SUPPLEMENTARY MATERIAL

Inactivating mutations of the histone methyltransferase gene *EZH2* in myeloid disorders

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Supplementary Table 1. Homozygous regions ≥20Mb in 148 patients

Patient ID	Disease	Chr	aUPD Localisation	Start (bp)	End (bp)	aUPD size (Mb)
#46*	aCML	**1**	1pter-1p13.3	1	108080000	108.08
#47	aCML		1pter-1p31.1	1	74760000	74.76
#48	MDS/MPN-U		1pter-1p32.3	1	53110000	53.11
#49	MDS/MPN-U		1pter-1p32.3	1	52962404	52.96
#72*	aCML		1q12-qter	143590000	247249719	103.66
#41*	CMML		1p22.2-1q24.2	91142736	165835065	74.69
#50	MDS/MPN-U	**3**	3p12.3-q13.12	77320000	108660000	31.34
#51*	aCML		3pter-3p24.1	4030000	29470000	25.44
#51*	aCML	**4**	4pter-4p15.1	7220000	31297000	24.08
#18	aCML		4q13.1-qter	63780000	191273063	127.49
#52*	CMML		4q21.21-qter	79610000	191273063	111.66
#53	CMML		4q21.21-qter	79893419	191273063	111.38
#54	aCML		4q21.23-qter	85000000	191273063	106.27
#10*	CMML		4q22.1-qter	91310000	191273063	99.96
#55	CMML		4q22.1-qter	92952251	191273063	98.32
#56*	MDS/MPN-U		4q22.1-q27	91566000	121860000	30.29
#51*	aCML	**5**	5p14.1-q11.2	28700000	56780000	28.08
#57	CMML		5q11.2-5q35.3	54490000	180857866	126.37
#58	CMML	**7**	7p11.2-q11.23	54926855	78029306	23.10
#01	CMML		7q11.1-qter	60000000	158821424	98.82
#02	MDS/MPN-U		7q11.1-qter	60000000	158821424	98.82
#03*	aCML		7q11.22-qter	68679165	158821424	90.14
#04	aCML		7q11.23-qter	77996776	158821424	80.82
#05*	aCML		7q11.23-qter	76362317	158821424	82.46
#06*	CMML		7q21.13-qter	90317358	158821424	68.50
#07	MDS/MPN-U		7q21.2-qter	92246265	158821424	66.58
#08	CMML		7q21.2-qter	92926362	158821424	65.90
#09*	CMML		7q21.3-qter	97302745	158821424	61.52
#10*	CMML		7q22.1-qter	98620286	158821424	60.20
#11*	MDS/MPN-U		7q22.3-qter	106052801	158821424	52.77
#12	MDS/MPN-U		7q22.3-qter	106721411	158821424	52.10
#51*	aCML	**8**	8p21.2-pter	1	24930000	24.93
#59	MDS/MPN-U		8q (whole arm)	43447222	146274826	102.83
#60	aCML	**9**	9pter-9p21.1	1	29158821	29.16
#61*	CMML		9p21.1-q21	32300000	85200000	52.90
#43	CMML	**10**	10q21.2-10q23.1	62337850	82562647	20.22
#62	aCML	**11**	11pter-11p12	1	40810000	40.81
#63	aCML		11p13-p15.3	12253357	36517371	24.26
#51*	aCML		11p12-q13.5	39840000	75690000	35.85
#46*	aCML		11q13.1-qter	72920000	134452384	61.53
#03*	aCML		11q13.1-qter	68420000	134452384	66.03
#64	aCML		11q13.1-qter	67161596	134449982	67.29
#65	CMML		11q13.1-qter	65823569	134452384	68.63
#15	aCML		11q13.2-qter	77480000	134452384	56.97
#05*	aCML		11q13.3-qter	69950000	134452384	64.50
#56*	MDS/MPN-U		11q12.1-14.3	57880000	91320000	33.44
#57	CMML		11p11.1-qter	49350000	134452384	85.10
#67	CMML		11q12-11q25	57941912	134452384	76.51

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#41*	CMML		11q13.1-11q25	65427086	134452384	69.03
#09*	CMML	**12**	12pter-12p11.1	1	37000000	37.00
#52*	CMML		12q13.11-qter	46960000	132349534	85.39
#23	aCML	**13**	whole chr	1	114142980	114.14
#68	aCML		13q12.1-qter	20767952	114142980	93.38
#19	CMML		13q12.1-qter	21977125	114142980	92.17
#06*	CMML	**14**	14q24.3-qter	73352955	106368585	33.02
#69	CMML	**15**	15q14-qter	36616462	100338915	63.72
#61*	CMML	**17**	17p12-q22	15100000	52170000	37.07
#70	CMML		17q11.2-17q25.3	24071616	78774742	54.70
#71	aCML	**18**	18q12.1-18q23	30600000	76117153	45.52
#72*	aCML	**21**	21q21.1-q22.2	1	38,869,972	38.87
#73	CMML	**22**	whole chr	1	49691432	49.69
#11*	MDS/MPN-U		whole chr	1	49691432	49.69
#33	CMML		whole chr	1	49691432	49.69

Cases in bold indicate those with *EZH2* mutations. Asterisks indicate cases with ≥2 copy number neutral homozygous SNP calls >20Mb

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Supplementary Table 2. Genes on chromosome 7q screened by direct sequencing

7q gene	Localisation	Genomic start	Genomic end	Coding exons	Screened exons
CDK6	7q21.2	92,072,175	92,301,148	2-8	3-8
SRPK2	7q22.2	104,544,060	104,816,583	1-16	3-16
PIK3CG	7q22.3	106,293,160	106,334,801	2-11	complete
HBP1	7q22.3	106,596,696	106,630,209	2-11	complete
HAKAI	7q22.3	107,171,822	107,187,260	1-6	complete
MET	7q31.2	116,099,682	116,225,676	2-21	complete
SMO	7q32.1	128,615,949	128,640,617	1-12	2-12
UBE2H	7q32.2	129,260,231	129,380,025	2-7	complete
HIPK2	7q34	138,907,915	139,123,984	1-15	complete
BRAF	7q34	140,080,751	140,271,033	1-18	2-18
EPHB6	7q34	142,262,914	142,278,967	5-20	complete
EPHA1	7q35	142,798,331	142,816,107	1-18	complete
CUL1	7q36.1	148,026,866	148,129,134	2-22	complete
GIMAP7	7q36.1	149,842,878	149,849,093	2	complete
NUB1	7q36.1	150,669,791	150,706,463	2-15	complete

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Supplementary Table 3. Summary of all *EZH2* mutations

