UNIVERSITY OF CALIFORNIA, SAN DIEGO

Functional characterization of the tumor suppressor RASSF2 in Acute Myelogenous Leukemia via CRISPR/Cas9-mediation

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in

Biology

by

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Stanley Lo
Yang Xu

2016

The thesis of Michael Bao Pu Wu is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2016

Dedication

This thesis is dedicated to my parents: Jackson Wu and Connie Chen

and to my mentors, Dr. Dong-Er Zhang and Samuel A. Stoner

and finally, to my truly amazing friends.

I would not have made it thus far without your **leading, loving, and guiding.**

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Figures included in the Introduction section, in full, will eventually be submitted for publication of the material. Stoner, Sam; Zhang, Dong-Er; Wu, Michael Bao Pu.

ABSTRACT OF THE THESIS

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Master of Science in Biology

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Professor Dong-Er Zhang, Chair

RASSF2 is a powerful pro-apoptotic K-Ras effector that is that is inactivated in many tumors via promoter methylation and has been shown to function as a tumor suppressor in lung, colorectal, and breast cell lines. RASSF2 belongs to the Ras-association domain family (RASSF) of proteins, which are able to engage in homo/hetero-dimerization and interact with common binding partners. In the context of

acute myelogenous leukemia (AML), RASSF2 is exclusively downregulated in t(8;21) AML, suggesting that its repression may be essential for t(8;21) leukemia development.

In order to further characterize RASSF2's tumor suppressive role in a leukemic context, we performed a CRISPR/Cas9-mediated knockout of RASSF2 in two non-t(8;21) AML cell lines: HL-60 and U937, generating single cell isolated clonal lines that are RASSF2 wild type, heterozygous, and knockout. Among the clonal cell lines, we assayed for changes in proliferation, apoptosis, cell cycle, and differentiation. We observed that heterozygous knockdown of RASSF2 in the U937 cells resulted in significantly higher ($p<.05$) proliferation when compared to wild type U937 clonal cells. However, mono- and bi-allelic knockout of RASSF2 did not result in significant differences in differentiation, apoptosis, or cell cycle arrest. In fact, clonal lines of the same genotype were observed to be characteristically variable. Taken together, we demonstrate that RASSF2 knockout is not critical in acute myelogenous leukemia since it does not manifest in a significant functional phenotype that overcame clonal characteristic variation. This suggests that loss of RASSF2 expression leads to the rescue of its function. We also prove that the process of single cell clonal isolation selects for clonal lines that are inherently variable in differentiation, which demonstrates that functional characterizations involving single cell clonal isolation must be performed with a larger pool of samples to account for the characteristic variability of clonal cell lines.

I. Introduction

Acute Myelogenous Leukemia

Leukemia is broadly defined as the cancer of the blood. The disease is marked by the malignant proliferation of abnormal white blood cells, which crowds out the bone marrow and eventually, disrupts the production of normal, mature blood cells by hematopoietic stem cells (Rubnitz et. Al). If the leukemia is marked by proliferation of B-cell precursors (lymphocytes), it is known as lymphoblastic; meanwhile, leukemia affected by proliferation of red blood cell precursors (myeloid) are known as "myelogenous leukemia." Depending on the degree of maturity of the abnormal white blood cells and the pace of disease progression, leukemia is defined as either "chronic" or "acute." (Kouchkovsky et. Al)

Acute Myelogenous Leukemia (AML), is a cancer of myeloid blood progenitors that is defined by abnormal proliferation of myeloblast cells that accumulate in the bone marrow, and ultimately disrupting the production of normal, differentiated blood cells (Döhner et. Al). AML can be sub-classified via cytogenetic abnormalities, cell lineage, or presence of hallmark cell surface markers. One established sub-classification system is the French-American-British (FAB) system, which divides AML into eight subtypes (M0-M7) based on the cell type and level of differentiation (Bennett et. Al). The M2 subtype, defined as "acute myeloblastic leukemia with granulocytic maturation," is the most common, accounting for 25% of adults with AML (Seiter et. Al). In this study, we will be comparing the knockout of a tumor suppressor in Acute Myelogenous Leukemia cell lines: HL-60 (FAB Subtype M2) and U937 (FAB Subtype M4/5) (Dalton et. Al;

Nestal de Moraes et. Al). Since we are comparing two cell lines with different FAB classifications, we can determine whether or not our tumor suppressor of interest has selective antitumor activity against AML cells with distinct morphological/cyto-chemical differences.

Significance of t(8;21) translocation

Under the M2 subtype, the most common cytogenetic abnormality is the translocation of the eighth and twenty-first chromosomes, leading to the genesis of the fusion oncoprotein RUNX1-ETO (also known as AML1-ETO) (Andrieu et. Al). *RUNX1* (*AML1*), located on the 21st chromosome, is a transcription factor that regulates critical processes in many aspects of hematopoiesis (e.g. blood cell differentiation). Specifically, in myeloid cells RUNX1 directly binds and regulates the promoter activities of genes related to myeloid cell function (Lam et. Al). Meanwhile *ETO*, located on the 8th chromosome, encodes a transcriptional co-repressor protein that associates with a multitude of transcriptional repressors, such as as the human nuclear co-repressor complex (HuN-CoR) (Davis et. Al). Interestingly, the sole existence RUNX1- ETO does not lead to leukemogenesis—additional mutations, or "hits" at other tumor suppressor or proto-oncogenes must occur in order for onset of cancer (Lam et. Al). Since the t(8;21) consequently fuses a hallmark hematopoietic transcription factor (RUNX1) to a transcriptional co-repressor (ETO), it is proposed that RUNX1-ETO (AML1-ETO) affects cancer development via repression of essential hematopoiesis-related genes. However, the relative functional significance of these individual, repressed genes is ill-defined. Thus, it is absolutely essential to identify the specific genes or pathways that are

downregulated in the presence of RUNX1-ETO in order to gain further molecular and functional insights into the progression of t(8;21) leukemia and to identify potential therapeutic targets.

RASSF2: A Potentially Important Target for AML Development

One specific gene that is significantly downregulated in t(8;21) leukemia is RASSF2. According to publically available gene expression datasets, RASSF2 is specifically downregulated (at least 2 fold) in total bone marrow of t(8;21) patients in comparison to non-t(8;21) FAB M2 Subtype patients (TCGA). In addition, a leukemia mouse model has previously shown that *rassf2* mRNA expression is 30 fold lower in t(8;21) leukemia cells when compared to wild-type Lin-Sca-cKit^{+} myeloblasts. Lin-Sca-cKit^{+} (LK) is a marker for mouse common myeloid progenitors and LK cells were chosen for this study since they have significantly faster leukemia-initiating capability than Lin^{-}/Sca1^{+}/cKit^{+} (LSK) mouse hematopoietic cells (Lo et. Al).

Meanwhile, unpublished work from our lab has confirmed aforementioned downregulation of RASSF2 in t(8;21) AML (ii) by comparing t(8;21) AML cell lines to control CD34+ hematopoietic progenitors and other AML cell lines. Also, we have demonstrated that introduction of ectopic AML-ETO in a non-t(8;21) cell line leads to significant decrease in RASSF2 mRNA expression (ii). Finally, our lab has demonstrated that RASSF2 has a tumor suppressive function in t(8;21) leukemia via retroviral transduction of t(8;21) cell lines with RASSF2 (shown below). With this preliminary understanding RASSF2's molecular role in a leukemia context and no knowledge of its

functional role in a blood context, we are motivated to further functionally characterize RASSF2.

Presence of AML1-ETO (RUNX1-ETO) leads to transcriptional repression of *RASSF2*

i

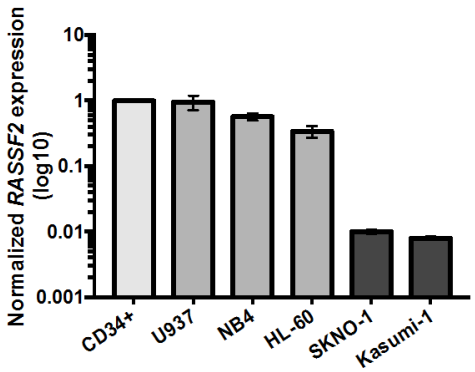


Figure i: Relative RASSF2 mRNA transcript expression was compared by RT-qPCR in primary $CD34^+$ cells isolated from human cord blood, two t(8;21) AML cell lines: SKNO-1 and Kasumi-1, and a FAB subtype M2 non-t(8;21) AML cell lines: HL-60, U937, and NB4. Data are normalized to expression in primary $CD34^+$ cell controls.

ii

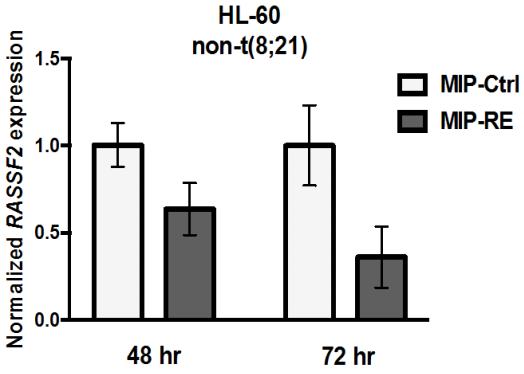


Figure ii: Relative RASSF2 mRNA transcript expression of HL-60 transduced with MIP-RUNX1-ETO compared to non-transduced HL-60 at 48 and 72 hour timepoints. Ct values are calculated based on comparison to the geometric mean of two independent reference genes, GAPDH and RNAPolII.

