Refractory anemia with ring sideroblasts and marked thrombocytosis cases harbor mutations in *SF3B1* or other spliceosome genes accompanied by *JAK2*V617F and *ASXL1* mutations

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doi:10.3324/haematol.2014.119032

Supplemental Information

for

Refractory anemia with ring sideroblasts and marked thrombocytosis (RARS-T) cases harbor mutations in *SF3B1* or other spliceosome genes accompanied by *JAK2*V617F and *ASXL1* mutations

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Short title: Mutational landscape of RARS-T

Patients

Of the 92 patients 87 samples were analyzed at diagnosis and 5 during the course of the disease. Three of these 5 patients were treated with hydroxyurea or anagrelide.

Isolation of Nucleic Acid

DNA or RNA from fresh bone marrow cells was isolated after Ficoll separation of mononucleated cells. DNA was isolated using the DSP DNA Midi Kit and the QIAsymphony instrument (Qiagen, Hilden, Germany). RNA was isolated using the MagNa Pure LC system with the corresponding mRNA HS Kit (Roche Applied Science, Mannheim, Germany). RNA was reverse transcribed with 500 U SuperScript II Reverse Transcriptase enzyme (Invitrogen, Carlsbad, CA) in a 50 µl reaction using random hexamers as primers.

Molecular Analyses

Sanger sequencing was performed with BigDye Term v1.1 chemistry (Applied Biosystems, Darmstadt, Germany). Hotspot regions of *ASXL1*, *SF3B1* and *SRSF2* were analyzed as published before.[1-3] For sequencing of *CALR* exon 9 (ENST00000316448) and whole coding region of *PRPF8* (ENST00000572621) on cDNA level following primers were used:

Gene	Exons	Forward sequence (5´- 3´)	Reverse sequence (5´- 3´)
CALR	08-09	GACCTCTGGCAGGTCAAGTC	TTCTCGAGTCTCACAGAGACATT
PRPF8	01-04	CTTTCTGCTGCGCTTCTTGT	GCATCCTCTTGAAATGCCTCC
	03-06	TGAGATTCCCTGGGTCATTGA	AGGCTGCCGGATGATAATCT
	05-10	GGCCCCAAATTTGAACCTCT	CTCCACCCAGTCCAGCTTT
	09-14	CCCCTAAGGCTCAAAAGAAGA	CTCCACTCGCTGCTTTGTTA
	13-17	CGATGGCTTGGCAACCTC	ATCAGACCTAGCTCCTCCCT
	16-19	GCTCATCTTGGCATTGGAGC	ATGACGACGTTGTTCTTGGC
	19-21	CAAGTGGTGTCAAGGCATCA	CCCGGCCTAAGTTAACATCA
	21-24	GTTACCTGACAGAGCACCCT	ACAGCCTCCCGAAAGTATGT
	23-26	AGATTCTCATGGCCTCTGGG	ACGGATTCTGCTTCAAAACCT
	26-30	CATTGCTCAGAACAGACGCC	ACTTTCGGGGATGGATTGTCT
	29-32	CGAGCTCACTTGTGGCAGAA	AAGGCTCAGTGGGTTCAGAT
	32-35	CCTCTCATACAACAGGCCATG	CCAGTCGTCATAGAGGTTGAA
	35-38	AGCTCCAACTCCCTTTCCAG	TGGGAAGGATGTAGGTGTAGC
	38-41	GGGTCAGGGCCATCTCTG	CCCCATTCGTAGCCACTGG
	40-42	TGAGTCCCCGCAGTTATCAC	GGCCAAACTGCTGAATGTCA

Sensitivity of Sanger sequencing for the aforementioned genes was about 10%. Melting curve analyses for *JAK2*V617F (sensitivity: 1%), *JAK2* exon12 (sensitivity: 10%; analyzed only in *JAK2*V617F negative cases) and *MPL*W515 (sensitivity: 5%) were performed as published previously.[4-6] The real-time quantitative approach for *JAK2*V617F was performed on DNA level using following primers and probes: forward primer 5´-AGTGCATCTTTATTATGGCAGAG-3´, reverse wild-type primer 5´-TTTTACTTACTCTCGTCTCCACATAC-3´, reverse mutated primer 5´-TTTTACTTACTCTCGTCTCCACATAA-3´, probes TCCTTAGTCTTTCTTTGAAGCAGCAAGTATG-fluorescein and LCRed640-GAGCAAGCTTTCTCACAAGCATTTGGTTT-phosphate. Analysis of genes using the 454 deep-sequencing approach (454 Life Sciences, Branford, CT) was performed as previously described and showed a sensitivity of 1-2% depending on the number of generated reads and type of molecular mutation.[7-10] The average coverage was 529 reads (range: 80-4111). Primers not published thus far are available on request. Cases being *SF3B1*wt by Sanger