Case	Diagnosis	Karyotype	SNP 6.0 array		*EZH2* mutation		Zygosity	*EZH2* exon
			aUPD	del 7q36.1	nucleotide	protein		
#01	CMML	n/a	7q11.1-qter	no	c.2054delA	p.Lys685fsX11	Hom	18
#03	aCML	46,XX	7q11.22-qter	no	c.982C>T	p.Gln328X	Hom	9
#04	aCML	n/a	7q11.23-qter	no	c.1992T>G	p.Asp664Glu	Hom	17
#05	aCML	n/a	7q11.23-qter	no	c.863G>A	p.Arg288Gln	Hom	8
#06	CMML	46,XY	7q21.13-qter	no	intron delA (2bp prior exon 8)	splice mutation	Hom	8
#07	MDS/MPN-U	+8	7q21.2-qter	no	c.2187dupT	p.Asp730X	Hom	19
#08	CMML	t(3;11)(p13;p15)	7q21.2-qter	no	c.619C>T	p.Arg207X	Hom	6
#11	MDS/MPN-U	46,XX	7q22.3-qter	no	c.1728C>G	p.Cys576Trp	Hom	15
#12	MDS/MPN-U	46,XY	7q22.3-qter	no	c.1992T>A	p.Asp664Glu	Hom	17
#13	aCML	46,XY	n/a	n/a	c.786_787het_insC	p.Asn263fsX7	Het	8
#14	CMML	46,XY	n/a	n/a	c.73C>T + c.1691het_delG	p.Arg25X + p.Gly564fsX110	Het	2, 15
#15	aCML	n/a	no	no	c.2068C>T	p.Arg690Cys	Het	18
#16	post-ET MF	-7	n/a	n/a	c.2050C>T	p.Arg684Cys	Hom	18
#17	aCML	n/a	no	400kb del(7q36.1)	c.1200_1203delAGAA	p.Lys400fsX22	Hom	10
#18	aCML	46,XY	no	no	c.188_189het_insA	p.Arg63fsX18	Het	3
#19	CMML	46,XY	no	no	c.574G>A	p.Asp192Asn	Het	6
#20	PMF	n/a	n/a	n/a	c.2079T>A	p.Asn693Lys	Het	18
#21	PMF	46,XY	n/a	n/a	c.2199C>A	p.Tyr733X	Het	20

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#22	PV	46,XX	n/a	n/a	c.1033G>A	p.Ala345Thr	Het	10
#23	aCML	46,XY	no	no	c.754C>G	p.Leu252Val	Het	8
#24	CMML	n/a	n/a	n/a	c.2138T>C	p.Ile713Thr	Het	19
#25	CMML	46,XX	no	no	c.1992_1997het_delTAAATA + c.2187_2188het_insT	p.del(Lys665+Tyr666) + p.Asp730X	Het	17, 19
#26	HES	n/a	n/a	n/a	c.727A>C	p.Lys243Gln	Het	7
#27	post-ET MF	46,XY	n/a	n/a	c.2069G>A	p.Arg690His	Hom	18
#28	aCML	46,XY, del(12)(p11.2),del(12) (q13q24.1)	no	no	c.763G>A	p.Ala255Thr	Het	8
#29	CMML	45,X -Y	n/a	n/a	c.444_445het_insGAA + c.2050C>T	p.ins_Glu149 + p.Arg684Cys	Het	5,18
#30	5q-syndrome	46,XY, del (5)(q14.3-q33.3)	n/a	n/a	c.1966het_delG	p.Ala656fsX18	Het	17
#31	RAEB-1	47XY, +8	n/a	n/a	c.745G>A + c.1967C>T	p.Glu249Lys + p.Ala656Val	Het	8, 17
#32	CMML	46,XY	n/a	n/a	c.755_756het_insC	p.Leu252fsX7	Het	8
#33	CMML	46,XY, t(2;12)(q31;p13)/47,idem,+21	no	no	c.2191T>G	p.Tyr731Asp	Het	19
#34	CMML	46,XX	no	no	c.172C>T + c.1712G>A	p.Gln58X + p.Cys571Tyr	Het	3, 15
#35	RA	n/a	n/a	n/a	c.730T>G + c.890A>G	p.Tyr244Asp + p.His297Arg	Het	8, 8
#36	RA	8+	n/a	n/a	c.216_217het_delTT + c.1978G>C	p.Thr72fsX8 + p.Gly660Arg	Het	3, 17
#37	RA	46,XX	n/a	n/a	c.434T>G	p.Phe145Cys	Het	5
#38	CMML	46, XY,-7,+21	no	no	c.1175_1176het_delAA	p.Glu392fsX6	Hom	10
#39	RARS-T	n/a	n/a	n/a	c.1774_1777het_delACTT	p.Thr592fsX82	Het	15
#40	CMML	47,XY,+8	no	no	c.965A>G	p.Asn322Ser	Het	9
#41	CMML	46,XY	no	no	c.1991A>T	p.Asp664Val	Het	17
#42	RA	46,XX	n/a	n/a	c.2078A>T	p.Asn693Ile	Hom	18
#43	CMML	47,XX,+8	no	no	c.949G>T	p.Glu317X	Het	9
#44	RA	46,XY	n/a	n/a	c.1513het_delG	p.Ala505fsX8	Het	13
#45	RAEB-1	45,XY,-7 [18] /46,XY	n/a	n/a	c.184C>T	p.Gln62X	Het	3
ELF153	Cell line	43,XY,-7,-14,-17,	no	no	c.1525A>G	p.Arg509Gly	Hom	13

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		de1(5)(q13q31), t(12;14)(p11.2;q11.2						
SKM-1	Cell line	hypodiploid with 8% polyploidy; 43(38-43) <2n>XY, +1, -12, -14, -20, -21, t(1;19)(q21;q13), del(2)(p11), del(9)(q12), add(17)(p1?)	no	no	c.1937A>G	p.Tyr646Cys	Hom	16

n/a, not available or failed

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Supplementary Table 4. Cell lines screened for *EZH2* mutations

Number	Cell line	*EZH2* status
1	FKH1	normal
2	MONOMAC6	normal
3	UT7	normal
4	MOLM13	normal
5	CNLBC1	normal
6	ELF153	R509G
7	F-36P	normal
8	MARIMO	normal
9	MB-02	normal
10	MDS-92	normal
11	MOLM-17	normal
12	MUTZ-8	normal
13	MUTZ-11	normal
14	OCI-M2	normal
15	SKK-1	normal
16	SKM-1	Y646C
17	K562	normal
18	HL60	normal
19	U937	normal
20	SIG-M5	normal
21	KMOE-2	normal

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Supplementary Table 5. Primer sequences

