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Graphical abstract

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A mitochondrial surveillance mechanism activated by SRSF2 mutations in hematologic malignancies

Xiaolei Liu,1 Sudhish A. Devadiga,1 Robert F. Stanley,2 Ryan M. Morrow,3 Kevin A. Janssen,3 Mathieu Quesnel-Vallières,4 Oz Pomp,5 Adam A. Moverley,6 Chenchen Li,1 Nicolas Skuli,1 Martin Carroll,1 Jian Huang,6 Douglas C. Wallace,3,7 Kristen W. Lynch,4 Omar Abdel-Wahab,2 and Peter S. Klein1,5

1Department of Medicine, Division of Hematology-Oncology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA. 2Molecular Pharmacology Program, Sloan Kettering Institute, Memorial Sloan Kettering Cancer Center, New York, New York, USA. 3Center for Mitochondrial and Epigenomic Medicine, Children’s Hospital of Philadelphia, Philadelphia, Pennsylvania, USA. 4Department of Biochemistry and Biophysics and 5Department of Cell and Developmental Biology, Institute for Regenerative Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA. 6Coriell Institute for Medical Research, Camden, New Jersey, USA. 7Department of Pediatrics, Division of Human Genetics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA.

Splicing factor mutations are common in myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML), but how they alter cellular functions is unclear. We show that the pathogenic SRSF2P95H+/− mutation disrupts the splicing of mitochondrial mRNAs, impairs mitochondrial complex I function, and robustly increases mitophagy. We also identified a mitochondrial surveillance mechanism by which mitochondrial dysfunction modifies splicing of the mitophagy activator PINK1 to remove a poison intron, increasing the stability and abundance of PINK1 mRNA and protein. SRSF2P95H+/−-induced mitochondrial dysfunction increased PINK1 expression through this mechanism, which is essential for survival of SRSF2P95H+/− cells. Inhibition of splicing with a glycogen synthase kinase 3 inhibitor promoted retention of the poison intron, impairing mitophagy and activating apoptosis in SRSF2P95H+/− cells. These data reveal a homeostatic mechanism for sensing mitochondrial stress through PINK1 splicing and identify increased mitophagy as a disease marker and a therapeutic vulnerability in SRSF2P95H mutant MDS and AML.

Introduction

Myelodysplastic syndrome (MDS) is a clonal hematopoietic disorder characterized by hyperproliferative bone marrow, dysplastic hematopoietic cells, peripheral cytopenias, and high risk of progression to acute myeloid leukemia (AML). Recurrent mutations in RNA splicing factors, including SF3B1, SRSF2, and U2AF1, account for 60%-70% of somatic mutations in MDS (1) and are common in myeloproliferative neoplasms (MPNs), the MDS/MPN overlap disorder chronic myelomonocytic leukemia (CMML), and AML (1–6). However, the mechanisms by which these mutations alter cellular functions or contribute to transformation remain unclear.

Mutations in distinct splicing factors cause myeloid disorders with similar phenotypic features but limited overlap in specific mRNAs that are aberrantly spliced. This may suggest that distinct mRNA targets are responsible for these myeloid malignancies or that shared pathways are affected downstream of aberrantly spliced mRNAs. Hotspot mutations in SRSF2 alter RNA binding site specificity and disrupt the splicing of mRNAs encoding hematopoietic regulators that are frequently mutated in AML (7, 8). For example, the SRSF2P95H+ mutation promotes inclusion of a poison exon in EZH2 mRNA, leading to nonsense-mediated decay and reduced levels of EZH2 (7). Thus, the number of patients with reduced EZH2 expression is higher than the number with EZH2 mutations (8). These studies support a pathogenic mechanism targeting the splicing of a single mRNA, but do not rule out additional contributions from other splicing events. Furthermore, transcriptomic analyses of different splicing factor mutants show that altered splicing of distinct mRNAs can affect common downstream pathways, including splicing itself, protein synthesis, and mitochondrial function (9).

Splicing factor mutations occur as mutually exclusive, heterozygous point mutations at specific residues (10, 11). The presence of a wild-type splicing factor is required for survival of MDS cells that express a mutant splicing factor, suggesting vulnerability associated with disrupted splicing factor activity. Consistent with this, hematopoietic cells with heterozygous splicing factor mutations are sensitized to additional perturbation of the splicing machinery by small-molecule splicing inhibitors (12, 13). This observation has led to clinical trials with splicing inhibitors in refractory leukemia patients (12, 14–18). However, these trials have been ineffective so far (14), and there are currently no FDA-approved therapies that target splicing factors in MDS or AML.

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Glycogen synthase kinase-3α (GSK-3α) and GSK-3β are multifunctional serine/threonine kinases encoded by 2 similar genes, GSK3A and GSK3B. GSK-3 functions downstream of Fli3, c-Kit, and Wnt signaling, all pathways associated with MDS and AML (19). Gsk3α/b knockdown (20) or Gsk3b deletion (21) in mouse bone marrow causes a pronounced myeloproliferative phenotype, and Gsk3α/b double-knockout (DKO) causes marked expansion of both mature granulocytes and primitive blasts, leading to an aggressive AML (21), but the substrates that mediate GSK-3 function in mouse and human hematopoietic malignancies have not been extensively characterized. Our recent phosphoproteomic analysis of GSK-3 substrates identified multiple core splicing factors implicated in MDS and AML (19, 22), consistent with prior studies supporting the importance of GSK-3 in splicing regulation, including evidence that GSK-3 phosphorylation of SRSF2 regulates splicing of MAPT (Tau) exon 10 (23–25). Both pharmacologic inhibition and genetic loss of GSK3 disrupt pre-mRNA splicing on a transcriptome-wide scale in diverse cell types (19, 26), raising the possibility that clinically well-tolerated GSK-3 inhibitors could be repurposed to treat splicing factor mutant MDS and AML.

In the present study, we assessed transcriptome-wide changes in splicing upon GSK-3 inhibition (GSK-3i) in leukemic cells. We find that GSK-3i disrupts splicing and selectively kills MDS and AML cells with splicing factor mutations. Mechanistically, GSK-3i induces a heightened splicing response in splicing factor mutant cells by repressing cassette exon inclusion and promoting intron retention. These findings led us to focus on SRSF2, as SRSF2-mutant cells show disrupted splicing of nuclear-encoded mitochondrial genes, impaired mitochondrial function, and an increase in mitophagy that is essential for their survival. Splicing of mRNA encoding the mitochondrial surveillance factor PINK1 is altered in response to mitochondrial stress in SRSF2 P95H/+ mutant cells, leading to a more stable splice form and reflecting what we believe to be a novel mechanism for sensing mitochondrial stress. GSK-3i disrupts splicing of PINK1, promoting a splice form with a premature stop codon and reducing overall PINK1 mRNA levels. The reduction in PINK1 is associated with reduced mitophagy and enhanced cell death in SRSF2 P95H/+ mutant cells, sparing cells with wild-type SRSF2. Our findings reveal a mechanism for mitochondrial surveillance and identify a new therapeutic vulnerability in SRSF2-mutant MDS and AML.

