USING A TRANSGENIC ZEBRAFISH MODEL TO IDENTIFY DOWNSTREAM THERAPEUTIC TARGETS IN HIGH-RISK, NUP98-HOXA9-INDUCED MYELOID DISEASE

by

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DEDICATION

To my friends and family, without you I could never have taken this journey –

Thank-you

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ABSTRACT

Acute myeloid leukemia (AML) is a genetic disease whereby sequential genetic aberrations alter essential white blood cell development leading to differentiation arrest and hyperproliferation. Pertinent animal models serve as essential intermediaries between *in vitro* molecular studies and the use of new agents in clinical trials. We previously generated a transgenic zebrafish model expressing human *NUP98-HOXA9* (*NHA9*), a fusion oncogene found in high-risk AML. This expression yields a pre-leukemic state in both embryos and adults. Using this model, we have identified the overexpression of *dnmt1* and the Wnt/β-catenin pathway as downstream contributors to the myeloproliferative phenotype. Targeted *dnmt1* morpholino knockdown and pharmacological inhibition with methyltransferase inhibitors rescues *NHA9* embryos. Similarly, inhibition of β-catenin with COX inhibitors partially restores normal hematopoiesis. Interestingly, concurrent treatment with a histone deacetylase inhibitor and either a methyltransferase inhibitor or a COX inhibitor, synergistically inhibits the effects of *NHA9* on embryonic hematopoiesis. Thus, we have identified potential pharmacological targets in *NHA9*-induced myeloid disease that may offer a highly efficient therapy with limited toxicity – addressing a major long-term goal of AML research.

LIST OF ABBREVIATIONS & SYMBOLS USED

Gene and protein symbol conventions, as represented by "*DNA (cytosine-5-)-methyltransferase 1"* gene

Species	Gene Symbol	Protein Symbol
Homo sapiens (human)	*DNMT1* *(UPPERCASE ITALICS)*	DNMT1 (UPPERCASE)
Mus musculus (mouse) *Rattus norvegicus* (rat)	*Dnmt1* (*Sentence case italics*)	Dnmt1 (Sentence case)
Danio rerio (zebrafish) *Xenopus* spp. (frog)	*dnmt1* (*lowercase italics*)	dnmt1 (lowercase)

Adapted from Wikipedia, http://en.wikipedia.org/wiki/Gene_nomenclature

Units

bp	base pairs
dpf	days post fertilization
g	gram
hpf	hours post fertilization
kb	kilo base pairs
kDa	kilo Dalton (atomic mass unit; 1 kDa = $1.660538782(83)x10^{-24}$ g)
L	litre
M	molarity (1 M = 1 mole per litre)
m_	milli_ (10^{-3})
µ_	micro_ (10^{-6})
n_	nano_ (10^{-9})
nt	nucleotide(s)
p_	pico_ (10^{-12})
%	percentage concentration of solution; solid in solvents (1%=1 g per 100 mL [w/v]), or liquid in solvents (1 mL per 100 mL [v/v])
rpm	rotations per minute
U	enzyme catalysis units (1 U = 1 µmole substrate consumed per minute)
v/v	volume by volume percentage solution
w/v	mass by volume percentage solution

Abbreviations and Symbols

AB	'wild-type' zebrafish strain, genotype designation
AGM	aorta-gonad-mesonephros
ALPM	anterior lateral plate mesoderm
AML	acute myeloid leukemia
AML1-ETO	*acute myeloid leukemia 1 – eight twenty-one* (fusion oncogene) Officially known as: RUNX1-MTG8 runt-related transcription factor 1— myeloid transforming gene on chromosome 8); or,

	RUNX1-RUNX1T1 (RUNX1— runt-related transcription factor 1; translocated to, 1)
APL	acute promyelocytic leukemia
As_2O_3	arsenic trioxide
ATRA	all-*trans*-retinoic acid
AZA	Azacitidine (5-azacytidine; 5-azaC)
BCR-ABL1	*breakpoint cluster region-v-abl Abelson murine leukemia viral oncogene homologue 1* (fusion oncogene)
CHT	caudal hematopoietic tissue
CML	chronic myeloid leukemia
CNS	central nervous system
COX	cyclooxygenase
Cre	*Cre* molecular recombinase (**C***ause* **re***combination*)
DAC	Decitabine (5-aza-2'-deoxycytidine; 5-azadC)
DIG	digoxogenin
DMSO	dimethyl sulphoxide
DNMT1	*DNA* (*cytosine-5-)methyltransferase 1*
EGFP	*enhanced green fluorescent protein*
EMP	erythro-myeloid progenitor
FACS	fluorescence-activated cell sorting
GMP	granulocyte/monocyte progenitor
gata1	*GATA-binding factor 1*
(mq)H_2O	(milli-Q) water
HDAC	histone deacetylase complex
HOXA9	*homeobox A9*
hs	heat-shock
HSC	hematopoietic stem cell
hsp70	*heat-shock protein 70*
ICM/ PLPM	intermediate cell mass / posterior lateral plate mesoderm
Indo	Indomethacin (1-[4-Chlorobenzoyl]-5-5-methoxy-2-methyl-3-indoleacetic acid)
IR	ionizing radiation
lcp1	*lymphocyte cytosolic protein 1*
lGl	*loxP-EGFP-loxP*
LSC	leukemia stem cell
loxP	*locus of X-over bacteriophage P1*
LPM	lateral plate mesoderm
lyz	*lysozyme*
MDS	myelodysplastic syndrome
MO	morpholino oligonucleotide
MPN	myeloproliferative neoplasm
NHA9	*NUP98-HOXA9*
NS-398	*N-2[-(Cyclohexyloxy)*-4-nitrophenyl]methanesulphonamide
NUP98	*nucleoporin 98 kDa*
PBI	posterior blood island
(qRT-)PCR	(quantitative reverse transcription) polymerase chain reaction

ProK	protein kinase
runx1	*runt-related transcription factor 1*
spi1	*spleen focus forming virus (SFFV) proviral integration oncogene*
TSA	trichostatin A; (7-[4-(dimethylamino)phenyl]-N-hydroxy-4,6-dimethyl-7-oxohepta-2,4-dienamide)
Tg	*transgene*
VPA	valproic acid; (2-propylpentanoic acid sodium; Sodium 2-propylpentanoate)
WISH	whole-mount RNA *in situ* hybridization
Wnt	wingless (Wg)-related mouse mammary tumour virus (MMTV) integration site (**Int**)
Zeb	Zebularine; 1-(β-D-Ribofuranosyl)-1,2-dihydropyrimidin-2-one, 2-Pyrimidone-1-β-D-riboside

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I have been told that it takes as much energy to wish for something as it does to plan for it; I suppose that's true. I wish I could say that everything has gone according to plan, but such is life, and such is the scientific world of research.

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Here's to the future and all it may bring!

CHAPTER 1: INTRODUCTION

1.1 Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is characterized as a heterogeneous disease, both biologically and clinically where a series of distinct genetic mutations prevents the ability of hematopoietic progenitor cells to differentiate/mature appropriately or respond to normal regulators of proliferation (Estey & Döhner, 2006; Horton & Huntly, 2012). This loss typically leads to fatal infection, bleeding and organ infiltration in the absence of treatment as the immature cells accumulate in the bone marrow and peripheral blood. AML is very aggressive in nature as rapid progression and lack of treatment results in patient mortality within weeks or months post diagnosis (Borthakur & Estey, 2010). As such, current therapy regimens are aggressive, yet consists of non-targeted and nonspecific cytotoxic drugs (Burnett *et al.*, 2011; Oki & Issa, 2010; Redaelli *et al.*, 2004). Consequently, AML treatment often leads to substantial morbidity and induction-related death (Borthakur & Estey, 2010), thus AML maintains less than a 60 percent overall survival (Gilliland & Tallman, 2002). It is therefore essential for AML research to focus on determining underlying molecular and genetic pathways in order to develop therapies that target specific abnormalities in leukemic cells and use this to establish more personalized and effective therapies for individual AML patients.

1.1.2 Environmental Risk Factors and Incidence Overview

The median age of AML is approximately 66 years old, however, incident rates vary greatly by age. AML affects 3.8-5.1 per 100,000 individuals in North American and accounts for nearly 30 percent of all adult leukemia cases (Howlader et al., 2012) (**Figure 1.1**). Despite being the most common adult leukemia, the overall 5-year survival rate of

adults with AML between 1996-2010 remained around 24 percent, the worst of all leukemias (Howlader et al., 2012). AML also affects a significant proportion of adolescents and young adults , ranging from 15 percent of new leukemia case for patients 14 years and younger to 25 percent for patients between 15-29 years of age (Leukemia and Lymphoma Society, 2013). However, this adolescents and young adult population has a notably higher, yet still poor 5-year survival of around 65 percent (Leukemia and Lymphoma Society, 2013). Though there is no clear reason as to why this may be, it likely has to do with the ability of the patients' bone marrow and other affected organs to recover from the aggressive therapy.

Of the worldwide population, there is a notable discordance between races with more whites than any other group being diagnosed with AML (Byrne *et al*., 2011; Matasar, *et al*., 2006). Interestingly, patients of African-American descent are younger at diagnosis than any other group with a median age of 57, while white patients have the highest median age and a significantly lower 5-year survival (Byrne *et al*., 2011). Additionally, there are 48 percent more males diagnosed annually worldwide than women (Dores, *et al*., 2012). Survival also differs by sex with females having a slightly higher overall 5-year survival (Dores *et al*., 2012). This gender difference is more significant at younger ages, however, there is a higher incidence rate of AML for women over 75 than men and their 5-year survival remains quite poor.

Environmental risk factors for developing AML include exposure to ionizing radiation (IR), benzene and cytotoxic chemotherapy (Estey & Döhner, 2006). There are many ways to be exposed to IR. Examples of IR exposure include medical procedures such as X-rays used for diagnosis or radiation therapy. More commonly, cosmic radiation

from the sun constitutes the largest contribution of IR exposure to the general population (Rubnitz *et al.*, 2008). Interestingly there is a less appreciated increase in IR exposure from extensive commercial flying (greater than 5,000 hours) (Gundestrup *et al*., 2000). Examples of benzene exposure include most notably cigarette smoking, as well as products made with benzene such as glues, paints, furniture wax, and detergents. Finally, exposure to alkylating cytotoxic chemotherapy drugs, such as cyclophosphamide, is usually seen from previous cancer treatment (Estey & Döhner, 2006).

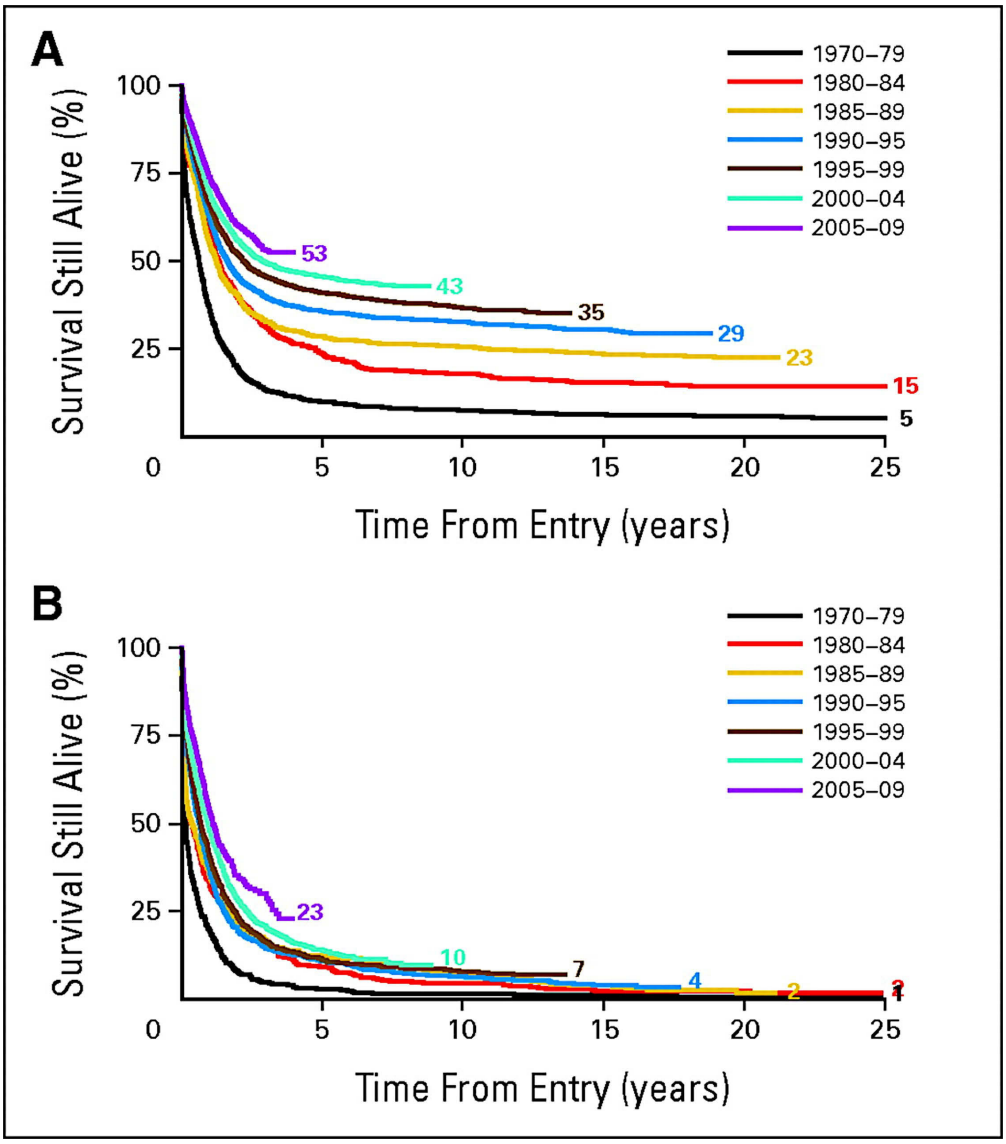


Figure 1.1.1 Representation of the overall AML survive with time (A) Change in survival of patients from the age 0-59. (B) Change in survival of patients 60 years and older. Adapted with permission from Burnett, *et al*., 2011 (*Appendix A*)

1.1.3 Subtypes and Clinical Diagnosis

Conventional classification of AML has been based on morphological characteristics and extent of cell maturation based on the French-American-British (FAB) classification system (Bennett *et al*., 1985). In this system AML is diagnosed when there is a blast-cell count of 30 percent or higher in the bone marrow (Bennett *et al*., 1985). Additionally, the FAB system classifies AML into eight different subgroups based on morphologic appearance and each associated with different prognoses. A ninth group is included for those cases not morphologically assigned to any of the other groups (**Table 1.1.1**).

Since the establishment of the FAB system, the World Health Organization (WHO) has established its own multidisciplinary approach to classification, in which morphologic and cytochemical information is incorporated with cytogenetics, molecular genetics, immunophenotypic, and clinical assessment (Vardiman, 2010; Vardiman *et al*., 2009) to define four major categories of AML. To note, the WHO classification reduces the blast count from 30 percent that is seen in FAB classification to 20 percent. Therefore many high-grade myelodysplastic syndromes from the FAB system are diagnosed as AML in the WHO system. Other criteria to be met for AML diagnosis include that the blasts must be shown to be myeloid in origin and at least 20 percent of the blasts must have surface antigens associated with myeloid differentiation, most commonly CD33 or CD13 (Vardiman et al., 2009).

The four WHO classifications include: AML with recurrent cytogenetic translocations; AML with multilineage dysplasia; AML with myelodysplastic syndrome, therapy related; and AML not otherwise categorized (Vardiman, 2010). Examples of

frequent genomic abnormalities in group one include balanced chromosomal translocations such as t(8;21) (q22;q22) found predominantly in FAB M2 subtype AML and t(15;17) (q22;q12) translocation that is pathognomonic of acute promyelocytic leukemia (Palanisamy, 2010). The second group defined by the presence of multilineage dysplasia (MDS) normally affects adults and is often characterized by loss or gain of major segments of chromosomes such as -5/-5q, -7/del(7q), +8, +9, +11, del(11q) and +21 (Verhaak & Valk, 2010). The third group includes chromosomal aberrations associated with exposure to mutagens and/or chemo-, radiotherapy (Vardiman et al., 2009). Most commonly found in this group are those individuals who have undergone previous cancer treatment with alkylating agents, usually for solid tumors and are 1-6 years post therapy (Borthakur & Estey, 2010). The fourth and final group is classified simply as those cases that do not meet any of the above criteria, thus it acts as the ninth subcategory from the FAB classification system.

Table 1.1.1 French-American-British system of classification of acute myeloid leukemia

FAB Subgroup	Type of Leukemia	Prognosis	Frequency in adult AML (%)	Morphology
M0	Acute myeloblastic leukemia without maturation	Poor	< 5	Immature myeloblasts, lacks definite myeloid differentiation by conventional morphologic or cytochemical analysis; myeloid differentiation evidenced by demonstration of peroxidase-positive granules and/or immunoreactivity
M1	Acute myeloblastic leukemia with minimal maturation	Intermediate	20	Immature myeloblasts predominate; <10% promyelocytes, myelocytes, or monocytes
M2	Acute myeloblastic leukemia with maturation	Intermediate	30	Immature myeloblasts predominate, but more maturation than in M1; <20% monocytic cells; most cells peroxisdase positive
M3 and M3v	Acute promyelocytic leukemia	Good	10	Promyelocytes predominate; marked granulation in more than 30% cells; granules not visible by light microscopy in M3v
M4 and M4EO	Acute myelomonocytic leukemia	Intermediate	25	Mixture of abnormal monocytic cells (>20%) and myeloblasts/ promyelocytes (>20%); 30% eosinophilic cells in M4EO
M5 a and b	Acute monocytic leukemia	Intermediate	10	Monocytic cells predominate (>80%); in M5a, >80% non-erythroid cells are immature monoblasts; in M5b, >20% are more mature

Table 1.1.2. Continued

FAB Subgroup	Type of Leukemia	Prognosis	Frequency in adult AML (%)	Morphology
M6	Acute erythroleukemia	Poor	<5	Myeloblasts and erythroblasts (>50%) predominate; abnormal multinucleated erythroblasts
M7	Acute megakaryoblastic leukemia	Poor	<5	Megakaryocytic cells as shown by platelet peroxidase activity on electron microscopy or by tests with platelet-specific antibodies
All others	Undifferentiated acute leukemia, mixed-lineage leukemia, hypocellular AML	Unknown	-	-

(Adapted from Abdul-hamid, 1999; Bennett *et al*., 1985; Valk *et al*., 2004)

1.1.4 Common genomic mutations and chromosomal translocations

AML is a very heterogeneous disease, both clinically and biologically, however, there are a number of recurrent chromosome abnormalities that are important in defining subgroups or categories of AML regarding prognosis and treatment. Interestingly, only one-percent of AML patients carry leukemic blast cells that are derived from different leukemic clones with completely different cytogenetic aberrations, suggesting that the disease phenotype in AML is brought on by the clonal expansion of a single defective cell (Palanisamy, 2010). Therefore these recurrent chromosomal aberrations can serve as markers for diagnosis and patient management. For example, the inversion of chromosome 16 [inv(16)] and the chromosomal translocation t(8;21) are characterized as having a favourable outcome while the chromosomal translocation t(7;11) carries a poor prognosis (Rubnitz *et al*., 2008). A summary of the chromosomal aberrations is listed in **Table 1.1.2**. However, although these chromosomal changes fit nicely within both the FAB and WHO systems of classification and do aid in determining prognosis of AML patients, there still remains a great deal of variation in overall treatment response and 5-year survival within the subgroups (Borthakur & Estey, 2010). This variation suggests that there is a need for additional molecular abnormality detection within the groups to further refine the AML prognosis in patients.

Our understanding of the pathophysiology of AML has improved greatly from relatively recent molecular and genetic work. A number of novel molecular markers have been identified and associated with AML prognosis (**Table 1.1.3**), which have been crucial in determining the prognosis for AML patients with normal karyotypes (Burnett *et al*., 2011). For instance, P53 mutations have been associated with poor prognosis as AML

patients harbouring these mutations have poor therapeutic responses (Trecca *et a*l., 1994). Conversely, a double mutation in CCAAT/enhancer-binding protein alpha's (CEBPA) N- and C- terminus is indicative of a highly favourable outcome (Pabst, *et al*., 2009). However, many of these identified molecular markers require further validation. For instance, high expression of Wilms tumor 1 (WT1), a well-known tumor suppressor, has been associated with poor prognosis in some studies (Miyoshi et al., 2002), yet has had mixed results in other studies (Kramarzova *et al*., 2012).

As our knowledge of these genetic markers expands, we will soon be able to use gene expression profiling effectively where gene expression levels will be measured by microarray techniques. This information will be combined with the traditional molecular abnormalities to create a comprehensive molecular classification of AML, which will hopefully support further individualization of treatment (Verhaak & Valk, 2010).

Table 1.1.2 Summary of chromosomal aberrations frequency associated with AML

	Genes	Morphological Association	Frequency in adult AML	Prognosis
Translocations/inversions				
inv(16)(p13;q22) or t(16;16)(p13;q22)	*CBFB;MYH11*	M4 and M4EO	25%	Good
t(8;21)(q22;q22)	*RUNX1;RUNX1*	M2	15%	Good
t(9;22)(q34;q11)	*BCR;ABL1*	M1 and M2	15%	
t(15;17)(q22;q21)	*PML;RARA*	M3/M3v	10%	Good
t(9;11)(p22;q23)	*MLL;AF9*	M5	5%	Intermediate
t(6;11)(p27;q23)	*MLL;AF6*	M4 and M5	5%	
inv(3)(q21;q26) or t(3;3)(q21;q26)	*EVI1;RPN1*	M1, M4, and M6	2%	Intermediate
t(7;11)(q22;q22)	*NUP98;HOXA9*	M2 and M4	2%	Poor
Chromosomal Imbalances				
+8	-	M2, M4 and M5	12%	Intermediate
-7/7q-	-	No FAB preference	7%	Intermediate/ Poor
-5/5q-	-	No FAB preference	5%	Poor
-17/17p-	*TP53*	No FAB preference	3%	Intermediate
+22	-	M4, M4EO	6%	Intermediate
+21	-	No FAB preference	8%	Intermediate
+11	-	M0, M1	<1%	Poor
Complex Karyotype			6%	Poor
Normal Karyotype			44%	Intermediate

Adapted from Alseraye et al., 2011; Palanisamy, 2010; Schaich *et al*., 2007; Soenen *et al*., 1998; Verhaak & Valk, 2010; Wan *et al.*, 1999; Woo *et al*., 2009; Xu *et al.*, 2008; Golub, 1999

Table 1.1.3 Molecular abnormalities associates with AML and their prospective frequency and associated prognosis

Gene	Abnormality	Prognosis	Frequency in adult AML (%)
FLT3	Internal tandem duplication	Poor	25
FLT3	Tyrosine kinase domain mutation	Variable; Poor → Favourable	10
MLL	Partial tandem duplications	Poor	8
CEBPA	Mutation	Good	5-10
TP53	Mutation	Poor	4
c-KIT	Mutation	Poor	4
N-*RAS*	Mutation	No significance; Intermediate	10
K-*RAS*	Mutation	No significance	4
MDR1	Overexpression	Poor	30
WT1	Overexpression	Variable; Poor	50
HOXA9	Overexpression	Poor	80
BAALC	Overexpression	Poor	50
ERG	Overexpression	Poor	25
EVI1	Overexpression	Poor	7

Adapted from Ahmad *et al.*, 2011; Bacher *et al.*, 2008; Bacher *et al.*, 2006; Golub, 1999; Kramarzova *et al.*, 2012; Miyoshi *et al*., 2002; Pabst *et al.*, 2009; Trecca *et al*., 1994; Verhaak & Valk, 2010; Yeh *et al.*, 2009

1.1.5 Myeloproliferative neoplasm and myelodysplastic syndromes

Myeloproliferative neoplasms (MPN) and MDS could be looked at as opposite sides of the same AML coin and as such, both diseases are classified as pre-leukemic disorders (Orazi & Germing, 2008). More specifically, frank AML represents the clinical endpoint of aggressive myeloid disease, characterized by both a block in differentiation as well as the ongoing ability of the leukemic blast cells to survive and proliferate (Horton & Huntly, 2012). MDS is a group of heterogeneous disorders with impaired peripheral blood cell production usually of a certain lineage (cytopenia) with dysplastic-appearing bone marrow (Kantarjian *et al*., 2006). By contrast, MPN is defined as the hyperproliferation of hematopoietic stem/progenitor cells leading to increased numbers of any combination of the blood cell lineages (Tonkin *et al*., 2012). Combined, these two myeloid diseases each represent one aspect of AML and is therefore likely that a second mutation (or number of mutations) in either MDS or MPN is necessary to give the pre-leukemic blast cells the ability to either survive and proliferate or the inability to differentiate, respectively. This second mutation would help progress the original myeloid clone to overt AML. A classic example would be of chronic myeloid leukemia a form of MPN in which the patient develops an additional mutation that inhibits the preleukemic cells from differentiating thus sending the patient into blast crisis and progressing to overt AML (Lavallade, 2013).

As pre-leukemic diseases, MPN and MDS have varying prognoses and latency periods as they progress to frank AML. Compared to MPN, MDS is a more aggressive myeloid disease with a significantly lower overall lifetime survival (Garcia-Manero, 2010; Kosmider *et al*., 2009; Thepot *et al*., 2010; Young, 2005). 20-30 percent of MDS

cases will result in refractory AML within a few months to a couple years after diagnosis and the median survival varies from mere months to a few years (Garcia-Manero, 2010; Kosmider *et al*., 2009; Thepot *et al*., 2010; Young, 2005). MPN however, is much less aggressive in its progression to AML. For instance, MPNs demonstrate a reduced rate of progression of leukemogenesis than MDS with longer latency periods of 30-76 months depending on the diagnosed subtype (Foucar, 2009; Heaney & Soriano, 2013). The discrepancy may be due to the nature of the disease, where MPN produces an overabundance of myeloid cells that are usually functionally competent (Miesner *et al*., 2010). Alternatively, MDS produces blast cells with both impaired morphology and maturation, which ultimately leads to a number of peripheral cytopenias in all the major blood groups (Miesner *et al*., 2010; Nimer, 2008), thus limiting the majority of hematopoietic processes.

Therapy options for MDS and MPNs present many challenges when considering an appropriate treatment strategy, including advanced age, comorbidities and an inability to tolerate certain types of intensive therapy (Kantarjian *et al*., 2006; Saba, 2007). For instance, treatment options for MDS may include supportive care, which involves red blood cell or platelet transfusions and to help restore normal peripheral blood counts and broad spectrum antibiotics to treat infections arising from leucopenia and neutropenia, specifically. However, more high-risk MDS patients may have to undergo intensive antileukemic chemotherapy, which have a number of negative toxicity effects on patients (*see section 1.1.6*) Therefore, a more disease-directed treatment model has been the long-term focus for MDS therapy.

Recently, the US Food and Drug Agency (FDA) has approved the use DNA demethylating agents, such as 5-azacitidine (AZA) and 5-aza-2-deoxycytidine (Decitabine; DAC) in the treatment of MDS (Kantarjian *et al*., 2006; Kuendgen *et al*., 2004; Saba, 2007). These demethylating agents have been designed to help restore expression levels of tumour-suppressor genes as well as genes involved in hematopoietic cell differentiation. In example, phase 2 studies of DAC in MDS patients yielded encouraging response rates, including overall responses (OR; complete response [CR] + partial response [PR]) of 26–45 percent and CR of 21–28 percent (Kihslinger & Godley, 2007; Saba, 2007; Wijerman *et al*., 2000; Wijermans *et al.*, 1997). These results led to a North American, multicenter phase 3 study of DAC compared with supportive care in 170 MDS patients, which formed the basis for the FDA approval of decitabine (Saba *et al* 2004; Kantarjian *et al*., 2006, 2007). The results of the phase 3 studies indicated that DAC was clinically effective in patients when given DAC in addition to supportive care than supportive care alone (17 percent OR versus 0 percent, respectively) (Kantarjian *et al*., 2006, 2007; Saba, 2007). As more is learned about the mechanism of hypomethylating agents, such as AZA and DAC, new roles as combination therapies will emerge for MDS as well as other hematologic malignancies such as MPN and AML.

In contrast to MDS, MPN treatment is focused on reducing the cellular burden of the increased proliferation of the specific cell lineage. Treatment may involve taking aspirin daily to avoid the risk of hyperviscosity or in the case of polycythemia vera (increased erythroid cells) treatment may be phlebotomy, where blood is drawn from a patient in order to reduce total cell number as well as reduce iron in the peripheral blood that can be used to produce new erythrocytes (Tonkin et al., 2012). Alternatively,

chemotherapeutics, such as hydroxycarbamide, can be used as cytoreductive agents that will lower the overall cellular burden in the peripheral blood (Tonkin et al., 2012). Overall, the current therapy options for MDS and MPN have minimal toxicity, however, this is not the case after the progression to overt AML.

1.1.6 Current therapy

As demonstrated earlier, AML is a very heterogenetic disease both clinically and biologically with differing outcomes based on a number of factors including leukemic karyotype, molecular abnormalities, response to therapy, age and overall health prior to an AML diagnosis. Although there has been a number of advancements in determining the biology of the disease as well as identification of prognostic molecular markers, the diversity of the disease presents a number of therapy challenges and ultimately the course of AML treatment has remained unchanged throughout the decades (Borthakur & Estey, 2010; Lehnertz et al., 2003; Rao et al., 2009). Further difficulty arises as many patients are considered to have minimal residual disease (MRD).

Conventional wisdom dictates that most, if not all, patients who obtain complete remission through treatment will still harbor a small amount of myeloid-disease sustaining cells (Lion, 1999; Negrin, 1998). These patients are considered to have a (MRD) as these cells are typically the cause of AML relapse (Estrov, 2010; Lion, 1999; Negrin, 1998). MRD combined with the diverse nature of AML has created a number of roadblocks in therapy advances through the years. **Figure 1.1.1** illustrates overall survival of treated AML patients 0-59 years of age (**Figure 1.1.1A**) and greater than 60 years (**Figure 1.1.1B**) since 1970. The figure divides time points into seven periods to demonstrate the increase in survival in the last 40 years (Burnett, *et al*., 2011).

It has only been the past few years that there has been any significant increase in the 5-year survival rate of elderly AML patients where 5-year survival prior to 2005 remained less than 15 percent and is now approximately 25 percent (Borthakur & Estey, 2010; Estey & Döhner, 2006; Rao *et al*., 2009). Young patients with AML however, have seen a steady increase in 5-year and overall survival rates since 1970 increasing from 10 percent to approximately 50 percent (Rao *et al*., 2009). This improvement however, may be due to advances in supportive care measures to better deal with complications arising from AML therapy rather than reflecting and direct treatment advances (Nottage *et al*., 2011).

Treatment for young AML patients has been standardized to include an induction round followed by post-remission treatment (Borthakur & Estey, 2010). The induction round involves seven days of a standard dose of cytarabine followed by 3 days of daunorubicin (Borthakur & Estey, 2010). Following this initial round of chemotherapy treatment, the patient's bone marrow and peripheral blood is checked to confirm complete remission (Borthakur & Estey, 2010). Post-induction remission rates range between 65-75 percent in patients between 18-60 years of age (Löwenberg *et al*., 2003). After four decades of this treatment combination there still has not been strong therapeutic candidates to replace these standard drugs, however, a more intensive regimen using these agents has been shown to increase remission rates (Nottage *et al*., 2011).

Complete remission described as the virtual absence of morphologic evidence of disease with fewer than 5% blasts in the marrow and a recovery of marrow function (Löwenberg *et al*., 2003). Some patients may respond well after the first induction, while

others may have incomplete remission with a blast count higher than 5% and will go through a second induction round. For those patients who have favourable or intermediate cytogenetic risk-factors (i.e., inversion 16) and achieve complete remission, they will then go through a few cycles of post-remission therapy (Borthakur & Estey, 2010). These cycles have been established in order to prolong remission and reduce the incidence of relapse. Currently, this comprises of further cycles of cytarabine with similar intensity to induction, however, the necessary number of post-remission courses remains unclear and is still debated (Burnett *et al*., 2010; Mayer *et al*., 1994). In contrast, patients with poor prognostic factors that reach CR after 1-2 rounds of induction often undergo an allogeneic hematopoietic stem cell transplant (HSCT; A. K. Burnett, 2012). By doing so, the chance of relapse is greatly reduced than that of chemotherapy alone (Nottage et al., 2011). Of course, HSCT requires finding a donor match, which presents additionally difficulties. For instance, many patients are not fortunate enough to have a family donor match and rely on the bone marrow donations from unrelated individuals (Burnett *et al*., 2011), which significantly increases the rate of graft-versus-host disease (GVHD) where the newly transplanted donor cells begin to attack health recipient tissues (Ferrar *et al.*, 2009). Additionally, due to the intensive combined radiation and chemotherapy treatments prior to and the chemical immunosuppression post HSCT results in a high treatment-related mortality, especially in the more elderly patients (Borthakur & Estey, 2010; Ferrar *et al*., 2009; Oki & Issa, 2010).

Regardless of the therapy option, there are a number of physical, psychological, emotional and sexual, short and long-term side effects of treatment, many of which increase in severity with increased age of the patient (Redaelli, *et al*., 2004). For example,

short-term, physical side effects may include some of the traditional cytotoxic events including nausea, vomiting and fatigue while long term side effects may include heart and liver damage (Redaelli *et al*., 2004). Unfortunately, even with the many side effects of current treatment there is still a surprising paucity of novel therapeutics that have been approved for treatment in AML. However, there are a few specific examples where identifying molecular mechanisms has yielded effective therapy alternatives.

One striking example is of the efficacy of targeted therapy for patients carrying the t(15;17)(q22;q21) translocation (**Table 1.1.2**), which yields the PML-RARA fusion oncogene. This translocation is found in approximately 10 percent of all AML cases and in more than 90 percent of cases classified as acute promyelocytic leukemia (APL) (Grignani *et al*., 1994; Löwenberg *et al*., 2003; Yoshida *et al*., 1996). In *PML-RARA*-induced APL, combination therapy with the targeted agents all-trans retinoic acid (RA) and arsenic trioxide (As_2O_3) yields an overwhelmingly positive response with predicted 5-year survival of approximately 70 percent and relapse rates as low as 30 percent (Grignani *et al*., 1994; Iland *et al*., 2012; Tallman *et al*., 1997). Outside of this treatment breakthrough most of the advancements in therapy have dealt with the refinement in the drug use protocol including determining therapy doses, therapy durations and combined therapy regimens (Burnett et al., 2011; Redaelli et al., 2004). Although these refinements have lead to increased overall survival, the protocols have not been able to reduce the therapeutic toxicity dramatically (Burnett et al., 2011; Redaelli et al., 2004). It is therefore crucial to find new anti-leukemia agents, particularly ones that avoid the overlapping toxicities with current chemotherapies, by identifying and targeting molecular abnormalities.

1.2 THE FAMILY OF HOMEOBOX GENES

Homeobox (*HOX*) genes encode highly conserved homeodomain transcription factors from *Drosophila* to mammals that bind to targeted regions of DNA (Argiropoulos & Humphries, 2007; Pillay *et al*., 2010) (**Figure 1.2.1**). There are 39 *HOX* genes found in each of the human and murine genomes, which are found on four different chromosomes (7, 17, 12, 2) and arranged in four clusters (A–D), respectively (Larhammar *et al*., 2002). The numbering of individual *HOX* genes follows homology between clusters to create 13 paralogs, with greatest similarity between HOX proteins of different groups with the same number (i.e., HOXA1 and HOXB1; Eklund, 2011). No cluster of *HOX* genes contains all 13 paralogs.

HOX genes are expressed in a temporal and spatial mode, which is known as the 'HOX code' (He *et al*., 2011). This HOX code is critical for the proper positioning of segmented structures along those axes, which include the vertebrate, limbs as well as the digestive and reproductive tracts (Argiropoulos & Humphries, 2007). As such, during mammalian embryogenesis, *HOX* paralogs 1–4 are most highly expressed in the head, *HOX* paralogs 5–7 in the thorax and *HOX* paralogs 8–11 in the abdomen and pelvis (Larhammar *et al*., 2002). This tight spatial and temporal regulation is thought to result in regulation of organ specific genes by the clusters of HOX proteins, however these genes have yet to be identified or associated to a high degree (He *et al*., 2011).

Most of what is known about the functions of *Hox* genes come from murine models, however identifying the precise functions of each gene has proven difficult in the past (Palmqvist *et al.*, 2007). Most paralogous genes (i.e., *HOXA*1, *HOXB*1 and *HOXD*1) have identical or similar domains of expression, suggesting that there is a great deal of

redundancy (Eklund, 2011; Eklund, 2006). As such, mutation or knockout of a single *Hox* gene yields no dramatic developmental phenotypes yet requires combined knockout of complete *Hox* gene clusters (He *et al*., 2011). For example, *Hoxa*3, *Hoxb*3, and *Hoxd*3 generally have very similar patterning and gene targeting has shown that members of this cluster compensate for each other when one of the paralogs is disrupted (Greer *et al*., 2000), yet when all three are simultaneously knocked out, this results in embryonic death due to improper anterior bone and cartilage development (Greer *et al*., 2000).

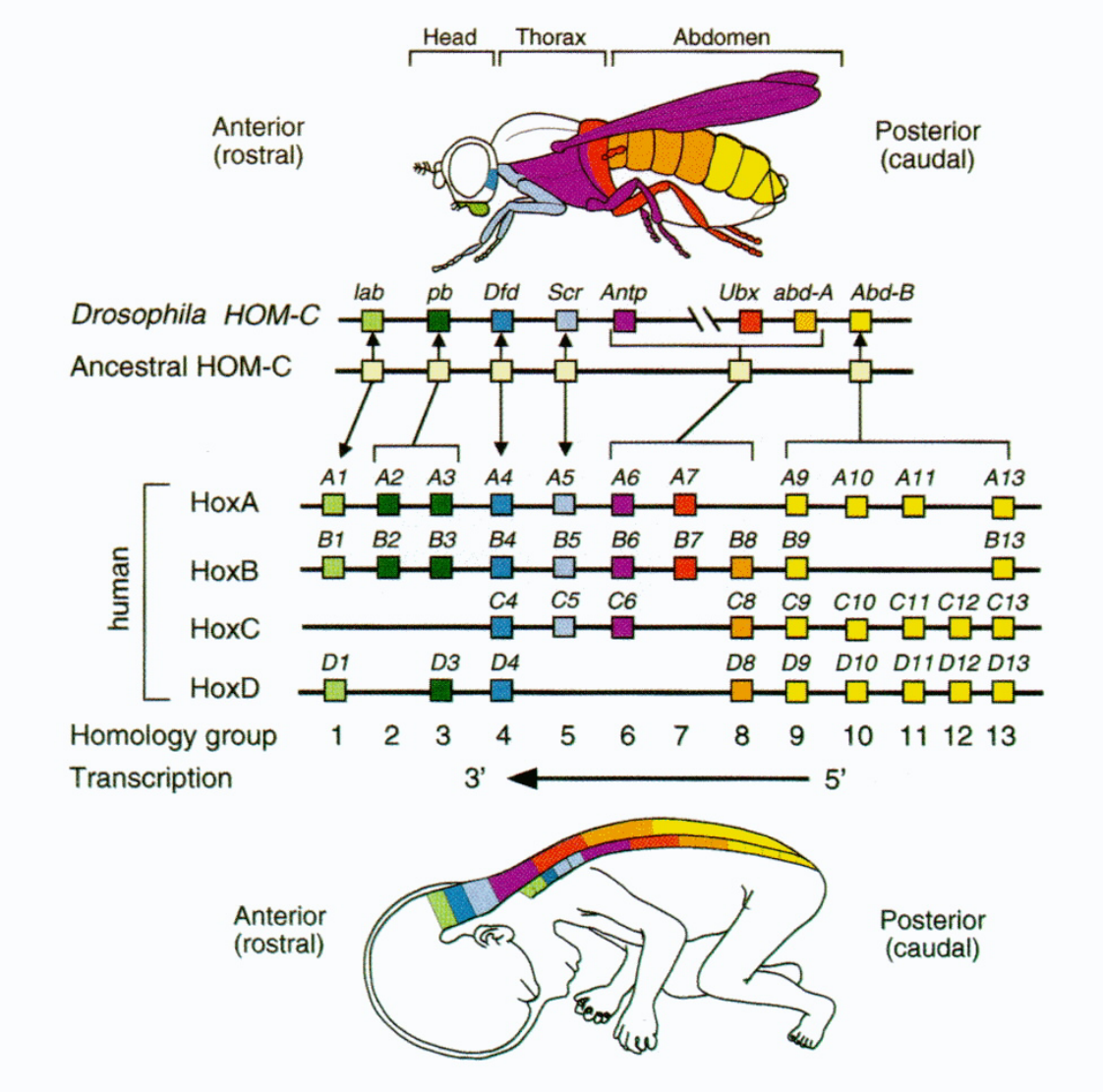


Figure 1.2.1. **Schematic representation demonstrate the genetic conservation of the homeodomain genes from *Drosophila* to mammals.** Hox genes are arranged on four chromosomes (7, 17, 12, 2) and are labeled A-D based on the chromosome there are found on respectively. Multiple hox genes are found on each chromosome and are labeled 1-13 based on sequential order of location on the chromosome. Adapted with permission from Mark, *et al.,* 1997 *Appendix B*.

1.2.1 HOX genes during normal hematopoiesis

Mammalian *HOX* genes, especially *HOXB7*, *HOXA*9, *HOXA*10 and *HOXD*9 are essential for normal hematopoiesis (Golub, 1999; Kroon *et al*., 1998; Larhammar *et al*., 2002) and as such their expression is predominantly confined to immature hematopoietic cells (Larhammar *et al*., 2002; Palmqvist *et al*., 2007). Hematopoiesis is defined as the creation of all mature blood cell types, arising from stem and early progenitor cells, passing through defined intermediate stages to functional blood cells (Simon, 1998). Similar to that of overall embryogenesis, the associated *HOX* genes during hematopoiesis are expressed in a tightly regulated and temporal manner (Cvejic *et al*., 2011). In early hematopoiesis, *HOX* paralogs 1–4 show a maximal expression level in the hematopoietic stem cells (HSC; Argiropoulos & Humphries, 2007). This expression level is then down-regulated as the HSCs begin to differentiate (Argiropoulos & Humphries, 2007). Later in hematopoiesis, maximal expression of *HOX* paralogs 7–11 is found within the immature, lineage-committed progenitors which again decreases in expression as differentiation continues (Palmqvist *et al*., 2007). Once hematopoietic cells reach functional maturity *HOX* genes are reduced to low or absent levels of expression (Magli *et al*., 1997).

The DNA-binding specificity of the blood-related HOX proteins is achieved through their interaction with other DNA-binding co-factors. For example, the Three Amino acid Loop Extension (TALE)-class homeodomain transcription factors, PBX and MEIS1, form heterodimeric and heterotrimeric proteins, then complex with HOXB7, HOXA9, HOXA10 and HOXD9 to regulate their DNA-binding affinity and specificity (Chang *et al*., 1995, 1997; Mann & Affolter, 1998). Together, these trimeric HOX complexes are capable of transcriptionally activating or repressing genes involved in the

hematopoietic pathway thereby influencing blood cell proliferation and differentiation (Bei *et al*., 2011; Bei *et al*., 2005; Mann *et al*., 2009).

1.2.2 HOX genes and leukemogenesis

Murine models have given the most insight into the *Hox* related leukemogenesis, yet expression overlap has made this difficult. For instance, mice deficient in *HoxA*9, *HoxA*10 or *HoxB*6 each have some skeletal abnormalities consistent with the patterns of expression of the specific gene during embryogenesis, yet do not produce prominent or severe hematologic abnormalities (Kappen, 2000; Lawrence *et al*., 1997; Satokata *et al*., 1995), making elusive the identification of the specific gene function. However, more recent overexpression studies have been able to define certain roles of *HOX* genes in abnormal hematopoiesis. For instance, retroviral-mediated overexpression of *HoxA9, HoxA10, HoxB4* or *HoxB8* in murine marrow cells leads to HSC expansion, conditional immortalization of myeloid progenitor cells and repression of differentiation - all hallmarks of acute myeloid disease (Helgason *et al*., 1996; Perkins & Cory, 1993; Sauvageau *et al*., 1994; Thorsteinsdottir *et al*., 1997; Thorsteinsdottir *et al*., 2002).

Both clinical assessment and murine model analysis of AML has helped to determine *HOX* gene expression during leukemogenesis. For instance, increased expression of *HOXB*3, *B*4, *A*7–11 is commonly identified in the most primitive blasts (Sauvageau *et al*., 1994; Unnur Thorsteinsdottir *et al*., 2002) with sustained aberrant overexpression of *HOXA*7–11 typically found in more mature blasts that have undergone some differentiation (Eklund, 2006). These aberrant expression patterns of *HOX* genes have strong prognostic associations, however, there is still limited understanding of the mechanisms involved in the HOX regulatory network (Cantile *et al.*, 2007; Frohling *et*

al., 2007; Plowright *et al.*, 2009). Regardless, *HOX* genes may be a poor option for targeted therapy as many aspects of their function are critical in regulating normal hematopoietic processes and treatment would undoubtedly lead to unacceptable global toxicities (Cantile *et al*., 2007). Fortunately, recent studies have identified a number of gene targets downstream of *HOX* clusters associated with leukemogenesis (He *et al*., 2011; Palmqvist *et al*., 2007). These findings are being used to help explain the crucial roles of the *HOX* gene family in both normal hematopoiesis as well as leukemogenesis, which may potentially lead to new molecular targets (He *et al*., 2011; Palmqvist *et al*., 2007). For instance, *HOXA*10 has been shown to activate *caudal type homeobox transcription factor 4* (*CDX4*) (Bei *et al*., 2011; Frohling *et al*., 2007). In turn, *CDX4* controls *HOX* expression and ectopic expression of murine *Cdx4* dysregulates a host of HOX factors involved in adult murine hematopoiesis creating a positive feedback loop (Bei *et al*., 2011; Frohling *et al*., 2007). These observations suggest that further identification of downstream genes affected by aberrant *Hox* expression will provide useful insights into hematopoiesis and myeloid leukemogenesis and are potentially more attractive therapeutic targets than the *HOX* genes themselves.

1.2.3 HOXA9 and MEIS1

HOXA9 is expressed in the posterior of the developing embryo and is believed to be necessary to enhance HSC activity, suppress myeloid differentiation and maintain survival of hematopoietic progenitors (Calvo *et al.*, 2001; Faber *et al*., 2009; Ohno *et al*., 2013; Thorsteinsdottir *et al*., 2002). In 1999, Golub *et al*. demonstrated that aberrant overexpression of *HOXA9* was found in approximately 80 percent of human AML cases as the single most highly correlating factor (of 6817 genes tested) with poor prognosis

(Golub, 1999). Overexpression of *HoxA9* in murine HSCs leads to the development of AML in the mouse after a latency period of 3-10 month post transplantation (Thorsteinsdottir *et al*., 2002). This latency period, although shorter than other *HOX* overexpression models (i.e., *HOXA7, HOXA10, HOXB3*, and *HOXB8*), suggests the requirement of an additional genetic or epigenetic alteration to occur for the leukemic progression (Ghannam et al., 2004; Nokamura et al., 1996; Yoshida et al., 1996).

In human AML, the *HOXA9* co-factor, *myeloid ecotropic integration site 1* (*MEIS1*) is commonly overexpressed (Hu *et al.*, 2009; Kroon *et al*., 1998; Thorsteinsdottir *et al.*, 2001) and co-overexpression in reconstituted mouse bone marrow leads to the direct establishment of AML after a brief latency phase of three months post transplantation (Iwasaki *et al*., 2005; Kroon *et al.*, 2001; Lawrence *et al.*, 1997; Y. Wang *et al.*, 2010). Therefore, co-overexpression of these two genes increases AML progression significantly over *HoxA9* overexpression alone and is now used as a powerful prognostic marker to differentiate between low-risk and high-risk AML cases clinically. For instance, patients with the prognostically favourable *AML1-ETO* and *PML-RARA* mutations typically maintain lower expression levels of *MEIS1* while patients harbouring higher-risk rearrangements of the mixed lineage leukemia gene (*MLL*) (i.e., *MLL-AF6, MLL-AF9; see table 1.1.2*) commonly have high expression of both *HOXA9* and *MEIS1* (Eklund, 2007; Faber *et al*., 2009; Lasa *et al*., 2004; Wang *et al*., 2011).

The frequency of *HOXA9* and *MEIS1* upregulation in human AML by a variety of fusion oncogenes (such as the *MLL*-rearrangements), and their association with high-risk, poor prognosis leukemia highlights a pressing need for research into the mechanisms of pathogenesis by *HOXA9*. Hopefully, such research may be able to identify conserved

druggable targets that could be used to treat the large number of AML cases with increased *HOXA9* expression.

1.2.4 The t(7;11)(p15;p15) NUP98-HOXA9 mutation

The overexpression of *HOXA9* is a common genetic aberration in human AML cases caused by a number of upstream gene rearrangements (i.e., *MLL*-rearrangements) or due to the t(7;11)(p15;p15) chromosomal translocation, which creates the *NUP98-HOXA9* (*NHA9*) fusion oncogene (**Table 1.1.2**). Here, the 5' region of the gene *nucleoporin 98 kiloDlaton* (*NUP98*) on chromosome 11p15 is fused in frame to the 3' coding region of *HOXA9* on chromosome 7p15 (Borrow *et al*., 1996; Nokamura *et al*., 1996; Ghannam *et al*., 2003; **Figure 1.2.1**). Clinically, *NHA9* is found in approximately two percent of cases and is associated with poor prognosis in *de novo* and treatment-related AML and in myelodysplastic syndrome (MDS) and results in the progression of chronic myeloid leukemia (CML) to blast crisis (Ahuja *et al.,* 2001; Borrow *et al*., 1996; Chou *et al*., 2009; Dash *et al*., 2002; Nakamura *et al.*, 1996; Nakamura, 2005), where CML is defined as a clonal MPN originating from a single pluripotent hematopoietic stem cell (*see section 1.1.2*) (Hehlmann *et al*., 2007; Lavallade, 2013).

In both retroviral-transduced and germline transgenic mice, *NHA9* expression induces a latency phase of a myeloproliferative neoplasm (MPN) then a progression to overt AML (Iwasaki *et al*., 2005; Kroon *et al.*, 2001). Similarly, in humans, *NHA9* alone has a long latency period which typically presents as MDS (Hatano *et al*., 1999). Specifically, during blast crisis of *breakpoint cluster region—v-abl Abelson murine leukaemia viral oncogene homolog 1* (*BCR-ABL1*)-induced CML, *NHA9* is associated with the inhibition myeloid terminal differentiation (Dash *et al*., 2002; Mayotte *et al.*,

2002). In this instance, it suggests that CML acts as the latency period before progression to AML. These latency periods, similar to that of *HOXA9* overexpression models, suggests a requirement for additional genetic or epigenetic aberrant events to occur in order for leukemogenesis.

HOXA9 functions as both a transcriptional activator and repressor (see section *1.2.3*) in order to maintain HSC and other blood progenitor cells however, the *NHA9* fusion event removes the N-terminal domain of *HOXA9*, which eliminates the transcriptional repression activity (Borrow *et al*., 1996; Kasper *et al*., 1999; Nokamura *et al.,* 1996). The NUP98 protein is a member of the nuclear pore complex and contains a FG-rich domain near the N-terminus which promotes the interaction of *NUP98* with prominent chromatin remodeling proteins such as cyclic AMP response element-binding (CREB)-binding protein (CREBBP) and E1A binding protein p300 (EP300) (Griffis *et al.,* 2004; Scandura *et al.*, 2002). Thus it is believed that, unlike *HOXA9*, *NHA9* acts predominantly as a transcriptional activator with little or no transcriptional repression capabilities.

