Supplementary materials

Methods

Antibodies and reagents

FITC-conjugated P-selectin, GPVI, GPIbα and α2 and R-phycoerythrin (PE)-conjugated JON/A antibodies were from Emfret Analytics (Eibelstadt, Germany); PE-conjugated anti-αIIb antibody from BD Biosciences (Oxford, UK); phalloidin-488 from Thermo Fisher (Loughborough, UK); rat anti-mouse CLEC-2 antibody from Serotec (Oxford, UK) and Alexa488-conjugated goat anti-rat antibody from Life Technologies (Paisley, UK). G6b-B specific antibodies were raised as previously described.[33] For immunoblotting, anti-Shp1 and anti-Shp2 antibodies were from Santa Cruz Biotechnology (Dallas, TX, USA); anti-phosphotyrosine (p-Tyr) from Merck Millipore (Nottingham, UK); Src phosphotyrosine-418 (Src p-Tyr418) from Life Technologies (Loughborough, UK) and Syk phosphotyrosine-525/26 (Syk p-Tyr525/526) from Cell Signaling Technology (Hitchin, UK). All other reagents were from Sigma-Aldrich (Poole, UK), unless stated otherwise. Custom monoclonal mouse anti-human G6b extracellular domain, monoclonal rabbit anti-mouse G6b extracellular domain and polyclonal rabbit anti-human and –mouse G6b-B antibodies were generated by Biogenes (Berlin, Germany) as previously described (Mazharian et al, Sci Sign, 2012). Custom rabbit polyclonal antibodies for human G6b-A were raised against the intracellular tail of G6b-A (Biogenes, Berlin, Germany).

Genotype confirmation using Sanger sequencing

The *G6b* locus (NM_138272.2) was amplified with a single primer pair long range (LR) F & LR R1 with Bio-RadBioRad iProof HF master mix (Bio-Rad Laboratories, Hercules, CA), and sequenced with the same LR primers as well as primers designed to flank each exon. A description of the primers and Sanger sequencing conditions is shown in Supplementary Table 2.

DNA constructs

The cDNA encoding the human G6b-B protein was amplified by PCR from a human cDNA library. This PCR fragment was first cloned into the pCR®-Blunt vector (Invitrogen, Paisley, UK), and then subcloned into the pcDNA3 vector, for expression of untagged G6b-B in heterologous cell systems. The construct expressing the p.Gly157Arg mutant was generated by site directed mutagenesis (Genscript, Piscataway, NJ, USA).

Cell culture

DT40 chicken B cells were grown in RPMI supplemented with 10% fetal bovine serum, 1% chicken serum, 100 units (U)/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol, and 20 mM glutamine. 2 μg of *G6b-B WT*, *G6b-B Gly157Arg* or empty pcDNA3 vector were transfected by electroporation at 350 V and 500 microfarads into 2 x 10^7 cells DT40 cells, (resuspended in 400 μl serum free medium). Twenty hours after transfection, live cells were counted by trypan blue exclusion, and G6b-B expression levels were analyzed via flow cytometry and western blotting. Flow cytometry data presented as media fluorescence intensity.

G6b humanized mouse

To create the humanized G6b mouse, a targeting vector containing the human *G6b* genomic locus*,* a puromycin resistance cassette flanked by Flp-recombinase targeting sites and two homology arms was produced and inserted into the genome of C57BL/6NTac mouse embryonic stem cells by homologous recombination. Validated clones were injected into mouse blastocysts, before implanting into pseudo-pregnant female mice. Removal of the puromycin resistance cassette by a Flp recombinase expressing mouse subsequently allowed for *WT* and *KI* mice to be distinguished by PCR targeting the residual Flp-recombinase recognition site (Figure S3D, E). This *KI* strategy effectively knocked-out mouse *G6b* and allowed for physiologically normal promotor driven expression of human *G6b* in its place.

Mouse hematology

ACD anticoagulated whole blood was collected and blood cells counted using an ABX Pentra 60 hematological counter (Horiba Medical, Northampton, UK). Platelet count, platelet volume, white blood cell count and lymphocyte count data were normalized using a modified Box-Cox power transformation:

$$y' = \frac{(y^{\lambda} - 1)}{\lambda} + \lambda$$

When λ = 1: y' = y (No transformation)

When λ = 0: y' = ln(y) (natural log transformation)

Transformed data were verified to be normally distributed using Browne-Forsythe test for equal variance before being analyzed by one-way ANOVA with Dunnett's post-hoc test to compared each genotype to *WT*.

Flow cytometry

ACD anticoagulated whole blood were incubated with indicated FITC- and PE-conjugated antibodies before measuring using an Accuri C6 flow cytometer (BD Biosciences, Oxford, UK).

Platelet functional assays

Washed platelets (2×10^7/mL) were allowed to adhere and spread on fibrinogen-coated coverslips as previously described.[47] All slides were imaged using a Zeiss Axiovert 200M microscope and blinded images quantified using ImageJ. Stages of platelet spreading were categorized as unspread, formation of filopodia, formation of lamellipodia and fully spread (Figure S12). Platelet aggregation and ATP release was measured as previously described using washed platelets (2×10^8/mL) and a Chronolog Model 700 aggregometer.[48] ADP sensitive platelets were prepared as previously described.[49] For flow adhesion glass coverslips were coated as previously described,[50] and anti-coagulated whole blood (final concentrations: 40 μM PPACK, 5 U/ml heparin and 50 U/ml fragmin) flowed over for 3.5

minutes immediately following collection of blood. Images were captured with an EVOS microscope (Life Technologies, Carlbad, CA, USA) and blinded images analyzed using Fiji (Figure S9).

Supplemental Data: Clinical Case Descriptions

Family 1

Patient 1-III-1

Patient 1-III-1 was the male child of consanguineous parents (first cousins) of Arabic decent from the United Arab Emirates (UAE). The boy was born full term via normal spontaneous vaginal delivery (NSVD) following an uneventful pregnancy. The neonatal period was complicated by prolonged jaundice and dehydration. He was subsequently diagnosed with salt wasting due to congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency as a consequence of a homozygous intron 2 mutation (c.293-13C>G) in the *CYP21A2* gene). He was started on hydrocortisone and fludrocortisone supplementation with good clinical improvement. He was doing well until 5 months of age when he presented with prolonged epistaxis. A complete blood count (CBC) revealed anemia with a hemoglobin (HGB) of 5.0 g/dL and thrombocytopenia with $20x10^9$/L. The white blood cell count (WBC) was not available. The infant received packed red blood cells (PRBC) and platelet transfusions. His platelet count transiently improved to ~$100x10^9$/L. He remained thrombocytopenic with platelet counts ranging between 6-24 $x10^9$/L and required intermittent platelet transfusions (6 total). He also had a microcytic anemia with HGB ranging between 5-8 g/dL requiring occasional PRBC transfusions (3 total). The patient's bleeding symptoms were mild with occasional episodes of mild epistaxis and an episode of oozing from the bone marrow (BM) biopsy site requiring PRBC and platelet transfusion.

A BM aspirate and biopsy performed at another institution at 8½ months of age was interpreted as refractory cytopenia with myeloid dysplasia with no excess in blasts. Cytogenetics were reported to be normal. Concurrent blood counts showed a WBC count of $12.8x10^9$/L, HGB 9.3 g/dL with a mean corpuscular volume (MCV) of 72 fl and platelets of $16x10^9$/L. Hematopoietic stem cell transplantation (HSCT) was considered at the time but sibling donor evaluation was logistically difficult and HSCT was deferred.

Additional workup revealed a normal fetal HGB, negative viral studies (cytomegalovirus (CMV), Epstein-Barr virus (EBV)), no evidence of paroxysmal nocturnal hemoglobinuria (PNH) by flow cytometry and normal diepoxybutane (DEB) induced chromosomal breakage making an underlying Fanconi Anemia unlikely. His IgG was 592, IgA 63, and IgM 102. His lactate dehydrogenase (LDH) was elevated at 1091 (normal reference range 500-920). His peripheral blood (PB) smear showed mild leukocytosis, microcytic anemia with anisopoikilocytosis showing target cells, ovalocytes and thrombocytopenia with enlarged platelets. A repeat BM aspirate and biopsy at 13 months showed a hypercellular marrow for age with minimal granulocytic and erythroid dysplasia with a decreased myeloid-to-erythroid ratio (1:1) and moderate megakaryocytic dysplasia. Blasts were not increased. The biopsy revealed marked reticulin fibrosis. An iron stain was negative for ringed sideroblasts. Concurrent cytogenetic studies showed a normal male karyotype of 46, XY and negative fluorescence in situ hybridization (FISH) for del5q and monosomy 7. Overall the findings were thought to be consistent with a myeloproliferative or myelodysplastic process, although *JAK2* mutation analysis was negative.

Based on his persistent thrombocytopenia and anemia with ongoing transfusion needs he was referred for HSCT. A suitable matched sibling, unrelated donor or alternative donor was not available. Therefore, a haploidentical peripheral blood stem cell transplant (PBSCT) from the mother was done, using a submyeloablative conditioning regimen with Campath 1H, Fludarabine $30mg/m^2x4$ doses and total body irradiation (TBI) 600cGy

(HIMSUM, clinicaltrials.gov NCT00058825). Documentation of the cell dose and graft manipulation procedures were not available. Unfortunately, the patient failed to engraft with less than 10% BM cellularity comprised of all male karyotype cells on day 20 after his HSCT. He subsequently developed gram-negative bacterial sepsis and underwent a second haploidentical PBSCT from his mother ~50 days after his first PBSCT. Unfortunately, he developed progressive multiorgan failure, sever upper gastrointestinal bleeding due to a duodenal ulcer requiring several surgical interventions, renal and respiratory failure, and acute respiratory distress syndrome (ARDS), and succumbed from transplant related complications.

Patient 1-III-5

Patient 1-III-5, the 5-year old younger brother of 1-III-1, was born at full term via NSVD. He was cared for in the UAE during the first 10 months of his life. He was diagnosed with congenital adrenal hyperplasia (CAH) in the first week of life and received supplementation with hydrocortisone and fludrocortisone. He presented with bruising and petechiae on his upper chest on the first day of life. Given the family history of thrombocytopenia, a complete blood count (CBC) was obtained and showed a platelet count of $23x10^9$/L. He received a platelet transfusion with a post-transfusion count of $141x10^9$/L but quickly dropped again to 71 $x10^9$/L. His HGB and WBC were normal for age. A direct Coombs test was negative. A BM aspirate and biopsy at birth resulted in a non-diagnostic specimen. His initial workup included serology studies for TORCH infections (Toxoplasmosis, Other (syphilis, varicella-zoster, parvovirus B19), Rubella, Cytomegalovirus (CMV), and Herpes infections), which were negative. Mutation analysis for Wiskott-Aldrich syndrome was negative.

Given recurrent thrombocytopenia at 10 days of life he was treated with IVIG 1mg/kg IV x 2 days which improved his platelet count from $13x10^9$/L to $61x10^9$/L. Serial CBCs over the next several months documented platelet counts between 50 $x10^9$/L and $90x10^9$/L. By 6½

months of age he had recurrent severe thrombocytopenia to $16x10^9$/L and a new anemia with a HGB of 6.9 g/dL and received blood and platelet transfusions. A repeat BM showed a hypocellular, hemodilute aspirate with frequent lymphocytes. The biopsy revealed increased megakaryocytes and associated reticulin fibrosis similar to that seen in his older brother 1-III-1.

