SUPPLEMENTARY APPENDIX

Slc35a1 deficiency causes thrombocytopenia due to impaired megakaryocytopoiesis and excessive platelet clearance in the liver

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Supplementary Data

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This supplementary file includes:

1. Materials and methods

2. A total of 11 supplementary figures and corresponding legends

Materials and methods

Platelet preparation

Platelets were prepared according to established methods in our previous publications.[1, 3, 4] Briefly, whole blood was obtained from inferior vena cava using sodium citrate as anticoagulant, diluted 1:1 with Tyrode's buffer, and centrifuged at 100 g for 10 minutes at room temperature. The supernatant was transferred into a new tube and centrifuged at 800 g for 8 minutes to obtain the platelet pellet, followed by two subsequent washes with Tyrode's buffer. Platelet activation status was routinely verified after preparation by flow cytometry based on their surface expression of established activation markers such as P-selectin.

Peripheral blood cell count

Peripheral blood samples were obtained from cheek vein in collection tubes containing EDTA. White blood cell (WBC) count, hemoglobin (Hb) content and platelet count were performed with an automated hematology analyzer (Sysmex, Illinois, USA).

Bleeding time

Analysis of bleeding time was performed as described previously.[5] Mice were anesthetized and the tail was cut at 3 mm from the tip and immediately immersed in saline at 37°C. Tail bleeding time was determined as the time point at which all visible signs of bleeding from the incision had stopped or at 10 minutes.

Immunofluorescence staining of bone marrow

Immunofluorescence staining was performed according to established methods in previous publications.[1, 3, 4] Femora were obtained from mice and fixed with 4%

paraformaldehyde (PFA) overnight at 4˚C followed by overnight decalcification with 2% PFA containing 5% formic acid. After washes with PBS, bones were immersed in 20% sucrose solution at 4˚C overnight. Treated tissues were embedded in OCT compound (Tissue Tek, Sakura, Alphen aan den Rijn The Netherlands) and stored at -80˚C. Eight μm-thick frozen bone marrow sections were blocked with PBS containing 3% bovine serum albumin (BSA) for one hour at room temperature. Anti-CD41 antibody (MWReg30, 1:200, Abcam, Cambridge, UK) and RCA I (1:500) were used to stain megakaryocytes. DAPI was used to counter stain the nuclei.

Determination of thrombopoietin (TPO) and free sialic acid (SA) level by enzyme-linked immunosorbent assay (ELISA)

Whole blood was obtained from inferior vena cava and centrifuged at 800 g for 8 minutes to isolate serum. RayBio® Mouse TPO ELISA kit (Ray Biotech, Georgia, USA) was used to detect serum TPO levels according to the manufacturer's instructions. Whole blood was obtained from inferior vena cava and kept at room temperature for 2 hours. Afterwards, serum was collected from supernatant for the detection of serum free SA levels using mouse SA ELISA kit (Jonln, Shanghai, China).

Flow cytometry

Platelet and bone marrow megakaryocyte sialylation profiles were measured by flow cytometry after staining with either biotinylated RCA 1 or biotinylated MAL II (Vector Lab, California, USA) for 15 minutes at room temperature, followed by a wash with Tyrode's buffer. [1] These platelets and megakaryocytes were then stained with PE-streptavidin (BioLegend, California, USA) and FITC-anti-CD41 antibody (MWReg30, BioLegend,

California, USA) for 15 minutes at room temperature in the dark, followed by a wash with Tyrode's buffer and flow cytometry analysis. As a positive control, platelets were pre-incubated with sialidase (Roche, Basel, Switzerland) for 10 min. Samples with PE-streptavidin only were set as a negative control. To avoid MPV effect, we analyzed the ratios of MFI of RCA 1/CD41 and MFI of MAL II/CD41.[6] Analysis of reticulated platelets was performed according to previous method.[1] Briefly, 4 μl whole blood was diluted in 1:30 in FACS buffer, then incubated with 0.1 μg/ml thiazole orange (TO, Sigma-Aldrich, Missouri, USA) and 2 μg/ml PE anti–mouse CD41 (MWReg30, Biolegend, California, USA) for 15 minutes at room temperature in the dark. The reaction was immediately stopped by paraformaldehyde fixation. The percentage of reticulated platelets was determined by flow cytometry.[7] For the levels of GPIbα and GPIIb-IIIa on platelet membrane, platelets were stained with FITC-anti-GPIbα (XiaG5, Emfret Analytics, Eibelstadt, Germany), PE-anti-CD41 (MWReg30, BioLegend, California, USA), FITC-anti-CD61 (BioLegend, California, USA), and isotype-matched control antibodies for 15 minutes at room temperature in the dark, followed by a wash with Tyrode's buffer and flow cytometry analysis. For detecting platelet associated IgG (PAIgG) on platelets, washed platelets were lysed with cell lysis buffer for 15 minutes followed by pull-down of PAIgG with protein A/G-beads. PAIgG was detected by western blotting using HRP-anti-mouse IgG (H+L). Sialylation status on PAIgGs was determined by MAL II lectin blotting on purified PAIgGs.

Multi-color flow cytometric analysis was performed for progenitor analysis.[8, 9] Bone marrow cells were flushed by injecting 0.5% human serum albumin (HSA) and 10 mM EDTA containing Hank's buffer into femur and tibia. After red blood cell lysis by adding

ACK buffer, bone marrow cells were stained with antibody cocktail containing PE-anti-CD4 (L3T4, BD pharmingen, California, USA), CD8a (53-6.7, BD pharmingen, California, USA), CD19 (1D3, BD pharmingen, California, USA), CD11b (M1/70, BD pharmingen, California, USA), Sca1 (D7, BioLegend, California, USA), IL-7Rα (A7R34, BioLegend, California, USA), Pacific blue-anti-c-Kit (2B8, BioLegend, California, USA), APC-anti-CD150 (TC15-12F12.2, BioLegend, California, USA), PerCP-Cy5.5-anti-CD41 (MWReg30, BioLegend, California, USA), and PE-Cy7-anti-CD16/CD32 (93, BioLegend, California, USA) for 30 min at room temperature. Final concentration of all antibodies for staining was 1 μg/ml. Dead cells were excluded from analysis by staining with propidium iodide (PI) (5 μg/ml, Sigma-Aldrich, Missouri, USA) for 5 min at room temperature. For progenitor analysis, doublet cells were excluded on FSC/SSC scatter plot at first. Fluorescence minus one (FMO) control on each fluorophore was set to determine each threshold of positivity of staining. Percentages of individual population in lineage negative (Lin^-), Sca1 negative, IL-7Rα negative and c-Kit positive population were determined. Detailed gating strategy is shown in Supplementary Figure 10.

