The Biological Influence of Mutation Order on Myeloproliferative Neoplasms

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Running title: Order of mutation acquisition impacts disease evolution

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ABSTRACT

Background: Cancers result from accumulation of somatic mutations and their properties are thought to reflect the sum of these mutations. However, little is known about the consequences of altering the order of mutation acquisition.

Methods: Mutation order was determined in myeloproliferative neoplasm patients by genotyping hematopoietic colonies or next generation sequencing. Stem and progenitor cells were isolated to study the effect of mutation order on mature and immature hematopoietic cells.

Results: Age of presentation, acquisition of JAK2V617F homozygosity and the balance of immature progenitors were all influenced by mutation order. Compared to TET2-first patients, JAK2-first patients had an increased likelihood of presenting with polycythemia vera than essential thrombocythemia, an increased risk of thrombosis and an increased sensitivity of JAK2-mutant progenitors to ruxolitinib *in vitro*. In studies of single hematopoietic stem and progenitor cells (HSPCs), mutation order influenced the proliferative response to JAK2V617F and the capacity of double-mutant HSPCs to generate colony-forming cells. Moreover the HSPC compartment was dominated by *TET2* single-mutant cells in TET2-first patients but by JAK2/TET2 double-mutant cells in JAK2-first patients. Prior mutation of *TET2* altered the transcriptional consequences of JAK2V617F in a cell-intrinsic manner, and prevented JAK2V617F from up-regulating genes associated with proliferation. These data demonstrate that mutation order influences progenitor proliferation and terminal cell expansion, thus influencing clinical presentation, thrombosis risk and progenitor response to targeted therapy.

Conclusions: The order in which *JAK2* and *TET2* mutations are acquired influences clinical features, stem/progenitor cell biology and clonal evolution in patients with myeloproliferative neoplasms.

Cancers evolve as a consequence of the stepwise accumulation of somatic lesions, with competition between subclones and sequential subclonal evolution[1,2]. Darwinian selection of variant subclones results in acquisition of biological attributes required for tumor formation[3]. Genetic interaction is central to this process, but it is unclear how mutated genes interact to generate the phenotypic hallmarks of cancer, and the influence of mutation order, if any, is unknown[4].

Co-operation between different genetic lesions has been demonstrated in cell line models of transformation (reviewed in [5]) and in mouse models of several cancers[6,7]. Moreover the consequences of an early lesion may influence the range of subsequent mutations able to confer a growth advantage, a concept borrowed from population genetics and termed functional buffering or genetic canalisation [4,8]. However several lesions can occur as either early or late events in the same tumor type[3,9], suggesting that the final malignant properties of a tumor reflect the sum of its driver mutations rather than the order in which they arose.

The myeloproliferative neoplasms are chronic myeloid malignancies with several tractable characteristics. It is possible to obtain purified stem and progenitor cells from peripheral blood and to grow clonal populations containing sufficient cells for genotyping and phenotypic analysis[10]. This allows direct comparison of genetically distinct subclones within a patient, thereby controlling for differences in age, sex, therapy, genetic background and other confounding variables. Myeloproliferative neoplasms also represent an early stage of tumorigenesis, inaccessible in most cancers, and their chronic clinical course permits longitudinal studies. Here, we have investigated the influence of mutation order in patients with myeloproliferative neoplasms carrying mutations in both *JAK2* and *TET2*; mutations in both genes are present in about 10% of patients.

METHODS

Patients and samples

246 patients with a JAK2V617F mutation were screened for mutations in *TET2*. Diagnoses were based on the British Committee for Standards in Haematology (BCSH) guidelines. See Supplemental Methods for details on follow-up patient cohort, ethical approval and sample collection.

HSPC isolation and clonal assays

Individual hematopoietic colonies were grown from peripheral blood mononuclear cells, plucked and genotyped by Sanger sequencing for *JAK2* and *TET2* mutations. Hematopoietic stem and progenitor cell fractions were isolated as previously described[11]. For single cell cultures, HSPCs were sorted into 96-well plates supplemented with cytokines previously shown to support progenitor expansion[12]. See Supplemental Methods for further details.

Gene expression analysis and mutation screening

BFU-E colonies were picked and genotyped and then pooled for expression array analysis (ArrayExpress accession E-MTAB-3086). To screen for recurrent driver mutations, previously published sequencing data[13] were used for 10 patients; 13 other patients were screened using targeted sequencing for 111 genes or genetic regions implicated in myeloid malignancies[14] (Supplementary Table 2).

