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Effect of Mutation Order on Myeloproliferative Neoplasms

Christina A. Ortmann, M.D., David G. Kent, Ph.D., Jyoti Nangalia, M.B.Chir., F.R.C.Path., Yvonne Silber, M.Sc., David C. Wedge, Ph.D., Jacob Grinfeld, M.B., Ch.B., F.R.C.Path., E. Joanna Baxter, Ph.D., Charles E. Massie, Ph.D., Elli Papaemmanuil, Ph.D., Suraj Menon, Ph.D., Anna L. Godfrey, F.R.C.Path., Ph.D., Danai Dimitropoulou, B.Sc., Paola Guglielmelli, M.D., Ph.D., Beatriz Bellosillo, Ph.D., Carles Besses, M.D., Ph.D., Konstanze Döhner, M.D., Claire N. Harrison, D.M., F.R.C.Path., George S. Vassiliou, F.R.C.Path., Ph.D., Alessandro Vannucchi, M.D., Peter J. Campbell, M.B., Ch.B., Ph.D., and Anthony R. Green, F.R.C.Path., F.Med.Sci.

ABSTRACT

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 thrombocythemia, 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.)

From the Cambridge Institute for Medical Research and Wellcome Trust–Medical Research Council Stem Cell Institute (C.A.O., D.G.K., J.N., Y.S., J.G., E.J.B., C.E.M., A.L.G., D.D., A.R.G.) and Department of Hematology (C.A.O., D.G.K., J.N., Y.S., J.G., E.J.B., C.E.M., A.L.G., D.D., G.S.V., P.J.C., A.R.G.), University of Cambridge, Department of Haematology, Addenbrooke's Hospital (C.A.O., J.N., J.G., E.J.B., A.L.G., G.S.V., P.J.C., A.R.G.), Wellcome Trust Sanger Institute (D.C.W., E.P., G.S.V., P.J.C.), and Cancer Research U.K. Cambridge Institute, Li Ka Shing Centre (S.M.), Cambridge, and Guy's and St. Thomas' National Health Service Foundation Trust, Guy's Hospital, London (C.N.H.) — all in the United Kingdom; Dipartimento di Medicina Sperimentale e Clinica, University of Florence, Florence, Italy (P.G., A.V.); Departments of Pathology (B.B.) and Hematology (C.B.), Hospital del Mar, Barcelona; and the Department of Internal Medicine III, University Hospital of Ulm, Ulm, Germany (K.D.). Address reprint requests to Dr. Kent or Dr. Green at the Cambridge Institute for Medical Research, Hills Rd., Cambridge CB2 0XY, United Kingdom, or at arg1000@cam.ac.uk or dgk23@cam.ac.uk.

Drs. Ortmann and Kent contributed equally to this article.

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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 biologic 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, if any, of the order in which mutations are acquired is unknown.[4]

Cooperation between different genetic lesions has been observed in cell-line models of transformation[5] and in mouse models of several cancers.[6,7] 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.[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 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.[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 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 colonies were grown 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 as described previously.[11] One population (lin^- $CD34^+CD38^-CD90^+CD45RA^-$) was enriched for both hematopoietic stem cells and hematopoietic progenitor cells (hereafter referred to as "hematopoietic stem and progenitor cells"). For single-cell cultures, hematopoietic stem and progenitor cells were sorted into 96-well plates supplemented with cytokines that were previously shown to support progenitor expansion.[12] (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[13] 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[14] (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.

Results

Stable Clonal Heterogeneity in Chronic-Phase Myeloproliferative Neoplasms

To identify patients who carried mutations in both *JAK2* and *TET2*, we sequenced all exons of the *TET2* gene in 246 patients (92 patients with essential

thrombocythemia, 107 with polycythemia vera, and 47 with myelofibrosis) who carried *JAK2* V617F. *TET2* mutations were identified in 24 patients (7 with essential thrombocythemia, 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 thrombocythemia 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 thrombocythemia 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 *TET2* mutation was acquired before the *JAK2* mutation (hereafter referred to as "*TET2*-first patients"), *JAK2* V617F was detected 12 to 112 months before the colony analysis. This indicates 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 obtained from 12 patients (4 with essential thrombocythemia, 4 with polycythemia vera, and 4 with myelofibrosis) after intervals of 1 to 3.7 years (Fig. 1D, and Fig. S2 and Table S2 in Supplementary Appendix 1). 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 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.

