Effect of Mutation Order on Myeloproliferative Neoplasms


BACKGROUND
Cancers result from the accumulation of somatic mutations, and their properties are thought to reflect the sum of these mutations. However, little is known about the effect of the order in which mutations are acquired.

METHODS
We determined mutation order in patients with myeloproliferative neoplasms by genotyping hematopoietic colonies or by means of next-generation sequencing. Stem cells and progenitor cells were isolated to study the effect of mutation order on mature and immature hematopoietic cells.

RESULTS
The age at which a patient presented with a myeloproliferative neoplasm, acquisition of JAK2 V617F homozygosity, and the balance of immature progenitors were all influenced by mutation order. As compared with patients in whom the TET2 mutation was acquired first (hereafter referred to as “TET2-first patients”), patients in whom the Janus kinase 2 (JAK2) mutation was acquired first (“JAK2-first patients”) had a greater likelihood of presenting with polycythemia vera than with essential thrombocytemia, an increased risk of thrombosis, and an increased sensitivity of JAK2-mutant progenitors to ruxolitinib in vitro. Mutation order influenced the proliferative response to JAK2 V617F and the capacity of double-mutant hematopoietic cells and progenitor cells to generate colony-forming cells. Moreover, the hematopoietic stem-and-progenitor-cell 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 JAK2 V617F in a cell-intrinsic manner and prevented JAK2 V617F from up-regulating genes associated with proliferation.

CONCLUSIONS
The order in which JAK2 and TET2 mutations were acquired influenced clinical features, the response to targeted therapy, the biology of stem and progenitor cells, and clonal evolution in patients with myeloproliferative neoplasms. ( Funded by Leukemia and Lymphoma Research and others.)

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Cancers evolve as a consequence of the stepwise accumulation of somatic lesions, with competition between subclones and sequential subclonal evolution. Darwinian selection of variant subclones results in acquisition of biologic attributes required for tumor formation. 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, if any, of the order in which mutations are acquired is unknown.

Cooperation between different genetic lesions has been observed in cell-line models of transformation and in mouse models of several cancers. Moreover, the consequences of an early lesion may influence the range of subsequent mutations that are able to confer a growth advantage; this concept, which is borrowed from population genetics, is termed functional buffering or genetic canalization. However, several lesions can occur as either early or late events in the same tumor type, 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 diseases with several tractable characteristics. It is possible to obtain enriched fractions of stem cells and progenitor cells from peripheral blood and to grow clonal populations containing a sufficient number of cells for genotyping and phenotypic analysis. 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 reflect an early stage of tumorigenesis that is inaccessible in most cancers, and their long-term clinical course permits longitudinal studies. We have investigated the influence of mutation order in patients with myeloproliferative neoplasms that carry mutations in both Janus kinase 2 (JAK2) and TET2; mutations in both genes are present in about 10% of patients with myeloproliferative neoplasms.

**Methods**

**Patients and Samples**

We screened 246 patients with a JAK2 V617F mutation for mutations in TET2. All patients provided written informed consent. Diagnoses were made in accordance with the guidelines of the British Committee for Standards in Haematology. (Details on the ethical approval and sample collection are provided in Supplementary Appendix 1, available with the full text of this article at NEJM.org.)

**Isolation of Hematopoietic Stem and Progenitor Cells and Clonal Assays**

Individual hematopoietic stem cells and progenitor cells from peripheral-blood mononuclear cells, picked, and genotyped with the use of Sanger sequencing for JAK2 and TET2 mutations. Hematopoietic stem-and-progenitor-cell fractions were isolated from peripheral-blood mononuclear cells, picked, and genotyped with the use of Sanger sequencing for JAK2 and TET2 mutations. Hematopoietic stem-and-progenitor cells were sorted into 96-well plates supplemented with cytokines that were previously shown to support progenitor expansion. (Further details are provided in the Methods section in Supplementary Appendix 1.)

**Analysis of Gene Expression and Mutation Screening**

Colonies of erythroid burst-forming units were picked, genotyped, and then pooled for expression-array analysis (ArrayExpress accession number, E-MTAB-3086). Previously published sequencing data were used to screen for recurrent driver mutations in 10 patients; 13 other patients were screened with the use of targeted sequencing for 111 genes or genetic regions implicated in myeloid cancers (Table S2 in Supplementary Appendix 1).

