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ORIGINAL ARTICLE

Hemopoietic-specific Sf3b1-K700E knock-in mice display the splicing defect seen in human MDS but develop anemia without ring sideroblasts

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Heterozygous somatic mutations affecting the spliceosome gene Sf3b1 drive age-related clonal hematopoiesis, myelodysplastic syndromes (MDS) and other neoplasms. To study their role in such disorders, we generated knock-in mice with hemopoietic-specific expression of Sf3b1-K700E, the commonest type of Sf3b1 mutation in MDS. Sf3b1-K700E/− animals had impaired erythropoiesis and progressive anemia without ringed sideroblasts, as well as reduced hematopoietic stem cell numbers and host-repopulating fitness. To understand the molecular basis of these observations, we analyzed global RNA splicing in Sf3b1-K700E/− hematopoietic cells. Aberrant splicing was associated with the usage of cryptic 3′ splice and branchpoint sites, as described for human Sf3b1 mutants. However, we found a little overlap between aberrantly spliced mRNAs in mouse versus human, suggesting that anemia may be a consequence of globally disrupted splicing. Furthermore, the murine orthologues of genes associated with ring sideroblasts in human MDS, including Abcb7 and Tmem14c, were not aberrantly spliced in Sf3b1-K700E/− mice. Our findings demonstrate that, despite significant differences in affected transcripts, there is overlap in the phenotypes associated with Sf3b1-K700E between human and mouse. Future studies should focus on understanding the basis of these similarities and differences as a means of deciphering the consequences of spliceosome gene mutations in MDS.

INTRODUCTION

The myelodysplastic syndromes (MDS) are clonal blood disorders characterized by dysplastic hematopoiesis and blood cytopenias. Somatic mutations affecting the genes Sf3b1, Srsf2, U2af1, Zrsb2 and Luc7l2, which code for components of the spliceosome, are found in more than half of MDS patients. SF3B1 mutations are the commonest among these and are associated with the presence of bone marrow ringed sideroblasts and a favorable prognosis. The mutations are missense, heterozygous and cluster strongly within HEAT domains 4–8 of the protein, suggesting that they may be neomorphic gain-of-function variants. Studies analyzing the subclonal composition of MDS indicate that SF3B1 mutations represent early/initiating lesions in MDS evolution, and this was corroborated by their recent identification in hematopoietic cell clones found in at least 2% of otherwise healthy individuals aged 50 years or older. Furthermore, in a significant proportion of SF3B1-mutant MDS this is the only identifiable driver mutation, suggesting that in the right context these mutations can impart a substantial fitness advantage on hematopoietic stem cells. However, the molecular effects of SF3B1 mutations and the mechanisms through which they drive clonal expansion and dyserythropoiesis remain obscure.

RNA splicing takes place at splice sites (ss) located at the 5′ and 3′ ends of introns, after the recognition of consensus nucleotide sequences by the spliceosome machinery. The U1 small nuclear ribonucleoprotein particle (snRNP) recognizes the 5′ ss, and at the 3′ ss the polypyrimidine (Py) tract and an invariant AG dinucleotide located at the intron-exon junction are recognized by the U2AF1–U2AF2 complex, while the proximal branch point sequence (BPS) is identified by the U2 snRNP, of which SF3B1 is a component. MDS cases associated with SF3B1 mutations show a distinct gene expression profile, including dysregulation of genes required for heme biosynthesis, such as Alsas and Abcb7. Furthermore, it was recently demonstrated in primary human cancers and cell lines that SF3B1 mutations are associated with aberrant splicing through recognition of alternative 3′ ss located around 10–25 nt upstream of the canonical 3′ ss. However, the consequences of these aberrations and their impact on hematopoiesis are not well understood.

