DNMT3A mutations occur early or late in patients with myeloproliferative neoplasms and mutation order influences phenotype

Somatic mutations in *JAK2*, *CALR* and *MPL* are found in the majority of myeloproliferative neoplasms (MPN) but many patients also harbor somatic mutations in epigenetic regulators of DNA methylation (*TET2*, *DNMT3A* and *IDH1/2*) or chromatin structure (*ASXL1* and *EZH2*). In MPN patients, mutations in *TET2*, *ASXL1* and *EZH2* occur either prior to or following the acquisition of *JAK2*^{V617F} [1] and, recently, the order of mutation acquisition for *JAK2*^{V617F} and *TET2* has been shown to influence hematopoietic stem/progenitor cell biology and clinical presentation.[2] *DNMT3A* is the next most frequently mutated gene in MPN after *TET2*, affecting 7-10% of patients.[3–5] However in contrast to other mutations, *DNMT3A* mutations (*DNMT3A*^{mut}) have only been reported early in myeloid disease: prior to acquisition of *JAK2*^{V617F} or in a separate clone in MPN;[1,6] prior to *NPM1* or *FLT3* mutations in *de novo* acute myeloid leukemia;[7] and prior to transformation of MPN or myelodysplasia into acute myeloid leukemia.[8] In this study we used clonal analysis of hematopoietic colonies from *DNMT3A*^{mut} MPN patients to investigate timing of mutation acquisition, subclonal evolution, and the influence of mutation order.

Thirteen *DNMT3A*^{mut} MPN patients were identified in whom viable material was available for clonal analysis (*Online Supplementary Table S1*): nine *JAK2*^{V617F}-mutated and two *MPL*-mutated patients identified by exome-sequencing, one *JAK2*^{exon12}-mutated patient identified by whole-genome sequencing, and one *CALR*-mutated patient identified by targeted gene screening. MPN diagnoses conformed to British Committee for Standards in Haematology. All samples were obtained following written informed consent and ethical approval. Peripheral blood mononuclear cells were cultured to obtain burst-forming units-erythroid (BFU-E) as described previously,[2] and 2991 individual colonies (average of 230 colonies/patient) were genotyped by Sanger sequencing for mutations in *DNMT3A*, *JAK2*, *CALR* and *MPL*.

DNMT3A^{mut} occurred prior to acquisition of *JAK2*^{V617F} in four patients ('*DNMT3A*-first' patients, Figure 1A) as evidenced by single-mutant colonies harboring *DNMT3A*^{mut} only and double-mutant colonies with *DNMT3A*^{mut} and *JAK2*^{V617F}. Single-mutant colonies represented a substantial proportion of BFU-E (mean 58%, range 27%-86%), consistent with mutant allele fractions from granulocyte whole-exome sequencing, and indicating the presence of significant 'pre-*JAK2*' multi-lineage clonal hematopoiesis in these patients. These results accord with observations of clonal hematopoiesis in patients with essential thrombocythemia despite low allele burdens of *JAK2*^{V617F},[9] and with reports of *DNMT3A*^{mut}-associated clonal hematopoiesis in normal individuals.[10,11] In three patients, *DNMT3A*^{mut} and *JAK2*^{V617F} colonies were mutually exclusive ('biclonal' patients, Figure 1B). In such patients, the two mutations arose either in separate cells downstream of a shared ancestral clone or in clonally unrelated cells. *DNMT3A*^{mut} and *JAK2*^{V617F} clones did not share any of the additional mutations identified by exome sequencing in the three biclonal patients (Figure 2 patients #25, #81, #27). Moreover, in one of the two female patients, *DNMT3A*^{mut} and *JAK2*^{V617F} clones harbored different active X-chromosomes (*Online Supplementary Figure S1*). These data demonstrate that *DNMT3A*^{mut} and *JAK2*^{V617F} clones in the same patient can represent clonally-unrelated expansions.