**Statistical Analysis**

Unless otherwise indicated, all comparisons were made with the use of the two-sided Student’s t-test. We used GraphPad Prism software, version 5.01, for all statistical analyses with the chi-square test, the Mann–Whitney test, and Student’s t-test. Multivariate analyses were performed with the use of R statistical software. Exome and targeted sequencing data were analyzed as described in Supplementary Appendix 1.
thrombocytopenia, 107 with polycythemia vera, and 47 with myelofibrosis) who carried JAK2 V617F. TET2 mutations were identified in 24 patients (7 with essential thrombocytopenia, 11 with polycythemia vera, and 6 with myelofibrosis) (Table S1 in Supplementary Appendix 1), from whom more than 7000 individual hematopoietic colonies were JAK2 and TET2 mutations (Fig. 1A) to establish mutation order (Fig. 1B). Subclones containing only the first mutation were more common in patients with polycythemia vera and in patients with essential thrombocytopenia than in patients with myelofibrosis (P=0.01 for the comparison between patients with polycythemia vera and those with myelofibrosis, and P=0.02 for the comparison between patients with essential thrombocytopenia and those with myelofibrosis, by the Mann–Whitney test); these findings are consistent with the fact that myelofibrosis is a more advanced disease (Fig. 1C).

We observed 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) (Fig. S1 and Table S2 in Supplementary Appendix 1), suggesting that double-mutant clones do not rapidly outcompete single-mutant clones. Second, in 9 patients in whom the mutation was acquired before the first time point, only 3 became undetectable at the later time point. To exclude the possibility that additional mutations contributed to the observed differences between JAK2-first and TET2-first patients, we performed exome13 or targeted14 sequencing in 23 of 24 patients. Only 4 patients harbored mutations known to be recurrent in myeloid cancers (Table S3 in Supplementary Appendix 1). These data show that the effects of mutation order were not confounded by other known oncogenic mutations, but they do not exclude the possibility that additional rare drivers might also influence clinical and pathologic phenotypes. Together these data indicate that the age at which patients present with myeloproliferative neoplasms, the acquisition of JAK2 V617F homozygosity, and the balance of immature progenitors are all influenced by mutation order.

TET2 MUTATIONS PRECEDING OR FOLLOWING JAK2 V617F, AND THE EFFECT OF MUTATION ORDER ON DISEASE BIOLOGY

JAK2 and TET2 mutations each occurred first in 12 of 24 patients (Fig. 2A). TET2 mutations arose in both JAK2 V617F–heterozygous and JAK2 V617F–homozygous cells, indicating that JAK2 V617F 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 patients in whom the JAK2 mutation was acquired first (referred to as “JAK2-first patients”) (mean age at diagnosis, 71.5 years vs. 59.2 years; P=0.004 by Student’s t-test) (Fig. S3A in Supplementary Appendix 1). The age difference remained significant after adjustment for disease phenotype (P=0.02 by two-way analysis of variance) and sex (P=0.007 by two-way analysis of variance). Although blood counts at presentation did not differ significantly between the JAK2-first and TET2-first patients, they were influenced by disease phenotype (Table S1 in Supplementary Appendix 1).

As compared with TET2-first patients, JAK2-first patients had a striking increase in the proportion of JAK2 V617F–homozygous colonies of erythroid burst-forming units (P<0.001 by the t-test) (Fig. 2B). We then studied more immature progenitors from 13 patients and 3 healthy persons (Fig. 2C, and Fig. S3B, S3C, and S3D in Supplementary Appendix 1). In TET2-first patients, there was a predominance of common myeloid progenitors over other progenitors within the CD34+CD38- compartment (P=0.001 by the t-test). By contrast, megakaryocyte and erythrocyte progenitors were more prevalent in JAK2-first patients (P<0.001 by the t-test).

To extend our findings to the hematopoietic stem-cell compartment, we studied the properties of individual hematopoietic stem and progenitor cells from patients with both single-mutant and double-mutant clones. We isolated and individually cultured single lin-CD34+CD38-
CD90<sup>+</sup>CD45RA<sup>−</sup> cells<sup>16</sup> for 10 days in conditions that were previously shown to support the growth of multipotent progenitors<sup>12</sup> (Fig. S4A in Supplementary Appendix 1). We then measured the proliferation, progenitor content, and genotype of individual clones.

