

Mis-splicing of Mitotic Regulators Sensitizes SF3B1-Mutated Human HSCs to CHK1 Inhibition



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ABSTRACT

Splicing factor *SF3B1* mutations are frequent somatic lesions in myeloid neoplasms that transform hematopoietic stem cells (HSCs) by inducing mis-splicing of target genes. However, the molecular and functional consequences of *SF3B1* mutations in human HSCs and progenitors (HSPCs) remain unclear. Here, we identify the mis-splicing program in human HSPCs as a targetable vulnerability by precise gene editing of *SF3B1* K700E mutations in primary CD34⁺ cells. Mutant *SF3B1* induced pervasive mis-splicing and reduced expression of genes regulating mitosis and genome maintenance leading to altered differentiation, delayed G2/M progression, and profound sensitivity to CHK1 inhibition (CHK1i). Mis-splicing or reduced expression of mitotic regulators *BUBR1* and *CDC27* delayed G2/M transit and promoted CHK1i sensitivity. Clinical CHK1i prexasertib selectively targeted *SF3B1*-mutant immunophenotypic HSCs and abrogated engraftment *in vivo*. These findings identify mis-splicing of mitotic regulators in *SF3B1*-mutant HSPCs as a targetable vulnerability engaged by pharmacological CHK1 inhibition.

SIGNIFICANCE: In this study, we engineer precise *SF3B1* mutations in human HSPCs and identify CHK1 inhibition as a selective vulnerability promoted by mis-splicing of mitotic regulators. These findings uncover the mis-splicing program induced by mutant *SF3B1* in human HSPCs and show that it can be therapeutically targeted by clinical CHK1 inhibitors.

INTRODUCTION

Change-of-function mutations in splicing factor *SF3B1* are recurrent and frequent somatic lesions in myeloid neoplasms, including up to 30% of patients with myelodysplastic syndrome (MDS; refs. 1, 2). *SF3B1* mutations are acquired in hematopoietic stem cells (HSCs) and commonly represent disease-initiating events (3, 4). *SF3B1* is a core component of the U2 spliceosome that mediates 3' splice site selection by binding to intronic branchpoint sites. Oncogenic mutations alter *SF3B1* branchpoint recognition leading to alternative 3' splice site selection (a3'ss), which commonly results in decreased expression of target genes (5–7). A number of genes mis-spliced by mutant *SF3B1* and their causal association with distinct pathological phenotypes have been identified. For instance, mis-splicing of erythroid genes *ABCB7*,

TMEM14C, *MAP3K7*, *COASY*, and *ERFE* has been linked with ineffective erythroid differentiation and formation of ring sideroblasts, erythroid precursors with perinuclear iron-laden mitochondria (8–11). However, much less is known about the mis-spliced gene repertoire and the concomitant molecular and functional consequences of *SF3B1* mutations in disease-initiating human hematopoietic stem and progenitor cells (HSPCs).

To sustain life-long hematopoiesis, HSCs encode developmentally divergent patterns of alternative splicing and gene expression. Transcriptional differences between HSCs, progenitors, and more differentiated cells suggest that mutant *SF3B1* may induce a distinct repertoire of mis-spliced transcripts in HSPCs (12). Several experimental models of *SF3B1* mutations have been developed, including K562 cells and patient-derived induced pluripotent stem cells (iPSC; refs. 8, 13). However, these cell types are transcriptionally distinct and may not recapitulate the splicing changes in human HSCs. MDS samples with *SF3B1* mutations are available but have heterogeneous mutational profiles, proliferate poorly *in vitro*, and display low engraftment *in vivo* (14). Engineering of precise mutations in human HSPCs is needed to understand the splicing alterations induced by mutant *SF3B1*, their functional consequences on self-renewal and differentiation, and to uncover therapeutic strategies against malignant HSPCs. However, efficient gene editing of precise mutations in human HSPCs has to date been technically challenging.

The central role of *SF3B1* in MDS pathogenesis makes it an attractive therapeutic target. Splicing factor mutations are almost always heterozygous and mutually exclusive of one another at a clonal level (15). This motivated the investigation of spliceosome modulation as a therapeutic strategy, by splicing inhibition or targeting of spliceosome accessory proteins and post-translational modifications (15–17). Despite promising preclinical results, the initial clinical trial of spliceosome inhibitor H3B-8800 did not result in objective responses (18)

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highlighting the need for alternative clinical strategies. Mis-splicing of multiple genes within a single biological pathway could sensitize spliceosome mutant cells to yet unidentified therapeutic vulnerabilities. *SF3B1*-mutant cells are sensitized to several inhibitors of DNA damage response, including PARP inhibitors and UCN-01 (bioRxiv 2021.05.26.445839; refs. 19–21), a non-specific inhibitor of multiple kinases including CHK1, a central regulator of DNA damage and cell cycle (22, 23). However, it is unclear whether CHK1 dependency is related to gene mis-splicing or other dependencies in spliceosome-mutant cells.

Here, we develop efficient gene editing to engineer *SF3B1* K700E mutation in primary human CD34⁺ HSPCs. By combining splicing analysis and single-cell transcriptomics, we identify pervasive mis-splicing of genes regulating cell cycle, mitosis, and genome maintenance in *SF3B1*-mutant HSPCs. Mis-splicing of mitotic regulators *BUBR1* and *CDC27* by mutant SF3B1 delays G2/M progression leading to mitotic activation of CHK1 and sensitizing *SF3B1*-mutant immunophenotypic HSCs to clinical CHK1 inhibitor prexasertib. Our study thus identifies mis-splicing of mitotic regulators as a targetable vulnerability in *SF3B1*-mutant myeloid malignancies.

RESULTS

Efficient Gene Editing of *SF3B1* K700E Mutation in Primary Human CD34⁺ HSPCs

SF3B1 mutations are initiating lesions in myeloid neoplasms acquired in normal HSCs (1–4). There are currently no models to understand the molecular and functional impact of mutant SF3B1 in human HSCs. To this end, we combined CRISPR/Cas9 gene editing and adeno-associated virus serotype 6 (AAV6)-mediated template delivery for homologous recombination to introduce the precise *SF3B1* K700E mutation at the endogenous locus in CD34⁺ umbilical cord blood (CB) or adult mobilized peripheral blood (PB) HSPCs (Fig. 1A and B). This system also allows introduction of BFP intronic marker for isolation and tracking of edited cells. We confirmed *SF3B1* K700E mutation (c.2098A>G) and a silent PAM mutation (to prevent re-editing) in sorted BFP⁺ cells (Supplementary Fig. S1A), and the absence of BFP expression without integration in AAV6-transduced cells (Supplementary Fig. S1B). Moreover, we confirmed heterozygous expression of the mutant *SF3B1* K700E allele in BFP⁺ cells (Fig. 1C), and that total *SF3B1* expression was not affected by the BFP cassette knock-in (Supplementary Fig. S1C). BFP cassette knock-in into the safe harbor AAVS1 locus was used as a control. Editing was efficient with ~20% of control- or *SF3B1*-edited CD34⁺ cells expressing BFP (Fig. 1B). These data demonstrate that AAV6-based homologous recombination efficiently introduces a precise *SF3B1* K700E mutation in CD34⁺ HSPCs.

In the IPSS-M prognostic classification for MDS, co-mutations in *RUNX1* or *STAG2* in *SF3B1*-mutant MDS confer poor prognosis and high risk of leukemic transformation (1, 2). To test the efficiency of multiplex editing to introduce these cooperating mutations, we combined *SF3B1* K700E knock-in with sgRNA-mediated targeting of *RUNX1* (abbreviated S-R) or *STAG2* (abbreviated S-S) or AAVS1 safe harbor locus as a control (abbreviated S-A). The efficiency of frame-shift mutations was 31.4% for AAVS1, 55.8% for *RUNX1*, and

59.7% for *STAG2* (Supplementary Fig. S1D), leading to ~50% reduction in *RUNX1* and ~90% reduction in *STAG2* expression (Supplementary Fig. S1E). These data show efficient multiplex editing to generate combinations of mutations in CD34⁺ HSPCs, which can be leveraged to test genotype-specific vulnerabilities in MDS and its clonal evolution.

To determine the functional impact of *SF3B1* mutations on hematopoietic differentiation, we expanded CB CD34⁺ edited cells in liquid culture supporting HSPC maintenance or erythroid differentiation. The proportion of *SF3B1*-mutant BFP⁺ cells declined modestly in HSPC culture (Fig. 1D), which was not affected by *RUNX1* or *STAG2* knockout (Supplementary Fig. S1F), while BFP⁺ cells expressing phenotypic HSC markers CD34⁺CD133⁺ were comparable to controls (Fig. 1D, right). By contrast, the proportion of *SF3B1* K700E BFP⁺ edited cells decreased dramatically during erythroid culture with markedly reduced growth and differentiation of CD235a⁺ erythroblasts (Fig. 1E and F). Lastly, we observed infrequent ring sideroblasts in terminally differentiated *SF3B1*-mutant erythroblasts (Fig. 1G). These findings suggest that the CD34⁺ gene editing model recapitulates ineffective erythropoiesis, a key clinical finding in patients with *SF3B1*-mutant MDS.

Pervasive Mis-splicing of Cell Division and Genome Maintenance Genes in CD34⁺ HSPCs

SF3B1 mutations promote aberrant splicing marked by recognition of alternative 3' splice sites (a3'ss; refs. 5–7). To understand splicing alterations directly induced by mutant SF3B1 in human HSPCs, we carried out bulk RNA sequencing and splicing analysis of edited CB and PB BFP⁺ CD34⁺ cells (Supplementary Fig. S2A; Supplementary Table S1). Splicing analysis showed that *SF3B1* K700E knock-in CB/PB HSPCs recapitulated a3'ss mis-splicing of canonical genes identified in patients with *SF3B1*-mutant MDS (6, 7), including *MAP3K7*, *TMEM14C*, *DYNLL1*, and *ORAI2* (Fig. 2A). These data suggest that the CD34⁺ editing model recapitulates splicing abnormalities associated with *SF3B1*-mutant MDS. To gain a more unbiased understanding of splicing alterations in human HSPCs, we performed pathway annotation of a3'ss mis-spliced genes. Strikingly, this analysis showed significant enrichment of gene ontology (GO) terms related to cell division, including mitosis and sister chromatid segregation (Fig. 2B). There was high overlap between PB and CB CD34⁺ cells with 186 conserved a3'ss events, which were similarly enriched for GO terms related to cell cycle and DNA damage response (Supplementary Fig. S2B). Cell cycle and DNA damage GO terms were also enriched albeit to a lesser extent in *SF3B1*-mutant K562 cells (Supplementary Fig. S2C; Supplementary Table S2). Overlap between PB, CB, and K562 a3'ss genes identified 37 conserved mis-spliced genes, including canonical mutant SF3B1 targets *BRD9*, *DPH5*, *MAP3K7*, *ORAI2*, *DYNLL1*, and *TMEM14C* (Fig. 2C). Protein-protein interaction showed a core network of mis-spliced genes in CD34⁺ HSPCs mediating cytoskeletal organization and chromosome alignment in mitosis, including *BUBR1* (*BUB1B*), *CDC27*, *DYNLL1*, *AURKB*, *CEP55*, *STIL* (Fig. 2D). These data show that mutant SF3B1 induces pervasive mis-splicing of genes related to cell division and genome maintenance in CD34⁺ HSPCs.

