

UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of SRSF2 Splicing Factor in Hematopoiesis

A Thesis submitted in satisfaction of the requirements

for the degree Master of Science

in

Biology

by

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Chair

University of California, San Diego

2013

## Dedication

I dedicate this thesis to my family for their love and support. I'm sorry for not being with you through these hard times, I love you.

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I would like to thank Dr. Zhang for the opportunity to join her lab and pairing me with Dr. Yukiko Komeno. Dr. Komeno had been an excellent mentor and friend. I had learned so much from her and from my time in the Zhang lab. I would also like to thank the entire Zhang lab for their selfless help with my project.

ABSTRACT OF THE THESIS

The Role of SRSF2 Splicing Factor in Hematopoiesis

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Professor Dong-Er Zhang, Chair

Acute myeloid leukemia (AML) is one of the deadliest forms of leukemia, a prominent cancer in the United States, with a five-year survival rate ranging from 15%-70%. Myelodysplastic syndromes (MDS) are a group of hematopoietic malignancies, which can lead to AML. Recently, it was found that mutations in the splicing factor SRSF2 are present in over 20% of MDS patients. To



understand the molecular mechanism of how *SRSF2* mutations contribute to blood disorders, we used *Srsf2* conditional knockout (KO) mice (*Srsf2* f/f) to examine the role of SRSF2 in steady-state and stress hematopoiesis. Blood cell-specific *Srsf2* KO (*Vav-Cre/Srsf2* f/f) was embryonic lethal between embryonic day 16 and 18. Furthermore, SRSF2 deficient fetal liver cells showed increased apoptosis and G1 cell cycle arrest. To study SRSF2 deficiency in hematopoiesis of adult mice, we created double stranded RNA poly-IC inducible KO mice by breeding *Mx1-Cre* with *Srsf2* f/f mice. Unexpectedly, after poly-IC injection *Mx1-Cre/Srsf2* f/f mice are alive. In addition, there is no phenotype in heterozygous *Srsf2* KO (*Srsf2* +/-) mice in steady state. To evaluate the effect of haploinsufficiency under stress condition, mice with *Srsf2*+/- blood cells were injected weekly with the chemotherapeutic drug 5-fluorouracil or underwent single sub-lethal dose of irradiation. Experiment that would elucidate the mechanism of the disease were performed as well, which include bone marrow transplantation of SRSF2 overexpressed cells, bone marrow transplantation of *Mx1-Cre/ Srsf2* f/f, and *Mx1-Cre/Srsf2* +/- cells followed by poly-IC injection, and RNA sequencing of lineage-depleted bone marrow cells that overexpress wild-type and mutant SRSF2. Through these in vitro and in vivo experiments we aim to analyze the role of SRSF2 in the blood system and in the development of MDS.

I

## Introduction

**Acute myeloid leukemia has a quick onset time and is often fatal**

Leukemia is one of the most prominent forms of cancer, ranking as one of the top cancers in the United States with more than 40,000 new cases of leukemia diagnosed each year <sup>[1]</sup>. Leukemia is a type of cancer involving the bone marrow and white blood cells and can be divided into four major subtypes: acute lymphoblastic leukemia (ALL), chronic lymphoblastic leukemia (CLL), acute myeloid leukemia (AML), and chronic myeloid leukemia (CML). This classification is based upon the onset of the disease (acute or chronic) as well as the white blood cell types affected <sup>[2]</sup>. Of these subtypes, acute leukemias are lethal and develop relatively quickly, with a median survival rate of three months <sup>[2]</sup>. Amongst the four major types of leukemia, AML is of particular interest as it remains highly lethal despite recent medical advances, with fatalities ranging from 50% in young adult patients and 90% in patients older than sixty-five<sup>[3]</sup>. As medical practice and technology became more advanced, disease characterization became more robust. In response to a portion of AML patients suffering from a period of anemia and abnormal blood cell production preceding the onset of AML, certain diseases were characterized as pre-leukemia diseases. It was not until recently in the late 20<sup>th</sup> century did many of these diseases receive an established and widely-accepted name<sup>[4]</sup>.

**Myelodysplastic syndrome is a pre-leukemia disease**

Myelodysplastic syndrome (MDS) is a hematopoietic stem cell disease characterized by ineffective hematopoiesis: low white blood cell count, anemia,

low platelet count in peripheral blood, and increase of dysplastic (abnormal shaped) cells in the bone marrow. MDS preferentially affects elderly population<sup>[5]</sup>. Some of the patients show progression to acute myeloid leukemia, a fatal malignant disease. Although chromosome abnormality, gene mutations, epigenetic changes are detected in MDS cells, the direct cause of MDS is still not clear. Current curative treatment of the disease is bone marrow transplantation; however, complications in elderly patients hamper aggressive chemotherapy and transplantation<sup>[5]</sup>. Coupled with its prominence of between 10,000 to 20,000 new cases each year in the United States, MDS is quickly becoming one of the most intractable blood diseases, and finding a new therapeutic target is of critical importance<sup>[6]</sup>.

### **The SRSF2 mutation was identified in MDS patients**

In 2011, by using next-generation sequencing technique, mutations in splicing machinery were found in MDS patients<sup>[7]</sup>. The *SRSF2* (serine-rich splicing factor 2, also known as SC35) mutations were preferentially found in chronic myelomonocytic leukemia (used to be a subtype of MDS), but also found in other MDS subtypes with lower frequency<sup>[7]</sup>. *SRSF2* mutations were present in over 10% of MDS patients. Interestingly, all *SRSF2* mutations involve a single amino acid change of proline at the 95 position into histidine (37%), leucine (25%), or arginine (13%) in the patients.

## The SRSF2 spliceosome

The SRSF2 protein is a spliceosome present in mammalian cells. Mutations in the splicing machinery had recently been associated to as much as 50-80% of the mutations in myeloid neoplasms, or abnormal growth of the myeloid cells<sup>[7]</sup>. Despite this knowledge, relatively little information is known about this splicing factor. SRSF2 has shown to be integral for T-cell development and required for alternative splicing of the T-cell marker CD45 in order for the T-cells to mature<sup>[8]</sup>. In an experiment done by Wang *et al* in 2001, the researchers created a *Srsf2* conditional knockout (cKO) mice strain. It was shown that when *Srsf2* was conditionally knocked out in the thymus, a significant number of T-cells failed to reach maturation in these cKO mice. Flow cytometry for CD4 and CD8, proliferation cell markers for T-cells discovered that compared to wild-type T-cells, *Srsf2* cKO mice's T-cells showed an almost 4 fold decrease in cells positive for CD4 or CD8<sup>[8]</sup>. However, other than its link to T-cells, there have been no past publications on SRSF2 and its relationship to the whole blood system, and the spliceosome's interaction with other cells of the hematopoietic system are not fully understood.

Another cKO model of SRSF2 focused on mice with SRSF2 knocked out in cardiomyocytes<sup>[9]</sup>. In this model, the cKO was not lethal and did not cause phenotypes until late in the developmental stage (5-6 weeks after birth). The cKO mice developed conditions similar to dilated cardiomyopathy (DCM) in human. Although mutant mice showed several changes in its heart structure from the

wild-type mice, their hearts retained relatively normal function and they exhibited similar lifespan as those of wild-type.

### **Multi-directional approach to SRSF2's role in MDS**

Due to the shortage of information regarding SRSF2 in the hematopoietic system, I sought to fill that gap in knowledge through multiple approaches. Unpublished data in Zhang's lab showed that *Srsf2* knock-out in the hematopoietic system was fatal for the mice embryos. This data establishes SRSF2's essential role in normal hematopoiesis. To study SRSF2's effect on hematopoiesis under stress, I used conditional *Srsf2* knock-out mice crossed with the transgenic *Vav-Cre* mice (expressed specifically in blood cells at E10.5) driver and the transgenic *Mx1-Cre* mice (inducibly activated by PIPC injection)<sup>[10][11]</sup>. To link *SRSF2* mutations to MDS development, I evaluated functional differences between wild type SRSF2 and its most common mutant form, SRSF2<sup>P95H</sup> by both in vitro and in vivo means.

## II Results

### **Srsf2 is indispensable for the development of hematopoietic system**

We first examined the role of SRSF2 in hematopoiesis by generating *Srsf2* null mutation in mouse blood cells via crossing conditional *Srsf2* knockout mice (*Srsf2<sup>fl/fl</sup>*) with blood cell-specific Cre transgenic mice (*Vav-Cre*). Cre expression driven by *vav* promoter is activated around E10-E11, which coincides with the establishment of fetal liver definitive hematopoiesis. The mutant mice produced significantly fewer definitive blood cells (10% of wild type controls) (Figure 1A), and exhibited increased apoptosis in the remaining blood cells (Figure 1B). Cell cycle analysis showed G1 arrest (Figure 1C). *Srsf2* null embryos died during embryonic development (E16-E18) (data not shown). These results indicate that SRSF2 is essential for hematopoiesis during embryonic development.

### **Mice heterozygous for SRSF2 in the whole blood have similar hematopoietic capabilities as wild-type mice after stress**

In *Vav-Cre<sup>+</sup>Srsf2<sup>w/f</sup>* fetal livers, hetero cells did not have any phenotypes in cell number, apoptosis, and cell cycle (unpublished data). Likewise, in adult mice, no difference was detected in blood cells between wild type and hetero mice (unpublished data). These results show that *Srsf2* is haplosufficient in steady state hematopoiesis.

To see whether there is any phenotype under stress condition, we administered a single dosage of sub-lethal X-ray irradiation (400 Rad) to the *Vav-Cre<sup>+</sup>* or *Vav-Cre<sup>-</sup>Srsf2<sup>w/f</sup>* mice, and evaluated the cell count recovery. Ionizing irradiation causes DNA damage, which results in cell death through the formation



of pyrimidine dimers, ultimately activating the *p53* apoptosis pathway<sup>[12]</sup>. In the previously published data, the white blood cells (WBC) counts of C57B6 mice are expected to drop rapidly within 7 days, then recover to normal levels 40-50 days post-irradiation<sup>[13]</sup>.

It is shown that both groups exhibited not only similar WBC depletion patterns immediately after irradiation, but also similar WBC recovery rates.

### **Mice heterozygous for *Srsf2* in the whole blood react similarly as wild-type mice in response to weekly 5-fluorouracil injections**

I tested another experiment to evaluate stress hematopoiesis. 5-FU is a nucleoside analog, and has been used as an anti-cancer drug. It blocks DNA synthesis through the inhibition of thymidylate synthase and prevents the production of dTMP, a critical component for DNA replication and repair, ultimately leading to cell death<sup>[14]</sup>. Mice heterozygous for *Srsf2* through the *Vav-Cre+* vs *Vav-Cre-* system were injected with weekly with 5-fluorouracil (5-FU) (150 mg/kg body weight) and the survival rate was evaluated (Figure 2). Similar to published literature, death resulting from the weekly chemotherapeutic drug injections can be seen around the third to fourth injections<sup>[15]</sup>. This experiment was repeated three times, and the difference was not significant. Together with the results of sublethal irradiation, it is shown that *Srsf2* is haplosufficient in the blood system.

### **Possible SRSF2 splicing targets were identified through RNA-mediated oligonucleotide annealing, selection, and ligation sequencing (RasL-seq)**

In MDS patients, the codon encoding proline 95 is the mutation hotspot of *SRSF2*<sup>[7]</sup>. The most frequent type of those mutations is missense mutation of P95H, which is caused by the nucleotide change from CCC to CAC. However, it is not clear whether those mutations are gain of function or loss of function. To gain insight into the functional changes caused by the mutations, I did Ras-Lseq analysis of BM cells overexpressing WT or mutant form of *Srsf2* to evaluate the difference in splicing target genes.

