The Histone H3, Lysine 79 Methyltransferase Activity of DOT1L is Necessary for Murine Yolk Sac Erythro-Myeloid Progenitor Self-Renewal and Erythroblast Survival

By

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ABSTRACT

Disruptor of Telomere silencing 1-Like (DOT1L), is a histone 3, lysine 79 (H3K79) methyltransferase that has been implicated in multiple processes, including activation of transcription, regulation of the cell cycle, leukemogenesis, and mouse embryonic development. Previous studies found that *Dot1L* deficiency results in an erythropoietic defect, leading to lethal anemia at around mid-gestation (Feng et al., 2010, Blood). The precise molecular mechanism(s) by which DOT1L regulates embryonic hematopoiesis has not yet been elucidated, however. DOT1L is a large protein (1540aa), and it is involved in several, diverse processes. However, its only documented activity has been as an intrinsic, histone methyltransferase. Additional functional domains of the protein might be responsible for its diverse activities, including murine hematopoiesis. This study sought to determine whether the methyltransferase activity of DOT1L is essential for hematopoiesis.

To test this hypothesis, a *Dot1L* methyltransferase mutant (*Dot1L* MM) mouse line was created. Using the Cas9/CRISPR system, a *Dot1L* point mutation was induced in cultured murine embryonic stem cells (mESC). These mESCs contained a wildtype allele, and the second had a single amino acid change in the methyltransferase domain of *Dot1L*, thereby eliminating its methyltransferase activity, but preserving the rest of the protein. These mutant mESCs were injected into blastocysts to produce chimeric mice. The chimeras possessing the methyltransferase mutation were back crossed onto the C57/BL6 background, producing male and female offspring heterozygous for the mutation. Through intercrosses of the F1 generation, it was found that the *Dot1L* MM mice display an embryonic lethality between embryonic days 10.5 and 13.5, similar to the *Dot1L* knockout mice, as reported in our previous studies.

Additionally, ex vivo blood differentiation assays and extensively self-renewing erythroblast (ESRE) cultures were performed using E10.5 yolk sacs from *Dot1L* MM and

knockout mice. The data showed that the *Dot1L* MM and knockout yolk sacs display similar phenotypes. In blood differentiation cultures, *Dot1L* knockout and MM yolk sac cells form the same types of hematopoietic colonies as in wildtype (erythroid, myeloid, and mixed), but there is a decrease in colony size and number compared to the wildtype. In the ESRE cultures, *Dot1L* knockout and MM yolk sac cells form significantly fewer ESREs and have increased cell death compared to wildtype. Strikingly, the cells in these cultures also exhibit a profound genomic instability, implicating DOT1L methyltransferase activity in maintenance of the genome as well as the viability of hematopoietic progenitors. These results suggest that the methyltransferase activity of DOT1L plays a predominant role in the activity of the protein as a whole, and is responsible for its function in facilitating early murine hematopoiesis.

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TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1 : Introduction

Formation of functional blood cells plays a crucial role in mammalian embryonic development. Red blood cells contain the protein hemoglobin, which carries and releases oxygen to tissues [1]. Oxygen supplied by red blood cells is necessary for embryo for growth and survival, and an absence of primitive erythrocytes (the first blood cells to form in an embryo) leads to embryonic lethality [2].

Previous studies in the Patrick Fields laboratory found that loss of the Disruptor of Telomere silencing 1-Like (*Dot1L*) gene, a histone H3 lysine 79 (H3K79) methyltransferase, in mice results in embryonic death at mid-gestation (E11-E13.5) [3]. Prior to their death, the mutant

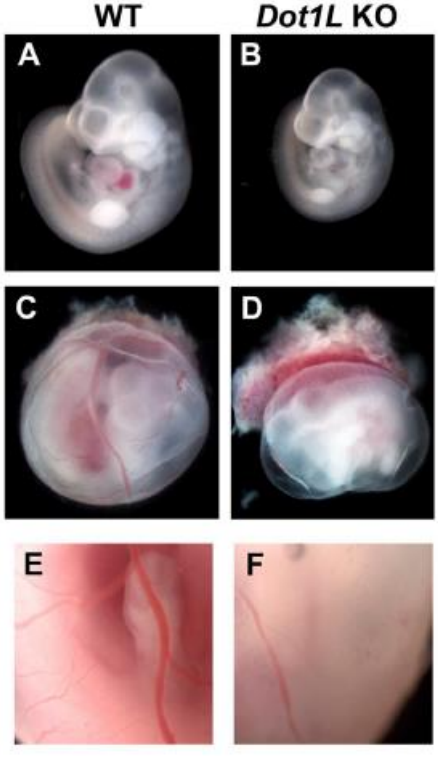

Figure 1.1 : Wildtype (WT) and *Dot1L* Knockout (KO) E10.5 mouse embryos.
(A,B) Whole embryos. (C,D) Surrounding yolk sac. (E,F) Enlarged view of blood vessels in yolk sac. Decreased embryo size and defective yolk sac vascular development is seen in *Dot1L* KO mice [3].

embryos are smaller in size, display abnormalities in the development of the heart and vasculature, and are severely anemic [3]. Although several aspects of the *Dot1L* knockout (KO) embryos' development are noticeably impacted by absence of DOT1L, the Fields lab decided to investigate blood development, since the anemia was striking. Significantly, when *Dot1L* KO yolk sacs from E8.5 and E10.5 embryos were dissociated and cultured, *ex vivo*, they formed erythroid colonies that were fewer in number and smaller than their wildtype (WT) counterparts. Myeloid colony formation was less affected by the absence of DOT1L, indicating that the enzyme predominantly affects erythroid growth and colony formation at the E8.5-E10.5 stage of murine development [3].

An examination of cell cycle progression of these cultured blood progenitors revealed that the DOT1L-deficient cells exhibited a significantly increased G_0/G_1 period and increased apoptosis compared to WT cells, indicating delayed or halted cell cycle progression and increased death [3]. These abnormalities in progenitor growth and survival could explain the profound decrease in erythroid potential that is observed in the DOT1L-deficient cells, ex vivo, as well as the pronounced anemia observed, in vivo. Since the overall number of erythroid progenitors is not affected in the *Dot1L*-deficient animals [3], the loss of DOT1L seems to impart a cell-autonomous alteration in erythroid potential that specifically affects the ability of YS hematopoietic progenitors to survive and expand. The precise molecular mechanism(s) by which DOT1L regulates embryonic hematopoiesis has not yet been elucidated and was the overall objective of this project.

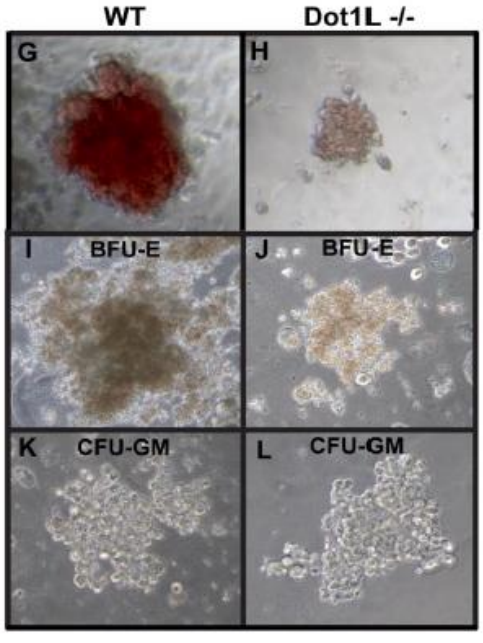

Figure 1.2 Blood differentiation assays of yolk sacs from E8.5 and E10.5 WT and *Dot1L* KO embryos.

(G,H) Primitive erythroid, (I,J) BFU-E, (K,L) CFU-GM colonies. Cells from *Dot1L* KO yolk sacs form fewer and smaller erythroid colonies [3].

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Chapter 2 : Literature Review

Murine Hematopoiesis

Wave One: Primitive Progenitor Emergence

Red blood cell development in the mouse begins around embryonic day 7.25 (E7.25), when primitive erythroid cells begin to form in the blood islands of the embryonic yolk sac [4]. These first red blood cells provide oxygen to support the rapidly growing embryo [5]. Primitive erythroid cells arising in the yolk sac also form in conjunction with megakaryocytes and macrophages [4]. It is thought that these erythroid progenitors form from posterior mesoderm and that Wnt/β-catenin signaling is required for this specification.

Blood islands of the yolk sac consist of an erythroid core surrounded by endothelial cells [6]. It has been hypothesized in the field for some time that these primitive erythroid and endothelial cells of the blood islands arise from a common precursor cell, termed the hemangioblast [6]. This hypothesis is based on several observations: primitive erythroid and endothelial cells appear in the mouse embryo around the same time and place, and primitive erythroid cells express the endothelial markers flk-1, vascular endothelial cadherin, tie-2, and PECAM-1 when they emerge from mesodermal cells [7]. Also, cultured embryonic stem cells and mouse embryos form blast colony forming cells (Blast-CFC), which have both hematopoietic and endothelial cell potential [7]. However, the hemangioblast argument lost some support once it was discovered that yolk sac blood islands were not actually islands but erythroid cells formed in a single belt shape around the yolk sac and not divided up by endothelial cells [6]. Additionally, lineage tracing studies discovered that yolk sac blood islands were polyclonal, indicating that primitive erythroblasts and angioblasts do not share a common progenitor [2]. Still, some studies in mouse embryos have added support to the hemangioblast hypothesis. Gastrulating embryos that were cultured in conditions that support the growth of Blast-CFCs

formed similar blast cell colonies, which differentiated into both hematopoietic and vascular cells [8]. Clonality of these colonies was also shown in cell mixing experiments using embryos with either hygromycin or neomycin transgenes [8]. These embryo hemangioblasts first appear in the posterior primitive streak portion of the embryo during the mid-streak phase of gastrulation. Their number peaks during the neural plate stage, and declines during the head fold stage [8]. They also co-express brachyury and Flk-1 just like Blast-CFCs from cultured ESCs, indicating that they represent a subpopulation of mesoderm [8]. The fact that hemangioblasts are not found in the yolk sac could indicate that they rapidly differentiate into the hematopoietic and vascular lineages after they leave the streak and then colonize the yolk sac as primitive erythroid, megakaryocyte-erythroid, and angioblast progenitors [2,8]. Others argue that these mesodermal hemangioblasts could just represent mesodermal cells with broad potential, outside of just hematopoietic and vascular [6].

Primitive erythroid progenitors continue to form in the yolk sac until E9.0 [2], and enter circulation after the heart begins beating (at E8.25) [9]. Primitive erythroid cells begin maturing after they enter the bloodstream. These progenitors lose their nucleoli between E9.5-10.5, become smaller in cell diameter and area between E10.5-11.5, condense their nuclei by 80% and ultimately lose their nuclei all together, forming primitive reticulocytes by E12.5-E16.5 [2,9]. These progenitors also begin to express cell adhesion markers from E12.5-14.5 [2]. Primitive erythrocytes can be found in the mouse for up to 3 weeks after birth, representing about 0.5% of all cells in the bloodstream [2]. Primitive erythropoiesis is vital for an embryo's early growth and survival, as embryos unable to carry out this process die by E9.5 [10].

Wave Two: Erythro-Myeloid Progenitor Emergence

Shortly after primitive erythroid cells form, a second wave of hematopoiesis begins in the yolk sac, around E8.25 [4,10]. These second-wave progenitors are termed erythro-myeloid

progenitors (EMP) [4]. EMPs can form definitive erythroid cells, (specifically burst-forming unit erythroid (BFU-E)), megakaryocytes, myeloid progenitors (including neutrophil, bi-potential granulocyte-macrophage [GM-CFC], and mast cell), and multipotent high proliferative potential (HPP-CFC) progenitors that give rise to macrophages, mast cells, basophils, and definitive erythroid progenitors [9,10]. They can also form Emix colonies, containing both erythroid and myeloid cells when cultured in semi-solid media [10]. EMPs contain the cell surface markers CD41 and c-kit at E8.25 and CD16/32 as well by E9.5 [4,10]. Primitive progenitors lack these markers and are contained in the c-kit^{lo}CD41^{lo} population at E8.5 [10]. EMP progenitors begin seeding the fetal liver around E10.5-E11.5 [2,4,10]. In the fetal liver, EMPs attach to macrophages in erythroblastic islands and differentiate [7]. Also here, BFU-E and the more mature colony forming unit-erythroid (CFU-E) progenitors expand exponentially [7]. EMPs provide the first circulating definitive erythrocytes and neutrophils for the embryo at E12 [4,10,11].

EMP-derived definitive erythroid cells differ from primitive erythroid cells in multiple ways. First, definitive erythroid cells are thought to form from lateral plate mesoderm, not from posterior mesoderm like primitive cells [2]. Second, definitive erythroid cells are smaller in size, and contain different globin genes: primitive cells predominantly contain βh1 and εγ-globin (embryonic globins), whereas definitive cells contain βmajor (β1) (adult globin) [2]. βh1 and εγ -globin have a higher affinity for oxygen than βmajor [12]. EMPs do, however, express low levels of βh1-globin in the E11.5 fetal liver [15]. Primitive cells begin to produce small amounts of βmajor at later stages, after E10.5 [2]. Third, primitive erythroid cells do not mature and enucleate until after they enter the blood stream, while definitive erythroid progenitors enucleate and mature in the fetal liver before entering circulation [7]. Primitive and definitive erythroid

cells also differ in their growth factor requirements [2], which will be addressed below (see section Growth and Transcription Factors Involved in Hematopoiesis).

Wave Three: Hematopoietic Stem Cell Emergence

A third wave of hematopoiesis, which culminates in the production of definitive hematopoietic stem cells (HSCs), occurs after yolk sac EMP formation. HSCs are thought to originate predominantly in the aorta-gonad-mesonephros (AGM) region of the embryo but also possibly in the major arteries and placenta, from E10.5-11.5 [2,4]. (It should be noted that some groups hypothesize that definitive HSCs arise earlier, around E7.5-9.5, in the embryonic para-aortic splanchnopleure [2]) HSCs are marked by c-kit, Sca1, and vascular endothelial cadherin (VEC) positivity and are Lineage negative [10,13,14]. Newly formed HSCs travel through the bloodstream and seed the fetal liver. HSCs are capable of forming all hematopoietic lineages, including B and T cell progenitors. HSCs proliferate and differentiate in the fetal liver for the second half of gestation, and then seed the spleen, thymus, and then finally the bone marrow around E18.5, which is where they reside throughout adulthood [2,5]. An ongoing hypothesis in the field is that HSCs only expand in the fetal liver, thymus, spleen, and bone marrow and do not arise anew [5]. It is thought that HSC expansion in the fetal liver is triggered by angiopoietin-like factors and the transcription factor Sox17 [6]. β1 integrins facilitate attachment and colonization of HSCs to the fetal liver [6].

While neither primitive nor EMP hematopoiesis is dependent on circulation for formation, HSC hematopoiesis is, as Ncx1 knockout mouse (which lack a heartbeat) yolk sacs contain normal amounts of erythrocytes and CFU-Cs, but an absence of progenitors in the embryo body [6]. These mice also die by E10.0 [6].

EMP- and HSC-derived definitive erythroid cells are identical in appearance but have some important differences. Most notably, EMPs cannot form lymphoid progenitors nor

repopulate adult or immunocompromised mice long-term, while HSCs can [4,10]. EMPs can, however, provide red blood cell reconstitution in adults transiently [10]. Also, EMPs can expand ex vivo, but HSC-bone marrow-derived erythroblasts can only expand temporarily [4]. Adult bone marrow HSCs produce erythroblasts that only express adult β1- and β2-globins, whereas EMP erythroblasts express adult β1-globin and small amounts of embryonic βh1-globin [4,15]. Even though HSCs begin to form in the embryo only a short time after EMPs, EMP presence is critical for embryo survival. Defective EMP development but presence of HSCs still leads to embryonic death by E15.5 [10]. Likewise, embryos missing HSCs but containing EMPs can survive until birth [4,10]. Interestingly, HSC emergence is dependent on primitive erythroblast and EMP presence. HSC development requires nitric oxide, produced by blood flow throughout the embryo, and oxidative signals, from myeloid cells [9]. Primitive and definitive erythroblasts from the yolk sac provide the blood flow, and EMPs generate the myeloid cells.

Growth and Transcription Factors Involved in Hematopoiesis

GATA1 and Associated Factors that Regulate Erythropoiesis

Both primitive and definitive erythropoiesis relies on GATA1 and EKLF. Both transcription factors incorporate into large protein complexes to control expression of erythroid-specific genes. EKLF/Klf1 promotes erythropoiesis and suppresses megakaryocyte formation [2]. It regulates primitive erythroblast proliferation and activates β globin gene expression [9]. Gata1 targets erythroid progenitors to differentiate into a more mature phenotype, and it activates βmajor globin gene expression [16].

GATA1 associates with FOG-1, CBP/p300, PIASy, and the SWI/SNF and Mediator complexes prior to transcription initiation [17]. FOG-1 (Friend of GATA-1) mediates GATA-1 activities, and it is necessary for GATA-1-dependent erythropoiesis [16]. Loss of FOG-1 results

in anemia and embryonic death midway through gestation [16]. CBP and p300 are acetyltransferases [16]. Aside from interacting with GATA-1, they also interact with p45/NF-E2, EKLF, and others [16]. CBP is involved in HSC self-renewal, while p300 is involved in differentiation [16]. Deletion of CBP in mice causes developmental delay and organ defects, such as impaired vasculogenesis and smaller amounts of primitive and definitive hematopoietic precursors. This impairment results in embryonic lethality [16]. GATA-1 associates with the Mediator complex, which is required for RNA polymerase II recruitment, via its subunit TRAP220 [17]. Deletion of TRAP220 in mice leads to embryonic lethality by E11.5 [17]. These mutant embryos display cardiac, placental, hepatic, and neural developmental defects as wells as defective erythroid and megakaryocyte differentiation [16]. Specifically, TRAP220-deficient E10.5 hematopoietic progenitors do not form BFU-E or CFU-E colonies [16]. A later study using TRAP220-conditional knockout mice found that absence of TRAP220 led to defective adult erythropoiesis as well but normal myelopoiesis and lymphopoiesis [17]. The defective erythropoiesis became apparent at the BFU-E and CFU-E stages, as the number of c-Kit+ and Sca-1+ hematopoietic stem/progenitor cells were equal between knockout and control mice [17]. Transcription factor analysis supported these phenotypic observations; GATA-1, EKLF, and NF-E2 were downregulated in TRAP220 knockout erythroblasts, and GATA-2, c-Kit, and c-Myb (all progenitor-specific genes) were slightly up-regulated in knockout proerythroblasts [17]. TRAP220 was also necessary for β-globin gene expression, as β-globin expression at the proerythroblast/erythroblast stage was absent in the TRAP220 knockouts [17].

GATA1 activity is inhibited by the transcription factor PU.1. PU.1 blocks erythropoiesis and signals hematopoietic progenitors to differentiate into monocytes, granulocytes, and B and T lymphocytes [16]. It does this by blocking Gata1 binding to DNA [16].

Transcription/Growth Factors Important for Definitive Hematopoiesis

Fetal liver erythropoiesis requires RUNX1,C-MYB, and ZBP-89. Primitive erythropoiesis does not depend on these three factors, but RUNX1 does play a role [2]. RUNX1 signals endothelial-to-hematopoietic transition [6]. Absence of RUNX1 leads to failure of EMPs and HSCs to form, and death by E12.5 [4,6]. When C-MYB is ablated, both EMP- and HSC-definitive erythroblasts fail to form, leading to embryonic death by E15.5. Primitive erythropoiesis remains unaffected [4].

