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UNIVERSITY OF CALIFORNIA, SAN DIEGO

# Functional characterization of the tumor suppressor RASSF2 in t(8;21) Acute Myeloid Leukemia

A thesis submitted in partial satisfaction of

the requirements for the degree Master of Science

in

Biology

by

Elizabeth Tara Andrews

Committee in charge:

Professor Dong-Er Zhang, Chair
Professor Eric Bennett
Professor James Kadonaga

2015

The thesis of Elizabeth Tara Andrews is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2015

**Dedication**

I dedicate this thesis to my dear parents, Mary and Leif,

and to the three best friends that anyone could have: Jarod, Sara, and Edrina.

Your love and support have been invaluable.

## Epigraph

Blut ist ein ganz besondrer Saft.

*Blood is a very special juice.*

-Johann Wolfgang von Goethe

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I would like to thank Sam Stoner for training me in the lab, as well as for being a patient, constant, unwavering source of guidance and support. Similarly, I would like to thank all of the members of the Zhang lab, for each of them provided me with valuable feedback, mentorship, and good will throughout my time in the lab.

I would also like to thank all of my friends and family who tolerated, supported, and encouraged me while I worked towards this graduate degree.

Figure 1, in full, will eventually be submitted for publication of the material. Stoner, Sam; Weng, Stephanie; Zhang, Dong-Er.

Figure 2, in full, will eventually be submitted for publication of the material. Stoner, Sam; Zhang, Dong-Er.

Figure 3, panel A, graphic is original work of thesis author, representing a protocol previously described by Liu et. al. 2008.

ABSTRACT OF THE THESIS

Functional characterization of the tumor suppressor RASSF2

in t(8;21) Acute Myeloid Leukemia

by

Elizabeth Tara Andrews

Master of Science in Biology

University of California, San Diego, 2015

Professor Dong-Er Zhang, Chair

Acute Myeloid Leukemia (AML) is a cancer of the myeloid blood cells. The most common karyotypic abnormality associated with AML is a translocation between chromosomes 8 and 21, resulting in the formation of the AML1-ETO (AE) fusion protein. AE is thought to stimulate leukemogenesis by aberrantly silencing the expression of different genes. Frequently, AE down-regulates genes that are important to normal

hematopoiesis, but this fusion protein also silences expression of many other targets unrelated to blood cell differentiation.

Using a mouse model of t(8;21) AML, our lab has identified the tumor suppressor, RASSF2, as a gene that is significantly down-regulated in the presence of AE, and has found that this down-regulation may be a critical step in the development of leukemia. Here, we have generated six RASSF2 variants in order to study which structural domains and corresponding functions are the most important to the tumor suppressive function of RASSF2 in t(8;21) AML. We have identified the SARAH domain as the most important for RASSF2 function, and we have found that cytoplasmic localization of RASSF2 may be uniquely important to blood cells. These findings implicate the Hippo pathway as important for repressing leukemogenesis, and also indicate that RASSF2 may play a previously unreported tumor suppressive role in the cytoplasm.

# I. Introduction

## Leukemia

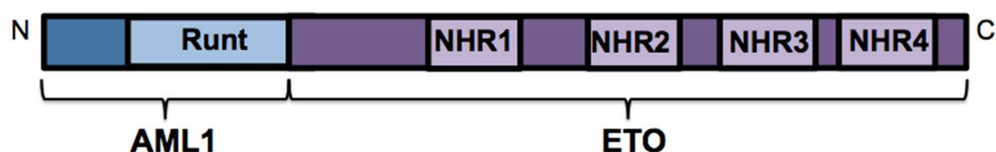
Leukemia is a form of cancer that originates in the bone marrow and results in the accumulation of abnormal blood cells that can build up to such high levels that they prevent normal hematopoietic stem cells (HSCs) from producing other important blood cells (Rubnitz et. al. 2010). In a healthy person, HSCs differentiate to give rise to all of the body's blood cells. This process begins with the differentiation of HSCs into either lymphoid progenitor cells or myeloid progenitor cells, then continues with lineage-specific differentiation into more specialized blood cells (Jagannathan-Bogdan et. al. 2013). Different leukemias are considered "myelogenous" or "lymphocytic" based on which progenitor-branch their excessively dividing blood cells belong to. Leukemias are considered "acute" or "chronic" based on how quickly their cancerous cells are dividing and stimulating disease progression (Vardiman et. al. 2010).

Accordingly, Acute Myeloid Leukemia (AML) describes a specific type of leukemia: "acute" because it is characterized by a sudden expansion of rapidly proliferating immature blood cells and "myeloid" because it is a cancer of the myeloid blood cells— e.g. the progenitor cells that normally differentiate into granulocytes such as basophils, neutrophils, eosinophils, monocytes, and mast cells (Rubnitz et. al 2010, Jagannathan-Bogdan et. al. 2013). The French American British (FAB) classification system divides AML into several subtypes—M0 through M7—based on the disease's level of cellular maturation and the type of blood cell the disease originated from (Vardiman et. al. 2010). Approximately 25% of AML patients are classified within the

M2 subtype; these leukemias display some limited cellular maturation and originate from myeloid progenitor cells (Vardiman et. al. 2010). The most common cytogenetic abnormality within the M2 subtype is a translocation between chromosomes 8 and 21, resulting in t(8;21) AML (Taj et. al. 1995).

**The AML1-ETO fusion protein**

The t(8;21) translocation results in the formation of an oncogenic fusion protein—shown below—known as AML1-ETO (Lam, et. al. 2012). While AML1-ETO severely disrupts hematopoiesis, it does not induce leukemia on its own. Studies in mouse models have found that expressing AML1-ETO in combination with random mutagen-induced mutations or intentional overexpression of oncogenes leads to the abnormal accumulation of immature myeloid blood cells indicative of AML (Higuchi et. al. 2002; Yuan et al 2001). In patients, AML1-ETO is thought to promote a “pre-leukemic” expansion of immature myeloid cells, which progresses to AML after the accumulation of additional secondary mutations (Steffen et. al. 2011).




Normally, AML1 (also known as RUNX1) is a transcription factor that serves as a key regulator of hematopoiesis, binding and activating expression of genes involved in blood cell differentiation. Normally, ETO plays an important but poorly understood role

in the central nervous system, interacting with numerous transcriptional-repressors, such as the Nuclear receptor corepressor-histone deacetylase (CoR-HDAC) complex (Lam et. al. 2012; Gelmetti et. al. 1998). As a result of the t(8;21) translocation, the DNA binding domain of AML1 is joined with the transcriptionally repressive domains of ETO. The primary accepted mechanism by which AML1-ETO promotes leukemia development is by binding AML1 target genes via AML1 and recruiting repressive proteins via ETO, ultimately repressing important hematopoiesis genes (Lam et. al. 2012). Therefore, identification of individual genes or biological pathways that are specifically disrupted in the presence of AML1-ETO will provide further molecular insight into the pathogenesis of t(8;21) AML.

**RASSF2: Key functional domains**

The AML1-ETO fusion protein represses a wide swath of different genes, but one that our lab has identified as particularly important is the tumor suppressor, RASSF2. RASSF2 is one protein of the ten-member Ras-Association Domain Family (RASSF) (Richter et. al., 2009). Numbered RASSF1-RASSF10, the family members are characterized by a common Ras-association (RA) domain, which allows RASSF proteins to bind Ras GTPases—a group of oncoproteins known to stimulate cell proliferation and cell survival (Chain et. al. 2013). While the specific RASSF-Ras interactions vary per family member, RASSF2 has been shown to interact with K-Ras to induce apoptosis. In 293T cells, expression of RASSF2 with K-Ras increases cellular apoptosis and cell cycle arrest (Vos et. al. 2003). In a prostate cancer model, RASSF2 plays a key role in

localizing prostate apoptosis response protein 4 (PAR-4) to the nucleus to induce apoptosis in response to activated K-Ras (Donninger et. al. 2010)

Though the RA domain is the only domain shared by all of the RASSF members, RASSF1-RASSF6 also contain a SARAH domain (Richter et. al. 2009), which is named after the drosophila tumor suppressor proteins **Sa**lvador (human: WW45), **Ra**ssf (human: RASSF1-6), and **H**ippo (human: MST1/2). These proteins form part of an evolutionarily conserved pathway responsible for controlling organ size by restraining cell growth and proliferation in response to intercellular adhesion signals and junction proteins (Zhao et. al. 2011).

The SARAH domain is responsible for many protein-protein interactions. It binds tightly and specifically to SARAH domains on other proteins such as WW45, MST1/2, and RASSF1-6 (Volokdo et. al. 2014). In thyroid cancer cell lines, mutating the SARAH domain of RASSF2 prevents binding with WW45 and RASSF members 1, 2, 3, 4, and 6, but doesn't interrupt the binding of RASSF2 with MST1 or RASSF5. Furthermore, in thyroid cancer cell lines, overexpression of wild type RASSF2 leads to cellular apoptosis, while overexpression of RASSF2 lacking a SARAH domain decreases induction of apoptosis (Volokdo et. al. 2014).

Additionally, RASSF2 contains its own unique nuclear localization signal (NLS) (Cooper, et. al., 2008). Mutation of the NLS' basic amino acids (R150A/R151A/R152A) disrupts nuclear import of RASSF2. Furthermore, in breast cancer cell lines ectopic expression of RASSF2 suppresses cellular growth, while ectopic expression of RASSF2 lacking its NLS displays much weaker suppressive ability (Cooper et. al. 2008). In addition, RASSF2 contains a nuclear export signal (NES), which is believed to play a

role in cell-cycle dependent RASSF2 regulation though little is known about its activity or importance (Kumari et. al. 2009).

While the general functional and structural properties of RASSF2 have been previously reported, it has not been studied in the context of leukemia development.

**RASSF2: Role in t(8;21) Acute Myeloid Leukemia**

Our lab has identified RASSF2 as a gene that is specifically down-regulated in the presence of the AML1-ETO fusion protein. Using a mouse model of leukemia, we found that RASSF2 mRNA is 30 fold lower in in t(8;21) leukemia cells as compared to wild-type Lin-Sca-cKit$^{+}$ (LK) myeloid progenitor cells (Lo et. al. 2012). Publically available gene expression datasets confirmed this finding, revealing that RASSF2 is down 2-4 fold in t(8;21) patient bone marrow samples as compared to non t(8;21) M2 AML patient samples (TCGA 2013). Similarly, RNA-seq data shows that knock down of AML1-ETO in Kasumi-1 cells by siRNA results in an increase in RASSF2 expression compared to a scrambled siRNA control (Ptasinska et. al. 2014).