The raw data consisted of different frequency of “hits” for the long and short isoforms of different genes. I set the signal threshold to 20, and removed genes that had an isoform “hit” of less than 20 in any of the nine samples (biological triplicate for overexpressed wild-type, mutant, and empty vectors). I then took the “hits” on the short isoform for each sample, and divided it by its “hits” on the long isoform to obtain nine short/long ratios for each gene. The short/long ratios for the three empty vector samples were then averaged to set a biological baseline for comparison. The triplicate ratios of the wild-type and mutant vectors were divided then by the empty vector average ratio to calculate fold change.

Interestingly, as shown in figure 3, cells overexpressing the wild-type and mutant *SRSF2* exhibited mostly different splicing targets. Of the genes with significant splicing pattern differences from the norm, we were particularly interested in the genes that were uniquely spliced by the *Srsf2*<sup>P95H</sup>.

overexpressed vector, since they are potentially important for MDS development. Some of the genes identified include published oncogenes and protooncogenes such as *Dnmt3b*, *Mapk14*, *Itga4*, *CDK19*. These results show clearly that the SRSF2<sup>P95H</sup> mutation is not only a gain-of-function mutation, but also a possible contributor to the onset of cancer.

### **Bone marrow transplantation of overexpressed wild-type and mutant SRSF2 vectors showed no symptoms of MDS development**

To see whether P95H mutant itself is sufficient to cause MDS phenotype, we overexpressed SRSF2<sup>WT</sup>, SRSF2<sup>P95H</sup> or empty MigR1 vector in mouse BM cells and performed transplantation.

Cell counts and GFP+ percentage in peripheral blood were followed up once a month. Three independent experiments were performed. GFP% was consistently lower in WT and P95H cells compared to MigR1 mice (Figure 6B). In six months, mice transplanted with bone marrow cells overexpressing the mutant SRSF2 showed no reproducible phenotypes relating to MDS development, such as leukopenia, myeloid shift in white blood cells, anemia, or thrombocytopenia.

These results show that overexpression of WT or mutant SRSF2 cause growth disadvantage, contrary to the expectation, and SRSF2 mutation itself is not sufficient to cause MDS in mouse BMT model.

### **In-vitro knockout and SRSF2 showed cell cycle arrest at G1 in adult bone marrow cells**

Transduction of Cre into *Srsf2*<sup>ff</sup> BM cells by retrovirus induced apoptosis (unpublished data). This is compatible with the enhanced apoptosis in *Srsf2*<sup>-/-</sup> fetal liver cells (Figure 1A). To confirm that this apoptosis is caused by knocking down SRSF2 itself, and to see the difference between WT and P95H mutant in rescuing the knockout effect, I transduced the *Srsf2*<sup>ff</sup> cells with SRSF2 WT, P95H mutant or empty MIP vector followed by Cre in MSCV-IRES-EYFP vector.

As shown in Figure 7B, P95H seemed to rescue the knockout effect more efficiently than WT.

### **In-vivo knockout of SRSF2 via the Mx1-Cre recombinase/PIPC system showed incomplete knockout but differences in certain lineages**

To see the effect of knocking out *Srsf2* in adult body after definitive hematopoiesis is established, *Srsf2*<sup>ff</sup> mice were crossed with transgenic Mx-Cre mice. *Mx1* promoter is inducibly activated upon introduction of PIPC in the mice body. Unlike *Vav-Cre* which is activated only in blood cells, *Mx-Cre* is activated in all cell types including blood cells. Of the different types of cells in the body, *Mx1-Cre* will see a near 100% expression in the hematopoietic system<sup>[16]</sup>.

PIPC was injected into *Mx1-Cre+ Srsf2*<sup>w/w</sup>, *Srsf2*<sup>w/ff</sup>, and *Srsf2*<sup>ff</sup> mice. Unexpectedly, *Srsf2*<sup>ff</sup> mice stayed alive for more than 6 months after PIPC injection. The genotyping of the peripheral blood revealed that the knockout was incomplete, although the dosage was enough to excise out *Srsf2* in hetero cells

(Figure 5A). I hypothesized that some differentiated cells can survive after knocking out *Srsf2*, but not the hematopoietic stem/progenitor cells, so that the cells with incomplete excision take over the population and support long-term hematopoiesis. To prove this hypothesis, the peripheral blood of the mice was collected weekly for genotyping analysis to determine the completeness of the SRSF2 knockout process. As expected, knockout band in *Srsf2*<sup>ff</sup> blood reached maximum level around 2 weeks, after which the floxed bands become more dominant than knockout bands.

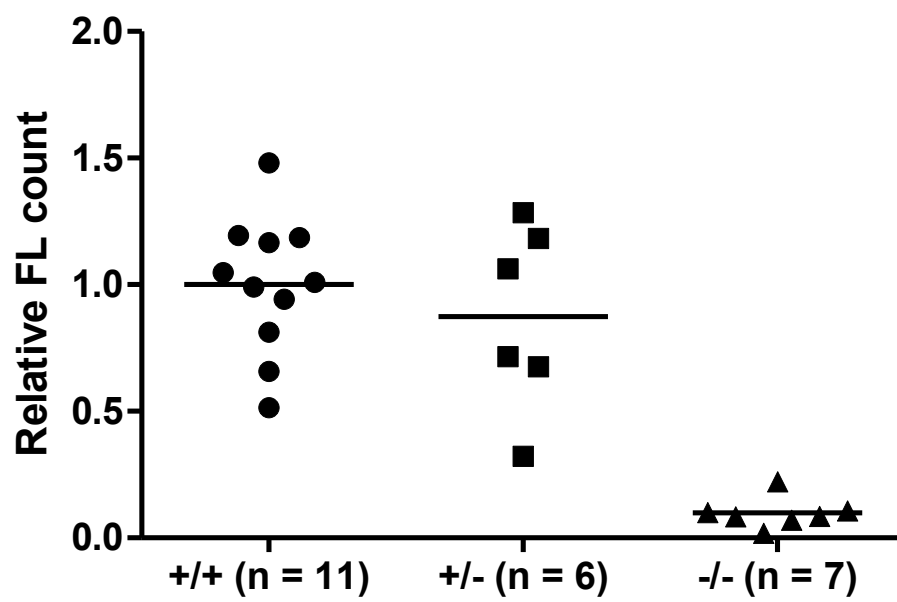
**Figure 1. Knock-out of SRSF2 is embryonically lethal for the mice.**

A) Blood cell counts in wild-type and *Srsf2* KO fetal livers. Normalized to average of wt counts. *Srsf2*<sup>-/-</sup> livers had significantly fewer cells compared to those of other genotypes.

B) Apoptosis assay of fetal liver cells. *Srsf2*<sup>-/-</sup> cells show enhanced apoptosis.

C) Cell cycle analysis of SRSF2<sup>+/+</sup>, SRSF2<sup>+/-</sup>, and SRSF2<sup>-/-</sup> embryos. Cell cycle analysis shows G<sub>1</sub> arrest for the SRSF2 KO embryos.

A)



B)

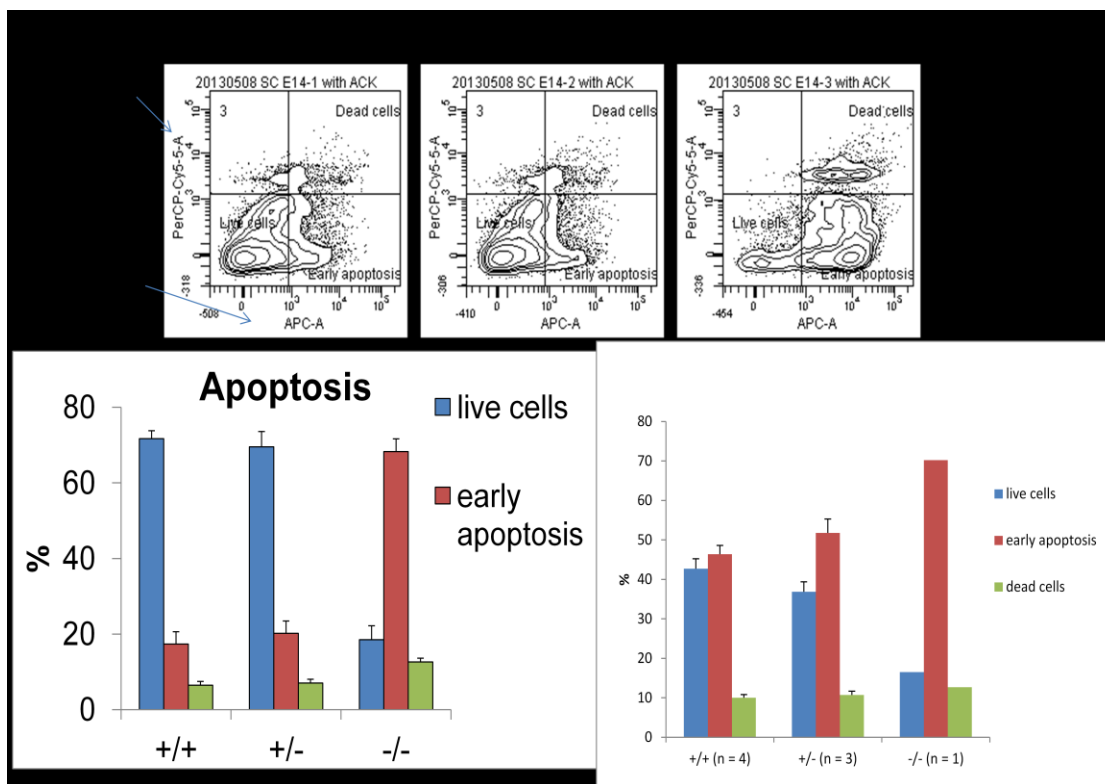


Figure 1. Knock-out of SRSF2 is embryonically lethal for the mice continued



C)

