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The Spliceosomal Component Sf3b1 is Essential for Hematopoietic Differentiation in Zebrafish

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Abstract

SF3B1 (*Splicing factor 3b, subunit 1*) is one of the most commonly mutated factors in myelodysplastic syndrome (MDS). Although the genetic correlation between *SF3B1* mutations and MDS etiology are quite strong, no *in vivo* model currently exists to explore how *SF3B1* loss alters blood cell development. Using zebrafish mutants, we show that proper function of Sf3b1 is required for all hematopoietic lineages. Similar to MDS patients, zebrafish *sf3b1* mutants develop a macrocytic anemia-like phenotype due to a block in maturation at a late progenitor stage. The mutant embryos also develop neutropenia as their primitive myeloid cells fail to mature and turn on differentiation markers such as *l-plastin* and *myeloperoxidase*. In contrast, production of definitive hematopoietic stem and progenitor cells (HSPCs) from hemogenic endothelial cells within the dorsal aorta is greatly diminished, while arterial endothelial cells are correctly fated. Notch signaling, imperative for the endothelial-to-hematopoietic transition, is also normal, indicating HSPC induction is blocked in *sf3b1* mutants downstream or independent of Notch signaling. The data demonstrate Sf3b1 function is necessary during key differentiation fate decisions in multiple blood cell types. Zebrafish *sf3b1* mutants offer a novel animal model to explore the role of splicing in hematopoietic development and provide an excellent *in vivo* system to delve into the why and how Sf3b1 dysfunction is detrimental to hematopoietic differentiation, which could enlighten MDS diagnosis and treatment.

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ADG, RCC, SN, SP, and TVB performed experiments and analyzed data. ADG, RCC, and TVB wrote and edited the manuscript. The authors have no conflicts to disclose.

Introduction

Transcription and RNA processing are interconnected processes that are essential for regulating gene expression. Genes involved in these processes are commonly mutated in hematological malignancies. Although many key transcription factors involved in both embryonic and adult hematopoiesis have been identified¹, the role of RNA processing factors is unclear.

Hematopoietic specification occurs in multiple waves. The first or primitive wave of hematopoietic induction consists mainly of erythrocytes and myeloid cells that sustain the embryo until adult cells are formed². The definitive wave, which occurs later in development, generates multipotent progenitors and hematopoietic stem and progenitor cells (HSPCs) that give rise to all the mature blood cells required throughout the lifetime of an organism³. The first HSPCs emerge from hemogenic endothelium in the ventral wall of the dorsal aorta and then colonize secondary organs, such as fetal liver, placenta, thymus and bone marrow in mammals and the caudal hematopoietic tissue, thymus, and kidney marrow in zebrafish (reviewed in¹⁻³). Much is known about the transcription factors, such as *runx1* (*runt-related transcription factor 1*), *gata2*, *scl* (*stem cell leukemia*), *gfi1* (*growth factor inducible-1*), *pu.1*, and *gata1* among others, that orchestrate developmental hematopoietic specification, but less is known about the role of RNA processing.

Splicing of pre-mRNA is a co-transcriptional event that diversifies the proteome and contributes to cell fate control. The spliceosome is a macromolecular machine comprised of five snRNPs (small nuclear ribonucleoproteins), each made of large, multicomponent protein complexes and snRNAs (small nuclear RNAs). Recent genome sequencing efforts identified mutations in several spliceosomal components in hematologic malignancies, such as acute myeloid leukemia and myelodysplastic syndrome (MDS)⁴⁻⁸. These clinical data suggest splicing regulation is important in hematopoiesis. Mutations in spliceosomal components are mutually exclusive in MDS, yet mutations in each factor correlate with a different disease phenotype. For example, mutations in the U2 snRNP component *SF3B1* (*Splicing factor 3b, subunit 1*) show a strong correlation with subtypes of MDS with refractory anemia and ringed sideroblasts⁹. In contrast, mutations in the splicing accessory protein *SRSF2* (*Serine/arginine-rich splicing factor 2*) or *U2AF1* (*U2 snRNP-associated factor-1*) correlate with poor prognosis and are found more frequently in more malignant forms of MDS, such as refractory anemia with excess blasts^{4-8,10}. These clinical data imply that splicing factors might employ different mechanisms and/or have different cell-type selective tropisms that lead to distinct MDS features. Thus, it is imperative to understand the specific roles of each splicing subunit.