Definitive erythropoiesis requires SCL/TAL1, GATA1, FOG-1, LDB1, and LMO2, as loss of any of these factors eliminates it and leads to embryonic death by E10.5 [4,9]. [These factors also regulate primitive erythropoiesis [9]. SCL is needed for HSC specification and formation but not for HSC and multipotent progenitor self-renewal or function [5,16]. In the absence of SCL, red blood cells do not form in mouse yolk sacs [16].

Another important transcription factor is the heterodimeric complex core binding factor (CBF). In its absence, yolk sac erythropoiesis is normal, but definitive progenitors are decreased and even absent, leading to embryonic hemorrhaging and death by E12.5 [18]. It is unknown whether CBF is required for definitive hematopoietic progenitor formation or for proliferation of these progenitors [18].

Definitive erythropoiesis also requires stem cell factor (SCF). Stem cell factor receptor (c-kit) is a proto-oncogene and anti-apoptotic factor that triggers proliferation of hematopoietic progenitors in the presence of other cytokines [18]. SCF and c-kit are needed for the proliferation and differentiation of BFU-Es to CFU-Es [19]. Mice lacking c-kit die of severe anemia due to an absence of definitive erythropoiesis [2]. Primitive erythroid progenitors express c-kit at low levels [10], but exogenous stem cell factor in culture doesn't affect their growth or survival [2]. Of note, definitive erythroid progenitors obtained from yolk sacs and fetal liver can proliferate in

ex vivo culture for several months in the presence of EPO, SCF, and dexamethasone [4]. Primitive erythroblasts are unable to do so, due to very low expression of the glucocorticoid receptor [4].

GATA2 is important for definitive erythroid progenitor proliferation and survival in response to growth [18,16]. Knockout of GATA2 results in severe fetal liver anemia and embryonic death E10.5-11.5 [18]. Primitive erythropoiesis is less affected but still reduced 2-7 fold [18]. Notably, GATA1 represses GATA2 during erythroid cell differentiation [16].

Also necessary for definitive hematopoiesis, specifically HSC production in the AGM region, is the protein NOTCH1. It regulates GATA2 expression, which regulates RUNX1 expression [6]. NOTCH1 also works in conjunction with Wnt signaling to increase HSC self-renewal and activity [5]. NOTCH1 does not seem vital for yolk sac hematopoiesis, however, as NOTCH1 knockout yolk sacs form normal amounts of colony forming units- culture (CFU-Cs). However, few of these form in the embryo body [6]. The H3K4 methyltransferase MLL protein is also necessary for HSC formation in the AGM [5].

The transcription factors SOX6 and BCL11a are also expressed in definitive erythroid cells [9]. SOX6 mediates embryonic and fetal globin gene repression and regulates erythroid maturation [9].

The growth factors VEGF,TFG-β, and FGF signal the somitic mesoderm (which contributes to the dorsal aorta) to form hematopoietic tissue, while TFG-α and EGF suppress hematopoiesis [5].

On the other hand, Transforming Growth Factor β (TGF-β) has been show to inhibit primitive progenitor growth at low concentrations and stimulate growth at higher concentrations, while decreasing definitive progenitor proliferation and inducing their differentiation [2].

Cytokines, Transcription/Growth Factors Important for Hematopoietic Progenitor Self-Renewal

Leukemia inhibitory factor (LIF) is a cytokine that plays a role in hematopoietic stem/progenitor cell self-renewal, as absence of LIF leads to a decrease in progenitors [18]. Interleukin-6 (IL-6) plays a similar role as LIF. Absence of IL-6 leads to a decreased in hematopoietic progenitor numbers as well [18]. IL-6 null HSCs not only lack self-renewal but transition to committed progenitors more rapidly [18].

Sox17 is necessary for fetal and early postnatal HSC self-renewal, but stops being needed 3-4 weeks postnatally as HSCs stop active cycling and enter quiescence [11]. This phenomenon is interesting because a defining feature of most stems cells is quiescence [11]. However, unlike adult, bone marrow mouse HSCs, mouse fetal HSCs actively cycle and expand but cease cycling around 3 weeks postnatally once they reach optimal numbers [11].

HSC expansion in the AGM is initiated by Bmp4, Shh, and the growth factor interleukin 3 (IL-3) [6]. BMPs play a role in maintenance of some other classes of stem cells, such as ESCs, intestinal and dental epithelium, and the hair follicle. Here, BMPs coordinate with Wnt proteins to regulate stem cell maintenance [11].

Erythropoietin Signaling

Erythropoietin (EPO) is a glycoprotein that acts on erythroid progenitors, hindering apoptosis and triggering their proliferation and terminal differentiation into erythrocytes [12,20]. EPO/EPO receptor (EPOR) binding activates JAK2/STAT5, which then activate Bcl-xl, PI3K/AKT, and/or MAPK and NF-κB pathways [21,22]. Importantly, EPO is not necessary for hematopoietic progenitor commitment to erythropoiesis, just growth and maturation [12,20]. EPO alone stimulates mature erythroid cells, whereas in combination with SCF and IL-3 or GM-CSF, it leads to proliferation of immature erythroid precursors [22].

Primitive erythroid cells in the E8.0 yolk sac blood islands express EPOR prior to fetal circulation initiation around E9.0 [20]. However, no EPO is detected in the yolk sac or embryo at that time [20]. Therefore, EPO is not needed for the initial formation or differentiation of primitive erythroid cells [20]. However, survival and expansion of primitive erythrocytes after fetal circulation begins is dependent on EPO [20]. EPOR knockout mice, which cannot respond to EPO, developed normally through E9.5 and have similar numbers of primitive erythrocytes in the embryo proper and yolk sac as wildtype [20,23]. The embryos did not become anemic until E10.5-12.5 [20,23]. At E10.5, EPO and EPOR knockout embryos had reduced numbers of erythrocytes in the yolk sac and embryo [20]. Additional studies found that exogenous EPO increased primitive erythroblast survival and proliferation, and mutation of EPO or EPOR resulted in accelerated maturation and a great decrease in the number of primitive erythroblasts [9].

In contrast to primitive erythropoiesis, the differentiation of definitive erythroid progenitors is dependent on EPO expression/EPOR activation [19,20]. By E11-12 (the time at which the fetal liver becomes the primary site of hematopoiesis), EPO and EPOR knockout mice have 5-10-fold fewer peripheral blood cells [19]. However, the fetal livers from these mice still contain BFU-E and CFU-E progenitors (though smaller and paler). BFU-E and CFU-E presence demonstrates that EPO/EPOR is not necessary for BFU-E proliferation or differentiation to CFU-Es, but is necessary for survival, proliferation, and terminal differentiation of CFU-E progenitors [19,23]. EPO and EPOR knockout mice die by E12.5-15 and display severe anemia, mostly strikingly in the fetal liver, but also in the yolk sac [19,24].

During the yolk sac stage of erythropoiesis, specifically E8.5-E11.5, EPO is produced by neural Epo-producing (NEP) cells within the neural crest and neuroepithelium [12,24]. It has been hypothesized that EPO from NEP cells reaches erythroid progenitors in the yolk sac after

E9.0 via the bloodstream, and then stimulates proliferation and maturation of primitive erythroid cells [24]. Hepatocytes of the fetal liver begin producing EPO around E9.5 [12,24]. At E10.5, the fetal liver produces more EPO than NEP cells [24]. In the adult, EPO is produced in the kidney by renal Epo-producing (REP) cells, which are located in the renal cortex and deep regions of the renal medulla [12]. As REP cell express the neural genes microtubule-associated protein 2 (MAP2) and nerve growth factor receptor (NGFR) and fibroblastic genes, it has been hypothesized that they originate from neural crest cells, specifically, NEP cells that migrate into the kidney [12,24].

DOT1L Background Information

Dot1/Dot1L Origins and Basic Cellular Functions

The Dot1 gene was first identified in a yeast screen for genes influencing telomere silencing [25]. Histone H3, Lysine 79 (H3K79) methylation, the enzymatic function of Dot1, was found to inhibit Sir (silent information regulator) protein binding at telomeres, preventing heterochromatin formation [25]. Dot1 is the only member of class II lysine methyltransferases [26]. In contrast to class I lysine methyltransferases, Dot1 does not contain a SET [Su(var)3-9, Enhancer of Zeste [E(Z)], and Trithorax (trx)] domain [26]. Dot1 belongs to the class I S-adenosylmethionine (SAM)-dependent methylases (i.e., Dot1 receives a donated methyl group from SAM to execute its methylation of H3K79). It mono-, di-, and tri-methylates Lysine 79, which lies within Histone H3's globular domain [26]. Interestingly, Dot1 methylates Histone H3 when it is incorporated into a nucleosome, rather than when it is free or part of a histone core [26,27]. This suggests that DOT1L may interact with other parts of the nucleosome outside of histone H3 Lysine 79 and that its enzymatic activity is regulated by other histone modifications/modifier proteins nearby, which several studies have supported [26]. No other

proteins are known to catalyze H3K79 methylation, as many groups have shown that absence of Dot1 in yeast, flies, and mice leads to loss of H3K79 methylation [3,26]. Also, Dot1 and its homologs (including the mammalian homolog DOT1L) all contain a conserved region found in SAM methyltransferases, which includes the sequence motifs I, post I, II, and III [26].

Dot1 and its homologs are involved in several cellular processes. Genome-wide analysis identified H3K79 methylation, and hence Dot1 activity, as a marker of active transcription in yeast, flies, mice, and humans [26]. Dot1 and DOT1L are also involved in transcriptional elongation, as increased H3K79 methylation correlates with increased transcriptional elongation [26]. In yeast, Dot1 activity is regulated by the Paf1 complex, which associates with RNA polymerase II [26]. In mammals, DOT1L forms elongation complexes with a variety of proteins including AF4, AF5q31, AF9, AF10, ENL, and P-TEFb [26]. These proteins recruit DOT1L to elongating RNA Pol II, where it methylates H3K79 to continue active transcription [26].

Dot1/DOT1L has also been implicated in the DNA damage response, specifically double strand break repair, though its specific role is not understood. Studies found that the yeast Rad9/human 53BP1 protein associated with methylated H3K79 then bound to DNA double strand breaks [26]. Likewise, DOT1L-knockdown in U2OS human osteosarcoma cells exposed to ionizing radiation caused the DNA strand break marker γH2AX to persist for longer periods of time than control [27]. Also, DOT1L-knockdown HEK293 cells and DOT1L-knockout mouse embryonic fibroblasts had low viability following ionizing radiation [27]. DNA damage in yeast utilizes Dot1-mediated H3K79 methylation to activate cell cycle checkpoints, such as the G1/S and pachytene [26]. However, the role of DOT1L in DNA damage-induced cell cycle checkpoint activation is less clear [26].

Dot1/DOT1L also plays a role in cell cycle regulation outside of DNA damage repair, though the H3K79 methylation states in each phase vary among organisms and tissues [26,27].

For example, in murine ESCs, knockdown and knockout of DOT1L led to a G2/M hold upon differentiation [25,26,28]. This cell cycle block led to apoptosis in knockout cells, but no apoptosis in the knockdown model [25,26,28]. In contrast, knockout of DOT1L in mice resulted in a G0/G1 hold in yolk sac erythroid progenitors and increased apoptosis [3,26].

DOT1L's Role in Embryonic Development

Loss of DOT1L and the resulting inability to methylate H3K79 leads to defects in organisms as a whole. Interestingly, H3K79 methylation is absent in *Drosophila* precellular blastoderm and mouse zygotes and does not substantially increase until after gastrulation in flies and after the blastocyst stage in mice [26]. DOT1L is then expressed ubiquitously in mouse embryos during embryonic development [25]. Mice containing *lacZ* under the control of the *Dot1L* promoter stain positive with X-gal ubiquitously from E7.5-E12.5 (earliest-latest time points tested) [25]. Mutations in Grappa (the fly ortholog of Dot1) lead to abnormal anterior-to-posterior parasegmentation and Wnt/Wingless signaling, which regulates wing morphogenesis [26]. Also, Grappa seems to play a role in *Drosophila* lifespan, as a partial loss-of-function mutation causes decreased lifespan [29]. Another species whose development is influenced by DOT1L is the frog species *Xenopus tropicalis*, where DOT1L plays a role metamorphosis, its transformation from tadpole to frog [29].

Knockdown of *Dot1L* in mESCs does not significantly affect proliferation or colony morphology [28]. However, once differentiation is induced with retinoic acid, the *Dot1L* deficient mESCs stop proliferating, while wildtype (WT) mESCs continue [28]. Likewise, although knockdown mESCs were capable of forming embryoid bodies, their morphology was different, and they were smaller in size than WT [28]. After differentiation, knockdown mESCs displayed increased G2/M phase cell cycle arrest as well as hyperploidy and abnormal mitotic spindle formation [28]. Interestingly, the differentiation-induced proliferation cessation was not

due to apoptosis [28]. Likewise, microarray analysis of WT and *Dot1L* deficient mESCs revealed that DOT1L-regulated genes are involved in cell cycle regulation, proliferation, and differentiation [28].

DOT1L is also involved in development of the nervous system. Absence of DOT1L activity via pharmacological inhibition leads to decreased proliferation and apoptosis in E14.5 murine cortical neural stem cells [30]. The observed apoptosis was found to be mediated by elevated transcription of endoplasmic reticulum stress genes upon DOT1L knockdown [30]. Specifically, H3K79 di-methylation was found to be enriched at ER stress genes, and this methylation mark suppresses expression of Atf4 and Ddit3, transcription factors associated with ER stress [30]. Therefore, when DOT1L is chronically inhibited, Atf4/Ddit3 transcription increases, leading to apoptosis via downregulation of BCL2 and activation of Caspase-3 [30].

Furthermore, DOT1L is expressed in developing limbs of mice during chondrogenesis [29]. A specific link for DOT1L in chondrogenesis was identified by a group that showed knockdown of DOT1L in ATDC5 chondrogenic mouse cells caused decreased collagen and proteoglycan content and mineralization, reflecting impaired chondrogenesis [29].

The Role of DOT1L in Murine Hematopoiesis

As discussed above, studies in the Fields laboratory have shown that the H3K79 methyltransferase DOT1L also plays an important role in murine erythropoiesis. Mice containing a truncated DOT1L protein, due to a gene trap insertion mutation within exon 13, displayed erythropoietic defects, leading to a lethal anemia [3]. Other groups have shown similar phenotypes in their DOT1L-mutant mouse models. Mice missing exons 5 and 6 of DOT1L, a region that encodes the catalytic domain of DOT1L, and H3K79 methyltransferase activity, died between E9.5 and E10.5 [25]. The embryos display impaired growth, heart enlargement, and abnormal yolk sac vasculogenesis [25]. ESCs obtained from blastocysts of these mice had

reduced proliferation, increased apoptosis, and G2 cell cycle arrest compared to WT [25]. They also displayed aberrant telomere elongation, aneuploidy, and less compacted chromatin at telomeres and centromeres [25]. Other groups have supported this cardiac defect. Cardiac-specific DOT1L knockout mice displayed dilated cardiomyopathy [26]. This group discovered that H3K79 methylation regulates *dystrophin* gene transcription, and ectopic expression of a functional form of dystrophin rescued the dilated cardiomyopathy phenotype [26]. This DOT1L-dystrophin signaling pathway appears to be present in humans as well, as DOT1L levels are reduced in the heart tissue of patients with idiopathic dilated cardiomyopathy [26].

A mouse model containing *Dot1L* deletion specifically in the hematopoietic compartment has also been developed. These *Dot1L* Vav-Cre mice (promoter is also expressed in endothelial cells [48]) were born at normal weights [31], in contrast to mice with ubiquitous *Dot1L* deletion. However, these mice had decreased white blood cell (WBC), neutrophil (ANC), lymphocyte (ALC), hemoglobin, and hematocrit counts at 3-6 weeks of age compared to littermate controls [31]. Colony forming assays with bone marrow hematopoietic progenitors showed no significant differences between *Dot1L* knockouts compared to controls except a significant decrease in the number of CFU-GM colonies in the knockouts, though not a complete loss [31]. However, *Dot1L* knockout mice contained decreased total bone marrow cellularity, and a moderate-to-severe reduction of LSK, GMP, and CMP progenitors compare to controls. The knockout mice also contained fewer MEP progenitors on average than controls, although the difference was not significant [31].

In order to investigate the role of DOT1L in adult murine hematopoiesis, an inducible knockout model is necessary, as *Dot1L* deletion is embryonic lethal. Two models have been developed. In one, a Cre-ER mouse model, *Dot1L* deletion and the loss of H3K79 di-methylation in adult mice led to bone marrow hypocellularity, pancytopenia, and death 8-12 weeks after

deletion of DOT1L [32]. When the bone marrow of these mice was examined more closely, the numbers of HSC, common lymphoid, megakaryocyte, granulocyte macrophage, and erythroid progenitors were all greatly decreased compared to control mice, with the HSC containing the greatest decrease [32]. In bone marrow transplantation studies, *Dot1l*-deleted HSCs were unable to repopulate recipient mice [32]. Another group used a different ER-Cre mouse model to delete *Dot1L* in adulthood and obtained similar results. In this model, *Dot1L* knockout mice displayed anemia, especially in the liver, and bleeding, especially in the brain, compared to wildtype [33]. The knockout mice also displayed bone marrow hypocellularity and greatly decreased numbers of common myeloid (CMP), granulocyte/monocyte (GMP), and megakaryocyte/erythrocyte (MEP) progenitors as well as terminally differentiated myeloid cells (Gr-1+ and Mac-1+ cells) [33]. Bone marrow transplant studies were also performed: recipient mice that received *Dot1L*-deleted cells contained significantly fewer total bone marrow cells than mice transplanted with wildtype cells [33]. Bone marrow reconstitution competition assays were also done; wildtype and *Dot1L*-deleted bone marrow cells were transplanted simultaneously at a 1:1 ratio into a single mouse. Similarly, wildtype cell percentages were significantly greater than the *Dot1L*-deleted cell percentages in the CMP, GMP, MEP, LSK HSC, long-term reconstituting HSC, terminally differentiated myeloid (Gr-1+/Mac-1+), and lymphoid (B220+/CD27+) populations [33]. Collectively, these studies indicate that DOT1L is not only vital for embryonic hematopoiesis but adult hematopoiesis as well.

DOT1L and MLL Leukemia

An area in which DOT1L has been getting a lot of attention recently is in the cancer field. DOT1L is thought to be involved in the formation and progression of mixed lineage leukemia (MLL)-rearranged leukemia [29]. MLL leukemias make up 5-10% of adult acute leukemias and 60-80% of infant acute leukemias [26]. The MLL protein regulates HOX gene transcription as

well as other developmental genes [34]. The N-terminal part of MLL contains a DNA-binding region, and the C-terminal contains a SET domain with H3K4 methyltransferase activity [34]. MLL leukemia occurs when the N-terminal portion the MLL protein fuses in-frame to a fusion partner (at least 70 or more have been identified), and the C-terminal methyltransferase domain is lost [29,34]. DOT1L is hypothesized to interact with some of these fusion partners, such as AF10, AF4, AF9, and ENL, where it is recruited to target genes, hypermethylates H3K79 on these genes, causing transcriptional activation/elongation, aberrant gene expression, and development of acute myeloid leukemia or acute lymphoblastic leukemia [26,29,34]. Notable target genes include the *HoxA* cluster and *Meis1* [29].

