A novel mechanism of thrombocytopenia by PS exposure through TMEM16F in sphingomyelin synthase 1 deficiency

Supplemental Materials and Methods: Establishment of Rosa-CreER, SMS1\(^{\text{fl/fl}}\), and tamoxifen-induced SMS1-deficient mice

The generation of mice bearing the Sgms1 flox allele (SMS1\(^{\text{fl/fl}}\)) and their cross with Rosa-CreER (stock no: 008463; Jackson Laboratories, Bar Harbor, ME, USA) has been described previously.\(^1\)\(^2\) To achieve postnatal deletion of the Sgms1 allele, 28-week-old mice were treated six times over 2 weeks with 200 mg/kg tamoxifen (Tmx) (Sigma-Aldrich, St. Louis, MO, USA) administered by intraperitoneal injection. One month after the final Tmx injection, mice were used for experiments. For genotyping of SMS1 deletion allele of the Sgms1 gene, genomic DNA was extracted from mice tails, and PCR was performed with the following primers: forward, 5′-AAA CAA CTT TGC CAG CTT CCC TCA CGT TCT-3′; and reverse, 5′-CTG GAG CAA TCT TGT AGC GAA CAG GCA ACA-3′.

Splenectomy

An incision was made on the left hypochondrium of an anesthetized mouse to expose the spleen. The splenic vessels were cauterized, and then the spleen was removed. After checking for any hemorrhages in the abdominal cavity, the peritoneum and skin were separately sutured.

Hematological analysis of peripheral blood

Peripheral blood was collected via inferior vena cava sampling into polypropylene tubes containing EDTA. The peripheral blood cells were counted using a pocH-100iV Diff hematology analyzer (Sysmex Corporation, Kobe, Japan).

Bleeding time assay

The tail of an anesthetized mouse was cut 2 mm from the tip. The tail tip was blotted with filter paper every 15 seconds. Tail bleeding times were defined as the time required for bleeding to stop, up to a maximum of 300 seconds.\(^3\)
Histology and immunohistochemistry

Murine spleens were fixed in 4% (w/v) paraformaldehyde (PFA) and embedded in paraffin. The bones, including the femora and tibiae, were fixed in 4% PFA, decalcified in citric-buffered formic acid, and then embedded in paraffin. Sections (4-µm thick) of spleen and bone were cut and stained with hematoxylin and eosin or anti-von Willebrand factor (vWF) antibody. Immunostained sections were counterstained with hematoxylin. The vWF-positive cells in several randomly chosen high-power fields were detected under a KEYENCE BZ9000 fluorescent microscope (Keyence, Osaka, Japan) and quantified. To check for the infiltration of macrophages and assess their co-localization with platelets or MKs, spleen sections were stained with anti-CD68-AlexaFluor 488 (BD Biosciences, San Jose, CA, USA) and anti-CD41-PE (BD Biosciences) antibodies. Images were taken under a TCS SP2 confocal laser microscope (Leica, Wetzlar, Germany).

Platelet collection and MK isolation

To collect platelets, the peripheral blood was centrifuged at 300 × g for 10 minutes at room temperature. Platelets were obtained as the platelet-rich plasma (PRP) between the erythrocyte-concentrated lower layer and upper plasma layer.

MKs were isolated from bone marrow by using a modified two-step separation method. Briefly, bone marrow cells were extracted from mouse femurs and tibias by flushing them with phosphate-buffered saline (PBS) containing 1% (w/v) bovine serum albumin (BSA) in a 26G needle (Terumo, Tokyo, Japan). The collected cells were then passed through a 200-µm mesh filter, centrifuged at 200 × g for 5 minutes at 4 °C, and resuspended in CATCH buffer (5.37 mM KCl, 0.44 mM KH₂PO₄, 0.14 M NaCl, 4.17 mM NaHCO₃, 0.134 mM Na₂HPO₄, 5.55 mM glucose, 1.4 mM adenosine, 2.74 mM theophylline, and 14.7 mM tri-sodium citrate dihydrate, pH 7.4). The solution was loaded on four discontinuous density layers of BSA solution with gravities ranging from 1.035–1.050 (pH 7.4) and subsequently subjected to ultracentrifugation at 10,000 × g for 30 minutes at 10 °C. The resulting supernatant was collected, mixed with an equal volume of CATCH buffer, and centrifuged at 300 × g for 10 minutes at 4 °C. The resulting cell pellet was resuspended with CATCH buffer, layered over the BSA gradient solution, and left for 30 minutes at room temperature. After removing the upper layer, CATCH buffer was added to the residual layers, and this mixture was subjected to centrifugation at 200 × g for 5 minutes at 4 °C. The resulting pellet was collected as the MK fraction.