Additional, unpublished work from our lab has confirmed RASSF2 as an important gene commonly down-regulated in t(8;21) AML, and has demonstrated the tumor suppressive function of RASSF2 in t(8;21) leukemia (Fig. 1B, Fig. 2A-E). Clearly a potentially important therapeutic target, the structural domains of RASSF2 and their functional significance in various cancer systems have been previously studied and reported. However, very little is known the role it plays in leukemogenesis and the blood system.

Here, we present data characterizing RASSF2 in the context of leukemia, furthering insights regarding the role it plays in t(8;21) AML. Exploring which domains are the most important for its tumor suppressive function in the presence of the AML1-ETO fusion protein, this work lays ground for future mechanism of action studies. In this work, we show that the SARAH domain of RASSF2—and presumably the corresponding ability of RASSF2 to interact with pro-apoptotic proteins of the Hippo pathway—is particularly important to the tumor suppressive function of RASSF2. Additionally, we show that Nuclear Export Signal of RASSF2 may serve a specific, functionally important, previously unexplored role in blood cells and the development of leukemia.

## II. Results

**RASSF2 is transcriptionally repressed in the presence of AML1-ETO**

Building upon previous work, which has identified RASSF2 as a gene commonly down-regulated in t(8;21) AML, we used RT-qPCR, to assess RASSF2 transcription in the presence and absence of AML1-ETO. We found that RASSF2 mRNA is 8-10 fold lower in the t(8;21) leukemia cell lines Kasumi-1 and SKNO as compared to the similar non-t(8;21) HL-60 leukemia cell line and to primary human CD34+ hematopoietic stem and progenitor cells (Fig. 1A). Similarly, we found ectopic expression of AML1-ETO in HL-60 or $CD34^+$ cells results in a decrease in RASSF2 mRNA expression (Fig. 1A). This data suggests that RASSF2 is a target for regulation by AML1-ETO. Based on these findings, we hypothesized that down regulation of RASSF2 by AML1-ETO may be a critical step in the development of t(8;21) leukemia.

**RASSF2 inhibits the self-renewal ability of AML1-ETO transformed hematopoietic cells by promoting apoptosis**

To examine this possibility, we assessed the effect RASSF2 expression has on the leukemic phenotype. AML1-ETO transduced primary mouse bone marrow cells gain the self-renewal capacity characteristic of leukemia cells—evidenced by the ability to serially replate for more than six weeks. We found that co-expression of RASSF2 with AML1-ETO significantly inhibits this long-term self-renewal capability, reducing colony number and abolishing the ability to replate after 3-4 weeks. (Fig 2B, 2D) This was due primarily to a dramatically increased rate of apoptosis in RASSF2 expressing cells. (Fig 2E)

This previous work has identified RASSF2 as an important gene commonly and dramatically down-regulated in t(8;21) AML. Furthermore, this work has demonstrated the tumor suppressive function of RASSF2 in t(8;21) leukemia cells.

**Generating RASSF2 domain deletions**

To explore which functions of RASSF2 are most important for its tumor suppressive function in leukemia, we generated six FLAG-tagged RASSF2 deletion variants. Four of these RASSF2 variants were designed lacking the RA domain, SARAH domain, NES, or NLS, and two additional variants were generated lacking arbitrary 50 amino acid segments in the N-terminus, where no functional domains have been previously characterized (Fig. 3B). These RASSF2 variants were cloned into MSCV-IRES-Puromycin (MIP) and MSCV-IRES-GFP (MIG) vectors. These variants were generated using a strategy described by Liu et. al. (2008). This strategy is outlined in Fig. 3A, and the functional significance of each domain is summarized in Fig. 3C.

**Effects of RASSF2 domain deletions**

Previous data has shown that expressing RASSF2 in leukemia cell lines using the GFP-encoding MIG vector leads to a significant decline in GFP+ cells over time compared to empty MIG controls, whose GFP percentage remains high over time. This suggests RASSF2 has a negative effect on leukemia cell lines, supporting the idea that RASSF2 is a tumor suppressor. In order to screen for the effect of each domain deletion on the tumor suppressive function of RASSF2, Kasumi-1 cells were transduced with the

MIG-RASSF2 deletion constructs. Following transduction, the percentage of $GFP^+$ cells was monitored over time.

Consistent with previous findings, the GFP percentage declined the most rapidly in cells expressing full length RASSF2 due to negative selection pressure. However, the mechanism of selection is gradual, with no clear increase in apoptosis or cell cycle arrest. These increases may be minor, and therefore difficult to detect in an impure population of $GFP^+$ and $GFP^-$ cells. Expression of a RASSF2 variant lacking the domain that is the most strongly selected against—i.e. the domain that is the most important to mediating the tumor suppressive function of RASSF2—results in a slower decline in GFP percentage over time, as the variant is not selected against as strongly as full length RASSF2.

**The SARAH domain appears most important for RASSF2 function**

The data suggests that the SARAH domain is the most strongly selected against. Cells expressing RASSF2ΔSARAH maintain the highest GFP percentage over time compared to full length RASSF2 and any other RASSF2 variant (Fig 4A). Similarly, cells expressing RASSF2dΔRA or RASSF2ΔN1 maintained higher GFP percentage over time compared to full length RASSF2, RASSF2ΔNLS, RASSF2ΔN2, and RASSF2ΔNLS (Fig. 4A). This suggests that the SARAH domain, the N1 domain, and the RA domain are the most important to the tumor suppressive function of RASSF2.

**Deletion of the NES alters RASSF2 function**

While these three deletions led to the most dramatic decrease in GFP percentage decline, it is important to note that deletion of the NES also reduced the negative effect of RASSF2 on Kasumi-1 cells. Furthermore, since the NES is a part of the RA domain, the

phenotype elicited by RASSF2ΔRA is likely due in part to deletion of the NES (Fig. 4A). Although the decrease elicited by the NES deletion is not as large as those elicited by SARAH, RA, or N1 deletions, this decrease is particularly interesting because RASSF2 has been previously reported to be a nuclear protein whose nuclear localization is necessary to its tumor suppressive function (Cooper et. al. 2008). This unexpected phenotype led us to hypothesize that RASSF2 may exist primarily in the cytoplasm in blood cells, where it may serve a novel function specific to the blood system.

**RASSF2ΔSARAH is overexpressed at the protein and mRNA level**

After observing the above phenotypes, we ensured that all of the RASSF2 variants were expressed at the protein level. We transduced Kasumi-1 cells with MIP-FLAG-RASSF2 deletion constructs, then subjected the lysates to immunobloting using an antibody against FLAG. All of the variant proteins were expressed at similar levels except for RASSF2ΔSARAH, which was expressed at a higher level (Fig 4B, 4C).

We hypothesized that deletion of the SARAH domain could stabilize the RASSF2ΔSARAH protein and therefore allow its accumulation. To test if RASSF2ΔSARAH overexpression was specific to the RASSF2ΔSARAH protein, we used RT-qPCR to check mRNA levels. RASSF2ΔSARAH mRNA was approximately three fold higher than full length RASSF2, while RASSF2ΔSARAH protein was approximately ten fold higher than full length RASSF2 (Fig. 4C, 4D). This suggests that the increased protein levels of RASSF2ΔSARAH could result from increased protein stability. Further biochemical analysis needs to be conducted to assess stability over time

and to identify proteins involved in the ubiquitin-proteasome system that may interact with the SARAH domain to facilitate degradation.

**RASSF2 localizes predominantly to the cytoplasm in blood cells**

Because deletion of the NES of RASSF2 led to a surprising decrease in RASSF2 function, we investigated the localization of full length RASSF2 as well as the RASSF2 variants.

We began by fractionating Kasumi-1 cells transduced with MIP-FLAG-RASSF2 deletion constructs into whole cell, nuclear, and cytoplasmic cell lysates, then subjected the lysates to immunoblotting using an antibody against FLAG (Ye, J. et. al. 1994). All variants except RASSF2ΔSARAH were undetectable in the nuclear fraction, localizing exclusively to the cytoplasm (Fig 5A). We confirmed these results via immunocytochemistry, again showing that RASSF2 was predominantly cytoplasmic (Fig 5B).

Surprised to see more RASSF2 in the cytoplasm than in the nucleus, we explored RASSF2 localization in different cell lines to see if this cytoplasmic expression was specific to Kasumi-1 cells. Western Blot analysis of another t(8;21) cell line, SKNO, transduced with MIP-FLAG-RASSF2 revealed predominantly cytoplasmic localization (Fig 5C). Western Blot analysis of a non-t(8;21) blood cell line, U937, transduced with MIP-FLAG again revealed predominantly cytoplasmic RASSF2 (Fig 5D). We confirmed this result by immunocytochemistry (Fig 5E).

To ensure that cytoplasmic localization of RASSF2 was not an unwanted result of overexpression from our MSCV promoter-based constructs, we ran a Western Blot for

endogenous RASSF2 protein with lysates from U937 cells and HL-60 cells—two blood cell lines with higher RASSF2 protein expression. These analyses again showed that all detectable RASSF2 was predominantly cytoplasmic (Fig 5E).

**RASSF2 localizes to both the cytoplasm and the nucleus in 293T cells**

In order to ensure that the protocols followed for our localization studies were not biasing our results by allowing RASSF2 to leak out of the nucleus, we studied localization in 293T cells, which are reported to express nuclear RASSF2. We transfected 293T cells with an empty MIP vector or MIP-RASSF2. 24 hours post-transfection, we cytospun 50,000 cells onto slides for immunocytochemistry and harvested the remaining cells for Western Blot analysis. In both cases, we probed with anti-FLAG antibodies to detect overexpressed RASSF2. These assays revealed both cytoplasmic and nuclear RASSF2 (Fig. 6A, 6B, 6C).