A. *EZH2* mutation screening

Exon	Forward primer	Reverse primer	Product length (bp)
2	GGTGATCATATTCAGGCTGG	AAACTTATTGAACTTAGGAGGGG	257
3	TTTCTCCTTTCCTCTCCTTCA	TCCAATAGCATAAACCAAAAGATG	251
4	GGCTACAGCTTAAGGTTGTCCT	CTGTCTTGATTCACCTTGACAAT	270
5	AAATCTGGAGAACTGGGTAAAGAC	TCATGCCCTATATGCTTCATAAAC	316
6	AGGCTATGCCTGTTTTGTCC	AAAAGAGAAAGAAGAAACTAAGCCC	332
7	CTGACTGGCATTCCACAGAC	AAGTGTAGTGGCTCATCCGC	380
8	CATCAAAAGTAACACATGGAAACC	TTGTAATAAATGATAGCACTCTCCAAG	348
9	TCCATTAATTGACTTTTCCAGTG	ACCTCCACCAAAGTGCAAAG	246
10	TTCTCTTCCATCAAAATGAGTTTTAG	TCCTCACAACACGAACTTTCAC	360
11	GAGTTGTCCTCATCTTTTCGC	CCAAGAATTTTCTTTGTTTGGAC	362
12	AAGAATGGTTTGCCTAAATAAGAC	CCTTGCCTGCAGTGTCTATC	212
13	TCTTGGCTTTAACGCATTCC	CAAATTGGTTTAACATACAGAAGGC	289
14	TGATCGTTTCCATCTCCCTG	AGGGAGTGCTCCCATGTTC	278
15	GAGAGTCAGTGAGATGCCCAG	TTTGCCCCAGCTAAATCATC	371
16	TTTTTGATGATGTGATTGTGTTTT	TGGCAATTCATTTCCAATCA	239
17	TTCTGTCAGGCTTGATCACC	CTCGTTTCTGAACACTCGGC	220
18	AGGCAAACCCTGAAGAACTG	TTCCAATTCTCACGTCAAAGGTA	217
19	CCGTCTTCATGCTCACTGAC	AAAAACCCTCCTTTGTCCAGA	204
20	CTTCAGCAGGCTTTGTTGTG	GGGGAGGAGGTAGCAGATG	163

B. *EZH2* SNP Pyrosequencing

EZH2_rs10488070_F	TTC CCT CAA GGT CAT GCA ATG TA
EZH2_rs10488070_R	[Btn]ACT GAA GCA TGT GTT CTT GAC TTG
EZH2_rs10488070_SEQ	AAG GAC TTC TAT CAA CAT TT
EZH2_rs6464926_F	[Btn]AAT GGT GGT GCA TGC TTG TA
EZH2_rs6464926_R	TTT GGG TTT CTC AAC ATT GTG T
EZH2_rs6464926_SEQ	CCT TTA GTC TAT TCA TGA GG
EZH2_rs740949_F	[Btn]CTA ATT CCC CAC TAA TGC TCA TG
EZH2_rs740949_R	TCT CTA AGG GCT TTT TCT ACT GGA
EZH2_rs740949_SEQ	GTT CTT TGC TTC TTT TGA

[Btn] = biotin

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C. RT-PCR

Ex6B cDNA_F	TTTATAAATGATGAAATTTTTGTGGAG
Ex9 cDNA_R	AAATGCTGGTAACACTGTGGTC
Exon 15 cDNA_F	ATTCAGCGGGGCTCCAAAAA
Exon 19 cDNA_R	TCGCCAGTCTGGATGGCTCT

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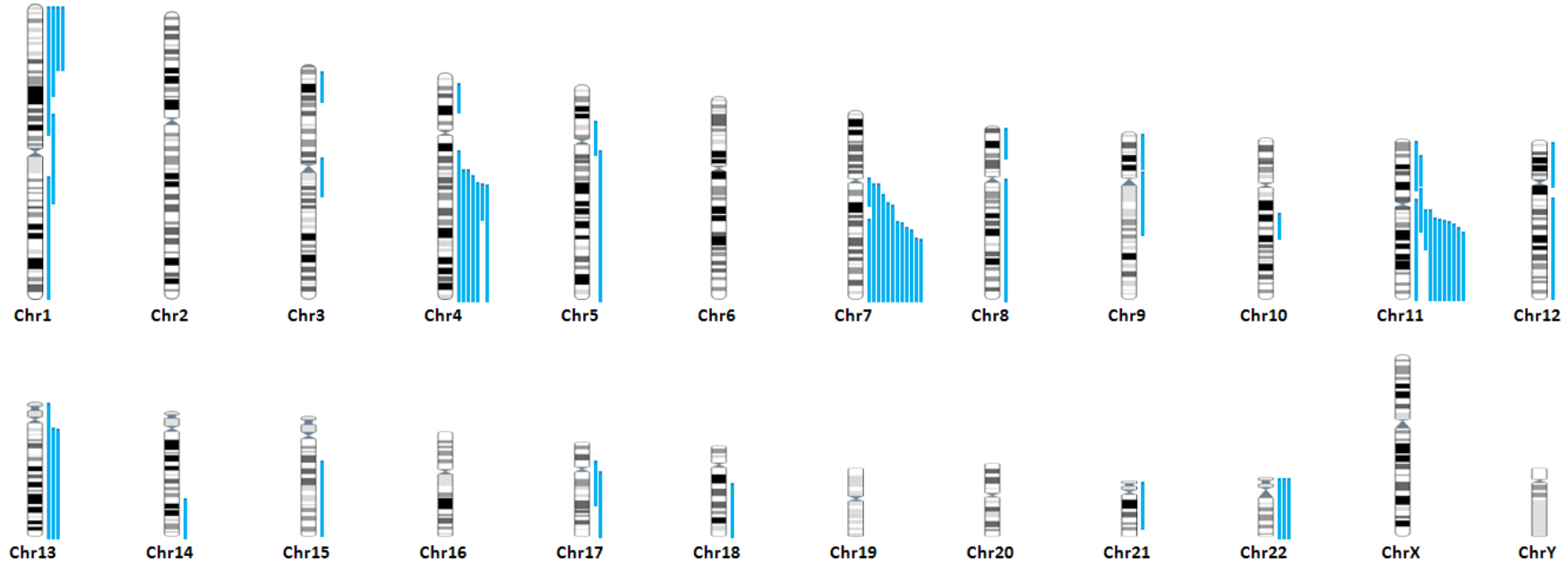

Supplementary Figure 1. Summary of acquired UPD. Copy number neutral homozygous SNP calls >20Mb in size are shown in the cohort of 148 cases. Analysis was performed by SNP 6.0 arrays and CNAG (version 3.2) using the AsCNAR algorithm and anonymous reference samples

