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14. ABSTRACT

This study focusses on ZRSR2, one of the recurrently mutated spliceosome genes in MDS. Our results demonstrate the importance of ZRSR2 to 012-type intron splicing, and identify candidate ZRSR2 targets that might mediate ZRSR2 pathogenic functions in MDS. We are using RNA-seq to comprehensively characterize the mis-splicing events driven by ZRSR2 mutations. To understand the mechanisms associated with ZRSR2 012-type intrans splicing, and the downstream pathways affected by ZRSR2 loss, we are identifying ZRSR2 binding partners, as well as pathways that can synergize with loss of ZRSR2 to promote MDS development. As ZRSR2 and TET2 mutations often cooccur in MDS, we are examining the mechanisms underpinning the cooperativity between ZRSR2 and TET2 mutations in myeloid transformation. Further, we are identifying and evaluating drugs that might selectively kill ZRSR2 mutated cells. Our long team goal is to provide a better understudying of the role of aberrant splicing in MDS, and to leverage this knowledge to develop new therapeutic modalities that specifically target cells with splicing factor mutations.

15. SUBJECT TERMS- Breast cancer, HSP90, small molecule inhibitors, lapatinib, oncogenic signaling

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1. INTRODUCTION

The discovery of widespread splicing factor mutations in myelodysplastic syndrome (MDS) created a new paradigm for the disease pathogenesis. In MDS, the vast majority of these mutations occur in one of four genes: *SF3B1*, *U2AF1*, *SRSF2* and *ZRSR2*. Although several studies have begun to elucidate the effects of splicing factor mutations on splicing and hematopoiesis, many questions remain open. In particular, the role of *ZRSR2* mutations, prevalent in MDS subtypes without ring sideroblasts and with chronic myelomonocytic leukemia (3-11%), is largely unknow. To our knowledge, our group is the only one studying this gene in detail in MDS. In contrast to mutations in *SF3B1*, *SRSF2* and *U2AF1* (heterozygous missense mutations at restricted residues, conferring an alteration of function), mutations in *ZRSR2* occur throughout the transcript, usually as nonsense or frameshift mutations (pattern consistent with loss-of-function). *ZRSR2* is located on the X chromosome, and *ZRSR2* mutations are found almost exclusively in males, signifying deficiency of ZRSR2 protein in such cases. Additionally, as shown by us and others, ZRSR2 is predominantly important for U12-type intron splicing, while the other three splicing factors regulate U2-type intron splicing. Although rare, U12-type introns are found in genes involved in vital cellular processes, and mutations in U12-splicing factor genes are associated with developmental and neurological disorders. Hence, understudying the role of ZRSR2 in splicing and hematopoiesis will lead to an overall better understanding of the underlying mechanisms of splicing dysregulation in MDS.

2. KEYWORDS

ZRSR2, myelodysplastic syndromes (MDS), splicing factor mutations, U12-type intron splicing

3. ACCOMPLISHMENTS

- **Major goals of the project**

Specific Aim 1: Determine how ZRSR2 mutations dysregulate downstream pathways, and contribute to molecular features of myelodysplastic cells.

Specific Aim 2: Study biological implications of loss of ZRSR2 in normal and MDS marrow cells.

Specific Aim 3: Investigate the role of ZRSR2 in myeloid transformation using *Zrsr2/Tet2*-deficent murine model.

- **Accomplishments under these goals**

Specific Aim 1: Determine how ZRSR2 mutations dysregulate downstream pathways, and contribute to molecular features of myelodysplastic cells.

1A. To investigate the role of ZRSR2 in vivo, we generated a mouse model of ZRSR2 deficiency. *Zrsr2* knockout (KO) murine embryonic stem cells were injected into blastocysts to produce chimeric mice, from which we obtained germline transmission of the *Zrsr2* KO allele (Fig. 1A). Male *Zrsr2* KO mice were obtained at expected frequency in breeding of heterozygous mice, and did not display any apparent morphological abnormalities. Deletion of *Zrsr2* locus was verified by absence of *Zrsr2* transcript in our PCR analysis of lymphoid organs, as well as in RNA-sequencing (RNA-seq) of bone marrow cells (Fig. 1).

To study the effect of ZRSR2 deficiency on splicing in vivo, we performed initial analysis on total bone marrow cells from *Zrsr2* wild-type (WT) and KO male mice. Analysis of RNA-sequencing (RNA-seq) data revealed increased mis-splicing in the KO cells compared with WT cells. Subsequently, we performed RNA-seq on purified common myeloid progenitors (CMPs; $Lin^-Kit^+Sca1^-CD34^+Fc\gamma RII/III^{lo}$), granulocyte monocyte progenitors (GMPs; $Lin^-Kit^+Sca1^-CD34^+Fc\gamma RII/III^{hi}$), and megakaryocyte erythroid progenitors (MEPs; $Lin^-Kit^+Sca1^-CD34^-Fc\gamma RII/III^-$) from three WT and three KO littermates. Preliminary analysis of the data showed a trend towards increased retention of U12-type introns in *Zrsr2* KO mice compared to WT mice (Fig. 2), similar to our findings in human MDS samples.

