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Characterization of a more clinically relevant human leukemia xenograft model to examine perturbation of MET/SAM metabolism as a novel therapeutic paradigm for MLL-R leukemia in vivo.

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CHARACTERIZATION OF A MORE CLINICALLY RELEVANT HUMAN LEUKEMIA XENOGRAFT MODEL TO EXAMINE PERTURBATION OF MET/SAM METABOLISM AS A NOVEL THERAPEUTIC PARADIGM FOR MLL-R LEUKEMIA *IN VIVO*

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A Dissertation Approved on

July 26th, 2019

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ABSTRACT

CHARACTERIZATION OF A MORE CLINICALLY RELEVANT HUMAN LEUKEMIA XENOGRAFT MODEL TO EXAMINE PERTURBATION OF MET/SAM METABOLISM AS A NOVEL THERAPEUTIC PARADIGM FOR MLL-R LEUKEMIA *IN VIVO*

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Acute myeloid leukemia (AML), is a heterogeneous clonal disorder characterized by an accumulation of malignant myeloid progenitors in the bone marrow (BM), hindering normal hematopoiesis. AML exhibits dramatic heterogeneity in terms of cytogenetics, morphology, and chemotherapeutic sensitivity. Therefore, the investigation of novel, efficacious AML therapeutics will require advanced preclinical *in vivo* model systems, capable of recapitulating patient specific disease heterogeneity, and induction chemotherapy outcomes. A major focus and eventual outcome of this work was the establishment and development of a more clinically relevant mouse xenograft model of patient AML, that efficiently harbors patient derived xenografts (PDXs), and unlike more

prevalent SCID models can tolerate more clinically relevant doses of DNA damaging induction chemotherapy.

We examined the functional utility of our newly established, advanced AML PDX model to confirm our *in vitro* findings that perturbation of methionine (Met) / S-adenosylmethionine (SAM) metabolism is uniquely cytotoxic to MLL-rearranged (MLL-R) leukemic cells, *in vivo*. We demonstrate here that perturbation of Met/SAM metabolism decreases intracellular methylation potential, alters global histone methylation dynamics, impairs the expression and function of the H3K79 methyltransferase DOT1L, and induces apoptosis in MLL-R leukemic cells. We show a significant extension in the survival of mice harboring aggressive patient MLL-R leukemias, when treated with a pharmacologic inhibitor of Met/SAM metabolism and induction therapy, as compared to induction alone. The work featured in this dissertation establishes a novel chemotherapy tolerant AML xenograft model, demonstrates its translational utility, and supports the continued investigation of targeted inhibition of Met/SAM metabolism against MLL-R leukemia.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

BACKGROUND AND INTRODUCTION

1.1 Acute Myeloid Leukemia

The highly heterogeneous clonal disorder, acute myeloid leukemia (AML), is defined by an accumulation of malignant immature myeloid progenitors in the bone marrow (BM) and peripheral blood (rarely the central nervous system), that exhibit a block in normal hematopoietic differentiation. The high degree of observed disease heterogeneity is dependent largely on which particular myeloid progenitor acquires oncogenic mutations, and during which stage of normal hematopoiesis the oncogenic lesions occur.[1] AML is the most prevalent form of adult leukemia and the second most common childhood leukemia, and the incidence of *de novo* AML has risen by 2.2% every year for the last 15 years. There will be an estimated 21,380 new cases of AML diagnosed this year in the U.S., many of them children. Modern remission induction chemotherapy of AML using an anthracycline (doxorubicin, daunorubicin, or idarubicin) combined with cytarabine, results in approximately 60-70% initial remission rates, but unfortunately the overall 5-year survival remains poor at only 26%, due largely to refractory relapse.[2] Clinical care of AML has stagnated since the advent of induction therapy in the 1970s and remains the only approved standard of care

(SOC) frontline therapy for AML, irrespective of the dramatic heterogeneity of patient AML in terms of cytogenetics, disease morphology, associated prognoses, and induction chemotherapeutic sensitivity. Therefore, novel patient-tailored therapies and/or treatment paradigms that are more efficacious, specific to patient cytogenetics, and better tolerated by elderly and infant patients should be investigated in conjunction with SOC therapy.[3]

The extreme heterogeneity of AML in terms of cytogenetics, morphology, and therapeutic sensitivity present a unique challenge for oncologists both in terms of treatment, and in classification. Initially, AML was classified according to the French-American-British (FAB) classification system, which groups various sub-types of AML based mainly on morphology, immunological phenotype, and basic cytochemical criteria into eight major subtypes (FAB M0 to M7).[4] The FAB classification system was initially established by light microscopy of malignant cells, as well as some limited cytogenetic characterization of recurrent genetic abnormalities. The numerical hierarchy of the classification system corresponds to the relative degree of maturity (lowest being the least differentiated), as well as the progenitor cell type from which the leukemia developed.

The FAB classification system was revolutionary in redefining our understanding of AML as a group of related neoplasms rather than a single disease, and clearly recognized distinct morphology and cytogenetics as key determinants of clinical outcomes and overall prognosis. However, this system was found to be somewhat oversimplified, and severely lacking in certain instances. The FAB classification system works well only in instances where the

observed genetic lesions directly induce the observed cellular morphology and cytochemical signaling. For example, in acute promyelocytic leukemia (APL, M4 subtype), or APL with abnormal eosinophils (M4Eo) the morphological characteristics directly predict the genetic abnormalities, making these classifications highly accurate.[5] However, as research and screening methodologies progressed and improved, it became evident that morphology/cytochemisty did not always correlate with or predict observed genetic abnormalities. Furthermore, patients diagnosed with the same proposed sub-type of AML by morphology and immunological phenotyping, often exhibit differences in observed genetic lesions. Additionally, there are many cases where there is absolutely no correlation between observed genetic defects and morphology, emphasizing the point that genetic profiling of AML provides a more accurate system of classification and prognostic prediction. While the FAB system was revolutionary in its recognition and characterization of AML as a group of related myeloid neoplasia, based on morphology and cytochemical heterogeneity, it does not accurately convey the genetic diversity and associated clinical outcomes of the disease sub-types.

In response, the World Health Organization (WHO) developed an alternate classification system in 2001, based on the central concept that the sub-types of AML are actually distinct diseases through correlation of genetic, morphologic, and clinical data. This new system of classification differs significantly from the FAB system as it defines cases of AML into unique sub-groups based on biological, clinical, and importantly genetic characteristics. The

WHO classification system was updated in 2008 to its current format which, like the FAB system, defines seven major AML subtypes: 1.) AML with recurrent genetic abnormalities, 2.) AML with myelodysplatic related changes, 3.) therapy-related myeloid neoplasms, 4.) Myeloid sarcoma, 5.) Myeloid proliferations related to Down Syndrome, 6.) Blastic plasmacytoid dendritic cell neoplasm, and 7.) AML otherwise not categorized.[6] Each major subtype is divided into specific subcategories based on commonly observed recurrent genetic abnormalities, detailed karyotype analyses, and molecular abnormalities, providing a much better representation of the vastly differing genetic landscape of AML even in morphologically similar cases. It is evident that the WHO system of classification is far superior to the FAB system in terms of scope and descriptiveness, and therefore has been universally adopted as the chosen classification methodology for AML.

1.2 Clinical Therapy of AML

Contemporary SOC cytotoxic chemotherapy for AML is divided into two distinct phases, namely induction therapy and consolidation therapy. The first phase is designed to dramatically reduce the leukemic blast burden in the periphery as well as the bone marrow, and eventually induce remission. Intensive induction therapy is effective in inducing complete remission (CR) in 60-70% of patients who are able to tolerate therapy. Sadly, over 45% of patients achieving initial CR have a less than three-year overall survival due to relapse of refractory disease. The SOC induction therapy regimen for young adults and healthy elderly patients is commonly referred to as “7+3”, and utilizes a continuous

intravenous (IV) infusion of a DNA damaging anthracycline (60 or 90mg/m^2) administered the first three days along with continuous infusion of cytarabine (Ara-C, 100mg/m^2) for all seven days of therapy. As aforementioned, the goal of induction therapy is to induce CR, which is clinically defined as less than 5% blasts in the spicule containing BM aspirate, as well as a platelet count greater than or equal to 100,000 cells/ml of blood and absolute neutrophil count greater than 1000 cells/ul.[6-8]

The second phase of AML therapy, consolidation therapy, aims to prevent subsequent disease relapse by reducing the minimal residual disease (MRD) burden, or the surviving leukemic blasts and founder cells which remain protected from cytotoxic drug exposure deep in the BM niche microenvironment. These cells, which survive the initial induction therapy, are the causative agents of refractory relapse resulting in patient mortality, and currently no FDA approved therapies exist for relapsed and/or refractory AML. Oncologists have two options for consolidation: 1.) HSC transplantation (HSCT) usually by allogenic BM transplant or 2.) Additional chemotherapy with intermediate dose cytarabine (1.5g/m^2) twice daily on days 1, 3, and 5 in 3-4 cycles. These strategies are used alone or in combination at the clinician's discretion based on the patient's ability to tolerate aggressive chemotherapy, the availability of compatible BM donors, and the patient's AML subtype. Chemotherapeutic consolidation therapy (3-4 cycles) alone has been shown to effectively improve survival and lengthen remission in patients less than 60 years of age, leaving HSC transplant as a last resort in the result of relapse. Comparisons of high dose (3 g/m^2) and

intermediate dose (1.5 g/m^2) cytarabine show equal benefit for patients under 60 years of age and therefore the intermediate dose became the standard of care, while the high dose regimen showed greater efficacy in patients with CBF AML [t(8:21); or inv(16)] or AML with mutated NPM1.[9,10]

In certain prognostic groups, HSCT remains the most effective long-term therapy for AML, with up to 60% of transplanted patients achieving CR. Yet, many patients are deemed ineligible for HSCT due to lack of suitable donors, co-morbidities, or a failure to achieve CR during the induction phase. Age, was initially thought to be the key determining factor in whether or not to use HSCT, but new evidence has conclusively shown that transplant success is actually dependent on remission status and co-morbidities. Improvements in conditioning regimens, supportive care, and haplo-identical/cord grafts have allowed for a larger percent of AML patients to become eligible for HSCT. The risk of relapse remains significantly higher in patients treated with HSCT alone, and again many patients are ineligible for transplant due to age or underlying co-morbidities.[11,12] Therefore, the investigation of new well-tolerated therapeutics, especially ones targeting patient specific genetic lesions, will be crucial to improving clinical therapy of AML.

1.3 Heterogeneity of AML presentation and common genetic abnormalities

Chromosomal irregularities (deletions, translocations, tandem repeats etc.) are observed in the majority of primary AML cases (approximately 50%), and are well recognized as causative events driving leukemic progression and transformation.[9] These chromosomal abnormalities are directly correlated with

varied patient survival, treatment outcomes, and remission duration making cytogenetic analysis of AML a critical factor in determining treatment modalities. For instance, AMLs harboring t(8;21)(q22;q22), inv(16)(p13.1q22), or t(15;17)(q22;q12) have a universally better prognosis, and prolonged survival time as compared to other irregularities, like alterations involving chromosomes 5 and 7 (complex karyotype AML) or 11q23, which are associated with shorter survival and diminished chemotherapeutic sensitivity.[13] Often, these genetic abnormalities can lead to the expression of characteristic aberrant fusion proteins (AML-ETO, PML-RARA, mixed lineage leukemia [MLL] fusions, DEK-CAN, etc.), most of which have been directly implicated in driving oncogenic transformation and blocking normal hematopoiesis through altered epigenetic regulation of gene expression. Interestingly, approximately 40-50% of all AML cases are actually cytogenetically normal (CN-AML) using conventional analysis,[14] demonstrating convincingly that AML associated genetic irregularities are not the only factor at play during the development of myeloid leukemia. Recent work is now starting to show that the presence or absence of specific mutations and/or altered regulation of gene expression can be used to further delineate various types of AML (especially CN-AML), and possibly provide novel avenues of targeted therapy.

Experts are only beginning to understand the effects of dysregulated gene expression patterns and mutation status, on transformation and leukemogenesis.[5] This is of special importance in people diagnosed with CN-AML, which appears normal according to karyotyping analysis, and lack obvious

genetic lesions found in other AML subtypes. As such, the prognostic value of cytogenetic analysis is limited in these patients, but with the advent of next-generation sequencing, the genetic portrait of CN-AML has become much clearer. It is now understood that on average CN-AMLs have 13 mutations, five of which are recurrent “driver” mutations (directly causing leukemogenesis) and eight are recurrent random “passenger” mutations.[15] These mutations result in molecular abnormalities in the proteins produced, and these abnormalities are now being identified as key mediators of AML pathogenesis, as well as valuable prognostic markers accurately predicting clinical outcome, and as novel druggable targets for new therapeutic approaches.[16] Mutations in several proteins namely, nucleophosmin 1 (NPM1), Fms-Like Tyrosine kinase (FLT3), and DNA methyltransferase 3A (DNMT3A) have come to the forefront of investigation due to their very high relative frequency in AML, and their influence on prognosis and treatment outcomes.

NPM1 mutations are the most recurrent molecular abnormality observed in AML patients, and can be detected in approximately 25-30% of patients.[17,18] These mutations result in the expression of a mutated protein lacking a folded C-terminal domain, which alters sub-cellular localization from the nucleus to the cytoplasm, and this transition has been shown to greatly promote myeloid proliferation and leukemogenesis. AMLs harboring these mutations generally present with monocytic morphology, and have generally favorable prognostic outcomes in the absence of other mutations (e.g. FLT3 internal tandem duplications [FLT3-ITD]).[19] AMLs with *NPM1* mutations have been conclusively

shown to have a greater sensitivity to SOC induction chemotherapeutics and the overall survival is greatly increased in both old and young patients as compared to other molecular abnormalities.[20] However, *NPM1* mutations are also associated with several other recurrent abnormalities like DNMT3A mutations, FLT3-ITDs, and isocitrate dehydrogenase mutations, most of which in conjunction with *NPM1* mutations actually worsen the prognosis.[21] Again, this is a clear demonstration of the inherent heterogeneity of AML, and the importance of cytogenetic analyses to examine potential molecular abnormalities, and guide therapeutic intervention.

FLT3 was first identified as a critical regulator of normal hematopoiesis, playing crucial roles in mediating proliferation and cell survival during hematopoiesis. FLT3-ITDs in the juxta-membrane coding domain or the second tyrosine kinase domain are detected in approximately 20% of all AML cases, and 30-45% of CN-AML. These mutations constitutively activate FLT3 promoting aberrant, unchecked proliferation of AML blasts.[22-24] Patients with AML expressing FLT3-ITDs exhibit extreme leukocytosis and characteristic nuclear invagination, and these mutations are universally associated with reduced survival, decreased induction therapeutic sensitivity, and an increased risk of relapse. When FLT3-ITDs occur in combination with other commonly recurring AML mutations they correlate with diminished survival and increased relapse rates, even when occurring with other normally favorable alterations, like NPM1 mutations. Finally, studies have shown that AML with mutations occurring in the non-juxta domain coding regions have a worse prognosis, again highlighting the

heterogeneity of AML and the clinical significance of mutational status as predictive biomarkers for patient outcome and to guide therapeutic approaches.[25]

Mutations of the *DNMT3A* gene are detectable in approximately 18-22% of all AML cases (34% in CN-AML), the most common being missense mutations affecting arginine codon 822.[21] These mutations have now been found to actually be pre-leukemic and arise very early in the evolution of the disease, but also persist even in times of CR. This finding is significant in that these mutations are perhaps the most common mutations involving proteins that regulate gene expression in AML.[26] DNMT3A is a *de novo* methylase that is capable of methylation of CpG dinucleotides in the promoter regions of target genes, thereby mediating transcriptional silencing by blocking access of transcriptional machinery to these promoters. This form of regulation, which is independent of the genetic sequence, is known as epigenetic regulation and only very recently have methylation and other epigenetic mechanisms become recognized as relevant to leukemogenesis.[27] Therefore it is logical that these mutations occur very early in leukemic development, and may even play a causative role in AML, by silencing genes required for maintenance of normal myeloid differentiation and hematopoiesis. Finally, the value of *DNMT3A* mutations as prognostic indicators remains hotly debated with some smaller studies finding a lowered overall survival (OS) and disease free survival (DFS) in patients expressing these mutations, while a much larger cohort study found no correlation between *DNMT3A* mutation status and clinical outcome.[21,28]

While these three mutations have the highest relative frequency of occurrence overall in AML and occur irrespective of cytogenetic irregularities, it is important to recognize that they represent only a small fraction of the vast array of combinations of molecular and genetic abnormalities that have been documented in AML. Furthermore, it becomes evident that AML in reality is a group of extremely heterogeneous related myeloid neoplasias in terms of morphology and cytogenetics, but more importantly these differences have been shown to result in drastically different therapeutic outcomes and overall survival. Therefore, the successful development of novel targeted AML therapeutics and treatment paradigms, will require advanced *in vivo* model systems capable of faithfully recapitulating the extreme genetic, epigenetic, and morphologic variation found in primary AML. Such advanced systems must also be able to replicate SOC induction therapy, not only as more relevant control (as opposed to vehicle only), but also as a more clinically applicable way to test experimental therapeutics and their translational/combinatorial efficacy.

1.4 Epigenetic dysregulation and heterogeneity in AML

Recently, epigenetic dysregulation has come to the forefront as a driving mechanism for the induction and maintenance of AML, and more than two thirds of patients with *de novo* AML have detectable epigenetic alterations.[29] Aberrant epigenetic regulation interferes with the physiological balance between gene activation/repression required for normal hematopoiesis and differentiation, resulting in the leukemic phenotype. The influence of epigenetic changes is only recently starting to be fully understood in the context of both normal and

malignant hematopoiesis. Early epigenetic changes are crucial to normal hematopoiesis as they regulate successive gene expression profiles that give rise to all immune and hematopoietic lineages.[30,31] For examples, DNA and histone methylation are required to silence the expression of transcription factors like RUNX1 and GATA3, which are required for self-renewal and pluripotency, thereby allowing differentiation.[32] Additionally, epigenomic analysis of normal hematopoietic stem cells (HSCs) has shown that promoter regions of transcription factors crucial for myeloid differentiation (C/EBPα, EBF1, PU.1, PAX5 etc.) show low levels of DNA methylation and are enriched for bivalently methylated histones (having both activating [H3K4me3] and repressing [H3K27me3] marks).[33] Dysregulation of these processes in AML is well documented and consistent aberrant DNA and histone methylation profiles are observed in a majority of analyzed patient samples. Interestingly, the various subtypes of AML have unique profiles of distinct aberrant methylation, findings that both highlight the importance of epigenetic regulation in the context of AML, and provide a new investigational avenue for targeted, patient specific therapy. Persuasive studies from several groups have demonstrated that many well defined AML genetic lesions, like chromosomal translocations resulting in characteristic oncogenic fusion proteins (e.g. PML-RARα, AML1-ETO, CBFβ-MYH11, MLL-fusions etc.), or mutations in key transcription factors or epigenetic regulators (e.g. DNMT3A, NPM1, TET2 etc.) can result in aberrant recruitment of a variety of epigenetic modifiers (DNMTs, HMTs, HDACs, etc.) to target genes

and associated promoter histones resulting in abnormal leukemic gene expression.[34,35]

These non-random chromosomal translocations and the resulting gene rearrangements and oncofusion protein expression, are exhibited by the majority of AML cases.[36] Many of these chromosomal translocations involve loci encoding transcriptional activators of myeloid differentiation with transcriptional effector proteins capable of interaction with co-activator or co-repressor complexes. The resultant fusion protein retains the DNA-binding ability of the wild-type transcriptional activator, but gains the capacity to mediate epigenetic regulation of gene expression through interaction with co-activator or co-repressor complexes through its fusion moiety.[37] AML therefore arises through a complex multi-stage process mediated by an accumulation of genetic and epigenetic changes, with the most common being recurrent genetic aberrations and their associated oncofusion protein expression.[38-40] Aberrant recruitment of epigenetic modifiers by oncofusion proteins to the promoters of embryonic genes or genes critical to myeloid differentiation, disrupts normal hematopoietic gene expression resulting in AML. Consequently, normal patterns of DNA methylation and histone modifications are disrupted in leukemic blasts, in a sub-type specific manner depending on their driving oncofusion protein.[41] Overall, the most common AML fusion proteins can be grouped based on those that gain repressor activity (PML-RARα, AML1-ETO, and CBFβ-MYH11) thereby silencing gene expression and those that activate gene expression (Mixed lineage leukemia [MLL]-fusions) through altered epigenetic regulation.

Fusion proteins that gain repressive function have been shown to do so through their interaction and recruitment of transcriptionally repressive epigenetic modifiers. For example, both PML-RARα and AML1-ETO fusions have been shown to suppress gene expression through the direct recruitment of DNMTs to various promoters, or the recruitment of co-repressor complexes (Sin3A, N-CoR, and SMRT) and HDACs resulting in gene silencing. Interestingly, the CBFβ-MYH11 fusion only mediates gene silencing through recruitment of Sin3A or N-CoR and HDAC8. PML-RARα can also silence gene expression through altered histone methylation mediated through recruitment of the Polycomb complex.[38] A common feature of these fusions is their abnormal ability to recruit repressive epigenetic machinery to the promoters of genes required for myelopoiesis thereby silencing their expression and driving AML.

Alternatively, MLL-fusions aberrantly activate and maintain gene expression at inappropriate times through a variety of epigenetic mechanisms including histone acetylation, histone methylation, or through interaction with the nucleosome remodeling complex SWI/SNF. The normal MLL (MLL1) protein is an activator of transcription, which works antagonistically to PcG proteins to maintain appropriate lineage and stage specific expression of the HoxA gene cluster, events crucial for proper hematopoietic differentiation. MLL can directly activate transcription by catalyzing H3K4 methylation through the HMT activity of its C-terminal SET domain[42,43], and has been shown to be fused in-frame with more than 60 different gene partners through its non-catalytic transactivatory N-terminus, in several types of leukemia. MLL rearrangement (MLL-R) correlates to

highly aggressive disease that is poorly treated by SOC induction with extremely high rates of relapse and low overall survival. MLL gene fusion partners are diverse and range from cytoplasmic proteins to nuclear factors, and are associated with AML, acute lymphoid leukemia (ALL), or biphenotypic/mixed lineage leukemias depending on the fusion moiety. However, approximately 80-90% of all MLL translocations in leukemia involve only one of six genes, all members of super elongation complex (SEC), namely AF4, AF6, AF9, AF10, ENL, or ELL.[44] The other detected fusion partners occur at an extremely low frequency, and many of them are identified in case studies only. Recently, published studies have suggested quite conclusively that the six most common MLL fusion partners share a common biochemical mechanism, by which they modulate aberrant promoter histone methylation leading to leukemogenic gene expression.