Beginning at 7-months of age, the patient became transfusion dependent for platelets, which he received every 10-days. He also required intermittent PRBC transfusions for a microcytic anemia. He never had leukopenia or neutropenia; his WBC was mildly elevated. He received a total of 3 IVIG treatment courses at day of life 10, and two- and four-months of age. He only showed a partial and transient improvement in his platelet count. His bleeding symptoms were mild to moderate with epistaxis, petechiae, frequent bruising and ecchymosis. He had one prolonged episode of epistaxis lasting for 12-hours, requiring transfusion support. The patient had normal growth and development and did not have hepatosplenomegaly on exam.

He transitioned care to our medical center at 10-months of age. A repeat BM aspirate and biopsy showed a cellular marrow with increased atypical megakaryocytes, occurring in clusters and associated marrow fibrosis. These findings confirmed the diagnoses of cMF and were consistent with those observed in his older brother. His genetic work up included negative mutation analysis for *MPL*, *JAK2* and *VPS45*.

The patient had the same identical human leukocyte antigen (HLA) type as his older brother (1-III-1). A repeat unrelated donor (URD) search did not reveal any acceptable HLA matched donors for HSCT consideration. In the meantime, the patient's aunt and uncle (also 1st cousins) had two additional children (1-III-8 and 1-III-9). 1-III-9 was found to be a 10/10 identical HLA match, but was also affected by CAH. She was considered as a related donor and underwent a work up to rule out cMF. Her CBC showed a mildly elevated WBC but no thrombocytopenia or anemia. Her marrow showed lymphoid aggregates with

mild fibrosis around the lymphoid aggregates. Her megakaryocytes were normal. Given the ongoing concern for an underlying inherited disorder we opted not to offer an upfront matched related donor (MRD) HSCT.

The patient remained transfusion dependent and treatment with a high dose steroid pulse (methylprednisone 30mg/kg x 3 days) followed by a slow taper was initiated. After 7-weeks he showed a partial response with platelet counts as high as $47x10^9$/L. A second steroid pulse with 1mg/kg methylprednisone was administered, to which his maximum platelet response was $89x10^9$/L. Unfortunately, the response was not sustained and his platelets decreased with the steroid taper, and ultimately reverted to $10x10^9$/L after he contracted primary varicella zoster infection.

Given that he remained transfusion-dependent, we re-considered the cousin (1-III-9) as the only MRD available. She underwent serial blood count and BM evaluations. Her blood counts remained normal and her BM on future studies was reassuring with normal megakaryocytes and minimal fibrosis in association with lymphocytes (see description below). None of those findings resembled those seen in the cousins and not diagnostic of cMF. The patient ultimately underwent a MRD HSCT from his cousin at 2-years of age. The patient was conditioned with a fully myeloablative conditioning regimen with busulfan and cyclophosphamide and received cyclosporine (CSA) and a short course of methotrexate (MTX) for graft-versus-host disease (GVHD) prophylaxis. Bone marrow was used as stem cell source with a cell dose of $4.32x10^8$ total cells/kg. His transplant course was uncomplicated without major infections, organ toxicity or GVHD. He engrafted myeloid cells on day +34 and became RBC transfusion independent on day +39. He had delayed platelet engraftment, becoming transfusion independent on day +60 and reached over $100x10^9$/L on day + 178. Blood counts at 21-months post-transplant were entirely normal with a WBC of 10.61 $x10^9$/L, HGB 13.7 g/dL, and platelets 343 $x10^9$/L. His MCV was normal at 80.5 fl (previously microcytic).

His BM aspirate and biopsy 4 and 8 months after HSCT showed a mildly hypocellular marrow with maturing trilineage hematopoiesis and a mildly increased myeloid-to-erythroid ratio. Megakaryocytes were present in normal numbers and morphology. Reticulin was minimally increased but markedly decreased from his pre-HSCT BM studies. All concurrent cytogenetic studies showed a normal female karyotype and negative FISH for monosomies 5 and 7, trisomy 8 and partial deletions of long arms of chromosomes 5, 7 and 20. Whole blood chimerism showed slight decrease in donor chimerism (93% donor, 7% recipient) but his blood counts remained normal.

Patient 1-III-6

During our evaluation of 1-III-5, the mother of 1-III-1 was pregnant with another male fetus. Amniocentesis confirmed that the fetus also had an intron 2G mutation in the *CYP21A2* gene and would be affected by CAH. HLA typing on the amniotic material showed the same homozygous HLA type found in his two older brothers (1-III-1 and 1-III-5). The baby was born full term and managed for his CAH. Given the family history of cMF, blood counts were followed closely. His platelet count at birth was $50x10^9$/L, but recovered to $132x10^9$/L and $270x10^9$/L at 2 and 4 weeks of life, respectively. A repeat evaluation at 5 months of age, however, showed leukocytosis, microcytic anemia and thrombocytopenia with a WBC of $13.79x10^9$/L, HGB 8.7 g/dL and platelets of 19 $x10^9$/L, raising concern that the infant was affected by cMF as well. A BM showed findings identical to his two affected brothers, including increased reticulin fibrosis and atypical megakaryocytes. Concurrent cytogenetics studies were normal. The patient, who was HLA identical to his brothers (1-III-1 and 1-III-5) and cousin (1-III-9) was carefully monitored while his older brother underwent a MRD HSCT from their cousin, 1-III-9. Between age 5 and 16 months of age his platelet count ranged from 15 to 25 $x10^9$/L. His WBC and HGB ranged from 6.2 to $17.17x10^9$/L and 7.8 to 9.7g/dL respectively. During this period of observation, he received three platelet transfusions and showed mild bleeding symptoms, including several hematomas and

episodes of epistaxis. He returned to the UAE, where, at 17-months of age, he had significant gum bleeding and epistaxis and received platelet transfusions, but did not show an improvement in his platelet count. He received a dose of IVIG, which transiently improved his platelets to $100x10^9$/L. A repeat BM at 14-months of age showed normocellular marrow for age (95% cellularity), increased atypical megakaryocytes often occurring in clusters, and worsening reticulin fibrosis (grade 3). His genetic work up included negative mutation analysis for *MPL, JAK2* and *VPS45*.

Given the successful HSCT of his older sibling from their fully matched related donor, and the lack of another related, unrelated or alternative donor source, we opted to perform the same HSCT for this patient. Prior to proceeding we confirmed that the donor remained unaffected with normal blood counts and a normal BM aspirate and biopsy. 1-III-6 underwent a fully MRD HSCT with myeloablative conditioning regimen comprised of busulfan and cyclophosphamide. GVHD prophylaxis consisted of cyclosporine A (CSA) and a short course of methotrexate (MTX). His transplant course was largely uncomplicated. He had delayed myeloid engraftment at day +45 and delayed platelet and red cell engraftment requiring intermittent platelet transfusion. A BM study on day +95 showed a mildly hypocellular marrow for age with normal myeloid-to-erythroid ratio and normal maturation. Megakaryocytes were mildly decreased but morphologically normal. Reticulin stain did not show evidence of reticulin fibrosis. Serial BM chimerism studies showed over 97% donor chimerism in all lineages (T-cells, B-cells and myeloid fraction). His laboratory studies showed evidence of hemolysis and it was felt his anemia and thrombocytopenia might be due to mild transplant-associated thrombotic microangiopathy (TA-TAM) due to CSA, and he was switched with Tacrolimus for GVHD prophylaxis. He received several doses of darbopoietin due to low erythropoietin levels.

His anemia and thrombocytopenia slowly improved beginning day +120 after his HSCT. His HGB level eventually normalized on day +136. Other than mild chronic oral GVHD at day

+150, for which he was treated with topical steroids and topical tacrolimus, he experienced no significant organ toxicities or infections.

Due to persistent thrombocytopenia a repeat marrow was performed 1 year post-HSCT and showed a mildly hypocellular marrow for age with maturing trilineage hematopoiesis and no evidence of reticulin fibrosis. Megakaryocytes were mildly decreased, occasional occurred in small clusters but morphologically normal appearing. His BM chimerism studies remained 100% donor.

Patient 1-III-9

1-III-9 is the cousin of proband 1-III-1 and his brothers 1-III-5 and 1-III-6. She is the product of a consanguineous relationship (first cousins) of Arabic decent from the United Arab Emirates. The parents of 1-III-1 and 1-III-9 are siblings to each other (Figure 1A).

Individual 1-III-9 was also diagnosed with CAH shortly after birth. She underwent surgical correction with vaginoplasty. HLA typing in search for a potential related donor for 1-III-5 showed an identical homozygous HLA type to the proband 1-III-1, 1-III-5 and 1-III-6. She was considered as a potential marrow donor for 1-III-5. Given the concern for an underlying inherited cause of the families' cMF she underwent careful diagnostic evaluation, which included serial CBCs that were all normal. A BM study at 14-months of age showed a normocellular marrow for age with numerous lymphoid aggregates that contained germinal centers. The myeloid-to-erythroid ratio was increased. Myeloid and erythroid elements showed normal maturation and no dysplasia. Megakaryocytes were normal in number and morphology. Reticulin stain showed a slight increase in reticulin in association with the lymphoid aggregates. Concurrent cytogenetic and FISH studies for monosomies 5 and 7 and trisomy 8 were negative. Lymphoid aggregates at this age can be seen in autoimmunity, but a detailed family history and screening laboratory studies for an autoimmune disorder were negative. Given the unknown significance of the lymphoid aggregates in the marrow, her donor status for her male cousin (1-III-5) was placed on hold.

A repeat BM at 16 months showed resolution of the lymphoid aggregates and no evidence of a cMF.

Her cousin 1-III-5 was treated with high dose steroids (see above) but did not show a sustained response and remained transfusion dependent. We therefore performed a reevaluation of 1-III-9. Blood counts remained entirely normal with WBC $8.39x10^9$/L, HGB 11.9 g/dL, platelet count $468x10^9$/L. A repeat BM at 2 years and 6 months showed a normocellular marrow for age with maturing trilineage hematopoiesis with relative erythroid and mild megakaryocytic hyperplasia.

Given the lack of features diagnostic of cMF and the ongoing transfusion needs of her cousin 1-III-5, we discussed treatment options with both families. After carefully consideration, informed consent regarding the theoretical risk for an underlying genetic diagnosis for which 1-III-9 could be a non- or minimally- expressing carrier, 1-III-9 was used as a marrow donor for 1-III-5. The transplant was successful with prompt myeloid engraftment and delayed platelet engraftment likely due to remodeling of hematopoietic niche due to reticulin fibrosis. Ultimately 1-III-5 achieved robust trilineage engraftment with normal blood counts and a normal marrow.

A follow up marrow was performed at 4-years of age to assess if she could serve as a donor for the other cousin, 1-III-6, who was also platelet transfusion dependent. The BM showed a mildly hypocellular marrow for age with maturing trilineage hematopoiesis and normal myeloid and erythroid maturation. Megakaryocytes were present in normal numbers and morphologically unremarkable. Reticulin fibrosis was not present. No lymphoid aggregates were noted. The blood counts remained normal.

Family 2

Patient 2-II-6

The proband (2-IV-6) is a child of a consanguineous family of Arabic decent; the parents are first cousins from Saudi Arabia (Figure 1). The patient was a male born at 36 weeks via caesarian section due to premature rupture of membranes and fetal distress. He required a brief period of non-invasive ventilation with a neonatal intensive care unit (NICU) stay of 1-month. During his first month of life, serial CBCs were performed, which showed mild thrombocytopenia with a platelet count of $112x10^9$/L with a normal HGB and WBC count. His platelet count improved to $161x10^9$/L by the time of discharge. He had a follow up CBC at 3 months of age, which showed a normal platelet count of $220x10^9$/L, HGB 10g/dL, and MCV of 97fl. WBC was reported as "normal". The patient was doing well until 6-months of age when a routine CBC showed severe thrombocytopenia (platelet count $10x10^9$/L) and microcytic anemia (HGB 5.4g/dL, MCV 65fl). He received RBC and platelets transfusion and was treated for a presumed immune mediated cytopenia and with IVIG and steroids. He showed some improvement in his blood counts with a maximum platelet count of $127x10^9$/L. Unfortunately, the improvement in his platelet count was never sustained and his platelet count dropped as soon as steroids were tapered. He received a total of 4 courses of steroids and several doses of IVIG. The patient required 6-8 RBC and approximately 10 platelet transfusions. His microcytic anemia was treated with iron supplementation, but did not lead to improvement in his anemia.