1B. Different spliceosome mutations in MDS cause distinct alterations in splicing, affecting largely non-overlapping gene sets. To assess how splicing alterations in *ZRSR2* mutated cells compare with splicing alterations caused by other common spliceosome mutations, we performed RNA-seq on MDS bone marrow harboring mutations in either *ZRSR2*, *SF3B1*, *SRSF2* or *U2AF1* (4-8 samples each) in a single cohort. Analysis of splice junctions across all samples demonstrated distinct mis-splicing events specific to each mutant group (Fig. 3A). Data were analyzed using our in-house developed analysis pipeline, with a parameter called Mis-Splicing Index (Madan et al., Nat. Nat Commun. 2015). Mis-spliced junctions largely clustered together in samples within each mutant group, except for *U2AF1* mutants (Fig. 3A). We did not detect significantly mis-spliced genes common to all four mutant groups; however, we identified genes whose splicing was affected in more than one mutant group. For example, increased usage of a cryptic splice site in intron 9 of *EZH2* was noted in both *SRSF2* and *U2AF1* mutant groups (Fig. 3B, upper panel). This aberrant splicing leads to a premature stop codon. We validated higher proportion of aberrant *EZH2* transcripts in *SRSF2* and *U2AF1* mutant samples using qPCR (Fig. 3B, lower panel). These results suggest that *EZH2* is a target of both spliceosome mutations. Loss-of-function mutations of *EZH2* frequently occur in MDS (10–13%), and are associated with poor overall and progression-free survival. In addition, *EZH2* is underexpressed in MDS. Our findings suggest that abnormal splicing of *EZH2* (due to spliceosome mutations) provides an additional mechanism for downregulation of *EZH2* levels in MDS.

1C. To identify genes and pathways that synergize with ZRSR2 loss, we are performed synthetic lethal screens using *ZRSR2*-deficient and control TF1 and K562 cells. For these experiments we used two parallel screening platforms: 1) A library of siRNAs directed against the entire tyrosine kinome (91 family members) as well as N-RAS, K-RAS, and control scrambled siRNAs, 2) a small-molecule panel (~130 drugs) of FDA-approved or late-stage development compounds. The screens identified several potentially interesting sensitivities in *ZRSR2* knockdown cell lines. For example, the knockdown cells exhibited increased sensitivity to siRNA targeting ERBB3/4, and to inhibitors of the MAPK pathway (RAF265, an inhibitor of WT and oncogenic RAF, and CI-1040, a MEK1/2 inhibitor) (Fig. 4). Encouragingly, our pathway analysis of mis-spliced genes in association with *ZRSR2* mutations in MDS patients showed enrichment of genes in the MAPK and ERBB signaling pathways (Madan et al., Nat. Nat Commun. 2015). Interestingly, a germline *ERBB3* variant was identified as a candidate for predisposition to erythroid MDS/erythroleukemia (Braunstein et al., Leukemia. 2016). Our results suggest strong dependencies of *ZRSR2*-deficient cells on MAPK and ERBB pathways for their viability; and, importantly, MAPK and ERBB inhibitors may be effective in patient with *ZRSR2* mutations.

1D. Compared to normal cells, cells with splicing factor mutation are preferentially sensitive to further perturbation of splicing. Hence, patients with splicing factor mutations may benefit from splicing modulators. E7107 is a small-molecule spliceosome modulator that binds SF3B1 and blocks early spliceosome assembly (SF3B1 is part of both U2- and U12-type spliceosome). Increased anti-cancer activity of E7107 in cells with splicing factor mutations was demonstrated both in vitro and in vivo. Colony assays of TF1 cells with silencing of *ZRSR2* (two different shRNAs) showed moderate decreased growth in the presence of E7017 (at 1-2 nM).

Specific Aim 2: Study biological implications of loss of ZRSR2 in normal and MDS marrow cells.

2A. To investigate the role of ZRSR2 in normal hematopoiesis and leukemogenesis, we are performing a detail analysis of the hematopoietic compartments of *Zrsr2* KO mice, including peripheral blood cell counts, and flow cytometry analysis of the bone marrow. Our initial analysis of peripheral blood of young (8-weeks old) WT and *Zrsr2* KO male mice revealed that red blood cells (RBCs) and white blood cells (WBCs) counts were largely unaffected in KO male mice, however, KO RBCs exhibited higher mean corpuscular volume (MCV) compared with WT mice (Fig. 5). The results are also in line with our RNA-seq data that identified altered intron retention in genes involved in erythroid differentiation in *ZRSR2* mutant cells. Of note, myeloid differentiation, and particularly erythroid differentiation are characterized by dynamic intron retention events (Edwards et al., Nucleic Acids Res. 2016). Also, a recent study reported that cases with myeloid disorders (MDS and clonal cytopenia of undetermined significance [CCUS]) harboring isolated loss-of-function mutations of *ZRSR2,* were characterized by macrocytic anemia (Fleishman et al., Leukemia Research, 2017).

Thus, ZRSR2 might play a key role in normal as well as leukemic erythroid and granulomonocytic differentiation.

We performed comprehensive flow cytometric analysis of stem/progenitor, as well as mature blood cell populations of *Zrsr2* KO mice. Bone marrow cellularity was unchanged in young KO male mice compared with WT littermates. Our preliminary experiments show that the proportion and absolute numbers of hematopoietic stem cells (HSCs; $Lin^-Sca1^+cKit^+$ [LSKs]), including long-term (LT-HSC; $CD34^-FLT3^-$LSK) and short-term (ST-HSCs; $CD34^+FLT3^-$ LSK) cells, as well as myeloid precursors (consisting of CMPs, GMPs and MEPs) were not significantly altered in the absence of *Zrsr2* (Figs. 6 and 7A-B). Similarly, frequency of mature myeloid cells in bone marrow and spleen of *Zrsr2* KO mice were largely conserved (Fig. 7C).

