SUPPLEMENTARY APPENDIX

Replication stress signaling is a therapeutic target in myelodysplastic syndromes with splicing factor mutations

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Supplemental table 1:

UPN	sex	age	WHO 2008 or 2016 (depending on time of diagnosis)	IPSS-R	cytogenetics	sf mutation	VAF of sf mutation	mutational analysis	other mutations (VAF)
non-sf mutated									
1	M	65	RAEB-1	very high	45,X,-Y[11]/45,X,-Y,del(9)(q21q32)[5]/ 45,X,-Y,del(9)(q13q34)[4]			WES	*KRAS* (25)
2	M	63	RCMD	very low	46,XY[20]			WES	*DDX41* (42), *TET2* (5)
3	M	80	EB-2/ AML	very high	complex aberrant			WES	*TP53* (29), *NRAS* (5)
4	M	83	RCMD	high	complex aberrant			WES	*TP53* (40)
5	W	86	EB-2	very high	44,XX,-3,del(5)(q13q31),-12[22]			WES	*TP53* (81), *EZH2* (56)
6	M	80	RCMD	low	46,XY[20]			diagnostic panel seq	*BCORL1* (55)
7	M	72	RAEB-2	high	46,XY[20]			diagnostic panel seq	no mutation detected
8	M	70	RAEB-2/ AML	very high	complex aberrant			WES	*TP53* (14), *DNMT3A* (12)
9	M	73	RCUD	very low	46,XY[20]			WES	*DNMT3A* (55), *DDX41* (5)
10	W	67	MLD	very low	46,XX[20]			WES	*ASXL1* (27)
11	M	76	EB-1	int	46,XY[20]			diagnostic panel seq	*TET2* (x2, 42 & 39)
12	W	66	RA	high	46,XX,del(5)(q14q34)[3]/ 46,idem,t(3;12)(q26;p13)[16]/46,XX[1]			WES	*EVI1-MECOM* fusion transcript
13	W	77	del(5q)	int	46,XX,del(5)(q14q34)[26]/46,XX[1]			diagnostic panel seq	*TET2* (13), *NF1* (15), *CEBPA* (12)
14	M	68	RAEB-1	int	46,XY[20]			WES	*TET2* (34)
15	W	73	del(5q)	int	46,XX,del(5)(q14q33)[12]/46XX [8]			WES	*DNMT3A* (13)
16	M	78	RAEB-II	very high	complex aberrant			WES	*TP53* (15, 38), *FLT3* (11)
sf mutated									
17	M	77	CMML-2	high	46,XY[20]	*SRSF2*	46	WES	*NPM1* (12), *IDH2* (48), *KRAS* (13), *NRAS* (14)
18	M	56	RCMD	low	46,XY[21]	*ZRSR2*	83	WES	*EZH2* (54), *SETBP1* (44)
19	M	83	RAEB-1	int	46, XY[30]	*SRSF2*	40	diagnostic panel seq	*IDH1* (35)
20	M	77	RCMD	very low	46,XY[20]	*SRSF2*	50	diagnostic panel seq	*TET2* (100)
21	W	83	RARS-T	low	46,XX[20]	*SF3B1*	49	WES	*JAK2* (13)
22	M	76	RA	low	46,XY[20]	*SF3B1*	50	diagnostic panel seq	no further typical MDS mutation
23	M	66	RCMD	low	46,XY[20]	*U2AF1*	43	WES	no further typical MDS mutation
24	M	60	s-AML		47,XY,+8[9]/47,XY,+21[3]/46,XY[11]	*SRSF2*	34	WES	*RUNX1* (21), *BCOR* (24), *DNMT3A* (35), *IDH2* (53)
25	M	65	RAEB-2	int	46,XY	*SRSF2*	47	WES	*ASXL1* (49), *IDH* (41)
26	W	63	RARS	low	46,XX[20]	*SF3B1*	50	WES	no further typical MDS mutation

