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The Role of RUNX2 in Mixed Lineage Leukemia

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THE ROLE OF RUNX2 IN MIXED LINEAGE LEUKEMIAS

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Abstract

RUNX transcription factors are a family of DNA-binding proteins that function in development and hematopoiesis. Mixed Lineage Leukemias (MLL) are dependent on RUNX1 for oncogenic activity, but it is unclear whether MLLs are also affected by RUNX2. In this project, RUNX2 was knocked down in a variety of MLL-derived cell lines using shRNAs, and the levels of leukemic cell apoptosis were monitored by FACS. If found to be required for MLL, this would identify RUNX2 as a new therapeutic target.

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Background

Leukemia Introduction

The leukemias are a group of related cancers that usually begin in the bone marrow and result in high numbers of abnormal, non-fully differentiated white blood cells. The symptoms may include bleeding and bruising, fatigue, fever, and an increased risk of infections.

There are four main types of leukemia: acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), and chronic myeloid leukemia (CML). The acute leukemias are characterized by a rapid increase in the number of immature blood cells, and are the most common forms of leukemia in children. Immediate treatment is often necessary because of the swift progression and accumulation of the malignant cells, which then can enter the bloodstream and metastasize to other organs. The chronic leukemias occur mostly in older people, and are characterized by a relatively slow buildup of relatively mature yet abnormal white blood cells, taking up to months or even years to develop. Additionally, the leukemias are subdivided according to lineage of affected cells. Lymphoblastic, or lymphocytic leukemia, occurs when the cancerous change takes place in marrow cells that form lymphocytes; myeloid, or myelogenous leukemia, occurs when leukemia develops in marrow cells that mature into red blood cells, platelets, etc.

Treatments of leukemia include chemotherapy, radiation therapy, targeted therapy, and bone marrow transplants. These treatment methods are often combined to maximize their effectiveness. Some chronic leukemias are managed with watchful waiting, or treating the symptoms of leukemia as they arise, in order to expose the patient to the minimum amount of

aggressive therapies as possible. The success of treatment depends on age and the type of leukemia, and success has improved in developed countries (GBD, 2015). The average five-year survival rate of people in the United States is approximately 57%. For children under the age of 15, the five-year survival rate is around 60-85%, and children that are cured are unlikely to relapse (World Cancer Report, 2014).

The causes for most cases of leukemia are unknown; the few known causes discovered to date account for a minority of the cases. The different leukemias likely have different causes, with both inherited and environmental (non-inherited) factors involved (Hutter, 2010). Among adults, the known causes include smoking, natural and artificial ionizing radiation, prior chemotherapy, viruses (such as human T-lymphotropic virus), DNA mutagenizing chemicals (such as benzene and alkylating agents), and Down's syndrome (Stass et al., 2000; Wiernik, 2001). Leukemia can also arise from chromosomal mutations such as inversions (where part of the chromosome is inverted) or translocations (the rearrangement of non-homologous chromosomes). In both cases, the chromosomal change can place two genes adjacent to each other in an abnormal position, causing a change in the protein coded by the genes such as a truncation or a fusion of two proteins (Muntean and Hess, 2012).

Protein MLL

Chromosome translocations that disrupt the mixed lineage leukemia 1 (*MLL1*) gene account for about 10% of all human acute leukemias (Muntean and Hess, 2012). The MLL-1 protein is also known as histone-lysine N-methyltransferase-2A (KMT2A), acute lymphoblastic leukemia-1 (ALL-1), or zinc finger HRX (HRX) protein (Ziemin-van der Poel et al., 1991). This histone methyltransferase associates with chromatin at regulatory elements, and is a positive

regulator of gene transcription. MLL is 3969 amino acids long (Muntean and Hess, 2012), and is a mammalian homolog of the *Drosophila* trithorax group (trxG) proteins involved in segmentation and development (Muntean and Hess, 2012). Like the trxG proteins, MLL regulates the expression of *Hox* genes that regulate embryonic transcription (Muntean and Hess, 2012). The *Hox* genes determine the regional identity of body parts along the anterior-posterior axis of the body during embryonic development (Nakamura et al., 2002). One of the specific aspects of development *Hox* genes are responsible for is normal hematopoiesis (Argiropoulos and Humphries, 2007). The exact mechanism by which *Hox* genes regulate hematopoiesis is unclear, but mis-regulation studies have shown that either up- or down-regulating *Hox* genes can lead to blocking differentiation and promoting leukemogenesis. As MLL is one of the regulators of these *Hox* genes, a mutation in MLL that impedes this regulation can cause leukemia (Argiropoulos and Humphries, 2007).

Post-translationally, MLL protein is cleaved into two proteins: the N-terminal domain, and a shorter C-terminal domain, which non-covalently dimerize in the presence of a large protein complex (Wilkinson et al., 2013). MLL regulates gene transcription epigenetically through its methyltransferase activity (Wilkinson et al., 2013). The protein dimer contains several functional domains (**Figure-1**). The AT-hooks of the N-terminal domain (red in the diagram) non-specifically bind the DNA minor groove. The DNA methyltransferase homology region (the CxxC domain) specifically binds un-methylated DNA. The SET domain of the C-terminal protein (blue in the diagram) is responsible for methylating the fourth lysine on Histone H3, which activates the transcription of target genes (Nakamura et al., 2002). In MLL leukemias (discussed in detail below), the binding of MLL to a translocation partner (diagram center, including AF4, AF6, AF9, or ENL) to form a fusion protein results in the loss of the C-terminal

domain of MLL. While the fusion protein likely retains the DNA-binding functions of the N-terminal domain, it must use other conserved sequences in the N-terminal domain and the translocation partner to attempt to bind and activate its targets, thus it mis-regulates its targets or aberrantly targets other genes (Muntean and Hess, 2012).

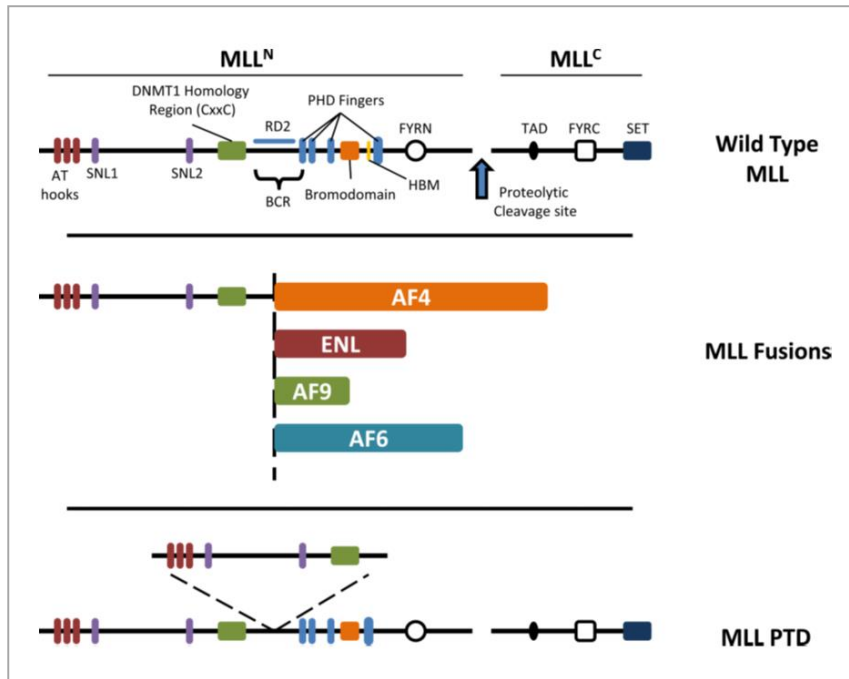


Figure-1: The MLL Protein. Shown are the domains of the WT MLL protein (diagram upper), and the structure of various MLL fusion proteins (diagram center) (Muntean and Hess, 2012).