1.6 MLL-fusion protein signaling and functional dependence on the histone methyltransferase DOT1L

Chromosomal translocations resulting in rearrangement of the *MLL1* gene have been associated with AML, ALL, and biphenotypic leukemia, and result in fusion of the catalytically inactive N-terminus of MLL to a variety of binding partners most commonly members of the SEC, suggesting an underlying general mechanism of action.[45] Over the last decade elegant work published by several groups has shown that expression of several different MLL-fusions alone is sufficient to drive leukemic transformation irrespective of the fusion partner, and without additional cooperating mutations through the induction of widespread

epigenetic dysregulation, again suggesting a common mechanism for MLL-fusion mediated leukemogenesis.[46-51] Analyses of gene expression patterns from patient leukemic blasts expressing several different MLL-fusions, have shown that MLL fusion proteins preferentially upregulate and maintain expression of a specific subset of wild-type MLL gene targets, most notably *MEIS1* and the *HOXA* cluster, thus disrupting the normal progression of hematopoietic gene expression. Taken together these findings demonstrated overall that MLL-fusion proteins likely function through a common underlying mechanism influencing gene expression through aberrant recruitment of epigenetic machinery to lineage-specific developmental genes leading to their constitutive expression thus driving leukemogenesis.[52-55]

Genome-wide histone methylation profiling of MLL-rearranged leukemias revealed profound abnormalities in the distribution of one particular histone modification, H3K79 methylation, further solidifying the idea that MLL-fusions drive leukemic progression and gene expression through epigenetic dysregulation. Early evidence further supported this conclusion as H3K79 methylation is enriched at actively transcribed promoters and gene bodies, and this modification was found at high levels at the promoters of the most prominent MLL-fusion targets *HOXA9* and *MEIS1* in primary patient samples, human hematopoietic progenitors transformed by MLL-ENL, or human MLL-rearranged cell lines.[56,57] These findings put particular emphasis on the importance of the histone methyltransferase Disruptor of telomeric silencing 1-like (DOT1L), the only known H3K79 methyltransferase, in MLL-fusion mediated leukemic gene

expression. Several MLL-fusions have been shown to interact with DOT1L, MLL-AF10 being the first identified to physically interact with DOT1L. Later seminal work by Bitoun et. Al and Muller et. Al reported that the other common binding partners of MLL (AF4, AF5, AF9, AF10 and ENL) exist in a complex that also contains DOT1L and the pTEFb complex (phosphorylates stalled RNA polymerase II [Pol II] activating transcription), dubbed the ENL-associated protein or EAP complex. Furthermore, several other groups independently identified may similar complexes like AEP (containing AF4, AF5, ENL and pTEFb), a complex composed of AF4, AF9, ENL, AFF4, ELL1 and pTEFb, and DotCom (containing DOT1L, AF9, ENL, AF10, AF17, several WNT modifiers), which have collectively been termed super elongation complex (SEC).[58-60] These discoveries prompted the now generally accepted hypothesis that when fused to the N-terminus of MLL retaining transactivation and DNA binding ability, each member of the SEC complex can subsequently miss-recruit the other SEC complex members including DOT1L to wild-type MLL target loci. Next, DOT1L presumably methylates H3K79 at MLL target promoters, inducing transcriptionally permissive open chromatin formation. Finally, pTEFb can recruit and stimulate the activation of RNA Pol II to allow aberrant constitutive transcription of several MLL gene targets, most notably *HOXA9* and *MEIS1*. **(Fig. 1.1)** The extremely strong correlation between increased H3K79 methylation and MLL-fusion target loci has been dubbed an “epigenetic lesion”, emphasizing the unique expressional dependence of these loci on aberrant histone methylation.[61]

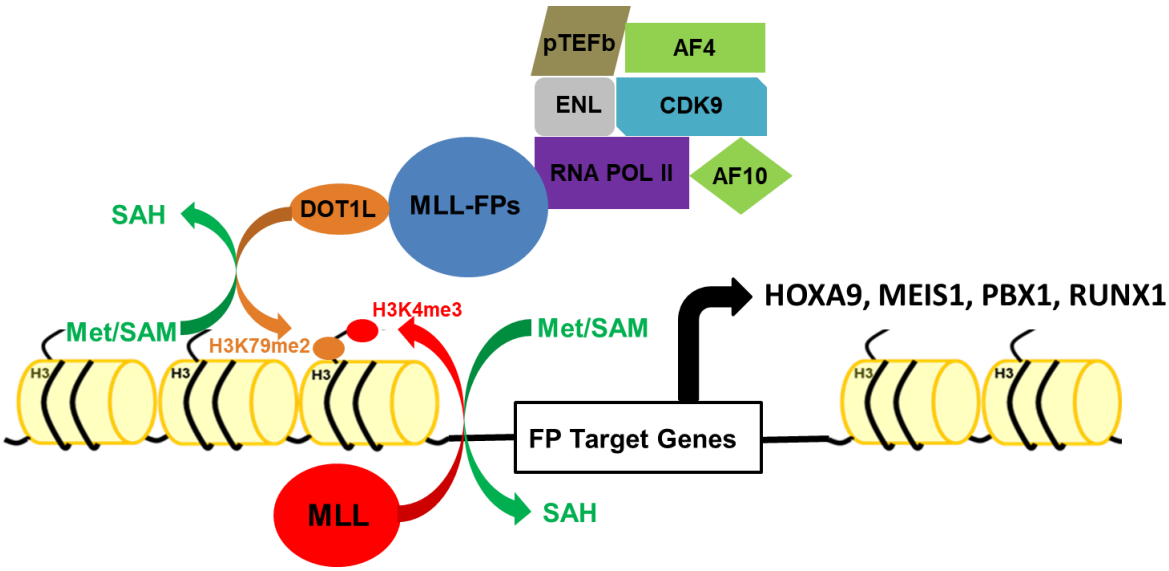

Figure 1.1 Mechanisms of DOT1L dependent MLL-R mediated leukemogenesis. Monoallelic translocations of the MLL gene result in the fusion of the catalytically inactive N-terminus in frame with a variety of partners, most commonly SEC complex members. These aberrant MLL fusion proteins (MLL-FPs) directly drive leukemogenic gene expression by mistargeting of the SAM-dependent activating H3K79 methyltransferase DOT1L to normally silenced developmental genes like the *HOXA* cluster and *MEIS1*, driving their irregular expression. Methylation of H3K79 by DOT1L at these promoters results in transcriptionally permissive conformational changes, and subsequently the functional MLL from the unfused allele can deposit the required transcriptionally activating H3K4me3. The SEC complex and RNA Pol II remain associated with the fusion partner portion of the MLL-FP, and can thus potently drive aberrant transcription of leukemogenic genes upon DOT1L mediated promoter euchromatin formation.

More recently, several groups have convincingly shown through various loss of function studies (shRNA-knockdown, pharmacologic inhibition, or conditional knockout) that DOT1L mediated activation of MLL gene targets like *HOXA9* or *MEIS1* through H3K79 methylation, is absolutely requisite for MLL-fusion protein mediated leukemic transformation, progression and maintenance *in vitro* and *in vivo*. Elegant studies published by several groups have shown that genetic loss of DOT1L function is cytotoxic to MLL-rearranged cells and inhibits MLL-fusion induced transformation (as does deletion of the *HOXA9* gene or mutation of H3K79), further supporting the currently accepted dogma of MLL-fusion mediated leukemogenesis.[62-64] Finally, these findings were confirmed by the *in vitro* and *in vivo* anti-proliferative/cytotoxic efficacy of several specific SAM competitive DOT1L small-molecule inhibitors against a variety of MLL rearranged cells, thereby confirming the results observed by genetic loss through pharmacologic inhibition of DOT1L catalytic activity.[65-68] Currently, it appears well-established that DOT1L activity is required for the initiation, progression, and maintenance of MLL-rearranged leukemias through aberrant H3K79 methylation, driving the continuous abnormal expression of leukemogenic genes like *HOXA9* or *MEIS1.* Given the now well-established and crucial role of DOT1L in MLL-rearranged leukemogenesis is mediated through the dysregulation of histone methylation dynamics, we next investigated the effects of perturbations to Met/SAM metabolism, a key metabolic pathway that determines the overall intracellular methylation potential, specifically in MLL-rearranged human leukemia *in vitro* and *in vivo*.

1.5 Methionine and S-adenosylmethionine metabolism

The methionine (Met) and S-adenosylmethionine (SAM) metabolic pathway is essential for cellular methylation reactions, including those involved in epigenetic gene regulation, and SAM serves as the primary methyl donor for the majority of cellular methylation reactions, including DNA, RNA, lipids and histone methylation (both arginine and lysine)[34,35]. In addition to being the principal methyl donor for all cellular reactions, SAM also serves as the sole source of the propylamine moiety required for polyamine biosynthesis.[69,70] The biologically usable SAM moiety is synthesized from methionine by the enzyme methionine adenosyltransferase 2A (MATIIA), and upon enzyme mediated donation of its methyl group SAM forms the product S-adenosylhomocysteine (SAH), which is further metabolized by SAH hydrolase (SAHH) to form homocysteine.[71] Homocysteine, is an obligatory intermediate in the transsulfuration pathway, and serves as the precursor for glutathione synthesis. Alternatively, homocysteine can be recycled back into methionine by the recycling enzyme methionine synthase (MTR), which catalyzes the final step in methionine regeneration by simultaneously converting 5-methyltetrahydrofolate to the biologically active cofactor tetrahydrofolate, while transferring a methyl moiety to homocysteine starting the cycle again.[72] Importantly, the intracellular ratio of SAM:SAH dictates the overall methylation potential of the cell, with SAH accumulation resulting in global feedback inhibition of cellular methylation reactions, including epigenetic methylation of DNA or histones **(Fig. 1.2)**.[73]

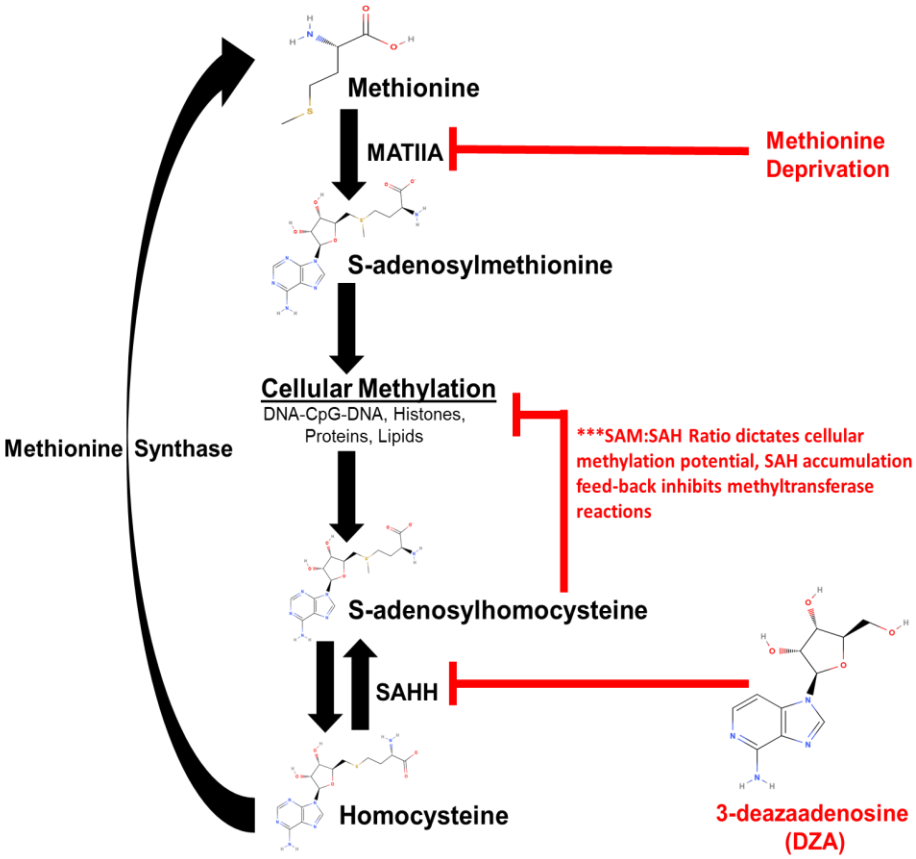

Figure 1.2 Simplified schematic of non-hepatic methionine and S-adenosylmethionine metabolism. Dietary methionine (Met) is first converted into the principal biologically active methyl donor S-adenosylmethionine (SAM), by the enzymatic action of methionine adenosyltransferase IIA (MATIIA). S-adenosylhomocysteine (SAH) is formed upon methyltransferase mediated donation of the SAM methyl moiety, and is further converted to homocysteine which can be recycled through methionine synthase mediated salvage. Importantly, the intracellular ratio of SAM:SAH determines the overall cellular methylation potential, therefore we chose to perturb Met/SAM metabolism and by extension methylation potential by either deprivation of exogenous methionine and/or DZA mediated SAHH inhibition and subsequent SAH accumulation.

Therefore, it is likely that Met/SAM metabolic flux and expression of the aforementioned enzymatic mediators are required at high levels selectively by MLL-rearranged leukemia cells, to maintain and enforce aberrant histone methylation contributing to the leukemic phenotype. Unlike genetic abnormalities, epigenetic modifications are frequently reversible, and therefore may provide new opportunities for targeted pharmacological therapies for MLL-rearranged AML. Published literature targeting this pathway specifically in MLL-rearranged leukemia is non-existent, and a very sparse body of work exists for targeting this pathway as a general anti-leukemic therapy, with studies limited to small *in vitro* studies using established human cell lines, and single agent pharmacological inhibition of MATIIA or SAHH. Thus, this dissertation will rigorously investigate methionine deprivation with and without inhibition of downstream enzymatic mediators of the biosynthetic pathway (SAHH) *in vitro* and *in vivo*, as a novel epigenetic treatment paradigm for MLL-rearranged leukemias, which have a unique dependence on aberrant histone methylation to activate and maintain enforced leukemic gene expression promoting leukemic survival and proliferation.

1.6 Advantages of *in vivo* pre-clinical modeling of AML

Animal models have consistently been shown to better predict the long-term success of experimental therapeutics in clinical trials, especially for novel AML therapeutics, as *in vivo* systems are capable of modeling the complex, highly dynamic interaction between heterogeneous populations of leukemic cells and the surrounding BM microenvironment.[74] Current evidence supports the

conclusion that specific niches within the BM microenvironment actually provide a protected sanctuary for specific subpopulations of leukemic cells capable of self-renewal and clonogenic proliferation, dubbed leukemic stem cells (LSCs). Seminal studies using early xenograft models were able to establish that only the most primitive fraction (Lin^-CD34+) of patient AML cells, and not the more mature blast population, were capable of transferring disease to primary and secondary NOD/SCID recipient mice.[75,76] It becomes evident that a hierarchy similar to normal hematopoiesis exists in AML, with a small population of self-renewing LSCs giving rise to a large population of mature, clonally expanded blasts. LSCs are able to evade normal induction chemotherapy, due to their low rate of proliferation and protection conferred by the BM microenvironment, and therefore are thought to be the cause of the high relapse rate and mortality associated with AML. Perhaps more alarming is the mounting data that these LSCs can actively induce changes in the BM microenvironment not only at the structural level, but in cytokine production and genetic/epigenetic abnormalities within supporting BM stromal cells.[77,78] Co-culture experiments have shown that HSCs cultured on BM stroma collected from AML patients have a pronounced impairment in both self-renewal and survival capacity, while LSCs lose neither attribute on AML or normal stroma.

Thus, LSCs induce changes to the BM microenvironment and supporting cells, which favor LSC survival and maintenance while simultaneously compromising the ability to support and maintain normal HSC survival and differentiation. These interactions add another layer of complexity, additional to

the dramatic heterogeneity of AML, which again must be accounted for in model systems that will ultimately be useful in the development of successful therapeutic paradigms that will translate to clinical care. Again, *in vivo* animal studies remain the only reliable way to examine the highly complex interplay between leukemic populations (LSC and blast populations) and the protected BM niche, and by extension the pharmacological efficacy of novel therapeutics. Finally, *in vivo* methodologies have been shown to have significant advantages in predicting success rates of experimental drugs, as compared to *in vitro* techniques which often show success in the lab, but due to the lack of an accurate organ specific extracellular environment, cross talk between hormones or different cell types, differential metabolism, and site specific delivery fail to show efficacy in clinical trials.[79] This remains especially true for AML, as effective novel therapeutics must be able to target the largely quiescent and protected LSC population residing in the bone marrow, or alternatively target the bone marrow microenvironment to make it unfavorable for leukemic growth or promote normal hematopoiesis.

1.7 Immune deficient mice strains in AML research

First developed in the 1960s, immune deficient mice overcame a major technical obstacle required for the successful xenotransplantation and durable engraftment of human tissues and cells. Highly immune deficient mice revolutionized the study of both normal and malignant hematopoietic processes and support the differentiation and continued growth of human xenografts *in vivo*. Characterized in 1962, nude or athymic mice, were the first immune-deficient

mice capable of accepting human cells and tissues, and since then there has been continued effort to improve patient sample engraftment through increased immune ablation.[80] Shortly thereafter severe combined immune deficient (SCID) mice were developed, which lack the ability to initiate or maintain effective adaptive immune responses due to an absence of functional B or T lymphocytes.[81] Immune ablation in these mice is achieved by a recessive mutation resulting in deficient activity of the enzyme protein kinase, DNA activated catalytic polypeptide (Prkdc), which impairs V(D)J recombination disrupting lymphocyte maturation. Prkdc is also required for effective DNA damage repair, and thus SCID mice are highly sensitive to DNA damaging chemotherapeutics. SCID mice were found to be far superior in terms of human tissue engraftment, and further refinement and immune ablation to these models came with the development of non-obese diabetic (NOD)/SCID mice. Generated through the introduction of the *Prdkc*^{scid} gene into an inbreeding NOD strain, NOD/SCID mice show consistently superior rates of human cell engraftment compared to SCID mice.[82,83] Around the same time similar strains of mice displaying a SCID like phenotype were developed bearing a defective recombination activated gene (RAG, can be either Rag1/2^{null}) and crossed with NOD mice resulting in NOD/Rag1/2^{null} mice. These mice show equivalent immune ablation and engraftment efficiency of human hematopoietic tissues compared to the more prevalent NOD/SCID mice, but retain a functional DNA damage repair response.[84,85] Further attempts were made to improve the durable long term engraftment, multilineage hematopoietic differentiation, and

proliferation of human hematopoietic xenografts through transgenic approaches allowing the expression of human hematopoietic cytokines in several iterations of NOD/SCID and NOD/RAG1/2^{null} mice.[86]

The early 2000s saw the arrival of a series of immune deficient mice with true humanization potential, capable of not only highly efficient engraftment of malignant human hematopoietic cells, but also well-differentiated multilineage expansion of normal human HSCs. These strains were developed by the introduction of a mutant *IL2ry* gene into NOD/SCID mice through respective backcross mating, generating perhaps the two most widely used immune deficient mice in hematological research, NOG and NSG mice, which are both NOD/SCID IL-2ry^{null} but differ in the location of the SCID mutation.[87,88] The IL-2ry is crucial to and B cell growth and is activated by several other interleukin cytokines including IL-2, IL-4, IL-7, IL-15, and IL-21. Thus, its inactivation allowed for enhance multilineage immune suppression and overcame inherent "leakiness" observed in NOD/SCID mice, which eventually develop both T and B lymphocytes with age.[89] Inactivation of IL-2ry was also introduced into NOD/Rag1/2^{null} mice, resulting in NRG [(NOD)/Rag1/2^{null} IL-2ry^{null}] mice whose equivalent immune impairment is dependent on hematopoietic specific RAG deficiency leaving DNA damage repair intact.[90]

Functionally NOG, NSG, and NRG mice were a revolutionary leap in murine xenograft model of human leukemia and show deficiencies in multiple immune lineages well beyond lymphocytic impairment. Defects in T, B, and natural killer (NK) cell lineages are observed in these mice, as well as markedly

reduced macrophage and dendritic cell function.[87] Dramatically enhanced engraftment rates of human HSCs, cell lines, and primary patient leukemia have been well documented in these strains, as well as well-defined multilineage expansion and maturation of several distinct human hematopoietic lineages upon normal human HSC xenotransplant when compared to parental strains. A particularly important finding was that these mice support the differentiation and proliferation of mature CD4 and CD8 single positive human T lymphocytes, which do not develop in NOD/SCID mice. These strains, particularly NSG mice, have become the predominant workhorses of hematological research focused on studying both human leukemogenesis and hematopoiesis, in an *in vivo* system that recapitulates many aspects of physiological conditions experience by normal and leukemic hematopoietic cells in the human body. Such advanced *in vivo* xenograft model systems are critical to effective preclinical investigation of novel experimental therapeutics that go on to have a high rate of success in clinical trials.

It is now well recognized that many mouse cytokines do not show cross reactivity with receptors expressed on human cells, another significant hurdle in the successful engraftment of many patient samples. Furthermore, this is a major shortcoming as cytokine mediated signaling is known to be required for normal and malignant hematopoiesis, and plays a role in leukemic progression and therapeutic outcomes. Additionally, cytokine signaling is required for reliable homing, engraftment, and proliferation of both patient leukemia samples and normal human HSCs, which are notoriously difficult to engraft.[91] In an effort to

overcome this problem several independent groups have developed various iterations of NOG and NSG mice expressing transgenes for a variety of human hematopoietic cytokines (e.g. hIL-2, hIL-3, hIL-6, hGM-CSF, etc.).[92,93] These developments further improved hematological xenograft models by allowing for efficient engraftment, differentiation and proliferation of human hematopoietic cells in a BM specific *in vivo* microenvironment along with limited human cytokine signaling.

Despite these great advances, the two most commonly utilized strains NOG and NSG mice have a major flaw in the context of investigation of novel leukemic therapeutics that is often overlooked. Mutation of the *Prkdc* gene, which impairs V(D)J recombination resulting in the SCID phenotype, also ablates its function in effective DNA damage repair. Therefore, these mice are highly intolerant to DNA damaging chemotherapeutics, which are the foundation for clinical therapy of leukemia, as they are unable to repair any drug induced DNA lesions. This poses a significant challenge especially for the investigation of novel AML therapeutics as the SOC frontline induction therapy combines an anthracycline with cytarabine, both DNA damaging compounds. Anthracyclines like doxorubicin or daunorubicin intercalate DNA thereby preventing macromolecular biosynthesis, while simultaneously stabilizing topoisomerase II promoting DNA strand breaks. Cytarabine (cytosine arabinoside, ara-C) interferes with DNA synthesis through rapid conversion to a toxic DNA damaging intermediate during S-phase DNA replication.[94,95] Mice harboring SCID mediated immune deficiencies are therefore severely intolerant to treatment with these

drugs, even at doses that are subtoxic to other mouse strains, thereby diminishing their utility in the investigation of novel and efficacious antileukemic drugs. Effective translational models must be able to closely mimic patient experience, including SOC therapy, not only as a more relevant control but also in the investigation of innovative combinatorial therapeutics.

