

AWARD NUMBER: W81XWH-16-1-0059

TITLE: Therapeutic targeting of spliceosomal-mutant acquired bone marrow failure disorders

PRINCIPAL INVESTIGATOR: Omar Abdel-Wahab MD

CONTRACTING ORGANIZATION: Sloan Kettering Institute for Cancer Research
New York, NY 10065-6007

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14. ABSTRACT Genes encoding core components of the RNA splicing machinery are the most common mutational targets in acquired bone marrow failure (BMF) due to myelodysplastic syndromes (MDS). The goals of this proposal were to identify (1) the subset of biologically and therapeutically relevant targets that link spliceosomal mutations to MDS and (2) therapeutic strategies that interfere with the altered function of mutant spliceosomal proteins. Since award of this grant we have found that different spliceosomal mutations, despite imparting distinct effects on splicing and gene expression, are negatively selected for when co-expressed in the same cell or in a homozygous state. At the same time, aberrant splicing of distinct targets/events by mutant SF3B1 and SRSF2 results in hyperactivated NF-κB signaling, thereby identifying convergent biological consequences of splicing factor mutations and the basis for their mutual exclusivity and heterozygous nature. In addition, we have completed both a negative selection shRNA screen and a genome-wide CRISPR dropout screen to identify genes selectively required in spliceosomal mutant cells. This effort has also highlighted a requirement for innate immune signaling in SF3B1-mutant MDS and has implicated a few specific proteins as potential novel therapeutic targets for spliceosomal mutant MDS.					
15. SUBJECT TERMS MDS, SF3B1, Splicing, SRSF2, U2AF1, ZRSR2.					
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1. INTRODUCTION:

Genes encoding core components of the RNA splicing machinery are the most common mutational targets in acquired bone marrow failure (BMF) due to myelodysplastic syndromes (MDS). MDS associated “spliceosomal mutations” most frequently affect *SF3B1*, *SRSF2*, and *U2AF1*. Mutations in each of these genes conspicuously occur as heterozygous point mutations at highly restricted residues and are mutually exclusive, suggesting that they are oncogenic gain-of-function alterations. While these data suggest that each RNA splicing factor mutation may share downstream targets, currently few mis-spliced genes have been conclusively identified as downstream targets of any mutant splicing protein, and no shared targets downstream of all three spliceosomal gene mutations have been identified. Thus, the goals of this proposal were to identify (1) the subset of biologically and therapeutically relevant targets that link spliceosomal mutations to MDS and (2) therapeutic strategies that interfere with the altered function of mutant spliceosomal proteins.

2. KEYWORDS:

MDS, SF3B1, Splicing, SRSF2, U2AF1, ZRSR2

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

The major goals of this project were as follows:

Specific Aim 1. Identify unifying molecular abnormalities across spliceosomal mutations using combined genomic and proteomic approaches.

Specific Aim 2. Identify additional genes that are required for the survival of cells carrying different spliceosomal mutations with synthetic lethality screens.

Specific Aim 3. Identify novel therapeutic strategies specifically targeting mutant spliceosomal protein function.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Aim 1 accomplishments: We identified that different spliceosomal mutations, despite imparting distinct effects on splicing and gene expression, are negatively selected for when co-expressed in the same cell or in a homozygous state. At the same time, aberrant splicing of distinct targets/events by mutant SF3B1 and SRSF2 results in hyperactivated NF- κ B signaling. Specifically, mutations in SF3B1 result in aberrant splicing and consequent downregulation of MAP3K7 while mutations in SRSF2 generate a stable truncated form of Caspase 8. Each of these alterations result in activation of NF κ -B signaling. These data identify convergent biological consequences of splicing factor mutations and the basis for their mutual exclusivity and heterozygous nature. Our shared manuscript on these data are in “Minor Revision” at *Cancer Cell* as follows (we described this manuscript in last year’s progress report but the review process has listed for ~12 months):

Stanley Chun-Wei Lee (co-first), Khrystyna Dilai (co-first), Eunhee Kim, Esther Obeng, Sydney X. Lu, Bo Liu, Daichi Inoue, Akihide Yoshimi, Michelle Ki, Xiao Jing Zhang, Min Kyung Kim, Young Rock Chung, Justin Taylor, Benjamin H. Durham, James Palacino, Michael Seiler, Silvia Buonamici, Peter G. Smith, Benjamin L. Ebert, **Robert K. Bradley** (co-corresponding), **Omar Abdel-Wahab** (co-corresponding). Synthetic lethal and convergent biological effects of cancer-associated spliceosomal gene mutations.

Aim 2 accomplishments: We previously identified that spliceosomal mutant cells are preferentially sensitive to genetic or pharmacologic perturbations to the splicing process. This work was published in the following paper which acknowledged this grant: Lee SC, Dvinge H, Kim E, Cho H, Micol JB, Chung YR, Durham BH, Yoshimi A, Kim YJ, Thomas M, Lobry C, Chen CW, Pastore A, Taylor J, Wang X, Krivtsov A, Armstrong SA, Palacino J, Buonamici S, Smith PG, Bradley RK, Abdel-Wahab O. Modulation of splicing catalysis for therapeutic targeting of leukemia with mutations in genes encoding spliceosomal proteins. *Nat Med.* 2016 Jun;22(6):672-8. doi: 10.1038/nm.4097. Epub 2016 May 2. PubMed PMID: 27135740; PubMed Central PMCID: PMC4899191.

Since publication of the above manuscript, we collaborated with H3B Biomedicine Inc. to evaluate a novel, orally bioavailable molecule which perturbs RNA splicing by binding to the SF3b complex and interfering with its interaction with RNA. This molecule is called H3B-8800. Across isogenic cell line models and patient-derived xenografts of patients with the MDS/myeloproliferative disorder known as CMML, we identified the H3B-8800 has preferentially toxic effects on spliceosomal mutant cells. In addition, we carefully evaluated the effects of this compound on splicing globally in spliceosomal mutant and wild-type cells. This work was published in the following publication which acknowledges this grant:

Seiler M, Yoshimi A, Darman R, Chan B, Keaney G, Thomas M, Agrawal AA, Caleb B, Csibi A, Sean E, Fekkes P, Karr C, Klimek V, Lai G, Lee L, Kumar P, Lee SC, Liu X, Mackenzie C, Meeske C, Mizui Y, Padron E, Park E, Pazolli E, Peng S, Prajapati S, Taylor J, Teng T, Wang J, Warmuth M, Yao H, Yu L, Zhu P, **Abdel-Wahab O**, Smith PG, Buonamici S. H3B-8800, an orally available small-molecule splicing modulator, induces lethality in spliceosome-mutant cancers. *Nat Med.* 2018 May;24(4):497-504. doi: 10.1038/nm.4493. Epub 2018 Feb 19. PubMed PMID: 29457796.

On the basis of the above work, H3B-8800 is now being tested in a multicenter phase I clinical trial for patients with MDS and related myeloid leukemias (AML, CMML). This is clinicaltrials.gov identifier NCT02841540.

Aim 3 accomplishments: We recently identified that a class of compounds known as “anticancer sulfonamides” which were recently demonstrated to kill cells by linking the ubiquitin ligase machinery in cells to degrade the splicing RBM39 has preferential effects on spliceosomal mutant cells. This led to a series of in vitro and in vivo experiments studying the biological and mechanistic effects of RBM39 loss in MDS and on splicing and gene expression. The data on these studies is currently being written in a manuscript to be submitted for publication.

Other Accomplishments: Omar Abdel-Wahab received the Seldon-Smith Award for Pioneering Research from the American Society of Clinical Investigation (ASCI) in 2017 and was elected as a member of ASCI in 2018.

What opportunities for training and professional development has the project provided?

Dr. Stanley Chun-Wei Lee (postdoctoral fellow in the Abdel-Wahab lab) received an NIH K99/R00 as well as a prestigious Scholar Award from the American Society of Hematology (ASH) on the basis of the work completed from this award.

Dr. Akihide Yoshimi (postdoctoral fellow in the Abdel-Wahab lab) received a prestigious Special Fellow Award from the Leukemia & Lymphoma Society on the basis of the work completed from this award.

Dr. Heidi Dvinge (former Bradley lab member) became appointed as an independent faculty member as an Assistant Professor in the Dept. of Biomolecular Chemistry at the University of Wisconsin, Madison.

Dr. Daichi Inoue and Stanley Chun Wei Lee (both Abdel-Wahab lab) presented their work at the 2017 FASEB Hematologic Malignancies Meeting.

Dr. Akihide Yoshimi presented his work at the 2017 ASH Annual Meeting.

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of

these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Our research funded by the U.S. Department of Defense Bone Marrow Failure Research Program (BMFRP) is highlighted on the Congressionally Directed Medical Research Programs (CDMRP) website.

What do you plan to do during the next reporting period to accomplish the goals?

If this is the final report, state “Nothing to Report.”

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Aim 2: We completed a genome-wide CRISPR dropout screen in isogenic myeloid cells with and without mutations in SF3B1 K700E and K666N. This identified a number of critical pathways required by SF3B1 mutant cells over wild-type include a preferential dependence on a variety of RNA processing steps as well as on protein translation. This finding then led us to identify that SF3B1 mutant cells may be preferentially sensitive to drugs which perturb translation such as rapamycin. We are now completing in vivo drug experiments with rapamycin and hope to conclude this work in a manuscript in the next 3 months.

Aim 3: As mentioned above, we are drafting a manuscript on the preferential effects of RBM39 degradation on spliceosomal mutant cells and the effects of RBM39 loss on splicing. This manuscript will be submitted this month for publication.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

One of the main findings from our work thus far is that cells which contains mutations in RNA splicing factors have enhanced sensitivity to drugs that further alter the RNA splicing process. This has led to a clinical trial of a novel RNA splicing inhibitor in patients with MDS and other forms of myeloid leukemia (clinicaltrials.gov identifier NCT02841540). This is a phase I clinical trial sponsored by the company H3 Biomedicine Inc. and is designed to test the safety of this approach and ability to block splicing in patients.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to report.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

None.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates

Significant changes in use or care of human subjects

None.

Significant changes in use or care of vertebrate animals.

None.

Significant changes in use of biohazards and/or select agents

None.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume; year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Stanley Chun-Wei Lee (co-first), Khrystyna Dilai (co-first), Eunhee Kim, Esther Obeng, Sydney X. Lu, Bo Liu, Daichi Inoue, Akihide Yoshimi, Michelle Ki, Xiao Jing Zhang, Min Kyung Kim, Young Rock Chung, Justin Taylor, Benjamin H. Durham, James Palacino, Michael Seiler, Silvia Buonamici, Peter G. Smith, Benjamin L. Ebert, Robert K. Bradley (co-corresponding), Omar Abdel-Wahab (cocorresponding).

Synthetic lethal and convergent biological effects of cancer-associated spliceosomal gene mutations. In Minor Revision at *Cancer Cell*.

Seiler M, Yoshimi A, Darman R, Chan B, Keaney G, Thomas M, Agrawal AA, Caleb B, Csibi A, Sean E, Fekkes P, Karr C, Klimek V, Lai G, Lee L, Kumar P, Lee SC, Liu X, Mackenzie C, Meeske C, Mizui Y, Padron E, Park E, Pazolli E, Peng S, Prajapati S, Taylor J, Teng T, Wang J, Warmuth M, Yao H, Yu L, Zhu P, **Abdel-Wahab O**, Smith PG, Buonamici S. H3B-8800, an orally available small-molecule splicing modulator, induces lethality in spliceosome-mutant cancers. *Nat Med*. 2018 May;24(4):497-504. doi: 10.1038/nm.4493. Epub 2018 Feb 19. PubMed PMID: 29457796.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

None.

Other publications, conference papers, and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Presentations by Dr. Omar Abdel-Wahab:

- 2017** New Horizons in Immune Dynamics & Translational Cancer Research, Weizmann Institute, Rehovot, Israel
- 2017** FASEB Hematologic Malignancies, Saxtons River, VT
- 2017** Dana Farber Cancer Institute, Bone Marrow Transplantation/Hematological Malignancies Grand Rounds, Boston, MA
- 2017** Edward P. Evans MDS Summit, Yale University, New Haven, CT
- 2017** Workshop on Myeloid Development, 2017 American Society of Hematology Annual Meeting, Atlanta, GA
- 2018** 12th International Workshop on molecular aspects of myeloid stem cell development and leukemia

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

None.

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

None.

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

None.

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *biospecimen collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

We have deposited our RNA-seq datasets at the Gene Expression Omnibus database under accession numbers GSE74064 and GSE97452.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Omar Abdel-Wahab
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	None
Nearest person month worked:	1
Contribution to Project:	Overseeing all experiments in conjunction with Dr. Bradley.
Funding Support:	US National Institutes of Health (NIH)-NHLBI grant R01 HL128239
Name:	Robert Bradley
Project Role:	co-PI
Researcher Identifier (e.g. ORCID ID):	None
Nearest person month worked:	1
Contribution to Project:	Overseeing all experiments in conjunction with Dr. Abdel-Wahab.
Funding Support:	US National Institutes of Health (NIH)-NHLBI grant R01 HL128239
Name:	Stanley Chun-Wei Lee
Project Role:	Postdoctoral fellow
Researcher Identifier (e.g. ORCID ID):	None
Nearest person month worked:	2
Contribution to Project:	Stanley has performed all in vivo experiments related to Specific Aim 1 and the shRNA screen in Aim 2.
Funding Support:	Leukemia and Lymphoma Society Special Fellow Award, NIH K99/R00, ASH Scholar
Name:	Bo Liu
Project Role:	Postdoctoral fellow
Researcher Identifier (e.g. ORCID ID):	None
Nearest person month worked:	2
Contribution to Project:	Bo generated the splicing reporters described in Aim 3.
Funding Support:	Josie Robertson Award (MSKCC)
Name:	Young Rock Chung
Project Role:	Sr. Research Assistant
Researcher Identifier (e.g. ORCID ID):	None
Nearest person month worked:	1
Contribution to Project:	Assisted postdoctoral fellows in the Abdel-Wahab lab with experiments.
Funding Support:	NIH (R01-CA201247), Pershing Square, W81XWH-16-1-0059
Name:	Khrystyna Dilai
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	None
Nearest person month worked:	3
Contribution to Project:	Khrystyna performed the informatics analyses to identify convergent effects of spliceosomal gene mutations in Aim 1.
Funding Support:	US National Institutes of Health (NIH)-NHLBI grant R01 HL128239

Name:	Akihide Yoshimi
Project Role:	Postdoctoral fellow
Researcher Identifier (e.g. ORCID ID):	None
Nearest person month worked:	2
Contribution to Project:	Dr. Yoshimi completed the experiments on H3B-8800.
Funding Support:	Leukemia and Lymphoma Society Special Fellow Award
Name:	Daichi Inoue
Project Role:	Postdoctoral fellow
Researcher Identifier (e.g. ORCID ID):	None
Nearest person month worked:	2
Contribution to Project:	Dr. Inoue is work on validating results from the CRISPR Screen in Aim 2 and the RBM39 work.
Funding Support:	Leukemia and Lymphoma Society Special Fellow Award

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

SEE APPENDIX FOR CHANGES IN ACTIVE SUPPORT

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name:

Location of Organization: (if foreign location list country)

Partner’s contribution to the project (identify one or more)

- Financial support;
- In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);
- Facilities (e.g., project staff use the partner’s facilities for project activities);

- Collaboration (e.g., partner's staff work with project staff on the project);
- Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and
- Other.

Organization Name: Fred Hutchinson Cancer Research Center

Location of Organization: (if foreign location list country): Seattle, WA

Partner's contribution to the project (identify one or more)

- Collaboration (e.g., partner's staff work with project staff on the project);

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

The appendix includes the following two publications (one published, the other submitted):

- Lee SC, Dvinge H, Kim E, Cho H, Micol JB, Chung YR, Durham BH, Yoshimi A, Kim YJ, Thomas M, Lobry C, Chen CW, Pastore A, Taylor J, Wang X, Krivtsov A, Armstrong SA, Palacino J, Buonamici S, Smith PG, Bradley RK, Abdel-Wahab O. Modulation of splicing catalysis for therapeutic targeting of leukemia with mutations in genes encoding spliceosomal proteins. *Nat Med.* 2016 Jun;22(6):672-8. doi: 10.1038/nm.4097. Epub 2016 May 2. PubMed PMID: 27135740; PubMed Central PMCID: PMC4899191.
- Stanley Chun-Wei Lee (co-first), Khristyna Dilai (co-first), Eunhee Kim, Esther Obeng, Sydney X. Lu, Bo Liu, Daichi Inoue, Akihito Yoshimi, Michelle Ki, Xiao Jing Zhang, Min Kyung Kim, Young Rock Chung, Justin Taylor, Benjamin H. Durham, James Palacino, Michael Seiler, Silvia Buonamici, Peter G. Smith, Benjamin L. Ebert, Robert K. Bradley (co-corresponding), Omar Abdel-Wahab (co-corresponding). Synthetic lethal and convergent biological effects of cancer-associated spliceosomal gene mutations. Under Review at *Cancer Cell*

PREVIOUS/CURRENT/PENDING SUPPORT

ABDEL-WAHAB, OMAR I.

PREVIOUS SUPPORT

1 R13 TR001695-01 (PI: Diamond) 8/18/16 - 8/17/17 0.24 calendar
NCATS

Fourth International Medical Symposium on Erdheim-Chester Disease

Goals: Because of the rarity of the Erdheim-Chester disease (ECD), clinical experience is limited worldwide and international collaboration has proven vital to advancement in research and clinical care for these patients. The proposed conference will be an international symposium to gather ECD specialists from the US and Europe to discuss clinical and research efforts, and specifically to derive a consensus approach to response criteria for implementation in ECD clinical trials.

Aims: Aim 1. To bring together clinicians and investigators from an international setting who treat and study patients with Erdheim-Chester disease (ECD). Aim 2. To assess the current and updated collective experience regarding genomic alterations and targeted therapies for ECD. Aim 3. To define, by consensus approach of ECD experts, response criteria for therapeutic clinical trials for ECD.

Agency Contact: Leslie Le, Grants Management Specialist, (e) leleslie@mail.nih.gov, **Overlap:** None

GC228236 (PI: J. Park) 7/1/14-6/30/17 0.30 calendar
Conquer Cancer Foundation

A Phase II Study of the BRAF Inhibitor, Vemurafenib, in Patients with Relapsed or Refractory Hairy Cell Leukemia (HCL): Evaluating Clinical Efficacy and Molecular Mechanisms of BRAF Inhibition in HCL **Aims:** Specific Aim 1: To determine the clinical efficacy of the BRAF inhibitor vemurafenib in patients with BRAFV600E+ relapsed or refractory HCL, as assessed by overall response rates, duration of response, kinetics of minimal residual disease (MRD), disease-free and overall survival

Specific Aim 2: To assess the effect of the BRAF inhibitor vemura

Agency Contact: grants@conquercancerfoundation.org

Overlap: None

GC226666 (PI: Abdel-Wahab) 1/1/16 - 12/31/16 0.00 calendar
Histiocytosis Association

MEK Inhibition in the Therapy of Histiocytic Neoplasms

Goals: We have recently completed a clinical trial of vemurafenib for the adult BRAFV600E-mutant patients and we now seek to identify novel therapies that will benefit those patients that are BRAFV600-wildtype. This grant would provide critical funding to (1) allow us to understand clinical responses in this clinical trial in greater depth and (2) provide critical support which will allow us to get additional data which will serve as the basis of a multi-PI R01 application with other already identified investigators on the biology and therapy of MAP2K1-mutant malignancies.

Aims: Specific Aim 1: Determine the clinical activity of cobimetinib in adult systemic histiocytic disorder patients as measured by radiologic (RECIST) response criteria, metabolic (FDG-PET) response criteria, and longitudinal assessment of somatic mutation burden in cell-free DNA (cfDNA). Specific Aim 2: Functionally characterize the novel kinase mutations and fusions identified in histiocytic disorders without the BRAFV600E mutation and evaluate the *in vitro* efficacy of MEK inhibition in the activating mutants. Results of these studies will be compared to clinical outcome data generated by the cobimetinib trial.

Agency Contact: Grants Office, grants@histio.org,

Overlap: None

GC223032 (PI: Abdel-Wahab) 10/1/14-9/30/16 0.0 calendar months*
The V Foundation

Understanding and Targeting Spliceosomal Mutations in Leukemia

Aims: Aim 1: Determine how SRSF2 mutations alter SRSF2's normal roles in pre-mRNA splicing Aim 2: Utilize Srsf2 P95H mutant murine models to further identify transcriptional events and splicing changes critical to hematopoietic alterations driven by SRSF2 mutations Aim 3: Identify and test therapeutic strategies for targeting leukemic clones with spliceosomal gene mutations.

Agency contact: Carole C. Wegner, Director of Grants, 106 Towerview Court, Cary, NC 27513 **Overlap:** None

*Effort commitment is not required per the sponsor.

GC221801 (PI: Abdel-Wahab) 10/1/13-9/30/14 0.50 calendar months

Erdheim-Chester Disease Global Alliance

Somatic Genetic Alterations in the Pathogenesis and Therapy of Histiocytic Disorders

Goals: Histiocytic disorders, including Langerhans cell histiocytosis (LCH) and Erdheim Chester disease (ECD), represent a wide range of rare diseases with heterogeneous clinical course and prognoses. The relative rarity and protean clinical nature of the histiocytic disorders has made it difficult to clearly delineate the pathophysiology of these conditions.

Agency Contact: Kathy Brewer, President, support@erdheim-chester.org.

Overlap: None

5 U10 CA021115-39 (PI: Comis) 2/1/14 - 1/31/15 0.92 calendar months NIH

Investigating the importance of MAP kinase signaling and novel mutations activating NOTCH signaling in CLL.

Goals: We propose to assess the status of somatic genetic abnormalities in CLL patient samples from the Intergroup trial E2997 as performed in our pilot experiments.

Aims: 1. Targeted resequencing and exome sequencing to identify novel somatic mutations that impact outcome and therapeutic response in CLL. 2. RNA-seq analysis to identify prognostically relevant expression changes in CLL. 3. Epigenetic studies to determine the impact of epigenomic alterations on CLL prognosis and therapeutic response.

Agency Contact: Debra Strandberg, Strandberg.debra@jimmy.harvard.edu

Overlap: None

1 U10 CA180827-01 (PI: Paietta) 4/14/14 – 2/28/15 0.01 calendar months NIH

Genetic and Epigenetic Determinants of response to therapy and outcome in patients with chronic lymphocytic leukemia

Goals: Perform targeted sequencing on a panel of ~300 recurrently mutated genes in genomic DNA from diagnostic CLL patient samples from E2997 clinical trial. Identification of samples with mutations activating MAP kinase signaling and NOTCH signaling will allow us to perform further functional analysis of the therapeutic and biological relevance of these 2 pathways in CLL.

Agency Contact: Mary LaRocca, 111 East 210th Street, Bronx, NY 10467, mlarocca@montefiore.org

Overlap: None

5 K08CA160647-05 (Abdel-Wahab) 9/20/11 – 8/31/16 6.0 calendar months* NIH/NCI

Clinical Scientist Research Career Development Award

“Role of ASXL1 mutations in myeloid malignancies.”

Aims: The specific aims of this grant were as follows: Specific Aim #1: Identify target genes of ASXL1 loss and alterations in chromatin state due to ASXL1 loss in normal and leukemic hematopoietic cells using *in vitro* assays. Specific Aim #2: Determine the *in vivo* effects of ASXL1 loss on hematopoiesis by targeted disruption of ASXL1. Specific Aim #3: Determine the role of ASXL1 mutations in the pathogenesis of MPN and AML

through studies in patient samples and in *in vivo* models in cooperation with known MPN and AML disease alleles.

Agency Contact: Esther Young, youngel@mail.nih.gov

* NIH granted approval for an effort reduction to 6 months to allow for 3 months of concurrent effort for 1 R01 HL128239-01.

Overlap: None

00003930 (PI: Janku)

1/1/16 - 12/31/16

0.60 calendar

Erdheim-Chester Disease Global Alliance

Understanding and targeting novel molecular alterations in Erdheim-Chester disease without the BRAFV600E mutation

Goals: We plan to extend our effort in furthering the treatment and understanding of histiocytic disorders through preclinical and clinical correlative studies to identify and target novel kinase alterations identified in BRAFV600E-wildtype ECD patients. These studies will be critical for (i) establishing diagnostic approaches to identifying the diverse, clinically actionable genomic alterations in ECD and (ii) promoting novel therapeutic studies for ECD patients.

Aims: 1) Perform genomic analysis of Erdheim Chester Disease (ECD) tissue biopsy samples. 2) Functionally characterize the novel kinase alterations recently identified in systemic histiocytic disorder. 3) Determine the sensitivity of kinase mutations present in BRAFV600E-wildtype ECD to targeted therapeutics in preclinical models.

Agency Contact: Jaime Farias, Assistant Director, OSP, 1515 Holcombe Boulevard, Unit 1676, Houston, TX 77030

Overlap: None

GC227311 (PI: Abdel Wahab)

1/1/16-12/31/16

0.00 calendar*

Hairy Cell Leukemia Foundation

HCL Grant Proposal 2015

Goals: To identify the spectrum of recurrent genomic alterations co-existing with BRAFV600E mutations in HCL and delineating their clinical relevance and functional contribution to HCL development

Aims: Specific Aim I: Identify genetic alterations co-existing with the BRAFV600E mutation in HCL patients and their clinical importance. Specific Aim II: Identify the functional importance of recurrent mutations co-existing with the BRAFV600E mutations in HCL pathogenesis.

Agency Contact: Michael Margolin, MD. President, 575 Madison Avenue, 10th Floor, New York, NY 10022

Overlap: None

*Effort commitment is not required per the sponsor.

I9 A9 059 (PI: Chan)

1/1/16-12/31/17

0.12 calendar

Starr Cancer Consortium

Personalized immunotherapy for the treatment of hematological malignancies

Goals: Test the hypothesis that mutations in CALR generate neo epitopes that can be harnessed for effective anti cancer therapy. Test the hypothesis that spliceosomal mutations generate novel epitopes that may be harnessed for anti cancer therapy.

Aims: Aim 1: Test the hypothesis that mutations in CALR generate neo-epitopes that can be harnessed for effective anti-cancer therapy. Specific Aim 2: Test the hypothesis that spliceosomal mutations generate novel epitopes that may be harnessed for anti-cancer therapy.

Agency Contact: Sylvie Le Blancq, PhD, Executive Director, Sloan Kettering Institute, 1275 York Avenue, New York, NY 10065, lesblanc2@mskcc.org

Overlap: None

I8-A8-075 (PI: Abdel-Wahab)

1/1/15-12/31/17 (NCE)

0.00 calendar months

Starr Cancer Consortium

Understanding and Targeting Spliceosomal-Mutation Hematopoietic Malignancies

Goals: We hypothesize that spliceosomal mutations impact the transcriptome in a manner that promotes hematopoietic transformation and that these alterations may be targeted therapeutically.

Aims: Specific Aim I: Determine the mechanistic basis for spliceosomal mutations in myeloid leukemias through comparison of their effects on the transcriptome, RNA-binding properties, and protein interactions. Specific Aim II: Develop novel therapeutic strategies targeting splicing-factor mutant cancers.

Agency Contact: Sylvie Le Blancq, Executive Director, The Starr Cancer Consortium, 1275 York Ave, Box 485, New York, NY 10065, leblancs@mskcc.org

Overlap: None

CURRENT SUPPORT

5R01HL128239-03 (PI: Bradley)

8/1/15 - 5/31/20

3.0 calendar months

NIH

Genetic and molecular basis for SRSF2 mutations in myelodysplasia

Goals: Measure the therapeutic efficacy of drugs that inhibit RNA splicing, and test whether drug-induced splicing inhibition specifically affects SRSF2 mutation-responsive exons versus causing widespread splicing failure.

Aims: Perform functional studies to determine the effect of specific mis-spliced isoforms on myeloid differentiation. Test the hypothesis that a wildtype copy of *SRSF2* is necessary for hematopoietic cell survival and maintenance of functional splicing. Determine whether *SRSF2* mutations do not co-occur with other spliceosomal mutations because they are functionally redundant, or whether multiple spliceosomal mutations are deleterious to the cell. Test the hypothesis that commonly co-occurring *SRSF2* and epigenetic factor mutations are synergistic, leading to more aggressive disease and/or more extreme splicing changes. Measure the therapeutic efficacy of drugs that inhibit RNA splicing, and test whether drug-induced splicing inhibition specifically affects *SRSF2* mutation-responsive exons versus causing widespread splicing failure. Generate cells to be utilized in CRISPR/Cas9 screen and perform validation of sgRNAs from the screen

Agency Contact: Kelly Stewart, Fred Hutchinson Cancer Res. Center, kstewart@fredhutch.org, 206-667-6925

Overlap: None

W81XWH-16-1-0059 (PI: Abdel-Wahab)

5/1/16 - 4/30/19 NCE

0.60 calendar

Congressionally Directed Medical Research

Programs

Therapeutic targeting of spliceosomal-mutant acquired bone marrow failure disorders

Goals: The purpose of the current DOD application is to test inhibitors of TGF-beta signaling pathway in preclinical mouse models as possible therapy for FA. Specific Aim 1. Identify unifying molecular abnormalities across spliceosomal mutations using combined genomic and proteomic approaches. Specific Aim 2. Identify additional genes that are required for the survival of cells carrying different spliceosomal mutations with synthetic lethality screens. Specific Aim 3. Identify novel therapeutic strategies specifically targeting mutant spliceosomal protein function.

Agency Contact: Christine Helman, Christine.e.helman.civ@mail.mil,

Overlap: None

2314-17 (PI: Abdel-Wahab)

7/1/16 - 6/30/21

*0.00 calendar

Leukemia and Lymphoma Society

Investigating and Targeting Diverse Kinase Alterations Driving Systemic Histiocytic Neoplasms

Goals: The discovery of BRAFV600E mutations in 50% of patients with systemic histiocytoses provided the first molecular target in these disorders. More recently, MAP2K1, ARAF, and N/KRAS mutations as well as kinase fusions have been identified in BRAFV600-wildtype patients. Here we propose to understand the clinical and biological importance of diverse kinase alterations in BRAFV600E-wildtype histiocytic neoplasms.

Aims: Specific Aim 1: Determine the clinical activity of cobimetinib in adult systemic histiocytic disorder patients as measured by radiologic (RECIST) response criteria, metabolic (FDG-PET) response criteria, and longitudinal assessment of somatic mutation burden in cell-free DNA (cfDNA). Specific Aim 2: Identify the

biological importance of mutations outside of *BRAFV600E* on histiocytosis pathogenesis and the molecular determinants of response to MEK inhibition.

Agency Contact: Lee Greenberger, Chief Scientific Officer, researchprograms@lls.org

*Sponsor has given approval to reduce effort to 0%

Overlap: None

GC231183 (PI: Abdel-Wahab)

7/1/16 - 6/30/19

0.00 calendar*

Pershing Square Sohn Cancer Research Alliance

Identification of novel transcripts, pathways, and therapeutic strategies to target spliceosomal-mutant malignancies

Aims: Aim I: Determine the functionally relevant splice isoforms linking spliceosomal gene mutations to cancer development. Aim II: Explore novel therapeutic approaches for spliceosomal-mutant cancers through targeting altered RNA binding preferences and new spliceosomal inhibitors. Aim III. Investigate the intersection of spliceosomal mutations and epigenetic alterations in myeloid malignancies.

Agency Contact: Olivia Tournay Flatto, Executive Director, otflatto@persq.org

Overlap: None

*Effort commitment is not required per the sponsor.

GC231754 (PI: Abdel-Wahab)

9/1/16 - 8/31/19

0.60 calendar Edward

P. Evans Foundation

Elucidating Critical Targets, Transcripts, and Collaborating Events in Spliceosomal-Mutant MDS

Goals: We propose to functionally characterize altered transcripts generated by mutant SRSF2 and/or U2AF1, test novel strategies for targeting spliceosome mutant cells, and study the implications of mutational co-occurrences between spliceosomal and epigenetic alterations in myeloid malignancies. The long-term goal of this project is to define genes and cellular pathways that cooperate with spliceosome gene mutations to induce MDS and to exploit them therapeutically.

Aims: Specific Aim 1. We will identify genes and pathways that cooperate with mutant U2AF1 to induce MDS and AML in vivo. Specific Aim 2. We will comprehensively define alterations in RNA splicing and expression throughout hematopoiesis, including in non-coding RNAs, induced by mutant U2AF1 and SRSF2.

Agency Contact: Michael D. Lewis, President, Edward P. Evans Foundation, mdl@epefoundation.org

Overlap: None

1 R01 CA201247-02 (PI: Abdel-Wahab / Park)

9/1/16 - 8/31/21

2.40 calendar months

NCI

Origins of BRAF-mutant hematologic malignancies and their therapeutic resistance

Goals: We expect our studies to elucidate mechanisms that underlying the development of these disparate blood cancers driven by a shared mutation, provide the first description of the genetic events that likely cooperate with the *BRAFV600E* mutation to drive these disorders and therapy resistance.

Aims: Aim 1. Determine the mechanistic basis for the divergent phenotypes of *BRAFV600E* mutant hematopoietic disorders. Aim 2. Determine whether recurrent mutations co-occurring with the *BRAFV600E* mutation HCL and SH alter the disease phenotype. Aim 3: Define mechanisms of vemurafenib resistance in HCL using patient samples and murine models of *BRAFV600E*-mutant hematopoietic malignancies.

Agency Contact: Funmi Elesinmogun, Grants Management Specialist, elesinmf@mail.nih.gov **Overlap:** None

I10-0074 (PI: Abdel-Wahab)

1/1/17 - 12/31/18

0.54 calendar

Starr Cancer Consortium

Dissecting micro-environmental contributions to the pathogenesis of myelodysplastic syndromes

Specific

Goals: Our proposal aims at highlighting the molecular alterations in the HMEV of MDS patients that could serve as novel therapeutic targets.

Aims: Aim 1: Identify the functional impact of molecular alterations in the microenvironment of MDS patients on hematopoiesis. Aim 2: Investigate the in vivo effects of molecular alterations in the MDS microenvironment on the kinetics of hematopoietic cells bearing MDS-associated mutations.

Agency Contact: Sylvie Le Blancq, PhD, Executive Director, Sloan Kettering Institute, 1275 York Avenue, New York, NY 10065, lesblanc2@mskcc.org

Overlap: None

GC232581 (PI: Abdel-Wahab)

1/15/17 - 1/14/20

0.60 calendar

Henry & Marilyn Taub Foundation

Understanding and manipulating pathologic splicing preferences and nonsense-mediated mRNA decay (NMD) in spliceosomal-mutant MDS.

Goals: Here we aim to develop two novel mechanism-based approaches targeting spliceosomal-mutant MDS based on: (1) selectively targeting the pathologic RNA-binding preferences of mutant spliceosomal proteins; and (2) restoring the expression of key transcripts targeted for nonsense-mediated decay (NMD) due to aberrant splicing. Understanding the mechanistic link between NMD and spliceosomal gene mutations will be critical for this latter aim.

Aims: Aim I. Determine the precise mechanism by which mutant SRSF2 promotes NMD. Aim II. Utilize antisense techniques to restore expression of mRNAs pathologically degraded by NMD in spliceosomal-mutant MDS cells. Aim III. Determine the therapeutic potential of oligonucleotide decoys that exploit the RNA-binding preferences of WT versus mutant SRSF2 in SRSF2-mutant malignant cells.

Agency Contact: Erin Johnstone, Program Officer, The Medical Foundation, ejohnstone@hria.org

Overlap: None

1 R01 HL138090-01 (PI: Geissmann)

8/16/17 - 6/30/21

1.80 calendar NHLBI

Lineages and pathophysiology of clonal histiocytic disorders

Goals: This project investigates the pathophysiology of clonal histiocytic disorders, exemplified by Langerhans Cell Histiocytosis (LCH) and Erdheim-Chester Disease (ECD). We made the novel hypothesis that somatic BRAF mutations in yolk sac hematopoietic progenitors during embryogenesis may be causative of these diseases, and we have developed in vivo models that allow this hypothesis to be tested experimentally. This work will help elucidate the pathophysiology and mechanism of histiocytoses.

Aims: We will (Aim 1) investigate the pathological, molecular and cellular mechanisms that underlie the development of the brain neurodegenerative disease we observe in our genetic model *Csf1rMeriCreMer; BRAFV600E; Rosa26LSL-YFP* in which we introduce a *BRAFV600E* allele in a small number of EMPs.

Next (Aim 2) we will determine the pathological consequences of targeting BRAFV600E outside the brain and in particular test whether environmental factors can influence the pathogenesis of histiocytosis in the liver. We will also investigate the extent to which lineage(s) of origin of histiocytoses determines pathological outcomes using a murine model to conditionally target BRAFV600E expression to a small fraction of HSCs. Finally (Aim 3) we will investigate the efficiency of BRAF inhibitor administration to prevent or treat the neurodegenerative disease in our models.

Agency Contact: Renee Livshin, livshinr@nhlbi.nih.gov,

Overlap: None

6533-18 (PI: Park)

10/1/17 - 9/30/20

0.60 calendar

Leukemia and Lymphoma Society

Chemotherapy-Free Targeted Therapeutic Approaches for New and Relapsed Hairy Cell Leukemia

Goals: This proposed study has a potential to challenge and change the current standard treatment practice of frontline HCL and offer chemotherapy-free, rationally targeted therapeutic options in HCL.

Aims: Aim 1: Determine the efficacy of combined RAF inhibition and anti-CD20 immunotherapy as frontline therapy for classical hairy cell leukemia (cHCL). Aim 2: Determine the safety and efficacy of ERK inhibition in relapsed/refractory (R/R) cHCL and hairy cell leukemia variant (HCLv).

Agency Contact: Director of Research Administration, 3 International Drive, Suite 200, Rye Brook, New York 10573, researchprograms@lls.org

Overlap: None

1R56AG052501-01A1 (PI: Liu)

9/15/2017 - 8/31/2018

0.34 calendar

NIH

Mutant p53 rejuvenates aged stem cells through modulating epigenetic regulators

Aims: Milestone Task 1: Determine if MDS cells expressing mutant TP53 will accelerate the onset of MDS in NSG-SGM3 mice. MDS cells are difficult to engraft immunodeficient mice. Milestone Task 2: Determine whether mutant SRSF2 collaborates with gain-of-function mutations in TP53 in vivo.

Agency Contact: Office of Research Administration, Lockefield 2232, 980 Indiana Ave., Indianapolis, IN 46202, spons2@iupui.edu

Overlap: None

LLS-01 (PI: Nimer)

10/1/17 - 9/30/22

0.36 calendar

Leukemia and Lymphoma Society

Interventional Epigenetics in Myeloid Malignancies

Goals: Our ultimate goal is to define the mechanistic epigenetic basis for the development and progression of myeloid malignancies and to develop novel and effective epigenetic-focused therapies for patients with these disorders.

Aims: Major Task 1: Determine genome-wide localization of ASXL1 versus ASXL2. Major Task 2: Characterize the effects of ASXL2 loss in the setting of AML1-ETO. Major Task 3: Determine the effect of p300 inhibition in AML1-ETO cells with or without Asxl2 loss. Major Task 4: Test LSD1 inhibition in ASXL1/2-deficient AML cells. Major Task 5: Identify genes which are synthetic lethal with ASXL1-mutant cells by performing functional genomic screens.

Agency Contact: Director of Research Administration, 3 International Drive, Suite 200, Rye Brook, New York 10573, researchprograms@lls.org

Overlap: None

170378 (PI: Varmus)

9/1/2017 - 8/31/2019

0.24 calendar Edward

P. Evans Foundation

The Roles of Mutant U2AF1 in the Initiation and Maintenance of MDS

Goals: Our work will improve our basic understanding of key genetic alterations in MDS & provide crucial insights into genetic targets for therapeutic intervention in MDS. Importantly, the majority of our proposed experiments will be conducted on primary human hematopoietic tissues from MDS patients or healthy donors, combining the pre-clinical and clinical expertise of the Varmus and Abdel-Wahab labs. Therefore, the knowledge gained will be directly relevant to MDS patients carrying U2AF1 mutations.

Aims: Specific Aim I: Determine if mutant U2AF1 is required for MDS disease maintenance. Specific Aim II: Determine the molecular targets of U2AF1(S34F) responsible for MDS development.

Agency Contact: Grants Administrator, P.O. Box 46 Casanova, VA 20139, grants@epefoundation.org

Overlap: None

GC231058 (PI: Abdel-Wahab)

12/31/2017 - 12/30/2018

0.60 calendar

Histiocytosis Association

Functional Evaluation of Immunologic Checkpoint Blockade in the Therapy of Histiocytosis

Goals: The recent success of immune checkpoint blockade therapy in other cancer types combined with the expression of immune checkpoint antigens and abundant immune infiltrates in histiocytosis lesions suggests that such therapies may be effective for histiocytosis patients. We therefore seek here to (1) test the efficacy of checkpoint inhibitors (anti-CTLA4, anti-PD-1/PD-L1) alone or in combination with BRAF inhibition in in vivo models of LCH and (2) determine molecular correlates of PD-L1 expression.

Role: PI

Agency Contact: Grants Office, grants@histio.org

Overlap: None

GC232041 (PI: Park) 1/1/2018 - 12/31/2018 *0.00 calendar
Hairy Cell Leukemia Foundation
Investigating the Basis of Hairy Cell Leukemia and Its Response to Therapy Through Single-Cell Approaches
Goals: The Abdel-Wahab lab will perform flow cytometric sorting of primary patient samples from HCL patients for single cell RNA sequencing studies. In addition, they will perform bulk cell RNA-sequencing in addition to mutational analysis by whole exome sequencing and/or MSKCC IMPACT targeted mutational analysis.
Role: Mentor
Agency Contact: Michael Margolin, MD. President, 575 Madison Avenue, 10th Floor, New York, NY 10022
Overlap: None
*Dr. Abdel-Wahab is a mentor and not contributing measurable effort to this project.

PENDING SUPPORT

GC231695 (PI: Liu) 3/1/2018 - 2/29/2020 0.60 calendar
Congressionally Directed Medical Research Programs
Understanding and Targeting Mutant p53 in Myelodysplastic Syndromes
Aims: Task 1. We will assist in the generation of patient-derived xenografts from patients with myelodysplastic syndromes (MDS). Task 2. We will generate p53R248W/+SRSF2P95H/+Mx1-Cre+ mice.
Role: Principal Investigator
Agency Contact: Christine Helman, Christine.e.helman.civ@mail.mil,
Overlap: None

BC171737 (PI: Abdel-Wahab) 9/30/2018 - 9/29/202 1.20 calendar
Congressionally Directed Medical Research Programs
Mechanistic and Therapeutic Implications of Spliceosomal Gene Mutations in ER+ Breast Cancer
Aims: Our grant aims to (1) identify what drives breast cancer growth; determine how to stop it and (2) revolutionize treatment regimens by replacing them with ones that are more effective, less toxic, and impact survival. Background: Aberrant splicing in
Role: Principal Investigator
Agency Contact: Christine Helman, Christine.e.helman.civ@mail.mil,
Overlap: None

GC232558 (PI: Landau) 4/1/2019 - 3/31/2024 0.60 calendar NIH
The role of DNA methylation modifiers in shaping the hematopoietic differentiation topology
Aims: The Abdel-Wahab will generate and maintain the murine models used in Aims 1-2 as well as perform purification of mouse and human cell populations in all aims of the grant. The Abdel-Wahab lab will also perform the cell transduction and differentiation experiments described in Aim 2.
Role: Principal Investigator
Agency Contact: 1300 York Avenue, Box 89, New York, NY 10065 subawards-wcmc@med.cornell.edu,
Overlap: None

GC232610 (PI: Padron) 9/1/2018 - 8/31/2023 0.60 calendar NCI
Developing and credentialing patient-derived xenograft models to advance therapeutic approaches for chronic myelomonocytic leukemia
Aims: Chronic myelomonocytic leukemia (CMML) is an aggressive blood cancer for which we unfortunately have no curative therapies. Our groups have now identified, for the first time, an effective way to grow cancer

cells from CMML patients in mice. We are proposing here to utilize this methodology to identify new drugs that may safely cure this disease.

