the anti-SCUBE1-neutralizing antibody blocked the SCUBE1-mediated cell aggregation in a transfected cell line and ristocetin-induced platelet agglutination (Figure XVf–XVh in the online-only Data Supplement). Moreover, the SCUBE1-neutralizing antibody protected +/+ mice against fatal thromboembolism induced by collagen and epinephrine (60% survival, Figure 6A). Consistently, SCUBE1 antibody treatment also significantly reduced arterial thrombosis formation induced by ferric chloride injury (Figure XVI in the online-only Data Supplement). Injection of saline or the isotype control antibody was ineffective in preventing fatal thromboembolism or arterial thrombosis in +/+ mice (Figure 6A–6D; Figure XVI in the online-only Data Supplement). In addition, SCUBE1-antibody-injected mice showed no sign of bleeding (Figure 6E). Together, these results indicate that functional neutralization of plasma SCUBE1 can effectively prevent thrombosis.

Discussion

Despite its original discovery in the endothelium,² SCUBE1 is also highly expressed in platelets.¹³ When overexpressed in

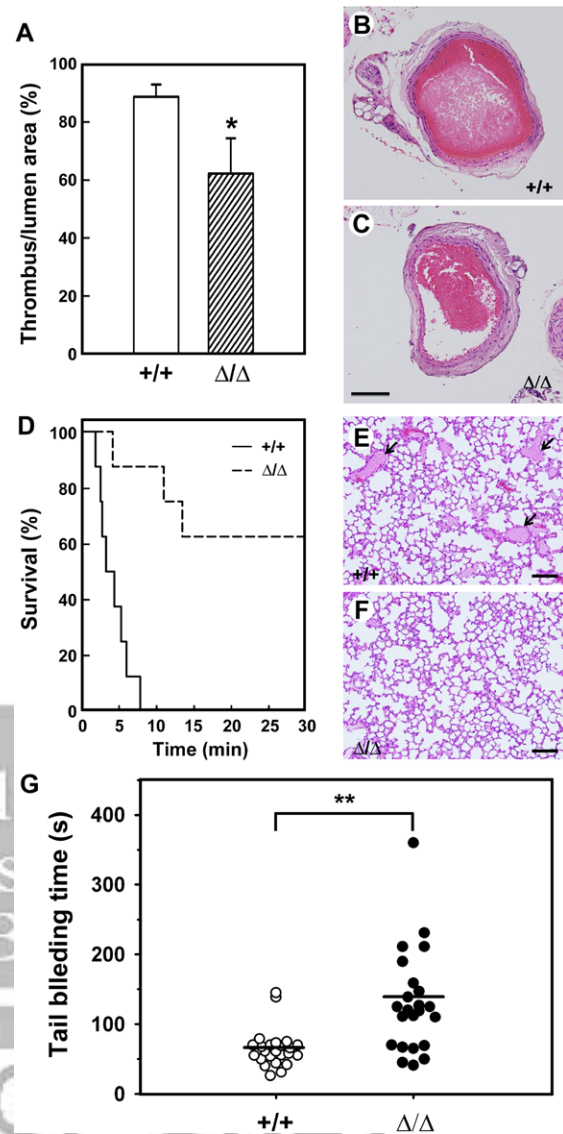


Figure 5. Plasma signal peptide-CUB-EGF domain-containing protein 1-deficient mice are protected against thrombosis and have a slightly prolonged bleeding time. **A–C**, Δ/Δ mice were protected against arterial thrombosis. Thrombus formation in the carotid artery was induced by placing a small piece of filter paper (1×2 mm) saturated with 5% FeCl_3 directly on the carotid artery for 3 minutes. Histological samples were prepared by perfusing the artery with 4% paraformaldehyde, then the artery was removed, fixed overnight in 4% paraformaldehyde solution, and embedded in paraffin. Thrombus in the carotid artery induced by FeCl_3 injury was smaller in Δ/Δ than +/+ mice (data are mean \pm SD, $n=5$ in each group; $*P < 0.05$). Representative cross sections of the carotid artery 3 minutes after injury of +/+ (B) or Δ/Δ mice (C). Bar, 50 μ m. **D–F**, Δ/Δ mice were protected against pulmonary thromboembolism. Systemic thromboembolism was induced by injection of a mixture of collagen (0.3 mg/kg) and epinephrine (60 μ g/kg). **D**, Data are percentage of mice still alive (survival) as a function of time ≤ 30 minutes ($n=8$ in each genotype; $P < 0.01$). **E** and **F**, Histological sections of lungs after collagen and epinephrine injection. Hematoxylin–eosin staining shows extensive platelet thromboembolism (arrows) in +/+ mice (E) but not in surviving Δ/Δ mice (F). Bar, 100 μ m. **G**, Tail bleeding time in +/+ and Δ/Δ mice. A 5-mm portion of the distal tail was removed from 8-week-old mice, and the cessation of bleeding time was recorded. Following a determination of bleeding time, genotype analysis was then performed. Horizontal lines represent the mean. Each point represents 1 mouse. $**P < 0.01$ ($n=24$ in each group).

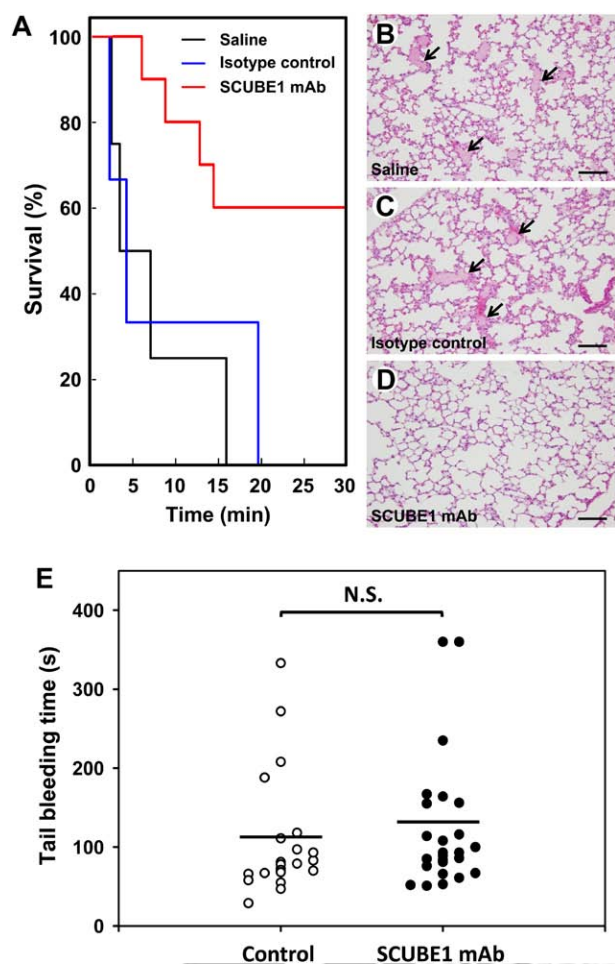


Figure 6. Inhibition of plasma signal peptide-CUB-EGF domain-containing protein 1 (SCUBE1) protects mice against lethal thromboembolism. **A**, Thromboembolism induced by collagen (0.3 mg/kg) and epinephrine (60 μ g/kg) treatment in $+/+$ mice injected with anti-SCUBE1 monoclonal antibody (mAb; 100 μ g each, $n=10$), saline (100 μ L each, $n=4$), or the isotype control antibody (100 μ g each, $n=3$, $P<0.05$). Representative hematoxylin-eosin staining of lung cross sections from each group revealing massive thromboembolism (arrows) in mice treated with saline control (**B**) or isotype control antibody (**C**) but not in surviving SCUBE1 mAb-injected mice (**D**). Bar, 100 μ m. **E**, Effect of treatment of the anti-SCUBE1-blocking antibody on tail bleeding time. The $+/+$ male mice (8-week-old) were injected with 100 μ g of anti-SCUBE1-neutralizing antibody ($n=23$) or the isotype control antibody ($n=25$), followed by tail bleeding time test as described in the figure. Horizontal lines represent the mean. Each point represents 1 mouse. N.S. indicates not significant.

human embryonic kidney-293T cells, recombinant SCUBE1 is a secreted glycoprotein that forms an oligomeric complex tethered to the cell surface.^{2,16} Consistent with the well-recognized role of the EGF-like domain in mediating homophilic cell-cell interaction and adhesion,^{14,28} our previous studies showed that protein fragments containing the NH_2 -terminal EGF-like repeats of SCUBE1 can enhance platelet adhesion and ristocetin-induced platelet agglutination.¹³ In addition, clinical studies by us and others showed significantly elevated plasma SCUBE1 content in patients with acute coronary syndrome, acute ischemic stroke, hypertension, and hemodialysis.²⁰⁻²² Thus, plasma SCUBE1 is a potential biomarker of platelet activation in acute thrombotic diseases.²⁰ However,

whether soluble SCUBE1 has a pathological role in thrombogenesis during progression of acute thromboembolic diseases remains largely unknown.

In this study, we provide genetic evidence that soluble plasma SCUBE1 is not only a marker but also an active participant of thrombosis. Plasma SCUBE1-deficient Δ/Δ mice were protected against arterial thrombosis but did not show spontaneous bleeding. The antithrombotic mechanism of plasma deficiency of SCUBE1 was at least in part attributed to defective platelet aggregation. Of note, the antithrombotic effect of lack of plasma SCUBE1 is comparable with that of aspirin when assayed in the same thromboembolism model.²⁹ Soluble SCUBE1 did not directly stimulate resting platelets, but it augmented the aggregation in response to known platelet agonists such as ADP (Figure XII in the online-only Data Supplement). Intriguingly, use of neutralizing antibodies against the EGF-like repeats at the NH_2 terminus of SCUBE1 protects mice against lethal thromboembolism without causing spontaneous bleeding. It is worth noting that tail bleeding time is unaltered after antibody treatment (Figure 6E), whereas it is modestly increased in Δ/Δ mice (Figure 5G). This difference between these 2 experiments may be the result of incomplete neutralization of plasma SCUBE1 by antibody treatment in vivo as opposed to a complete genetic ablation.