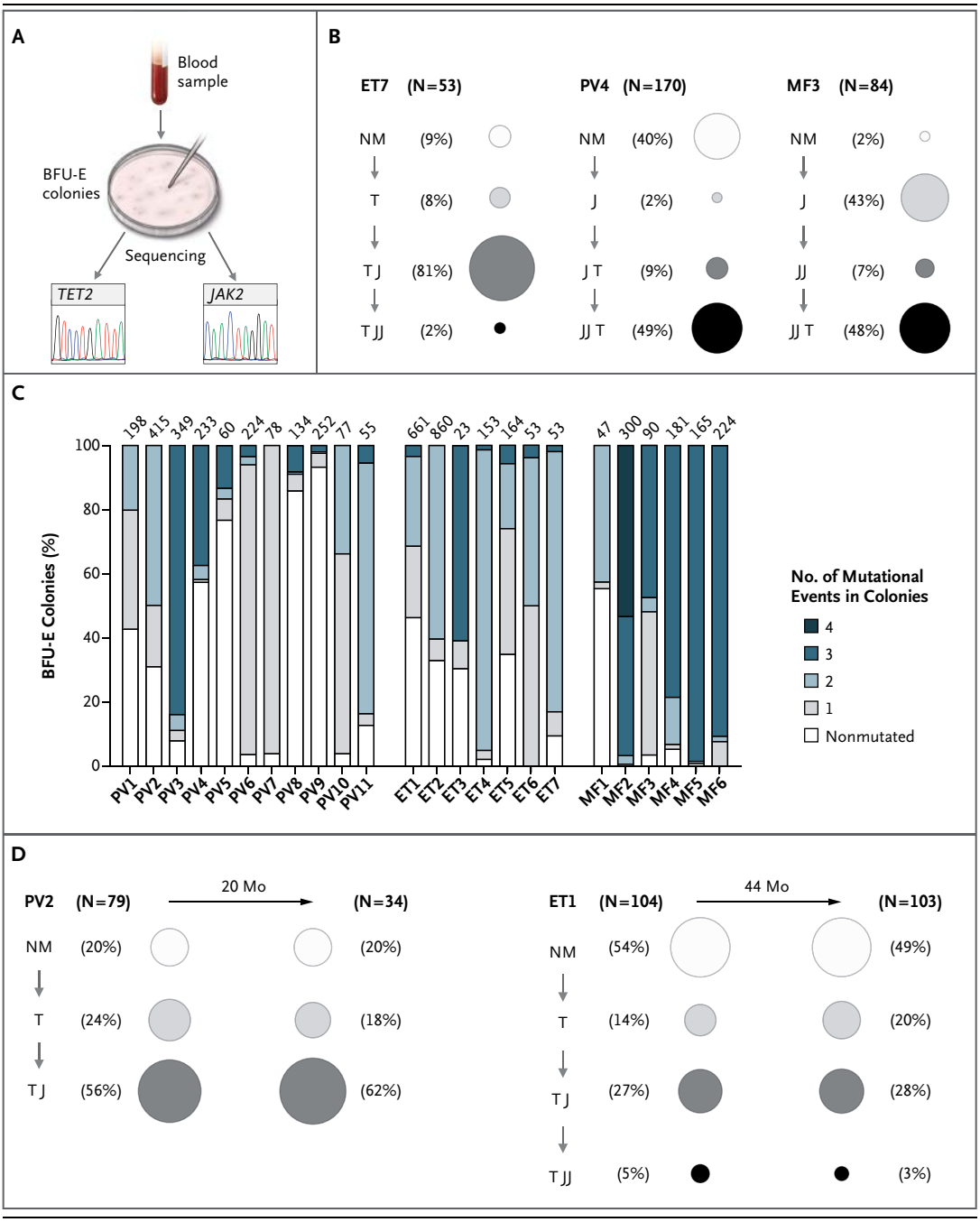
At presentation, *TET2*-first patients were, on average, 12.3 years older than patients in whom the *JAK2* mutation was acquired first (referred to as "*JAK2*-first patients") (mean age at diagnosis, 71.5 years vs. 59.2 years; $P=0.004$ by Student's t-test) (Fig. S3A in Supplementary Appendix 1). The 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 exclude the possibility that additional mutations contributed to the observed differences between *JAK2*-first and *TET2*-first patients, we performed exome[13] or targeted[14] sequencing in 23 of 24 patients. Only 4 patients harbored mutations known to be recurrent in myeloid cancers (Table S3 in Supplementary Appendix 1).[15] 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.