Given the suggested central role of *HOXA9* in both hematopoiesis and leukemogenesis, the similar transcriptional activity of *NHA9* to that of *HOXA9,* as well as the similar kinetics of *HOXA9* and *NHA9* with regard to the preleukemic latency period and the progression to AML, identifying the downstream activities of *NHA9* has the potential to uncover universal mechanisms of AML pathogenesis.

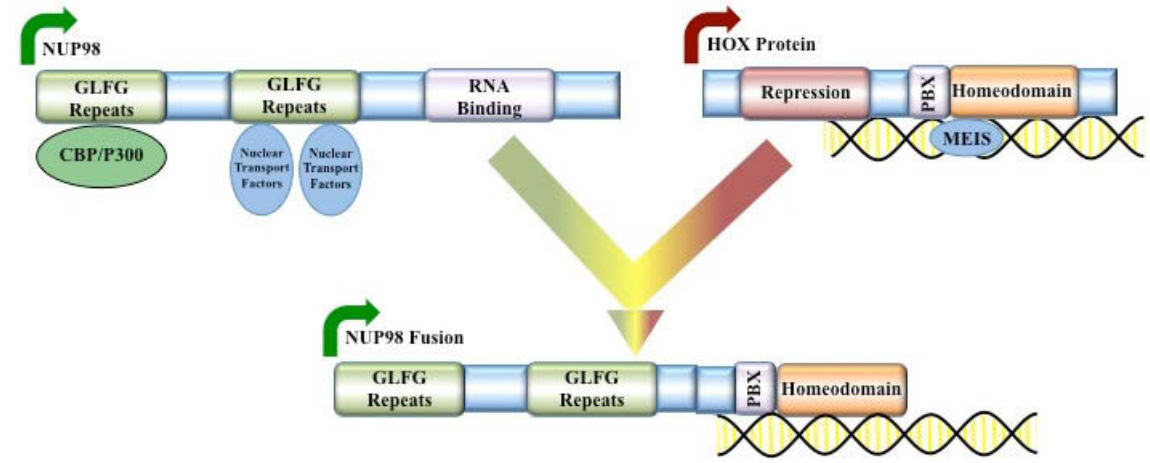


Figure 1.1.2 The t(7;11)(p15;p15) translocation yielding fusion oncogene NHA9.

The 5' region of *NUP98* on chromosome 11p15 is fused in frame to the 3' coding sequence of *HOXA9* on chromosome 7p15. The NHA9 fusion oncoprotein possesses the DNA-binding homeodomain of the HOXA9 peptide and gains novel transcriptional activation domains from the N-terminus of the NUP98 peptide.

1.3 THE LEUKEMIA STEM CELL – THEORIES AND CONCEPTS

1.3.1 Hematopoietic stem cell

The process of hematopoiesis is initiated with a multipotent hematopoietic stem cell (HSC) (Jones *et al.*, 1990; Morrison *et al.*, 1995; Sharkis *et al.*, 1997; Weissman, 2000) A HSC is a self-renewing cell, which sits at the pinnacle of a hierarchy of downstream multilineage and unilineage progenitor cells that systematically differentiate into fully mature blood cells (Estrov, 2010; Horton & Huntly, 2012). More specifically, HSCs are thought to be rare quiescent cells that divide once every 40 weeks (range 25-50 weeks) until, upon demand, give rise to progenitor cells, which then proceed to differentiate into the necessary blood cells (Burns *et al.*, 2005; Catlin *et al.*, 2011; Goessling *et al*., 2007; Thorsteinsdottir *et al*., 2002).

The concept of the HSC was first proposed by Till and McCulloch in 1961 when they found that a rare, self-renewing population of cells in the mouse bone marrow could form myelo-erythroid colonies when transplanted into the spleens of other irradiated mice (Till & McCulloch, 1961; Moore & Metcalf, 1973; McCulloch & Till, 1971). This finding led to the eventual isolation of mouse HSCs in 1988 where monoclonal antibody to cell surface markers, such as Thy1 (CD90), Sca1 (a CD59/Ly6 family protein) and c-kit (CD117), were used in combination with prospective isolation by fluorescence-activated cell sorting (FACS) (Spangrude *et al.*, 1988) and then to human HSCs using similar techniques on $CD34^+$ cells (Civin *et al*., 1984; Spangrude *et al*., 1988; Weissman & Shizuru, 2008).

There are three different types of HSC division. These include; 1) HSC depletion where the division produces two identical blood progenitor cells with decreased self-

renewal and differentiation capabilities, 2) HSC expansion where the division produces two identical sister HSCs with the same multipotent and self-renewal capacity or 3) HSC maintenance which asynchronously produces one identical HSC with the same multipotent and self-renewal capacity and one blood progenitor cell with decreased self-renewal and differentiation capabilities (John, 2003; Jones *et al*., 1990; Reya *et al.*, 2001; Weissman, 2000). These processes are tightly regulated and complex, as many necessary cell divisions are required, which produce 10^{11} red cells, granulocytes, and platelets each day (Catlin *et al*., 2011). However, like all known cell replication, it is inherently error-prone.

1.3.2 Leukemia Initiating Cell

There are two prominent models of leukemogenesis that have been long proposed - stochastic and hierarchal (Estrov, 2010). In the stochastic model, leukemia consists of a homogenous population of immature cells and a few cells that can either self-renew or proliferate in a stochastic manner (Horton & Huntly, 2012; Reya *et al*., 2001; Welch *et al*., 2012). Conversely, the hierarchal model suggests that leukemia consists of a heterogeneous population, within which only a small percentage of stem cells generate leukemic clones and sustain the disease (Ding *et al*., 2012; Ravandi & Estrov, 2006; Walter *et al*., 2012; Welch *et al*., 2012). The latter of the two introduces the theory of leukemia having a cancer stem cell that maintains the central role in leukemia pathogenesis.

The cancer stem cell hypothesis was proposed almost 150 years ago by Rudolf Virchow and Julius Cohnheim, who argued that cancer results from the activation of dormant embryonic tissue remnants (Estrov, 2010; Pérez-Caro & Sánchez-García, 2006;

Perez-Losada & Balmain, 2003). The leukemia stem cell (LSC) originates from this hypothesis and emerged from experiments performed in the early 1970s (Minden *et al.*, 1979; Sutherland *et al.*, 1990; Welch *et al*., 2012). Initial discoveries concluded that a only a small subset of leukemia cells were capable of *in vitro* proliferation (Moore & Metcalf, 1973). Additionally, cells were isolated from an acute myeloid leukemia (AML) patient and it was determined that only the most primitive Lin- (lineage negative) $CD34^{+}CD38^{-}$ fraction of cells and not the more mature Lin^{-} $CD34^{+}CD38^{+}$ or $CD34^{+}$ cells were the ones capable of propagating the disease in a NOD/SCID immunosuppressed mouse (Bonnet & Dick, 1997; Hope *et al*., 2004). In these experiments, the Lin^{-} $CD34^{+}CD38^{-}$ cells were able to differentiate into leukemic blasts and recapitulated the same disease phenotype in the mouse as was observed in the patient (Bonnet & Dick, 1997) as well as propagate the myeloid disease to secondary recipients (Bonnet & Dick, 1997; Hope *et al*., 2004; Horton & Huntly, 2012). These findings suggest that the LSCs are capable of self-renewal and the creation of heterogeneous populations of leukemic cells (Hope *et al.*, 2004; Horton & Huntly, 2012). Thus, similar to normal HSCs, LSCs are based on a hematopoietic hierarchy in which a small population of self-renewing cells gives rise to a larger, heterogeneous population of more mature blasts with the reduced capacity to self-renew (Estrov, 2010; Weissman, 2000). There is, however, still much controversy over this theory.

Retroviral insertion of oncogenes into mouse bone marrow cells *in vitro* has shown that LSCs in a number of myeloid leukemias originate from lineage-committed cells instead of HSCs. For AMLs driven by *MLL-AF9* (Krivtsov *et al*., 2006), *MLL-ENL* (Cozzio *et al*., 2003) and *MOZ-TIF2* (Huntly *et al.*, 2004) fusion oncogenes,

granulocyte/monocyte precursors (GMPs) are the LSCs. In most of these studies the transformed GMPs maintained their differentiated state but re-acquired the stem-like capacity to self-renew and were able to inappropriately active at the Wnt/β-catenin (Wnt/β-cat) pathway (*see section 1.4*) (Muller-Tidow *et al*., 2004).

Even more convincing, in acute promyelocytic leukemia (APL), there is an accumulation of cells at the promyelocytic stage at the expense of neutrophil granulocytes (*see section 1.1.3*) (de Thé & Chen, 2010). In 90 percent of APL cases there is a chromosomal translocation that produces *PML-RARA*, a mutant transcription factor. *PML-RARA* works through direct and epigenetic dysregulation of transcriptional programing and avoidance of differentiation signals from retinoic acid in the stem cell niche, to keep target cells in an immature state and allow them to re-acquire stem-like self-renewal properties (Martens *et al*., 2010; Palanisamy, 2010; Voss *et al.*, 2009; Wang, K *et al*., 2010). For example, in *PML-RARA*-transgenic mice, prior to the onset of APL, the phenotypically and morphologically normal promyelocytes demonstrate the capacity to self-renew and form colonies, whereas promyelocytes from wild-type mice do not (Guibal *et al*., 2009; Wojiski *et al*., 2009). Most surprisingly, treatment with all-*trans* retinoic acid (ATRA) induced the differentiation of the myeloid cells, which demonstrated a strong proof-of-concept that chemically targeting LSC can forcibly eradicate or at least inhibit self-renewal and other stem-like properties (Guibal *et al*., 2009; Wojiski *et al*., 2009). Therefore, although these findings challenge the theory that LSCs originate from mutated HSCs it does strengthen the argument that targeting the LSCs in AML can be a safe and effective therapy option.

1.3.3 Clinical Significance

Despite an aggressive therapy regimen, AML still maintains a high relapse rate with a poor overall survival (*see section 1.1* **Acute Myeloid Leukemia**). Additionally, conventional wisdom dictates that patients who achieve complete remission with AML treatment still harbor MRD, a small amount of myeloid-disease sustaining cells that can lead to AML relapse (*see section 1.6.3*) (Estrov, 2010; Lion, 1999; Negrin, 1998). Therefore, there is a great effort to detect, quantify and eradicate the disease-sustaining cells with the hope that it will increase treatment outcomes (Estrov, 2010). However, the cells are able to survival current chemotherapy treatments, thus relapse rates remain high (Faderl *et al.*, 1999; Talpaz *et al*., 1994).

As stated earlier, normal HSCs are mostly quiescent and, because of that, are protected from the cell-cycle-dependent chemotherapy reagents (Ravandi & Estrov, 2006). Similar to HSC, LSCs have been found to be up to 96 percent quiescent, therefore resistant to both endogenous and exogenous apoptotic stimuli (Dean *et al.*, 2005; Guzman, 2001; Konopleva *et al*., 2002). Therefore, similar to normal HSCs, LSCs are resistant to cell-cycle-dependent chemotherapy. Additionally, it is thought that high expression of ATP-associated transporters help protect quiescent cells against non-cell-dependent chemotherapies (Dean *et al*., 2005). Here, ATP binding cassettes (ABC) are capable of actively transporting chemotherapy toxins outside of the cell (Dean et al., 2005), thus LSCs may inherently possess drug resistant mechanisms and therefore, cause MRD and eventual relapse. Additionally, LSCs undergo mutational events, which may further contribute to their intrinsic drug resistant properties.

1.3.4 Mutations in HSCs and LSCs

Most mutations in cancer are thought to be acquired after the initiating event, which may increase the cell's proliferative or anti-apoptotic advantage or, increase genomic instability (Bacher *et al.*, 2008; Ding *et al*., 2012; Horton & Huntly, 2012; Oki & Issa, 2010; Walter *et al*., 2012; Wang *et al*., 2010; Welch *et a*l., 2012). In this sense, isolating the genetic mutations and determining their downstream molecular pathways would yield important information on the pathogenesis of the disease as well as help determine potential therapeutic targets. However, recent data suggests that most of the mutations in LSCs in AML are from randomly occurring events that occurred in the HSC before they acquired an initiating mutation (Welch *et al*., 2012). In this study, Welch *et al.*, determined that all AML blasts have nearly identical mutational history as normal HSCs and in most cases only two to three mutations are required to produce a malignant LSC (Ding *et al*., 2012; Welch *et al*., 2012). DNA replication is inherently error prone as well, and HSCs live for a long time and sit at the top of a hierarchy to produce all blood cells (Catlin *et al*., 2011). It is therefore logical that non-detrimental mutations would arise through time at a linear rate and propagate through all generations of blood cells. Furthermore, if a mutational event arises, which gives an HSC an increased self-renewal capacity the potential to accumulate more mutations would also greatly increase.

1.3.5 Regulatory Molecular Pathways

The most defining feature of stem cells is their self-renewal capacity therefore, this capacity has been extensively studied and several regulatory genes have been identified (Estrov, 2010). The genes *SCL, GATA2, LMO2* and *RUNX1* have been determined to govern transcriptional regulation of blood specific genes in early HSCs

(McCulloch, 1993; Narula *et al*., 2013), yet have also been demonstrated in leukemogenesis (Estrov, 2010; Horton & Huntly, 2012). For example, the chromosomal translocation t(8;21)(q22;q22), which produces the fusion oncogene *AML1-ETO* (*see* **Table 1.1.2**) causes abnormal activation of *RUNX1*, thus causing a subsequent increase self-renewal properties of that stem cell (Bäsecke *et al*., 2005; Yeh *et al.*, 2008). This chromosomal abnormality does not inhibit normal differentiation or cause AML pathogenesis alone, however the possible increase in self-renewal capacity could allow for more rapid development of secondary genetic aberrations (Bäsecke *et al*., 2005; Yeh *et al*., 2008).

Of interest, the gene *phosphatase and tensin homologue deleted on chromosome ten* (*PTEN*)-dependence has been demonstrated to be a way of distinguishing between HSCs and LSCs (Yilmaz *et al*., 2006). PTEN acts as a negative regulator of the *phosphatidylinositol 3 kinase* (*PI3K*) pathway (Zhang *et al*., 2006), which essentially plays a critical role in restricting the activation of HSCs in lineage fate determination (Costello *et al*., 2000; Yilmaz *et al*., 2006). Inactivation of *PTEN* promotes HSC proliferation and leads to an aggressive myeloproliferative disease and rapid development of AML in transplanted mice (Yilmaz *et al.*, 2006).

Finally, the Wnt-pathway has been determined to have a central role on leukemogenesis (Y. Wang *et al*., 2010). This pathway is critical for the development of many organ systems but also plays an important role in the regulation of hematopoietic stem cell and progenitor cell function (MacDonald *et al.*, 2010; Reya *et al*., 2003; Staal & Clevers, 2005). Overexpression of β-catenin, a downstream transcriptional activator in the Wnt-pathway, results in HSC expansion as well as increased expression of other

transcriptional factors and cell-cycle regulators important to HSC renewal (Reya *et al*., 2001; Reya *et al*., 2003; Wang *et al*., 2010).

1.4 THE WNT/β-CATENIN PATHWAY AND LEUKEMOGENESIS

1.4.1 Wnt/β-Catenin pathway

The conical *Wnt/β-catenin* (*Wnt/β-cat*) pathway is involved in the self-renewal of normal hematopoietic stem cells (HSCs) and activation of this pathway is a common and important event to achieve acute transformation in acute myeloid leukemia (AML) (Eaves & Humphries, 2010; Heidel, Mar, & Armstrong, 2011; F. J. T. Staal & Clevers, 2005; Y. Wang et al., 2010). β-catenin (β-cat; encoded by *CTNNB1*; *catenin* [*cadherin-associated protein*], *beta 1*), is the pathway's central effector molecule (Heidel *et al*., 2012, 2011; Reya & Clevers, 2005) (**Figure 1.4.1**). When the pathway is inactive, β-cat is inhibited via phosphorylation by a multi-protein complex (Heidel *et al*., 2011; Reya & Clevers, 2005). This complex includes adenomatous polyposis coli (APC), axis inhibitor (AXIN), glycogen synthase kinase 3 beta (GSK-3β) and casein kinase 1 (CK1) (Behrens *et al*., 1998; Rubinfeld *et al*., 1996), which binds to β-cat to in order to phosphorylate and unbiquitinate in order to mark it for proteasomal degradation (Heidel *et al*., 2011). The method of β-cat activation in the canonical model includes the binding of the Wnt ligand to the Frizzled (FZD) receptor, which in turns actives Dishevelled (DVL) (Heidel *et al*., 2011; Reya & Clevers, 2005). DVL inhibits AXIN and GSK3β and allows newly synthesized β-cat to freely enter the nucleus (Heidel *et al*., 2011; Reya & Clevers, 2005). Once β-cat enters the nucleus it initiates transcription by displacing transcriptional repressors, such as Transducin-like enhancer of split (TLE-Groucho) and histone deacetylase complexes (HDACs) at the target gene site (Heidel *et al*., 2011; Reya *et al.*,

2001). It then drives transcription via transcription factor/lymphoid enhancer-binding factor (TCF/LEF) and cyclic AMP response element-binding (CREB)-binding protein (CREBBP) (Heidel *et al.*, 2011; Reya & Clevers, 2005; Staal & Clevers, 2005). Alternatively, β-cat can be activated by prostaglandin E_2 (PGE_2) through production of cyclic AMP (cAMP) by protein kinase A (PKA), therefore allowing β-cat to once again enter the nucleus and initiate transcription (Heidel *et al*., 2011; Staal & Clevers, 2005).

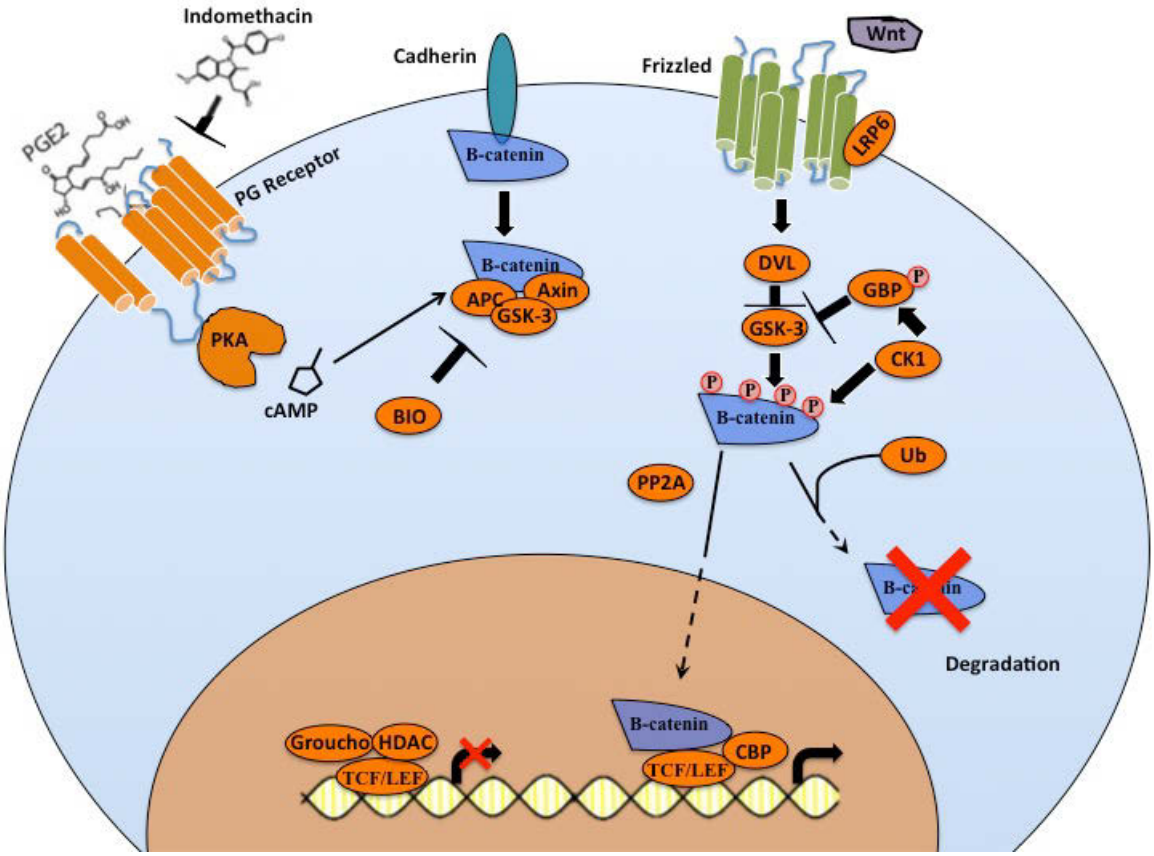


Figure 1.4.1 The canonical Wnt/β-catenin signaling pathway

β-catenin (β-cat) is naturally in a multiprotein complex with Axin, APC, GSK3β, which phosphorylates and targets β-cat for degradation. Wnt ligands binds to FZD, activates DVL or PGE_2 binds to PG receptor to activate PKA, releasing β-cat to the nucleus, which initiates transcription of target genes via TCF/LEF transcription factors. Inhibition of COX by Indomethacin, inhibits PGE_2 synthesis and inhibits the pathway. (Axin = axis inhibitor: APC = adenomatous polyposis coli; GSK3β = glycogen synthase kinase 3 beta; FZD = Frizzled; DVL = Dishevelled; PGE_2 = prostaglandin E_2; PG receptor = prostaglandin receptor; PKA = protein kinase A; TCF/LEF = transcription factor/lymphoid enhancer-binding factor 1; COX = cyclooxygenase

1.4.2 Wnt/β-Catenin in hematopoiesis

The Wnt/β-cat is not normally active in any progenitor or fully mature hematopoietic cell (Koch *et al*., 2008; Luis *et al.*, 2012), however, in mice, knockout of *β-cat* is embryonic lethal during gastrulation due to the lack of mesoderm organization (Haegel *et al.*, 1995). Furthermore, the first mammalian HSCs are believed to derive from the embryonic mesoderm (Staal & Clevers, 2005), therefore it is not surprising that pathways involved in mesodermal organization, such as Wnt and Notch also have an essential role in hematopoiesis (i.e., HSC commitment, differentiation or self-renewal).

During hematopoiesis, the Wnt pathway activity is thought to be required in the bone marrow niche to regulate HSC proliferation and preserve self-renewal capacity (Fleming *et al.*, 2008). As the HSCs exit the bone marrow niche, they encounter retinoic acid and different colony stimulating factors, which work to induce differentiation and expand progenitor cell populations (Crosnier *et al.,* 2006). At this time it is believed that the Wnt-pathway switches from self-renewal to maintenance of proliferation (Crosnier *et al.*, 2006), however, there has been much controversy over the matter.

Recent experimental animal models have produced contradictory findings regarding the importance of Wnt signals for normal hematopoiesis (Staal & Sen, 2008). For example, the first few studies on a role for Wnt signaling in the hematopoietic system focused on modeling loss-of-function, which determined the crucial role of Wnt during T-lymphocyte development in the thymus (Mulroy *et al.*, 2002; Staal *et al*., 2001). Subsequent studies on HSCs initially used overexpression models, where transplantation of HSCs with constitutively active *β-cat* retroviral overexpression into lethally irradiated mice exhibited an increase in the proliferation and repopulation capacity (Reya *et al.*,

2003). However, conditional overexpression of a stable form of *β-cat* in a transgenic mouse led to a block in multilineage differentiation and a transient expansion of the HSC pool, which was followed by the exhaustion of long-term HSCs (Kirstetter *et al.*, 2006; Scheller *et al*., 2006). As conflicting as this data appears, it should be noted that Wnt proteins are normally present in carefully controlled gradients within tissues, and responses to them can be concentration dependent (Reya & Clevers, 2005).

Studies with reporter strains of mice suggest that levels of Wnt signaling are particularly high in intestine and skin, whereas lower levels are present in breast and the central nervous system and even more modest levels are found in hematopoietic organs (Aoki, 2008; Grigoryan *et al.*, 2008). Additionally, Luis *et al.* recently identified that Wnt signaling affects HSCs in a dose-dependent manner where slightly elevated levels of Wnt/ β-cat enhanced HSC proliferation and HSC reconstitution was increased (Tiago C Luis et al., 2011). However, once HSCs were forced to express high levels of Wnt signaling they lost almost all capability of reconstituting in lethally irradiated mice (Tiago C Luis et al., 2011). It therefore appears that indeed the Wnt/β-cat pathway plays a crucial role in HSC development.

1.4.3 Wnt/β-Catenin in leukemogenesis

Tumour cells that reactivate the Wnt/β-cat pathway acquire self-renewal capabilities, which reprogram already specialized cells to confer stem-like properties (Kosinski *et al*., 2007). For instance, inactivation of the negative regulator, APC, in endothelial cells is associated with cellular expansion and is considered a hallmark in the pathogenesis of colon cancer (Reya & Clevers, 2005). Similarly, in t(9;22)(q34;q11); (*BCR-ABL1*)-induced chronic myeloid leukemia (CML) (*see* **Table 1.1.2**) Wnt signaling

is activated during blast crisis (Jamieson *et al*., 2004). Here, the CML granulocyte–macrophage progenitors (GMP), which demonstrated higher nuclear *β-cat* levels, took on stem cell-like properties such as self-renewal and the ability to produce replatable myeloid colonies (Jamieson *et al*., 2004). Furthermore, when the CML-GMPs were enforced *in vitro* to ectopically express a *β-cat* antagonist, *AXIN1,* the self-renewal capacity was significantly reduced (Jamieson *et al*., 2004). This suggests that *BCR-ABL1* may not be able to confer self-renewal on its own and may require time to acquire secondary mutations (Huntly *et al*., 2004).

Wang *et al.* demonstrated that, similar to *BCR-ABL1*-induced CML, *HOXA9 + MEIS1* overexpression-induced and fusion oncogene *MLL-AF9*-induced AML (*see* **Table 1.1.2**) requires active Wnt/β-cat (Y. Wang et al., 2010). In this study, HSCs that have naturally active Wnt/β-cat were co-transduced to overexpress *HoxA9* and *Meis1* and injected into recipient mice. Injections yielded a staggering penetrance of 93 percent progression to AML (**Figure 1.4.2**). In contrast, when *HoxA9+Meis1* co-transduced GMPs, which have a naturally silent Wnt/β-cat pathway, were injected there was only a 4 percent progression to AML. Yet, if those same cells were retrovirally transduced to express constitutively active *β-cat*, there was once again high penetrance of AML. Thus, it appears that leukemia-initiating cells have an increased requirement for Wnt/β-cat activity.

This idea is further supported by recent work with *MLL-AF9*-induced leukemia cells. Lane *et al.* used a *MLL-AF9* mouse model in order to produce and identify normal HSCs, established *MLL-AF9*-LSCs and *MLL-AF9*-pre LSCs (Lane *et al*., 2011). They discovered that population of pre-LSCs contained immortalized HSC and progenitors

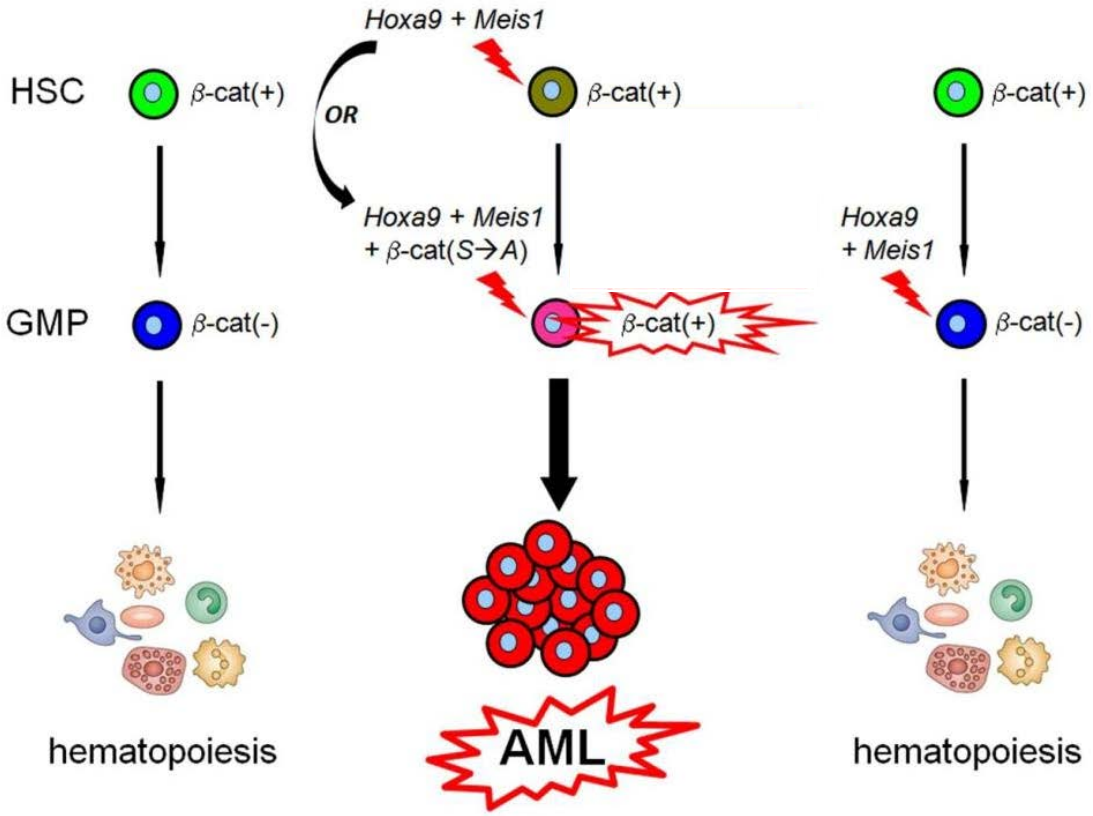


Figure 1.4.2 Activation of the Wnt/beta-catenin pathway is necessary for the generation of *Hoxa9* + *Meis1*-induced AML.

HSCs intrinsically possess Wnt/beta-catenin activity and co-overexpression of *Hoxa9* + *Meis1* produces AML. However, GMPs with *Hoxa9*+*Meis1* co-overexpression require the addition of exogenous, hyperactive beta-catenin to generate AML. Adapted from Forrester, 2012; data derived from Wang *et al*., 2010.

with normal self-renewal properties. These cells were eventually able to give rise to AML yet only after acquiring a secondary genetic aberration often resulting in intrinsic activation of Wnt/β-cat, as seen in previous studies (Mikesch *et al.*, 2007). Interestingly though, they determined that pre LSCs and HSCs occupied distinct niches within the bone marrow with distinct differences in niche-derived Wnt/β-cat signaling; the pre-LSC niche had overall elevated concentrations of external Wnt signals versus the HSC niche. Thus it appears that the pre-LSCs are dependent on external Wnt/β-cat signaling until they are able to obtain their own intrinsic signaling, establishing the role of β-cat in leukemogenesis.

Given the current evidence, Wnt/β-cat signaling appears to be capable of conferring stem cell-like properties on LSCs. Therefore, in contrast to most conventional chemotherapy that often is less affective at targeting leukemia-initiating cells than the bulk of the malignant cells, targeting aberrant Wnt/β-cat signaling may be a means to directly affect LSCs.

1.4.4 The conserved role of Wnt/β-Catenin in zebrafish

Using zebrafish and chemical screens the COX-PGE_2 signaling axis was identified as an important modifier in HSC formation and survival (Goessling *et al*., 2009; North *et al*., 2007). Zebrafish embryos that were treated with the broad-spectrum COX inhibitor, Indomethacin (Indo), demonstrated a loss of *c-myb*- and *runx1*-expressing HSCs in the aorta-gonad-mesonephros (AGM) hematopoietic region (North *et al*., 2007). Alternatively when embryos were treated with PGE_2 to increase their intrinsic amount so that it was higher than normal, there was an increase in HSC formation (North *et al*., 2007). As demonstrated earlier, PGE_2 ultimately drives the activity of the Wnt/β-cat

pathway in blood cells and is also conserved in the zebrafish. For example, overexpression of *dickkopf 1b* (*dkk1b*), a negative regulator of *β-cat*, eliminated normal HSC formation in zebrafish embryos and dampened the response to PGE_2 treatments (Goessling *et al*., 2009) which was corroborated in mouse cell culture.

Interestingly, a transgenic line of zebrafish that expresses the fusion oncogene *AML-ETO* (*see section 1.1.2*) produces an embryonic hematopoietic phenotype where there is an expansion of primitive granulocytes at the expense of erythrocytes, a similar phenotype seen in AML patients with the same fusion (Yeh *et al.*, 2008). Using this line of zebrafish it was determined that *AML1-ETO* upregulates the COX-PGE_2 signaling axis and the *AML1-ETO*-induced myeloproliferative phenotype could be inhibited by using chemical COX inhibiters, such as indomethacin (Yeh *et al*., 2009). Based on the work done in zebrafish and mouse cell lines, a phase III clinical trials are currently underway for a long-acting, 16,16-dimethyl derivative of PGE_2 ($dmPGE_2$) that promotes HSC regeneration, survival, and proliferation (Lord *et al.*, 2007). This therapy has the potential to aid in the reconstitution of the native hematopoietic stem cell, therefore of the entire hematopoietic system following chemotherapy or radiation or greatly increase the engraftment success in HSCT (Goessling *et al*., 2011).

1.5 EPIGENETICS, METHYLATION AND CLINICAL MYELOID DISEASE

1.5.1 DNA methylation

If the DNA sequence is what provides the inheritable "blueprints" for building all necessary proteins, then the epigenetic patterning would provide the information of how much, where and when to act on these "blueprints" (Holliday, 2006). More precisely, epigenetic regulation changes the accessibility of chromatin to transcriptional regulation both locally and globally through modifications of the DNA and by modification or rearrangement of nucleosomes (Esteller, 2008; Holliday, 2006; Robertson, 2001). One form of epigenetic regulation is DNA methylation or the stable, reversible epigenetic mark that silences gene expression (Esteller, 2008). This occurs when a DNA methyltransferase enzyme (DNMT [DNMT1, DNMT3A, DNMT3B]) covalently binds a methyl group at the C5 position of cytosine (Esteller, 2008). 5-methylcytosine constitutes approximately 3-8% of the total cytosine residues in the mammalian genome. In mammals, it is found predominantly in the short canonical sequence 5' -CpG-3', and rarely at non-CpG sites (Fatemi *et al.,* 2002). These CpG dinucleotides are underrepresented in the human genome, with their occurrence being 5- to 10- fold lower than statistically expected (Esteller, 2008; Holliday, 2006; Lund & van Lohuizen, 2004). These CpG dinucleotides are also unevenly distributed; in mammalian genomes they are clustered in regions, which are called CpG islands. CpG islands are short regions (0.5 to 4kb in length), which are made up of approximately 60-70 percent CG content (Holliday, 2006; Robertson *et al.*, 2000). These islands are also unevenly distributed along the genome most commonly being found around the promoter region of housekeeping genes (Esteller, 2008; Holliday, 2006).

Once DNA methylation is established in a CpG dinucleotide, it is maintained after cell division through the activity of DNMTs, which localize to replication foci to work on newly synthesized hemi-methylated DNA (Li *et al.*, 1970; Robertson, 2001). Recent studies suggest that DNA methylation status is determined through complex mechanisms where DNMTs interact with each other and with other proteins to induce DNA methylation and gene silencing (Clements *et al*., 2012; Fuks, Burgers *et al.*, 2000; Rountree *et al.*, 2000). For example, after methylation of a cytosine residue the binding of methyl-binding proteins to DNA is triggered, which attracts histone deacetylases and histone methylases that eventually modify the structure of histones into a condensed chromatin state (Nan *et al*., 1998) (**Figure 1.5.1**). Condensation of the chromatin prevents specific transcription factors or DNA-dependent RNA polymerase from having access to the promoter region to cause gene silencing (Bird, 2002; P. a Jones & Baylin, 2002). However, this and other processes of epigenetic regulation are prone to errors. For instance, aberrant methylation of CpG islands present in the promoter region of a gene has often been seen associated with silencing of an off-target gene (Costello *et al*., 2000; Rai *et al*., 2006; Robertson *et al*., 2000). This epigenetic, hypermethylation-mediated silencing of several genes is thought to be major contributing factor to heart disease, developmental disorders and various forms of cancer (Esteller, 2008; He *et al.*, 2011; Holliday, 2006; Lund & van Lohuizen, 2004; Oki & Issa, 2010). However, DNA methylation is not the sole component to epigenetic gene regulation machinery.

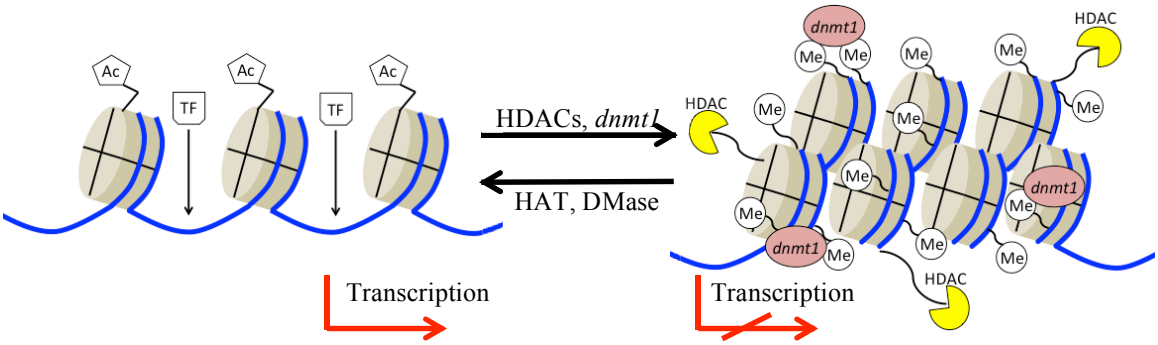


Figure 1.5.1 Schematic representation of chromatin remodeling

Chromatin remodeling is the process reversible gene activation or repression without changes to the DNA sequence. Schematic represents epigenetic regulation of HDACs and DNMT1, which work together to repress transcription. HATs and Dmase reverse this process to allow for transcription factors to bind to DNA and allow for gene activation. (HDAC - histone deacetylase complex, DNMT1 – DNA methyltransferase 1, HAT – histone acetyl transferase, DMase – DNA demethylase)

1.5.2 Histone Modification

DNA is organized into a condensed structure called chromatin. Within the chromatin, 146 base pairs of DNA wrap around an octamer of core proteins called histones that all condense together to form nucleosomes. Post translational modifications of these core histone proteins creates changes in the local chromatin architecture, which alters the accessibility of transcription factors to bind to the DNA that in turn alters gene expression (Felsenfeld & Groudine, 2003). In general, histones have 'tails' that are able to be modified through methylation, acetylation, phosphorylation, ubiquitination and sumoylation (Felsenfeld & Groudine, 2003; Khorasanizadeh, 2004). These histone tail modifications are collectively considered as the 'histone code' and work to characterize gene transcription (Khorasanizadeh, 2004).

Unmodified histones have a positively charged N-terminus, which is attracted to the negative charge of the DNA phosphate backbone (Oki & Issa, 2010). Histone acetylation, the most common form of histone modification, neutralizes the positive charge of the histone, thus lowering the attraction to DNA and relaxing the chromatin (**Figure 1.5.1**) (Felsenfeld & Groudine, 2003). Thus, increased acetylation is associated with increased transcriptional activity, whereas removal of the acetyl group by histone deacetylase complexes (HDACs) is associated with repression of gene expression (Felsenfeld & Groudine, 2003). DNA methylation and histone modification use similar reversible processes to regulate gene expression. However, each of these systems can be altered, causing aberrant gene expression that has the potential to be tumourigenic.

1.5.3 Epigenetic regulation and leukemia

AML is a heterogeneous disease with a series of distinct genetic mutations; however, there are still a high proportion of AML cases that present with a normal cytogenetic profile (*see section 1.1 Acute Myeloid Leukemia*). As such, it has long been hypothesized that aberrant epigenetic regulation could be associated with certain subtypes of AML (Oki & Issa, 2010) and a large effort has been focused on determining the potential epigenetic roles. For example, *in vitro* work demonstrates that there are coordinated changes in expression level of various genes during hematopoietic differentiation (*see section 1.3*). Similarly, observations have identified patterns of DNA methylation that are also required for differentiation into specific hematopoietic lineages (Ji *et al*., 2010). During differentiation, gene promoters required for specific hematopoietic cell fates are often demethylated, whereas genes that are responsible for maintaining a stem or progenitor state such as *MEIS1* and *HOXA9* become increasingly methylated (Faber *et al*., 2009; Ji *et al*., 2010; Shih *et al.*, 2012). As discussed in *section 1.2.3*, aberrant expression of *HOXA* and *MEIS1* has been shown to cause the pathogenesis of AML. Therefore, altered epigenetic patterning that fails to inhibit the expression of these transcriptional regulators may 'prime' the HSC for additional mutagenesis and eventual leukemogenesis.

In 2010, Figueroa and colleagues conducted a genome-wide methylation profiling on AML patient samples (Figueroa *et al*., 2010), which concluded that indeed distinct subgroups of AML patients had similar DNA methylation patterns. Interestingly, samples obtained from patients with myelodysplastic syndromes (MDS) had notably different aberrant hypermethylated regions as compared to patients with *de novo* AML (Figueroa

et al., 2010). Combined with other functional studies on AML pathogenesis, this suggests that the classical two-hit model of myeloid malignancies must be modified to account for novel classes of mutant disease alleles, most notably mutations in epigenetic modifiers (Chen *et al.*, 2010; Lamprecht *et al.*, 2010; Oki & Issa, 2010; Robertson, 2001; Shih *et al.*, 2012).

Mutations in epigenetic modifier genes include *tet methylcytosine dioxygenase 2* (*TET2), isocitrate dehydrogenase 1* (*IDH1*), *IDH2*, *additional sex combs-like 1* (*ASXL1*), *enhancer of zeste homologue 2* (EZH2) and *DNA methyltransferase 3A* (*DNMT3A*), which recently demonstrated to have biological, clinical and potentially therapeutic relevance in AML (Andersson *et al*., 2011; Wen-Chien Chou *et al*., 2010; Holmfeldt & Mullighan, 2011; Ley *et al*., 2010; Roche-Lestienne *et al*., 2011). For instance in 2010, Ley and colleagues used whole genome sequencing techniques and determined that 22 percent of AML patients from one cohort expressed a mutation in one copy of *DNMT3A*, a key player in *de novo* DNA methylation. These patients presented with a higher peripheral white blood cell count and had additional mutations associated with leukemogenesis including *nucleophosmin 1* (*NPM1*), *FMS-related tyrosine kinase 3* (*FLT3*), and *IDH1* (Ley *et al*., 2010). A subsequent study revealed that patients harbouring a *DNMT3A* mutation along with other various cytogenetic aberrations maintained a poor prognosis with a low overall survival (Thol *et al*., 2011). Combined, these findings suggest that drugs that target altered processes in the epigenetic machinery may prove to be an effective treatment strategy in AML.

1.5.4 Epigenetic therapy in AML

There are a number of U.S. Food and Drug Administration approved drugs that function to inhibit aspects of epigenetic regulation. For example Azacitidine (5-azacytidine; 5-azaC; AZA) and its deoxy-derivative, Decitabine (5-aza-2'-deoxycytidine; 5-azadC; DAC) are both in use for the clinical treatment of MDS and AML. These are two nucleoside analogues that have been studied most extensively as they target the activity of DNMT1, the major maintenance DNA methyltransferase in mammals that epigenetically regulates the expression of terminal differentiation genes in various tissues (Brown & Robertson, 2007; Song *et al.*, 2012). Interestingly, single-copy deletion of DNMT1 has little to no effect on normal HSC function, but loss of DNMT1 in LSCs inhibits LSC self-renewal capabilities (Trowbridge et al., 2012).

DAC enters the cell where it is phosphorylated by deoxycytidine kinase (Christman, 2002) Eventually, DAC becomes decitabine triphosphate, which is incorporated into newly synthesized DNA (Christman, 2002). AZA, by contrast, is phosphorylated then activated by uridine–cytidine kinase and is mainly incorporated into RNA, which markedly inhibits protein synthesis (Christman, 2002). Excessive concentrations of DAC oversaturate the DNA and inhibit DNA synthesis (Čihák *et al.*, 1985; Li *et al*., 1970; Li *et al*., 1970 (B)), however at lower concentrations, DNA incorporated decitabine triphosphate covalently binds to DNMT1 (Creusots *et al.*, 1982; Martienssen & Richards, 1995). This covalent bond eventually causes degradation of DNMT1 without causing DNA synthesis arrest (Creusots *et al.*, 1982; Martienssen & Richards, 1995), leading to induction of hypomethylation and ultimately gene reactivation (Christman, 2002).

There are also a myriad of HDAC inhibitors that are used to reverse the deacetylation of histone tails and activate the expression of selected genes (Marks *et al*., 2001). HDAC inhibitors were originally discovered through screens for agents that induce cellular differentiation *in vitro*, however, there are now several structural classes that have been recognized and some agents have been evaluated in clinical trials (Marks *et al*., 2001; Oki & Issa, 2010). For instance, valproic acid (VPA) is an antiepileptic agent that has demonstrated the ability to inhibit HDAC activity at low concentrations (Cinatl *et al.*, 1997; Göttlicher *et al*., 2001; Werling *et al.*, 2001). In a phase I study, elderly patients (age 60-78) with intermediate and high-risk forms of MDS were giving VPA as a monotherapy and yielded a surprising 44 percent response rate (Kuendgen *et al*., 2004). VPA combined with ATRA treatment was then used for patients with either secondary or *de novo* AML (Bellos & Mahlknecht, 2008). There was a positive response rate, with a limited amount of overall toxicity, however, the response rate remained lower than for those patients with MDS (Bellos & Mahlknecht, 2008). Additionally, a phase I clinical trial with DAC treatment combined with VPA in AML also did not yield as promising results (Blum *et al*., 2007). In this study, 25 patient with the median age of 70 years were enrolled, of which 12 had untreated AML and 13 had relapsed AML (Blum *et al*., 2007). DAC was given as monotherapy to14 patients for 10 consecutive days while 11 patients received DAC for 10 consecutive days plus VPA on day 5 through day 21. As expected from other clinical studies, there was a positive response from the patients treated with the DAC alone, however an high percentage of the patients with combinational therapy began to develop encephalopathy (Blum *et al*., 2007). However, four patients did reach both morphologic and cytogenetic complete remission, two from monotherapy and two

from combination therapy. Interestingly, the two patients with complete remission after monotherapy reached complete remission after 3-4 courses of treatment, however the two patients given combination therapy, reached complete remission after only 1 course of treatment (Blum *et al*., 2007). These findings were encouraging and warrants additional study on combination therapy, yet perhaps with an alternative HDAC inhibitor, such as Trichostatin A (TSA) (Kosugi *et al*., 1999). Based on these studies and other trials, there remains an interest in developing drugs that impact epigenetic pathways, such as methyl binding proteins, histone methylation, and other histone deacetylases in order to find less toxic, more efficient therapy options.

Interestingly, many of these epigenetic regulators have been identified in zebrafish and through close analysis of the mechanism by which the proteins interact, many of the functions remain well conserved (Bird, 2002; Chu *et al.*, 2012; Gros *et al*., 2012; He *et al*., 2011; Rai, 2006; Rai *et al*., 2006; Robertson *et al*., 2000; Tittle *et al*., 2011). For instance, it has been determined that dnmt1, the maintenance methyltransferase in mammals, functions similarly in the zebrafish (Rai *et al*., 2006; Tittle *et al*., 2011). Similarly to murine models, knockdown of the *dnmt1* gene in zebrafish during embryogenesis cause genome wide hypomethylation and embryonic death during gastrulation, while inhibition at later developmental stages still yields hypomethylation as well as developmental defects in organ systems, such as the intestine and exocrine pancreas (Rai, 2006; Rai *et al*., 2006; Tittle *et al*., 2011). Given the conserved epigenetic mechanisms and that zebrafish are becoming a well used an recognized system to model human disease, this may be an idea system to utilize in determining potential drugs that work to correct epigenetic function.

1.6 Zebrafish as a Cancer Model

1.6.1 Zebrafish background

The zebrafish (*Danio rerio*) is a small, tropical bony fish that originates from the Ganges and surrounding bodies of water of India and Burma. Zebrafish were made popular in the 1960s in Oregon when they were used as a new vertebrate model system for studying biological development but were quickly used as a cancer model to determine the effects of certain carcinogens (Grunwald & Eisen, 2002; Streisinger *et al.*, 1981). Early researchers found that water-soluble carcinogens can be added directly to the water, exposing the zebrafish to the toxins and inducing tumor formation (Amatruda *et al.*, 2002; Beckwith *et al.,* 2000; Berghmans *et al.*, 2005; Majorova *et al.*, 2004; Soares *et al.*, 2012). Additionally, these chemically induced tumors demonstrated a high degree of similarity to human carcinogenesis including a high rate of proliferation, low degree of cellular differentiation and an overall likeness in the gene signatures involved in the regulation of DNA damage/repair, cell cycle progression and apoptosis (Lam & Gong, 2006). This set the framework for subsequent disease modeling in the zebrafish.

Zebrafish have now been firmly established as a reliable *in vivo* tool for modeling human leukemia (Langenau *et al*., 2003; Sabaawy *et al*., 2006; Yeh *et al*., 2008). This has been made possible by a number of features: zebrafish share 85 percent of conserved genetics and cell biology with humans; there is a relative ease of husbandry, and genetic manipulation; embryonic development is rapid and external; embryos maintain a high optical transparency; there is a large library of transgenic and mutant lines; and zebrafish have an inherent capacity for high-throughput chemical screens with lower costs and time commitments than current murine models. Furthermore, zebrafish reach sexual maturity

at three months of age, reproduce weekly and produce upwards of 200-300 embryo offspring per female (Jing & Zon, 2011; Zon, 1999), thus allowing rapid and repeated analysis of conserved developmental pathways.

1.6.2 Four waves of hematopoiesis in zebrafish embryos

Similar to mammals, there are four distinct waves of hematopoiesis during zebrafish embryogenesis that each occur in a distinct spatiotemporal manner with their own cell specification (Bertrand *et al*., 2007). There are two waves of 'primitive' hematopoiesis, which help to influence the morphology of the developing embryo as well as produce circulating erythrocytes and primitive macrophages (Baumann & Dragon, 2005). More specifically, primitive macrophages arise and differentiate directly from mesenchymal progenitor cells at 12 hpf in the anterior lateral plate mesoderm (**Figure 1.6.1**) (Hume, 2006). These macrophages, identified by early expression of *spi1,* help to remodel early tissues by shaping ECM with matrix metalloproteinase 9, assisting in tissue vascularization and eliminating apoptotic cells (Bennett *et al*., 2001; Herbomel *et al*., 1999; Warga *et al.*, 2009). Primitive erythrocytes, the second wave of hematopoiesis form along the bilateral stripes in the posterior lateral plate mesoderm (PLPM) at around 12hpf (Orkin & Zon, 2008), which fuse together between 16-18 hpf to form the intermediate cell mass (ICM). These primitive erythrocytes initially express *gata1* and facilitate tissue oxygenation during the rapid embryonic growth and enter into circulation around 24 hpf (Orkin & Zon, 2008).

