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

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Supplementary Appendix

Construction of phylogenetic hierarchies by genotyping of individual haematopoietic colonies

Haematopoietic colonies (BFU-E) were grown and individually genotyped using Sanger sequencing or Fluidigm SNP Genotyping for all the mutations that had been previously identified by next-generation sequencing in each patient. Colony genotyping for patients #81, #03, #42 and #25 was by PCR and Sanger sequencing. Colony genotyping for #052 and #27 was by Fluidigm SNP Genotyping.

Fluidigm genotyping primers were designed according to the manufacturer's recommendations, and obtained from Fluidigm (Fluidigm Corp, CA, USA). The Fluidigm SNPType Genotyping Reagent Kit (Fluidigm Corp, CA, USA) was used according to the manufacturer's instructions. Colony DNA was first amplified using Specific Target Amplification as per the manufacturer's instructions, to enrich for the DNA sequences for subsequent PCR. Genotyping reactions were then performed on a nanofluidic 192.24 Dynamic Array Integrated Fluid Circuit (IFC; Fluidigm Corp, CA, USA), which automatically assembles PCR reactions. End-point fluorescent images were acquired on a Biomark system and analysed using the Biomark SNP Genotyping Analysis software version 3.1.2. Colonies with ambiguous genotype results underwent repeat PCR and Sanger sequencing.

Primer sequences for Sanger sequencing were as follows:

Gene	Forward primer	Reverse Primer
JAK2^{V617F}	TTTCCTTAGTCTTTCTTTGAAGCAGC	TAGTTTACACTGACACCTAGCTGTGATCC
JAK2^{exon12}	CTCCTCTTTGGAGCAATTCA	CATCTAACACAAGGTTGGCATA
DNMT3A^{R882}	AATACTCCTTCAGCGGAGCGAAGA	AAGATTCGGCAGAACTAAGCAGGC
DNMT3A^{Y660F}	GTCCCCGACGTACATGATCT	GACTTGGGCCTACAGCTGA
DNMT3A$^{splice(\#81)}$	CAGGACGTTTGTGGAAAACA	CTCCATAAAGCAGGGCAAA
DNMT3A$^{splice(\#052)}$	TGCCCTCATTTACCTTCTGG	CTCAGAGTCTGGCCTTGAGC
DNMT3A^{S770L}	ATGAAGCAGCAGTCCAAGG	TTGAGTTCTACCGCCTCCTG
DNMT3A^{Y908*}	CGCCTCTGTGGTTTTTGTTT	AGTCATCCGCCACCTCTTC
DNMT3A^{D279fs*1}	CACCTCGTACTCTGGCTCGT	CAGGAATGAATGCTGTGGAA
DNMT3A^{F731V}	ATGAAGCAGCAGTCCAAGG	GGCTTTCTCTTCCGACCTCT
DNMT3A^{I705T}	CAAGGAGGAAGCCTATGTG	CTTCCTGTCTGCCTCTGTCC
TET2^{N281fs*0}	CACATGGTGAACTCCTGGAA	TCAGCATCATCAGCATCACA
TET2^{Q744fs*10}	GAGCAGATTCCCAAACTGAAA	CTTTGGGGGTGAGGAAAAGT
TET2^{K1785*}	TGGTGAACATCATTCACCTTCT	TGGTGAACATCATTCACCTTCT
CALR	CCTGCAGGCAGCAGAGAAAC	ACAGAGACATTATTTGGCGCG
MPL	TGACCGCTCTGCATCTAGT	TACAGGCCTTCGGCTCCA
POLK	TGTTCTAGTCTCCCAAGCAAGTC	AAGCAAACATCCACATGCAC
PLD1	CATCCCCAGGAAGTCACTGT	CATTCGAGCTGAGGAGGAAC
MRPL49	CGCCCAGCCTTTTATTTATG	GGGATCTGGGATCCTGGTAG
APLP2	GACGTCACTGCCTCTGTCCT	GCGAGGCAGGACTTACTCAT
TMEM87A	AATGCCAGACTATTAGATTTCAGAAC	TTGAAGCCATCTGAGGCTAA
RFTN2	TAAAACCCCCATGTGTCCTT	CGGGGCTATTCATCCTGTTA
SH2B3	ACCACCTTTGCTGCTACCAC	CCCACCTTGGTTAAGGGAAT
LANCL3	GCTCACGGCTTGTCGTCTAT	CAGTGCACCAGCTCATTCTC