RESULTS

Stable clonal heterogeneity in chronic phase myeloproliferative neoplasms

To identify patients who carry mutations in both *JAK2* and *TET2*, all exons of the *TET2* gene were sequenced in 246 patients (92 essential thrombocythemia, 107 polycythemia vera, and 47 myelofibrosis) carrying JAK2V617F. *TET2* mutations were identified in 24 patients (7 essential thrombocythemia, 11 polycythemia vera and 6 meylofibrosis) (Supplementary Table 1) from whom >7000 individual hematopoietic colonies were genotyped for *JAK2* or *TET2* mutations (Figure 1A) to establish mutation order (Figure 1B). Subclones containing only the first mutation were more common in polycythemia vera (p=0.012, Mann-Whitney-

test) and essential thrombocythemia (p=0.016, Mann-Whitney-test) than in myelofibrosis, consistent with myelofibrosis representing more advanced disease (Figure 1C).

Several observations indicated considerable clonal stability within individual patients. First, clones carrying either mutant *TET2* alone or mutant *JAK2* alone were readily detected in all 24 patients (median disease duration 7.3 years) (Supplementary Figure 1 and Supplementary Table 2) suggesting that double-mutant clones do not rapidly outcompete single-mutant clones. Second, in 9 patients in whom the *TET2* mutation was acquired before the *JAK2* mutation (TET2-first patients), JAK2V617F was detected 12-112 months before colony analysis, demonstrating that *TET2* single-mutant and *TET2*/*JAK2* double-mutant clones coexisted for at least these periods (data not shown). Third, it was possible to repeat colony assays in new samples from 12 patients (4 essential thrombocythemia, 4 polycythemia vera, 4 myelofibrosis) after intervals of 1 – 3.7 years (Figure 1D; Supplementary Figure 2 and Supplementary Table 2). The clonal pattern of most patients was very stable; of 44 subclones identified at the first time point only 3 became undetectable at the later time point.

TET2 mutations can precede or follow JAK2V617F and mutation order influences disease biology

Mutation of *JAK2* and *TET2* each occurred first in 12/24 patients (Figure 2A). *TET2* mutations arose in both JAK2V617F-heterozygous and homozygous cells indicating that JAK2V617F homozygosity is not required for, nor does it prevent, subsequent acquisition of a *TET2* mutation.

At presentation TET2-first patients were on average 12.3 years older than JAK2-first patients (mean age at diagnosis 71.5 versus 59.2 years; p=0.0043, t-test) (Supplementary Figure 3A). The age difference remained significant after correction for disease phenotype (p=0.0195, two-way ANOVA) and sex (p=0.0068, two-way ANOVA). Blood counts at presentation were

not significantly different between JAK2-first and TET2-first patients, but rather were influenced by disease phenotype (Supplementary Table 1).

Compared to TET2-first patients, JAK2-first patients had a striking increase in the proportion of JAK2V617F homozygous BFU-E (Figure 2B; $p<0.001$, t-test). We then studied more immature progenitors from 13 patients and 3 normal individuals (Figure 2C and Supplementary Figure 3B-D). TET2-first patients showed a predominance of common myeloid progenitors over other progenitors within the $CD34^+CD38^+$ compartment ($p=0.001$, t-test). By contrast, megakaryocyte-erythrocyte progenitors were more prevalent in JAK2-first patients ($p<0.001$, t-test).

To exclude the possibility that additional mutations contribute to the observed differences between JAK2-first and TET2-first patients, exome[13] or targeted sequencing[14] was performed in 23/24 patients. Only 4 patients harbored mutations known to be recurrent in myeloid malignancies (Supplementary Table 3). These data demonstrate that mutation order effects are not confounded by other known oncogenic mutations, but do not exclude the possibility that additional rare drivers might also influence clinical/pathological phenotypes.

Together these data indicate that the age of presentation, the acquisition of JAK2V617F homozygosity and the balance of immature progenitors are all influenced by mutation order.

Mutation order influences proliferation and progenitor generation by single HSPCs
To extend our findings to the hematopoietic stem and progenitor cell (HSPC) compartment we studied the properties of individual HSPCs from double-mutant patients. Single lin^- $CD34^+CD38^-CD90^+CD45RA^-$ cells[15] were isolated and individually cultured for 10 days in conditions previously shown to support the growth of multi-potent progenitors[12] (Supplementary Figure 4A). Proliferation, progenitor content and genotype of individual clones were then measured.