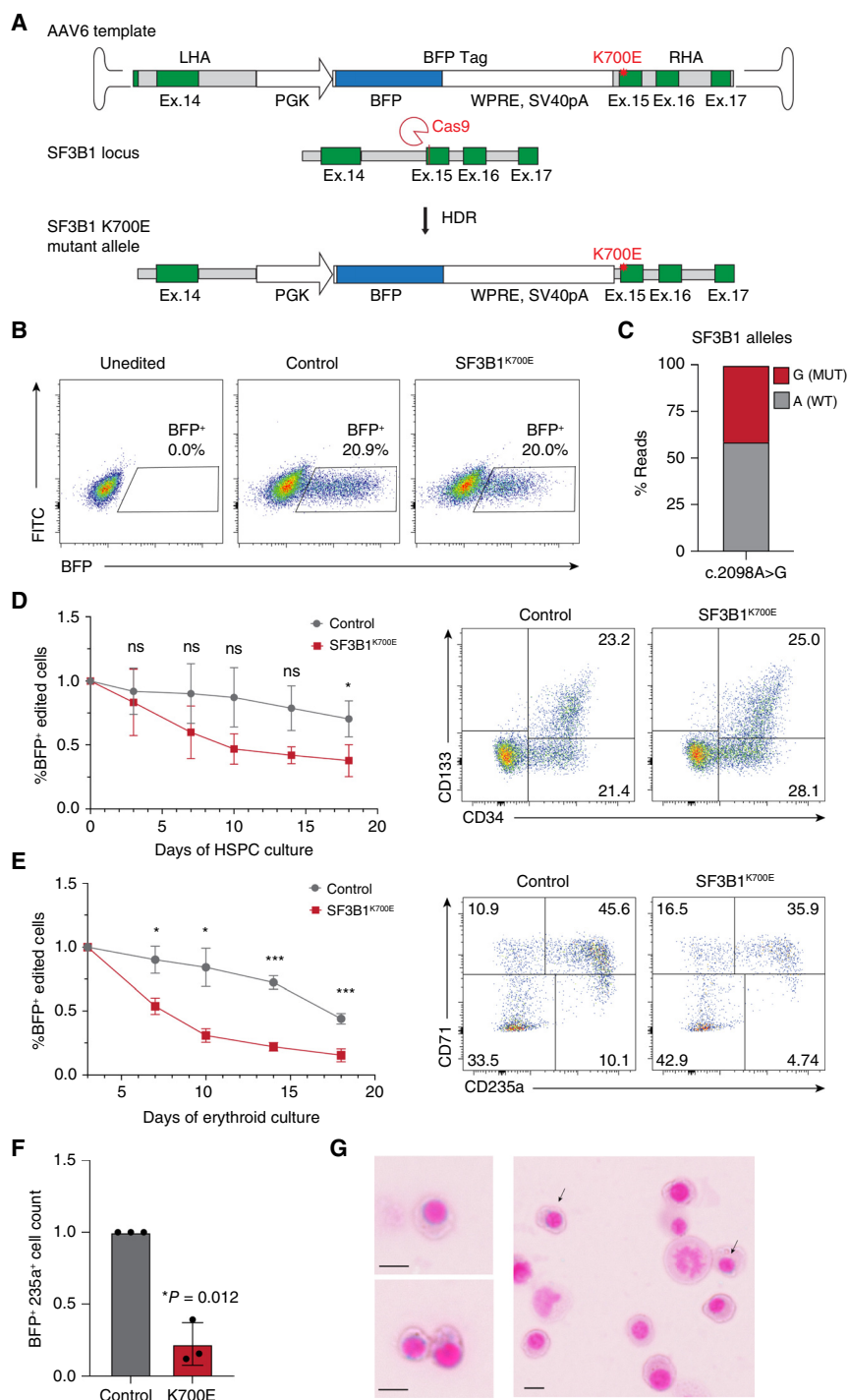


Figure 1. Efficient editing of *SF3B1* K700E mutation in primary CD34⁺ HSPCs. **A**, Schematic of the *SF3B1* K700E gene editing strategy showing the design of the AAV6 repair template encoding the BFP transgene, unedited *SF3B1* locus showing exons (green), introns (gray), the sgRNA cut site in exon 15, and the edited *SF3B1* locus after homology-directed recombination. LHA, RHA: left and right homology arms; HDR: homology-directed repair. **B**, Representative gene editing efficiency as measured by BFP transgene expression 3 days after AAVS1 or *SF3B1* gene editing in CD34⁺ HSPCs. FITC: empty channel. **C**, Percent of *SF3B1* WT and K700E transcripts based on RNA-seq reads in *SF3B1*-edited BFP⁺ HSPCs, confirming heterozygous expression of the mutant allele. **D**, Percent of *SF3B1* K700E or K700K control edited cells over 18 days of HSPC liquid culture (left) and representative flow staining for CD34⁺CD133⁺ HSC markers on day 10 (right). % BFP was normalized to day 0 for each experiment. Mean $\pm$ SD of $n = 3$ experiments with independent CB donors; paired t test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. **E**, Percent of *SF3B1* K700E or control edited cells over 18 days of erythroid differentiation culture (left), and representative flow staining for CD235a⁺CD71⁺ erythroblasts on day 10 (right). % BFP was normalized to day 3 for each experiment. Mean $\pm$ SD of $n = 3$ experiments with independent CB donors; paired t test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. **F**, Number of *SF3B1* K700E mutant CD235a⁺ erythroid cells after 18 days of erythroid culture normalized to control. Mean $\pm$ SD of $n = 3$ experiments with independent CB donors; one-sample t test. **G**, Representative Prussian blue staining for ring sideroblasts (arrows) of *SF3B1* mutant BFP⁺ erythroid cells on day 18 of differentiation. Scale bar, 10 μ m.

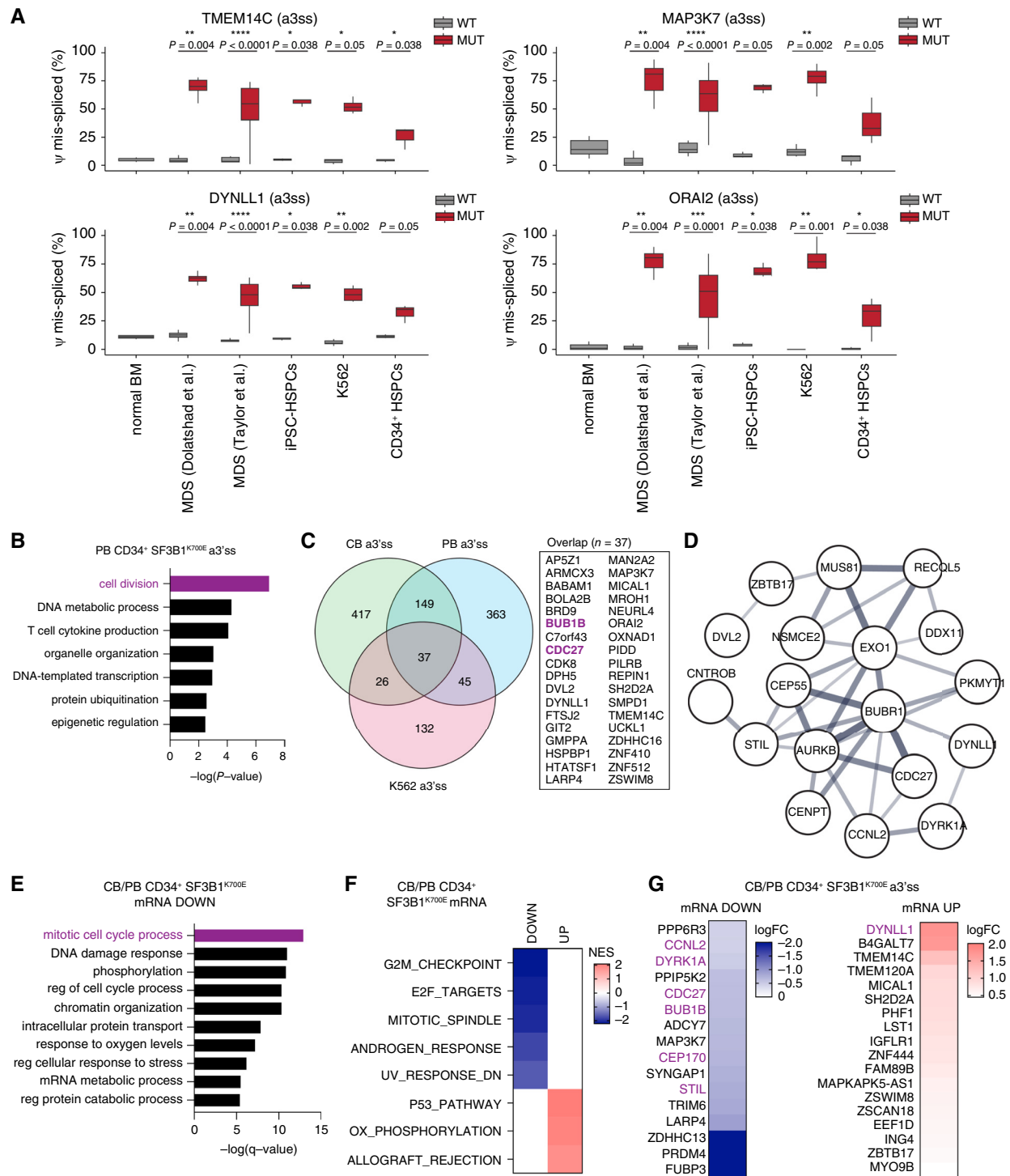


Figure 2. Pervasive mis-splicing of cell division and genome maintenance genes in CD34⁺ HSPCs. **A**, Percent mis-splicing of *TMEM14C*, *MAP3K7*, *DYNLL1*, and *ORAI2* mRNA in patients with *SF3B1* WT or mutant (MUT) MDS, iPSC-derived HSPCs, K562 cells, and edited CD34⁺ CB and PB HSPCs, or normal BM. Median and range; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; one-sided Mann-Whitney U test. **B**, Gene ontology (GO) analysis of a3'ss mis-splicing targets in *SF3B1* K700E edited PB CD34⁺ HSPCs. GO performed using Metascape showing summary terms; $\geq 10\%$ mis-splicing, Bayes factor ≥ 5 . **C**, Overlap between mis-spliced a3'ss events in CD34⁺ CB, CD34⁺ PB and K562 *SF3B1* K700E cells, and list of recurrently mis-spliced genes in all three cell types (right); $\geq 10\%$ mis-splicing, Bayes factor ≥ 5 . **D**, STRING protein-protein interaction network of CB/PB a3'ss mis-spliced genes in the cell division GO category; disconnected nodes removed, line thickness proportional to interaction score > 0.40 . **E**, GO analysis of genes downregulated in *SF3B1* K700E CD34⁺ CB/PB HSPCs ($\log_2$ fold change ≤ -0.5 ; $P < 0.05$). GO performed using Metascape showing summary terms. **F**, Hallmark pathways upregulated or downregulated in *SF3B1* K700E vs. control edited CD34⁺ CB/PB HSPCs; > 25 genes, FDR < 0.01 . NES, normalized enrichment score. **G**, Significantly down- or up-regulated genes among 186 genes with conserved a3'ss mis-splicing in both CB and PB CD34⁺ HSPCs from Supplementary Fig. S2B. Expression ranked by $\log_2$ fold change in *SF3B1* K700E vs. control edited CD34⁺ HSPCs, $P < 0.1$. Mitosis-related terms are shown in purple color.

To understand if the mis-splicing program induced by mutant *SF3B1* results in concordant transcriptional changes, we performed differential gene expression analysis of *SF3B1*-mutant HSPCs. GO and Hallmark categories related to cell division, including mitotic spindle, chromosome segregation and G2/M checkpoint, were most significantly enriched among the downregulated genes (Fig. 2E and F). DNA damage response genes were also downregulated, while p53 pathway and oxidative phosphorylation were upregulated (Fig. 2F). DNA damage response genes included *BRCA1*, *RAD50*, *ERCC6L*, and *FANC* genes, consistent with the reported defects in DNA repair in *SF3B1*-mutant cells (19–21, 24). We next evaluated the overlap between mis-splicing and transcriptional changes. Of the 186 genes with conserved a3'ss events identified in both CB and PB datasets (Supplementary Fig. S2B), 34 (18.3%) were significantly differentially expressed (Fig. 2G). As expected, mis-splicing was associated with both transcript downregulation and upregulation. Mis-spliced downregulated genes included multiple core mitotic network genes *BUBR1*, *CDC27*, *CEP170*, *DYRK1A*, *CCNL2*, and *STIL* (Fig. 2G). Taken together, combined splicing and transcriptomic analysis identified mis-splicing and concomitant reduced expression of genes regulating cell division and genome maintenance in *SF3B1*-mutant cells.