MLL Leukemogenesis

MLL translocations account for about 10% of all acute leukemias in humans (Muntean and Hess, 2012). MLL has a wide range of translocation partners, including both nuclear and cytoplasmic proteins (Muntean, 2013). Although more than 60 different translocation partners are known, nine predominant partners make up about 90% of MLL translocation leukemias: AF-1, AF-4, AF-6, AF-9, AF-10, AF-17, ENL, ELL, and SEPT-6 (Muntean and Hess, 2012). The distribution of these nine translocation partners in pediatric and adult MLL leukemias is shown

in **Figure-2**. For example, 85% of adult ALL leukemias result from MLL-AF4 translocations, while 35% of adult AML leukemias result from MLL-AF-9 translocations.

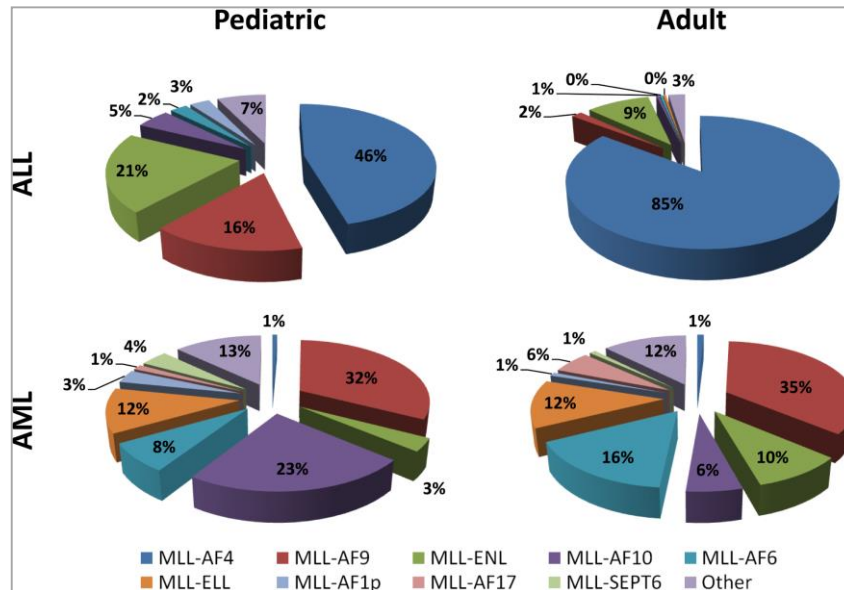


Figure-2: Translocations in MLL-Type Leukemias. Shown are the frequency of translocation partners in pediatric and adult MLL leukemias (Muntean and Hess, 2012).

Patients with MLL have mixed prognoses depending on the translocation partner, but in general, there is a low survival rate and high risk, so aggressive treatments are often used (Muntean and Hess, 2012).

Leukemogenesis is the induction and development of leukemia in bone marrow. MLL fusion proteins can transform hematopoietic stem cells and intermediate progenitor cells into leukemic stem cells (Muntean, 2013). Leukemogenesis does not occur by simply truncating the MLL protein, indicating that the translocation partner is necessary for the disease phenotype (Muntean, 2013). Additionally, *in vivo* experiments have shown that MLL mutations require a latency period before leukemogenesis occurs, suggesting that additional mutations are necessary

for leukemogenesis. This supports the two hit theory that leukemia occurs from a combination of mutations in activated kinase pathways and DNA-binding proteins. In support of this theory, 30% of pediatric MLL cases also involve Ras mutations (Muntean, 2013).

MLL leukemias up-regulate *Hox* genes, especially Cluster-A *Hox* genes. Up-regulation of *HoxA9* by MLL fusion proteins is the most highly correlated gene with poor prognosis for patients. In fact, an *in vivo* study showed that *HoxA9* up-regulation was essential for the transformation of leukemic cells, showing that MLL fusion proteins are “Hox-dependent gain-of-function mutants” (Ayton and Cleary, 2003).

The RUNX Family of Transcription Factors

The Runt-related family of transcription factors (RUNX, including RUNX1, RUNX2 and RUNX3) derive their name, and are homologous to *runt*, a segmentation gene in *Drosophila* (Ito, 2004). The RUNX factors help regulate the differentiation of a variety of tissues, including the regulation of cell fate determination decisions of hematopoietic stem and progenitor cells into mature blood cells. The family is also called core binding factor- α (CBF α). RUNX proteins form heterodimeric complexes with CBF β which confers increased DNA binding and stability to the complexes.

The three *RUNX* genes (**Figure-3**) share a common DNA-binding domain known as the Runt Homology domain (red in the diagram), and their proteins form heterodimers with their cofactor CBF β (Cameron and Neil, 2004). *In vivo* data has shown that CBF β is required for the proper function of all three RUNX proteins. CBF β knockout embryos have null Runx function, and die at mid-gestation (at about day 11.5 after conception) with a lack of normal hematopoiesis (Wang et al., 1996; Cameron et al., 2003). All three RUNX proteins, along with their cofactor

CBFb, regulate development of various types of progenitor cells at key decision points in the cell cycle, regulating the decision between proliferation, differentiation, and cell-death (Cameron and Neil, 2004).

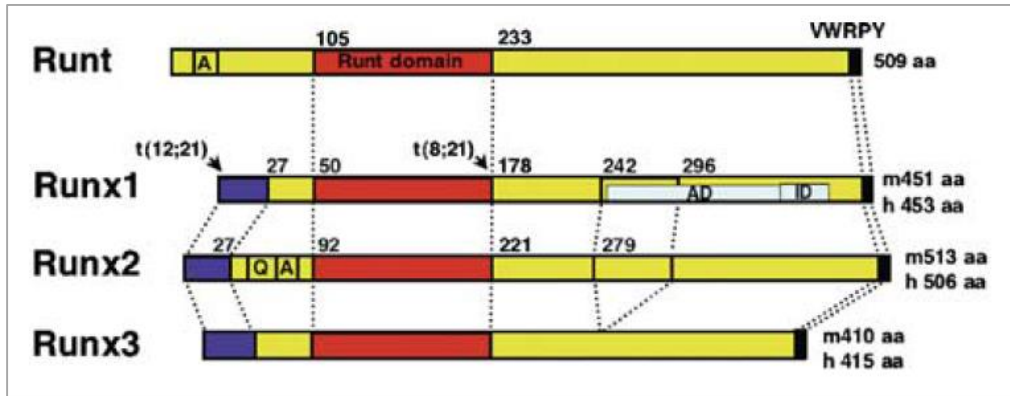


Figure-3: Structure of the Three RUNX Family Transcription Factor Genes. The Runt domain common to all RUNX family members is shown as red in the diagram (Ito, 2004).

