Myelodysplastic syndrome-associated spliceosome gene mutations enhance innate immune signaling

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SUPPLEMENTARY METHODS

Spliceosome gene inhibition and overexpression

RNAi was performed as described[1, 2]. Briefly, siRNAs targeting spliceosome genes and control non-targeting siRNAs (Dharmacon, Lafayette, CO, USA) were transfected into RAW264.7 cells using an Amaxa 96-well shuttle transfection system (Lonza, Basel, Switzerland); 24 hours later, the cells were stimulated with LPS for 6 hours. Supernatant cytokine levels were measured by ELISA (R&D Systems, Minneapolis, MN, USA). Cells were used to prepare RNA for qPCR or were analyzed for viability using fluorescein diacetate, as described previously[3, 4].

Plasmids expressing *SF3B1-wt* and *SF3B1-K700E* were from Addgene[5]. *SRSF2-wt* and *SRSF2-P95L* were synthesized commercially (Blue Heron Biotech, Bothell, WA, USA) and cloned into pCMV-AC6. Plasmids expressing *U2AF1-wt* and *U2AF1-S34F* were from M. Meyerson[6]. RAW264.7 cells plated at 200,000 cells per well in 24 well format were transfected with three expression plasmids using Fugene HD (Roche, Basel, Switzerland, USA): (1) wild type or mutant spliceosome genes, (2) NFκB-luciferase (Promega, Madison, WI, USA) or IL-6-luciferase[7] reporters, and (3) SV40-rluc reporter for normalization (Promega). Transfection conditions for the NFκB reporter were 75 ng NFκB-luc, 40 ng SV40-rluc, and either 600 ng (*SF3B1* and *SRSF2*) or 885 ng (*U2AF1*) plasmid DNA. Transfection conditions for the IL-6-luc reporter plasmids were either 160 ng IL-6-luc, 80 ng SV40-rluc, and 760 ng spliceosome gene (*SF3B1* and *SRSF2*) or 160 ng IL-6-luc, 40 ng SV40-rluc, and 800 ng spliceosome gene (*U2AF1*). 24 hours after transfection, cells were stimulated with LPS for 6 hours. Luciferase activity was monitored using the Dual Luciferase Assay kit (Promega). Expression of the spliceosome genes was monitored in parallel transfection studies using qPCR primers that amplify both the endogenous mouse gene and the human transgene. *U2AF1-wt*, *U2AF1-S34F*,

SRSF2-wt, and *SRSF2-P95L* were overexpressed 87.7±51.5, 62.9±45.6, 81.9±6.2, and 82.6±20.5 –fold compared to the endogenous genes (mean ± SEM), respectively. SF3B1 overexpression could not be assessed as the transgene cDNA sequence has been resynthesized completely[5]; thus, no primers can amplify both the endogenous gene and transgene.

K562 cell lines with spliceosome mutations

K562 leukemia cells expressing either *U2AF1-S34F*, *SRSF2-P95H*, or *SF3B1-K700E* were generated using CRISPR/Cas with guide RNAs targeting the relevant codon regions and repair templates. CRISPR guides and homology repair templates were ordered as gBlocks (Integrated DNA Technologies, Coralville, IA, USA) (sequences in Supplementary Table S2). gGuide RNA templates were cloned into a pBluescript backbone and grown in *E. coli*. The isolated CRISPR guide plasmids, a Cas9 containing plasmid, a GFP and puromycin resistance containing plasmid, and the homology repair template were introduced into K562 cells by electroporation using the Amaxa nucleofector II and nucleofection kit V (Lonza). Transformed K562 cells were grown for 2 days in RPMI 1640 medium supplemented with 10% FBS, L-glutamine and Pen-Strep before being subjected to puromycin selection. When the culture reached 90% GFP positive cells, individual cells were plated by dilution into 96-well plates. Clones established after about 7 days were re-plated and checked for the presence of the desired mutation by Sanger sequencing. All data is from independent biological replicates performed on cells derived from a single clone with each spliceosome mutation. Variant allele frequencies (VAF) in the mutant cell lines were monitored by next-gen sequencing. *U2AF1-S34F* and *SRSF2-P95H* were present at a VAF of approximately 33% and 17%, respectively (consistent with these loci being multiploid in K562 cells) and were stable. *SF3B1-K700E* was present at a VAF of approximately 50%; however, *SF3B1-K700E* was not stable and reverted to wild type in K562 cells; thus, RNA was prepared and analyzed from early-passage *SF3B1-*

K700E clones. Spliceosome mutations in MDS patients are typically heterozygous; our prior studies suggest that pre-mRNA splicing is very sensitive to spliceosome perturbation; thus, differences in allele frequencies could have effects on splicing of specific genes in different contexts.