For polyploidy analysis, bone marrow cells were stained with PerCP-Cy5.5-anti-CD41 (MWReg30, BioLegend, California, USA) for 30 min at room temperature followed by additional staining with hoeschst33342 (10 μg/ml, Sigma-Aldrich, Missouri, USA) for 10 min at room temperature. After washing, polyploidy analysis was carried out by gating on large CD41 positive cells. Detailed gating strategy is shown in Supplementary Figure 11.

Isolation of megakaryocytes

Bone marrow megakaryocytes were isolated as previously described.[10] Briefly, bone marrow was isolated from femora and tibias of mice (8-12 weeks) with red blood cells lysed using RBC lysis buffer (Sangon Biotech, Shanghai, China). After wash, the remaining cells were labeled with anti-CD41 microbeads (Miltenyi Biotec, California, USA) and then loaded onto a MACS column in a MACS separator (Miltenyi Biotec, California, USA). The magnetically-retained megakaryocytes were then eluted using attached plundger.

Quantitative reverse transcription PCR (qRT-PCR) and reverse transcription PCR (RT-PCR)

RNA was extracted from megakaryocytes/platelets/liver by using a RNeasy Mini Kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was generated using a reverse transcription kit (Takara, Shiga, Japan) according to manufacturer's instructions. qRT-PCR was performed using an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, California, USA) with Fast SYBR Green Master Mix (Applied Biosystems, California, USA); the Ct index for each reaction was obtained. The following primer sets were used for the SYBR Green method: *Actb*-F: 5'-GCTCCTAGCACCATGAAGAT-3', *Actb*-R: 5'-GTGTAAAACGCAGCTCAGTA-3'; *Slc35a1*-F: 5'-GAACTGGCGAAGTTGAGTGTG-3', *Slc35a1*-R: 5'-TAGGTCACCTGGTACACTGCT-3'; *Mpl*-F: 5'-CCCACCTGGGAGAAATGTGAAGAG-3', *Mpl*-R: 5'-CCGGTGTAGGTCTGGAAGCGAGGG-3'. Levels of *Actb* transcripts were used as the references for all qRT-PCR experiments. The average of three duplicates was used to calculate the relative Ct against *Actb* ($\Delta Ct = Ct_{gene} - Ct_{Actb}$) for each sample. The average of

ΔCt from three samples was used to calculate ΔΔCt of experimental samples. RT-PCR was performed using an Applied Biosystems Veriti 96-Well Thermal Cycler (Applied Biosystems, California, USA). The following primer set was used: *Actb*-F: 5' GCTCCTAGCACCATGAAGAT3', *Actb*-R: 5'GTGTAAAACGCAGCTCAGTA3'; *Slc35a1*-F2: 5'-GCGAAGTTGAGTGTGCCATC-3', *Slc35a1*-R2: 5'-ACGTCACAACGATCCCTGAC-3' (from Exon 3 to Exon 6). RT-PCR system (total 20 μl) included 2×PCR Mix (Takara, Shiga, Japan) 10 μl, primer-F 1 μl, primer-R 1 μl, cDNA 3 μl and DEPC water 5 μl. Liver cDNA was set as a positive control.

Histology

Adult mice were anesthetized and femora were carefully dissected before fixing in 4% PFA at 4˚C overnight. The femora were washed with PBS for three times (30 minutes for each time), and immersed in 2% PFA containing 5% formic acid at 4˚C overnight for decalcification. Decalcified bones were processed and embedded in paraffin. Bones were cut into 5-μm thick sections and stained with hematoxylin and eosin (H&E). Megakaryocyte numbers were counted per image (40X) under Olympus light microscope (CX31, Olympus, Tokyo, Japan). Other tissues including liver, spleen, heart and kidney were fixed in 10% formalin. All tissues were then dehydrated, cleared, waxed and embedded in paraffin. Paraffin sections were cut into 5-μm thick sections and stained with H&E. Images were captured using Olympus light microscope with 100X oil immersion or 10X or 40X objective lenses, a digital camera (BX41, Olympus), and the controller software.

Assay of colony forming unit-megakaryocyte (CFU-MK)

CFU-MK was performed as previously described.[11] Briefly, bone marrow cells were suspended in IMDM (Gibco, Thermo Fisher Scientific, Massachusetts, USA) with 10% fetal bovine serum (FBS), stem cell factor (20 ng/ml, Peprotech, Rocky Hill, NJ, US), TPO (50 ng/ml, Peprotech, Rocky Hill, NJ, US), and antibiotics. A total of 5×10^5 bone marrow cells were mixed with methycellulose medium (MethoCult M3231, STEM CELL, Florida, USA), seeded onto 35 mm dishes, and cultured for 5 to 7 days in a cell culture incubator (37˚C, 5% CO_2). Colonies were picked up and stained with anti-CD41 antibody to confirm megakaryocyte colonies by flow cytometry. Megakaryocyte progenitor colonies were analyzed under Olympus light microscope (CX31, Olympus, Tokyo, Japan) and imaged with a digital light camera and its software.

