

Splicing factor gene mutations in the myelodysplastic syndromes: impact on disease phenotype and therapeutic applications

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Abstract

Splicing factor gene mutations are the most frequent mutations found in patients with the myeloid malignancy myelodysplastic syndrome (MDS), suggesting that spliceosomal dysfunction plays a major role in disease pathogenesis. The aberrantly spliced target genes and deregulated cellular pathways associated with the commonly mutated splicing factor genes in MDS (*SF3B1*, *SRSF2* and *U2AF1*) are being identified, illuminating the molecular mechanisms underlying MDS. Emerging data from mouse modelling studies indicate that the presence of splicing factor gene mutations can lead to bone marrow hematopoietic stem/myeloid progenitor cell expansion, impaired hematopoiesis and dysplastic differentiation that are hallmarks of MDS. Importantly, recent evidence suggests that spliceosome inhibitors and splicing modulators may have therapeutic value in the treatment of splicing factor mutant myeloid malignancies.

Keywords: myelodysplastic syndromes, splicing factor gene, mutations, RNA splicing

Introduction

The myelodysplastic syndromes (MDS) represent a heterogeneous group of myeloid malignancies that originate from a neoplastic hematopoietic stem cell (HSC) in the bone marrow (Heaney and Golde, 1999; Jhanwar, 2015; Pellagatti and Boultonwood, 2015; Tefferi and Vardiman, 2009). Patients with MDS suffer from ineffective hematopoiesis resulting in peripheral blood cytopenias, and many cases show an increasing number of malignant blasts in the bone marrow over time (Heaney and Golde, 1999; Jhanwar, 2015; Pellagatti and Boultonwood, 2015; Tefferi and Vardiman, 2009). Approximately 30-40% of MDS patients progress to acute myeloid leukemia (AML) (Heaney and Golde, 1999; Tefferi and Vardiman, 2009). MDS is as frequent as *de novo* AML, and the incidence is 4-5 per 100,000 people per year (Polednak, 2013). Patient survival in MDS is variable, but cases with excess blasts (RAEB1 and RAEB2) have a median overall survival of less than 24 months, demonstrating the poor prognosis of patients with advanced MDS.

Current treatments for low-risk MDS are erythropoietin (EPO) and lenalidomide (MDS with the 5q-), and for high-risk MDS azacitidine, decitabine and chemotherapy (Garcia-Manero, 2014). Allogeneic bone marrow transplantation is the only curative treatment for MDS and is generally considered only appropriate for a small proportion of patients. There is clearly a need for more effective treatments for MDS (Jonas and Greenberg, 2015; Steensma, 2015).

The molecular landscape of MDS has been illuminated in recent years and splicing factor gene mutations, which occur in over half of all patients, have been shown to be the most common molecular abnormality found in this disorder (Graubert et al., 2012; Haferlach et al., 2014; Makishima et al., 2012; Papaemmanuil et al., 2011; Papaemmanuil et al., 2013; Walter et al., 2013; Yoshida et al., 2011). Splicing factor gene mutations are found in other myeloid malignancies including AML, but are strongly associated with the phenotype of MDS and closely related conditions.

The splicing of pre-mRNA by the excision of intronic sequences results in the production of mature mRNAs and is an essential process for the expression of >95% of human genes (Boultonwood et al., 2014; Hoskins and Moore, 2012; Pan et al., 2008). Splicing of mRNA plays a major role in protein diversity since it enables the generation of multiple protein isoforms from a single pre-mRNA transcript. For the vast majority of human genes, splicing is performed by the major spliceosome, a complex of five small nuclear ribonucleoproteins (snRNPs), U1, U2, U4, U5 and U6, and a myriad of associated proteins. During the splicing process the formation of the active spliceosome occurs in a number of discrete stages involving the ordered assembly of distinct factors on the pre-mRNA substrate (Hoskins and Moore, 2012). *SF3B1*, *U2AF1*, *SRSF2* and *ZRSR2* are the most frequently mutated splicing factor genes in MDS (Haferlach et al., 2014; Papaemmanuil et al., 2013) and are part of the E/A splicing complex that orchestrates 3' splice site recognition during the early phase of pre-mRNA processing (Yoshida et al., 2011). In this process, SF1 binds to the branch point upstream of the 3' splice site, and SRSF2 and ZRSR2 bind to the exon splicing enhancer (ESE) site of the next exon to aid the binding and stability of U2AF1 (U2AF35) and U2AF2 (U2AF65) (complex E) (Hoskins and Moore, 2012). The SF3B1 protein is a core component of the U2 snRNP and is involved in the recognition of the branch point, targeting of the U2 snRNP to the 3' splice site (complex A) (Schellenberg et al., 2011). It is recognized that the common splicing factor mutations in MDS result in aberrant 3' splice site recognition and spliceosome dysfunction is a major feature of MDS (Yoshida et al., 2011).

SF3B1 gene mutations are the most frequent mutations found in MDS and are more common in low-risk MDS (Malcovati et al., 2011). In many studies it has been shown that approximately 80% of MDS patients with refractory anemia with ring sideroblasts (RARS) and refractory cytopenia with multilineage dysplasia and ring sideroblasts (RCMD-RS) carry mutations of *SF3B1* (Malcovati and Cazzola, 2016; Malcovati et al., 2011; Papaemmanuil et al., 2011). *U2AF1* and *SRSF2* mutations are more common in high-risk MDS, and are associated with an increased risk of transformation to AML (Graubert et al., 2012; Thol et al., 2012).

