

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

Short title: Aberrant splicing in splicing factor mutant MDS

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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.

Abstract

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 impact 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 MDS patients. 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, whilst several others have not been previously associated with MDS, such as sirtuin signaling. We identified aberrantly spliced events associated with clinical variables, and isoforms which 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 impact of the common spliceosome mutations on the MDS phenotype and provides novel insights into disease pathophysiology.

Introduction

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

Splicing factor gene mutations occur in >50% of all MDS patients, 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} whilst *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 ageing human BM. HSCs expressing splicing factor mutations show a compromised repopulation capacity in mice compared with wildtype 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 impact of *SF3B1*, *SRSF2* and *U2AF1* mutations on pre-mRNA splicing in purified BM stem and progenitor cells from a large cohort of MDS patients. The major cellular processes and dysregulated pathways were identified in the CD34⁺ cells and in precursors of MDS-affected lineages of SFmut MDS patients. We have determined the associations between aberrantly spliced isoforms and clinical variables and patient survival. Furthermore, we studied the functional impact 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 MDS patients (Table S1) and 8 healthy controls using MACS columns (Miltenyi Biotec, Germany). Of the 84 MDS cases, 28 were *SF3B1* mutant (*SF3B1*mut), eight *SRSF2* mutant (*SRSF2*mut), six *U2AF1* mutant (*U2AF1*mut), two *ZRSR2* mutant (with co-mutation of *SRSF2*) and 40 had no mutations in splicing factor genes (SFwt), as determined by targeted next-generation sequencing data⁸ (Table S1, Table S2, Figure S1A).

Granulocytic, monocytic and erythroid precursors were purified from the BM samples of 11 MDS patients (seven *SF3B1*mut and four *SRSF2*mut) and five healthy controls. 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 José, CA, USA), 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 >95%. Here, the mutational profile of the MDS patients was determined using a next-generation sequencing-based myeloid gene panel²⁰ (Table S3, Figure S1B).

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, UK) 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>) (Data Supplement 1). Differential splicing was assessed using rMATSV3.2.2beta^{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 rMATSV consistently in the comparisons of SFmut MDS both to SFwt MDS and to healthy controls (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 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, Hb; white blood cell counts, WBC; absolute neutrophil count, ANC; platelet count, Plt; BM blasts; and age) and a Kruskal-Wallis test for categorical variables (gender, International Prognostic Scoring System, IPSS; 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 transfect CD34⁺ cells from healthy donors as described previously.⁴³

Additional method details are described in the Supplementary 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 MDS patients and 8 healthy controls. 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 controls and with SFwt patients, identifying >200 misregulated splicing events for each splicing factor (Figure 1A-C, Data Supplement 2). *SF3B1* mutations were associated with a higher proportion of A3SS and RI events, while *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 controls (Figure 1G). Similarly, *SRSF2*mut cases clustered together (Figure 1H). Most *U2AF1* mutations affect the S34 and R156/Q157 codons within the two zinc finger (ZF) domains.⁹ All *U2AF1*mut cases clustered together, with the S34 mutants and the R156/Q157 mutants in two sub-clusters (Figure 1I).

Among the top 40 aberrant splicing events in *SF3B1*mut MDS (Table S4), 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 (Table S4) and *U2AF1*mut MDS (Table S4), 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 of 147, 132 and 181 genes were aberrantly spliced distinctly in association with *SF3B1*mut, *SRSF2*mut and *U2AF1*mut MDS cases, respectively (Data Supplement 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 (i.e. *STAG2*^{47,48}), or in *SRSF2*mut and *U2AF1*mut cases (i.e. *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- and *U2AF1*mut-associated aberrantly spliced genes (Data Supplement 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 (Figure S2A and 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 (Figure S2C). 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 to median log2 fold -0.03 for NMD-insensitive events), albeit this did not reach statistical significance (Figure S2D).

Gene ontology analysis of aberrantly spliced genes in SFmut MDS

We performed gene ontology (GO) analysis on the genes showing significant aberrant splicing events in SFmut MDS (Data Supplement 4). A marked convergence of significant GO themes was identified, with 74 GOs common to all SFmut MDS groups (Figure 2A, Data Supplement 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 (Figure S3A). 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 (Figure S3B).

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, Table S5-7). Interestingly, all three mutated splicing factors shared six common significant biological pathways, with the Sirtuin Signaling Pathway as the top ranking pathway (Figure 2B, Figure S4). 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, Table S5), while Hypoxia Signaling was dysregulated in *SRSF2*mut MDS (Figure 2B, Table S6).