As DOT1L's methyltransferase activity is needed for MLL leukemia [31], drugs inhibiting this enzymatic activity are being developed for clinical treatment. Some examples include EPZ004777, SGC0946, and EPZ-5676, which is currently in clinical trials [29]. All three of these drugs work by competitively inhibiting SAM, which donates the methyl group used by DOT1L for H3K79 methylation [29]. These drugs are similar in structure to SAM but differ slightly from each other. SGC0946 is a brominated version of EPZ004777 [35]. This additional bromine groups allows SGC0946 to fit better into DOT1L's SAM-binding pocket [35]. SGC0946 was found to be more potent than EPZ004777, having a lower K_D and IC_{50} than EPZ004777 [35]. Both compounds are selective, as they were found to be inactive against 12 different protein methyltransferases (PMTs) and DNA methyltransferase I (DNMT1) [35]. As EPZ004777 has poor pharmacokinetic properties that limit its clinical use, EPZ-5676 was investigated [34]. EPZ-5676 is more potent, having a lower K_i and IC_{50}, has greater selectivity against other PMTs, and greater drug-target residence time than EPZ004777 [34]. Studies using these drugs on MLL-rearranged leukemia cells found that they downregulated the expression of MLL fusion target genes, including *HoxA9* and *Meis1* [29,34,35]. These drugs also halted

proliferation, induced G1 cell cycle arrest, and induced apoptosis in MLL-rearranged leukemia cells [27,29,34]. Importantly, proliferation was less affected in non-MLL-rearranged leukemia cell lines [27,29,34], showing that these DOT1L inhibitors are specific for MLL-rearranged leukemia. *In vivo* experiments with EPZ004777 and EPZ-5676 found that the inhibitors increased survival in mice and rats containing xenografts of MLL-rearranged leukemia cells [29,34]. EPZ-5676 shrunk tumors and prevented them from re-growing for over 30 days in rat models [29,34]. Tumors from the inhibitor-treated rats had decreased mRNA levels of *HOXA9* and *MEIS1* [34].

These DOT1L inhibitor drugs may also be therapeutic in other types of cancer. For instance, DOT1L knockdown studies in A549 and NCI-H1299 lung cancer cells resulted in decreased growth, G1 cell cycle arrest, and aneuploidy [29]. Additionally, The DOT1L inhibitors SYC-522 and EPZ004777 have been found to decrease H3K79 di-methylation and inhibit the growth of DNMT3A-mutant AML cells [36]. DOT1L inhibition in DNMT3A-mutant AML lines also led to apoptosis, sub-G1 cell cycle arrest, and differentiation induction, shown by increased expression of CD14, a marker of mature monocytes [36].

The side effect profiles of these drugs continue to be investigated, however, as DOT1L is necessary for normal adult hematopoiesis. For example, one study exposed healthy mice to EPZ004777 for 2 weeks. These mice displayed negative hematopoietic side effects, shown by a mild-to-moderate reduction in hematopoietic progenitor cells [29]. Also, healthy mice treated with the drug had a significant increase in their white blood cell count [29]. However, these mice did not display nearly as severe anemia and pancytopenia as seen in *Dot1L* knockout mice [27,29]. Importantly, rats containing xenografts of the MLL-AF4 leukemia cell line MV4-11 that were treated with EPZ-5676 tolerated all doses and did not experience significant weight loss [34]. This finding indicates that EPZ-5676 may not be too toxic to an organism, at least not in

the short term or at certain doses. More extensive studies are necessary before any definitive conclusions can be made.

Chapter 3 : Methods & Materials

Primers

Methyl Mutation Fwd WT: 5'-AATAACTTTGCCTTTGGTCCT-3'

Methyl Mutation Rev (new): 5'-CTCCACAAGGGACAGCATGT-3'

Methyl Mutation Fwd 3bp: 5'-GCTAACTTTGCTTTTGGACCA-3'

Methyl Mutation Fwd-Tail 3bp: 5'-
GCTAACTTTGCTTTTGGACCAGAGGTGGATCACCAGCTGAA-3'

Methyl Mutation Fwd 4bp: 5'-GCTAACTTCGCTTTTGGACCA-3'

Methyl Mutation Fwd-Tail 4bp: 5'-
GCTAACTTCGCTTTTGGACCAGAGGTGGATCACCAGCTGAA-3'

Methyl Mutation Fwd 5bp: 5'-GCTAATTTCGCTTTTGGACCA-3'

Methyl Mutation Fwd-Tail 5bp: 5'-
GCTAATTTCGCTTTTGGACCAGAGGTGGATCACCAGCTGAA-3'

Methyl Mutation Fwd 6bp: 5'-GCTAATTTCGCTTTCGGACCA-3'

Methyl Mutation Fwd-Tail 6bp: 5'-
GCTAATTTCGCTTTCGGACCAGAGGTGGATCACCAGCTGAA-3'

Cas9 Dot1L MM Repair Template (200bp): 5'-
CCCTCTGGAGAGGGCGGTGGTACGAGGCCTGTGGATGCGTTACCTTCCTTCATGTTT
GCGAATCGCTCCTTCAGCTGGTGATCCACCTCTGGTCCGAAAGCGAAATTAGCCACA
AATATAACACTGCAAAACAATGTTCCAATTCAGTAAAACTGAGGCTGTGCATGGAA
GTCCCAGCCTGGACACAGCCTGCCGCCGGT-3'

MM Short Repair Template (141bp): 5'-
TGTGGATGCGTTACCTTCCTTCATGTTTGCGAATCGCTCCTTCAGCTGGTGATCCACC

TCTGGTCCGAAAGCGAAATTAGCCACAAATATAACACTGCAAAACAATGTTCCAATT

CAGTAAAACTGAGGCTGTGCATGGAA-3’

M13F(-20): 5’-GTAAAACGACGGCCAG-3’

Repair Template Seq Fwd: 5’-TTTGGCTCGGGGCTTGCAGC-3’

Repair Template Seq Rev: 5’-TGCTGGCTGCCTGGGATATG-3’

Dot1L GT Forward: 5’-TGGACACTTACCCAGCACTTCC-3’

Dot1L GT WT Rev: 5’-GAGGGAGATGGCTTTTTGACAGTA-3’

Dot1L GT KO Rev: 5’-TTTGAGCACCAGAGGACATCCG-3’

Methyltransferase Mutation Creation using CRISPR/Cas9

CRISPR/Cas9 Vector

The CRISPR vector PX330 (derived in the Feng Zhang lab) was purchased from Addgene. *Cas9 Dot1L MM Oligo #1 Forward (5’-CACCGTTTGTGAATAACTTTGCCTT-3’)* and *Cas9 Dot1L MM Oligo #1 Reverse (5’-AAACAAGGCAAAGTTATTCACAAAC-3’)* were purchased from Integrated DNA Technologies (IDT; Coralville, Iowa).

Oligo Selection

The guide sequence oligo inserts for the CRISPR vector target the Cas9 protein to cut *Dot1L* Exon 9 about 10bp downstream of the point mutation site (asparagine241). Specific details and requirements involved in CRISPR/Cas9 mechanisms and vector creation were based on previous studies [37]. The following sequence is our area of interest in *Dot1L* Exon9: TTTGTG*AAT*AACTTTGCCTT**TGG**TCCT. The oligo sequence (underlined) is upstream of a PAM site “TGG” (labeled in bold type). Asparagine241 is labeled in italics.

Anneal Oligos

DNA oligos were annealed: Add 1µl of each oligo (100uM) to 1µl of 10X T4 PNK Buffer (NEB), 1µl T4 PNK Enzyme (NEB), and 6µl water. Incubate at 37°C for 30 minutes, 95°C for 5 minutes, ramp temperature down 5°C/minute until 25°C is reached, hold at 25°C for 2 minutes, and hold at 4°C indefinitely.

Cloning

The CRISPR vector was modified to contain a gene encoding enhanced Green Fluorescent Protein (eGFP). The vector was digested with Bbs1 restriction enzyme (New England BioLabs). 50ng of digested CRISPR vector was added to 1µl annealed oligo mix (diluted 1:10 in water after annealing), 5µl Instant Sticky-end Ligase Master Mix (NEB), and water, up to 10µl. 3µl of this mixture was transformed into competent bacteria [Add 50µl bacteria to 3µl of vector/oligo mix and incubate on ice for 30 minutes. Heat shock the bacteria at 42°C for 1 minute and then place on ice for 2 minutes. Next, add 450µl LB medium and incubate at 37°C for 1 hour, shaking at 225rpm. Centrifuge the cells at 6,000xg for 2 minutes, aspirate 350µl LB medium, and plate remaining 150µl cell suspension onto pre-warmed LB agar plates containing 100µg/ml ampicillin antibiotic. Incubate plates at 37°C overnight. Place plates at 4°C in the morning.].

Sequencing CRISPR vector

The cloned CRISPR vector was sequenced to make sure the guide sequence oligos were inserted correctly. Five bacteria colonies were picked from the ampicillin LB agar plates and grown up in 3ml LB medium containing 100µg/ml ampicillin overnight at 37°C, shaking at 225rpm. Plasmid DNA was isolated using the Qiagen QIAprep Spin Miniprep Kit (catalog# 27104). Plasmid DNA concentration was measured using a NanoDrop spectrophotometer. Plasmid DNA was sent to GENEWIZ (South Plainfield, NJ) for Sanger Sequencing. The

sequencing primer (ssDNA oligo purchased from IDT) used was the *hU6 promoter sequence (5'-AAAGGACGAAACACC-3')*. Sequencing data received from GENEWIZ was analyzed using Vector NTI Advance 11 programs ContigExpress, AlignX, and Vector NTI Explorer (Invitrogen).

Repair Template Design

Methyltransferase mutant mESCs were created by introducing a point mutation in the methyltransferase domain of *Dot1L* in a germline-competent mESC line (R1). Exon 9 of *Dot1L*, which is part of the SAM binding pocket, was targeted. The point mutation consisted of a single amino acid change from asparagine241 to alanine (AAT→GCT). A previous study showed that this specific single amino acid substitution changes the conformation of the SAM binding pocket, preventing SAM binding and eliminating DOT1L histone methyltransferase activity [38]. The repair template was transfected along with the GFP-CRISPR vector containing this amino acid change plus 6 additional silent nucleotide changes downstream (one silent mutation in each of the 6 subsequent codons). The additional changes enabled screening for clones possessing the point mutation via polymerase chain reaction (PCR). In order to determine how many silent mutations should be made for accurate PCR screening, we created a series of primers that contained 20 nucleotides complementary to exon 9, located 5 codons downstream of the amino acid change, with 5' tails that contained silent mutations in 3, 4, 5, or 6 of these five codons [Tail primers are labeled *Methyl Mutation Fwd-Tail 3bp/4bp/5bp/6bp*]. PCR reactions were then done using these 4 forward primers and *Methyl Mutation Fwd WT* and a reverse primer matching wildtype mouse genome in exon 9 (*Methyl Mutation Rev (new)*). The same reverse primer was used for all 5 forward primers. Three reactions were done for each primer pair.

<u>25ul PCR Reaction-NEB Taq DNA Polymerase with ThermoPol® Buffer</u>:

10X ThermoPol reaction buffer: 2.5ul

10mM dNTPs: 0.5ul

10uM Fwd primer: 0.5ul

10uM Rev primer: 0.5ul

Taq DNA polymerase: 0.125ul

Wildtype mouse DNA: 2 ul

Water: 18.875ul

- Thermocycler Program: 95°C for 30 seconds, 12 cycles of 95°C for 15 seconds→62°C, -1°C/cycle for 15 seconds→68°C for 20 seconds, 18 cycles of 95°C for 15 seconds→55°C for 15 seconds→68°C for 20 seconds, 68°C for 5 minutes, hold at 4°C

PCR reaction products were purified using the Qiagen "QIAquick PCR Purification Kit" (catalog# 28104). DNA concentrations of purified products were measured using a NanoDrop spectrophotometer. Ran products out on 3% agarose gel in o.5X TAE to make sure products were present and correct size (~450bp). A second PCR reaction was done using the PCR products created above as the template DNA. The purpose of this second reaction was to test how specific the wildtype forward primer was for the wildtype DNA sequence and not the DNA sequences with silent mutations. The primers used in the second reaction were *Methyl Mutation Fwd WT (5'-AATAACTTTGCCTTTGGTCCT-3')* and *Methyl Mutation Rev (new) (5'-CTCCACAAGGGACAGCATGT-3')*. The same 25ul PCR Reaction protocol listed above was used, except annealing temperature was done on a gradient from 65°C-78°C (same temperature for 30 cycles), with 65°C producing the best primer specificity. The wildtype forward primer had the least amount of cross-reactivity with the DNA products containing 6 silent mutations, so we chose to design the methyltransferase mutation repair template to have 6 silent mutations downstream of the single amino acid change. Our repair template consisted of a 200bp (*Cas9*

Dot1L MM Repair Template: 5'-

CCCTCTGGAGAGGGCGGTGGTACGAGGCCTGTGGATGCGTTACCTTCCTTCATGTTTGCG

*AATCGCTCCTTCAGCTGGTGATCCACCTC**TGGTCCGAAAGCGAAATTAGC**CACAAATATA*

ACACTGCAAAACAATGTTCCAATTCAGTAAAACTGAGGCTGTGCATGGAAGTCCCAGCCTG

GACACAGCCTGCCGCCGGT-3') or 141bp (*MM Short Repair Template: 5'-*

*TGTGGATGCGTTACCTTCCTTCATGTTTGCGAATCGCTCCTTCAGCTGGTGATCCACCTC**T***

***GGTCCGAAAGCGAAATTAGC**CACAAATATAACACTGCAAAACAATGTTCCAATTCAGTAAA*

ACTGAGGCTGTGCATGGAA-3') ssDNA oligo purchased from IDT. The 21bp region of *Dot1L* Exon 9 containing the Asn241Ala change and the subsequent 6 silent mutations was located in the middle of the oligos (shown in bold), with remaining 89/60 nucleotides on either side of the 21bp-mutated-region being homologous to the innate *Dot1L* Exon 9 sequence.

CRISPR/Cas9 Transfection of mESCs

R1 mESCs were grown on gelatin-coated 6-well tissue culture plates containing mitotically inactivated (via irradiation: Faxitron RX-650 x-ray cabinet) mouse embryonic fibroblast (MEF) feeder cells. mESCs were grown until the well was ~50% confluent. Cells were trypsinized, and 0.4μg of CRISPR/Cas9 vector and 3μg of repair template were transfected into cells using Lipofectamine 3000 (ThermoFisher, catalog# L3000001) or Effectene Transfection Reagent (Qiagen, catalog# 301425) according to the manufacturer's instructions. Transfected cells were FACS sorted 24-48 hours later for eGFP-positive cells. About 2,000-3,000 eGFP-positive mESCs were plated onto a gelatin-coated 10cm tissue culture dish containing mitotically inactivated MEFs, and individual cells formed clonal colonies. Colonies were isolated with a 10μl pipette (coat 10cm dish in PBS, then just suction up cell colony) and then plated into 1 well of a 96-well plate along with 20μl 0.25% trypsin-EDTA to dissociate cells. After a few minutes, trypsin was de-activated with 100μl ESC medium [For 50ml: 7.5ml Performance Plus FBS

(ThermoFisher #16000044), 0.5ml Sodium Pyruvate (100mM), 0.5ml Penicillin-Streptomycin (10,000U/ml), 0.5ml Glutamine (200mM), 0.5ml Non-Essential Amino Acids (100X), 50µl Leukemia Inhibitor Factor (LIF, 10µg/ml), 43µl MTG (diluted 1:100 in DMEM), 40.4ml High-Glucose DMEM]. Cells were then moved into 1 well of a gelatin- and MEF- coated 96-well tissue culture plate and expanded.

Screening for Dot1L Methyltransferase Mutant mESCs

Each mESC clone was grown in 2 wells of gelatin-coated 48well tissue culture plate until well was overgrown. Cells were dissociated from tissue culture plate with 0.25% trypsin-EDTA. Trypsin-cell mixture was placed in a 1.5ml Eppendorf tube and centrifuged at 20,000xg for 10 minutes. Trypsin was aspirated and cells were washed with 0.5ml PBS and centrifuged again at 20,000xg for 5 minutes. PBS was aspirated, and cell pellets were put at -20°C. DNA was extracted from mESC pellets using the Invitrogen PureLink® Genomic DNA Mini Kit (ThermoFisher, catalog# K182001). Two separate PCR reactions were then done. The first reaction screened the mESC clones for the presence of the Asn241Ala and 6 subsequent silent mutations (shown in bold) and used the primers *Methyl Mutation Fwd 6bp* (*5'-**GCT**AA**T**TT**C**GC**T**TT**C**GG**A**CC**A**-3'*) and *Methyl Mutation Rev (new)* (*5'-CTCCACAAGGGACAGCATGT-3'*). The second reaction screened the mESC clones for the presence of the wildtype sequence of amino acids 241-247 and used the primers *Methyl Mutation Fwd WT* (*5'-AATAACTTTGCCTTTGGTCCT-3'*) and *Methyl Mutation Rev (new)* (*5'-CTCCACAAGGGACAGCATGT-3'*). Both PCR reactions produce bands ~450bp in length. Clones that did not produce bands from either reaction were presumed to be knockout clones that contained insertions or deletions within the 21bp forward primer binding region (amino acids 241-247).

25ul PCR Reaction-NEB Taq DNA Polymerase with ThermoPol® Buffer:

10X ThermoPol reaction buffer: 2.5µl

10mM dNTPs: 0.5µl

10uM Fwd primer: 0.5µl

10uM Rev primer: 0.5µl

Taq DNA polymerase: 0.125µl

ESC clone DNA: 4 µl

Water: 16.875µl

- Thermocycler Program: 95°C for 30 seconds, 30 cycles of 95°C for 15 seconds→59°C for 15 seconds→68°C for 20 seconds, 68°C for 5 minutes, hold at 12°C

mESC Clone Sequencing

Clones that the above PCR reactions showed were positive for containing the methyltransferase mutation and clones that were negative for containing the methyltransferase mutation and negative for a wildtype allele were then sequenced via Sanger Sequencing (GENEWIZ) for confirmation of precise DNA sequence. First, a PCR reaction was done to amplify a 450bp region of *Dot1L* that included the 200bp repair template region. The primers used were *Repair Template Seq Fwd* (*5'-TTTGGCTCGGGGCTTGCAGC-3'*) and *Repair Template Seq Rev* (*5'-TGCTGGCTGCCTGGGATATG-3'*).

