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JAK2 V617F stimulates proliferation of erythropoietin-dependent erythroid progenitors and delays their differentiation by activating Stat1 and other non-erythroid signaling pathways

Jiahai Shi^{1,3}, Bingbing Yuan¹, Wenqian Hu^{1,4}, and Harvey Lodish^{1,2,*}

¹Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, Massachusetts 02142, USA

²Massachusetts Institute of Technology (MIT), Departments of Biology and Biological Engineering, Cambridge, Massachusetts 02139, USA

³City University of Hong Kong, Departments of Biomedical Sciences, Hong Kong S.A.R

Introduction

Janus Kinase 2 (JAK2) is a non-receptor tyrosine kinase that transduces the signal from several cytokine receptors including the erythropoietin (Epo), G-CSF, and thrombopoietin (Tpo) receptors. Depending on the receptor to which it is bound, JAK2 can activate multiple signal transduction pathways, including activation of several STAT proteins as well as the PI-3' kinase/Akt, Ras/MAPK, and other signaling pathways essential for cytokine-mediated cell survival, proliferation, and differentiation (1). The N-terminal JAK2 FERM domain is critical for binding of JAK2 to receptors, followed by an SH2 domain that may play a role in JAK2 activation (2). The pseudokinase domain normally inhibits the C-terminal kinase domain (3).

Polycythemia Vera (PV), Essential Thrombocythemia (ET), and Primary Myelofibrosis (PMF) are myeloproliferative neoplasms (MPN) caused by the abnormal clonal expansion of hematopoietic precursors and/or panhyperplasia of myeloid cells. Progression leads to bone marrow fibrosis and extramedullary hematopoiesis (4). A somatic mutation in JAK2, V617F, is found in more than 90% of patients with PV and half of those with ET or PMF (5–8). The pathogenicity of JAK2 V617F was further confirmed by JAK2 V617F knock-in mouse

To whom Correspondence should be addressed: Harvey Lodish, Departments of Biology and Biological Engineering, Massachusetts Institute of Technology and Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, Massachusetts 02142, USA, Tel.: (+1) 617 258 5216; Fax: (+1) 617 258 6768; lodish@wi.mit.edu.

⁴Present address: Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN 55905, USA

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J.S. and H.F.L. designed the research and wrote the manuscript; J.S., W.H., and B.Y. performed experiments and bioinformatics analysis; J.S. analyzed and interpreted results.

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models that developed MPN-like symptoms (9–12). The pathogenic V617F mutation is located in the pseudokinase domain, disrupting kinase inhibition by the pseudokinase domain; this triggers constitutive activation of JAK2 but only in cells co-expressing a dimeric cytokine receptor such as the EpoR, TpoR, or G-CSFR(13–15). In particular, JAK2 V617F binds to the EpoR and triggers hyper-activation of downstream signaling pathways such as STAT5, ERK, and AKT both in the absence and presence of low Epo levels (9,12).

The effects of JAK2 V617F on early hematopoietic development are many. Hasan et al. found that JAK2 V617F promotes amplification of early hematopoietic cells in a mouse knock-in model (16), while Kent et al. found that JAK2 V617F reduces self-renewal of single hematopoietic stem cells (HSCs) (17). However, Mullally et al. suggested that HSCs expressing JAK2 V617F does not have a significant competitive advantage over wild-type HSCs (12,18).

JAK2 V617F does simulate some abnormal signaling pathways in early hematopoietic development. JAK2 V617F binds to PRMT5, a type II arginine methyltransferase, more tightly than JAK2, and activates PRMT5 by phosphorylation. Activated PRMT5 alters chromatin modifications and impairs hematopoietic differentiation, resulting in an elevated number of BFU-Es (19). JAK2 V617F also increases expression of CDC25A, a key regulator of the cell cycle, and NF-E2, an erythroid important transcription factor. Overexpression of these genes causes abnormal expansion of myeloid and erythroid progenitors (18–21). In addition, JAK2 V617F hyper-activates Stat3 and Stat5 in bone marrow cells and CD34⁺ cells from patients, and activates Stat1, which is normally inactive, in mature erythroblasts derived from erythroid progenitors from ET patients (22–24). However, there has been no systematic study of signal transduction pathways or gene expression in cells expressing JAK2 V617F during Epo-dependent terminal erythroid differentiation.

While knock-in mouse models that express JAK2 V617F have been developed and develop MPN-like symptoms(9–12), purification of Epo- dependent erythroid progenitors at the same developmental stage from normal and JAK2 V617F knock-in mice has not been accomplished. Thus for our studies we used lineage negative (Lin⁻) fetal liver cells from E14.5 C57BL/6J mice that are enriched for Epo- dependent erythroid progenitors, mainly Colony- Forming Units Erythroid (CFU-Es). When normal Lin- murine fetal liver progenitors are placed in culture containing only Epo, they undergo a stereotypic differentiation process involving 4–5 cell divisions over 2–3 days, induction of globins, heme synthesizing enzymes, and ~400 other essential erythroid genes, chromatin condensation, and enucleation in a manner similar to differentiation of erythroblasts in vivo (25).

Here we show that Lin- fetal liver progenitors ectopically expressing JAK2 V617F produce many more RBCs than normal. Expression of JAK2 V617F does not affect the initial rate of cell division but the cells continue to undergo ~6 rather than ~4 cell divisions. After 48 hours in culture, all control cells or cells ectopically expressing wild-type JAK2 have exited the cell cycle, are fully hemoglobinized, and most have enucleated. In contrast, at 48 hours cells expressing JAK2 V617F are larger and less hemoglobinized than normal and express higher

levels of Myb and other transcription factors characteristic of early erythroid progenitors. These cells express many genes not normally activated in terminally differentiating erythroid cells and that are characteristic of other hematopoietic lineages. Over the next two days these cells divide ~2 additional times and eventually accumulate normal amounts of hemoglobin, condense their nuclei, and enucleate. Thus expression of JAK2 V617F in Epo-dependent progenitors delays but does not prevent terminal differentiation.

Strikingly, global gene expression profiling and western blotting of JAK2 V617F expressing cells indicate that in addition to signaling pathways normally activated by EpoR, several signal transduction proteins not normally activated during terminal erythroid proliferation and differentiation, including Stat1 and Stat3 are activated and Stat5 is hyperactivated. We also show that these fetal liver erythroid progenitors cells express many cytokine receptors additional to the EpoR. It is likely that binding of JAK2 V617F to some of these non-erythroid receptors activates non-erythroid-signaling pathways that delays terminal erythroid differentiation and permits extended numbers of cell divisions. Inhibiting the activation of Stat1 by Fludarabine blocks erythroid hyper-proliferation in cells expressing JAK2 V617F. We hypothesize that these abnormal signaling pathways interfere with normal EpoR-JAK2 signaling, delay cell cycle exit and terminal differentiation, and thus lead over time to more RBCs produced from each CFU-E progenitor.

Material and methods

Plasmids

The bicistronic MSCV vectors expressing JAK2 or JAK2 V617F and GFP were gifts from Prof. Wei Tong at the University of Philadelphia.

Antibodies

The antibody for Jak2 was from EMD Millipore (06-1310). The antibody for Gapdh was from Santa Cruz (FL-335). The antibodies for Stat1(14994), phospho-Stat1 (9167), Stat3(12640), phospho-Stat3 (9145), Stat5 (9363), phospho-Stat5 (4322), Stat6 (5397), CSF2RB (3432), GP130 (3732) and CXCR4 (97680) were from Cell Signaling. The antibodies for IFNGR1 (ab61179), phospho-IFNGR1 (ab61062) and phospho-CXCR4 (ab74012) were from Abcam.

Murine fetal erythroid progenitor cell purification and differentiation

Enriched erythroid progenitors were purified from E14.5 C57BL/6J mouse embryos, and underwent *in vitro* differentiation following a protocol described previously in detail (26,27). Briefly, Day 14.5 pregnant C57BL/6J mice were euthanized by carbon dioxide. The embryos were isolated and the entire fetal livers were collected in Phosphate Buffered Saline (PBS) containing 2% Fetal Bovine Serum (FBS) and 100 μ M EDTA. After repeated suspension through pipette tips and filtration through a 70 μ m filter (BD), the fetal liver cells were incubated with an Ammonium Chloride Solution (Stemcell) for lysis of red blood cells. After 10 minutes the remaining fetal liver cells were centrifuged at 1500 RPM for 5 minutes and re-suspended in PBS. Following the manufacturer's protocol, lineage negative cells were obtained after magnetic depletion of lineage positive cells using the BD Biotin Mouse

Lineage Panel (559971) and BD Streptavidin Particles Plus – DM (557812). These lineage negative fetal liver cells were enriched more than 90% for erythroid progenitors.

Retrovirus production and infection

MSCV-based retroviruses were produced and used to infect erythroid progenitors following a previously described protocol (27). Briefly, after isolation, lineage negative fetal liver cells were plated in 24-well plates with 100,000 cells per well, covered by 1 ml virus containing supernatant, and centrifuged at 2000 RPM for 90 min at 37°C. After spin-infection, the virus supernatant was replaced with erythroid maintenance medium (StemSpan-SFEM (StemCell Technologies) with added recombinant mouse stem cell factor (100 ng/ml SCF, R&D), recombinant mouse IGF-1 (40 ng/ml, R&D), dexamethasone (100 nM, Sigma), and erythropoietin (2 u/ml, Amgen)). The cells were cultured overnight for recovery and expression of transgenes. The next morning, the infected cells were pooled and sorted for green fluorescence by FACS sorting at a flow rate 2. Following our published protocol for culture and terminal erythroid differentiation (26, 27), the GFP⁺ cells were cultured in Epo-only erythroid differentiation medium (Iscove modified Dulbecco's medium (IMDM) containing 15% FBS (Stemcell), 1% detoxified bovine serum albumin (BSA) (Stemcell), 500 µg/mL holo-transferrin (Sigma-Aldrich), 0.5 U/mL Epo (Amgen), 10 µg/mL recombinant human insulin (Sigma-Aldrich), 2 mM L-glutamine (Invitrogen) and 1× Pen Strep (Invitrogen)).

Number of cell divisions

Fetal liver lineage negative erythroid progenitors were labeled with PKH26 as described previously (28); we calculated the number of cell division based on the mean fluorescence intensity of PKH26 measured at different times of culture.

Microarray analysis

Agilent one-color arrays were quantile normalized. If a gene was represented by multiple probes in an array, its average probe expression value was used. Differential expression analysis was performed using a moderated t-test, as implemented in the limma package of Bioconductor, with P-value correction for false discovery rate. Log₂ ratios of mean expression levels (treatment/control) and false discovery rate (FDR)-adjusted P-values were used for further analysis. Hierarchical clustering was obtained using hclust method with complete linkage and Euclidean distance in R. Analysis of gene expression across 30 tissues and cells followed the protocol used previously(29).