In support of these clinical observations, zebrafish spliceosomal mutants display some overlapping and distinct defects in hematopoiesis¹¹⁻¹⁶. For example, mutants in the RNA helicases *ddx18*^{hi1727} (*DEAD-box protein 18*) and *dhx8*^{hg9} (*DEAH-box protein 8*) have greatly reduced primitive myeloid and erythroid cells, while *ddx46*^{hi2137} (*DEAD-box protein 46*) mutants have impaired primitive erythropoiesis as well as defects in definitive HSPCs maintenance¹¹⁻¹³. In contrast, mutants in the U5 snRNP component *prpf8*^{gl1} (*pre-mRNA processing factor 8*), have defective myeloid lineage development, but no defects in

erythropoiesis or definitive HSPC induction¹⁴. The U2 snRNP-associated factor U2af1 is also critical for hematopoietic development, as zebrafish *u2af1*^{hi199} mutants have diminished erythroid differentiation and fewer HSPCs¹⁶. In contrast, mutants for the U1 snRNP component *snrnp70*^{hi3018} (small nuclear ribonucleoprotein, 70kDa) and the U2 snRNP member *sf3a3*^{hi1950} (*splicing factor 3A, subunit 3*) show diminished HSPC formation, but no defects in primitive hematopoiesis¹⁵. These data show that dysfunction in numerous components of the spliceosome negatively impact hematopoiesis, but also demonstrates differential cell-type sensitivity depending on the spliceosomal constituent mutated.

SF3B1 is the most commonly mutated spliceosomal component in MDS⁴⁻¹⁰, yet its *in vivo* consequences on developmental hematopoiesis are unknown. Homozygous *Sf3b1*-null mice are embryonic lethal pre-implantation, precluding analysis of development¹⁷. Adult heterozygous *Sf3b1* mutants develop macrocytic anemia and have HSC self-renewal defects, demonstrating cell-type selective susceptibilities to *Sf3b1* dosage¹⁸⁻²⁰. To explore the *in vivo* outcomes of *sf3b1* depletion on developmental hematopoiesis, we characterized a recently described zebrafish *sf3b1* mutant^{21,22}. These mutants contain a loss-of-function mutation that is partially compensated by retention of maternally-deposited wild-type protein and mRNA²¹. The *sf3b1* mutants show increased neuronal cell death, similar to many other spliceosomal mutants, and have defects in neural crest cell induction²¹. Using the *sf3b1*^{hi3394a} loss-of-function allele, we defined an essential role for *sf3b1* in blood development. The primitive erythroid and myeloid cells are specified normally, but then fail to mature. In the definitive wave, HSPCs do not form, although the precursor endothelial cells are present. Notch signaling, a critical mediator of hemogenic endothelium and HSPC induction, also appears unaffected in the dorsal aorta in *sf3b1* mutants. These data indicate *sf3b1* is required during hemogenic specification downstream or independent of Notch. Overall, our findings show *sf3b1* function is critical for key hematopoietic cell fate decisions during early development and establish *sf3b1* zebrafish mutants as an *in vivo* model to delineate how *Sf3b1* dysfunction alters hematopoietic decisions, which could impact MDS diagnosis and treatment.

Materials and methods

Zebrafish

Zebrafish were maintained as described.^{23,24} All fish were maintained according to IACUC approved protocols in accordance with Albert Einstein College of Medicine research guidelines.

Zebrafish lines used

The *sf3b1* mutant line *hi3394a* contains a viral insertion between the 1st and 2nd exons of the gene *sf3b1*, resulting in a premature stop codon in exon 2^{21,22}. Wild-type and heterozygous embryos are phenotypically indistinguishable from one another, thus for all of the experiments presented in the paper, wild-type and *sf3b1* heterozygous embryos are cumulatively referred to as wild type. As *sf3b1* homozygous mutants are developmentally delayed and show defects in melanocyte development²¹, mutant embryos were age-matched for all analyses according to morphological features. In addition, analyses of each

hematopoietic population were done for at least two developmental time points to control for any variation in stage matching. Mutant embryos die between 2-3dpf, precluding analysis of later stages²¹. For fluorescent imaging and flow cytometry analysis, we crossed *sf3b1^{hi3394a}* heterozygous animals to the following transgenic lines: Tg(*gata1:gfp*)²⁵, Tg(*kdr1:dsRed*)²⁶, Tg(*cmyb:gfp*)²⁷, Tg(*lyz:dsRed*)^{nz50 28} and Tg(*tp1bglob:eGFP*)(*tp1:eGFP*)²⁹.