50ul PCR Reaction- NEB Phusion® High-Fidelity DNA Polymerase:

5X HF Buffer: 10µl

10mM dNTPs: 1µl

10uM Forward Primer: 2.5µl

10uM Reverse Primer: 2.5µl

Phusion DNA polymerase: 0.5µl

DNA: 3µl

Water: 30µl

- Thermocycler Program: 98°C for 3 minutes, 12 cycles of 98°C for 10 seconds→ 65°C, -1°C/cycle for 15 seconds→ 72°C for 15seconds, 18 cycles of 98°C for 10 seconds→ 55°C for 15 seconds→ 72°C for 15seconds, 72°C for 10 minutes, hold at 12°C

These PCR products (2µl) were cloned into pCR-BluntII-TOPO vector using the Invitrogen Zero Blunt® TOPO® PCR Cloning Kit, with One Shot® TOP10 Chemically Competent E. coli cells (ThermoFisher, catalog# K280020) and according to the manufacturer's recommendations. Transformed bacteria were plated onto pre-warmed LB agar plates containing 50ug/ml kanamycin antibiotic. Subsequently, 10-12 bacteria colonies were picked from LB plates and grown overnight in 3ml LB medium containing 50ug/ml kanamycin at 37°C, shaking at 225rpm. Plasmid DNA was collected from the bacteria the following day with the Qiagen QIAprep Spin Miniprep Kit (catalog# 27104). DNA concentration was measured using a NanoDrop spectrophotometer. Plasmid DNA was sent to GENEWIZ (South Plainfield, NJ) for Sanger Sequencing. The sequencing primer (ssDNA oligo purchased from IDT) used was the *M13 Forward (-20) priming site (5'-GTAAAACGACGGCCAG-3').* Sequencing data received from GENEWIZ was analyzed using the Vector NTI Advance 11 programs ContigExpress, AlignX, and Vector NTI Explorer (Invitrogen).

LB agar plates: Add 8g LB agar (Sigma) to 250ml Milli-Q water in a 500ml Erlenmeyer flask, cover top of flask with foil, and autoclave on "Liquid" setting. Let agar cool until cool-to-the-touch. Add kanamycin at a concentration of 50ug/ml and quickly pour into 10cm Petri dishes

until liquid covers the bottom of the dish. Allow dishes to cool at room temperature for 24 hours then place at 4°C upside-down.

Cell Culture

Reagents

MEF Medium (for 50ml): 39ml High-Glucose Dulbecco's Modified Eagle Medium (DMEM; 4500mg/L glucose), 0.5ml Penicillin-Streptomycin (10,000U/ml), 0.5ml Glutamine (200mM), 10ml Fetal Bovine Serum (FBS), 43ul 1-Thioglycerol (MTG; diluted 1:100 in DMEM)

Freezing Medium: 50% FBS, 40% Cell Medium (varies depending on cell type), 10% Dimethyl sulfoxide (DMSO)

Embryonic Stem Cell Medium (for 50ml): 7.5ml Performance Plus FBS (ThermoFisher #16000044), 0.5ml Sodium Pyruvate (100mM), 0.5ml Penicillin-Streptomycin (10,000U/ml), 0.5ml Glutamine (200mM), 0.5ml Non-Essential Amino Acids (100X), 50ul Leukemia Inhibitor Factor (LIF, 10ug/ml), 43ul MTG (diluted 1:100 in DMEM), 40.4ml High-Glucose DMEM

Pre-Differentiation Medium *(for in vitro hematopoietic differentiation of mESCs*; 50ml): 7.5ml Performance Plus FBS (ThermoFisher, catalog# 16000044), 0.5ml Sodium Pyruvate (100mM), 0.5ml Penicillin-Streptomycin (10,000U/ml), 0.5ml L-Glutamine (200mM), 0.5ml Non-Essential Amino Acids (100X), 50ul Leukemia Inhibitor Factor (LIF, 10ug/ml), 43ul MTG (diluted 1:100 in IMDM), 40.4ml Iscove's Modified Dulbecco's Medium (IMDM)

Primary Differentiation Medium *(for in vitro hematopoietic differentiation of mESCs*; 90ml): 40ml Basic Methylcellulose (Stem Cell Technologies, catalog# 03120), 15ml ES-Cult FBS (Stem Cell Technologies, catalog# 06900), 1ml L-Glutamine (200mM), 0.124ml MTG (diluted 1:100 in IMDM), 0.4ml murine Stem Cell Factor (mSCF, 10ug/ml), 33.476ml IMDM

Hematopoietic Differentiation Medium *(for in vitro hematopoietic differentiation of mESCs*; 100ml): 40ml Basic Methylcellulose (Stem Cell Technologies, catalog# 03120), 15ml ES-Cult FBS (Stem Cell Technologies, catalog# 06900), 1ml L-Glutamine (200mM), 0.124ml MTG (diluted 1:100 in IMDM), 20ml BIT 9500 Serum Substitute (Stem Cell Technologies, catalog# 09500), 1.6ml mSCF (10ug/ml), 0.3ml murine Interleukin-3 (10ug/ml), 0.3ml human Interleukin-6 (10ug/ml), 300U human Erythropoietin, IMDM to final volume of 100ml

mESC Hematopoietic Differentiation Assays- Exon 9 mutant mESCs (of the R1 cell line)

Wildtype (CRISPR/Cas9 transfected), *Dot1L* methyltransferase mutant, and knockout mESC clones were differentiated into hematopoietic colonies in vitro according to the Stem Cell Technologies "In Vitro Hematopoietic Differentiation of mESCs & miPSCs" technical manual, version 3.1.0 [39]. *Dot1L* methyltransferase mutant mESCs contained the single amino acid change Asn241Ala in *Dot1L* exon 9 in both alleles. The *Dot1L* knockouts contained the same amino acid change plus a single nucleotide deletion 22-53 nucleotides downstream of it in both alleles. In short, mutant and wildtype mESCs were grown on gelatin-coated 6 well plates with irradiated DR4 mouse embryonic fibroblasts in Embryonic Stem Cell Medium and then passaged once in Pre-Differentiation Medium about 48 hours prior to differentiation. Next, mESCs were plated into Primary Differentiation Medium at 4 different concentrations: 4000, 8000, 12000, and 16000 cells/ml/35mm dish, where they differentiated into embryoid bodies (EBs). EBs were counted and collected on day 8. EBs were dissociated into a single cell suspension: 0.5ml 0.25% trypsin-EDTA and 0.5ml 0.25% collagenase were added to each EB sample, cells were incubated at 37°C for ~45 minutes, and passaged through a 21g needle mid-way through incubation and at the end. Dissociated cells were then plated into Hematopoietic Differentiation Medium at a concentration of $7.5x10^4$ cells/ml/35mm dish. Primitive Erythroid, Blast Forming Unit-Erythroid (BFU-E, a definitive erythroid cell colony), Myeloid (granulocyte/macrophage

bipotential and individual colonies), and Mixed (multipotential, containing both erythroid and myeloid progenitors) colonies were counted and area measured on day 10.

Dot1L Methyltransferase Mutant Mouse Line

Karyotyping and Blastocyst Injection

R1 mESCs that were confirmed by PCR and Sanger Sequencing to contain the methyltransferase mutation in one allele and wildtype sequence in the second were submitted to the Transgenic and Gene Targeting Institutional Facility (TGIF) at KUMC for karyotyping. One clone that was karyotyped normal was then injected into blastocysts of C57/BL6 females (by TGIF), producing 3 male chimeras. These chimeras were back crossed onto the C57/BL6 background, producing male and female offspring heterozygous for the mutation. Intercrosses of the F1 generation were done to determine if/at what time point the methyltransferase mutation caused embryonic lethality.

Mouse Matings

For colony expansion, 2 females were placed into a cage with 1 male for two weeks. Females were then separated from the male and placed into their own cages until after pups were born and weaned (P21). For timed matings, a heterozygous female was placed with a heterozygous male in the afternoon and allowed to mate overnight. Females were separated from males in the morning when a vaginal plug was seen (1-4 days after pairing with male). As conception is thought to happen at 12am, females were sacrificed, and embryos were collected 10-13 days after separation around 12pm.

Mouse Genotyping

Mice were ear-tagged and ear-punched (ear tags obtained from National Band & Tag Company). Ear punches were digested overnight in a digestion buffer according to a protocol

previously described [40]. In short, ear punches were incubated in 150ul of digestion buffer and 0.4mg proteinase K/ 1ml digestion buffer overnight at 55°C. Digestion buffer consisted of 5mM EDTA pH 8.0, 200mM NaCl, 100mM Tris pH 8.0, 0.2% SDS, in water. The following day, ear punches were vortexed for 15 seconds and then centrifuged for 6 minutes at 12,000rpm to pellet hair. Supernatant was moved to a new 1.5ml Eppendorf tube, and 0.5ml 100% ethanol was added, and solution vortexed. Samples were centrifuged for 10 minutes at 16,000xg to pellet DNA. Supernatant was poured off, and DNA pellets were washed in 0.5ml 70% ethanol. Samples were centrifuged at 16,000xg for 5 minutes. Supernatant was aspirated, and DNA pellets were dried for 5 minutes. DNA pellets were then resuspended in 30-75ul water depending on amount of DNA. Optimal DNA concentration was 50ng/ul. PCR reactions were then done using the primers *Methyl Mutation Fwd 6bp* (5'-GCTAATTTCGCTTTCGGACCA-3') and *Methyl Mutation Rev (new)* (5'-CTCCACAAGGGACAGCATGT-3'), *Methyl Mutation Fwd WT* (5'-AATAACTTTGCCTTTGGTCCT-3') and *Methyl Mutation Rev (new)* (5'-CTCCACAAGGGACAGCATGT-3') to show presence of methyltransferase mutant allele and/or wildtype allele, respectively. Both PCR reactions produce bands ~450bp in length.

<u>25ul PCR Reaction-NEB Taq DNA Polymerase with ThermoPol® Buffer</u>:

10X ThermoPol reaction buffer: 2.5ul

10mM dNTPs: 0.5ul

10uM Fwd primer: 0.5ul

10uM Rev primer: 0.5ul

Taq DNA polymerase: 0.125ul

Ear DNA: 3 ul

Water: 17.875ul

- Thermocycler Program: 95°C for 30 seconds, 30 cycles of 95°C for 15 seconds→59°C for 15 seconds→68°C for 20 seconds, 68°C for 5 minutes, hold at 12°C

Dot1L Gene Trap Mouse Line

Mouse Matings

The *Dot1L* Gene Trap Mouse line used to generate *Dot1L* KO mice is the same as described by Feng et al., 2010 (26). In summary, C57Bl/6 heterozygous mice contain one wildtype allele and the second a gene trap integration within exon 13 of the *Dot1L* gene. For E10.5 timed matings, a heterozygous male and a heterozygous female were placed together in the afternoon and allowed to mate overnight. Females were separated from males in the morning when a vaginal plug was seen (1-4 days after pairing with male). Embryos were collected 10 days after separation around 12pm. For colony expansion, wildtype,C57Bl/6 females were bred with heterozygous males.

Mouse Genotyping

Mice were ear-tagged and ear-punched. Ear punches were digested in 125ul of 50mM NaOH for 10 minutes at 95°C, vortexed, and then mixed with 25ul 1M Tris, pH 8.0. The solution was centrifuged at 12,000rpm for 6 min to pellet undigested tissue. PCR reactions were then done using the primers *Dot1L GT Forward* (5'-TGGACACTTACCCAGCACTTCC-3'), *Dot1L GT WT Rev* (5'-GAGGGAGATGGCTTTTTGACAGTA-3'), *Dot1L GT KO Rev* (5'-TTTGAGCACCAGAGGACATCCG-3')

<u>25ul PCR Reaction-NEB Taq DNA Polymerase with ThermoPol® Buffer</u>:

10X ThermoPol reaction buffer: 2.5ul

10mM dNTPs: 0.5ul

10uM Dot1L GT Fwd, Dot1L GT KO, and Dot1L GT WT Rev primers: 1ul

Taq DNA polymerase: 0.125ul

Ear DNA: 3 ul

Water: 17.875ul

- Thermocycler Program: 95°C for 3 minutes, 35 cycles of 95°C for 30 seconds→61°C for 30 seconds→68°C for 30 seconds, 72°C for 5 minutes, hold at 12°C

Western Blotting

Mouse Embryonic Fibroblast (MEF) Harvesting

E10.5 Methyltransferase Mutant and *Dot1L* GT embryos were split into head and body portions, and the body portion was dissociated in 1ml MEF medium [for 50ml: 39ml High-Glucose Dulbecco's Modified Eagle Medium (DMEM; 4500mg/L glucose), 0.5ml Penicillin-Streptomycin (10,000U/ml), 0.5ml Glutamine (200mM), 10ml Fetal Bovine Serum (FBS), 43µl 1-Thioglycerol (MTG; diluted 1:100 in DMEM)] by pipetting several times. Cells were plated into gelatin-coated 12 well plates. Supernatant was discarded the following day, and adherent cells were expanded. Cells were trypsinized and histone and whole protein extracted for western blotting.

Histone Extraction for Western Blotting of H3K79-me2 and H3

Histones were collected from mESCs and E10.5 MEFs (harvested from *Dot1L* MM or KO mice) according to Abcam's "Histone Extraction Protocol." In summary, cells were tryspinized off of tissue culture plates and washed twice with PBS. Cells were resuspended in Triton Extraction Buffer [PBS with 0.5% Triton X-100, 2mM phenylmethylsulfonyl fluoride (PMSF), 0.02% NaN3, and protease inhibitor cocktail (Roche)] at a density of $1x10^7$cells/ml. Cells were then lysed on ice for 10 minutes, vortexed every 2-3 minutes. Cells were centrifuged

for 10 minutes at 2000rpm and 4°C then washed in half the volume of TEB as before. Cells were spun down again, resuspended in 0.2N HCl at a density of $4x10^7$cells/ml, and histones were acid extracted overnight at 4°C. Cells were centrifuged at 2000rpm for 10 minutes at 4°C, and supernatant (containing histones) was collected and stored at -80°C. Protein concentration was measured using the Bradford Assay.

Whole Cell Protein Extraction for Western Blotting of DOT1L and GAPDH

Whole cell protein was collected from mESCs and E10.5 MEFs (harvested from *Dot1L* MM or KO mice) according to Abcam's "Sample Preparation for Western Blot" protocol. In summary, cells were tryspinized off of tissue culture plates and washed twice with PBS. Cells were resuspended in RIPA buffer [150mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50mM Tris-pH 8.0, 1mM PMSF, and protease inhibitor cocktail (Roche)] at a density of $4x10^7$cells/ml. Cells were lysed for 45 minutes at 4°C, under constant agitation. Cell lysate was centrifuged for 20 minutes at 12,000 rpm at 4°C. Supernatant (containing protein) was collected and stored at -80°C. Protein concentration was measured using the Bradford Assay.

H3 and H3K79-me2 Expression Analysis

Fish gelatin Blocking Buffer (2X): For 500ml: 10ml 10X PBS, 2.5ml Fish skin gelatin, 0.5g Casein hydrolysate amino acid (MP biomedicals catalog# 101290), 2ml 10% Sodium Azide, bring volume up to 500ml with Milli-Q water

TBS 10X: Add 24.23g Trizma HCl to 80.06g NaCl. Mix in 800ml ultra pure water and pH to 7.6 with HCl. Bring volume to 1L.

TBST: Add 100ml 10X TBS to 900ml ultra pure water and add 1ml Tween20.

Tris-Glycine Buffer (Running Buffer): 25mM Tris base, 190mM glycine, 0.1% SDS

Transfer Buffer: 25mM Tris base, 190mM glycine, 0.1% SDS, 20% methanol

Histone samples were Western Blotted for H3K79 di-methylation and H3. 2µg protein/sample was diluted in 2x Laemmli Sample Buffer (Bio-Rad, catalog# 1610737) and loaded into Bio-Rad 12% Mini-PROTEAN® TGX™ Precast Protein Gels, 10-well, 30 µl (catalog# 4561043). Gels were run in a Bio-Rad Mini-PROTEAN® Tetra Vertical Electrophoresis Cell for Mini Precast Gels (catalog# 1658004) in Tris-Glycine Buffer at 100V until bands were reached the end of the gel. Gels were then transferred to PVDF membrane (pre-soaked in methanol) in Transfer Buffer at 30V for 1 hour using an Invitrogen XCell II™ Blot Module (ThermoFisher, catalog# EI9051) and according to manufacturer's instructions. PVDF membranes were blocked in Fish gelatin Blocking Buffer overnight, shaking at 4°C. The next morning, membranes were washed in TBST 3 times, for 10 minutes, shaking at room temperature. Membranes were blocked in rabbit polyclonal Histone H3 (di methyl K79) primary antibody (Abcam, catalog# ab3594) at a dilution of 1:1000 in TBST or in Histone H3 (D1H2) XP® rabbit monoclonal antibody (Cell Signaling Technology, catalog# 4499) at a dilution of 1:500 in TBST for 1 hour, shaking at room temperature. Membranes were washed in TBST 3 times, for 10 minutes, shaking at room temperature. Membranes were blocked in Goat Anti-Rabbit HRP-Conjugated secondary antibody (Pierce, catalog# 1858415) at a dilution of 1:5000 in TBST for 1 hour, shaking at room temperature. Membranes were blocked in ECL Western Blotting Substrate (Promega, catalog# W1001) for 1 minute, shaking at room temperature, and bands were visualized on autoradiography film (GeneMate, catalog# F-9023-5X7). Exposure time ranged from 30 seconds to 45 minutes depending on age of primary antibodies and ECL substrate.

DOT1L Expression Analysis

Fish gelatin Blocking Buffer (2X): For 500ml: 10ml 10X PBS, 2.5ml Fish skin gelatin, 0.5g Casein hydrolysate amino acid (MP biomedicals catalog# 101290), 2ml 10% Sodium Azide, bring volume up to 500ml with Milli-Q water

TBS 10X: Add 24.23g Trizma HCl to 80.06g NaCl. Mix in 800ml ultra pure water and pH to 7.6 with HCl. Bring volume to 1L.

TBST: Add 100ml 10X TBS to 900ml ultra pure water and add 1ml Tween20.

Tris-Glycine Buffer (Running Buffer): 25mM Tris base, 190mM glycine, 0.1% SDS

Transfer Buffer: 25mM Tris base, 190mM glycine, 0.1% SDS

Whole protein cell lysates were Western Blotted for DOT1L. 50µg protein/sample was diluted in 2x Laemmli Sample Buffer (Bio-Rad, catalog# 1610737) and loaded into Bio-Rad 4-15% Mini-PROTEAN® TGX™ Precast Protein Gels, 10-well, 50 µl (catalog# 4561084). Gels were run in a Bio-Rad Mini-PROTEAN® Tetra Vertical Electrophoresis Cell for Mini Precast Gels (catalog# 1658004) in Tris-Glycine Buffer at 50V for 2 hours, then 100V for 15 minutes until bands were reached the end of the gel. Gels were then transferred to PVDF membrane (pre-soaked in methanol) in Transfer Buffer at 30V for 1.5 hours using an Invitrogen XCell II™ Blot Module (ThermoFisher, catalog# EI9051) and according to manufacturer's instructions. PVDF membranes to be stained with DOT1L primary antibody from Novus were blocked in Fish gelatin Blocking Buffer overnight, shaking at 4°C. PVDF membranes to be stained with DOT1L primary antibody from Abcam were blocked in 1% milk in TBST overnight, shaking at 4°C. The next morning, membranes were washed in TBST 3 times, for 10 minutes, shaking at room temperature. Membranes were blocked in rabbit polyclonal KMT4/Dot1L primary antibody (Abcam, catalog# ab64077) at a dilution of 1:200 in TBST or rabbit polyclonal Dot1L primary

antibody (Novus Biologicals, catalog#NB100-40845) at a dilution of 1:500 in TBST for 1.5 hours, shaking at room temperature. Membranes were washed in TBST 3 times, for 10 minutes, shaking at room temperature. Membranes were blocked in Goat Anti-Rabbit HRP-Conjugated secondary antibody (Pierce, catalog# 1858415) at a dilution of 1:5000 in TBST for 1 hour, shaking at room temperature. Membranes were washed in TBST 1 time, for 10 minutes, shaking at room temperature. Membranes were blocked in ECL Western Blotting Substrate (Promega, catalog# W1001) for 1 minute, shaking at room temperature, and bands were visualized on autoradiography film (GeneMate, catalog# F-9023-5X7). Exposure time ranged from 10-45 minutes depending on age of primary antibodies and ECL substrate.