MDS patient samples

Isolation and analysis of peripheral blood monocytes from MDS patients and age-matched healthy control subjects was described previously[8]; these data were re-analyzed here to stratify by spliceosome gene mutation status (demographic data in Supplemental Table S1). We did not further analyze the data based on mutation allele frequency because most of these patient samples predate the use of our Next-gen sequencing panel, and thus these data were not available. This study was approved by the National Jewish Health and University of Colorado Institutional Review Boards. All subjects or an appropriate proxy gave informed written consent.

U2AF1 mouse studies

Mouse experiments were approved by the National Jewish Health Animal Care and Use Committee (IACUC). The U2AF1-wt, rtTA and U2AF1-S34F, rtTA transgenic mice were described previously[9]. U2AF1 expression was induced by feeding mice chow containing doxycycline (Teklad diet TD.09761) for five days prior to bone marrow (BM) isolation. BM HSPC populations, Gr-1+ myeloid cells, and monocytes were surface stained and isolated by double sorting on a FACSAria Fusion (Becton-Dickenson, Franklin Lakes, NJ, USA) as previously described[9, 10]. Bone marrow derived macrophages (BMDM) were generated as described[11]; transgene expression was induced in differentiated BMDM by adding 150 ng/ml doxycycline (Sigma, Burlington, MA, USA) to the cultures for five days prior to analysis.

qPCR and ELISAs

RNA was purified from cells using RNAeasy mini or micro kits (Qiagen). qPCR was performed using the Quantitect SYBR-Green RT-PCR kit (Qiagen, Hilden, Germany) on a QuantStudio 7 Flex (Applied Biosystems, Foster City, CA, USA). Data were analyzed using the ddCt method and normalized to βactin. Oligonucleotides used are listed in Supplementary Table S3. ELISAs were performed on cell supernatants using kits from (R&D Biosystems).

Statistical analysis

All data represents at least three independent biological replicates. qPCR data and luciferase data were normalized such that the expression of wild type genes or control genes were averaged to 1. Data were analyzed using t-tests in Graphpad Prism 5 (GraphPad Software, San Diego, CA, USA). Statistical significance is defined as $p<0.05$, although we also note a few experiments in which p values approached but did not reach significance ($p<0.10$).

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Supplementary Table S1. Patient Demographics Related to Figure 2J

	Wild Type	Splicing Mutant
N	9	8
Age[a]	61.8 ± 14.1	63.8 ± 19.5
Gender		
Female	4	2
Male	5	6
Race/Ethnicity		
White	8	8
Hispanic	1	0
Mutations		
SF3B1	0	3
U2AF1	0	2
SRSF2	0	3
Selective Disease Features		
High grade MDS[b]	6	3
Treatment Related MDS	1	0
del5q MDS	1	0
IPSS-R Risk Categories		
Low (≤3)	3	5
Intermediate (>3-4.5)	1	1
High (>4.5)	5	2

[a]Age displayed as mean ± stdev

[b]Blasts account for 5-19% of marrow cells and/or 3-19% of blood cells

Supplementary Table S2. Sequences relevant to K562 CRISPR line construction

SF3B1-K700E gRNA #1	GGATGAGCAGCAGAAAGTT
SF3B1-K700E gRNA #2	AGACCTACAAAACCAAACAC
SF3B1-K700E Repair Template	TGT TGG GGC ATA GTT AAA ACC TGT GTT TGG TTT TGT AGG TCT TGT GGA TGA GCA GCA GGA AGT TCG AAC CAT CAG TGC TTG GGC CAT TGC TGC CTT GGC TGA AGC AGC AAC TCC TTA TGG T
U2AF1-S34F gRNA	GTCATGGAGACAGGTGCTCT
U2AF1-S34F Repair Template	CTC TCA TTT TCC CTT ACA GAG TCA ACT GTT CAT TTT ATT TCA AAA TTG GAG CAT GTC GTC ATG GAG ACA GGT GCT TTC GGT TGC ACA ATA AAC CGA CGT TTA GCC AGG TTT GTT TGC CTT TTT TTC ATG TAA ATT ATA AAA ACT TCA TGT TCT TTT CAA AGA CAG T
SRSF2-P95H gRNA	GCGCGCTACGGCCGCCCCC
SRSF2-P95H Repair Template	GGG GCC GTG CTG GAC GGC CGC GAG CTG CGG GTG CAA ATG GCG CGC TAC GGC CGC CAC CCG GAC TCA CAC CAC AGC CGC CGG GGA CCG CCA CCC CGC AGG TAC GGG GGC GGT GGC TAC GGA C