Single-Cell Transcriptomic Analysis of *SF3B1*-Mutant HSPCs

To gain an unbiased understanding of how *SF3B1* mutations shape human HSPC differentiation and gene expression, we isolated CB-derived BFP⁺CD34⁺ control and *SF3B1*-mutant cells and performed single cell RNA sequencing. Following filtering, normalization, and cell cycle regression, unsupervised clustering analysis identified 15 clusters (Fig. 3A). We assigned cell identity to each cluster based on expression of lineage-specific genes (Fig. 3A and B). HSCs expressing *AVP*, *HLF*, and *CD133/PROM1* mapped to cluster 3, while a larger population of early HSPCs based on *CD34* and *PROM1* mapped to clusters 3 and 7. HSC/early HSPCs differentiated into progenitors of granulocyte-monocyte (GMP) lineages expressing *MPO*, *ELANE*, *S100A8/9* (cluster 9), megakaryocyte-erythroid (MEP) lineages expressing *TFRC*, *KLF1*, and *GATA1* (clusters 4, 8), or *ITGA2B* and *GP9* (clusters 10, 14), mast cell (MCP) lineages expressing *MITF*, *KIT*, *KRT1* (clusters 0, 1, 5), and eosinophil-basophil (EoBP) lineages expressing *IL3RA*, *IL5RA*, and *FCER1A* (clusters 2, 6, 13). Interestingly, under our culture conditions, control HSPCs predominantly differentiated along MCP and EoBP lineage trajectories (Fig. 3C). By contrast, *SF3B1*-mutant HSPCs were retained in the early HSPC compartment and preferentially differentiated along the MEP trajectory (Fig. 3C). These data are consistent with preferential commitment of *SF3B1*-mutant HSPCs to the erythroid lineage with ineffective erythroid differentiation (25).

To next identify transcriptional changes induced by mutant *SF3B1*, we manually annotated progenitor populations based on lineage-specific genes (Supplementary Fig. S2D) and performed differential expression analysis. GO and Hallmark categories related to cell division, mitotic spindle organization, and DNA damage repair were selectively downregulated

in *SF3B1*-mutant immature HSPCs (Fig. 3D and E; Supplementary Fig. S2E). Hypoxia and glycolysis genes were downregulated in *SF3B1*-mutant MEPs, cell activation and inflammatory response in GMPs, while oxidative phosphorylation and MYC targets were broadly upregulated (Fig. 3D and E). These results are concordant with our bulk transcriptomic analysis and show that mitotic genes are selectively downregulated in early HSPCs. Downregulated mitotic genes included regulatory (e.g., *BUB3*, *BUBR1*, *CDC27*) and structural (e.g., *STIL*, condensin, CENP proteins) components of the kinetochore and mitotic checkpoint complex (Supplementary Fig. S2F). A number of transcription factors that regulate hematopoietic differentiation, including *MECOM*, *MLLT3*, *CBFB*, and *CEBPD* were differentially expressed, consistent with altered lineage commitment (Supplementary Fig. S2F). Taken together, our single-cell data shows that *SF3B1* mutations alter HSPC differentiation and downregulate gene programs associated with cell division and genome maintenance and is a resource for further interrogation of molecular changes in human HSPCs.

SF3B1-Mutant Cells Have Delayed G2/M Cell Cycle Progression

To investigate if mis-splicing by mutant *SF3B1* and reduced expression of genes regulating cell division and genome maintenance leads to cell cycle changes, we performed EdU/DAPI analysis in *SF3B1* K700E mutant and wild-type (WT) K562 cells, a useful model for biochemical and mechanistic studies. *SF3B1*-mutant cells had a reduced proportion in S phase and increased proportion in G2/M consistent with delayed progression through G2/M (Fig. 4A). We observed concordant cell cycle changes in *SF3B1* K700E edited CB cells expressing HSC marker CD133 (Fig. 4B). The finding of G2/M delay and mis-splicing of core mitotic regulators in *SF3B1*-mutant cells prompted us to further assess mitotic transit. An increased proportion of *SF3B1*-mutant cells were in mitosis as marked by phosphorylated histone H3 (pH3), suggesting an intramitotic delay (Fig. 4C). To further elucidate the rate of mitotic progression, we synchronized K562 cells at the G2/M border using the CDK1 inhibitor RO-3306 and tracked pH3-positive cells after RO-3306 release (26). *SF3B1*-mutant cells showed a marked delay in mitotic progression, starting to exit mitosis 90 minutes after release compared to 60 minutes for WT cells (Fig. 4D). After 2 hours, nearly all WT cells exited mitosis, while more than half of *SF3B1*-mutant cells remained in mitosis ($P = 0.003$; Fig. 4D). These data show that *SF3B1*-mutant cells accumulate in G2/M and show a marked delay in mitotic progression.

To identify mis-spliced genes that induce G2/M delay, we considered 37 genes with conserved a3'ss mis-splicing events in CD34⁺ CB, PB, and K562 cells (Fig. 2C), which demonstrated consistent cell cycle changes (Fig. 4A and B). Of these, *BUB1B* (*BUBR1*) and *CDC27* were part of the core mitotic network (Fig. 2D). *BUBR1* is a mitotic kinase with dual function in chromosome alignment and mitotic checkpoint complex that monitors the spindle assembly (27, 28). *CDC27* is a component of the anaphase-promoting complex that mediates metaphase-to-anaphase transition following correct spindle attachment to kinetochores (29). *BUBR1* and *CDC27* were

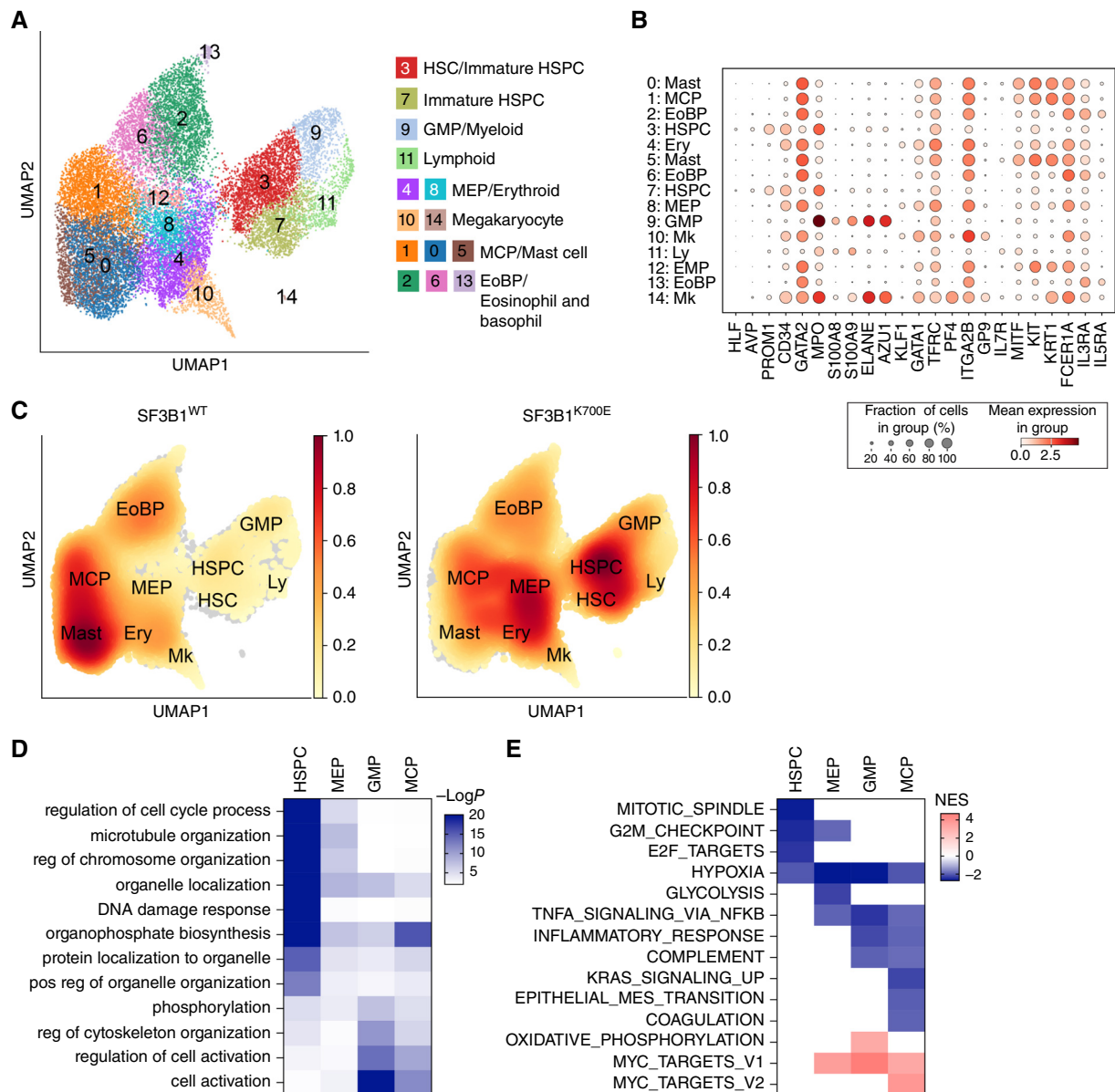


Figure 3. Single-cell transcriptome analysis of *SF3B1*-mutant HSCs. **A**, Combined UMAP of *SF3B1* K700E and control AAVS1 edited (WT) CB BFP⁺CD34⁺ cells showing unsupervised clustering in Scanpy. Clusters were annotated based on the expression of lineage-specific genes in **B**. **B**, Relative expression of lineage-specific marker genes across clusters 0-14: HSPC; GMP; MEP; MCP; EoBP; Ly, lymphoid; Ery, erythroid; Mk, megakaryocyte. Also see Supplementary Fig. S2D. **C**, UMAP density plot of control (left) and *SF3B1*-mutant (right) cell distribution with cell type designations superimposed from **A**. **D**, GO analysis of genes downregulated (log₂ fold change < -0.25, adj. *P* < 0.05) in *SF3B1*-mutant vs. control HSPC, MEP, GMP, and MCP. GO annotation was performed using Metascape multisample mode showing summary terms. **E**, Hallmark pathways upregulated or downregulated in *SF3B1* K700E vs. control HSPC, MEP, GMP, and MCP. Annotation was performed using GSEA with gene lists ranked by log₂ fold change (adj. *P* < 0.05). Only significant terms are shown (>25 genes, FDR < 0.01). NES, normalized enrichment score.

recurrently mis-spliced in CD34⁺ HSPCs, K562 cells, and patients with MDS with *SF3B1* mutation (Fig. 4E; Supplementary Fig. S3A; refs. 6, 7). *BUBR1* and *CDC27* mis-splicing events lead to inclusion of 15 and 18 bp of intronic sequence, respectively, not predicted to influence the susceptibility to nonsense mediated decay of the parent transcript, as neither disrupts the open reading frame nor causes inclusion of a premature termination codon. However, *BUBR1* and *CDC27* were downregulated at both the transcript (Supplementary Fig. S3B)

and protein level (Fig. 4F) by ~50% in *SF3B1*-mutant cells. To test if reduced expression of *BUBR1* or *CDC27* altered cell cycle progression, we transduced WT CD34⁺ cells and K562 cells with individual shRNAs targeting *BUBR1*, *CDC27*, or validated luciferase control. We achieved 75% to 90% knockdown of *BUBR1* and ~60% knockdown of *CDC27* (Supplementary Fig. S3C). Knockdown of *BUBR1* or *CDC27* in CD34⁺ cells or K562 cells increased the proportion of cells in G2/M phase (Fig. 4G; Supplementary Fig. S3D) recapitulating cell cycle