Inhibition of Stat1 and Stat3 activation by small molecule inhibitors

The Stat1 inhibitor Fludarabine (Santa Cruz Biotechnology, sc-204755) and the Stat3 inhibitor Stattic (Santa Cruz Biotechnology, sc-202818) were dissolved in dimethyl sulfoxide (DMSO) at 200 mM and 50 mM, respectively, and added to the RBC differentiation medium at a final concentration of 1.5 µM.

Cell apoptosis assay measured by Annexin V

Apoptosis of cell expressing Jak2 V617F after Stat1 or Stat3 inhibition was measured by the BD Pharmingen™ PE Annexin V Apoptosis Detection Kit following the manufacturer's manual. Briefly, two million cells were collected and washed twice with cold phosphate buffered saline (PBS). After resuspended in 1* Binding Buffer at a concentration of 2 million cells/ml, ten percent of the sample was incubated with 5 µl of PE Annexin V and 5 µl 7-AAD for 15 mins at room temperature while covered by aluminum foil. The cells were then washed by PBS and analyzed by flow cytometry to detect Annexin V positive cells.

Colony forming assay

Cell concentration was determined using a hemocytometer (Hausser Scientific) after staining with Trypan Blue. Two hundred cells were mixed with 3 ml methylcellulose-based medium (Stemcell, MethoCult™ M3334), and plated in a 35 mm culture dish. Each experimental point contained two duplicate culture dishes plus one dish filled with water that were held in one 10 cm plate, and cultured at 37°C with 5% carbon dioxide for five days before examination under a microscope.

Standard techniques of hematology

Standard techniques used in hematology were performed following the protocols described before (25–27). These included flow cytometry (FACS) sorting and analysis, western blotting, colony forming assay, cell staining and microscopic analysis, hemoglobin measurement, RNA extraction and Quantitative RT-PCR analysis.

Results

Ectopic expression of JAK2 V617F promotes proliferation of Epo- dependent erythroid progenitors

We infected lineage negative fetal liver cells with bicistronic retroviral vectors expressing GFP and either JAK2 or JAK2 V617F (30). After an overnight culture (termed 0 hour) to allow expression of the transgenes, JAK2 and JAK2 V617F infected erythroid progenitors expressed a similar low level of GFP, suggesting a moderate expression level of JAK2 or JAK2 V617F. This was confirmed by Western blots, showing a 2 and 2.2-fold increased expression of JAK2 and JAK2 V617F, respectively, compared to endogenous JAK2 levels (Figure 1a). We then cultured these cells in medium containing only Epo for 96 hours. As normal terminal erythropoiesis is completed by 48 hours, we compared the expression levels of JAK2 and JAK2 V617F at 24 and 48 hours by Western Blots. Based on the ratio of JAK2 expression to that of the internal control GAPDH, the expression level of ectopic JAK2 remained similar at 24 hours, but decreased at 48 hours, likely because the protein expression machinery had switched to produce hemoglobin. In contrast, the expression level of JAK2 V617F decreased greatly at 24 and 48 hours, although ectopic expression of JAK2 and JAK2 V617F were driven by the same promoter. The decrease in JAK2 V617F might be due to feedback of aberrant JAK2 V617F signaling pathways (Figure 1a).

During the first 48 hours of culture, all cells expanded 16-fold with a similar 11–12 hour doubling time. But after 72 hours the number of control and JAK2 expressing cells

decreased due to lysis of enucleated reticulocytes. In contrast, cells expressing JAK2 V617F continued proliferating and reached a peak at 96 hours, resulting in a four-fold increase in the number of cells produced from each progenitor (Figure 1b).

Moreover, we labeled control, JAK2 and JAK2 V617F expressing progenitors at 0 hour with PKH26, a red fluorescent cell membrane dye, and monitored by flow cytometry the fluorescent densities of these cells during differentiation (28). Since the amount of PKH26 decreases by one half after each cell division, the decrease in PKH26 fluorescence corresponds to the number of cell division. Control and JAK2- expressing cells divided 4 times in the first 48 hours and then stopped dividing, but cells expressing JAK2 V617F later underwent nearly two extra divisions (Figure 1 c and d).

Ectopic expression of JAK2 V617F delays cell cycle exit and increases the number of colony forming units – erythroid (CFU-Es)

Cell cycle exit is a hallmark of terminal erythropoiesis (31). As expected, cells expressing the control vector or JAK2 exited the cell cycle by 48 hours of culture, as the majority of cells were in the G0/G1 phase. In contrast, cells expressing JAK2 V617F were actively proliferating at 48 hours as judged by the high percentage in the S and G2/M phases; and did not exit the cell cycle until 96 hours (Figure 1 e, f and S2). Importantly, most of cells expressing JAK2 V617F, JAK2, and control vector were committed erythroid progenitors, as evidenced by cell surface expression of Ter-119 at as early as 24 hours of culture (Figure S1).

CFU-Es are defined as committed erythroid progenitors able to give rise to colonies containing 8–32 fully hemoglobinized cells after 2–3 days of *in vitro* culture in medium containing only Epo. CFU-Es quickly lose their colony forming potential during terminal erythroid development. Of 200 Lineage- negative fetal liver progenitors plated, as expected about 100 were functional CFU-Es. Also as expected, cultures of progenitors expressing either a control vector or JAK2 lost colony-forming potential after 24 hr. In contrast, cultures of CFU-Es expressing JAK2-V617F exhibited a four -fold increase in functional CFU-Es after 24 hours in culture with Epo. Since these cells divided twice during the first 24 hr of culture, most if not all of the cells produced at 24 hr were, like the parental cell, a CFU-E. The loss of functional CFU-Es during the subsequent 24 hr. culture period (Figure 1g) is consistent with terminal erythroid differentiation, as documented below. Representative erythroid colonies from cultures expressing control, JAK2, and JAK2-V617F vectors at 0 hour, and cultures expressing JAK2-V617F at 24 hours of culture (Figure 1h) exhibit normal morphology.

Ectopic expression of JAK2 V617F delays terminal erythropoiesis

To determine whether JAK2 V617F expression delays terminal erythroid differentiation, we studied the morphology of cells expressing control, JAK2, and JAK2 V617F at 48 hours, and JAK2 V617F at 96 hours of culture with Epo. At 48 hours normal erythroid progenitors and cells expressing JAK2 have, as expected, decreased in cell size, accumulated ~31 pg/cell hemoglobin, and undergone enucleation (26). At 48 hours cells expressing JAK2 V617F were larger and paler in color than normal (Figures 2a and S3) and had reduced amounts of

hemoglobin (Figure 2d); none had undergone enucleation (Figure 2c). Moreover, cells expressing JAK2 V617F expressed much lower than normal levels of many erythroid-important genes and retained higher than normal expression levels of Myb and other transcription factors characteristic of early erythroid progenitors (Figure 2b). Nevertheless, cells expressing JAK2 V617F eventually differentiated. At 96 hours they had undergone enucleation at a slightly lower- than normal frequency, and accumulated normal hemoglobin levels (31 pg/cell), forming reticulocytes that were indistinguishable in size from controls (Figure 2c–f).

Expression of the constitutively active mutant EpoR R129C does not affect terminal erythroid proliferation or differentiation

To determine whether JAK2 V617F mediated terminal erythroid hyper-proliferation is driven only by the same pathways as normally activated by Epo, we overexpressed the disulfide-linked homodimeric and constitutively active EpoR R129C mutant in erythroid progenitors, and cultured them in erythroid differentiation medium(32). When cultured in the presence of Epo, cells expressing EpoR R129C underwent normal proliferation and differentiation, and enucleation at 48 hours. The sizes of the reticulocytes formed were normal (Figure 3 a–c). Without Epo, expression of EpoR R129C supported normal proliferation and differentiation of erythroid progenitors whereas normal erythroid progenitors did not divide and underwent apoptosis (Figure 3 d and e). These results suggest that constitutive hyper-activation of the EpoR neither disrupts terminal erythropoiesis nor delays erythroid differentiation.

As revealed by microarray analysis JAK2 V617F activates non-erythroid signaling pathways in Epo-dependent differentiating erythroblasts

To understand the molecular mechanisms by which expression of JAK2 V617F affects terminal erythroid proliferation and differentiation, we collected RNA samples from erythroid progenitors expressing the control vector, JAK2, or JAK2 V617F at 0, 24, 48 hours of culture, and two additional samples from cells expressing JAK2 V617F at 72 and 96 hours. Global gene expression profiling was analyzed using Agilent Mouse Gene Expression Microarrays (Figure S4). Hierarchical clustering revealed that these cell populations segregated into four transcriptionally distinct groups: (1) All progenitor cells at 0 hours of culture, that is after the overnight pre-incubation to allow expression of the transgenes; (2) Control and JAK2 expressing cells at 24 hours; (3) Control and JAK2 expressing cells at 48 hours together with JAK2 V617F expressing cells at 72 hours and 96 hours. (4) JAK2 V617F expressing cells at 24 hours and 48 hours (Figure 4a). Groups 1, 2, and 3 correspond to normal erythroid progenitors, differentiating nucleated erythroblasts, and terminal erythroblasts and reticulocytes, respectively.

To confirm that JAK2 V617F delays terminal erythropoiesis, we selected the top 600 genes upregulated in Ter119⁺ erythroblasts compared to CFU-Es as markers for erythroid maturation(27). Half of these genes were expressed specifically in the erythroid lineage (Figure S5). Expression of these marker genes increased dramatically at 48 hours in cells expressing JAK2, but not in those expressing JAK2 V617F until 72 and 96 hours (Figure 4b). Interestingly, cells expressing control or JAK2 exhibit nearly identical patterns of gene

expression at all-time points; only 0, 2 and 25 genes were differentially expressed greater than 2 fold in JAK2 expressing cells relative to control cells at 0, 24 and 48 hours, respectively. This extends data in Figures 1 and 2 showing that the slight overexpression of wild- type JAK2 does not affect terminal erythroid proliferation or differentiation. Consistent with our finding that cells expressing JAK2 V617F eventually underwent normal hemoglobinization and enucleation at 96 hours, the expression profile of cells expressing JAK2 V617F at 72 and 96 hours was virtually indistinguishable from those of control or JAK2 expressing terminally differentiated cells at 48 hours; the R^2 of linear regression for 36,551 mRNA expression values in JAK2 V617F expressing cells at 96 hours plotted against JAK2 cells at 48 hours was 0.9482 (Figure 4d).