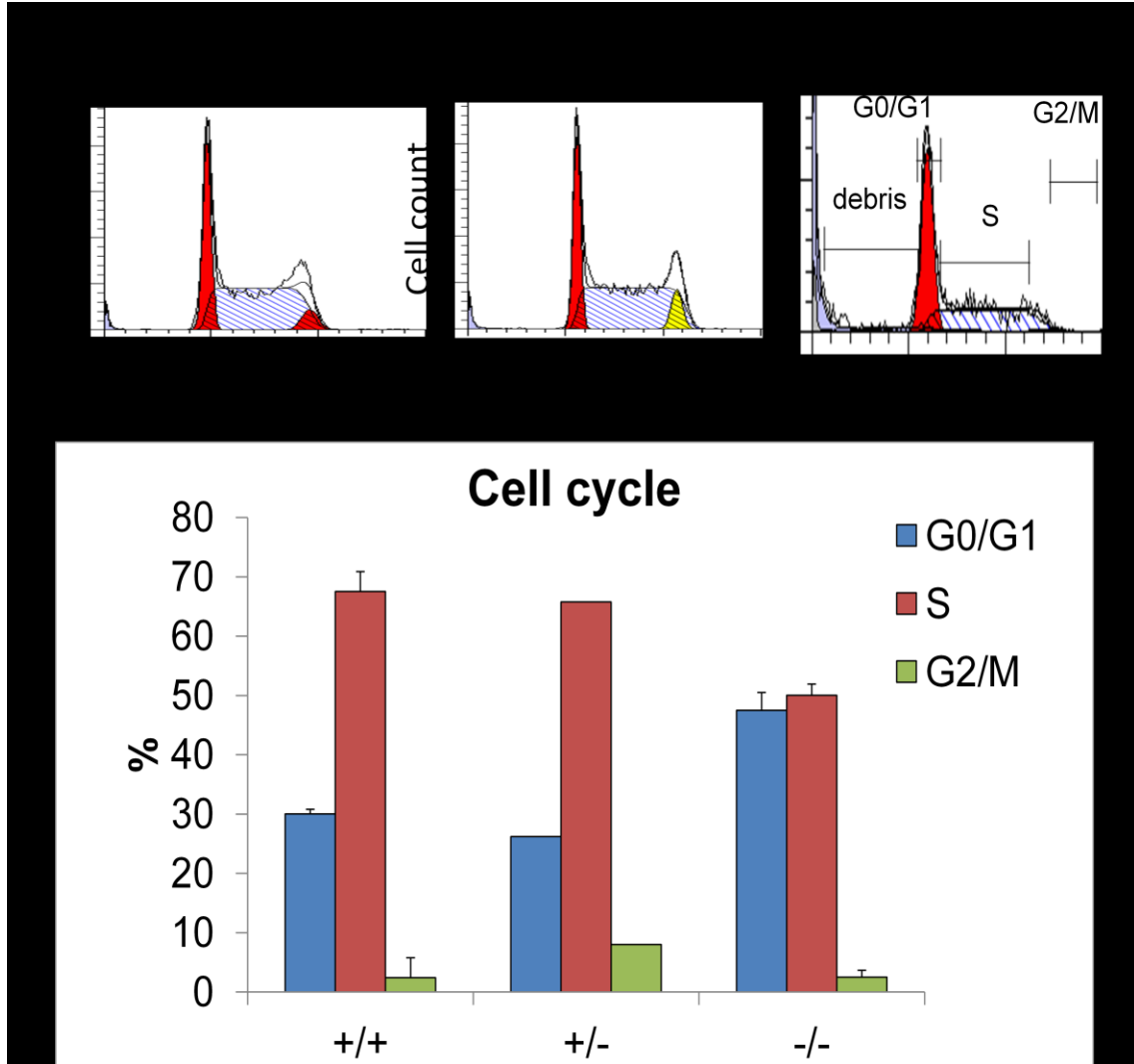
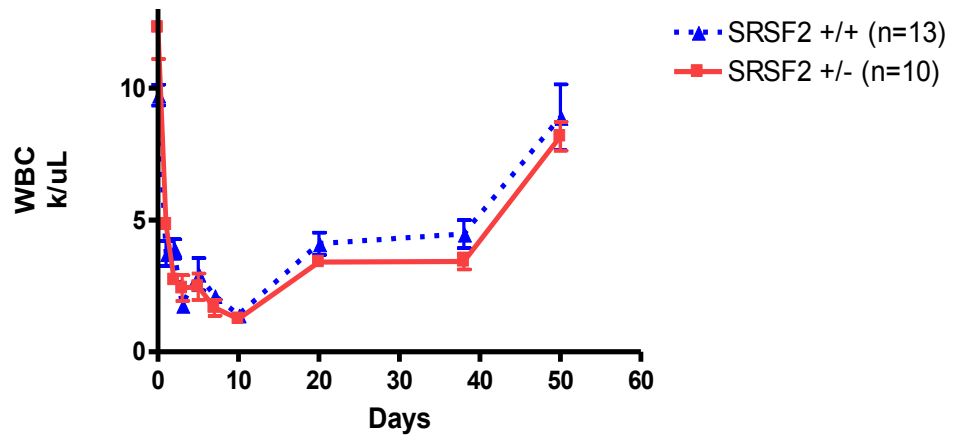


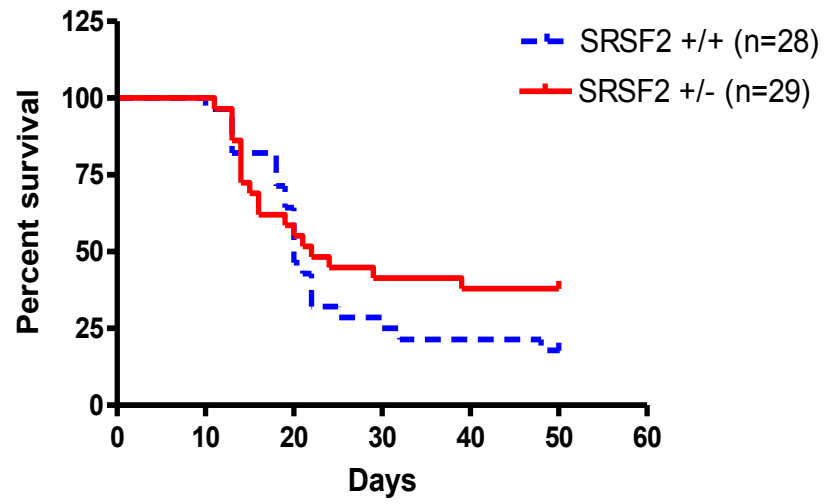
Figure 1. Knock-out of SRSF2 is embryonically lethal for the mice continued

**Figure 2. Mice heterozygous for SRSF2 shows no significant WBC recovery rate after a single dosage of sub-lethal irradiation.** Mice received sublethal dose of irradiation (400 rad), and cell count recovery was followed up. The combined data of two independent experiments.

**WBC Recovery Rate post-sublethal irradiation on SRSF2 heterozygous mice**

**Figure 3. Mice heterozygous for *Srsf2* in the blood via the *vav*-Cre system exhibited no significant difference in weekly treatment of the chemotherapeutic drug 5-Fluorouracil. *Vav-Cre*<sup>+</sup> or *Vav-Cre*<sup>-</sup> *Srsf2*<sup>wf</sup> mice were injected with 5-FU (150 mg/kg body weight) weekly, and survival rate was evaluated. The combined data of three independent experiment is shown (p = 0.2643)**

### Survival of SRSF2 Heterozygous mice after weekly 5FU injections

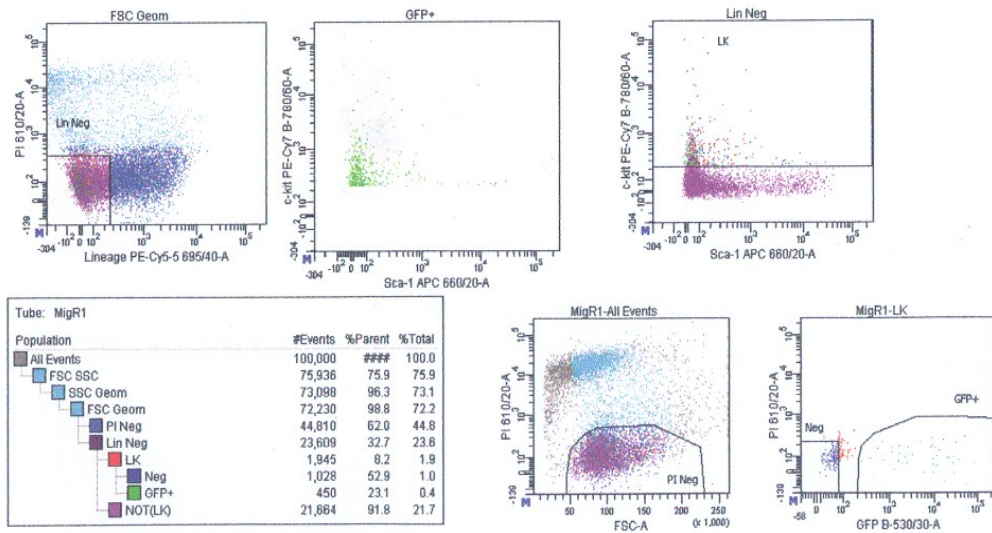


**Figure 4. Potential targets of SRSF2 were identified by RasL-seq**

- A) Representative gating for sorting. Murine lineage-negative bone marrow cells overexpressing MIG-SRSF2, MIG-SRSF2<sup>P95H</sup>, or empty MIG vectors were sorted via FACS for RasL-seq.
- B) Strategy of data analysis. Raw data from RASL seq showed the frequencies of probe hits for the short and long isoforms of a particular gene.
- C) Details of identified target genes.
- D) Pathway analysis of the target genes. Oncogenes or proto-oncogenes are highlighted in red.

A)

MiG vector only



B)

	A	B	C	D	E	F	G	H	I	J
1				5030_1		5030_2		5030_3		5031_1
2	event_id	gene_symbol	event_type	short_isoform	long_isoform	short_isoform	long_isoform	short_isoform	long_isoform	short_isoform
3	3755	1700029G01Rik	Cassette	0	0	0	5	2	15	4
4	3942	Acan	Cassette	0	0	0	0	0	0	0
5	3969	Rapgef1	Cassette	0	0	33	0	16	0	18
6	4001	Epb4.1l3	Cassette	0	0	8	0	2	0	4
7	4044	Jarid1c	Cassette	0	1	549	1573	465	1415	305
8	4204	Erc2	Cassette	0	0	0	0	0	0	0
9	4205	Cln3	Cassette	0	0	0	25	0	51	0
10	4206	Mtap4	Cassette	0	0	0	121	0	153	0
11	4218	Asph	Cassette	0	0	95	0	96	0	79
12	4219	Qpct	Cassette	0	0	7	746	3	735	2
13	4220	H13	Cassette	0	1	0	15	0	1	0
14	4234	Ankrd15	Cassette	0	0	0	1	0	0	0
15	4241	Zfp236	Cassette	0	0	127	9	137	1	98
16	4317	D15Wsu169e	Cassette	0	0	5	390	4	343	2



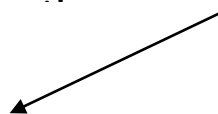
Divided short isoform hits by long isoform hits for short/long ratio



Averaged the MigR1 samples' short/long ratio



Normalized *SRSF2* and *SRSF2*<sup>P95H</sup> ratio to MigR1



Identified genes of interest based on significance and change

**Figure 4. Potential targets of SRSF2 were identified by RasL-seq continued**



C)