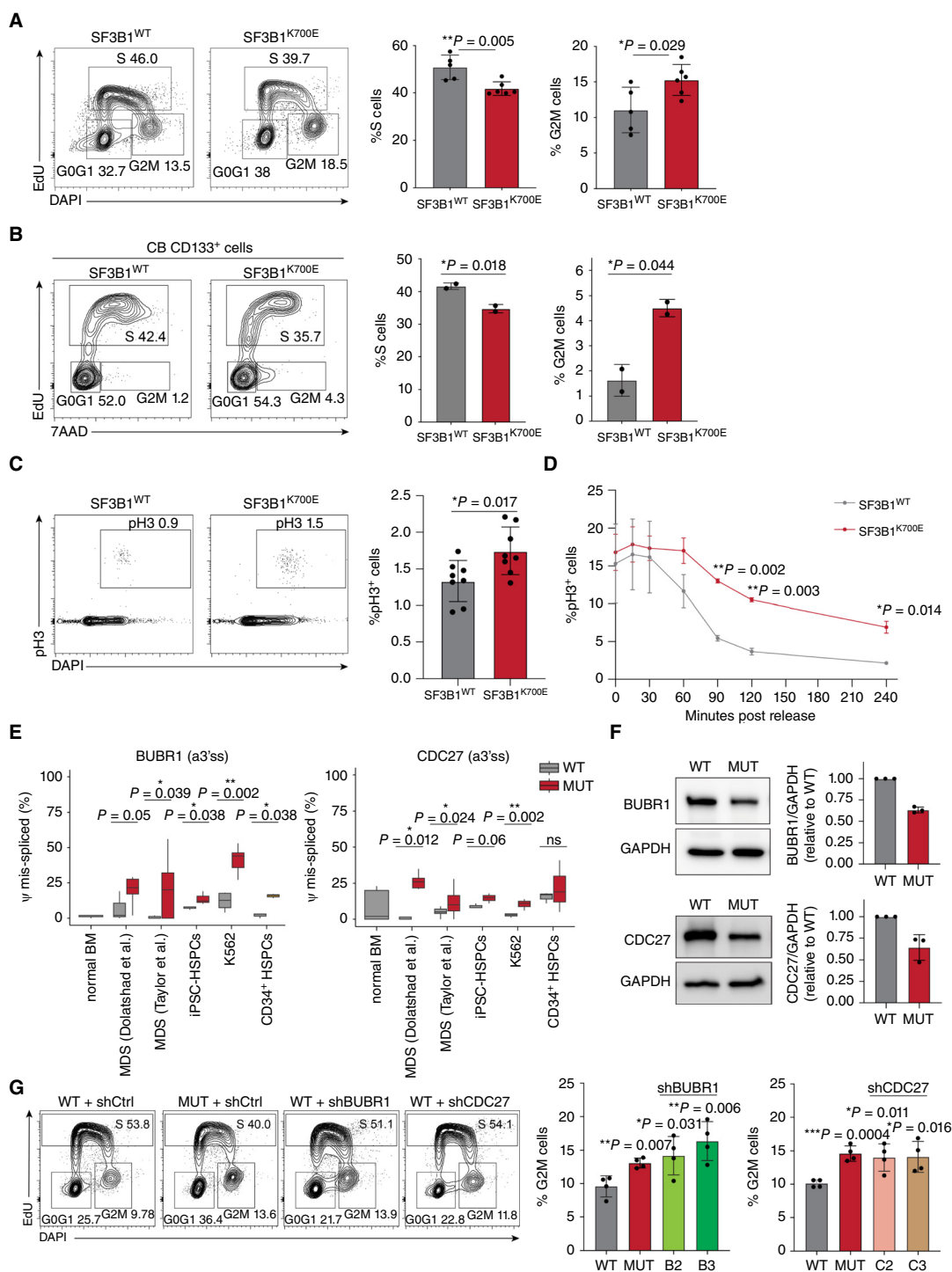


Figure 4. SF3B1-mutant cells have delayed G2/M cell cycle progression. **A**, EdU cell cycle analysis of WT and SF3B1 K700E K562 cells. Representative flow plots (left) and quantitation (right) of the proportion in S or G2/M phase. Mean $\pm$ SD, $n = 6$ independent experiments, unpaired t test. **B**, EdU cell cycle analysis of control or SF3B1 K700E edited CB cells expressing HSC marker CD133. Representative flow plots (left) and quantitation (right). Mean $\pm$ SD, $n = 2$ independent experiments, paired t test. **C**, Proportion of WT and SF3B1 K700E K562 cells positive for pH3, a marker of mitosis. Representative flow plots (left) and quantitation (right). Mean $\pm$ SD, $n = 8$ experiments, unpaired t test. **D**, Proportion of pH3-positive mitotic WT and SF3B1-mutant cells after release from G2/M block using CDK1 inhibitor R0-3306. $n = 2$ independent time-course experiments, unpaired t test. **E**, Mis-splicing of BUBR1 and CDC27 in SF3B1 WT or mutant (MUT) MDS patients, iPSC-HSPCs, K562 cells, and edited CB/PB CD34⁺ HSPCs, or normal bone marrow (BM); significance shown using 1-sided Mann-Whitney U test. **F**, Western Blot analysis of BUBR1 (top) and CDC27 (bottom) protein level in SF3B1-mutant (MUT) and WT K562 cells. Expression normalized to GAPDH and shown as fold change relative to WT; $n = 3$ experiments, mean $\pm$ SD. **G**, EdU cell cycle analysis of WT K562 cells transduced with control luciferase (WT), BUBR1 (B2, B3), or CDC27 (C2, C3) shRNAs, or SF3B1-mutant K562 cells transduced with control shRNA (MUT). Left: representative EdU cell cycle flow plots. Right: proportion of BUBR1 (left) or CDC27 (right) knockdown K562 cells in G2/M phase. Mean $\pm$ SD, $n = 4$ experiments; unpaired t test.

changes in *SF3B1*-mutant cells (Fig. 4A). These data indicate that decreased expression of *BUBR1* or *CDC27* in *SF3B1*-mutant cells delays G2/M progression.

SF3B1-Mutant Cells Are Selectively Sensitized to CHK1 Inhibition

We next sought to determine whether the mis-splicing program and dysregulated mitotic transit of *SF3B1*-mutant HSPCs can be therapeutically targeted. To this end, we first performed an unbiased screen of 165 FDA-approved and investigational compounds. To identify compounds that target *SF3B1*-mutant cells irrespective of cooperating mutations, which can modify drug responses (30), we screened K562 lines with *SF3B1* K700E alone or high-risk co-mutations in *RUNX1* or *STAG2* (Supplementary Fig. S4A). This screen identified CHK1 inhibitor (CHK1i) AZD-7762 as the top hit (Supplementary Fig. S4B; Supplementary Table S3). *SF3B1*-mutant cells were previously shown to respond to inhibitors of DNA damage response, including PARP inhibitors and UCN-01, a nonspecific inhibitor of multiple kinases including CHK1 (bioRxiv 2021.05.26.445839; refs. 19–22).

To ascertain the selective role of CHK1, we first tested a panel of selective inhibitors. *SF3B1*-mutant K562 cells displayed ~7-fold lower IC₅₀ to AZD-7762 that targets CHK1/2 (IC₅₀ Ctrl 384 nmol/L, S-A 57 nmol/L, S-R 65 nmol/L, S-S 42 nmol/L; Fig. 5A; Supplementary Fig. S4C), and ~5–7-fold lower IC₅₀ to specific CHK1 inhibitors MK-8776 (IC₅₀ WT 1.7 μmol/L, K700E 0.4 μmol/L) and prexasertib (IC₅₀ Ctrl 24 nmol/L, S-A 3.0 nmol/L, S-R 3.5 nmol/L, S-S 3.5 nmol/L; Fig. 5B; Supplementary Fig. S4D and S4E), a selective CHK1 inhibitor currently in clinical trials for solid and hematological malignancies. Consistently, *SF3B1*-mutant cells were sensitized to ATR inhibitors VE-821 (IC₅₀ WT 6.6 μmol/L, K700E 1.7 μmol/L) and ceralasertib (IC₅₀ WT 2.7 μmol/L, K700E 1.3 μmol/L; Supplementary Fig. S4E). By contrast, there was no response to CHK2 inhibitor BML277 (Supplementary Fig. S4E). Interestingly, consistent with the mitotic defect there was a modest sensitivity to the spindle poison taxol (Supplementary Fig. S4F). As a reference, spliceosome inhibitor pladienolide B showed ~3-fold lower IC₅₀ in *SF3B1*-mutant cells (IC₅₀ Ctrl 5.1 nmol/L, S-A 1.7 nmol/L, S-R 1.9 nmol/L, S-S 1.4 nmol/L; Fig. 5C; Supplementary Fig. S4G). Consistent with CHK1 activation, *SF3B1*-mutant cells also had increased CHK1 phosphorylation at S345, which was enhanced by CHK1i prexasertib (Fig. 5D). To validate the selective requirement for CHK1, we ablated the *CHEK1* locus by CRISPR/Cas9 targeting with ~75% knockout efficiency (Fig. 5E). Growth of *SF3B1*-mutant but not WT K562 cells was significantly reduced by CHK1 knockout confirming a selective requirement for CHK1 in *SF3B1*-mutant cells (Fig. 5E). These data suggest that *SF3B1*-mutant cells are dependent on CHK1 function and highly sensitized to CHK1 inhibition.

We next tested the response to prexasertib and pladienolide B in primary CB CD34⁺ HSPCs. Edited cells were treated for 7 days with 2.5 nmol/L prexasertib or pladienolide B and the number of BFP⁺ cells quantified by flow cytometry. Prexasertib treatment reduced *SF3B1*-mutant CD34⁺ cells by ~50% compared to 20% for WT CD34⁺, similar to spliceosome inhibitor pladienolide B (Fig. 5F). Notably, this response was unchanged

by introduction of *RUNX1/STAG2* high-risk co-mutations. These data show that *SF3B1*-mutant cells display CHK1 activation and are highly sensitized to clinical CHK1i prexasertib.

Mis-splicing of BUBR1 and CDC27 Promotes Sensitivity to CHK1 Inhibition

Although CHK1 is best studied in the context of DNA damage response, CHK1 also has a critical function in mitotic spindle assembly checkpoint (27, 31, 32). As *SF3B1*-mutant cells show delayed mitotic transit (Fig. 4C and D), we tested if reduced expression of mitotic regulators *BUBR1* and *CDC27* contributed to CHK1 inhibitor sensitivity. To determine if depletion of mitotic regulators induced CHK1 activation, we probed phosphorylation of CHK1 at S345 following knockdown of *BUBR1* or *CDC27*. Knockdown of either *BUBR1* or *CDC27* induced CHK1 phosphorylation, which was further enhanced by CHK1i prexasertib (Supplementary Fig. S5A and S5B). These data suggest that depletion of *BUBR1* or *CDC27* leads to CHK1 activation, consistent with a role in mitotic checkpoint response. We next sought to determine if depletion of *BUBR1* or *CDC27* sensitized WT cells to prexasertib. *SF3B1*-mutant cells transduced with control shRNA maintained increased sensitivity to CHK1i (IC₅₀ WT 14.0 nmol/L, MUT 3.1 nmol/L). Knockdown of *BUBR1* with two independent shRNAs partially sensitized WT cells to prexasertib (IC₅₀ shRNA-B3 4.2 nmol/L, shRNA-B2 6.4 nmol/L; Fig. 5G; Supplementary Fig. S5C). Similarly, knockdown of *CDC27* with two independent shRNAs partially sensitized WT cells to prexasertib (IC₅₀ shRNA-C3 5.5 nmol/L, shRNA-C2 8.0 nmol/L; Fig. 5G; Supplementary Fig. S5C). By contrast, candidate *SF3B1* targets involved in genome maintenance, *DYNLL1* and *GEN1* (bioRxiv 2021.05.26.445839), did not affect prexasertib sensitivity (Supplementary Fig. S5D–S5H). Taken together, these findings show that reduced expression of *BUBR1* and *CDC27* delays G2/M progression, induces compensatory CHK1 activation, and partially contributes to CHK1 inhibitor sensitivity.

