Concurrent Zrsr2 mutation and Tet2 loss promote myelodysplastic neoplasm in mice

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INTRODUCTION

Genetic mutations in splicing factors are observed in circa 50% of human myelodysplastic neoplasm (MDS) and about 45% of cases carry mutations in epigenetic regulators. Importantly, a subgroup of patients (25%) presents mutations in both splicing factors and epigenetic regulators, indicating possible cooperation among both functional categories in the pathomechanism of the disease.

The splicing factor ZRSR2 is mutated in about 5% of patients with MDS and appears associated with mutations in the epigenetic regulator TET2 [1]. ZRSR2 is implicated in the minor spliceosome and acts on U2-type introns (99.7% of introns in the human genome) as well as on U12-type introns (only present in approximately <0.3% of introns of all human genes) [2]. Mutations in ZRSR2 are very diverse and comprise nonsense, frameshift, missense, and splice-site mutations. While different hotspots are affected in other mutated splicing factors, mutations in ZRSR2 distribute across the entire coding region leading to protein loss-of-function [3]. ZRSR2 mutations affect predominantly males and cause abnormal splicing via intron retention of U12-dependent introns [4–7]. Apart from MDS, ZRSR2 mutations have also been reported in other hematological malignancies including chronic myelomonocytic leukemia (CMML) [3], myeloproliferative neoplasms (MPN) [3], and blastic plasmacytoid dendritic cell neoplasm (BPCDN) [8]. Functionally, shRNA-mediated ZRSR2 silencing in AML cell lines induced aberrant retention of U12-type introns, diminished cell growth, and altered myeloid differentiation [4]. Recently, two in vivo Zrsr2 knockout mouse models have been developed. Constitutive germline deletion of Zrsr2 in mice demonstrated that Zrsr2 is dispensable for hematopoietic development and that absence of a major phenotype is due to a Zsr1 compensatory mechanism [6]. In contrast, Zrsr2 hematopoietic conditional knockout mice resulted in enhanced hematopoietic stem cell (HSC) self-renewal in vitro and in vivo [7].

On the other hand, TET2 promotes DNA demethylation by oxidizing the methyl group to 5-hydroxymethylcytosine. TET2 is the most frequently mutated gene in MDS (25–35%) [9], and biallelic TET2 gene inactivation is frequently observed in myeloid neoplasms [10]. In addition, TET2 mutations are highly prevalent in clonal hematopoiesis of indeterminate potential [11], suggesting that TET2 lesions are early driver events with the potential to
predispose for further malignant transformation. TET2 is involved in the epigenetic control of gene regulation, HSC self-renewal, and myeloid lineage commitment [12]. Deletion of TET2 in mice leads to sporadic development of MDS/CMML-like disease [13–16] and co-expression of mutated TET2 with additional oncogenic mutations, including Axl1, EzH2, Jak2, c-Kit, Flt3, Bcor, Sf3b1, and $AML1:ETO$, promotes disease in mice [12, 17, 18]. A recent work explored the co-occurrence of Zrsr2 and Tet2 mutations in the context of hematopoiesis and MDS. Moreover, we identify global gene expression changes and aberrant mRNA splicing of key biological pathways. Notably, 20–25% of double-mutant mice developed a more severe phenotype characterized by cytopenias (thrombocytopenia with or without anemia), splenomegaly with extramedullary hematopoiesis, and multi-lineage dysplasia, signs reminiscent of MDS. Moreover, we identify global gene expression changes and aberrant mRNA splicing of key biological pathways. Taken together, we demonstrate that mutations in Zrsr2 and Tet2 promote MDS with reduced penetrance.

**MATERIALS AND METHODS**

**Animals**

Novel Zrsr2 mutant mice were created by CRISPR/Cas9 technology. In particular, exon 10 was targeted and a 17-nucleotide deletion was introduced, which generated a frameshift mutation [19]. Further details are provided in Supplementary information. Mice were then crossed to obtain homozygous mutants (Zrsr2<sup>+/−</sup> and Zrsr2<sup>−/−</sup>) and wild-type (Zrsr2<sup>+/+</sup>) animals. Both female and male Zrsr2 mutant mice were used for experiments. To simplify nomenclature, we referred to all of them with the female genotypes. To generate animals carrying alterations in both Zrsr2 and Tet2, Zrsr2 mutant mice were crossed with a Tet2 KO line previously described [20]. Both female and male double mutant animals were used for experiments. Mice were genotyped by standard PCR using Zrsr2 and Tet2 specific primers (Supplementary Table 1). CD45.1 mice, used as recipients in transplantation experiments, were purchased from Charles Rivers (Wilmington, MA, USA). Pilot preliminary experiments were performed for sample size estimates. No animals were excluded from the analysis. Regarding randomization, in all cases where multiple experimental groups were analyzed, mice were not randomly distributed but rather allocated in such a way that each group was evenly matched with regard to age range and frequency of mice of each sex. Investigators were not blinded to the mouse genotypes. This study was approved by the Regional Vaenical Ministry of Agriculture (permit 2017/7SC/PEA/00200) and the Ethics Committee at IIS La Fe (permit 2016/0756).