Importantly, the transcriptional profile of cells expressing JAK2 V617F at 24 and 48 hours was very different from that of any normal differentiating erythroid cell (Figure 4a). Specifically, 423, 875 and 3868 genes were differentially expressed greater than 2 fold in JAK2 V617F cells at 0, 24 and 48 hours, relative to JAK2 expressing or control cells, respectively. We then used Gene Set Enrichment Analysis (GSEA) to identify differentially expressed gene sets using JAK2 V617F and JAK2 expressing cells at 0, 24 and 48 hours. As expected, the “DNA replication” gene set was enriched in JAK2 V617F expressing cells at 48 hours, consistent with the delay of cell cycle exit and the continued cycling of these cells compared to control cells (Figure 4c). At 0 hours there were 20 gene sets significantly enriched in JAK2 V617F expressing erythroid progenitors, including “cytokine & cytokine receptor interaction”, “JAK-STAT signaling,” and many other immunological and inflammatory pathways that are not normally activated during erythropoiesis (Table 1). Leading edge analysis of these signaling pathways revealed that signaling molecules including TNF α , interleukin, interferon, TGF β , EGF, and their receptors and downstream signal transduction proteins were common molecules among these signaling pathways (Figure 4e and Table S1).

Consistent with this, the 423 genes unregulated in erythroid progenitors expressing JAK2 V617F were predominantly expressed in other hematopoietic lineages, including T cells, B cells, macrophages and megakaryocytes (Figure S6). During differentiation of JAK2 V617F cells, the expression of most of these 423 genes decreased gradually. (Table S1); as a consequence, most of the non-erythroid signaling pathways (13/20) were no longer enriched in erythroblasts expressing JAK2 V617F at 48 hours of differentiation (Table 1). The drop in expression of these non-erythroid genes in JAK2 V617F expressing cells is consistent with the eventual normal terminal differentiation of these cells.

JAK2 V617F activates abnormal STAT signaling in erythroid differentiation

Interestingly, in purified murine fetal liver Epo- dependent erythroid progenitors, which are characterized by low Ter119 and high CD71 expression, many cytokine receptors additional to EpoR, JAKs additional to JAK2, as well as several STATs additional to Stat5, are also expressed (Figure 5a) (25). Western blotting shows that, after being placed in medium containing only Epo for 2.5 hours, cells expressing JAK2 V617F hyper-phosphorylated Stat5 compared to control cells, consistent with previous findings(9,14). Strikingly, overexpression and activation of Stat1 and Stat3 were found only in cells expressing JAK2

V617F, but not in controls. STAT6 was also found in all cells, though no phosphorylated Stat6 was detected (Figure 5b). STAT2 and STAT4 were not detectable by western blotting.

Indeed, Stat1 and Stat3 likely activate a number of genes normally expressed in other hematopoietic lineages. By overlapping the chip-seq data of Stat1 in T cells and Stat3 in macrophages with our microarray data (33–35) we identified 203 potential Stat1-activated genes and 171 potential Stat3-activated genes that were specifically upregulated at 0 hour in JAK2 V617F cells, compared to cells expressing JAK2 (Figure S7 a and b). Most of these Stat1 and Stat3 activated genes were abundantly expressed in other hematopoietic lineages, but not normally in erythroid cells (Figure S7 c and d). In contrast, only the JAK2-Stat5 signaling pathway is activated during normal CFU-E terminal erythropoiesis and prevents progenitor apoptosis and is essential for terminal proliferation and differentiation(36). It is highly likely that the non-erythroid genes and pathways activated by Stat1 and Stat3 interfered with normal JAK2-Stat5 signaling pathways and blocked erythroid differentiation. Normal maturation of erythroblasts expressing JAK2 V617F could only occur after expression of these Stat1 and Stat3 activated genes had decreased (Figure S7).

Unlike JAK2, JAK2 V617F can bind to and activate dimeric cytokine receptors in the absence of cytokine stimulation, and activate downstream signaling pathways (14,15). Expression of four cytokine receptors – interferon gamma receptor 1 (IFNGR1), C-X-C chemokine receptor type 4 (CXCR4), Glycoprotein 130 (GP130), and cytokine receptor common beta-chain (CSF2RB) – were identified by western blotting as being expressed in erythroid progenitors after culture in Epo-only medium for 2.5 hours. More importantly, IFNGR1 and CXCR4 were phosphorylated only in cells expressing JAK2 V617F, but not in differentiating control cells in the presence of Epo (Figure 5b). These results suggest that JAK2 V617F associates with and activates several cytokine receptors in CFU-Es in the absence of the normal activating cytokine, and subsequently activates non-erythroid downstream signaling pathways.

JAK2 V617F mediated erythroid terminal differentiation is Epo dependent

To examine whether Epo is indeed essential for JAK2 V617F terminal erythropoiesis, we cultured JAK2 V617F expressing progenitors in medium with or without Epo. Without Epo, the proliferation rate of JAK2 V617F expressing cells was much lower than with Epo (Figure 6a); only half of the cells became committed Ter-119 positive erythroblasts and only a few underwent enucleation (Figure 6 b and c). These results suggest that some Epo is essential for terminal erythroid differentiation despite the expression of JAK2 V617F.

Inhibition of activated Stat1 disrupts JAK2 V617F mediated erythroid hyper-proliferation

To examine whether Stat1 or Stat3 was essential for erythroid hyper-proliferation, we treated cells expressing JAK2 V617F with Stattic, a chemical inhibitor of Stat3, and Fludorabine, an inhibitor of Stat1, respectively. JAK2 V617F – expressing cells treated with 1.5 μ M Fludorabine exhibited a significant less proliferation (Figure 7a) with increased apoptosis measured by Annexin V staining (Figure 7b). Western blot confirmed the reduction of phosphorylated Stat1 in the presence of Fludorabine comparing to cells treated with DMSO (Figure 7c). However inhibiting Stat3 activity by Stattic only slightly affected cell

proliferation. Our data suggests JAK2 V617F mediated erythroid terminal hyper-proliferation depends on Stat1 phosphorylation by the constitutively active JAK2 V617F kinase.

Discussion

Our principal result is that expression of JAK2 V617F in murine fetal liver erythroid progenitors and cultured in the presence of Epo does not affect the rate of cell doubling – about 12 hours – but JAK2 V617F- expressing cells divide ~6 rather than the normal ~4 times. Whereas normal terminally differentiating erythroid cells exit the cell cycle after 3 – 4 divisions and undergo nuclear condensation and enucleation, those expressing JAK2 V617F delay cell cycle exit for ~2 additional divisions. But JAK2 V617F expressing erythroid progenitors eventually differentiate into apparently normal reticulocytes.

Microarray analyses comparing JAK2 and JAK2 V617F – expressing erythroblasts indicate that JAK2 V617F not only activates EpoR-JAK2 signaling pathways, but also transiently induces non-erythroid- signaling molecules including TNF α , interleukin, interferon, TGF β , EGFR, receptors and downstream signal transduction proteins. We further show the overexpression and activation of Stat1 and Stat3 signaling pathways only in progenitors expressing JAK2 V617F, but not in normal progenitors or those expressing JAK2.

Our explanation for this relates to the fact that JAK2 is essential for signal transduction by a large number of cytokine receptors, including the Epo, Tpo, G-CSF, prolactin, IL-3, IL-5, GM-CSF, IL-6, IL-12, and interferon gamma receptors (1). Of these, we show that fetal erythroid progenitor cells normally express several cytokine receptors additional to the Epo receptor, JAK1 and JAK3 additional to JAK2, and other STATs additional to STAT5 (25). Specifically, we show the expression of IFNGR1, CXCR4, GP130, and CSF2RB in erythroid progenitors by Western blotting; it is surprising for us to see how many cytokine receptors, in addition to the EpoR, were expressed in our mouse fetal liver erythroid cells (25,27).

Importantly, IFNGR1 and CXCR4 are activated (phosphorylated) only in cells expressing JAK2 V617F, but not in control or JAK2- expressing differentiating cells. IFNGR1 forms heterodimers with IFNGR2 and is associated with both JAK1 and JAK2 (37). CXCR4, a G protein-coupled receptor, is expressed on the surface of many cell types. Upon binding to CXC chemokine stromal-derived factor 1 (SDF-1 α), CXCR4 activates the downstream JAK2/STAT3 signaling pathway (38). Additionally, a low level of terminal erythropoiesis can be stimulated by Tpo (39) and it is likely that some or all erythroid progenitors express low and possibly variable numbers of some of these other receptors. JAK2 V617F is known to bind to and activate the Epo, Tpo, IGF, and prolactin receptors(40) and likely can bind to and activate other cytokine receptors in the absence of the cognate cytokine and activate signaling pathways characteristic of these receptors as discussed previously (41,42).

The exact signaling pathways activated in any particular cell by JAK2 V617F will depend on the expression pattern of multiple cytokine receptors, including those normally activated in these cells, e.g. the EpoR in erythroid cells, and those not, e.g. IFNGR1 and CXCR4.

Proliferation and differentiation of erythroid Epo- dependent progenitors depends on the regulated, sequential activation and then inactivation of the EpoR-JAK2 signaling pathway and presumably the absence of activation of Stat1 or Stat3(36). Since JAK2 V617F activates several non-erythroid signaling pathways including Stat1 in the early stages of erythroid differentiation, it is likely that activation of these non-erythroid signaling proteins interferes with normal erythroid signaling and blocks erythroid differentiation.

Indeed, we show that JAK2 V617F expressing cells express over 400 genes not normally expressed during terminal erythroid differentiation. Many of these genes are expressed predominantly in other hematopoietic lineages, including T cells, B cells, macrophages and megakaryocytes. Of these 400 genes 203 are abundantly expressed in macrophages and are activated by Stat1 and 171 are activated by Stat3; it is likely that in JAK2 V617F expressing cells these genes are also activated by Stat1 or Stat3. These non-erythroid pathways could be all or part of the explanation why erythroid progenitors expressing JAK2 V617F undergo excess numbers of early cell divisions. During differentiation of JAK2 V617F cells, the expression of most of these ~400 genes decreased gradually and were no longer enriched in erythroblasts expressing JAK2 V617F at 48 hours of differentiation. The drop in expression of these non-erythroid genes in JAK2 V617F expressing cells and the induction of normal erythroid important genes is consistent with the eventual normal terminal differentiation of these cells.

Importantly, activated Stat1 signaling has been found in mature erythroid cells from JAK2 V617F ET patients (24), suggesting strong Stat1 signaling in terminal erythropoiesis. We also showed that JAK2 V617F expressing cells exhibit hyperactivation of Stat5. Consistent with this finding, depletion of Stat5 in JAK2 V617F knock-in mice disrupts hyperactive Epo-EpoR signaling and reverses production of excess red blood cells. Thus hyperactivation of Stat5 could be another reason why JAK2 V617F expressing erythroid progenitors undergo an excessive number of cell divisions.

