Single cell approaches identify the molecular network driving malignant hematopoietic stem cell self-renewal in myeloproliferative neoplasms

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Running title: Single cell approaches identify molecular regulators of HSC self-renewal in MPNs

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Key points:

1. Single cell approaches identify regulators of malignant HSC self-renewal
2. Identification of novel roles for *Bmi1*, *Pbx1* and *Meis1* in myeloproliferative neoplasms

Abstract

Recent advances in single cell technologies have permitted the investigation of heterogeneous cell populations at previously unattainable resolution. Here we apply such approaches to resolve the molecular mechanisms driving disease in mouse hematopoietic stem cells (HSCs), using JAK2V617F mutant myeloproliferative neoplasms (MPNs) as a model. Single cell gene expression and functional assays identified a subset of JAK2V617F mutant HSCs that display defective self-renewal. This defect is rescued at the single HSC level by crossing JAK2V617F mice with mice lacking TET2, the most commonly co-mutated gene in MPN patients. Single cell gene expression profiling of JAK2V617F-mutant HSCs revealed a loss of specific regulator genes, some of which were restored to normal levels in single TET2/JAK2 mutant HSCs. Of these, *Bmi1* and, to a lesser extent, *Pbx1* and *Meis1,* overexpression in JAK2-mutant HSCs could drive a disease phenotype and retain durable stem cell self-renewal in functional assays. Together, these single cell approaches refine the molecules involved in clonal expansion of MPNs and have broad implications for deconstructing the molecular network of normal and malignant stem cells.

Introduction

Billions of blood cells are produced and destroyed each day[1] and hematopoietic stem cells (HSCs) must provide sufficient progenitors for hematopoiesis while also maintaining their numbers. Malignancy results from disturbances in this balance, leading to the production and expansion of HSC clones with differentiation and/or proliferation abnormalities[2-4]. Genetic mutations often drive these changes and have been a major focus for cancer researchers[5]

The molecular function of individual mutations and their role in disease is less clear and the combinatorial action of multiple mutations is nearly completely uncharted. In most malignancies, there are numerous recurrent driver mutations, resulting in a wide array of mutation combinations, presenting significant challenges for discerning disease relevant biology[6]. The myeloproliferative neoplasms (MPNs) are genetically less complicated than the vast majority of malignancies[7] and are an excellent model for delineating the impact of mutation combinations in early tumorigenesis. A single acquired JAK2V617F mutation was reported to be present in most MPN patients[8-11] and subsequent studies identified a number of recurrent mutations found to collaborate with JAK2V617F[12-14], suggesting that on its own JAK2V617F is insufficient to initiate disease.

Several groups have developed JAK2V617F knock-in mouse models to study the effect of physiological levels of JAK2V617F (reviewed in [15]). Although not phenotypically identical, these models uniformly develop an MPN phenotype with increased myeloid cell production in the erythrocytic and/or megakaryocytic lineage with some increases in granulocytic/monocytic lineages[15,16]. These phenotypes are transplantable and can transform to more severe forms of disease (e.g., myelofibrosis and/or AML). When HSC self-renewal was tested in serial transplantation experiments, HSCs from JAK2V617F knock-in models did not outcompete wild-type HSCs, again supporting the notion that JAK2V617F on its own was insufficient to initiate disease[17-19].

The most commonly co-mutated gene in JAK2V617F-positive MPNs is *TET2* where loss-of-function mutations are thought to give a clonal advantage to the HSCs[13,20,21]. Recently, both a transgenic and a heterozygous JAK2V617F knock-in model were crossed with a *Tet2* knock-out and formally demonstrated that loss of TET2 could confer a self-renewal advantage on JAK2V617F-mutant HSCs, resulting in a robust serially transplantable disease[22,23]. The molecular basis for this self-renewal advantage in long-term HSCs (LT-HSCs) remains undetermined and is complicated by heterogeneity in the HSC compartment.

In order to resolve this heterogeneity and to identify which molecules drive

the increased self-renewal capacity of a malignant clone, we undertook a single cell approach. By profiling homozygous JAK2V617F-mutant HSCs in cell biological and molecular assays, we identify key HSC self-renewal regulators that were lower or absent in a subset of single-mutant HSCs. Our results highlight the power of single cell approaches for identifying molecular profiles and for interrogating essential components responsible for each aspect of disease phenotype.

Methods

Mice

Homozygous JAK2V617F (JAK HOM) knock-in mice[19] were crossed with *Tet2* KO (TET HOM) mice from Ko et al., 2011[20]. Full details are available in Supplemental Methods.

Isolation of E-SLAM HSCs, *in vitro* assays, and expression profiling

E-SLAM cells were isolated as described previously[24] and full details of their isolation and culture are found in the Supplemental Methods. Single-cell gene expression analysis was performed as described previously[28] using the Fluidigm BioMark system with full details in the Supplemental Methods.