The splicing factor mutations found in MDS are typically heterozygous and occur in a mutually exclusive manner (Haerlach et al., 2014; Papaemmanuil et al., 2013). Mutations in *SF3B1*, *U2AF1* and *SRSF2* are considered to be change of function/neomorphic or gain of function mutations due to the presence of hotspots and the absence of nonsense or frameshift changes, while mutations in *ZRSR2* are loss-of-function (Papaemmanuil et al., 2011; Yip et al., 2016; Yoshida et al., 2011). While splicing factor mutations can be found in isolation (Mian et al., 2013), several studies have shown that splicing factor mutations show specific associations with epigenetic regulators and also oncogenes mutated in MDS (Haerlach et al., 2014; Mian et al., 2013; Papaemmanuil et al., 2013), indicative of epistatic interactions involving these genes. These findings are of great interest as they link pathways involved in MDS pathophysiology. Co-mutation of *ASXL1* and *U2AF1*, and *SF3B1* and *DNMT3A*, for example are frequently reported in MDS, whilst co-mutation of *TET2* with *SRSF2* or *ZRSR2* is highly predictive of a CMML disease phenotype (specificity of 98%) (Haerlach et al., 2014; Malcovati et al., 2014; Papaemmanuil et al., 2013). Thus, co-operation between splicing factor mutations and mutations in other genes is likely required for the development of the MDS phenotype. The relationship between the proteins encoded by these genes and how they interact and contribute to the MDS phenotype remains poorly understood, however.

There is evidence to suggest that splicing factor mutations are founder mutations in MDS (Mian et al., 2015; Mian et al., 2013). Cross-sectional studies involving large MDS patient cohorts (Haerlach et al., 2014; Papaemmanuil et al., 2013) and more recent studies involving the analysis of serial bone marrow samples from MDS patients (Mossner et al., 2016; Pellagatti et al., 2016b) have shown that splicing factor and epigenetic regulator gene mutations are typically early events in the disease course, whilst mutations in transcriptional regulator and signal transduction genes are predominantly late events and are associated with disease evolution. Early occurrence of splicing factor gene mutations may also determine the trajectories of clonal evolution by influencing subsequent gene mutations, thus leading to distinct disease phenotypes (Papaemmanuil et al., 2013). Several studies have shown that the presence of splicing factor gene mutations significantly impact patient survival in MDS (Bejar, 2016).

A wealth of transcriptome-based studies have shown that the splicing factor gene mutations found in MDS result in aberrant expression and pre-mRNA splicing of many downstream target genes. Whilst the commonly mutated splicing factor genes in MDS all play a role in 3' splice site recognition, it has been shown that their target genes are largely different and this may explain why these mutations are associated with some distinct clinical features (Darman et al., 2015; Gerstung et al., 2015). The dysregulation of 3' splice site recognition resulting from splicing factor gene mutation in MDS may be expected to result in widespread missplicing, and indeed a large number of target genes have been identified (Dolatshad et al., 2016; Shiozawa et al., 2015). However the number of target genes affected is relatively small (in the order of hundreds rather than thousands) and there is specificity in the pathways affected (Dolatshad et al., 2016; Shiozawa et al., 2015).

The identification of key downstream target genes of splicing factor gene mutations and affected pathways is shedding new light on the disease mechanisms underlying MDS and AML (Colla et al., 2015; Dolatshad et al., 2015; Dolatshad et al., 2016; Kim et al., 2015; Madan et al., 2015; Shirai et al., 2015). Deregulated cellular processes/pathways include genome stability and the DNA damage response, epigenetic regulation and RNA processing and splicing (Dolatshad et al., 2015; Dolatshad et al., 2016; Kim et al., 2015; Przychodzen et al., 2013). Furthermore, evidence from human and mouse modeling studies suggests that splicing factor gene mutations can drive hematopoietic stem/myeloid progenitor cell expansion in the bone marrow and aspects of the dysplastic differentiation and ineffective hematopoiesis characteristic of MDS (Dolatshad et al., 2016; Kim et al., 2015; Shirai et al., 2015).

The pre-mRNA splicing process has been extensively described elsewhere (Matera and Wang, 2014; Wahl et al., 2009) and therefore will not be discussed in detail here. In this review, we will describe the role of the common splicing factor gene mutations in MDS (and related disorders) and discuss possible therapeutic implications.

SF3B1 mutations

SF3B1 is a component of the U2snRNP complex that replaces SF1 on the branch point, leading to the formation of the A complex (Gao et al., 2008). SF3B1 also interacts with other splicing regulatory proteins including U2AF2 (Gozani et al., 1998). In contrast to most other spliceosome components that play an early role in intron recognition and then depart, SF3B1 remains associated with the spliceosome after full assembly to B complex and through activation and catalysis (Agafonov et al., 2011; Ilagan et al., 2013).

Approximately 20-28% of all MDS patients harbor *SF3B1* mutations (Malcovati et al., 2011; Papaemmanuil et al., 2011; Yoshida et al., 2011) and the presence of this mutation is strongly associated with the ring sideroblast phenotype (positive predictive value of 97.7%) (Malcovati et al., 2011). Importantly, it has recently been shown that *SF3B1* mutation in MDS patients with ring sideroblasts can arise from the rare HSC compartment and is an initiating event in this disorder (Mian et al., 2015). Using xenotransplantation assays, it was demonstrated that the *SF3B1* mutant clone alone or in combination with other molecular abnormalities results in a clonal growth advantage over ‘normal’ cohabitating cells in NSG mice (Mian et al., 2015). *SF3B1* mutant MDS patients show higher platelet counts and lower bone marrow blast percentage compared to MDS patients with wild-type *SF3B1* (Seo et al., 2014). The presence of *SF3B1* mutations has been associated with better overall survival (Malcovati et al., 2011; Mian et al., 2013) and leukemia free survival (Haferlach et al., 2014; Papaemmanuil et al., 2013) in MDS. A recent study of 243 patients with myeloid neoplasms and 1% or more ring sideroblasts demonstrated that *SF3B1* mutant cases had significantly better survival and a reduced risk of disease progression compared to wild-type cases (Malcovati et al., 2015). In this study it was suggested that *SF3B1* mutation identifies a distinct subset of MDS with ring sideroblasts characterized by indolent clinical course and favorable outcome (Malcovati et al., 2015).