Role: Principal Investigator

Agency Contact: Funmi Elesinmogun, Grants Management Specialist, elesinmf@mail.nih.gov, **Overlap:** None

GC232644 (PI: Shlush)

7/1/2018 - 6/30/2023

2.40 calendar

Rising Tide Foundation

Early diagnosis and treatment of pre-leukemia

Aims: Successful completion of our aims could have major ramifications on how subjects with ARCH are evaluated and treated, which could, in turn, have a major impact on development of MDS and AML. We now propose to utilize this knowledge to treat high-risk individuals with ARCH, at a time point before they have developed disease, by targeting the driving alterations most associated with AML development.

Role: Principal Investigator

Agency Contact: Eveline Mumenthaler, Director of RTFCCR, Eveline.mumenthaler@risingtide.ch **Overlap:** None

GC231504 (PI: Silberstein)

3/1/2018 - 4/30/2021

0.60 calendar

Leukemia and Lymphoma Society

Protection from proliferative stress to restore hematopoiesis in MDS

Aims: Omar Abdel-Wahab, co-principal investigator from MSKCC, will be responsible for the following aspects of the project: 1) Generation of MDS patient-derived xenografts (PDX). 2) Analysis of PDX models pre- and post-treatment. 3) Generation of single-cell RNA-sequencing data.

Role: Principal Investigator

Agency Contact: Director of Research Administration, 3 International Drive, Suite 200, Rye Brook, New York 10573, researchprograms@lls.org

Overlap: None

1 UG1 CA233332-01 (PI: Levine)

3/1/2019 - 2/28/2025

0.60 calendar

NCI

ECOG-ACRIN Integrated Leukemia Translational Science Center (LTSC)

Advances in the treatment of hematologic malignancies have been disappointing. The ECOG-ACRIN Leukemia Translational Science Center will create, support, and synergize partnerships between clinical and laboratory investigators and foster the continuous, near-term translation of state-of-the art laboratory studies into the clinical context to improve outcomes for leukemia patients.

Aims: **1.** To identify the molecular signatures which define clinical, prognostic, and therapeutically distinct leukemia subtypes, and can be used to develop risk-stratified therapeutic protocols and/or to therapy with mechanism-based therapies for biomarker-defined disease subsets.

2. To evaluate the mechanism of action, therapeutic efficacy, and predictors of response/resistance of mechanism based therapies for leukemia patients.

3. To perform dynamic, biomarker based molecular assessment of response to leukemia therapies which can be used to monitor disease response, inform mechanisms of sensitivity and resistance to leukemia therapies, and to design clinical trials which incorporate dynamic biomarker profiling into clinical trial design and clinical care.

Role: Co-Investigator

Agency Contact: N/A

Overlap: None

GC233106 (PI: Figueroa)

9/1/2018 - 8/31/2020

0.24 calendar

Edward P. Evans Foundation

Determining the Contribution of MDS-associated RNA Splicing Factor Mutations to Altered DNA Methylation

Goals: As part of this collaboration, our lab will perform analyses to integrate DNA methylation and

hydroxy-cytosine methylation data with RNA splicing data. We will also attempt to understand how alterations in DNA methylation alter splicing through focused studies of splicing and expression of potential regulators of DNA methylation. In addition, we will perform locus-specific biochemical experiments to understand how alterations in the epigenome impact splicing.

Aims: Aim 1: Evaluate the association between mutations in RNA-splicing factors and altered DNA cytosine modifications in MDS.

Aim 2: Determine the contribution of altered DNA cytosine modifications on RNA splicing in spliceosomal mutant MDS.

Overlap: None

GC232855 (PI: List)

10/1/2018 - 9/30/2023

0.60 calendar

Leukemia and Lymphoma Society

Regulation and Targeting of Inflammatory Circuits in Myelodysplastic Syndromes

Aims: Aim 1: Determine the mechanistic basis for hyper-activated innate immune signaling in spliceosome-mutant MDS bearing mutations in RNA splicing factors. Aim 2: Define the therapeutic potential for MAP3K7, p38/MAPK, and TGF- β pathway inhibition in spliceosome mutant MDS. Aim 3: Define the mechanistic basis for preferential dependency on IFN-JAK-STAT signaling in SF3B1 mutant cells.

Overlap: None

GC231826 (PI: Zhang)

7/1/2018 - 6/30/2020

0.36 calendar

Congressionally Directed Medical Research

Programs

Muscarinic Acetylcholine Receptor Signaling in the Pathogenesis and Treatment of MDS

Aims: Major Task 1. Evaluate the effects of genetic depletion and pharmacologic inhibition of Chrm4 in multiple murine genetic models of MDS. Major Task 2. Evaluate genetic predictors of response to CHRM4 antagonists to improve anemia using primary MDS patient materials.

Overlap: None

*If BC171737 is funded, Dr. Abdel-Wahab will reduce effort from 1.8 to 0.6 on 1 R01 HL138090-01.

Current/Pending/Previous Support: BRADLEY, R. K.

CURRENT

BMFRP – FY15 (PIs: Abdel-Wahab & Bradley)
Department of Defense

(NCE to 4/30/2019)

04/01/2016 – 04/30/2018)

0.6 CPM

Therapeutic targeting of spliceosomal--- mutant acquired bone marrow failure disorders

Dr. Bradley will be primarily responsible for the genomics studies in Aim 1, including SILAC experiments and computational data analysis, and CRISPR/Cas9 screens in Aim 2. Dr. Bradley will also contribute to compound library screening and subsequent hit validation in Aim 3. All studies conducted by Dr. Bradley will involve cell lines or computational analysis of data from cell lines, murine models, or primary patient samples.

Grant Specialist: Elena G. Howell, elena.g.howell.civ@mail.mil

R01 DK103854 (PI: Bradley)

12/01/2015 – 11/30/2019

3.0 CPM

NIH/NIDDK

U2AF1 mutations in myelodysplastic syndromes: from mechanism to therapy

The goal of this project is to determine how myelodysplasia-associated mutations in *U2AF1* alter the RNA splicing process to contribute to MDS pathophysiology. There is no research or budgetary overlap between this grant and the DoD proposal.

Program Official: Cindy Roy, cindy.roy@nih.gov

(NCE to 4/30/2018)

P01 CA163227 (PI: Balk, Beth Israel)

05/24/2013 – 04/30/2018

0.6 CPM NCI

Androgen Receptor Action in Castration Resistant Prostate Cancer

In recent years, multiple studies have identified isoforms of the androgen receptor that are capable of activating transcription even in the absence of canonical androgen signaling. These isoforms, generated by alternative splicing of the androgen receptor gene, are associated with castration-resistant cancer. We seek to determine the regulatory mechanisms governing their expression, with the ultimate goal of identifying promising targets for the development of new therapeutics.

Program Official: Neeraja Sathyamoorthy, ns61r@nih.gov

R01 HL128239 (PI: Bradley)

08/01/2015 – 05/31/2020

3.0 CPM NIH/

NHLBI

Genetic and molecular basis for SRSF2 mutations in myelodysplasia

The goal of this project is to determine how MDS-associated mutations affecting the splicing factor SRSF2 alter SRSF2's normal function to contribute to MDS pathophysiology. There is no research or budgetary overlap between this grant and the DoD proposal.

Program Official: Elizabeth Wagner, wagnere@mail.nih.gov

P01 NS069539-06 (PI: Tapscott)

09/30/2015 – 09/29/2020

NINDS

The Pathogenesis of Facioscapulohumeral Muscular Dystrophy

The studies proposed under this program project grant combine genetic, epigenetic, transcriptional and developmental approaches to defining the epigenetic modifications associated with FSHD. Together they will determine whether repeat-mediated epigenetic silencing and repetitive element expression represent specific pathways that can be targeted for therapeutic intervention in FSHD. Results from this research will provide the basis for clinical therapeutic trials in FSHD.

PPG Project #2

3.0 CPM Repeat

derepression and RNA-mediated toxicity in FSHD

The broad and long-term goal of this project is to identify molecular pathways downstream of D4Z4 derepression that may be targeted to slow disease progression or improve muscle function. The major hypothesis of this project is that repetitive and other aberrant RNAs contribute to DUX4 cytotoxicity and

modify FSHD severity. The specific goal of the project is to identify the mechanistic origins of aberrant RNA production and cytotoxicity, and determine whether these toxic RNAs modify FSHD penetrance
Program Official: Glen Nuckolls, nuckollg@mail.nih.gov

Career Development Scholar Award (PI: Bradley) 07/01/17-06/30/22 6.39 CPM
Leukemia & Lymphoma Society

The biological and therapeutic consequences of SF3B1 mutations in myelodysplastic syndromes
The studies proposed will combine experimental and computational techniques to determine the functional consequences of SF3B1 mutations and identify therapeutic opportunities for selectively killing SF3B1 mutant cells. Aim 1, Determine the mechanistic consequences of SF3B1 mutations for RNA splicing. Aim 2, Identify molecular pathways that connect SF3B1 mutations to dysplastic hematopoiesis. Aim 3, Identify therapeutic opportunities to selectively target SF3B1 mutant cells.

Grants Manager: Erin Salvo, researchprograms@lls.org

NEW PENDING

Discovery Research Award (PI: Bradley) 07/01/18 – 06/30/19 0.60 CPM
Evans Foundation

Leveraging the minor spliceosome to understand and treat myelodysplastic syndromes In this proposal, we will combine experimental and computational tools to determine how a common genetic change gives rise to MDS. Mutations affecting the gene ZRSR2, which encodes a protein involved in a molecular process called RNA splicing, are common in MDS. Although ZRSR2 mutations occur frequently, it is not known why they prevent normal blood production and give rise to MDS. We will determine how ZRSR2 mutations drive the development of MDS and search for new ways to specifically target and kill cells carrying ZRSR2 mutations. The long-term goals of this research are to further our understanding of basic MDS biology and identify potential new therapies for this disease.

Edward P. Evans Foundation, President: Michael D. Lewis, grants@epefoundation.org

PREVIOUS

Damon Runyon Cancer Research Fnd (PI: Bradley) 01/01/2012 – 12/31/2013 1.2 CPM
Dale F. Frey Award for Breakthrough Scientists

Alternative Splicing Regulation and Tumor Biology

The intent of this funding is to support the establishment of a research program as a new investigator with the ultimate goal of procuring independent research funding.

Award Programs Administration, Director: Clare M. Cahill, awards@damonrunyon.org

PNW Prostate Cancer Spore – FHCRC (PI: Bradley) 09/01/2012 – 08/31/2013 0.6 CPM
FHCRC Award Number P50CA097186

Regulation of ligand-independent isoforms of the androgen receptor

Androgen deprivation therapy is a common treatment for prostate cancer. However, despite an initial period of regression, the tumor may ultimately become castration-resistant, with low patient survival rates. This study investigates regulatory mechanisms governing the expression of ligand-independent isoforms of the androgen receptor.

Program Official: Andrew Hruszkewycz, hruskea@mail.nih.gov

Hartwell Innovation Fund (PIs: Bradley & Ramakrishnan) 09/01/2012 – 08/31/2013 1.2 CPM
Splicing dysregulation in myelodysplastic syndromes

Specific spliceosomal proteins are commonly mutated in myelodysplastic syndromes (MDS). We will conduct preliminary experiments to test whether these mutated proteins drive dysplastic hematopoiesis. The goal is to obtain preliminary data to secure long-term NIH funding.

Administrative Coordinator: Danielle Parsons, dparsons@fredhutch.org,

SCC Breast SPORE CDP– FHCRC (PI: Bradley)
FHCRC Award Number P50 CA138293

09/01/2012 – 08/31/2014

0.6 CPM

Synthetic interactions between spliceosomal RNAs and breast cancer therapeutics

This project proposes to (1) develop a protocol for in vitro knock-down of snRNA levels, a requirement for foundational experimental investigation, and (2) test for interactions between snRNA knock-down and targeted breast cancer therapeutics, using triple negative breast cancer cell lines displaying differential drug sensitivity.

Program Official: Igor Kuzmin, kuzmini@mail.nih.gov

AG-NS-1030-13 (PI: Bradley)
Ellison Medical Foundation

09/01/2013 – 08/31/2017

0.6 CPM

Inhibition of RNA quality control in differentiation and aging cells

This application proposes to use the developing and aging heart as a tractable and biomedically relevant system to determine the mechanistic origins and global consequences of RNA surveillance inhibition. The proposed research will provide the first characterization of a novel cellular dysfunction that may contribute to aging-associated disorders ranging from age-related macular degeneration to neurodegenerative diseases.

Ellison Medical Foundation, Executive Director: Kevin J. Lee, klee@ellisonfoundation.org

Basic Sciences Research Grant 2015 (PI: Bradley)
EvansMDS Foundation

09/01/2015 – 08/31/17

0.6 CPM

Biological mechanisms and therapeutic opportunities for MDS with spliceosomal mutations

We propose to use a combination of experimental and computational approaches to determine how mutations that affect RNA splicing give rise to MDS. We will use this knowledge to conduct drug discovery experiments, wherein we will screen thousands of molecules to identify compounds that specifically target malignant cells carrying these mutations. The long-term goal of this research is to lay the foundation for drug development to create new therapies for MDS. There is no research or budgetary overlap between this grant and the DoD proposal.

Edward P. Evans Foundation, President: Michael D. Lewis, grants@epefoundation.org

OVERLAP

There is scientific overlap between Aims 2 and 3 of R01 HL128239 and Aims 2 and 3 of the Leukemia & Lymphoma Society Scholar Award. Three calendar months is concurrent between the two.

There is also scientific overlap between Aims 2 and 3 of R01 DK103854 and Aims 2 and 3 of the Leukemia & Lymphoma Society Scholar Award. Three calendar months is concurrent between the two.

There is no budgetary overlap among any of the above awards.

Cancer Cell

Synthetic lethal and convergent biological effects of cancer-associated spliceosomal gene mutations

--Manuscript Draft--

Manuscript Number:	CANCER-CELL-D-17-00340R3
Full Title:	Synthetic lethal and convergent biological effects of cancer-associated spliceosomal gene mutations
Article Type:	Research Article
Keywords:	Myelodysplastic Syndromes, NF- κ B, SF3B1, Splicing, SRSF2, U2AF1
Corresponding Author:	Omar Abdel-Wahab New York, UNITED STATES
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Abstract:	Mutations affecting RNA splicing factors are the most common genetic alterations in

	myelodysplastic syndrome (MDS) patients and occur in a mutually exclusive manner. The basis for the mutual exclusivity of these mutations and how they contribute to MDS is not well understood. Here we report that although different spliceosome gene mutations impart distinct effects on splicing, they are negatively selected for when co-expressed due to aberrant splicing and downregulation of regulators of hematopoietic stem cell survival and quiescence. In addition to this synthetic lethal interaction, mutations in the splicing factors SF3B1 and SRSF2 share convergent effects on aberrant splicing of mRNAs that promote NF-κB signaling. These data identify shared consequences of splicing factor mutations and the basis for their mutual exclusivity.
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	Seishi Ogawa
	Jaroslav Maciejewski
Additional Information:	
Question	Response
If your paper is accepted, we encourage you to publish the original, unprocessed data alongside your Cell Press paper through Mendeley Data. For more information, please refer to our detailed instructions.	I do not wish to publish my original data.

Monday, May 21, 2018

Harmony Turk, Ph.D.
Scientific Editor, *Cancer Cell*

Dear Dr. Turk,

It is a pleasure for us to resubmit our manuscript for your consideration entitled “Synthetic lethal and convergent biological effects of cancer-associated spliceosomal gene mutations” (CANCER-CELL-D-17-00340R2). We thank you and the Reviewers for their consideration and comments. The insightful comments from you and the three Reviewers have substantially improved the content and clarity of the manuscript since initial submission.

We have now addressed the remaining comments from Reviewer #2 and a point-by-point response is included below. The majority of the comments from Reviewer #2 concern statements made in the paper that the Reviewer felt were either overstated or not emphasized enough. We have now revised the manuscript text in the locations noted by the Reviewer to make these points more clear.

We thank you again for considering our manuscript,

Omar Abdel-Wahab and Robert Bradley on behalf of all coauthors.

Reviewer's comments:

Reviewer #1: Thank you for addressing my comments

REPLY: We would like to thank the Reviewer for the insightful comments through the revision process that contributed to a much-improved manuscript.

Reviewer #2:

The authors did an excellent job of addressing many of my previous concerns. I am impressed by their rigorous analysis and remain enthusiastic for the potential implications on the biology of splicing factors in hematologic malignancies. The current manuscript is significantly improved but still has a complicated and at times overstated narrative. As noted before, the focus on two independent observations distracts from the overall impact and major findings. The clinically-relevant observations are related to the finding that SF3B1 and SRSF2 mutant cells exhibit increased sensitivity to inflammatory stressors. As suggested before, either separating the two observations or further clarification of several intertwined concepts may address this issue.

REPLY: We thank the Reviewer for these insightful comments and suggestions and the continual enthusiasm on the findings and implications of this study. We agree with the Reviewer that we can further improve the link between the findings presented in the manuscript. We have now made more edits to the manuscript text to improve the link (described in detail below).

The new and previous data clearly demonstrate that NFkB is activated in SF3B1 and SRSF2 mutant cells and more sensitive to inflammatory stress via TLR-LPS. As indicated by the authors, the current data shows that SF3B1-K700E cells are sensitized to inflammatory signaling but that loss of MAP3K7 expression is not responsible for the hematopoietic defects (i.e., myeloid skewing) observed in the absence of inflammation (Figures 1-2). Indeed, re-expression of MAP3K7 suppresses LPS-induced NFkB activation in SF3B1-K700E cells, however expression of MAP3K7 does not affect pp65 (Figures 7C, D, F) nor restore the hematopoietic defect (figure in response, and Supp Figure 6L) in the absence of LPS stimulation indicating that baseline NFkB activation in SF3B1-K700E cells occurs through a different mechanism. An alternative possibility is that splicing alterations observed in SF3B1 and SRSF2 mutant cells result in ER stress which in turn activates NFkB. This is supported by the observation that the double mutant mice have a stronger signature associated with NFkB signaling (Figure 5C) and that re-expression of MAP3K7 does not suppress baseline NFkB activation.

REPLY: Regarding the Reviewer's comments on the differential contribution of *MAP3K7* to the various hematopoietic and inflammatory phenotypes in *SF3B1K700E* mutant cells, we did not intend to give the impression that *MAP3K7* mis-splicing alone is the sole contributing factor for *SF3B1K700E* mutant phenotypes. Although we performed in-depth analysis of *MAP3K7* mis-splicing in the context of *SF3B1* mutation, we are not ruling out the possible involvement of other targets mis-spliced by mutant SF3B1. To make this point more clear, we have further emphasized that MAP3K7 alone does not fully explain enhanced inflammatory signaling in SF3B1 mutant cells with new text on pages 16 and 21 of the revised manuscript. To this end, the manuscript describes hundreds to thousands of mRNAs that are altered in splicing and/or expression in *SF3B1K700E* mutant cells beyond MAP3K7 (see Figures 3C-E, the entire Figure 4, Figure 5A, Figure 6A, the entire Figure S4, Figure S5A-E, and Tables S2-7).

As already mentioned in the manuscript text, we focused on *MAP3K7* for two major reasons: 1) it is one of the most robustly and consistently mis-spliced genes in both human and mouse; and 2) *MAP3K7* is a central regulator of innate immunity and inflammation upstream of NF- κ B signaling, whose loss of function effects are evident in *Sf3b1K700E* cells. In support of our claims, we have provided robust experimental evidence that *MAP3K7* plays a critical role in driving such phenotypes. For example, *SF3B1* wildtype cells with *MAP3K7* loss exhibited stronger phospho-p65 induction at steady state (Figure S6J), similar to *SF3B1K700E* cells (Figures 6D-G). Moreover, *MAP3K7* re-expression significantly suppressed phospho-p65 induction in the context of *SF3B1K700E* mutation, at both steady state and post LPS-stimulation (Figures 7D & 7F).

Based on our current and prior studies, the hematopoietic defects observed in *Sf3b1*^{K700E/+} mice are caused by dysregulation of a multitude of genes and pathways, among them *Map3k7*. It is therefore not surprising that restoring only one of the many targets mis-spliced by mutant SF3B1 can only partially rescue the phenotypic defects. Based on the above evidence, we therefore conclude that *MAP3K7* dysregulation is one of several contributors to *SF3B1*K700E-driven phenotype. We have made further clarification of this concept in the revised manuscript text.

The Reviewer notes an interesting possibility that SF3B1 and SRSF2 mutations result in ER stress. We are now studying this possibility but there are no data in the manuscript to clearly support this hypothesis (in contrast to the substantial data presented on robust aberrant splicing events in *MAP3K7* and *CASP8* activating NF-κB). We had hoped to include a description of the possibility of splicing mutations activating ER stress in the Discussion; however, major limitations on space in the manuscript text limited our ability to discuss this possibility given the other clarifications to the text made in this revision.

The differential requirement of MAP3K7 for myeloid hematopoiesis and inflammation in SF3B1-mutant cells remains unclear, which is eluded to in the rebuttal but not sufficiently clear in the manuscript. Moreover, the shared consequences of splicing mutations and the basis for their mutual exclusivity does not appear to be related to NFκB (yet this is suggested in the manuscript - see page 5 as one example).

REPLY: We appreciate the Reviewer's point about the need to more clearly state the importance of evaluating the differential requirement of MAP3K7 for hematopoiesis in SF3B1-mutant versus wild-type cells. We have now emphasized this point in the revised Discussion. As mentioned in the prior response to review where we provided substantial *in vivo* data on this point, this is now a major focus of our efforts and there is evidence of a differential requirement of MAP3K7 for hematopoiesis SF3B1 mutant versus wild-type cells.

Regarding the sentence on page 5 noted by the Reviewer, we have revised this sentence to remove the phrase "in addition to hyperactivated innate immune signaling." We also removed a similar sentence that was previously the final sentence on page 14 of the Results section.

Missplicing of MAP3K7 and CASP8 appear to increase the sensitivity of splicing factor mutant cells but are not responsible for the inflammatory signaling observed in the absence of LPS. These concepts need to be explicitly stated and effectively clarified in the manuscript.

REPLY: As suggested by the Reviewer, we have now explicitly stated in the revised manuscript that "Mis-splicing of *MAP3K7* and *CASP8* increase the sensitivity of splicing factor mutant cells to inflammatory stimuli but are not solely responsible for the inflammatory signaling observed in the absence of LPS." This change was made on page 21 of the Discussion. In addition, we would like to emphasize to the Reviewer that we did not attempt to claim that mis-splicing of any single transcript is solely responsible for enhanced inflammatory signaling in spliceosomal mutant cells. Finally, the fact that restoration of MAP3K7 expression does not fully restore inflammatory signaling is not evidence that MAP3K7 loss does not contribute to inflammatory signaling observed in the absence of LPS. Rather, it is evidence that there are multiple contributors to inflammatory signaling in SF3B1 mutant cells in the absence of LPS.

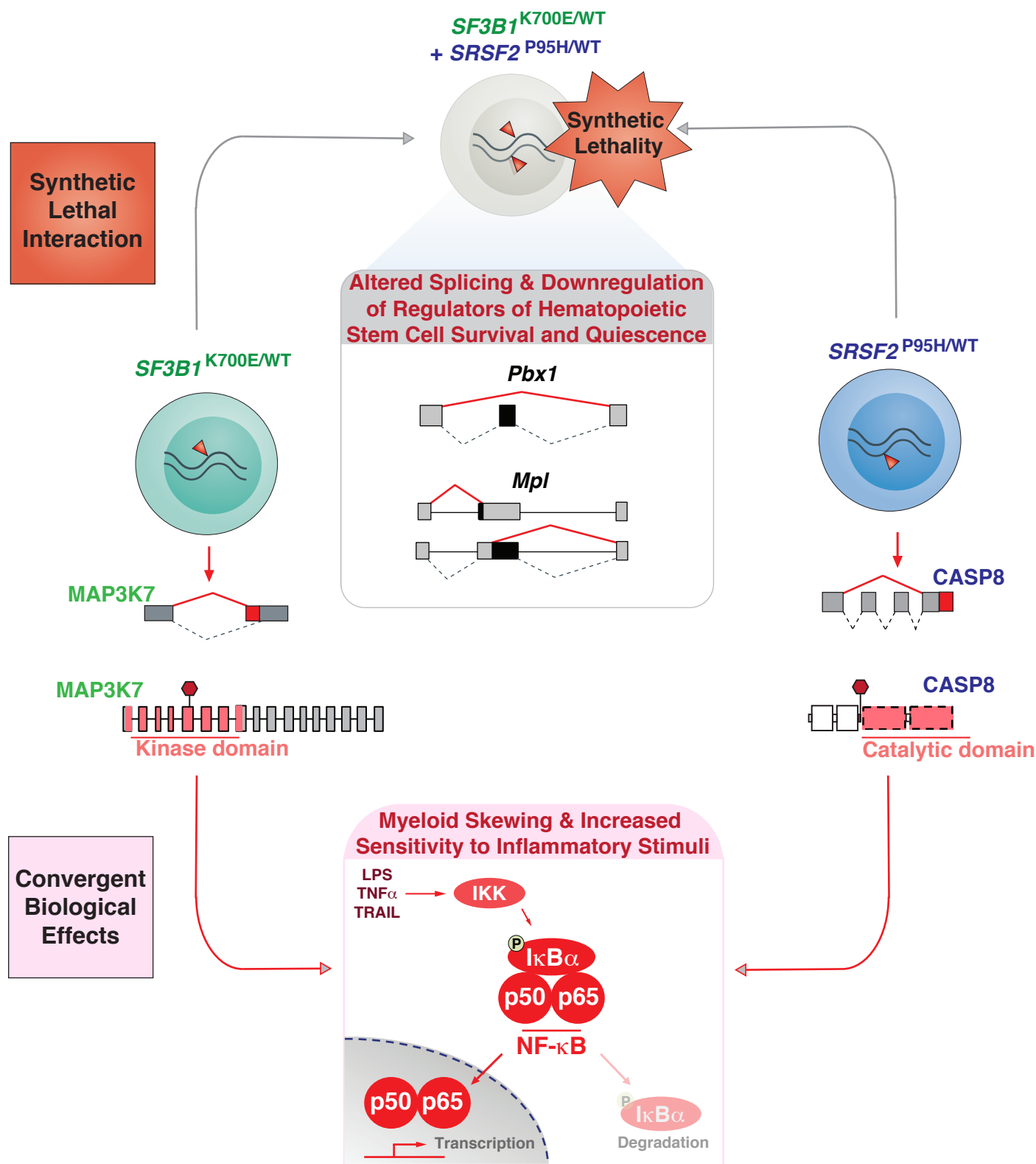
Minor comments:

1. Page 29: "Overall, these data reveal that the inability of the hematopoietic system to tolerate combined expression of mutations in SRSF2 and SF3B1 is not due to widespread failure of splicing catalysis, but rather due to impaired splicing and expression of key regulators of HSPC survival and quiescence". - This is overstated as it has not been formally demonstrated that only key regulators of HSPC are responsible for the hematopoietic defects observed in the mutant mice.

REPLY: We thank the Reviewer for this point and we have revised this sentence accordingly. We also revised several sentences on the first page of this discussion to emphasize that we have not formally demonstrated that only key regulators of HSPC survival and quiescence are responsible for the hematopoietic defects observed in the double-mutant mice.

2. Supplemental Figure 6A. The figure labels are unclear.

REPLY: We have modified the axis labels and legend labels in Figure 6A to clarify the data presentation.



Synthetic lethal and convergent biological effects of cancer-associated spliceosomal gene mutations

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Summary

Mutations affecting RNA splicing factors are the most common genetic alterations in myelodysplastic syndrome (MDS) patients and occur in a mutually exclusive manner. The basis for the mutual exclusivity of these mutations and how they contribute to MDS is not well understood. Here we report that although different spliceosome gene mutations impart distinct effects on splicing, they are negatively selected for when co-expressed due to aberrant splicing and downregulation of regulators of hematopoietic stem cell survival and quiescence. In addition to this synthetic lethal interaction, mutations in the splicing factors SF3B1 and SRSF2 share convergent effects on aberrant splicing of mRNAs that promote NF- κ B signaling. These data identify shared consequences of splicing factor mutations and the basis for their mutual exclusivity.

Keywords: Myelodysplastic Syndromes, NF- κ B, SF3B1, Splicing, SRSF2, U2AF1

Significance

Mutual exclusivity of different mutations that affect a single pathway in cancer is commonly thought to indicate convergent effects of these mutations. RNA splicing factor mutations constitute the most common class of alterations in patients with myelodysplastic syndromes (MDS) and are also frequent in several additional cancer types and occur as heterozygous mutations at restricted residues in a mutually exclusive manner. The mutual exclusivity of spliceosomal mutations suggests synthetic lethal and/or convergent biological effects of these mutations; however, there is currently no functional evidence supporting either of these possibilities. Here we report that individual spliceosomal mutations have non-overlapping effects on splicing and are mutually exclusive due to both synthetic lethal interactions and convergent effects on hyperactivation of innate immune signaling.

Highlights

- Mutations in *SF3B1* and *SRSF2* have a synthetic lethal interaction.
- Mutations in RNA splicing factors are not tolerated in a homozygous state.
- Mutations in *SF3B1* and *SRSF2* have distinct effects on pre-mRNA splicing.
- Both *SF3B1* and *SRSF2* mutations result in hyperactive NF- κ B signaling.

INTRODUCTION

Recently, recurrent somatic mutations in genes encoding components of the spliceosome have been identified through large-scale DNA sequencing across multiple cancer types. Spliceosomal gene mutations most commonly occur in myeloid neoplasms including myelodysplastic syndromes (MDS), chronic myelomonocytic leukemia (CMML), and acute myeloid leukemia (AML) (Graubert et al., 2012; Papaemmanuil et al., 2011; Yoshida et al., 2011), chronic lymphocytic leukemia (CLL) (Quesada et al., 2012; Wang et al., 2011), and uveal melanoma (Harbour et al., 2013; Martin et al., 2013). *SF3B1*, *U2AF1*, and *SRSF2*, encoding RNA splicing factors that promote recognition of 3' splice sites and exons, are the most commonly mutated genes. Mutations in each of these genes are consistently heterozygous and occur as point mutations at highly restricted residues, suggesting that these are oncogenic gain-of-function alterations. In addition, mutations in splicing factor genes are mutually exclusive with one another, presumably due to redundant oncogenic effects and/or a limit on cellular tolerance of disrupted spliceosome function.

Despite recent insights into the individual effects of each of these mutations on pre-mRNA splicing, gene expression, and hematopoiesis, the mechanistic basis for the mutual exclusivity of these mutations and any functionally convergent effects they may have are currently unknown. For example, recent work has identified that mutations affecting the core RNA splicing factor *SF3B1* are associated with cryptic 3' splice site (ss) selection and altered branchpoint recognition (Alsafadi et al., 2016; Darman et al., 2015; DeBoever et al., 2015; Obeng et al., 2016; Wang et al., 2016). In contrast, mutations affecting *SRSF2*, an auxiliary splicing factor that binds to exonic splicing enhancers to promote splicing, alter its RNA binding preferences in a sequence-specific manner and thereby alter the efficiency of exon inclusion (Kim et al., 2015; Zhang et al., 2015). Finally, mutations affecting *U2AF1*, the small subunit of the *U2AF* heterodimer that binds the AG dinucleotide at the 3' ss, promote or repress 3' ss based on sequences flanking the AG dinucleotide (Fei et al., 2016; Ilagan et al., 2014; Okeyo-Owuor et al., 2015; Shirai et al., 2015). Given that the reported effects of *SF3B1*, *U2AF1*, and *SRSF2* mutations on splicing mechanisms are distinct, it is unclear why mutations affecting these three factors are mutually exclusive with one another. Moreover, a direct comparison of the effects of these

individual mutations on RNA splicing and gene expression in an isogenic manner has not been performed.

Here we directly evaluated the functional basis for the mutually exclusive and heterozygous occurrence of mutations affecting RNA splicing factors using murine models expressing single and/or double splicing factor mutations simultaneously. Evaluation of mice with inducible expression of mutations in *Srsf2* or *Sf3b1*, alone or together, and in a heterozygous, homozygous, or hemizygous state, definitively demonstrated that each mutation affects RNA splicing in a distinct manner and are only tolerated when expressed alone in a heterozygous state. Mutations in *Sf3b1* and *Srsf2* were not tolerated when co-expressed in the same cell due to aberrant splicing and impaired expression of key regulators of hematopoietic stem cell survival and quiescence. Remarkably, although mutant SRSF2 and SF3B1 alter RNA splicing through different mechanisms, hematopoietic cells expressing either mutant splicing factor resulted in hyperactivate NF- κ B signaling via aberrant splicing of distinct signaling intermediates. These data provide a functional explanation for the genetic configuration of mutations affecting RNA splicing factors and identify a convergent biological effect of these mutations in a pathway of established relevance to MDS pathogenesis.

RESULTS

Simultaneous expression of cancer-associated mutations in *Srsf2* and *Sf3b1* is incompatible with hematopoiesis

Evaluation of sequencing data from >4,000 patients with MDS and related myeloid neoplasms (Bejar et al., 2012; Damm et al., 2012; Haferlach et al., 2014; Lasho et al., 2012; Makishima et al., 2012; Meggendorfer et al., 2012; Papaemmanuil et al., 2013; Patnaik et al., 2013; Thol et al., 2012; Yoshida et al., 2011; Zhang et al., 2012) revealed that while ~48% of patients (1,935/4,032) have a mutation in an RNA splicing factor, only ~2% of MDS patients (86/4,032) have mutations in >1 splicing factor (**Figure 1A** and **Table S1**). To understand the functional basis of this apparent mutual exclusivity, we generated mice to permit inducible heterozygous expression of two of the most common mutations in RNA splicing factors (the *SF3B1*K700E and *SRSF2*P95H mutations) simultaneously (*Mx1*-Cre *Srsf2*^{P95H/+} *Sf3b1*^{K700E/+}) (**Figure 1B**). We performed noncompetitive bone marrow transplantation (BMT), wherein each mutation was induced, either alone or together, following stable engraftment in recipient mice (**Figure 1B**). Bone marrow (BM) cells from mice co-expressing *Srsf2*^{P95H} and *Sf3b1*^{K700E} mutations had severe defects in multi-lineage reconstitution and severe leukopenia compared to other groups (**Figures 1C-D** and **Figure S1A-D**; chimerism analysis and evaluation for recombination prior to administration of polyinosinic-polycytidylic acid (pIpC) confirmed minimal spontaneous excision occurred prior to transplantation and that pre-transplant chimerism was indistinguishable between groups (**Figure S1E-F**)). Similarly, in competitive BMT assays (**Figure 1E**), simultaneous expression of *Sf3b1*^{K700E} and *Srsf2*^{P95H} mutations resulted in severe defects in the repopulating capacity of hematopoietic stem and progenitor cells (HSPCs), which were more readily outcompeted by CD45.1 competitor bone marrow cells relative to wild-type (WT) and single-mutant controls. Analyses of hematopoietic organs six months post-BMT revealed a near-complete absence of *Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} double-mutant cells, which was distinct from expression of the *Srsf2*^{P95H} or *Sf3b1*^{K700E} mutation alone (**Figures 1E-F** and **Figure S1**). These data provide functional evidence that co-expression of these change-of-function mutations affecting RNA splicing factors are not tolerated in the same cell.

Expression of cancer-associated RNA splicing factor mutations in a homozygous or hemizygous state is incompatible with hematopoiesis

Prior data from epithelial and hematopoietic cells identified that cells bearing mutant splicing factors require the WT allele for survival (Fei et al., 2016; Lee et al., 2016; Zhou et al., 2015). While these observations potentially explain the heterozygous nature of RNA splicing factor mutations in patients, the copy number of the RNA splicing factor was not maintained in these experiments. To test whether these mutations can exist in a homozygous state, we generated mice with conditional homozygous expression of the *SRSF2*^{P95H} mutation (*Mx1-Cre Srsf2*^{P95H/P95H}). In competitive BMT assays, HSPCs from these mice showed severe defects in self-renewal as well as multi-lineage reconstitution relative to heterozygous mutant (*Mx1-Cre Srsf2*^{P95H/+}) or control (*Mx1-Cre Srsf2*^{+/+}) HSPCs, analogous to the defects seen with hemizygous *Srsf2*^{P95H} expression (*Mx1-Cre Srsf2*^{P95H/fl}) (**Figures 1G-H and Figure S2A-C**). These data firmly establish that neither hemizygous nor homozygous expression of splicing factor mutations is tolerated by hematopoietic cells, further highlighting the unique dependency of spliceosome-mutant cells on residual function of WT spliceosome components.

Simultaneous expression of mutations in *Srsf2* and *Sf3b1* resulted in increased apoptosis and reduced quiescence of HSPCs

In order to identify the cellular basis for why simultaneous expression of *Srsf2*^{P95H} and *Sf3b1*^{K700E} resulted in severe disadvantage of HSPC function, we next examined the effects of expression of both mutations together versus single-mutant and WT controls on cell cycle and cell death. Two-weeks following induction of recombination *in vivo*, there was a significant increase in the proportion of lineage-negative Sca-1⁺ c-Kit⁺ (LSK) cells undergoing apoptosis (as indicated by Annexin V positivity) and in the S-phase of cell cycle in double-mutant mice (*Mx1-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+}) relative to all controls (**Figure 2A-B**). These data suggest that co-expression of *Srsf2*^{P95H} and

Sf3b1^{K700E} mutations severely impairs hematopoiesis due to a simultaneous loss of HSPC quiescence as well as increased apoptosis.

Selection against co-expression of two splicing factor mutations was also strongly supported by the fact that mice with hematopoietic-specific expression of both *Srsf2*^{P95H} and *Sf3b1*^{K700E} (*Vav-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+}) mutations were associated with 100% lethality at weaning, in contrast to mice with expression of either mutation alone, which were present at expected Mendelian ratios (**Figure 2C**). Timed sacrifice of mid-gestation female mice and genotyping of 139 embryos revealed that *Vav-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} fetuses were detectable at the expected frequency at embryonic day 14.5 (E14.5) (**Figure S3A-B**). Analysis of HSPCs from E14.5 embryos revealed that *Vav-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} fetal livers were marked by increased frequency and number of LSK cells along with phenotypically-defined long-term hematopoietic stem cell (LT-HSC, LSK CD150⁺ CD48⁻), multi-potent progenitor (MPP, LSK CD150⁻ CD48⁻), HPC-1 (LSK CD150⁻ CD48⁺) and HPC-2 (LSK CD150⁺ CD48⁺) populations compared to all other genotypes (**Figure 2D-E** and **Figure S3C-G**). Despite a significant increase in the number of phenotypically defined HSPCs, E14.5 fetal liver cells from *Vav-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} embryos demonstrated significantly impaired ability to form progeny *in vitro* (**Figure 2F**) and were marked by increased proportion of cells in the active phase of cell cycle as well as undergoing apoptosis (**Figures 2G-I**). These derangements in HSPC survival and clonogenic capacity were manifest in embryos by E18.5 where *Vav-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} fetuses were characterized by near absence of myeloid hematopoietic cells in the developing bone marrow space as well as increased proportions of apoptotic cells expressing cleaved Caspase-3 in the fetal liver (**Figure S3H-I**). Taken together, multiple complementary genetic systems here conclusively demonstrate that co-expression of MDS-associated RNA splicing factor mutations severely disrupts hematopoiesis, driven in part by aberrant cell cycle progression and increased apoptosis of HSPCs.

***Srsf2* and *Sf3b1* mutations have largely distinct effects on gene expression**

To understand the mechanistic basis for mutual exclusivity of *SF3B1* and *SRSF2* mutations, we performed RNA-seq of lineage⁻ c-Kit⁺ (LK) cells from *Mx1-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} mice, as well as

the respective single-mutant and WT control cells, two weeks after conditionally expressing each mutation alone or together (**Figure 3A**). The mean allelic ratio of *Sf3b1*^{K700E} and *Srsf2*^{P95H} expressed in double-mutant mice was 20.7% and 33.5%, markedly lower than the near 50% expression seen in single-mutant controls, illustrating the intolerability of combined *SF3B1* and *SRSF2* mutations (**Figure 3B**). Expression of either mutation resulted in dysregulation of hundreds of coding genes (**Figure 3C** and **Table S2**). Double-mutant (*Srsf2*^{P95H/+} *Sf3b1*^{K700E/+}) cells exhibited significantly more marked gene dysregulation than did single-mutant cells, consistent with their more dramatic biological phenotype (**Figure 3D**). Double-mutant cells shared a greater proportion of differentially expressed genes with each single-mutant genotype than single-mutants shared with one another (**Figure 3E**). Gene Ontology (GO) enrichment analysis of coding genes that were differentially expressed within each genotype relative to WT control revealed strong signatures of processes associated with impaired hematopoiesis in double-mutant cells, consistent with the multi-lineage defects in hematopoietic reconstitution evident in these cells noted above (**Figure 3F** and **Table S3**).

We next tested whether mutations in *SF3B1* and *SRSF2* have independent effects on gene expression: that is, whether double-mutant cells recapitulated the gene dysregulation observed for each single-mutant genotype relative to WT cells. Approximately 80% and 40% of genes that were dysregulated in *Sf3b1*^{K700E/+} and *Srsf2*^{P95H/+} single-mutant cells were also dysregulated in *Sf3b1*^{K700E/+} *Srsf2*^{P95H/+} double-mutant cells (**Figure 3E** and **Table S2**). That degree of recapitulation of gene dysregulation was highly similar to and statistically indistinguishable ($p = 0.90$ and 0.44 for *Sf3b1* and *Srsf2* mutations, respectively) from the expected recapitulation under the assumption of independence (**Figure S4A**). We therefore conclude that *SF3B1* and *SRSF2* mutations have independent effects on gene expression, even when present in the same cell. Interestingly, in addition to recapitulating the dysregulation of specific genes expected based on single-mutant cells, double-mutant cells exhibited additional gene dysregulation (**Figures S4B-C**), consistent with the dramatic hematopoietic phenotype of these cells. These results suggest that *SF3B1* and *SRSF2* mutations have independent but compound effects on gene expression when present in the same cell.

***Srsf2* and *Sf3b1* mutations have distinct and independent effects on RNA splicing**

We next assessed the consequences of single versus double mutations in *SF3B1* and *SRSF2* on RNA splicing (**Table S4**). Mutations in *SF3B1* have been proposed to preferentially alter 3' splice site recognition (Alsafadi et al., 2016; Darman et al., 2015; DeBoever et al., 2015). In contrast, mutations in *SRSF2* alter exon recognition via preferential recognition of C-rich exonic splicing enhancer (ESE) motifs relative to G-rich ESEs, while WT *SRSF2* recognizes both classes of ESEs (Kim et al., 2015; Zhang et al., 2015). Our current understanding of *SF3B1* and *SRSF2* mutations therefore indicates that they induce distinct changes in RNA splicing. However, the consequences of these mutations for splicing have not yet been compared in an isogenic context.

We used our double-mutant system to directly compare the consequences of *SF3B1* and *SRSF2* mutations on RNA splicing mechanisms. Consistent with prior studies (Alsafadi et al., 2016; Darman et al., 2015; DeBoever et al., 2015; Kim et al., 2015; Zhang et al., 2015), *Sf3b1* and *Srsf2* mutations preferentially affected 3' splice site and cassette exon recognition, respectively (**Figure 4A-B**). These preferential effects of *Sf3b1* and *Srsf2* mutations on 3' splice site recognition and cassette exon recognition were statistically significant (**Figure S4D-E**). We observed a modest enrichment of adenosines upstream of intron-proximal 3' splice site promoted by the *Sf3b1*K700E mutation, independent of the presence or absence of *Srsf2*P95H. This enrichment was absent from cells expressing *Srsf2*P95H alone (**Figure S4F**). Cassette exons that were promoted versus repressed in cells expressing *Srsf2*P95H were respectively enriched for the CCNG and GGNG ESEs, independent of the presence or absence of *Sf3b1*K700E, but were absent from cells expressing *Sf3b1*K700E alone (**Figure 4C** and **Figure S4G**). These sequence analyses are consistent with previous reports that *SF3B1* and *SRSF2* mutations affect branchpoint recognition and ESE preference, respectively, and furthermore demonstrate that these changes in splicing mechanisms are specific to *SF3B1* and *SRSF2* mutations.