Our study focuses on JAK2 V617F function at the terminal erythropoiesis stage. However, activation of abnormal signaling pathways in the HSCs and early progenitors also disturbs early hematopoietic development. Over-expression of hyperactive EpoR R129C in early hematopoietic progenitors, inducing strong Epo signaling, indeed leads to severe leukemia (43). JAK2 V617F alters the expression of several genes, including NF-E2, in early hematopoietic progenitors. Transgenic mouse models with elevated or mutated NF-E2 levels develop MPN-like phenotypes and leads to leukemic transformation (44,45). Moreover, JAK2 V617F has been shown to affect the self-renewal and differentiation of HSCs (12,16–18). Therefore, JAK2 V617F ET patients or JAK2 V617F positive chronic granulocytic patients may have other defects in early hematopoiesis that results in erythroid progenitor cell defects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Leonard WJ, O'Shea JJ. Jaks and STATs: biological implications. *Annual review of immunology*. 1998; 16:293–322.
2. Huang LJ, Constantinescu SN, Lodish HF. The N-terminal domain of Janus kinase 2 is required for Golgi processing and cell surface expression of erythropoietin receptor. *Mol Cell*. 2001; 8:1327–1338. [PubMed: 11779507]
3. Ungureanu D, Wu J, Pekkala T, Niranjana Y, Young C, Jensen ON, Xu CF, Neubert TA, Skoda RC, Hubbard SR, Silvennoinen O. The pseudokinase domain of JAK2 is a dual-specificity protein kinase that negatively regulates cytokine signaling. *Nature structural & molecular biology*. 2011; 18:971–976.
4. Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A, Harris NL, Le Beau MM, Hellstrom-Lindberg E, Tefferi A, Bloomfield CD. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood*. 2009; 114:937–951. [PubMed: 19357394]
5. Baxter EJ, Scott LM, Campbell PJ, East C, Fourouclas N, Swanton S, Vassiliou GS, Bench AJ, Boyd EM, Curtin N, Scott MA, Erber WN, Green AR, Cancer Genome P. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet*. 2005; 365:1054–1061. [PubMed: 15781101]
6. James C, Ugo V, Le Couedic JP, Staerk J, Delhommeau F, Lacout C, Garçon L, Raslova H, Berger R, Bennaceur-Griscelli A, Villeval JL, Constantinescu SN, Casadevall N, Vainchenker W. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature*. 2005; 434:1144–1148. [PubMed: 15793561]
7. Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweg JR, Tichelli A, Cazzola M, Skoda RC. A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med*. 2005; 352:1779–1790. [PubMed: 15858187]
8. Levine RL, Wadleigh M, Cools J, Ebert BL, Wernig G, Huntly BJ, Boggon TJ, Wlodarska I, Clark JJ, Moore S, Adelsperger J, Koo S, Lee JC, Gabriel S, Mercher T, D'Andrea A, Frohling S, Dohner K, Marynen P, Vandenberghe P, Mesa RA, Tefferi A, Griffin JD, Eck MJ, Sellers WR, Meyerson M, Golub TR, Lee SJ, Gilliland DG. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell*. 2005; 7:387–397. [PubMed: 15837627]
9. Akada H, Yan D, Zou H, Fiering S, Hutchison RE, Mohi MG. Conditional expression of heterozygous or homozygous Jak2V617F from its endogenous promoter induces a polycythemia vera-like disease. *Blood*. 2010; 115:3589–3597. [PubMed: 20197548]
10. Li J, Spensberger D, Ahn JS, Anand S, Beer PA, Ghevaert C, Chen E, Forrai A, Scott LM, Ferreira R, Campbell PJ, Watson SP, Liu P, Erber WN, Huntly BJ, Ottersbach K, Green AR. JAK2 V617F impairs hematopoietic stem cell function in a conditional knock-in mouse model of JAK2 V617F-positive essential thrombocythemia. *Blood*. 2010; 116:1528–1538. [PubMed: 20489053]
11. Marty C, Lacout C, Martin A, Hasan S, Jacquot S, Birling MC, Vainchenker W, Villeval JL. Myeloproliferative neoplasm induced by constitutive expression of JAK2V617F in knock-in mice. *Blood*. 2010; 116:783–787. [PubMed: 20472827]
12. Mullally A, Lane SW, Ball B, Megerdichian C, Okabe R, Al-Shahrour F, Paktinat M, Haydu JE, Housman E, Lord AM, Wernig G, Kharas MG, Mercher T, Kutok JL, Gilliland DG, Ebert BL. Physiological Jak2V617F expression causes a lethal myeloproliferative neoplasm with differential

- effects on hematopoietic stem and progenitor cells. *Cancer Cell*. 2010; 17:584–596. [PubMed: 20541703]
13. Bandaranayake RM, Ungureanu D, Shan Y, Shaw DE, Silvennoinen O, Hubbard SR. Crystal structures of the JAK2 pseudokinase domain and the pathogenic mutant V617F. *Nature structural & molecular biology*. 2012; 19:754–759.
 14. Lu X, Huang LJ, Lodish HF. Dimerization by a cytokine receptor is necessary for constitutive activation of JAK2V617F. *J Biol Chem*. 2008; 283:5258–5266. [PubMed: 18158285]
 15. Pradhan A, Lambert QT, Griner LN, Reuther GW. Activation of JAK2-V617F by components of heterodimeric cytokine receptors. *The Journal of biological chemistry*. 2010; 285:16651–16663. [PubMed: 20363735]
 16. Hasan S, Lacout C, Marty C, Cuingnet M, Solary E, Vainchenker W, Villeval JL. JAK2V617F expression in mice amplifies early hematopoietic cells and gives them a competitive advantage that is hampered by IFN α . *Blood*. 2013; 122:1464–1477. [PubMed: 23863895]
 17. Kent DG, Li J, Tanna H, Fink J, Kirschner K, Pask DC, Silber Y, Hamilton TL, Sneade R, Simons BD, Green AR. Self-renewal of single mouse hematopoietic stem cells is reduced by JAK2V617F without compromising progenitor cell expansion. *PLoS biology*. 2013; 11:e1001576. [PubMed: 23750118]
 18. Mullally A, Poveromo L, Schneider RK, Al-Shahrour F, Lane SW, Ebert BL. Distinct roles for long-term hematopoietic stem cells and erythroid precursor cells in a murine model of Jak2V617F-mediated polycythemia vera. *Blood*. 2012; 120:166–172. [PubMed: 22627765]
 19. Liu F, Zhao X, Perna F, Wang L, Koppikar P, Abdel-Wahab O, Harr MW, Levine RL, Xu H, Tefferi A, Deblasio A, Hatlen M, Menendez S, Nimer SD. JAK2V617F-mediated phosphorylation of PRMT5 downregulates its methyltransferase activity and promotes myeloproliferation. *Cancer cell*. 2011; 19:283–294. [PubMed: 21316606]
 20. Gautier EF, Picard M, Laurent C, Marty C, Villeval JL, Demur C, Delhommeau F, Hexner E, Giraudier S, Bonneville N, Ducommun B, Recher C, Laurent G, Manenti S, Mansat-De Mas V. The cell cycle regulator CDC25A is a target for JAK2V617F oncogene. *Blood*. 2012; 119:1190–1199. [PubMed: 22065597]
 21. Mutschler M, Magin AS, Buerge M, Roelz R, Schanne DH, Will B, Pilz IH, Migliaccio AR, Pahl HL. NF-E2 overexpression delays erythroid maturation and increases erythrocyte production. *British journal of haematology*. 2009; 146:203–217. [PubMed: 19466964]
 22. Teofili L, Martini M, Cenci T, Petrucci G, Torti L, Storti S, Guidi F, Leone G, Larocca LM. Different STAT-3 and STAT-5 phosphorylation discriminates among Ph-negative chronic myeloproliferative diseases and is independent of the V617F JAK-2 mutation. *Blood*. 2007; 110:354–359. [PubMed: 17376889]
 23. Oh ST, Simonds EF, Jones C, Hale MB, Goltsev Y, Gibbs KD Jr, Merker JD, Zehnder JL, Nolan GP, Gotlib J. Novel mutations in the inhibitory adaptor protein LNK drive JAK-STAT signaling in patients with myeloproliferative neoplasms. *Blood*. 2010; 116:988–992. [PubMed: 20404132]
 24. Chen E, Beer PA, Godfrey AL, Ortmann CA, Li J, Costa-Pereira AP, Ingle CE, Dermitzakis ET, Campbell PJ, Green AR. Distinct clinical phenotypes associated with JAK2V617F reflect differential STAT1 signaling. *Cancer Cell*. 2010; 18:524–535. [PubMed: 21074499]
 25. Wong P, Hattangadi SM, Cheng AW, Frampton GM, Young RA, Lodish HF. Gene induction and repression during terminal erythropoiesis are mediated by distinct epigenetic changes. *Blood*. 2011; 118:e128–138. [PubMed: 21860024]
 26. Zhang J, Socolovsky M, Gross AW, Lodish HF. Role of Ras signaling in erythroid differentiation of mouse fetal liver cells: functional analysis by a flow cytometry-based novel culture system. *Blood*. 2003; 102:3938–3946. [PubMed: 12907435]
 27. Flygare J, Rayon Estrada V, Shin C, Gupta S, Lodish HF. HIF1 α synergizes with glucocorticoids to promote BFU-E progenitor self-renewal. *Blood*. 2011; 117:3435–3444. [PubMed: 21177435]
 28. Sankaran VG, Ludwig LS, Sicinska E, Xu J, Bauer DE, Eng JC, Patterson HC, Metcalf RA, Natkunam Y, Orkin SH, Sicinski P, Lander ES, Lodish HF. Cyclin D3 coordinates the cell cycle during differentiation to regulate erythrocyte size and number. *Genes & development*. 2012; 26:2075–2087. [PubMed: 22929040]

