

Reviewed Preprint

v1 • March 16, 2026

Not revised

For correspondence:

alexandra.mazharian@inserm.fr

Competing interests:

No competing interests declared

Funding:

See [page 25](#)

Reviewing editor:

Seth J Corey, Cleveland Clinic, United States

© 2026, Barré et al. This article is

distributed under the terms of the

[Creative Commons Attribution](#)

[License](#), which permits unrestricted use and redistribution provided that the original author and source are credited.

Synergistic effects of deleting the tyrosine phosphatases Shp1 and Shp2 on megakaryopoiesis and thrombopoiesis in mice

Elsa Barré¹, Marc-Damien Lourenco-Rodrigues¹, Lucie Zimmermann¹, Marion Pugliano¹, Cécile Loubière¹, Fabienne Proamer¹, Jean-Yves Rinckel¹, Anita Eckly¹, Zihan Qu², Jinmin Miao³, Zhong-Yin Zhang^{2,3,4}, Yotis A Senis¹, Alexandra Mazharian¹ ✉

¹Institut National de la Santé et de la Recherche Médicale Unité Mixte de Recherche - S 1255, Etablissement Français du Sang Grand Est, Université de Strasbourg, Strasbourg, France • ²James Tarpo Jr. and Margaret Tarpo Department of Chemistry, Purdue University, West Lafayette, United States • ³Borch Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, United States • ⁴Institute for Drug Discovery, Purdue University, West Lafayette, United States

eLife Assessment

This manuscript provides an **important** contribution to the field of platelet biogenesis, and the **convincing** evidence will advance our understanding of signal transduction driving the development of late megakaryopoiesis and platelet reactivity that results in bleeding diathesis. The paper is noteworthy for analyzing two related, either singly or in combination, tyrosine phosphatases in this conditional, stage development gene knockout. Because SHP1 is a negative regulator and SHP2 is an activator, the synergistic effects found in the double knockout were surprising.

<https://doi.org/10.7554/eLife.110053.1.sa4>

Abstract

The Src homology 2 (SH2) domain-containing non-transmembrane protein-tyrosine phosphatases 1 and 2 (Shp1 and Shp2) have been implicated in regulating signaling from a variety of receptors and cell types, including the thrombopoietin (Tpo) receptor Mpl in megakaryocytes (MKs) and platelets. We previously showed that deletion of Shp1 and Shp2 in the MK/platelet lineage in mice using the *Pf4-Cre* transgene/*loxP* system impairs megakaryopoiesis and thrombopoiesis. However, we also observed unexpected phenotypes including a *motheaten-like* phenotype in Shp1-deficient mice and severe myelofibrosis in mice lacking both phosphatases. To determine whether these were lineage-specific effects, we utilized the *Gp1ba-Cre* transgenic mouse to delete *loxP*-flanked Shp1 and Shp2 in mice. Bone marrow-derived MKs from these mice expressed approximately 20-25% of Shp1 and Shp2, whereas platelets contain 5-10% of each phosphatase compared with controls. Minor MK/platelet defects were observed in mice lacking either Shp1 or Shp2 alone, however mice lacking both Shp1 and Shp2 exhibited macrothrombocytopenia, mild bleeding following tail injury, and impaired GPVI-mediated platelet aggregation and Syk phosphorylation, associated with reduction GPVI and integrin $\alpha 2$ subunit expression. Reduced Shp1 and Shp2 expression resulting in a significant reduction in ploidy, a block in MK maturation and proplatelet-producing MKs. Tpo-mediated Ras/MAPK signaling was reduced in Shp1/2-deficient MKs. Treatment of MKs with structurally distinct Shp2 allosteric inhibitors recapitulated key aspects of the Shp2-deficient phenotype, including aberrant megakaryopoiesis and reduced Mpl signaling. Our study highlights the synergistic functions of Shp1 and Shp2 in the MK/platelet lineage, and identifies Shp2 as a potential therapeutic target in myeloproliferative neoplasms.

Introduction

Platelets, or thrombocytes are small, anucleate blood cells derived from megakaryocytes (MKs) that are essential for hemostasis and thrombosis.¹ MK development, or megakaryopoiesis and platelet production, or thrombopoiesis are tightly regulated processes that take place in the bone marrow (BM), spleen and lungs, but the molecular mechanisms regulating these inter-related processes remain incompletely defined.^{2,3} The primary driver of megakaryopoiesis, which encompasses differentiation of hematopoietic stem and progenitor cells (HSPCs) to MKs, proliferation, endomitosis and maturation is the cytokine thrombopoietin (Tpo) produced by the kidneys, liver and BM acting on its receptor, myeloproliferative leukemia protein (Mpl) expressed throughout the MK/platelet lineage.⁴ Tpo does not however explain which MKs undergo proplatelet formation and thrombopoiesis, which occurs when MKs come in contact with sinusoidal blood vessels. Several other cytokines and growth factors, extracellular matrix (ECM) proteins and chemokines are also required for optimal megakaryopoiesis and thrombopoiesis, as evidenced in mice lacking either Tpo or Mpl, which still produce MKs and platelets, albeit at much lower levels than normal mice.^{5,6}

The three primary signaling pathways downstream of Mpl are Janus kinase 2 (JAK2)/Signal transducer and activator of transcription (STAT), Ras/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/AKT, all of which culminate in nuclear translocation of signaling/transcription factors and gene regulation.⁷ Cytoskeletal remodeling via the RhoA/Rho-associated protein kinases (ROCK) pathway is also an integral part of megakaryopoiesis and thrombopoiesis, notably during endomitosis⁸, but the molecular link with the Mpl receptor is undefined. Clonal gain-of-function mutations in the Mpl/JAK2/STAT pathway in HSPCs are the most common causes of myeloproliferative neoplasms (MPN)⁹, characterized by increase MK/platelet production (essential thrombocythemia, ET), increased red blood cell production (polycythemia vera, PV) and associated fibrotic deposits (primary myelofibrosis, PMF).^{10–12} Notably, the valine 617 phenylalanine (V617F) mutation in the pseudo-kinase domain of JAK2, resulting in increased catalytic active is the most prevalent, found in 60% of MPN cases, followed by C-terminal deletions in calreticulin, resulting in the release of truncated calreticulin and it acting as a ligand of Mpl in 30% of the cases, and mutations in Mpl resulting in increased expression in <5% of the cases.¹³ JAK2 and MAPK kinase (MEK) inhibitors are effective in some instances of MPN¹⁴, however there is a tendency of resistance and relapse, requiring alternative and improved therapeutics.

The Src homology 2 (SH2) domain-containing non-transmembrane protein-tyrosine phosphatases (PTPs) 1 and 2 (Shp1 and Shp2) are involved in hematopoietic cell differentiation, proliferation, function and survival, including megakaryopoiesis and thrombopoiesis.¹⁵ Intra- and inter-molecular interactions of the tandem SH2 domains with the catalytic domain and phosphotyrosine binding partners regulate the activity and compartmentalization of both phosphatases, respectively. Tandem phospho-tyrosine residues in the C-terminal tails of Shp1 and Shp2 are implicated in substrate binding and regulation.¹⁶ Despite high structural similarities, the two phosphatases have distinct biological functions. Shp1, encoded by the *PTPN6* gene, is primarily expressed in hematopoietic cells and is a negative regulator of immune receptor signaling, particularly the immunoreceptor tyrosine-based activation motif (ITAM)/Spleen tyrosine kinase (Syk)/phospholipase pathway.¹⁷ Shp2, encoded by the *PTPN11* gene, is ubiquitously expressed and is a positive regulator of cytokine and growth factor signaling, specifically the Ras/MAPK and PI3K/AKT pathways.¹⁸ However, their functions go beyond these pathways and are context dependent. Downstream substrates and effectors of both phosphatases remain elusive.

We previously showed that targeted deletion of Shp1 and Shp2 in the MK/platelet lineage using the *Pf4-Cre* transgene/*loxP* system impairs megakaryopoiesis and thrombopoiesis in mice.¹⁵ However, we also found that these mice exhibited unexpected, non-MK/platelet-related defects, including a *motheaten-like* phenotype in Shp1-deficient mice, and severe myelofibrosis in mice lacking both phosphatases. Intriguingly, the *motheaten-like* phenotype was lost in Shp1/2 double-deficient mice, suggesting that Shp1 and Shp2 have opposing effect in this pathology. To determine whether these were in fact MK/platelet lineage-specific effects, we utilized the *Gp1ba-Cre* transgenic mouse to

delete *loxP*-flanked Shp1 and Shp2 in mice. In contrast to *Pf4-Cre* transgenic mice, conditional deletion of Shp1 or Shp2 using the *Gp1ba-Cre* transgene had little if any detectable effect on megakaryopoiesis or thrombopoiesis in these mice under steady state conditions. However, deletion of both Shp1 and Shp2 using *Gp1ba-Cre* resulted in macrothrombocytopenia and aberrant MK development and maturation, due to a block in *Mpl*-mediated Ras/MAPK signaling. GPVI-induced platelet aggregation was also impaired due to reduced GPVI and $\alpha 2$ integrin subunit expression. Similar effects were seen with the structurally-distinct allosteric Shp2 inhibitors, SHP099 and RMC-4550.¹⁹ Findings demonstrate synergistic effects of deleting Shp1 and Shp2 in the MK/platelet lineage in mice by disrupting distinct signaling pathways, and highlight Shp2 as a potential therapeutic target in MPN.^{20,21}

Materials and Methods

Mouse models

All mice used were on a C57BL/6 background. *Ptpn6*^{fl/fl}, *Ptpn11*^{fl/fl} and *Gp1ba-Cre*^{+/*KI*} mice were generated, as previously described.^{22–24} MK/Platelet specific Shp1 and Shp2 knockout (KO) mice were generated by crossing *Ptpn6*^{fl/fl} and *Ptpn11*^{fl/fl} mice with the *Gp1ba-Cre* transgenic deleter mouse *Gp1ba-Cre*^{KI/+} mice. *Gp1ba-Cre*^{KI/+} were used as control mice (CT) (Supplemental Figure S1 [↗](#)). All animal experiments were conducted in accordance with the CREMEAS Committee on the Ethics of Animal Experiments of the University of Strasbourg (Permit Number: E67-482-10, Project approval number: APAFIS#28221-2020111714459066).

Inhibitors

SHP099 and RMC-4550 Shp2 allosteric inhibitors were purchased from MedChemExpress. M029 is a covalent allosteric Shp1 inhibitor (Z.Q. and Z.Y.Z., manuscript in preparation) and F2Ac is a reversible active site directed Shp1 inhibitor (J.M. and Z.Y.Z., manuscript in preparation).

Platelet preparation and functional assays

Blood was collected from the aorta of anesthetized mice into 1/10 acid-citrate-dextrose (ACD) anticoagulant. Platelet counts were measured from peripheral blood samples using an automated hematology analyzer (Element HT5 counter, Heska company). Washed platelets were prepared as previously described.²⁵ Platelet counts were normalized and used for aggregation (2×10^8 /ml) or biochemical analysis (5×10^8 /ml). Platelet aggregation was measured using the lumi-aggregometer APACT®4004. Surface glycoprotein expression was measured in whole blood by flow cytometry using fluorescein isothiocyanate (FITC)-conjugated antibodies. Resting and activated platelets were fixed and stained with anti-P-selectin antibody.

Megakaryocyte culture and functional assays

Mature MKs from mouse BM were cultured and analysed, as previously described.²⁶ BM cells were obtained from femora, tibiae and iliac crests of C57BL6 mice for pharmacological study and *Gp1ba-Cre* mice for other experiments by flushing, and Lin-cells obtained after immunomagnetic sorting were cultured for 3 days at 37°C 5% CO₂ at a concentration of 1×10^6 cells/well in a Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS), 50 ng/ml Tpo, 100 U/ml Hirudin, 1% PSG.²⁶ MK ploidy was assessed after 3 days of culture, using FITC-coupled anti-CD41 antibody and DNA staining with propidium iodide (PI). Samples were acquired using the BD Fortessa X20 and analyzed using BD FACSDiva software. The ability of MKs to form proplatelets was assessed *in vitro*, and *ex vivo* using the explant assay.²⁷

Immunoblotting

Platelet and MK whole cell lysates (WCLs) were prepared and analyzed by automated capillary-based immunoassay (ProteinSimple Jess), prepared according to manufacturer's instructions, as previously described.²⁸ The High Dynamic Range profile was used for chemiluminescent and

fluorescent multiplexing signal detection. Optimized antibody dilutions and sample concentrations used are provided in [Supplemental Table 1](#).

Immunohistochemistry

Spleens from KO and litter-matched CT mice were fixed in buffered formalin and embedded in paraffin. Sections (5 μ m) were H&E stained and examined by light microscopy using a 40 $\times$ objective.

Electron microscopy

Maturation stage of MKs was assessed by electron microscopy (EM). BM samples obtained by flushing mouse femora with 0.1 M sodium cacodylate buffer were fixed in 2.5% glutaraldehyde and embedded in Epon as previously described.²⁹ Thin sections were stained with uranyl acetate and lead citrate, and examined under a JEOL 2100Plus transmission electron microscope at 120 kV (Jeol, Tokyo, Japan). MKs at stages I, II and III were identified using distinct ultrastructural characteristics: stage I, a cell 10–15 μ m in diameter with a large nucleus; stage II, a cell 15–30 μ m in diameter containing platelet-specific granules; stage III, 40 μ m, a MK containing a well-developed demarcation membrane system defining cytoplasmic territories and a peripheral zone. Samples from at least three mice of each genotype were examined in each case.

Platelet recovery assay

An intraperitoneal injection of anti-GPIIb antibody (Emfret) at 2 mg/kg is performed at D0. Blood samples were collected and platelet counts measured prior to injection, and at D5, D10, D15 and D20.³⁰

HSPC colony-forming unit (CFU) assay

Cells are obtained by flushing and were resuspended in IMDM and plated at a density of 1×10^4 cells per dish in methylcellulose-based medium (MethoCult™ GF M3434, Stemcell Technologies) containing appropriate cytokines to support multilineage hematopoietic colony formation. Cultures were incubated at 37 °C with 5% CO₂ in a humidified incubator for 10–14 days. Colonies were scored under an inverted microscope and classified based on morphological criteria into CFU-G (granulocyte), CFU-M (macrophage), CFU-GM (granulocyte-macrophage), BFU-E (erythroid), and CFU-GEMM (granulocyte, erythrocyte, macrophage, megakaryocyte). Only colonies containing ≥ 50 cells were counted. Results were expressed as the mean colony number $\pm$ SEM from $n = 3$ independent experiments.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9 software. Data were expressed as mean $\pm$ standard error mean (SEM). Statistical significance was analyzed by one- or two-way ANOVA followed by the appropriate *post hoc* test, or Kruskal-Wallis test for nonparametric data, a Bonferroni correction was applied for multiple comparisons, as indicated in figure legends. P-values < 0.05 were considered statistically significant.

Results

Deletion of Shp1 and Shp2 in the MK/platelet lineage

To validate the efficiency of protein ablation of Shp1 and Shp2 proteins in MKs/platelets using the *Gp1ba-Cre* transgenic mouse strain, we employed a quantitative capillary-based immunoassay. This was performed on cells derived from *Ptpn6^{fl/fl};Gp1ba-Cre^{+/-}* (Shp1 KO), *Ptpn11^{fl/fl};Gp1ba-Cre^{+/-}* (Shp2 KO) and *Ptpn6^{fl/fl};Ptpn11^{fl/fl};Gp1ba-Cre^{+/-}* double-knockout (DKO) mice, with *Gp1ba-Cre^{+/-}* littermates serving as controls.