In three patients, *DNMT3A*^{mut} occurred after acquisition of mutated *JAK2* or *MPL* (*JAK2/MPL*-first patients, Figure 1C) but these patients were more difficult to identify. In patient #650, the order of mutation acquisition was initially unclear as the majority of colonies were double-mutant for *DNMT3A* and *JAK2*^{exon12}, and no antecedent single-mutant colonies were detected (Figure 1C). However, we confirmed that the *JAK2*^{exon12} mutation arose first, and that heterozygous-*JAK2*^{exon12} colonies with wild-type *DNMT3A* became undetectable following acquisition of *DNMT3A*^{mut}, because colonies grown from an earlier time-point showed heterozygous-*JAK2*^{exon12} colonies with wild-type *DNMT3A* (*Online Supplementary Figure S2A*). Furthermore, whole genome sequencing of a diagnostic sample and one taken at a later time-point demonstrated mutated *JAK2*^{exon12} at both times but *DNMT3A*^{mut} at only the later time-point (*Online Supplementary Figure S2B*). Consistent with this mutation order, the two homozygous-*JAK2*^{exon12} subclones present in this patient (Figure 1C, h1 and h2) carried different mitotic recombination breakpoints (*Online Supplementary Figure S2C*). In two further *JAK2/MPL*-first patients, antecedent *JAK2/MPL* single-mutant colonies were detected but were present only at low levels (~3% of total colonies, Figure 1C). Overall, considering MPN patients with sequential acquisition of mutations within the same clone, single-mutant *JAK2/MPL* subclones were significantly smaller than single-mutant *DNMT3A*^{mut} and double-mutant subclones (P=0.03 for both comparisons, t-test, *Online Supplementary Figure S3*). To exclude a confounding effect of mutations in other genes known to be recurrently mutated in myeloid malignancies, whole exome or genome sequencing data were interrogated for all 13 patients. Four patients had additional mutations in *TET2*, *CBL* or *SH2B3*. Delineation of full phylogenetic hierarchies using Sanger sequencing or Fluidigm single nucleotide polymorphism genotyping (see *Online Supplementary Methods*) of individual colonies from these patients did not identify any preferential association of these mutations with either single or double-mutant subclones (Figure 2). Our data therefore suggest that *JAK2/MPL* single-mutant subclones may have a competitive disadvantage compared with *DNMT3A*^{mut} subclones, in which case *JAK2/MPL*-first patients may be enriched among those in whom mutation order could not be determined by colony assay (Figure 1D). To investigate competition between *JAK2/MPL*-mutated and *DNMT3A*^{mut} subclones further, colonies were grown from paired samples obtained at different time-points (median separation 35 months; range, 6-179 months) in ten patients (3 biclonal, 3 *JAK2/MPL*-first, 3 *DNMT3*-first, 1 order unclear). In six patients who harbored single-mutant *DNMT3A*^{mut} colonies (2 patients receiving hydroxycarbamide, 1 patient receiving pipobroman, 2 patients receiving interferon-α, and 1 patient not receiving cytoreduction), the proportions did not change significantly between time-points (Figure 3A,B; blue shading). Similarly, in seven patients with double-mutant colonies (4 patients receiving hydroxycarbamide, 1 patient receiving pipobroman and 2 patients not receiving cytoreduction) there was no significant change in the proportions over time (Figure 3A,B; purple shading). In contrast, in all six patients with single-mutant *JAK2/MPL* colonies (1 patient receiving hydroxycarbamide, 2 patients receiving interferon and 3 patients not receiving cytoreduction), the proportions of single-mutant colonies fell significantly over time (Figure 3A,B; red shading, P=0.027, Wilcoxon signed rank test,). While the observed reduction of *JAK2/MPL* colonies could have been influenced by interferon treatment in two patients (#81 and #25), two patients in whom this pattern was also observed had not

received cytoreduction (#27 and #650), supporting the notion that single-mutant *JAK2/MPL* subclones have a competitive disadvantage. We found no associations between subclonal changes over time and treatment responses (measured in accordance with European LeukemiaNet guidelines) in patients, possibly because individual patients harbored multiple and differing combinations of the various clones.