Although the three RUNX proteins are very similar, they each have independent functions; knockout mice for any member either die during fetal development or are severely compromised from developmental defects (Cameron et al., 2003). Perhaps the best characterized family member is RUNX1. RUNX1, also known as acute myeloid leukemia-1 protein (AML1) or core-binding factor subunit alpha-2 (CBFA2), is a transcription factor that is required for the development of normal hematopoiesis, and for the differentiation of myeloid progenitors into granulocytes. It is also the most frequent target of chromosome translocations associated with AML (Ito, 2004). RUNX1 was initially shown to have a role in leukemogenesis when it was found to be a component of chromosomal translocation t(8;11), which occurs in acute myeloid leukemia (AML) (Erickson et al., 1992; Nisson et al., 1992; Miyoshi et al., 1993). Since this discovery, as many as ten other translocation fusion partners have been identified for RUNX1,

all resulting in a fusion and deactivation of the RUNX1 C-terminal transactivation domain (Cameron et al., 2003). In all cases, the Runt Homology Domain, that binds DNA and CBF β , is always conserved as part of the leukemia fusion protein.

RUNX2 is required for the normal development of osteoblasts (Ito, 2004). RUNX2 has not yet been shown to be involved in any human cancers, however its deficiency has been linked to the development of cleidocranial dysplasia, a human bone disease (Ito, 2004). RUNX3 is involved in regulation of mature DC8+ T cells and in the development of the gastric epithelium (Cameron et al., 2004).

The Roles of RUNX in Leukemogenesis

RUNX proteins can participate in cancer either as direct fusion proteins or as targets of fusion proteins. And RUNX genes can act both as oncogenes and tumor suppressors (Cameron and Neil, 2004). Tumor suppressor genes protect cells from the induction of cancer, and their functions are typically lost in cancer cells. Oncogenes are dominant genes whose functions promote cancer by activating mutations and transcriptional deregulations (Cameron and Neil, 2004). In some instances, RUNX1 can act as a tumor suppressor; RUNX1 mutations caused by translocations that inactivate the protein have been found in cases of myeloid leukemia (Cameron et al., 2003). On the other hand, in a study of 41 children with acute lymphoblastic leukemia, enhanced RUNX1 levels were found in 43.9% of cases, showing that RUNX1 over-expression might be an important factor in leukemogenesis (Mikhail et al., 2002). In this case, the over-expressed RUNX1 acts as an oncogene. Similarly, it was found that experimental over-expression of wildtype RUNX proteins has an oncogenic effect (Cameron et al., 2003). Cameron et al. showed that RUNX2 can also act as an oncogene in a dose-dependent way. After

amplifying RUNX2 expression using retroviral insertional mutagenesis to activate an enhancer, 7% of mice heterozygous for the transgene developed lymphomas within 12 months, while 64% of homozygous mice developed lymphomas within 7 months (Cameron et al., 2003).

RUNX1 has the dual ability to function as a tumor suppressor and pro-survival factor, and this function is dose-dependent in leukemogenesis (**Figure-4**). Wildtype cells (left side of the diagram) require a certain level of RUNX1 for normal hematopoiesis, and as stated before, over-expression of RUNX1 can have an oncogenic effect on cells (Cameron et al., 2003). When RUNX1 levels are reduced from wildtype levels (right side of the diagram), myeloid differentiation is blocked, leading to leukemia (diagram lower). This illustrates the tumor suppressor role of RUNX1 in cell development and maintenance that is lost with a loss-of-function RUNX1 mutation (Goyama et al., 2013). However, it was found that a certain level of RUNX is necessary for the maintenance of leukemic cells; completely knocking out RUNX1 leads to cell cycle arrest and death (diagram right). The cancer cells' dependency on RUNX1 shows the role of RUNX1 as a pro-survival factor, and identifies it as a potential therapeutic target (Goyama et al., 2013).

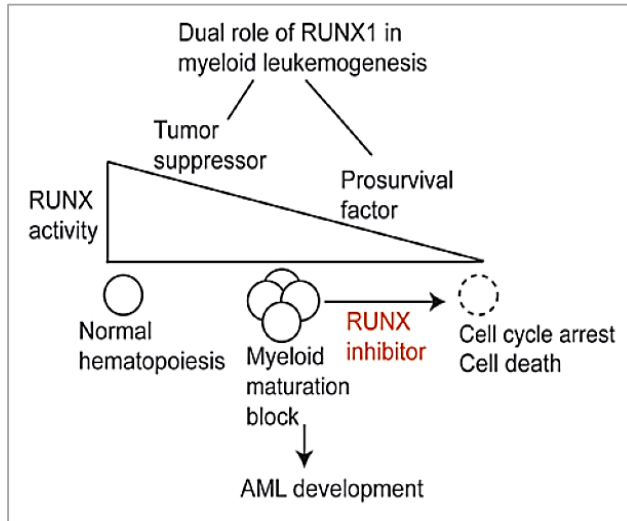


Figure-4: The Dose-Dependent Function of RUNX1 in Leukemogenesis. Shown is the dual role of RUNX1 as a tumor suppressor and pro-survival factor (Goyama et al., 2013).

In MLL-AF4 leukemia (**Figure-5**), RUNX1 is over-expressed and is directly activated by the MLL-AF4 fusion protein (diagram left). RUNX1 then interacts with MLL-AF4 to activate its downstream targets (diagram right) (Wilkinson et al., 2013). Since RUNX1 promotes the survival of MLL-AF4 cells by activating target genes and regulating them, knocking down RUNX1 reduces the survival of these cells (Wilkinson et al., 2013).

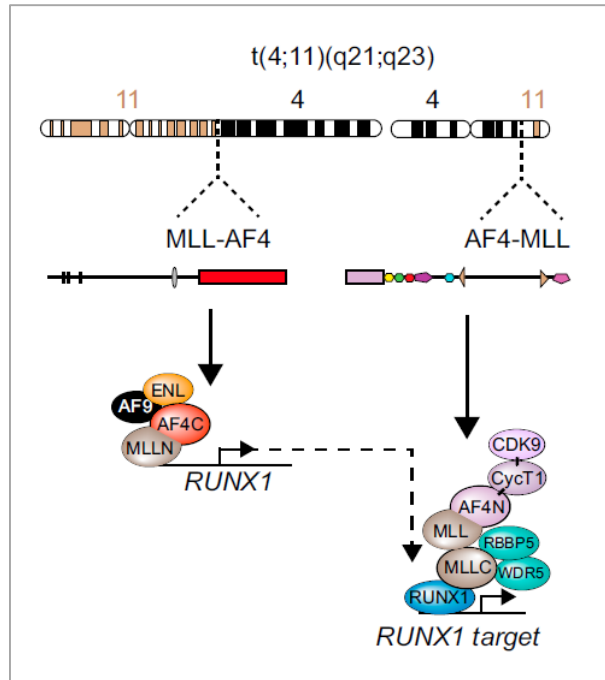


Figure-5: The Role of RUNX1 in MLL-AF4 Leukemia. A chromosome translocation creates the MLL-AF4 fusion protein which activates RUNX1 (diagram left). RUNX1 then interacts with MLL-AF4 and other proteins to activate its downstream targets (diagram right) (Wilkinson et al., 2013).

Similarly, RUNX2 is a target of MLL-AF4 and AF9 fusion proteins, and is upregulated by binding the fusion protein to a proximal promoter of RUNX2 (Guenther et al., 2008; Bernt et al., 2011). If RUNX2 acts in a similar manner to RUNX1, it could be a potential therapeutic target to treat MLL-AF4 and –AF9 leukemias.