2B. We began to assess the reconstitution potential of *Zrsr2* KO bone marrow cells by transplanting sorted *Zrsr2* KO HSCs in lethally irradiated congenic recipients. Our initial analysis of competitive repopulation assay at 4-weeks shows that the proportion of donor-derived ($CD45.2^+$) myeloid cells in recipients transplanted with *Zrsr2* KO cells is comparable with the WT cells (Fig. 7D).

2C. Splicing activity is orchestrated by the spliceosome, a large macromolecular complex composed of numerous splicing factors and auxiliary proteins. Our previous studies demonstrated a key role of ZRSR2 in splicing of U12-type introns. However, which proteins interact with ZRSR2 during spliceosome assembly to facilitate U12-type intron splicing, is largely unknown. ZRSR2 has been shown to interact with the U2AF1-U2AF2 heterodimer and SRSF2, and to function in the recognition of 3' splice sites. As these interactions are not specific to the U12-spliceosome, we hypostasized that other ZRSR2 binding partners might be required for its unique role in U12-splicing. To identify ZRSR2 binding partners, we used two parallel affinity purification approaches, combined with mass spectrometry:

1) BioID. This method relies on fusion of the protein of interest with a biotin ligase (BirA*) that biotinylates proximal proteins promiscuously.
2) FLAG-immunoprecipitation (IP). In this method a FLAG-tagged protein of interest is immuneprecipitated using a FLAG antibody.

The Flp-In T-REx 293 cell line allows moderate and tetracycline-inducible bait protein expression from isogenic cell clones. We transfected Flp-In T-REx 293 cells with either ZRSR2-BirA*-FLAG or BirA*-FLAG (control) vectors to establish stable and inducible isogenic cell lines with or without ZRSR2-tagged expression. Expression of ZRSR2-BirA*-FLAG fusion protein was verified in cells treated with doxycycline (tetracycline derivative) (Fig. 8A). For FLAG-IP experiments, stable Flp-In 293 T-Rex cells were treated with doxycycline for 24 hours, and cell lysates were harvested for FLAG pull-downs. For BioID experiments, cells were treated with doxycycline and biotin for 24 hours, lysates were prepared and used in streptavidin pull-downs (to detect biotinylated proteins). ZRSR2 binding proteins were identified by mass spectrometry. As expected, this analysis identified several splicing factors, including U2AF1, U2AF2 and members of the SF3B complex in ZRSR2 complexes. We validated ZRSR2 binding to U2AF2 and SF3B2 (component of SF3B complex) using co-IP and Western blot analyses (Fig. 8B). In addition, several putative novel interactions were detected. One of the interesting new candidates is C1QBP (also known as p32), a protein with roles in metabolism and tumorigenesis, which was shown to associate with the splicing factor SRSF1, and inhibit its RNA binding and phosphorylation. Binding of C1QBP to ZRSR2 was verified (Fig. 8B).

Specific Aim 3: Investigate the role of ZRSR2 in myeloid transformation using *Zrsr2/Tet2*-deficent murine model.

3A. In MDS, mutations of *ZRSR2* frequently co-occur with loss-of function mutations of *TET2*. To understand the cooperativity between these two genetic aberrations in the pathogenesis of myeloid disorders, we are obtaining *Zrsr2/Tet2* double KO (DKO) mice. We will assess hematopoiesis and development of leukemia in the in the DKO mice.

3B. To further assess gene expression and splicing changes caused by combined loss of *TET2* and *ZRSR2* in human cells, we performed RNA-seq on MDS bone marrow harboring either *ZRSR2* mutation alone, *TET2* mutation alone or *ZRSR2* + *TET2* mutations. We are currently analyzing the data using our Mis-Splicing Index.

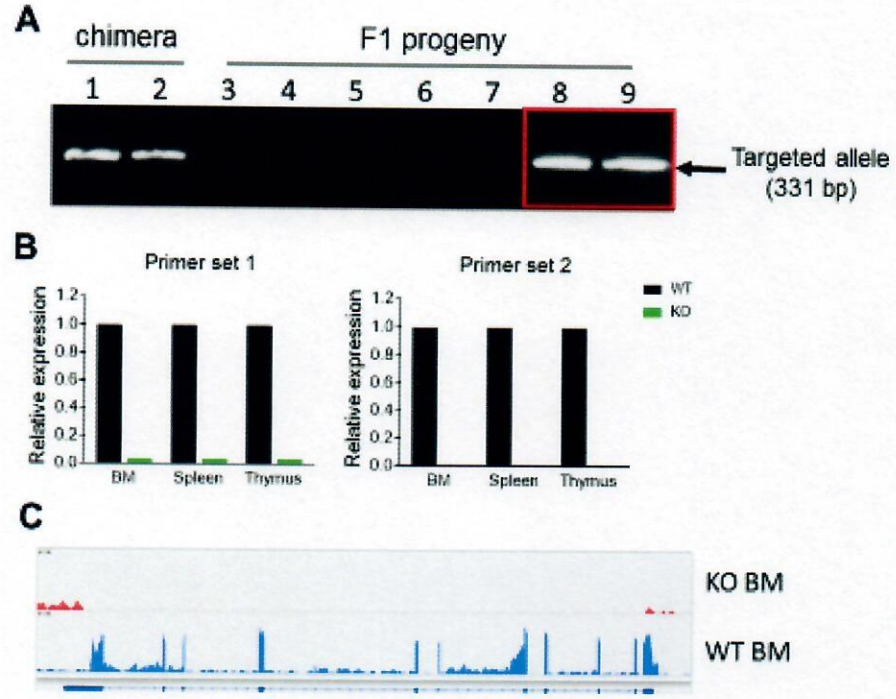

Figure 1. (A) Germline transmission of *Zrsr2* knockout (KO) allele. PCR analysis indicates band corresponding to deleted *Zrsr2* allele (331 bp) in offspring generated from backcross of chimera. (B) Quantitative PCR analysis for *Zrsr2* transcript levels using two primer pairs in bone marrow of wild-type (WT) and KO mice. (C) Integrative Genomics Viewer (IGV) snapshot (RNA-Seq) of *Zrsr2* locus in bone marrow cells from WT and *Zrsr2* KO male mice.