Results

Preferential sensitivity of spliceosomal mutant leukemia to GSK-3i. Heterozygous splicing factor mutations sensitize leukemic cells to additional perturbation of the core splicing machinery (13). As GSK-3 regulates splicing in other cell types (22–26), we tested whether GSK-3i would enhance cell death in leukemic cells with heterozygous knockins of recurrent driver mutations in SRSF2 and SF3BI. Isogenic SRSF2 P95H/+ and SF3BI K700E/+ knockin K562 cells (13) were cultured with the GSK-3 inhibitor CHIR99021 (CHIR) or vehicle control, and proliferation was monitored at 2 and 4 days. Both SRSF2 P95H/+ and SF3BI K700E/+ cells were preferentially sensitive to CHIR (Figure 1A and Supplemental Figure 1A); supplemental material available online with this article; https://doi.org/10.1172/JCI175619DS1) and the clinically well-tolerated GSK-3 inhibitor lithium (Supplemental Figure 1B) compared with isogenic cells with wild-type (WT), SRSF2, and SF3BI. A flow cytometric assay for apoptosis revealed that GSK-3i promoted both early and late apoptosis (Figure 1B) in splicing factor mutant cells compared with parental cells but had little impact on their cell cycle status (Supplemental Figure 1C). Collectively, these data demonstrate that GSK-3i preferentially kills spliceosomal mutant leukemias.

As an alternative approach to compare the effect of mutated and WT SRSF2 in otherwise genetically identical cells, we introduced SRSF2 P95H or SRSF2 WT into parental TF-1 or K562 cells along with mCherry (Supplemental Figure 1, D and E), and then followed proliferation of mCherry-positive and untransduced cells. Expression of SRSF2 P95H resulted in a competitive disadvantage in comparison with WT-expressing cells (Supplemental Figure 1F), consistent with prior reports (7, 27). Further, GSK-3i markedly increased apoptosis in TF-1 and K562 cells expressing SRSF2 P95H compared with parental cells and cells overexpressing SRSF2 WT (Figure 1G and Supplemental Figure 1G). Therefore, SRSF2 P95H confers sensitivity to GSK-3i whether introduced as a heterozygous knockin or overexpressed in a genetically identical background.

We next tested whether GSK-3i selectively kills splicing factor mutant cells in vivo in mice bearing xenografts of isogenic WT and SRSF2 P95H/+ K562 cells (Figure 1D). Daily administration of CHIR slowed the growth of xenografts with SRSF2 P95H/+ (P = 0.0036) but had no significant effect on SRSF2 WT xenografts (Figure 1E and Supplemental Figure 1, H and I).

GSK-3i induces cell death in splicing factor mutant cells from patients with hematologic malignancies. Based on the promising results with leukemic cell lines (Figure 1), we evaluated the preferential cytotoxicity of GSK-3i in primary cells from patients with AML or the MDS/MPN overlap disorder CMML. GSK-3i induced apoptosis in primary cells from patients with CMML or AML with SRSF2, SF3BI, or U2AF1 mutations, but not in leukemic cells with WT splicing factors or in CD34+ cells from healthy subjects (Figure 2A and Supplemental Table 1).

Given the heterogeneity of primary human AML cells, we also tested the preferential cytotoxicity of GSK-3i in an isogenic context by expressing SRSF2 WT or SRSF2 P95H with mCherry in primary blasts from AML patients with WT splicing factors and then assessing the loss of mCherry-positive cells over time with or without CHIR treatment (Figure 2B and Supplemental Table 1). Primary AML cells expressing SRSF2 P95H were preferentially depleted upon CHIR exposure (Figure 2C). In contrast, GSK-3i had no substantial effect on primary AML cells expressing WT SRSF2 (Figure 2C). Cells expressing mutant SRSF2 also displayed a higher level of apoptosis in response to CHIR treatment relative to controls transduced with WT SRSF2 (Figure 2D and Supplemental Figure 2). These data confirm that SRSF2 P95H confers sensitivity to GSK-3i in primary AML cells.

GSK-3i alters global gene expression and splicing in human leukemic cells. To understand the functional consequences of GSK-3i for gene expression and splicing, we performed deep RNA sequencing (RNA-Seq; 106 reads per replicate sample) of parental K562 and isogenic SRSF2 P95H/+ and SF3BI K700E/+ lines treated with or without CHIR (Figure 3A and Supplemental Figure 3). CHIR treatment had distinct effects on the transcriptomes of WT, SRSF2 P95H/+, and SF3BI K700E/+ mutant cells (Supplemental Figure 3, B and C, and Supplemental Table 2). Additionally, GSK-3i altered the expression of multiple BCL2 family genes in a manner that may contribute to cell death, including decreased expression of the antiapoptotic
BCL-xL (encoded by BCL2L1) and increased expression of the pro-apoptotic genes BAK1, BCL2L11, and BIK regardless of splicing factor mutation status (Supplemental Figure 3D).

We then performed genome-wide analysis of splicing using the rMATS pipeline (28). We measured the change in percentage spliced in (APS1 or dPS1) values across 5 main types of alternative splicing events (skipped exon, alternative 5′ splice site exon, alternative 3′ splice site exon, mutually exclusive exons, and retained introns) in CHIR-treated versus control cells (Supplemental Table 3). Using a false discovery rate (FDR) less than 0.05 and dPSI greater than 10% (Figure 3B), we identified 694 alternative splicing events in CHIR-treated versus control cells, 705 in SRSF2 P95H/+ cells, 710 in SF3B1 K700E/+ cells, and 872 in SF3B1 K700E/+ cells. However, only 32 differentially spliced mRNAs were found in all 3 genotypes over GSK-3i, suggesting that GSK-3i has distinct and independent consequences for splicing in WT and splicing factor mutant cells (Figure 3C and D). The predominant change in mRNA splicing upon CHIR treatment in SRSF2 P95H/+, and SF3B1 K700E/+ cells was increased cassette exon skipping relative to DMSO-treated controls, while approximately equal proportions of exon inclusion and exclusion with CHIR exposure were observed in parental cells (Figure 3E). SF3B1 normally facilitates 3′ splice site recognition and is involved in the splicing of most introns by binding to the branchpoint (29). GSK-3i in SF3B1 K700E/+ cells resulted in a higher proportion of repressed alternative 3′ splice site (Figure 3F) and an increased number of retained intron events (Figure 3B). Consistent with the distinct effects of GSK-3i on splicing in cells with WT versus mutant splicing factors, multiple regulators of hematopoietic cell survival and proliferation were alternatively spliced only in splicing factor mutant K562 cells (Figure 3G and Supplemental Figure 3E). Among them, GSK-3i enhanced exon skipping in DEPDC1 pre-mRNA in SRSF2 P95H/+, and SF3B1 K700E/+ cells compared with WT counterparts, an event that was further validated by transcript-specific quantitative reverse transcriptase PCR (RT-qPCR) (Figure 3G). Taken together, these data show that inhibition of GSK-3 further reduces splicing fidelity in splicing factor mutant hematopoietic cells, likely explaining the selective toxicity of GSK-3i to splicing factor mutant leukemias over WT counterparts.