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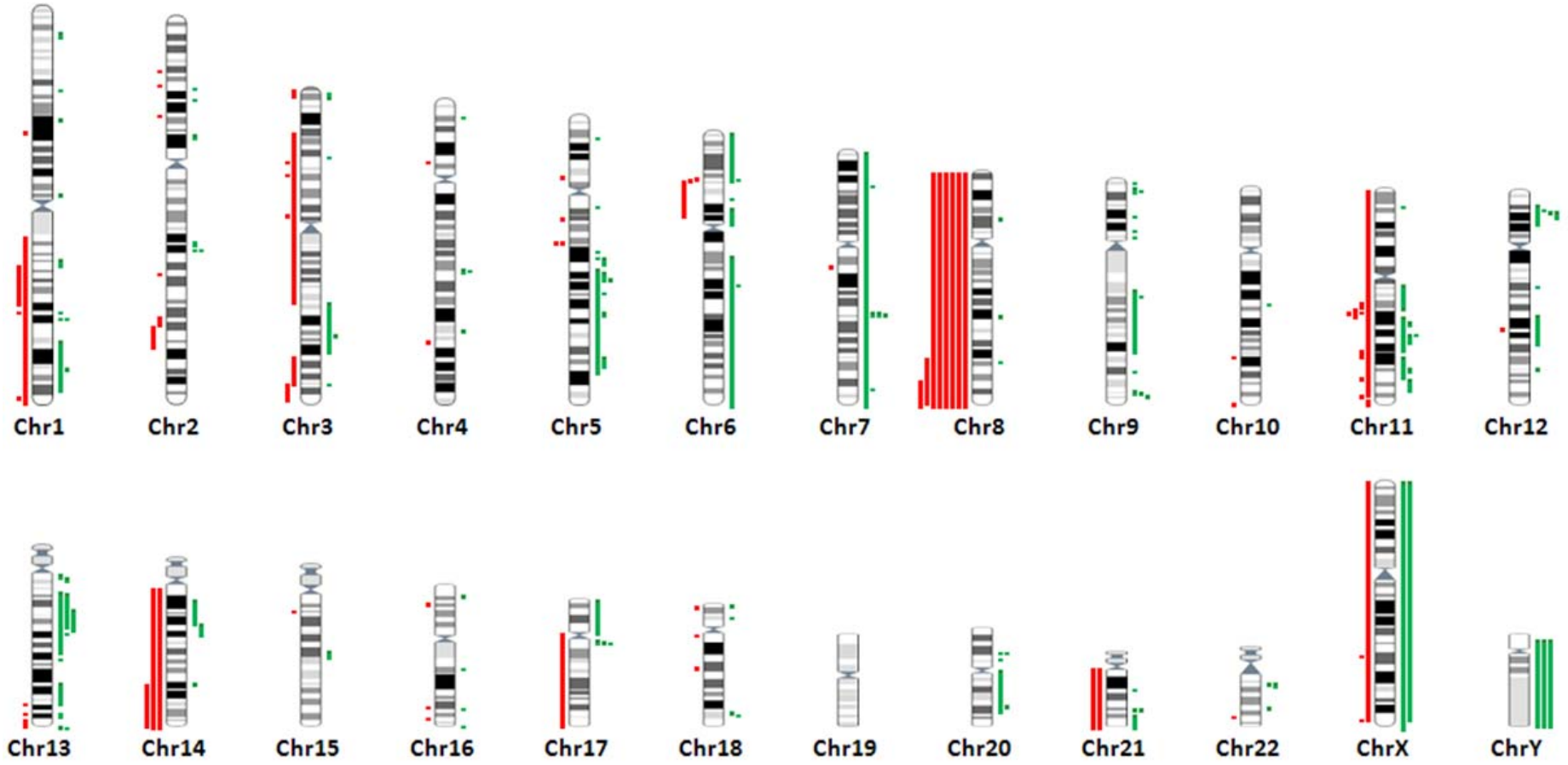

Supplementary Figure 2. Copy number changes. Gains (red) and losses (green) in the same cohort are shown. Only those changes detected by the Partek Genomics Suite (version 6.5 beta) and verified by CNAG using anonymous references and the Hidden Markov Model algorithm are shown.

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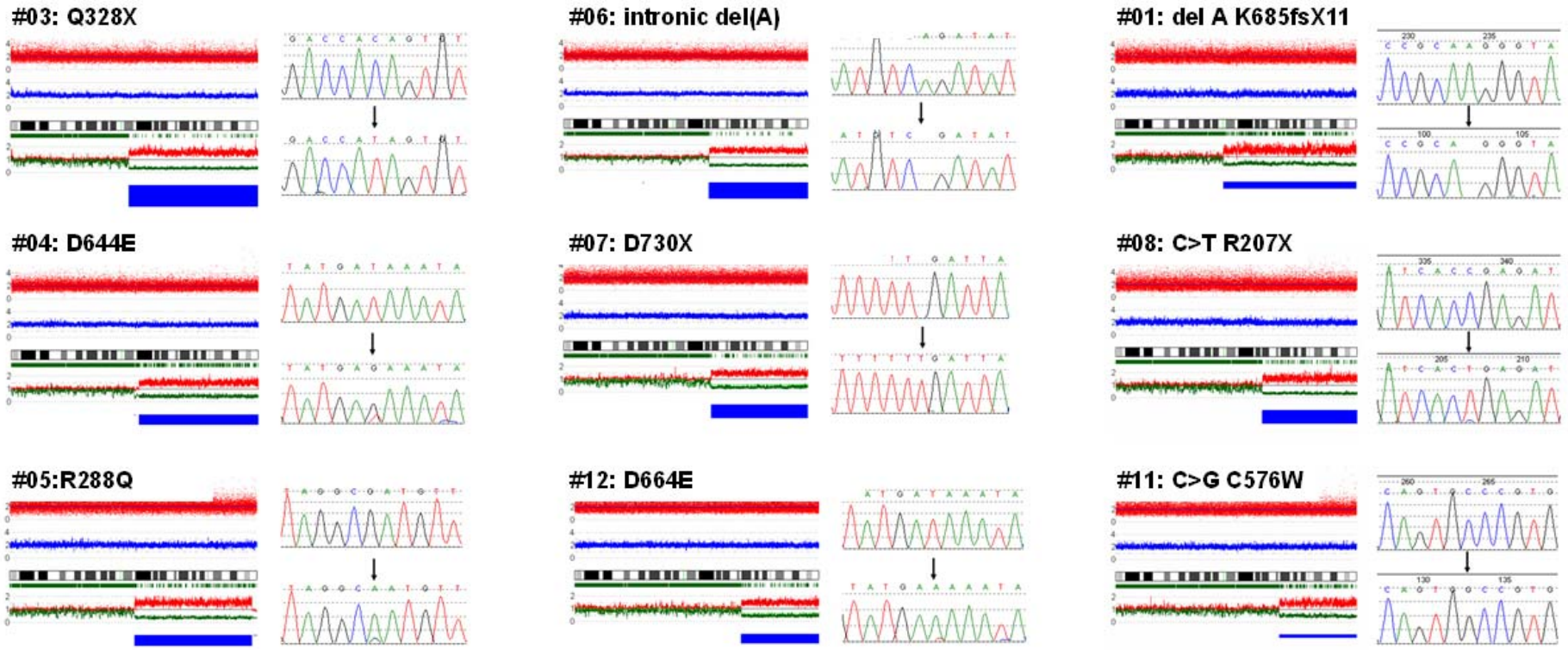

Supplementary Figure 3. *EZH2* mutations are associated with aUPD. Regions of aUPD and associated sequence changes for all nine mutated cases. The left panels show the copy number calls (red dots), averaged copy number calls (blue traces), allele-specific copy number calls (red and green traces). Acquired UPD is indicated by the solid blue bars and corresponds to divergence of the allele-specific copy number calls.