From a clinical perspective, he showed objective signs of bleeding including mucosal bleeding (lower gastrointestinal and oral mucosa), easy bruising and oozing from a bone marrow procedure site, requiring a transfusion. He had mild decrease in energy due to anemia. Three BM studies were performed at 12-, 14- and 24-months and overall showed a normocellular marrow with trilineage hematopoiesis and normal numbers of megakaryocytes. Reticulin stain at 24-months was negative.

A follow up marrow at 3 years of age showed a hypercellular marrow for age with maturing trilineage hematopoiesis and increased lymphocytes, including young forms and hematogones and moderate myelofibrosis (reticulin fibrosis grade 1-2+).

His work-up included normal blood chemistries, liver function tests, iron studies, ferritin 45, iron saturation 45% and elevated hepcidin of 437.5 ng/ml. Vitamin B_{12}, Vitamin D, and folate were normal. Platelet autoantibodies were detected against GP IIb/IIIa and Ib/IX. An autoimmune work up (ANA, auto-antibody screen) and autoimmune lymphoproliferative syndrome (ALPS) panel was negative. Lymphocyte subsets and immunoglobulins were normal, except IgM was decreased at 30 mg/dL. A gene sequencing panel for inherited BM failure syndromes, globin disorders and myeloproliferative neoplasms (*JAK2, MPL, CALR*) and *VPS45* was negative. Platelet function and aggregation studies could not be performed due to low platelet count.

Due to concern for a congenital disorder and the potential need for HSCT, HLA typing was performed revealing a homozygous HLA type, although different from that present in Families 1 and 3.

Patient 2-II-5

Patient 2-IV-5 is the older brother of 2-IV-6 who was noted to have macrothrombocytopenia on a CBC following his brother's diagnosis. He has a stable platelet count, ranging from 45 to $107x10^9$/L. His blood counts and smear showed mild leukopenia, no neutropenia, aniso- and poikilocytosis with a HGB of 8.8g/dL.

All of the siblings of the other siblings are reported to have iron deficiency anemia with a HGB of <10 g/dL. All of the father's sisters are reported to have iron deficiency anemia and are reported to be on iron supplementation. Platelet counts in those relatives are thought to be normal. The family history was also significant for diabetes and hypertension in the grandparents, liver cancer in the father's aunt and gastric cancer in the grandmother. There is no history of bleeding disorders or thrombocytopenia.

Family 3

Patient 3-II-4

The proband (3-IV-4) was referred to our medical center at 18-months of age for work up of cMF. She was born at full-term and is the 4th living child of a consanguineous family (parents are first cousins) from the UAE (Figure 1). At birth, the patient had ambiguous genitalia and was diagnosed with CAH due to 21-hydroxylase deficiency. She has been managed with hydrocortisone and fludrocortisone. She was doing well until 15 months of age when she was found to be pale. Her blood counts showed leukocytosis (WBC 19.2 cells x10^9/L), microcytic anemia (HGB 5.1g/dL, MCV 71.3, reticulocytes 1.5%) and thrombocytopenia 81 x10^9/L. She did not have any hepatosplenomegaly, lymphadenopathy or dysmorphic features. The peripheral blood smear demonstrated anemia with marked anisopoikilocytosis (target cells, schistiocytes) and occasional nucleated red blood cells. Leukocytosis with left myeloid cells and immature forms and thrombocytopenia with variable sized platelets including larger forms was noted. Platelet granulation was normal.

A BM aspirate and biopsy showed a mildly hypercellular marrow for age (90% cellularity) with a relative myeloid hyperplasia. Myeloid and erythroid elements matured normally. Megakaryocytes were increased, showed variable morphologic abnormalities including small hypolobated forms, and occurred in clusters. Blasts were not increased. Scattered small lymphocytes appeared normal. Mildly increased eosinophils were noted. A reticulin stain showed mild to moderately increased fibrosis (grade 1-2 out of 3). Immunohistochemistry (IHC) for CD61 showed increased megakaryocytes, focally clustering. The BM aspirate was hypocellular and aspicular and likely not representative, due to marrow fibrosis.

Her work-up included a normal chest x-ray with no bony abnormalities. She underwent an abdominal ultrasound to evaluate possible hepatosplenomegaly and was found to have asplenia. Laboratory testing was negative for nutritional deficiencies (iron, B_{12}, folate).

1,25-dihydroxy Vitamin D was mildly elevated at 89.2. Chemistry studies including liver function tests, electrolytes, and creatinine were normal. Her LDH was elevated at 446 with a negative direct Coombs test. Her immunology workup showed normal immunoglobulin levels (IgG, IgA, IgM) and a largely unremarkable lymphocyte subsets with a slight increase in CD19+ B-cells and mild decrease in CD3+ T-cells). Her molecular work up included sequencing analysis for *JAK2, MPL, CALR* and *VPS45*, all of which were negative.

The patient has 3 full biological siblings ages 3-, 7- and 8- years old, all of which are reported to be healthy but could not be fully assessed given that they were in their home country at the time of evaluation at our center. There is no family history of thrombocytopenia or anemia. A now 27-year-old aunt on the maternal side was diagnosed with acute leukemia at age 8 years and has been in remission since.

Family 4

Patient 4-II-2

Patient 4-II-2 is the second male child of consanguineous parents (first cousins) of Arabic decent from Algeria. The boy was born full term via NSVD following an uneventful pregnancy. He was seen at 18 months when a CBC revealed a severe microcytic anemia (5.1 g/dL) with low ferritin level (4ng/mL) and thrombocytopenia ($96x10^9$/L). His anemia improved to 11.5 g/dL with hemoglobin F level (4%). He has a normal WBC of $6.44x10^9$/L but a persistent severe thrombocytopenia with a platelet count between $10\text{-}30x10^9$/L. The patient continued to be followed with supportive care and is now 25-years old at last follow up. He continues to have persistent thrombocytopenia with platelet counts under $10x10^9$/L. He received IVIG and steroid therapy with no response, but has transient improvements in his platelet count after a platelet transfusion. The peripheral blood smear showed hypogranulated and gray platelets with a normal mean platelet volume. The red cells showed microcytosis, anisiopoikylocytosis with leptocytes, spherocytes, dacrocytes and occasional Howell-Jolly bodies. A partial deficiency in alpha granules in a subset of the

platelets was confirmed by electronic microscopy. The levels of platelet glycoproteins GPIb, GPIIbIIIa, GPIV studied by flow cytometry were normal. The patient did not show any clinical bleeding. He did have a cerebral cavernous malformation (cavernoma) but no other syndromic abnormalities. Two BM biopsies performed at 13 and 15 years of age were similar and showed erythroid dysplasia without blasts excess, dysmegakaryopoiesis with numerous hypobulated and small megakaryocytes. Reticulin staining showed marked reticulin fibrosis (stage II). Additional investigations revealed normal BM cytogenetics and a normal diepoxybutane induced chromosomal breakage studies. Molecular sequencing analysis was negative for mutations in *WAS, GATA1, FOG, c-MPL, RUNX1, NBLEA2, TERC, TERT, TINF2* and *DKC1*. The patient's maternal grandfather deceased at the age of 65 of a myelodysplastic syndrome with blasts excess.

Patient 4-II-3

The proband's (4-II-2) sister presented with thrombocytopenia in her first year of life but was not diagnosed until 11 years of age. Her peripheral blood cell count showed leukocytosis (WBC 17 $X10^9$/L), a normal hemoglobin level (12.5 g/dL) with a normal hemoglobin F level, and mild thrombocytopenia with a platelet count of $97x10^9$/L. The patient continued to be followed and was 23-years old at the last follow up. She had persistent mild to moderate thrombocytopenia with platelet counts between 80-$140X10^9$/L. She has not had any bleeding symptoms despite three surgical procedures. Her PB smear showed anisopoikylocytosis with target cells, rare schistocytes, leptocytes, dacryocytes and occasional Howell-Jolly bodies. The majority of her platelets showed a normal platelet volume (91%) with a small subset showing an increased platelet volume (9%). Some platelets showed decreased granulation (gray platelets, 8%). Platelet function assays were normal. A BM biopsy revealed a hypercellular marrow with dysmegakaryopoiesis and numerous micromegakaryocytes, moderate erythroid dysplasia without blast excess and normal granulopoiesis. A marked reticulin fibrosis stage II was detected.

Table S1

Genotype	Observed frequency	Predicted frequency
WT (+/+)	38 (26%)	37 (25%)
Het (+/hu)	72 (50%)	72 (50%)
Hom (hu/hu)	35 (24%)	36 (25%)

Data collected from 10 breeding pairs, average litter size 6 pups.

Table S2

Amplicon Primers (5’-3’)	
MPIG6B LR_F	ATCACAATCCCCTACAAAACAGG
MPIG6B LR_R1	GGAGAGGGATTATGGGGAGC
Sequencing Primers (5’-3’)	
MPIG6B 1F	GTTCTCCCCACGCCTAACTT
MPIG6B 1R	ATCTTCCCACACACAACCCA
MPIG6B 2F	GAAAGAGGAGACGGGATGGA
MPIG6B 2R	CCTCCTCCGGGTATGTGTC
MPIG6B 3F	CTGGCTGTCCCTCGTCAAA
MPIG6B 3R	TTTTGCGAAGGTTCTGGTCC
MPIG6B 4_5F	TGTCGGTGGGGTAGAGTCTA
MPIG6B 4_5R	GGAGAGGGATTATGGGGAGC

