CREATION OF A NOVEL ETV6-CRE REPORTER MOUSE MODEL FOR ETV6-RELATED FUSION PROTEIN ANALYSIS

AND

CHARACTERIZATION OF INK4AB/VAV-CRE TRANSGENIC MICE LINES

by

DANIEL WILLIAM JONES

A Thesis submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

and

The Graduate School of Biomedical Sciences

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Master of Science

Graduate Program in Cell and Developmental Biology

Written under the direction of

Dr. Hatem Sabaawy

And approved by

_____

_____

_____

New Brunswick, New Jersey

October, 2015

ABSTRACTS OF THE THESIS

Creation of a Novel ETV6-Cre Reporter Mouse Model for ETV6-Related Fusion Protein Analysis

By DANIEL WILLIAM JONES

Thesis Director:

Dr. Hatem Sabaawy

The ETV6 gene is one of the most commonly targeted genes for chromosomal translocation in leukemia. ETV6 has been found to play a key role in hematopoiesis and maintenance of embryonic vascular development, yet its role in B-cell precursor acute lymphoblastic leukemia has yet to be fully determined in respect to both gene expression localization and interactions with other proteins. In this study, we focus on the creation of a novel transgenic line of ETV6-mCherry-CreER^{T2} mice that can be used both as a reporter for ETV6 expression and to drive ETV6-specific expression of Cre-ER^{T2} in mice with ETV6-related fusions using recombineering strategies.

ABSTRACTS OF THE THESIS

Characterization of Ink4ab/ Vav-Cre Transgenic Mice Lines

By DANIEL WILLIAM JONES

Thesis Director:

Dr. Hatem Sabaawy

The Ink4ab genetic locus encodes for three essential proteins involved in tumor suppression, designated p15, p16, and p19 in mice. Full knockout models of this locus have been shown to exhibit accelerated tumorigenesis in multiple forms of cancer (Krimpenfort et al., 2007). By combining the Vav promoter, a promoter that first targets fetal liver hematopoietic stem cells, with a Cre-Lox system to allow full knock out of Ink4ab in only hematopoietic stem cells, we theorize the possibility of creating a hematopoietic system-based malignancy.

Dissections have shown that Ink4ab$^{+/-}$; Vav-Cre+ and Ink4ab$^{-/-}$; Vav-Cre+ mice have splenic hyperplasia, tumor growth in both liver and spleen, and ascites in the peritoneal cavity. Peripheral blood analysis of Ink4ab$^{-/-}$; Vav-Cre+ mice showed the presence of lymphoblasts, abnormal erythrocytes, and apoptotic neutrophils. Immunohistochemical staining has shown that Ink4ab$^{-/-}$; Vav-Cre+ mice have increased apoptosis in T-cells, decreased mature B-cell and T-cell presence, increased cellular proliferation, deregulated cell cycle, and leukemia and lymphoma formation. These studies reveal a novel model for studying hematopoietic cell development, leukemia formation, and lymphoma formation.

ACKNOWLEDGMENT:

I am very grateful to Dr. Hatem Sabaawy, PhD, MD for providing me the opportunity to work in his lab at the Cancer Institute of New Jersey.

I thank Dr. Chong Xu, PhD for his work on and design of the cloning component and for his propagation and maintenance of the Ink4ab; Vav-Cre line used for this project.

I thank Dr. Shamila Yusuff, PhD for her assistance with mouse maintenance, genotyping design, analysis of the ES cell and chimera mice, and western blotting for this project.

I thank my thesis committee members, Dr. Richard Padgett, PhD, Dr. Ping Xie, PhD, and Dr. Hatem Sabaawy, PhD, MD for their input and willingness to participate in my thesis defense.

I thank Dr. Mantu Bhaumik, PhD and the members of the Transgenic/Knockout Mouse core for their work in producing the chimera mice for this project.

I thank Dr. Lei Cong, PhD and the members of the Histology core for their work in producing the immunohistochemical stains for this project.

I also thank the members of the Sabaawy Lab (Eric Huselid, BS, Katie Flaherty, MS, Dr. Mohammad Shawky, MD, and Dr. Monica Bartucci, PhD) for their input and assistance at numerous points throughout the project.

Last but not least, I would like to thank my family for all their love, support, and encouragement.

TABLE OF CONTENTS:

PROJECT 2: CHARACTERIZATION OF INK4AB/ VAV-CRE TRANSGENIC MICE LINES

LIST OF TABLES

PROJECT 2: CHARACTERIZATION OF INK4AB/ VAV-CRE TRANSGENIC MICE LINES

LIST OF FIGURES

PROJECT 1: CREATION OF A NOVEL ETV6-CRE REPORTER MOUSE MODEL FOR ETV6-RELATED FUSION PROTEIN ANALYSIS

PROJECT 2: CHARACTERIZATION OF INK4AB/ VAV-CRE TRANSGENIC MICE LINES

PROJECT 1: Creation of a Novel ETV6-Cre Reporter Mouse Model for ETV6-Related Fusion Protein Analysis

CHAPTER 1: INTRODUCTION

1.1 ETV6 Structure and Function:

ETV6, previously referred to as TEL1 or TEL/ABL, is a member of the ETS transcription factor family. There are 29 genes of this family in humans and 28 genes in mice, making this family one of the largest families of transcription factors. An ETS transcription factor family member is identified solely through the presence of ETS domains. The ETS domain is responsible for sequence-specific DNA-binding and protein-protein interactions (De Braekeleer et al., 2012). Since the only conserved domain in ETS transcription factors is the ETS domain, the members of the ETS family can vary greatly in their remaining sequences and structures they encode.

Human ETV6 is a 250 kb, 8 exon gene that plays a crucial role in hematopoiesis and maintenance of embryonic vascular development. It contains two major domains: a helix-loop-helix (HLH) domain and an ETS domain. The HLH domain is encoded by exons 3 and 4 and plays a role in homodimerization. The ETS domain is encoded by exons 6, 7, and 8 and plays a role in sequence-specific DNA binding and protein-protein interactions (De Braekeleer et al., 2012). The ETV6 gene encodes a 452 amino acid protein that has homology at both the 5' and 3' ends to other ETS family members (De Braekeleer et al., 2012).

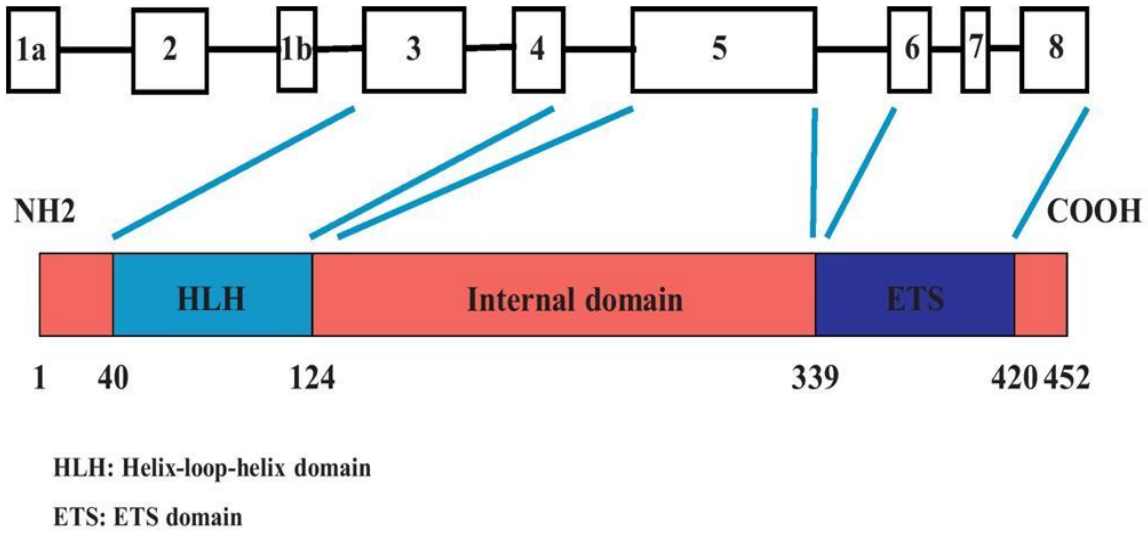


Figure 1: The structure of the ETV6 gene and protein. The above diagram indicates the gene, with the numbers designating the exons. The lower numbers indicates the ETV6 protein structure, with the numbers designating the amino acids (De Braekeleer et al., 2012).

1.2 ETV6 and Leukemia:

One of the most common chromosomal aberrations in human leukemia is at chromosomal band 12p13. ETV6 is located on this chromosomal band at position 13.1. ETV6 is a 250 kb, 8 exon gene that plays a crucial role in hematopoiesis and maintenance of embryonic vascular development. The importance of ETV6 in early development is such that full knockout of the gene is embryonically lethal in mice between day 10.5 and 11.5 of embryonic development. Outside of embryonic development, ETV6 has been shown to be essential for the initiation of hematopoiesis in adult bone marrow. Outside of the bone marrow, ETV6 knockout/knockdown does not impact hematopoietic cells that are committed to a specific lineage (De Braekeleer et al., 2012). There are many fusion proteins that the ETV6 gene can form during leukemia and lymphoma development, but one of the most frequent and least understood ETV6 fusion genes is ETV6-RUNX1, which has an incidence of approximately 25% in all childhood B-cell precursor acute lymphoblastic leukemias (BCP-ALL) (Linka et al., 2013).

1.3 ETV6-RUNX1 Fusion Protein Structure:

The ETV6-RUNX1 fusion protein is comprised of the first 5 exons of the ETV6 gene fused to the full coding region of the RUNX1 gene. This layout overrides the functional binding domain of the ETV6 gene by removing exons 6 through 8 of the ETV6 gene, also known as the ETS domain. This causes the ETV6 gene to bind to both RUNX1 targets and self-bind via homodimerization. However, simply creating ETV6-RUNX1 fusion mice does not cause a leukemic phenotype, and many ETV6-RUNX1 fusion mice models are not successful in producing fusion proteins in a timely fashion (Linka et al., 2013). Moreover, to date there have been no transgenic mice created combining a knockdown of the ETV6 gene with the presence of a Cre protein (Bohlander, 2005).

1.4 Homologous Recombination and Transgenic Knockdown Models:

Homologous recombination is the exchange of a linear DNA construct such that it excises, and thereby overrides, a target chromosomal gene. Gene sequences can be replaced through homologous recombination if the flanking regions of the sequence to be replaced and the construct to be introduced have the same sequence and the bacteriophage lambda-derived *Redαβδ* protein are present. Homologous arms are efficient in recombination down to 40-50 base pair sequences (Muyrers et al., 1999).

The largest benefit of homologous recombination is that no restriction enzymes are necessary for transformation of the construct into the target sequence. This allows for the modification of large target sequences such as Bacterial Artificial Chromosomes without the risk of disrupting sequences outside of the targeted sequence.

1.5 Project Goals:

For this project, we focus on the creation of a transgenic line of ETV6-knockdown mice that can be used for successful ETV6-RUNX1 and other ETV6-related fusion proteins creation via the Cre-Lox system, as well as studying the functions of the ETV6 gene. The construct that we intend to introduce into the mice contains an mCherry-T2A-Cre sequence that will homologously recombine over exons 5 through 6 or exons 5 through 7. The intent is that the mCherry protein will allow for expression tracing and the T2A fusion protein coupled to the Cre protein will allow for a tamoxifen-induced introduction of a RUNX1 protein to the end of the ETV6 protein, creating an ETV6-RUNX1 fusion protein. Other fusion protein examples that can be made from this construct include ABL1, ABL2, JAK2, FGFR3, SYK, and LYN, as shown below (De Braekeleer et al., 2012).

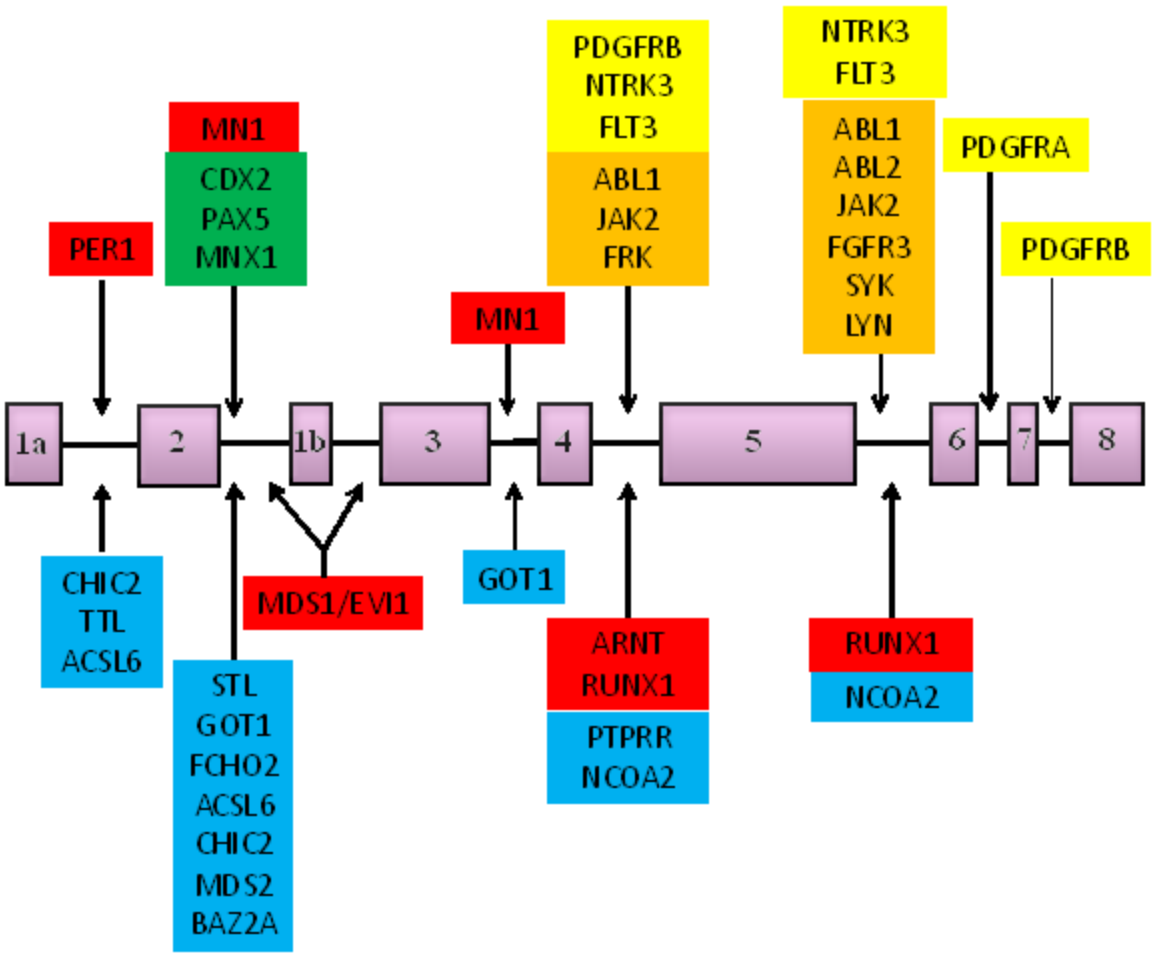


Figure 2: The breakpoints for various fusion proteins on the ETV6 gene. Expression of all the above fusion proteins is viable through this project (De Braekeleer et al., 2012).

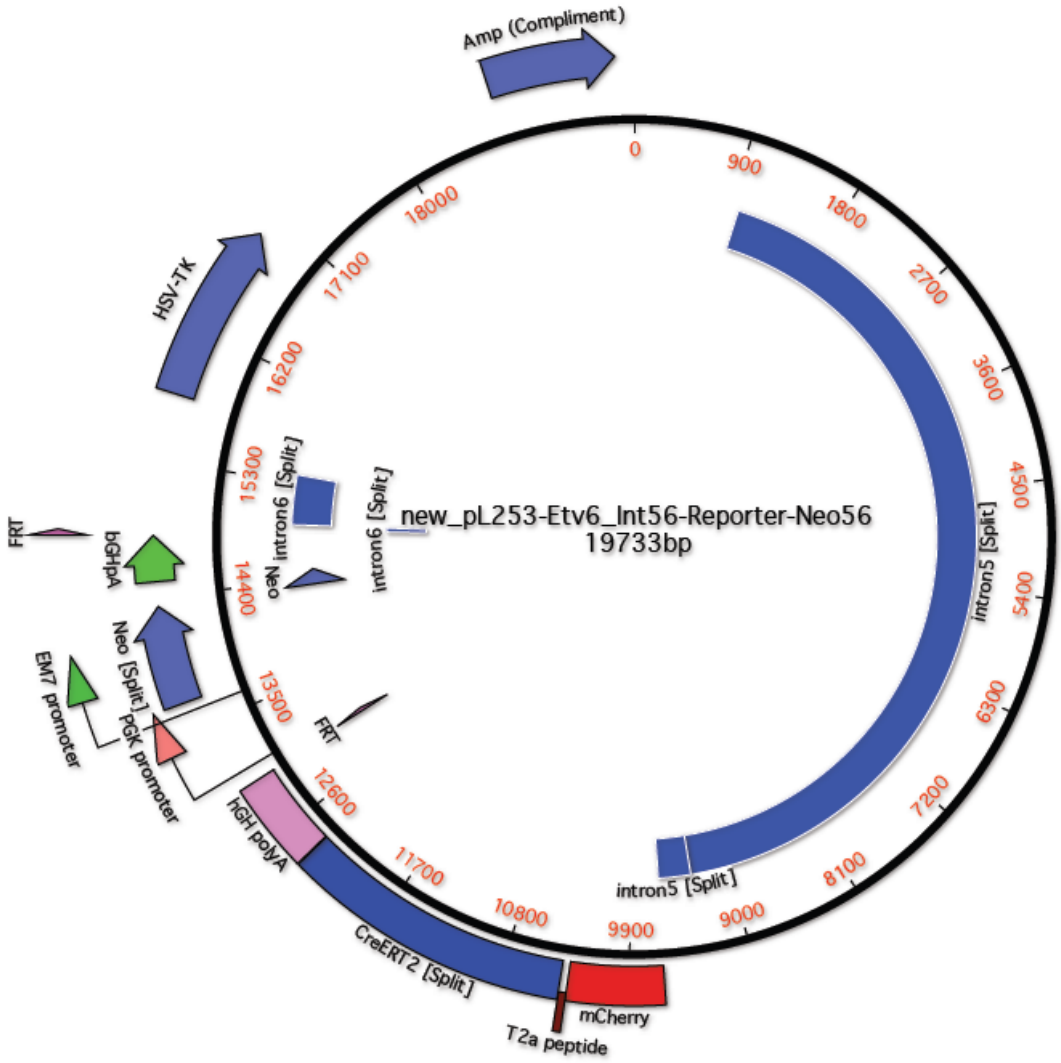


Figure 3: The final construct design for ETV6 knockdown over exons 5 through 6. Homologous regions of introns 5 and 6 flank the mCherry-CreER^{T2}-hGH and FRT-Neomycin-bGh-FRT inserts so that the binding domain of the chromosomal ETV6 gene is overridden.