Project Purpose

The RUNX transcription factors are a family of DNA-binding proteins that function in development and hematopoiesis. Mixed Lineage Leukemias (MLL), involving translocations of the protein MLL, are dependent on RUNX1 for oncogenic activity, but it is unclear whether MLLs are also affected by RUNX2. In MLL-AF4 leukemia, RUNX1 is directly activated by the MLL-AF4 fusion protein, and RUNX1 interacts with MLL-AF4 to activate downstream targets (Wilkinson et al., 2013). Since RUNX1 promotes the survival of MLL-AF4 cells by activating target survival genes, inhibiting RUNX1 reduces the survival of these cells (Wilkinson et al., 2013). Similarly, RUNX2 has been shown to be a target of MLL-AF4 and AF9 fusion proteins, and is upregulated by binding of the fusion protein to a proximal promoter of RUNX2 (Guenther et al., 2008; Bernt et al., 2011), but it is not known whether the leukemic cells also depend on RUNX2 for survival. In this project, the overarching hypothesis is that RUNX2 is directly upregulated by fusion protein MLL-AF9 and plays a role in the subsequent block of differentiation and the survival of the AML cells. We have reduced (knocked down) RUNX2 expression in three MLL-derived cell lines using shRNAs, and the level of leukemic cell apoptosis was monitored by FACS for early apoptosis marker Annexin-V. If found to be required for MLL, this would identify RUNX2 as a new therapeutic target to treat MLL-AF4 and -AF9 leukemias.

Methods

Cell Culture

The three leukemic cell lines used for this study were MOLM-13, THP-1, and RS4;11 cells, all were obtained from the ATCC. The MOLM-13 cell line originated from the peripheral blood of a patient with acute myeloid leukemia expressing the MLL-AF9 fusion. THP-1 cells are human monocytes with acute monocytic leukemia expressing MLL-AF9. RS4;11 cells are human lymphoblasts with acute lymphoblastic leukemia expressing the MLL-AF4 fusion. All three cell lines were grown as suspensions in T-25 flasks in complete RPMI medium (Gibco) containing 10% FBS (Clonotect) and 1% Pen-Strep (Gibco). Cultures were incubated at 37°C in 5% CO₂. Cells were sub-cultured every 2-3 days, with the media changed and cells split as needed to maintain a concentration of 0.5-2.0 x 10⁶ cells/mL.

shRNA Sequencing

Two samples of plasmid DNA containing the RUNX2 shRNAs [sh1 (TRCN0000013656) and sh3 (TRCN0000013654)] were obtained from the TRC library of RNAi core (UMMS). One plasmid containing a scrambled control insert (Scr, Plasmid #1864) was obtained from Addgene repository. In these plasmids, the puromycin marker was replaced by green fluorescent protein (GFP) in the Castilla lab for sorting purposes. The sequences of the two RUNX2 and scrambled shRNAs were originally designed and distributed by Dharmacon (Now Thermo Fischer Scientific, Inc.) and sequenced by us before using them in this project to verify their inserts. Samples of all three plasmids were sent to Eton Biosciences to be sequenced. These plasmid constructs were further used for making lentiviral particles. The sequences of sh1 and sh2 are shown below.

Sh1: TRCN0000013656 TAAGACTGGTCATAGGACCAC

Sh3: TRCN0000013654 AATGCGCCCTAAATCACTGAG

Lentivirus Production

HEK293T cells were transfected with the shRNA plasmids and lentiviral packaging plasmids to produce transgenic lentiviruses containing the desired shRNAs. For each of the three plasmids (sh1, sh3, and scrambled control), three plates of 5×10^6 293T cells were transfected as follows: for each plate, 1.8 mL of Opti-MEM (Gibco) and 50 μ L FuGENE transfection reagent (Promega) were added to a DNA mix made of 6 μ g of psPAX lentiviral packaging plasmid, 3 μ g MD2.G envelope-expressing plasmid, and 6 μ g of the plasmid of interest. The mix was incubated for 15 minutes at room temperature, and then was added dropwise to the plate of cells. The plates were incubated overnight, and the medium from each plate was harvested and replaced with fresh complete DMEM medium every morning for the next 3 days. After all the media were harvested, 1 mL of Lentiviral concentrator (Clonetechn) was added for every 3 ml virus supernatant, and it was incubated overnight at 4°C. The next day, the media samples were spun for 45 minutes at 1300 rpm in a Beckman Coulter Allegra 6KR centrifuge and re-suspended in 4 mL fresh DMEM. The virus-containing medium was split into 1 mL aliquots and stored at -80°C.

Transduction of Cell Lines

2×10^6 cells were plated in each well of a 6-well plate. 45 μ L HEPES buffer (Gibco), 1.5 μ L Polybrene, and a 1 mL aliquot of the virus containing the shRNA of interest were added to each well, and RPMI medium was added to bring the total volume of the well to 3 mL. Each

plate was spun for 90 minutes at 2500 rpm at 30°C, and then incubated for 5 hours at 37°C. After 5 hours, the medium of each well was replaced with fresh RPMI medium.

Sorting of Transduced Cells

Cells were sorted by FACS at 48 hours post-transduction. They were spun for 5 minutes at 1300 rpm and resuspended in 500 μ L sterile FACS buffer (sterile PBS with 0.1% BSA) per 1×10^6 cells. Cells were filtered through a 37 micron filter mesh and kept on ice until sorting. 7-AAD fluorophore, a dead cell marker, was added to cells at a concentration of 7 μ L 7-AAD per 500 μ L FACS buffer. Cells were sorted on a “BD FACSAria” FACS machine. Cells that were GFP positive (containing shRNA plasmid) and 7-AAD negative (viable) were selected and collected in PBS solution. Sorted cells were seeded in 6-well plates at a concentration of 2×10^6 cells per well, and fresh RPMI medium was added to bring the well volume to 3 mL. Cells were kept in the 37°C incubator and the medium was changed every 2 days.

Dead Cell Removal

Dead cells were removed from the cultures of sorted cells 24 hours after sorting using the Miltenyi Biotec Dead Cell Removal Kit following the manufacturer’s procedure.

Annexin-V Assay

After cell sorting, approximately 1×10^6 cells were cultured in 6-well plates. Cells were analyzed by FACS every 48 hours starting 24 hours after sorting (72 hours after transduction). Levels of GFP and an early-apoptosis marker, Annexin-V, were analyzed to monitor levels of apoptosis in cells transduced with the RUNX2 shRNAs until nine days after transduction. Cells were analyzed by FACS as discussed above. Samples containing approximately 10^5 cells were

taken for cultures transduced with sh1, sh3, or the scrambled control, and non-transduced (NT) cells as a negative control. Medium was removed from each sample, and they were washed twice with 1 mL cold PBS. Cells were resuspended in 100 μ L of 1X binding buffer and stained for 15 minutes with 5 μ L of PE-Annexin V and 5 μ L of 7-AAD. After 15 minutes, cells were spun for 5 minutes at 1300 rpm and resuspended in 200 μ L fresh binding buffer. An Unstained control was made with a sample of NT cells to which no PE-Annexin V or 7-AAD was added. Single-stained compensation controls were prepared by taking a sample of sh1-transduced cells that remained unstained (to control for GFP), and taking two samples of NT cells and staining with 5 μ L of either PE-Annexin V or 7-AAD. Cells were kept on ice after staining until analyzed on the FACS machine (BD LSRII).