27	W	67	MLD	int	46,XX[20]	*SRSF2*	47	WES	*ASXL1* (39), *IDH2* (42), *STAG2* (36)
28	M	76	RAEB-2	very high	46,XY,i(17)(q10)[4]/46,XY[16]	*SRSF2*	45	WES	*ASXL1* (x2, 28 & 19), *ETV6* (48)
29	W	75	MLD	very low	46,XX[20]	*SRSF2*	31	WES	*TET2* (x2, 35 & 44), *MPL* (25)
30	W	84	del(5q)	int	46,XX,del(5q)(q13q33)[13]/46,XX[7]	*SF3B1*	31	WES	*TP53* (5)
31	M	78	RCUD	low	47XY,+8[15]/46,XY[5]	*SF3B1*	52	WES	no further typical MDS mutation
32	M	77	RAEB-2	int	46,XY[20]	*SRSF2*	48	WES	*IDH1* (25), *IDH2* (40), *JAK2* (25)
33	M	69	RS	low	46,XY[20]	*SF3B1*	34	diagnostic panel seq	no further typical MDS mutation
34	M	53	RARS-T	high	complex aberrant	*SF3B1*	46	WES	*KMT2A* deletion (40)
35	M	71	MLD	int	46,XY,r(6)(p21q26)[14]/46,XY[6]	*SRSF2*	50	WES	*RUNX1* (42), *TET2* (92), *ASXL1* (45)
36	W	82	RARS	low	46,XX[20]	*SF3B1*	44	WES	*DNMT3A* (55), *TET2* (x3, 17 & 10 & 9), *JAK2* (5)
37	M	66	RAEB-2	int	46,XY[20]	*SRSF2*	51	WES	*IDH1* (11), *RUNX1* (43), *STAG2* (43)
38	M	72	RCMD	very low	46,XY[20]	*SRSF2*	18	WES	*NF1* (8), *TET2* (x2, 38 & 16)
39	M	75	RCMD	very low	46,XY	*SRSF2*	50	WES	*TET2* (38)
40	M	77	MDS/MPN	high	46,XY,t(2,2)(p23;q32)[10]/ 47,XY,t(2,2)(p23;q32),+8[2]/46,XY[9]	*SRSF2*	47	WES	*STAG2* (5), *RUNX1* (85), *ASXL1* (42)
41	M	74	RCMD	int	46,XY[20]	*SRSF2*	47	WES	*TET2* (x2, 47 & 39), *STAG2* (77), *ASXL1* (46), *BCOR* (32)
42	M	73	RS-MLD	low	46,XY[20]	*U2AF1*	11	diagnostic panel seq	*JAK2* (7), *ASXL1* (8)
43	M	66	RS-MLD	low	46,XY[21]	*SF3B1*	44	WES	no further typical MDS mutation
44	M	63	EB-1	high	46,XY,+1,der(1;16)(q10;p10)[18]/46,XY[2]	*SRSF2*	40	WES	*IDH2* (13), *RUNX1* (40)
45	W	74	MLD	int	46,XX[20]	*SRSF2*	41	WES	*ASXL1* (19), *KRAS* (9), *KIT* (ratio:0.102), *TET2* (72)
46		80	EB2	high	47,XY,+8[2]/46,XY[19]	*SRSF2*	47	WES	*ASXL1* (46), *BCOR* (32), *STAG2* (77), *TET2* (x2, 47 & 39)
47	M	73	RCMD-RS	low	46,XY [20]	*SF3B1*	57	WES	no further typical MDS mutation
48	M	61	RCMD	int	46,XY [20]	*ZRSR2*	69	WES	*NF1* (28), *RUNX1* (11), *STAG2* (32)
49	W	76	RARS-T		46,XX [20]	*SF3B1*	46	WES	*JAK2*, *SETBP1* (5)
50	M	79	MLD	int	46,XY [20]	*SRSF2*	53	WES	*TET2* (41), *ASXL1* (x3, 5 & 4 & 44), *TP53* (6), *NF1* (13)
51	W	77	RARS	low	46,XX,del(11)(q14q25)[17]/46,XX[3]	*SF3B1*	46	WES	no further typical MDS mutation
52	W	83	RARS	low	46,XX [20]	*SF3B1* & *U2AF1*	40 & 9	WES	no further typical MDS mutation
53	M	77	MLD	int	46,XY [20]	*SF3B1*	30	WES	*TET2* (10)

Int, intermediate; sf, splicing factor; UPN, unique patient number; VAF, variant allele frequency; WES, whole exome sequencing

Supplemental figure 1

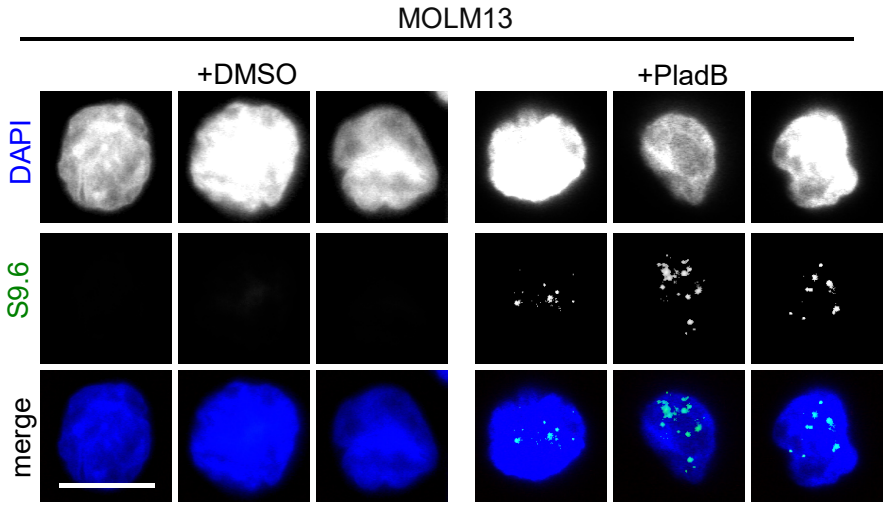


Figure S1. Association of splicing perturbations induced by Pladienolide B (PladB) with the accumulation of R-loops in cell line cells. Representative immunofluorescence images of R-loops using antibody S9.6 (Kerafast, Boston) in MOLM13 cells after treatment with PladB (50nM) or DMSO only. Scale bar, 5 μm.

