# Short-term administration of JAK2 inhibitors reduces splenomegaly in mouse models of β-thalassemia intermedia and major

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# Material and Methods

**Animal models**. All animals (2.5 to 3.5-month old male all in C57BL/6 background) were bred at the mouse facility of the Children's Hospital of Philadelphia. We used $Hbb^{th3/+}$ mice (Jackson Laboratories) as a model of β-thalassemia intermedia and C57-$FLC^{th3/th3}$ animals as model for β-thalassemia-major. C57-$FLC^{th3/th3}$ were generated by transplanting $Hbb^{th3/th3}$ fetal liver cells (FLC) from embryos collected at 14.5 to 15.5 days of gestation into irradiated WT (C57BL/6) 2-month old syngeneic animals. Transfusions started 1-month after transplant, when the phenotype was fully established. Based on previous experiments, we did not expect any difference between genders treated with JAK2i. For this study only males were utilized.

**Drug preparation and administration.** INCB018424 (Ruxolitinib) and TG101348 (Fedratinib, SAR302503) were purchased from Chemietek and were administered for 10 days, twice daily by oral gavage, at a dose of 180 and 120 mg/Kg respectively. Stock solution was in DMSO.

**Transfusion**. $Hbb^{th3/+}$ and C57-$FLC^{th3/th3}$ mice were infused weekly, for a total of three weeks (starting one week before the JAK2i administration) via retro-orbital venous plexus with 300 µL freshly harvested blood from normal healthy GFP-C57BL/6 animals. GFP blood was collected from the retro-orbital plexus into acid citrate dextrose (7 volumes of blood for 1 volume of acid citrate dextrose), under anesthesia.

**Measurement of tissue iron content and serum iron parameters**. Serum parameters (iron, Transferrin saturation) were measured using the Iron/TIBC Reagent Set (BioPacific Diagnostic Inc). Serum EPO was analyzed using a "Mouse Erythropoietin Quantikine Elisa Kit" (R&D Systems) following manufacturer's instructions. Serum HEPCIDIN was measured using the "Hepcidin-Murine-Compete" kit (Intrinsic Lifesciences) following manufacturer's instructions. Liver and spleen iron content was determined by non-heme iron analysis. Perls' Prussian Blue staining on liver and spleen sections were prepared at the Pathology core laboratories of the Children's Hospital of Philadelphia.

Images were captured using a Leica DM4000B upright scope paired with a Spot RT/ SE Slider camera (10x/N.A. 0.40 objective) and then acquired using the Spot 5.1 software.

**FACS analysis:** Single cell suspensions of BM and spleen cells ($1*10^6$ cells per sample) were incubated with anti-mouse CD71, anti-mouse Ter119 antibodies (BD Biosciences — Pharmingen) and anti-mouse CD44 marker (BD Biosciences — Pharmingen) to study the erythroid compartment. Analysis of the cells using CD44 and Ter119 allows the separation of erythroid cells into distinct populations (indicated as 1 to 5). For all analyses, cells were sorted using a FACSCalibur instrument (BD) and the results analyzed with FlowJo software (Tree Star Inc.).

**Statistics.** Statistical analysis was performed using One-way ANOVA with Tukey multiple comparison adjustment or Two-way ANOVA with Sidak's multiple comparison adjustment. WT values data are displayed as a reference guide but are not included in the test comparisons. All data were analyzed using GraphPad Prism version 6 (Microsoft GraphPad Software, La Jolla California USA).