CHK1 Inhibition Selectively Targets SF3B1-Mutant HSCs

We next sought to determine if prexasertib can eradicate disease-propagating HSCs with *SF3B1* mutation. To this end, we edited CD34⁺ cells with *SF3B1* mutation alone or with high-risk *RUNX1* or *STAG2* co-mutations. Edited cells were treated for 7 days with 2.5 nmol/L prexasertib or pladienolide B and the number of phenotypic HSCs was determined by flow cytometry for CD34, CD38, and CD133 (Fig. 6A). The proportion of WT CD34⁺CD133⁺ phenotypic HSCs increased after prexasertib treatment, suggesting that normal HSCs are resistant to prexasertib (Fig. 6B; Supplementary Fig. S6A). Consistently, the number of WT phenotypic HSCs was not affected by prexasertib treatment, while *SF3B1*-mutant phenotypic HSCs derived from CB or adult PB were depleted by ~50% (Fig. 6C). This response was unchanged by introduction of *RUNX1/STAG2* co-mutations, indicating that high-risk HSCs have similar response to prexasertib. In addition, prexasertib depleted primary CD34⁺ cells from a panel of patients with *SF3B1*-mutant MDS (Supplementary Fig. S6B). By contrast, spliceosome inhibitor pladienolide B was toxic to

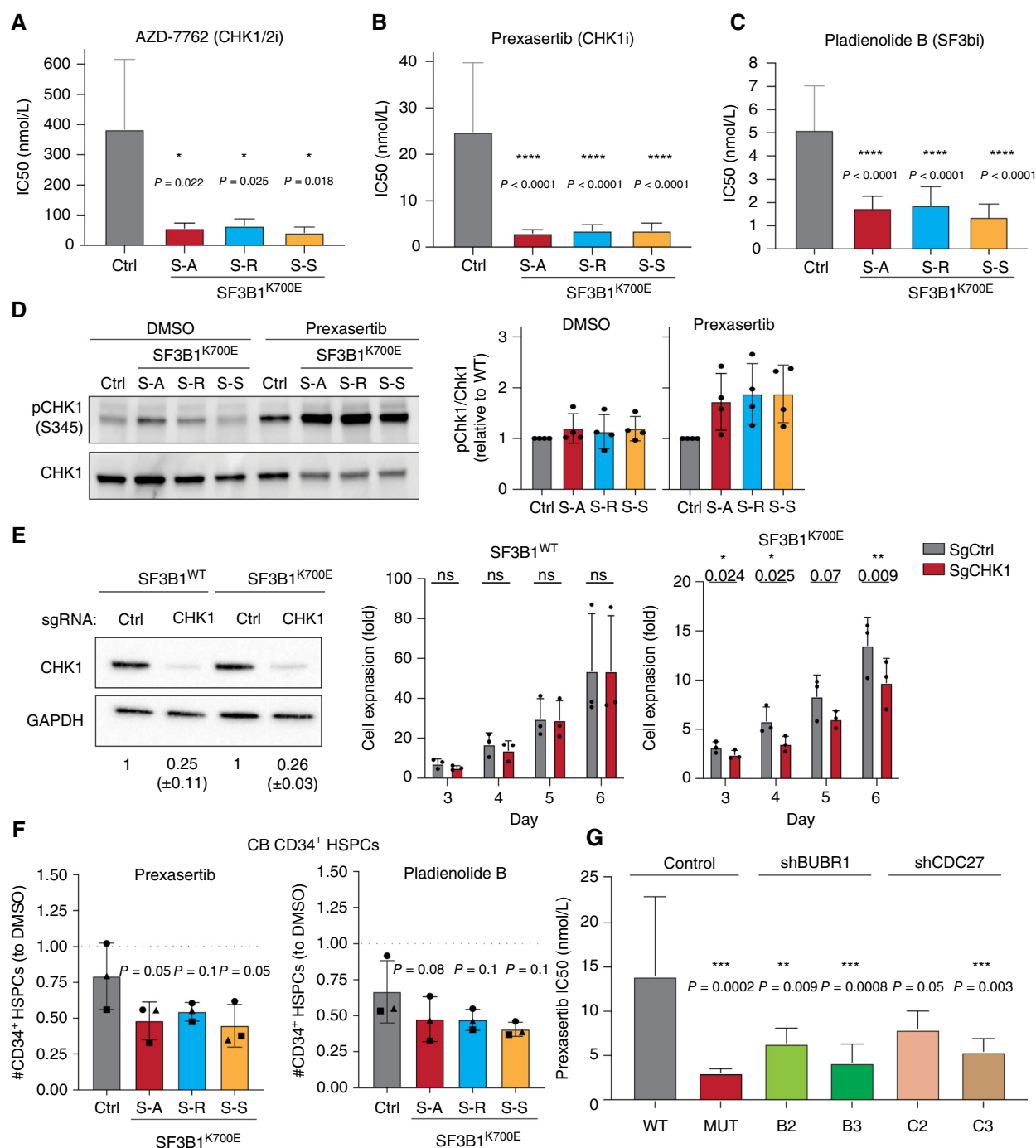


Figure 5. *SF3B1*-mutant cells are selectively sensitized to CHK1 inhibition. **A–C**, IC₅₀ value of CHK1 inhibitor AZD-7762 (**A**), CHK1 inhibitor prexasertib LY2606368 (**B**), or SF3B inhibitor pladienolide B (**C**) in WT (Ctrl) or *SF3B1*-mutant (S-A, S-R, S-S) K562 cells. Cells were treated with drugs in a dose-response format for 5 days. Mean $\pm$ SD, of four independent experiments, one-way ANOVA with Dunnett's correction for multiple comparisons. **D**, CHK1 phosphorylation in WT (Ctrl) or *SF3B1*-mutant (S-A, S-R, S-S) K562 cells. Left: Western blot analysis of pCHK1 (S345) and total CHK1 treated for 5 hours with DMSO or 10 nmol/L CHK1i prexasertib. Right: Ratio of pCHK1 to total CHK1 in *SF3B1*-mutant normalized to WT cells. $n = 2$ independent experiments, two technical replicates. **E**, Cell growth of WT and *SF3B1*-mutant K562 cells after CRISPR-mediated ablation of *CHK1*. Left: CHK1 protein level after control AAVS1 (sgCtrl) or *CHK1* (sgCHK1) knockout. Right: Cell growth of WT and *SF3B1*-mutant cells with sgCHK1 or sgCtrl at 3–6 days of culture, relative to day 0 (72 hours after editing). $n = 3$ independent experiments, ratio paired *t* test. **F**, Number of CB-derived control (Ctrl) or *SF3B1* K700E-edited (S-A, S-R, S-S) CD34⁺ HSPCs after treatment with 2.5 nmol/L pladienolide B (right) or 2.5 nmol/L CHK1i prexasertib (left) for 7 days. Fold change of CD34⁺ cell number after drug treatment was calculated relative to vehicle (DMSO). Mean $\pm$ SD, of $n = 3$ independent experiments; paired *t* test. **G**, IC₅₀ value of CHK1i prexasertib in WT K562 cells transduced with control luciferase (WT), *BUBR1* (B2, B3), or *CDC27* (C2, C3) shRNAs, or *SF3B1*-mutant K562 cells transduced with control shRNA (MUT). Cells were treated with prexasertib in a dose response format for 5 days; two independent shRNAs, two sets of lines independently generated for each condition. Mean $\pm$ SD, of three independent experiments, one-way ANOVA with Dunnett's correction for multiple comparisons.

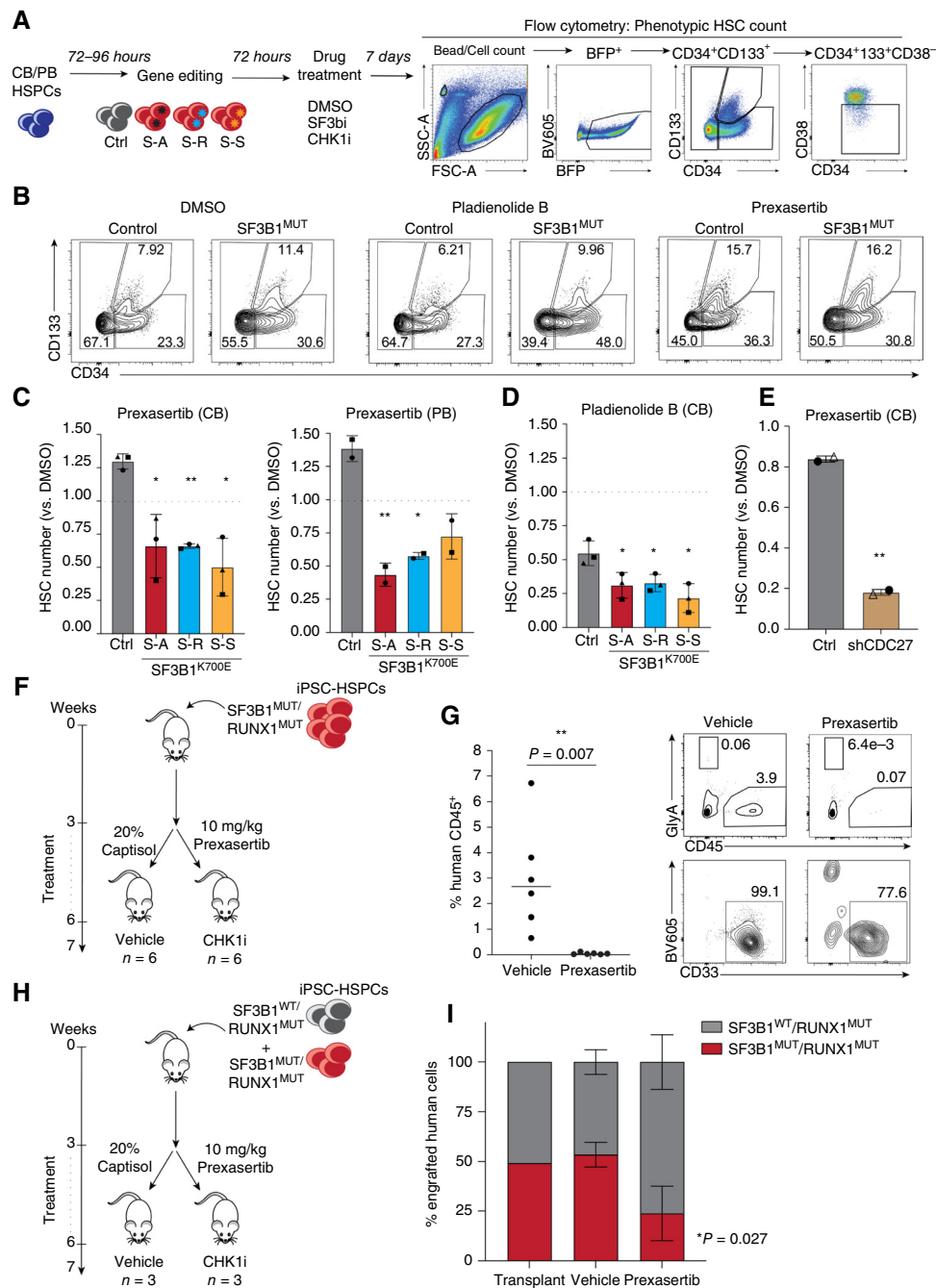


Figure 6. CHK1 inhibition selectively targets *SF3B1*-mutant HSCs. **A**, Experimental strategy to determine the number of WT (Ctrl) or *SF3B1* K700E-edited (BFP⁺) CD34⁺CD133⁺(CD38⁻) phenotypic HSCs after drug treatment; BV605: empty channel. **B**, Representative flow plots of the % CD34⁺CD133⁺ cells after 7 days of treatment with vehicle (DMSO), 2.5 nmol/L pladienolide B, or 2.5 nmol/L prexasertib. **C**, Number of CD34⁺CD133⁺ phenotypic HSCs edited for control (Ctrl) or *SF3B1* K700E (S-A, S-R, S-S) after 7 days of treatment with 2.5 nmol/L prexasertib. CD34⁺ cells derived from CB (left) or PB (right) donors. Fold change of CD34⁺CD133⁺ phenotypic HSC number after drug treatment was calculated for each genotype, relative to vehicle (DMSO). Mean $\pm$ SD, of $n = 3$ independent experiments; *, $P < 0.05$; **, $P < 0.01$; paired t test. **D**, Same as **C** with 2.5 nmol/L pladienolide B (CB). **E**, Number of normal CB CD34⁺CD133⁺ phenotypic HSCs transduced with control or *CDC27* shRNA after 7 days of treatment with 2.5 nmol/L prexasertib. Mean $\pm$ SD, of $n = 2$ independent experiments; paired t test. **F**, Experimental approach for *in vivo* treatment with prexasertib CHK1i. NSG-SGM3 mice were transplanted with *SF3B1*/RUNX1 mutant MDS patient iPSC-derived HSPCs and treated with vehicle (Captisol) or 10 mg/kg prexasertib hydrochloride for 3 weeks. **G**, Engraftment of *SF3B1*/RUNX1 mutant iPSC-HSPCs in NSG-SGM3 mice treated with vehicle or CHK1i for 3 weeks. Right: representative flow plots and gating strategy to determine human engraftment. Human engraftment was determined as % of human CD45⁺CD33⁺ cells in the injected femur 7 weeks after transplantation. Mean $\pm$ SD, of $n = 6$ vehicle and $n = 6$ CHK1i mice, unpaired t test. **H**, Experimental approach for *in vivo* treatment with prexasertib CHK1i of mice co-transplanted with *SF3B1*-WT and mutant cells. NSG-SGM3 mice were transplanted with *SF3B1*-WT/RUNX1-mutant and *SF3B1*/RUNX1 double-mutant iPSC-HSPCs in a 1:1 ratio and treated with vehicle (Captisol) or 10 mg/kg prexasertib hydrochloride for 3 weeks. **I**, Percentage of *SF3B1*-mutant iPSC-HSPCs of human engraftment in NSG-SGM3 mice co-transplanted with *SF3B1*-WT and mutant cells and treated with vehicle or CHK1i for 3 weeks. Human CD45⁺CD33⁺ cells were isolated from individual mice and % of *SF3B1* K700E cells determined by amplicon next generation sequencing. Mean $\pm$ SD, of $n = 3$ vehicle and $n = 3$ CHK1i mice, unpaired t test.

normal phenotypic HSCs (Fig. 6D). Importantly, while normal phenotypic HSC were completely resistant to prexasertib killing, knockdown of *CDC27* was sufficient to render normal HSCs sensitive to prexasertib (Fig. 6E). These data indicate that prexasertib selectively eradicates *SF3B1*-mutant HSCs irrespective of co-mutations while preserving normal HSCs.