Supplementary Table S3. Oligonucleotides used for qPCR

Gene	Forward Primer	Reverse Primer
m βactin	CTGAACCCCAAGGCCAACCG	CCGTCACCGGAGTCCATCAC
m IL-6	CTTGGGACTGATGCTGGTGAC	GCCTCCGACTTGTGAAGTGGTATAGACAGG
m MyD88-L	CCACCCTTGATGACCCCCTAGGACAAAC	GTCTGTTCTAGTTGCCGGATCATCTCCTGCAC
m MyD88-S	GGAGCTGAAGTCGCGCATCGGACAAAC	GTCTGTTCTAGTTGCCGGATCATCTCCTGCAC
h βactin	CTGAACCCCAAGGCCAACCG	CCGTCACCGGAGTCCATCAC
h IL-6	GTCCTGATCCAGTTCCTGCAGAAAAAGGCAAAG	TGTGGTTGGGTCAGGGGTGGTTATTGCATCTAG
h TNF	AACCCCGAGTGACAAGCCTGTAGCCCATGTTG	AGGGCATTGGCCCGGCGGTTCAGCCACTGG
h IL-8	GAGGGTTGTGGAGAAGTTTTTGAAGAGGGC	CACTGGCATCTTCACTGATTCTTGGATACC
h MyD88-L	GGCGCCTCTGTAGGCCGACTGCTCGAGCTG	GCTTCAAGATATACTTTTGGCAATCCTCCTCAATG
h MyD88-S	GGCGCCTCTGTAGGCCGACTGCTCGAGCTG	AACGCTCAGGCATATGCCCAATG
h MAP3K7 canonical	CTGAAGGGGGCTCTTTATATAATGTGCTGCATG	CCTGTGAATTAGCGCTTTGGGTTGCATGCTGTG
h MAP3K7 alternate	GGGCTCTTTATATAATGTTTGTGCCTTTCTTTCG	CCTGTGAATTAGCGCTTTGGGTTGCATGCTGTG
h CASP8 canonical	GGAAGTCCTGATGAATTTTCA AATGGGGAGGA	CTGTGATTCACTATCCTGTTCTCTTGGAGAGTCCGAG
h CASP8Δ6	GGAAGTCCTGATGAATTTTCAAATGACTTTGGAC	GTGCTTTTGCAAAATTGTGATTGTTGATGATCAGAC
h CAPS8 long	GTGCGTCCACTTTCTGGGCACGTGAGG	CTGCTGAAGTCCATCTTTTTAAAAGGCAGGAGAATATAA
h CASP8 short	TCCACTTTCTGGGCACGTGAGGTTGGGCC	CATAAAGATTTCTGCTGAAGTCCATCTCAATATTCC
mh U2AF1	TGGCCTCCATCTTCGGCACC	CTGTCTCCATGACGACATGCTCC
mh SRSF2	CCGCGGGACCGCTACACCAAGGAGTCC	TGCACCCGCAGCTCGCGGCCGTC

m indicates mouse gene; h indicates human gene. mh indicates primers that amplify the indicated gene in both mice and humans.

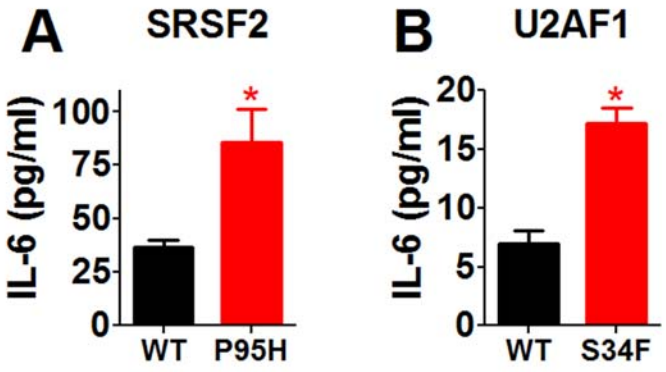

Figure S1. Spliceosome gene mutations found in MDS patients enhance inflammatory cytokine production in cancer-relevant myeloid cells. IL-6 proteins levels were assayed by ELISA in K562 cells carrying the indicated spliceosome gene mutations (red) or wild type control genes (black). Data represent mean, SEM. * indicates $p<0.05$.