As *SF3B1* and *SRSF2* mutations cause distinct changes in splicing mechanisms, even when simultaneously present in a cell, we hypothesized that the effects of *SF3B1* and *SRSF2* mutations on splicing were likely independent. Consistent with this hypothesis, double-mutant cells recapitulated the preferential effects of *Sf3b1* and *Srsf2* mutations on 3' splice site recognition and cassette exon recognition,

respectively, that we observed in single-mutant cells (**Figure S4D**). We did not observe signs of widespread decreases in splicing efficiency, such as increased retention of constitutive introns, in double-mutant cells (**Figure S4H**). Instead, *Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} cells exhibited modest increases in the number of mis-spliced genes relative to cells bearing single mutations in *Sf3b1* or *Srsf2* (**Figures 4A-B**). *Sf3b1*^{K700E/+} *Srsf2*^{P95H/+} cells recapitulated approximately 40% and 32% of splicing dysregulation driven by expression of the single mutations in *Sf3b1*K700E and *Srsf2*P95H (**Figure S5A**). We identified only seven genes that were mis-spliced in both *Srsf2*^{P95H/+} and *Sf3b1*^{K700E/+} single-mutant as well as double-mutant cells, and only six genes that were mis-spliced in both *Srsf2*^{P95H/+} and *Sf3b1*^{K700E/+} single-mutant but not double-mutant cells. Such genes might represent cases where mutant *SF3B1* and *SRSF2* have opposing or convergent consequences for splicing. However, these genes were ten-fold less abundant than genes that were shared between either single-mutant and the double-mutant cells. We therefore conclude that *SF3B1* and *SRSF2* mutations typically have distinct and independent consequences for RNA splicing.

In order to confirm the effects of co-expressing *Srsf2*P95H and *Sf3b1*K700E mutations on splicing and gene expression using an alternate system, we next performed RNA-seq analysis on purified LK cells from E14.5 *Vav-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} double-mutant fetal liver and all controls. At this timepoint, the allelic frequencies of both mutations in double-mutant cells were near 50% across all replicates and comparable to that seen in *Vav-Cre* single-mutant controls (**Figure 4D**). Similar to the effects seen in the *Mx1-Cre* system, the greatest alterations in gene expression relative to *Vav-Cre* WT controls was seen in *Vav-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} HSPCs compared to *Vav-Cre* single-mutant controls, and double-mutant cells recapitulated much of the gene expression dysregulation observed in single-mutant cells (**Figures 4E-F**). GO enrichment analysis again revealed signatures associated with impaired hematopoiesis in double-mutant cells, consistent with those seen in *Mx1-Cre* mice and in the functional analyses of HSPCs from *Vav-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} embryos (**Figure S5B** and **Table S5**). Moreover, similar effects on splicing were seen in HSPCs from *Vav-Cre* mice as were seen in the *Mx1-Cre* system, including preferential effects of *Sf3b1* and *Srsf2* mutations on 3' splice site recognition and cassette exon recognition (**Figure S5C-D**).

Combined expression of *Srsf2* and *Sf3b1* mutations impairs expression of regulators of HSPC survival and increases sensitivity to inflammatory activation

We next sought to understand the molecular basis for the impaired HSPC survival, increased apoptosis, and increased cell cycling seen when *Srsf2*^{P95H/+} and *Sf3b1*^{K700E/+} mutations were co-expressed. Given the largely non-overlapping effects of *Srsf2*^{P95H/+} and *Sf3b1*^{K700E/+} mutations on gene expression and splicing, we systematically identified and evaluated transcripts that exhibited concomitant dysregulation in both gene expression and splicing in the *Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} double-mutant state (**Figure 5A** and **Table S6**). This analysis revealed that multiple well-known regulators of HSPC survival and quiescence were subject to severe alterations in splicing as well as significantly reduced expression in *Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} double-mutant HSPCs. This included aberrant splicing and decreased expression of the thrombopoietin receptor *Mpl*, integrin α IIb (*Itga2b*, CD41, GPIIb), and the transcription factor *Pbx1* (**Figure 5A** and **S5E**). After confirming reduced expression of each of these mRNAs in LK cells in an independent cohort of *Mx1-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} mice (**Figure 5B**), we evaluated the functional consequences of shRNA-mediated depletion of these mRNAs in WT HSPCs after confirmation of efficient knockdown of each of these shRNAs (**Figure S5F-G**). Consistent with prior published data on the effects of loss of these factors on HSPC function (Alexander et al., 1996; Ficara et al., 2008; Gekas and Graf, 2013; Qian et al., 2007; Yoshihara et al., 2007), silencing any of these individual genes was associated with severe impairment of HSPC clonogenicity (**Figure S5H**). Overall, these data reveal that the inability of the hematopoietic system to tolerate combined expression of mutations in *SRSF2* and *SF3B1* is not due to widespread failure of splicing catalysis.

In addition to gene expression changes consistent with impaired hematopoietic processes in *Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} double-mutant cells (**Figure 3F**), GO analysis also revealed a strong signature associated with immune signaling that was augmented in double- relative to single-mutant cells (**Figure 5C** and **Figure S5B**). To functionally evaluate these gene expression changes we studied the response of *Srsf2* and *Sf3b1* mutant cells to inflammatory stimulus from lipopolysaccharide (LPS). HSPCs from *Vav-Cre* spliceosomal mutant mice exhibited increased baseline NF- κ B activation (marked by

increased nuclear accumulation of phosphorylated p65 (phospho-p65)) in *Srsf2* or *Sf3b1* single-mutant relative to WT cells (**Figure 5D-E**), and this was further enhanced following *ex vivo* LPS stimulation in double-mutant cells relative to single-mutant and wild-type cells (**Figure S5I-J**).

Given prior data identifying that chronic exposure to inflammation impairs quiescence and repopulation potential of HSCs (Esplin et al., 2011; Zhao et al., 2013), we next evaluated the effects of chronic inflammatory insult on the repopulating potential of *Srsf2* or *Sf3b1* mutant BM HSPCs (**Figure 5F**). *Vav-Cre* WT, *Vav-Cre Srsf2*^{P95H/+} and *Vav-Cre Sf3b1*^{K700E/+} mice were treated with LPS (1 mg/kg) via intra-peritoneal injection every second day for 30 days (Esplin et al., 2011) followed by serial BMT into lethally irradiated syngeneic recipient mice. Chronic LPS treatment had a mild effect on the repopulating potential of WT HSPCs evidenced by similar peripheral blood and BM HSPC chimerism in primary transplant recipients (**Figure 5G-H**). In contrast, LPS-treated *Srsf2*^{P95H/+} and *Sf3b1*^{K700E/+} BM HSPCs showed significant reduction in their repopulating ability relative to the respective vehicle-treated counterparts, and this defect was further exacerbated following serial transplantation into secondary recipient mice (**Figure 5G-H**).

The above observations suggest that *Srsf2*- and *Sf3b1*-mutant HSPCs exhibit intrinsic hyperactivation to inflammatory signaling which contributes to defective hematopoietic function similar to features found in human MDS patients. In addition, chronic LPS administration resulted in splenomegaly in both *Srsf2*^{+/+} and *Srsf2*^{P95H/+} mice while BM cellularity was significantly reduced in *Srsf2*^{+/+} mice (**Figure S6A**). *Vav-Cre Srsf2*^{P95H/+} mice treated with LPS showed enhanced myeloid skewing with reduced B-lymphopoiesis relative to either vehicle treated mice or *Vav-Cre Srsf2*^{+/+} mice similarly treated with LPS (**Figure S6A**). These data reveal that chronic inflammatory stimulation promotes myeloid disease phenotypes in *Vav-Cre Srsf2*^{P95H/+} mice *in vivo*. Of note, acute LPS stimulation *ex vivo* of purified BM LSK cells from *Vav-Cre Srsf2*^{P95H/+} mice also resulted in significant increase in nuclear translocation of phospho-p65 relative to *Vav-Cre Srsf2*^{+/+} LSK cells (**Figure S6B-C**).

The enhanced response to inflammatory stimuli across *Srsf2* and *Sf3b1* mutant mice was evident *in vivo* upon exposure to LPS-induced sepsis. Intraperitoneal injection of primary *Mx1-Cre* WT, *Mx1-Cre Sf3b1*^{K700E/+}, and *Mx1-Cre Srsf2*^{P95H/+} mice with LPS (15 mg/kg) 8 weeks

following induction of mutant allele expression resulted in accelerated death in both *Srsf2*- and *Sf3b1*-mutant mice relative to WT controls (**Figure 5I**).

***SF3B1* mutations promote mis-splicing of *MAP3K7*, resulting in hyperactivation of NF- κ B signaling**

The above results identify that spliceosomal gene mutations are intolerable when co-expressed but that they also share convergent effects on activation of NF- κ B signaling. As innate immune signaling has been implicated in MDS pathogenesis (Basiorka et al., 2016; Fang et al., 2014; Fang et al., 2017; Varney et al., 2015; Wei et al., 2013), we next attempted to understand precisely how *SF3B1* and *SRSF2* mutations activate convergent immune signaling pathways. We first focused on events that were mis-spliced within *SF3B1*-mutant hematopoietic cells in both human and mouse (**Table S7**). Although recent studies reported very few such shared mis-spliced transcripts (Mupo et al., 2016; Obeng et al., 2016), we identified a larger set of 205 mRNAs that were mis-spliced in both *Sf3b1*-mutant murine hematopoietic cells and *SF3B1*-mutant MDS patient samples (**Figure 6A**). One of the most dramatically mis-spliced mRNAs in MDS patients with *SF3B1* mutations was *MAP3K7* (also known as TAK1; TGF β -activated kinase 1), for which an intron-proximal 3' ss was promoted by mutant *SF3B1* in both human and mouse hematopoietic cells. This same aberrant isoform of *MAP3K7* has been previously noted in *SF3B1*-mutant CLL (DeBoever et al., 2015) and uveal melanoma (Darman et al., 2015) patient samples. Our re-analysis of RNA-seq data from these cohorts and isogenic human leukemia cell lines with endogenous mutations in *SF3B1*, as well as directed RT-PCR and Sanger sequencing of cDNA from *SF3B1*-mutant cells, validated this aberrant isoform (**Figures 6B-C** and **Figure S6D**). This aberrant 3' ss recognition occurred in exon 5 of *MAP3K7*, which encodes part of the kinase domain, and is predicted to result in an out-of-frame transcript that undergoes nonsense-mediated decay (NMD; **Figure S6E**). Consistent with induction of NMD (which was experimentally confirmed through mRNA half-life measurements in isogenic cells with or without endogenous *SF3B1* K700E mutation; **Figure S6F**), we observed reduced *MAP3K7* protein levels in isogenic cell lines expressing *SF3B1*K700E (**Figure 6D-E**) as well as primary patient BM mononuclear cells (MNCs) from

MDS patients (**Figure 6F**), and peripheral blood MNCs from CLL patients (**Figure 6G**) with *SF3B1* mutations versus patients lacking any splicing factor mutations.

MAP3K7 encodes a kinase that mediates TNF α , IL-1 β , LPS, TRAIL, and toll-like receptor (TLR) signaling through the NF- κ B, JNK, and p38 MAPK pathways. The biological effects of MAP3K7 loss have been extensively studied across a variety of immune cell types and can result in either loss or promotion of inflammatory signaling depending on the cellular context (Ajibade et al., 2012; Lamothe et al., 2012; Sato et al., 2005; Tang et al., 2008; Vink et al., 2013; Xin et al., 2017). Here, we observed that *SF3B1*K700E human myeloid (K562) or lymphoid (NALM-6) leukemia cells stimulated with LPS had enhanced NF- κ B activation (marked by increased level of nuclear phospho-p65 and simultaneous decrease in I κ B- α level) compared with *SF3B1* WT controls (**Figure 6D-E** and **Figure S6G-H**). Increased phospho-p65 was also evident in baseline in these isogenic cells as well as in primary patient BM MNCs from *SF3B1*-mutant MDS and PB MNCs from *SF3B1*-mutant CLL relative to spliceosomal WT counterpart (**Figures 6F-G**). Enhanced NF- κ B transcriptional activation and phospho-p65 levels in *SF3B1*K700E cells compared with *SF3B1* WT cells were also evident in the same cells by increased p-p65 nuclear translocation (**Figure 7A**) and NF- κ B transcriptional activation using an NF- κ B transcriptional reporter assay (DeDiego et al., 2014; Hernandez et al., 2015; Simoes et al., 2015) (**Figures 7B**; background leakiness of this reporter was excluded using a version of the reporter where NF- κ B binding elements in the reporter were mutagenized (**Figure S6I**)). We next assessed whether hyperactive NF- κ B signaling in *SF3B1*-mutant cells resulted in the induction of known NF- κ B target genes that are associated with inflammatory responses. Quantitative RT-PCR (qRT-PCR) analysis revealed marked induction in NF- κ B target genes IL-1 β and TNF in response to LPS stimulation in *SF3B1*K700E NALM-6 cells relative to parental and *SF3B1*K700K control cells (**Figure 7C**).

Given that a number of aberrant splicing and gene expression events occur in *SF3B1*-mutant cells beyond aberrant splicing of *MAP3K7*, we next sought to understand the contribution of MAP3K7 loss to hyperactivated NF- κ B signaling. First, hyperactive NF- κ B signaling was confirmed using shRNA-mediated downregulation of *MAP3K7* in NALM-6 and K562 parental cells at both the level of phospho-p65 signaling and NF- κ B transcriptional activity at both baseline and post LPS stimulation (**Figure 7B**

and **Figure S6J**). In addition, restored expression of *MAP3K7* cDNA in K562 *SF3B1*K700E cells resulted in a significant decrease in p65 phosphorylation in both resting state as well as following LPS exposure based on immunoblot analysis, NF-κB luciferase reporter, and immunofluorescence of nuclear phospho-p65 (**Figures 7D-F** and **Figure S6K**). At a biological level, however, restoration of *Map3k7* expression in *Sf3b1*^{K700E/+} HSPCs resulted in mild rescue of HSPC clonogenic capacity (**Figure S6L**). Overall, these data suggest that the effects of *SF3B1*K700E mutation on induction of NF-κB signaling are indeed, in part, mediated through aberrant splicing of *MAP3K7* (these data do not exclude the possibility that other aberrant splicing events also contribute to increased NF-κB signaling in *SF3B1* mutant cells).

***SRSF2* mutations promote aberrant splicing of caspase-8 (*CASP8*), resulting in expression of a truncated protein that hyperactivates NF-κB signaling**

Consistent with the distinct effects of *SF3B1* and *SRSF2* mutations on splicing, *MAP3K7* aberrant splicing was restricted to *SF3B1*-mutant cells and not seen in *SRSF2*-mutant cells (**Figure S6M**). We therefore searched for aberrant splicing events in *SRSF2*-mutant cells that might impact NF-κB signaling. One such event was aberrant splicing of *caspase-8* (*CASP8*), which was recurrently mis-spliced in AML and CMML patients with *SRSF2* mutations, but not in cells bearing *SF3B1* mutations (**Figure 8A** and **Figure S6N**). Caspase-8 is a cysteine protease that initiates death receptor-mediated apoptosis (Shu et al., 1997; Thome et al., 1997) in addition to playing a role in necroptosis (Kearney and Martin, 2017; Weinlich et al., 2017) and serving as a key activator of NF-κB during the early response to antigenic stimuli (Chaudhary et al., 2000; Hu et al., 2000; Shikama et al., 2003). *SRSF2* mutations repressed a cassette exon of *CASP8*, as was evident by RNA-seq, RT-PCR, and direct sequencing of cDNA from cell lines and primary patient samples (**Figures 8A-B** and **Figures S7A-C**). Exclusion of this cassette exon results in an mRNA encoding a truncated caspase-8 protein lacking the C-terminal catalytic domains, which was readily detectable in *SRSF2*-mutant cells by immunoblotting using an N-terminal anti-caspase-8 antibody, including a newly generated genetically-modified myeloid leukemia cell line (K562 cells) with knockin of the P95H mutation in the *SRSF2* endogenous locus, and

primary patient samples bearing endogenous mutations in *SRSF2* (**Figure 8C-D**). *CASP8* normally encodes a 54/55 kDa protein of 480 amino acids containing two death-effector domains (DED) at the N-terminus and a C-terminal catalytic domain. Although several caspase-8 isoforms have been described which lack the C-terminal catalytic domain (Xu et al., 2009; Yuan et al., 2012), the specific isoform of *CASP8* detected in *SRSF2*-mutant cells is distinct from those previously described.

Previously described DED-only forms of caspase-8 have been suggested to serve either as competitive inhibitors or promoters of apoptosis (Xu et al., 2009; Yuan et al., 2012). Given this, we first tested the effect of expressing this *SRSF2*-mutant-specific truncated isoform (hereafter referred to as *CASP8 TR*) in an *SRSF2* WT cell line (K562 cells) followed by stimulation with TRAIL, a potent agonist of death-receptor mediated cell death. Overexpression of both *CASP8* full-length (*CASP8 FL*) and *CASP8 TR* isoforms resulted in the robust expression of caspase-8 proteins at the expected size (**Figure 8E** and **Figure S7D**) with no apparent effect on growth of K562 cells (**Figure S7E**). Moreover, both isoforms promoted TRAIL-mediated cell death in K562 cells, suggesting that the truncated isoform did not impart a clear differential effect of modulating cell death relative to the *CASP8 FL* isoform (**Figure S7F**). Given this, we next evaluated the effect of overexpressing *CASP8 TR* relative to *CASP8 FL* or an empty vector control on NF- κ B transcriptional activity, signaling, and nuclear localization. In cells with endogenous *CASP8* expression, overexpression of the *CASP8 TR*, but not the *CASP8 FL* isoform, resulted in the robust induction of NF- κ B activity upon increasing concentration of TRAIL exposure. This was demonstrated in both K562 and HAP1 (a near-haploid cell line derived from chronic myeloid leukemia cells KBM7 (Carette et al., 2011)) cells with overexpression of *CASP8 TR* or *CASP8 FL* (**Figure 8E-F** and **Figures S7G-H**). These data were also confirmed in K562 cells with or without endogenous *SRSF2*P95H mutation (**Figure 8G** and **Figure S8A-B**). To rule out the effect of the endogenous caspase-8 in K562 and HAP1 cells, we next assessed the effect of *CASP8 TR* on the cell death and NF- κ B activity in *CASP8* knockout (*CASP8 KO*) HAP1 cells. Consistent with the lack of the catalytic domain, HAP1 *CASP8 KO* cells expressing *CASP8 TR* isoform were unable to undergo TRAIL-mediated cell death (**Figure S8C-D**); however, the *CASP8 TR* isoform was able to induce robust

NF- κ B signaling in the absence of WT caspase-8, evidenced by elevated NF- κ B transcriptional activity, increased phospho-p65 and a concomitant decrease in total I κ B- α level (**Figure 8F** and **Figures S8F**).

DISCUSSION

Mutations affecting RNA splicing factors are the most common genetic alterations in patients with MDS (Graubert et al., 2012; Papaemmanuil et al., 2011; Yoshida et al., 2011) but the biological basis for their significant enrichment in this disease remains largely unexplained. Here we identify that two of the most commonly mutated RNA splicing factors in MDS converge on activation of innate immune signaling through aberrant splicing of mRNAs encoding distinct enzymes. These findings were identified using genetically engineered mouse models and cell lines to evaluate the effect of expressing each RNA splicing factor mutation in an isogenic context, and by comparing the effects of each mutation on gene expression and splicing across mouse and human cells.

Although the initial description of spliceosomal gene mutations predicted that the mutually exclusive pattern of these mutations might be due to a common impact of these mutations on MDS pathogenesis (Yoshida et al., 2011), the data presented here identify that these mutations are mutually exclusive, in part due to synthetic lethal interaction. This provides one of the few examples of mutually exclusive oncogenic alterations due to synthetic lethal interactions in cancer. Interestingly, co-expression of *SF3B1*K700E and *SRSF2*P95H mutations resulted in impaired HSPC self-renewal, differentiation and survival, but this phenotype is not due to widespread inhibition of splicing efficiency.. Instead we identified aberrant splicing and impaired expression of key regulators of HSPC function in *SF3B1/SRSF2* double-mutant cells, including the thrombopoietin receptor *Mpl*, the homeodomain transcription factor *Pbx1* and integrin α IIb (amongst additional aberrant splicing events). Hematopoietic-specific deletion of these factors individually has previously been shown to result in failure of hematopoiesis due to reduced HSC self-renewal, increased apoptosis, and loss of quiescence (Ficara et al., 2008; Gekas and Graf, 2013; Qian et al., 2007; Yoshihara et al., 2007), all features which were characteristic of the *Srsf2*^{P95H/+} *Sfb31*^{K700E/+} double-mutant state. Of note, these specific aberrant splicing events in the double-mutant state were from mice and thereby may not address the precise basis of potential synthetic lethal effects of combined *SRSF2/SF3B1* mutations in patients.

Multiple lines of evidence implicate innate immune signaling in MDS pathogenesis (Basiorka et al., 2016; Fang et al., 2014; Fang et al., 2017; Varney et al., 2015; Wei et al., 2013), including

experiments demonstrating that increased innate immune signaling contributes to MDS development *in vivo* (Fang et al., 2017; Varney et al., 2015). In particular, increased activation of TLR and IL-1R signaling with downstream activation of the MAPK and NF- κ B pathways is widely reported in MDS, but studies of the mechanistic basis for this activation have mostly been restricted to MDS with deletion of chromosome 5q (Fang et al., 2014; Fang et al., 2017; Starczynowski, 2014; Varney et al., 2015). Here we provide data identifying a mechanism for hyperactivated NF- κ B signaling in a wider spectrum of MDS patients carrying mutations in *SF3B1* and *SRSF2*. Physiologic effects of hyperactivated innate immune signaling driven by mutant splicing factors was associated with both enhanced myeloid skewing of hematopoiesis upon chronic exposure to inflammatory stimuli as well as accelerated death due to septic shock from acute inflammatory stimuli. It will be interesting to understand if *EZH2* downregulation also contributes to aberrant NF- κ B activation as reported in other cancer types recently (Lee et al., 2011; Yamagishi et al., 2012), given the *EZH2* mis-splicing driven by mutant *SRSF2* as well as loss-of-functions mutations in *EZH2* in myeloid neoplasms (Kim et al., 2015).

Although recent studies of *SF3B1*K700E mutations have identified relatively few mRNAs that are mis-spliced in both mouse and human cells (Mupo et al., 2016; Obeng et al., 2016), we identified here a much greater overlap of aberrantly spliced transcripts shared across mouse and human hematopoietic cells with an *SF3B1*K700E mutation than has been previously reported. This includes aberrant splicing of *MAP3K7* through the use of an alternative 3' ss promoted by mutant *SF3B1*, an event that was conserved between mouse and human cells (Mupo et al., 2016; Obeng et al., 2016). As partial downregulation of *Map3k7* within myeloid cells *in vivo* has been previously shown to promote development of myeloid neoplasms (Ajibade et al., 2012; Eftychi et al., 2012; Lamothe et al., 2012; Vink et al., 2013), this finding is likely to be relevant to the role of mutant *SF3B1* in MDS pathogenesis. For example, downregulation of *Map3k7* through shRNA knockdown *in vivo* resulted in splenomegaly, myeloproliferation, extramedullary hematopoiesis as well as increased immune activation that was exacerbated by LPS challenge (Vink et al., 2013). Similarly, myeloid-specific deletion of *Map3k7* results in heightened response to inflammatory stimuli and these mice succumb to a clonal myeloid leukemia (Ajibade et al., 2012; Eftychi et al., 2012; Lamothe et al., 2012; Xin et al., 2017). Interestingly, the pro-

inflammatory and leukemogenic effects of *Map3k7* loss appear to be restricted to the myeloid lineages (Ajibade et al., 2012), which is in stark contrast to pan-hematopoietic-deletion of *Map3k7*, which results in complete failure of hematopoiesis (Tang et al., 2008). Exactly how *Map3k7* loss results in cell-type specific effects on signaling and cell survival is currently not defined. It will also be important to understand if there is a differential requirement for hematopoiesis in *SF3B1* mutant cells given the partial loss of *MAP3K7* induced by mutant *SF3B1*.

While aberrant splicing of *MAP3K7* and consequent downregulation of *MAP3K7* expression was specific to cells expressing mutant *SF3B1*, cells expressing mutant *SRSF2* shared a similar transcriptional profile of elevated innate immune signaling and heightened sensitivity to LPS-induced inflammation as did *SF3B1*-mutant cells. This led us to identify a novel gain-of-function effect of *SRSF2* mutations through the generation of a novel *CASP8* isoform that promotes NF- κ B signaling. This observation can be partly attributed to a specific splicing aberration in *CASP8*, whereby mutant *SRSF2* promoted a cassette exon exclusion event resulting in the production of a previously unannotated transcript that stably expresses a truncated form of caspase-8 lacking the C-terminal effector domains. This is consistent with prior work showing that N-terminal prodomain-only containing isoforms of procaspase-8 activate NF- κ B signaling through interactions with upstream regulators of NF- κ B, a function not mediated by the canonical full-length caspase-8 (Chaudhary et al., 2000; Hu et al., 2000; Shikama et al., 2003). While the experiments here are focused on mutations in *SF3B1* and *SRSF2*, it is also noteworthy that mutations in *U2AF1* also drive *CASP8* mis-splicing (Ilagan et al., 2014). Finally, it is important to note that mis-splicing of *MAP3K7* and *CASP8* increase the sensitivity of splicing factor mutant cells to inflammatory stimuli but are not solely responsible for the inflammatory signaling observed in the absence of LPS.

Together, our data demonstrate that different splicing factor mutations alter distinct set of targets at the level of pre-mRNA splicing that nonetheless converge on the same downstream signaling node to drive hyperactive innate immune signaling, a phenomenon that has been previously implicated in MDS pathogenesis. In addition to identifying the functional basis for the mutual exclusivity and convergent consequences of splicing factor mutations, our study also highlights new avenues for

potential therapeutic development. The universal requirement for the expression of the WT allele of a splicing factor in cells with an existing spliceosomal mutation motivated the use of compounds that inhibit basal splicing catalysis to specifically target mutant cells (Fei et al., 2016; Lee et al., 2016; Obeng et al., 2016; Shirai et al., 2017; Zhou et al., 2015). The identification that hematopoietic cells with expression of either of two mutated splicing factors simultaneously or in the homozygous state, further reinforces the requirement of a defined level of basal WT splicing catalysis in cells with heterozygous expression of a mutant RNA splicing factor.

In addition to therapeutic implications regarding targeting RNA splicing, it is important to note that while partial downregulation of *Map3k7* can drive the development of myeloid neoplasms in murine models, complete *Map3k7* loss across hematopoietic cells results in eradication of hematopoiesis (Tang et al., 2008). Thus, we speculate that the convergent effects downstream of splicing factor mutations on immune signaling may suggest new therapeutic opportunities through inhibition of residual MAP3K7 function in *SF3B1*-mutant cells or NF- κ B signaling in *SF3B1*- or *SRSF2*-mutant cells. In particular, efforts to develop potent and specific small-molecule inhibitors of MAP3K7 may be especially important to study in the context of spliceosomal mutant cancers (Bosman et al., 2014; Kilty and Jones, 2015; McNew et al., 2016; Singh et al., 2012). Finally, this study also highlights the advantage of functionally interrogating aberrantly spliced transcripts to identify the potentially pathogenic events that can be targeted by novel strategies. The use of oligonucleotide-based approaches to target aberrant splicing choices mediated by mutant splicing factors or specific mRNAs encoding aberrant isoforms, such as the *CASP8 TR* isoform, may be another therapeutic avenue for targeting cells bearing RNA splicing factor mutations.

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Author Contributions

S.C.-W.L., K.N., E.K., R.K.B. and O.A.-W. designed the study. S.C.-W.L., K.N., E.K., E.J., S.X.L., B.L., D.I., M.K., M.Y., H.C., and Y.J.K. performed experiments. X.Z., M.K.K., Y.R.C. provided technical assistance. S.C.-W.L., K.N., E.K., E.J., S.X.L., B.L., D.I. and A.P. analyzed data. B.H.D. and S.M. analyzed histology data. K.N., J.P., M.S., S.B., P.G.S. and R.K.B. performed RNA-seq analysis. A.Y. and J.T. provided critical feedback and discussion, including reviewing and editing the manuscript. E.O. and B.L.E. provided the *Sf3b1*K700E mutant mouse. S.C.-W.L., K.N., R.K.B. and O.A.-W. prepared the manuscript with help from all co-authors.

Competing financial interests

J.P., M.S., S.B., and P.G.S. are employees of H3 Biomedicine, Inc.

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Figure Legends

Figure 1. Simultaneous expression of cancer-associated mutations in *Srsf2* and *Sf3b1* or expression of a single RNA splicing factor mutation in the homozygous state is incompatible with hematopoiesis. (A) Heatmap representation of the four most commonly mutated genes encoding pre-mRNA splicing factors across 11 sequencing studies in patients with myeloid malignancies (Bejar et al., 2012; Damm et al., 2012; Haferlach et al., 2014; Lasho et al., 2012; Makishima et al., 2012; Meggendorfer et al., 2012; Papaemmanuil et al., 2013; Patnaik et al., 2013; Thol et al., 2012; Yoshida et al., 2011; Zhang et al., 2012). Each column represents a single patient, and each colored bar represents the presence of the specified mutation in an RNA splicing factor. **(B)** Experimental schema of competitive and noncompetitive bone marrow transplantation (BMT) experiments using bone marrow mononuclear cells (BM MNCs) from 8-12 week old *Mx1-Cre* wild-type (WT), *Mx1-Cre Srsf2*^{P95H/+}, *Mx1-Cre Sf3b1*^{K700E/+}, *Mx1-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+}, *Mx1-Cre Srsf2*^{P95H/P95H} and *Mx1-Cre Srsf2*^{P95H/fl} mice. Polyinosinic-polycytidylic acid (plpC) was administered to recipient mice 4-weeks post-transplantation to induce expression of mutant alleles. **(C)** Percentage of CD45.2⁺ (donor-derived) chimerism in the peripheral blood of CD45.1 recipient mice (n=8-10 mice per genotype) in noncompetitive BM transplantation. **(D)** Representative FACS plots of CD45.2⁺ (donor-derived) cells in the peripheral blood of CD45.1 recipient mice 52 weeks post plpC administration. **(E)** Percentage of CD45.2⁺ (donor-derived) chimerism in the peripheral blood of CD45.1 recipient mice (n=10 mice per genotype) in competitive BM transplantation from *Mx1-Cre* WT, *Mx1-Cre Srsf2*^{P95H/+}, *Mx1-Cre Sf3b1*^{K700E/+}, *Mx1-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} mice. **(F)** Analysis of CD45.2 chimerism in the BM, spleen, thymus and blood 20 weeks post-plpC administration. **(G)** Percentage of CD45.2⁺ (donor-derived) chimerism in the peripheral blood of CD45.1 recipient mice (n= 5-10 mice per genotype) in competitive BM transplantation from *Mx1-Cre Srsf2*^{+/+}, *Mx1-Cre Srsf2*^{P95H/+}, *Mx1-Cre Srsf2*^{P95H/P95H} and *Mx1-Cre Srsf2*^{P95H/fl} mice. **(H)** Analysis of CD45.2 chimerism in the BM, spleen, thymus, and blood 20 weeks post plpC administration. Error bars represent means ± standard deviation. Analysis of variance followed by Tukey's post-hoc test was used to compare differences between groups, and each *p*-value shown has

been adjusted to account for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus WT group; ^ $p < 0.05$, ^^ $p < 0.01$, ^^^ $p < 0.001$ versus *Mx1-Cre Sf3b1*^{K700E/+} mice; ## $p < 0.01$, ### $p < 0.001$ versus *Mx1-Cre Srsf2*^{P95H/+} mice. See also **Figure S1-2** and **Table S1**.

Figure 2. Combined expression of mutations in *Srsf2* and *Sf3b1* results in hematopoietic stem and progenitor cell apoptosis and loss of quiescence. Percentage of (A) Bromodeoxyuridine⁺ (BrdU⁺) or (B) Annexin-V⁺ PI⁻ lineage-negative c-Kit⁺ Sca-1⁺ (LSK) cells from *Mx1-Cre* wild-type (WT), *Mx1-Cre Srsf2*^{P95H/+}, *Mx1-Cre Sf3b1*^{K700E/+} and *Mx1-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} mice 2-weeks following plpC administration *in vivo*. (C) Number of mice present at weaning (Day 14) per genotype from crossing *Sf3b1*^{K700E/+} x *Vav-Cre Srsf2*^{P95H/+} or *Srsf2*^{P95H/+} x *Vav-Cre Sf3b1*^{K700E/+} mice. A two-sided Chi-square test was used to determine statistical significance (** $p = 0.0064$). Percentage of phenotypic (D) LSK and (E) long-term hematopoietic stem cells (LT-HSC; LSK CD150⁺ CD48⁻) in embryonic day 14.5 (E14.5) fetal livers from *Vav-Cre Srsf2*^{+/+} *Sf3b1*^{+/+} wild-type (WT), *Vav-Cre Srsf2*^{P95H/+} (P95H), *Vav-Cre Sf3b1*^{K700E/+} (K700E) and *Vav-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} double knock-in (DKI) fetuses and from *Vav-Cre*-negative controls. (F) Numbers of colonies formed from E14.5 fetal liver cells from WT, P95H, K700E, and DKI fetuses and from *Vav-Cre*-negative controls. (G) Representative flow cytometric plots and quantitation of (H) BrdU⁺ and (I) Annexin V⁺ PI⁻ LSK cells from *Vav-Cre* WT, *Vav-Cre Srsf2*^{P95H/+} (P95H), *Vav-Cre Sf3b1*^{K700E/+} (K700E) and *Vav-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} (DKI) fetuses. Error bars represent means $\pm$ standard deviation. Analysis of variance followed by Tukey's post-hoc test was used to compare differences between groups, and each p -value shown has been adjusted to account for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. See also **Figure S3**.

Figure 3. *Srsf2* and *Sf3b1* mutations have distinct and independent effects on gene dysregulation. (A) Experimental schema of sample collection for RNA-seq performed in biological triplicate. (B) Expression of *Srsf2*^{P95H} and *Sf3b1*^{K700E} alleles as percentage of mRNAs expressed from *Srsf2* and *Sf3b1*. Color indicates genotype; the three biological replicates are A-C from left to right. (C) Scatter plots comparing coding gene expression in *Mx1-Cre Srsf2*^{P95H/+}, *Mx1-Cre Sf3b1*^{K700E/+}, and *Mx1-*

Cre *Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} cells relative to *Mx1-Cre Srsf2*^{+/+} *Sf3b1*^{+/+} wild-type (WT) cells for replicate B. Red and blue indicate coding genes that were significantly up- or down-regulated, respectively, in mutant relative to wild-type cells. TPM, transcripts per million (TMM-normalized). **(D)** Bar plots comparing percentage of significantly dysregulated coding genes (% of total coding genes expressed) in *Mx1-Cre Srsf2*^{P95H/+}, *Mx1-Cre Sf3b1*^{K700E/+}, and *Mx1-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} cells relative to *Mx1-Cre Srsf2*^{+/+} *Sf3b1*^{+/+} WT cells for replicate B. A two sided binomial proportion test was used to compare differences between groups. *** $p < 0.0001$. **(E)** Venn diagram showing the overlap between coding genes that were significantly dysregulated in *Mx1-Cre Srsf2*^{P95H/+}, *Mx1-Cre Sf3b1*^{K700E/+}, and *Mx1-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} cells relative to *Mx1-Cre Srsf2*^{+/+} *Sf3b1*^{+/+} WT cells for replicate B. **(F)** Gene Ontology (GO) enrichment analysis of *Mx1-Cre Srsf2*^{P95H/+}, *Mx1-Cre Sf3b1*^{K700E/+}, and *Mx1-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} cells relative to wild-type *Mx1-Cre Srsf2*^{+/+} *Sf3b1*^{+/+} WT cells for replicate B. Circle size indicates the magnitude of the p -value for each term and genotype comparison. See also **Figure S4** and **Table S2-3**.

Figure 4. *Srsf2* and *Sf3b1* mutations have distinct and independent effects on RNA splicing.

(A) Scatter plots of cassette exon inclusion in *Mx1-Cre Srsf2*^{P95H/+}, *Mx1-Cre Sf3b1*^{K700E/+}, and *Mx1-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} cells relative to *Mx1-Cre Srsf2*^{+/+} *Sf3b1*^{+/+} WT cells for replicate B. Axes indicate the fraction of mRNAs containing each cassette exon in the indicated sample. Red and blue indicate cassette exons whose inclusion is promoted or repressed, respectively, in mutant relative to WT cells. **(B)** As (A), but for competing/alternative 3' splice site events. Axes indicate the fraction of mRNAs that use the intron-proximal 3' splice site in the indicated sample. Red and blue indicate intron-proximal 3' splice sites whose usage is promoted or repressed, respectively, in mutant relative to wild-type cells. **(C)** Plots illustrating the spatial distribution of the CCNG and GGNG (N = any nucleotide) exonic splicing enhancers adjacent to differentially spliced cassette exons that are promoted versus repressed in mutant relative to wild-type cells. Vertical axis indicates the relative frequency of each motif, averaged over all promoted versus repressed cassette exons for the indicated genotype comparisons. **(D)** Expression of *Srsf2*^{P95H} and *Sf3b1*^{K700E} alleles as percentage of mRNAs expressed from *Srsf2* and

Sf3b1 in lineage-negative (Lin⁻) c-Kit⁺ cells from fetal livers of *Vav*-Cre mice at embryonic day 14.5 (E14.5). Color indicates genotype; the three biological replicates are A-C from left to right. **(E)** Scatter plots comparing mean coding gene expression for all replicates in *Vav*-Cre *Srsf2*^{P95H/+}, *Vav*-Cre *Sf3b1*^{K700E/+}, and *Vav*-Cre *Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} cells relative to *Vav*-Cre *Srsf2*^{+/+} *Sf3b1*^{+/+} WT cells. Red and blue indicate coding genes that were significantly up- or down-regulated, respectively, in mutant relative to WT cells. TPM, transcripts per million (TMM-normalized). **(F)** Venn diagram showing the overlap between coding genes that were significantly dysregulated in *Vav*-Cre *Srsf2*^{P95H/+}, *Vav*-Cre *Sf3b1*^{K700E/+}, and *Vav*-Cre *Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} cells relative to *Vav*-Cre *Srsf2*^{+/+} *Sf3b1*^{+/+} WT cells in all replicates. See also **Figures S4-5** and **Table S4-5**.

Figure 5. Co-expression of *Srsf2* and *Sf3b1* mutations results in aberrant splicing and impaired expression of key regulators of hematopoietic stem cell survival and quiescence. **(A)** Venn diagram of the overlap between genes that were differentially expressed and spliced in *Mx1*-Cre *Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} cells relative to *Mx1*-Cre *Srsf2*^{+/+} *Sf3b1*^{+/+} wild-type (WT) cells in any replicate. **(B)** Relative expression by quantitative RT-PCR of *Mpl*, and *Itga2b* and *Pbx1* in purified lineage-negative c-Kit⁺ (LK) cells from *Mx1*-Cre *Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} mice relative to other control groups (n = 8-10 mice per genotype). **(C)** Gene Ontology enrichment analysis of mouse hematopoietic stem and progenitor cells from *Mx1*-Cre mice revealed that differentially expressed genes were enriched for innate immune and inflammatory pathways in *Sf3b1*^{K700E/+}, *Srsf2*^{P95H/+} and *Sf3b1*^{K700E/+} *Srsf2*^{P95H/+} cells relative to WT control. **(D)** Immunofluorescence analysis of nuclear phosphorylated p65 (phospho-p65) in LK cells from *Vav*-Cre *Srsf2*^{+/+} *Sf3b1*^{+/+} WT, *Vav*-Cre *Srsf2*^{P95H/+}, *Vav*-Cre *Sf3b1*^{K700E/+}, and *Vav*-Cre *Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} mice *ex vivo*. Scale bar: 10 μm. **(E)** Violin plots showing quantitation of phospho-p65 intensity in the nucleus of LK cells from (D). Analysis of variance using Kruskal-Wallis ranked test was performed. Multiple pair-wise comparisons were adjusted by false-discovery rate. **(F)** Experimental schema of competitive (1:10) bone marrow (BM) transplantation experiments using bone marrow mononuclear cells from *Vav*-Cre WT, *Vav*-Cre *Srsf2*^{P95H/+} and *Vav*-Cre *Sf3b1*^{K700E/+} mice that were subjected to chronic LPS treatment (1 mg/kg; every second day for 30 days). **(G)** Percentage of

CD45.2⁺ (donor-derived) chimerism in the peripheral blood of CD45.1 recipient mice (n=4-5 mice per group) from primary and secondary competitive BM transplantation. Donor mice were treated with either vehicle (PBS) or LPS (1 mg/kg) outlined in (F). **(H)** Chimerism in BM lineage⁻ Sca-1⁺ c-Kit⁺ (LSK) or myeloid progenitor (MP; lineage⁻ Sca-1⁻ c-Kit⁺) fractions from primary transplant recipients 14-weeks post BM transplantation. **(I)** Kaplan-Meier survival analysis of *Mx1-Cre* WT, *Mx1-Cre Srsf2*^{P95H/+} and *Mx1-Cre Sf3b1*^{K700E/+} mice following a single dose of LPS (15 mg/kg) *in vivo*. Log-ranked Mantel-Cox test was used to compare median survival difference. ** *p*=0.0055. Analysis of variance followed by Tukey's post-hoc test was used to compare differences between groups, and each *p*-value shown has been adjusted to account for multiple comparisons. * *p*<0.05, ** *p*<0.01, *** *p*<0.001, **** *p*<0.0001 versus wild-type (“#” is versus *Srsf2*P95H and “^” is versus *Sf3b1*K700E). See also **Figure S5** and **Table S6**.

Figure 6. *SF3B1* mutations promote mis-splicing of *MAP3K7*, resulting in hyperactivation of NF- κ B signaling. **(A)** Venn diagram illustrating the overlap of differentially spliced genes across MDS patient samples that were mutant versus wild-type (WT) for *SF3B1* and murine bone marrow hematopoietic progenitors (lineage⁻ c-Kit⁺) in *Sf3b1*^{K700E/+} versus *Sf3b1*^{+/+} mice. **(B)** From top to bottom, exon-intron structure of *MAP3K7*, conservation of mouse and human sequences adjacent to the competing 3' splice site affected by *SF3B1* mutations, and RNA-seq coverage plots of this region in human and mouse samples with or without *SF3B1* mutations. **(C)** RT-PCR of the *MAP3K7* competing 3' splice site in primary patient MDS and CLL samples with or without *SF3B1* mutations as well as in isogenic human and mouse cells. Immunoblot analysis of phosphorylated p65 (phospho-p65), I κ B- α , MAP3K7 (using a C-terminal anti-MAP3K7 antibody), and loading controls in isogenic **(D)** K562 and **(E)** NALM-6 leukemia cells expressing WT or mutant *SF3B1* from its endogenous locus (“Time (h)” refers to hours following LPS (5 μ g/mL) exposure). Immunoblot analysis of phospho-p65, MAP3K7, and loading controls in **(F)** bone marrow mononuclear cells (MNCs) from MDS patients with or without *SF3B1* mutation and **(G)** peripheral blood MNCs from CLL patients with or without *SF3B1* mutation. See also **Figure S6** and **Table S7**.

Figure 7. MAP3K7 loss is responsible for hyperactivated NF-κB signaling in SF3B1-mutant cells.

(A) Immunofluorescence analysis of phosphorylated p65 (phospho-p65) in K562 cells with or without *SF3B1*K700E mutation following stimulation with lipopolysaccharide (LPS). Quantitation of phospho-p65 intensity is shown on the right. Scale bar: 10 μm. **(B)** Heatmap representation of NF-κB luciferase reporter signal in SF3B1 mutant knockin NALM-6 cells (left two panels) or parental NALM-6 cells with anti-*MAP3K7* shRNAs (right two panels) following stimulation with LPS or TNFα. **(C)** Quantitative RT-PCR (qRT-PCR) analysis of NF-κB target genes IL-1β and TNF 8 hours post LPS stimulation in NALM-6 *SF3B1*-isogenic cells (two independent experiments performed, each in technical triplicate). **(D)** Immunoblot analysis of phospho-p65 in isogenic K562 cells with and without *SF3B1*K700E knockin and with and without Flag-*MAP3K7* cDNA expression and/or LPS (5 μg/mL) exposure. **(E)** Quantitation of NF-κB luciferase reporter signal in cells from (D; from a biological replicate of three independent experiments). **(F)** Immunofluorescence analysis of nuclear phospho-p65, Flag, and DAPI in cells from (D; quantitation of phospho-p65 intensity is shown on the right). Scale bar: 10 μm. Error bars represent means ± standard deviation. Analysis of variance followed by Tukey's post-hoc test was used to compare differences between groups, and each *p*-value shown has been adjusted to account for multiple comparisons. * *p*<0.05, ** *p*<0.005, *** *p*<0.0002, **** *p*<0.0001. See also **Figure S6**.