We next assessed whether order of acquisition of *DNMT3A*^{mut} influenced *JAK2*^{V617F}-mutated MPN phenotype. In the eight patients in whom mutation order was established, all four *DNMT3A*^{mut}-first patients had essential

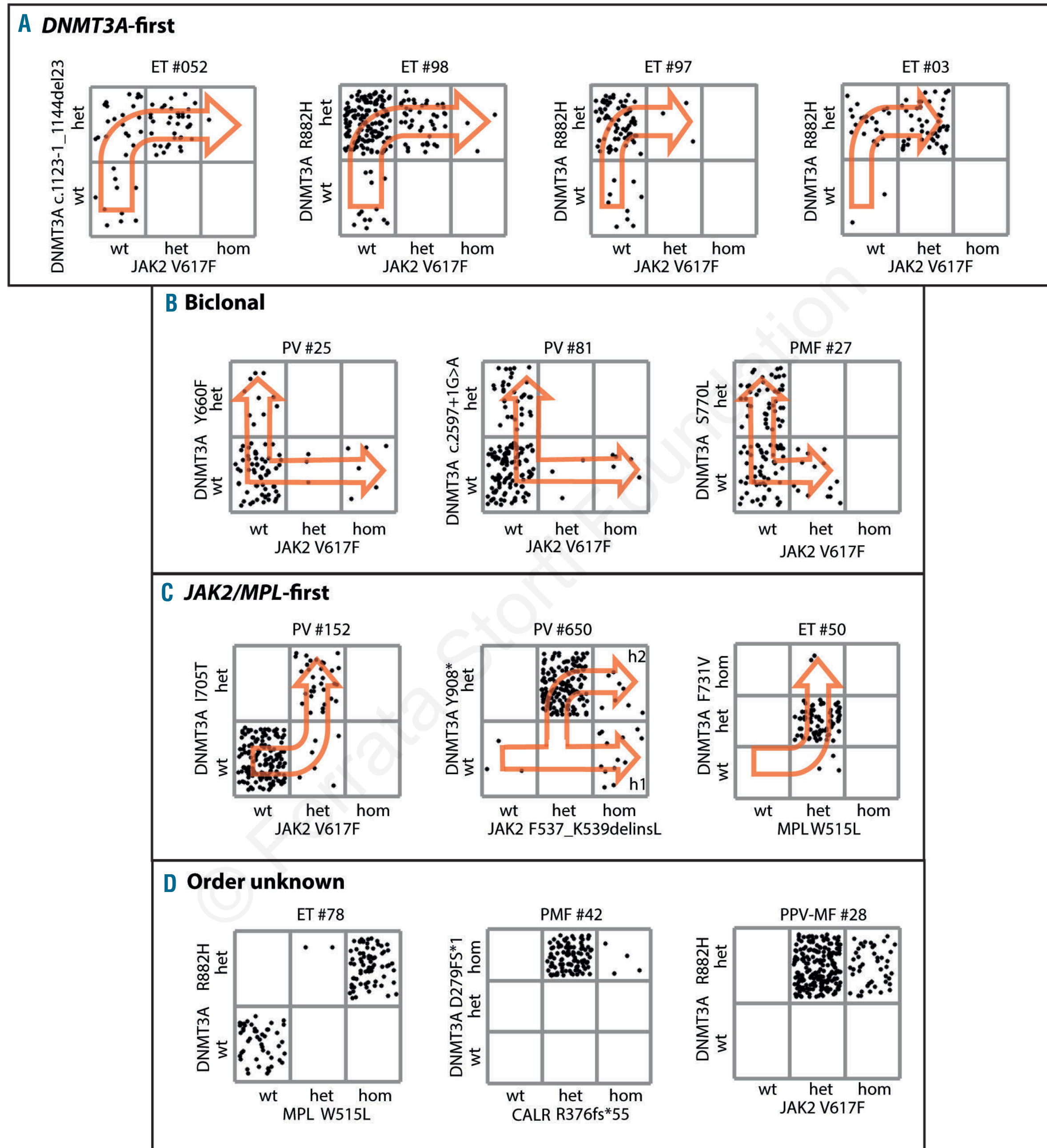

Figure 1. Timing of acquisition of *DNMT3A* mutations. Single cell-derived hematopoietic erythroid colonies (BFU-E) were grown *in vitro* from peripheral blood-derived mononuclear cells and individually genotyped for mutations using Sanger sequencing. Plots in (A), (B), (C) and (D) show colony genotyping results for mutations in *DNMT3A* and *JAK2*, *MPL* or *CALR* for each patient and the order of mutation acquisition. Within each plot, each dot represents a single colony and its quadrant placement shows the corresponding genotype of *DNMT3A* (vertical axis) and *JAK2/MPL/CALR* (horizontal axis). Solid red arrows within quadrants show the confirmed path of clonal evolution. Wt, wild-type; het, heterozygous mutation; hom, homozygous mutation; PV, polycythemia vera; ET, essential thrombocythemia; PMF, primary myelofibrosis; PPV-MF, post-PV myelofibrosis (A) *DNMT3A*-first patients: four patients in whom mutated *DNMT3A* occurred prior to the acquisition of *JAK2*^{V617F}. (B) Biclonal patients: three patients in whom *DNMT3A*^{mut} and *JAK2*^{V617F} were in separate clones. (C) *JAK2/MPL*-first patients: three patients in whom mutated *JAK2* or *MPL* occurred prior to the acquisition of mutated *DNMT3A*. (D) Order unknown: three patients in whom the order of mutation acquisition of *DNMT3A* and *JAK2/MPL/CALR* could not be delineated as only wild-type colonies and/or double mutant colonies were detected.

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thrombocythemia, and of four patients in whom *JAK2*^{V617F} occurred on a *DNMT3A*-non-mutated background (1 *JAK2*^{V617F}–first and 3 biclonal patients), three had polycythemia vera and one had myelofibrosis. To expand this cohort, an additional 918 