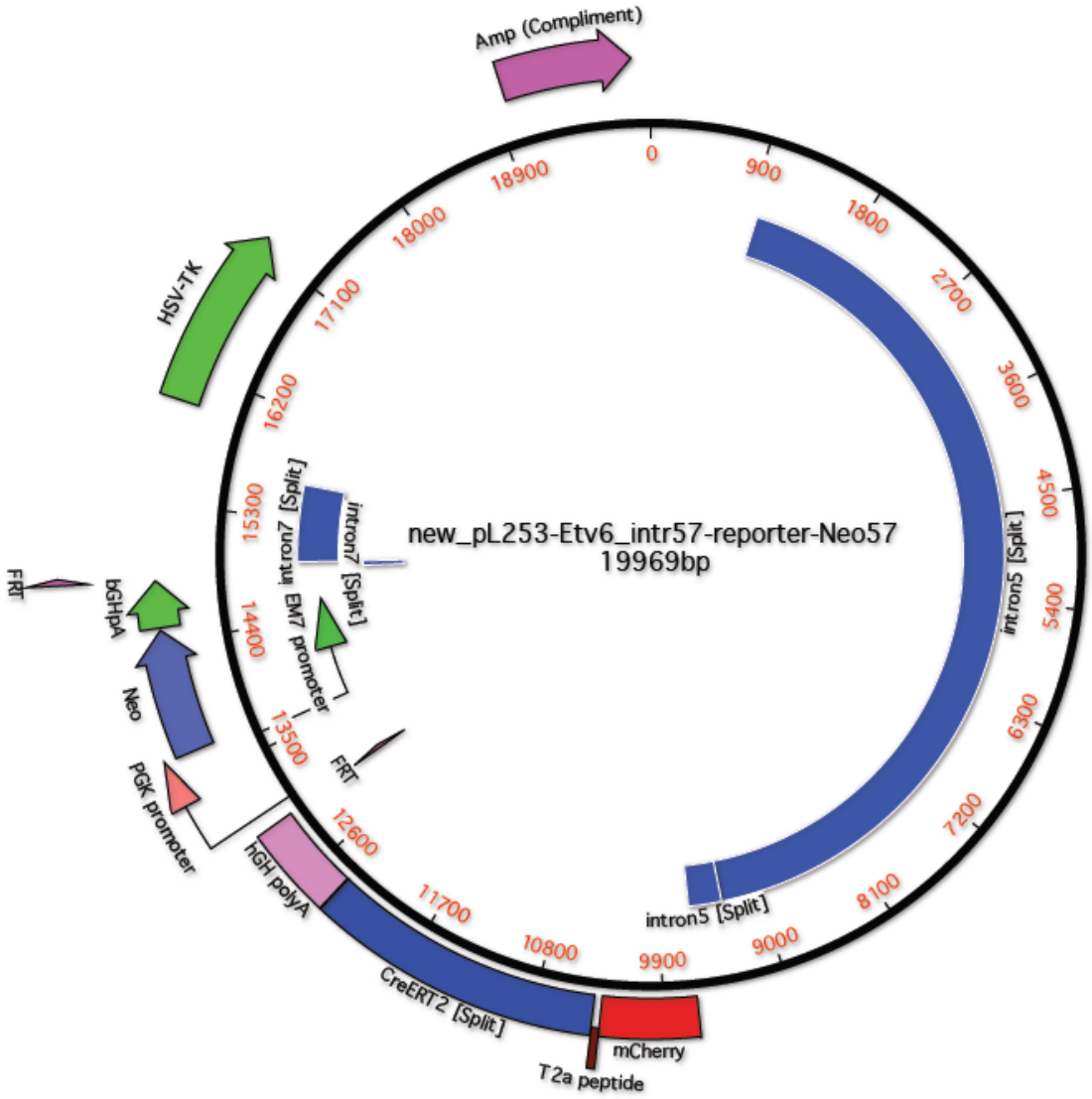


Figure 4: The final construct design for ETV6 knockdown over exons 5 through 7. Homologous regions of introns 5 and 7 flank the mCherry-CreER^{T2}-hGH and FRT-Neomycin-bGh-FRT inserts so that the binding domain of the chromosomal ETV6 gene is overridden.

CHAPTER 2: METHODS

2.1 Creation of glycerol stock of *E. coli* Electrocompetent Competent Cells:

E. coli strains were selected from modified DH10B strains obtained from the Biological Research Branch at NCI at Frederick. Strains were kept on dry ice for the duration of the first-day procedure. Culture tubes were first filled with 5mL of lysogeny broth (LB), using a bunsen burner on the lip of the uncapped LB broth prior to and after use. The bunsen burner was then used to pre-heated a loop, which was used to scratch the top of the frozen bacteria and transfer the bacteria directly into the LB broth. The loop can then be reheated and reused for any remaining samples. Samples were grown on a shaker at 37^{o}C overnight. The initial bacteria stock can then be stored in its respective environment.

The cultured samples were obtained after overnight growth, and new culture tubes were set up and labeled for 1:5 dilution. Each tube was filled with 4mL of fresh LB broth and 1mL of cultured samples of their respective overnight culture. The 1:5 dilution tubes were placed on a shaker at 37^{o}C for between 2-4 hours.

500µL of autoclaved 80% glycerol stock solution and 500µL of 1:5 diluted culture were pipetted into a new cryo tube. The cryo tubes were then flash-frozen in an ethanol-dry ice bath and finally stored in a -80^{o}C freezer until needed for transformation.

2.2 Culturing of *E. coli* Electrocompetent Competent Cells:

The SW106 *E. coli* strain was selected from among the modified DH10B strains obtained from the Biological Research Branch at NCI at Frederick for this procedure. The glycerol stock was kept frozen on dry ice for the duration of this procedure.

After glycerol stock preparation, a preheated loop was used to scratch the frozen SW106 strain and transfer it to a 300mL Erlenmeyer flask containing 50mL of LB broth (with no antibiotics). The culture was grown overnight at 30^{o}C.

2.3 Heat-Shock Activation of SW106 *E. coli* Electrocompetent Competent Cells (Activation of the λ Prophage Redαβδ):

The SW106 *E. coli* strain was chosen for recombination due to its presence of the λ prophage Redαβδ. This defective bacteriophage protein from bacteriophage λ mediates recombination primarily through the use of its endogenous enzyme Exo and endogenous proteins Beta and Gam. The λ prophage is designed to fully express these proteins at 42^{o}C by way of a temperature-sensitive λ cI-repressor. Exo is an exonuclease that degrades the 5' ends of both the insert and the double-strand break on the target vector. λ Beta binds to the open 3' overhang to protect and prepare the homologous regions for strand invasion. λ Gamma inhibits the RecBCD nuclease from degrading the linear insert (Yu et al., 2000).

Using a SW106 culture grown overnight, we performed a 1:5 dilution and grew the samples at 30^{o}C for another 2-3 hours. Samples were then heat shocked by first placing the flasks into a 42^{o}C water bath, with shaking, for 15 minutes. They were then immediately transferred to a wet ice bath and shaken to ensure the temperature drops. The flasks were left in the ice water bath for 10-20 minutes. The culture was then transferred to multiple centrifuge tubes and centrifuged at 5000rpm for 6 minutes at 0^{o}C. The cell pellets were then resuspended in 1mL ice-cold dH_2O times the number of samples that were collected (for example, 9mL for 9

samples). 888µL aliquots were transferred from the centrifuge tubes into individual Eppendorf tubes, which were then centrifuged for 20 seconds at 17,000rpm at 4°C. The supernatant was removed and another 888µL of ice-cold H_2O was added to each Eppendorf tube and centrifuged again for 20 seconds at 17,000rpm at 4°C. After the second supernatant was removed, a third and final 888µL of ice-cold H_2O was added to each Eppendorf tube and centrifuged for 20 seconds at 17,000rpm at 4°C. The supernatant was removed and each pellet was then resuspended in 50µL of 10% glycerol solution. The Eppendorf tubes were then flash frozen using a dry ice and ethanol bath and stored in a -80°C freezer until use.

2.4 Transformation of Bacterial Artificial Chromosomes into SW106 recombinant strains via Electroporation:

Four strains of frozen competent SW106 cells were obtained and allowed to thaw over ice. Three strains of BAC plasmids (F15, B07, and F22) were obtained from Source BioScience. 50µL of competent SW106 cells and 3µL of one of the BAC plasmids were placed into separate electroporation cuvettes. The cuvettes were then chilled on ice for approximately 5 minutes. Electroporation was then run on the cuvettes under the following conditions: 1.75kV, 25µF, pulse controller set at 200 ohms, time constant approximately 4.0.

After electroporation, 1mL of S.O.C. Medium was added to each cuvette. Each solution was then transferred to a separate culture tube and incubated at 30°C for 1 hour. Finally, 200µL of each solution was transferred to a separate chloramphenicol plate, spread with a cell spreader, and grown overnight at 30°C.

2.5 Selection and Growth via Patching of Competent Cell Colonies:

After overnight growth of bacterial culture, a new agar plate containing the selected antibiotic resistance was obtained. Using a pipette tip, a single isolated colony was chosen from the grown plate and lightly scratched on the new agar plate to create a large patch. The new agar plate was then grown at 37^{o}C overnight to obtain a more purified colony selection.

After patching, select colonies from the patched plate were removed via pipette tip and added to individual 5mL of LB broth containing a 1X concentration of antibiotic (Antibiotic concentrations vary based on the chosen treatment. The concentrations for the antibiotics used for this project are as follows: Ampicillin at 100µg/mL, Chloramphenicol at 25µg/mL, and Kanamycin at 50µg/mL). The culture tubes were then grown with shaking overnight at 37^{o}C to prepare for miniprep.

2.6 Miniprep of Select Colonies:

All minipreps were performed using the Fermentas Miniprep kit. After colony selection and overnight growth, the bacteria cultures were harvested via centrifugation at 2,000rpm for 15 minutes at room temperature. All supernatant was then removed, leaving only the cell pellets. The pelleted cells were resuspended in 250µL of resuspension buffer, pipetting to ensure that the cell pellets were fully dissolved. The cell solutions were then each transferred into their own individual microcentrifuge tube. 250µL of Lysis Solution was added to each tube and mixed thoroughly via inversion so that the solution becomes viscous and slightly clear. 350µL of Neutralization Buffer was then added to each tube and inverted immediately 4 to 6 times to mix. The microcentrifuge tubes were then centrifuged for 5 minutes at 17,000rpm to pellet the cell debris and chromosomal DNA. The supernatant was then transferred to a spin column and centrifuged for 1 minute at 17,000rpm. The flow-through was discarded and the column was placed back into the same collection tube. 500µL of the Wash Solution was added to the spin column and the samples were then centrifuged for 1 minute at 17,000rpm. The flow-through was discarded and the column was placed back into the same collection tube. 500µL of the Wash Solution was added to the spin column and the samples were then centrifuged for 1 minute at 17,000rpm. The flow-through was discarded and the column was placed back into the same collection tube. The spin columns were then transferred into a fresh 1.5mL microcentrifuge tube. 50µL of Elution Buffer was added to the center of each of the spin column membranes and allowed to incubate for 2 minutes at room temperature. Each sample was then centrifuged at 17,000rpm for 2 minutes at room temperature. Finally, the column for each sample was discarded and the purified samples were stored at -20^{o}C until use.

2.7 Restriction Enzyme Digestion:

Restriction enzymes were chosen from the Fermentas and New England Biolabs. Using a final solution volume of 40µL, a mix of 4µL of 10X buffer from the respective company, a sufficient quantity of vector for digest (to reach between 50ng/µL and 100ng/µL final concentration), 0.5µL of each restriction enzyme used, and a sufficient quantity of dH_2O needed to bring the final volume up to 40µL was combined in an Eppendorf tube. The mixture was then incubated for approximately 12 hours at 37^{o}C to allow for full digestion to take place, but to avoid star activity.

2.8 Preparation of an Agarose Gel and Gel Electrophoresis:

Agarose gels for this project were made in two sizes requiring different gel apparatuses for each: small and large. For small gels, 0.7g of agarose powder was boiled in 70mL TAE in a 1L glass bottle. For large gels, 1.2g of agarose powder was boiled in 120mL TAE in a 1L glass bottle. The resulting mixture in each case was allowed to briefly cool and 5µL of ethidium bromide was added to the solution and mixed. The solution was then poured into the gel mold with combs to create wells and allowed to cool until solid. The combs were then removed and the gel was oriented so that the electricity will flow down the length of the gel. TAE buffer was added to the gel apparatus such that the gel was fully covered and the samples for analysis were added to their respective wells, along with a well containing 5µL of DNA ladder containing dye.

Gel electrophoresis was then run under the following conditions: 120V, 400mA, 30 minutes. The gel was then analyzed via a UV gel imager and rerun for an extended amount of time and reimaged so that the band(s) in question were sufficiently separated to render a clear result.

2.9 PCR of Select DNA Fragments:

PCR was performed according to various protocols, depending on the amplification needed. These protocols can be broken down as follows:

2.9.1 PCR amplification of intron fragments from mouse Bacterial Artificial Chromosomes :

Mouse Bacterial Artificial Chromosomes containing the ETV6 gene (B07, F15, and F22) were used as the template for the amplification of fragments of introns 5, 6, and 7 from ETV6. The following mixtures were made per BAC template for each of introns 5, 6, and 7: 6µL of dH_2O, 2µL of 10X QIAGEN PCR Buffer, 4µL of 5X QIAGEN Q-Buffer, 1µL of $MgCl_2$, 2µL of 5µM Forward Primer, 2µL of 5µM Reverse Primer, 1µL of dNTP solution, 0.1µL of QIAGEN Taq DNA Polymerase, and 2µL of chosen BAC Template.

The primers used for intron 5 are as follows:

Forward Primer: $^{5'}$ACT CGA ATT CTT CAT TTG CAG GTA CAG C$^{3'}$

Reverse Primer: $^{5'}$GAA CGG TAC CGT CTA CAG AGG AGA GAG G$^{3'}$

The primers used for intron 6 are as follows:

Forward Primer: $^{5'}$ACT CAA GCT TTT CTT TGG TTT CTC CTC ACT GGG$^{3'}$

Reverse Primer: $^{5'}$GAA CGG ATC CGC GTG GTC TAA TCT CCT TAT GTG TC$^{3'}$

The primers used for intron 7 are as follows:

Forward Primer: $^{5'}$ACT CAA GCT TCT TGG TCA ACA ACA GCC ACT TTC$^{3'}$

Reverse Primer: $^{5'}$GAA CGG ATC CCC CCC TAT GCC ATC CTC ATT ATC$^{3'}$

The PCR parameters for this amplification are as follows:

Cycles (Stage 2): 35

Stage One: 95^{o}C for 3 minutes

Stage Two: 95^{o}C for 30 seconds

58^{o}C for 30 seconds

72^{o}C for 2 minutes

Stage Three: 72^{o}C for 5 minutes

4^{o}C until samples are removed from PCR instrument

2.9.2 Amplification of mCherry-CreER^{T2} from cDNA3.1:

The steps of generation of ETV6-mCherry-CreER^{T2} targetting vectors are displayed in figures 5 through 8.

A cDNA3.1 vector previously made in the Sabaawy lab, containing a mCherry-CreER^{T2} construct, was used as the template for the following PCR. The following mixtures were made per sample in a PCR tube: 40.5µL of dH_2O, 5µL of 10X Accuprime Pfx Polymerase Mix, 1.5µL of 5µM Forward Primer, 1.5µL of 5µM Reverse Primer, 1µL of Accuprime Pfx High-fidelity DNA Polymerase, and 0.5µL of cDNA3.1 template.