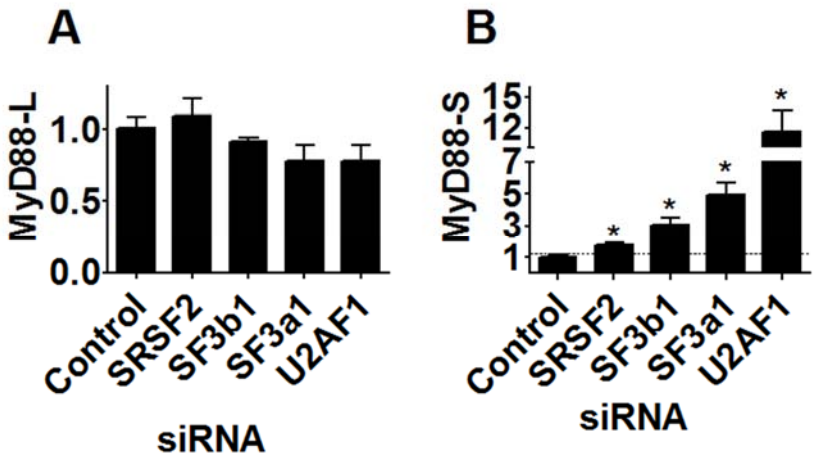

Figure S2. Inhibition of spliceosome genes alters splicing of MyD88. siRNA targeting the indicated spliceosome genes or a control non-targeting siRNA were transfected into RAW264.7 mouse macrophages, the cells were then stimulated with 20 ng/ml LPS for 6 hr, and the indicated mRNA isoforms were monitored by qPCR. Data represent mean, SEM. * indicates $p<0.05$. If no p value is indicated, then that comparison was not significantly different.

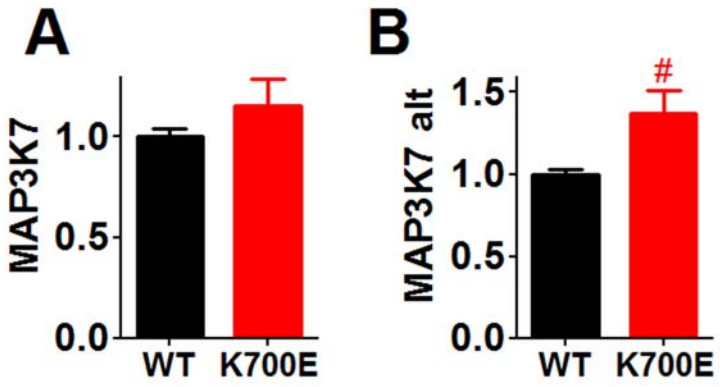

Figure S3. Trend towards increased MAP3K7 alternative isoform usage when SF3B1-K700E is overexpressed in RAW264.7 cells. Plasmids expressing the indicated *SF3B1-wt* (black) or *SF3B1-K700E* (red) genes were transfected into RAW264.7 cells (along with an IL-6 luciferase reporter construct and a SV40-rluc construct as outlined in Figure 1). The cells were then stimulated with 20 ng/ml LPS for six hours, RNA was prepared, and qPCR was used to assess canonical *MAP3K7* and alternate *MAP3K7* isoform levels. Data represent mean, SEM. # indicates $p<0.10$. If no p value is indicated, then that comparison was not significantly different.

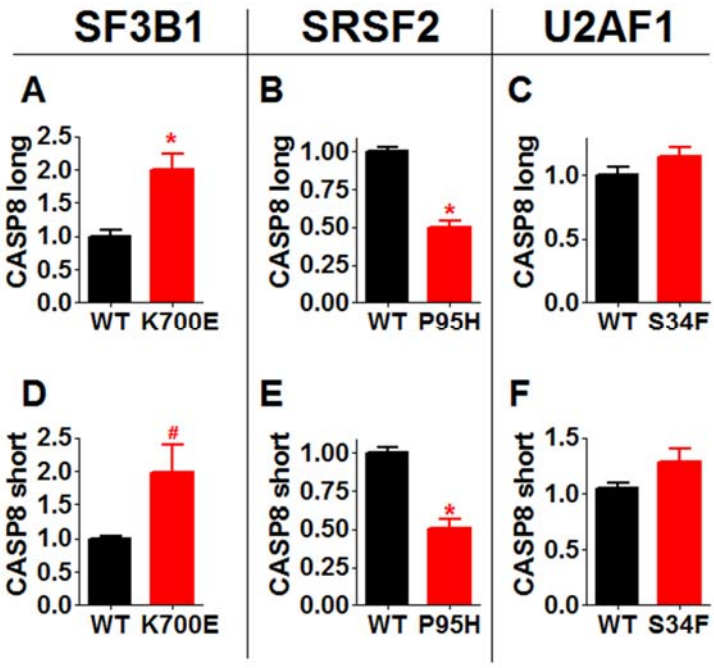

Figure S4. MDS-associated Spliceosome gene mutations alter splicing of CASP8. CASP8 "long" and "short" isoforms, as defined in the methods section of Fei et al.[12], were monitored by isoform-specific qPCR on RNA isolated from K562 cells carrying the indicated spliceosome gene mutations (red) or wild type control genes (black). Data represent mean, SEM. * indicates $p<0.05$. # indicates $p<0.10$. If no p value is indicated, then that comparison was not significantly different.