For FACS analysis, the unstained sample was run first to identify the cell population by forward (FSC-A) and side scatter (SSC-A) and to adjust the voltage levels as needed. Next, the three single control samples were run to determine the level of spectral overlap between the three fluorophores and to adjust the compensation for these overlaps. Finally, each of the four stained samples were run and the fluorescence was recorded.

The FACS results were analyzed by first gating for live cells on a plot of forward scatter vs side scatter. Single cells were gated on a plot of FSC-A vs FSC-H. A gate for GFP positive cells was created on a plot of FITC-A vs FSC-A, where FITC-A is the channel on which GFP fluoresces. Lastly, a plot of Per-C-P5.5 vs PE-A was used to calculating the percentage of cells in early apoptosis by calculating the population of PE-Annexin V positive and 7-AAD negative cells.

Western Blot

After cell sorting, approximately 1×10^6 cells for each group (NT, scrambled, sh1, and sh3) of RS4;11 and THP-1 cell lines were lysed in 100 μL RIPA lysis buffer. The concentration of total protein in each lysate was assayed using a Bradford assay. Approximately 20 μg of protein was added to 5 μL of 5X SDS-PAGE dye, and samples were heated for 15 minutes at 95°C . Samples were loaded onto a 10% PAGE gel, with 10 μL of a prestained protein marker (Broad Spectrum, Life Technologies) loaded alongside the samples. The gel was run at 60 V until samples were about halfway down the gel, at which point the voltage was increased to 100 V, and the electrophoresis was continued until the marker dye reached the bottom of the gel. The protein was transferred from the gel to a PVDF membrane by trans-blotting at 100 V for 90 minutes in 1X transfer buffer at 4°C . The blot was then blocked for 1 hour with 0.5% milk protein in 0.1% TbsT, and then probed for RUNX2, RUNX1, or ERK (as a loading control), using 10 μL of each primary antibody purchased from Cell Signaling Technologies and 0.2 μL of anti-rabbit IgG secondary antibody. Blots were processed and developed on an X-ray film using enhanced chemiluminescent solutions (Millipore).

Results

To test the hypothesis that knocking down RUNX2 will increase the levels of apoptosis in MLL leukemic cells, RUNX2 was knocked down via shRNAs in one MLL-AF4 and two MLL-AF9 cell lines. The approach used was to: 1) sequence the shRNA RUNX2 plasmids to verify the inserts, 2) determine the level of RUNX2 knockdown using western blots, and 3) analyze the cell lines by FACS for changes in apoptosis and cell death.

shRNA Sequencing

First, the two shRNA-containing plasmids used for RUNX2 knockdown (sh1 and sh3) were obtained from Emmanuel Bikorimana, a postdoc in the Castilla laboratory, and were then sent to Eton Biosciences. The results of the sequencing are shown in **Figure-6**.

```
sh1: TAA GAC TGG TCA TAG GAC CAC
NNGATTTCGATTTCTTGGCTTTATATATCTTGTGGAAGGACGAAACACCGG GTGGTCCTATGACCAGTCTTACTCGA
GTAAGACTGCCCATAGGACCAC CCGGTGTTTCGCCCTTCAACAAGATTTATAAAGCCACTAAATCGATATACTTTC
ATGTTCC CGTTAACCTATGATAGTCATTTTTCTCTCATAGATTTCTTCA

sh3: AAT GCG CCC TAA ATC ACT GAG
AGGATTTTCGATTTCTTGGCTTTATATATCTTGTGGAAGGACGAAACACCGG CTAGTGATTTAGGGCGCATT CTCA
AG AATGACCACCAAATCTAAGTG TTGCCGTTTCAGAGTTTTATTGATACTGAAGTTACTTTTCGTAATATGCAGATG
TGGACTT TTAACCTCTAGGCAATTTTTCTCTGAAGATTCACC
```

Figure-6: Sequence of RUNX2 shRNA Inserts for Plasmids sh1 and sh3. Shown are the desired sequences of sh1 and sh3 (blue), and the results of the sequencing experiment of the plasmids containing each insert (black). The forward shRNA sequence is shown in yellow, and the reverse complement (to make the hairpin) is shown in green.

Since shRNAs have a hairpin turn to be functional, they contain a palindrome repeat, so there should be a forward shRNA sequence and its reverse complement. The sequences of the forward sequence are shown in yellow, and the reverse complement strands of the shRNAs are

highlighted in green. As can be seen, both of the sequences highlighted in green are the exact reverse complements of the forward shRNA sequences. The forward sequence for sh1 almost exactly matches the known desired sequence of sh1 (blue in the diagram). Since both the forward and reverse complementary sequences are almost exact matches of the known sequence, this can be considered a reasonably positive identification result, indicating that the plasmid thought to contain sh1 indeed contains the sh1 insert. The forward (yellow) sequence for sh3 is not an exact match for the desired sequence, but over half of the nucleotides match. Since the reverse complement was an exact match of the known sequence, this can also be considered a reasonably positive identification of the sh3 insert. It is possible that the two insert sequences did not exactly match the known desired sh1 and sh3 sequences due to mutations that incurred in our lab during cloning and amplification of the plasmids, but we deem this unlikely because the plasmid sequences originally obtained in our lab matched perfectly the desired sequence. We deem it more likely that the rapid sequencing done here was not entirely accurate, especially as it had to be done multiple times to get a relatively clean read. In any case, the sequences were considered close enough to warrant continuing ahead with the knockdown experiments, since the purpose of the sequencing was merely to identify the shRNA plasmids as not being the scrambled control.

Quantification of RUNX2 Knockdown

Three MLL-fusion cell lines (THP-1, MOLM-13, and RS4;11) were transduced with sh1, sh3, a scrambled control, or the pLKO vector control as described in Methods. After sorting for transduced cells on Day-2 by GFP FACS and removing dead cells using a dead cell removal kit on Day-3, approximately 1×10^6 cells per condition were obtained. These cells were lysed in RIPA buffer, and total cellular protein was analyzed on a Western blot to assay the level of

RUNX2 protein knockdown induced by the shRNAs. 1×10^6 cells of each non-transduced cell line were also analyzed as a control to show the levels of WT RUNX2 expression. The resulting blots are shown in **Figure-7** for the RS4;11 and THP-1 cell lines; there were not enough MOLM-13 cells recovered from the sorting after setting aside 1×10^6 cells for FACS analysis to run a blot.

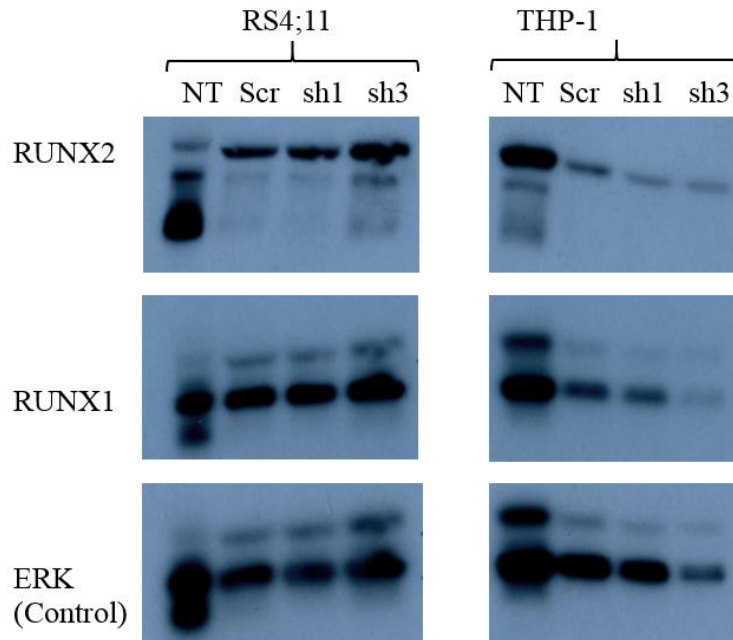


Figure-7: Western Blot Analyses for Transduced Cell Lines to Measure RUNX2 Knockdown. Shown are Western blots for RUNX2, RUNX1, and ERK Control for non-transduced cells (lane NT), and cells transduced with scrambled plasmid (Scr), sh1, and sh3, for RS4;11 cells (left column) and THP-1 cells (right column). Cells transduced with the pLKO vector control were not included on these blots.