Expression of downregulated RASSF2 in t(8;21) cell lines demonstrates tumor suppressive function

iii

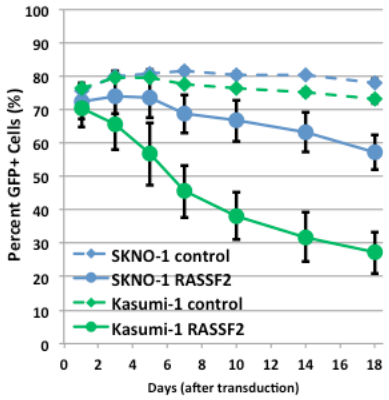


Figure iii: t(8;21) AML cell lines retrovirally transduced with MSCV-Ires-eGFP (MIG-control) or MSCV-RASSF2-Ires-eGFP (MIG-RASSF2). After transduction the percentage of GFP+ cells was monitored over a two-week period. Data are plotted as mean +/- S.E.M.

RASSF2: a potential tumor suppressor in various contexts

RASSF2 has been identified as a potential novel tumor suppressor and is a pro-apoptotic effector of K-Ras. It is part of the RASSF family of proteins, which comprises 10 members that all possess the RAS association (RA) domain (Cooper et. Al). Since RASSF family members share common domains, they are able to heter/homodimerize and interact with common binding partners. RASSF2 is frequently inactivated in a variety of primary tumors, such as human colorectal cancer, by promoter methylation (Akino et. Al). RASSF2 has also been shown to promote apoptosis and cell cycle arrest in embryonic kidney cell lines and is frequently down-regulated in lung tumor cell lines (Vos et. Al). RASSF2 has also been shown to stabilize and co-localize with MST1/2 proapoptotic kinases to induce apoptosis in 293T cells (Cooper et. Al).

One of the most convincing studies that demonstrates RASSF2's functional significance in a blood context is an *in vivo Rassf2* knockout mouse model (Song et. Al).

In this study, *Rassf2* knockout mice exhibited a unique systematic phenotype that demonstrates that RASSF2 may also have a unique physiological function in the human context. A significant finding of this study is that *Rassf2* knockout mice display systemic lymphopenia, which is defined as "having an abnormally low level of lymphocytes." There are three important pieces of evidence that pointed to this: first of all, total cell numbers in the thymus, spleen, bone marrow, and peripheral blood was significantly reduced; secondly, the authors observed greatly reduced numbers of B cells in the spleen and T cells in the thymus and in peripheral blood; finally, it was observed that in *Rassf2* knockout mice, both mouse hematopoietic LSK cells and mouse Myeloid Progenitor cells composed of a significantly smaller percentage of Bone Marrow (BM) cells when compared to their wild type counterparts. In addition, *Rassf2* knockout mice exhibited a severe osteoporotic phenotype, inhibition of NF-kB signaling, and severe bone retardation. In order to investigate whether or not systemic lymphopenia was due to an intrinsic defect in the repopulation capacity of hematopoietic stem cells, BM cells from *Rassf2* knockout mice or WT mice were mixed in a 2:1 ratio with control BM cells and transplanted into lethally irradiated WT mice. Surprisingly, no difference was observed in hematopoietic reconstitution between WT and *Rassf2* knockout donors, suggesting that a RASSF2 knock in hematopoietic cells would not have a dramatic effect. However, the authors conclude that this evidence alone cannot discount *Rassf2*'s role in hematopoiesis and that tissue-specific depletion of *Rassf2* will be required to identify the true roles played by RASSF2 during hematopoietic development.

CRISPR as a tool for RASSF2 Functional Characterization

CRISPR (**C**lustered **r**egularly **i**nterspaced **s**hort **p**alindromic **r**epeats) is a precise genome editing system that uses engineered nucleases adapted from the microbial adaptive immune system (Wang et. Al). The system is composed of a Cas9 nuclease and a CRISPR guide RNA (gRNA), which directs it to a specified cleavage site in the genome (gRNA is homologous to this site) and thus generating a double stranded break. The break is then repaired via error-prone non-homologous end joining (NHEJ), which generally results in a frameshift or premature stop indel mutation. CRISPR-Cas9 mediate gene editing is extremely precise, and when combined with single cell clonal isolation, is able to produce effective knockout models (Ran et. Al).

In order to further functionally characterize RASSF2 as a tumor suppressor in a leukemic context, we decided to generate an AML RASSF2 Knockout via CRISPR-Cas9 mediation. Although we currently have a vague understanding of RASSF2's tumor suppressive function, it is not yet fully understood how RASSF2 gene inactivation contributes to tumor development. We have chosen to perform this knockout in HL-60 (FAB M2 Subtype) and U937 (FAB M4/M5 Subtype) Acute Myelogenous Leukemia cell lines specifically since they are AML cell lines with relatively normal RASSF2 expression levels (i). Given the extensive evidence of RASSF2's tumor suppressive function in other cancers, we hypothesize that the knockout of RASSF2 will lead to the acquisition of a more cancerous phenotype, such as inhibition of apoptosis, inhibition of differentiation ability, and increase in proliferation.

Figures included in the Introduction section, in full, will eventually be submitted for publication of the material. Stoner, Sam; Zhang, Dong-Er; Wu, Michael Bao Pu.

II. Results

Generating clonal cell lines with various RASSF2 genotypes in U937 and HL-60 cell lines via CRISPR-Cas9 mediation

To determine the functional significance of RASSF2's tumor suppressive role in a leukemic context, we used two independent CRISPR construct systems to generate stable RASSF2 knockout, heterozygote, and control (WT) cell lines in HL-60 acute promyelocytic leukemia and U937 histolytic lymphoma cancer cells. HL-60 RASSF2 clonal cell lines were generated with tetracycline-inducible, double vector system targeting the 1st and 2nd exons, respectively (Figure 1A); U937 clonal cell lines were generated with single vector system targeting the 3rd and 7th exons (Figure 1B), respectively. For each cell line, we generated 2 clonal lines that are RASSF2 homozygous knockout, 2 clonal lines that are RASSF2 heterozygotes, and 2 clonal lines that are RASSF2 wild type (Figure 1A-B). Specific locations of sgRNA targets are shown in Figure 1C.

Double vector, Tet-Inducible system used in HL-60 exhibited uninduced FLAG-Cas9 expression and subsequently, uninduced cleavage at respective gRNA target sites

The tet-inducible, two vector approach was utilized to address the concern that Cas9 could possibly be toxic when consitutively expressed in our cell lines. Cas9 has been demonstrated to be toxic in single cell algae *Chlamydomonas reinhardtii* (Jiang et. Al) Interestingly, WB Blot confirmation of FLAG-Cas9 expression in Cas9 lentiviral

construct revealed that pCW-Cas9 transduced HL-60 pool exhibited ~10-20% Cas9 expression, even without doxycycline treatment (Figure 1A). Meanwhile, T7 Endonuclease Assay of sgRNA vector-transduced, Cas9 expression HL-60s revealed cleavage at guide RNA target sites without doxycycline treatment (Figure 1B). We can rule out the possibility of basal FLAG expression by HL-60 cells since leaky FLAG-Cas9 expression produced leaky cleavage gRNA targets. Thus, it is possible that either vector system may be faulty or cell culture medium contained a tetracycline analog.

CRISPR/Cas9 mediated gene editing and single cell clonal isolation produced successful RASSF2 knockout, heterozygous, and wild type cell lines

Following their respective retroviral transductions and subsequent puromycin selections (and GFP+ expression confirmation for HL-60s), HL-60 and U937 pools were respectively single cell seeded to isolate cells with bi-allelic knockout (knockouts) and mono-allelic knockouts (heterozygotes). Pools tranduced with control vectors were also seeded in order to ensure consistent conditions. Bi-allelic and mono-allelic knockouts were identified via Western Blot screen, probed with RASSF2 antibody. RASSF2 expression of clonal lines identified as “knockout,” “heterozygote,” and “wild type” confirmed via Western Blot (Figure 3A-B). Genotypes of clonal lines confirmed via PCR of gRNA target sites and TA-Cloning, followed by Sanger sequencing. Sequencing revealed that our HL-60 cell line exhibits tetraploidy—however, genotypes are consistent with RASSF2 expression.

Heterozygotic expression of RASSF2 in U937 confers enhanced cell proliferation when compared to U937 RASSF2 wild type clonal cell lines

U937 cells with heterozygotic RASSF2 expression (Het-1, Het-2) exhibited statistically significant ($P<0.05$) enhanced proliferation compared to control cells (WT-1, WT-2) (Figure 4). Although in HL-60 clonal lines heterozygotes clonal lines do also exhibit increased proliferation, here is no statistically significant difference in relative proliferation when compared to wild type clonal lines. This suggests that partial loss of RASSF2 expression may result in a more proliferative phenotype. Interestingly, Knockout HL-60 and U937 clonal lines were consistently less proliferative than their heterozygotic counterparts. This in itself insinuates that loss of RASSF2 expression is not critical in myeloid cancer cells.