Here, to investigate the molecular and phenotypic consequences of SF3B1 mutations on hematopoiesis, we generate and

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Impaired splicing in Sf3b1<sup>K700E</sup> knock-in mice

A Mupo et al

Competitive hematopoietic progenitor transplantation
C57BL/6 mice (n = 4 per group) were irradiated with a lethal dose of 2 × 500 rad. They were then injected into the tail vein with a combination of 250,000 lineage negative bone marrow cells (lin<sup>−</sup>) from C57BL/6.SJL (Sf3b1<sup>K700E/+</sup>) mice and 250,000 lin<sup>−</sup> cells from Sf3b1<sup>K700E/+</sup> (CD45.2) mice plus 250,000 whole bone marrow (BM) rescue cells from C57BL/6.SJL (CD45.1/CD45.2) mice. Control animals were injected with 1 × 10<sup>8</sup> whole BM cells from C57BL/6.SJL (CD45.1/CD45.2). Blood counts and FACS analysis were performed after 1, 2 and 4 months. Samples were stained with CD45.1-APC (Becton Dickinson, Oxford, UK) and CD45.2-FITC (Becton Dickinson) antibodies run on Becton Dickinson LSR Fortessa and analyzed with Flowjo 7.6.5 (FlowJo, LLC, San Diego, CA, USA).

**Materials and Methods**

Generation of the Sf3b1-K700E targeting construct

The targeting construct was generated using gateway and recombining recombination technologies as described previously. Briefly, a pool of two BACs containing the region of interest of the Sf3b1 locus, RP24-64H9 and RP24-439B17, were used to generate the gateway-adapted intermediate plasmid by inserting the ‘U’ and ‘G’ cassettes. The latter were generated by PCR and the ‘U’ cassette inserted into the BAC first so that the ‘G’ cassette then retrieved the relevant portion of the Sf3b1 locus into a gateway-adapted intermediate plasmid. The endogenous exon 15 was then replaced with a synthetic one bearing the A > G mutation encoding for the K700E variant. The synthetic DNA was made by GeneArt (Life Technologies, Regensburg, Germany), and was subcloned into the intermediate vector exploiting two KpnI sites in introns 14 and 16 (Supplementary Figure 1). The splice trap cassette encoding an Engrail-2 splice acceptor site, codon-optimized exons 12–15, intron 15, codon-optimized exons 15–19, intron 19 and native exons 20–25 (including the 3′ UTR) was synthesized by GeneArt (Life Technologies) and subcloned into the pL1L2_BactP vector previously modified to remove the EnSA-IRES-lacz-P-AloxP and subsequently the 3′ LoxP site by digestion. The final targeting vector was built using the two vectors above and the pL34L-DTA vector in a multi-vector gateway reaction, and was linearized prior to electrotransfection using the Asil restriction site (Supplementary Figure 2). Sequences of primers used to generate and validate the targeting construct are given in Supplementary Table 1.

ES cell culture and validation of clones

JM8 ES cells were used for electroporation of the final targeting vector. Cells were grown for 9 days after electroporation in a feeder-coated plate with DMEM (Invitrogen, Hemptead, UK) supplemented with 10% FBS (Biosera, UK), 1% BME (Sigma-Aldrich, Haverhill, UK), 1000 U/ml leukemia inhibitory factor (ESGRO, Millipore, UK) and G418 (Sigma-Aldrich) 180 µg/ml. Clones were picked and replated in a 96-well plate with feeders, split in replica plates after 5 days, and lysis for DNA extraction was performed after seven additional days. Clones were screened by 5′ long-range PCR (Supplementary Figure 3). Three clones with successful 5′ and 3′ long-range PCR (Supplementary Figure 3). Three clones with successful 5′ and 3′ amplification, were and all showed that the splice-trap cassette was indeed functional (Supplementary Figure 4a and b). ES cell clones post exposure to FLP and Cre recombinases were validated for excision of the positive selection marker and cDNA splice-trap cassette, respectively (Supplementary Figure 4c). To confirm that the K700E mutation was not expressed in basal conditions but only after Cre expression, cDNA from clone D9 pre- and post- excision of the splice trap cassette was sequenced (Supplementary Figure 4d).

**Hematological measurements and mouse survival**

Blood counts were performed using a VetABC analyzer (Horiba ABX, Montpellier, France). Mice were monitored daily and culled if they showed signs of illness or suffering. All animal studies were performed according to the Animals Scientific Procedures Act 1986 (ASPAB), as recently revised to transpose European Directive 2010/63/EU on the protection of animals used for scientific purposes. Mouse cohort numbers were estimated to detect (P < 0.05) a difference in survival of 2 months or greater with a power of 0.8, assuming a standard deviation of 2.2 months (http://www.biomath.info/power/ttest.htm).