A

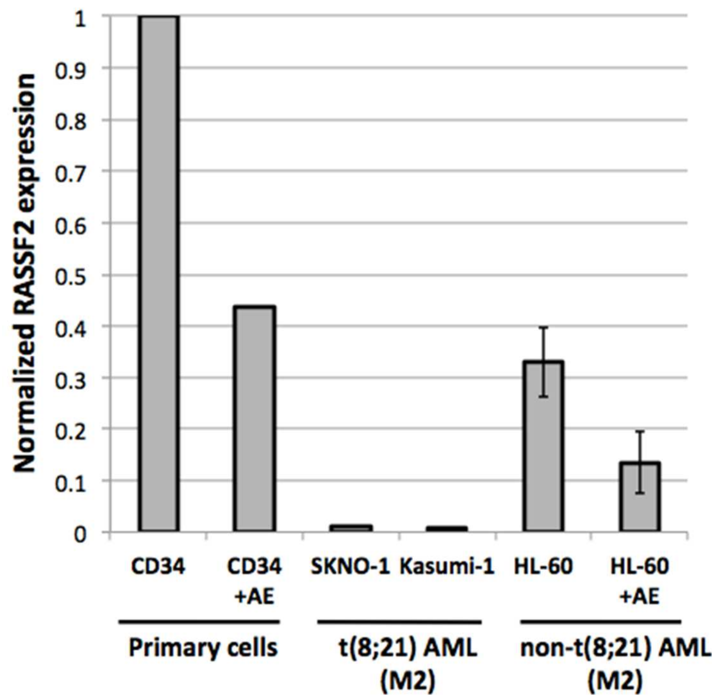



**Figure 1. RASSF2 is transcriptionally repressed in the presence of AML1-ETO**
(A) Relative RASSF2 mRNA transcript expression was compared by RT-qPCR in primary $CD34^+$ cells isolated from human cord blood (with or without transduction of AML1-ETO), two t(8;21) AML cell lines: SKNO-1 and Kasumi-1, and a FAB subtype M2 non-t(8;21) AML cell line: HL-60 (with or without transduction of AML1-ETO). For AE transduction experiments cells were transduced with empty vector controls or MIG-AE / MIP-AE expression constructs, cultured for 3 days, and $GFP^+$ or puromycin-resistant cells were selected for RNA isolation. Ct values are calculated based on comparison to the geometric mean of two independent reference genes, GAPDH and RNAPolII. Data are normalized to expression in primary $CD34^+$ cell controls

Figure 1, in full, will eventually be submitted for publication of the material. Stoner, Sam; Weng, Stephanie; Zhang, Dong-Er.

A

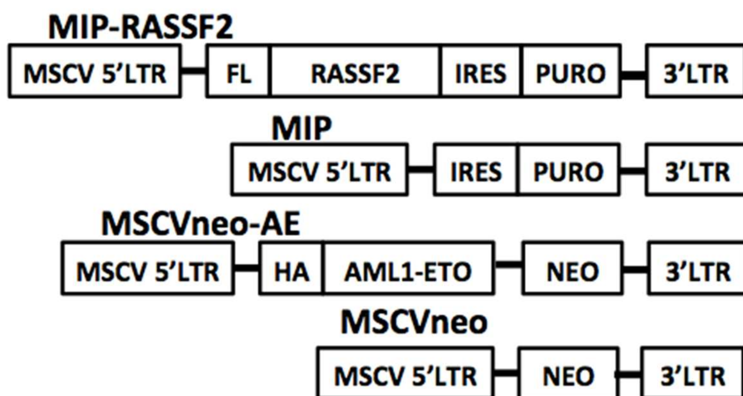



**Figure 2. RASSF2 inhibits the self-renewal ability of AML1-ETO transformed hematopoietic cells via promotion of apoptosis**
(A) For serial replating assay primary murine bone marrow was co-retrovirally transduced to express constructs shown.

B

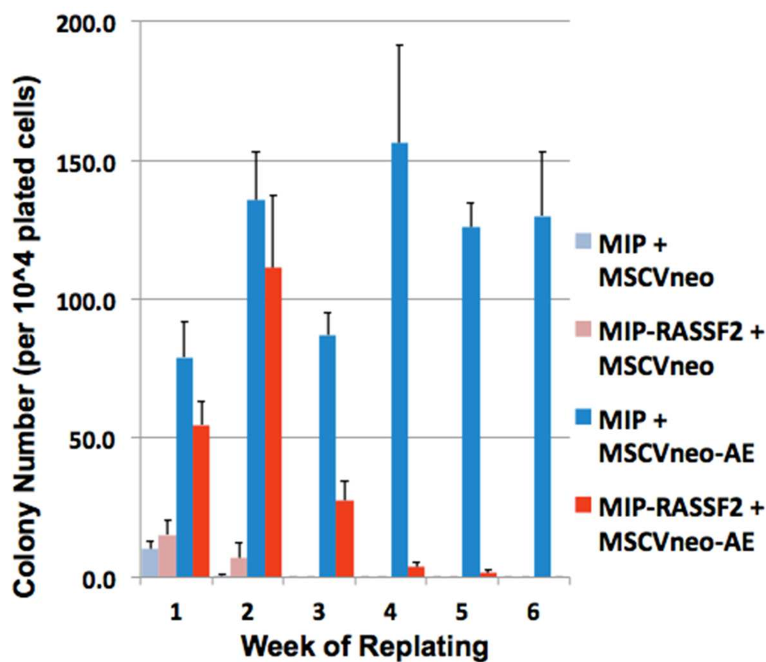



**Figure 2. (Continued) RASSF2 inhibits the self-renewal ability of AML1-ETO transformed hematopoietic cells via promotion of apoptosis**
(B) Following 1 week of drug selection 10,000 cells were seeded in semi-solid methylcellulose medium for weekly serial replatings. The colony number was counted each week.

C

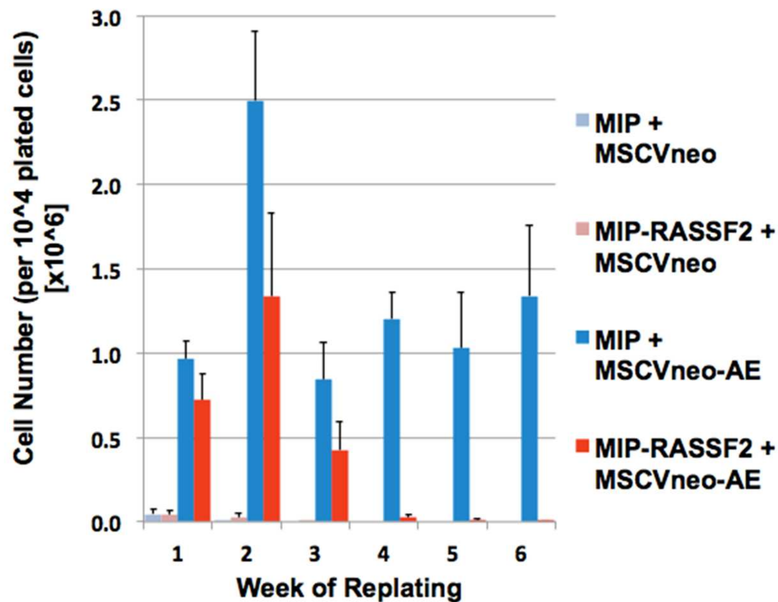



**Figure 2. (Continued) RASSF2 inhibits the self-renewal ability of AML1-ETO transformed hematopoietic cells via promotion of apoptosis**
(C) Following 1 week of drug selection, 10,000 cells were seeded in semi-solid methylcellulose medium for weekly serial replatings. The number of viable cells was counted each week.

D

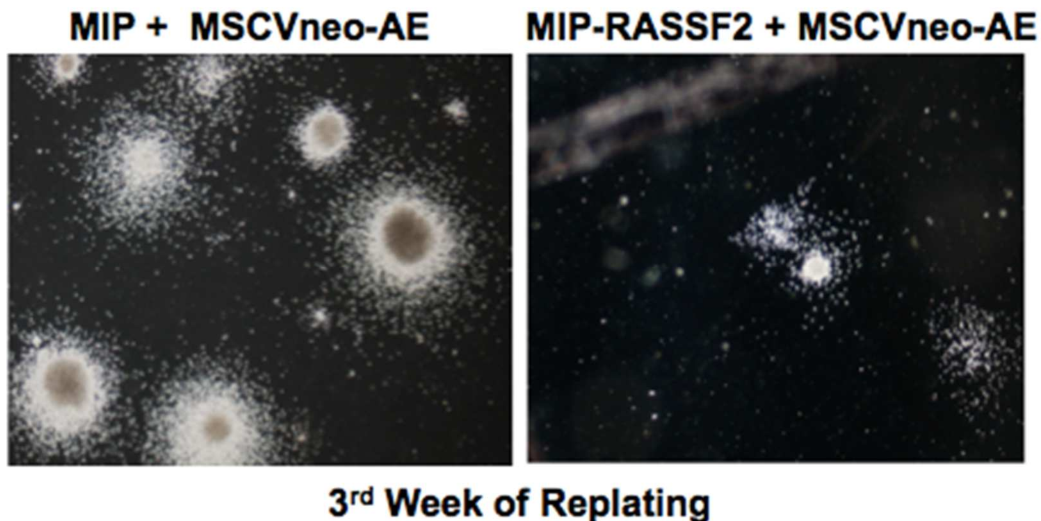



**Figure 2. (Continued) RASSF2 inhibits the self-renewal ability of AML1-ETO transformed hematopoietic cells via promotion of apoptosis**
(D) Representative colonies from the 3rd week of replating.

E

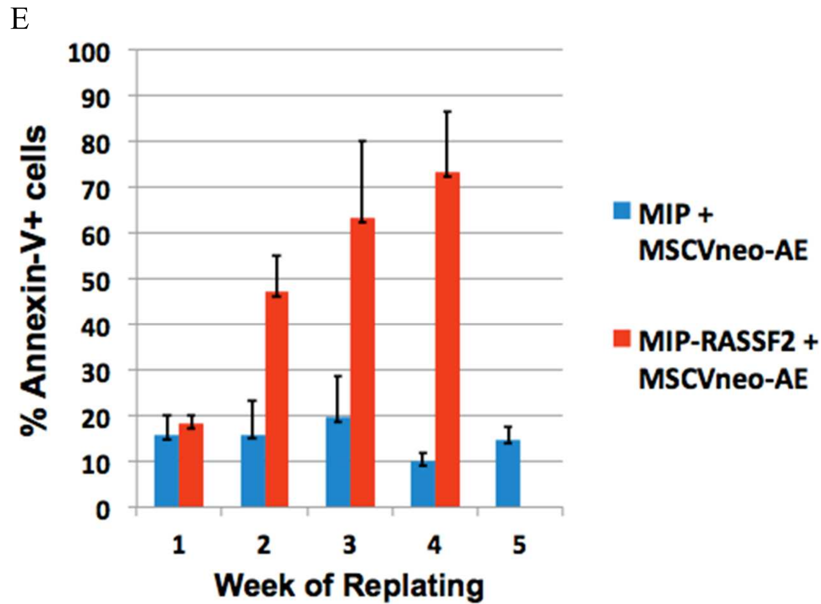



**Figure 2. (Continued) RASSF2 inhibits the self-renewal ability of AML1-ETO transformed hematopoietic cells via promotion of apoptosis**
(E) Percent of apoptotic cells (measured via Annexin-V staining) were monitored each week. All data are presented as the mean +/- s.e.m for three independent biological replicates.

Figure 2, in full, will eventually be submitted for publication of the material.

Stoner, Sam; Zhang, Dong-Er.