Reticulated platelet measurement

Reticulated platelet counts were determined as previously described. Briefly, PRP was resuspended
with Tyrode’s buffer (138 mM NaCl, 2.9 mM KCl, 20 mM HEPES, 1 mM MgCl2, 3.3 mM Na2HPO4, and 5.6 mM glucose, pH 7.4), stained with Thiazole orange (Retic-Count™, BD Biosciences) for 15 minutes at room temperature, and subsequently fixed with 1% PFA. Reticulated platelets were detected with the MoFlo XDP cell sorter and analyzer system (Beckman Coulter, Miami, FL, USA) and are represented as percentages.

**Platelet life-span assay**

The analysis of platelet life-span was conducted as described previously.6,7 In brief, mice were intravenously injected with 600 µg of N-hydroxysuccinimide ester (NHS)-conjugated biotin (Sigma-Aldrich) dissolved in buffer containing 140 mM NaCl. Whole blood was collected at 0, 24, 48, 72, and 96 hours after injection and mixed with a 1/7 volume of acid-citrate-dextrose (ACD) (2.5% (w/v) sodium citrate, 2.0% (w/v) D-glucose, 1.5% (w/v) citric acid). A 5-µl sample of whole blood was mixed with 45 µl of modified Tyrode’s buffer containing 0.1% BSA and then stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD41 antibody and allophycocyanin (APC)-conjugated streptavidin (BioLegend, San Diego, CA, USA) at room temperature for 1 hour. The percentage of biotinylated CD41-positive platelets was detected by flow cytometry.

**Flow cytometry**

The measurements of membrane SM and PS were conducted as described previously.8,9 For SM detection, PRP was stained with lysenin-conjugated Venus (Venus-lysenin) and phycoerythrin (PE)-conjugated anti-CD41 antibody (anti-CD41-PE) for 30 minutes at room temperature. For measuring PS exposure, PRP and cells were suspended in Annexin V-binding buffer (BD Biosciences) and then stained with FITC-conjugated annexin V (FITC-annexin V; BD Biosciences) with or without anti-CD41-PE for 30 minutes at room temperature. For A23187 (Sigma-Aldrich) treatment, PRP was stained with FITC-annexin V and anti-CD41-PE with 10 µM A23187. Detection of the intracellular calcium (Ca2+) level was performed using the fluorometric dye fluo-4 acetoxymethyl ester (Fluo-4 AM) (Dojindo Lab, Kumamoto, Japan). PRPs were incubated with 5 µM Fluo-4 AM for 20 minutes at 37 °C, and then stained with anti-CD41-PE for 10 minutes at 37 °C. Stained PRP and cells were analyzed by Gallios (Beckman Coulter). Erythrocytes were collected from the lower layer of blood after centrifugation, and then suspended with PBS including 1% FBS and detected by APC-conjugated anti-Ter119 antibody. Regarding lymphocytes, spleens were isolated from mice into normal saline solution, homogenized, and then filtered with a nylon cell strainer (70-µm aperture). Filtered cells were centrifuged at 600 ×g for 10 min at 4 °C, re-suspended in PBS, and stained with PE/Cyanine7-conjugated anti-CD3 antibody. For the detection of PS in platelets by lactadherin, PRPs were pre-treated with or without 20 mM EGTA for
15 minutes on ice and then stained with FITC-conjugated lactadherin (Haematologic Technologies, Essex Jct., VT, USA) and anti-CD41-PE. For staining PS in tMEFs, the cells were pre-incubated with or without 20 mM EGTA for 30 minutes at 37 °C. After being washed with PBS, the cells were harvested and stained with FITC-conjugated lactadherin for 20 minutes. Stained PRP and cells were analyzed with Gallios (Beckman Coulter). Fluorescence intensity was quantified with Kaluza software (Beckman Coulter) and is represented as the mean fluorescence intensity (MFI).

