Distinct Clinical Phenotypes Associated with *JAK2V617F* Reflect Differential STAT1 Signaling

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SUMMARY

The *JAK2V617F* mutation is associated with distinct myeloproliferative neoplasms, including polycythemia vera (PV) and essential thrombocythemia (ET), but it remains unclear how it generates disparate disorders. By comparing clonally-derived mutant and wild-type cells from individual patients, we demonstrate that the transcriptional consequences of *JAK2V617F* are subtle, and that *JAK2V617F*-heterozygous erythroid cells from ET and PV patients exhibit differential interferon signaling and STAT1 phosphorylation. Increased STAT1 activity in normal CD34-positive progenitors produces an ET-like phenotype, whereas downregulation of STAT1 activity in *JAK2V617F*-heterozygous ET progenitors produces a PV-like phenotype. Our results illustrate the power of clonal analysis, indicate that the consequences of *JAK2V617F* reflect a balance between STAT5 and STAT1 activation and are relevant for other neoplasms associated with signaling pathway mutations.

INTRODUCTION

The myeloproliferative neoplasms (MPNs) are hematological malignancies characterized by a chronic clinical course and a risk of thrombosis and transformation to acute leukemia. These disorders are an attractive model for understanding the earliest stages of cancer development because many patients present at an early stage with an incidental abnormal blood count, the landscape of causative somatic mutations underpinning the disorders is increasingly well understood and tumor cells can be grown and differentiated in vitro from single progenitor cells. The two most common MPNs are polycythemia vera (PV), in which patients present with a raised red cell mass sometimes associated with increased platelet and white cell counts, and essential thrombocythemia (ET), which is defined by an elevated platelet count but normal red cell mass.

The MPNs result from transformation of a multipotent hematopoietic progenitor (Adamson et al., 1976; Delhommeau et al., 2007; Fialkow et al., 1981; Jamieson et al., 2006). In 95% of patients with PV and 60% of those with ET, an identical somatically acquired mutation is found in the tyrosine kinase, *JAK2* (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005a; Levine et al., 2005). This V617F mutation results in dysregulated kinase activity of JAK2 and produces ligand-independent activation of receptor signaling in cytokine-dependent cell lines. It has been suggested that expression of a homodimeric type I cytokine receptor provides a scaffold necessary for optimal signaling by mutant JAK2 (Lu et al., 2008; Lu et al., 2005) and several signaling cascades are activated by mutant JAK2, including the STAT5, MAPK, and PI3K pathways (James et al., 2005; Kralovics et al., 2005a; Laubach et al., 2009; Levine et al., 2005; Oku et al., 2010). Of these, the STAT5 pathway

Significance

Our results reveal a central role for STAT1 activation in the pathogenesis of *JAK2V617F*-positive MPNs and indicate that the phenotypic consequences of *JAK2V617F* reflect a balance between hematopoietic effects of STAT1 and STAT5 activation. In addition to providing a molecular explanation for this long-standing conundrum, several aspects of our data are of general relevance: (1) the transcriptional consequences of *JAK2V617F* are less than normal interindividual variation, a finding with implications for other somatic mutations; (2) tumors harboring an identical mutation can exhibit strikingly different activity of associated signaling pathways, an observation with implications for targeted therapy; and (3) our results demonstrate the power of comparing clonally-derived mutant and wild-type cells obtained from the same patient and grown under identical conditions.
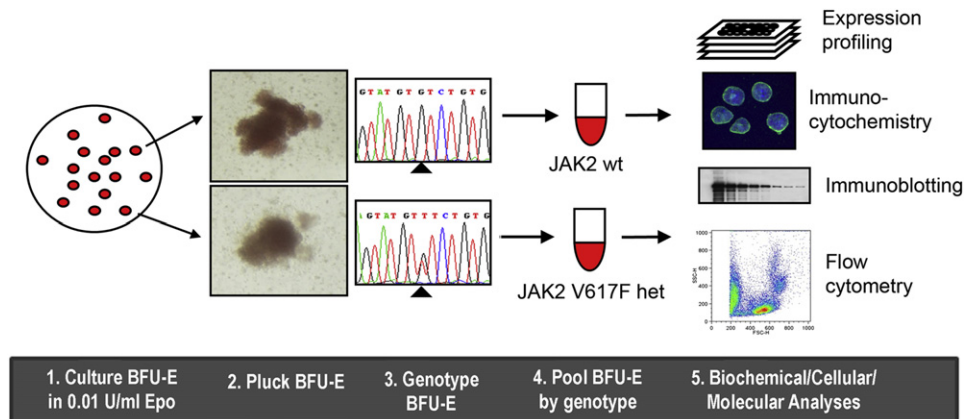
Figure 1. Strategy for Analysis of Paired Normal and *JAK2V617F* Samples from MPN Patients

BFU-E colonies were grown from 20 ET and 16 PV patients in methylcellulose media supplemented with 0.01 U/ml erythropoietin, individually plucked and genotyped for *JAK2V617F* mutation burden. Up to 20 wild-type and V617F-heterozygous colonies were pooled and subjected to further analysis. See also Figure S1.

has been shown to be necessary and sufficient for at least some aspects of the MPN phenotype in vitro and in vivo (Funakoshi-Tago et al., 2010; Garcon et al., 2006; Grebien et al., 2008; Ilaria et al., 1999). *Drosophila* studies have also revealed a noncanonical pathway by which JAK2 regulates chromatin (Shi et al., 2006; Shi et al., 2008) and a direct nuclear function for JAK2 as a histone H3 kinase has recently been reported (Dawson et al., 2009). The *JAK2V617F* mutation is sufficient to cause an MPN phenotype in mouse models (Akada et al., 2010; Lacout et al., 2006; Li et al., 2010; Marty et al., 2010; Mullally et al., 2010; Tiedt et al., 2008; Wernig et al., 2006; Xing et al., 2008; Zaleskas et al., 2006) and, taken together, existing data demonstrate that mutation of *JAK2* plays a central and early role in MPN pathogenesis.

One fundamental, unresolved question is how the same mutation in *JAK2* can give rise to phenotypically distinct disorders such as ET and PV. Homozygosity for the mutation is common in *JAK2V617F*-positive PV but rare in *JAK2V617F*-positive ET (Scott et al., 2006), thus raising the possibility that increased signaling through *JAK2V617F* may be responsible for the PV phenotype. However, attempts to identify different signaling consequences in ET and PV have not yielded consistent results using western blotting (Heller et al., 2006), immunohistochemistry (Grimwade et al., 2009; Teofili et al., 2007), or expression profiling (Berkofsky-Fessler et al., 2010; Goerttler et al., 2005; Kralovics et al., 2005b; Laubach et al., 2009; Pellagatti et al., 2003; Puigdecanet et al., 2008; Slezak et al., 2009; Tenedini et al., 2004). This is likely to reflect several issues that complicate interindividual comparisons including constitutional variation in transcript levels among normal individuals (Stranger et al., 2005; Stranger et al., 2007), the widely varying proportions of wild-type and mutant clones present in samples obtained from different patients (Dupont et al., 2007; Scott et al., 2006) and treatment differences across patient cohorts. Such difficulties undoubtedly confound similar laboratory studies of other cancers as well, but are generally difficult to identify or quantify and have therefore often not been formally evaluated.

