Dnmt3a DELETION COOPERATES WITH THE *Flt3-ITD* MUTATION TO DRIVE LEUKEMOGENESIS IN A MURINE MODEL

By

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Abstract

The advent of next generation sequencing has facilitated the establishment of an exhaustive catalog of recurrent mutations in Acute Myeloid Leukemia (AML). We know that FLT3 is the most commonly mutated gene in the disease, it is activated most commonly through internal tandem duplication (ITD) within the juxtamembrane domain, and at a lesser frequency via point mutations in the kinase domain. *Flt3*$^{ITD/+}$ knock-in mice develop a fatal myeloproliferative neoplasm (MPN) but fail to fully transform, indicating that additional mutations are necessary for leukemogenesis. Genetically engineered mouse strains provide a powerful platform to investigate what these cooperating mutations might be, and identify signaling differences in these mice.

While ITD and mutations within the kinase domain (most commonly D835Y) both constitutively activate the kinase, these mutations confer distinct prognoses and signaling differences according to survival analyses and *in vitro* studies, respectively. In addition to our *Flt3*$^{ITD/+}$ model, we generated a *Flt3*$^{D835Y/+}$ knock-in and observed several differences between the two mutant strains, including differences in the stem cell compartments and disease spectra. As in human subjects, *Flt3*$^{D835Y/+}$ conferred a more indolent disease with longer median survival than *Flt3*$^{ITD/+}$. Additionally, in agreement with previous *in vitro* studies Stat5 was preferentially phosphorylated in progenitor cells from *Flt3*$^{ITD/+}$ bone marrow compared with *Flt3*$^{D835Y/+}$. To further characterize signaling difference between these genotypes, we performed expression arrays using progenitor cells from each genotype, and identified a number of differentially expressed genes, and dysregulated pathways. A number of these candidate genes were validated by qPCR.

As previously stated, studies in *Flt3*$^{ITD/+}$ mice suggest that additional mutations are necessary for transformation. Pairwise comparisons of large datasets have identified a number of potentially cooperating mutations, including DNMT3A and FLT3-ITD, which co-occur in a significant proportion of patients, portending a poor prognosis. We examined the potential cooperativity by breeding a substrain of our *Flt3*$^{ITD/+}$ mice with a conditional knock-out of *Dnmt3a* and find that the two mutations do, indeed cooperate to drive leukemia development, expansion of multiple progenitor pools, and enhanced self-renewal. Interestingly, we found that *Dnmt3a* dosage significantly affected a number of these parameters, indicating the importance of *Dnmt3a* stoichiometry in hematopoiesis, and transformation in the context of *Flt3*$^{ITD/+}$.

Among DNMT3A^{mut};FLT3-ITD patients, it was recently discovered that a significant subset of these patients also harbor a mutant NPM1 allele (NPM1c+). We hypothesized that the addition of a mutant Nucleophosmin to our *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/f}$ model might lead to a more aggressive and uniform disease, since patients with all three mutations cluster together based on a number of other molecular parameters. While data is very preliminary, we find that the additional of NPMc+ doesn't shorten survival, but increases disease aggressiveness, with a larger percentage of blasts in the bone marrow at the time of sacrifice.

This work underscores the power and utility in discerning functionally characterizing mutations relevant to human disease. Using isogenic mouse strains, we have begun to elucidate signaling differences underlying various activating FLT3 mutations, demonstrated for the first time that mutant DNMT3A and FLT3 cooperate to drive leukemia development, and the addition of mutant NPMc+ enhances disease severity. Taken together, these mice can be used a powerful tool to discover underlying disease mechanisms, and a platform for transplantation studies to test novel therapeutics.

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Preface

I am profoundly grateful for the opportunity that I've had to conduct this research, and for the many special people that I've met along the way. First and foremost, I thank and acknowledge my thesis advisor and mentor, Don Small. I previously studied leukemia for several years using a forward genetics approach, mapping the breakpoints of novel chromosomal rearrangements in patient samples. I feel extremely fortunate that my interests and passions aligned with Don's, giving me the opportunity to continue studying leukemia through functionally characterizing similar disease associated mutations in murine models. Through this work, I vastly expanded my skill set, and benefited from Don's extensive knowledge of the field, which guided and shaped my project. His humor and patience, as a friend and a mentor, will stay with me throughout my career.

A number of people in the Small lab have contributed to the work described in this dissertation. Emily Bailey generated the *Flt3*$^{D835Y/+}$ knock-in mice, and performed the microarray experiments that I analyzed and interpreted in Chapter 2. Li Li greatly assisted in those efforts, and generated the *Flt3*$^{ITD/+}$ knock-in mice described throughout this report. She has been a tremendous resource throughout my training, performing all my transplant experiments and assisting me whenever I needed help. She is truly the lab sensei, and deserves a great deal of gratitude. Diane Heiser was instrumental in establishing the colony of mice used in chapter 3, which yielded extremely exciting results. I also want to thank Amy Duffield for applying her extensive training as a pathologist and scientist to accurately diagnose the mice in chapters 3 and 4. I thank Hayley Ma, Bao Nguyen, and Courtney Shirley for their intellectual contributions and assistance in these chapters. To the entire lab: I couldn't have asked for a better group of people

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Our collaborator Chris Gamper has long been interested in the role of Dnmt3a function in the immune system, and provided the *Dnmt3a*$^{f/f}$ mice used in chapters 3 and 4, as well as primers, advice, laughs, and moral support. Sarah Wheelan and Vasan Yegnasubramanian also contributed valuable input regarding future experiments to assess methylation and expression changes in these mice. I thank Pat Brown for providing moral support and scientific insight during my graduate career, and fostering a collaborative lab environment. The NPMc+ mice used in chapter 4 were kindly provided Pat's talented physician scientist Rachel Rau, while a number of other Brown lab members, especially Colleen Annesley, never hesitated to share reagants and guidance in my mouse studies. These collaborations grew into friendships, and are among the most rewarding aspects of my graduate experience.

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It has been a great honor and privilege to train in the Human Genetics PhD program, among such talented and passionate scientists. The rigorous training I received was supported through the efforts of program director Dr. Valle, co-director Dr. Smith, and administrator Sandy Muscelli. I thank them for fostering an environment for personal and scientific growth, and the personal attention they provided whenever I needed it. I also thank the many faculty members,

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This dissertation is dedicated to my father, James A. Poitras, whose unfortunate lymphoma diagnosis sparked my interest in cancer research and genetics. He worked third shift 3 days a week, and still managed to have dinner with us every night, help us with our homework, and attend parent teacher conferences. His perseverance, selflessness, and encouragement are responsible for creating the person I've become, and I would never have made it this far without him. If someone told me in high school that I'd one day complete a PhD at Johns Hopkins, I never would've believed it. My father wouldn't have been surprised because he always made sure I knew that he believed I was smart enough and capable of anything, even when I didn't think I was good enough. This one's for you, dad.

"When there was no dream of mine, you dreamed of me"

-The Grateful Dead

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1. CHAPTER 1. INTRODUCTION

1.1 Overview of AML

Acute Myeloid Leukemia (AML) is an aggressive neoplastic condition, leading to accumulation of immature myeloid cells and ineffective hematopoiesis. In 2014, the American Cancer Society projected around 19,000 newly diagnosed Acute Myeloid Leukemia (AML) cases, and around 11,000 deaths. AML arises when mutations occur in a primitive progenitor cell within the bone marrow niche, which are then propagated to the cellular progeny. Additional genetic changes are acquired, conferring a selective advantage to these cells, and subsequent clonal outgrowth of immature, abnormally functioning, rapidly dividing stem cells. This step wise genetic progression of cancer development was first proposed by Peter Nowell (Nowell,P.C. 1976), however thorough investigation and identification of what these mutations might be wouldn't be feasible until decades later, with the introduction of next generation sequencing (Bentley,D.R. 2008).

Over the last several decades, the WHO and FAB classifications of leukemia subtypes were descriptive, relying on morphological changes to classify patients. The advent and affordability of whole genome sequencing, (WGS) presented an opportunity to completely annotate the leukemia genome, identifying a number of genetic perturbations (sequence variants, expression differences and methylation changes) that might correlate with clinical outcomes and aid in the understanding of disease pathogenesis. With an extensive list of mutations, we have identified particular mutations, including internal tandem duplications within FLT3 (FLT3-ITD), which portend a particularly poor prognosis. Understanding the functional impact of these

mutations through in vitro and in vivo studies is essential in identifying new, effective therapeutic targets.

1.2 FLT3 in normal hematopoiesis

FLT3 is a receptor tyrosine kinase, which is expressed in the earlies stages of hematopoiesis, and is turned off during differentiation in a lineage specific manner (Matthews,W. 1991). Expression of FLT3 is essential to maintaining healthy hematopoeisis, as targeted disruption of the gene results in defective stem cell engraftment and B cell progenitor defects in the bone marrow (Mackarehtschian,K. 1995). In development of lymphoid cells, FLT3 is expressed in common lymphoid progenitors (CLP), but is shut off during later points of lymphocyte development. FLT3 expression in myeloid cells is restricted to a smaller population wtihin the common myeloid progenitor (CMP) compartment, but is extremely important in facilitating dendridic cell development (63 D'Amico,A. 2003).

Normally, FLT3 is stimulated by its ligand, cuing the cell to grow by activating multiple downstream signaling pathways including MAPK and PI3K. This stimulation drives cells to rapidly divide and differentiate into more restricted progenitors, predominantly cells within the myeloid lineage. Knock-out of the ligand (*Flt3-L*) in mice results in even more severe defects than Flt3 deletion, underscoring the importance of FLT3 in normal blood development (McKenna,H.J. 2000).

1.3 The role of FLT3 and FLT3 mutations in AML

FLT3 is now recognized as the most frequently mutated gene in AML, where activating mutations in either the juxtamembrane or kinase domain of the protein predict distinct clinical outcomes. About 25% of AML patients harbor an internal tandem duplication within the juxtamembrane of FLT3, which confer reduced overall survival and increased rates of relapse

(Shen,Y. 2011). These in-frame mutations vary in size, from 9-300 base pairs, and lead to ligand independent activation of downstream oncogenic pathways, including MAPK and PI3K. Additionally, phosphorylated STAT5 seems to be particularly prominent in this context, suggesting this transcription factor is important in the disease. Less commonly, point mutations occur in the kinase domain (most often D835Y), and predict a more favorable prognosis than the FLT3-ITD.

Given the mutation frequency and poor prognosis associated with FLT3-ITD, our lab previously sought to understand if this mutation alone was sufficient to drive leukemia development using a knock-in mouse model (Li,L. 2008). $Flt3^{ITD/+}$ mice succumb to a fatal myeloproliferative neoplasm (MPN) with a median survival of around 350 days but failed to develop AML, suggesting additional cooperating mutations are necessary to drive leukemogenesis.

Subsequent studies in these mice have revealed potential underlying roles of Flt3-ITD in leukemogenesis, including defective DNA repair and stem cell abnormalities. For example, knock-in of the same Flt3-ITD mutation results in a block in differentiation of the B cell lineage, as Pro-B cells exhibit increased double strand breaks induced by VDJ recombination machinery, but ineffective repair via non-homologous end joining (NHEJ) (Li,L. 2011). This leads to a reduction in the number of B cells and a block in differentiation. These findings have important implications about the contribution of FLT3-ITD to leukemia development, as these mutations may compromise the integrity of the genome within the bone marrow. Murine studies have also implicated FLT3 as a critical regulator of quiescence, as knock-in of the ITD mutation leads to hyperactive signaling through PI3K, MAPK, and Stat5, directing an abnormal exit from

quiescence, driving cells to proliferate and differentiation toward a myeloid fate (Small,D. 1994; Chu,S.H. 2012).

1.4 Sequencing efforts to identify cooperating mutations

The commercial availability of next generation sequencing in 2008 enabled researchers all over the world to sequence tumor samples in a high throughput manner. By sequencing whole genomes and exomes of leukemic and matched normal samples, we were suddenly able to extensively catalog and annotate the leukemia genome. These studies yielded important findings regarding the frequency of each mutation among AML patients, and established 8-13 as the median number of mutations in a given leukemia, which is decidedly lower than most other solid tumors (Vogelstein,B. 2013).

Given that numerous mutations are present within a typical AML sample, it is not surprising that while *FLT3* mutations are the most frequent, commonly *FLT3-ITD* (16 Cancer Genome Atlas Research Network 2013; 15 Welch,J.S. 2012), these mutations are insufficient to alone cause leukemia in murine models and likely require additional genetic events to fully transform (Li,L. 2008; Bailey,E. 2013). Using the wealth of whole genome sequencing data, we can determine which mutations are concurrently mutated to hypothesize what these cooperating events might be.

In a 2012 study, Patel et al. performed targeted sequencing on a cohort of 400 AML patients, examining fifty of the most frequently mutated genes (Patel,J.P. 2012). By performing pairwise comparisons, they determined which genes were commonly co-mutated in patients. Once again, *FLT3* was established as the most frequently mutated gene, and these patients also harbored concomitant *DNMT3A* mutations. Additionally, when stratifying patients by FLT3-ITD mutation status, those with comcomitant *DNMT3A* mutations exhibited poorer overall survival

compared with other genotypes, underscoring the likelihood that mutant *FLT3* and *DNMT3A* represent synergistic and cooperating mutational events in leukemia.

1.5 DNMT3A in stem cell function and transformation

Beyond changes to the DNA sequence, epigenetic modifications, including DNA methylation and histone modifications, have long been established as important regulators of gene expression and function. DNA methyltransferases covalently link methyl groups to cytosines (or adenines) to control gene expression via two different mechanisms. Maintenance methyltransferases, such as DNMT1, ensure stable transmission of methylation from a dividing cell to the resulting daughter cells. De novo methyltransferases, including DNMT3A and DNMT3B, produce labile modifications to the DNA, responding to environmental cues to methylate novel loci, controlling gene expression as necessary. Recent Crispr/Cas9 disruption of *DNMT1* in human embryonic stem cells resulted in rapid cell death, while mutating *DNMT3A* and/or *DNMT3B* resulted in global hypomethylation, but less severe cellular defects (Liao,J. 2015). In the hematopoietic system, conditional knock-out of *Dnmt1* in the stem cell compartment leads to stem cell depletion, cell cycle abnormalities, and defects in self-renewal (Trowbridge,J.J. 2009). Conversely, conditional ablation of *Dnmt3a* in murine bone marrow results in expansion of the long term hematopoietic stecm cell (LT-HSC) pool through enhanced self-renewal (Challen,G.A. 2011). These opposing phenotypes indicate that DNMT1 and DNMT3A play distinct functions in the hematopoietic system. These experiments provide a biological basis for the preponderance of mutations in DNMT3A, and near absense of DNMT1 mutations found in leukemias (Cancer Genome Atlas Research Network 2013).