**MK immunocytochemistry**

After staining with Venus-lysenin or FITC-annexin V and anti-CD41-PE, MKs were cytopspun onto glass slides and fixed with 3% (w/v) PFA for 10 minutes at room temperature. After being washed with PBS, the nuclei were counterstained with 4,6-diamidino-2-phenyindole. Images were obtained with an LSM710 confocal microscope (Carl Zeiss, Jene, Germany) with a 63× objective lens and analyzed using ZEN software (Carl Zeiss).

**Cell lines**

Mouse embryonic fibroblasts immortalized by SV40 large T antigen (tMEFs) were previously established from WT, SMS1-KO, and SMS2-KO mice.\(^{10}\) TMEM16F knockout SMS1-KO tMEFs (1KO/16FKO) were established via insertion of the single guide RNA targeting TMEM16F exon 7 (5′-CCC TCG GAG TAT ATA CAA GAA GC-3′) via the lentiCRISPRv2 vector (Addgene #52961).\(^{11}\) Briefly, SMS1-KO tMEFs were transfected with TMEM16F-CRISPR vector by using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer’s protocol, and the resulting single clones were analyzed by sequencing their genomic DNA. As a control, SMS1-KO tMEFs were transfected with an empty vector (1KO/vec). All tMEFs were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum.

**Calcium imaging**

The Ca\(^{2+}\) influx of tMEFs was measured with Fura-2 AM (Dojindo Lab) in accordance with the manufacturer’s protocol. Briefly, 1×10\(^5\) tMEFs were seeded on a 35-mm glass-bottomed dish and incubated in loading buffer containing 5 μM Fura-2 AM for 1 hour at 37 °C. After being washed with PBS, the dishes were placed on the stage of an ECLIPSE TE300 inverted microscope (Nikon, Tokyo, Japan) equipped with a 20× S-fluor objective. Fluorescence images were recorded and analyzed by the ARGUS/HiSCA video image analysis system (Hamamatsu Photonics, Hamamatsu, Japan). Image pairs
were captured at 10-second intervals. Fura-2 AM fluorescence was recorded at a 510-nm emission wavelength and at 340-nm and 380-nm excitation wavelengths. The 340–380 nm fluorescence ratio (R_{340nm/380nm}) was measured in ten regions of interest (ROIs).

**DRM fractionation and western blot analysis**

tMEFs were lysed with buffer containing 1% (w/v) Brij 58, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM Na_{3}VO_{4}, and a protease inhibitor cocktail, and then centrifuged at 1,000 \times g for 5 minutes. The resulting supernatant was collected, adjusted to 40% (w/v) sucrose, and placed into the bottom of an OpitiPrep™ gradient containing 5% and 30% (w/v) iodixanol solutions (Axis-Shield Alere Technologies, Oslo, Norway). The gradients were centrifuged at 100,000 \times g for 16 hours at 4 °C. Fractions (500 µl/fraction) were collected, and fractions 8–10 were used as the DRM fraction. The proteins within each fraction were separated via SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were incubated in Blocking One solution (Nakalai Tesque, Kyoto, Japan) for 10 minutes to block non-specific binding and then incubated overnight at 4 °C with anti-TMEM16F antibody, which was kindly provided by S. Nagata (Osaka University). The DRM fraction was confirmed using anti-flotillin antibody (BD Biosciences), which is a well-known DRM marker. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Promega, Madison, WI, USA). Immunoactive protein bands were detected with SuperSignal West Dura extended duration substrate (Thermo Fisher Scientific) and the LAS4000 system (Fujifilm, Tokyo, Japan).

**Flipping assay**

The flipping assay was conducted as described previously. Briefly, WT and SMS1-KO tMEFs were washed with Hanks’ balanced salt solution HBSS (Wako, Tokyo, Japan), gently scraped, and harvested via centrifugation. After being kept on ice for at least 10 minutes, the cells were incubated with 0.1 µM of nitrobenzoxadiazole (NBD)-conjugated PS (Avanti Polar Lipids, Alabaster, AL, USA) or NBD-conjugated phosphatidylethanolamine (NBD-PE) (Avanti Polar Lipids) for 10 minutes at 15 °C. The cells were then resuspended in HBSS containing 3.0 mg/ml BSA to stop the reaction and finally analyzed with flow cytometry. NBD fluorescence was detected by using a 488-nm laser for excitation with an emission wavelength of 525 nm.