**Study approval**. All the animal studies were conducted under protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the Children's Hospital of Philadelphia.
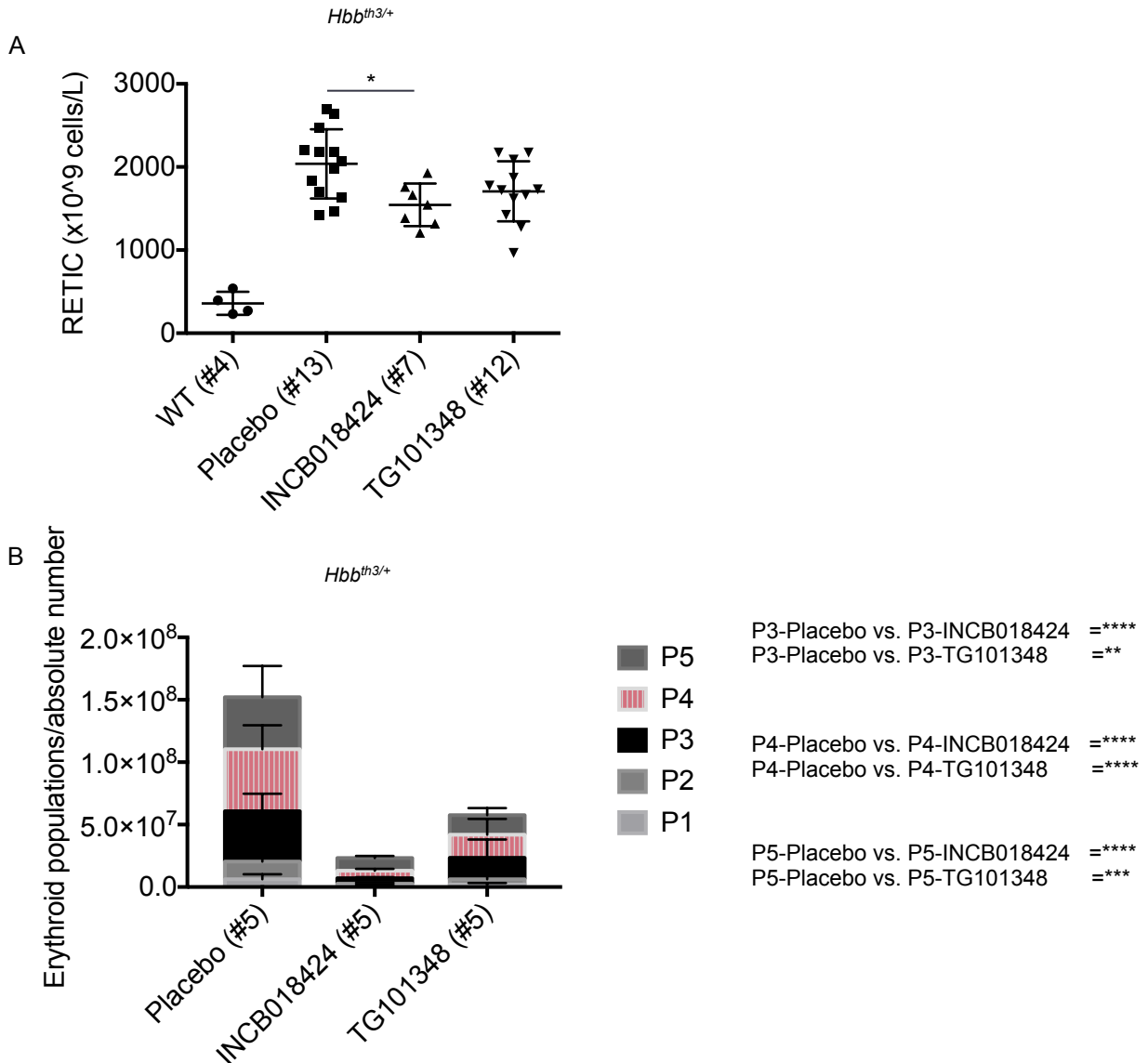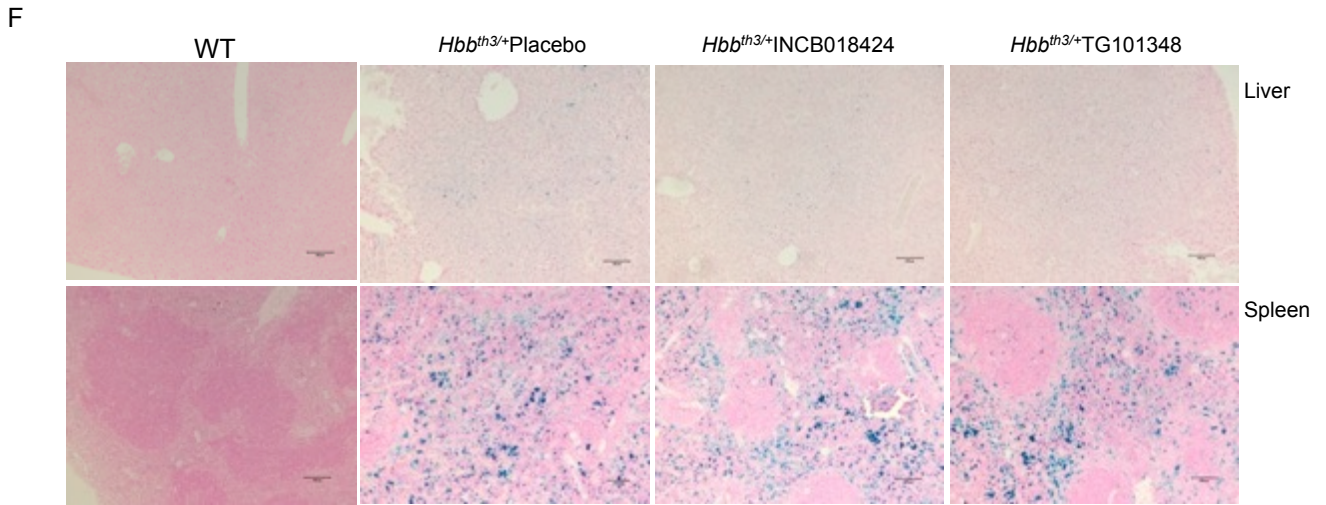
**Supplementary Figure 1. Ineffective erythropoiesis in the spleen was improved after Jak2i treatment.** Reduction in spleen size after JAK2i administration was associated with a significant decrease in reticulocyte numbers (A) and erythropoiesis in the spleen (B) when compared with placebo. The different splenic erythroid populations were analyzed by taking in consideration the absolute number of cells. Individual sub-populations were compared among each group of animals. End point analysis revealed a substantial reduction for all of 5 populations, showing significance for population 3 and 4 which represent Polychromatic erythroblast (3) and Ortho/Retic (4). Analysis was performed using Two-way ANOVA with Tukey multiple comparison adjustment. Results represent mean ± SD, ****P <0.0001, ***P < 0.001, **P < 0.01,