Supplemental figure 2

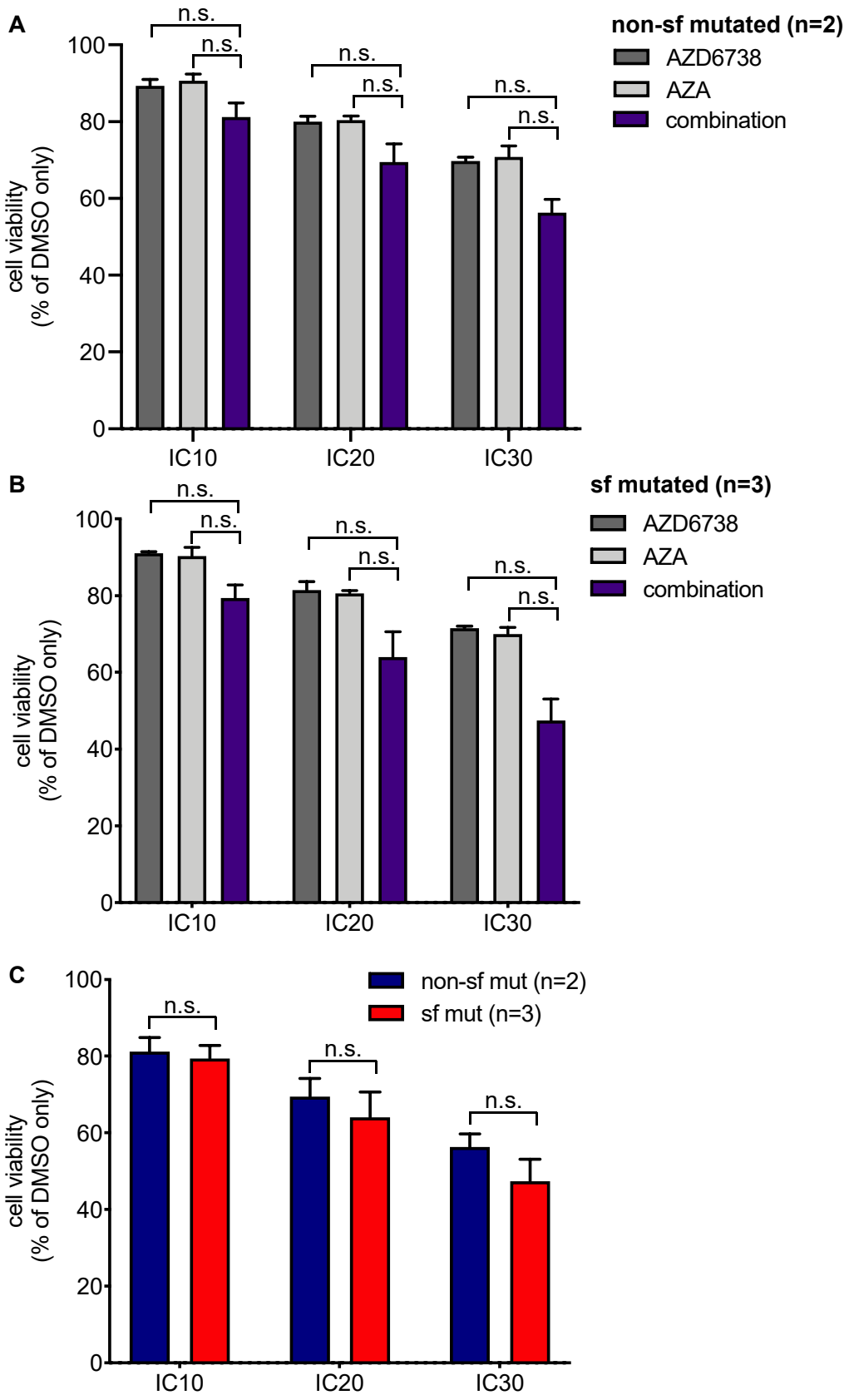


Figure S2. **Effects of AZD6738 in combination with 5-Azacytidine. (A-B)** Cytotoxicity tests of AZD6738 and AZA, alone and in combination in non-sf mutated (n=2) **(A)** and sf mut (n=3) **(B)** CD34+ cells of MDS patients. **C.** Direct comparison of values obtained in both AZD6738 and AZA treated samples. Cell viability was determined by Celltiter-Glo® after 48hr. Shown are IC10, IC20, and IC30 values of dose-response experiments. Data are means +/-SD. AZA, 5-Azacytidine; mut, mutated; n.s.= not significant; sf, splicing factor.