Following these primitive waves, the first wave of definitive hematopoiesis occurs at 24 hpf in posterior blood island (PBI) region, which produce progenitor cells that have the dual potential to form cells of the erythroid or myeloid fates (**Figure 1.6.2**)

(Bertrand *et al*., 2007). When these erythroid-myeloid progenitors (EMP) are in their undifferentiated state, they are marked by the combined expression of *gata1* and *LIM domain only 2* (*lmo2*) (Bertrand *et al*., 2007). This wave continues to produce erythrocytes and produces the first definitive myeloid cells after the primitive waves are completed. However, the EMPs are transient and production of these progenitors declines as the final wave of hematopoiesis begins at around 30-36 hpf. During this time, HSCs start to bud directly from hemogenic endothelium in the ventral wall of the dorsal aorta (the mammalian equivalent of the aorta-gonad-mesonephros [AGM])(Bertrand *et al*., 2007). These stem cells, which express *integrin, alpha 2b* (*platlet glycoprotein IIb of IIb/IIIa complex*)*, antigen CD41B* (*itga2b*), *v-mub myeloblastosis viral oncogene homolog* (*avian*) (*c-myb*) and *runt-related transcription factor 1* (*runx1*) migrate to the caudal hematopoietic tissue (CHT) where they begin to divide and give rise to all hematopoietic lineages of adult blood cells (Kissa & Herbomel, 2010). Eventually, the HSCs will migrate and populate the adult kidney and thymus, the analogous hematopoietic organs to the mammalian bone marrow and thymus, respectively.

1.6.3 Conserved genetics and cell biology of zebrafish hematopoiesis

Model systems with conserved genetic and cell biology have been difficult to identify and often expensive to use. However, the zebrafish is a highly efficient model system for studying both the conserved pathways of blood cell development and of leukemogenesis (Berman *et al*., 2003;Berman *et al.*, 2005; Feng *et al*., 2007; Langenau *et al*., 2003, 2007; Sabaawy *et al*., 2006). For instance, the hematopoietic hierarchy and all the major hematopoietic cell lineages are homologous in the zebrafish and the basic genetic mechanisms that control hematopoiesis remain well conserved (Berman *et al*.,

2003; Berman *et al*., 2005; Forrester *et al*., 2011). Additionally, a number of studies have determined that myelopoiesis in mammals is closely mimicked in zebrafish in terms of neutrophil, macrophage and mast cell development (Bennett *et al*., 2001; Berman *et al.,* 2005; Da'as *et al*., 2012; Da'as *et al.*, 2011; Lieschke *et al.*, 2001). Additionally, the transcriptional regulation during blood cell development is also similarly conserved. For instance, key myeloid genes such as *myeloperoxidase* (*mpx*) (C. M. Bennett et al., 2001; Lieschke et al., 2001), *lymphocyte cytosolic protein 1* (*lcp1*) (Herbomel *et al.*, 1999; Le Guyader *et al*., 2008; Mathias *et al*., 2009), and *lysozyme* (*lyz*) (Hall *et al.*, 2007; Liu & Wen, 2002) demonstrate analogous roles compared to that of murine models and humans. Whereas *lcp1* and *lyz* were previously considered to specify monocytic and granulocytic differentiation in the mouse, respectively, subsequent data has revealed that these genes show a pan-myeloid expression pattern in zebrafish embryos and may continue to mark progenitor cells through 48 hours post fertilization (hpf) (Hall *et al*., 2007; Le Guyader *et al*., 2008). Additionally, the classical antagonism between the myeloid transcription factor SPI1 (spleen focus forming virus [SFFV] proviral integration oncogene) and the erythroid transcription factors, GATA1 (GATA binding factor 1), which compete to specify whether a blood progenitor cell is destined to a myeloid or erythroid fate, respectively, also plays a similar role in zebrafish (Galloway *et al.*, 2005; Lyons *et al.*, 2002; Rhodes *et al*., 2005). Combined, these findings have helped to justify modeling human myeloid disease in the zebrafish.

1.6.4 The SPI1 versus GATA1 paradigm

The SPI1 AND GATA1 proteins directly interact with each other to inhibit transcription of target genes and thus, compete to specify myeloid or erythroid fate

(Nerlov *et al.*, 2000; Rekhtman *et al*., 1999; Stopka *et al.*, 2005; Zhang *et al*., 2000). More specifically, *SPI1* is an ETS family transcription factor that is responsible for specifying the myeloid cell while *GATA1* encodes a zinc finger transcription factor that specifies erythroid cell fate (McKercher *et al*., 1996; Scott *et al.*, 1994; Zhang *et al*., 1996). In mammals, overexpression of *GATA1* in myeloid cells induces a switch from a myeloid cell fate to a megkaryoctic/erythroid cell fate (H. Iwasaki et al., 2003). In zebrafish embryos, inhibition of *gata1* expression reduced the number of circulating erythrocytes while promoting the expansion of neutrophil and macrophage populations (Galloway *et al*., 2005; Lyons *et al*., 2002; Rhodes *et al*., 2005). In contrast, *spi1* knockdown in zebrafish embryos reduces myeloid cell populations and results in ectopic expression of *gata1*in the anterior lateral plate mesoderm an anatomic region where myeloid cells normally predominate (**Figure 1.6.1***; see section 1.6.6*) (Rhodes *et al.*, 2005). Collectively, this conserved antagonistic pathway as well as the conserved cellular and genetic hematopoietic pathways in the zebrafish justify their use in modeling human myeloid disease.

1.6.5 Zebrafish as a model system to study cancer

Zebrafish have emerged as a reliable and powerful tool in modeling human cancers (**Table 1.6.1**) (Berghmans *et al*., 2005; Yang *et al*., 2004). Initiated by early carcinogenesis studies it has been determined that many of the oncogenes and tumour suppressor genes that play roles in human malignancies have homologues in the zebrafish. In addition, the critical pathways that regulate cell growth, proliferation, apoptosis and cell differentiation appear well conserved (Feitsma & Cuppen, 2008; Liu & Leach, 2011; Mione & Trede, 2010; Stoletov & Klemke, 2008; Yang *et al*., 2004).

Technological advancements and the increasing number of mutant/transgenic zebrafish lines have enabled the generation of tumors in the zebrafish that resemble human malignancies both histologically and genetically (S. Liu & Leach, 2011). For example, a zebrafish model of *Myc*-induced T-cell acute lymphoblastic leukemia (T-ALL) uses the lymphoid specific, zebrafish *rag2* promoter to overexpress *Myc*, a dual function oncogene that is involved in cell proliferation and apoptosis (Langenau *et al*., 2003). This overexpression in the zebrafish demonstrates a rapid onset of T-ALL, which resembles the human leukemia in as early as 21 days post fertilization (dpf) (Feng *et al*., 2007; Langenau *et al*., 2003). Other examples include *tp53*-induced malignant peripheral neural sheath tumors (zMPNST) (Berghmans *et al*., 2005), *K-RAS*-induced rhabdomyosarcoma (Langenau *et al.*, 2007), *TEL-AML1*-induced pre-B-ALL (Sabaawy *et al*., 2006), *EWS-FLI1*-induced Ewing's sarcoma (Leacock *et al*., 2012), *B-RAF*-induced melanoma (Patton & Zon, 2005) and *MYCN*-induced neuroblastoma (Zhu *et al*., 2012). The power of the zebrafish as a cancer model is also highlighted when it is used to rapidly screen for drugs that ameliorate leukemia thus complementing and bridging the gap between leukemia cell line studies and mouse models (K. Wang *et al*., 2012).

1.6.6 Genetic and chemical modifier screens in zebrafish

Chemical modifier screens can be conducted rapidly and efficiently *in vivo* in zebrafish, which can be used as efficient tool to bridge the gap from cell line studies to mouse models (Sola & Gornung, 2001; Streisinger *et al*., 1981). For instance, chemical screens in cell lines typically produce a large number of 'hits' that require *in vivo* validation. In the past, the model of choice has been the mouse, yet high-throughput drug validation is ineffective due to the biological complexity and prohibitive costs associated

with these types of assays (Molina *et al.,* 2007). Alternatively, the zebrafish is able to validate drugs in a manner that is highly time efficient and at a fraction of the cost. The 'hits' from this first-line *in vivo* testing will reduce the list of potential drugs and only those that demonstrate promising results would be pushed through for further validation in a mammalian model, thus reducing the time and financial burden of large-scale *in vivo* drug testing.

The first drug discovery screen in zebrafish identified compounds which effectively restore normal vascular development in a zebrafish model of aortic coarctation (Peterson *et al*., 2004), while subsequent screens have found novel retinoic acid-like compounds that effect embryogenesis (Das *et al.*, 2010), inhibitors of lymphatic growth (Karpanen & Schulte-Merker, 2011) and behavior modifying neurochemicals (Kokel *et al*., 2010). Two recent chemical screens (North et al., 2007; Yeh et al., 2009) both identified PTGS/COX (prostaglandin-endoperoxide synthase (prostaglandin G/H synthase and cyclooxygenase) inhibitors, such as Indomethacin (Indo) and NS-398, as pharmacological modifiers of hematopoietic differentiation. Recently, the Biobide laboratory in Spain developed an automated high-throughput platform for *in vivo* chemical screenings on zebrafish embryos that includes automated methods for embryo dispensation, compound delivery, incubation, imaging and analysis of the results using fluorescent reporter lines (Letamendia *et al*., 2012). Successful validations using known positive and negative compounds for cardiotoxic compounds and angiogenesis inhibitors have been conducted, which has also led to detection of some interesting, previously unknown anti-angiogenic compounds (Letamendia *et al*., 2012). Though this system has

been validated using only two florescent report lines, the potential to use other lines, such as those with fluorescent HSCs, red blood cells or myeloid cells, is yet to be realized.

The zebrafish has also contributed greatly to our understanding of hematopoietic transcription factor networks in vertebrates. For example, forward genetic screens using large-scale mutagenesis identified a number of mutant lines with bloodless phenotypes such as *kugleig* (*kgg*^{tv205}*; cd4*$^{-/-}$) (Davidson & Zon, 2004; Davidson *et al.*, 2003; de Jong *et al*., 2010), *riesling*, *merlot*, *cabernet*, and *shiraz* (Ransom *et al*., 1996), *moonshine* (*mon*^{tg234}*; trim33/tif1γ*$^{-/-}$*)* (Bai *et al*., 2010; Monteiro *et al.*, 2011; Ransom *et al.*, 2004)*,* *cloche* (*clo*^{m39}) (Challen *et al*., 2012; Lin *et al*., 2005; Stainier *et al.*, 1995), and *vlad tepes* (*vlt*^{m651}) (Lyons *et al*., 2002). In addition, Wang and colleagues performed a very large scale, forward genetics screen and isolated 52 hematopoietic-mutant zebrafish lines (Kun Wang et al., 2012) Using specific hematopoietic markers, they were able to determine putative mutations in genes that help regulate relevant aspects of hematopoiesis including macrophage development, early granulopoiesis, embryonic myelopoiesis and definitive erythropoiesis/lymphopoiesis (Kun Wang et al., 2012). Although these mutants require further study and categorization, this forward genetic screen has set the groundwork to help unveil potentially conserved, unknown molecular pathways regulating hematopoiesis.

Table 1.6.1 Table of Zebrafish cancer models

Model Type	Primary Function	*In vivo* Imaging	Advantages	Disadvantages
Chemical carcinogenesis	To determine carcinogenic activity of chemical compounds or mutant fish lines with developmental abnormalities	Low magnification of tumor; high magnification of fixed samples	No need to specific zebrafish strains; easy to perform	Low incidence and delayed onset of tumourigenesis
Mutant lines	To screen for chemical or genetic factors that promote tumourigenesis	Low magnification of tumor; high magnification of fixed samples	Fair tumourigenesis rate	Required to develop/use specific fish lines
Transgenic lines	Enables the study of progression/ initiation of specific cancer types; aids in visualization in specific zebrafish cell components	Low magnification of tumor; high magnification of fixed samples Fluorescent tagging of transgene possible to enable high resolution imaging	Ability to control time of transgene expression; able to induce tissue or organ specific tumourigenesis; high incidence of tumourigenesis	Required to develop/use specific fish lines; transgenic lines may be genetically unstable
Xenotransplant (30-day-old)	To study the microenvironments and tumor cell interactions	Able to tag injected cells fluorescently for high resolution *in vivo* imaging	Number, type and location of injected cells is tightly controlled; large number of fish can be injected at once; all organ systems are developed	Either zebrafish need to be immune-suppressed or embryonic stages used; tumor may only survive a few weeks
Xenotransplant (2–5-days-old)	To study the microenvironments and tumor cell interactions	Able to tag injected cells fluorescently for high resolution *in vivo* imaging	Number, type and location of injected cells is tightly controlled; large number of fish can be injected at once; immunosuppression not required	Tumor may only survive a few weeks; gross organ systems still undergoing development

Adapted from Amatruda *et al*., 2002; Berghmans *et al*., 2005; Lam & Gong, 2006; Langenau *et al*., 2003, 2007; Patton & Zon, 2005; Stoletov & Klemke, 2008; Yang *et al*., 2004

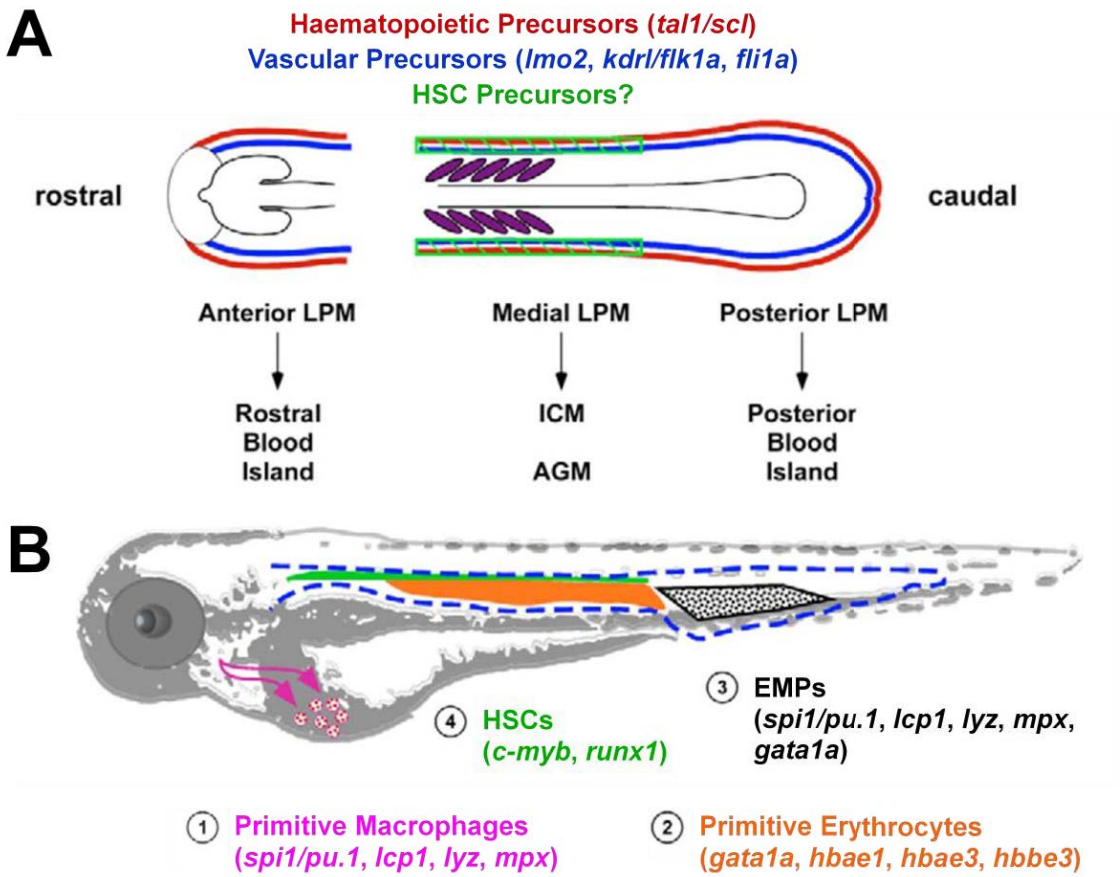


Figure 1.6.1 Depiction of the four waves of hematopoiesis in the zebrafish embryo.

Gene markers of cell populations noted in parentheses. (***A***) Drawing depicting a flat-mount, dorsal view of a five-somite-stage embryo at approximately 12 hpf. The lateral plate mesoderm (LPM) gives rise to anatomically distinct regions of blood cell precursors. The blue bilateral stripes of LPM depict vasculature while the red depict hematopoietic regions. (***B***) There are four waves to embryonic hematopoiesis. The number dictates the temporal order of the functional cells from each subset. Primitive macrophages arise in the ALPM first and migrate over the yolk before spreading throughout the embryo. Primitive erythrocytes form second and begin to differentiate within the ICM, which is formed from the fusion of bilateral stripes in the PLPM and enter at approximately 24 hpf. The first definitive progenitors arise as the third wave of hematopoiesis. They arise as EMPs, which generate the first definitive myeloid cells and a new wave of erythroid cells and are formed within the PBI. The final wave of hematopoiesis marks the production of multipotent HSCs between the axial vessels in the zebrafish equivalent of the AGM region. (ALPM / PLPM = anterior / posterior LPM; ICM = intermediate cell mass; PBI = posterior blood island; EMP = erythromyeloid progenitor; HSC = hematopoietic stem cell; AGM = aorta-gonad-mesonephros.) Adapted with permission from **Bertrand, J. Y., Kim, A. D., Violette, E. P., Stachura, D. L., Cisson, J. L., & Traver, D. (2007). Definitive hematopoiesis initiates through a committed erythromyeloid progenitor in the zebrafish embryo. Development (Cambridge, England), 134(23), 4147–56. doi:10.1242/dev.012385**.

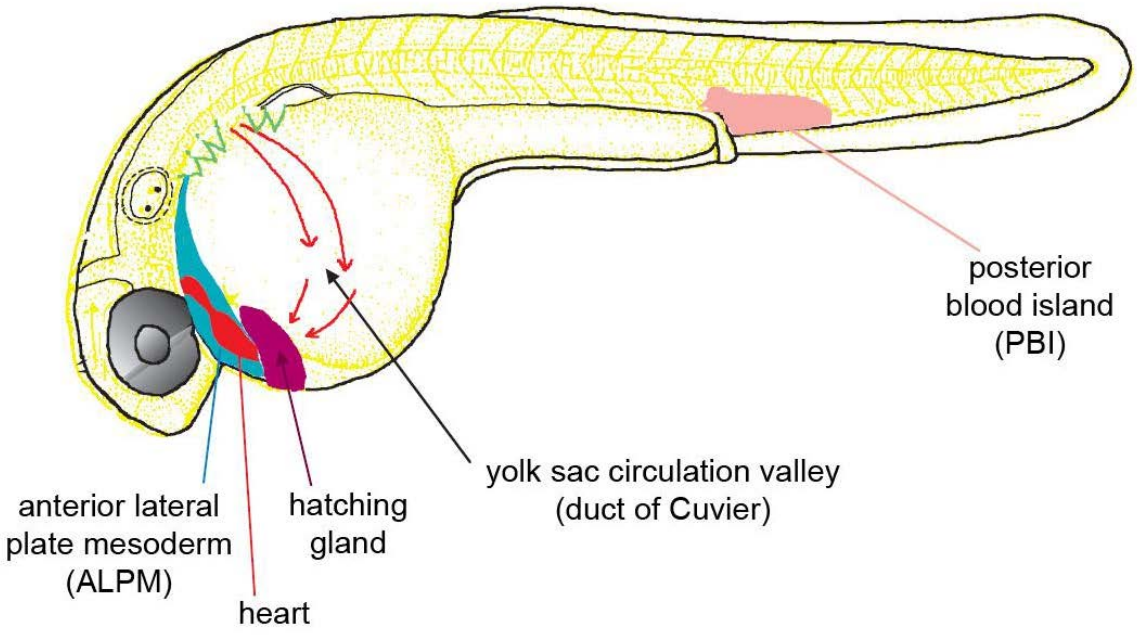


Figure 1.6.2 Relevant hematopoietic regions in a zebrafish embryo at 28 to 30hpf.

Zebrafish embryo, lateral view depicting the anterior region to the left. The ALPM (light blue), heart (red), hatching gland (purple) and PBI (pink) are visible. Red arrows on the yolk sac indicate venous blood flow in a depression of the yolk surface illustrated as the yolk sac circulation valley (duct of Cuvier). Adapted with permission from **Herbomel, P., Thisse, B., & Thisse, C. (1999). Ontogeny and behaviour of early macrophages in the zebrafish embryo. Development (Cambridge, England), 126(17), 3735–45**.

1.7 *NUP98-HOXA9* transgenic zebrafish

Not surprisingly, zebrafish are being used more frequently to help determine the underlying molecular mechanisms of cancer. Mutations in zebrafish tumour-suppressor genes as well as human and mouse oncogenes are being introduced into the zebrafish at early embryonic stages to help investigate potential primary gene collaborators as well as the sequential progression to malignant transformation. There are a large number of transgenic mouse cancer models, which have been used in the past to identify collaborating mutations with the potential to accelerate leukemogenesis, however this approach has proven to often be difficult and expensive. As such, the high genetic/cellular conservation in zebrafish, as the reproducible defects in the early embryogenesis and the relative ease to identify the potential contributing genes in cancer, has clearly demonstrated the power of the zebrafish to study human cancers.

1.7.1 NUP98-HOXA9 inducible expression in zebrafish

HOXA9 is upregulated in approximately 80 percent of human AML cases and is associated with poor prognosis (Golub, 1999). One way in which *HOXA9* can be overexpressed is through the t(7;11)(p15;p15) chromosomal translocation, yielding the *NUP98-HOXA9* (*NHA9*) fusion oncogene (*see section 1.2.4*). The molecular mechanisms by which the *HOXA9* and *NHA9* oncogenes promote the development and progression of high-risk AML remain largely unknown despite a number of *in vitro* studies (Calvo *et al.*, 2001; Calvo *et al.*, 2002; Chung *et al*., 2006; Ghannam *et al*., 2003; Kasper *et al*., 1999), and *in vivo* studies with mammalian models (Iwasaki *et al*., 2005; Kroon *et al*., 1998; Kroon *et al.*, 2001; Thorsteinsdottir *et al*., 2002; Thorsteinsdottir *et al.*, 2001). The Berman lab has created a transgenic zebrafish model by which the human *NHA9* fusion

oncogene has been expressed under the zebrafish 9.1kb *spi1* promoter (Forrester *et al*., 2011; Forrester, 2012). Zebrafish *spi1* is expressed in myeloid cells during embryogenesis as well as at low levels in the adult kidney marrow, the site of adult hematopoiesis in the fish (*see section 1.6.5*) (Hsu *et al*., 2004).

NHA9 was cloned downstream of a Cre/*lox*-inducible expression cassette with an enhanced green florescent protein (EGFP) tag (**Figure 1.7.1A**). This stop cassette allows the expression of *NHA9* to be induced at a specific time point, thus allowing the essential developmental processes of gastrulation to complete without perturbation. Fluorescence microscopy was used to observe the expression of the floxed *EGFP* (*lGl*) cassette in order to confirm germline transmission of the *NHA9* transgene. This can only be performed prior to activation, given that *Cre*-mediated recombination removes this cassette and thus turns off expression of *EGFP*. EGFP can be observed as early as the two-cell stage in the embryo and punctate GFP^+ blood cells could be observed at the APLM in the head region, the ICM in the mid-body region and in the PBI in the tail region (**Figure 1.7.1Bi**) (Forrester et al., 2011). Analysis of cell morphology by Wright-Giemsa stain confirmed that the *NHA9* transgene was being expressed in myeloid lineage at multiple stages of cell development, including immature precursors and mature neutrophils (**Figure 1.7.1Bii**) (Forrester *et al*., 2011; Forrester, 2012).

To activate the expression of the oncogene, *NHA9* adults were outcrossed to the *Tg*(*hsp70::Cre*) activator line, which expresses *Cre* under the *heat shock protein 70* (*hsp70*) promoter. To activate *Cre* and therefore excise the *lGl* cassette, *NHA9;Cre* heterozygous embryos were heat-shocked by incubating the embryos at 37-39^{o}C for one hour (Forrester et al., 2011; Forrester, 2012). Some expected activation was observed in

NHA9 embryos that were not heat-shocked, due to 'leakiness' of the *Cre* zebrafish line (Le *et al*., 2007).

1.7.2 NUP98-HOXA9 induces a myeloproliferative neoplasm in zebrafish

NHA9;Cre heterozygous embryos were heat-shocked 24hpf to excise the *lGl* cassette then grown to adulthood. Between 19 and 23 months post-fertilization, many activated *NHA9* zebrafish presented with abdominal masses and laboured swimming. Whole fish histological sections identified that 23 percent of the adult *NHA9* fish had kidney hypertrophy with evidence of malignant infiltrates of pleomorphic, mitotically active myeloid cells. By contrast, no AB wild-type fish or *Cre*;AB heterozygotes presented with this phenotype (Forrester et al., 2011; Forrester, 2012). Periodic-acid Schiff (PAS) and hematoxylin and eosin (H/E) staining confirmed that a large proportion of the infiltrates were eosinophils, mast cells and neutrophils. Furthermore, granulation in the eosinophils and neutrophils appeared normal, which suggests that the cells were not the result of inflammation or infection.

The kidneys of the infected embryos also had a near absence of normal kidney structures, such as glomeruli and tubules, compared to that of the kidneys in the AB wild-type control fish. These results, combined with the apparent hyperproliferation of mature myeloid cells demonstrate that the *NHA9*-transgenic fish had developed a myeloproliferative neoplasm (MPN) (Forrester *et al*., 2011; Forrester, 2012) Interestingly, this kidney pathology is quite reminiscent of transgenic mice where *NHA9* expression lead to polyclonal MPN after a long latency, which then subsequently progresses to the onset of overt AML (Iwasaki *et al*., 2005; Kroon *et al*., 2001). However, unlike the murine model, none of the activated *NHA9*-transgenic fish progressed to frank

AML. For this reason, it is postulated that a longer latency period may be required in order to produce additional genetic aberrations that will cause the progression of leukemogenesis (Forrester, 2012). As the zebrafish reproduce weekly and yield up to 200 embryos per female, identifying an embryonic phenotype then using it to find additional mechanistic data in *NHA9*-mediated disease, may be a fast and efficient method of identifying these potential secondary genetic aberrations that will lead to overt AML.

1.7.3 NUP98-HOXA9 inhibits apoptosis and cell cycle arrest after DNA damage

NHA9-associated human leukemia demonstrates cellular resistance to conventional chemotherapy drugs, such as DNA damaging agents (Giles et al., 2002). Similarly, *NHA9*-transgenic zebrafish embryos demonstrate reduced cell-cycle arrest and apoptotic responses after induction of DNA damage by ionizing radiation (Forrester *et al*., 2011; Forrester, 2012). The number of phosphorylated histone H3 (pH3)-labeled cells, a marker of mitotically active cells undergoing the G2-M transition, were decreased in heat-shocked *NHA9* embryos (A Michael Forrester et al., 2011) only demonstrated a 1.63-fold decrease (Forrester *et al*., 2011; Forrester, 2012).

Fluorescent microscopy with acridine orange staining confirmed a reduced apoptotic response in activated *NHA9* embryos compared to that of AB wild-type (Forrester *et al.*, 2011; Forrester, 2012). Similarly, there was a reduction in the activation of the caspase 3 (Forrester *et al*., 2011; Forrester, 2012). This reduced caspase activity implies impaired caspase-mediated apoptosis as the cause of a decreased apoptotic response in irradiated *NHA9* embryos. These experiments, however, were examining more global effects of *NHA9* expression in the embryos and experiments to determine the specific effects on blood development were necessary.

1.7.4 NUP98-HOXA9 perturbs hematopoiesis in the zebrafish embryos

Overexpression of *HOXA9* promotes immortalization and inhibits the differentiation of committed myeloid progenitor cells and of HSCs *in vitro* (Calvo *et al*., 2002; Takeda *et al.*, 2006; Thorsteinsdottir *et al*., 2002). Therefore, Forrester *et al.*, sought to determine *NHA9*'s effects on embryonic hematopoiesis (Forrester *et al*., 2011; Forrester, 2012). Initial focus was on the primitive waves of zebrafish hematopoiesis by heat-shocking at 12 hpf (*see section 1.6.5*). Whole-mount RNA *in situ* hybridization (WISH) for the early gene marker, *spi1*, demonstrated an increase in primitive myeloid cells of the *NHA9* embryos during this first wave of hematopoiesis. However, when other myeloid gene markers, such as *lcp1* and *lyz*, which represent more mature myeloid cells (Hall *et al.*, 2007; Le Guyader *et al.*, 2008), were examined, there was an overall decrease compared to AB wild-type controls. These findings suggest that *NHA9* may be working to inhibit terminal differentiation of primitive myeloid cells, but fails to elucidate how *NHA9* induces a MPN in adults, as this is a transient wave of hematopoiesis.

The first definitive wave of hematopoiesis occurs between 24 to 32hpf, which marks the emergence of the dual-potential EMPs in the PBI (*see section 1.6.5*). Upon WISH assessment of activated *NHA9* embryos, there was a decrease in *gata1* suggesting nearly a 20-fold decrease in erythrocyte production compared to AB wild-type embryos (**Figure 1.7.4B**) (Forrester *et al*., 2011; Forrester, 2012). Concurrently, WISH demonstrated an increase in *lcp1*, 2.79-fold increase in *lyz* and a 1.41-fold increase in *mpx*, suggesting that *NHA9* promotes the development of myeloid cells at the expense of erythroid cells during the first wave of definitive hematopoiesis (Forrester *et al*., 2011; Forrester, 2012). A BrdU assay, which assesses the overall number of cells undergoing

the S phase of the mitotic cell cycle and a pH3 assay confirmed that *NHA9* was not increasing global cell proliferation (Forrester *et al*., 2011; Forrester, 2012). Furthermore, neither *spi1*-, nor *lcp1*-expressing cells demonstrated increased cell proliferation capabilities (Forrester *et al*., 2011; Forrester, 2012) suggesting that *NHA9* is affecting hematopoietic cell fate determination and not proliferation. However, this does not entirely explain the adult *NHA9*-transgenic phenotype, as the impact of *NHA9* at later time points and more specifically, HSCs has not been explored yet.

The molecular mechanisms by which the *HOXA9* and *NHA9* oncogenes promote the development and progression of high-risk AML remain largely unknown despite a number of studies. Furthermore, current therapies to combat AML remain largely ineffective, contributing to morbidity and high relapse rates. However, this model of *NHA9*-induced myeloid disease in the zebrafish provides a novel tool to interrogate downstream molecular mechanisms in the pathogenesis of *NHA9*-mediated AML. These discoveries may ultimately be translated into new treatment strategies for AML, which would be of particular benefit to this high-risk group of affected patients.

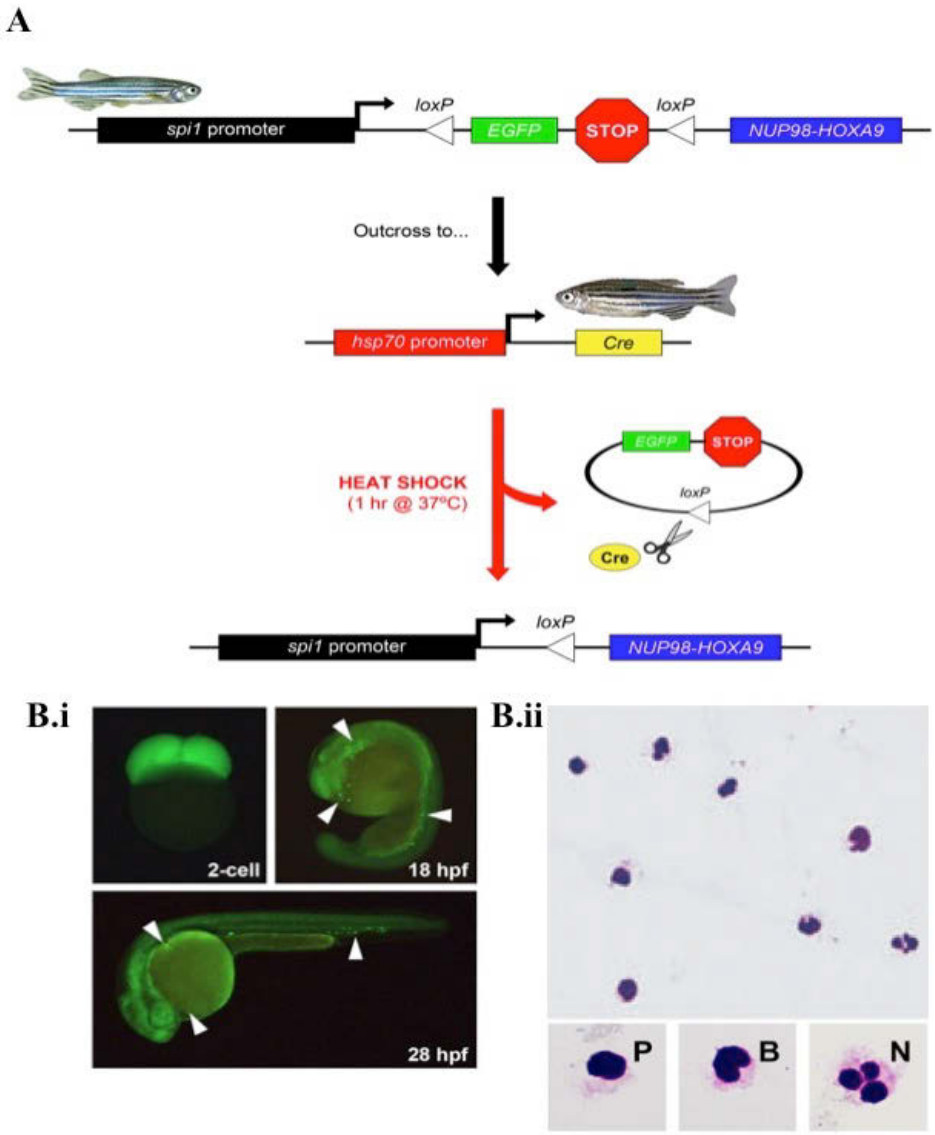


Figure 1.7.1 Transgenic zebrafish express inducible human *NUP98-HOXA9* under the 9.1 kb zebrafish *spi1* promoter

(A) A schematic of the Tg(*spi1::lGl::NUP98-HOXA9*) expression vector. The Cre/*lox*-inducible, EGFP tagged expression cassette inhibits expression of NUP98-HOXA9 buy can be removed by outcross to Tg(*hsp70::Cre*), followed by heat-shock for 1 hour at 37^{o}C. (B) (i) NUP98-HOXA9-transgenic embryos demonstrate EGFP expression. Ubiquitous EGFP observed as early as the 2-cell stage; blood cell, CNS and musculature expression can be distinguished by 18 hpf, and are still present at 28 hpf. Punctate blood cells (white arrowheads) are shown at the APLM spreading over the yolk sac and at the PBI in the tail region. (ii) Cytospin of FACS-sorted GFP+ cells, stained with Wright-Giemsa stain (top, 40X magnification), demonstrate characteristic myeloid morphologies in the NUP98-HOXA9-transgenic embryos, such as precursors (P), band form (B) and segmented neutrophil (N) (bottom, 100X magnification) Adapted from Forrester *et al*., 2011; Forrester, 2012

1.8 RATIONAL OF STUDIES AND HYPOTHESES

There are a large number of advantages with adopting zebrafish as an *in vivo* model. Here we use zebrafish to assist in our research goal of improving survival in high-risk forms of human AML. More specifically, we wish to elucidate molecular pathways and potential collaborating genes in order to develop targeted therapies that promote less toxicity and greater efficacy than currently used treatment options.

I hypothesize that the already established *NHA9*-transgenic zebrafish model can serve as a tool to help identify potential downstream drug targets in *NHA9*-induced disease in directed, gene-seeking and unbiased, whole-genome approaches. I also hypothesize that by targeting identified proteins either by genetic knockdown or chemical inhibition, we will be able to inhibit the effects of *NHA9* on blood cell development. Additionally, by combining treatment of these potential drug targets, I hypothesize that we will be able to identify pathways that synergize to help ameliorate *NHA9*-induced disease.

A number of genes have been identified to have a collaborative role in *HOXA9*-induced AML, including *β-catenin*. The *Wnt/ β-catenin* signaling pathway is required for self-renewal of LSCs that are derived from either HSCs or more differentiated granulocyte-macrophage progenitors *in vitro*. We aim to investigate whether or not this collaboration is similar in *NHA9*-induced disease and if *β-catenin* targeted therapy will help restore normal hematopoiesis in the transgenic embryo. Additionally, we aim to investigate whole-genome changes by microarray analysis in *NHA9*-transgenic embryos compared to wild-type embryos with the hope that potentially targetable microarray 'hits'

will be further explored. Thus the goal of my research project was to investigate genes downstream of *NHA9* that could be used to inhibit *NHA9* disease progression.

Given their conserved genetics, ease of genetic manipulation and feasibility of acquiring large sample sizes, zebrafish are being used more frequently to help determine the underlying molecular mechanisms of cancer. Mutations in zebrafish tumour-suppressor genes as well as human and mouse oncogenes are being introduced into the zebrafish at early embryonic stages to help investigate potential primary gene collaborators as well as the sequential progression to malignant transformation. There are, however a large number of transgenic mouse models of cancer that have also been used to identify collaborating mutations that may accelerate carcinogenesis (Gonzalez-Rodriguez *et al.*, 2012; Hu *et al.*, 2012; Rivina & Schiestl, 2013; Sugihara & Saya, 2013), however these classical mouse models have often proven to be difficult and expensive. As such, the high genetic/cellular conservation in zebrafish as well as the reproducible defects in the early embryogenesis and the relative ease to identify genetic driver mutations in carcinogenesis, has clearly demonstrated the power of the zebrafish to study human cancers.

CHAPTER 2 MATERIALS AND METHODS

2.1 ZEBRAFISH HUSBANDRY AND HOUSING

Zebrafish (*Danio rerio*) were maintained according to established standard protocols (Westerfield, 2000). The use of zebrafish in this study was approved by the Dalhousie University Animal Care Committee, under protocol #11-129 (expires on December 1st, 2013). Adult fish were kept at 28.5^{o}C under 14:10 light:dark schedule and feed twice a day with cultured brine shrimp, *Artemia spp.* (INVE Aquaculture Nutrition, Salt Lake City, UT, USA). Fish water was purified using reverse osmosis and supplemented with Instant Ocean® sea salt (Spectrum Brands, Inc., Madison WI, USA) and sodium bicarbonate (Aquatic Eco-Systems, Inc., Apopka, FL, USA) at a pH between 6.8 and 7.5.

Embryos were collected and grown at 28^{o}C in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.4 mM $CaCl_2$, and 0.16 mM $MgSO_4$) at a pH of 7.5. Methylene Blue was also added to the egg water to inhibit the growth of mold (1 x 10^{-5}%[v/v]). Embryos were kept in egg water for up to 7 days before being placed on the fish system in adult tanks. Egg water supplemented with 0.003% (w/v) 1-phenyl-2-thiourea (PTU; Sigma-Aldrich, St. Louis, MO, USA) to produce embryos without pigmentation. For early dechorination, embryos were treated with 10 mg/mL of stock Pronase for 15-25 minutes at 35^{o}C (Roche Applied Science).

2.2 TRANSGENIC LINE CONSTRUCTION, GENOTYPING AND ACTIVATION

2.2.1 NUP98-HOXA9

T human *Nup98-HoxA9* (*NHA9*) fusion gene was kindly provided by DG Gilliland and cloned down stream of the 9.1 kb zebrafish spi1 promoter and a loxP-

EGFP-loxP (lGl) ‘strong STOP’ cassette (*spi1::lGl::NHA9*). The *NHA9* vector construction, microinjection, screening and line generation were by performed by C Grabher and F-B Kai using published methods (Forrester *et al*., 2011; Grabher, *et al.* 2004). For these *NHA9*-transgenic fish, the excision of the lGl ‘strong STOP’ is necessary to induce transgene expression. To achieve this, *NHA9* fish were outcrossed to the *Tg*(*heat shock protein 70 [hsp70]::Cre*; [Cre]) line. Embryos were then incubated at 39^{o}C for one hour (‘heat shocked’), at indicated time points, to induce the Cre recombinase. Cre excised the lGl ‘strong STOP’ cassette thus allowing expression of *NHA9* (*Tg[spi1::NHA9]*).

2.2.2 runx1

The transgenic NHA9 and Cre zebrafish lines were crossed to two different transgenic reporter lines; the transgenic zebrafish reporter line *Tg(runx1::EGFP)* (*runx1::GFP*) was graciously donated by the KE Crosier lab (University of Auckland School of Medicine, Auckland, New Zealand) and marks discrete sites of progenitor blood cells. The reporter lines were outcrossed to the *NHA9* and *Cre* lines to produce two F_1 heterozygous generations (*Tg(spi1::lGl::NHA9;runx1::EGFP;* [*NHA9;runx1*]*);; Tg(hsp70::Cre;runx1::EGFP; [Cre;runx1]).* The F_1 generations were then crossed and screened to produce homozygous F_2 lines for *runx1::EGFP*.

To produce the (*Tg(spi1::lGl::NHA9;runx1::EGFP)* F_2 line, embryos from the F_1 incross were screened by fluorescent microscopy at 24 hours post fertilization (hpf) for brightest transmission of GFP from the NHA9 construct, then screened again at 48 hpf for brightest transmission of GFP from the *runx1* construct. Expression of *spi1* diminishes after 30hpf and is again expressed strongly later during embryogenesis. This

reduced expression of *spi1* reduces the *NHA9* construct GFP expression, thus allowing screening of *runx1* at 48hpf. The embryos were allowed to grow to approximately three months post fertilization then were individually outcrossed to wild-type, non-reporter line. Embryos were once again screened for GFP at 24 and 48 hpf for *NHA9*, and runx1. Only F_2 parents that produced 90-100% GFP positive fish at both time points were kept and used for further study.

To produce the *Tg(hsp70::Cre;runx1::EGFP)* F_2 lines from the F_1 incross were screened at 48 hpf for brightest GFP expression. Embryos were allowed to grow to approximately three months post fertilization then were individual outcrossed to a wild-type, non-reporter line. Progeny were once again screened for GFP at 48 hpf. If embryos were GFP positive (GFP^+) they were genotyped using the REDExtract-N-Amp™ Tissue PCR Kit (Sigma-Aldrich), according to manufacturer's instructions for Cre. For genotyping, 10 embryos were used and only the F_2 fish that produced 9-10 positive Cre and 90-100% GFP positive embryos were kept and used for further study.

2.3 MORPHOLINO OLIGONUCLEOTIDE (MO)

Morpholino oligonucleotides were purchased at GeneTools LLC Philomath, OR, USA). The MOs listed in **Table 2.1** work to selectively suppress gene expression by inhibiting ribosome binding to the mRNA and thus protein translation (this appears as atg/5'UTR'). MO were diluted in sterile milliQ water to designated concentrations, heat activated at 65°C for 10 minutes, suspended in 0.1% (w/v) phenol red then injected into embryos at 1-2 cell stage. Gene inhibition through this technique has been demonstrated to last up to seven days post injection, but does rely on the specific gene of which is being targeted.

Table 2.1 Morpholino sequences for blocking gene expression

Gene	Target site	MO sequence	Injected concentration
dnmt1[1]	atg5'/UTR	ACAATGAGGTCTTGGTAGGCATTTC	0.75 mM
urfh1[2]	atg5'/UTR	CACCTGAATCCACATGGCGGCAAAC	0.5 mM

[1] (Rai et al., 2006)
[2] (Chu et al., 2012)

2.4 WHOLE MOUNT RNA *IN SITU* HYBRIDIZATION (WISH)

2.4.1 Bacterial Cloning

To amplify plasmids, the DH5α™ (Invitrogen Corporation, Carlsbad, CA, USA) strain of chemically-competent *Escherichia coli* (*E. coli*) were used for transformation, plating and culturing. For transformations, 1-5 µL of plasmid suspended in sterile milliQ water were incubated with 35-40 µL of *E. coli* for 10 min on ice then heat shocked at 42°C for 45 seconds. Transformed bacteria were then incubated in 250 µL of Super Optimal broth with Catabolite repression for one hour at 37°C. After, 50-200 µL of culture was spread-plated on Lysogeny/Luria broth agar supplemented with antibiotic (either 50 µg/mL kanamycin or 100 µg/mL ampicillin), placed inverted at 37°C overnight. A colony was selected using a sterile pipette and placed in 2-5 mL of LB broth with the appropriate antibiotic, placed on a shaker at 37°C and left overnight. To make a glycerol stock of the colony, 0.5-1 mL of LB liquid culture was added to 100-200 µL 80% (v/v) glycerol and stored at -80°C. Remaining LB culture was purified using GelElute™ Plasmid Mini-prep Kit (Sigma-Aldrich) according to the manufacturer's instructions. Purified plasmid was stored at -20°C.

2.4.2 RNA Probe Synthesis

RNA probes for *spi1/pu.1, lcp1, gata1* and *dnmt1* were designed using published techniques. cDNA vectors were linearized post bacterial cloning and mini-prep

purification, then using T7 or SP6 polymerase with digoxigenin RNA labeling kits, according to manufacturer's instructions (Roche), RNA anti-sense probes were synthesized. Template DNA was digested using TURBO^{TM} DNase (AB/Ambion) and probes were purified using NucAway^{TM} Spin Columns (AB/Ambion). To confirm proper synthesis, 2 µL of purified probe was run on a 1% agarose gel at 110 volts for 30 minutes with an appropriate ladder. Only probe that produced one solid band at the correct size was used for further experiments

2.4.3 Embryo Preparation

Post microinjection, heat shock and/or chemical treatment, embryos were fixed in 4% (w/v) paraformaldehyde (PFA) for 24-48 hours at directed time points. To help permeabilize embryos post fixation, embryos were washed 3.x5 minutes in 1x phosphate buffered solution plus 10% Tween 20 (PBS-T) then incubated in Proteinase K (ProK; Roche) for indicated times (**Table 2.2**). Following ProK treatment, embryos were washed for 5 minutes in PBS-T then placed back into PFA for a minimum of 20 minutes.

Table 2.2 ProK permeabilization times for zebrafish embryos at various times of development.

Embryonic Stage	Length of treatment (min)	ProK Concentration (µg/mL)
18 hpf	0	N/A
24 hpf	20	1
30 hpf	5	10
36 hpf	10	10
48 hpf	20	10
60 hpf	10	50
72 hpf	20	50

Adapted from Talbot JC, https://wiki.zfin.org/display/prot/Triple+Fluorescent+In+Situ

2.4.4 WISH Protocol

The WISH protocol was adapted from the published protocol (Westerfield, 2000). Fixed embryos were washed in pre-hybridization buffer (Hyb–; 5X saline sodium citrate buffer with 0.1% (v/v) Tween 20 (SSC-T), 50% (v/v) formamide) for 15 minutes at 65^{o}C, and then blocked in hybridization buffer (Hyb+; same formulation as Hyb–, supplemented with 5 mg/mL torula (yeast) RNA type IV, 50 µg/mL heparin) for 1 hour at 65^{o}C. Embryos were incubated overnight at 65^{o}C in labeled anti-sense RNA probes (1:100 to 1:200 dilution). Embryos were then washed 1 x 30 and 1 x 15 minutes in 2X SSC-T/50% (v/v) formamide, 1 x 15 minutes in 2X SSC-T, and 1 x 30 and 1 x 15 minutes in 0.2X SSC-T (all washes at 65^{o}C). Next, embryos were washed 3 x 5 minutes in 1X maleic acid buffer (MAB-T; 100mM maleic acid, 150mM NaCl, 10% [w/v] Tris, 0.1% [v/v] Tween-20, pH 7.5) at room temperature, and incubated in WISH blocking solution (2% [w/v] blocking reagent [Roche], 10% [v/v] heat-inactivated FBS in 1X MAB-T) at room temperature for 1 hour. Embryos were incubated overnight at 4^{o}C in sheep anti-digoxigenin/-fluorescein-AP, Fab fragments (Roche Applied Science) (2^{o}) antibody (1:10,000). At room temperature, embryos were then washed 1 x 15 minutes in WISH blocking solution and 2 x 15 minutes in 1X MAB-T.

For chromogenic development with BCIP/NBT (Vector Laboratories, Inc., Burlingame, CA, USA), embryos were washed 4 x 5 minutes in 0.1M Tris, pH 9.5 staining buffer. Staining was performed according to manufacturer's instructions for 1-4 hours (length of time depending on specific probe) at room temperature in the dark. To stop the staining, embryos were washed in 1X PBS-T for 5 minutes. Embryos were then

de-stained in 100% methanol for 1-5 minutes, and transferred back to 1X PBS-T for imaging. For long-term storage at 4^{o}C, embryos were placed in 4% (w/v) PFA.

2.5 EXPERIMENTAL CHEMICAL COMPOUNDS

2.5.1 β-catenin Studies

The Wnt/β-catenin (β-cat) pathway was both inhibited and stimulated in the course of this study. The β-cat pathway was stimulated using varying doses of 16, 16-dimethyl-Prostaglandin E_2 (dmPGE$_2$; Santa Cruz) and inhibited using varying doses of either Indomethacin (Indo; 1-[4-Chlorobenzoyl]-5-methoxy-2-methyl-3-indoleacetic acid; Sigma-Aldrich) or NS-398 (N-[2-(Cyclohexyloxy)-4-nitrophenyl] methanesulfonamide; Sigma-Aldrich) in dimethyl sulfoxide. To ensure long-term stability, drugs were resuspended in 20 mM stock solution in dimethyl sulfoxide (DMSO). Remaining embryos were treated in 0.3% DMSO to served as a vehicle control.

Chemical treatments were conducted on dechorinated embryos staged at 24 hpf. Embryos were arrayed into groups of 25-30 and placed into a sterile 6-well plate with 6 mL of pure E3 medium (Methylene Blue-free, PTU-free). Chemicals were then added to the wells at indicated concentrations and the plate entire plate was concurrently heat-shocked for one hour. Post heat shock, the plate was moved to a 28^{o}C incubator. Embryos were kept in treatment until 30hpf.

2.5.2 dnmt1 and HDAC Studies

The function of dnmt1 was inhibited using either Decitabine (5-aza-2'-deoxycytidine; DAC; Sigma-Aldrich) or Zebularine (1-(β-D-Ribofuranosyl)-1,2-dihydropyrimidin-2-one, 2-Pyrimidone-1-β-D-riboside; Zeb; Sigma-Aldrich) (Bradbury,

2004; Gros et al., 2012; Rai, 2006). To ensure long-term stability, drugs were resuspended in 20 mM stock solution in DMSO.

To inhibit the function of histone deacetylase complexes (HDACs) chemically, embryos were treated with either Valproic Acid Sodium Salt (2-propylpentanoic acid sodium; VPA; Sigma-Aldrich) or Trichostatin A ([R-(E,E)]-7-[4-(Dimethylamino)phenyl]-N-hydroxy-4,6-dimethyl-7-oxo-2,4-heptadienamide; TSA; Sigma-Aldrich). As with previously listed chemicals, TSA was resuspended in DMSO at 5 mM stock concentration, however, VPA was resuspended in sterile milliQ water at 300 mM stock concentrations. Treatment protocols were identical to that of the β-cat studies and consisted of adding embryos to a 6-well plate, adding the indicated concentration of chemical then heat shocking and growing the embryos at 28^{o}C until 28-30 hpf.