Figure 8. SRSF2 mutations promote aberrant splicing of caspase-8 (CASP8), resulting in

expression of a truncated protein that hyperactivates NF-κB signaling. **(A)** RNA-seq coverage

plots of *CASP8* splicing in chronic myelomonocytic leukemia (CMML) and acute myeloid leukemia (AML) patients wild-type (WT) or mutant (MUT) for *SRSF2*. *SRSF2* mutations repress the illustrated *CASP8* cassette exon. AML and CMML RNA-seq data are from Kim *et al* (Kim et al., 2015). **(B)** RT-

PCR analysis of the *CASP8* splicing event in human leukemia cell lines WT (TF1 and K562) or mutant (K052) for *SRSF2* and a diagram of the corresponding *CASP8* isoforms. Immunoblot analysis of

caspase-8 using an N-terminal anti-caspase-8 antibody in **(C)** isogenic K562 cells with or without

*SRSF2*P95H mutation expressed from the endogenous *SRSF2* locus, or **(D)** human leukemia cells (left panel) and primary AML patient samples (right panel) wild-type or mutant for *SRSF2*. **(E)** Immunoblot analysis of phosphorylated p65 (phospho-p65), I κ B- α , Caspase-8, and loading controls in K562 cells expressing cDNA constructs encoding empty vector (EV), full-length *CASP8* (*CASP8^{FL}*), or the aberrantly spliced *caspase-8* truncated isoform (*CASP8^{TR}*) following exposure to TRAIL (50 ng/mL). **(F)** Heatmap representation of NF- κ B luciferase reporter signal following TRAIL stimulation in K562, *CASP8* WT HAP1, or *CASP8* KO HAP1 cells expressing cDNA constructs encoding empty vector, full-length *caspase-8* (*CASP8^{FL}*), or the aberrantly spliced *caspase-8* truncated isoform (*CASP8^{TR}*). **(G)** Immunoblot analysis of phospho-p65, I κ B- α , and loading controls in K562 cells with or without *SRSF2*P95H mutation expressed from the endogenous *SRSF2* locus following exposure to TRAIL (50 ng/mL). See also **Figures S7-8**.

STAR Methods

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Omar Abdel-Wahab (abdelwao@mskcc.org).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

All animals were housed at Memorial Sloan Kettering Cancer Center (MSKCC). All animal procedures were completed in accordance with the Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committees at MSKCC. Generation and genotyping of the *Srsf2*^{P95H/+}, *Sf3b1*^{K700E/+} and *Srsf2*^{fl/+} as well as the *Mx1*-Cre and *Vav*-Cre transgenic mice have been previously described (Kim et al., 2015; Obeng et al., 2016). 8-week-old female CD45.1 C57BL/6J mice (The Jackson Laboratory) were used as recipients for bone marrow transplantation assays.

Primary human MDS and CLL samples

Studies were approved by the Institutional Review Boards of Memorial Sloan Kettering Cancer Center and conducted in accordance to the Declaration of Helsinki protocol. Primary human de-identified MDS and CLL samples derived from whole peripheral blood or BM mononuclear cells were utilized.

Cell lines

The NALM-6 isogenic cell lines (NALM-6 cells engineered to express the single mutations *SF3B1*K700E or *SF3B1*K700K from the endogenous locus) were cultured in RPMI/10% FCS and K562 isogenic cell lines (engineered to express *SF3B1*K700E or *SRSF2*P95H mutations from each respective endogenous locus) were cultured in IMDM/10% FCS. HAP1 and *CASP8*-KO HAP1 cells (obtained from Horizon Discovery) were cultured in IMDM/10% FCS.

METHOD DETAILS

Peripheral blood analysis

Blood was collected by submandibular bleeding using heparinized microhematocrit capillary tubes (Thermo Fisher Scientific). Automated peripheral blood counts were obtained using a ProCyt Dx Hematology Analyzer (IDEXX).

Bone marrow transplantation assays

Primary mouse bone marrow (BM) cells were isolated from *Mx1-Cre*, *Mx1-Cre Srsf2*^{P95H/+}, *Mx1-Cre Sf3b1*^{K700E/+}, *Mx1-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+}, *Mx1-Cre Srsf2*^{P95H/P95H} or *Mx1-Cre Srsf2*^{P95H/fl} mice (aged 8-12 weeks) into cold phosphate-buffered saline (PBS), without Ca²⁺ and Mg²⁺, and supplemented with 2% bovine serum albumin (BSA) to generate single cell suspensions. Red blood cells (RBCs) were removed using ammonium chloride-potassium bicarbonate (ACK) lysis buffer, resuspended in PBS/2% BSA, and filtered through a 40µm cell strainer. Total nucleated cells were quantified by the Vi-Cell XR cell counter (Beckman Coulter). For competitive transplantation experiments, a total of 1.8 x 10⁶ BM cells from donor mice (*Mx1-Cre*, *Mx1-Cre Srsf2*^{P95H/+}, *Mx1-Cre Sf3b1*^{K700E/+}, *Mx1-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+}, *Mx1-Cre Srsf2*^{P95H/P95H}, *Mx1-Cre Srsf2*^{P95H/fl}, *Vav-Cre* WT, *Vav-Cre Srsf2*^{P95H/+}, and *Vav-Cre Sf3b1*^{K700E/+} CD45.2⁺ mice were mixed with 0.2 x 10⁶ wild-type CD45.1⁺ BM and transplanted via tail vein injection into 8-week old lethally irradiated (2x 450 cGy) CD45.1⁺ recipient mice. For noncompetitive transplantation experiments, 2 x 10⁶ total BM cells from *Mx1-Cre*, *Mx1-Cre Srsf2*^{P95H/+}, *Mx1-Cre Sf3b1*^{K700E/+}, or *Mx1-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} mice were injected into lethally irradiated (2 x 450 cGy) CD45.1⁺ recipient mice. To induce the conditional alleles on *Mx1-Cre* background, mice were treated with 3 doses of polyinosinic:polycytidylic acid (plpC; 12mg/kg/day; GE Healthcare) every other day via intra-peritoneal injection. Peripheral blood chimerism of mature blood cell lineages was assessed routinely by flow cytometry.

In vivo LPS stimulation experiment

For *in vivo* LPS stimulation, *Escherichia coli* 055:B5 LPS (Sigma Aldrich) was used. For chronic LPS exposure, Vav-Cre WT, Vav-Cre *Srsf2*^{P95H/+} and Vav-Cre *Sf3b1*^{K700E/+} received intra-peritoneal injection of LPS (1 mg/kg) every other day for 30 days. For acute LPS exposure, *Mx1*-Cre wild-type (WT), *Mx1*-Cre *Srsf2*^{P95H/+} and *Mx1*-Cre *Sf3b1*^{K700E/+} mice were given a single dose of LPS (15 mg/kg) via intra-peritoneal injection.

***In vitro* colony-forming assays**

Single-cell suspension was prepared from E14.5 fetal livers, and 25,000 cells from each embryo were plated in duplicates in cytokine-supplemented methylcellulose medium (MethoCult™ GF M3434; StemCell Technologies), and colonies were enumerated 10-14 days later. To assess the effect of shRNA-mediated knockdown of target genes, 8-12 week-old C57BL/6 male mice were treated with 5-fluorouracil (150 mg/kg) via intra-peritoneal injection. Five days after injection, BM cells were harvested from the legs (femora and tibiae) and hip bones, and lineage-depletion was performed with biotin-conjugated antibodies against B220 (RA3-6B2), CD19 (1D3), CD3 (17A2), CD4 (GK1.5), CD8a (53-6.7), Gr-1 (RB6-8C5), Mac-1 (M1/70), NK1.1 (PK136) and Ter119, labeled with anti-biotin MicroBeads (130-090-485; Miltenyi Biotec), and lineage-negative (Lin⁻) cells were magnetically separated using MACS columns according to the manufacturer's instructions. Lin⁻ BM cells were cultured overnight in IMDM/10% FCS supplemented with mIL-3 (10 ng/mL), mIL-6 (10 ng/mL) and mSCF (50 ng/mL). The next day, cells were subjected to spinfection (2,700 rpm for 1 hour) with retroviral supernatants containing shRNAs or cDNAs of interests in the presence of polybrene (5 µg/mL). 24 hours after spinfection, cells that were successfully infected with retrovirus were marked with GFP, and were purified by flow cytometry. FACS-sorted cells were cultured in cytokine-supplemented methylcellulose medium (MethoCult™ GF M3434; StemCell Technologies), and colonies were enumerated 10-14 days later.

Flow cytometry analyses

Cells were incubated with antibodies in PBS/2% BSA (without Ca^{2+} and Mg^{2+}) for 30-45 minutes on ice. For hematopoietic stem and progenitor cell analysis from adult mouse bone marrow, cells were stained with a lineage cocktail of monoclonal antibodies including B220 (RA3-6B2), CD19 (1D3), CD3 (17A2), CD4 (GK1.5), CD8a (53-6.7), Gr-1 (RB6-8C5), Mac-1 (M1/70), NK1.1 (PK136) and Ter119, allowing for mature lineage exclusion from the analysis. For fetal liver analysis, Mac-1 (M1/70) was excluded from the lineage-depletion cocktail. Cells were also stained with antibodies against c-Kit (2B8), Sca-1 (D7), FcγRII/III (93), CD34 (RAM34), CD45.1 (A20), CD45.2 (104), CD48 (HM48-1) and CD150 (9D1). DAPI was used to exclude dead cells. The composition of mature hematopoietic cell lineages in the bone marrow, spleen, thymus and peripheral blood was assessed using a combination of antibodies against Mac-1, Gr-1, B220, CD19, CD3, CD4, CD8a, CD25 (PC61.5), CD44 (IM7), IgM (II/41), CD43 (S11). All FACS sorting was performed on FACS Aria, and analysis was performed on an LSR Fortessa (BD Biosciences). Data analysis was performed using the FlowJo software.

Cell cycle and apoptosis analyses

For apoptosis assays, freshly harvested bone marrow or fetal liver cells were first stained with antibodies against cell surface markers of interests, and then stained with FITC-conjugated Annexin V in Annexin V binding buffer (BD Pharmingen) according to manufacturer instructions. For assessment of cell cycle status in adult bone marrow HSPCs, BrdU (1 mg/kg) was administered via intra-peritoneal injection to adult mice 48 hours prior to sacrifice. For cell cycle analysis of E14.5 fetal HSPCs, BrdU (1 mg/kg) was administered to pregnant mice via intra-peritoneal injection 3 hours prior to harvesting fetal livers. Assessment of BrdU incorporation was performed following manufacturer instructions (BD Pharmingen) and data was acquired on a LSR Fortessa (BD Biosciences).

Histological analysis

Tissues, embryos and pups were fixed in 4% paraformaldehyde, processed routinely in alcohol and xylene, embedded in paraffin, sectioned at 5-micron thickness, and stained with hematoxylin-eosin (H&E). Multiple sections were obtained through the head in the coronal plane, trunk in the transverse

plane, and fore and hind limbs in the longitudinal plane. Immunohistochemistry (IHC) was performed on a Leica Bond RX automated stainer (Leica Biosystems, Buffalo Grove, IL). Following HIER at pH 6.0, the primary antibody against mouse CD45 (BD Pharmingen; 550539), myeloperoxidase (Dako; A0398), and cleaved caspase-3 (Cell Signaling; 9661) were applied at a concentration of 1:250, 1:1000, and 1:250 respectively, followed by application of a polymer detection system (DS9800, Novocastra Bond Polymer Refine Detection, Leica Biosystems) in which the chromogen was 3,3 diaminobenzidine tetrachloride (DAB) and counterstain was hematoxylin. For quantification of cleaved caspase-3 by image analysis, whole slide digital images were generated on a slide scanner (Pannoramic 250 Flash III, 3DHistech, 20x/0.8NA objective, Budapest, Hungary) at a resolution of 0.2431 μm per pixel. Staining quantification was performed with QuPath 0.1.2 software (Centre for Cancer Research & Cell Biology, Queen's University Belfast, UK). The region of interest (ROI) was defined as the liver parenchyma. The number of DAB positive cells per mm^2 was measured with the positive cell detection algorithm. ROI selection and algorithm optimization and validation, and qualitative examination of all H&E and IHC slides were performed by a board-certified veterinary pathologist (S.M.).

Immunoblot

For immunoblotting, the following antibodies were used: NF- κ B/p65 (CST; 8242), phospho NF- κ B/p65-Ser536 (CST; 3033), I κ B- α (CST; 9242), TAK1/MAP3K7 (CST; 5206), Caspase-8, N-terminal (Abcam; clone E6), Caspase-8, C-terminal (Enzo Life Science; clone 12F5), Flag (Sigma-Aldrich; F-1804), β -actin (Sigma-Aldrich; A-5441).

Immunofluorescence

Following stimulation with LPS (Sigma-Aldrich), TNF α (PeproTech), or TRAIL (Enzo Life Science), cells were fixed with 4% paraformaldehyde/PBS for 10 minutes at room temperature (RT), permeabilized with PBS-T (PBS/1% BSA/0.2% Triton-X) for 15 minutes, blocked with PBS-T/5% goat serum (ThermoFisher PCN500) for 1 hour at RT, and incubated with primary antibodies (1:50 dilution for phospho-p65 and 1:100 for Flag) in PBS-T/5% goat serum overnight at 4°C. Cells were washed three

times with PBS-T for 10 minutes at RT with gentle agitation, and were incubated with goat anti-rabbit Alexa Fluor 594 (ThermoFisher Scientific; A-11012) or goat anti-mouse Alexa Fluor 488 (ThermoFisher Scientific; A-11001) secondary antibodies (1:500 dilution in PBS-T/5/% normal goat serum) for 2 hours at RT in the dark. Cells were then washed twice with PBS-T for 10 minutes at RT, and counter-stained with DAPI (0.5 µg/mL; Sigma-Aldrich; D-9542) for 20 minutes at RT. Cells were coverslipped with ProLong Gold anti-fade (ThermoFisher Scientific; P36930). Images were captured using either a confocal microscope (Leica TCS SP5, Upright; Leica Microsystems), or were digitally scanned with Panoramic Confocal Scanner (3DHistech, Budapest Hungary) using a 20x/0.8NA objective. The projected images were exported into tif format using CaseViewer software (3DHistech) and analyzed with ImageJ/FIJI. A macro was written that segments each nucleus using the DAPI channel and measures the nuclear phospho-p65 intensity within the nucleus after appropriate threshold was set.

Caspase 8 and MAP3K7 constructs

MSCV-Flag-CASP8^{FL}-IRES-GFP, MSCV-Flag-CASP8^{TR}-IRES-GFP, MSCV-Flag-hMAK3K7-IRES-GFP, MSCV-Flag-mMap3k7-IRES-GFP and MSCV-IRES-GFP empty vector constructs were used for overexpression studies. Retroviral supernatants were produced by transfecting 293 GP11 cells with cDNA constructs and the packaging plasmid VSV.G using XtremeGene9 (Roche), and were used to transduce HAP1, CASP8 KO HAP1 and K562 parental and K562 SF3B1K700E isogenic cells in the presence of polybrene (5 µg/mL; Millipore). Successfully transduced cells expressing GFP were purified by flow cytometry. Cells were stimulated with LPS (Sigma-Aldrich), TNFα (PeproTech) or TRAIL (Enzo Life Science).

mRNA stability assay

For mRNA half-life measurement using qRT-PCR, UPF1 shRNA and shControl lentivirus infected K562 SF3B1K700E cells were treated with 2.5 µg/ml Actinomycin D (Life Technologies) and harvested at 0, 2, 4, 6, and 8 hours (following protocols established previously (t Hoen et al., 2011)). MAP3K7 inclusion, MAP3K7 exclusion and 18s rRNA mRNA levels were measured by qRT-PCR.

Luciferase reporter assay

We generated K562, NALM-6 *SF3B1* isogenic cells, HAP1 and *CASP8* knockout (KO) HAP1 cells expressing the luciferase reporter for NF- κ B response elements by following the manufacturer instructions (Cignal™ Reporter Assay, Qiagen). Cells were stimulated with LPS, TNF α or TRAIL as described above, and NF- κ B activity was assessed by luciferase intensity using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer instructions. To verify that the luciferase reporter assay was not aberrantly activated by basal leakiness, we used another NF- κ B reporter plasmids (Promega; N1111) with known NF- κ B response elements (RE), and performed mutagenesis in the NF- κ B-RE using the QuiKChange II Site-Directed Mutagenesis Kit (Agilent Technologies; #200522). There are five putative NF- κ B-RE binding sites in this reporter plasmid (5'-GGGRNTTTC-3', where R is a purine, Y is a pyrimidine and N is any nucleotide). To mutagenize the binding sequence, the "TTTC" sequence was mutated to "AAAA". The primers used to create the two mutant plasmids are:

Mutant-Fwd:

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GGTACCTGAGCTCGCTAGCGGGAACGCGGGACAAAACGCGGGAACGCGGGACAAAACGCGG  
AAAAACAGATCTGGCCTCGGCGGCCAAGCTTA
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Mutant-Rev:

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TAAGCTTGGCCGCCGAGGCCAGATCTGTTTTTCCCGTTTTGTCCCGTTTTTCCCGTTTTGTCC  
CCGTTTTTCCCGCTAGCGAGCTCAGGTACC
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RT-PCR and quantitative RT-PCR (qRT-PCR)

Total RNA was isolated using RNeasy Mini kit (Qiagen). For cDNA synthesis, total RNA was reverse transcribed to cDNA with SuperScript VILO cDNA synthesis kit (Life Technologies). The resulting cDNA was diluted 10-20 fold prior to use. Quantitative RT-PCR (qRT-PCR) was performed in 10 μ L reactions

with either SYBR Green PCR Master Mix or Taqman Gene Expression Master Mix with AmpErase (ThermoFisher Scientific). All qRT-PCR analysis was performed on an Applied Biosystems QuantStudio 6 Flex Cyclor (ThermoFisher Scientific). Relative gene expression levels were calculated using the comparative CT method.

Primers used in RT-PCR reactions were:

MAP3K7 (human) – Fwd: GATGGAATATGCTGAAGGGG, Rev: CACTCCTTGGGAACACTGTA

Map3k7 (mouse) – Fwd: GATGGAATATGCAGAGGGGG, Rev: CACTCCTTGGGAACACTGTA

CASP8 (human) – Fwd: GAACTTCAGACACCAGGC, Rev: CTTTGTCCAAAGTCTTTGCTG

Primers used in qRT-PCR reactions were:

CASP8 exclusion isoform (aberrant):

Fwd: GATGAATTTTCAAATGACTTTGGAC

Rev: TGATCAGACAGTATCCCCGAG

CASP8 inclusion isoform (canonical):

Fwd: TGATGAATTTTCAAATGGGGAGGA

Rev: ATCCTGTTCTCTTGGAGAGTCC

MAP3K7 mRNA half-life experiment (human):

Fwd (common): GCGTTTATTGTAGAGCTTCGG

Rev (canonical): GCACCATGCAGCACATTATATAAAG

Rev (aberrant): CATGCAGCACTGCGAAAGAAAG

Taqman probes were used for gene expression analysis of *TNF* (Hs00174128_m1), *IL-1 β* (Hs01555410_m1), *GAPDH* (Hs02786624_g1), *Mpl* (Mm00440310_m1), *Pbx1* (Mm04207617_m1), *Itga2b* (Mm00439741_m1), and *Hprt* (Mm03024075_m1).

shRNA experiments

NALM-6 parental cells carrying the NF- κ B luciferase reporter were transduced with a doxycycline-inducible lentiviral vector, T3G-dsRED-mirE-PGK-Neo-IRES-rtTA3 (Fellmann et al., 2013), expressing shRNAs for *MAP3K7* or a non-targeting renilla or firefly luciferase control. Transduced cells were selected with G418 (0.5 mg/mL; ThermoFisher Scientific), and the short hairpins were induced with the addition of doxycycline (2.0 μ g/mL; Sigma Aldrich). All mouse shRNAs used in clonogenic assays were cloned into the retroviral pMSCV-LTR-mirE-PGK-SV40-IRES-GFP (MLS-E) backbone (Fellmann et al., 2013). All shRNAs were designed using the SplashRNA algorithm (Pelossof et al., 2017). The short hairpin sequences are:

sh.*MAP3K7*.748: TTAGGTAAATTTTTTATCAGTG

sh.*MAP3K7*.1041: TTTTCAACAATTTTGATTCTAA

sh.Luciferase control: TTAATCAGAGACTTCAGGCGGT

sh.*Ren*.713 control: CAGGAATTATAATGCTTATCTA

sh.*Mpl*.2121: TTATATAATAAACAGTGTCTAA

sh.*Mpl*.2368: TCAAATAAATAGATGACAGCAA

sh.*Pbx1*.824: TTCATCCAAACTCTGGTCTGTG

sh.*Pbx1*.1393: TCATTGAGAATTTCTGTGGCTT

sh.*Itga2b*.2279: TTCTCTTTCTTCTGAGTGCAGA

sh.*Itga2b*.3380: TTAGGAAAAGGGATGCACCCGG

sh.*UPF1*(TRCN0000022254): CCGGGCATCTTATTCTGGGTAATAACTCGAGTTATTACCCAGAATAA
GATGCTTTTT

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses

Statistical significance was determined by analysis of variance (ANOVA) after testing for normal distribution and equal variance, followed by Tukey's post-hoc test for multiple group comparisons. For

non-normally distributed data, non-parametric test (Kruskal-Wallis) was used, followed by multiple group comparisons using false-discovery rate (FDR). For Kaplan Meier survival analysis, Mantel-Cox log-ranked test was used to determine statistical significance. For offspring frequency analysis, a Chi-Square test was performed to test the difference between observed and expected frequencies from different genotypes. Data were plotted using GraphPad Prism 7 software as mean values, and error bars represent standard deviation unless otherwise stated.

mRNA isolation, sequencing, and analysis

RNA was extracted from sorted mouse cell populations using Qiagen RNeasy columns. Poly(A)-selected, unstranded Illumina libraries were prepared with a modified TruSeq protocol. 0.5X AMPure XP beads were added to the sample library to select for fragments <400 bp, followed by 1X beads to select for fragments >100 bp. These fragments were then amplified with PCR (15 cycles) and separated by gel electrophoresis (2% agarose). 300 bp DNA fragments were isolated and sequenced on the Illumina HiSeq 2000 (~100M 101 bp reads per sample).

Genome annotations

Genome annotations for the human (NCBI GRCh37/UCSC hg19) and mouse (NCBI GRCm38/UCSC mm10) genomes were created as previously described (Dvinge et al., 2014). Genome annotations from the Ensembl (Flicek et al., 2013) and UCSC (Meyer et al., 2013) databases were merged with splicing event annotations from MISO v2.0 (Katz et al., 2010). An additional annotation of all possible combinations of annotated 5' and 3' splice sites found in the merged annotation was created for read mapping. Constitutive introns were defined as those whose associated 5' and 3' splice sites were alternatively spliced in the UCSC annotation.

RNA-seq read mapping

RNA-seq reads were sequentially mapped to the transcriptome and genome as previously described (Dvinge et al., 2014). Reads were first mapped to the transcriptome using Bowtie v1.0.0 (Langmead et

al., 2009) and RSEM v1.2.4 (Li and Dewey, 2011). The resulting read alignments were then filtered to require that reads spanning splice junctions overlapped the flanking exons by at least six nt. The remaining unaligned reads were then mapped to the genome and splice junctions using TopHat v2.0.8b (Trapnell et al., 2009), where reads were only allowed to align to the splice junctions present in the file of all possible combinations of annotated 5' and 3' splice sites described above. The resulting read alignments were then merged with the output of RSEM's alignment to create a final file of aligned reads.

Differential gene expression analysis

Gene expression analysis was performed using the gene expression estimates computed by RSEM in units of transcripts per million (TPM). Those estimates were then further normalized using the TMM method (Robinson and Oshlack, 2010), with a reference set of all protein-coding genes. Differentially expressed genes were defined as those with an associated Bayes factor ≥ 100 (computed using Wagenmakers's Bayesian framework (Wagenmakers et al., 2010)) and an associated fold-change ≥ 1.5 .

Differential splicing analysis

Isoform ratios for annotated splicing events (cassette exons, competing 5' and 3' splice sites, and annotated retained introns) were calculated using MISO v2.0 (Katz et al., 2010). Splicing of constitutively spliced introns and junctions was quantified using only junction-spanning reads, as previously described (Hubert et al., 2013). Differential splicing in the murine data was identified by comparing samples from different genotypes for a single replicate in a pairwise fashion. The analysis was restricted to splicing events with at least 20 informative reads, where an informative read is defined as a read that distinguishes between isoforms. Differentially spliced events were defined as those with an associated Bayes factor was ≥ 5 (computed using Wagenmakers's Bayesian framework (Wagenmakers et al., 2010)) and absolute change in isoform ratio of $\geq 10\%$. Differential splicing in the human patient cohorts was identified using a group statistical test to identify differences between

patient samples with or without defined splicing factor mutations. Differentially spliced events were defined as those with an associated p -value ≤ 0.05 (computed using the Wilcoxon rank-sum test) and an absolute change in median per-group isoform ratio of $\geq 10\%$.

Gene Ontology (GO) enrichment analysis

GO enrichment analysis was performed using the GOrseq method (Young et al., 2010) to correct for sequencing depth biases. The background set of genes was defined as all protein-coding genes. False discovery rates were calculated using the Wallenius method and corrected using the Benjamini-Hochberg method. We restricted reporting of enriched terms to those with at least two ancestors and fewer than 500 associated genes.

Motif enrichment analysis and sequence logos

The relative enrichment of different ESEs was computed by comparing motif occurrence within and adjacent to cassette exons that were promoted versus repressed in cells or samples with versus without defined splicing factor mutations. These analyses were performed using all cassette exons that were differentially spliced in at least one mouse replicate for a given genotype comparison. 95% confidence intervals were calculated by bootstrapping with 500 resampling steps. Sequence logos centered on intron-proximal or intron-distal 3' splice sites were created using all competing 3' splice sites that were differentially spliced in at least one mouse replicate for a given genotype comparison. The analysis was restricted to events with canonical GT and AG dinucleotides at the 5' and 3' splice sites. These analyses relied upon the GenomicRanges package in Bioconductor (Huber et al., 2015).

Analysis of expected and observed gene expression convergence

Expected gene expression programs for *Mx1-Cre/Srsf2*^{P95H/+}/*Sf3b1*^{K700E/+} samples were calculated by computing the mean of individual gene expression levels between *Mx1-Cre/Srsf2*^{P95H/+} and *Mx1-Cre/Sf3b1*^{K700E/+} samples for each replicate. This method of computing the expected gene expression program was motivated by the assumption that mutations in *Srsf2* and *Sf3b1* have independent

consequences for individual gene expression. Gene dysregulation for the expected gene expression program was then calculated as described previously for the actual, observed gene expression values. The numbers of dysregulated genes that were convergent between the expected gene expression program and the two *Mx1-Cre/Srsf2*^{P95H/+} and *Mx1-Cre/Sf3b1*^{K700E/+} single mutants was then determined for each replicate, again using a method identical to that for the actual, observed gene expression values (**Figure S4A**). A one-sided binomial proportion test was then used to test whether there were more dysregulated genes that were observed than expected (**Figure S4B**).

DATA AND SOFTWARE AVAILABILITY

Publicly available RNA-seq data

FASTQ files from published RNA-seq studies of patients with MDS (Dolatshad et al., 2015) and CLL (Darman et al., 2015) were downloaded from GEO series GSE63569 and GSE72790.

Accession codes

Gene Expression Omnibus: All newly generated RNA-seq data are deposited into the GEO database (accession number GSE97452).

KEY RESOURCES TABLE

See separate document.

Table S1: Mutational analysis of *SF3B1*, *SRSF2*, *U2AF1*, and *ZRSR2* in patients with myelodysplastic syndromes and related myeloid neoplasms.

Table S2: Differentially expressed genes in spliceosomal mutant mouse hematopoietic progenitors.

Table S3: Gene Ontology (GO) terms significantly enriched in the comparison of genes differentially expressed in *Mx1-Cre Srsf2*^{P95H/+}, *Mx1-Cre Sf3b1*^{K700E/+}, and *Mx1-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} cells relative to wild-type *Mx1-Cre Srsf2*^{+/+} *Sf3b1*^{+/+} cells

Table S4: Percent spliced in (Psi) values for differentially spliced events across all replicates.

Table S5: Gene Ontology (GO) terms significantly enriched in the comparison of genes differentially expressed in *Vav-Cre Srsf2*^{P95H/+}, *Vav-Cre Sf3b1*^{K700E/+}, and *Vav-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} cells relative to wild-type *Vav-Cre Srsf2*^{+/+} *Sf3b1*^{+/+} cells

Table S6: mRNAs differentially spliced and differentially expressed in genes in *Mx1-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} cells relative to wild-type *Mx1-Cre Srsf2*^{+/+} *Sf3b1*^{+/+} cells in any replicate.

Table S7: Human splicing events corresponding to differentially spliced genes in both human and mouse samples with a mutation in *SF3B1*.

Figure S1. Severe self-renewal disadvantage of hematopoietic stem cells from *Srsf2*^{P95H/+}

Sf3b1*^{K700E/+} double-mutant mice.** (A) Representative FACS plots of CD45.2⁺ (donor-derived) cells of both lymphoid and myeloid lineages in the peripheral blood of CD45.1 recipient mice reconstituted with bone marrow (BM) mononuclear cells (MNCs) from *Mx1-Cre*, *Mx1-Cre Srsf2*^{P95H/+}, *Mx1-Cre Sf3b1*^{K700E/+} and *Mx1-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} mice 20 weeks post plpC administration. (B) Percentage of CD45.2 (donor-derived) chimerism in the B-, T- or myeloid lineages in peripheral blood (PB) of CD45.1 recipient mice (n=10 mice per genotype) in competitive BM transplantation. (C) Analysis of CD45.2 chimerism in the various cellular lineages in the bone marrow, spleen and thymus 20 weeks post plpC administration in CD45.1 recipient mice. Error bars represent means $\pm$ standard deviation. (D) White blood cell (WBC) count of *Mx1-Cre*, *Mx1-Cre Srsf2*^{P95H/+}, *Mx1-Cre Sf3b1*^{K700E/+} and *Mx1-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} mice 52 weeks post plpC administration. (E) Representative FACS plots of BM MNC samples prior to noncompetitive transplantation. (F) Representative genotyping PCR gel of PB MNCs of *Mx1-Cre*, *Mx1-Cre Srsf2*^{P95H/+}, *Mx1-Cre Sf3b1*^{K700E/+} and *Mx1-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} mice pre- and post-plpC administration. Analysis of variance followed by Tukey's post-hoc test was used to compare differences between groups, and each *p*-value shown has been adjusted to account for multiple comparisons. **p*<0.05, ***p*< 0.01 and *p*< 0.001 versus *Mx1-Cre* mice; ^*p*<0.05, ^^*p*<0.01 and ^^^*p*<0.001 versus *Mx1-Cre Sf3b1*^{K700E/+} mice; #*p*< 0.05, ##*p*< 0.01 and ###*p*< 0.001 versus *Mx1-Cre Srsf2*^{P95H/+} mice.

Figure S2. Severe self-renewal disadvantage of hematopoietic stem cells from *Srsf2*^{P95H/P95H}

homozygous mutant mice. (A) Representative FACS plots of CD45.2⁺ (donor-derived) cells of both lymphoid and myeloid lineages in the peripheral blood of CD45.1 recipient mice reconstituted with bone marrow (BM) mononuclear cells (MNCs) from *Mx1-Cre*, *Mx1-Cre Srsf2*^{P95H/+}, *Mx1-Cre Srsf2*^{P95H/P95H} and *Mx1-Cre Srsf2*^{P95H/fl} mice 20 weeks post plpC administration. (B) Percentage of CD45.2 (donor-derived) chimerism in the B-, T- or myeloid lineages in peripheral blood of CD45.1 recipient mice (n=5-10 mice per genotype) in competitive BM transplantation. (C) Analysis of CD45.2 chimerism in the various cellular lineages in the bone marrow, spleen and thymus 20 weeks post plpC administration in CD45.1

recipient mice. Error bars represent means $\pm$ standard deviation. Analysis of variance followed by Tukey's post-hoc test was used to compare differences between groups, and each p -value shown has been adjusted to account for multiple comparisons. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus *Mx1-Cre* mice; # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ versus *Mx1-Cre Srsf2*^{P95H/+} mice.

Figure S3. Co-expression of *Srsf2* and *Sf3b1* mutations results in death before birth in *Vav-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} mice. (A) Number of embryos detected at embryonic day 14.5 (E14.5) per genotype from crossing *Sf3b1*^{K700E/+} x *Vav-Cre Srsf2*^{P95H/+} or *Srsf2*^{P95H/+} x *Vav-Cre Sf3b1*^{K700E/+} mice. (B) Photographs of representative fetuses with each genotype at E14.5 and E18.5. (C) Number of viable fetal liver mononuclear cells (MNCs) and (D) LSK (lineage-negative Sca-1⁺ c-Kit⁺) cells from E14.5 embryos from (A). Frequency of (E) MPP (LSK CD48⁻ CD150⁻), (F) HPC-1 (LSK CD48⁺ CD150⁻), and (G) HPC-2 (LSK CD48⁺ CD150⁺) cells amongst fetal liver MNCs at E14.5. (H) Photomicrographs of hematoxylin and eosin (H&E), anti-CD45, and anti-myeloperoxidase (MPO) immunohistochemical stains of E18.5 marrow cavity cells of the mice in (B). (I) Photomicrographs of cleaved caspase-3 immunohistochemical stains of E18.5 fetal liver cells of the mice in (B) with quantitation on right. Error bars represent means $\pm$ standard deviation. Analysis of variance followed by Tukey's post-hoc test was used to compare differences between groups, and each p -value shown has been adjusted to account for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Figure S4. Co-occurring *Srsf2* and *Sf3b1* mutations do not cause global splicing failure. (A) Table illustrating the numbers of expected versus observed coding genes exhibiting dysregulated expression that were shared between the illustrated comparisons between genotypes ("A", "B", and "C" refer to replicates of RNA-seq). The set of expected coding genes exhibiting dysregulation was defined assuming that the *Sf3b1* and *Srsf2* mutations were independent, such that the double-mutant gene expression program was simply the mean of the two single-mutant gene expression programs. p -values were computed using the one-sided binomial proportion test. (B) Table illustrating the numbers of expected versus observed coding genes that were dysregulated in double-mutant cells relative to wild-

type cells. The set of expected coding genes exhibiting dysregulation was defined as in (A). **(C)** Bar plots showing % of expected versus observed coding genes exhibiting dysregulated expression in each replicate of *Mx1-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} cells relative to *Mx1-Cre Srsf2*^{+/+} *Sf3b1*^{+/+} wildtype (WT) cells. *** $p < 0.0001$. **(D)** Bar plot illustrating the percentage of dysregulated splicing events that corresponded to cassette exon events (se; left) or competing 3' splice site events (a3ss; right) for each illustrated genotype relative to wild-type cells. p -values were computed using a two sided binomial proportion test where ** $p < 0.01$ and *** $p < 0.001$. **(E)** Diagram illustrating whether there was a statistically significant difference in the fraction of dysregulated splicing events corresponding to cassette exon events (se; left) or competing 3' splice site events (a3ss; right). p -values were computed using the binomial proportion test and are represented by the thickness of the lines. **(F)** Sequence logos associated with competing 3' splice sites that are differentially spliced in *Mx1-Cre Srsf2*^{P95H/+}, *Mx1-Cre Sf3b1*^{K700E/+}, and *Mx1-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} cells relative to *Mx1-Cre Srsf2*^{+/+} *Sf3b1*^{+/+} wildtype (WT) cells in any replicate. The left- and right-hand columns contain sequence logos created for the intron-proximal and intron-distal 3' splice sites. **(G)** Bar plots illustrating enrichment of different variants of the SSNG (S = C or G; N = any nucleotide) exonic splicing enhancer in *Mx1-Cre Srsf2*^{P95H/+}, *Mx1-Cre Sf3b1*^{K700E/+}, and *Mx1-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} cells relative to *Mx1-Cre Srsf2*^{+/+} *Sf3b1*^{+/+} wildtype (WT) cells. Enrichment computed using the alternatively spliced sequence for cassette exons that are differentially spliced in the indicated genotype comparisons. **(H)** Scatter plots of constitutive intron splicing in *Mx1-Cre Srsf2*^{P95H/+}, *Mx1-Cre Sf3b1*^{K700E/+}, and *Mx1-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} cells relative to *Mx1-Cre Srsf2*^{+/+} *Sf3b1*^{+/+} wild-type (WT) cells for replicate B. Axes indicate the fraction of mRNAs for which each constitutive intron is removed. Red and blue indicate constitutive introns that are preferentially spliced or retained, respectively, in mutant relative to wild-type cells.

Figure S5. *Srsf2* and *Sf3b1* mutations have distinct and independent effects on RNA splicing.

(A) Venn diagram showing the overlap between splicing events that were significantly dysregulated in *Mx1-Cre Srsf2*^{P95H/+}, *Mx1-Cre Sf3b1*^{K700E/+}, and *Mx1-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} cells relative to *Mx1-Cre Srsf2*^{+/+} *Sf3b1*^{+/+} wildtype (WT) cells for replicate B. Example genes illustrated below are indicated with

arrows. **(B)** Gene Ontology (GO) enrichment analysis of *Vav-Cre Srsf2*^{P95H/+}, *Vav-Cre Sf3b1*^{K700E/+}, and *Vav-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} cells relative to *Vav-Cre Srsf2*^{+/+} *Sf3b1*^{+/+} wildtype (WT) cells. Circle size indicates the magnitude of the *p*-value for each term and genotype comparison. **(C)** Scatter plots of retained introns (top), competing 3' splice sites (middle), and cassette exon inclusion (bottom) in *Vav-Cre Srsf2*^{P95H/+}, *Vav-Cre Sf3b1*^{K700E/+}, and *Vav-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} cells relative to wild-type *Vav-Cre Srsf2*^{+/+} *Sf3b1*^{+/+} cells. Axes indicate the fraction of mRNAs containing each cassette exon in the indicated sample. Red and blue indicate cassette exons whose inclusion is promoted or repressed, respectively, in mutant relative to wild-type cells. **(D)** Venn diagram showing the overlap between splicing events that were significantly dysregulated in *Vav-Cre Srsf2*^{P95H/+}, *Vav-Cre Sf3b1*^{K700E/+}, and *Vav-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} cells relative to *Vav-Cre Srsf2*^{+/+} *Sf3b1*^{+/+} wildtype (WT) cells. **(E)** RNA-seq read coverage across the genomic loci containing the illustrated differentially spliced events in *Mpl*, *Itga2b* and *Pbx1* for all genotypes. Yellow indicates the differentially spliced sequence for each event. **(F)** Schema of evaluation of the effects of shRNA mediated knockdown of *Mpl*, *Itga2b* and *Pbx1* on 5-fluorouracil-treated, lineage-depleted bone marrow (BM) cells from 8-12 week-old C57BL/6 WT mice. **(G)** Quantitative RT-PCR (qRT-PCR) for *Mpl*, *Itga2b* or *Pbx1* in lineage-negative (lin⁻) bone marrow cells treated with shRNA or renilla-luciferase control (sh-Control). **(H)** Colony numbers from lin⁻ HSPCs following shRNA-mediated knockdown of *Mpl*, *Itga2b* and *Pbx1* cells. Error bars represent means ± standard deviation. Analysis of variance followed by Tukey's post-hoc test was used to compare differences between groups, and each *p*-value shown has been adjusted to account for multiple comparisons. ** *p*<0.01 and **** *p*<0.0001. **(I)** Immunofluorescence analysis of nuclear phospho-p65 in LK cells from *Vav-Cre Srsf2*^{+/+} *Sf3b1*^{+/+} wild-type, *Vav-Cre Srsf2*^{P95H/+}, *Vav-Cre Sf3b1*^{K700E/+}, and *Vav-Cre Srsf2*^{P95H/+} *Sf3b1*^{K700E/+} mice following stimulation with lipopolysaccharide (LPS; 200 ng/mL; 15 minutes) *ex vivo*. Scale bar: 10 μm. **(J)** Violin plots showing quantitation of nuclear phospho-p65 intensity of LK cells following LPS stimulation from (I). Analysis of variance using Kruskal-Wallis ranked test was performed. Multiple pair-wise comparisons were adjusted by false-discovery rate.

Figure S6. *SF3B1* mutations promote mis-splicing of *MAP3K7* in *SF3B1*-mutant cells. (A)

Phenotypic analysis of bone marrow, spleen, and blood from *Vav-Cre Srsf2^{+/+}* and *Vav-Cre Srsf2^{P95H/+}* mice following chronic LPS or vehicle exposure. Error bars represent means $\pm$ standard deviation. Analysis of variance followed by Tukey's post-hoc test was used to compare differences between groups, and each *p*-value shown has been adjusted to account for multiple comparisons. **p* < 0.05 and ****p* < 0.001. **(B)** Immunofluorescence of phosphorylated p65 (phospho-p65) intensity from FACS-purified LSK cells following acute exposure to LPS *ex vivo*. Scale bar: 10 μ m. **(C)** Violin plots showing quantitation of phospho-p65 intensity in the nucleus of LSK cells following LPS stimulation of the following groups: *Srsf2^{+/+}* Veh (n = 5499 cells), *Srsf2^{+/+}* LPS (n = 4923 cells), *Srsf2^{P95H/+}* Veh (n = 7234 cells), *Srsf2^{P95H/+}* LPS (n = 4906 cells). Analysis of variance using Kruskal-Wallis ranked test was performed. Multiple pair-wise comparisons were adjusted by false-discovery rate. **p* < 2.2e⁻¹⁶. **(D)** RT-PCR analysis and Sanger sequencing traces of the normal and aberrantly spliced *MAP3K7* isoforms in NALM6 *SF3B1* isogenic cells. **(E)** Protein diagram of MAP3K7 indicating region of aberrant 3' splice site usage in *SF3B1* mutant cells. **(F)** mRNA half-life measurements of *MAP3K7* 3' splice site inclusion (left) and exclusion (right) isoforms in *SF3B1^{K700E/WT}* knockin K562 cells. UPF1 knockdown efficiency in these cells is shown. **(G)** Quantitation of immunoblot densitometry of Figure 6E. **(H)** Quantitation of immunoblot densitometry of Figure 6F. **(I)** Fold-change in luciferase activity of NALM-6 isogenic cells transfected with a wildtype NF- κ B luciferase reporter or a version of the reporter where all five NF- κ B response elements were mutagenized. **(J)** Immunoblot of phospho-p65, I κ B- α , MAP3K7, and loading controls in K562 cells treated with anti-*MAP3K7* shRNAs. Quantitation of immunoblot densitometry (bottom). **(K)** Quantitation of immunoblot densitometry of Figure 7D. Analysis of variance followed by Tukey's post-hoc test was used to compare differences between groups. **(L)** Numbers of hematopoietic colonies 10-14 days after *Map3k7* cDNA expression in lineage-negative cells from *Vav-Cre Sf3b1^{K700E/+}* and *Vav-Cre WT* E14.5 fetal liver cells (Left). Immunoblot analysis of Flag-tagged *Map3k7* cDNA from hematopoietic colonies derived from *Sf3b1^{+/+}* and *Sf3b1^{K700E/+}* cells (Right). **(M)** RNA-seq coverage plots of *MAP3K7* in acute myeloid leukemia (AML) and chronic myelomonocytic leukemia (CMML) patient samples wild-type (WT) or mutant (MUT) for *SRSF2*. **(N)** RNA-seq coverage plots of *CASP8* in

normal human bone marrow (BM) cells as well as the NALM-6 cell line and cells from myelodysplastic syndrome (MDS) and chronic lymphocytic leukemia (CLL) patients wild-type (WT) or mutant (MUT) for *SF3B1*. Error bars represent means $\pm$ standard deviation. Analysis of variance followed by Tukey's post-hoc test was used to compare differences between groups. *** $p < 0.001$ versus sh.Control or parental, **** $p < 0.0001$ versus sh.Control or parental, ## $p < 0.01$ versus unstimulated, ### $p < 0.001$ versus unstimulated, ^^^ $p < 0.0001$ parental.