Microsatellite marker analysis

Microsatellite analysis was used to determine the extent of chromosome 9p loss-of-heterozygosity. A panel of microsatellite markers along chromosome 9p were selected from the GeneLoc database. The PCR reaction contained 1.25µl 10x ReddyMix PCR buffer IV, 0.75µl 25mM magnesium chloride solution, 0.125µl Thermoprime plus polymerase (Thermo Scientific, MA, USA), 0.1µl 100mM dNTPs, 0.06µl 100µM forward primer, 0.06µl 100µM reverse primer, and 0.5-2µl DNA, in a total reaction volume of 12.5µl. PCR conditions were 11 minutes at 95^{o}C, followed by 38 cycles of 30 seconds at 95^{o}C, 30 seconds at 57^{o}C and 1 minute at 72^{o}C, followed by a final extension at 72^{o}C for 10 minutes. The PCR reaction was diluted between 1:5 and 1:40 in sterile water. Fragment detection was performed on a 3730xl Genomic analyser and results were analysed using the Peak Scanner software version 1.0 (Applied Biosytems, CA, USA). Heterozygosity for these markers was initially analysed using constitutional DNA obtained from a buccal swab. Informative markers were then taken forwards for testing on tumour DNA from individual homozygous *JAK2*^{exon12}-mutated colonies to ascertain the extent of loss-of-heterozygosity in heterozygous microsatellite markers.

Primer sequences were as follows:

Microsatellite marker	Primer name	Sequence (5'-3')
D9S288	D9S288_F	GTTTCTTAGCAACCTCAACAGGG
	D9S288_R	6-FAM-AATCATCCAGAAAGGCCA
D9S1852	D9S1852_F	GTTTCTTGAATCACAACATACACCCAC
	D9S1852_R	6-FAM-GAAACATTCTTTTACAAGTAACATT
D9S235	D9S235_F	6-FAM-CTGTATGGAGAGAGAATACG
	D9S235_R	GTTTCTTGGTCTCTCCGGTATACTCA
D9S925	D9S925_F	6-FAM-TGTGAGCCAAGGCCTTATAG
	D9S925_R	GTCTGGGTTCTCCAAAGAAA
D9S162	D9S162_F	6-FAM-GCAATGACCAGTTAAGGTTC
	D9S162_R	AATTCCCACAACAAATCTCC
D9S161	D9S161_F	6-FAM-TGCTGCATAACAAATTACCAC
	D9S161_R	GTTTCTTCATGCCTAGACTCCTGATCC
D9S43	D9S43_F	6-FAM-TTCTGATATCAAAACCTGGC
	D9S43_R	AAGGATATTGTCCTGAGGA
D9S1817	D9S1817_F	6-FAM-AGCTGTAGTGAGCCCTGAT
	D9S1817_R	CGTTAGGAGCCTTGAGACTT
D9S1791	D9S1791_F	6-FAM-GTAATCTTGGGCAACCTATGTATG
	D9S1791_R	TCAAAATAAGTCTGGGACAAAACC
D9S2148	D9S2148_F	6-FAM-TCAATCAACATCTGTCTATTCATC
	D9S2148_R	ACATCTGGCACTCTGGAGAG
D9S176	D9S176_F	6-FAM-AGCTGGCTGTTGGAGAAA
	D9S176_R	TGACCAATGGCAGGGTAT