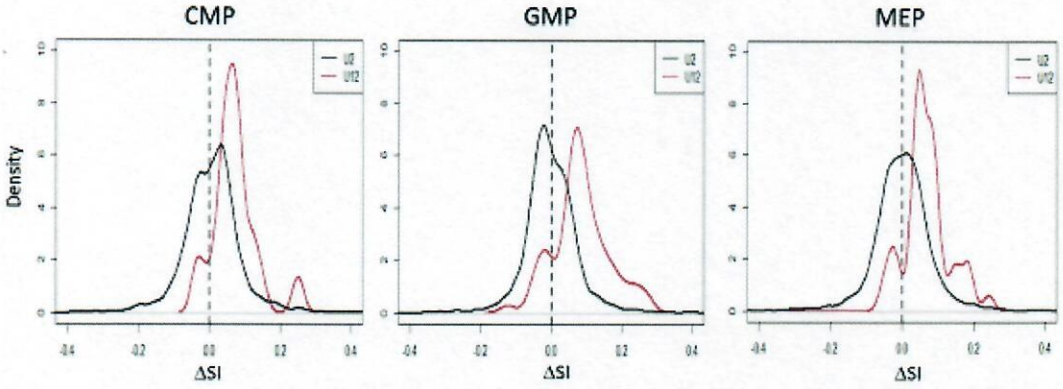

Figure 2. Density plots depict retention of U2- (black) and U12-type introns (red) in *Zrsr2* KO CMP, GMP and MEP cells compared with the wildtype cells. RNA-Seq was performed on sorted cells from three KO and WT littermates. ΔMSI; difference in Mis-Splicing Index.

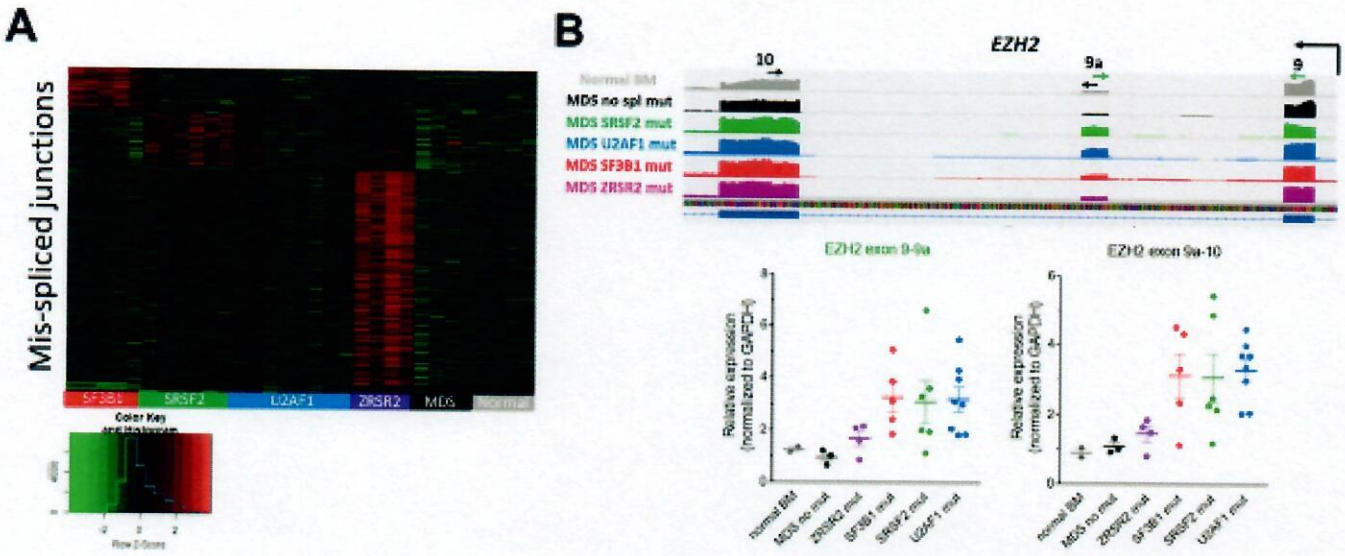

Figure 3. (A) Heat map depicts significantly mis-spliced junctions in MDS bone marrow harboring mutations of either *SF3B1*, *SRSF2*, *U2AF1* or *ZRSR2*, and control samples (Normal bone marrow and MDS without spliceosome mutations). (B) IGV image shows RNA-seq reads at *EZH2* locus in spliceosome mutant MDS and control samples (upper). qPCR validation of mis-splicing involving *EZH2* intron 9 (lower).