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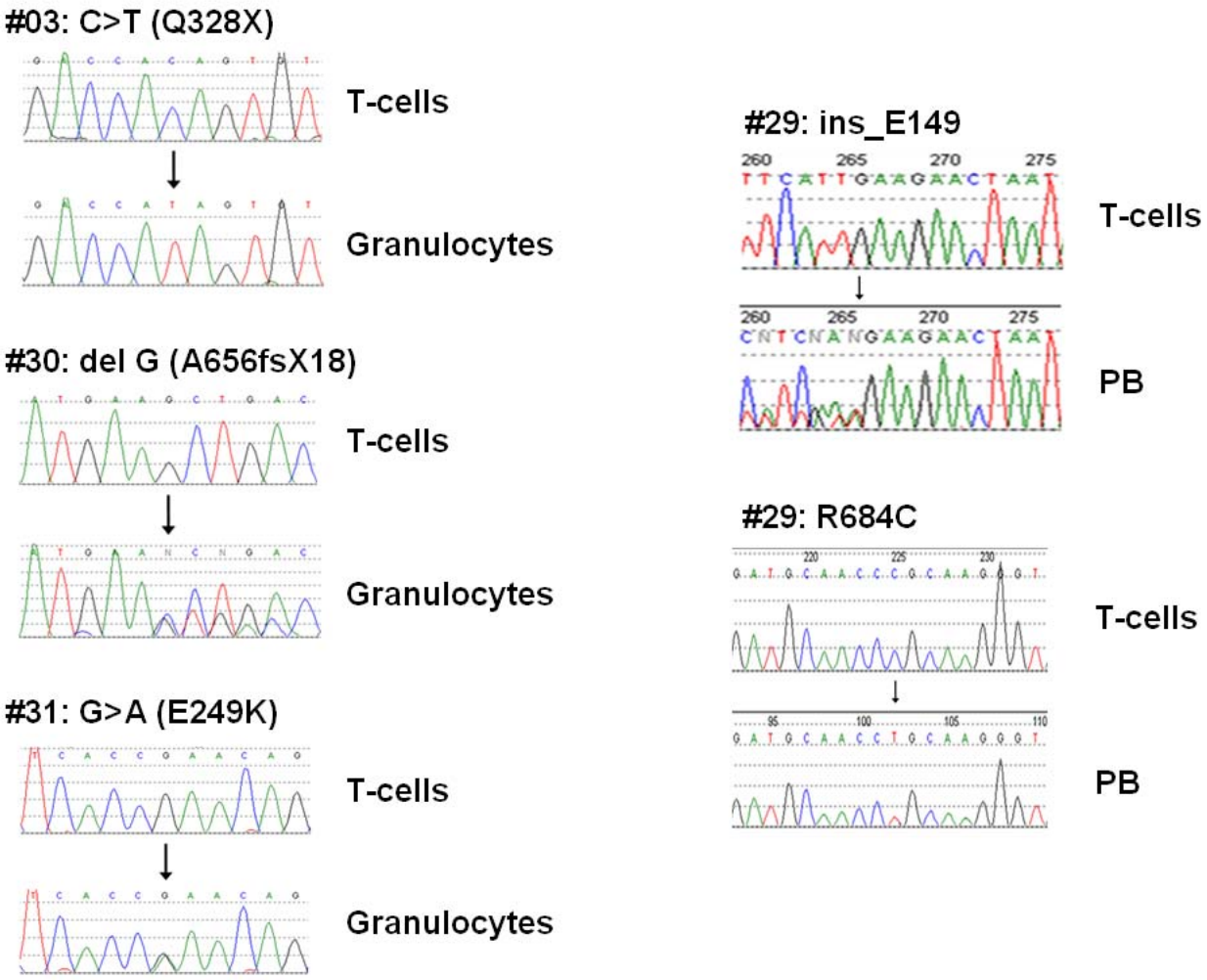

Supplementary Figure 4. *EZH2* mutations are acquired. Analysis of peripheral blood leukocytes (PB), granulocytes and T-cells demonstrates that 5 of 5 *EZH2* mutations tested were somatically acquired.

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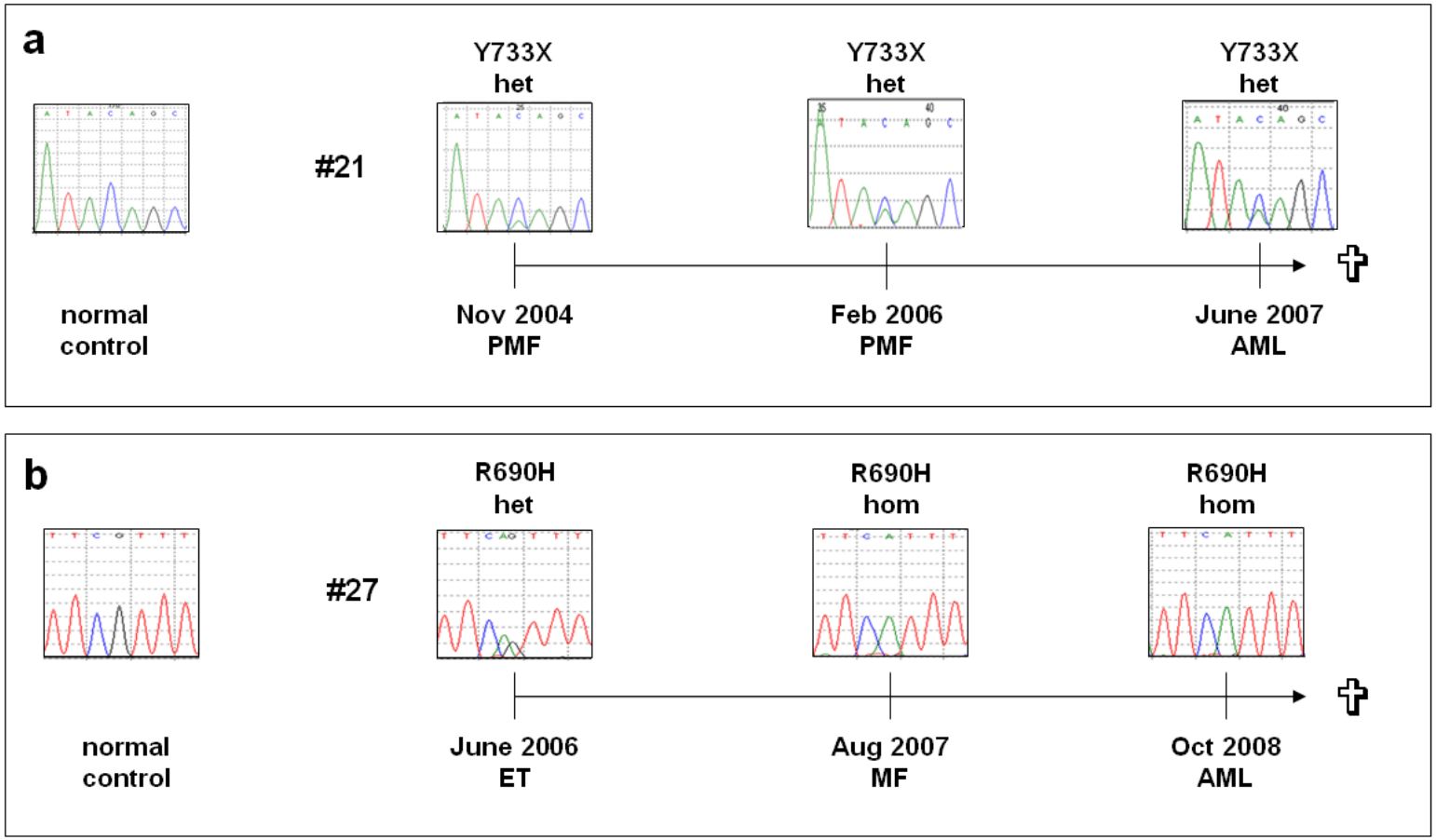