patients were screened by targeted gene sequencing, and a further 33 patients with *DNMT3A*^{mut} and *JAK2*^{V617F} were identified. Copy-number corrected variant allele fractions were used to determine the order of mutation acquisition as recently described,[2] and mutation order was unambiguously assigned in eight further patients: two *DNMT3A*-first patients had essential thrombocythemia, and six *JAK2*-first patients comprised three with polycythemiva vera, one with post-polycythemia vera myelofibrosis, one primary myelofibrosis and one essential thrombocythemia. Combining both cohorts, all six *DNMT3A*-first patients presented with essential thrombocythemia. By contrast, of ten patients in whom *JAK2*^{V617F} arose on a wild-type *DNMT3A* background, seven presented with polycythemia vera and only one with essential thrombocythemia (P=0.003, chi-squared test). There were no significant differences in other clinical features (*Online Supplementary Table S2*) in this cohort. Our results therefore indicate that mutation order influences clinical presentation, not only in MPN patients with *JAK2*^{V617F} and *TET2* mutations, as previously described,[2] but also in patients with *JAK2*^{V617F} and *DNMT3A* mutations. Mutations in either *DNMT3A* or *TET2* are associated with an essential thrombocythemia phenotype when acquired prior to *JAK2*^{V617F}. By contrast, acquisition of *JAK2*^{V617F} prior to mutation of *DNMT3A* or *TET2* is associated with polycythemia vera. '*TET2*-first' patients were older at presentation in our previous study,[2] but no difference in age was identified between *DNMT3A*-first and *JAK2*-first patients. This may be due to the smaller number of patients in the current study or may reflect a real difference in the age at which *TET2* and *DNMT3A* mutations are acquired.

In summary, we demonstrate that in MPN, *DNMT3A*^{mut} can follow *JAK2* and *MPL* mutations, and that *JAK2/MPL* single-mutant subclones have a competitive disadvantage