GSK-3 regulates PINK1 splicing. To test whether genes that are alternatively spliced following GSK-3i contribute to the preferential killing of spliceosome-mutant cells, we evaluated transcripts that exhibit concomitant dysregulation in gene expression and splicing with CHIR treatment (Figure 4A). We focused our attention initially on PINK1, because it is a serine/threonine kinase that serves as a critical sensor of mitochondrial damage and activates mitophagy to maintain mitochondrial homeostasis (30). GSK-3i reduces the abundance and alters the splicing of PINK1 mRNA in WT, SRSF2 P95H/+, and SF3B1 K700E/+ K562 cells. A sashimi plot shows that GSK-3i enhances the retention of intron 6, which includes a premature termination codon (PTC) that is predicted to promote mRNA degradation through nonsense-mediated decay (NMD) (Figure 4B). RT-qPCR confirms that GSK-3i reduces the abundance of PINK1 in WT, SRSF2 P95H/+, and SF3B1 K700E/+
cells (Figure 4C). This change in PINK1 abundance was not due to a change in transcription, as detection of primary transcripts with PCR primers that detect intron 1 or intron 5 showed no significant difference in nascent transcripts with GSK-3i in WT or splicing factor mutant cells (Figure 4D). Qualitative RT-PCR with primers that span exons 6 and 7 confirmed that GSK-3i with CHIR (Figure 4E) or AR-A014418 (Supplemental Figure 4) impaired excision of intron 6, increasing intron 6 retention (568 bp), in SRSF2+/−, SRSF2+/−/−, and SF3B1+/−/− K562 cells. CHIR also promoted retention of intron 6 in primary cells from patients with AML or CMML and CD34+ cells from healthy donors (Figure 4F). To confirm that CHIR impairs PINK1 splicing through inhibition of GSK-3, we...
Figure 3. GSK-3i is associated with global alterations in gene expression and splicing in human leukemic cells. (A) Schematic of deep RNA-Seq in WT, SRSF2<sup>P95H</sup>/+, and SF3B1<sup>K700E</sup>/+ K562 cells treated with DMSO or 3 μM CHIR for 24 hours. (B) Bar graphs show numbers of differential splicing events (FDR < 0.05, dPSI > 10%) in WT, SRSF2<sup>P95H</sup>/+, and SF3B1<sup>K700E</sup>/+ K562 cells treated with CHIR versus DMSO controls. A3SS, alternative 3′ splice site; ASSS, alternative 5′ splice site; MXE, mutually exclusive exon; SE, skipped exon. (C) Venn diagram showing the number of overlapping alternatively spliced genes between CHIR and DMSO in WT, SRSF2<sup>P95H</sup>/+, and SF3B1<sup>K700E</sup>/+ K562 cells. (D) Heatmap of PSI values for overlapping alternatively spliced genes comparing CHIR and DMSO in WT, SRSF2<sup>P95H</sup>/+, and SF3B1<sup>K700E</sup>/+ K562 cells. (E) Scatterplots of cassette exon inclusion in WT, SRSF2<sup>P95H</sup>/+, and SF3B1<sup>K700E</sup>/+ K562 cells treated with 3 μM CHIR relative to DMSO-treated controls. Numbers in brown (left) and purple (right) indicate number of cassette exons whose inclusion is repressed or promoted, respectively, in CHIR- relative to DMSO-treated cells. P values were determined by 1-way ANOVA with Šidák’s multiple-comparison test. (F) Bar graphs show numbers of alternative 3′ splice site events upon CHIR treatment compared with DMSO in WT, SRSF2<sup>P95H</sup>/+, and SF3B1<sup>K700E</sup>/+ K562 cells. Purple and orange indicate intron-proximal 3′ splice sites whose usage is repressed or enhanced, respectively, in CHIR- relative to DMSO-treated cells. (G) Left: Sashimi plots of DEPDC1 in WT and SRSF2<sup>P95H</sup>/+ cells treated with DMSO or CHIR. Right: Bar plots show quantification of percentage of exon inclusion based on rMATS analysis (top) and on isoform-specific qPCR validation (bottom). Percentage of exon inclusion was quantified by RT-qPCR analysis of mRNA levels containing the cassette exons normalized to total mRNA levels. Data are presented as the mean ± SD. P values were determined by 2-way ANOVA with Šidák’s multiple-comparison test.
tested PINK1 splicing in WT and GSK3A/B-DKO cells. Similarly to CHIR and AR-A014418, GSK3A/B DKO impaired intron 6 excision. In contrast, overexpression of GSK3B in DKO cells restored excision of intron 6 (Figure 4G). Blocking NMD by knockdown of UPF1, a core NMD factor, increased the mRNA stability and expression of the intron 6-retained form of PINK1 (Figure 4H). Taken together, these data show that GSK-3 regulates PINK1 splicing and that CHIR reduces PINK1 mRNA abundance specifically by inhibiting GSK-3 and promoting retention of an intron containing an in-frame PTC.