sequencing were reanalyzed with a more sensitive (about 2%) NGS approach, but no additional mutation was detected. The pan-myeloid NGS gene panel included information on following additional genes: *BCOR, BRAF, FLT3*-TKD*, GATA1, GATA2, KIT, KRAS, NRAS, PHF6, TP53, WT1*.

Statistical Analyses

Statistical analyses were performed with SPSS software version 19.0.0 (SPSS by IBM Corporation, Armonk, NY) using Fisher´s exact test and Student´s t-test.

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Table S1: Chromosomal aberrations in 15 RARS-T patients. All remaining patients had a normal karyotype.

Case	Karyotype
1	47,XX,+8[4]/46,XX[14]
2	47,XX,+8[3]/46,XX[17]
3	47,XX,+8[9]/46,XX,del(11)(q21q23)[15]/46,XX[1]
4	47,XY,+8[4]/46,XY,del(6)(q16q27)[3]/46,XY[16]
5	47,XX,+9[9]/46,XX[11]
6	45,X,-Y[20]/46,XY[1]
7	45,X,-Y[19]/46,XY[1]
8	45,X,-Y[18]/46,XY[2]
9	45,X,-Y,del(6)(q13q16)[18]/46,XY[2]
10	45,X,-X[10]/46,XX[10]
11	46,XX,del(11)(q14q24)[17]/46,XX[3]
12	46,XY,del(13)(q14q31)[14]/46,XY[6]
13	46,XX,t(11;12)(q25;q14)[20] *
14	46,X,t(X;11)(p22;p13)[9]/46,XX[12]
15	46,XY,der(18)t(15;18)(q22;q22)[2]/46,XY[18]

*probably represents a constitutional aberration

Table S2: Frequency of different gene mutations in RARS-T.

Gene	Analyzed cases	Mutated cases	%
SF3B1	92	83	90.2
JAK2	92	54	58.7
TET2	90	21	23.3
DNMT3A	78	13	16.7
ASXL1	91	13	14.3
EZH2	79	6	7.6
SRSF2	88	5	5.7
U2AF1	89	4	4.5
IDH2	79	3	3.8
CBL	91	3	3.3
ETV6	79	2	2.5
RUNX1	81	2	2.5
ZRSR2	83	2	2.4
MPL	92	2	2.2
IDH1	78	0	0
NPM1	79	0	0
CALR	91	0	0

Table S3: Distribution of gene mutations according to numbers of mutations (n) in 75 patients analyzed for 14 genes. One patient showed no gene mutation (not depicted). Colors are graded by 20% steps.

	n	1	2	3	4	5	6	7
Total cohort	75	19%	43%	23%	11%	1%	1%	1%
***SF3B1*mut**	70	21%	47%	25%	4.3%	1.4%	0	1.4%
***JAK2*mut**	44	0	44%	30%	18.2%	2.3%	2.3%	2.3%
***MPL*mut**	2	0	50%	50%	0	0	0	0
***DNMT3A*mut**	13	0	45%	55%	0	0	0	0
***CBL*mut**	3	0	33%	0	0	0	33%	33%
***TET2*mut**	19	0	26%	53%	16%	0	0	5%
***ASXL1*mut**	11	0	9%	18%	55%	9%	9%	0
***ZRSR2*mut**	2	0	0	50%	50%	0	0	0
***U2AF1*mut**	4	0	0	25%	75%	0	0	0
***SRSF2*mut**	5	0	0	0	60%	20%	20%	0
***EZH2*mut**	5	0	0	0	60%	0	20%	20%
***ETV6*mut**	2	0	0	0	50%	0	0	50%
***IDH2*mut**	3	0	0	0	33%	33%	33%	0
***RUNX1*mut**	1	0	0	0	0	0	0	100%

Table S4: Median and mean numbers of gene mutations in 75 patients analyzed for all genes with at least 10 mutations.