Bone marrow transplantation assays and analysis

Primary and secondary transplantation assays were performed as described previously[19] and full details of HSC frequencies, method of transplantation and peripheral blood analysis are in the Supplemental Methods.

Overexpression Assays

Small pools (1500-3600 cells) of $CD45^{+}Lin^{-}CD150^{+}CD48^{-}$ hematopoietic stem and progenitor cells (HSPCs) were isolated and transduced with candidate genes as described in the Supplemental Methods.

Patient stem and progenitor cell assays

Fresh blood samples were collected from MPN patients with JAK2 and TET2 mutations diagnosed according to British Committee for Standards in Haematology (BCSH) guidelines. 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. Details of HSPC isolation, *in vitro* assays and gene expression studies are found in the Supplemental Methods.

Results

JAK2^{V617F} drives increased proliferation and differentiation of HSCs *in vitro*

To determine the impact of JAK2V617F expression on single HSCs we isolated single CD45$^+$EPCR$^+$CD48$^-$CD150$^+$ (E-SLAM) cells (40-50% HSCs by long-term transplantation studies[24]) from a knock-in mouse model expressing either one (JAK HET) or two (JAK HOM) copies of human JAK2V617F. HSCs were cultured in conditions that support HSC self-renewal (Figure 1A)[35], in which cell division kinetics, colony size, and mature cell production were assessed. JAK HET HSCs did not show significant differences in early divisional kinetics or cell cycle entry but did give rise to significantly larger clones (Supplemental Figure 1A), in agreement with previous data in the inducible MX1Cre knock-in model[25]. However, when compared to WT cells, JAK HOM HSCs exited quiescence faster and had shorter cell cycle transit time, with an increased proportion of HSCs (~20%) having completed the first and second division at 48 hours compared to WT controls (Figure 1B-C) ($p<0.0001$). These data were supported by single cell time-lapse imaging and tracking of HSCs isolated from WT and JAK HOM animals (n = 2 mice, data not shown). Assessment of clonal progeny of individual HSCs showed that colonies from JAK HOM HSCs were on average larger (Supplemental Figure 1A), more differentiated (Figure 1D) and contained proportionally fewer progenitor cells (KSL: c-Kit$^+$, Sca1$^+$, Lin$^-$) (Figure 1E) than clones grown from HSCs obtained from WT littermate controls.

We next undertook Cobblestone-area-forming cell (CAFC) assays, an *in vitro* surrogate for HSC transplantation where test cells are cultured on a stromal cell line for >5 weeks and assessed for the presence of haematopoietic colonies[26]. HSCs with less durable self-renewal tend to form colonies at early time points[36]. HSCs from JAK HOM mice gave rise to an increased number of early-CAFCs and a reduced number of mid- and late-CAFCs (Figure 1F), suggesting that JAK HOM HSCs have altered stem-cell function, in agreement with transplantation experiments showing reduced numbers of HSCs in JAK HOM mice[19]. Taken together, these data show that JAK2V617F provides an advantage for progenitor cells resulting in overproduction of differentiated cells, and that this expansion comes at the expense of LT-HSC self-renewal.

Single cell expression profiling identifies mutant HSCs with reduced expression of self-renewal regulators

To determine the molecular drivers of HSC functional abnormalities, we first performed gene expression arrays on sorted E-SLAM HSCs from WT, JAK HET and

JAK HOM mice. While the hyper-proliferation observed in progenitors could be potentially explained by modest changes in genes regulating the cell cycle, no clear differences emerged on a bulk level that could account for the loss of HSC self-renewal observed (ArrayExpress accession E-MTAB-6878). Since the data in Figure 1 identified a fraction of JAK2V617F-homozygous HSCs with aberrant behavior *in vitro,* single cell gene expression profiling was performed to determine whether some mutant HSCs had altered expression of self-renewal genes. Single phenotypic HSCs from 3 JAK HOM (n=277) and 3 WT (n=465) mice were analyzed for the expression of 39 transcription factors and self-renewal regulators by multiplexed qPCR (genes listed in Supplemental Table 1). Principal component analysis (Figure 1G) revealed a subset of cells, in which JAK HOM cells are over-represented, that are marked by reduced expression of *Meis1*, *Smarcc2*, *Bmi1*, *Pbx1*, *Sfpi1*, *Runx1*, *Hoxb4*, *Myb*, *Lmo2*, as indicated by loadings plot shown in Supplemental Figure 2A. Single cell analysis further shows that this subset of cells has reduced expression of most, if not all, of these regulators in each cell compared to cells in the larger cluster (Supplemental Figure 2B), suggesting that reduced expression of these genes might contribute to the loss of competitive reconstitution ability observed in JAK2V617F HSCs.