Recently, in 2014, our collaborators at Cincinnati Children's Hospital developed a new mouse strain by crossing NRG mice with NSGS mice, which are transgenic NSG mice harboring the SGM3 triple co-injected transgenes coding for expression of human IL-3, GM-CSF, and stem cell factor (SCF). After genotyping the offspring, subsequent selective breeding together or with NRG mice allowed for the removal of the SCID mutation resulting in NRGS mice, essentially SGM3 transgene expressing NRG mice.[96] NRGS mice have no mature T, B, or NK cells due to hematopoietic RAG1 inactivation (as opposed to a SCID mutation) and also express the three aforementioned human cytokines, which are important for both the engraftment and support of human myeloid cells. Since NRGS mice lack a SCID mutation they have functional DNA damage repair and should therefore be tolerant to higher, more clinically relevant doses of induction chemotherapeutics. Indeed, published studies from our collaborators have shown that NRG mice are tolerant of doses of induction therapeutics that are highly toxic to NSG mice, and maintain the inherent radioresistance that is a hallmark of NRG mice.[79] NRGS mice are truly cutting edge and uniquely suited to modeling both normal and malignant human myelopoiesis, the influence of the *in*

vivo hematopoietic microenvironment, human myeloid cytokine signaling, and aggressive SOC induction therapy and dose escalation.

OVERALL GOALS AND SPECIFIC AIMS

It is now well established that *in vivo* xenograft models using immune deficient mice consistently better predict the success of experimental therapeutics, especially those against AML, through their ability to model complex and dynamic interactions between leukemic cells and the protected BM niche. Animal models of human leukemia have continued to improve over time, with increasing immune ablation and transgenic human cytokine expression boosting xenograft establishment and engraftment. However, despite decades of research, approved clinical therapy of AML has remained unchanged since the advent of cytotoxic induction therapy in the 1970, and no new AML therapeutics have been approved for frontline therapy (except in the case of APL) singly or in combination with the SOC. Furthermore, the majority of investigators utilize NOD/SCID IL-2Rγnull immune deficient xenograft models, whose SCID mutation and defective DNA repair render them intolerant to DNA damaging induction therapeutics like doxorubicin and cytarabine. It is therefore crucial to develop and establish a highly advanced, robust xenograft model of patient AML, is capable of highly efficient engraftment of patient cells, modeling the influence of human cytokines, and most importantly modeling therapeutic outcomes using a clinically similar induction therapy regimen.

Thus, the overall goals of this dissertation are to establish such a more clinically relevant advanced xenograft model of both cell line derived (CDX) and patient derived xenograft (PDX) human AML, and optimize a clinically similar, well-tolerated, and efficacious SOC induction therapy regimen to recapitulate clinical therapy. Furthermore, we examined the comparative utility of each strain in the investigation of CDXs, PDXs, response to SOC induction chemotherapeutics, and humanization potential. The final goal of this dissertation is to utilize such an advanced preclinical model to investigate therapeutic efficacy of targeted inhibition of Met/SAM metabolism against MLL-rearranged AML *in vivo*. Preliminary *in vitro* data from our group has shown that perturbation of Met/SAM metabolism is cytotoxic to MLL-translocated leukemic cells and alters global histone methylation dynamics and gene expression, specifically decreasing the expression and function of the histone methyltransferase DOT1L. These findings must be confirmed *in vivo* to merit further investigation into the therapeutic potential of targeting Met/SAM metabolism. The outcomes of this study would aid in future preclinical investigation of successful AML experimental therapeutics *in vivo*, while simultaneously modeling therapeutic outcomes in combination with SOC therapy greatly enhancing the likelihood of success in clinical trials.

Aim 1: Examine comparative engraftment efficiency of both malignant and normal hematopoietic cells, including humanization potential plus disease presentation of cell-line derived xenografts (CDXs) and primary and

secondary patient derived xenografts (PDXs) in unconditioned NRG and NRGS mice.

Profound differences were observed in both engraftment rates and disease presentation in CDXs and PDXs in NRG and NRGS mice. As expected, unconditioned NRG mice were incapable of harboring different AML PDXs, and were determined to be ideal for CDX studies, showing predictable disease latency and diffuse clinically similar presentation in the blood and hematopoietic organs. Unconditioned NRGS mice showed high rates of PDX engraftment from secondary transplants and primary AML, but developed clinically dissimilar disease and solid tumor formation in CDXs, likely due to constitutive transgenic human cytokine expression. Thus, we have established and optimized the functional usefulness of both NRG and NRGS mice in human AML CDXs and PDXs. Our findings conclude that both NRG and NRGS mice are powerful tools for the investigation of malignant hematopoiesis, each with their own strengths and limitations, as determined contextually by the experimental design.

Immune deficient mice, while indispensable to hematological research, have the inherent flaw of lacking functional immunity. Moreover, patient PDX models, which recapitulate patient specific disease lack the component of functional human immunity still present in patients, thereby limiting investigation of therapeutic efficacy, especially for novel immune therapies. Thus, we wanted to examine the differential humanization potential of unconditioned NRG and NRGS mice xenografted with normal enriched human umbilical cord blood (UBC) derived CD34+ HSCs. Our findings demonstrate that only unconditioned NRGS

mice, but not unconditioned NRG mice, are capable of durable long-term engraftment and multilineage expansion of human HSCs. Interestingly, our data showed that contrary to others findings, expression of human cytokines are not detrimental to human HSC engraftment and proliferation. Our findings suggest that the differences observed in other studies were likely attributable to the intense ablative pre-conditioning of the mice prior to xenograft, which we avoided. Thus, our data indicates that unconditioned NRGS mice support normal human hematopoiesis, and may indicate that ablative pre-conditioning of mice prior to humanization may be unnecessary and counterproductive.

Aim 2: Develop and optimize a clinically similar, effective dosing regimen of induction therapeutics that is well tolerated in NRG and NRGS mice.

The only approved frontline therapy for AML remains revolves around using a combination of an anthracycline and cytarabine, and has remained unchanged since the 1970s. However, the large majority of published pre-clinical studies involving experimental AML therapeutics compare efficacy only to a vehicle control, and sometimes rarely a single agent (usually cytarabine). In reality, the efficacy of new experimental therapeutics should be tested and compared to approved SOC therapeutic outcomes, which remains problematic due to the prevalence of SCID dependent xenograft model systems. To address these issues, we utilized SCID-independent NRG and NRGS mice harboring human AML CDXs and PDXs, and treated them with a modified 5+3 bolus IV induction regimen (3 mg/kg doxorubicin for the first three days and 75 mg/kg cytarabine all five days), using doses of chemotherapeutics that SCID mice

cannot tolerate, but more closely recapitulate patient experience. Additionally, we conducted dose escalation and treatment timing modulation studies using increased doses of either drug or starting treatment 7 or 25 days post xenograft. Our data indicate that NRG and NRGS mice can tolerate significantly higher and more clinically relevant doses of DNA damaging induction therapeutics, and furthermore our regimen is efficacious in prolonging the survival of mice harboring aggressive human AML xenografts.

Aim 3: Investigate inhibition of Met/SAM metabolism as a novel therapeutic paradigm against MLL-rearranged leukemia *in vivo*, in an advanced xenograft model of patient AML, both against SOC induction and in combination.

Preliminary *in vitro* data from our group has shown that perturbation of Met/SAM metabolism through methionine deprivation or pharmacological inhibition of downstream metabolism induces apoptosis in MLL-rearranged leukemia, alters global histone methylation dynamics, and decreases expression and function of the crucial histone methyltransferase DOT1L. DOT1L expression and activity are absolutely required to maintain proliferation and leukemic potential of MLL-rearranged leukemia, through mis-localization to normally silenced embryonic gene promoters driving expression. Loss of DOT1L expression or function is sufficient to stop MLL fusion protein induced leukemic transformation and inhibits the proliferation and survival of MLL-translocated leukemia cells. Inhibition of downstream Met/SAM metabolism significantly prolonged the life of mice xenografted with aggressive pediatric MLL-translocated

leukemia samples in combination with SOC induction therapy. Thus, confirmation of our *in vitro* findings *in vivo* may reveal targeted inhibition of Met/SAM metabolism through dietary or pharmacologic methods, as a novel therapeutic paradigm against MLL-translocated leukemia, meritorious of further investigation.

CHAPTER 2

ESTABLISHING A MORE CLINICALLY RELEVANT MOUSE MODEL OF PATIENT AML AND STANDARD OF CARE CHEMOTHERAPY, TO INVESTIGATE THE THERAPEUTIC POTENTIAL OF ALTERATIONS TO MET/SAM METABOLISM

INTRODUCTION

The study of normal and malignant hematopoietic processes has truly been revolutionized by immune deficient mice, capable of supporting the growth and differentiation of human hematopoietic cells and tissues without rejection.[97-99] More importantly, *in vivo* xenograft models of human cancers consistently better predict the success of experimental chemotherapeutics in clinical trials, likely due to the complex and dynamic interplay existing between the BM microenvironment and heterogenous populations of leukemic cells.[100] Several seminal studies using early human leukemia xenograft models showed that only the most primitive and undifferentiated fraction of patient leukemic cells (Lin^-,$CD34^+$), but not the mature blast population, was capable of transmitting disease to primary and secondary immune deficient mice. These cells were dubbed LSCs, and exist at the apex of a hierarchy similar to HSCs during normal hematopoiesis.[76] This small population of slowly proliferating, self-renewing LSCs reside within the BM niche microenvironment, and can eventually give rise to a

large population of rapidly proliferating, clonally expanded blasts. Furthermore, a large body of evidence supports the notion that specific niches within the BM provide a chemotherapeutic protected sanctuary which the LSCs occupy, thereby eventually driving lethal refractory relapse in the majority of patients who experience initial remission.[101,102] Logically, it becomes evident that xenograft models of are the most reliable preclinical method capable of modeling the highly complex interplay among patient specific heterogenous leukemic populations, the BM microenvironment, clincal therapeutic outcomes, and by extension the true pharmacological efficacy and utility of novel investigational chemotherapeutics.[103-105] Despite the great advances made in murine leukemic xenograft models many samples of patient AML fail to sufficiently engraft, likely due to the following factors: 1.) Improper homing to BM nice 2.) Residual mouse innate immunity, 3.) Absence of human cytokines, 4.) Lack of appropriate stromal support, or 5.) AML sample intrinsic factors.

Transgenic approaches have dramatically enhanced the engraftment efficiency of human AML, by allowing the development of several iterations of immune deficient mice capable of expressing several human myeloid cytokines (IL-3, GM-CSF, and SCF), most frequently on NOD/SCID IL-2Ry^{null} background mice in hematologic research. (NSGS, NSS, etc.). Human myeloid cytokine expression not only supports myelopoiesis and engraftment of a variety of patient AML samples, but also the establishment and multilineage expansion of mature human hematopoietic cells from CD34^{+} human HSCs.[87,88,106,107] These advances, while indeed highly significant, are hindered by the reliance on SCID-

mediated immune deficiency (*Prdkc*^{scid}), which also impairs DNA damage repair, making these strains extremely intolerant to DNA damaging AML therapeutics like anthracyclines and cytarabine.[79,100] Thus, it becomes impossible to model patient xenograft response to SOC therapy in the most commonly used strains in hematologic research, despite such data being an absolutely necessary component of more physiologically and clinically relevant in preclinical investigation of new efficacious AML therapeutics. In 2003, Schultz et. Al developed another strain of comparably immune deficient mice with an intact DNA damage response dubbed NRG mice (NOD/Rag1/2^{null} IL-2Ry^{null}), whose immune ablation is maintained through a defective RAG gene.

Recently in 2014, in an effort to further improve engraftment rates of human AML samples in DNA damaging chemotherapeutic tolerant mice, NRG mice were crossed with human cytokine expressing NSGS (NSG-hIL3, h-GM-CSF, hSCF) mice and the SCID mutation removed through selective backcrossing with NRG mice. The resultant strain called NRGS (NRG-SGM3) mice express human myeloid cytokines, display a comparable degree of immune ablation to SCID mice, and can likely tolerate higher doses of SOC therapeutics in a clinically similar dosing regimen.[79] NRGS mice show consistent highly efficient engraftment of human hematopoietic cells, and can model the influence of the BM microenvironment, human cytokine signaling, and therapeutic outcomes. Given the recent development of these strains, and their relative lack of use in AML research, we next conducted an in-depth analysis of the comparative utility of unconditioned NRG and NRGS mice for the study of CDXs,

patient derived xenografts (PDXs), response to SOC chemotherapeutic treatment in clinically similar dosing regimen, and finally humanization potential using purified human HSCs.

MATERIAL AND METHODS

Cell Culture and Patient Samples. Established human AML cell lines K562, MV411, and U937 were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in RPMI medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C with 5% CO_2. The patient derived xenograft models (CCHMC-23, CCHMC-7, CCHMC-9, and CCHMC-35), were established at Cincinnati Children's Hospital Medical Center from specimens acquired under an IRB-approved protocol following informed consent. These primary specimens were established and expanded in NSGS mice at CCHMC and BM aspirates obtained from leukemic mice were frozen at -80°C in RPMI with 10% FBS and 10% DMSO until xenograft. Primary AML blasts were isolated from de-identified diluted whole marrow aspirate acquired from JGBCC biorepository with informed consent and under IRB guidelines (IRB#13.0188), by Histopaque gradient and centrifugation at 500 x g for 20 minutes at room temperature. Human CD34+ hematopoietic stem cells purified from umbilical cord blood were obtained from AllCells, Alameda, California, USA).

***In vivo* studies.** NRG (NOD/RAG1/2$^{-/-}$IL2Rγ$^{-/-}$, stock no: 007799) and NRGS (NOD/RAG1/2$^{-/-}$IL2Rγ$^{-/-}$Tg[CMV-IL3,CSF2,KITLG]1Eav/J, stock no: 024099) mice producing 2-4 ng/ml of human IL-3, GM-CSF, and SCF[108] were obtained

from Jackson laboratories, and bred and maintained under standard conditions in the University of Louisville Rodent Research Facility (RRF) on a 12-hour light/12-hour dark cycle with food and water provided ad libitum. Animal procedures were approved by the Institutional Animal Care and Use Committee. For all cell line derived xenograft studies and the human HSC study mice received 5 x 10^5 cells, and for all passaged patient derived xenograft studies 1.25 x 10^5 human cells suspended in 200µl PBS per mouse by bolus intravenous injection. Similarly, mice engrafted with the primary patient sample JGB-AML1 received 2 x 10^6 cells in 200 µl per mouse by intravenous bolus. Mouse health was monitored for characteristic signs of leukemia like scruffiness, and hind-limb paralysis, at which time mice were euthanized. Analysis of human cell engraftment was conducted on a case-by-case basis when mice became moribund and humane endpoints were reached.

5+3 Induction Chemotherapy Regimen. Using previously published data we established a similar 5+3 induction therapy regimen using intravenous bolus injection of doxorubicin (3 mg/kg, Days 1-3) combined with cytarabine (75 mg/kg, Days 1-5). Mice began treatment seven (or twenty five) days after transplantation of human leukemic cells with all mice receiving injections balanced to a final volume of 200ul PBS. Patients receiving standard 7+3 induction therapy (60 mg/m^2 doxorubicin and 100 mg/m^2 cytarabine) get approximately 1.5 mg/kg doxorubicin and 2.5 mg/kg cytarabine. Therefore, similar to previously published work, our dose of doxorubicin was doubled and the cytarabine concentration increased by 20-fold in an attempt to achieve necessary plasma concentrations

of the drugs using a bolus injection as compared to continuous infusion given in clinical care.[79]

Chemotherapeutics. Clinical formulations of both doxorubicin and cytarabine were obtained from the James Graham Brown Cancer Center as self-sealing vials containing 20 mg/10 ml or 2g/20ml respectively dissolved in saline, manufactured by APP a division of Fresenius Kabi USA LLC (Lake Zurich, IL).

FACS Analysis. The spleen and BM cells were analyzed by FACS as previously described.[109] Briefly cells were isolated from tissues, red blood cells were lysed and blocked for 10 mins at 4°C with Fc Block (#553142 BD Biosciences, Miami, FL, USA). The samples were then stained with the appropriate fluorophore conjugated anti-human antibody (Alexa 700-CD45, APC-CD11b, APC-CD19, PE-CD3, PE-Cy.5-CD34, and PE-Cy.5-CD33) from BioLegend (San Diego, CA) for 20 mins at 4° C and then analyzed on a Becton Dickinson FACScan with FlowJo.

Statistical analysis. All statistics were performed using GraphPad Prism 6 software. Unless specified below significance was determined by one-way ANOVA, followed by Tukey tests, using a cut off of $P < 0.05$. For all survival curves the log rank (Mantel-Cox) test was used, with a cut off of $P < 0.05$.

RESULTS

Cell line derived xenografts (CDXs) recapitulate clinical disease presentation in unconditioned NRG mice, but not unconditioned NRGS mice.

We first wanted to examine the differential engraftment efficiency of several commonly used, well- characterized human leukemic cell lines (MV411, K562, and U937) in unconditioned NRG and NRGS mice. All established cell lines showed high rates of successful engraftment in both strains, leading to leukemic disease progression and eventual euthanasia upon achievement of humane endpoints. **(Fig. 2.1A)** Interestingly, clinically disparate disease presentation was observed selectively in NRGS mice, which developed large intraperitoneal (IP) and lymphatic solid tumors with all cell lines tested. Conversely, NRG mice developed more clinically similar diffuse disease with the expected signs of leukemic disease progression like splenomegaly, upon xenograft of all established human cell lines. Necropsy of NRGS mice revealed disparity in preferential location of solid tumor formation with MV411 cells forming both IP and lymphatic tumors **(Fig. 2.1B, bottom right)**, K562 cells had an extremely high potential to form tumors within the kidney **(Fig. 2.1B, bottom left)**, and U937 cells formed only intra-splenic solid tumors **(Fig. 2.1B, bottom middle)**. All mice showed high levels of engraftment with any of these cell lines, and showed pronounced leukemic infiltration of hematopoietic organs **(Fig. 2.2A and 2.2B)**, but only NRG mice selectively failed to develop IP masses or hematopoietic solid tumors and showed a much more clinically relevant disease progression. Therefore, these studies provide compelling evidence that NRG mice, but not NRGS mice, are well suited for experimental studies involving CDXs.

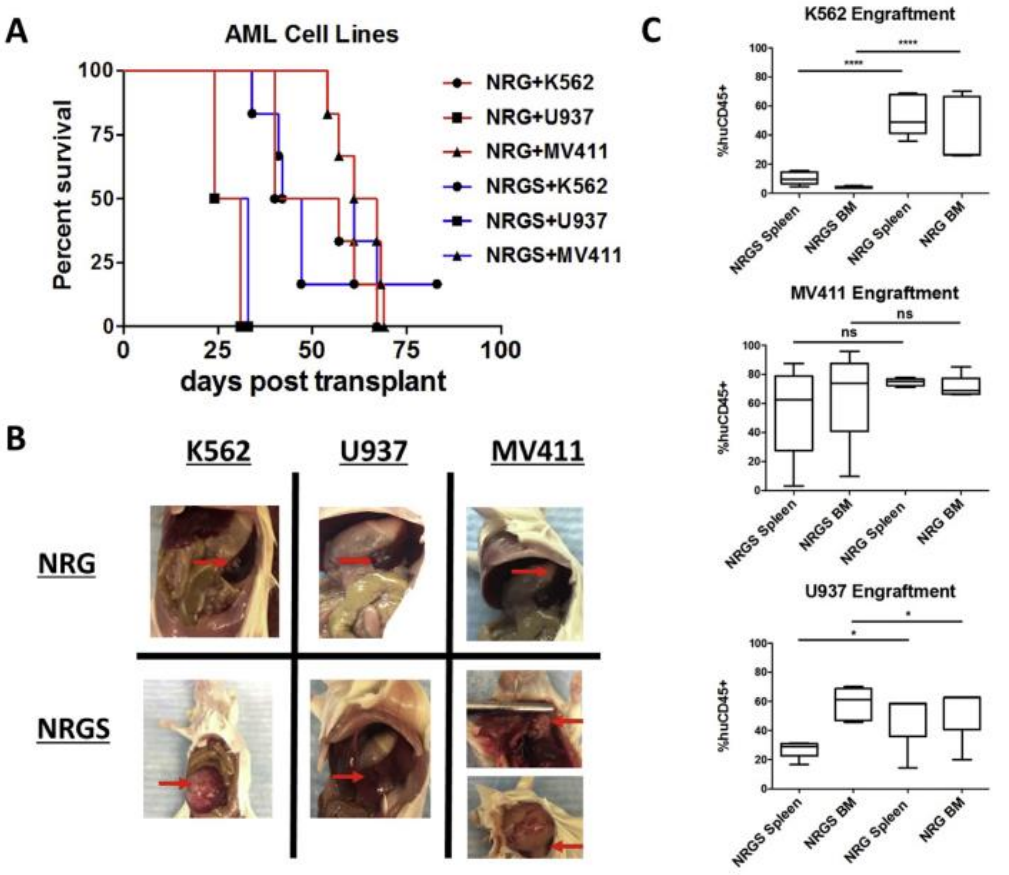

Figure 2.1. Engraftment of established human AML cell lines in NRG and NRGS mice. (A) Survival curves of unconditioned NRG and NRGS mice post IV xenograft of 5 x 10^5 of each respective cell line, K562, U937, and MV411. (B) NRG mice transplanted with human AML cell lines showed normal leukemic progression with diffuse disease and splenomegaly (as depicted), whereas NRGS mice developed solid splenic tumors (U937), solid IP tumors (K562, MV411), and solid lymphatic tumors (MV411). (C) Box-and-whisker plots showing the percentage of detectable human cells in the spleens and BM of NRG (n = 5) and NRGS (n = 5) mice, as quantified by flow cytometry for human CD45. AML, acute myeloid leukemia; BM, bone marrow; IP, intraperitoneal.

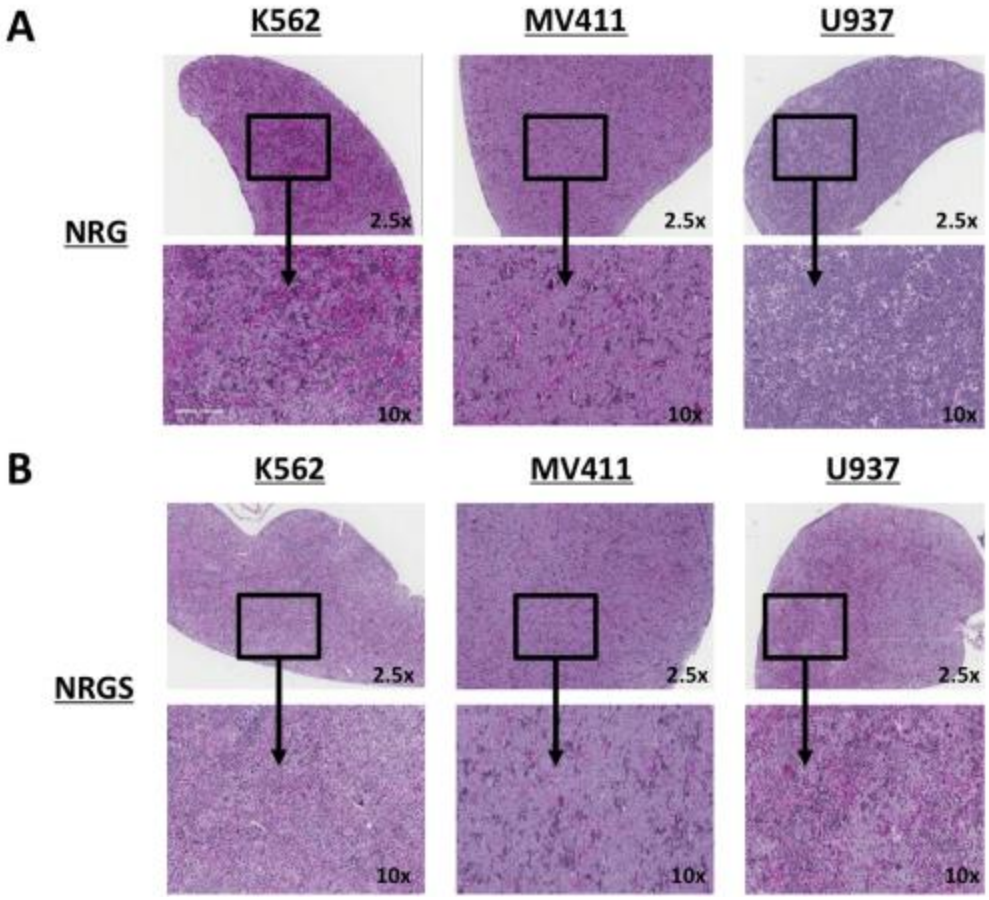

Figure 2.2 H&E staining of spleen sections from NRG and NRGS mice xenografted with established human cell lines. (A) H&E-stained sections of representative formalin-fixed spleens from unconditioned NRG mice IV xenografted with 5 x 10^5 K562, MV411, or U937 cells per mouse suspended in 200 mL of PBS. (B) Representative spleens from unconditioned NRGS mice IV xenografted with 5 x 10^5 K562, MV411, or U937 cells per mouse suspended in 200 mL of PBS. PBS, phosphate-buffered saline.