Figure S1

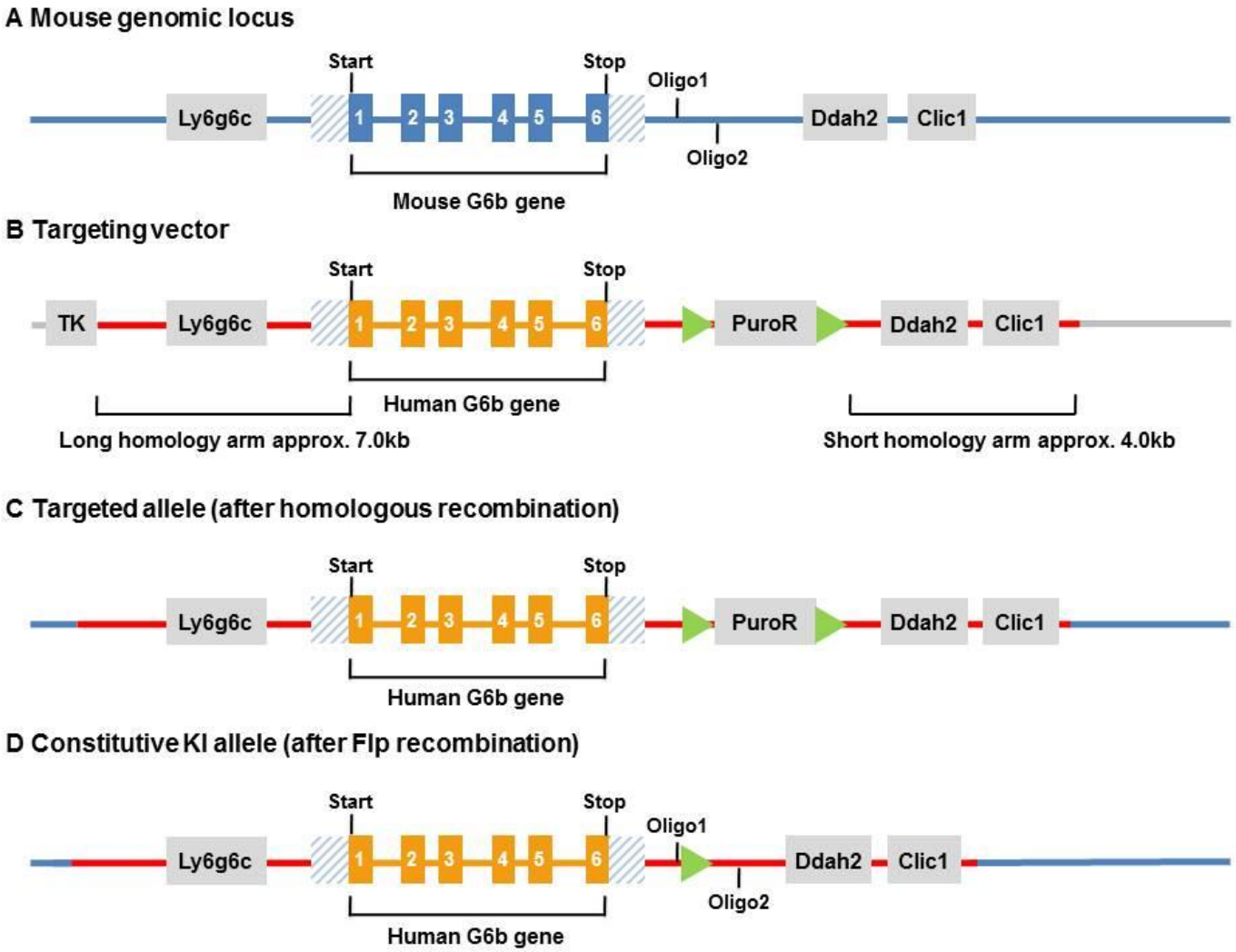

A Mouse genomic locus
Start
Stop
Oligo1
Ly6g6c
1
2
3
4
5
6
Ddah2
Clic1
Oligo2
Mouse G6b gene
B Targeting vector
Start
Stop
TK
Ly6g6c
PuroR
Ddah2
Clic1
Human G6b gene
Long homology arm approx. 7.0kb
Short homology arm approx. 4.0kb
C Targeted allele (after homologous recombination)
Start
Stop
Ly6g6c
PuroR
Ddah2
Clic1
Human G6b gene
D Constitutive KI allele (after Flp recombination)
Start
Stop
Oligo1
Ly6g6c
Ddah2
Clic1
Oligo2
Human G6b gene
G6b exon
G6b untranslated region
Human G6b exon
Neighbouring gene
Mouse genomic region
Vector knock-in region
Vector region
Human genomic region
Flippase recognition target
Oligo PCR primer target
Start Start codon
Stop Stop codon

Figure S1

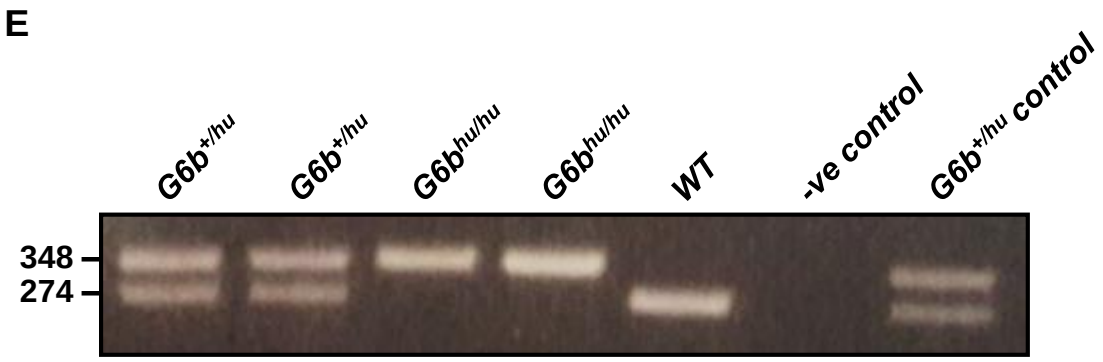


Figure S1. A constitutive knock-in strategy was used by Taconic to insert the human *G6b* allele into (A) the genomic locus of mouse *G6b*. (B) A targeting vector containing human *G6b* and a puromycin resistance cassette flanked by Flp recombinase sites was produced. (C) The two homology arms of the targeting vector flanking the human gene provided sites for homologous recombination to insert human *G6b* into the C57BL/6NTac embryonic stem cell genome. (D) The puromycin resistance was removed by crossing mice expressing the human *G6b* allele with a Flpe recombinase expressing mouse producing the final constitutive knock-in allele. (E) Oligos 1 and 2 were used to distinguish *WT* and *KI* mice by PCR.

Figure S2

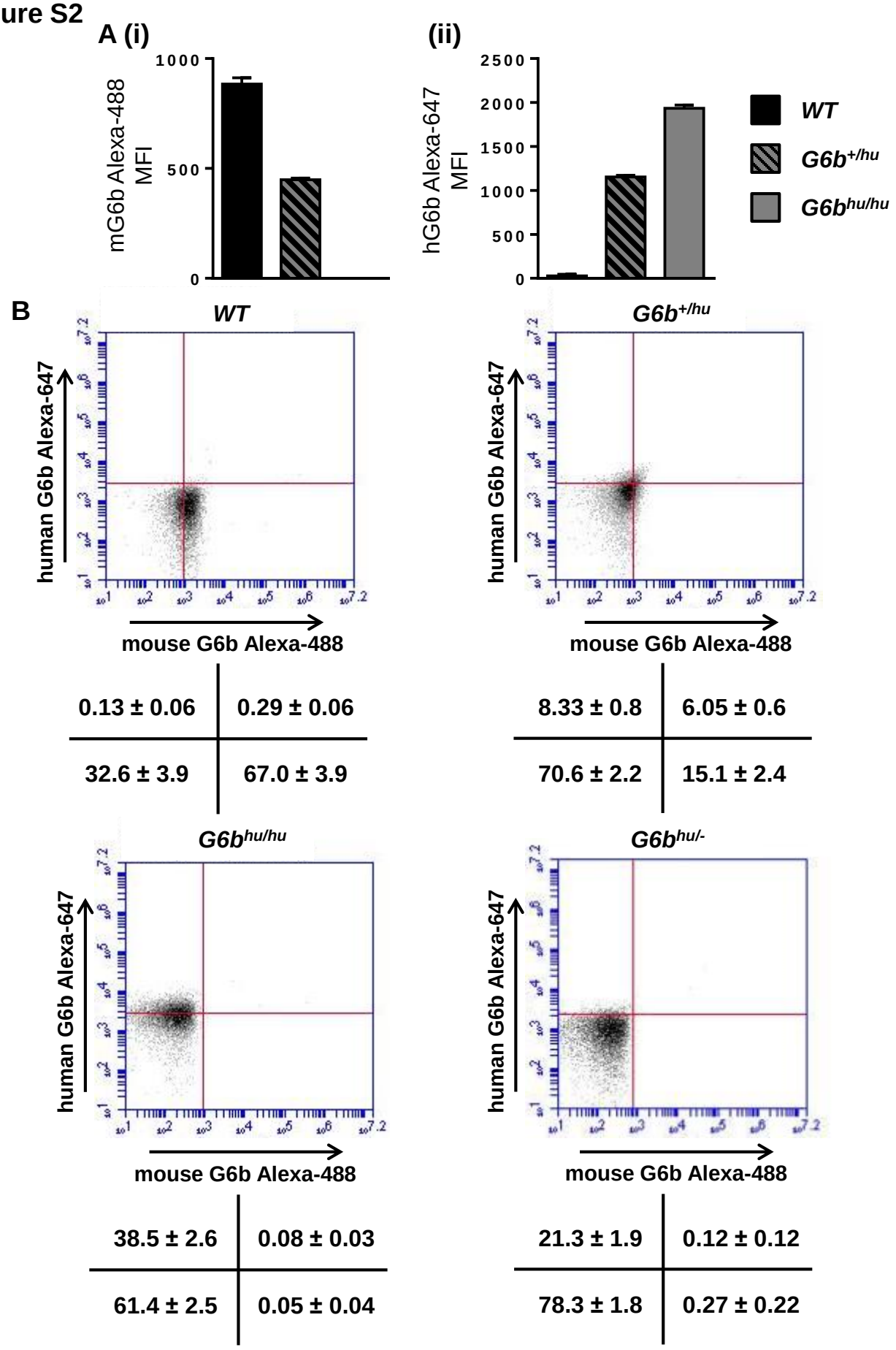


Figure S2. (A) mouse (m)G6b and human (h)G6b median fluorescence intensity (MFI), mean ± SEM, n=6. (B) Representative density plots and mean ± SD of percentage of events in each quadrant (n=6).2

Figure S3

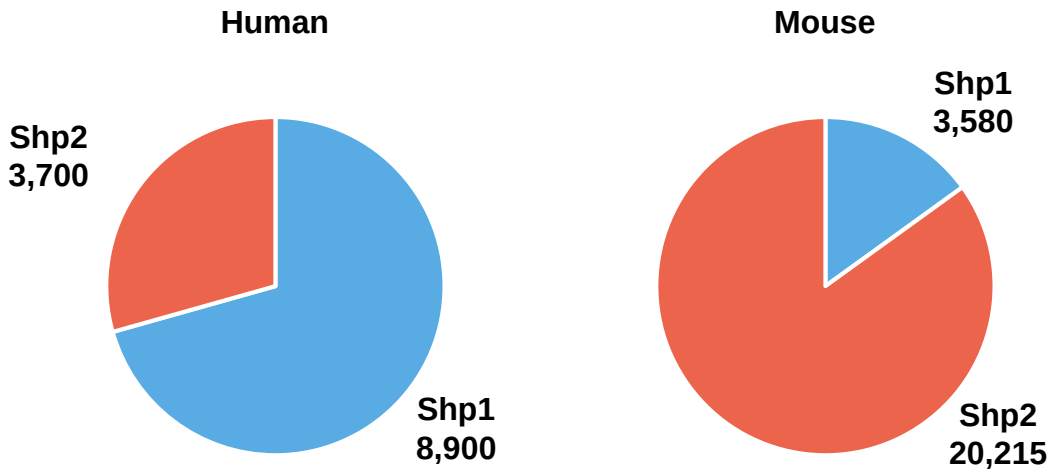


Figure S3. Relative proportions of Shp1 (blue) and Shp2 (red) expression in human and mouse platelets. Numbers indicate copy number per platelet as measured by quantitative proteomic studies (Burkhart et al, 2012 and Zeiler et al, 2014)