Compound loss of *Tet2* does not reverse JAK2V617F-induced HSC hyper-proliferation

Loss of *Tet2* is the most common collaborating mutation observed in JAK2V617F positive MPNs[12]. Previous studies have shown that loss of *Tet2* leads to increased re-plating capacity *in vitro* and increased self-renewal *in vivo*[20,21]. When heterozygous or transgenic JAK2V617F knock-in mice were crossed with TET2 knockout mice, an accelerated MPN phenotype was observed and BM transplantations showed a strong self-renewal advantage over WT and JAK2V617F single-mutant controls[22,23]. Homozygous JAK2V617F knock-in mice have been previously documented to have a more substantial loss of HSC self-renewal[19] and have not previously been combined with other mutations. To first test whether loss of TET2 function could rescue the HSC cycling and hyper-proliferation phenotypes observed in Figure 1, we crossed the homozygous JAK2V617F knock-in model[19] with a TET2 knock-out model[20] (Figure 2A).

Loss of TET2 alone did not alter exit from quiescence (Figure 2B), divisional kinetics (Figure 2C), colony size (Figure 2D) or the proportion of differentiated/progenitor cells (Figure 2E-F) compared to WT controls. However when loss of TET2 was combined with JAK2V617F homozygosity, entry into cell

cycle was accelerated (Figure 2B), cell cycle transit time was increased (Figure 2C), and average clone size was increased (Figure 2D), similar to the phenotypes observed in JAK2V617F-homozygous HSCs. Clones derived from double-mutant HSCs also had an increased proportion of mature lineage marker positive cells (Figure 2E) and a decreased proportion of primitive progenitor (KSL) cells (Figure 2F). Overall therefore, compound loss of TET2 did not rescue the JAK2V617F-induced cycling and hyper-proliferation phenotypes observed in single *in vitro* HSC functional assays.

In order to validate these findings in patient samples, we isolated human $Lin^-CD34^+CD38^-CD90^+CD45RA^-$ cells (human HSCs) from MPN patients. Single human HSCs were cultured in stem cell maintenance conditions and divisional kinetics tracked for 10 days. Individual clones were plated in a colony forming cell (CFC) assay for a further 14 days before being harvested for genotyping (Figure 3A), allowing retrospective assignment of mutational status. No difference was observed between TET2-mutant and non-mutant (WT) cells in the time to 1^{st} or 2^{nd} cell division (Figure 3B-C) and both JAK2 single and JAK2/TET2 double mutant cells had faster divisional kinetics compared to non-mutant cells, with an increased proportion having completed 1^{st} and 2^{nd} divisions at 72 hours (JAK $p<0.01$; JAK TET $p<0.001$) (Figure 3B-C). However, JAK2 single and double-mutant clone size was not significantly affected compared to non-mutant cells (Figure 3D), perhaps due to the slower speed of human HSCs that take several days to begin dividing. Notably, day 10 clones derived from human HSCs with a TET2 mutation were on average smaller than non-mutant and JAK2-mutant cells (Figure 3D).

Together these data show in both mouse models and patient samples that JAK2V671F increases proliferation of primary HSCs in the presence or absence of a TET2 mutation, and that TET2 loss on its own does not confer a proliferation advantage on HSCs *in vitro*.

Homozygous JAK2V617F combined with loss of TET2 drives a robust transplantable myeloproliferative phenotype *in vivo*

Competitive transplantation was undertaken to assess the relative ability of bone marrow (BM) cells from each genotype to initiate an MPN phenotype. Despite a red cell phenotype in the steady state JAK HOM mouse, an MPN phenotype is not observed in transplantation experiments using low cell numbers[19], likely due to low levels of donor cell repopulation. Loss of *Tet2* on its own was also insufficient to give an MPN phenotype, although donor cell repopulation was quite high throughout the experiment. In combination, low cell numbers of double-mutant BM

were sufficient to drive a robust serially transplantable MPN phenotype, where hyper-proliferation combined with high donor chimerism to give persistently high hematocrit (Figure 4A) and hemoglobin (Figure 4B) levels even at limiting cell doses (Supplemental Figure 3) and the phenotype was sustained in secondary transplantations (Supplemental Figure 4). No consistent significant differences were observed in other blood cell parameters throughout the transplantation experiments, although recipient numbers were not sufficient to exclude the possibility of mild differences or heterogeneous phenotypes.

