Plenary Paper

MYELOID NEOPLASIA

CME Article

Impact of spliceosome mutations on RNA splicing in myelodysplasia: dysregulated genes/pathways and clinical associations

Andrea Pellagatti,[1,2,]* Richard N. Armstrong,[1,2,]* Violetta Steeples,[1,2,]* Eshita Sharma,[3] Emmanouela Repapi,[4] Shalini Singh,[1,2] Andrea Sanchi,[1,2] Aleksandar Radujkovic,[5] Patrick Horn,[5] Hamid Dolatshad,[1,2] Swagata Roy,[1,2] John Broxholme,[3] Helen Lockstone,[3] Stephen Taylor,[4] Aristoteles Giagounidis,[6] Paresh Vyas,[7-9] Anna Schuh,[10] Angela Hamblin,[10] Elli Papaemmanuil,[11] Sally Killick,[12] Luca Malcovati,[13,14] Marco L. Hennrich,[15] Anne-Claude Gavin,[15] Anthony D. Ho,[5] Thomas Luft,[5] Eva Hellström-Lindberg,[16] Mario Cazzola,[13,14] Christopher W. J. Smith,[17] Stephen Smith,[18] and Jacqueline Boultwood[1,2]

[1]Nuffield Division of Clinical Laboratory Sciences, Radcliffe Department of Medicine, University of Oxford, Oxford, United Kingdom; [2]Haematology Theme Oxford Biomedical Research Centre, Oxford, United Kingdom; [3]Wellcome Trust Centre for Human Genetics and [4]The Computational Biology Research Group, Weatherall Institute of Molecular Medicine (WIMM), University of Oxford, United Kingdom; [5]Department of Internal Medicine V, University Hospital Heidelberg, Germany; [6]Clinic for Oncology, Hematology, and Palliative Medicine, Marien Hospital Düsseldorf, Germany; [7]Medical Research Council Molecular Hematology Unit, WIMM, University of Oxford, United Kingdom; [8]Haematology Theme Oxford Biomedical Research Centre, Oxford, United Kingdom; [9]Department of Hematology, Oxford University Hospitals NHS Foundation Trust, Oxford, United Kingdom; [10]Molecular Diagnostics Centre, Oxford University Hospitals NHS Foundation Trust, Oxford, United Kingdom; [11]Department of Epidemiology-Biostatistics, Center for Molecular Oncology, Memorial Sloan-Kettering Cancer Center, New York, NY; [12]Department of Haematology, Royal Bournemouth Hospital, Bournemouth, United Kingdom; [13]Department of Molecular Medicine, University of Pavia, Pavia, Italy; [14]Department of Hematology Oncology, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy; [15]European Molecular Biology Laboratory Structural and Computational Biology Unit, Heidelberg, Germany; [16]Center for Hematology and Regenerative Medicine, Karolinska University Hospital Huddinge, Sweden; and [17]Department of Biochemistry and [18]Department of Pathology, University of Cambridge, Cambridge, United Kingdom

KEY POINTS

- **RNA-seq analysis of CD34+ cells identifies novel aberrantly spliced genes and dysregulated pathways in splicing factor mutant MDS.**

- **Aberrantly spliced isoforms predict MDS survival and implicate dysregulation of focal adhesion and exosomes as drivers of poor survival.**

SF3B1, *SRSF2*, and *U2AF1* are the most frequently mutated splicing factor genes in the myelodysplastic syndromes (MDS). We have performed a comprehensive and systematic analysis to determine the effect of these commonly mutated splicing factors on pre-mRNA splicing in the bone marrow stem/progenitor cells and in the erythroid and myeloid precursors in splicing factor mutant MDS. Using RNA-seq, we determined the aberrantly spliced genes and dysregulated pathways in CD34+ cells of 84 patients with MDS. Splicing factor mutations result in different alterations in splicing and largely affect different genes, but these converge in common dysregulated pathways and cellular processes, focused on RNA splicing, protein synthesis, and mitochondrial dysfunction, suggesting common mechanisms of action in MDS. Many of these dysregulated pathways and cellular processes can be linked to the known disease pathophysiology associated with splicing factor mutations in MDS, whereas several others have not been previously associated with MDS, such as sirtuin signaling. We identified aberrantly spliced events associated with clinical variables, and isoforms that independently predict survival in MDS and implicate dysregulation of focal adhesion and extracellular exosomes as drivers of poor survival. Aberrantly spliced genes and dysregulated pathways were identified in the MDS-affected lineages in splicing factor mutant MDS. Functional studies demonstrated that knockdown of the mitosis regulators *SEPT2* and *AKAP8,* aberrantly spliced target genes of *SF3B1* and *SRSF2* mutations, respectively, led to impaired erythroid cell growth and differentiation. This study illuminates the effect of the common spliceosome mutations on the MDS phenotype and provides novel insights into disease pathophysiology. (*Blood*. 2018;132(12):1225-1240)

Medscape Continuing Medical Education online

In support of improving patient care, this activity has been planned and implemented by Medscape, LLC and the American Society of Hematology. Medscape, LLC is jointly accredited by the Accreditation Council for Continuing Medical Education (ACCME), the Accreditation Council for Pharmacy Education (ACPE), and the American Nurses Credentialing Center (ANCC), to provide continuing education for the healthcare team.

JOINTLY ACCREDITED PROVIDER™
INTERPROFESSIONAL CONTINUING EDUCATION

Introduction

The myelodysplastic syndromes (MDS) are myeloid malignancies arising from bone marrow (BM) hematopoietic stem cells (HSCs).[1-5] The MDS are characterized by ineffective hematopoiesis resulting in peripheral blood cytopenias. Patients with MDS show increasing numbers of BM blasts over time, and 30% to 40% of cases progress to acute myeloid leukemia (AML).[1-4]

Splicing factor gene mutations occur in more than 50% of all patients with MDS, implicating spliceosome dysfunction as a key driver of disease pathophysiology.[6] *SF3B1*, *SRSF2*, *U2AF1*, and *ZRSR2* are the most frequently mutated splicing factor genes in MDS.[7,8] These splicing factors are involved in recognition of 3′ splice sites during pre-mRNA splicing.[9] SF3B1 stabilizes U2 snRNP binding to the branch point sequence,[6,10] SRSF2 promotes exon recognition by binding mRNA exonic splicing enhancer motifs,[6,11,12] whereas U2AF1 recognizes the AG dinucleotide at the 3′ splice sites.[6,13] These mutated genes have different prognostic impacts and to some degree define distinct clinical phenotypes in MDS.[8,14-17] *SF3B1* mutations are strongly associated with the presence of ring sideroblasts.[16,18]

Splicing factor mutations are typically early events in MDS,[19,20] and some are found in association with clonal hematopoiesis of indeterminate potential,[21-23] suggesting that the presence of splicing factor mutations can result in clonal expansion in aging human BM. HSCs expressing splicing factor mutations show a compromised repopulation capacity in mice compared with wild-type HSCs, however,[24-28] and precisely how splicing factor mutations confer a positive selection advantage in the BM remains a mystery.

Mouse models expressing splicing factor mutations show some features of MDS, including expansion of stem and progenitor cells in the BM and leukopenia.[24,26-28] Although these models have significantly advanced our understanding of how splicing factor mutations lead to particular hematological phenotypes, the differences in intronic sequences and consensus-binding motifs between mice and humans mean that the splicing abnormalities observed in the mice often differ from those observed in patients.[25,26]

Aberrant RNA splicing occurs as a consequence of splicing factor gene mutations in several human malignancies.[29-33] Some studies have investigated aberrant splicing in MDS and AML patients with splicing factor mutations,[24,28,34-38] but included small numbers of splicing factor mutant (SFmut) cases and/or analyzed unfractionated BM or mononuclear cell samples.

Here, we performed a comprehensive and systematic analysis to determine the effect of *SF3B1*, *SRSF2*, and *U2AF1* mutations on pre-mRNA splicing in purified BM stem and progenitor cells from a large cohort of patients with MDS. The major cellular processes and dysregulated pathways were identified in the CD34+ cells and in precursors of MDS-affected lineages of SFmut patients with MDS. We have determined the associations between aberrantly spliced isoforms and clinical variables and patient survival. Furthermore, we studied the functional effect of aberrant splicing of key target genes on erythroid cell growth and differentiation.

Materials and methods

Patients and samples

CD34+ cells were isolated from the BM samples of 84 patients with MDS (supplemental Table 1, available on the Blood Web site) and 8 healthy control individuals using MACS columns (Miltenyi Biotec, Germany). Of the 84 MDS cases, 28 were *SF3B1* mutant (SF3B1mut), 8 *SRSF2* mutant (SRSF2mut), 6 *U2AF1* mutant (U2AF1mut), and 2 *ZRSR2* mutant (with comutation of *SRSF2*), and 40 had no mutations in splicing factor genes (SFwt), as determined by targeted next-generation sequencing data[8] (supplemental Table 1; supplemental Table 2; supplemental Figure 1A).

Granulocytic, monocytic, and erythroid precursors were purified from the BM samples of 11 patients with MDS (7 *SF3B1*mut and 4 *SRSF2*mut) and 5 healthy control individuals. Mononuclear cells were isolated from BM aspirates using density gradient

centrifugation (Biocoll, Biochrom, Germany). After staining with CD34-APC (8G12), CD45-FITC (2D1), and CD14-PE (MφP9) (all from BD Biosciences, San Jose, CA), monocytes/macrophages restricted precursors (CD34$^-$CD45$^+$SSC^{med}CD14$^+$), granulocytic precursors (CD34$^-$CD45^{med}SSC^{high}), and erythroid precursors (CD34$^-$CD45$^-$) were isolated by FACS using a FACSAria cell sorter and the FACSDIVA software (BD Biosciences). After sorting, morphology and purity of the isolated cell populations were determined and the purity was higher than 95%. Here, the mutational profile of the patients with MDS was determined using a next-generation sequencing-based myeloid gene panel[20] (supplemental Table 3; supplemental Figure 1B).

The research was approved by the relevant institutional review boards or ethics committees, and all participants gave written informed consent.

RNA sequencing and data analysis

Total RNA was extracted using TRIzol (Thermo Scientific), with a linear acrylamide carrier, treated with DNase I (Life Technologies) and purified using Agencourt RNAClean XP beads (Beckman Coulter). RNA quality was assessed using a 2100 Bioanalyzer (Agilent), and RNA integrity numbers were greater than 8 for all samples. cDNA libraries were produced using a SMARTer library preparation protocol (Clontech). Sequencing was performed on an Illumina HiSeq4000 with 100-bp paired-end reads. The reads were mapped to human genome GRCh37 using HISAT2 version 2.0.0-beta.[39] Uniquely mapped read pairs were counted using featureCounts,[40] included with subread v1.5.0.[41] Quality control was performed on the mapped files using Picard CollectRnaSeqMetrics (http://broadinstitute.github.io/picard; supplemental Data 1). Differential splicing was assessed using rMATS v3.2.2beta,[34,42,43] with the BAM alignments generated by HISAT2. Aberrant splicing events associated with each mutated splicing factor were identified on the basis of the overlap between the lists of significant events identified by rMATS consistently in the comparisons of SFmut MDS both with SFwt MDS and with healthy control individuals (false discovery rate [FDR], <0.05 and inclusion level difference of >0.1 or <−0.1). The data discussed in this article have been deposited in the NCBI's Gene Expression Omnibus (GEO) repository (GEO accession number: GSE114922).