DNMT3A, like *FLT3*, is one of the most frequently mutated genes in AML. Missense mutations in *DNMT3A* are nearly always heterozygous in this context, often occuring in the methyltransferase domain. Within this hot spot, an amino acid substitution altering Arginine 882 to Histidine is most common, and several lines of evidence suggest these heterozygous alterations result in a dominant negative loss of function (Zhang,J. 2012; Walter,M.J. 2011; Tadokoro,Y. 2007; Holz-Schietinger,C. 2012). *In vitro* experiments have shown the R882H mutation significantly inhibits tertramerization, and processive methylation of promoters (Holz-Schietinger,C. 2012; Kim,S.J. 2013). These findings were corroborated when Tim Ley's group mined the TCGA data set, finding R882H mutant patient samples exhibited significant global hypomethylation, and again, resulted in ineffective tetramer formation (Russler-Germain,D.A. 2014). Ideally, a mouse harboring a heterozygous mutation knocked into the orthologous residue (R878H) would present the ideal tool to rigorously perform in vivo experiments, no such model is currently available. Given the overwhelming evidence that the common R882H mutation results in a dominant negative loss of function, conditionally knocking out *Dnmt3a* in a bone marrow specific manner presents the best approach to biologically model these mutations.

Extensive work using inducible, hematopoietic specific knock-out of *Dnmt3a* has defined the gene as essential in regulating self-renewal, as its deletion results in significant expansion of LT-HSCs (Challen,G.A. 2011). Global methylation analysis of LT-HSCs revealed that Dnmt3a loss resulted in hypomethylation and increased expression of important genes known to maintain stemness. These genes failed to be appropriately methylated in downstream, differentiated progeny, where this stem cell signature continued to be aberrantly expressed. Upon further analysis of these data, it became clear that the genomes of LT-HSCs is partitioned into larger segments termed "canyons", where a lack of methylation is flanked by hypermethylated regions

and concomitant H3K27 trimethylation (Jeong,M. 2014). Dnmt3a deletion often led to expansion of these canyons, often containing stem cell related genes, indicating that Dnmt3a is important in maintaining appropriate expression of these genes through site specific CpG methylation, as well as maintaing appropriate methylation at canyon borders. Loss of these methylation patterns therefore leads to uncontrolled expression of these genes, enhanced stem cell self-renewal, and expansion of this progenitor pool.

In a study similar to the one we previously performed with our *Flt3-ITD* knock-in model, these *Dnmt3a* conditional knock-out mice (*Dnmt3a*$^{f/f}$) were watched over time for disease development to assess the ability for *Dnmt3a* deletion alone to cause leukemia (Mayle,A. 2014). Unlike our *Flt3-ITD* mice, which solely develop MPN, developed a spectrum of neoplasms, including AML, T-ALL, and preleukemic conditions. However, median survival was around 300 days, which is significantly long for a mouse model of leukemia. We hypothesized that breeding this model with our *Flt3-ITD* knock-in, representing two of the most frequently co-mutated genes in AML, would cooperate to shorten survival and drive AML development, thus mimicking human disease.

1.6 NPM1 as a potential cooperating event

Along with *DNMT3A* and *FLT3*, *NPM1* (Nucleophosmin) is among the most frequently mutated genes in AML, and occurs at a similar frequency (Patel,J.P. 2012). As a nucleolar protein, NPM1 normally shuttle between the nucleus and cytoplasm to exert a number of critical cellular functions, including ribosomal biogenesis, controlling cell growth through transcriptional regulation, and maintaining genomic stability (Grisendi,S. 2006). In the setting of normal karyotype AML (NK-AML), *NPM1* mutations are heterozygous. Referred to as NPMc+, these mutations disrupt the nucleolar localization signal in the cytoplasmic portion of the protein,

and replace it with a novel nuclear export motif (66 Falini 2006). Abnormal nuclear export leads to accumulation of the protein in the cytoplasm resulting in failed p53-dependent cell cycle arrest due to the inability of mutant NPM1 to interact with p19Arf (Falini,B. 2009).

Several transgenic mouse models have been generated to study NPMc+ function, but the model discussed in chapter 4 harbors a mutant NPMc+ transgene driven by the human, myeloid specific promoter MRP8 (hMRP8-NPMc+) (69 Cheng,K. 2010). Characterization of these mice found, yet again, that while these mutations are common in AML, they alone appear to be insufficient to result in full blown leukemia, as these mice succumb to a fatal MPN with incomplete penetrance. As NPMc+ mutations occur with the FLT3-ITD mutation at an appreciable frequency, and their co-occurrence is a poor prognostic indicator (Meyer,S.C. 2014), $Flt3^{ITD/+}$ mice were bred with the (hMRP8-NPMc+) model and followed for survival (8 Rau 2014). These mutations did indeed cooperate to generate myriad neoplasms, including AML, MPN, and T-ALL, however the median survival was extremely long at 420 days. As previously mentioned, the median number of mutations in an AML sample is between 8-13, indicating that additional cooperating mutations may be necessary to accelerate the disease in the murine setting.

A recent TCGA publication identified a unique patient population harboring mutations in DNMT3A, FLT3, and NPMc+ concurrently, exhibiting common clinical features (16 Cancer Genome Atlas Research Network 2013). Breeding the hMRP8-NPMc+ onto the $Flt3^{ITD/+}$;$Dnmt3a^{f/f}$ background presents the an opportunity to study the potential cooperativity of the three mutations in vivo.

2. CHAPTER 2. KNOCK-IN OF SPECIFIC *FLT3* MUTATIONS IN MICE FACILITATES STUDY OF SIGNALING DIFFERENCES

2.1 Introduction

FLT3 is well established as the most frequently mutated gene in AML, with two predominant classes of mutations constitutively activating the kinase (Patel,J.P. 2012; Meshinchi,S. 2009; Cancer Genome Atlas Research Network 2013). Internal tandem duplications of the juxtamembrane domain (FLT3-ITD) are more common, occuring in 25% of patients, portending a worse prognosis than point mutations in the kinase domain (FLT3-KD), which are less common, occurring in 10% of patients. FLT3-ITD mutations induce ligand independent of downstream pathways PI3K, RAS, ERK, as well as enhanced STAT5 phosphorylation. FLT3-KD mutants activate the same signaling cascades, however STAT5 is only weakly activated, indicating distinct pathways are important for disease development, which may also account for differing prognoses between the two mutations (Meshinchi,S. 2009).

Our lab previously generated a $Flt3^{ITD/+}$ knock-in harboring an 18 base pair ITD cloned from a patient. These mice developed a fatal MPN with a median survival of 400 days. To assess the effects of FLT3-KD in vivo in a similar manner, the lab generated a knock-in of the most common mutation D835Y (the orthologous mouse residue is D838, which was altered to D838Y, but these mice will be referred to as $Flt3^{D835Y/+}$ for simplicity's sake) (Bailey,E. 2013). Similar to the $Flt3^{ITD/+}$ model, the D835Y mutation alone was insufficient to leukemia development, as these mice developed MPN, histiocytic sarcomas, and B and T cell lymphomas. Of note, median survival for $Flt3^{D835Y/+}$ mice was 678 days, vs. 394 days in the $Flt3^{D835Y/+}$ mice. Additionally, western blot experiments on the bone marrow showed that Stat5 phosphorylation was far less

prominent in the *Flt3*$^{D835Y/+}$ mice compared with *Flt3*$^{ITD/+}$. The extended survival and attenuated Stat5 phosphorylation seen in *Flt3*$^{D835Y/+}$ mice mimics phenotypic differences seen in patients with FLT3-KD and FLT3-ITD mutations, indicating that these mouse models present a powerful tool to assess the diverging signaling pathways responsible for eliciting these differences.

2.2 Results

2.2.1 Expression array identifies differentially expressed genes in Flt3$^{ITD/+}$ and *Flt3*$^{D835Y/+}$ progenitors

The MouseWG-6 v2.0 Microarray allowed us to analyze over 45,200 transcripts simultaneously in LSK cells from wild type, *Flt3*$^{ITD/+}$, and *Flt3*$^{D835Y/+}$ mice at an early time point, prior to disease presentation in *Flt3*$^{ITD/+}$ mice. After normalizing the data, hierarchical clustering was performed to determine similarities between individual samples. As expected, biological replicates from each genotype clustered with eachother (figure 2-1A). Initially, 4 samples per genotype were included in the study. Initial hierarchical clustering aligned one of the *Flt3-ITD* samples in the wild type cluster. The outlying samples was therefore excluded, leaving only 3 biological replicates from *Flt3*$^{ITD/+}$ mice for analysis. Additionally, wild type and D835Y samples are members of the same bifurcating arm on the dendogram, while ITD samples are separated to the left. These results indicate that expression patterns separate ITD samples from both other genotypes, while D835Y and wild type samples were more similar to eachother.

After hierarchical clustering, a Benjamini-Hochberg correction was used to derive adjusted p values, and identified 1301 probes in D835Y samples and 1471 probes in ITD samples were differentially expressed compared to wild type mice. To reduce the number of targets, we used a $\log_2$ fold change cut off of 1 (absolute fold change of 2), and an adjusted p

value of less than 0.05. Establishing these cut offs identified 185 genes overall that were differentially expressed over both Flt3 mutant genotypes (figure 2-1B). In keeping with the hierarchical clustering data, these thresholds identified more differentially expressed genes in the ITD samples than the D835Y samples; 162 genes vs 45 genes respectively (figure 2-1C). 22 of these genes were differentially expressed in both mutant populations, and just one of these genes, *Dntt*, was reciprocally expressed as it was upregulated in D835Y and downregulated in ITD compared to wild type (figure 2-1D).

2.2.2 Validation of candidate genes in murine progenitors by qPCR

To confirm the *Dntt* expression patterns identified in the microarray, we performed qPCR on lineage negative bone marrow from each genotype, using mice ranging in age from 6 to 20 weeks. At each time point, expression patterns in qPCR experiments validated the dysregulation seen in the array (figure 2-2). By expanding the threshold to include any reciprocally expressed gene above $\log_2$ fold change of 0.5 (absolute fold change ~1.41) we identified (X) number of differentially expressed genes, (X) of which were reciprocally expressed. We additionally validated two of these genes (*Gata2* and *Kdm7a*) in the same manner as with *Dntt*, and corroborated the findings from the microarray, with Kdm7a being overexpessed in D835Y and underexpressed in ITD samples compared to wild type, and Gata2 being downregulated in both genotypes.

One gene in particular seemed to display the predicted reciprocal expression in an age dependent manner. The results from the microarray predict *Cox6a2* to be upregulated in D835Y and downregulated in ITD samples. Reciprocal expression isn't observed until 20 weeks of age (figure 2-3A). This progressive dysregulated expression of *Cox6a2* may represent an important event in disease progression, as ITD mice develop disease more rapidly than D835Y mice, and

down regulation occurs in ITD samples at 20 weeks of age, a time point more similar to the time point assayed in the microarray.

To assess protein levels of Cox6a2 between genotypes, we performed western blotting on protein samples from Ba/F3 cells transduced with human wild type FLT3, FLT3-D835Y, or FLT3-ITD overexpression constructs (figure 2-3B). While Cox6a2 protein levels failed to exhibit lower levels in FLT3-ITD expressing cells compared with wild type FLT3 expressing cells, protein expression is extremely elevated in FLT3-D835Y cells, in agreement with the array results. The failure of protein expression to be depressed in FLT3-ITD cells may reflect the nature of the cell line used. Ba/F3 cells are a lymphoid cell line, and the expression data was derived from primary mouse progenitor cells. To examine this possibility, we performed western blot experiments to detect Cox6a2 protein in lineage negative cells from 6 week old mice (figure 2-3C). While protein expression was lower in cells from *Flt3*$^{ITD/+}$ mice compared with *Flt3*$^{D835Y/+}$ samples, the lack of a wild type control here makes it difficult to draw comparisons relative to the normal progenitor population. Additionally, 20 week old mice were not available at the time this experiment was performed. Given that qPCR validation at this later time point more closely resembled the microarray expression levels, future experiments in primary cells should be performed in that later time point.

We hypothesized that since Cox6a2 is a protein expressed in the mitochondrial as one of the most terminal enzymes in the electron transport chain, we hypothesized perhaps the downregulation of *Cox6a2* seen in *Flt3*$^{ITD/+}$ mice may lead to uncoupling of the electron transport chain, disrupted membrane potential, and increased ROS production, as the ITD mutation has been associated with increased reactrive oxygen species previously (Sallmyr,A. 2008). To assess ROS levels, we incubated lineage negative cells from each genotype at 20

weeks of age with a cell-permeant dye that fails to fluoresce in its reduced state, but once oxidized by ROS, exhibits bright fluorescence. After incubation, flow cytometry revealed extremely high fluorescence levels in Lin- cells from *Flt3*$^{ITD/+}$ mice, and no real difference between wild type and *Flt3*$^{D835Y/+}$ samples (figure 2-3D). ROS levels assessed at earlier time points were marginally increased in both Flt3 mutant genotypes compared to wild type (figure 2-3E). The exaggerated ROS production at 20 weeks specifically correlated with depressed Cox6a2 in the microarray, and qPCR experiments from ITD mice at this time point, indicating that dysregulation of this mitochondrial protein may be playing a role. Further experiments are necessary to define a causative role.

2.3 Discussion

Discovering the causative signaling differences in which dictate differing clinical outcomes in FLT3-ITD and FLT3-TK mutated AMLs is important in providing insight into disease development, and subsequently informs targetable pathways to more effectively treat the disease. Our knock-in mice of each mutation recapitulate the survival variations and signaling differences seen in patients, including variable STAT5 phosphorylation. Performing expression analysis within the progenitor compartments in these mice presents a genetically pure system to investigate the contribution of each mutation to downstream signaling activation.

The attenuated phenotype in the *Flt3*$^{D835Y/+}$ mice correlated with expression findings, as expression patterns were more similar to wild type mice compared *Flt3*$^{ITD/+}$ mice. When considering all differentially expressed genes reaching statistical significance and a log fold change of 2, *Dntt*, otherwise known at Tdt, was upregulated in LSK cells from *Flt3*$^{D835Y/+}$ mice, and downregulated in *Flt3*$^{ITD/+}$ samples. This specialized polymerase is expressed in immature B and T cells, and is responsible to repairing DNA breaks created to generate junctional diversity

in these cell populations (Mahajan,K.N. 1999; Mahajan,K.N. 2002). The overexpression of this gene in *Flt3*$^{D835Y/+}$ progenitors may account for the T and B lymphomas that develop in these knock-in mice. Conversely, repressed *Dntt* expression in *Flt3*$^{ITD/+}$ may be responsible for solely driving these mice toward a myeloid fate, and provide a mechanism for the defective NHEJ observed in these mice (Li,L. 2011). We attempted to perform intracellular staining and flow cytometry to correlate protein expression with mRNA levels, however these experiments are difficult to perform, and presented a number of challenges. Optimization of the protocol, and/or western blotting for the protein will be necessary to further confirm the microarray results.

Unlike *Dntt*, which was exhibited similarly dysregulated expression levels at each time point assayed from primary mouse cells, *Cox6a2* exhibited dysregulation most similar to microarray experiments at 20 weeks of age. Since 12-16 week old mice were used for the initial microarray experiments, the 20 week time point more accurately represents the primary data we are attempting to validate. Decreased expression in the *Flt3*$^{ITD/+}$ mice may lead to increased ROS production in these mice, as inhibited COX protein expression is associated dissipation in mitrochondrial membrane potential, oxidative stress and dysfunction, and resultant ROS production (Lecoeur,H. 2012). Knockdown and overexpression experiments in these cells will be necessary to begin linking *Cox6a2* expression to ROS production.