	Median mutated	**Mean mutated vs. wild-type**	**p-value**
SF3B1	2.0	2.3 vs. 3.7	0.002
DNMT3A	3.0	2.6 vs. 2.4	n.s.
JAK2	3.0	3.0 vs. 1.6	<0.001
TET2	3.0	3.1 vs. 2.1	0.002
ASXL1	4.0	3.9 vs. 2.1	<0.001

Figure S1: Associations of different gene mutations. Red: negatively associated ($p<0.05$) and green: positively associated ($p<0.05$) gene mutations. Gray: mutations that did not occur concomitantly (n.s.). Blue: the gene mutation (row) is always accompanied by gene mutation seen in the column (n.s.).

Figure S2: Mutation burdens of exemplary patients with four to seven gene mutations including *SF3B1*mut. Each line defines an individual patient.

Figure S3: Mutation burdens of all detected gene mutations in 6 of the 7 *SF3B1*wt cases. Each line defines an individual patient.

Figure S1

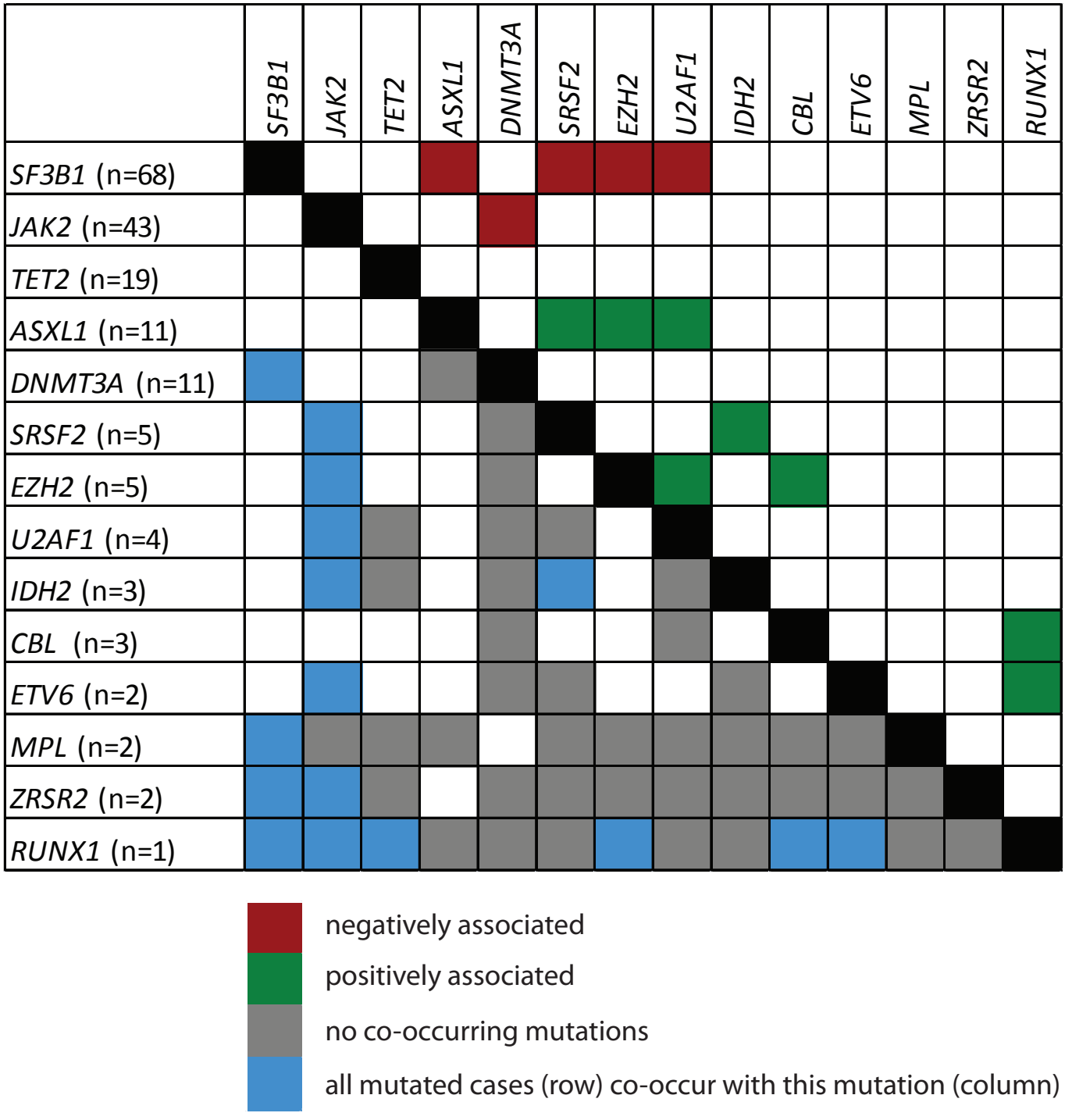
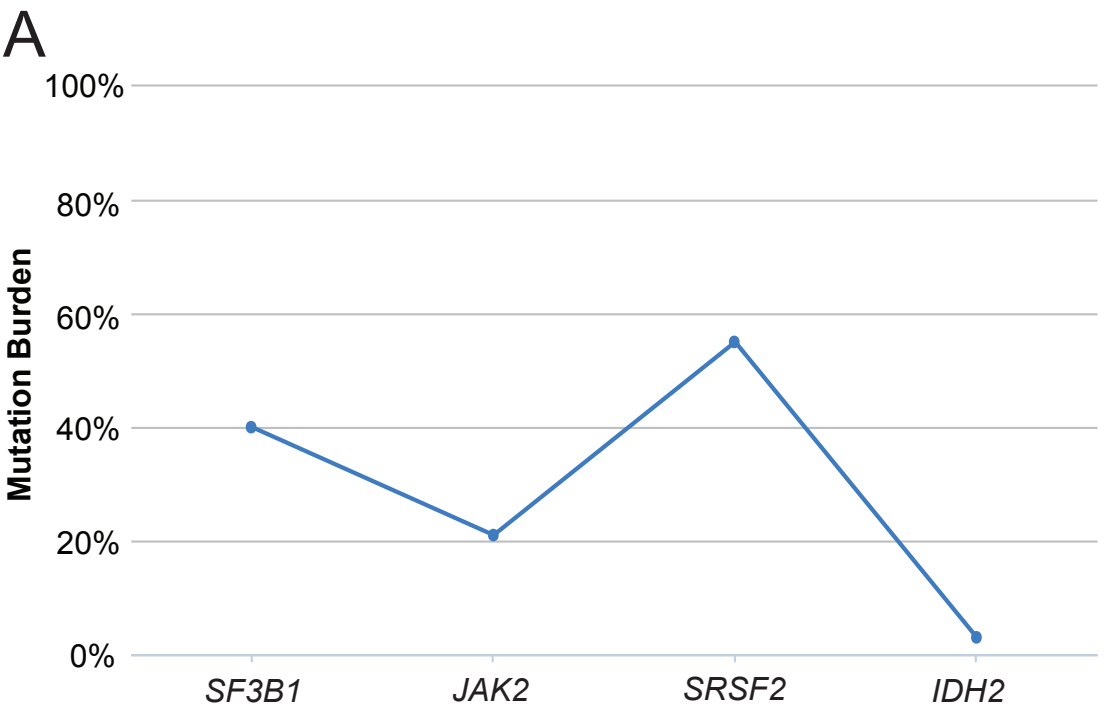
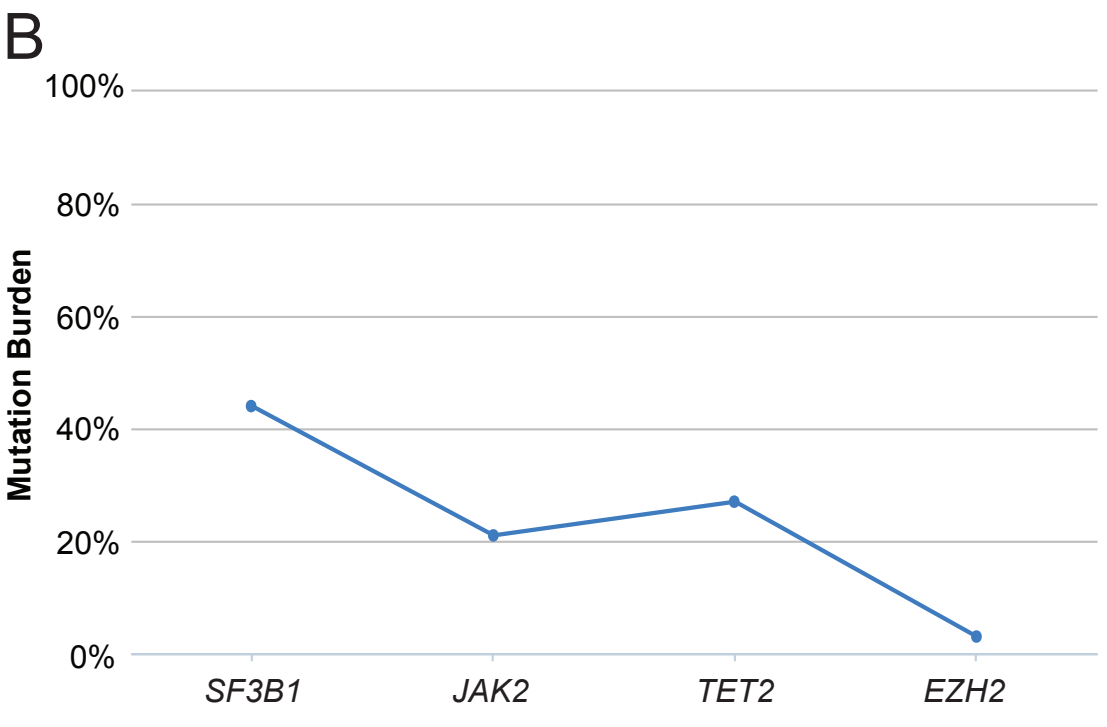