Figure S4

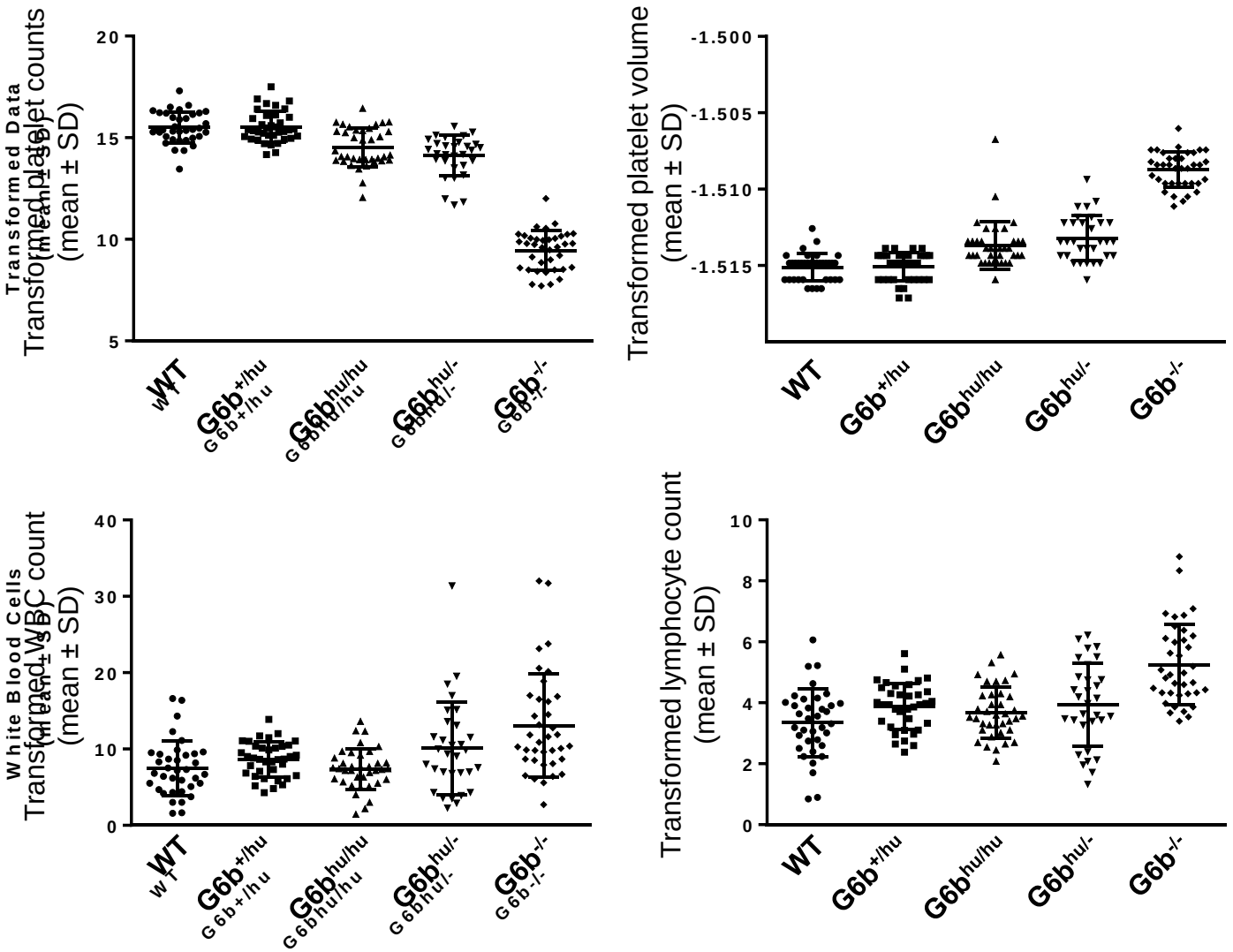
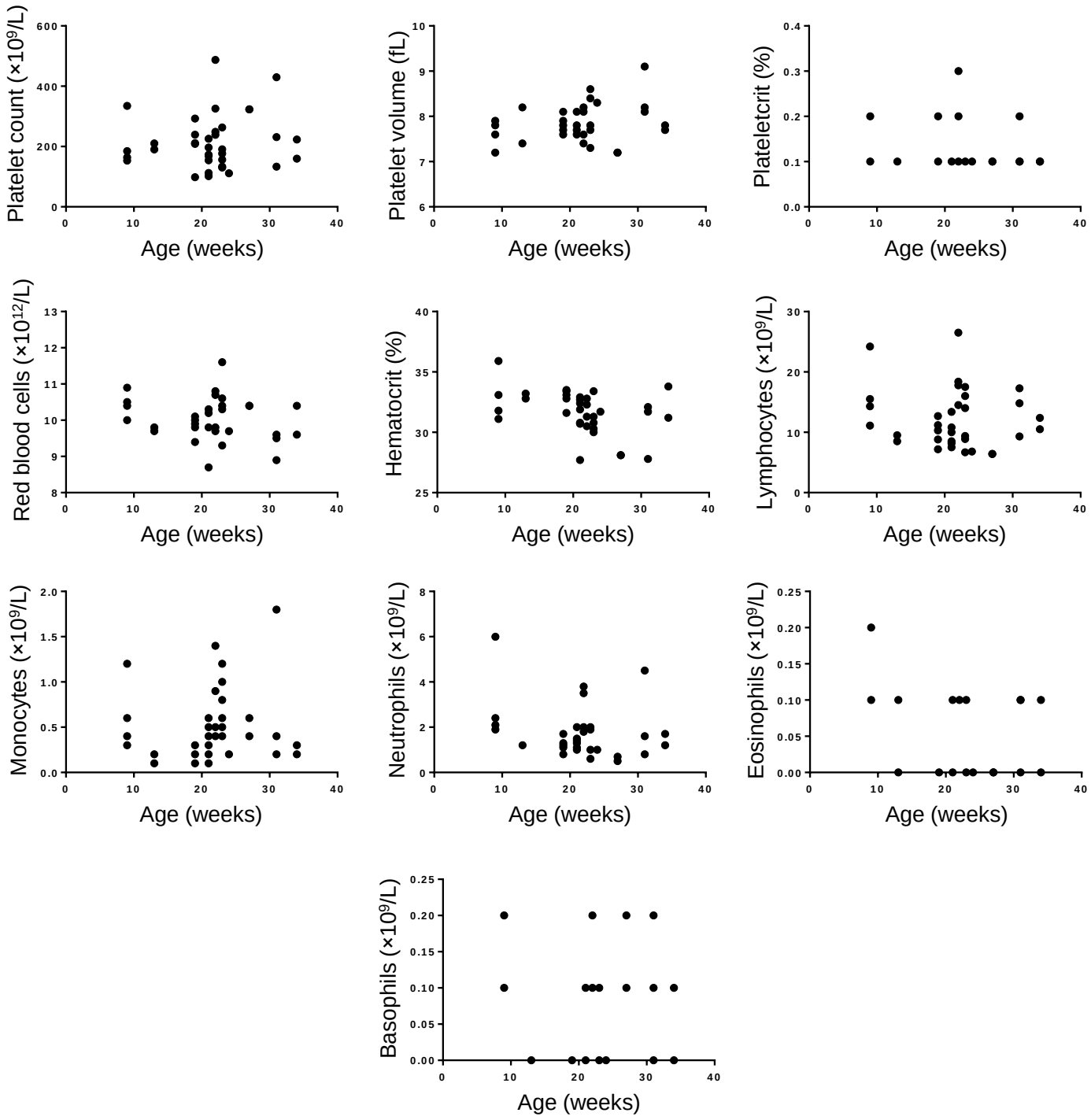


Figure S4. Distribution of transformed blood cell counts used for statistical analysis. Platelet count, platelet volume, white blood cell (WBC) count and lymphocyte count data were transformed using a modified Box-Cox power transformation and normality of distribution verified using Browne-Forsythe test for equal variance. Transformed data were used for analysis by one-way ANOVA and Dunnett's post-hoc test to compare all genotypes to *WT*.

Figure S5

A



B

	Platelet count	Platelet volume	Plateletcrit	Red blood cells	Hematocrit	Lymphocytes	Monocytes	Neutrophils	Eosinophils	Basophils
R^2	0.01	0.06	0.00	0.04	0.14	0.02	0.01	0.05	0.07	0.00
P-value	0.50	0.15	0.84	0.21	0.02	0.40	0.58	0.17	0.11	0.08

Figure S5. Blood cell counts variation is independent of age in *G6b KO* mice. (A) Blood cell counts were plotted against age for *G6b KO* mice (n=37). (B) Regression analysis indicates that there is no correlation between age and hematological parameters.

Figure S6

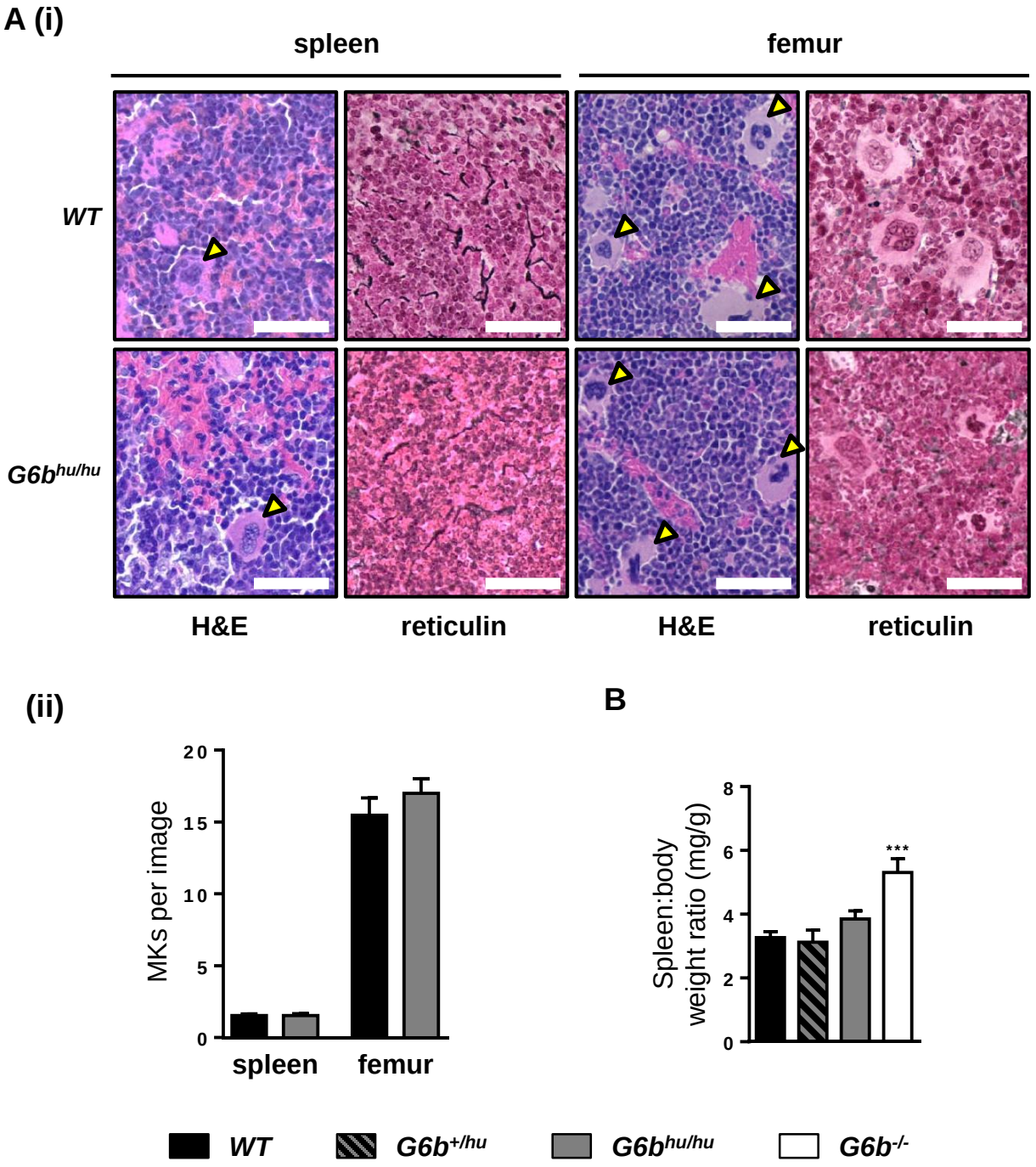


Figure S6. (A) Representative images of H&E and reticulin stained *WT* and *G6b*$^{hu/hu}$ mouse spleens and femurs and quantification of megakaryocytes (MKs) in spleen and femur sections (n=6). (B) Spleen to body weight ratio of indicated genotypes (n=3-30). All data represented as mean ± SEM. Scale bar: 50 μm.