2.5.3 Drug optimization and dose curves using hematopoietic dysregulation as phenotypic readout

The experimental design in this thesis for drug curves was not based on global zebrafish toxicity but focused on changes to hematopoiesis at 30hpf as assessed by WISH for the myeloid blood marker *lcp1* and the erythroid blood marker *gata1*. The objective of the dose curves was to determine at what maximum dose AB wild-type embryos could be treated without any changes to normal hematopoiesis. Once the dose was narrowed down, smaller dose treatments were conducted with activated *NHA9* and *Cre* (control) embryos. The dose that represented the most prominent inhibition of the *NHA9*-induced hematopoietic phenotype through assessment of WISH while not affecting *Cre* control embryos is the specified dose in the respective sections.

Two different dose treatment strategies were used: 1) a linear combination strategy was used to see if the drugs had an linear effect on the *NHA9* phenotype by using specific percentages of the monotherapy concentrations of each drug where a 20 percent reduction in concentration from 'drug A' was followed by a 20 percent increase in concentration from 'drug B' and 2) the synergistic combination strategy by which both drug 'A' and 'B' are reduced simultaneously. If the combined drug treatment response is linear, then 50 percent of the monotherapy concentration of drug A combined with 50 percent of drug B would have a similar effect as the drugs given as a monotherapy. Conversely, if there is a synergistic effect, then combining the drugs at levels that are below 100 percent (i.e., 20 percent concentration of drug A and B) should yield a greater rescue of the phenotype than expected (i.e., greater than a 40 percent recovery).

2.6 STEM CELL QUANTIFICATION

2.6.1 Experimental design

The effects of *NHA9* on stem cells was explored using both *runx1::GFP* and *CD41::GFP* transgenic lines. Here, *NHA9;runx1* and *NHA9;CD41* were crossed with *Cre;runx1* and *Cre;CD41*, respectively, while *runx1::GFP* and *CD41::GFP* were incrossed as GFP controls. Both groups were grown to 24 hpf then heat-shocked and left to develop to 48 hpf, then euthanized and dissociated into single cell suspension to be used in FACS analysis (*see section 2.6*). An AB wild-type, non-reporter line of zebrafish was also used as a control for auto-fluorescence. Embryos were heat-shocked at 24 hpf then allowed to develop to 48 hpf at 28^{o}C.

2.6.2 Whole embryo dissociation and fluorescence-activated cell sorting (FACS)

To quantify the number of GFP^+ stem cells, 25-30 embryos were euthanized by a Tricaine overdose at either 48 hpf. Embryos were transferred to 15 mL falcon tubes, tricaine removed and 1.2 mL of pre-warmed protease solution (0.25% trypsin, 1mM EDTA and 45 mL 1X PBS) added. To the protease solution 27 µL of collagenase (Sigma-Aldrich) at 100 mg/mL was added. Falcon tubes were then vortexed and placed at 35^{o}C for 15 minutes. After embryos were dissociated using glass Pasteur pipettes by drawing the solution up and down, forcibly, 10 times and vortexing for 10 seconds, then placed back at 35^{o}C for 5 minutes. This process was repeated as necessary until visual inspection of the solution revealed fully dissociated embryos, which took approximately 45 minutes. Once dissociation was complete, 600 µL of STOP solution (30% FBS, 6 mM $CaCl_2$, PBS, 6 mL sterile milliQ water) was added to each Falcon tube to stop the enzymatic reaction between dissociation solution and the embryos. Next, 40 µm filter nets were placed over each well of a 6-well plate. After 5 minutes of adding the STOP solution, the suspension was transferred over the net using the same Pasteur pipette, to filter out un-dissociated cells and the embryo spines. Next, the filtered cell suspensions were transferred to 1.5 mL eppendorf tubes at centrifuged at 2000 rpm at 4^{o}C for 5 minutes to create a single-cell pellet. The tubes were placed on ice and the supernatant removed and was replaced with 200 µL of chilled PBS-FBS solution. Tubes were then quickly vortexed to resuspended the cells for FACS quantification. Cell suspensions were sorted using a FACSAria^{TM} I (BD Biosciences, Mississagua, ON) at 4^{o}C at GFP^+ stem cells were gated for GFP^{HI} (509 nm).

2.7 MICROARRAY ANALYSIS

Microarray studies employing Agilent oligo-arrays were performed in collaboration with Dr. Stephen Lewis at the Atlantic Cancer Research Institute in Moncton, NB. Extraction of mRNA was performed using the Trizol + RNeasy method. In Dr. Lewis's laboratory, the purified mRNA was then reverse transcribed to generate cDNA that was differentially labeled 80 with fluorescent dyes using the SuperScript Plus Direct cDNA Labeling System (Invitrogen). Specifically, groups of mRNA were labeled with Alexa Fluor® 555 (orange fluorescent dye, false-coloured green for analyses) or with Alexa Fluor® 647 (far-red fluorescent dye) (Invitrogen). Any samples with low concentrations of RNA were subjected to whole genome amplification using Amino Allyl MessageAmp II aRNA Amplification Kit (AB/Ambion) with Alexa Fluor® 555 and Alexa Fluor® 647 reactive dye packs (Invitrogen). Equal amounts of labeled cDNA were mixed and competitively hybridized to a zebrafish oligonucleotide genome array containing 43,803 probes (Agilent) using standard procedures. The hybridized array was scanned and analyzed using a GenePix 4200AL autoloader microarray scanner (Molecular Devices, Japan) and associated software (GenePix Pro 6.0, Acuity 4.0). The microarrray experiments were performed in triplicate for each sample pair. In addition, 'dye-swap' experiments, in which the fluorescent label for each sample is switched, were performed in triplicate to eliminate any variation due to labeling efficiency and/or dye properties. The mRNAs whose expression changed ≥2-fold were considered significant.

2.8 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

2.8.1 Primer design

Primers (Table 2.3) for zebrafish transcripts were designed using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/, Rozen and Skaletsky 2000) with modified parameters (Dorak 2006):

- PCR product size: 70 – 150 bp;
- Primer must span an exon-exon junction (Exon at 5'side: 7, Exon at 3' side: 4);
    - *OR*, Primer must be separated by at least one intron (Min: 1000, Max: 1000000);
- Database: Refseq RNA;
- Misprimed product size deviation: 250 bp;
- Splice variant handling: YES;
- Primer GC content: 40 – 60%;
- GC clamp: 1;
- Max Poly-X: 4;
- Max 3' end complementarity: 2.0

2.8.2 Reagents, thermocycler conditions, controls & analysis

qRT-PCR was performed using QuantiFast™ SYBR® Green PCR Kit (QIAGEN) with a Stratagene Mx3000P™ QPCR thermocycler (Agilent Technologies, Inc., Santa Clara, CA, USA). Two-step thermocycler conditions were: 1) 1 cycle – 95°C, 5:00 to activate; 2) 40 cycles – 95°C, 0:10 to denature, 60°C, 0:30 combined annealing and extension, with collection of fluorescence data; 3) 1 cycle for melting curve analysis – 95°C, 1:00 to denature, 55°C, 0:30 climb to 95°C, 0:30, with collection of fluorescence

data. Reverse-transcription was performed on 0.5 – 2 μg of RNA. Four-fold dilutions of cDNA were made. For each primer set, two dilutions per genotype were plated in triplicate; in effect, each primer set per genotype had six representations for each independent round of amplification. Negative controls for qRT-PCR were: 1) no template controls (NTC) for each primer set; 2) no RT for each independent RNA

2.9 IMAGING

For whole-mount *in situ* hybridization (WISH), embryo images were captured on a Leica MZ16F microscope (5X objective) with a Leica DFC 490 color camera running Leica Application Suite, Version 2.4.0 [Build:795] (Leica). An inverted Zeiss AxioObserver.Z1 microscope (5X, 10X and 40X objectives) equipped with a Colibri LED light source and a Zeiss AxioCam HRm camera running Zeiss AxioVision, Release 4.7.1 software (Carl Zeiss Microimaging GmbH, Gottingen, Germany), was used to capture fluorescent images.

2.10 WISH QUALITATIVE ANALYSIS

For the analysis of WISH, embryo samples were organized in a randomized manor by an unbiased, external research associate in order to limit any sort of biased classification. A qualitative analysis was conducted by comparing normal, wild-type expression levels of the respective gene to the expression levels of the embryos in each sample. A predetermined, categorized ranking system, consisting of of high, normal, low-normal and low expression was given to each embryo. Samples were averaged together to produce the percentage of embryos with the corresponding expression levels (i.e., 28 percent of embryos had high *lcp1* expression).

2.11 STATISTICAL ANALYSIS

Micrographs are representative of at least 2 independent trials with a minimum of 15 embryos per genotype, per trial. Data reported as mean values +/- SEM.

CHAPTER 3 RESULTS

3.1 MICROARRAY ANALYSIS OF *NUP98-HOXA9*-TRANGENIC ZEBRAFISH EMBRYOS

NUP98-HOXA9 (*NHA9*)-transgenic zebrafish develop both a myeloproliferative neoplasm (MPN) in adults as well as embryonic defects in hematopoiesis (*see section 1.7*). In order to identify downstream genes involved in *NHA9*-mediated disease, past lab member, Dr. Michael Forrester performed a microarray analysis to assess global changes in gene expression in *NHA9*-transgenic zebrafish embryos. To maximize accuracy, the *Cre*-activated *NHA9* embryos (the mating result of homozygous *Tg*[*spi1::lGl::NHA9*] (*lGl::NHA9*) fish with *Tg*[*hsp70::Cre*] (*Cre*)homozygous) were compared to unactivated *NHA9* embryo controls (the mating result of homozygous *Tg*[*spi1::lGl::NHA9*] fish with wild-type AB fish). Both sets of embryos were heat-shocked at 24hpf, then euthanized at 28 hpf, after which RNA extraction was performed and samples were shipped to Dr. Stephen Lewis at the University of New Brunswick for cDNA labeling and microarray hybridization.

The Wnt/β-catenin (Wnt/β-cat) pathway has been well demonstrated to be involved in the pathogenesis of AML (*see section 1.4.3*) As such, our hypothesis was that we would observe abnormal gene expression for members of the PTGS/COX-PGE_2 signaling pathway which is involved in the activation of β-catenin (β-cat) in the canonical Wnt/β-cat pathway in blood cells (Goessling *et al*., 2009; North *et al*., 2007) (refer back to **Figure 1.4.1**). Therefore, we anticipated microarray hits for zebrafish genes, such as PGE2 synthase, *ptgs1*, which was identified in a microarray analysis of human NHA9 cell culture (Ghannam *et al*., 2004); the zebrafish isoforms of the COX2 enzyme, *ptgs2a* and *ptgs2b*, which were identified in a separate human NHA9 microarray (Takeda *et al.,*

2006), and were also upregulated in *AML1-ETO*-transgenic zebrafish (Yeh *et al*., 2009); or the zebrafish *prostaglandin E receptor, subtype EP1a* (*ptger1a*), which was upregulated in the mouse model of *Hoxa9;Meis1*-induced AML (Wang *et al*., 2010). However, analysis of the microarray data did not find any of these genes to have altered gene expression levels. However, the microarray analysis did yield data identifying other genes of interest in AML pathogenesis. For example, zebrafish *gli2a* and *mitochondrial ribosomal protein L53* (*mrpl53*) were two of the most down-regulated hits (>3-fold); whereas *dnmt1* and *fos* were two of the most upregulated genes (>7-fold), which were confirmed by qRT-PCR. In this thesis, I will focus on determining the potential role of *dnmt1* as well as assess the potential of dnmt1 as a druggable target in *NHA9*-mediated myeloid disease (**Figure 3.1**).

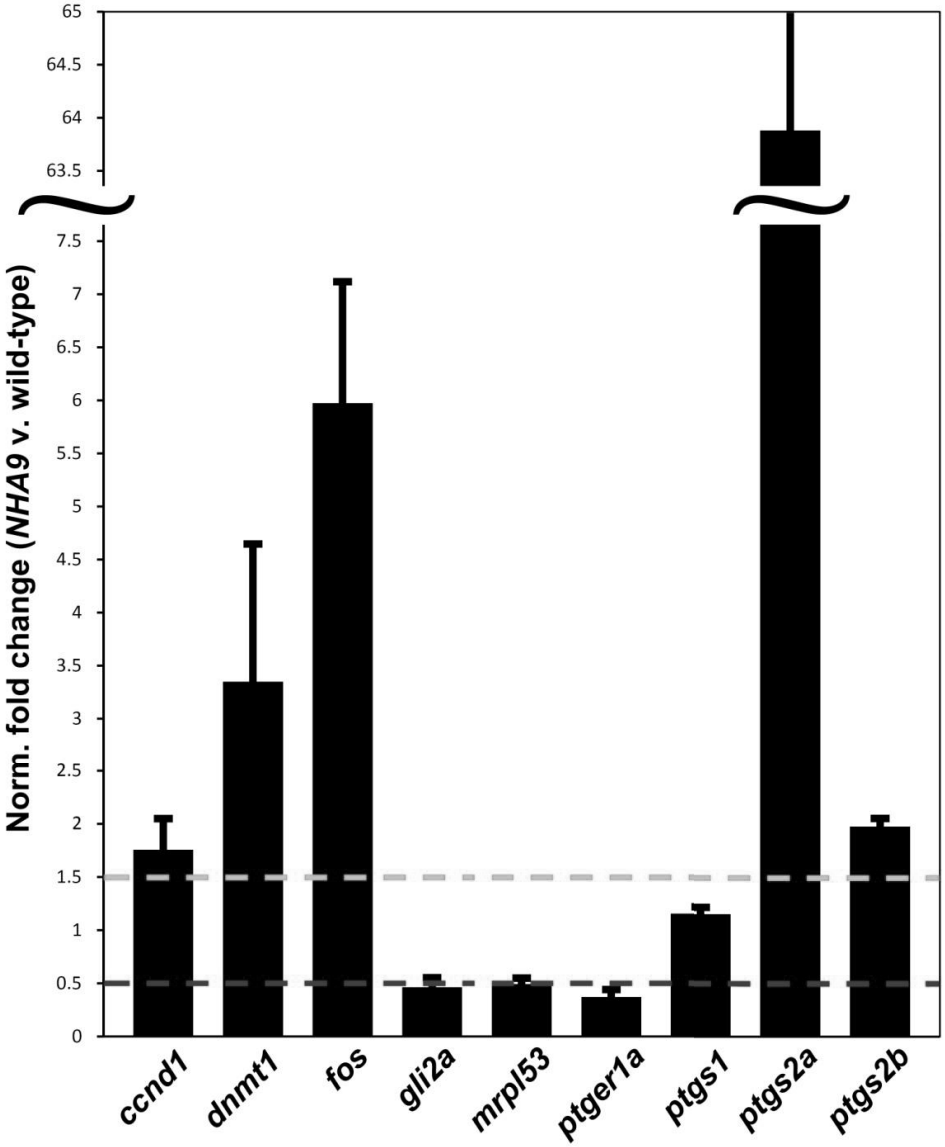


Figure 3.1 qRT-PCR from *NUP98-HOXA9* embryos depicting confirmation of microarray results and Wnt/beta-catenin investigations.

RNA extracted from wild-type and *NHA9* embryos at 28 hpf. qRT-PCR results show the following changes to gene expression: *ccnd1*, 1.75 0.29-fold increase (n=3); *dnmt1*, 3.35 1.30-fold increase (n=3); *fos*, 5.97 1.15-fold increase (n=3); *gli2a*, 2.16 0.43-fold decrease (n=2); *mrpl53*, 2.04 0.25-fold (n=2); *ptger1a,* 2.71 0.56-fold decrease (n=2); *ptgs1*, 1.15 0.07-fold wild-type-like (n=6); *ptgs2a*, 63.89 6.01-fold increase (n=3); *ptgs2b*, 1.98 0.08-fold increase (n=4).
(Bar graphs denote fold change in *NHA9* embryos compared to wild-type, normalized to *ef1a*. Black and gray dotted lines represent the lower and upper boundaries [0.5X and 1.5X expression of wild-type, respectively] for real change in expression.)
RNA extraction performed together with Dr. Forrester, qRT-PCR performed by Dr. Forrester

3.2 *NHA9* UPREGULATES *DNMT1* IN *NHA9*-TRANSGENIC EMBRYOS

The microarray analysis identified *dnmt1* to be upregulated in *NHA9*-transgenic embryos. Subsequently, qRT-PCR and WISH against *dnmt1* was used to examine changes to gene expression, which confirmed that *dnmt1* was indeed upregulated 3.35 ± 1.30-fold (n=3) (**Figure 3.1; Figure 3.2.1**). *DNMT1* encodes the major maintenance DNA methyltransferase in animals (Song *et al.*, 2012), which epigenetically regulates the expression of terminal differentiation genes in various tissues (Anderson *et al*., 2009; Rai, 2006; Rai*et al*., 2006; Tittle *et al*., 2011). Upregulation of *DNMT1* has been associated with loss-of-function *CCAAT/enhancer binding protein alpha* (*C/EBPA*) and *RUNX1* mutations in human AML, which leads to hyperactivity of MYC and a repression of terminal hematopoietic differentiation (*see section 1.5.4*). However, increased expression of *DNMT1* has not previously been associated with *NHA9*-mediated disease. Given this role in myeloid disease, further investigation of *dnmt1* seemed warranted. Moreover, a translation-blocking morpholino (MO) targeted to zebrafish *dnmt1* was readily available and validated in a number of past studies (Anderson et al., 2009; Rai, 2006; Rai et al., 2006; Tittle et al., 2011) as well as US FDA approved pharmacological dnmt1 inhibitors that are currently in clinical trials to combat various myeloid diseases.

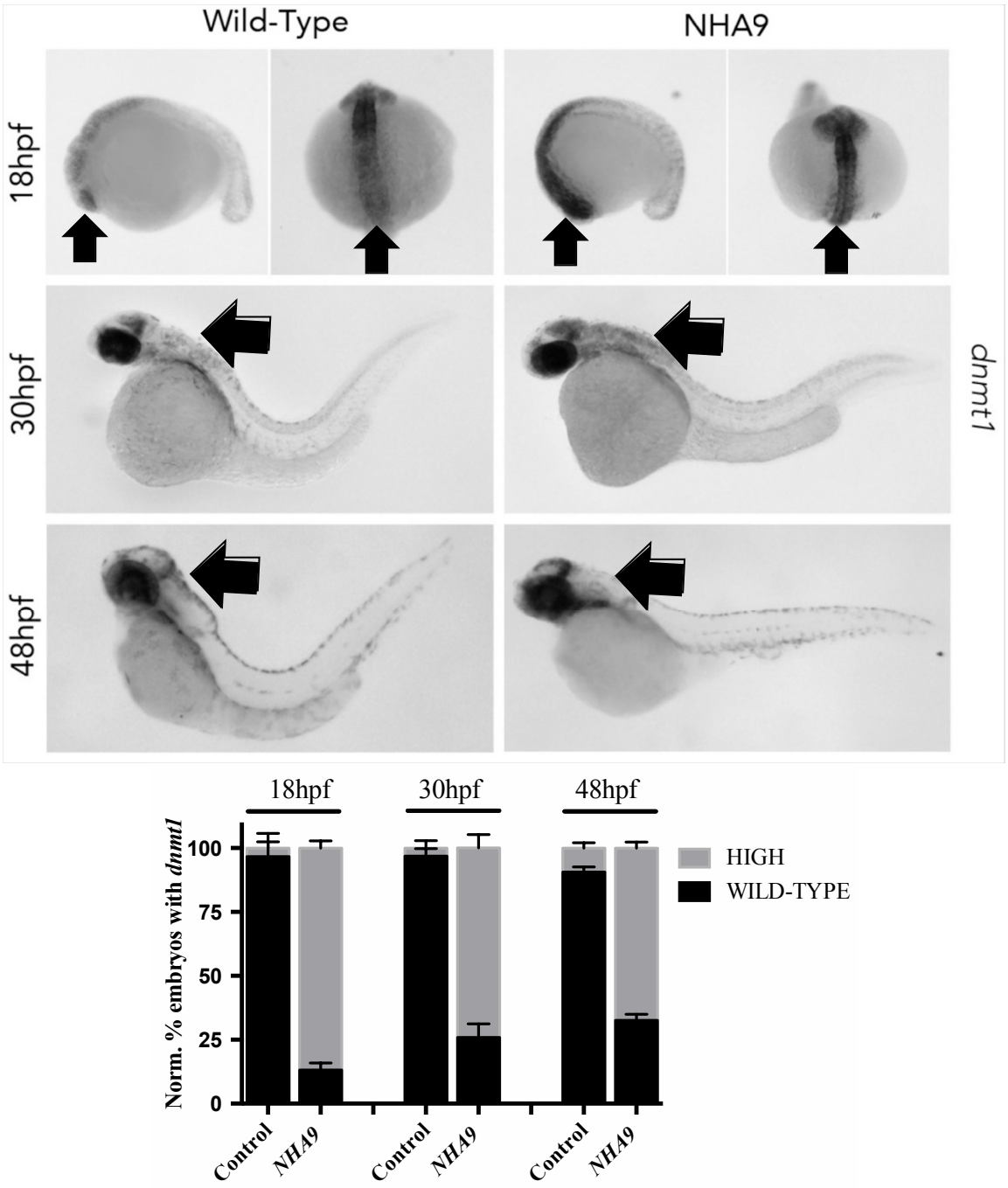


Figure 3.2.1 Activation of NHA9 in zebrafish embryos upregulates dnmt1 expression.

Embryos stained by WISH for the expression of the DNA methyltransferase *dnmt1*. Dark staining is *dnmt1* expression tissues where it is being expressed, indicated by black arrows. Arrows indicate anterior regions and around the eye lens where *dnmt1* expression is most prominent. Embryos displayed in side profile, anterior to the left. Embryos scored for *dnmt1* expression relative to *Cre* wild-type, normalized to a percentage value of the total number of embryos of that genotype and quantified in bar graph at the developmental time points indicated. . Values are reported as the mean +/- the SEM.

3.2.1 Loss of zebrafish dnmt1 activity inhibits myeloproliferation in NHA9-transgenic embryos

We hypothesized that inhibition of zebrafish *dnmt1* could restore wild-type levels of myeloid cells and by leveraging the published myeloproliferative phenotype in our *NHA9*-transgenic zebrafish embryos we could measure its oncogenic activity. We began by first knocking down the expression of *dnmt1* through translation-blocking MO injection. I performed *dnmt1* MO injections along with research associate, Andrew Coombs and past lab member, Dr. Forrester. *Cre* controls as well as *NHA9* embryos were injected at the one-cell stage with 0.75 mM of MO while uninjected embryos from each group served as controls. We allowed the embryos to grow to 12 and 24 hpf, then heat-shocked for 1 hour and fixed the embryos at 18 and 30 hpf respectively while uninjected embryos from each groups served as controls. We allowed the embryos to grow to 12 and 24hpf, then heat-shocked them for 1 hour and fixed the embryos at 18 and 30 hpf respectively to asses myeloid expression by (*see section 2.3*) WISH analysis against myeloid gene marker *lcp1* and erythroid gene marker *gata1* measured effect of MO on hematopoiesis.

In *Cre* controls, injecting *dnmt1* MO appeared to mildly decrease the global *lcp1* expression at 30 hpf (**Figure 3.2.2A,C**), however there is a known developmental toxicity with this MO (Rai *et al*., 2006). In uninjected, activated *NHA9* zebrafish, approximately 80 percent of the embryos had increased number of *lcp1* expressing cells at 30 hpf, as previously published (Forrester *et al*., 2011; Forrester, 2012). However, in *dnmt1* MO injected, *NHA9* activated embryos, there was a prominent decrease in *lcp1* expression. Additionally, *gata1* expression is greatly reduced in approximately 80 percent of

uninjected, *NHA9* activated embryos After *dnmt1* MO injection, *Cre* controls had a mild decrease in *gata1*expression, which again is most likely a result of the known developmental delay resulting from injection of this MO (Rai *et al*., 2006) while injected *NHA9* activated embryos demonstrated a remarkable restoration of *gata1* expression to that near wild-type levels at both 18 and 30 hpf (**Figure 3.2.2B, D and E**). Furthermore, at 18 hpf, the expression level of *gata1* was increased more robustly in *NHA9* activated embryos than it was at 30 hpf, while the decrease in *gata1* expression demonstrated in *Cre* controls at 30 hpf was limited at 18 hpf (**Figure 3.2.2E**). This robust increase in *gata1* at 18hpf in *NHA9* embryos while mild decrease of *gata1* in *Cre* controls at 18 hpf suggests that the reduced developmental time is sufficient to reduce the *dnmt1* MO toxicity demonstrated at later time points.

In order to ensure that the phenotypic rescue was due to the knockdown of *dnmt1* methyltransferase activity and not due to any other functions the dnmt1 enzyme has in the zebrafish, I injected *Cre* control embryos and *NHA9* embryos with an *ubiuitin-like, containing PHD and RING finger domains, 1(uhrf1*) MO. Similar to *dnmt1* MO studies, *uhrf1* MO was injected at the one-cell stage and the embryos were fixed at 30 hpf. The uhrf1 protein binds to DNA just before a gene promoter region and works to anchor the dnmt1 enzyme to the DNA so that it may start its methylation process. Knocking down the expression of *uhrf1* has been demonstrated to reduce the ability of dnmt1 to methylate DNA (Chu *et al*., 2011; Tittle *et al*., 2011). Therefore, if restoration of normal hematopoiesis in the *NHA9* activated embryos is similarly observed following knockdown of *uhrf1*, this would suggest that the rescue demonstrated with *dnmt1* MO knockdown was due to reduced genomic methylation.

Previous studies have demonstrated that roughly 60 percent of embryos injected at the one-cell stage with *uhrf1* MO die at 50 percent epiboly (~5hpf) (Chu *et al*., 2011). In keeping with this finding, approximately 65 and 73 percent of *Cre* and *NHA9* activated embryos survived to 24 hpf, respectively following *uhrf1* MO injection. However, of the embryos that did survive, approximately 80 percent appeared developmentally normal. Similar to *dnmt* MO studies, *uhrf1* MO was injected at the one-cell stage and fixed at 30 hpf. Uninjected *Cre* and *NHA9* activated embryos served as controls compared to the *uhrf1* MO injected groups. Due to the high percentage of embryonic death after *uhrf1* MO injection, only *gata1* expression analysis was conducted using WISH. Upon assessment, the level of *gata1* expression in *uhrf1* MO injected, *Cre* control embryos appeared slightly lower than the uninjected controls. However, this minor change in *gata1* expression is mostly likely due to the developmental delay of the injected embryos (**Figure 3.2.2B, D**). Encouragingly, preliminary results of *gata1* expression in *NHA9* activated embryos suggest that there is a slight recovery in erythropoiesis, although this recovery does not appear as significant as *dnmt1* MO. Although this experiment was only conducted twice, the overall embryo numbers were low and additional experiments need to be performed in order to better analyze the effects of *uhrf1* inhibition on *NHA9* activated embryos. Additionally, WISH analysis will need to be performed in order to address the changes in myelopoiesis, using *lcp1* and *spi1in situ* probes. Regardless, these preliminary data suggest that *dnmt1* DNA methylation is indeed affecting hematopoiesis in the transgenic *NHA9* zebrafish.

Next, I wanted to test if pharmacological inhibition of zebrafish dnmt1 enzymatic activity with DNA methyltransferase inhibitors such as Decitabine (DAC) would have

similar effects on embryonic hematopoiesis while reducing the global toxicities demonstrated by *dnmt1* MO knockdown. *Cre* (controls) and *NHA9* embryos were grown to 24 hpf when they were concurrently heat shocked for 1 hour and treated pharmacologically with either DAC or Zeb (*see section 2.5.2*). Treatment with 0.3 percent DMSO served as a vehicle control. The embryos were exposed to the chemicals for 5 hours post heat-shock, then fixed at 30 hpf, respectively to assess myeloid and erythroid development using the same WISH protocol and probes as used in *dnmt1* MO injected embryos. Drug optimization was not based on global zebrafish toxicity but rather the maximum dose AB wild-type embryos could tolerate without any changes to normal hematopoiesis. The dose that represented the most prominent inhibition of the *NHA9*-induced hematopoietic phenotype through assessment of WISH while not affecting *Cre* control embryos was selected as the specified dose.

Drug optimization (*see section 2.5.3*) demonstrated that 75 μM of DAC is the maximum tolerated dose before significant hematopoietic dysregulation is demonstrated (**Figure 3.2.3A**). At 30hpf, 75 μM DAC did not appear to affect the global expression of *lcp1,* or *gata1* in *Cre* control embryos (**Figure 3.2.3**), which is consistent with previous findings that human *DNMT1* may be dispensable for normal HSC function (Trowbridge *et al*., 2012). With DMSO treatment, we observed the expected increase of *lcp1*-expressing cells at 30 hpf in the *NHA9* activated embryos compared to the *Cre* controls, though the percentage of embryos displaying the phenotype for these experiments (71% *lcp1*) was slightly less than the published 80 percent (**Figure 3.2.3C, E**) (Forrester *et al*., 2011; Forrester, 2012). However, treatment with DAC results in the return to normal, wild-type *lcp1* expression in *NHA9* embryos at 30hpf. Assessment of *gata1* expression at

30 hpf demonstrated additional encouraging results. At both time points, 75 μM DAC had a mild effects on *Cre* controls where *gata1* was slightly reduced, yet robustly increased the expression of *gata1* in *NHA9* activated embryos to that of normal, wild-type levels (**Figure 3.2.3D, F**). Taken together with WISH evaluation of *lcp1* expression, these data demonstrate that *dnmt1* is an important downstream activator of *NHA9*-induced myeloproliferation and that targeting DNMT1 enzyme activity may be a novel therapeutic option in human AML patients with the *NHA9* fusion oncogene. However, DAC treatment has been associated with a number of toxicities in patients, including genome-wide hypomethylation.

Currently, there are a number of other DNA methyltransferase inhibitors available. These inhibitors, however, are not yet US FDA approved for human treatment, but demonstrate lower overall toxicities in murine models (Champion *et al*., 2010; Cheng *et al.*, 2004; Lund & van Lohuizen, 2004). I therefore, wanted to determine if any other methyltransferase inhibitors would provide an effective alternative to DAC. Zebularine (Zeb) has recently demonstrated strong DNA methyltransferase inhibitory functions with reduced global toxicity effects both *in vitro* and with *in vivo* mouse models, when compared to DAC (Bradbury, 2004; Champion *et al*., 2010; Cheng *et al*., 2003, 2004; Herranz *et al*., 2006). Based on these recent findings, I hypothesized that Zeb would be an appropriate therapy alternative to combat *NHA9*-mediated disease in the zebrafish.

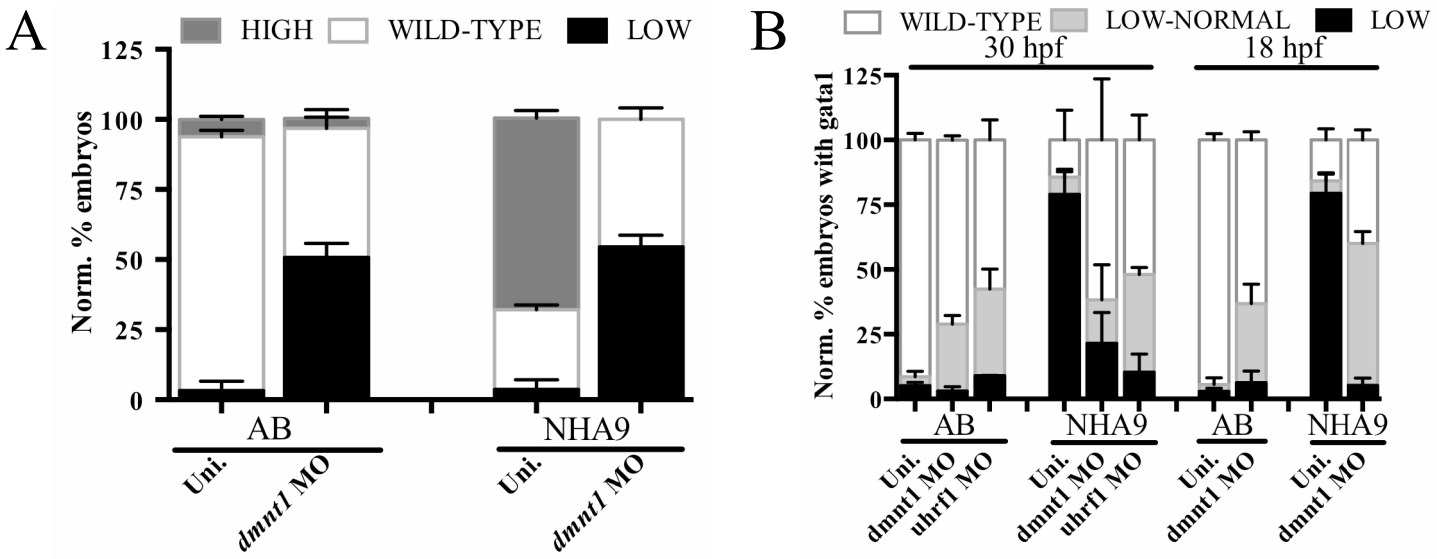
Treatment protocols were similar to the DAC treatment and WISH assessment of *lcp1* and *gata1* expression was again conducted at 30 hpf. Dose optimization and WISH analysis determined that at 250 μM of Zeb had very little effect on AB wild-type fish (**Figure 3.2.3B**). Zeb had very little effect on the number of *lcp1* expressing cells in *Cre*

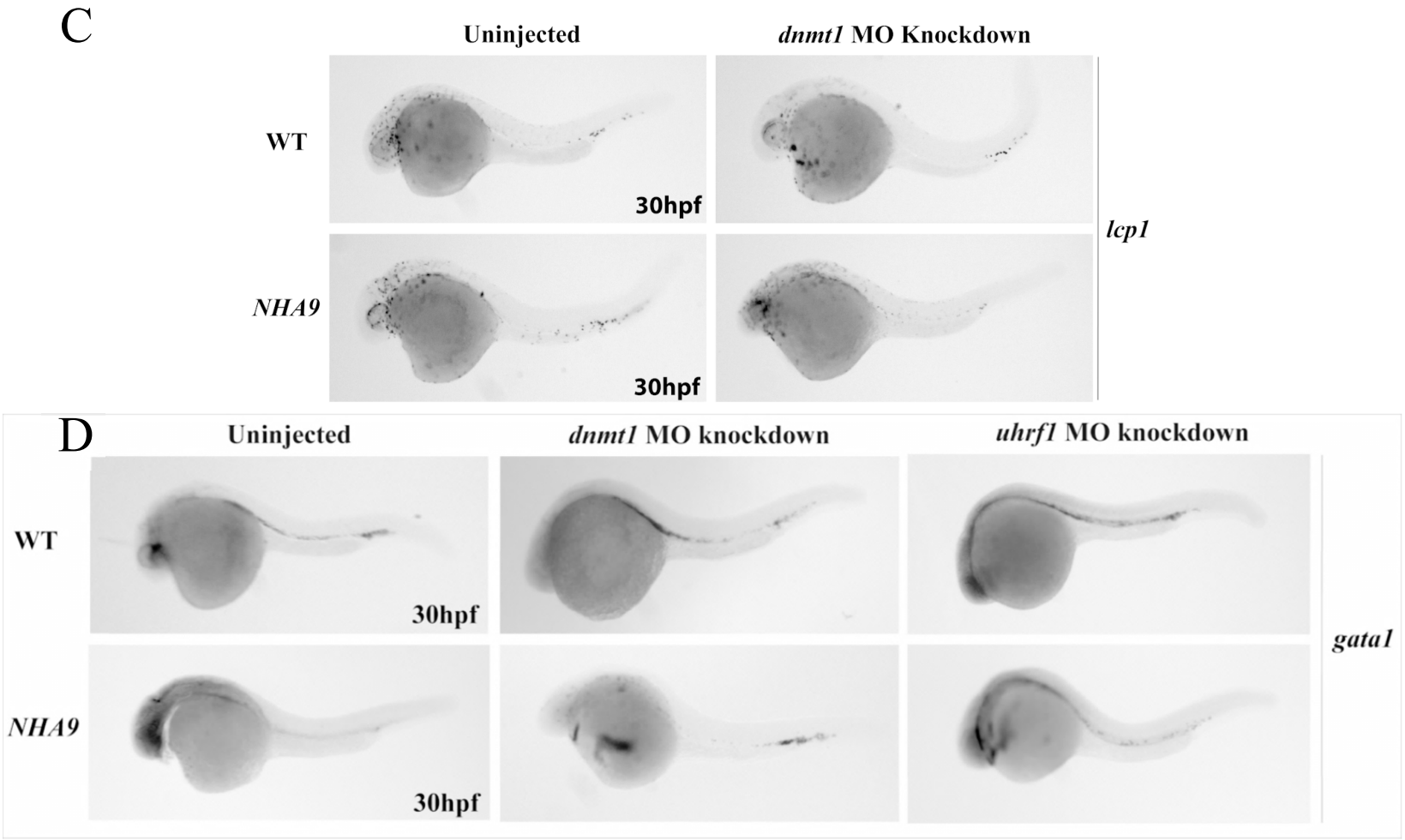
control embryos, yet partially restored normal hematopoiesis in *NHA9* activated embryos to near wild-type expression levels (**Figure 3.2.3C, E**). Additionally, examination of *gata1* expression at 30 hpf in *Cre* controls demonstrated very little effect after treatment with 250 μM Zeb, while in *NHA9* activated embryos, expression of *gata1* was increased significantly and appeared similar to that of the controls (**Figure 3.2.3D, F**). These findings suggest that not only is *dnmt1* important in *NHA9*-induced disease, but also may be inhibited using a number of targeted therapies.

Figure 3.2.2 Zebrafish dnmt1 knockdown inhibits myeloproliferation and restores erythropoiesis

Embryos stained by WISH for the expression of the myeloid gene*, lcp1* and erythroid genes *gata1* at indicated time points. Embryos displayed in side profile, anterior to the left. Dark, punctate staining represents *lcp1* expression in the PBI indicated by black arrows. Embryos were scored for their level of *lcp1* and *gata1* expression relative to *Cre* wild-type, normalized to a percentage value of the total number of embryos of that genotype, and quantified in bar graph for *lcp1* (A), and *gata1* (B). Embryos at the one-cell stage were either uninjected or injected with 0.75 mM translation-blocking *dnmt1* MO. Embryos then heat-shocked at 24 hpf and assessed at 30 hpf for. Each experiment was replicated three times with 15-25 embryos in each group per replicate. *lcp1* (C), and *gata1* (D). . Values are reported as the mean +/- the SEM.

Acknowledgements: Dr. Michael Forrester and Andrew Coombs aided with the morpholino injections




C
Uninjected
dnmt1 MO Knockdown
WT
NHA9
30hpf
30hpf
lcp1
D
Uninjected
dnmt1 MO knockdown
uhrf1 MO knockdown
WT
NHA9
30hpf
30hpf
gata1

E

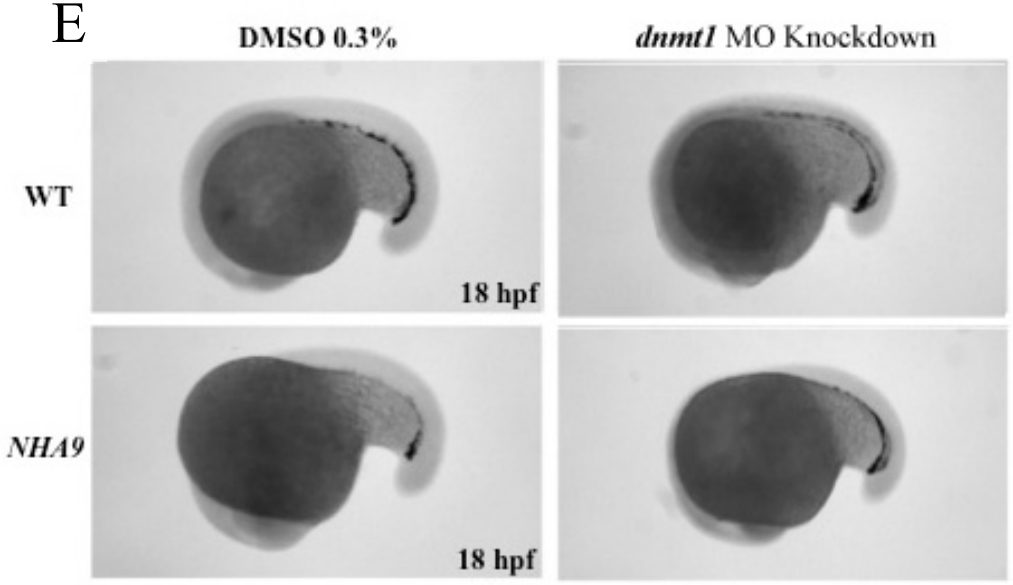
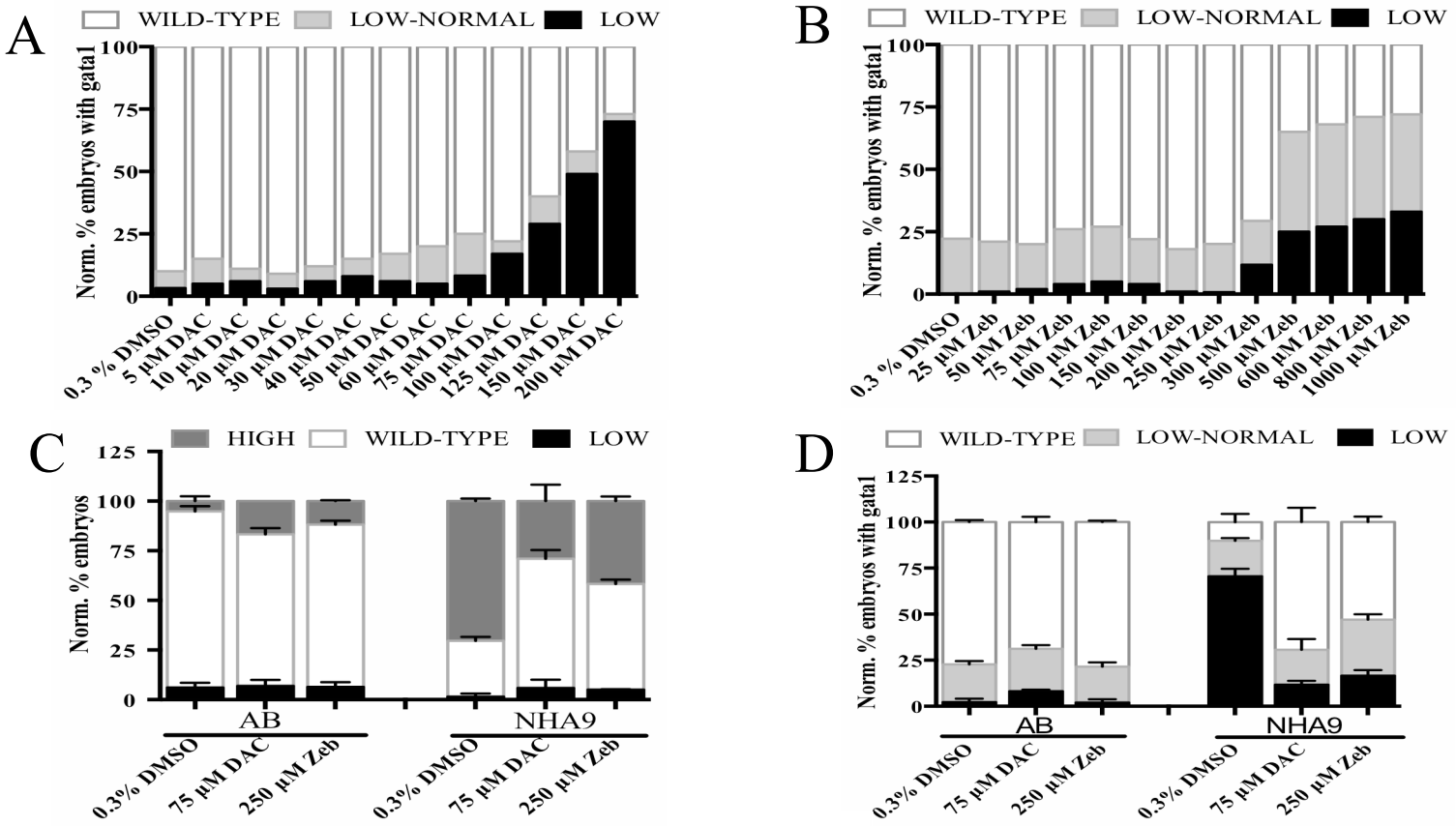
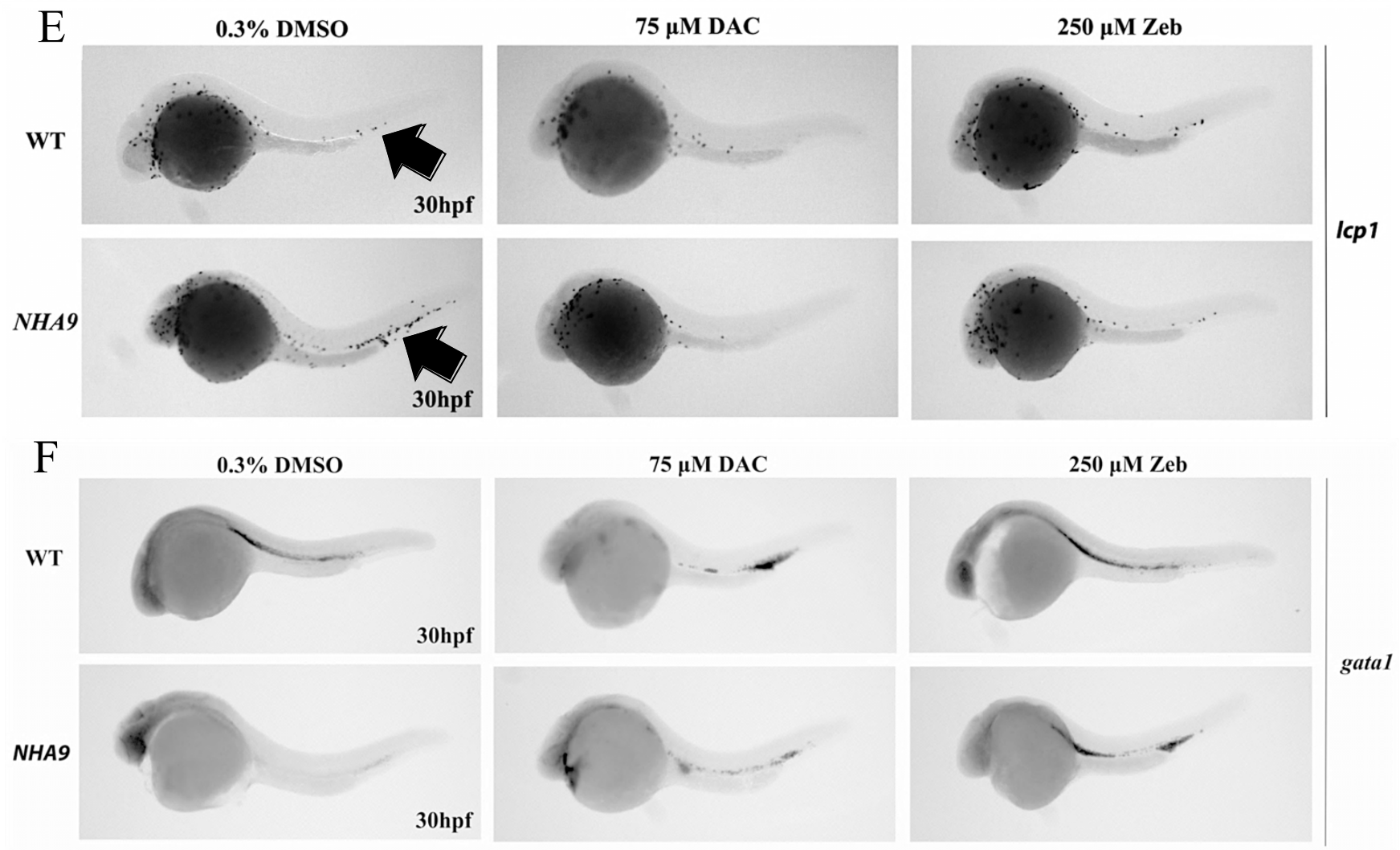


Figure 3.2.3 Pharmacological inhibition of zebrafish dnmt1 enzyme activity with DAC and Zeb restores normal hematopoiesis in *NUP98-HOXA9* transgenic embryos

Embryos stained by WISH for the expression of the myeloid gene, *lcp1* and erythroid gene, *gata1*, at indicated time points. Embryos displayed in side profile, anterior to the left. Dark, punctate staining represents *lcp1* expression in the PBI indicated by blue arrows. Wild-type embryos were treated with DAC (A) and Zeb (B) at 24 hpf and assessed by WISH and represented by graphs (*below*) for *gata1* expression in order to determine optimal treatment dose. *NHA9* embryos were then scored for their level of *lcp1* and *gata1* expression relative to *Cre* wild type, normalized to a percentage value of the total number of embryos of that treatment group, and quantified in bar graph for *lcp1* (C), and *gata1* (D) and represented by micrographs (E and F, respectively). Embryos were treated either with 0.3% DMSO vehicle control 75 µM DAC or 250 µM Zeb, two demethylating agents and inhibitors of dnmt1. Embryos then heat-shocked at 24 hpf and assessed at 30 hpf. Each experiment was replicated three times with 15-25 embryos in each group per replicate. . Values are reported as the mean +/- the SEM.




E
0.3% DMSO
75 µM DAC
250 µM Zeb
WT
30hpf
NHA9
30hpf
lcp1
F
0.3% DMSO
75 µM DAC
250 µM Zeb
WT
30hpf
NHA9
30hpf
gata1

3.2.2 Histone deacetylase complex inhibitors partially restore normal hematopoiesis in NHA9 transgenic zebrafish

Work has been completed in both *in vitro* and *in vivo* mouse models, as well as in the clinical setting to determine the effects of histone deacetylase complex (HDAC) inhibitors in patients with MDS and AML, as well as trials evaluating the treatment effects of combined HDAC and DNA methyltransferase inhibition (Bellos & Mahlknecht, 2008; Blum *et al.*, 2007; Fuks *et al.*, 2000; Göttlicher*et al*., 2001; Marks *et al*., 2001; Rountree *et al.*, 2000; Savickiene *et al.*, 2012). More recent studies have suggested that HDAC and DNA methyltransferase inhibitors may work together to restore normal epigenetic regulation in patients with myeloid disease. As there are a number of studies indicating significant toxicity using DNA methyltransferase inhibitors, such as DAC as a monotherapy, looking for potential synergistic effects between epigenetic modifying drugs may prove to be advantageous by reducing the dose of drug necessary and potentially reducing global toxicity effects. To examine this phenomenon in the zebrafish, I first wanted to see if HDAC inhibitors as monotherapy would work to inhibit *NHA9*-induced myeloproliferation. Fortunately, there are a number of HDAC inhibitors, some of which are in Phase 3 clinical trials and others that are already used in routine clinical practice. Valproic acid (VPA) for instance, is a class I HDAC inhibitor and has found clinical use as an anticonvulsant and mood-stabilizing drug, primarily in the treatment of epilepsy and bipolar disorder, respectively. Additionally it has been used in early clinical trials to combat myeloid disease (Bellos & Mahlknecht, 2008; Blum *et al*., 2007; Cinatl *et al*., 1997; Göttlicher*et al*., 2001; Kuendgen*et al*., 2004; Werling *et al.*,

2001; Wiltse, 2005). Due to the number of studies as well as its current US FDA approval, VPA appeared to be a good HDAC inhibitor drug candidate.