Gene ontology (GO) analysis was performed on the genes showing significant aberrant splicing events (FDR <0.05), using goseq.[44] A weighted bias correction based on the number of exons in each gene from Ensembl-GRCh37 was applied. Pathway analysis and upstream regulator analysis was performed on the genes showing significant aberrant splicing events (FDR <0.05) using Ingenuity Pathway Analysis (IPA) software (Qiagen).

Association of aberrant splicing with clinical variables

Clinical variables were analyzed for correlation with the rMATS-generated event inclusion levels for each MDS sample. Association between aberrant splicing events and clinical variables was determined using Spearman correlation for continuous variables (hemoglobin, white blood cell counts, absolute neutrophil count [ANC], platelet count [Plt], BM blasts, and age) and a Kruskal-Wallis test for categorical variables (sex, International Prognostic Scoring System, and transfusion dependence), with Bonferroni multiple hypothesis correction.

Functional studies

CD34$^+$ cells from healthy donors (Lonza) were cultured in erythroid differentiation media for 14 days, as described previously.[45] SEPT2 and AKAP8 genes were knocked down individually, using Mission shRNA lentiviral vectors (Sigma-Aldrich). Lentiviruses were produced and used to transduce CD34$^+$ cells from healthy donors, as described previously.[43]

Additional method details are described in the supplemental Information.

Results

Aberrant pre-mRNA splicing in CD34$^+$ cells of MDS cases with *SF3B1*, *SRSF2*, and *U2AF1* mutations

To identify aberrantly spliced transcripts associated with *SF3B1*, *SRSF2*, and *U2AF1* mutations in MDS hematopoietic stem and progenitor cells, we performed RNA-seq on CD34$^+$ cells from 82 patients with MDS and 8 healthy control individuals. The rMATS bioinformatics pipeline[34,42,43] was used to detect alternative (including cryptic) splicing events and categorize them as alternative 3′ splice site (A3SS) usage, alternative 5′ splice site (A5SS) usage, exon skipping (SE), mutually exclusive exons (MXE), or retained introns (RI).

*SF3B1*mut, *SRSF2*mut, or *U2AF1*mut patients were compared both with healthy control individuals and with SFwt patients, identifying more than 200 misregulated splicing events for each mutated splicing factor (Figure 1A-C; supplemental Data 2). *SF3B1* mutations were associated with a higher proportion of A3SS and RI events, whereas *SRSF2* mutations and *U2AF1* mutations were associated with a higher proportion of SE and RI events (Figure 1D-F).

Hierarchical clustering performed using the aberrant splicing events identified for each mutated splicing factor showed that *SF3B1*mut cases clustered together and separately from SFwt cases and healthy control individuals (Figure 1G). Similarly, *SRSF2*mut cases clustered together (Figure 1H). Most *U2AF1* mutations affect the S34 and R156/Q157 codons within the 2 zinc finger (ZF) domains.[9] All *U2AF1*mut cases clustered together, with the S34 mutants and the R156/Q157 mutants in 2 subclusters (Figure 1I).

Among the top 40 aberrant splicing events in *SF3B1*mut MDS (supplemental Table 4), the largest proportion was A3SS (n = 21/40), followed by RI (n = 11/40). In contrast, among the highest-ranking aberrant splicing events in *SRSF2*mut (supplemental Table 4) and *U2AF1*mut MDS (supplemental Table 4), the largest proportion was SE (n = 31/40 and n = 27/40, respectively).

Mutations in splicing factor genes define distinct clinical phenotypes in MDS.[6,14] We investigated the overlap of the aberrantly spliced genes identified in *SF3B1*mut, *SRSF2*mut, and *U2AF1*mut patients. The aberrant splicing events associated with each mutated splicing factor mainly affected different genes, although some overlap was observed (Figure 1J). A total
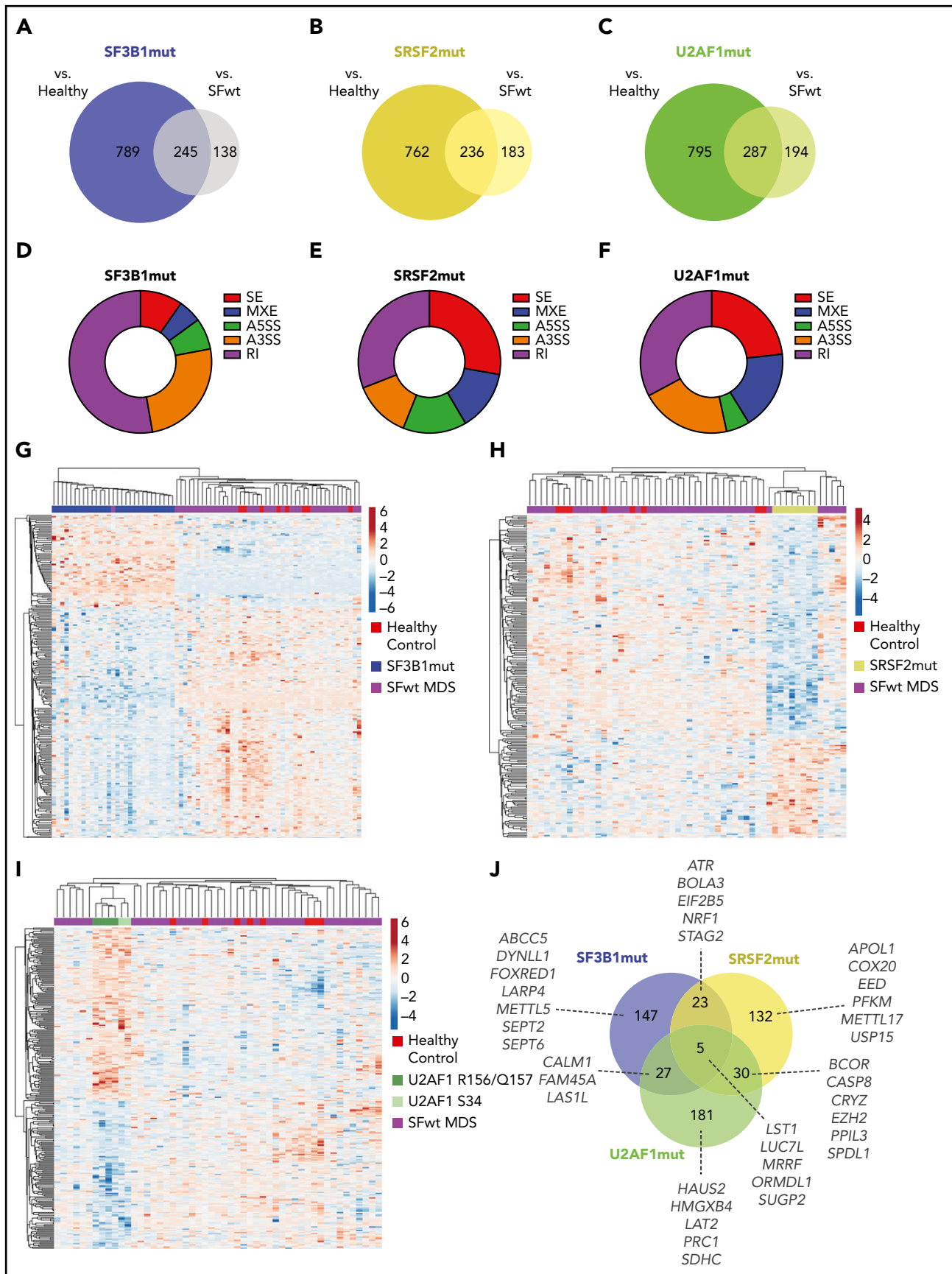
Figure 1.

of 147, 132, and 181 genes were aberrantly spliced distinctly in association with *SF3B1*mut, *SRSF2*mut, and *U2AF1*mut MDS cases, respectively (supplemental Data 3). These aberrantly spliced genes include *SEPT2* and *DYNLL1* in *SF3B1*mut cases, *PKFM* and *METTL17* in *SRSF2*mut cases, and *HMGXB4* and *LAT2* in *U2AF1*mut cases. An aberrant splicing event (RI) in the caspase 1 (*CASP1*) gene, the key effector of pyroptosis,[46] was observed in *SF3B1*mut MDS cases in our study. Five genes (*LST1*, *LUC7L*, *MRRF*, *ORMDL1*, *SUGP2*) were common to *SF3B1*mut, *SRSF2*mut, and *U2AF1*mut MDS. Some recurrently mutated genes in MDS were aberrantly spliced in *SF3B1*mut and *SRSF2*mut cases (ie, *STAG2*[47,48]), or in *SRSF2*mut and *U2AF1*mut cases (ie, *EZH2*[49] and *BCOR*). We found increased inclusion of a "poison" cassette exon of *EZH2* in *SRSF2*mut cases, as previously reported,[24] and observed the same aberrant splicing event in *U2AF1*mut cases.

Significantly dysregulated gene expression was observed for 33 (16%) of the aberrantly spliced genes identified in *SF3B1*mut MDS cases, with a lower percentage observed for *SRSF2*mut-associated and *U2AF1*mut-associated aberrantly spliced genes (supplemental Data 3). We did not observe a difference in gene expression levels between nonsense-mediated mRNA decay (NMD)-sensitive and NMD-insensitive events associated with *SRSF2* mutations and *U2AF1* mutations (supplemental Figure 2A-B). These data are in agreement with other studies.[24,35] For events associated with *SF3B1* mutations, gene expression levels were overall lower for NMD-sensitive events (supplemental Figure 2C). Given the known function of SF3B1, we examined the aberrant A3SS events associated with *SF3B1* mutation and found a more pronounced reduction of gene expression levels for NMD-sensitive events (median log2 fold -0.2 compared with median log2 fold -0.03 for NMD-insensitive events), although this did not reach statistical significance (supplemental Figure 2D).