**Flow cytometry analysis and cell sorting**

Mice were sacrificed, autopsied, and bones (femurs, tibias, and cristae), spleen, and thymus were collected for further analysis. Red blood cells were lysed with Gey’s Solution (NH4Cl 8.3 g/L, NaHCO3 1.0 g/L, EDTA 37 mg/L) and RBC-depleted cells were stained using monoclonal antibodies for 15 min at 4 °C. PB samples were lysed with BD FACSTM Lysing Solution (BD, Franklin Lakes, NJ, USA) and stained with monoclonal antibodies for 15 min at 4 °C. Lin ‘Sca-1’-c-Kit’- (LSK) cells were sorted from a pool of 3 mice for whole transcriptome analysis and transplantation assays. BM cells were lysed with Gey’s solution, stained with anti-mouse CD117 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and enriched using LS columns (Miltenyi Biotec). c-Kit<sup>+</sup> cells were subsequently stained with monoclonal antibodies for cell separation. Please refer to the Supplementary Information for additional details on antibodies and marker combinations.

**In vivo transplantation assays**

For HSPC transplantations, LSK cells were FACs-sorted from a pool of 3 donor mice, and 8000 LSK cells (CD45.2) were transplanted along with 2×10<sup>5</sup> support unfractionated bone marrow cells (CD45.1) into each lethally irradiated (12 Gray, split dose) mouse (CD45.1). Recipient mice were given enrofloxacin (Bayer, Leverkusen, Germany) in the drinking water for 4 weeks post-irradiation. PB engraftment was monitored by flow cytometry.

**Gene expression analysis by RT-qPCR**

Total RNA was extracted by RNeasy Mini Kit (QIAGEN, Hilden, Germany) from 10×10<sup>6</sup> bone marrow or spleen cells previously lysed in RLT buffer (QIAGEN) supplemented with 1% β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) and stored at −80 °C. RNA was treated with DNA-free DNase Treatment and Removal Reagents (Ambion, Austin, TX, USA). DNase-treated total RNA (1 μg) was reverse transcribed with oligo(dT) and TaqMan Reverse Transcription Reagents (Thermo Fisher Scientific, Waltham, MA, USA). The resulting cDNA was diluted tenfold before use. Real-time quantitative PCR was performed in technical triplicates using AceQ...
SYBR qPCR Master Mix (Vazyme Biotech Co., Nanjing, China) and the corresponding primers (Supplementary Table 1) using a Viia 7 Real-Time PCR System (Thermo Fisher Scientific). All RT-qPCR analyses were performed on an Applied Biosystems QuantStudio Software V1.3 (Thermo Fisher Scientific). Primer set efficiency was firstly calculated in a standard curve experiment. Relative gene expression levels were calculated using the comparative Ct method. Since target and housekeeping primer set efficiencies differed >10%, we applied the corrective formula described in [21] to calculate the relative expression ratio. Gene expression levels were then normalized to that of Hprt.

**RNA sequencing**

LSK cells (50,000 per sample) from pools of WT (n = 4), Zrsr2m/m (n = 5), Tet2−/− (n = 5), and Zrsr2m/mTet2−/− (n = 6) were sorted into RLT buffer supplemented with 1% β-mercaptoethanol. Total RNA extraction was performed using an RNEasy Micro kit (QIAGEN). DNA was then removed using on-column DNase treatment. RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Samples with an RNA Integrity Number value ≥7 were selected for further analysis. Low input directional RNA-seq libraries were prepared using NEBNext Ultra Directional RNA Library Prep Kit for Illumina (Illumina Inc., Cat N°. 7420, San Diego, CA, USA). Puriﬁed libraries were paired-end sequenced on the Illumina NovaSeq 6000 (Novogene, Beijing, China).