In JAK2-first patients *JAK2* single-mutant clones were significantly larger than wild-type (p=0.047, t-test) and double-mutant clones (Figure 3A p=0.044, t-test) This demonstrates that HSPC proliferation is enhanced by acquisition of a *JAK2* mutation on a *TET2*-wildtype background (i.e. JAK2-first patients) but not on a *TET2*-mutant background (i.e. TET2-first patients). We next assessed the number of progenitors created in the 10-day cultures by secondary colony assays (Figure 3B). JAK2V617F reduced progenitor formation when acquired after a *TET2* mutation (p=0.002), but *TET2* mutations increased progenitor expansion when acquired after JAK2V617F (p=0.012). Progenitor expansion of individual double-mutant HSPCs was therefore starkly different in TET2-first and JAK2-first patients.

The HSPC compartment is dominated by single-mutant clones in TET2-first patients, but not in JAK2-first patients

We next genotyped clones derived from single HSPCs and found that the HSPC compartment was dominated by single-mutant cells in TET2-first patients but by double-mutant cells in JAK2-first patients (Figure 3C). These results could reflect a longer interval between acquisition of the two mutations in TET2-first patients. However, in TET2-first patients, the JAK2V617F mutation was detectable in the earliest available DNA sample (mean 5 years before HSPC assay), showing that the double-mutant clone had at least this amount of time to expand. Furthermore, individual double-mutant HSPCs from JAK2-first patients created more progenitors *in vitro* compared to HSPCs from TET2-first patients, suggesting an increased intrinsic ability to expand at the stem/progenitor cell level (Figure 3B). We therefore favor an interpretation that acquisition of a *TET2* mutation enhances the fitness of *JAK2* single-mutant HSCs whereas acquisition of a *JAK2* mutation does not enhance the fitness of *TET2* single-mutant HSCs.

Genotyping the BFU-E compartment from the same patients revealed that the frequencies of single-mutant and double-mutant colonies in JAK2-first patients resembled those seen in the HSPC compartment (compare Figure 3D and 3C). However in TET2-first patients, double-mutant erythroid colonies were more prevalent than *TET2* single-mutant colonies (p=0.003,

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t-test), a result that contrasts with the genotype distribution in HSPCs. The clonal architecture of purified progenitor fractions were studied in four patients (Supplemetary Figure 4B). JAK2-first patients retained the same genotype distributions throughout the hematopoietic hierarchy, whereas in TET2-first patients, the proportion of double-mutant cells was increased in megakaryocyte-erythroid progenitors and BFU-Es compared to earlier progenitors. These results indicate that in TET2-first patients, acquisition of JAK2V617F, although not associated with HSPC expansion, does give rise to expansion of committed erythroid progenitors.

Our results suggest that, in TET2-first patients, *TET2* single-mutant HSPCs expand but do not give rise to excess differentiated megakaryocytic/erythroid cells until subsequent acquisition of a *JAK2* mutation. By contrast in JAK2-first patients, *JAK2* single-mutant HSPCs do not expand until acquisition of a *TET2* mutation, but are able to generate increased numbers of megakaryocytic/erythroid cells.

Prior mutation of *TET2* alters the transcriptional response to JAK2V617F

To study the molecular mechanisms underlying the biological differences associated with distinct mutation orders, we performed transcriptional profiling on individual wild-type, single-mutant and double-mutant colonies erythroid colonies from 7 patients (4 TET2-first, 3 JAK2-first). Over 500 colonies were plucked and pooled according to JAK2/TET2 genotype, with at least 10 colonies of each genotype in each sample. This strategy allows direct comparison of genetically distinct cells within a patient, thus controlling for differences in age, sex, treatment, genetic background and other confounding variables.[10]

Mutation of *JAK2* or *TET2* was associated with altered patterns of gene expression that were strikingly dependent on the antecedent genotype (Supplementary Figure 5A; Appendix 1). For example, most genes up-regulated or down-regulated when JAK2V617F was acquired on a TET2-wildtype background were not altered when JAK2V617F was acquired on a TET2-mutant background (Figure 3E). The most enriched clusters across all

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comparisons were up-regulation of translational machinery when JAK2V617F was acquired on a wild-type background (Set 1) and down-regulation of cell cycle progression genes when JAK2V617F was acquired on a TET2-mutant background (Set 8).