CRISPR-mediated knockout of RASSF2 does not result in significant difference in cell differentiation, apoptosis, or cell cycle and produces characteristic variation between knockout, heterozygote, and wild-type clonal cell lines

RASSF2 clonal cell lines exhibit variable levels of apoptosis

Among HL-60 *RASSF2* clonal cell lines, extremely variable apoptosis levels were observed between wild type (WT-1, WT-2) and knockout (KO-1, KO-2) cell lines while U937 *RASSF2* clonal cell lines exhibited more consistent levels of apoptosis (Figure 5A). Levels of apoptosis were determined through Annexin-V Staining. No significant differences were determined between clonal lines. This suggests that single cell isolation

may have isolated clonal cell lines that are inherently variable in their apoptotic profiles, regardless of RASSF2 expression.

RASSF2 clonal cell lines exhibit variable levels of cell differentiation

Both HL-60 and U937 clonal cell lines did not exhibit significant difference in CD11b+ differentiation upon treatment with All-Trans Retinoic Acid (Figure 5B-C). CD11b+ is a granulocyte surface marker that effectively identifies differentiated myeloid cells. No significant trends were observed at either concentration (0.1 micromolar, 1 micromolar ATRA) of treatment. However, as observed in our apoptosis assay, differentiation was also extremely variable within clonal lines of each genotype. This suggests that single cell isolation may have isolated clonal cell lines that are inherently variable in cell differentiation patterns, regardless of RASSF2 expression.

RASSF2 clonal cell lines do not exhibit notable differences in Cell Cycle Arrest of Progression

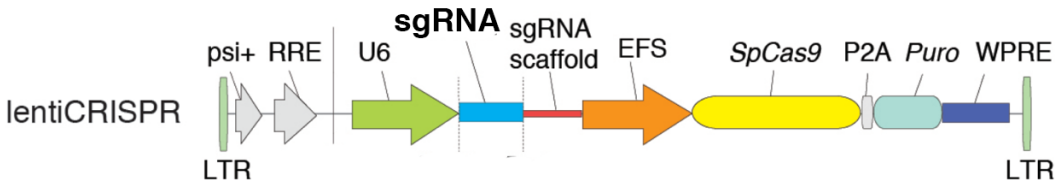
In order to align the cell cycles of our clonal cell lines, they were serum starved for 24 hours. Following reinstatement of serum (10% Fetal Bovine Serum), samples were fixed with ethanol at 12, 24, and 36 hour timepoints. (Figure 5D-G). Cell Cycle Analysis of both our HL-60 and U937 RASSF2 clonal cell lines revealed that in general, partial or total loss of RASSF2 expression did not notably alter arrest from or entry into G2/Mitotic phase. Rather, high levels of variability in cell cycle were observed at the 12 and 24 hr timepoints. This suggests that RASSF2 may not be essential for cell cycle progression

and that single cell isolation may have isolated clonal cell lines that are inherently variable in cell cycle progression.

Process of CRISPR clonal cell line generation itself may be the cause of phenotype variability within and between cell lines of various *RASSF2* genotypes.

Characterization of HL-60 and U937 *RASSF2* clonal cell lines revealed extremely variable differentiation and apoptotic patterns, even between cell lines with identical genotypes. One possible explanation is that single cell clonal isolation may lead to isolation of characteristically different cells. To gain further insight, we single cell isolated non-transduced, wild-type U937 cells and expanded them. 72 hours after treatment of each “clonal line” with 0, 0.1, and 1 micromolar All-Trans Retinoic Acid (ATRA), “clonal lines” exhibited extremely variable CD11b+ differentiation. This demonstrates that clonal isolation may be the reason variable phenotypes are observed.

A



Guide RNA No.	Guide RNA (5'→3')	Target Exon	Cell Lines
1	GCATCTGCAGGCGAATGGGC	3	KO-1, KO-2, HET-1,
2	TCAGATTGAGAATTCAGCAG	7	HET-2
3	*No Insert*	No Target	WT-1, WT-2

Figure 1: Generating clonal cell lines with various RASSF2 genotypes in U937 and HL-60 cell lines via CRISPR-Cas9 mediation

A) Single vector system used to transduce U937 Cell Line. **B)** Double Vector System with sgRNA and Cas9 located on separate vectors used to transduce HL-60 Cell Line. **C)** Schematic outlining the RASSF2 genomic locus labeled to indicate sites targeted by respective gRNAs.

B

Guide RNA No.	Guide RNA (5'-3')	Target Exon	Cell Lines
4	CAAACGTCCCTAGTCCCATGTGG	1	KO-1, HET-1, HET-2
5	AGAATTTACAGCTCCGGCACCGG	2	KO-2
6	*No Insert*	No Target	WT-1, WT-2

C

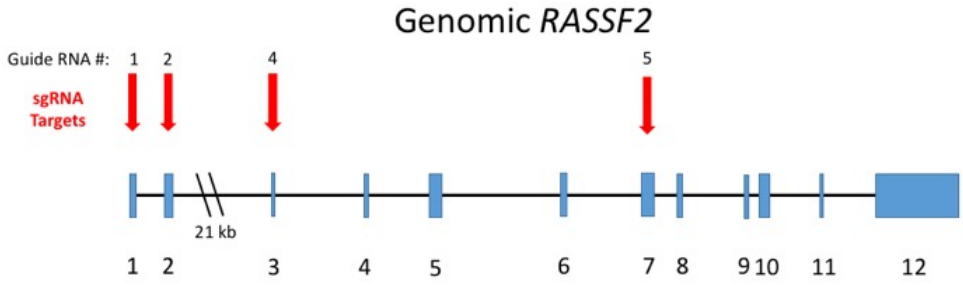


Figure 1: Generating clonal cell lines with various RASSF2 genotypes in U937 and HL-60 cell lines via CRISPR-Cas9 mediation, Continued.

A

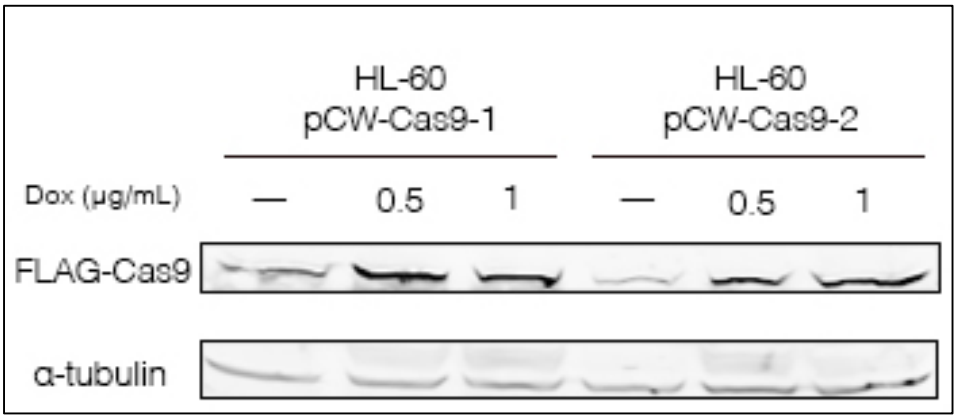


Figure 2: Double vector, Tet-Inducible system used in HL-60 exhibited leaky FLAG-Cas9 expression and subsequently, leaky cleavage at respective gRNA target sites

A) Western Blot confirmation of FLAG-Cas9 expression in Cas9 lentivral construct **Figure 1b** infected HL-60 cells treated with 0.5, 1 μM doxycycline compared to non treated cells. pCW-Cas9-1 and pCW-Cas9-2 are two midi-preps of the same construct. Lentiviral construct shown to exhibit leaky expression of Cas9 **B)** T7E1 endonuclease assay–based confirmation of target site cleavage at *RASSF2* genomic loci for guide RNA 4 (exon 1) and Guide RNA 5 (exon 2) indicated in **Figure 1b** in HL-60. Dox-treated pool compared to non-Doxycycline treated, control sgRNA infected, non-sgRNA infected, and negative control (Kasumi) pools.