SRSF2P95H increases PINK1 expression and mitophagy. In contrast to GSK-3i, the SRSF2P95H mutation markedly increased PINK1 mRNA abundance in comparison with parental and SF3B1K700E/− cells (Figure 4C). Nascent PINK1 transcripts were not increased in SRSF2P95H/− cells (Figure 4D), indicating that the increase in mRNA abundance was due to stabilization of the PINK1 transcript rather than increased transcription. In support of this, excision of intron 6 was increased in SRSF2P95H/− cells compared with parental cells or SF3B1K700E/− cells (Figure 4E). Increased excision of intron 6 was further validated in TF-1 cells overexpressing SRSF2P95H compared with cells expressing SRSF2WT (Supplemental Figure 5A). PINK1 activates mitophagy, and in parallel with the increase in PINK1 levels, expression of other mitophagy-related genes, including OPTN, ULK1, TOMM7, CALCOCO2, NBR1, and TAXIBP1, was significantly increased in SRSF2P95H/− cells (Figure 5A). The increase in PINK1 and other mitophagy markers was associated with a substantial increase in mitophagy specifically in SRSF2P95H/− cells, as determined by the colocalization of TOMM20 (mitochondrial marker) and LAMP1 (lysosomal marker) (Figure 5B). Immunofluorescent staining demonstrated that PINK1 colocalizes with TOMM20 (Supplemental Figure 5B) and PARKIN (Supplemental Figure 5C), suggesting that PINK1 was stabilized on impaired mitochondria in SRSF2P95H/− cells. Transmission electron microscopy (TEM) showed accumulation of autophagic vacuoles with defective mitochondria with swollen matrix and collapsed cristae enclosed by a double membrane in SRSF2P95H/− cells (Figure 5C). As SRSF2P95H and GSK-3i appear to have opposing effects on PINK1 splicing and abundance, we tested the effect of CHIR on mitophagy in SRSF2P95H/− cells and found that GSK-3i impaired mitophagy in SRSF2P95H/− cells (Figure 5, B and C), in parallel with the reduction in PINK1 mRNA. We then assessed mitophagic flux by evaluating the accumulation of mitochondria with Mitotracker Green (MTG) staining in the presence of chloroquine (CQ), which blocks the final step of autophagy/mitophagy, fusion with the lysosome, by inhibiting lysosomal acidification. CQ increased mitochondrial mass, as expected (Supplemental Figure 5D). The increase was significantly higher in SRSF2P95H/− cells than in SRSF2−/− cells. Mitophagic flux was then determined by subtraction of the MTG value for untreated cells from the value for cells treated with CQ (31). The SRSF2P95H/− mutation significantly enhanced mitophagic flux (Figure 5D). A similar increase in mitophagic flux was observed with Lys05, an alternative inhibitor of lysosomal acidification (Figure 5D). GSK-3i also increased mitochondrial mass in both SRSF2−/− and SRSF2P95H/− cells, with or without CQ or Lys05 (Supplemental Figure 5, D and E), consistent with reduced PINK1 levels and impaired mitophagy. Western blot analysis confirmed that PINK1 protein was increased in SRSF2P95H/− cells and decreased upon CHIR treatment (Figure 5E).

To address whether an increase in mitophagy is a feature of primary cells from patients with hematologic malignancies, we queried The Cancer Genome Atlas (TCGA) database for expression of markers of mitophagy in hematologic malignancies with or without the SRSF2 mutation. Expression of the canonical mitophagy markers OPTN (P = 0.0015) and TOMM7 (P = 0.0004) was significantly increased in patients with SRSF2P95H/− (n = 43) compared with patients with WT SRSF2 (n = 538) (Figure 5F). No significant difference was observed between patients with WT and mutant SF3B1 (Supplemental Figure 5F). These data show elevated mitophagy marker expression specifically in SRSF2-mutant MDS and AML, and we therefore focused on the impact of SRSF2P95H on mitochondrial function and the role of GSK-3-dependent PINK1 splicing in the regulation of mitophagy in SRSF2P95H/− cells.

The SRSF2 mutation is associated with accumulation of defective mitochondria. This increase in mitophagy indicates that the SRSF2P95H mutation causes mitochondrial dysfunction, which could arise through mis-splicing of nuclear mRNAs encoding mitochondrial proteins. We analyzed splicing variations in SRSF2P95H/− cells compared with SRSF2−/− cells (Supplemental Figure 6A). Gene Ontology (GO) analysis of differentially spliced genes in SRSF2P95H−/− cells revealed enrichment of processes related to regulation of protein targeting to mitochondrion organization, mitochondrial genome maintenance, mitochondrial respiratory chain complex I assembly, and oxidative phosphorylation (Figure 6A and Supplemental Table 4). We identified 12 alternatively spliced mRNAs that are associated with mitochondrial respiratory chain complex I (Figure 6A and Supplemental Figure 6B). These findings are consistent with GO analyses in SRSF2-mutant cells from primary MDS (9), CMML (Figure 6B) (7), and AML patients (Figure 6B) (7, 32), which similarly showed enrichment of mitochondrial genes in the set of alternatively spliced mRNAs.

To further assess the consequences of SRSF2 mutation for the cellular proteome in myeloid neoplasms, we performed quantitative mass spectrometry (qMS) with a focus on the mitochondrial proteome by using human proteome (UniProtKB) and mitochondrial (MitoCarta3.0) databases for protein identification (Figure 6C). We identified 163 mitochondrial proteins whose levels are affected by SRSF2 mutation (Supplemental Table 5). The 84 proteins that showed significantly reduced levels in SRSF2P95H/− cells included multiple mitochondrial ribosomal proteins (Figure 6D). Of the 6 respiratory mitochondrial complex I proteins detected by qMS, 5 were significantly downregulated by SRSF2 mutation (Figure 6D).

Evaluation of the mitochondrial properties of SRSF2P95H/− cells revealed elevated mitochondrial mass in comparison with SRSF2−/− cells based on MTG staining (Figure 6E) and immunostaining of the outer membrane protein TOMM20 (Supplemental Figure 6C). In contrast, mitochondrial mass was not increased in SF3B1K700E/− cells (Supplemental Figure 6D). Overexpression of SRSF2P95H also increased mitochondrial content in K562, TF-1, and primary AML cells when compared with overexpression of WT SRSF2 (Supplemental Figure 6, E–G). Mitochondrial DNA (mtDNA) copy number, which correlates with mitochondrial biogenesis, significantly increased (Figure 6F), suggesting that elevated mitophagy in SRSF2P95H cells is balanced by increased mitochondrial biogenesis. This dynamic ultimately leads to increased mitochondrial content.
Mitochondrial membrane potential (MMP) per unit mitochondrial mass as assessed by tetramethylrhodamine ethyl ester (TMRE)/MTG ratio and mitochondrial reactive oxygen species (mtROS) levels were significantly higher in SRSF2P95H+/+ cells (Figure 6, G and H). This chronic oxidative stress may damage the mitochondria and mtDNA. To test mitochondrial function, we subjected parental and SRSF2P95H+/+ cells to high-resolution respirometry. Surprisingly, both the basal and the maximal respiratory capacity were significantly lower in SRSF2P95H+/+ cells (Figure 6I). As SRSF2 mutation dramatically altered the splicing of complex I–related genes and reduced complex I protein levels, we further tested complex I respiratory capacity. Complex I–linked respiration, which was measured by sequential addition of the complex I–linked substrates pyruvate, malate, and glutamate (P/M/G) and ADP, was also significantly lower in SRSF2P95H+/+ cells relative to SRSF2+/+ cells (Figure 6I). The elevated mitochondrial mass and MMP in SRSF2P95H+/+ cells therefore could not maintain high respiratory capacity. These functional data together with the increase in mitophagy demonstrate a marked defect in mitochondrial function associated with the leukemogenic SRSF2P95H mutation.