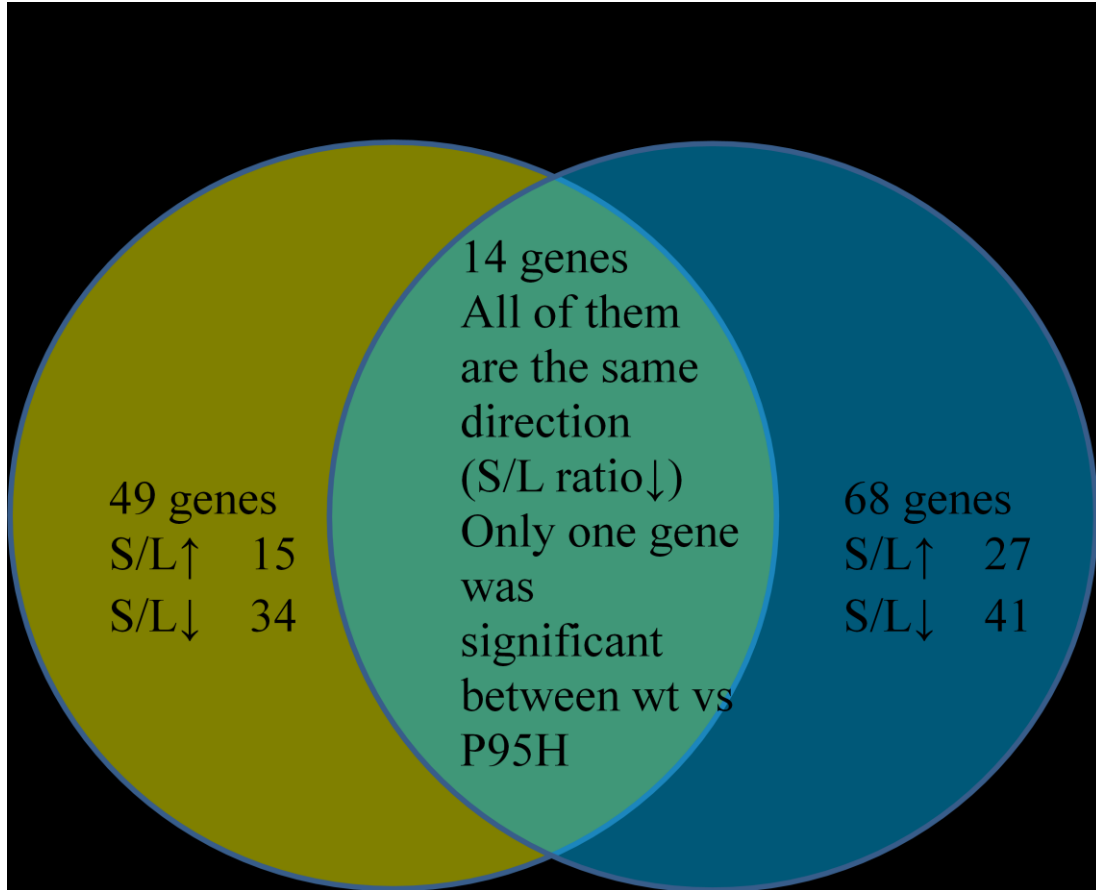
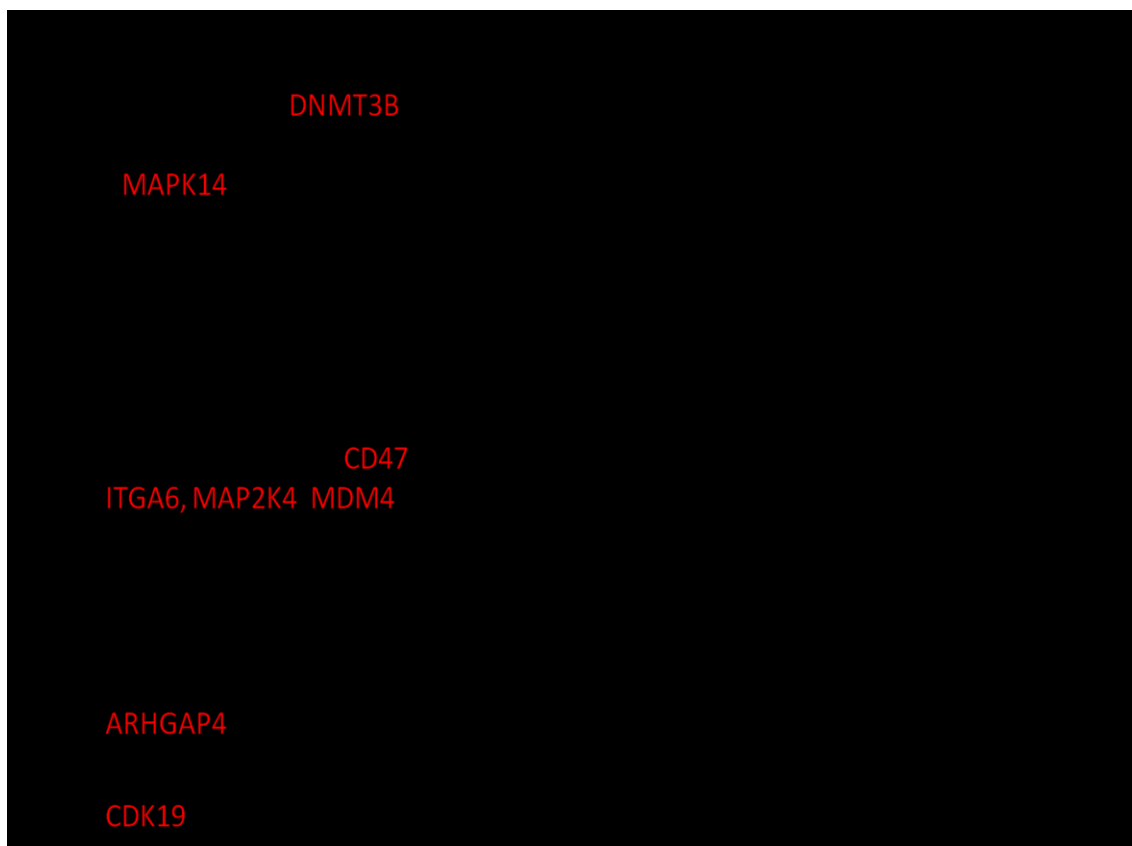


Figure 4. Potential targets of SRSF2 were identified by RasL-seq continued

D)

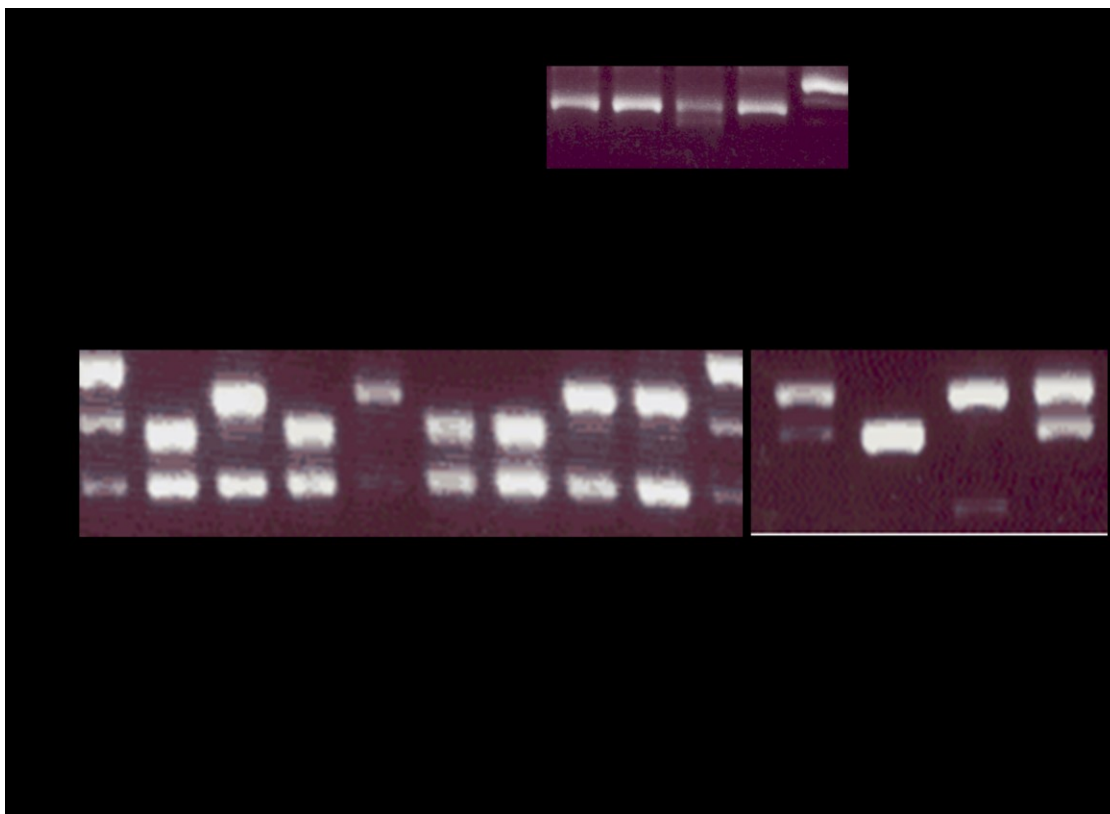


**Figure 4. Potential targets of SRSF2 were identified by RasL-seq continued**

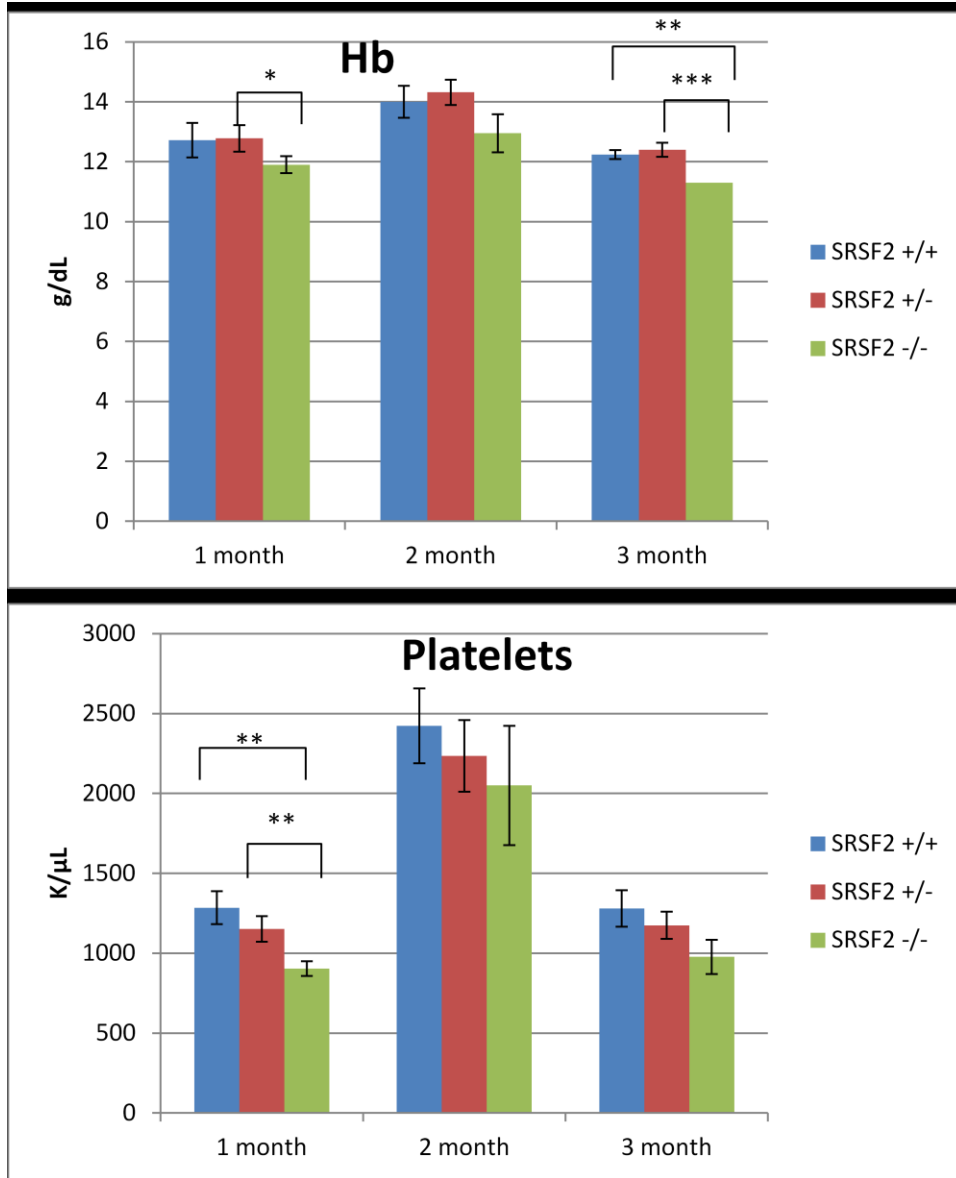
**Figure 5. In-vivo knockout were incomplete, but mice exhibited phenotypes following a decreasing cell count trend for *Srsf2* KO mice.**

- A) Genotyping results of *Srsf2* KO mice before and after PIPC treatment. Two weeks after treatment, *Srsf2* KO was seemingly complete
- B) *Srsf2* KO mice (incomplete) exhibited lower population of lymphocytes. *Srsf2* KO mice also showed a decrease in certain erythrocytes and leukocyte lineages. (B, T, Granulocyte population from flow cytometry data, other data from cell counts; *Srsf2*<sup>+/+</sup> n=5 , *Srsf2*<sup>+/-</sup> n=5 , *Srsf2*<sup>-/-</sup> n=2).
- C) Cells with incomplete excision seemingly has a growth advantage. The “recovery” rate of the floxed band was faster in *Srsf2*<sup>-/-</sup> mice than in the *Srsf2*<sup>+/-</sup> mice.