Although the specific binding target(s) for the released SCUBE1 remains to be identified, our in vitro pull-down assay showed that SCUBE1 can interact with PEAR1 through its EGF-like repeats and the CUB domain, suggesting PEAR1 as a potential receptor for SCUBE1 binding. However, further studies are required to verify this hypothesis and elucidate its signaling relevance in platelet biology. Interestingly, surface-bound SCUBE1 can be induced to form cell aggregates by the addition of recombinant SCUBE1 protein fragment containing the EGF-like repeats (Figure XIII in the online-only Data Supplement). In agreement with these findings, rSCUBE1 protein further enhanced ADP-stimulated platelet aggregation (Figure XII in the online-only Data Supplement). Together, our data suggest that soluble SCUBE1 acts through its EGF-like domain to form an oligomeric structure as a key adhesive protein forming trans-homophilic bridges between the surface-bound SCUBE1 on activated platelets during platelet thrombus formation.

In our plasma and platelet switching experiments, Δ/Δ or $+/+$ platelets aggregated less in Δ/Δ plasma than in normal plasma (Figure 3A) and the addition of rSCUBE1 improved ex vivo platelet aggregation in Δ/Δ mice (Figure 3C). These findings clearly indicate that lack of SCUBE1 in plasma is responsible for reduced platelet activity in Δ/Δ mice. Because SCUBE1 is also stored in the α -granules,¹³ it is likely that a local concentration of SCUBE1 released after platelet activation and degranulation might further promote platelet aggregation and thrombus growth at the sites of vascular injury. However, this notion was challenged by our aggregation studies with washed platelets that showed no difference between $+/+$ and Δ/Δ platelets (Figure IX in the online-only Data Supplement). Thus, locally secreted SCUBE1 derived from platelets seems not to contribute to aggregation. On the other hand, the addition of rSCUBE1 at normal plasma level

(150 ng/mL) or a higher concentration (data now shown) within the range of elevated plasma SCUBE1 levels observed in patients experiencing acute coronary syndrome or acute ischemic stroke²⁰ further enhanced platelet aggregation of +/- PRP and possibly promoted pathological thrombosis (Figure X11A in the online-only Data Supplement). In addition, a recent study showed a significant association of SCUBE1 polymorphism and venous thromboembolism,²³ which underscores SCUBE1 as a promising susceptible candidate gene for this type of disease.

However, the shortcoming of the global mutant mice makes it difficult to distinguish to which extent the platelet or the endothelium (or other cell types) contributes to the antithrombotic phenotype. We are currently generating a flox conditional allele that will be crossed within the Pf4-Cre (megakaryocyte/platelet-specific) or Tie2-Cre (endothelium-specific) mouse line to further dissect the relative contribution of platelets or the endothelium on the observed phenotype. Because the SCUBE protein family contains 3 different members (ie, SCUBE1 to 3) and because these molecules can be secreted and form heteromeric complexes when overexpressed,²⁷ further investigation with genetic intercrosses among *Scube2*- or *Scube3*-deficient mice is needed to confirm whether soluble forms of other SCUBEs are also involved in platelet adhesion and thrombogenesis. In addition, we noticed that triple-knock-out mice deficient in other platelet adhesion proteins, including plasma fibrinogen, von Willebrand factor, and fibronectin, could still undergo platelet aggregation and thrombus formation after vascular injuries.³⁰ Thus, additional molecules with plasma SCUBE1 as a potential candidate may be involved in these processes.

Mouse models deficient in the ADP receptor P2Y₁ or P2Y₁₂,^{31–35} von Willebrand factor receptor glycoprotein Ib-IX-V complex,³⁶ or the GTP-binding Gαq³⁷ show similar protection against fatal thromboembolism induced by collagen and epinephrine. However, in contrast to these models, our mouse model of plasma SCUBE1 deficiency did not show greatly increased bleeding time after tail clipping (Figure 5G). Thus, plasma SCUBE1 seems redundant for baseline hemostasis but constitutes an important platelet adhesive system in pathological thrombosis. Our findings indicate that genetic loss or inhibition of plasma SCUBE1 protected mice against fatal thromboembolism with a modest effect on bleeding. Hence, targeting plasma SCUBE1 might provide a novel therapeutic strategy to prevent thrombosis.

Acknowledgments

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Sources of Funding

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Disclosures

None.

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Significance

Signal peptide-CUB-EGF domain-containing protein 1 is a novel platelet adhesive protein that promotes platelet–platelet interaction and supports platelet–matrix adhesion. Genetic loss or antibody blockade of plasma signal peptide-CUB-EGF domain-containing protein 1 protects mice against thrombosis, pointing to signal peptide-CUB-EGF domain-containing protein 1 as a novel antithrombotic target.

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Materials and Methods

Mice

Our investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All experimental procedures were performed according to the protocol approved by the Institutional Animal Care and Utilization Committee, Academia Sinica (Protocol RMIIBMYR2010063). The generation of *Scube1*^{Δ/Δ} mice was as follows. The targeting vector (as described in Figure I in online-only Data Supplement) was electroporated into the ES cell line R1. The correctly targeted ES cell clones were injected into blastocysts and implanted into pseudopregnant females. Heterozygous global *Scube1*^{Δ/+} mice were obtained by crossing male *Prm-Cre; Scube1*^{Flox/+} mice to wild-type female mice. The targeted allele was backcrossed with C57BL/6 females for at least 5 generations before experiments. *Scube1*^{Δ/Δ} mouse colonies were established by interbreeding heterozygotes.

RT-PCR

Total RNA was prepared from +/+ and Δ/Δ platelet (1×10⁹ platelets) using TRIzol reagents (Invitrogen). First-strand cDNA synthesis using RevertAid H Minus reverse transcriptase (Fermentas) was prepared on 5 μg of total RNA. The first-strand cDNA reaction was used for each PCR as template. Semi-quantitative RT-PCR primers specific for mouse ADP receptors P2Y1 (*P2ry1*) and P2Y12 (*P2ry12*) were listed in Table I in the online-only Data Supplement. For PCR reactions, 25 μl of total reaction containing 2.5 μl of 10X PCR buffer, 0.5 μl of dNTP mix, 0.5 μl each 10 μM forward and 10 μM reverse primer, 0.5 μl *Taq* polymerase and 2 μl cDNA were incubated at 94°C for 5 min, then run for 30 cycles with the following steps: 30 sec at 94 °C, 30 sec at 55 °C, 1 min at 72 °C and final extension at 72 °C for 5 min.

Confocal immunofluorescence microscopy

Glass coverslips were coated with 50 μg/ml fibrinogen in PBS (pH 7.4) at 37 °C for 2 h and blocked with 1% bovine serum albumin (BSA) for 1 h. Washed platelets (300 μl of 10⁸/ml) were allowed to adhere for 30 min, and then fixed with 1% paraformaldehyde for 15 min. Platelets were permeabilized with 0.5% Triton X-100 for 10 min and incubated in PBS containing 1% BSA for 1 h. Platelets were incubated with anti-SCUBE1 monoclonal antibody for 1 h at room temperature, followed by incubation with Alexa 594-conjugated secondary antibody and FITC-labeled anti-vWF antibody for 1 h at room

temperature. Coverslips were mounted and sealed on glass slides and analyzed with a Zeiss LSM510 confocal microscope (Carl Zeiss, Thornwood, NY).

Plasma SCUBE1 assay

Heparinized blood samples were centrifuged at 2,500 g and plasma was collected to measure the concentration of SCUBE1 as described below. High-binding, flat-bottom polypropylene 96-well plates (NUNC, Naperville, IL) were coated overnight at 4 °C with 50 µl of anti-SCUBE1 monoclonal Ab #701 (5 µg/ml) developed in our lab.¹ The coated plates were washed (0.05% Tween-20 in PBS), blocked (0.5% BSA in PBS), and incubated for 1 h with 50 µl of recombinant SCUBE1 or plasma samples in triplicate. After washing, a second HRP-conjugated SCUBE1 mAb #701 (8 µg/ml) was incubated for 1 hr, then washed, and developed with tetramethyl benzidine (KPL, Gaithersburg, MD) followed by 1N HCl. Then the absorbance at 450 nm was determined (SpectraMax 340PC, Molecular Devices, Sunnyvale, CA). The minimum detection limit by this method was 50 ng/ml.

Cell aggregation

A2058 control (vector-transfected) cells or stable cells expressing HA-tagged SCUBE1-E4-9 were detached by trypsin-EDTA and suspended in Dulbecco's minimal essential medium containing 5 mM CaCl₂ at 1 x 10⁶ cells/ml in polystyrene tubes. Cells were incubated with isotype control (IgG_{2b}) or anti-SCUBE1 monoclonal antibody (20 µg/ml) on a rotating platform (10 rpm) at 37 °C for 9 h. The extent of cell aggregation was viewed microscopically and photographed. For quantitation, cell clusters of more than 4 cells were considered aggregated. Data are mean ± S.D. of 3 experiments performed in duplicate.

Immunoprecipitation and western blot analysis

Two days after transfection, cell lysates were clarified by centrifugation at 10,000 xg for 20 min at 4 °C. Samples were incubated with 1 µg of the indicated antibody and 20 µl of 50% (v/v) Protein A-agarose (Pierce) for 2 h with gentle rocking. After 3 washes with lysis buffer, precipitated complexes were solubilized by boiling in Laemmli sample buffer, fractionated by SDS-PAGE, and transferred onto PVDF membranes. The membranes were blocked with phosphate buffered saline (pH 7.5) containing 0.1% gelatin and 0.05% Tween 20 and blotted with the indicated antibodies. After 2 washes, the

blots were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) for 1 h. After washing the membranes, the reactive bands were visualized by use of the VisGlow™ chemiluminescent substrate, horseradish peroxidase system (Visual Protein).

Flow cytometry

Whole blood or washed platelets from +/+ and Δ/Δ mice were stimulated with a variety of agonists as indicated. After incubation for 1 min at 37 °C without stirring, unstimulated or activated platelets were stained with appropriate fluorophore-conjugated antibodies for 15 min at room temperature. The reaction was stopped by adding 350 μ l phosphate-buffered saline (PBS), and samples were analyzed with a FACSCalibur flow cytometer (BD Biosciences). For Fibrinogen (Fg) binding, +/+ and Δ/Δ washed platelets were preincubated with rSCUBE1 protein (up to 1000 ng/ml) or unlabeled Fg (100 μ g/ml) for 3 min and followed by ADP (10 μ M) stimulation for 1 min at 37 °C. Resting or activated platelets were stained with FITC-conjugated Fg (100 μ g/ml) for 15 min and samples were analyzed by a FACSCalibur flow cytometer.