GAPDH Expression Analysis

Fish gelatin Blocking Buffer (2X): For 500ml: 10ml 10X PBS, 2.5ml Fish skin gelatin, 0.5g Casein hydrolysate amino acid (MP biomedicals catalog# 101290), 2ml 10% Sodium Azide, bring volume up to 500ml with Milli-Q water

TBS 10X: Add 24.23g Trizma HCl to 80.06g NaCl. Mix in 800ml ultra pure water and pH to 7.6 with HCl. Bring volume to 1L.

TBST: Add 100ml 10X TBS to 900ml ultra pure water and add 1ml Tween20.

Tris-Glycine Buffer (Running Buffer): 25mM Tris base, 190mM glycine, 0.1% SDS

Transfer Buffer: 25mM Tris base, 190mM glycine, 0.1% SDS, 20% methanol

Whole protein cell lysates were Western Blotted for GAPDH. 50µg protein/sample was diluted in 2x Laemmli Sample Buffer (Bio-Rad, catalog# 1610737) and loaded into Bio-Rad 12% Mini-PROTEAN® TGX™ Precast Protein Gels, 10-well, 30 µl (catalog# 4561043). Gels were run in a Bio-Rad Mini-PROTEAN® Tetra Vertical Electrophoresis Cell for Mini Precast Gels (catalog# 1658004) in Tris-Glycine Buffer at 50V for 2 hours, then 100V for 15 minutes until bands were reached the end of the gel. Gels were then transferred to PVDF membrane (pre-

soaked in methanol) in Transfer Buffer at 30V for 1.5 hours using an Invitrogen XCell II™ Blot Module (ThermoFisher, catalog# EI9051) and according to manufacturer's instructions. PVDF membranes blocked in Fish gelatin Blocking Buffer overnight, shaking at 4°C. The next morning, membranes were washed in TBST 3 times, for 10 minutes, shaking at room temperature. Membranes were blocked in Anti-GAPDH primary antibody (Abcam, catalog#ab9485) at a dilution of 1:1000 in TBST for 1.5 hours, shaking at room temperature. Membranes were washed in TBST 3 times, for 10 minutes, shaking at room temperature. Membranes were blocked in Goat Anti-Rabbit HRP-Conjugated secondary antibody (Pierce, catalog# 1858415) at a dilution of 1:5000 in TBST for 1 hour, shaking at room temperature. Membranes were washed in TBST 1 time, for 10 minutes, shaking at room temperature. Membranes were blocked in ECL Western Blotting Substrate (Promega, catalog# W1001) for 1 minute, shaking at room temperature, and bands were visualized on autoradiography film (GeneMate, catalog# F-9023-5X7). Exposure time ranged from 10-45 minutes depending on age of primary antibodies and ECL substrate.

Embryo Experiments

Yolk Sac Digestion

Yolk sacs and their corresponding embryo tissue were obtained from E10.5 timed matings between Dot1L Methyltransferase Mutant and Dot1L Gene Trap heterozygous mice. Yolk sacs were digested in 0.5ml digestion buffer [20% FBS, 10% Collagenase (0.25% obtained from Stem Cell Technologies, catalog# 07902), and 70% PBS] for 30 minutes at 37°C. Yolk sacs were then aspirated through 27-gauge needles to obtain a single cell suspension. Cell were washed in 3.5ml IMDM with 5% FBS and filtered through 70μm nylon mesh (Celltreat). Yolk sac cells were then centrifuged at 300xg for 7 minutes. Supernatant was discarded and cells were

resuspended in either 3ml IMDM with 2% FBS or IMDM alone depending on the following analysis and centrifuged at 300xg for 7 minutes. DNA was extracted from corresponding embryo tissue and used for genotyping.

Embryo Genotyping

Embryo tissue was dissociated by pipetting several times in PBS. Dissociated tissue was spun down at 16,000xg for 10 minutes, and supernatant was discarded. Tissue was resuspended in 300ul digestion buffer and 0.4mg proteinase K/ 1ml according to previously described protocol [40] and digested for at least 1 hour at 55°C. Tissue was vortexed and pipetted several times, and undigested tissue was removed. 1ml 100% ethanol was added, and the solution was vortexed. Samples were centrifuged for 10 minutes at 16,000xg to pellet DNA. Supernatant was poured off, and DNA pellets were washed in 1ml 70% ethanol. Samples were centrifuged at 16,000xg for 5 minutes. Supernatant was aspirated, and DNA pellets were dried for 5 minutes. DNA pellets were then resuspended in 30-75ul water depending on amount of DNA. DNA concentration was measured on a Nanodrop spectrophotometer and concentration was adjusted to about 50ng/ul. PCR reactions were then done as described above for the Methyltransferase Mutant and *Dot1L* Gene Trap Mouse lines.

E10.5 YS Hematopoietic Differentiation Assays- Definitive Erythroid, Myeloid, and Mixed Colony Formation Analysis

Digested E10.5 yolk sacs were resuspended in 175ul IMDM with 2% FBS and counted on a hemocytometer using Trypan Blue exclusion. Cell concentrations were brought to $2.5x10^5$ cells/ml in IMDM with 2% FBS. Cells were diluted 1:10 into M3434 (for erythroid, myeloid, mixed progenitor analysis) or M3334 (for mature BFU-E and CFU-E analysis) methylcellulose medium (obtained from Stem Cell Technologies). 1ml of medium containing $2.5x10^4$ cells was plated into a 35mm dish. 35mm dishes were placed into a 15cm dish along with an uncovered

35mm dish containing 3ml water. Cells were incubated at 37°C, and colonies were identified and counted on day 7, and colony area was measured on day 10. Pictures of colonies were taken Olympus IX71 inverted fluorescent microscope with camera, at 4X and 10X magnification, on bright field setting. Colony area was measured in pixels using ImageJ software (National Institutes of Health) and converted to μM in Microsoft Excel using a scatter plot and linear regression analysis.

Extensively Self-Renewing Erythroblasts Assays

Digested E10.5 yolk sacs were washed in IMDM alone, resuspended in 0.5ml Expansion media for ESRE (extensively self-renewing erythroblasts) according to England et al., 2011 [41], and plated into gelatin-coated 24 well plates. Expansion medium consisted of StemPro34 (Gibco/BRL) expansion media supplemented with nutrient supplement (Gibco/BRL), 2 U/ml human recombinant EPO (University of Kansas Hospital Pharmacy), 100 ng/ml SCF (PeproTech), 10^{-6} M dexamethasone (Sigma), 40 ng/ml insulin-like growth factor-1 (Pepro Tech) and penicillin-streptomycin (Invitrogen). After 1 day of culture, the nonadherent cells were aspirated, spun down, resuspended in fresh ESRE medium, and transferred to a new gelatin coated well. After 3 days in culture, cells were counted on a hemocytometer using trypan blue exclusion. Day 3 after isolation is day 0 of expansion culture. Cells were then spun down, resuspended in fresh ESRE medium, and plated into a new gelatin-coated well. Cells were counted every other day, resuspended in fresh medium, and plated into 24 well plates. Cells were diluted via partial medium changes to keep concentration at ≤ 2 x106 cells/ml. Fold expansion was calculated relative to day 0 (day 3 after isolation).

For studies using the DOT1L inhibitor drug SGC0946, equal numbers of day 2 (day 5 after isolation) wildtype ESREs (from either the Methyltransferase Mutant or *Dot1L* GT mouse line) from the same yolk sac were plated into 2 wells of a 12 or 6 well plate. One well contained

ESRE medium, and the other contained ESRE medium with 250-400nM SGC0946. Cells were counted every other day, resuspended in fresh medium with and without SGC0946, and plated into 12 or 6 well plates. Cell concentration was kept at ≤ 2 x106 cells/ml. Fold expansion was calculated relative to day 0 of inhibitor treatment.

DNA Damage Analysis- Comet Assays

Alkaline Comet Assays were performed on E10.5 yolk sac ESREs using the Trevigen CometAssay® Kit (catalog# 4250-050-K) according to manufacturer's recommendations. DNA was stained with SYBR Gold (ThermoFisher) and photographed with an Olympus IX71 inverted fluorescent microscope with camera, at 10X magnification, with a FITC (fluorescein isothiocyanate) fluorochrome. Comet tails (%DNA in Tail) were measured using CometScore software (TriTek Corp.).

Apoptosis Analysis- AnnexinV Staining

E10.5 yolk sac ESREs and erythroid/myeloid/mixed hematopoietic progenitors (obtained from E10.5 yolk sac hematopoietic differentiation assays using M3434 methylcellulose medium) were analyzed for apoptosis. 0.5-1x10^5 cells were stained with Annexin V-FITC and propidium iodide using the BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I, according to the manufacturer's recommendations, and analyzed on a BD LSRII Flow Cytometer. Percentage of apoptotic cells was determined by calculating the number of Annexin V-positive cells.

Cell cycle analysis- Propidium Iodide Staining

E10.5 yolk sac ESREs and erythroid/myeloid/mixed hematopoietic progenitors (obtained from E10.5 yolk sac hematopoietic differentiation assays using M3434 methylcellulose medium) were analyzed for cell cycle progression. 0.5-2x10^5 cells were washed in PBS and then resupsended in 250µl 0.9% NaCl. Cells were vortexed at low speed, and ice cold 90% ethanol (625µl) was added dropwise. Cells were placed at 4°C for 1-30 days for fixation. For cell cycle

analysis, cells fixed cells were spun down and resuspended in 50ug/ml propidium iodide in PBS (400ul) and 100ug/ml RNase A. Cells were incubated at 37°C for 30 minutes and analyzed on a BD LSRII Flow Cytometer.

Chapter 4 : DOT1L methyltransferase activity is essential for *ex vivo* hematopoiesis in a murine embryonic stem cell (mESC)-based culture system

Introduction

The Fields laboratory initially described the phenomenon of defective blood development using hematopoietic progenitors isolated from the yolk sacs (YS) of E10.5 embryos [3] The YS is considered a major site of embryonic hematopoiesis, and the majority of hematopoietic progenitors reside in this anatomic location at E10.5. Even though that is the case, the Fields laboratory has found that it is only possible to isolate ~500 of these progenitors from a single YS. This severe limitation in cell number represents a major obstacle in the ability to conduct detailed mechanistic studies. In order to begin to decipher the molecular mechanisms by which DOT1L regulates hematopoiesis, a system other than *ex vivo* culture of yolk sac (YS) cells is needed. Hence, we sought to develop a model where a large number of hematopoietic progenitor cells can be generated, *in vitro*.

Murine Embryonic Stem Cells (mESC) are an excellent tool in which to study blood development *in vitro*, as they are easily expandable and manipulable genetically. One example of how mESCs can be genetically altered is by use of the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated) system. This system evolved in bacteria and archaea as a defense system against invading viruses and plasmids [42]. It involves short RNAs that contain matching sequences to the invading nucleotides in complex with Cas proteins that cleave the invading nucleic acids [42]. In the case of genome editing, the guide CRISPR RNA is in complex with the Cas9 enzyme, which enables the targeting of the enzyme to the genome at a specific site (defined by a consensus sequence of $GN_{20}GG$). At this site, the enzyme creates a double strand break in the DNA usually about 3bp upstream of the PAM (protospacer-adjacent motif) sequence -NGG [37]. Insertions and deletions are incorporated at

the cut site via Non-Homologous End Joining [37,43]. Since these insertions and deletions are random, disruption of the coding sequence of the targeted area is possible, generating a nonfunctional (or degraded) mRNA. The CRISPR/Cas9 system can also be utilized to introduce a point mutation in the genome if the CRISPR/Cas9 vector is transfected into cells along with a single stranded DNA oligonucleotide repair template. Cas9 creates a double-stranded break, and the repair template is integrated into the cut site via homologous recombination [37,43]. This technique allows one to seamlessly introduce a point mutation (or several nucleotide substitutions) into any region of the genome.

Embryonic Stem Cells are also useful for developmental studies, since they can be cultured *in vitro* to differentiate into hematopoietic progenitors, as demonstrated in numerous studies [10,44,45]. These studies found that mESC differentiation events parallel those found in the embryo, including similar gene expression [44,45]. Most importantly, in terms of hematopoiesis, ESCs form the different blood cell lineages in a similar manner (both temporally and in terms of growth factor responsiveness and cell surface markers) to those developing in the murine yolk sac [10,44].

In this study, mESCs were used to address two hypotheses. The first is that *Dot1L* knockout mESCs induced to undergo hematopoiesis would produce similar hematopoietic defects as those observed *in vivo* in *Dot1L* knockout mice. The second is that the enzymatic function of DOT1L, H3K79 methylation, is dispensable for blood development, and may be involved in separable functions in normal cells. Despite the considerable size of DOT1L (1540aa), only its methyltransferase activity has been documented. Its involvement in several, diverse processes ranging from embryonic development, to transcriptional activation to leukemogenesis suggests that additional domains of the protein may be responsible for its activities. In order to test these hypotheses, mESCs were targeted by the CRISPR/Cas9 system

to introduce an out-of-frame deletion in *Dot1L*, thus creating a stop codon and truncated DOT1L protein. In addition, a cell containing a point mutation in the methyltransferase domain of *Dot1L* was generated. This point mutation eliminates DOT1L methyltransferase activity but preserves the remainder of the protein. *In vitro* hematopoietic differentiation assays with these mutant mESCs showed similar blood development defects to those seen in the *Dot1L KO* mouse embryos. These results indicate that mESCs represent an effective model with which to study early mammalian hematopoiesis *in vitro*. They also indicate that the methyltransferase activity of DOT1L plays a predominant role in the activity of the protein as a whole, and is responsible for its function in facilitating early, murine hematopoiesis.

Methods & Materials

(See Chapter 3-Methods & Materials sections "Methyltransferase Mutation Creation using CRISPR/Cas9," "Cell Culture," and "Western Blotting: Histone Extraction and H3 and H3K79-me2 Expression")

Results

Mutant mESC Generation via CRISPR/Cas9

Using the CRISPR/Cas9 system, two different mutations in *Dot1L* in cultured mESCs were created: a *Dot1L* point mutant and *Dot1L* knockout. The *Dot1L* point mutation resulted in a single amino acid change from asparagine to alanine at amino acid 241, which lies within exon 9 and the methyltransferase domain of *Dot1L*. This point mutation was shown by another group to eliminate Histone H3 methylation without changing the protein's structure [38]. In addition to the single amino acid change, 6 single nucleotide, silent mutations were induced in the six codons immediately downstream of aa241 to enable clone screening via polymerase chain

reaction. These mutations were induced in both alleles. In effect, this point mutation eliminates DOT1L methyltransferase activity but preserves the rest of the protein. The *Dot1L* knockouts (2 clones) contained the same amino acid change and 6 silent mutations as the *Dot1L* point mutant as well as a single nucleotide deletion 22 and 53 nucleotides downstream of the amino acid change, in both alleles.This single nucleotide deletion created a stop codon in both mutants 12aa and 19aa, respectively, downstream of the point mutation. The DNA sequences of the point mutant and knockout mESC clones were verified by Sanger sequencing. A schematic of these *Dot1L* mutant mESC clones is shown in Figure 4.1.

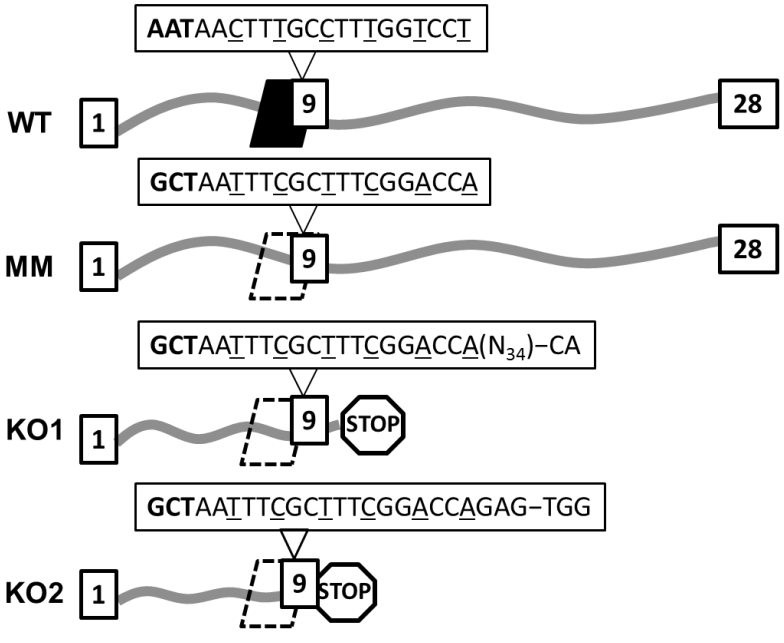

Figure 4.1: Wildtype (WT), Methyltransferase Mutant (MM), and Knockout (KO) mESC clones.
Murine ESCs were targeted with CRISPR/Cas9 to induce a point mutation Asp241Ala within exon 9 of Dot1L. The MM clone contains this point mutation (in bold) in addition to 6 silent nucleotide changes (underlined). KO1 and KO2 clones contain the point mutation and silent nucleotide changes as well as a single nucleotide deletion 53bp and 22bp downstream of the point mutation, respectively. These single nucleotide deletions create a stop codon shortly thereafter.

Histones were extracted from wildtype, methyltransferase mutant, and knockout mESC clones, and Western blot analysis was performed to assess DOT1L methyltransferase activity. Specifically, histones were labeled with antibodies against histone H3 and H3K79 di-

methylation. The methyltransferase mutant and both knockout clones showed near absent H3K79 methylation relative to histone H3 and wildtype, as seen in Figure 4.2. These results show that the Asp241Ala point mutation did in fact eliminate DOT1L's histone methyltransferase activity.

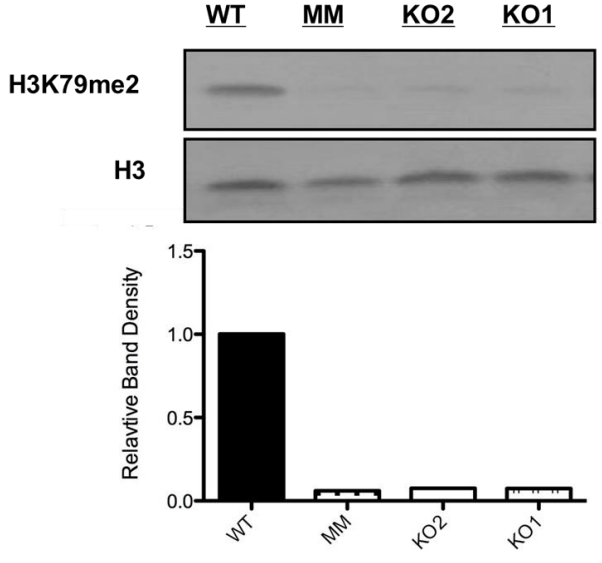

Figure 4.2: Dot1L MM & KO clones have near absent H3K79 di-methylation.