A)

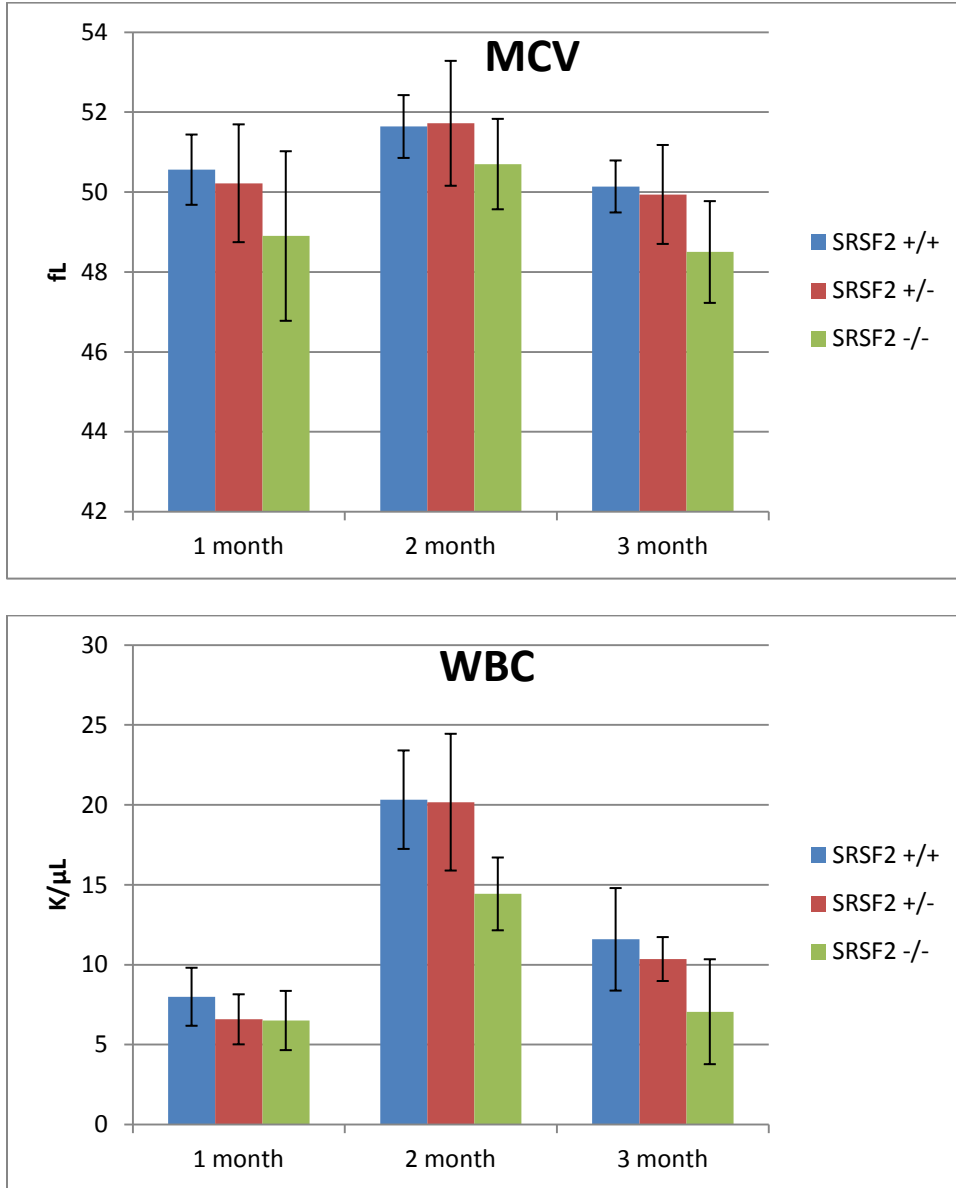


B)



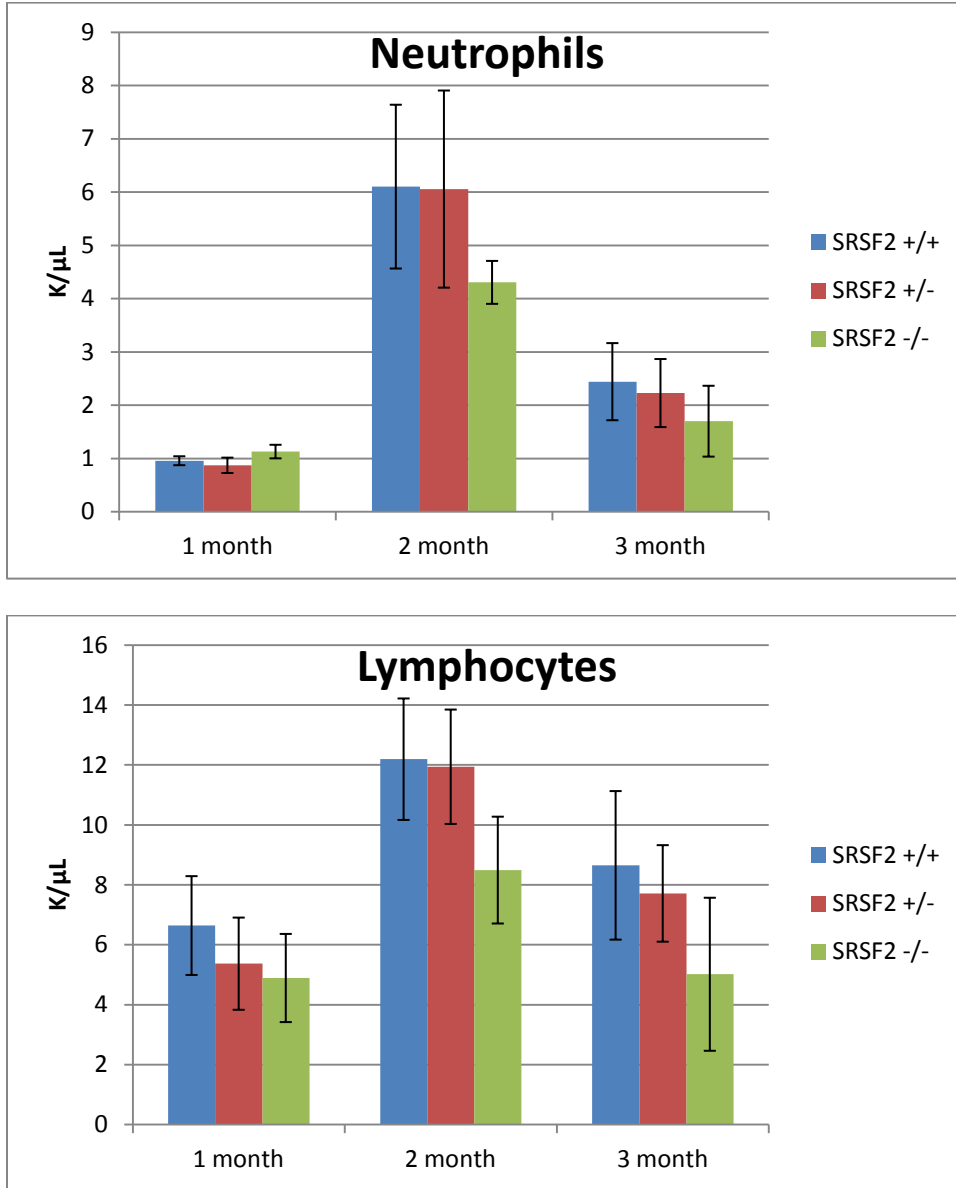
**Figure 5. In-vivo knockout were incomplete, but mice exhibited phenotypes following a decreasing cell count trend for *Srsf2* KO mice continued**

B)



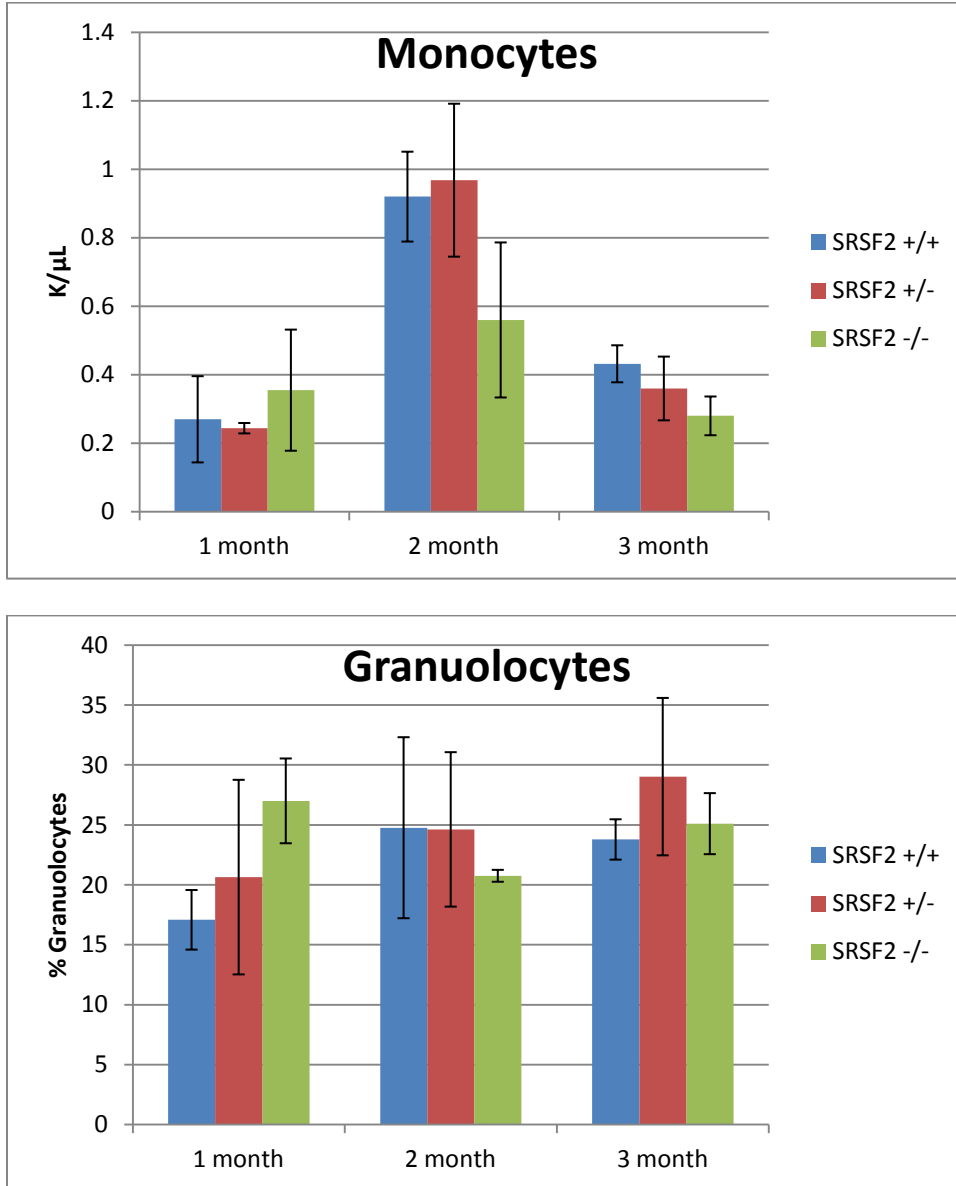
**Figure 5. In-vivo knockout were incomplete, but mice exhibited phenotypes following a decreasing cell count trend for *Srsf2* KO mice continued**

B)



**Figure 5. In-vivo knockout were incomplete, but mice exhibited phenotypes following a decreasing cell count trend for *Srsf2* KO mice continued**