Figure S7. *SRSF2* mutations promote aberrant splicing of *caspase-8* (*CASP8*) resulting in expression of a stable truncated protein that hyperactivates NF- κ B signaling. (A) RT-PCR of the *CASP8* splicing event in human leukemia cell lines and primary human AML patient samples wild-type or mutant for *SRSF2* with diagram of predicted effects of *CASP8* mRNA. The red box highlights the *CASP8* isoform promoted by mutant *SRSF2*. **(B)** Sanger sequencing traces of the aberrant *CASP8* isoform (*CASP8* *TR*) in *SRSF2*-mutant leukemia cell line (K052) and *SRSF2*-mutant primary AML patient samples. **(C)** Quantitative RT-PCR (qRT-PCR) analysis of the aberrant *CASP8* isoform expression in *SRSF2* wild-type and *SRSF2* mutant patient AML samples. **(D)** Immunoblot analysis of caspase-8 in K562 cells (left panel) and *CASP8* knockout (KO) HAP1 cells (right panel) expressing cDNA constructs encoding empty vector (EV), full-length *CASP8* (*CASP8*^{FL}), or the aberrantly spliced *caspase-8* truncated isoform (*CASP8*^{TR}). **(E)** Growth assay of K562 cells overexpressing empty vector control (EV), full-length *caspase-8* (*CASP8*^{FL}) and the aberrant *caspase-8* truncated isoform (*CASP8*^{TR}). **(F)** Cell viability assay using CellTiter-Glo in K562 cells expressing empty vector control (EV), *CASP8*^{FL}, and *CASP8*^{TR} isoforms 48 hours post-TRAIL stimulation. **(G)** Quantitation of Western blot densitometry of Figure 8E. **(H)** Immunofluorescence analysis of phosphorylated p65 (phospho-p65) in K562 cells from Figure 8E with quantitation of results shown on right. Scale bar: 10 μ m. Analysis of variance followed by Tukey's post-hoc test was used to compare differences between groups.

Figure S8. Effects of *SRSF2*P95H mutations or *CASP8* truncated isoform on NF-κB signaling. (A) Immunofluorescence analysis of phosphorylated p65 in isogenic genetically modified K562 cells with or without *SRSF2*P95H mutation expressed from the endogenous *SRSF2* locus with and without TRAIL stimulation (50 ng/mL). Scale bar: 10 μm. (B) Quantitation of immunoblot densitometry of Figure 8G. Cell viability assay using CellTiter-Glo in (C) *CASP8* wild-type (WT) and (D) *CASP8* knockout (KO) HAP1 cells expressing EV control, *CASP8*^{FL}, and *CASP8*^{TR} isoforms 48 hours post TRAIL stimulation. Analysis of NF-κB signaling following TRAIL stimulation (50 ng/mL) in (E) *CASP8* wild-type and (F) *CASP8* KO HAP1 cells expressing EV, *CASP8*^{FL}, and *CASP8*^{TR} isoforms. Quantification of immunoblot signals using densitometry is shown on right. Analysis of variance followed by Tukey's post-hoc test was used to compare differences between groups.

Figure 1

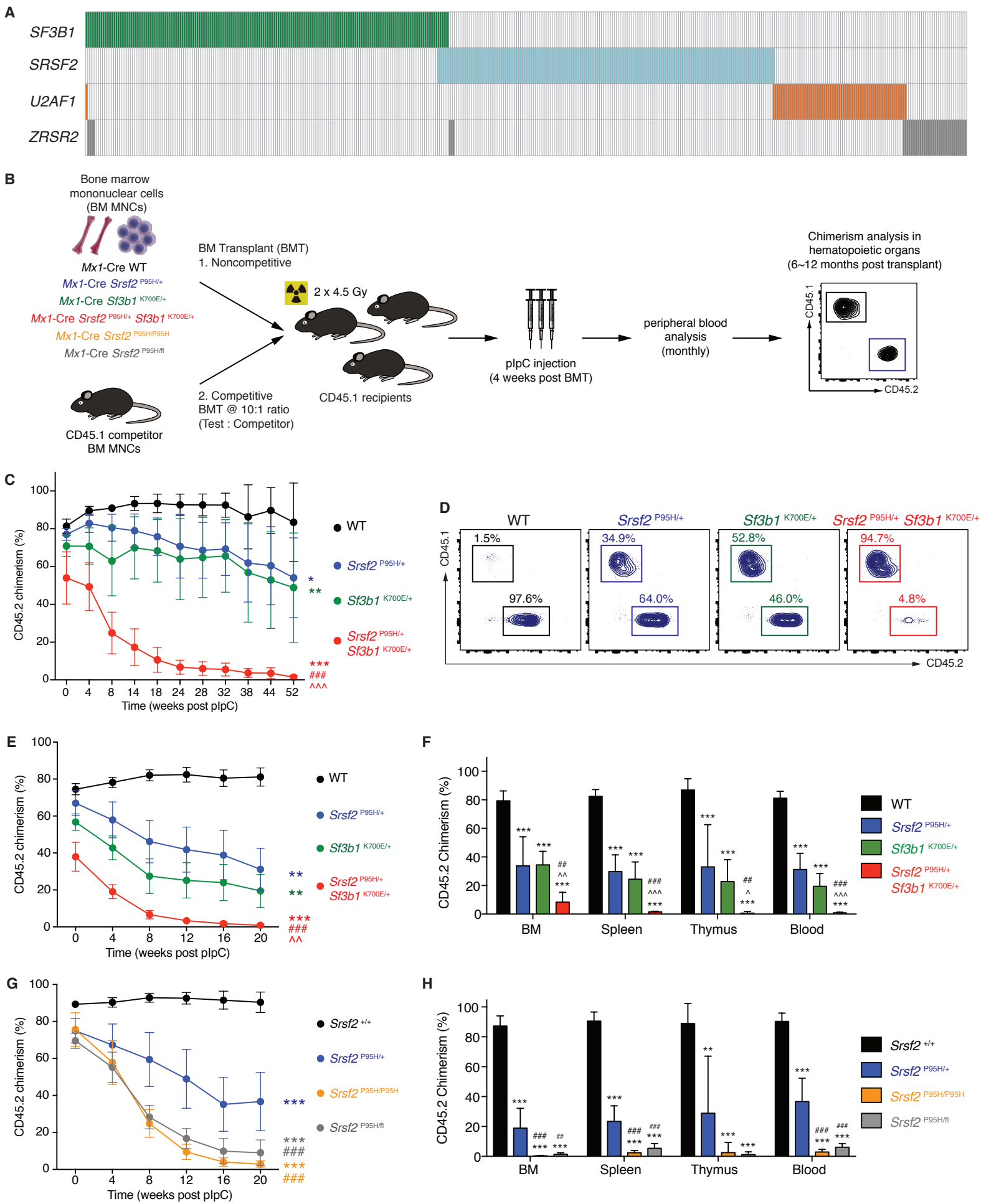


Figure 2

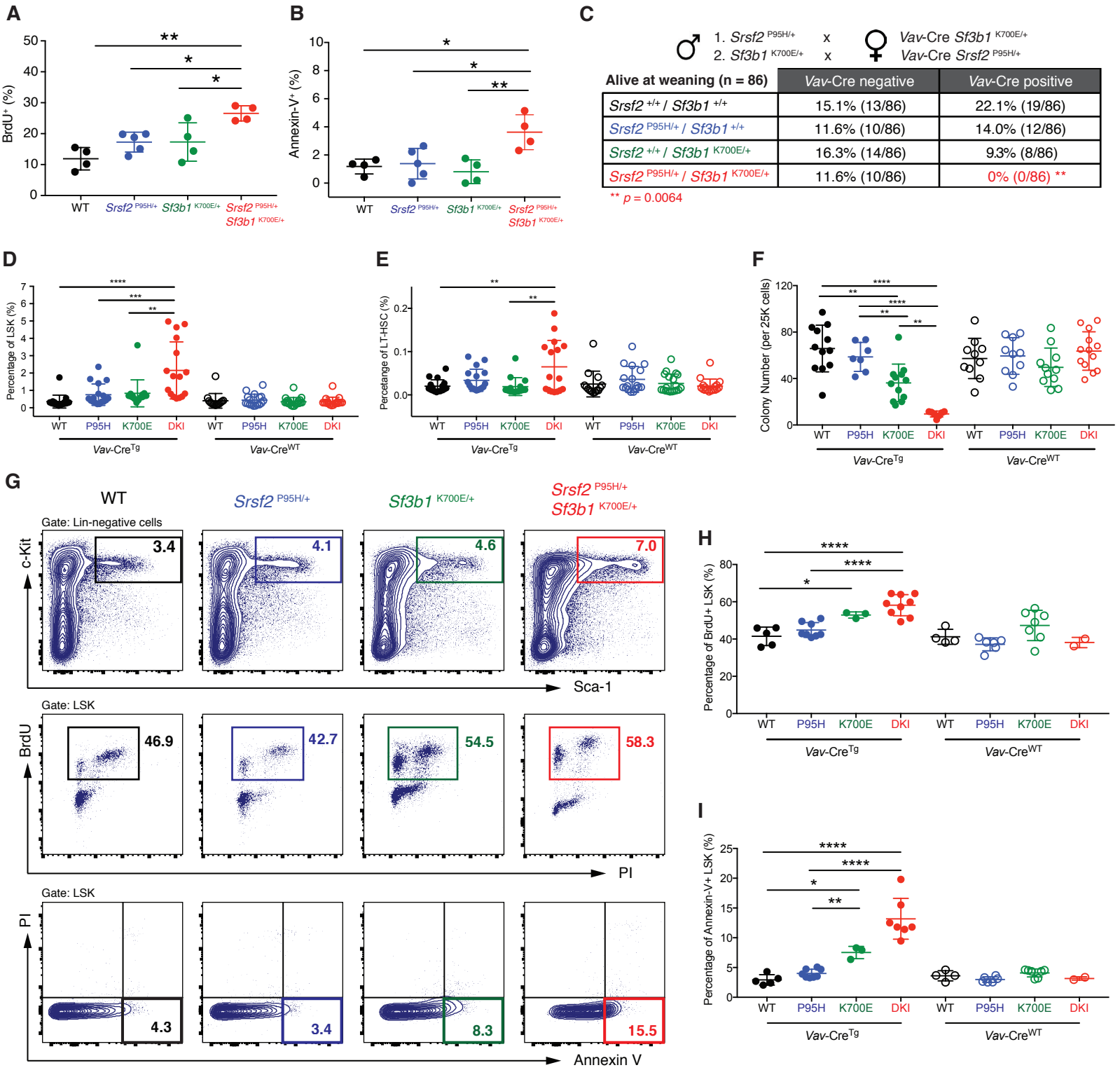


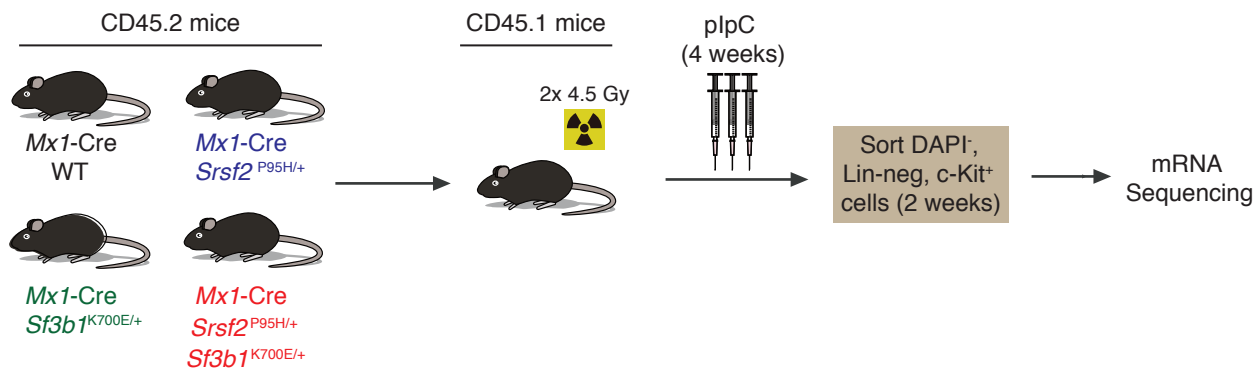
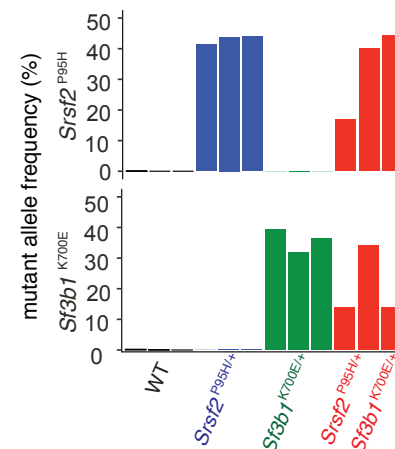
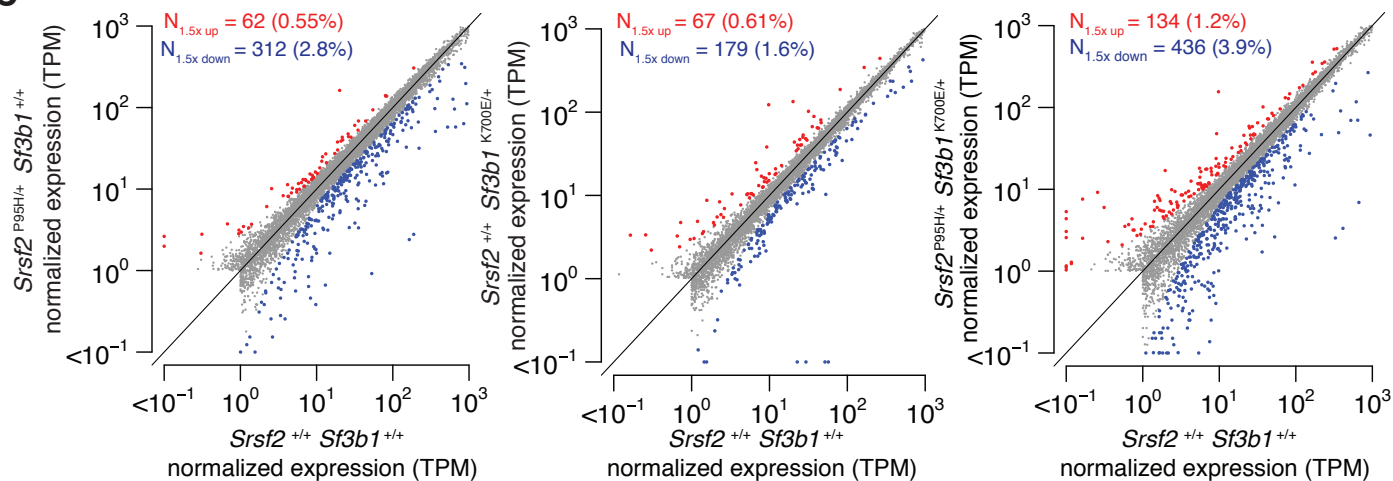
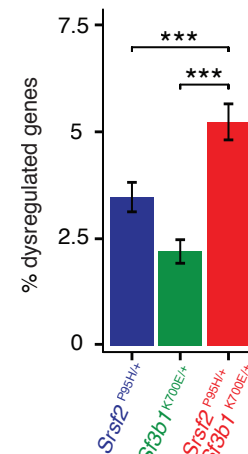
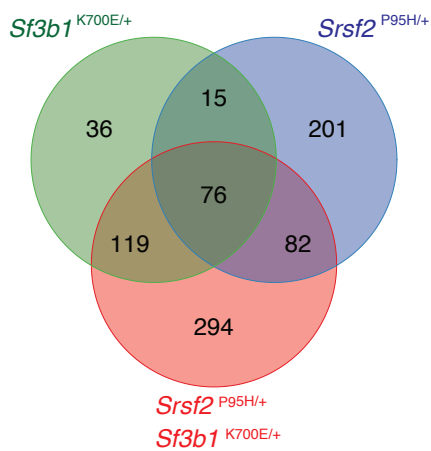
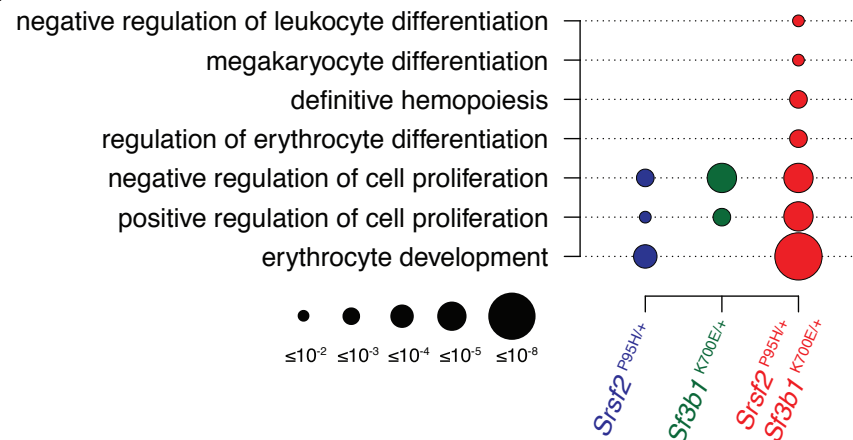
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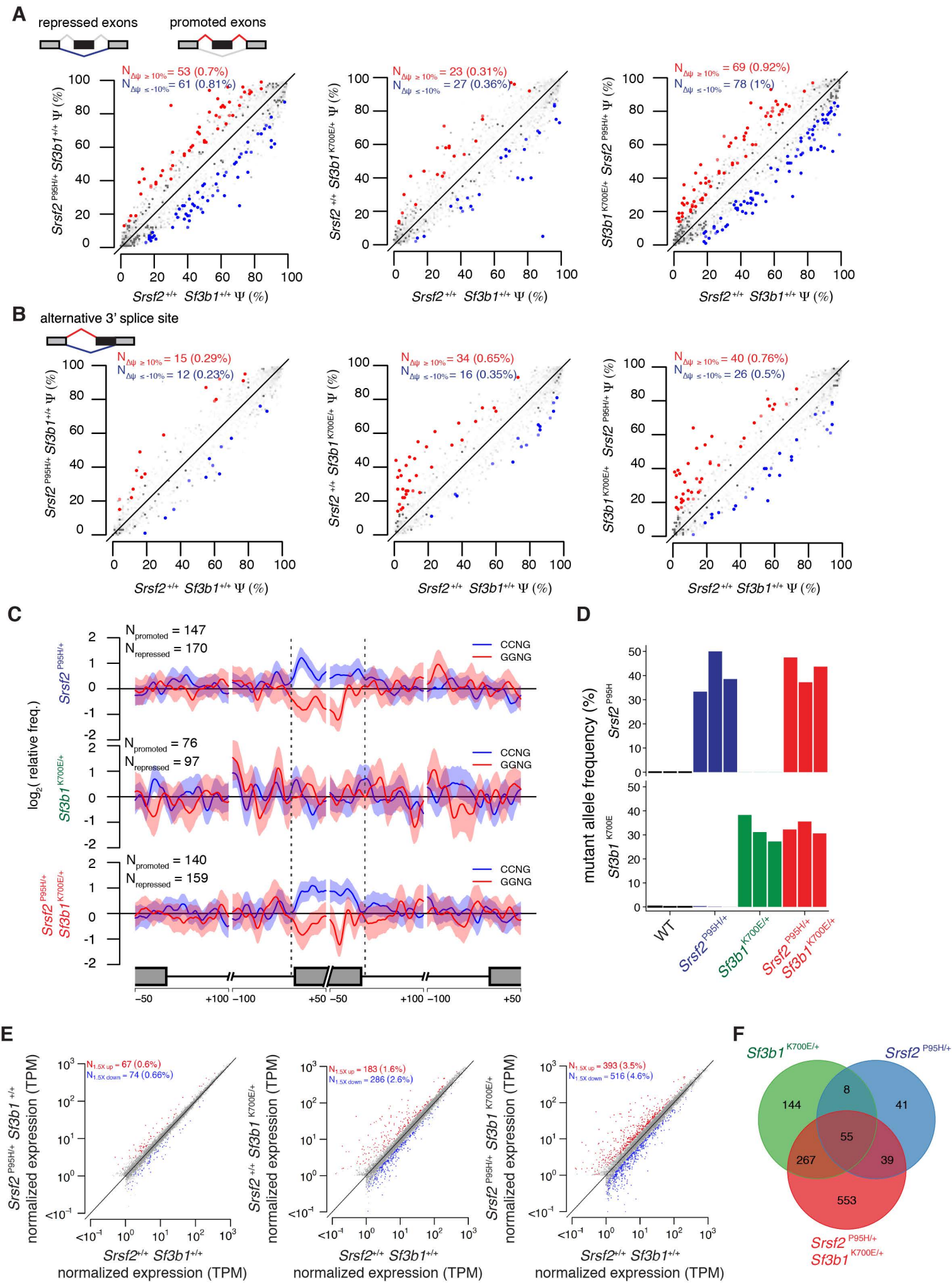
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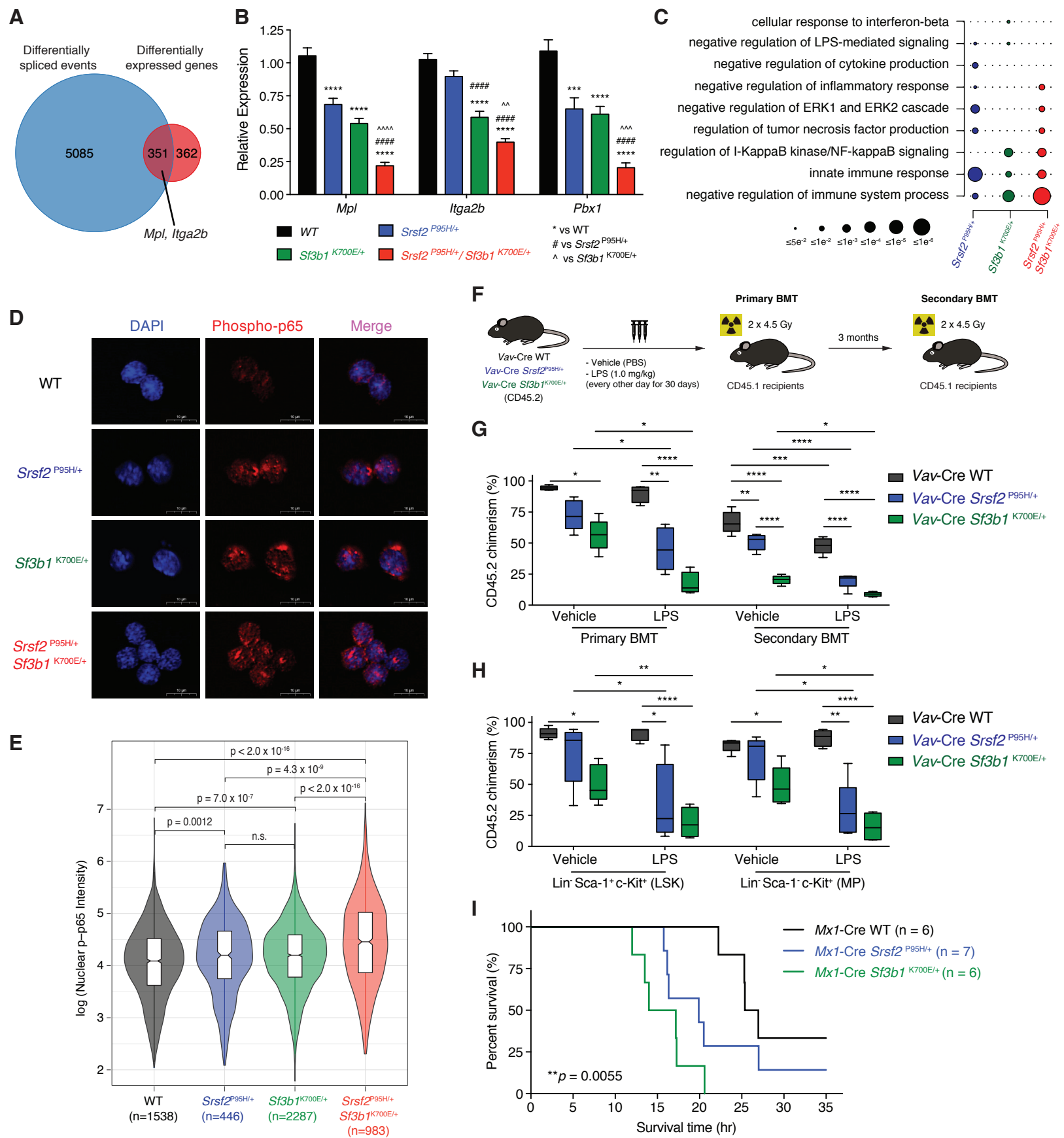
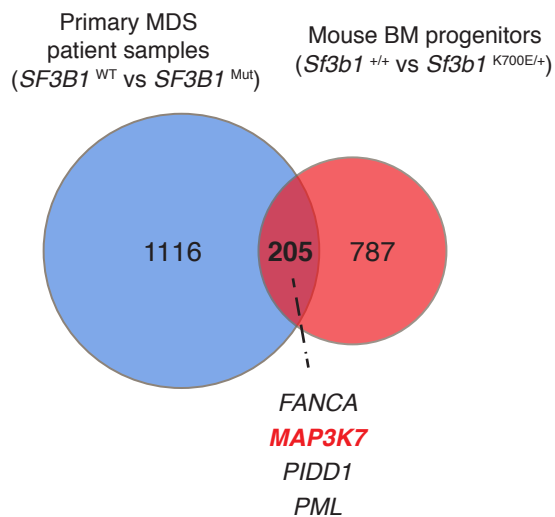
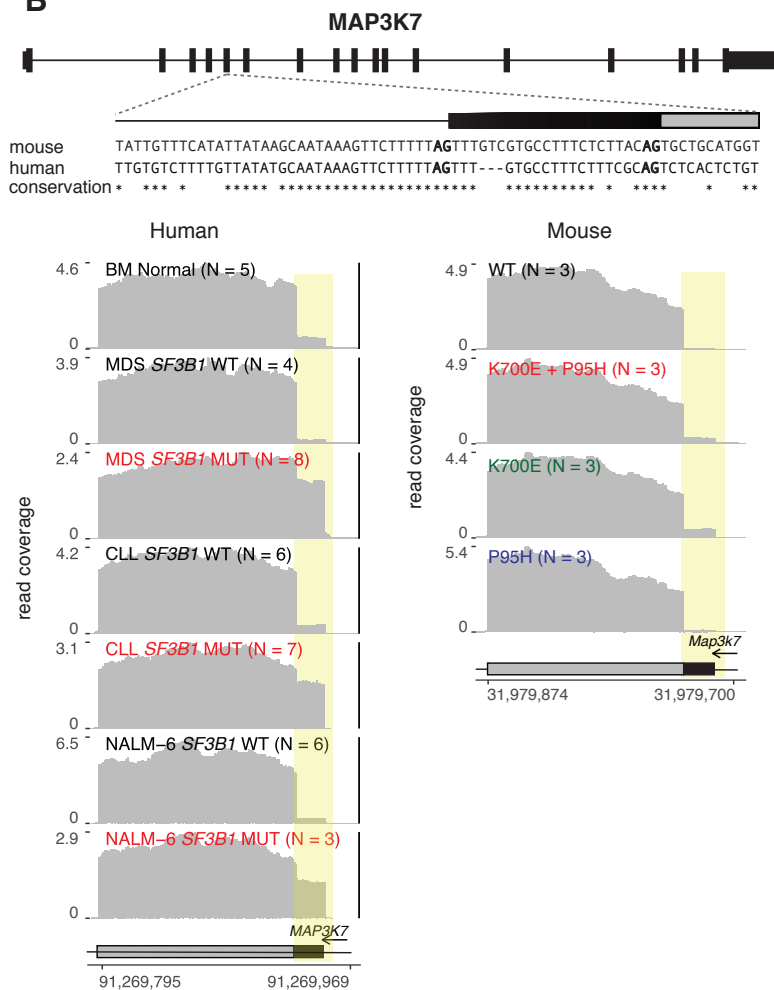
Figure 5

Figure 6

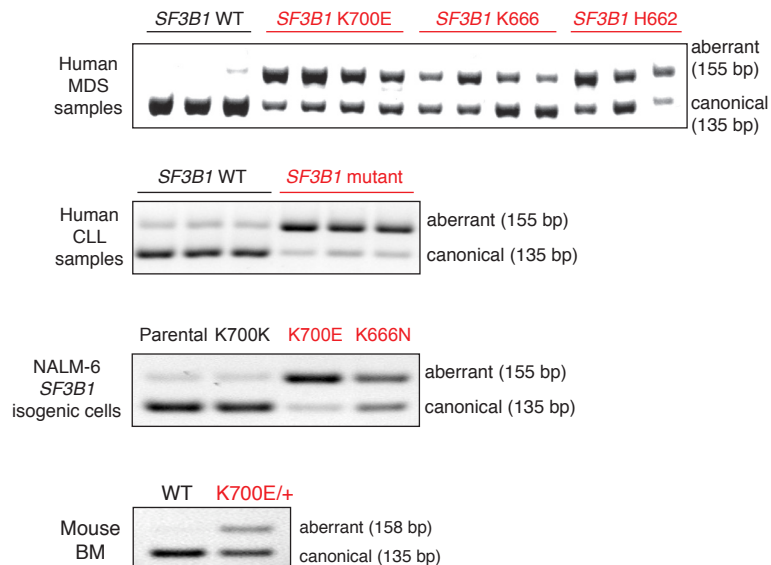
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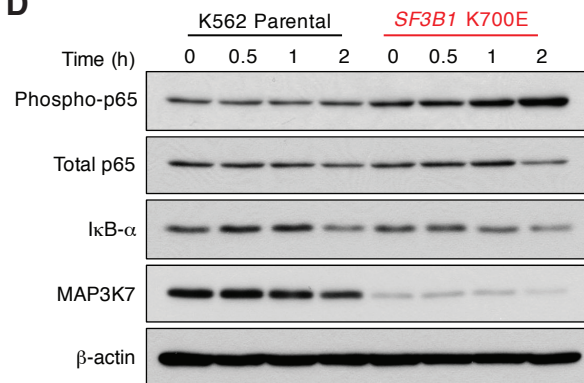
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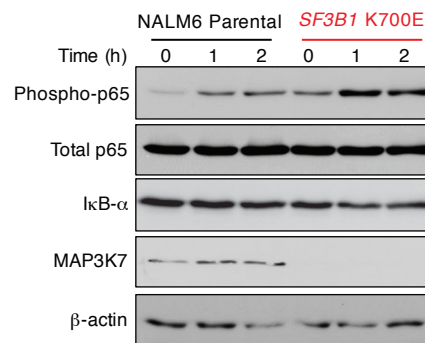
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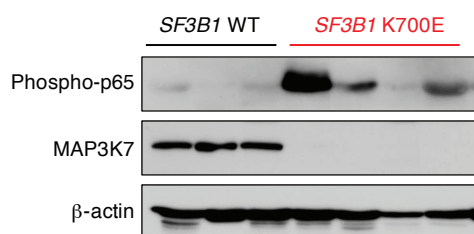
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E



F



G

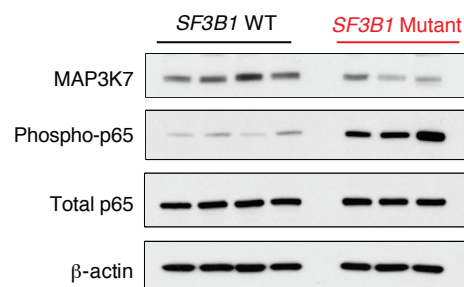


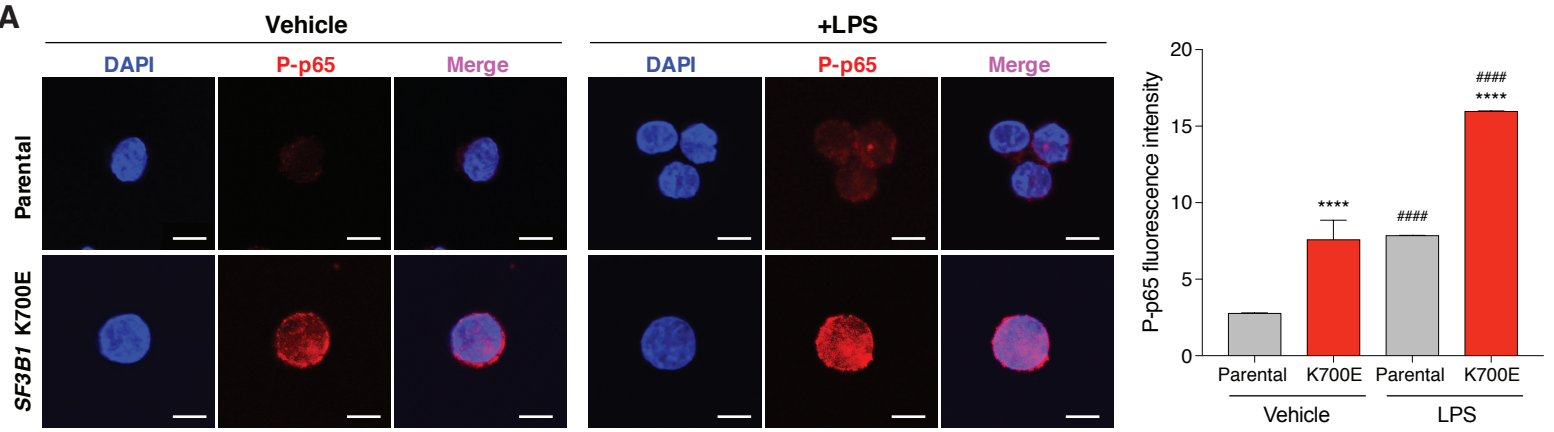
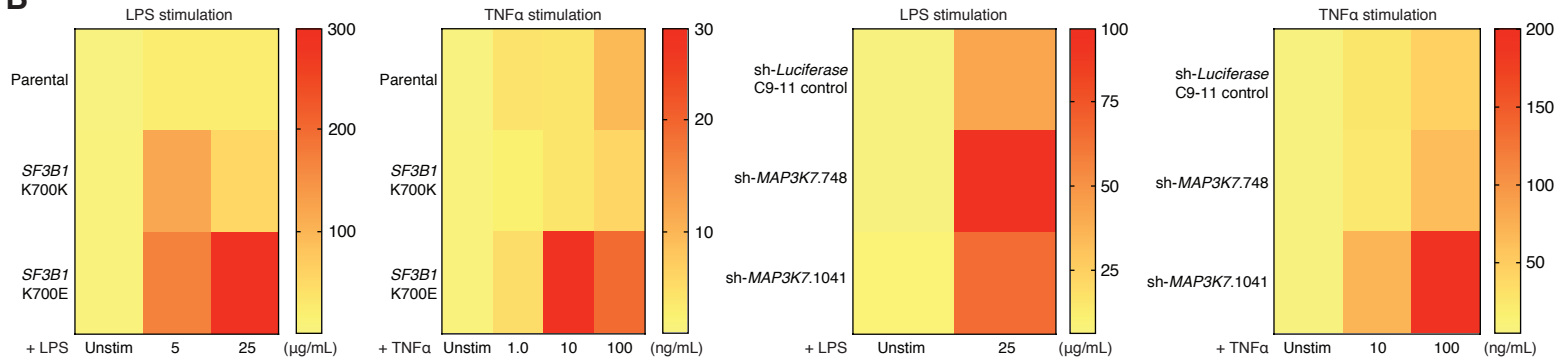
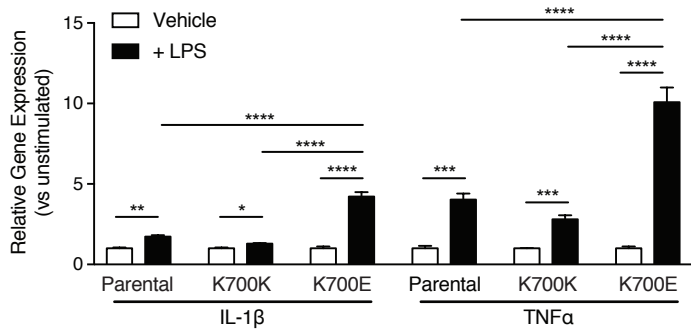
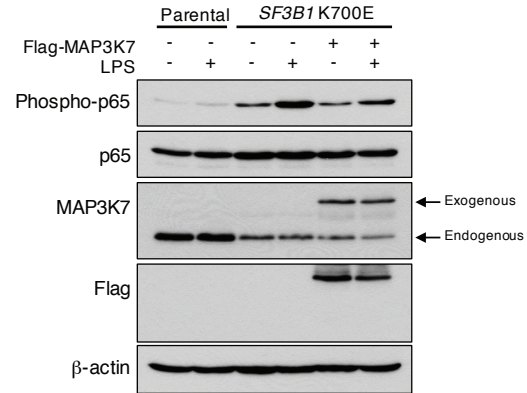
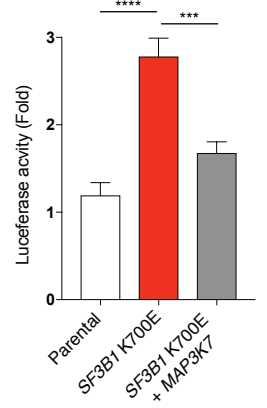
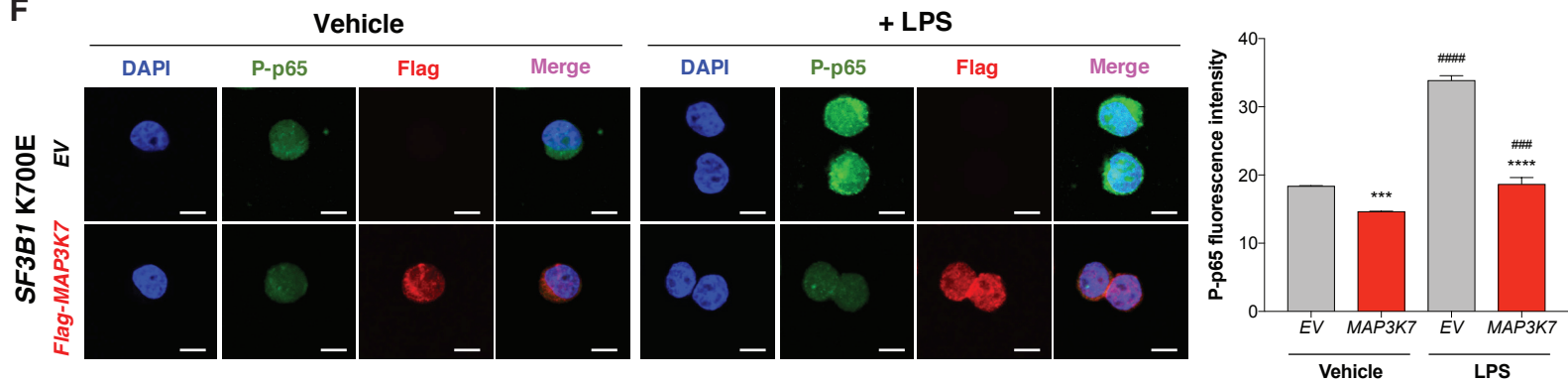
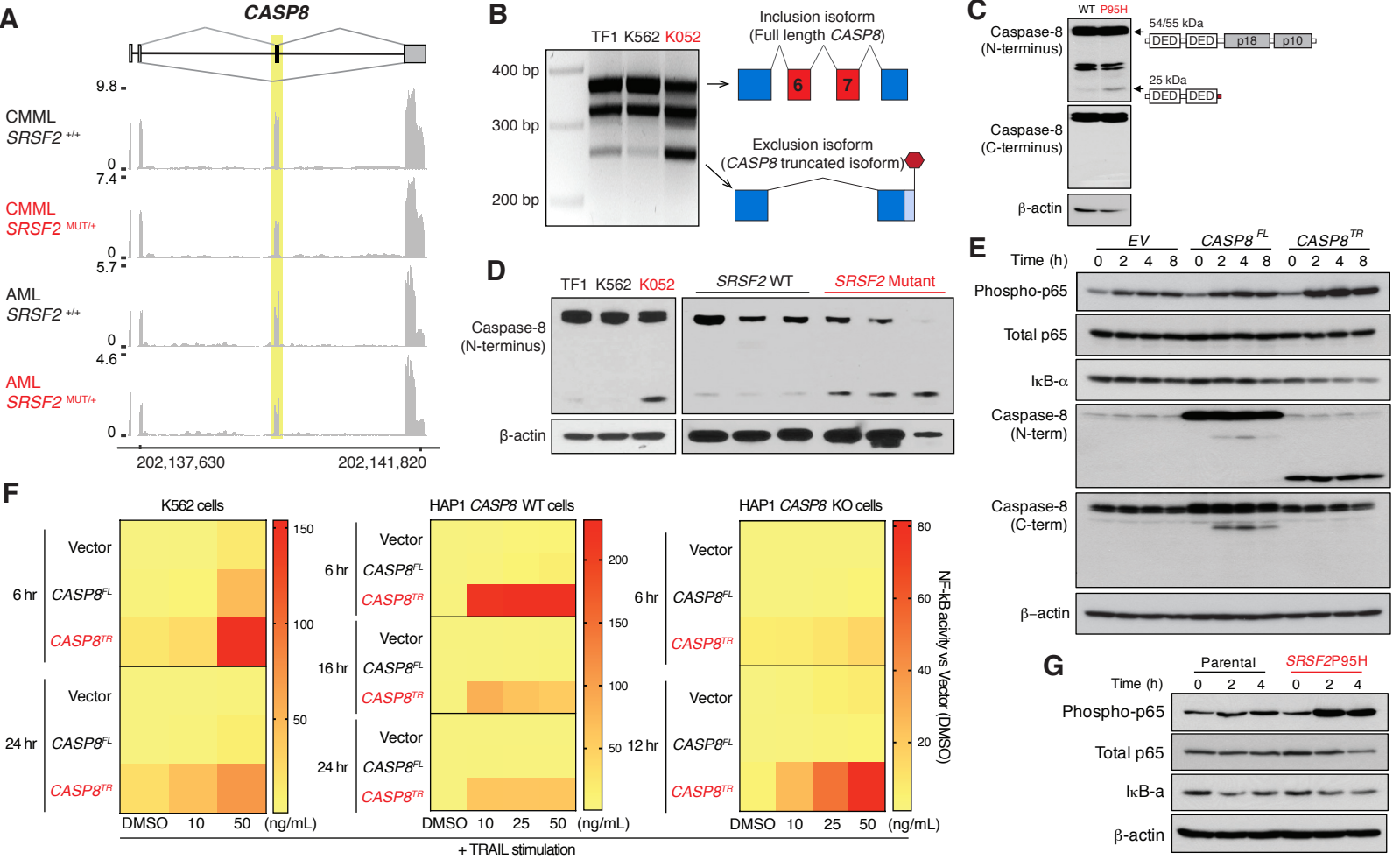
Figure 7**A****B****C****D****E****F**