We firstly evaluated Shp1 and Shp2 levels in MK-sorted progenitors (MKPs) in order to obtain a pure population of matures MKs. Under these conditions, we quantified protein loss of Shp1 and Shp2 in MKs, as shown in Figure 1Ai-iii [\[1\]](#), with significant 70% and 82% reduction of Shp1 protein in Shp1 KO and Shp1/2 DKO mice, respectively and 78% and 75% reduction of Shp2 protein in Shp2 KO and Shp1/2 DKO mice, respectively compared to controls.

We next evaluated protein levels in washed platelets and found efficient deletion in Shp1 KO and Shp2 KO platelets, with a significant 90% and 92% reduction of Shp1 protein in Shp1 KO and Shp1/2 DKO mice respectively, and 95% and 96% reduction of Shp2 protein in Shp2 KO and Shp1/2 DKO mice respectively, compared to controls (Figure 1Bi-iii [\[1\]](#)). In addition, there was no upregulation of Shp1 in Shp2-deficient MKs/platelets and vice versa. These results indicate that the *Gp1ba-Cre* transgene efficiently deleted *loxP*-flanked *Ptpn6* and *Ptpn11*, resulting in the significant loss of Shp1 and Shp2 in MKs/platelets.

Macrothrombocytopenia and increased bleeding in Shp1/2 DKO mice

Deletion of Shp1 or Shp2 using the *Gp1ba-Cre*-mouse strain did not affect platelet counts and volumes as shown in Figure 2Ai-ii [\[1\]](#) and Supplemental Table 2 [\[1\]](#). However, Shp1/2 DKO mice were macrothrombocytopenic, with a 40% reduction in platelet count and 15% increase in platelet volume (Figure 2Ai-ii [\[1\]](#)). Monocyte, neutrophil and eosinophil counts were marginally increased in Shp1/2 DKO mice, suggesting possible immune responses. (Supplemental Table 2 [\[1\]](#)).

The primary function of platelets is to prevent blood loss following injury. Therefore, we investigated the effect of the loss of Shp1 and Shp2 on hemostasis. Shp1/2 DKO exhibited a significant increase in bleeding following tail injury as shown in Figure 2Bi-ii [\[1\]](#). Bleeding time was mildly prolonged compared to control mice, suggesting impaired hemostasis. Quantitative measurement of blood loss revealed a substantial increase in hemoglobin levels (Figure 2Bi-ii [\[1\]](#)).

Aberrant functional responses of Shp1/2-deficient platelets

We next investigated platelet receptor expression by flow cytometry. Receptor expression was normal in Shp1- and Shp2-deficient platelets. However, we found that the collagen activation receptor GPVI and the $\alpha 2$ subunit of the collagen integrin $\alpha 2\beta 1$ were reduced by 25% and 13%, respectively, in Shp1/2-deficient platelets (Figure 2Ci-ii [\[1\]](#) and Supplemental Table 3 [\[1\]](#)). All other major surface receptors analyzed were unaltered in these platelets (Supplemental Figure S2 [\[1\]](#) and Supplementary Table 3 [\[1\]](#)).

We next checked platelet aggregation by light transmission aggregometry. Shp1- and Shp2-deficient platelets responded normally to all agonists tested (Figure 3A-C [\[1\]](#)). However, consistent with reduced GPVI and $\alpha 2$ surface expression, Shp1/2-deficient platelets exhibited a marginal reduction in aggregation to a low and intermediate concentration of the GPVI-specific agonist CRP (1 and 3 $\mu\text{g/mL}$), and collagen (3 $\mu\text{g/mL}$), which signals via GPVI and binds with high affinity to the integrin $\alpha 2\beta 1$ (Figure 3Ai-ii [\[1\]](#)). However, they responded normally to thrombin (0.06 and 0.1 U/ml) (Figure 3Bi-ii [\[1\]](#)), antibody-mediated cross-linking of the hem-ITAM-containing podoplanin receptor CLEC-2 (3 $\mu\text{g/mL}$) and ADP (3 μM) (Figure 3Ci-ii [\[1\]](#)).

We also measured P-selectin surface expression in whole blood, as a marker of α -granule secretion. Consistent with reduced GPVI expression, Shp1/2-deficient platelets did not respond to 1, 3 and 10 $\mu\text{g/mL}$ CRP (Figure 3Di [\[1\]](#)), but aggregated normally to 100 and 500 μM PAR-4 peptide (Figure 3Dii [\[1\]](#)).

Aberrant ITAM signaling in Shp1- and Shp2-deficient platelets

Since Shp1 and Shp2 have been implicated in regulating ITAM-containing receptor signaling, we investigated GPVI and CLEC-2 signaling in Shp1- and Shp2-deficient platelets. We focused on SFKs, which phosphorylate tyrosine residues within the ITAM of GPVI-associated FcR γ -chain and hemi-ITAM of the CLEC-2 receptor, and Syk, which docks to both via its tandem SH2 domains, and mediates downstream effects. SFK activation was indirectly measured as *trans*-

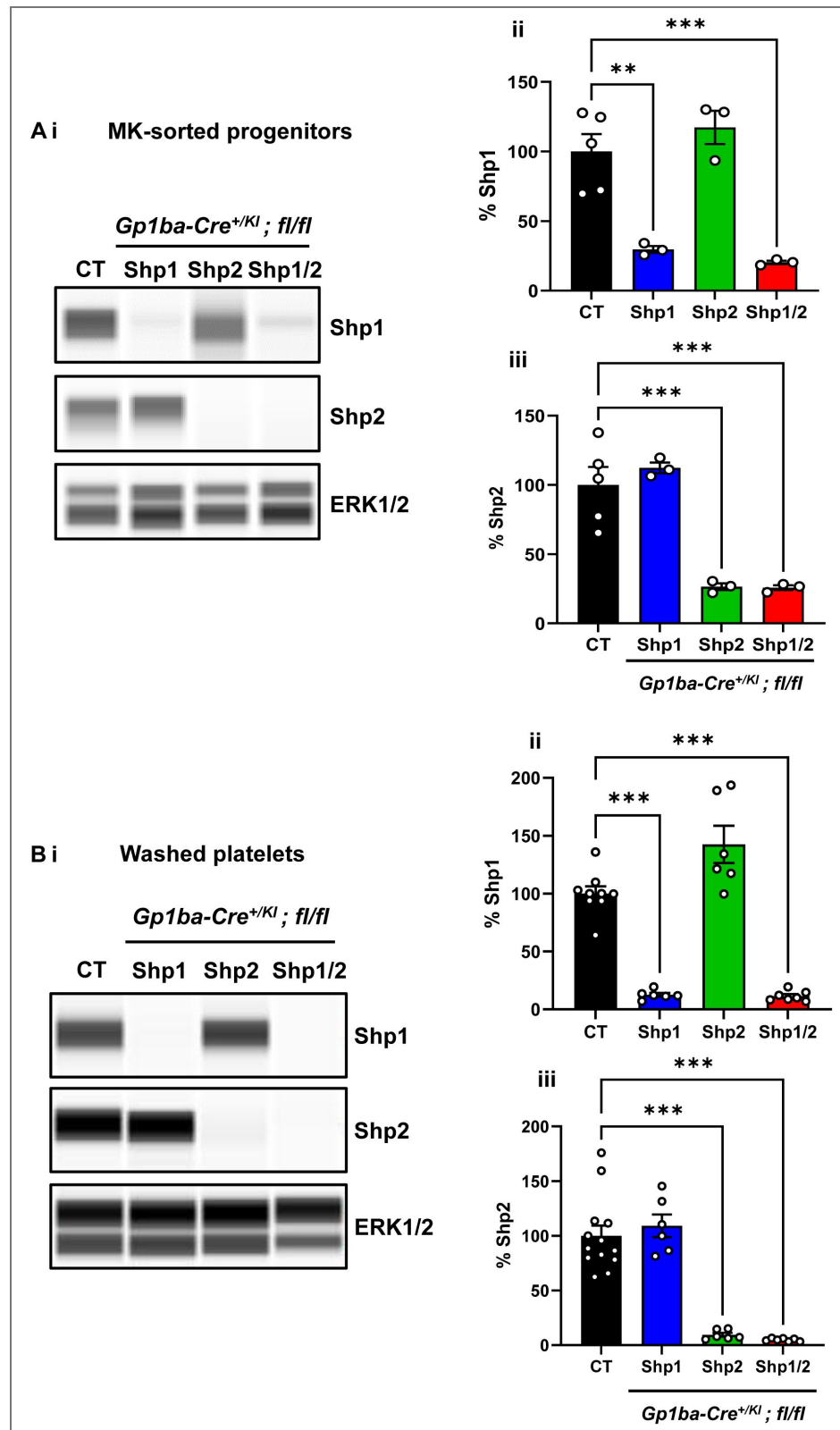


Figure 1. Protein expression of Shp1 and Shp2 in megakaryocytes and platelets from *Gp1ba-Cre* mice.

(i) Representative blots of Shp1 and Shp2 expression levels from CT, Shp1, Shp2 and Shp1/2 DKO mice by capillary immunoassays with the respective antibodies in (A) sorted-MK progenitors and (B) washed platelets. Percentage of residual level of (ii) Shp1 and (iii) Shp2 in Shp1 KO (blue), Shp2 KO (green) and Shp1/2 DKO (red) mice. $n = 3-6$ mice per genotype. Mean $\pm$ SEM; one way-ANOVA; ** $P < 0.01$, *** $P < 0.001$.

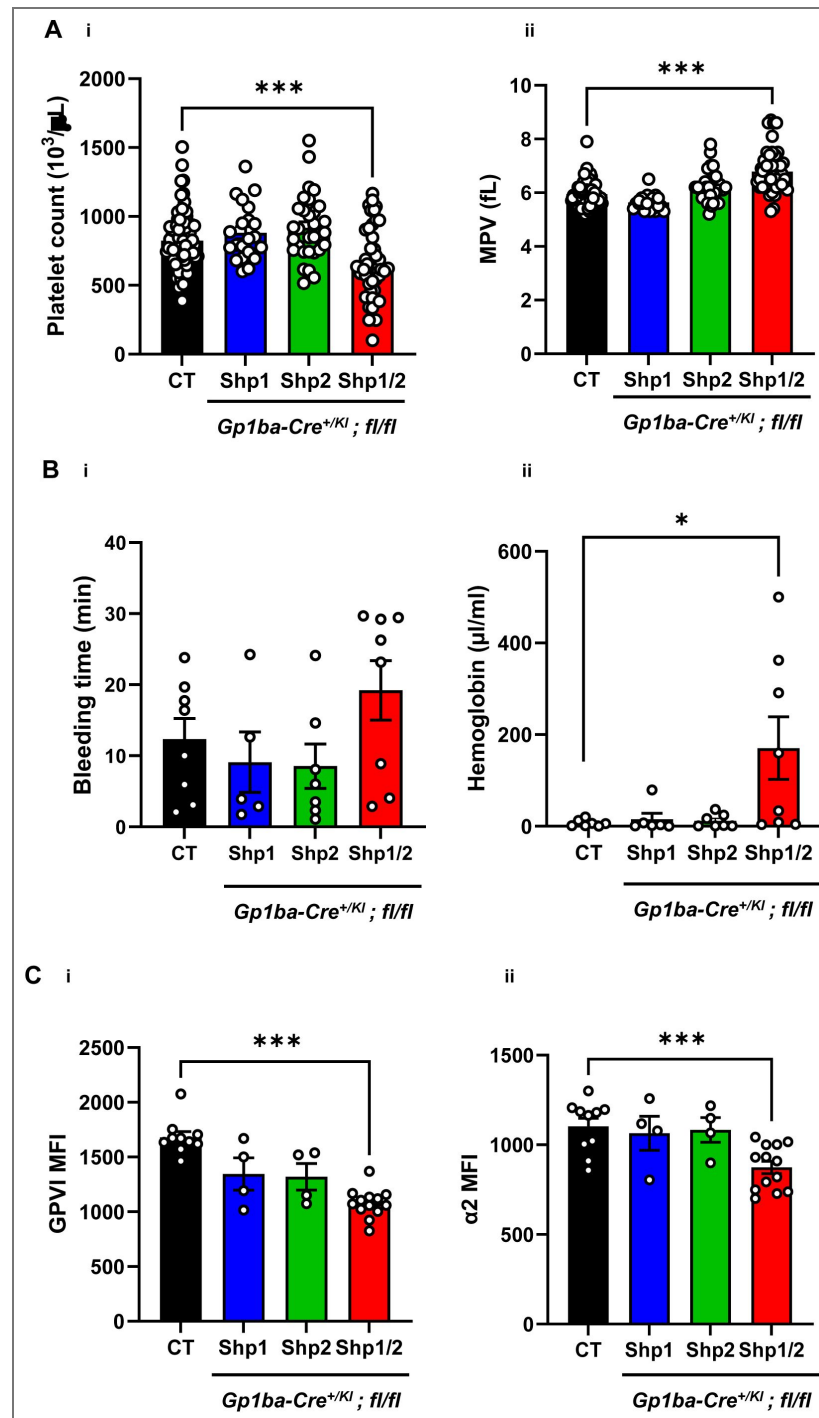


Figure 2. Macrothrombocytopenia and mild increased bleeding in Shp1/2 DKO mice.

(A) Platelet counts (i) and platelet volumes (ii) of control (CT) ($n=75$), Shp1 KO ($n=21$), Shp2 KO ($n=29$) and Shp1/2 DKO ($n=46$) mice. Mean $\pm$ SEM; one way-ANOVA; *** $P < 0.001$. **(B)** (i) Cumulative bleeding time was recorded (ii) and the volume of blood loss was measured over 30 minutes. Mean $\pm$ SEM; one way-ANOVA; * $P < 0.05$. **(C)** Mean fluorescence intensity (MFI) of platelet (i) GPVI and (ii) $\alpha 2$ integrin expression in CT ($n=10$), Shp1 KO ($n=4$), Shp2 KO ($n=4$) and Shp1/2 DKO ($n=13$) mice. Mean $\pm$ SEM; one way-ANOVA; *** $P < 0.001$.