2.4 Conclusions

The data presented in this chapter begins to reveal expression differences between *Flt3*$^{ITD/+}$and *Flt3*$^{D835Y/+}$ progenitors that may be responsible for the survival differences between the two models, and ultimately patients with the two mutations. Expression patterns were more similar in *Flt3*$^{D835Y/+}$ and wild type samples compared with *Flt3*$^{ITD/+}$, consistent with the indolent

phenotype in D835Y mice, and known expression differences that remain similar to wild type as previously described. For the first time, we identified reciprocally expressed genes between the two mutant genotypes that may account for phenotypes diverging phenotypes observed in mice. Further investigation is necessary to define extensive pathway maps to indicate how these reciprocally expressed genes may fit into targetable pathways.

2.5 Materials and methods

2.5.1 RNA extraction and expression array

Whole mouse bone marrow was extracted from *Flt3*$^{ITD/+}$, *Flt3*$^{D835Y/+}$, and wild type mice (4 mice per genotype) at 12-16 weeks of age, prior to overt disease onset. LSK cells (Lin$^-$c-Kit$^+$Sca-1$^+$) were sorted using the FACS Aria (BD Biosciences). RNA was extracted from LSK cells using the RNeasy Micro Kit (Qiagen), and quality and concentration were subsequently assessed using the Bioanalyzer (Agilent). Samples were then hybridized to the MouseWG-6 v2.0 Microarray (Illumina) in the JHMI Deep Sequencing and Microarray Core.

2.5.2 Analysis of expression array data

Analysis of raw data. Text files containing the raw data were processed and analyzed using the Limma package in R**.** All code for the these analyses can be found in Appendix 1. The heatmap in figure 2-1B represents the 185 genes that are differentially expressed among wild type, *Flt3*$^{ITD/+}$, and *Flt3*$^{D835Y/+}$ LSK cells. These genes were identified by establishing a fold change cut off of 2, adjusted p value of 0.05. P values were adjusted using the Benjamani Hochberg method. The differentially expressed genes are represented in the Venn diagram in figure 2-1C. The 22 differentially expressed genes that are common to both mutant genotypes are shown in the heatmap in figure 2-1D.

2.5.3 Validation of candidate genes

Target validation of reciprocally expressed genes. (Figures 2-2 and 2-3) RNA was extracted from Lineage negative bone marrow from wild type, ITD, and D835Y mice using TRIzol (Invitrogen), and reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative RT-PCR was performed using the iCycler iQ multicolor real-time PCR system (Bio-Rad), and transcript levels were normalized to *Rps16*. Primers used are in (Table 2-1). Fold changes were caldulated using the delta-delta Ct method.

2.5.4 Analysis of Reactive Oxygen Species

Lineage negative bone marrow cells were collected at the specified time points, and seeded at 100,000 cells per well in 12 well dishes in RPMI 1640. Cells were incubated with CellROX deep Red reagant (ThermoFisher) at a final concentration of 5uM for 30 minutes at 37^{o}C. Cells were washed, and subjected to subjected to flow cytometry. The MFI graphed in figure 2-3E is calculated by subtracting MFI(CellROX)-MFI(unstained)

2.5.5 BaF3 cell culture

BaF3 cells were cultured and maintained as previously described (Williams,A.B. 2012).

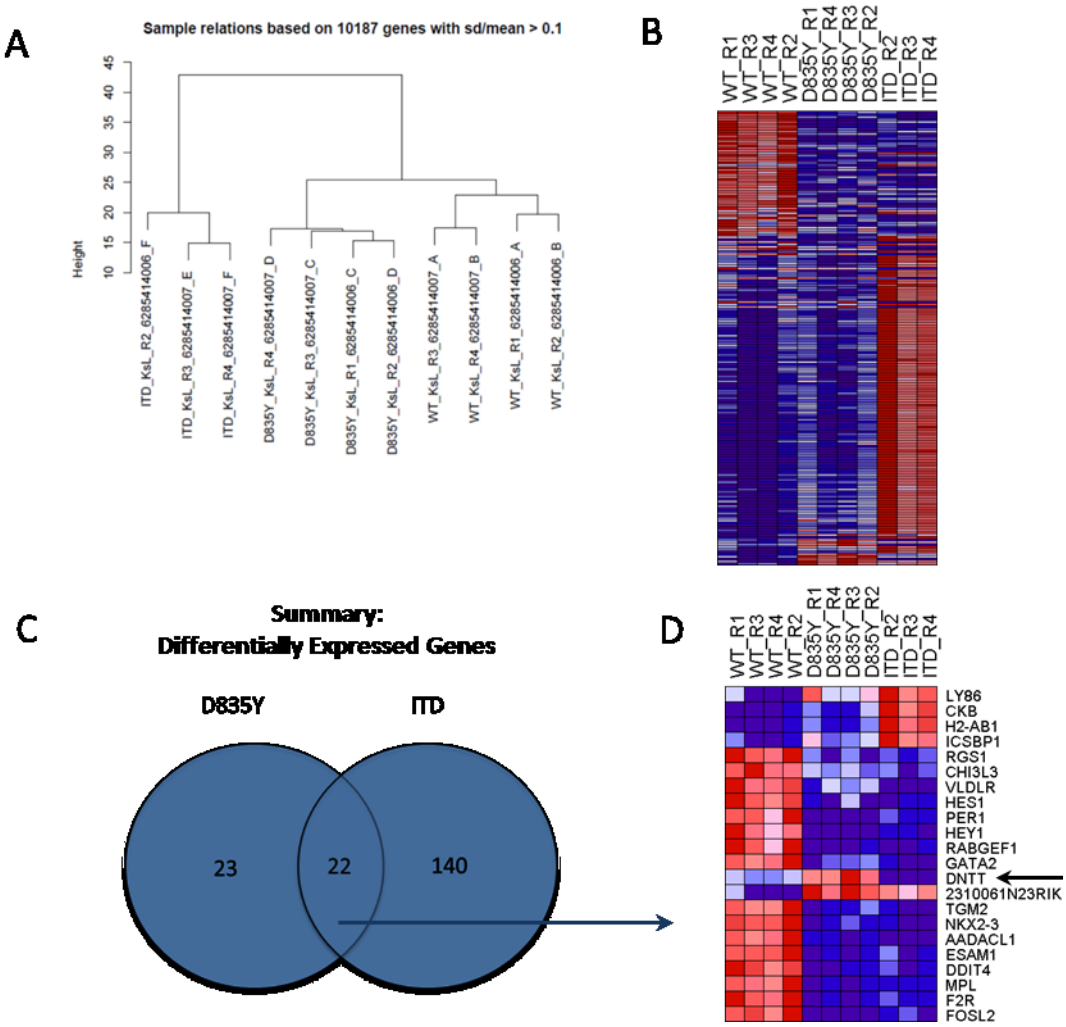
2.6 Tables: Chapter 2

Table 2-1. Primers for target validation

Primer	Sequence
Socs2 F	GAG CTC AGT CAA ACA GCA TGG
Socs2 R	CGG TCC AGC TGA CGT CTT A
Dntt F	GAA GCC ACA GAG GAT GAA GAG
Dntt R	GAC TCT AAG ATG TCG CAG TAC AA
Il7R F	AGT TGG AAG TGG ATG GAA GTC
Il7R R	CAG CTT GTT AAG AGT TAG GCA TTT
Cox6a2 F	GAC CTT TGT GCT GGC TCT T
Cox6a2 R	CGA AGG GCT TGG TTC GG
Sgk1 F	CCA AGT CCC TCT CAA CAA ATC A
Sgk1 R	CCT AGC CAG AAG AAC CTT TCC
Rps16 F	CCA ATT TGA ATG TGC CTG GA
Rps16 R	ACT GAG GTG TGG CTG TGA TG

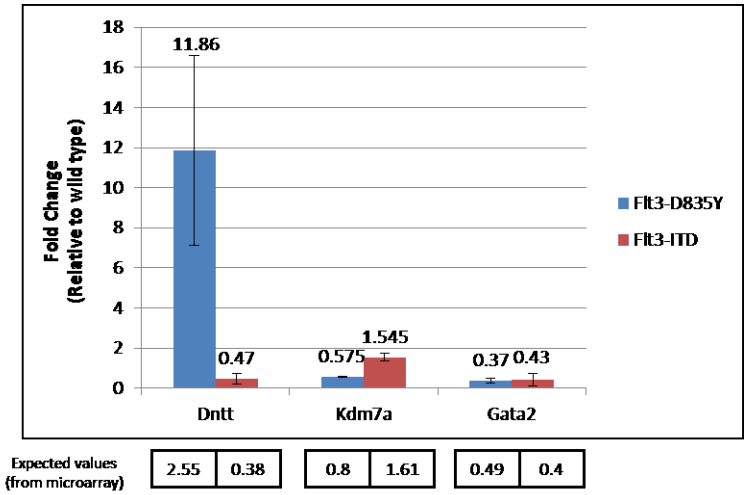
2.7 Figures: Chapter 2

Figure 2-1. Hierarchical clustering and heatmap of differentially expressed genes



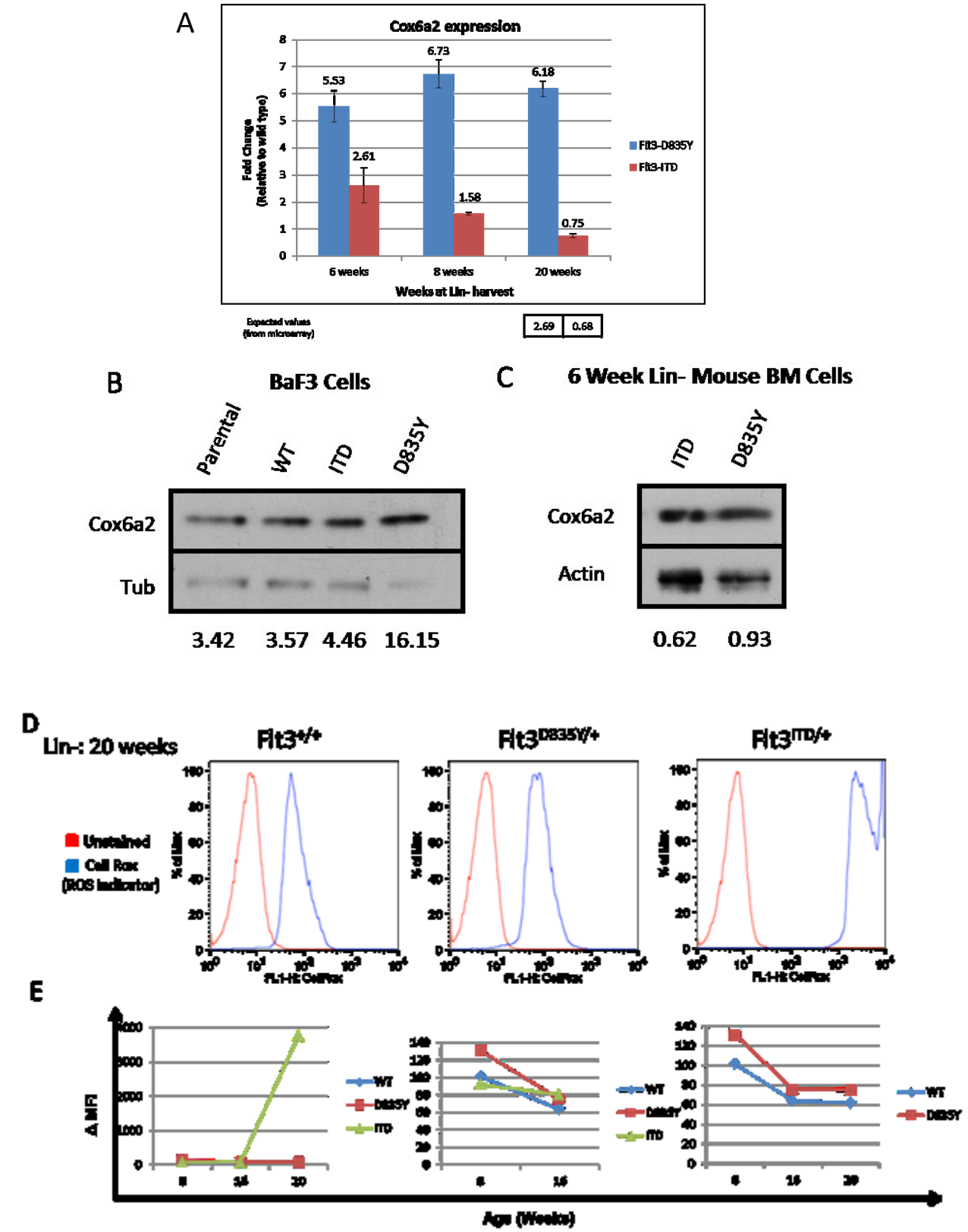
A) Hierarchical clustering dendrogram depicting relatedness of *Flt3*$^{ITD/+}$, *Flt3*$^{D835Y/+}$, and wild type LSK cells based on expression patterns. Each arm represents a sample used in the microarray. One of the *Flt3*$^{ITD/+}$ samples had to be excluded due to poor performance. B) Heatmap of the differentially upregulated (red) and downregulated (blue) genes meeting cut off criteria. C) Venn diagram with numbers of differentially expressed genes per genotype. D) Heatmap showing 22 differentially expressed genes in both genotypes.

Figure 2-2. qPCR validation of candidate loci



Lineage negative bone marrow from 8-20 week old mice of each genotype was subjected to qPCR. Expected fold changes from the microarray are in boxes beneath each gene. Reciprocally expressed genes *Dntt* and *Kdm7a* are validated, as well as *Gata2*, which is predicted to be down regulated in both genotypes.

Figure 2-3. Increasing Cox6a2 expression correlates with increased ROS productiion



(A) qPCR amplification of *Cox6a2* from lineage negative bone marrow from mice of each genotype at varying ages. Boxes underneath the 20 week time point indicate the expected results from the microarray, as this later time point is most representative of those values. (B) Western blot using lysates from BaF3 cells shows increased Cox6a2 expression in D835Y cells, however ITD expressing cells don't exhibit a decrease in protein levels as expected (C) Western blots from lineage negative cells of mice 6 weeks of age D835Y levels of Cox6a2 were increased compoared to the ITD, but we lacked a WT control. (D) Representative flow plots from lineage negative cells at 20 weeks of age assayed for ROS levels. (E) Mean fluorescence intensity (MFI) plotted for the same experiments for 8,16, and 20 weeks.

