Title: Distinct effects of concomitant Jak2V617F expression and Tet2 loss in mice combine to promote disease progression in myeloproliferative neoplasms

Short title/ Running title: Tet2 loss accelerates Jak2V617F MPN

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Word Count: 4171 (excluding figure legends & references)
Abstract word count = 200
References = 41
Key Points

- Tet2 loss of function confers a strong functional competitive advantage to Jak2V617F-mutant hematopoietic stem cells
- Jak2V617F expression and Tet2 loss generate distinct and non-overlapping transcriptional programs in hematopoietic stem cells
Abstract

Signaling mutations (e.g. JAK2V617F) and mutations in genes involved in epigenetic regulation (e.g. TET2) are the most common co-occurring classes of mutations in myeloproliferative neoplasms (MPN). Clinical correlative studies have demonstrated that TET2 mutations are enriched in more advanced phases of MPN such as myelofibrosis and leukemic transformation, suggesting that they may co-operate with JAK2V617F to promote disease progression. To dissect the effects of concomitant Jak2V617F expression and Tet2 loss within distinct hematopoietic compartments in vivo, we generated Jak2V617F/Tet2 compound mutant genetic mice. We found that the combination of Jak2V617F expression and Tet2 loss resulted in a more florid MPN phenotype than that seen with either allele alone. Concordant with this, we found that Tet2 deletion conferred a strong functional competitive advantage to Jak2V617F-mutant hematopoietic stem cells (HSC). Transcriptional profiling revealed that both Jak2V617F expression and Tet2 loss were associated with distinct and non-overlapping gene expression signatures within the HSC compartment. In aggregate, our findings indicate that Tet2 loss drives clonal dominance in HSC and Jak2V617F expression causes expansion of downstream precursor cell populations, resulting in disease progression through combinatorial effects. This works provides insights into the functional consequences of JAK2V617F-TET2 co-mutation in MPN, particularly as it pertains to HSC.
Introduction

Whole-genome and whole-exome sequencing studies have provided important insights into the somatic genetic lesions that drive myeloid neoplasms\textsuperscript{1-3}. Although much can be inferred from the patterns of genetic alterations identified in such studies, we still have an incomplete understanding of the functional significance of these relationships, particularly in how different driver mutations collaborate in the transformation of the hematopoietic stem cell (HSC).

In myeloproliferative neoplasms (MPN), the majority of driver mutations can be broadly classified within two categories\textsuperscript{4}. First, virtually all MPN patients are now known to harbor mutations that confer hyperactive JAK-STAT signaling. By far, the \textit{JAK2V617F} mutation is the most frequent of these mutations\textsuperscript{5-8} with a minority of patients also harboring mutations in exon 12 of \textit{JAK2}\textsuperscript{9}, \textit{MPL}\textsuperscript{10}, \textit{LNK}\textsuperscript{11} or \textit{c-CBL}\textsuperscript{12}. Recently, mutations in the gene encoding the ER chaperone, calreticulin (\textit{CALR}) have been identified in the majority of \textit{JAK2}-unmutated MPN patients\textsuperscript{3,13}, with early evidence suggesting that mutant \textit{CALR} also causes constitutive JAK-STAT signaling and cytokine-independent growth\textsuperscript{13}.

The second major class of somatic alterations in the MPN cancer genome is in genes encoding epigenetic regulators\textsuperscript{14}. In particular, deletions or loss-of-function mutations of the \textit{TET2} methylcytosine dioxygenase occur in approximately 7.5\% to 17\% of MPN and are enriched in myelofibrosis as compared to essential thrombocytemia (ET)\textsuperscript{15,16} and in more aggressive forms of mastocytosis\textsuperscript{17}. Outside of \textit{JAK2V617F} and mutations in \textit{CALR}, \textit{TET2} is the most common somatically altered gene in MPN and the most commonly co-mutated gene with \textit{JAK2V617F}\textsuperscript{18}. Although \textit{JAK2V617F} and \textit{CALR} mutations are mutually exclusive, \textit{TET2} mutations co-occur with both\textsuperscript{19} suggesting that \textit{TET2} impacts distinct downstream oncogenic pathways from those affected by \textit{JAK2V617F} or mutant \textit{CALR}.
MPN animal models accurately re-capitulate human disease in mice and have been an important tool for the study of MPN biology and therapy\textsuperscript{20,21}. Genetically engineered Jak2V617F and Tet2 animal models generated by ourselves and others\textsuperscript{20,21} have permitted a detailed examination of the functional effects of these genetic alterations in different hematopoietic compartments. In this study, we sought to model the co-occurrence of JAK2V617F and TET2 mutations in MPN patients by investigating the consequences of concomitant Jak2V617F expression and Tet2 loss \textit{in vivo}. We provide new insights into the impact of Tet2 loss on (i) disease progression in Jak2V617F-mediated MPN, (ii) Jak2V617F-mutant hematopoietic stem and progenitor cell (HSPC) function and (iii) the transcriptional program of Jak2V617F-mutant MPN stem cells.