B

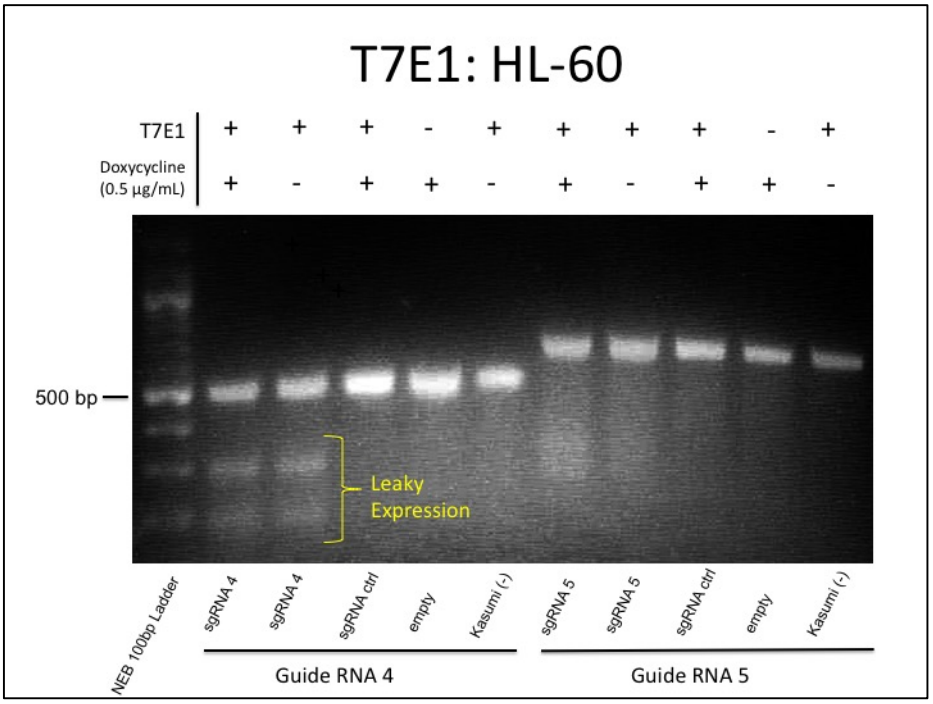


Figure 2: Double vector, Tet-Inducible system used in HL-60 exhibited leaky FLAG-Cas9 expression and subsequently, leaky cleavage at respective gRNA target sites, Continued.

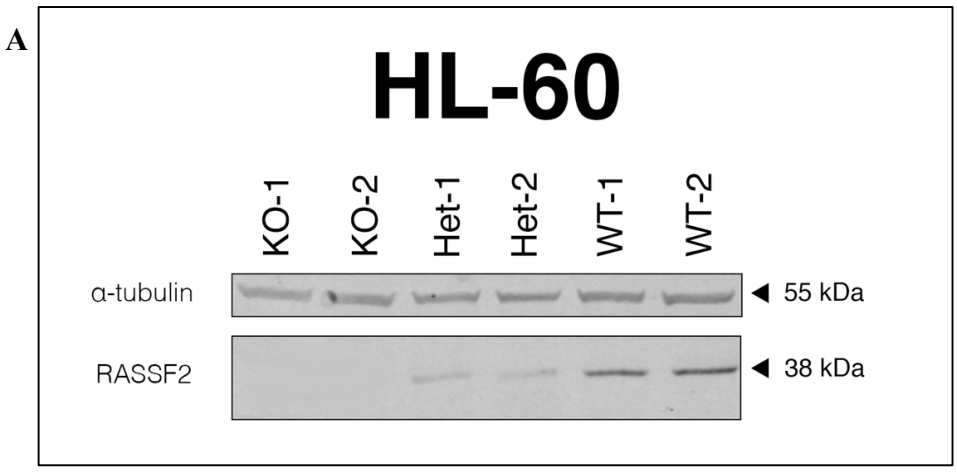


Figure 3: CRISPR/Cas9 mediated gene editing and single cell clonal isolation produced successful RASSF2 knockout, heterozygous, and wild type cell lines

Western Blot Analysis analysis of human RASSF2 expression in **A)** HL-60 RASSF2 clonal cell line lysates and **B)** U937 RASSF2 clonal cell line lysates. Blot probed with RASSF2 and α-Tubulin. **C)** Sequencing results of HL-60 and U937 *RASSF2* Clones at respective target sites, listed in **Figure 1**. Human promyelocytic HL-60 cell line is tetraploid.

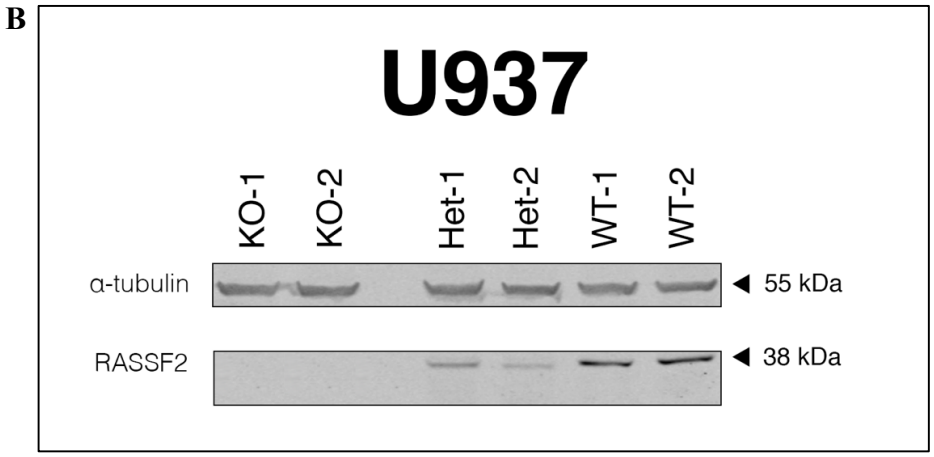


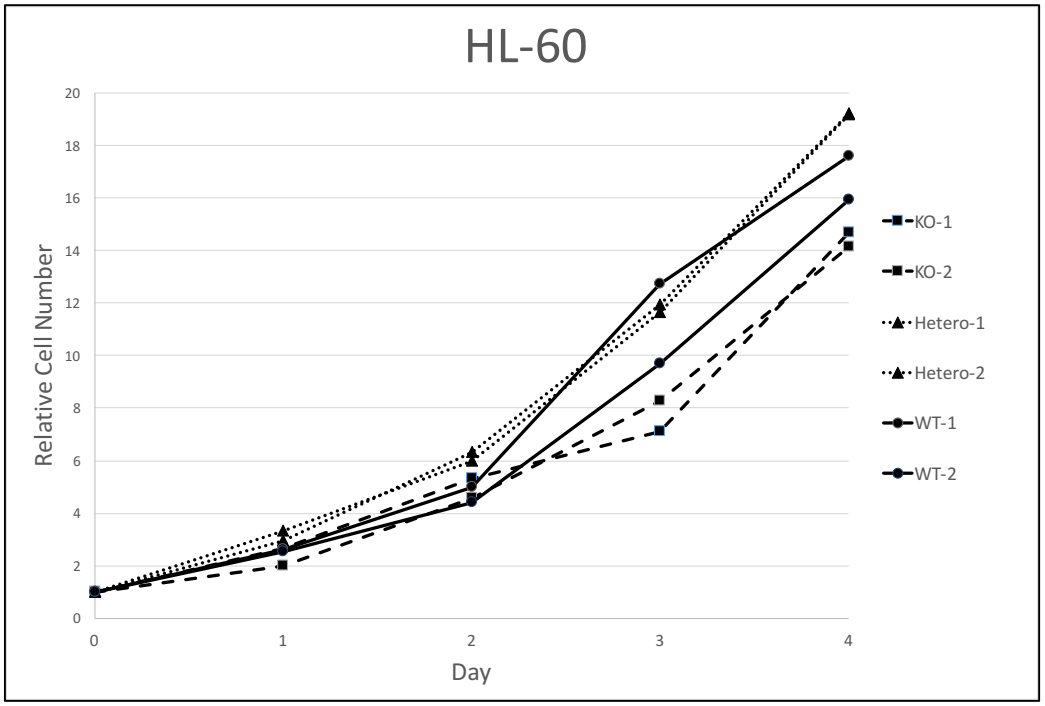
Figure 3: CRISPR/Cas9 mediated gene editing and single cell clonal isolation produced successful RASSF2 knockout, heterozygous, and wild type cell lines, Continued.