**SM depletion and supplementation**
Membrane SM depletion and exogenous SM supplementation were performed as previously described. For the depletion of membrane SM by bacterial sphingomyelinase (BSM) (Sigma-Aldrich), WT tMEFs and platelets were incubated with 20 mU/ml of BSM for 10 minutes. For exogenous SM supplementation, SMS1-KO tMEFs and platelets were treated with 5 µM C6-SM (Matreya, Pleasant Gap, PA, USA) for 10 minutes. The membrane SM, PS exposure, and Ca2+ influx were then analyzed with flow cytometry.

Quantitative real-time PCR

Total RNA was extracted from tMEFs and platelets using a RNeasy mini kit (Qiagen, Hilden, Germany), and cDNA was generated from the total RNA by using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). Quantitative real-time PCR (qPCR) was performed using the QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific) with TaqMan probes (Thermo Fisher Scientific) for Tmem16f (Mm00614693_m1) and Xkr8 (Mm01187363_m1) and the TaqMan master mix (Thermo Scientific). The expression of each gene was normalized with Actb (β-actin; Mm00607939_s1) expression and is indicated as the fold increase of Tmem16f expression in WT tMEFs and platelets.

REFERENCES

1. Ohnishi T, Hashizume C, Taniguchi M, et al. Sphingomyelin synthase 2 deficiency inhibits the induction of murine colitis-associated colon cancer. *FASEB J*. 2017;31(9):3816-3830.
2. Matsumoto G, Hashizume C, Watanabe K, Taniguchi M, Okazaki T. Deficiency of sphingomyelin synthase 1 but not sphingomyelin synthase 2 reduces bone formation due to impaired osteoblast differentiation. *Mol Med*. 2019;25(1):56.
3. Dejana E, Quintana A, Callioni A, de Gaetano G. Bleeding time in laboratory animals. III - Do tail bleeding times in rats only measure a platelet defect? (the aspirin puzzle). *Thromb Res*. 1979;15(1-2):199-207.
4. Levine RF, Fedorko ME. Isolation of intact megakaryocytes from guinea pig femoral marrow. Successful harvest made possible with inhibitions of platelet aggregation; enrichment achieved with a two-step separation technique. *J Cell Biol*. 1976;69(1):159-172.
5. Kienast J, Schmitz G. Flow cytometric analysis of thiazole orange uptake by platelets: a diagnostic aid in the evaluation of thrombocytopenic disorders. *Blood*. 1990;75(1):116-121.
6. Nayak MK, Kulkarni PP, Dash D. Regulatory role of proteasome in determination of platelet life span. *J Biol Chem*. 2013;288(10):6826-6834.
7. Zhao L, Liu J, He C, et al. Protein kinase A determines platelet life span and survival by regulating apoptosis. *J Clin Invest*. 2017;127(12):4338-4351.
8. Taniguchi M, Tsuchida T, Ninomiya H, et al. Sphingomyelin generated by sphingomyelin synthase 1 is involved in attachment and infection with Japanese encephalitis virus. *Sci Rep*. 2016;6:37829.
9. Taniguchi M, Ogiso H, Takeuchi T, Kitatani K, Umehara H, Okazaki T. Lysosomal ceramide generated by acid sphingomyelinase triggers cytotoxic cathepsin B-mediated degradation of X-linked inhibitor of apoptosis protein in natural killer/T lymphoma cell apoptosis. *Cell Death Dis*. 2015;6:e1717.
10. Asano S, Kitatani K, Taniguchi M, et al. Regulation of cell migration by sphingomyelin synthases: sphingomyelin in lipid rafts decreases responsiveness to signaling by the CXCL12/CXCR4 pathway. *Mol Cell Biol*. 2012;32(16):3242-3252.

11. Sanjana NE, Shalem O, Zhang F. Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods*. 2014;11(8):783-784.

12. Fujii T, Sakata A, Nishimura S, Eto K, Nagata S. TMEM16F is required for phosphatidylserine exposure and microparticle release in activated mouse platelets. *Proc Natl Acad Sci U S A*. 2015;112(41):12800-12805.