GO analysis of aberrantly spliced genes in SFmut MDS

We performed GO analysis on the genes showing significant aberrant splicing events in SFmut MDS (supplemental Data 4). A marked convergence of significant GO themes was identified, with 74 GOs common to all SFmut MDS groups (Figure 2A; supplemental Data 5). We refined these overlapping GOs using REVIGO, which removes redundant GO terms and visualizes the most informative ontology themes. This analysis showed a strong cluster of GOs associated with mRNA metabolism, including RNA splicing, RNA processing, and mRNA translation (Figure 2A). Another strong GO cluster identified was associated with ribonucleoprotein complex biogenesis, which includes mitochondrial organization and translation. REVIGO treemap analysis of the 65 overlapping GO terms associated with *SF3B1*mut and *SRSF2*mut identified ontologies associated with ribonucleoprotein complex assembly, ligase activity (including regulation of mitotic cell cycle phase transition), and cellular response to DNA

damage (supplemental Figure 3A). The REVIGO treemap of 118 GO terms associated only with *SF3B1*mut MDS showed multiple GO terms associated with cellular response to DNA damage stimulus and cell cycle processes (supplemental Figure 3B).

Pathway analysis of aberrantly spliced genes in SFmut MDS

Pathway analysis was performed using IPA on the genes showing significant aberrant splicing events in SFmut MDS (Figure 2B; supplemental Tables 5-7). Interestingly, all 3 mutated splicing factors shared 6 common significant biological pathways, with the sirtuin signaling pathway as the top ranking pathway (Figure 2B; supplemental Figure 4). Among the significant pathways uniquely dysregulated in one SFmut MDS group, the regulation of eIF4 and p70S6K signaling pathway (a key pathway for translational regulation) was dysregulated in *SF3B1*mut MDS (Figure 2B; supplemental Table 5), whereas hypoxia signaling was dysregulated in *SRSF2*mut MDS (Figure 2B; supplemental Table 6).

Next, using IPA, we performed an analysis of upstream transcriptional regulators (including transcription factors), aiming to evaluate the significance of the overlap between the significant aberrantly spliced genes associated with each mutant splicing factor and genes regulated by a transcriptional regulator. This analysis showed a significant overlap for all 3 mutated splicing factors with several transcriptional regulators, including HNF4A, RICTOR, E2F1, MYC, and RB1 (Figure 2C).

A similar approach identified drugs/chemicals that can affect the expression of the genes found to be aberrantly spliced in SFmut MDS. Two retinoid drugs (CD437 and ST1926), the nucleotide antagonist 5-fluorouracil, and the mTOR inhibitor sirolimus (rapamycin) were significant for all 3 mutated splicing factors (Figure 2D), indicating that a significant proportion of the target genes of these compounds are aberrantly spliced in SFmut MDS.

Aberrantly spliced genes involved in heme metabolism and iron processing in SFmut MDS

*SF3B1*mut, *SRSF2*mut, and *U2AF1*mut MDS cases showed significant dysregulation of the heme biosynthesis II pathway (Figure 2B). Given the striking phenotypic association between ring sideroblasts and *SF3B1* mutation in MDS,[16,18] we performed an extensive investigation of the influence of splicing factor mutations on heme metabolism or iron processing. Of 200 and 150 genes involved in heme metabolism or in iron homeostasis and transport (supplemental Methods), respectively, we found several showing aberrant splicing events in *SF3B1*mut, *SRSF2*mut, and *U2AF1*mut MDS cases, with a higher number of events occurring in *SF3B1*mut MDS cases (supplemental Data 6).

We have previously reported aberrant splicing of the iron transporter *ABCB7* in MDS RARS,[34] and here we have found the

Figure 1. Aberrant splicing events in CD34+ cells of patients with SFmut MDS. (A-C) Venn diagrams showing the aberrant splicing events identified in *SF3B1* (A), *SRSF2* (B), and *U2AF1* (C) mutant MDS cases vs healthy control individuals and patients with SFwt MDS. (D-F) Doughnut charts showing the distribution of the aberrant splicing events identified in *SF3B1* (D), *SRSF2* (E), and *U2AF1* (F) mutant MDS cases by event type. For each category, the number of significant aberrant splicing events was normalized to the total number of events identified by the rMATS pipeline. (G-I) Hierarchical clustering of *SF3B1* (G), *SRSF2* (H), and *U2AF1* (I) mutant MDS samples, with wild-type MDS and healthy control samples using the rMATS-calculated inclusion levels of the 245, 236, and 287 aberrant splicing events identified.

Figure 2. Gene ontology and Ingenuity pathway analysis of aberrantly spliced genes in SFmut MDS. (A) Venn diagram showing the overlap of significant GOs identified in *SF3B1*, *SRSF2*, and *U2AF1* mutant MDS, and visualization of the significant BP GO terms common to all splicing factor mutant MDS using a REVIGO treemap. REVIGO panel sizes are inversely proportional to enrichment *P* values. (B-D) Ranked heat maps, as determined by collective significance across all splicing factor mutation groups, showing the significant dysregulated pathways (B), top 10 transcriptional regulators (C), and top 6 drug/chemical gene sets (D) in *SF3B1*, *SRSF2*, and *U2AF1* mutant MDS. Only heat map tiles with a -log10pvalue > 1.3 (*P* value < .05) are shown. Within each heat map, dysregulated pathways, transcriptional regulators and drug/chemical names are ranked by the lowest *P*-value identified in the SFmut group.
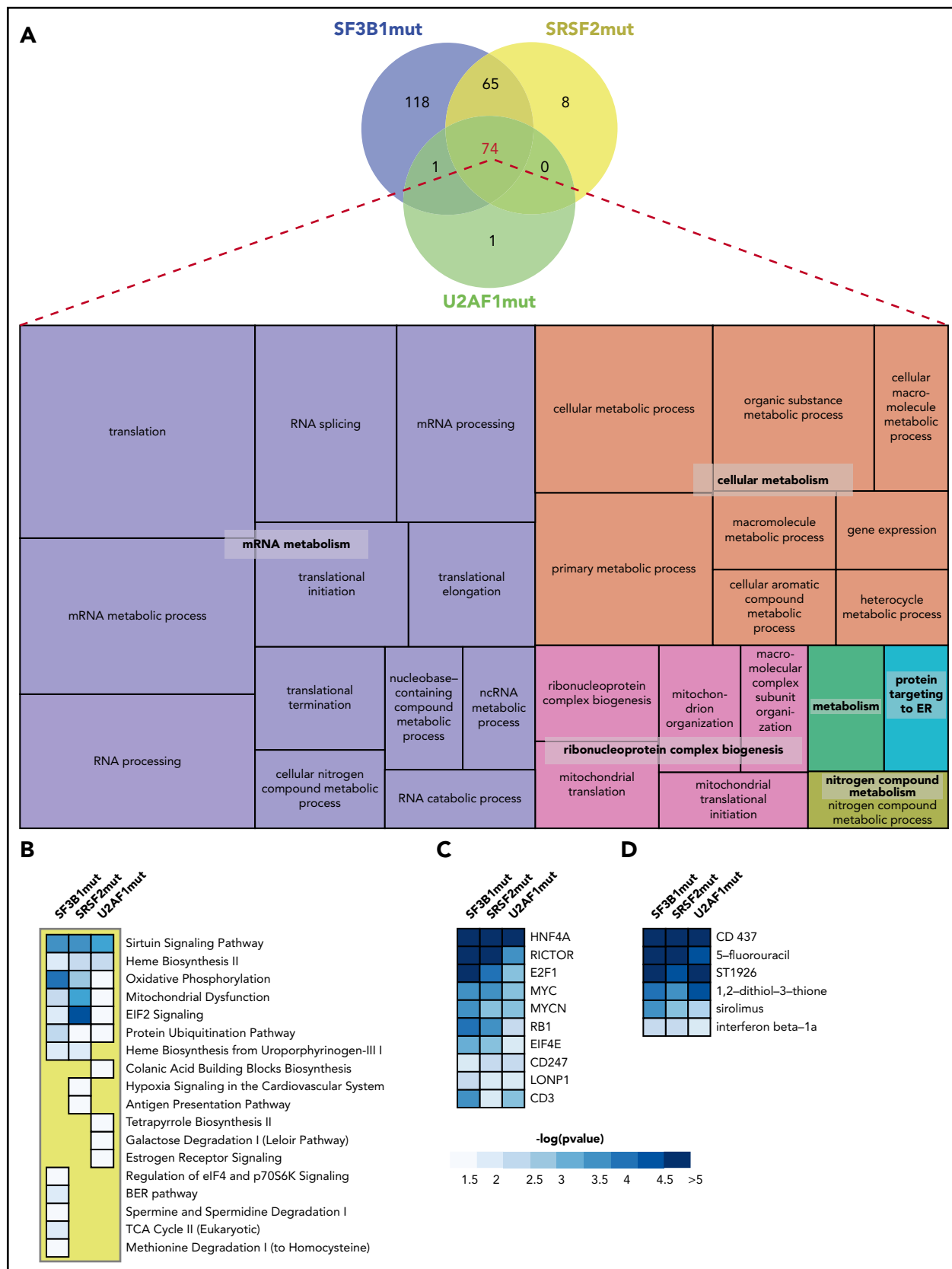
PELLAGATTI et al

Table 1. List of aberrant splicing events, identified in *SF3B1*mut MDS, significantly correlated with a clinical variable

Event ID	Gene	Event type	Chr	Strand	Start position	End position	Spearman correlation variable	cor_estimate	P	adj.P
6795	PARVG	A3SS	22	+	44582456	44583758	BM blast %	0.58	4.38E-07	.003031
5420	RPRD1A	RI	18	−	33605560	33607038	BM blast %	0.58	6.21E-07	.004292
3146	DOM3Z	RI	6	−	31938382	31938924	ANC	−0.57	1.00E-06	.006916
12280	CXXC1	SE	18	−	47811694	47811721	ANC	−0.56	1.28E-06	.00888
3305	AP1G2	RI	14	−	24031170	24031624	ANC	−0.56	1.68E-06	.011579
2362	SNRPN	A3SS	15	+	25219434	25219603	ANC	−0.55	3.14E-06	.021689
5460	TCEA2	A3SS	20	+	62703210	62703294	ANC	0.54	3.24E-06	.022393
6627	NICN1	A3SS	3	−	49462381	49462579	Plt	−0.54	3.80E-06	.026281
6153	ABCC5	A3SS	3	−	183703091	183703243	ANC	−0.54	4.23E-06	.029229
518	ERCC3	RI	2	−	128046912	128047400	ANC	−0.54	4.71E-06	.032551
2359	SNRPN	A3SS	15	+	25219457	25219603	ANC	−0.54	5.02E-06	.03468
7563	PPOX	A3SS	1	+	161137128	161137276	ANC	0.53	5.99E-06	.041411
4975	GPR108	A3SS	19	−	6730997	6731122	ANC	−0.53	6.43E-06	.044443
5816	PSTPIP1	A3SS	15	+	77328142	77328276	ANC	−0.53	6.49E-06	.044832
4728	NICN1	RI	3	−	49462381	49462871	Plt	−0.53	6.75E-06	.046655

ANC, absolute neutrophil count; BM blast %, bone marrow blast percentage; Plt, platelet count.

same aberrant splicing event in the *ABCB7* mRNA (FDR=5.20 × 10⁻¹¹ in *SF3B1*mut MDS vs healthy control individuals and FDR=0 in *SF3B1*mut MDS vs SFwt MDS) and significant *ABCB7* downregulation in *SF3B1*mut cases (adjusted $P = 1.48 \times 10^{-12}$ and fold change = −2.4 in *SF3B1*mut MDS vs healthy control individuals, and adjusted $P = 3.65 \times 10^{-31}$ and fold change = −2.23 in *SF3B1*mut MDS vs SFwt MDS).