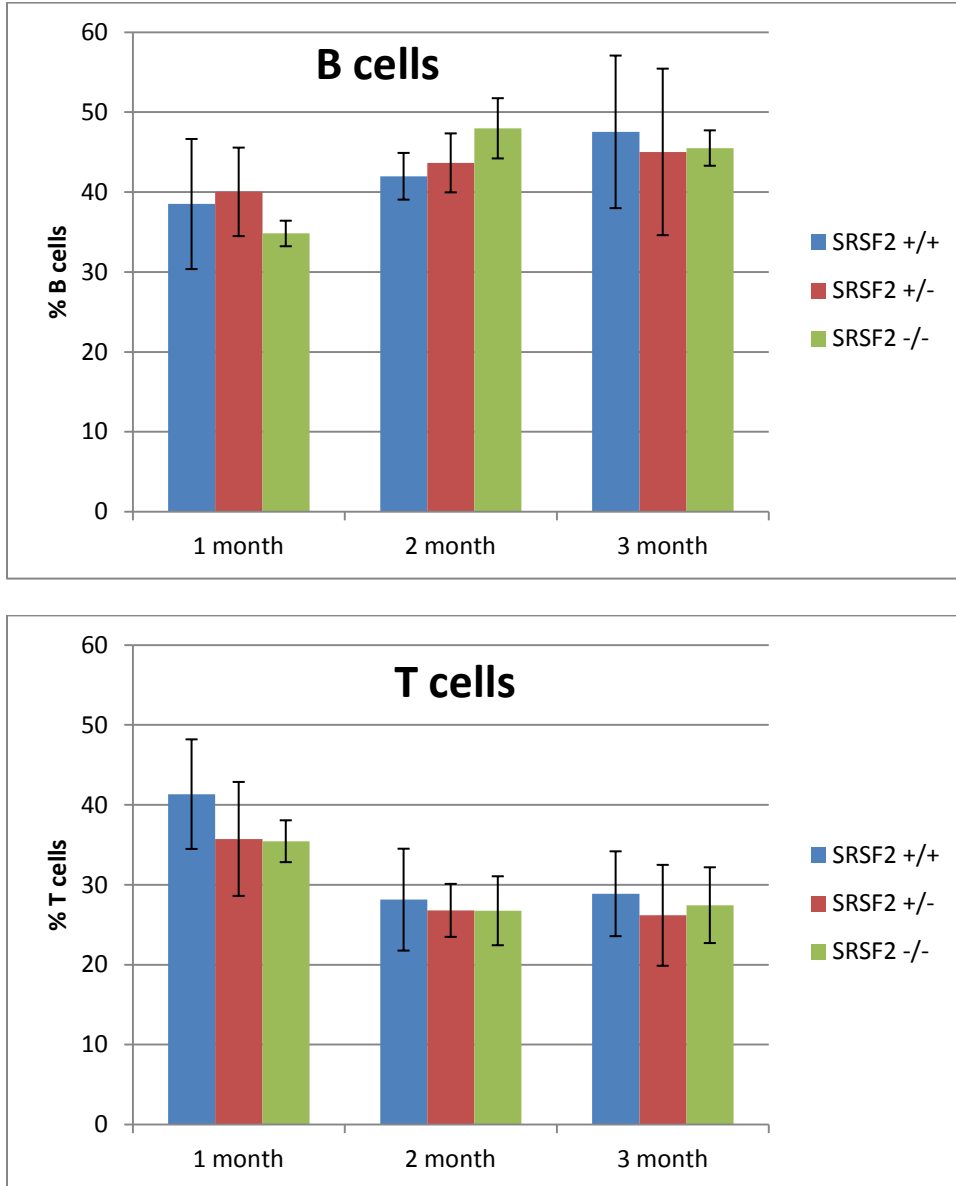
B)



**Figure 5. In-vivo knockout were incomplete, but mice exhibited phenotypes following a decreasing cell count trend for *Srsf2* KO mice continued**

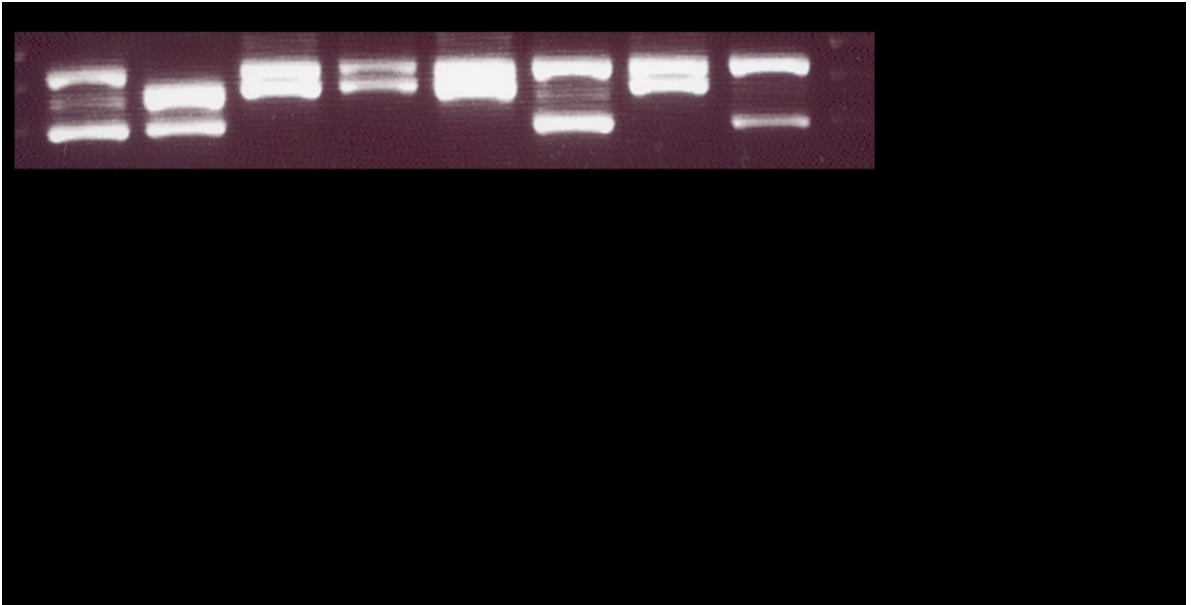


B)



**Figure 5. In-vivo knockout were incomplete, but mice exhibited phenotypes following a decreasing cell count trend for *Srsf2* KO mice continued**

C)

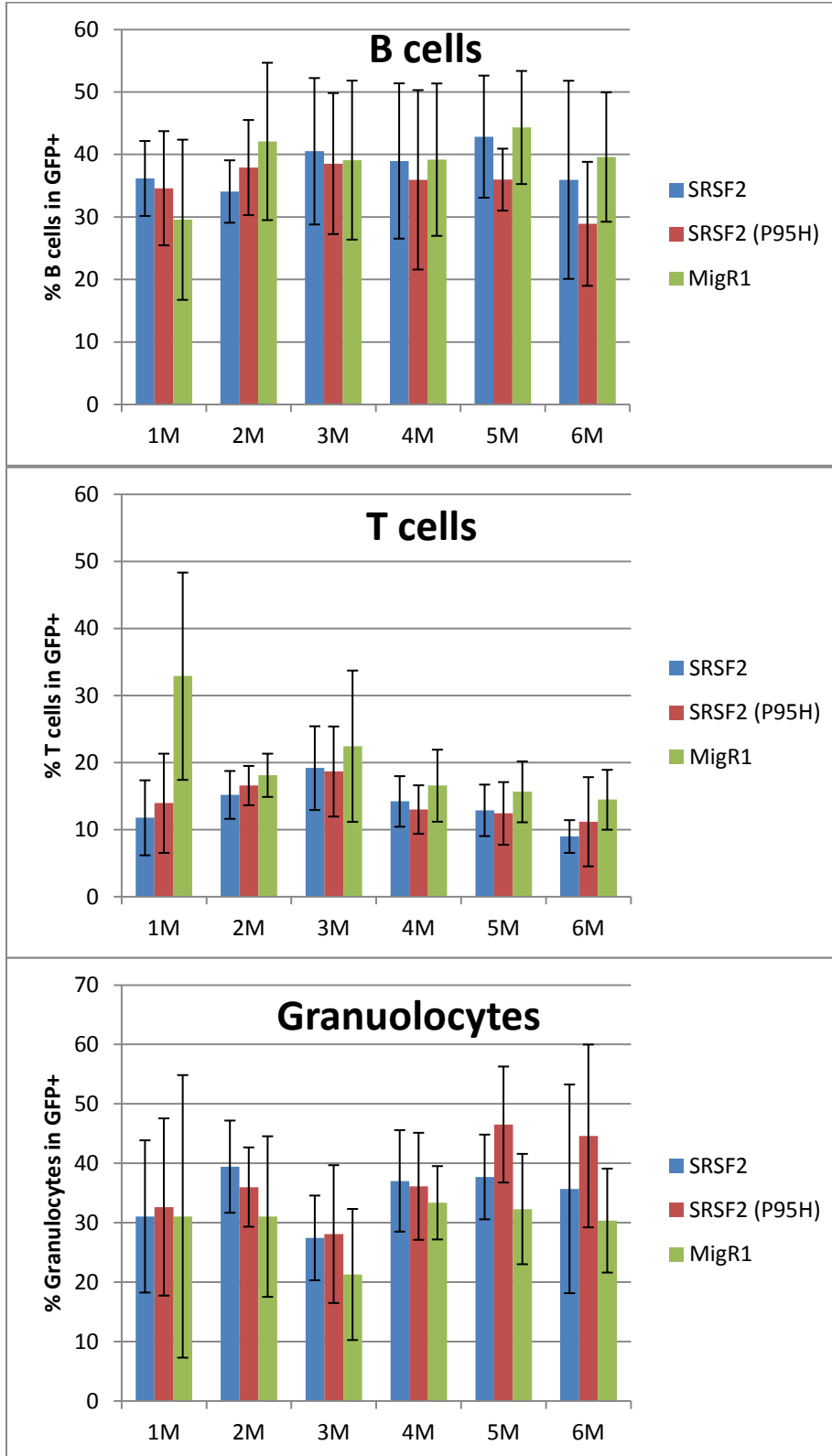


**Figure 5. In-vivo knockout were incomplete, but mice exhibited phenotypes following a decreasing cell count trend for *Srsf2* KO mice continued**

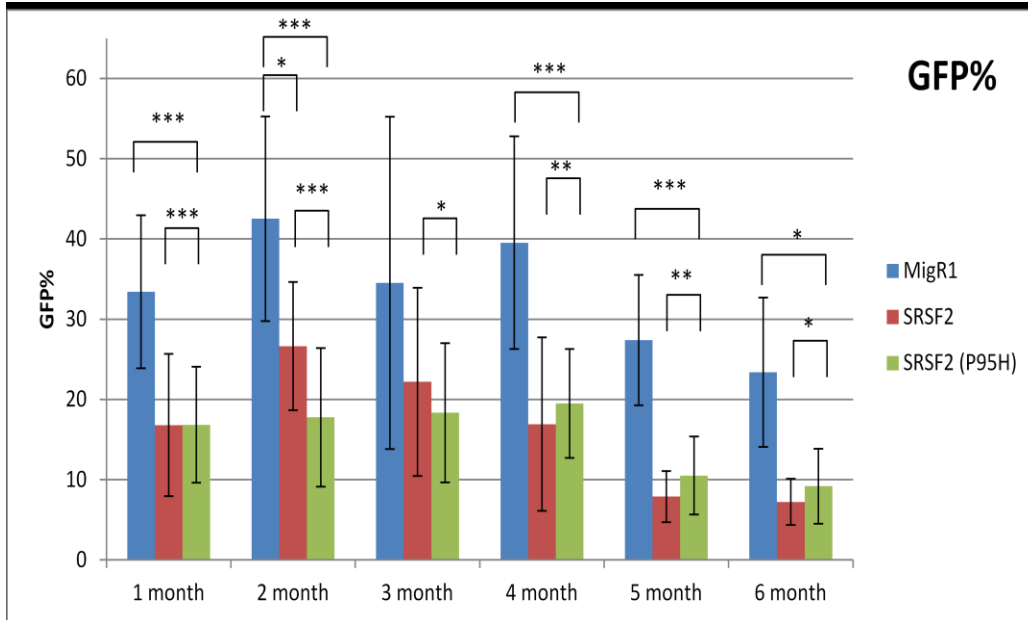
**Figure 6. Bone marrow cells overexpressing wild-type SRSF2 and SRSF2<sup>P95H</sup> have a competitive disadvantage.**

- A) WBC differentiation pattern of the GFP+ lymphocyte population in recipient mice after specific time points
- B) GFP% of recipient mice peripheral blood. Low GFP% at different time points after transplantation indicate competitive disadvantage.