A

*Hbb^{th3/+}*



B

*Hbb^{th3/+}*

C

*Hbb^{th3/+}*



D

*Hbb^{th3/+}*

E

*Hbb^{th3/+}*

F
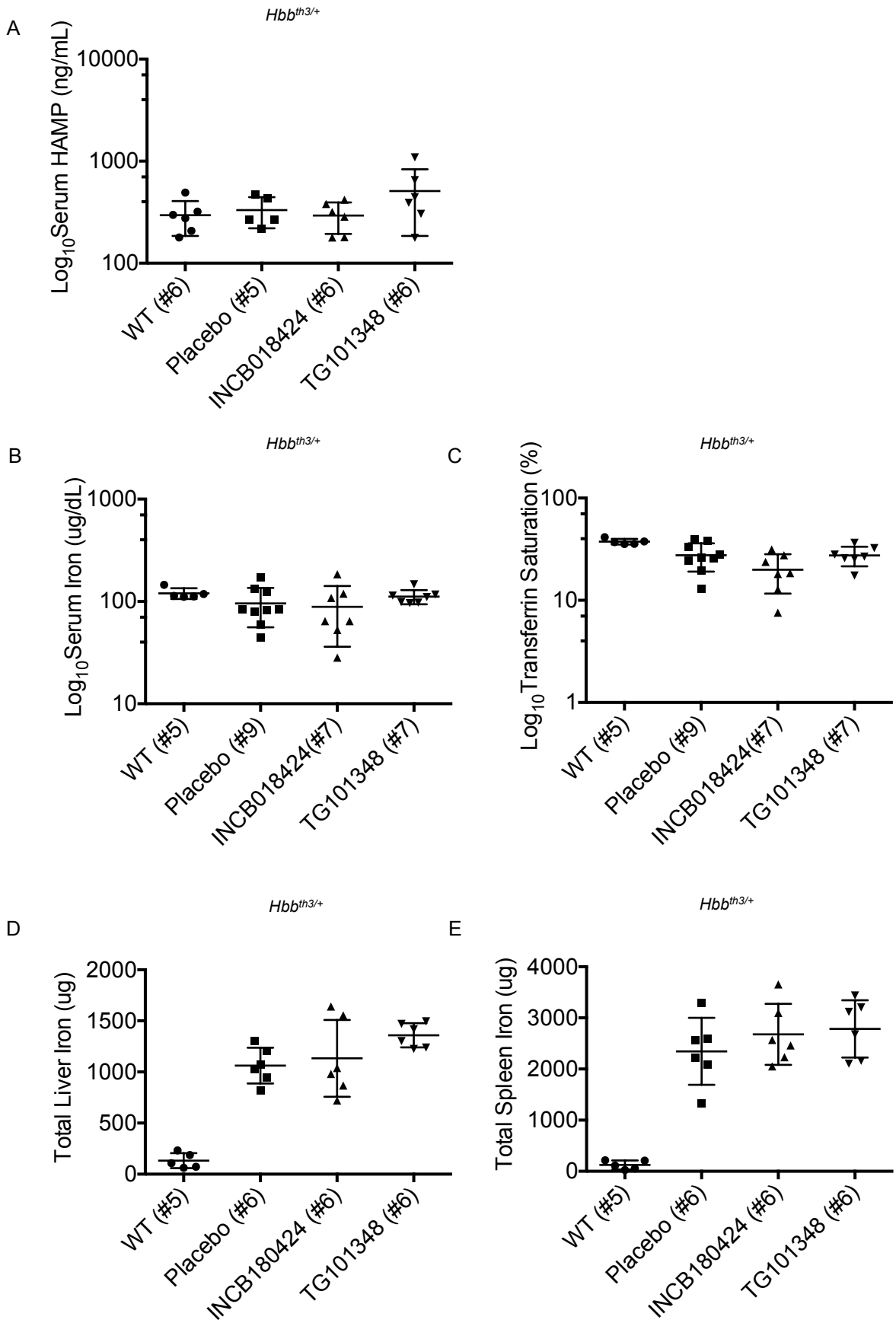


**Supplementary Figure 2. Iron parameters were unchanged after ten days of treatment with JAK2i.** No differences were observed in serum HEPCIDIN levels (A) as well as other iron parameters such as serum iron and transferrin saturation (B-C). Total liver and spleen iron content of animals receiving the inhibitors did not change when compared with mice receiving the placebo (D-E). These findings were confirmed by Perls' Prussian Blue staining of liver and spleen sections (F). Analysis was performed using One-way ANOVA with Tukey multiple comparison adjustment. Results represent mean ± SD.
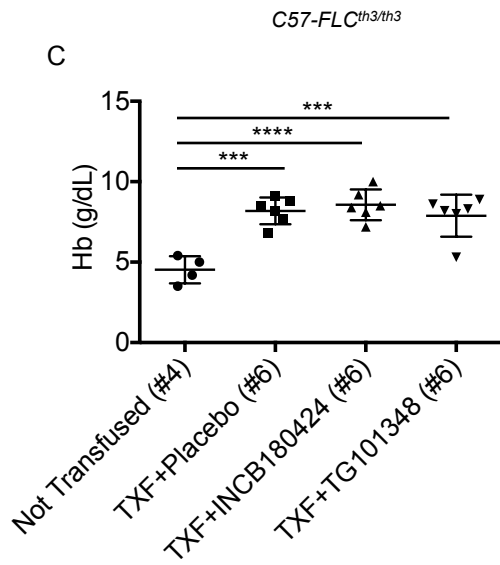
A