Supplemental figure 3

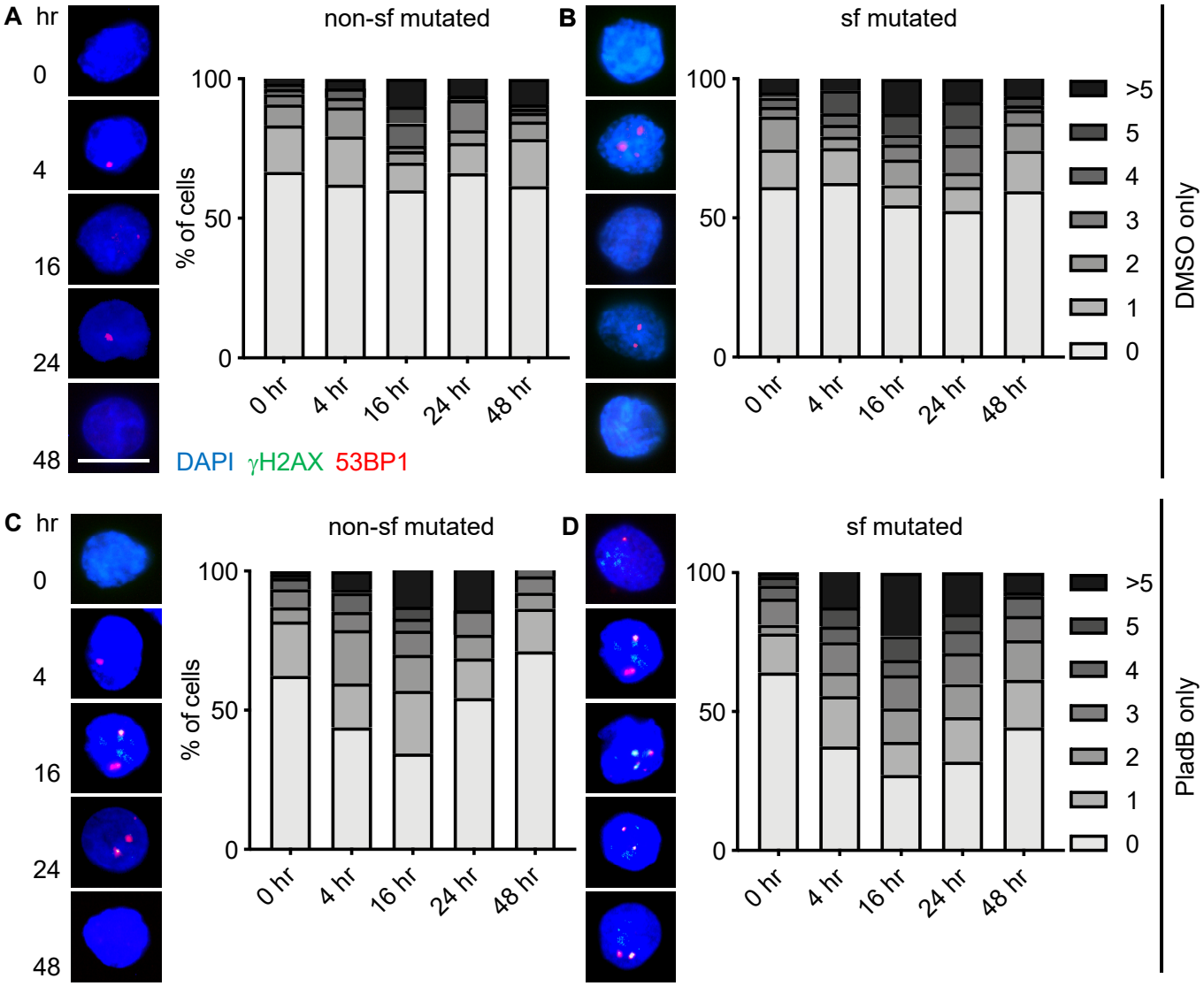


Figure S3. Unresolved DNA damage leads to elevated cytotoxicity in splicing factor mutated MDS CD34+ cells treated with AZD6738 and Pladienolide B. (A-B) Representative immunofluorescence images of DNA repair kinetics following exposure to vehicle (DMSO) **(A, C)** or 5nM PladB **(B, D)** in cultured splicing factor non-mutated **(A, B)** or mutated **(C, D)** MDS CD34+ cells (2 (non-sf mutated) and 3 (sf mutated) independent experiments, respectively; 25-130 cells were analyzed per timepoint and experiment). Scale bar, 5 μm; sf, splicing factor.