To investigate further whether prior mutation of *TET2* influences the transcriptional response to JAK2V617F, we compared genes up-regulated when JAK2V617F was acquired by TET2-wildtype cells with those down-regulated when JAK2V617F was acquired by TET2-mutant cells. This approach identified 10 genes discordantly regulated by JAK2V617F depending on the *TET2* genotype (Figure 3F), results validated for all 6 genes tested (Supplementary Figure 5B). Remarkably, six of these genes have been implicated in DNA replication[16] (MCM2, MCM4, MCM5) or regulation of mitosis (AURKB[17], FHOD1[18], TK1[19]). These results accord with our functional studies of single HSPCs which revealed increased proliferation when JAK2V617F was acquired by TET2-wildtype but not TET2-mutant cells (Figure 3A).

Together these data demonstrate that acquisition of a prior *TET2* mutation dramatically alters the transcriptional consequences of JAK2V617F in a cell-intrinsic manner, and in particular prevents JAK2V617F from up-regulating genes associated with proliferation.

Mutation order influences clinical presentation, risk of thrombosis, and sensitivity to JAK-inhibition

In our initial patient cohort, the ratio of patients with polycythemia vera to those with essential thrombocythemia appeared greater in JAK2-first patients compared to TET2-first patients (Figure 2A). To explore this further, a follow-up cohort (918 patients) was screened to identify 90 patients who harbored both JAK2 and TET2 mutations. Copy-number corrected variant allele fractions for both mutations were used to identify 24 patients (18 JAK2-first, 6 TET2-first) in whom mutation order could be unambiguously determined (see supplementary methods). To interrogate the clinical relevance of mutational order we combined both cohorts (total 48 patients) for further analyses. Compared to TET2-first patients, JAK2-first patients presented at a younger age (mean 60.71 years versus 71.17

years; p=0.0015, Figure 4A), were more likely to present with polycythemia vera (p=0.046; Figure 4B) and, despite presenting at a younger age, were more likely to develop a thrombotic event (p=0.002 by multivariate analysis; Figure 4C). Reassuringly, patients in whom mutation order could not be unambiguously determined had an intermediate risk of thrombosis-free survival. Although this small retrospective cohort study requires confirmation in a prospective study, these data suggest that mutation order influences clinical presentation and outcome.

To explore the implications of mutation order for therapy, we studied the effect of ruxolitinib (a JAK1/2 inhibitor) on colony formation. Although not specific for mutant JAK2[20], ruxolitinib has been shown to inhibit increased proliferation of splenocytes and erythroblasts in a mouse model[21]. The proportions of single-mutant colonies from all 4 JAK2-first patients were reduced by ruxolitinib, as were the proportions of double-mutant colonies in 3 of 4 patients (Figure 4D). By contrast, in all 4 TET2-first patients, the proportions of single-mutant and double-mutant colonies were essentially unchanged by ruxolitinib. These results indicate that mutant progenitors from JAK2-first patients are more sensitive to JAK2 inhibition. Together, these data support a model in which mutation order affects progenitor proliferation and terminal cell expansion, thus influencing clinical presentation, thrombosis risk and response *in vitro* to targeted therapy (Figure 4E).

DISCUSSION

We provide evidence that the order of acquiring somatic mutations influences stem/progenitor cell behavior and clonal evolution together with clinical presentation and thrombotic risk in patients with myeloproliferative neoplasms. Prior mutation of *TET2* dramatically influences the transcriptional program activated by JAK2V617F, thus providing a molecular basis for the effect of mutation order. These results have clinical implications for patients including the prediction that mutation order may influence response to therapy.

Mutation in either *TET2* or *JAK2* may occur first in all three myeloproliferative neoplasm subtypes, but JAK2-first patients are significantly more likely to have polycythemia vera. These data confirm and extend previous studies of small numbers of patients reporting TET2-first patients[22], JAK2-first patients[23] or both[24]. The fitness of clones *in vivo* may not always directly relate to their *in vitro* capacity, and cell surface markers may be altered by individual *JAK2* or *TET2* mutations. However, the consequences of mutation order that we describe in primary cells from patients accord with several lines of *in vivo* evidence. *TET2* mutations give rise to clonal expansions in elderly individuals with normal blood counts[25], and in xenograft studies of two patients, a double mutant TET2/JAK2 clone was outcompeted by its *TET2* single-mutant ancestor[22]. Moreover in genetically-modified mice, inactivation of *TET2* causes HSC expansion with no effect on erythropoiesis[26,27], whereas JAK2V617F causes increased erythropoiesis and progenitor expansion but either no HSC advantage[28,29], or a disadvantage[30,31] in serial repopulation experiments.