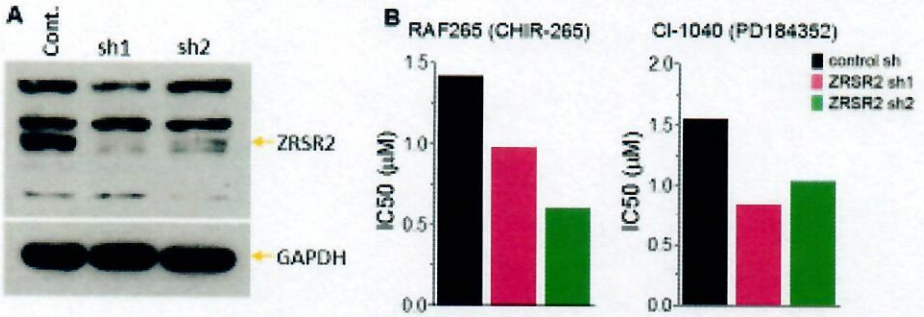

Figure 4. Increased sensitivity of *ZRSR2* TF1 knockdown cells to inhibitors in a small-molecule screen. (A) *ZRSR2* knockdown by western blotting in TF1 cells stably transduced with either control vector or *ZRSR2* shRNA vectors. GAPDH was used as endogenous loading control. (B) Shown are small-molecule screen results for RAF265 (an inhibitor of WT and V600E mutant BRAF) and CI-1040 (an ATP non-competitive MEK1/2 inhibitor).

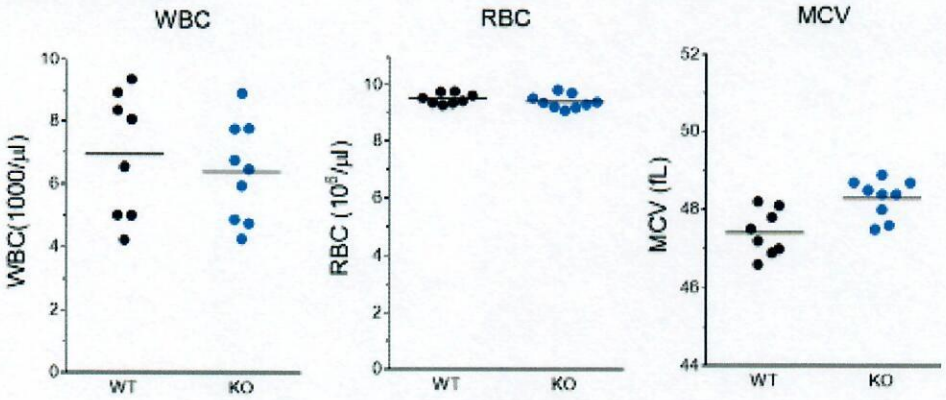

Figure 5. Number of WBCs, RBCs and mean corpuscular volume (MCV) of RBCs in the peripheral blood of 8-weeks old WT and *Zrsr2* KO male mice.

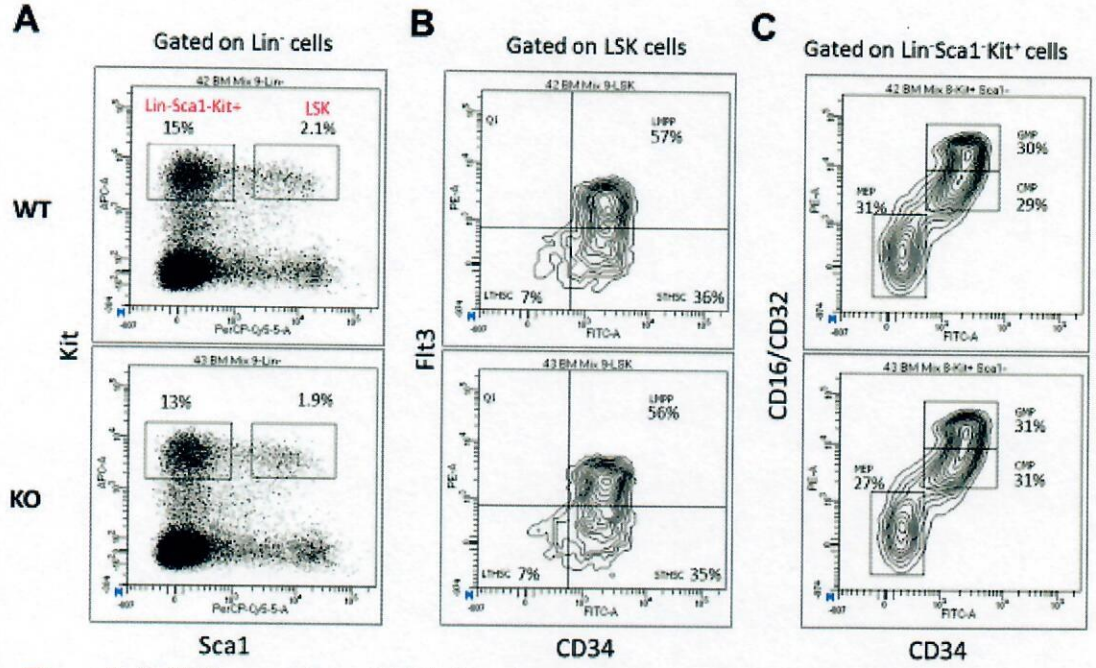

Figure 6. (A-C) Representative FACS plots show proportions of HSCs (A,B) and myeloid precursors (C) in the bone marrow of young WT and *Zrsr2* KO male mice.