INFLUENCE OF MUTATION ORDER ON PROLIFERATION OF HEMATOPOIETIC STEM AND PROGENITOR CELLS

To extend our findings to the hematopoietic stem-and-progenitor-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^{+}CD45RA^{-} cells[16] for 10 days in conditions that were previously shown to support the growth of multipotent progenitors[12] (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 (Fig. 3A) (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-

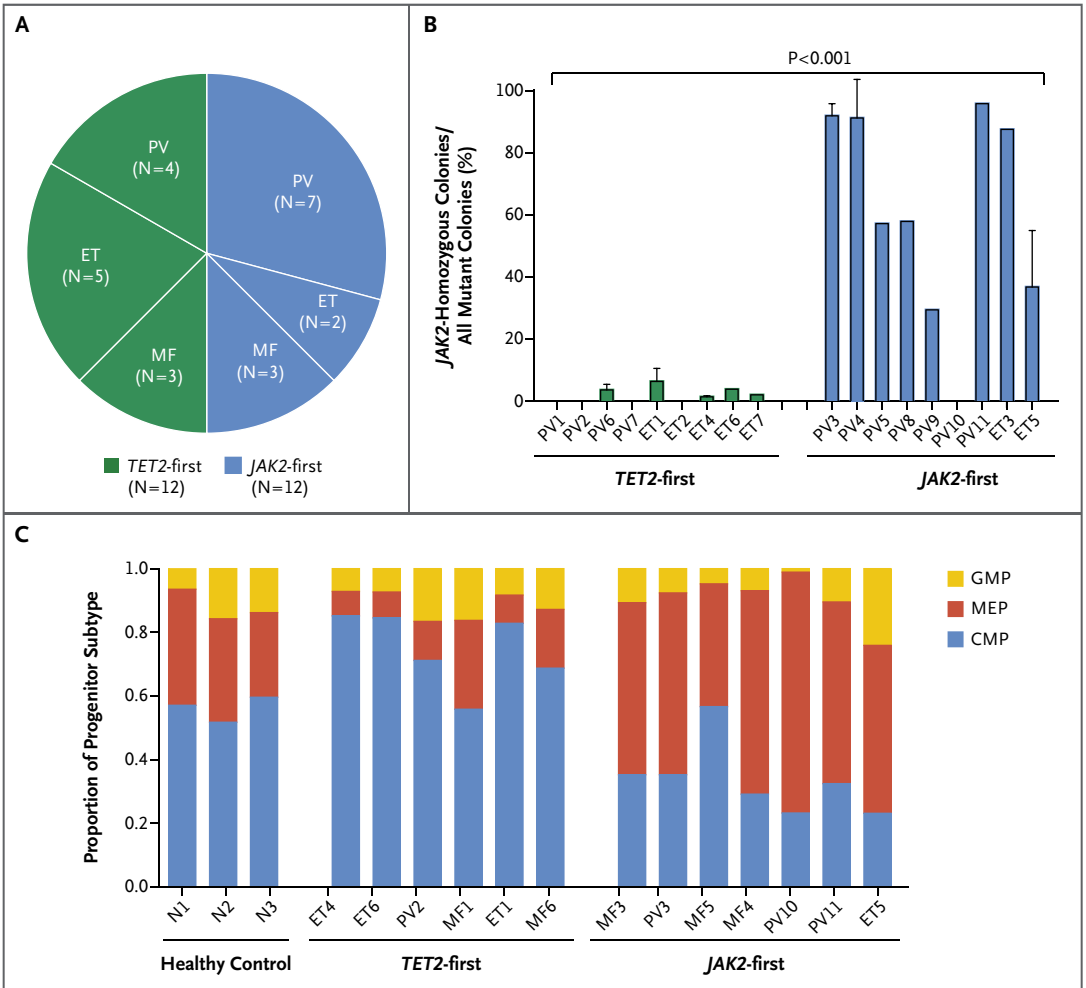


Figure 2. Effect of Mutation Order on Disease Phenotype.