Table S1: Clinical features of MPN patients with *DNMT3A* mutations

			At Diagnosis											At Follow-up	
MPN	Patient	Sex	Age	Hb g/dl	WCC x10^9/l	Plts x10^9/l	Spleen	Karyotype	Therapy during study	Past therapy	Disease (yrs)	*DNMT3A* mutation	*JAK2*/*MPL*/*CALR* status	Death	Thrombosis
ET	#052	F	76	14.5	7.7	804	N	FISH normal	HU	HU	7	c.1123-1_1144del23$	JAK ^{V617F}	N	N
ET	#98	M	84	15.0	10.7	1285	N	Karyotype normal	P	HU	14	p.R882H	JAK ^{V617F}	Y	N
ET	#8697	F	66	12.0	36.3^	1034	N	13q del	HU	HU	5	p.R882H	JAK ^{V617F}	N	N
ET	#03	M	54	14.3	16.4	953	N	FISH normal	HU	IFN	11	p.R882H	JAK ^{V617F}	N	Y
PV	#152	M	72	18.6	18.8	750	N	Del Y	HU	HU	15	p.I705T	JAK ^{V617F}	Y	Y
PV	#650	F	53	18.5	5.1	308	N	Karyotype normal	nil	nil	19	p.Y908*	JAK2 $^{exon\ 12\$\$\$}$	N	N
ET	#50	M	75	11.9	7.1	488	N	NA	nil	nil	5	p.F731V	MPL ^{W515L}	N	N
ET	#78	M	66	14.8	9.1	983	N	NA	HU	HU	6	p.R882H	MPL ^{W515L}	N	N
PPV-MF	#28	F	45	10.4	10.4	229	Y	NA	nil	nil	8	p.R882H	JAK ^{V617F}	N	Y
PV	#25	F	45	16.2	17.6	654	Y	NA	IFN	IFN	4	p.Y660F	JAK ^{V617F}	N	Y
PV	#81	M	33	17.7	11.2	853	Y	Karyotype normal	IFN	IFN	16	*G>A*, c.2597+1$$	JAK ^{V617F}	N	N
PMF	#27	F	57	10.7	5.2	293	N	FISH normal	nil	Thal	12	p.S770L	JAK ^{V617F}	N	N
PMF	#42	F	62	12.6	15	238	Y	NA	Rux	Imatinib	1	p.D279fs*1	CALR ^{R376fs*55}	N	N

MPN, myeloproliferative neoplasm; Hb, hemoglobin; WCC, white cell count; Plts, platelet count; PV, polycythemia vera; PMF, primary myelofibrosis; ET, essential thrombocythemia; PPV-MF, post PV myelofibrosis; HU, hydroxyurea; P, pipobroman; IFN, interferon alpha; Rux, Ruxolitinib; NA, not available; Y, yes; N, no, NA, not available; $ cDNA annotation of *DNMT3A* deletion affecting intron-exon boundary; $$ cDNA annotation of *DNMT3A* mutation affecting an essential splice site; $$$ *JAK2* exon 12 mutation p.F537_539_delinsL; * stop codon. No transformation events to myelodysplasia, acute myeloid leukemia or polycythemia vera occured during the follow-up period.^ Significant leucocytosis due to concurrent chronic lymphocytic leukemia.

Table S2

	JAK2^{V617F}-first*	*DNMT3A*-first	P-value
Total	10	6	
PV (n)	6	0	0.002
ET (n)	1	6	
MF (n)	3**	0	
Median age (years)	54.6	70.5	0.40
Mean WBC (x10^{9}/l)	9.6	14.4	0.57
Thrombosis (n)	2	1	NP
Progression (n)	0	0	NP
Death (n)	1	1	NP
Median follow-up (days)	4170 (863-5866)	3149 (777-5036)	0.52

Clinical data for *JAK2*^{V617F} and *DNMT3A*^{mut} patients with an established order of mutation acquisition (8 patients in whom order was determined from initial colony analysis and 8 further patients in whom mutation order was determined using data from targeted next-generation sequencing). Data show number of patients or events (n) in the different mutation hierarchy groups. *includes three biclonal patients where *JAK2*^{V617F} was also acquired on a *DNMT3A*-wildtype background. **includes one patient with post-PV MF. P-values show results of Chi-squared analysis for categorical variables and t-test for continuous variables. NP, not performed.