\textbf{Material and Methods}

\textit{Experimental mice}

We have previously described Jak2V617F (Jak2\textsuperscript{VF}) conditional knockin and Tet2 conditional knockout mice\textsuperscript{22,23}. In this study, we used VavCre transgenic mice to target Cre recombinase expression to the hematopoietic lineage\textsuperscript{24} and delete Tet2 in the hematopoietic compartment of Jak2V617F mice (Supp. Figure 1). We generated Jak2\textsuperscript{VF} mice that were wild-type or nullizygous for Tet2 (Jak2\textsuperscript{VF} or Jak2\textsuperscript{VF}/Tet2\textsuperscript{null} respectively). We also generated mice that were wild-type for Jak2 and nullizygous for Tet2 (Tet2\textsuperscript{null}). For controls, we used VavCre positive mice that were wild-type for both Jak2 and Tet2 (WT). We maintained all mice in pathogen free facilities at Children’s Hospital Boston (CHB). The institutional ethics committee of CHB approved all mouse experiments on protocol 13-04-2393R.

\textit{Blood analysis}
Blood was collected into EDTA-coated containers and was analyzed on a Hemavet 950 analyzer (Drew Scientific, FL).

**Flow cytometry**

Bone marrow, spleen or peripheral blood were collected and prepared for staining by red blood cell lysis (BD Pharmlyse, BD Biosciences) and homogenization through a 70micron filter. Erythroid precursor cell stainings were not pretreated with red cell lysis. All samples were analyzed by flow cytometry using FACS Canto cytometer or LSR II (BD Biosciences). All staining steps were performed in ice-cold PBS containing 2% FBS. Post-acquisition analysis of data was performed with FlowJo software V9.2.3 (Treestar, CA). For peripheral blood chimerism studies, the following antibodies were used (clone in parentheses): CD45.1 (A20) and CD45.2 (104). For erythroid precursor cells, the following antibodies were used: CD71 (RI7217) and Ter119 (Ter-119). For stem cell and progenitor analysis, the following antibodies were used: lineage cocktail containing CD3ε (145-2C11), CD5 (53-7.3), Ter-119 (TER-119), Gr-1 (RB6-8C5), Mac-1 (M1/70) and B220 (30-F11); Kit (2B8), Sca-1 (D7), CD150 (TC15-12F12.2), CD48 (HM48-1), CD135 (A2F10), CD34 (Ram34), CD16/32 (93) in addition to CD45.1 (A20) and CD45.2 (104). For dead cell discrimination Sytox blue (Invitrogen) was used. For cell cycle analysis, cells were first stained for lineage, stem and progenitor markers, then fixed and permeabilized and stained with Ki67, followed by staining with Hoechst 33342. For cell sorting experiments, lineage positive cells were first either depleted with Dynabeads (Invitrogen), or c-Kit positive cells were enriched using CD117 mouse microbeads (MACS Miltenyi). The remaining cells were then stained with biotinylated lineage cocktail antibodies (clones listed above) followed by Streptavidin-ApcCy7, cKit (2B8), Sca-1 (D7) and CD150 (TC15-12F12.2), CD48 (HM48-1) where appropriate and then sorted on a FACS Aria (Becton Dickinson).

**Histopathology**
Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin (HE) or with reticulin, to assess for fibrosis. For megakaryocyte analysis, megakaryocytes were counted in 10 high power fields (HPF, 1000X with oil) per tibia from four biological replicates. Images of histological slides were obtained on a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan) equipped with a SPOT RT color digital camera model 2.1.1 (Diagnostic Instruments, Sterling Heights, MI).

**Colony-Forming Unit (CFU) Assays**

Myeloid colony-plating assays were performed in methylcellulose-based medium supplemented with complete cytokine mix (MethoCult GF M3434; Stem Cell Technologies). We plated unfractionated bone marrow cells in methylcellulose-based medium, seeded 1x10^4 cells per plate in triplicate and scored for colony formation 7–10 days later. For serial re-plating assays, we re-suspended, pooled and washed the remaining cells of the same genotype in phosphate-buffered saline (PBS, Gibco). We then counted the cells and re-plated 1x10^4 cells in triplicate under the same culture conditions as previously and scored colonies 7-10 days later. We performed serial re-plating a total of 4 times.

**Bone Marrow Transplantation**

Bone marrow cells were re-suspended in Hanks balanced salt solution (Gibco) and injected into lethally irradiated (1 x 10 Gy [1000 rads]) wild-type recipient mice by either lateral tail vein or retro-orbital injection.