In both the primary (Figure 4C) and secondary (Figure 4D) transplantations, recipients of JAK HOM BM have low donor chimerism at 16 weeks post-transplantation, in line with previous data where low numbers (e.g., 1-5 x 10^5) of BM cells were transplanted[19]. Recipients of TET HOM BM on the other hand were successfully repopulated through both primary and secondary transplantations, comparable to WT BM transplantation. Recipients of double mutant (JAK HOM TET HOM) BM also show persistent donor cell contribution similar to WT, demonstrating that TET2 loss rescues the HSC disadvantage observed in JAK HOM BM. Of note, the frequency of phenotypic E-SLAM HSCs in donor cell suspensions was highest in TET KO mice

These transplantation data were further supported by the number of phenotypic HSCs present in BM samples in primary and secondary recipients (Figure 4E-F). Notably, although primary recipients of JAK HOM BM had a normal number of phenotypic HSCs, these cells were clearly unable to repopulate secondary recipients (Figure 4F). Finally, we performed a limiting dilution transplantation assay to determine the minimal number of double-mutant cells required to initiate disease. Chimerism levels were similar to transplantations of WT BM cells (Supplemental Figure 3A) and doses of 100,000 and 50,000, but not 10,000 double-mutant cells were able to sustain a red cell phenotype up to 3 months (Supplemental Figure 3B). These results estimate the disease-initiating cell frequency as ~1 in 33,000 double mutant WBM cells, a number similar to the expected LT-HSC frequency in normal BM (1 in 20,000).

Single- and double-mutant HSCs have distinct molecular profiles of self-renewal regulators

In order to understand the molecular mechanism of increased self-renewal that TET2 loss confers on JAK2 mutant HSCs, single cell gene expression profiling was performed on TET2 single mutant (TET HOM, n=73) and double mutant (JAK HOM TET HOM, n=99) HSCs and these were compared to WT (n=561) and JAK HOM

(n=376) HSCs (Supplemental Figures 5). Hierarchical clustering shows five broad molecular clusters of cells (Supplemental Figure 5A). Clusters I and II are significantly enriched ($p < 0.05$ and $p < 0.005$) for JAK HOM HSCs, Cluster III is significantly enriched ($p < 0.001$) for TET HOM HSCs and Cluster IV is significantly enriched for WT ($p < 0.05$) and double mutant cells ($p < 0.05$). Interestingly, Cluster II does not have any TET-single or double-mutant HSCs, a result re-enforced by the PCA in individual mice (Supplemental Figure 6).

Single HSCs of all genotypes were next displayed on a single PCA plot and t-SNE analysis to help visualise molecular clusters and identify the genes driving the differences (Figure 5A and Supplemental Figure 7A). WT HSCs existed in all regions of the molecular landscape, whereas JAK HOM HSCs were over-represented in a specific region (top left, Figure 5B) and this region was almost completely devoid of cells lacking TET2 expression, suggesting that it is a unique molecular region exclusive to a minority of WT HSCs and a larger proportion of JAK HOM HSCs. The majority of these cells are also found in Cluster II described above. The single-mutant TET HOM HSCs (Figure 5C) are predominantly found in a single area of the PCA plot (top right) and the double-mutant HSCs are enriched in a different region (bottom right, Figure 5D), although a small fraction of double-mutant cells are also found with the single-mutant TET HOM HSCs. The genes negatively associated with each of these regions are indicated in the loadings plot (Figure 5E) implicating genes such as *Bmi1*, *Runx1*, and *Pbx1* as being involved in the self-renewal of HSCs (i.e., associated with TET HOM and double-mutant genotypes) rather than proliferation (i.e., associated with JAK HOM HSCs). The individual expression patterns of these genes across genotypes is displayed by violin plots in Supplemental Figure 7B. We also assessed gene expression levels in $CD34^{+}CD38^{-}$ HSC-enriched cell fractions from primary patient samples bearing JAK2V617F and/or TET2 mutations for these candidates and did not observe genotype-specific increases in these candidates (Supplemental Figure 8). This mirrors our results in the mouse where bulk studies could not detect differences and is also complicated by the large number of non-HSCs and non-mutated cells in the $CD34^{+}CD38^{-}$ fraction.

The single cell profiling provides molecular evidence of distinct HSC states and they parallel the unique functional features of HSCs with different genotypes – JAK HOM HSCs can exist in a hyper-proliferative, primed state that exits quiescence more rapidly, TET HOM HSCs are in a slow-dividing, durably self-renewing state, and the majority of double-mutant HSCs are both highly proliferative and possess durable self-renewal (Figure 5F). Since each distinct molecular profile could be

identified in WT HSCs – although sometimes at a low frequency – these data suggest that WT HSCs are a heterogeneous mix of cells in distinct proliferative and self-renewing states. JAK2 and TET2 mutations disrupt the balance of HSC molecular subtypes by restricting the number of HSC states and subsequently, via their downstream progeny, lead to an MPN phenotype.