Supplementary Figure Legends

Figure S1. X chromosome inactivation status assessment by analysis of expression patterns of heterozygous SNPs in LANCL-3 and MPP1 in the two female patients (#25 and #27). Hematopoietic colonies were pooled by mutation status for *JAK2* and *DNMT3A.* Pooled colonies underwent cDNA extraction followed by SNP genotyping. Sequencing traces are of cDNA derived from colonies sharing the same genotype of *JAK2*^{V617F} and *DNMT3A*. #27 (upper panel) has the same X chromosome inactivation status in all subclones and is not informative. #25 (lower panel) has differing expression patterns in *JAK2*^{V617F}-mutated and *DNMT3A*-mutated colonies confirming their independent origins.

Figure S2 (a) Colony genotyping from an early sample from #650 showing colonies with heterozygous mutated *JAK2* and wildtype *DNMT3A* **(b)** Whole genome sequencing reads from #650. Forward and reverse DNA reads are shown in blue and yellow, respectively, and mismatched bases (compared with the reference genome) are shown in red. Left panels shows a region in *JAK2* exon 12 and right panels shows a region in *DNMT3A.* Lower panels show an early bone marrow sample from diagnosis and upper panels show a peripheral blood sample from 14 years later. *JAK2* exon 12 mutation (p. F537_K539delinsL) is present in both early and late timepoints, however, *DNMT3A* mutation (p. Y990*) is not present in the earlier time point. **(c)** Microsatellite mapping of 9p loss-of-heterozygosity (LOH) in individual hematopoietic colonies (BFU-Es) to determine the breakpoint region leading to acquired uniparental disomy in colonies with homozygous mutations in *JAK2* exon 12 from patient #650. The panel of microsatellite markers is shown along the bottom in relation to their position from the telomere and centromere on chromosome 9. Non-informative markers are shaded grey, heterozygous markers are shaded in black and markers showing LOH are shaded in red. Colony numbers genotyped in the two homozygous clones h1 and h2 are shown on the right. Differing lengths of LOH in h1 and h2 confirm distinct breakpoints in the two homozygous clones.

Figure S3 Differences in the proportions of *JAK2/MPL/CALR*-only, *DNMT3A*-only and double-mutant subclones in patients where mutations have been acquired sequentially (ie. excluding biclonal patients). * $p<0.05$ Students t-test performed on pairwise comparisons.