Measurement of platelet dense granule secretion

Dense granule secretion by platelet was evaluated by measuring ATP release with the ATP determination kit as described.² +/+ or Δ/Δ washed platelets were adjusted to 3×10^8 platelets/ml. Platelets were stimulated with indicated agonists for 2 min at 37°C with stirring. To stop reaction, 3 mM EDTA and 0.1% formaldehyde were added to fix platelets for 1 h, followed by centrifugation for 1 min at 1,3000 rpm. A mixture (12.5 μ l) of supernatants and ethanol (equal volume of each) was used to measure ATP content by using a bioluminescence assay kit (Roche).

Construction of expression plasmid and cell transfection

The expression plasmid encoding the SCUBE1- Δ mutant (lacking residues 563-794) was constructed essentially as described.³ Human embryonic kidney (HEK)-293T cells were transfected with the plasmid by use of Lipofectamine 2000 (Invitrogen).

Antibodies

Anti-FLAG M2 and anti-Myc 9E10 monoclonal antibodies were obtained from Sigma and Covance, respectively. Monoclonal antibodies conjugated to FITC or PE against the high-affinity conformation of mouse integrin $\alpha_{IIb}\beta_3$ or other

mouse platelet surface proteins were purchased from EMFRET Analytics (Eibelstadt, Germany). Anti-SCUBE1 monoclonal antibodies were produced by using the recombinant protein fragments containing the EGF-like domains as immunogens as described.¹ The specificity of anti-SCUBE1 monoclonal antibodies was verified by western blot analysis or flow cytometry as shown in Figure VII in the online-only Data Supplement.

Platelet aggregation assay

WT and Δ/Δ mice were anesthetized and blood was drawn from the heart into a tube containing 3.2% sodium citrate (1/9, vol/vol). Platelet-rich plasma (PRP) was obtained by centrifugation at 250g for 5 min. Platelet-poor plasma (PPP) was prepared by further centrifugation at 900g for 20 min. PPP contained < 1,000 platelets/ml. PRP was adjusted to 2×10^8 platelets/ml with the use of autologous PPP. Platelet aggregation in response to different concentrations of agonists including ADP (Sigma), collagen (Chrono-Log, Havertown, PA), and protease-activated receptor 4 (PAR-4)-activating peptide (Bachem, Torrance, CA) was measured in 200 μ l PRP at 37°C with the Chrono-log aggregometer (model 490). In some experiments, purified recombinant murine SCUBE1 protein was added to PRP at a final concentration of 150 ng/ml before the addition of platelet agonist. Washed platelets were isolated from PRP and sedimented by centrifugation at 1300g for 5 min. After removal of supernatant plasma, the platelet pellet was washed twice with 1 ml Tyrode's buffer containing 0.5 μ M PGI₂ and 0.02 U/ml apyrase and incubated for 30 min at 37°C in 0.5 ml Tyrode's buffer containing 0.02 U/ml apyrase and adjusted to 2×10^8 /ml. In another set of experiments, washed platelet aggregations in Tyrode's buffer were stimulated with different platelet agonists.

FeCl₃-induced carotid arterial thrombosis

Mice were anesthetized by intraperitoneal injection of ketamine/xylazine (100/15 mg/kg). Thrombus formation in the carotid artery was induced by placing a small piece of filter paper (1 \times 2 mm) saturated with 5% FeCl₃ directly on the carotid artery for 3 min. Histological samples were prepared by perfusing the artery with 4% paraformaldehyde, then the artery was removed, fixed overnight in 4% paraformaldehyde solution, and embedded in paraffin.

Pulmonary thromboembolism model

To induce acute systemic pulmonary thromboembolism, a mixture of collagen (0.3 mg/kg) and epinephrine (60 μ g/kg) was injected into the tail vein as

described.⁴ Mice were monitored for 30 min; those alive for more than 30 min after challenge were considered survivors. For antibody blocking experiments, mice received injection of 100 µg anti-SCUBE1 antibody (directed against the EGF-like repeats of SCUBE1), control isotype-matched antibody, or saline 15 min before collagen-epinephrine challenge.

Tail bleeding time in mice

Mouse tail bleeding times were determined as described.⁵ Briefly, 5 mm of the distal tail was removed by use of a surgical blade and immediately immersed in physiological saline maintained at 37°C. Complete cessation of bleeding was defined as the bleeding time. If bleeding did not cease within 6 min, the tail was cauterized and 360 sec was noted as the bleeding time.

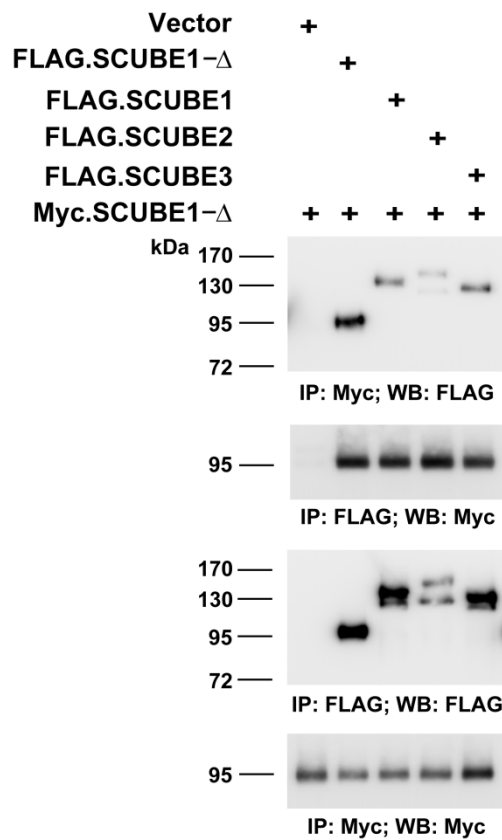
Statistical analysis

Tail bleeding time data were analyzed by non-parametric Log-rank test. Pulmonary thromboembolism data underwent survival analysis followed by the Log-rank test. A $P < 0.05$ was considered statistically significant. Statistical analyses involved use of the package “coin” within the open-source statistical software R (<http://www.R-project.org>).

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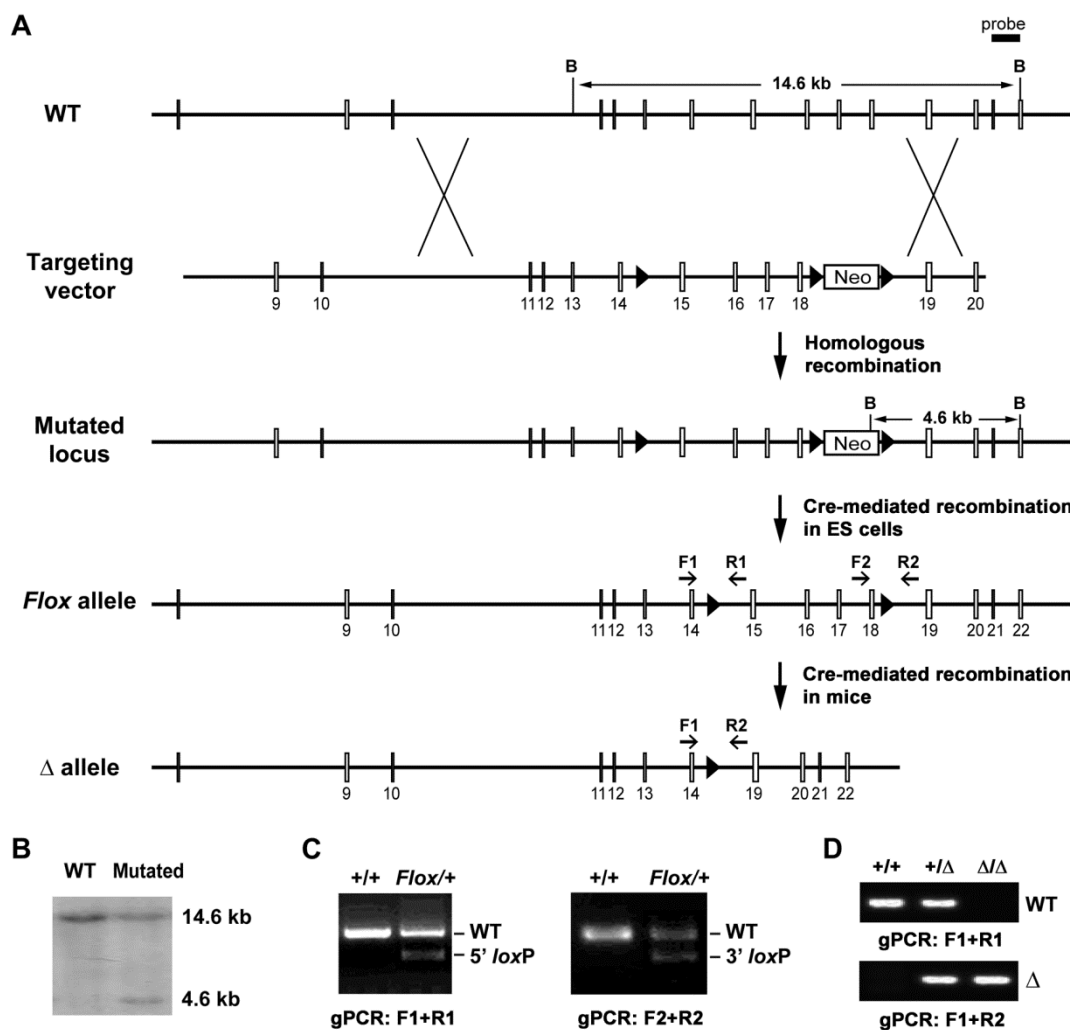
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Suppl. FIG. I



Supplemental Figure I. SCUBE1-Δ can form an oligomeric complex with itself or other SCUBE protein members. The SCUBE1-Δ expression construct (Myc-tagged) was co-transfected with the expression plasmids encoding FLAG-tagged SCUBE1-Δ or full-length SCUBE1 to 3 in HEK-293T cells. Two days later, cell lysate underwent immunoprecipitation (IP) and Western blot (WB) with antibodies as indicated to determine the protein-protein interactions.