Figure S7

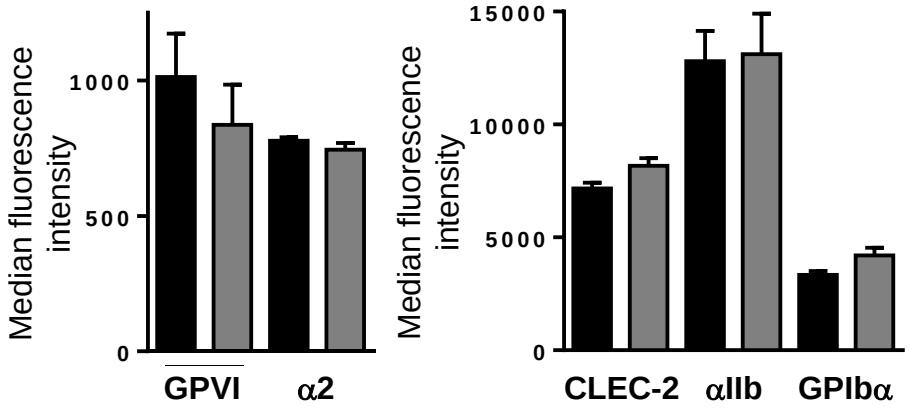


Figure S7. Surface expression of the major platelet receptors in *WT* and *G6b*$^{hu/hu}$ mice. All data represented as mean ± SEM.

Figure S8

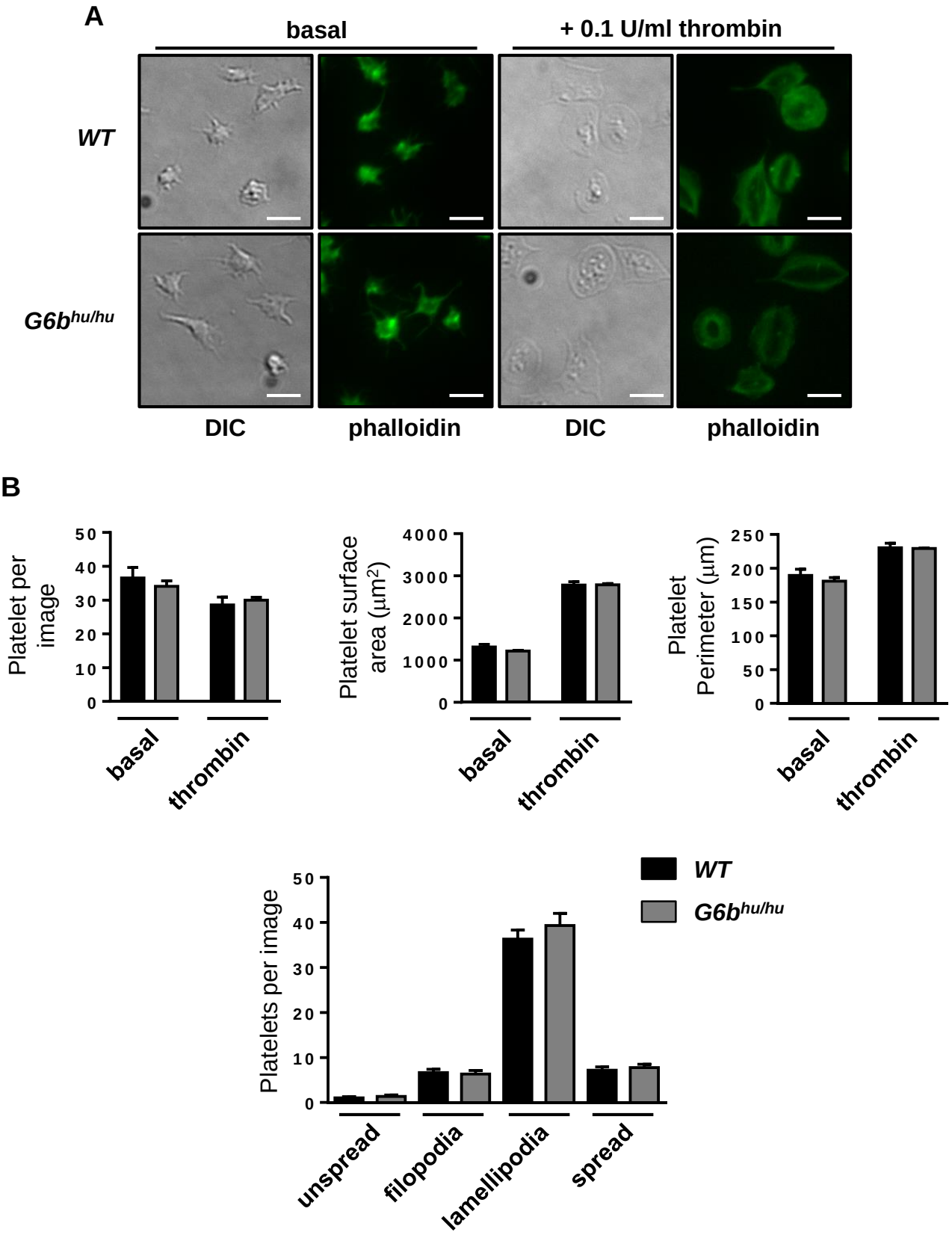


Figure S8. (A) Representative differential interference contrast (DIC) and Alexa-488-conjugated phalloidin stained images of fibrinogen spread washed platelets (2×10^7/mL) under both resting and 0.1 U/ml thrombin pre-activated conditions. (B) Quantification of DIC images in indicated genotypes. Mean ± SEM, n=3.

Figure S9

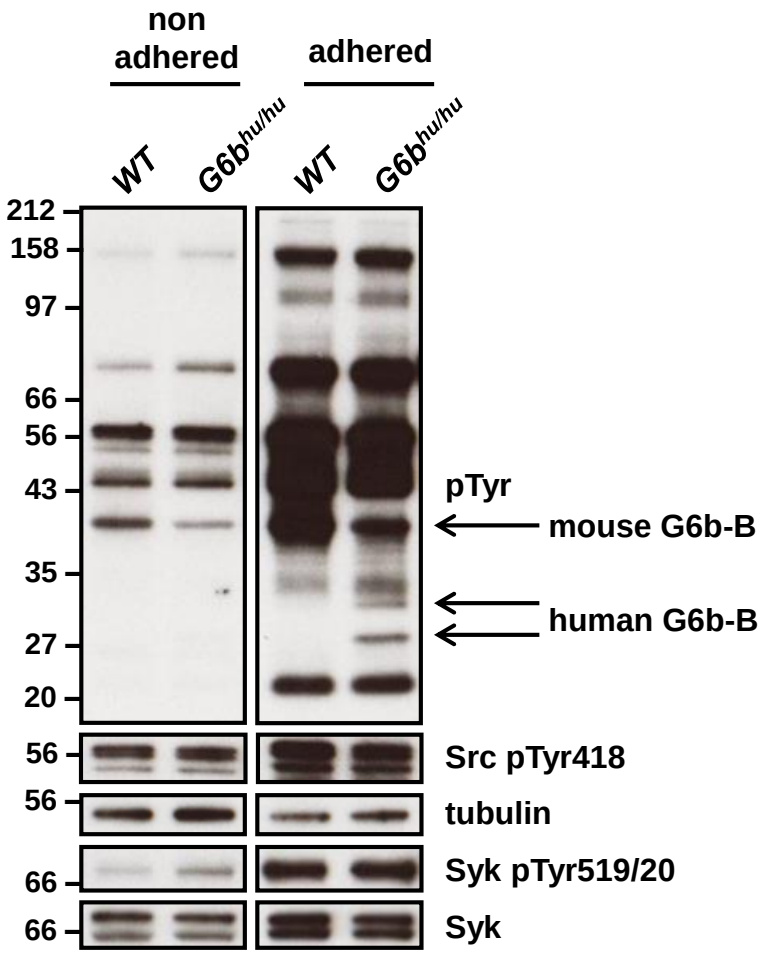


Figure S9. Normal signalling in fibrinogen adhered *G6b*$^{hu/hu}$ washed platelets (4 × 10^8/mL).

Figure S10

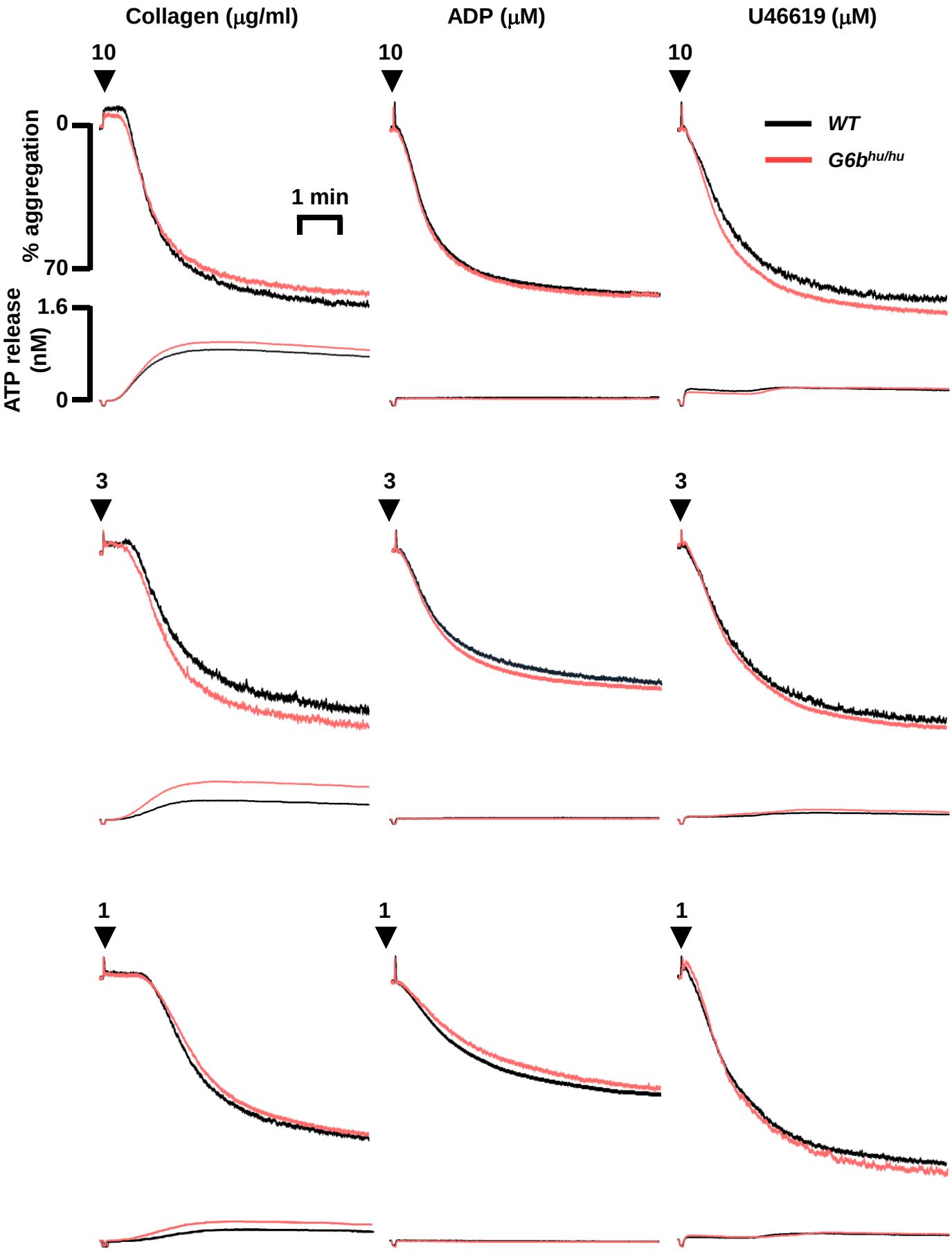

Collagen (μg/ml)
ADP (μM)
U46619 (μM)
10
3
1
% aggregation
0
70
ATP release (nM)
1.6
0
1 min
WT
G6b^hu/hu