In Panel A, the pie chart shows the order of acquisition of mutations in 24 patients. Half the patients with myeloproliferative neoplasms acquired the *JAK2* mutation first (*JAK2*-first) and half acquired the *TET2* mutation first (*TET2*-first). Both orders of acquisition of mutations occur in each disease subtype. ET denotes essential thrombocythemia, MF myelofibrosis, and PV polycythemia vera. In Panel B, the bar graph shows the proportion of *JAK2* V617F–homozygous colonies in the total number of mutant colonies. Patients in whom *JAK2* V617F was acquired first had a greater proportion of homozygous colonies in the mutant compartment (mean percentage of homozygous mutant colonies in *JAK2*-first patients, 57.8%, vs. 1.4% in *TET2*-first patients; P<0.001 by the t-test). Patients are indicated by their patient-identification numbers. T bars indicate standard errors. In Panel C, the bar graph shows the relative number of progenitor cells in the *TET2*-first patients and *JAK2*-first patients. The three bars represent the relative number of common myeloid progenitors (CMPs) ($lin^-CD34^+CD38^+CD90^-CD10^-FLK2^+CD45RA^-$), granulocyte–macrophage progenitors (GMPs) ($lin^-CD34^+CD38^+CD90^-CD10^-FLK2^+CD45RA^+$), and megakaryocyte–erythroid progenitors (MEPs) ($lin^-CD34^+CD38^+CD90^-CD10^-FLK2^-CD45RA^-$). *TET2*-first patients had a uniform predominance of CMPs over other progenitors within the $CD34^+CD38^+$ compartment (P=0.001 by the t-test). By contrast, in *JAK2*-first patients, MEPs were more prevalent than CMPs or GMPs (P<0.001 by the t-test). When the samples were obtained, the ages of the *TET2*-first and *JAK2*-first patients were similar (mean age, 78 years [range, 66 to 86] vs. 72 years [range, 50 to 84]).

throid 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 double-mutant erythroid colonies in samples obtained from seven patients (four *TET2*-first patients and three *JAK2*-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.

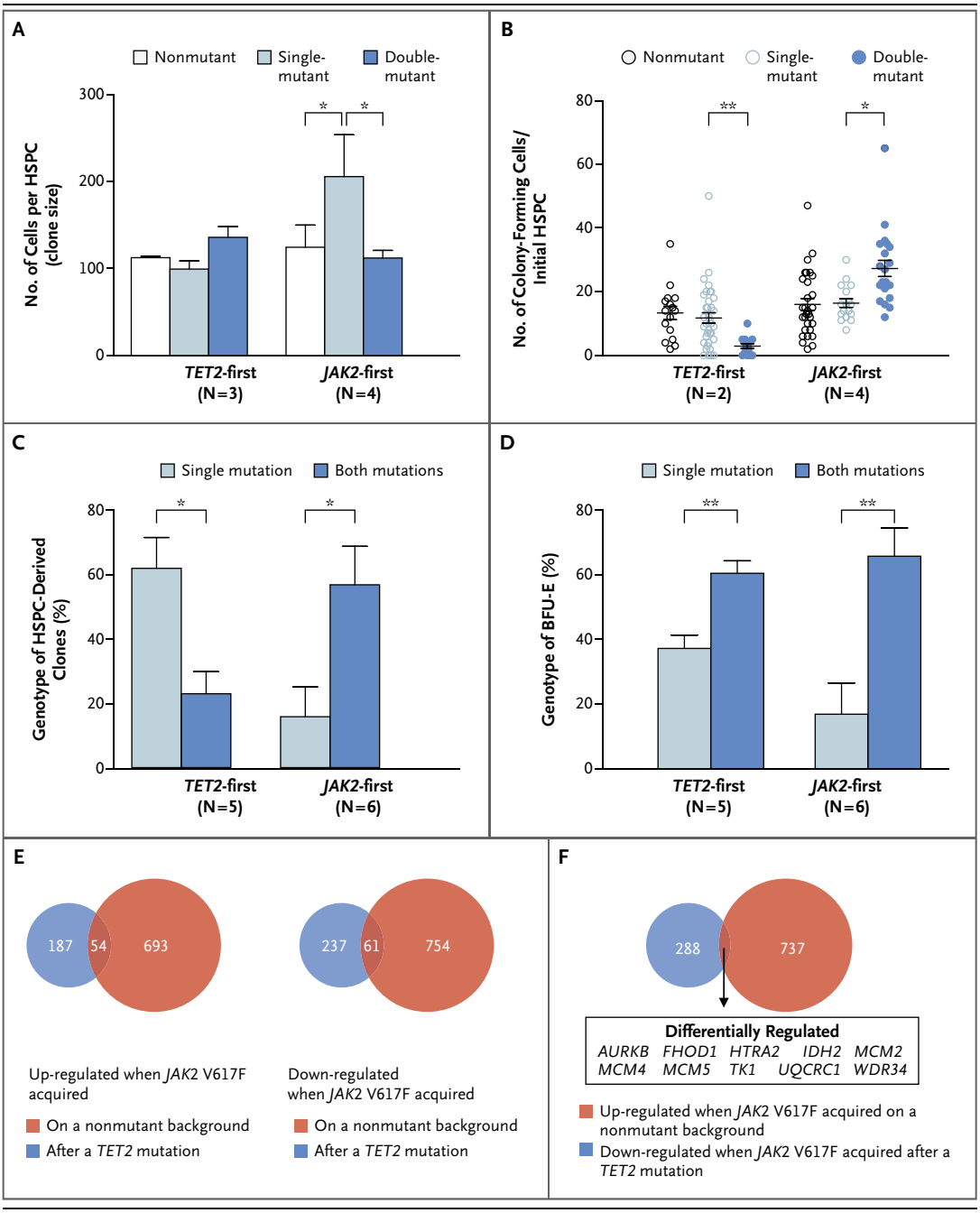
To investigate further whether prior mutation of *TET2* influences the transcriptional response to *JAK2* V617F, we compared genes that were up-regulated when *JAK2* V617F was acquired by TET2-nonmutant cells with those that were down-regulated when *JAK2* V617F was acquired by TET2-mutant cells. This 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 replication[17] (*MCM2*, *MCM4*, and *MCM5*) or regulation of mitosis (*AURKB*,[18] *FHOD1*,[19] and *TK1*[20]). 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 splanchnic-vein thrombosis, 1 had deep-vein thromboses, and 1 had calf-vein thromboses (1 patient had a transient ischemic attack, myocardial infarction, and splanchnic-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-