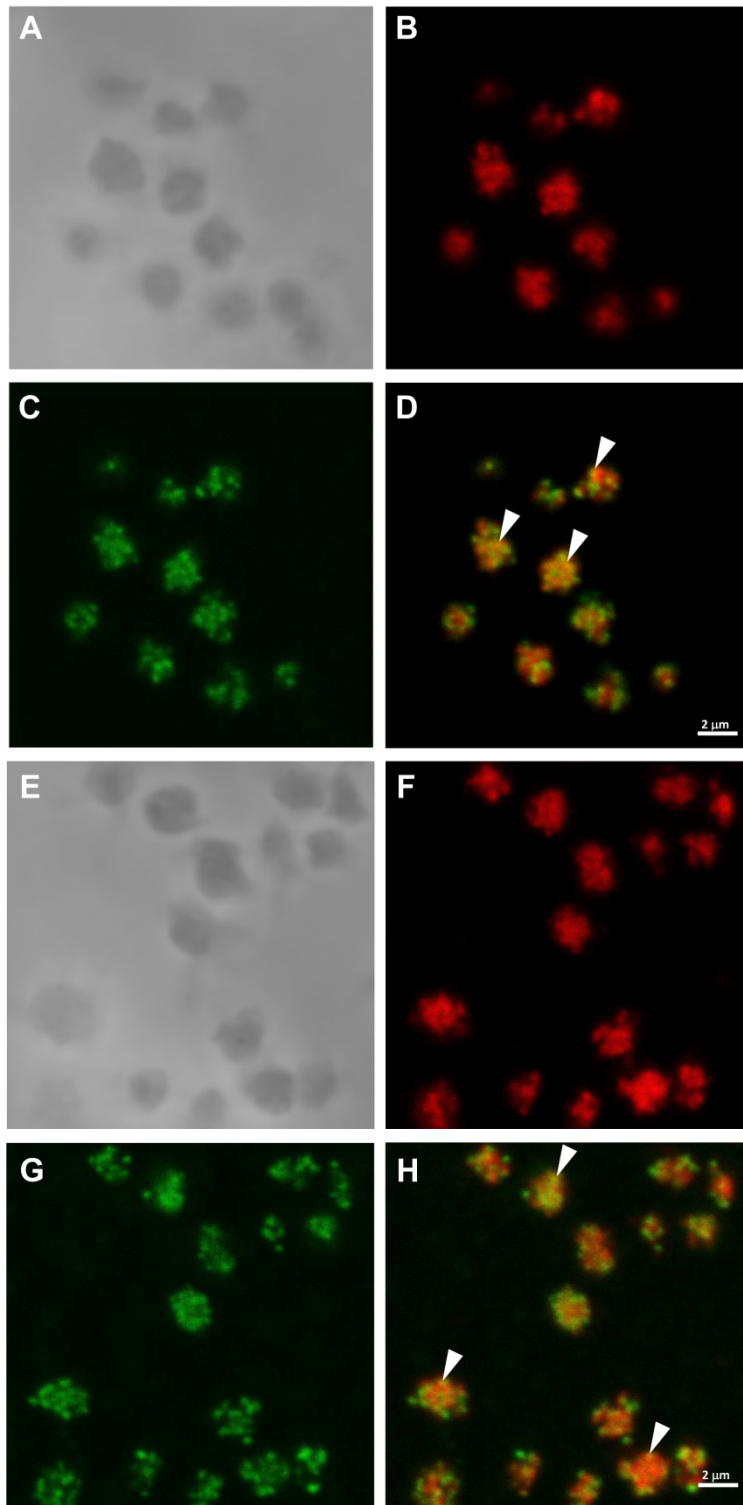
Suppl. FIG. II



Supplemental Figure II. Generation of targeted *loxP*-flanked (*Flox*) and deletion (Δ) alleles of *Scube1* gene. A, The scheme for gene targeting and Cre-mediated recombination. We generated conditional *Scube1* mutant mice with one targeting vector that introduced 2 *loxP* sites flanking exons 15 to 18 and coding for a part of the spacer region and complete 3 CR repeat motifs of *Scube1*. The external probe for Southern blot analysis is indicated by a thick line, and the restriction site used (B, *Bam*HI) is indicated. We then removed the neomycin resistance gene cassette by transfection of the targeted ES cells with a Cre-recombinase–encoding expression plasmid. The final targeted *Flox* allele contained a *loxP* site upstream of exon 15 and another *loxP* site downstream of exon 18 in the *Scube1* gene. ES cells were injected into C57BL/6 blastocysts to generate chimera. Germline transmission of the *Flox* allele was confirmed by PCR analysis. Heterozygous global *Scube1*^{*Flox*/+} mice were generated by crossing male Prm-Cre; *Scube1*^{*Flox*/+} mice to wild-type female mice. Small arrows indicate primers (F1, R1, F2 and R2) used in PCR analyses to confirm

gene targeting or Cre-mediated recombination or for genotyping. Exons are numbered under open boxes. Filled triangles indicate *loxP* sites. Neo, neomycin resistance gene cassette. WT, wild-type. B, Genomic Southern blot analysis to detect the targeted ES clones with *Bam*HI digestion using the external probe. C, Genotyping of the *Flox* allele by PCR analysis. PCR of genomic DNA from wild-type (+/+) or heterozygous (*Flox*+) mice with the primers F1+R1 or F2+R2. Because an *Eco*RI restriction site was introduced along with the *loxP* site, digestion of *Eco*RI resulted in smaller PCR products indicating the presence of the *loxP* site. gPCR, genomic DNA PCR analysis. D, Genotyping of the Δ allele. Genomic DNA PCR analyses from +/+, Δ /+, and Δ / Δ mice with the primers F1 and R2 showed that exons 15 to 18 were deleted in the Δ allele.

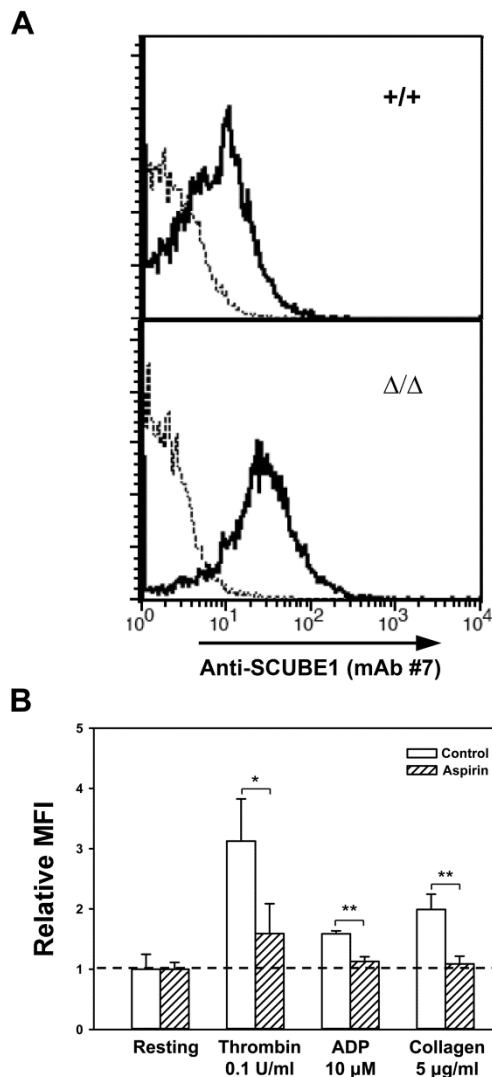
Suppl. FIG. III



Supplemental Figure III. SCUBE1 co-localizes with vWF in platelet α -granules by confocal immunofluorescence microscopy. Mouse $+/+$ (A-D) or Δ/Δ (E-H) platelets spread over a fibrinogen matrix was paraformaldehyde-fixed and Triton-permeabilized. (A, E) The phase-contrast images. SCUBE1 localization was detected with anti-SCUBE1 CUB monoclonal antibody (see Fig. 1A) and Alexa Fluoro

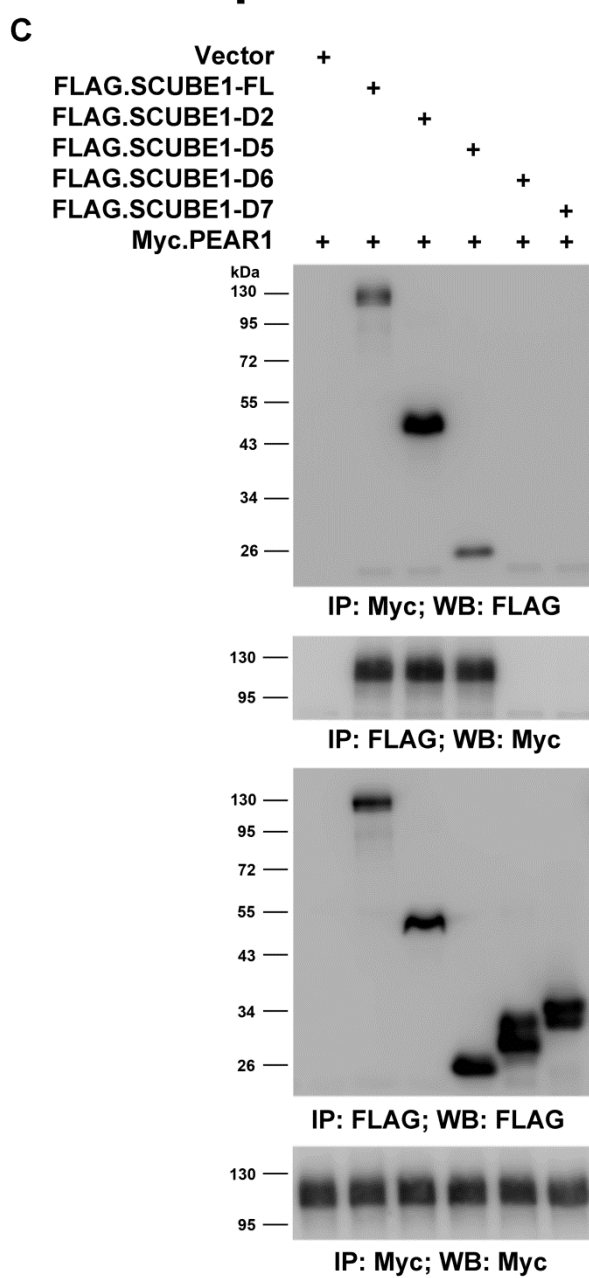
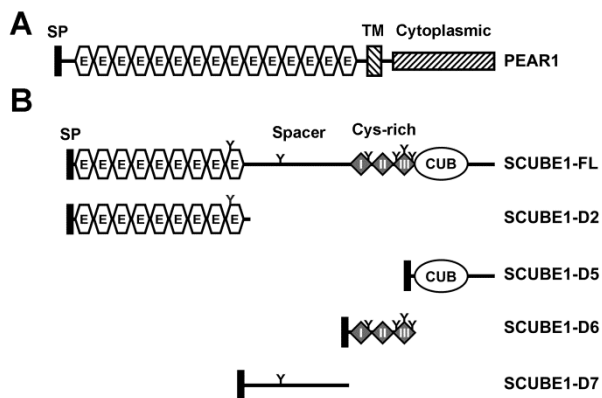
594-conjugated goat anti-mouse IgG (B and F, red). vWF, a marker of α -granules, was detected with FITC-labeled rabbit polyclonal antibody (C and G, green). Co-localization of SCUBE1 and vWF in the intracellular granular compartments (arrowheads) was shown by a merged image (D and H, yellow). Bar, 2 μ m.