Supplementary Figure 5. Sequential analysis of *EZH2* mutations. (a) Case #21 presented in November 2004 with PMF, a normal karyotype and a relatively low level heterozygous c.2199C>A (Y733X) *EZH2* mutation. The mutation level increased slightly but never became predominant even at transformation to AML in June 2007. (b) Case #27 presented with ET in June 2006 with a heterozygous c.2069G>A (R690H) mutation. Fourteen months later he progressed to secondary MF at which time the mutation had become homozygous. The mutation remained homozygous at transformation to AML 18 months later. Both cases showed a normal male karyotype throughout their disease course. Retrospective results were obtained from DNA extracted from bone marrow smears or bone marrow-derived fixed cytogenetics suspensions.

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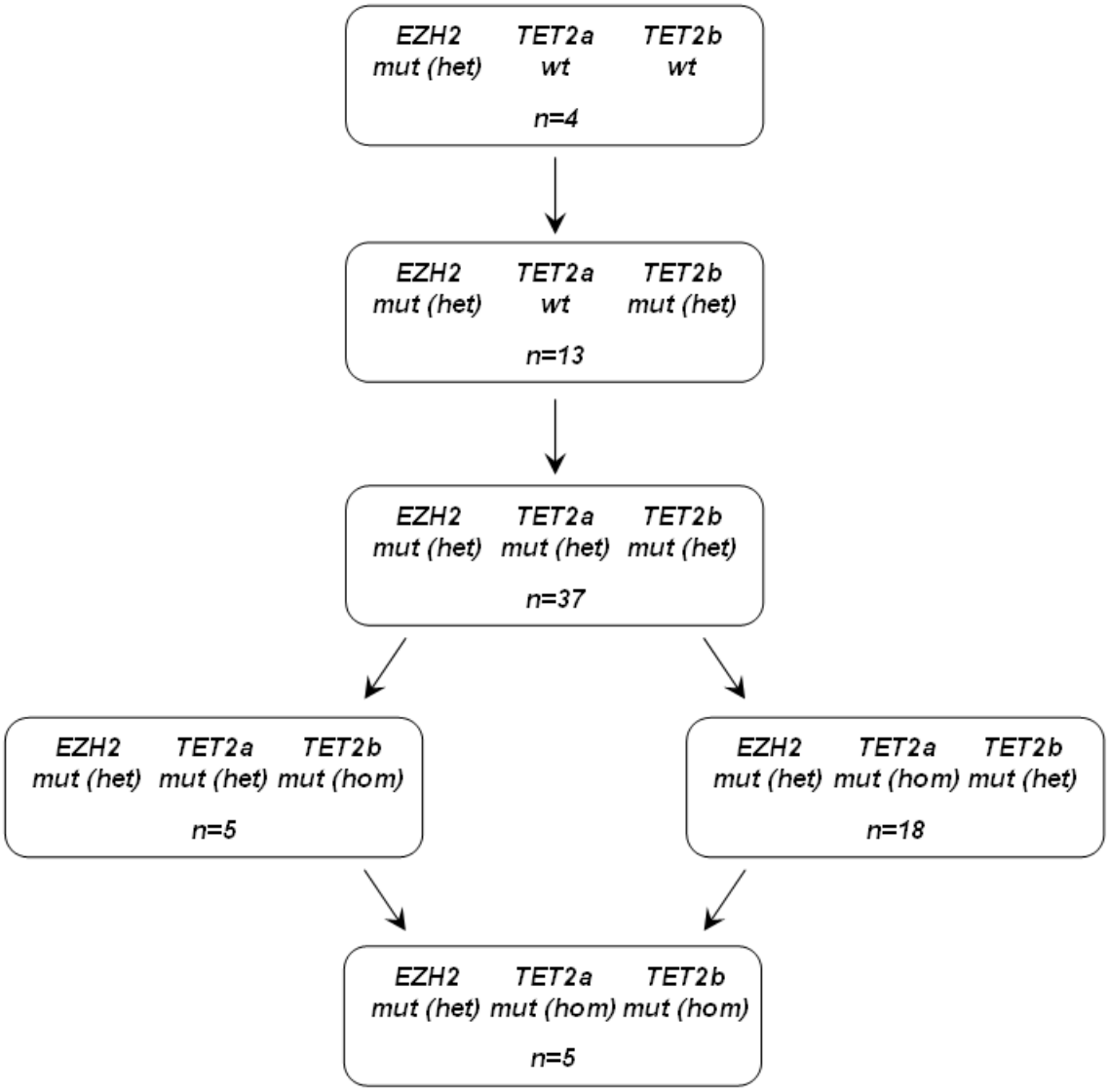

Supplementary Figure 6. Analysis of *EZH2* and *TET2* mutations in case #29. Individual CFU-GM were plucked and tested for the *EZH2* c.444_445het_insGAA mutation (p.ins_Glu149) and two *TET2* mutations: c.4171G>GC (designated *TET2a*; p.1262R>R/P) and c.4288C>CA (designated *TET2b*; p.1301A>A/D. The *EZH2* mutation was found in all colonies (but was nevertheless acquired; see Supplementary Figure 2) whereas one or both *TET2* mutations were found in either a heterozygous (het) or homozygous (hom) form in a subset of colonies, thus indicating that the *EZH2* mutation preceded both *TET2* mutations.

