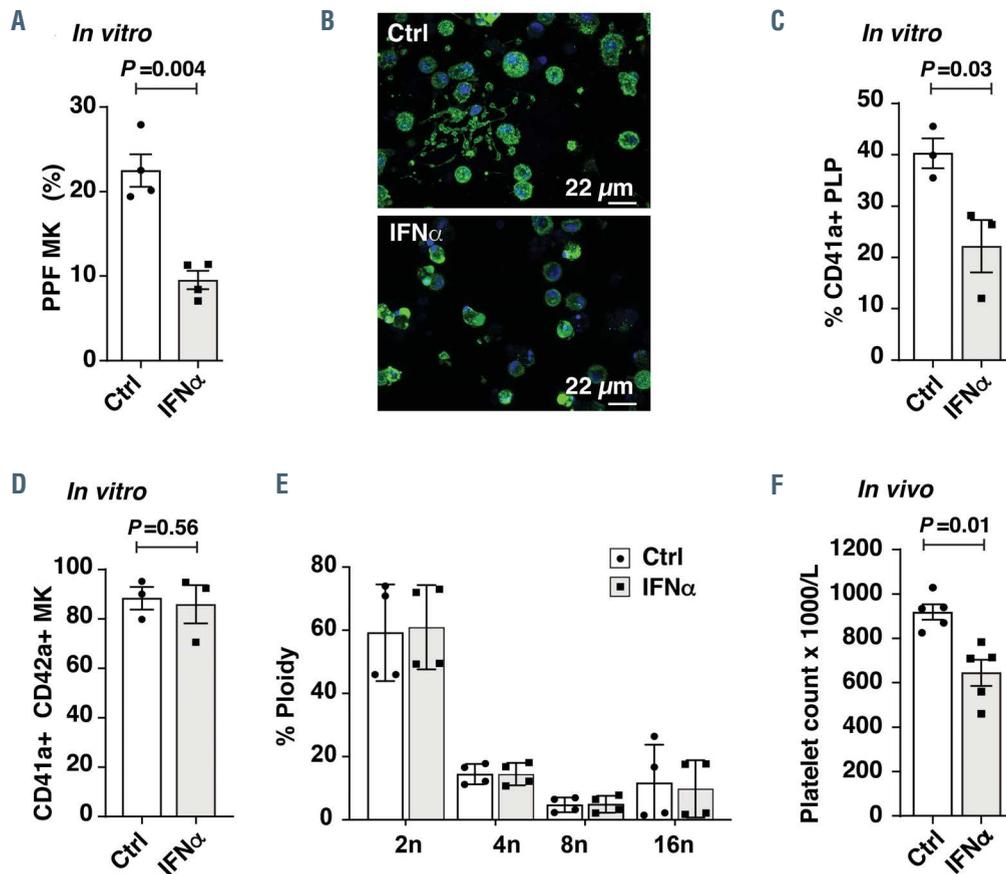


## Interferon $\alpha$ -induced SAMHD1 regulates human cultured megakaryocyte apoptosis and proplatelet formation

Megakaryocyte (MK) growth, differentiation and maturation are required for thrombopoiesis and platelet production. Most studies of megakaryocytopoiesis have utilized *in vitro* culture systems expected to model a healthy human condition. However, consistent with the ability of MK to respond to inflammatory mediators, chronic inflammatory conditions often induce thrombocytosis, whereas acute inflammation can result in thrombocytopenia. Furthermore, there is an increasing awareness of the role MK play in innate and adaptive immunity.<sup>1</sup> Type 1 interferons (IFN-1), including IFN $\alpha$ , IFN $\beta$  and IFN $\omega$  are a family of cytokines that bind to the IFN-1 receptor and trigger transcription of diverse genes. IFN-inducible genes regulate resistance to viral infections, enhance innate and adaptive immunity, and modulate

normal and tumor cell survival and death.<sup>2</sup> MK express the IFN-1 receptor that signals through Janus kinases/signal transducer and activator of transcription proteins (JAK/STAT) pathway in response to IFN-1 cytokines.<sup>3</sup> IFN $\alpha$ , an IFN-1 cytokine, has been effectively utilized in the treatment of myeloproliferative neoplasms and viral hepatitis. Thrombocytopenia is a common adverse effect of IFN $\alpha$  therapy that can require dose reduction. Although there are inconsistent reports regarding IFN $\alpha$  suppression of colony forming units of megakaryocyte progenitors (CFU-MK) in cultures of human CD34+ cells, there are consistent findings to support a mechanism of decreased platelet production rather than reduced platelet life span.<sup>4-7</sup> However, the molecular mechanisms regulating IFN $\alpha$ -induced decrease in platelet production and peripheral blood thrombocytopenia are poorly understood. The major conclusions in this report are (i) using genome-wide gene expression profiling we show that IFN $\alpha$  upregulates the