Overexpression of *Bmi1*, *Pbx1*, or *Meis1* can sustain an MPN phenotype in JAK HOM HSCs *in vivo*

Since these experiments were performed on single HSCs, the clustering diagram and differential enrichment by genotype (Supplemental Figure 5A-B) in combination with the loadings plot (Figure 5E) can be used to identify which genes might define molecular subtypes and identify candidates for re-balancing the HSC pool. For example, double-mutant cells (which have restored self-renewal but still proliferate rapidly) cluster away from JAK HOM single-mutant cells (which have high proliferation but lack durable self-renewal), allowing us to narrow the list of candidate genes that drive the self-renewal advantage (e.g., increased *Bmi1*, *Pbx1*, *Runx1*, and *Gfi1* and decreased *Gfi1b* and *Dnmt3a*). Assessment of individual cells in the clustering diagram (Supplemental Figure 5A) confirm these genes are down-regulated or absent in single JAK HOM HSCs.

To test which genes might modulate the self-renewal of JAK HOM HSCs, we performed lentiviral overexpression and transplantation assays. $CD45^{+}Lin^{-}CD150^{+}CD48^{-}$ hematopoietic stem and progenitor cells (HSPCs) isolated from either JAK HOM or WT mice were transduced with a lentivirus containing a GFP reporter and the *Bmi1, Pbx1, Runx1* or *Meis1* genes. Three days post-infection, cells were re-sorted for presence of GFP and transplanted into recipient mice to monitor for disease phenotype (Figure 6A).

The transplantation of limiting doses of JAK2-mutant cells gives a phenotype (higher hematocrit and hemoglobin) that fades over time as donor chimerism is reduced due to an HSC self-renewal defect[19]. As expected, both WT and JAK HOM HSPCs infected with an empty vector lentivirus had a comparable engraftment (Figure 6B-C, purple line) but the JAK HOM chimerism started to decrease after 16 weeks post-transplantation and no phenotype was visible at late timepoints (Figure 6D-E), most likely as a result of donor HSC exhaustion. *Runx1* overexpression drove both WT and JAK HOM cells to differentiate, with 8 of 10 mice showing no almost donor cell contribution at 24 weeks post-transplantation (Figure 6B-C).

Some mice transplanted with JAK2-mutant cells overexpressing *Meis1* (1 of 5) or *Pbx1* (2 of 5) did not sustain donor repopulation out to 24 weeks whereas all

mice over-expressing *Bmi1* (5 of 5) were successfully repopulated. Since 3-day HSPC-derived cultures represent a mix of HSCs and progenitors, it is possible that the *Meis1* and *Pbx1* failed grafts were due to an inability to drive disease from a transduced progenitor cell, whereas *Bmi1* may be able to sustain donor repopulation from a broader range of cell targets. Overexpression of *Meis1*, *Pbx1* or *Bmi1* in JAK2-mutant HSPCs resulted in hematopoietic phenotypes and in some cases, resulted in the premature death of recipient animals (1 *Bmi1*, 1 *Pbx1*). Blood samples taken prior to death revealed high hematocrit and hemoglobin in both cases and post-mortem examination revealed splenomegaly in the mouse receiving *Bmi1*-transduced cells. Repopulated mice from both *Meis1*-transduced and *Bmi1*-transduced JAK2-mutant cells sustained a red cell phenotype as indicated by significant increases in hematocrit (0.028 *Bmi1*; 0.012 *Meis1*) and hemoglobin (0.056 *Bmi1*; 0.044 *Meis1*). Together, these data show that increased *Bmi1* expression, and to a lesser extent *Meis1* and *Pbx1,* can co-operate with JAK2V617F to initiate and sustain a myeloproliferative phenotype.

Discussion

For a leukemia to develop and maintain itself from a single HSC, the HSC and its progeny must be able to thrive relative to the endogenous set of non-malignant HSCs. The self-renewal and proliferation capacity of each HSC type are therefore critical properties to understand. Using single cell gene expression profiling in combination with single cell *in vitro* assays and genetic rescue experiments, this study identifies which self-renewal regulators are involved in partnering with JAK2V617F to drive a myeloid malignancy. We identify distinct molecular profiles that correlate with observed HSC functional properties in mouse models and primary patient samples where JAK2V617F drives hyper-proliferation, TET2 loss increases self-renewal and double mutant HSCs have increased self-renewal and hyper-proliferation. Finally, we test several candidates functionally and reveal a novel role for *Bmi1* in sustaining the malignant HSC population.

Several knock-in mouse models have been used to study the impact of JAK2V617F on self-renewal of HSCs by undertaking secondary transplantation experiments[17-19]. Each of these studies showed that heterozygous JAK2V617F on its own did not confer a self-renewal advantage to HSCs in competitive secondary transplantations. Three possible explanations for the HSC self-renewal defect uniquely observed in our JAK HOM model are 1) this is the only model completely lacking WT JAK2 in the HSCs tested in competitive serial transplantations; 2) our model expresses human, not mouse, JAK2 V617F which might result in different