Figure 8



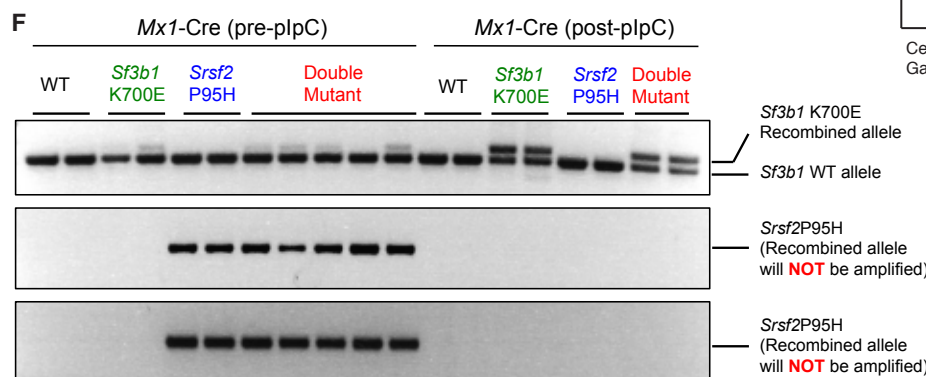
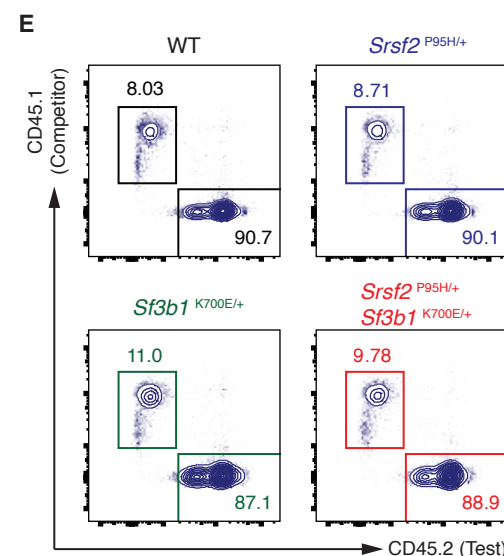
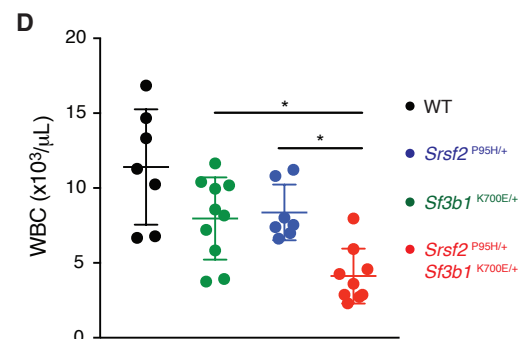
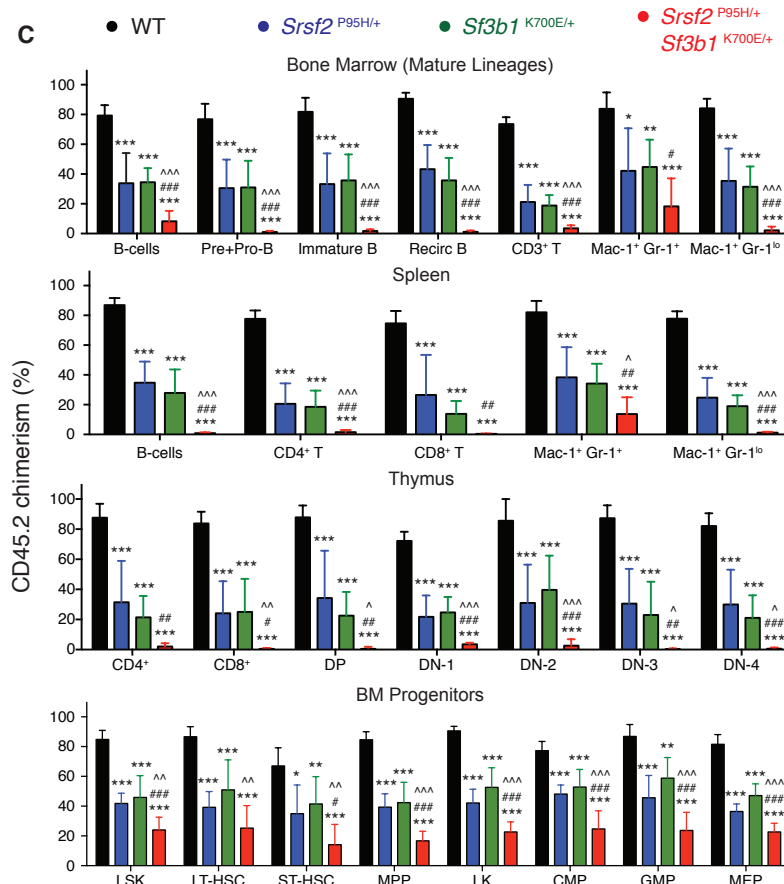
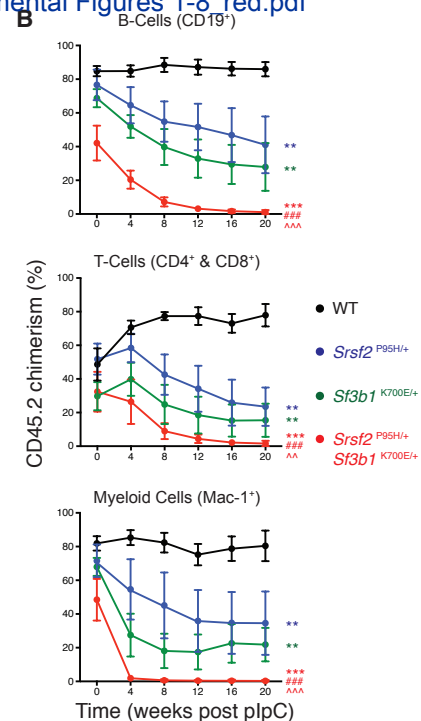
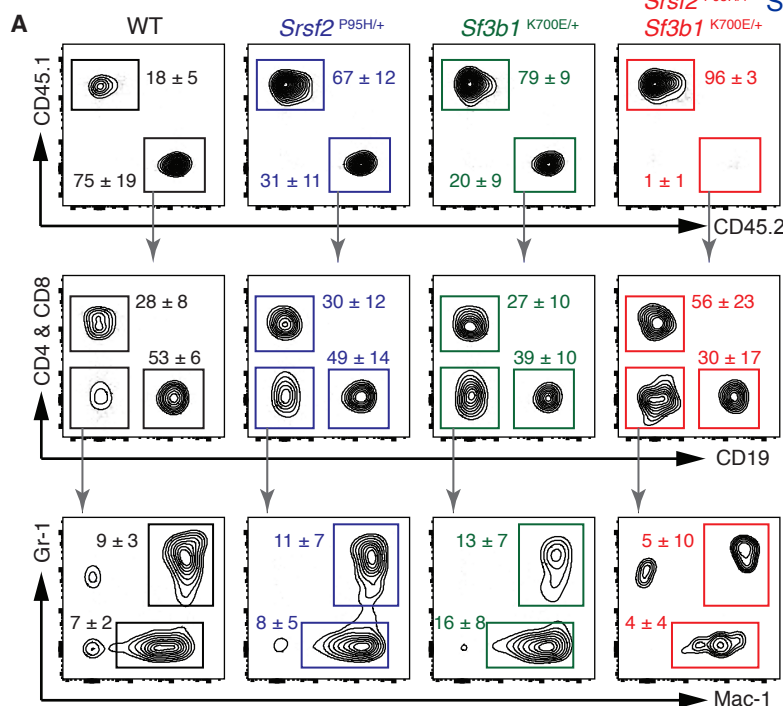


Figure S2

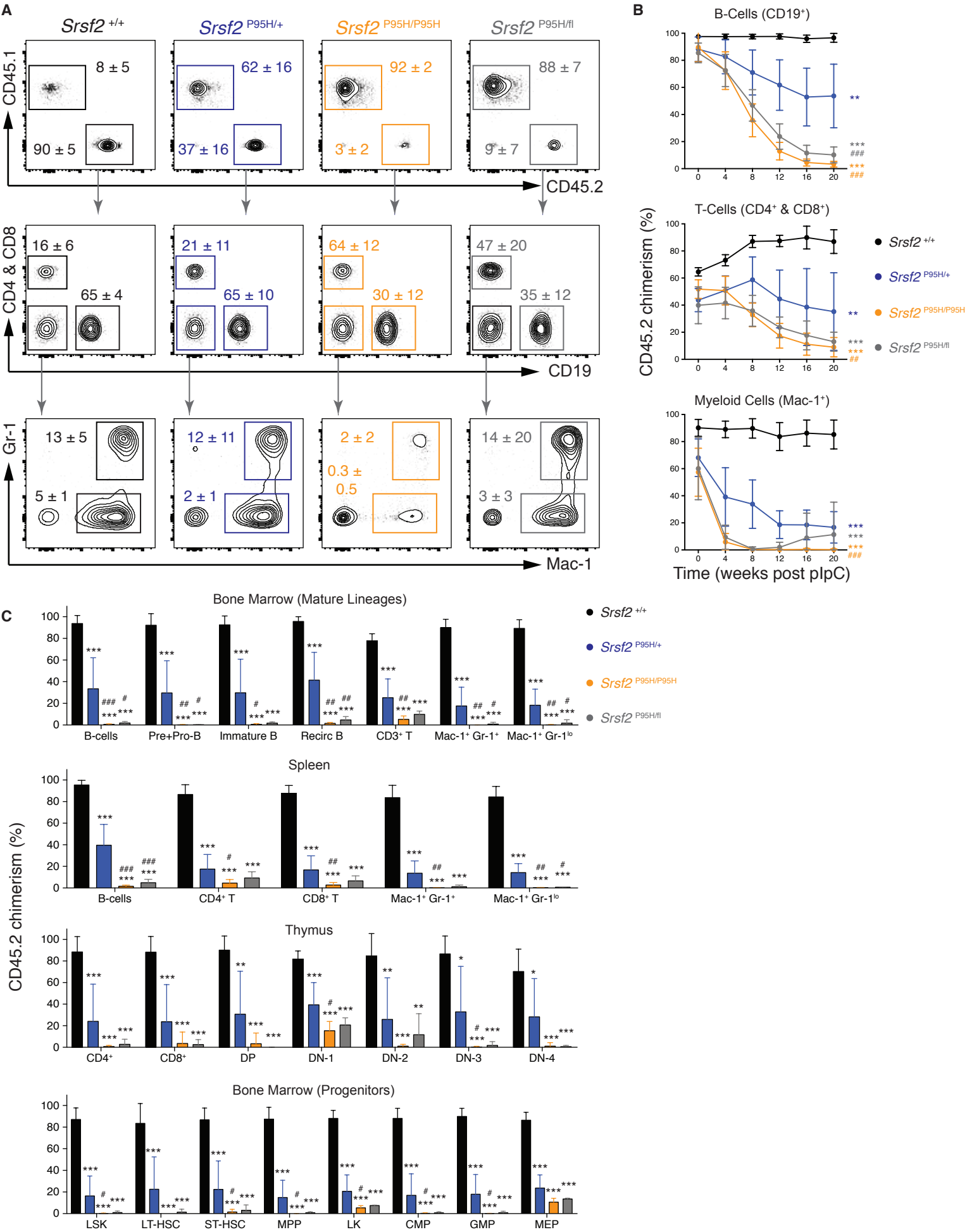


Figure S3**A**

E14.5 embryos (n = 139)	Vav-Cre negative	Vav-Cre positive
<i>Srsf2</i> ^{+/+} / <i>Sf3b1</i> ^{+/+}	10.8% (15/139)	14.3% (20/139)
<i>Srsf2</i> ^{P95H/+} / <i>Sf3b1</i> ^{+/+}	13.7% (19/139)	13.7% (19/139)
<i>Srsf2</i> ^{+/+} / <i>Sf3b1</i> ^{K700E/+}	12.2% (17/139)	10.8% (15/139)
<i>Srsf2</i> ^{P95H/+} / <i>Sf3b1</i> ^{K700E/+}	13.7% (19/139)	10.8% (15/139)

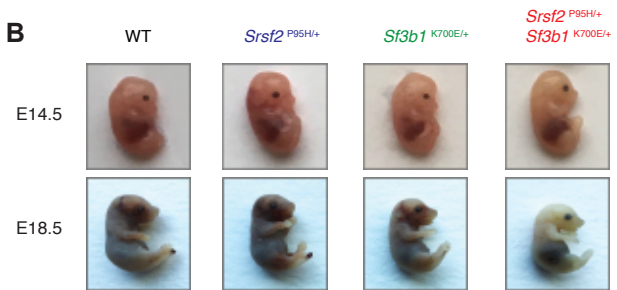
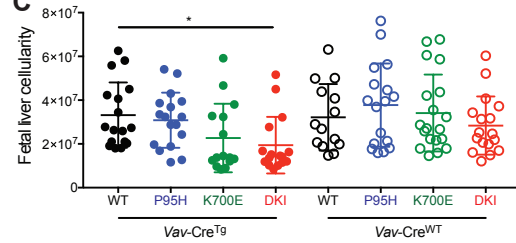
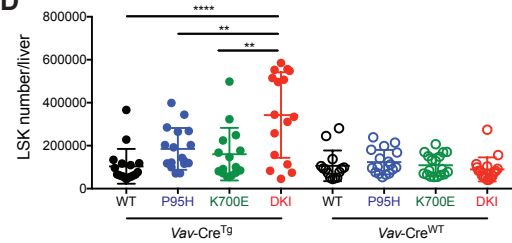
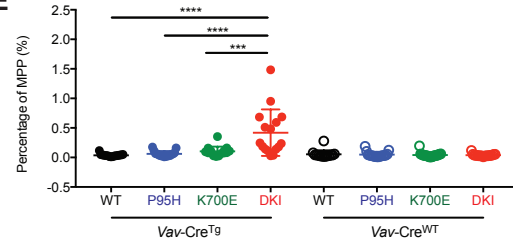
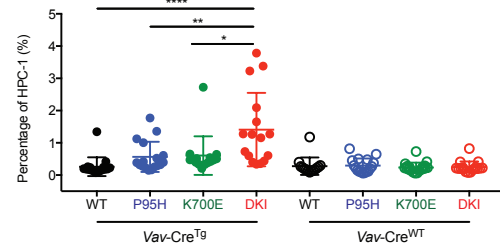
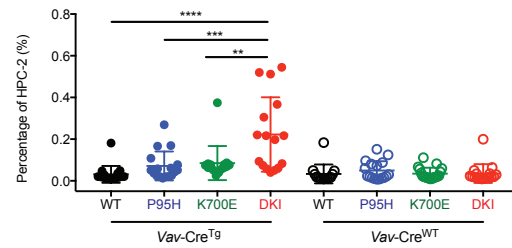
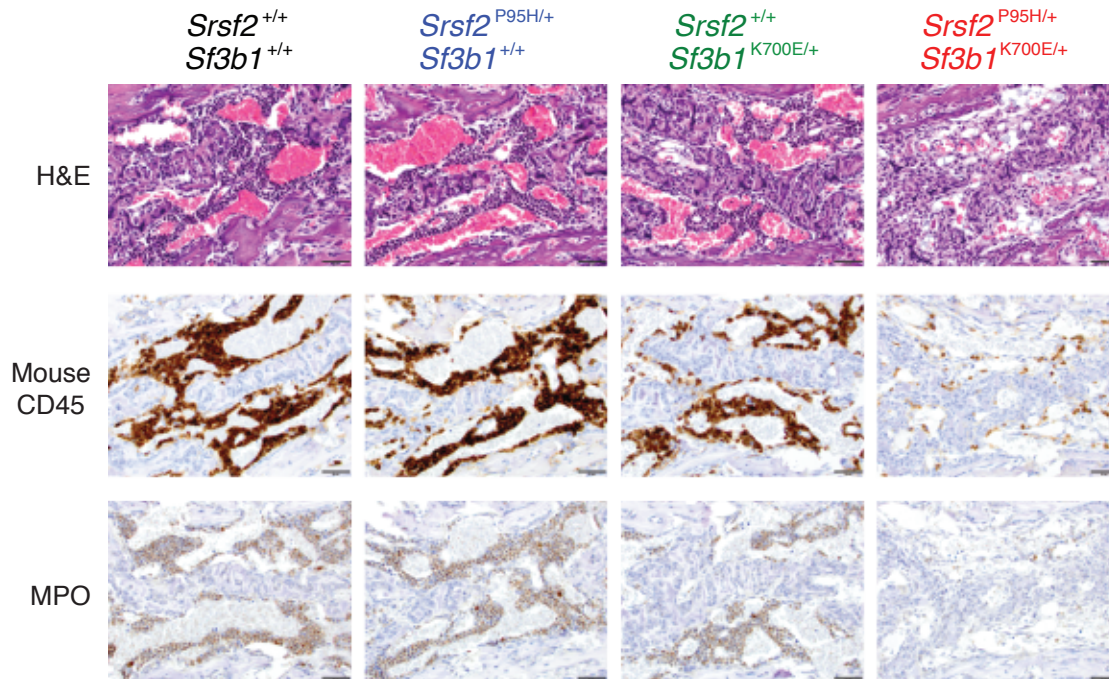
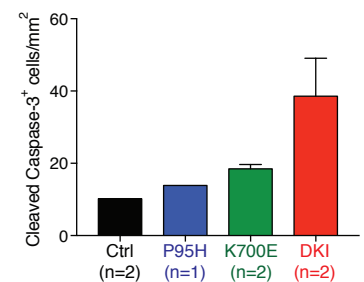
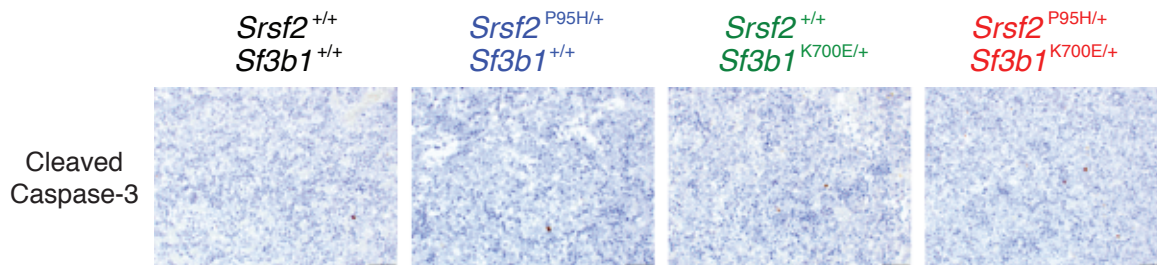
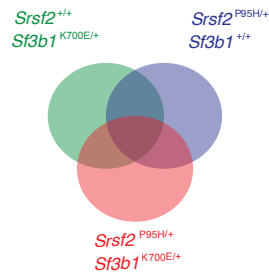
B**C****D****E****F****G****H****I**

Figure S4

A

	Genotypes Compared	Expected		Observed		p-value
		Convergent	Total	Convergent	Total	
A	<i>Srsf2</i> ^{+/+} <i>Sf3b1</i> ^{K700E/+} <i>Srsf2</i> ^{P95H/+} <i>Sf3b1</i> ^{K700E/+}	186	431	197	429	0.23
	<i>Srsf2</i> ^{P95H/+} <i>Sf3b1</i> ^{+/+} <i>Srsf2</i> ^{P95H/+} <i>Sf3b1</i> ^{K700E/+}	143	232	135	236	0.81
B	<i>Srsf2</i> ^{+/+} <i>Sf3b1</i> ^{K700E/+} <i>Srsf2</i> ^{P95H/+} <i>Sf3b1</i> ^{K700E/+}	108	248	90	241	0.90
	<i>Srsf2</i> ^{P95H/+} <i>Sf3b1</i> ^{+/+} <i>Srsf2</i> ^{P95H/+} <i>Sf3b1</i> ^{K700E/+}	161	385	161	378	0.44
C	<i>Srsf2</i> ^{+/+} <i>Sf3b1</i> ^{K700E/+} <i>Srsf2</i> ^{P95H/+} <i>Sf3b1</i> ^{K700E/+}	121	239	87	233	0.99
	<i>Srsf2</i> ^{P95H/+} <i>Sf3b1</i> ^{+/+} <i>Srsf2</i> ^{P95H/+} <i>Sf3b1</i> ^{K700E/+}	108	198	99	198	0.79



Definitions:

Expected - number of genes predicted to be differentially expressed assuming *Sf3b1* and *Srsf2* independence

Observed - number of genes observed to be differentially expressed in the data

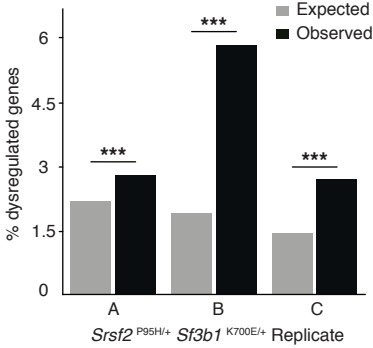
Convergent - number of differentially expressed genes shared by the the genotypes compared

Total - number of differentially expressed genes in the first genotype listed per category

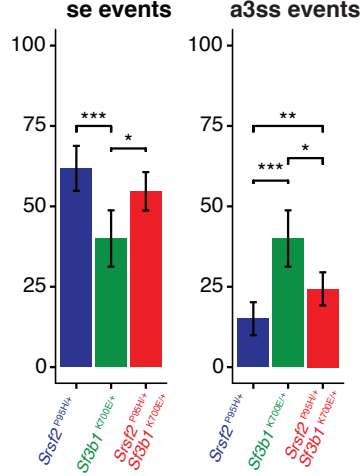
B

	Expected		Observed		p-value
	Dysregulated	Expressed Coding	Dysregulated	Expressed Coding	
A	217	11031	275	10943	3.6×10^{-3}
B	187	11007	563	10759	2.2×10^{-16}
C	143	11026	261	10793	5.5×10^{-10}

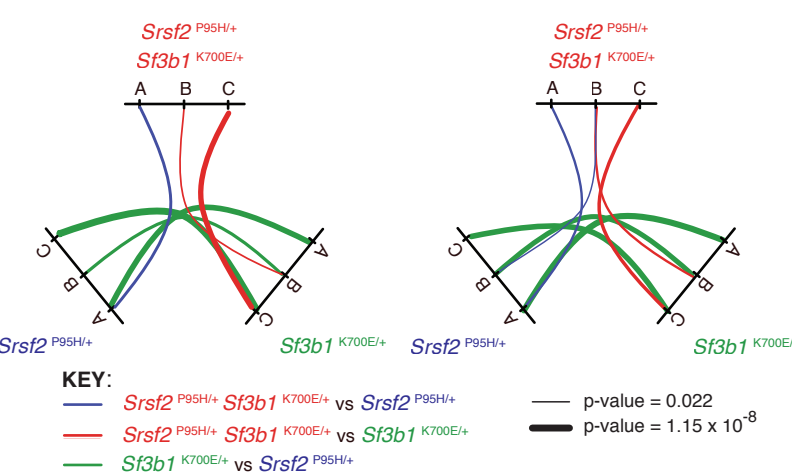
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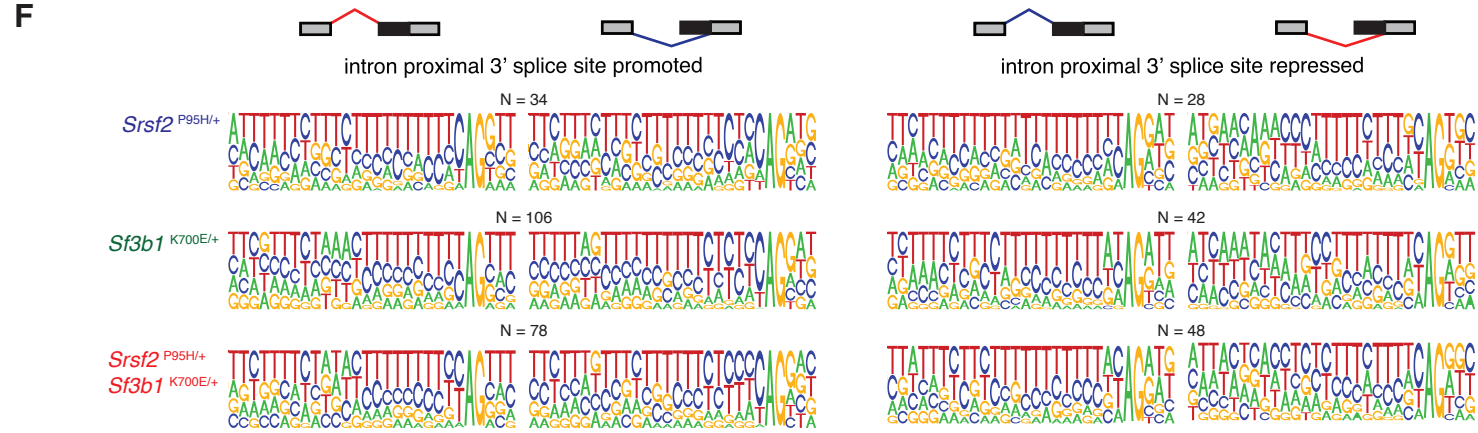
D



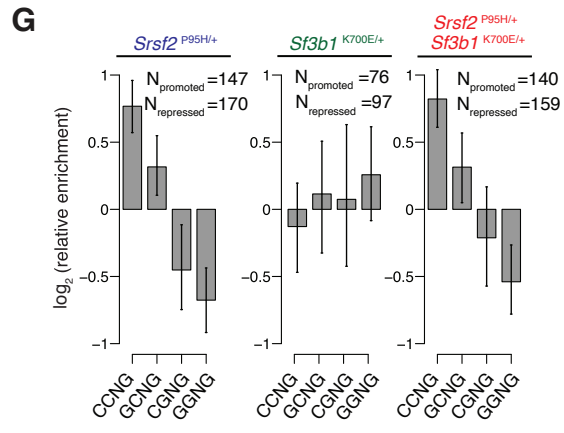
E



F



G



H

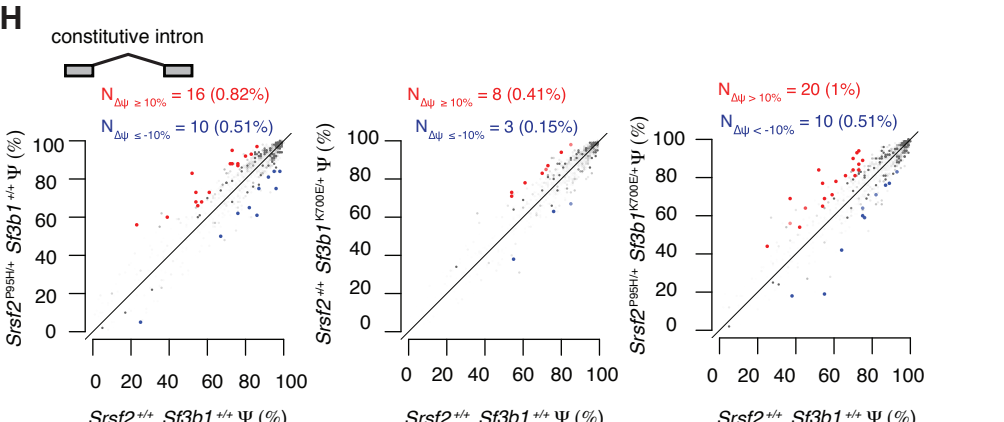
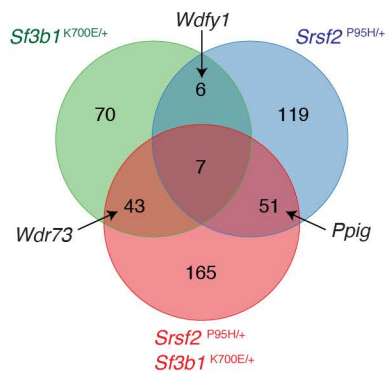
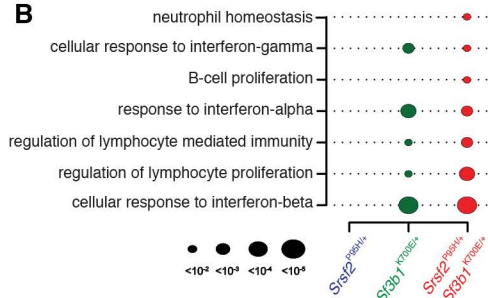


Figure S5

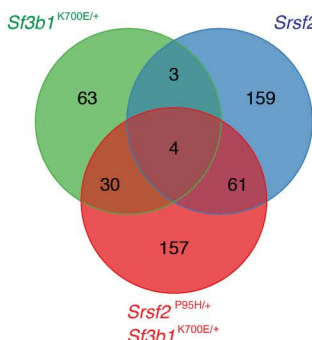
A



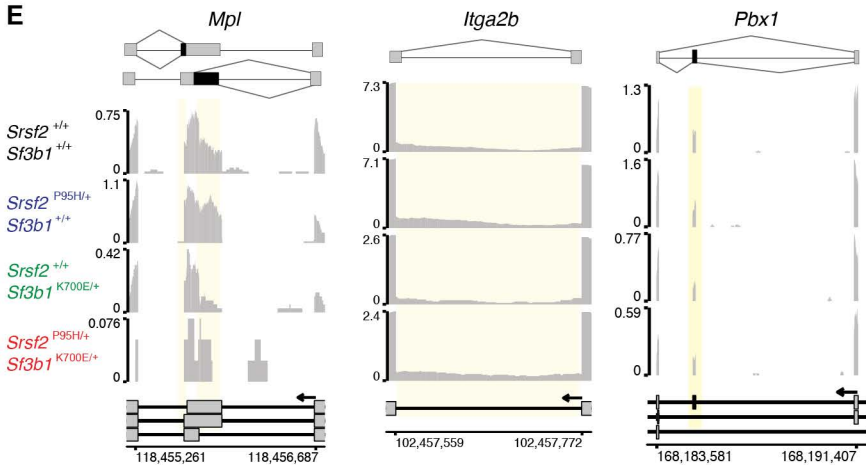
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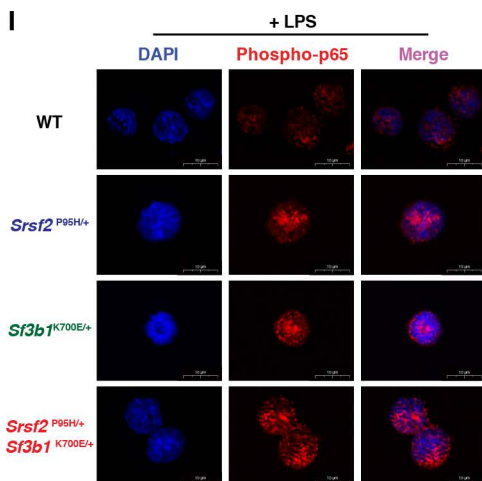
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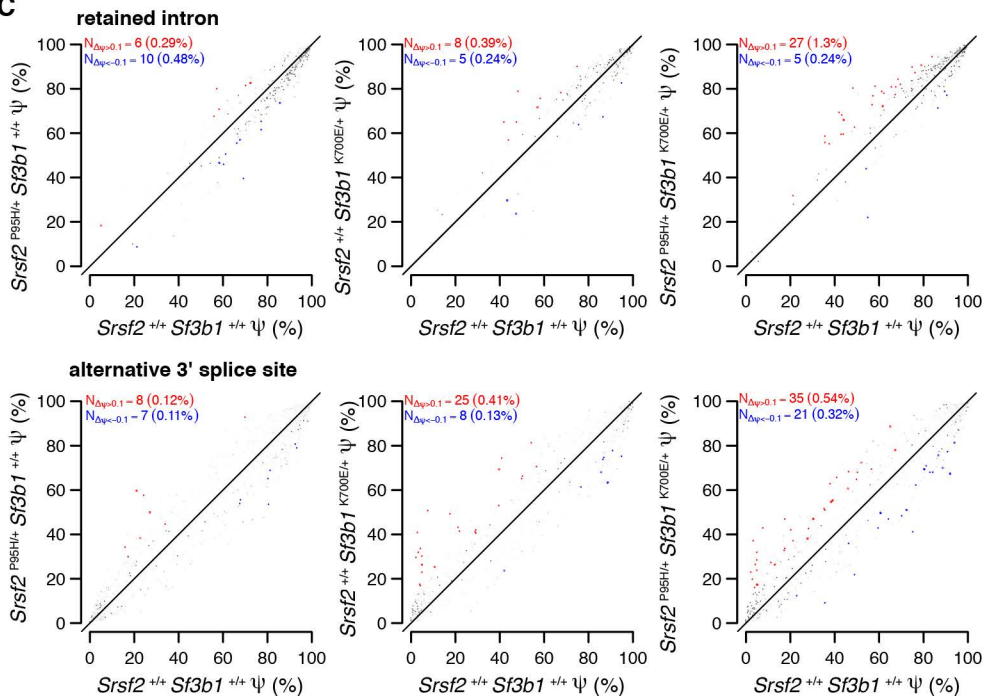
E



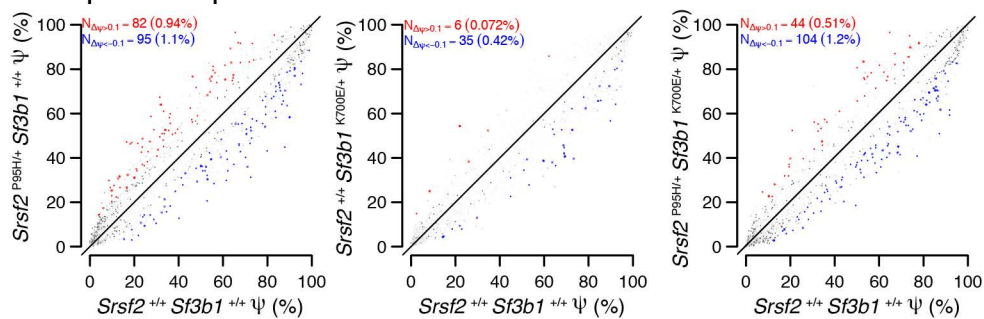
I



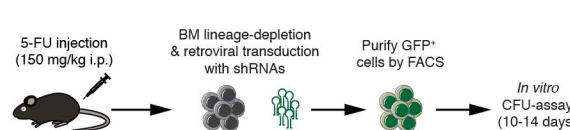
C



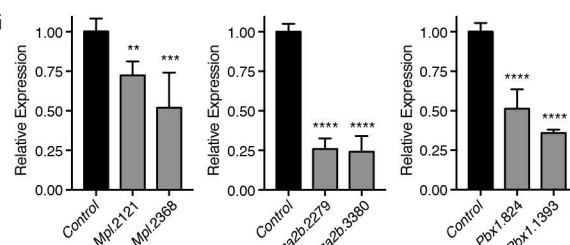
repressed and promoted exons



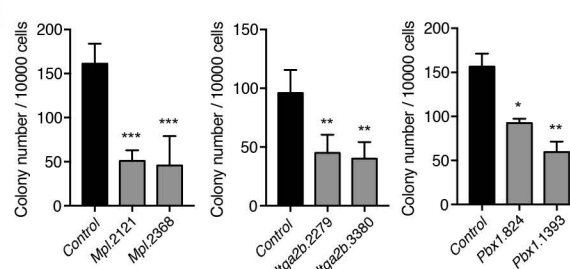
F



G



H



J

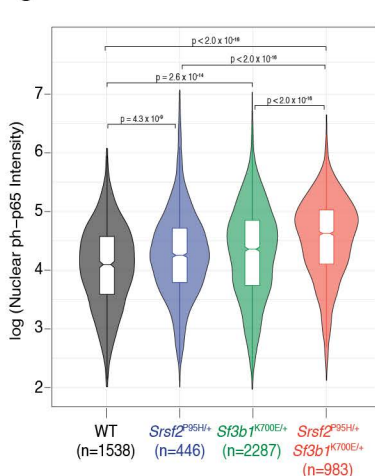


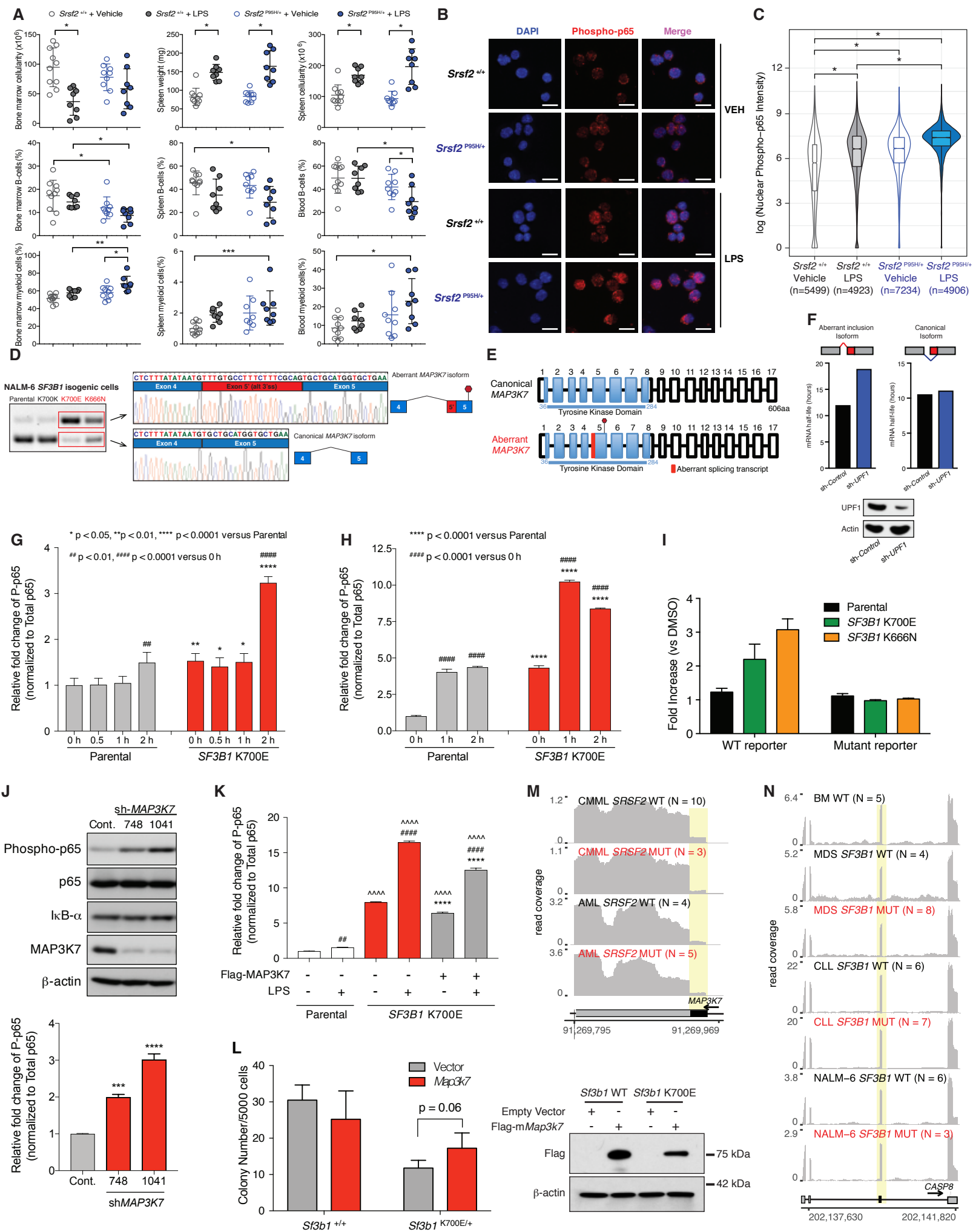
Figure S6

Figure S7

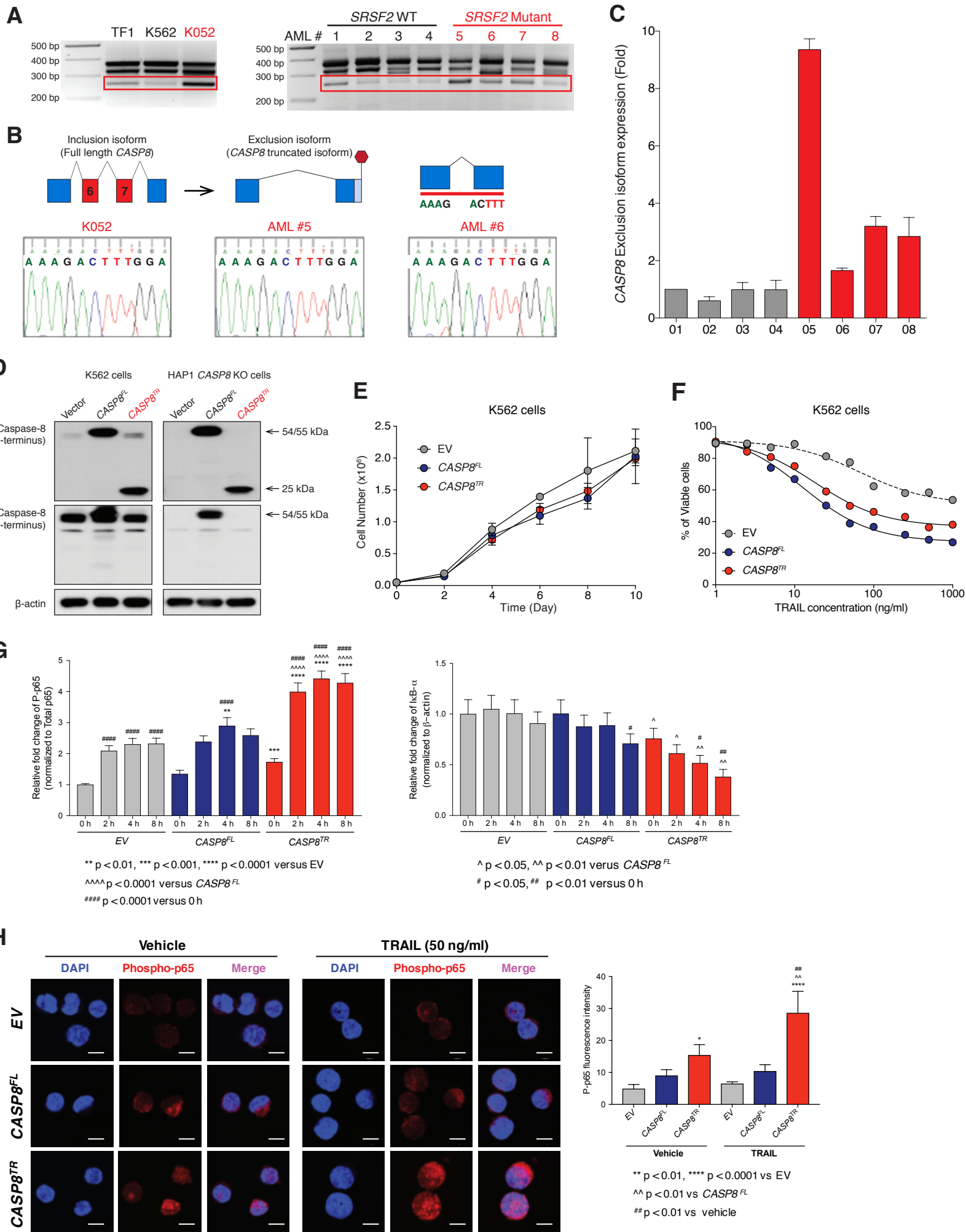
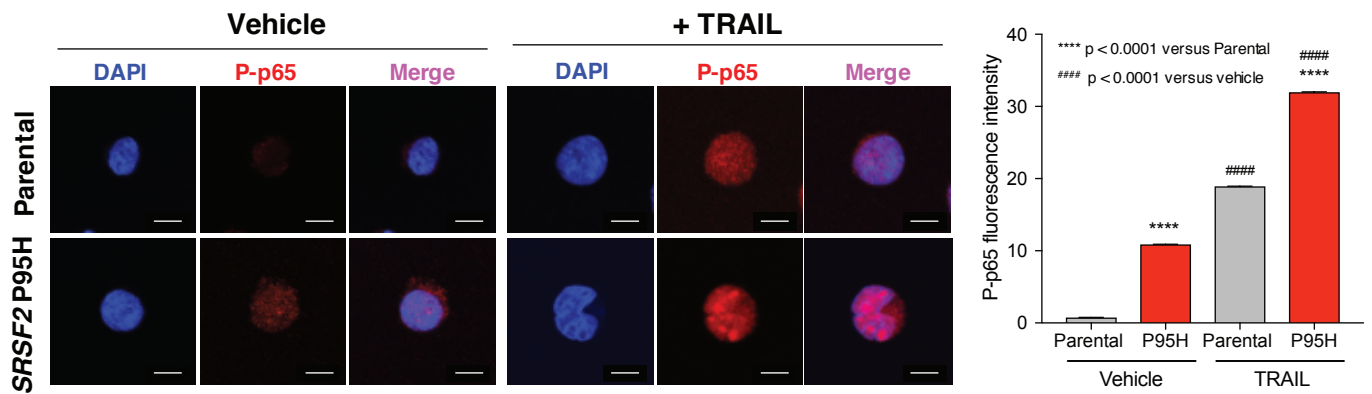
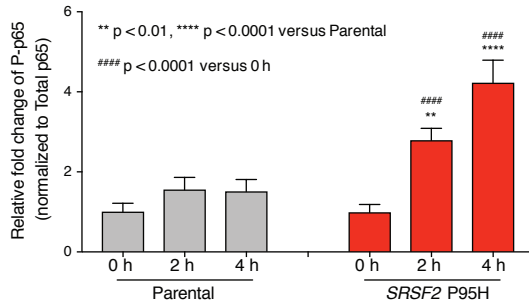