Competitive transplantation experiments were performed using lineage^low^Kit^high^Sca1^+^ (LSK) cells purified from Jak2^VF^, Tet2^null^ or Jak2^VF^/Tet2^null^ mice (n = 2 pooled for each genotype) and wild-type LSK cells isolated from 45.1 mice. LSK cells were mixed and then injected into lethally irradiated 45.1 SJL recipients (n = 5 in each recipient group). On a single mouse basis, 2.5 x 10^4
Jak2\textsuperscript{VF} LSK cells were competed against 1.43 \times 10^4 WT 45.1 LSK cells (ratio 1.7:1; 64% Jak2\textsuperscript{VF}, 36% WT); 1.65 \times 10^4 LSK Tet2\textsuperscript{null} LSK cells were competed against 2.3 \times 10^4 WT 45.1 LSK cells (ratio 0.7:1; 42% Tet2\textsuperscript{null}, 58% WT); 2.0 \times 10^4 Jak2\textsuperscript{VF}/Tet2\textsuperscript{null} LSK cells were competed against 2.0 \times 10^4 WT 45.1 LSK cells (ratio 1:1; 50% Jak2\textsuperscript{VF}/Tet2\textsuperscript{null}, 50% WT). Bone marrow derived from \textit{Jak2V617F} and \textit{Tet2} mice expressed the CD45.2 antigen and WT competitor bone marrow cells expressed CD45.1. Of note, since recipient mice also expressed CD45.1, residual recipient hematopoietic cells also contributed to hematopoiesis post transplantation (at an irradiation dose of 10Gy we expect approximately 10-20% residual recipient hematopoiesis).

Purified bone marrow subpopulation transplants were performed using 2.2 \times 10^3 short-term (ST) ST-HSC (CD150\textsuperscript{-} CD48\textsuperscript{-} LSK) or 5.0 \times 10^3 multipotent progenitor (MPP) (CD48\textsuperscript{+} LSK) donor cells from Jak2\textsuperscript{VF} or Jak2\textsuperscript{VF}/Tet2\textsuperscript{null} mice (n = 2 pooled for each genotype) plus 4 \times 10^5 supportive wild-type bone marrow cells injected into lethally irradiated 45.1 SJL recipients (n = 5 in each recipient group). Bone marrow derived from \textit{Jak2V617F} and \textit{Tet2} mice expressed the CD45.2 antigen, recipient mice and supportive wild-type bone marrow cells both expressed CD45.1.

For BMT experiments percentage chimerism was defined as the proportion of \textit{Jak2V617F} or Tet2 or \textit{Jak2V617F}/Tet2 cells as a percentage of total cells. That is, (\%CD45.2)/(\%CD45.2 + \%CD45.1 WT) \times 100\%.

\textit{Gene Expression Profiling}

3-5 \times 10^4 LSK cells per mouse were isolated from WT (n = 4), Tet2\textsuperscript{null} (n = 3), Jak2\textsuperscript{VF} (n = 3) or Jak2\textsuperscript{VF}/Tet2\textsuperscript{null} (n = 4) mice. RNA was extracted using Picopure RNA extraction kit according to the manufacturer’s instructions (Invitrogen). The samples were amplified with the Illumina Total Prepamp kit (Invitrogen) and hybridized on Illumina MouseRef-8 v2.0 gene expression arrays. The data were analyzed with Gene Pattern online analysis software including quantile
normalization⁵. Gene set enrichment analysis (GSEA) was performed across the complete list of genes ranked by signal-to-noise ratio according to their differential expression⁶. STAT5A and HSC self-renewal signatures were extracted from previously published gene expression data⁷,⁸. The microarray dataset reported in this article has been deposited in the ArrayExpress repository at EBI (http://www.ebi.ac.uk/arrayexpress/) and are accessible through ArrayExpress accession number E-MTAB-2986.

Statistical Analysis

Graphpad Prism (Graphpad Software, Inc. La Jolla, CA) was used to analyze results and create graphs. All comparisons represent 2-tailed unpaired t-test analysis (with Welch’s correction where appropriate) unless otherwise specified.

Results

**Tet2 loss accelerates the MPN phenotype of Jak2V617F mice**

We have previously described a conditional knock-in, which expresses Jak2V617F under the control of the endogenous Jak2 promoter (Jak2VF mice)²². The phenotype of the model closely recapitulates many of the clinical features of human MPN, including prominent splenomegaly as a result of extra-medullary hematopoiesis. To evaluate the effect of concomitant Jak2V617F expression and Tet2 loss on MPN phenotype, we generated Jak2VF/Tet2null mice and analyzed the splenic phenotype. As previously reported, Jak2VF mice exhibited marked splenomegaly (Figure 1A). Strikingly, the spleens of Jak2VF/Tet2null mice were significantly larger than those of Jak2VF mice (Figure 1A, B). We next performed histopathological examination of the spleen and found expansion of the white pulp and enlarged partially confluent lymphoid follicles in Tet2null mice as compared to WT mice (Figure 1C). In contrast, the spleens of Jak2VF and Jak2VF/Tet2null mice showed marked effacement of the white pulp and tri-lineage hyperplasia consistent with
MPN (Figure 1C). Similar to Jak2VF mice\textsuperscript{22}, the phenotype of Jak2VF/Tet2null mice was apparent at the time of genotyping (1 month) and mice were followed until 6 months of age.