A)



B)



**Figure 6. Bone marrow cells overexpressing wild-type SRSF2 and SRSF2<sup>P95H</sup> have a competitive disadvantage continued**

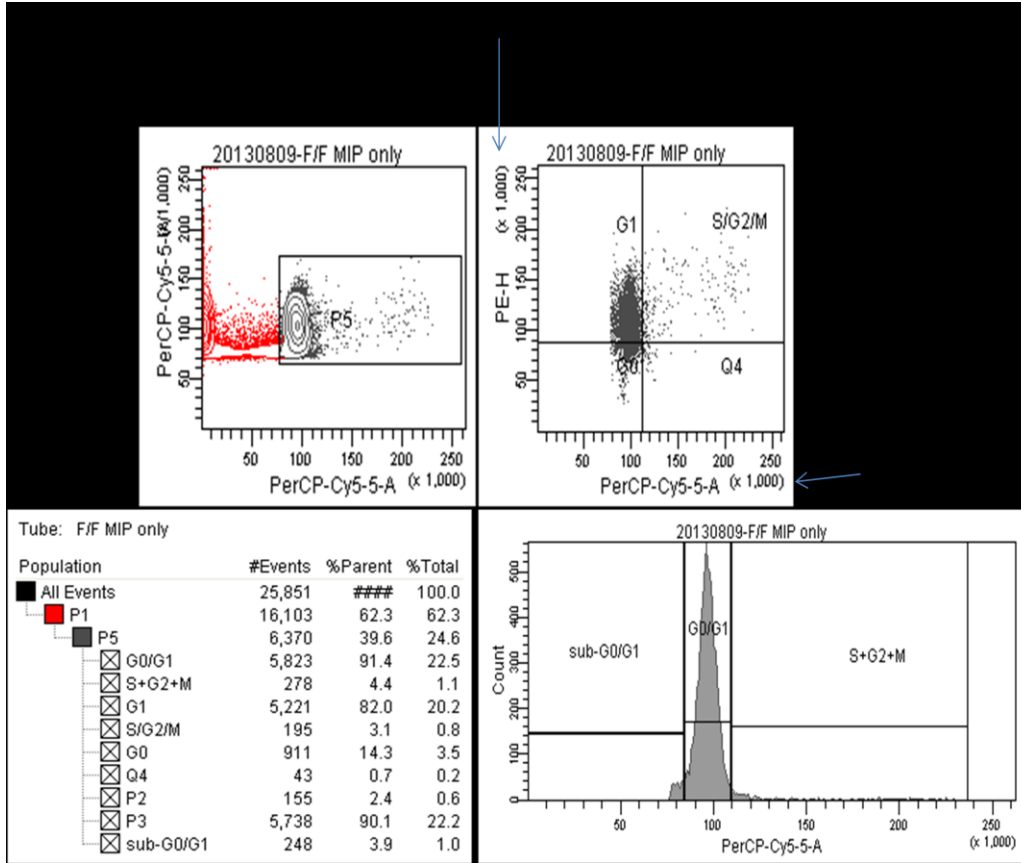
**Figure 7. In-vitro knockout of *Srsf2* cause G<sub>1</sub>/G<sub>0</sub> arrest in murine bone marrow cells.**

A) Gating for cell cycle analysis

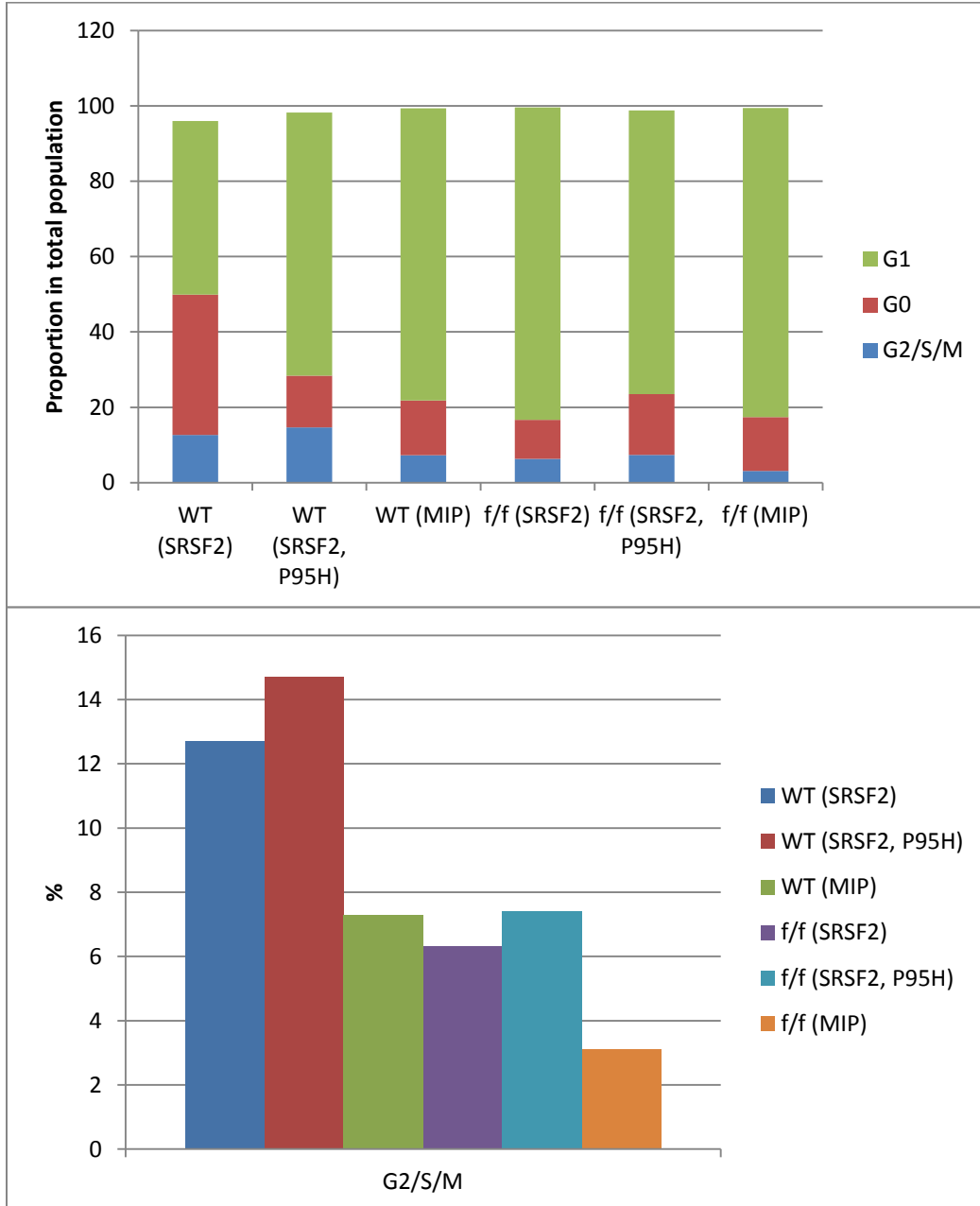
B) Cell cycle analysis of *Srsf2*<sup>WT</sup> or KO bone marrow cells

overexpressing SRSF2 or SRSF2<sup>P95H</sup>. *Srsf2*<sup>-/-</sup> bone marrow cells showed less cells in the G<sub>2</sub>/M/S stage than the rescued cells.

A)



B)



**Figure 7. In-vitro knockout of *Srsf2* cause G<sub>1</sub>/G<sub>0</sub> arrest in murine bone marrow cells continued**



### III

## Discussion

Myelodysplastic syndrome (MDS) is one of the most intractable diseases in hematology, affecting mainly elderly population. In search of the novel therapeutic targets, genome-wide sequencing revealed that splicing machinery mutation is a new category of gene aberrations in MDS. *SRSF2* is one of those genes. However, the physiological and pathological roles of *SRSF2* are not yet clear.

Here, I have used conditional knockout mice to evaluate roles of *SRSF2* in steady state or stress hematopoiesis. I also used in vitro system to overexpress WT and mutant forms of *SRSF2* to clarify the functional changes caused by the mutation.

I used lineage-negative murine bone marrow cells to identify genes whose splicing patterns were affected significantly when *SRSF2*<sup>P95H</sup> was overexpressed (Fig 4). Using different experimental conditions (single irradiation for WBC recovery rate and weekly 5-FU injections for survival rate) I have shown that mouse heterozygous for *Srsf2* do not exhibit a significant phenotype under hematopoietic stress (Fig 2, 3). I have shown that in bone marrow transplantation recipient mice, bone marrow cells overexpressing *SRSF2* and *SRSF2*<sup>P95H</sup> have a competitive disadvantage versus normal bone marrow cells (Fig 6). Although I was unable to achieve complete *Srsf2* knockout through the PIPC-*Mx* driver system, I was able to observe different phenotypes in the *Srsf2* incomplete knockout mice (Fig 5). I was also able to show that through the PIPC-*Mx* driver system, *Srsf2* KO cells have a disadvantage in growth, and that the mice requires at least one allele of normal *Srsf2* in order to survive (Fig 4C). Finally, I

was able to show that in-vitro, *Srsf2* KO mice experiences more G<sub>1</sub>/G<sub>0</sub> arrest and that *Srsf2* KO can be rescued from the cell cycle arrest through overexpression of the gene prior to knockout (Fig 7).

### **Mice heterozygous for *Srsf2* behave similarly to wild-type mice under hematopoietic stress**

Since *Srsf2* hetero FL cells during development and adult PB or BM cells in steady state did not show phenotypes, I tried to evaluate hematopoiesis under stress conditions. Mice that were heterozygous for *Srsf2* via the Vav-Cre driver system exhibited similar survival rates in weekly 5-FU injections as well as similar WBC recovery rates after sublethal irradiation. These results show that one allele of *Srsf2* is sufficient for normal cellular function. This hypothesis is further supported by the genotyping data of the PIPC-injected mice (Fig 5C). The data showed that *Srsf2*<sup>w/f</sup> mice, which are supposed to become *Srsf2*<sup>+/-</sup> after PIPC injection, show almost complete excision of *Srsf2*. For the *Srsf2*<sup>fl/fl</sup> mice, however, the intensity of the floxed band equaled that of the KO band, indicating a selective advantage in the mice for cells that did not experience *Srsf2* knockout.

### **Knockout of *Srsf2* showed a trend of decrease in certain lineages**

From the genotyping data, it can be seen that *Srsf2* knockout in mice via the PIPC-*Mx*-Cre driver was incomplete. For mice with supposedly complete knockouts, roughly 50% of the cells had recovered to the floxed state. However,

the remaining 50% of cells still maintain its *Srsf2* KO status and may exhibit phenotypes that could be observed. Peripheral blood count confirmed slight differences among leukocyte lineages, with the *Srsf2* KO mice showing a lesser amount of erythrocytes and leukocytes while the composition of these lineages remain largely the same as wild-type. Hemoglobin and platelet population was significantly decreased in *Srsf2* KO mice. These results suggest that without *Srsf2*, certain lineages of the hematopoietic system such as platelets cannot differentiate and mature completely.