A

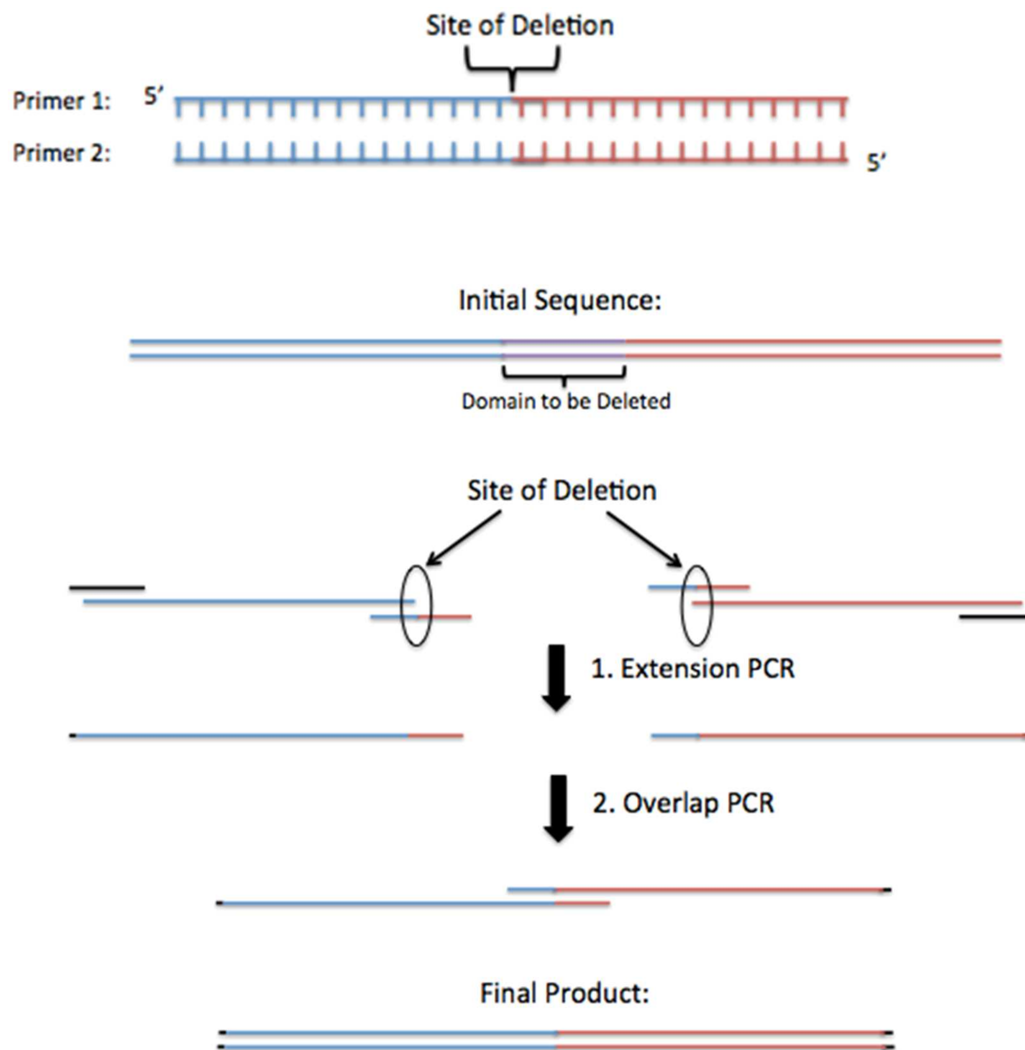



**Figure 3. Generating RASSF2 deletion constructs**

(A) Strategy employed to generate deletion constructs. Perfectly complimentary primers were designed to overlap each domain to be deleted, bridging the two flanking regions to be brought together (top). Extension PCR was performed with these primers as well as with primers complimentary to the 5' and 3' ends of RASSF2, containing a BamHI and EcoRI restriction site, respectively. Purified products were used for Overlap PCR in which products primed each other to generate final products.

Figure 3, panel A, graphic is original work of thesis author, representing a protocol previously described by Liu et. al. 2008.

B

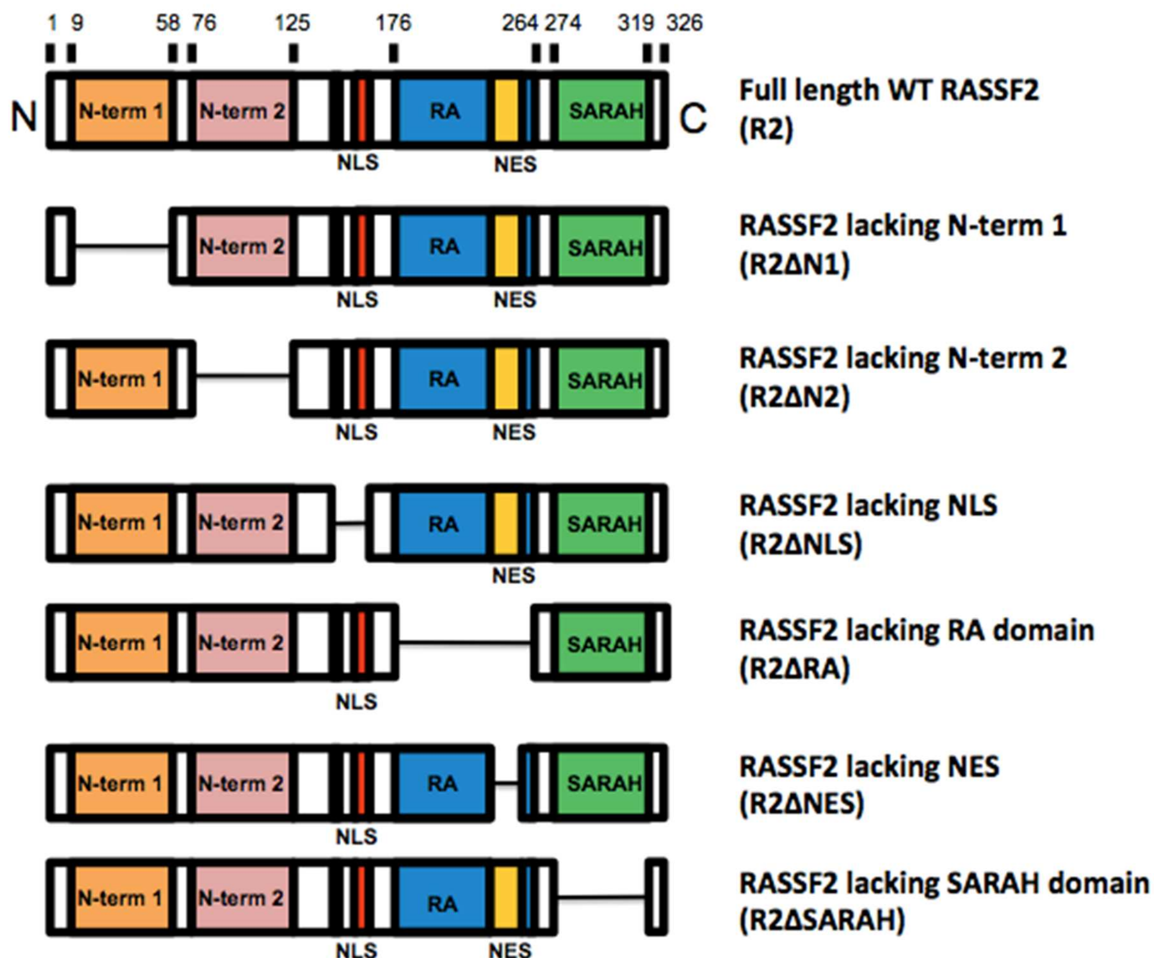



**Figure 3. (Continued) Generating RASSF2 deletion constructs**
(B) Schematic representing the six RASSF2 variants generated. Numbers above schematic indicate amino acid position. RASSF2 variants were cloned into MSCV-IRES-Puro (MIP) and (MSCV-IRES-GFP) MIG vectors.

C

| Domain | Amino Acids | Function | Reported Significance |
|---|---|---|---|
| **N1** | 9-58 | None reported | None |
| **N2** | 76-125 | None reported | None |
| **NLS** | 151-152, 162-167 | Nuclear localization | Breast cancer cells transduced with RASSF2 display a compromised ability for anchorage-independent growth as compared to empty vector. Cells transduced with RASSF2 with a mutant NLS display an increased ability for anchorage-independent growth as compared to WT RASSF2 (Cooper et. al. 2008). |
| **RA** | 176-264 | Interaction with Ras proteins, specifically K-Ras | Expression of RASSF2 in lung cancer cells leads to increased apoptosis and cell cycle arrest. Expression of RASSF2 in the presence of K-Ras exaggerates this phenotype, leading to larger increases in apoptosis and cell cycle arrest (Vos et. al. 2003). |
| **NES** | 240-260 | Nuclear export | Mutation of NES ablated nucleo-cytoplasmic shuttling of RASSF2, leading to only nuclear localization (Kumari et. al. 2009). |
| **SARAH** | 274-319 | Interaction with other SARAH domain-containing proteins: MST1/2, self, or other RASSF proteins | In thyroid carcinoma cells, expression of RASSF2 increased apoptosis as compared to empty vector, while expression of RASSF2 lacking a SARAH domain led to decreased apoptosis compared to wildtype RASSF2. (Schagdarsurengin et. al. 2010) |

**Figure 3. (Continued) Generating RASSF2 deletion constructs**
(C) Summary of the functions of different RASSF2 domains as well as the published significance of domains.