The mutations in *SF3B1* predominantly cluster within the C-terminal HEAT domains (residues 622–781), with the most frequently mutated residue being K700 (Inoue et al., 2016; Papaemmanuil et al., 2011; Yoshida et al., 2011). *SF3B1* mutations have been shown to co-occur with mutations of genes involved in the regulation of DNA methylation, in particular the

methytransferase *DNMT3A* and the methylcytosine dioxygenase *TET2*. *SF3B1* mutations have also been associated with the presence of mutation in the tyrosine kinase *JAK2* (Haferlach et al., 2014; Mian et al., 2013; Papaemmanuil et al., 2013). Interestingly, RARS-T has been shown to develop from RARS through the emergence of a subclonal driver mutation in *JAK2* in the founder *SF3B1* mutated clone (Malcovati et al., 2011).

SF3B1 mutations have been shown to promote usage of 3' cryptic splice sites in several cancers, including chronic lymphocytic leukemia (DeBoever et al., 2015). A recent study has shown that aberrant 3' splice site usage is the most common splicing defect in *SF3B1* mutant tumor samples, and that mutant SF3B1 uses a different branch point from the wild-type SF3B1 but still requires the canonical 3' splice site to enable splicing (Darman et al., 2015). Aberrant 3' splice site usage can result in the introduction of premature termination codons in a transcript that may be subject to nonsense-mediated decay (Brognia and Wen, 2009), and in this study around half of the aberrantly spliced mRNAs were indeed shown to be targeted by nonsense-mediated decay resulting in down-regulation of the affected genes (Darman et al., 2015). Thus the presence of *SF3B1* mutations may impact the phenotypes observed in various cancers through aberrant splicing that leads to reduced protein levels of affected target genes (Darman et al., 2015).

A heterozygous conditional knock-in mouse model of the common *SF3B1* K700E mutation was recently generated (Obeng et al., 2014). These mice develop a macrocytic anemia, with rare ring sideroblasts in the bone marrow, and show normal neutrophil and platelet counts. This study shows that *Sf3b1* heterozygous mutations result in aberrant erythroid maturation and ineffective hematopoiesis in mice, consistent with the clinical picture observed in RARS patients. It was noted that *Sf3b1* K700E HSCs have a competitive disadvantage in competitive transplantation assays (Obeng et al., 2014).

Target genes of *SF3B1* mutations

Several RNA sequencing (RNA-Seq)-based studies have been performed to study the effects of *SF3B1* mutations on global gene expression and splicing in the bone marrow cells of MDS patients (Dolatshad et al., 2015; Dolatshad et al., 2016; Makishima et al., 2012; Visconte et al., 2015; Visconte et al., 2012). An early study investigating bone marrow mononuclear cell samples from *SF3B1* mutant MDS patients identified differential exon usage (indicating alternative splicing) in several genes recurrently mutated in MDS, including *ASXL1* and *CBL* (Visconte et al., 2012). Dolatshad et al showed differential expression and/or exon usage in many genes involved in several cellular processes/pathways, many of which are relevant to the known RARS pathophysiology, in *SF3B1* mutant MDS CD34⁺ cells (Dolatshad et al., 2015). Importantly, several aberrantly spliced genes identified in this study, including *TMEM14C*, *UQCC1* and *CRNDE* also showed the same splicing abnormality in other cancers with the *SF3B1* mutation, suggesting that these represent common downstream target genes (Dolatshad et al., 2015; Furney et al., 2013; Maguire et al., 2015).

In a subsequent study, using a bioinformatics pipeline designed to detect alternative (including cryptic) splicing events, Dolatshad et al identified aberrant/cryptic mRNA splicing events in many key downstream target genes, including *TMEM14C*, *ABCB7*, *SEPT6*, *DYNLL1* and *ABCC5* in the HSCs of *SF3B1* mutant MDS patients (Dolatshad et al., 2016). The significant main ontology themes found to be enriched in *SF3B1* mutant MDS include RNA processing and RNA splicing, showing that RNA splicing itself is a major downstream target of the *SF3B1* mutation. This may be predicted to result in an exacerbation of aberrant pre-mRNA

splicing in MDS. Usage of cryptic 3' splice sites was found to be a frequent event in MDS cases with *SF3B1* mutation, as previously shown in other cancers.

Ring sideroblasts are characterized by an excess accumulation of iron in the mitochondria of erythroblasts (Cazzola et al., 2003). The link between *SF3B1* mutations and ring sideroblasts indicates a causal relationship, making this the first gene to be strongly associated with a specific morphological feature of MDS. It is unclear how *SF3B1* mutations lead to the formation of ring sideroblasts, however. Interestingly, a strong relationship between increasing percentage of bone marrow ring sideroblasts and decreasing expression levels of *ABCB7*, a mitochondrial iron exporter, was first described in MDS several years ago (Boulton et al., 2008). A later integrative analysis of mutation and gene expression data in MDS HSCs identified a strong association between the presence of *SF3B1* mutations and marked downregulation of *ABCB7* (Gerstung et al., 2015), indicating a three-way association among *SF3B1* mutation, *ABCB7* downregulation and the occurrence of ring sideroblasts. Elegant functional studies implicated *ABCB7* in the phenotype of RARS (Nikpour et al., 2013).

Thus downregulation of *ABCB7* has been recognized as an important event in MDS patients with RARS for several years, but the mechanism causing the downregulation of this gene has remained unknown. Aberrant splicing of *ABCB7*, due to usage of an alternative 3' splice site, was recently found to occur in *SF3B1* mutant MDS patient samples by Dolatshad et al (Dolatshad et al., 2016). The aberrantly spliced *ABCB7* transcript contained a premature termination codon and was shown to be targeted by nonsense-mediated RNA decay (NMD) leading to downregulation of this gene. Similar findings in MDS patient samples harboring *SF3B1* mutations have recently been described by others (Malcovati and Cazzola, 2016). The results of these studies support a model in which mutant *SF3B1* causes aberrant splicing of the iron transporter *ABCB7* resulting in *ABCB7* downregulation, and the subsequent mitochondrial iron accumulation observed in the erythroblasts of MDS patients with ring sideroblasts (Boulton et al., 2008; Dolatshad et al., 2016; Gerstung et al., 2015; Nikpour et al., 2013).