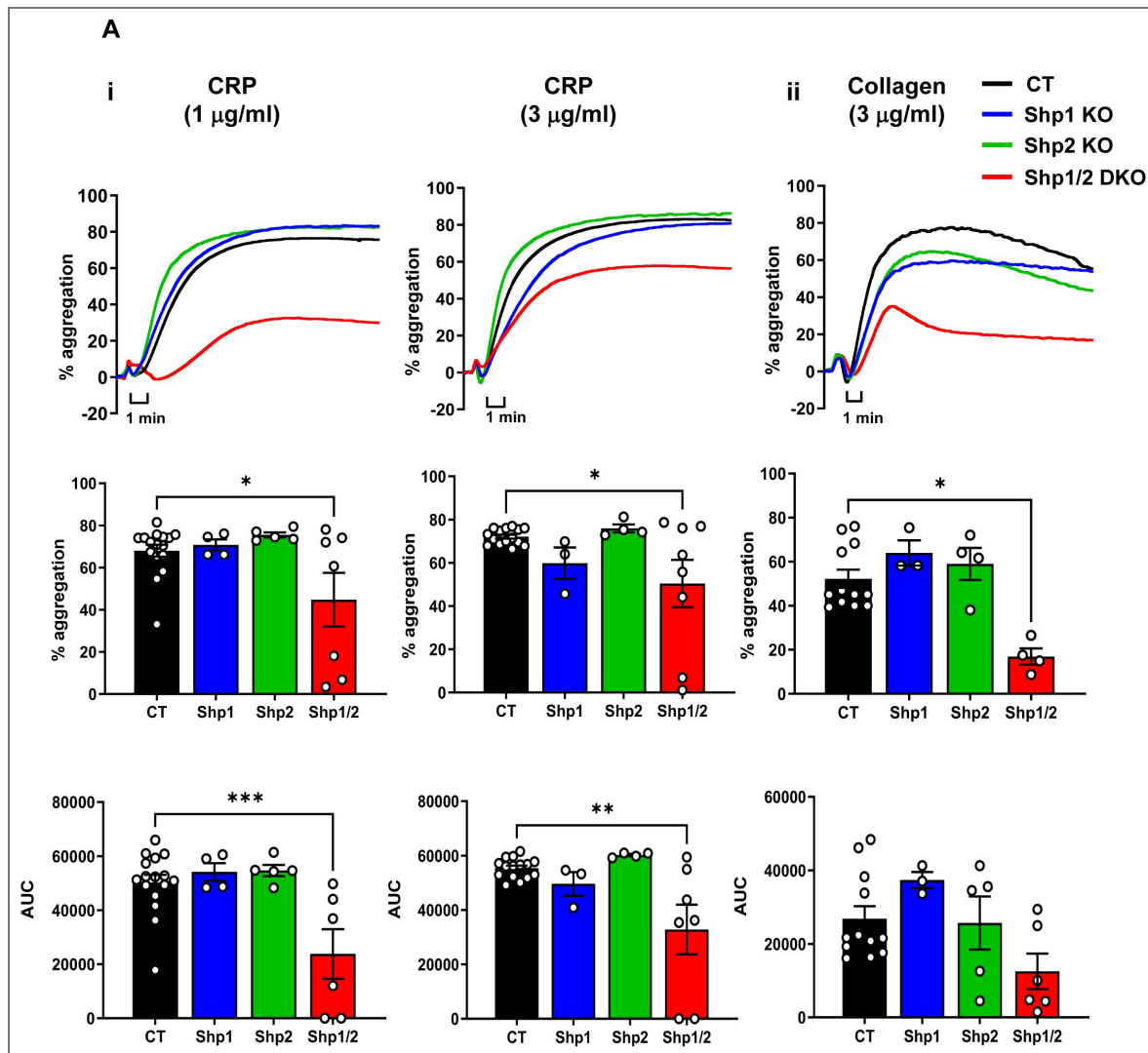


Figure 3. Aberrant platelet function in Shp1/2 DKO mice.

(A-C) Aggregation of washed platelets were measured by lumi-aggregometry in response to agonists indicated. Representative traces, $n = 4-8$ mice/genotype per condition, percentage of aggregation at 5 minutes and area under the curve (AUC) quantification. Mean $\pm$ SEM, $n = 5-17$ mice/genotype per condition, one way-ANOVA, * $P < 0.05$. (D) Mean fluorescence intensity (MFI) of P-selectin expression of control (CT), Shp1 KO, Shp2 KO and Shp1/2 DKO platelets in whole blood in response to (i) 1 and 3 $\mu\text{g/ml}$ CRP and (ii) 100 and 500 μM PAR4 peptide (PAR4p). Mean $\pm$ SEM, $n = 5-9$ mice/genotype per condition, two way-ANOVA; * $P < 0.05$, *** $P < 0.001$.

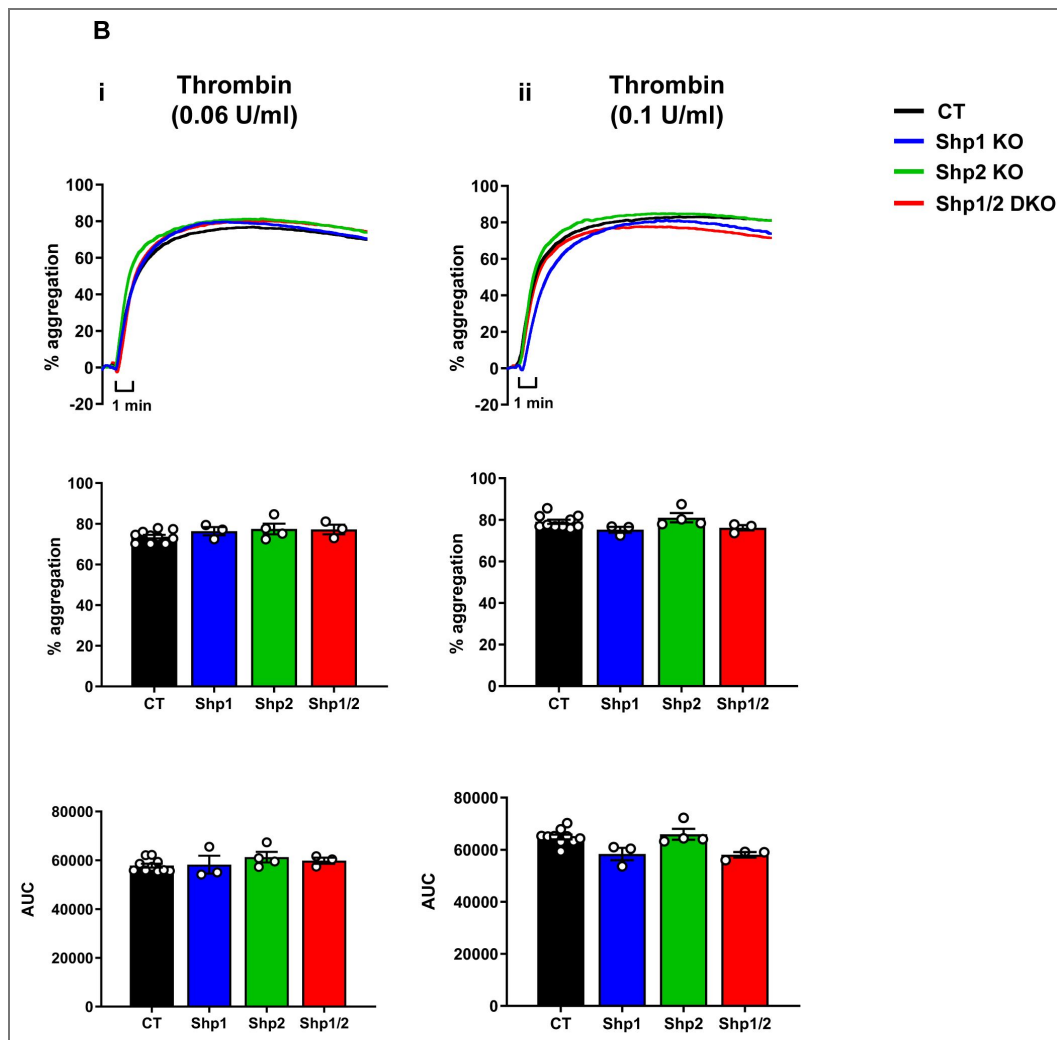


Figure 3. (continued)

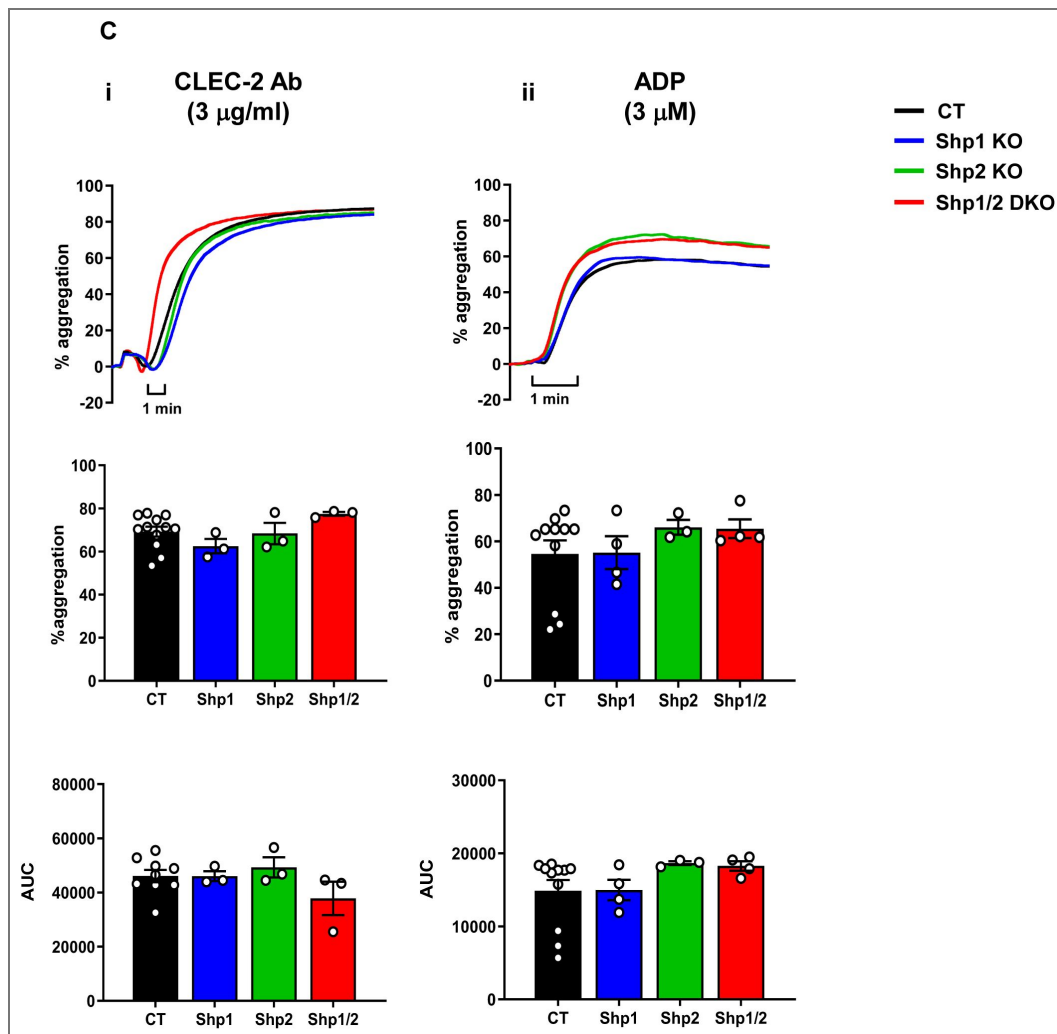


Figure 3. (continued)

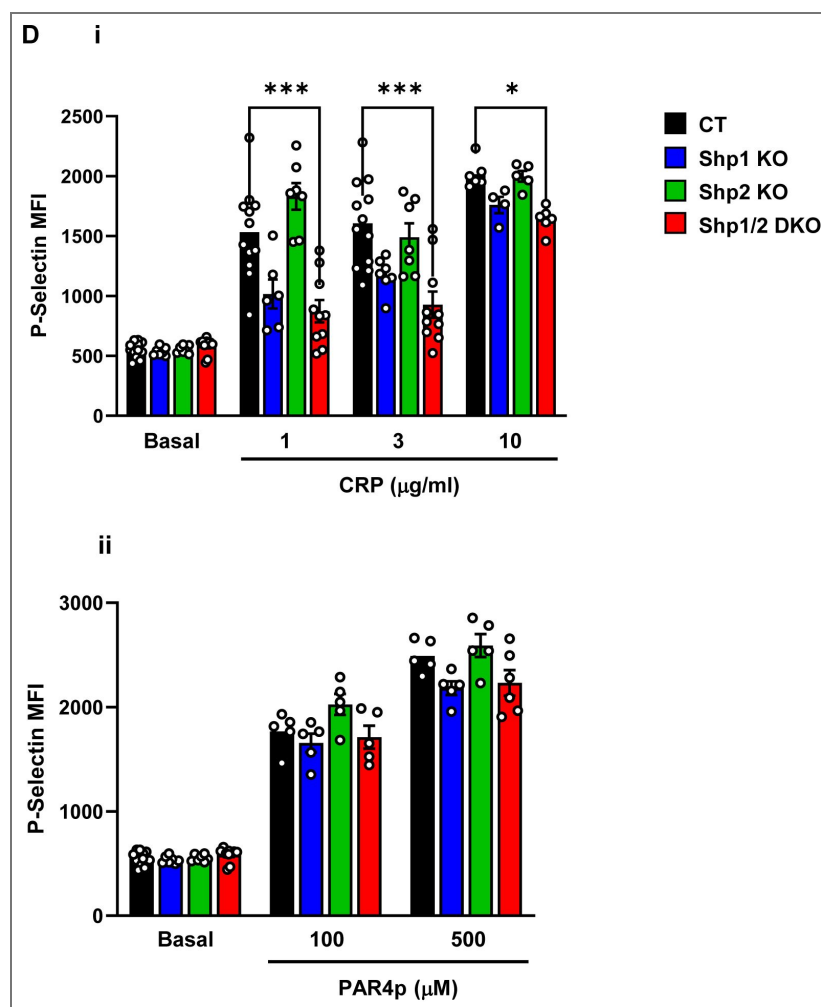


Figure 3. (continued)

autophosphorylation of the highly conserved tyrosine residue 418 in Src (Src p-Tyr418) and Syk activation was measured as *trans*-autophosphorylation of tyrosine residues 519/520 (p-Tyr519/20), which directly correlate with activity. Western blot analysis demonstrated that Syk p-Tyr519/520 was significantly decreased in CRP-stimulated platelets from Shp1/2-deficient platelets (Figure 4Ai [↗](#)), and marginally reduced in CLEC-2-stimulated Shp1 KO and Shp2 KO deficient platelets. (Figure 4Bi [↗](#)). Notably, Src p-Tyr418 was not altered under any conditions (Figure 4Aii [↗](#) and Bii [↗](#)), suggesting that Shp1 and Shp2 modulate Syk-dependent signaling downstream of GPVI and CLEC-2.

Impaired megakaryocyte differentiation and function

To determine the cause of thrombocytopenia in Shp1/2 DKO mice, we measured platelet recovery following antibody-mediated depletion as an indicator of platelet turnover. As expected, administration of the anti-GPIIb antibody resulted in extreme and sustained thrombocytopenia, confirming efficient depletion of circulating platelets (Supplemental Figure S3 [↗](#)). No differences were observed the different phases or kinetics of platelet clearance or recovery in any of the mouse models. Interestingly, spleen size and weight were significantly increased in Shp1/2 DKO mice (Supplemental Figure S4 [↗](#)), suggesting compensatory extramedullary hematopoiesis. However, HSPC colony-forming unit (CFU) assays performed on BM cells showed no significant difference in the frequency or composition of HSPCs, including MKPs between genotypes *in vitro* (Supplemental Figure S5 [↗](#)).

Despite the maintenance of normal platelet turnover *in vivo*, *in vitro* analyses revealed pronounced defects in MK development, maturation and function of Shp1/2-deficient MKs (Figure 5A-C [↗](#)). BM-derived MKs from DKO mice exhibited a severe defect in polyploidization, as shown by a significant increase in 2-8N immature MKs, and decreased 16-64N mature MKs (Figure 5Ai-ii [↗](#)). Moreover, proplatelet formation was severely impaired, with a significantly lower percentage of MKs extending cytoplasmic projections compared to controls (Figure 5B [↗](#)).