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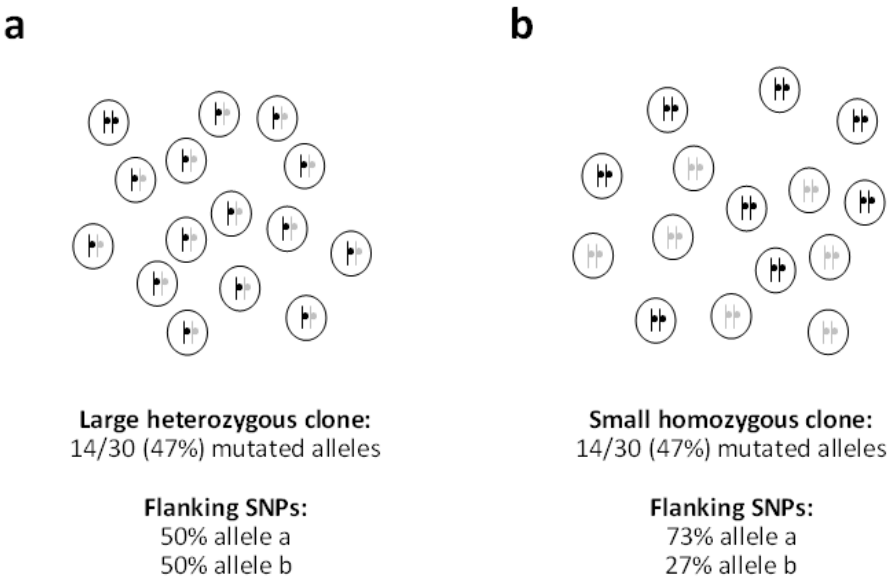

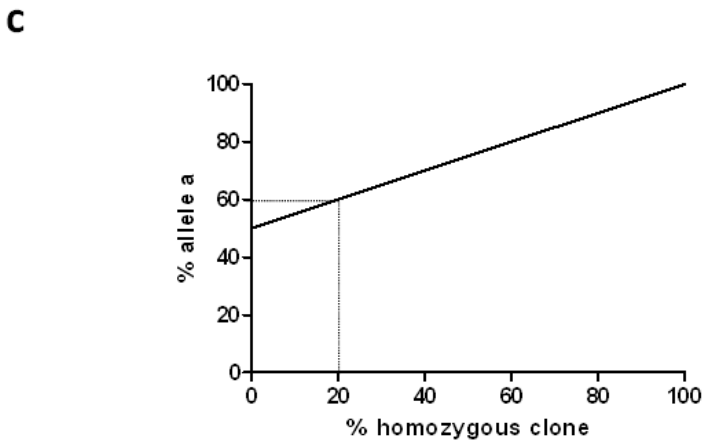

Supplementary Figure 7. Detection of small homozygous clones using flanking SNPs. Panel A illustrates a case with a large heterozygous mutant clone and panel B illustrates a case with a small homozygous clone brought about through acquired UPD. Fifteen cells with two chromosomes (wild type, black; mutant, grey) each are shown for each clone. The proportion of mutant: wild type chromosomes is identical in both scenarios and thus measurement of the mutation level cannot distinguish between them. Measurement of the allele ratios of flanking SNPs, however, clearly distinguishes the two. C. Graph illustrating the relationship between homozygous clone size and skewed allele ratios of heterozygous flanking SNPs. Pyrosequencing can reliably detect differences in allelic ratios of 10% and therefore a homozygous clone that comprises at 20% or more of the total cell population can be detected. The percentage of mutant alleles depends on the size of both the homozygous and the heterozygous mutated clones, but in the extreme situation of 80% normal cells, 20% homozygous and 0% heterozygous mutated cells the total percentage of mutated alleles is only 20%. Thus, measurement of flanking SNPs can detect a small homozygous clone in cases with only ≥20% mutated alleles.

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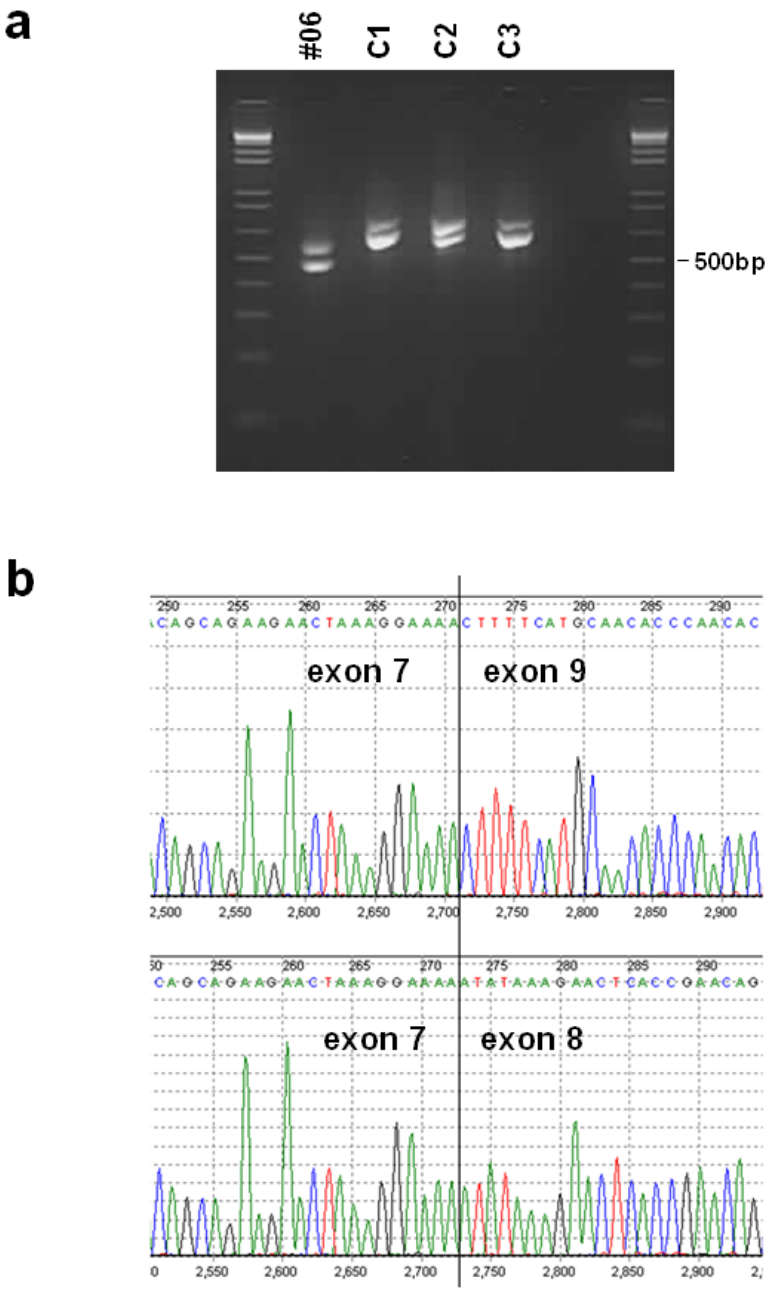

Supplementary Figure 8. Analysis of a candidate splicing mutation. Case #6 had a deletion of an 'A' 2bp prior to exon 8. (A) RT-PCR amplification using primers to exon 6 (5'-GGAGTTGGTGAATGCCCTTGG-3') and exon 10 (5'-CCCCGTTTCAGTCCCTGCTT-3') produced smaller size bands for #06 compared to three control MPNs without *EZH2* mutations (C1-C3). (B) Sequence analysis showed the smaller bands in #06 were a consequence of skipping of exon 8. Each case showed a double band as a consequence of the inclusion or exclusion of an alternatively spliced exon:
agCCACTCCTACCTAGGAACTAAATGGGTATATATTGCCTGTTGGATTTTGGACAGAAGATTCATTGAATGG
CACCTGCAGAAGgt.
This exon is absent from EZH2-004 (ENST00000320356) but is present in EZH2-003 (ENST00000492143).