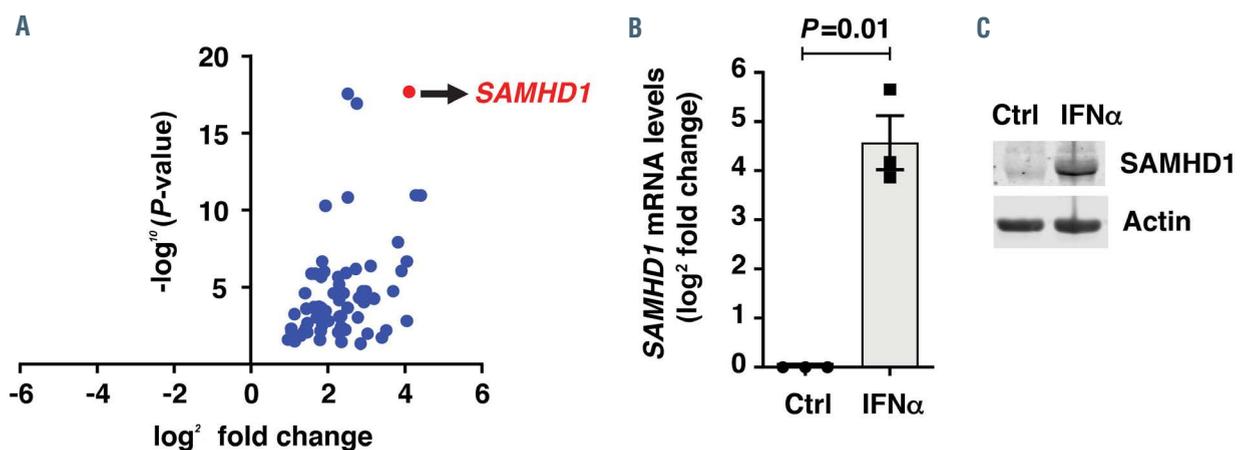
**Figure 1. Interferon $\alpha$  regulates platelet production.** (A to E) CD34+ hematopoietic stem cells and progenitor cells were isolated by immunomagnetic separation from human umbilical cord blood. Cells were cultured for 13 days in stem cells expansion media supplemented with thrombopoietin that promotes megakaryocyte (MK) differentiation. 1,000 units/milliliter (U/mL) of human interferon  $\alpha$  (IFN $\alpha$ ) and phosphate-buffered saline (PBS) (used as a negative control) was added at day 9 and further incubated until day 13. All assays mentioned in panels A to E were performed at day 13. (A) MK proplatelet formation (PPF) was counted blinded as to the IFN $\alpha$  treatment. At least 200 cells were counted per culture ( $n=4$ ). (B) Representative images of day 13 cultured MK treated with PBS or IFN $\alpha$ . Treated MK were plated on fibrinogen on day 12 overnight, and fixed with 4% paraformaldehyde, stained with Alexa Fluor 488 Phalloidin (green) and a nuclear stain, DAPI (blue). Images were taken by a confocal microscope at 40X oil objective lens. (C) Platelet-like particles (PLP) were collected from IFN $\alpha$  or PBS-treated MK cultures and stained with APC labeled anti-CD41a antibody at 37 °C for 10 minute and measured by flow cytometry ( $n=3$ ). PLP were gated based on human peripheral blood platelets. (D) MK were stained with APC-labeled CD41a and PE-labeled CD42a antibodies, and CD41a+ CD42a+ MK (a marker for MK maturation) were assessed by flow cytometry ( $n=3$ ). (E) Cultured MK treated with IFN $\alpha$  or PBS were stained with APC-labeled CD41a and propidium iodide, and ploidy was assessed by flow cytometry ( $n=3$ ). The quantification of the ploidy distribution is shown on the y-axis by calculating the percentage of cells with 2n, 4n, 8n and 16n. Apoptotic population were gated out. Statistical significance was determined by two-tailed paired t-test (A to E). Error bars represent mean  $\pm$  standard error of mean. (F) 25,000 units of murine IFN $\alpha$  or PBS (negative control) were administered intraperitoneally in wild-type mice for consecutive three days ( $n=5$  per group). On day 4, mice blood was harvested by cardiac puncture and platelet count was measured by Hemavet. Statistical significance was determined by two-tailed unpaired t-test with Welch's correction. Error bars represent mean  $\pm$  standard error of mean.