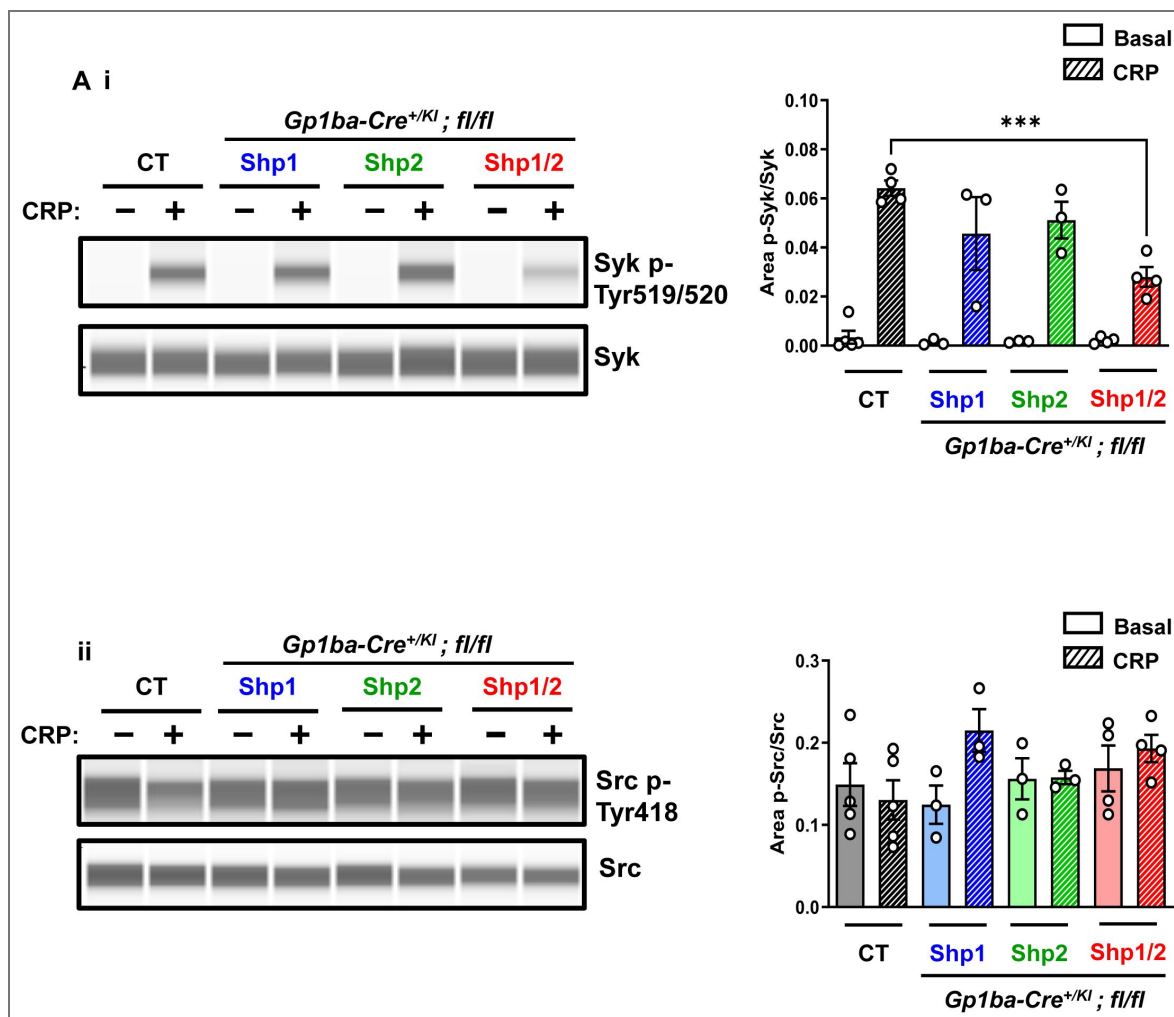
Collectively, these data suggest that while homeostatic and compensatory thrombopoiesis is preserved *in vivo*, potentially supported by extramedullary hematopoiesis, Shp1 and Shp2 are required for efficient megakaryopoiesis *in vitro*, highlighting a critical role in late-stage megakaryopoiesis under defined conditions.

Reduced Tpo signaling in Shp1/2-deficient MKs

To understand the molecular basis of impaired megakaryopoiesis in Shp1/2 DKO mice, we assessed Tpo-mediated signaling in BM-derived MKs. We previously demonstrated that Shp2 is a positive regulator of ERK1/2 downstream of Mpl. Tpo-mediated ERK1/2 activation was normal in Shp1-deficient MK and significantly reduced in Shp2- and Shp1/2-deficient MKs. However, other downstream effectors, including AKT and STAT3, showed modest or variable changes (Figure 5C [↗](#)). These findings provide a mechanistic explanation for the impaired MK development and maturation observed *in vitro*, and defective Tpo signaling through the ERK1/2 pathway in Shp1/2-deficient MK, underscoring the importance of these phosphatases in late-stage megakaryopoiesis.

Defective proplatelet formation and MK maturation in Shp1/2-deficient mice

To further characterize the functional consequences of Shp1 and Shp2 deficiency on megakaryopoiesis, we evaluated proplatelet formation *ex vivo* and examined MK morphology *in situ*. We first performed *ex vivo* proplatelet formation assays using BM explants. The results revealed a marked reduction in proplatelet formation in Shp1/2 DKO mice compared with controls, with an increased number of round MKs and a decreased number of MK-forming proplatelets in Shp1/2 DKO mice (Figure 6A-B [↗](#)). To assess MK maturation *in situ*, we performed EM and ploidy analysis in BM. In control mice, MK displayed characteristic features of terminal maturation, including large polyploid nuclei, demarcation membrane system (DMS) organization, and extensive cytoplasmic development. Shp1/2 DKO MK exhibited a maturation arrest, with



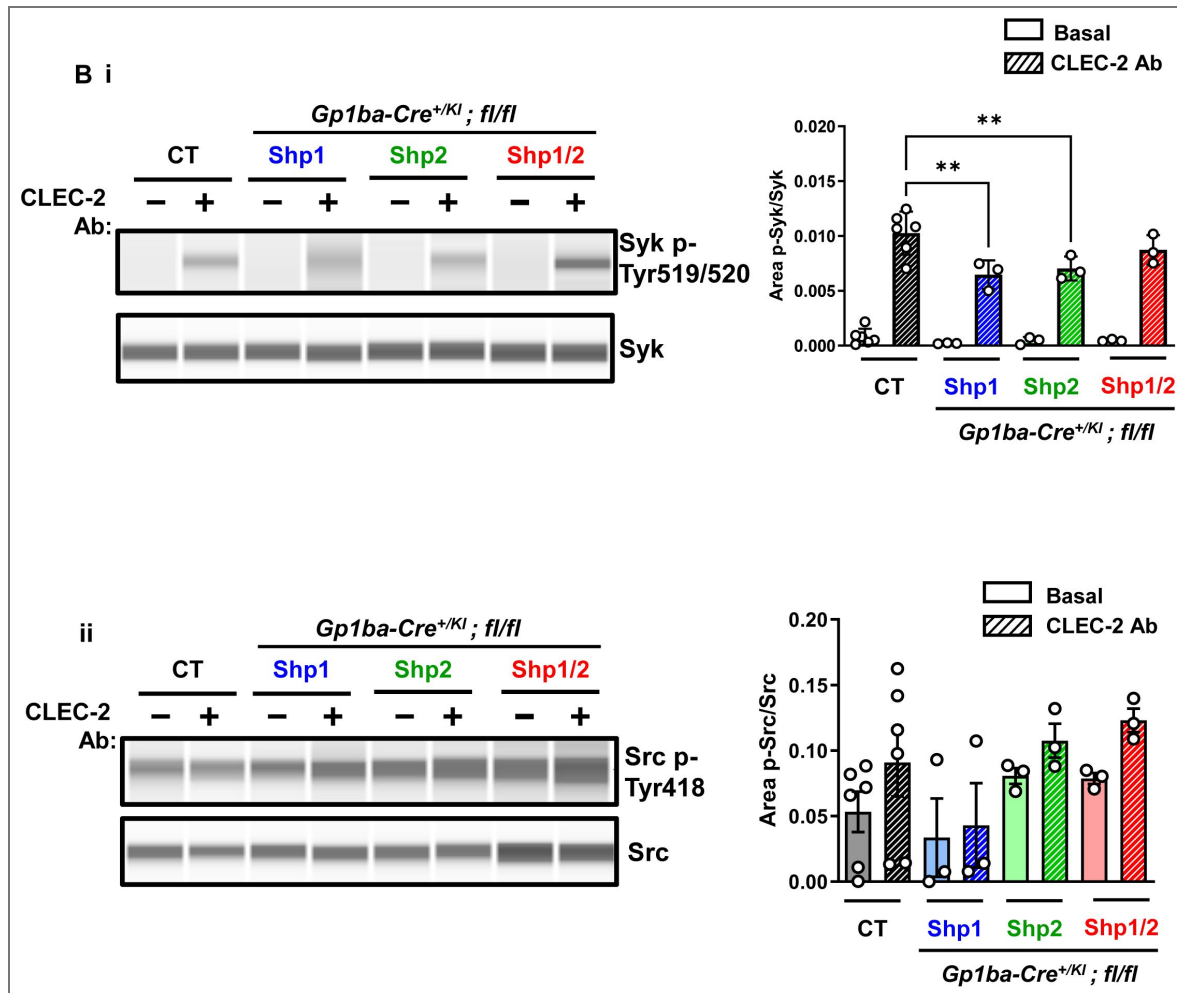


Figure 4. (continued)

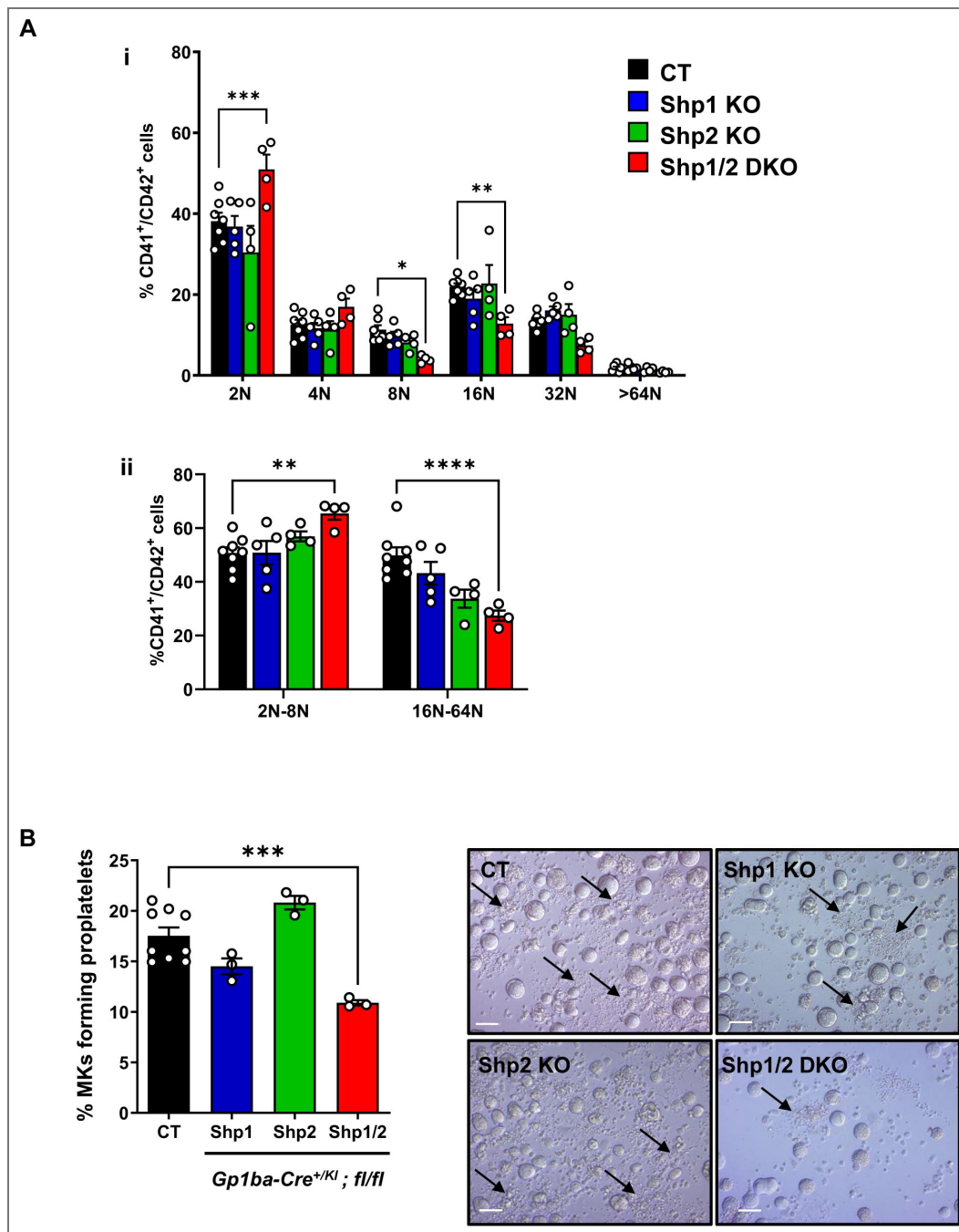


Figure 5. Defects in megakaryocyte maturation and Tpo signaling in Shp1/2 DKO.

(A) Mature BM-derived MKs from Shp1 KO, Shp2 KO, Shp1/2 DKO, and litter-matched CT mice were stained with propidium iodide and ploidy of cells was quantified by flow cytometry. **(i-ii)** The percentage of 2-4N and 8-128N ploidy cells was quantified ($n = 4-6$ mice/genotype; mean $\pm$ SEM; two-way ANOVA, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). **(B)** *Ex vivo* proplatelet formation. Percentage of MKs forming proplatelet was quantified in culture. Mean $\pm$ SEM, two-way ANOVA, *** $P < 0.001$. **(C)** Mature BM-derived MKs from Shp1 KO, Shp2 KO, Shp1/2 DKO and litter-matched CT mice were stimulated with 50 ng/mL thrombopoietin (Tpo) for 10 min at 37°C. Whole cell lysates were western blotted with indicated antibodies. Representative blots capillary-based immunoassays and quantification of peak areas from $n = 3$ independent experiments/genotype; two-way ANOVA, *** $P < 0.001$.