29. Alvarez-Dominguez JR, Hu W, Yuan B, Shi J, Park SS, Gromatzky AA, van Oudenaarden A, Lodish HF. Global discovery of erythroid long non-coding RNAs reveals novel regulators of red cell maturation. *Blood*. 2013
30. Bersenev A, Wu C, Balcerek J, Tong W. Lnk controls mouse hematopoietic stem cell self-renewal and quiescence through direct interactions with JAK2. *The Journal of clinical investigation*. 2008; 118:2832–2844. [PubMed: 18618018]
31. Pop R, Shearstone JR, Shen Q, Liu Y, Hallstrom K, Koulis M, Gribnau J, Socolovsky M. A key commitment step in erythropoiesis is synchronized with the cell cycle clock through mutual inhibition between PU.1 and S-phase progression. *PLoS biology*. 2010; 8
32. Pharr PN, Hankins D, Hofbauer A, Lodish HF, Longmore GD. Expression of a constitutively active erythropoietin receptor in primary hematopoietic progenitors abrogates erythropoietin dependence and enhances erythroid colony-forming unit, erythroid burst-forming unit, and granulocyte/macrophage progenitor growth. *Proceedings of the National Academy of Sciences of the United States of America*. 1993; 90:938–942. [PubMed: 7679218]
33. Ng SL, Friedman BA, Schmid S, Gertz J, Myers RM, Tenover BR, Maniatis T. IkappaB kinase epsilon (IKK(epsilon)) regulates the balance between type I and type II interferon responses. *Proc Natl Acad Sci U S A*. 2011; 108:21170–21175. [PubMed: 22171011]
34. Liao W, Lin JX, Wang L, Li P, Leonard WJ. Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. *Nature immunology*. 2011; 12:551–559. [PubMed: 21516110]
35. Hutchins AP, Poulain S, Miranda-Saavedra D. Genome-wide analysis of STAT3 binding in vivo predicts effectors of the anti-inflammatory response in macrophages. *Blood*. 2012; 119:e110–119. [PubMed: 22323479]
36. Porpiglia E, Hidalgo D, Koulis M, Tzafiriri AR, Socolovsky M. Stat5 signaling specifies basal versus stress erythropoietic responses through distinct binary and graded dynamic modalities. *PLoS biology*. 2012; 10:e1001383. [PubMed: 22969412]
37. Thiel DJ, le Du MH, Walter RL, D'Arcy A, Chene C, Fountoulakis M, Garotta G, Winkler FK, Ealick SE. Observation of an unexpected third receptor molecule in the crystal structure of human interferon-gamma receptor complex. *Structure*. 2000; 8:927–936. [PubMed: 10986460]
38. Ahr B, Denizot M, Robert-Hebmann V, Brelot A, Biard-Piechaczyk M. Identification of the cytoplasmic domains of CXCR4 involved in Jak2 and STAT3 phosphorylation. *The Journal of biological chemistry*. 2005; 280:6692–6700. [PubMed: 15615703]
39. Kieran MW, Perkins AC, Orkin SH, Zon LI. Thrombopoietin rescues in vitro erythroid colony formation from mouse embryos lacking the erythropoietin receptor. *Proceedings of the National Academy of Sciences of the United States of America*. 1996; 93:9126–9131. [PubMed: 8799165]
40. Staerk J, Kallin A, Royer Y, Diaconu CC, Dusa A, Demoulin JB, Vainchenker W, Constantinescu SN. JAK2, the JAK2 V617F mutant and cytokine receptors. *Pathologie-biologie*. 2007; 55:88–91. [PubMed: 16904848]
41. Mullally A, Ebert BL. Janus reveals another face: the biologic rationale for targeting Janus kinase 2 in lymphoma. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*. 2012; 30:4168–4170. [PubMed: 22965959]
42. Vainchenker W, Favale F. Myelofibrosis, JAK2 inhibitors and erythropoiesis. *Leukemia*. 2013; 27:1219–1223. [PubMed: 23739260]
43. Longmore GD, Pharr PN, Lodish HF. A constitutively activated erythropoietin receptor stimulates proliferation and contributes to transformation of multipotent, committed nonerythroid and erythroid progenitor cells. *Molecular and cellular biology*. 1994; 14:2266–2277. [PubMed: 8139532]
44. Kaufmann KB, Grunder A, Hadlich T, Wehrle J, Gothwal M, Bogeska R, Seeger TS, Kayser S, Pham KB, Jutzi JS, Ganzenmuller L, Steinemann D, Schlegelberger B, Wagner JM, Jung M, Will B, Steidl U, Aumann K, Werner M, Gunther T, Schule R, Rambaldi A, Pahl HL. A novel murine model of myeloproliferative disorders generated by overexpression of the transcription factor NF-E2. *The Journal of experimental medicine*. 2012; 209:35–50. [PubMed: 22231305]
45. Jutzi JS, Bogeska R, Nikoloski G, Schmid CA, Seeger TS, Stegelmann F, Schwemmers S, Grunder A, Peeken JC, Gothwal M, Wehrle J, Aumann K, Hamdi K, Dierks C, Kamar Wang W, Dohner K,

Jansen JH, Pahl HL. MPN patients harbor recurrent truncating mutations in transcription factor NF-E2. *The Journal of experimental medicine*. 2013; 210:1003–1019. [PubMed: 23589569]

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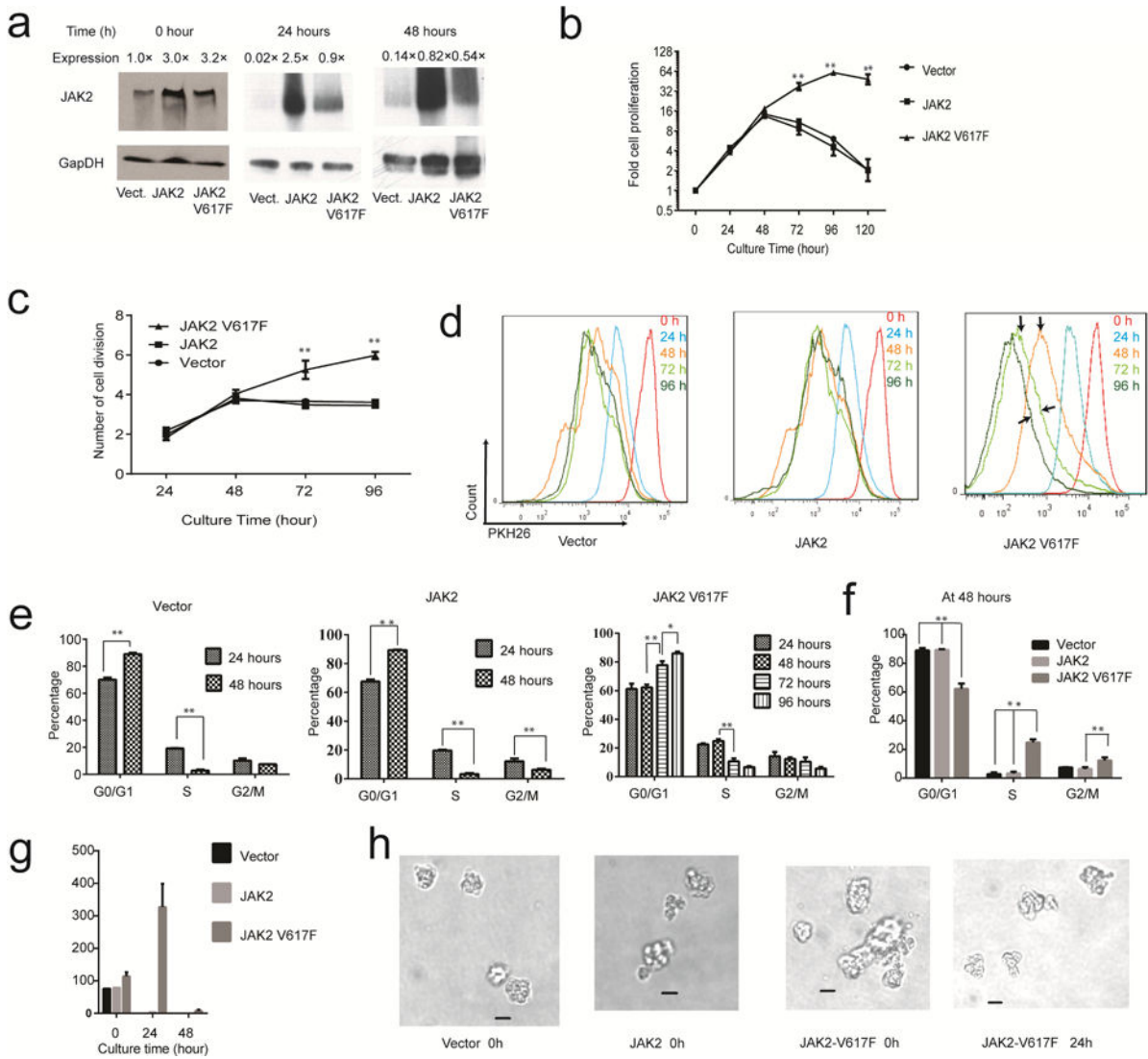


Figure 1. Ectopic expression of JAK2 V617F promotes proliferation of murine erythroid Epo-dependent progenitors, delays cell cycle exit and increases the number of CFU-Es
 (a) Western blot shows the protein expression levels of JAK2 or JAK2 V617F in erythroid progenitors at 0, 24 and 48 hours after retroviral infection. GAPDH was used as a loading control. Numbers above the panels denote the ration of JAK2 to GAPDH. (b) Progenitors expressing Vector (circle) and JAK2 (square) expanded sixteen fold after 48 hours and then stopped dividing, while progenitors expressing JAK2 V617F (triangle) expanded four times more, resulting at 96 hours in a sixty-four fold increase in cell numbers. (c) As measured by PKH26 labeling, cultured Vector- and JAK2- expressing erythroid progenitors divided four times, whereas JAK2 V617F expressing erythroid progenitors underwent two additional divisions (**: $p < 0.01$). (d) Plots of PKH26 fluorescence used to calculate the data in Panel c. (e) Cell cycle analyses were performed on erythroid progenitors expressing Vector, JAK2 and JAK2 V617F during terminal differentiation. (f) The cell cycle analysis for erythroid cells expressing Vector, JAK2, and JAK2 V617F at 48 hours are replotted to more directly demonstrate the differences. (g) CFU-E colony assays utilizing a Methylcellulose-Based

Medium containing only Epo. Of 200 plated (0 hour control) lineage negative cells expressing Vector, JAK2 or JAK2 V617F, one half (100 cells) formed CFU-E colonies and thus were CFU-Es. Although cultures expressing only Vector or JAK2 exhibited no CFU-Es after 24 hours, erythroid progenitors expressing JAK2-V617F exhibited a four-fold increase in numbers of colony-forming CFU-Es after 24 hours. (h) Micrographs of typical erythroid colonies generated by zero-hour erythroid progenitors expressing only Vector or JAK2 or JAK2 V617F, as well as JAK2-V617F expressing progenitors after 24 hours of culture. Scale bar is 10 μ m