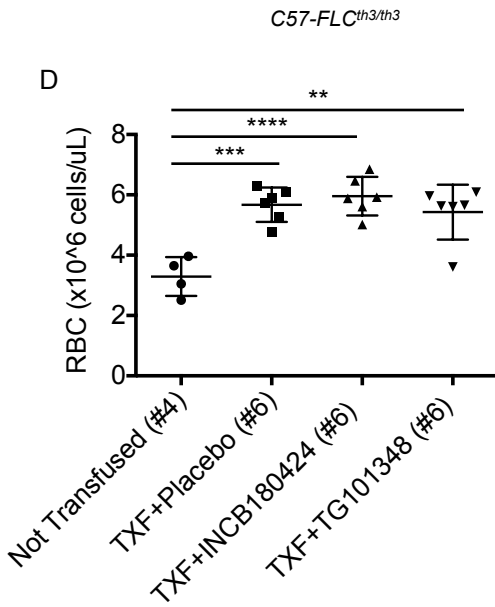$Hbb^{th3/+}$



B

$Hbb^{th3/+}$



C

$C57\text{-}FLC^{th3/th3}$



D

$C57\text{-}FLC^{th3/th3}$
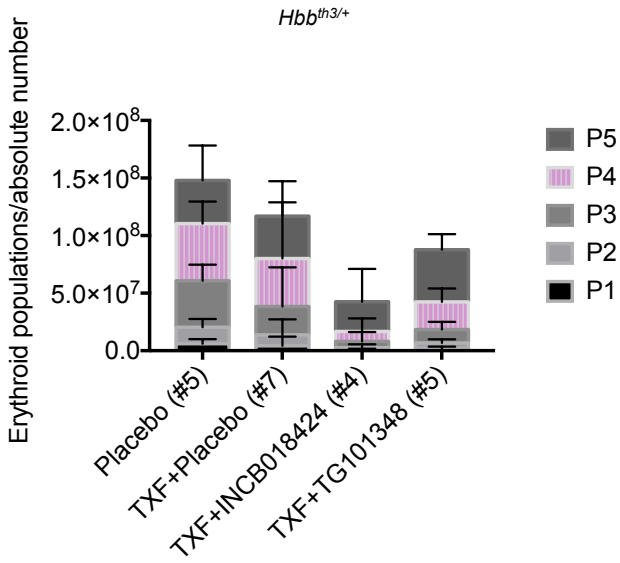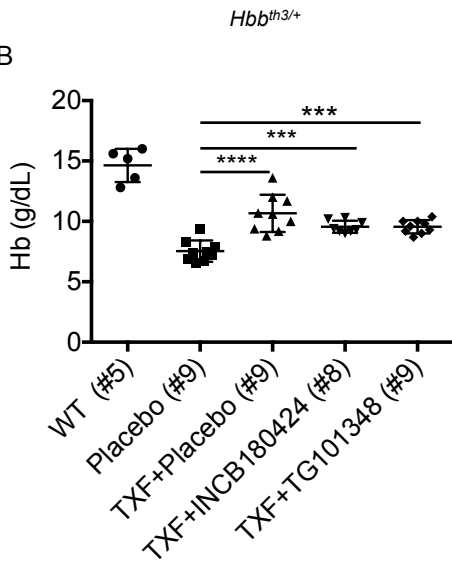
**Supplementary Figure 3**. **JAK2i treatment combined with transfusion significantly improved ineffective erythropoiesis in the spleen without detrimental effect on anemia.** NTDT mice that received co-administration of transfusion (indicated with TXF in the graphs) and JAK2i showed reduction in ineffective erythropoiesis. This is indicated by the same trend in reduction of erythroid progenitor cells observed in thalassemic animals that did not undergo transfusion  (A). Individual sub-populations were compared among each group of animals. Analysis was performed using Two-way ANOVA with Tukey multiple comparison adjustment. Animals that received JAK2i associated with the transfusion did not show any significant differences in Hb level when compared with animals receiving transfusion alone (B). In a mouse model of TDT, combination of blood transfusion and JAK2i did not have any negative effect on the efficacy of the transfusion but did not result in any increase in Hb levels or RBC numbers. (C-D). Analysis was performed using One-way ANOVA with Tukey multiple comparison adjustment. Results represent mean ± SD, ****P <0.0001, ***P < 0.001, **P < 0.01.