3. CHAPTER 3. *Dnmt3a* DELETION COOPERATES WITH THE *Flt3-ITD* MUTATION TO DRIVE LEUKEMOGENESIS IN A MURINE MODEL

3.1 Introduction

Large scale genomic studies have revealed the complex and heterogeneous genomic underpinnings of Acute Myeloid Leukemia (AML), identifying *FLT3* as the most frequently mutated gene in the disease (Cancer Genome Atlas Research Network 2013; Welch,J.S. 2012; Patel,J.P. 2012), occurring in ~30% of AML patients. Internal tandem duplication (ITD) of the juxtamembrane domain is the most common of these mutations, and predicts poor clinical outcomes (Patel,J.P. 2012). Previous studies in knock-in mice illustrate that $Flt3^{ITD/+}$ alone generates myeloproliferative disease (MPN) and is insufficient to drive leukemogenesis, suggesting additional mutations are necessary for full transformation (Li,L. 2008; Lee,B.H. 2007). Global genomic sequencing studies have identified a substantial subset of patients in which *FLT3/ITD* and *DNMT3A* mutations are concomitantly present (Patel,J.P. 2012; Cancer Genome Atlas Research Network 2013). Moreover, the co-occurrence of these mutations is significantly associated with adverse clinical outcomes (Patel,J.P. 2012). Typically in AML patients, sequencing studies have demonstrated 8-13 mutations but it is unclear how many of these mutations are drivers vs. passengers, leaving in doubt the number of mutations required for full transformation of primary cells (Vogelstein,B. 2013). Based on these observations, investigating the potential cooperativity of these mutations in a murine model serves to answer important questions regarding the underlying biology of the disease, while serving as a powerful drug discovery tool with significant clinical impact.

DNMT3A mutations are among the most common alterations in AML, just behind *FLT3/ITD* (Cancer Genome Atlas Research Network 2013; Welch,J.S. 2012; Ley,T.J. 2010). While AML-associated mutations have been identified throughout the body of the gene, the overwhelming majority are heterozygous missense mutations within the catalytic domain, often affecting Arginine 882 (Yang,L. 2015). *In vitro* studies suggest the R882H mutation leads to reduced methyltransferase activity, and acts in a dominant negative manner by impairing tetramer formation (Russler-Germain,D.A. 2014; Kim,S.J. 2013; Holz-Schietinger,C. 2012).

In accordance with mounting evidence that *DNMT3A* mutations result in a loss of function, conditional knock-out mice have been used extensively to evaluate the effects of Dnmt3a loss on stem cell function and leukemia development (Mayle,A. 2014; Challen,G.A. 2011). These mice harbor floxed *Dnmt3a* alleles ($Dnmt3a^{f/f}$), and a hematopoietic specific Cre transgene (Mx1-Cre+), which is induced upon injection with polyinosinic-polycytidylic acid (pIpC) (Kaneda,M. 2004). Recent work demonstrated that upon serial transplantation, Dnmt3a ablation in hemaotpoietic stem cells (HSC) promotes self-renewal and expansion of the LT-HSC pool, with progressive differentiation and methylation defects of downstream progeny (Challen,G.A. 2011; Jeong,M. 2014). As DNMT3A mutations are thought to represent one of the earliest founding events in AML (Xie,M. 2014; Shlush,L.I. 2014), these functional studies may explain how mutant DNMT3A initiates the disease, through expanding the primitive cell pool, thereby increasing the probability of acquiring additional deleterious mutations. Conversely, *Flt3/ITD* disrupts LT-HSC quiescence, resulting in depletion of this compartment (Chu,S.H. 2012). Given the co-occurrence of *FLT3/ITD* and *DNMT3A* mutations in AML patients, we hypothesized that *Dnmt3a* deletion may cooperate with *Flt3/ITD* to induce leukemia, potentially through rescuing the LT-HSC depletion seen in $Flt3^{ITD/+}$ mice.

Since *DNMT3A* mutations seem to represent an initiating event, breeding *Dnmt3a*$^{f/f}$ mice to our *Flt3*$^{ITD/+}$ knock-in mice presents a significant challenge, as mutant *Flt3* is expressed at the earliest stages of hematopoeisis, preceding *Dnmt3a* deletion. To partially circumvent this issue, we used a substrain of our *Flt3*$^{ITD/+}$ knock-in mice which retains a floxed Neomycin (Neo) selection cassette from the initial targeting (*Flt3*$^{ITDneo/+}$; referred to hereafter as *Flt3*$^{ITD/+}$ for simplicity's sake). The presence of the cassette greatly reduces expression of the mutant allele (Li,L. 2008). *Flt3*$^{ITD/+}$ expression is fully restored upon excision of the cassette following Cre induction, effectively "knocking in" the *Flt3/ITD* mutation and knocking out *Dnmt3a* simultaneously (Figure 3-1A). This novel approach allowed us to perform long term survival studies and disease characterization in a biologically relevant context.

3.2 Results

3.2.1 Dnmt3a deletion reduces survival of Flt3$^{ITD/+}$ knock-in mice in a dose dependent manner and drives leukemogenesis

In accordance with mounting evidence that *DNMT3A* mutations result in a loss of function (Russler-Germain,D.A. 2014), we used a mouse model harboring floxed *Dnmt3a* alleles (*Dnmt3a*$^{f/f}$) (Kaneda,M. 2004), and a lymphocyte specific Cre transgene (Mx1-Cre), which is activated upon injection with polyinosinic-polycytidylic acid (pIpC). These mice were bred to a substrain of our *Flt3*$^{ITD/+}$ knock-in mice, which retains a floxed PGK-Neo selection cassette from the initial targeting (Figure 3-1A). The presence of the cassette reduces transcription of the mutant allele, and full expression is restored upon Cre excision (Li,L. 2008). Since *DNMT3A* mutations are thought to represent one of the earliest events in leukemia development (Xie,M. 2014; Shlush,L.I. 2014; Welch,J.S. 2012), this approach provides the unique advantage of temporally restraining *Flt3*^{ITD} expression until *Dnmt3a* is lost; effectively "knocking in" the

Flt3^{ITD} mutation, and knocking out *Dnmt3a* simultaneously upon pIpC injection. Mx1-Cre expressing mice harboring *Flt3*$^{ITD/+}$ and homozygous floxed *Dnmt3a* alleles (*Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/f}$), heterozygous (*Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/+}$), or wild type alleles (*Flt3*$^{ITD/+}$), as well as litter mate controls (*Flt3*$^{+/+}$;*Dnmt3a*$^{f/f}$ or *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/+}$ lacking the Mx1-Cre transgene) were injected with pIpC intraperitoneally at 8 weeks of age and monitored for disease development. Peripheral blood was collected every 1-2 months after injection to monitor changes in the differential white blood count, and to collect genomic DNA to confirm effective loxP recombination (table 3-1).

Interestingly, deletion of *Dnmt3a* significantly reduced median survival of *Flt3*$^{ITD/+}$ mice in a dose-dependent manner, with median survival of 162 days and 256 days for *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/f}$ and *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/+}$, respectively (Figure 3-1B). Both genotypes confer a significantly shorter survival time compared to *Flt3*$^{ITD/+}$ mice alone, which have a median survival of 412 days, consistent with our previous findings (Greenblatt,S. 2012; Bailey,E. 2013; Li,L. 2008). *Dnmt3a* dosage was also associated with a number of other parameters, including splenomegaly (Figure 3-1C) and leukocytosis (Figure 3-1D). While the trending increase in spleen weight and WBC is evident, only *Flt3*$^{ITD/+}$ mice with complete loss of *Dnmt3a* (*Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/f}$) displayed increases reaching statistical significance compared to *Flt3*$^{ITD/+}$ alone.

Dnmt3a deletion cooperates with Flt3$^{ITD/+}$ *to induce a broad spectrum of neoplasms*

As expected from previous work, mice with *Flt3*$^{ITD/+}$ alone developed MPN, while loss of one or both *Dnmt3a* alleles cooperates with the *Flt3*^{ITD} mutation to elicit leukemia development of varying lineages including AML, MPN, and T lymphoblastic lymphoma/leukemia (T-LL) (Figure 3-2A). Histological analysis of bone marrow derived from

mice with myeloid diseases revealed predominance of blasts and immature myeloid cells in AML samples (Figure 3-2B). While T-LLs displayed variable immunophenotypes, including double or single positive for CD4 or CD8 (data not shown), the immunophenotype among myeloid mice was fairly uniform, displaying an increase in blasts that are FcrG+, CD34+, Mac1+/lo, consistent with a Myelomonocytic leukemia (Figure 3-2C).

In FLT3/ITD+ AML, loss of heterozygosity of the wild type allele is sometimes observed, and is associated with poorer survival (Meshinchi,S. 2006; Whitman,S.P. 2001; Thiede,C. 2002). To investigate whether or not this phenomenon also occurred in our model, genomic DNA was extracted from bone marrow and matched tail tissue from leukemic mice, and PCR amplification was performed using primers flanking the ITD mutation (Figure 3-3A). Loss of the wild type allele was observed in 36% (5/14) of mice developing AML (both *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/f}$ and *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/+}$), while no evidence of LOH was observed in any of the 13 mice developing lymphoid neoplasms (Figure 3-3B). These findings are consistent with previous studies in *Flt3*$^{ITD/+}$ knock-in mice, where loss of the wild type allele is restricted to AML, and absent in T-LL (Rau,R. 2014; Greenblatt,S. 2012), indicating a selective advantage for these events specifically in the myeloid lineage.

3.2.2 T-LL samples exhibit enhanced transplantability compared with other neoplasms

To investigate transplantability of primary neoplasms developing in our mice, cells from each leukemic donor were transplanted into three sublethally irradiated recipients and monitored for survival (Figure 3-3C). Engraftment and survival were variable based on disease subtype, as all 4 of the AML samples from *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/f}$ donors engrafted, with a median survival of 174 days, while only 2 of 4 *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/+}$ AML samples engrafted, displaying slightly prolonged (but not statistically significant) median survival. Transplantation of MPNs derived

from *Flt3*$^{ITD/+}$ mice failed to engraft, consistent with previous findings (Li,L. 2008). In stark contrast, T-LL samples from both *Flt3*$^{ITD/+}$*;Dnmt3a*$^{f/f}$ and *Flt3*$^{ITD/+}$*;Dnmt3a*$^{f/+}$genotypes were much more aggressive, killing recipients within one month.

3.2.3 Lymphocytic neoplasms exhibit Notch1 mutations and repressed Flt3 expression

Since LOH of the ITD allele was found to be a disease specific finding, associated with AML samples specifically, we were interested in identifying lymphoid specific mutations as well. Activating mutations in *NOTCH1* are the most frequent mutation in T-ALL (Liu,H. 2011; Liu,H. 2010). To detect Notch1 mutations in our samples, we performed RT-PCR, and amplified hot spots for common mutation sites within the heterodimerization and PEST domains, sending them for sequencing. In all samples where adequate material was available, we identified mutations which are predicted to be activating (table 3-2). These mutations were lymphoid disease specific, as expected.

Previous sequencing endeavors have identified *DNMT3A* and *FLT3* as frequently mutated genes in AML, both individually and concurrently, and at lower frequencies in T-ALL (Neumann,M. 2013; Grossmann,V. 2013; Neumann,M. 2012). While lymphocytic leukemias haven't previously been observed in our *Flt3*$^{ITD/+}$ knock-in model, previous work has demonstrated that ~15% of *Dnmt3a*$^{f/f}$ mice develop T-ALL (Mayle,A. 2014), with median survival of 246 days, compared with 162 days in our *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/f}$ mice. Taken together, these findings suggest that perhaps the *Flt3/ITD* mutation is dispensable for maintenance of lymphoid malignancies in *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/f}$ mice, but may play an important role in expanding the progenitor pool giving rise to these lymphoid neoplasms, thereby accelerating disease development.

To examine *Flt3* expression, we performed qPCR on whole bone marrow from wild type and *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/f}$ mice with varying diagnoses. *Flt3* expression was elevated in myeloid neoplastic samples, especially those exhibiting LOH, with expression levels at about 11 fold higher than wild type bone marrow samples (Figure 3-3D). Interestingly, T-LL samples expressed virtually no *Flt3*, supporting the hypothesis that once full transformation occurs in these cells, *Flt3* is turned off, as in normal lymphoid development (De Obaldia,M.E. 2013).

3.2.4 Myeloid progenitors are expanded in double mutant mice at an early time point, underlying observed relative monocytosis and disease variability

Recent work has demonstrated that *Dnmt3a* deletion in hematopoietic stem cells (HSC) promotes self-renewal and expansion of the LT-HSC pool (Challen,G.A. 2011), resulting in leukemia with incomplete penetrance and prolonged time to disease development (Mayle,A. 2014). Conversely, *Flt3*$^{ITD/+}$ disrupts LT-HSC quiescence, resulting in depletion of this compartment, and failure to develop full blown leukemia in the absence of cooperating mutations (Chu,S.H. 2012). We hypothesized that enhanced self-renewal and expansion in the LT-HSC compartment conferred by *Dnmt3a* deletion might "rescue" the LT-HSC depletion seen in *Flt3*$^{ITD/+}$ mice, thereby increasing this primitive pool and the opportunity for additional mutations necessary to drive either myeloid or T cell leukemia to develop. To investigate the frequencies of progenitor populations well prior to disease onset, mice were sacrificed at 8 weeks post pIpC injection and the stem cell compartment was examined by flow cytometry.

While overt leukemia was absent at this early time point, pathological changes were evident in the bone marrow and peripheral blood. None of the genotypes exhibited leukocytosis (Figure 3-4A), but a relative monocytosis was seen, as expected, in *Flt3*$^{ITD/+}$ mice, and was exaggerated in the *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/+}$ and *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/f}$ mice, with an increase in the

percentage of monocytes, and concomitant decrease in percent lymphocytes (Figure 3-4B). While *Dnmt3a* ablation alone had no effect on spleen size at 8 weeks, splenomegaly was noted in *Flt3*$^{ITD/+}$ mice, which was again more pronounced in double mutant mice (Figure 3-4C). Flow cytometric analysis of whole bone marrow displayed varying anomalies, including aberrant CD34 expression and expansion within the myeloid lineage (Figure 3-4D). This immunophenotypic variability at an early time point likely underlies the ultimate variability in neoplasms developing in moribund mice. Interestingly, while percentage shifts favoring myeloid cell types in the peripheral blood were similar between *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/+}$ and *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/f}$ mice, appreciable shifts in myeloid progenitor populations were only present in the *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/f}$ bone marrow samples, where obvious increases in common myeloid (CMP) and granulocyte-macrophage (GMP) progenitors were observed with a decrease in megakaryocyte-erythrocyte progenitors (MEP) (Figure 3-4E).

3.2.5 Loss of both copies of Dnmt3a is necessary to elicit LT-HSC expansion

Upon examining the stem cell compartment, we confirmed the previously described expansion in *Dnmt3a* knock-out mice and depletion in *Flt3*$^{ITD/+}$ mice. The data suggest that loss of one *Dnmt3a* allele is sufficient to restore the LT-HSC (Lin$^-$ c-kit$^+$ Sca-1$^+$ Flt3$^-$CD34$^-$) pool of *Flt3*$^{ITD/+}$ mice to wild type proportions, while loss of both *Dnmt3a* alleles together with *Flt3/ITD* expression results in a dramatic expansion, beyond that of *Dnmt3a*$^{f/f}$ alone (Figure 3-5A). Given the previous findings that *Dnmt3a* deletion expands the LT-HSC compartment through enhanced quiescence, the observed minor decrease in the immediate downstream ST-HSC (Lin$^-$ c-kit$^+$ Sca-1$^+$ Flt3$^-$CD34$^+$) (Figure 3-5B) and MPP (Lin$^-$ c-kit$^+$ Sca-1$^+$ Flt3$^+$CD34$^+$) (Figure 3-5C) populations in *Dnmt3a*$^{f/f}$ mice is not surprising. Likewise, as aberrant exit from quiescence driven by *Flt3*$^{ITD/+}$ alone resulted in LT-HSC depletion, these cells are driven to mobilize and

differentiate, as evidenced by an observed increase in the ST-HSC (Figure 3-5B) and MPP (Figure 3-5C) pools. Similar to the LT-HSCs, haploinsufficiency of *Dnmt3a* in *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/+}$ mice was sufficient to bring the fraction of ST-HSCs back towards the wild-type levels. In contrast, loss of both *Dnmt3a* alleles in *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/f}$ mice resulted in a dramatic increase in the ST-HSC compartment (Figure 3-5B). *Dnmt3a* deletion in the *Flt3*$^{ITD/+}$ context conferred an increase in the MPP population in a dose dependent manner (Figure 3-5C).