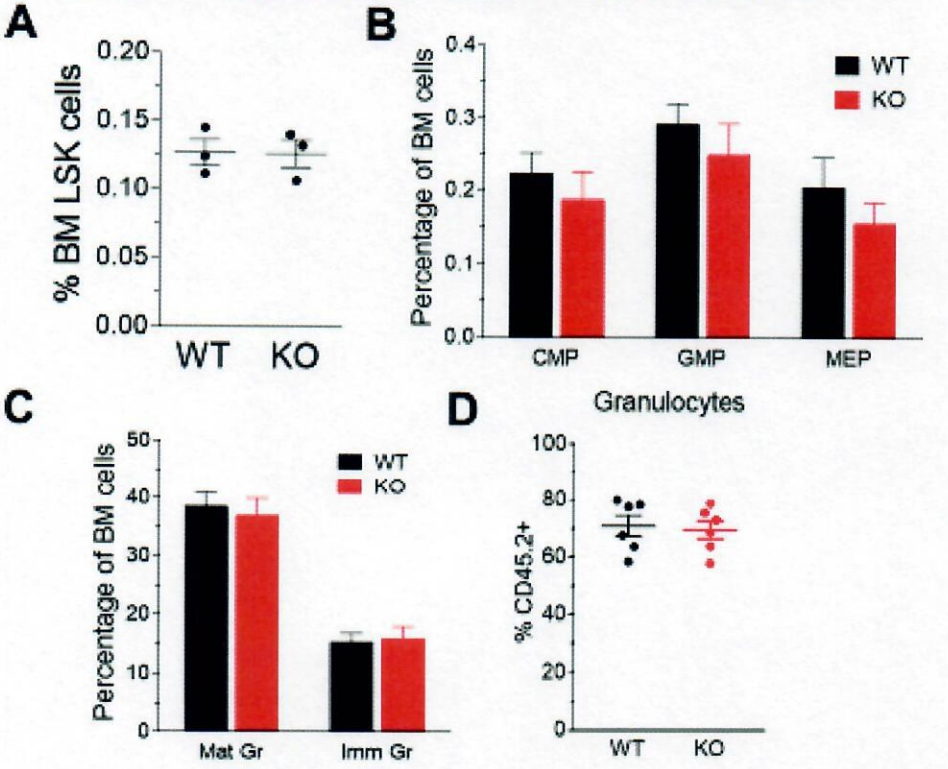

Figure 7. (A-B) Percentages of LSK (A) and myeloid precursors (CMP, GMP and MEP) (B) in the bone marrow of 8-weeks old WT and *Zrsr2* KO male mice. (C) Proportion of immature (Imm) and mature (Mat) granulocytes in the bone marrow of WT and *Zrsr2* KO male mice. (D) Competitive repopulation assay. Percentage of donor-derived (CD45.2+) granulocytes in peripheral blood of recipient mice 4 weeks after transplantation.

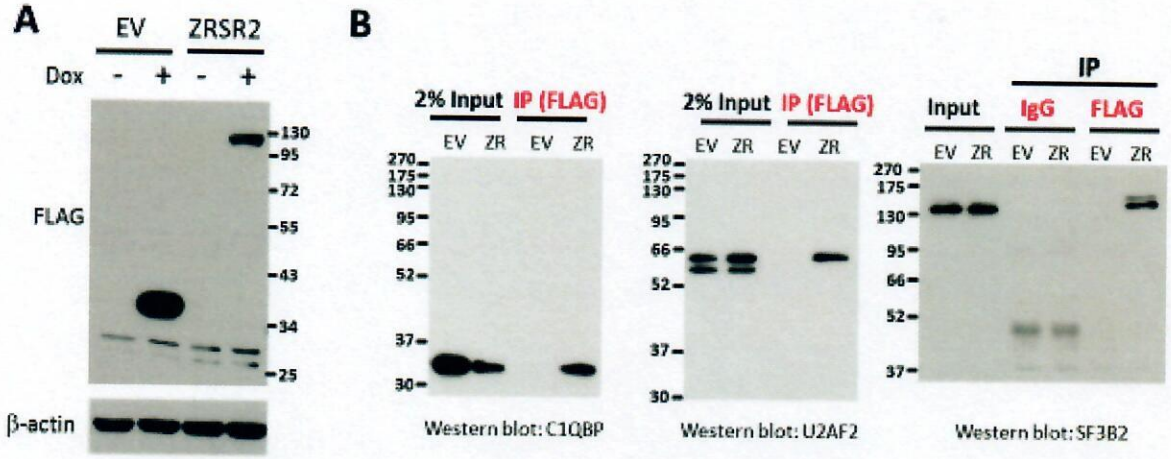

Figure 8. (A) Expression of ZRSR2-BirA-FLAG fusion in Flp-In-T-Rex 293 cells treated with 1 µg/ml doxycycline for 24 hours. Western blot was performed with FLAG antibody. EV: empty vector, Dox: doxycycline. (B) C1QBP, U2AF2 and SF3B2 interact with ZRSR2. Immunoprecipitation was performed using FLAG antibody on nuclear extracts from Flp-In-T-Rex 293 cells stably expressing ZRSR2-BirA-FLAG fusion (ZR) or empty vector (EV) 24 hours after induction with 1 µg/ml doxycycline. Blots were probed with either C1QBP (left), U2AF2 (center) or SF3B2 (right) antibodies.

- **Training and professional development opportunities**

Nothing to Report

- **Results dissemination**

Nothing to Report

- **Plans for the next reporting period**

Specific Aim 1: Determine how ZRSR2 mutations dysregulate downstream pathways, and contribute to molecular features of myelodysplastic cells.