Supplemental figure 4

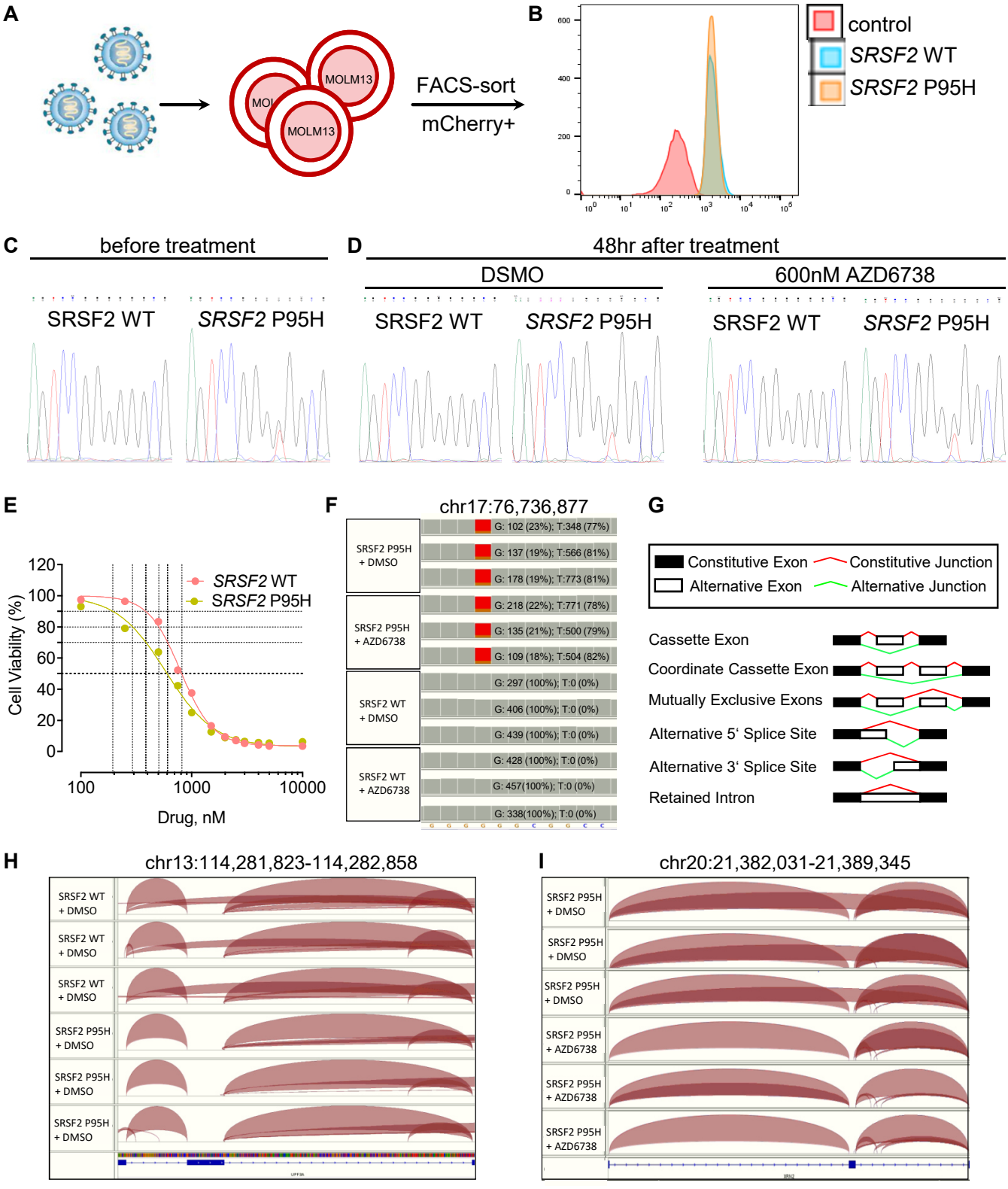
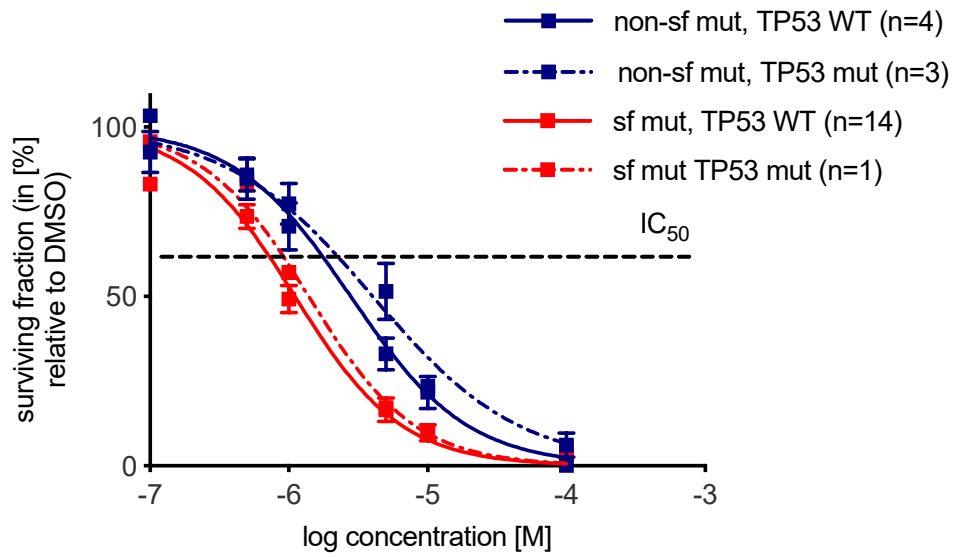