Figure S1

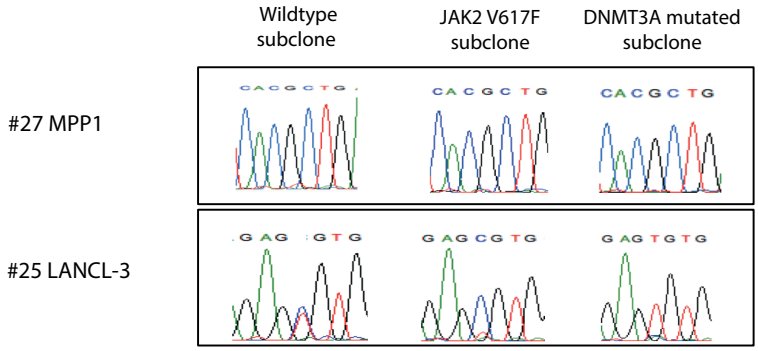


Figure S2

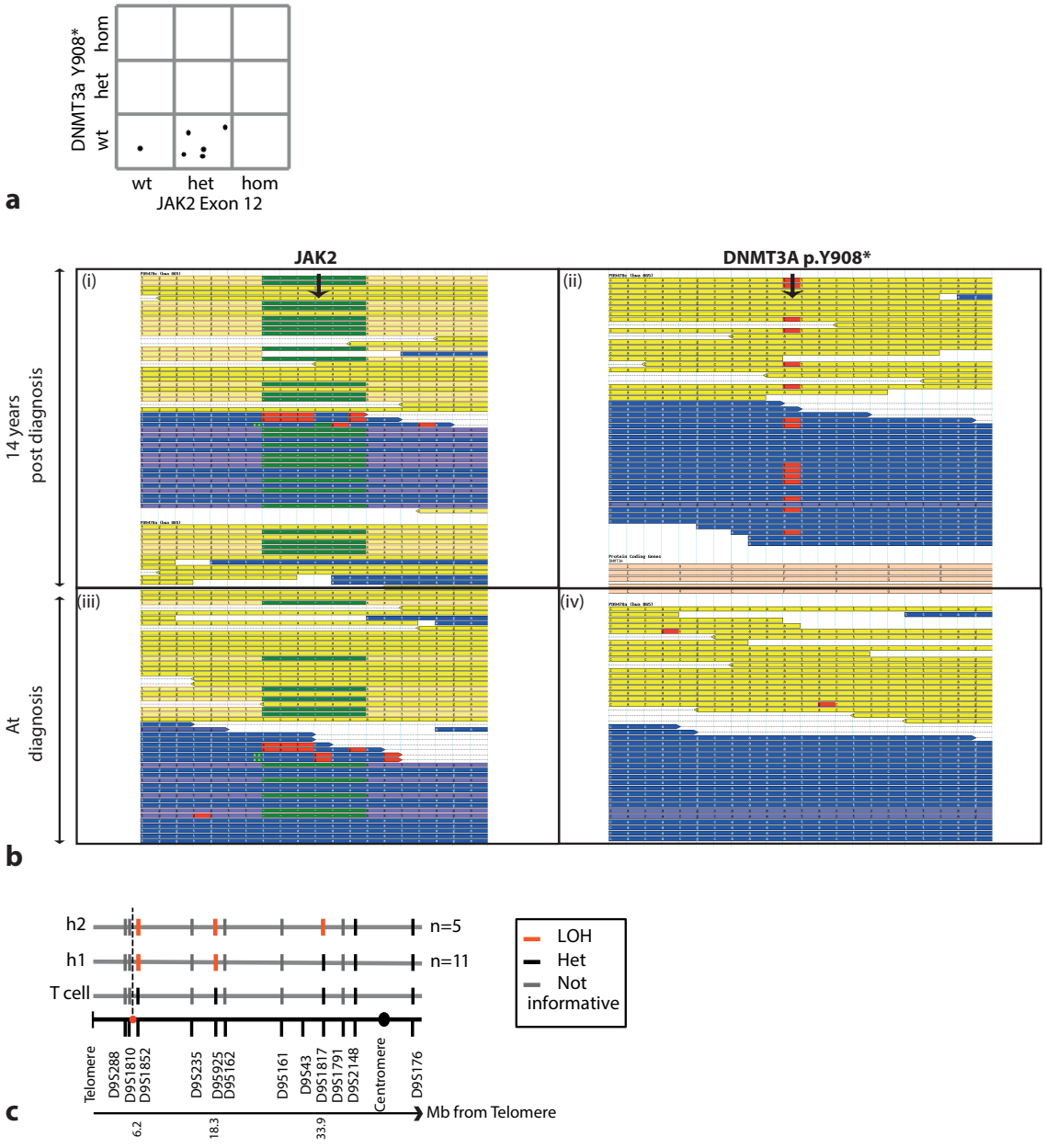
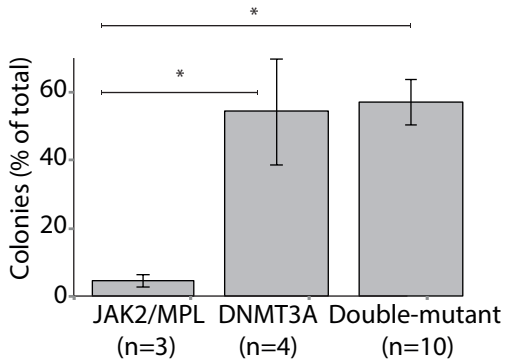


Figure S3


*
*
Colonies (% of total)
60
40
20
0
JAK2/MPL
(n=3)
DNMT3A
(n=4)
Double-mutant
(n=10)