C

Clonal Cell Line	Sequence (5' -> 3')
HL60 *RASSF2* (-/-)_01 (270+)	ALLELE: TACAGCCACCAAACGTCCCTAGTCCCCATGTGGACAAGATA ALLELE: TACAGCCACCAAACGTCCCTAGTCCCCATGTGGACAAGATA ALLELE: TACAGCCACCAAACGTCCCTAGTCCCCATGTGGACAAGATA ALLELE: TACAGCCACCAAACGTCCCTAGTCCCCATGTGGACAAGATA
HL60 *RASSF2* (-/-)_ 02	ALLELE: ---------- ALLELE: GAAGGCCAGAATTTACAG----------GTAAGGA ALLELE: GAAGGCCAGAATTTACAG----------GTAAGGA ALLELE: GAAGGCCAGAATTTACAG----------GTAAGGA
HL60 *RASSF2* (+/-)_ 01	ALLELE: TACAGCCACCAAACGTCCCTAGT-CCATGTGGACAAGATA ALLELE: TACAGCCACCAAACGTCCCTAGT-CCATGTGGACAAGATA ALLELE: TACAGCCACCAAACGTCCCTAGTCCCCATGTGGACAAGATA ALLELE: TACAGCCACCAAACGTCCCTAGTCCCATGTGGACAAGATA
HL60 *RASSF2* (+/-)_ 02	ALLELE: TACAGCCACCAAACGTCCCTA-------GTGGACAAGATA ALLELE: TACAGCCACCAAACGTCCCTAGT-------GGACAAGATA ALLELE: TACAGCCACCAAACGTCCCTAG-------TGGACAAGATA ALLELE: TACAGCCACCAAACGTCCCTAGTCCCATGTGGACAAGATA
HL60 *RASSF2* (+/+)_01	ALLELE: TACAGCCACCAAACGTCCCTAGTCCCATGTGGACAAGATA ALLELE: TACAGCCACCAAACGTCCCTAGTCCCATGTGGACAAGATA ALLELE: TACAGCCACCAAACGTCCCTAGTCCCATGTGGACAAGATA ALLELE: TACAGCCACCAAACGTCCCTAGTCCCATGTGGACAAGATA
HL60 *RASSF2* (+/+)_02	ALLELE: GAAGGCCAGAATTTACAGCTCCGGCACCGGGAGGTAAGGA ALLELE: GAAGGCCAGAATTTACAGCTCCGGCACCGGGAGGTAAGGA ALLELE: GAAGGCCAGAATTTACAGCTCCGGCACCGGGAGGTAAGGA ALLELE: GAAGGCCAGAATTTACAGCTCCGGCACCGGGAGGTAAGGA
U937 *RASSF2* (-/-)_01	ALLELE: TTTTGCTCCTGCTTCAGA----------GGAGTT ALLELE: TTTTGCTCCTGCTTCAGATTGAGAATTCAG-AGAGGAGTT
U937 *RASSF2* (-/-)_02	ALLELE: ----------AGGAGTT ALLELE: TTTTGCTCCTGCTTCAGATTGAGA---------AGGAGTT
U937 *RASSF2* (+/-)_01	ALLELE: TTTTGCTCCTGCTTCAGATTGAGAATT---CAGAGGAGTT ALLELE: TTTTGCTCCTGCTTCAGATTGAGAATTCAG---AGGAGTT
U937 *RASSF2* (+/-)_02	ALLELE: GGGGCCTGCGC---CCCATTCGCCTGCAGATGCAGGATGA ALLELE: GGGGCCTGCGCCGG------CG------GATGCAGGATGA
U937 *RASSF2* (+/+)_01	ALLELE: TTTTGCTCCTGCTTCAGATTGAGAATTCAGCAGAGGAGTT ALLELE: TTTTGCTCCTGCTTCAGATTGAGAATTCAGCAGAGGAGTT
U937 *RASSF2* (+/+)_02	ALLELE: GGGGCCTGCGCCGGCCCATTCGCCTGCAGATGCAGGATGA ALLELE: GGGGCCTGCGCCGGCCCATTCGCCTGCAGATGCAGGATGA

Figure 3: CRISPR/Cas9 mediated gene editing and single cell clonal isolation produced successful RASSF2 knockout, heterozygous, and wild type cell lines, Continued.

A



Standard Error of Mean*

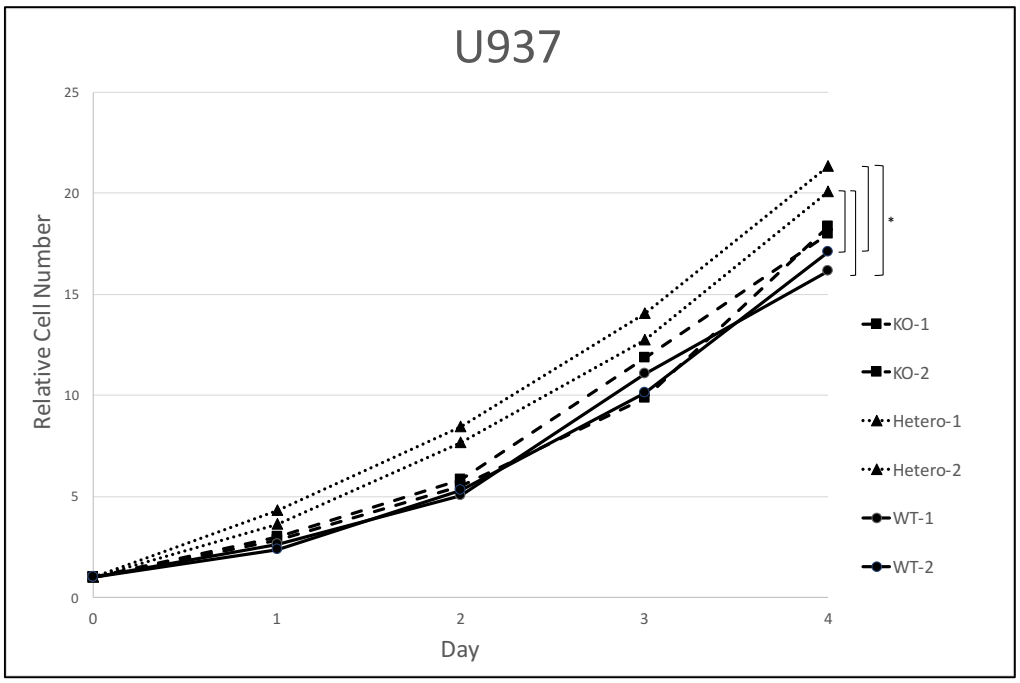
Day	KO-1	KO-2	Het-1	Het-2	WT-1	WT-2
1	0.55	0.22	0.66	0.22	0.37	0.27
2	0.9	0.24	1.13	1.42	0.39	0.17
3	1.49	1.14	0.87	0.89	3.5	2.34
4	2.14	2.87	2.97	3.49	5.13	3.44

Figure 4: Heterozygotic expression of RASSF2 in U937 confers enhanced cell proliferation when compared to U937 RASSF2 wild type clonal cell lines

After successful clonal isolation and growth of RASSF2 clonal cell lines, 100,000 cells of each clonal line was seeded and observed for difference in relative cell proliferation. **A)** HL-60 RASSF2 clonal cell lines did not exhibit significant differences in proliferation, however **B)** U937 RASSF2 Heterozygotes Clonal Lines exhibited significantly higher proliferation when compared to RASSF2 wild type clonal lines. Brackets indicate a significant difference in proliferation, determined by paired two-tailed t-test ($p<.05$). All data are presented as the mean, with s.e.m listed separately for three independent biological replicates performed, each performed as technical duplicates.

*Standard Error was divided by 100,000 in reference to relative cell number magnitude. Standard Error rounded to the second decimal place.

B



Standard Error of Mean*

Day	KO-1	KO-2	Het-1	Het-2	WT-1	WT-2
1	1.06	0.5	0.27	0.29	0.44	0.49
2	0.46	0.01	0.89	1.78	0.12	0.1
3	2.23	1.15	1.58	2.67	2.07	1.98
4	2.8	2.92	2.63	4.22	2.24	2.89

Figure 4: Heterozygotic expression of RASSF2 in U937 confers enhanced cell proliferation when compared to U937 RASSF2 wild type clonal cell lines, Continued.

*Standard Error was divided by 100,000 in reference to relative cell number magnitude. Standard Error rounded to the second decimal place.

A

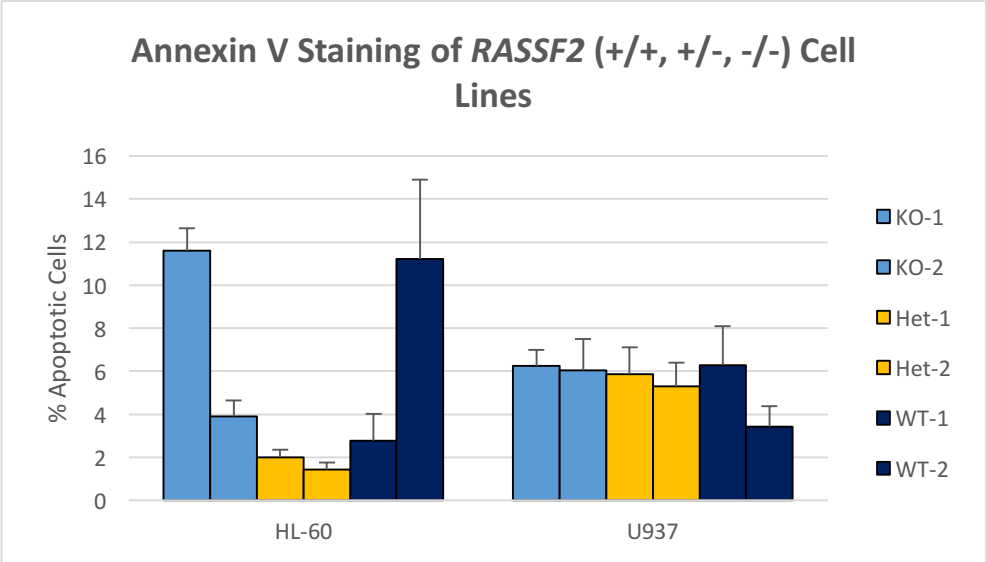


Figure 5: CRISPR-mediated knockout of RASSF2 does not result in significant difference in cell differentiation, apoptosis, or cell cycle and produces characteristic variation between knockout, heterozygote, and wild-type clonal cell lines

A) Percent of apoptotic cells (via Annexin-V staining) were monitored in U937 and HL-60 RASSF2 clonal cell lines at least 3 weeks after clonal isolation. All data presented as the mean +/- s.e.m for three independent biological replicates.