The blots were probed for RUNX2 to show levels of knockdown, RUNX1 to see if there was any effect on another RUNX family member, and ERK 1/2 as a loading control to show the relative amounts of protein loaded on the gel; expression of ERK should not change due to RUNX2 knockdown. Unfortunately, the levels of ERK were not the same for each condition in either cell line. So, either ERK was a poor choice of loading control, or there were problems

loading the same amounts of total protein. More protein was loaded for the non-transduced cells than the other conditions, and there appears to be less protein loaded for the sh3 condition for THP-1 cells. Since there were varying amounts of protein loaded between conditions, the amount of information that can be gained from this blot is limited. Due to time constraints, a better blot was not able to be produced. In the future, a blot with the same amounts of total protein load per lane should be done, or a different load marker should be tested.

Aside from the differences in loaded protein, there appears to be a decrease in the level of RUNX2 expression in the sh1 group of THP-1 cells compared to the scrambled control. These two conditions appear to have approximately the same level of ERK, so it is likely that RUNX2 was successfully knocked down by sh1. Sh3 also appears to be knocked down compared to the scrambled control in THP-1 cells, but there was a much lower level of ERK for sh3, so this apparent knockdown might be just a lower level of protein loaded. Similarly, there is no apparent change in levels of RUNX2 in RS4;11 cells, or of RUNX1 in either cell line, except for the NT lane showing some degradation of RUNX2 for an unknown reason, but the varying levels of ERK show that these results might be faulty. While the results of this blot are difficult to interpret, they provide preliminary evidence that sh1 knocked down RUNX2 in the THP-1 cells. Since the loading of the blot was faulty, quantification of the knockdown levels cannot be determined.

GFP and Annexin-V FACS Assays

Three leukemic cell lines (THP-1, MOLM-13, and RS4;11) were transduced with sh1, sh3, the scrambled control, or the pLKO vector control as described in Methods. After sorting for transduced cells via GFP and removing dead cells, approximately 1×10^6 cells per condition per

cell line were obtained. These cells were placed in culture, and starting on Day-3 (72 hours after transduction), cells were assayed for levels of apoptosis using FACS for Annexin-V every 48 hours until Day-9. The experiment was originally performed without sorting for GFP-positive cells on Day-2, but it was impossible to tell from the initial set of data whether the apoptosis levels resulted from RUNX2 knockdown. So, the GFP sort was introduced to remove the background from cells that were not successfully transduced.

The experiment was repeated using the GFP pre-sorting procedure for transduced cells. Time did not allow the sorted experiment to be done in triplicate, so only data from N=1 is reported here for the three cell lines. First, the percentage of GFP-positive (GFP+) cells was analyzed by FACS (**Figure-8**). The Y-axis represents GFP (Comp-FITC-A, the fluorophore associated with GFP), and the X-axis represents FSC-A (the amount of light scattered by the cell, which corresponds to cell size). The population of GFP+ cells was gated to exclude most, if not all, of the cells in the non-transduced condition. The THP-1 cells show approximately 91.1% transduction after sorting.

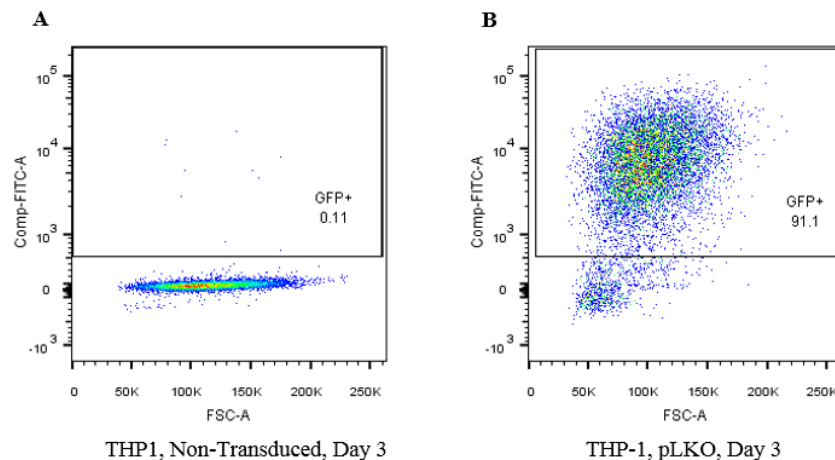


Figure-8: Analysis of Percent Transduced Cells. Non-transduced THP-1 cells (left panel) and cells transduced with plasmid pLKO (right panel) were analyzed on Day-3 by FACS for GFP (Comp-FITC fluorophore associated with GFP) (Y-axis), and for FSC-A (light scattering, X-axis, corresponding to cell size).

Graphs of GFP (Comp-FITC-A) vs. light scatter (FSC-A) for all conditions of all three cell lines on Days 3, 5, 7, and 9 were gated for GFP in the manner described above, and the percentage of cells defined as GFP+ were recorded (**Figure-9**).

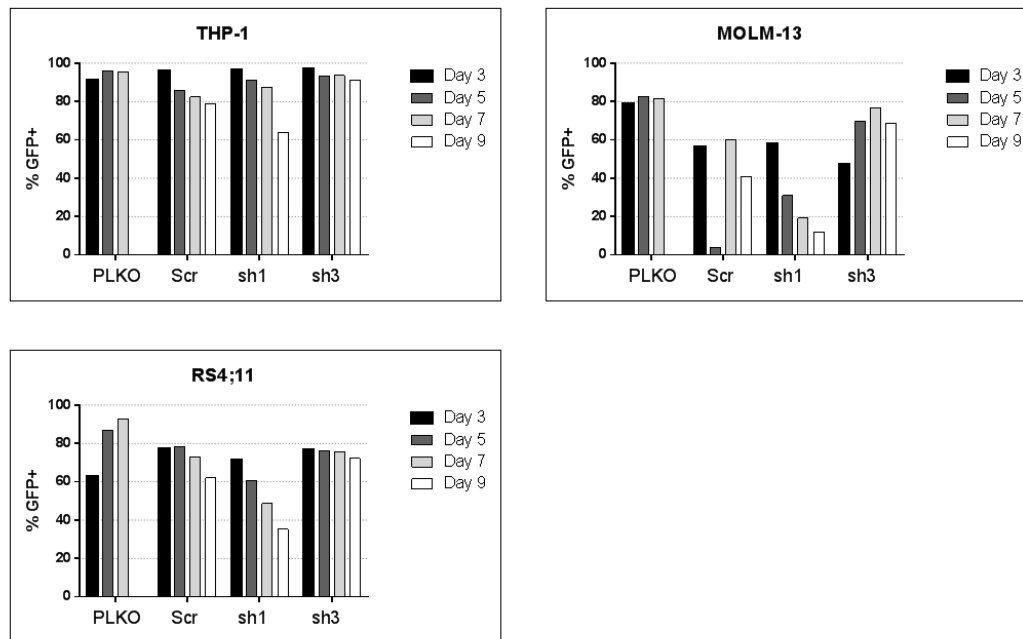


Figure-9: Percentage of GFP+ Cells for Each Cell Line. Shown is the GFP+ FACS analysis of three leukemic cell lines (THP-1, MOLM-13, and RS4;11) measured on Days 3, 5, 7, and 9 post-transduction with the plasmid listed on the X-axis. Note: The Day-9 data for the pLKO control group for each cell line were not able to be collected, so were omitted from these graphs. Each histogram represents N=1.