1A. RNA-seq data of *Zrsr2* sorted KO vs WT myeloid cells will be analyzed. Since our earlier work pointed to the significance of U12-type introns in ZRSR2 mutant MDS, we will primarily focus on U12-type introns annotated in the U12 database (U12DB). We will select individual mis-spliced genes for validation studies that will include: 1) qualitative and quantitative isoform-specific PCR, 2) Sanger sequencing of full-length isoforms and the aberrant splice junctions, and 3) rescue experiments in 293T cells with *ZRSR2* knockdown/overexpression. These experiments will confirm alternative splicing of specific genes by ZRSR2. The murine RNA-seq data will be compared to our RNA-seq data from *ZRSR2* mutant MDS samples and *ZRSR2* knockdown TF1 cells, as well as published data (e.g. Gault CM et al., Proc Natl Acad Sci U S A. 2017). Identification of significantly dysregulated splicing events across different datasets will help prioritize potential key ZRSR2 target genes. To gain insights into the functional consequences of ZRSR2 loss, the lists of ZRSR2 splicing target genes, and differentially expressed genes will be submitted to Ingenuity Pathway Analysis (IPA, Qiagen), including Canonical Pathways, Causal Networks, and Diseases and Functions. This functional annotation will identify the biological pathways that are the most significant to cells with *ZRSR2* mutations. IPA software and data analysis are available to Cedars investigators. Statistical analysis and data mining will be done with the help of our longtime collaborator, Prof. H. Yang (Head of Bioinformatics Core, CSI, National University of Singapore), as well as Qian Yang (bioinformatician lab member) and the Biostatistics & Bioinformatics Core at Cedars Sinai Medical Center.

We will select several mis-spliced genes (~10) for further studies. We will use antisense oligonucleotides to induce mis-splicing in specific genes. We will design steric hindrance antisense oligonucleotides to induce specific intron retention events in selected ZRSR2 target genes. Antisense oligonucleotides will be

electroporatred into TF1 and K562 cells and their ability to trigger specific alternative splicing will be evaluated using PCR. Oligonucleotides-transfected cells will be studied for proliferation (Cell Titer Glo, Promega), differentiation (flow cytometry analysis in the presence of either EPO, GM-CSF or TPA), clonogenic potential, and apoptosis. As an alternative approach, we will design shRNAs to specifically silence selected isoforms in TF1 and K562, and study the cells as described above. Results will show whether induction of specific splicing events can mimic the phenotype of loss of ZRSR2. Additional studies will depend on the nature of the candidate gene and may include phosphorylation analysis (for kinases), ChIP-seq (for transcription factors and epigenetic modifiers) and specific pathway analysis.

1B. Analysis of RNA-seq data of MDS bone marrow harboring mutations in either *ZRSR2*, *SF3B1*, *SRSF2* or *U2AF1* will continue. We will validate candidate genes commonly mis-spliced in more than one mutant group. Subsequently, we will study the significance of shared mis-spliced genes in MDS. As we identified abnormal splicing of *EZH2* in *SRSF2* and *U2AF1* mutated cells, we are currently investigating the significance of this altered splicing in myeloid cells. Further analyses will focus on functional annotation of mis-spliced genes and identification of convergent biological pathways enriched in more than one spliceosome-mutant groups. These studies will provide new insights into pathogenesis of MDS and identify potentially new pathways for the design of novel therapeutic interventions.

1C. We will continue to validate and characterize candidate genes identified in our small-molecule and siRNA screens that can cooperate with loss of ZRSR2 to drive myelodysplasia. This analysis will provide insights into the underlying molecular mechanisms for MDS development in the context of *ZRSR2* mutations. Importantly, these studies have the added benefit of identifying clinically relevant drugs and drug targets in MDS.

1D. Additional splicing modulators (e.g. anticancer sulfonamides) and other rational drugs (e.g. NF-κB and HDAC inhibitors, anthracyclines) will be evaluated in TF1 cells, and other leukemic cell lines with *ZRSR2* knockdown. Promising drugs will be further tested in murine cells with *Zrsr2* KO (colony assays). In future experiments we will also evaluate the effect of splicing modulators on *Zrsr2* deficient cells in vivo, using our *Zrsr2* KO mice.

Specific Aim 2: Study biological implications of loss of ZRSR2 in normal and MDS marrow cells.

2A. As MDS is a disease associated with aging, it is plausible that *Zrsr2* KO mice will develop impaired myelopoiesis with increasing age. Therefore, we will assess blood cell counts, myeloid dysplasia and other parameters every 8-weeks in peripheral blood of *Zrsr2* KO male mice. We will also perform flow cytometric analysis on bone marrow HSCs/myeloid precursors in mice > 6-month, and > 1-year old to assess signs of myeloid hyperplasia and leukemic transformation.

2B. We will continue to evaluate the reconstitution potential of *Zrsr2* KO bone marrow cells by using both competitive and non-competitive repopulation assays with sorted *Zrsr2* KO HSCs from 8-, 12- and 16-week old mice. Further, we will examine the reconstitution every four weeks in peripheral blood. 16-weeks after transplantation, recipient mice will be euthanized and donor chimerism will be assessed in bone marrow and lymphoid organs. In addition, $CD45.2^{+}$ donor bone marrow cells will be sorted from the primary recipients and used for secondary competitive repopulation assay.

2C. We will continue to identify and characterize novel ZRSR2 binding partners. We will study the role of C1QBP and other novel interactors of ZRSR2 in ZRSR2-induced splicing by knocking down their expression in TF1 cells and performing: 1) PCR analysis on selected U12 and U2 introns and 2) RNA-seq for global splicing analysis. Our studies will help to characterize the interactome of ZRSR2 and reveal mechanisms associated with ZRSR2 unique functions in U12 introns splicing.

Specific Aim 3: Investigate the role of ZRSR2 in myeloid transformation using *Zrsr2/Tet2*-deficent murine model.