Patient derived xenografts (PDXs) in unconditioned NRG and NRGS mice.

We next examined the comparative engraftment efficiency of several patient-derived AML samples in both NRG and NRGS mice. Patient-derived samples were acquired as secondary transplants from NSGS mice harboring pediatric patient leukemia taken upon relapse, and three of the four samples displayed MLL translocations, whereas the final sample tested was cytogenetically normal (**Fig. 2.3A)**. Leukemic disease progression eventually requiring euthanasia occurred in all NRGS mice irrespective of sample origin, while all NRG mice survived until the 100-day study endpoint without overt signs of disease **(Fig. 2.3B)**. As expected efficient engraftment and leukemic infiltration of the hematopoietic compartment occurred with all four patient-derived samples in NRGS mice, whereas three of the four patient samples tested (CCHC-7, CCHC-23, and CCHC-35) showed virtually no useful engraftment (<5%) in NRG mice **(Fig. 2.3C)**. Interestingly, large numbers of human cells were detected in the hematopoietic organs of NRG mice engrafted with CCHC-9 cells, but without symptomatic leukemic disease progression. Furthermore, no IP or lymphatic solid tumors formed in NRGS mice harboring PDXs, thus providing convincing evidence that NRGS mice, but not NRG mice, are uniquely suited for studies involving patient-derived leukemia cells.

A

Sample ID	Age of Patient	Cytogenetics	Status
CCHC-9	19 months	MLL-AF6, FLT3 ITD	Relapse
CCHC-7	16 years	MLL-AF9	Relapse
CCHC-23	23 months	MLL-SEPT6	Relapse
CCHC-35	3 years	Normal	Relapse

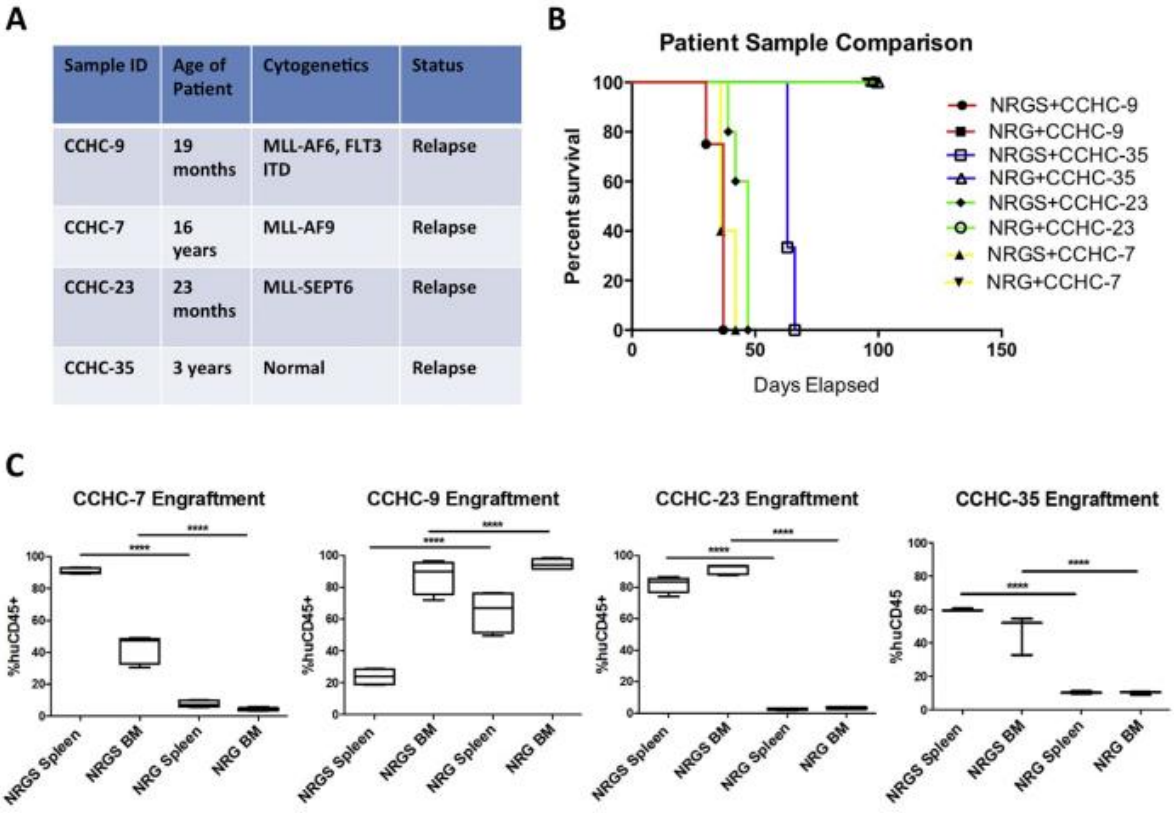

Figure 2.3. Engraftment of patient-derived AML cells in NRG and NRGS mice. (A) All patient samples were initially obtained from pediatric patients upon relapse and passaged once through NSGS mice. All xenograft experiments performed in these studies utilized the BM of NSGS mice harboring high levels of each respective patient leukemic cells, as quantified by flow cytometry for human CD45. (B) Survival curves of NRG and NRGS mice after IV xenograft of 1.25 x 10^5 human cells of each respective patient sample. Mice showing no signs of disease were euthanized 100 days after xenograft, the end-point of the study. (C) Box-and-whisker plots showing the percent of detectable human cells in the spleens and BM of NRG (n = 5) and NRGS (n = 5) mice, as quantified by flow

cytometry for human CD45. AML, acute myeloid leukemia; BM, bone marrow.

Humanization potential of unconditioned NRG and NRGS mice using purified human CD34$^+$ HSCs.

Given the recent push for the development of humanized mice bearing functional human immunity, we next examined the differential engraftment potential and the multilineage expansion of normal human CD34$^+$ HSCs. CD34$^+$ cells purified from umbilical cord blood **(Fig. 2.4A)** were transplanted into unconditioned NRG and NRGS mice, but only NRGS mice showed significant, durable engraftment of human hematopoietic cells 105 days later. Human cells were still detected in the hematopoietic organs of NRG mice, albeit at an extremely low percentage. Hematoxylin and eosin staining of spleen sections from NRGS mice revealed distinct patches of human cells absent in NRG mice, likely human HSCs undergoing extramedullary hematopoiesis **(Fig. 2.4B)** Multilineage expansion of human myeloid cells, pre-B cells, and T cells was observed selectively in NRGS mice, as characterized by surface expression of human CD19, CD33, and CD3 respectively **(Fig. 2.4C)**. In contrast to previously published work indicating transgenic human cytokine expression is counterproductive to human HSC engraftment, we show that unconditioned NRGS mice retain high levels of human HSCs (huCD45$^+$/huCD34$^+$) in the spleen **(Fig. 2.5A)** and BM **(Fig. 2.5B)** even 105 days after xenotransplantation.

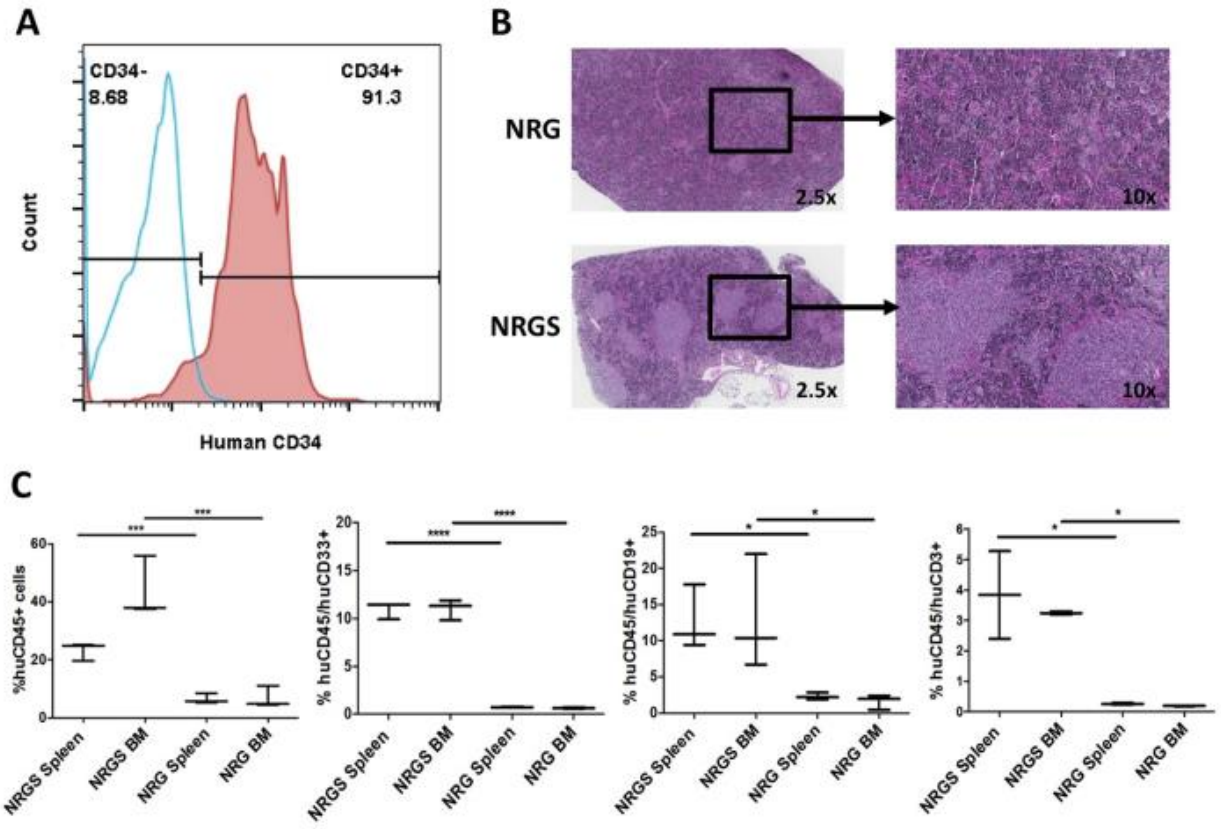

Figure 2.4. Comparative humanization potential of NRG and NRGS mice using human umbilical cord-derived CD34+ HSCs. (A) Histogram showing the percentage of human CD34+ cells purified from UCB before xenotransplantation. Blue indicates unstained cells and red indicates cells stained with antihuman CD34-PE/Cy5. (B) Immunohistochemistry analysis with H&E staining of representative NRG and NRGS spleens. Distinct patches of human cells are clearly visible only in NRGS spleens. (C) Box-and-whisker plots showing the percentage of human cells in the spleens and BM of NRG (n = 3) and NRGS (n = 3) mice 105 days after transplantation of 5 x 10^5 human CD34+ cells and lineage analysis by FACS staining using antihuman CD45-Alexa Fluor 700 (human pan-

hematopoietic), CD19-APC (pan-B cell), and CD33-PE/Cy5 (pan-myeloid). UCB, umbilical cord blood.

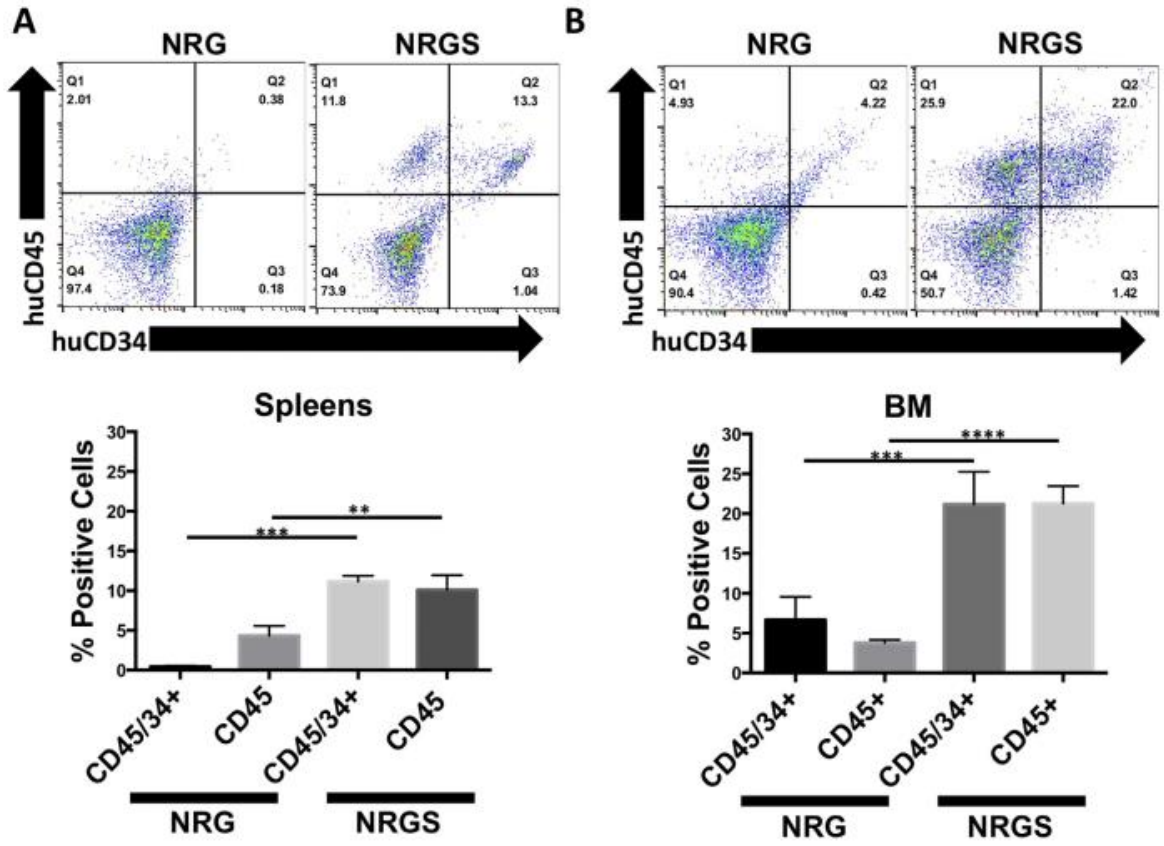

Figure 2.5. Comparison of long-term engraftment of mature and stem human hematopoietic cells in unconditioned NRG and NRGS mice. (A) Representative flow plots and relative percentages of human stem (huCD45+/huCD34+) and mature (huCD45+/huCD34-) hematopoietic cells in the spleens of unconditioned NRG (n = 5) and NRGS (n = 5) mice 105 days after transplantation of 5 x 10^5 human CD34+ UBC cells, indicating significantly higher engraftment of both stem and mature human hematopoietic cells. (B) Representative flow plots and relative percentages of mature and stem human hematopoietic cells detected in the BM of unconditioned NRG and NRGS mice showing highly significant enrichment of both stem and mature human hematopoietic cells selectively in NRGS mice.

Establishment and comparison of clinically similar induction therapy dosing regimen in unconditioned NRG and NRGS mice.

Truly useful AML xenograft models also require the ability to model SOC therapeutic outcomes, which in current clinical practice relies solely on the cytotoxic combination of an anthracycline and cytarabine. Therefore, we next examined the tolerability and efficacy of an adapted 5+3 induction regimen in both NRG and NRGS mice xenografted with human leukemic cells. Xenograft of the established cell lines MV411 and K562 resulted in solid tumor formation and clinically disparate disease presentation in NRGS mice. Mice injected with U937 cells show similar survival trajectories, and engraftment efficiency in both strains, with NRGS solid tumor formation restricted to the spleen. Further, given the previous findings that PDX samples fail to engraft efficiently in NRG mice we chose U937 cells to examine comparative SOC therapeutic outcomes in both unconditioned NRG and NRGS mice. NRG and NRGS mice were able to tolerate our adapted 5+3 bolus IV induction therapy regimen, and all treated mice showed highly significant enhancement in survival as compared to vehicle-injected controls **(Fig 2.6A)**. Our findings indicate that both strains are tolerant of a more clinically similar induction dosing regimen, and furthermore induction therapy in efficacious in treating disease **(Fig 2.6B)**. Comparison of PDX induction sensitivity in NRG and NRGS mice proved a challenge as none of the four patient samples (CCHC-7, CCHC-9, CCHC-23, and CCHC-35) gave rise to leukemia in NRG mice at the time points examined. Although the inherent limitations of NRG prevented comparative examination of SOC therapeutic

outcomes with patient-derived AML xenografts, we still felt that it was important to test the efficacy of our regimen against AML PDXs. Therefore, unconditioned NRG and NRGS mice were engrafted with the patient sample CCHC-23 and 5+3 induction therapy was initiated 7 days post xenograft. Again, 5+3 induction therapy significantly prolonged the life of all treated NRGS mice compared to vehicle-injected controls **(Fig 2.6C)**, and therapy significantly reduced the leukemic burden. NRG mice in this study again failed to develop disease and survived until the experimental endpoint. Similarly, our adapted 5+3 induction therapy regimen was also efficacious in significantly prolonging the life of NRGS mice harboring either CCHC-7 or CCHC-9 PDXs **(Fig 2.6D)**. Take together these findings indicate that our newly established adapted 5+3 induction therapy regimen is efficacious against several different patient-derived AMLs of varied etiology, at rates comparable to those observed in the aforementioned study using the established U937 cell line. These data are crucial to establishing the feasibility and efficacy of modeling SOC induction therapeutic outcomes in mice bearing AML PDXs.

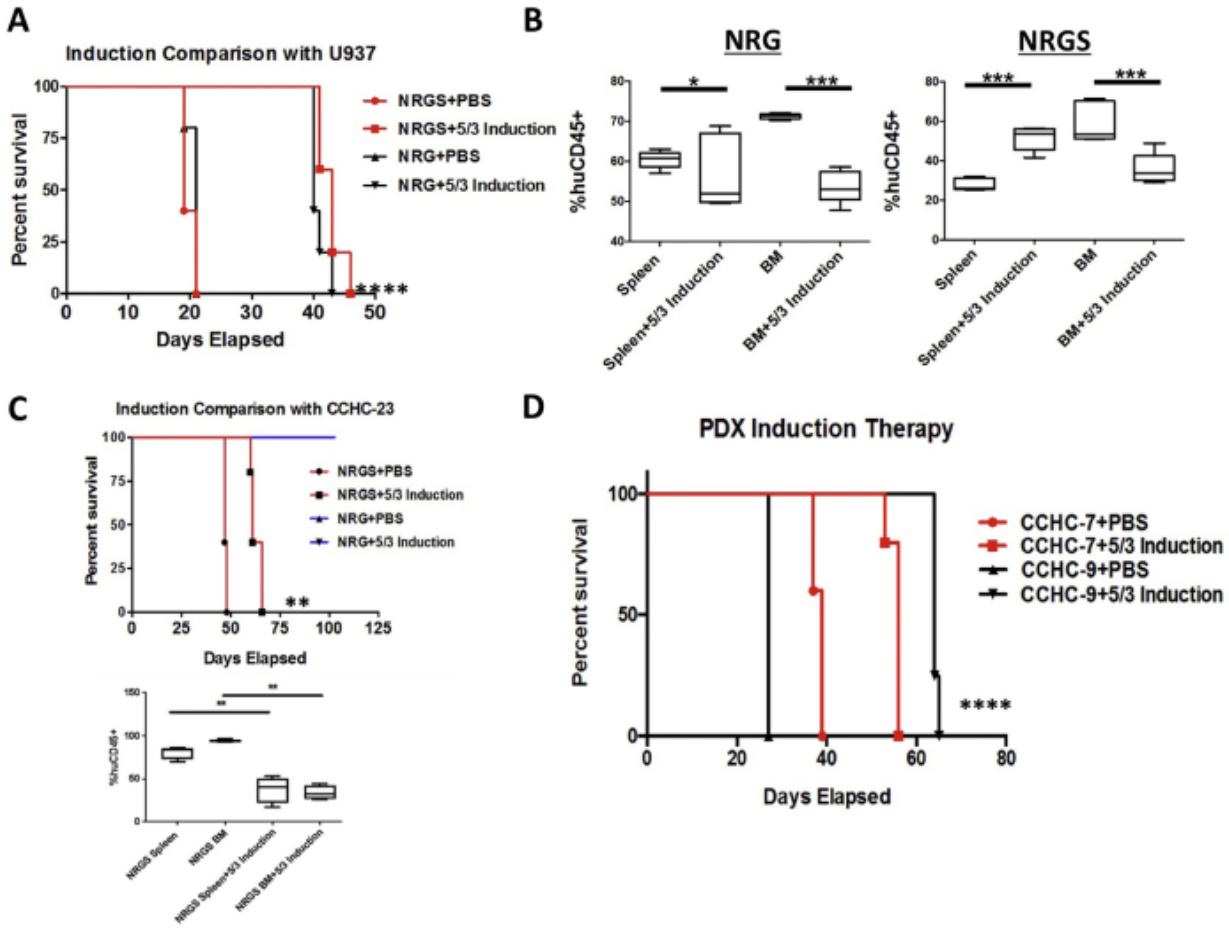

Figure 2.6 Comparison of standard of care chemotherapeutic response using 5+3 induction regimen in NRG and NRGS mice harboring cell line or patient-derived leukemia. (A) Survival curves for unconditioned NRG (n = 5) and NRGS (n = 5) mice engrafted with 5 x 10^5 U937 cells and subsequently treated with a 5+3 induction regimen [3 mg/kg doxorubicin (days 1-3) and 75 mg/kg cytarabine (all 5 days)] or the vehicle (PBS) by IV injection 7 days after xenograft. (B) Box-and-whisker plots showing significant differences in the percentage of human cells detected in the spleens and BM of the NRG or NRGS mice from the study in (A). (C) Survival curves and box-and-whisker plots showing the percentage of human cells engraftment in unconditioned NRG (n = 5

per group) and NRGS (n = 5 per group) mice xenografted with 1.25 x 10^5 CCHC-23 cells, and subsequently treated with 5+3 induction or the vehicle 7 days after xenograft. NRG mice failed to develop disease and survived until the endpoint of the study with or without induction therapy. (D) Survival curves showing significantly prolonged survival of unconditioned NRGS mice treated with 5+3 induction using two additional patient xenografts, CCHC-7 and CCHC-9, to confirm therapeutic efficacy in multiple PDX samples. PBS, phosphate-buffered saline.