The primers used for this procedure are as follows:

Forward Primer: $^{5'}$CGA GCA GGT ACC ATG GCC ATC ATC AAG GAG TTC$^{3'}$

Reverse Primer: $^{5'}$CAT CAT CTT AAG TCA AGC TGT GGC AGG GAA ACC$^{3'}$

The PCR parameters for this amplification are as follows:

Cycles (Stage 2): 35

Stage One: 95^{o}C for 3 minutes

Stage Two: 95^{o}C for 30 seconds

58^{o}C for 30 seconds

68^{o}C for 2 minutes and 45 seconds

Stage Three: 68^{o}C for 7 minutes

4^{o}C until samples are removed from PCR instrument

2.9.3 Amplification of Intron 5 Splicing Acceptor from pL253-ETV6 (5-7):

The gap-repaired pL253-ETV6 vector containing introns 5 through 7 of ETV6 was used as the template for the following PCR. The following mixtures were made per sample in a PCR tube: 40.5µL of dH_2O, 5µL of 10X Accuprime Pfx Polymerase Mix, 1.5µL of 5µM Forward Primer, 1.5µL of 5µM Reverse Primer, 1µL of Accuprime Pfx High-fidelity DNA Polymerase, and 0.5µL of pL253-ETV6(5-7) template.

The primers used for this procedure are as follows:

Forward Primer: $^{5'}$ACT CGA ATT CTT CAT TTG CAG GTA CAG C$^{3'}$

Reverse Primer: $^{5'}$GAA CGG TAC CGT CTA CAG AGG AGA GAG G$^{3'}$

The PCR parameters for this amplification are as follows:

Cycles (Stage 2): 35

Stage One: 95^{o}C for 3 minutes

Stage Two: 95^{o}C for 20 seconds

58^{o}C for 20 seconds

68^{o}C for 30 seconds

Stage Three: 68^{o}C for 7 minutes

4^{o}C until samples are removed from PCR instrument

2.9.4 Amplification of Intron 5 Splicing Acceptor + mCherry-CreER^{T2} + hGh from p3xFlag-CMV14 + Reporter Cassette:

A p3xFlag-CMV14 vector containing Intron 5 Splicing Acceptor, mCherry-CreER^{T2}, and hGh sequences was used as the template for the following PCR. The following mixtures were made per sample in a PCR tube: 40.5µL of dH_2O, 5µL of 10X Accuprime Pfx Polymerase Mix, 1.5µL of 5µM Forward Primer, 1.5µL of 5µM Reverse Primer, 1µL of Accuprime Pfx High-fidelity DNA Polymerase, and 0.5µL of p3xFlag-CMV14 + Reporter Cassette template.

The primers used for this procedure are as follows:

Forward Primer: $^{5'}$ACT CGA ATT CTT CAT TTG CAG GTA CAG C$^{3'}$

Reverse Primer: $^{5'}$GAA CCA TAT GAC AAT TCA ACA GGC ATC TAC TG$^{3'}$

The PCR parameters for this amplification are as follows:

Cycles (Stage 2): 35

Stage One: 95^{o}C for 3 minutes

Stage Two: 95^{o}C for 30 seconds

58^{o}C for 30 seconds

68^{o}C for 4 minutes

Stage Three: 68^{o}C for 7 minutes

4^{o}C until samples are removed from PCR instrument

2.9.5 Amplification of Neo Cassette from pL451:

A pL451 vector containing a Neo sequence flanked by two FRT sequences was used as the template for the following PCR. Depending on the end intron, two sequence ranges were used: introns 5 through 6 (5-6) and introns 5 through 7 (5-7). The following mixtures were made per sample in a PCR tube: 17.5µL of dH_2O, 25µL of 2X KOD Xtreme Hot Start DNA Polymerase Buffer, 0.5µL of 5µM Forward Primer, 0.5µL of 5µM Reverse Primer, 1µL of KOD Xtreme Hot Start DNA Polymerase, and 1.5µL of pL451 template.

The primers used for targeting introns 5 through 6 are as follows:

Forward Primer: $^{5'}$GGA ATT CCA TAT GGA ATT CCC GAA GTT CCA TTC TCT AGA$^{3'}$

Reverse Primer: $^{5'}$AGT CAG CCC GGG ATC CGG GCC CCC TTC CTT CAT AGC TAC CTA$^{3'}$

The primers used for targeting introns 5 through 7 are as follows:

Forward Primer: $^{5'}$GGA ATT CCA TAT GGA ATT CCC GAA GTT CCA TTC TCT AGA$^{3'}$

Reverse Primer: $^{5'}$AGT CAG CCC GGG ATC CGG GCC CCG TTT AAC TCC ATC GGA CAC$^{3'}$

The PCR parameters for this amplification are as follows:

Cycles (Stage 2): 26

Stage One: 94^{o}C for 2 minutes

Stage Two: 98^{o}C for 20 seconds

60^{o}C for 30 seconds

68^{o}C for 2 minutes

Stage Three: 68^{o}C for 7 minutes

4^{o}C until samples are removed from PCR instrument

2.9.6 Amplification of Intron 5 Splicing Acceptor + mCherry-CreER^{T2} + hGh + Neo Cassette insert from pUC-19 + Intron 5 Splicing Acceptor + mCherry-CreER^{T2} + hGh + Neo Cassette:

A pUC-19 vector containing intron 5 splicing acceptor, reporter cassette, and neo cassette sequences was used as the template for the following PCR. The following mixtures were made per sample in a PCR tube: 40.5µL of dH_2O, 5µL of 10X Accuprime Pfx Polymerase Mix, 1.5µL of 5µM Forward Primer, 1.5µL of 5µM Reverse Primer, 1µL of Accuprime Pfx High-fidelity DNA Polymerase, and 0.5µL of pUC-19 + Intron 5 Splicing Acceptor + mCherry-CreER^{T2} + hGh + Neo Cassette template.

The primers used for this procedure are as follows:

Forward Primer: $^{5'}$ACT CGA ATT CTT CAT TTG CAG GTA CAG C$^{3'}$

Reverse Primer: $^{5'}$AGT CAG CCC GGG ATC CGG GCC CCC TTC CTT CAT AGC TAC CTA$^{3'}$

The PCR parameters for this amplification are as follows:

Cycles (Stage 2): 35

Stage One: 95^{o}C for 3 minutes

Stage Two: 95^{o}C for 30 seconds

58^{o}C for 30 seconds

68^{o}C for 6 minutes

Stage Three: 68^{o}C for 7 minutes

4^{o}C until samples are removed from PCR instrument

2.10 Gel Extraction Protocol:

Gel extraction was performed using a QIAquick Gel Extraction Kit. The first step was to excise the DNA fragment from a 1% agarose gel via a scalpel under a UV light to view the band in question. The gel slice was added to an Eppendorf tube and weighed. For each 100mg of gel, 300µL of Buffer QG was added. The tubes were then incubated at 50^{o}C for either 10 minutes or until the gel dissolved, with vortexing every 2 minutes. The solution of the dissolved gel was then checked. If the solution was orange or purple, then the pH balance of the solution was off. 10µL of 3M sodium acetate (pH 5.0) was added to an unbalanced pH solution and mixed to change it to the required yellow color. 1 volume's worth of isopropanol was then added to the sample and mixed. The resultant solution was transferred to a QIAgen spin column and centrifuged for 1 minute at 17,000rpm. The flow-through was then discarded and the column was placed back in the same collection tube. 500µL of Buffer QG was added to the column and centrifuged for 1 minute at 17,000rpm. The flow-through was then discarded and the column was placed back in the same collection tube. 750µL of Buffer PE was added to the column and allowed to stand for 5 minutes. The column was centrifuged for 1 minute at 17,000rpm. The flow-through was then discarded and the column was placed in a clean 1.5mL Eppendorf tube.

50µL of Buffer EB was added to the center of the column filter and allowed to stand for 4 minutes. The resultant solution was then centrifuged for 1 minute at 17,000rpm. Finally, the column was disposed of and the Eppendorf tubes were labeled and stored in -20^{o}C until use.

2.11 Ligation Protocol:

After successful digestion of inserts and vector, the ligation protocol was performed according to the following mix: 2µL of 10X ligation buffer, 5µL of vector, 6µL of each insert to be added to digested vector, 1µL of T4 ligase, and a sufficient quantity of dH_2O needed to bring the final volume of the mixture up to 20µL. The mixture was made in Eppendorf tubes, mixed by flicking the tube briefly, and then incubated at 16^{o}C overnight.

This protocol was used in the formation of constructs pL253-ETV6 (Intron5-6), pL253-ETV6 (Intron5-7), p3xFlag-CMV14 + Reporter Cassette, and pUC19 + Reporter Cassette + Neo 5-6/5-7.

2.12 Embryonic Stem (ES) Cell Work to Create Genetic Knockdown Mice:

Embryonic stem cell work was performed by the Transgenic/Knock-out Mouse Shared Resource at the Cancer Institute of New Jersey at Rutgers. Services provided included the culturing and electroporation of ES cells, selection, picking, and freezing of ES clones, mating of mice to produce blastocysts, injection of targeted ES cells into blastocysts, surgical re-implantation of injected blastocysts into pseudopregnant female hosts, mating of high-grade male chimeras, and screening of progeny mice for germline transmission by PCR of tail tip DNA.

2.13 Crossing of Chimera Mice to FvB lines to obtain an FvB background:

Chimera mice were obtained from the Transgenic/Knock-out Mouse Shared Resource at the Cancer Institute of New Jersey at Rutgers. After allowing sufficient aging of 21 days to occur, chimera mice were placed into cages such that either one chimera male was with two FvB

wildtype female mice or one chimera female was with one FvB wildtype male mouse. Approximately 21 days were allowed for mating to occur and a new litter of pups to be born.

Once born, the male mouse was removed to a separate cage, and the pups were kept with mother for 21 days. At 21 days, pups were separated by gender, ear-tagged, and genotyped via tail-clipping.

2.14 Genotyping for ETV6-mCherry-CreER^{T2} mice:

Genotyping was performed on 21 day old pups that showed sufficient growth such that they could be safely tail-clipped and separated from their mother. A section of approximately 1cm of the tail tip was removed from each pup via sterilized scissors and placed into their respective labeled Eppendorf tube. Eppendorf tubes were then centrifuged at 17,000rpm for 30 seconds to ensure tail fragments are at the bottom of the Eppendorf tubes.

75mL of Alkaline Lysis solution (1.25M NaOH, 10mM EDTA, pH=12) was added to each individual Eppendorf tube and the tubes were then boiled at 100^{o}C for 30 minutes. Samples were then allowed to cool to room temperature and centrifuged at 17,000rpm for 1 minute. 75mL of Neutralization buffer (2M TRIS-HCl, pH=5) was then added to each sample and stored at 4^{o}C until used in genotyping PCR.

Genotyping PCR reactions were performed using the prepared mouse tail DNA as the template. The following mixtures were made per sample in a PCR tube: 9µL of dH_2O, 4µL of 5X Promega GoTaq Buffer, 1µL of 10µM Forward Primer, 1µL of 10µM Reverse Primer, 0.1µL of Promega GoTaq DNA Polymerase, and 2µL of prepared mouse tail DNA template.

The primers used for this procedure are as follows:

Forward Primer: $^{5'}$CAT CAA GTT GGA CAT CAC CTC CC$^{3'}$

Reverse Primer: $^{5'}$GGT ATG CTC AGA AAA CGC CTG G$^{3'}$

The PCR parameters for this amplification are as follows:

Cycles (Stage 2): 32

Stage One: 95^{o}C for 4 minutes

Stage Two: 95^{o}C for 20 seconds

58^{o}C for 20 seconds

72^{o}C for 30 seconds

Stage Three: 72^{o}C for 5 minutes

4^{o}C until samples are removed from PCR instrument

A successful genotype band is to be found as a 286bp PCR product

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Schematic Representations of Cloning Protocols:

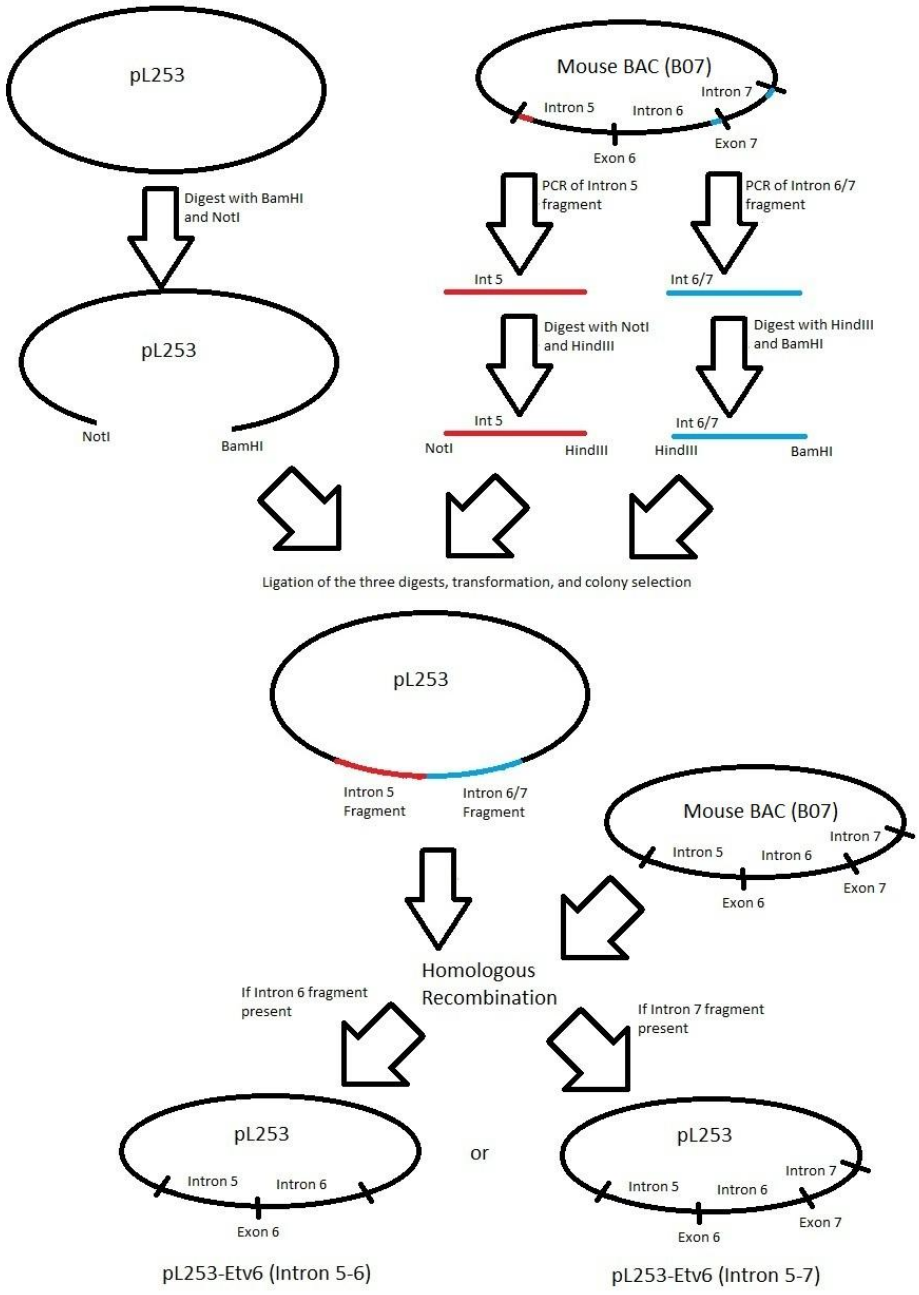


Figure 5: Schematic representation of the creation of the pL253-Etv6 (intron 5-6) and pL253-Etv6 (intron 5-7) constructs containing the full Etv6 gene from intron 5 to either intron 6 or 7.

Please note that “homologous recombination” indicates the introduction of heat shock activated SW106 *E. coli* strain, whereas “transformation” refers to the use of DH10B strain *E. coli*.

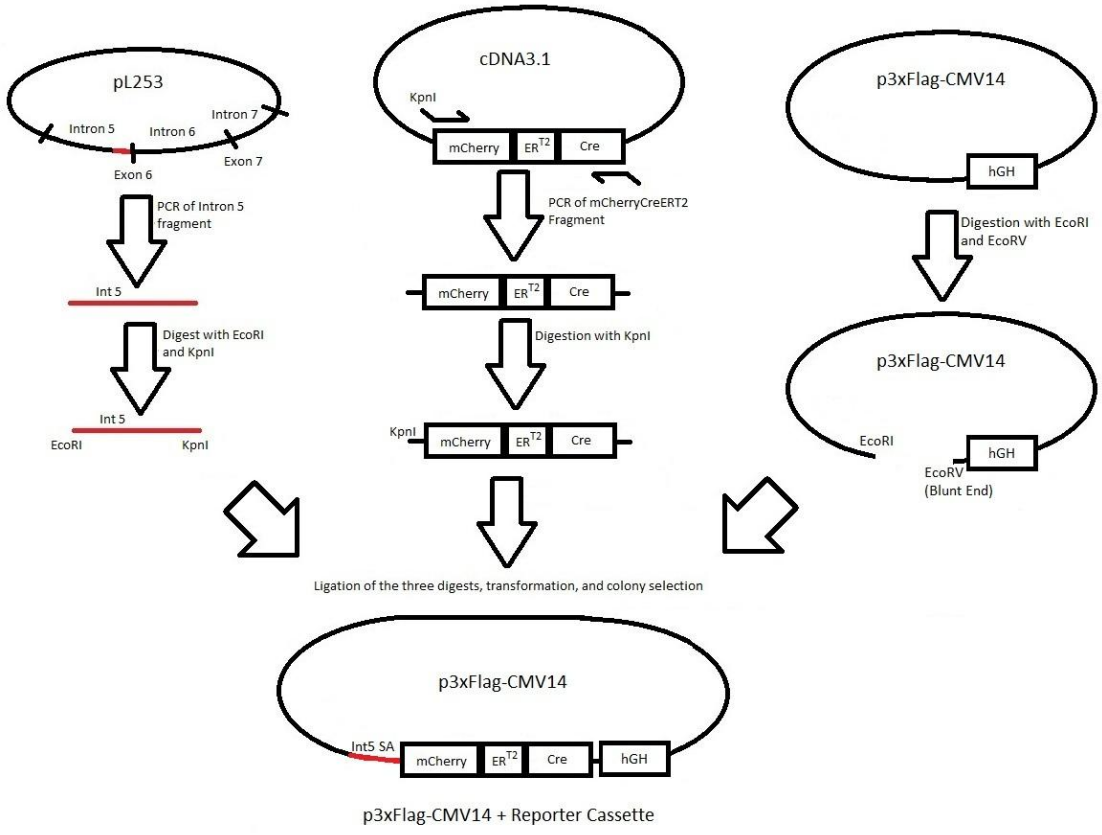


Figure 6: Schematic representation of the creation of the Reporter Cassette (Intron 5 splicing acceptor + mCherry + ER^{T2} + Cre + hGH) through traditional digestion, ligation, and DH10B transformation strain *E. coli*.