Other cellular processes disrupted by the *SF3B1* mutations in MDS include the DNA damage response (DDR). Savage et al identified a DNA damage-induced BRCA1 protein complex containing BCLAF1 and SF3B1 (BRCA1–BCLAF1–SF3B1 complex) that regulates pre-mRNA splicing of genes involved in DNA damage signaling and repair (Savage et al., 2014). Interestingly, several genes regulated by the BRCA1–BCLAF1–SF3B1 complex show differential expression and/or exon usage in *SF3B1* mutant MDS patients. It is possible, therefore, that this complex may be impaired in *SF3B1* mutant MDS patients, impacting the efficiency of the DNA damage repair process (Dolatshad et al., 2015).

SRSF2 mutations

SRSF2 (*SC35*) encodes a serine/arginine-rich protein that promotes exon recognition during the splicing process by interacting with ESE sequences of 5' or 3' splice sites via its RNA recognition motif (RRM) domain (Chen and Manley, 2009; Graveley and Maniatis, 1998; Liu et al., 2000; Schaal and Maniatis, 1999). U2AF is then recruited to the upstream 3' splice site and U1 snRNP to the downstream 5' splice site, promoting splice site usage (Chen and Manley, 2009; Graveley and Maniatis, 1998; Liu et al., 2000; Schaal and Maniatis, 1999).

Mutations of *SRSF2* occur in 12-15% of MDS patients and are especially common in CMML (28-47% of cases) (Haferlach et al., 2014; Papaemmanuil et al., 2013; Thol et al., 2012;

Yoshida and Ogawa, 2014; Yoshida et al., 2011). The majority of *SRSF2* mutations affect the P95 codon (Yoshida et al., 2011), which is located close to the RRM domain. Patients harboring *SRSF2* mutations have a significantly shorter overall survival and shorter time to transformation to AML compared with patients wild-type for this gene (Damm et al., 2012; Makishima et al., 2012; Thol et al., 2012).

A conditional *Srsf2* knock-in mouse model with P95H mutation has been generated (Kim et al., 2015). Heterozygous *Srsf2* P95H mice display leukopenia, macrocytic anemia with peripheral blood and bone marrow myeloid dysplasia, and show hematopoietic stem and progenitor cell expansion and increased apoptosis (Kim et al., 2015). These phenotypes resemble those seen in human MDS, indicating that the *SRSF2* P95H mutation is important in the development of MDS. It was noted that the *Srsf2* P95H mice did not develop AML (Kim et al., 2015; Obeng and Ebert, 2015), however, and the *Srsf2* P95H mutant stem cells had a competitive disadvantage compared with wild-type stem cells in competitive repopulation assays (Kim et al., 2015).

The effects of the *SRSF2* P95H mutation on RNA splicing were investigated in the HSC and myeloid progenitor populations of the *Srsf2* P95H mice and in the K562 cell line ectopically expressing this mutation (Kim et al., 2015). The mutant *SRSF2* showed differential recognition activity in the ESE of promoted and repressed exons (Kim et al., 2015). It is known that wild-type *SRSF2* recognizes the consensus binding sequences CCNG and GGNG within exons with the same affinity (Daubner et al., 2012). The *SRSF2* P95H mutation was shown to affect the sequence specificity of the *SRSF2* RRM domain, resulting in increased binding affinity towards CCNG and a decreased binding affinity towards GGNG (Kim et al., 2015). These findings were confirmed in a recent study in which the *SRSF2* P95H mutation was introduced into the K562 cell line using CRISPR/Cas9 genome editing (Zhang et al., 2015). Taken together, these studies indicate suggest that the *SRSF2* P95H mutation confers a gain/change of function.

Interestingly, abnormalities of *SRSF2* have been associated with genomic instability. Reduced levels of *SRSF2* can result in DNA damage and genomic instability through the formation of R loops (RNA:DNA hybrids), which trigger activation of the DDR (Chabot and Shkreta, 2016; Xiao et al., 2007).

Target genes of *SRSF2* mutations

Several important aberrantly spliced target genes associated with the *SRSF2* P95H mutation have been identified by comparing different RNA-Seq datasets comprising HSCs and myeloid progenitors from the *Srsf2* P95H mouse model, and primary samples from AML and/or CMML patients (Kim et al., 2015). Some of these downstream target genes are known to be recurrently mutated in MDS and AML, including for example the epigenetic regulator *EZH2* (Haferlach et al., 2014; Papaemmanuil et al., 2013). The presence of *SRSF2* mutations leads to the retention of a so-called “poison exon” that introduces a premature stop codon in the *EZH2* transcript, resulting in NMD and gene down-regulation (Kim et al., 2015). Moreover, reduced *EZH2* protein expression levels and histone H3 lysine 27 (H3K27) trimethylation levels were observed in the *SRSF2* mutant K052 AML cell line (Kim et al., 2015). Importantly, the hematopoietic defects associated with mutant *SRSF2* could be partially rescued by the overexpression of full-length *EZH2* mRNA (Kim et al., 2015). It is recognized that connections between splicing and epigenetic factors may play a role in the oncogenic process in myeloid malignancies and this study shows that *EZH2* is a critical downstream target of the *SRSF2* mutation (Kim et al., 2015). *SRSF2* and *EZH2* mutations are known to be mutually exclusive in

MDS (Haferlach et al., 2014; Papaemmanuil et al., 2013), and the reduced EZH2 expression associated with the mutant SRSF2 observed in the study by Kim et al may explain this mutual exclusivity (Kim et al., 2015).

The study of isogenic cells can be valuable for the identification of aberrant splicing events associated with splicing factor gene mutations (Pellagatti et al., 2016a). The *SRSF2* P95H mutation has been introduced into the K562 cell line using CRISPR/Cas9 genome editing and several aberrantly spliced downstream target genes were identified, including the tyrosine kinase *FYN* and the putative tumor suppressor gene *ARMC10* (Zhang et al., 2015).