Aberrantly spliced genes involved in *R*-loop formation and DNA repair in SFmut MDS

Dysregulation of splicing factor genes has been associated with the formation of R-loops (structures resulting from the invasion of nascent RNA into DNA) and activation of the DNA damage response (DDR).[50-52] Several significant GOs dysregulated in SFmut MDS in our study have been associated with increased R-loop formation, including mitochondrial dysfunction,[53,54] aberrant RNA processing,[52,55,56] and defective ribonucleoprotein particle biogenesis.[57] Of 41 genes described in the literature as involved in suppression/regulation of R-loop formation and in the DDR,[58-63] we found aberrant splicing of 4 genes (*ERCC3*, *FANCI*, *SETX*, and *ATR*) in *SF3B1*mut cases, 3 genes (*CHEK1*, *SETX*, and *ATR*) in *SRSF2*mut cases, and 2 genes (*ERCC8* and *FANCM*) in *U2AF1*mut cases (supplemental Table 8).

Properties of dysregulated splice sites

We analyzed various properties of the major misregulated splicing events. *SF3B1*mut-misregulated A3SSs showed varying sequence profiles between canonical and cryptic sites (supplemental Figure 5A) and the characteristic 16 nt peak separation of upstream cryptic sites from their associated canonical sites (supplemental Figure 5B).[34,64-66] Exons downregulated in association with *U2AF1* mutations in the ZF1 and ZF2 domains (S34 and R156/Q157, respectively) showed altered 3′ splice site (3SS) logos (supplemental Figure 5C) consistent with contact by ZF1 and ZF2 at the −3 and +1 positions.[35] *SRSF2*mut-misregulated exons showed no alterations in splice site compositions (supplemental Figure 5D), but consistent with previous observations,[24] CCNG motifs were enriched in upregulated cassette exons and GGNG motifs in downregulated exons (supplemental Figure 5E).

Retained introns were the most common aberrant event in *SF3B1*mut cells, with the majority showing decreased retention (ie, increased splicing) in *SF3B1*mut MDS (supplemental Figure 6A). We observed that among the most affected RI events, a number used 3SSs that were also annotated as the upstream of a pair of A3SSs (supplemental Figure 6B-C). For example, *ERCC3* showed reduced retention of intron 10 in conjunction with use of an A3SS 18 nt upstream of the canonical 3SS, with the change in intron retention exceeding the change in A3SS use (supplemental Figure 6B). *DOM3Z* showed reduced intron retention using an upstream A3SS, although in this case there was no observed switching in A3SS use (supplemental Figure 6C). This novel observation suggests that reduced intron retention might be linked to the ability of *SF3B1* mutants to select an upstream A3SS.
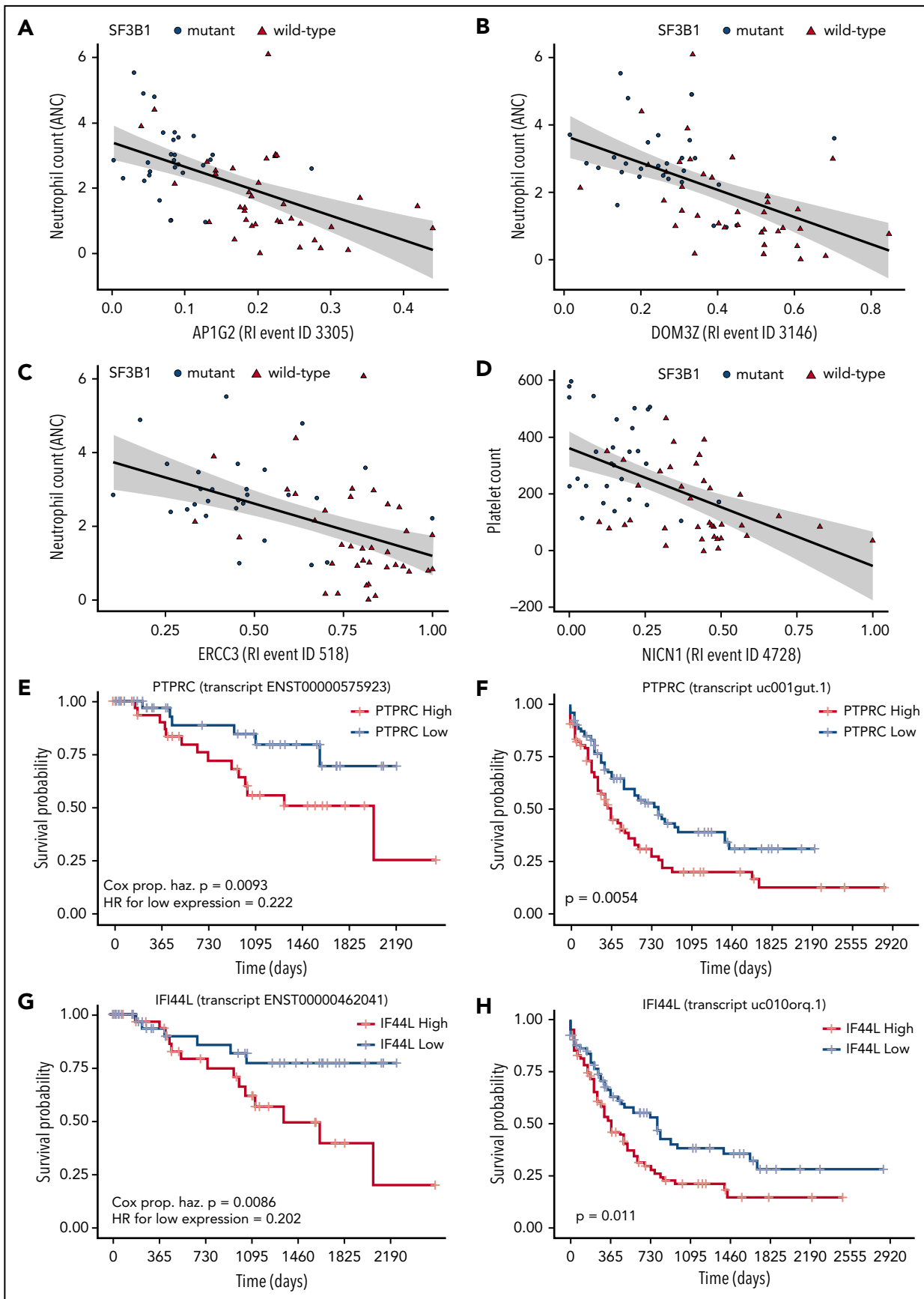
Figure 3.

Table 2. Genes with isoforms that significantly predicted survival in MDS in multivariate models

Gene	Associated splice factor mutation or mutations	Function/pathway	P (multivariate survival)
CAP1	SRSF2	Focal adhesion & extracellular exosomes	.0044
PTPRC	U2AF1 (S34)	Focal adhesion & extracellular exosomes	.0093
IFI44	SRSF2, U2AF1 (S34)	Interferon, extracellular exosomes	.012
IFI44L	U2AF1 (S34)	Interferon, extracellular exosomes	.0086
CD46	U2AF1 (S34)	Focal adhesion & extracellular exosomes	.039
CRTC2	SF3B1	Extracellular exosomes	.035
FCGR2A	U2AF1 (S34)	Extracellular exosomes	.016
PPOX	SF3B1, U2AF1 (R156/Q157)	Heme biosynthesis	.031
AHSA2	SF3B1	HSP90 ATPase	.029
DHP5	SF3B1	Translation elongation factor 2 modification	.026
MECR	U2AF1 (S34 & R156/Q157)	Mitochondrial reductase	.022
NASP	U2AF1 (S34)	HSP90 binding	.014
PFDN5	U2AF1 (R156/Q157)	Prefoldin subunit	.042
PABPC4	U2AF1 (R156/Q157)	NMD mRNA decay	.036

Associations between aberrant splicing and clinical variables or patient survival in MDS

We investigated the correlations between aberrantly spliced events and clinical variables. Clinical variables showing a significant difference between SFmut and SFwt patients with MDS were lower percentage of BM blasts and higher number of white blood cells, ANC, and Plt in the *SF3B1*mut group (supplemental Table 9). Fifteen aberrant splicing events, all identified in *SF3B1*mut MDS, were significantly correlated with a clinical variable (BM blasts, Plt, or ANC) (Table 1; Figure 3A-D). These include RI events of *AP1G2*, *DOM3Z*, and *ERCC3* with ANC, and a RI event of *NICN1* with Plt, indicating increased aberrant splicing of these genes in MDS cases with higher ANC and higher platelets, respectively.

We investigated the association of expression of isoforms produced by aberrant splicing events identified earlier with patient survival. Isoforms arising from significantly differentially regulated splicing events between SFmut and both SFwt patients with MDS and healthy control individuals were identified. Multivariate Cox proportional hazard modeling of survival of patients stratified by median expression of individual isoforms identified 14 genes with isoforms that significantly predicted survival in MDS (Table 2; Figure 3E,G; supplemental Table 10; supplemental Figure 7). In all cases, the isoforms that predicted survival were ones that would be generated because of the

aberrant splicing event identified. The affected isoforms showed striking enrichment (7/14) in genes involved in the formation of extracellular exosomes and focal cellular adhesion. Isoforms from the genes (*CRTC2*, *CAP1*, *IFI44*, *IFI44L*, *CD46*, *FCGR2A*, and *PTPRC*) from these pathways were investigated further using the AML data from the Cancer Genome Atlas. Patients were stratified by median expression of individual isoforms of the genes identified in the MDS cohort. Of the 7 extracellular exosome/focal adhesion genes identified as significant survival predictors in MDS, 5 genes (*CAP1*, *IFI44*, *IFI44L*, *PTPRC*, *FCGR2A*) also expressed isoforms that were significantly predictive of survival in the AML Cancer Genome Atlas cohort (Figure 3F,H).