Our data suggest a model for the effects of mutation order on myeloproliferative neoplasm biology (Figure 4E). In TET2-first patients, *TET2* single-mutant HSPCs expand but do not give rise to excess differentiated megakaryocytic/erythroid cells until subsequent acquisition of a *JAK2* mutation. By contrast in JAK2-first patients, *JAK2* single-mutant HSPCs do not expand until acquisition of a *TET2* mutation, but are able to generate increased numbers of megakaryocytic/erythroid cells. This model is consistent with the early presentation of JAK2-first patients, since they more rapidly generate excess megakaryocytic/erythroid cells and abnormal blood counts, and is also consistent with the altered HSPC behavior that we observe.

At least three mechanisms, not mutually exclusive, may contribute to the influence of mutation order. First, the initial mutation may alter the cellular composition of the neoplastic clone including early stem/progenitors and their differentiated progeny. As a consequence, following acquisition of the second mutation, the double-mutant subclone will find itself in a cellular environment determined by the identity of the first mutation. Cellular interactions

between genetically distinct subclones may conceivably take several forms including direct competition for available niches and feedback effects of differentiated cells on stem and progenitor compartments[32].

Second, the initial mutation may mandate distinct cellular pathways as targets for subsequent mutations able to provide a growth advantage. The order in which *TET2* and *JAK2* are acquired may therefore dictate co-mutation preferences and thus influence disease pathogenesis.

Third, the initial mutation may modify the epigenetic program of HSPCs and thus alter the consequences of the second mutation. TET2 alters the epigenetic landscape by converting 5-methylcytosine to 5-hydroxymethylcytosine[33,34], and also promotes the addition of N-acetylglucosamine to histones[35]. Our results demonstrate that prior mutation of *TET2* alters the transcriptional consequences of JAK2V617F in a cell-intrinsic manner, and prevents JAK2V617F up-regulating a proliferative program. The frequency with which epigenetic regulators are mutated in hematological[36] and non-hematological[37] cancers raises the possibility that mutation order influences the biology of multiple cancers.

Authorship Contributions:

Experiments were designed by DGK and CAO; Phenotypic patient data was compiled and analyzed by CAO with assistance from DGK, EJB, CEM, JN, JG, PG, and PC; BB, KD, CB, PG, CNH, and AV provided patient samples; validation cohort collection, targeted sequencing and mutation analysis was performed by JN with assistance from EP, CEM, EJB, PG, AV, CNH; Sample preparation, colony assays, and sequencing were carried out by CAO, YS, DD, and DGK; HSPC and progenitor isolation and flow cytometric analyses were carried out by DGK with assistance from CAO and YS; single cell in vitro and secondary

colony analyses were performed by DGK with assistance from CAO; microarray studies were completed by CAO and GSV with analyses done by SM and DGK; DGK and CAO performed qPCR experiments; DGK performed inhibitor studies; CAO, EJB, JN, and EP prepared samples and performed exome sequencing studies; DGK, CAO and ARG wrote the paper, and ARG directed the research.

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Figure Legends

Figure 1: JAK2V617F can precede or follow *TET2* mutations, and chronic phase patients exhibit stable clonal heterogeneity.

A) Hematopoietic colonies (BFU-E) were grown from peripheral blood mononuclear cells in semisolid media from MPN patients who carried mutations in *TET2* and *JAK2*. Colonies (20 to 200 per patient, n>7000 total) were plucked and individually sequenced to determine the clonal composition and order of mutation acquisition.

B) Three representative MPN patients with longstanding disease are shown. The time from diagnosis in each patient is 61 months (ET7), 67 months (PV4), and 16 months (MF3). A similar level of clonal heterogeneity is present in the majority of 24 MPN patients with a mean total disease duration of 80, 97 and 85 months in PV, ET and MF, respectively. WT = wild-type, J = JAK2V617F mutation, JJ = JAK2V617F homozygosity, T = *TET2* mutation, PV = polycythemia vera, ET = essential thrombocythemia, MF = myelofibrosis

C) The stacked column plot displays the mutational status of colonies in PV, ET and MF patients (n= total number of colonies per patient; average percentages are shown in case of repeated assays). Heterozygous and homozygous acquisition of mutations are each counted as separate mutational events. Subclones containing only the first mutation were more common in PV (p=0.012, Mann-Whitney-test) and ET (p=0.016, Mann-Whitney-test) than in MF.