To circumvent these issues, we took advantage of the tractable clinical and laboratory features of MPNs to devise a strategy based on analysis of clonally-derived cells genotyped for *JAK2* mutation status. This approach allows comparison of pure populations of phenotypically equivalent mutant and wild-type cells grown from the same patient under identical conditions, and has allowed us to address the mechanisms responsible for the different phenotypic consequences of the *JAK2* mutation in ET and PV.

RESULTS

JAK2V617F-Associated Transcriptome Changes Are Less Marked than Those Due to Interindividual Differences and Can Be Identified by Clonal Analysis

In patients with MPNs, erythroid colonies containing thousands of cells can be grown by in vitro culture in semisolid media. Each colony represents the in situ expansion of a single erythroid progenitor cell and so reflects the genetic landscape of the initiating cell. Because *JAK2* mutant and wild-type progenitor cells coexist in the peripheral blood of patients with ET and PV, colonies from both genotypes can be grown in the same experiment, individually picked and typed for presence or absence of the V617F mutation, pooled by genotype, and studied for phenotypic differences (Figure 1). This strategy enables direct comparison of mutant and wild-type cells within a patient, thereby controlling for differences in age, sex, treatment, germline genetic background, experimental factors, and other confounding variables. Laboratory assays that can be applied to phenotype such colonies are flexible and wide-ranging: in this study, we present results of transcriptional profiling, conventional and intracellular flow cytometry, western immunoblotting, as well as immunohistochemistry.

A total of 5302 BFU-E colonies from 36 MPN patients (20 ET and 16 PV; Table S1 available online) were grown, collected, genotyped, and pooled based on presence or absence of the *JAK2V617F* mutation. For each patient a pool of *JAK2V617F*-heterozygous erythroid colonies was compared to a pool of *JAK2V617F*-negative colonies using gene expression microarrays. We chose to study erythroid cells because it is the presence or absence of a raised red cell mass that is the key distinction between PV and ET. The high erythropoietin

concentrations normally used in erythroid colony assays may mask transcriptional consequences of the *JAK2* mutation, and so colonies were grown in 0.01 U/ml erythropoietin, a concentration shown to maximize the difference in expression of a known JAK/STAT target (*PIM1*) between mutant and wild-type colonies (Figure S1A). Comparison of colonies with and without the *JAK2V617F* mutation revealed no overt differences in colony morphology or size (Figure S1B), and no detectable differences in the degree of erythroblast differentiation (assessed by GPA and CD71 expression and morphological analysis of cytospins) (Figures S1C and S1D), demonstrating that colonies from the two genotypes represented comparable stages of erythroid development. Gene set enrichment analysis did not show any enrichment of erythroid differentiation genes among genes upregulated in mutant colonies compared to wild-type colonies (p = 0.2) or among mutant PV colonies compared to mutant ET colonies (p = 0.19).

Unsupervised clustering analysis of expression profiles from the two classes of colony demonstrated that *JAK2* mutant colonies were more closely related to wild-type colonies from the same patient than to mutant colonies from other patients (Figure 2A). There was no clustering based on other parameters, such as diagnosis, therapy, age, or gender. To ensure that this pattern of clustering did not reflect simultaneous processing of paired samples from a given patient, repeat blood samples were drawn from four individuals and subjected to the entire process ab initio. The resulting expression profiles still revealed close clustering between samples derived from the same patient, with samples of the same genotype from the same patient clustering closer yet (Figure S2A). Moreover, the ratio of gene expression between the V617F-heterozygous and wild-type colonies for all genes revealed strong concordance in experiments carried out independently (Figure S2B). These results demonstrate first that variability attributable to interassay differences was minimal, and second that the transcriptional consequences of the *JAK2* mutation are less marked than normal interindividual variation.

To identify V617F-associated genes common to both PV and ET, expression profiles derived from all 36 patients were examined to identify genes consistently dysregulated in V617F-heterozygous colonies relative to autologous wild-type colonies. After adjustment for age, gender, and therapy, and controlling the false discovery rate (FDR) at 10% for multiple hypothesis testing, expression levels of 201 genes were increased in V617F-heterozygous cells and those of 22 genes were reduced (minimum fold change, 1.3; p values ≤ 0.0034; Figures S2C and S2D). Gene set enrichment analysis (GSEA) was applied to a preranked gene list ordered according to the significance with which they were differentially expressed in mutant and wild-type colonies. Components of the erythropoietin signaling pathway (net enrichment score [NES] = 1.51; q = 0.02) and previously reported targets of STAT5A signaling (NES = 1.40; q = 0.09) (Olthof et al., 2008) were enriched among genes upregulated in mutant colonies (Figure 2B). Array results from individual patients for known JAK/STAT targets (*PIM1* and *CISH*) showed upregulation associated with presence of the JAK2 mutation in most (but not all) patients and were confirmed by quantitative RT-PCR in at least five PV and five ET patients (Figure 2C). Consistent with these data, nuclear pSTAT5 was increased in *JAK2V617F*-

heterozygous but not wild-type erythroblasts from both PV and ET patients (Figure 2D).

Together these data demonstrate that the effect of the V617F mutation on the transcriptome is surprisingly subtle, and less than the influence of interindividual variation. However, comparison of clonally-derived mutant and wild-type cells from each patient permits identification of mutation-associated expression changes.

JAK2V617F-Heterozygous Erythroid Cells from ET and PV Patients Exhibit Differential Interferon Signaling and STAT1 Phosphorylation

We next investigated whether gene expression changes associated with *JAK2V617F*-heterozygous erythroid colonies were the same in patients with ET and PV. After adjustment for potential confounders including age, gender, and therapy, a linear mixed effects (LME) modeling algorithm was applied to the data set to identify genes for which a significant interaction existed between *JAK2* mutation status and MPN subtype (i.e., ET or PV). This type of analysis has the advantage of allowing for correction of interindividual variability without the need to explicitly model it (Li et al., 2004). Controlling the FDR at 10%, a total of 171 genes exhibited significant interaction, all of which fell into one of four basic patterns of gene behavior (Figure S3A): (1) genes upregulated in *JAK2* mutant erythroblasts specifically in ET but not PV (n = 83); (2) genes downregulated in *JAK2* mutant erythroblasts specifically in ET but not PV (n = 21); (3) genes upregulated in *JAK2* mutant erythroblasts specifically in PV but not ET (n = 40); and (4) genes downregulated in *JAK2* mutant erythroblasts specifically in PV but not ET (n = 24) (p ≤ 0.0028). A hierarchical clustering showing these combined 171 genes and their ability to subdivide the PV patients from the ET patients is shown in a heat map (Figure 3A). Linear discriminant analysis and leave-one-out cross-validation using the 12 most statistically significant genes resulted in correct classification of 33/36 (92%) of the patients; an identical classification rate was determined using the Stanford PAMR software. Taken together, these data demonstrate cell-intrinsic differences in *JAK2V617F*-heterozygous erythroblasts from PV and ET.