In an identical fashion to DAC and Zeb treatment, *Cre* controls as well as transgenic *NHA9* embryos underwent concurrent heat-shock for 1 hour and drug treatment for 6 hours. Embryos treated in 0.3% DMSO served as controls. Dose optimization on AB wild-type embryos determined 250 μM of VPA to be the maximum dose with little effect on normal hematopoiesis (**Figure 3.2.4A**). Following treatment with 250 μM VPA, *Cre* control embryos demonstrated no substantial change in *lcp1* or *gata1* gene expression at 30 hpf (**Figure 3.2.4C-F**). Interestingly, *NHA9* activated embryos demonstrated a partial recovery of erythropoiesis as demonstrated by a mild increase in *gata1* (**Figure 3.2.4D, F**) expression, as well as a reduction in myeloproliferation as demonstrated by decreased *lcp1* expression (**Figure 3.2.4C, E**). Although there was not a complete recovery in the *NHA9* activated embryo, these were still surprising data, as the microarray analysis did not indicate any altered expression in genes that are involved with HDAC activity. Regardless, I wanted to see if this phenotype could be duplicated or improved using an alternative HDAC inhibitor.

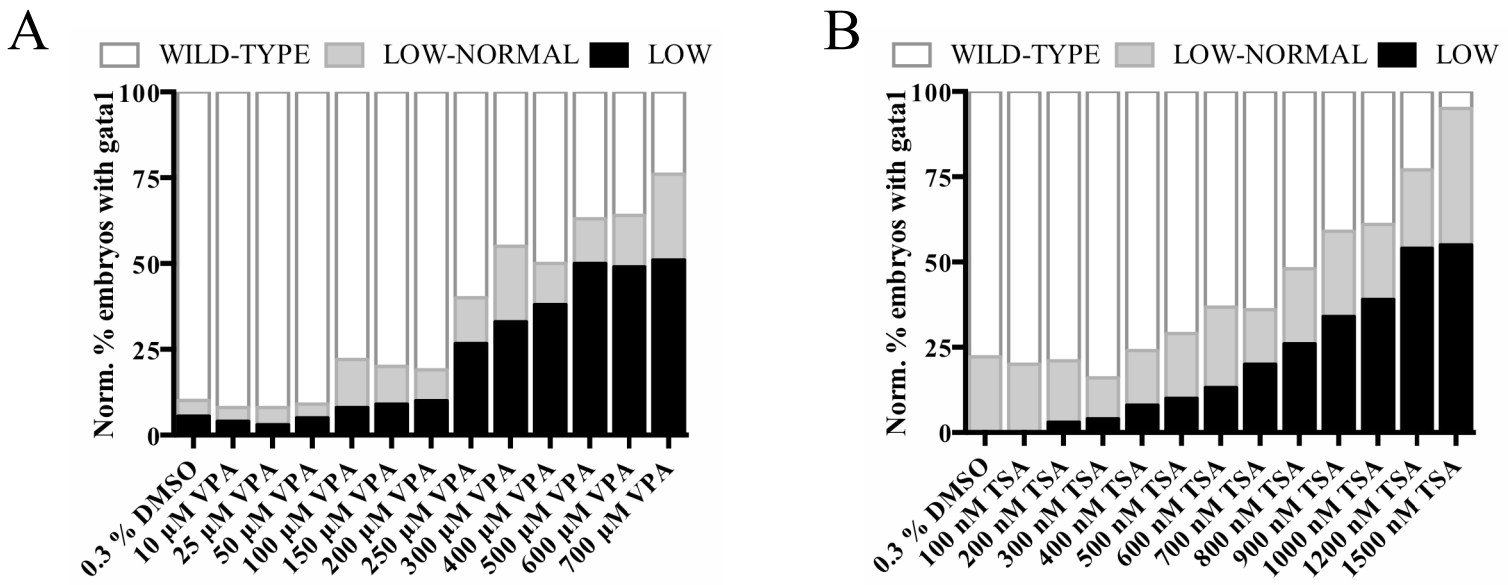
Trichostatin A is a class I and II HDAC inhibitor (*see section 1.5.2*) that has been demonstrated to inhibit proliferation, induce apoptosis and induce drug sensitivity to breast cancer cells both *in vitro* with human breast cancer cells and *in vivo* in mouse breast cancer models (Drzewiecka & Jagodzinski, 2012; Jang *et al*., 2004; Tavakoli-Yaraki *et al.*, 2013; Vigushin *et al.*, 2001). Additionally, in recent studies, TSA has demonstrated strong antileukemic activity *in vitro* with human leukemia cell lines

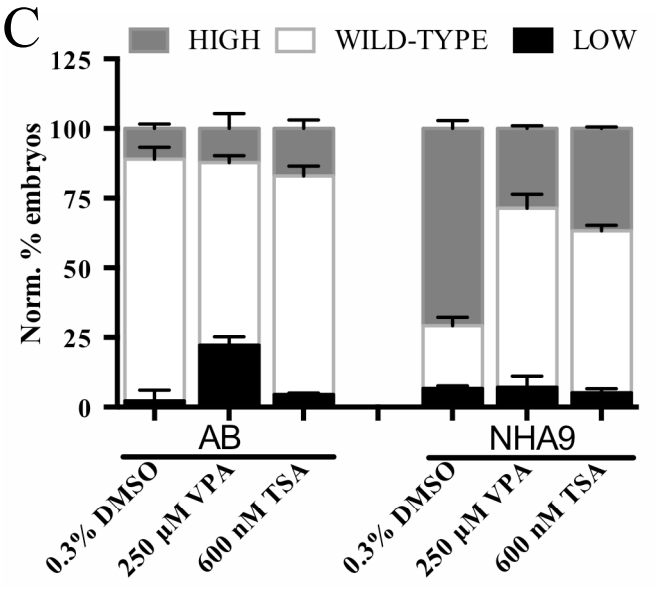
(Savickiene *et al*., 2012; Tran *et al*., 2013; Yu *et al.*, 2008). As such, this agent appeared an ideal alternative HDAC inhibitor to test in our transgenic *NHA9* zebrafish line.

The treatment protocol using TSA was identical to that of DAC, Zeb and VPA treatments. Following heat-shock and 6 hours of treatment with TSA, *Cre* controls and *NHA9* activated embryos were fixed at 30hpf and analyzed using WISH for *lcp1* and *gata1* (**Figure 3.2.4**). *Cre* and activated *NHA9* embryos treated with 0.3% sterile milliQ H_2O served as controls. Dose optimization suggested a maximum dose of 600 nM TSA should be used (**Figure 3.2.4B**). Surprisingly, at 600 nM TSA, there was little to no effect on expression of *lcp1* or *gata1* in *Cre* embryos, however a reduction in expression of *lcp1* and small increase in *gata1* in the *NHA9* activated embryos (**Figure 3.2.4C, E**). The phenotype observed, however, was not a restoration of normal hematopoiesis, but a stronger recovery than that demonstrated by VPA. The stronger recovery may be due to the fact that TSA inhibits both class I and II HDACs while VPA solely inhibits class I HDACs. Regardless, these studies demonstrated that both VPA and TSA were strong potential candidates for combined treatment with DNA methyltransferase inhibitors, such as DAC.

Figure 3.2.4 Histone deacetylase complexes inhibitors partially restore normal hematopoiesis in NHA9 transgenic zebrafish

Embryos stained by WISH for the expression of the myeloid gene, *lcp1* and erythroid gene, *gata1*, at indicated time points. Embryos displayed in side profile, anterior to the left. Dark, punctate staining represents *lcp1* expression in the PBI indicated by black arrows. Wild-type embryos were treated with VPA (A) and TSA (B) at 24 hpf and assessed by or *gata1* expression as represented by graphs below, in order to determine optimal treatment dose. *NHA9* embryos were then scored for their level of *lcp1* and *gata1* expression relative to *Cre* wild type, normalized to a percentage value of the total number of embryos of that treatment group, and quantified in bar graph for *lcp1* (C), and *gata1* (D) and represented by micrographs (E and F, respectively). Embryos were treated either with 0.3% DMSO vehicle control, 250 μM VPA, or 600 nM TSA, two histone deacetylase complex inhibitors. Embryos then heat-shocked at 24 hpf and assessed at 30 hpf. Each experiment was replicated three times with 15-25 embryos in each group per replicate. . Values are reported as the mean +/- the SEM.

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HIGH
WILD-TYPE
LOW
Norm. % embryos
125
100
75
50
25
0
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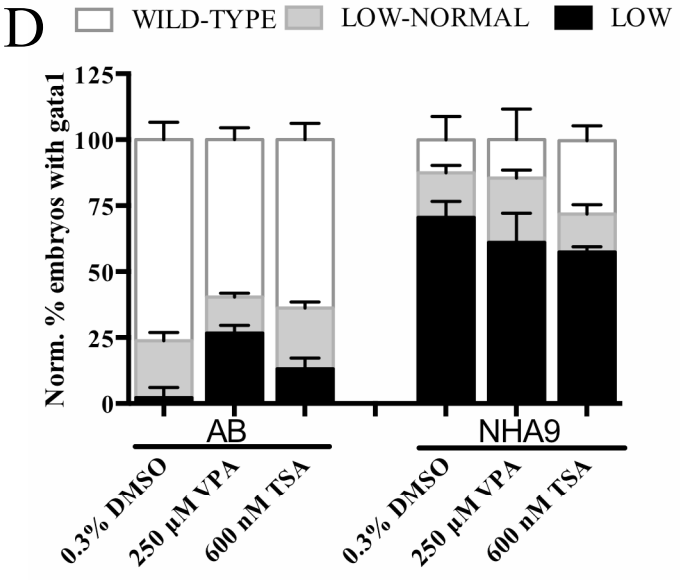
600 nM TSA
0.3% DMSO
250 µM VPA
600 nM TSA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Norm. % embryos with gata1
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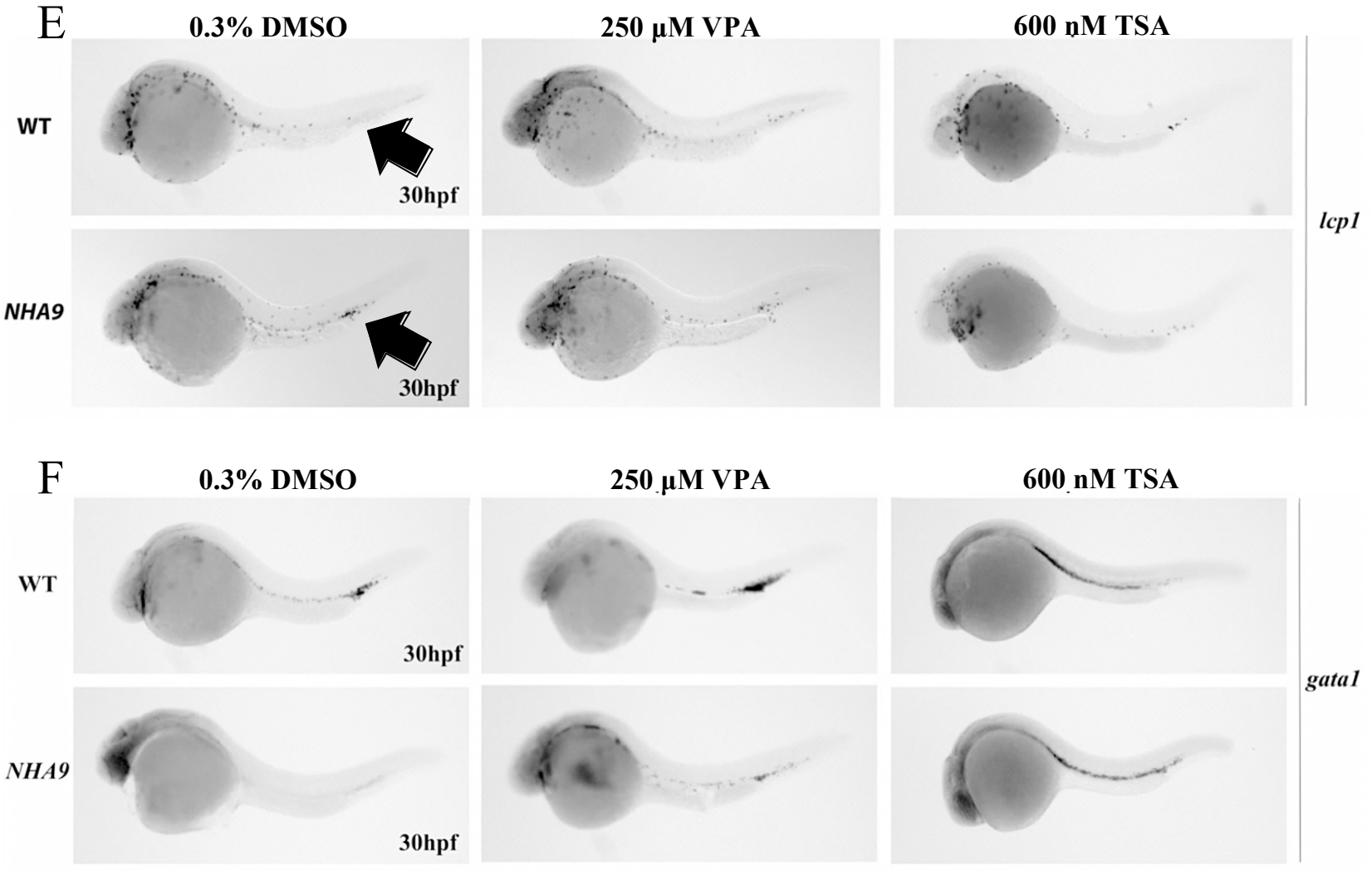
600 nM TSA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WT
NHA9
30hpf
30hpf
lcp1
F
0.3% DMSO
250 μM VPA
600 nM TSA
WT
NHA9
30hpf
30hpf
gata1

3.2.3 Histone deacetylase complex inhibitors function synergistically with methyltransferase inhibitors to restore hematopoiesis in NHA9 transgenic zebrafish

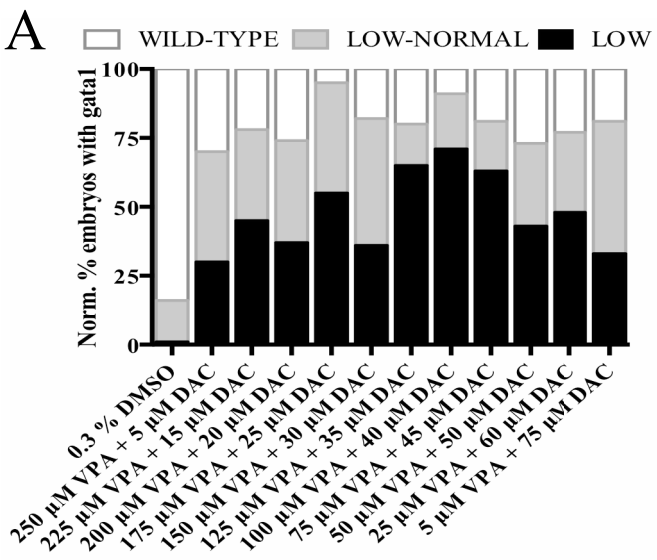
Given the strong evidence that *dnmt1* is involved in *NHA9*-mediated myeloid disease in the zebrafish as well as the evidence that chemical inhibition of HDACs help to reduce the impact of *NHA9* on zebrafish hematopoiesis, I sought out to determine if targeting both of these mechanisms of epigenetic regulation would yield a strong therapeutic response. Ideally I wanted to find a drug combination, which produced a complete response in the zebrafish at doses that were significantly less than the concentration of the drugs used as monotherapy. The linear combination strategy using HDAC inhibitor, VPA and dnmt1 inhibitor, DAC yielded unacceptable developmental toxicity in the zebrafish (*see section 2.5.3*) (**Figure 3.2.5A**). At 30 hpf both *Cre* and *NHA9* activated embryos demonstrated excessive death, developmental delay and reduced expression of both *lcp1* and *gata1* as assessed by WISH. These findings were similar to the combined treatment of the HDAC inhibitor, TSA and DAC, therefore I abandoned the additive strategy to determine if there is treatment synergy at lower concentrations.

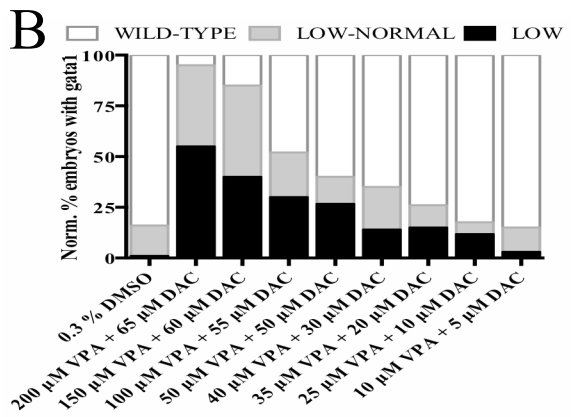
Indeed, when VPA and DAC were combined at concentrations below 50 percent of each monotherapy there was a reduced amount of toxicity to *Cre* embryo controls (**Figure 3.2.5B**). More specifically, when *Cre* embryos were treated with 50 μM VPA and 50 μM DAC there was neither developmental delay nor any alteration to *lcp1* or *gata1* expression levels as determined by WISH (**Figure 3.2.5D-G**). Remarkably, *NHA9* activated embryos demonstrated reduced *lcp1* expression (**Figure 3.2.5D, F**) and an increase in *gata1* (**Figure 3.2.5E, G**) expression after treatment with VPA and DAC at

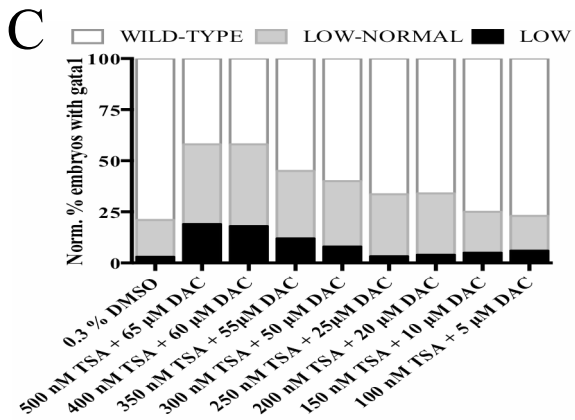
concentrations as low as 25 μM of VPA and 10 μM DAC. This is a substantial lower concentration than their relative monotherapy concentrations (10 and 13 percent of monotherapy doses, respectively), demonstrating a strong synergy between these two drugs. Similarly, combined treatment of TSA with DAC also demonstrated synergy, where there was a strong restoration of normal hematopoiesis, however the concentrations used remained higher than that of VPA and DAC. For example, at a concentration of 250 nM TSA and 25 μM DAC (42 and 33 percent of monotherapy doses, respectively), *Cre* control embryos demonstrated very little change to *lcp1* and *gata1* expression levels as determined by WISH, while *NHA9* activated embryos demonstrated a decrease in *lcp1* expression and an increase in *gata1* expression to that near wild-type levels (**Figure 3.2.5D-G**). However, at combined concentrations at higher doses, there were toxicity effects to both *Cre* controls and *NHA9* activated embryos, while lower concentrations did not yield any effects on *Cre* hematopoiesis, however did not show a convincing rescue in the *NHA9* activated embryos (**Figure 3.2.5**). Taken together, it appears that targeting different aspects of the epigenetic pathway through treatment with HDAC and methyltransferase inhibitors is an effective treatment option to *NHA9*-induced disease.

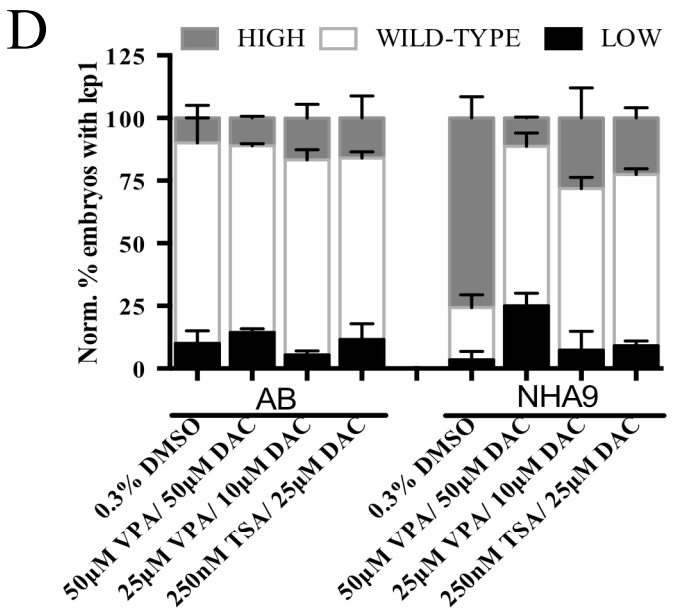
Figure 3.2.5 Histone deacetylase complex inhibitors function synergistically with methyltransferase inhibitors to restore hematopoiesis in NHA9 transgenic zebrafish

Embryos stained by WISH for the expression of the myeloid gene, *lcp1* and erythroid gene, *gata1*, at indicated time points. Embryos displayed in side profile, anterior to the left. Dark, punctate staining represents *lcp1* expression in the PBI indicated by black arrows. Wild-type embryos were treated with combined doses of VPA and DAC in a linear fashion (A) and in a synergistic fashion (B) and treated with combined doses of TSA and DAC in a synergistic fashion (C) at 24 hpf and assessed for *gata1* expression in order to determine optimal treatment dose. *NHA9* embryos were scored for their level of *lcp1* and *gata1* expression relative to *Cre* wild type, normalized to a percentage value of the total number of embryos of that treatment group, and quantified in bar graph for *lcp1* (D) and *gata1* (E) and represented by micrographs (F and G, respectively). Embryos were treated either with 0.3% DMSO vehicle control, 50 µM DAC + 50 µM VPA, 10 µM DAC + 25 µM VPA or 25 µM DAC + 250 nM TSA. Embryos then heat-shocked at 24 hpf and assessed at 30 hpf. Each experiment was replicated three times with 15-25 embryos in each group per replicate. . Values are reported as the mean +/- the SEM.





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WILD-TYPE
LOW
Norm. % embryos with lcp1
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100
75
50
25
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AB
NHA9
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50μM VPA/ 50μM DAC
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250nM TSA/ 25μM DAC
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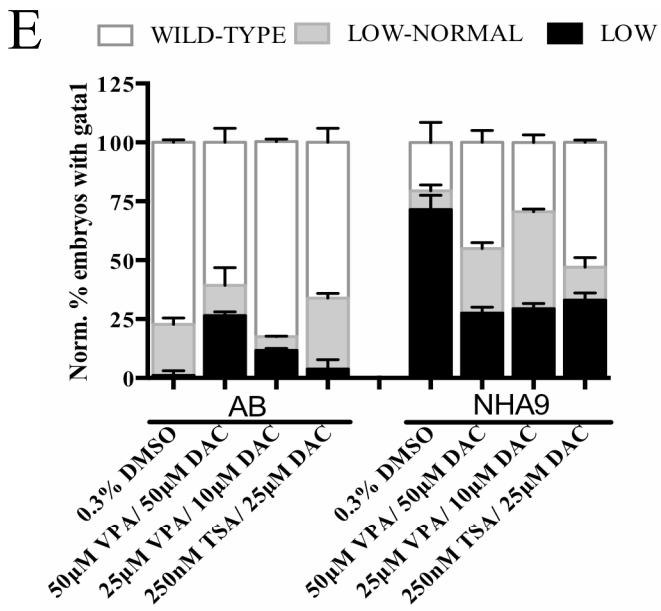
50μM VPA/ 50μM DAC
25μM VPA/ 10μM DAC
250nM TSA/ 25μM DAC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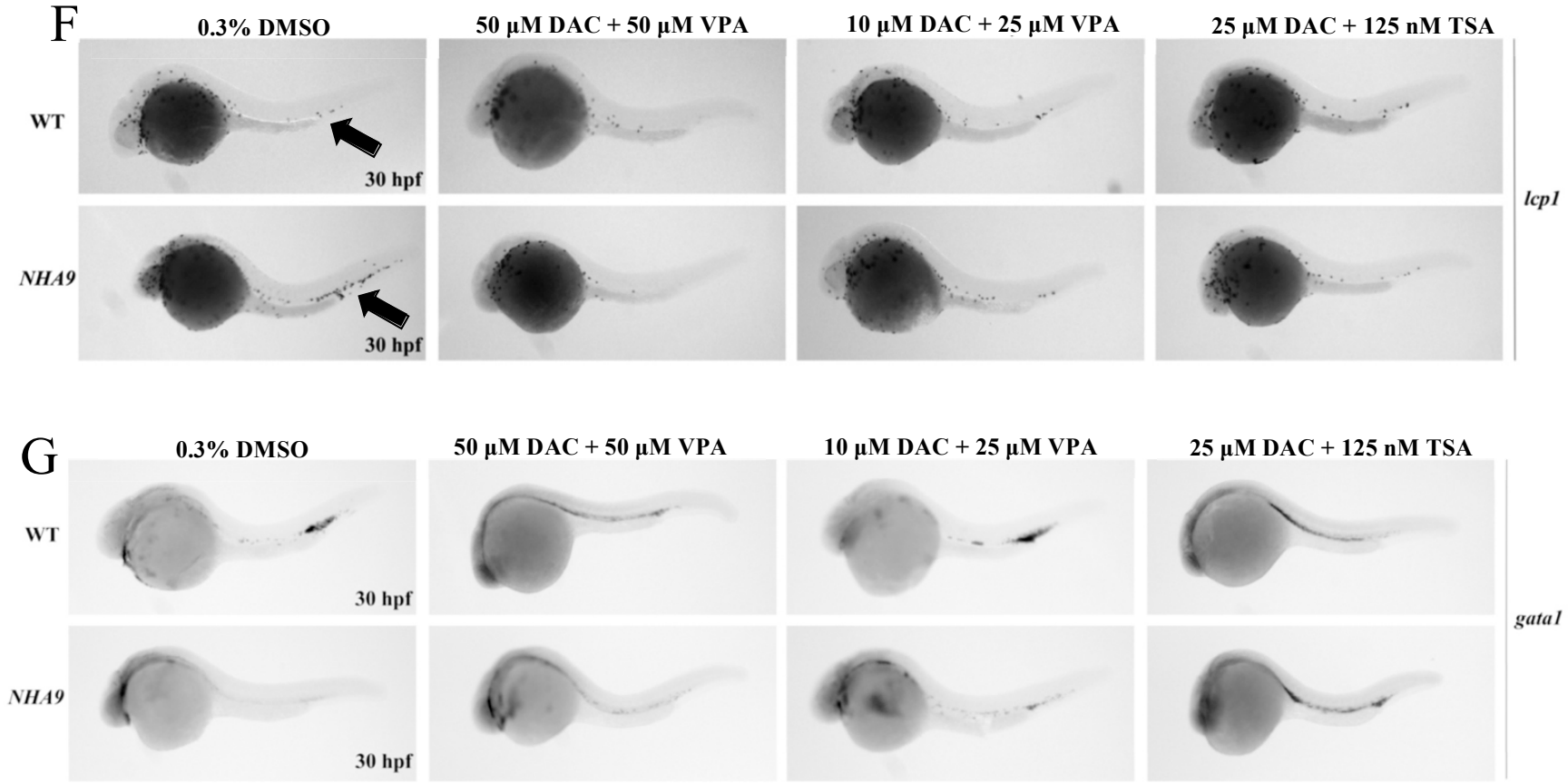
LOW-NORMAL
LOW
Norm. % embryos with gata1
125
100
75
50
25
0
AB
NHA9
0.3% DMSO
50μM VPA/ 50μM DAC
25μM VPA/ 10μM DAC
250nM TSA/ 25μM DAC
0.3% DMSO
50μM VPA/ 50μM DAC
25μM VPA/ 10μM DAC
250nM TSA/ 25μM DAC


F
0.3% DMSO
50 µM DAC + 50 µM VPA
10 µM DAC + 25 µM VPA
25 µM DAC + 125 nM TSA
WT
NHA9
30 hpf
30 hpf
lcp1
G
0.3% DMSO
50 µM DAC + 50 µM VPA
10 µM DAC + 25 µM VPA
25 µM DAC + 125 nM TSA
WT
NHA9
30 hpf
30 hpf
gata1

3.3 MANIPULATING THE NUP98-HOXA9 MYELOPROLIFERATION PHENOTYPE BY TARGETING WNT/β-CATENIN

As demonstrated earlier, our *NHA9* zebrafish embryos show similar hematopoietic defects with increased myelopoiesis at the expense of erythropoiesis as the *Tg*(*hsp70::AML-ETO*) zebrafish line (*see section 1.4.4*) (Yeh *et al*., 2008). That same group also determined that the *AML-ETO* fusion oncogene upregulates the COX-PGE_2 signaling axis, which drives the Wnt/β-cat pathway in blood cells (Goesslin*et al*., 2009; North *et al*., 2007; Yeh*et al*., 2009). Subsequently, we hypothesized that our microarray in *NHA9* embryos would identify genes involved in the COX-PGE_2 signaling axis. However, our microarray demonstrated neither up- nor down-regulation of any of these genes. Despite the absence, we still wanted to determine whether there was any collaboration of the Wnt/β-cat pathway and the *NHA9* fusion oncogene.

Using qRT-PCR (**Figure 3.1**), completed by former lab member Dr. Forrester, we observed that zebrafish *ptgs1*, the COX1 homolog, was expressed at near wild-type levels (n=3), however *ptgs2a*, a COX2 isoform, in *NHA9* embryos revealed a massive upregulation (63.89 ± 6.01-fold, n=3). Encouraged by these results, we hypothesized that direct modulation of Wnt/β-cat pathway would influence the activity of *NHA9* in zebrafish embryonic hematopoiesis. More specifically, as was demonstrated in previous studies (Wang *et al*., 2010; Yeh *et al*., 2009), we determined whether inhibiting the Wnt/β-cat pathway we could rescue normal hematopoiesis in *NHA9*-transgenic embryos and whether stimulating the pathway we could accelerate the hematopoietic effects.

To examine the effects of inhibiting the Wnt/β-cat pathway, we used the drugs Indomethacin (Indo) and NS-398, a COX1 and COX2 inhibitor, respectively, at 10 μM

concentrations (*see section 2.5.1*). A previous pharmacological treatment protocol was used to treat embryos with Indo and NS-398. Treatment with 0.3 percent DMSO served as vehicle controls. At 18 and 30hpf, we fixed the embryos and performed WISH for *spi1, lcp1* and *gata1*.

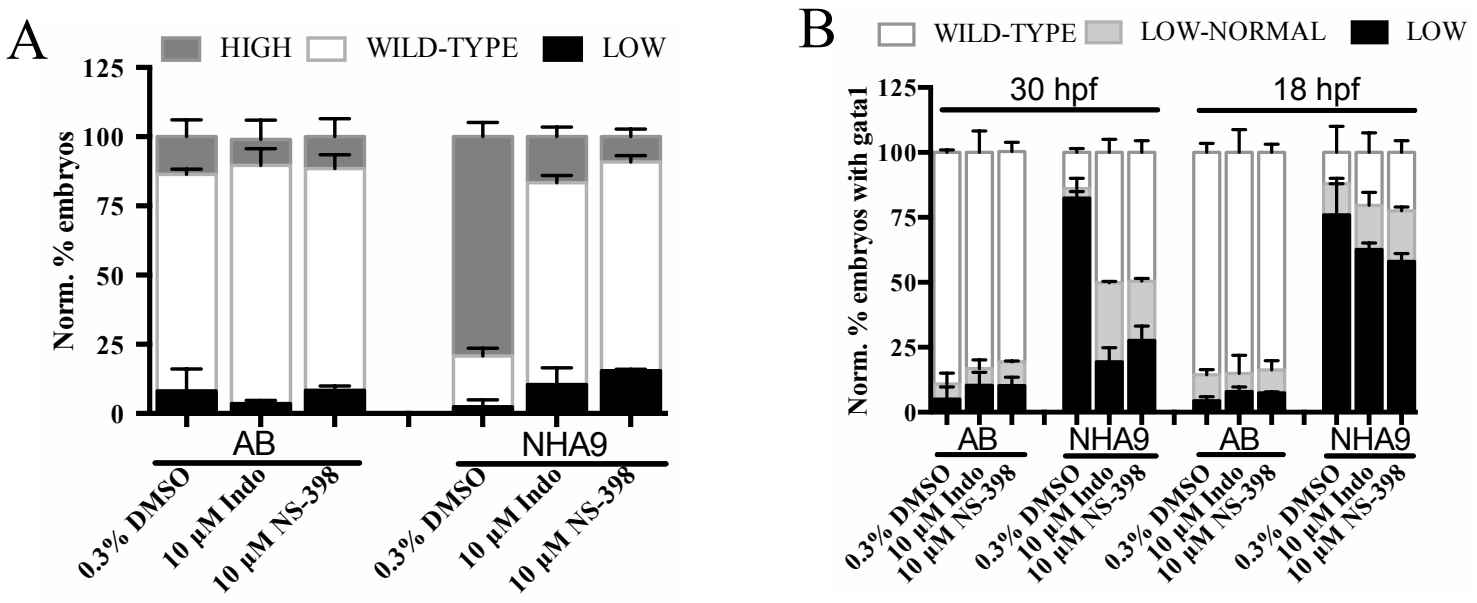
Using 10 μM concentration, neither Indo nor NS-398 appeared to affect the expression of *lcp1* in *Cre* control embryos (**Figure 3.3.1A, C**), which is consistent with the previous reports (Goessling *et al.*, 2009; Wang *et al*., 2010; Yeh *et al.*, 2009). Similar to previous chemical treatments in the *NHA9* transgenic line, approximately 80 percent of activated *NHA9* embryos demonstrated an increase in both *spi1* and *lcp1* expression at 30 hpf. However, treatment with Indo resulted in a near complete return of normal *lcp1* expression, while NS-398 treatment demonstrated an even greater response where *lcp1* expression in almost all of the embryos examined appeared normal at 30 hpf.

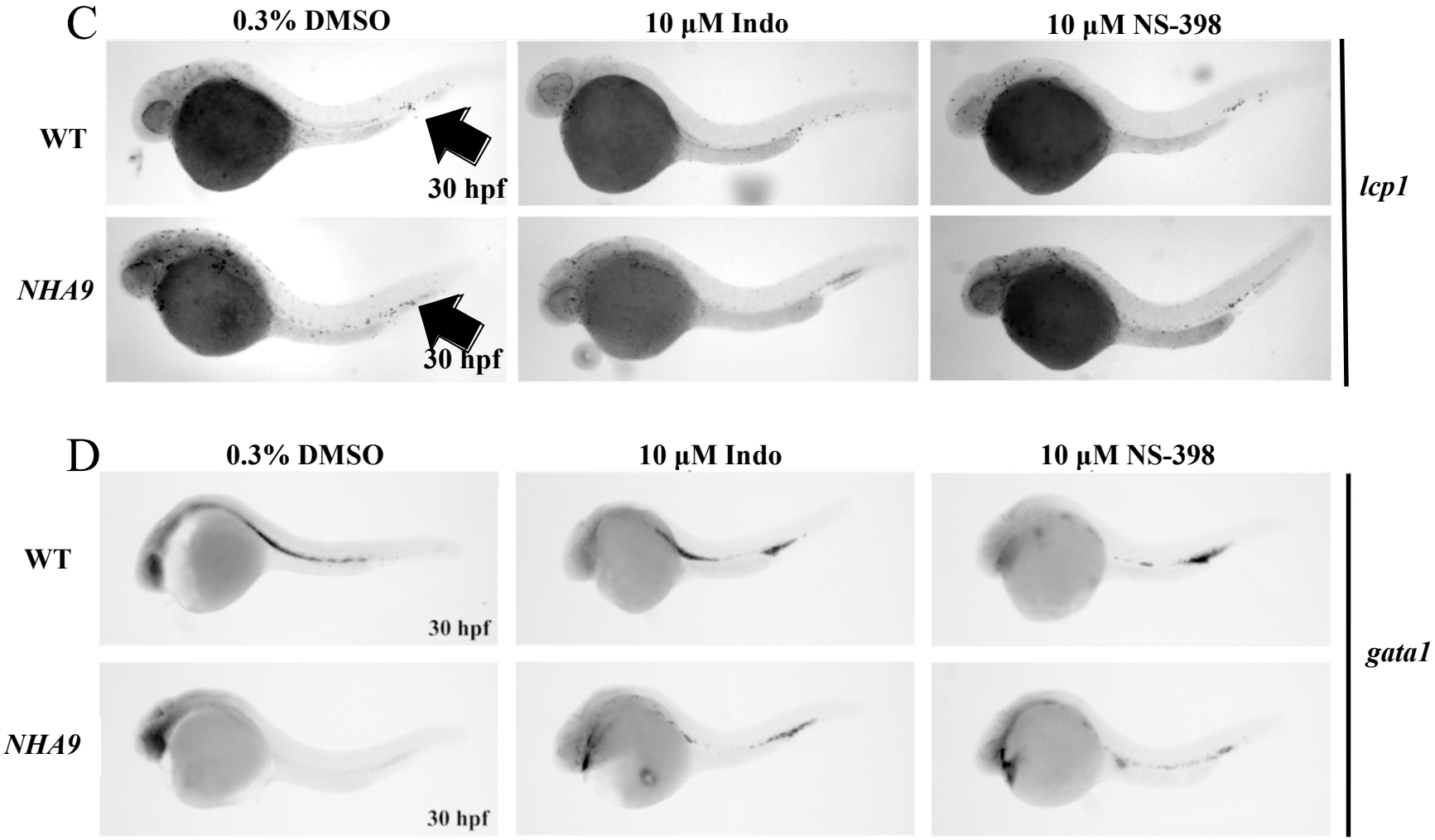
Interestingly, *gata1* expression in *Cre* control embryos was not altered by treatment with Indo or NS-398 at 30 hpf, however, in *NHA9* activated embryos, both drugs demonstrated a return of *gata1* expression to that near wild-type levels (**Figure 3.3.1B, D and E**). Previous zebrafish studies treated embryos at 12 hpf to look at the effects of Indo and NS-398 at 18 hpf (Yeh *et al*., 2009). As such *NHA9* activated embryos were treated with either Indo or NS-398 and fixed at 18 hpf. However, this resulted in only a weak rescue of wild-type *gata1* expression, while not affecting the *gata1* expression in *Cre* controls at all (**Figure 3.3.1E**). These findings are interesting and may indicate that during earlier, primitive erythropoiesis that Indo and NS-398 have very little effect, however during the first definitive wave of hematopoiesis, derived from EMPs, the drugs are more effective at restoring normal hematopoiesis. Additionally,

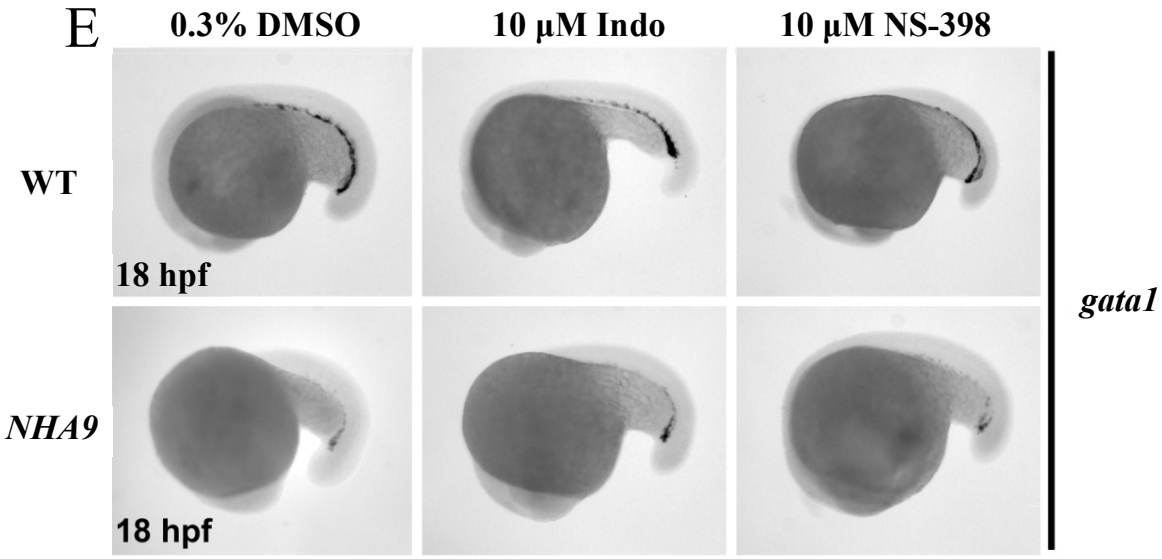
despite the prominent anti-myeloid effect of Indo and NS-398, the lack of COX-PGE_2 axis signaling gene hits in our microarray as well as the mild affect on early *gata1* expression levels in the *NHA9* embryos demonstrates that the Wnt/β-cat may not be directly involved in *NHA9*-mediated disease and that the drugs may be operating under a different mechanism to help restore hematopoiesis.

Figure 3.3.1 The Wnt/beta-catenin pathway may play a limited role on the hematopoietic phenotype in NHA9 embryos

Embryos stained by WISH for the expression of the myeloid gene, lcp1 and erythroid gene, gata1, at indicated time points. Embryos displayed in side profile, anterior to the left. Dark, punctate staining represents lcp1 expression in the PBI indicated by black arrows. Embryos were scored for their level of lcp1 and gata1 expression relative to Cre wild type, normalized to a percentage value of the total number of embryos of that treatment group, and quantified in bar graph for lcp1 (A) and gata1 (B) and represented by micrographs (C and D, respectively). Embryos were treated either with 0.3% DMSO vehicle control, 10 µM Indo or 10 µM NS-398 to inhibit Wnt/beta-catenin signaling. Embryos then heat-shocked at 24 hpf and assessed at 30 hpf. Each experiment was replicated three times with 15-25 embryos in each group per replicate. . Values are reported as the mean +/- the SEM




C
0.3% DMSO
10 μM Indo
10 μM NS-398
WT
NHA9
30 hpf
30 hpf
lcp1
D
0.3% DMSO
10 μM Indo
10 μM NS-398
WT
NHA9
30 hpf
30 hpf
gata1


E
0.3% DMSO
10 µM Indo
10 µM NS-398
WT
NHA9
18 hpf
18 hpf
gata1

3.4 CHEMICAL INHIBITION OF HISTONE DEACETYLASE COMPLEX AND COX FUNCTION TOGETHER TO RESTORE HEMATOPOIESIS IN NHA9 TRANSGENIC ZEBRAFISH

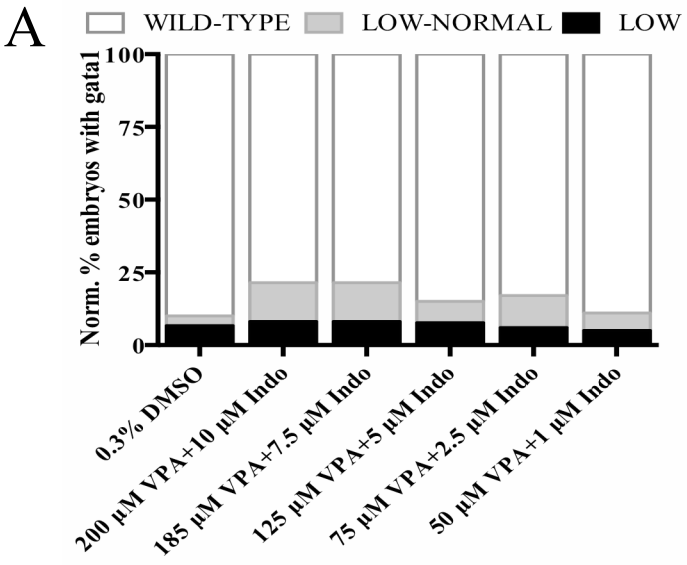
Interestingly, treatment of *NHA9* transgenic embryos with the HDAC inhibitors, VPA or TSA, partially restores normal hematopoiesis. However, it has been determined that both VPA and TSA activate the Wnt/β-cat pathway (Wiltse, 2005), a pathway that has been described to collaborate with other genetic aberrations to induce leukemogenesis (Wang *et al*., 2010; Yeh*et al*., 2009; Yeung*et al.*, 2010). I therefore, hypothesized that the mechanisms by which HDAC and COX inhibitors functions correspond to similar pathways. As such, I speculated that the partial hematopoietic rescue in *NHA9* activated embryos with treatment of the HDAC inhibitors, VPA and TSA, could potentially be increased by combining the epigenetic therapy with drugs that inhibit the Wnt/β-cat pathway.

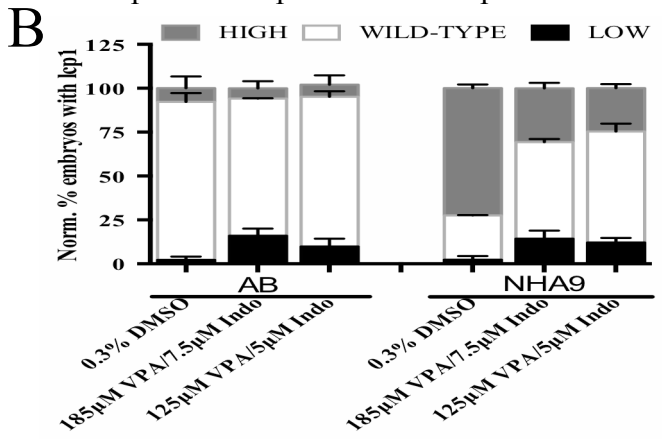
I conducted a number of treatments to produce an accurate dose curve as demonstrated in *section 3.2.3* (**Figure 3.4.1A**). Treatment with 0.3 percent DMSO served as a vehicle control. At doses less than 75 percent of each of VPA or Indo monotherapy concentrations (185 and 7.5 μM, respectively), there was little to no change in the expression of *lcp1* or *gata1* at 30 hpf in the *Cre* control embryos (**Figure 3.4.1B-F**). Interestingly, at 18 hpf, the *Cre* control embryos did demonstrate reduced *gata1* expression after treatment of 185 μM VPA and 7.5 μM Indo, suggesting that there may be some toxicity to the embryos at the earlier stages of development (**Figure 3.4.1C, F**). In *NHA9* activated embryos approximately 75 percent demonstrated increased *lcp1* expression and reduced *gata1* expression as seen in previous studies. However, when treated with combined treatments of between 50 and 75 percent the concentration of the

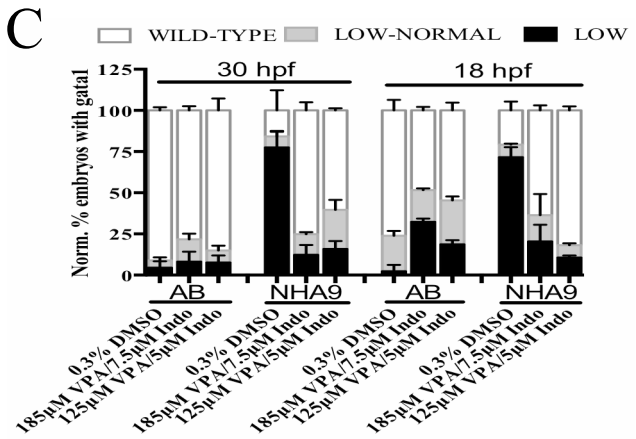
monotherapy of each drug (i.e., 125 μM VPA with 5 μM Indo to 185 μM VPA with 7.5 μM Indo), there was a decrease in *lcp1* expression at 30 hpf in the *NHA9*-activated embryos to that near wild-type levels (**Figure 3.4.1A, C**). Additionally, at 30 hpf treatments in the same range of combined treatment concentrations of VPA and Indo resulted in an increase in *gata1* expression to near wild-type levels in the *NHA9* activated embryos (**Figure 3.4.1C, E**). Interestingly, at 18 hpf *gata1* expression in the *NHA9* embryos, using the same combined therapy was also increased to near wild-type levels, despite the fact that some *Cre* control embryos at this time demonstrated a decrease in *gata1* expression (**Figure 3.4.1C, F**). Taken together, these findings are very promising as neither drug treatment alone yielded full restoration of hematopoiesis in the *NHA9*-activated embryos, however combined at dose concentrations lower than monotherapy, there was a more complete response. For instance, VPA had a partial recovery of both *lcp1* and *gata1* expression levels in *NHA9* embryos, and Indo demonstrated a strong inhibition of myeloproliferation, however did not fully rescue *gata1* expression at 30 hpf and had very little effect on *gata1* expression at earlier time points. Yet, combined treatment demonstrates a full rescue of *lcp1* and *gata1* in the *NHA9* activated embryos at 30hpf and the rescue of normal *gata1* expression at 18 hpf. Therefore, these findings suggest that by targeting aspects of both the epigenetic and the COX pathways may be a novel therapeutic options in human AML patients with the *NHA9* fusion oncogene.

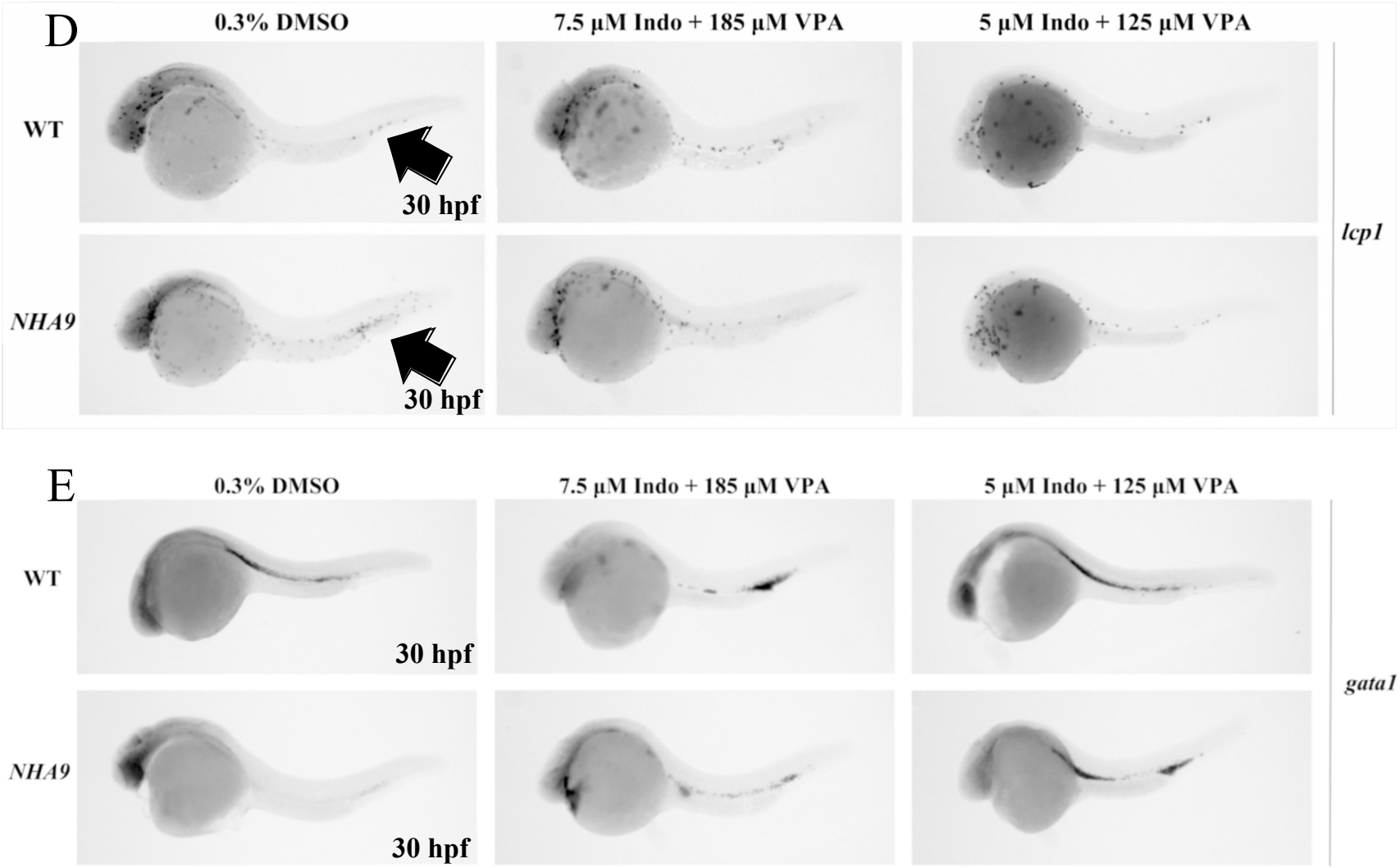
Figure 3.4.1. Chemical inhibition of histone deacetylase complex and COX, function together to restore hematopoiesis in *NUP98-HOXA9* transgenic embryos

Embryos stained by WISH for the expression of the myeloid gene, *lcp1* and erythroid gene, *gata1*, at indicated time points. Embryos displayed in side profile, anterior to the left. Dark, punctate staining represents *lcp1* expression in the PBI indicated by black arrows. Wild-type embryos were treated with combined doses of VPA and Indo at 24 hpf in a synergistic fashion and assessed by WISH for *gata1* expression, represented by graph (A) in order to determine optimal treatment dose. *NHA9* embryos were scored for their level of *lcp1* and *gata1* expression relative to *Cre* wild type, normalized to a percentage value of the total number of embryos of that treatment group, and quantified in bar graph. Embryos were treated either with 0.3% DMSO vehicle control, 7.5 µM Indo + 185 µM VPA, or 5 µM Indo + 125 µM VPA. Embryos then heat-shocked at 24 hpf and assessed at 30 hpf. Each experiment was replicated three times with 15-25 embryos in each group per replicate. Values are reported as the mean +/- the SEM.