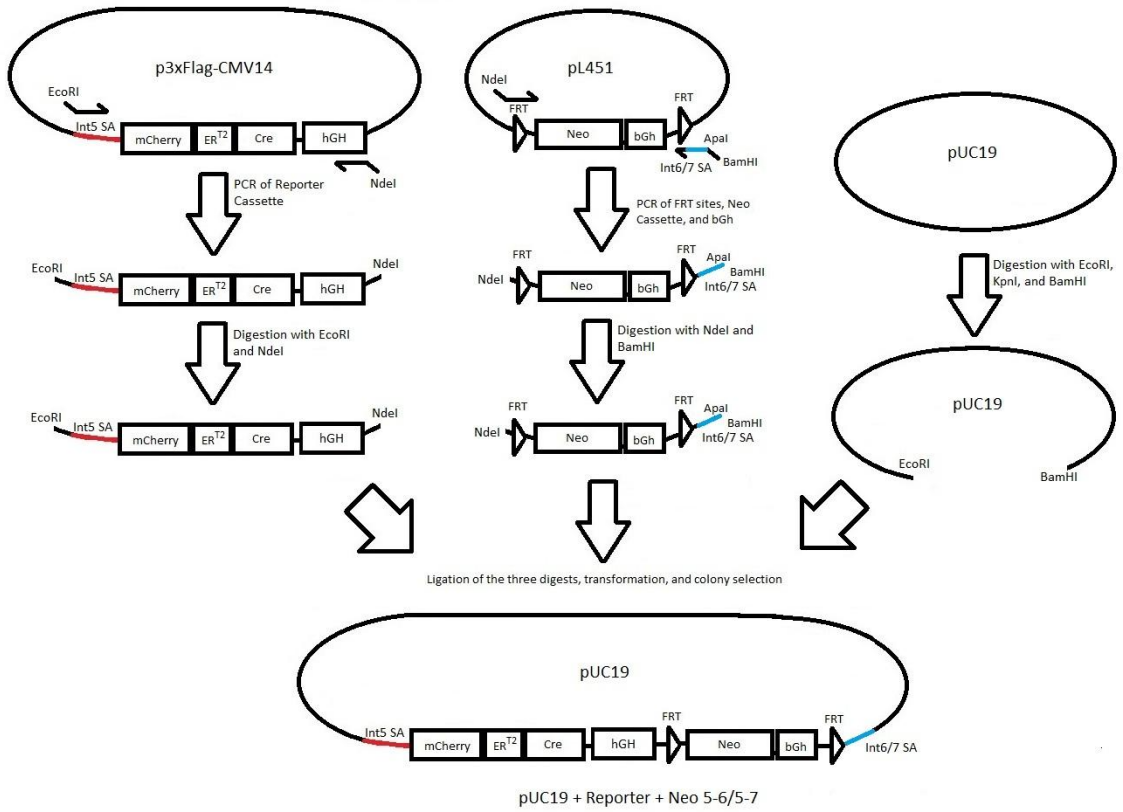


Figure 7: Schematic representation of the creation of the Reporter Cassette (Intron 5 splicing acceptor + mCherry + ER^{T2} + Cre + hGH poly A tail) + Neo Cassette (FRT + Neo + bGh poly A tail + FRT) in the pUC19 vector.

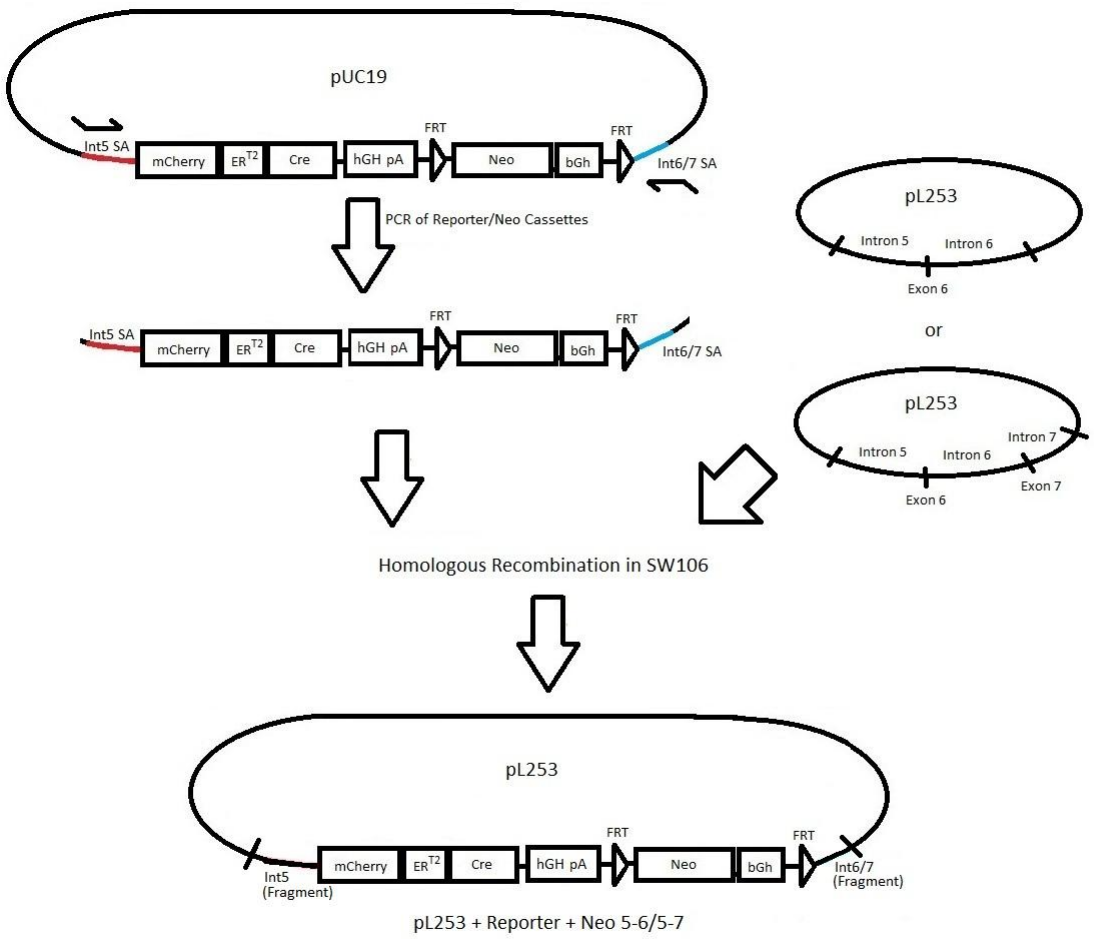


Figure 8: Schematic representation of the introduction of the Reporter Cassette (Intron 5 splicing acceptor + mCherry + ER^{T2} + Cre + hGH poly A tail) and Neo Cassette (FRT + Neo + bGh poly A tail + FRT) into the final pL253-Etv6 (5-6 or 5-7) targeting vector.

3.2 Creation of pL253 with Introns 5 and Intron 6 or 7:

The first step of this project was the introduction of fragments of ETV6 introns 5, 6, and 7 into the pL253 recombineering vector to create pL253-intron5-intron6 and pL253-intron5-intron7 DNA constructs. The fragments of the ETV6 introns were obtained through PCR of mouse bacteria artificial chromosome B07, which contains the ETV6 gene.

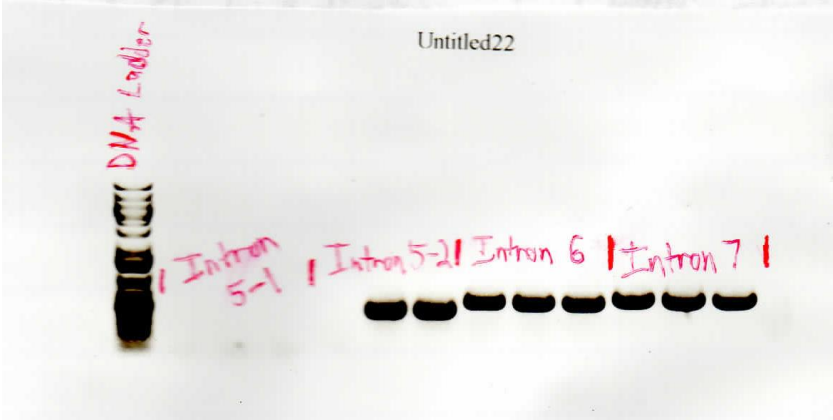


Figure 9: PCR amplification of fragments of introns 5, 6, and 7 from mouse bacterial artificial chromosome B07. The resultant bands were gel extracted, purified, and then digested as per the first schematic.

The pL253-intron5-intron6 and pL253-intron5-intron7 constructs were created through a three piece ligation using NotI, HindIII, and BamHI. After transformation and colony selection, these pL253 constructs were then linearized with HindIII as to separate their two respective intron fragments.

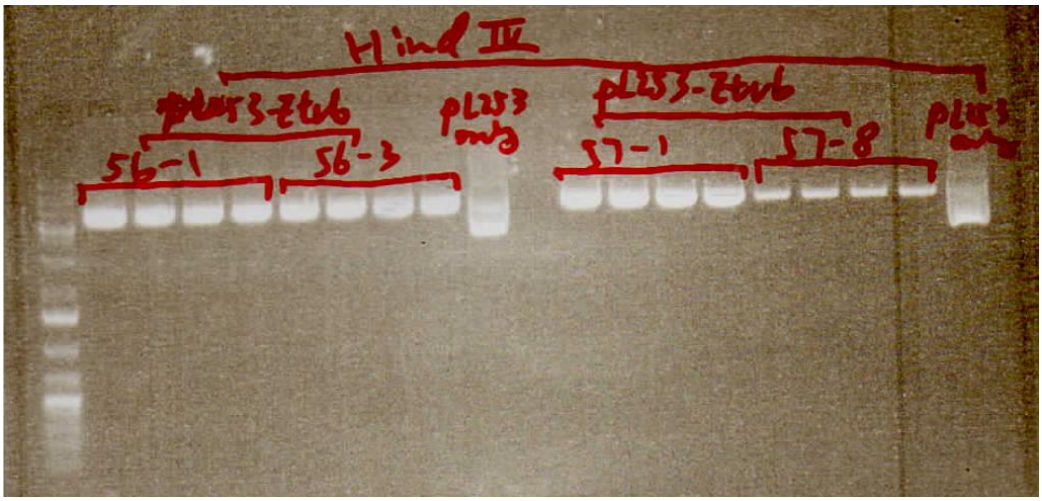


Figure 10: The linearized constructs of pL253 containing either ETV6 intron 5 and 6 fragments (designated 5-6) or ETV6 intron 5 and 7 fragments (designated 5-7). The "pL253 only" lanes indicate a digested but unmodified pL253 vector.

3.3 Homologous Recombination of SW106 + BAC B07 with pL253 + Intron 5/6 or 5/7 to Create pL253-ETV6 Construct:

The linear construct was then transformed into activated *E. coli* strain SW106 with mouse bacteria artificial chromosome B07 and homologous recombination was instigated. The resultant "gap-repair" homologous recombination created pL253 vectors with the entire ETV6 gene between introns 5 and 6 or introns 5 and 7, depending on the intron fragments in the initial pL253 vector-fragment construct. The resultant constructs were labeled pL253-int5/6 and pL253-int5/7 respectively, and analyzed via restriction enzymes.

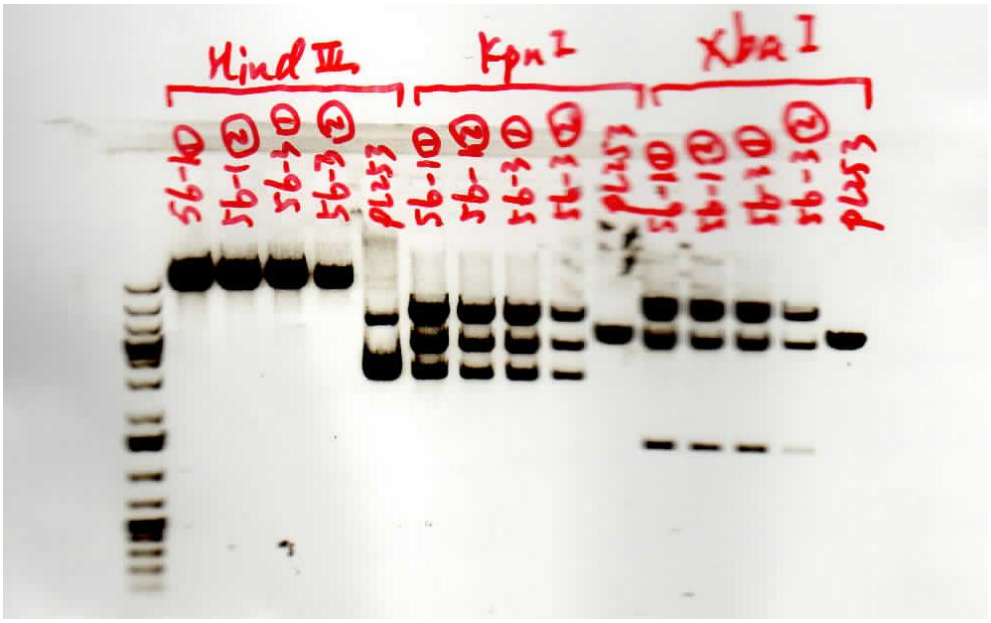


Figure 11: Restriction digestion analysis of four selected colonies of potential pL253-Etv6(5-6) candidates and an unmodified pL253 control plasmid. Expected bands for the above digestions were approximately as follows:

HindIII digestion (in basepairs): 6000 and 9000

KpnI digestion (in basepairs): 2800, 4300, and 8000

XbaI digestion (in basepairs): 800, 1300, 1400, and 9000

Figure 12: Restriction digestion analysis of four selected colonies of potential pL253-Etv6(5-7) candidates and an unmodified pL253 control plasmid. Expected bands for the above digestions were approximately as follows:

KpnI digestion (in basepairs): 2300, 2800, 3700, and 8000

SacII digestion (in basepairs): 4000 and 14000

XbaI digestion (in basepairs): 800, 1300, 5700, and 9200

3.4 Introduction of Intron 5 Splicing Acceptor and mCherry-CreER^{T2} into p3xFlag-CMV14 Vector:

The next step is the creation of the reporter cassette, comprised of the sequences for an intron 5 slicing acceptor, mCherry, ER^{T2}, Cre, and human growth hormone (hGH). The intron 5 splicing acceptor was digested out of the pL253-int5/7 construct, the mCherry-ER^{T2}-Cre construct was digested out of a cDNA3.1- mCherry-ER^{T2}-Cre previously created in the Sabaawy Lab, and the remaining hGH was added when the intron 5 splicing acceptor-mCherry-CreER^{T2} was introduced into the p3xFlag-CMV14 vector, which contained an hGH sequence, via three-

piece ligation and transformation. The final result of this stage is designated p3xFlag-CMV14-Int5 SA-mCherry-CreER^{T2}-hGH and was confirmed via restriction enzymes.