SF3B1
JAK2
TET2
ASXL1
DNMT3A
SRSF2
EZH2
U2AF1
IDH2
CBL
ETV6
MPL
ZRSR2
RUNX1
SF3B1 (n=68)
JAK2 (n=43)
TET2 (n=19)
ASXL1 (n=11)
DNMT3A (n=11)
SRSF2 (n=5)
EZH2 (n=5)
U2AF1 (n=4)
IDH2 (n=3)
CBL (n=3)
ETV6 (n=2)
MPL (n=2)
ZRSR2 (n=2)
RUNX1 (n=1)
negatively associated
positively associated
no co-occurring mutations
all mutated cases (row) co-occur with this mutation (column)

Figure S2


A
100%
80%
60%
40%
20%
0%
Mutation Burden
SF3B1
JAK2
SRSF2
IDH2


B
100%
80%
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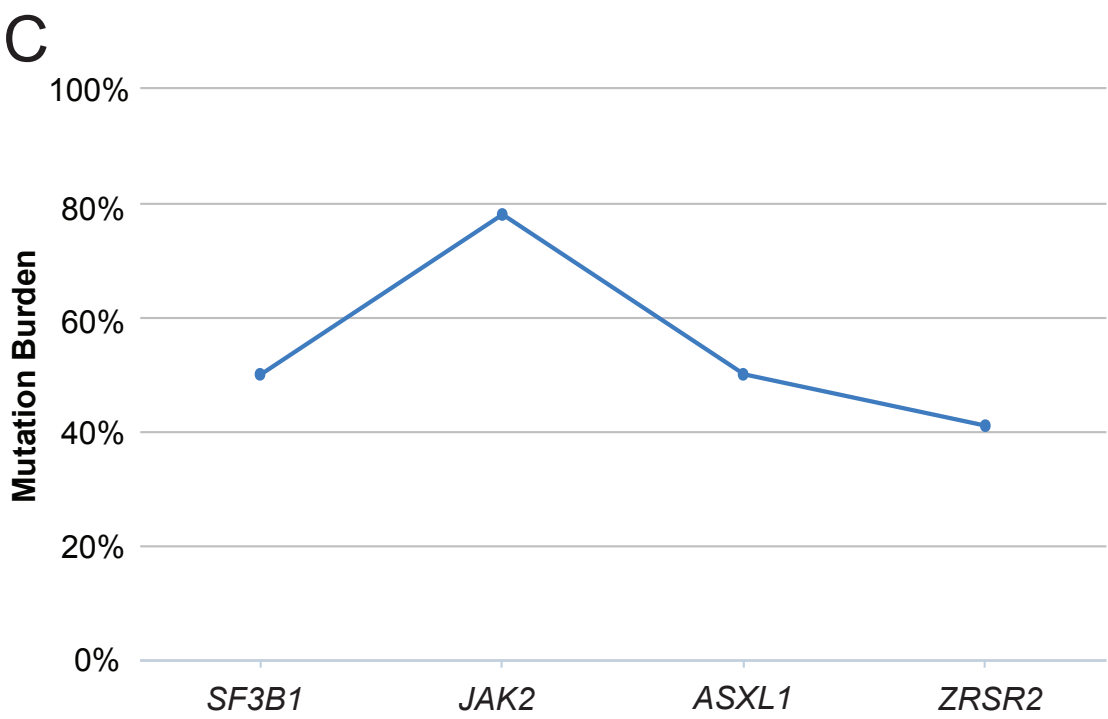
Mutation Burden
SF3B1
JAK2
TET2
EZH2