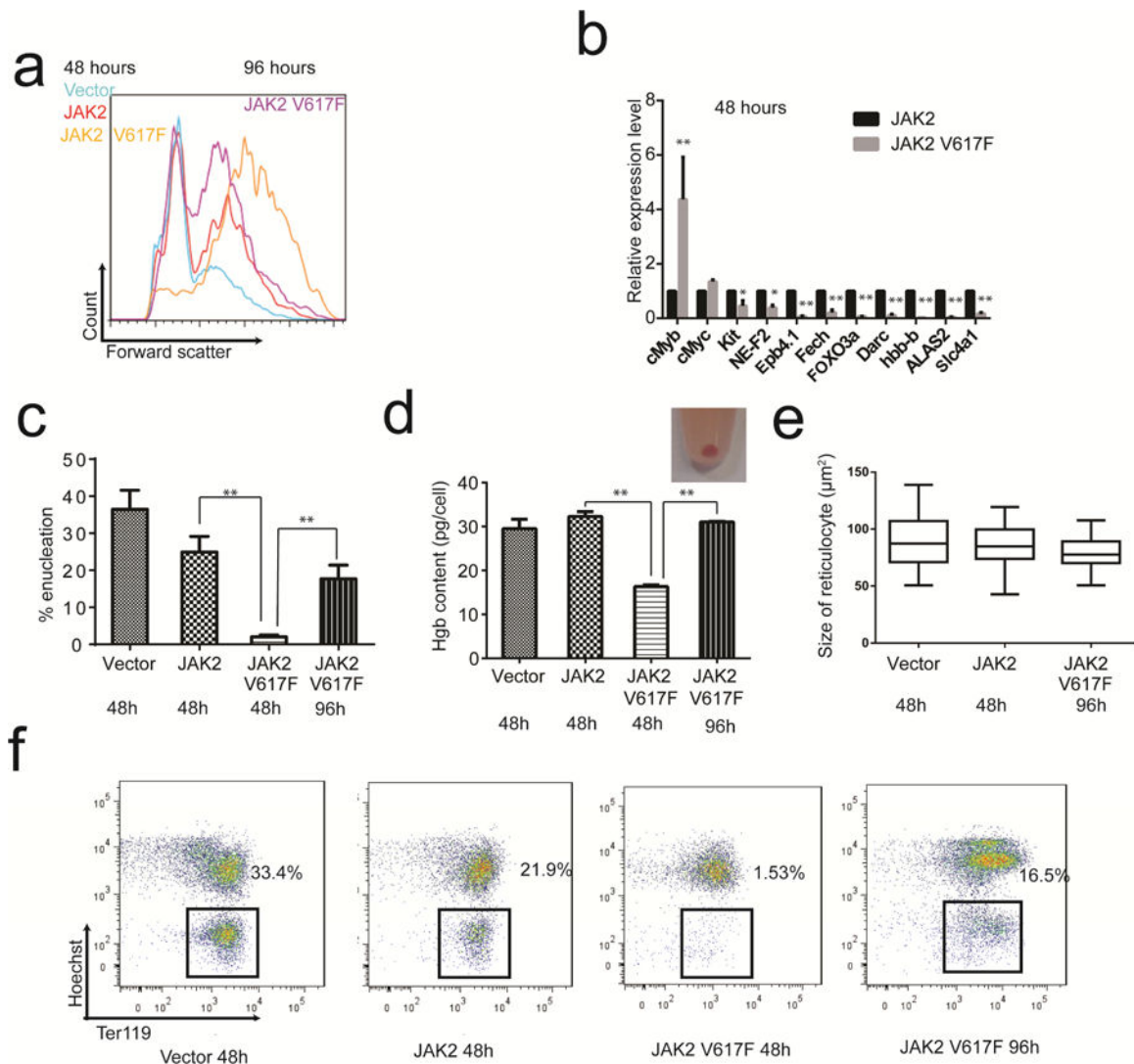


Figure 2. Erythroid progenitors expressing JAK2 V617F require a longer time to differentiate into normal reticulocytes as characterized by several criteria

At 48 hours of differentiation, erythroid progenitors expressing JAK2 V617F were undifferentiated compared to normal. (a) The size of JAK2 V617F-expressing cells at 48 hr. was larger than those of control vector or JAK2 cells, while the size of JAK2 V617F expressing cells at 96 hours was similar to that of control cells at 48 hr, as measured by forward scatter flow cytometry. (b) After 48 hr of culture JAK2 V617F-expressing cells also had lower erythroid-specific gene expression levels than JAK2 cells, as analyzed by quantitative PCR.

At 96 hour of differentiation, JAK2 V617F erythroid progenitors had differentiated to mature reticulocytes by several criteria. (c) JAK2 V617F erythroblasts had undergone enucleation, although the extent of enucleation was slightly lower than Vector and JAK2-expressing erythroblasts at 48 h. (d) Hemoglobin content per cell of total JAK2 V617F-expressing erythroblasts was similar to that of Vector or JAK2-expressing erythroblasts at 48 hr, which was ~ 31 pg/cell. The cell pellet of 1.5 million JAK2 V617F erythroblasts at 96 hours was red, as shown. (e) The size of JAK2 V617F reticulocytes formed at 96 hr was

similar to that of Vector and JAK2- expressing cells at 48 hr. (f) Flow cytometry measurement of enucleation of erythroblasts expressing Vector, JAK2 or JAK2 V617F at 48 h, and JAK2 V617F at 96 h are shown. (*: $p < 0.05$; **: $p < 0.01$).

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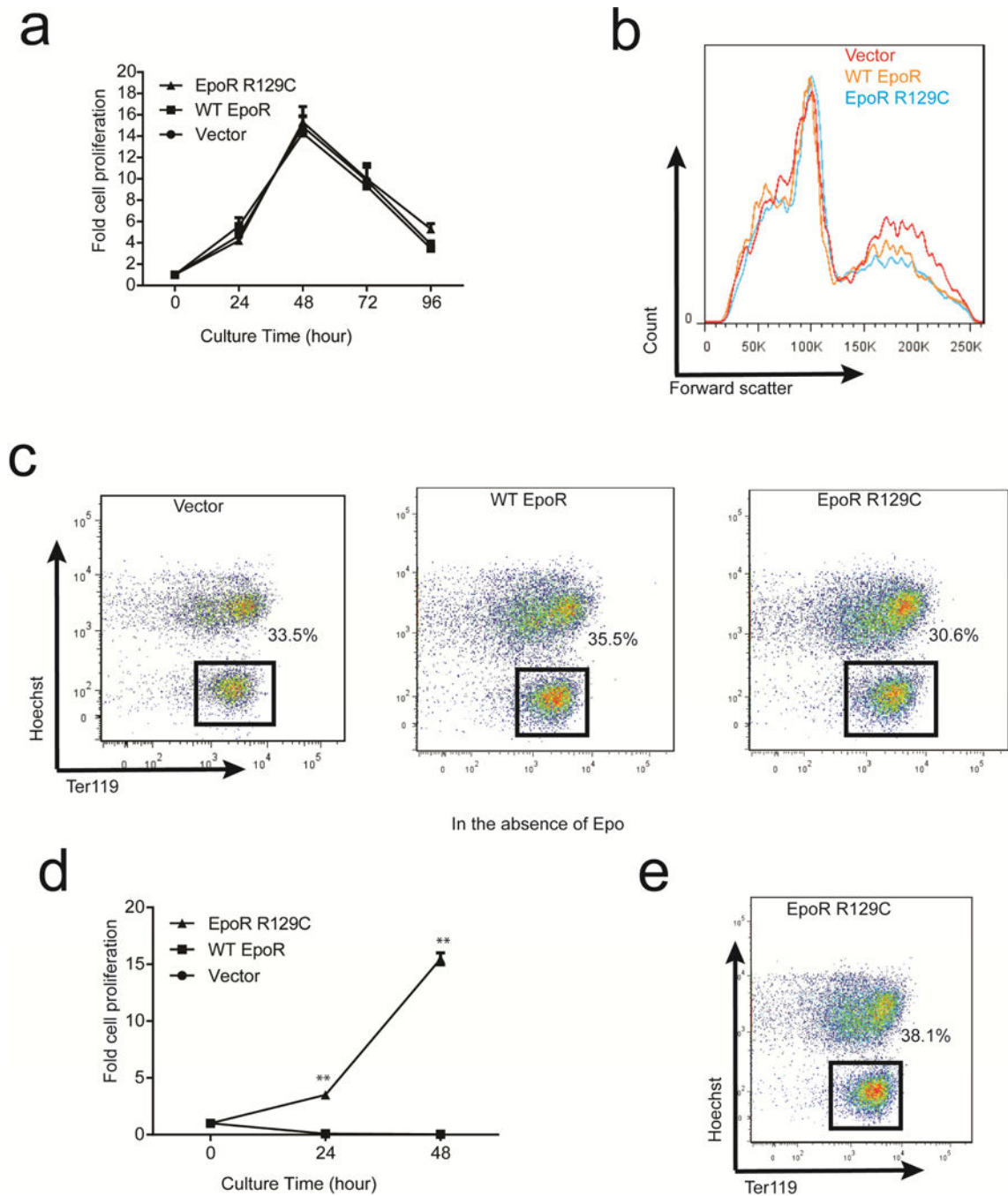


Figure 3. Expression of mutant Epo receptor R129C did not delay erythroid differentiation
 In the presence of Epo, erythroid progenitors expressing EpoR R129C underwent proliferation (a) and enucleation at 48 hr (c) at almost the same rate as those expressing Vector or wild-type (WT) EpoR. Importantly, the size of progenitors and enucleated reticulocytes expressing EpoR R129C at 48 hr of culture are similar to those of cells expressing Vector or WT EpoR (b), as measured by flow cytometry forward scatter. In the absence of Epo, only erythroid progenitors expressing EpoR R129C underwent proliferation (d) and enucleation (e) after 48 hr culture. (**: $p < 0.01$).