The quality of raw RNA-seq data was assessed in fastq data ﬁles using Fastp. Reads were mapped to the reference mouse genome GRCh 38 (mm10), with genome annotations from GENCODE vM24, using the splicing aware mapping software STAR v2.5 [22]. Quantification of reads was calculated with HTSeq v0.6.1 [23]. Differential gene expression was determined using DESeq2 v2.16.3 [24] with adjusted P value ≤0.05,

**Fig. 2** Zrsr2 mutation and Tet2 loss expand LT-HSC and impair myeloid-erythroid differentiation. a Flow cytometry analysis of the LSK compartment from 3-months-old WT (n = 21), Zrsr2m/m (n = 18), Tet2−/− (n = 9), and Zrsr2m/mTet2−/− mice (n = 18). Representative dot plots are shown in Supplementary Fig. 2. b Flow cytometry analysis of myeloid-erythroid progenitors from 3-months-old WT (n = 21), Zrsr2m/m (n = 18), Tet2−/− (n = 9), and Zrsr2m/mTet2−/− (n = 18) mice. Representative dot plots are shown in Supplementary Fig. 2. c Left and middle panel: GM-CFU and BFU-E forming capacity of unﬁnated BM from 3-month-old WT (n = 18), Zrsr2m/m (n = 12), Tet2−/− (n = 10), and Zrsr2m/m Tet2−/− (n = 17) mice. Right panel: Serial replating capacity of unﬁnated BM from 3-months-old WT (n = 3), Zrsr2m/m (n = 3), Tet2−/− (n = 3), and Zrsr2m/mTet2−/− (n = 6) mice cultured under myeloid conditions. Data represent mean ± SEM.
RESULTS

Development of a germline Zrsr2 mutant mouse

To gain insight into the role of Zrsr2 in normal and malignant hematopoiesis, we generated a mutant allele targeting Zrsr2 in vivo by CRISPR/Cas9 technology (Fig. 1a). This approach generated a 17-nucleotide deletion in exon 10 of Zrsr2 DNA sequence (Supplementary Fig. 1a), which resulted in a frameshift mutation. Consequently, the mutated Zrsr2 allele lacks the second zinc finger domain and the serine-rich domain (Fig. 1a). Seventeen nucleotide deletion was confirmed in different hematopoietic tissues (Supplementary Fig. 1b). Mutant bone marrow and spleen cells expressed levels of Zrsr2 mRNA equivalent to WT controls, suggesting that the mutated allele escapes the nonsense-mediated mRNA decay (Fig. 1b). In addition, 17-nucleotide deletion, in turn, caused skipping of exon 10, spliced event that was confirmed by RT-PCR in cDNA from LSK cells (Supplementary Fig. 1c). Due to difficulties to detect ZRSR2 protein in murine tissue, we tagged the WT and truncated mutant protein with FLAG and over-expressed it in HEK293T cells. Both WT and mutant ZRSR2 forms were detected, indicating that the truncated protein product is stable (Fig. 1c). Hemizygous Zrsr2m/y or homozygous Zrsr2m/mTet2 mice were mated with Tet2 knockout mice to produce Zrsr2m/mTet2−/− double mutant mice. Zrsr2 mutant mice had a normal lifespan (24 months) while double mutant mice died at earlier ages (average 14 months). Zrsr2m/m females were infertile.

Zrsr2 mutation and Tet2 loss expand LT-HSC and MPP2 compartments and impair myelo-erythroid progenitor differentiation in steady-state

We analyzed the stem and myelo-erythroid progenitor compartments at 12-13 weeks. Fractionation of the HSC (LSK) compartment using CD150 and CD48 expression showed expansion of some phenotypic HSC subsets, such as the long-term HSC (LT-HSC, LSK CD150−CD48−) and the multipotent MPP2 progenitors (LSK CD150+CD48−, erythroid, and megakaryocyte biased progenitors) [29, 30] (Fig. 2a, Supplementary Fig. 2a). At the progenitor level, we observed a significant increase in the early precursors of granulo-monocytic (PreGM) and erythroid (PreCFU-E) progenitors, an increase in megakaryocytic progenitors (MkP) and a decrease in the more committed granulo-monocytic (GMP), and erythroid (CFU-E) progenitors, suggestive of a differentiation block at the early stages of myelo-erythroid lineage commitment [31] (Fig. 2b, Supplementary Fig. 2b). These phenotypic alterations were accompanied by an increase in GM-CFU and BFU-E colony-forming units in Zrsr2m/mTet2−/− mice (Fig. 2c). Zrsr2m/mTet2−/− myeloid progenitors exhibited properties of self-renewal as