We next assessed the cellular composition of the spleen using flow cytometry and found marked expansion of CD71+ Ter119+ erythroid precursor cells in Jak2VF and Jak2VF/Tet2null mice (Figure 1D) and additional expansion of Mac1+ Gr1+ myeloid precursor cells in the spleens of Jak2VF/Tet2null mice as compared to Jak2VF animals (Figure 1E). Given the marked tri-lineage hyperplasia that we observed in the spleens of Jak2VF and Jak2VF/Tet2null mice, we performed a quantitative assessment of the splenic HSC compartment. Compared to WT or Tet2null mice, we found expansion of long-term (LT)-HSC, short-term (ST)-HSC and multi-potent progenitors (MPP) in the spleens of Jak2 mutant mice irrespective of Tet2 genotype (Figure 1F). Given that the spleens of Jak2VF/Tet2null mice were significantly larger than those of Jak2 VF animals (Figure 1A), this indicates that the splenic LSK compartment of Jak2 VF/Tet2null mice was expanded absolutely as compared to that of Jak2 VF animals. In aggregate, these data indicate that Tet2 loss augments the extra-medullary hematopoiesis phenotype of Jak2V617F mice through additional LSK and myeloid precursor cell expansion, resulting in enhanced splenomegaly.

**Tet2 loss influences hematopoietic differentiation within the myeloid progenitor compartment of Jak2V617F mice but is insufficient to induce leukemic transformation**

To evaluate the effect of Tet2 loss on the hematopoietic differentiation of Jak2V617F mice, we began by analyzing peripheral blood counts. As previously reported, Jak2V617F mice demonstrate leukocytosis (Figure 2A), erythrocytosis (Figure 2B, Supp. Figure 2B) and thrombocytosis (Figure 2C, Supp. Figure 2C - D) when compared to WT animals. Homozygous loss of Tet2 led to a trend towards a further elevation in white cell count in mutant Jak2 mice (Figure 2A), while hematocrit and platelet numbers were similarly elevated in both Jak2VF and Jak2VF/Tet2null animals (Figure 2B - C). More marked leukocytosis developed in Jak2VF/Tet2null
mice that were older than 6 months (Supp. Figure 2A). Finally, we reviewed peripheral blood smears and found that platelets from Jak2^{VF}/Tet2^{null} mice were large and showed signs of dysplasia including increased basophilia of the cytoplasm, a tendency to aggregate, abnormal shape and pseudopod formation (Supp. Figure 2D).

Next, we focused on the bone marrow. We have previously reported that Jak2^{V617F} induces erythroid skewing in the myeloid progenitor compartment. Consistent with these findings we found a relative decrease in common myeloid progenitor (CMP) cells and a relative increase in megakaryocyte-erythroid progenitor (MEP) cells in the myeloid progenitor bone marrow compartment of Jak2^{VF} mice as compared to WT mice, which was not seen in Jak2^{VF}/Tet2^{null} mice (Figure 2D – F). These findings indicate that Tet2 loss reduces Jak2^{V617F}-induced erythroid skewing in the myeloid progenitor compartment. We also performed a quantitative analysis of HSC in the bone marrow and found a significant expansion of LT-HSC in Tet2^{null}, Jak2^{VF} and Jak2^{VF}/Tet2^{null} mice as compared to WT animals and a significant expansion of MPP in Jak2^{VF}/Tet2^{null} mice as compared to WT animals (Supp. Figure 3A).

*TET2* loss-of-function mutations have been associated with leukemic transformation in MPN. Therefore, we compared the propensity for leukemic transformation in Jak2^{VF}/Tet2^{null} mice relative to control animals. In the bone marrow of both Jak2^{VF} and Jak2^{VF}/Tet2^{null} mice, we found tri-lineage hyperplasia consistent with MPN, with more prominent myeloid expansion and less prominent erythroid hyperplasia observed in Jak2^{VF}/Tet2^{null} marrow relative to Jak2^{VF} marrow (Figure 2G, H). However, we did not see histopathological evidence of acute myeloid leukemia (AML) in the bone marrow of Jak2^{VF}/Tet2^{null} mice aged up to 6 months old (n=12). Because Jak2^{VF} mice die prematurely at approximately 6 months and Jak2^{VF}/Tet2^{null} mice have a comparable survival it was not possible to follow Jak2^{VF}/Tet2^{null} animals for an extended period beyond 6 months. To further evaluate for leukemic transformation we measured the
percentage of circulating c-Kit+ cells using flow cytometry and found no difference between Jak2VF and Jak2VF/Tet2null animals (Supp. Figure 3B).