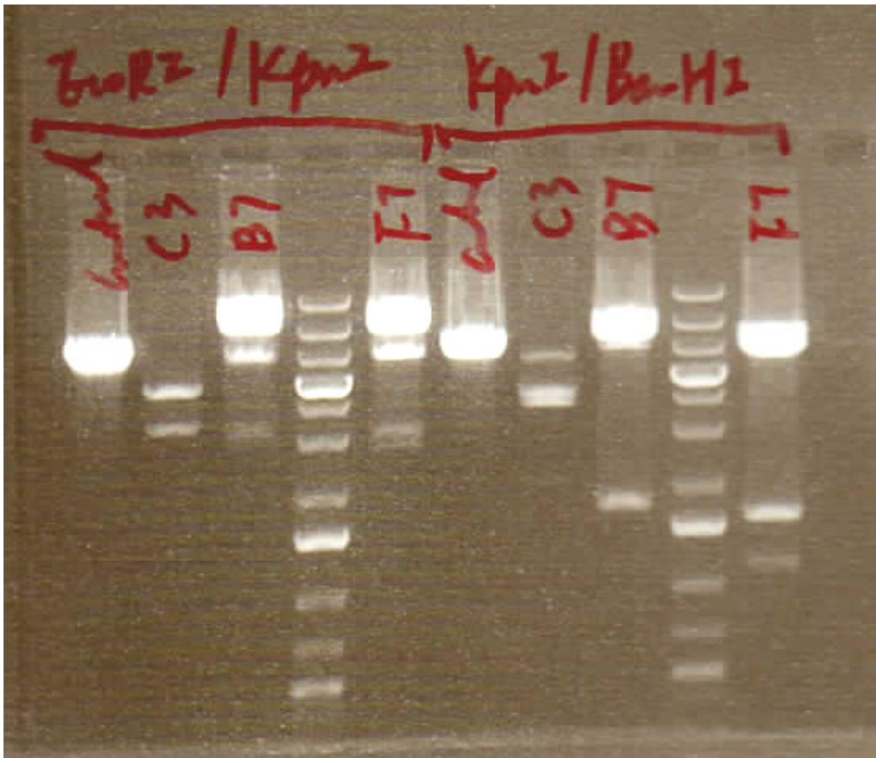


Figure 13: Restriction digestion analysis of two selected colonies of potential p3xFlag-CMV14-Int5 SA-mCherry-CreER^{T2}-hGH candidates and an unmodified p3xFlag-CMV14 control plasmid. Of the two samples, only F7 showed all the expected bands. The expected bands for the above digestions were as follows:

EcoRI/KpnI digestion (in basepairs): 312, 2742, and 6282

BamHI/KpnI digestion (in basepairs): 15, 1095, 1647, and 6579

3.5 Creation of pUC19 vector with Reporter Cassette (Intron 5 Splicing Acceptor and mCherry-CreER^{T2}) from p3xFlag-CMV14 and Neo Cassette (FRT-Neo-bGH-FRT) from pL451:

The next step was to add a neomycin cassette(FRT-Neomycin-bGh-FRT) and the reporter cassette (Int5 SA-mCherry-CreER^{T2}-hGH) into a pUC19 backbone. PCR was used to extract both the reporter cassette from the p3xFlag-CMV14-Int5 SA-mCherry-CreER^{T2}-hGH construct and the

neomycin cassette from a pL451 recombineering vector. The pUC19 recombineering vector, reporter cassette, and neomycin cassette were digested, three-piece ligated, and transformed to create the resultant pUC19-Int5 SA-mCherry-CreER^{T2}-hGH-FRT-Neomycin-bGh-FRT construct.

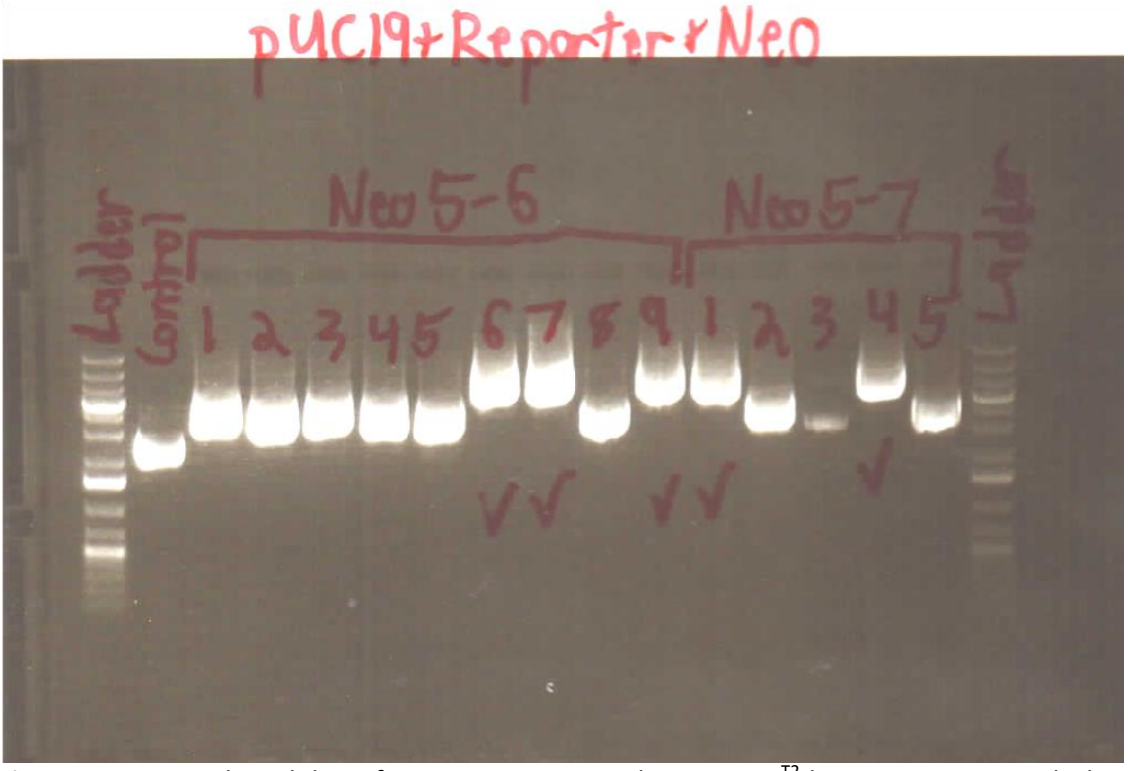


Figure 14: Potential candidates for pUC19-Int5 SA-mCherry-CreER^{T2}-hGH-FRT-Neomycin-bGh-FRT candidates. Five candidates were indicated with checkmarks and further analysis was performed to confirm the reporter and neo cassettes were present.

3.6 Recombination of Intron 5 Splicing Acceptor + mCherry-CreER^{T2} + hGh + Neo Cassette (Introns 5 to 6) (from pUC-19) into pL253-ETV6:

The final step was to place the reporter and neo cassettes into the pL253-int5/6 or pL253-int5/7 recombineering vector. This is accomplished by first using PCR to amplify the Int5 SA-mCherry-CreER^{T2}-hGH-FRT-Neomycin-bGh-FRT sequence out of the pUC19-Int5 SA-mCherry-CreER^{T2}-hGH-FRT-Neomycin-bGh-FRT construct. The reporter cassette and neo cassette was then homologously recombined into pL253-ETV6 (5-6) or pL253-ETV6 (5-7) such that the

reporter cassette and neo cassette replaces the ETV6 gene between exons 6 through 8. This creates the final constructs of pL253-ETV6 (5-6)-Int5 SA-mCherry-CreER^{T2}-hGH-FRT-Neomycin-bGh-FRT and pL253- ETV6 (5-7)-Int5 SA-mCherry-CreER^{T2}-hGH-FRT-Neomycin-bGh-FRT.

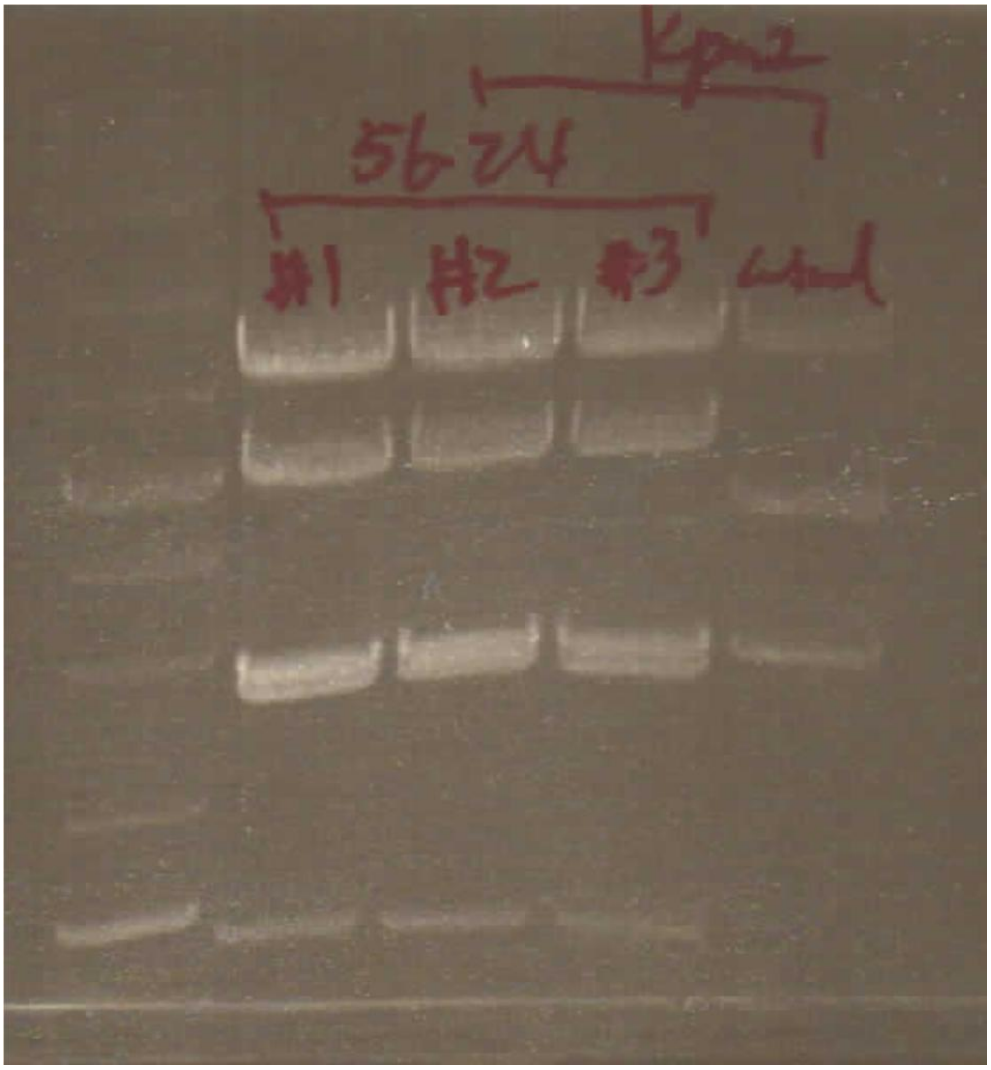


Figure 15: Restriction digestion analysis of final construct pL253-ETV6 (5-6) + Intron 5 Splicing Acceptor + mCherry-CreER^{T2} + hGh + Neo Cassette by KpnI. All three digestions confirmed colony #24 contained the full reporter and neo cassette inserts. The expected bands for the above digestion were as follows (in basepairs): 1384, 2742, 2820, 5490, and 8357.

3.7 Embryonic Stem Cell Work to create Transgenic ETV6 Knockdown Mice:

After sequencing to confirm the presence of the correct construct, repairing the insert, and introducing the repaired construct into pL253-ETV6 (5-7) (See Appendix 1), the Int5 SA-mCherry-CreER^{T2}-hGH-FRT-Neomycin-bGh-FRT insert was isolated, linearized via NdeI, and sent to the Transgenic/Knock-out Mouse Shared Resource at the Cancer Institute of New Jersey at Rutgers for implantation into mouse embryonic stem cells and grown into chimera mice. The resultant chimera mice were genotyped for confirmation of construct presence and bred with wildtype mice in an attempt to find a founder and create a stable genetic line. As of current, we have reached the F2 generation of this transgenic knock-in line and are attempting to find a founder that meets all genotyping requisites.

3.8 Future Goals:

The key future goal of this project is the introduction of a timely, traceable ETV6 fusion protein into mice, specifically the ETV6-RUNX1 fusion protein. This introduction will be accomplished through crossing a successful ETV6-reporter mouse with a floxed RUNX1 or similar gene that overrides the ETS (DNA binding) domain of the ETV6 gene. The mCherry insert will allow for a quick visual confirmation that the ETV6-RUNX1 protein has been translated, and the CreER^{T2} fusion protein will allow for an inducible Cre-Lox recombination with the addition of tamoxifen.

The construct also has the added benefit of creating a knockdown of ETV6, since one of the two chromosomal copies has a disrupted ETS domain. This can add to the understanding of the normal function of ETV6 and whether any level of gene expression reduction has an impact on the phenotype of the modified mouse model.

Current research surrounding ETV6-RUNX1 shows that the fusion protein causes a partial impairment of blood cell maturation, downregulation of PI3K/AKT/mTOR signaling, and

upregulation of genes involved in immune response, apoptosis, signal transduction, development, and differentiation (Fischer et al., 2005 and Fuka et al., 2011). At least 777 genes have been identified to interact with ETV6-RUNX1 (Fuka et al., 2011).

ETV6-RUNX1 has also recently been show to induce overexpression of MDM2, the key negative regulator of tumor suppressor protein p53. This is an interesting note in that p53 is mutated in nearly 50% of all cancers and silenced in many more, yet acute lymphoblastic leukemia rarely has any modifications of the p53 protein or its encoding gene TP53 (Kaindl et al., 2014). Inversely, it has also been recently shown that the RUNX1 transcription factor stimulates p53 under cellular stress (Wu et al., 2013). Activated oncogenes have also been found to activate the transcription of the Ink4ab ARF protein, which translocates MDM2 away from p53 and into the nucleolus (Moll and Petrenko, 2003), preventing the degradation of p53 and causing the cell to undergo a stress-like response.

Under normal physiological conditions, p53 is kept at very low levels due to continual degradation by MDM2. Under stresses such as DNA damage and oncogene activation, a key response is the rapid stabilization of p53 by blocking degradation via MDM2 (Moll and Petrenko, 2003). This high level of p53 can lead to the killing of the cell through both transcription-dependent and transcription-independent pathways (Moll and Petrenko, 2003). Based on the above findings, I would hypothesize that ETV6-RUNX1 has the potential to cause a “silent development” of leukemia by removing the potential to have a p53-related stress response due to overexpression of MDM2, thereby allowing for the development and propagation of cells carrying mutated and damaged DNA. I feel that the traceable construct created in this project has the potential to add to the understanding of ETV6-RUNX1 and its interaction with oncogenic stress and the p53 pathway through bypassing the embryonic lethality of ETV6 knockout mice, and allowing a successful and timely production of an ETV6-RUNX1 mouse model.

REFERENCES

Bohlander SK. ETV6: A versatile player in leukemogenesis. Seminars in Cancer Biology (2005); **15**; 162-174

De Braekeleer E, Douet-Guilbert N, Morel F, Le Bris MJ, Basinko A, and De Braekeleer M. ETV6 fusion genes in hematological malignancies: A review. Leukemia Research (2012); **36**; 945-961

Fischer M, Schwieger M, Horn S, Niebuhr B, Ford A, Roscher S, Bergholz U, Greaves M, Löhler J and Stocking C. Defining the oncogenic function of the TEL/AML1 (ETV6/RUNX1) fusion protein in a mouse model. *Oncogene* (2005); **24**; 7579-7591

Fuka G, Kauer M, Kofler R, Haas OA, and Panzer-Grümayer R. The Leukemia-Specific Fusion Gene ETV6/RUNX1 Perturbs Distinct Key Biological Functions Primarily by Gene Repression. *PLoS ONE* (2011); DOI: 10.1371/journal.pone.0026348

Kaindl U, Morak M, Portsmouth C, Mecklenbräuker A, Kauer M, Zeginigg M, Attarbaschi A, Haas OA and Panzer-Grümayer R. Blocking ETV6/RUNX1-induced MDM2 overexpression by Nutlin-3 reactivates p53 signaling in childhood leukemia. *Leukemia* (2014); **28**; 600-608

Linka Y, Ginzel S, Krüger M, Novosel A, Gombert M, Kremmer E, Harbott J, Thiele R, Borkhardt A, and Landgraf P. The impact of TEL-AML1 (ETV6-RUNX1) expression in precursor B cells and implications for leukaemia using three different genome-wide screening methods. *Blood Cancer Journal* (2013); **3**; doi:10.1038/bcj.2013.48

Moll UM and Petrenko O. The MDM2-p53 Interaction. *Mol Cancer Res* (2003); **1**; 1001-1008

Muyrers JP, Zhang Y, Testa G, and Stewart AF. Rapid modification of bacterial artificial chromosomes by ET- recombination. *Nucleic Acids Res* (1999); **27**; 1555-1557

Wu D, Ozaki T, Yoshihara Y, Kubo N and Nakagawara A. Runt-related Transcription Factor 1 (RUNX1) Stimulates Tumor Suppressor p53 Protein in Response to DNA Damage through Complex Formation and Acetylation. *The Journal of Biological Chemistry* (2013); **288**; 1353-1364.