To investigate pathways associated with JAK2V617F expression in ET and PV, GSEA was applied to the entire gene list ranked in order from those "most significantly up-regulated in association with *JAK2V617F* in ET, compared to PV" to those "most significantly up-regulated in association with *JAK2V617F* in PV, compared to ET." At a FDR cut-off of 20%, 23 gene sets were enriched for genes upregulated in ET, of which seven were related to interferon signaling whereas 0/25 gene sets enriched for genes upregulated in PV were interferon related (Figure 3B, Table S2). We then looked for interferon-regulated genes among the genes significantly upregulated by JAK2V617F in an ET-selective or in a PV-selective manner. Interferon regulated genes were defined by their presence in the Interferome database (Samarajiwa et al., 2009), a group of manually curated gene sets comprising known IFNα and IFNγ target genes. Of the 83 genes upregulated in an ET-selective manner, 26 were IFN targets (8 IFNγ targets, 2 IFNα targets, and 16 targets of both). By contrast, only one of 40 genes upregulated in a PV-selective manner were targets of either interferon (Table S4).
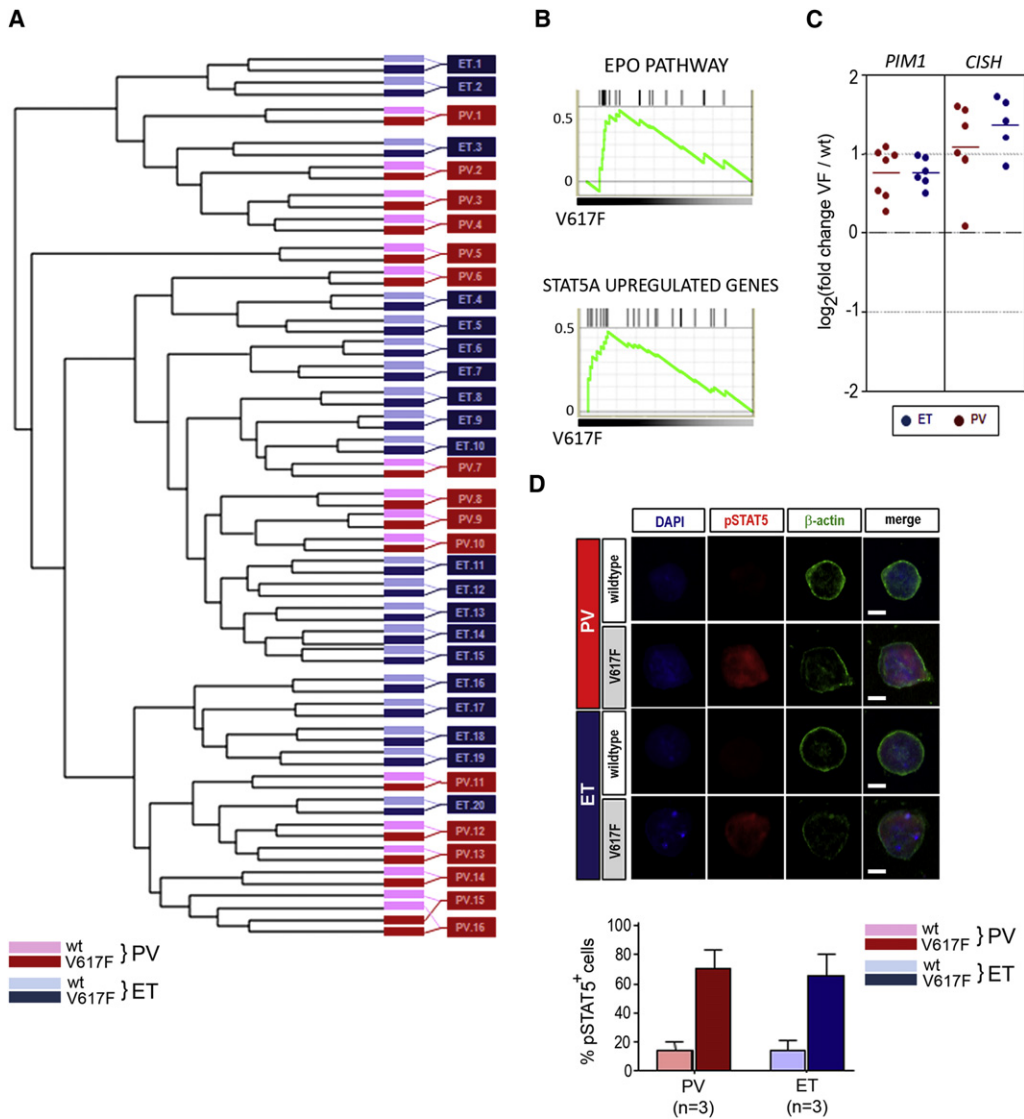
Figure 2. *JAK2V617F*-Associated Transcriptional Changes Are Less than Those Due to Interindividual Differences and Include Activation of STAT5 Signaling

(A) Dendrogram constructed from unsupervised hierarchical clustering of all 72 data sets (paired wild-type and V617F-heterozygous data sets from 20 ET and 16 PV patients) using Pearson correlation. Data sets from PV patients (listed PV.1–PV.16) are depicted as light red for expression profiles from wild-type erythroblasts and dark red for expression profiles from V617F-heterozygous erythroblasts, with each patient connected by a line to their two-paired expression profiles. Data sets for ET patients (ET.1–ET.20) are similarly depicted as light blue for expression profiles from wild-type erythroblasts and dark blue for expression profiles from V617F-heterozygous erythroblasts.

(B) Gene set enrichment analysis for genes significantly associated with V617F mutation across all MPN patients. Enrichment was seen for gene sets comprised of components of the erythropoietin pathway and for *STAT5A* targets.

(C) qPCR validation of *PIM1* and *CISH* upregulation in V617F-heterozygous erythroblasts. Fold increase represents the ratio of gene expression in V617F-heterozygous compared to wild-type erythroblasts, with each data point representing an individual ET (blue) or PV (red) patient.

(D) Immunocytochemical staining of cytospins of wild-type and V617F-heterozygous BFU-E pools from PV and ET patients (left panel). Histograms show significantly higher numbers of cells positive for pY694-STAT5 in V617F-heterozygous colonies compared to wild-type colonies in both PV and ET (right panel). Scale bars represent 5 μm. Results represent the mean ± SD for three PV and three ET patients. See also Figure S2.

Real-time qPCR confirmed increased transcript levels of five known IFNγ target genes (*IFI44L*, *GBP2*, *IRF1*, *HLA-F*, *IFITM3*) in V617F-heterozygous relative to autologous wild-type erythroblasts in 10 ET patients but not in 9 PV patients (Figure 3C). These results demonstrate that JAK2V617F is associated with enhanced IFN signaling, predominantly involving the IFNγ

pathway, in erythroblasts from patients with ET but not in those from patients with PV.