expression of MK sterile  $\alpha$  motif (SAM) and histidine-aspartate (HD) domain containing deoxynucleoside triphosphate triphosphohydrolase 1 (SAMHD1) and (ii) SAMHD1 expression inhibits cultured human MK proplatelet formation (PPF) and promotes apoptosis. This is the first identification of SAMHD1 in human MK and report of a dNTP hydrolase regulating platelet production.

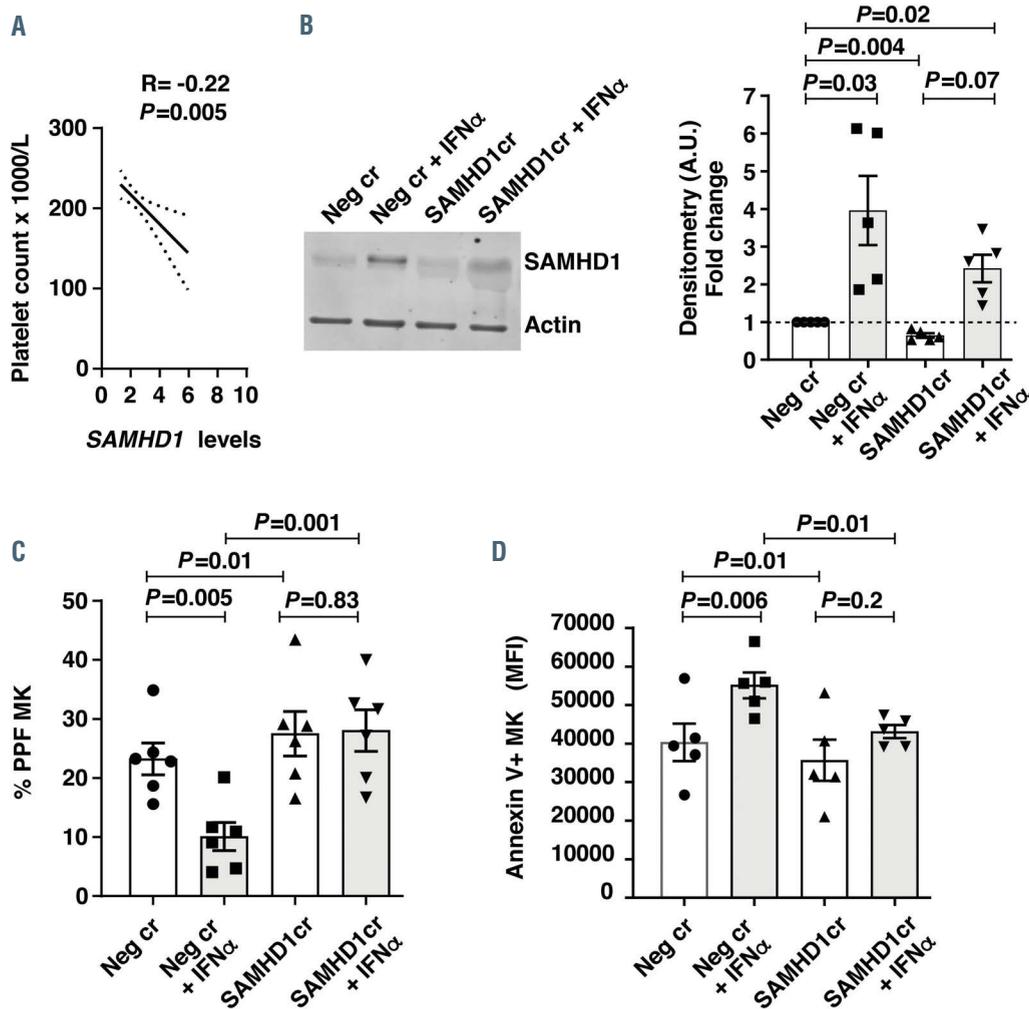
In order to pursue studies on the effects of inflammation on megakaryocytopoiesis, we used CD34+ hematopoietic stem cells derived from human umbilical vein cord blood. IFN $\alpha$  significantly decreased day 13 PPF and platelet-like particles (Figure 1A to C), but did not affect the percentages of MK or polyploidy (Figure 1D to E). Importantly, we also showed that exogenous IFN $\alpha$  induces thrombocytopenia in wild-type mice (Figure 1F), consistent with studies in immunodeficient mice.<sup>4</sup> In order to begin to understand how IFN $\alpha$  regulates late-stage megakaryopoiesis and platelet production, we used an unbiased, transcriptome-wide approach and performed RNA sequencing (RNA-seq) on CD61-purified, day 13 cultured MK stimulated with IFN $\alpha$ . Our analyses identified 201 transcripts that were differentially expressed at a nominal significance threshold ( $P < 0.05$ ). Adjusting for multiple comparisons and setting a false discovery rate (FDR) threshold of  $< 0.05$ , we found that 66 of the 201 transcripts were upregulated by IFN $\alpha$  (Online Supplementary Table S1). Increased mRNA expression in response to IFN $\alpha$  was validated by real-time polymerase chain reaction (PCR) analysis for all five genes tested (SAMHD1, PHF11, ISG20, IFITM3 and TAP2) (Online Supplementary Figure S1). Gene ontology analysis indicated that the differentially expressed genes were associated with the type 1 interferon signaling pathway, defense response to virus, and negative regulation of viral genome replication. Subsequent studies focused on SAMHD1, whose abundance increased more than 16-fold with IFN $\alpha$  induction (FDR =  $2.0 \times 10^{-18}$ )