Proplatelet formation assay

Megakaryocyte separation and proplatelet formation assay were adapted from previous publication.[12] Bone marrow cells were aseptically harvested from mouse femurs and tibias by flushing the bone marrow cavity with 3 ml Hank's medium (Procell, Hubei, China). The derived cells were gently triturated into single-cell suspension, and filtered through a 40-μm cell strainer (Corning, New York, USA). Three milliliters of bone marrow cell suspension were added to 4 ml of the 50% Percoll solution (Sigma-Aldrich, Missouri, USA) and centrifuged at 200 g for 20 minutes at 20°C. Cells were harvested from the interface, washed with Hank's medium at 90 g for 10 minutes at 4°C, and resuspended in 3 ml of the same medium. Next, enriched megakaryocytes were purified using density-gradient centrifugation with discontinuous BSA gradients (5 ml of 16%, 4%, and 3% BSA/Hank's with 10 ml of 2% BSA/Hank's medium). Cells were harvested from interface between 16% and 4%

BSA/Hank's medium. The derived cells were washed with Hank's medium, resuspended in Iscove's modified Dulbecco's medium (IMDM, Procell, Hubei, China), and cultured in a cell culture incubator (37˚C, 5% CO_2). Coverslips (NEST, New Jersey, USA) were coated with fibrinogen (100 μg/ml, Sigma-Aldrich, Missouri, USA) for 3 hours at 37°C. After two washes with PBS, the coverslips were blocked with BSA (5 mg/ml) for 1 hour at room temperature, followed by two washes with PBS. Then megakaryocytes were plated onto these fibrinogen-coated coverslips for 4 hours at 37°C. Cells were fixed with 4% PFA and permeabilized with Triton X-100 (0.1%) for 5 minutes. After blocking with 5% goat serum, these cells were stained with anti-β-tubulin (5 μg/ml, Sigma-Aldrich, Missouri, USA) and anti-CD41 antibodies (MWReg30, Abcam, Cambridge, UK). Goat anti-mouse IgG H&L (Alexa Fluor® 594, Abcam, Cambridge, UK) and donkey anti-rat IgG H&L (Alexa Fluor® 488, Abcam, Cambridge, UK) were used as secondary antibodies, respectively. DAPI (Beyotime, Shanghai, China) was used to counter stain the nuclei. Isotype control antibodies were normal rat IgG (Santa Cruz Biotechnology, Texas, USA) and mouse IgG (Beyotime, Shanghai, China). Cells were then analyzed using a confocal laser scanning microscope (Leica TCS SP8, Wetzlar, Germany).

Western blot analysis

Freshly isolated megakaryocytes or platelets were washed with PBS, and the pellet was re-suspended in cell lysis buffer (Cell Signaling Technology, Massachusetts, USA) containing protease inhibitor (100 ng/ml, Cell Signaling Technology, Massachusetts, USA). After centrifugation for 20 minutes at 12000 rpm and 4˚C, protein lysates were obtained from the supernatant. Proteins were transferred onto a nitrocellulose membrane and blocked with 3%

BSA for one hour at room temperature. For megakaryocyte samples, the nitrocellulose membrane was incubated with primary antibodies to Mpl (Abcam, Cambridge, UK) and β-Actin (Beyotime, Shanghai, China) at 4˚C overnight. For platelet samples, the nitrocellulose membrane was incubated with primary antibodies to GPIbα (Emfret Analytics, Eibelstadt, Germany) and β-Actin (Beyotime, Shanghai, China) at 4˚C overnight. After washes, the membrane was incubated with a relevant secondary antibody (Cell Signaling Technology, Massachusetts, USA) for one hour at room temperature. Images were captured after washes with TBST. For detection of platelet associated IgG, same number of platelets obtained from retro orbital sinus of mice were individually lysed with cell lysis buffer containing protease inhibitor followed by incubation with protein A/G-conjugated beads (Santa Cruz Biotechnology, Texas, USA) for 6 hrs at 4˚C to obtain PAIgG. After protein separation by SDS-PAGE, PAIgG was detected using HRP-conjugated anti-mouse IgG. Sialylation of PAIgG was detected using biotinylated MAL II (Vector Lab, California, USA) followed by blotting with HRP-Streptavidin (BioLegend, California, USA).

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Supplementary Figure Legends

Supplementary Figure 1. *Slc35a1*$^{-/-}$ platelets are deficient of functional *Slc35a1* transcripts. *Slc35a1* transcripts of WT and *Slc35a1*$^{-/-}$ platelets were analyzed by RT-PCR. cDNA from 5 mice were analyzed, respectively. Liver cDNA was used as a positive control. Arrowhead indicates a PCR amplicon.

Supplementary Figure 2. Optimization for flow cytometric analysis of platelet lectin staining. Platelets were stained with serially diluted biotinylated lectins for 15 min. After washing, platelets were further stained with PE-streptavidin for 15 min. After washing, lectin binding was analyzed by flow cytometry.

Supplementary Figure 3. Flow chart of MALDI-TOF/MS-MS analysis of glycan structures.

Supplementary Figure 4. Annotated MALDI-TOF/MS of permethylated *N*- and *O*-glycans from WT platelets or *Slc35a1*$^{-/-}$ platelets in negative mode. All annotated ions were $[M+Na]^+$. The number indicated above the peak in the spectra is the m/z value of the ion that has been detected by the mass spectrometer. (A) *N*-glycans on platelets, (B) *O*-glycans on platelets.

Supplementary Figure 5. Exogenous sialylated IgG are detected in WT and Plt *Slc35a1*$^{-/-}$ platelet lysates. Representative western blotting of platelet associated IgGs (reduced SDS-PAGE electrophoresis). IgG was purified with Protein A/G-beads from platelet lysates, and analyed by HRP-anti-mouse IgG (H+L). Input control is platelet lysates. Sialylation status on IgG was determined by biotinylated MALII lectin blotting followed by blotting with HRP-streptavidin. Blotting with HRP-streptavidin only is negative control (n = 3 mice per

genotype).

Supplementary Figure 6. Free sialic acid levels are comparable in WT and Plt *Slc35a1*$^{-/-}$ sera. Serum sialic acid concentration measured by ELISA. Data represent means ± SD. n = 10 mice/group. NS, no significance.

Supplementary Figure 7. Plt *Slc35a1*$^{-/-}$ mice exhibit macrothrombocytopenia. (A) The MPV of platelets was determined by Sysmex analyzer from WT (n = 14) and Plt *Slc35a1*$^{-/-}$ mice (n = 14). (B) Bleeding time of WT (n = 10) and Plt *Slc35a1*$^{-/-}$ mice (n = 10) was analyzed. Data represent means ± SD. NS, no significance. (C) Peripheral blood white blood cells (WBC) counts in WT (n = 10) and Plt *Slc35a1*$^{-/-}$ (n = 10) mice. (D) Hemoglobin content in peripheral blood from WT (n = 10) and Plt *Slc35a1*$^{-/-}$ (n = 10) mice. Data represent means ± SD. ***P <0.001. NS, no significance.