C
100%
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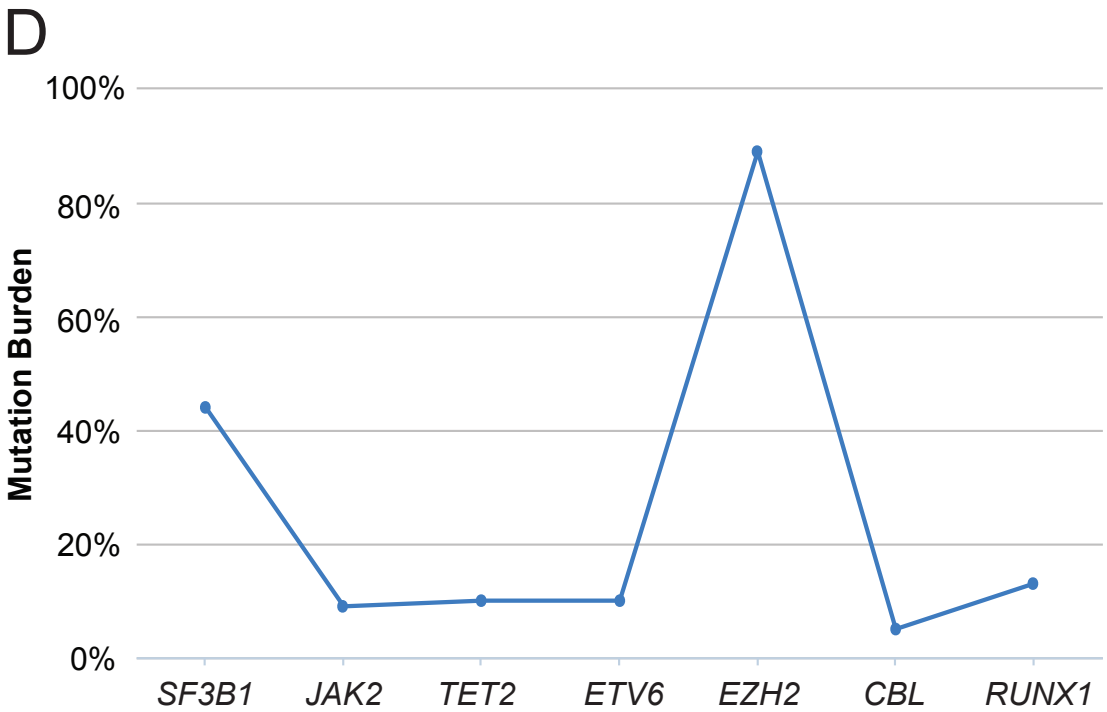
60%
40%
20%
0%
Mutation Burden
SF3B1
JAK2
ASXL1
ZRSR2