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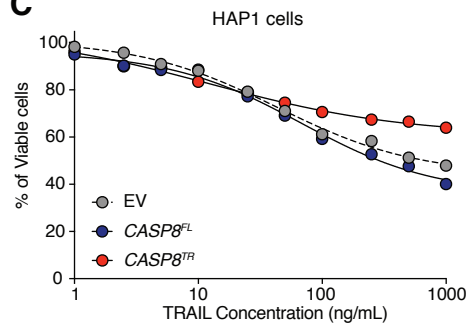
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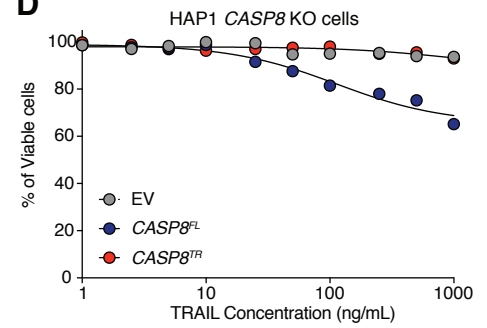
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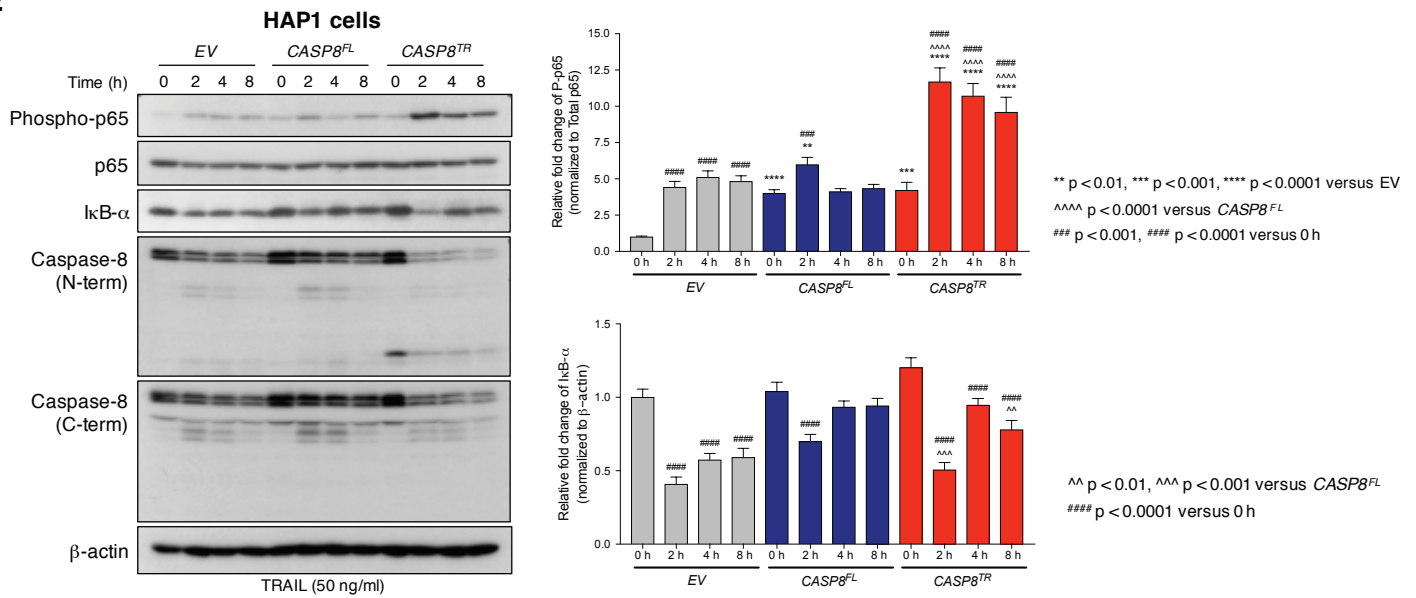
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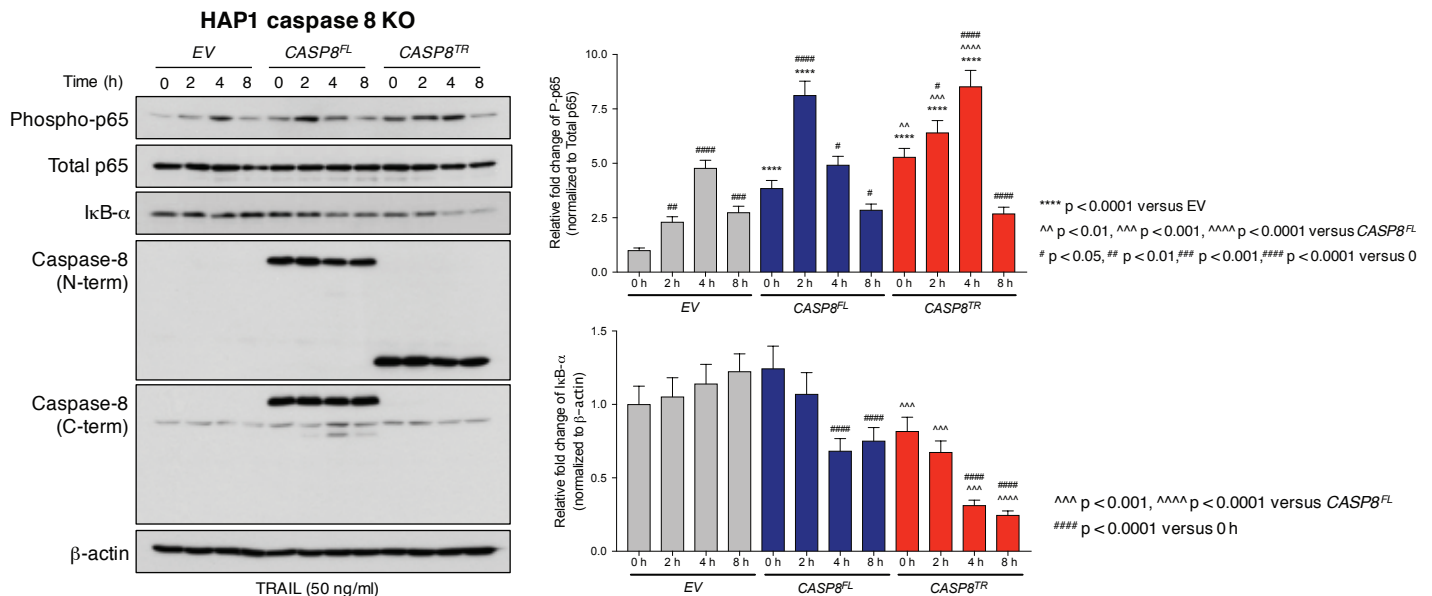
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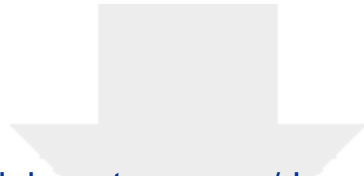


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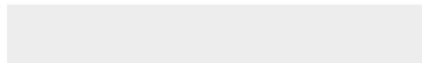
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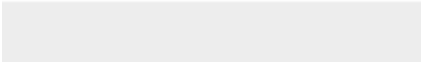
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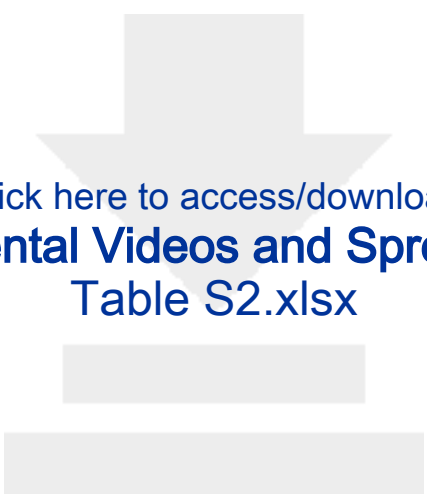




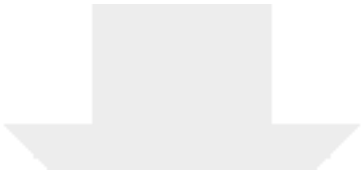
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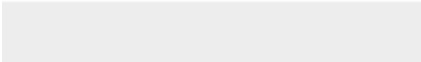


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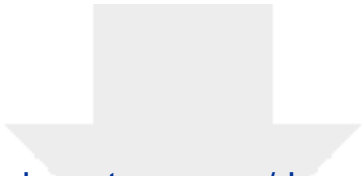
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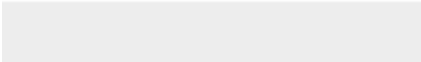


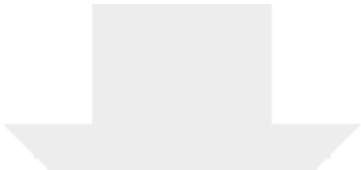
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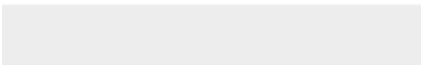
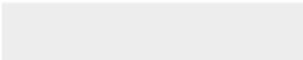
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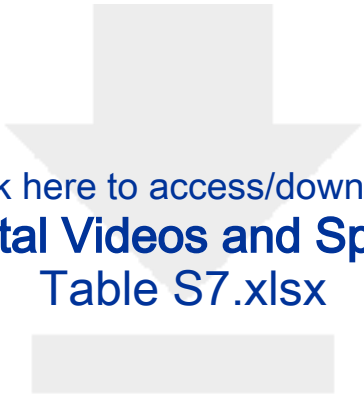




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H3B-8800, an orally available small-molecule splicing modulator, induces lethality in spliceosome-mutant cancers

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Genomic analyses of cancer have identified recurrent point mutations in the RNA splicing factor–encoding genes *SF3B1*, *U2AF1*, and *SRSF2* that confer an alteration of function^{1–6}. Cancer cells bearing these mutations are preferentially dependent on wild-type (WT) spliceosome function^{7–11}, but clinically relevant means to therapeutically target the spliceosome do not currently exist. Here we describe an orally available modulator of the SF3b complex, H3B-8800, which potently and preferentially kills spliceosome-mutant epithelial and hematologic tumor cells. These killing effects of H3B-8800 are due to its direct interaction with the SF3b complex, as evidenced by loss of H3B-8800 activity in drug-resistant cells bearing mutations in genes encoding SF3b components. Although H3B-8800 modulates WT and mutant spliceosome activity, the preferential killing of spliceosome-mutant cells is due to retention of short, GC-rich introns, which are enriched for genes encoding spliceosome components. These data demonstrate the therapeutic potential of splicing modulation in spliceosome-mutant cancers.

Heterozygous hotspot mutations in the genes encoding the RNA splicing factors splicing factor 3B subunit 1 (SF3B1), U2 small nuclear RNA auxiliary factor 1 (U2AF1), and serine and arginine rich splicing factor 2 (SRSF2) are among the most common mutations in patients with myelodysplastic syndromes (MDS)^{1,12}, chronic myelomonocytic leukemia (CMML)¹, and chronic lymphocytic leukemia (CLL)², and they also occur in a number of solid tumors (including breast⁶, lung⁴, uveal melanoma³, and pancreatic carcinomas⁵). RNA splicing is the process through which introns are removed from precursor mRNAs (pre-mRNAs) to produce mature mRNAs^{13,14}. Introns typically feature a short consensus sequence at the 5' splice site and three elements, namely the branchpoint sequence (BPS), a polypyrimidine tract (Py-tract), and an AG dinucleotide at the intron–exon junction,

at the 3' splice site. Recent work has demonstrated that mutations in the genes encoding SF3B1, U2AF1, and SRSF2 alter their functions in a manner distinct from that underlying loss of function^{15–17}. Specifically, *SF3B1* mutations induce cryptic 3' splice site selection through reduction of branchpoint fidelity^{17,18}. In contrast, mutations in *SRSF2* alter its RNA-binding preferences in a sequence-specific manner, thereby changing the efficiency of exon inclusion^{15,19}. Although the mechanisms through which these effects in RNA splicing promote cancer are still being investigated, several groups have demonstrated that cells with a mutant splicing factor are exquisitely sensitive to genetic or pharmacologic perturbation of splicing^{7–11}. However, the splice modulators used in these preclinical studies were not optimized to induce preferential lethality in spliceosome-mutant cells, are not orally bioavailable, and are not progressing for clinical development^{11,20}.

To address these issues, we specifically set out to identify orally bioavailable small molecules with preferential cytotoxicity in spliceosome-mutant cells. Through an iterative medicinal chemistry effort starting from a scaffold based on pladienolide, we identified the small molecule H3B-8800, which potently competed with pladienolide for binding to SF3b complexes containing either WT or mutant SF3B1 (Fig. 1a and Supplementary Fig. 1a). The competitive binding between pladienolide and H3B-8800 was similar for SF3b complexes containing WT SF3B1 and those containing each of eight different cancer-associated SF3B1 mutants (Fig. 1b, Supplementary Fig. 1b, and Supplementary Table 1). In accordance with these binding data and similarly to E7107, a pladienolide analog²⁰, H3B-8800 demonstrated dose-dependent inhibition of both canonical splicing and aberrant splicing induced by mutant SF3B1 in *in vitro* assays of biochemical splicing (Fig. 1c,d and Supplementary Fig. 1c,d). These results indicate that H3B-8800 potentially binds and inhibits splicing catalysis by both WT SF3B1 and its mutant forms *in vitro*.

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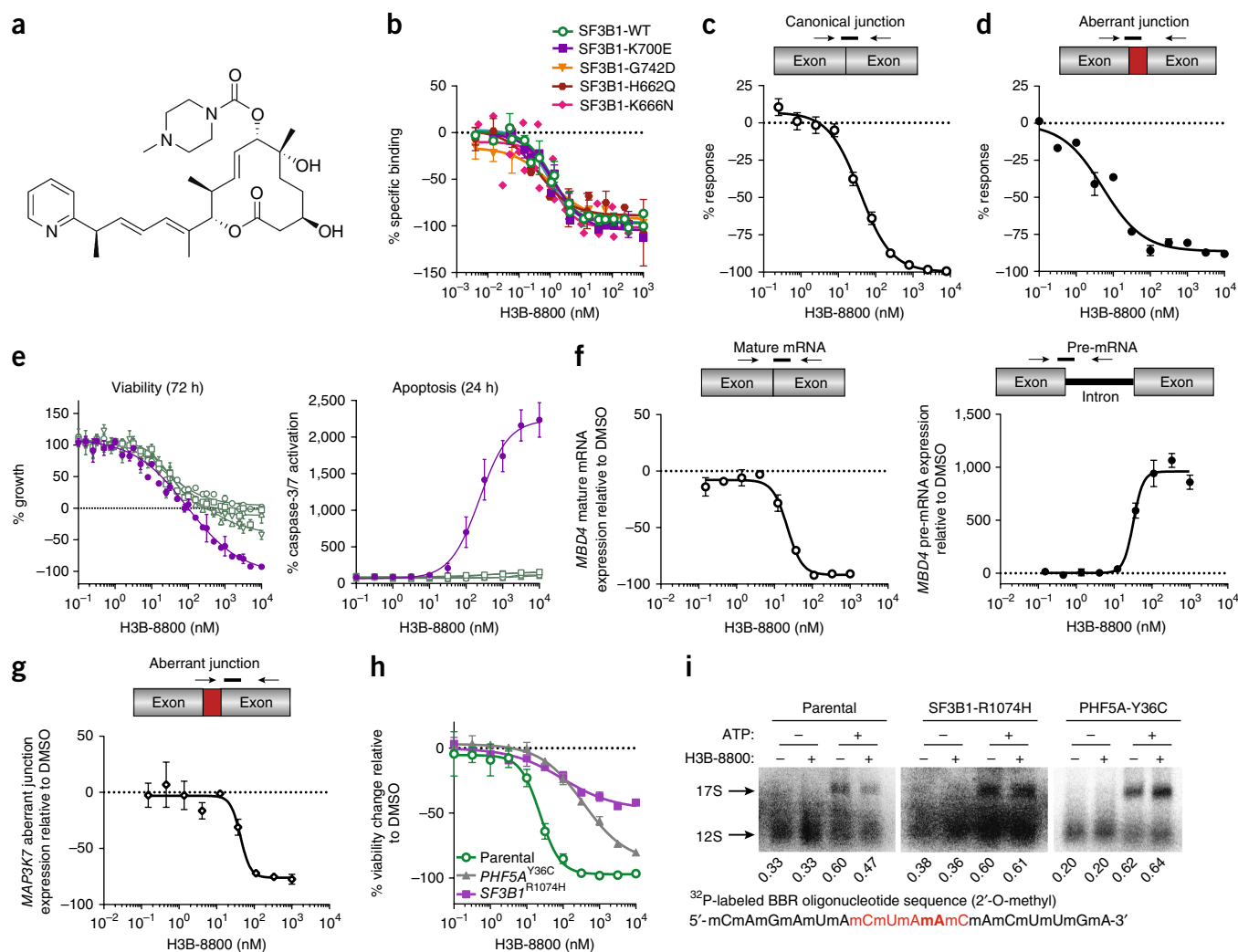


Figure 1 H3B-8800 modulates splicing of WT and mutant SF3B1 spliceosomes *in vitro* and preferentially kills SF3B1-mutant cells. **(a)** The chemical structure of H3B-8800. **(b)** Competitive binding of increasing concentrations of H3B-8800 to SF3b complexes isolated from cells overexpressing WT or mutant SF3B1 in the presence of 1 nM pladienolide B. The y axis represents the percentage change (% response) of specific binding relative to the DMSO control of 0%. Data are represented as mean $\pm$ s.d.; $n = 3$ experimental replicates. **(c,d)** Quantification of canonical **(c)** and aberrant **(d)** junctions using the Ad2-ZDHC16 pre-mRNA substrate, in which the original Ad2 intron was substituted with intron 9 of *ZDHC16*, in an *in vitro* splicing assay using nuclear extracts from 293FT cells overexpressing SF3B1^{K700E} and treated with increasing concentrations of H3B-8800. The y axis represents the percent response relative to the DMSO control (0%). Data are represented as mean $\pm$ s.d.; $n = 3$ technical replicates. **(e)** Viability and apoptosis of pancreatic cells bearing WT (Panc10.05, HAPFII, CFPAC1, and Panc04.03 in green) or mutant (Panc05.04 in violet) SF3B1 treated with increasing concentrations of H3B-8800. Data are represented as mean $\pm$ s.d.; $n = 3$ technical replicates. **(f,g)** Effect of increasing concentrations of H3B-8800 on splicing of *MBD4* mature mRNA (open circles) and *MBD4* pre-mRNA (black circles) **(f)** and *MAP3K7* aberrant junction expression (open diamonds) **(g)** in Panc05.04 cells as quantified by TLDA after 6 h of treatment with H3B-8800. Data are represented as mean $\pm$ s.e.m.; $n = 3$ technical replicates. **(h)** Viability of HCT116 parental cells and clones carrying the pladienolide-resistance mutation SF3B1^{R1074H} or PHF5A^{Y36C}. Data are represented as mean $\pm$ s.d.; $n = 3$ technical replicates. **(i)** Binding of U2 snRNA to the canonical branchpoint sequence (2'-O-methyl BBR oligonucleotide in red with the branchpoint adenosine bolded) in nuclear extracts from the same HCT116 cells as in **h**. Nuclear extracts were incubated in the presence or absence of ATP and H3B-8800; band quantification is shown below each lane. The functional 17S U2 snRNP was formed via ATP-dependent binding of the SF3b complex to the nonfunctional 12S U2 snRNP particle.

We next investigated the cellular activity of H3B-8800 using a panel of SF3B1 WT or mutant pancreatic cancer cell lines. H3B-8800 treatment for 72 h resulted in cellular lethality only in the SF3B1-mutant cell line Panc05.04; this lethality was accompanied by caspase-3/7 cleavage as early as 24 h following incubation (**Fig. 1e**). In contrast, E7107 did not show clear differential effects in the same cell panel (**Supplementary Fig. 1e**), suggesting that H3B-8800 has increased selectivity for SF3B1-mutant cells. Next, we tested the ability of H3B-8800 to modulate canonical and aberrant splicing in Panc05.04 (SF3B1-K700E) cells using a TaqMan Low Density Array

(TLDA), which is designed to evaluate expression of pre-mRNA and mature mRNA isoforms impacted by SF3B1 modulation as well as aberrant RNA splicing events induced by mutations in SF3B1¹⁷ (Online Methods). H3B-8800 modulated both canonical and aberrant splicing catalysis as revealed by a dose-dependent decrease in the levels of mature methyl-CpG binding domain 4 (*MBD4*) mRNA and an accumulation of *MBD4* pre-mRNA in addition to decreased SF3B1-mutant-induced aberrant splicing of *MAP3K7* (**Fig. 1f,g**). Similar results were observed for additional mRNAs in the pancreatic cell lines tested as well as for K562 cells expressing SF3B1^{WT} or SF3B1^{K700E} at the SF3B1

endogenous locus (Supplementary Fig. 2). In addition, H3B-8800, but not E7107, demonstrated preferential killing of K562 cells bearing *SF3B1*^{K700E} as compared to their isogenic *SF3B1*^{WT} counterparts (Supplementary Fig. 1f).

To verify the selectivity of H3B-8800 for the SF3b complex, we tested the activity of H3B-8800 in HCT116 cells with acquired resistance to pladienolides²¹ due to mutations in *SF3B1* or *PHF5A*, which encode components of the SF3b complex involved in branchpoint recognition²². *SF3B1*-R1074H or *PHF5A*-Y36C mutants conferred resistance to H3B-8800, whereas resistance to bortezomib, a compound whose mechanistic effects are not splicing dependent, was not observed in cells with these mutants (Fig. 1h and Supplementary Fig. 1g). These data indicate the selectivity of H3B-8800 for the SF3b complex and suggest that H3B-8800 may function through interfering with the interaction between the SF3b complex and the branchpoint. To test this hypothesis, we incubated nuclear extract from HCT116 parental cells with H3B-8800, ATP, and a ³²P-2'-O-methyl oligonucleotide complementary to the U2 small nuclear RNA (snRNA) branchpoint binding region (BBR). H3B-8800 inhibited ATP-dependent 17S U2 small nuclear ribonucleoprotein (snRNP) complex formation (Fig. 1i), providing evidence that H3B-8800 abolishes precatalytic spliceosome assembly, as was previously proposed for E7107 (ref. 23). This inhibition was not observed for nuclear extracts from the two H3B-8800-resistant cell lines (Fig. 1i), suggesting that H3B-8800 modulates splicing through interfering with the interaction between the SF3b complex and the branchpoint region.

We next tested the antitumor effects of H3B-8800 *in vivo* in NOD-SCID interleukin-2 receptor gamma (*IL2Rγ*)^{null} (NSG) mice bearing xenografts of isogenic K562 cells with *SF3B1*^{WT} or *SF3B1*^{K700E} (p.K700E was introduced through genetic modification of the endogenous *SF3B1* locus). Oral administration of 2 or 4 mg H3B-8800 per kg body weight (mg/kg) daily slowed the growth of xenografts with *SF3B1*^{K700E} ($P < 0.003$, two-way ANOVA followed by Dunnett's multiple comparison test) but had no effect on *SF3B1*^{WT} xenografts. Although a dose of 8 mg/kg did slow the growth of *SF3B1*^{WT} tumors, it completely abrogated growth of *SF3B1*^{K700E} tumors ($P < 0.003$; Fig. 2a,b). H3B-8800 similarly demonstrated significant antitumor activity against xenografts of human HNT-34 acute myeloid leukemia (AML) cells bearing the endogenous mutation encoding *SF3B1*-K700E (Supplementary Fig. 3a). H3B-8800 reached similar concentrations in plasma as well as in tumors of mice harboring *SF3B1*^{WT} or *SF3B1*^{K700E} xenografts, and H3B-8800 modulated both canonical and aberrant splicing in a dose-dependent manner (Fig. 2c and Supplementary Fig. 4). Splicing modulation was observed as early as 1 h following treatment, and splicing returned to pretreatment levels within 24 h following treatment, in accordance with the H3B-8800's pharmacokinetic profile (Fig. 2c).

H3B-8800 treatment resulted in antileukemic efficacy and splicing modulation in mice bearing AML patient-derived xenografts (PDXs) with a mutation in *SF3B1* but had little effect in mice bearing *SF3B1*^{WT} AML PDXs. In this experiment, NSGS mice (NSG mice with transgenic expression of human granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, and SCF) were engrafted with AML cells from a patient with AML harboring *SF3B1*^{K700E}, *NRAS*^{A59T}, and *inv(3)(q21q26.2)* or from a patient with AML harboring variations in *KRAS*, *WT1*, and *MLL1* but lacking any mutations in spliceosome-related genes (Supplementary Table 2). In mice engrafted with the *SF3B1*^{K700E} PDX, 10 d of H3B-8800 treatment significantly reduced leukemic burden relative to that in vehicle-treated mice, but this treatment had negligible effects in the *SF3B1*^{WT} PDX recipients (Fig. 2d–g

and Supplementary Fig. 5a–c). Analysis of human leukemic cells purified from the mice 3 h after the last dose of H3B-8800 revealed reduced mature mRNA levels and accumulation of pre-mRNA resulting from canonical splicing events in all H3B-8800-treated mice, regardless of *SF3B1* genotype. In contrast, reduced levels of aberrant splicing events induced by a mutation in *SF3B1* were observed only in the *SF3B1*-mutant model (Fig. 2h and Supplementary Fig. 5d).

Given the activity and preferential efficacy of H3B-8800 on *SF3B1*-mutant human AML xenografts *in vivo*, we next tested H3B-8800 in mouse models with mutated *SRSF2*. After observing clear antitumor activity of H3B-8800 in mice bearing xenografts comprising *SRSF2*^{P95H} K052 cells (Supplementary Fig. 3b), we generated six PDX models derived from patients with CMML, three of whom harbored *SRSF2* mutations (Supplementary Table 2). A PDX model from each patient was generated, and the models were randomized to receive vehicle or H3B-8800 (8 mg/kg orally for 10 d) once human CD45 (hCD45)⁺ cells in the peripheral blood made up >3% of total mononuclear cells or the concentration of hemoglobin was ≤11 g/dl (Fig. 3a). Of note, leukemic burden was equivalent in mice bearing *SRSF2*^{P95H} tumors and those with *SRSF2*^{WT} tumors at the onset of treatment (Supplementary Fig. 6a). H3B-8800 treatment reduced human leukemic burden in peripheral blood and all organs examined in PDX models with *SRSF2*^{P95H} but not in those with *SRSF2*^{WT} (Fig. 3b–h and Supplementary Figs. 6b and 7). Moreover, we observed splenomegaly and hepatomegaly due to leukemic cell infiltration, and H3B-8800 treatment for 10 d substantially reduced spleen and liver size only in the *SRSF2*^{P95H} PDX models (Fig. 3d,e). In accordance with the reduced number of mature leukemic cells observed in H3B-8800-treated PDX models of CMML bearing a mutant spliceosome, but not in those with a WT spliceosome, H3B-8800 treatment reduced the number of immunophenotypically defined leukemia-initiating cells only in spliceosome-mutant PDX models of CMML (Supplementary Fig. 8). Evaluation of RNA splicing in hCD45⁺ cells 3 h after the last dose of vehicle or H3B-8800 revealed that *in vivo* H3B-8800 treatment modulated aberrant splicing mediated by mutant *SRSF2* (Fig. 3i,j). In addition, H3B-8800 treatment resulted in decreased levels of mature *MBD4* mRNA and accumulation of *MBD4* pre-mRNA (Fig. 3j) as noted for the cell lines treated with H3B-8800 (Fig. 1f). These data further suggest the efficacy and preferential activity H3B-8800 in treating spliceosome-mutant malignancy *in vivo*.

To further understand the preferential effects of H3B-8800 on spliceosome-mutant cells, we performed RNA-seq analysis of isogenic *SF3B1*^{WT} and *SF3B1*^{K700E} K562 cells treated with DMSO or H3B-8800. We observed enrichment of alternative 3' splice sites in DMSO-treated K562 *SF3B1*^{K700E} cells as compared to DMSO-treated *SF3B1*^{WT} cells (Supplementary Fig. 9a and Supplementary Table 3), consistent with prior reports on the effects of *SF3B1* mutations on splicing^{17,18}. At a concentration of 13 nM, the half-maximal inhibitory concentration (IC₅₀) of H3B-8800 in a cell viability assay at 72 h following treatment (Supplementary Fig. 1f), H3B-8800 treatment for 6 h primarily induced exon-skipping and intron-retention events in K562 cells expressing *SF3B1*-K700E as compared to untreated (DMSO control) cells (Supplementary Fig. 9c and Supplementary Table 3). Genes in which splicing events in these categories occurred ($n = 4,353$) were more likely to show downregulated expression following H3B-8800 treatment as compared to DMSO treatment ($P = 2.1 \times 10^{-26}$, binomial proportion test; Supplementary Fig. 9d,e and Supplementary Table 4). Expression of genes with intron-retention events that were predicted to induce degradation through the nonsense-mediated decay (NMD) pathway were further downregulated as compared to

genes with other types of intron-retention events ($P = 6.37 \times 10^{-22}$, Kruskal–Wallis H test; **Supplementary Fig. 9h**). Notably, when we compared the effects of treatment with 13 nM H3B-8800 in K562 cells with *SF3B1*^{K700E} and in those with *SF3B1*^{WT}, we observed few effects on alternative 3' splice site usage but a clear increase in exon-skipping

and intron-retention events; however, the magnitude of these changes was minimal overall (75th quartile < $(2 \times \log_2(\text{fold change}))$; **Supplementary Fig. 9b** and **Supplementary Table 4**). These results suggest that the splicing changes induced by H3B-8800 treatment are similar in WT and *SF3B1*^{K700E} K562 cells.

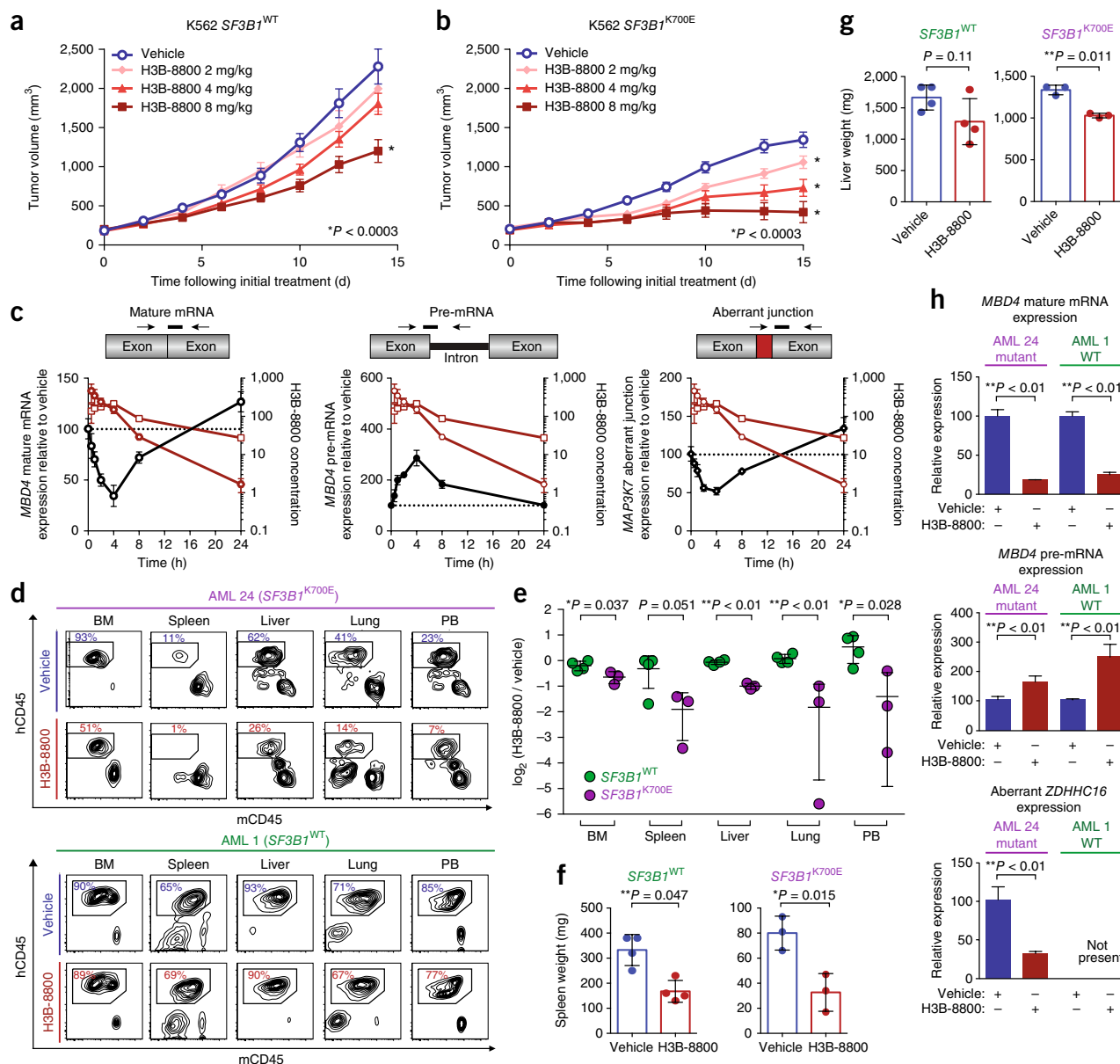


Figure 2 H3B-8800 modulates splicing and selectively kills *SF3B1*-mutant leukemia cells *in vivo*. (**a,b**) Mean tumor volume in NSG mice subcutaneously implanted with K562 isogenic cells with (**b**) or without (**a**) endogenous mutations in *SF3B1*. Mice were orally treated with vehicle or H3B-8800 daily at the indicated doses. Mean tumor volumes $\pm$ s.e.m. are shown; $n = 8$ mice per group. (**c**) Splicing of *MBD4* mature mRNA (black open circles), *MBD4* pre-mRNA (black circles), and *MAP3K7* aberrant junction expression (black open diamonds) in K562 *SF3B1*^{K700E} tumors after a single oral dose of H3B-8800 (8 mg/kg) as quantified by TLDA. The drug concentration in H3B-8800-treated mice was determined in plasma (red open circles) and tumor (red open squares). Data are represented as mean $\pm$ s.e.m.; $n = 4$ mice per group. The H3B-8800 concentration is shown in ng/ml for plasma and ng/g for tumor. (**d**) Results from flow cytometry analysis of human versus mouse hematopoietic cells (hCD45 cells versus mCD45 cells) in bone marrow (BM), spleen, liver, lung, and peripheral blood (PB) of PDX-bearing mice with (top) or without (bottom) mutation in *SF3B1* following 10 d of vehicle or H3B-8800 treatment (8 mg/kg). Cells were gated on DAPI⁺Ter119⁺ cells, and the percentages of live Ter119⁺ cells are shown. (**e**) Quantification of human leukemic cells (hCD45), expressed as log₂ of the percentage of hCD45⁺ cells in H3B-8800-treated mice divided by the percentage of hCD45⁺ cells in vehicle-treated mice, in each tissue compartment in all PDX models treated with vehicle or H3B-8800. Data are represented as mean $\pm$ s.d.; $n = 3$ mice for *SF3B1*^{K700E} and 4 for *SF3B1*^{WT}. (**f,g**) Spleen (**f**) and liver (**g**) weight in PDX mice treated with vehicle or H3B-8800 (8 mg/kg). Data are represented as mean $\pm$ s.d.; $n = 3$ mice for *SF3B1*^{K700E} and 4 for *SF3B1*^{WT}. (**h**) Effects of H3B-8800 (8 mg/kg) on mature mRNA and pre-mRNA *MBD4* levels (canonical splicing) and aberrant *ZDHHC16* splicing in human cells isolated from PDX mice. The cells were isolated 3 h after the final dose of H3B-8800. Data are represented as mean $\pm$ s.d.; $n = 3$ mice per group. All P values were calculated using a two-tailed Student's *t*-test.

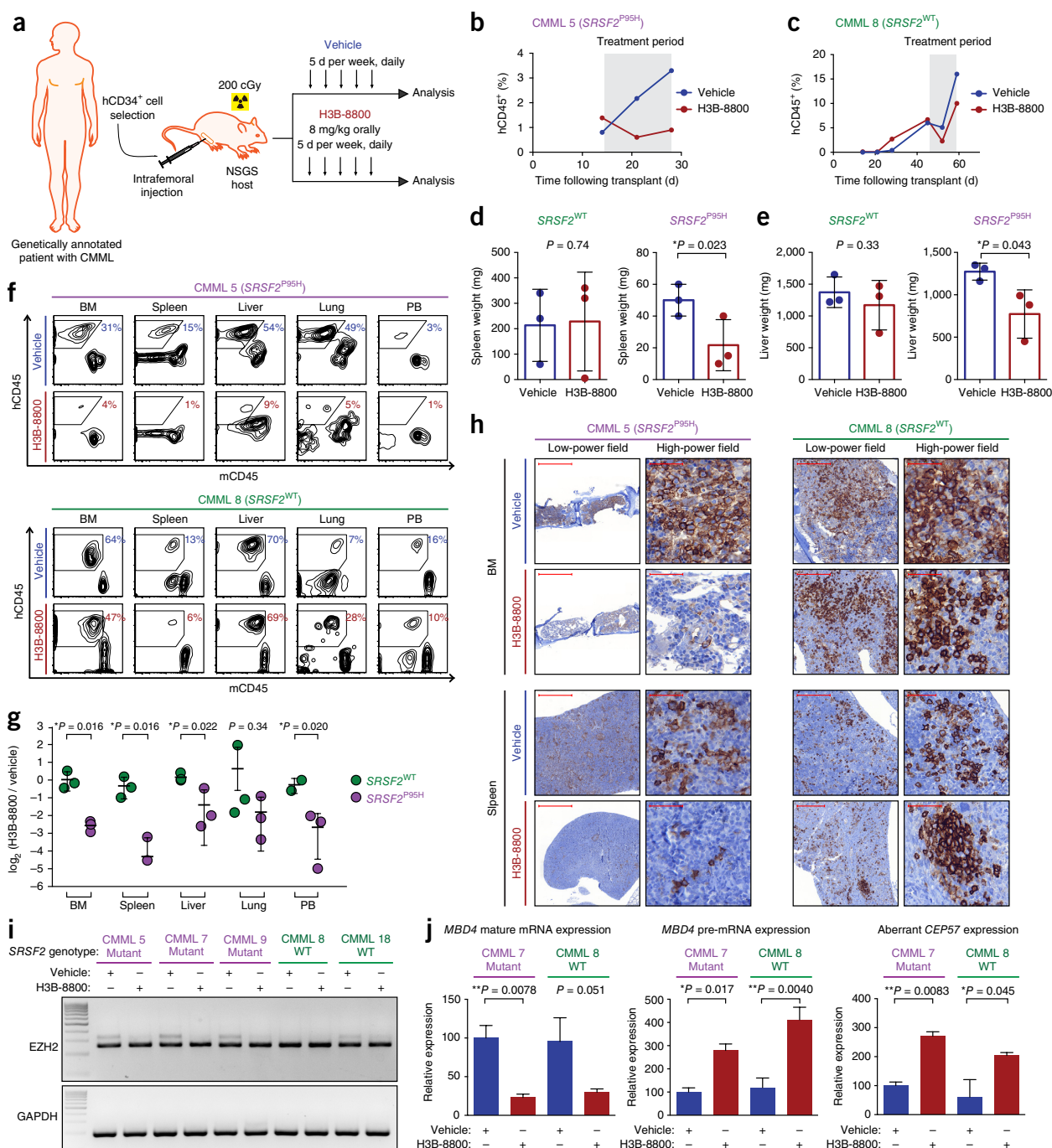


Figure 3 H3B-8800 demonstrates preferential activity on SRSF2-mutant leukemia in PDX mice. (a) Schematic of H3B-8800 testing in CMML PDX mice. Mice were treated orally with 8 mg/kg of H3B-8800 or vehicle for 10 d (in 2 intervals of 5 d each, separated by 2 d). Each sample was engrafted into two NSGS recipient mice, which were then treated with vehicle or H3B-8800 once human CD45⁺ (hCD45⁺) cells in peripheral blood were >3% of total peripheral blood mononuclear cells or hemoglobin was ≤11 g/dl. The mice were killed 2 h after the last dose and were analyzed. (b,c) The effect of H3B-8800 on the percentage of peripheral blood hCD45⁺ cells in spliceosome-mutant (b) and WT (c) PDX models engrafted with an individual spliceosome-mutant or WT tumor (patient numbering is shown in **Supplementary Table 2**). The treatment period is marked in gray. (d,e) The effect of H3B-8800 on spleen (d) and liver (e) weight in spliceosome-mutant and WT PDX models bearing tumors derived from 3 patients per genotype. Data are represented as mean ± s.d.; *n* = 3 mice per group. (f) Flow cytometric analysis of hCD45 versus mCD45 cells in bone marrow, spleen, liver, lung, and peripheral blood of vehicle- or H3B-8800-treated mice engrafted with an individual spliceosome-mutant or WT tumor (cells were gated on DAPI-mTer119⁻ cells, and the percentages of live Ter119⁻ cells are shown). (g) Quantification of the proportion of human leukemic cells in H3B-8800- versus vehicle-treated mice (expressed as log₂ of the percentage of hCD45⁺ cells in H3B-8800-treated mice divided by the percentage of hCD45⁺ cells in vehicle-treated mice) in each tissue compartment in all PDX models. Data are represented as mean ± s.d.; *n* = 3 mice per group. (h) Immunohistochemical stains for hCD45⁺ cells in bone marrow or spleen from H3B-8800- versus vehicle-treated mice. Scale bars, 1,000 μm (CMML 5 low-power field), 200 μm (CMML 8 low-power field) and 50 μm (high-power field). (i,j) *EZH2* aberrant junction formation as assessed through RT-PCR (i), and mature mRNA and pre-mRNA *MBD4* levels as well as levels of *CEP57* aberrant junction expression (j) in cDNA from hCD45⁺ cells isolated 3 h following the last dose of vehicle or H3B-8800. Data are represented as expression relative to GAPDH; mean ± s.d.; *n* = 3 technical replicates. All *P* values were calculated using a two-tailed Student's *t* test.