Dose escalation and modulation of adapted 5+3 induction regimen in AML PDX bearing NRGS mice.

Having established the tolerability of NRG and NRGS mice to our adapted 5+3 induction therapy regimen, we next wanted to test the limits of our model system by doubling the doses of both chemotherapeutics. Comparable to studies in human patients, the dose-limiting agent in this study was determined to be the anthracycline doxorubicin. Cohorts of mice receiving double the dose of doxorubicin showed widespread toxicity and mortality (7 of 10 mice). Interestingly, doubling of the cytarabine dose of cytarabine showed no overt increase in signs of toxicity as determined by weight loss, with no change in the survival trajectory compared to our standard 5+3 induction therapy **(Fig. 2.7A, top)**. Perhaps, most intriguingly the few mice that survived treatment with double the dose of doxorubicin (6 mg/kg) showed no signs of leukemia at the endpoint of the study **(Fig. 2.7A, bottom)**. When taken together these data are highly significant as they suggest that with proper optimization of drug concentration and dosing schedule it may be possible to achieve a “remission-like” phenotype in murine PDX models, a feature that closely recapitulates patient experience of AML. Up to 75% of *de novo* AML patients experience complete remission with only one cycle of induction therapy, with the majority of mortality being driven by refractory relapsed disease, again highlighting the importance of our findings in the accurate modeling of patient AML outcomes. Finally, in an effort to more faithfully recapitulate clinical therapy of AML we examined how modulation of treatment initiation timing affected therapeutic efficacy of our 5+3 induction

therapy 7 days and 25 days post leukemic xenograft. Concurrent with previously published reports we found that survival was significantly different between the cohorts receiving induction therapy 7 days or 25 days post xenograft, with mice receiving chemotherapy earlier surviving longer **(Fig 2.7B)**. Finally, further investigation and optimization of treatment schedules may allow the development of an AML PDX model that can also accurately recapitulate clinical treatment outcomes, including minimal residual disease, remission, and relapse.

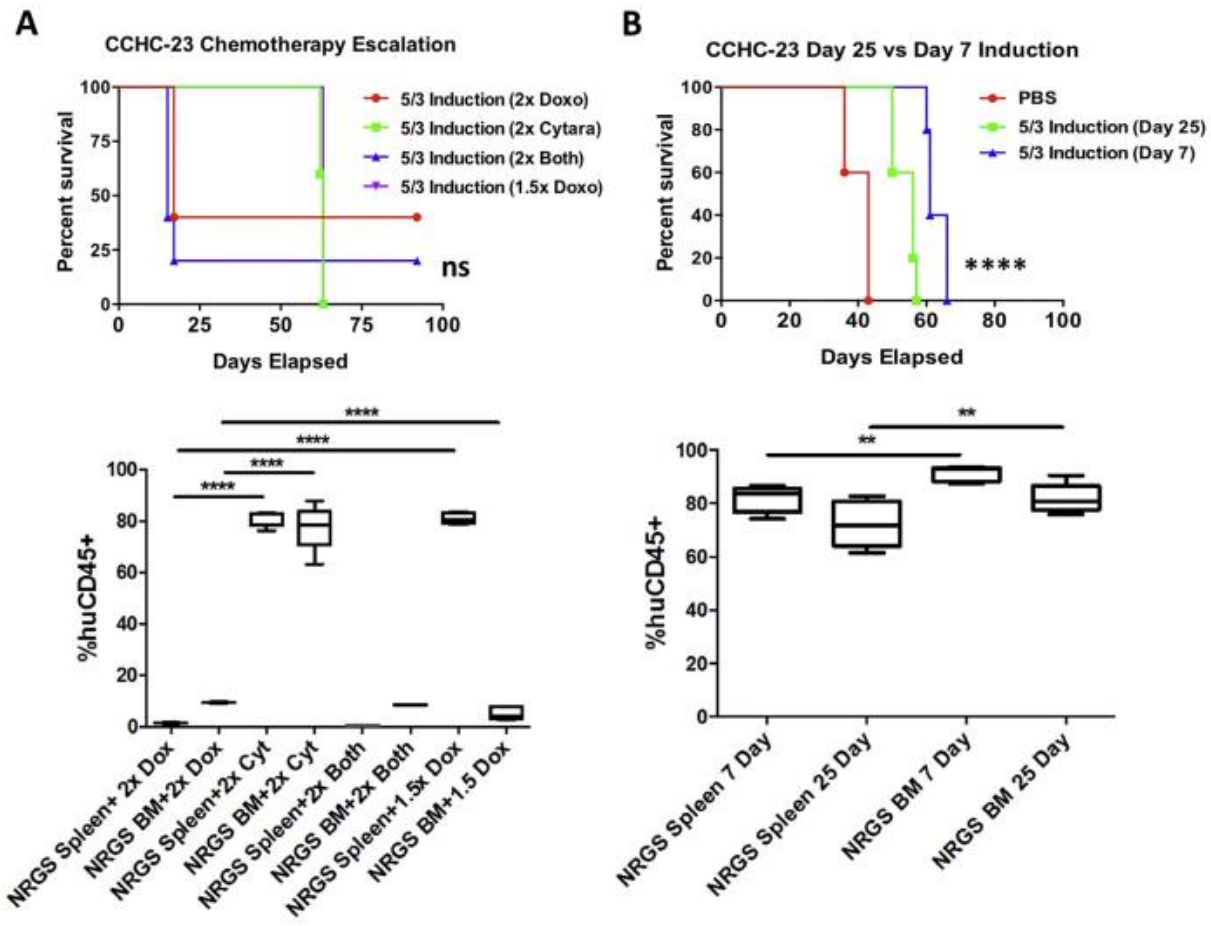

Figure 2.7. Escalation of 5+3 induction chemotherapy and modulation of timing of chemotherapy initiation. (A) Survival curves of unconditioned NRGS mice (n = 5 per group) xenografted with 1.25 x 10^5 CCHC-23 cells and subsequently treated with 5+3 induction therapy this time with either double the dose of both drugs (6 mg/kg doxorubicin, 150 mg/kg cytarabine), double the dose of each drug singly without manipulation of concentration of the other or 1.5X the standard dose of doxorubicin (4.5 mg/kg, 75 mg/kg cytarabine). Box-and-whisker plots show the percentage of human cells detected in the spleen or BM in each treatment escalation group. (B) Survival curves of unconditioned NRGS mice (n = 5 per group) xenografted with 1.25 x 10^5 CCHC-23 cells and treated with 5+3

induction 7 days after xenotransplantation or 25 days after xenotransplantation. Box-and-whisker plots show the percentage of human cells detected in the spleens and BM of NRGS mice upon disease progression and euthanasia after 5+3 induction therapy initiated either 7 days or 25 days after xenograft. BM, bone marrow.

Primary patient sample engraftment in unconditioned NRG and NRGS mice.

The final line of investigation in this chapter examines the differential engraftment efficiency of an un-passaged primary patient AML sample **(for detailed description, see Fig. 2.8A)** obtained from a fresh BM aspirate from The James Graham Brown Cancer Center **(Fig. 2.8B).** 2 x 10^6 nucleated cells obtained from purification of the leukemic BM aspirate were injected into unconditioned NRG or NRGS mice, and all mice were euthanized 100 days post xenograft at which time the spleens and BM were analyzed for human CD45 expression by flow cytometry. On average, NRGS mice had consistently higher rates of both spleen and BM engraftment as compared to NRG mice **(Fig. 2.8C)**. Rates of splenic engraftment were consistently low in both NRG and NRGS mice (NRG average %huCD45= 3.07%, NRGS average %huCD45= 5.87%), with NRGS mice harboring approximately twice as many spleen resident human cells. H&E staining of spleen sections further confirmed these findings and revealed a higher degree of leukemic infiltration in NRGS mice compared to NRG mice. **(Fig. 2.8D)**

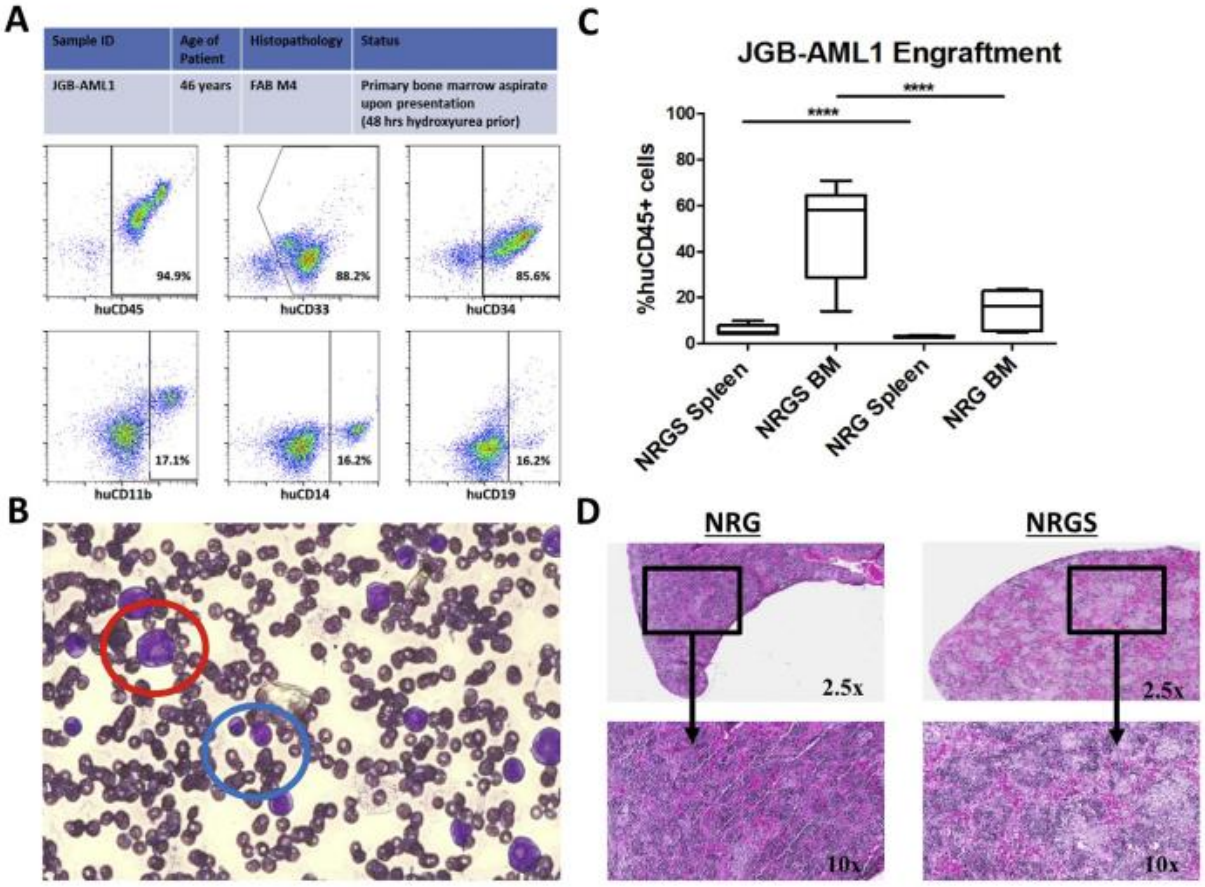

Figure 2.8. Engraftment of primary patient-derived AML in NRG and NRGS mice. (A) Initial characterization of primary patient AML sample upon acquisition of BM aspirate and subsequent Ficoll gradient separation of mononucleated cells by flow cytometry for human CD45, CD34, CD33, CD19, CD14, and CD11b. (B) BM aspirate slide smear stained with Protocol Hema 3 showing both leukemic myeloblasts (red) and monoblasts (blue) (photograph acquired at 40X on Zeiss Observer AX10 microscope connected to a Sony Nex-5n HD camera) typical for M4 subtype AML. (C) Box-and-whisker plot showing the percentage of human cells detected in the spleens and BM of unconditioned NRG (n = 5) and NRGS (n = 5) mice 100 days after xenograft of 2 x 10^6 mononucleated cells. (D)

Immunohistochemistry of representative formalin-fixed, H&E-stained NRG and NRGS spleens xenografted with 2 x 10^6 mononuclear cells obtained from Ficoll gradient separation of clinical BM aspirate.

CHAPTER 3

PERTURBATION OF MET/SAM METABOLISM INDUCES APOPTOSIS, ALTERS HISTONE METHYLATION DYNAMICS, AND DECREASES DOT1L EXPRESSION IN MLL-TRANSLOCATED LEUKEMIA PROLONGING SURVIVAL IN ADVANCED PATIENT AML XENOGRAFT MODEL

INTRODUCTION

Mixed lineage leukemia 1 (MLL) gene rearrangement is a defining feature of a unique group of particularly aggressive and chemotherapy resistant acute leukemias. Rearrangement of the *MLL* gene on chromosome 11q23 results in fusion to over 60 different partner genes, the large majority of MLL fusions occurring with just six common partners. Furthermore, MLL-rearrangement (MLL-R) is detectable in 10% of leukemia cases, which have the overall worst prognosis among cytogenetically abnormal leukemias.[110] Leukemias arising from MLL-R result in the aberrant expression of oncogenic MLL fusion proteins (MLL-FPs), and typically manifest as either acute myeloid or acute lymphoid leukemias (AML or ALL), accounting for 10% of adult AML cases, and 70-80% of infant leukemias.[111] Modern highly aggressive multiagent cytotoxic chemotherapy and hematopoietic stem cell transplantation (HSCT), has extended the overall disease-free survival of pediatric leukemia patients to approximately 90%. Unfortunately, patients diagnosed with MLL-R leukemia have a particularly poor

prognosis with an overall survival less than 50%, and this prognosis is not improved by allogenic HSCT.[112] Great advances have been made in understanding the unique molecular mechanisms that mediate MLL-R driven leukemogenesis, and recently studies have suggested MLL-R leukemias are largely driven and maintained through epigenetic dysregulation. The HMT disruptor of telomeric silencing 1-like (DOT1L) has come to the forefront as a critical mediator of MLL-FP mediated leukemogenesis and has been shown to be required for the development the development, maintenance, and progression of MLL-R leukemias. Despite our improved understanding of the unique signaling mechanisms underlying MLL-FP mediated leukemogenesis, these leukemias are generally still treated with cytotoxic remission induction chemotherapeutic regimens resulting in high rates of initial remission, but the 5-year event-free survival rates remains under 50% due to refractory relapse. Thus, novel treatment paradigms must be investigated specifically for the therapy MLL-R leukemias, alone and in combination with SOC cytotoxic induction chemotherapeutics.

DOT1L, the HMT which catalyzes the sequential methylation of H3K79 at the promoters of actively transcribed genes, has been strongly implicated as a requisite driver of MLL-FP mediated leukemic transformation and progression. Chromosomal translocations cause the in-frame fusion of MLL to a variety of fusion partners, most frequently members of the SEC complex namely, AF4, AF6, AF9, AF10, and ENL.[113] These oncogenic fusions can interact directly and indirectly with Dot1L, promoting aberrant recruitment to leukemogenic gene

promoters like the HoxA cluster or Meis1.[56] Dot1L causes local hypermethylation of H3K79 at these gene promoters inducing their aberrant expression causing leukemic transformation resulting in either AML or ALL.[57] Several independent studies have shown through pharmacologic and genetic methods that Dot1L expression and function are required for both leukemic transformation and maintenance *in vitro* and *in vivo*. Consistent with this paradigm genetic deletion of DOT1L stops the leukemic transformation of normal HSCs transfected to express MLL fusions and new small molecule inhibitors of Dot1L are selectively toxic to MLL fusion driven leukemia cells *in vitro* and in xenograft models.[62,65,67,114,115] The investigation of regulatory mechanisms controlled by Dot1L (cell cycle, development, transcription, DNA damage repair etc.) is exhaustive, however the regulation of Dot1L's own expression remains unstudied and elusive. DOT1L mRNA is expressed ubiquitously in all organs at varying levels, and its level is dynamically regulated during the cell cycle as are the levels of H3K79me1,2,3.[116,117] DOT1L-knockout cells transformed with MLL-fusion proteins, DOT1L siRNA transfected human lung cancer cells, and DOT1L-knockout murine yolk sac-derived erythroid progenitors all undergo G1 cycle arrest and eventual cell death.[118-120] Given the unique dependence of MLL-FPs on epigenetic dysregulation to drive leukemogenesis through aberrant activating histone methylation at leukemogenic promoters, we sought to investigate perturbation of Met/SAM metabolism as a novel therapeutic strategy for MLL-R leukemias.

The Met/SAM metabolic pathway is essential for cellular methylation reactions, including those involved in epigenetic gene regulation, as SAM serves as the primary methyl donor for most cellular methylation reactions, including DNA, RNA, lipids and histones (both arginine and lysine).[34,35] In addition to being the principal methyl donor, SAM also serves as the sole source of the propylamine moiety required for polyamine biosynthesis.[69,70] The biologically usable SAM moiety is synthesized from methionine by the enzyme MATIIA, and upon enzyme mediated donation of the SAM methyl group forms the product SAH, which is further metabolized by SAH hydrolase (SAHH) to form homocysteine.[71] Homocysteine is an obligatory intermediate in the transsulfuration pathway, and serves as the precursor for glutathione synthesis, and alternatively homocysteine can be recycled back into methionine by the enzyme MTR, which catalyzes the final step in methionine regeneration by simultaneously converting 5-methyltetrahydrofolate in the biologically active cofactor tetrahydrofolate while transferring a methyl moiety to homocysteine starting the cycle again.[72] Importantly, the intracellular ratio of SAM:SAH dictates the overall methylation potential of the cell, with SAH accumulation resulting in global feedback inhibition of cellular methylation reactions, including epigenetic methylation of DNA or histones.[73]

Therefore, we hypothesized that Met/SAM metabolic flux and expression of the aforementioned enzymatic mediators are required at high levels selectively by MLL-R leukemia cells, to maintain and enforce aberrant histone methylation contributing to the leukemic phenotype. Unlike genetic abnormalities, epigenetic

modifications are frequently reversible, and therefore may provide new opportunities for targeted pharmacological therapies for MLL-R leukemias. Published literature targeting this pathway specifically in MLL-R leukemia is non-existent, and a very sparse body of work exists for targeting this pathway as a general antileukemic therapy, with studies limited to small in vitro studies using established human cell lines, and single agent pharmacological inhibition of MATIIA or SAHH.[121] Thus, we rigorously investigated methionine deprivation with and without inhibition of enzymatic mediators of the biosynthetic pathway *in vitro* and in combination with SOC cytotoxic induction therapy *in vivo*, as a novel epigenetic treatment paradigm for MLL-R leukemias.

Here, we show for the first time to our knowledge that perturbation of Met/SAM metabolism is cytotoxic to MLL-FP expressing cells and induces apoptosis and DNA damage. Furthermore, perturbation of this critical metabolic pathway through methionine restriction or pharmacologic inhibition of SAH metabolism resulted in alteration of intracellular metabolite pools and overall methylation potential. Finally, we found that perturbation of Met/SAM metabolism and subsequently methylation potential, alters histone methylation dynamics globally and specifically at the DOT1L promoter, decreasing its expression and function in MLL-FP expressing cell lines and patient cells. *In vivo* pharmacologic perturbation of Met/SAM metabolism through SAHH inhibition, significantly prolonged the survival of mice harboring aggressive MLL-R patient leukemia in combination with a clinically similar regimen of cytotoxic induction therapy

combining doxorubicin and cytarabine. Thus, targeting Met/SAM metabolism may provide a novel therapeutic vulnerability against MLL fusion driven leukemia.

MATERIAL AND METHODS

Cell culture and Patient Samples. All established human leukemia cell lines MV411, RS411, and K562 were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in standard RPMI medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C with 5% CO_2. Patient derived xenografts (CCHC-7, CCHC-9, and CCHC-23) were established and expanded at Cincinnati Children's Hospital Medical Center in NSGS mice from primary pediatric specimens aquired under an IRB-approved protocol following informed consent at time of relapse. Following engraftment and expansion in NSGS mice, resulting in terminal leukemic disease and euthanasia, we received the harvested BM aspirates from leukemic mice frozen at -80°C in RPMI with 10% FBS and 10% DMSO until xenograft. CCHC-7 cells were also cultured *in vitro* in standard RPMI medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and human cytokines (SCF, FLT3L, TPO, IL-3, and IL-6) at 37°C with 5% CO_2.

Annexin V/Propidum Iodide Staining for Apoptotic Cells. Cell death was analyzed and quantified by FACS staining for Annexin V and propidium iodide (PI). Briefly, cells were thoroughly washed twice with ice cold PBS following exposure to experimental conditions and resuspended in 300ul of 1X Annexin

binding buffer. Next cells were incubated with 1µL of anti-Annexin V antibody conjugated to APC (catalog #640920, BioLegend San Diego, CA, USA) and 4µL of 1 mg/ml PI solution (Sigma-Aldrich St. Louis, MO, USA) for 15 min at 4°C, followed by analysis on a Becton Dickinson FACScan using FlowJo software.

Protein Isolation/Quantification and Western Blot Analysis. Protein was isolated from cells in CHAPS lysis buffer, and quantified as previously described.[122] Western blot analysis was then conducted as previously described using 30µg of protein for experiments involving total protein lysates and 15µg of protein for experiments involving purified histones, using 1:5000 or 1:2000 dilutions respectively for primary antibodies and 1:20000 dilution of secondary antibodies. Proteins of interest were then detected by addition of chemiluminescence substrate.

SAM/SAH Reverse Competition ELISA. Intracellular metabolites were isolated on ice by sonication of 10 x 10^6 cells per timepoint in 1ml of ice cold PBS using a 30 kHz sonnicator with probe at 30% amplitude for three 20 second cycles with one minute breaks between sonication. Cell debris was removed by high speed centrifugation and the resultant supernatant was snap frozen in liquid nitrogen and immediately stored at -80°C to prevent metabolite degradation. Quantification of intracellular SAM and SAH concentration was then conducted using the SAM/SAH ELISA Combo Kit from Cell Biolabs, INC. (catalog #STA-671-C, San Diego, California, USA) following the manufacturers' protocol.