Morpholino injections

To knockdown *sf3b1* levels, we utilized a published translation-inhibiting morpholino²¹. Wild-type embryos were injected with 0.1ng (0.5 nl of 0.2µg/µl stock) and compared to non-injected sibling controls.

Whole-mount *in situ* hybridization

In situ hybridization steps were performed as described previously by Thisse *et al.*³⁰ with minor modifications: before proteinase K permeabilization, embryos older than 28 hours post fertilization (hpf) were bleached after re-hydration to remove pigmentation. The bleaching was done for 5-10 minutes using a bleaching solution of 0.8% KOH, 0.9% H₂O₂ and 0.1% Tween 20. The following probes were used: *β-globin*³¹, *ephrinb2*³², *flt4*³², *kdr*³³, *l-plastin*³⁴, *mpx*³⁵, *notch1b*³², *notch3*³², *pu.1*³⁶, *runx1*³⁷, *gata2b*³⁸ and *scf*³⁹. Embryos were then scored manually, imaged and genotyped. Separate PCR reactions were used for detecting wild-type and mutant *sf3b1* alleles. A common reverse primer (5'-GACAATCACCCACGGCCATG-3') was paired with unique forward primers for the wild-type (5'-GATCGCCAAAACACATGATG-3') or mutant alleles (5'-CTGTCCATCTGTTCTGAC-3').

Flow cytometry

For generation of single cell suspensions, 10-20 embryos were first removed from their chorions using pronase (Roche), and then homogenized by manual dissociation using a sterile razor blade followed by digestion with Liberase (Roche). For the digestion, dissociated embryos were resuspended in 3 ml 1× Dulbeccos-PBS (D-PBS) (Life Technologies) supplemented with a 1:65 dilution (46 µl) of 5 mg/ml Liberase and then incubated at 37°C for 8 minutes. The reaction was topped with 5% (157µl) fetal bovine serum (FBS) (Life Technologies). The cells were then filtered twice through 40-µm cell strainers (Falcon) and pelleted by centrifugation at 3000 rpm for 5 minutes. Cell pellets were resuspended in 1-2 ml FACS buffer (0.9× D-PBS, 5% FBS, 1% Penn/Strep (Life Technologies)). DAPI (4',6-diamidino-2-phenylindole) was added to a final concentration of 1 µg/ml to facilitate exclusion of dead cells from the analysis. Samples were analyzed with a LSRII flow cytometer (BD Biosciences) and FlowJo version 10.0.8.

Fluorescent imaging

Embryos (24-36 hpf) were anesthetized with tricaine and oriented in a drop of 3% wt/vol methylcellulose and then mounted in 1% agarose in 35-mm imaging dishes (MatTek) as previously described⁴⁰. Fluorescent imaging was performed with a Zeiss Axio Observer A1 with an AxioCam ICM1 camera and Zeiss Zen 2 software. Fluorescence was detected with GFP and Texas-Red filters.

May-Grunwald Giemsa staining of primitive erythroid cells

Two-day old embryos were placed on poly-L-lysine coated slides in a drop of 1× D-PBS + 1% bovine serum albumin (Sigma). Blood cells were released from the embryo by puncturing the pericardial sac and upper yolk sac with fine forceps. The slides were air-dried at room temperature prior to staining. For staining, slides were immersed in undiluted May-Grunwald stain (Eng Scientific May-Grunwald stain solution 1, Fisher Scientific) for 2 minutes and briefly rinsed in ddH₂O. Slides were then immersed in diluted Giemsa stain (diluted 1:4 with milliQ water) for 20 minutes (Eng Scientific May-Grunwald stain solution II, Fisher Scientific) and briefly rinsed in ddH₂O. Once slides were dry, a drop of Permount solution (Fisher Scientific) was added and slides were covered with cover slips and left overnight to dry. Once slides were dry, the cells were visualized with a 63× oil-immersion lens.