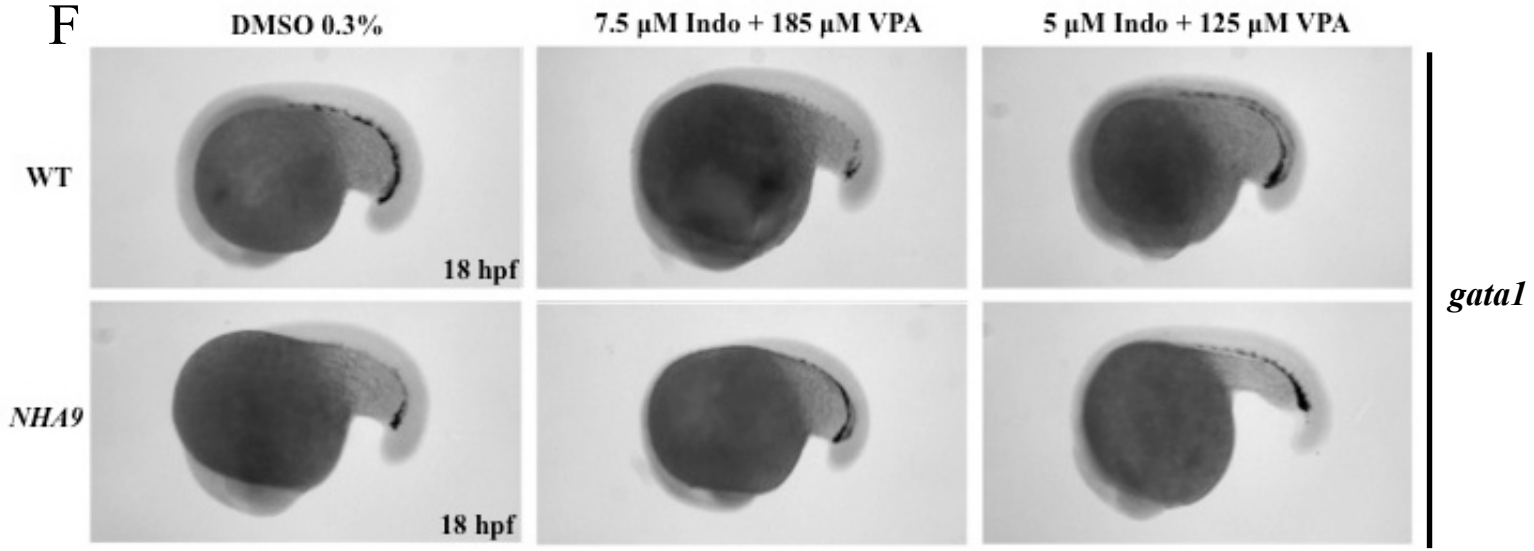







D
0.3% DMSO
7.5 µM Indo + 185 µM VPA
5 µM Indo + 125 µM VPA
WT
NHA9
30 hpf
30 hpf
lcp1
E
0.3% DMSO
7.5 µM Indo + 185 µM VPA
5 µM Indo + 125 µM VPA
WT
NHA9
30 hpf
30 hpf
gata1

F


DMSO 0.3%
7.5 µM Indo + 185 µM VPA
5 µM Indo + 125 µM VPA
WT
NHA9
18 hpf
18 hpf
gata1

3.5 *NUP98-HOXA9* DRIVES HEMATOPOIETIC STEM CELL EXPANSION IN THE ZEBRAFISH

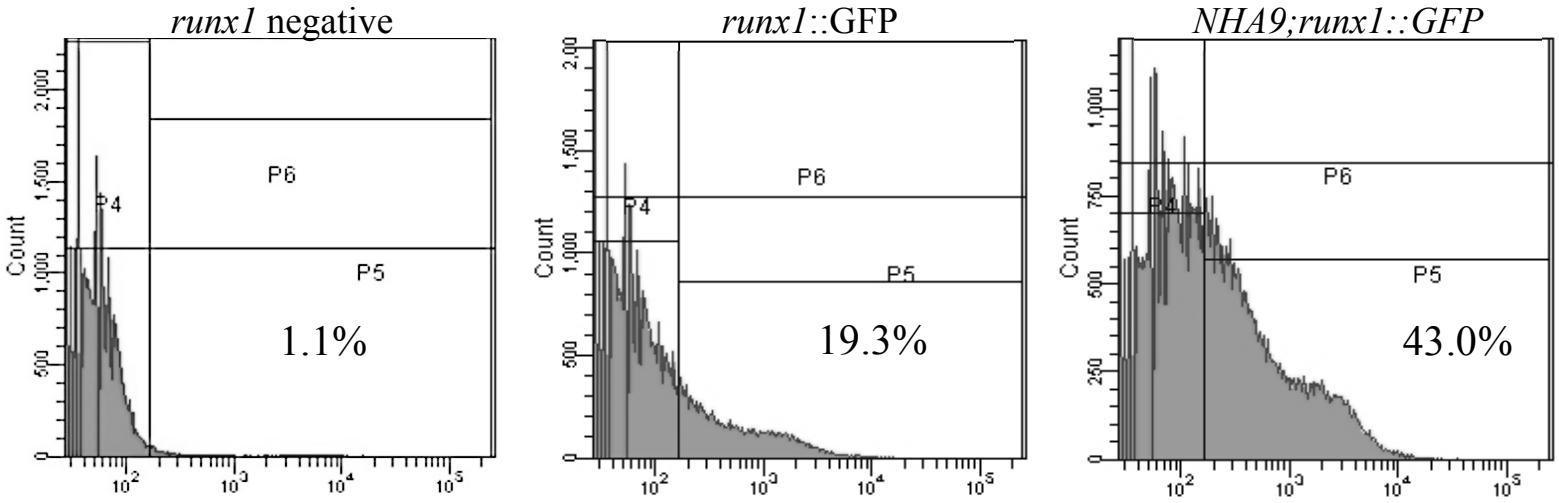
During early hematopoiesis, the *HOX* gene clusters 1–4 show maximal expression level in HSCs (Argiropoulos & Humphries, 2007), which is down-regulated as HSCs begin to differentiate (Argiropoulos & Humphries, 2007). However, later in hematopoiesis, immature, lineage-committed progenitors demonstrate maximal expression of *HOX* gene clusters 7–11 (Palmqvist, *et al.* 2007). Interestingly, some of these *HOX* genes have been associated with leukemogenesis (*see section 1.2.2*).

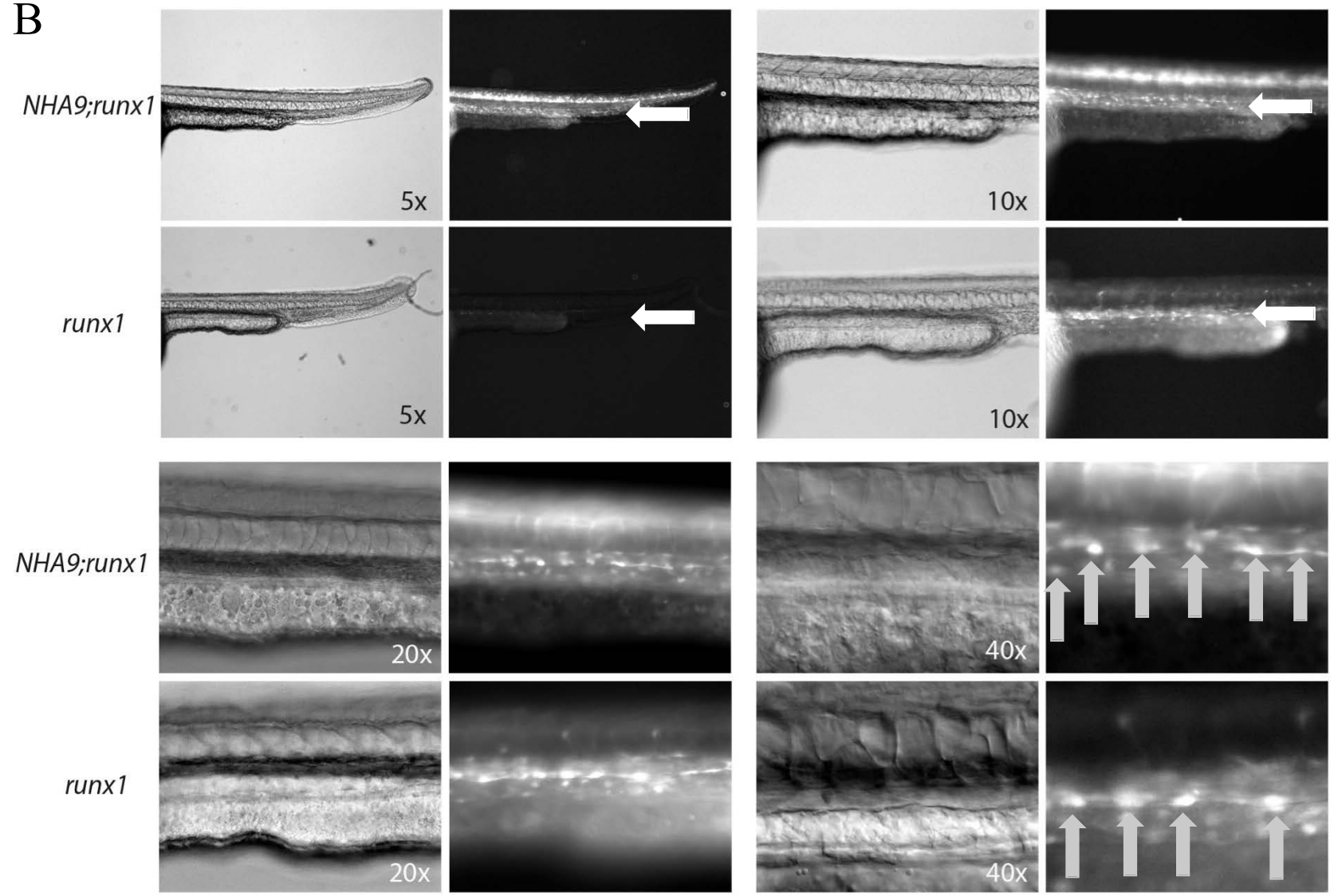
Transgenic *NHA9* embryos express the fusion oncogene under the *spi1* promoter, which is activated early during hematopoiesis. As such, during the final wave of hematopoiesis, zebrafish HSCs express *HOXA9* as part of the *NHA9* fusion oncogene (*see section 1.2.4*). I therefore hypothesized that the *NHA9* hematopoietic phenotype extends beyond the primitive blood cells and EMPs as previously published to also affect the HSCs, thereby implicating this population as the cell-of-origin responsible for the MPN phenotype seen later in development. To study the contribution of HSCs to myeloid proliferation, I made use of the *NHA9*;*runx1* zebrafish line, which I generated by crossing *tg(runx1::eGFP)*, which develop fluorescently labeled HSCs with the NHA9 line (*see section 2.2.2*). To assess the effects of *NHA9* on HSC development, *NHA9;runx1* zebrafish were mated to *Cre* fish. For controls, *Cre* were crossed to *runx1*, wild-type zebrafish. Both groups were grown to 12 hpf, then heat-shocked and left to develop to 36 hpf at 28^{o}C at which time embryos were euthanized and dissociated into single-cell suspension and run through FACS (*see section 2.6*) Interestingly, preliminary results indicate that compared to *Cre;runx1* controls, *NHA9;runx1* activated zebrafish expressed a 2-fold increase in GFP+ cells (**Figure 3.5.1A**) (n=3), suggesting that activation of

NHA9 has a direct effect on HSC development. However, the number of GFP^+ cells in both the *Cre;runx1* and *NHA9*;*runx1* activated embryos was much higher than anticipated (approximately 20 percent and 40 percent, respectively) (**Figure 3.5.1B**). Upon closer examination, off-target *runx1*::*GFP* expression was located in the hindbrain and in the myelin of the CNS, which has been previously described (Lam *et al*., 2009). Therefore, it is difficult to confirm that the increase in GFP^+ cells is due to an increase in HSCs alone, or developmental changes to the CNS. For this reason, additional testing using other HSC reporter lines and *runx1*/*c-myb* WISH needs to be performed for verification. If additional testing confirms these preliminary findings, this would then suggest that the HSC might in fact be the cell-of-origin in *NHA9*-induced myeloid disease.

Figure 3.5. NUP98-HOXA9 drives hematopoietic stem cell expansion in the zebrafish

Reporter runx1::GFP zebrafish embryos with fluorescently labeled hematopoietic stem cells were dissociated run through FACS. A GFP negative wild-type line was used as the negative control. Embryos were heat-shocked at 12 hpf then imaged and assessed at 36 hpf. Each experiment was replicated three times with 40-50 embryos in each group per replicate. Graphs below depict average percentage of GFP+ cells in each group. Micrograph demonstrates a greater number of fluorescently labeled cells along the AGM (white arrows) and in the caudal hematopoietic tissues (gray arrows).




B
NHA9;runx1
runx1
NHA9;runx1
runx1
5x
5x
10x
10x
20x
20x
40x
40x

CHAPTER 4 - DISCUSSION

4.1 TRANSGENIC ZEBRAFISH CAN BE USED TO IDENTIFY DOWNSTREAM EFFECTOR PATHWAYS IN *NUP98-HOXA9*-INDUCED MYELOID DISEASE

The NHA9-fusion oncogene is associated with high-risk AML in humans. Moreover, in humans, mice and recently in zebrafish, *NHA9* has been demonstrated to promote a myeloid disease on its own. *NHA9*-transgenic zebrafish exhibit MPN with some maturation in 23% of fish between 19 to 23 months of life (Forrester *et al*., 2011; Forrester, 2012). Despite evidence of myeloid proliferation and delayed cell maturation, no transgenic animals were identified with overt AML (Forrester *et al*., 2011; Forrester, 2012). The long latency and low penetrance of overt AML in our zebrafish model could be, in part, due to the *spi1* promoter. For instance, the expression of zebrafish *spi1* is normally downregulated during terminal myeloid differentiation and is active in only ~2 percent of adult hematopoietic kidney marrow cells (Hsu e*t al*., 2004). This lack of *spi1* at later time points could account for the incidence rate of MPN in our *NHA9* transgenic model and the lack of progression to overt AML. However, my work has lead to new mechanistic insights regarding disease pathogenesis at the embryonic level. Our findings present a more comprehensive understanding of *NHA9* -induced myeloid disease in the zebrafish.

NHA9 activated embryos demonstrated altered hematopoiesis with increased myeloid development marked by *spi1, lcp1,* and *lyz,* at the expense of erythroid development marked by *gata1* (Forrester *et al*., 2011; Forrester, 2012). Microarray analysis of *NHA9* activated versus AB wild-type embryos produced a number of

interesting 'hits' including the maintenance DNA methyltransferase, *dnmt1*. By leveraging the embryonic phenotype, I have been able to determine the contribution of genetic/epigenetic pathways downstream of *NHA9*, including COX signalling, histone acetylation and DNA methylation. Furthermore, we have been able to identify potential pharmacological targets in *NHA9*-induced myeloid disease that may offer high efficiency with limited toxicity – addressing a major long-term goal of AML research.

4.2 NUP98-HOXA9 AND WNT/β-CATENIN

Our *NHA9*-transgenic zebrafish model develops both a myeloproliferative neoplasm (MPN) in adults as well as embryonic defects in hematopoiesis (*see section 1.7*), which are similar to that of the *AML1-ETO*-transgenic zebrafish AML model (*see section 1.4.4*). There is already much known about the downstream genetic contributors of *AML1-ETO*, but none of those that assist in *NHA9*-mediated disease.

I found that *NHA9* embryos demonstrated an upregulation of *ptgs2a* and that myeloproliferation could be reduced with a broad-spectrum COX inhibitor or a specific COX2 inhibitor, Indo and NS-398, respectively. This is similar to what was observed for K562 cells that have been additionally transformed with *AML1-ETO*, which show increased expression of *PTGS2*, encoding the COX2 enzyme and inhibition of erythroid differentiation (Yeh *et al.*, 2009). Interestingly, these cells could be rescued by treatment with NS-398, which suppresses the canonical Wnt/β-cat pathway. In the *AML1-ETO* transgenic zebrafish lines, embryos treated with NS-398 or Indo similarly rescued wild-type levels of myeloid *mpx* and erythroid *gata1* expression. Additionally, rescue was also seen by injecting the *AML1-ETO* embryos with single MOs to zebrafish *ptgs1, ptgs2a, ptgs2b, ctnnb1,* or *ctnnb2* (Yeh *et al*., 2009). In contrast, treating these embryos with

supraphysiologic amounts of PGE_2, the enzymatic product of COX2, which increases the activation of the canonical Wnt/β-cat pathway, accelerated the *mpx* myeloproliferation and inhibited the ability of NS-398 and Indo to rescue *gata1* when treated concurrently. These findings place activation of the Wnt/β-cat pathway as a primary function of *AML1-ETO* expression *in vivo*.

Previous work by Dr. Forrester in our *NHA9* transgenic zebrafish line, demonstrated that treatment with PGE_2 in embryos treated at 24-30hpf did not accelerate the *lcp1* myeloproliferation which suggests that the Wnt/β-cat may not be as central in *NHA9*-induced disease compared to *AML1-ETO* (Forrester, 2012). However, this finding appears inconsistent with the massive upregulation of *pgt2a* that I detected in *NHA9* activated embryos, and has been reported in *NHA9*-transformed human cells (*see section 3.1*) (Takeda *et al.*, 2006). Nevertheless, PGE_2 positively regulates its own expression (Araki *et al*., 2003), which allows us to speculate that the level of $PGE_2$is already quite high in *NHA9* activated embryos and therefore, accounts for the fact that I did not see the accelerated phenotype demonstrated in *AML1-ETO* zebrafish. This data suggests that the Wnt/β-cat may still be involved in *NHA9*-mediated disease and should be looked at in our transgenic embryos.

Although inconsistent with PGE_2 treatments, we did observe some inhibition of myeloproliferation and a mild rescue of erythropoiesis in *NHA9* activated embryos treated with NS-398 or Indo, similar to that observed in *AML1-ETO* zebrafish. At the earlier time point of 18 hpf, there was a very mild increase in *gata1*, however at the later time point, 30 hpf, there was a much stronger increase of *gata1*. This difference in restored expression may suggest that Wnt/β-cat does not play a large role in primitive

erythropoiesis, but has a greater influence during definitive red blood cell production. Additionally, the most encouraging result was the dramatic decrease in myeloproliferation at 30 hpf, suggesting a high sensitivity to COX inhibitors during definitive myeloid cell development. Taken together, these findings suggest that the role of the Wnt/β-cat pathway in *NHA9*-mediated disease may be more complex as its role appears minor and not the sole or major pathway as it has been demonstrated to be in *AML1-ETO*.

4.2.1 Future Directions – Zebrafish Wnt/β-catenin In NHA9 Embryos

The suggested complexity of Wnt/β-cat signaling in *NHA9*-mediated disease demonstrates a need to consider both upstream and downstream factors along the pathway. For example, it has been demonstrated by microarray that *NHA9*-transformed $CD34^+$ cells had a downregulation of *WNT5B* (Chung *et al*., 2006). *WNT5A* and *WNT5B* encode secreted ligands that activate the non-canonical Wnt pathway involved in calcium-mediated remodeling of the actin cytoskeleton (Wang *et al*., 2011). This may also promote apoptosis in myeloid leukemia. For instance, K652 cells demonstrate an increase in *WNT5B*, as well as non-canonical *FZD4, FZD5,* and *FZD7* ligand receptors when cells are treated with *BCR-ABL1* inhibitor, imatinib mesylate and cytotoxic etoposide (Sercan *et al.*, 2007). Previously, Forrester *et al.* demonstrated a decrease in apoptosis of *NHA9* activated embryos compared to that of wild-type controls after DNA damage causing radiation (Forrester *et al*., 2011; Forrester, 2012). This apoptotic inhibition may be the result of the non-canonical Wnt/β-cat pathway suppression in *NHA9*-mediated disease.

The downregulation of *WNT5B* and therefore activation of the non-canonical Wnt/β-cat pathway, as observed in *NHA9*-transformed human cells, may also reflect the inhibition of myeloid differentiation (Pukrop, 2006; Taki *et al*., 2003; Weeraratna *et al*., 2002). Moreover, this downregulation may even remove competition between canonical and non-canonical signaling, therefore permitting an increase in signaling through the canonical Wnt/β-cat pathway. I could use our transgenic zebrafish line to study this hypothesis by A) quantifying *wnt8a* expression by qRT-PCR, B) injecting *wnt8a* mRNA at the one-cell stage and measure the effects on hematopoiesis and C) injecting *wnt8a* MO to determine if there is any acceleration of the *NHA9*-induced hematopoietic phenotype.

There are cross-reactive antibodies available for the transcriptionally, hypophosphorylated form of β-cat (Wang *et al*., 2010). Western blot analysis can be conducted with the antibodies to confirm whether the activation of *NHA9* truly activates the canonical Wnt/β-cat pathway. Additionally, inhibiting β-cat by increasing mRNA levels of upstream ligands that mark β-cat for phosphorylation and destruction, such as the zebrafish homologue of *APC, axin* or *GSK3,* may counteract the increased expression of *ptgs2* and inhibit *NHA9*-induced hematopoietic phenotype. Inversely, if *NHA9* does not demonstrate an increase in activation of β-cat by Western blot, we can inject MO against the zebrafish homologue of *axin* or *gsk3* to reduce β-cat phosphorylation and therefore, induce its activation to determine if indeed, the canonical pathway contributes to the NHA9 phenotype.

It would be interesting if hyperactivation of β-cat leads to an increase in *NHA9*-induced disease in the zebrafish as we could then conduct additional microarray analysis.

Using the previous microarrays and the *NHA9;β-cat* microarray we may be able identify downstream events from Wnt/β-cat, which will allow us to further understand downstream genetic events that lead to in *NHA9*-induced myeloid leukemia.

4.3 *NUP98-HOXA9* AND *DNMT1*

Fortunately, only one *DNMT1* homologue has been identified in zebrafish, compared to six DNMT3 homologues (Smith *et al.*, 2011). As such, I've demonstrated that MO knockdown of *dnmt1* as well as chemical inhibition of dnmt1 using DAC and Zeb inhibits the myeloproliferative effects of *NHA9* in transgenic zebrafish embryos, as well as rescue erythropoiesis. Additionally, MO knockdown of *uhrf1*, which encodes the protein that recruits dnmt1 to DNA, demonstrated a similar effect to that of *dnmt1* MO knockdown. In human and murine cell lines UHRF1 knockdown leads to global genomic hypomethylation (Arita *et al.*, 2008; Hervouet *et al.*, 2010). Furthermore, these hypomethylated effects cannot be corrected by overexpression of any DNMTs (Arita *et al*., 2008; Bostick *et al*., 2007; Hervouet *et al*., 2010; Unoki *et al.*, 2009). Together, these data help to increase our confidence that our microarray, MO and *dnmt1* chemical inhibitor findings in *NHA9* embryos would apply to human myeloid disease.

4.3.1 Targeting epigenetic regulation in clinical myeloid disease

DAC and its chemical cousin, Azacitidine (AZA) are both used as effective treatments for high-risk MDS, than best supportive care (BSC), which consists of red blood cell transfusions, erythropoiesis-stimulating agents, platelet transfusions, and colony-stimulating factors (*see section 1.1.5*). However, for overt AML, these agents may be more efficacious when used in combination with cytotoxic chemotherapy.

MDS is characterized by a failure of cell differentiation, leading to myeloid

cytopenia, but does not necessarily involve the proliferation of progenitor cells. *DNMT1* exerts epigenetic control over cell differentiation, highlighting at least part of the therapeutic mechanism of AZA and DAC in human MDS. Both AZA and DAC similarly decrease the levels of heterogeneous nuclear ribonucleoproteins (hnRNPs) (Buchi *et al*., 2012). Myeloid diseases, such as those driven by *AML1-ETO* and *BCR-ABL1*, generally overexpress hnRNPs, which dysregulates mRNA splicing and metabolism (Ohshima *et al*., 2003). These events are associated with downregulation of MYC and FOS signaling, of the late differentiation gene, *C/EBPA*, and may impact mixed lineage leukemia (MLL) histone methyltransferase activity (Eiring *et al*., 2008, 2010; Jing *et al.*, 2011). MYC and FOS are also associated with DNMT1 where increased FOS expression is associated with overexpression of *DNMT1* and DNMT1 epigenetic gene silencing antagonizes *MYC* expression, while MLL is an upstream activator of HOXA9 (Mishra *et al*., 2008; Saunthararajah *et al*., 2012; L.-L. Smith *et al*., 2011). Taken together, the activities of hnRNPs potentially create a large interactive network with *NHA9* and *DNMT1* in the regulation of genome epigenetics. These various findings suggest that zebrafish *dnmt1* is a druggable target in our *NHA9* embryos, which could have relevance to human myeloid disease, and that hnRNP activity could be further downstream of *dnmt1* in our *NHA9* embryos, possibly to limit expression of *c/ebpa*.

4.3.2 Possible mechanisms in NHA9-induced epigenetic dysregulation

Recently, Clements *et al.* demonstrated that DNMT1 has non-catalytic, non DNA methyltransferase activity that also epigenetically represses gene activity (Clements et al., 2012). In this study, they produced three colon cancer cell lines: one cell line with wild-type DNMT1; another cell line with mutated *DNMT1*, which produced DNMT1 without

its catalytic domain; and the last cell line with no *DNMT1*expression. They were able to find downstream repressive targets of DNMT1 by identifying genes upregulated in the *DNMT1*$^{-/-}$ hypomorphic cells. Then by allowing for production of catalytically inactive DNMT1 in the cell line, they were able to determine that some of the DNMT1 target genes were still being repressed although DNMT1 was not able to methylate newly synthesized DNA. This suggested an alternative means of transcriptional repression by DNMT1. Interestingly, the use of DNA methyltransferase inhibitors, such as DAC, AZA and Zeb also induce dnmt1 degradation and depletion from the nucleus, suggesting that broad functions of dnmt1 are inhibited by the pharmacological therapy (Ghoshal *et al*., 2005; Patel *et al*., 2010).

This concept of catalytically inactive DNMT1 still repressing gene expression invites further investigation of the sites at which DNMT1 functions and the types of protein complexes in which it is a component in order to repress transcription. For instance, in the previous DNMT1 study, the authors determined that DNMT1 interacts with a range of histone modifiers such as H3-K4me2, H3-K4me3 and H3-K9Ac (Clements *et al*., 2012). In addition to interaction with H3-K4 demethylases (KDMs), DNMT1 has been described to interact with HDAC s(Fuks *et al.*, 2000; Robertson *et al*., 2000; Rountree *et al.*, 2000), which have been described previously to be components of many repressive epigenetic complexes (*see section 1.5.2*).

A zebrafish study has demonstrated that *dnmt1* works in concert with another epigenetic co-factor, *suv39h1a* (*suppressor of variegation 3-9 homolog 1a*), which selectively methylates specific lysine residues on histones rather than the cytosines in DNA (Rai, 2006; Rai *et al.*, 2006). It has been proposed that suv39h1a also binds to DNA

that has recently been methylated by dnmt1. This subsequently leads to trimethylation of histone H3 at lysine 9 (H3K9) to recruit HDACs. In the zebrafish *suv39h1a* MO injection at the one-cell stage mimicked the same differentiation defects seen with the loss of *dnmt1*(Rai *et al*., 2006). These results suggest that dnmt1 methylating activity is linked to broader epigenetic silencing machinery and that *dnmt1-suv39h1a* activity may regulate the hematopoietic differentiation defects in *NHA9* embryos.

4.3.3 Epigenetic combination therapy synergistically inhibits NUP98-HOXA9-induced myeloid disease in transgenic zebrafish embryos

Importantly, treatment with DNA methyltransferase inhibitors, such as DAC and Zeb restores expression of late differentiation genes, but may be insufficient to cure myeloid disease when used alone as a monotherapy (Sauntharajah et al., 2012). There is also a risk that DAC treatment is itself carcinogenic, as genome-wide DNA hypomethylation can lead to genomic instability, chromosome rearrangements, and secondary tumours in animals (Maslov *et al*., 2012). This cautions against the indiscriminate use of demethylating agents and asserts the value in searching for effective combination chemotherapy regimens. Additionally, the sequential link between DNA methylation and histone deacetylation further suggests that DNA methyltransferase inhibitors combined with HDAC inhibitors are an effective means to combat human myeloid disease.

Clinical trials using DAC and VPA as a combination therapy alternative to current chemotherapy have been conducted with mixed results in terms of response to treatment and toxicity to the patient (*see section 1.5.2*). Here, I have demonstrated that *NHA9* is uniquely susceptible to the combined pharmacological treatment of DAC + VPA or DAC

+ TSA in transgenic zebrafish embryos. These are encouraging results as patients with *NHA9*-induced myeloid disease are at high risk and generally respond quite poorly to current therapies (Borrow *et al*., 1996; Forrester *et al*., 2011; Iwasaki *et al*., 2005). This specific susceptibility may offer the ability to sensitize AML blast cells to current chemotherapy treatment therefore enabling increased survival in patients with *NHA9*-associated myeloid disease.

4.3.3.1 Possible mechanisms for combined histone deacetylase complex inhibitors and DNA demethylating agents

Natural DNA repair mechanisms are able to identify and extract DAC and other nucleoside analogues and repair the affected sequence of DNA. HDAC inhibitors, however, have been demonstrated to help reduce the cells ability to remove those nucleoside analogues (Chai *et al*., 2008). This reduced ability of a cell to maintain its methylation pattern may be one mechanism of action that contributes to the synergistic effect of DAC and VPA/TSA combination therapy observed in the *NHA9* zebrafish. If so, HDAC inhibitors greatly reduce not only the amount of DAC or other DNA methyltransferase inhibitors required for therapy but limit the number of treatments necessary as the HDAC inhibitor would keep the nucleoside analogue from being degraded, thus limiting patient toxicity. Alternatively, as discussed previously, DNMT1 interacts with many proteins involved in chromatin remodeling and epigenetically represses gene expression through more than one regulator pathway. Therefore, the combination of DAC with an HDAC inhibitor may disrupt the interactions of DNMT1 with HDACs or other epigenetic complexes, thus reducing its methylating capabilities (Robertson *et al*., 2000; Rountree *et al*., 2000; Savickiene *et al.,* 2012). This inhibition of

DNMT1 to bind to other epigenetic complexes through HDAC inhibitor treatment may also explain the synergy demonstrated when I used VPA in combination with the COX inhibitors Indo or NS-398 (*see section 4.3.4*).

4.3.4 Histone deacetylase inhibitors and COX inhibitors work to combat NUP98-HOXA9-induced myeloid disease in transgenic zebrafish

DNMT1 is dispensable in normal hematopoiesis, but required in LSCs (Trowbridge *et al.*, 2012). Moreover, a recent zebrafish study linked Wnt/β-cat signaling to the regulation of DNA methylation (Rai *et al*., 2010). This group showed that APC, a negative regulator of β-cat, subsequently regulates methylation and demethylation machinery. Similarly, in human colorectal cancer cells, reactivation of APC reduced the expression of *DNMT1*(Campbell & Szyf, 2003). Therefore, Wnt/β-cat and *DNMT1* regulation share a similar pathway of activation.

Interestingly, treatment with VPA inhibits GSK3 in human HSC lines, which leads to the activation of the Wnt/β-cat pathway (Jung *et al*., 2008; Z. Wang *et al*., 2010; Wiltse, 2005). However, this seems contradictory to the current understanding of the treatment effects of VPA and the effects of the canonical Wnt/β-cat pathway. For instance, Wnt/β-cat activation has been demonstrated to reduce differentiation, increase proliferation and self-renewal, while VPA treatment has been demonstrated *in vitro* in leukemia blast cell lines*, in vivo* studies in zebrafish and murine leukemia models and clinical trials in patients with myeloid disease, to induce differentiation and reduce proliferation (Bellos & Mahlknecht, 2008; Blum *et al.,* 2007; Cinatl *et al.*, 1997; Göttlicher *et al*., 2001; Jung *et al*., 2008; Kuendgen *et al*., 2004). Thus, it appears that VPA may have differential effects on HSCs as compared with leukemia blast cells.

However, the effect on LSCs rather compared to slightly more mature leukemic blasts has not directly been investigated.

Laq824 and CG1521, two potent HDAC inhibitors, which induce differentiation and/or apoptosis in leukemia cell lines, share the effect of VPA on HSCs (Ivanenkov *et al.*, 2008). There is a relationship between the differentiation level and the response to HDAC inhibitors, meaning that very immature cells respond to HDAC inhibitors with a down-regulation of *p21*and cell cycle progression, whereas at a more advanced differentiation stage, cells respond to VPA and Laq824 with a down-regulation of *p21*and differentiation or apoptosis (Aldana-Masangkay *et al*., 2011; Gurvich *et al.*, 2004). As such, VPA may increase self-renewal of HSCs by ‘‘transcriptional reprogramming’’ of these cells through its capacity to induce histone acetylation as well as DNA demethylation in a dose-dependent manner (Detich *et al.*, 2003), while by contrast inducing slightly more mature leukemia blast cells to differentiate or undergo apoptosis.

Combining VPA with either a general COX inhibitor or a specific COX2 inhibitor demonstrated a strong inhibition of the *NHA9* hematopoietic phenotype in transgenic zebrafish embryos. As these therapies seem to work antagonistically, my findings need to be reconciled. Firstly VPA might inhibit some hematopoietic defects by impacting the efficiency of dnmt1 ability to methylate DNA, while the Indo or NS-398 treatment reduces global canonical Wnt/β-cat activity. Thus, the downstream effects of *NHA9* on *dnmt1*are reduced and the *NHA9* expressing myeloid cells, which may have more sensitivity to β-cat expression, are reduced, inhibiting the myeloproliferative effect of *NHA9.* Alternatively, Indo and NS-398 treatment could be reducing the effects of the large increase in *ptgs2* in the EMPs and HSCs of the embryo, while VPA simultaneously

reduces the methylating ability of dnmt1 and induces differentiation in the more mature myeloid cells. Regardless of the means of rescue, it appears that the combination of a HDAC inhibitor with a COX inhibitor may be an effective therapy in *NHA9*-induced myeloid disease.

4.3.5 dnmt1 future directions

DNMT1 methylates cytosine residues in DNA, so an established methylation analysis of zebrafish genomic DNA (Anderson et al., 2009) may be used to confirm the upregulation of *dnmt1* in *NHA9* embryos. For instance, genomic DNA from *NHA9* activated and *Cre* (control) embryos would be isolated and digested with methylation-sensitive HpaII, or methylation insensitive MspI restriction enzymes. Samples would undergo Southern blot analysis using probes targeted against the consensus *DANA* sequence, a short interspersed nuclear element (SINE) that comprises ~10% of the zebrafish genome and is methylated by dnmt1 (Izsvák *et al.*, 1996). In the absence of dnmt1, the hypomethylated *DANA* sequence is digested, and widespread genomic hypomethylation results in smearing of high molecular weight DNA. Thus, *NHA9* embryos should present little to no digestion of the *DANA* sequence and the absence of high molecular weight smearing.

Furthermore, repeated *uhrf1* MO injections are necessary to help determine its effects on hematopoiesis in *NHA9* activated embryos on both erythropoiesis as well as myelopoiesis. Preliminary results suggest that *urf1* MO knockdown phenocopies the rescue of *gata1* expression in the *NHA9* activated embryos seen with *dnmt1* MO knockdown. However, how *uhrf1* MO knockdown affects the myeloproliferative phenotype needs to be assessed. Additionally, *suv39h1a,* which works in tandem with

dnmt1, could be knocked down by MO injection, to corroborate my experiments with *dnmt1* MO injections. In contrast, I can also inject *uhrf1* and *suv39h1a* mRNA in attempt to augment *dnmt1* activity and possibly accelerate the *NHA9*-induced hematopoietic phenotype. Additionally, cross-reactive antibodies are available for zebrafish dnmt1 and uhrf1 proteins, therefore Western blot analysis could be performed in order to denote the efficiency of the MO and mRNA injections into the embryos.

Finally, MDS and AML are both associated with *DNMT1* overexpression, but *DNMT3* family genes, which encode *de novo* DNA methyltransferases, are often found to be downregulated or mutated in AML (Challen *et al.*, 2012; Ley *et al*., 2010; Thol *et al*., 2011; Yan *et al*., 2011). DNMT3 proteins are *de novo* DNA methyltransferases, but work in concert with DNMT1 to regulate HDACs and ultimately epigenetic silencing. DAC and AZA are nucleoside analogues, so part of their therapeutic action is incorporation into cellular nucleic acids leading to genotoxic stress. Interestingly, DAC treatment is known to lack efficacy in cells that lack DNMT3A and DNMT3B, suggesting that incorporated DAC is first targeted by *de novo* DNA methyltransferases before binding to DNMT1 (Patel *et al*., 2010). Thus, an investigation into the role of the *DNMT3* gene family in *NHA9* embryos may be warranted. However, in zebrafish, there are six genes that constitute the *dnmt3* family. The current consensus is that *dnmt6* and *dnmt8* primarily account for dnmt3a enzyme activity, while *dnmt4* accounts for dnmt3b enzyme activity (TSmith *et al.*, 2011). Using qRT-PCR on *NHA9* embryos, one could begin by measuring expression for *dnmt4, dnmt6* and *dnmt8* then look to MO to knockdown the expression and determine its effects in *NHA9* activated embryos.

4.4 *NUP98-HOXA9* EXPRESSION IN TRANSGENIC ZEBRAFISH DEMONSTRATES AN EXPANSION OF HSCS

It has been long hypothesized AML is caused by an elusive LSC that accumulates genetic/epigenetic mutations, which leads to selective advantages, such as reduced apoptotic response, differentiation inhibition and increase proliferation/self-renewal (Horton & Huntly, 2012; Mikesch *et al.*, 2007; Reya *et al.*, 2001; Wojiski *et al.*, 2009; Zhang *et al.*, 2006). Additionally, these LSCs are believed to give rise to all leukemic blast cells, therefore represent ideal therapeutic targets in AML. Not surprisingly, I found that transgenic zebrafish embryos expressing the *NHA9* fusion oncogene demonstrate an approximate 2-fold expansion of HSCs.

Overexpression of *HoxA9* in murine marrow cells leads to HSC expansion, conditional immortalization of myeloid progenitor cells and repression of differentiation - all hallmarks of acute myeloid disease (Helgason *et al*., 1996; Perkins & Cory, 1993; Sauvageau *et al*., 1994; Thorsteinsdottir *et al*., 1997; Thorsteinsdottir *et al*., 2002). In *NHA9* activated in transgenic zebrafish embryos, the transcriptional activation properties of *HOXA9* are prompted in the HSCs under the *spi1* promoter, therefore potentially bestowing additional expansion capabilities. Recently, it has been determined that HSCs acquire mutations at a linear rate with time (Welch *et al.*, 2012). Moreover, it is hypothesized that LSCs in AML are from randomly occurring events that occurred in the HSC before they acquired an initiating mutation (Welch *et al*., 2012). If in *NHA9*-mediated myeloid disease, HSCs acquire an increased ability to self-renew/proliferate, this would increase the rate of mutations over time, increasing the likelihood of acquiring a second mutation 'hit', thus increasing the chance of leukemogenesis.

4.4.1 Hematopoietic stem cell – future directions

This *NHA9*-induced hematopoietic expansion of HSCs could be leveraged to determine potential therapeutics to combat *NHA9*-mediated myeloid disease. For instance, treatment with DNA methyltransferase inhibitors, HDAC inhibitors and COX inhibitors, which I have demonstrated to help rescue *NHA9*-induced disease at early time points during embryogenesis, could be used to treat embryos at later time points to measure their effect on HSC expansion. This would prove useful in determining if these treatment alternatives transfer well to treatment of adult LSCs. Additionally, these treatments can be carried over to the previously published zebrafish transplant model.

As the HSCs are the part of the definitive waves of hematopoiesis during embryogenesis and produce all blood cell lineages in adults, they may be the cells of origin in *NHA9*-induced MPN in transgenic zebrafish. It would be of interest to determine if the HSCs expressing *NHA9* have selective advantages to normal HSCs. To do this juvenile wild-type *runx1::GFP* and *NHA9;runx1::GFP* zebrafish could be dissociated and GFP^+ cells sorted using FACS then put through a cytospin to filter out the GFP^+ HSCs. Cells can then be injected into the yolk sac of 2-day-old wild-type zebrafish embryos using the established cellular transplant protocols (Langenau *et al*., 2013; Traver *et al.*, 2004). Using established proliferation and migration assays in the embryos, we can determine if the *NHA9* cells have a selective advantage to wild-type HSCs in their proliferative or migratory capabilities. Afterwards, using drugs previously demonstrated in this thesis to have an effect on *NHA9*-mediated hematopoiesis, transplanted embryos can be treated to see if there is a proliferative or migratory inhibition on injected GFP^+

cells. This may be able to determine if *NHA9*expressing HSCs have an increased sensitivity to the selected therapies compared to wild-type HSCs.

Interestingly, it has been demonstrated that ascorbic acid (vitamin C) promotes specific DNA demethylation of the epigenome in human embryonic stem cells (hESC) (T.-L. Chung *et al.*, 2010). They determined that 1,847 genes are hypomethylated just outside of the promoter transcription start sites after hESCs are grown in 50mg/mL Vitamin C rich media. Furthermore, it has recently been demonstrated that Vitamin C activates TET enzymes, which is most likely the cause of the hypomethylation (Blaschke *et al*., 2013; Wossidlo *et al*., 2011). TET enzymes convert methylated cytosines (5mC) to hydroxymethylated cytosines (5hmC), which prevents DNMTs from recognizing the methylation pattern after DNA synthesis and therefore prevents the maintenance of methylation. Interestingly, there are a number of hematologic malignancies, including MDS, MPN, and de novo and secondary AML that are associated with TET mutations (Blaschke *et al.*, 2013; Holmfeldt & Mullighan, 2011; Kosmider *et al*., 2009; Nikoloski *et al.*, 2012; Roche-Lestienne *et al.*, 2011). Notably, up to 50 percent of chronic myelomonocytic leukemia (CMML), a mixed MDS/MPN condition with a propensity to progress to AML, harbor TET2 mutations. It would therefore be of interest to treat embryos with Vitamin C to determine its effects on embryo hematopoiesis.

Vitamin C treatment has already been established in zebrafish embryos and increased Vitamin C has lead to an increase in cellular energy production (Kirkwood *et al*., 2012). This treatment can be mimicked in wild-type *runx1::GFP* and *NHA9;runx1::GFP* activated embryos to determine its effect on HSC expansion. Vitamin

C demethylation could help inhibit the effects of the increased expression of *dnmt1*, therefore reducing the *NHA9*-mediated defect in hematopoiesis.

4.5 A ZEBRAFISH XENOTRANSPLANTATION MODEL USING HUMAN MYELOID LEUKEMIA CELLS TO EXAMINE DRUG RESPONSES

In a similar manner to the zebrafish HSC transplant protocol, using zebrafish as a xenotransplantation model is a potential complimentary strategy to my work in *NHA9* transgenic zebrafish. Two groups, including ours, have exploited xenotransplantation for the study of myeloid leukemias (Corkery *et al.*, 2011; Pruvot *et al*., 2011). K562, erythroleukemia, and NB4, acute promyelocytic leukemia, cell lines were fluorescently labeled with CM-DiI and injected into the yolk sac of 48 hpf zebrafish embryos. At this time point, xenograft rejection is minimized as the adaptive immune system has yet to develop. The embryos were then treated with chemotherapeutics by addition of the compound to the water at low enough concentrations to prevent embryo toxicity. Embryos with the K562 cells, which harboured the *BCR-ABL1* fusion (*see section 1.1.3*) and NB4 cells, which harbour the *PML-RAR*α fusion oncogene were treated with either imatinib mesylate or all-*trans*-retinoic acid (ATRA), respectively.

The number of K562 xenografted cells after exposure to imatinib was reduced in a dose-dependent manner and there was death of the NB4 xenografted cells after ATRA treatment (Corkery *et al.*, 2011; Pruvot *et al.*, 2011). Most importantly, when the treatment was swapped and applied against the opposite cell line, there was no proliferative inhibition, suggesting that the human cancer cells can be specifically targeted in the zebrafish xenotransplantation model. Ideally, using K562 cells transfected with the *NHA9* fusion oncogene, we can use the same xenotransplantation model and the

treatment protocols I have developed in the *NHA9* transgenic zebrafish line to compare if the therapy is transferable to human *NHA9* leukemia cells.

At 48 hpf, the mutant zebrafish line, *casper,* which lacks all pigment for optical transparency, can be microinjected with transfected *NHA9* K562 cells. The cell line will be fluorescently labeled with CM-DiI tracking dye and approximately 25-50 cells will be microinjected into each embryo. Embryos can then be screened by fluorescent microscopy for the presence of a fluorescent mass at the site of injection. Positive embryos will then be divided into two groups; one group will act as a control and not be treated with any compound while the other group is exposed to the potential therapeutics (i.e., DAC, Zeb, VPA, ect) in a single or combined manner. At the end of a given time point post injection, the embryos can be dissociated into a single cell suspension and the number of fluorescent cells can be counted using a semi-automated macro in ImageJ (NIH, Bethesda, MD). The number of fluorescent cells present in the control embryos divided by the number of cells in the embryos treated with the pharmacological drugs would represent the fold change in the cell number. Ideally, this work would further extend the use of zebrafish in identifying novel therapeutic strategies to combat human leukemia with improved anti-cancer potency and reduced toxicity.

4.6 LIMITATIONS OF THE ZEBRAFISH MODEL

The zebrafish offers exciting opportunities to study a number of diseases in a relatively short time frame that is scarcely rivaled by traditional animal models. The zebrafish has specific utility to elucidate molecular mechanisms underlying oncogenesis by virtue of ease of genetic manipulations; observations of invasive cellular phenotypes, due to embryonic transparency and unique imaging; and as a relatively high-throughput

cost-effective first pass *in vivo* platform to evaluate drug responses to prospective anti-cancer agents. However, there remain several biological limitations when using zebrafish as a model for human cancers.

In zebrafish, there is a redundancy of genes as a result of a whole genome duplication (Amores *et al*., 1998). This genome duplication could mask some changes to gene expression. For example, the mammalian genome has three forms of *DNMT3* (*DNMT3A, DNMT3B, DNMT3L*), however the zebrafish genome consists of six *dnmt3* paralogues (Campos, *et al.,* 2012), which reduces the feasibility of using reverse genetic techniques to identify specific phenotypes. Additionally, as a newer model system the zebrafish genome is less annotated compared to mammals and there are less nucleotide 'spots' on a zebrafish microarray chip (Lewis S, personal communication), which means that not all possible homologous genes can be measured with current technology. Also, rigorous analytical tools will be required to compare the gene expression signatures obtained from multiple experimental conditions, which means that some important changes to gene expression could be screened out. Moreover, the newer, more novel technology, such as RNA-seq, is first designed and used in mammalian modeling systems and requires time before being implemented into zebrafish work,

Gene knockdown has been performed by injection of gene specific morpholino oligonucleotides, which transiently targets mRNA to either prevent proper splicing or translation into protein. However, this approach has its limitations, not the least of which the potential for non-specific 'off target' effects of the reagents along with the consequent need to perform careful titration and multiple controls for specificity of the injected oligonucleotides. Additionally, the transient nature of the inhibition of gene function

achieved by MO injection effectively limits their application to only early stages of development preventing the use of gene knockdown for the establishment of models of late onset disease (Huang, *et al.,* 2012). However, as a newer yet, promising *in vivo* model, techniques to combat these limitations are continuously being investigated and put into experimental practice such as TALENs, Zinc-fingers and CRISPRs, which enable us to modify the zebrafish genome and knock-out genes for long term reverse genetic analysis.

4.7 CONCLUSION

In summary, this research has demonstrated some of the attributes that make the zebrafish a novel and versatile model for studying human cancers. We have developed a transgenic zebrafish line for studying *NUP98-HOXA9*-mediated, high-risk AML with a specific emphasis on identifying pharmacologically targetable genetic and epigenetic downstream contributors. We have identified *dnmt1* and the Wnt/β-cat pathway as possible targets, and have demonstrated ways in which to use these targets to specifically inhibit the hematopoietic effects of *NHA9* while allowing for normal hematopoiesis to continue, undisturbed. I have also been able to demonstrate how a combination of current FDA approved drugs that target epigenetic regulatory pathways, can be used to treat *NHA9*-mediated disease synergistically at surprisingly low concentrations. As such, I believe that the future of this transgenic model could easily be applied to study additional downstream contributors of *NHA9* in the zebrafish, in order to determine new and promising treatment targets that can further increase the efficacy of current therapy as well as reduce the toxicity to the patients, therefore satisfying the long-term goal of AML research.