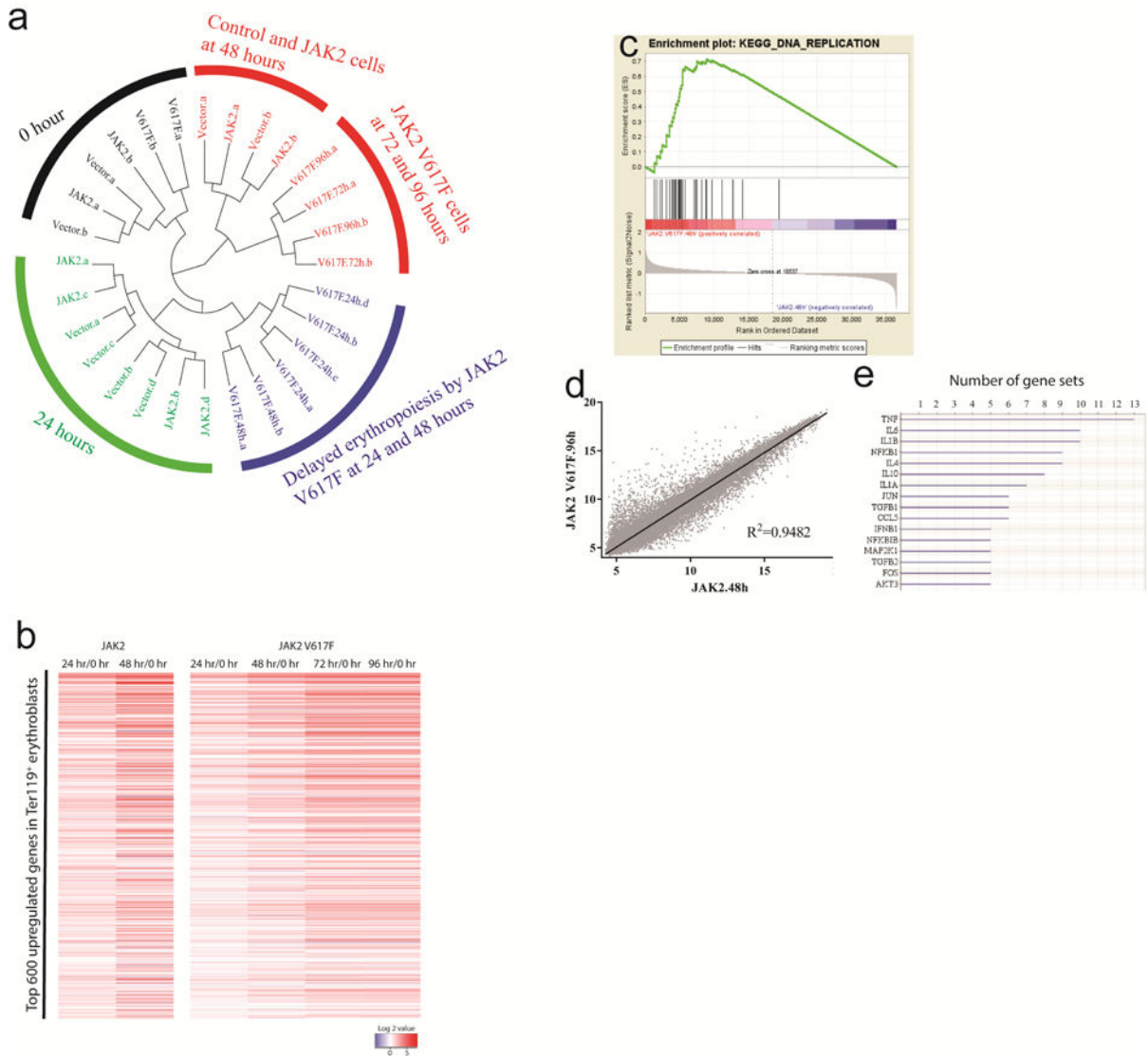


Figure 4. Microarray analysis of erythroid progenitors expressing Vector, JAK2 and JAK2 V617F undergoing terminal differentiation

(a) Hierarchical clustering of global gene expression profiles for JAK2- expressing, JAK2 V617F- expressing, and control cells during terminal erythropoiesis. (b) Expression of top 600 genes upregulated in normal Ter119⁺ erythroblasts, relative to expression at 0 hour, in cells expressing either wild type JAK2 or JAK2 V617F. Induction of these genes is delayed in cells expressing JAK2 V617F. Color intensity indicates Log₂ fold changes. (c) Enrichment plot of gene set “DNA replication” between JAK2 and JAK2 V617F cells at 48 hours. (d) Log₂ expression value of 36,551 mRNAs in JAK2 cells at 48 hours and JAK2 V617F cells at 96 hours are plotted on a scatter plot with linear regression shown in black. (e) Leading edge analysis on top 20 gene sets significantly enriched in JAK2 V617F cells compared with JAK2 cells at 0 hour, showing the most common genes in the top gene sets.

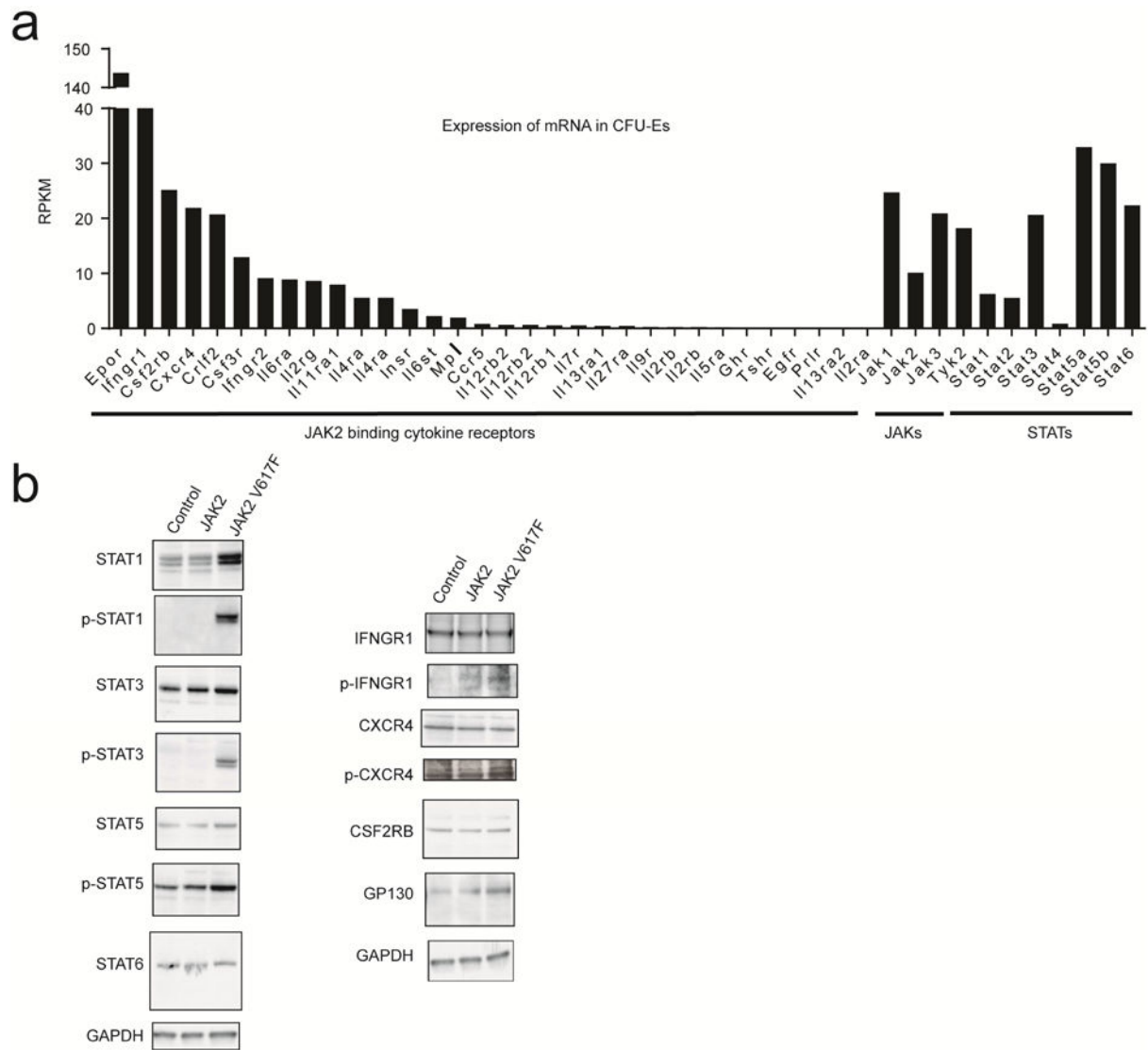


Figure 5. Activation of non-erythroid signaling pathways in erythroid progenitors expressing JAK2 V617F

(a) mRNAs encoding several cytokine receptors additional to EpoR, other JAKs additional to JAK2 and other STATs additional to STAT5 are highly expressed in purified murine fetal liver CFU-Es, characterized by Ter119 low and CD71 high. Data from Wong et. al.(25) (b) Western blots show expression of STAT1, STAT3, STAT5 and STAT6 in control, JAK2-expressing, and JAK2 V617F-expressing erythroid progenitors at 2.5 hours after Epo stimulation. Strikingly, hyper expression and activation (phosphorylation) of STAT1, STAT3 and STAT5 were only found in progenitors expressing JAK2 V617F. Moreover, Western blots show expression of several cytokine receptors, including Glycoprotein 130 (GP130), cytokine receptor common beta chain (Csf2rb), Interferon gamma receptor 1(IFNGR1) and chemokine (C-X-C motif) receptor 4 (CXCR4) in erythroid progenitors at 2.5 hours culture after Epo stimulation. Importantly, phosphorylation of IFNGR1 and CXCR4 were only found in progenitors expressing JAK2 V617F. GAPDH was used as loading control.

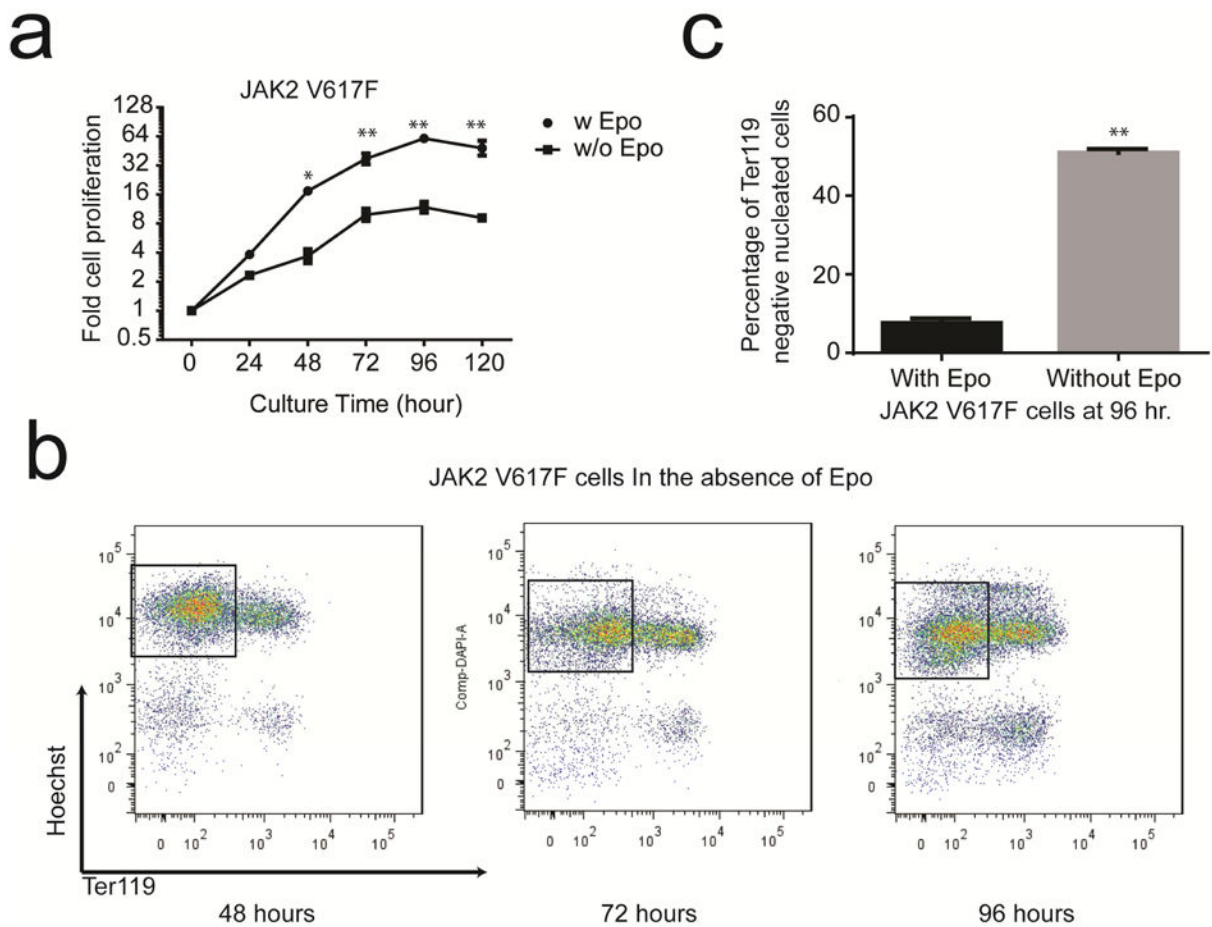
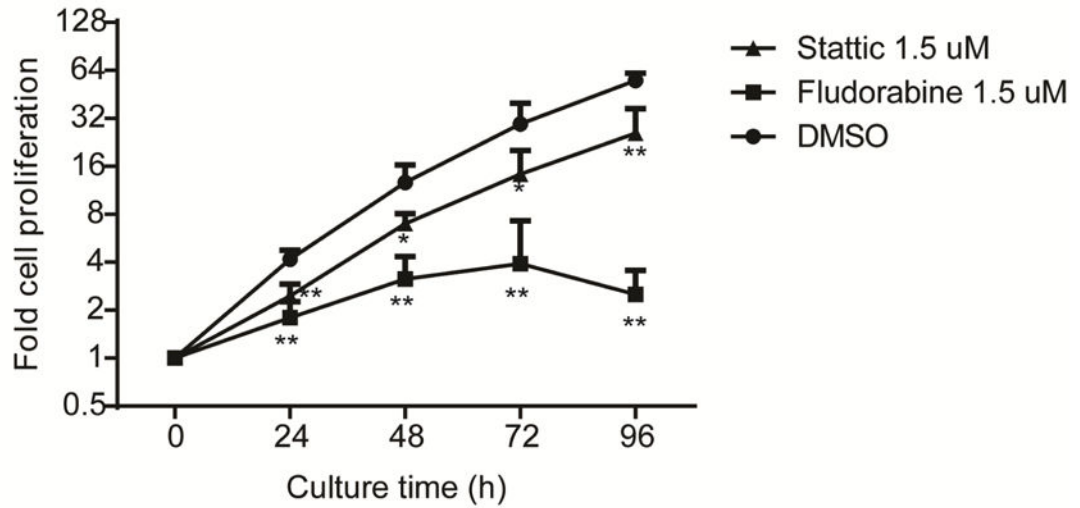