Immunphenotyping

For mouse phenotyping peripheral blood and bone marrow from femurs were collected from animals of different genotypes and processed as previously described. For flow analysis, samples were stained with Gr1-PE (Miltenyi Biotec, Woking, UK), CD11b (Mac1)-FITC (Becton Dickinson), CD71-PE (Becton Dickinson), Ter119-Fitc (Becton Dickinson). For analysis of progenitor populations BM cells were lysed with Red Blood Cell buffer (NH4, CI) and then enriched with the EasySep Hematopoietic Progenitor Cell Isolation Kit (StemCell Technologies, Cambridge, UK) followed by staining with following antibodies Sca1-PB, c-KIT-Acy7, CD34-FITC, Flk2-PeCy5, Cd16/32-PE, CD150-PeCy7, CD48-APC.

RNA isolation and analysis

Whole BM cells were harvested from femurs of Sf3b1<sup>+/+</sup> and Sf3b1<sup>K700E/+</sup> animals. lin<sup>−</sup> cells were isolated with MACS separation system (Milteny Biotec) following standard protocol. RNA from whole BM was isolated using an RNA isolation Kit (Qiagen, Manchester, UK) and RNA from lin<sup>−</sup> cells with PicoPure RNA Isolation System (Thermo Fisher, Hemepstead, UK). Sequencing was performed on the Hiseq2000 V4 sequencing platform (llumina, San Diego, CA, USA). Sequence data have been deposited in ArrayExpress with the accession number E-ERAD-379. All RNA-seq data were analyzed as described previously. RNA-seq datasets have been deposited at the NCBI GEO and are available under accession number GEO: GSE72790. STAR alignment software was used to align raw sequence fragments to the hg19 human reference genome. 72% Junction percent spliced-in (PSI) was calculated for any two or more splice junctions that share a junction boundary and have alternative end points by dividing the raw count of each junction species by the total sum of junction counts that predicted were compared using the Kruskal-Wallis test (http://www.biomath.info/power/ttest.htm). We used the Gencode v19 transcriptome annotation to evaluate whether junction boundaries were equivalent to known exon boundaries (that is, junction novelty). Gene counts were obtained using the Sailfish package, and differential gene expression calculated using edgeR. All P-values were corrected using the Benjamini–Hochberg procedure and q-values < 0.05 were considered significant.

**Nested PCR for lariat sequencing**

Gene-specific reverse transcription of the endogenous lariat region was done using Superscript IV (Invitrogen) and primer C (Supplementary Table 2). The resulting cDNA was used for nested PCR reactions using Platinum Taq DNA polymerase (Invitrogen) and two sets of primers, outer primers (C and D) followed by inner primers (A and B) (Supplementary File 3). The PCR product from the second reaction was run on 2% agarose gel and gel-extracted for Sanger sequencing. The sequencing data were analyzed using Mutation Surveyor software.

**Statistical analyses**

Blood counts and cellular compartment sizes were compared using unpaired t-test. Mouse survival was compared using the Kaplan Meier estimator. Adenine counts near canonical vs aberrant splice junctions and expression levels of transcripts that were NMD-predicted vs non-NMD predicted were compared using the Kruskal H test (Figure 3). Comparison of RNA-seq transcript levels was performed using edgeR (Figure 4).

Leukemia (2017) 720 – 727
RESULTS

Conditional Sf3b1^flox-K700E/+ mice were generated by targeted modification of the Sf3b1 locus in JM8 mouse embryonic stem cells (ESCs)\(^1\),\(^2\) (Figure 1a and Supplementary Figures 1 and 2). To ensure that normal mRNA splicing was retained, the targeted allele was designed to express normal SF3B1 protein via a part-codon optimized Sf3b1 exon 12-25 cDNA (Figure 1a and Supplementary Figure 3). After germline transmission, a downstream neomycin cassette was removed in vivo through mating with Rosa26-FLPe mice,\(^2\) to generate the conditional Sf3b1^flox–K700E allele (Supplementary Figure 3). The Sf3b1^K700E mutant allele could then be expressed after Cre-loxP recombination (Figure 1a and Supplementary Figure 4).