Suppl. FIG. IV



Supplemental Figure IV. Surface expression of platelet SCUBE1 after stimulation. (A) Washed platelets derived from +/+ or Δ/Δ mice were stimulated with thrombin (0.1 U/ml) for 3 min. Surface expression of SCUBE1 was revealed by staining with mouse anti-SCUBE1 antibodies prebound with the FITC-conjugated goat anti-mouse IgG (thick line). Resting platelets represent the baseline expression (dotted line). (B) Platelet SCUBE1 surface expression stimulated by indicated agonists **in the absence or presence of aspirin** were assessed by flow cytometry as described above. For quantifying purpose, relative MFI of agonist-induced SCUBE1 surface expression was normalized to the MFI of the resting platelets. Data are means \pm S.D. (n=5 mice per group). *, P < 0.05; **, P < 0.01.

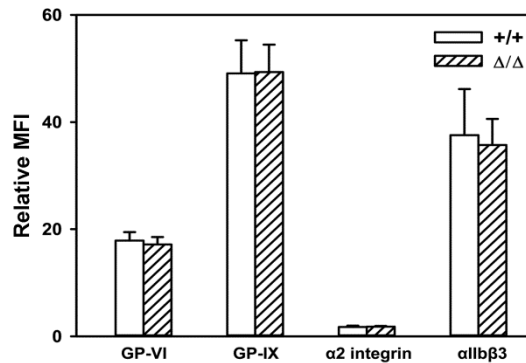
Suppl. FIG. V



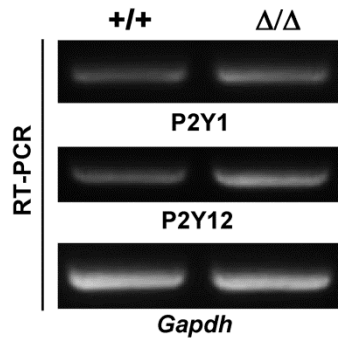
Supplemental Figure V. SCUBE1 binds PEAR1 (platelet endothelial aggregation receptor-1) through heterophilic EGF-like repeat interactions. (A) Schematic representation of the domain structure of PEAR1. Human PEAR1 is composed of a signal peptide (SP) sequence at the NH₂-terminus, an extracellular 15 EGF-like (E) repeats, a transmembrane domain (TM) and a cytoplasmic domain. PEAR1 is a novel EGF-like repeat-containing transmembrane receptor involved in platelet contact-induced activation. (B) Domain organization of the SCUBE2 expression plasmids used in this study. FL, amino acids 1-988; D2, amino acids 1-411; D5, amino acids 791-988; D6, amino acids 628-788; D7 amino acids 402-627. SP, signal peptide; E, EGF-like repeat; Cys-rich, cysteine-rich motif; CUB, CUB domain. (C) Interaction between SCUBE1 and PEAR1. The PEAR1 expression construct (Myc-tagged) was transfected alone or in combination with the expression plasmids encoding indicating FLAG-tagged SCUBE1 proteins in HEK-293T cells. Two days later, cell lysate underwent immunoprecipitation (IP) and Western blot (WB) with antibodies as indicated to determine the protein-protein interactions.

Suppl. FIG. VI

A

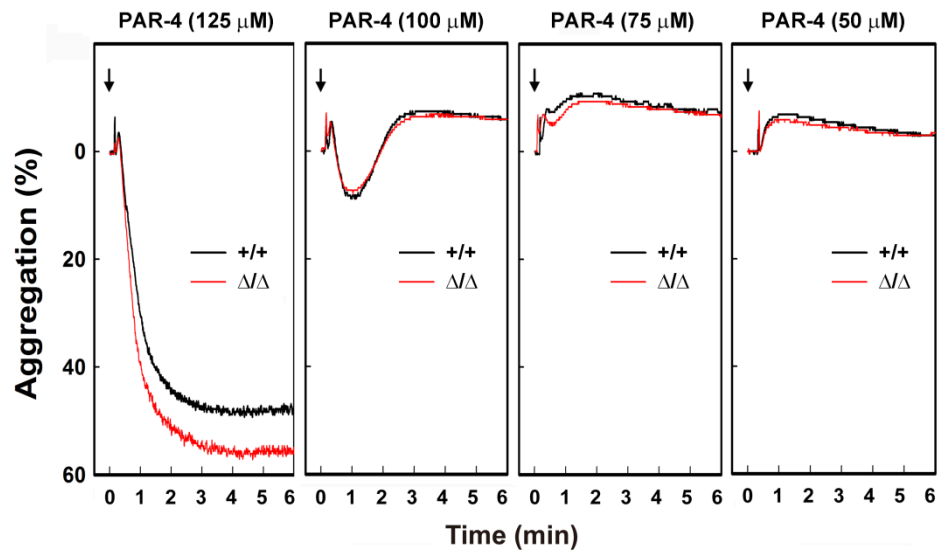


B



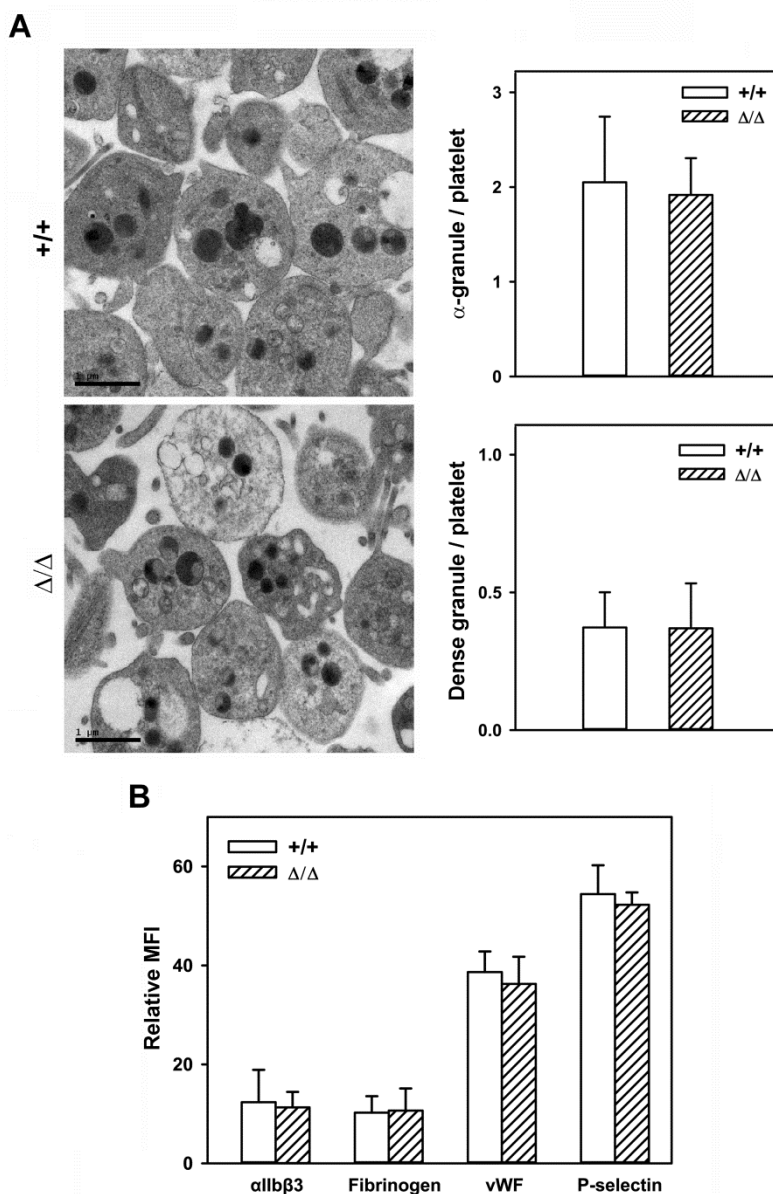
Supplemental Figure VI. Platelet expression of major signaling receptors is similar between +/+ and Δ/Δ platelets. (A) Flow cytometry of the indicated glycoproteins or integrin proteins in +/+ and Δ/Δ platelets. Platelets were gated by forward scattering/side scattering characteristics. For quantifying purpose, relative mean fluorescence intensity (MFI) of each surface protein expression on platelets was indicated. The relative MFI is defined as the MFI of each surface protein detected by corresponding antibody divided by the MFI of the control IgG in each sample. Results are expressed as means ± S.D. (n=5 mice per group; not significant). (B) RT-PCR analysis of mRNA expression of two ADP receptors (P2Y₁ and P2Y₁₂) for +/+ and Δ/Δ platelets.