Figure S10

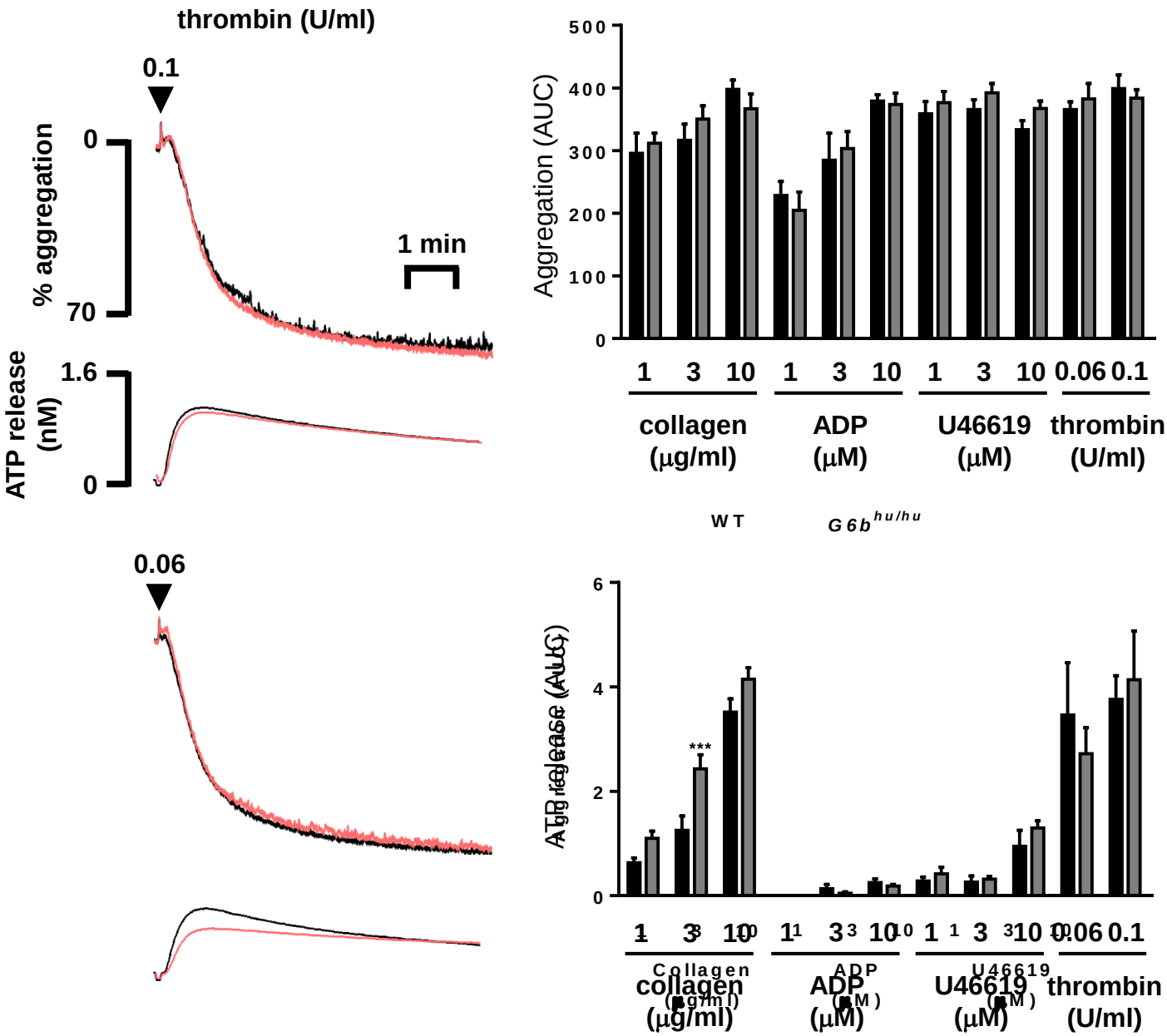
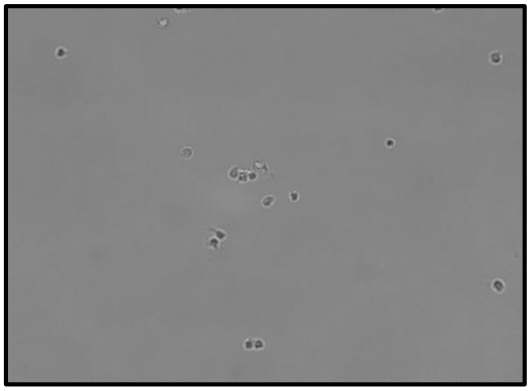
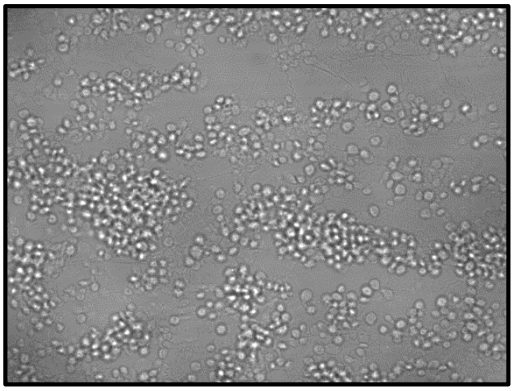


Figure S10. Mean aggregation and ATP release traces for *WT* and *G6b*$^{hu/hu}$ washed platelets (2×10^8/mL) activated with indicated agonists. Area under the curve (AUC) was compared for each of the traces (n=4-6, mean ± SEM, *** P<0.001).

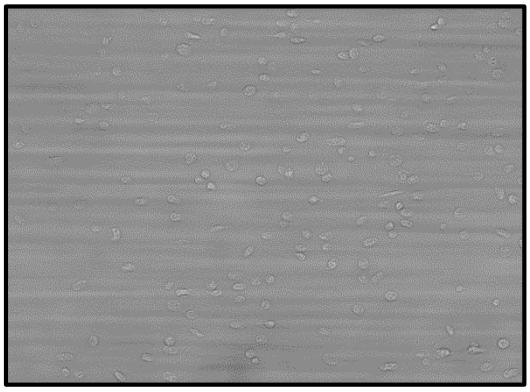
Figure S11



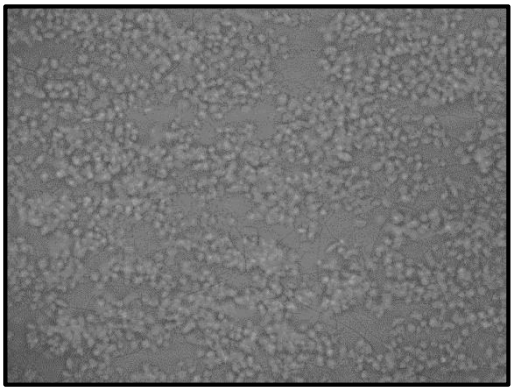
Morphological score – 0
Contraction score – 0
Multilayer score – 0



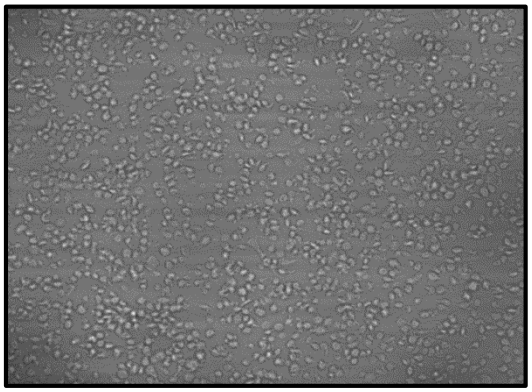
Morphological score – 3
Contraction score – 1
Multilayer score – 1



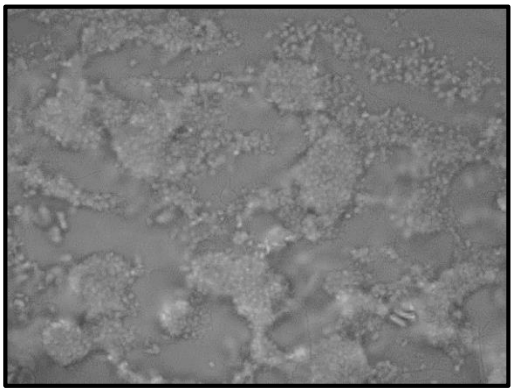
Morphological score – 1
Contraction score – 0
Multilayer score – 0