Finally, given that TET2 mutations are enriched in myelofibrosis as compared to ET16 and that megakaryocytes are a key cellular driver of fibrotic transformation in MPN32, we focused on megakaryocytes within the bone marrow of Jak2VF/Tet2null mice. We found that the number of megakaryocytes was increased in Jak2VF/Tet2null as compared to Jak2VF mice and that there was greater heterogeneity in megakaryocyte size in Jak2VF/Tet2null as compared to Jak2VF animals (Supp. Figure 4A - B). We next performed a detailed histopathological analysis and noted emperipolesis and abnormal clustering of megakaryocytes consistent with MPN in the bone marrow of both Jak2VF and Jak2VF/Tet2null mice (Figure 2I). With more detailed morphological analysis, we further noted that Jak2VF/Tet2null megakaryocytes showed additional atypical features, including pyknotic nuclei, atypical mitotic figures and were apoptotic appearing. In aggregate, these findings suggest higher megakaryocyte turnover in Jak2VF/Tet2null as compared to Jak2VF animals. (Figure 2I, Supp. Figure 4C). Finally, we performed reticulin staining but did not see evidence of reticulin fibrosis in the bone marrow (Supp. Figure 4D) of Jak2VF/Tet2null mice aged up to 6 months old (n=12).

Tet2 loss confers enhanced self-renewal to Jak2V617F bone marrow cells in vitro

Next, we turned to the functional effects of concomitant Jak2V617F expression and Tet2 loss on HSPC by performing serial re-plating CFU assays. We compared CFU assay formation using unfractionated bone marrow cells derived from Jak2VF, Tet2null or Jak2VF/Tet2null mice. We found that from the third re-plating onwards Tet2null and Jak2VF/Tet2null cells had markedly enhanced re-plating activity compared to Jak2VF or WT cells (Figure 3A). These findings indicate that homozygous Tet2 loss confers increased self-renewal potential in vitro to Jak2V617F-expressing hematopoietic cells.
Tet2 loss confers a competitive repopulating advantage to Jak2V617F HSC

To further evaluate the functional impact of Tet2 loss on Jak2V617F HSC in vivo, we performed competitive bone marrow transplantation experiments. We transplanted LSK cells derived from Jak2VF, Tet2null or Jak2VF/Tet2null mice into lethally irradiated congenic recipients, together with an approximately equal number of 45.1 wild-type competitor LSK cells (see methods for precise ratios). The blood counts were broadly similar in all recipient groups (Supp. Figure 5A - C). We assessed peripheral blood chimerism in total white blood cells (WBC), myeloid cells (Gr1+), B-lymphoid cells (B220+) or T-lymphoid cells (CD3+) and found a competitive advantage over WT cells for the Jak2VF/Tet2null and Tet2null cells, in each of these compartments (Figure 3B – D, Supp. Figure 5D). This competitive advantage was present at 4 weeks post transplantation and was sustained at 17 weeks. We assessed chimerism in the bone marrow and spleen at 18 weeks post BMT and found a strong competitive advantage for Jak2VF/Tet2null and Tet2null cells (Figure 3E - F). Chimerism in the LSK compartment at 18 weeks showed that > 90% of the cells were derived from Jak2VF/Tet2null or Tet2null donors in these groups (Figure 3G). We next determined the cell cycle status of LSK cells from primary mice from each of the genotypes. Consistent we our previously published findings, we found a more activated cell cycle in Jak2VF LSK cells as compared to WT LSK cells (Supp. Figure 5G). We also found a more activated cell cycle in both Jak2VF/Tet2null LSK and LK cells as compared to WT LSK and LK cells (Supp. Figure 5H). We have previously demonstrated that MPN disease-propagating cells are contained exclusively in the long-term (LT) HSC (CD150+ CD48- LSK) compartment of Jak2V617F mice and thatJak2V617F expression in short-term (ST) HSC and multi-potent progenitor (MPP) cells does not confer long-term self-renewal capability to these cell populations. To determine if Tet2 loss enhanced the self-renewal of Jak2V617F-mutant ST-HSC or MPP, we compared the competitive repopulation of ST-HSC (CD150- CD48+ LSK) or MPP (CD48+ LSK) purified from Jak2VF or Jak2VF/Tet2null mice using bone marrow
transplantation and found no difference in self-renewal capacity between the groups at 19 weeks (Supp. Figure 3E - F). In aggregate, these data demonstrate that (i) homozygous Tet2 loss confers a competitive advantage to Jak2V617F-mutant LT-HSC similar to that seen in Tet2 deficient LT-HSC that are wild-type for Jak2 and that (ii) deleting Tet2 in Jak2V617F-mutant ST-HSC or MPP does not confer long-term self-renewal capability to these cell populations.