3.2.6 Dnmt3a ablation enhances colony formation of Flt3ITD/+ bone marrow in serial replating experiments

After determining that *Dnmt3a* loss and the *Flt3/ITD* mutation do indeed cooperate to greatly expand the LT-HSC pool, we sought to investigate the mechanism underlying this observation. To assess self-renewal, lineage negative bone marrow cells were plated on methylcellulose, and serially re-plated twice more. The number of colonies was recorded before each re-plating. Since the methylcellulose used in these experiments contains cytokines directing myeloid differentiation, persistence of colony forming cells in the third plating suggests a block in differentiation and enhanced self-renewal. As expected, wild type bone marrow displays a significant decrease in colony formation at the secondary plating and an absence of colonies at the tertiary re-plating. In agreement with previous observations, *Dnmt3a*$^{f/f}$ cells continued to re-plate through the tertiary plating, exhibiting enhanced self-renewal. Only one tertiary re-plating colony was observed of all plates analyzed from *Flt3*$^{ITD/+}$ mice, congruent with disrupted quiescence (Figure 3-5D). However, when *Dnmt3a* loss is added to the *Flt3*$^{ITD/+}$ background, partial restoration of the enhanced self-renewal phenotype is observed. These experiments suggest that the expansion in the LT-HSC pool of *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/f}$ mice is achieved, at least in part, by enhanced self-renewal conferred by *Dnmt3a* deletion.

3.3 Discussion

Throughout the last decade, thorough analysis of the leukemia genome has generated an extensive compendium of AML-associated mutations (Welch,J.S. 2012; Cancer Genome Atlas Research Network 2013). These data have formed the basis for subsequent functional studies in mice, using knock in, knock out, or transgenic strains. Mutation of the orthologous mouse gene represents the most biologically relevant experimental approach, yet many of the most common driver mutations do not, in isolation, lead to fully penetrant, rapidly fatal leukemias (Lee,B.H. 2007; Li,L. 2008; Bailey,E. 2013; Li,Z. 2011; Moran-Crusio,K. 2011; Moran-Crusio,K. 2011; Chan,G. 2009). These results are not surprising, as elegant studies dissecting the clonal evolution of leukemia indicate several mutations occur in concert, often chronologically, and are likely necessary for transformation.

Integrative genomic profiling has identified a significant proportion of AML patients with concomitant *FLT3-ITD* and *DNMT3A* mutations. Concurrently, these mutations stratify patients into a poorer prognostic category, predicting inferior clinical outcomes and overall survival (Patel,J.P. 2012). While *Flt3/ITD* knock-in alone fails to fully transform and recapitulate human leukemia, ablation of *Dnmt3a* alone is sufficient to predispose HSCs to malignant transformation, resulting in a spectrum of neoplasms with a prolonged time to disease development (Mayle,A. 2014). We hypothesized that breeding $Flt3^{ITD/+}$ mice and the conditional *Dnmt3a* knock-out would result in shortened survival compared to either mutation alone, cooperating to drive AML development in a greater proportion of mice than $Dnmt3a^{f/f}$ alone.

The data reported here confirm that indeed, $Flt3^{ITD/+}$ and bone marrow specific *Dnmt3a* deletion cooperate to result in shortened survival due to fatal hematopoietic neoplasms, including AML. Interestingly, we found that *Dnmt3a* dosage significantly impacts survival and the

spectrum of neoplasms developing in *Flt3*$^{ITD/+}$ mice. The observation that loss of a single allele is sufficient to shorten survival and elicit leukemia development highlights the importance of *Dnmt3a* stoichiometry in maintaining appropriate hematopoietic stem cell function. This finding is especially relevant in light of a recent meta-analysis of the TCGA cohort, which revealed a significant focal loss of CpG methylation throughout the genomes of AML patients harboring *DNMT3A* mutations (Russler-Germain,D.A. 2014). Mean beta values were also further reduced in DNMT3A^{R882} compared to DNMT3A$^{non\text{-}R882}$ samples, supporting *in vitro* evidence that R882 mutations impair methyltransferase activity more severely than mutations at other residues. Future assessment and comparison of these *Flt3*$^{ITD/+}$*;Dnmt3a*$^{f/+}$ and *Flt3*$^{ITD/+}$*;Dnmt3a*$^{f/f}$ mice with human FLT3^{ITD};DNMT3A$^{non\text{-}R882}$ and FLT3^{ITD};DNMT3A^{R882} AML methylomes may reveal differentially methylated loci that are dosage, rather than sequence context dependent.

Unlike AML, which is characterized by heterozygous *DNMT3A* mutations primarily in the methyltransferase domain, the gene is frequently biallelically inactivated in T-ALL, with mutations occurring throughout the body of the gene (Yang,L. 2015). Homozygous ablation of *Dnmt3a* in our mice may mimic biallelic inactivation seen in patients, accounting for the incidence of the disease in these mice. While *Flt3* is virtually unexpressed in T-LL derived from our double mutant mice, this does not preclude the possibility that Flt3/ITD is important for expansion of a lymphocytic progenitor, with cooperating somatic mutations and epigenetic changes favoring transformation within a later compartment, where FLT3 activity is dispensible (De Obaldia,M.E. 2013). The identification of FLT3 mutations in a subset of T-ALL patients raises important questions that warrant further investigation (Neumann,M. 2013; Neumann,M. 2012).

Variance in phenotype and delay in disease development may indicate that cells in the context of both mutations are still predisposed to transformation, but require additional mutations. Although leukemic mice meet the diagnostic criteria consistent with AML, including >20% blasts, inconsistent engraftment makes it difficult to perform transplantation experiments and *in vivo* drug treatments. A recent study in AML1-ETO driven murine leukemia illustrates the utility and power of cross referencing genomic data from murine studies with the enormous repository of publicly available data from human leukemia samples sharing the same genetic underpinnings (Hatlen,M.A. 2015). Using a similar approach in our model, we hope to identify cooperative orthologous genomic events which we can introduce into $Flt3^{ITD/+};Dnmt3a^{f/f}$ bone marrow, thereby creating an increasingly relevant model of the disease, increasing aggressiveness and facilitating *in vivo* TKI and epigenetic combination therapy testing.

This model presents a platform to answer important questions regarding Dnmt3a dosage in the context of Flt3/ITD. Prior to leukemia formation, the incremental expansion of the LT-HSC pool indicates that differentially methylated loci important in maintaining quiescence can be determined. It will be important to assay whether or not dosage simply influences methylation density within the same discrete genomic regions, or perhaps stoichiometry directs repressive complexes to novel loci.

3.4 Conclusions

The data presented in this report assert, for the first time, that conditional deletion of Dnmt3a and simultaneous "knock in" of $Flt3^{ITD/+}$, cooperate to drive leukemia development at a faster rate than *Dnmt3a* loss alone. Loss of heterozygosity of the Flt3 allele in our double mutant AMLs further substantiates our model as a powerful tool to study human leukemogenesis, as these events also spontaneously occur in FLT3/ITD+ patients. An exhaustive genomic survey of

the stem cell compartment at an early time point, double mutant AML blasts, and comparisons with human FLT3-ITD/DNMT3A mutant AML will reveal common core pathways and therapeutic targets, providing a vital resource to investigate clinically relevant therapies.

3.5 Materials and methods

3.5.1 Mice.

Mice harboring floxed *Dnmt3a* alleles (*Dnmt3a*$^{f/f}$) and an inducible hematopoietic specific Cre transgene (Mx1-Cre) (Kaneda,M. 2004) were bred to a substrain of our *Flt3*$^{ITD/+}$ knock-in mice (Li,L. 2008) which retain a floxed PGK-Neo cassette from the initial targeting (Figure 3-1A). *Dnmt3a* knock-out, and excision of the PGK-Neo cassette within the *Flt3*$^{ITD/+}$ allele, were achieved by two intraperitoneal injection of pIpC (250ug/mouse in PBS; Invivogen) every other day. Genomic DNA was prepared from peripheral blood eight weeks after pIpC injection to confirm loxP recombination at both alleles (Table 3-1) Primers used for genotyping and confirmation of loxP recombination (Flox F & Flox R) can be found in Table 3-1. Diagnoses and classification of hematopoietic neoplasms were made based on the previously established Bethesda proposals (Kogan,S.C. 2002; 27 Morse,H.C.,3rd 2002). All animal experiments were performed according to protocols approved by the Animal Care and Use Committee of Johns Hopkins University in accordance with guidelines set forth by the National Institutes of Health.

3.5.2 Disease characterization.

Complete peripheral blood cell count and cytology. Mice were monitored and sacrificed when they exhibited signs of disease development (lethargy, ruffled coat, abnormal complete blood count (CBC) differential). Peripheral blood was collected from the facial vein and subjected to complete blood cell counting, and a WBC differential was performed using the Hemavet950 system (Drew Scientific). Bone marrow cytospins and peripheral blood smears were stained

using a modified Wright-Giemsa protocol (Sigma-Aldrich), and representative images were acquired using a Zeiss Axioskop upright microscope (Carl Zeiss).

Flow cytometry. Diagnostic flow cytometric analysis was performed as previously described {{6 Li,L. 2008;}}. Data were analyzed using FlowJo Version 9.3.3 software (TreeStar).

Transplantation. 1x10^6 whole bone marrow cells from leukemic mice were transplanted into sub-lethally irradiated C57Bl/6-CD45.1 recipients (7.5 cGy) by tail vein injection. Blood was collected from the facial vein every 3-4 weeks for CBCs and flow cytometric analysis of engraftment. Additionally, recipients were monitored for visible signs of disease development.

3.5.3 RT-PCR and mutation analysis

Loss of Heterozygosity (LOH) analysis of *Flt3* allele. Genomic DNA was extracted from tail tissue, whole bone marrow, thymus, or lymph node where appropriate, using the Wizard Genomic DNA Purification Kit (Promega). PCR amplification was performed using 50ug of genomic DNA with *Flt3* genotyping primers flanking the ITD mutation Table 3-1.

RT-PCR and *Flt3* expression analysis. RNA was extracted from whole bone marrow, thymus, or lymphnode where appropriate using TRIzol (Invitrogen), and reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative RT-PCR was performed using the iCycler iQ multicolor real-time PCR system (Bio-Rad), and transcript levels were normalized to *Rps16*. Primers spanning exons 16 and 17 were used to detect *Flt3* expression Table 3-1.

***Notch1* mutation analysis.** RNA extraction and RT-PCR were performed as above. To detect *Notch1* mutations, primers specific for the heterodimerization and PEST domains were used to amplify the cDNA from appropriate hematopoietic tissues. PCR products were TA cloned

(Invitrogen) and 10 colonies per product were picked and Sanger sequenced. Sequences for all primers can be found in the Table 3-1.

3.5.4 Stem cell quantification and analysis.

Quantification of progenitors. Eight weeks post pIpC injection, whole bone marrow was isolated, and stem cell and progenitor populations were quantified as previously described {{4 Chu,S.H. 2012;}}. Phenotypic definitions of these compartments are as follows: LSK: $Lin^{-}Sca\text{-}1^{hi}c\text{-}KIT^{hi}$; LT-HSC: $Lin^{-}Sca\text{-}1^{hi}c\text{-}KIT^{hi}CD34^{-}CD135^{-}$; ST-HSC: $Lin^{-}Sca\text{-}1^{hi}c\text{-}KIT^{hi}CD34^{+}CD135^{-}$; MPP: $Lin^{-}Sca\text{-}1^{hi}c\text{-}KIT^{hi}CD34^{+}CD135^{+}$; CMP: $Lin^{-}Sca\text{-}1^{-}c\text{-}KIT^{hi}CD34^{+}CD16^{mid}$; GMP: $Lin^{-}Sca\text{-}1^{-}c\text{-}KIT^{hi}CD34^{+}CD16^{hi}$; MEP: $Lin^{-}Sca\text{-}1^{-}c\text{-}KIT^{hi}CD34^{-}CD16^{-}$.

Colony formation assays. Bone marrow was isolated from mice 8 weeks post pIpC injection, and subjected to lineage depletion using the MACS cell separation system (Miltenyi Biotec). 10,000 lineage negative bone marrow cells were plated on Methocult M3434 (Stemcell Technologies), and analyzed at day 9-11. Colonies were disaggregated, and 200 cells were re-plated and scored for subsequent secondary and tertiary colony formation. Three mice were assayed per genotype, and experiments were performed in triplicate.

3.6 Tables: Chapter 3

Table 3-1. Genotyping and Sequencing Primers.