biological consequences; and 3) HSC regulator expression (e.g., *Bmi1*, *Pbx1*, *Meis1*, etc) could be different in other knock-in models. Overall, the lack of advantage in competitive serial transplantations suggests that collaborating hits are required to give a long-term advantage over non-mutant HSCs. These data were further supported by patient-derived xenograft experiments which showed that HSCs from patient samples were able to sustain long term engraftment if they had both TET2 and JAK2 mutations compared to those with a JAK2 mutation alone[13]. Evidence for genetic collaboration with JAK2V617F in mouse models was recently published[22], where TET2 knockout mice were crossed with heterozygous JAK2V617F knock-in mice[37], resulting in a serially transplantable, highly competitive myeloproliferative disease, but the molecular and cellular basis for this interaction in HSCs remained unclear. On its own, homozygous JAK2V617F expression in knock-in mice was shown to induce an MPN phenotype at steady-state, but disease could not be sustained in a transplantation setting due to a strong self-renewal defect[19]. Our study demonstrates that even this strong HSC self-renewal defect could be rescued by loss of TET2 and reveals the cellular consequences of JAK2 and/or TET2 mutations at single cell resolution.

Our single cell approach further delineates the key molecular players for maintaining malignant HSCs and JAK2V617F-induced disease, while isolating for the first time the specific molecular drivers of mutant HSC proliferation and self-renewal. Approximately 25% of the JAK HOM HSCs lack the key self-renewal regulators identified in this study meaning that, as a population, there is a constant interplay between these two states. One potential explanation for the increased proportion of cells lacking HSC self-renewal regulators in the JAK2 homozygous mouse is that the phenotypic LT-HSC gate (E-SLAM) captures a different proportion of HSCs compared to contaminating progenitors than its wildtype littermates (e.g., there are more contaminating non-HSCs). Several lines of evidence suggest this would not be the case including the similar reduction in HSC number using an alternative sorting gates ($Lin^-Sca1^+Kit^+CD34^-Flk2^-$[19]) and the observation that HSCs with similar molecular programmes are present in WT mice (albeit at a lower frequency) but not in the TET KO or double-mutant cells (Figure 5). In either case, since our molecular study represents a static picture taken at a single developmental stage (3-4 months) and does not give any information regarding the dynamics of the HSC compartment, it is not currently possible to resolve the relationship of impaired to non-impaired HSCs and we cannot yet ask questions of primacy or relatedness.

Functional studies *in vivo* showed that *Runx1* overexpression resulted in

HSC exhaustion in both WT and JAK HOM cells. In contrast, each of *Meis1*, *Pbx1* and *Bmi1* showed some form of hematopoietic phenotype when over-expressed in JAK HOM cells ranging from a lethal hematologic disease to sustained increases in hematocrit and hemoglobin. *Bmi1* overexpression in JAK HOM cells was the most robust, resulting in a more sustained HSC repopulation and a consistent MPN phenotype, aligning with previous studies which demonstrated that *Bmi1* overexpression drives a robust self-renewal increase in primary HSCs[38]. Notably, the phenotype is not as strong as the JAK2/TET2 double-mutant mouse which may indicate that a combination of genes is required or that TET2 loss alters the epigenetic landscape necessary for MPN development.

Of note, *Bmi1* expression was not changed in the $CD34^{+}CD38^{-}$ HSC enriched fraction of MPN patient samples with JAK2 and TET2 mutations and this could be due to 1) the vast majority of cells being non-HSCs; and 2) Non-mutant cells with normal expression levels masking any changes in *Bmi1*; and 3) *Bmi1* levels only changing upon transformation to more severe disease (e.g., AML). The latter would be consistent with the fact that *Bmi1* is upregulated in blast crisis CML, MDS, and AML and is associated with a worse prognosis[39-42], suggesting that increased *Bmi1* expression may play a role in advanced disease stages.

Our data suggest that a JAK2V617F mutation would not be sufficient to initiate disease on its own; however, a substantial cohort of JAK2V617F-positive MPN patients with no known additional driver mutation do exist[12]. If JAK2V617F does not give an intrinsic self-renewal advantage, some additional factor(s) likely influence a JAK2-mutant HSC's outgrowth relative to the other HSCs in the body. Several possible explanations exist including: 1) additional genetic or epigenetic drivers not yet identified by exome sequencing (e.g., lncRNAs, enhancer elements, etc); 2) inherited genetic risk factors where some patients are more susceptible to cells gaining a clonal advantage due to defective non-mutant HSCs; and 3) micro-environmental factors (the physical niche itself or secreted cytokines) that encourage the outgrowth of mutant subclones[43,44]. It is also interesting to note that JAK2V617F mutations are found in a substantial percentage of older individuals with no obvious blood phenotype[45-47]. This latter finding is consistent with a low number of JAK2V617F HSCs without a significant clonal advantage giving rise to more mature cells on a per-HSC basis, but an insufficient number to create an observable phenotype in an individual.