Aberrant pre-mRNA splicing in CD34⁺ cells of MDS cases with *ZRSR2* mutations

ZRSR2 is another recurrently mutated splicing factor gene in MDS.[7,8] We performed RNA-seq on CD34⁺ cells from 2 MDS cases with *ZRSR2* mutations to determine the aberrant splicing events in these samples. As these 2 *ZRSR2* mutant cases also harbored a *SRSF2* mutation, we compared them with *SRSF2* mutant cases (without *ZRSR2* mutations) and with healthy control individuals to identify the aberrant splicing events that can be attributed to the presence of the *ZRSR2* mutations. Using rMATS, a total of 137 misregulated splicing events were identified, including 11 A3SS, 6 A5SS, 51 RI, 63 SE, and 6 MXE events (supplemental Figure 8A; supplemental Data 7). Pathway analysis

Figure 3. Associations between aberrant splicing and clinical variables or patient survival. (A-C) Scatterplots of aberrant splicing values in *AP1G2* (A), *DOM3Z* (B), and *ERCC3* (C) and neutrophil counts (ANC) in patients with MDS. (D) Scatterplot of aberrant splicing values in *NICN1* and platelet counts in patients with MDS. (E-H) Kaplan-Meier survival plots for individual isoforms of *PTPRC* and *IFI44L* in our MDS cohort (E and G, respectively), and the Cancer Genome Atlas AML cohort (F and H, respectively).
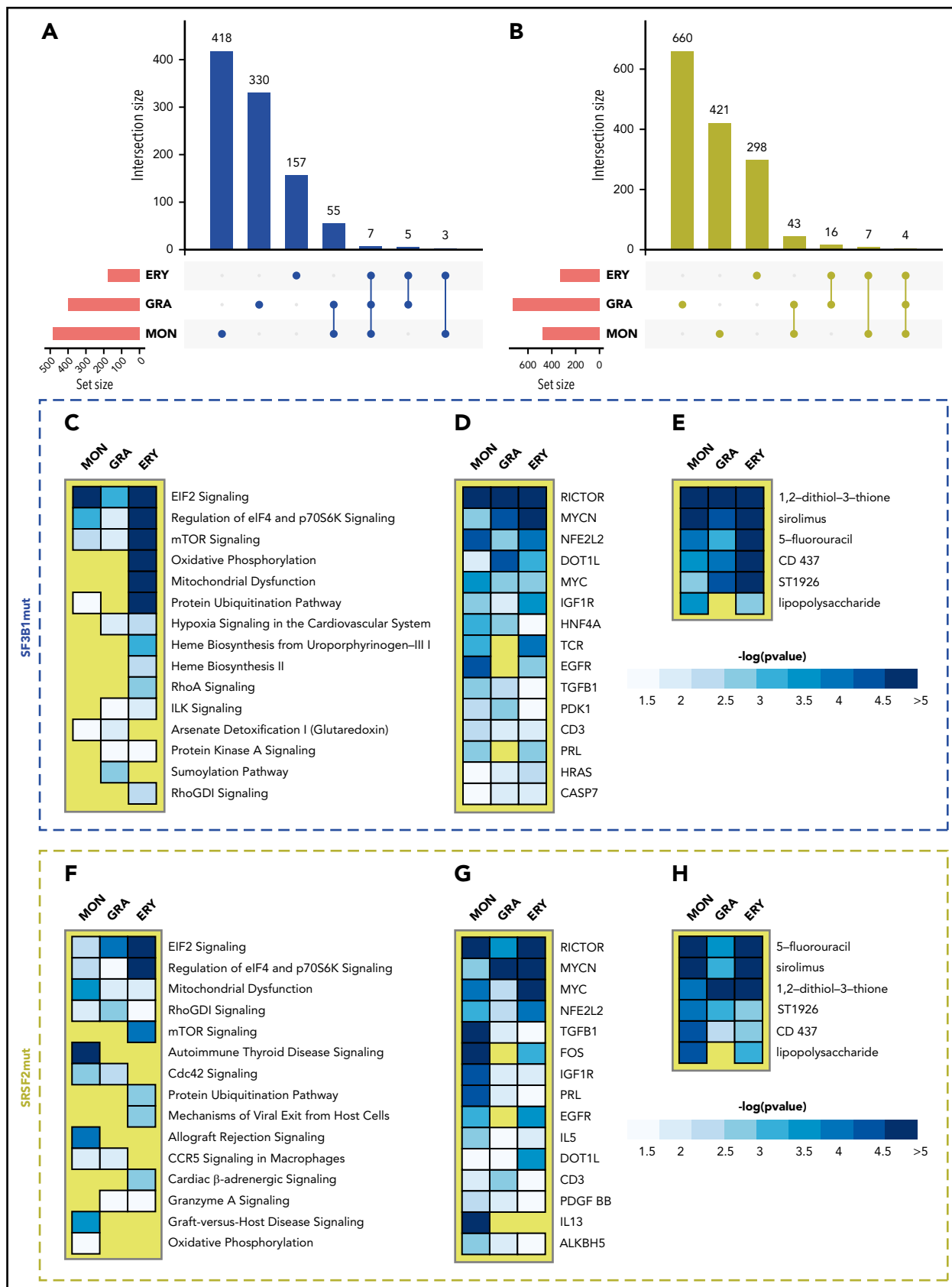
Figure 4. Aberrant splicing in BM cell populations of SFmut MDS. (A-B) UpSet plots showing the overlap of aberrant splicing events identified in monocyte (MON), granulocyte (GRA), and erythroid (ERY) precursor cell populations isolated from *SF3B1* (A) and *SRSF2* (B) mutant MDS patient samples. (C-H) Ranked heat maps showing the top 15 dysregulated pathways (C,F), top 15 transcriptional regulators (D,G), and top 6 drug/chemical gene sets (E,H) in MON, GRA, and ERY populations of *SF3B1* mutant and *SRSF2* mutant patients with MDS. Only heat map tiles with a -log10pvalue > 1.3 (*P* value < .05) are shown. Within each heat map, dysregulated pathways, transcriptional regulators and drug/chemical names are ranked by the IPA ranking score.

PELLAGATTI et al

performed on the genes showing significant aberrant splicing events showed dysregulation of pathways including sirtuin signaling and protein ubiquitination (supplemental Figure 8B; supplemental Table 11).

Aberrant splicing in BM cell populations of SFmut MDS

We performed RNA-seq on the granulocytic, monocytic, and erythroid precursors purified from BM of *SF3B1*mut and *SRSF2*mut patients with MDS and healthy control individuals (supplemental Table 3). Principal component analysis showed clustering of all samples by cell type (supplemental Figure 9).

rMATS analysis identified many unique aberrant splicing events in each cell population for *SF3B1*mut or *SRSF2*mut samples compared with healthy control individuals, with a limited overlap between cell populations (Figure 4A-B; supplemental Data 8). This suggests that *SF3B1* and *SRSF2* mutations affect splicing in a hematopoietic cell type-specific manner. Pathway analysis of the genes showing significant aberrant splicing events showed that 2 pathways involved in translation, EIF2 signaling and regulation of eIF4 and p70S6K signaling, were significantly dysregulated in all cell populations in both *SF3B1*mut and *SRSF2*mut MDS (Figure 4C,F). The analysis of upstream transcriptional regulators and drugs and chemicals is shown in Figure 4D-E,G-H for *SF3B1*mut and *SRSF2*mut MDS, respectively.

We analyzed panels of 120, 164, and 85 genes described in the literature as involved in erythroid,[67] granulocyte[68] and monocyte[69] function (supplemental Data 9). Aberrantly spliced genes include *GYPB* and *HMBS* in the erythroid precursors of *SF3B1*mut and *SRSF2*mut MDS cases, and *CSF1R* and *CSF3R*, respectively, in the monocyte and granulocyte precursors of *SRSF2*mut cases.

We determined the overlap between the aberrant splicing events identified in the granulocytic, monocytic, and erythroid precursors of *SF3B1*mut and *SRSF2*mut patients with MDS, and the aberrant splicing events identified in the CD34⁺ cells of *SF3B1*mut and *SRSF2*mut patients with MDS. Many aberrant splicing events were common between CD34⁺ cells and 1 or more of the granulocytic, monocytic, and erythroid precursor populations for *SF3B1* mutations or *SRSF2* mutations (supplemental Figure 10). Three genes (*SEPT2*, *DDX24*, and *DYNLL1*) and 1 gene (*RIMKLB*) were aberrantly spliced in common to all 4 cell populations for *SF3B1* mutations or *SRSF2* mutations, respectively.

Functional effects of splicing aberrations associated with splicing factor mutations

We validated selected aberrant splicing events identified in SFmut MDS using RT-PCR (supplemental Figure 11A-J). Of these, the *AKAP8* SE and *SEPT2* A3SS events identified generate premature termination codons predicted to trigger NMD and were associated with significant downregulation in *SRSF2*mut and *SF3B1*mut MDS, respectively (supplemental Figure 11B,F). *AKAP8* and *SEPT2* play important roles in the regulation of mitosis and cell growth.[70,71] Cell growth is dysregulated in MDS,[1-4] and thus we selected *AKAP8* and *SEPT2* for functional studies.

Patients with MDS suffer from anemia.[1] The effect of the splicing abnormalities of *AKAP8* and *SEPT2* on erythroid cell growth and differentiation was studied by knocking down these genes individually in human BM CD34⁺ cells subsequently differentiated toward the erythroid lineage. Erythroid cells with *AKAP8* or *SEPT2* knockdown showed significantly impaired growth and G1/S transition arrest compared with the scramble control (Figure 5A-C,H-J). Knockdown of these genes resulted in a significant decrease in the intermediate erythroid cell population (CD71⁺CD235a⁺ and CD36⁺CD235a⁺) on day 11 (Figure 5D-E, K-L) and the late erythroid cell population (CD71⁻CD235a⁺) on day 14 of culture (Figure 5F,M), and in a significant reduction in the number of BFU-E and CFU-E in colony-forming cell assays (Figure 5G,N), compared with the scramble control.

Discussion

The processes and pathways by which splicing factor mutations exert their effects are not established in the stem/progenitor cells and erythroid and myeloid precursors in MDS.

The analysis of a large dataset allowed us for the first time to compare and contrast the effects of different mutated splicing factor genes in MDS CD34⁺ cells. Many significant aberrant splicing events associated with *SF3B1*, *SRSF2*, and *U2AF1* mutations were identified, including A3SS, RI, and SE. These events may alter isoform expression and affect protein levels and function: some A3SS can result in a frameshift leading to stop codon generation and gene downregulation via NMD; RI events can lead to NMD or to an increase in mRNA stability;[72] and SE events result in alternative isoform generation, often not expected to alter gene expression levels.[6,24,35,50,73] We present the first evidence of a common mechanistic pathway underlying retention of intron sequences and the use of A3SSs in cells harboring *SF3B1* mutations. This event occurred, for example, in *ERCC3*, encoding the XPB component of TFIIH, which has roles as a transcription factor and in DNA repair. Loss of XPB activity contributes to R-loop-mediated DNA damage.[62] Use of the upstream A3SS in the upregulated intron would insert 6 amino acids between the helicase and ResIII domains and may affect protein function.