13. Tsuchiya M, Hara Y, Okuda M, et al. Cell surface flip-flop of phosphatidylserine is critical for PIEZO1-mediated myotube formation. *Nat Commun*. 2018;9(1):2049.
Supplemental Figure Legends:

**Supplemental figure S1. Platelet numbers in mice with postnatal SMS1 deletion.** (A) *Sgms1* flox allele targeting strategy. The structures of the wildtype (WT) allele (top), *Sgms1* flox allele (middle), and *Sgms1* exon 6 deletion allele (Δ*Sgms1*) (bottom) after Cre-mediated recombination are shown. Arrows indicate the primer position to detect the Δ*Sgms1* allele. (B) Identification of Cre recombination of the Δ*Sgms1* allele. Genomic DNA from the tail was used for PCR. M, size marker. (C) Peripheral blood was collected and counted with CBC. Values represent the means ± S.D. *p < 0.05, **p < 0.005.

**Supplemental figure S2. Measurement of the ceramide levels in platelets.** The same platelets used to generate the data shown in Figure 3C were measured by LC-MS/MS to detect ceramide and hexocylceramide (HexCer) levels. Values show the means ± S.D.

**Supplemental figure S3. PS exposure in T lymphocytes and erythrocytes.** (A) Splenocytes were isolated from WT, SMS1-KO, and SMS2-KO mouse spleens (n = 6 per group). T lymphocytes were stained with PE/Cyanine7-conjugated anti-CD3 antibody. (B) Peripheral blood was collected from WT, SMS1-KO, and SMS2-KO mice (n = 3 per group). Erythrocytes were subjected to staining by APC-conjugated anti-Ter119 antibody. Cells were pre-treated with A23187 for 10 minutes before membrane PS was stained with FITC-annexin V and detected by flow cytometry. The percentages of FITC-annexin V-positive cells are presented. Values show the means ± S.D. *p < 0.05.

**Supplemental figure S4. Effect of splenectomy on platelet and red blood cell counts in WT and SMS1-KO mice.** Peripheral blood was collected after splenectomy and counted with complete blood count analysis. (A) Fold increase of platelets relative to day 0 in WT or SMS1-KO mice. The corresponding platelet numbers are indicated in Figure 4D. Values show the means ± S.D. *p < 0.05, **p < 0.005. (B) Numbers of red blood cells. Values show the means ± S.D. *p < 0.05, **p < 0.005.

**Supplemental figure S5. Lifespans of WT and SMS1-KO mouse platelets.** Details of the platelet lifespan assay used to generate the data shown in Figure 4E. At 0, 24, 48, 72, and 96 hours after the injection of NHS-conjugated biotin, whole blood was collected and stained with anti-CD41-PE and APC-conjugated streptavidin. The percentage of biotinylated platelets (CD41⁺) was analyzed by using flow cytometry.
Supplemental figure S6. Ca\textsuperscript{2+} influx in platelets from WT or SMS1-KO mice. The Ca\textsuperscript{2+} influx of the cells used to generate the data shown in Figure 4E was analyzed with flow cytometry. Platelets were detected by staining with anti-CD41-PE.

Supplemental figure S7. Detection of PS exposure with lactadherin in platelets and tMEFs. (A–B) Membrane PS on platelets (A) and tMEFs (B) of wildtype (WT) or SMS1-KO mice were stained with FITC-conjugated lactadherin. To examine whether PS externalization is Ca\textsuperscript{2+}-dependent or -independent, platelets and cells were pre-treated with or without 20 mM EGTA. PS was then stained with FITC-conjugated lactadherin and analyzed with flow cytometry. Values show the means ± S.D. *p < 0.05, **p < 0.005.

Supplemental figure S8. Flipping assay of NBD-PS and NBD-PE in WT or SMS1-KO tMEFs. Flow cytometry analysis of inward translocation (flipping) activity of NBD-PS and NBD-PE in WT or SMS1-KO tMEFs. NBD fluorescence was detected at an emission wavelength of 525 nm by performing flow cytometry with a 488-nm laser for excitation. Values show the means ± S.D. **p < 0.005.