In JAK2-first patients, JAK2 single-mutant clones were significantly larger than nonmutant clones (P = 0.047 by the t-test) and double-mutant clones (P = 0.04 by the t-test). This shows that proliferation of hematopoietic stem and progenitor cells was enhanced by acquisition of a JAK2 mutation on a TET2-nonmutant 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 with the use of secondary colony assays (Fig. 3B). JAK2 V617F reduced progenitor formation when it was acquired after a TET2 mutation (P = 0.002), but TET2 mutations increased
Figure 1 (facing page). JAK2 V617F Preceding or Following TET2 Mutations in Patients with Chronic-Phase Myeloproliferative Neoplasms.

As shown in Panel A, colonies of erythroid burst-forming units (BFU-E) were grown in a semisolid medium from peripheral-blood mononuclear cells obtained from patients with myeloproliferative neoplasms who carried mutations in TET2 and JAK2. Colonies (20 to 200 per patient, >7000 total) were picked and individually sequenced to determine the clonal composition and order in which mutations were acquired. In Panels B and D, the letters and numbers at the top of each column are patient-identification numbers for individual patients. Numbers beside the patient-identification numbers indicate the total number of colonies in that patient genotyped, with the percentages of total colonies of each genotype shown below. The circles beside these percentages are proportional to the percentages. In each of these 3 patients with myeloproliferative neoplasms who had long-standing disease, the time from diagnosis was 61 months (in a patient with essential thrombocythemia [ET], left column), 67 months (in a patient with polycythemia vera [PV], middle column), and 16 months (in a patient with myelofibrosis [MF], right column). A similar level of clonal heterogeneity was present in the majority of 24 patients with myeloproliferative neoplasms (see Fig. 1 in Supplementary Appendix 1). The mean total duration of disease was 80 months in patients with polycythemia vera, 97 months in patients with essential thrombocythemia, and 85 months in patients with myelofibrosis. J denotes JAK2 V617F mutation, JJ JAK2 V617F homozygosity, NM nonmutated, and T TET2 mutation. In Panel C, the stacked column plot indicates the mutational status of colonies in patients with polycythemia vera, essential thrombocythemia, and myelofibrosis. The total number of colonies per patient is shown at the top of each column; average percentages are shown in cases of repeated assays. Heterozygous and homozygous acquisitions of mutations were each counted as separate mutational events. Subclones containing only the first mutation were more common in patients with polycythemia vera (P = 0.01 by the Mann–Whitney test) and essential thrombocythemia (P = 0.02 by the Mann–Whitney test) than in patients with myelofibrosis. The clonal composition was determined in 12 patients with myeloproliferative neoplasms at intervals of 12 to 44 months; 2 representative patients are shown in Panel D. Disappearance of clones over the observed time period was rare, with just 3 of 44 clones falling below detection in follow-up samples.

progenitor expansion when they were acquired after JAK2 V617F (P = 0.01). Progenitor expansion of individual double-mutant hematopoietic stem and progenitor cells was therefore starkly different in TET2-first patients and JAK2-first patients.

EFFECT OF MUTATION ORDER ON CLONAL COMPOSITION OF THE HEMATOPOIETIC STEM-AND-PROGENITOR-CELL COMPARTMENT

We next genotyped clones derived from single hematopoietic stem and progenitor cells and found that the hematopoietic-stem-and-progenitor-cell compartment was dominated by single-mutant cells in TET2-first patients but by double-mutant cells in JAK2-first patients (Fig. 3C). These results could reflect a longer interval between acquisition of the two mutations in TET2-first patients. However, in TET2-first patients, the JAK2 V617F mutation was detectable in the earliest available DNA sample (a mean of 5 years before the hematopoietic-stem-cell and progenitor-cell assay). This shows that the double-mutant clone had at least this amount of time to expand. Furthermore, individual double-mutant hematopoietic stem and progenitor cells obtained from JAK2-first patients created more progenitors in vitro than did hematopoietic stem and progenitor cells obtained from TET2-first patients (Fig. 3B); this suggests an increased intrinsic ability to expand at the level of stem and progenitor cells. We therefore favor an interpretation that acquisition of a TET2 mutation enhances the fitness of JAK2 single-mutant hematopoietic stem cells, whereas acquisition of a JAK2 mutation does not enhance the fitness of TET2 single-mutant hematopoietic stem cells.