O-dianisidine and apoptotic marker analysis

O-dianisidine staining was performed as described previously³⁵. Dechorionated, live embryos were soaked in o-dianisidine staining solution (0.62mg/ml o-dianisidine (Sigma), 10.9 μM sodium acetate, and 0.65% H₂O₂) for 20 min in the dark. Acridine Orange staining was performed as previously described⁴¹. Dechorionated, live embryos were soaked in a 2μg/ml acridine orange (Sigma) solution for one hour in the dark. Immunofluorescence analysis was performed for active caspase 3 (BD Biosciences) at 24 hpf as previously described⁴².

Treatment with SNAP

Embryos from an incross of *sf3b1* mutants were treated with the Nitric Oxide (NO) donor SNAP (S-Nitroso-N-Acetyl-D,L-Penicillamine) from 7 hpf until 28 hpf. Doses tested were based on previous studies⁴³ and included 10μM and 30μM. Control embryos were treated with 0.06% DMSO as a vehicle control.

Statistics

For pairwise comparisons, the Student's t-test (unpaired, two-tailed) was employed. For comparison of population distributions, Chi-squared analysis was performed. Statistical test utilized is designated in the figure legend for each analysis.

Results

Erythroid maturation is hindered in *sf3b1* mutants

To characterize the blood compartments in *sf3b1* zebrafish, we first assessed primitive hematopoietic induction. Primitive erythroid and myeloid cells arise from *scf*-positive mesoderm^{3,39}. We observed no difference in *scf* expression between *sf3b1* mutants and wild-type siblings at the 12 somite stage (ss)/14hpf (Figure 1A). Gata1 is an erythroid-specific transcription factor that is essential for erythropoiesis⁴⁴, thus to determine if erythroid cell identity was properly specified in *sf3b1* mutants, we performed flow cytometry analysis of *sf3b1* Tg(*gata1*:eGFP) embryos at 24 hpf. We observed a subtle, but significantly higher percentage of *gata1* positive cells in *sf3b1* mutants compared to wild-type siblings (Figure

1B-D). At 48 hpf the number of *gata1*-positive hematopoietic cells remained high in mutants, but was reduced in wild-type siblings (Figure 1E). The reduction in *gata1*-positive cells in wild-type embryos is consistent with previous studies that showed a down-regulation of *gata1* mRNA at 36-48 hpf thought to be associated with erythroid differentiation or maturation^{45,46}. The perdurance of high *gata1*-expressing cells in *sf3b1* mutants might be a reflection of a block in differentiation or maturation.

To investigate the differentiation status of erythroid cells in *sf3b1* mutants, we examined the expression of the differentiation marker β -globin by *in situ* hybridization. Expression of β -globin, a component of oxygen-carrying hemoglobin, was normal in *sf3b1* mutants at 24 hpf (Figure 1F). Functional hemoglobin can be detected in wild-type red blood cells beginning around 36-48 hpf using o-dianisidine staining⁴⁷. In *sf3b1* mutants at 36 hpf, we observed little o-dianisidine-positive erythroid cells (Figure 1G). Mutants display some developmental delay that becomes more severe as the embryos get older, thus we also looked at o-dianisidine staining at 48 hpf and noted no improvement to hemoglobin function (Figure 1H). Moreover, the level of o-dianisidine staining in the 48 hpf embryos is lower than wild types at 36 hpf indicating the decrease in o-dianisidine is unlikely solely due to developmental delay. To validate the findings in the mutants, we also tested hemoglobin function in *sf3b1* morpholino-injected embryos²¹, and noted a similar decrease in o-dianisidine staining (Figure S1A). To further characterize the defects in primitive erythropoiesis in *sf3b1* mutants, we analyzed the morphology of isolated red blood cells using May-Grunwald-Giemsa staining (Figure 1I). Mutants had a higher percentage of proerythroblasts and a corresponding decrease in more mature erythroid precursors at 48 hpf (Figure 1J). Furthermore, the later stage orthochromatophilic erythroblasts in the *sf3b1* mutants showed a megaloblastoid-like phenotype compared to wild type (Figure 1I). Together, our results indicate *sf3b1* mutant erythroid cells have a late-stage block in differentiation.