Supplemental figure S9. Effects of membrane SM modification on PS exposure and Ca\textsuperscript{2+} influx. (A–C) WT tMEFs were treated with or without 20 mU/ml bacterial sphingomyelinase (BSM) for 10 minutes. (D–F) SMS1-KO tMEFs were supplemented with 5 μM C\textsubscript{6}-SM for 10 minutes. (A and D) Membrane SM was stained with Venus-lysenin. (B and E) Cell surface PS was detected by APC-conjugated annexin V. (C and F) The intracellular Ca\textsuperscript{2+} levels were examined by Fluo-4 AM staining. Fluorescence was analyzed with flow cytometry, and the fluorescence intensity is presented as MFI. (G) WT platelets were stained with or without 20 mU/ml of BSM for 10 minutes. (H) SMS1-KO platelets were treated with 5 μM of C\textsubscript{6}-SM for 10 minutes. Platelets were stained with anti-CD41-PE and FITC-annexin V, and the percentages of FITC-annexin V-positive platelets (CD41\textsuperscript{+}) are presented. Values show the means ± S.D. *p < 0.05, **p < 0.005.

Supplemental figure S10. Expression of Tmem16f and Xkr8 in tMEFs and platelets. (A–B) Tmem16f and Xkr8 transcripts in tMEFs (A) and platelets (B) were analyzed with qPCR. Each mRNA level was normalized with β-actin mRNA and is presented as the fold increase of Tmem16f expression.
in WT tMEFs and platelets, respectively. Values show the means ± S.D. **p < 0.005.

**Supplemental figure S11. Cytosolic Ca\(^{2+}\) level in SMS1-KO tMEFs treated with a Ca\(^{2+}\) channel inhibitor.** (A–B) The relative R\(_{340\text{nm}/380\text{nm}}\) value of SMS1-KO tMEFs treated with the T-type Ca\(^{2+}\) channel inhibitor Pimozide (20 μM) (A) or the store-operated Ca\(^{2+}\) entry blocker YM58483 (3 μM) (B). Cytosolic Ca\(^{2+}\) levels were measured with Fura-2 AM and are presented as the fold increase over that in vehicle (DMSO)-treated controls. Values show the mean ± SD.
**Supplemental Figure S1**

**A**

- **Sgms1 wt allele**
  - BamHI
  - 9.8kb
  - AseI

- **Sgms1 flox allele**
  - BamHI
  - loxP
  - frt
  - 9.4kb
  - AseI

- **ΔSgms1 allele (+Cre)**
  - BamHI
  - loxP
  - frt
  - Recombination by Cre

  After recombination: 400 bp

**B**

- M
- 500bp
- **Rosa-Cre; SMS1^f/f^**
- Tmx
- **ROSA-Cre; SMS1^f/f^**
  - + Tmx
  - + Tmx

**C**

- **Platelets (x10^4/μL)**
  - **SMS1^f/f^**
  - **ROSA-Cre; SMS1^f/f^**
  - - +
  - - +:Tmx

- **Red blood cells (x10^5/μL)**
  - **SMS1^f/f^**
  - **ROSA-Cre; SMS1^f/f^**
  - - +
  - - +:Tmx

- **White blood cells (x10^2/μL)**
  - **SMS1^f/f^**
  - **ROSA-Cre; SMS1^f/f^**
  - - +
  - - +:Tmx

**Graphs**

- Black bars represent control groups.
- White bars represent treatment groups.

**Statistical Significance**

- ****
- ****
- ****
Supplemental Figure S4

A

**Platelet (fold increase of day 0)**

| Days after splenectomy | WT  | SMS1-KO |
|------------------------|-----|---------|
| 0                      | 1.0 | 0.5     |
| 2                      | 1.5 | 1.0     |
| 4                      | 2.0 | 1.5     |

B

**Red blood cells (10^5 cells/ml)**

| Days after splenectomy | WT  | SMS1-KO |
|------------------------|-----|---------|
| 0                      | 80  | 40      |
| 2                      | 100 | 80      |
| 4                      | 120 | 100     |
Supplemental Figure S6

CD41-PE

SS

WT

[CD41+ platelets]

SMS1-KO

CD41-PE

Counts

Fluo-4
Supplemental Figure S10

A

Tmem16f/Actin
Xkr8/Actin

Relative mRNA levels (Compared with Tmem16f of WT)

WT
SMS1-KO

B

Platelets

Tmem16f/Actin
Xkr8/Actin

Relative mRNA levels (Compared with Tmem16f of WT)