Yu D, Ellis HM, Lee EC, Jenkins NA, Copeland NG, and Court DL. An efficient recombination system for chromosome engineering in Escherichia coli. *Proc Natl Acad Sci USA*(2000); **97;** 5978-5983

APPENDIX

Repair of pL253-ETV6 (5-6)-Int5 SA-mCherry-CreER^{T2}-hGH-FRT-Neomycin-bGh-FRT

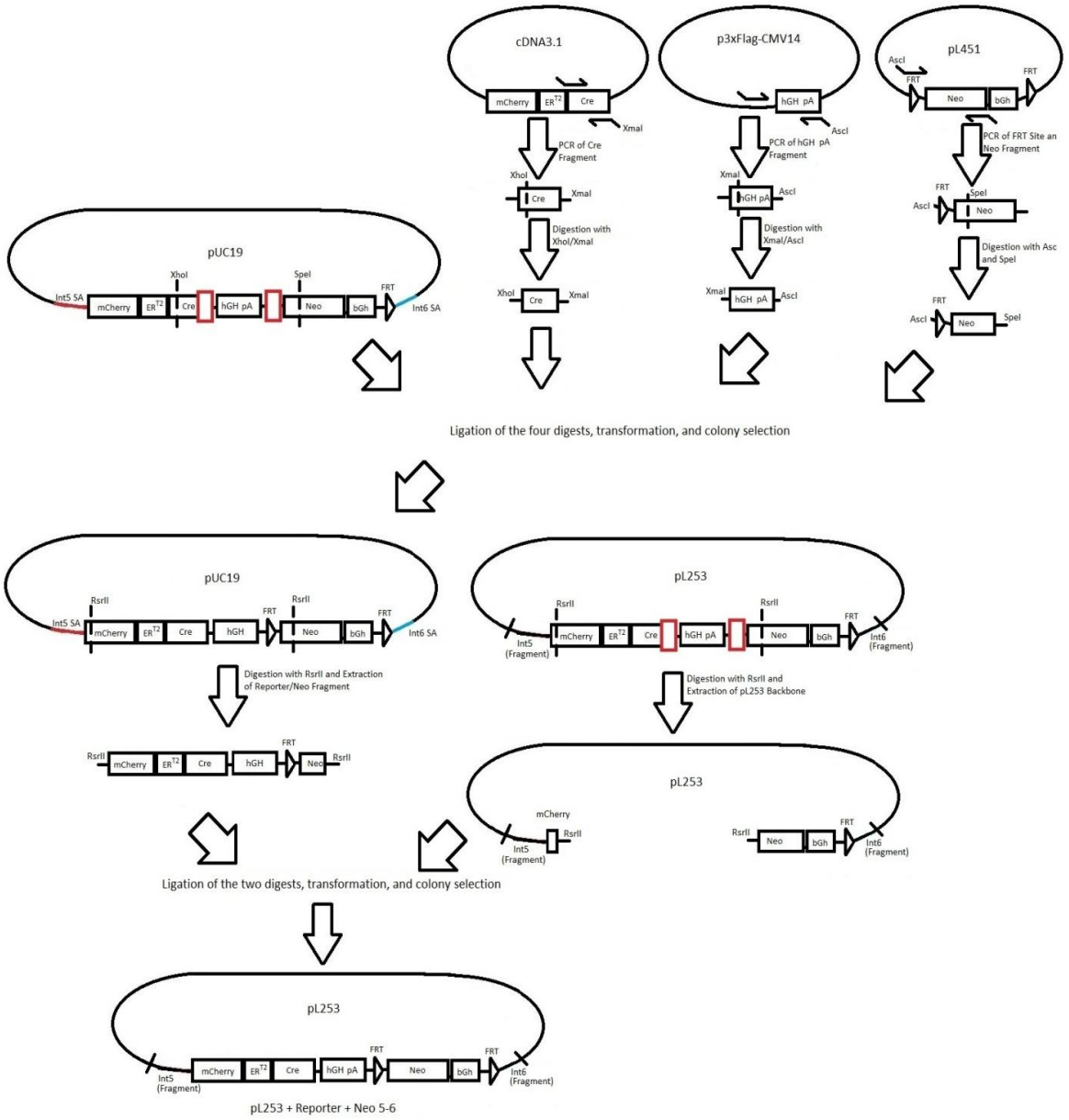


Figure 16: Schematic representation of the repairing of the pL253-ETV6 (5-6) + Reporter + Neo construct. After sequencing, it was found that the Reporter + Neo insert was missing part of the Cre sequence and the first FRT sequence flanking the Neo cassette (shown as red boxes above).

Repair was performed by using PCR to amplify the fragments of the insert that contained errors, digested, and four-piece ligated such that the insert was repaired in the pUC19 (5-6) + Reporter + Neo construct. The resultant corrected insert was then removed via RsrII digestion and inserted into a pL253-ETV6 (5-6) + Reporter + Neo construct that had the same missing sequence. The repaired pUC19 (5-6) + Reporter + Neo construct was also used to insert the correct construct into the pUC19 (5-7) + Reporter + Neo construct via traditional digestion, ligation, and transformation. From this repaired pUC19 (5-7) + Reporter + Neo construct, homologous recombination was performed with activated SW106 and pL253-ETV6 (5-7) to introduce the final insert into pL253 and create the final pL253-ETV6 (5-7) + Reporter + Neo construct (schematic not shown).

PROJECT 2: Characterization of Ink4ab/ Vav-Cre Transgenic Mice Lines

CHAPTER 1: INTRODUCTION

1.1 Hematopoiesis:

Hematopoiesis is the process through which all white and red blood cells are made. It is organized as a hierarchy, with hematopoietic stem cells as the source of all blood cellular components. Starting with multipotential hematopoietic stem cells (HSCs) in the bone marrow, these HSCs can differentiate into myeloid or lymphoid progenitor cells. These progenitor cells can then develop further towards lymphocytes, erthyrocytes, neutrophils, basophils, eosinophils, macrophages, and platelets, depending on the transcription factors present and the progenitor cell involved. As hematopoiesis is based on a hierarchy, disrupting higher up on the pathway can lead to a higher degree of impact on normal hematopoietic function.

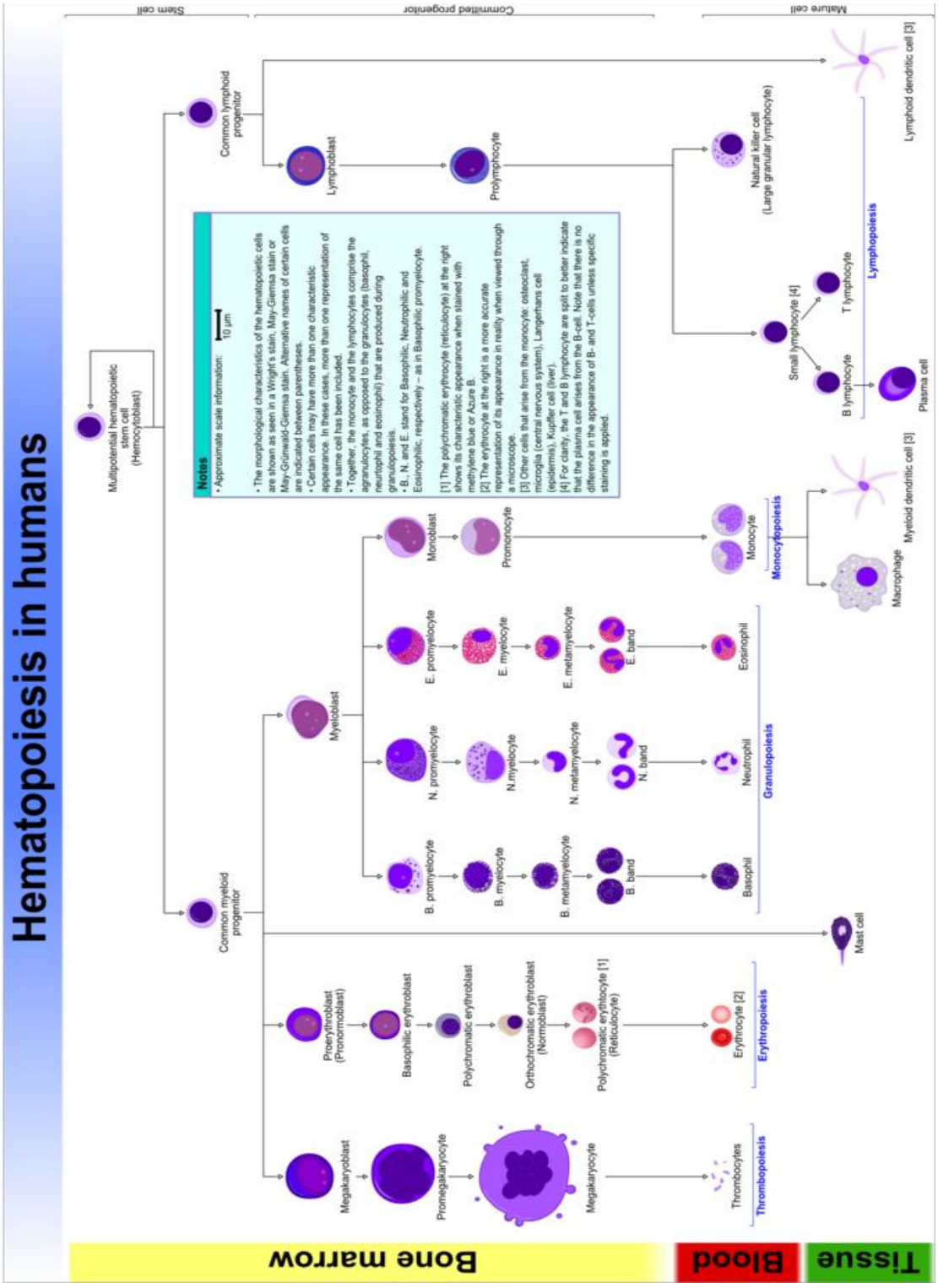


Figure 1: The hierarchy of hematopoiesis in humans (Rad, 2006). Depending on the level of precursor cell impacted, one or multiple linages may be disrupted.

1.2 Hematopoietic Stem Cells and Leukemia:

Leukemia is a cancer that impacts the bone marrow and its capacity to make mature white and/or red blood cells and their variants. It frequently results in high numbers of abnormal white blood cells within the bone marrow, spleen, and peripheral blood. There are multiple forms of leukemia, derived from the hierarchy and speed of onset. Leukemias can be either chronic or acute. Chronic leukemias cause a long-term reduction of fully-mature white blood cells. Due to their low growth of the disease, chronic leukemias can be identified and treated prior to the development of any serious symptoms. Acute leukemias cause a short-term, near complete removal of white blood cell maturation. This accelerated impact causes acute leukemias to be significantly more deadly than their chronic counterparts. Lymphoproliferative leukemias disrupt common lymphoid progenitor cells whereas myeloproliferative leukemias disrupt common myeloid progenitor cells.

Research performed by Dr. Dominique Bonnet and Dr. John E. Dick connected acute myeloid leukemia to a hematopoietic stem cell source. By injecting "SCID leukemia-initiating cells" (SL-IC cells) from human patients into NOD/SCID mice, they studied the cells' characteristics in comparison to their normal stem cells by testing the cells' differentiation, proliferative, self-renewal capacities, and surface markers CD34 and CD38. They concluded that the phenotype of the SL-ICs "was similar to that of normal stem cells and the same in every patient" regardless of the impact of the leukemic blast cells or AML cells (Bonnet and Dick, 1997). This discovery led to the coining of "cancer stem cells": cancer cells that possess self-renewal and differentiation capabilities that allows for tumor growth and relapse.

1.3 Ink4ab Locus Structure and Function:

The Ink4b-ARF-Ink4a genetic locus encodes three proteins that greatly impact the cell cycle. Ink4a and Ink4b encode two proteins, CDKN2A and CDKN2B respectively, which inhibit Cdk4 activity and therefore inhibit the entry of quiescent HSCs from G_0 to G_1 phase of the cell cycle, designated p15 and p16 in both humans and mice (Pietras et al., 2011). The ARF sequence encodes p14 in humans and p19 in mice, both of which interact by inhibiting mdm2, which then promotes transcription of the p53 tumor suppressor. The p53 tumor suppressor acts as a tumor suppressor by initiating cell cycle arrest and apoptosis.

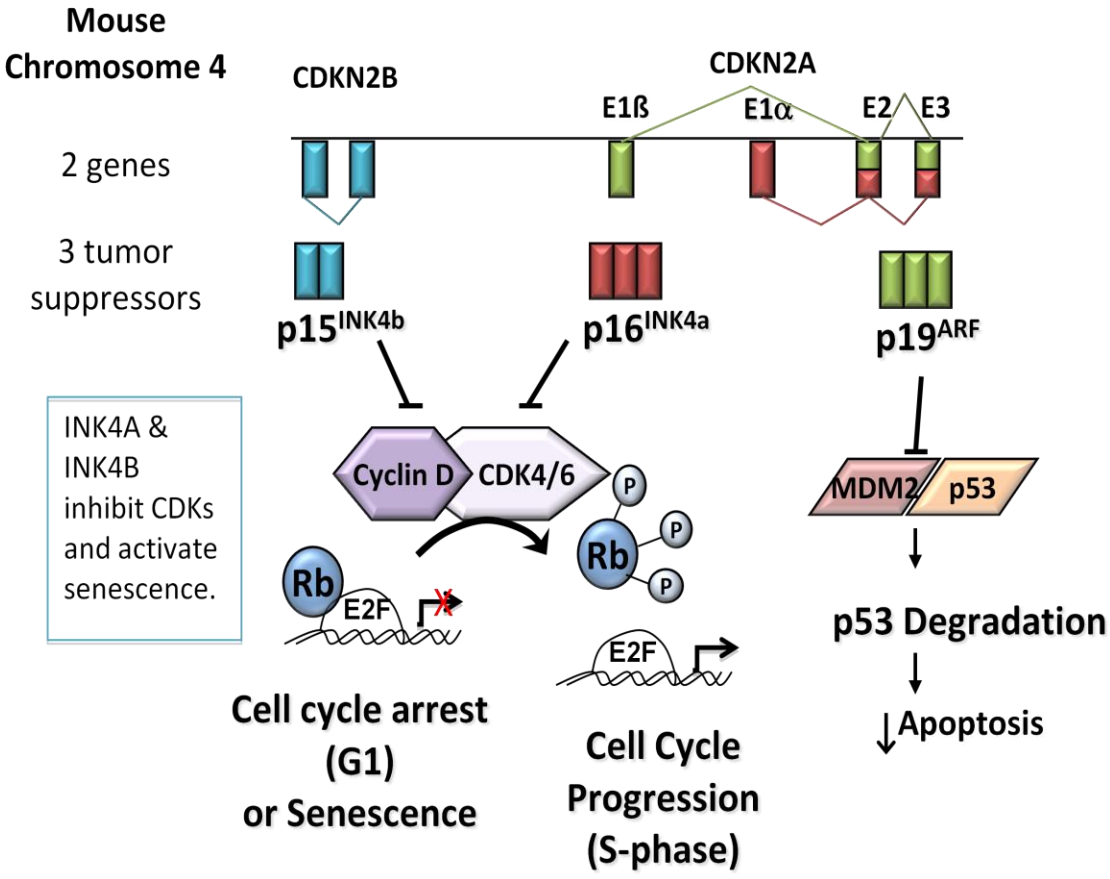


Figure 2: The pathways of Ink4ab in mice and their impact on the cell cycle (Davis and Sabaawy, 2013).

1.4 Ink4ab Knockout within mice:

The Ink4b-ARF-Ink4a locus on chromosome 9p21 in humans and chromosome 4 in mice is frequently lost in many cancers. Full knockouts of Ink4ab and ARF, designated Cdkn2ab$^{-/-}$, in mice were created using a transgenic line to remove the Ink4a and Ink4b sequences and the Cre-Lox recombination system to remove the ARF sequence. These mouse models have shown a significant increase in tumor growth and diversity of tumors formed, even when compared to Ink4a knockout mouse models. Of note, there is a propensity for developing skin tumors and soft tissue sarcomas when ubiquitous Cre lines were used (Krimpenfort et al., 2007). This increased tumor formation has shown that there is a compensatory effect between Ink4a, Ink4b, and ARF mutations, and has confirmed that Ink4b can act as critical tumor suppressors in the absence of Ink4a (Krimpenfort et al., 2007).

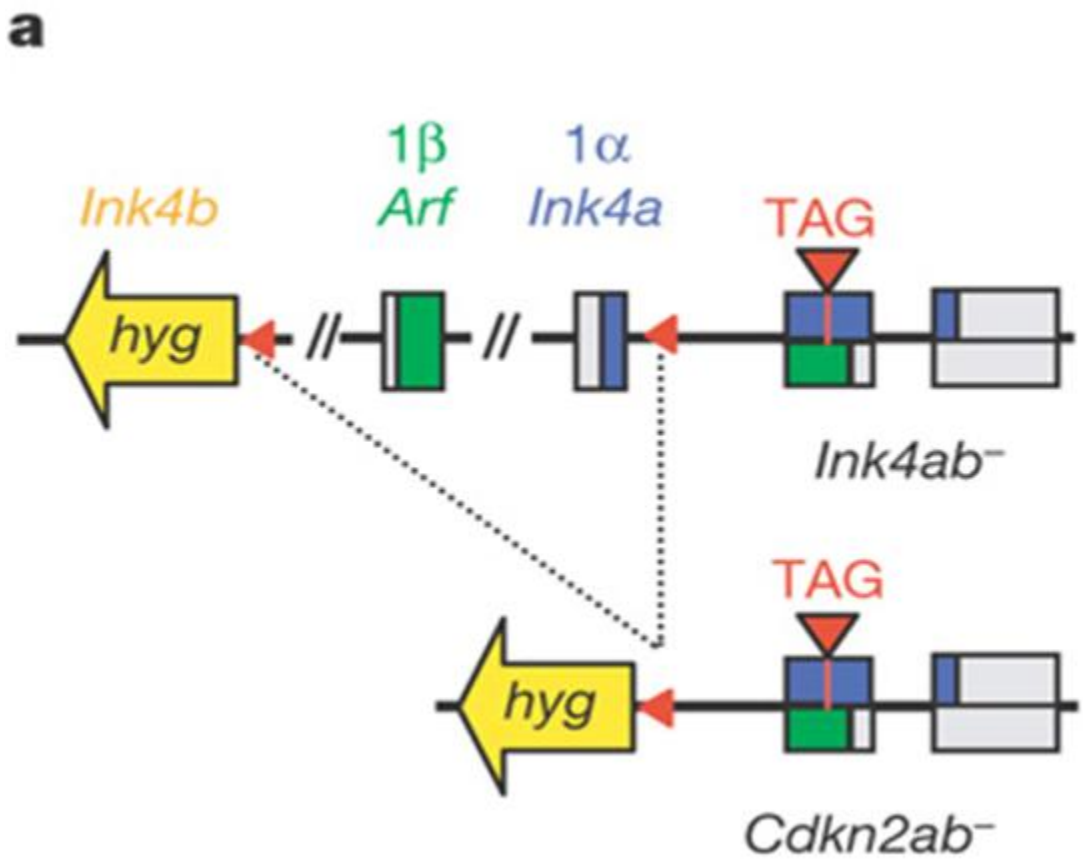


Figure 3: The construct for Cdkn2ab knockout mice. The Ink4a and Ink4b sequences are transgenically knocked out, with the ARF sequence flanked by LoxP sequences for a Cre-Lox induced knockout (Krimpenfort et al., 2007).