(Figure 2A). Figure 2B and C shows that IFN $\alpha$  treatment of cultured MK greatly increased the abundance of SAMHD1 mRNA and protein (n=3 independent biological replicates). SAMHD1 is a hydrolase, the activated form of which degrades the intracellular pool of deoxynucleoside triphosphates (dNTPase) into deoxynucleosides and inorganic triphosphates, and is known to restrict viral replication of the human immunodeficiency virus type-1.<sup>8</sup> In addition to viral restriction, SAMHD1 is required for cellular functions including replication fork progression, cell proliferation, apoptosis and DNA damage repair.<sup>9</sup> IFN $\alpha$  stimulation induces SAMHD1 expression in human monocytes,<sup>10</sup> astrocytes, microglia,<sup>11</sup> HEK293T and HeLa cells,<sup>12</sup> but there are no prior reports of SAMHD1 expression and/or function in MK or platelets.

Platelet RNA And eXpression 1 (PRAX1) data<sup>13</sup> demonstrated that SAMHD1 transcript levels are negatively associated with platelet count in healthy human subjects (Figure 3A), suggesting a possible inhibitory role of SAMHD1 in platelet production. Since SAMHD1 modulates the intracellular levels of dNTP, we hypothesized that an increase in the abundance SAMHD1 upon IFN $\alpha$  stimulation leads to decreased MK proliferation, maturation and DNA synthesis (MK polyploidy). However, deletion of SAMHD1 by CRISPR/Cas9 gene editing in cultures promoting unilineage MK differentiation (Figure 3B) did not affect MK maturation (Online Supplementary Figure 2A and B) or ploidy (Online Supplementary Figure 2C). This suggests SAMHD1 effects thrombopoiesis rather than megakaryocytopoiesis. Similar to Figure 1, IFN $\alpha$  stimulation caused a significant decrease in MK PPF MK without CRISPR modification (Figure 3C, first 2 bars). The effect of IFN $\alpha$  on PPF was abolished when SAMHD1 was deleted (Figure 3C, second 2 bars). Lastly, IFN $\alpha$  is well-established as pro-apoptotic.<sup>2</sup> MK must restrain apoptosis to survive and progress safely through PPF and platelet shedding.<sup>14,15</sup>