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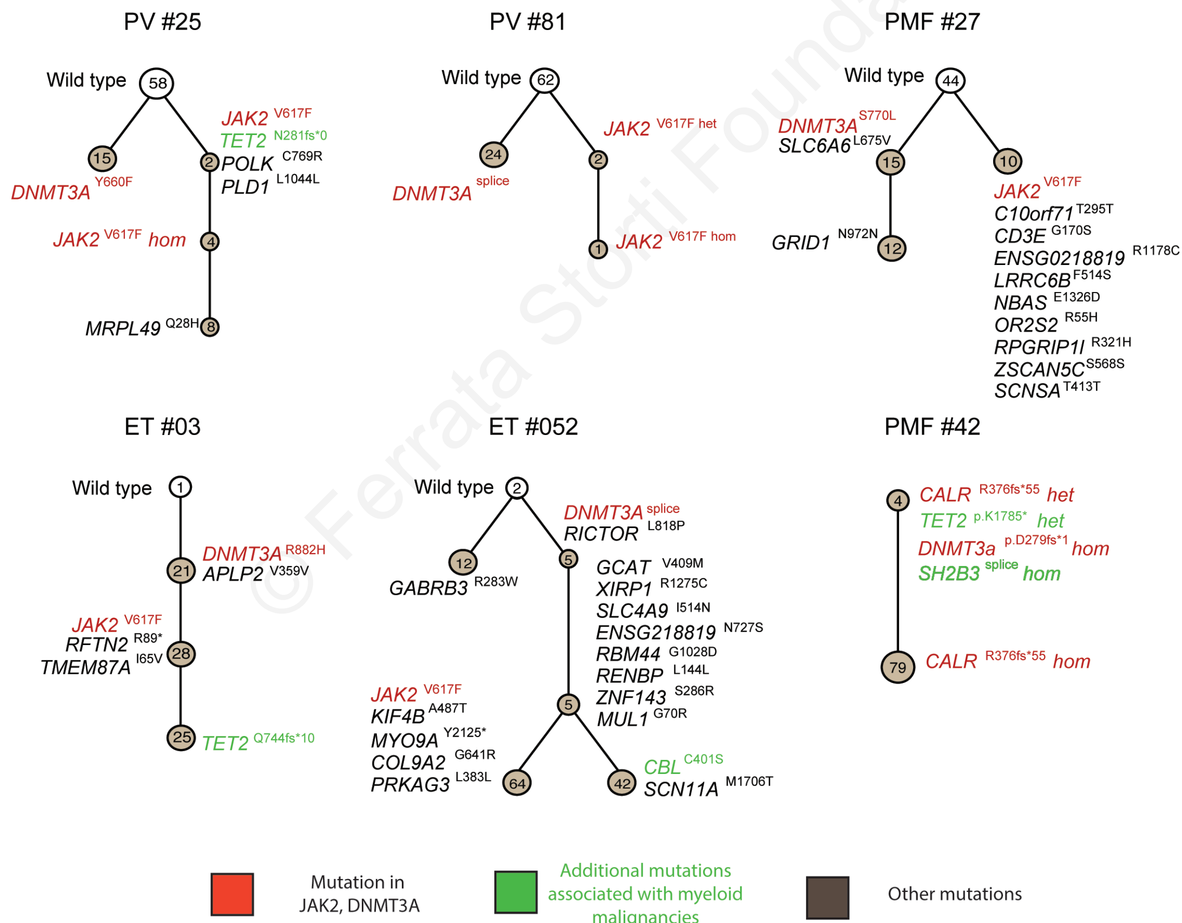

Figure 2. Clonal structures of *DNMT3A*-mutated MPN. Individual single cell-derived hematopoietic erythroid colonies (BFU-E) from six patients were genotyped using Sanger sequencing or Fluidigm single nucleotide polymorphism genotyping for their respective somatic variants identified previously by whole exome sequencing. Genotyping results from individual colonies were then used to construct phylogenetic trees. Circles represent the subclones; wild-type (white); mutated (brown). The earliest detectable clone is represented at the top of each structure, with subsequent subclones shown below. Somatic mutations acquired in each subclone are indicated beside respective circles, and represent those that are acquired in addition to mutations present in earlier clones. Numbers of colonies identified for each subclone are shown inside circles. Mutations in *JAK2*, *MPL*, *CALR* and *DNMT3A* are highlighted in red. Additional mutations in genes known to be recurrently mutated in myeloid malignancies are highlighted in green. ET: essential thrombocythemia; PMF: primary myelofibrosis; PV: polycythemia vera; het: heterozygous mutation; hom: homozygous mutation.