The large majority of aberrantly spliced genes associated with each mutated splicing factor were different, although some overlap was observed. Aberrant splicing of 5 genes was observed in *SF3B1*mut, *SRSF2*mut, and *U2AF1*mut MDS, and thus represent common targets. Of these, *LUC7L* and *SUGP2* encode proteins involved in splicing,[74,75] with *LUC7L* associated with the U1 snRNP splicing complex involved in 5′ splice site selection.[75] Dysregulation of the *MRRF* gene, involved in mitochondrial translational, leads to changes in mitochondrial morphology and function.[76]

Several biological processes were significantly affected in all 3 SFmut MDS groups, including RNA splicing, RNA processing, mRNA translation, and mitochondrial translation. Thus, splicing factor mutations affect cellular processes that are fundamental for the flow of information from the genome to proteins. Tightly controlled protein synthesis is critical for HSC (and progenitor) function and serves as a tumor suppressive mechanism,[77] implicating the aberrant splicing of multiple translation-related genes in SFmut MDS in disease pathogenesis.

Several commonly dysregulated pathways were associated with all 3 mutated splicing factors, some of which are relevant to the
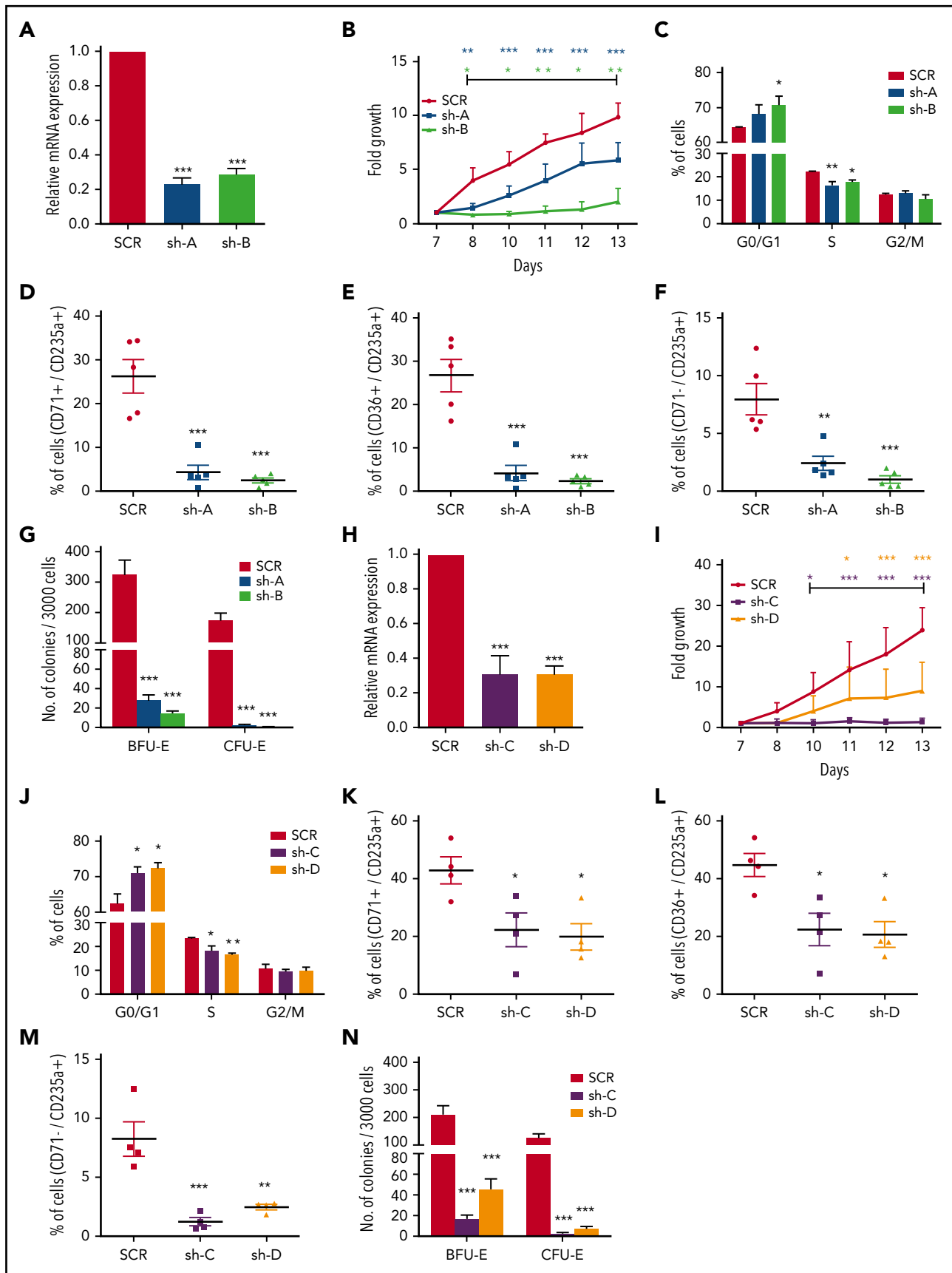
Figure 5.

known MDS pathophysiology, including mitochondrial dysfunction, oxidative phosphorylation, and heme biosynthesis, linking aberrant splicing with impaired mitochondrial function in MDS. Mitochondrial dysfunction is a well-recognized feature of MDS,[78,79] and mice with mitochondrial dysfunction develop some MDS features including macrocytic anemia.[80] The most significant dysregulated pathway across the 3 mutated splicing factors was sirtuin signaling, a pathway not previously associated with MDS. Sirtuins are histone deacetylases, some of which reside in the mitochondria,[81] with diverse roles in regulating metabolism, inflammation, genome stability, and cell proliferation, and have been implicated in aging, cancer and survival.[82,83]

Dysregulation of splicing factors leads to R-loop formation with associated genomic instability, resulting in activation of the DDR.[50-52] Elevated R-loops have been shown in Srsf2(P95H) mice,[51] and SRSF2 is involved in maintaining genomic stability.[51,52] Thus, R-loop formation and activation of the DDR are tightly linked cellular processes, and interestingly, regulators of these processes show aberrant splicing in our study. Biological processes associated with DDR and cell cycle regulation were significantly affected in both SF3B1mut and SRSF2mut MDS. SF3B1, SRSF2, and other splicing factors are components of a DNA damage-induced mRNA splicing complex,[84] linking splicing factors to the DDR. Several genes regulated by this complex, including LIG1 and BRCC3,[85,86] showed aberrant splicing in SFmut cases in our study. Several genes involved in the suppression/regulation of R-loop formation were aberrantly spliced in SFmut MDS in our study, including SETX and ATR.[87-89] SETX resolves R-loops[89] and its loss leads to aberrant R-loop accumulation.[87] Aberrant splicing of R-loop-related genes may contribute to increased formation of R-loops in SFmut MDS. This might be expected to result in an increased mutation frequency and explain in part the clonal advantage of SFmut MDS HSCs.

Dysregulation of cell growth is a feature of MDS.[1] We found that genes regulated by the transcriptional regulators HNF4A, RICTOR, E2F1, MYC, MYCN, and RB1, all major controllers of cell growth/cell cycle,[90-93] showed significant enrichment for aberrantly spliced genes. HNF4A inhibition promotes tumorigenesis in solid cancers.[94] Our data suggest for the first time a role of HNF4A in SFmut MDS disease pathophysiology.

Taken together, these data demonstrate that splicing factor mutations in MDS result in different mechanistic alterations in splicing and largely affect different genes, but these converge in overlapping (approximately one third of the total number) dysregulated pathways and cellular processes, suggesting common mechanisms of action. Many of the dysregulated pathways and cellular processes identified can be linked to the known disease pathophysiology and to the phenotypes associated with splicing factor mutations in MDS, whereas several have not previously been associated with MDS.

The association of aberrantly spliced isoforms with clinical parameters and with patient survival in MDS is an important aspect that has not been previously investigated. We identified several aberrant splicing events associated with clinical features for SF3B1mut MDS, demonstrating that specific splicing events may contribute directly to aspects of the disease phenotype. The first evidence that disparate splicing mutations in MDS may affect survival via effects on a common pathway involving focal adhesion and extracellular exosomes is presented here. We showed that the isoform changes that are induced in MDS significantly affect patient survival in multivariate models, and the same genes significantly affect survival in AML. Focal adhesion and extracellular exosomes play a role in cancer and leukemia,[95,96] and our data implicate their dysregulation as drivers of poor survival in both MDS and AML.

The effect of splicing factor mutations remains largely unexplored in the different lineages affected in MDS,[43] and we showed aberrant splicing of important genes for erythroid, granulocyte, and monocyte function in the respective cell population in SFmut MDS. For example, we have shown aberrant splicing of CSF3R, a critical regulator of granulopoiesis,[97] in SRSF2mut granulocyte precursors, and of CSF1R, a controller of macrophage production and function,[98] in SRSF2mut monocyte/macrophage precursors. Pathways playing critical roles in translational regulation were significantly dysregulated in all cell populations in both SF3B1mut and SRSF2mut MDS, suggesting that this process may be impaired in the erythroid and myeloid precursors of SFmut MDS.

To date, very few of the aberrantly spliced isoforms identified in SFmut malignancies have been functionally characterized.[6] We identified aberrant splicing of the mitosis regulators SEPT2[99,100] and AKAP8,[71] leading to their downregulation in the CD34+ cells of SF3B1mut and SRSF2mut MDS, respectively. Anemia is a hallmark of MDS,[1] and we found that knockdown of SEPT2 or AKAP8 in human hematopoietic progenitors resulted in markedly impaired erythroid cell growth and differentiation. These results suggest that aberrant splicing of SEPT2 or AKAP8 may lead to impaired erythropoiesis in association with SF3B1 and SRSF2 mutations in MDS.

This comprehensive study provides novel insights into SFmut MDS disease pathophysiology, with newly identified clinical associations, and dysregulated genes and pathways representing potential new therapeutic targets.

Figure 5. Functional effects of AKAP8 and SEPT2 knockdown on erythroid differentiation. (A,H) Real-time quantitative PCR showing the mRNA knockdown of AKAP8 (A) and SEPT2 (H) in erythroid cells. (B,I) Growth curves for erythroid cells with knockdown of AKAP8 (B) and SEPT2 (I). (C,J) Cell cycle analysis of erythroid cells with knockdown of AKAP8 (C) and SEPT2 (J) on day 11 of culture. (D-F and K-M) Flow cytometry quantification of erythroid differentiation. (D and K) Percentage of CD71+CD235a+ cells in erythroid cultures with knockdown of AKAP8 (D) and SEPT2 (K) on day 11. (E and L) Percentage of CD36+CD235a+ cells in erythroid cultures with knockdown of AKAP8 (E) and SEPT2 (L) on day 11. (F and M) Percentage of CD71−CD235a+ cells in erythroid cultures with knockdown AKAP8 (F) and SEPT2 (M) on day 14. (G and N) Number of BFU-E and CFU-E obtained from CD34+ progenitors with knockdown of AKAP8 (G) and SEPT2 (N) after 14 days in methylcellulose (colony-forming cell assays). Results shown in A-G were obtained from 5 independent experiments, except for C (3 replicates). Results shown in H-N were obtained from 4 independent experiments. Data represent the mean ± SEM. All P-values were obtained by 1-way ANOVA with Bonferroni's posttest with the exception of G and N, in which 2-way ANOVA with Bonferroni's posttest was used. *P < .05; **P < .01; ***P < .001.