Wild-type JAK2 binds the IFNRG1 component of the IFNγ heterodimeric receptor (Ihle, 1994; Parganas et al., 1998; Silvennoinen et al., 1993), and our results suggest that JAK2V617F enhances activity of this pathway in *JAK2* mutant erythroblasts
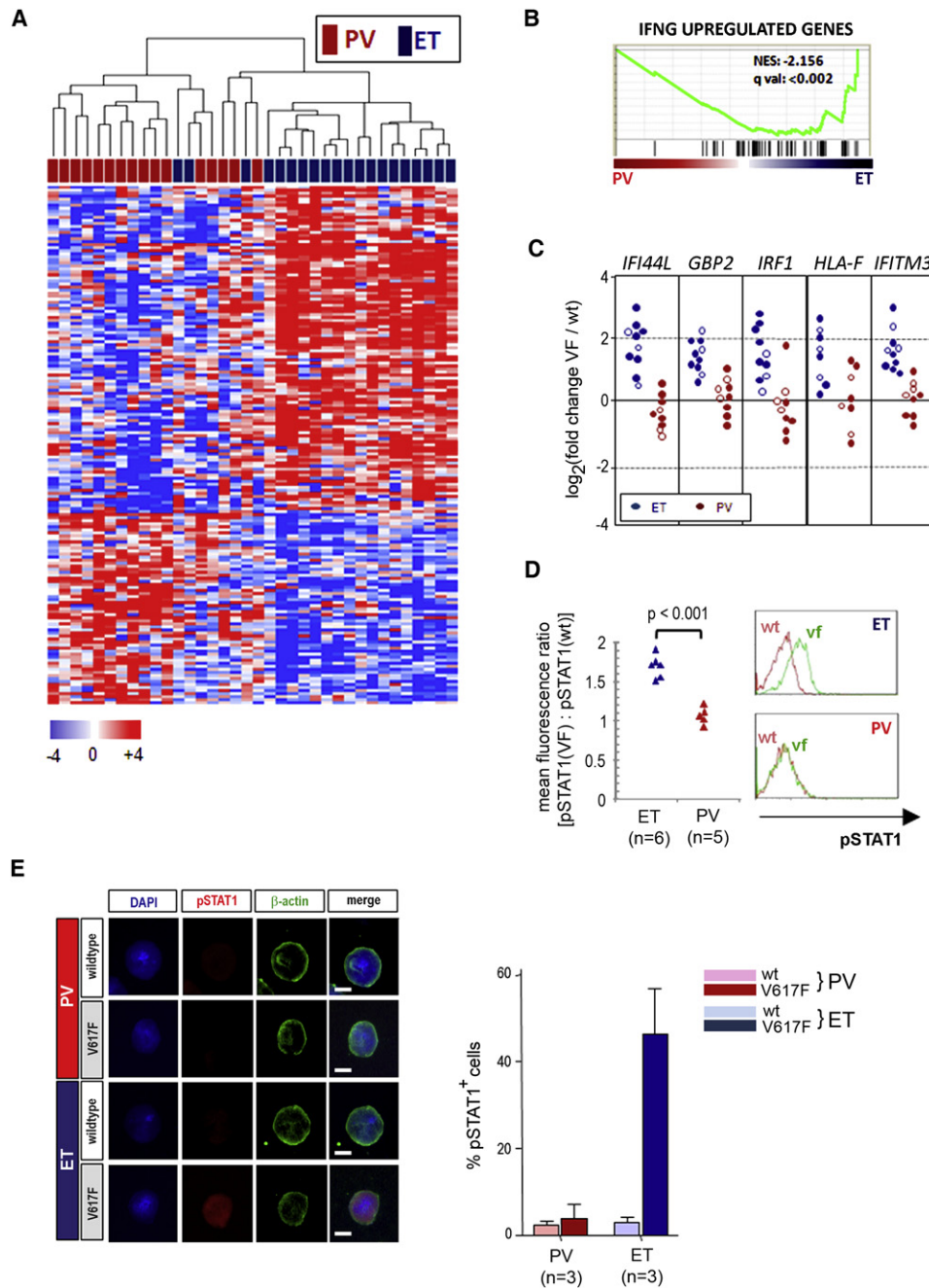
Figure 3. . Activation of Interferon Signaling and STAT1 Phosphorylation in *JAK2V617F*-Heterozygous Erythroblasts from Patients with ET but Not Those with PV

(A) Hierarchical clustering of V617F-associated expression profiles of all 36 patients according to the 171 genes that showed significant interaction between the V617F mutation and each disease class (i.e., PV or ET). Each gene is expressed as a ratio of expression in V617F-heterozygous to autologous wild-type sample. Bars at the top of the graph display disease phenotype for each patient (blue: ET; red: PV).

(B) GSEA demonstrates that targets of interferon signaling are enriched among genes upregulated by *JAK2V617F* in ET but not PV.

(C) Real-time quantitative RT-PCR analysis of five IFNγ target genes (*IFI44L*, *GBP2*, *IRF1*, *HLA-F*, *IFITM3*) in V617F-heterozygous and wild-type colonies from ET patients (n = 10) and PV patients (n = 9). Fold increase represents the ratio of gene expression in V617F-heterozygous relative to autologous wild-type erythro-blasts, with each data point representing an individual ET (blue) or PV (red) patient. Closed points represent patients used in the gene expression profiling; open points represent a patient from an independently derived validation cohort.

(D) Intracellular flow cytometry shows elevated expression of pY701-STAT1 in V617F-heterozygous BFU-E colonies relative to autologous wild-type colonies from ET patients (n = 6), and not in PV patients (n = 5).

(E) pY701-STAT1 staining of cytospins of wild-type and V617F-heterozygous BFU-E pools from PV and ET patients (left panel). Histogram depicts increase in percentage of cells positive for pSTAT1 in V617F-heterozygous colonies from ET patients only compared to PV (right panel). Scale bars represent 5 μm. Results represent the mean ± SD for three PV and three ET patients. See also Figure S3.

from ET patients. Indeed, relative to expression of wild-type JAK2, expression of JAK2V617F in γ2A cells (which lack endogenous JAK2) resulted in increased STAT1 phosphorylation on the tyrosine-701 residue (pSTAT1) in response to IFNγ (Figure S3B). Similar results were obtained using 293T cells (Figure S3C). These data demonstrate that, analogous to its well-documented effects in conferring cytokine hypersensitivity on type I homodimeric cytokine receptors (Lu et al., 2008; Lu et al., 2005), JAK2V617F also enhances signaling from IFNγ receptors.

To investigate the mechanisms responsible for the striking lack of interferon signaling in *JAK2V617F*-heterozygous erythroblasts from PV patients, we focused on STAT1 because it is essential for IFNγ receptor signaling (Gough et al., 2010). Levels of pSTAT1 were measured in mutant and wild-type erythroblasts from ET and PV patients using intracellular flow cytometry. Relative to autologous erythroblasts, elevated expression of pSTAT1 was seen in *JAK2V617F*-heterozygous erythroblasts from all six ET patients tested but not in any of five PV patients (Figure 3D). Consistent with these results, pSTAT1 was detected by immunofluorescence in the nucleus of JAK2 mutant erythroblasts from ET but not PV patients (Figure 3E). Immunoblotting revealed no differences in total STAT1 levels between mutant and wild-type erythroblasts in either disease (Figure S3D).

These data demonstrate that *JAK2V617F*-heterozygous PV erythroblasts lack detectable pSTAT1 and therefore provide a mechanism for the differential activation of IFN signaling pathways in ET and PV.

Increased STAT1 Activity Produces an ET-Like Phenotype with Enhanced Megakaryocytic Differentiation and Restrained Erythroid Differentiation

The results described above raised the possibility that differences in the level of STAT1 activity may contribute to the distinct biological features of ET and PV. ET is characterized by increased megakaryopoiesis whereas the dominant feature of PV is enhanced erythropoiesis. We therefore assessed the effect of altering STAT1 activity on megakaryocytic and erythroid differentiation.