Histone Isolation. Histones were isolated from cellular nuclei by acid extraction as follows. First cells were harvested and washed twice in ice cold PBS and then resuspended in Triton Extraction Buffer(TEB: PBS containing 0.5% Triton X 100, 2 mM phenylmethylsulfonyl fluoride (PMSF), 0.02% NaN3) at a cell density of 10^7 cells per ml per timepoint. Cells were lysed by ten minute incubation in TEB buffer at 4°C with gentle stirring followed by centrifugation at 6500 x g for ten minutes at 4°C to pellet the nuclei. The supernatant was discarded and the nuclei resuspended and washed in half the original volume of TEB buffer, followed by centrifugation as before. The supernatant was discarded and the nuclei pellet was resuspended in 100µL of 0.2 N HCl and placed at 4°C overnight to acid extract histones. The following day samples were centrifuged at 6500 x g for 10 min to pellet debris and the supernatant was collected and HCl neutralized by addition of 10µL of 2M NaOH. Protein content was then determined using the BCA assay.

Chromatin Immunopreciptation (ChIP). Chromatin fixation, isolation, digestion, and immunoprecipitation was performed as previously described in detail.[123] ChIP antibodies detecting anti-H3K4me3, anti-H3K27me3, and anti-H3K79me2 were used for immunoprecipitation followed by ChIP quantitative RT-PCR as described earlier using DOT1L promoter specific primers. Further, similar quantitative RT-PCR was performed on immunoprecipitated chromatin using primers designed against a non-specific region approximately 6 kb upstream of the transcription start site (TSS) as a control to ensure that differential results are truly DOT1L promoter specific and not technique driven. DOT1L promoter

specific primers were designed just upstream of putative transcription factor consensus sites as determined by MotifMap software, and a detailed promoter map including primer binding sites can be found in Fig. 5A.

RT-PCR. RT-PCR was performed on immunoprecipitated genomic DNA (as detailed above) or total isolated RNA reverse transcribed to cDNA as previously described.[122] Primers for qPCR were designed against the promoter sequence or transcribed sequence using Primer Express 3.0 software (Applied Biosystems, Foster City, CA) per the manufacturer's instructions for SYBR green dye assays. RT-PCR was performed using iTaq Universal Sybr Green Supermix (Bio-Rad) and relative expression levels were analyzed using ΔΔCT method data was normalized to 12.5% total chromatin inputs for RT-PCR following ChIP or β-actin expression for experiments on reverse transcribed mRNA. PCR reactions were analyzed on a BioRad CFX96, and a list of primer sequence and amplicon lengths can be found in Supplementary Table 1.

Clinical Chemotherapeutics. Clinical formulations of both doxorubicin and cytarabine were obtained from the James Graham Brown Cancer Center as self-sealing vials containing 20 mg/10 ml or 2g/20ml respectively dissolved in saline, manufactured by APP a division of Fresenius Kabi USA LLC (Lake Zurich, IL).

***In vivo* xenograft studies.** As previously described NRGS (NOD/RAG1/2$^{-/-}$IL2Rγ$^{-/-}$Tg[CMV-IL3,CSF2,KITLG]1Eav/J, stock no: 024099) mice producing 2-4 ng/ml of human IL-3, GM-CSF, and SCF[108] were obtained from Jackson laboratories, and bred and maintained under standard conditions in

the University of Louisville Rodent Research Facility (RRF) on a 12-hour light/12-hour dark cycle with food and water provided ad libitum. For all xenograft studies NRGS mice received 1.25 x 10^5 human cells (CCHC-7 or CCHC-9) suspended in 200µl PBS per mouse by bolus IV injection and n=5 for all study cohorts. Mice were then injected with an isovolumetric bolus of the vehicle (PBS), or treated with our previously described high intensity 5+3 induction regimen with or without 25 mg/kg DZA dissolved in 200µl.[124]

Statistical analysis. All statistics were performed using GraphPad Prism 8 software. Unless specified below significance was determined by one-way ANOVA, followed by Tukey tests, using a cut off of $P < 0.05$. For all survival curves the log rank (Mantel-Cox) test was used, with a cut off of $P < 0.05$.

RESULTS

Perturbation of Met/SAM metabolism impairs cellular viability and induces apoptosis in MLL-FP expressing cell lines.

We first sought to examine changes in cellular viability induced by alterations to Met/SAM metabolism on two MLL-AF4 expressing cell lines representing distinct leukemic lineages, MV411 and RS411 (AML and T-ALL respectively), as well as BCR-ABL driven K562 cells. We chose to target two distinct nodes of Met/SAM metabolism namely the synthesis of SAM by restriction of exogenous methionine, and downstream metabolism through pharmacological inhibition of SAH hydrolase using the competitive inhibitor 3-deazaadenosine (DZA), singly or in combination. Inhibition of downstream

Met/SAM metabolism by DZA induced a dose and time dependent impairment of cellular viability as quantified by Alamar Blue assay selectively in the MLL-AF4 expressing cell lines, but not MLL-FP independent K562 cells **(Fig. 3.1A)**. Similarly, decreasing concentrations of exogenous methionine also impair cellular viability **(Fig. 3.1B**). Using the data obtained from both dose curves we determined an appropriate concentration (15uM) of DZA, and timeframe to investigate methionine deprivation without overt toxic effects, for all further studies. Annexin V staining revealed that methionine deprivation and/or inhibition of SAH metabolism potently induced apoptosis MV411 cells, with the combination producing an additive increase in apoptosis. Interestingly, in the timeframe investigated RS411 cells only underwent apoptosis in the presence of DZA, but additive effects were still observed with simultaneous methionine deprivation and DZA treatment. **(Fig. 3.1C, middle)** Conversely, K562 cells lacking MLL-FP expression, were resistant to apoptosis induction through either methionine deprivation or inhibition of downstream SAHH mediated metabolism **(Fig. 3.1C, right).** Changes in protein expression corresponding the induction of apoptosis, like cleavage of PARP-1 and Caspase-3 were observed selectively in the MLL-AF4 expressing cell lines upon perturbation of Met/SAM metabolism, and consistent with the aforementioned Annexin V staining data RS411 cells only underwent apoptosis upon inhibition of downstream SAHH mediated metabolism, while MV411 cells underwent apoptosis upon deprivation of exogenous methionine or by SAHH inhibition **(Fig. 3.1D)**.

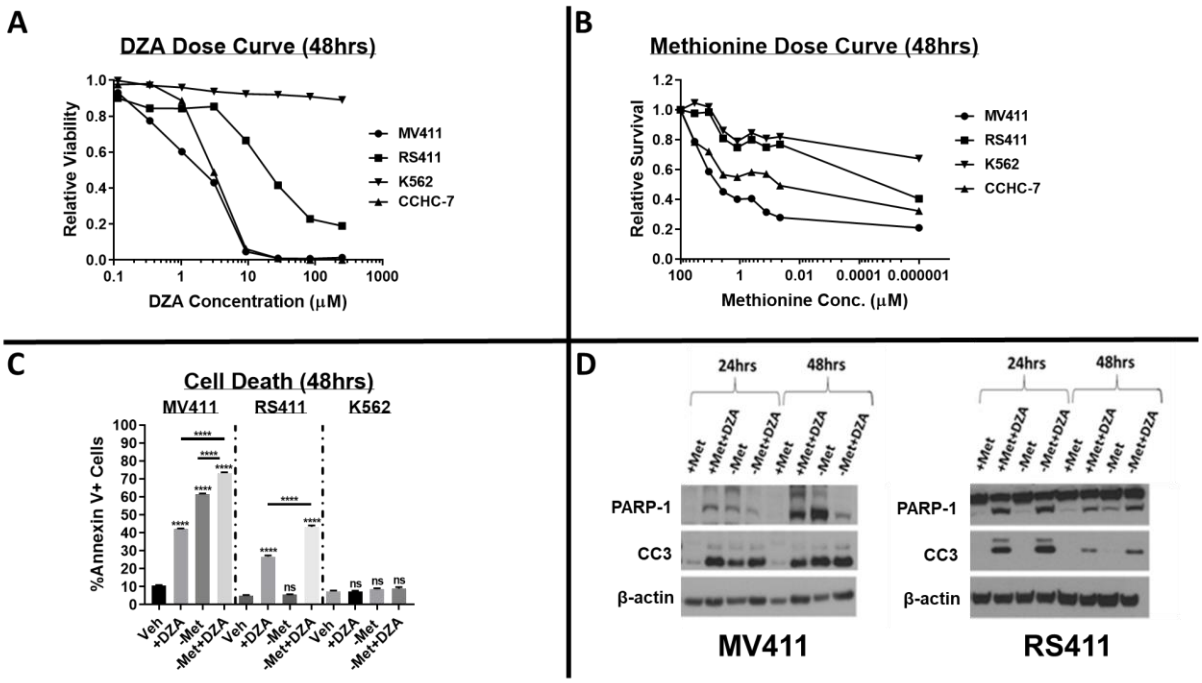

Figure 3.1 Perturbation of Met/SAM metabolism potently induces apoptosis and reduces cell viability in established MLL-R leukemia cell lines . (A) Dose and time dependent reduction in relative survival of MLL-AF4 expressing MV411 (AML) and RS411 (ALL) cells treated with increasing concentrations DZA as quantified by Alamar Blue vitality assay 48 hours post treatment. K562 cells lacking MLL-fusion expression show no reduction in relative survival upon DZA treatment. (C) From left to right; Methionine deprivation or exposure to 15μM DZA potently induced apoptosis at 48 hours in MV411 cells singly, and the combination showed an additive increase in apoptosis as determined by Annexin V+ staining. RS411 cells only undergo apoptosis upon exposure to 15μM DZA and this effect is amplified by methionine deprivation. K562 cells lacking MLL-R show no induction of apoptosis 48 hour post DZA exposure or methionine deprivation. (D) Changes in protein expression corresponding with apoptosis

induction (PARP-1 and Caspase-3 cleavage) were observed under all experimental conditions in MV411 cells (left), while RS411 cells only undergo apoptosis upon DZA mediated SAHH inhibition (right).

Perturbation of Met/SAM metabolism influences intracellular metabolite pools, causing an increase in intracellular SAH and a decrease in methylation potential in MLL-AF4 expressing cells.

After confirming that impairment of Met/SAM metabolism was selectively toxic to MLL-FP expressing cells, negatively impacted cellular viability, and induced apoptotic cell death we next sought to examine the impact on relevant intracellular metabolites, namely SAM and SAH, using a commercially available ELISA kit. These metabolites are of crucial importance as the ratio of intracellular SAM and SAH determines the overall cellular methylation potential, and the accumulation of SAH globally inhibits all methyltransferase reactions including those required for epigenetic regulation of gene expression **(Fig. 3.2A)**. 8 hour exposure to 15uM DZA and/or methionine deprivation was sufficient to significantly increase intracellular SAH in MV411 cells, with a likely synergistic increase in SAH concentration upon simultaneous DZA treatment and methionine deprivation. By 24 hours MV411 cells showed a significant increase in intracellular SAH concentration under all conditions even methionine deprivation alone, however the increase is least pronounced with simultaneous methionine deprivation and DZA treatment, likely due to reduced metabolic flux. **(Fig. 3.2B, left)** Similarly, RS411 cells also showed a significant increase in intracellular SAH after 8hr or 24hr of exposure to DZA, but not simply by methionine deprivation, and no apparent synergy was observed with combined DZA treatment and methionine deprivation. **(Fig. 3.2B, right)** Interestingly, no significant change in intracellular SAM was observed in either cell line under any

of the experimental conditions including methionine deprivation suggesting these cells exhibit some capacity to recycle Met/SAM from other intracellular metabolite pools like homocysteine or 5-methylthioadenosine **(Fig. 3.2C)**. Finally, we determined the overall cellular methylation potential, expressed as the ratio of intracellular SAM:SAH concentration normalized to untreated cells in methionine rich media, and found that 8 or 24hrs of methionine deprivation or exposure to DZA was sufficient to significantly lower overall cellular methylation potential in MV411 cells **(Fig. 3.2D, left)**. RS411 cells show no significant changes in methylation potential upon short-term exposure to any experimental conditions, but show significant changes at 24hrs. As expected treatment with DZA significantly decreased cellular methylation potential with or without methionine, but methionine deprivation alone actually significantly increased cellular methylation potential in RS411 cells **(Fig. 3.2D, right)**.

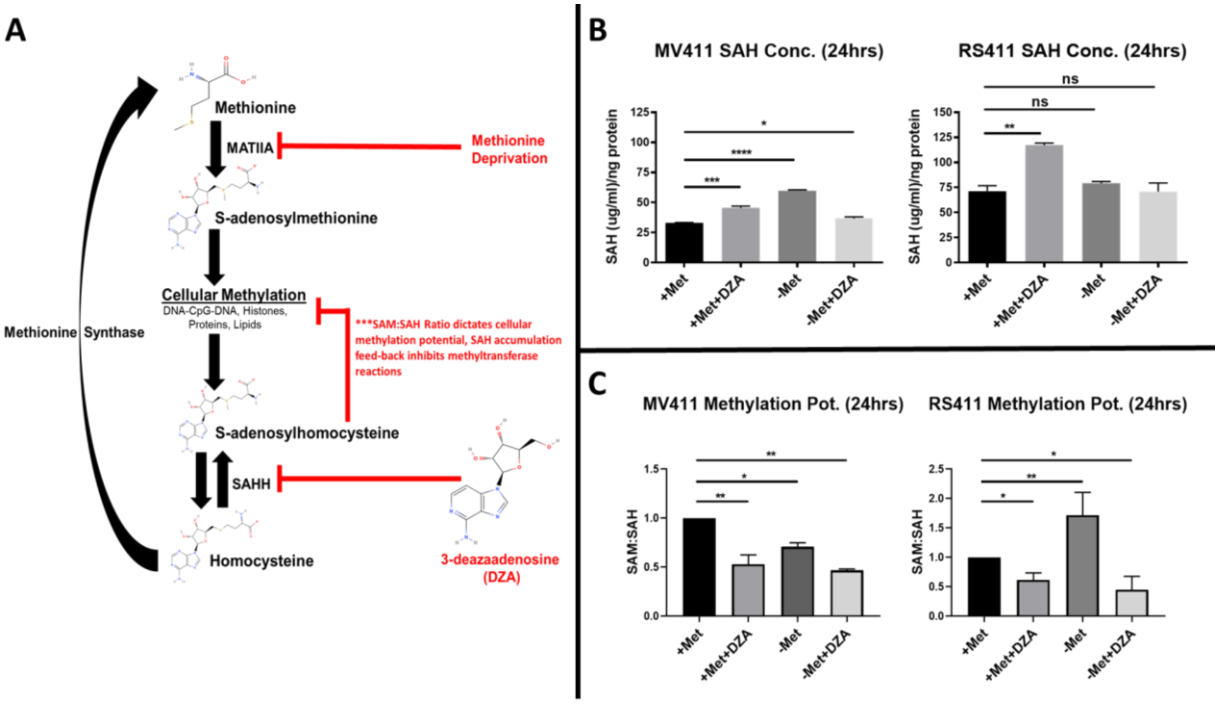

Figure 3.2 Disruption of Met/SAM metabolism increases intracellular SAH concentration and decreases overall methylation potential. (A) Simplified schematic of Met/SAM metabolism showing important enzymatic mediators, substrates and products. Deregulation of Met/SAM metabolism and subsequently methylation potential was achieved by targeting two distinct nodes, either by reducing synthesis of the methyl donor moiety SAM by methionine deprivation or pharmacological inhibition of downstream SAH metabolism and methionine recycling. (B) From left to right; Intracellular SAH concentration was increased in MV411 cells under all experimental conditions and timepoints, while only DZA mediated inhibition of SAH metabolism sufficiently increased intracellular SAH concentration in RS411 cells. (C) No significant changes were detected in the intracellular SAM concentration as compared to vehicle treated cells in methionine rich media, in either MV411 or RS411 cells under any experimental

conditions. (D) Overall cellular methylation potential (SAM:SAH) was significantly reduced in MV411 cells 24 hours post exposure to experimental conditions. RS411 cells only show significant reduction in methylation potential following 24 hour exposure to DZA, and conversely a significant increase in methylation potential was observed following 24 hours of methionine deprivation.

Methionine deprivation or inhibition of downstream SAH metabolism alters global histone methylation dynamics in MLL-AF4 expressing leukemic cell lines.

Next, we examined how perturbation of Met/SAM metabolism impacted global histone methylation dynamics in both MV411 and RS411 cells, by Western blot analysis on purified histone extracts. Initially, we chose to examine the most common activating histone modification H3K4Me3, which is heavily enriched at transcribed promoters, and the most common repressive modification H3K27me3. Methionine deprivation and/or SAHH inhibition through DZA treatment severely diminished global H3K4me3 levels in MV411 cells, while only treatment with DZA resulted in a global increase of the repressive modification H3K27me3 at all timepoints examined **(Fig. 3.3A)**. RS411 cells show decreased levels of global H3K4me3 only in conditions of methionine deprivation, and when combined with DZA a more potent reduction is seen in the short term **(Fig. 3.3B)**. Similar to MV411 cells, RS411 cells show a time dependent increase in global H3K27me3 only upon SAHH inhibition with or without methionine. MLL-R leukemias have been shown to be uniquely dependent on the misdirected enzymatic activity of the methyltransferase DOT1L to catalyze aberrant H3K79 methylation and activation of leukemogenic gene expression. We next examined how perturbation of Met/SAM metabolism and methylation potential would impact global levels of H3K79me2, a modification required for the survival of MV411 and RS411 cells. Disruption of Met/SAM metabolism at either node dramatically reduced global H3K79me2 at all examined timepoints in MV411 cells, indicating

that they require sufficiently high levels of Met/SAM metabolic flux to maintain global levels of H3K79me2 **(Fig. 3.3C)**. Global H3K79me2 was also reduced in RS411 cells at all timepoints examined **(Fig. 3.3D)**

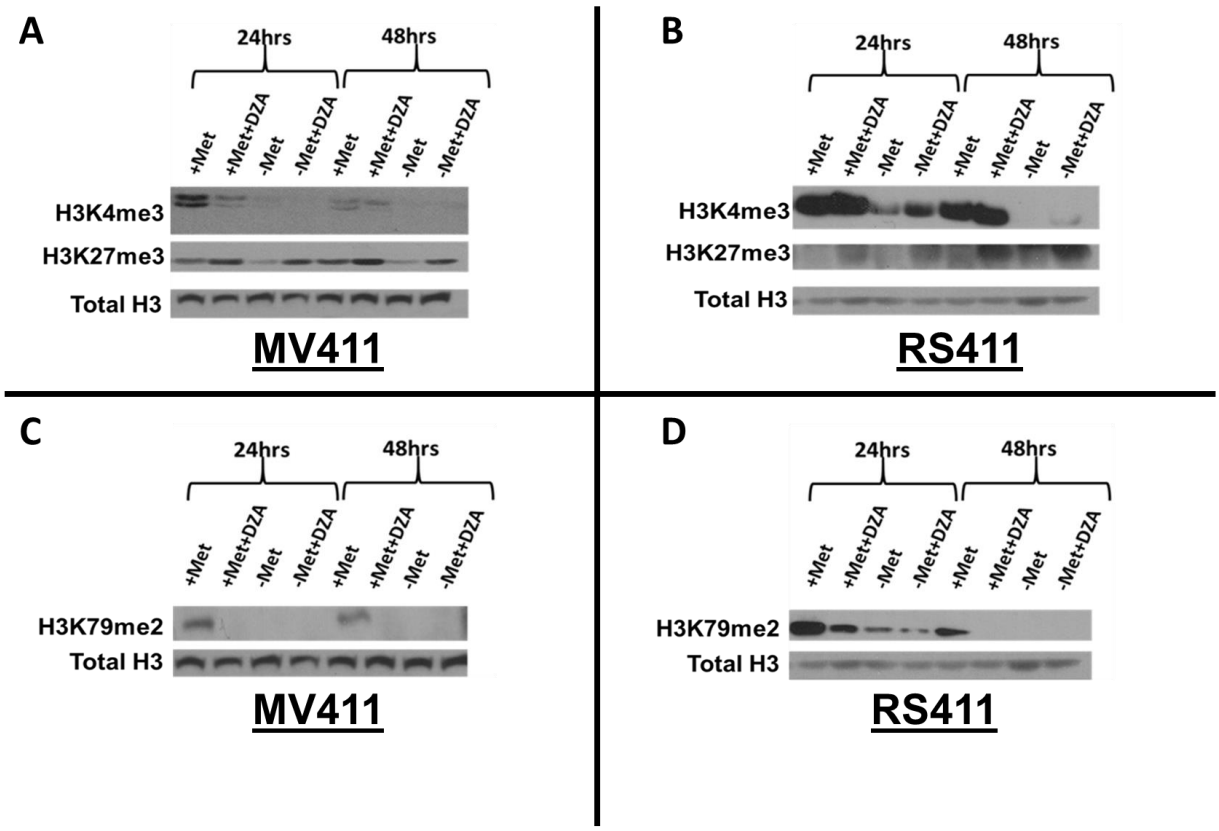

Figure 3.3 Perturbation of Met/SAM metabolism alters global histone methylation dynamics and decreases DOT1L dependent global H3K79me2 in MLL-R leukemia cell lines. (A) MV411 cells display a dramatic reduction in global levels of the activating H3K4me3 modification following methionine deprivation or DZA treatment, and a global increase in the repressive H3K27me3 modification, only upon DZA mediated SAHH inhibition. (B) RS411 cells only display reduced levels of H3K4me3 only under conditions of methionine deprivation, but similar to MV411 cells show increased H3K27me3 after DZA mediated inhibition of SAH metabolism (right). (C) MV411 cells have profoundly reduced levels of MLL-R required H3K79me2 at all timepoints and experimental conditions examined. (D) RS411 cells also show a loss in global H3K79me2 with

differential reduction upon methionine deprivation and/or DZA treatment, but loss of this modification occurs more slowly as compared to MV411 cells.

Inhibition of SAH metabolism and/or methionine deprivation reduces mRNA expression and protein levels of the H3K79 methyltransferase DOT1L, and induces DNA damage.

Perturbation of Met/SAM metabolism significantly decreased global levels of the required activating H3K79me2 modification in MV411 and RS411 cells. Therefore, we next examined how alterations to Met/SAM metabolism impacted the expression of the H3K79 methyltransferase DOT1L, at the mRNA and protein level. MV411 cells show significantly reduced DOT1L mRNA expression following 8hr treatment with DZA, with or without methionine deprivation, and by 24hrs all three conditions significantly diminish DOT1L mRNA expression **(Fig. 3.4A, left)**. Consistent with the mRNA expression data, protein levels of DOT1L are also diminished in MV411 cells, and interestingly DNA damage was observed under conditions of DOT1L depletion **(Fig. 3.4A, right)**. In alignment with previous cytotoxicity data a decrease in DOT1L mRNA was only observed upon DZA treatment of RS411 cells at 8 or 24hrs post exposure to experimental conditions, however by 36hrs DOT1L mRNA levels are decreased in RS411 cells upon methionine deprivation **(Fig. 3.4B, left)**. Protein expression of DOT1L was reduced upon exposure to DZA at 8 and 24hrs post treatment, and under all experimental conditions by 48hrs, and again DNA damage was observed by pH2.AX foci formation **(Fig. 3.4B, right)**. Conversely, DOT1L mRNA expression was significantly increased or remained unchanged in non-MLL rearranged K562 cells, and very modest changes were observed in overall DOT1L protein levels **(Fig. 3.4C)**. Finally, to further demonstrate correlation between Met/SAM

metabolic status, DOT1L expression, and apoptosis induction we either DZA treated RS411 cells for 24 hours followed by washout, or deprived RS411 cells of methionine for 48hrs followed by re-plating in methionine rich media, and tracked DOT1L and cleaved caspase-3 protein expression. As expected exposure to DZA for 24hrs or methionine deprivation for 48hrs decreased DOT1L expression and induced caspase-3 cleavage, followed by the subsequent increase in DOT1L expression and decrease in caspase-3 cleavage during recovery in drug free methionine rich media **(Fig. 3.4D)**.