### **Lineage-negative bone marrow cells overexpressing SRSF2 and SRSF2<sup>P95H</sup> show different splicing targets via RasL-seq**

I took advantage of the novel technique RasLseq established by our collaborator to detect the changes in the splicing patterns in 3800+ genes. RasL-seq is more sensitive but less costly compared to RNAseq to evaluate splicing events. The purpose of using lineage-depleted bone marrow cells was to use cells that were not too far down its differentiation path to maximize the effect observed on splicing when SRSF2 or its mutant form is expressed. It was interesting to note that when compared to the cells overexpressing only the MIG vector, cells overexpressing MIG-SRSF2<sup>WT</sup> and MIG-SRSF2<sup>P95H</sup> had only 14 common splicing targets while the majority of the splicing targets are unique to each vector. This result clearly shows that P95H mutant is gain-of function mutation, and it is compatible with the fact that SRSF2 has a mutation hot spot, and *SRSF2* mutation is heterozygous in MDS patients. We need to validate the

splicing patterns by RT-PCR. Further studies on the splicing targets of the mutant that show the most dramatic splicing pattern changes may elucidate the role of SRSF2<sup>P95H</sup> or other mutant forms in MDS as well as its mechanism and interaction with other oncogenes critical to the development of MDS. However, there is a possibility that overexpression of SRSF2 and its mutant may not cause the same reactions in the cell as endogenous SRSF2 and its mutant. In this sense, knock-in model is the most ideal, however, not available in our lab. The RASL-seq experiment therefore serves as a source of data for preliminary targets for future studies.

**Bone marrow cells overexpressing SRSF2 and SRSF2<sup>P95H</sup> have a competitive disadvantage in recipient mice**

From the low GFP positive population in the peripheral blood in the recipient mice, it could be observed that the bone marrow cells overexpressing MIG-SRSF2<sup>WT</sup> and MIG-SRSF2<sup>P95H</sup> have marked disadvantage when compared to BM cells overexpressing the MIG empty vector. On the other hand, flow cytometry data of the GFP+ population showed no particular trend amongst the three vectors in terms of white blood cell differentiation patterns. Furthermore, after 8 months the recipient mice did not exhibit any symptoms of MDS, pointing to the possibility of *SRSF2* mutation alone is not sufficient to induce the onset of MDS.

### **SRSF2 KO bone marrow cells exhibit cell cycle arrest at the G<sub>0</sub>/G<sub>1</sub> stage**

*Srsf2*<sup>-/-</sup> fetal liver cells show enhanced apoptosis and cell cycle arrest at G1 phase (unpublished data). However, we cannot exclude the possibility of non-hematopoietic liver cell contamination. To confirm the cell cycle arrest in pure blood cells, bone marrow cells were first collected from *Srsf2*<sup>w/w</sup> and *Srsf2*<sup>f/f</sup> mice. These bone marrow cells were then infected with MIP, MIP-SRSF2, MIP-SRSF2<sup>P95H</sup>. The purpose of the overexpression prior to knockout is to prevent any irreversible apoptotic pathway taken by the cell in the case of *Srsf2* KO. *Srsf2* KO is then induced with the infection of MIY-Cre. From the cell cycle analysis, it was observed that *Srsf2* KO BM cells had a 50% decrease in cells present in the S/M/G<sub>2</sub> stage. This data is compatible with G1 arrest in *Srsf2* KO fetal liver cells. It is interesting to note that mouse embryonic fibroblast cell line show G2 arrest when SRSF2 expression is turned off<sup>[17]</sup>. Cells of different types may be using different mechanisms to cause cell cycle arrest. Further studies from the data of the RASL-seq experiment may shed light on the cause for the cell cycle arrest.

In summary, I revealed physiological and pathological roles of SRSF2 in hematopoiesis, and functional differences of wild type and mutant forms of SRSF2, by using in vitro and in vivo models.

IV.  
Methods and Materials

## Mice

Conditional knockout *Srsf2* f/f mice of C57Bl/6 background were provided by Dr. Xiang-Dong Fu. Vav-cre and Mx-cre mice were obtained from Jackson Laboratory. 6-12 week-old mice were used for experiments. All the procedures were approved by the UCSD IACUC.

## Cell Culture

293T cells were cultured in DMEM containing 10% bovine calf serum, 1 % penicillin/streptomycin and 1 % glutamine.

Mouse bone marrow (BM) cells were collected from femurs and tibiae. Mouse fetal liver (FL) cells were collected from E14-16 embryos. Primary BM and FL cells were treated with red blood cell lysis buffer (ACK: 150 mM NH<sub>4</sub>Cl, 1 mM KHCO<sub>3</sub>, 0.1 mM EDTA) and cultured overnight in “2x BM media” ( Isocove’s Modified Dulbecco’s Medium (IMDM) , 20 % fetal bovine serum, 4 % IL3 conditioned media (IL3-CM, culture supernatant of X63 AG-653 myeloma cell line), 4 % SCF-conditioned media (SCF-CM, culture supernatant of BHK-MKL cell line), 1 % penicillin/streptomycin and 1 % glutamine), prior to infection.

## Constructs

Wild type human *SRSF2* construct was provided by Dr. Xiang-Dong Fu. EcoRI restriction sites were added to 5'- and 3'- ends of C-terminally HA-tagged human *SRSF2* sequence by PCR. The *SRSF2* wild type or mutant fragments



were subcloned into EcoRI sites of MSCV-IRES-GFP (MigR1) and MSCV-IRES-puro (MIP) vectors.

### **Retroviral Infection**

To produce retrovirus, 293T cells were transfected with 5 µg of MSCV-IRES-PURO (MIP), MSCV-IRES-GFP (MIG), MIG-SRSF2, MIG-SRSF2<sup>P95H</sup>, MSCV-IRES-EYFP (MIY), MIY-Cre vectors and with 5 µg of Ecopac vector by using polyethylenimine (PEI) reagent (Sigma). The 293T media was changed from DMEM to 6 mL of IMDM 10 hours post transfection. Retrovirus supernatants were harvested 48 hours after transfection and were filtered through a 0.45 µm filter. The retrovirus supernatant from the vectors was then added to primary bone marrow cells, respectively, along with 4 % IL3-CM, 4 % SCF-CM, 1 % HEPES and 0.1 % polybrene (final concentration 4 µg/mL). The cells were spinoculated at 3000 rpm (1200 x g) for 3 hr at 32° C then cultured at 37° C with 5 % carbon dioxide. Infections were performed twice on consecutive days. Infection efficiency of MIG virus was evaluated by flow cytometry. The cells infected with MIP were selected in 1 µg/ml puromycin for 3 days before analysis.

### **5-Fluorouracil Injection**

5-FU (Intas Pharmaceuticals Ltd.) was diluted in PBS, and was injected intraperitoneally (*i.p.*) at the dosage of 150 mg/kg body weight. The Vav-Cre-/*Srsf2*<sup>w/f</sup>, Vav-Cre-/*Srsf2*<sup>f/f</sup>, Vav-Cre+/*Srsf2*<sup>w/f</sup>, Vav-Cre+/*Srsf2*<sup>f/f</sup> mice were injected

once a week. The survival was shown in Kaplan-Meyer curves, and evaluated by Log-rank test.

### **Sub-lethal Irradiation**

$Vav-Cre+/Srsf2^{w/f}$  and  $Vav-Cre-/Srsf2^{w/f}$  mice underwent a single dosage of sub-lethal irradiation (400 Rad). Peripheral blood was collected from tails at indicated time points to evaluate white blood cell count recovery using the Hemavet cell counter (DREW scientific).

### **PIPC Injection**

Polyinosinic:polycytidylic acid (PIPC) (Sigma) was injected *i.p.* into mice to induce Cre recombinase expression under *Mx* promoter. Two different dosages were used: 600  $\mu$ g every other day for a total of seven injections; or 250  $\mu$ g every other day for a total of three injections.

### **Bone Marrow Transplantation**

Bone marrow donor C57BL/6 mice were injected with 150 mg/kg body weight 5FU 5 days prior to bone marrow harvest. Bone marrow (BM) cells were harvested and infected with MIG, MIG-SRSF2, MIG-SRSF2<sup>P95H</sup> vectors. GFP percentage was adjusted to 17 % by using uninfected cells. Infected BM cells were injected into lethally irradiated recipient mice (900 rad) through the tail veins. Recipient mice were given acidic water (pH 4) for three weeks following the bone marrow transplant.

### **Peripheral Blood Collection**

Peripheral blood collection was done according to IACUC protocol S07271. Prior to the protocol revision on 8/2/2013, mice were anesthetized and peripheral blood was collected retro-orbitally for a total of 45-60  $\mu$ L of blood per mice. After the protocol revision, peripheral blood was collected submandibularly for a similar volume of blood per mice.

### **Flow cytometry**

Peripheral blood was treated with ACK at room temperature for 5 minutes. After washing with PBS, the cells were stained for B cell lineage (APC-conjugated B220), T cell lineage (PerCP-Cy5.5-conjugated CD4 and CD8a), or granulocyte lineages (PE-conjugated Gr1 and CD11b) (all antibodies from eBioscience) in 100  $\mu$ L of PBS at 4°C for 20 minutes. After washing with PBS, the data were collected on FACSCanto (BD), and analyzed by FACSDiva software (BD). As for apoptosis assay, Annexin V-APC apoptosis kit (BD) was used. Cell cycle analysis was performed as previously described<sup>[18]</sup>.

### **Preparation of Peripheral Blood for Genotyping**

Peripheral blood samples were suspended in 700  $\mu$ L of lysis buffer (0.32 M sucrose, 10 mM Tris-HCL pH 7.5, 5 mM MgCl<sub>2</sub>, 1 % v/v Triton X-100) for 3 minutes, and spun down at 4000 rpm for 5 minutes. The supernatant was removed and the pellet was resuspended in 200  $\mu$ L of lysis buffer for another 3

minutes. This process was repeated for 5 times or until there were no more red in the solution. After a final centrifugation and the removal of the supernatant, the pellet was digested in 100  $\mu$ L of PCR Buffer with Nonionic Detergents (PBND) buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM MgCl<sub>2</sub>, 0.1 mg/mL gelatin, 0.45% v/v Nonidet P40, 0.45% v/v Tween 20) with 60  $\mu$ g/mL of protease K at 60°C overnight. The samples were incubated at 97°C for 10 minutes to inactivate the protease K, and 5  $\mu$ L were used in the genotyping PCR reactions.

### **Sample preparation for RasL-seq**

Lineage-negative bone marrow cells were sorted by magnetic beads (Mouse Lineage depletion kit, Miltenyi). The cells were transduced with *SRSF2* and *SRSF2*<sup>P95H</sup> both in MIG vectors as well as empty MIG vector. Lineage-negative GFP<sup>+</sup> cells were sorted by FACSArialI (BD). The lineage antibody cocktail consisted of CD3, CD4, CD8a, B220, CD19, Gr1, and Ter119. The sorted cells underwent total RNA extraction and were sent to our collaborators at Xiang-Dong Fu's lab for RasLseq (RNA-mediated oligonucleotide Annealing, Selection, Ligation with next-generation SEQuencing) analysis.

### **Primer design for RasL-seq data validation**

Primers were designed using the UCSC Genomic Library. Forward and reverse primers were chosen to anneal to the exons of the genes. The annealing site of the primers were based on the isoforms' exon configuration.

**RasL-seq data analysis**

From the short/long isoform ratios of these genes for the mutant samples, we divided each of the three ratios with the empty vector ratio average to obtain the fold changes of the short/long isoform ratios. These fold changes were then averaged, and ratios that show a number of 2 or above (increase of two-fold or more) or 0.5 or below (decrease of two-fold or more) were identified. Ingenuity pathway analysis software was used to identify the affected signaling pathways.

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