Histones were extracted from wildtype (WT), methyltransferase mutant (MM), and knockout (KO) mESC clones, and western blots were performed to probe for H3K79 di-methylation (H3K79me2) and histone H3. H3K79 di-methylation band intensity is shown relative to histone H3 and WT.

Dot1L Mutation has varying effects on Embryoid Body Formation

In order to differentiate mESCs into hematopoietic cells, mESCs are grown on tissue culture plates, then passaged into a primary methylcellulose-based medium containing murine Stem Cell Factor (mSCF), where they differentiate into pluripotent, three dimensional structures called embryoid bodies (EBs) [39,44]. EBs are then dissociated into a single cell suspension via a collagenase/trypsin mixture and re-plated into a secondary methylcellulose-based medium containing cytokines that promote myeloid and erythroid differentiation (Interleukin (IL)-3, IL-6, and erythropoietin (EPO)) [39,44].

Methyltransferase mutant, both knockout, and WT mESC clones were grown on mouse embryonic fibroblasts to prevent differentiation and then plated into the primary methylcellulose medium containing mSCF at 3 different concentrations: 4000, 8000, and 12000 cells/35mm dish, where they differentiated into embryoid bodies (EB). Embryoid bodies were counted on day 8.

Wildtype and methyltransferase mutant mESC clones formed similar numbers of EBs at all 3 concentrations of mESCs plated. The knockout1 clone formed fewer EBs on average at all 3 concentrations than wildtype and methyltransferase mutant clones; however the difference was not statistically significant. Interestingly, the knockout2 clone grew hardly any EBs, at any cell concentration, and the difference relative to wildtype mESCs was noticeably significant (Figure 4.3A). This growth deficiency only became apparent at the EB stage, as undifferentiated knockout2 mESCs had similar morphology and growth rates as the knockout1 mESCs.

Phenotypically, wildtype, methyltransferase mutant, and knockout1 EBs resembled each other in size and shape. However, knockout2 EBs were not only fewer in number relative to the other 3 mESC clones, but they were extraordinarily small, as can been seen in Figure 4.3B.

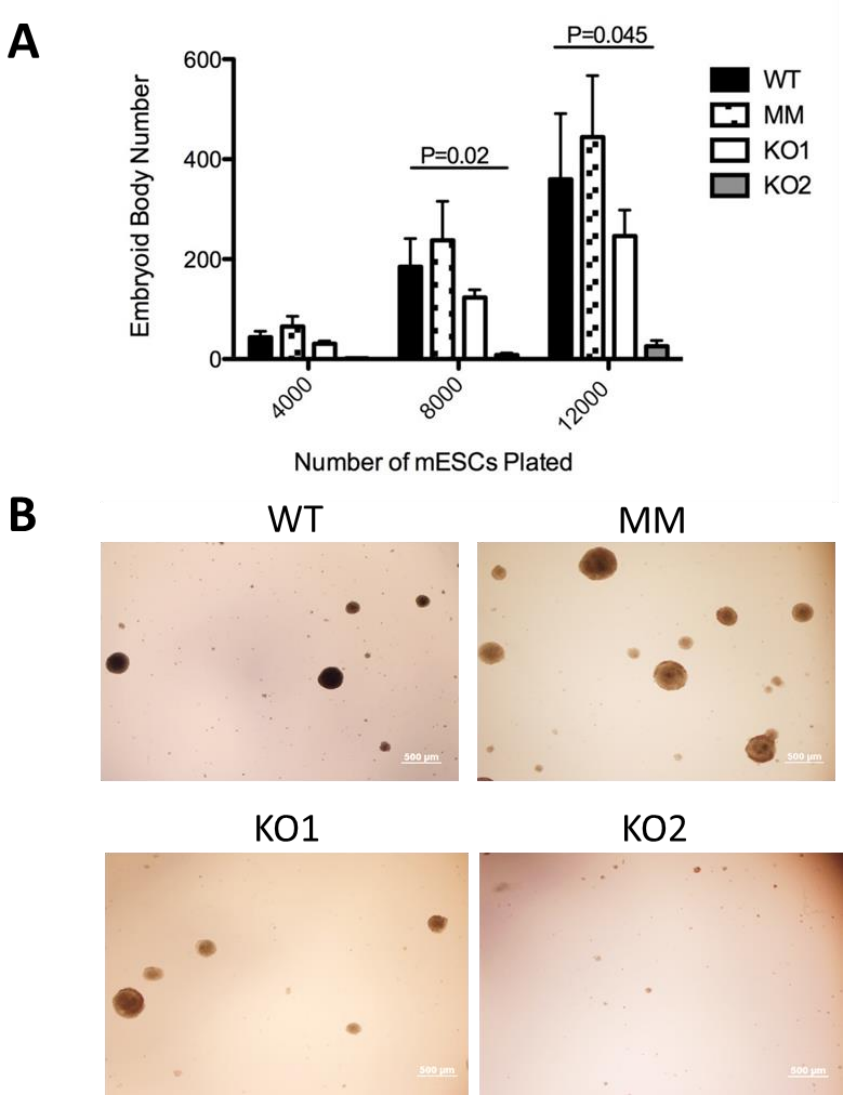

Figure 4.3: WT, *Dot1L* MM and KO mESCs form Embryoid Bodies to varying degrees.

Wildtype (WT), methyltransferase mutant (MM), and knockout (KO) mESC clones were cultured in methylcellulose medium containing mSCF for 8 days, where they differentiated into Embryoid Bodies (EB). (A) EB numbers were counted on day 8. KO2 mESCs formed fewer EBs than WT, MM, and KO1 clones. No significant differences were found between WT, MM, and KO1 clones' ability to form EBs. (B) Representative pictures of EBs derived from each mESC clone are shown.

Dot1L Mutants display impaired hematopoiesis in vitro

Dot1L knockout1, methyltransferase mutant, and wildtype EBs (day 8) were dissociated into a single cell suspension and plated into a secondary methylcellulose medium. This medium contained cytokines and growth factors that promote erythroid and myeloid hematopoietic differentiation. Day 8 EB cells have been shown to be ideal for formation of Blast Forming Unit-Erythroid (BFU-E, a definitive erythroid cell colony), myeloid colonies (both granulocyte and macrophage progenitors), and mixed colonies (multipotential colony containing both erythroid and myeloid progenitors) [39,44]. They also have been shown to form immunophenotypic erythro-myeloid progenitors (EMPs) and at peak levels along with definitive erythroid progenitors in some ES cell lines [10]. As EMPs and definitive erythropoiesis emerge in the yolk sac between E8.25-E11, day 8 embryoid bodies are a good *in vitro* representation of the E10.5 yolk sac hematopoietic differentiation assays used to study *Dot1L* knockout mouse embryos [3]. Primitive erythroid colonies (EryP) can also form from day 8 embryoid bodies, but they are not as predominant. Primitive erythropoiesis is better measured from day 6 embryoid bodies [10]. However, primitive erythroid colonies were noted if present in these cultures. Insufficient numbers of knockout2 EBs grew to be plated into the hematopoietic differentiation cultures.

The data showed that this protocol leads to similar erythropoietic defects as those observed *in vivo*. *Dot1L* knockout EBs formed fewer primitive erythroid and BFU-E colonies, on average, than wildtype. The difference was not statistically significant, but this could be due to the low number of replicates (n=3) (Figure 4.4A). However, the knockout BFU-E colonies that did form were significantly smaller than wildtype (Figure 4.4B). Knockout EBs formed significantly fewer and smaller myeloid and mixed colonies than wildtype (Figure 4.4A,C).

Likewise, *Dot1L* methyltransferase mutant EBs formed fewer and smaller primitive erythroid and BFU-E colonies, on average, than wildtype (Figure 4.4A,B). Mutant EBs also formed significantly fewer and smaller myeloid and mixed colonies compared to wildtype (Figure 4.4A,C). Interestingly, methyltransferase mutant BFU-E colonies were larger on average than the knockout. Myeloid and mixed colony sizes were similar between the two mutant clones.

Phenotypically, wildtype, methyltransferase mutant, and knockout EBs formed BFU-E and myeloid colonies that were similar to wildtype in appearance, just smaller in size and with reduced cellularity (Figure 4.4D-I). Mixed colonies derived from mutant and knockout clones, however, were almost unrecognizable compared to wildtype (Figure 4.4J-L). The mutant and knockout mixed colonies contained both erythroid and myeloid cells, but at strikingly reduced numbers.

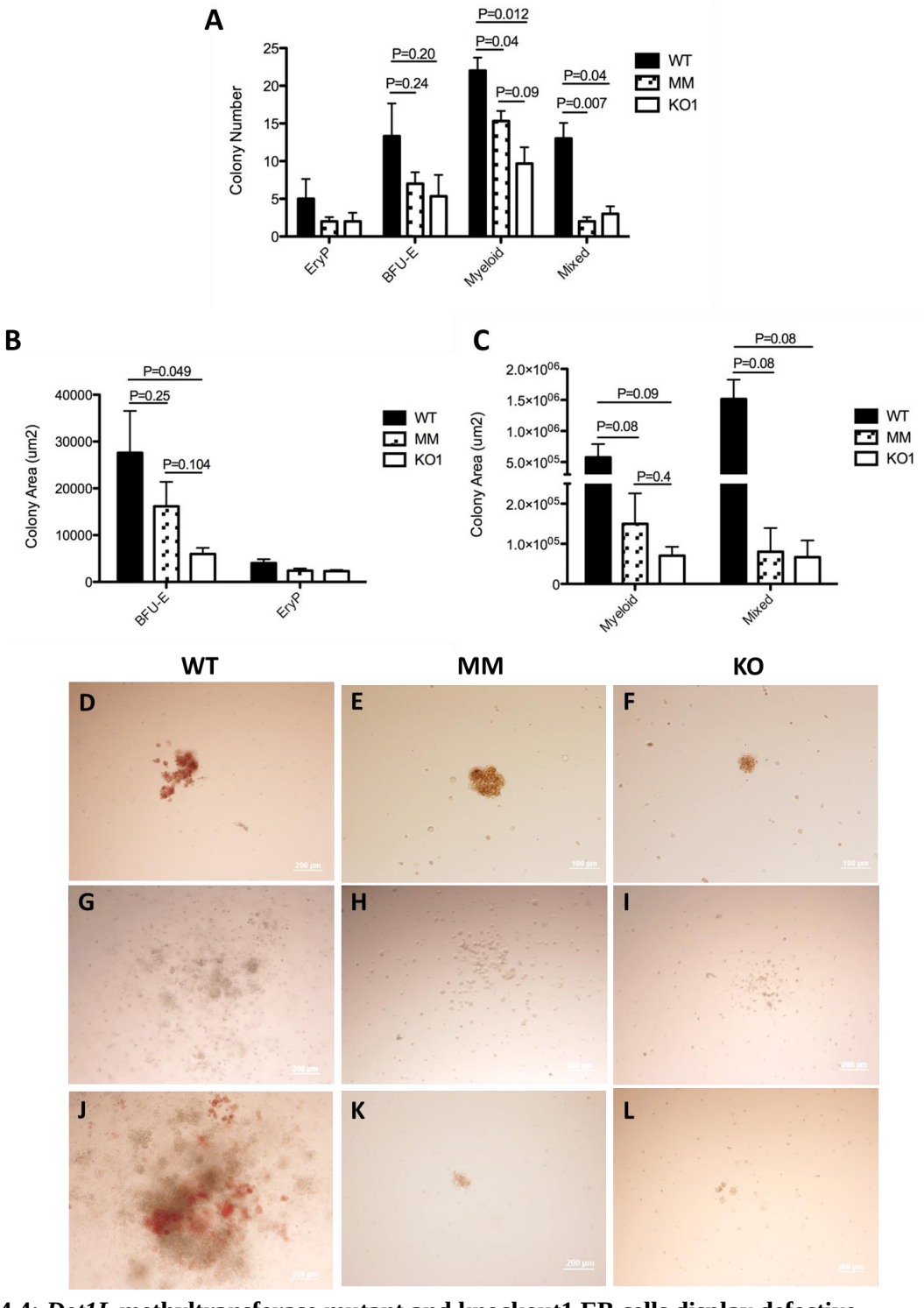

Figure 4.4: *Dot1L* methyltransferase mutant and knockout1 EB cells display defective hematopoiesis in vitro.

Equal numbers of wildtype (WT), methyltransferase mutant (MM) and knockout1 (KO1) day 8 EB cells were cultured in methylcellulose medium containing cytokines that promote primitive erythroid (EryP), definitive erythroid (BFU-E), myeloid (granulocyte, macrophage), and mixed (multipotential, erythroid and myeloid) differentiation. EryP, BFU-E, myeloid, and mixed colonies were counted (A) and area measured (B &C) on day 10. Dot1L MM & KO mESCs, on average, form fewer and smaller hematopoietic colonies compared to WT. Representative pictures of BFU-E (D,E,F), myeloid (G,H,I), and mixed (J,K,L) colonies are shown.

Discussion

In this study, murine stem cells were used to examine the hypotheses that *Dot1L* knockout mESCs induced to undergo hematopoiesis would produce similar hematopoietic defects as those observed *in vivo* in *Dot1L* knockout mice and that the enzymatic function of DOT1L, H3K79 methylation, is dispensable for blood development. In other words, it was probable that the erythroid progenitor development defect seen in *Dot1L* knockout mice was due to loss of some other functional domain of the DOT1L protein, not its methyltransferase domain or its histone methyltransferase activity.

The results of these *in vitro* experiments did support the first hypothesis, that *Dot1L* knockout mESCs induced to undergo hematopoietic differentiation *in vitro* have similar blood development defects as E10.5 yolk sacs from *Dot1L* knockout mice. The knockout1 mESC clone produced fewer BFU-E colonies on average than wildtype, and the colonies were significantly smaller, similar to the results obtained by Feng et al., 2010 [3]. In these *in vitro* cultures, myeloid colony formation was affected more so than in the yolk sac cultures, but this discrepancy could be due to differences in progenitor populations. Previous studies have shown that mESCs differentiated in culture undergo hematopoiesis in a very similar manner to those developing in the murine yolk sac [10,44]. However, it is difficult to discriminate at which point *in vitro* BFU-E colonies emerge from EMPs versus hematopoietic stem cells (which begin to emerge around E10.5 in the AGM, shortly after EMP emergence in the yolk sac) [10]. Some ES cell lines produce peak EMP levels from day 8 EBs (PC13 ESCs), while others produce peak levels from day 7 EBs (W4 ESCs) [10]. The mESCs used in this study (the R1 line) have not been directly tested. Regardless, there could still be overlap of EMP and HSC emergence in EB differentiation due to the even more truncated time frame. HSC activity in *Dot1L* knockout embryos was not

directly tested in hematopoietic differentiation assays by Feng et al. [3], so differentiation assays using this progenitor could result in defective myelopoiesis.

The second hypothesis, that DOT1L methyltransferase activity would be dispensable for blood development, received mixed support. On one hand, methyltransferase mutant mESCs produced fewer and smaller erythroid, myeloid, and mixed colonies compared to wildtype. However, the differences in erythroid colony number and size were not as great as those observed by the knockout1 mESCs, and the differences were not significant. Likewise, when examining EB formation, the methyltransferase mutant mESCs consistently formed greater numbers of EBs than knockout1 mESCs. The low number of replicates most likely explains the lack of P value significance. Also, if knockout1 EB numbers are averaged with those of the knockout2 mESCs, which grew hardly any EBs, the methyltransferase mutant clone proliferates and differentiates significantly better than the knockout.

Discrepancies in the two knockout clones' ability to differentiate into EBs could be explained by a number of things. First of all, the knockout2 mESC clone contains an out-of-frame deletion 31 nucleotides upstream to the deletion in knockout1. This results in a slightly smaller DOT1L protein. A vital binding partner domain could exist in this region of exon 9, with deletion of it resulting in defective cell differentiation. Also, CRISPR/Cas9 transfection and FACS, clone screening, and analysis is a very taxing process, especially on sensitive cell lines such as mESCs. It is possible that other growth/proliferation/DNA damage repair pathways could have been mutated in this process, resulting in a sicklier phenotype. It should also be noted that biological variability exists, in general. In the *Dot1L* knockout mice, for example, some knockout embryos survived for longer periods of time than others. Around two-thirds of knockout embryos survived to E11.5 and a smaller percentage living even longer, as shown by the presence of dead embryos yet to be resorbed by E13.5 [3].

Overall, these results indicate the utility of this mESC *in vitro* model for the production of hematopoietic progenitors, and confirm the initial rationale for developing this system, both conceptually and technically. The results also indicate that the methyltransferase activity of DOT1L plays a predominant role in the activity of the protein as a whole, and is responsible for its function in facilitating early, murine hematopoiesis. However, the differences seen in methyltransferase mutant and knockout clones ability to form EBs and BFU-E colonies suggests that other functional domains, downstream of the DOT1L methyltransferase domain are playing some kind of role in hematopoiesis and cooperate with histone methylation to effectively direct development.

Chapter 5 : Development of a Methyltransferase Mutant mouse line: the methyltransferase activity of DOT1L is essential for hematopoiesis

Introduction

Red blood cell development in the mouse begins around embryonic day 7.25 (E7.25), when primitive erythroid cells begin to form in blood islands of the embryonic yolk sac [4]. These first red blood cells provide oxygen to support the rapidly growing embryo [5]. Primitive erythroid cells arising in the yolk sac also form in conjunction with megakaryocytes and macrophages [4]. These erythroid cells predominantly contain βh1 and εγ-globin (embryonic globin) [2], which have a higher affinity for oxygen than the adult globin βmajor [12]. They continue to form in the yolk sac until E9.0 [2], and enter circulation after the heart begins beating (at E8.25) [9]. Primitive erythropoiesis is vital for early growth and survival of the embryo, as embryos unable to carry out this process die by E9.5 [10].

Shortly after primitive erythroid cells form in the yolk sac, a second wave of hematopoiesis begins in the yolk sac, around E8.25 [4,10]. These second-wave progenitors are termed erythro-myeloid progenitors (EMPs) [4]. EMPs can form definitive erythroid cells, megakaryocytes, myeloid progenitors, and multipotent progenitors that give rise to macrophages, mast cells, basophils, neutrophils, and definitive erythroid progenitors [9,10]. The definitive erythroid progenitors produced from EMPs contain the adult globin βmajor (β1) [2] but also have been found to express low levels of βh1-globin in the E11.5 fetal liver [15]. EMPs contain the cell surface markers CD41 and c-kit at E8.25 and CD16/32 as well by E9.5 [4,10]. Primitive progenitors lack these markers and are contained in the c-kit^{lo}CD41^{lo} population at E8.5 [10]. EMPs begin seeding the fetal liver around E10.5-E11.5 [2,4,10], where they attach to macrophages in erythroblastic islands and differentiate [7]. Also in the fetal liver, BFU-E and the more mature colony forming unit-erythroid (CFU-E) progenitors expand exponentially [7].

EMPs provide the first circulating definitive erythrocytes and neutrophils for the embryo at E12 [4,10,11].