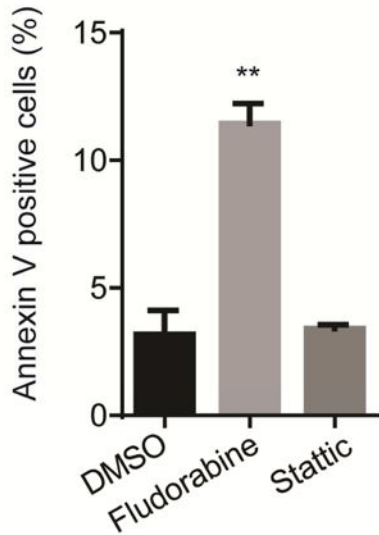
Figure 6. In the absence of Epo erythroid progenitors expressing JAK2 V617F proliferate slower and do not differentiate

Without Epo, the proliferation rate of erythroid progenitors expressing JAK2 V617F is significantly lower than that in the presence of Epo (a). At 48, 72 and 96 hours, JAK2 V617F cells cultured without Epo were stained with Hoechst and Ter119 antibody followed by flow cytometry showing the non-erythroid cells did not undergo enucleation, in contrast to JAK2 V617F- expressing cells cultured in the presence of Epo. The non-erythroid nucleated Ter119 negative cells were gated by black boxes (b). At 96 hours of differentiation without Epo, the percentage of Ter119 negative nucleated cells was significantly higher than in cultures with Epo. (**: $p < 0.01$).

a



b



c

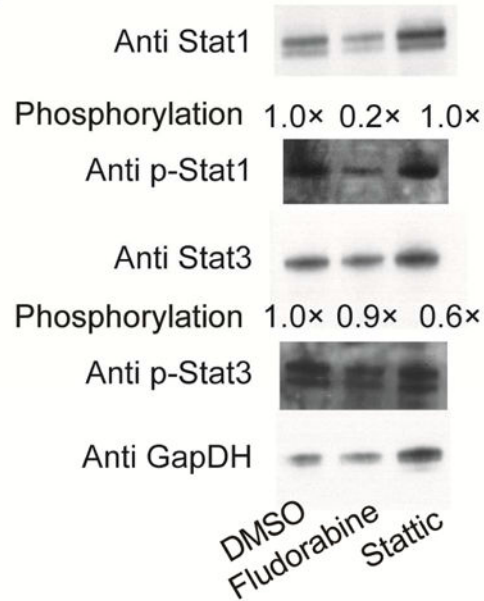


Figure 7. Inhibition of Stat1 disrupted the erythroid hyper-proliferation of cells expressing JAK2 V617F

(a) A Stat1 inhibitor, Fludorabine, blocked the proliferation of cells expressing JAK2 V617F, leading to a significant decrease in cell numbers during differentiation, while the Stat3 inhibitor, Stattic, only moderately disturbed the proliferation of JAK2 V617F cells, comparing to control cells treated with DMSO. (b) Compared with cells treated with DMSO and Stattic, more cells treated with Fludorabine were Annexin V positive, suggesting more cells underwent apoptosis. (c) Western blots showed the Stat1 phosphorylation decreased in cells treated with Fludorabine, while Stat3 phosphorylation was also slightly reduced in cells

treated with Stattic, comparing to cells treated with DMSO. Western blot quantifications were done by Image J. The degree of Stat1 and Stat3 phosphorylation were represented by the ratios of pStat1 to Stat1 and pStat3 to Stat3, respectively, which were normalized to those in cells treated with DMSO (set to 1.0). (*: $p < 0.05$; **: $p < 0.01$).

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Table 1

Gene sets enriched in JAK2-V617F cells compared with JAK2 cells

| | 0 hour | 24 hours | 48 hours |
|----|--|--|---|
| 1 | CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION | CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION | DNA_REPLICATION |
| 2 | JAK_STAT_SIGNALING_PATHWAY | TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY | SPLICEOSOME |
| 3 | CYTOSOLIC_DNA_SENSING_PATHWAY | CYTOSOLIC_DNA_SENSING_PATHWAY | PYRIMIDINE_METABOLISM |
| 4 | TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY | RIG_I_LIKE_RECEPTOR_SIGNALING_PATHWAY | RNA_DEGRADATION |
| 5 | RIG_I_LIKE_RECEPTOR_SIGNALING_PATHWAY | NOD_LIKE_RECEPTOR_SIGNALING_PATHWAY | BASE_EXCISION_REPAIR |
| 6 | GRAFT_VERSUS_HOST_DISEASE | JAK_STAT_SIGNALING_PATHWAY | MISMATCH_REPAIR |
| 7 | LEISHMANIA_INFECTION | LEISHMANIA_INFECTION | HOMOLOGOUS_RECOMBINATION |
| 8 | ALLOGRAFT_REJECTION | CELL_ADHESION_MOLECULES_CAMS | RIBOSOME |
| 9 | INTESTINAL_IMMUNE_NETWORK_FOR_IIGA_PRODUCTION | APOPTOSIS | RNA_POLYMERASE |
| 10 | EPITHELIAL_CELL_SIGNALING_IN_HELICOBACTER_PYLORI_INFECTION | PROTEASOME | NUCLEOTIDE_EXCISION_REPAIR |
| 11 | PRION_DISEASES | PATHOGENIC_ESCHERICHIA_COLI_INFECTION | SELENOAMINO_ACID_METABOLISM |
| 12 | TYPE_I_DIABETES_MELLITUS | TYPE_I_DIABETES_MELLITUS | DRUG_METABOLISM_OTHER_ENZYMES |
| 13 | AUTOIMMUNE_THYROID_DISEASE | GRAFT_VERSUS_HOST_DISEASE | PROTEASOME |
| 14 | PROTEASOME | NATURAL_KILLER_CELL_MEDIATED_CYTOTOXICITY | TYROSINE_METABOLISM |
| 15 | COMPLEMENT_AND_COAGULATION_CASCADES | ASTHMA | GRAFT_VERSUS_HOST_DISEASE |
| 16 | MAPK_SIGNALING_PATHWAY | EPITHELIAL_CELL_SIGNALING_IN_HELICOBACTER_PYLORI_INFECTION | HISTIDINE_METABOLISM |
| 17 | NOD_LIKE_RECEPTOR_SIGNALING_PATHWAY | PHENYLALANINE_METABOLISM | CYTOSOLIC_DNA_SENSING_PATHWAY |
| 18 | CELL_ADHESION_MOLECULES_CAMS | MAPK_SIGNALING_PATHWAY | AMINOACYL_TRNA_BIOSYNTHESIS |
| 19 | ASTHMA | TYROSINE_METABOLISM | NOD_LIKE_RECEPTOR_SIGNALING_PATHWAY |
| 20 | T_CELL_RECEPTOR_SIGNALING_PATHWAY | PRION_DISEASES | RENIN_ANGIOTENSIN_SYSTEM |
| | | HISTIDINE_METABOLISM | BASAL_TRANSCRIPTION_FACTORS |
| | | T_CELL_RECEPTOR_SIGNALING_PATHWAY | ASTHMA |
| | | FC_GAMMA_R_MEDIATED_PHAGOCYTOSIS | GLYCOLYSIS_GLUCCONEGENESIS |
| | | FC_EPSILON_RI_SIGNALING_PATHWAY | ARGININE_AND_PROLINE_METABOLISM |
| | | COMPLEMENT_AND_COAGULATION_CASCADES | PEROXISOME |
| | | ALLOGRAFT_REJECTION | ALLOGRAFT_REJECTION |
| | | ANTIGEN_PROCESSING_AND_PRESENTATION | CYSTEINE_AND_METHIONINE_METABOLISM |
| | | HEMATOPOIETIC_CELL_LINEAGE | GLYCINE_SERINE_AND_THREONINE_METABOLISM |
| | | INTESTINAL_IMMUNE_NETWORK_FOR_IIGA_PRODUCTION | PURINE_METABOLISM |
| | | ERBB_SIGNALING_PATHWAY | |
| | | RNA_DEGRADATION | |
| | | REGULATION_OF_ACTIN_CYTOSKELETON | |