Suppl. FIG. VII



Supplemental Figure VII. Effects of PAR-4 peptide on +/+ and Δ/Δ PRP aggregation. PRP samples derived from +/+ or Δ/Δ mice were stimulated by a titration of PAR-4 concentration (50 to 125 μM). Traces are representative of data obtained from at least 3 independent experiments. Arrows indicate the addition of PAR-4.

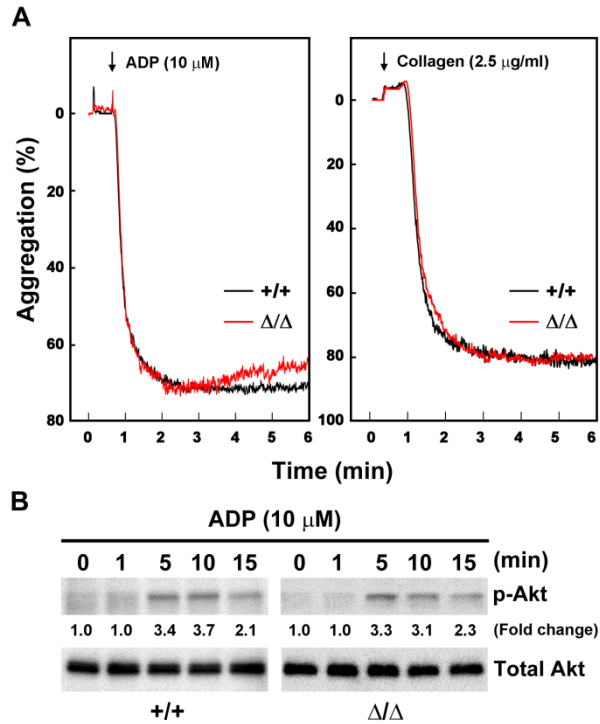
Suppl. FIG. VIII



Supplemental Figure VIII. Structure and activation signaling in +/+ and Δ/Δ platelets. (A) Morphology and structure are comparable between the +/+ and Δ/Δ platelets as revealed by electron microscopy and enumeration of platelet α- and dense granules. Unstimulated platelets freshly isolated from the +/+ and Δ/Δ mice were immediately fixed in glutaraldehyde and further processed for electron microscopic observations. Magnification x25,000 (left panel). The numbers of platelet α-granules and dense granules were evaluated using electron microscopic sections. The total numbers of platelet in 12 equivalent-sized fields were quantified, and granules numbers are expressed as granules / platelet (right panel). Data are means ± S.D. (not significant). Bars = 1 μm. (B) Flow cytometry revealed no significant

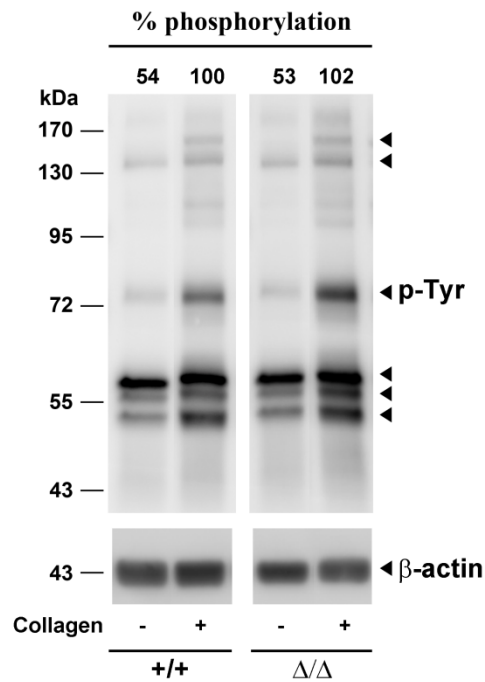
difference in inside-out activation of β_3 integrin and the α -granules secretion of fibrinogen (Fg), von Willebrand factor (vWF), or P-selectin between +/+ and Δ/Δ platelets . Activation of β_3 integrin, P-selectin and vWF surface expression on +/+ or Δ/Δ washed platelets by thrombin (0.1 U/ml) was examined by use of JON/A (PE-conjugated), anti-P-selectin or anti-vWF (FITC-conjugated) antibody. Mouse whole blood was used to measured platelet surface expression of fibrinogen after stimulation with 10 μ M ADP+1 μ M TXA₂ by staining with its corresponding antibodies (FITC-conjugated). For quantifying purpose, relative MFI of each surface protein upon platelet activation was normalized to the MFI of the resting platelets. Bars represent means \pm S.D. (n=5 animals per group; not significant).

Suppl. FIG. IX



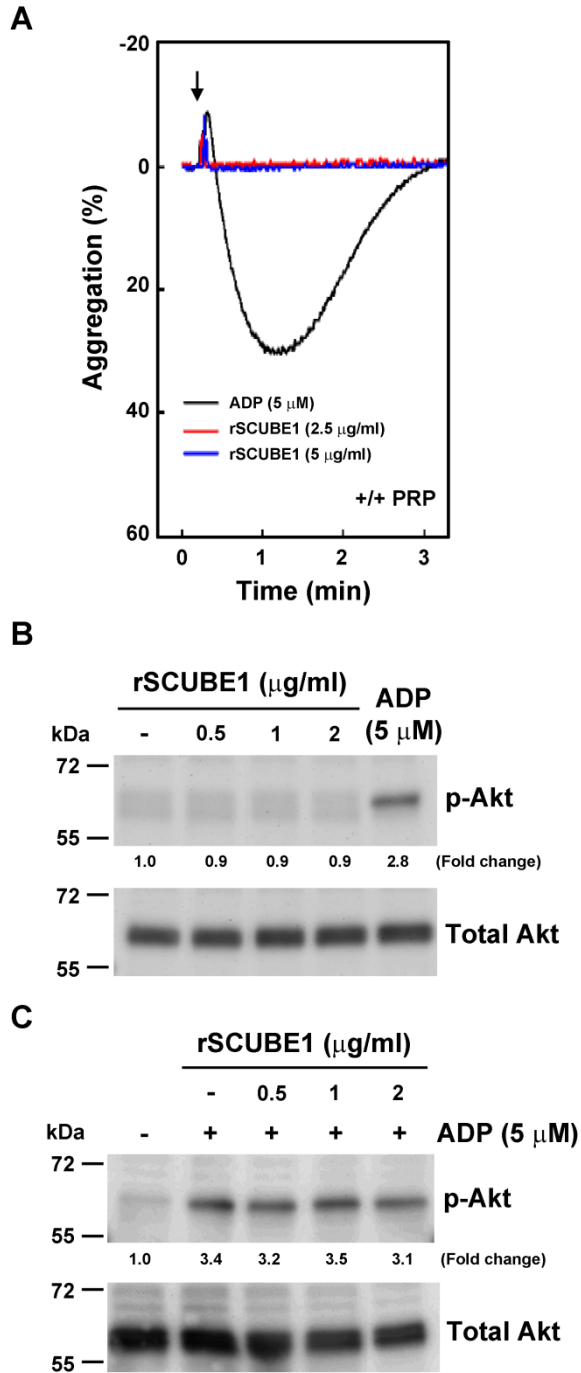
Supplemental Figure IX. Washed platelets from +/+ and Δ/Δ mice show comparable agonist-induced aggregation and downstream signaling. (A) Washed platelets from +/+ and the Δ/Δ mice were stimulated with ADP (together with 250 μ g/ml fibrinogen) or collagen. Experiments were repeated 3 times with similar results. (B) Western blot analysis of the kinetics of the phosphorylation status of ADP-induced Akt phosphorylation in +/+ and Δ/Δ platelets for different times (0-15 min). Platelet lysates were used to determine the levels of phosphorylated Akt (Ser⁴⁷³) or total Akt by Western blotting with appropriate antibodies, respectively. Quantified data of relative p-Akt level in each time point were given as “fold change” below the anti-p-Akt blot (quantified by desitometric scanning and normalized by the total amount of Akt). Results are representative of 3 independent experiments.

Suppl. FIG. X



Supplemental Figure X. Total platelet tyrosine phosphorylation profiles between +/+ and Δ/Δ mice during collagen-induced aggregation. Washed +/+ or Δ/Δ platelets were left untreated (-) or stimulated with collagen (2.5 $\mu\text{g}/\text{ml}$) with stirring for 1 min. Platelets were lysed in SDS denaturing buffer and proteins were subjected to SDS-polyacrylamide gel electrophoresis and transferred to PVDF. Membranes were probed with anti-phosphotyrosine (p-Tyr) 4G10 platinum antibody to visualize the total tyrosine phosphorylation profiles. The levels of total p-Tyr were quantified by densitometric scanning and normalized by the total amount of protein loading. The Western blot shown is representative of 3 independent experiments.