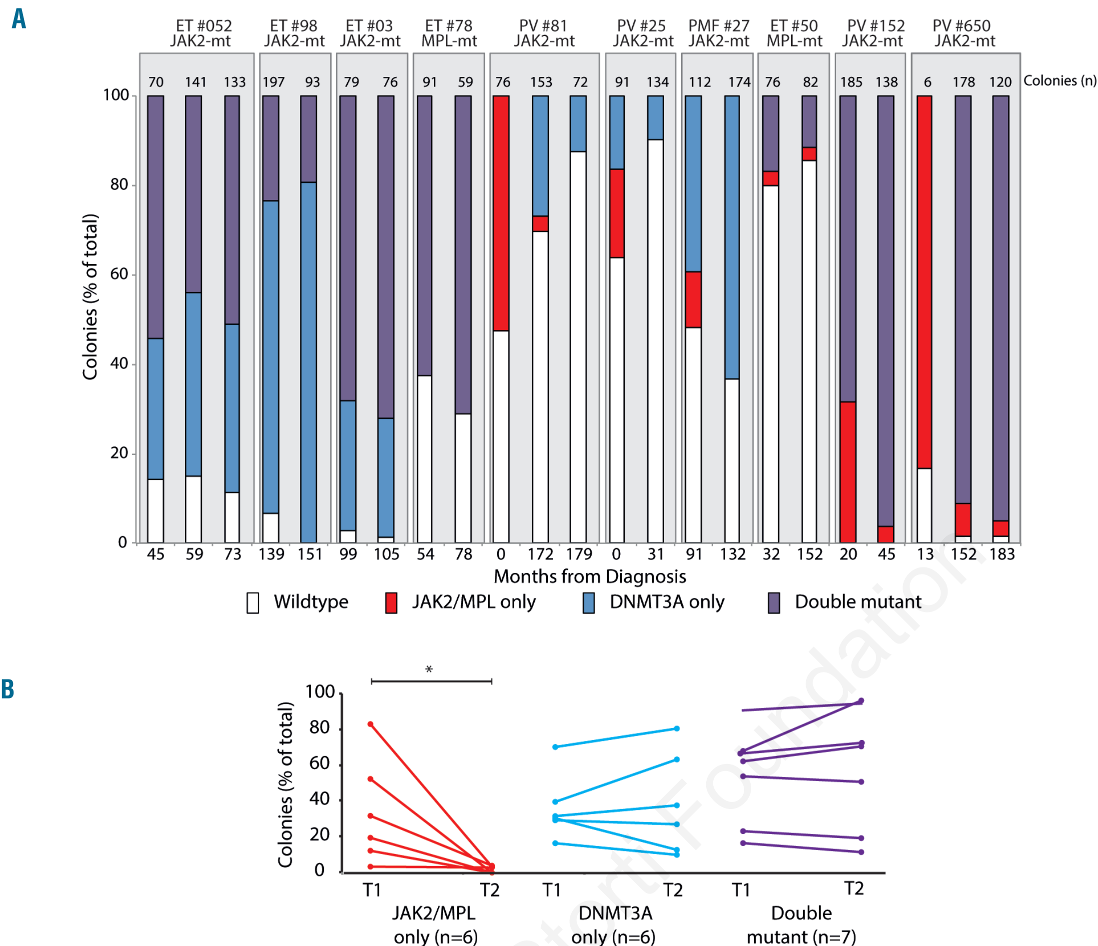

Figure 3. Evolution of subclones in *DNMT3A*-mutated MPN. (A) Colonies grown from paired samples obtained at different time-points (median separation 35 months; range, 6-179 months) in ten patients. Vertical axis shows the percentage of total colonies and columns are shaded to represent the proportions of the different genotypes (red, *JAK2/MPL*-only; blue, *DNMT3A*-only; purple, double mutant). Numbers of colonies genotyped per patient are shown above columns and the timings of sample acquisition (months from diagnosis) are shown below. (B) Changes in subclonal proportions over time for the ten patients in (A) for a total of 19 subclones. red, *JAK2/MPL*-only; blue, *DNMT3A*-only; purple, double mutant; T1 and T2 represent the earliest and latest time-points sampled for the patients. The median interval between T1 and T2 did not differ significantly between the different subclones (one-way analysis of variance). * <0.05 Wilcoxon ranked sum test.