B

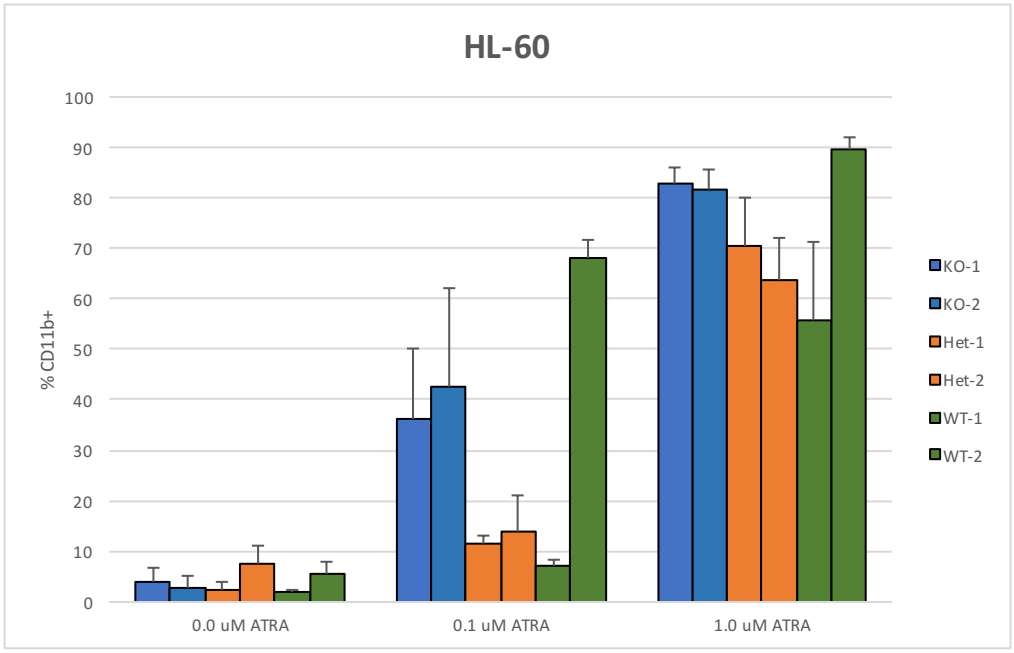


Figure 5: CRISPR-mediated knockout of RASSF2 does not result in significant difference in cell differentiation, apoptosis, or cell cycle and produces characteristic variation between knockout, heterozygote, and wild-type clonal cell lines, Continued.

72 hr ATRA-induced differentiation of **B)** HL-60 and **C)** U937 clonal cell lines of various RASSF2 Genotypes (+/+, +/-, -/-). Cells treated with concentrations of 0, 0.1, and 1 micromolar All-Trans Retinoic Acid . All data presented as the mean of +/- s.e.m for three independent experiments.

C

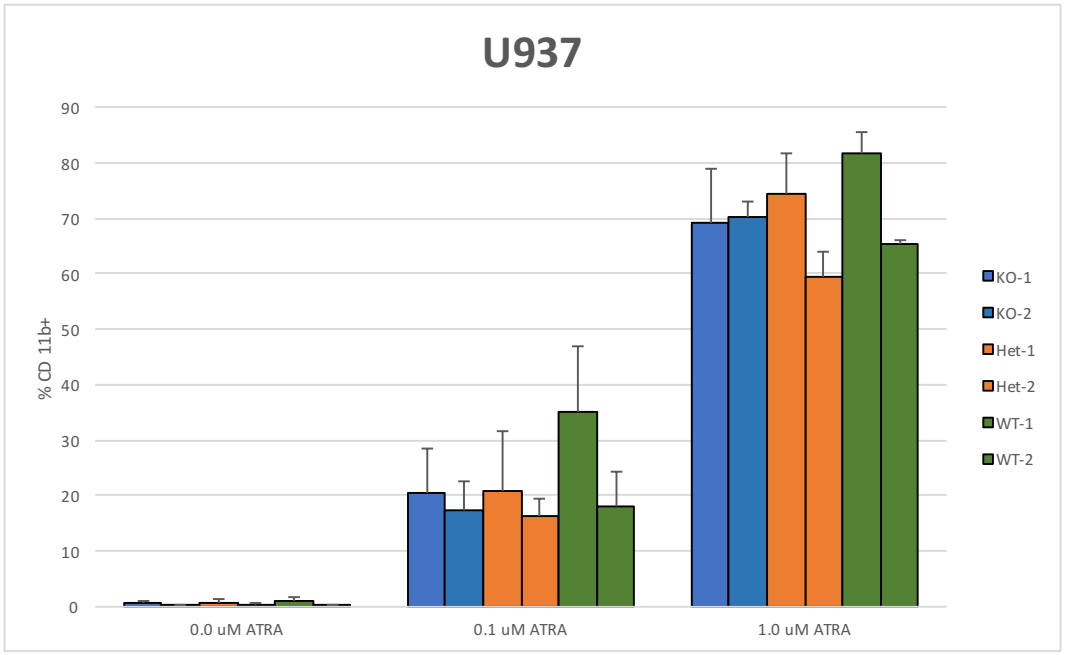


Figure 5: CRISPR-mediated knockout of RASSF2 does not result in significant difference in cell differentiation, apoptosis, or cell cycle and produces characteristic variation between knockout, heterozygote, and wild-type clonal cell lines, Continued.

D

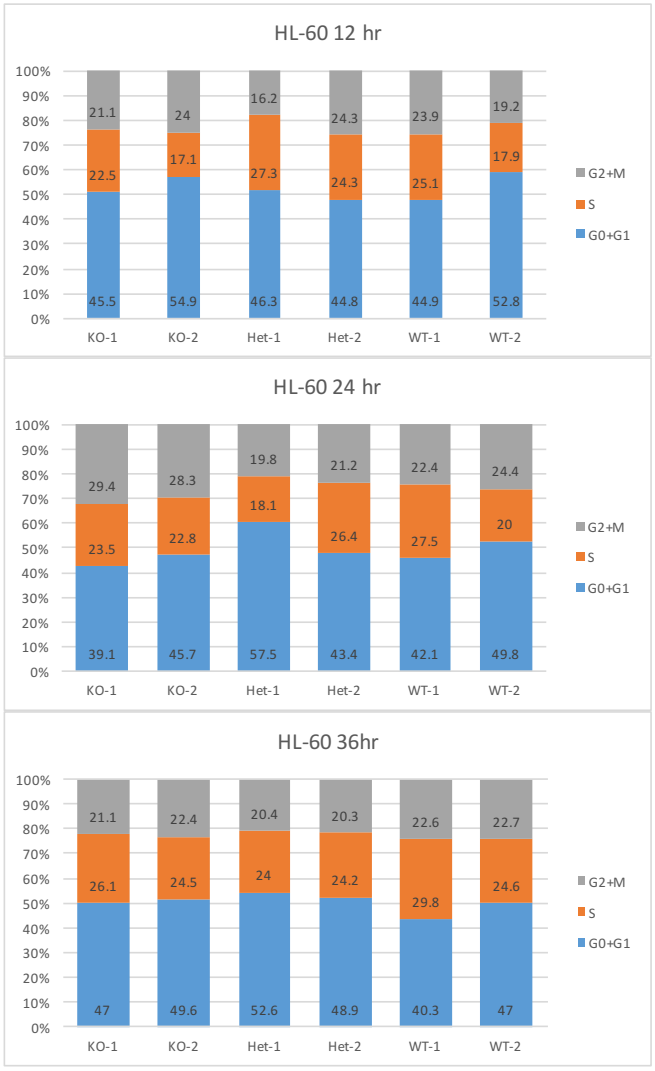


Figure 5: CRISPR-mediated knockout of RASSF2 does not result in significant difference in cell differentiation, apoptosis, or cell cycle and produces characteristic variation between knockout, heterozygote, and wild-type clonal cell lines, Continued.

Loss or downregulation of RASSF2 expression does not shift cell cycle distribution. Percentage of cells in each phase of the cell cycle using gates from **Figure 5f and Figure 5g** for **D)** HL-60 and **E)** U937 RASSF2 Clonal cell lines at 12, 24, and 36 hour time points after resuspension in media with serum (10% Fetal Bovine Serum). Histograms of PI staining in **F)** HL-60 and **G)** U937 RASSF2 clonal cell lines 12, 24, and 36 hrs after serum starving.