Figure 3 (facing page). Influence of Mutation Order on Proliferation of Stem and Progenitor Cells and Expression of Progenitor Genes.

In Panel A, the bar graph shows the average number of cells (clone size) that were present at 10 days in cultures of hematopoietic stem and progenitor cells (HSPCs) of different genotypes. Bars represent nonmutant, single-mutant, and double-mutant (both *TET2* and *JAK2*) clones. In the three *TET2*-first patients, the size of *TET2* single-mutant clones did not differ significantly from that of nonmutant clones ($P=0.24$ by the t-test) or double-mutant clones ($P=0.07$ by the t-test). By contrast, in the four *JAK2*-first patients, *JAK2* single-mutant clones were significantly larger than nonmutant clones ($P=0.05$ by the t-test) and double-mutant clones ($P=0.04$ by the t-test). T bars indicate standard errors, single asterisks $P<0.05$, and double asterisks $P<0.01$. In Panel B, the number of secondary colony-forming cells created per HSPC is shown for two patients who had a *TET2* mutation followed by a *JAK2* V617F mutation and four patients who had a *JAK2* mutation followed by a *TET2* mutation. HSPCs that acquire *JAK2* V617F on a TET2-mutant background, as compared with TET2 single-mutant HSPCs, produce fewer colony-forming cells (mean, 3 colony-forming cells vs. 12 colony-forming cells; $P=0.002$ by the t-test), whereas those that acquire *TET2* mutations on a JAK2 single-mutant background, as compared with JAK2 single-mutant HSPCs, produce more colony-forming cells (mean, 27 colony-forming cells vs. 16 colony-forming cells; $P=0.01$ by the t-test). In Panel C, each bar graph shows the percentage of total HSPCs of each genotype measured in five *TET2*-first patients and six *JAK2*-first patients. The percentage of HSPCs that retained the first mutation alone was higher in *TET2*-first patients than in *JAK2*-first patients. In Panel D, each bar graph shows the percentage of BFU-E colonies of each genotype in five *TET2*-first patients and six *JAK2*-first patients. In contrast to the proportion of mutant HSPCs (Panel C), the *TET2*-first BFU-E compartment is skewed toward the double-mutant clone. The *JAK2*-first BFU-E compartment is similar to the HSPC compartment. In Panel 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) when *JAK2* V617F is acquired on different backgrounds. In Panel F, the Venn diagram shows the numbers of genes that are up-regulated when *JAK2* V617F is acquired on a nonmutant background overlapped with those that are down-regulated when *JAK2* V617F is acquired on a TET2-mutant background. The 10 genes that followed this pattern are listed in the box.