1.5 Vav Promoter Function:

The Vav gene encodes a guanine nucleotide-exchange protein. This protein is expressed in adult mice almost exclusively in hematopoietic stem cells. The Vav gene is also expressed in testicular germ cells, developing teeth, and placental trophoblasts (Georgiades et al., 2002).

1.6 Project Goals:

By using a Vav promoter to drive the Cre-Lox recombination for the Cdkn2ab knockout shown in figure 3, we hope to obtain mouse models that have tumor growth solely within the hematopoietic system. If successful, we intend to diagnose the specific hierarchy impacted and the form or forms of cancer present within the line.

CHAPTER 2: METHODS

2.1 Genotyping for Ink4ab mice:

Genotyping was performed on 21 day old pups that showed sufficient growth such that they could be safely tail-clipped and separated from their mother. A section of approximately 1cm of the tail tip was removed from each pup via sterilized scissors and placed into their respective labeled Eppendorf tube. Eppendorf tubes were then centrifuged at 13,300rpm for 30 seconds to ensure tail fragments are at the bottom of the Eppendorf tubes.

75mL of Alkaline Lysis solution (1.25M NaOH, 10mM EDTA, pH=12) was added to each individual Eppendorf tube and the tubes were then boiled at 100^{o}C for 30 minutes. Samples were then allowed to cool to room temperature and centrifuged at 13,300rpm for 1 minute. 75mL of Neutralization buffer (2M TRIS-HCl, pH=5) was then added to each sample and stored at 4^{o}C until used in genotyping PCR.

Genotyping PCR reactions were performed using the prepared mouse tail DNA as the template. The following mixtures were made per sample in a PCR tube: 6.9µL of dH_2O, 4µL of 5X Promega GoTaq Buffer, 2µL of 5µM Forward Primer, 2µL of 5µM Reverse Primer, 0.1µL of Promega GoTaq DNA Polymerase, and 2µL of prepared mouse tail DNA template.

The primers used for this procedure are as follows:

Forward Primer: $^{5'}$GCA GTG TTG CAG TTT GAA CCC$^{3'}$

Reverse Primer: $^{5'}$TGT GGC AAC TGA TTC AGT TGG$^{3'}$

The PCR parameters for this amplification are as follows:

Cycles (Stage 2): 38

Stage One: 94^{o}C for 3 minutes

Stage Two: 94^{o}C for 30 seconds

58^{o}C for 30 seconds

72^{o}C for 45 seconds

Stage Three: 72^{o}C for 7 minutes

4^{o}C until samples are removed from PCR instrument

A successful genotype band for Ink4ab$^{-/-}$ mice is a 600bp PCR product. A successful genotype for Ink4ab$^{+/-}$ mice contains 472bp and 600bp PCR products. A successful genotype band for wildtype mice is a 472bp PCR product.

2.2 Genotyping for Vav-Cre mice:

Genotyping was performed on 21 day old pups that showed sufficient growth such that they could be safely tail-clipped and separated from their mother. A section of approximately 1cm of the tail tip was removed from each pup via sterilized scissors and placed into their respective labeled Eppendorf tube. Eppendorf tubes were then centrifuged at 13,300rpm for 30 seconds to ensure tail fragments are at the bottom of the Eppendorf tubes.

75mL of Alkaline Lysis solution (1.25M NaOH, 10mM EDTA, pH=12) was added to each individual Eppendorf tube and the tubes were then boiled at 100^{o}C for 30 minutes. Samples were then allowed to cool to room temperature and centrifuged at 13,300rpm for 1 minute. 75mL of Neutralization buffer (2M TRIS-HCl, pH=5) was then added to each sample and stored at 4^{o}C until used in genotyping PCR.

Genotyping PCR reactions were performed using the prepared mouse tail DNA as the template. The following mixtures were made per sample in a PCR tube: 6.9µL of dH_2O, 4µL of 5X Promega GoTaq Buffer, 2µL of 5µM Forward Primer, 2µL of 5µM Reverse Primer, 0.1µL of Promega GoTaq DNA Polymerase, and 2µL of prepared mouse tail DNA template.

The primers used for this procedure are as follows:

Forward Primer: $^{5'}$AGA TGC CAG GAC ATC AGG AAC CTG$^{3'}$

Reverse Primer: $^{5'}$ATC AGC CAC ACC AGA CAC AGA GAT C$^{3'}$

The PCR parameters for this amplification are as follows:

Cycles (Stage 2): 35

Stage One: 94^{o}C for 1 minute

Stage Two: 94^{o}C for 30 seconds

64^{o}C for 45 seconds

72^{o}C for 45 seconds

Stage Three: 72^{o}C for 2 minutes

4^{o}C until samples are removed from PCR instrument

A successful genotype band is to be found as a 236bp PCR product

2.3 General Dissection for selected mice:

Dissections were performed on chosen mice candidates that were either at a reasonable age for analysis or showed signs of significant disease progression. Select mice were euthanized with carbon dioxide and immediately tail clipped for peripheral blood analysis. The arms and legs were then pinned to a dissection stage with thumbtacks so that the mouse was on its back and its abdomen was visible.

The mouse was then sprayed with 70% ethanol and opened in an X-pattern with autoclaved scissors and forceps. The interior of the mouse was analyzed and any organs of interest were extracted.

Organs that were found to be impacted were extracted for sectioning and immunohistochemical staining. Extracted organs were placed in 10% formalin overnight and then transferred to 70% ethanol for long-term storage until transfer for immunohistochemical staining.

2.4 Peripheral Blood Analysis of selected mice:

Directly after mouse euthanasia, a drop of blood was obtained on each of multiple microscope slides previously cleaned with 70% ethanol wipes. The droplet of blood was then smeared across the slide using a cover slide so that a single layer of blood cells would be obtained. The blood smears were then allowed to dry before being transported to the lab for Wright-Giemsa staining.

Prior to Wright-Giemsa staining, the following five solutions were made fresh: 36mL methanol, 36mL Wright-Giemsa Stain, 6mL Wright-Giemsa Stain$^{(1)}$ + 30mL Sorensen Buffer, 4mL Wright-Giemsa Stain$^{(1)}$ + 32mL dH_2O, and a pool (with slide holder) of dH_2O. For all Wright-Giemsa-using solutions, the Wright-Giemsa stain was run through a 3 micron filter when added.

The dry slides were then submerged in the following order, removing excess solution between each step by holding the slide on its side on a paper towel: methanol for a few seconds, 100% Wright-Giemsa stain for 4 minutes, Wright-Giemsa + Sorensen for 4 minutes, Wright-Giemsa + dH_2O for 5 minutes, and pure dH_2O for 5 minutes. The slides were allowed to air dry overnight, cover slipped, and then analyzed.

2.5 Heart Blood Extraction for Complete Blood Counts:

After mouse euthanasia, a 1cc syringe was inserted under the rib cage prior to dissection to reach the heart. Approximately between 250µL and 500µL of heart blood was obtained per specimen and transferred to its respective BD Microtainer Tube with EDTA. The Microtainer Tubes were then sent to ANTECH Diagnostics for Complete Blood Counts.

2.6 Immunohistochemical Staining of Selected Sections from mice:

Parafin blocking, sectioning, and immunohistochemical staining was performed by the histology core at the Cancer Institute of New Jersey at Rutgers University. Successful immunohistochemical staining on mouse spleens was performed using the following primary antibodies:

Caspase: Cell Signaling, 1:200 Dilution

CD3: Ventana, Pre-Diluted

B220: Novus Biologicals, 1:100 Dilution

pCNA: Sigma, 1:40,000 Dilution

pH3: Millipore, 1:300 Dilution

Ki67: Abcam, 1:400 Dilution

Cyclin D1: Ventana, Pre-Diluted

H3K9me: Active Motif, 1:500 Dilution

HP1 Gamma: Bioss, 1:200 Dilution

CHAPTER 3: RESULTS

3.1 Ink4ab$^{-/-}$; Vav-Cre+ Mice Show a Significantly Reduced Lifespan compared to Heterozygous and Wildtype Mice:

Using a survival log of the Ink4ab; Vav-Cre mouse line over the span of multiple years, the following figure from different genotypes was created using GraphPad Prism:

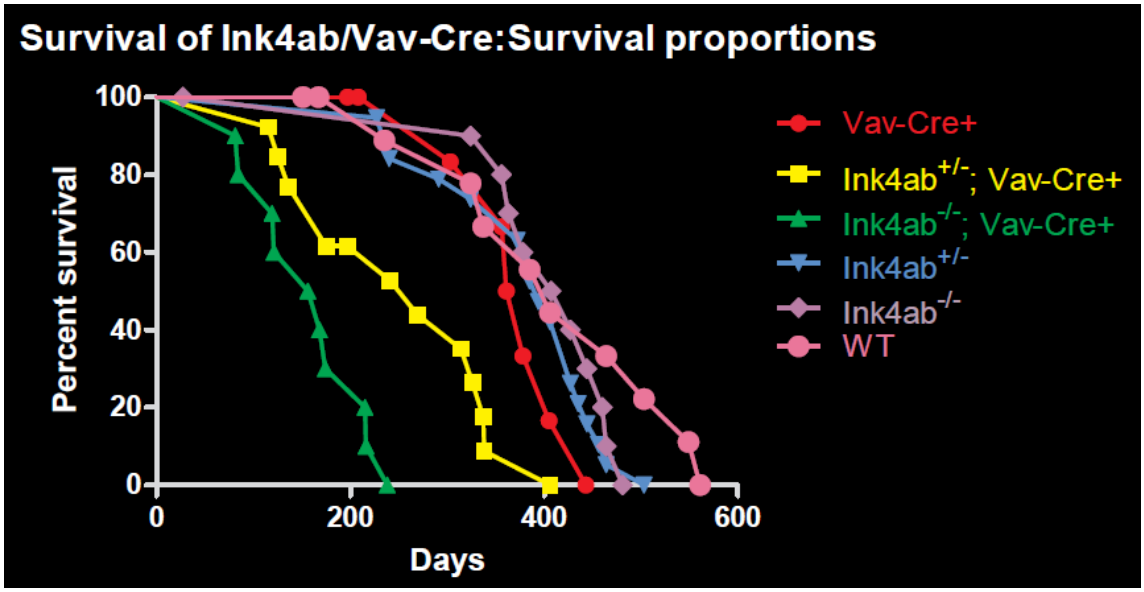


Figure 4: Survival of Ink4ab; Vav-Cre mice lines. The lowest number for a mouse line analyzed is n=8, a value that is sufficiently above the recommended n=4 for statistical significance.

3.2 Peripheral Blood Analysis of Ink4ab; Vav-Cre Line:

Analysis of the slides obtained from Ink4ab$^{-/-}$; Vav-Cre+ mice showed the presence of lymphoblasts, abnormal erythrocytes, and apoptotic neutrophils. While the apoptotic neutrophils are indicative of Ink4ab knockdown and knockout, the presence of lymphoblasts and abnormal erythrocytes indicate the potential for a blood-born cancer, specifically leukemia or lymphoma:

	Wildtype		Vav-Cre+		Ink4ab$^{+/-}$	
	Mean	±SD	Mean	±SD	Mean	±SD
Neutrophils	74.08	±4.66	68.84	±8.42	65.33	±1.98
Blast-Like Cells	0.00	±0.00	0.00	±0.00	0.05	±0.10
Lymphocytes	15.43	±4.11	20.17	±7.29	23.47	±2.69
Monocytes	7.50	±0.59	8.65	±1.42	8.46	±2.21
Eosinophils	2.84	±1.20	2.16	±0.62	2.54	±0.21
Basophils	0.15	±0.19	0.18	±0.36	0.15	±0.20

	Ink4ab$^{-/-}$		Ink4ab$^{+/-}$; Vav-Cre+		Ink4ab$^{-/-}$; Vav-Cre+	
	Mean	±SD	Mean	±SD	Mean	±SD
Neutrophils	65.74	±2.73	64.61	±0.97	65.95	±8.77
Blast-Like Cells	0.00	±0.00	0.05	±0.10	6.43	±12.87
Lymphocytes	21.74	±1.62	22.39	±0.27	17.76	±3.26
Monocytes	10.76	±1.30	10.74	±1.13	8.20	±1.75
Eosinophils	1.60	±0.26	2.02	±0.50	1.50	±0.30
Basophils	0.16	±0.20	0.20	±0.23	0.16	±0.21

Tables 1-2: A preliminary diagnosis of leukemic phenotypes was made to judge the Ink4ab; Vav-Cre line. For each line, peripheral blood samples of four mice were quantified by identification of 500 white blood cells per sample and reported in percentages above. Mice that contained over 20% lymphoblastic cells were designated as potential leukemia/lymphoma models. Student's T-test comparing the blast-like cell percentage between Ink4ab$^{-/-}$; Vav-Cre+ and Wildtype mice (t=0.999) returned a P value of 0.356275, which indicates that the difference is not statistically significant by conventional criteria ($P < 0.05$).

	Wildtype	Ink4ab$^{+/-}$; Vav-Cre+	Ink4ab$^{-/-}$; Vav-Cre+
White Blood Cells	8.4 x 10^3/µL	10.0 x 10^3/µL	10.2 x 10^3/µL
Red Blood Cells	10.8 x 10^6/µL	2.2 x 10^6/µL	3.1 x 10^6/µL
Hemoglobin	15.5 g/dL	4.0 g/dL	5.2 g/dL
Hematocrit	52%	13%	23%
Mean Corpuscular Volume (MCV)	48 fL	59 fL	74 fL
Mean Corpuscular Hemoglobin (MCH)	14.4 pg	18.2 pg	16.8 pg
Mean Corpuscular Hemoglobin Concentration (MCHC)	29.8 g/dL	30.8 g/dL	22.6 g/dL
Platelet Count	1091 x 10^3/µL	151 x 10^3/µL	86 x 10^3/µL

Table 3: Complete Blood Count (CBC) comparing extracted heart blood from wildtype mice, Ink4ab$^{+/-}$; Vav-Cre+, and Ink4ab$^{-/-}$; Vav-Cre+. While most of the values are within nominal range, the red blood cell, hemoglobin, hematocrit, and platelet counts are significantly reduced in Ink4ab$^{+/-}$; Vav-Cre+ and Ink4ab$^{-/-}$; Vav-Cre+ mice.

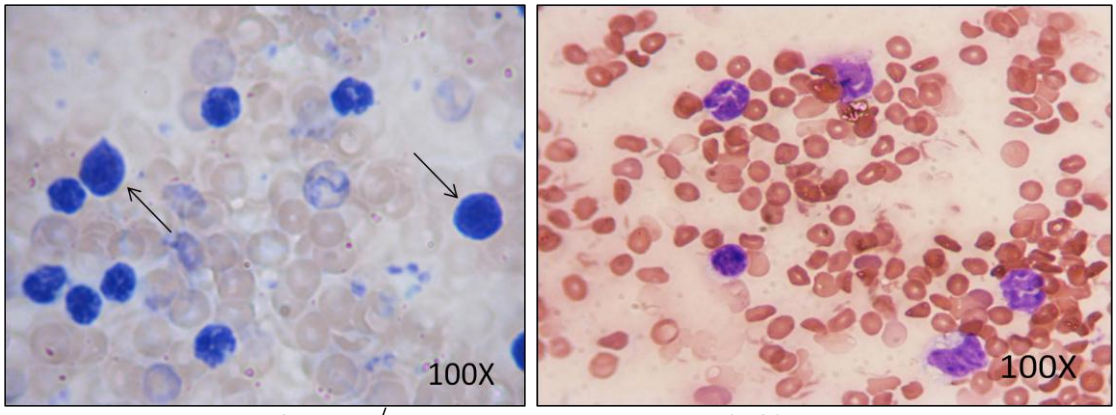


Figure 5: Comparison of Ink4ab$^{-/-}$; Vav-Cre+ peripheral blood (left) to wildtype peripheral blood (right). The Ink4ab$^{-/-}$; Vav-Cre+ image appears to contains multiple lymphoblastic cells (marked by arrows), whereas the wildtype image contains multiple neutrophils but no lymphoblastic cells.