Acknowledgments

Authorship

Contribution: A.P., R.N.A., V.S., S. Singh, H.D., and S.R. performed experiments; A.P., A.R., P.H., A.G., P.V., S.K., L.M., M.L.H., A.-C.G., A.D.H., T.L., E.H.-L., M.C., and J. Boultwood provided essential patient samples and clinical data; A.P., R.N.A., V.S., E.S., E.R., A. Sanchi, J. Broxholme, H.L., S.T., A. Schuh, A.H., E.P., C.W.J.S., S. Smith, and J. Boultwood analyzed data; A.P., C.W.J.S., S. Smith, and J. Boultwood designed the research; A.P., R.N.A., V.S., S. Singh, C.W.J.S., S. Smith, and J. Boultwood wrote the paper; and all authors approved the manuscript and the submission.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

ORCID profiles: A.P., 0000-0002-6122-0221; R.N.A., 0000-0001-7744-5890; J.B., 0000-0002-4330-2928.

Correspondence: Jacqueline Boultwood, Bloodwise Molecular Haematology Unit, Nuffield Division of Clinical Laboratory Sciences, Radcliffe Department of Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom; e-mail: jacqueline.boultwood@ndcls.ox.ac.uk.

Footnotes

REFERENCES

1. Heaney ML, Golde DW. Myelodysplasia. *N Engl J Med*. 1999;340(21):1649-1660.

2. Pellagatti A, Boultwood J. The molecular pathogenesis of the myelodysplastic syndromes. *Eur J Haematol*. 2015;95(1):3-15.

3. Sperling AS, Gibson CJ, Ebert BL. The genetics of myelodysplastic syndrome: from clonal haematopoiesis to secondary leukaemia. *Nat Rev Cancer*. 2017;17(1):5-19.

4. Tefferi A, Vardiman JW. Myelodysplastic syndromes. *N Engl J Med*. 2009;361(19):1872-1885.

5. Woll PS, Kjällquist U, Chowdhury O, et al. Myelodysplastic syndromes are propagated by rare and distinct human cancer stem cells in vivo [published correction appears in *Cancer Cell*. 2014;25(6):861]. *Cancer Cell*. 2014;25(6):794-808.

6. Yip BH, Dolatshad H, Roy S, Pellagatti A, Boultwood J. Impact of splicing factor mutations on pre-mRNA splicing in the myelodysplastic syndromes. *Curr Pharm Des*. 2016;22(16):2333-2344.

7. Haferlach T, Nagata Y, Grossmann V, et al. Landscape of genetic lesions in 944 patients with myelodysplastic syndromes. *Leukemia*. 2014;28(2):241-247.

8. Papaemmanuil E, Gerstung M, Malcovati L, et al; Chronic Myeloid Disorders Working Group of the International Cancer Genome Consortium. Clinical and biological implications of driver mutations in myelodysplastic syndromes. *Blood*. 2013;122(22):3616-3627.

9. Yoshida K, Sanada M, Shiraishi Y, et al. Frequent pathway mutations of splicing machinery in myelodysplasia. *Nature*. 2011;478(7367):64-69.

10. Gao K, Masuda A, Matsuura T, Ohno K. Human branch point consensus sequence is yUnAy. *Nucleic Acids Res*. 2008;36(7):2257-2267.

11. Chen M, Manley JL. Mechanisms of alternative splicing regulation: insights from molecular and genomics approaches. *Nat Rev Mol Cell Biol*. 2009;10(11):741-754.

12. Liu HX, Chew SL, Cartegni L, Zhang MQ, Krainer AR. Exonic splicing enhancer motif recognized by human SC35 under splicing conditions. *Mol Cell Biol*. 2000;20(3):1063-1071.

13. Wu S, Romfo CM, Nilsen TW, Green MR. Functional recognition of the 3′ splice site AG by the splicing factor U2AF35. *Nature*. 1999;402(6763):832-835.

14. Damm F, Kosmider O, Gelsi-Boyer V, et al; Groupe Francophone des Myélodysplasies. Mutations affecting mRNA splicing define distinct clinical phenotypes and correlate with patient outcome in myelodysplastic syndromes. *Blood*. 2012;119(14):3211-3218.

15. Makishima H, Visconte V, Sakaguchi H, et al. Mutations in the spliceosome machinery, a novel and ubiquitous pathway in leukemogenesis. *Blood*. 2012;119(14):3203-3210.

16. Malcovati L, Papaemmanuil E, Bowen DT, et al; Chronic Myeloid Disorders Working Group of the International Cancer Genome Consortium and of the Associazione Italiana per la Ricerca sul Cancro Gruppo Italiano Malattie Mieloproliferative. Clinical significance of SF3B1 mutations in myelodysplastic syndromes and myelodysplastic/myeloproliferative neoplasms. *Blood*. 2011;118(24):6239-6246.

17. Patnaik MM, Lasho TL, Hodnefield JM, et al. SF3B1 mutations are prevalent in myelodysplastic syndromes with ring sideroblasts but do not hold independent prognostic value. *Blood*. 2012;119(2):569-572.

18. Papaemmanuil E, Cazzola M, Boultwood J, et al; Chronic Myeloid Disorders Working Group of the International Cancer Genome Consortium. Somatic SF3B1 mutation in myelodysplasia with ring sideroblasts. *N Engl J Med*. 2011;365(15):1384-1395.

19. Mossner M, Jann JC, Wittig J, et al. Mutational hierarchies in myelodysplastic syndromes dynamically adapt and evolve upon therapy response and failure. *Blood*. 2016;128(9):1246-1259.

20. Pellagatti A, Roy S, Di Genua C, et al. Targeted resequencing analysis of 31 genes commonly mutated in myeloid disorders in serial samples from myelodysplastic syndrome patients showing disease progression. *Leukemia*. 2016;30(1):247-250.

21. Xie M, Lu C, Wang J, et al. Age-related mutations associated with clonal hematopoietic expansion and malignancies. *Nat Med*. 2014;20(12):1472-1478.

22. Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med*. 2014;371(26):2488-2498.

23. Genovese G, Kähler AK, Handsaker RE, et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *N Engl J Med*. 2014;371(26):2477-2487.

24. Kim E, Ilagan JO, Liang Y, et al. SRSF2 mutations contribute to myelodysplasia by mutant-specific effects on exon recognition. *Cancer Cell*. 2015;27(5):617-630.

25. Kon A, Yamazaki S, Nannya Y, et al. Physiological *Srsf2* P95H expression causes impaired hematopoietic stem cell functions and aberrant RNA splicing in mice. *Blood*. 2018;131(6):621-635.

26. Mupo A, Seiler M, Sathiaseelan V, et al. Hemopoietic-specific Sf3b1-K700E knock-in mice display the splicing defect seen in human MDS but develop anemia without ring sideroblasts. *Leukemia*. 2017;31(3):720-727.

27. Obeng EA, Chappell RJ, Seiler M, et al. Physiologic expression of Sf3b1(K700E) causes impaired erythropoiesis, aberrant splicing, and sensitivity to therapeutic spliceosome modulation. *Cancer Cell*. 2016; 30(3):404-417.

28. Shirai CL, Ley JN, White BS, et al. Mutant U2AF1 expression alters hematopoiesis and pre-mRNA splicing in vivo. *Cancer Cell*. 2015;27(5):631-643.

29. Biankin AV, Waddell N, Kassahn KS, et al; Australian Pancreatic Cancer Genome Initiative. Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. *Nature*. 2012;491(7424):399-405.

30. Furney SJ, Pedersen M, Gentien D, et al. SF3B1 mutations are associated with alternative splicing in uveal melanoma. *Cancer Discov*. 2013;3(10):1122-1129.

31. Scott LM, Rebel VI. Acquired mutations that affect pre-mRNA splicing in hematologic malignancies and solid tumors. *J Natl Cancer Inst*. 2013;105(20):1540-1549.

32. Ferreira PG, Jares P, Rico D, et al. Transcriptome characterization by RNA sequencing identifies a major molecular and clinical subdivision in chronic lymphocytic leukemia. *Genome Res*. 2014;24(2):212-226.

33. Maguire SL, Leonidou A, Wai P, et al. SF3B1 mutations constitute a novel therapeutic target in breast cancer. *J Pathol*. 2015;235(4): 571-580.

34. Dolatshad H, Pellagatti A, Liberante FG, et al. Cryptic splicing events in the iron transporter ABCB7 and other key target genes in SF3B1-mutant myelodysplastic syndromes. *Leukemia*. 2016;30(12):2322-2331.

35. Ilagan JO, Ramakrishnan A, Hayes B, et al. U2AF1 mutations alter splice site recognition in hematological malignancies [published correction appears in *Genome Res* 2015; 25(1):14-26]. *Genome Res*. 2015;25(1): 14-26.

36. Madan V, Kanojia D, Li J, et al. Aberrant splicing of U12-type introns is the hallmark of ZRSR2 mutant myelodysplastic syndrome. *Nat Commun*. 2015;6(1):6042.

37. Visconte V, Avishai N, Mahfouz R, et al. Distinct iron architecture in SF3B1-mutant myelodysplastic syndrome patients is linked to an SLC25A37 splice variant with a retained intron. *Leukemia*. 2015;29(1):188-195.

38. Visconte V, Rogers HJ, Singh J, et al. SF3B1 haploinsufficiency leads to formation of ring sideroblasts in myelodysplastic syndromes. *Blood*. 2012;120(16):3173-3186.

39. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. *Nat Methods*. 2015;12(4): 357-360.

40. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*. 2014;30(7): 923-930.

41. Liao Y, Smyth GK, Shi W. The Subread aligner: fast, accurate and scalable read mapping by seed-and-vote. *Nucleic Acids Res*. 2013;41(10):e108.

42. Shen S, Park JW, Lu ZX, et al. rMATS: robust and flexible detection of differential alternative splicing from replicate RNA-Seq data. *Proc Natl Acad Sci USA*. 2014;111(51): E5593-E5601.

43. Yip BH, Steeples V, Repapi E, et al. The U2AF1S34F mutation induces lineage-specific splicing alterations in myelodysplastic syndromes. *J Clin Invest*. 2017;127(6): 2206-2221.

44. Young MD, Wakefield MJ, Smyth GK, Oshlack A. Gene ontology analysis for RNA-seq: accounting for selection bias. *Genome Biol*. 2010;11(2):R14.

45. Yip BH, Pellagatti A, Vuppusetty C, et al. Effects of L-leucine in 5q- syndrome and other RPS14-deficient erythroblasts. *Leukemia*. 2012;26(9):2154-2158.

46. Basiorka AA, McGraw KL, Eksioglu EA, et al. The NLRP3 inflammasome functions as a driver of the myelodysplastic syndrome phenotype. *Blood*. 2016;128(25):2960-2975.