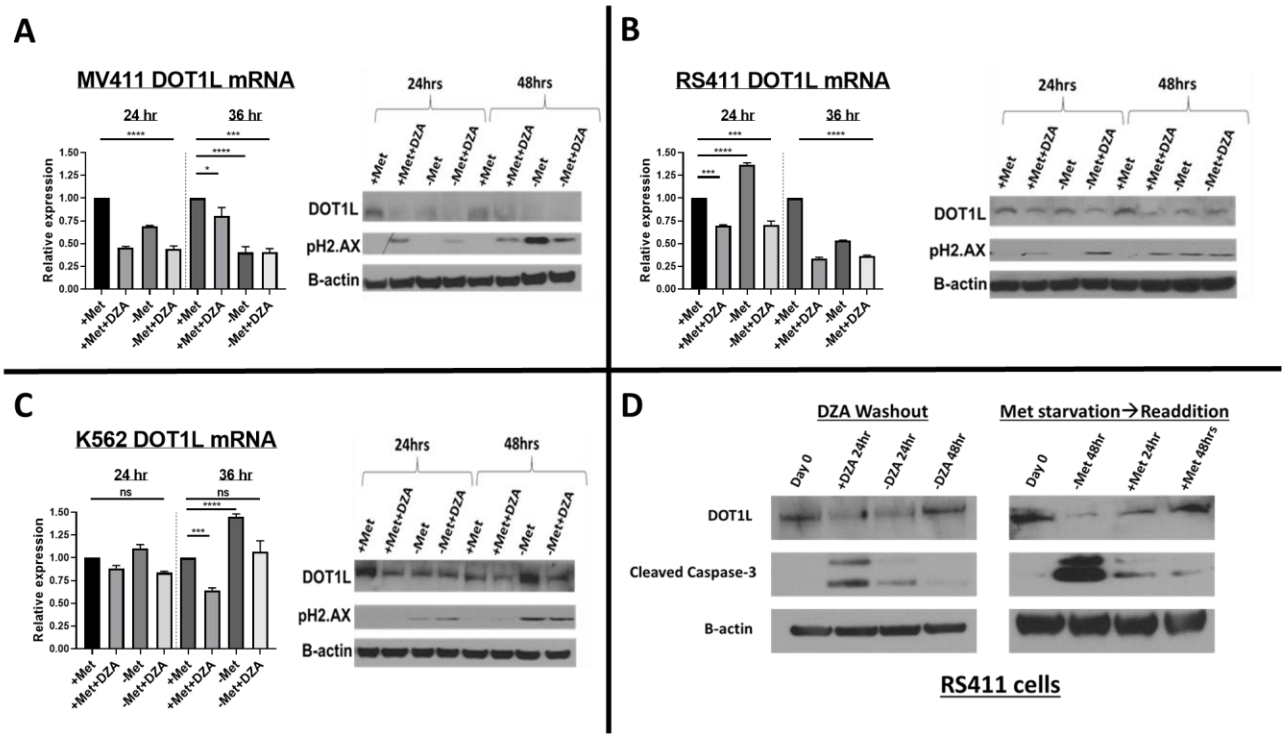

Figure 3.4 Alteration of Met/SAM metabolism reduces mRNA and protein expression of the required H3K79 methyltransferase DOT1L selectively in MLL-R leukemia cells. (A) DZA treatment or methionine deprivation of MV411 cells significantly reduced DOT1L mRNA following 24 or 36 hour exposure to experimental conditions (left) and these changes are reflected in DOT1L protein levels (right). Reduction in DOT1L expression also correlated with the induction of DNA damage as determined by pH2.AX formation, likely due to the critical role DOT1L plays in regulation of the DNA damage repair response. (B) RS411 cells display a significant reduction in DOT1L mRNA (left) and protein levels (right) following DZA mediated SAHH inhibition, but not with methionine deprivation alone at the timepoints studied. Eventually, methionine deprivation alone significantly reduced DOT1L mRNA and protein expression in RS411 cells, and again DNA damage induction was detected only under conditions of DOT1L

reduction. (C) Non-MLL-R K562 cells lacking functional dependence on DOT1L show no significant changes in DOT1L mRNA expression (left) 24 hours post study initiation, however DOT1L mRNA was significantly reduced 36 hours after DZA exposure, but was significantly increased following 36 hours of methionine deprivation. Interestingly, modulation of DOT1L protein levels was observed even in K562 cells (right), and pH2.AX foci formation was also detected only under conditions of methionine deprivation in K562 cells. These results may suggest that appropriate regulation of Met/SAM metabolism and one-carbon sensing may directly play a more universal role in regulation of DOT1L expression and function in multiple cell types, regardless of MLL-R mediated functional dependence on DOT1L and H3K79 methylation.

Decreased expression of DOT1L is correlated to changes in histone methylation dynamics specifically at the DOT1L promoter.

Chromatin immunoprecipitation (ChIP) was conducted on MV411 and RS411 cells under all experimental conditions, using antibodies against H3K4me3 (data not shown) and H3K27me3 followed by RT-PCR using DOT1L promoter specific primers. Promoter specific primer sets were designed against a DOT1L promoter region just upstream of two major putative transcription factor binding sites as predicted by MotifMap software. Control primers were also designed at a distal arbitrary location as an internal control to ensure that observed results were not due to technical bias **(Fig. 3.5A)**. We found that inhibition of SAH metabolism and/or deprivation of exogenous methionine significantly elevated levels of the repressive H3K27me3 modification at the DOT1L promoter in MV411 cells 24hrs and 48hrs post exposure to experimental conditions, and there is likely a synergistic increase in H3K27me3 48hrs post combined methionine deprivation and DZA treatment **(Fig. 3.5B)**. As we expected RS411 cells only showed increased levels of DOT1L promoter H3K27me3, upon DZA mediated inhibition of SAH metabolism, again matching with our previous cytotoxicity and expressional data in RS411 cells **(Fig. 3.5C)**

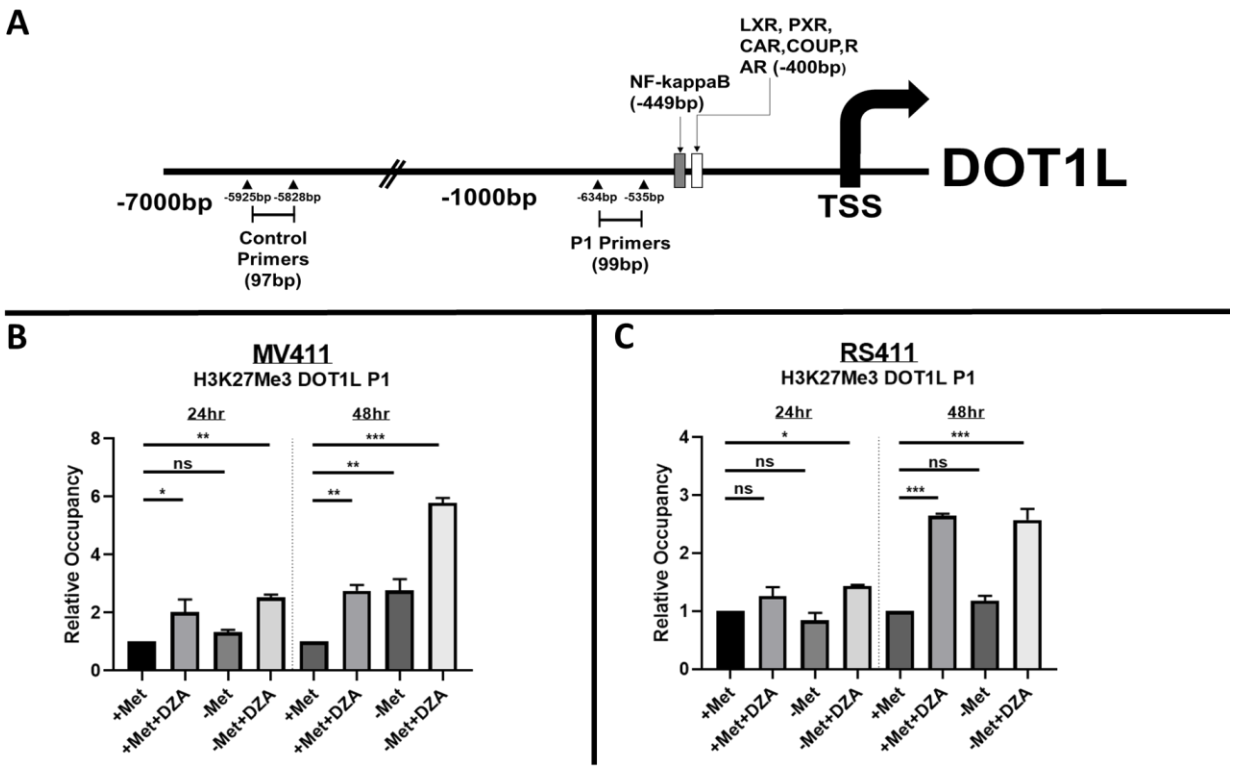

Figure 3.5 Decreased expression of DOT1L is correlated to changes in histone methylation dynamics specifically at the DOT1L promoter. (A) DOT1L promoter map illustrating putative transcription factor binding sites as determined by MotifMap software, as well as the specific binding sites of the DOT1L promoter specific primers (P1) and the distal control primers. (B) MV411 cells show significantly elevated levels of H3K27me3 occupancy at the DOT1L promoter following DZA treatment or methionine deprivation and by 48 hours an additive increase was observed under simultaneous methionine deprivation and DZA mediated SAHH inhibition. (C) RS411 cells exhibit significantly increased DOT1L promoter H3K27me3 occupancy following 24 hours of simultaneous methionine deprivation and DZA exposure, and by 48 hours DZA treatment but not methionine deprivation alone is sufficient to significantly elevate DOT1L

promoter H3K27me3 occupancy. (D) RS411 cells show a significant decrease in mRNA levels of HOXA9 and MEIS1, two well established DOT1L targets known to be required to maintain MLL-R mediated leukemogenesis, only upon DZA mediated inhibition of SAH metabolism but not methionine deprivation alone in agreement with our previous cytotoxicity and DOT1L expressional data in RS411 cells.

Patient derived MLL-FP expressing leukemic blasts, similar to established cell lines, are sensitive to alterations in Met/SAM metabolism, and show corresponding changes in global and promoter specific histone methylation dynamics.

We next examined if similar changes in histone methylation dynamics and DOT1L expression and function could be observed in patient derived MLL-FP expressing blasts (CCHC-7). CCHC-7 cells, bear an MLL-AF9 rearrangement and grow under standard culture conditions with human hematopoietic cytokines. We therefore plated these cells under all the previous experimental conditions and similarly examined apoptotic effects, alterations to global histone methylation dynamics, and changes in DOT1L expression. Like MV411 and RS411 cells, CCHC-7 cells undergo apoptosis, as determined by immunoblotting for caspase-3 and PARP-1 cleavage **(Fig. 3.6A)**. However, the cytotoxic effects of perturbation of Met/SAM metabolism in CCHC-7 cells more closely resemble RS411 cells, in that only DZA induced inhibition of SAHH with or without methionine resulted in apoptosis induction. Furthermore, we observed a decrease in overall DOT1L protein expression and similar changes in global histone methylation dynamics in the patient derived CCHC-7 cells, as compared to the tested MLL-FP cell lines **(Fig. 3.6B)**. Interrogation of the DOT1L promoter by ChIP using a H3K27me3 specific antibody followed by RT-PCR using DOT1L promoter specific primers, showed a significant increase in H3K27me3 occupancy at the DOT1L promoter in CCHC-7 cells **(Fig. 3.6C).** These results are highly similar to our *in vitro* studies utilizing the established MLL-AF4

expressing cell lines, we therefore felt confident in moving forward to *in vivo* studies.

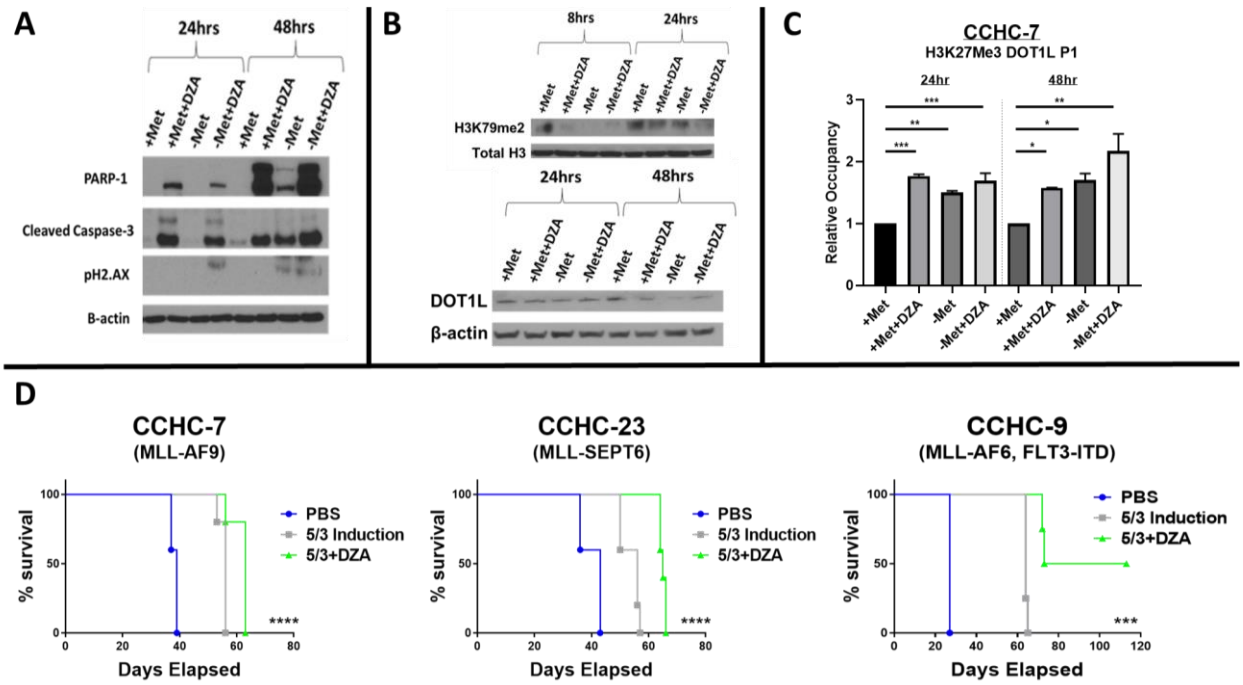

Figure 3.6 Patient derived MLL-R leukemic blasts are sensitive to perturbation of Met/SAM metabolism, and show changes in global and DOT1L promoter specific histone methylation dynamics consistent with tested MLL-R cell lines. Combining DZA with 5+3 induction therapy significantly prolonged the life of mice harboring several different MLL-R patient derived xenografts. (A) Patient derived CCHC-7 cells behave similarly to the RS411 cell line and only undergo apoptosis following DZA mediated SAHH inhibiton with or without methionine deprivation as determined by PARP-1 and caspase-3 cleavage. Methionine deprivation for 48 hours was also sufficient to induce apoptosis and PARP-1 and caspase-3 cleavage. (B) CCHC-7 cells show a profound early loss in global H3K79me2 levels following 8 hours of exposure to experimental conditions, and this reduction was more modest 24 hours post exposure to either DZA or methionine deprivation alone. However, the

combination of simultaneous DZA mediated SAHH inhibition and methionine deprivation was still sufficient to decrease global H3K79me2 even after 24hrs (top). DOT1L protein expression was also reduced in CCHC-7 cells by methionine deprivation and/or DZA treatment (bottom). (C) CCHC-7 cell display significantly elevated levels of DOT1L promoter H3K27me3 occupancy 24 or 48 hours after exposure to all experimental conditions and these changes likely correlate with the observed reduction in DOT1L protein expression and function (decreased H3K79me2). (D) NRGS mice (n=5 for all cohorts in all studies) were xenografted with 1.25 x 10^5 CCHC-7, CCHC-23, or CCHC-9 patient leukemic cells each bearing a unique MLL-R, and 7 days post xenotransplant mice began bolus intravenous infusion of isovolumetric amounts of 5+3 induction therapy (3 mg/kg doxorubicin, Days 1-3 and 75 mg/kg cytarabine, Days 1-5), 5+3 induction plus 25 mg/kg DZA (Days 1-5), or the vehicle PBS alone. DZA in combination with 5+3 induction significantly prolonged the life of these mice as compared to either 5+3 induction therapy or vehicle treatment alone in all xenograft studies regardless of particular patient MLL-R, and excitingly two of the five mice bearing CCHC-9 xenografts completely failed to develop leukemic disease and survived till the endpoint of our study (far right).

Pharmacologic inhibition of SAH metabolism by DZA significantly prolongs the survival of mice xenografted with patient MLL-FP expressing leukemia, in combination with SOC induction therapy.

Patient derived CCHC-7 cells were found to be highly sensitive to pharmacologic disruption of downstream SAH metabolism *in vitro*, and also displayed similar changes in DOT1L expression, and both DOT1L promoter specific and global histone methylation dynamics as compared to established MLL-AF4 expressing cell lines. Thus, we finally examined the *in vivo* efficacy of DZA mediated SAHH inhibition in a highly advanced and clinically relevant xenograft model of patient MLL-R leukemia. Cohorts of transgenic NRGS mice (n=5 per group) were xenografted with three induction refractory pediatric patient AML samples, each bearing a different MLL rearrangement. Seven days post xenograft the mice were given isovolumetric bolus intravenous injections of either saline, induction chemotherapeutics, or induction chemotherapeutics combined with 25 mg/kg DZA in a clinically similar and previously defined 5+3 regimen.[124] Combined treatment using 5+3 induction and DZA significantly prolonged the survival of NRGS mice xenografted with aggressive patient derived MLL-FP expressing leukemia, albeit to varying degrees. A modest but significant survival extension was seen in mice engrafted with either CCHC-7 or CCHC-23 cells, as compared to 5+3 induction therapy alone **(Fig. 3.6D left, center)**. Excitingly, combinatorial therapy of mice harboring CCHC-9 leukemia significantly prolonged their survival, and two of five mice in the (5+3) + DZA cohort survived to the endpoint of the study without developing disease **(Fig. 3.6D right).**

CHAPTER 4

OVERALL DISCUSSION AND SUMMARY

AML, and more broadly leukemia in general, is in actuality a heterogenous group of related hematopoietic dysplasias characterized by a block in hematopoietic differentiation, resulting in aberrant growth and proliferation. Disease presentation of human leukemia is uniquely heterogenous among cancers, and displays highly patient specific disparities in terms of morphology, cytogenetics, and epigenetics. However, approved frontline clinical induction therapy revolving around the cytotoxic combination of an anthracycline and cytarabine remains the only FDA approved therapy for AML, and treats AML in a “one size fits all” manner, regardless of patient specific disease etiology. Current preclinical investigation of novel AML therapeutics very rarely involves comparison to SOC chemotherapy. Furthermore, it has been well documented that preclinical *in vivo* animal models of patient disease consistently better predict the successful outcomes of novel experimental therapeutics in clinical trials. Therefore, the overall goal of this dissertation was two-fold, first to develop a more clinically relevant model of CDX or PDX AML that is capable of efficient engraftment of patient AML, humanization, can model the influence of human myeloid cytokine signaling, and most importantly tolerate an efficacious more

clinically similar regimen of induction chemotherapy compared to the much more prevalent SCID-dependent AML xenograft models.

Secondly, we sought to use our newly established advanced PDX model of MLL-translocated pediatric leukemia to confirm preliminary *in vitro* findings that perturbations of Met/SAM metabolism are cytotoxic to MLL-rearranged leukemia cells and alter global and promoter specific histone methylation dynamics decreasing the function and expression of the required H3K79 methyltransferase DOT1L.

To address the first overall goal of developing a more advanced and clinically relevant murine xenograft model, we compared the usefulness of the relatively new and under-utilized SCID-independent immunodeficient NRG mice, and the related human myeloid cytokine expressing NRGS strain without preconditioning, in modeling of both malignant and normal human hematopoiesis. Initially, we examined comparative engraftment efficiency of human leukemic CDXs and PDXs in both NRG and NRGS mice. As expected, we found that human primary and secondary PDX samples engraft with high efficiency selectively in NRGS mice, but not NRG mice, likely due to their transgenic expression of human myeloid cytokines whose signaling is required for efficient engraftment. Remarkably, human CDXs showed irregular engraftment and clinically disparate disease progression in NRGS mice resulting in the formation of large IP and intra-lymphatic solid tumors, but showed canonical diffuse hematopoietic restricted disease presentation and progression in NRG mice. These effects are likely due to the constitutive whole body

expression of human myeloid cytokines driving the rapid growth and proliferation of established human leukemic CDXs in non-hematopoietic organs. Thus, our findings highlight the importance of selecting the correct strain of mice as contextually determined by the experimental design.

Importantly, we also examined therapeutic outcomes and tolerability of SOC chemotherapeutic induction therapy, in a clinically similar 5+3 treatment regimen, and showed therapeutic efficacy against human CDXs and PDXs. Furthermore, these mice were tolerant of significantly higher doses of induction chemotherapeutics, as compared to more commonly used SCID mice, and these doses more closely recapitulate clinical patient therapy. Xenograft mouse models have consistently been shown to better predict the success of experimental chemotherapeutics in clinical trials, however most preclinical studies fail to also model SOC therapy (usually only vehicle or less frequently single agent therapy are used as study controls), both as a more relevant experimental control and additionally in the study of combinatorial therapies. Our findings validate the feasibility of modeling highly aggressive and clinically similar 5+3 induction therapy in RAG1 deficient (SCID independent) NRG and NRGS mice, and similar model systems may enhance investigation of highly efficacious novel single-agent or combinatorial chemotherapeutics.

In addition to modeling malignant human hematopoiesis, we also felt it was important to examine the utility of these two under used mouse strains in modeling normal hematopoiesis. Astonishingly, we found that even without expensive and/or time-consuming ablative preconditioning NRGS mice were able

to support the efficient durable engraftment and multilineage expansion of human CD34+ UBC derived HSCs. These results are highly significant as they contradict previous studies which demonstrate transgenic human hematopoietic cytokine production is counterproductive and diminishes human HSC engraftment and expansion. However, in contrast with the aforementioned studies for the sake of ease, cost, and high-throughput efficiency we chose to forgo the intense ablative preconditioning (pharmacological agents or irradiation) utilized in these studies, opting instead to used unconditioned animals. Ablative conditioning may render the murine BM microenvironment inhospitable to human HSC seeding or promote hematopoietic differentiation and loss of self-renewal in human HSCs. Thus, our findings indicate that not only is myeloablative conditioning unnecessary in NRGS mice, and may in fact be detrimental to long-term durable engraftment of human HSCs.