**Jak2V617F expression and Tet2 loss cause distinct and non-overlapping gene expression changes**

To interrogate the molecular pathways responsible for the different HSC phenotypes of Jak2VF and Jak2VF/Tet2null mice, we performed gene expression profiling of LSK cells derived from WT, Jak2VF, Tet2null or Jak2VF/Tet2null animals (n= 2-4 mice per group). Unsupervised hierarchical clustering of the global gene expression signatures revealed two main branches in the gene expression hierarchy, with WT and Tet2null samples clustering closely along one branch, and Jak2VF and Jak2VF/Tet2null samples comprising the other (Figure 4A). This indicates that Jak2V617F expression imparts a larger effect on global gene expression patterns than Tet2 loss. We successfully identified 17 unique transcripts were differentially regulated by Jak2V617F expression and/or Tet2 loss (false discovery rate < 10%, minimum fold change relative to WT samples = 1.3) (Figure 4B, Supp. Table S1). These included transcripts unique to LSK cells harboring the Jak2VF allele (n=2), Tet2null allele (n=2) or the Jak2VF/Tet2null alleles (n=8), as well as transcripts that were common to Jak2VF and Jak2VF/Tet2null LSK cells (n=5) (Figure 4B-C).

The paucity of differentially expressed genes in multiple comparisons and the modest magnitudes of the fold change suggested to us that individually these genes were unlikely to be the sole drivers of the phenotypic differences in the Jak2VF, Tet2null or Jak2VF/Tet2null animals. Therefore, we used gene set enrichment analysis (GSEA) to identify more subtle perturbations downstream of Jak2V617F expression or Tet2 loss in LSK cells by leveraging the dataset in its
entirety for the presence of specific gene signatures (Table S2-3). We found that targets of STAT5A signaling were enriched amongst genes that were differentially expressed in Jak2VF and Jak2VF/Tet2null LSK cells but not in genes differentially expressed in Tet2null LSK cells (Figure 4D). In addition, we found enrichment of genes that harbor putative STAT5 binding sites (defined by the motif NAWTTCYN within -2kb and 2kb of transcriptional start site) only in mice harboring a mutant Jak2 allele but not in Tet2 deleted LSK cells (Supp. Figure 6A). Concomitantly, increased STAT5A signatures were correlated with enrichment of gene signatures from erythroid-, megakaryocytic- and granulocyte-macrophage-committed precursors, a feature of Jak2VF and Jak2VF/Tet2null (but not Tet2null) LSK cells (Supp. Figure 6B). Taken together, these findings demonstrate that the LSK compartment of mice harboring mutant Jak2 exhibit genetic signatures indicative of robust activation of the Jak2-Stat5 signaling axis.

Next, we performed GSEA analysis using a 363 gene signature that has previously been shown to be associated with a murine leukemic stem cell self-renewal signature. We found significant enrichment of a HSC self-renewal signature in LSK cells from Jak2VF/Tet2null and Tet2null mice but not in LSK cells from Jak2VF animals (Figure 4E). These findings are consistent with our in vitro and in vivo data demonstrating both increased serial re-plating capacity and enhanced competitive repopulation activity for Tet2-deficient LSK cells, irrespective of Jak2 status.

Discussion

MPN are primarily disorders of activated intracellular signaling, with the majority of patients harboring mutations in genes that encode proteins which regulate cytokine signaling. The recent identification of CALR mutations provides further evidence that MPN are diseases driven by aberrant signal transduction. Mutations in genes involved in epigenetic regulation (e.g. TET2, ASXL1, DNMT3A, EZH2) are the most frequently co-mutated genes with signaling...
mutations in MPN suggest that genetic co-operation may occur between these two classes of genes in MPN. *TET2* mutations are known to occur within the HSC compartment of MPN patients and while an initial clonal analysis of patient samples demonstrated that *TET2* mutations can precede or follow the *JAK2V617F* mutation, a more recent larger study has shown that *TET2* mutations are predominantly acquired prior to *JAK2V617F*. Initial functional studies in immunodeficient mice demonstrated that *TET2-JAK2V617F* co-mutated CD34+ HSC have increased re-populating capacity over *JAK2V617F*-mutated CD34+ HSC. In this study, using syngeneic genetic murine models that we have developed, we determined the individual and combinatorial effects of Jak2V617F expression and Tet2 loss on (i) disease phenotype, (ii) HSPC function and (iii) HSC signaling and self-renewal transcriptional signatures.

We found that homozygous Tet2 loss accelerated the MPN phenotype of Jak2V617F mice, as evidenced by expansion of the splenic HSC compartment, enhanced extra-medullary hematopoiesis and splenomegaly. Although mutations in epigenetic regulators are enriched in more advanced phases of MPN such as myelofibrosis and secondary AML, in our study, combining a mutant Jak2 allele with a Tet2-null allele was insufficient to induce fibrotic or leukemic transformation at 6 months. The fact that AML that arises out of *JAK2V617F*-mutant MPN retains the *JAK2V617F* allele only approximately 50% of the time suggests that only a subset of genetic lesions seen in secondary AML co-operate with *JAK2V617F* and that cell non-autonomous mechanisms of transformation may also occur. One additional point of note is that in our study we used VavCre to target Cre recombinase to the hematopoietic lineage, and in so doing Jak2V617F expression and Tet2 loss occurred simultaneously in VavCre positive cells. In MPN patients, *JAK2V617F* and *TET2* mutations typically occur sequentially and it is possible that the order in which these mutations are acquired influences the disease phenotype. Since VavCre is non-inducible we were unable to address the impact of the temporal order of mutation acquisition on MPN phenotype.
In functional studies, we found a strong competitive advantage for Jak2\textsuperscript{VF}/Tet2\textsuperscript{null} compound mutant HSPC, together with marked expansion of Jak2\textsuperscript{VF}/Tet2\textsuperscript{null} myeloid and erythroid precursor cell populations resulting in an enhanced MPN phenotype in Jak2\textsuperscript{VF}/Tet2\textsuperscript{null} mice as compared to Jak2\textsuperscript{VF} or Tet2\textsuperscript{null} animals. These findings are consistent with a model where Tet2 loss drives clonal dominance in HSC and Jak2V617F expression causes expansion of downstream progenitor and precursor cell populations, resulting in disease progression through combinatorial effects. Furthermore, these findings are consistent with the fact that the JAK2V617F allele burden in the HSC compartment of polycythemia vera (PV) and ET patients is low and that JAK2V617F-mutant HSC predominate in more advanced phases of MPN such as myelofibrosis, where epigenetic mutations are enriched\textsuperscript{41}.