As primary MDS samples engraft poorly in mice, we utilized an MDS iPSC-based model to test the efficacy and selectivity of prexasertib *CHK1i* *in vivo*. For these experiments, we reprogrammed primary CD34⁺ cells from a patient with high-risk MDS with *SF3B1* K700E and *RUNX1* P359fs mutations. We isolated two iPSC lines corresponding to distinct genetic subclones in this patient: *SF3B1*-WT/*RUNX1*-mutant, *SF3B1*/*RUNX1* double-mutant, and derived expandable HSPCs through conditional expression of five transcription factors (33). We first transplanted *SF3B1*/*RUNX1*-mutant iPSC-derived HSPCs into NOD/SCID-IL2R^γ null mice transgenic for human SCF, GM-CSF, and IL-3 (NSGS). Three weeks post-transplant, mice were treated with a standard preclinical regimen of 10 mg/kg prexasertib or vehicle control (34) and CD45⁺CD33⁺ human myeloid engraftment quantified after 7 weeks (Fig. 6F). All of the (6/6) vehicle-treated mice were engrafted (3.0% ± 2.1%). Strikingly, none (0/6) of the prexasertib-treated mice were engrafted above the 0.2% threshold (Fig. 6G). Treatment was well tolerated, with slightly reduced RBC and increased platelet PB counts (Supplementary Fig. S6C). To test whether prexasertib selectively ablated *SF3B1*-mutant MDS cells *in vivo*, we next co-transplanted isogenic *SF3B1*/*RUNX1*-mutant and *SF3B1*-WT/*RUNX1*-mutant iPSC-derived HSPCs in a 1:1 ratio (Fig. 6H). Three weeks post-transplant, mice were treated with prexasertib or vehicle control as above, and the proportion of *SF3B1*-mutant cells in the CD45⁺CD33⁺ human engraftment was quantified after 7 weeks. Vehicle-treated mice had comparable engraftment of *SF3B1*-mutant and *SF3B1*-WT cells (Fig. 6I). Prexasertib treatment significantly decreased the percentage of *SF3B1*-mutant relative to *SF3B1*-WT cells compared to vehicle control ($P = 0.027$; Fig. 6I). These data suggest that prexasertib is well-tolerated and selectively eradicates *SF3B1*-mutant MDS cells *in vivo*.

DISCUSSION

SF3B1 mutations are acquired in HSCs and generate a repertoire of mis-spliced transcripts providing valuable therapeutic targets (3, 4, 6, 7, 10). However, molecular and functional consequences of *SF3B1* mutations in human HSPCs remain poorly understood limiting the discovery of therapeutic strategies. Here, we have developed efficient gene editing of *SF3B1* mutations in human HSPCs and show that *SF3B1* mutations induce pervasive mis-splicing and downregulation of cell division and genome maintenance genes leading to delayed mitotic progression and profound sensitivity to *CHK1* inhibition. We identify mis-splicing of mitotic regulators *BUBR1* and *CDC27* as one of the mutant *SF3B1* dependencies that contributes to *CHK1i* sensitivity. Our study identifies the mis-splicing program in human HSPCs as a clinically relevant vulnerability in *SF3B1*-mutant myeloid malignancies.

The pathogenic role of *SF3B1* change-of-function mutations have been modeled in mice and human iPSCs (8, 13, 35). However, these models are transcriptionally distinct from

human HSCs and may have a different repertoire of mis-spliced target genes. Here, we have sought to develop a gene editing approach to introduce *SF3B1* K700E mutations in human CD34⁺ cells. Our gene editing approach uses the AAV6 homology template, first developed for multiplexed genetic engineering to knockout driver genes (36). Our approach introduces a precise *SF3B1* K700E mutation at the endogenous locus with an intronic BFP cassette for long-term tracking and isolation of edited cells. Our single-cell and functional data consistent with studies in *SF3B1*-mutant clonal hematopoiesis (25), suggest that *SF3B1* mutations interfere with HSPC differentiation promoting preferential commitment to the erythroid lineage and ineffective erythroid differentiation. Mutant *SF3B1* induces widespread mis-splicing and quantitatively reduced expression of genes regulating cell division and genome maintenance leading to altered cell cycle progression with delayed mitotic transit. We propose that these broad molecular changes sensitize *SF3B1*-mutant cells to compounds targeting cell cycle and DNA damage response (bioRxiv 2021.05.26.445839; refs. 19–22), and show that *SF3B1*-mutant human phenotypic HSCs are potentially and selectively targeted by clinical *CHK1* inhibitors. Although multiple mutant *SF3B1* dependencies likely promote *CHK1i* sensitivity (19–21, 24, 37), we identify mis-splicing of mitotic regulators as one of the mechanisms that contribute to heightened *CHK1i* response.

CHK1 has distinct roles in DNA damage response and mitotic surveillance. In response to DNA damage during interphase, ATR phosphorylates *CHK1* to engage the S and G2/M checkpoints (23, 31). Spliceosome mutant cells incur several types of DNA damage, notably R loops, hybrid DNA:RNA structures associated with defective splicing (21, 24, 37). Accumulation of R loops has been associated with activation of ATR/*CHK1* signaling in *SF3B1*-mutant cells (21). However, other reports have not detected increased R loops in *SF3B1*-mutant cells (38). In addition, *CHK1* has a critical but poorly understood role in the spindle assembly checkpoint, which arrests mitotic progression until chromosomes are properly attached to the spindle (23, 27). *CHK1* S345 phosphorylation increases in mitosis and its mitotic role has been proposed to be uncoupled from DNA damage (31, 32). Our findings suggest that *SF3B1* mutations engage this mitotic function of *CHK1*. Consistent with our data, expression of G2/M genes, including *BUBR1*, is highly predictive of prexasertib response across a large panel of cancer cell lines and xenografts (39). We propose a model in which mis-splicing of *BUBR1* and *CDC27* by mutant *SF3B1* reduces the efficiency of chromosome alignment and anaphase entry leading to activation of *CHK1*, which is likely required for proper error correction to occur and resumption of mitosis. Although further investigation is needed to establish the mechanism of *CHK1* function in mitosis and the relative contributions of DNA damage and mitotic surveillance pathways to *CHK1* dependency of *SF3B1*-mutant cells, we present evidence for a novel mechanism wherein mis-splicing of mitotic regulators underlies *CHK1* dependency in *SF3B1*-mutant HSPCs.

Targeted therapeutic strategies for *SF3B1*-mutant MDS and acute myeloid leukemia remain limited. Despite promising preclinical results, the clinical trial of spliceosome inhibitor H3B-8800 in myeloid neoplasms, including spliceosome-mutant patients, did not lead to objective responses (18). We show

that SF3b inhibition does not effectively target *SF3B1*-mutant HSCs because normal human phenotypic HSCs are sensitized to spliceosome inhibition, which could explain the lack of clinical responses (18). A previous study has shown that *SF3B1*-mutant cells are sensitized to UCN-01, which inhibits multiple kinases including CHK1 (21, 22). However, preclinical efficacy of selective CHK1i in *SF3B1*-mutant myeloid neoplasms has not been tested. We identified prexasertib, a specific inhibitor of CHK1 kinase as a promising therapeutic (22). Prexasertib selectively targeted *SF3B1*-mutant phenotypic HSCs *in vitro* irrespective of co-mutations, was well-tolerated *in vivo* and selectively abrogated engraftment of *SF3B1*-mutant MDS iPSC-derived HSPCs in a human xenograft model. Phase I/II clinical trials with prexasertib in solid tumors have displayed acceptable tolerability and toxicity (40). Taken together, our findings demonstrate that prexasertib is highly selective and efficacious at targeting *SF3B1*-mutant HSCs *in vitro* and *in vivo* and may be a promising strategy for patients with *SF3B1*-mutant MDS and its advanced stages.

In conclusion, we develop precise gene editing of human HSPCs to reveal mis-splicing of mitotic genes as a targetable vulnerability in *SF3B1*-mutant HSPCs engaged by CHK1 inhibition. The safety profiles displayed in clinical trials make prexasertib a suitable agent for clinical investigation in *SF3B1*-mutant myeloid malignancies.

METHODS

AAV Generation: Vector Constructs and AAV Production

Gene editing vectors were derived from pAAV-MCS2 [a gift from Steve Jackson (Addgene plasmid #46954)]. pAAV.SF3B1K700E.BFP plasmid was generated with *SF3B1* homology arms containing a total of 1.6 kb of homology comprising coordinates chr2:198,266,105-198,267,704 (hg37). Each homology arm was 800 bp in length and was synthesized as gBlocks (IDT). The right homology arm contains the K700E mutation at genomic coordinate 198,266,834 (A>G, hg37) and a synonymous PAM mutation at genomic coordinate 198,266,826 (G>C, hg37). For pAAV.SF3B1K700K.BFP control plasmid, the right homology arm contains only the synonymous PAM mutation. The PGK.BFP.WPRE.SV40 cassette was generated by gene synthesis and amplified using primers FP: 5'-GGGTTGCGCCTTTTCCAAG and RP: 5'-TAAGATACATTGATGAGTTTGGACAA. pAAV-MCS2 was digested with NotI-HF and MluI-HF (NEB) and Gibson Assembly (NEB) was used to insert the two homology arms and the PGK.BFP.WPRE.SV40 cassette.

pAAV.AAVS1.BFP plasmid was generated with *AAVS1* homology arms containing a total of 1.6 kb of homology comprising coordinates chr19:55,626,320-55,627,919 (hg37). A>T mutation was introduced at genomic coordinate 55,627,159 (hg37). Homology arms synthesis, PGK.BFP.WPRE.SV40 cassette generation and cloning were done as for pAAV.SF3B1.BFP plasmid. AAV stocks were produced by double transfection of pAAV.SF3B1K700E.BFP, pAAV.SF3B1K700K.BFP, or pAAV.AAVS1.BFP vector and pDGM6 (a gift from David Russell, Addgene plasmid #110660) in HEK293T cells. Transfected cells were collected 48 hours later, lysed by freeze-thaw, benzonase-treated, and purified over iodixanol density gradient, as previously described (41).

Isolation, Culture, and Gene Editing of Primary Human CD34⁺ HSPCs

Umbilical CB was obtained from Bloodworks Northwest following guidelines approved by the institutional review board at the University of Washington. Mobilized PB CD34⁺ cells were purchased from the

Fred Hutchinson Cancer Center CCEH core. Samples were collected from both male and female recipients. All reported experiments were replicated with two or more independent donors unless otherwise indicated.

CB mononuclear cells were collected by centrifugation with Ficoll-Paque Plus (GE), followed by red cell lysis with ammonium chloride (StemCell Technologies). CD34⁺ cells were extracted using the Miltenyi CD34 Microbead kit according to manufacturer's instructions and viably cryopreserved in 10% DMSO, 40% FBS. Cryopreserved CD34⁺ HSPCs were thawed following the Lonza Poietics protocol (www.lonza.com) and cultured for 72 to 96 hours in HSPC expansion media: StemSpan SFEM II (StemCell Technologies), 100 U/mL penicillin/streptomycin (Fisher), 100 ng/mL SCF, 100 ng/mL FLT3, 100 ng/mL TPO, 100 ng/mL IL6 (all Peprotech), 35 nmol/L UM171 (Apexbio Technology), and 0.75 μ M SR1 (Cellagen Technology). Cell density was maintained between 4×10^5 and 1×10^6 per mL.