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 three mutated splicing factors with several transcriptional regulators, including HNF4A, RICTOR, E2F1, MYC and RB1 (Figure 2C).

A similar approach identified drugs/chemicals that can impact 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 three 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 (Supplementary 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 (Data Supplement 6).

We have previously reported aberrant splicing of the iron transporter *ABCB7* in MDS RARS³⁴ and here we have found the same aberrant splicing event in the *ABCB7* mRNA (FDR=5.20x10⁻¹¹ in *SF3B1*mut MDS vs healthy controls and FDR=0 in *SF3B1*mut MDS vs SFwt MDS) and significant *ABCB7* downregulation in *SF3B1*mut cases (adjusted p=1.48x10⁻¹² and fold change = -2.4 in *SF3B1*mut MDS vs healthy controls, and adjusted p=3.65x10⁻³¹ 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 four genes (*ERCC3*, *FANCI*, *SETX* and *ATR*) in *SF3B1*mut cases, three genes (*CHEK1*, *SETX* and *ATR*) in *SRSF2*mut cases and two genes (*ERCC8* and *FANCM*) in *U2AF1*mut cases (Table S8).

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 (Figure S5A) and the characteristic 16 nt peak separation of upstream cryptic sites from their associated canonical sites (Figure S5B).^{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 (Figure S5C) 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 (Figure S5D) but, consistent with previous observations,²⁴ CCNG motifs were enriched in upregulated cassette exons and GGNG motifs in downregulated exons (Figure S5E).

Retained introns were the most common aberrant event in *SF3B1*mut cells, with the majority showing decreased retention (i.e. increased splicing) in *SF3B1*mut MDS (Figure S6A). 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 (Figure S6B-C). For example, *ERCC3* showed reduced retention of intron 10 in conjunction with use of an A3SS 18 nt upstream of the canonical 3'SS with the change in intron retention exceeding the change in A3SS use (Figure S6B). *DOM3Z* showed reduced intron retention using an upstream A3SS, although in this case there was no observed switching in A3SS use (Figure S6C). This novel observation suggests that reduced intron retention might be linked to the ability of *SF3B1* mutants to select an upstream A3SS.

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 MDS patients were lower percentage of BM blasts and higher number of WBC, ANC and Plt in the *SF3B1*mut group (Table S9). 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 examples 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 above with patient survival. Isoforms arising from significantly differentially regulated splicing events between SFmut and both SFwt MDS patients and healthy controls were identified. Multivariate Cox proportional hazard modeling of survival of patients stratified by median expression of individual isoforms identified 14 genes with isoforms which significantly predicted survival in MDS (Table 2, Figure 3E and G, Table S10, Figure S7). In all cases the isoforms which predicted survival were ones which would be generated due to 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 (TCGA). Patients were stratified by median expression of individual isoforms of the genes identified in the MDS cohort. Of the seven extracellular exosome/focal adhesion genes identified as significant survival predictors in MDS, five genes (*CAP1*, *IFI44*,

IFI44L, *PTPRC*, *FCGR2A*) also expressed isoforms which were significantly predictive of survival in the AML TCGA cohort (Figure 3F and 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 have performed RNA-seq on CD34⁺ cells from two MDS cases with *ZRSR2* mutations to determine the aberrant splicing events in these samples. As these two *ZRSR2* mutant cases also harbored a *SRSF2* mutation, we have compared them with *SRSF2* mutant cases (without *ZRSR2* mutations) and with healthy controls in order 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 (Figure S8A, Data Supplement 7). Pathway analysis performed on the genes showing significant aberrant splicing events showed dysregulation of pathways including Sirtuin Signaling and Protein Ubiquitination (Figure S8B, Table S11).