Single cell technologies at the cell biological and molecular level have now reached a level where more sophisticated questions about the molecules operating individually or collaboratively can be asked in mouse models of disease and, to a

lesser extent, in patient samples. Combinatorial studies of HSCs from mouse models with distinct and easily assayable properties permit dissection of molecular programs of each biological phenotype. These studies set the stage for targeting molecules that might guide HSC fate choice at the population level to restore balance throughout the hematopoietic system.

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Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: MSS, DGK, NKW, JuL, DL, TS, ARG. Performed the experiments: MSS, JuL, JiL, DL, JF, MB, CAO, JCMP, DCP, TLH, JF, NKW, DGK. Analyzed the data: MSS, DGK, NKW, DCP, JuL, JiL. Contributed reagents/materials/analysis tools: AR. Wrote the paper: MSS DGK with input from DL, BG, ARG, TS.

Figure Legends

Figure 1: JAK2V617F induces increased proliferation and differentiation in single HSC-derived clones.

(A) Schematic of single cell *in vitro* cultures. Single $CD45^+EPCR^+CD48^-CD150^+$ (E-SLAM) cells were sorted into individual wells and cultured for 10-14 days in STEMSPAN with 10% FCS, 300ng/ml SCF and 20ng/ml IL-11 and assessed for proliferation, cell cycle kinetics and differentiation. (B, C) Daily cell counts revealed that JAK2V617F homozygous HSCs (red line) display faster cell cycle kinetics as indicated by a shorter time to first and second division, $p<0.0001$ at 48 hours (three independent experiments). (D, E) Homozygous JAK2V617F HSCs (red bars) give rise to an increased number of differentiated cells (positive for $Gr1^+/Mac1^+$; Lin^+) ($p=<0.0001$) and a reduced number of stem/progenitor cells ($Gr1^-$, $Mac1^-$, c-Kit^+, $Sca1^+$, LSK) ($p<0.0001$). WT n=100, JAK HET n=189, JAK HOM n=154 (three independent experiments). (F) Compared to WT HSCs, JAK HOM HSCs have increased early forming cobblestone area forming cells (CAFCs) ($p= 0.0086$). WT n=61, JAK HOM n=62 (two independent experiments). Asterisks indicate significant differences by Student t test for D+E, and by chi-squared for B, C and F (*$p< 0.05$; **$p< 0.01$, *** $p <0.001$). Data are shown as mean ± SEM. (G) Principal component (PC) analysis of all HSCs calculated from the 39 genes analyzed. PC1 and PC2 account JAK2V617F HSCs are indicated by red circles and WT HSCs are indicated by blue diamonds. A cluster of cells on the right hand side of the graph is enriched for JAK2V617F HOM HSCs and have reduced expression of several important hematopoietic genes (*Meis1*, *Smarcc2*, *Bmi1*, *Pbx1*, *Sfpi1*, *Runx1*, *Hoxb4*, *Myb*, *Lmo2*). Axes are in arbitrary units. WT n=465 JAK HOM n=277.

Figure 2: Loss of Tet2 does not reverse JAK2V617-induced hyper-proliferation in single HSCs.

(A) Table summarising the mutations in each genotype and the colour code for the remainder of the figures. (B, C) The cumulative times to first (B) and second (C) division are shown and were determined by manual daily cell counts, scoring the presence of 1, 2, or 3-4 cells. Loss of *Tet2* on its own (green line) does not affect cell cycling compared to WT (blue line), whereas JAK2V617F on its own (red line) increases proliferation ($p<0.0001$). Compound mutants (JAK HOM TET HOM, orange line) behave like JAK2V617F single mutant HSCs with a faster time to first and second division respectively ($p<0.0001$). Data are shown as mean ± SEM. WT n=572, JAK HOM n=439, TET HOM n=566, JAK HOM TET HOM n=435 and are from 3-6 independent experiments (D) Colony size after 9 days in culture. JAK2V617F

and double mutant HSCs give rise to a higher frequency ($p<0.0001$ and <0.0001) of large colonies compared to WT controls. Colonies were categorised as very small (>50), small (51-500), medium (501-10,000) or large (>10,000). WT n=572, JAK HOM n=439, TET HOM n=566, JAK HOM TET HOM n=435. (E, F) After 10 days in culture, colonies were analyzed by flow cytometry for differentiation markers. Colonies with JAK2V617F (JAK HOM (red bars) and JAK HOM TET HOM (orange bars)) have an increased frequency of Lin^+ cells ($Ly6g^+$, $Mac1^+$)(JAK HOM $p<0.0001$, JAK HOM TET HOM $p=0.0177$). Double mutants (orange bars) and JAK HOM (red bars) show a reduced frequency of KSL ($Ly6g^-$, $Mac1^-$, $c\text{-}Kit^+$, $Sca1^+$) cells compared to WT (blue bars) and TET HOM (green bars) (JAK HOM TET HOM $p=0.001$, JAK HOM $p<0.0001$). WT n=165, JAK HOM n=178, TET HOM n=154, JAK HOM TET HOM n=145 (three independent experiments).