We next treated WT and SRSF2P95H+/+ K562 cells with a mitochondrial uncoupler, carbonyl cyanide m-chlorophenylhydrazone (CCCP), and then measured mitochondrial depolarization occurring during apoptosis using JC-1 staining (33). Although WT cells responded to CCCP treatment with reduced MMP, the SRSF2 mutation further decreased MMP, suggesting a higher demand for efficient mitophagy in response to mitochondrial stress in SRSF2P95H+/+ cells (Figure 6J). Overall, these data support a model in which splicing defects associated with the SRSF2 mutation disrupt mitochondrial function and lead to compensatory increased mitochondrial content and turnover.
sensing mitochondrial stress through modulation of $PINK1$ splicing, leading to increased expression of $PINK1$ mRNA and protein. Increased mitophagy is a therapeutic vulnerability in SRSF2-mutant hematologic malignancies. GSK-3i reduces $PINK1$ expression, impairs mitophagy, and is selectively lethal to $SRSF2^{P95H/+}$ cells. Given that $PINK1$-mediated mitophagy is required for the survival of normal hematopoietic stem cells (HSCs) (34) and acute myeloid leukemia stem cells (35, 36), we hypothesized that a greater dependency on $PINK1$-mediated mitophagy upon stress may be the basis for the selective toxicity of GSK-3i to $SRSF2^{P95H/+}$ cells. To explore this hypothesis, we evaluated the effects of GSK-3i on
mitochondria. GSK-3i increased MMP (Figure 7A) and promoted accumulation of mitochondrial mass in K562 cells with WT SRSF2 (Figure 7B and Supplemental Figure 7A), consistent with previous reports in mouse cardiomyocytes and HSCs (37, 38). GSK-3i in SRSF2P95+/- cells impaired mitophagy (Figure 5, B and C) and further increased MMP (Figure 7A) and mitochondrial content (Figure 7B and Supplemental Figure 7, A-D) in comparison with WT counterparts. Gene set enrichment analysis (GSEA) revealed that GSK-3i with CHIR strongly enriched for genes representing enhanced mitochondrial biogenesis and oxidative phosphorylation, a mitochondrial stress that may increase the requirement for enhanced mitochondrial biogenesis and oxidative phosphorylation, which can increase the requirement for mitophagy, and selectively kills cells with the SRSF2P95+/- mutation. Inhibition of mitophagy by targeting of lysosomal function is also lethal to SRSF2P95+/- cells. These data therefore reveal a dependency of splicing factor mutant MDS and AML and identify potential therapeutic targets for these hematologic malignancies.

The altered splicing of nuclear-encoded mitochondrial mRNAs observed here fits well with previous work identifying altered splicing of mRNAs involved in mitochondrial function in primary cells from MDS and AML patients with the SRSF2P95 mutation (7, 9, 32). We also find that markers of mitophagy are robustly increased in AML and MDS patients with the SRSF2P95+/- mutation in the TCGA data set. Mitochondrial turnover is required for self-renewal of normal HSCs (34, 43, 44) and leukemic stem cells (36, 44, 45), and was recently shown to mediate resistance to BH3 mimetics in AML cells (46–49). Thus, while inhibition of mitophagy or autophagy has been shown to be toxic to AML cells (36, 40, 45, 46), a mechanism for increased mitophagy in AML has not previously been defined. Our data demonstrate significantly greater sensitivity to mitophagy/autophagy inhibitors in SRSF2P95+/- cells, reveal a mechanism connecting this leukemogenic splicing factor mutation to mitochondrial dysfunction and increased mitophagy, and identify PINK1 splicing as a targetable vulnerability in SRSF2-mutant AML and MDS.

The data presented here reveal a homeostatic mechanism for the regulation of mitochondrial clearance. Prior work has established that, under basal conditions, PINK1 protein is degraded within mitochondria with a high MMP, whereas low MMP associated with mitochondrial dysfunction allows PINK1 protein stabilization and initiation of mitophagy (50). The homeostatic mechanism described here functions by altering PINK1 expression of GSK-3i in SRSF2P95+/- cells impaired mitophagy (Figure 5, B and C) and further increased MMP (Figure 7A) and mitochondrial content (Figure 7B and Supplemental Figure 7, A-D) in comparison with WT counterparts. Gene set enrichment analysis (GSEA) revealed that GSK-3i with CHIR strongly enriched for genes representing enhanced mitochondrial biogenesis and oxidative phosphorylation, a mitochondrial stress that may increase the requirement for mitophagy, and selectively kills cells with the SRSF2P95+/- mutation. Inhibition of mitophagy by targeting of lysosomal function is also lethal to SRSF2P95+/- cells. These data therefore reveal a dependency of splicing factor mutant MDS and AML and identify potential therapeutic targets for these hematologic malignancies.

The altered splicing of nuclear-encoded mitochondrial mRNAs observed here fits well with previous work identifying altered splicing of mRNAs involved in mitochondrial function in primary cells from MDS and AML patients with the SRSF2P95 mutation (7, 9, 32). We also find that markers of mitophagy are robustly increased in AML and MDS patients with the SRSF2P95+/- mutation in the TCGA data set. Mitochondrial turnover is required for self-renewal of normal HSCs (34, 43, 44) and leukemic stem cells (36, 44, 45), and was recently shown to mediate resistance to BH3 mimetics in AML cells (46–49). Thus, while inhibition of mitophagy or autophagy has been shown to be toxic to AML cells (36, 40, 45, 46), a mechanism for increased mitophagy in AML has not previously been defined. Our data demonstrate significantly greater sensitivity to mitophagy/autophagy inhibitors in SRSF2P95+/- cells, reveal a mechanism connecting this leukemogenic splicing factor mutation to mitochondrial dysfunction and increased mitophagy, and identify PINK1 splicing as a targetable vulnerability in SRSF2-mutant AML and MDS.