47. Kon A, Shih LY, Minamino M, et al. Recurrent mutations in multiple components of the cohesin complex in myeloid neoplasms. *Nat Genet*. 2013;45(10):1232-1237.

48. Thota S, Viny AD, Makishima H, et al. Genetic alterations of the cohesin complex genes in myeloid malignancies. *Blood*. 2014;124(11):1790-1798.

49. Ernst T, Chase AJ, Score J, et al. Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. *Nat Genet*. 2010;42(8):722-726.

50. Chabot B, Shkreta L. Defective control of pre-messenger RNA splicing in human disease. *J Cell Biol*. 2016;212(1):13-27.

51. Chen L, Chen JY, Huang YJ, et al. The augmented R-loop is a unifying mechanism for myelodysplastic syndromes induced by high-risk splicing factor mutations. *Mol Cell*. 2018;69(3):412-425.

52. Li X, Manley JL. Inactivation of the SR protein splicing factor ASF/SF2 results in genomic instability. *Cell*. 2005;122(3):365-378.

53. Brown TA, Tkachuk AN, Clayton DA. Native R-loops persist throughout the mouse mitochondrial DNA genome. *J Biol Chem*. 2008;283(52):36743-36751.

54. Akman G, Desai R, Bailey LJ, et al. Pathological ribonuclease H1 causes R-loop depletion and aberrant DNA segregation in mitochondria. *Proc Natl Acad Sci USA*. 2016; 113(30):E4276-E4285.

55. Paulsen RD, Soni DV, Wollman R, et al. A genome-wide siRNA screen reveals diverse cellular processes and pathways that mediate genome stability. *Mol Cell*. 2009;35(2): 228-239.

56. Wahba L, Amon JD, Koshland D, Vuica-Ross M. RNase H and multiple RNA biogenesis factors cooperate to prevent RNA:DNA hybrids from generating genome instability. *Mol Cell*. 2011;44(6):978-988.

57. Santos-Pereira JM, Aguilera A. R loops: new modulators of genome dynamics and function. *Nat Rev Genet*. 2015;16(10):583-597.

58. Eksioglu E, Chen X, Cheng P, et al. The S100A9/Fto axis induces formation of RNA: DNA hybrids that serve as damps initiating myeloid skewing & genetic injury in MDS [abstract]. *Blood*. 2017;130(Suppl 1):52.

59. García-Benítez F, Gaillard H, Aguilera A. Physical proximity of chromatin to nuclear pores prevents harmful R loop accumulation contributing to maintain genome stability. *Proc Natl Acad Sci USA*. 2017;114(41): 10942-10947.

60. Schwab RA, Nieminuszczy J, Shah F, et al. The Fanconi anemia pathway maintains genome stability by coordinating replication and transcription. *Mol Cell*. 2015;60(3): 351-361.

61. Sollier J, Cimprich KA. Breaking bad: R-loops and genome integrity. *Trends Cell Biol*. 2015;25(9):514-522.

62. Sollier J, Stork CT, García-Rubio ML, Paulsen RD, Aguilera A, Cimprich KA. Transcription-coupled nucleotide excision repair factors promote R-loop-induced genome instability. *Mol Cell*. 2014;56(6):777-785.

63. Wei S, Eksioglu EA, Chen X, et al. Inflammaging-associated metabolic alterations foster development of the MDS genotype [abstract]. *Blood*. 2015;126(23):144.

64. Alsafadi S, Houy A, Battistella A, et al. Cancer-associated SF3B1 mutations affect alternative splicing by promoting alternative branchpoint usage. *Nat Commun*. 2016;7: 10615.

65. Darman RB, Seiler M, Agrawal AA, et al. Cancer-associated SF3B1 hotspot mutations induce cryptic 3′ splice site selection through use of a different branch point. *Cell Reports*. 2015;13(5):1033-1045.

66. DeBoever C, Ghia EM, Shepard PJ, et al. Transcriptome sequencing reveals potential mechanism of cryptic 3′ splice site selection in SF3B1-mutated cancers. *PLOS Comput Biol*. 2015;11(3):e1004105.

67. Tan JS, Mohandas N, Conboy JG. High frequency of alternative first exons in erythroid genes suggests a critical role in regulating gene function. *Blood*. 2006;107(6): 2557-2561.

68. Naranbhai V, Fairfax BP, Makino S, et al. Genomic modulators of gene expression in human neutrophils. *Nat Commun*. 2015;6(1): 7545.

69. Gren ST, Rasmussen TB, Janciauskiene S, Håkansson K, Gerwien JG, Grip O. A single-cell gene-expression profile reveals inter-cellular heterogeneity within human monocyte subsets. *PLoS One*. 2015;10(12): e0144351.

70. Spiliotis ET, Kinoshita M, Nelson WJ. A mitotic septin scaffold required for Mammalian chromosome congression and segregation. *Science*. 2005;307(5716):1781-1785.

71. Bieluszewska A, Weglewska M, Bieluszewski T, Lesniewicz K, Poreba E. PKA-binding domain of AKAP8 is essential for direct interaction with DPY30 protein. *FEBS J*. 2018; 285(5):947-964.

72. Jacob AG, Smith CWJ. Intron retention as a component of regulated gene expression

programs. *Hum Genet.* 2017;136(9): 1043-1057.

73. Brooks AN, Choi PS, de Waal L, et al. A pan-cancer analysis of transcriptome changes associated with somatic mutations in U2AF1 reveals commonly altered splicing [published correction appears in *PLoS ONE* 9(4): e96437]. *PLoS One.* 2014; 9(1):e87361.

74. Sampson ND, Hewitt JE. SF4 and SFRS14, two related putative splicing factors on human chromosome 19p13.11. *Gene.* 2003; 305(1):91-100.

75. Tufarelli C, Frischauf AM, Hardison R, Flint J, Higgs DR. Characterization of a widely expressed gene (LUC7-LIKE; LUC7L) defining the centromeric boundary of the human alpha-globin domain. *Genomics.* 2001; 71(3):307-314.

76. Rorbach J, Richter R, Wessels HJ, et al. The human mitochondrial ribosome recycling factor is essential for cell viability. *Nucleic Acids Res.* 2008;36(18):5787-5799.

77. Signer RA, Magee JA, Salic A, Morrison SJ. Haematopoietic stem cells require a highly regulated protein synthesis rate. *Nature.* 2014;509(7498):49-54.

78. Malcovati L, Cazzola M. Recent advances in the understanding of myelodysplastic syndromes with ring sideroblasts. *Br J Haematol.* 2016;174(6):847-858.

79. Tehranchi R, Invernizzi R, Grandien A, et al. Aberrant mitochondrial iron distribution and maturation arrest characterize early erythroid precursors in low-risk myelodysplastic syndromes. *Blood.* 2005;106(1): 247-253.

80. Chen ML, Logan TD, Hochberg ML, et al. Erythroid dysplasia, megaloblastic anemia, and impaired lymphopoiesis arising from mitochondrial dysfunction. *Blood.* 2009; 114(19):4045-4053.

81. Carrico C, Meyer JG, He W, Gibson BW, Verdin E. The mitochondrial acylome

emerges: proteomics, regulation by sirtuins, and metabolic and disease implications. *Cell Metab.* 2018;27(3):497-512.

82. Roth M, Wang Z, Chen WY. Sirtuins in hematological aging and malignancy. *Crit Rev Oncog.* 2013;18(6):531-547.

83. Roth M, Chen WY. Sorting out functions of sirtuins in cancer. *Oncogene.* 2014;33(13): 1609-1620.

84. Savage KI, Gorski JJ, Barros EM, et al. Identification of a BRCA1-mRNA splicing complex required for efficient DNA repair and maintenance of genomic stability. *Mol Cell.* 2014;54(3):445-459.

85. Wang B, Elledge SJ. Ubc13/Rnf8 ubiquitin ligases control foci formation of the Rap80/Abraxas/Brca1/Brcc36 complex in response to DNA damage. *Proc Natl Acad Sci USA.* 2007;104(52):20759-20763.

86. Das-Bradoo S, Nguyen HD, Wood JL, Ricke RM, Haworth JC, Bielinsky AK. Defects in DNA ligase I trigger PCNA ubiquitylation at Lys 107. *Nat Cell Biol.* 2010; 12(1):74-79.

87. Becherel OJ, Yeo AJ, Stellati A, et al. Senataxin plays an essential role with DNA damage response proteins in meiotic recombination and gene silencing. *PLoS Genet.* 2013;9(4):e1003435.

88. Maréchal A, Zou L. DNA damage sensing by the ATM and ATR kinases. *Cold Spring Harb Perspect Biol.* 2013;5(9): a012716.

89. Skourti-Stathaki K, Proudfoot NJ, Gromak N. Human senataxin resolves RNA/DNA hybrids formed at transcriptional pause sites to promote Xrn2-dependent termination. *Mol Cell.* 2011;42(6):794-805.

90. Hallstrom TC, Mori S, Nevins JR. An E2F1-dependent gene expression program that determines the balance between proliferation and cell death. *Cancer Cell.* 2008; 13(1):11-22.

91. Huang R, Cheung NK, Vider J, et al. MYCN and MYC regulate tumor proliferation and tumorigenesis directly through BMI1 in human neuroblastomas. *FASEB J.* 2011;25(12): 4138-4149.

92. Indovina P, Pentimalli F, Casini N, Vocca I, Giordano A. RB1 dual role in proliferation and apoptosis: cell fate control and implications for cancer therapy. *Oncotarget.* 2015;6(20):17873-17890.

93. Jebali A, Dumaz N. The role of RICTOR downstream of receptor tyrosine kinase in cancers. *Mol Cancer.* 2018;17(1):39.

94. Walesky C, Apte U. Role of hepatocyte nuclear factor 4α (HNF4α) in cell proliferation and cancer. *Gene Expr.* 2015;16(3): 101-108.

95. Boyiadzis M, Whiteside TL. The emerging roles of tumor-derived exosomes in hematological malignancies. *Leukemia.* 2017; 31(6):1259-1268.

96. Maziveyi M, Alahari SK. Cell matrix adhesions in cancer: The proteins that form the glue. *Oncotarget.* 2017;8(29): 48471-48487.

97. Maxson JE, Tyner JW. Genomics of chronic neutrophilic leukemia. *Blood.* 2017;129(6): 715-722.

98. Bonifer C, Hume DA. The transcriptional regulation of the Colony-Stimulating Factor 1 Receptor (csf1r) gene during hematopoiesis. *Front Biosci.* 2008;13(13): 549-560.

99. Cerveira N, Correia C, Bizarro S, et al. SEPT2 is a new fusion partner of MLL in acute myeloid leukemia with t(2;11)(q37;q23). *Oncogene.* 2006;25(45):6147-6152.

100. Santos J, Cerveira N, Bizarro S, et al. Expression pattern of the septin gene family in acute myeloid leukemias with and without MLL-SEPT fusion genes. *Leuk Res.* 2010; 34(5):615-621.