As can be seen in the graphs, all three cell lines showed a decrease in GFP+ cells in the sh1 group: THP-1 cells showed a 33% decrease in GFP+ cells by Day-9 (compared to the levels of GFP+ cells in the sh1 group on Day-3), MOLM-13 cells showed a 46% decrease, and RS4;11 cells showed a 36% decrease. There was no significant change in the percentage of GFP+ cells in the other groups in any cell line. The scrambled control group for MOLM-13 cells shows a decrease in GFP+ cells on Day 5 and 9, but since not many viable cells were recovered in this

group, these decreases may represent error than an actual loss of GFP+ cells. Additionally, there was no significant decrease in % GFP+ cells in the sh3 groups of any of the cell lines, indicating that sh3 was likely not as effective at knocking down RUNX2 levels, as was suggested by the Western blot data described above. Assuming the percent GFP+ cells is high in strongly viable cells, the GFP+ FACS data also allow indirect conclusions to be made about cell viability. In each cell line, the cells transduced with sh1 show a greater decrease in % GFP+ cells than either of the control groups. Since the GFP+ cells represent the cells that were successfully transduced and express the shRNA, this decrease in % GFP+ cells shows that when RUNX2 is knocked down in cells, they are less viable than cells without RUNX2 knockdown. This supports the hypothesis that decreasing levels of RUNX2 in MLL cells results in cell death.

Next, the levels of cells in apoptosis were analyzed by FACS after staining with Annexin-V, an early apoptosis marker, and 7-AAD, a dead cell marker. The percentage of cells that were Annexin-V positive and 7-AAD negative (in apoptosis but not dead yet) were recorded for each condition on Days 3, 5, 7, and 9 (**Figure-10**). The FACS data shows Comp-PerCP-Cy5-5-A (the fluorophore associated with 7-AAD) on the y-axis, and Comp-PE-A (the fluorophore associated with Annexin-V) on the x-axis. Gates were set for both axes based on the fluorescence for non-transduced cells not stained with either marker, so most cells should lie underneath each gate. These gates were maintained for all subsequent experiments. As shown in the right panel, most of the leukemic cells in the scrambled control group appeared in the Annexin+, 7-AAD- quadrant, indicating that they were in early apoptosis, as expected.

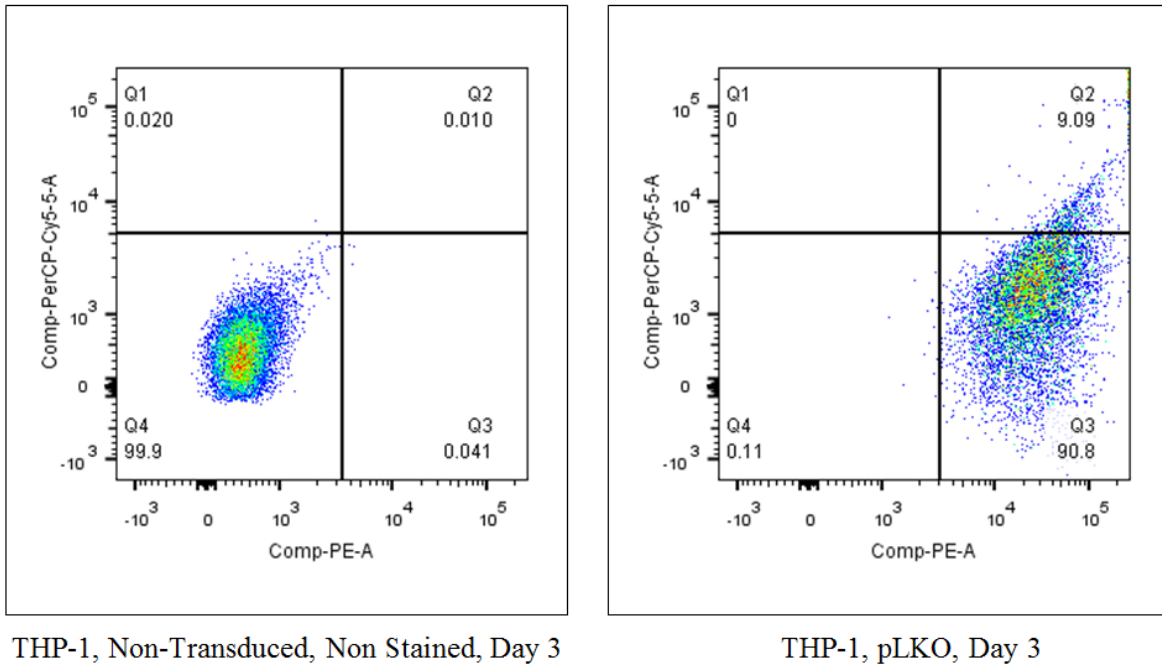


Figure-10: FACS Analysis of Annexin-V and 7-AAD. Shown are non-transduced THP-1 cells (left panel) and cells transduced with control plasmid pLKO (right panel) on Day-3. The Y-axis represents Comp-PerCP-Cy5-5-A (the fluorophore associated with 7-AAD, a cell death stain), and the X-axis denotes the fluorescence of Comp-PE-A (the fluorophore associated with Annexin-V, early apoptosis marker).

Graphs of Comp-PerCP-Cy5-5-A (7-AAD) vs. Comp-PE-A (Annexin-V) for all conditions in 3 cell lines were analyzed on Days 3, 5, 7, and 9, using the gates described above. The percentage of cells defined as Annexin-V+ and 7-AAD- (early apoptosis) were determined (**Figure-11**) (data from the final experiment done). As can be seen in the figure, there was no significant change in the percentage of Annexin-V+, 7-AAD- cells in any condition of any of the cell lines, suggesting that none of the RUNX2 shRNA plasmids affected apoptosis. The only notable change was that the sh1 group of THP-1 cells shows a 14% decrease in Annexin-V+, 7-AAD- cells on Day-9 compared to Day-3. Also, it is important to note that the control plasmid pLKO group of RS4;11 cells shows much lower levels of Annexin-V+, 7-AAD- cells than the other conditions, suggesting the empty vector itself may increase apoptosis as well as the sh

RNAs. However, very few viable cells were recovered from this sorting, so the data cannot be considered highly accurate.