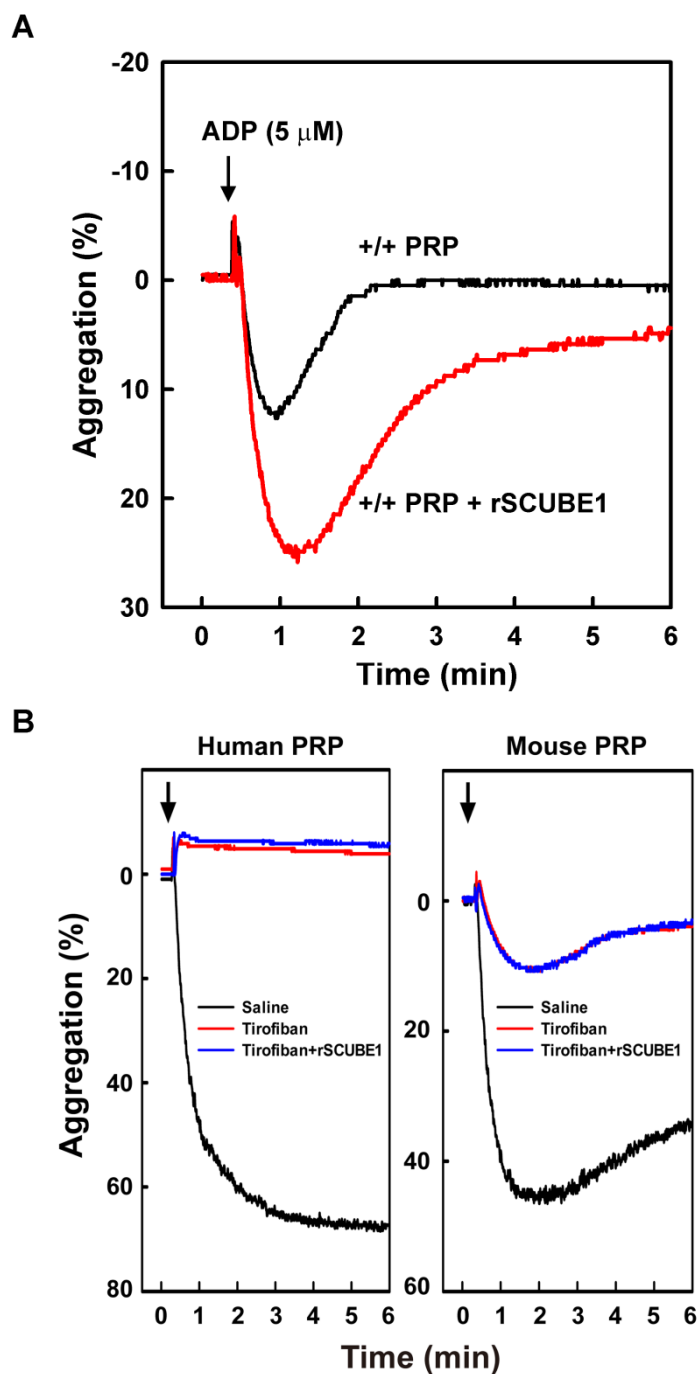
Suppl. FIG. XI



Supplemental Figure XI. Effects of rSCUBE1 on platelet aggregation, signaling and activation induced by ADP in +/+ mice. (A) rSCUBE1 protein alone has no effect on platelet aggregation. rSCUBE1 (up to 5 μ g/ml) or ADP (5 μ M) as a positive control was added to stirred PRP from +/+ mice. Arrow indicates the addition of rSCUBE1 or ADP. Traces are representative of data obtained from 3 independent experiments. (B, C) Effect of rSCUBE1 alone or together with ADP on platelet

signaling and activation. Mouse washed platelets were incubated with an increasing concentration of rSCUBE1 in the absence (B) or presence of ADP (C) for 10 or 5 min, respectively. Platelet lysates were used to determine the levels of phosphorylated Akt (Ser⁴⁷³) or total Akt by Western blotting with appropriate antibodies, respectively. Quantified data of relative p-Akt level given as fold change were shown below the anti-p-Akt blot (quantified by densitometric scanning and normalized by the total amount of Akt).

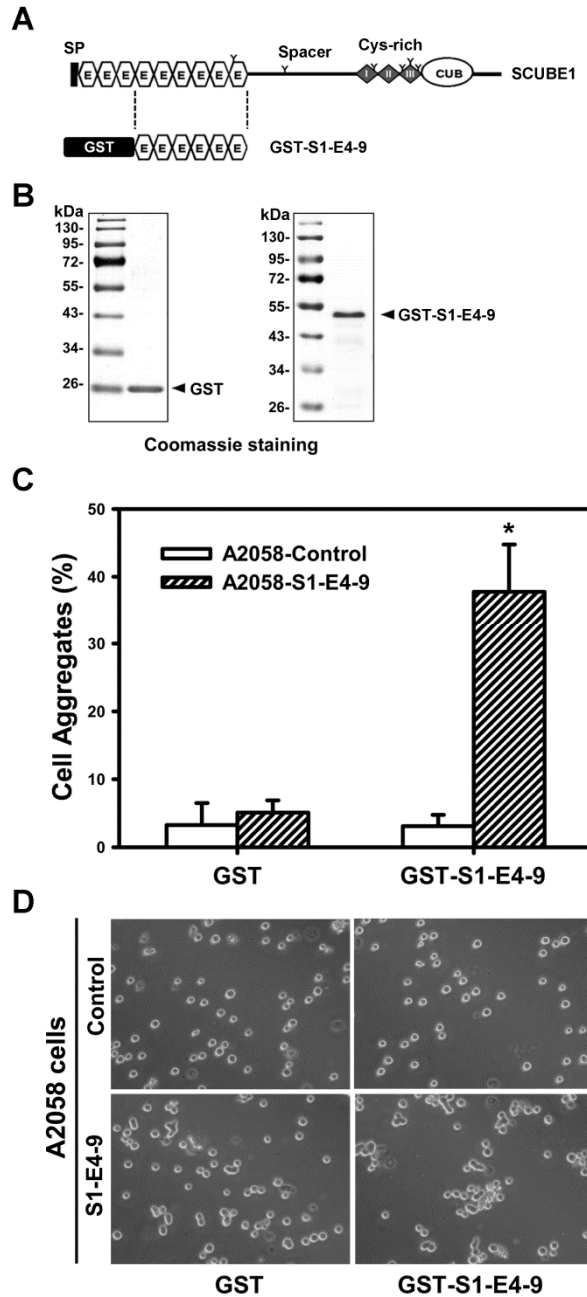
Suppl. FIG. XII



Supplemental Figure XII. Recombinant murine SCUBE1 protein (rSCUBE1) augments ADP-induced aggregation in +/+ PRP, but not when $\alpha_{IIb}\beta_3$ function was inhibited. (A) The addition of rSCUBE1 (500 ng/ml) further enhances platelet aggregation in response to adenosine diphosphate (ADP; 5 μ M). (B) Human and +/+ mouse PRP (2.5×10^8 platelets/mL) were pre-incubated with saline, $\alpha_{IIb}\beta_3$ inhibitor (tirofiban, 2.8 μ M to human PRP; 8.55 μ M to mouse PRP) alone or together rSCUBE1 (1 μ g/ml) for 3 min as indicated. Arrows indicate application of platelet agonists (20

$\mu\text{M ADP}$). Traces are representative of data obtained from at least 3 independent experiments.

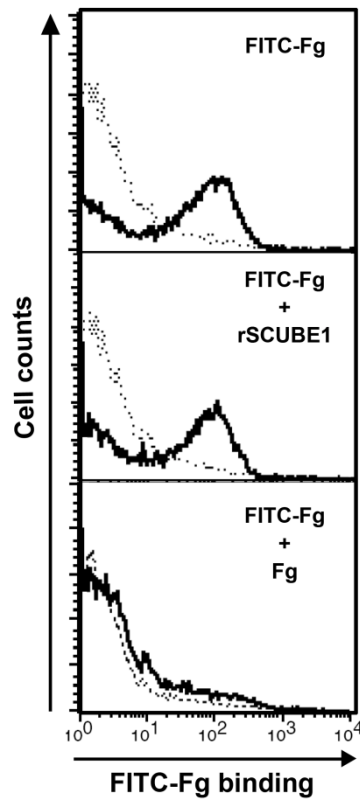
Suppl. FIG. XIII



Supplemental Figure XIII. Purified recombinant GST-fusion protein containing the EGF-like repeats of SCUBE1 promotes cell aggregations through cell-surface homophilic EGF-like repeat interactions. (A) Production of a GST-fusion protein containing the EGF-like repeats of SCUBE1. Schematic representation of SCUBE1 and the GST-fusion construct containing the EGF-like repeats 4-9 of SCUBE1 (GST-S1-E4-9). (B) Purity of GST and GST-S1-E4-9 protein. Purified GST alone or recombinant GST-S1-E4-9 (1 μ g of each) were verified by Coomassie staining. (C) Addition of GST-S1-E4-9 enhances homophilic aggregations

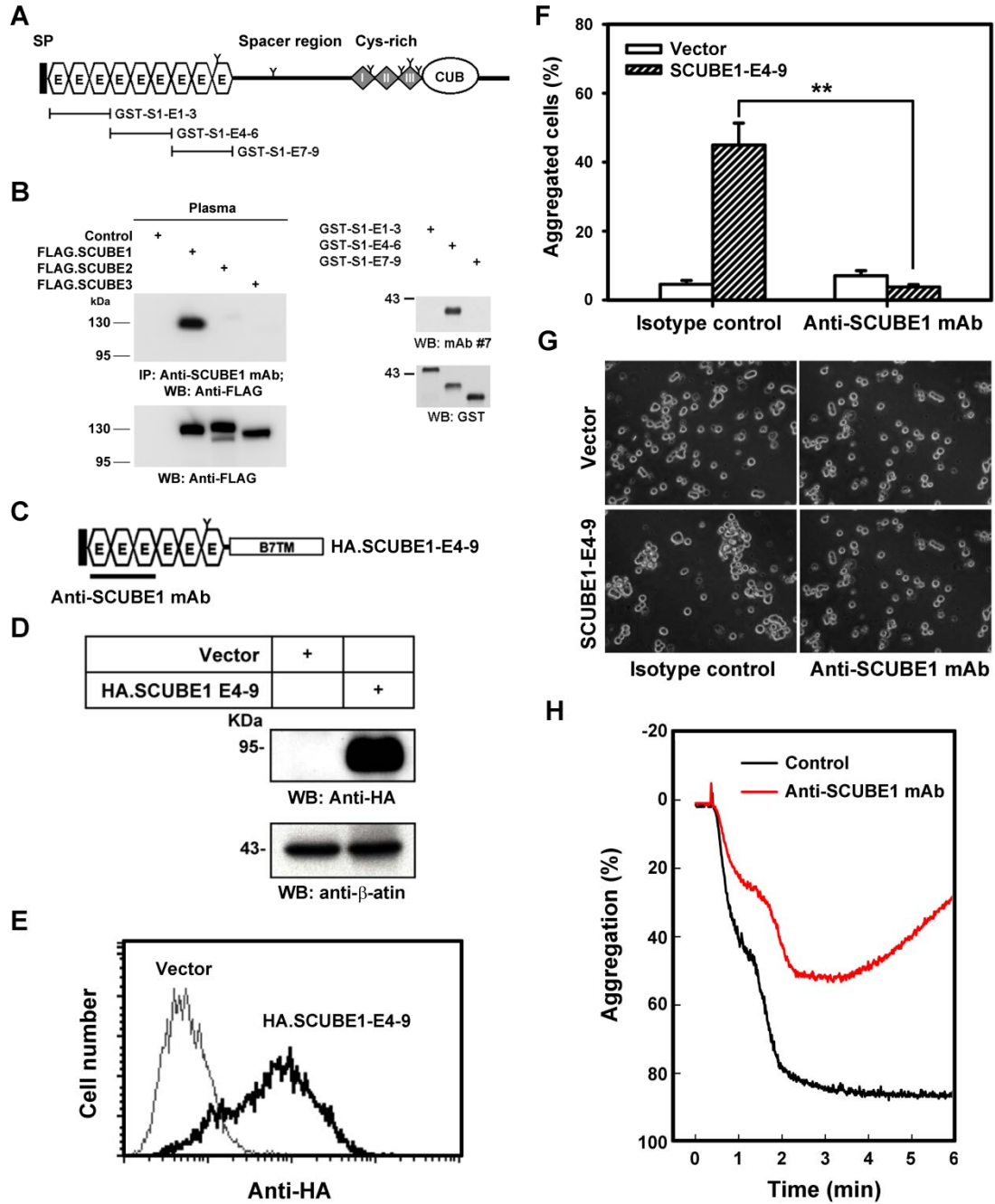
of A2058 cells expressing the SCUBE1 EGF-like repeats 4-9 on surface. Parental (Control) or S1-E4-9 stable cells were detached and allowed to aggregate in suspension culture in the presence of GST or GST-S1-E4-9 (1 µg/ml) in aggregation medium for 9 h. Aggregates were defined as clusters of four or more cells. *. P < 0.01. (D) Representative micrographs of aggregates formed by parental (Control) or S1-E4-9 stable line. The experiments were performed three times in duplicates with similar results. Original magnification, x100.