U2AF1 mutations

U2AF1 (U2AF35) is an auxiliary factor of the U2snRNP complex that recognizes the 3' AG dinucleotide at the splice acceptor site (Ruskin et al., 1988; Wu et al., 1999). *U2AF1* mutations occur in MDS and AML (Haferlach et al., 2014; Papaemmanuil et al., 2013; Thol et al., 2012; Yoshida et al., 2011). Approximately 7-11% of MDS patients harbor mutations in *U2AF1* (Haferlach et al., 2014; Papaemmanuil et al., 2013; Thol et al., 2012; Yoshida et al., 2011). The majority of *U2AF1* mutations affect the S34 and Q157 codons located within the two zinc finger domains of the protein (Yoshida et al., 2011). Mutations of *U2AF1* have been shown to be associated with shorter survival in MDS in some but not all studies (Bejar et al., 2012; Graubert et al., 2012; Makishima et al., 2012; Thol et al., 2012; Wu et al., 2016; Wu et al., 2013). *U2AF1* mutations have been associated with a higher risk of transformation to AML (Graubert et al., 2012; Thol et al., 2012; Walter et al., 2013).

Aberrant cassette exon splicing is the most frequent abnormal splicing event occurring in bone marrow samples of MDS and AML patients with *U2AF1* mutations (Brooks et al., 2014; Ilagan et al., 2015; Przychodzen et al., 2013). Mutations in *U2AF1* have been found to alter the 3' splice site recognition activity of the protein in a sequence specific manner. It has been shown that differentially spliced exons have different consensus nucleotides at the -3 and +1 positions flanking the AG dinucleotide of the 3' splice site (Brooks et al., 2014; Ilagan et al., 2015; Okeyo-Owuor et al., 2015; Przychodzen et al., 2013). The U2AF1 S34F mutant protein recognizes the thymidine (uridine) at the -3 position of the 3' splice site less frequently than cytosine compared with wild-type U2AF1 (Brooks et al., 2014; Ilagan et al., 2015; Okeyo-Owuor et al., 2015). These observations underlie the *U2AF1* S34F mutation-associated aberrant cassette exon splicing and alternative 3' splice site events (Brooks et al., 2014; Ilagan et al., 2015; Okeyo-Owuor et al., 2015). The *U2AF1* Q157 mutation is instead associated with alterations at the +1 position relative to the AG dinucleotide of the 3' splice site (Ilagan et al., 2015). The presence of an adenosine suppressed cassette exon recognition (Ilagan et al., 2015). The *U2AF1* Q157 mutation is associated with preferential recognition of guanine instead of adenosine at the +1 position (Ilagan et al., 2015). The different recognition preference for 3' splice site consensus sequences may provide an explanation for the different effects of the S34 and Q157 mutations on pre-mRNA splicing (Okeyo-Owuor et al., 2015).

A doxycycline-inducible transgenic mouse model of the *U2AF1* S34F mutation has been generated (Shirai et al., 2015), and these mice show some phenotypes associated with MDS, including leukopenia, expansion of myeloid progenitor cells, abnormal mature cell lineage distribution and increased apoptosis in the bone marrow (Shirai et al., 2015). In contrast to the *Srsf2* P95H mouse model that developed morphological dysplasia (Kim et al., 2015), the *U2AF1* S34F mice did not show dysplasia and did not develop MDS or AML (Shirai et al.,

2015). As observed with other splicing factor mutant HSCs (Kim et al., 2015), U2AF1 S34F mutant stem cells show a competitive disadvantage compared with wild-type stem cells in competitive repopulation assays in mice (Shirai et al., 2015). This observation is somewhat puzzling given that splicing factor mutations are thought to be founder mutations in myeloid malignancies.

Targets genes of *U2AF1* mutations

A number of studies have analyzed the RNA-Seq data derived from bone marrow cells of AML patients with *U2AF1* mutations (Brooks et al., 2014; Ilagan et al., 2015; Przychodzen et al., 2013). Aberrant cassette exon splicing events were identified in *U2AF1* mutant AML samples in several genes known to be mutated in cancer and in many genes involved in specific cellular pathways, including RNA processing, cell cycle and the DNA damage response (Przychodzen et al., 2013). Similarly, another study showed that *U2AF1* mutations in AML result in differential splicing of genes involved in these and other cellular processes such as epigenetic regulation (Ilagan et al., 2015).

Shirai et al. have identified several aberrantly spliced genes, including *H2AFY*, *BCOR*, *PICALM* and *GNAS*, that are common to three RNA-Seq datasets comprising *U2AF1* mutant AML patients, CMPs from the U2AF1 S34F mouse model and primary human CD34⁺ overexpressing S34F (Shirai et al., 2015). The significant main ontology themes found to be enriched were RNA processing, RNA splicing, RNA localization/transport, and RNA binding, protein translation processes and ribosomal pathways. Moreover, spliceosome genes and genes recurrently mutated in MDS and AML were also enriched among the aberrantly spliced genes (Shirai et al., 2015). Aberrant splicing of *GNAS*, *H2AFY*, *STRAP* and *PICALM* has also been observed in lung adenocarcinoma patients and in K562 cells expressing the *U2AF1* S34F mutation (Brooks et al., 2014; Ilagan et al., 2015; Przychodzen et al., 2013).