Genotyping of the compartment of erythroid burst-forming units from the same patients revealed that in JAK2-first patients, the frequencies of single-mutant and double-mutant colonies in that compartment resembled those seen in the hematopoietic-stem-and-progenitor-cell compartment (Fig. 3D and 3C). However, in TET2-first patients, double-mutant erythroid colonies were more prevalent than TET2 single-mutant colonies (P = 0.003 by the t-test), a result that contrasts with the genotype distribution in hematopoietic stem and progenitor cells. The clonal architecture of purified progenitor fractions was studied in four patients (Fig. S4B in Supplementary Appendix 1). In JAK2-first patients, the same genotype distributions throughout the hematopoietic hierarchy were retained, whereas in TET2-first patients, the proportion of double-mutant cells was increased in megakaryocyte and erythroid progenitors and erythroid burst-forming units as compared with earlier progenitors. These results indicate that in TET2-first patients, acquisition of JAK2 V617F, although not associated with expansion of hematopoietic stem and progenitor cells, does give rise to expansion of committed erythroid progenitors.

Our results suggest that in TET2-first patients, TET2 single-mutant hematopoietic stem and progenitor cells expand but do not give rise to excess differentiated megakaryocytic and ery-
The thyroid cells until subsequent acquisition of a JAK2 mutation. By contrast, in JAK2-first patients, JAK2 single-mutant hematopoietic stem and progenitor cells do not expand until acquisition of a TET2 mutation, but they are able to generate increased numbers of megakaryocytic and erythroid cells.

Prior Mutation of TET2 and an Altered Transcriptional Response to JAK2 V617F

To study the molecular mechanisms underlying the biologic differences associated with distinct mutation orders, we performed transcriptional profiling on individual nonmutant, single-mutant, and homozygous mutant colonies.
and double-mutant erythroid colonies in samples obtained from seven patients (four JAK2-first patients and three TET2-first patients). More than 500 colonies were picked and pooled according to JAK2 or 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 (Fig. S5A in Supplementary Appendix 1, and Supplementary Appendix 2, available at NEJM.org). For example, most genes that were up-regulated or down-regulated when JAK2 V617F was acquired on a TET2-nonmutant background were not altered when JAK2 V617F was acquired on a TET2-mutant background (Fig. 3E). The most up-regulated gene cluster across all comparisons was translational machinery when JAK2 V617F was acquired on a TET2-nonmutant background, and the most down-regulated gene cluster was cell-cycle progression when JAK2 V617F was acquired on a TET2-mutant background.

To investigate further whether prior mutation of TET2 influences the transcriptional response to JAK2 V617F, we compared genes that were up-regulated when JAK2 V617F was acquired by TET2-nonmutant cells with those that were down-regulated when JAK2 V617F was acquired by TET2-mutant cells. This approach identified 10 genes that were discordantly regulated by JAK2 V617F, depending on the TET2 genotype (Fig. 3F). These results were validated for all six genes tested (Fig. S5B in Supplementary Appendix 1). Six of the genes have been implicated in DNA replication17 (MCM2, MCM4, and MCM5) or regulation of mitosis (AURKB,18 FHOD1,19 and TK120). These results are consistent with those in our functional studies of single hematopoietic stem cells and progenitor cells, which revealed increased proliferation when JAK2 V617F was acquired by TET2-nonmutated but not TET2-mutated cells (Fig. 3A).

Together these data show that acquisition of a prior TET2 mutation dramatically altered the transcriptional consequences of JAK2 V617F in a cell-intrinsic manner. In particular, it prevented JAK2 V617F from up-regulating genes associated with proliferation.

**Influence of Mutation Order on 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 to be greater among JAK2-first patients than among TET2-first patients (Fig. 2A). To explore this further, a follow-up cohort involving 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 patients and 6 TET2-first patients) in whom mutation order could be unambiguously determined (see the Supplementary Methods section in Supplementary Appendix 1). To investigate the clinical relevance of mutational order, we combined both cohorts (a total of 48 patients) for further analyses. As compared with TET2-first patients, JAK2-first patients presented at a younger age (mean, 60.71 years vs. 71.17 years; P=0.002) (Fig. 4A), were more likely to present with polycythemia vera (P=0.05) (Fig. 4B), and, despite presenting at a younger age, were more likely to have a thrombotic event (P=0.002 by multivariate analysis) (Fig. 4C). In the JAK2-first group, 4 patients had cardiac events (3 had myocardial infarction, and 1 had unstable angina), 2 had a transient ischemic attack, 2 had portal-vein thromboses, 2 had splanchic-vein thrombosis, 1 had deep-vein thromboses, and 1 had calf-vein thromboses (1 patient had a transient ischemic attack, myocardial infarction, and splanchic-vein thrombosis). In the TET2-first group, 1 patient had deep-vein thromboses and 1 had a cardiac event (unstable angina). Patients in whom mutation order could not be unambiguously determined had an intermediate rate 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 (an inhibitor of JAK1 and JAK2) on colony formation. Although ruxolitinib is not specific for mutant JAK2,21 it has been shown to inhibit increased proliferation of splenocytes and erythroblasts in a mouse model.22 The proportions of single-mutant colonies from all four JAK2-first patients were reduced after the administration of ruxolitinib, as were the proportions of double-mutant colonies in
three of four patients (Fig. 4D). By contrast, in all four TET2-first patients, the proportions of single-mutant and double-mutant colonies were essentially unchanged after the administration of ruxolitinib. These results indicate that mutant progenitors from JAK2-first patients are more sensitive to JAK2 inhibition.