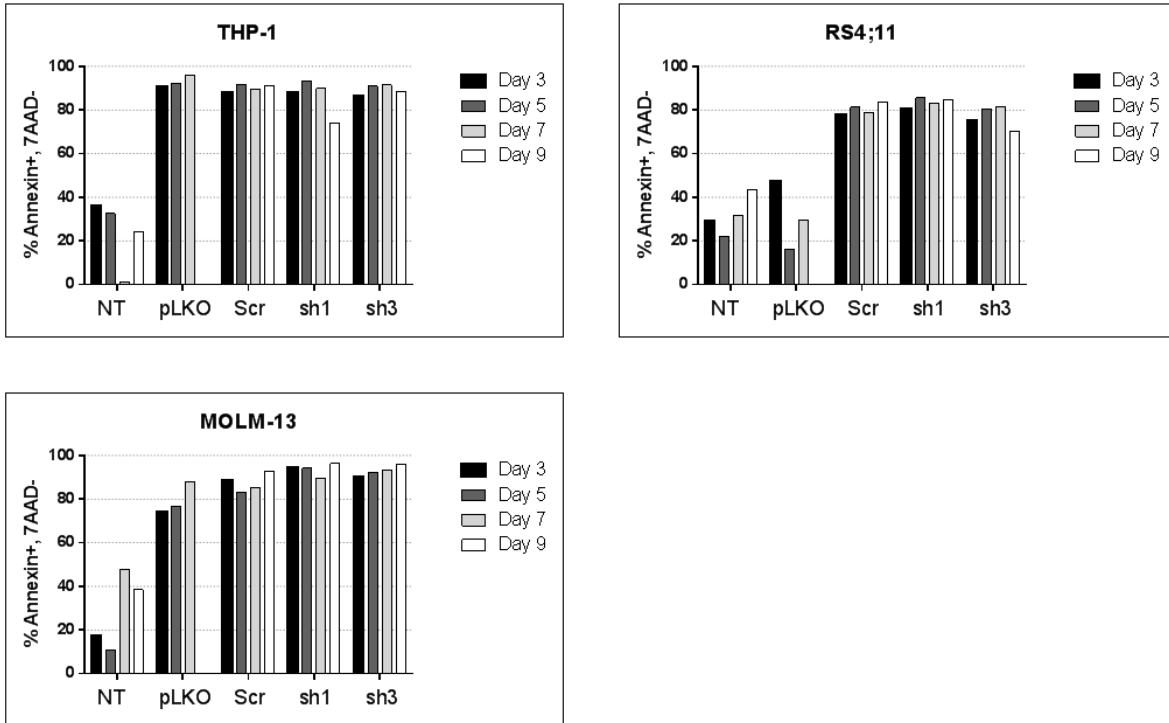


Figure-11: Percentage of Annexin-V-Positive and 7-AAD-Negative Cells in Early Apoptosis for Three Leukemic Cell Lines. Shown are the FACS percentages of Annexin-V+, 7-AAD- cells for each leukemic cell line (THP-1, MOLM-13, and RS4;11) measured on Days 3, 5, 7, and 9 post-transduction. Note: The Day-9 data points for the pLKO group for each cell line were not able to be collected and so were omitted from these graphs

The data analyzed from this part of the experiment does not support the hypothesis that knocking down RUNX2 causes an increase in cell apoptosis. None of the sh1 or sh3 groups of any of the cell lines showed a significant increase in the percentage of cells that bound Annexin-V, a marker of early apoptosis, compared to cells transduced with scrambled plasmid. It is interesting to note that although there was no apparent change in apoptosis levels, all conditions except the non-transduced cells showed very high levels of apoptosis from Day-3. The non-

transduced cells started between 20-40% Annexin-V+, 7-AAD- cells on Day-3, while the other groups started at 80-90% of Annexin-V+, 7-AAD- cells. These high levels of apoptosis from the start of analysis leaves little room for any further increases in apoptosis. It was expected that the control groups transduced with pLKO plasmid would show similar levels of apoptosis to the non-transduced cells, but the pLKO groups show much higher levels, indicating the control pLKO vector or the transduction process itself may cause cell toxicity.

Conclusions and Future Experiments

Leukemia is a devastating disease, and the search for better treatments is imperative. It has previously been found that while under-expression of RUNX1 contributes to leukemogenesis, those leukemic cells depend on the existing, yet low, level of RUNX1 for survival, and inhibiting RUNX1 entirely can cause cell cycle arrest and cell death (Goyama et al., 2013). RUNX1 functions in this manner in certain types of MLL leukemia, and is actually a target of the MLL fusion protein in MLL-AF4 (Wilkinson et al., 2013). This project aimed to determine whether RUNX2, a target of MLL-AF4 and -AF9, also functions as a pro-survival factor for the leukemic cells; if so, it could be a potential therapeutic target.

Two RUNX2 shRNAs (sh1 and sh3) cloned into pLKO plasmids were used to attempt to knock down RUNX2 in three MLL leukemic cell lines. The results showed that cells transduced with sh1 likely knocked down for RUNX2, and die more rapidly in culture than cells transduced with the scrambled control shRNA or empty pLKO vector. However, analyzing the levels of apoptosis markers expressed by the cells showed equally high levels of apoptosis in all groups, both control and experimental, suggesting that the pLKO vector or the method of transduction is inducing apoptosis and killing the cells. This was not the expected result, and neither supports nor refutes the hypothesis, since the effect of RUNX2 knockdown on apoptosis could not be determined.

There are several ways in which this experiment could be optimized or changed to better test the hypothesis. First, since even the vector control showed high levels of apoptosis, a different plasmid vector could be used to transduce cells with the shRNAs. There are a multitude of different commercially available expression cloning vectors to use other than the pLKO

plasmid used here. In addition, the cells could be tested with the transduction procedure without any plasmid to determine whether the procedure itself significantly increases apoptosis.

Another way to solve the vector control problem would be to use a different method entirely of knocking down RUNX2. For example, instead of using a shRNA, an inhibitor could be used to treat cells to inhibit the expression or activity of RUNX2. When using an inhibitor, the time it takes for the compound to inhibit the target protein, the length of time of inhibition, and the half-life of the compound must be taken into account, but inhibitors can often be effective in studies such as this. However, there are no commercially available RUNX2 inhibitors at the moment, so until one becomes available another method must be used. Another method such as using CRISPR technology could be used to completely excise the *RUNX2* gene. This could be a more effective, if not more time consuming, way to ensure lower expression of RUNX2 in the experimental groups compared to the controls. Another approach would be to use either CRISPR or shRNAs to knock down RUNX2 levels using an inducible expression system that allows the cells to initially recover from the transduction procedure, and then later initiate the knock down using an inducing compound.

In addition to resolving the problem with the controls, further experiments need to be done to analyze the effects of different levels of knockdown on apoptosis. As is shown in Figure 4 in the Background illustrating the effects of decreasing levels of RUNX expression, lower levels of RUNX in leukemic cells can cause cell death. By inducing varying levels of RUNX2 knockdown in cells, perhaps by using different doses of an inhibitor, it could be determined whether the level of RUNX2 expression is proportional to the amount of cell death or if there is some threshold level of RUNX2 that separates whether cells survive or apoptose. This knowledge could inform future studies of RUNX2 inhibitors, if RUNX2 is indeed a therapeutic

target, of the level of RUNX2 inhibition needed to kill cells. Additionally, *in vivo* studies and studies using primary leukemic cell samples could be done to show the effects of RUNX2 knockdown in a tumor microenvironment closer to that of a patient. These experiments might provide results that lead to better therapies for MLL leukemias.

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