A

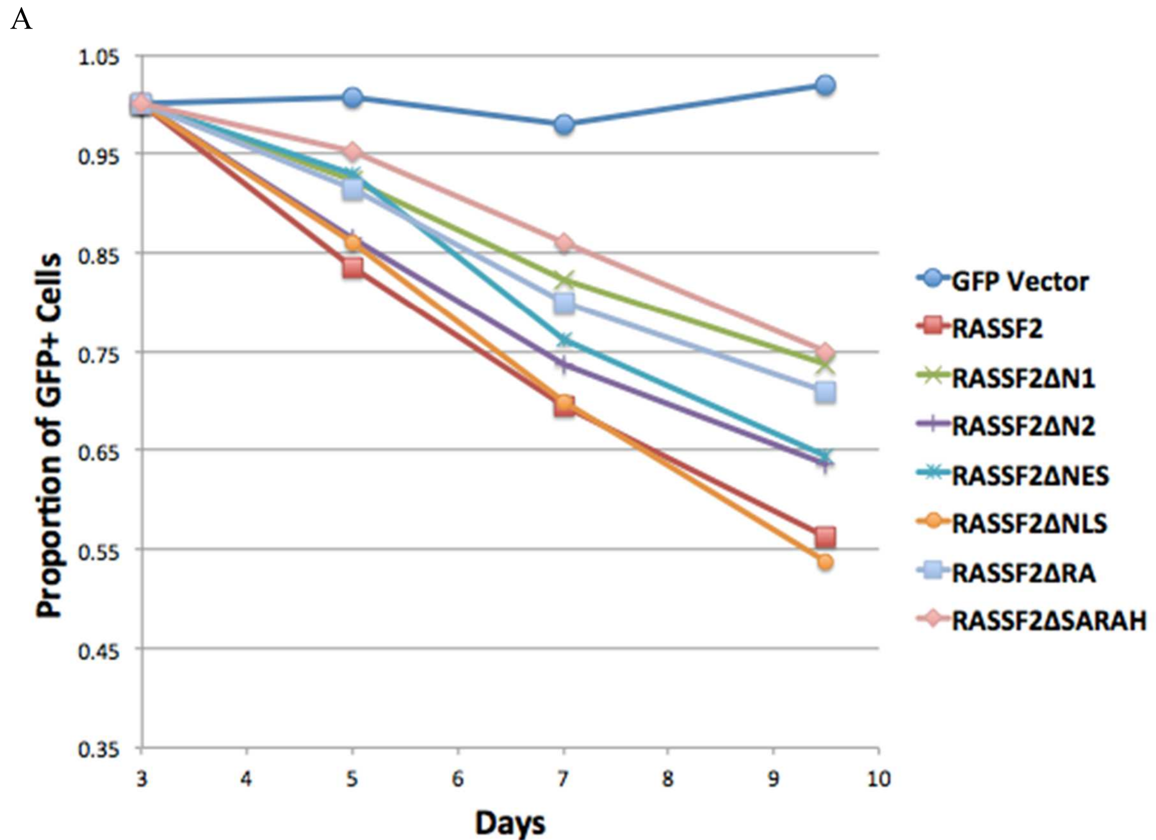



**Figure 4. Effects of RASSF2 domain deletions on expression over time**
(A) Kasumi-1 cells were stably transduced with MIG-RASSF2 deletion constructs so that the cells expressing RASSF2 would be $GFP^+$. Following infection, the percentage of $GFP^+$ cells was monitored over time. The GFP percentage observed three days following infection was set to 100%, and the GFP percentage observed on following days are represented as a percentage of that initial value. Data are averages of two independent experiments.

B

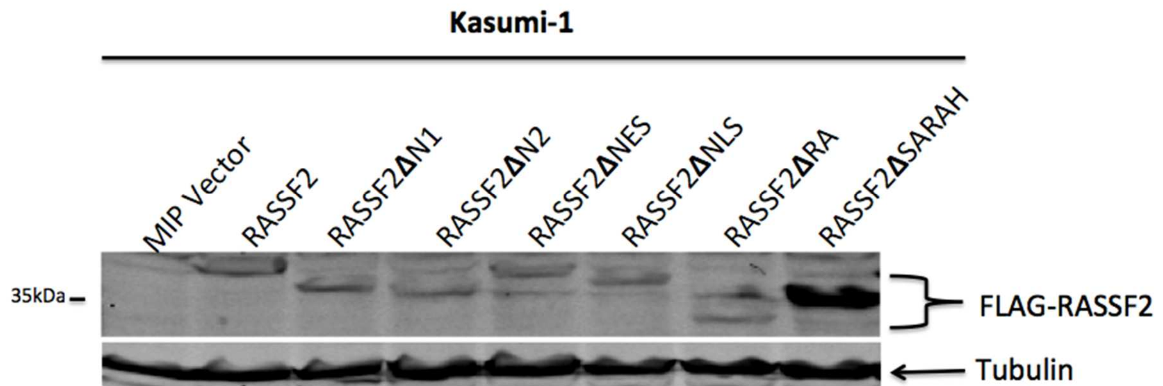



**Figure 4. (Continued) Effects of RASSF2 domain deletions on expression over time** (B) Western blot analysis of Kasumi-1 cells transduced with different MIP-FLAG-RASSF2 deletion constructs. Blot was immunoprobed with anti-FLAG to detect RASSF2, as well as anti-tubulin as a loading control.

C

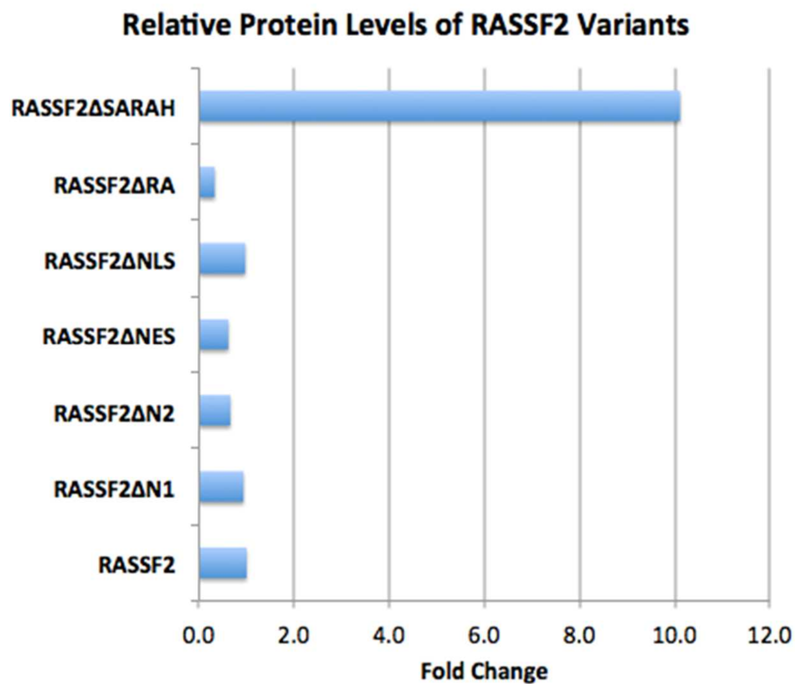



**Figure 4. (Continued) Effects of RASSF2 domain deletions on expression over time** (C) Quantification of protein expression. Western Blot in Fig. 4B was analyzed using LiCor Odyssey software. Relative expression normalized to that of full length RASSF2.

D

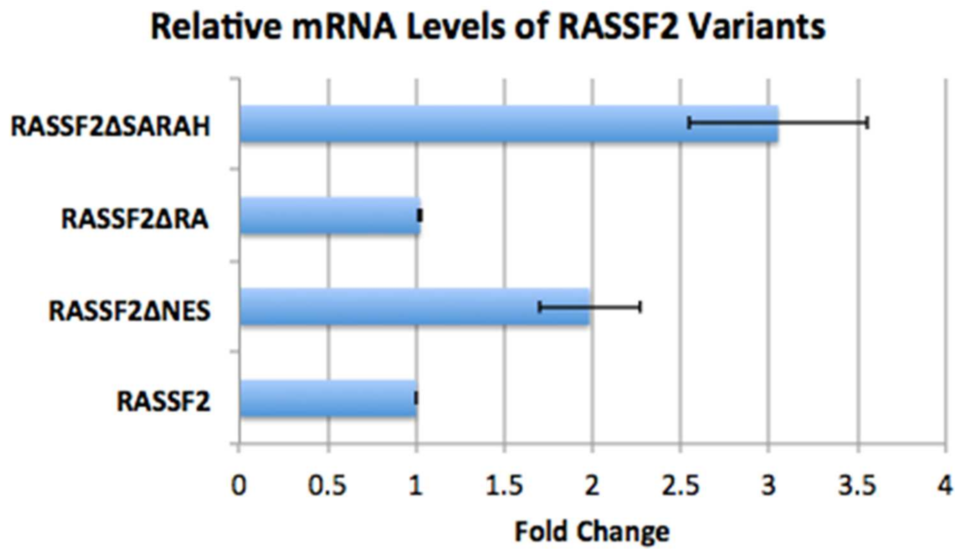



**Figure 4. (Continued) Effects of RASSF2 domain deletions on expression over time** (D) RT-qPCR analysis showing mRNA levels are increased in RASSF2delSARAH. Ct values are calculated based on comparison to the geometric mean of two independent reference genes, GAPDH and RNAPolII. Fold change expressed relative to full length WT RASSF2. Values represent average of two biological replicates, +/- s. e. m.

A

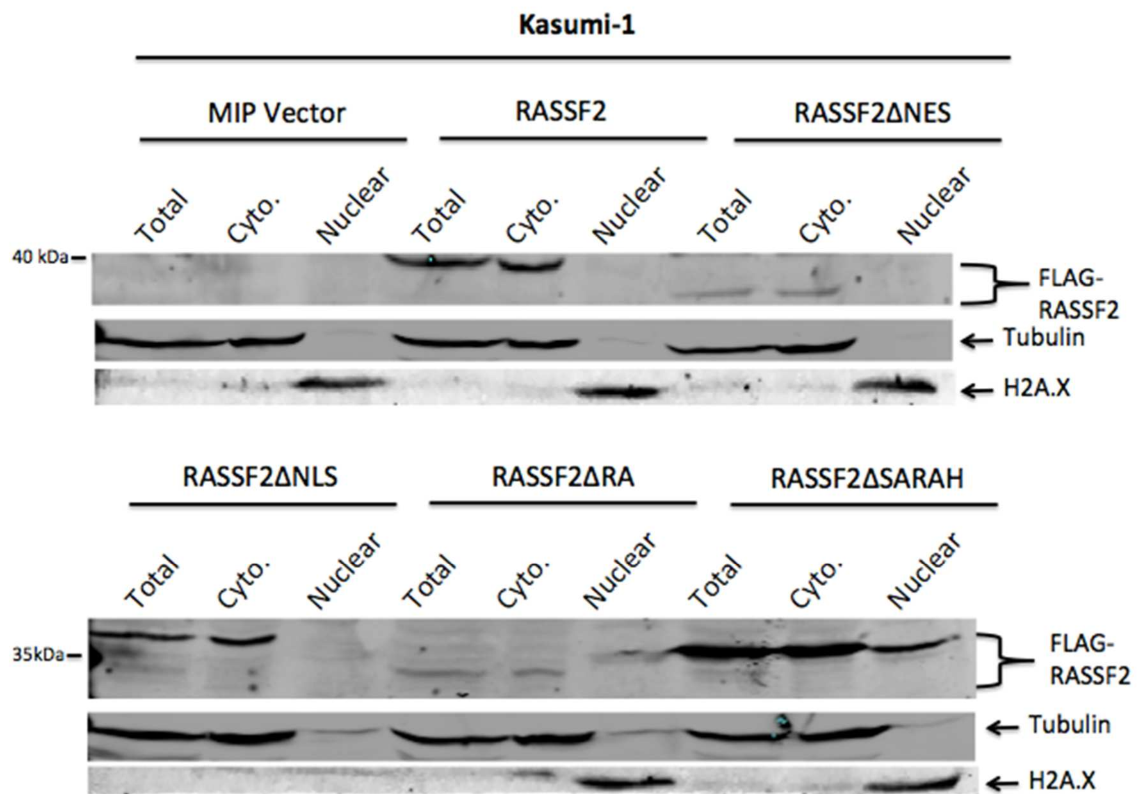



**Figure 5. RASSF2 localizes predominantly to the cytoplasm in blood cell lines**
(A) Western blot analysis of Kasumi-1 cells transduced with different MIP-FLAG-RASSF2 deletion constructs, then drug selected in 1ug/ml puromycin for 3 days. Cytoplasmic and nuclear lysates were fractionated via the protocol described previously by Ye, J. et. al. 1994. Total protein was quantified by Bradford assay, and equal amounts of total protein were loaded. Blots were immunoprobed with anti-FLAG as well as anti-tubulin, which serves as a loading/contamination control for the cytoplasmic fraction. Same lysates were run on another gel and probed with anti-Lamin B as a loading/contamination control for the nuclear fraction.