A third wave of hematopoiesis, which culminates in the production of definitive hematopoietic stem cells (HSCs), occurs after yolk sac EMP formation. HSCs are thought to originate predominantly in the aorta-gonad-mesonephros (AGM) region of the embryo but also possibly in the major arteries and placenta, from E10.5-11.5 [2,4]. HSCs are marked by c-kit, Sca1, and vascular endothelial cadherin (VEC) positivity and are Lineage negative [10,13,14]. Newly formed HSCs travel through the bloodstream and seed the fetal liver. HSCs are capable of forming all hematopoietic lineages, including B and T cell progenitors. HSCs proliferate and differentiate in the fetal liver for the second half of gestation, and then seed the spleen, thymus, and then finally the bone marrow around E18.5, which is where they reside throughout adulthood [2,5]. HSCs produce erythroblasts that express adult β1- and β2-globins [4,15]. Also, unlike EMPs, HSCs can repopulate adult and immunocompromised mice long-term [4,10].

Effective murine embryonic hematopoiesis depends on the activity of several growth and transcription factors and histone modifiers. One such modifier is the histone H3 lysine 79 (H3K79) methyltransferase Disruptor of Telomere silencing 1-Like (DOT1L). Mice containing a truncated DOT1L protein, due to a gene trap insertion mutation within exon 13, displayed erythropoietic defects, leading to a lethal anemia by E11-E13.5 [3]. Prior to their death, the mutant embryos were smaller in size, and displayed abnormalities in heart development, altered vasculature, and a severe anemia [3]. When *Dot1L* knockout yolk sacs from E8.5 and E10.5 embryos were dissociated and cultured, *ex vivo*, the erythroid colonies were fewer in number and smaller than their wildtype counterparts. An examination of cell cycle progression of these cultured blood progenitors revealed that the DOT1L-deficient cells exhibited a significantly increased G_0/G_1 period and increased apoptosis compared to wildtype cells, indicating delayed or

halted cell cycle progression and increased death [3]. Other groups have shown similar phenotypes in their DOT1L-mutant mouse models. Mice missing exons 5 and 6 of DOT1L, a region which contains the DOT1L catalytic domain, and H3K79 methyltransferase activity, died between E9.5 and E10.5 [25]. They displayed impaired growth, heart enlargement, and abnormal yolk sac vasculogenesis [25]. ESCs obtained from blastocysts of these mice had decreased proliferation, increased apoptosis, and G2 cell cycle arrest compared to WT [25]. They also displayed aberrant telomere elongation, aneuploidy, and less compacted chromatin at telomeres and centromeres [25]. Absence of DOT1L not only leads to defective hematopoiesis in murine embryos, but in adults as well, as shown in conditional *Dot1L* knockout mouse models. Consistently, these adult mutant mice display defects in hematopoiesis, such as bone marrow hypocellularity with decreased amounts of common myeloid (CMP), granulocyte/monocyte (GMP), megakaryocyte/erythrocyte (MEP), and HSC progenitors, pancytopenia, and eventual death [32,33]

DOT1L has several functions outside of hematopoiesis as well, including transcriptional elongation, DNA double strand break repair [26], and cell cycle regulation [26,27]. An area in which DOT1L has been getting a lot of attention recently is in the cancer field. DOT1L has been implicated in the formation and progression of mixed lineage leukemia (MLL)-rearranged leukemia [29]. MLL leukemia occurs when the N-terminal portion the MLL protein fuses in-frame to a fusion partner (at least 70 or more have been identified), and the C-terminal methyltransferase domain is lost [29,34]. DOT1L is hypothesized to interact with some of these fusion partners, such as AF10, AF4, AF9, and ENL, where it is recruited to target genes, hypermethylates H3K79 on these genes, causing transcriptional activation/elongation, aberrant gene expression, and development of acute myeloid leukemia or acute lymphoblastic leukemia [26,29,34]. Notable target genes include the *HoxA* cluster and *Meis1* [29].

Interestingly, the DOT1L protein is very large, composed of 1540 amino acids in mice [27], but, its only documented activity is as a histone H3K79 methyltransferase. Given the involvement of DOT1L in multiple, varied processes, including embryonic development, transcriptional activation, and leukemogenesis, it is likely that the protein possesses additional functional domains that might contribute to these diverse activities. This study sought to determine if the enzymatic function of DOT1L, H3K79 methylation, is dispensable for blood development. If this is the case, then the observed blood development defect in *Dot1L* knockout mice is a result of the loss of some function of the DOT1L protein that is distinct from its methyltransferase activity. Currently, the frontrunners in the treatment of MLL-rearranged leukemias are small molecule competitive inhibitors of DOT1L's methyl-donating cofactor S-adenosyl methionine (SAM) [29]. As SAM is needed for the methyltransferase activity associated with DOT1L, blocking its binding prevents H3K79 methylation. However, these inhibitors might pose a risk to human health if they also have an impact on other processes associated with DOT1L, such as transcription and normal hematopoiesis. A better understanding of the specific functions associated with other putative domains of the DOT1L protein is critical, and will allow better targeted therapy with reduced side effects.

Here, a *Dot1L* methyltransferase mutant mouse line was created. Mutant mice contained an intact DOT1L protein that lacks H3K79 methyltransferase activity. Methyltransferase mutant mice displayed defects in embryonic hematopoiesis, including a decreased ability to form definitive erythroid, myeloid, and multipotent, mixed blood progenitors in *ex vivo* cultures, as well as embryonic lethality around mid-gestation. These results indicate that the histone methyltransferase activity of DOT1L plays a predominant role in the protein as a whole, at least in murine embryonic hematopoiesis. However, the phenotype of these *Dot1L* methyltransferase mutant mice was not quite as severe as that observed in *Dot1L* knockout mice. This discrepancy

indicates that DOT1L may in fact possess some activities separable from its function as a histone H3 lysine 79 methyltransferase.

Methods & Materials

See Chapter 3 sections "Methyltransferase Mutation Creation using CRISPR/Cas9," "*Dot1L* Methyltransferase Mutant Mouse Line," "*Dot1L* Gene Trap Mouse Line," "Western Blotting," "Embryo Experiments"

Results

Generation of Dot1L Methyltransferase Mutant Mouse Line

In order to test whether the methyltransferase activity of DOT1L is necessary for hematopoiesis, a methyltransferase mutant mouse line was created. Using the CRISPR/Cas9 system, a point mutation consisting of a single amino acid substitution from asparagine to alanine was made in Exon 9 of *Dot1L* in mESCs. Exon 9 lies within the catalytic SAM-binding pocket of DOT1L, and previous studies have found that the Asp241Ala substitution abolishes histone H3 methyltransferase activity [38]. Six additional silent, single nucleotide changes were induced, one in each of the 6 codons immediately following aa241, to enable clone screening via PCR. A schematic is shown in Figure 5.1A. Two different forward primers, one with wildtype and one with the methyltransferase mutant DNA sequence, and one reverse were created. An example of PCR genotyping of wildtype, heterozygous, and methyltransferase mutant embryos is shown in Figure 5.1B.

These mutations were made in one allele, with the other allele remaining wildtype. mESC clones were sequenced to verify mutation accuracy. Mutated mESCs were karyotyped and injected into blastocysts to create male chimera offspring. Chimeras were backcrossed to

C57/Bl6, and heterozygous methyltransferase mutant offspring were bred together. Histones and whole protein lysates were extracted from E10.5 embryonic fibroblasts and western blotted for histone H3, histone H3 lysine 79 (H3K79) di-methylation, DOT1L, and GAPDH. As shown in Figure 5.1C, methyltransferase mutant mice had near absent H3K79 di-methylation, but the DOT1L protein was still present.

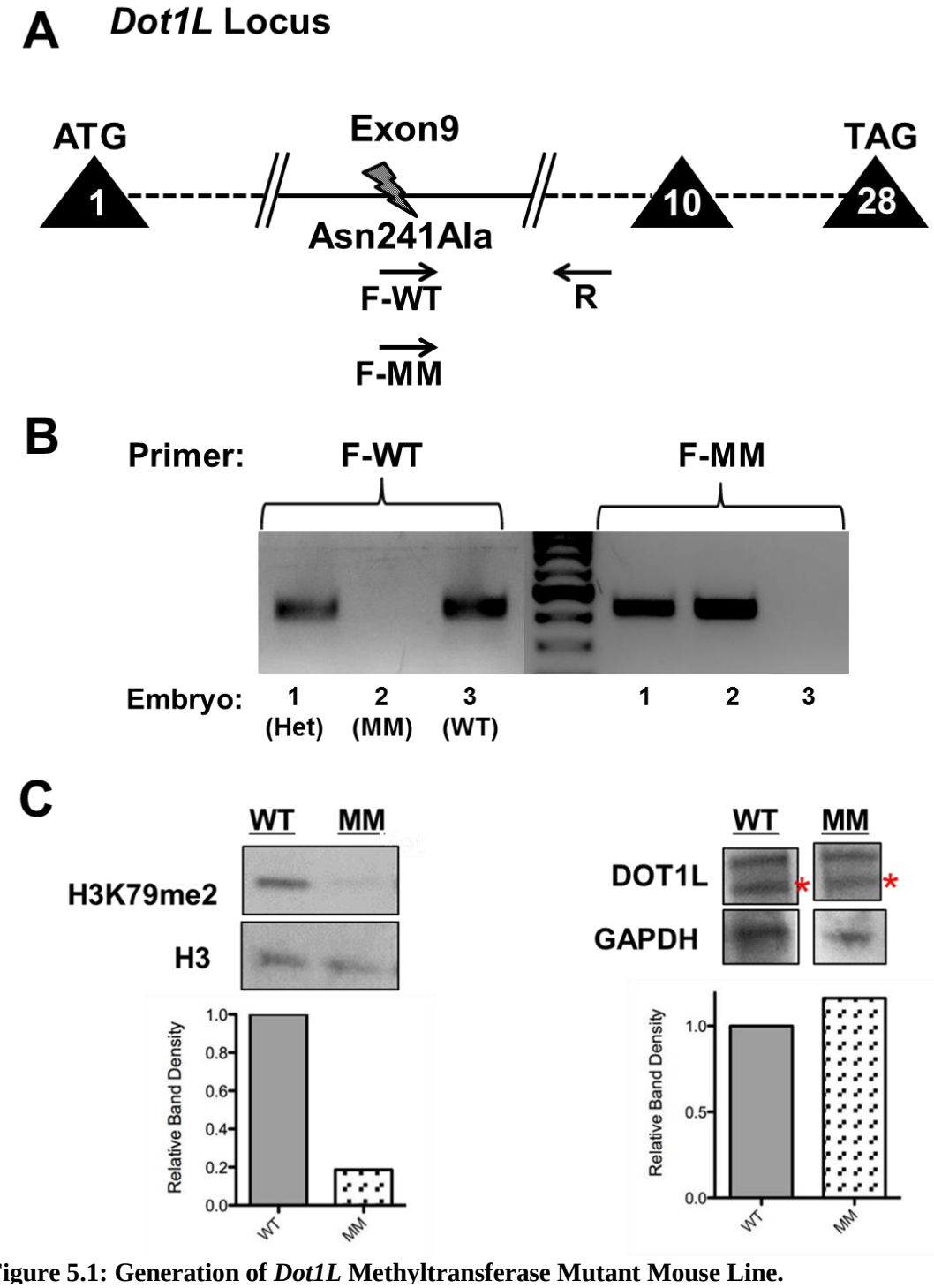

Figure 5.1: Generation of *Dot1L* Methyltransferase Mutant Mouse Line.

(A) Schematic showing region of Dot1L targeted by CRISPR/Cas9 to induce the point mutation Asn241Ala that eliminates H3K79 methyltransferase activity and the primers used to screen for mutants. (B) PCR genotyping using the primers shown in (A) to identify wildtype (WT), heterozygous (Het), and methyltransferase mutant (MM) embryos. Two separate PCR reactions are run: one with F-WT and R primers (left), and a second with F-MM and R primers (right). (C) Histones and whole protein lysates were extracted from E10.5 MEFs and western blotted for histone H3, histone H3 lysine 79 di-methylation (H3K79-me2) and DOT1L, GAPDH, respectively. Band densities are shown relative to loading controls (histone H3 and GAPDH) and wildtype.

Dot1L methyltransferase mutant mice display embryonic lethality

Heterozygous methyltransferase mutant mice of the F1 generation were bred together, and viability studies were performed. Offspring were genotyped at 21 days post-birth (P21), and embryonic days E10.5, E12.5, and E13.5. No mutant embryos were alive at P21 or E13.5, and 80% were dead at E12.5. Mutant embryos were, however, at Mendelian ratios at E10.5 (Figure 5.2A). Thus, most mutant embryos die sometime between E11-E12.5.

A ***Dot1L* Methyltransferase Mutation Survival**

	E10.5	E12.5	E13.5	P21
Total embryos	133	25	38	41
***Dot1L* WT**	27 (20%)	6 (24%)	10 (26%)	13 (32%)
***Dot1L* Het**	78 (59%)	14 (56%)	25 (66%)	28 (68%)
***Dot1L* MM**	28 (21%)	1 (4)* (20%)	0 (3)* (8%)	0 (0%)

**Embryos were dead but not resorbed.*

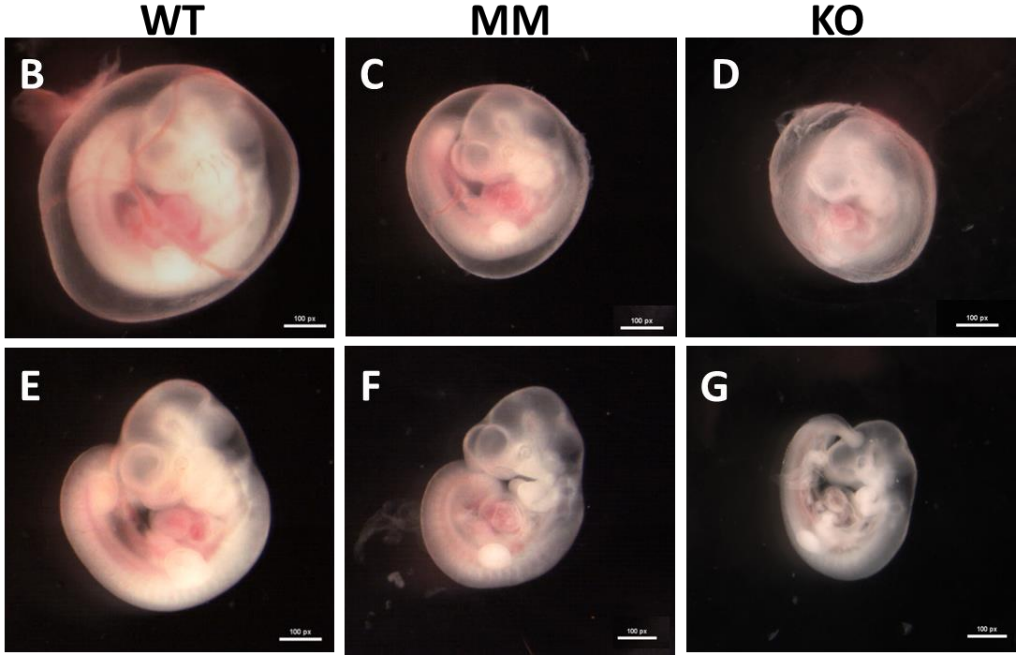

Figure 5.2: *Dot1L* Methyltransferase Mutant embryos display embryonic lethality and slight defects in growth and hematopoiesis.
(A) Table showing the percentage of wildtype (WT), heterozygous (Het), and methyltransferase mutant (MM) embryos alive at embryonic days 10.5, 12.5, and 13.5, and 21 days after birth. Methyltransferase mutant embryos die between E10.5 and E13.5. (B-G) Representative pictures of WT, MM, and KO E10.5 embryos. MM and KO yolk sacs have less prominent blood vessels than WT (B,C,D). MM embryos are slightly smaller and more anemic than WT (E,F), but are not as small or anemic as KO (G).

Methyltransferase mutant embryos at E10.5 were slightly smaller than their wildtype littermates. They contained blood, which can be seen in the heart and AGM region, but not to the same extent as wildtype (Figure 5.2E,F). The mutant embryos, however, were not as small or anemic as *Dot1L* knockout embryos (Figure 5.2G). The mutant embryo yolk sacs were vascularized and contained blood, but the blood vessels were not as prominent as those seen in wildtype yolk sacs (Figure 5.2B,C). Mutant yolk sacs were, however, more vascularized than knockout (Figure 5.2D). In conclusion, E10.5 *Dot1L* methyltransferase mutant embryos display a slight anemia and developmental defects, but not as severe as those seen in E10.5 *Dot1L* knockout embryos.

Defective yolk sac hematopoiesis in Dot1L methyltransferase mutant embryos

E10.5 yolk sacs were dissociated into a single cell suspension and plated in methylcellulose medium containing cytokines that promote definitive erythroid, myeloid, and mixed progenitor colony formation. Cells were cultured for 10 days, at which point colony number and area were assessed. Erythroid colonies included megakaryocyte/erythroid progenitors (MEP), blast forming unit-erythroid (BFU-E), and colony forming unit-erythroid (CFU-E). Myeloid colonies included bi-potential granulocyte/macrophage (CFU-GM) as well as singularly granulocyte and macrophage (CFU-G and CFU-M). Mixed colonies were multipotent, containing both erythroid and myeloid progenitors.

Methyltransferase mutant yolk sacs formed smaller numbers of erythroid colonies than wildtype, on average, but the difference was not statistically significant. Mutant cells, however, formed about 50% fewer myeloid colonies and about 90% fewer mixed colonies than wildtype, and these differences were significant (Figure 5.3A). Interestingly, colony area measurements showed the opposite trend. Mutant YS cells formed significantly smaller erythroid colonies than wildtype. Also, despite the fact that mutant YS cells formed fewer myeloid and mixed colonies,

the colonies that did grow reached similar sizes as the wildtype (Figure 5.3B). Representative pictures of all 3 colony types are shown in Figure 5.3C. (Dot plots showing the distribution of individual colony sizes are shown in Figure 5.4A,B,C)

Compared to *Dot1L* knockout E10.5 YS cells, methyltransferase mutants displayed similar hematopoietic defects in terms of erythroid, myeloid, and mixed colony number and size. There was one difference, however, in erythroid colony size. *Dot1L* methyltransferase mutant YS cells grew larger erythroid colonies than knockout, on average, although this difference was not quite significant (P=0.26) (Figure 5.3B).

The ability of YS progenitors to differentiate into mature erythroid colonies was also assessed. E10.5 YS cells were plated in methylcellulose medium containing cytokines that promote mature erythroid colony formation (mature BFU-E and CFU-E only). Interestingly, no differences were found in colony number between *Dot1L* methyltransferase mutant and wildtype cells (Figure 5.5A). After 4 days in culture, mature erythroid cells were labeled with Annexin V and analyzed via flow cytometry for apoptosis. No differences were found in the percentage of cells undergoing apoptosis between mutant and wildtype (Figure 5.5B). These data are different than that of E10.5 knockout YS cells. Knockout cells cultured in mature erythroid medium formed significantly fewer erythroid colonies and compared to wildtype, and a greater percentage of knockout erythroid cells were Annexin V-positive [3].