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Fig. 3 Concomitant Zrsr2 mutation and Tet2 loss result in PB cytopenias and multilineage dysplasia. a Hematological parameters from 3-month-old WT (n = 28), Zrsr2m/m (n = 21), Tet2−/− (n = 12), and Zrsr2m/mTet2−/− mice (n = 26). WBC: white blood cells, RBC: red blood cells, HGB: hemoglobin, PLT: platelets. Boxes show mice presenting cytopenias. b Peripheral blood smears and bone marrow cytopsins from 3-month-old WT and Zrsr2m/mTet2−/− mice stained with May-Grünwald Giemsa. Normal RBC from WT (Ai) and dysplastic RBC from Zrsr2m/mTet2−/− mice (Aii) showing RBC anisocytosis, polychromasia, and presence of nucleated erythrocytes. Normal platelets from WT (Bi) and dysplastic platelets from Zrsr2m/mTet2−/− mice (Bii) showing anisocytosis and giant size. Normal neutrophil from WT (Ci), hypersegmented neutrophil (Cii) and hyposegmented neutrophil with pseudo Pelger-Huët anomalies (Di-Dii) from Zrsr2m/mTet2−/− mice. Nuclear fragmentation and karyorrhexis of granulocytes (Ei-Eii). Erythrophagocytosis (Fi) and binucleated erythroid precursors (Fii) in BM of Zrsr2m/mTet2−/− mice. Normal megakaryocyte precursors from WT (Gi) and dysplastic megakaryocyte precursors with cytoplasm vacuolization from Zrsr2m/mTet2−/− mice (Gii). Normal megakaryocyte in WT (Hi) and giant megakaryocyte in Zrsr2m/mTet2−/− mice (Hii). Dysplastic features are indicated with arrows.
Notably, within the cohort of Zrsr2<sup>−/−</sup>Tet2<sup>−/−</sup> mice analyzed, a subset of mice (25%; 6 out of 24) showed a more aggressive hematological phenotype. These mice presented cytopenias (thrombocytopenia with or without anemia) (Fig. 3a). Evaluation of peripheral blood phenotype. These mice presented cytopenias (thrombocytopenia of mice (25%, 6 out of 24) showed a more aggressive hematological dysplasia was present in the form of neutrophil hypersegmentation and splenic anomalies (Fig. 3b/Di, Dii), nuclear fragmentation and karyorrhexis of neutrophils, and abnormal vacuolization in myeloid precursors (Fig. 3b/Gii). Macroscopic analysis of Zrsr2<sup>−/−</sup>Tet2<sup>−/−</sup> spleens showed splenomegaly (Fig. 4a). Histopathological analysis of splenic sections demonstrated disruption of red and white pulp in Zrsr2<sup>−/−</sup>Tet2<sup>−/−</sup> mice (Fig. 4a). A closer examination revealed infiltration by megakaryocyte and erythroid (Ter119<sup>+</sup>) cells and a decrease in B cells (B220<sup>+</sup>) as assessed by flow cytometry (Fig. 4b). Signs of extramedullary hematopoiesis were evidenced by the presence of GM-CFU and BFU-E activity in the spleen of Zrsr2<sup>−/−</sup>Tet2<sup>−/−</sup> mice (Fig. 4c). Overall, a subset of Zrsr2<sup>−/−</sup>Tet2<sup>−/−</sup> mice showed features consistent with myelodysplasia [32].

Since MDS is a disease of the elderly, we also analyzed the impact of mutations in aged control (24 months), Zrsr2<sup>−/−</sup> (24 months), and Zrsr2<sup>−/−</sup>Tet2<sup>−/−</sup> mice (18 months). Old Zrsr2<sup>−/−</sup>mice presented anemia and one out three cases had leukocytosis, thrombocytopenia, and splenomegaly with disrupted spleen histology. Zrsr2<sup>−/−</sup>Tet2<sup>−/−</sup> mice displayed anemia and thrombocytopenia (Supplementary Fig. 3a), and exhibited erythroid and myeloid dysplasia (Supplementary Fig. 3b). This was accompanied by splenomegaly with disrupted splenic architecture (Supplementary Fig. 3c, d). Notably, aged Zrsr2<sup>−/−</sup>Tet2<sup>−/−</sup> mice were analyzed at earlier time-points (18 months) due to evident signs of illness (reduced mobility,