Figure 3: JAK2 V617F-mutant patient HSCs proliferate faster than TET2-single mutant and non-mutant cells.

(A) Schematic of experimental design. Single $Lin^-CD34^+CD38^-CD90^+CD45RA^-$ cells were sorted from MPN patient peripheral blood samples (n=12, 3 ET, 5 PV. 4 sMF) into 96 well plates and cell counts were performed daily for 10 days. On day 9-10, clones were harvested and plated in a colony forming cell assay and the colonies produced were harvested for genotyping. Several patient assays were originally performed for a previous study[34] and were pooled together with newly generated data. (B, C) Human HSCs with a TET mutation alone (n=2 patients, green line) have similar divisional kinetics to non-mutant HSCs (blue line), whereas cells with a JAK2 V617F mutation alone (n=10 patients, red line) or both mutations (orange lines) had a reduced time to first (B) and second (C) divisions (higher proportion completed division at 72hrs, JAK $p<0.01$, JAK TET $p<0.001$). WT n=114, JAK n=108, TET n=40, JAK TET n=183. Patients with genotyped colonies: WT N=11, TET alone N=2, JAK alone N=11, JAK TET together N=8. (D) Compared to WT cells, cells with TET mutations gave rise to a higher proportion of very small clones (<50 cells) ($p<0.0001$). WT n=114, JAK n=108, TET n=40, JAK TET n=183.

Figure 4: Double JAK/TET mutant cells have a clonal advantage and result in a sustained myeloproliferative phenotype in transplantation.

Bone marrow transplantations were performed and peripheral blood was analyzed for donor cell chimerism and blood parameters. Only recipients of double mutant bone marrow have persistently high haematocrit (A) ($p<0.01$ at 16 weeks) and haemoglobin (B) ($p<0.01$ at 16 weeks). Donor cell chimerism at 16 weeks in

primary (C) and secondary (D) transplantations is displayed. Whereas JAK2V617F cells (red bars) are less competitive relative to WT cells (blue bars) ($p<0.01$), those with a single TET mutation (green bars) or double mutants (JAK HOM TET HOM, orange bars) were similar to WT cells, indicating no deficiency in self-renewal. (E, F) Mean proportion of E-SLAM HSCs in donor cells in bone marrow, 24 weeks post transplantation, from primary (E) and secondary (F) recipients. In double mutant recipients, E-SLAM numbers were not different compared to WT, whereas JAK2 single mutant recipients have lost both phenotypic and functional HSCs.

Figure 5: Single cell gene expression profiling reveals distinct molecular clusters of single and double mutant HSCs with altered self-renewal and proliferation.
Cells from all four genotypes were assessed by single cell multiplexed qPCR and each PCA plot displays all single cells analyzed (with one population highlighted in each plot) (A-D) Principal component analysis displays on a single plot, HSCs from WT (n=561, blue circles), JAK HOM (n=376, red circles), TET HOM (n=73, green circles), JAK HOM TET HOM (n=99, orange circles). Notably, WT cells are present across the entire molecular landscape whereas single- and double-mutant HSCs are enriched in specific regions. (E) Loadings plot for PCA indicating the key defining molecular features of each region with the distance from the centre indicating the negative correlation with a cell type (e.g., cells in the top right lack *Vwf*). (F) Illustration depicting the different cell characteristics associated with each region of the molecular landscape.

Figure 6: Overexpression of *Bmi1*, *Meis1*, and *Pbx1* in JAK2-mutant HSCs enhance MPN-like phenotype *in vivo*. (A) Schematic of candidate gene overexpression transplants. Bulk $CD45^+Lin^-CD48^-CD150^+$ (HSPC) cells (300-800 per mouse) were sorted from WT and JAK HOM mice and infected with lentivirus carrying no gene (empty vector) or lentivirus carrying genes to overexpress *Bmi1*, *Pbx1*, *Runx1*, or *Meis1* (two independent experiments). Three days after infection GFP^+ cells (300-2000) were isolated and transplanted into recipient mice. Serial analysis of peripheral blood was performed to assess chimerism (B and C, n=4-5 recipients) and blood cell parameters (D and E). Hematocrit (Hct) and hemoglobin (Hgb) at 20 weeks post-transplantation for JAK HOM cells over-expressing each gene, compared to WT cells transduced with the same construct (D and E). Successfully repopulated JAK HOM cells over-expressing either of *Meis1* or *Bmi1* display a strong erythrocytic phenotype compared to JAK HOM cells transduced

with the empty vector with * indicating $p<0.05$ and a dark circle indicating a mouse which died prior to the 20-week timepoint (unrelated to transplantation in the mice receiving Runx1-transduced and the non-phenotypic Pbx1-transduced cells).

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