Genotyping	
Primer	**Sequence**
ITD F	TGC AGA TGA TCC AGG TGA CT
ITD R	CTC TCG GGA ACT CCC ACT TA
Flox F	GTG GAG CAC TGA ACA GCA AG
Flox R	CCT GAA GCA CTG GAA AGG AC
Dnmt3a F	TGG GGA TTT GAG AGG TGA AG
Dnmt3a R	GTG GAG CAC TGA ACA GCA AG
Mx1-Cre F	CAC AAA GAA CAG GAG CAC GA
Mx1-Cre R	CTC TCG GGA ACT CCC ACT TA
Dnmt3a LoxPF	ATA GCA TGA TGG GGC CAC TA
2LoxPR	GTG GAG CAC TGA ACA GCA AG
1LoxPR	TGC AGA TGA TCC AGG TGA CT
qPCR	
Primer	**Sequence**
Flt3 F	TGG GAC ACC ATG ACA ACA TC
Flt3 R	GGA ATT TGA ATG TGC CTG GA
Notch1 HD F	AAC AGT GCC GAA TGT GAG TG
Notch1 HD R	CAC AAA GAA CAG GAG CAC GA
Notch1 PEST-1F	AGT CAC CCC ATG GCT ACT TG
Notch1 PEST-1R	ACT GAG GTG TGG CTG TGA TG
Notch1 PEST-2F	ATA GCA TGA TGG GGC CAC TA
Notch1 PEST-2R	CCT GAA GCA CTG GAA AGG AC
mS16 F	CCA ATT TGA ATG TGC CTG GA
mS16 R	ACT GAG GTG TGG CTG TGA TG

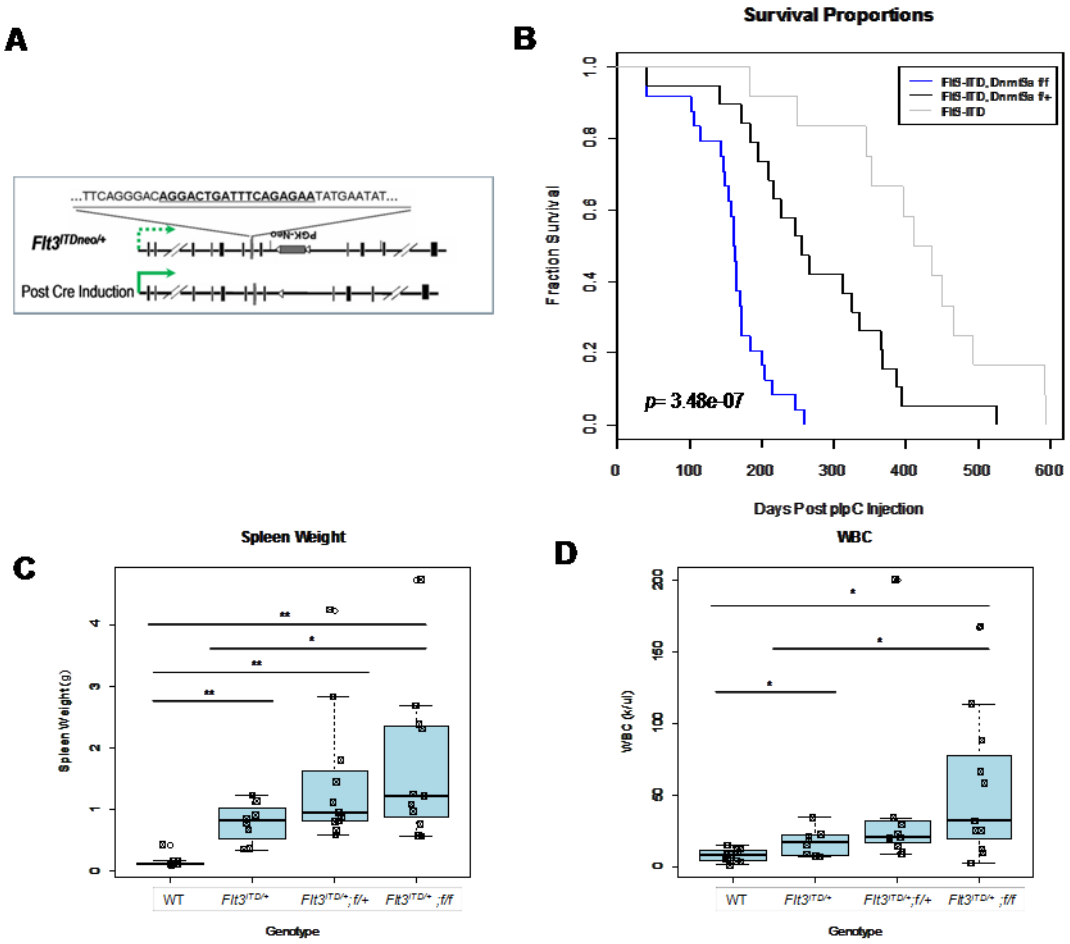
Table 3-2. Notch1 mutations in lymphoid neoplasms.

Genotype	Immunophenotype	Domain	Mutation
Flt3$^{ITD/+}$;*Dnmt3a*$^{f/+}$	CD4+	PEST	c.7539delG (p.2439fs*2451)
Flt3$^{ITD/+}$;*Dnmt3a*$^{f/+}$	DP*	HD	c.T5040G, 5040insGAG (p.F1592L, 1592insE)
Flt3$^{ITD/+}$;*Dnmt3a*$^{f/f}$	DP	PEST	c.7354insAGGGGGG (p.2356fs*2494)
Flt3$^{ITD/+}$;*Dnmt3a*$^{f/f}$	DP	PEST	c.7154del(22) (p.2285fs*2301)
Flt3$^{ITD/+}$;*Dnmt3a*$^{f/f}$	CD4+	HD	c.G5179A (p.A1639T)
Dnmt3a$^{f/f}$	DP	PEST	c.7539delG (p.2439fs*2451)

*DP-CD4+CD8+ double positive

3.7 Figures: Chapter 3

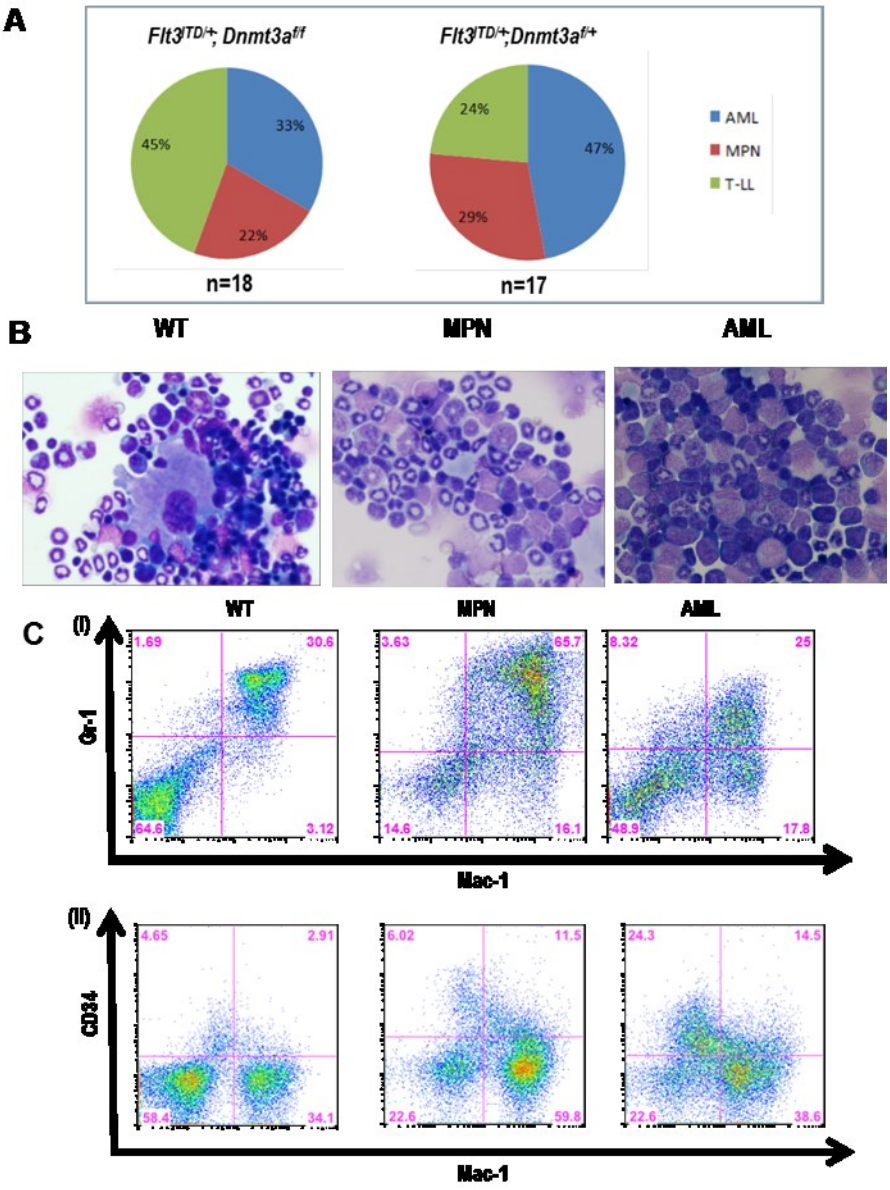
Figure 3-1. Dnmt3a deletion cooperates with $Flt3^{ITD/+}$ to shorten survival in a dose dependent manner.



(A) Strategy for temporally controlling Flt3/ITD expression using a substrain of our $Flt3^{ITD/+}$ knock-in mice. The substrain ($Flt3^{ITDneo}$/+) retains the PGK-Neo cassette inserted at the mutant allele (referred to hereafter as $Flt3^{ITD/+}$). The presence of the cassette reduces expression of the mutant allele (dashed green arrow), and $Flt3^{ITD/+}$ expression is fully restored upon excision of the cassette following cre induction (solid green arrow). (B) Kaplan-Meier Survival Curve. Median survival is 162 days for $Flt3^{ITD/+}$;$Dnmt3a^{f/f}$ (n=24), 260 days for $Flt3^{ITD/+}$;$Dnmt3a^{f/+}$ (n=20), and 412 days for $Flt3^{ITD/+}$ alone (n=12). (C) Spleen weights, and (D) White blood cell counts (WBC) of mice developing myeloid neoplasms at the time of sacrifice. Wildtype (WT); Flt3-

ITD alone (*Flt3*$^{ITD/+}$); *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/+}$ (*Flt3*$^{ITD/+}$;$^{f/+}$); *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/f}$(*Flt3*$^{ITD/+}$;$^{f/f}$). (*, $p < 0.05$; **, $p < 0.01$).

Figure 3-2. Dnmt3a deletion cooperates with Flt3$^{ITD/+}$ to induce a broad spectrum of neoplasms.

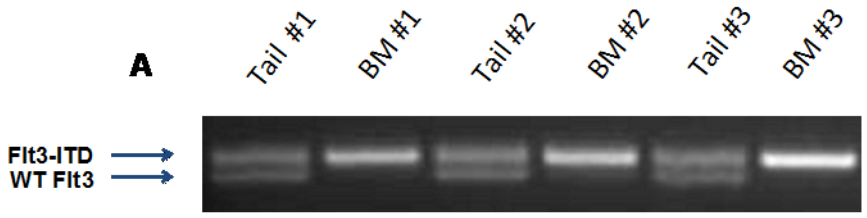


A) Disease distribution of *Flt3*$^{ITD/+}$ mice lacking both (left) or a single (right) *Dnmt3a* allele. (B) Hematoxylin and Eosin stained bone marrow cytospins from a representative wild type mouse (WT, left column), a mouse with a Myeloproliferative Neoplasm (MPN, center column), and a mouse with Acute Myeloid Leukemia (AML, right column). (C) Flow cytometric analysis of bone marrow. (i) The MPN mouse displays the typical Mac1+Gr1+ expansion, while the

leukemic mouse on the right displays an abnormal Mac1+Gr1lo/- population. (ii) The wild-type mouse (left), displays the normal CD34 positivity, while MPNs exhibit increased immature CD34+, cells which are Mac-$1^{lo/-}$, but fail to reach and exceed the 20% requirement for an AML diagnosis. This same population exceeds 20% in the representative AML plot on the right. That same Blasts appear to be Mac1lo,CD34+.

Figure 3-3. Loss of Heterozygosity (LOH) and Flt3 expression are present in a disease specific manner.

A



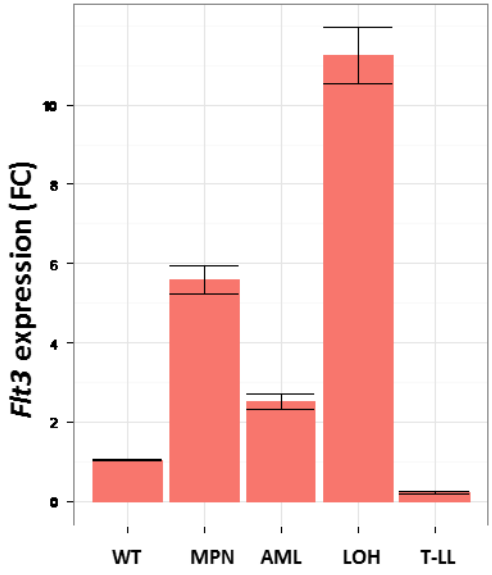
B

Genotype	Disease	Fraction LOH+
Flt3$^{ITD/+}$; *Dnmt3a*$^{f/+}$	AML	3/8
Flt3$^{ITD/+}$; *Dnmt3a*$^{f/+}$	T-LL	0/5
Flt3$^{ITD/+}$; *Dnmt3a*$^{f/f}$	AML	2/6
Flt3$^{ITD/+}$; *Dnmt3a*$^{f/f}$	T-LL	0/8

C

Genotype	Disease	# Recipients	# Engrafted	Median Survival
Flt3-ITD, Dnmt3a f/f	AML	12	12	174 days
Flt3-ITD, Dnmt3a f/+	AML	12	6	210 days
Flt3-ITD	MPN	6	0	
Flt3-ITD, Dnmt3a f/f	T-LL	6	6	29 days
Flt3-ITD, Dnmt3a f/+	T-LL	3	3	16 days

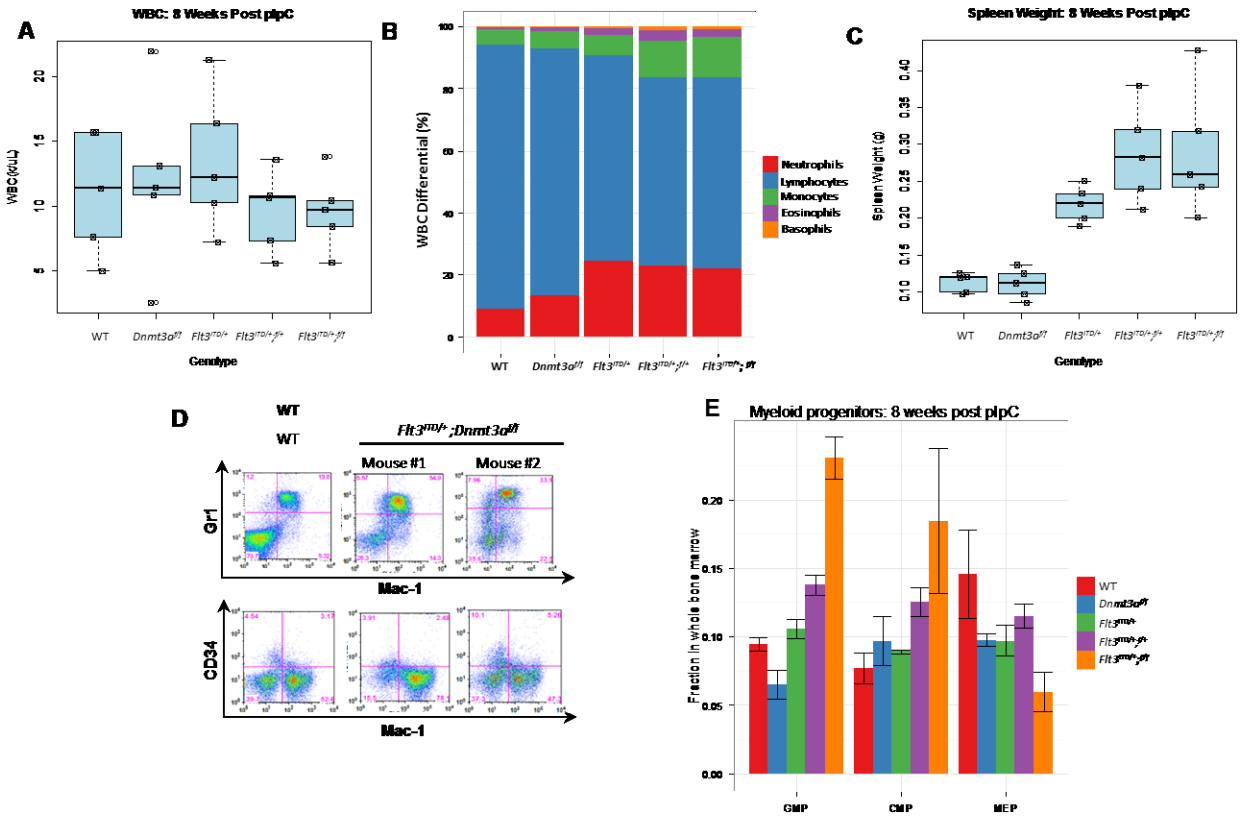
D



(A) Representative samples exhibiting bone marrow specific LOH at the *Flt3* locus. Primers

surrounding the ITD mutation were used to amplify genomic DNA prepared from bone marrow and matched tail tissue from leukemic mice. (B). Loss of Heterozygosity (LOH) of the wild type Flt3 allele occurs exclusively in mice developing AML, in both $Flt3^{ITD/+};Dnmt3a^{f/f}$ and $Flt3^{ITD/+};Dnmt3a^{f/+}$ genotypes. Summary of samples exhibiting loss of the wild type Flt3 allele (C) Survival summary of transplantation experiments. Diseases of donors and median survival of all engrafted recipients are noted. (D) T-ALLs are driven by Flt3/ITD independent factors. qPCR of leukemic tissue displays silencing of *Flt3* in lymphocytic leukemia samples. RNA was extracted from whole bone marrow of Wildtype (WT), or $Flt3^{ITD/+};Dnmt3a^{f/f}$ mice developing MPN, AML without LOH, AML with LOH at the *Flt3* locus, or T-LL.