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Zrsr2 mutation and Tet2 loss in HSPC enhance BM repopulation capacity and cause cell-autonomous hematopoietic alterations

Since there is evidence of HSPC involvement in MDS, we evaluated hematopoietic stem cell function in Zrsr2 mutant and Zrsr2<sup>−/−</sup> Tet2<sup>−/−</sup> mice. We performed transplantation assays using 8000 LSK cells (CD45.2) along with 2 x 10<sup>5</sup> support unfractonated bone marrow cells (CD45.1) (Fig. 5a). 8–12 weeks after transplantation, the reconstitution of the recipient (CD45.1) hematopoietic system by the donor cells was verified by flow cytometric analysis of the peripheral blood. We observed that Zrsr2<sup>−/−</sup> Tet2<sup>−/−</sup> cells displayed an increased repopulation capacity and retained multilineage reconstitution ability (Supplementary Fig. 4a). In the donor-derived CD45.2+ fraction, we detected an increase in erythroid-megakaryocytic biased MPP2 progenitors and a decrease in PreMegE, suggestive of a differentiation block along the erythroid lineage at an earlier step (Supplementary Fig. 4b, c). As occurred in steady-state, we also observed an accumulation of PreGM progenitors and a decrease in GMP (Supplementary Fig. 4c). LT-HSC were reduced, suggesting a push of LT-HSC towards more committed HSPC in a transplantation context. Further, Zrsr2<sup>−/−</sup> Tet2<sup>−/−</sup> mice presented cytopenias (anemia and thrombocytopenia) (Fig. 5b) and myeloid and erythroid dysplasia (Fig. 5c). Remarkably, transplanted Zrsr2<sup>−/−</sup> Tet2<sup>−/−</sup> animals were moribund at the time of sacrifice (15 months post-transplantation).

Whole transcriptome sequencing in HSPC shows an alteration of lineage-specific hematopoietic genes and an enrichment of inflammatory pathways

Next, we performed RNA sequencing experiments to get insight into the molecular changes of Zrsr2<sup>−/−</sup> Tet2<sup>−/−</sup> HSPC. Transcriptome analysis resulted in distinct gene expression signatures (Fig. 6a). Differential gene expression analysis in pair-wise comparisons against WT control by DESeq2 [24] yielded 571 differentially expressed genes (DEG) in Zrsr2<sup>−/−</sup> Tet2<sup>−/−</sup> mutant, 1203 DEG in Zrsr2<sup>−/−</sup> and 2952 DEG in Zrsr2<sup>−/−</sup> Tet2<sup>−/−</sup> LSK cells (adjusted P-value ≤ 0.05). To evaluate the impact of the combined dysfunction of Zrsr2 and Tet2...
on DNA methylation, we performed global epigenomic profiling of WT, Tet2−/−, and Zrsr2m/mTet2−/− mice using Infinium Mouse Methylation Bead Chip array (285K, Illumina). Principal component analyses (PCA) revealed that Zrsr2m/mTet2−/− and Tet2−/− hematopoietic cells had distinct epigenomic profiles from those of WT (Supplementary Fig. 5a). We observed that 8747 CpG, corresponding to 531 murine genes, were found differentially methylated in Zrsr2m/mTet2−/− cells in comparison to WT cells (Supplementary Table 2). Next, we performed a Venn diagram analysis to identify the overlapping genes in both transcriptomic and epigenomic analyses. We detected that circa 3% of DEG presented alterations in DNA methylation levels (Supplementary Fig. 5b, Supplementary Table 4a).

Within Zrsr2m/mTet2−/− LSK, 1327 genes were up-regulated and 1625 genes were down-regulated. Molecularly, Zrsr2m/mTet2−/− LSK were primed towards a myeloid fate since transcripts related to common lymphoid progenitors (MPP2) within the LSK compartment. Enrichment with the increased proportion of erythroid-megakaryocytic biased erythroid priming originates in the immature HSPC in concordance with potential deregulated by DNA methylation changes (Supplementary pathway were down-regulated (Fig. 6b) while transcripts associated with inflammation, 