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in vivo compared with *DNMT3A*^{mut} subclones. This concept is consistent with observations that *DNMT3A* and *TET2* mutations confer an advantage to hematopoietic stem/progenitor cells,[2,12–14] whereas this is not the case for *JAK2*^{V617F} in some mouse models.[15] Furthermore, we show that mutation order of *JAK2*^{V617F} and *DNMT3A*^{mut} is associated with differences in MPN phenotype. This emphasizes the importance of the pattern of acquisition of *JAK2*^{V617F} with respect to mutations in epigenetic modifiers in influencing the phenotype of *JAK2*^{V617F}-mutated MPN.

Jyoti Nangalia,[1,2,3] Francesca L. Nice,[1] David C. Wedge,[3] Anna L Godfrey,[1,2] Jacob Grinfeld,[1,2,3] Clare Thakker,[1] Charlie E. Massie,[1] Joanna Baxter,[2,4] David Sewell,[2,4] Yvonne Silber,[1] Peter J. Campbell,[1,2,3] and Anthony R. Green[1,2]

[1]Department of Haematology, Cambridge Institute for Medical Research and Wellcome Trust/MRC Stem Cell Institute, University of Cambridge; [2]Department of Haematology, Addenbrooke's Hospital, Cambridge; [3]Wellcome Trust Sanger Institute, Hinxton, Cambridge; and [4]Cambridge Blood and Stem Cell Bank, University of Cambridge, UK

Correspondence: arg1000@cam.ac.uk
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The online version of this letter has a Supplementary Appendix.

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References

1. Lundberg P, Karow A, Nienhold R, et al. Clonal evolution and clini-

cal correlates of somatic mutations in myeloproliferative neoplasms. Blood. 2014;123(14):2220–2228.
2. Ortmann CA, Kent DG, Nangalia J, et al. Effect of mutation order on myeloproliferative neoplasms. N Engl J Med. 2015;372(7):601–612.
3. Stegelmann F, Bullinger L, Schlenk RF, et al. DNMT3A mutations in myeloproliferative neoplasms. Leukemia. 2011;25(7):1217–1219.
4. Abdel-Wahab O, Pardanani A, Rampal R, Lasho TL, Levine RL, Tefferi A. DNMT3A mutational analysis in primary myelofibrosis, chronic myelomonocytic leukemia and advanced phases of myeloproliferative neoplasms. Leukemia. 2011;25(7):1219–1220.
5. Nangalia J, Massie CE, Baxter EJ, et al. Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. N Engl J Med. 2013;369(25):2391–2405.
6. Rao N, Butcher CM, Lewis ID, et al. Clonal and lineage analysis of somatic DNMT3A and JAK2 mutations in a chronic phase polycythemia vera patient. Br J Haematol. 2012;156(2):268–270.
7. Shlush LI, Zandi S, Mitchell A, et al. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. Nature. 2014;506(7488):328–333.
8. Fried I, Bodner C, Pichler MM, et al. Frequency, onset and clinical impact of somatic DNMT3A mutations in therapy-related and secondary acute myeloid leukemia. Haematologica. 2012;97(2):246–250.
9. Kralovics R, Teo S-S, Li S, et al. Acquisition of the V617F mutation of JAK2 is a late genetic event in a subset of patients with myeloproliferative disorders. Blood. 2006;108(4):1377–1380.
10. Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. N Engl J Med. 2014;371(26):2488–2498.
11. Genovese G, Kähler AK, Handsaker RE, et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. N Engl J Med. 2014;371(26):2477–2487.
12. Challen GA, Sun D, Jeong M, et al. Dnmt3a is essential for hematopoietic stem cell differentiation. Nat Genet. 2012;44(1):23–31.
13. Ko M, Bandukwala HS, An J, et al. Ten-eleven-translocation 2 (TET2) negatively regulates homeostasis and differentiation of hematopoietic stem cells in mice. Proc Natl Acad Sci USA. 2011;108(35):14566–14571.
14. Moran-Crusio K, Reavie L, Shih A, et al. Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation. Cancer Cell. 2011;20(1):11–24.
15. Li J, Kent DG, Chen E, Green AR. Mouse models of myeloproliferative neoplasms: JAK of all grades. Dis Model Mech. 2011;4(3):311–317.

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