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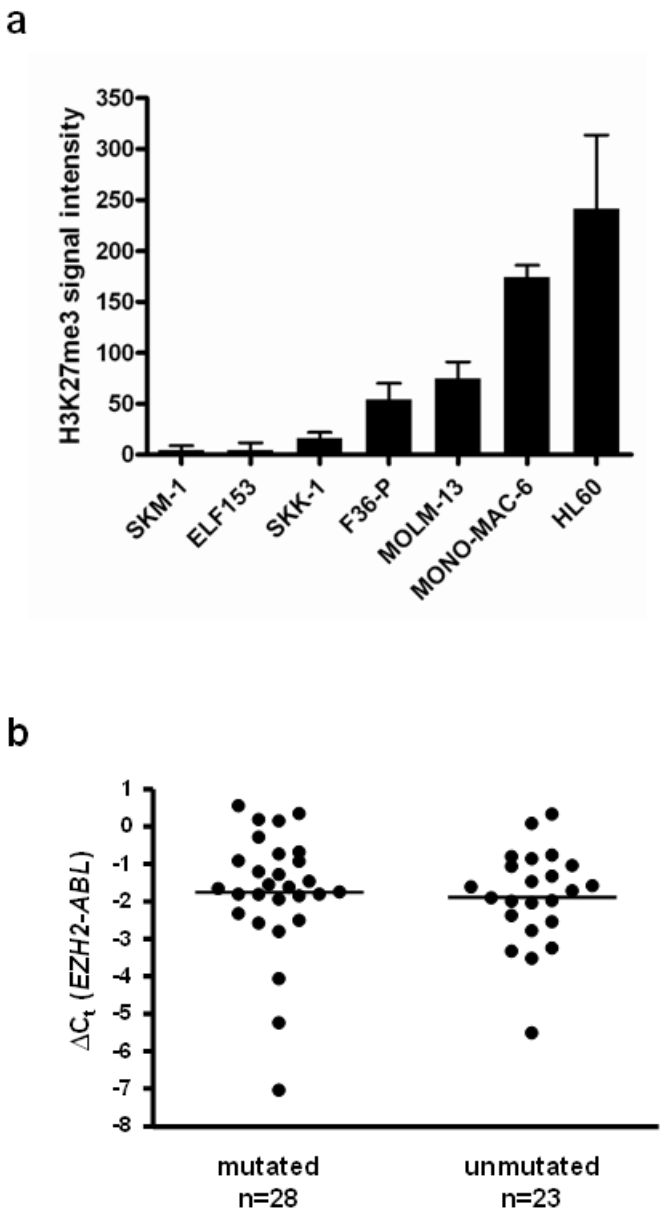

Supplementary Figure 9. Levels of H3K27me3 in cell lines and expression levels of *EZH2* in patients. (A) Dot blot analysis of H3K27me3 levels in cell lines. SKM-1 and ELF153 had *EZH2* mutations; all other lines had wild type *EZH2*. (B) *EZH2* mRNA levels in mutated and unmutated patients were analyzed by real time quantitative PCR and normalized to expression of *ABL*. No difference in expression was seen between the two groups.

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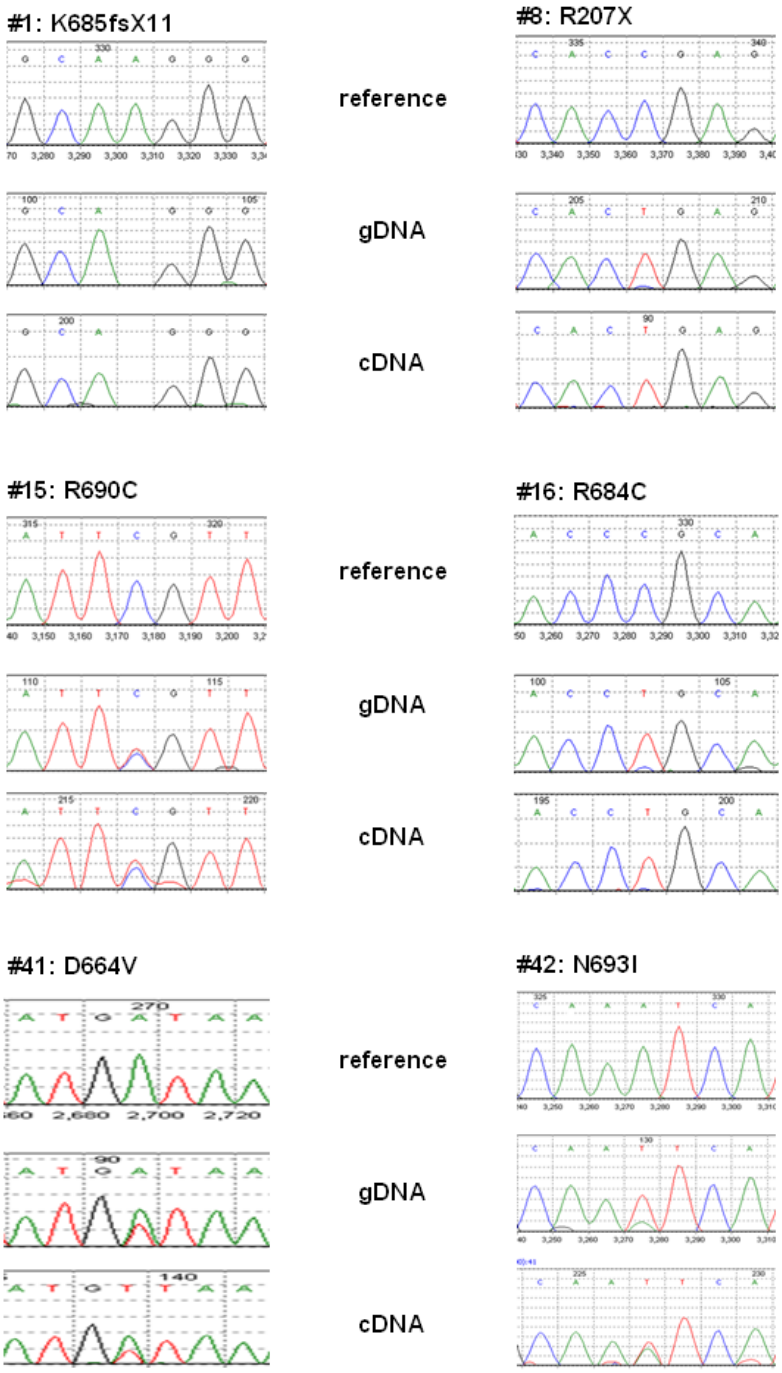

Supplementary Figure 10. *EZH2* mutants are expressed. Sequence traces are shown for 6 cases (two with frameshift mutations, four with missense mutations). For each case the top trace is the *EZH2* reference sequence, gDNA is the sequence of the relevant exon amplified from genomic DNA and cDNA is the corresponding region amplified using primers specific for cDNA.

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