The data presented here reveal a homeostatic mechanism for the regulation of mitochondrial clearance. Prior work has established that, under basal conditions, PINK1 protein is degraded within mitochondria with a high MMP, whereas low MMP associated with mitochondrial dysfunction allows PINK1 protein stabilization and initiation of mitophagy (50). The homeostatic mechanism described here functions by altering PINK1 expression of GSK-3i in SRSF2P95+/- cells impaired mitophagy (Figure 5, B and C) and further increased MMP (Figure 7A) and mitochondrial content (Figure 7B and Supplemental Figure 7, A-D) in comparison with WT counterparts. Gene set enrichment analysis (GSEA) revealed that GSK-3i with CHIR strongly enriched for genes representing enhanced mitochondrial biogenesis and oxidative phosphorylation, a mitochondrial stress that may increase the requirement for mitophagy, and selectively kills cells with the SRSF2P95+/- mutation. Inhibition of mitophagy by targeting of lysosomal function is also lethal to SRSF2P95+/- cells. These data therefore reveal a dependency of splicing factor mutant MDS and AML and identify potential therapeutic targets for these hematologic malignancies.
splicing: mitochondrial dysfunction, whether caused by direct pharmacologic disruption of the MMP or indirectly by the SRSF2 mutation, promotes the excision of a poison intron to yield a more stable PINK1 mRNA. Although the SRSF2*Wt mutation could directly alter the splicing of PINK1 mRNA, we propose that the mechanism is indirect. SRSF2 typically regulates cassette exon selection, whereas we observed enhanced intron excision, and, importantly, CCCP alters PINK1 splicing in cells with WT SRSF2, supporting a homeostatic mechanism that is sensitive to but independent of the SRSF2 mutation. Although the altered splicing of PINK1 mRNA described here is distinct from the well-established regulation of PINK1 protein stability by MMP, both mechanisms result in increased PINK1 protein abundance under conditions of mitochondrial stress. Our data thus support a mechanism for sensing mitochondrial stress through modulation of PINK1 splicing, increasing PINK1 expression to support an increased demand for mitophagy in the setting of mitochondrial dysfunction.

Splicing out of the poison intron requires GSK-3, which phosphorylates multiple core splicing factors, including SRSF2, and regulates splicing at a transcriptome-wide level in diverse cell types (22–26). Thus, pharmacologic inhibition or GSK3 knock-out impairs splicing of PINK1, leading to retention of the poison and reduction in overall levels of PINK1 mRNA and protein. Altered splicing of PINK1 explains the lethality of GSK-3 inhibitors in SRSF2*Wt cells, as survival in the presence of a GSK-3 inhibitor is rescued by expression of PINK1 cDNA. Furthermore, chloroquine and Lys05, which target autophagy downstream of PINK1, also preferentially kill SRSF2*Wt cells, supporting the hypothesis that these splicing factor mutant cells are dependent on mitophagy for survival and suggesting an actionable therapeutic target in splicing factor mutant myeloid neoplasms.

Currently there are no FDA-approved drugs targeting splicing factor mutant malignancies. Here we identify mitophagy as a therapeutic vulnerability specifically in AML and MDS driven by hotspot mutations in the splicing factor SRSF2. We have also uncovered a mechanism for mitochondrial surveillance that is mediated through GSK-3-dependent alternative splicing of PINK1 and show significantly increased sensitivity to GSK-3 or autophagy inhibition in splicing factor mutant cells. Several GSK-3 inhibitors are either in wide clinical use or have been shown to be safe in phase I–III clinical trials (51–54) and could be repurposed to treat MDS. Hence targeting mitophagy with GSK-3 inhibitors or general autophagy inhibitors may provide a new opportunity to treat SRSF2-mutant MDS and AML.

Methods

Sex as a biological variable. This study used deidentified primary patient cells from male and female patients. However, sex was not considered as a biological variable because the study was not powered to distinguish sex differences. Xenografts of human cells into murine hosts were performed using female mice only. Sex of the host was not considered as a biological variable in these xenograft experiments.

Cell lines, primary human samples, constructs, and nucleofection. Bone marrow– or peripheral blood–derived mononuclear cells (MNCs) from AML or CMML patients were obtained from the Stem Cell and Xenograft Core Facility at the University of Pennsylvania (RRID: SCR_010035). Detailed patient characteristics are listed in Supplemental Table 1. CD34+ cells were purified from CMML patients through immunomagnetic selection (Miltenyi Biotech) according to the manufacturer’s instructions. CD34+ cells from healthy donors were purchased from STEMCELL Technologies. Cryopreserved AML MNCs were resuspended in IMDM supplemented with 15% BIT (BSA, insulin, transferrin; STEMCELL Technologies), 100 ng/mL SCF, 50 ng/mL FLT3L, 20 ng/mL IL-3, and 20 ng/mL G-CSF. CD34+ cells enriched from CMML patients or healthy donors were cultured in StemSpan SFEM II medium (STEMCELL Technologies) supplemented with 10% FBS, 1% L-glutamine, 10 ng/mL IL-3, 10 ng/mL IL-6, and 25 ng/mL SCF. TF-1 cells were maintained in RPMI supplemented with 10% FBS and 2 ng/mL hGcSCF (PeproTech). K562 cells were cultured in IMDM supplemented with 10% FBS. Cells were maintained at 37°C and 5% CO₂. PINK1 (pLenti6-DEST PINK1-V5 WT, 13320), shUPEF1 (PLKO.1-UPE1-CDS, 136037), WT (pRRL_SRSF2WT_mCherry, 84020), and P95H SRSF2 (pRRL_SRSF2_P95H_mCherry, 84023) lentiviral overexpression constructs were purchased from Addgene. Lentiviruses were produced and used to transduce TF-1, K562, and primary AML cells, as described previously (55). Primary AML MNCs (2 × 10⁵ cells) were transduced with WT and P95H SRSF2 lentivirus by spin infection in growth medium containing 4% LentiBlast (Oz Biosciences). Transduction efficiency was 30–60%. HEK293T cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. GS3A/B-DKO HEK293T cells were generated by lentiviral delivery of Cas9 and guide RNA sequence targeting GS3-A (GCTGAGGTT-GGCTACGACTG) and GS3-B (AGATGAGGTCTATCTTAATC) followed by single clone selection as previously described (51). GS3A/B-DKO HEK293T cells were transfected using Lipofectamine 3000 (Invitrogen) according to the manufacturer’s instructions.

Flow cytometry, apoptosis assay, and cell cycle analysis. For flow cytometric apoptosis assay, K562, TF-1, primary AML blast, or CD34+ cells purified from CMML patients or healthy donors were suspended in Annexin V Binding buffer (BioLegend 422201) and incubated with anti-annexin V antibody (BioLegend 640920) and
Immunoblotting. Cells were lysed in RIPA buffer plus protease inhibitor cocktail (Sigma-Aldrich P8340) and phosphatase inhibitor cocktail #2 (Sigma-Aldrich P0044) used 1:100 each. Supernatants were collected after centrifugation at 20,800g for 15 minutes at 4°C, adjusted to 1× Laemmli sample buffer, and subjected to SDS-PAGE and then immunoblotted as described previously (51). Antibodies for biochemical studies included anti-GAPDH (Cell Signaling Technology [CST] 2118), anti–cleaved caspase-8 (CST 9496), anti–cleaved caspase-3 (CST 9661), anti-FLAG tag (CST 14793), anti-V5 tag (CST 13202), and anti–β-catenin (CST 9562). Other antibodies included antibodies against PINK1 (Invitrogen PA5-86941) and β-actin (Sigma-Aldrich A5441).