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 MDS patients and healthy controls (Table S3). Principal component analysis showed clustering of all samples by cell type (Figure S9).

rMATS analysis identified many unique aberrant splicing events in each cell population for *SF3B1*mut or *SRSF2*mut samples compared with healthy controls, with a limited overlap between cell populations (Figure 4A-B, Data Supplement 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 two 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 and F). The analysis of upstream transcriptional regulators and drugs and chemicals is shown in Figure 4D-E and 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 granulocyte⁶⁸ and monocyte⁶⁹ function (Data Supplement 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 have determined the overlap between the aberrant splicing events identified in the granulocytic, monocytic and erythroid precursors of *SF3B1*mut and *SRSF2*mut MDS patients, and the aberrant splicing events identified in the CD34⁺ cells of *SF3B1*mut and *SRSF2*mut MDS patients. Many aberrant splicing events were common between CD34⁺ cells and one or more of the granulocytic, monocytic and erythroid precursor populations for *SF3B1* mutations or *SRSF2* mutations (Figure S10). Three genes (*SEPT2*, *DDX24* and *DYNLL1*) and one gene (*RIMKLB*) were aberrantly spliced in common to all four 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 (Figure S11A-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 (Figure S11B and F). *AKAP8* and *SEPT2* play important roles in the regulation of mitosis and cell growth.^{70,71} Cell growth is dysregulated in MDS¹⁻⁴ and thus we selected *AKAP8* and *SEPT2* for functional studies.

MDS patients suffer from anemia.¹ The impact 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 towards the erythroid lineage. Erythroid cells with *AKAP8* or *SEPT2* knockdown showed significantly impaired growth and G1/S transition arrest compared to the scramble control (Figure 5A-C and 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 and K-L) and the late erythroid cell population (CD71⁻CD235a⁺) on day 14 of culture (Figure 5F

and M), and in a significant reduction in the number of BFU-E and CFU-E in colony-forming cell assays (Figure 5G and N), compared to 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 impact protein levels and function: some A3SS can result in a frameshift leading to stop codon generation and gene down-regulation 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 six 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 five 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 three SFmut MDS groups, including RNA splicing, RNA processing, mRNA translation, and mitochondrial translation. Thus splicing factor mutations impact cellular processes which 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 three mutated splicing factors, some of which are relevant to the 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 three 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, 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 DNA damage response (DDR).⁵⁰⁻⁵² Elevated R-loops occur 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 *SF3B1*mut and *SRSF2*mut 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-loops⁸⁹ 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, while 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 which has not been previously investigated. We identified several aberrant splicing events associated with clinical features for *SF3B1*mut 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 which are induced in MDS significantly impact on patient survival in multivariate models, and the same genes significantly impact on 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 impact of splicing factor mutations remain 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 *SRSF2*mut granulocyte precursors, and of *CSF1R*, a controller of macrophage production and function,⁹⁸ in *SRSF2*mut monocyte/macrophage precursors. Pathways playing critical roles in translational regulation were significantly dysregulated in

all cell populations in both *SF3B1*mut and *SRSF2*mut 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 *SF3B1*mut and *SRSF2*mut 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* leads 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.

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Authorship

Contribution: A.P., R.N.A., V.S., S. Singh, H.D., and S.R. performed experiments; A.P., 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. Boulwood 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. Boulwood analyzed data; A.P., C.W.J.S., S. Smith, and J. Boulwood designed the research; A.P., R.N.A., V.S., S. Singh, C.W.J.S., S. Smith, and J. Boulwood wrote the paper; and all authors approved the manuscript and the submission.

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

<i>Event ID</i>	<i>Gene</i>	<i>Event type</i>	<i>Chr</i>	<i>Strand</i>	<i>Start position</i>	<i>End Position</i>	<i>Spearman Correlation Variable</i>	<i>cor_estimate</i>	<i>pvalue</i>	<i>adj.p</i>
6795	PARVG	A3SS	22	+	44582456	44583758	BM blasts %	0.58	4.38E-07	0.003031
5420	RPRD1A	RI	18	-	33605560	33607038	BM blasts %	0.58	6.21E-07	0.004292
3146	DOM3Z	RI	6	-	31938382	31938924	ANC	-0.57	1.00E-06	0.006916
12280	CXXC1	SE	18	-	47811694	47811721	ANC	-0.56	1.28E-06	0.00888
3305	APIG2	RI	14	-	24031170	24031624	ANC	-0.56	1.68E-06	0.011579
2362	SNRPN	A3SS	15	+	25219434	25219603	ANC	-0.55	3.14E-06	0.021689
5460	TCEA2	A3SS	20	+	62703210	62703294	ANC	0.54	3.24E-06	0.022393
6627	NICN1	A3SS	3	-	49462381	49462579	Plt	-0.54	3.80E-06	0.026281
6153	ABCC5	A3SS	3	-	183703091	183703243	ANC	-0.54	4.23E-06	0.029229
518	ERCC3	RI	2	-	128046912	128047400	ANC	-0.54	4.71E-06	0.032551
2359	SNRPN	A3SS	15	+	25219457	25219603	ANC	-0.54	5.02E-06	0.03468
7563	PPOX	A3SS	1	+	161137128	161137276	ANC	0.53	5.99E-06	0.041411
4975	GPR108	A3SS	19	-	6730997	6731122	ANC	-0.53	6.43E-06	0.044443
5816	PSTPIP1	A3SS	15	+	77328142	77328276	ANC	-0.53	6.49E-06	0.044832
4728	NICN1	RI	3	-	49462381	49462871	Plt	-0.53	6.75E-06	0.046655