plasms. Prior mutation of *TET2* dramatically influenced the transcriptional program activated by *JAK2* V617F; this provides 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 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,[23] *JAK2*-first patients,[24] or both.[25] 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,[26] and in xenograft studies involving two patients, a double-mutant *TET2*–*JAK2* clone was outcompeted by its *TET2* single-mutant ancestor.[23] Moreover, in genetically modified mice, expression of *JAK2* V617F resulted in increased erythropoiesis, whereas inactivation of *TET2* had no effect on erythropoiesis.[27,28] 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[29,30] or a disadvantage.[31,32]

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

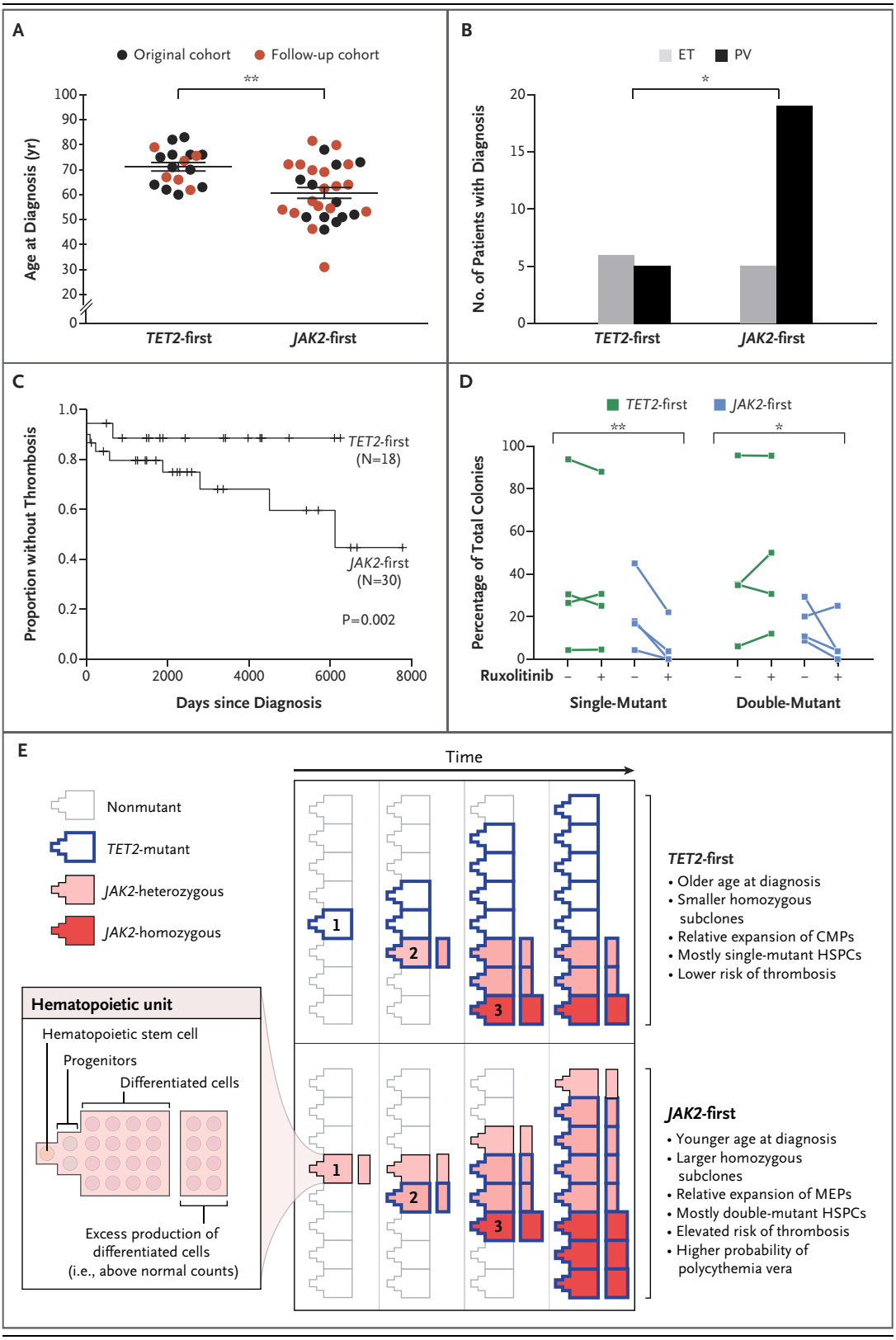


Figure 4 (facing page). Clinical Significance of Mutation Order in Myeloproliferative Neoplasms.
In Panel A, the mean age at presentation of 48 patients with myeloproliferative neoplasms in whom mutation order was determined is shown. On average, *TET2*-first patients presented 10.46 years later than *JAK2*-first patients (P=0.002 by the t-test; P=0.02 by two-way analysis of variance accounting for order of acquisition of mutation, sex, white-cell count, and phenotype of the myeloproliferative neoplasm). I bars indicate standard errors, and double asterisks P<0.01. In Panel B, histograms represent the number of patients who received a diagnosis of essential thrombocythemia (ET) or polycythemia vera (PV). *JAK2*-first patients were more likely to receive a diagnosis of PV (P=0.05 by the chi-square test). The single asterisk indicates P<0.05. In Panel C, a Kaplan–Meier curve shows thrombosis-free survival among 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 by multivariate analysis for mutational order and P=0.01 by Cox proportional-hazards model for age taking into account age, prior thrombosis, sex, subtype of myeloproliferative neoplasm, white-cell count, receipt of cytoreductive therapy, and order of mutations). In Panel D, the graph shows the relative sensitivity of colonies with distinct genotypes to ruxolitinib in a colony-forming cell assay (4 *TET2*-first patients and 4 *JAK2*-first patients, 35 to 66 colonies picked per patient). In *TET2*-first patients, single-mutant colonies had a *TET2* mutation alone, and in *JAK2*-first patients, single-mutant colonies had a *JAK2*-heterozygous or *JAK2*-homozygous