**Fig. 6 Gene expression dysregulation in Zrsr2m/mTet2−/− LSK cells leads to transcriptional myeloid priming and enrichment of inflammatory pathways.** a Heatmap visualizing the differentially expressed genes (DEG) identified in WT, Zrsr2m/mTet2−/−, and Zrsr2m/mTet2−/− LSK cells from 3-months-old mice (red, up-regulated genes; blue, down-regulated genes). n = 4-6 replicates/genotype. b Gene expression levels of CLP, PreGM, and PreMegE and MkP associated genes. c KEGG enrichment analysis of DEG in LSK from WT vs Zrsr2m/mTet2−/− mice.
spliced genes (Supplementary Fig. 7b). We found that the MAPK kinase family and the Fanconi anemia pathway were the most affected targets by mRNA aberrant splicing in Zrsr2m/mTet2−/−. Importantly, a total of nine genes related to the MAPK pathway were identified as mis-spliced, from which we validated 3, Dusp1, Tgfbr2, and Fgf11 (Fig. 7b). Strikingly, we observed that these targets converged into a particular kinase, the MAPK14 (Fig. 7c). We also identified and corroborated exon skipping events in Per1 and Tbk2 (in Zrsr2m/mTet2−/−), and Frrs1 (in Zrsr2m/m) transcripts, involved in circadian regulation, cell migration/motility, and iron metabolism, respectively (Supplementary Fig. 7c). Remarkably, mis-splicing of these targets was also found in human ZRSR2 and ZRSR2-TET2 co-mutated samples (unpublished observations).

DISCUSSION

Here we show that mutations of Zsr2 and Tet2, genes commonly co-mutated in human MDS, resulted in impairment of myeloid-erythroid differentiation and the development of thrombocytopenia and anemia, multi-lineage dysplasia, and splenomegaly with extramedullary hematopoiesis. These features, manifested with incomplete penetrance, are compatible with MDS [32]. Further, Zsr2 and Tet2 mutations were associated with an expansion of LT-HSC and MPP2 compartments and an increase of CFUs, with CFU-GM cells acquiring self-renewal abilities. Moreover, the phenotype observed in steady-state mice was reproduced in transplanted mice, thus indicating that these alterations were cell-autonomous. Altogether, Zsr2 and Tet2 mutations cause a phenotype that recapitulates critical features of myelodysplasia.

Prior studies exploring in vivo Zsr2 function in hematopoiesis provided somewhat contrasting results. Zsr2 constitutive knockout mice exhibited normal myeloid development and regular repopulating ability [6]. Data from mice with hematopoietic-specific conditional deletion of Zsr2 showed that Zsr2 loss promotes HSC self-renewal [7]. In our study, young adult mice expressing truncated ZSR2 (Zrsr2m/m) exhibited normal myeloid development and regular repopulating ability, similar to the phenotype described in Zsr2 constitutive knockout [6]. However, old Zsr2m/m mice did display several hematological alterations, such as reduced RBC and hemoglobin levels akin to patients with isolated ZSR2 mutations showing refractory macrocytic anemias [35]. One aspect for which our model differs from previous studies is
that we did not generate a knockout but a stable truncated protein. Many Zrsr2 mutations observed in the clinics are likely analogous to the Zrsr2 mutant allele that we describe here in that they generate a loss of the C-terminal region [3]. Other models of MDS based on mutations in recurrently mutated genes have been described. Sfib1<sup>+/−<sup>fl</sup>Tet2<sup>−/−</sup> mice [18] showed similar characteristics to the ones we report here, such as anemia, expansion of the LT-HSC compartment, and erythroid and megakaryocyte dysplasia. Other studies recapitrating MDS/MPN-like disease also showed splenomegaly with extramedullary hematopoiesis [36]. Loss of Tet2 itself promotes myeloid-biased hematopoiesis and HSC self-renewal [15]. Correspondingly, we show that concurrent deletion of Tet2 enhanced myeloid-biased differentiation, as reflected by increased MPP2 progenitors and increased myelo-erythroid lineage priming in LSK HSCP. Further, Tet2 loss confers growth advantage and self-renewal capacity to Zrsr2 mutant progenitors, as evidenced by clonogenic and serial replating assays. This increased output of colonies has also been shown in an Asxl1<sup>−/−</sup>Tet2<sup>−/−</sup> model [37].