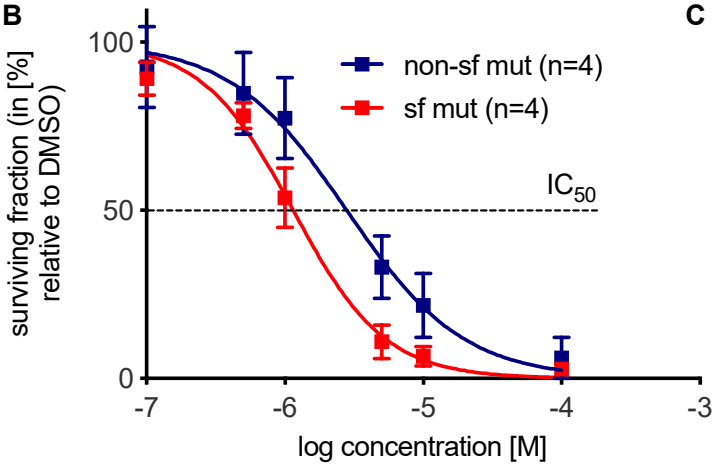
Figure S4. Mechanistic validation of RNA-Seq experiment. (A) Experimental procedure of lentivirus-mediated overexpression of SRSF2 WT and SRSF2 P95H in MOLM13 cells. **(B)** mCherry expression post lentiviral transduction. **(C-D)** Sanger sequencing of the *SRSF2* locus in transduced and sorted mCherry+ MOLM13 cells before treatment **(C)** and after treatment with 600nM AZD6738 or DMSO **(D). (E)** Dose-response experiments of AZD6738 in MOLM13 cells transduced with *SRSF2* WT or P95H. **(F)** Values of *SRSF2* P95H VAF in MOLM13 cells after *in vitro* treatment with DMSO or 600nM AZD6738 (= IC50 for MOLM13 *SRSF2* P95H cells) for 48hr. **(G)** Illustration of possible alternative splicing events driven by the presence of mutant *SRSF2* **(H-I)** Examples of alternative junction use driven by the *SRSF2* P95H mutation **(H)** and by treatment with AZD6738 **(I)**. Results from n=3 experiments are shown.

Supplemental figure 5

A



B



C

UPN	WHO	IPSS-R	sf mutation	VAF
2	RCMD	very low		
11	EB-1	int		
13	del(5q)	int		
14	RAEB-1	int		
29	MLD	very low	*SRSF2*	31
30	del(5q)	int	*SF3B1*	31
32	RAEB-2	int	*SRSF2*	48
45	MLD	int	*SRSF2*	41

Figure S5. **Influence of TP53 and IPSS-R risk group on the cytotoxic effect of AZD6738. (A)** Dose-response experiments measuring cell viability with Celltiter-Glo® at 48hr after continuous *in vitro* drug exposure with ATR inhibitor AZD6738 in CD34+ cells from patients with no sf mutation and WT TP53 (n=4), no sf mutation and TP53 mutation (n=3), sf mutation and WT TP53 (n=14), and sf mutation and TP53 mutation (n=1). **(B)** Dose-response experiments measuring cell viability with Celltiter-Glo® at 48hr after continuous *in vitro* drug exposure with ATR inhibitor AZD6738 in CD34+ cells from sf mutated and non-sf mutated patients matched by IPSS-R risk group. **(C)** Short description of characteristics of patients analyzed in **(B).** mut, mutated; sf, splicing factor.

Online Methods section

Patient material

Bone marrow of MDS patients was obtained from residual diagnostic material. Healthy CD34+ cells were isolated from hip replacement surgery bone specimen (healthy old CD34+ cells) or collected by iliac crest puncture of healthy volunteers (healthy young CD34+ cells). Furthermore, healthy CD34+ cells were isolated from cord blood (for lentiviral transduction experiments). Human material in this study was used only after informed written consent and following Institutional Review Board approval by the Medical Ethics committee II of the Medical Faculty Mannheim, University of Heidelberg, Germany in accordance with the declaration of Helsinki. Mononuclear cells (MNCs) were isolated using Ficoll density centrifugation. CD34+ cell enrichment from MNCs was performed using MACS columns (Miltenyi Biotech). CD34+ cells were routinely analyzed by flow cytometry in order to ensure purity >90%. Isolated CD34+ cells were either used directly or stored in liquid nitrogen until use.