mutation. Single-mutant clones in *JAK2*-first patients were more sensitive to ruxolitinib (left side, P<0.01 by the one-tailed t-test). Double-mutant clones (those bearing both *JAK2* and *TET2* mutations) in *JAK2*-first patients were also more sensitive to ruxolitinib as compared with clones from *TET2*-first patients (right side, P=0.03 by the one-tailed t-test). In Panel E, the way in which the order of mutation acquisition influences the evolution of disease is shown. This model depicts the manner in which single hematopoietic units (left), consisting of stem cells, progenitors, and differentiated cells, acquire mutations over time. Some units are hyperproliferative and produce excess differentiated cells that contribute to the disease phenotype. The numbers represent the acquisition of the first mutation (1), second mutation (2), and *JAK2* V617F homozygosity (3). Patients who acquire a *TET2* mutation first gain a self-renewal advantage but do not overproduce downstream progeny. The expansion of the *TET2*-alone clone (bold borders) without excess differentiated cells leads to clonal expansion without immediate clinical presentation. Hematopoietic stem cells that acquire a secondary *JAK2* mutation (pink fill) compete with the *TET2*-alone clone, and their increased proliferation at the progenitor level drives an overproduction of terminal cells. When homozygosity is acquired as a third event (red fill), this clone has limited space to expand because of the high self-renewal activity of *TET2*-alone and *TET2–JAK2*–heterozygous clones. Patients who acquire a *JAK2* mutation first (pink fill, lower panel) produce excess differentiated cells in the absence of a distinct self-renewal advantage in the hematopoietic stem cells. When a secondary *TET2* mutation is acquired, hematopoietic stem cells obtain a self-renewal advantage and JAK2–TET2–mutant cells expand at the stem-cell level. Hematopoietic stem cells with loss of heterozygosity of *JAK2* V617F (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 polycythemia vera and the elevated risk of thrombotic events in *JAK2*-first patients.

environment that is 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-cell and progenitor compartments.[33]

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 hematologic[37] and nonhematologic[38] cancers raises the possibility that mutation order influences the biology of many different cancers.

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