Morphological score – 4
Contraction score – 2
Multilayer score – 2



Morphological score – 2
Contraction score – 0
Multilayer score – 0

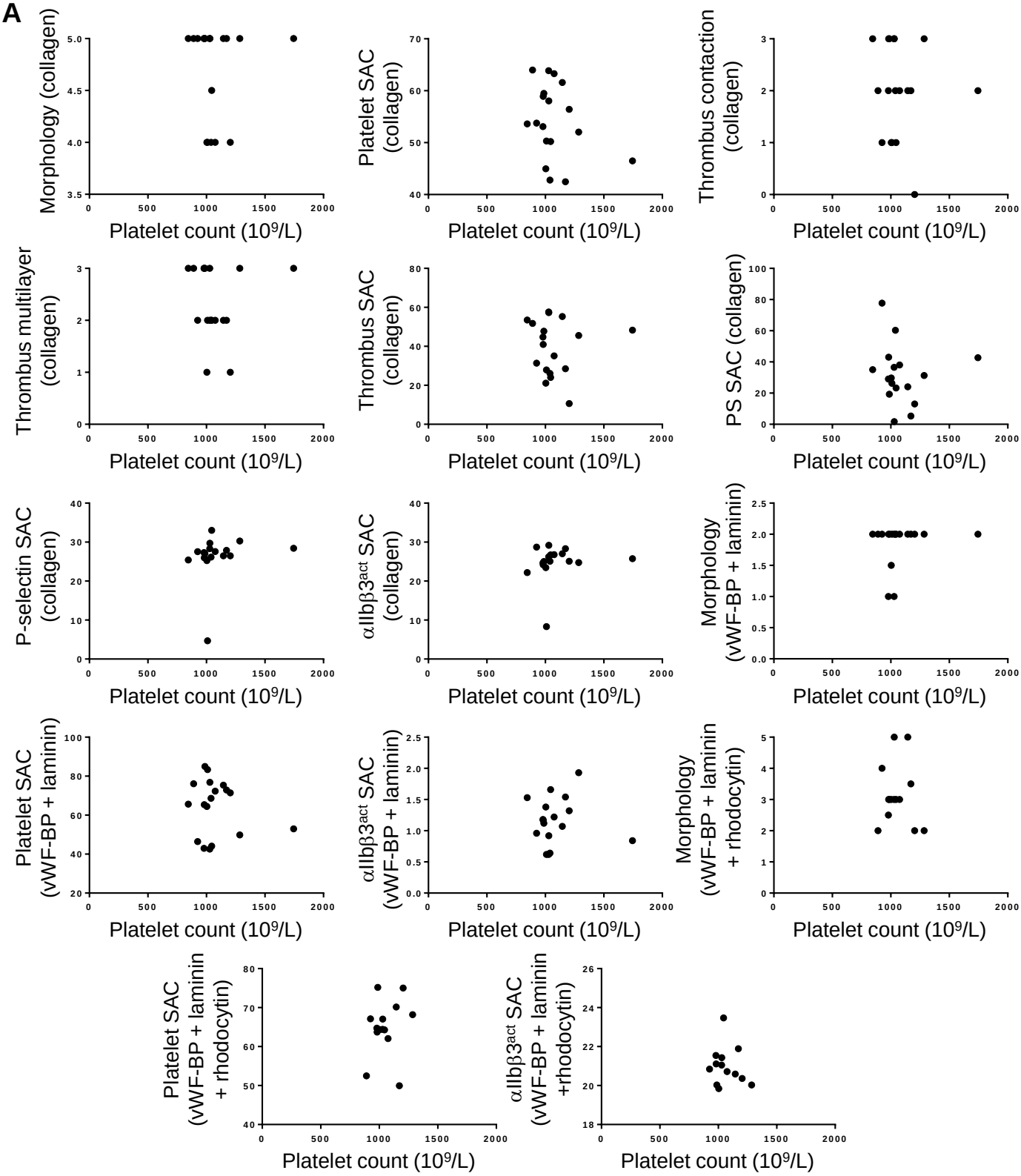


Morphological score – 5
Contraction score – 3
Multilayer score – 3

Figure S11. Platelet flow adhesion morphological scoring representative images

Figure S12

A



B

	Morphology	Platelet SAC	Thrombus contraction	Thrombus multilayer	Thrombus SAC	PS SAC	P-selectin SAC	αIIbβ3act SAC	Morphology	Platelet SAC	αIIbβ3act SAC	Morphology	Platelet SAC	αIIbβ3act SAC
R^2	0.002	0.1	0.006	9×10^{-4}	3×10^{-4}	0.006	0.032	0.02	0.026	0.029	1×10^{-5}	0.007	0.042	0.026
P value	0.858	0.202	0.763	0.905	0.947	0.763	0.492	0.591	0.52	0.5	0.989	0.778	0.482	0.601
	Collagen								vWF-BP + Laminin			vWF-BP + Laminin + Rhodocytin		

Figure S12. (A) Scatter plots of measured parameters of platelet adhesion and activation against platelet count in a cohort of *WT* mice with platelet counts ranging between 845-1745 × 10^9/L (n=14-18). (B) R^2 and P-values of linear regression models, demonstrating platelet count is not a good predictor of any of the parameters measured.

Figure S13

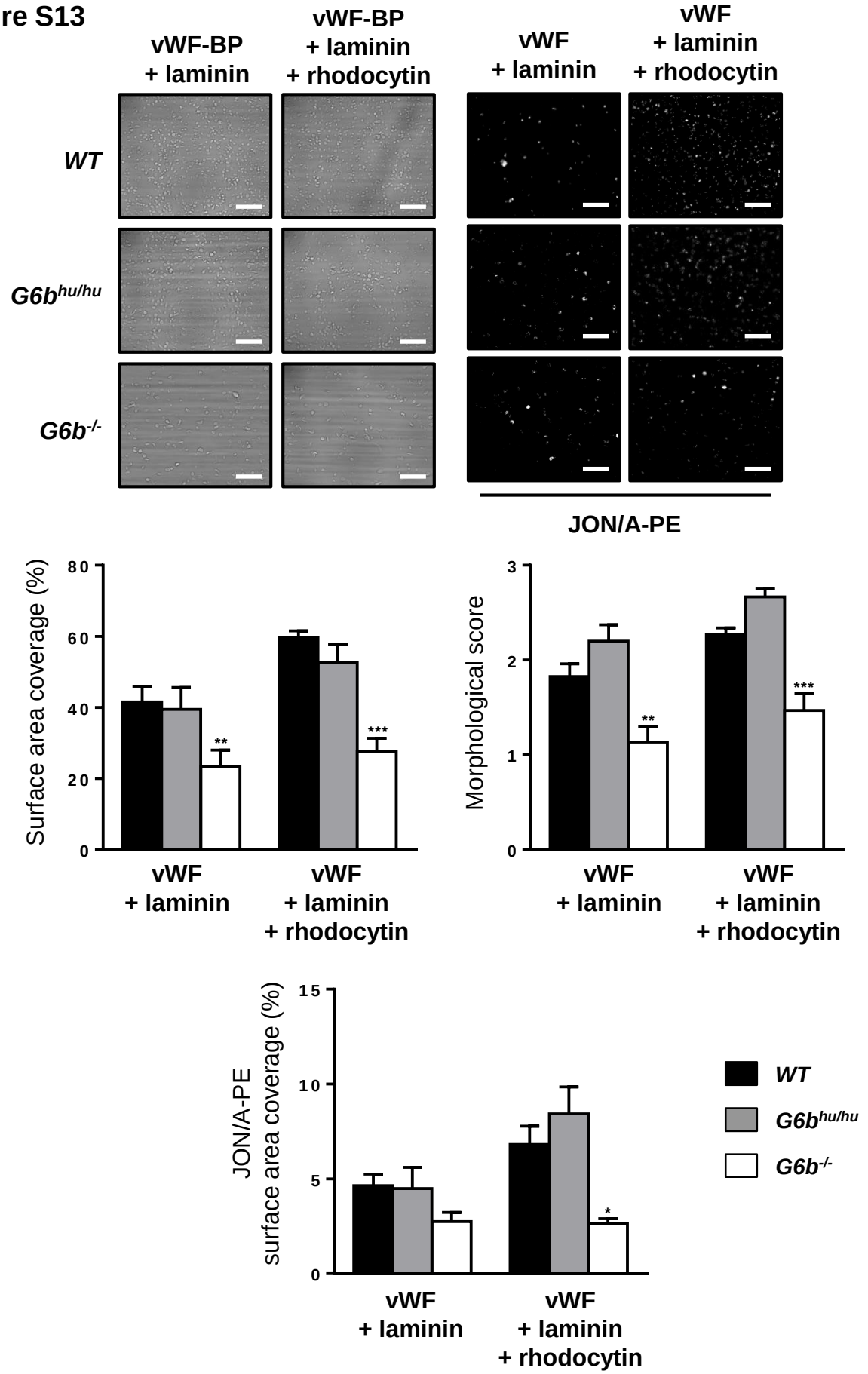


Figure S13. Representative images and quantification of total platelet surface area coverage, morphological score and JON/A-PE surface area coverage of heparin-PPACK-fragmin anticoagulated whole blood flowed over collagen coated coverslips. n=5-6, mean ± SEM, * P<0.05, ** P<0.01, *** P<0.001.

Figure S14

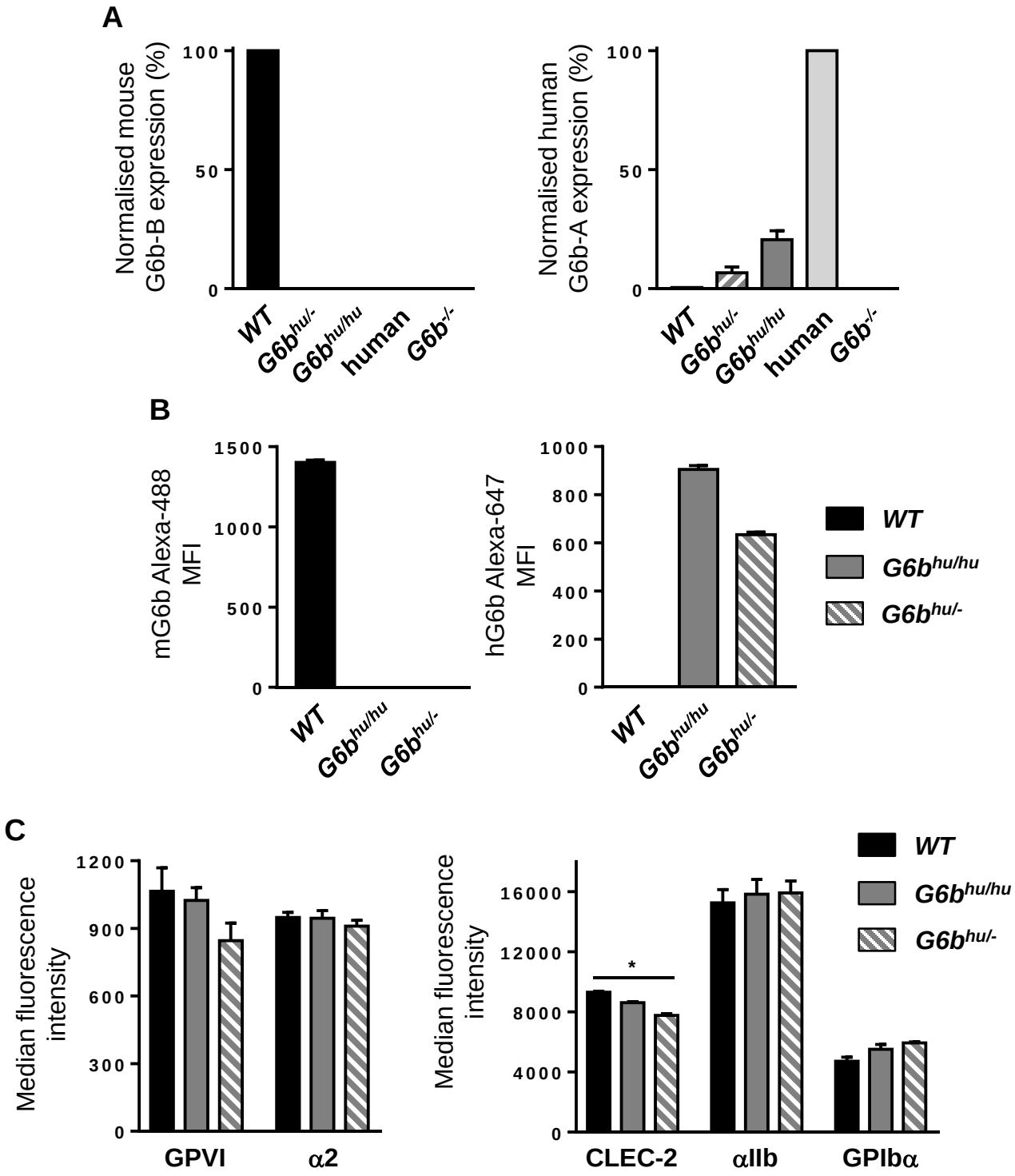


Figure S14. (A) Quantification of mouse and human G6b-A total protein levels in lysates prepared from washed platelets (4×10^8/mL). (B) Surface expression levels of mouse and human G6b. (C) Surface expression levels of major platelet receptors.

Figure S15

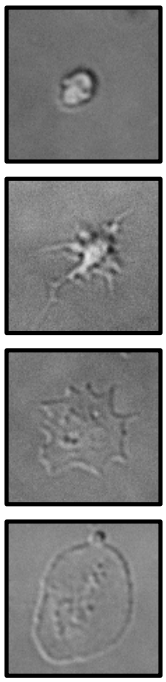


Figure S15. Washed platelet spreading on fibrinogen scoring categories