In the transplantation setting, Zrsr2<sup>−/−</sup>Tet2<sup>−/−</sup> cells showed higher repopulation capacity than Zrsr2 mutant and WT cells, in accordance with the clonal advantage typical of MDS HSC. In addition, we observed an increased reconstitution to megakaryocytic-erythroid biased MPP2 progenitors, LSK, and PreGM in mice transplanted with Zrsr2<sup>−/−</sup>Tet2<sup>−/−</sup> cells. Similarly, previous work in bone marrow chimeras transplanted with Zrsr2 and Tet2 double knockout cells reported an increase in LSK and myeloid progenitors (CMP, GMP, and MEP) [8].

Deregulated DNA methylation is one of the hallmarks of MDS [38], although in our study only circa 3% of genes that were differentially expressed in Zrsr2<sup>−/−</sup>Tet2<sup>−/−</sup> LSK may potentially be regulated by an epigenetic mechanism such as DNA methylation. Importantly, transcriptional profiling followed by GO enrichment analysis indicated that genes upregulated in Zrsr2<sup>−/−</sup>Tet2<sup>−/−</sup> LSK were associated with ribosome, inflammation, and cell migration/motility. Interestingly, alterations in ribosome biogenesis are associated with MDS and defective erythropoiesis [39, 40]. On the other hand, deregulation of inflammatory signaling plays a central role in the pathogenesis of MDS [34]. In fact, MDS patients present higher levels of pro-inflammatory cytokines in serum [41]. In this regard, splicing factor mutations and Tet2 deficiency have been associated with increased inflammatory cytokine production [42, 43]. Cytokine multiplex assay reveals that Zrsr2<sup>−/−</sup>Tet2<sup>−/−</sup> mice with more aggressive MDS phenotype display higher levels of pro-inflammatory cytokines. This suggests a possible role of inflammation favoring MDS.

Since mutations in ZRSR2 alter RNA splicing and HSC are the cell-of-origin of MDS, we performed an alternative splicing analysis in LSK cells. Unexpectedly, this analysis showed that exon skipping but not intron retention was the category most affected in Zrsr2 mutant mice. This contrasts with most of the literature associating ZRSR2 to intron retention of U12-type introns [4–8]. However, our data are in agreement with one study reporting that ZRSR2 mutations in humans cause more exon skipping than intron retention [44]. One of the most affected pathways by mis-splicing in Zrsr2<sup>−/−</sup>Tet2<sup>−/−</sup> LSK cells was MAPK signaling. Among the nine genes that were found to be aberrantly spliced in this pathway, we confirmed Dusp1, Tgfb2, and Fgfg1. Interestingly, we found that these MAPK-related targets converged into MAPK14, an essential kinase for definitive erythropoiesis in mice [45]. In addition, MAPK14 has a key role in regulating pro-inflammatory cytokine production via MK2 and MSK1/2. Aberrant mRNA splicing in this pathway, thus, may deregulate MAPK14 function and explain, in combination with other transcriptional changes, the level of pro-inflammatory cytokines found in Zrsr2<sup>−/−</sup>Tet2<sup>−/−</sup> mice. This finding coincides with previous work showing MAPK14 as a down-regulated target downstream of ZRSR2 mutation [6].

Collectively, these data provide a unique insight into the mechanisms by which Zrsr2 and Tet2 mutations affect MDS pathogenesis. Our results indicate that concomitant mutations in Zrsr2 and Tet2 can initiate MDS in mice. We identify alterations in HSC function and myelo-erythroid differentiation as well as transcriptional and splicing changes in relevant biological pathways (MAPK family, Fcanci Anemia pathway) that may contribute to MDS tumorigenesis. Given these findings, MAPK modulators, anti-inflammatory agents, and drugs aiming to restore normal myelo-erythroid differentiation may be tested in our model. This is particularly important given the unmet clinical need to find new curative approaches beyond HSCP transplantation. Since co-occurrence of ZRSR2 and TET2 mutations have also been observed in BPDCN, our model could also be exploited to explore the biology of dendritic leukemia. Overall, this study expands on mouse models based on recurrent mutations in splicing and epigenetic regulators and offers an opportunity to elucidate further the mechanisms governing tumorigenesis in minor spliceosome-mutated MDS.

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AUTHOR CONTRIBUTIONS

ASP designed the study; CGR and ASP designed experiments; CGR and CMV performed experiments; AGA, RFG and EP generated the mouse lines; CGR, CMV, LC, JS, and ASP analyzed data; AL, JC, JS and AGA performed critical reading of the manuscript; CGR and ASP wrote the manuscript; ASP supervised the study.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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