To activate Sf3b1^K700E in hematopoietic stem cells, we crossed Sf3b1^fllox–K700E/+ and Mx1-Cre\(^2\) mice, treated 20 double transgenic and 20 control (wild type and Sf3b1^fllox–K700E/+; Mx1-Cre+), henceforth referred to as WT 4- to 6-week-old mice with polyinosinic-polycytidylic acid (pIpC) and monitored the animals longitudinally. Monthly blood counts from mutant mice (henceforth referred to as Sf3b1^K700E/+)) showed a progressive normocytic anemia compared to wild type, but no significant differences in white blood cell (WBC) or platelet (PLT) counts (Figure 1b). Cytological examination of the bone marrow of Sf3b1^K700E/+ mice showed no significant dysplasia and no ring sideroblasts (Figure 1c). Bone marrow (BM) histology did not reveal any significant morphological differences between WT and Sf3b1^K700E/+ mice, but did show a moderate overall increase in iron deposits in the latter (Figure 1d). At a median follow-up of 83 weeks, mutant animals did not show altered survival or increased signs of illness when compared to WT controls (Supplementary Figure 5a).

To investigate the consequences of Sf3b1^K700E expression on hematopoiesis in greater detail, we analyzed equal numbers of WT and mutant bone marrow cells harvested 4 weeks after pIpC injection by flow cytometry. We found that Sf3b1^K700E/+ mice showed a significant decrease in the number of phenotypically defined hematopoietic stem cells (HSCs), but no change in the size of the LMPP, GMP, CMP and MEP progenitor compartments compared to WT littermates (Figure 2a). By contrast, Sf3b1^K700E/+ mice...
Impaired splicing in SF3b\textsuperscript{K700E} knock-in mice  
A Mupo et al

Figure 2. Effects of mutant SF3b\textsuperscript{K700E/+} on hematopoietic stem and progenitor cells. (a) Flow cytometric analysis of bone marrow cells from SF3b\textsuperscript{K700E/+} mice showed a significant decrease of phenotypically defined HSCs compared to WT animals, but no differences in LMPP, GMP, CMP or MEP progenitor numbers (n = 4). (b) Early erythroid cells (Ter119+/CD71+) were unchanged in number, but there was a reduction in mature Ter119+/CD71+ erythroid cells in SF3b\textsuperscript{K700E/+} animals. (c) Competitive transplantation of bone marrow lin− cells from WT (CD45.1) and SF3b\textsuperscript{K700E/+} (CD45.2) into young (2 months) or old (1 year) syngeneic (C57BL/6) recipients (n = 4 per group). Results shows that, after good initial engraftment (1 month), SF3b\textsuperscript{K700E/+} progenitor cells were steadily outcompeted by co-transplanted WT (CD45.1) cells. Bars in (a, b) and datapoints in (c) show mean ± standard error of the mean.

animals showed a small but significant increase in the number of Gr1+/Mac1+ double-positive bone marrow cells (Supplementary Figure 5b), and in the erythroid compartment there were no differences in the frequency of Ter119+/CD71+ early erythroid cells, but a relative decrease of the more mature Ter119+/CD71\textsuperscript{low}/FSC\textsuperscript{low} population\textsuperscript{24} (Figure 2b), indicative of impaired terminal erythroid differentiation similar to that observed in human MDS\textsuperscript{25}.