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

<i>Gene</i>	<i>Associated splice factor mutation(s)</i>	<i>Function/Pathway</i>	<i>p-value (multivariate survival)</i>
CAP1	SRSF2	Focal adhesion & extracellular exosomes	0.0044
PTPRC	U2AF1 (S34)	Focal adhesion & extracellular exosomes	0.0093
IFI44	SRSF2, U2AF1 (S34)	Interferon, extracellular exosomes	0.012
IFI44L	U2AF1 (S34)	Interferon, extracellular exosomes	0.0086
CD46	U2AF1 (S34)	Focal adhesion & extracellular exosomes	0.039
CRTC2	SF3B1	Extracellular exosomes	0.035
FCGR2A	U2AF1 (S34)	Extracellular exosomes	0.016
PPOX	SF3B1, U2AF1 (R156/Q157)	Heme biosynthesis	0.031
AHSA2	SF3B1	HSP90 ATPase	0.029
DHP5	SF3B1	Translation elongation factor 2 modification	0.026
MECR	U2AF1 (S34 & R156/Q157)	Mitochondrial reductase	0.022
NASP	U2AF1 (S34)	HSP90 binding	0.014
PFDN5	U2AF1 (R156/Q157)	Prefoldin subunit	0.042
PABPC4	U2AF1 (R156/Q157)	NMD mRNA decay	0.036

Figure 1: Aberrant splicing events in CD34⁺ cells of SFmut MDS patients. **A-C**, Venn Diagrams showing the aberrant splicing events identified in *SF3B1* (**A**), *SRSF2* (**B**) and *U2AF1* (**C**) mutant MDS patients versus healthy controls and SFwt MDS patients. **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 event 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 wildtype MDS and healthy control samples using the rMATS-calculated inclusion levels of the 245, 236 and 287 aberrantly spliced 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 heatmaps, as determined by universal significance across all splicing factor mutation groups, showing the significant dysregulated pathways (**B**), top 10 transcriptional regulators (**C**), and top 6 drug/chemical genesets (**D**) in *SF3B1*, *SRSF2* and *U2AF1* mutant MDS. Only heatmap tiles with a -log₁₀pvalue >1.3 (p value <0.05) are shown. Transcriptional regulators and drug/chemical names, heatmaps are ranked by the lowest p-value identified in the SFmut group.

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 MDS patients. **D**, Scatterplot of aberrant splicing values in *NICN1* and platelet counts in MDS patients. **E-H**, Kaplan-Meier survival plots for individual isoforms of *PTPRC* and *IFI44L* in our MDS cohort (**E** and **G** respectively), and the TCGA 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-E**, Ranked heatmaps showing the top 15 deregulated pathways (**C** and **F**), top 15 transcriptional regulators (**D** and **G**), and top 6 drug/chemical

genesets (**E and H**) in MON, GRA and ERY populations of *SF3B1* mutant and *SRSF2* mutant MDS patients. Only heatmap tiles with a $-\log_{10}p\text{value} > 1.3$ ($p\text{ value} < 0.05$) are shown. Heatmap pathways, transcriptional regulators and drug/chemical names are ranked by the IPA ranking score.

Figure 5: Functional effects of *AKAP8* and *SEPT2* knockdown on erythroid differentiation. (**A and H**) Real-time quantitative PCR showing the mRNA knockdown of *AKAP8* (**A**) and *SEPT2* (**H**) in erythroid cells. **B and I**, Growth curves for erythroid cells with knockdown of *AKAP8* (**B**) and *SEPT2* (**I**). **C and 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 panels A-G were obtained from 5 independent experiments, except for panel C (3 replicates). Results shown in panels H-N were obtained from 4 independent experiments. Data represent the mean \pm SEM. All p-values were obtained by 1-way ANOVA with Bonferroni's post-test with the exception of panels **G and N** where 2-way ANOVA with Bonferroni's post test was used. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.