Supplementary Figure 8. No significant difference in histology analysis of organs between WT and Plt *Slc35a1*$^{-/-}$ mice. Hematoxylin-eosin (H&E)-stained sections from the liver (A), spleen (B), heart (C) and kidney (D) of WT and (n = 5) and Plt *Slc35a1*$^{-/-}$ mice (n = 5). For each panel, representative images on the left are with 10 × magnification (Bar, 160 μm) and images on the right are with 40× magnification (Bar, 40 μm).

Supplementary Figure 9. CD42b expression in WT and Plt *Slc35a1*$^{-/-}$ mouse platelets. The expression of CD42b on *Slc35a1*$^{-/-}$ (n=3) platelets is decreased compared with WT (n=3) platelets by Western blot analysis. β-actin was used as a loading control.

Supplementary Figure 10. Gating strategy for analysis of progenitors in bone marrow. Bone marrow cells collected from tibia and femur were treated with ACK buffer for hemolysis. After staining with antibody cocktail for 20 min in the dark followed by additional

staining with propidium iodine (PI) for 5 min, washed cells were analyzed by flow cytometry. FMO control, samples that contain all the antibodies minus one of them.

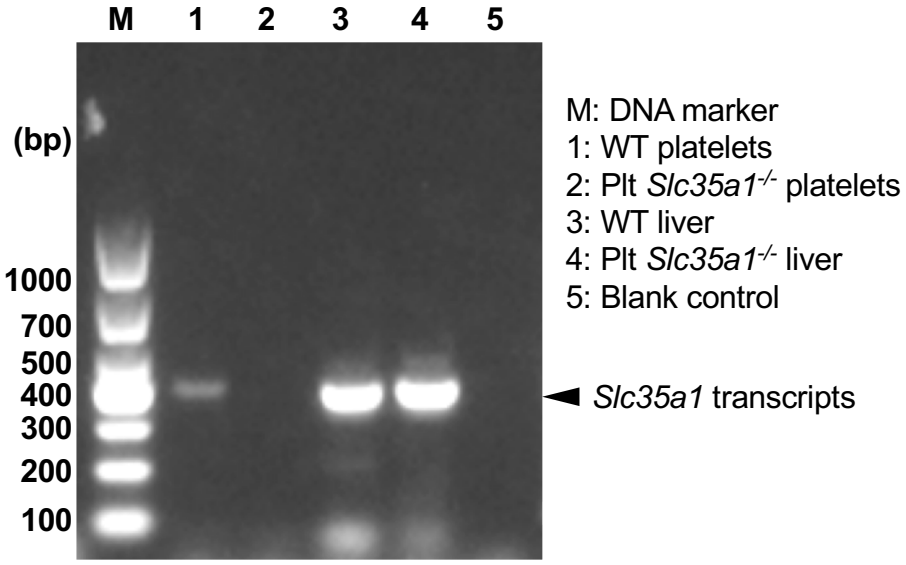
Supplementary Figure 11. Gating strategy for analysis of ploidy in bone marrow megakaryocytes. Bone marrow cells collected from tibia and femur were treated with ACK buffer for hemolysis. After staining with PerCP-Cy5.5-anti-CD41 antibody for 20 min in the dark followed by additional staining with hoechst33342 for 10 min, washed cells were analyzed by flow cytometry.

Supplementary Table 1

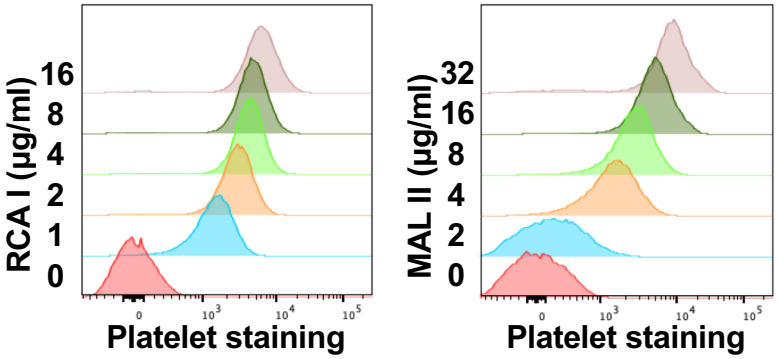
Summary of reduction of major sialylated *N*- and *O*-linked glyco-forms of *Slc35a1*$^{-/-}$ platelets compared with WT platelets based on the relative abundance of mass spectrometry

N-glycans		*O*-glycans	
m/z	*Slc35a1*$^{-/-}$ vs WT	m/z	*Slc35a1*$^{-/-}$ vs WT
774	0.830	669	0.191
1034	0.286		
1304	0.375		
1438	0.333		
1525	0.7		