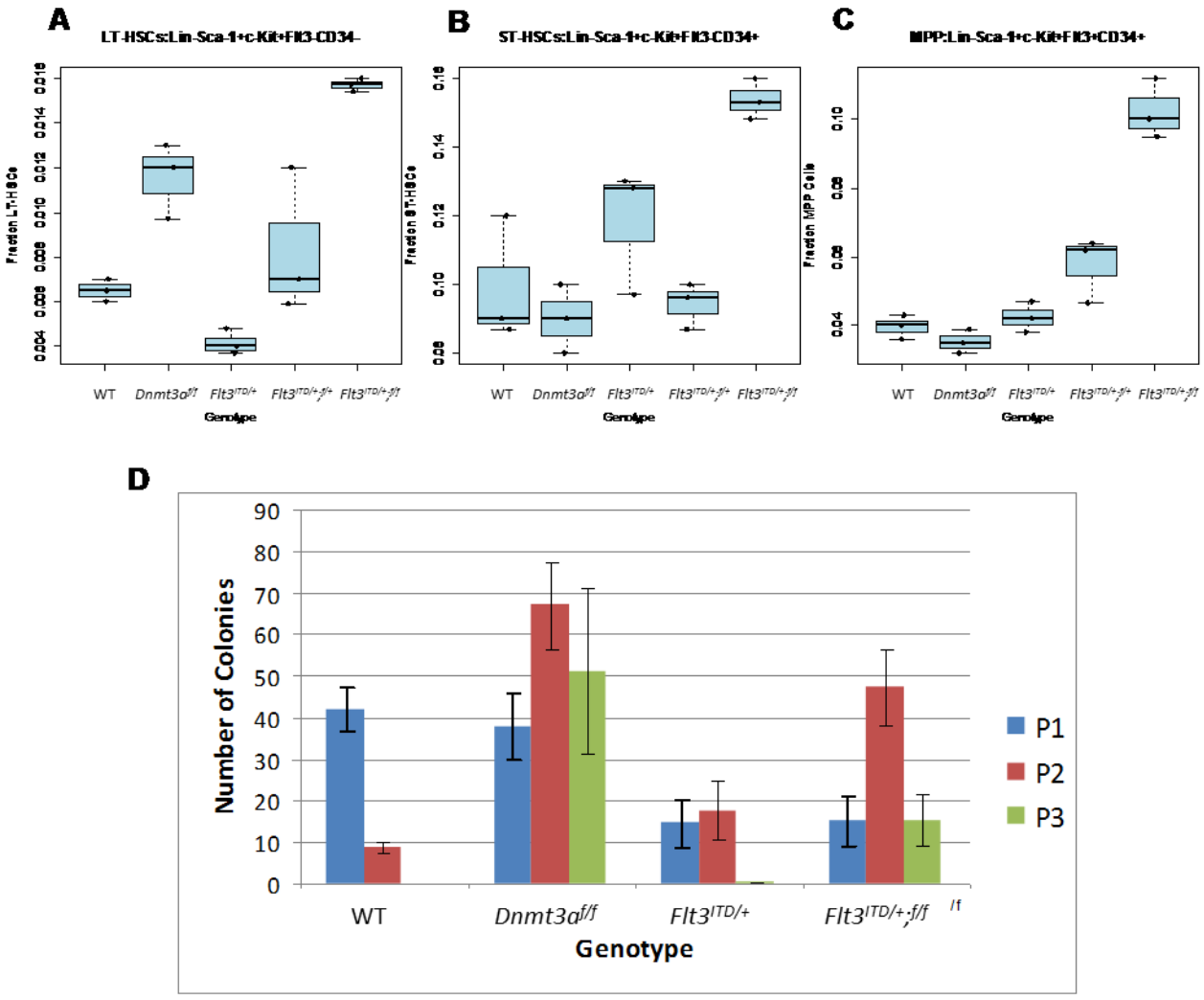
Figure 3-4. Early signs of disease development and myeloid expansion are present at 8 weeks post pIpC injection.



(A)White Blood Cell counts (WBC) remain wtihin the normal range for all genotypes. (B) Relative monocytosis is observed upon CBC, despite normal WBC. WT and *Dnmt3a*$^{f/f}$ exhibit normal leukocyte ratios, while *Flt3*$^{ITD/+}$ mice display an increase in percentage of neutrophils and concomitant decrease in percentage of lymphocytes. When these mice lose one or both Dnmt3a alleles, the phenotype is exaggerated with further decrease in relative lymphocytes and an increase in the percentage of monocytes.(C)Spleen weight is increased only in mice harboring a Flt3/ITD mutation. Splenomegaly is further enhanced with loss of one or both Dnmt3a alleles. (D) Flow cytometric analysis of bone marrow at an early time point reveals dysplastic changes . Two representative mutant mice are shown. (E) Shifts in the myeloid progenitor compartment

are present. Wildtype (WT); Flt3-ITD alone (*Flt3*$^{ITD/+}$); *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/+}$ (*Flt3*$^{ITD/+}$;$^{f/+}$); *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/f}$ (*Flt3*$^{ITD/+}$;$^{f/f}$). n=5 mice per genotype in each experiment.

Figure 3-5. Dnmt3a deletion rescues the LT-HSC depletion phenotype seen in Flt3$^{ITD/+}$ knock-in mice.



(A) LT-HSC, (B) ST-HSC, and (C) MPP populations were quantified by flow cytometric analysis of whole bone marrow of mice at 8 weeks post pIpC n=3 per genotype. (D) Serial replating of Lineage negative bone marrow at 8 weeks post pIpC from 3 mice per genotype. Each plating was performed in triplicate. Wildtype (WT); Flt3-ITD alone (*Flt3*$^{ITD/+}$); *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/+}$ (*Flt3*$^{ITD/+;f/+}$); *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/f}$ (*Flt3*$^{ITD/+;f/f}$).

4. CHAPTER 4. ADDITION OF MUTANT NPM1 INCREASES DISEASE AGGRESSIVENESS IN *Flt3$^{ITD/+}$;Dnmt3a$^{f/f}$* MICE

4.1 Introduction

Nucleophosmin family member 1 (NPM1) is a nucleolar protein which shuttles between the nucleus and cytoplasm to exert a number of critical cellular functions, including ribosomal biogenesis, controlling cell growth through transcriptional regulation, and maintaining genomic stability (Grisendi,S. 2006), and represents one of the frequently mutated genes in leukemia (Falini,B. 2009; Patel,J.P. 2012; Grisendi,S. 2006). Similar to the *DNMT3A* mutations that have been identified, *NPM1* mutations are thought to result in a loss function in several leukemia subtypes. Reciprocal translocations have been identified in certain subsets of leukemia and lymphoma, resulting in haploinsufficiency, as only one wild type copy remains intact (Grisendi,S. 2006). As a transcriptional regulator, the decrease in functional NPM1 protein is thought to drive oncogenesis through failing to control expression of genes important in restricting cellular growth.

In the setting of normal karyotype AML (NK-AML), *NPM1* mutations are heterozygous. Referred to as NPMc+, these mutations disrupt the nucleolar localization signal in the cytoplasmic portion of the protein, and replace it with a novel nuclear export motif (66 Falini 2006). Abnormal nuclear export leads to accumulation of the protein in the cytoplasm resulting in failed p53-dependent cell cycle arrest due to the inability of mutant NPM1 to interact with p19Arf (Falini,B. 2009). Given the frequent co-occurrence of FLT3 and NPMc+ mutations (Patel,J.P. 2012), our collaborators recently bred our classical *Flt3$^{ITD/+}$* mice with a transgenic NPMc+ which overexpresses the human mutant NPM cDNA under a myeloid specific promotor

(Rau 2014). While a proportion of these mice do develop AML, the lag time is sufficiently long such that additional cooperating events may be necessary to accelerate disease development and result in a greater proportion of animals developing AML.

A recent TCGA publication identified a unique patient population harboring mutations in DNMT3A, FLT3, and NPMc+ concurrently, exhibiting common clinical features (16 Cancer Genome Atlas Research Network 2013). Breeding the hMRP8-NPMc+ onto the *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/f}$ background presents the an opportunity to study the potential cooperativity of the three mutations in vivo. We hypothesized that these mutations would result in a shorter median survival compared to the 162 day survival observed in *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/f}$ mice in the previous chapter. The results presented in this chapter are extremely preliminary, and additional experiments are currently underway.

4.2 Results

4.2.1 Flt3$^{ITD/+}$;Dnmt3a$^{f/f}$;NPMc+ mice exhibit shortened survival and AML

Just as in the previous chapter, mice were injected with pIpC and followed for signs of disease development. Data for this chapter is admittedly sparse, but interestingly, *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/f}$;*NPMc*+ mice was almost uniformly died at 152 days, which was also the median survival in this population (n=9) (Figure 4-1A). While this shortens survival by about ten days compared to *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/f}$ mice, the difference didn't reach statistical significance. Additionally, while the sample size is too small to draw any conclusions about disease spectra, 64% of mice develop AML, while 36% develop T-ALL (Figure 4-1B). Of note, unlike *Flt3*$^{ITD/+}$;*Dnmt3a*$^{f/f}$ which develop MPN in addition to the other full blown leukemias, addition of the NPMc+ mutant transgene seems to drive full transformation.

4.3 Discussion

In this report, we have begun to analyze, for the first time, the contribution of three, commonly concurrently mutated genes. Thus far, we only have appreciable data for *Flt3*$^{ITD/+}$*;Dnmt3a*$^{f/f}$*;NPMc+* mice, and have determined so far that they almost uniformly die at 152 days, with the majority developing AML. As a corollary, the addition of mutant NPMc+ seems to drive these mice down a myeloid fate, with a more aggressive disease, increasing the number of blasts present in these mice (data not shown). It will be interesting to assess whether Dnmt3a dosage will affect survival and stem cell properties in the presence of mutated NPM1. Additionally, it is known the *DNMT3A* and *NPM1* are concurrently mutated (with wild type FLT3). We have included this genotype as well (*Dnmt3a*$^{f/f}$*;NPMc+*) in our analysis, as no one has performed this experiment. These mice particularly provide an important resource to look at the contribution of mutant *Flt3* to disease development, survival, and genomic changes on the molecular level. Additional studies are underway to provide insight into loss of just one *Dnmt3a* allele in the triple mutant mice,

4.4 Conclusions

To our knowledge, we are the only group investigating a model in which Flt3

Dnmt3a, and Npm1 are all mutated, representing a potential new took to model a new subtype of patients that has recently been recognized in which all three genes are mutated. These studies are ongoing to increase the number mice in each genetic cohort, and broaden our understanding of each gene's contribution, alone or in tandem, to disease development

4.5 Material and methods

4.5.1 Mice.

Transgenic mice expressing Flag-tagged human NPMc+ mutant driven by human MRP8 promoter (hMRP8-NPMc+) were generated as previously reported (Cheng,K. 2010). These mice were bred with the mice described in section 3.5.1, and pIpC injections were performed as described in this section as well. Genotyping primers and those to assess effective knock-out are described in Table 3-1. Additionally, to genotype for the NPMc+ transgene the following primers were used NPM874F-GGT TCT CTT CCC AAA GTG GAA GC, MRP8R- GAG GTA TTG ATG ACT TTA TTA TTC TGC AGG. Diagnoses and classification of hematopoietic neoplasms were made based on the previously established Bethesda proposals (Kogan,S.C. 2002; Morse,H.C.,3rd 2002). All animal experiments were performed according to protocols approved by the Animal Care and Use Committee of Johns Hopkins University in accordance with guidelines set forth by the National Institutes of Health.

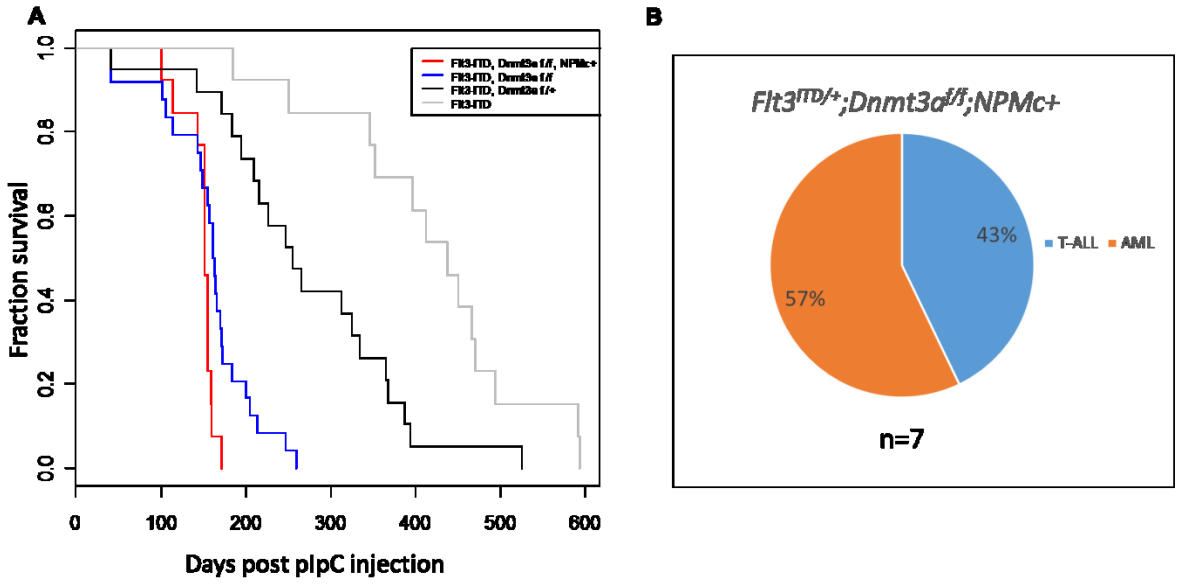
4.5.2 Disease characterization.