The results of the functional studies outlined above are supported by our findings from the gene expression profiling of LSK cells. In this study, we found that both Jak2V617F expression and Tet2 loss were associated with distinct and non-overlapping gene expression signatures in the HSC compartment. Using GSEA, we found that a STAT5A signature was enriched only in Jak2\textsuperscript{VF} LSK cells and an HSC self-renewal signature was enriched only in Tet2\textsuperscript{null} LSK cells, but that both signatures were enriched in Jak2\textsuperscript{VF}/Tet2\textsuperscript{null} LSK cells. These data demonstrate that Jak2V617F expression and Tet2 loss each exert a specific effect on the transcriptional program of LSK cells. Specifically, Jak2V617F facilitates increased STAT5 signaling which is known to potentiate erythroid differentiation, while Tet2 loss gives rise to a leukemic stem cell transcriptional program that is associated with increased HSC self-renewal. The combined effects of these transcriptional changes in the HSC compartment drives the development of a more florid MPN phenotype (Jak2\textsuperscript{VF}/Tet2\textsuperscript{null} mice), as compared to the phenotype that arises when either transcriptional program manifests alone (Jak2\textsuperscript{VF} or Tet2\textsuperscript{null} mice).
In conclusion, we report the effects of homozygous Tet2 loss on Jak2V617F-mediated MPN. Overall, we find accelerated myeloproliferation but no overt fibrotic or leukemic transformation. In aggregate, this work elucidates the functional effects of combined Jak2V617F expression and Tet2 loss in distinct hematopoietic compartments in vivo and provides insights into the mechanisms of clonal dominance and disease progression in MPN.
Acknowledgements

This work was supported by the NIH (K08 HL109734 to AM), the MPN Foundation (AM) and the Leukemia Research Foundation (AM). A.M. has received support from the Jeanne D. Housman Fund for Research on Myeloproliferative Disorders and is a recipient of a Damon Runyon clinical investigator award. EC is a recipient of a Lady Tata Memorial Trust Award and EAR is a recipient of an American Society of Hematology HONORS Award.

Authorship Contributions

AM, EC, RL and BLE designed experiments and interpreted data. AM, LJB, EAR, LP, SE, AK and KB performed experiments. EC performed experiments and analyzed gene expression data. RKS performed experiments and reviewed, interpreted and photographed histopathology. AM and EC wrote the manuscript. All authors reviewed the manuscript.

Disclosure of Conflicts of Interest

None.
References

Figure Legends

**Figure 1. Tet2 loss accelerates the MPN phenotype of Jak2V617F mice**

(A) Spleen weights of age-matched WT, Tet2null, Jak2VF and Jak2VF/Tet2null mice aged 24-30 weeks (mean +/- SEM; n=4 in each group).

(B) Photograph from spleens from Jak2VF and Jak2VF/Tet2null mice.

(C) Histopathologic (HE) sections of spleen from representative WT, Tet2null, Jak2VF and Jak2VF/Tet2null mice.

(D) Frequency of CD71+, Ter119+ erythroid precursor cells in spleen from age-matched WT, Tet2null, Jak2VF and Jak2VF/Tet2null mice (mean +/- SEM; n=4 in each group).

(E) Frequency of Mac1+, Gr1+ myeloid precursor cells in spleen from age-matched WT, Tet2null, Jak2VF and Jak2VF/Tet2null mice (mean +/- SEM; n=4 in each group).

(F) Frequency of CD150+ CD48-lineage^low^Kit^high^Sca1^+^ (LSK) cells (LT-HSC), CD150^−^CD48^−^LSK cells (ST-HSC) and CD48^+^LSK cells (MPP) in spleen from age-matched WT, Tet2null, Jak2VF and Jak2VF/Tet2null mice (mean +/- SEM; n=4 in each group). *p < 0.05, **p < 0.005, ***p < 0.001.