These data suggest that mutation order affects the proliferation of progenitors and terminal cell expansion, thereby influencing clinical presentation, the risk of thrombosis, and the response in vitro to targeted therapy (Fig. 4E).

**DISCUSSION**

We found that the order in which somatic mutations were acquired influenced the behavior of stem and progenitor cells and clonal evolution, as well as the clinical presentation and risk of thrombosis among patients with myeloproliferative neo-
Mutation in either TET2 or JAK2 may occur first in all three subtypes of myeloproliferative neoplasms, but JAK2-first patients are significantly more likely to have polycythemia vera. These data confirm and extend the results of previous studies involving small numbers of TET2-first patients, JAK2-first patients, or both. The fitness of clones in vivo may not always relate directly 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 are consistent with several lines of in vivo evidence. TET2 mutations have been shown to give rise to clonal expansions in elderly persons with normal blood counts, and in xenograft studies involving two patients, a double-mutant TET2-JAK2 clone was outcompeted by its TET2 single-mutant ancestor. Moreover, in genetically modified mice, expression of JAK2 V617F resulted in increased erythropoiesis, whereas inactivation of TET2 had no effect on erythropoiesis. In experiments of serial repopulation, inactivation of TET2 was shown to cause hematopoietic stem-cell expansion, whereas expression of JAK2 V617F was associated with either no hematopoietic stem-cell advantage or a disadvantage.

Our data suggest a model for the effects of mutation order on the biology of myeloproliferative neoplasms (Fig. 4E). In TET2-first patients, TET2 single-mutant hematopoietic stem and progenitor cells expand but do not give rise to excess differentiated megakaryocytic and erythroid cells until subsequent acquisition of a JAK2 mutation. By contrast, in JAK2-first patients, JAK2 single-mutant hematopoietic stem and progenitor cells do not expand until acquisition of a TET2 mutation, but they are able to generate increased numbers of megakaryocytic and erythroid cells. This model is consistent with the early clinical presentation of JAK2-first patients, since they have a more rapid generation of excess megakaryocytic and erythroid cells and abnormal blood counts. It is also consistent with the altered behavior of hematopoietic stem and progenitor cells that we observed.

At least three mechanisms that are 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 cells and progenitors and their differentiated progeny. As a consequence, after acquisition of the second mutation, the double-mutant subclone will find itself in a cellular
Ruxolitinib

Nonmutant TET2-mutant JAK2-homozygous JAK2-heterozygous

Time

• Older age at diagnosis
• Smaller homozygous subclones
• Relative expansion of CMPs
• Mostly single-mutant HSPCs
• Lower risk of thrombosis

• Younger age at diagnosis
• Larger homozygous subclones
• Relative expansion of MEPs
• Mostly double-mutant HSPCs
• Elevated risk of thrombosis
• Higher probability of polycythemia vera
Second, the initial mutation may mandate distinct cellular pathways as targets for subsequent mutations that are able to provide a growth advantage. The order in which TET2 and JAK2 are acquired may therefore dictate which additional mutations are acquired and thus influence the pathogenesis of disease.

Third, the initial mutation may modify the epigenetic program of hematopoietic stem cells and progenitor cells and thus alter the consequences of the second mutation. TET2 alters the epigenetic landscape by converting 5-methylcytosine to 5-hydroxymethylcytosine,34,35 and it also promotes the addition of N-acetylglucosamine to histones.36 Our results indicate that prior mutation of TET2 alters the transcriptional consequences of JAK2 V617F in a cell-intrinsic manner and prevents JAK2 V617F from up-regulating a proliferative program. The frequency with which epigenetic regulators are mutated in hematologic37 and nonhematologic38 cancers raises the possibility that mutation order influences the biology of many different cancers.

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