D) The clonal composition was determined in 12 MPN patients at intervals of 12-44 months and two representative patients are shown. Disappearance of clones over the observed time period was rare, with just 3 of 44 clones falling below detection in follow-up samples.

Figure 2: Order of acquisition impacts disease phenotype: TET2-first patients present later, have fewer JAK2V617F homozygous clones and a distinct progenitor cell composition.

A) The pie chart shows the order of acquisition for 24 patients. Half of the MPN patients are "JAK2-first" (blue) and half are "TET2-first" (green) with both mutational orders found in each disease subtype.

B) The bar graph shows the proportion of JAK2V617F homozygous colonies out of total mutant colonies. Notably, patients who acquire JAK2V617F first have a greater proportion of homozygous colonies in the mutant compartment (mean % of homozygous mutant colonies: JAK2-first 57.8% versus TET2-first 1.4%; p<0.001, t-test)

C) The bar graph shows the relative number of progenitor cells in TET2-first and JAK2-first patients. Individual patients or normal controls are shown along the bottom and the three bars represent the relative number of CMPs (lin$^-$CD34$^+$CD38$^+$CD90$^-$CD10$^-$FLK2$^+$CD45RA$^-$, blue), GMPs (lin$^-$CD34$^+$CD38$^+$CD90$^-$CD10$^-$FLK2$^+$CD45RA$^+$, green), and MEPs (lin$^-$CD34$^+$CD38$^+$CD90$^-$CD10$^-$FLK2$^-$CD45RA$^-$, red). TET2-first patients showed a uniform predominance of CMPs over other progenitors within the CD34$^+$CD38$^+$ compartment (p=0.001, t-test). By contrast MEPs were more prevalent than CMPs or GMPs in JAK2-first patients (p<0.001, t-test). CMP = common myeloid progenitor, MEP = megakaryocyte-erythroid progenitor, GMP = granulocyte-macrophage progenitor. At the time of sample acquisition, the ages of the TET2-first and JAK2-first patients were similar (mean age 78 years, range 66-86, compared to 72 years, range 50-84).

Figure 3: Mutation order influences proliferation and progenitor generation by single HSPCs and prior loss of TET2 alters the transcriptional response to JAK2V617F.

A) The bar graph shows the average number of cells (clone size) that were present at 10 days from cultures of HSPCs of different genotypes with non-mutant clones shown as white bars, first mutation (single mut.) as grey bars and both mutations (T + J mut.) as black bars. In TET2-first patients *TET2* single-mutant clones were not different in size compared to WT (p=0.24, t-test) or double mutant clones (p=0.07, t-test). By contrast in JAK2-first patients *JAK2* single-mutant clones were significantly larger than WT (p=0.047, t-test) and double-mutant clones (p=0.044, t-test). n = number of patients examined.

B) The number of secondary colony forming cells created per HSPC is shown for patients who had a *TET2* mutation followed by a JAK2V617F mutation and those who had a *JAK2* mutation followed by a *TET2* mutation. HSPCs that acquire JAK2V617F on a TET2-mutant background produce fewer CFCs (mean 3 versus 12, p=0.002, t-test) whereas those that acquire *TET2* mutations on a *JAK2* single-mutant background produce more CFCs (mean 27 versus 16 CFCs p=0.012, t-test). n = number of patients examined.

C) Each bar graph shows the percentage of total HSPCs of each genotype measured in TET2-first (left) and JAK2-first (right) patients. Notably the percentage of HSPCs retaining the first mutation alone is higher in TET2-first patients compared to JAK2-first patients. n = number of patients examined.

D) Each bar graph shows the percentage of BFU-E colonies of each genotype in TET2-first (left) or JAK2-first (right) patients. The TET2-first BFU-E compartment is skewed toward the double-mutant clone compared to the proportion of mutant HSPCs (Figure 3C). The JAK2-first BFU-E compartment is similar to the HSPC compartment. n = number of patients examined.