Suppl. FIG. XIV



Supplemental Figure XIV. The rSCUBE1 protein does not compete with fibrinogen (Fg) for binding to activated platelets. Washed +/+ platelets were preincubated with rSCUBE1 (1 $\mu\text{g/ml}$) or unlabelled Fg (100 $\mu\text{g/ml}$) for 3 min, followed by stimulation with ADP (10 μM) for 1 min or left unstimulated. Fg binding was measured by incubation with fluorescein isocyanate (FITC)-conjugated Fg (FITC-Fg) for additional 15 min. Resting, unstimulated platelets were served as the basal level for Fg binding (dotted line) when compared to activated platelets (thick line).

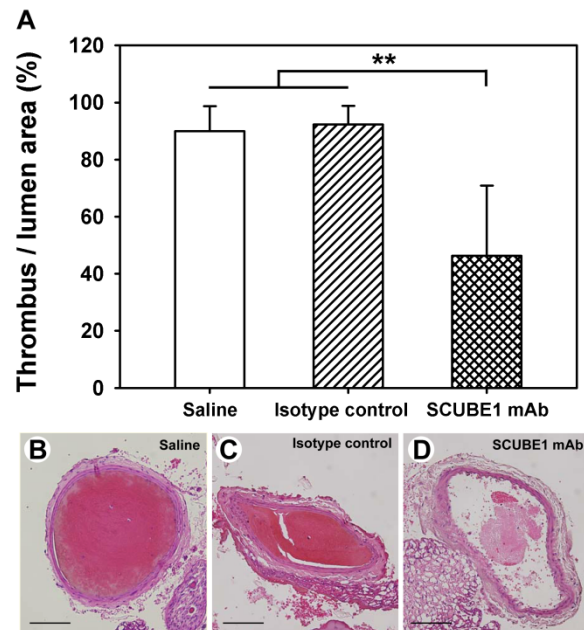
Suppl. FIG. XV



Supplemental Figure XV. Production of anti-SCUBE1 monoclonal antibody (mAb) and its effect on SCUBE1-mediated cell aggregation and platelet aggregation. (A) Diagram of the domain structure of SCUBE1 shows the location of the recombinant NH₂-terminal EGF-like repeat fragments fused with glutathione S-transferase (GST): GST-S1-E1-3 (amino acids 26-165), GST-S1-E4-6 (amino acids 157-285), and GST-S1-E7-9 (amino acids 284-412). The potential N-glycan sites were marked by “Y”. (B) Specificity of anti-SCUBE1 monoclonal antibody (mAb #7) by a pull-down assay or Western blot analysis. Recombinant SCUBE proteins

(FLAG-tagged) produced from HEK-293T cells were mixed with human plasma and subjected to a pull-down assay followed by Western blot analysis using anti-FLAG antibody, demonstrating that anti-SCUBE1 mAb #7 could specifically recognize soluble SCUBE1 protein but not SCUBE2 or SCUBE3 in the presence of plasma (left panel). Further epitope mapping showed that mAb #7 could bind to only the GST fusion protein containing the EGF-like repeats 4-6 of SCUBE1 (right panel). Molecular masses of marker proteins in kDa are indicated at the left margin. (C) Domain composition of SCUBE1 EGF-like repeats 4-9 (HA.SCUBE1-E4-9). EGF-like repeats 4-9 (residues 157-412) were fused to the B7 transmembrane (B7TM) to target the chimeric protein on the plasma membrane. An HA epitope tag was added to the NH₂ terminus for monitoring the protein expression. (D) Western blot analysis of the protein expression of the HA.SCUBE1-E4-9 construct. Protein lysates isolated from A2058 melanoma cells stably expressing HA.SCUBE1-E4-9 or stable integration of the empty vector (Vector) were probed with anti-HA antibody to verify the expression of HA.SCUBE1-E4-9 protein. (E) Flow cytometry of the A2058 stable line with anti-HA antibody to confirm the surface targeting of HA.SCUBE1-E4-9. (F) Anti-SCUBE1 mAb inhibited the homophilic SCUBE1-mediated cell aggregation. A2058 control (Vector) or HA.SCUBE1-E4-9 stable line was detached and allowed to aggregate in suspension culture in the presence of isotype control antibody or anti-SCUBE1 mAb (20 μg/ml) in aggregation medium for 2 h. Aggregates were defined as clusters of 4 or more cells. (G) Representative micrographs of aggregates formed by A2058 control (Vector) or HA.SCUBE1-E4-9 stable line after incubation with isotype control or anti-SCUBE1 mAb. The experiments were performed twice in duplicate with similar results. Data are mean ± S.D. **, P < 0.01. (H) Suppressive effect of anti-SCUBE1 mAb on human platelet aggregation. Human PRP was incubated with anti-SCUBE1 mAb (50 μg/ml) or saline (Control) for 5 min at 37 °C before stimulation with agonist ristocetin (1.25 mg/ml).

Suppl. FIG. XVI



Supplemental Figure XVI. Treatment of SCUBE1 antibody markedly suppressed arterial thrombosis. (A) The +/+ mice pretreated with saline (100 μ l, n=3), the isotype control antibody (100 μ g, n=3), or anti-SCUBE1 antibody (100 μ g, n=4) were induced to form thrombosis in the carotid artery by FeCl₃ injury for 3 min. Data are mean \pm S.D.; *, P < 0.01. Representative cross-sections of the carotid artery 3 min after injury of each group (B, saline; C, isotype control; D, SCUBE1 mAb) were shown at the bottom panel. Bar, 100 μ m.

Supplemental Table I. Primers used in this study

Gene	Forward	Reverse	Assay
<i>Scube1</i> (F1+R1)	CCT AGG TGA GCG GTG GGT AAT	GGT TGC CTG AGG ACA TAA GA	Genotyping
<i>Scube1</i> (F2+R2)	GGT GAG CGC TGG GTAATG GA	GAG GCA GGT GGA TCT TTG TGA GT	Genotyping
<i>Scube1</i> (F3+R3)	CCC CCA CCA CCC CTA TCA GAC AGA	TTG GGG GAA CGG GAG GTA AAG	RT-PCR
<i>Scube1</i> (F4+R4)	CGG CGG CGA ACT TGG TGA CTA CA	TTG ATA AAG GAC CGG GGG AAC AT	RT-PCR
<i>Scube2</i>	TGA CTA CCT GGT GAT GCG GAA AAC	CAG TGG CGT GTG GGA AGA GTC A	RT-PCR
<i>Scube3</i>	TGC TCC CCG GGC CAC TAC TAT	AGC GCT GTT GGC CTC ACT GGT CTT	RT-PCR
<i>Gapdh</i>	ATC ATC CCT GCA TCC ACT GGT GCT G	TGA TGG CAT TCA AGA GAG TAG GGA G	RT-PCR
<i>P2ry1</i> (P2Y1)	TCC AGA CCC CAG AAA TGT GT	GAA AAA CCC TTG AGC TTG GAA TGG	RT-PCR
<i>P2ry12</i> (P2Y12)	TGA GAA GAA AGC AAC AGA TG	ATG AAG GCC CAG ATG ACA ACA	RT-PCR
CD45	GGA GCA CAT GAG TCA TTA GAC ACA CTG ATG	GCA CAG CTG ATC TCC AGA TAT GAC CAT GGG T	RT-PCR
GP1b α	GGG TGG AAG GAA AGT GAG AAT	CAG CAG GAA GAG CAA GAT GAG	RT-PCR

Supplemental Table II. Hematological and coagulation parameters in +/+ and Δ/Δ mice

Hematological / coagulation parameters	+/+	Δ/Δ
Platelets ($10^{10}/L$)	102.7 \pm 9.8	98.1 \pm 7.2
White blood cells ($10^9/L$)	5.9 \pm 2.0	5.1 \pm 1.8
Red blood cells ($10^{12}/L$)	10.5 \pm 0.3	10.4 \pm 0.2
Hematocrit (%)	53.9 \pm 2.1	53.2 \pm 1.1
Hemoglobin (g/dL)	15.7 \pm 0.5	15.5 \pm 0.4
MPV (fL)	5.8 \pm 0.3	5.6 \pm 0.3
PT (s)	10.2 \pm 0.1	9.9 \pm 0.1
aPTT (s)	25.4 \pm 0.2	26.1 \pm 0.4

Data are mean \pm S.D. (n = 5). Data did not significantly differ between groups (P > 0.05). MPV, mean platelet volume; PT, prothrombin time; aPTT, activated partial thromboplastin time.