Sequencing

Sf mutations were either assessed in diagnostic myeloid panel sequencing performed at the Munich Leukemia Laboratory (Munich, Germany) (n=9), or, if sequencing data were incomplete, the respective samples were subjected to whole exome sequencing (WES) (n=44) according to the following protocol: WES was performed either from DNA of CD34+ cells or bone marrow MNCs. Genomic DNA from *in vitro* expanded bone marrow derived mesenchymal stroma cells (MSCs) served as germline control. 250ng of high molecular gDNA was subjected to the Nextera DNA Flex Kit (Illumina) and subsequent hybrid capture using the xGen Exome Research Panel (IDT) according to the manufacturers' protocols. The final library pools were sequenced on an S4 NovaSeq flow cell with 150bp PE (Illumina). For mutational calling, raw sequencing data was subjected to an exome-sequencing bioinformatical pipeline including fastq-trimming (trimmomatic v0.39), alignment (bwa v.0.7.9) and PCR deduplication (picard MarkDuplicates v2.20.5). BAM files were realigned and recalibrated with the gatk bundle (v.3.8). Potential overlapping forward and reverse reads were softclipped by bamUtil clipOverlap (v1.0.14). Somatic mutations were called by gatk Mutect2 (gatk v4.1.3.0). Only variants, which passed FilterMutectCalls were further annotated with annovar.[1] On average, a median coverage of 84.7-fold was achieved for MNC/CD34+ and germline samples.

Tissue culture and cell-based assays

Primary hematopoietic cells were cultured in StemSpan SFEM II supplemented with myeloid expansion supplement containing SCF, TPO, G-CSF, and GM-CSF (Stemcell Technologies). MOLM13 cells were obtained from the DSMZ German Collection of Microorganisms and Cell Cultures GmbH and cultured in RPMI-1614 supplemented with 10% heat-inactivated fetal calf

serum. VE-821 (TargetMol), AZD6738 (Selleckchem), and Pladienolide B (PladB) (R&D Systems) were diluted in DMSO as recommended by the manufacturers. 5-Azacytidine (AZA, Sigma) was freshly diluted in water before each use. The substances or DMSO (vehicle) were added to the culture media in concentrations as indicated.

Cell viability assay

Cell viability in response to treatment was determined by using CellTiter-Glo® assay (Promega) according to the manufacturer's recommendation.

Cell apoptosis assay

Apoptosis was determined by flow cytometry using AnnexinV/ Propidium Iodide (PI) staining according to the manufacturer's protocol (BD Bioscience). In brief, after treatment with AZD6738, PladB (alone and in combination), or vehicle for 24-48 hours cells were washed and stained with FITC-conjugated AnnexinV and PI for 15 min at room temperature (RT) in the dark. Cells were analyzed on a FACSMelody Flow Cytometer (BD Bioscience).

Flow cytometry

Differential bone marrow fractions were isolated from MNCs stained with fluorochrome-conjugated antibodies against CD33, CD19, CD3, CD34 and CD38 by flow cytometry-based cell sorting (FACS). Cell isolation was performed on a BD FACSMelody Flow Cytometer using double sorting to ensure maximum purity.

Immunofluorescence

Cells were pipetted onto Polysine® coated slides (Thermo Fisher Scientific), fixed with 4% paraformaldehyde (PFA, 10 min at RT), permeabilized in 0.15% Triton® X-100 (2 min at RT) and blocked in 1% BSA/PBS. Slides were then incubated overnight at 4°C in 1% BSA/PBS containing the following antibodies alone or in combination: anti-phospho-H2A.X (Ser139) (Millipore, 1:500), anti-53BP1 (Novus Biologicals, 1:400), anti-S9.6 (Kerafast, 1:500) and anti-RPA (pSer33) (Novus Biologicals, 1:2000). After washing, slides were incubated for 2 hr at RT in 1% BSA/PBS with Alexa Fluor® 488-conjugated goat anti-mouse and Alexa Fluor® 594-conjugated goat anti-rabbit secondary antibodies (Thermo Fisher Scientific) before being mounted in VectaShield containing 1 µg/ml DAPI (Vector Laboratories). Images were acquired on a DMi8 Leica inverted microscope (10x, 20x or 100x objective) and processed using LasX software (Leica). Quantification of mean fluorescence intensity (MFI) was performed using the following formula: mean fluorescence of selected cell – (area of selected cell x mean fluorescence of background readings). Values are displayed as arbitrary units (A.U.).