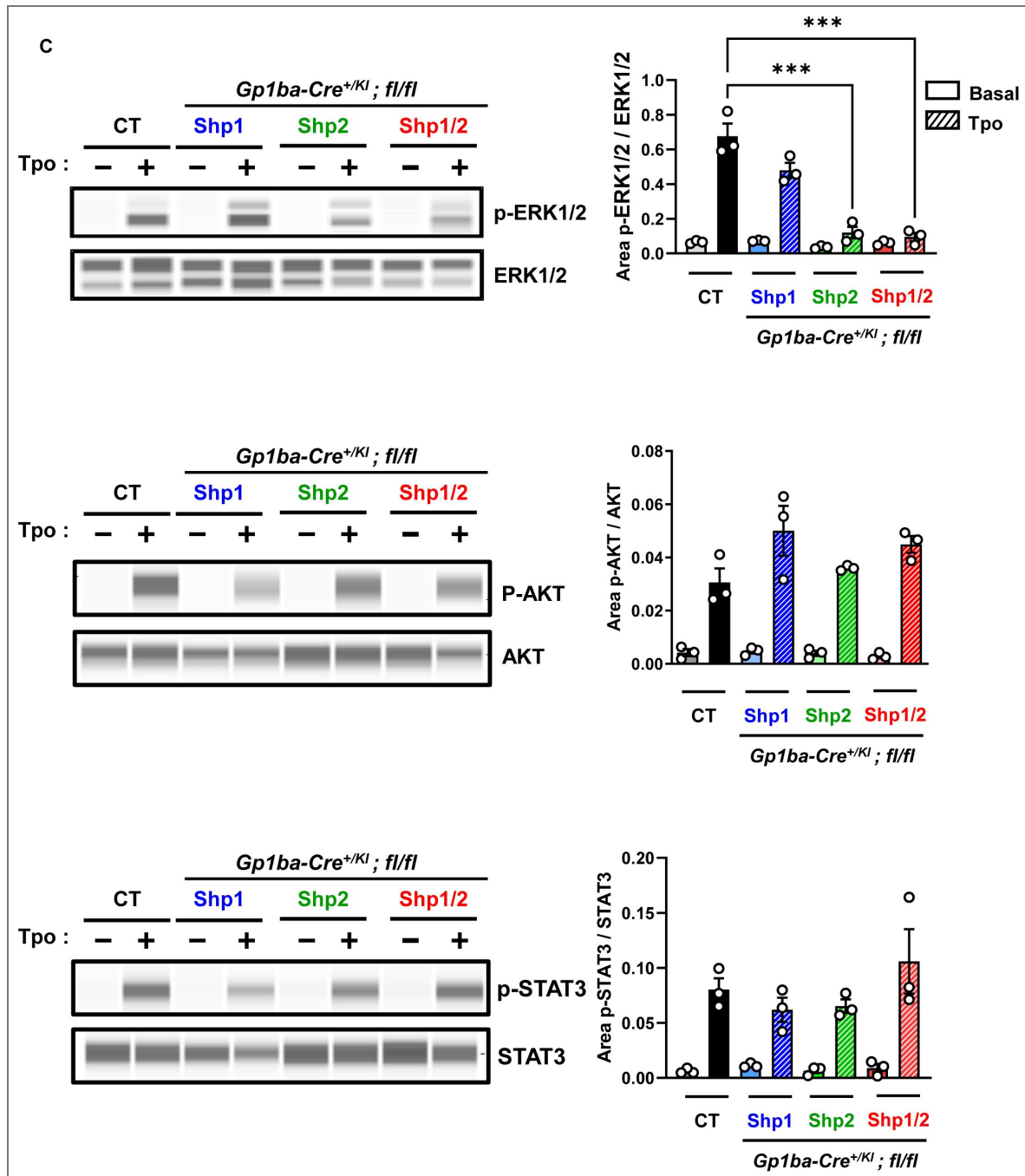


Figure 5. (continued)

increased frequency of stage III MK, defined by incomplete DMS formation, and limited cytoplasmic granularity (Figure 6Ci-ii). This was further supported by flow cytometric analysis of DNA content, showing lower ploidy level (4N–8N) in Shp1/2 DKO MK (Figure 6D). These findings provide further evidence of the involvement of Shp1 and Shp2 in late-stage MK maturation and proplatelet formation, absence of which results in the accumulation of immature, stage III MK in the BM and a functional failure to complete thrombopoiesis.

Shp2 allosteric inhibitors impair megakaryopoiesis and Tpo signaling

To further delineate the respective roles of Shp1 and Shp2 in MK and Tpo signaling, we employed two pharmacologically distinct inhibitors for each phosphatase to ensure specificity and validate findings from genetically modified mouse models.

For Shp1, we used M029, an covalent allosteric inhibitor that induces conformational changes that block Shp1 activity (Z.Q. and Z.Y.Z., manuscript in preparation), and F2Ac, a reversible inhibitor, which directly target the catalytic site (J.M. and Z.Y.Z., manuscript in preparation). Treatment of murine BM-derived MK with these Shp1 inhibitors showed no significant effects on cell viability, proliferation and MK ploidy (Figure 7Ai-iii), nor on Tpo-mediated signaling (Figure 7B), correlating with what was observed in Shp1-deficient MKs. Similarly, Shp2 was inhibited using SHP099 and RMC-4550, both selective allosteric inhibitors that stabilize Shp2 in its inactive conformation³¹. Addition of Shp2 inhibitors resulted in a marginal decrease in cell viability and a marked reduction of cell proliferation, both established functions of Shp2 in other lineages (Figure 7Ci-ii). Moreover, treatment with either SHP099 or RMC-4550 resulted in a significant inhibition of ploidy with a reduction in the percentage of cells achieving >8N (Figure 7Ciii), and a significant reduction in proplatelet formation (Figure 7Civ). This was accompanied by a severe impairment in Tpo-mediated ERK1/2 and AKT phosphorylation (Figure 7D). This dual-inhibitor profiling reveals that Shp2, but not Shp1, is critical for Tpo-driven MK proliferation, survival, endomitosis and Mpl signaling, highlighting Shp2 as a key regulator of late stage megakaryopoiesis and suggest that its inhibition disrupts thrombopoietic pathways.

Discussion

In this study, we used the *Gp1ba-Cre* transgenic mouse model to achieve conditional deletion of the tyrosine phosphatases Shp1 and Shp2 in the MK/platelet lineage. Our results demonstrate efficient deletion of Shp1 and Shp2 in MKs and more completely in platelets, confirming the utility of this mouse model for studying protein involvement in megakaryopoiesis and thrombopoiesis. Residual Shp1 and Shp2 in MKs allowed us to study the phenotype of DKO MKs more thoroughly compared with DKO MKs on the *Pf4-Cre* background, which were severely compromised developmentally and did not survive *in vitro*.¹⁵ Additionally, the less severe MK defects meant fewer platelet anomalies, which contained lower levels of Shp1 and Shp2 than the MKs, suggesting different half-lives of the phosphatases in MKs versus platelets. The *Gp1ba-Cre* transgenic mouse allowed us to reveal distinct and synergistic functions of Shp1 and Shp2 in these processes, without confounding effects of deleting Shp1 and Shp2 in non-MK/platelet lineages observed with the *Pf4-Cre* transgene mouse.

Deletion of Shp1 or Shp2 alone did not significantly affect platelet counts or volumes, while combined deletion resulted in macrothrombocytopenia characterized by a 40% reduction in platelet counts and marginal increase in platelet volume. This phenotype was accompanied by prolonged bleeding and increased blood loss following tail tip excision, indicating impaired hemostatic function. These defects correlated with reduced GPVI and $\alpha 2$ expression, GPVI-mediated platelet aggregation and P-selectin expression. GPVI signaling was also impaired in Shp1/2-deficient platelets, with reduced Syk phosphorylation despite normal SFK activation, suggesting that Shp1 and Shp2 modulate ITAM/Syk proximal signaling. This is further supported by the marginal reduction in Syk activation downstream of the hemi-ITAM-containing CLEC-2 receptor.

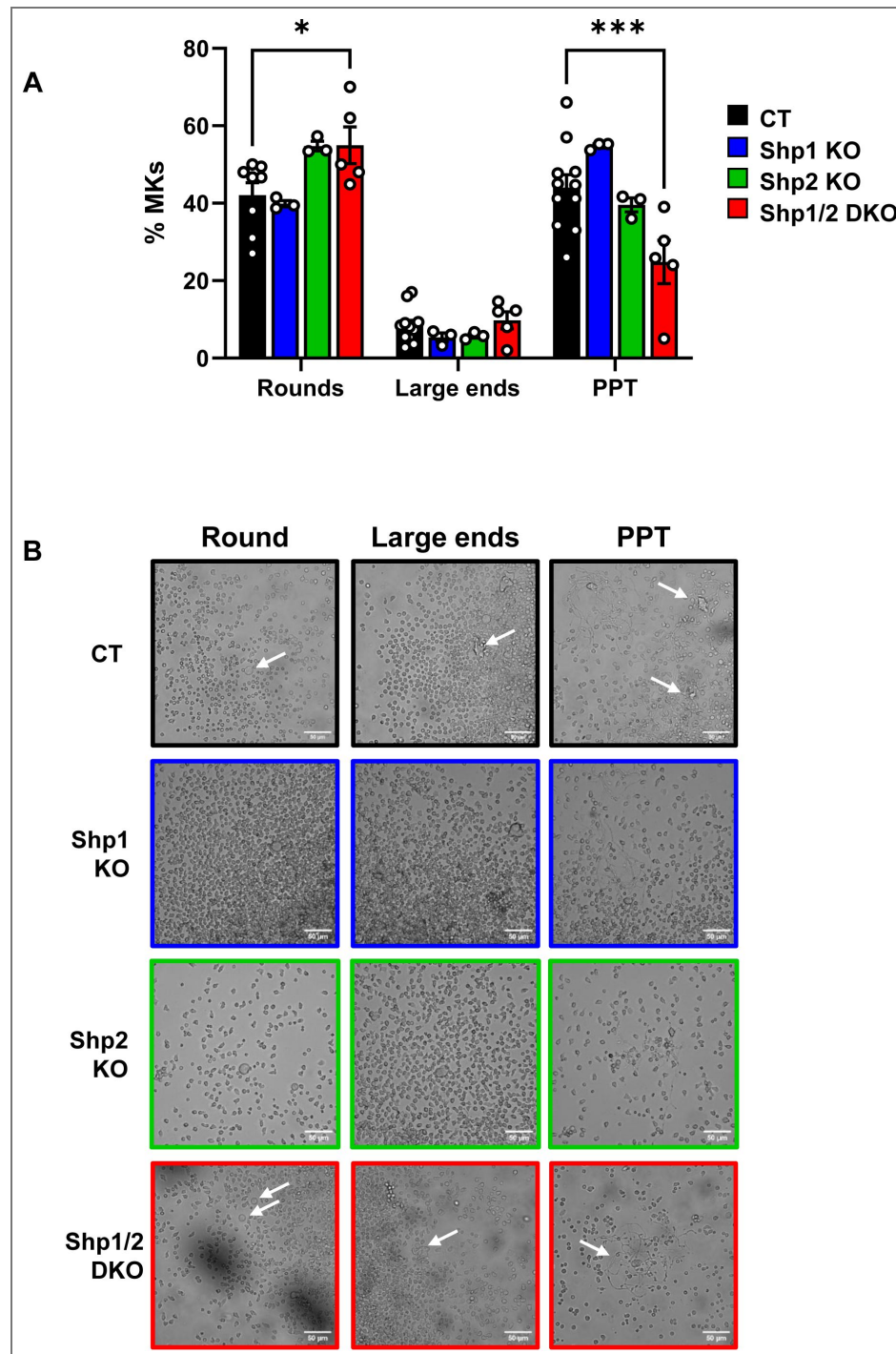


Figure 6. Defects in *ex vivo* proplatelet formation and *in vivo* MK maturation in Shp1/2 DKO mice.

Bone marrow explants. Proportion of MKs from Shp1 KO, Shp2 KO, Shp1/2 DKO and litter-matched CT mice extending proplatelets at 6h of observation were observed. Bars represent the mean $\pm$ SEM of six independent experiments. **(A)** Quantification and **(B)** representatives' images of round MKs, MKs with large ends and proplatelet (PPT) forming MKs. Scale bar: 50 μ m. Mean $\pm$ SEM, one-way ANOVA, * $P < 0.1$, *** $P < 0.001$. **(C)** Classification of the MK according to their maturation stage: stage I (absence of granules), stage II (granules and developing demarcation membrane system (DMS) not yet organized), stage III (DMS organized in cytoplasmic territories). Data are reported as the percentage of the total number of MK, **(i)** of all stage MK and **(ii)** only stage 3 MK. Bars represent the mean $\pm$ SEM in three BM samples, *** $P < 0.001$. **(D)** Ploidy of *in situ* BM MKs measured by flow cytometry. Mean $\pm$ SEM, two-way ANOVA, * $P < 0.1$, *** $P < 0.001$.

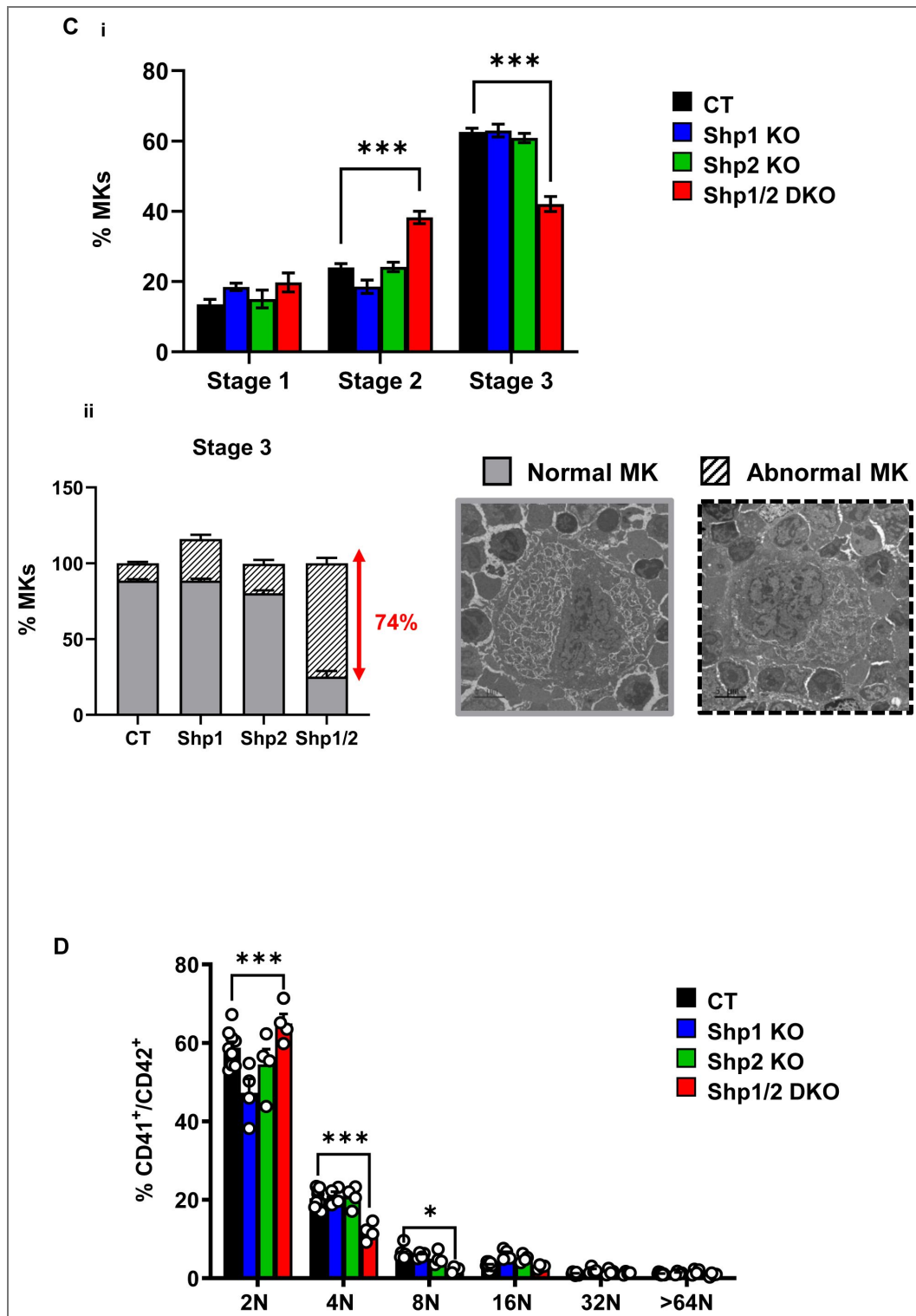


Figure 6. (continued)