One key unresolved question is how splicing factor gene mutations may contribute to clonal expansion and disease transformation in MDS. A recent study by Park et al has revealed a model of oncogenic activity for *U2AF1* mutations (Park et al., 2016). RNA-Seq analysis of a murine cell line stably expressing the *U2AF1* S34F mutation showed that the most common type of aberrant RNA processing event associated with the mutant U2AF1 was increased usage of a distal cleavage and polyadenylation (CP) site. Importantly, in addition to the analysis of alternative splice site usage, an algorithm testing for alternative use of CP sites was used in this study. Aberrant processing of the pre-mRNA of *Atg7*, a gene encoding an important autophagy factor (Komatsu et al., 2005), was highlighted as an important finding, since loss of essential autophagy factors has been implicated in tumorigenesis (Liu et al., 2013; Mathew et al., 2007). Furthermore, mice with conditional depletion of *Atg7* in hematopoietic cells develop an MDS-like syndrome (Komatsu et al., 2005; Mortensen et al., 2011). In the study by Park et al, increased use of the *Atg7* distal CP site in cells expressing the *U2AF1* S34F mutation was shown to result in a longer 3' UTR, which leads to inefficient translation of the mRNA and decreased ATG7 protein levels. Importantly, increased use of the *ATG7* distal CP site was observed in bone marrow cells of MDS and AML patients harboring the *U2AF1* S34F mutation. U2AF1 S34F expressing cells had defective autophagy associated with mitochondrial dysfunction and increased reactive oxygen species production, leading to genomic instability and an increased frequency of spontaneous mutations (Park et al., 2016). This study illuminated the mechanisms by which *U2AF1* mutations may promote disease transformation in myeloid malignancies.

Mutations of other splicing factor genes

In addition to the three most commonly mutated splicing factor genes in MDS (*SF3B1*, *SRSF2* and *U2AF1*), several other splicing factor genes have been found to be mutated in myeloid malignancies, including MDS and AML, at a lower frequency. These genes include *ZRSR2*, *PRPF8*, *U2AF65*, *SF1*, *SF3A1*, *PRPF40B* and *LUC7L2* (Kurtovic-Kozaric et al., 2015; Makishima et al., 2012; Yoshida et al., 2011).

ZRSR2 is an essential component of the minor spliceosome assembly and plays an important role in the splicing of U12-type introns (Madan et al., 2015; Shen et al., 2010; Tronchere et al., 1997). U12-type introns represent only about 0.5% of all human introns (Turunen et al., 2013). *ZRSR2* mutations have been reported in 3-11% of patients with MDS (Damm et al., 2012; Haferlach et al., 2014; Papaemmanuil et al., 2013; Yoshida et al., 2011). Missense, nonsense and frameshift mutations of *ZRSR2* have been described and are distributed across the entire length of the coding region, suggesting that *ZRSR2* mutations are loss-of-function mutations (Ogawa, 2012; Yoshida et al., 2011).

Knockdown of *ZRSR2* in human CD34⁺ cells has been shown to result in impaired erythroid growth and differentiation and an increase in the numbers of myeloid cells (Madan et al., 2015). RNA-Seq analysis on bone marrow cells of *ZRSR2* mutant MDS patients and on TF-1 cells with *ZRSR2* knockdown showed that both mutations and knockdown of *ZRSR2* resulted in aberrant retention of U12-type introns while U2-dependent splicing was mainly unaffected (Madan et al., 2015). A number of aberrantly spliced target genes were identified in *ZRSR2* mutant MDS samples, including genes involved in the MAPK signaling pathway and E2F transcription factor signaling (Madan et al., 2015).

PRPF8 is the largest and most evolutionarily conserved protein of the spliceosome (Grainger and Beggs, 2005). Recurrent mutations or hemizygous deletions of *PRPF8* have been identified in 1-5% of patients with myeloid malignancies (Kurtovic-Kozaric et al., 2015). Molecular abnormalities of *PRPF8* have been associated with increased myeloblasts, the presence of ring sideroblasts (in cases without *SF3B1* mutations) and missplicing defects in myeloid malignancies (Kurtovic-Kozaric et al., 2015).

Interestingly, inherited and somatic defects in *DDX41* have been identified in myeloid malignancies (Polprasert et al., 2015). There is evidence to suggest that *DDX41* interacts with core spliceosome components and *DDX41* lesions were found to cause altered pre-mRNA splicing and RNA processing in affected patients (Polprasert et al., 2015).

The relationship between splicing factor mutant MDS and splicing factor mutant AML

Mutations in splicing factor genes are far more frequent in MDS (>50% cases) than de novo AML (>10% cases) (Rose et al., 2015; Yoshida et al., 2011). This has led to speculation that splicing factor gene mutations may be key factors driving the phenotypic differences between these related conditions.

In study by Taskesen et al, that sought to compare the impact of splicing factor gene mutations in MDS and AML, DNA from the bone marrow of 47 RAEB cases, 29 AML cases with low marrow blast cell count (AML-LBC), and 325 other AML patients was sequenced for

mutations in the splicing factor genes *SF3B1*, *U2AF1*, and *SRSF2* (Taskesen et al., 2014). Splicing factor gene mutations were identified in 10 RAEB cases, 12 AML-LBC cases, and 25 other AML cases. Importantly, it was found that these three patient groups were highly similar in their clinical characteristics and shared highly similar phenotypes, raising the possibility that RAEB, AML-LBC, and AML cases with splicing factor mutations might be considered a distinct leukemic subset. This suggestion was further supported by the observation that AML patients with *SF3B1*, *U2AF1* or *SRSF2* mutations showed significantly lower BM blast percentages, were older, showed significantly lower WBC counts and had higher erythroblasts percentages compared to AML cases without splicing factor mutations. Splicing factor-mutant RAEB/AML therefore constitutes a related disorder overriding the separation between AML and MDS and transcending the boundaries of these two myeloid malignancies (Taskesen et al., 2014). Similar observations were made recently by Papaemmanuil et al in a retrospective study that analyzed somatic driver mutations in >1500 patients with AML (Papaemmanuil et al., 2016). The AML subgroup harboring chromatin/spliceosome mutations were, on average, found to be older with lower blast counts and higher rates of a preceding MDS or dysplasia-related morphologic features than patients in other subgroups (Papaemmanuil et al., 2016).