Next, as SF3B1-K700E behaves like an initiating mutation in MDS\textsuperscript{7} and can also drive clonal hematopoiesis in elderly individuals\textsuperscript{8}, we wanted to investigate if it confers a fitness advantage to murine HSCs. To test this, we performed competitive transplantation assays in lethally irradiated syngeneic (C57BL/6) mice. These experiments were performed in both young (2 months) and old (12 months) animals to investigate the possibility that age-related changes in recipient mice may influence the repopulating fitness of SF3b\textsuperscript{K700E/+} mutant stem cells\textsuperscript{26}. Experimental mice were transplanted with an equal number (2.5 × 10\textsuperscript{5}) of bone marrow lineage negative (lin−) progenitor cells from WT (CD45.1) and SF3b\textsuperscript{K700E/+} (CD45.2) mice, as well as 2.5 × 10\textsuperscript{6} whole bone marrow (rescue) cells from SF3b\textsuperscript{+/+} (CD45.1/CD45.2) animals. The control group was transplanted with rescue cells from whole bone marrow (CD45.1/CD45.2 = 1 × 10\textsuperscript{6}). We found that although SF3b\textsuperscript{K700E/+} cells did show good engraftment at 1 month post transplantation, they displayed a significant decline in their white blood cell progeny after 4 months compared to WT cells. This was true for both young and old recipients (Figure 2c). Similar results were obtained using a different SF3b\textsuperscript{K700E/+} donor mouse.

Since SF3B1 mutations affect pre-mRNA splicing and consequently gene expression\textsuperscript{13}, we performed mRNA sequencing from whole bone marrow (BM) and lin− progenitors of SF3b\textsuperscript{K700E/+} and WT animals. Firstly, we observed that in SF3b\textsuperscript{K700E/+} mice transcript levels of the mutant (K700E) and wild-type SF3b\textsuperscript{1} mRNAs were very similar (Supplementary Table 3), confirming that expression from the targeted allele was not altered by genetic modification of the locus. We next sought to identify aberrant splicing events associated with SF3b\textsuperscript{K700E} using splice site analysis of RNAseq data from SF3b\textsuperscript{K700E/+} and WT BM cells as we described before\textsuperscript{13} (also see Materials and Methods). Compared to WT we identified 719 aberrant events in lin− and 293 in whole BM cells from SF3b\textsuperscript{K700E/+} mice. The dominant type of aberrant splicing event was increased usage of cryptic 3′ splice sites (3′ ss)
Reconstruction of annotated transcripts that used aberrant 3’ splice sites in the lin~ RNaseq dataset revealed that approximately 42% of affected genes gain a premature termination codon in all isoforms, which is predicted to result in mRNA degradation through the nonsense-mediated decay (NMD) pathway. Genes for which NMD was predicted (n = 190) showed significantly lower mRNA expression levels than those for which NMD was not predicted (n = 225) (P = 3.92 x 10^{-13}, Kruskal H test, Figure 3f). Furthermore, looking at the transcriptome as a whole, we found that most (408 of 511) differentially expressed genes in lin~ cells were downregulated (discussed below). These findings demonstrate that *Sf3b1−/−* is associated with abnormal splicing in hematopoietic stem/progenitor cells, primarily through the use of aberrant 3’-ss that are thought to result in NMD, and this is associated with significant downregulation of a large number of mRNAs.

Unbiased gene set enrichment analysis (GSEA) of the genes differentially expressed in *Sf3b1−/−* revealed an enrichment of
Impaired splicing in Sf3b1K700E knock-in mice
A Mupo et al

Figure 4. Mouse and human SF3B1-K700E share aberrant splicing properties, but affect different mRNA transcripts. (a) Comparison of consensus sequences upstream of aberrant and canonical 3′ ss shows a striking preservation of these motifs between mouse Sf3b1K700E/− lin− cells and several human SF3B1-mutant cancers. (b) Distribution of distances of aberrant 3′ ss (AG) from the downstream canonical AG is also strikingly similar between mouse Sf3b1K700E/− lin− cells and human cancer samples. (c) By contrast, a comparison of genes found to be aberrantly spliced in human vs mouse samples shows minimal overlap. (d) Poor sequence preservation in the mouse of the aberrant splice sites affecting human genes TMEM14C and ABCB7 that are thought to trigger the formation of ring sideroblasts in MDS. (e) Volcano plots comparing mRNA expression between Sf3b1K700E/− vs Sf3b1+/+ BM and lin− cells. The mRNA expression of genes Sfk25a37, Tmem14c, Alas2 and Abcb7, thought to be involved in the formation of ring sideroblasts, is not significantly altered by the K700E mutation in mice. The number of aberrant events used to generate images in a and b is indicated in b (brackets). BRCA, breast cancer; CLL, chronic lymphocytic leukemia; MDS, myelodysplastic syndromes; SKCM, skin cutaneous melanoma.