**Figure 2.** Interferon  $\alpha$  upregulates dNTP hydrolase enzyme SAMHD1 in cultured megakaryocytes. CD34+ hematopoietic stem cells and progenitor cells were isolated by immunomagnetic separation from human umbilical cord blood. Cells were cultured in megakaryocyte (MK) promoting conditions by supplementation with thrombopoietin for 13 days. 1,000 units per milliliter (U/mL) of interferon  $\alpha$  (IFN $\alpha$ ) and phosphate-buffered saline (PBS) (negative control) was added in 3 independent cultures at day 13 for 8 hours. (Dose and time course studies were performed in Meg-01 to select optimal concentrations and times, data not shown). CD61+ MK were separated by immunomagnetic beads and total RNA was obtained by mirVana kit. RNA samples with RIN score of  $> 8$  were used for RNA sequencing. The library preparation was done using TruSeq standard total RNA sample preparation kit with RNA depletion (Illumina Inc.). RNA sequencing was performed using NextSeq500 instrument with read length of 50 basepair single-end read. On average 68.9 million reads were obtained from each sample and genome mapping was on average 94.5% for all samples. (A) Volcano plot shows the relationship between the P-values and the fold change in normalized expression for IFN $\alpha$  or PBS treated cultured human MK. Differentially expressed genes (false discovery rate [FDR]  $< 0.05$ ) on IFN $\alpha$  treatment was plotted. SAMHD1 is shown in red. (B) Real-time polymerase chain reaction analysis validates the upregulation of SAMHD1 in IFN $\alpha$ -treated CD61+ MK compared to PBS control. Actin was used as housekeeping gene. Log<sub>2</sub> fold change of SAMHD1 mRNA levels is plotted for IFN $\alpha$  vs. PBS control (n=3). Statistical significance was determined by one sample t-test. (C) Representative immunoblot shows increase in SAMHD1 protein in IFN $\alpha$ -treated MK compared to PBS control. Actin is used as loading control.



**Figure 3.** Interferon  $\alpha$ -induced SAMHD1 expression regulates human megakaryocyte (MK) proplatelet formation and MK apoptosis. (A) Plot of Pearson correlation (R) between platelet count and SAMHD1 mRNA levels in 154 healthy donors. Dotted lines represent 95% confidence intervals. (B to D) CRISPR/Cas9 knock-down of SAMHD1 in CD34+ derived human umbilical cord cells were performed at day 3. Cells were cultured in MK differentiation promoting conditions, and treated with 1,000 U/mL interferon  $\alpha$  (IFN $\alpha$ ) and phosphate-buffered saline (PBS) control. On day 13, MK treated with IFN $\alpha$  or PBS were assayed as mentioned below. (B) Representative immunoblot of SAMHD1 after CRISPR-Cas9 knock-down (denoted as "SAMHD1 cr" throughout the figure) in day 13 human CD34+ derived cultured MK with or without IFN $\alpha$ . Guide RNA not targeting known genes are used as negative control with or without IFN $\alpha$  ("neg cr" throughout the figure). Fold changes of densitometric quantification of SAMHD1 immunoblots, normalized to actin is shown on right (n=5). (C) The percentage of proplatelet formation (PPF) MK, scored blinded, for SAMHD1 cr vs neg cr, with or without IFN $\alpha$  is shown (n=6). (D) The mean fluorescence intensity (MFI) of annexin V binding (a marker of apoptosis-induced phosphatidylserine expression) on IFN $\alpha$  stimulation in SAMHD1 cr vs. neg cr MK, with or without IFN $\alpha$  is plotted (n=5). Statistical significance was determined by paired t-test (B to D). Error bars represent mean  $\pm$  standard error of mean.

Although viral infections induce MK apoptosis,<sup>15</sup> we are not aware of *in vitro* studies assessing IFN $\alpha$ -induced MK apoptosis. Under the culture conditions described in Figure 3C, we assessed annexin V binding as a measure of MK apoptosis, and observed a significant increase in response to IFN $\alpha$  stimulation (Figure 3D, first 2 bars). Next, because SAMHD1 also promotes apoptosis,<sup>9</sup> we tested the effects of SAMHD1 deletion on IFN $\alpha$ -induced MK apoptosis. Deletion of SAMHD1 caused a modest reduction in annexin V binding (Figure 3D, bar 1 vs. bar 3), and significantly reduced IFN $\alpha$ -induced MK annexin V binding (Figure 3D, compare bars 2 and 4), supporting a role for SAMHD1 as a mediator of IFN $\alpha$ -induced MK apoptosis.

In summary, our study indicates that IFN $\alpha$  leads to reduced platelet production and thrombocytopenia through apoptosis, and that IFN $\alpha$ -induced SAMHD1 is at least partially responsible for these effects on late-

stage platelet production by MK. Prior work has shown that expression of three candidate MK transcription factors is inhibited by IFN $\alpha$ ,<sup>4</sup> and perhaps SAMHD1 is also regulated at a transcriptional level in MK. Post-transcriptional mechanisms may also be at play, since the enzymatic ability of SAMHD1 to maintain dNTP homeostasis in other cells requires protein phosphorylation. Future studies in MK will be need to address these issues.

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