After 72 to 96 hours of stimulation, HSPCs were CRISPR-edited using the Lonza 4D Nucleofection system and the Human Primary Cell Nucleofection Kit (Lonza). RNP complexes were generated by combining Cas9 protein and sgRNA (Synthego) at 1:2.5 molar ratio in P3 Primary Cell Nucleofector Solution with Supplement 1 (Lonza). For simultaneous gene editing of multiple loci, Cas9 was complexed with multiple sgRNAs maintaining 1:2.5 molar ratio for each individual sgRNA.

The sgRNA sequences are as follows:

SF3B1	5'-UGGAUGAGCAGCAGAAAGUU-3'
AAVS1	5'-GGGCCACUAGGGACAGGAU-3'
RUNX1#C	5'-GAGCCCAGGCAAGAUGAGCG-3'
STAG2#B	5'-AUACCUUGUGAUAGCAUGU-3'

Complex was incubated for 20 minutes at room temperature. Between 4×10^5 and 1×10^6 HSPCs per condition were resuspended in 17.5 μ L P3 Buffer and added to the RNP complex. CD34⁺ HSPCs were electroporated using Lonza 4D (DZ-100 program) and returned to HSPC media containing AAV particles comprising <20% culture volume, as recommended (36). HSPCs were washed 16 to 24 hours later and plated in HSPC media for 48 hours. Editing efficiency was assessed by flow cytometry and PCR amplification of edited loci, followed by Sanger sequencing and inference of CRISPR edits (ICE) analysis (Synthego).

Erythroid Differentiation of Human Cord Blood HSPCs

Differentiation protocol was adapted based on Clough and colleagues (8). Gene-edited HSPCs were placed into erythroid stage 1 media 72 hours after gene editing. Stage 1 (6 days): Iscove's modified Dulbecco's medium (IMDM) + 1% bovine serum albumin (Gibco), 20% fetal bovine serum (FBS; Sigma), 1 mmol/L L-glutamine (ThermoFisher), penicillin/streptomycin (Fisher), 500 μ g/mL holo-transferrin (Sigma), 10 μ g/mL human insulin (CellSciences), 6 U/mL Epo (Procrit), 100 ng/mL SCF, and 5 ng/mL IL-3 (both Peprotech). Cells were seeded at a density of 2 to 3×10^5 cells/mL in 24-well plates. Stage 2 (12 days): media were changed to IMDM + 1% bovine serum albumin, 20% FBS, 1 mmol/L L-glutamine, penicillin/streptomycin, 500 μ g/mL holo-transferrin, 10 μ g/mL human insulin, 6U/mL Epo, and 50 ng/mL SCF. Cells were seeded at a density of 3×10^5 cells/mL in 24-well plates as cell number permitted. During erythroid differentiation, cells were maintained at a density of $<2 \times 10^6$ /mL. For larger numbers of cells, plates and dish sizes were scaled up accordingly to maintain the 3×10^5 cells/mL starting density. Erythroid differentiation was carried out until day 18 (6 days stage 1 + 12 days stage 2) and differentiation efficiency evaluated by flow cytometry.

Prussian blue iron staining was performed on day 18 of erythroid culture. 5×10^4 to 1×10^5 cells were collected onto microscope slides with a cytocentrifuge (Thermo Scientific) and air dried. Cells were fixed in -20°C methanol for 12 minutes and air dried prior to

iron staining. Slides were stained with Prussian Blue kit (Abcam, ab150674) as per the manufacturer's instructions. Slides were incubated in water for 30 seconds prior to a 12-minute incubation in iron stain followed by a 30-second water wash. Cells were counter-stained in nuclear fast red for 5 minutes and rinsed for 4 × 30-second washes in water. Slides were preserved with Vectamount (Vector laboratories) and coverslipped. Images were collected with an EVOS M5000 microscope (ThermoFisher Scientific).

Lentiviral Vectors and Preparation

For lentivirus-mediated CRISPR/Cas9 editing, oligos were cloned into pLentiCRISPR-v2-mCherry vector (#99154; a gift from Agata Smogorzewska) following the cloning vector protocol (Addgene).

sgRNA oligonucleotide sequences are as follows:

AAVS1	5'-GGGCCACTAGGGACAGGAT-3'
RUNX1#A	5'-CACTTCGACCGACAAACCTG-3'
RUNX1#C	5'-GAGCCCAGGCAAGATGAGCG-3'
STAG2#C	5'-AATACTAACCTTGAACCGAC-3'

For lentivirus-mediated gene knockdown, shRNA oligonucleotides were cloned in the TRC cloning vector pLKO.1 (Addgene, plasmid #10878) following the pLKO.1 TRC cloning vector protocol (Addgene). shRNA sense oligonucleotide sequences for BUBR1, CDC27, and GEN1 were selected from the GPP web portal (Broad Institute). A shRNA targeting luciferase was used as a control. shRNA sequences used are as follows:

Control Luciferase (M1)	5'-CTTACGCTGAGTACTTCGAC-3'
BUBR1#2 (B2)	5'-GAGACAACCTAACTGCAAATT-3'
BUBR1#3 (B3)	5'-GTGGAACACTGAACTGTATG-3'
CDC27#2 (C2)	5'-CAAGTACCTAATCATAGTTTA-3'
CDC27#3 (C3)	5'-GCCTATAACAGTGACTTGATT-3'
GEN1#1 (G1)	5'-GCCTTTAAAGGAACGAATATT-3'
GEN1#2 (G2)	5'-CAATACTTCTGTCCCTTATTC-3'

For lentivirus-mediated gene overexpression, open reading frame (ORF) for DYNLL1 was purchased from the Mission TRC3 Human LentiORF Collection and subcloned into the pSMAL overexpression vector (Addgene, Plasmid #161785). A luciferase ORF was synthesized and subcloned into pSMAL as the control.

Virus was prepared using third generation packaging plasmids pMDLg/RRE, pRSV/Rev, and pMD.G for VSV-G pseudotyping. Plasmids were transfected into HEK293T cells with calcium phosphate (Takara). Lentivirus was concentrated via ultracentrifugation at 23,000 rpm for 2:15 hours, resuspended in StemSpan SFEM II (Stem-Cell Technologies), and stored at -80°C. All viruses were titered by serial dilution on 293T cells.

Lentiviral Transduction of Human CD34⁺ HSPCs

After 4 to 5 days of stimulation, 10⁵ HSPCs were transduced on Retrofectin-coated (Takara), non-tissue culture-treated 96-well plates in complete HSPC expansion media with polybrene at multiplicity of infection (MOI) = 25 and a total volume of 150 µL. Virus was concentrated onto cells by centrifuging the plate at 2,300 rpm for 30 minutes at room temperature, prior to incubation at 37°C. After 16 to 24 hours, cells were washed in PBS and cultured in HSPC expansion media for 48 hours.

K562 Culture, Infection, and Gene Editing

SF3B1-mutant (K700E) and wild-type control K562 cells (8) were grown in IMDM with 10% FBS and 100 U/mL penicillin/streptomycin (Fisher), and cultures were maintained at <1.5 × 10⁶ cells/mL. For lentivirus infection, K562 cells were transduced in a 48-well plate in standard media with polybrene. A total of 5 × 10⁵ cells were transduced at an MOI = 1 and washed 16 to 24 hours later. Transduced cells

were purified on a BD FACS Aria III sorter. SF3B1 WT control cells with AAVS1 editing and SF3B1-mutant genotypes (S-A, S-R, S-S) were established as single cell-derived clones, and two clones per genotype were used for downstream experiments.

For CHK1 genetic targeting, K562 cells were CRISPR-edited using the Lonza 4D Nucleofection system and the SF Cell Line Nucleofector X Kit (Lonza). RNP complexes were generated by combining Cas9 protein and sgRNA (Synthego) at 1:2.5 molar ratio in SF Cell Line Nucleofector Solution with Supplement (Lonza). The sgRNA sequences are as follows:

AAVS1	5'-GGGCCACUAGGGACAGGAU-3'
CHK1	5'-AACCUCAAAAUCUCAGACUU-3'

Complex was incubated for 20 minutes at room temperature. 4.5 × 10⁵ K562 cells per condition were resuspended in SF Buffer and added to the RNP complex. K562 cells were electroporated using Lonza 4D (FF 120 program) and returned to culture. After 72 hours, knockout efficiency was assessed by Western blot and 1.5 × 10⁵ cells/mL were plated for analysis of cell growth.

In vitro Drug Treatment

For high-throughput drug screening, K562 cells were plated into 6 × 384-well plates plus one clear bottom plate and placed at 37°C, 5% CO₂. Serial dilutions of each compound were added 24 hours after plating, and cells were incubated for 72 hours. Viability was measured with Promega's CellTiter Glo 2.0 Lot# 000472252. Data from wells of each plate were normalized for 32-plate blank values and 16 DMSO solvent control wells with the formula: Well %Viability = [(Well Relative Light Unit - Plate Blank)/(DMSO Control - Plate Blank)] × 100. IC₅₀s were determined for each genotype from fitted curves of viabilities at serial dilution of each compound (Supplementary Table S3).

For targeted drug testing, K562 cells were plated into 96-well tissue culture-treated plates at a density of 5,000 cells per 150 µL per well. Cells were treated with serial dilutions of SF3b inhibitor pladienolide B (0–100 nmol/L, Tocris); CHK1/CHK2 inhibitor AZD-7762 (0–1 µmol/L, SelleckChem); CHK1 inhibitors LY2606368 prexasertib HCl (0–100 nmol/L, SelleckChem) and MK-8776 (SCH 900776, SelleckChem; 0–1 µmol/L); ATR inhibitors VE-821 (0–40 µmol/L, SelleckChem) and ceralasertib (AZD6738; 0–10 µmol/L, SelleckChem); DNA-PK inhibitor AZD7648 (0–40 µmol/L, SelleckChem); and taxol (0–20 nmol/L, Tocris). After 5 to 6 days, cell viability read-out was performed using the CellTiter-Glo 2.0 Cell Viability Assay (Promega, G9242) as per the manufacturer's instructions. The proportion of viable cells with drug treatment was calculated relative to DMSO control. A three-parameter nonlinear fit of log(inhibitor) versus response was performed in GraphPad Prism v7.0 (GraphPad Software, San Diego, CA) to determine IC₅₀ values.

For testing SF3b and CHK1 inhibitor in CD34⁺ HSPCs, cells were cultured in HSPC expansion media with 2.5 nmol/L or pladienolide B, 2.5 nmol/L prexasertib, or DMSO vehicle control for 7 days. Fresh media and drugs were added at day 4. Total viable cell counts and proportion of HSPC populations were determined by flow cytometry, as described below. For CHK1i testing in BM-MNC from patients with SF3B1-mutant MDS, cells were cultured in HSPC expansion media with 2.5 nmol/L prexasertib or DMSO vehicle control for 4 days. Total viable HSPC counts were determined by flow cytometry, as described below.

iPSC-HSPC Generation and Culture

Bone marrow mononuclear cells were obtained from a patient with MDS with excess of blasts, according to the institutional guidelines approved by the University of Washington and Fred Hutchinson Cancer Research Center Institutional Review Board.

Written informed consent was received per the Declaration of Helsinki. Clinical sequencing was performed using a TruSeq Custom Amplicon panel. iPSC reprogramming, hematopoietic differentiation, and iPSC-HSPCs generation was performed as previously described (8, 33). iPSC-HSPCs cells were cultured in StemSpan SFEM II (StemCell Technologies) with 50 ng/mL SCF, 50 ng/mL FLT3, 50 ng/mL TPO, 50 ng/mL IL-6, 10 ng/mL IL-3 (all Peprotech), and 100 U/mL penicillin/streptomycin (Fisher). Doxycycline was added at 2 µg/mL (Sigma). Cultures were maintained at $<1.5 \times 10^6$ cells/mL and media were exchanged every 3 to 4 days.

Mouse Transplantation, Drug Treatment, and Evaluation of Human Cell Engraftment

NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}Tg(CMV-IL3,CSF2,KITLG)1Eav/MlozSzJ (NSGS) mice were purchased from Jackson Labs and housed at the University of Washington animal care facility. Animal experiments were performed in accordance to institutional guidelines approved by UW animal care committee. Intra-femoral transplants were performed as previously described (33). Briefly, 8 to 14-week-old mice were irradiated at 275 rads 24 hours before transplant. To ensure consistency, only female mice were used. Prior to transplantation, mice were temporarily sedated with isoflurane. A 27g needle was used to drill the right femur (injected femur), and 5×10^6 iPSC-HSPCs were transplanted in a 25 µL volume using a 29.5g insulin needle. For co-transplantation, 2.5×10^6 SF3B1-mutant and 2.5×10^6 SF3B1-WT isogenic iPSC-HSPCs were transplanted together as above. Baytril was administered in drinking water to prevent infections after irradiation. Doxycycline (1.0 mg/mL) was added to the drinking water to maintain transgene expression *in vivo*, as previously described (33).