Genes involved in RNA processing, splicing and transcription. Interestingly, similar enrichment was observed in human MDS11 (Supplementary Figure 7).

Broad transcriptomic changes were observed in human cancers with SF3B1 mutations including MDS,11,27 and we recently described the aberrant splicing patterns in such cancers.13 Here we perform a similar analysis on RNAseq data from Sf3b1K700E/− mice and report that for aberrant 3′ splice sites with upstream cryptic AGs, splicing abnormalities are strikingly similar to those seen in human cancer samples, including the aberrant splice site consensus sequence (Figure 4a) and distance of aberrant 3′ splice sites from the canonical ones (Figure 4b). However, despite these striking similarities, there was minimal overlap between aberrantly spliced genes in mouse vs human cells. In fact, comparing the two datasets we found that only ~5% (17/360) of the aberrantly spliced genes in mouse Sf3b1K700E/− lin− cells were also aberrantly spliced in human samples (Figure 4c). To look at this more closely, we compared the nucleotide sequences at the location of the aberrant 3′ splice sites identified in human SF3B1-mutant cancers,11,13 including MDS, and those identified in our Sf3b1K700E/− mouse BM and lin− cells with the equivalent positions in the genome of the other species, examining the conservation of the invariant AG dinucleotides at the splice sites and that of the upstream 35nt. We first examined genes thought to have a role in the formation of ring sideroblasts in human SF3B1-mutant MDS, namely TMEM14C25 and ABCB7,26,29 for which aberrant 3′ splicing was observed in humans, but not in Sf3b1K700E/− mice. The human TMEM14C aberrant splice site AG was not conserved in mice, while for ABCB7, although the AG dinucleotide was conserved, it was located 2 nt upstream and was preceded by a poorly conserved sequence that deviated significantly from the aberrant splicing consensus (Figure 4d). We then looked at the 360 canonical junctions with aberrant upstream 3′ splice sites in Sf3b1K700E/− lin− cells and found that 268 were conserved in the human genome.
Of the 268 human junctions only 125 contained both a potential cryptic AG in the range preferred by SF3B1/SF3B1 mutants (-5 to -20nt) and a potential branchpoint adenosine at -8 to -18 from the cryptic AG, indicating that less than 35% of mouse junctions with aberrant 3' splice sites had the hypothetical capacity for aberrant splicing in human cells. Of these 125 potential aberrant junctions, only 53 (14.7% of total) were found to be spliced in any human MDS sample, indicating that the limited conservation between mouse and human intronic sequences is responsible for the differences in misspliced mRNAs in the two species. We also noted that both Tmem14C and Abcb7 as well as the Alas2 (heme biosynthesis) and SLC25A37 (mitochondrial iron importer) genes that are downregulated in human SF3B1-mutant MDS did not display altered expression in SF3B1-K700E/lin- or whole BM cells (Figure 4e).

**DISCUSSION**

In order to understand the molecular and phenotypic consequences of SF3B1 mutations on hematopoiesis and their role in the pathogenesis of clonal blood disorders, we generated a knock-in conditional mouse model of SF3B1-K700E, the most common mutation in human MDS. Our conditional allele preserved wild-type SF3B1 expression, thus avoiding any confounding effects of reduced SF3B1 levels on hematopoiesis or other developmental effects. Conditional activation by Mx1-Cre of the SF3B1-K700E mutation in SF3B1-K700E/+/fox-/- mice then enabled direct comparison to wild-type (WT) isogenic hematopoietic progenitors at the molecular and phenotypic level. At the molecular level, we report that the splicing defect seen in SF3B1-K700E/+/ hematopoietic progenitors closely mimicked that described in human MDS, in sharing a strikingly similar splice-site consensus sequence and distance from the aberrant splice site AG. Furthermore, as was recently described for human cancer samples, we show that aberrant BP recognition is central to the disrupted function of mutant SF3B1 in our mice. However, despite the closely similar properties of human and mouse SF3B1 mutants, we found that misspliced transcripts differed significantly between mouse and human as a result of the relatively poor interspecies conservation of intronic sequences able to function as aberrant splice sites. Interestingly, a recent study describing a murine model of U2AF1-S34F, another MDS-associated splicing gene mutation associated with aberrant 3' splice acceptor site recognition, also showed limited mouse-human overlap of misspliced transcripts. The mouse–human overlap appeared greater for a mouse model of SRSF2 mutations (SRSF2-P95H), another commonly mutated gene in human MDS, possibly because the splicing aberration involves alternative exonic sequences, which are more likely than intronic ones to be conserved.