3A. *Tet2* deficiency leads to increased stem cell self-renewal and myeloproliferation in mice. *Zrsr2/Tet2* DKO mice will be utilized to determine how the loss of *Zrsr2* affects the myeloproliferative and increased stem cell activity observed in *Tet2* deficient mice. We will analyze the HSC and myeloid compartments of the mice using flow cytometry, as well as we perform competitive repopulation assays to examine HSC function. Blood cell counts, bone marrow cellularity and infiltration of hematopoietic cells in liver will be examined in DKO mice and compared with the single KO mice. Peripheral blood will be analyzed periodically for morphological dysplasia of circulating myeloid cells. *Zrsr2/Tet2* DKO mice will be monitored for at least 12 months for clinical signs of leukemia, and moribund mice will be analyzed. In addition, we will perform re-plating assays (methylcellulose colony assays) to determine if concomitant loss of *Zrsr2* and *Tet2* enhances the clonogenic potential of stem/progenitor *Zrsr2/Tet2* DKO cells in vitro.

To understand the molecular basis of cooperativity between RNA splicing and epigenetic pathways in MDS, we will examine splicing and expression changes in *Zrsr2/Tet2* DKO HSC/progenitor cells compared with single KO and WT controls using RNA-seq.

3B. We will perform Gene Ontology and Gene Set Enrichment Analysis (GSEA) on differentially expressed and differentially spliced genes in MDS samples with either *ZRSR2* mutation alone, *TET2* mutation alone or *ZRSR2* + *TET2* mutations. Results from the human and murine cells will be compared. This analysis will help identify crucial biological pathways affected by loss of both *ZRSR2* and *TET2*, which will provide insights into molecular pathogenesis of MDS harboring these mutations. More broadly, these studies will help decipher molecular links between the spliceosome and epigenetic machineries, the two most frequently altered pathways in MDS.

4. IMPACT

- **Impact on the development of the principal discipline(s) of the project**

Our work will help clarify the mechanistic basis for the pervasive nature of splicing disruption in MDS, leading to an overall better understanding of the disease. Over the longer term, this study may help guide design of novel therapeutic strategies that will improve the health and lives of patients with MDS and related diseases.

- **Impact on other disciplines**

Nothing to Report

- **Impact on technology transfer**

Nothing to Report

- **Impact on society beyond science and technology?**

Knowledge gained from this study may be harnessed to develop rational, effective treatments for MDS patients with splicing mutations.

5. CHANGES/PROBLEMS

To study consequences of *Zrsr2* deficiency in murine myeloid cells, we used three independent shRNAs to knockdown *Zrsr2* expression in flow cytometry sorted $Lin^{-}Kit^{+}$ (early myeloid precursors) murine bone marrow cells. Cells were cultured in the presence of IL3, IL6 and SCF and RNA was extracted for splicing and expression analysis. Although, a significant knockdown of *Zrsr2* transcript levels was observed, qPCR analysis showed only minor changes in splicing of U12-type introns. Lack of a substantial effect on splicing in *Zrsr2*-deficient myeloid precursors can possibly be due to either residual ZRSR2 levels (still ~50% of WT) or other compensatory mechanisms. As we obtained *Zrsr2* KO mice, we are using their bone marrow cells for murine cell studies.

To evaluate the effect of loss ZRSR2 on global protein isoforms, we performed a quantitative mass spectrometry analysis coupled with Stable Isotope Labeling by Amino acids in Cell culture (SILAC). However, in SILAC experiments with *ZRSR2*-deficient and control TF1 and K562 cells we did not detect major changes in protein abundance between *ZRSR2*-silnced and control cells. A likely explanation for this result is that *ZRSR2* deficiency induces changes in low abundance proteins such as signaling proteins and transcription factors. Moreover, it has been shown by us and others that only a relatively small fraction of alternatively spliced events is affected in cells with spliceosome mutations.

As an alternative approach to study the pathways that are downstream ZRSR2 and cooperate with loss of *ZRSR2* to drive MDS, we performed synthetic lethal screens against TF1 and K562 cells with knockdown of *ZRSR2*. A summary of our initial results is described under the accomplishment section. As shown, some of the positive hits from the screen correlate with our RNA-seq data on *ZRSR2* mutated MDS samples, suggesting that specific mis-splicing events (e.g. in ERBB genes) lead to altered protein expression. We will continue to validate the results and evaluate whether genes commonly identified in both the functional screens and RNA-seq have changes in their protein expression.

- **Changes that had a significant impact on expenditures**

Nothing to Report

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.**

Nothing to Report

6. PRODUCTS

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

H. Phillip Koeffler, PI "no change"

Sigal Gery, Research Scientist "no change"

Li Chen, Post-Doc "no change"

Name	Sulin Lim, Ph.D.
Project Role:	Post Doc
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Dr. Lim will analyze and validate the RNA-seq data. She will perform functional studies to evaluate the significance of ZRSR2 downstream targets in MDS. Will identify and characterize ZRSR2 binding partners. Will study the effect of candidate drugs on cells with loss of ZRSR2. She will study the role of ZRSR2 in myeloid transformation using *Zrsr2/Tet2*-deficent mouse model. Dr. Lin will be involved in experimental design, and manuscripts preparation.
Funding Support:	

Name	Dechen Lin, Ph.D.
Project Role:	Research Scientist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.2
Contribution to Project:	Dr. Lin will use antisense and shRNA to target specific mis-spicing events generated in ZRSR2 mutated cells. Will do animal work, including harvesting bone marrow and bone marrow transplantations. Will also perform in vitro functional studies with drugs and candidate genes. Dr. Lin will help with designing experiments, data analysis and manuscripts preparation.
Funding Support:	

8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report

9. APPENDICES

Nothing to Report