E

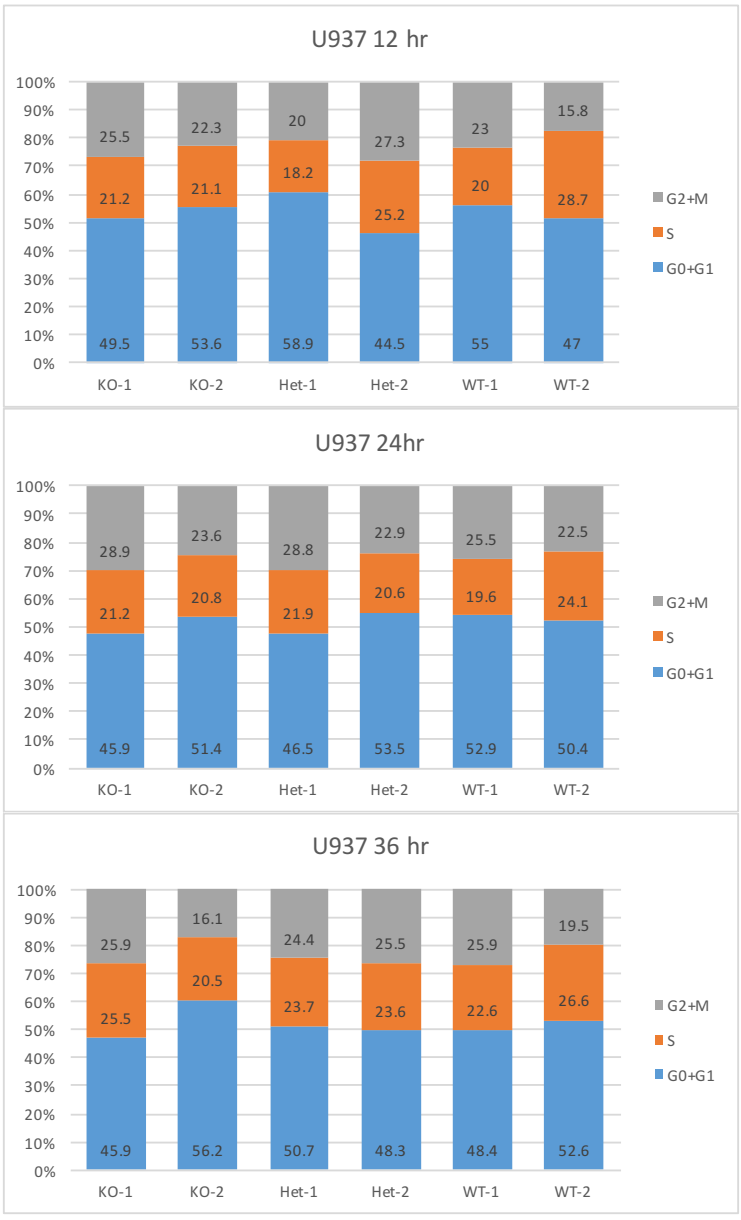


Figure 5: CRISPR-mediated knockout of RASSF2 does not result in significant difference in cell differentiation, apoptosis, or cell cycle and produces characteristic variation between knockout, heterozygote, and wild-type clonal cell lines, Continued.

F

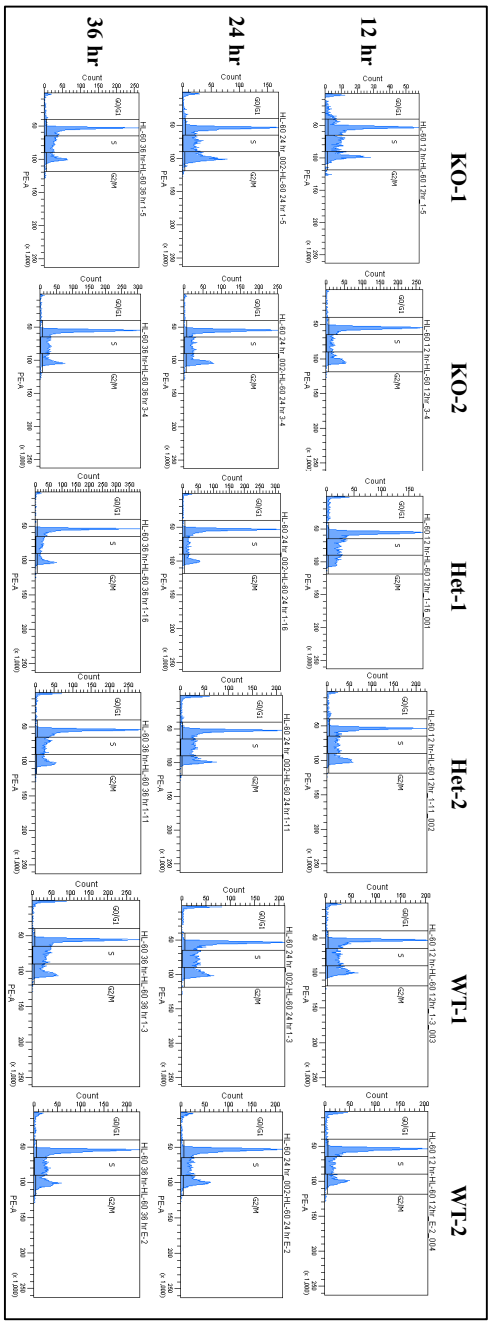


Figure 5: CRISPR-mediated knockout of RASSF2 does not result in significant difference in cell differentiation, apoptosis, or cell cycle and produces characteristic variation between knockout, heterozygote, and wild-type clonal cell lines, Continued.

G

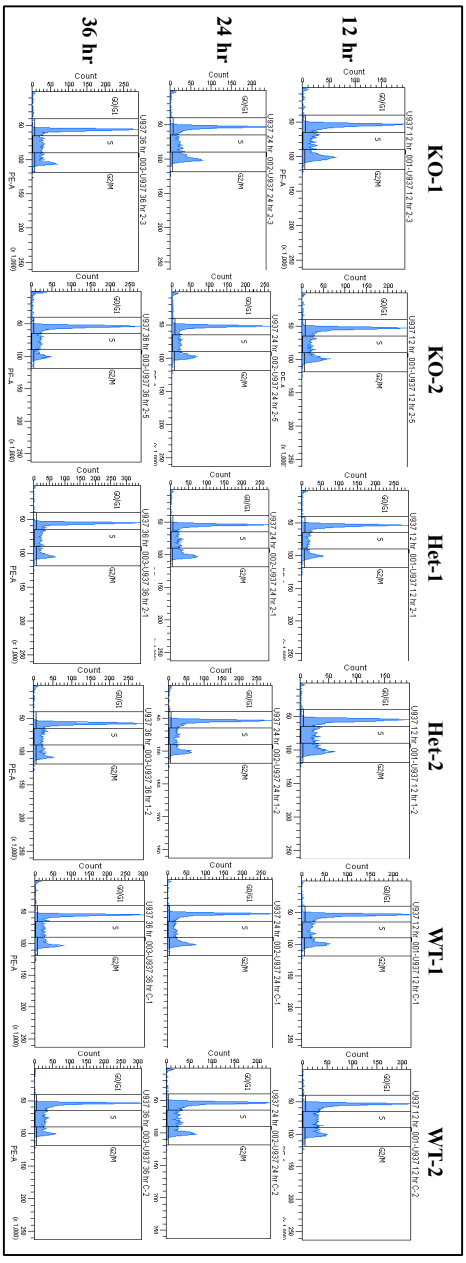


Figure 5: CRISPR-mediated knockout of RASSF2 does not result in significant difference in cell differentiation, apoptosis, or cell cycle and produces characteristic variation between knockout, heterozygote, and wild-type clonal cell lines, Continued.

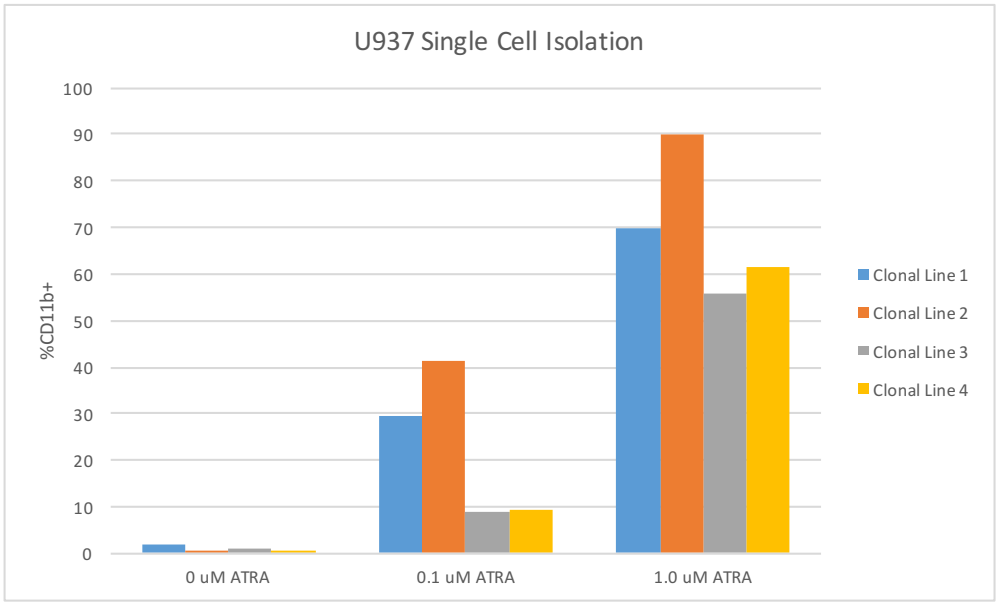


Figure 6: Process of CRISPR clonal cell line generation itself may be the cause of phenotype variability within and between cell lines of various *RASSF2* genotypes.

U937 cells, without treatment, are clonally isolated via single cell seeding. 72 hr ATRA-induced differentiation of 4 U937, single-seeded clonal lines. Cells treated with concentrations of 0, 0.1, and 1 micromolar All-Trans Retinoic Acid (ATRA).