Lentiviral constructs were used to express wild-type or constitutively active STAT1 (STAT1C) (Liddle et al., 2006) in the hematopoietic progenitor cell line K562, which was then induced to undergo megakaryocytic or erythroid differentiation by exposure to phorbol 12-myristate 13-acetate (PMA) or hemin respectively. Transduction efficiencies for all constructs were >95% as determined by GFP positivity by FACS, and expression levels of exogenous STAT1 and STAT1C were equivalent but pSTAT1 levels were only increased in cells expressing STAT1C (Figure 4A). Low concentrations of PMA (insufficient to generate detectable megakaryocytic differentiation of unmodified K562 cells) induced substantial megakaryocytic differentiation of K562 cells expressing STAT1C but not those expressing STAT1. K562 cells expressing STAT1C upregulated the megakaryocytic markers CD41 (Figure 4B), CD61 (Figure S4A) and GPIX (Figure 4C) and also developed increased DNA ploidy (Figure 4D and Figure S4B). Exposure to hemin induced erythroid differentiation as indicated by increased levels of γ-globin transcripts and hemoglobinization. However, the increases in γ-globin transcripts and hemoglobin levels were both inhibited in K562 cells expressing STAT1C (Figures 4E and 4G). These results demonstrate that

increased STAT1 activity in K562 cells results in enhanced megakaryocytic and repressed erythroid differentiation.

To confirm our results in primary hematopoietic progenitors, purified CD34+ cord blood cells were infected with lentiviruses expressing STAT1 or STAT1C and then grown in conditions promoting either megakaryocytic or erythroid differentiation. At day 3 postinfection, pSTAT1 was readily detected in cells expressing STAT1C but not in those expressing STAT1 (Figure 5A). No differences in growth kinetics were detected in cells expressing either STAT1 isoform when cultured in either megakaryocytic or erythroid conditions (Figure 5B). However, expression of STAT1C increased the proportion of cells expressing the megakaryocytic markers CD41 (Figure 5C and Figure S5A), CD61 (Figure 5D and Figure S5B) and GPIX (Figure 5E), and also increased the proportion of polyploid cells (Figure 5F and Figure S5C). Moreover, expression of STAT1C resulted in a reduced proportion of GPA+CD71+ erythroid cells (Figure 5G and Figure S5D) and reduced levels of γ-globin transcripts (Figure 5H). These results accord with our K562 data and demonstrate that increased STAT1 activity in primary hematopoietic progenitors is sufficient to produce an ET-like phenotype with enhanced megakaryocytic and reduced erythroid differentiation.

Downregulation of STAT1 Activity in JAK2V617F+ Progenitors from ET Patients Results in a PV-Like Phenotype with Increased Erythroid and Reduced Megakaryocytic Differentiation

To test the hypothesis that increased STAT1 activity constrains erythropoiesis in patients with ET, a dominant negative form of STAT1 (STAT1DN) was expressed in CD34+ hematopoietic stem and progenitor cells derived from patients diagnosed with ET.

To perform these experiments at a clonal level, CD34+ progenitors were isolated from two ET patients, infected with a lentivirus containing an empty vector (VA) or expressing a cDNA encoding a dominant negative form of STAT1 (STAT1DN) in which the Tyr701 residue was mutated to a phenylalanine (Walter et al., 1997). Infected cells were subsequently sorted at one cell per well into media capable of supporting both erythroid and megakaryocytic differentiation. After 7 days in culture each clone was genotyped to identify *JAK2V617F* mutant clones, which were subsequently expanded in fresh media for an additional 7–14 days (Figure 6A). Within 3 weeks, three distinct categories of GFP+ JAK2V617F+ clones were evident: GPA-expressing erythroid clones, CD41-expressing megakaryocytic clones, and mixed erythromegakaryocytic clones comprised of cells expressing GPA or CD41 (Figure 6B). In both ET patients examined, the percentage of GFP+ erythroid clones derived from cells infected with the STAT1DN lentivirus was increased and that of the megakaryocytic clones was decreased compared to cells infected with the empty vector (p < 0.05, Fisher-Freeman-Halton test) (Figure 6C). These results demonstrate that STAT1 activity is necessary to repress erythropoiesis in *JAK2V617F*-heterozygous ET progenitors, and that reducing STAT1 activity results in a switch to a PV-like phenotype with increased erythroid and reduced megakaryocytic differentiation.