REFERENCES

Abdul-hamid, G. (1999). Classification of Acute Leukemia. *University of Aden/Hematology Unit Yemen*. Retrieved from www.intechopen.com

Ahmad, E. I., Gawish, H. H., Al Azizi, N. M., & Elhefni, A. M. (2011). The prognostic impact of K-RAS mutations in adult acute myeloid leukemia patients treated with high-dose cytarabine. *OncoTargets and therapy*, *4*(4), 115–21. doi:10.2147/OTT.S12602

Ahuja, H. G., Popplewell, L., Tcheurekdjian, L., & Slovak, M. L. (2001). NUP98 gene rearrangements and the clonal evolution of chronic myelogenous leukemia. *Genes, chromosomes & cancer*, *30*(4), 410–5. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11241795

Aldana-Masangkay, G. I., Rodriguez-Gonzalez, A., Lin, T., Ikeda, A. K., Hsieh, Y.-T., Kim, Y.-M., … Sakamoto, K. M. (2011). Tubacin suppresses proliferation and induces apoptosis of acute lymphoblastic leukemia cells. *Leukemia & lymphoma*, *52*(8), 1544–55. doi:10.3109/10428194.2011.570821

Alseraye, F. M., Zuo, Z., Bueso-Ramos, C., Wang, S., Medeiros, L. J., & Lu, G. (2011). Trisomy 11 as an isolated abnormality in acute myeloid leukemia is associated with unfavorable prognosis but not with an NPM1 or KIT mutation. *International journal of clinical and experimental pathology*, *4*(4), 371–7. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3093062&tool=pmcentrez&rendertype=abstract

Amatruda, J. F., Shepard, J. L., Stern, H. M., & Zon, L. I. (2002). Zebrafish as a cancer model system. *Cancer cell*, *1*(3), 229–31. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12086858

Amores, A., Force, A., Yan, Y. L., Joly, L., Amemiya, C., Fritz, A., … Postlethwait, J. H. (1998). Zebrafish hox clusters and vertebrate genome evolution. *Science*, *282*(5394), 1711–4. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9831563

Anderson, R. M., Bosch, J. A., Goll, M. G., Hesselson, D., Si, P. D., Shin, D., … Stainier, D. Y. R. (2009). Loss of Dnmt1 catalytic activity reveals multiple roles for DNA methylation during pancreas development and regeneration. *Developmental biology*, *334*(1), 213–223. doi:10.1016/j.ydbio.2009.07.017.Loss

Andersson, a K., Miller, D. W., Lynch, J. a, Lemoff, a S., Cai, Z., Pounds, S. B., … Downing, J. R. (2011). IDH1 and IDH2 mutations in pediatric acute leukemia. *Leukemia*, *25*(10), 1570–7. doi:10.1038/leu.2011.133

Aoki K, T. M. M. (2008). Tissue-specific transgenic, conditional knockout and knock-in mice of genes in the canonical Wnt signaling pathway. *Methods in molecular biology*, *468*, 307–331.

Araki, Y., Okamura, S., Hussain, S. P., Nagashima, M., He, P., Shiseki, M., & Miura, K. (2003). Regulation of Cyclooxygenase-2 expression by the Wnt and Ras pathways. *Cancer research*, *63*, 728–734.

Argiropoulos, B., & Humphries, R. K. (2007). Hox genes in hematopoiesis and leukemogenesis. *Oncogene*, *26*(47), 6766–76. doi:10.1038/sj.onc.1210760

Arita, K., Ariyoshi, M., Tochio, H., Nakamura, Y., & Shirakawa, M. (2008). Recognition of hemi-methylated DNA by the SRA protein UHRF1 by a base-flipping mechanism.pdf. *Nature*, *455*.

Bacher, U., Haferlach, C., Kern, W., Haferlach, T., & Schnittger, S. (2008). Prognostic relevance of FLT3-TKD mutations in AML: the combination matters--an analysis of 3082 patients. *Blood*, *111*(5), 2527–37. doi:10.1182/blood-2007-05-091215

Bacher, U., Haferlach, T., Schoch, C., Kern, W., & Schnittger, S. (2006). Implications of NRAS mutations in AML: a study of 2502 patients. *Blood*, *107*(10), 3847–53. doi:10.1182/blood-2005-08-3522

Bai, X., Kim, J., Yang, Z., Jurynec, M. J., Akie, T. E., Lee, J., … Zon, L. I. (2010). TIF1gamma controls erythroid cell fate by regulating transcription elongation. *Cell*, *142*(1), 133–43. doi:10.1016/j.cell.2010.05.028

Bäsecke, J., Schwieger, M., Griesinger, F., Schiedlmeier, B., Wulf, G., Trümper, L., & Stocking, C. (2005). AML1/ETO promotes the maintenance of early hematopoietic progenitors in NOD/SCID mice but does not abrogate their lineage specific differentiation. *Leukemia & lymphoma*, *46*(2), 265–72. doi:10.1080/10428190400010767

Baumann, R., & Dragon, S. (2005). Erythropoiesis and red cell function in vertebrate embryos. *European journal of clinical investigation*, *35 Suppl 3*, 2–12. doi:10.1111/j.1365-2362.2005.01524.x

Beckwith, L. G., Moore, J. L., Tsao-Wu, G. S., Harshbarger, J. C., & Cheng, K. C. (2000). Ethylnitrosourea induces neoplasia in zebrafish (Danio rerio). *Laboratory investigation; a journal of technical methods and pathology*, *80*(3), 379–85. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10744073

Behrens, J., Jerchow, B.-A., Wurtele, M., Grimm, J., Wirtz, R., Kuhl, M., … Kohl, M. (1998). Functional Interaction of an Axin Homolog, Conductin, with β-Catenin, APC, and GSK3β. *Science*, *280*(5363), 596–599.

Bei, L., Huang, W., Wang, H., Shah, C., Horvath, E., & Eklund, E. (2011). HoxA10 activates CDX4 transcription and Cdx4 activates HOXA10 transcription in myeloid cells. *The Journal of biological chemistry*, *286*(21), 19047–64. doi:10.1074/jbc.M110.213983

Bei, L., Lu, Y., & Eklund, E. A. (2005). HOXA9 activates transcription of the gene encoding gp91Phox during myeloid differentiation. *The Journal of biological chemistry*, *280*(13), 12359–70. doi:10.1074/jbc.M408138200

Bellos, F., & Mahlknecht, U. (2008). Valproic acid and all-trans retinoic acid: meta-analysis of a palliative treatment regimen in AML and MDS patients. *Onkologie*, *31*(11), 629–33. doi:10.1159/000160599

Bennett, C. M., Kanki, J. P., Rhodes, J., Liu, T. X., Paw, B. H., Kieran, M. W., … Look, T. A. (2001). Myelopoiesis in the zebrafish, Danio rerio. *Blood*, *98*(3), 643–651. doi:10.1182/blood.V98.3.643

Bennett, J. M., Catovsky, D., Daniel, M. T., Flandrin, G., Galton, D. A. G., Gralnick, H. R., & Sultan, C. (1985). Proposed Revised Criteria for the Classification of Acute Myeloid Leukemia. *Annual Iternal Medicine*, *103*, 626–629.

Berghmans, S., Langenau, D., Hsu, K., Stewart, R., T, L., Kanki, J., & Jette, C. (2005). Making waves in cancer research: new models in the zebrafish. *BioTechniques*, *39*(2), 227–237.

Berman, J., Hsu, K., & Look, T. A. (2003). Zebrafish as a model organism for blood diseases. *British journal of haematology*, *123*(4), 568–76. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14616958

Berman, J. N., Kanki, J. P., & Look, T. A. (2005). Zebrafish as a model for myelopoiesis during embryogenesis. *Experimental hematology*, *33*(9), 997–1006. doi:10.1016/j.exphem.2005.06.010

Bertrand, J. Y., Kim, A. D., Violette, E. P., Stachura, D. L., Cisson, J. L., & Traver, D. (2007). Definitive hematopoiesis initiates through a committed erythromyeloid progenitor in the zebrafish embryo. *Development (Cambridge, England)*, *134*(23), 4147–56. doi:10.1242/dev.012385

Bird, A. (2002). DNA methylation patterns and epigenetic memory. *Genes & development*, *16*(1), 6–21. doi:10.1101/gad.947102

Blaschke, K., Ebata, K. T., Karimi, M. M., Zepeda-Martinez, J. A., Goyal, P., Mahapatra, S., … Ramalho-Santos, M. (2013). Vitamin C induces Tet-dependent DNA demethylation and a blastocyst-like state in ES cells. *Nature*, *Advance on*(ePub). Retrieved from http://dx.doi.org/10.1038/nature12362

Blum, W., Klisovic, R. B., Hackanson, B., Liu, Z., Liu, S., Devine, H., … Marcucci, G. (2007). Phase I study of decitabine alone or in combination with valproic acid in acute myeloid leukemia. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, *25*(25), 3884–91. doi:10.1200/JCO.2006.09.4169

Bonnet, D., & Dick, J. E. (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Medicine*, *3*(7), 730–737.

Borrow, J., Shearman, A. A., Stanton, V. P., Becher, R., Collins, T., Williams, A. J., … Housman, D. E. (1996). The t(7;11)(p15;p15) translocation in acute myeloid leukmaemia fuses the genes for nucleoporin NUP98 and class 1 homeoprotein HOXA9. *Nature genetics*, *12*, 159–167.

Borthakur, G., & Estey, E. E. (2010). Therapy of Acute Myelogenous Leukemia in Adults. In L. Nagarajan (Ed.), *Acute Myelogenous Leukemia: Genetics, Biology and Therapy* (Vol. 145, pp. 257–270). New York, NY: Springer New York. doi:10.1007/978-0-387-69259-3

Bostick, M., Kim, J. K., Estève, P.-O., Clark, A., Pradhan, S., & Jacobsen, S. E. (2007). UHRF1 plays a role in maintaining DNA methylation in mammalian cells. *Science*, *317*(5845), 1760–4. doi:10.1126/science.1147939

Bradbury, J. (2004). Zebularine : a candidate for epigenetic cancer therapy Stapled peptide induces cancer cell death. *Drug Discoveries and Therapeutics*, *9*(21), 1–2.

Brown, K. D., & Robertson, K. D. (2007). DNMT1 knockout delivers a strong blow to genome stability and cell viability. *Nature. Genetics*, *39*(3), 289–290. doi:10.1083/jcb.200610062

Buchi, S. E., Masala, E., Gozzini, A., Sanna, A., Bosi, A., Ferrari, G., & Santini, V. (2012). Proteomic analysis identifies differentially expressed proteins in AML1/ETO acute myeloid leukemia cells treated with DNMT inhibitors azacitidine and decitabine. *Leukemia Research*, *36*(5), 607–618.

Burnett, A. K. (2012). New induction and postinduction strategies in acute myeloid leukemia. *Current opinion in hematology*, *19*(2), 76–81. doi:10.1097/MOH.0b013e3283500a92

Burnett, A. K., Hills, R. K., Milligan, D. W., Goldstone, A. H., Prentice, A. G., McMullin, M.-F., … Wheatley, K. (2010). Attempts to optimize induction and consolidation treatment in acute myeloid leukemia: results of the MRC AML12 trial. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, *28*(4), 586–95. doi:10.1200/JCO.2009.22.9088

Burnett, A., Wetzler, M., & Löwenberg, B. (2011). Therapeutic advances in acute myeloid leukemia. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, *29*(5), 487–94. doi:10.1200/JCO.2010.30.1820

Burns, C. E., Traver, D., Mayhall, E., Shepard, J. L., & Zon, L. I. (2005). Hematopoietic stem cell fate is established by the Notch-Runx pathway. *Genes & development*, *19*(19), 2331–42. doi:10.1101/gad.1337005

Byrne, M. M., Halman, L. J., Koniaris, L. G., Cassileth, P. a, Rosenblatt, J. D., & Cheung, M. C. (2011). Effects of poverty and race on outcomes in acute myeloid leukemia. *American journal of clinical oncology*, *34*(3), 297–304. doi:10.1097/COC.0b013e3181dea934

Calvo, K R, Knoepfler, P. S., Sykes, D. B., Pasillas, M. P., & Kamps, M. P. (2001). Meis1a suppresses differentiation by G-CSF and promotes proliferation by SCF: potential mechanisms of cooperativity with Hoxa9 in myeloid leukemia. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(23), 13120–5. doi:10.1073/pnas.231115398

Calvo, Katherine R, Sykes, D. B., Pasillas, M. P., & Kamps, M. P. (2002). Nup98-HoxA9 immortalizes myeloid progenitors, enforces expression of Hoxa9, Hoxa7 and Meis1, and alters cytokine-specific responses in a manner similar to that induced by retroviral co-expression of Hoxa9 and Meis1. *Oncogene*, *21*(27), 4247–56. doi:10.1038/sj.onc.1205516

Campbell, P. M., & Szyf, M. (2003). Human DNA methyltransferase gene DNMT1 is regulated by the APC pathway. *Carcinogenesis*, *24*(1), 17–24.

Campos, C., Valente, L. M. P., & Fernandes, J. M. O. (2012). Molecular evolution of zebrafish dnmt3 genes and thermal plasticity of their expression during embryonic development. *Gene*, *500*(1), 93–100. doi:http://dx.doi.org/10.1016/j.gene.2012.03.041

Cantile, M., Schiavo, G., Terracciano, L., & Cillo, C. (2007). The HOX Gene Network as a Potential Target for Cancer Therapy. *Current Cancer Therapy Reviews*, *3*(1), 17–24.

Catlin, S. N., Busque, L., Gale, R. E., Guttorp, P., & Abkowitz, J. L. (2011). The replication rate of human hematopoietic stem cells in vivo. *Blood*, *117*(17), 4460–6. doi:10.1182/blood-2010-08-303537

Chai, G., Li, L., Zhou, W., Wu, L., Zhao, Y., Wang, D., … Zhu, W.-G. (2008). HDAC inhibitors act with 5-aza-2'-deoxycytidine to inhibit cell proliferation by suppressing removal of incorporated abases in lung cancer cells. *PloS one*, *3*(6), e2445. doi:10.1371/journal.pone.0002445

Challen, G. a, Sun, D., Jeong, M., Luo, M., Jelinek, J., Berg, J. S., … Goodell, M. a. (2012). Dnmt3a is essential for hematopoietic stem cell differentiation. *Nature genetics*, *44*(1), 23–31. doi:10.1038/ng.1009

Chang, C. P., Jacobs, Y., Nakamura, T., Jenkins, N. a, Copeland, N. G., & Cleary, M. L. (1997). Meis proteins are major in vivo DNA binding partners for wild-type but not chimeric Pbx proteins. *Molecular and cellular biology*, *17*(10), 5679–87. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=232416&tool=pmcentrez&rendertype=abstract

Chang, C. P., Shen, W. F., Rozenfeld, S., Lawrence, H. J., Largman, C., & Cleary, M. L. (1995). Pbx proteins display hexapeptide-dependent cooperative DNA binding with a subset of Hox proteins. *Genes & Development*, *9*(6), 663–674. doi:10.1101/gad.9.6.663

Chen, J., Odenike, O., & Rowley, J. D. (2010). Leukemogenesis: More than Mutant Genes. *Nature review. Genetics*, *10*(1), 23–36. doi:10.1038/nrc2765.Leukemogenesis

Chou, W-C, Chen, C.-Y., Hou, H., Lin, L.-I., Tang, J.-L., Yao, M., … Tien, H.-F. (2009). Acute myeloid leukemia bearing t(7;11)(p15;p15) is a distinct cytogenetic entity with poor outcome and a distinct mutation profile: comparative analysis of 493 adult patients. *Leukemia*, *23*(7), 1303–10. doi:10.1038/leu.2009.25

Chou, Wen-Chien, Huang, H.-H., Hou, H.-A., Chen, C.-Y., Tang, J.-L., Yao, M., … Tien, H.-F. (2010). Distinct clinical and biological features of de novo acute myeloid leukemia with additional sex comb-like 1 (ASXL1) mutations. *Blood*, *116*(20), 4086–94. doi:10.1182/blood-2010-05-283291

Christman, J. K. (2002). 5-Azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene*, *21*(35), 5483–95. doi:10.1038/sj.onc.1205699

Chu, J., Loughlin, E. a, Gaur, N. a, SenBanerjee, S., Jacob, V., Monson, C., … Sadler, K. C. (2012). UHRF1 phosphorylation by cyclin A2/cyclin-dependent kinase 2 is required for zebrafish embryogenesis. *Molecular biology of the cell*, *23*(1), 59–70. doi:10.1091/mbc.E11-06-0487

Chung, K. Y., Morrone, G., Schuringa, J. J., Plasilova, M., Shieh, J.-H., Zhang, Y., … Moore, M. a S. (2006). Enforced expression of NUP98-HOXA9 in human CD34(+) cells enhances stem cell proliferation. *Cancer research*, *66*(24), 11781–91. doi:10.1158/0008-5472.CAN-06-0706

Chung, T.-L., Brena, R. M., Kolle, G., Grimmond, S. M., Berman, B. P., Laird, P. W., … Wolvetang, E. J. (2010). Vitamin C promotes widespread yet specific DNA demethylation of the epigenome in human embryonic stem cells. *Stem cells (Dayton, Ohio)*, *28*(10), 1848–55. doi:10.1002/stem.493

Čihák, A., Veselý, J., & Sǩoda, J. (1985). Azapyrimidine nucleosides: metabolism and inhibitory mechanisms. *Advances in Enzyme Regulation*, *24*(0), 335–354. doi:http://dx.doi.org/10.1016/0065-2571(85)90085-8

Cinatl, J., Driever, P. H., Kotchetkov, R., Pouckova, P., Kornhuber, B., & Schwabe, D. (1997, November). Sodium valproate inhibits in vivo growth of human neuroblastoma cells. *Anti-cancer drugs*. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9436639

Civin, C. I., Strauss, L. C., Brovall, C., Fackler, M. J. O., Schwartz, J. F., & Shaper, J. H. (1984). Antigenic Analysis of Hematopoiesis: III. A Hematopoietic Progenitor Cell Surface Antigen Defined by a Monoclonal Antibody Raised against KG-1a Cells. *The Journal of Immunology*, *133*(1), 151–160.

Clements, E. G., Mohammad, H. P., Leadem, B. R., Easwaran, H., Cai, Y., Van Neste, L., & Baylin, S. B. (2012). DNMT1 modulates gene expression without its catalytic activity partially through its interactions with histone-modifying enzymes. *Nucleic acids research*, *40*(10), 4334–46. doi:10.1093/nar/gks031

Corkery, D. P., Dellaire, G., & Berman, J. N. (2011). Leukaemia xenotransplantation in zebrafish--chemotherapy response assay in vivo. *British journal of haematology*, *153*(6), 786–9. doi:10.1111/j.1365-2141.2011.08661.x

Costello, J. F., Frühwald, M. C., Smiraglia, D. J., Rush, L. J., Robertson, G. P., Gao, X., … Plass, C. (2000). Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. *Nature genetics*, *24*(2), 132–8. doi:10.1038/72785

Cozzio, A., Passegué, E., Ayton, P. M., Karsunky, H., Cleary, M. L., & Weissman, I. L. (2003). Similar MLL-associated leukemias arising from self-renewing stem cells and short-lived myeloid progenitors. *Genes & development*, *17*(24), 3029–35. doi:10.1101/gad.1143403

Creusots, F., Acs, G., & Christmang, J. K. (1982). Inhibition of DNA methyltransferase and induction of Friend erythroleukemia cell differentiation by 5-Azacytidine. *Journal of Biological Chemistry*, *257*, 2041–2048.

Crosnier, C., Stamataki, D., & Lewis, J. (2006). Organizing cell renewal in the intestine: stem cells, signals and combinatorial control. *Nature reviews. Genetics*, *7*(5), 349–59. doi:10.1038/nrg1840

Cvejic, A., Serbanovic-Canic, J., Stemple, D. L., & Ouwehand, W. H. (2011). The role of meis1 in primitive and definitive hematopoiesis during zebrafish development. *Haematologica*, *96*(2), 190–8. doi:10.3324/haematol.2010.027698

Da'as, S. I., Coombs, a. J., Balci, T. B., Grondin, C. a., Ferrando, a. a., & Berman, J. N. (2012). The zebrafish reveals dependence of the mast cell lineage on Notch signaling in vivo. *Blood*, *119*(15), 3585–3594. doi:10.1182/blood-2011-10-385989

Da'as, S., Teh, E. M., Dobson, J. T., Nasrallah, G. K., McBride, E. R., Wang, H., … Berman, J. N. (2011). Zebrafish mast cells possess an FcεRI-like receptor and participate in innate and adaptive immune responses. *Developmental and comparative immunology*, *35*(1), 125–34. doi:10.1016/j.dci.2010.09.001

Das, B. C., McCartin, K., Liu, T.-C., Peterson, R. T., & Evans, T. (2010). A forward chemical screen in zebrafish identifies a retinoic acid derivative with receptor specificity. *PloS one*, *5*(4), e10004. doi:10.1371/journal.pone.0010004

Dash, A. B., Williams, I. R., Kutok, J. L., Tomasson, M. H., Anastasiadou, E., Lindahl, K., … Gilliland, D. G. (2002). A murine model of CML blast crisis induced by cooperation between BCR/ABL and NUP98/HOXA9. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(11), 7622–7. doi:10.1073/pnas.102583199

Davidson, A. J., Ernst, P., Wang, Y., Dekens, M. P. S., Kingsley, P. D., Palis, J., … Zon, L. I. (2003). Cdx4 Mutants Fail To Specify Blood Progenitors and Can Be Rescued By Multiple Hox Genes. *Nature*, *425*(6955), 300–6. doi:10.1038/nature01973

Davidson, A. J., & Zon, L. I. (2004). The "definitive" (and "primitive") guide to zebrafish hematopoiesis. *Oncogene*, *23*(43), 7233–46. doi:10.1038/sj.onc.1207943

De Jong, J. L. O., Davidson, A. J., Wang, Y., Palis, J., Opara, P., Pugach, E., … Zon, L. I. (2010). Interaction of retinoic acid and scl controls primitive blood development. *Blood*, *116*(2), 201–9. doi:10.1182/blood-2009-10-249557

De Thé, H., & Chen, Z. (2010). Acute promyelocytic leukaemia: novel insights into the mechanisms of cure. *Nature reviews. Cancer*, *10*(11), 775–83. doi:10.1038/nrc2943

Dean, M., Fojo, T., & Bates, S. (2005). Tumour stem cells and drug resistance. *Nature reviews. Cancer*, *5*(4), 275–84. doi:10.1038/nrc1590

Detich, N., Bovenzi, V., & Szyf, M. (2003). Valproate induces replication-independent active DNA demethylation. *The Journal of biological chemistry*, *278*(30), 27586–92. doi:10.1074/jbc.M303740200

Ding, L., Ley, T. J., Larson, D. E., Miller, C. A., Daniel, C., Welch, J. S., … Graubert, T. A. (2012). Clonal evolution in relapsed acute myeloid leukemia revealed by whole genome sequencing. *Nature*, *481*(7382), 506–510. doi:10.1038/nature10738.Clonal

Dores, G. M., Devesa, S. S., Curtis, R. E., Linet, M. S., & Morton, L. M. (2012). *Acute leukemia incidence and patient survival among children and adults in the United States, 2001-2007. Blood* (Vol. 119, pp. 34–43). doi:10.1182/blood-2011-04-347872

Eaves, C. J., & Humphries, R. K. (2010). Acute Myeloid Leukemia and the Wnt Pathway. *The New England journal of medicine*, *362*(24), 2326–2327.

Eiring, A., Neviani, P., Garton, C., Oaks, J., Spizzo, R., Liu, S., … Harb, J. G. (2010). miR-328 functions as an RNA decoy to modulate hnRNP E2 regulation of mRNA translation in leukemic blasts. *Cell*, *140*(5), 652–665.

Eiring, A., Santhanam, R., Oaks, J., Chang, J., Notari, M., Willis, W., … Neviani, P. (2008). Identification of novel posttranscriptional targets of the BCR/ABL oncoprotein by ribonomics: requirement of E2F3 for BCR/ABL leukemogenesis. *Blood*, *111*(2), 816–828.

Eklund, E. a. (2007). The role of HOX genes in malignant myeloid disease. *Current opinion in hematology*, *14*(2), 85–9. doi:10.1097/MOH.0b013e32801684b6

Eklund, E. A. (2006). The role of HOX genes in myeloid leukemogenesis. *Current opinion in hematology*, *13*(2), 67–73. doi:10.1097/01.moh.0000208467.63861.d6

Eklund, E. A. (2011). The role of HOX proteins in leukemogenesis; Insights into key regulatory events in hemtopoiesis. *Critical Reviews in Oncogenes*, *16*(1-2), 65–76.

Esteller, M. (2008). Epigenetics in cancer. *The New England journal of medicine*, *358*(11), 1148–59. doi:10.1056/NEJMra072067

Estey, E., & Döhner, H. (2006). Acute myeloid leukaemia. *Lancet*, *368*, 1894–1907.

Estrov, Z. (2010). The Leukemia Stem Cell. In L. Nagarajan (Ed.), *Acute Myelogenous Leukemia: Genetics, Biology and Therapy* (1st ed., pp. 1–17). New York, NY: Springer New York. doi:10.1007/978-0-387-69259-3

Faber, J., Krivtsov, A. V, Stubbs, M. C., Wright, R., Davis, T. N., van den Heuvel-Eibrink, M., … Armstrong, S. a. (2009). HOXA9 is required for survival in human MLL-rearranged acute leukemias. *Blood*, *113*(11), 2375–85. doi:10.1182/blood-2007-09-113597

Faderl, S., Talpaz, M., Kantarjian, H. M., & Estrov, Z. (1999). Should polymerase chain reaction analysis to detect minimal residual disease in patients with chronic myelogenous leukemia be used in clinical decision making? *Blood*, *93*(9), 2755–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10216068

Fatemi, M., Hermann, A., Gowher, H., & Jeltsch, A. (2002). Dnmt3a and Dnmt1 functionally cooperate during de novo methylation of DNA. *European Journal of Biochemistry*, *269*(20), 4981–4984. doi:10.1046/j.1432-1033.2002.03198.x

Feitsma, H., & Cuppen, E. (2008). Zebrafish as a cancer model. *Molecular cancer research : MCR*, *6*(5), 685–94. doi:10.1158/1541-7786.MCR-07-2167

Felsenfeld, G., & Groudine, M. (2003). Controlling the double helix. *Nature*, *421*(6921), 448–53. doi:10.1038/nature01411

Feng, H., Langenau, D. M., Madge, J. a, Quinkertz, A., Gutierrez, A., Neuberg, D. S., … Look, a T. (2007). Heat-shock induction of T-cell lymphoma/leukaemia in conditional Cre/lox-regulated transgenic zebrafish. *British journal of haematology*, *138*(2), 169–75. doi:10.1111/j.1365-2141.2007.06625.x

Ferrar, J. L. M., Levine, J. E., Reddy, P., & Holler, E. (2009). Graft-versus-host disease. *Lancet*, *373*(9674), 1550–1561. doi:10.1016/S0140-6736(09)60237-3.Graft-versus-Host

Figueroa, M. E., Lugthart, S., Li, Y., Erpelinck-Verschueren, C., Deng, X., Christos, P. J., … Melnick, A. (2010). DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia. *Cancer cell*, *17*(1), 13–27. doi:10.1016/j.ccr.2009.11.020

Fleming, H. E., Janzen, V., Lo Celso, C., Guo, J., Leahy, K. M., Kronenberg, H. M., & Scadden, D. T. (2008). Wnt signaling in the niche enforces hematopoietic stem cell quiescence and is necessary to preserve self-renewal in vivo. *Cell stem cell*, *2*(3), 274–283.

Forrester, A Michael, Grabher, C., McBride, E. R., Boyd, E. R., Vigerstad, M. H., Edgar, A., … Berman, J. N. (2011). NUP98-HOXA9-transgenic zebrafish develop a myeloproliferative neoplasm and provide new insight into mechanisms of myeloid leukaemogenesis. *British journal of haematology*, *155*(2), 167–81. doi:10.1111/j.1365-2141.2011.08810.x

Forrester, Alexander Michael. (2012). *Zebrafish tools to combat acute myeloid leukaemia - the nucleoporin 98 kilodalton-homebox A9 (NUP98-HOXA9) fusion ongogene drive myeloproliferation by upregulating DNA (Cytosine-5-)-methyltransferase 1 (DNMT1)*. Dalhousie University.

Foucar, K. (2009). Myelodysplastic/myeloproliferative neoplasms. *American journal of clinical pathology*, *132*(2), 281–9. doi:10.1309/AJCPJ71PTVIKGEVT

Frohling, S., Scholl, C., Bansal, D., & Huntly, B. J. P. (2007). HOX Gene Regulation in Acute Myeloid Leukemia: CDX Maks the Spot? *Cell Cycle*, *6*(18), 2241–2245.

Fuks, F., Burgers, W. a, Brehm, a, Hughes-Davies, L., & Kouzarides, T. (2000). DNA methyltransferase Dnmt1 associates with histone deacetylase activity. *Nature genetics*, *24*(1), 88–91. doi:10.1038/71750

Galloway, J. L., Wingert, R. a, Thisse, C., Thisse, B., & Zon, L. I. (2005). Loss of gata1 but not gata2 converts erythropoiesis to myelopoiesis in zebrafish embryos. *Developmental cell*, *8*(1), 109–16. doi:10.1016/j.devcel.2004.12.001

Garcia-Manero, G. (2010). Prognosis of Myelodysplastic Syndromes. *American Society of Hematology Education Program*, 330–337.

Ghannam, G., Takeda, A., Camarata, T., Moore, M. A., Viale, A., & Haseen, N. R. (2004). The Oncogene Nup98-HOXA9 Induces Gene Transcription in Myeloid Cells. *Journal of Biological Chemistry*, *279*(2), 866–875. doi:10.1074/jbc.M307280200

Ghoshal, K., Datta, J., Majumder, S., Bai, S., Kutay, H., Motiwala, T., & Jacob, S. T. (2005). 5-Aza-Deoxycytidine induces selective degradation of DNA Methyltransferase 1 by a proteasomal pathway that requires the KEN Box, Bromo-Adjacent Homology Domain, and nuclear localization signal. *Molecular and cellular biology*, *25*(11), 4727–4741. doi:10.1128/MCB.25.11.4727

Giles, F. J., Keating, A., Goldstone, A. H., Avivi, I., Willman, C. L., & Kantarjian, H. M. (2002). Acute myeloid leukemia. *Hematology / the Education Program of the American Society of Hematology. American Society of Hematology. Education Program*, (Table 1), 73–110. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12446420

Gilliland, D. G., & Tallman, M. S. (2002). Focus on acute leukemias. *Cancer cell*, *1*(5), 417–20. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12124171

Goessling, W., Allen, R. S., Guan, X., Jin, P., Uchida, N., Dovey, M., … North, T. E. (2011). Prostaglandin E2 enhances human cord blood stem cell xenotransplants and shows long-term safety in preclinical nonhuman primate transplant models. *Cell stem cell*, *8*(4), 445–58. doi:10.1016/j.stem.2011.02.003

Goessling, W., North, T. E., Loewer, S., Lord, A. M., Lee, S., & Stoick-, C. L. (2007). Supplemental Data Genetic Interaction of PGE2 and Wnt Signaling Regulates Developmental Specification of Stem Cells and Regeneration, *136*.

Goessling, W., North, T. E., Loewer, S., Lord, A. M., Lee, S., Stoick-cooper, C. L., … Zon, L. I. (2009). Genetic interaction of PGE2 and Wnt signaling regulates developmental specification of stem cells and regeneration. *Cell*, *136*(6), 1136–1147. doi:10.1016/j.cell.2009.01.015.Genetic

Golub, T. R. (1999). Molecular Classification of Cancer: Class Discovery and Class Prediction by Gene Expression Monitoring. *Science*, *286*(5439), 531–537. doi:10.1126/science.286.5439.531

Gonzalez-Rodriguez, D., Guevorkian, K., Douezan, S., & Brochard-Wyart, F. (2012). Soft matter models of developing tissues and tumors. *Science*, *338*(6109), 910–7. doi:10.1126/science.1226418

Göttlicher, M., Minucci, S., Zhu, P., Krämer, O. H., Schimpf, a, Giavara, S., … Heinzel, T. (2001). Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *The EMBO journal*, *20*(24), 6969–78. doi:10.1093/emboj/20.24.6969

Grabher, C., Joly, J.-S., & Wittbrodt, J. (2004). Highly Efficient Zebrafish Transgenesis Mediated by the Meganuclease I-SceI. *METHODS IN CELL BIOLOGY*, (77), 381–402.

Greer, J. M., Puetz, J., Thomas, K. R., & Capecchi, M. R. (2000). Maintenance of functional equivalence during paralogous Hox gene evolution. *Nature*, *403*(6770), 661–5. doi:10.1038/35001077

Griffis, E. R., Craige, B., Dimaano, C., Ullman, K. S., & Powers, M. A. (2004). Distinct Functional Domains within Nucleoporins Nup153 and Nup98 Mediate Transcription-dependent Mobility. *Molecular biology of the cell*, *15*(April), 1991–2002. doi:10.1091/mbc.E03

Grignani, F., Fagioli, M., Alcalay, M., Longo, L., Pandolfi, P. P., Donti, E., … Pelicci, P. G. (1994). Acute promyelocytic leukemia: from genetics to treatment. *Blood*, *83*(1), 10–25. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10498878

Grigoryan, T., Wend, P., Klaus, A., & Birchmeier, W. (2008). Deciphering the function of canonical Wnt signals in development and disease: conditional loss- and gain-of-function mutations of beta-catenin in mice. *Genes & development*, *22*(17), 2308–41. doi:10.1101/gad.1686208

Gros, C., Fahy, J., Halby, L., Dufau, I., Erdmann, A., Gregoire, J.-M., … Arimondo, P. B. (2012). DNA methylation inhibitors in cancer: Recent and future approaches. *Biochimie*, *94*(11), 2280–96. doi:10.1016/j.biochi.2012.07.025

Grunwald, D. J., & Eisen, J. S. (2002). Headwaters of the zebrafish - emergence of a new model vertebrate. *Nature reviews. Genetics*, *3*(9), 711–7. doi:10.1038/nrg891

Guibal, F. C., Alberich-Jorda, M., Hirai, H., Ebralidze, A., Levantini, E., Di Ruscio, A., Tenen, D. G. (2009). Identification of a myeloid committed progenitor as the cancer-initiating cell in acute promyelocytic leukemia. *Blood*, *114*(27), 5415–25. doi:10.1182/blood-2008-10-182071

Gundestrup, M., Andersen, M. K., Sveinbjornsdottir, E., Rafnsson, V., Storm, H. H., & Pedersen-bjergaard, J. (2000). Cytogenetics of myelodysplasia and acute myeloid leukaemia in aircrew and people treated with radiotherapy For personal use only . Not to be reproduced without permission of The Lancet . *The Lancet*, *356*, 2158.

Gurvich, N., Tsygankova, O. M., Meinkoth, J. L., & Klein, P. S. (2004). Histone deacetylase is a target of Valproic Acid-mediated cellular differentiation. *Cancer research*, *64*, 1079–1086.

Guzman, M. L. (2001). Nuclear factor-kappaB is constitutively activated in primitive human acute myelogenous leukemia cells. *Blood*, *98*(8), 2301–2307. doi:10.1182/blood.V98.8.2301

Haegel, H., Larue, L., Ohsugi, M., Fedorov, L., Herrenknecht, K., & Kemler, R. (1995). Lack of beta-catenin affects mouse development at gastrulation. *Development (Cambridge, England)*, *121*(11), 3529–37. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8582267

Hall, C., Flores, M. V., Storm, T., Crosier, K., & Crosier, P. (2007). The zebrafish lysozyme C promoter drives myeloid-specific expression in transgenic fish. *BMC developmental biology*, *7*, 42. doi:10.1186/1471-213X-7-42

Hatano, Y., Miura, I., Nakamura, T., Yamazaki, Y., Takahashi, N., & Miura, a B. (1999). Molecular heterogeneity of the NUP98/HOXA9 fusion transcript in myelodysplastic syndromes associated with t(7;11)(p15;p15). *British journal of haematology*, *107*(3), 600–4. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10583265

He, H., Hua, X., & Yan, J. (2011). Epigenetic regulations in hematopoietic Hox code. *Oncogene*, *30*(4), 379–88. doi:10.1038/onc.2010.484

Heaney, M. L., & Soriano, G. (2013). Acute myeloid leukemia following a myeloproliferative neoplasm: clinical characteristics, genetic features and effects of therapy. *Current hematologic malignancy reports*, *8*(2), 116–22. doi:10.1007/s11899-013-0154-5

Hehlmann, R., Hochhaus, A., & Baccarani, M. (2007). Chronic myeloid leukaemia. *Lancet*, *370*(9584), 342–50. doi:10.1016/S0140-6736(07)61165-9

Heidel, F. H., Bullinger, L., Feng, Z., Wang, Z., Neff, T. a, Stein, L., … Armstrong, S. a. (2012). Genetic and pharmacologic inhibition of β-catenin targets imatinib-resistant leukemia stem cells in CML. *Cell stem cell*, *10*(4), 412–24. doi:10.1016/j.stem.2012.02.017

Heidel, F. H., Mar, B. G., & Armstrong, S. a. (2011). Self-renewal related signaling in myeloid leukemia stem cells. *International journal of hematology*, *94*(2), 109–17. doi:10.1007/s12185-011-0901-0

Helgason, C. D., Sauvageau, G., Lawrence, H. J., Largman, C., & Humphries, R. K. (1996). Overexpression of HOXB4 enhances the hematopoietic potential of embryonic stem cells differentiated in vitro. *Blood*, *87*(7), 2740–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8639890

Herbomel, P., Thisse, B., & Thisse, C. (1999). Ontogeny and behaviour of early macrophages in the zebrafish embryo. *Development (Cambridge, England)*, *126*(17), 3735–45. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10433904

Hervouet, E., Vallette, F. M., & Cartron, P.-F. (2010). Dnmt1/Transcription factor interactions: an alternative mechanism of DNA methylation inheritance. *Genes & cancer*, *1*(5), 434–43. doi:10.1177/1947601910373794

Holliday, R. (2006). Epigenetics: A Historical Overview. *Epigenetics*, *1*(2), 76–80.

Holmfeldt, L., & Mullighan, C. G. (2011). The role of TET2 in hematologic neoplasms. *Cancer cell*, *20*(1), 1–2. doi:10.1016/j.ccr.2011.06.025

Hope, K. J., Jin, L., & Dick, J. E. (2004). Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. *Nature immunology*, *5*(7), 738–43. doi:10.1038/ni1080

Horton, S. J., & Huntly, B. J. P. (2012). Recent advances in acute myeloid leukemia stem cell biology. *Haematologica*, *97*(7), 966–74. doi:10.3324/haematol.2011.054734

Howlader, N., Noone, A., Krapcho, M., Garshell, J., Neyman, N., Altekruse, S., … Cronin, K. (2012). SEER Cancer Statistics Review 1975-2010.

Hsu, K., Traver, D., Kutok, J. L., Hagen, A., Liu, T.-X., Paw, B. H., … Look, a T. (2004). The pu.1 promoter drives myeloid gene expression in zebrafish. *Blood*, *104*(5), 1291–7. doi:10.1182/blood-2003-09-3105

Hu, J., Van Valckenborgh, E., Menu, E., De Bruyne, E., & Vanderkerken, K. (2012). Understanding the hypoxic niche of multiple myeloma: therapeutic implications and contributions of mouse models. *Disease models & mechanisms*, *5*(6), 763–71. doi:10.1242/dmm.008961

Hu, Y.-L., Fong, S., Ferrell, C., Largman, C., & Shen, W.-F. (2009). HOXA9 modulates its oncogenic partner Meis1 to influence normal hematopoiesis. *Molecular and cellular biology*, *29*(18), 5181–92. doi:10.1128/MCB.00545-09

Huang, P., Zhu, Z., Lin, S., & Zhang, B. (2012). Reverse genetic approaches in zebrafish. *Journal of genetics and genomics = Yi chuan xue bao*, *39*(9), 421–33. doi:10.1016/j.jgg.2012.07.004

Hume, D. a. (2006). The mononuclear phagocyte system. *Current opinion in immunology*, *18*(1), 49–53. doi:10.1016/j.coi.2005.11.008

Huntly, B. J. P., Shigematsu, H., Deguchi, K., Lee, B. H., Mizuno, S., Duclos, N., … Gilliland, D. G. (2004). MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors. *Cancer cell*, *6*(6), 587–96. doi:10.1016/j.ccr.2004.10.015

Iland, H., Bradstock, K., Seymour, J., Hertzberg, M., Grigg, A., Taylor, K., … Reynolds, J. (2012). Results of the APML3 trial incorporating all-trans-retinoic acid and idarubicin in both induction and consolidation as initial therapy for patients with acute promyelocytic leukemia. *Haematologica*, *97*(2), 227–34. doi:10.3324/haematol.2011.047506

Ivanenkov, Y. A., Balakin, K. V, & Tkachenko, S. E. (2008). New Approaches to the treatment of inflammatory disease: Focus on small-molecule inhibitors of signal transduction pathways. *Drug in R&D*, *9*(6), 397–434.

Iwasaki, H., Mizuno, S., Wells, R. a, Cantor, A. B., Watanabe, S., & Akashi, K. (2003). GATA-1 converts lymphoid and myelomonocytic progenitors into the megakaryocyte/erythrocyte lineages. *Immunity*, *19*(3), 451–62. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14499119

Iwasaki, M., Kuwata, T., Yamazaki, Y., Jenkins, N. a, Copeland, N. G., Osato, M., … Nakamura, T. (2005). Identification of cooperative genes for NUP98-HOXA9 in myeloid leukemogenesis using a mouse model. *Blood*, *105*(2), 784–93. doi:10.1182/blood-2004-04-1508

Izsvák, Z., Ivics, Z., Garcia-Estefania, D., Fahrenkrug, S. C., & Hackett, P. B. (1996). DANA elements: a family of composite, tRNA-derived short interspersed DNA elements associated with mutational activities in zebrafish (Danio rerio). *Proceedings of the National Academy of Sciences of the United States of America*, *93*(3), 1077–81. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=40033&tool=pmcentrez&rendertype=abstract

Jamieson, C. H. M., Ailles, L. E., Dylla, S. J., Muijtjens, M., Jones, C., Zehnder, J. L., … Weissman, I. L. (2004). Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. *The New England journal of medicine*, *351*(7), 657–67. doi:10.1056/NEJMoa040258

Ji, H., Ehrlich, L. I. R., Seita, J., Murakami, P., Doi, A., Lindau, P., … Feinberg, A. P. (2010). Comprehensive methylome map of lineage commitment from haematopoietic progenitors. *Nature*, *467*(7313), 338–42. doi:10.1038/nature09367

Jing, L., & Zon, L. I. (2011). Zebrafish as a model for normal and malignant hematopoiesis. *Disease models & mechanisms*, *4*(4), 433–8. doi:10.1242/dmm.006791

John, D. E. (2003). Self-renewal write in blood. *Nature*, *423*(6937), 231.

Jones, P. a, & Baylin, S. B. (2002). The fundamental role of epigenetic events in cancer. *Nature reviews. Genetics*, *3*(6), 415–28. doi:10.1038/nrg816

Jones, R. J., Wagner, J. E., Celano, P., Zicha, M. S., & Sharkis, S. J. (1990). Separation of pluripotent haematopoietic stem cells from spleen colony-forming cells. *Nature*, *347*(6289), 188–9. doi:10.1038/347188a0

Jung, G.-A., Yoon, J.-Y., Moon, B.-S., Yang, D.-H., Kim, H.-Y., Lee, S.-H., … Choi, K.-Y. (2008). Valproic acid induces differentiation and inhibition of proliferation in neural progenitor cells via the beta-catenin-Ras-ERK-p21Cip/WAF1 pathway. *BMC cell biology*, *9*, 66. doi:10.1186/1471-2121-9-66

Kantarjian, H., Issa, J.-P. J., Rosenfeld, C. S., Bennett, J. M., Albitar, M., DiPersio, J., … Saba, H. (2006). Decitabine improves patient outcomes in myelodysplastic syndromes: results of a phase III randomized study. *Cancer*, *106*(8), 1794–803. doi:10.1002/cncr.21792

Kantarjian, H., Oki, Y., Garcia-Manero, G., Huang, X., O'Brien, S., Cortes, J., … Issa, J.-P. J. (2007). Results of a randomized study of 3 schedules of low-dose decitabine in higher-risk myelodysplastic syndrome and chronic myelomonocytic leukemia. *Blood*, *109*(1), 52–7. doi:10.1182/blood-2006-05-021162

Kappen, C. (2000). Disruption of the homeobox gene Hoxb-6 in mice results in increased numbers of early erythrocyte progenitors. *American journal of hematology*, *65*(2), 111–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10996827

Karpanen, T., & Schulte-Merker, S. (2011). Zebrafish provides a novel model for lymphatic vascular research. In *Methods in cell biology* (Third Edit., Vol. 105, pp. 223–38). Elsevier Inc. doi:10.1016/B978-0-12-381320-6.00009-6

Kasper, L. H., Brindle, P. K., Schnabel, C. a, Pritchard, C. E., Cleary, M. L., & van Deursen, J. M. (1999). CREB binding protein interacts with nucleoporin-specific FG repeats that activate transcription and mediate NUP98-HOXA9 oncogenicity. *Molecular and cellular biology*, *19*(1), 764–76. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=83933&tool=pmcentrez&rendertype=abstract

Khorasanizadeh, S. (2004). The Nucleosome : From Genomic Organization to Genomic Regulation. *Cell*, *116*(Electron micrographs first confirmed that the eukary- otic genome is organized into repeating disk-shaped nucleosomal units composed of histones and their associated DNA. Those images made clear the func- tion of the nucleosome in packaging and condensing), 259–272.

Kihslinger, J. E., & Godley, L. a. (2007). The use of hypomethylating agents in the treatment of hematologic malignancies. *Leukemia & lymphoma*, *48*(9), 1676–95. doi:10.1080/10428190701493910

Kirkwood, J. S., Lebold, K. M., Miranda, C. L., Wright, C. L., Miller, G. W., Tanguay, R. L., … Stevens, J. F. (2012). Vitamin C deficiency activates the purine nucleotide cycle in zebrafish. *The Journal of biological chemistry*, *287*(6), 3833–41. doi:10.1074/jbc.M111.316018

Kirstetter, P., Anderson, K., Porse, B. T., Jacobsen, S. E. W., & Nerlov, C. (2006). Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. *Nature immunology*, *7*(10), 1048–56. doi:10.1038/ni1381

Kissa, K., & Herbomel, P. (2010). Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature*, *464*(7285), 112–5. doi:10.1038/nature08761

Koch, U., Wilson, A., Cobas, M., Kemler, R., Macdonald, H. R., & Radtke, F. (2008). Simultaneous loss of beta- and gamma-catenin does not perturb hematopoiesis or lymphopoiesis. *Blood*, *111*(1), 160–4. doi:10.1182/blood-2007-07-099754

Kokel, D., Bryan, J., Laggner, C., White, R., Cheung, C. Y. J., Mateus, R., … Peterson, R. T. (2010). Rapid behavior-based identification of neuroactive small molecules in the zebrafish. *Nature chemical biology*, *6*(3), 231–237. doi:10.1038/nchembio.307

Konopleva, M., Zhao, S., Hu, W., Jiang, S., Snell, V., Weidner, D., … Andreeff, M. (2002). The anti-apoptotic genes Bcl-X(L) and Bcl-2 are over-expressed and contribute to chemoresistance of non-proliferating leukaemic CD34+ cells. *British journal of haematology*, *118*(2), 521–34. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12139741

Kosinski, C., Li, V. S. W., Chan, A. S. Y., Zhang, J., Ho, C., Tsui, W. Y., … Chen, X. (2007). Gene expression patterns of human colon tops and basal crypts and BMP antagonists as intestinal stem cell niche factors. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(39), 15418–23. doi:10.1073/pnas.0707210104

Kosmider, O., Gelsi-Boyer, V., Cheok, M., Grabar, S., Della-Valle, V., Picard, F., … Fontenay, M. (2009). TET2 mutation is an independent favorable prognostic factor in myelodysplastic syndromes (MDSs). *Blood*, *114*(15), 3285–91. doi:10.1182/blood-2009-04-215814

Kosugi, H., Towatari, M., Hatano, S., Kitamura, K., Kiyoi, H., Kinoshita, T., … Naoe, T. (1999). Histone deacetylase inhibitors are the potent inducer/enhancer of differentiation in acute myeloid leukemia: a new approach to anti-leukemia therapy. *Leukemia*, *13*(9), 1316–24. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10482980

Kramarzova, K., Stuchly, J., Willasch, a, Gruhn, B., Schwarz, J., Cermak, J., … Boublikova, L. (2012). Real-time PCR quantification of major Wilms' tumor gene 1 (WT1) isoforms in acute myeloid leukemia, their characteristic expression patterns and possible functional consequences. *Leukemia*, *26*(9), 2086–95. doi:10.1038/leu.2012.76

Krivtsov, A. V, Twomey, D., Feng, Z., Stubbs, M. C., Wang, Y., Faber, J., … Armstrong, S. a. (2006). Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. *Nature*, *442*(7104), 818–22. doi:10.1038/nature04980

Kroon, E., Krosl, J., Thorsteinsdottir, U., Baban, S., Buchberg, a M., & Sauvageau, G. (1998). Hoxa9 transforms primary bone marrow cells through specific collaboration with Meis1a but not Pbx1b. *The EMBO journal*, *17*(13), 3714–25. doi:10.1093/emboj/17.13.3714

Kroon, E., Thorsteinsdottir, U., Mayotte, N., Nakamura, T., & Sauvageau, G. (2001). NUP98-HOXA9 expression in hemopoietic stem cells induces chronic and acute myeloid leukemias in mice. *The EMBO journal*, *20*(3), 350–61. doi:10.1093/emboj/20.3.350

Kuendgen, A., Strupp, C., Aivado, M., Bernhardt, A., Hildebrandt, B., Haas, R., … Gattermann, N. (2004). Treatment of myelodysplastic syndromes with valproic acid alone or in combination with all-trans retinoic acid. *Blood*, *104*(5), 1266–9. doi:10.1182/blood-2003-12-4333

Lam, S. H., & Gong, Z. (2006). Modeling liver cancer using zebrafish: a comparative oncogenomics approach. *Cell Cycle*, *5*(6), 573–577.

Lane, S. W., Wang, Y. J., Lo Celso, C., Ragu, C., Bullinger, L., Sykes, S. M., … Williams, D. a. (2011). Differential niche and Wnt requirements during acute myeloid leukemia progression. *Blood*, *118*(10), 2849–56. doi:10.1182/blood-2011-03-345165

Langenau, D. M., Ferrando, A. A., Traver, D., Kutok, J. L., Hezel, D., Kanki, J. P., … Zont, L. (2013). zebrafish in transgenic and engraftment of T cell development , In vivo tracking.

Langenau, D. M., Keefe, M. D., Storer, N. Y., Guyon, J. R., Kutok, J. L., Le, X., … Zon, L. I. (2007). Effects of RAS on the genesis of embryonal rhabdomyosarcoma. *Genes & development*, *21*(11), 1382–95. doi:10.1101/gad.1545007

Langenau, D. M., Traver, D., Ferrando, A. A., Kutok, J. L., Aster, J. C., Kanki, J. P., … Look, A. T. (2003). Myc-Induced T Cell Leukemia in Transgenic Zebrafish. *Science*, *299*(5608), 887–890.