Real-time qPCR and RT-PCR. RNA was extracted from TF-1, K562, and primary cells using an RNeasy Kit (QIAGEN) according to the manufacturer’s instructions. cDNA was synthesized using SuperScript III (Life Technologies) according to the manufacturer’s instructions. For detection of PINK1 mature mRNA, the following primers were used: forward, 5′-GCCTCATCGAGGAAAAACAGG-3′; reverse, 5′-GTCTCGTGTC\_CAACGGGGTC-3′. For detection of PINK1 pre-mRNA, the following primers were used: intron 5 forward, 5′-CCTTTGCCTGGGGATTTTGC-3′; 7-amino-actinomycin D (7-AAD; BioLegend 420403) for 15 minutes at room temperature in the dark. Immunophenotypes of viable cells or cells in early apoptosis or late apoptosis were defined as annexin V7-AAD', annexin V7-AAD', or annexin V7-AAD', respectively. For cell cycle analysis, cells were fixed and permeabilized in 70% ethanol at -20°C for 1 hour, washed in cold FACS buffer, and then stained with anti-Ki67 (BD Biosciences 350506) for 30 minutes on ice. After staining, cells were washed twice in FACS buffer and resuspended in FACS buffer containing 10 μM DAPI (BioLegend 422801). Stained cells were then tested by flow cytometry. Differences in apoptosis between culture conditions were analyzed by 2-sided χ² test.

Cell growth assay. Triplicates of WT and SRSF2P95H/+ K562 cells were seeded at 10,000 cells per well in 96-well plates and treated with indicated concentrations of CHIR (Cayman 13122) or chloroquine (CQ; Sigma-Aldrich C6628). Four days after culture, MTS reagent (Abcam ab197010) was added to cell medium at a final concentration of 0.5 mg/mL for 3 hours at 37°C, and cell viability was measured per the manufacturer’s instructions. IC₅₀ was determined with GraphPad Prism 8 using baseline correction (by normalizing to vehicle control), the asymmetric (5-parameter) equation, and least-squares fit.
introns 5 reverse, 5′-GGGGCCTTAAGTGCTGTGT-3′; intron 1 forward, 5′-GGGCCAGGGGTCCTTTAAAGC-3′; intron 1 reverse, 5′-TCCGACAGGAGGCTTAAGCG-3′. For detection of the PINK1 aberrant junction, the following primers were used: forward, 5′-GGTATGCGAGATTTGTCGT-3′; reverse, 5′-GCCGGAGAGGTTGAGG-3′. The primers used for DEPDCl isomeric-specific qPCR analysis were: BCL-XL forward, 5′-CATCAATGGCAACCCATCTGG-3′; reverse, 5′-GACCTTCAACTCTGCGCT-3′; BCL-XS forward, 5′-GACCTTTGAACAGGATACTTCTTG-3′; reverse, 5′-TTCCGACTGAAAGTGG-3′. The primers used for DEPDCl isomeric-specific qPCR analysis were: forward, 5′-TGTGACGTAATGGTACAGG-3′; reverse, 5′-TTCCACGGAAGCCTCATCA-3′; DEPDCl long isoform forward, 5′-GAACTCGGAGAGCTGTCG-3′; reverse, 5′-CATCGATGCCAACCCCTCTCT-3′. The efficiency of UPF1 knockdown was measured at the mRNA level by RT-qPCR: forward, 5′-AATTGTTGAAGAGAATGCGG-3′; reverse, 5′-TCAAGGAGCCCTTGTGACG-3′.

Mitophagy measurement and fluorescence microscopy. WT and SRSF2P95H/WT K562 cells were treated with DMSO or 3 μM CHIR for 44 hours, then seeded on poly-d-lysine–coated slides for 4 hours at 37°C. After incubation, cells were fixed with 4% paraformaldehyde (pH 7.4) for 15 minutes, permeabilized with 0.1% Triton X-100 for 15 minutes, and blocked with 2% BSA in PBS for 1 hour at room temperature. Cells were immunostained with mouse anti-TOMM20 antibody (1:100 dilution; clone 4F3, Sigma-Aldrich, WH0009804M1) and rabbit anti-LAMP1 antibody (1:100 dilution; Abclonal A2582) overnight at 4°C in a humidified chamber. Cells were incubated with secondary anti–rabbit AF568 (Invitrogen, A11001) and anti–mouse AF488 (1:1,000 dilution; Invitrogen, WH0009804M1) antibodies at room temperature and rinsed in dH2O before en bloc staining with 2% uranyl acetate. After dehydration through a graded ethanol series, the tissue was infiltrated and embedded in EMBed-812 (Electron Microscopy Sciences). Thin sections were stained with uranyl acetate and lead citrate and examined with a JEOL 1010 electron microscope fitted with a Hamamatsu digital camera and AMT Advantage image capture software. Images of single cells were saved as separate image files, and an observer blind to the identity of each cell counted vesicles per cell.

Measurement of mitochondrial respiration. Oxygen consumption was measured using high-resolution respirometry Oxygraph-2k (Oroboros Instruments) with a polarographic oxygen electrode and two 2-mL chambers allowing for parallel measurements. Briefly, K562 WT and SRSF2P95H/WT cells in MiR05 buffer (0.5 mM EGTA, 3 mM MgCl2, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES, 110 mM d-sucrose, 1 g/L BSA) were added into the closed chamber through a small capillary tube. Oxygen concentration (μmol/L) and oxygen flux [μmol/L/s·106 cells] were simultaneously recorded in real time. During the assay, 5 μg/mL digitonin, 5 mM pyruvate, 2 mM malate, 10 mM glucose, 2.5 mM ADP, 5 μM oligomycin, 0.5 μM FCCP, 0.5 μM rotenone, and 2.5 μM antimycin A were added sequentially.

K562 xenograft model. K562 WT or SRSF2P95H/WT cells (5 × 106) were subcutaneously implanted into the flank of female NSG mice of 6–8 weeks of age. Mice were then treated with 30 mg/kg body weight CHIR or vehicle (10% DMSO, 45% PEG400, and 45% PBS, injected subcutaneously) daily for the duration of the implantation period. Tumor size was measured 3 times a week using a caliper. Tumor volume was calculated by the ellipsoid formula: (length × width2) /2.