DISCUSSION

In this study, we address the apparent paradox that an identical *JAK2* mutation is associated with different clinical phenotypes.
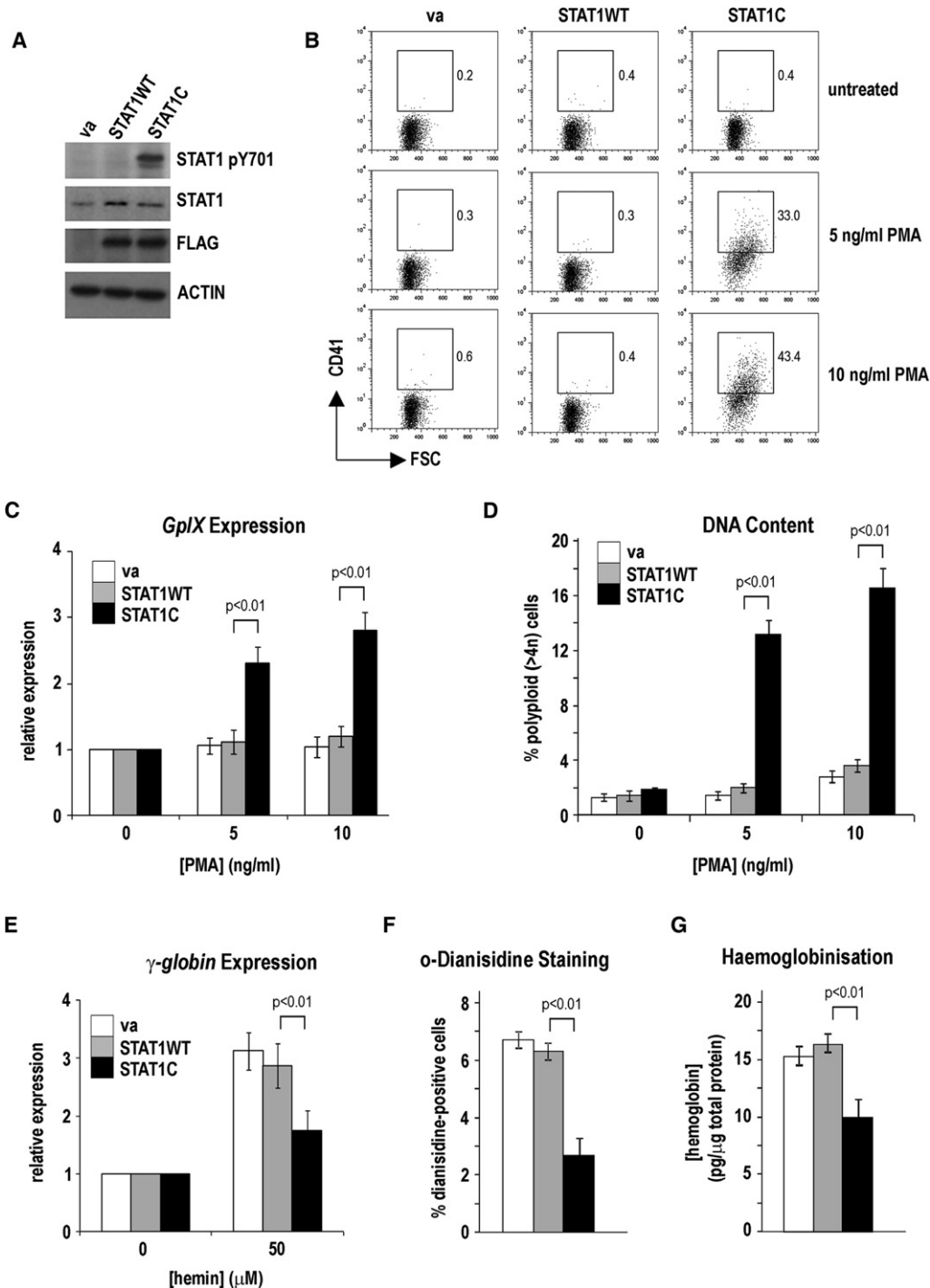
Figure 4. STAT1 Activation Enhances Megakaryocytic Differentiation and Inhibits Erythroid Differentiation in K562 Cells
(A) Western immunoblot shows increased pY701-STAT1 expression in K562 cells transduced with a constitutively active form of STAT1 (STAT1C), compared to cultures transduced with an empty vector (VA) or wild-type STAT1 cDNA (STAT1WT).
(B) Representative FACS profiles showing that PMA treatment results in increased numbers of CD41-expressing cells in the STAT1C-infected. Also note concomitant increase in cell size as assessed by forward scatter (FSC), consistent with a megakaryocytic-like phenotype. Results are representative of three independent experiments.
(C) Real-time quantitative RT-PCR of PMA-treated K562 cells shows increased expression of *GpIX* transcripts in STAT1C-infected K562 cultures relative to VA and STAT1WT-infected control cultures. Error bars represent standard deviation for three independent experiments.
(D) PMA treatment results in increased numbers of polyploid (>4n) cells in STAT1C-infected K562 cultures. Error bars represent standard deviation for three independent experiments.
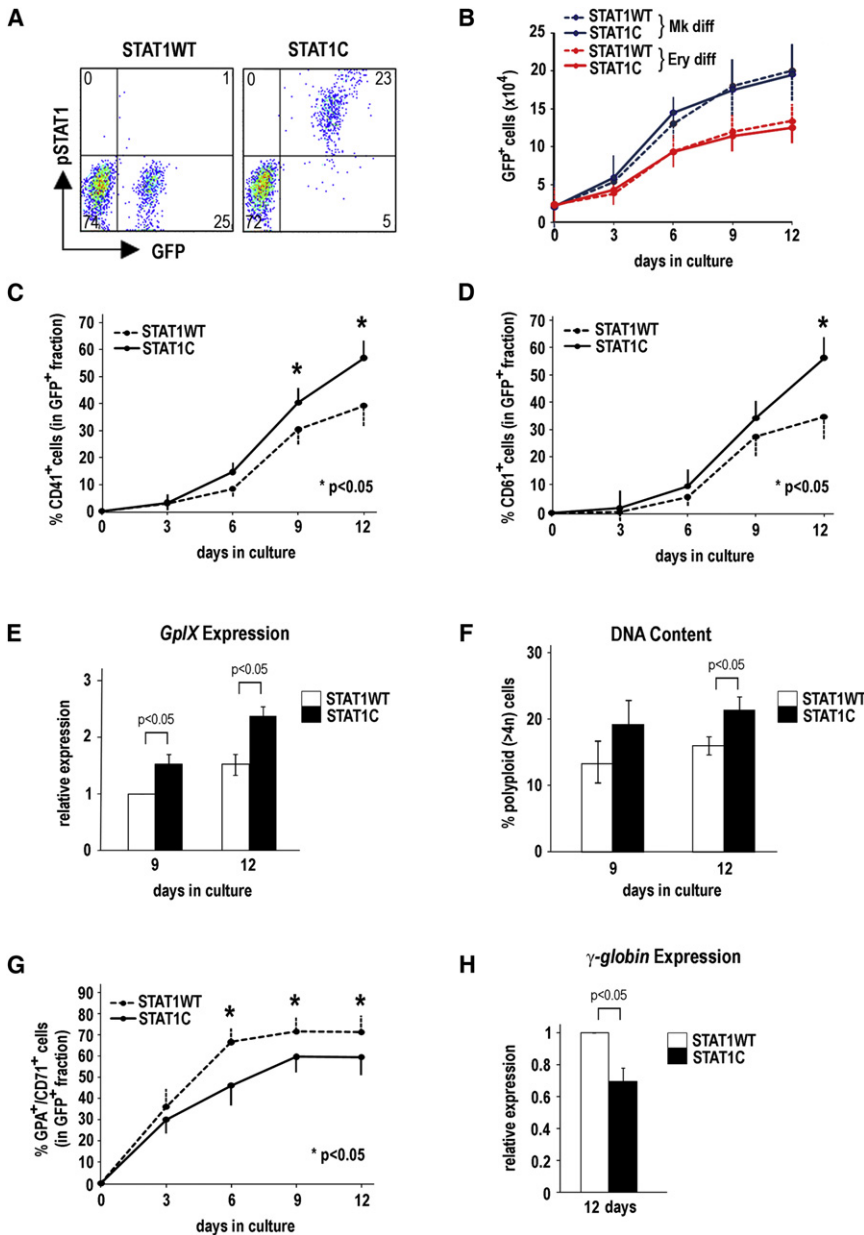
Figure 5. STAT1 Activation Enhances Megakaryocytic Differentiation and Inhibits Erythroid Differentiation in Normal Human Progenitors

(A) Intracellular flow cytometry to detect pSTAT1 in cord blood CD34^{+} cells after transduction with lentiviruses expressing wild-type STAT1 (STAT1WT) or constitutively active STAT1 (STAT1C). Presence of pY701-STAT1 was detected in GFP[+] subpopulation of STAT1C-transduced cultures only.

(B) STAT1WT- and STAT1C-infected cells were cultured in megakaryocytic differentiation conditions (blue) or erythroid differentiation conditions (red) (refer to Supplemental Experimental Procedures). Total numbers of GFP^{+} cells during 12 days of culture under both differentiation conditions are shown. Error bars represent standard deviation for three independent cultures.

(C–F) Expression of STAT1C in cord blood-derived CD34^{+} cells grown in conditions supporting megakaryocyte differentiation result in increased numbers of GFP^{+}CD41^{+} cells (C), increased numbers of GFP^{+}CD61^{+} cells (D), increased expression of *GpIX* transcripts (E), and increased numbers of polyploid (>4n) cells (F). Error bars represent standard deviation for three independent cultures. The data are representative of two independent experiments.