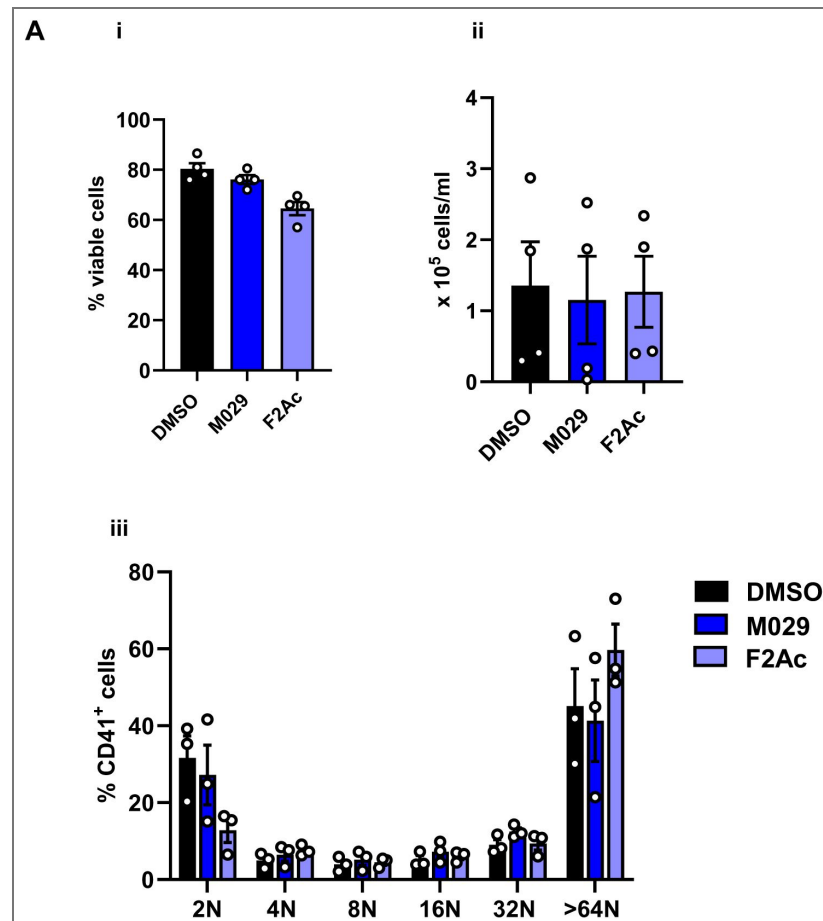


Figure 7. Inhibition of Shp2 activity impairs megakaryopoiesis and Mpl signaling.

(A) Effects of two selective Shp1 allosteric inhibitors 10 μ M M029 and 10 μ M F2Ac were tested on megakaryopoiesis. **(i)** Viability, **(ii)** proliferation, and **(iii)** MK ploidy. Quantification from $n = 3$ -4 independent experiments/condition. **(B)** Whole cell lysates of resting and 50 ng/mL Tpo-stimulated MKs were western blotted with the indicated antibodies. Representative blots capillary-based immunoassays and quantification of peak areas from $n = 3$ independent experiments/genotype. **(C)** Effects of two selective Shp2 allosteric inhibitors 10 μ M SHP099 and 10 μ M RMC-4550 were tested on megakaryopoiesis. **(i)** Viability, **(ii)** proliferation, **(iii)** MK ploidy and **(iv)** percentage of MKs forming proplatelets. Quantification from $n = 3$ -6 independent experiments/condition. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, two-way ANOVA. **(D)** Whole cell lysates of resting and 50 ng/mL Tpo-stimulated MKs were western blotted with the indicated antibodies. Representative blots capillary-based immunoassays and quantification of peak areas from $n = 3$ independent experiments/genotype. * $P < 0.05$; ** $P < 0.01$; two-way ANOVA.

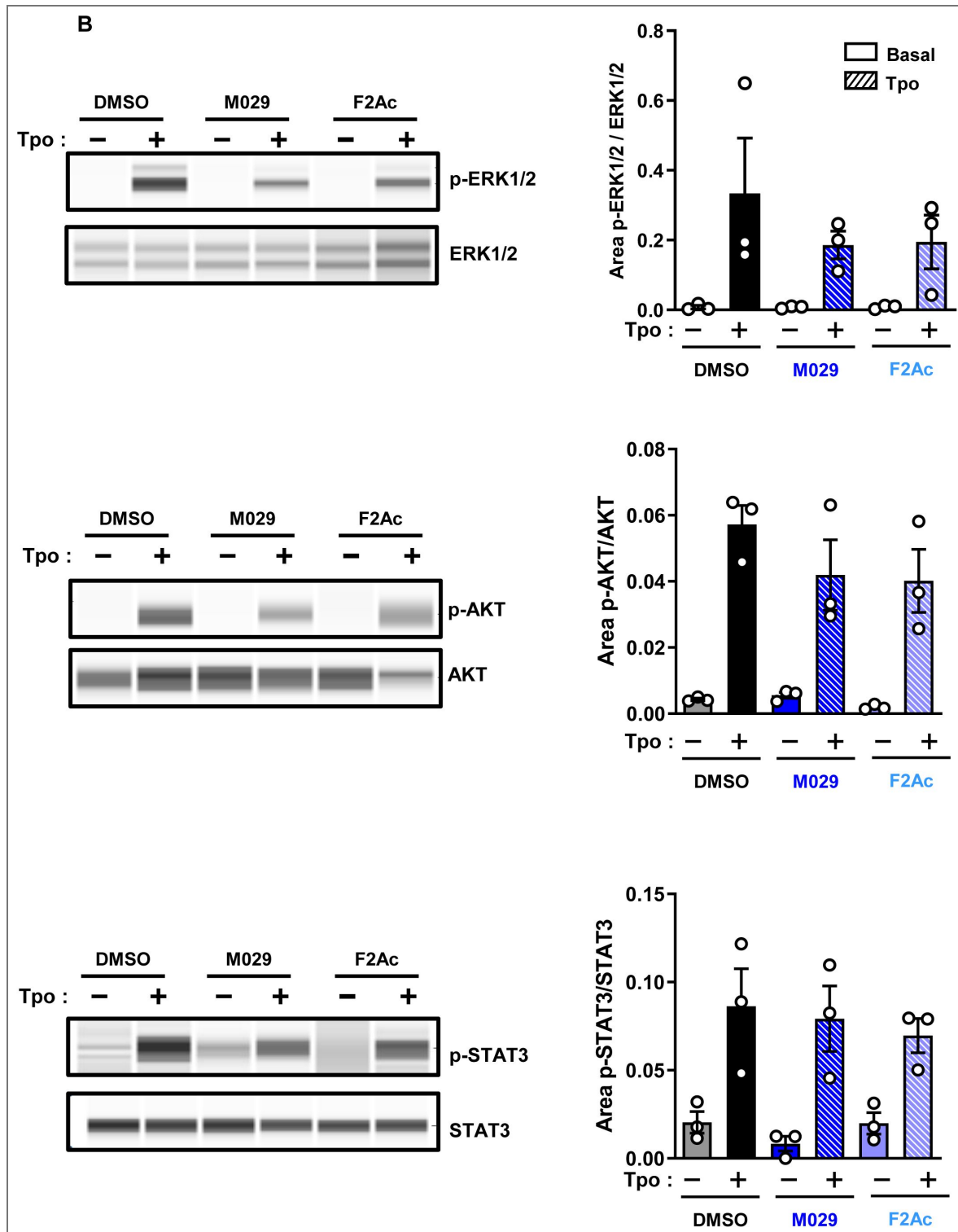


Figure 7. (continued)

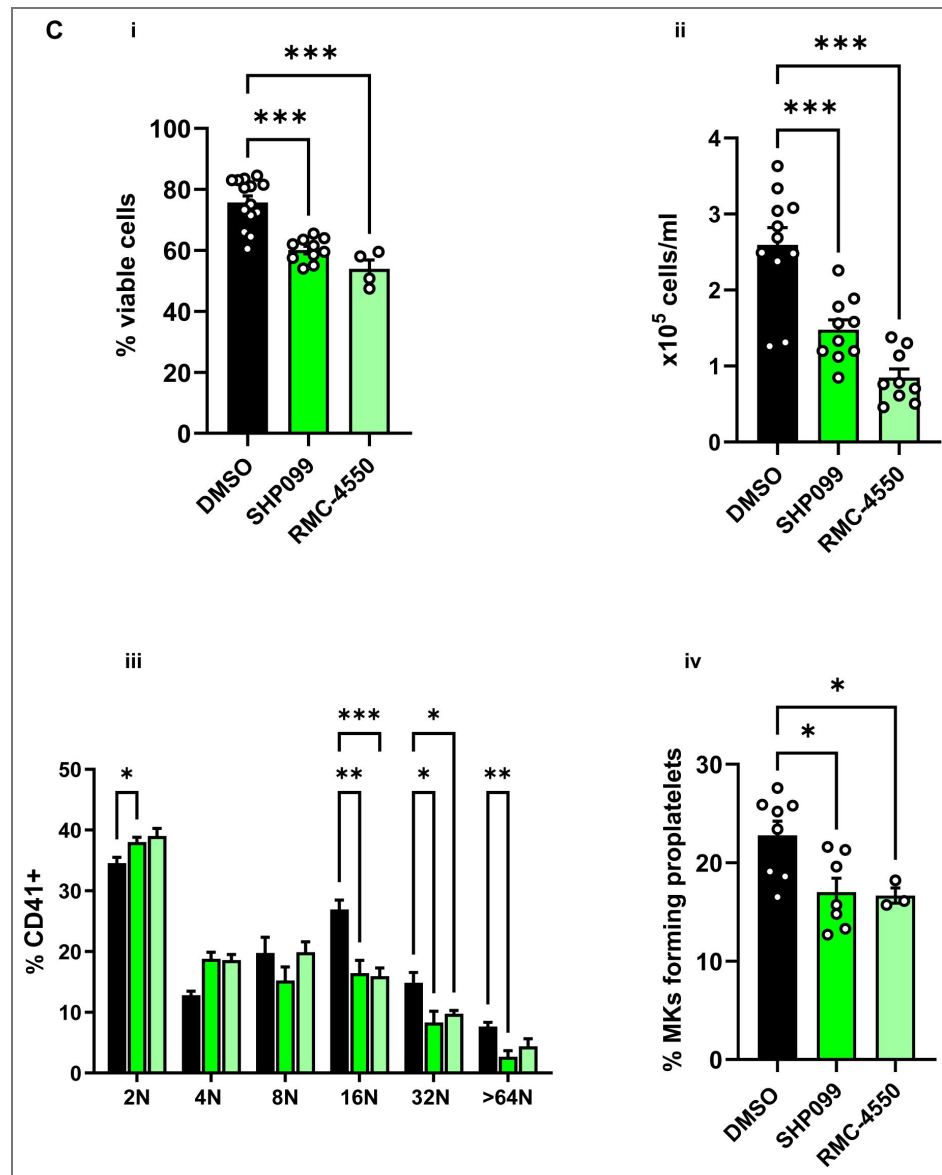


Figure 7. (continued)

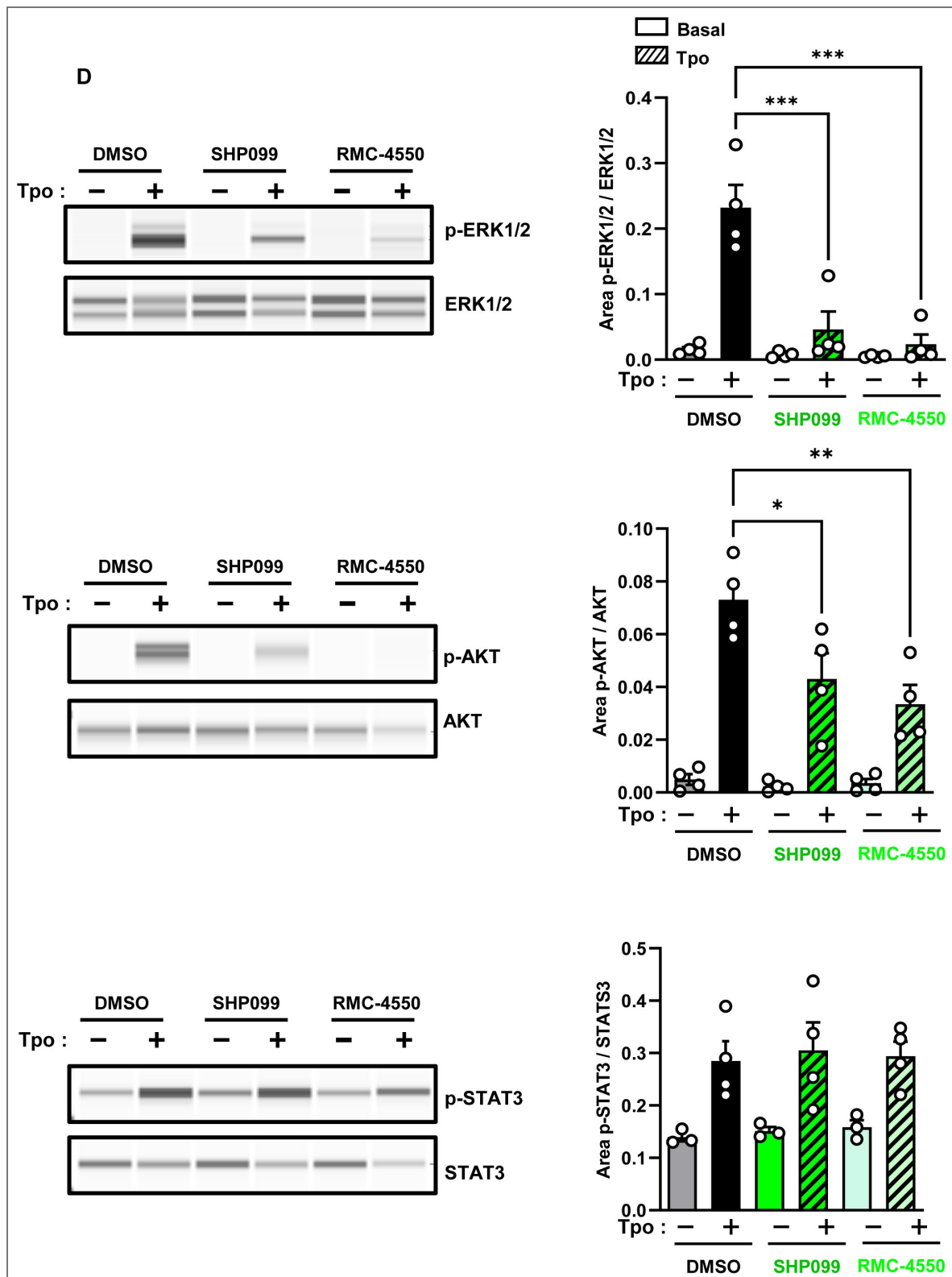


Figure 7. (continued)

Similar phases and kinetics of platelet recovery following immune-induced platelet clearance suggest that thrombocytopenia in Shp1/2 DKO mice is not due to increased platelet clearance, but rather to impaired MK development, maturation and function. *In vitro* assays showed significant defects in MK polyploidization and proplatelet formation, while *in vivo* analysis demonstrated an accumulation of immature MKs with incomplete demarcation membrane system formation and lower ploidy levels. These findings point to a critical requirement for Shp1 and Shp2 in late-stage MK maturation, a process tightly regulated by Tpo signaling through Mpl. Consistently, Tpo-mediated activation of the ERK1/2 pathway was markedly reduced in Shp2- and Shp1/2-deficient MKs, while changes in AKT and STAT3 pathways were modest, underscoring the prominent role of Shp2 in facilitating Tpo-driven Ras/MAPK signaling. Based on these findings, the synergistic effects of deleting both Shp1 and Shp2 suggests that Shp1 acts through another, as yet undefined pathway vital for megakaryopoiesis and thrombopoiesis. RhoA/ROCK is an obvious candidate that Shp1 has been implicated in regulating in other lineages and is essential for cytoskeletal remodeling during endomitosis.