B

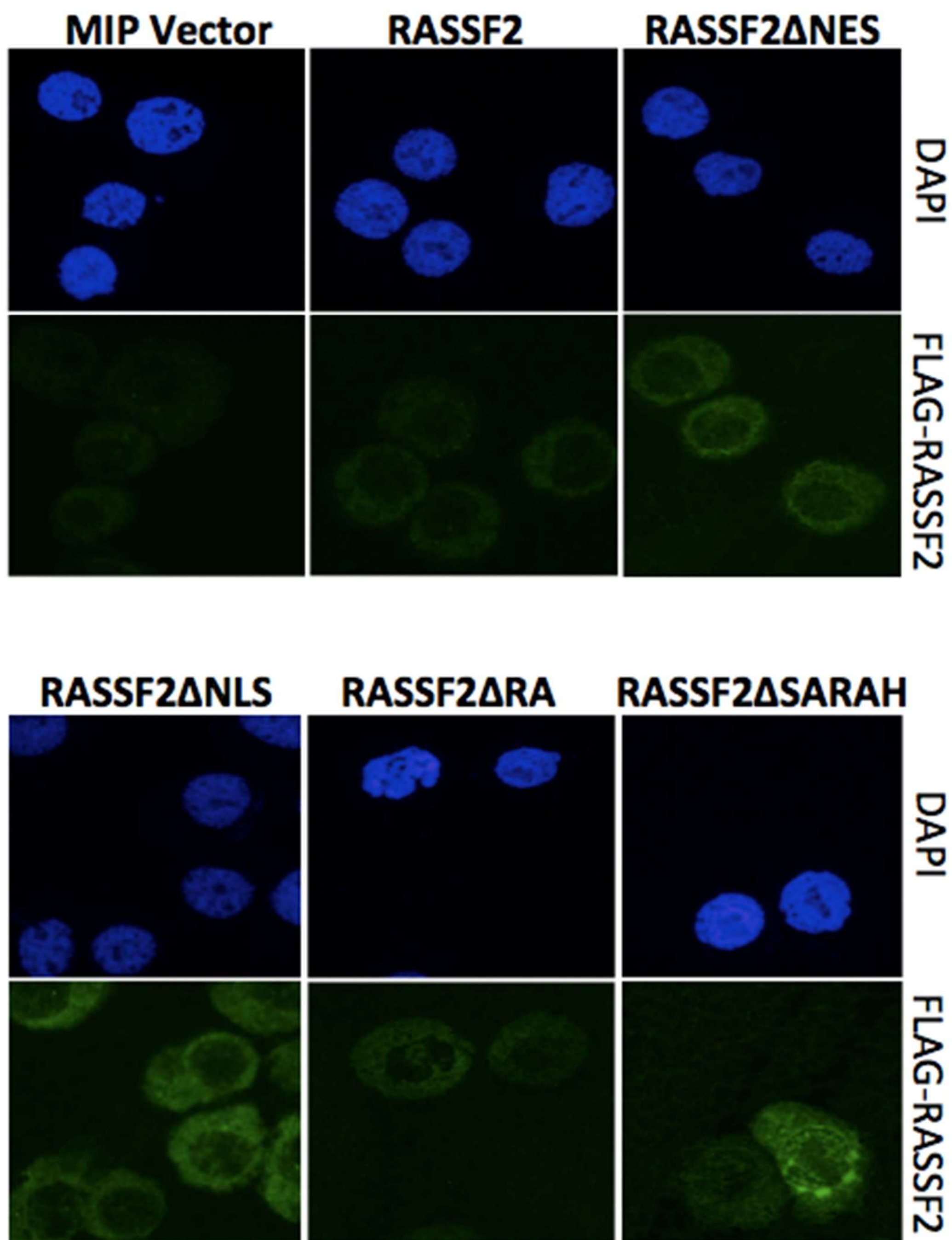



**Figure 5. (Continued) RASSF2 localizes predominantly to the cytoplasm in blood cell lines**

(B) Immunofluorescence microscopy was performed to detect FLAG-RASSF2 variants (green) with DAPI staining (blue) in Kasumi-1 cells.

C

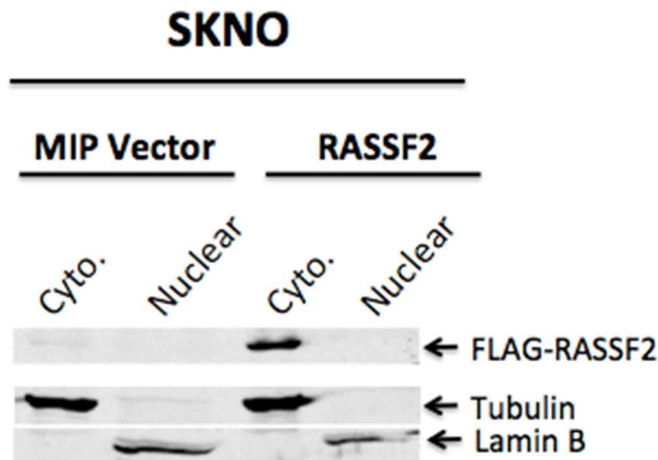



**Figure 5. (Continued) RASSF2 localizes predominantly to the cytoplasm in blood cell lines**
(C) Western blot analysis of SKNO cells transduced with MIP-FLAG-RASSF2, then drug selected in 1 ug/ml puromycin for 3 days. Cytoplasmic and nuclear lysates immunoprobed with anti-FLAG as well as anti-tubulin and anti-Lamin B as a loading/contamination control for the cytoplasmic and nuclear fractions, respectively.

D

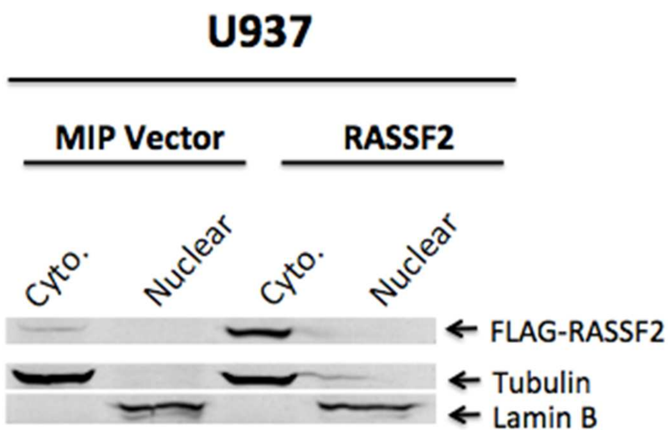



**Figure 5. (Continued) RASSF2 localizes predominantly to the cytoplasm in blood cell lines**
(D) Western blot analysis of U937 cells transduced with MIP-FLAG-RASSF2, then drug selected in 1.5 ug/ml puromycin for 3 days. Cytoplasmic and nuclear lysates were immunoprobed with anti-FLAG as well as anti-tubulin and anti-Lamin B as a loading/contamination control for the cytoplasmic and nuclear fractions, respectively.

E

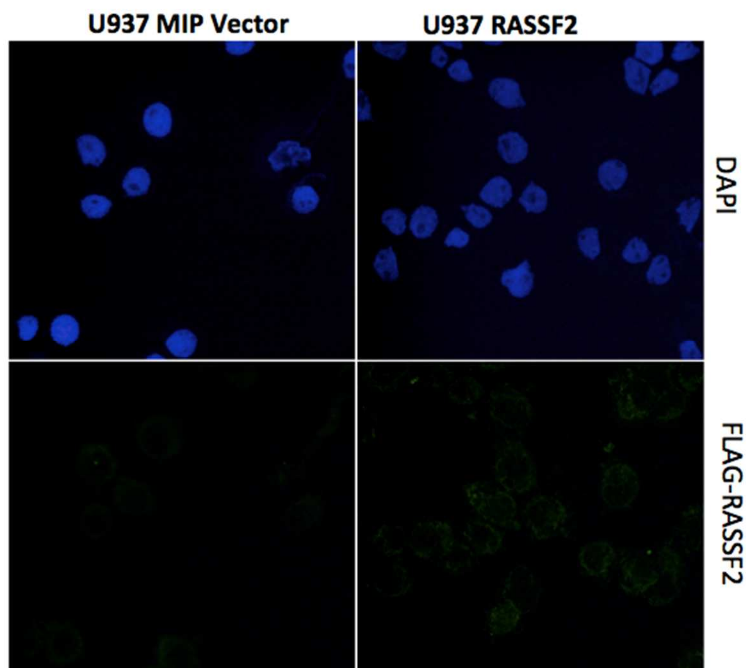



**Figure 5. (Continued) RASSF2 localizes predominantly to the cytoplasm in blood cell lines**
(E) Immunofluorescence microscopy was performed to detect FLAG-RASSF2 (green) with DAPI staining (blue) in U937 cells.