Single-molecule DNA replication analyses

Replication track analyses were adapted from a published protocol.[2] In brief, CD34+ cells were cultured for 36-48 hr, pulsed for 30 min with 25 µM 5-Chloro-2′-Deoxyuridine (CldU), washed 1x with media, pulsed for 30 to 60 min with 250 µM 5-Iodo-2′-Deoxyuridine (IdU), washed with cold PBS and spotted onto SuperFrost® microscope slides. Cells were lysed for 6 min with 7 µl of fiber spreading buffer (0.2 M Tris pH 7.4, 50 mM EDTA, 0.5% SDS). Slides were tilted at a 15° angle to allow DNA to spread, fixed in 3:1 volume absolute methanol:glacial acetic acid for 10 min and air-dried. DNA was denatured with 2.5 M HCl for 80 min at RT. Slides were blocked in 0.1% Tween20/3% BSA/PBS (blocking buffer) for 1 hr at RT. Cells were incubated for 1 hr at RT with rat anti-BrdU (clone BU1/75) and mouse anti-BrdU (clone B44) antibodies to detect CldU and IdU, respectively. Slides were fixed using 4% PFA for 10 min at RT and - after PBS wash- incubated for 90 min at RT with Alexa Fluor® 488-conjugated goat anti-mouse, Alexa Fluor® 594-conjugated goat anti-rat and Alexa Fluor® 647-conjugated goat anti-mouse secondary antibodies. Slides were mounted in Vectashield. Tracks were imaged on a Zeiss AXIO microscope and fork rate was calculated based on the length of the IdU tracks measured using ImageJ software and the following formula: fork rate (kb/min) = [(2.59 (kb/micrometer) x length (micrometer))/pulse time (min)].

Lentiviral transduction of CD34+ cells and confirmation of transduced cells

Lentiviral constructs expressing *SRSF2* WT and *SRSF2* P95H were transiently transfected into 293T cells together with a third-generation lentiviral packaging mix (AMS.P904, Amsbio). Lentiviral supernatants were concentrated (Lenti-X concentrator, Clontech) and used to infect cord-blood derived CD34+ cells by spin-infection (2,000xg, 33°C, 90 min). mCherry-positive cells were isolated 48-72 hr later on a BD FACSMelody Flow Cytometer using double sorting to ensure maximum purity. mCherry+ cells were then expanded using StemSpan SFEM II supplemented with myeloid expansion supplement (Stemcell Technologies) (for cytotoxicity assays) or put onto Polysine® slides (for immunofluorescence experiments). pRRL_SRSF2_WT_mCherry and pRRL_SRSF2_P95H_mCherry were a gift from Robert Bradley (Addgene plasmids # 84023; http://n2t.net/addgene:84023; RRID:Addgene_84023 and Addgene plasmid # 84020; http://n2t.net/addgene:84020; RRID:Addgene_84020). Sanger sequencing of genomic DNA isolated from mCherry+ CD34+ cells was performed using the following – mutation-spanning - *SRSF2*-specific primers: forward: 5'-TCCCGCGGCTTCGCCTTCGTTC, reverse: 5'-TACCTGCGGCTCCGGCGTCCGTA

Lentiviral transduction of MOLM13 cells and RNA-Sequencing (RNA-Seq) experiment

MOLM13 cells were transduced with the same lentiviral particles mentioned above encoding *SRSF2* WT and *SRSF2* P95H. mCherry positive cells were FACS-sorted 48 hr later and expanded in culture. For the dose-response experiment, $2.5x10^3$ *SRSF2* WT and *SRSF2* P95H expressing MOLM13 cells were FACS-sorted from expanded cultures and incubated with a

range of AZD6738 concentrations (0-10000 nM) in 96-well plates for 48 hr followed by cell viability determination using the CTG assay. The presence of the *SRSF2* mutation was checked before and after treatment by Sanger sequencing **(supplemental Figures 4C, D)**. For RNA sequencing, $1x10^5$ *SRSF2* WT and *SRSF2* P95H expressing MOLM13 cells were FACS-sorted from expanded cultures and treated for 48 hr with 600nM AZD6738 (IC_{50} for MOLM13 *SRSF2* P95H expressing cells, **supplemental Figure 4E)** or DMSO in triplicates. Total DNA and RNA were extracted using the Qiagen AllPrep DNA/RNA Mikro kit (Hilden, Germany). Genomic DNA was subjected to Sanger sequencing to validate the P95H mutation before and after ATR treatment. For library preparation the Illumina TruSeq Stranded mRNA kit (San Diego, USA) with 500 ng RNA as input was used. Sequencing was performed with 100bp paired end on an Illumina NovaSeq device. Sequencing reads were aligned to hg38 using Hisat2 v2.1.0 mapper.[3] Cufflinks v2.2.1[4] was used to reconstruct transcripts with GRCh38 release 84 as reference. The assemblies were merged and taken as reference for alternative splicing analysis. This was performed with the JuncBASE v0.6 package,[5] which was run with default settings to identify alternative splicing events. Assessment of differential splicing was performed using a two-sided t-test. Correction for multiple testing was done by the Benjamini-Hochberg method with a cut-off q-value of <0.1.

Statistical analysis

Data were analyzed using Prism 8 (GraphPad Software, La Jolla, CA) using t-test or one-way analysis of variance (ANOVA), or Mann-Whitney U test. p value was considered significant at values less than 0.05 (n.s.: not significant, statistically significant *p<0.05, **p<0.01, ***<0.001).

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