E) Each Venn diagram shows the overlap in genes that are commonly up-regulated (left diagram, 54 common genes) or down-regulated (right diagram, 61 common genes) upon addition of JAK2V617F on different backgrounds (WT background = red circles, TET2 mutant background = blue circles)

F) This Venn diagram shows the genes which are upregulated when JAK2V617F is acquired on a WT background (red circle) overlapped with those that are down-regulated when JAK2V617F is acquired on a TET2-mutant background (blue circle). There were 10 genes that followed this pattern and they are listed in the box.

Figure 4: Clinical significance of mutation order in MPNs.

A) The mean age at presentation of 48 MPN patients (original cohort, black; follow-up cohort, red) in whom mutation order was determined. On average, TET2-first patients present

10.46 years later than JAK2-first patients (p=0.0015 t-test, p=0.02 two-way ANOVA accounting for order, sex, WBC, and MPN phenotype).

B) Histograms represent the number of patients diagnosed with essential thrombocythemia (ET) or polycythemia vera (PV). JAK2-first patients are more likely to have a PV diagnosis (p=0.046, chi-squared test)

C) Kaplan-Meier curve showing thrombosis-free survival for all 48 patients in whom mutation order was determined. JAK2-first status was identified as an independent risk factor for thrombotic events in addition to the known risk factor of age (p=0.002 for mutational order and p=0.01 for age, multivariate analysis by Cox proportional hazards model taking into account age, prior thrombosis, sex, MPN subtype, WBC, cytoreductive therapy and mutational order). JAK2-first group - 4 cardiac events (3 myocardial infarction (MI), 1 unstable angina), 2 transient ischemic attack (TIA), 2 portal vein thromboses, 2 splanchnic vein thromboses, 1 deep vein thrombosis, and 1 calf vein thrombosis (N.B. one patient had TIA, MI and splanchnic vein thrombosis). TET2-first group - 1 deep vein thrombosis, 1 cardiac event (unstable angina)

D) This graph shows the relative sensitivity of colonies with distinct genotypes to ruxolitinib in a colony-forming cell assay (n = 4 TET2-first and 4 JAK2-first patients, 35-66 colonies picked per patient). In the case of TET2-first patients, single mutant colonies have a TET2 mutation alone, and in JAK2-first patients, a JAK2-heterozygous or homozygous mutation. Single mutant clones from JAK2-first patients were more sensitive to ruxolitinib (left panel, p<0.01, one-tailed t-test). Double mutant clones (those bearing both JAK2 and TET2 mutations) from JAK2-first patients were also more sensitive to ruxolitinib compared to clones from TET2-first patients (p=0.029, one-tailed t-test).

E) The order of mutation acquisition influences disease evolution. This model depicts how single hematopoietic units (see legend at left hand side), consisting of stem cells (HSC), progenitors (Prog) and differentiating cells (Diff), acquire mutations over time. Some units are hyperproliferative (Excess) and produce excess differentiated cells that contribute to disease phenotype. The mutational status of each unit is coded by fill and border and the

numbers represent the acquisition of the first mutation (1), second mutation (2) and JAK2V617F homozygosity (3).

TET2-first: Patients who acquire a *TET2* mutation first (green border, upper panel) gain a self-renewal advantage but do not over-produce downstream progeny. The expansion of the *TET2*-alone clone (bold borders) without excess differentiated cells leads to clonal expansion without immediate clinical presentation. HSCs that acquire a secondary *JAK2* mutation (pink fill) compete with the *TET2*-alone clone and their increased proliferation at the progenitor level now drives an overproduction of terminal cells (as indicated by the box in the "excess" section). When homozygosity is acquired as a third event (red fill), this clone has limited space to expand due to the high self-renewal activity of *TET2*-alone and *TET2*/*JAK2* heterozygous clones.

JAK2-first: Patients who acquire a *JAK2* mutation first (pink fill, lower panel) produce excess differentiated cells in the absence of a distinct HSC self-renewal advantage. When a secondary *TET2* mutation is acquired (green border), HSCs obtain a self-renewal advantage and *JAK2*/*TET2* mutant cells (pink fill with green border) expand at the stem cell level. HSCs with loss of heterozygosity of JAK2V617F (acquired before or after the *TET2* mutation) (red fill) also have space to expand and result in a more pronounced excess of differentiated cells. This excess production would explain both the presentation as a PV and the elevated risk of thrombotic events in JAK2-first patients.