RNA-Seq sample preparation. K562 isogenic lines were treated with 3 μM CHIR for 24 hours. After treatment, the cells were washed with cold PBS, and RNA was isolated using an RNeasy Kit (QIAGEN) according to the manufacturer’s instructions. cDNA library preparation, sequencing, and raw read filtering methods were described previously (22).

Quantification of RNA-Seq data. Raw sequence reads (106 reads per replicate sample) were aligned to the human reference sequence hg38 by STAR 2.4.2a using 2-pass alignment. Raw gene counts were compiled into total gene counts, then analyzed using edgeR 3.20.2 to assess the significance of changes between cohorts. Gene changes with an adjusted P value less than 0.05 were considered significant. Significant gene expression changes are provided.
in Supplemental Table 2. Heatmaps for BCL2 family gene expression and mitophagy-related gene expression were generated using the Morpheus tool (Broad Institute, https://software.broadinstitute.org/morpheus/). Briefly, normalized expression values were transformed to z-scored log, expression by subtraction of row mean and then division by row standard deviation. RNA-Seq data were deposited in GEO (GSE235600) and are accessible as described below.

Identification of differential splicing events. Alternative splicing analyses relied on RNA-Seq reads mapped to the reference human genome as described using rMATS v4.1.1 with the default parameters (28). Events were defined as significant if (a) the FDR-corrected P value was smaller than 0.05 and (b) the dPSI was larger than 10%. Examples of splicing and mis-splicing events were visualized with the Integrative Genomics Viewer (Broad Institute). The heatmap for alternative splicing events between WT and SRSF2P95H/+ cells was generated by Morpheus. Hierarchical clustering was performed using metric one minus Pearson’s correlation.

Gene Ontology analysis. Gene Ontology analysis was performed with Metascape (https://metascape.org) using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and GO-specific signatures according to the manual.

GSEA analysis. GSEA was performed using GSEA version 4.3 (Broad Institute). Normalized read values produced from gene expression analysis were formatted into GCT files containing expression values for genes in different biological states. CLS files were manually built to label biological states involved in each study. The following parameters were used: number of permutations = 1,000, permutation type = gene set, ChiP platform = human Ensembl gene ID MSigDB. Other parameters were used at default values.

Mass spectrometry. SRSF2+/− (WT) and SRSF2P95H/+ KS62 cell pellets were resuspended in a solution of 100 mM ammonium bicarbonate and 8 M urea. Samples were sonicated for 15 seconds, then placed on ice for 15 seconds 10 times, and then centrifuged at 20,000g for 5 minutes at 4°C. Protein concentration in supernatants was measured by bicinchoninic acid assay. For each sample, 100 μg of protein was diluted to 50 μL at a final concentration of 10 mM DTT and incubated at 56°C for 30 minutes to reduce cysteines to cysteines. Alkylation was performed by addition of 5.5 μL of 0.5 M iodoacetamide and incubation at room temperature in the dark for 40 minutes. Samples were diluted to 250 μL with 50 mM Tris-HCl (pH 8.3), 2 μg of sequencing-grade modified trypsin (Promega) was added, and samples were incubated at 37°C overnight. Samples were acidified to 0.1% trifluoroacetic acid and immediately desalted using C18 StageTips (Thermo Fisher Scientific, 13-110-018), washed with 0.1% formic acid, and eluted with 0.1% formic acid in 60% acetonitrile. Peptides were dried in a Savant SpeedVac and then resuspended in 20 μL of 0.1% trifluoroacetic acid. UV absorption at 280 nm was recorded in 20 μL of 0.1% trifluoroacetic acid. UV absorption at 280 nm was measured to normalize injection volumes, and samples were run on a Dionex UltiMate 3000 nanoLC and Q Exactive HF (Thermo Fisher Scientific). Peptides were loaded on a C18 trap column (Thermo Fisher Scientific), washed with buffer A (0.1% formic acid), and then separated using an analytical column (75 μm x 15 cm) packed in-house with C18 resin (Dr. Maisch GmbH), using an analytical gradient of 5% buffer B (0.1% formic acid in 80% acetonitrile) to 25% over 90 minutes, and then 25% to 45% over 30 minutes. Mass spectrometric detection was performed using data-independent acquisition with 24 m/z windows. Data were searched using DIA-NN (https://github.com/vdemichev/DiaNN) with a UniProt FASTA digest library-free search and without heuristic protein inference. The mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium via the PRIDE (56) partner repository with the data set identifier PXD043213.

AML TCGA analysis. Gene expression analysis of OPTN, ULK1, and TOMM7 in AML patients from the TCGA project (57) was retrieved from cBioPortal for Cancer Genomics (https://www.cbioportal.org) (58), and visualized using Prism (GraphPad). GEPIA (Gene Expression Profiling Interactive Analysis; http://geopia.cancer-pku.cn) was used for overall survival analysis based on high and low expression of OPTN in publicly available TCGA data sets.

Statistics. Statistical analysis was performed using Prism version 8 software. Statistical differences between 2 groups were determined by 2-tailed Student’s t test. To assess the statistical significance of differences between more than 2 treatments, 1-way or 2-way ANOVA followed by Sidák’s multiple-comparison test was used. P less than 0.05 was considered significant.

Study approval. All mouse studies were carried out through the Stem Cell and Xenograft Core under a protocol approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Data availability. RNA-Seq data were deposited in the NCBI’s Gene Expression Omnibus (GEO) database (59) and are accessible through GEO Series accession number GSE235600 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE235600). The mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium via the PRIDE (56) partner repository with the data set identifier PXD043213. Supporting data values for all figures are provided in the Supporting Data Values file.

Author contributions. XL and PSK conceptualized the study. XL, MQV, NS, MPC, RM, and KJ established methodology. PSK, OAW, KWL, and DCW supervised the study and acquired funding. XL, SAD, RFS, RM, KJ, MQV, OP, AAM, and CL performed investigation. PSK, RFS, OAW, NS, MPC, JH, KWL, and DCW provided resources. XL, PSK, RFS, RM, KJ, and NS wrote and/or edited the manuscript. XL, OP, and AAM performed visualization. PSK, OAW, KWL, DCW, MPC, and JH supervised the study. PSK and KWL acquired funding.

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Address correspondence to: Peter S. Klein, 9-103 Smilow, Perelman School of Medicine at the University of Pennsylvania, 3400 Civic Center Boulevard, Philadelphia, Pennsylvania 19104, USA. Phone: 215.898.2179; Email: pklein@pennmedicine.upenn.edu.
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