F

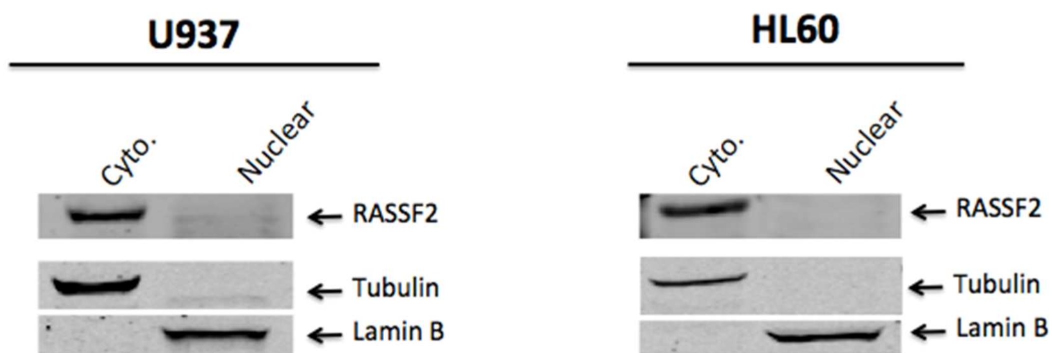



**Figure 5. (Continued) RASSF2 localizes predominantly to the cytoplasm in blood cell lines**
(F) Western blot analysis of U937 cells and HL-60 cells. Cytoplasmic and nuclear lysates were immunoprobed with anti-RASSF2 as well as anti-tubulin and anti-lamin B as a loading control/contamination control for the cytoplasmic and nuclear fractions, respectively.

A

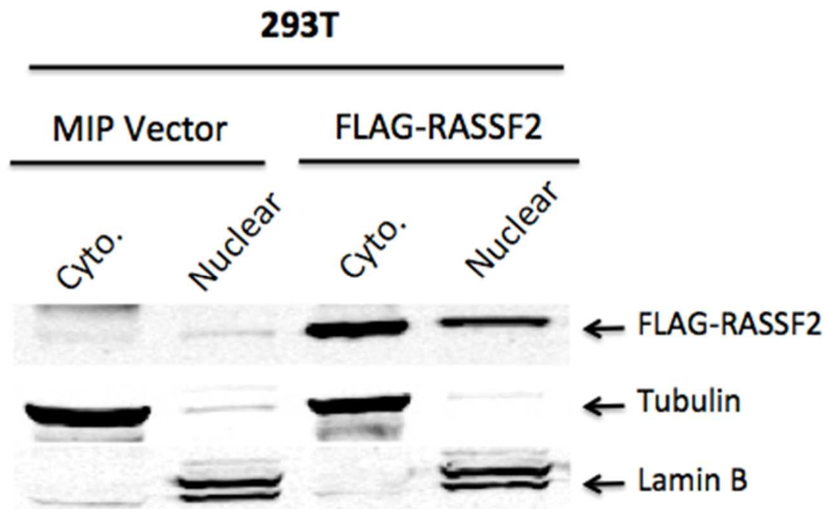



**Figure 6. RASSF2 appears more nuclear in 293T cells**
(A) Western blot analysis of 293T cells transfected with MIP-FLAG-RASSF2, then harvested 24 hours after transfection. Cytoplasmic and nuclear lysates were immunoprobed with anti-FLAG, anti-tubulin, and anti-Lamin B, which serve as loading/contamination controls for the cytoplasmic fraction and nuclear fraction, respectively.

B

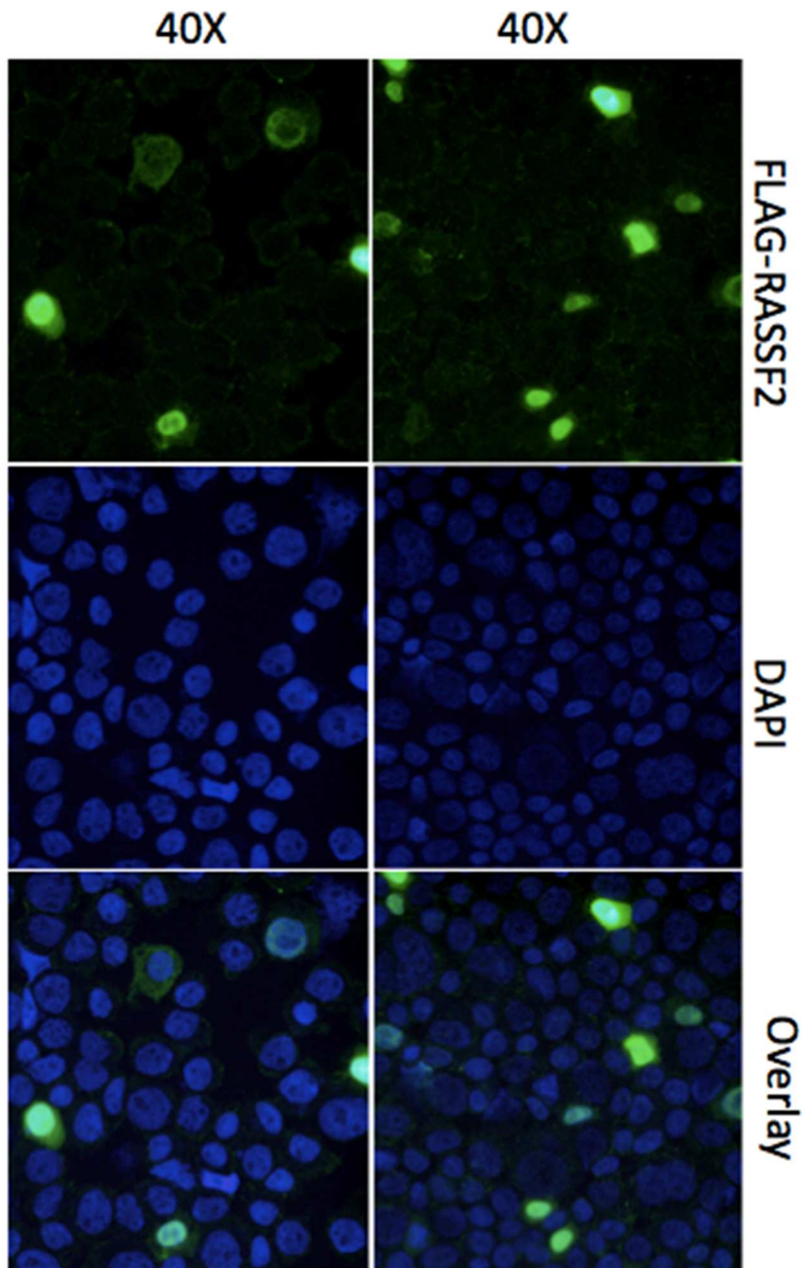



**Figure 6. (Continued) RASSF2 appears more nuclear in 293T cells**
(B) Two representative images of immunofluorescence microscopy at 40X. 293T cells were transfected with MIP-FLAG-RASSF2, then cytospun onto slides 24 hours after transfection. Cells were immunoprobed with FLAG-RASSF2 (green) with DAPI staining (blue).

C

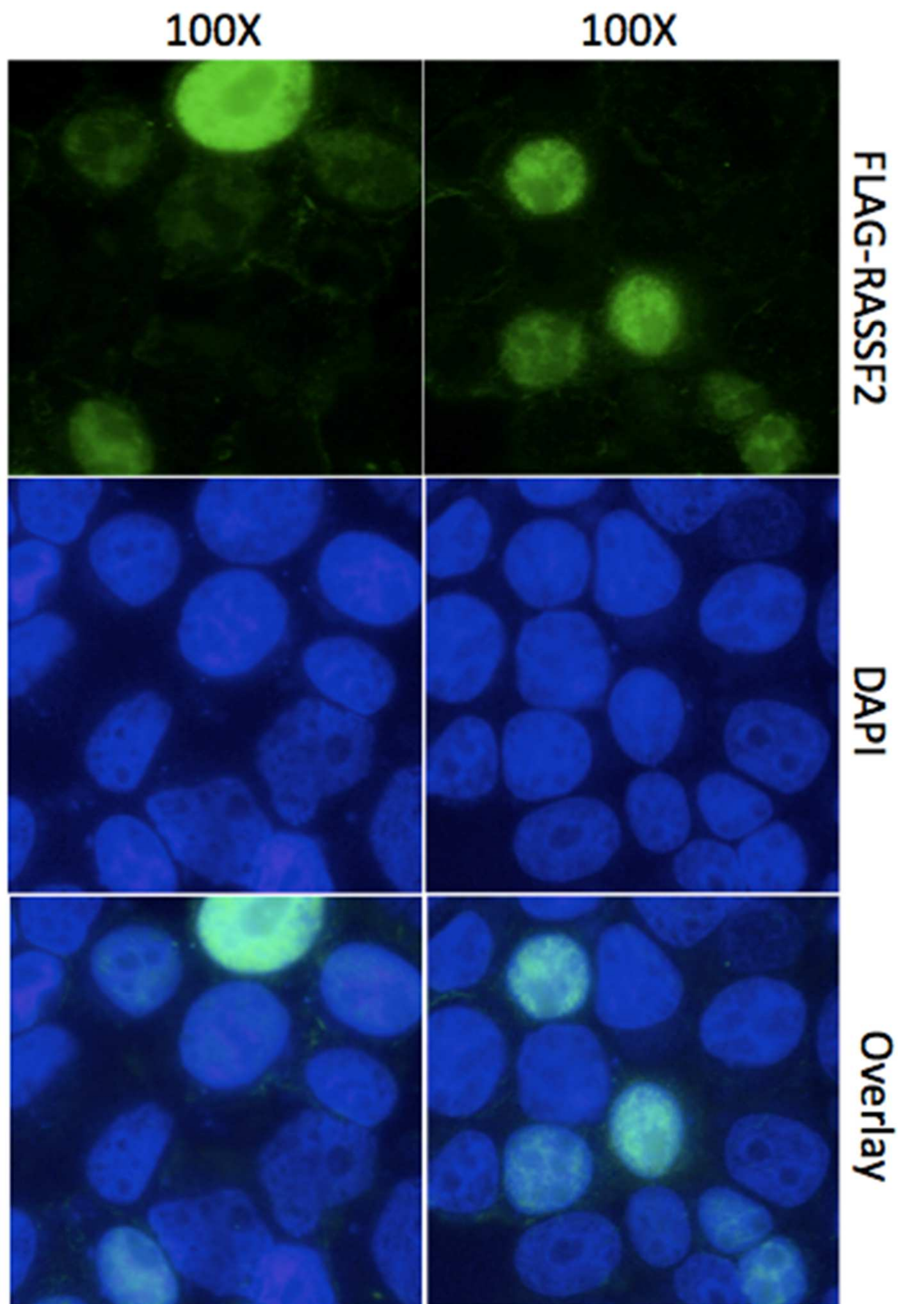



**Figure 6. (Continued) RASSF2 appears more nuclear in 293T cells**
(C) Two representative images of immunofluorescence microscopy at 100X. 293T cells were transfected with MIP-FLAG-RASSF2, then cytospun onto slides 24 hours after transfection. Cells were immunoprobed with FLAG-RASSF2 (green) with DAPI staining (blue).