Pharmacological inhibition corroborated genetic findings. Selective inhibition of Shp2 with allosteric inhibitors SHP099 and RMC-4550 significantly impaired MK viability, proliferation, polyploidization, proplatelet formation, and Tpo signaling, while Shp1 inhibitors M029 and F2Ac had no significant effects. This demonstrates a dominant role for Shp2 in regulating MK proliferation and differentiation downstream of Tpo, whereas Shp1 is dispensable for these functions. The pharmacological data thus confirm the functional specificity of the two phosphatases and provide further mechanistic insights into their differential contributions. The milder nature of the platelet and hemostasis phenotypes in Shp1/2 DKO platelets suggests that inhibiting Shp2 will not have any bleeding consequence in patients.

The relatively milder, lineage-specific phenotype of *Gp1ba-Cre*-driven Shp1/2 DKO mice provides distinct advantages to the previously described *Pf4-Cre* mouse model^{32,33} (Supplemental Table 4³²). *Pf4-Cre*-mediated deletion induces recombination earlier during megakaryopoiesis, starting in MK progenitors, whereas *Gp1ba-Cre* is activated predominantly in mature MKs and platelets. This temporal difference allows normal early MK development and platelet production, resulting in less severe thrombocytopenia and functional defects. Additionally, our protein quantification data show significant, but incomplete ablation of Shp1 and Shp2 in BM-derived MKs, suggesting residual phosphatase activity is sufficient under steady-state conditions. In contrast, *Pf4-Cre* models generally achieve near-complete deletion at earlier stages of development, leading to profound thrombocytopenia and often lethality. Furthermore, compensatory extramedullary hematopoiesis observed in our *Gp1ba-Cre* model may further alleviate thrombocytopenia. Importantly, this milder phenotype allowed us to study the Shp1/2 DKO mice in detail, which was impossible with the *Pf4-Cre* model due to the severity of the phenotype and cell viability. Findings further establish the utility of the *Gp1ba-Cre* strain for dissecting the distinct roles of Shp1 and Shp2 in late-stage megakaryopoiesis and platelet biology.

Collectively, Shp2 is a critical positive regulator of Mpl signaling and downstream Ras/MAPK pathways, essential for MK proliferation, polyploidization, and proplatelet formation, whereas Shp1 plays a more modulatory role in ITAM-containing receptor signaling in platelets without directly impacting MK maturation. The combined deletion of Shp1 and Shp2 reveals additive effects on platelet function and MK development, underscoring the importance of coordinated phosphatase regulation in maintaining platelet homeostasis. This study provides the first detailed analysis of Shp1 and Shp2 functions in megakaryopoiesis and platelet biology using a late-stage MK/platelet-specific deletion model, highlighting the indispensable role of Shp2 in megakaryopoiesis and thrombopoiesis, and distinct contributions of Shp1 in platelet signaling. It also emphasizes the utility of the *Gp1ba-Cre* mouse for exploring protein function in the MK/platelet lineage. These insights may have broader implications for understanding phosphatase regulation in hematopoiesis and could inform therapeutic targeting of Shp2 in hematologic diseases.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgements

This work was supported by Inserm, the Agence Nationale pour la Recherche (AM, ANR-22-REMYS-CE14-0061-01 (E. Barre, PhD candidate), and YS, ANR-23-TARGITT-CE18-0020-01). Z.Q., J. M. and Z.Y.Z are supported by NIH RO1 CA069292. The authors acknowledge all members of the animal facility at UMR_S1255 for maintenance of mouse colonies and the flow cytometry platform (CytoTriCS).

Additional information

Author Contributions

E.B. Performed experiments, analyzed data, revised the manuscript.

MD.L.R. Performed experiments, analyzed data, revised the manuscript.

L.Z. Performed experiments and analyzed data.

M.P. Performed experiments and analyzed data.

C.L. Performed experiments and analyzed data.

F.P. Performed experiments and analyzed data.

J.Y.R. Performed experiments and analyzed data.

A.E. Analyzed data.

Z.Q. Provided reagents.

J.M. Provided reagents.

Z.Y.Z. Provided reagents and revised the manuscript.

Y.A.S. Designed experiments, analyzed data, wrote and revised the manuscript.

A.M. Conceptualized, designed experiments, analyzed data, wrote and revised the manuscript.

Funding

Funder	Grant reference number	Author
Agence Nationale de la Recherche (ANR)	ANR-22- REMYS-CE14-0061-01	Marion Pugliano
		Elsa Barré
		Alexandra
		Mazharian
Agence Nationale de la Recherche (ANR)	ANR-23-TARGITT-CE18-0020- 01	Yotis A Senis
		Marion Pugliano
		Cécile Loubière
HHS National Institutes of Health (NIH)	RO1 CA069292	Zihan Qu
		Zhong-Yin Zhang
		Jinmin Miao

Author ORCID iDs

Elsa Barré: <https://orcid.org/0009-0000-5056-1349>

Marc-Damien Lourenco-Rodrigues: <https://orcid.org/0000-0001-6572-1798>

Anita Eckly: <https://orcid.org/0000-0001-9620-4961>

Zihan Qu: <https://orcid.org/0009-0004-8446-8459>

Jinmin Miao: <https://orcid.org/0000-0001-7471-0847>

Zhong-Yin Zhang: <https://orcid.org/0000-0001-5527-7910>

Yotis A Senis: <https://orcid.org/0000-0002-0947-9957>

Alexandra Mazharian: <https://orcid.org/0000-0002-0204-3325>

Additional files

[Supplemental Figures](#) 

References

1. **Machlus KR, Italiano JE** (2013) The incredible journey: From megakaryocyte development to platelet formation. *The Journal of cell biology* **201**:785-796
2. **Asquith NL, Carminita E, Camacho V, et al.** (2024) The bone marrow is the primary site of thrombopoiesis. *Blood* **143**:272-278
3. **Lefrancais E, Looney MR** (2019) Platelet Biogenesis in the Lung Circulation. *Physiology* **34**:392-401
4. **Kaushansky K** (1995) Thrombopoietin: the primary regulator of megakaryocyte and platelet production. *Thrombosis and haemostasis* **74**:521-525
5. **Alexander WS, Roberts AW, Nicola NA, Li R, Metcalf D** (1996) Deficiencies in progenitor cells of multiple hematopoietic lineages and defective megakaryocytopoiesis in mice lacking the thrombopoietic receptor c-Mpl. *Blood* **87**:2162-2170
6. **Kimura S, Roberts AW, Metcalf D, Alexander WS** (1998) Hematopoietic stem cell deficiencies in mice lacking c-Mpl, the receptor for thrombopoietin. *Proceedings of the National Academy of Sciences of the United States of America* **95**:1195-1200
7. **Hitchcock IS, Kaushansky K** (2014) Thrombopoietin from beginning to end. *British journal of haematology* **165**:259-268
8. **Italiano JE Jr, Payne C, Bekendam RH** (2025) Looking Under the Hood at the Cytoskeletal Engine of Platelet Production. *Arteriosclerosis, thrombosis, and vascular biology* **45**:186-197
9. **Vainchenker W, Kralovics R** (2017) Genetic basis and molecular pathophysiology of classical myeloproliferative neoplasms. *Blood* **129**:667-679
10. **Marty C, Lacout C, Martin A, et al.** (2010) Myeloproliferative neoplasm induced by constitutive expression of JAK2V617F in knock-in mice. *Blood* **116**:783-787
11. **Pikman Y, Lee BH, Mercher T, et al.** (2006) MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. *PLoS medicine* **3**:e270
12. **Klampfl T, Gisslinger H, Harutyunyan AS, et al.** (2013) Somatic mutations of calreticulin in myeloproliferative neoplasms. *The New England journal of medicine* **369**:2379-2390
13. **Gango A, Mozes R, Boha Z, et al.** (2018) Quantitative assessment of JAK2 V617F and CALR mutations in Philadelphia negative myeloproliferative neoplasms. *Leukemia research* **65**:42-48
14. **Stivala S, Codilupi T, Brkic S, et al.** (2019) Targeting compensatory MEK/ERK activation increases JAK inhibitor efficacy in myeloproliferative neoplasms. *The Journal of clinical investigation* **129**:1596-1611
15. **Mazharian A, Mori J, Wang YJ, et al.** (2013) Megakaryocyte-specific deletion of the protein-tyrosine phosphatases Shp1 and Shp2 causes abnormal megakaryocyte development, platelet production, and function. *Blood* **121**:4205-4220
16. **Dushek O, Goyette J, van der Merwe PA** (2012) Non-catalytic tyrosine-phosphorylated receptors. *Immunological reviews* **250**:258-276
17. **Brockdorff J, Williams S, Couture C, Mustelin T** (1999) Dephosphorylation of ZAP-70 and inhibition of T cell activation by activated SHP1. *European journal of immunology* **29**:2539-2550
18. **Chan G, Cheung LS, Yang W, et al.** (2011) Essential role for Ptpn11 in survival of hematopoietic stem and progenitor cells. *Blood* **117**:4253-4261

19. **Petrocchi A**, Ciammaichella A (2024) A patent review of SHP2 allosteric inhibitors (2018-present). *Expert opinion on therapeutic patents* **34**:383-396
20. **Mali RS**, Chan R, Kapur R (2012) Targeting SHP2 phosphatase in myeloproliferative neoplasms. *Oncotarget* **3**:1049-1051
21. **Pandey G**, Mazzacurati L, Rowsell TM, et al. (2024) SHP2 inhibition displays efficacy as a monotherapy and in combination with JAK2 inhibition in preclinical models of myeloproliferative neoplasms. *American journal of hematology* **99**:1040-1055
22. **Fornaro M**, Burch PM, Yang W, et al. (2006) SHP-2 activates signaling of the nuclear factor of activated T cells to promote skeletal muscle growth. *The Journal of cell biology* **175**:87-97
23. **Pao LI**, Lam KP, Henderson JM, et al. (2007) B cell-specific deletion of protein-tyrosine phosphatase Shp1 promotes B-1a cell development and causes systemic autoimmunity. *Immunity* **27**:35-48
24. **Nagy Z**, Vogtle T, Geer MJ, et al. (2019) The Gp1ba-Cre transgenic mouse: a new model to delineate platelet and leukocyte functions. *Blood* **133**:331-343
25. **Cazenave JP**, Ohlmann P, Cassel D, Eckly A, Hechler B, Gachet C (2004) Preparation of washed platelet suspensions from human and rodent blood. *Methods in molecular biology* **272**:13-28
26. **Strassel C**, Eckly A, Leon C, et al. (2012) Hirudin and heparin enable efficient megakaryocyte differentiation of mouse bone marrow progenitors. *Experimental cell research* **318**:25-32
27. **Guinard I**, Lanza F, Gachet C, Leon C, Eckly A (2021) Proplatelet Formation Dynamics of Mouse Fresh Bone Marrow Explants. *Journal of visualized experiments : JoVE* **171**
28. **Nagy Z**, Mori J, Ivanova VS, Mazharian A, Senis YA (2020) Interplay between the tyrosine kinases Chk and Csk and phosphatase PTPRJ is critical for regulating platelets in mice. *Blood* **135**:1574-1587
29. **Eckly A**, Strassel C, Cazenave JP, Lanza F, Leon C, Gachet C (2012) Characterization of megakaryocyte development in the native bone marrow environment. *Methods in molecular biology* **788**:175-192
30. **Mazharian A**, Wang YJ, Mori J, et al. (2012) Mice lacking the ITIM-containing receptor G6b-B exhibit macrothrombocytopenia and aberrant platelet function. *Science signaling* **5**:ra78
31. **Liu Q**, Qu J, Zhao M, Xu Q, Sun Y (2020) Targeting SHP2 as a promising strategy for cancer immunotherapy. *Pharmacol Res* **152**
32. **Tiedt R**, Schomber T, Hao-Shen H, Skoda RC (2007) Pf4-Cre transgenic mice allow the generation of lineage-restricted gene knockouts for studying megakaryocyte and platelet function in vivo. *Blood* **109**:1503-1506
33. **Gollomp K**, Poncz M (2019) Gp1ba-Cre or Pf4-Cre: pick your poison. *Blood* **133**:287-288

Peer reviews

Reviewer #1 (Public review):

Barré et al. investigated the role of Shp1 and Shp2 in megakaryocytes (MKs) and platelets by conditional knock-out of Shp1, Shp2, or both under the control of the Gp1ba promoter. Deletion of Shp1 and Shp2 in MKs and platelets was almost complete. The Shp1/Shp2 double knock-out mice displayed macrothrombocytopenia and increased bleeding, whereas the single knock-outs did not show significant defects. Platelet function was aberrant in DKO, but not in single knock-outs, and so was ligand-induced signaling, particularly Syk phosphorylation.

Megakaryocyte maturation was impaired in Shp1/Shp2 DKO mice. Ligand-induced signaling was impaired in Shp2 knock-out and DKO. Ex vivo formation of platelets and in vivo maturation of MKs were impaired in DKO mice. Pharmacological inhibitors of Shp1 and Shp2 had largely similar effects as observed in the single knock-outs. The authors conclude that Shp1 and Shp2 have synergistic functions in the MK/platelet lineage, and that Shp2 may be a potential therapeutic target in myeloproliferative neoplasms.

Strengths:

The data clearly show effects of the Shp1/Shp2 double knock-out on MKs and platelets.

Weaknesses:

There appears to be a discrepancy between the results with the Shp2 single knock-out and the Shp2 inhibitor: the Shp2 knock-out does not affect MKs and platelets, except Erk1/2 signaling, whereas the Shp2 inhibitors appear to affect MK function.

This work is interesting and may have potential from a therapeutic point of view.