**Figure 2. Tet2 loss influences hematopoietic differentiation within the myeloid progenitor compartment of Jak2V617F mice but is insufficient to induce leukemic transformation**

(A - C) WBC count, hematocrit and platelet counts of age-matched WT, Tet2null, Jak2VF and Jak2VF/Tet2null mice aged 24-30 weeks (mean +/- SEM; n=4 in each group).

(D - F) Relative frequency of common myeloid progenitor (CMP), granulocyte macrophage progenitor (GMP) and megakaryocyte erythroid (MEP) cells in bone marrow from age-matched WT, Tet2null, Jak2VF and Jak2VF/Tet2null mice (mean +/- SEM; n=4 in each group).

(G – I) Histopathologic (HE) sections of bone marrow from representative WT, Tet2null, Jak2VF and Jak2VF/Tet2null mice.
Figure 3. Tet2 loss confers enhanced self-renewal to Jak2V617F HSPC in vitro and in vivo

(A) Colony forming unit (CFU) assays from unfractionated bone marrow derived from wildtype (WT), Jak2VF, Tet2null and Jak2VF/Tet2null mice. Results represent average of triplicate assays (mean +/- SEM). P values for each of the comparisons are indicated in the figure.

(B) % 45.2 donor chimerism assessed in peripheral blood (PB) total WBC from lethally irradiated secondary recipients of Jak2VF, Tet2null or Jak2VF/Tet2null LSK cells competed against an approximately equal number of 45.1 wild-type (WT) LSK cells, measured 4-17 weeks post transplantation (mean +/- SEM; n = 5 in each group).

(C) % 45.2 donor chimerism assessed in peripheral blood (PB) Gr1+ cells from lethally irradiated secondary recipients of Jak2VF, Tet2null or Jak2VF/Tet2null LSK cells competed against an approximately equal number of 45.1 wild-type (WT) LSK cells, measured 4-17 weeks post transplantation (mean +/- SEM; n = 5 in each group).

(D) % 45.2 donor chimerism assessed in peripheral blood (PB) B220+ cells from lethally irradiated secondary recipients of Jak2VF, Tet2null or Jak2VF/Tet2null LSK cells competed against an approximately equal number of 45.1 wild-type (WT) LSK cells, measured 4-17 weeks post transplantation (mean +/- SEM; n = 5 in each group).

(E) % 45.2 donor chimerism assessed in whole bone marrow cells from lethally irradiated secondary recipients of Jak2VF, Tet2null or Jak2VF/Tet2null LSK cells competed against an approximately equal number of 45.1 wild-type (WT) LSK cells, measured 18 weeks post transplantation (mean +/- SEM; n = 5 in each group).

(F) % 45.2 donor chimerism assessed in whole spleen cells from lethally irradiated secondary recipients of Jak2VF, Tet2null or Jak2VF/Tet2null LSK cells competed against an approximately equal number of 45.1 wild-type (WT) LSK cells, measured 18 weeks post transplantation (mean +/- SEM; n = 5 in each group).

(G) % 45.2 donor chimerism assessed in LSK bone marrow cells from lethally irradiated...
secondary recipients of Jak2^{VF}, Tet2^{null} or Jak2^{VF}/Tet2^{null} LSK cells competed against an approximately equal number of 45.1 wild-type (WT) LSK cells, measured 18 weeks post transplantation (mean +/- SEM; n = 5 in each group).

**Figure 4. Jak2V617F expression and Tet2 loss cause distinct and non-overlapping gene expression changes**

(A) Dendrogram constructed from unsupervised hierarchical clustering of all 13 datasets from WT (n=4), Tet2^{null} (n=2), Jak2^{VF} (n=3) and Jak2^{VF}/Tet2^{null} (n=4) LSK cells using Pearson correlation.

(B) Venn diagrams depicting differentially expressed genes in LSK cells from Jak2^{VF}, Tet2^{null} and Jak2^{VF}/Tet2^{null} mice (FDR = 10%, minimum fold change relative to WT samples = 1.3)

(C) Hierarchical clustering of expression profiles of all 12 datasets according to the 17 genes differentially expressed in either Jak2^{VF}, Tet2^{null} or Jak2^{VF}/Tet2^{null} mice relative to wildtype controls. A red-blue color scale depicts normalized gene expression levels (Red: high, Blue: low). Dendrograms were constructed using Pearson correlation.

(D) GSEA demonstrating enrichment for STAT5A target genes in Jak2^{VF} and Jak2^{VF}/Tet2^{null} LSK cells but not in Tet2^{null} LSK cells (Top row = STAT5A targets UP; bottom row = STAT5A targets DOWN).

(E) GSEA demonstrating enrichment of a HSC self-renewal signature in Tet2^{null} and Jak2^{VF}/Tet2^{null} LSK cells but not in Jak2^{VF} LSK cells.
Figure 2
Figure 3
Figure 4
Distinct effects of concomitant Jak2V617F expression and Tet2 loss in mice combine to promote disease progression in myeloproliferative neoplasms