Myeloid cell development is impaired in *sf3b1* mutants

During development, primitive myelopoiesis originates in both the anterior and posterior lateral plate mesoderm from *scf*-expressing cells². As mentioned above, *scf* expression was unchanged in *sf3b1* mutants, either in the anterior or posterior blood islands (Figure 1A). The transcription factor *pu.1* is a master regulator of myeloid cell fate and is first expressed in developing zebrafish around 12 hpf/6ss³⁶. In *sf3b1* mutants, *pu.1*-expressing cells are detected at 22 hpf, but are slightly diminished and less dispersed across the yolk, suggesting a potential delay in differentiation (Figure 2A). We next examined the expression of markers of mature myeloid cells. Differentiation of myeloid cells is marked by expression of genes such as *l-plastin* or *myeloperoxidase* (*mpx*), beginning around 16 hpf and 19 hpf, respectively. At 24 hpf, expression of *l-plastin* and *mpx* are greatly diminished in *sf3b1* mutants (Figure 2B,C). To rule out a contribution of developmental delay for the lack of mature myeloid marker expression in *sf3b1* mutants, we examined the expression of *l-plastin* and *mpx* at 28 hpf. The markers were still diminished in the mutants, suggesting a block in myeloid cell differentiation (Figure 2D,E). We confirmed the decrease in *mpx* at 28 hpf in *sf3b1* morpholino-injected embryos (Figure S1B). To further test this hypothesis, we used flow cytometry to quantify the frequency of *lyz:dsRed* myeloid cells at 36 hpf. *Lyz:dsRed*

myeloid cells were over 22-fold decreased in *sf3b1* mutants compared to wild-type siblings (Figure 2F,G). Erythroid-myeloid progenitors are also arising in zebrafish embryos at this time during development^{48,49}. To characterize this cell population, we examined *pu.1* at 26 hpf and *l-plastin* and *mpx* at 30 hpf within the posterior blood island (PBI) (Figure 3). As in the primitive wave, we observed some *pu.1*-positive cells in the PBI, but greatly diminished expression of more differentiated markers. Taken together, these results show myeloid specification initiates normally in the mutants, but myeloid cell differentiation is hindered.

HSPC formation is inhibited in *sf3b1* mutants

We next examined if there were changes to definitive HSPC formation. HSPCs emerge from hemogenic endothelial cells that reside along the ventral wall of the dorsal aorta^{3,50}. One of the earliest markers of HSPCs is *runx1*³⁷. Expression of *runx1* is nearly absent within the aorta of 28 hpf *sf3b1* mutants, but is readily detected in wild-type siblings (Figure S2A). Mutant *sf3b1* embryos have a variable reduction in blood flow and decrease in aortic lumenization, thus we wanted to determine if these defects were the main contributors to the observed HSPC loss in mutants. Although blood flow is needed for HSPC formation, it is dispensable for the early induction of *runx1* expression at 24 hpf⁵¹. To determine if the decrease in HSPC gene expression is present at this earlier stage, we examined expression of *runx1* and another HSPC marker, *gata2b*³⁸, at 24 hpf. In agreement with the results at 28 hpf, we observed diminished expression of both markers at 24 hpf (Figure 4A, B). We confirmed the decrease in *runx1* expression at 24 hpf in *sf3b1* morpholino-injected embryos (Figure S1C). Newly-born HSPCs co-express markers of hematopoietic and endothelial cells⁵². This property can be visualized in embryos that are double-transgenic for *c-myb:gfp* and *kdr1:dsred* (Figure 4C). We incrossed *c-myb:gfp;kdr1:dsred* transgenic *sf3b1* heterozygotes and quantified HSPC number at 38 hpf in mutant and wild-type siblings. Wild-type embryos had 5.4 *c-myb:gfp;kdr1:dsred* double positive cells on average, while *sf3b1* mutants had only 1.4 (Figure 4C,D). Due to the variable decrease in blood flow and aortic lumen size observed in *sf3b1* mutants, we quantified *c-myb:gfp;kdr1:dsred* double positive cells in embryos with different lumen sizes, but noted no significant difference in the number of double positive cells, suggesting the defect in HSPC formation is independent of these defects. To further confirm that the decreased HSPC production was not due to loss of signals from blood flow, we treated embryos with the nitric oxide donor SNAP (S-Nitroso-N-Acetyl-D,L-Penicillamine), which was previously shown to restore HSPC levels in mutants lacking blood flow^{43,51}. Treatment with SNAP had no effect on *runx1* levels in mutant embryos (Figure S2B). Combined, these data demonstrate a severe defect in HSPC formation in *sf3b1* mutants independent of the effects on blood flow and aortic lumenization.