Prexasertib HCl (SelleckChem) was resuspended in 20% Captisol (Captisol). Prexasertib or vehicle was administered subcutaneously at a standard dose of 10 mg/kg twice daily for 3 consecutive days followed by 4 days of rest, repeated for 3 weeks, as previously described (34). Drug administration was started 3 weeks post-transplant; mice were sacrificed after 7 weeks, and injected femur, and uninjected femur and tibiae were collected. Single-cell suspension was prepared using standard flushing and cell dissociation techniques in 1 mL of IMDM. From that suspension, 100 µL of injected femur, 50 µL uninjected marrow were stained in a total volume of 300 µL staining buffer. Samples were not lysed with red blood cell lysis buffer as not to lyse human erythrocytes. Samples were stained with the 1:80 dilution of the following antibodies: CD33-PE (WM53; BD), CD45-APC (HI30; BD), CD235a (11E4B-7-6; Coulter). DAPI (ThermoFisher) was used to discriminate live/dead cells. For quantification of SF3B1 K700E variant allele frequency, gDNA was extracted from FACS purified CD45⁺CD33⁺ cells using QIAamp DNA Micro Kit (Qiagen) as per manufacturer instructions. SF3B1 locus was PCR amplified with the primers below and analyzed by next generation sequencing (Amplicon-EZ, Genewiz).

Fwd: 5'-CTGCAGTTTGGCTGAATAGTTGA-3'
Rev: 5'-ATGGCCAAAGCACTGATGGT-3'

Flow Cytometry and Cell Sorting

Erythroid differentiation was analyzed using antibodies CD71 APC (M-A712; BD) and CD235a/Glycophorin A PE-Cy7 (11E4B-7-6; Coulter). HSPC populations were analyzed using stem cell markers CD34 AF700 (S81; BD), CD38 PE-Cy7 (HB7; BD), and CD133 APC or FITC (AC133; Miltenyi). Primary BM-MNCs were stained with SytoxGreen live-dead stain (ThermoFisher), CellTrace Calcein Violet (ThermoFisher), CD45 APC (HI30; BD), and CD34 PE-Cy7 (S81; BD). For quantifying drug response, CountBright Absolute Counting Beads (Invitrogen) were added to cells resuspension as per the manufacturer's instructions. For all staining panels, cells were incubated for 20 minutes at room temperature in PBS with 2% FBS with 1:100

antibody dilution (1:50 for CD133). For cell cycle analysis, cultured cells were incubated in 20 µmol/L EdU for 2.5 hours. Cells were then harvested and fixed (BD Cytofix) for 15 minutes at room temperature, permeabilized with BD Perm/Wash buffer for 15 minutes (BD Biosciences), followed by EdU detection as per Click-iT EdU cell proliferation kit protocol (ThermoFisher cat. #C10340). For G2/M synchronization, cells were treated for 17 hours with 9 µmol/L RO-3306 (SelleckChem), washed three times and plated in complete medium. Samples were harvested at 0, 15, 30, 60, 90, 120, and 240 minutes after plating and fixed/permeabilized. Mitotic cells were stained for 1 hour at room temperature with Phospho-Histone H3 (Ser10) PE-conjugated antibody (Cell Signaling Technologies #5764) diluted 1:100 in BD Perm/Wash buffer. For DNA detection, cells were stained with DAPI (ThermoFisher) or 7-AAD (BD Biosciences) and acquired in linear mode. Acquisition was performed on the BD FACSLyric, BD LSR II, and Symphony A3 cytometers. Cell sorting was performed on BD Aria III flow sorter. All data were analyzed using FlowJo software (10.7.0).

gDNA Extraction, PCR Amplification, and Sanger Sequencing

gDNA was extracted from gene-edited cells using QIAamp DNA Micro Kit (Qiagen) as per the manufacturer's instructions. Edited loci were PCR amplified using Taq 5X Master Mix (NEB) or PrimeStar GXL Polymerase (Takara). PCR products were purified using NucleoSpin gel and PCR Clean-Up kit (Macherey-Nagel) and submitted to Genewiz for Sanger sequencing. Percentage of frameshift mutations was estimated from Sanger sequencing by ICE analysis. Primer details are as follows (* indicates sequencing primer):

AAVS1: *F 5'-ATCCTCTCTGGCTCCATCGT-3'
R 5'-CCGGTTAATGTGGCTCTGGT-3'
RUNX1: *F 5'-AAGGCCCTGAACGTGTATG-3'
R 5'-GGCCAGTACCTTGAAGCGA-3'
STAG2: *F 5'-GGCAGTTTCTCTCTGTCTCT-3'
R 5'-AACAAACTATGCACGAAGTAAGA-3'
SF3B1: F 5'-GCTGCTGGTCTGGCTACTAT-3'
*R 5'-TCTATCCAAAGCCATCCTGTGC-3'

Quantitative RT-PCR

RNA was extracted from 50,000 to 300,000 cells using TRIzol (ThermoFisher) and cDNA synthesis performed using iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. Quantitative PCR (qPCR) was carried out using SYBR Green Master Mix (ThermoFisher). Primer details are as follows:

GAPDH: F 5'-TCCTGCACCACTGCTTA-3'
R 5'-TCTTCTGGGTGGCAGTGATGG-3'
RUNX1: F 5'-AGATGAGCGAGGCGTTG-3'
R 5'-CGGAGCAGAGGAAGTTGG-3'
STAG2: F 5'-GGATAGCATGTGGGACTGTG-3'
R 5'-GCACTCTCTTGCCTATCTGTT-3'
GEN1: F 5'-TCCCCTTGCCTAATCTTGGTG-3'
R 5'-TCCAGAAGACCCATACCGAGA-3'

Western Blotting

Protein lysates were prepared by resuspending 100,000 washed cells/10 µL of RIPA buffer (Thermo Fisher) with protease and phosphatase inhibitor (Sigma). Lysates were resolved by 4% to 20% SDS-PAGE (Bio-Rad) and immunoblotted with antibodies for GAPDH (1:10,000, Abcam, ab9485), HSP90 (1:10,000, BD Biosciences #610419), RUNX1 (1:1,000, Santa Cruz sc-365644), STAG2 (1:1,000, Santa Cruz sc-81852), BUBR1 (1:1,000, BD Biosciences #612502), CDC27 (1:500, Cell Signaling Technologies #12530), pCHK1 S345 (1:1,000, Cell Signaling Technologies #2348), CHK1 (1:1,000, Cell Signalling Technology #2360), DYNLL1 (1:5,000, abcam #51603).

Membranes were washed in TBST and then incubated with HRP-conjugated secondary antibodies for visualization on a Bio-Rad ChemiDoc or Azure c600 (Azure biosystems). Images were exported to ImageJ for analysis.

Bulk RNA Sequencing and Splicing Analysis

FACS-sorted BFP⁺CD34⁺ gene edited HSPCs from CB and PB and clonally derived K562 cells were resuspended in TRIzol (ThermoFisher, cat. #15596026), and RNA extracted per manufacturer protocol. RNA sequencing was performed at Omega Bioservices with TruSeq Stranded mRNA kit with polyA selection. PB BFP⁺CD34⁺ gene edited HSPCs were additionally sequenced with SMART-Seq v4 Ultra Low Input RNA Kit. Samples were processed on the Illumina HiSeq platform in the 2 × 150 bp configuration with 25 to 50 million reads per sample. For gene expression analysis, reads were pseudoaligned to hg38 reference genome using kallisto (42). Differential gene expression was determined using the DESeq2 package (43). Gene set enrichment analysis (GSEA) was performed on the Gene Ontology datasets in the Broad MsigDB. For splicing analysis, isoform expression levels were estimated as previously described (8). In brief, a transcriptome annotation for the hg19/GRCh37 human genome assembly was created by merging gene annotations from Ensembl v71.1, the UCSC knownGene track, and MISO v2.0 isoform annotations (44). RSEM v1.2.4 (45) was used to map all RNA-seq reads to this transcriptome annotation. Remaining unaligned reads were then mapped to a database of all possible junctions between annotated 5' and 3' splice sites of the transcriptome annotation, as well as to the genome sequence, with TopHat v2.0.8b (46), and the resulting aligned reads were merged with the RSEM output. The expression levels of isoforms annotated in MISO v2.0's annotation were estimated with MISO v2.0. Events that were differentially spliced in *SF3B1*-mutant versus control samples were identified as previously described (8). Briefly, we defined the metric deltaPSI (Δ PSI) as the isoform ratio (absolute percentage of mRNA) in *SF3B1*-mutant samples—isoform ratio in control cells, and computed a significance statistic using Wagenmakers's Bayesian framework (47) for single-sample comparisons or the Mann–Whitney *U* test for group comparisons. An event was classified as differentially spliced if it exhibited an absolute change of Δ PSI $\geq$ 10% and Bayes factor $\geq$ 5 or $P \leq$ 0.05.

Single-Cell RNA Sequencing and Analysis

Gene-edited CD34⁺ HSPCs were flow-sorted 72 hours after gene editing. Single-cell sequencing was performed using the 10X Chromium Next GEM Single Cell 3' Reagents kit v3.1 on a Chromium Controller. Data were aligned to hg38 reference genome using Cell Ranger (48). The resulting count matrix was used to create the Anndata object for subsequent data analysis, which was performed in Scanpy (49). Data preprocessing involved removing cells with <200 expressed genes, UMI < 5,000, mitochondrial reads >10%, and genes expressed in <3 cells. Doublets were removed using Scrublet. Filtered data were log-normalized. Cell cycle bias was evaluated using principal component analysis and uniform manifold approximation and projection for dimension reduction (UMAP), and cell cycle regression performed using S and G2M gene lists in Seurat (50). UMAP generation and clustering was performed using the Leiden algorithm with 20 principal components, 80 neighbors, random seed set to 84. Cluster identity was assigned based on lineage-specific gene expression. Cell types were annotated based on the average expression of the following lineage-specific genes: HSC (*PROM1*, *AVP*, *HLF*), early HSPC (*PROM1*, *CD34*), GMP/myeloid (*MPO*, *ELANE*, *AZU1*, *S100A8/9*), MEP/erythroid (*KLF1*, *TFRC*, *GATA1*), MEP/megakaryocyte (*PF4*, *ITGA2B*, *GP9*), and MCP/mast (*KIT*, *KRT1*). This was done with replacement such that some cells may be assigned to more than one population. Differential gene expression analysis for each cell type was conducted on filtered and log-normalized raw data using *t* test with FDR multiple testing

correction. Output genes were filtered using the following criteria: expressed in >25% of control or *SF3B1*-mutant cells, FDR < 0.05, and log fold change as indicated.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism software. Data are shown as the mean with standard deviation unless noted. For all analyses, $P < 0.05$ was considered statistically significant, unless otherwise noted. Investigators were not blinded to the different groups.

Data Availability

Single-cell and bulk RNAseq data have been deposited in Gene Expression Omnibus under accession numbers: GSE263299 (bulk RNAseq and splicing analysis) and GSE263300 (single-cell RNAseq).

Authors' Disclosures

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Authors' Contributions

M. Sarchi: Conceptualization, formal analysis, investigation, visualization, writing—original draft, writing—review and editing. **C.A. Clough:** Formal analysis, investigation, methodology. **E.I. Crosse:** Data curation, software, formal analysis. **J. Kim:** Formal analysis, investigation, visualization. **L.D. Baquero Galvis:** Investigation, visualization, methodology. **N. Aydinian:** Data curation, software, formal analysis. **R. Wellington:** Data curation, software, formal analysis. **F. Yang:** Investigation. **A. Galli:** Data curation. **J.P. Creamer:** Data curation. **S. Stewart:** Investigation. **R.K. Bradley:** Conceptualization, resources, data curation, supervision, funding acquisition, project administration, writing—review and editing. **L. Malcovati:** Conceptualization, resources, supervision, funding acquisition, project administration, writing—review and editing. **S. Doulatov:** Conceptualization, resources, formal analysis, supervision, funding acquisition, investigation, project administration, writing—review and editing.

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