In another study targeted mutational screening was performed on bone marrow samples obtained from 93 cases of AML secondary to MDS (s-AML) and the data generated compared to genetic data available for 180 cases of non-M3 *de novo* AML reported in The Cancer Genome Atlas (Lindsley et al., 2015). It was shown that the presence of a mutation in eight genes comprising *SRSF2*, *SF3B1*, *U2AF1*, *ZRSR2*, *ASXL1*, *EZH2*, *BCOR* or *STAG2* was >95% specific for the diagnosis of s-AML. These findings further highlight the link between the MDS phenotype and the presence of splicing factor mutations and suggest that these mutations may primarily drive the ineffective hematopoiesis and dysplastic differentiation that is so characteristic of MDS (Lindsley et al., 2015).

Use of spliceosome inhibitors and splicing modulators in the treatment of myeloid malignancies

Spliceosome inhibitors

Several compounds are known to act as inhibitors of pre-mRNA splicing (Bonnal et al., 2012; Webb et al., 2013). The first splicing inhibitors identified were FR901464 and its acetylated derivate spliceostatin A (Corrionero et al., 2011; Osman et al., 2011). Spliceostatin A binds to and inhibits SF3B1, impairing the ability of the U2snRNP complex to bind to the branch point and destabilizing it (Corrionero et al., 2011). This prevents the transition of the spliceosome complex A to B, inhibiting normal splicing (Corrionero et al., 2011). More recently it has been shown that SF3B1 inhibitors also interfere with later events in pre-mRNA splicing, including exon ligation (Effenberger et al., 2016), observations that are consistent with a requirement for SF3B1 throughout the splicing process (Agafonov et al., 2011; Ilagan et al., 2013). The treatment of cancer cell lines with Spliceostatin A results in alternative splicing changes in selected target genes including genes important for cell division, such as cyclin A2 and Aurora A kinase (Corrionero et al., 2011; Furumai et al., 2010), and this drug exhibits anti-tumor effects on several xenograft models harboring human and murine tumors (Nakajima et al., 1997; Webb et al., 2013). Several synthetic analogs of spliceostatin A have been developed, most notably, Meayamycin B (MAMB) Sudemycin and the Pladienolide analog E7107 and these compounds have been shown to have similar splicing inhibitory properties, and anti-tumor cell activity in culture, as spliceostatin A (Corrionero et al., 2011; Osman et al., 2011;

Webb et al., 2013). For example, Pladienolide B has been shown to induce mRNA intron retention in primary leukemia cells derived from patients with CLL and in leukemia-lymphoma cell lines, concomitant with increased apoptosis in the malignant cells compared to normal lymphocytes (Kashyap et al., 2015). It has also been recently shown that sudemycin induces a specific antitumor response in NSG mice engrafted with primary cells from CLL patients (Xargay-Torrent et al., 2015).

The splicing factor gene mutations found in MDS and other myeloid malignancies are invariably heterozygous, and only very rarely co-occur with other splicing factor mutations (Papaemmanuil et al., 2013), suggesting that homozygous splicing factor mutations may be lethal to the cell and, furthermore, that only a partial decrease in normal pre-mRNA splicing can be tolerated. These observations have led to the suggestion that spliceosome inhibitors might be more toxic to malignant cells harboring heterozygous splicing factor mutations than normal cells, and therefore result in the preferential killing of the splicing factor-mutant cells.

Lee et al have recently investigated the therapeutic potential of splicing inhibitors in splicing factor mutant leukemia (Lee et al., 2016), testing the hypothesis that cells with heterozygous splicing factor mutations may be especially sensitive to further disruption of normal splicing. These studies concerned the use of the spliceosome inhibitor E7107, a semi-synthetic derivative of pladienolide B that targets SF3b and blocks spliceosome assembly by preventing tight binding of U2 snRNP to pre-mRNA (Folco et al., 2011). Engineered mice that conditionally expressed the *Srsf2*^{P95H} mutation in a hemizygous manner in hematopoietic cells were shown to develop fatal bone marrow failure, indicating that *Srsf2*-mutant hematopoietic cells are dependent on the wild-type *Srsf2* allele for survival. It was then demonstrated that E7107 treatment of an isogenic mouse leukemia model, generated by retroviral overexpression of the *MLL-AF9* fusion oncogene in *Srsf2*^{+/+} or *Srsf2*^{P95H/+} bone marrow cells, resulted in a reduction in disease burden and survival benefit in *Srsf2*^{P95H/+} mice, with no impact on overall survival of *Srsf2*^{+/+} mice. E7107 treatment resulted in global splicing inhibition in both genotypes, with frequent intron retention and cassette exon skipping, however the extent of splicing inhibition was greater in *Srsf2*^{P95H/+} versus *Srsf2*^{+/+} mice (Lee et al., 2016).

Next, in order to determine *in vivo* splicing factor inhibitor effects in primary human AML cells patient-derived xenografts from patients with primary AML with and without a spliceosomal gene mutation were generated and the mice treated with E7107. All splicing factor-mutant AML patient-derived xenografts showed significant reductions in human leukemic burden in response to E7107 treatment, while AMLs that were WT for splicing factor mutations showed less reduction in leukemic cell burden, and the preferential sensitivity to E7107 in splicing factor-mutant AML was associated with a marked increase in apoptosis. These findings establish a link between sensitivity to E7107 and the mutational status of splicing factor genes in primary human AMLs (Lee et al., 2016).

These findings provide genetic and drug-based evidence that myeloid leukemias with splicing factor gene mutations are preferentially susceptible to additional splicing modulations *in vivo* in comparison to leukemia without splicing factor gene mutations. Splicing factor inhibitors may thus have therapeutic potential in splicing factor gene mutant MDS or AML (Lee et al., 2016).

The splicing inhibitor E7107 induces cell-cycle arrest at G1 and G2/M phases and apoptosis and has been shown to inhibit the growth of tumor cell lines *in vitro* and to cause tumor regression in most human cancer xenograft models (Hong et al., 2014; Mizui et al., 2004).