With regard to erythropoiesis, SF3B1-K700E/+ mice showed impaired terminal erythroid differentiation, associated with progressive normocytic anemia without ring sideroblasts. In human SF3B1-mutant MDS, ring sideroblasts are thought to arise as a result of aberrant splicing of key genes involved in heme biosynthesis, such as ABCC7, TMEM14C, ALAS2 and SLC25A37. In keeping with the mouse–human differences in target transcripts, we did not find the splicing or expression levels of any of these genes to be noticeably disrupted by SF3B1-K700E, and, consistent with this, did not identify ring sideroblasts in the bone marrow of mutant mice. Of note, ring sideroblasts were not observed in single-gene knock-out mice for either Alas2 or Abcb7, suggesting that sideroblast formation may require simultaneous reduction in the levels of multiple proteins involved in both heme biosynthesis (ALAS2, TMEM14C) and mitochondrial iron transport (ABCC7 and SLC25A37), and may therefore be difficult to replicate in single-gene targeting models. In this light, the development of anemia in SF3B1-K700E/+ mice may be at least in part due to globally disrupted splicing rather than effects on particular genes. Aberrant splicing leads to the introduction of premature termination codons, which can in turn lead to the generation of truncated proteins with potential toxic effects on cells, leading in this instance to anemia and impaired iron utilization manifested as excess hemosiderin deposition in the bone marrow of SF3B1-K700E/+ mice. An alternative or additional explanation may reside in the fact that both mouse and human cells displayed aberrant expression of genes involved in splicing and RNA processing itself (Supplementary Figure 7), which may be a response to, rather than a result of, globally disrupted splicing. In fact there is evidence that when their intracellular concentrations become too high, some splicing factors regulate their own expression by targeting their mRNAs for NMD.

Beyond erythropoiesis, SF3B1-K700E/+ mice had reduced numbers of HSCs and displayed a myeloid cell bias. Furthermore, investigation of the self-renewal potential of SF3B1-K700E/+ HSCs as determined by their repopulating ability in competitive transplantation assays into either young or old recipient mice revealed a fitness disadvantage of mutant over wild-type HSCs. This contrasts with observations that mutant SF3B1 can drive clonal hematopoiesis and can even be the sole identifiable driver mutation in human MDS. However, observations that SF3B1 mutations appear to selectively impart a clonal advantage on HSCs of elderly, but not young, individuals suggest that the phenomenon is context dependent even in humans.

In this light, the development of anemia in MDS (e.g. anemia, ring sideroblasts, clonal expansion) may have different phenotypic effects of spliceosome gene mutations, and our data propose that factors other than somatic mutations may be operative in the development of MDS.

The fact that disruption of a process as central to cellular function as RNA splicing can have detrimental effects on cells is unsurprising and further augments the conundrum of how particular spliceosome gene mutations can instead impart a survival/fitness benefit upon human HSCs, albeit in a context-dependent manner. This remains the least-well-understood feature of spliceosome gene mutations, and our data propose that the different phenotypic effects of spliceosome gene mutations in MDS (e.g. anemia, ring sideroblasts, clonal expansion) may have distinct molecular causes. Future studies aimed at deciphering the mechanisms underlying these phenotypes should exploit both the similarities and the differences in the transcriptional consequences of these mutations between mice and humans, as a means of identifying their respective etiologies.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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