<https://doi.org/10.7554/eLife.110053.1.sa3>

Reviewer #2 (Public review):

Summary:

In this manuscript, Barré et al. investigate the roles of the phosphatases Shp1 and Shp2 in the megakaryocyte and platelet lineage using genetic depletion in mice. By employing Gp1ba-Cre-based models, the study builds on the authors' previous work and addresses some limitations associated with earlier Pf4-Cre approaches. The authors report relatively mild alterations in megakaryocyte and platelet parameters in mice lacking either Shp1 or Shp2 alone, whereas combined deletion of both phosphatases results in macrothrombocytopenia, mild bleeding, and impaired GPVI-dependent platelet aggregation accompanied by reduced Syk phosphorylation. The functional platelet defects are linked to reduced expression of GPVI and integrin $\alpha 2$, while thrombocytopenia is associated with impaired megakaryocyte maturation, reduced ploidy, defective proplatelet formation, and altered TPO-dependent Ras/MAPK signaling. Similar effects on megakaryopoiesis are also observed in vitro following treatment with newly developed Shp2 inhibitors.

Strengths and Weaknesses:

The study addresses an important biological question and presents a substantial dataset that could contribute to a better understanding of Shp1 and Shp2 function in platelet biology. However, several aspects of data presentation and interpretation would benefit from additional clarification. In particular, while the authors conclude that single genetic deletion or pharmacological inhibition of Shp1 has a limited impact and that the major phenotypes are specific to combined Shp1/2 deletion or Shp2 inhibition, some of the data suggest more nuanced effects that may warrant further discussion.

<https://doi.org/10.7554/eLife.110053.1.sa2>

Reviewer #3 (Public review):

Summary:

In this manuscript, Barré et al utilize the Gp1ba-Cre transgenic mouse model to build upon previous findings in a Pf4-Cre system to investigate the effects of individual and combined Shp1 and Shp2 deletion in megakaryocytes and platelets. They report decreased megakaryocyte maturation, macrothrombocytopenia, and increased bleeding primarily in association with the Shp1/Shp2 double-knockout condition. The authors further show that this phenotype appears to be driven primarily by Shp2 and implicate dysregulation of Mpl signaling and downstream Ras/MAPK pathways, including ERK1/2. Given the key role of these pathways in human diseases such as myeloproliferative neoplasms and the challenges

associated with modulating such a central pathway, identification of a specific regulator of Mpl signaling poses intriguing questions for future studies on clinical applicability.

Strengths:

Overall, the experiments combine *in vitro*, *in vivo*, and *ex vivo* approaches and appear to have been carefully designed and carried out, with multiple technical and biological replicates where relevant. The authors make a compelling argument for using the Gp1ba-Cre as opposed to the Pf4-Cre system and demonstrate both the dose- and stage-dependent effects of Shp1 and Shp2 on megakaryopoiesis and thrombopoiesis. They find that Shp1 and Shp2 are required in late-stage megakaryocyte maturation and that even low levels of expression compared to baseline are likely sufficient to yield generally normal megakaryocytes. Their findings also lead to specific future directions, such as the mechanism by which Shp1 regulates megakaryopoiesis and thrombopoiesis that is distinct from TPO-mediated signaling.

Weaknesses:

While the experiments have been thoughtfully designed and carried out, there is limited background explanation on relatively complex or niche pathways/mechanisms, such as the relationship between P-selectin, CRP, and PAR4p; the interactions between SFK, Syk, GPVI, and CLEC-2; and TPO, MPL, ERK1/2, AKT, and STAT3, which, while likely intuitive to experts in their respective fields, may be less obvious to a reader approaching this manuscript with a global interest in megakaryopoiesis/thrombopoiesis and thus detract from the impact of the findings.

With regard to the science itself, some of the conclusions feel premature based on the available data.

(1) The section "Aberrant ITAM signaling in Shp1- and Shp2-deficient platelets" is challenging to follow for those not well-versed in ITAM signaling and associated pathways, and may take additional outside reading to follow the conclusion that Syk-dependent signaling is modulated downstream of GPVI and CLEC-2 based on lack of change in Src p-Tyr418, especially considering that Src p-Tyr418 was previously introduced as a measure of SFK rather than Syk. In the introduction, Shp1 is specifically mentioned as a negative regulator of the ITAM/Syk/phospholipase pathway. However, in Figure 4Ai and Bi, Syk phosphorylation/activation in Shp1 knockout cells did not appear to be different from Shp2 knockout cells, and is lower than the control, which is surprising for a negative regulator. It is also not clear why, in the section (Figure 4A-B), there is reduced Syk activation in Shp1 and Shp2 single knockout cells upon CLEC2 stimulation (but apparently not with CRP) when there was no difference in response to CLEC2 (but a difference in response to CRP) in the previous section (Figure 3A, C).

(2) In the section "Reduced Tpo signaling in Shp1/2-deficient MKs," only Western blot data for (p)ERK1/2, AKT, and STAT3 are presented before concluding that decreased ERK1/2 activity is a mechanistic explanation for thrombocytopenia seen in the Shp1/2 double-knockout condition. Such a statement would benefit from additional experiments, such as protein or transcriptional levels of ERK1/2 targets specifically relevant to megakaryopoiesis, such as ETS, FOS, and JUN, to assess the consequences of decreased phosphorylated ERK1/2.

(3) Suggesting that "inhibiting Shp2 will not hav[e] any bleeding consequence in patients" and that Shp2 may be a therapeutic target in myeloproliferative neoplasms when none of these studies have been carried out in a human model is a bold conclusion. There are no data presented on, for example, whether Shp2 inhibition can help reverse the MPL/JAK/STAT pathway in the setting of gain-of-function mutations specifically associated with myeloproliferative neoplasms.

<https://doi.org/10.7554/eLife.110053.1.sa1>

Author response:

eLife Assessment

This manuscript provides an important contribution to the field of platelet biogenesis, and the convincing evidence will advance our understanding of signal transduction driving the development of late megakaryopoiesis and platelet reactivity that results in bleeding diathesis. The paper is noteworthy for analyzing two related, either singly or in combination, tyrosine phosphatases in this conditional, stage development gene knockout. Because SHP1 is a negative regulator and SHP2 is an activator, the synergistic effects found in the double knockout were surprising.

We thank the reviewer for acknowledging the importance and novelty of our findings.

Public Reviews:

Reviewer #1 (Public review):

Barré et al. investigated the role of Shp1 and Shp2 in megakaryocytes (MKs) and platelets by conditional knock-out of Shp1, Shp2, or both under the control of the Gp1ba promoter. Deletion of Shp1 and Shp2 in MKs and platelets was almost complete. The Shp1/Shp2 double knock-out mice displayed macrothrombocytopenia and increased bleeding, whereas the single knock-outs did not show significant defects. Platelet function was aberrant in DKO, but not in single knock-outs, and so was ligand-induced signaling, particularly Syk phosphorylation.

Megakaryocyte maturation was impaired in Shp1/Shp2 DKO mice. Ligand-induced signaling was impaired in Shp2 knock-out and DKO. Ex vivo formation of platelets and in vivo maturation of MKs were impaired in DKO mice. Pharmacological inhibitors of Shp1 and Shp2 had largely similar effects as observed in the single knock-outs. The authors conclude that Shp1 and Shp2 have synergistic functions in the MK/platelet lineage, and that Shp2 may be a potential therapeutic target in myeloproliferative neoplasms.

Strengths:

The data clearly show effects of the Shp1/Shp2 double knock-out on MKs and platelets.

Weaknesses:

There appears to be a discrepancy between the results with the Shp2 single knock-out and the Shp2 inhibitor: the Shp2 knock-out does not affect MKs and platelets, except Erk1/2 signaling, whereas the Shp2 inhibitors appear to affect MK function.

This work is interesting and may have potential from a therapeutic point of view.

Pharmacological effects do not always correlate with congenital anomalies arising for genetic defects. The Shp2 allosteric inhibitors used in our study only inhibit catalytically inactive Shp2, whereas targeted deletion of *Ptpn11* results in a loss of total Shp2 expression, including catalytic and non-catalytic related functions, with developmental consequences. Further, *Gp1ba-Cre⁺;Shp2^{fl/fl}* megakaryocytes express approximately 22% of normal Shp2 level, which likely also contributes to differences observed between pharmacological inhibition and genetic ablation of Shp2.

We thank the reviewer for recognizing the therapeutic potential of our findings.

Reviewer #2 (Public review):

Summary:

In this manuscript, Barré et al. investigate the roles of the phosphatases Shp1 and Shp2 in the megakaryocyte and platelet lineage using genetic depletion in mice. By employing Gp1ba-Cre-based models, the study builds on the authors' previous work and addresses some limitations associated with earlier Pf4-Cre approaches. The authors report relatively mild alterations in megakaryocyte and platelet parameters in mice lacking either Shp1 or Shp2 alone, whereas combined deletion of both phosphatases results in macrothrombocytopenia, mild bleeding, and impaired GPVI-dependent platelet aggregation accompanied by reduced Syk phosphorylation. The functional platelet defects are linked to reduced expression of GPVI and integrin $\alpha 2$, while thrombocytopenia is associated with impaired megakaryocyte maturation, reduced ploidy, defective proplatelet formation, and altered TPO-dependent Ras/MAPK signaling. Similar effects on megakaryopoiesis are also observed in vitro following treatment with newly developed Shp2 inhibitors.

Strengths and Weaknesses:

The study addresses an important biological question and presents a substantial dataset that could contribute to a better understanding of Shp1 and Shp2 function in platelet biology. However, several aspects of data presentation and interpretation would benefit from additional clarification. In particular, while the authors conclude that single genetic deletion or pharmacological inhibition of Shp1 has a limited impact and that the major phenotypes are specific to combined Shp1/2 deletion or Shp2 inhibition, some of the data suggest more nuanced effects that may warrant further discussion.

We thank the reviewer for raising this point. The manuscript is being revised accordingly, including highlighting the potential role of Shp1 in megakaryopoiesis and thrombopoiesis under steady-state and stressed conditions, requiring more detailed investigation.

Reviewer #3 (Public review):

Summary:

In this manuscript, Barré et al utilize the Gp1ba-Cre transgenic mouse model to build upon previous findings in a Pf4-Cre system to investigate the effects of individual and combined Shp1 and Shp2 deletion in megakaryocytes and platelets. They report decreased megakaryocyte maturation, macrothrombocytopenia, and increased bleeding primarily in association with the Shp1/Shp2 double-knockout condition. The authors further show that this phenotype appears to be driven primarily by Shp2 and implicate dysregulation of Mpl signaling and downstream Ras/MAPK pathways, including ERK1/2. Given the key role of these pathways in human diseases such as myeloproliferative neoplasms and the challenges associated with modulating such a central pathway, identification of a specific regulator of Mpl signaling poses intriguing questions for future studies on clinical applicability.

We thank the reviewer for acknowledging the importance and novelty of our findings.

Strengths:

Overall, the experiments combine in vitro, in vivo, and ex vivo approaches and appear to have been carefully designed and carried out, with multiple technical and biological replicates where relevant. The authors make a compelling argument for using the Gp1baCre as opposed to the Pf4-Cre system and demonstrate both the dose- and stagedependent effects of Shp1 and Shp2 on megakaryopoiesis and thrombopoiesis. They find that Shp1 and Shp2 are required in late-stage megakaryocyte maturation and that even low levels of expression compared to baseline are likely sufficient to yield generally normal megakaryocytes. Their findings also lead to specific future directions,

such as the mechanism by which Shp1 regulates megakaryopoiesis and thrombopoiesis that is distinct from TPO-mediated signaling.

Weaknesses:

While the experiments have been thoughtfully designed and carried out, there is limited background explanation on relatively complex or niche pathways/mechanisms, such as the relationship between P-selectin, CRP, and PAR4p; the interactions between SFK, Syk, GPVI, and CLEC-2; and TPO, MPL, ERK1/2, AKT, and STAT3, which, while likely intuitive to experts in their respective fields, may be less obvious to a reader approaching this manuscript with a global interest in megakaryopoiesis/thrombopoiesis and thus detract from the impact of the findings.

We thank the reviewer for raising this point. The manuscript is being revised, to better explain the rationale and molecular mechanisms linking these pathways and functions.

With regard to the science itself, some of the conclusions feel premature based on the available data.

(1) The section "Aberrant ITAM signaling in Shp1- and Shp2-deficient platelets" is challenging to follow for those not well-versed in ITAM signaling and associated pathways, and may take additional outside reading to follow the conclusion that Syk-dependent signaling is modulated downstream of GPVI and CLEC-2 based on lack of change in Src p-Tyr418, especially considering that Src p-Tyr418 was previously introduced as a measure of SFK rather than Syk. In the introduction, Shp1 is specifically mentioned as a negative regulator of the ITAM/Syk/phospholipase pathway. However, in Figure 4Ai and Bi, Syk phosphorylation/activation in Shp1 knockout cells did not appear to be different from Shp2 knockout cells, and is lower than the control, which is surprising for a negative regulator. It is also not clear why, in the section (Figure 4A-B), there is reduced Syk activation in Shp1 and Shp2 single knockout cells upon CLEC2 stimulation (but apparently not with CRP) when there was no difference in response to CLEC2 (but a difference in response to CRP) in the previous section (Figure 3A, C).

We thank the reviewer for raising these important points. The manuscript is being revised accordingly, including clarifying the roles of SFKs, Shp1 and Shp2 in the ITAM-Syk-PLCg2 signaling pathway.

Briefly, SFKs are essential for phosphorylating ITAMs, allowing SH2-dependent docking of Syk. Reduced reactivity of Shp1/2 DKO platelets to CRP and collagen is likely due to downregulation of the ITAM-containing GPVI-FcR g-chain complex and integrin $\alpha 2$ subunit, and concomitant reduction in Syk phosphorylation.

However, the marginal albeit significant reduction in Syk phosphorylation downstream of CLEC-2 in Shp1 and Shp2 KO platelets was not determined and was insufficient to impact CLEC-2-mediated platelet aggregation under the conditions tested.

Differences in the stoichiometry and docking of Syk to phosphorylated GPVI-FcR g-chain and CLEC-2 likely contribute to the differences in platelet reactivity and Syk phosphorylation downstream of the two receptors in the absence of Shp1 and Shp2.

(2) In the section "Reduced Tpo signaling in Shp1/2-deficient MKs," only Western blot data for (p)ERK1/2, AKT, and STAT3 are presented before concluding that decreased ERK1/2 activity is a mechanistic explanation for thrombocytopenia seen in the Shp1/2 doubleknockout condition. Such a statement would benefit from additional experiments, such as protein or transcriptional levels of ERK1/2 targets specifically relevant to megakaryopoiesis, such as ETS, FOS, and JUN, to assess the consequences of decreased phosphorylated ERK1/2.

We thank the reviewers for these constructive comments. Further experiments are being planned to determine the biological and transcriptional consequences of reduced ERK1/2 phosphorylation during megakaryopoiesis and thrombopoiesis.

(3) Suggesting that "inhibiting Shp2 will not have any bleeding consequence in patients" and that Shp2 may be a therapeutic target in myeloproliferative neoplasms when none of these studies have been carried out in a human model is a bold conclusion. There are no data presented on, for example, whether Shp2 inhibition can help reverse the MPL/JAK/STAT pathway in the setting of gain-of-function mutations specifically associated with myeloproliferative neoplasms.

This conclusion is being tempered in the revised manuscript. Genetic- and pharmacological-based approaches will be used to establish the therapeutic potential of inhibiting Shp1 and Shp2 in mouse models of MPN, including Jak2 gain-of-function mice. Bleeding and thrombotic complications of inhibiting Shp1 and Shp2 will be explored as part of these studies.

<https://doi.org/10.7554/eLife.110053.1.sa0>