Aortic specification and Notch signaling are unperturbed in *sf3b1* mutants

Hemogenic endothelial cells in the ventral wall of the dorsal aorta are the immediate precursor to HSPCs, thus proper vasculature formation is an essential prerequisite for HSPC induction⁵⁰. We next analyzed the expression pattern of endothelial markers in *sf3b1* mutants and wild-type siblings. The pan-endothelial marker *kdr1* is expressed in the dorsal aorta, cardinal vein, and intersomitic vessels in both wild-type and mutant siblings at 24 and 28 hpf (Figure 5A and S3A). Proper artery-vein identity precedes HSPC induction^{3,50}. To

determine if vessel identity was correctly initiated in *sf3b1* mutants, we examined artery and vein-specific markers. The vein marker *flt4* shows the presence of a well-formed posterior cardinal vein in 24 and 28 hpf mutant embryos (Figure 5B and S3B). Aorta-specific markers *ephrinb2*, *notch1b*, and *notch3* are expressed in the aorta of both wild type and mutants at 24 and 28 hpf, demonstrating proper specification of aortic endothelium (Figure 5C and S3C). The Notch pathway gives arterial identity to endothelial cells, and is a critical signal for HSPC specification^{29,50}. Correct expression of *notch1b* and *notch3* within the dorsal aorta suggests an intact signaling network, but does not test if the pathway is active. Notch pathway activation can be visualized using *tp1:eGFP* Notch reporter fish²⁹. At 36 hpf, mutant embryos show the same pattern of Notch signaling within the aorta and intersegmental vessels compared to their wild-type siblings (Figure 5D). These data indicate the lack of HSPC formation is not due to a lack of Notch signaling within arterial cells.

The results suggest that the hematopoietic defects in *sf3b1* mutants are likely due to problems transitioning between different cellular states, but cell death cannot be excluded as a contributing factor. We assessed apoptosis in *sf3b1* mutants using acridine orange staining and antibody staining for active caspase-3^{41,42}. While a clear increase in cell death in neuronal tissues, such as the brain and spinal cord, was observed, we noted no significant increase in apoptosis in other tissues in *sf3b1* mutants compared to wild-type siblings (Figure S4). These results support the model that splicing controls hematopoietic differentiation decisions.

Discussion

Recent clinical findings and discoveries in zebrafish development indicate that normal functioning of the spliceosome is critical for hematopoiesis. These studies also implied that the ensuing hematopoietic defects were somewhat distinct depending on the spliceosomal component affected. The most commonly mutated spliceosomal factor in MDS is *SF3B1*, yet there is currently no *in vivo* model describing what aspects of hematopoiesis are affected when this factor is lost. Here we demonstrated that depletion of *sf3b1* during zebrafish development results in deficits in primitive and definitive hematopoiesis. Primitive erythroid cells in *sf3b1* mutants arise and initially differentiate normally, but then arrest at the orthochromatophilic stage and take on an abnormal megaloblastoid-like morphology. Primitive myelopoiesis also initiates normally, but then *pu.1*-expressing progenitors fail to mature. These data indicate *sf3b1* is needed for maturation of myeloid and erythroid progenitors. In contrast, induction of definitive hematopoiesis is severely impaired. Expression of the hemogenic endothelial and HSPC markers *runx1* and *gata2b* is hampered as early as 24 hpf although the specification of non-hemogenic aortic endothelial cells at the same time and place in development appears mostly unaltered. These data suggest that *sf3b1* is differentially required in distinct endothelial cell populations and that hematopoiesis is acutely sensitive to perturbations in the spliceosome.

A common feature among the hematopoietic defects in *sf3b1* mutants is inhibited differentiation and maturation. Recent RNA-seq studies have revealed a complex orchestration of splicing throughout erythroid and myeloid differentiation⁵³⁻⁵⁷. The functional importance of these splicing events remains unclear. In erythroid maturation,

splicing changes are greatest in the later stages of differentiation^{55,56}. Our data provide a functional corollary to the importance of this observation as erythroid differentiation in *sf3b1* mutants is blocked at a late progenitor stage. Unlike erythroid and myeloid splicing patterns, the splicing events occurring during the endothelial-to-hematopoietic transition are unknown. The lack of induction of expression of the key hemogenic transcription factors *runx1* and *gata2b* demonstrate splicing is a regulator of definitive HSPC origins. Further exploration of the splicing aberrations in *sf3b1* endothelial cells will help decipher which specific splice isoforms are important in HSPC development.