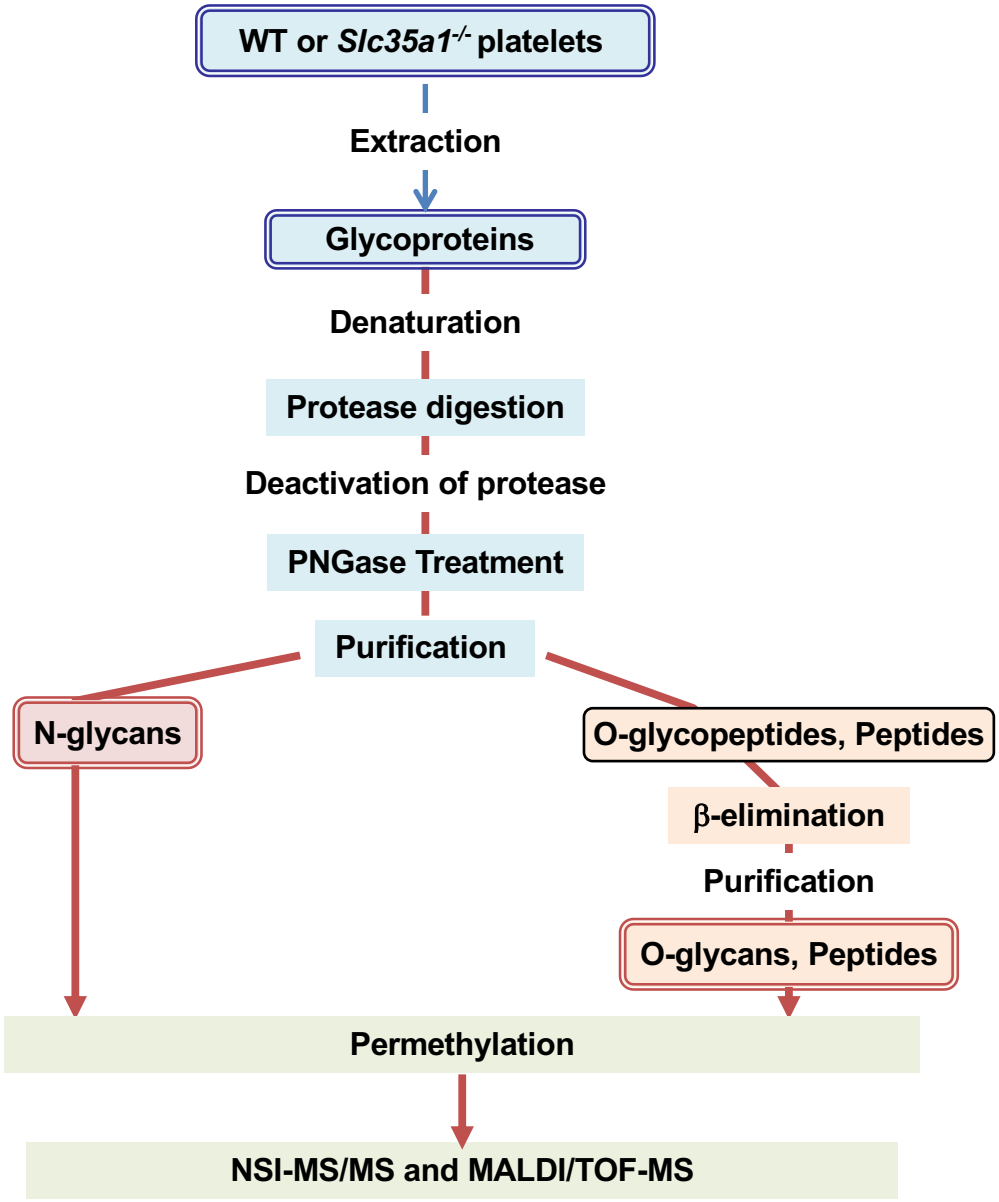
Supplementary Fig. 1



Supplementary Fig. 2

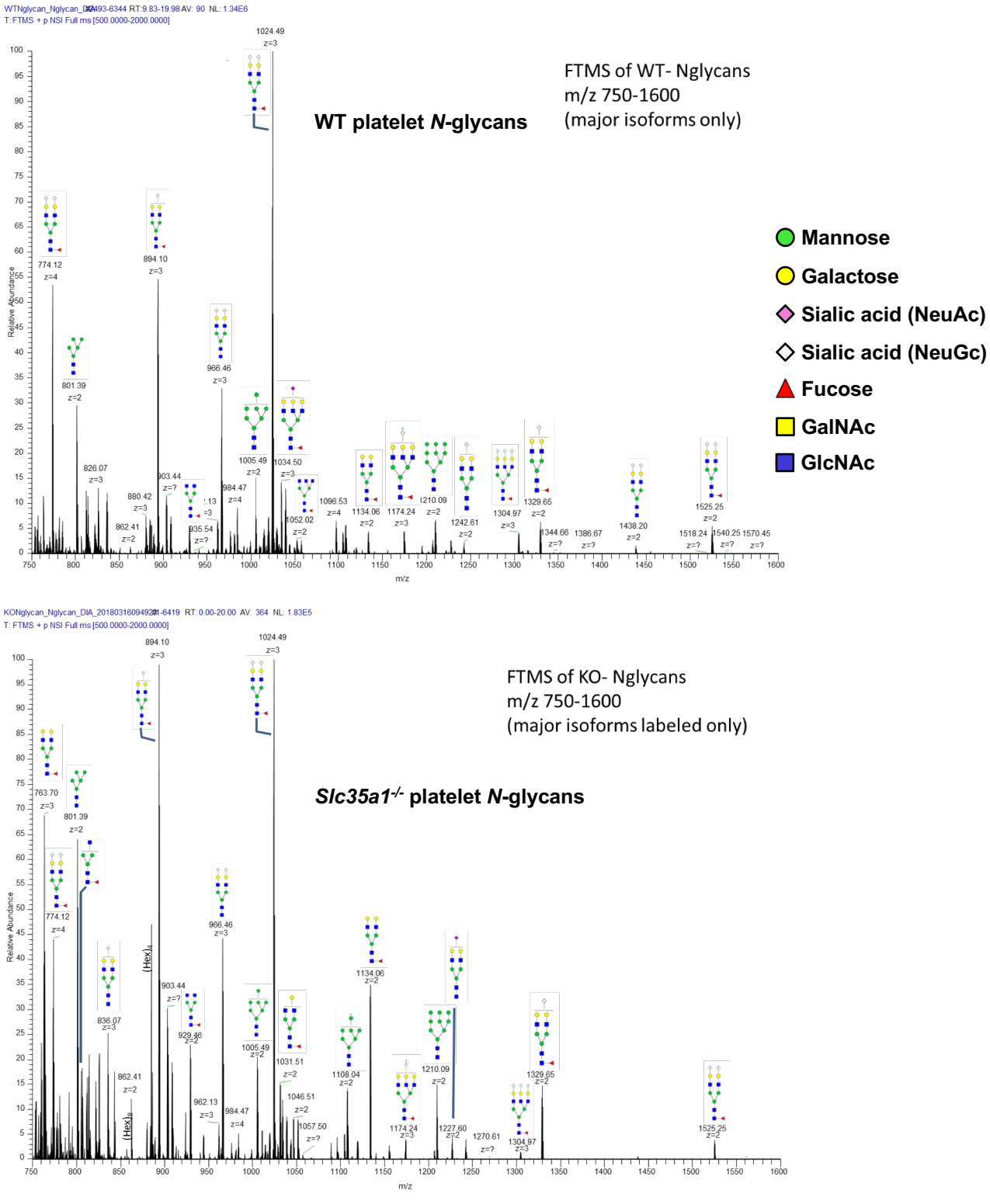


Supplementary Fig. 3

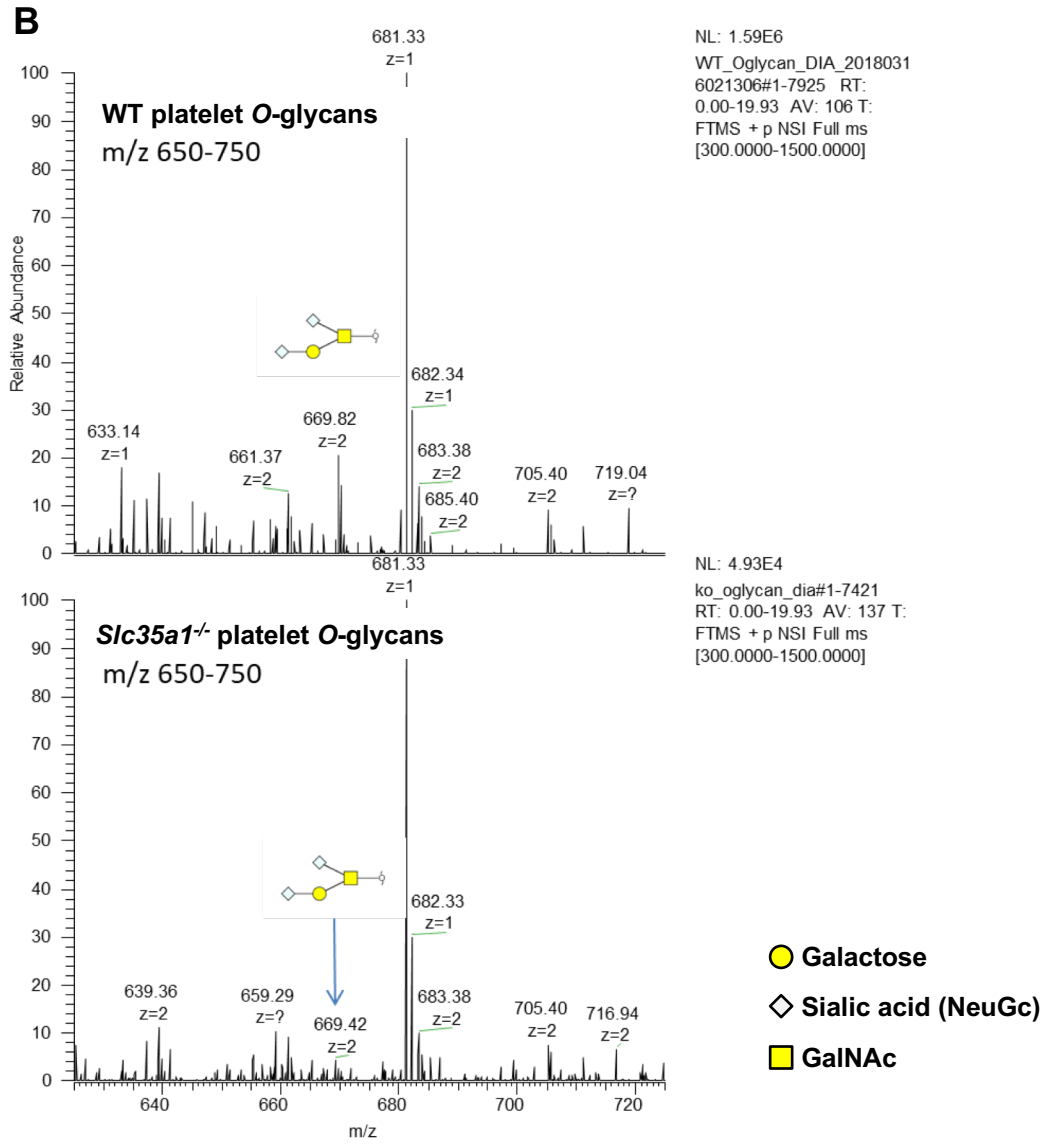