Larhammar, D., Lundin, L.-G., & Hallböök, F. (2002). The human Hox-bearing chromosome regions did arise by block or chromosome (or even genome) duplications. *Genome research*, *12*(12), 1910–20. doi:10.1101/gr.445702

Lasa, a, Carnicer, M. J., Aventín, a, Estivill, C., Brunet, S., Sierra, J., & Nomdedéu, J. F. (2004). MEIS 1 expression is downregulated through promoter hypermethylation in AML1-ETO acute myeloid leukemias. *Leukemia*, *18*(7), 1231–7. doi:10.1038/sj.leu.2403377

Lavallade, H. De. (2013). Chronic myeloid leukaemia. *Medicine*, *41*(5), 275–277. doi:10.1016/j.mpmed.2013.03.001

Lawrence, H. J., Helgason, C. D., Sauvageau, G., Fong, S., Izon, D. J., Humphries, R. K., & Largman, C. (1997). Mice bearing a targeted interruption of the homeobox gene HOXA9 have defects in myeloid, erythroid, and lymphoid hematopoiesis. *Blood*, *89*(6), 1922–30. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9058712

Le Guyader, D., Redd, M. J., Colucci-Guyon, E., Murayama, E., Kissa, K., Briolat, V., … Herbomel, P. (2008). Origins and unconventional behavior of neutrophils in developing zebrafish. *Blood*, *111*, 132–141. doi:10.1182/2007-06-095398.An

Le, X., Langenau, D. M., Keefe, M. D., Kutok, J. L., Neuberg, D. S., & Zon, L. I. (2007). Heat shock-inducible Cre/Lox approaches to induce diverse types of tumors and hyperplasia in transgenic zebrafish. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(22), 9410–5. doi:10.1073/pnas.0611302104

Leacock, S. W., Basse, A. N., Chandler, G. L., Kirk, A. M., Rakheja, D., & Amatruda, J. F. (2012). A zebrafish transgenic model of Ewing's sarcoma reveals conserved mediators of EWS-FLI1 tumorigenesis. *Disease models & mechanisms*, *5*(1), 95–106. doi:10.1242/dmm.007401

Lehnertz, B., Ueda, Y., Derijck, A. A. H. A., Braunschweig, U., Perez-burgos, L., Kubicek, S., … Peters, A. H. F. M. (2003). Suv39h-Mediated Histone H3 Lysine 9 Methylation Directs DNA Methylation to Major Satellite Repeats at Pericentric Heterochromatin. *Current Biology*, *13*, 1192–1200. doi:10.1016/S

Letamendia, A., Quevedo, C., Ibarbia, I., Virto, J. M., Holgado, O., Diez, M., … Callol-Massot, C. (2012). Development and validation of an automated high-throughput system for zebrafish in vivo screenings. *PloS one*, *7*(5), e36690. doi:10.1371/journal.pone.0036690

Ley, T. J., Ding, L., Walter, M. J., Mclellan, M. D., Lamprecht, T., Larson, D. E., … Wilson, R. K. (2010). DNMT3A Mutations in Acute Myeloid Leukemia. *The New England journal of medicine*, *363*(25), 2424–2433. doi:10.1056/NEJMoa1005143.DNMT3A

Li, L. H., Olin, E. J., Buskirk, H. H., & Reineke, L. M. (1970). Cytotoxicity and Mode of Action of 5-Azacytidine on L1210 Leukemia. *Cancer research*, *30*, 2760–2769.

Li, L. H., Olin, E. J., Fraser, T. J., & Bhuyan, B. K. (1970). Phase Specificity of 5-Azacytidine against Mammalian Cells in Tissue Culture Phase Specificity of 5-Azacytidine against Mammalian Cells in Tissue Culture1. *Cancer research*, *30*, 2770–2775.

Lieschke, G. J., Oates, A. C., Crowhurst, A. C., Ward, A. C., & Layton, J. E. (2001). Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish. *Blood*, *98*(10), 3087–3096. doi:10.1182/blood.V98.10.3087

Lin, H.-F., Traver, D., Zhu, H., Dooley, K., Paw, B. H., Zon, L. I., & Handin, R. I. (2005). Analysis of thrombocyte development in CD41-GFP transgenic zebrafish. *Blood*, *106*(12), 3803–10. doi:10.1182/blood-2005-01-0179

Lion, T. (1999). Minimal Residual Disease. *Current opinion in hematology*, *6*(6), 406–410.

Liu, F., & Wen, Z. (2002). Cloning and expression pattern of the lysozyme C gene in zebrafish. *Mechanisms of development*, *113*(1), 69–72. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11900976

Liu, S., & Leach, S. D. (2011). Zebrafish models for cancer. *Annual review of pathology*, *6*, 71–93. doi:10.1146/annurev-pathol-011110-130330

Lord, A. M., North, T. E., & Zon, L. I. (2007). Prostaglandin E2: Making more of your bone marrow. *Cell Cycle*, *6*(24), 3054–3057.

Löwenberg, B., Griffin, J. D., & Tallman, M. S. (2003). Acute Myeloid Leukemia and Acute Promyelocytic Leukemia. *American Society of Hematology Education Program*, 82–101.

Luis, T C, Ichii, M., Brugman, M. H., Kincade, P., & Staal, F. J. T. (2012). Wnt signaling strength regulates normal hematopoiesis and its deregulation is involved in leukemia development. *Leukemia*, *26*(3), 414–21. doi:10.1038/leu.2011.387

Luis, Tiago C, Naber, B. a E., Roozen, P. P. C., Brugman, M. H., de Haas, E. F. E., Ghazvini, M., … Staal, F. J. T. (2011). Canonical wnt signaling regulates hematopoiesis in a dosage-dependent fashion. *Cell stem cell*, *9*(4), 345–56. doi:10.1016/j.stem.2011.07.017

Lund, A. H., & van Lohuizen, M. (2004). Epigenetics and cancer. *Genes & development*, *18*(19), 2315–35. doi:10.1101/gad.1232504

Lyons, S. E., Lawson, N. D., Lei, L., Bennett, P. E., Weinstein, B. M., & Liu, P. P. (2002). A nonsense in zebrafish gata1 causes the mutation bloodless phenotype in viad tepes. *National Academy of Sciences*, *99*(8), 5454–5459.

MacDonald, B. T., Tamai, K., & He, X. (2010). Wnt/β-catenin signaling: components, mechanisms, and diseases. *Developmental Cell*, *17*(1), 9–26. doi:10.1016/j.devcel.2009.06.016.Wnt/

Magli, M. C., Largman, C., & Lawrence, H. J. (1997). Effects of HOX homeobox genes in blood cell differentiation. *Journal of cellular physiology*, *173*(2), 168–77. doi:10.1002/(SICI)1097-4652(199711)173:2<168::AID-JCP16>3.0.CO;2-C

Majorova, I., Gorodinskaya, V., Khudoley, V., Revskoy, S., & Mizgireuv, I. (2004). Carcinogenic Effect of N-Nitrosodimethylamine on Diploid and Triploid Zebrafish (Danio rerio). *Toxicologic Pathology*, *32*(5), 514–518. doi:10.1080/01926230490496311

Mann, R. S., & Affolter, M. (1998). Hox proteins meet more partners. *Current opinion in Genetics and Development*, *8*, 423–429.

Mann, R. S., Lelli, K. M., & Joshi, R. (2009). Hox Specificity: Unique Roles of Cofactors and Collaborators. *Current Topics in Developmental Biology*, *88*, 63–101. doi:10.1016/S0070-2153(09)88003-4.Hox

Mark, M., Rijli, F. M., & Chambon, P. (1997). Homeobox Genes in Embryogenesis and Pathogenesis. *Pediatric Research*, *42*, 421–429. doi:10.1203/00006450-199710000-00001

Marks, P., Rifkind, R. a, Richon, V. M., Breslow, R., Miller, T., & Kelly, W. K. (2001). Histone deacetylases and cancer: causes and therapies. *Nature reviews. Cancer*, *1*(3), 194–202. doi:10.1038/35106079

Martens, J. H. a, Brinkman, A. B., Simmer, F., Francoijs, K.-J., Nebbioso, A., Ferrara, F., … Stunnenberg, H. G. (2010). PML-RARalpha/RXR Alters the Epigenetic Landscape in Acute Promyelocytic Leukemia. *Cancer cell*, *17*(2), 173–85. doi:10.1016/j.ccr.2009.12.042

Martienssen, R. a, & Richards, E. J. (1995). DNA methylation in eukaryotes. *Current opinion in genetics & development*, *5*(2), 234–42. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7613094

Maslov, a Y., Lee, M., Gundry, M., Gravina, S., Strogonova, N., Tazearslan, C., … Vijg, J. (2012). 5-aza-2'-deoxycytidine-induced genome rearrangements are mediated by DNMT1. *Oncogene*, *31*(50), 5172–9. doi:10.1038/onc.2012.9

Matasar, M. J., Ritchie, E. K., Consedine, N., Magai, C., & Neugut, A. I. (2006). Incidence rates of the major leukemia subtypes among US Hispanics, Blacks, and non-Hispanic Whites. *Leukemia & lymphoma*, *47*(11), 2365–70. doi:10.1080/10428190600799888

Mathias, J. R., Dodd, M. E., Walters, K. B., Yoo, S. K., Erik, A., & Huttenlocher, A. (2009). Characterization of zebrafish larval inflammatory macrophages. *Developmental and comparative immunology*, *33*(11), 1212–1217. doi:10.1016/j.dci.2009.07.003.Characterization

Mayer, R. J., Davis, R. B., Schiffer, C. A., Deborah, B. T., Powell, B. L., Schulman, P., Frei, E. I. (1994). Intensive postremission chemotherapy in adults with acute myeloid leukemia. *The New England journal of medicine*, *331*, 896–903.

Mayotte, N., Roy, D.-C., Yao, J., Kroon, E., & Sauvageau, G. (2002). Oncogenic interaction between BCR-ABL and NUP98-HOXA9 demonstrated by the use of an in vitro purging culture system. *Blood*, *100*(12), 4177–84. doi:10.1182/blood-2002-04-1244

McCulloch, E. A. (1993). Stem cell renewal and determination during clonal expansion in normal and leukaemic haemopoiesis. *Cell proliferation*, *26*(5), 399–425. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9087532

McCulloch, E. A., & Till, J. E. (1971). Regulatory Mechanisms Acting on Hemopoietic Stem Cells. *American journal of pathology*, *65*(3), 601–619.

McKercher, S. R., Torbett, B. E., Anderson, K. L., Henkel, G. W., Vestal, D. J., Baribault, H., … Maki, R. A. (1996). Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *The EMBO journal*, *15*(20), 5647–58. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=452309&tool=pmcentrez&rendertype=abstract

Miesner, M., Haferlach, C., Bacher, U., Weiss, T., Macijewski, K., Kohlmann, A., … Haferlach, T. (2010). Multilineage dysplasia (MLD) in acute myeloid leukemia (AML) correlates with MDS-related cytogenetic abnormalities and a prior history of MDS or MDS/MPN but has no independent prognostic relevance: a comparison of 408 cases classified as "AML not otherwis. *Blood*, *116*(15), 2742–51. doi:10.1182/blood-2010-04-279794

Mikesch, J.-H., Steffen, B., Berdel, W. E., Serve, H., & Müller-Tidow, C. (2007). The emerging role of Wnt signaling in the pathogenesis of acute myeloid leukemia. *Leukemia*, *21*(8), 1638–47. doi:10.1038/sj.leu.2404732

Minden, D., Buick, R. N., & Mcculloch, E. A. (1979). Separation of Blast Cell and T-Lymphocyte Progenitors in the Blood of Patients with Acute Myeloblastic Leukemia. *Blood*, *54*(1), 186–195.

Mione, M. C., & Trede, N. S. (2010). The zebrafish as a model for cancer. *Disease models & mechanisms*, *3*(9-10), 517–23. doi:10.1242/dmm.004747

Mishra, M. V, Bisht, K. S., Sun, L., Muldoon-Jacobs, K., Awwad, R., Kaushal, A., … Gius, D. (2008). DNMT1 as a molecular target in a multimodality-resistant phenotype in tumor cells. *Molecular cancer research : MCR*, *6*(2), 243–9. doi:10.1158/1541-7786.MCR-07-0373

Miyoshi, Y., Ando, A., Egawa, C., Taguchi, T., Tamaki, Y., Tamaki, H., … Noguchi, S. (2002). High expression of Wilms' tumor suppressor Gene Predicts poor prognosis in breast cancer patients. *Clinical cancer research*, *8*, 1167–1171.

Molina, G. a, Watkins, S. C., & Tsang, M. (2007). Generation of FGF reporter transgenic zebrafish and their utility in chemical screens. *BMC developmental biology*, *7*, 62. doi:10.1186/1471-213X-7-62

Monteiro, R., Pouget, C., & Patient, R. (2011). The gata1/pu.1 lineage fate paradigm varies between blood populations and is modulated by tif1γ. *The EMBO journal*, *30*(6), 1093–103. doi:10.1038/emboj.2011.34

Moore, M. A., & Metcalf, D. (1973). Cytogenetic analysis of human acute and chronic myeloid leukemic cells cloned in agar culture. *International journal of Cancer*, *11*, 143–152.

Morrison, S. J., Uchida, N., & Weissman, I. L. (1995). The biology of Hematopoietic stem cells. *Annual Review of Cell Developmental Biology*, *11*, 35–71.

Muller-Tidow, C., Steffen, B., Cauvet, T., Tickenbrock, L., Ji, P., Diederichs, S., … Serve, H. (2004). Translocation Products in Acute Myeloid Leukemia Activate the Wnt Signaling Pathway in Hematopoietic Cells. *Molecular and cellular biology*, *24*(7), 2890–2904. doi:10.1128/MCB.24.7.2890

Mulroy, T., McMahon, J. a, Burakoff, S. J., McMahon, A. P., & Sen, J. (2002). Wnt-1 and Wnt-4 regulate thymic cellularity. *European journal of immunology*, *32*(4), 967–71. doi:10.1002/1521-4141(200204)32:4<967::AID-IMMU967>3.0.CO;2-6

Nakamura, T. (2005). NUP98 fusion in human leukemia: dysregulation of the nuclear pore and homeodomain proteins. *International journal of hematology*, *82*(1), 21–7. doi:10.1532/IJH97.04160

Nakamura, T., Largaespada, D. A., Shaghnessy, J. D. J., Jenkins, N. A., & Copeland, N. G. (1996). Cooperative activation of Hoxa and Pbx1-related genes in murine myeloid leukaemias. *Nature genetics*, *12*, 149–153.

Nan, X., Ng, H. H., Johnson, C. a, Laherty, C. D., Turner, B. M., Eisenman, R. N., & Bird, a. (1998). Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature*, *393*(6683), 386–9. doi:10.1038/30764

Narula, J., Williams, C. J., Tiwari, A., Marks-Bluth, J., Pimanda, J. E., & Igoshin, O. a. (2013). Mathematical model of a gene regulatory network reconciles effects of genetic perturbations on hematopoietic stem cell emergence. *Developmental biology*, *379*(2), 258–269. doi:10.1016/j.ydbio.2013.04.016

Negrin, R. S. (1998). Minimal residual disease. *Current opinion in hematology*, *5*, 488–493.

Nerlov, C., Querfurth, E., Kulessa, H., & Graf, T. (2000). GATA-1 interacts with the myeloid PU.1 transcription factor and represses PU.1-dependent transcription. *Blood*, *95*(8), 2543–51. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10753833

Nikoloski, G., van der Reijden, B. a, & Jansen, J. H. (2012). Mutations in epigenetic regulators in myelodysplastic syndromes. *International journal of hematology*, *95*(1), 8–16. doi:10.1007/s12185-011-0996-3

Nimer, S. D. (2008). Myelodysplastic syndromes. *Blood*, *111*(10), 4841–51. doi:10.1182/blood-2007-08-078139

Nokamura, T., Largaespada, D. A., Lee, M. P., Johnson, L. A., Ohyashiki, K., Toyama, K., … Shaughnessy, J. D. J. (1996). Fusion of the nucleoporin gene NUP98 to HOXA9 by the chromosome translocation t(7;11)(p15;p15) in human myeloid leukemia. *Nature Genetics*, *12*, 154–158.

North, T. E., Goessling, W., Walkley, C. R., Lengerke, C., Kopani, K. R., Lord, A. M., Zon, L. I. (2007). Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. *Nature*, *447*(7147), 1007–11. doi:10.1038/nature05883

Nottage, K., Lanctot, J., Li, Z., Neglia, J. P., Bhatia, S., Hammond, S., … Armstrong, G. T. (2011). Long-term risk for subsequent leukemia after treatment for childhood cancer: a report from the Childhood Cancer Survivor Study. *Blood*, *117*(23), 6315–8. doi:10.1182/blood-2011-02-335158

Ohno, Y., Yasunaga, S., Janmohamed, S., Ohtsubo, M., Saeki, K., Kurogi, T., … Takihara, Y. (2013). Hoxa9 transduction induces hematopoietic stem and progenitor cell activity through direct down-regulation of geminin protein. *PloS one*, *8*(1), e53161. doi:10.1371/journal.pone.0053161

Ohshima, K., Karube, K., Hamasaki, M., Tutiya, T., Yamaguchi, T., Suefuji, H., … Kikuchi, M. (2003). Differential Chemokine, Chemokine Receptor and Cytokine Expression in Epstein-Barr Virus-associated Lymphoproliferative Diseases. *Leukemia & Lymphoma*, *44*(8), 1367–1378.

Oki, Y., & Issa, J. J. (2010). Epigenetic Mechanisms in AML – A Target for Therapy. In L. Nagarajan (Ed.), *Acute Myelogenous Leukemia: Genetics, Biology and Therapy* (1st ed., pp. 19–40). New York, NY: Springer New York. doi:10.1007/978-0-387-69259-3

Orazi, a, & Germing, U. (2008). The myelodysplastic/myeloproliferative neoplasms: myeloproliferative diseases with dysplastic features. *Leukemia*, *22*(7), 1308–19. doi:10.1038/leu.2008.119

Orkin, S. H., & Zon, L. I. (2008). Hematopoiesis: an evolving paradigm for stem cell biology. *Cell*, *132*(4), 631–44. doi:10.1016/j.cell.2008.01.025

Pabst, T., Eyholzer, M., Fos, J., & Mueller, B. U. (2009). Heterogeneity within AML with CEBPA mutations; only CEBPA double mutations, but not single CEBPA mutations are associated with favourable prognosis. *British journal of cancer*, *100*(8), 1343–6. doi:10.1038/sj.bjc.6604977

Palanisamy, N. (2010). Chromosomal Translocations in AML : Detection and Prognostic Significance. In L. Nagarajan (Ed.), *Acute Myelogenous Leukemia: Genetics, Biology and Therapy* (1st ed., pp. 41–58). New York, NY: Springer New York. doi:10.1007/978-0-387-69259-3

Palmqvist, L., Pineault, N., Wasslavik, C., & Humphries, R. K. (2007). Candidate genes for expansion and transformation of hematopoietic stem cells by NUP98-HOX fusion genes. *PloS one*, *2*(8), e768. doi:10.1371/journal.pone.0000768

Patel, K., Dickson, J., Din, S., Macleod, K., Jodrell, D., & Ramsahoye, B. (2010). Targeting of 5-aza-2'-deoxycytidine residues by chromatin-associated DNMT1 induces proteasomal degradation of the free enzyme. *Nucleic acids research*, *38*(13), 4313–24. doi:10.1093/nar/gkq187

Patton, E. E., & Zon, L. I. (2005). Taking human cancer genes to the fish: a transgenic model of melanoma in zebrafish. *Zebrafish*, *1*(4), 363–368.

Pérez-Caro, M., & Sánchez-García, I. (2006). Killing time for cancer stem cells (CSC): discovery and development of selective CSC inhibitors. *Current medicinal chemistry*, *13*(15), 1719–25. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16787215

Perez-Losada, J., & Balmain, A. (2003). Stem-cell hierarchy in skin cancer. *Nature reviews. Cancer*, *3*(6), 434–43. doi:10.1038/nrc1095

Perkins, a C., & Cory, S. (1993). Conditional immortalization of mouse myelomonocytic, megakaryocytic and mast cell progenitors by the Hox-2.4 homeobox gene. *The EMBO journal*, *12*(10), 3835–46. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=413667&tool=pmcentrez&rendertype=abstract

Peterson, R. T., Shaw, S. Y., Peterson, T. a, Milan, D. J., Zhong, T. P., Schreiber, S. L., Fishman, M. C. (2004). Chemical suppression of a genetic mutation in a zebrafish model of aortic coarctation. *Nature biotechnology*, *22*(5), 595–9. doi:10.1038/nbt963

Pillay, L. M., Forrester, a M., Erickson, T., Berman, J. N., & Waskiewicz, A. J. (2010). The Hox cofactors Meis1 and Pbx act upstream of gata1 to regulate primitive hematopoiesis. *Developmental biology*, *340*(2), 306–17. doi:10.1016/j.ydbio.2010.01.033

Plowright, L., Harrington, K. J., Pandha, H. S., & Morgan, R. (2009). HOX transcription factors are potential therapeutic targets in non-small-cell lung cancer (targeting HOX genes in lung cancer). *British journal of cancer*, *100*(3), 470–5. doi:10.1038/sj.bjc.6604857

Pruvot, B., Jacquel, A., Droin, N., Auberger, P., Bouscary, D., Tamburini, J., … Solary, E. (2011). Leukemic cell xenograft in zebrafish embryo for investigating drug efficacy. *Haematologica*, *96*(4), 612–6. doi:10.3324/haematol.2010.031401

Pukrop, T. (2006). Wnt 5a signaling is critical for macrophage-induced invasion of breast cancer cell lines. *Proceedings of the National Academy of Sciences Proceedings of the National Academy of Sciences*, *103*(14), 5454–5459.

Rai, K. (2006). Roles of DNA and histone methyltransferases during zebrafish development. *The Univeristy of Utah*, (December).

Rai, K., Jafri, I. F., Chidester, S., James, S. R., Karpf, A. R., Cairns, B. R., & Jones, D. a. (2010). Dnmt3 and G9a cooperate for tissue-specific development in zebrafish. *The Journal of biological chemistry*, *285*(6), 4110–21. doi:10.1074/jbc.M109.073676

Rai, K., Nadauld, L. D., Chidester, S., Manos, E. J., James, S. R., Karpf, A. R., … Jones, D. a. (2006). Zebra fish Dnmt1 and Suv39h1 regulate organ-specific terminal differentiation during development. *Molecular and cellular biology*, *26*(19), 7077–85. doi:10.1128/MCB.00312-06

Ransom, D. G., Bahary, N., Niss, K., Traver, D., Burns, C., Trede, N. S., … Zon, L. I. (2004). The zebrafish moonshine gene encodes transcriptional intermediary factor 1gamma, an essential regulator of hematopoiesis. *PLoS biology*, *2*(8), E237. doi:10.1371/journal.pbio.0020237

Ransom, H. P., Odenthal, J., Brownlie, A., Vogelsang, E., Kelsh, R. N., Brand, M., … Granato, M. D. G. (1996). Characterization of zebrafish mutants with defects in embryonic hematopoiesis. *Development*, *123*, 311–319.

Rao, A. V, Valk, P. J. M., Metzeler, K. H., Acharya, C. R., Tuchman, S. a, Stevenson, M. M., … Löwenberg, B. (2009). Age-specific differences in oncogenic pathway dysregulation and anthracycline sensitivity in patients with acute myeloid leukemia. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, *27*(33), 5580–6. doi:10.1200/JCO.2009.22.2547

Ravandi, F., & Estrov, Z. (2006). Eradication of leukemia stem cells as a new goal of therapy in leukemia. *Clinical cancer research*, *12*(2), 340–4. doi:10.1158/1078-0432.CCR-05-1879

Redaelli, A., Stephens, J. M., Brandt, S., Botteman, M. F., & Pashos, C. L. (2004). Short- and long-term effects of acute myeloid leukemia on patient health-related quality of life. *Cancer treatment reviews*, *30*(1), 103–17. doi:10.1016/S0305-7372(03)00142-7

Rekhtman, N., Radparvar, F., Evans, T., & Skoultchi, A. I. (1999). GATA-1 : functional antagonism in erythroid cells Direct interaction of hematopoietic transcription factors PU.1 and GATA-1 : functional antagonism in erythroid cells. *Genes & development*, *13*, 1398–1411.

Reya, T., & Clevers, H. (2005). Wnt signalling in stem cells and cancer. *Nature*, *434*(7035), 843–50. doi:10.1038/nature03319

Reya, T., Duncan, A. W., Ailles, L., Domen, J., Scherer, D. C., Willert, K., … Weissman, I. L. (2003). A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature*, *423*(6938), 409–14. doi:10.1038/nature01593

Reya, T., Morrison, S. J., Clarke, M. F., & Weissman, I. L. (2001). Stem cells, cancer, and cancer stem cells. *Nature*, *414*(6859), 105–11. doi:10.1038/35102167

Rhodes, J., Hagen, A., Hsu, K., Deng, M., Liu, T. X., Look, a T., & Kanki, J. P. (2005). Interplay of pu.1 and gata1 determines myelo-erythroid progenitor cell fate in zebrafish. *Developmental cell*, *8*(1), 97–108. doi:10.1016/j.devcel.2004.11.014

Rivina, L., & Schiestl, R. (2013). Mouse models for efficacy testing of agents against radiation carcinogenesis—a literature review. *International journal of environmental research and public health*, *10*(1), 107–43. doi:10.3390/ijerph10010107

Robertson, K D. (2001a). DNA methylation, methyltransferases, and cancer. *Oncogene*, *20*(24), 3139–55. doi:10.1038/sj.onc.1204341

Robertson, K D. (2001b). DNA methylation, methyltransferases, and cancer. *Oncogene*, *20*(24), 3139–55. doi:10.1038/sj.onc.1204341

Robertson, K D, Ait-Si-Ali, S., Yokochi, T., Wade, P. a, Jones, P. L., & Wolffe, a P. (2000). DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. *Nature genetics*, *25*(3), 338–42. doi:10.1038/77124

Robertson, Keith D, Wolffe, A. P., & Regulation, E. G. (2000). DNA Methylation in Health and Disease. *Nature review. Genetics*, *1*(October), 1–9.

Roche-Lestienne, C., Marceau, a, Labis, E., Nibourel, O., Coiteux, V., Guilhot, J., … Preudhomme, C. (2011). Mutation analysis of TET2, IDH1, IDH2 and ASXL1 in chronic myeloid leukemia. *Leukemia*, *25*(10), 1661–4. doi:10.1038/leu.2011.139

Rountree, M. R., Bachman, K. E., & Baylin, S. B. (2000). DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. *Nature genetics*, *25*(3), 269–77. doi:10.1038/77023

Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S., & Polakis, P. (1996). Binding of GSK3beta to the APC-beta-catenin complex and regulation of complex assembly. *Science*, *272*(5264), 1023–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8638126

Rubnitz, J. E., Gibson, B., & Smith, F. O. (2008). Acute myeloid leukemia. *Pediatric clinics of North America*, *55*(1), 21–51, ix. doi:10.1016/j.pcl.2007.11.003

Saba, H. I. (2007). Decitabine in the treatment of myelodysplastic syndromes. *Therapeutics and clinical risk management*, *3*(5), 807–17. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2376088&tool=pmcentrez&rendertype=abstract

Sabaawy, H. E., Azuma, M., Embree, L. J., Tsai, H.-J., Starost, M. F., & Hickstein, D. D. (2006). TEL-AML1 transgenic zebrafish model of precursor B cell acute lymphoblastic leukemia. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(41), 15166–71. doi:10.1073/pnas.0603349103

Satokata, I., Benson, G., & Maas, R. (1995). Sexually dimorphic sterility phenotypes in Hoxa10-deficient mice. *Nature*, *374*(6521), 460–3. doi:10.1038/374460a0

Saunthararajah, Y., Triozzi, P., Rini, B., Singh, A., Radivoyevitch, T., Sekeres, M., … Bolwell, B. (2012). P53-independent, normal stem cell sparing epigenetic-differentation therapy for myeloid and other malignancies. *Seminars in Oncology*, *39*(1), 97–108. doi:10.1053/j.seminoncol.2011.11.011.P53-INDEPENDENT

Sauvageau, G., Lansdorp, P. M., Eaves, C. J., Hogge, D. E., Dragowska, W. H., Reid, D. S., … Humphries, R. K. (1994). Differential expression of homeobox genes in functionally distinct CD34+ subpopulations of human bone marrow cells. *Proceedings of the National Academy of Sciences of the United States of America*, *91*(25), 12223–7. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=45409&tool=pmcentrez&rendertype=abstract

Savickiene, J., Treigyte, G., Borutinskaite, V.-V., & Navakauskiene, R. (2012). Antileukemic activity of combined epigenetic agents, DNMT inhibitors zebularine and RG108 with HDAC inhibitors, against promyelocytic leukemia HL-60 cells. *Cellular & molecular biology letters*, *17*(4), 501–25. doi:10.2478/s11658-012-0024-5

Scandura, J. M., Boccuni, P., Cammenga, J., & Nimer, S. D. (2002). Transcription factor fusions in acute leukemia : variations on a theme. *Oncogene*, *21*, 3422–3444. doi:10.1038/sj/onc/1205315

Schaich, M., Schlenk, R. F., Al-Ali, H. K., Döhner, H., Ganser, A., Heil, G., … Ehninger, G. (2007). Prognosis of acute myeloid leukemia patients up to 60 years of age exhibiting trisomy 8 within a non-complex karyotype: individual patient data-based meta-analysis of the German Acute Myeloid Leukemia Intergroup. *Haematologica*, *92*(6), 763–70. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17550848

Scheller, M., Huelsken, J., Rosenbauer, F., Taketo, M. M., Birchmeier, W., Tenen, D. G., & Leutz, A. (2006). Hematopoietic stem cell and multilineage defects generated by constitutive beta-catenin activation. *Nature immunology*, *7*(10), 1037–47. doi:10.1038/ni1387

Scott, E. W., Simon, M. C., Anastasi, J., & Singh, H. (1994). Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science*, *265*(5178), 1573–1577.

Sercan, P. M., Simsek, O., Ates, H., & Sercan, Z. (2007). Induction of apoptosis increases expression of non-canonical WNT genes in myeloid leukemia cell lines. *Oncology reports*, *18*(6), 1563–1570.

Sharkis, S. J., Collector, M. I., Barber, J. P., Vala, M. S., & Jones, R. J. (1997). Phenotypic and functional characterization of the hematopoietic stem cell. *Stem Cells: Hematopoietic Stem Cells*, *15*(Suppl 1), 41–4. doi:10.1002/stem.5530150807

Shih, A. H., Abdel-Wahab, O., Patel, J. P., & Levine, R. L. (2012). The role of mutations in epigenetic regulators in myeloid malignancies. *Nature reviews. Cancer*, *12*(9), 599–612. doi:10.1038/nrc3343

Simon, M. C. (1998). PU.1 and hematopoiesis: lessons learned from gene targeting experiments. *Seminars in immunology*, *10*(2), 111–8. doi:10.1006/smim.1998.0112

Smith, L.-L., Yeung, J., Zeisig, B. B., Popov, N., Huijbers, I., Barnes, J., … So, C. W. E. (2011). Functional crosstalk between Bmi1 and MLL/Hoxa9 axis in establishment of normal hematopoietic and leukemic stem cells. *Cell stem cell*, *8*(6), 649–62. doi:10.1016/j.stem.2011.05.004

Smith, T. H. L., Collins, T. M., & McGowan, R. A. (2011). Expression of the dnmt3 genes in zebrafish development: similarity to Dnmt3a and Dnmt3b. *Development Genes and Evolution*, *220*(11-12), 11–12.

Soares, J., Castro, L. F. C., Reis-Henriques, M. a, Monteiro, N. M., & Santos, M. M. (2012). Zebrafish (Danio rerio) life-cycle exposure to chronic low doses of ethinylestradiol modulates p53 gene transcription within the gonads, but not NER pathways. *Ecotoxicology (London, England)*, *21*(5), 1513–22. doi:10.1007/s10646-012-0905-4

Society, L. and lymphoma. (2013). Leukemia and Lymphoma Society Spring 2013 facts. *Fighting Blood Cancers*, *Spring 201*.

Soenen, V., Preudhomme, C., Roumier, C., Daudignon, a, Laï, J. L., & Fenaux, P. (1998). 17p Deletion in acute myeloid leukemia and myelodysplastic syndrome. Analysis of breakpoints and deleted segments by fluorescence in situ. *Blood*, *91*(3), 1008–15. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9446663

Sola, L., & Gornung, E. (2001). Classical and molecular cytogenetics of the zebrafish, Danio rerio (Cyprinidae, Cypriniformes): an overview. *Genetica*, *111*(1-3), 397–412. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11841183

Song, J., Teplova, M., Ishibe-Murakami, S., & Patel, D. J. (2012). Structure-based mechanistic insights into DNMT1-mediated maintenance DNA methylation. *Science*, *335*(6069), 709–12. doi:10.1126/science.1214453

Spangrude, G. J., Heimfeld, S., & Weissman, I. L. (1988). Purification and characterization of mouse hematopoietic stem cells. *Science*, *241*(4861), 58–62.

Staal, F. J., Meeldijk, J., Moerer, P., Jay, P., van de Weerdt, B. C., Vainio, S., … Clevers, H. (2001). Wnt signaling is required for thymocyte development and activates Tcf-1 mediated transcription. *European journal of immunology*, *31*(1), 285–93. doi:10.1002/1521-4141(200101)31:1<285::AID-IMMU285>3.0.CO;2-D

Staal, F. J. T., & Clevers, H. C. (2005). WNT signalling and haematopoiesis: a WNT-WNT situation. *Nature reviews. Immunology*, *5*(1), 21–30. doi:10.1038/nri1529

Staal, F. J. T., & Sen, J. M. (2008). The canonical Wnt signaling pathway plays an important role in lymphopoiesis and hematopoiesis. *European journal of immunology*, *38*(7), 1788–94. doi:10.1002/eji.200738118

Stainier, D. Y., Weinstein, B. M., Detrich, H. W., Zon, L. I., & Fishman, M. C. (1995). Cloche, an early acting zebrafish gene, is required by both the endothelial and hematopoietic lineages. *Development (Cambridge, England)*, *121*(10), 3141–50. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7588049

Stoletov, K., & Klemke, R. (2008). Catch of the day: zebrafish as a human cancer model. *Oncogene*, *27*(33), 4509–20. doi:10.1038/onc.2008.95

Stopka, T., Amanatullah, D. F., Papetti, M., & Skoultchi, A. I. (2005). PU.1 inhibits the erythroid program by binding to GATA-1 on DNA and creating a repressive chromatin structure. *The EMBO journal*, *24*(21), 3712–23. doi:10.1038/sj.emboj.7600834

Streisinger, G., Walker, C., Dower, N., Knauber, D., & Singer, F. (1981). Production of clones of homozygous diploid zebra fish (Brachydanio rerio). *Nature*, *291*, 293–296.

Sugihara, E., & Saya, H. (2013). Complexity of cancer stem cells. *International journal of cancer. Journal international du cancer*, *132*(6), 1249–59. doi:10.1002/ijc.27961

Sutherland, H. J., Lansdorp, P. M., Henkelman, D. H., Eaves, a C., & Eaves, C. J. (1990). Functional characterization of individual human hematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layers. *Proceedings of the National Academy of Sciences of the United States of America*, *87*(9), 3584–8. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=53946&tool=pmcentrez&rendertype=abstract

Takeda, A., Goolsby, C., & Yaseen, N. R. (2006). NUP98-HOXA9 induces long-term proliferation and blocks differentiation of primary human CD34+ hematopoietic cells. *Cancer research*, *66*(13), 6628–37. doi:10.1158/0008-5472.CAN-06-0458

Taki, M., Yokoyama, K., Fujimoto, R., Tsutsumi, S., Nagayama, M., & Kamata, N. (2003). Down-regulation of Wnt-4 and up-regulation of Wnt-5a expression by epithelial-mesenchymal transition in human squamous carcinoma cells. *Cancer science*, *94*(7), 593–597.

Tallman, M. S., Andersen, J. W., Schiffer, C. A., Appelbaum, F. R., Feusner, J. H., Ogeden, A., … Wiernik, P. H. (1997). All-Trans-Retinoic Acid in Acute Promyelocytic Leukemia. *The New England journal of medicine*, *337*(15), 1021–1028.

Talpaz, M., Estrov, Z., Kantarjian, H., Ku, S., Foteh, a, & Kurzrock, R. (1994). Persistence of dormant leukemic progenitors during interferon-induced remission in chronic myelogenous leukemia. Analysis by polymerase chain reaction of individual colonies. *The Journal of clinical investigation*, *94*(4), 1383–9. doi:10.1172/JCI117473

Thepot, S., Itzykson, R., Seegers, V., Raffoux, E., Quesnel, B., Chait, Y., … Adès, L. (2010). Treatment of progression of Philadelphia-negative myeloproliferative neoplasms to myelodysplastic syndrome or acute myeloid leukemia by azacitidine: a report on 54 cases on the behalf of the Groupe Francophone des Myelodysplasies (GFM). *Blood*, *116*(19), 3735–42. doi:10.1182/blood-2010-03-274811

Thol, F., Damm, F., Lüdeking, A., Winschel, C., Wagner, K., Morgan, M., … Heuser, M. (2011). Incidence and prognostic influence of DNMT3A mutations in acute myeloid leukemia. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, *29*(21), 2889–96. doi:10.1200/JCO.2011.35.4894

Thorsteinsdottir, U, Sauvageau, G., Hough, M. R., Dragowska, W., Lansdorp, P. M., Lawrence, H. J., … Humphries, R. K. (1997). Overexpression of HOXA10 in murine hematopoietic cells perturbs both myeloid and lymphoid differentiation and leads to acute myeloid leukemia. *Molecular and cellular biology*, *17*(1), 495–505. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=231774&tool=pmcentrez&rendertype=abstract

Thorsteinsdottir, Unnur, Kroon, E., Jerome, L., Blasi, F., & Sauvageau, G. (2001). Defining Roles for HOX and MEIS1 Genes in Induction of Acute Myeloid Leukemia. *Molecular and cellular biology*, *21*(1), 224–234. doi:10.1128/MCB.21.1.224

Thorsteinsdottir, Unnur, Mamo, A., Kroon, E., Jerome, L., Bijl, J., Jeffrey, L. H., … Sauvageau, G. (2002). Overexpression of the myeloid leukemia-associated Hoxa9 gene in bone marrow cells induces stem cell expansion. *Blood*, *99*(1), 121–129. doi:10.1182/blood.V99.1.121

Till, J. E., & McCulloch, E. A. (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Rediation Research*, *14*(43), 213–222.

Tittle, R. K., Sze, R., Ng, A., Nuckels, R. J., Swartz, M. E., Anderson, R. M., … Gross, J. M. (2011). Uhrf1 and Dnmt1 are required for development and maintenance of the zebrafish lens. *Developmental biology*, *350*(1), 50–63. doi:10.1016/j.ydbio.2010.11.009

Tonkin, J., Francis, Y., Pattinson, A., Peters, T., Taylor, M., Thompson, R., & Wallis, L. (2012). Myeloproliferative neoplasms: diagnosis, management and treatment. *Nursing standard (Royal College of Nursing (Great Britain) : 1987)*, *26*(51), 44–51. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/22953404

Traver, D., Winzeler, A., Stern, H. M., Mayhall, E. a, Langenau, D. M., Kutok, J. L., … Zon, L. I. (2004). Effects of lethal irradiation in zebrafish and rescue by hematopoietic cell transplantation. *Blood*, *104*(5), 1298–305. doi:10.1182/blood-2004-01-0100

Trecca, D., Longo, L., Biondi, A., Cro, L., Calori, R., Grignani, F., … Neri, A. (1994). Analysis of p53 gene mutations in acute myeloid leukemia. *American journal of hematology*, *46*(4), 304–309. Retrieved from http://onlinelibrary.wiley.com/doi/10.1002/ajh.2830460409/abstract?deniedAccessCustomisedMessage=&userIsAuthenticated=false

Trowbridge, J. J., Sinha, A. U., Zhu, N., Li, M., Armstrong, S. A., & Orkin, S. H. (2012). Haploinsufficiency of Dnmt1 impairs leukemia stem cell function through derepression of bivalent chromatin domains. *Genes & development*, *26*, 344–349. doi:10.1101/gad.184341.111.344

Unoki, M., Brunet, J., & Mousli, M. (2009). Drug discovery targeting epigenetic codes: the great potential of UHRF1, which links DNA methylation and histone modifications, as a drug target in cancers and toxoplasmosis. *Biochemical pharmacology*, *78*(10), 1279–88. doi:10.1016/j.bcp.2009.05.035

Valk, P. J. M., Verhaak, R. G. W., Beijen, M. A., Erpelinck, C. a J., Barjesteh van Waalwijk van Doorn-Khosrovani, S., Boer, J. M., … Delwel, R. (2004). Prognostically useful gene-expression profiles in acute myeloid leukemia. *The New England journal of medicine*, *350*(16), 1617–28. doi:10.1056/NEJMoa040465

Vardiman, J. W. (2010). The World Health Organization (WHO) classification of tumors of the hematopoietic and lymphoid tissues: an overview with emphasis on the myeloid neoplasms. *Chemico-biological interactions*, *184*(1-2), 16–20. doi:10.1016/j.cbi.2009.10.009

Vardiman, J. W., Thiele, J., Arber, D. a, Brunning, R. D., Borowitz, M. J., Porwit, A., … Bloomfield, C. D. (2009). The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood*, *114*(5), 937–51. doi:10.1182/blood-2009-03-209262

Verhaak, R. G. W., & Valk, P. J. M. (2010). Genes Predictive of Outcome and Novel Molecular Classification Schemes in Adult Acute Myeloid Leukemia. In L. Nagarajan (Ed.), *Acute Myelogenous Leukemia: Genetics, Biology and Therapy* (1st ed., pp. 67–83). New York, NY: Springer New York. doi:10.1007/978-0-387-69259-3

Voss, A. K., Collin, C., Dixon, M. P., & Thomas, T. (2009). Moz and retinoic acid coordinately regulate H3K9 acetylation, Hox gene expression, and segment identity. *Developmental cell*, *17*(5), 674–86. doi:10.1016/j.devcel.2009.10.006

Walter, M. J., Shen, D., Ding, L., Shao, J., Koboldt, D. C., Chen, K., … Graubert, T. A. (2012). Clonal Architecture of Secondary Acute Myeloid Leukemia. *New England Journal of Medicine*, *366*(12), 1090–1098. doi:10.1056/NEJMoa1106968.Clonal

Wan, T. S., Au, W. Y., Chan, J. C., Chan, L. C., & Ma, S. K. (1999). Trisomy 21 as the sole acquired karyotypic abnormality in acute myeloid leukemia and myelodysplastic syndrome. *Leukemia research*, *23*(11), 1079–83. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10576514

Wang, Kankan, Wang, P., Shi, J., Zhu, X., He, M., Jia, X., … Zhang, J. (2010). PML/RARalpha targets promoter regions containing PU.1 consensus and RARE half sites in acute promyelocytic leukemia. *Cancer cell*, *17*(2), 186–97. doi:10.1016/j.ccr.2009.12.045

Wang, Kun, Huang, Z., Zhao, L., Liu, W., Chen, X., Meng, P., … Zhang, W. (2012). Large-scale forward genetic screening analysis of development of hematopoiesis in zebrafish. *Journal of genetics and genomics = Yi chuan xue bao*, *39*(9), 473–80. doi:10.1016/j.jgg.2012.07.008

Wang, Q.-F., Wu, G., Mi, S., He, F., Wu, J., Dong, J., … Thirman, M. J. (2011). MLL fusion proteins preferentially regulate a subset of wild-type MLL target genes in the leukemic genome. *Blood*, *117*(25), 6895–905. doi:10.1182/blood-2010-12-324699

Wang, Y., Krivtsov, A. V, Sinha, A. U., North, T. E., Goessling, W., Feng, Z., … Armstrong, S. a. (2010). The Wnt/beta-catenin pathway is required for the development of leukemia stem cells in AML. *Science*, *327*(5973), 1650–3. doi:10.1126/science.1186624

Wang, Z., Xu, L., Zhu, X., Cui, W., Sun, Y., Nishijo, H., … Li, R. (2010). Demethylation of specific Wnt/β-catenin pathway genes and its upregulation in rat brain induced by prenatal valproate exposure. *Anatomical record*, *293*(11), 1947–53. doi:10.1002/ar.21232

Warga, R. M., Kane, D. A., & Ho, R. K. (2009). Fate mapping embryonic blood in zebrafish: multi- and unipotential lineages are segregated at gastrulation. *Developmental cell*, *16*(5), 744–55. doi:10.1016/j.devcel.2009.04.007

Weeraratna, A., Hostetter, G., Rosenblatt, K., Duray, P., Bittner, M., Trent, J., & Jiang, Y. (2002). Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma. *Cancer cell*, *1*(3), 279–288.

Weissman, I. L. (2000). Translating Stem and Progenitor Cell Biology to the Clinic: Barriers and Opportunities. *Science*, *287*(5457), 1442–1446. doi:10.1126/science.287.5457.1442

Weissman, Irving L, & Shizuru, J. a. (2008). The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases. *Blood*, *112*(9), 3543–53. doi:10.1182/blood-2008-08-078220

Welch, J. S., Ley, T. J., Link, D. C., Miller, C. a, Larson, D. E., Koboldt, D. C., … Xia, J. (2012). The origin and evolution of mutations in acute myeloid leukemia. *Cell*, *150*(2), 264–78. doi:10.1016/j.cell.2012.06.023

Werling, U., Siehler, S., Litfin, M., Nau, H., & Göttlicher, M. (2001). Induction of differentiation in F9 cells and activation of peroxisome proliferator-activated receptor delta by valproic acid and its teratogenic derivatives. *Molecular pharmacology*, *59*(5), 1269–76. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11306712

Westerfield, M. (2000). *The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio)*. (4th ed.). Eugene: Univ. of Oregon Press.

Wijerman, P., Lubbert, M., Verhoef, G., Bosly, A., Ravoet, C., Andre, M., & Ferrant, A. (2000). Low-Dose 5'-Aza-2'-Deoxycytidine, a DNA Hypomethylating Agent, for the Treatment of High-Risk Myelodysplastic Syndrome: A Multicenter Phase II Study in Elderly Patients. *Journal of clinical oncology*, *18*(5), 956–962.

Wijermans, P. W., Krulder, J. W., Huijgens, P. C., & Neve, P. (1997). Continuous infusion of low-dose 5-Aza-2'-deoxycytidine in elderly patients with high-risk myelodysplastic syndrome. *Leukemia*, *11 Suppl 1*(June 1996), S19–23. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9130687

Wiltse, J. (2005). Mode of Action: Inhibition of Histone Deacetylase, Altering WNT-Dependent Gene Expression, and Regulation of Beta-Catenin—Developmental Effects of Valproic Acid. *Critical Reviews in Toxicology*, *35*(8-9), 727–738. doi:10.1080/10408440591007403

Wojiski, S., Guibal, F. C., Kindler, T., Lee, B. H., Jesneck, J. L., Fabian, a, … Gilliland, D. G. (2009). PML-RARalpha initiates leukemia by conferring properties of self-renewal to committed promyelocytic progenitors. *Leukemia*, *23*(8), 1462–71. doi:10.1038/leu.2009.63

Woo, K.-S., Kim, K.-E., Kim, K.-H., Kim, S.-H., Park, J.-I., Shaffer, L. G., & Han, J.-Y. (2009). Deletions of chromosome arms 7p and 7q in adult acute myeloid leukemia: a marker chromosome confirmed by array comparative genomic hybridization. *Cancer genetics and cytogenetics*, *194*(2), 71–4. doi:10.1016/j.cancergencyto.2009.04.017

Wossidlo, M., Lepikhov, K., Marques, C., Zakhartchenko, V., Boiani, M., Arand, J., … Nakamura, T. (2011). 5-Hydroxymethylcytosine in the mammalian zygote is linked with epigenetic reprogramming. *Nature communications*, *2*(241).

Xu, W., Zhou, H.-F., Fan, L., Qian, S.-X., Chen, L.-J., Qiu, H.-R., … Li, J.-Y. (2008). Trisomy 22 as the sole abnormality is an important marker for the diagnosis of acute myeloid leukemia with inversion 16. *Onkologie*, *31*(8-9), 440–4. doi:10.1159/000141917

Yan, X.-J., Xu, J., Gu, Z.-H., Pan, C.-M., Lu, G., Shen, Y., … Chen, S.-J. (2011). Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. *Nature genetics*, *43*(4), 309–15. doi:10.1038/ng.788

Yang, H. W., Kutok, J. L., Lee, N. H., Piao, H. Y., Fletcher, C. D. M., Kanki, J. P., & Look, A. T. (2004). Targeted Expression of Human MYCN Selectively Causes Pancreatic Neuroendocrine Tumors in Transgenic Zebrafish Targeted Expression of Human MYCN Selectively Causes Pancreatic Neuroendocrine Tumors in Transgenic Zebrafish. *Cancer research*, *64*, 7256–7262.

Yeh, J.-R. J., Munson, K. M., Chao, Y. L., Peterson, Q. P., Macrae, C. a, & Peterson, R. T. (2008). AML1-ETO reprograms hematopoietic cell fate by downregulating scl expression. *Development (Cambridge, England)*, *135*(2), 401–10. doi:10.1242/dev.008904

Yeh, J.-R. J., Munson, K. M., Elagib, K. E., Goldfarb, A. N., Sweetser, D. a, & Peterson, R. T. (2009). Discovering chemical modifiers of oncogene-regulated hematopoietic differentiation. *Nature chemical biology*, *5*(4), 236–43. doi:10.1038/nchembio.147

Yilmaz, O. H., Valdez, R., Theisen, B. K., Guo, W., Ferguson, D. O., Wu, H., & Morrison, S. J. (2006). Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. *Nature*, *441*(7092), 475–82. doi:10.1038/nature04703

Yoshida, H., Kitamura, K., Tanaka, K., Omura, S., Miyazaki, T., Hachiya, T., … Naoe, T. (1996). Accelerated Degradation of PML-Retinoic Acid Receptor α (PML-RARA) Oncoprotein by All-trans-Retinoic Acid in Acute Promyelocytic Leukemia : Possible Role of the Proteasome Pathway Advances in Brief Accelerated Degradation of PML-Retinoic Acid Receptor a. *Cancer research*, *56*, 2945–48.

Young, N. S. (2005). Aplastic Anemia, Myelodysplasia, and Related Bone Marrow Failure Syndromes. In D. L. Longo, A. S. Fauci, D. L. Kasper, S. L. Hauser, J. L. Jameson, & J. Loscalzo (Eds.), *Harrison's Principles of Internal Medicine* (16th ed.). New York, NY: McGraw-Hill.

Zhang, D., Voso, M., Chen, H., Smith, L., Hetherington, C., Tenen, D., & Hohaus, S. (1996). Function of PU.1 (Spi-1), C/EBP, and AML1 in early myelopoiesis: regulation of multiple myeloid CSF receptor promoters. *Current topics in microbiology and immunology*, *211*, 137–147.

Zhang, J., Grindley, J. C., Yin, T., Jayasinghe, S., He, X. C., Ross, J. T., … Li, L. (2006). PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. *Nature*, *441*(7092), 518–22. doi:10.1038/nature04747

Zhang, P., Xiaobo, Z., Iwama, A., Yu, C., Smith, K. A., Mueller, B. U., … Tenen, D. G. (2000). PU.1 inhibits GATA-1 function and erythroid differentiation by blocking GATA-1 DNA binding. *Blood*, *96*, 2641–2648.

Zhu, S., Lee, J.-S., Guo, F., Shin, J., Perez-Atayde, A. R., Kutok, J. L., … Look, T. A. (2012). Activated ALK Collaborates with MYCN in Neuroblastoma Pathogenesis. *Cancer cell*, *21*(3), 362–373. doi:10.1016/j.ccr.2012.02.010.Activated

Zon, L. I. (1999). Zebrafish : A New Model for Human Disease. *Genome research*, *9*, 99–100. doi:10.1101/gr.9.2.99

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