To address the second overall goal of this dissertation, we next sought to utilize the aforementioned, and now well-characterized NRGS model system to further investigate our *in vitro* findings that perturbations of Met/SAM metabolism are cytotoxic, and deregulates the misdirected and required DOT1L / H3K79me signaling axis required to maintain survival and leukemic potential in MLL-R leukemia cells. We were able to show that perturbation of Met/SAM metabolism by targeting two distinct nodes, namely through deprivation of exogenous methionine or DZA mediated pharmacologic inhibition of downstream SAH metabolism, induced apoptosis in established MLL-R cell lines and patient derived MLL-R blasts, but not in MLL-R independent BCR-ABL expressing K562

cells. Furthermore, we determined that perturbation of this critical metabolic pathway also resulted in changes in global and promoter specific histone methylation dynamics including the global loss of the activating H3K4me3 and H3K79me2, and an increase in the repressive H3K27me3 globally and specifically at the DOT1L promoter, only in MLL-R leukemic cells. Interestingly, we also observed that perturbation of Met/SAM metabolism even reduced both mRNA and protein expression of the DOT1L methyltransferase, thus suggesting that some degree of feed-back one-carbon metabolic regulation exists controlling DOT1L expression and function in MLL-R leukemia cells. **(Fig. 4.1)**

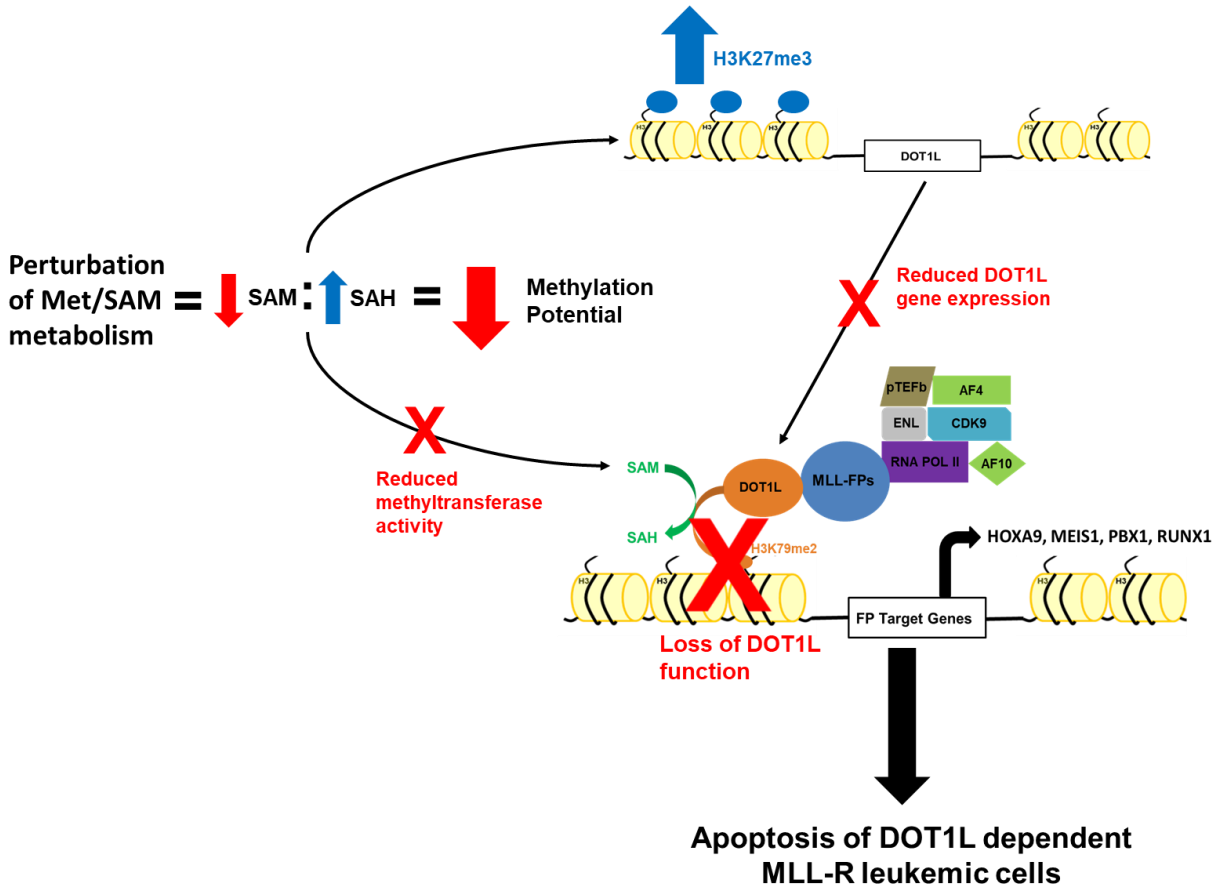

Figure 4.1 One proposed mechanism by which perturbation of Met/SAM metabolism induces apoptosis in DOT1L dependent MLL-R leukemia cells.

Our findings suggest that perturbation of Met/SAM metabolism reduces methylation potential and negatively impacts both expression and function of the requisite H3K79 methyltransferase DOT1L in MLL-R cell lines and patient blasts. Furthermore, under conditions of diminished methylation potential we observed a significant decrease in global H3K79me2 and an increase in the repressive H3K27me3 modification specifically at the DOT1L promoter, as well as a reduction in DOT1L mRNA and protein. Given the unique dependence of MLL-R leukemia on sufficiently high levels of DOT1L expression and function, our findings suggest that perturbation of Met/SAM metabolism may be a novel

therapeutic vulnerability specifically targeting the high levels of aberrant activating histone methylation required to maintain MLL-R mediated leukemogenic gene expression and phenotype.

We next confirmed that similar changes in histone methylation dynamics and cytotoxic effects occur in patient derived MLL-R leukemic blasts *in vitro* and therefore felt confident in testing this novel therapeutic paradigm against MLL-R PDXs, in our highly advanced *in vivo* human leukemia xenograft model. Excitingly, the addition of 25 mg/kg DZA to our standard 5+3 induction therapy regimen significantly prolonged the survival of NRGS mice bearing several unique MLL-R patient samples as compared to vehicle or 5+3 induction alone treated mice, and additionally no overt toxic effects were observed by weight loss. These results are even more promising as all patient PDX cells were acquired at the time of induction refractory relapse suggesting that perturbation of Met/SAM metabolism may be a valuable therapeutic adjuvant, especially during subsequent rounds of induction and/or consolidation therapy. Our studies provides *in vivo* proof of concept that targeting Met/SAM metabolism may be a unique vulnerability specifically against aggressive and poorly treated MLL-R leukemia, by deregulating the expressional axis controlled by the MLL-R epigenetic lesion. Finally, our findings also demonstrate the utility of our newly established highly advanced PDX leukemia model, especially in the investigation of novel combinatorial therapeutic treatment paradigms.

In total, work outline in this dissertation highlights the need for implementation of more clinically relevant animal models of human leukemia, that model not only physiologically relevant disease progression, but also response to SOC cytotoxic induction therapy. Using our newly established mouse model of human leukemia in NRG and NRGS mice, we were able to test the *in vivo* utility

of targeting Met/SAM metabolism as a new treatment strategy specifically against MLL-R leukemias, and were able to show significant life extension in all mice treated in combination with DZA and 5+3 induction as compared to 5+3 induction alone. In conclusion, our findings demonstrate the utility of our newly established murine PDX model, and furthermore provide support for the continued investigation of perturbation of Met/SAM metabolism as a potential new therapeutic approach against MLL-R leukemia in combination with SOC cytotoxic chemotherapy.

STRENGTHS AND LIMITATIONS

STRENGTHS

The work presented in this dissertation, to our knowledge, is the first comprehensive study of the utility of the under-utilized SCID-independent NRG and NRGS mice in modeling malignant and normal human hematopoiesis. Previous preclinical studies on experimental leukemia therapeutics have depended heavily of chemotherapeutic intolerant SCID mouse models and therefore fail to account for SOC treatment outcomes, as either a more relevant control or in the investigation of adjuvant therapeutics. Our data conclusively shows for the first time that both NRG and NRGS mice can tolerated relevant doses of induction therapeutics in a clinically similar treatment regimen, and therapy is efficacious in delaying disease progression and prolongs life of mice harboring human CDXs or PDXs. Thus, our data challenges the hematological

field to abandon the widely favored SCID dependent murine model systems, in favor of RAG deficient strains tolerant of induction therapy, therefore not only model the unique heterogeneity of patient leukemic disease, but more importantly therapeutic response to SOC induction therapy. Truly efficacious experimental anti-leukemia therapeutics must be judged in comparison to SOC therapeutic outcomes, and additionally our new model system allows for investigation of novel combinatorial therapies.

We next were the first to identify perturbation of Met/SAM metabolism as a novel vulnerability *in vitro* and *in vivo* against the now well-defined MLL-R epigenetic lesion, which maintains leukemogenic potential through aberrant activating histone methylation and subsequent anomalous expression of embryonic genes like the *HOXA* cluster or *MEIS1*. Our novel approach was to investigate perturbation of Met/SAM metabolism, which controls both methylation potential and methylation substrate availability, as a new therapeutic vulnerability given MLL-R leukemia's necessity to maintain high levels of aberrant histone methylation to activate leukemogenic gene expression patterns crucial for survival and disease progression. Perturbation of this metabolic pathway was indeed toxic to MLL-R cells, induced apoptosis, and deregulated global and DOT1L promoter specific histone methylation dynamics, providing support for our initial hypothesis. We further demonstrated this point *in vivo* by showing significant survival extension of mice when treated in combination with SOC induction and the competitive SAHH inhibitor DZA as compared to induction alone, in our newly described NRGS PDX model system.

LIMITATIONS

While we believe our newly characterized, highly advanced, and more clinically relevant human leukemia xenograft model is significantly superior to more commonly used SCID dependent preclinical models, there are still limitations to our NRG/NRGS model system. First and foremost, we demonstrate that the utility of each of these relatively new mouse strains is contextually limited by individual experimental design, more specifically NRG mice are well suited only for CDX studies while NRGS mice develop clinically disparate CDX disease progression, and should be used exclusively for highly efficient engraftment and progression of PDXs. Secondly, while our collaborators at Cincinnati Children's Hospital have directly examined plasma levels of induction therapeutics in mice and calibrated their doses based on mouse pharmacokinetics and clinical patient plasma concentrations from literature, we have never directly examined plasma drug concentrations in our mice, instead basing our doses on their published studies. Regardless, our findings demonstrate the tolerability of these mice to aggressive chemotherapy, to be double that of more commonly used SCID dependent strains, and furthermore treatment is efficacious in prolonging survival and human CDX and PDX disease latency.

Our work is the first to identify targeting of Met/SAM metabolism as a potential therapeutic vulnerability against MLL-R leukemias, by deregulating the required high levels of aberrant, activating DOT1L mediated H3K79 histone methylation to maintain survival and leukemogenic potential. However, our studies fail to elaborate on the essential nature of Met/SAM metabolism in a

myriad of other critical cellular processes like purine/pyrimidine biosynthesis, glutathione synthesis, 5-THF regeneration, etc. Perhaps our effects are mediated more profoundly through decreased metabolic flux through these pathways resulting in cytotoxicity and apoptosis induction. Furthermore, it is possible that methionine deprivation induces other starvation related effects, like a global decrease in all protein synthesis, and this may explain our observed decrease in DOT1L expression. However, MLL-R independent K562 cells show no significant change in global H3K79me2 levels and/or DOT1L mRNA and protein expression, again suggesting that these effects only occur in the context of MLL-R leukemia. Furthermore, differential effects were observed in sensitivity to targeting different nodes of Met/SAM metabolism and may suggest that inhibition of this pathway is only highly cytotoxic in MLL-R leukemias of myeloid origin or with other favorable cell type intrinsic characteristics.

Finally, we were able to use our newly established NRGS PDX model system to demonstrate significant life extension in mice treated with a combination of SOC induction therapy and the SAHH inhibitor DZA, but never investigated single-agent DZA therapeutic outcomes, and our studies were limited by exclusively focusing on survival. Thus, we have no data from patient leukemic cells isolated from PDX bearing NRGS mice, showing concurrent changes in histone methylation dynamics as compared to our *in vitro* studies. Furthermore, perturbation of Met/SAM metabolism may likely show additive and/or synergistic effects with DNA-damaging induction chemotherapeutics, a possibility that remained uninvestigated in our studies. These limitations were

taken into consideration, however considering this was the first preliminary investigation into targeting of Met/SAM metabolism in MLL-R leukemia, we simply wanted to investigate potential differential adjuvant therapeutic outcomes to determine whether this treatment paradigm was meritorious of future investigation. Furthermore, no clinically useable inhibitors of any Met/SAM metabolic enzymes exist, and most preclinical investigation, including this dissertation, relies on relatively non-specific substrate competitive inhibitors (cycloleucine, DZA, DZNep, adenosine dialdehyde, etc.). This changed in 2017, when a group from Pfizer discovered and characterized PF-9366 the first novel allosteric inhibitor of MATIIA, the enzyme responsible for the synthesis of SAM from methionine, capable of inhibition at physiologically reachable concentrations. Our findings now support the continued investigation of potent targeted inhibitors of Met/SAM metabolism, as a unique vulnerability specific to MLL-R leukemias.

FUTURE DIRECTIONS

While we have worked to extensively characterize and validate the utility of NRG and NRGS mice in modeling both normal and malignant human hematopoiesis, and further utilized our newly established model system to confirm our *in vitro* findings that perturbation of Met/SAM metabolism may be a unique therapeutic vulnerability in MLL-R leukemia. However, several important underlying mechanisms and questions remain unanswered in our studies, and thus the future directions are outlined below:

1. **Optimization of both induction therapeutic dosing and timing in NRG and NRGS mice.**

   We have shown that NRG and NRGS mice are tolerant of aggressive 5+3 induction with 3 mg/kg doxorubicin and 75 mg/kg cytarabine, but widespread toxicity and decreased survival was observed when the concentration of doxorubicin was doubled to 6 mg/kg, irrespective of cytarabine dosage. Interestingly, the few mice that did survive our dose escalation study actually survived until the 100 day study endpoint with no symptomatic leukemic disease progression. These findings are highly exciting as they suggest that with careful optimization of the dose limiting anthracycline, we may be able to model the most predominant clinical outcome of leukemia, initial remission. Further, aging of the PDX harboring mice that receive remission inducing induction therapy, may actually allow for the development of refractory patient disease, even further increasing the preclinical modeling utility of the NRG/NRGS model system. In the future we also plan to examine how modulation of induction timing affects therapeutic outcomes, and to model other approved induction regimens like 7+3 therapy.

2. **Investigate the *in vivo* therapeutic potential of dietary methionine restriction in mice harboring human leukemia xenografts.**

   Data outlined in Chapter 3 indicate that perturbation of Met/SAM metabolism by pharmacologic inhibition of SAHH using 25 mg/kg DZA significantly prolonged the survival of NRGS mice harboring aggressive patient MLL-R leukemia, in combination with 5+3 induction. However, the

therapeutic potential of dietary methionine restriction and 5+3 induction remains unstudied. In order to examine these potentially beneficial effects, we have established and treated two cohorts of NRGS mice harboring the same MLL-R PDX, and each cohort is fed either isocaloric chow containing 0.86% or 0.11% methionine respectively. Previously published studies have shown that modulation of dietary methionine is sufficient to alter histone methylation dynamics in mice and humans.[125,126] Additionally, we may also observe toxicity ameliorative or chemotherapy enhancing effects, as several other studies have shown beneficial effects of methionine restriction on chemotherapeutic drug efficacy and tumor regression.[127-129]

3. **Examine therapeutic efficacy of combined DZA treatment and methionine restriction with 5+3 induction against patient MLL-R xenografts.**
   Our final line of future investigation aims to combine the observed beneficial effects of DZA treatment along with 5+3 induction, with the potential benefits of dietary methionine restriction. These studies may demonstrate even further enhanced improvement in treatment outcomes and amelioration of chemotherapeutic toxicity, especially if beneficial effects are seen in the studies described above combining dietary methionine restriction and 5+3 induction therapy. Further investigation may reveal targeting of Met/SAM metabolism as a bona fide therapeutic vulnerability inhibiting the aberrant activating histone methylation driven by the MLL-R epigenetic lesion.

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ABBREVIATIONS

AML	Acute myeloid leukemia
BM	Bone marrow
SOC	Standard of care
FAB	French-American-British
APL	Acute promyelocytic leukemia
WHO	World Health Organization
CR	Complete Remission
IV	Intravenous
Ara-c	Cytarabine
MRD	Minimal residual disease
CN-AML	Cytogenetically normal AML
NPM1	Nucleophosmin 1
FLT3	Fms-like tyrosine kinase
DNMT3A	DNA methyltransferase 3A
FLT3-ITD	FLT3 Internal Tandem Duplication
OS	Overall survival
DFS	Disease free survival
HSC	Hematopoietic stem cell
HMT	Histone Methyltransferase
HDAC	Histone Deacetylase
MLL	Mixed-lineage leukemia
ALL	Acute lymphocytic leukemia

SEC	Super elongation complex
DOT1L	Disruptor of telomeric silencing 1-like
MLL-FP	MLL fusion protein
SAM	S-adenosylmethionine
Met	Methionine
MATIIA	Methionine adenosyltransferase IIA
SAH	S-adenosylhomocysteine
SAHH	S-adenosylhomocysteine hydrolase
MTR	Methionine synthase
LSC	Leukemic stem cell
SCID	Severe combined immunodeficiency
Prkdc	DNA-activated catalytic polypeptide
NOD	Non-obese diabetic
RAG	Recombination activated gene
NK	Natural killer
SF	Steel factor
SCF	Stem cell factor
UBC	Umbilical cord blood
RRF	Rodent research facility
IP	Intraperitoneal
HSCT	Hematopoietic stem cell transplant
ChIP	Chromatin immunoprecipitation
PI	Propidium iodide
TSS	Transcription start site

CURRICULUM VITAE

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POSTER PRESENTATIONS

- *Alterations to Methionine/S-adenosylmethionine metabolism regulate Dot1L expression and function; a novel vulnerability in MLL fusion leukemia.*

14th Midwest Blood Club- St. Jude Children's Research Hospital (2019).

- *Targeting S-adenosylmethionine Dependent Trans-Methylation and Metabolism as a Novel Treatment Paradigm for AML.*
  12th International Workshop On Molecular Aspects Of Myeloid Stem Cell Development And Leukemia (2018).
- *Effects of Alterations on SAM/Met Pathway on Normal HSPCs.*
  Research!Louisville (2018).
- *Targeting S-adenosylmethionine Dependent Trans-Methylation and Metabolism as a Novel Treatment Paradigm for AML.*
  11th Annual Symposium Mechanisms and Models of Cancer- Salk Institute (2017).
- *Targeting S-adenosylmethionine Dependent Trans-Methylation as a Novel Treatment Paradigm for AML.*
  Research!Louisville (2017).
- 13th Midwest Blood Club (2015)
- *Exploring novel differentiation therapies for acute myeloid leukemia*.
  Research!Louisville (2015)

PUBLICATIONS AND ABSTRACTS

Publications

- **Barve, A.**, Shah, P., Ghare, S., Wunderlich, M., Mulloy, JC., Beverly LJ. *Perturbation of Met/sam metabolism is cytotoxic to MLL-translocated leukemia and impairs Dot1l expression and function; a novel vulnerability in vitro and in vivo.* (Under review at American Journal of Hematology)
- **Barve, A.**, Casson, L., Krem, M., Wunderlich, M., Mulloy, JC., Beverly, LJ. *Comparative utility of NRG and NRGS mice for the study of normal hematopoiesis, leukemogenesis, and therapeutic response.* Experimental Hematology. 2018 Nov;67:18-31. doi: 10.1016/j.exphem.2018.08.004. Epub 2018 Aug 17.
- Saforo, D., Omer, L., Smolenkov, A., **Barve, A.**, Casson, L., Boyd, N., Clark, G., Siskind, LJ., Beverly, LJ. *Primary lung cancer samples cultured under microenvironment-mimetic conditions enrich for mesenchymal stem-like cells that promote metastasis.* Scientific Reports. 2019; 9: 4177.Published online 2019 Mar 12. doi: 10.1038/s41598-019-40519-4
- Ketchem, CJ., Kucera, C., **Barve, A.**, Beverly, LJ. *The Antiarrhythmic Drug, Amiodarone, Decreases AKT Activity and Sensitizes Human Acute Myeloid Leukemia Cells to Apoptosis by ABT-263*. American Journal of the Medical Sciences. 2018 May;355(5):488-496. doi: 10.1016/j.amjms.2018.01.011. Epub 2018 Feb 6.

- Jani, TS., Gobijishvili, L., Hote, PT., **Barve, AS.**, Joshi-Barve, S., Suttles, J., Chen, T., McClain, CJ., Barve, S. *Inhibition of methionine adenosyltransferase II induces FasL expression, Fas-DISC formation and caspase-8-dependent apoptotic death in T leukemic cells.* Cell Research.

Abstracts

- **Barve, A.**, Beverly L. *Establishing a clinically relevant mouse model of human AML to test novel transmethylation inhibitors.*
- **Barve, A.**, Stepp, M., Doll, M., Zhang J., Arteel, GE., Barve, S., Hein, D. *Hepatocarcinogenic Effects of 4,4 methylenedianaline (MDA) and Obesogenic Dietary Components.*
- Kucera, C., **Barve, A.**, Beverly, LJ. *Novel drug combination in leukemia reduces AKT activity and enhances cytotoxicity of Bcl-2 inhibitor.*
- Shah, P., Beverly, LJ., **Barve, A**. *Ubiquilin proteins are novel tumor suppressors in breast cancer*
- Shah, C., **Barve, A.**, Beverly, LJ. *Effects of Alterations on SAM/Met Pathway on Normal HSPCs.*
- **Barve, A.**, Shah, P., Ghare, S., Wunderlich, M., Mulloy, JC., Beverly LJ. *Targeting S-adenosylmethionine Dependent Trans-Methylation and Metabolism as a Novel Treatment Paradigm for AML*
- Shah, P., Beverly, LJ., **Barve, A.** *Novel Role Of UBQLN1/2 As A Potential Tumor Suppressor In Breast Cancer.*
- **Barve, A.**, Shah, P., Ghare, S., Wunderlich, M., Mulloy, JC., Beverly LJ. *Alterations to Methionine/S-adenosylmethionine metabolism regulate Dot1L expression and function; a novel vulnerability in MLL fusion leukemia.*

AWARDS AND HONORS

2018

University of Louisville Institute for Molecular Diversity and Drug Design (IMD3) Travel Award

2017

University of Louisville Institute for Molecular Diversity and Drug Design (IMD3) Travel Award

2014-2016

- IPIBS Integrated Programs in Biomedical Sciences (IPIBS) fellowship award
- NCI R25 Fellowship in Cancer Education Program

2007

Intel International Science and Engineering Fair- 3rd place Overall in Medicine and Health Sciences