Supplementary Fig. 4

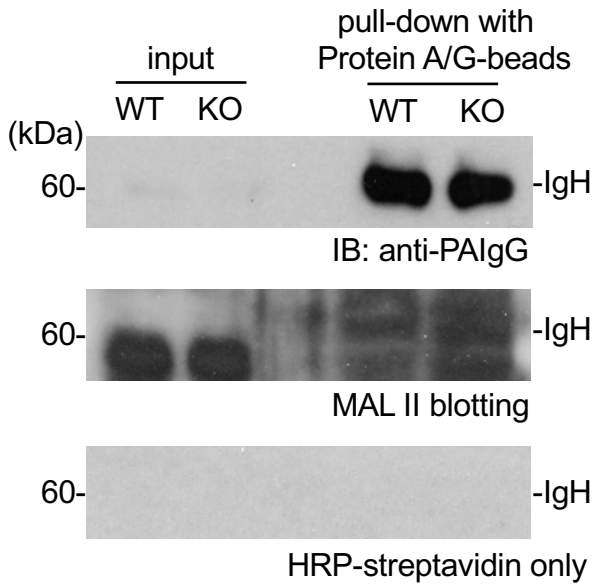
A



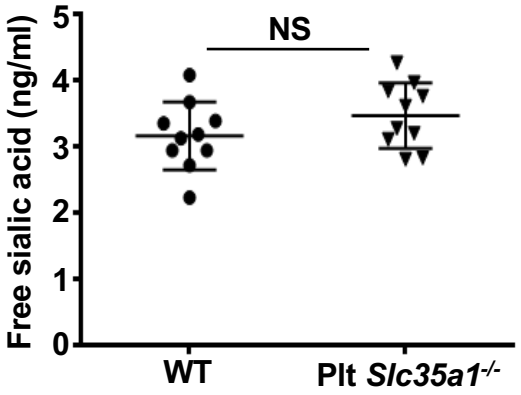
Supplementary Fig. 4



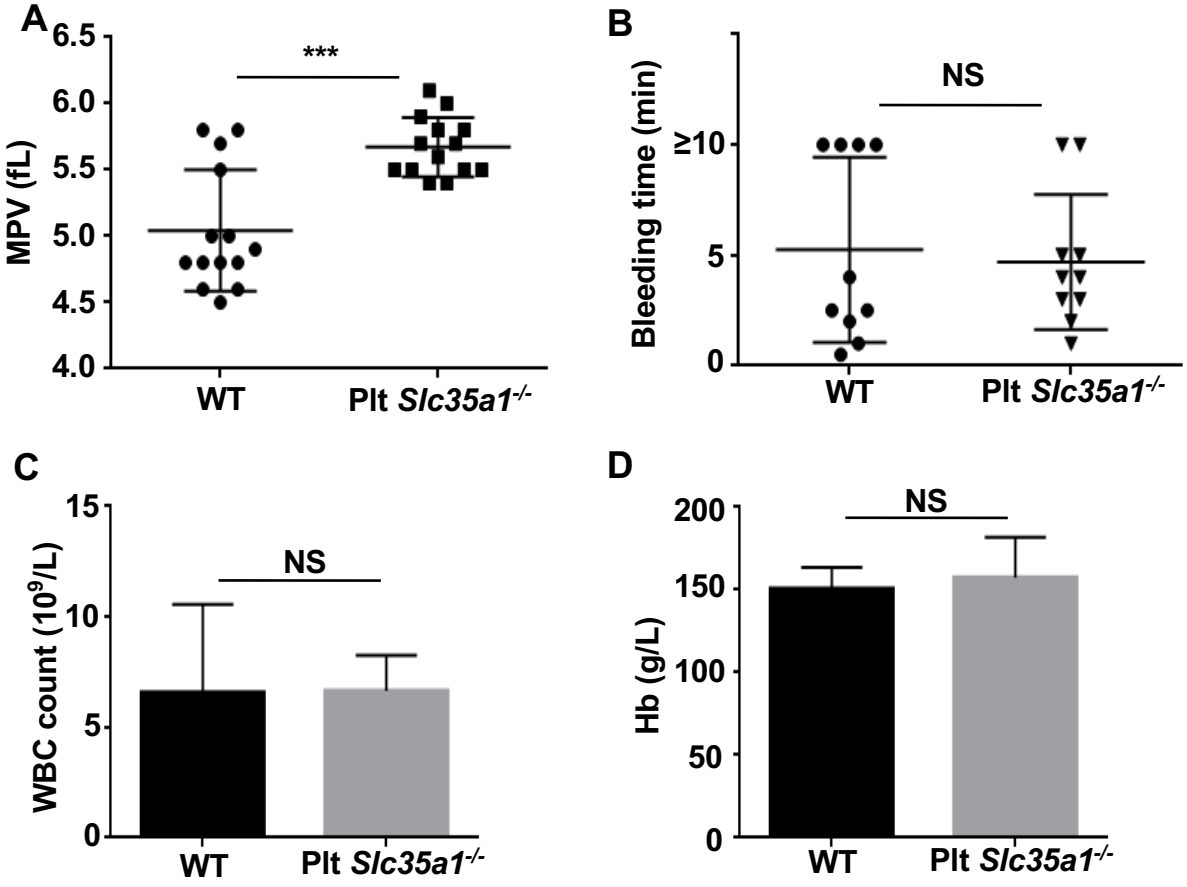
Supplementary Fig. 5