(G–H) Expression of STAT1C in CD34^{+} cells grown in conditions supporting erythroid differentiation resulted in decreased numbers of GFP^{+}GPA^{+}CD71^{+} cells (G), and decreased expression of γ-globin (H). Results represent mean ± SD for three independent cultures. The data are representative of two independent experiments. See also Figure S5.

the same individual than to mutant cells from other patients. This observation demonstrates that normal interindividual variation in gene expression is greater than the transcriptional consequences of the *JAK2* mutation, a finding with relevance for many somatic genetic lesions associated with cancer. Indeed, it is increasingly recognized that quantitative differences in transcript levels are under genetic control in both man (Stranger et al., 2005; Stranger et al., 2007) and mouse (Breitling et al., 2008; Gerrits et al., 2009). Our results, therefore, emphasize the power of comparing clonally-derived, phenotypically equivalent cell populations from the same individual, together with the importance of using physiological levels of cytokines that do not swamp the signaling pathways of interest.

Using a strategy based on analysis of clonally derived and genotypically defined cell populations, we have circumvented the problems associated with interindividual comparison of expression profiles. Our results illuminate the pathogenesis of the MPNs and are also of broad relevance for cancer biology.

We report the surprising finding that, in the vast majority of PV and ET patients, the expression profiles of JAK2V617F-positive erythroid cells are more closely related to wild-type cells from

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(E) Real-time quantitative RT-PCR demonstrating lower levels of γ-globin transcripts in STAT1C-infected K562 cultures relative to VA and STAT1WT-infected control cultures after erythroid differentiation with hemin. Results represent mean ± SD for three independent experiments.

(F) *o*-Dianisidine staining showing decreased numbers of hemoglobin-positive cells in STAT1C expressing K562 cells differentiated with hemin. Results represent mean ± SD for three independent cultures.

(G) Increased hemoglobin levels in STAT1C-expressing cells differentiated with hemin. Results represent the mean ± SD for three independent cultures. See also Figure S4.
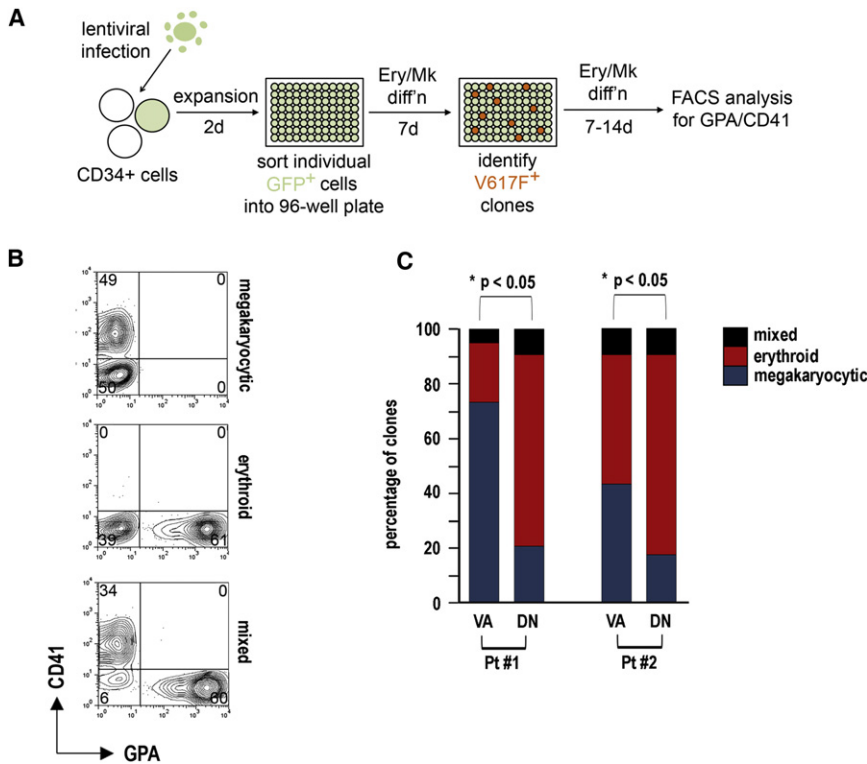
Figure 6. Downregulation of STAT1 Activity in *JAK2V617F*-Heterozygous Progenitors from ET Patients Results in a PV-Like Phenotype with Increased Erythroid and Reduced Megakaryocytic Differentiation

(A) Strategy for assessing erythroid/megakaryocytic differentiation potential of CD34+ cells from ET patients after expression of an empty vector (VA) or a dominant negative STAT1 (DN).

(B) Typical FACS profiles illustrating the three clone types generated: megakaryocytic, erythrocytic, and mixed.

(C) Histogram showing the relative proportions of the three different types of GFP+ V617F+ clones derived from cells infected with virus expressing empty vector (VA) or the dominant negative STAT1 (DN). Increased percentages of GFP+ erythroid clones and decreased percentages of GFP+ megakaryocytic clones were derived from cells infected with the DN lentivirus compared to cells infected with the VA. Testing for statistical significance was carried out using a Fisher-Freeman-Halton test.

Our results also have implications for the strategy of individualized cancer therapy, an approach based on the concept of identifying genetic lesions in each tumor and using this information to select targeted therapies. Mutant JAK2 activates multiple receptor-associated pathways and we show that some of these, such as STAT5 and STAT1, can have competing consequences. Our results also demonstrate that patients can carry an identical *JAK2* mutation but harbor striking differences in the degree to which the mutation activates the STAT1 pathway. The consequences of inhibiting a particular activated kinase may therefore be different for individual tumors and difficult to predict without a detailed knowledge of the signaling environment within a given tumor. Moreover, because some pathways downstream of an activated kinase may, like STAT1 activation, restrain disease evolution, therapeutic kinase inhibition could potentially result in paradoxical tumor-enhancing effects.

The mechanism whereby a single *JAK2* mutation can give rise to distinct diseases has been a major lacuna in our understanding of MPN pathogenesis, and distinguishing *JAK2V617F*-positive ET from PV represents a diagnostic challenge when patients have borderline hemoglobin levels. Patients with *JAK2V617F*-positive ET exhibit an increased erythroid drive but lack overt erythrocytosis (Campbell et al., 2005), indicating the existence of additional mechanisms that either constrain erythropoiesis in *JAK2* mutation-positive ET or enhance it in PV. We believe the data presented here demonstrate that STAT1 is activated in association with *JAK2V617F* in ET but not PV and that pSTAT1 levels provide a molecular marker that distinguishes these disorders. Importantly, we demonstrate that inhibition of STAT1 signaling in ET progenitors resulted in enhanced erythropoiesis and reduced megakaryopoiesis. Together, our results indicate that the clinical phenotype developed by a given individual reflects the opposing effects of STAT5 and STAT1 signaling. In ET, the intact pSTAT1 response to JAK2V617F constrains erythroid and promotes megakaryocytic differentiation—the reduced pSTAT1 response in PV removes the "brake" on erythropoiesis, thus allowing the development of an overt erythrocytosis, and also reduces megakaryopoiesis (Figure 7). This model is consistent with previous studies of the effects of STAT5 and
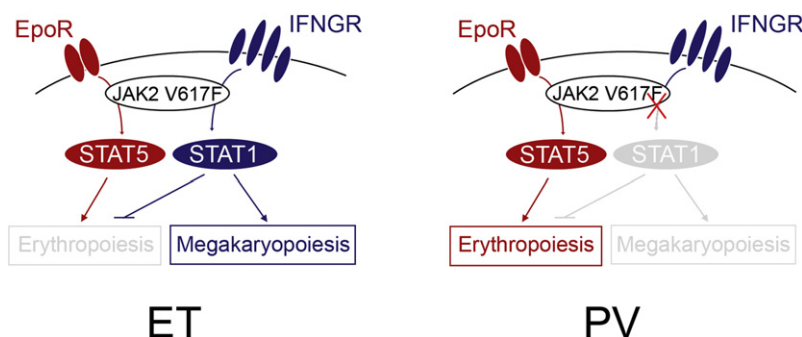

Figure 7. Model for the Different Effects of *JAK2V617F* in ET and PV

In ET, JAK2V617F induces simultaneous activation of both STAT5 and STAT1 signaling pathways. Activation of pSTAT1 constrains erythroid and promotes megakaryocytic differentiation. In PV, reduced pSTAT1 response to JAK2V617F removes the "brake" on erythropoiesis, thus allowing the development of an overt erythrocytosis, and also reduces megakaryopoiesis.