D
100%
80%
60%
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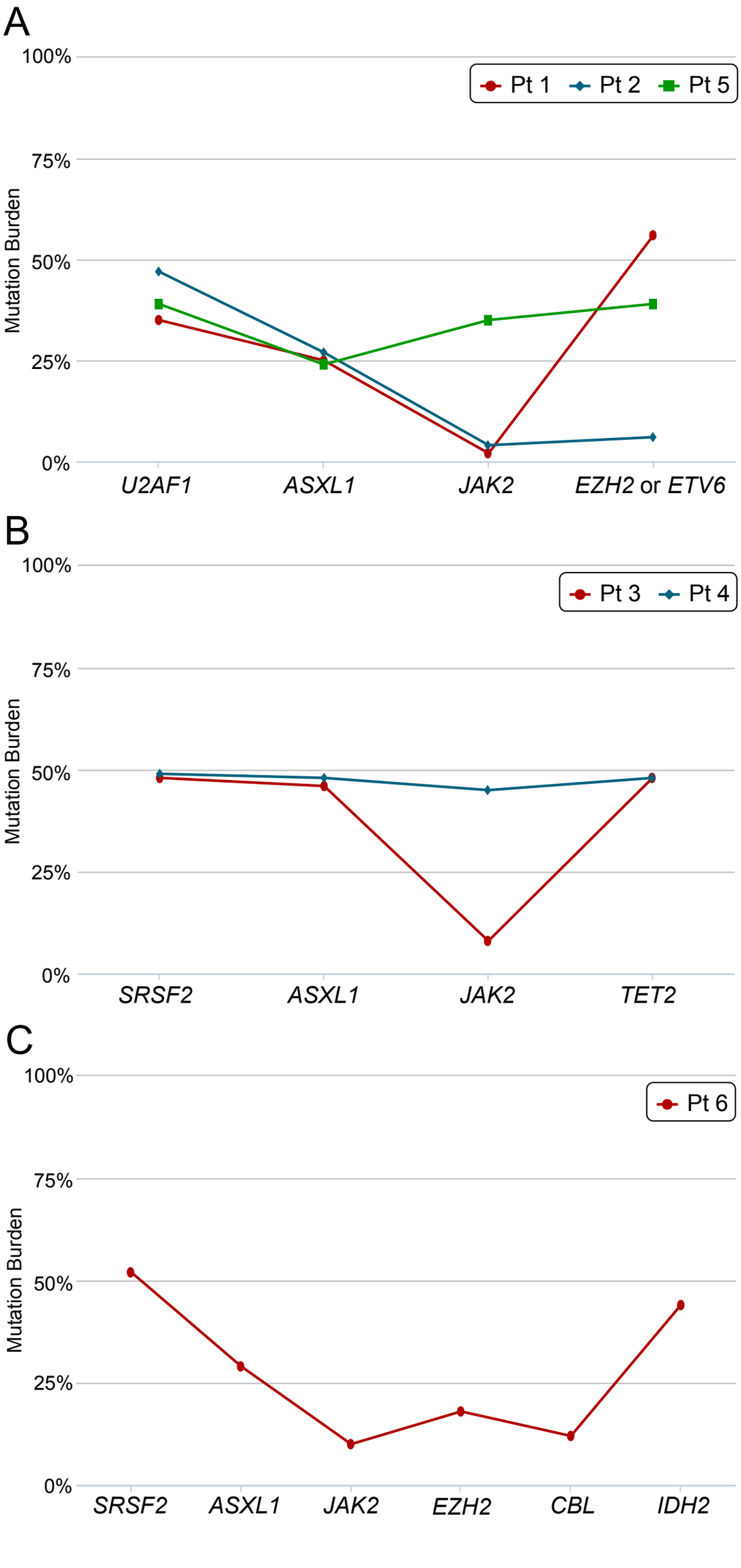
20%
0%
Mutation Burden
SF3B1
JAK2
TET2
ETV6
EZH2
CBL
RUNX1

Figure S3


A
100%
75%
50%
25%
0%
Mutation Burden
Pt 1
Pt 2
Pt 5
U2AF1
ASXL1
JAK2
EZH2 or ETV6
B
100%
75%
50%
25%
0%
Mutation Burden
Pt 3
Pt 4
SRSF2
ASXL1
JAK2
TET2
C
100%
75%
50%
25%
0%
Mutation Burden
Pt 6
SRSF2
ASXL1
JAK2
EZH2
CBL
IDH2