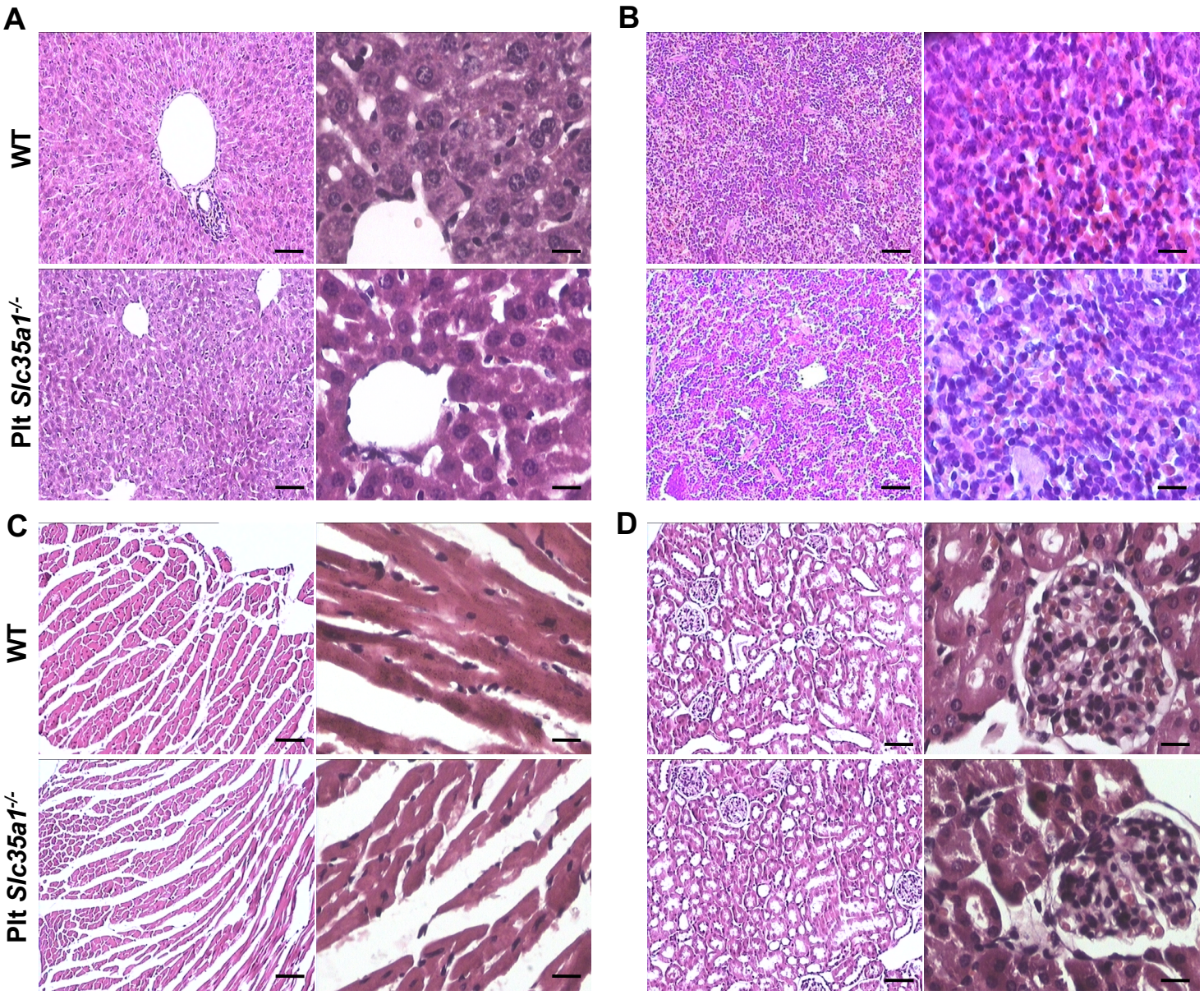
Supplementary Fig. 6



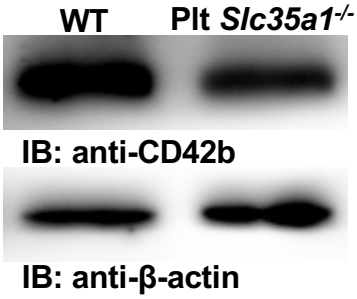
Supplementary Fig. 7



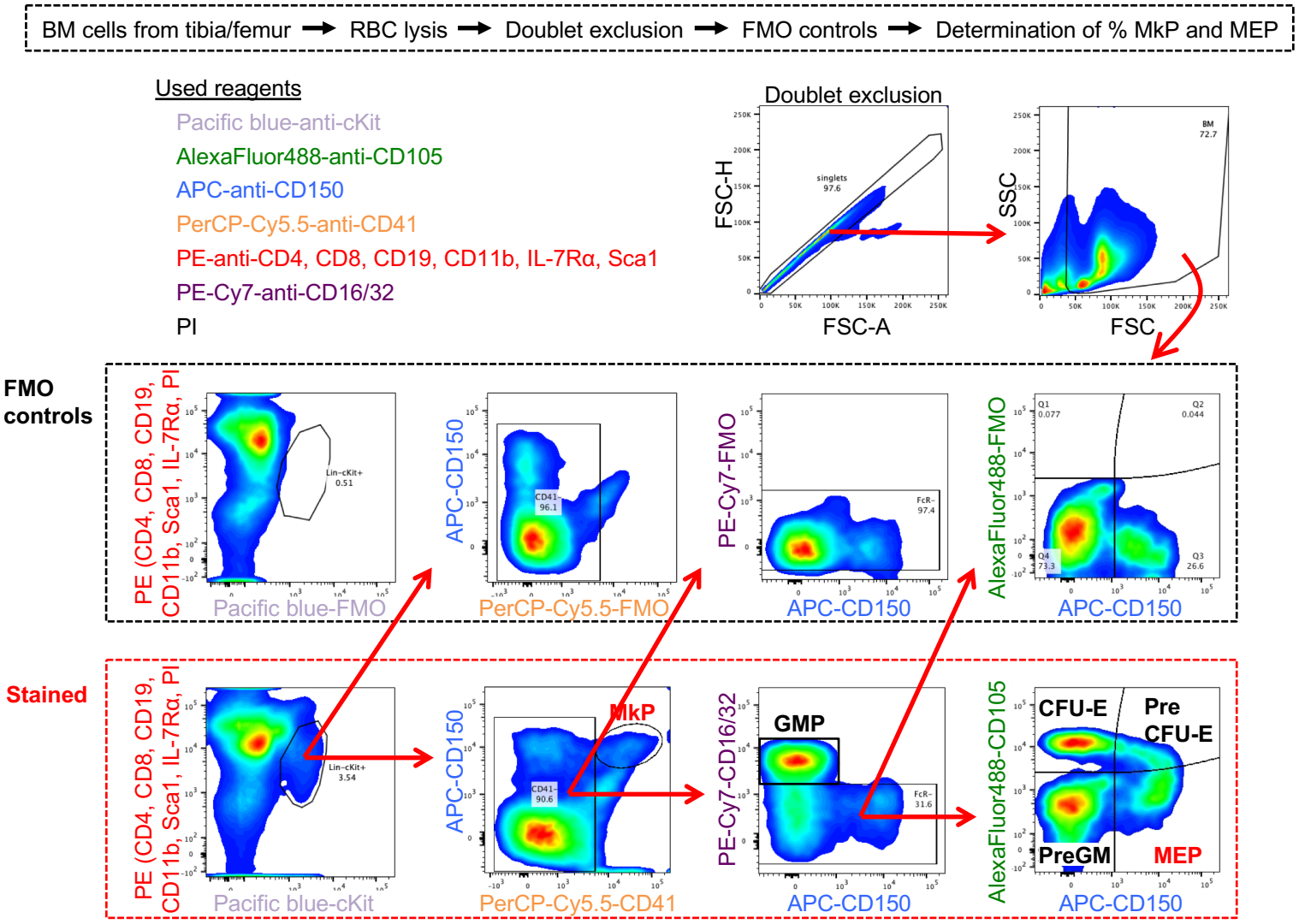
Supplementary Fig. 8



Supplementary Fig. 9



Supplementary Fig. 10



Supplementary Fig. 11

BM cells from tibia/femur → RBC lysis → Gate large CD41⁺ cells → FMO controls → Determination of % each ploidy

Used reagents

PerCP-Cy5.5-anti-CD41

Hoechst33342