STAT1 on hematopoietic differentiation (Huang et al., 2007; Olthof et al., 2008; Socolovsky et al., 1999).

Homozygosity for the *JAK2* mutation is common in PV but rare in ET (Scott et al., 2006), and has been suggested as a potential mechanism for increased erythropoiesis in PV (Dupont et al., 2007) Our results show that reduced pSTAT1 levels precede homozygosity and enhance erythropoiesis, but do not exclude a subsequent role for *JAK2V617F* homozygosity. Once the pSTAT1 pathway is repressed, homozygosity of *JAK2V617F* would be predicted to enhance erythroid differentiation by increasing pSTAT5 signaling. By contrast, in the presence of intact STAT1 signaling, *JAK2V617F* homozygosity may fail to confer any growth or selective advantage (or even result in a disadvantage). We are collecting *JAK2V617F*-homozygous colonies to investigate the transcriptional consequences of homozygosity.

The process responsible for loss of STAT1 phosphorylation in PV remains unclear. Our data do not distinguish between constitutional or acquired mechanisms. However, the pSTAT1 response to IFNγ is normal in T cells from patients with PV (data not shown), indicating that there is no constitutional block to STAT1 activation. Moreover, the fact that some patients with high-risk ET develop overt PV transformation (Harrison et al., 2005) argues for an acquired genetic modifier in at least a subset of patients, a concept consistent with the observation that 10% of *JAK2V617F* knock-in mice also develop PV transformation (Li et al., 2010).

Our results also have implications for interferon therapy, HSC behavior, and clonal evolution in the MPNs. *JAK2* mutant erythroblasts from PV patients have markedly reduced pSTAT1 levels (relative to ET patients), and yet both categories of patients respond to therapy with IFNα. This suggests that such responses may reflect non cell-intrinsic mechanisms, that reduced STAT1 phosphorylation in PV can be overcome by pharmacological doses of IFNα, or that IFNα elicits its effects through STAT1-independent pathways (Lu et al., 2010). Normal HSC function is regulated by basal IFN tone (Baldridge et al., 2010; Essers et al., 2009; Zhao et al., 2010) and increased IFNγ or IFNα signaling results in HSC exhaustion (Trumpp et al., 2010). Hence, within a *JAK2V617F*-positive population, a subclone that acquires a defect in STAT1 activation may not only give rise to erythrocytosis but also display a competitive advantage within the HSC compartment, thus providing a potential mechanism for clonal evolution.

EXPERIMENTAL PROCEDURES

Patients and Samples

A total of 36 MPN cases (18 men/18 women) with a median age of 64 (ranging from 12 to 89 years), diagnosed with either essential thrombocythemia or polycythemia vera according to the World Health Organization (WHO) criteria were recruited for this study from the MPN clinic at the Addenbrooke's Hospital in Cambridge, UK. All patients were shown to possess the *JAK2V617F* mutation in granulocyte DNA using allele-specific PCR. Two PV patients and one ET patient also possessed mutations in *TET2*. None harbored *MPL* or *JAK2* exon 12 mutations. The clinical and biological features of all patients at diagnosis are listed in Table S1. All patients gave informed written consent before participating in the study. The study was approved by the Cambridge and Eastern Region Ethics Committee, and was carried out in accordance with the principles of the Declaration of Helsinki. Venous blood samples (20 ml) were collected from each patient, and peripheral blood mononuclear cells were isolated using Lymphoprep (Axis Shield PLC) according to the manufac-

turer's protocols, and plated in Methocult (H4531; Stem Cell Technologies) supplemented with 0.01 U/ml erythropoietin at a density of 3×10^5 cells/ml. Cultures were incubated at 37°C for 14 days.

RNA Extraction

For each patient, individual BFU-E colonies were plucked into 100 μl of Buffer RLT (QIAGEN). A portion of the sample in Buffer RLT for each colony was used for genomic DNA (gDNA) extraction, followed by genotyping by real-time qPCR to determine its JAK2 mutational status, as described previously (Levine et al., 2006). Colonies with V617F percentages below 10% were designated "wild-type" and those between 40% and 60% were designated "heterozygous." Colonies not fulfilling any of the two criteria were discarded. Up to 20 colonies of each *JAK2* genotype were pooled, and total cellular RNA from each sample was isolated using the QIAGEN RNeasy kit following the manufacturer's protocols. RNA quality was assessed by nano electrophoresis using the Pico lab-on-a-chip assay (Bioanalyzer, Agilent Technologies) and by agarose gel electrophoresis.

Microarray Analysis

Total RNA (100 ng) obtained from wild-type and V617F-heterozygous BFU-E colonies from each patient were reverse transcribed into cDNA and amplified by in vitro transcription into biotinylated cRNA using the Illumina TotalPrep RNA Amplification Kit (Ambion). For each sample, 1.5 μg of amplified cRNA was used for hybridization onto Illumina Human-6 v2.0 Expression BeadChips, comprising ~48,000 oligonucleotide probes.

Single Cell Erythroid/Megakaryocyte Differentiation Cultures

Peripheral blood mononuclear cells were obtained from peripheral blood of patients diagnosed with ET over a Ficoll gradient, and CD34+ cells were then selected using a magnetic cell sorting system (Miltenyi Biotech), according to manufacturer's instructions. Purified CD34+ cells were immediately infected with either an empty vector or a dominant negative STAT1-expressing lentiviruses by spinoculation for 2 hr in the presence of 4 μg/ml polybrene, followed by culturing for 2 days in SFEM medium supplemented with 100 ng/ml Flt3 ligand and 10 ng/ml rhTPO. Subsequently, cells were sorted based on GFP positivity and seeded as single cells in a well of a 96-well plate with SFEM media supplemented with 25 ng/ml rhSCF, 0.5 U/ml rhEPO, 100 ng/ml rhTPO, 30 μg/ml holo-transferrin, 10 nM β-mercaptoethanol, and 4 μg/ml dexamethasone to support both erythroid and megakaryocytic differentiation. After 7 additional days in culture, 10% of the cells were removed for *JAK2* genotyping as described above. After an additional 7–14 days of culture, V617F-heterozygous clones were analyzed for GPA and CD41 expression.

ACCESSION NUMBERS

All expression profiling data have been deposited into ArrayExpress under accession number E-MTAB-384.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, five figures, and four tables, and can be found with this article online at doi:10.1016/j.ccr.2010.10.013.

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