Although many spliceosomal mutants characterized to date have hematopoietic perturbations, they are not entirely overlapping, even in factors that are within the same complex. U2 snRNP is a large macromolecular complex made up of nearly 20 factors. Zebrafish mutants of three of these factors (*sf3a3*¹⁵, *ddx46/prp5*¹², and now *sf3b1*) have been characterized, and all display defects in the hematopoietic system. Mutants in *sf3a3* have normal primitive erythropoiesis and myelopoiesis, but show defects in HSPC formation, while *ddx46* mutants initiate HSPC induction normally, but have problems maintaining their stem cell pool. These studies lead to two non-mutually exclusive hypotheses for the differences observed: 1) different splicing events are affected in each spliceosomal mutants and 2) the diverse phenotypes could be due to non-spliceosomal functions of each splicing factor. Indeed, recent studies have found that *SF3B1* has some non-splicing functions in responding to various cellular stressors^{58,59}.

These differences can also be observed clinically. MDS is a pleiotropic hematopoietic disease marked by perturbations to the erythroid and myeloid lineages. U2AF1 is an essential factor that associates with the U2 snRNP complex to help recruit it to splice sites. Despite the interconnection between these two complexes in splicing, mutations in *U2AF1* and *SF3B1* are associated with different features of MDS. Mutations in *U2AF1* segregate with MDS with poor overall survival, while mutations in *SF3B1* show a strong correlation with refractory anemia with ringed sideroblasts (RARS) and an overall favorable prognosis⁴⁻¹⁰. Erythroid cells in RARS are poorly differentiated and show defects in iron usage resulting in hemoglobinization problems. Reminiscent of this type of MDS, zebrafish *sf3b1* mutants have poorly differentiated red blood cells and low levels of functional hemoglobin, but they do not show symptoms of myeloproliferation associated with more malignant forms of MDS. Whether these differences are due to differences in splicing abnormalities or in non-splicing functions of U2AF1 and SF3B1 requires further exploration.

Combined, the clinical indications and differences in developmental hematopoietic deficiencies among spliceosomal mutants show that there is still much to learn about spliceosome protein function in metazoans. The zebrafish is an excellent model for addressing this problem. Maternal contribution of essential factors like spliceosomal components allows survival of embryos to later stages during organogenesis permitting comparison across many tissues. Zebrafish are highly amenable to genetic and chemical perturbations, which can be employed to define the functionally relevant pathways for each tissue type affected. Genome-wide studies of blood diseases, such as MDS, have revealed a number of correlations between gene mutation and disease phenotype, which are difficult to

dissect due to the complexity of the mutational landscape within the heterogenous mix of cells analyzed. The use of *in vivo* systems, such as the zebrafish, provides a facile genetic approach for testing the contribution of single gene perturbations. We have now established numerous phenotypic similarities between zebrafish *sf3b1* mutants and *SF3B1*-mutated MDS. Future studies will take advantage of this model to uncover the molecular and cellular origins of the hematopoietic defects arising from deficiencies in *sf3b1*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Erythroid and myeloid differentiation is inhibited in *Sf3b1* zebrafish mutants.
- *Sf3b1* is required for hematopoietic stem and progenitor cell emergence (HSPC).
- Loss of HSPCs is independent to defects in endothelial cell specification.
- Notch signaling in the aorta does not require *Sf3b1*.

Figure 1. *sf3b1* mutants have normal erythroid cell development but lack mature erythrocytes
A. *In situ* hybridization of the primitive hematopoietic marker *scl* at 12 ss (14 hpf) in wild type (top) and *sf3b1* mutants (bottom). Number shown in the lower left corner denotes the number of embryos of each genotype that displayed a similar phenotype to the image. B. Flow cytometry analysis of *gata1:eGFP* cells from wild type (left) and *sf3b1* mutants (right). Averages $\pm$ standard deviation of 3 replicates are shown. For each replicate, 10-20 embryos were pooled for the analysis. C. Graph showing the percentage of *gata1:eGFP* positive cells shown in B. Student's t-test **p=0.007. D,E. Schematic showing area of the

embryo shown in images below. Fluorescent images of *gata1:eGFP* wild type (top) and *sf3b1* mutants (bottom) at 24 hpf (D.) and 48 hpf (E.), scale bar: 100 μ m. F. *In situ* hybridization of the erythroid marker β -globin at 24 hpf in wild type (top) and *sf3b1* mutants (bottom). G,H. O-dianisidine staining of functional hemoglobin in mature primitive erythrocytes in wild type (top) and *sf3b1* mutants (bottom) at 36 hpf (G.) and 48 hpf (H.). I. Representative images of orthochromatophilic erythroblasts stained with May Grunwald-Giemsa from wild type (left) and *sf3b1* mutants (right), scale bar: 20 μ m. J. Number of cells that were classified by morphology at each differentiation stage with percentages shown in brackets for wild type and *sf3b1* mutants. Chi-squared test ****p=0.0001.