LETTERS

Considering that H3B-8800 has preferential cytotoxic effects on spliceosome-mutant cells as compared to spliceosome-WT cells despite similar overall effects on splicing as observed in the RNA-seq analysis, we next sought to understand the effects of H3B-8800 on splicing in more detail. Characterization of introns retained in K562 *SF3B1*^{K700E} cells following treatment with H3B-8800 revealed that 27.2% of all significantly retained introns ($q < 0.05$) were short (≤ 300 nt) and GC rich ($\geq 60\%$ GC content) relative to 6.1% in all genes with detectable spliced introns ($P = 2.2 \times 10^{-16}$; Fisher's exact test; **Fig. 4a**). A similar preferential retention of short, GC-rich introns was also seen following H3B-8800 treatment of NALM-6 *SF3B1*^{K700E} cells, an effect that was much less evident for E7107 treatment at a similar

concentration (**Supplementary Fig. 9f**). A recent report suggested that short, GC-rich introns represent a specialized class of introns characterized by minimal differences in GC content between the intron and the following exon²⁴. We therefore examined these features in introns retained by H3B-8800 versus all introns from expressed genes. In the results from this analysis, introns retained after H3B-8800 treatment were enriched for GC content along their length, and there was an equal enrichment for GC content along the length of the following exon, with local enrichment in GC content at the 5' and 3' splice sites (**Fig. 4b**). The largest divergence in GC content from all genes with detectable spliced introns occurred at the 3' splice site, where the U2AF2-binding Py-tract is found²⁵. This result implies that

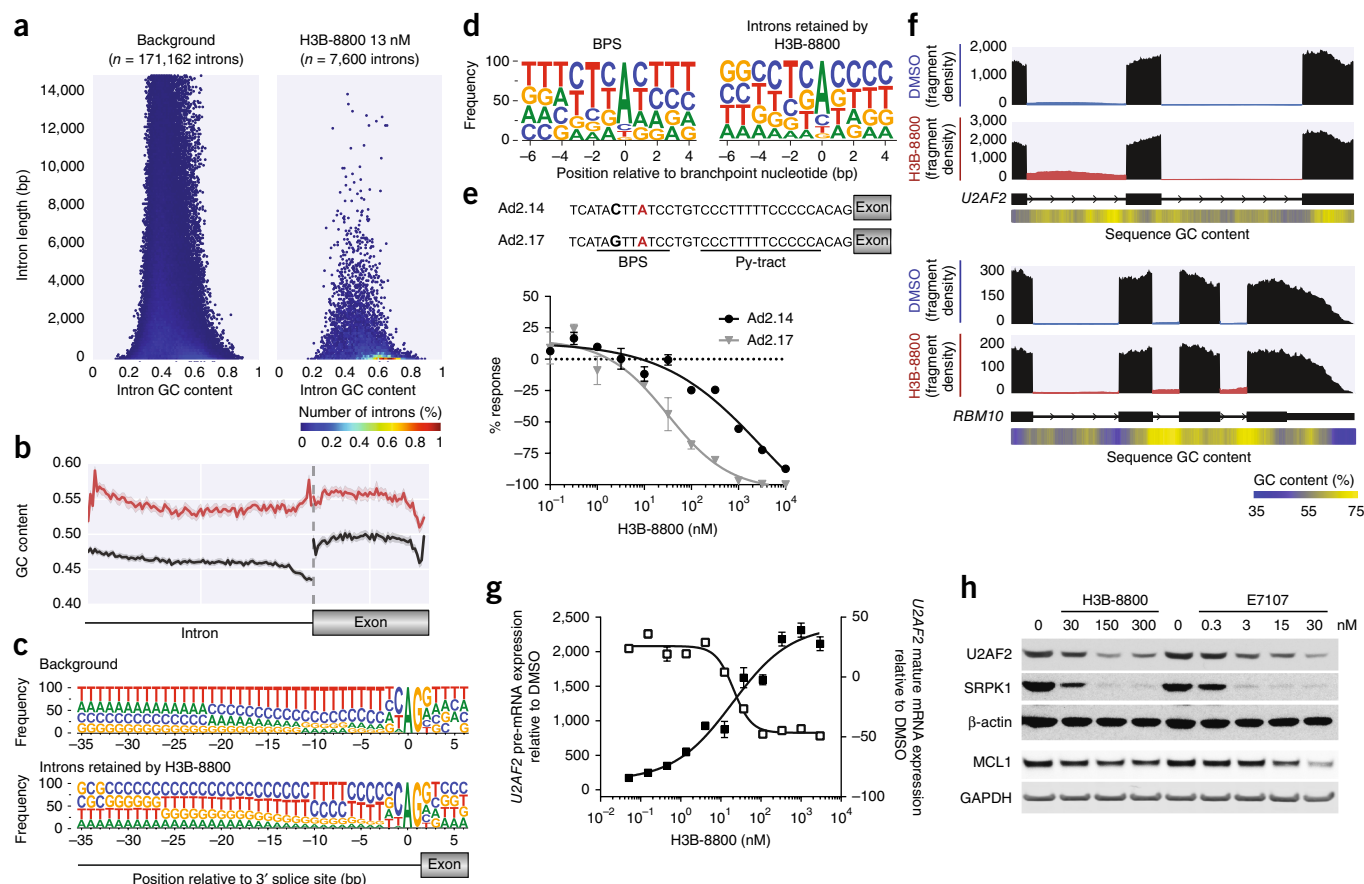


Figure 4 H3B-8800 inhibits splicing of short, GC-rich introns and affects splicing of mRNAs encoding spliceosome components. **(a)** Hexbin plot showing relative abundance of introns based on length (y axis) and GC content (x axis) of all introns of expressed genes in K562 cells (background, left) versus introns retained in *SF3B1*^{K700E} cells after H3B-8800 treatment (13 nM, right). The percentage of the total intron count contained within each hex is indicated by its color. **(b)** GC content within introns retained after H3B-8800 treatment of K562 *SF3B1*^{K700E} cells (red; $n = 5,788$ introns) versus a randomly selected subset of introns from background (black; $n = 10,000$ introns). The solid line represents the mean GC content, and the shaded area surrounding the solid line represents the 95% confidence interval of that measurement. **(c)** The 3' splice site motif of introns retained in K562 *SF3B1*^{K700E} cells following H3B-8800 treatment as compared to background. **(d)** Branchpoint sequence motif (extending from 6 nt upstream of the branchpoint nucleotide to 4 nt downstream of the branchpoint) representing all branchpoints sequenced by Mercer *et al.*²⁶ (left) and only those branchpoints within introns retained in K562 *SF3B1*^{K700E} cells following H3B-8800 treatment (right). **(e)** Top, depiction of two Ad2-derived pre-mRNA substrates: Ad2.14, in which the Py-tract contains five cytosines and five thymines, and Ad2.17, which also contains an alteration in the BPS to reduce binding affinity to U2 snRNP. The branchpoint nucleotide is labelled in red, and the nucleotide that was changed between Ad2.14 and Ad2.17 to alter branchpoint strength is bolded. Bottom, the results from *in vitro* splicing using these two pre-mRNA substrates. The y axis represents the percent response relative to DMSO control (0%). Data are represented as mean $\pm$ s.d.; $n = 3$ technical replicates. **(f)** Sashimi plot representing the average read density of three technical replicates in introns 3 and 4 of *U2AF2* (top) and introns 20, 21, and 22 of *RBM10* (below) of RNA-seq samples from K562 *SF3B1*^{K700E} cells treated with DMSO or H3B-8800. Exons are shown in black, and introns are shown in blue (DMSO treated) or red (H3B-8800 treated). Relative GC content (derived using a 100-bp sliding window) is depicted in a heat map below the reference sequence. **(g)** The effect of an increasing concentration of H3B-8800 on the level of *U2AF2* pre-mRNA (black squares) and mature mRNA (open squares) in K562 *SF3B1*^{K700E} cells as quantified by qPCR. Data are represented as mean $\pm$ s.e.m.; $n = 3$ technical replicates. **(h)** Western blot analysis of U2AF2, SRPK1, and MCL1 protein levels following treatment of K562 *SF3B1*^{K700E} cells with H3B-8800 or E7107 at the indicated concentrations. β -actin and GAPDH were used as loading controls.

the Py-tract of introns retained by H3B-8800 might be predominantly cytosine-rich although it was expected to be thymine-rich; we confirmed this inference via sequence motif analysis (**Fig. 4c**). In contrast, introns retained by E7107 treatment were less GC-rich than those retained by H3B-8800, and they demonstrated a clear increase in GC content in the following exon (**Supplementary Fig. 9g**).

As the 3' region of the intron also contains the BPS, we next explored whether the BPS in introns retained following H3B-8800 treatment might also be GC rich. Using branchpoint location data from deep sequencing of K562 cells in Mercer *et al.*²⁶, we discovered that, within introns retained by H3B-8800, the branchpoint nucleotide was less likely to be the preferred adenosine (68.8% versus 78.7%; $P = 4.32 \times 10^{-9}$; Fisher's exact test; **Fig. 4d**). This result strongly suggests that the BPS might play a role in determining which introns are more likely to be retained following H3B-8800 treatment. To test this hypothesis, we designed two substrates, Ad2.14 and Ad2.17, based on the Ad2 construct²⁷; both contain a partially cytosine-rich Py-tract conforming to the observed 3' splice site sequence motif (**Fig. 4e**). In addition, a base substitution in the BPS of in Ad2.17 was created to reduce binding fidelity to the U2 snRNP. Using these constructs, we observed that H3B-8800 exhibited a greater modulation of splicing for Ad2.17, the substrate with a weakened BPS (**Fig. 4e**), suggesting that H3B-8800 selectively modulates splicing of introns with a GC-rich 3' splice site and a weak BPS. In contrast, in the same experiment, E7107 showed a much smaller shift in IC_{50} values (**Supplementary Fig. 10a**). Results from similar experiments using two other sets of Ad2 constructs, each with identical pairs of weak and strong BPS sequences but with differing 3' splice sites, revealed selective modulation by H3B-8800 of the construct with a weakened BPS in sequences with a Py-tract composed completely of cytosine (**Supplementary Fig. 10c**) or with a partially cytosine-rich Py-tract that was dissimilar to the observed 3' splice site sequence motif (**Supplementary Fig. 10e**). In contrast, E7107 inhibited splicing of both sets of constructs equally (**Supplementary Fig. 10b,d**). Moreover, in constructs in which the BPS was changed to increase fidelity for binding with the U2 snRNP, E7107 showed potent splicing inhibition (IC_{50} , 8.8 nM), whereas H3B-8800 showed no inhibition (**Supplementary Fig. 11a,b**). Taken together, these results demonstrate that H3B-8800 exhibits sequence-specific properties that modulate splicing that are distinct from those of E7107.

To test whether genes containing short, GC-rich introns retained following H3B-8800 treatment are involved in pathways that might explain the preferential killing of spliceosome-mutant cells exhibited by the drug, we performed pathway enrichment analysis of genes whose splicing was affected by H3B-8800 treatment in the K562 isogenic cell lines. Pathways involved in mRNA splicing were among those significantly enriched for such genes (**Supplementary Table 5**). Considering the prior data showing that WT spliceosome components are necessary for the survival of spliceosome-mutant cells^{7–11}, we focused on the disruption of mRNA splicing as a possible mechanism underlying the preferential lethality of H3B-8800 against spliceosome-mutant cells. First, we curated a complete list of genes encoding spliceosome components using information from several studies^{28–30} (**Supplementary Table 6** and Online Methods). Short, GC-rich introns retained by H3B-8800 were present in 41 of 404 genes encoding proteins in spliceosome complexes ($P = 0.017$; binomial proportion test; **Supplementary Fig. 11c**), including genes encoding RNA binding motif protein 10 (RBM10) and the essential splicing factor U2AF2 (**Fig. 4f** and **Supplementary Fig. 11d,f**). H3B-8800 treatment induced intron retention of *U2AF2* in a dose-dependent manner (**Fig. 4g**), indicating that H3B-8800 directly modulated the

splicing of genes encoding proteins required for splicing catalysis, and this modulation resulted in a corresponding loss of U2AF2 protein expression in K562 cells (**Fig. 4h**). Despite the effects of H3B-8800 on *U2AF2* splicing and expression, stable reintroduction of full-length *U2AF2* cDNA was unable to rescue H3B-8800-induced cell death (**Supplementary Fig. 12**), likely because H3B-8800 treatment affects expression of many additional splicing factors. For example, H3B-8800 treatment also induced a dose-dependent reduction in protein expression of SRPK1, a kinase involved in splicing factor regulation. Although both H3B-8800 and E7107 treatment reduced U2AF2 and SRPK1 protein expression, only E7107 treatment induced intron retention of *MCL1* and reduced MCL1 protein expression (**Fig. 4h** and **Supplementary Fig. 13**), further indicating that E7107 has a more pleiotropic effect on splicing modulation than H3B-8800.

The data presented here identify H3B-8800 as a small molecule that selectively modulates splicing to induce preferential lethality in cancer cells bearing a mutant spliceosome. Recent work has shown that short, GC-rich introns are recognized by an intron-defined splicing mechanism that is associated with intron retention observed in cells under normal conditions²⁴. We demonstrate that H3B-8800 preferentially induces retention of these introns, which are characterized by short length, high GC content, and low differentiation in GC content compared with the following exon. Together, these findings suggest that H3B-8800 treatment selectively modulates a pre-existing mechanism for splicing recognition, and this selectivity is not shared with other small-molecule modulators of splicing. The enrichment for intron retention in mRNAs encoding RNA splicing factors provides an explanation for H3B-8800's preferential killing of spliceosome-mutant tumor cells, which are already deficient in splicing, as compared to spliceosome-WT cells. As the expression of numerous RNA splicing factors was influenced by H3B-8800 treatment, the killing effects of the drug are not attributable to altered expression of any single target.

Despite the fact that the global alterations in splicing conferred by mutant SF3B1 (refs. 17,18), SRSF2 (refs. 15,19), and U2AF1 (ref. 16) are distinct from one another, cells bearing any of the corresponding mutations have been shown to exhibit dependency on the remaining WT spliceosome^{7–11}. It is therefore predicted that expression of any of these cancer-associated RNA splicing factor mutations would confer sensitivity to H3B-8800. The preferential vulnerability of SRSF2- and SF3B1-mutant cells to H3B-8800 results in the differential lethality observed *in vitro* and *in vivo*. The selective mechanism of action of H3B-8800 in combination with its unique pharmacological profile among previously described direct modulators of splicing supports clinical testing of H3B-8800 in genetically defined subsets of cancer with RNA splicing factor mutations.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the [online version of the paper](#).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

G.K., S. Prajapati, J.W., and X.L. guided and performed the medicinal chemistry efforts that led to identification of H3B-8800. E. Park, A.A.A., B.C., and P.F. performed the *in vitro* biochemical assays. A.Y., A.C., B.C., R.D., C.K., L.L., P.K., C. Mackenzie, Y.M., T.T., H.Y., P.Z., P.G.S., and S.B. performed experiments and analyses of H3B-8800 performance in *in vitro* cellular assays. M.T., E.S., G.L., E. Pazolli, C. Meeske, P.G.S., M.W., and S.B. performed studies and analyzed data from the cell line xenografts. V.K. and E. Padron provided patient materials used for PDX models. A.Y., S.C.-W.L., J.T., and O.A.-W. generated PDX models and performed PDX experiments and analyses. M.S., S. Peng, and L.Y. performed RNA-seq analyses. O.A.-W., M.S., A.A.A., P.G.S., and S.B. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the [online version of the paper](#).

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ONLINE METHODS

Cell culture. The cell lines HCT-116, Panc05.04, Panc10.05, HPAF-II, CFPAC1, and Panc04.03 were obtained from the American Type Culture Collection (ATCC) or from RIKEN BioResource Center and cultured as instructed. The isogenic paired cell lines (K562 and NALM-6 *SF3B1*^{K700E} and *SF3B1*^{WT}) were generated at Horizon Discovery and were cultured in the presence of Geneticin (0.7 mg/ml, Life Technologies) for selection. HCT116 clones resistant to H3B-8800 were generated by treating cells with 4 nM of splicing modulator for 2 weeks. Splicing modulator was refreshed every 4 d. When needed, confluent cells were split in a 1:3 ratio and were allowed to recover overnight without splicing modulator treatment after reseeding. At the end of the 2 weeks of compound selection, individual clones were transferred to 12-well plates and further expanded without splicing modulator. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit from Qiagen. The resistance mutations *SF3B1*^{R1074H} and *PHF5A*^{Y36C} were identified by whole exome sequencing.

Cell viability and apoptosis assays. Pancreatic cancer cells were seeded at 750 cells per well in a 384 well plate and treated with compound on day 2. K562 isogenic cells (*SF3B1*^{K700E} and *SF3B1*^{K700K}) were seeded at 10,000 cells per well in 96-well plates and treated with compound 4 h later. The relative numbers of viable and apoptotic cells were measured via luminescence using CellTiter-Glo or Caspase-Glo 3/7 (Promega) at the indicated time points as instructed. Bortezomib was obtained from Selleckchem.

Expression plasmids. The plasmids used in the scintillation proximity assay were generated through cloning the cDNA of *mxSF3B1* (cloned by ligating the N-terminal half of the product of the mouse *Sf3b1* open reading frame (ORF) and the C-terminal half of the product of the *Xenopus Sf3b1* ORF, followed by replacement of three amino acid residues in the product of the *Xenopus* ORF with three human amino acid residues) into the HindIII and KpnI sites of pFlag-CMV2 (Sigma). The mutations corresponding to *mxSF3B1*-E622D, *mxSF3B1*-R625L, *mxSF3B1*-H662Q, *mxSF3B1*-K666E, *mxSF3B1*-K666N, *mxSF3B1*-K666R, *mxSF3B1*-K700E, and *mxSF3B1*-G742D were introduced using a site-directed mutagenesis kit (QuikChange II XL, Agilent). Mammalian U2AF2 expression plasmids were generated using Gateway cloning technology (Life Technologies). N-terminal hemagglutinin (HA)-tagged U2AF2 (NP_009210.1) was cloned into the pLenti6.3-V5 Dest vector (Life Technologies), which was used to transfect LentiX293 cells (Promega). Viral supernatants from these cells were then used to infect K562 isogenic cells. These isogenic K562 cells were selected for construct expression using 4 µg/ml blasticidin.

Nuclear extract preparation. Nuclear extracts were prepared from either HeLaS3 or 293F cells expressing pFLAG-*mxSF3B1* plasmids, 293FT cells expressing *mxSF3B1*, or HCT116 cells. Expression of *mxSF3B1* was achieved in 293F cells by transfecting pFLAG-CMV-2-*mxSF3B1* plasmids using Lipofectamine 2000 (Life Technologies) and in 293FT cells using the PiggyBac transposon system. Cell pellets were resuspended in hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 mM dithiothreitol (DTT)). The suspension was brought up to a total of 5 packed cell volumes (PCV). After centrifugation (3,000 r.p.m. for 5 min at 4 °C), the supernatant was discarded, and the cells were brought up to 3 PCV with hypotonic buffer and incubated on ice for 10 min. Cells were lysed using a dounce homogenizer and then centrifuged (4,000 r.p.m. for 15 min at 4 °C). The supernatant was discarded, and the pellet was resuspended with ½ packed nuclear volume (PNV) of low-salt buffer (20 mM HEPES pH 7.9, 1.5 mM MgCl₂, 20 mM KCl, 0.2 mM EDTA, 25% glycerol, 0.2 mM PMSF, 0.5 mM DTT), followed by ½ PNV of high-salt buffer (same as low salt buffer except 1.4M KCl was used). The nuclei were gently mixed by rotating for 30 min before centrifuging (10,000 r.p.m. for 30 min at 4 °C). The supernatant (nuclear extract) was then dialyzed into storage buffer (20 mM HEPES pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.2 mM PMSF, 0.5 mM DTT). Protein concentration was determined using a NanoDrop 8000 UV-Vis spectrophotometer (Thermo Scientific).

In vitro splicing reaction. The Ad2-derived³¹ sequence was cloned into the pGEM-3Z vector (Promega) using 5' EcoRI and 3' XbaI restriction sites. The

Ad2 variants (Ad2.12–Ad2.17; all modifications are described in Fig. 4e and Supplementary Fig. 10) were cloned into the pcDNA3.1(+) vector (Promega) using the same restriction sites. The Ad2-ZDHC16 hybrid substrate, which contained exons from the Ad2 vector and intron 9 from ZDHC16, was cloned into the pcDNA3.1(+) vector using the same restriction sites. The plasmids were linearized using XbaI and then were used as DNA templates in the *in vitro* transcription reactions. The Ftz plasmid that lacked introns (FtzΔi) was obtained from Luo & Reed³² and linearized using EcoRI. All RNAs were *in vitro* transcribed using the MEGAScript T7 kit and then purified using the MegaClear kit (Invitrogen).

For splicing reactions using pre-mRNAs of Ad2 variants, reactions were prepared using 8 µg of HeLa S3 nuclear extract, 2 ng pre-mRNA, 0.2 ng control, and varying concentrations of H3B-8800, E7107, or DMSO to reach a final reaction volume of 1 µl in this high-throughput assay. After a 15-min preincubation at 30 °C, 1 µl of splicing-activation buffer (0.5 mM ATP, 20 mM creatine phosphate, 1.6 mM MgCl₂) was added, and the reactions were incubated for 90 min at 30 °C. The reactions were then quenched with 13 µl DMSO, and 25 nl was used for RT-qPCR.

For splicing reactions using pre-mRNA of the Ad2-ZDHC16 hybrid, 1 µl reactions were prepared using 10 µg nuclear of extract prepared from 293FT PiggyBac cells, 8 ng pre-mRNA, and varying concentrations of H3B-8800, E7107, or DMSO. After a 15-min preincubation at 30 °C, 2 µl splicing-activation buffer was added, and the reactions were incubated for 90 min at 30 °C. RNA was extracted using a modified protocol from a RNeasy 96 Kit (Qiagen). The splicing reactions were quenched in 350 µl Buffer RLT Plus (Qiagen), and 1.5 volume of ethanol (555 µl) was added. 100 nl of quenched splicing reactions was used for RT-qPCR.

RT-qPCR reactions were prepared using the TaqMan RNA-to-C_T 1-step kit (Life Technologies), RNA from splicing reactions, Ad2 canonical junction (forward: 5'-ACTCTCTTCCGCATCGCTGT-3'; reverse: 5'-CCGACGGGTTCGATCCAA-3'; probe: 5'-CTGTTGGGCTCGCGTTG-3') or Ad2 aberrant junction (probe: 5'-CCAGCTGTGGGTCTTCGCC-3') and Ftz (forward: 5'-TGGCATCAGATTGCAAAGAC-3'; reverse: 5'-ACGCCGGGTGATGTATCTAT-3'; probe: 5'-CGAAACGCACCGTCAGACG-3') mRNA primer-probe sets.

Competitive binding assay. SF3B complexes were immunoprecipitated from nuclear extracts prepared from 293F cells overexpressing Flag-tagged SF3B1. First, batch immobilization of antibody to beads was performed through incubating 80 µg of anti-SF3B1 antibody (MBL International D221-3, Anti-Sap155 monoclonal antibody) and 24 mg anti-mouse PVT scintillation proximity assay (SPA) beads (PerkinElmer) for 30 min. After centrifugation (14,000 r.p.m. for 5 min at 4 °C), the antibody-bead mixture was resuspended in PBS supplemented with PhosSTOP phosphatase inhibitor cocktail (Roche) and cOmplete ULTRA protease inhibitor cocktail (Roche). Nuclear extracts were prepared through diluting 40 mg into a total volume of 16 ml PBS with phosphatase and protease inhibitors, and the mixture was centrifuged (14,000 r.p.m. for 10 min at 4 °C). The supernatant was transferred into a clean tube, and the antibody-bead mixture was added and incubated for 2 h. The beads were collected via centrifuging, washed twice with PBS + 0.1% Triton X-100, and resuspended with 4.8 ml of PBS. 100 µl binding reactions were prepared using slurry and varying concentrations of H3B-8800. After 15 min preincubation at room temperature, 1 nM ³H-probe used in Kotake *et al.*²⁰ was added. The mixture was incubated at room temperature for 15 min, and luminescence signals were read using a MicroBeta2 Plate Counter (PerkinElmer).

RNA-seq sample preparation. NALM-6 and K562 isogenic cell lines were treated with H3B-8800 or E7107 at the indicated doses (in Fig. 4) for 6 h. After treatment, the cells were washed with PBS, and RNA was isolated using PureLink (Life Technology) according to the manufacturer's instructions. cDNA library preparation, sequencing, and raw read filtering methods were described previously¹⁷.

U2 snRNP complex assembly assay. This assay was performed as previously described by Folco²³. The BBR 2'-O-methyl oligonucleotide complementary

to the branch point binding region (5'-mCmAmGmAmUmAmCmUmAmAmCmAmCmUmUmGmA-3') and the control oligonucleotide (5'-mAmCmUmGmGmCmAmGmCmAmAmUmGmAmCmUmG-3') were ³²P-labeled using γ-ATP (6,000 Ci/mmol, Perkin Elmer) and T4 polynucleotide kinase. For all assays, ATP was depleted from nuclear extracts through incubation for 20 min at room temperature. Reaction mixtures (25 μl) containing the depleted nuclear extract (15 μl) were preincubated with or without 1 μM H3B-8800 for 5 min at 30 °C, followed by incubation with 0.5 mM ATP, 3.2 mM MgCl₂, and 20 mM creatine phosphate (di-Tris salt) for 5 min at 30 °C. For reactions carried out in the absence of ATP, water was used to supplement the reaction mixture. The radiolabeled BBR or control 2'-O-methyl oligonucleotide (30 ng of each) was added to the reaction mixture last, and the reaction mixture was incubated for 5 min at 30 °C. G50 columns (GE Healthcare) were used to purify the samples before adding 6× DNA loading dye and running 1.2% native low-melting agarose (Invitrogen) gels in Tris/borate/EDTA (TBE) buffer at 70 V for 2 h at room temperature to resolve the 17S and 12S U2 snRNPs. Gels were dried, exposed overnight to a PhosphorImager screen, and imaged.

Real time qPCR and RT-PCR. RNA was extracted from K562 cells using PureLink (Life Technology) according to the manufacturer's instructions. cDNA was synthesized using the SuperScript VILO II (Life Technologies) according to the manufacturer's instructions. cDNA was diluted to 20 ng input for qPCR using the ViiA7 system (Life Technologies). For detection of *U2AF2* mature mRNA, the following primer–probe set was used: forward: 5'-GAGCAGGTGGACTGATTC-3'; reverse: 5'-GGTGATGTGCTCAAAGCCT-3'; and probe: 5'-CCGCCACGAGAAGAAGAAGGT-3'. For detection of *U2AF2* pre-mRNA, the following primer–probe set was used: forward: 5'-TGTTGGTCAGACTGAGGTG-3'; reverse: 5'-GTGGCACGTCCCAGTATTTA-3'; and probe: 5'-CCGCCACGAGAAGAAGAAGGT-3'.

RNA was extracted from patient material and PDX models and was used to generate cDNA as previously described¹¹. RT-PCR analyses for expression of *EZH2* and *GAPDH* were also performed using methods and primers as previously described¹¹. For detection of the *CEP57* aberrant junction, the following primer–probe set was used: forward: 5'-TGAGACTGGTCTAGAAACAAA-3'; reverse: 5'-GCTAGGGCTTGTGGACTTT-3'; and probe: 5'-ACTCCGTGTGTTCCCAATGCAAGA-3'. Statistical comparison of the expression of mRNA isoforms as determined through qPCR data was performed using the Student's *t*-test.

TaqMan low-density arrays. K562 isogenic cell lines were seeded at 1×10^5 cells per well in 96-well plates, and Panc05.04 and Panc10.05 cells were seeded at 1.5×10^4 cells per well in a 96-well plate and treated with various concentrations of H3B-8800 for 6 h. RNA (0.5 μg for *in vitro* studies and 2 μg for *in vivo* studies) was reverse transcribed using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific, CA, USA) following the standard protocol. The resulting cDNA was diluted, and the equivalent of 100 ng was used to prepare a sample specific PCR mix by combining with 50 μl TaqMan Gene Expression Master Mix (Thermo Fisher Scientific, CA, USA) and water up to a final volume of 100 μl. The PCR mix was loaded into the sample fill port of a 24-gene custom developed TaqMan Low-Density Array Card (Thermo Fisher Scientific, CA, USA), which contained two printed technical replicates of each assay per fill port. The TLDA was loaded with eight individual samples and prepared and run on the ViiA7 Real-Time PCR system following the manufacturer's recommendations (Thermo Fisher Scientific, CA, USA). Threshold PCR cycle (Ct) was determined by the ViiA7 RUO software v 1.2.3 (Thermo Fisher Scientific, CA, USA) using a relative threshold. A Ct greater than or equal to 35 was considered undetectable. Two technical replicates were averaged, and the geometric mean of the expression of five housekeeping genes (*B2M*, *EEF1A1*, *GAPDH*, *RPLP0*, *TUBB*) was calculated and used to normalize data as the first step in the comparative ΔΔCt method followed for analysis. Data shown represent three experimental replicates. The list of primers and probes included in the TLDA are reported in **Supplementary Table 7**.

K562 isogenic lines, HNT-34 and K052 xenograft models efficacy and pharmacokinetic/pharmacodynamic modeling. 1×10^7 K562 isogenic, HNT-34,

or K052 cells were subcutaneously implanted into the flank of female NSG or CB17-SCID mice of 6–8 weeks of age. Mice were treated with H3B-8800 (10% ethanol, 5% Tween-80, 85% saline) or vehicle control. For the efficacy studies, the mice were orally dosed daily, and the mice were monitored until they reached either of the following endpoints: (i) excessive tumor volume (≥ 20 mm in its longest diameter), which was measured three times a week (tumor volume calculated by using the ellipsoid formula: (length $\times$ width²) / 2), or (ii) development of any health problem, such as paralysis or excessive body weight loss. The differences in tumor volume during the study period between the vehicle-treated and H3B-8800-treated groups were analyzed by two-way analysis of variance (ANOVA) followed by the Dunnett's multiple comparison test. For the pharmacokinetic/pharmacodynamic modeling (PK/PD) studies, the mice were treated with one dose, and the tumors were collected at the indicated times (in **Figs. 2** and **3**) after treatment for further analysis. RNA was isolated using RiboPure RNA purification kit (Ambion) and used for TLDA. All mouse studies were carried out under protocols approved by the Institutional Animal Care and Use Committee at H3 Biomedicine.

Patient samples. Diagnostic bone marrow or peripheral blood aspirates were obtained from a total of 6 patients with CMML and 2 patients with AML. This includes 5 patients with CMML who were treated at the Memorial Sloan Kettering Cancer Center (MSKCC) and 1 from the Moffitt Cancer Center as well as 2 patients with AML treated at the MSKCC. Patient samples were collected after obtaining written informed consent. The use of human materials was approved by the Institutional Review Board of MSKCC, the Moffitt Cancer Center Scientific Review Committee, and the University of South Florida Institutional Review Board in accordance with the Declaration of Helsinki. Detailed patient characteristics are listed in **Supplementary Table 2**.

Generation of PDX models. Generation of CMML PDX models was performed as described in detail by Yoshimi *et al.*³³. Briefly, 6- to 10-week-old NSGS (stock no. 013062) female mice were purchased from the Jackson Laboratory and sublethally irradiated (200 cGy) 3–24 h before transplantation followed by direct intrafemoral injection. CD34⁺ cells were purified from viably frozen patient bone marrow mononuclear cells (MNCs) through immunomagnetic selection (Miltenyi Biotec). CBC analysis was performed on peripheral blood collected from submandibular bleeding or cardiac puncture post-euthanasia using a Procyte Dx Hematology Analyzer (IDEXX Veterinary Diagnostics). Each of the CMML PDX models used has been previously described³³. Genomic analysis of patient bone marrow MNCs and FACS-purified hCD45⁺ cells from corresponding xenografts was performed using a targeted next-generation sequencing assay as previously described³⁴.

AML PDX models were generated as previously described¹¹, with the exception that NSGS mice were used as recipients (as was done for CMML PDX models described above). All mouse studies were carried out according to the MSKCC Guide for the Care and Use of Laboratory Animals.

Flow cytometric analysis of PDX models. All flow cytometric antibodies (for both human and mouse studies) were purchased from BD Pharmingen, Biolegend, or eBioscience. Mononuclear cells (MNCs) from patient samples, xenografted tissues, or PB were stained with a combination of antibodies against human CD3 (hCD3, clone: OKT3, purchased from eBioscience), hCD14 (61D3, eBioscience), hCD16 (3G8, Biolegend), hCD19 (HIB19, eBioscience), hCD33 (WM-53, eBioscience), hCD34 (4H11, eBioscience), hCD38 (HB-7, Biolegend), hCD45 (2D1, eBioscience), hCD45RA (HI100, Biolegend), hCD90 (5E10, Biolegend), and hCD123 (7G3, BD Bioscience). Antibodies against mouse CD45.1 (A20, eBioscience) and Ter119 (catalog no. 116223, Biolegend) were used to exclude host-derived cells.

Quantification of RNA-seq data. Raw sequence reads were aligned to the human reference sequence hg19 by STAR 2.4.2a using two-pass alignment³⁵, and isoform quantification was performed using kallisto 0.42.4 (ref. 36) against GENCODE annotation v 25 mapped to GRCh37. Estimation of the count of retained introns was performed by defining 6 nt (–3 nt from the splice site and +3 following the splice site) exon–intron boundary regions and counting

alignments, which fully overlapped these regions, as previously described¹⁷. All raw junction counts, including those from exon–intron boundaries, were pooled over all technical replicates per cohort.

Identification of differential splicing events. Differential junction usage was assessed using a binomial z test for differences in proportion between treated and untreated sample pools. Proportions were calculated on the basis of all splice junctions (and exon–intron boundaries) which shared a splice site, similar to the percent spliced in (PSI) measurement¹⁷. In order to preserve Gaussian assumptions, only those shared splice sites with total junction count (sum raw count over all junctions sharing that splice site) greater than or equal to 10 reads in both treated and untreated cohorts were considered for analysis. z -scores corresponding to a false discovery rate (FDR)-corrected q value of ≤ 0.05 for treated ('aberrant') junctions and ≤ 0.20 for untreated ('canonical') junctions were considered significant.

Two or more splice junctions that share a splice site and have at least one junction that is significantly upregulated in treated and/or mutant samples or at least one junction significantly upregulated in untreated WT samples were considered aberrant or canonical junctions, respectively. Both aberrant and canonical junctions were required to be present in an event for it to be considered differential splicing. In the case of intron-retention events, both exon–intron boundaries (5' and 3' splice sites) as well as the junction bridging the two were required to be significant. We report all differential splicing events in **Supplementary Table 3**.

Gene expression analysis. Raw gene counts provided by Kallisto v 0.42.4 (ref. 36) were compiled into total gene counts, then analyzed using EdgeR 3.20.2 (ref. 37) to assess the significance of changes between cohorts. Gene changes with a q value < 0.05 were considered significant. Significant gene expression changes are provided in **Supplementary Table 4**. Prediction of splice variation leading to degradation via the nonsense mediated decay (NMD) pathway was performed as previously described¹⁷. For each gene, all associated coding transcripts were assessed to determine whether any annotated exons shared a splice site with a junction found to be significant in treated samples that would modify the protein sequence. If the modified sequence of any transcript would result in a stop codon found >50 nt from the last exon–exon junction—the so-called 'NMD rule'¹⁷—that transcript is predicted to be targeted by the NMD pathway. Genes for which all transcripts were predicted to be degraded were marked as NMD targeted, and genes for which no transcripts were predicted to be targeted were not. Ambiguous genes, for which some, but not all, transcripts were predicted to be degraded, were ignored. All NMD calls for genes with splicing events are found in **Supplementary Table 4**.

Analysis of short, GC-rich introns. A hexbin plot was produced using the matplotlib v 2.0.2 library³⁸. Each hex corresponds to a region of length (y axis) and average overall GC content (x axis) space where introns are found. All colored hexes have at least one intron that matches length and GC content within the bounds defined by the hexagon shape. The color of each hex corresponds to the fraction of the total number of introns represented by introns found in that hex alone.

GC content across an intron and following an exon was assessed by fractionating all intronic and exonic sequences into 100 and 50 bins, respectively, of equal sequence length. The average GC content of each bin was assessed, and then the 95% confidence interval was derived using a permutation test by bootstrapping these averages 5,000 times each. Only introns with sequence length ≥ 100 nt with at least one known following exon (via RefSeq) of length ≥ 50 nt were considered. If more than one following exon could be used, one was randomly selected. The resulting figure was drawn using the tsplot function from the seaborn library (<https://zenodo.org/record/12710#.WmeMaHlOmUk>).

For all sequence analyses, background introns were sampled from a random subset of all genes with at least one intron expressed in the untreated cellular context ($n = 6,405$ and $n = 5,500$ for K562 and NALM-6, respectively) that have an expression distribution that is not significantly different from those genes that contained intron retention events in the treated context, measured by Kruskal H test ($P = 0.12$ and $P = 0.55$ for K562 and NALM-6, respectively). Genes with

matching expression levels were identified using Matching Quantiles Estimation (MQE) in seaborn³⁹. Ideally, background transcripts would also be matched on the basis of transcript turnover rate, however to our knowledge, a comprehensive database in the presented cellular contexts is not currently available.

Pathway analysis. Overrepresentation analysis of genes containing short (≤ 300 nt), GC-rich ($\geq 60\%$) introns retained by H3B-8800 in K562 SF3B1-K700E cells in the most current Reactome⁴⁰ pathway reference database was accomplished using PANTHER 11.1 (ref. 41). Pathways significant after correction ($q < 0.1$) using the Benjamini–Hochberg procedure with at least a twofold enrichment over background are reported in **Supplementary Table 5**. To build a complete database of spliceosome-related genes, we collected 1,512 spliceosome and splicing-related proteins from three sources: (i) 244 spliceosome proteins provided by Hegele *et al.*²⁹, (ii) 255 spliceosome proteins annotated by Barbosa-Morais *et al.*³⁰, and (iii) 1,100 proteins from SpliceosomeDB²⁸. Genes were considered spliceosome-related if they were found in (i) or (ii). Genes found in (iii) were considered only if they were found in the same protein family as any protein in (i) or (ii) or they were clearly annotated with specific splicing roles. The final table of 404 spliceosome genes is given in **Supplementary Table 6**. Enrichment of intron-retention events among 404 spliceosome genes was accomplished by adding these genes as a separate pathway to the existing Reactome database and performing a binomial proportion test followed by Bonferroni correction as previously described⁴¹.

Western blotting. SF3B1-WT or mutant SF3B1-K700E K562 isogenic cell lines were lysed in RIPA buffer (Boston BioProducts) or IP Lysis Buffer (Thermo Fisher Scientific) plus protease-inhibitor cocktail (Mini-complete, EDTA-free, Roche). Forty to fifty micrograms of protein were loaded per well in SDS-PAGE gels (Novex, Life Technologies). Gels were transferred to nitrocellulose membranes using iBlot (Life Technologies). Membranes were blocked in Odyssey Blocking Buffer (LiCor) or 5% milk in Tris-buffered saline and 0.1% Tween20 and were probed overnight at 4 °C with antibodies against the following targets: U2AF2 (1:5,000, Sigma, U4758), HA-Tag (1:1,000, Abcam, 18181), SRPK1 (1:1,000, Bethyl, A302-461A), MCL1 (1:1,000, Cell Signaling Technologies, 5453), β -Actin (1:1,000; Sigma-Aldrich, A5441) or GAPDH (1:5,000, Sigma, G8795 and G9545). After washing, blots were probed at room temperature with secondary antibodies labeled with LI-COR IRDye fluorescent dye or conjugated to HRP. Target proteins were detected using the LI-COR system or the ImageQuant (GE) for enhanced chemiluminescence. Uncropped blots are available in **Supplementary Figure 14**.

Statistical analysis. For all results, the relevant statistical tests are provided in the figure legends as well as the total number of samples (n) in each case and control condition. Unless otherwise specified, $P < 0.05$ was considered significant. Multiple testing correction was performed where applicable using the Benjamini–Hochberg FDR correction, and $q < 0.05$ was considered significant unless otherwise specified.

Life Sciences Reporting Summary. Further information on experimental design is available in the **Life Sciences Reporting Summary**.

Data availability statement. All RNA sequencing data used in this manuscript are available in GEO under accession number [GSE95011](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE95011).

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► Experimental design

1. Sample size

Describe how sample size was determined.

For the in vivo experiments the number of animals included in each arm of the efficacy study was decided based on the variability of the tumor growth within the animals. The variability of tumor growth was evaluated during the model development study for each model. For the RNAseq experiments 3 replicates were included for each condition.

2. Data exclusions

Describe any data exclusions.

No data excluded

3. Replication

Describe whether the experimental findings were reliably reproduced.

The experiments were repeated a minimum of 3 times for all the in vitro experiments.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

For the efficacy study the animals were randomized to have a mean tumor volume and weight similar across the different groups.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

No blinding was used in these studies as the person that performed the experiment also analyzed the data.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or the Methods section if additional space is needed).

n/a Confirmed

- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- ☐ ☒ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly.
- ☐ ☒ A statement indicating how many times each experiment was replicated
- ☐ ☒ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
- ☐ ☒ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- ☐ ☒ The test results (e.g. p values) given as exact values whenever possible and with confidence intervals noted
- ☐ ☒ A summary of the descriptive statistics, including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- ☐ ☒ Clearly defined error bars

See the web collection on [statistics for biologists](#) for further resources and guidance.

► Software

Policy information about [availability of computer code](#)

7. Software

Describe the software used to analyze the data in this study.

Kallisto 0.42.4, STAR 2.4.2a, EdgeR 3.20.2, matplotlib 2.0.2, PANTHER 11.1

For all studies, we encourage code deposition in a community repository (e.g. GitHub). Authors must make computer code available to editors and reviewers upon request. The *Nature Methods* [guidance for providing algorithms and software for publication](#) may be useful for any submission.

► Materials and reagents

Policy information about [availability of materials](#)

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

All materials will be available by request and MTA will be required

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

SF3B1 (MBL International D221-3, Anti-Sap155 mAb), U2AF2 (Sigma, U4758), HATag (Abcam, 18181), SRPK1 (Bethyl, A302-461A), MCL1 (Cell Signaling Technologies, #5453), beta-Actin (Sigma-Aldrich, A5441) or GAPDH (Sigma G8795 and G9545).

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

HCT-116, Panc05.04, Panc10.05, HPAF-II, CFPAC1 and Panc04.03 were obtained from the American Type Culture Collection (ATCC). The isogenic paired cell lines (K562 and Nalm-6 SF3B1K700E and SF3B1WT) generated at Horizon Discovery.

b. Describe the method of cell line authentication used.

An aliquote of each cell lines were authenticated using ATCC DNA fingerprinting.

c. Report whether the cell lines were tested for mycoplasma contamination.

All cell lines were tested at IDEXX using IMPACT1 testing.

d. If any of the cell lines used in the paper are listed in the database of commonly misidentified cell lines maintained by [ICLAC](#), provide a scientific rationale for their use.

No commonly misidentified cell lines were used.

► Animals and human research participants

Policy information about [studies involving animals](#); when reporting animal research, follow the [ARRIVE guidelines](#)

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Female NSG mice were purchased from Charles river and female CB17-SCID or NSGS mice from the Jackson Laboratory (6-10 weeks old).

Policy information about [studies involving human research participants](#)

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

The study did not involve human research participants.

Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

► Data presentation

For all flow cytometry data, confirm that:

- ☒ 1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- ☒ 2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- ☒ 3. All plots are contour plots with outliers or pseudocolor plots.
- ☒ 4. A numerical value for number of cells or percentage (with statistics) is provided.

► Methodological details

5. Describe the sample preparation.

The bone marrow (BM), spleen, liver, lung, and peripheral blood (PB) from NSGS xenograft mice were used for flow cytometric analysis. BM, spleen, and PB were lysed with ACK (Ammonium-Chloride-Potassium) lysing buffer to collect mononuclear cells and then washed with ice-cold PBS. The samples were stained with a combination of antibodies against human CD3 (hCD3, SK7/HIT3a), hCD14 (61D3/MφP9), hCD16 (3G8), hCD19 (HIB19), hCD33 (WM-53/P67.6), hCD34 (4H11/581), hCD38 (HIT2), hCD45 (HI30), hCD45RA (HI100), hCD90 (5E10), and hCD123 (7G3) in PBS with 2% BSA for 30 min on ice. Antibodies against mouse CD45.1 (A20) and Ter119 were used to exclude host-derived cells. DAPI was used to exclude dead cells. The final staining volume was 100 µl, and the final concentration for all antibodies used was 1:200.

6. Identify the instrument used for data collection.

All FACS sorting was performed on FACS Aria, and analysis was performed on an LSRII or LSR Fortessa (BD Biosciences).

7. Describe the software used to collect and analyze the flow cytometry data.

FlowJo Ver.9 was used for analysis of flow cytometry data.

8. Describe the abundance of the relevant cell populations within post-sort fractions.

To check the purity of human CD45 (hCD45) positivity in post-sort samples, the sorted samples were analyzed for hCD45 in FACS Aria (BD Biosciences) and samples with >99% hCD45 cells were used for RNA extraction.

9. Describe the gating strategy used.

The FSC/SSC gates of the starting cell population was set in order to include all the lineages of human and mouse hematopoietic cells such as granulocytes, monocytes, and lymphocytes. Then doublet cells were excluded by SSC-H vs SSC-W and FSC-H vs FSC-W gating. The boundaries between "positive" and "negative" staining cell population were defined by using unstained and single color-stained controls that were prepared by staining the whole BM mononuclear cells from B6 mice at 8-12 weeks with antibodies against mouse CD11b. "Positive" staining cell population was defined as CD11b+ population and "negative" staining cell population was defined by unstained control. The boundary for each fluorescence was set between these "positive" and "negative" staining cell populations.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. ☒