## III. Discussion

RASSF2 has been well characterized as an important tumor suppressor gene that is commonly down-regulated in a wide variety of cancers. Previous data generated by our lab has shown that RASSF2 is specifically down-regulated in the presence of the AML1-ETO fusion protein and that expression of RASSF2 in AML1-ETO transduced primary mouse bone marrow cells represses the leukemic phenotype. In order to further these studies, our most recent work—as discussed in this thesis—has focused on characterizing RASSF2 by discovering which functional domains are the most important to its tumor suppressive function in the context of leukemia.

Based on RASSF2 variants lacking the N1, N2, NES, NLS, RA, or SARAH domain, we have identified the SARAH domain as the most important to the tumor suppressive function of RASSF2 in the presence of the AML1-ETO fusion protein. Previous studies have characterized the pro-apoptotic Hippo pathway, which keys on SARAH domain containing proteins to limit organ size in response to extracellular signals such as cell-cell contact (Plouffe et. al. 2015). The identification of the SARAH domain as important to the tumor suppressive function of RASSF2 in leukemia suggests it may serve a novel function in blood cells, as circulating blood cells do not make adherens or tight junctions, which often interact directly with members of the Hippo pathway (Plouffe et. al. 2015).

Additionally, we found that deletion of the NES of RASSF2 had a modest but surprising effect on its function. This finding led us to investigate the localization of RASSF2. Subsequent studies revealed that RASSF2 localizes predominantly to the

cytoplasm in various blood cell lines, while it localizes to both the cytoplasm and the nucleus in the 293T cell line. Though interesting, these findings are far from definitive and can be interpreted in several ways.

**RASSF2 localization is cell-type dependent**

First, they can be interpreted as indicating that RASSF2 preferentially localizes to the cytoplasm of blood cells. A similar phenomenon has been documented by Fas-associated death domain (FADD), a pro-apoptotic protein that localizes to the nucleus in a spectrum of cell lines (such as HeLa, HEK 293, and A549), but localizes to the cytoplasm in Jurkat T-lymphocytes (Gomez-Angelats et. al. 2003). Predominantly cytoplasmic localization of RASSF2 would have numerous implications for the way we think about its function. Previous research discussing the interaction between RASSF2 and Ras have established that RASSF2 may act as a common Receptor Tyrosine Kinase effector, interacting with GTPases present in the cytoplasm (Vos et. al. 2003). However, previous studies have emphasized the importance of eventual nuclear localization, suggesting RASSF2 helps shuttle pro-apoptotic proteins such as PAR-4 into the nucleus (Cooper, et. al., 2008; Donninger et. al. 2010). These studies have largely ignored the protein interactions that RASSF2 may facilitate within the cytoplasm.

Ultimately, the idea that RASSF2 preferentially localizes to the cytoplasm suggests that the tumor suppressive role RASSF2 plays in leukemia is mechanistically different than the role it plays in other cancers. It raises the question: does RASSF2 have a novel function in the blood system previously unexplored in other cell lines? BioGPS analysis reveals that RASSF2 expression increases dramatically with myeloid blood cell differentiation—a phenomenon specifically relevant to leukemia

(http://biogps.org/#goto=genereport&id=9770). Considering potential blood cell-specific functions of RASSF2, it seems likely that RASSF2 may facilitate interactions between cytoplasmic proteins in order to promote cellular differentiation.

It is important to note that the localization of RASSF2 may be expression-level dependent rather than cell-type dependent. RASSF2 may only shuttle into the nucleus in response to abnormally high levels of expression, which we could not achieve or detect in our blood cell line system.

**RASSF2 localization depends on sensitivity to extracellular signals**

As mentioned, RASSF2 has been reported to localize to the nucleus in response to treatment with TRAIL or expression of activated K-Ras (Donninger et. al. 2010). Considering RASSF2 often localizes to the nucleus in response to specific extracellular signals, our localization studies suggest that leukemia cells may be less sensitive to these signals than 293T cells. If found true, this finding may change the way we think about disease progression and the role tumor suppressors play in the onset of leukemia. This would suggest that blood cells respond differently to extracellular stress than other cell types do, which could have important clinical implications.

**RASSF2 localizes to the cytoplasm as a result interaction with MST2**

RASSF2 has been reported to localize to the cytoplasm as a result of binding with activated MST2 (Cooper et. al. 2009). Taking this into consideration, it seems likely that blood cells may express a higher level of activated MST2, which would result in more cytoplasmic localization of RASSF2.

**One important consideration: Expression level**

These conclusions are far from definitive because of a key technical issue we encountered during our localization studies: modulating RASSF2 expression level between different cell types. RASSF2 manifested as a nuclear protein in experiments in which its expression was at its highest: in 293T cells transfected with RASSF2 and in Kasumi-1 cells transduced with RASSF2ΔSARAH. This raises the possibility that localization of RASSF2 to the nucleus is an artefact resulting from extreme overexpression. This possibility needs to be explored further with overexpression studies of full length RASSF2 in blood cells, but a promoter must be identified that will drive RASSF2 expression up to a level comparable to that which is achieved by transfection.

## Future directions

While these studies do not point to any definitive conclusions, they have laid important ground for future work. There are many avenues to explore in continuing to study the function of cytoplasmic RASSF2 in blood cell lines, as well as investigating the role the SARAH domain plays in RASSF2 function and the increased protein stability conferred by its deletion.

### RASSF2 localization

Future studies regarding RASSF2 localization will focus on resolving unequal expression in different cell lines, checking endogenous RASSF2 localization in a wider spectrum of cell lines, and measuring expression of activated MST2. Additional studies will measure RASSF2 localization in blood cells in response to apoptosis-inducing ligands—such as TRAIL—in pursuit of more mechanistic studies.

### The SARAH domain

Future studies regarding the functional significance of the SARAH domain in t(8;21) AML will assay the effect of this deletion on the leukemic phenotype through replating. Studies examining the stability conferred by the SARAH domain deletion will assess protein stability by monitoring expression over time after cyclohexamide treatment. Additionally, stability studies will work to identify proteins that interact with the SARAH domain and are involved in the ubiquitin-proteasome system. Since increased expression of RASSF2ΔSARAH was seen at the mRNA level as well as the protein level, it is possible that overexpression occurs because expression of RASSF2ΔSARAH exerts weaker tumor suppressive effects than full length RASSF2. In this case, our efforts will be funneled into more functional studies.

**Exploring downstream signaling pathways**

Additional studies assaying the effects of specific RASSF2 domain deletions on previously reported pro-apoptotic pathways will lend themselves to further mechanism of action studies. Since we believe RASSF2 may localize differently in blood cells than in previously explored systems, this work will focus on the protein-interaction domains: RA and SARAH.

Previous studies of the SARAH-domain containing RASSF5 have monitored the effects of RASSF5 knock down on the activation of MST1 and on cellular sensitivity to Tumor Necrosis Factor-α (TNF-α) mediated apoptosis, ultimately concluding that RASSF5 is necessary for MST1 activation in response to various extracellular signals (Park et. al. 2010). A similar approach could be taken to study RASSF2, comparing MST1 activation and TNF-α-mediated apoptosis in the presence of full length RASSF2 and RASSF2ΔSARAH.

Previous characterization of RASSF2 has identified a direct interaction between the RA domain and K-Ras, which appears integral in stimulating apoptosis in response to activated K-Ras (Vos et. al. 2003). It would be interesting to assess the apoptotic effects RASSF2ΔRA elicits upon co-expression with activated K-Ras. These studies will help elucidate the role RASSF2 plays in t(8;21) AML.

## IV. Materials and Methods

### Generation of RASSF2 Deletion Constructs

The RASSF2 variants (RASSF2, RASSF2delN1, RASSF2delN2, RASSF2delNES, RASSF2delNLS, RASSF2delRA, RASSF2delSARAH) were generated using serial PCR reactions. Primers were designed to be complimentary to one another, flanking and excluding the desired region of RASSF2. This PCR strategy is outlined elsewhere (Liu et. al. 2009) as well as in Figure 3A of this thesis. All RASSF2 deletion PCR products were sequenced to verify the success of each deletion. The resulting fragments (Fig. 3C) were cloned into the EcoRI and BamHI sites in the polylinker region of the MSCV-IRES-GFP (MIG) and MSCV-IRES-Puromycin (MIP) constructs.

### Cell Culture

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

### Flow Cytometry

Cells were transduced with various MIG-FLAG-RASSF2 deletion constructs. At specific time points after transduction, GFP percentage of cell populations was analyzed by flow cytometry.

**Western Blots**

Blood cell lines (Kasumi-1, SKNO, U937) were stably transduced with various FLAG-RASSF2 deletion constructs. Cells were drug selected in 1ug/ml puromycin for 3 days, then lysed in RIPA buffer to obtain whole cell lysates, or fractionated according to protocol outlined by Ye, J. et. al. (1994) to obtain nuclear and cytoplasmic lysates. Total protein was quantified by Bradford assay, and equal amounts of total protein were loaded. Blots were probed with anti-FLAG (1:1000, SIGMA) as well as anti-tubulin (1:10,000, SIGMA) and anti-H2A-X (1:2000, Upstate) or anti-Lamin B (1:1000, Oncogene) to confirm equal loading and that fractions were free of contamination.

293T cells were transfected with MIP-FLAG-RASSF2. Cells were allowed to grow for 24 hours, then fractionated according to protocol outlined by Ye, J. et. al. (1994) to obtain nuclear and cytoplasmic lysates. Total protein was quantified by Bradford assay, and equal amounts of total protein were loaded. Blots were probed with anti-FLAG (1:1000) as well as anti-Lamin B (1:1000), and anti-tubulin (1:10,000) to confirm equal loading and that fractions were free of contamination.

**Immunocytochemistry**

Kasumi-1 cells and U937 cells were transduced with various MIP-FLAG-RASSF2 constructs and selected for 3 days in the 1 ug/ml puromycin. 293T cells were transfected with various MIP-FLAG-RASSF2 constructs and allowed to grow for 24 hours. Cells were cytospun onto slides and fixed with 3.7% formaldehyde. Cells were permeabilized 5 min in 0.1% Triton X, then stained with anti-FLAG antibodies (1:100).

Slides were coverslipped with anti-fade mounting medium with DAPI. Samples were viewed with the Olympus BX51 microscope and images were recorded using the Olympus DP71 camera.

**Replating**

Mouse bone marrow cells stably transduced with MSCV-neo-AML1-ETO and various MIP-FLAG-RASSF2 deletion constructs. Cells were plated on semi-solid methylcellulose with penicillin/streptomycin, and drug selected in G418 and puromycin. Each week, colonies were counted, and then an equal number of cells were resuspended in IMDMEM and seeded in fresh methylcellulose.

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