Most splicing inhibitors reduce normal splicing by inhibiting SF3B1 function and E7107 is the only such compound to date that has been tested in human cancer patients. There are two completed phase 1 dose-escalation studies for E7107 in patients with a wide range of advanced solid cancers, including colon, pancreatic and lung cancers (Eskens et al., 2013; Hong et al., 2014). A dose-dependent reversible inhibition of pre-mRNA processing of target genes was recorded in patients treated with E7107, confirming proof-of-principle activity of the drug (Eskens et al., 2013). The best tumor response was stable disease in eight patients (Hong et al., 2014). The drug was generally well tolerated and the dose-limiting toxicity was mainly gastrointestinal related. Visual impairment was reported in 3/66 patients, however, and the studies were discontinued (Eskens et al., 2013; Hong et al., 2014). Further clinical trials with other/newly identified splicing factor inhibitors are needed to determine the therapeutic efficacy and safety of this strategy in cancer patients.

It has long been recognized that increased expression or aberrant activation of the transcription factor MYC is known to play an important role in leukemogenesis, and is frequently activated in AML and other myeloid malignancies (Hoffman et al., 2002). MYC is known to regulate the splicing of specific genes through induction of alternative splicing factors or components of the spliceosome (David et al., 2010) and, intriguingly, it has recently been shown that the spliceosome is a major target of oncogenic stress in MYC-driven cancers (Hsu et al., 2015). MYC hyperactivation was shown to elicit an increase in total pre-mRNA synthesis, resulting in a higher burden on the core spliceosome to process pre-mRNA. Partial inhibition of the spliceosome in MYC-hyperactivated cells was shown to result in global intron retention and defects in pre-mRNA maturation. MYC-dependent cancer cell lines (breast cancer and B-cell derived) were significantly more sensitive to the splicing inhibitor SD6, a small molecule inhibitor of SF3B1, *in vitro* than cancer cell lines with normal MYC expression, suggesting that oncogenic MYC may lead to hyperdependency on the spliceosome in many tumor types. Moreover, pharmacological inhibition of the spliceosome by SD6 in MYC-dependent breast cancer cell xenografts reduced tumor growth and also impaired lung metastatic expansion and increased survival. These findings show that oncogenic MYC engenders a stress on splicing, and suggests that components of the spliceosome may also be therapeutic targets for MYC-driven cancers (Hsu et al., 2015).

Importantly, this study has demonstrated that the inhibition of components of the spliceosome is deleterious in cancer cell lines without splicing factor mutations, suggesting that other drivers of cancer (including MYC) are determinants of sensitivity to splicing inhibitors. It is possible that this observation may be of relevance to cases of AML and MDS that do not harbor splicing factor mutations.

Splicing modulators

The aberrantly spliced genes associated with splicing factor gene mutations in myeloid malignancy may be targeted using splicing modulation approaches. Strategies involving the use of antisense/splice site switching oligonucleotides (ASO/SSO) aim to manipulate the balance between mRNA isoforms in order to restore normal splicing conditions or to preferentially express specific isoforms, with potential therapeutic effects (Kole et al., 2012). ASO/SSO are short oligonucleotides that exert their effect by annealing to a target sequence that spans splice sites or splicing regulatory sequences and preventing their recognition by the spliceosome or by splicing regulatory factors (Daguenet et al., 2015), thus interfering with the splicing process. For example, ASO/SSO may prevent access of the splicing machinery to a specific exon-intron junction, resulting in redirection of the splicing machinery to an adjacent

splice site. ASO/SSO can also be designed to target splicing enhancer or silencer sequences, interfering with the binding of trans-acting regulatory factors and promoting exon skipping or inclusion (Bonomi et al., 2013). ASO/SSO have been shown to be effective for splicing modulation in several *in vivo* studies in mouse, and are currently being in clinical trials for the treatment of Duchenne Muscular Dystrophy and of spinal muscular atrophy (Havens and Hastings, 2016).

Another approach called targeted oligonucleotide enhancers of splicing (TOES) uses bifunctional ASOs to induce the inclusion of exons that would otherwise be skipped (Bonomi et al., 2013; Hua et al., 2007). TOES are modified antisense RNA oligonucleotides composed of two regions: a segment complementary to a target exon sequence and a non-complementary tail containing exonic splicing enhancer (ESE) motifs. TOES recruit serine/arginine-rich (SR) protein and act as enhancers that promote exon inclusion (Bauman and Kole, 2011; Havens et al., 2013). TOES have been shown to induce inclusion of exon 7 of the *SMN2* gene in the fibroblasts of patients with spinal muscular atrophy (Skordis et al., 2003).

The use of splicing modulators may be of value in the treatment of myeloid malignancies with splicing factor gene mutation.

Conclusions

The aberrantly spliced downstream target genes and affected pathways associated with the common splicing factor mutations in MDS are being identified and their impact on human and mouse hematopoiesis is being defined, illuminating the role played by these mutations on MDS disease pathogenesis. RNA-Seq studies in HSC and erythroid and myeloid precursor cell fractions obtained from bone marrow samples of MDS patients with splicing factor gene mutations are, however, required in order to identify lineage-specific aberrantly spliced downstream target genes in MDS. Further functional studies are also necessary to fully determine how the aberrantly spliced target genes identified contribute to the MDS phenotype. Moreover, it is important to determine how the mutated splicing factor genes and other co-mutated genes such as epigenetic regulators interact in MDS disease pathogenesis. Exciting recent data suggests that splicing inhibitors may have therapeutic potential in the treatment of patients with myeloid malignancies with splicing factor gene mutations, offering new hope to such patients.

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Figure 1. Splicing modulation using antisense/splice site switching oligonucleotides (ASO/SSO). The top panel shows a pre-mRNA and the enhancer/silencer sequences (ESE and ISS) recognized by splicing enhancers or inhibitors, with the resulting effects on mRNA splicing. The bottom left panel shows an ASO/SSO targeting an intronic splicing silencer sequence and preventing the binding of a splicing inhibitor, resulting in the inclusion of exon 2 in the mature mRNA. The bottom right panel shows an ASO/SSO targeting an exonic splicing enhancer sequence and preventing the binding of a splicing enhancer, resulting in the skipping of exon 2 which is therefore not included in the mature mRNA.