3.3 General Dissection of Sick Mice Show Significant Increase in Spleen Size, Tumor Growth in both Liver and Spleen, and Ascites in the Peritoneal Cavity:

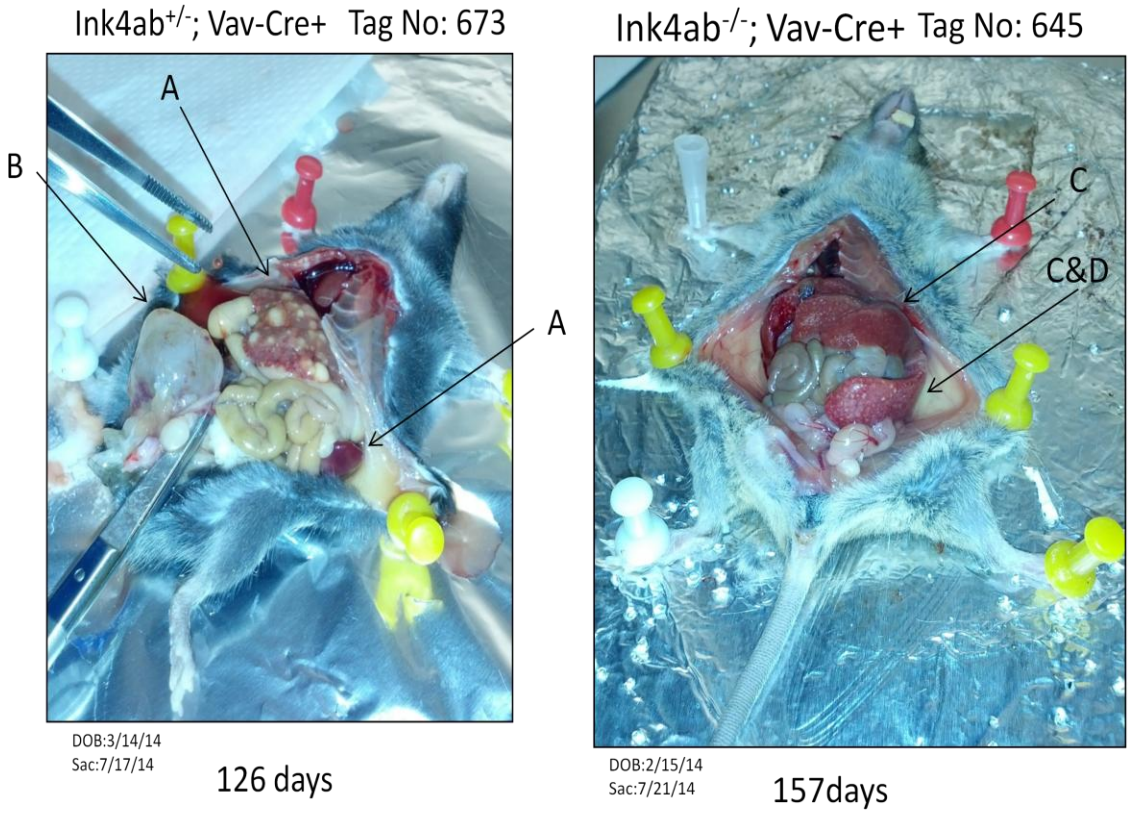
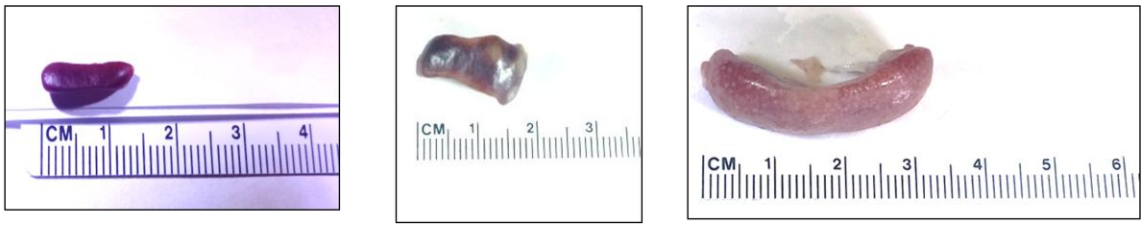


Figure 6: Dissection of Ink4ab$^{+/-}$; Vav-Cre+ and Ink4ab$^{-/-}$; Vav-Cre+ mice. Of note are the tumor formation on the liver and spleen (A) and what appears to be an obstructed urinary tract (B) in the Ink4ab$^{+/-}$; Vav-Cre+ mouse and the tumor formation on the liver and spleen (C) and the splenomegaly (D) present in the Ink4ab$^{-/-}$; Vav-Cre+ mouse.



Ink4ab$^{+/+}$; Vav-Cre+

(Tag no: 408)

Length: 1.5cm Weight: 0.075g

Ink4ab$^{+/-}$; Vav-Cre+

(Tag No:673)

Length: 2cm Weight: 0.382 g

Ink4ab$^{-/-}$; Vav-Cre+

(Tag No:645)

Length: 3cm Weight: 1.499g

Figure 7: Spleen comparison between a wildtype (Vav-Cre+) mouse control and the above two dissected mice. Tumor formation is visible in both Ink4ab$^{+/-}$; Vav-Cre+ and Ink4ab$^{-/-}$; Vav-Cre+ spleens above.

3.4 Immunohistochemical Staining of Dissected Mice Spleen show increased apoptosis, decreased mature T-cell and B-cell presence, increased cellular proliferation, decreased cell cycle inhibition, and decreased genetic repression compared to their wildtype counterparts:

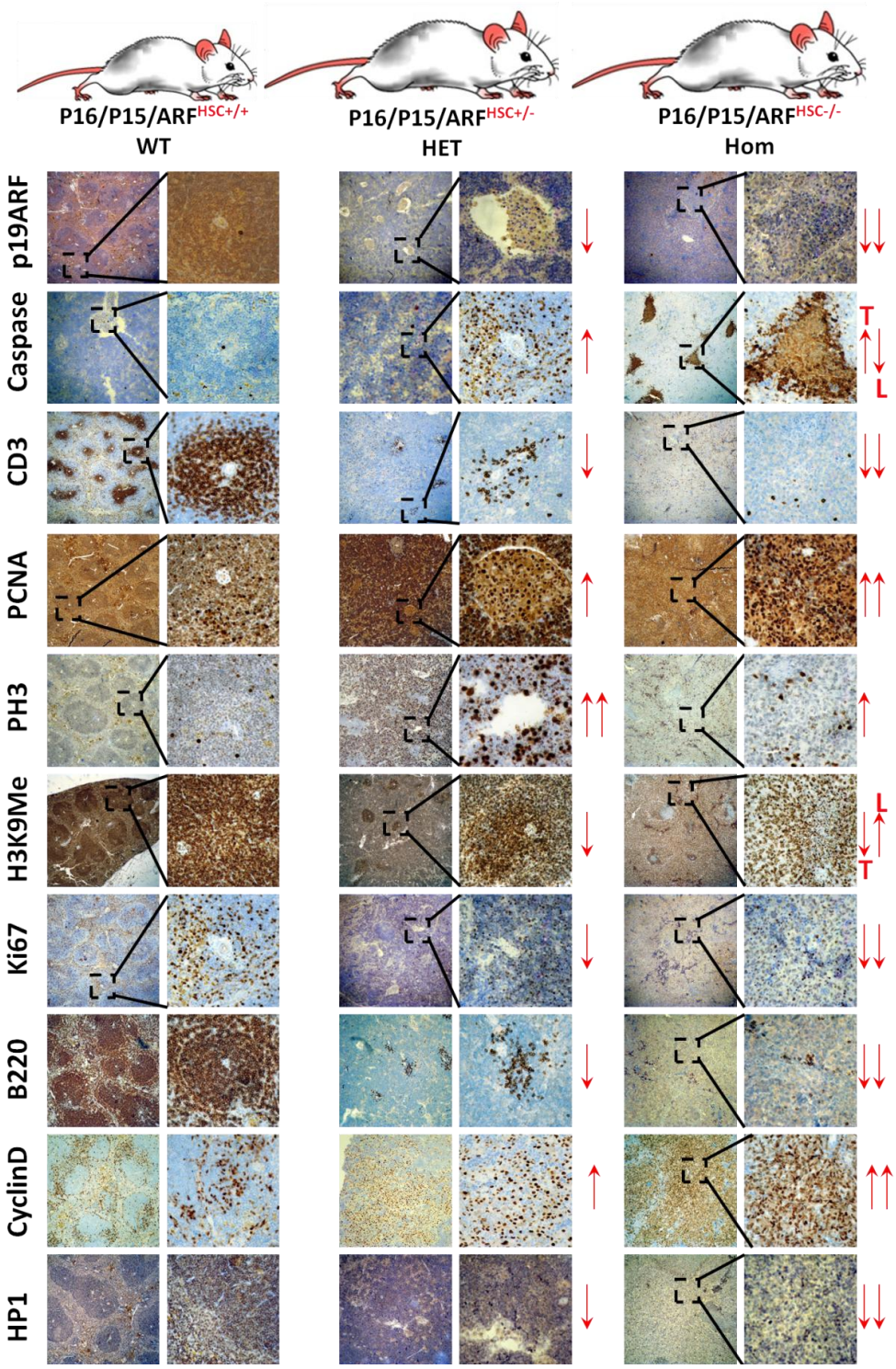


Figure 8: Immunohistochemical staining comparing multiple factors across the Ink4ab; Vav-Cre line (Flaherty et al., 2014). Antibodies successfully used for spleen cell analysis include the following:

- Caspase to test for cellular apoptosis
- CD3 to test for mature T-cell lymphocytes
- B220 to test for mature B-cell lymphocytes
- PCNA, PH3, Ki67, and Cyclin D to test for cellular proliferation
- H3K9me to test for cell cycle inhibition and delay of cell cycle and proliferation
- HP1 to test for genetic repression and transcriptional arrest

Arrows indicate the relative increase or decrease of expression in comparison to the wildtype control samples. "T" indicates T-cells and "L" indicates lymphoma cells.

3.5 H&E Staining Reveals a Diffused Structure and White Pulp Necrosis in Ink4ab$^{-/-}$; Vav-Cre+ Spleens:

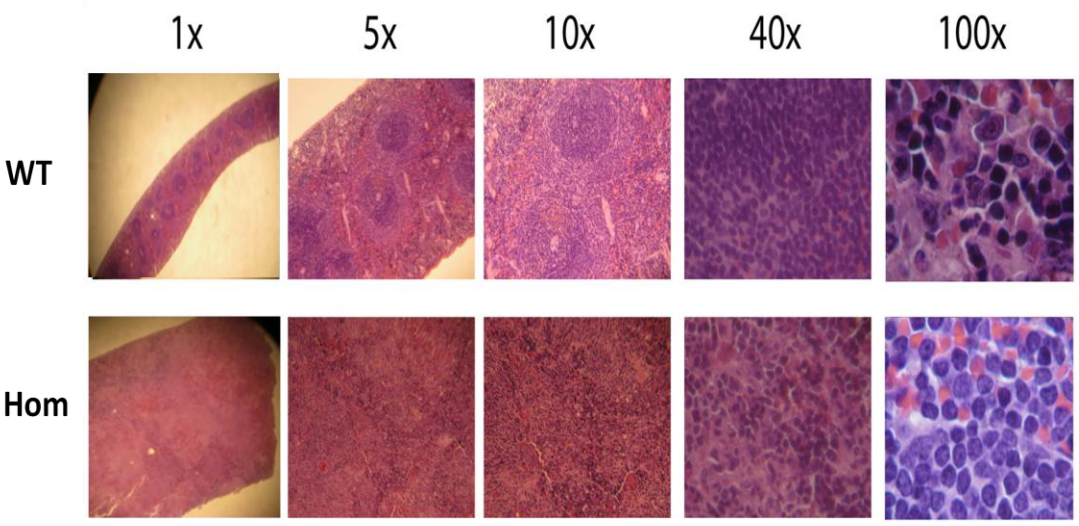


Figure 9: H&E staining comparison between wildtype (designated "WT") and Ink4ab$^{-/-}$; Vav-Cre+ (designated "Hom") spleens (Flaherty et al., 2014). Of note is the lack of white pulp structures and significant increase in leukemia/lymphoma blast-like cells in the Ink4ab$^{-/-}$; Vav-Cre+ spleen.

CHAPTER 4: DISCUSSION

4.1 Diagnosis of Ink4ab$^{+/-}$; Vav-Cre+ and Ink4ab$^{-/-}$; Vav-Cre+ mice spleen necrosis indicates multiple potential cancerous origins:

Splenic hyperplasia can be traced to two types of cancers, leukemia and lymphoma. As both are blood-based cancers, the distinction of its source is required for proper diagnosis. Leukemia starts within the bone marrow, whereas lymphoma is the cancer of the lymph system. While bone marrow has been isolated and fixed to microscope slides for viewing, it has of current not been Wright-Giemsa stained for analysis. There is also the consideration that lymphomas can begin at almost any point in the body. Both cancers can impact the spleen and the liver, which are two organs that have shown clearly visible tumor formation during dissections.

Going by the design of the Ink4ab; Vav-Cre construct, it can best be explained as a deregulation of cell cycle inhibition within the blood line. This result has been supported through both the dissections and the immunohistochemical staining of the Ink4ab$^{+/-}$; Vav-Cre+ and Ink4ab$^{-/-}$; Vav-Cre+ mice, showing increased apoptosis, decreased mature B-cell presence, increased cellular proliferation, and deregulated cell cycle compared to their wildtype counterparts.

A key fact of the above findings is the decrease in mature B-cell presence. The lack of mature B-cells may hint to a disruption of the B-cell lymphocyte hierarchy. If there is a distinct lack of mature B-cells, then the diagnosis can be directed towards B-cell precursor acute lymphoblastic leukemia (BCP-ALL), a cancer that has yet to be fully modeled and understood in mice (Van der Weyden et al., 2011). However, proper diagnosis of BCP-ALL requires confirmation that the bone marrow is the source of the malignancy, which would possibly require an Ink4ab$^{+/-}$; Vav-Cre+ or Ink4ab$^{-/-}$; Vav-Cre+ mouse that has significant lymphoblast

production prior to splenic and liver necrosis and tumor formation. Timing to obtain such a mouse would prove very difficult due to the rate of disease progression.

There is also the consideration that one of the four Ink4ab$^{-/-}$; Vav-Cre+ mice in the peripheral blood analysis was found to have a leukemic phenotype. While the dissections of the Ink4ab$^{-/-}$; Vav-Cre+ line showed tumor formation and splenic hyperplasia in multiple mice, the peripheral blood analysis for those same mice displayed both leukemic and non-leukemic lymphocyte morphologies.

4.2 Regarding the Rate of Disease Onset:

A challenge faced in the analysis of the Ink4ab; Vav-Cre mouse line was the rate of onset of the disease, compared to the window of time that symptoms would be visible within the mouse when dissected. Early signs that were looked for included shivering and enlargement of the abdomen, yet most mice would obtain severe symptoms and die within approximately one to two weeks from initial notice. This is mainly due to the Vav promoter targeting adult HSCs rather than all HSCs throughout the mouse's development (Georgiades et al., 2002). This created an acute onset of the disease within seemingly random and short intervals, depending on the maturation of the mouse in question.

If a mouse were to be dissected prior to severe symptoms, there was the potential that the spleen or other usually impacted organs would not show as aggressive of a result. This created a challenge that required a very careful balance between consistent observation and correct estimation of lifespan remaining of the target mouse. As such, some mice were unable to be analyzed due to them dying over a weekend or few-days stretch.

4.3 Future Goals:

The main future goal of this project is determination of the source, and thereby confirming the disease present, whether leukemias or lymphomas, in the Ink4ab$^{+/-}$; Vav-Cre+ and Ink4ab$^{-/-}$; Vav-Cre+ lines. One of the primary methods for confirmation includes Wright-Giemsa staining on previously obtained bone marrow samples for Ink4ab$^{+/-}$; Vav-Cre+ and Ink4ab$^{-/-}$; Vav-Cre+ mice. Should Ink4ab$^{+/-}$; Vav-Cre+ and Ink4ab$^{-/-}$; Vav-Cre+ bone marrow show significantly increased lymphoblast production and presence, with phenotypes defined by flow cytometry, especially prior to disrupting spleen and liver function, then leukemia would be able to be successfully diagnosed.

The phenotype displayed suggests the development of non-B, non-T lymphomas in noted spleens. However, accurate assessment of the phenotype requires further analyses.

Western blotting has been attempted on splenic extractions, yet has been met with some complications. The main issue has been finding a successful housekeeping gene that expresses equally within apoptotic and non-apoptotic cells. As of current, β-actin, nucleolin, and vinculin have been attempted as baseline antibodies, with all three giving unequal expression throughout the Ink4ab; Vav-Cre line (data not shown). Since a uniform baseline has not been established, expression comparisons between other antibodies on the Ink4ab; Vav-Cre line cannot be considered reliable. Should a uniformly expressed antibody be found, western blotting can be used as another tool for diagnostic analysis of the Ink4ab; Vav-Cre line.

Dissections of further diseased Ink4ab$^{+/-}$; Vav-Cre+ and Ink4ab$^{-/-}$; Vav-Cre+ mice can provide analysis of organs that were previously not considered in diagnoses. Most notably is the extraction and observation of lymph nodes, which would clearly distinguish leukemia from lymphoma. Should the lymph nodes be significantly enlarged along with the previously found splenic and liver tumor formation, lymphoma can be reliably diagnosed.

REFERENCES

Bonnet D and Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med (1997); **3**: 730-737

Davis S and Sabaawy H. Tumor suppressor functions of the zebrafish ink4ab: A novel cyclin-dependent kinase inhibitor. Cancer Research (2013); **73**: Supplement 1

Flaherty K, Jones D, Yussuf S, Davis S, Huselid E, Wang W, Bartucci M, and Sabaawy HE. Conditional mouse and zebrafish models of INK4-mediated tumor suppression reveal ARF-independent regulation of cellular senescence. American Association for Cancer Research (2014); Abstract

Geogiades P, Ogilvy S, Duval H, Licence DR, Charnock-Jones DS, Smith SK, and Print CG. vavCre transgenic mice: a tool for mutagenesis in hematopoietic and endothelial lineages. Genesis (2002); **34:** 251-256

Krimpenfort P, IJpenberg A, Song JY, van der Valk M, Nawijn M, Zevenhoven J, and Berns A. p15Ink4b is a critical tumour suppressor in the absence of p16Ink4a. Nature (2007); **448**: 943-946

Pietras EM, Warr MR, and Passegué E. Cell cycle regulation in hematopoietic stem cells. J. Cell Bio (2011); **195**: 709-720

Rad A. Hematopoiesis (human) diagram. Wikimedia Commons (2006); http://commons.wikimedia.org/wiki/File:Hematopoiesis_(human)_diagram.png

Van der Weyden L, Giotopoulos G, Rust AG, Matheson LS, van Delft FW, Kong J, Corcoran AE, Greaves MF, Mullighan CG, Huntly BJ, and Adams DJ. Modeling the evolution of ETV6-RUNX1-induced B-cell precursor acute lymphoblastic leukemia in mice. Blood (2011); **118:** 1041-1051