Complete peripheral blood cell count and cytology. Mice were monitored and sacrificed when they exhibited signs of disease development (lethargy, ruffled coat, abnormal complete blood count (CBC) differential). Peripheral blood was collected from the facial vein and subjected to complete blood cell counting, and a WBC differential was performed using the Hemavet950 system (Drew Scientific). Bone marrow cytospins and peripheral blood smears were stained using a modified Wright-Giemsa protocol (Sigma-Aldrich), and representative images were acquired using a Zeiss Axioskop upright microscope (Carl Zeiss).

Flow cytometry. Diagnostic flow cytometric analysis was performed as previously described (Li,L. 2008). Data were analyzed using FlowJo Version 9.3.3 software (TreeStar).

4.6 Figures: Chapter 4

Figure 4-1. Preliminary survival and disease distribution in $Flt3^{ITD/+}$;$Dnmt3a^{f/f}$;NPMc+ mice



(A) Kaplan-Meier Survival Curve. This curve is identical to the survival curve in figure 3-1B, with the addition of the red line, representing *$Flt3^{ITD/+}$;$Dnmt3a^{f/f}$;NPMc+* with a median survival of 152 days (n=9). (B)) Disease distribution of *$Flt3^{ITD/+}$;$Dnmt3a^{f/f}$;NPMc+* mice.

5. CHAPTER 5. CONCLUDING REMARKS

5.1 Knock-in of specific FLT3 mutations in mice facilitates study of signaling differences

Chapter 2 recounts the underlying differences between AML patients bearing FLT3-ITD and FLT3-KD mutations. With significant prognostic differences in these populations, murine models bearing mutations in the respective endogenous murine loci present an opportunity to study the contributions of each of these mutations in a genetically pure setting. We previously saw that the Flt3-ITD knock-in mice solely develop MPN, and succumb to disease much more rapidly (350 days vs 600 days) than the Flt3-KD model, recapitulating the patient survival data. The fact that Flt3-KD mice develop lymphoid diseases, and others MPN, may point to key differences between the two mutations and the pathways they act upon.

After performing an expression array, it was clear that expression signature of progenitor cells from the Flt3-KD mice were similar to the Flt3-ITD samples. These results are not surprising since the disease course for FLT3-ITD patients is far more aggressive. In analyzing the data and performing additional validation, I determined that Dntt (or Tdt), a protein essential to creating receptor diversity in the lymphocytes, was reciprocally expressed in LSK cells from our $Flt3^{D835Y/+}$ and $Flt3^{ITD/+}$ mice compared to wild type samples. The downregulation in the ITD mice is expected, since they exhibit a shift toward the myeloid lineage and a lack of lymphoid cells. Additionally, there is an obvious defect in DNA repair, so it follows that perhaps the downregulation of this protein contributes to faulty repair of double strand breaks and increased DNA damage. Further studies are necessary to examine expression patterns and abundance of

double strand breaks within non-lymphoid cell types that may be contributing to increased DNA damage seen in ITD bearing patients and knock-in mice.

We also confirmed that Cox6a2 exhibited reciprocal expression in the two mutant models, with lower expression in the *Flt3*$^{ITD/+}$ and higher expression in *Flt3*$^{D835Y/+}$ compared to wild type. As a protein expressed in the mitochondrial membrane, Cox6a2 is a cytochrome c oxidase gene involved in the terminal steps of the electron transport chain, transferring electrons from reduced cytochrome c to oxygen. The observed down regulation with age in ITD mice indicates the loss of expression may be important in disease progression and development. The same cell population also exhibits and increase in reactive oxygen species with age. If membrane potential and permeability are compromised as a result of Cox6a2 repression, this may contribute to the increase in ROS accumulation, and DNA damage observed in these mice.

5.2 *Dnmt3a* deletion cooperates with the *Flt3-ITD* mutation to drive leukemogenesis in a murine model

With the advent of affordable whole genome sequencing, we have established an ever expanding compendium of leukemia associated mutations. The field is now faced with the arduous task of defining the functional contributions of these mutations, both in vitro and in vivo. FLT3-ITD and DNMT3A mutations frequently occur together in patients and portend a poor prognosis. While we have a knock-in model of the FLT3-ITD mutation, attempt to create a knock-in of the most frequent DNMT3A mutation (R882H) have been challenging (knowledge gained through personal communications with Tim Ley's group). Since the most common R882H mutation is widely thought to behave in a dominant negative manner, bone marrow specific, conditional knock-outs are currently utilized as a surrogate.

Chapter 3 defines, for the first time, a model of cooperativity between *Flt3*$^{ITD/+}$ and *Dnmt3a* deletion, wherein mice with both mutations exhibit shortened survival, and leukemia development in a proportion of mice. The fact that loss of just one Dnmt3a allele reduces survival dramatically compared with *Flt3*$^{ITD/+}$ alone, and also leads to leukemia, illustrates the importance of stoichiometry of the protein within the cell. We also determined the importance of both mutations in expanding the stem cell pool, and preliminary results suggesting that both are important in enhancing stemness via clonogenicity assays. While we used a modified *Flt3*$^{ITD/+}$ mouse, where expression of the mutant allele was repressed, then restored simultaneously with *Dnmt3a* deletion, this still doesn't perfectly model the order of events in clonal evolution in human samples (Shlush,L.I. 2014; Welch,J.S. 2012). The ideal model would elicit expression of the mutant Dnmt3a allele first (rather than a knock-out), then induce expression of Flt3-ITD. These mice would more accurately mimic the step wise progression of mutations we know to occur through sequencing studies, however no such model currently exists.

5.3 Addition of mutant NPM1 increases disease aggressiveness in *Flt3*$^{ITD/+}$*;Dnmt3a*$^{f/f}$ mice

The work presented in chapter 4 is extremely preliminary, but aims to answer a very important question. The recent TCGA report identified a new subset of leukemia patients that harbored DNMT3A, FLT3, and NPM1 mutations simultaneously. While I was already working on the project discussed in chapter 3, I was breeding in the NPMc+ transgene. After about a year, I finally had mice that were suitable for experiments, harboring the *NPMc+* transgene, the *Mx1-Cre* transgene, the *Flt3-ITD*, and wild type, one, or two floxed *Dnmt3a* alleles. Preliminary data suggests that mutant NPM1 in the context of the *Flt3*$^{ITD/+}$*;Dnmt3a*$^{f/f}$ mice does increase disease aggressiveness,with increased blasts in the marrow and a slight (10 day) decrease in survival.

While the decrease is not significant, it is extremely predictable at about 150 days. This work is ongoing to include many more mice and additional genotypes.

5.4 Additional opportunities on the horizon

The wealth of publicly available data has increased dramatically during my training, and serves as a rich resource to draw conclusions from mouse studies, and inform future experiments. The ultimate goal of each chapter presented in this dissertation is to functionally characterize the contributions of each common leukemia associated mutation in a genetically pure system. By perturbing the endogenous mouse locus of a given gene, we can study how that one gene, in isolation, contributes to cellular and disease phenotypes.

Given the long lag time to disease development in *Flt3*$^{ITD/+}$*;Dnmt3a*$^{f/f}$ mice from chapter 3, for example, there are likely a number of cooperating genetic events that must occur during disease progression to finally result in transformation. One of our future goals is to perform exome sequencing to define somatic changes that may be common among these mice (or fall into common pathways).

More immediately, we aim to take LSK cells from wild type, *Dnmt3a*$^{f/f}$, *Flt3*$^{ITD/+}$, and *Flt3*$^{ITD/+}$*;Dnmt3a*$^{f/f}$ at 100 days post pIpC injection and perform MBD-seq (or RRBS as funds dictate) and RNA-Seq to look at the changes each genetic abnormality is responsible for. By using the LSK population at a time point prior to leukemia development, we can compare between genotypes, as *Flt3*$^{ITD/+}$ mice never exhibit leukemia. We will not only be able to correlate expression changes with epigenetic modifications, but we will also be able to use these data as a powerful resource to discover "essential" or targetable genes. For example, recently, Ross Levine's group bred the *Flt3*$^{ITD/+}$ model to a *Tet2*$^{-/-}$ and following disease characterization,

performed bisulfite sequencing and gene expression analysis (Shih,A.H. 2015). They saw that *Gata2* was hypermethylated and downregulated in double mutant mouse samples, as well as patients with both mutations as analyzed through public data and independent sequencing efforts. By transducing $Flt3^{ITD/+}$;$Tet2^{-/-}$ bone marrow with *Gata2*, they found re-expression of Gata2 enforced differentiation and attenuated disease. These findings indicate that Gata2 silencing is an important event in disease progression, and perhaps reactivating the gene therapeutically would be effective in treating AML. We hope to implore a similar strategy with our $Flt3^{ITD/+}$;$Dnmt3a^{f/f}$ samples and identify similarities between mouse and human commutated samples that may represent essential, and targetable genes and pathways.

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Appendix 1. R code for expression analysis in chapter 2

```
> library(limma)
> x <- read.ilmn(files="probe new.txt",ctrlfiles="control probe
new.txt",other.columns="Detection")
Reading file probe new.txt ... ...
Reading file control probe new.txt ... ...
> options(digits=3)
> targets <- readTargets("targets.txt")
> y <- neqc(x)
> y <- y[expressed,]
> ct <- factor(targets$gtype)
> design <- model.matrix(~0+ct)
> colnames(design) <- levels(ct)
> fit <- lmFit(y,design)
> contrasts <- makeContrasts(D835Y-WT, ITD-WT,levels=design)
> contrasts.fit <- eBayes(contrasts.fit(fit, contrasts))
> summary(decideTests(contrasts.fit, method="global"))
> fit2 <- contrasts.fit(fit, contrasts)

DECIDE<- decideTests(fit2,method="global",adjust.method="BH",p.value=0.05)
```

Curriculum Vitae

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EDUCATION

Johns Hopkins School of Medicine, Baltimore, MD

PhD, Human Genetics, December 2015 (Expected)

Thesis: "*Dnmt3a* Deletion Cooperates with the *Flt3-ITD* Mutation to Drive Leukemogenesis in a Murine Model"

University of Connecticut, Storrs, CT

BS, Diagnostic Genetic Sciences, May 2006

RESEARCH EXPERIENCE

Johns Hopkins School of Medicine, Baltimore, MD

PhD Candidate, August 2009-Present

- Thesis Advisor: *Don Small*, MD, PhD
- Characterized a clinically relevant mouse model of Acute Myeloid Leukemia to serve as a powerful tool to test novel therapeutic strategies
- Generated mice harboring mutations in both *Flt3* and *Dnmt3a* (genes that are often concurrently mutated in patients) and illustrated that these mice develop leukemia mimicking human disease
- Diagnosed mice by performing routine peripheral blood counts, flow cytometry, and histology on bone marrow, spleen, and lymphnode tissues
- Transplanted leukemic cells into irradiated recipient mice and investigated drug combinations with curative potential
- Performed analyses and created graphs using Excel and R to examine large gene expression data sets and identified upregulated genes that could represent therapeutic targets.

Brigham and Women's Hospital/Harvard Medical School, Boston, MA

Cytogenetics Research Technologist, July 2006-July 2009

- Laboratory of *Cynthia C. Morton*, PhD
- Mapped breakpoints of novel chromosomal rearrangements in leukemia patient samples to identify new genes critical for cancer development
- Described a novel *SSBP2-JAK2* fusion gene, representing an additional target that may respond to JAK2 inhibitors
- Worked independently, performing fluorescence *in situ* hybridization, RNA and DNA isolation, array CGH, polymerase chain reaction, electrophoresis, plasmid preparation, cloning and subcloning
- Conducted literature and relevant database searches i.e. UCSC genome browser, Uniprot, Ensembl
- Supervised rotation students and colleagues

Brigham and Women's Hospital, Boston, MA

Clinical Internship, July 2005-December 2005

- Performed independent research investigating the prevalence of tetraploid metaphases in chorionic villi and amniotic fluid *in situ* cultures
- Cultured and karyotyped amniotic fluid, chorionic villi, products of conception, tumor, and bone marrow samples

PUBLICATIONS

Poitras JL. Reflections on Janet Rowley, MD. Journal of the Association of Genetic Technologists. 2014; 40(1): 29.

Poitras JL, Costa D, Kluk MJ, Amrein PC, Stone RM, Lee C, Dal Cin P, Morton CC. Genomic alterations in myeloid neoplasms with novel, apparently balanced translocations. Cancer Genetics. 2011; 204(2): 68-76.

Poitras JL, Dal Cin P, Aster JC, DeAngelo DJ, Morton CC. Novel *SSBP2-JAK2* fusion gene resulting from a t(5;9)(q14.1;p24.1) in Pre-B Acute Lymphocytic Leukemia. Genes, Chromosomes & Cancer. 2008; 47:884-889.

PRESENTATIONS **Poitras JL,** Heiser D, Li L, Nguyen B, Duffield AS, Gamper C, Small D. "Dnmt3a Deletion Cooperates with the Flt3-ITD mutation to drive leukemogenesis in a murine model". 56th American Society of Hematology Annual Meeting and Exposition, San Francisco, CA, December 8th, 2014.

- Poster

Poitras JL, Dal Cin P, Aster JC, DeAngelo DJ, Morton CC. Novel *SSBP2-JAK2* fusion gene resulting from a t(5;9)(q14.1;p24.1) in Pre-B Acute Lymphocytic Leukemia: A TGAP Endeavor. Cytogenetics Laboratory Conference at the Department of Pathology, Brigham and Women's Hospital, Boston, MA, June 11, 2008.

- Invited Presentation

Poitras JL. The Prevalence of Tetraploid metaphases in *in situ* cultures. Cytogenetics Laboratory Conference at the Department of Pathology, Brigham

and Women's Hospital, Boston, MA, Dec. 12, 2005

- Undergraduate research talk

HONORS AND AWARDS

Poitras JL, Heiser D, Li L, Nguyen B, Duffield AS, Gamper C, Small D. "Dnmt3a Deletion Cooperates with the Flt3-ITD mutation to drive leukemogenesis in a murine model". 56th American Society of Hematology Annual Meeting and Exposition, San Francisco, CA, December 8th, 2014.

- Abstract Achievement Award

VOLUNTEER EXPERIENCE

Volunteering Untapped, **Volunteer,** February 2014-Present

- Volunteering Untapped partners with a different nonprofit organization in Baltimore each month, providing young professionals with an opportunity to give back to the community.

Maryland State House, Advocate/Lobbyist, February 2015

Independent Higher Education Day

- Met with elected officials in support of higher education funding through the Sellinger Program

Delete Blood Cancer, Registry Drive Host, September 2014

- Hosted a bone marrow registry drive at Hampdenfest (a local street festival in Baltimore, MD)
- Recruited 100 new potential donors, while raising public awareness.

Charlestown Lacrosse and Learning Center, Tutor/Reading group leader

Charlestown, MA, April 2007-May 2009

- Led summer weekly reading groups for ages 8-12
- Tutored children grade 5-12 weekly