Figure 2. Primitive myeloid cell development is impaired in *sf3b1* mutants

A-E. *In situ* hybridization for myeloid markers. Images of wild type are shown on the left and *sf3b1* mutants are shown on the right. Number shown in the lower right corner denotes the number of embryos of each genotype that displayed a similar phenotype to the image. A. The myeloid progenitor marker *pu.1* at 22 hpf. B,D. The myeloid differentiation marker *l-plastin* at 24 hpf (B.) and 28 hpf (D.). C,E. The neutrophil marker *mpx* at 24 hpf (C.) and 28 hpf (E.). F. Flow cytometry analysis of *lyz:dsRed*-positive myeloid cells from wild type (left) and *sf3b1* mutants (right) at 36 hpf. Averages $\pm$ standard deviation of 3 replicates are

shown. For each replicate, 10-20 embryos were pooled for the analysis. G. Graph showing the percentage of *Iyz*.dsRed positive cells shown in F. Student's t-test ****p =0.0001.

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Figure 3. Erythroid-myeloid progenitor development is impaired in *sf3b1* mutants

A-C. *In situ* hybridization for myeloid markers. Images of wild type are shown on the left and *sf3b1* mutants are shown on the right. Number shown in the lower right corner denotes the number of embryos of each genotype that displayed a similar phenotype to the image. Inset to the right shows a higher magnification view of the PBI region of the embryo boxed in the image on the left. A. The myeloid progenitor marker *pu.1* at 26 hpf. B. The myeloid differentiation marker *l-plastin* at 30 hpf. C. The neutrophil marker *mpx* at 30 hpf.

Figure 4. *sf3b1* mutants have diminished HSPCs

A,B. *In situ* hybridization of the HSPC markers *runx1* (A.) and *gata2b* (B.) at 24 hpf in wild type (top) and *sf3b1* mutants (bottom). Inset to the right shows a higher magnification view of the AGM region of the embryo boxed in the image on the left with aorta marked with arrowheads. Number shown in the lower left corner denotes the number of embryos of each genotype that displayed a similar phenotype to the image. C. Representative fluorescent images of *cmvb:gfp+*; *kdr:dsred+* HSPCs (arrows) from wild type (top) and *sf3b1* mutants (bottom) at 38 hpf. Middle image depicts a mutant with a less inflated aorta and the bottom image shows a mutant with a more inflated aorta. Scale bar: 50μM. Schematic on the right shows the area of the embryo shown in the fluorescent images. D. Graph showing quantification of HSPCs (*cmvb:gfp+*; *kdr:dsred+* cells) in wild type and *sf3b1* mutants at 38 hpf. Each dot represents the number of HSPC per embryo and the line denotes the mean. Dark blue dots are HSPC counts for mutants with less inflated aorta and the light blue dots are HSPC counts for mutants with more inflated aorta. Student's t-test **** $p=6\times 10^{-11}$.

Figure 5. Endothelial markers are expressed in the correct vessels in *sf3b1* mutants

A-C. *In situ* hybridization for vascular markers at 24 hpf. Images of wild type are shown on the left and *sf3b1* mutants are shown on the right. Number shown in the lower right corner denotes the number of embryos of each genotype that displayed a similar phenotype to the image. Inset to the right shows a higher magnification view of the AGM region of the embryo boxed in the image on the left with aorta or vein marked with arrowheads. A. The pan-endothelial marker *kdr1*. B. The vein marker *flt4*. C. The aorta-specific markers *ephrinb2*, *notch1b* and *notch3*. D. Schematic showing area of the embryo shown in images

below. Representative fluorescent images of *tp1:eGFP* (Notch reporter) wild type (left) and *sf3b1* mutants (right) at 36 hpf. Arrowhead denotes the aorta.

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