III. Discussion

Building off previous studies that have characterized RASSF2 as a tumor suppressor, we aimed to functionally define RASSF2's role in the leukemic context. The work presented in this thesis planned to fulfill that goal by functionally characterize RASSF2 in myeloid cell lines via CRISPR/Cas9 genome editing. From our results, we are able to posit that RASSF2 deletion has no detectable unexpected results HL-60 and U937 cell lines. The results have also provided us the unexpected opportunity to reflect on the possibilities of CRISPR.

Functional Characterizations involving single cell clonal isolation must be performed with a larger pool of samples to account for the characteristic variability of clonal cell lines.

With the exception of our cell proliferation assays, the results from our characterization experiments have been extremely variable, between and within clonal lines of various RASSF2 genotypes. This variation may be attributed to the process of clonal isolation, which was used to generate our clonal cell lines. Single cell isolation is an extremely taxing process that may inadvertently select for expansion of single cells that are most robust and senescence resistant. Single cell seeding may also isolate individual cells that may have acquired additional leukemic mutations. Moreover, due to the amount of time required for clonal expansion, clonal lines may also have the opportunity to acquire additional mutations during this time. All of these factors may lead to variability between clonal lines.

Our suspicions were confirmed after single cell clonal expansion of U937 cells yielded extremely variable differentiation when treated with ATRA (Figure 6). For example, at 0.1 μL ATRA treatment the range of %CD11b+ was 9.1-41.4 while at 1.0 μL ATRA treatment, the range is 55.7-89.8 (for HL-60 clonal lines: 7.2-68.2, 55.0-89.6; U937 clonal lines: 18.0-35.1, 59.3-81.8). Additionally, the act of isolating single cells within a population inherently amplifies the existing cell to cell characteristic variation within our respective cell lines. In order to address this problem, future functional characterizations involving single cell clonal isolation must utilize a larger pool of samples in order to account for characteristic variation of clonally expanded cell lines.

Important Consideration: Off Site Targeting

Another possible explanation for our variable results is the possibility that our CRISPR guide RNAs may have had off site targets. As demonstrated in a recent mass genetic screen, guide RNAs were shown to have an average of ~2.2 off site targets (Wang et. Al). Although the majority of off site targets are in the intronic regions of the genome, occasionally gRNAs may accidentally target a gene that may have functional significance. This possibility, however, is highly unlikely in the context of our study because variable results were observed with clonal lines using the same guide RNA.

Drawing connections to an *in vivo* model

In 2012, a *Rassf2* knockout mouse model was generated to discern Rassf2's role in a systematic context (Song et. Al). Although hematopoietic anomalies were observed, bone marrow transplantation from knockout mice to irradiated wild-type mice

demonstrated that *Rassf2* knockout cells had normal hematopoietic reconstitution. This suggests that knocking out RASSF2 in blood cells would not be so significant since the knockout cells from the mouse model were able to retain the essential function of establishing a healthy HSC population and differentiating normally. Since our clonal lines did not exhibit notable differences in apoptosis nor cell differentiation, our data supports the implications of the mouse study that RASSF2 may not be essential for blood cell function.

Interpreting the significance of RASSF2 Heterozygosity in a Leukemic Context

One surprising result that came from our study is that RASSF2 heterozygous U937 clonal lines exhibited significantly higher proliferation rates ($p<.05$) when compared to wild type and knockout clonal lines. Primarily, this suggests that loss of RASSF2 expression may not be critical for leukemic development. However, it also suggests that RASSF2's role may be similar in its role in other cancers. On the genomic level, *RASSF2* is downregulated via promoter methylation in a variety of cancers—this leads to a "heterozygous" phenotype. This, combined with our data suggests that RASSF's tumor suppressive function is most greatly repressed when RASSF2 expression is low, but not completely absent.

This finding also suggests that loss of RASSF2 expression may be functionally ameliorated by due to two separate, but likely possibilities. The first possibility is that the cell lines used (the clonal lines generated) may have acquired additional mutations that make them less sensitive to the RASSF2 tumor suppressive pathway. The second possibility is that expression of other RASSFs could be compensating for the deletion of

RASSF2. As mentioned before, RASSF2 is part of a ten member RASSF family that share identical domains (e.g. RA domain) and are able to bind to similar effector proteins. Recent studies have shown that RASSF2 in involved with the stabilization of downstream pro-apoptotic kinases MST1 and MST2 (Cooper et. Al; Song et. Al). It could be possible that in a blood context, loss of RASSF2 expression could result in another RASSF family member stabilizing MST1/2, and thus restoring its pro-apoptotic function. However, in the context of RUNX1-ETO, these RASSF members may also be downregulated in concert with RASSF2, leading to RASSF2's pronounced significance in t(8;21) leukemia.

Future Directions

Although results from this study have not demonstrated a significant functional role for RASSF2 in a leukemic context, it has provided us with exceptional insight into the limitations of CRISPR/Cas9 in functional studies. With this being said, it would be prudent to alter our experimental model and repeat our study using shRNA interference. This approach will allow us to answer our original research question in a much timelier, cost effective manner and avoid the logistical obstacles that a CRISPR/Cas9 workflow presents. Another approach would be using CRIPSR interference (CRISPRi) in order to repress *RASSF2* expression. In this system, the mutated Cas9 is unable to cleave due to a modified enzymatic site, resulting in a gRNA-guided transcription block. Both of these approaches may be more appropriate than a CRISPR knockout since they allow us to simulate the manner in which RASSF2 is down regulated in primary cancer cells—via promoter methylation.

IV. Materials and Methods

Generation of lentiviral CRISPR/Cas9 constructs

Guide RNAs were designed using the MIT optimized CRISPR design tool (http://crispr.mit.edu/). Each target site is adjacent to a PAM sequence that contains the canonical trinucleotide NGG. sgRNA oligos synthesized by ETON biosciences and cloned into the BsmBI sites in the pLKO5.sgRNA.EFS.GFP (gRNAs targeting exon 1 and 2) and lentiCRISPR (gRNAs targeting exon 3 and 7) constructs. Detailed diagrams listed in Figure 1.

Cell Culture

U937 and HL-60 cells were maintained in RPMI supplemented with 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin and. 293T cells were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% Bovine Calf Serum or Fetal Bovine Serum and 1% penicillin/streptomycin.

Lentiviral Transduction of CRISPR constructs

HEK-293T cells transfected with CRISPR constructs to generate viral supernatant (used to transduce blood cells). U937 cells were transduced with lentiCRISPR contructs containing sgRNAs targeting the 3rd and 7th exon, respectively. HL-60 cells were transduced first with pCW-Cas9 construct and afterwards, transduced with pLKO5.sgRNA.EFS.GFP containing sgRNAs targeting the 1st and 2nd exons. Both cell

lines transduce with respective control constructs containing no guide RNAs. Transduced cells drug selected in 1ug/ml purmomycin for 5 days.

T7 Endonuclease Assay

Genomic DNA isolated from HL-60 infected with pCW-Cas9 and pLKO5.sgRNA.EFS.GFP using Tail Lysis Buffer. sgRNA target sites are amplified via PCR and PCR purified to a concentration of 20 ng/uL. Afterwards, purified PCR products were re-annealed via a heteroduplex formation reaction in the thermocycler. PCR products are then digested with T7 Endonuclease and incubated for 30 minutes at 37 degrees Celsius. Digested products are run on agarose gel to determine if cleavage occurred at gRNA target site.

Western Blots

HL-60 cells transduced with pCW-Cas9 were transduced were lysed in RIPA Buffer to obtain protein lysates. Protein concentration was determined by Bradford Assay and equal amounts of protein were loaded. Blots were probed with anti-FLAG (1:1000 dilution) and anti-tubulin (1:10,000 dilution).

For the RASSF2 confirmation WB, 3,000,000 cells are lysed for each clonal line. Samples are lysed directly in 1X Loading Buffer and immediately loaded onto gel to prevent degradation of RASSF2. Blots were probed with anti-RASSF2 (1:4000 dilution) and anti-tubulin (1:10,000 dilution).

Single Cell Clonal Isolation by dilution

After puromycin selection, HL-60 and U937 CRISPR/gRNA transduced pools are serially diluted to a concentration of 0.5 cell per 100 microliters to reduce the likelihood of having multiple cells per well. Cells are plated in 96-well plate using a multi-channel pipette. Colonies are inspected 1 week after plating to identify wells containing single cells. Cells are then allowed to clonally expand for 1 month before replenishment with fresh media. Viable cells are transferred to larger volumes to be grown up. WB Screen using RASSF2 antibody was used to identify 2 wild type clonal lines, 2 Heterozygote clonal lines, and 2 knockout clonal lines in both HL-60 and U937.

Sanger Sequencing to determine clonal cell line genotypes

To ensure high DNA fidelity, genomic DNA of HL-60/U937 KO-1, KO-2, Het-1, Het-2, WT-1, WT-2 cell lines obtained through Phenol/Chloroform DNA extraction. PCR are respective gRNA target sites performed with high-fidelity KODHS polymerase and A-Tailed with TAQ polymerase. After ligation into pGEM-T-Easy vector, TA cloned constructs are transformed into DH5alpha and individual colonies are mini-prepped. Sanger sequencing performed by Eton Biosciences.

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