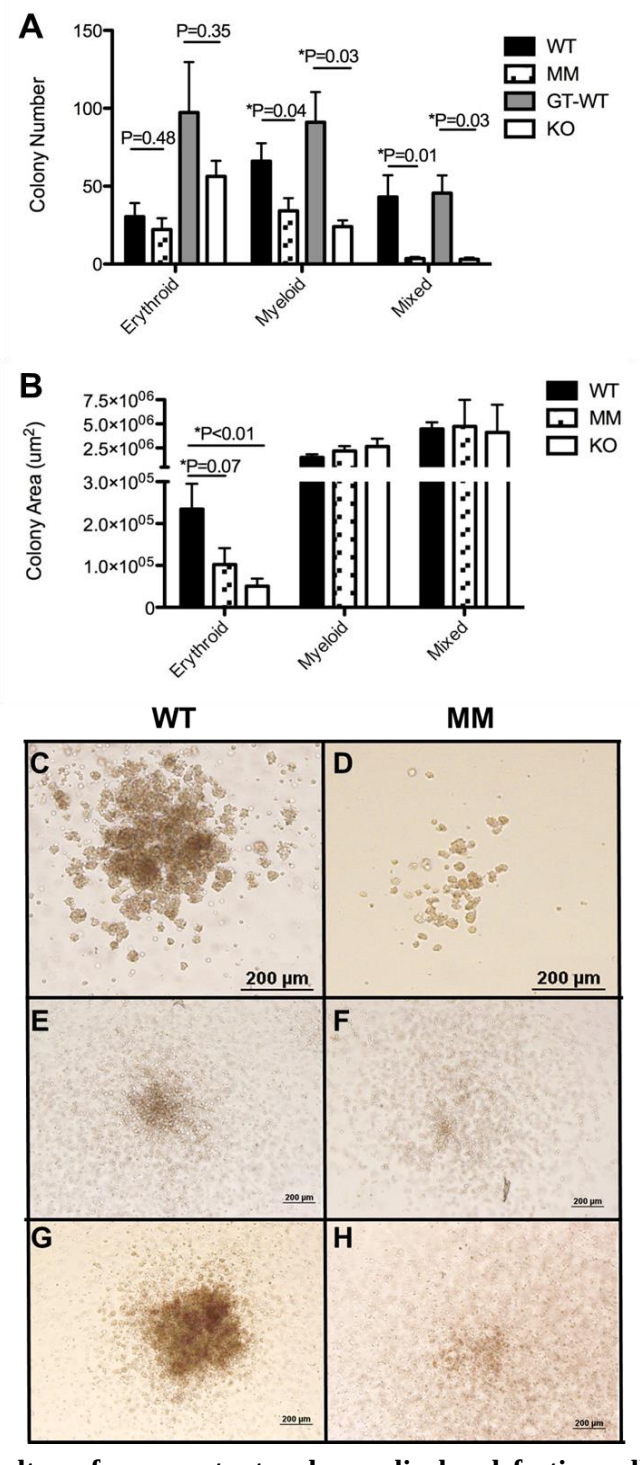

Figure 5.3: *Dot1L* methyltransferase mutant embryos display defective yolk sac hematopoiesis. Equal numbers of wildtype (WT) and methyltransferase mutant (MM), wildtype (WT-GT; from Dot1L Gene Trap Mouse line) and knockout (KO) E10.5 yolk sac cells were cultured in methylcellulose medium containing cytokines that promote definitive erythroid (megakaryocyte/erythroid, BFU-E, and CFU-E progenitors), myeloid (granulocyte/macrophage progenitors), and mixed (multipotential, erythroid and myeloid progenitors) differentiation. Erythroid, myeloid, and mixed colonies were counted (A) and area measured (B) on day 10. Dot1L MM YS form similar numbers of erythroid colonies as WT, but they're significantly smaller in size. Dot1L MM YS form significantly fewer myeloid and mixed colonies at WT, but the few colonies that do form reach similar sizes as WT. Representative pictures of BFU-E (C,D), myeloid (E,F), and mixed (G,H) colonies are shown.

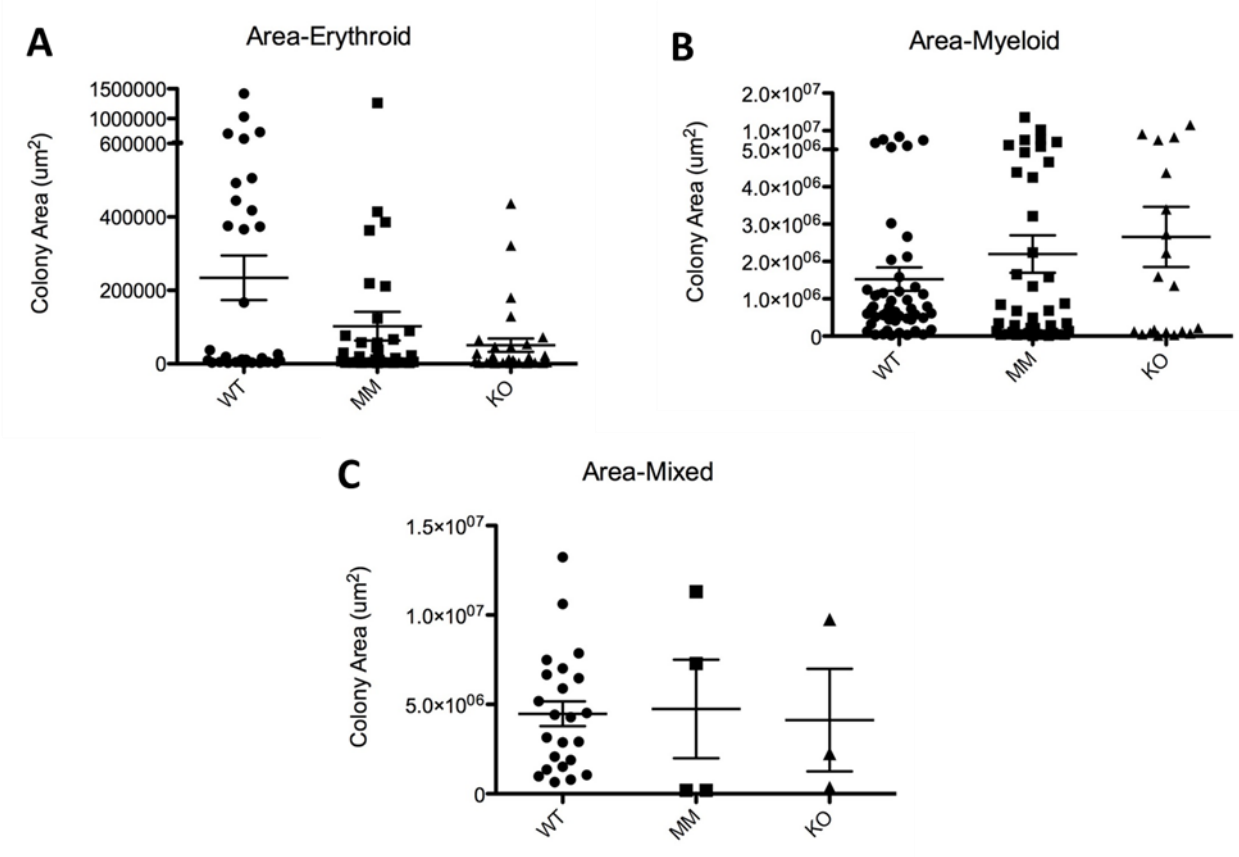

Figure 5.4: Dot plots showing the distribution of individual hematopoietic colony area.
(A) erythroid, (B) myeloid, and (C) mixed colony areas from E10.5 WT, MM, and KO yolk sac hematopoietic differentiation assays.

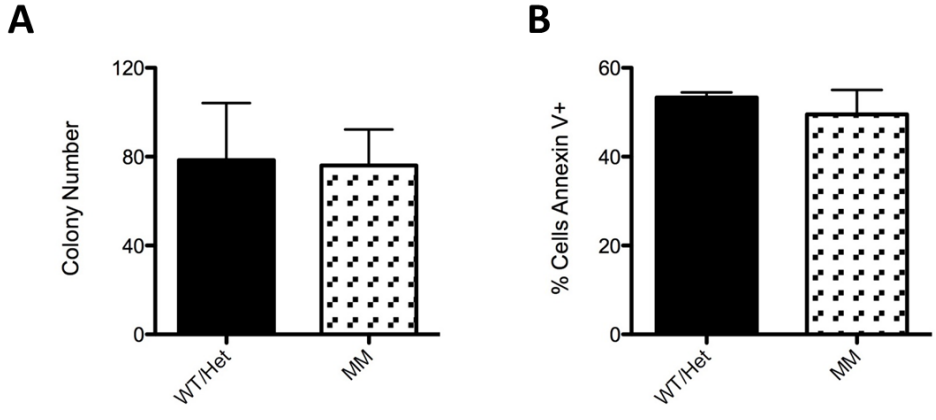

Figure 5.5: *Dot1L* methyltransferase mutant yolk sac blood progenitors do not display apparent defects in the ability to form mature erythroid colonies.

Equal numbers of wildtype (WT), heterozygous (Het), and methyltransferase mutant (MM) E10.5 yolk sac cells were cultured in methylcellulose medium containing cytokines that promote definitive erythroid (mature BFU-E and CFU-E progenitors) differentiation. (A) Erythroid colonies were counted on day 3. (B) Cells were collected on day 4 and stained with Annexin V to assess apoptosis. Cells were analyzed via flow cytometry. *Dot1L* MM YS blood progenitors formed similar numbers of mature erythroid colonies as WT and Het, and displayed equal levels of apoptosis.

Dot1L methyltransferase mutant yolk sac blood progenitors have increased apoptosis and defects in cell cycle progression

E10.5 yolk sacs were dissociated into single cell suspensions and plated in methylcellulose medium containing cytokines that promote definitive erythroid, myeloid, and mixed progenitor colony formation. Cells were cultured for 4 days, labeled with Annexin V and propidium iodide, and analyzed by flow cytometry for apoptosis and cell cycle phase, respectively. A greater percentage of *Dot1L* methyltransferase mutant and knockout YS hematopoietic progenitors were undergoing apoptosis compared to wildtype (Figure 5.6A). Also, mutant YS cells displayed a G0/G1 cell cycle arrest, compared to wildtype (Figure 5.6B). These results suggest that the mutant YS blood progenitors are not proliferating or surviving in hematopoietic differentiation cultures to the same extent as wildtype.

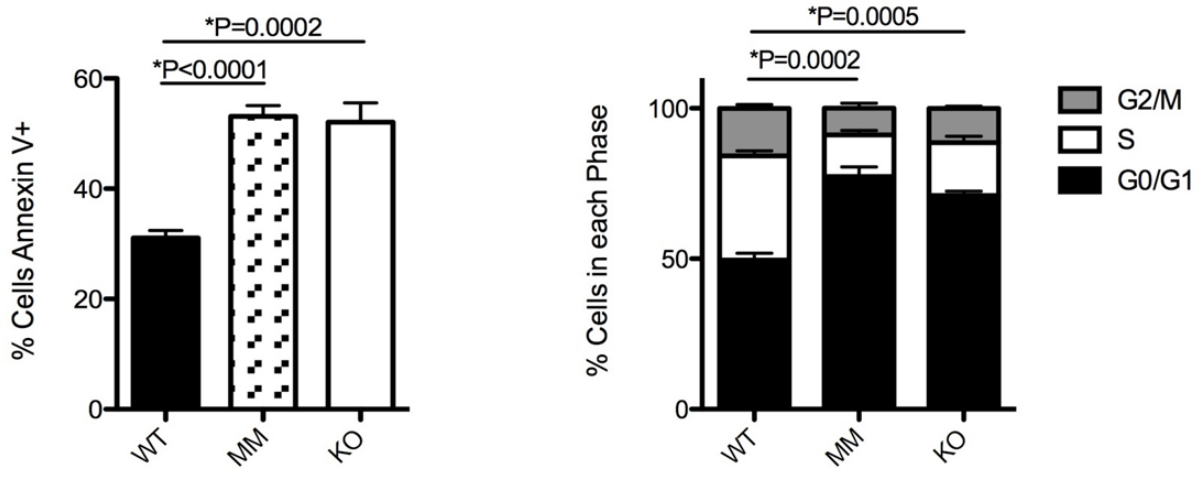

Figure 5.6: *Dot1L* methyltransferase mutant and knockout yolk sac hematopoietic progenitors display increased apoptosis and defects in cell cycle progression.

E10.5 yolk sac cells were cultured in methylcellulose medium containing cytokines that promote definitive erythroid, myeloid, and mixed progenitor differentiation. Cells were collected on day 4 and stained with Annexin V to assess apoptosis (A) and stained with propidium iodide to assess cell cycle progression (B). Cells were analyzed via flow cytometry. Dot1L MM and KO YS hematopoietic progenitors display increased apoptosis levels (A) and a G0/G1 cell cycle arrest (B).

Dot1L mutant erythroblasts display defective proliferation and survival

Dot1L methyltransferase mutant and knockout E10.5 yolk sacs were cultured in expansion medium and differentiated into extensively self-renewing erythroblasts (ESREs) as previously described [41]. Erythroblasts were counted every other day for 2 weeks to calculate expansion. *Dot1L* methyltransferase mutant and knockout erythroblasts had severely blunted proliferation in comparison to wildtype (Figure 5.7A). Day 6-7 *Dot1L* mutant and knockout erythroblasts also displayed increased apoptosis compared to wildtype (Figure 5.7B), as shown by increased percentages of Annexin V-positive cells. Cell cycle analysis via propidium iodide staining was performed, but no significant differences were found between mutant and wildtype, as all three groups displayed a G0/G1 cell cycle arrest (Figure 5.7C). Also, alkaline comet assays were performed on Day 6-7 *Dot1L* mutant, knockout, and wildtype ESREs. *Dot1L* mutant and knockout ESREs contained significantly more DNA damage than wildtype (Figure 5.7D).

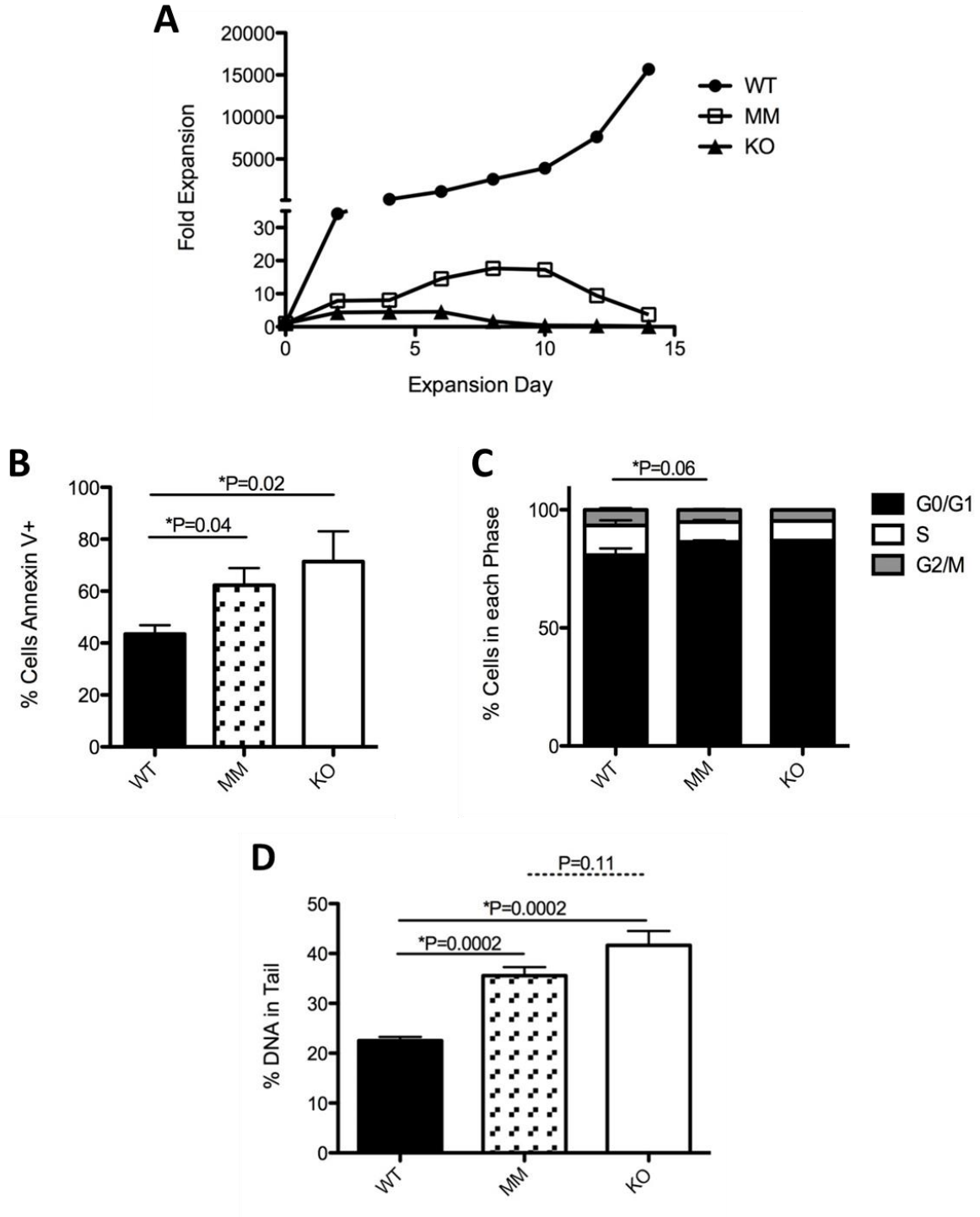

Figure 5.7: *Dot1L* methyltransferase mutant and knockout extensively self-renewing erythroblasts (ESREs) display defective proliferation and survival.

E10.5 Dot1L MM, KO, and WT yolk sacs were cultured in expansion medium for ESREs. (A) Cell number was counted via trypan blue exclusion every 2 days for 14 days, and fold expansion was calculated relative to day 0 (day 3 after isolation). On day 6-7 of counting, cells were labeled for Annexin V and propidium iodide and analyzed via flow cytometry for apoptosis (B) and cell cycle phase (C). (D) Alkaline Comet assays were performed on day 6-8 ESREs to assess DNA damage. Dot1L MM and KO ESREs contained greater DNA damage than WT.

DOT1L-inhibitor treated erythroblasts display defective proliferation and survival

Wildtype E10.5 yolk sacs were cultured in expansion medium with 250-400nM of the DOT1L-inhibitor SGC0946 and differentiated into extensively self-renewing erythroblasts (ESREs) as previously described [41]. After 7 days of inhibitor treatment, histones were extracted from these cells and Western blotted for histone H3 and histone H3 lysine 79 (H3K79) di-methylation. As shown in Figure 5.8A, SGC0946-treated ESREs had near absent H3K79 di-methylation relative to histone H3 and wildtype.

Erythroblasts were counted every other day for 14 days to calculate expansion. DOT1L-inhibitor treated erythroblasts had severely blunted proliferation in comparison to wildtype (Figure 5.8B). Day 7 inhibitor-treated erythroblasts displayed slightly increased apoptosis compared to wildtype, but the difference became significantly larger by day 14 (Figure 5.8C), as shown by increased percentages of Annexin V-positive cells. Cell cycle analysis was performed on days 7 and 14 (Figure 5.8D). Inhibitor-treated ESREs displayed a slight G0/G1 arrest at day 7. Interestingly, at day 14, inhibitor-treated ESREs displayed a G2/M arrest. Also, alkaline comet assays were performed on day 7 inhibitor-treated and wildtype ESREs. At this time point, inhibitor-treated ESREs contained significantly more DNA damage than wildtype (Figure 5.8E).

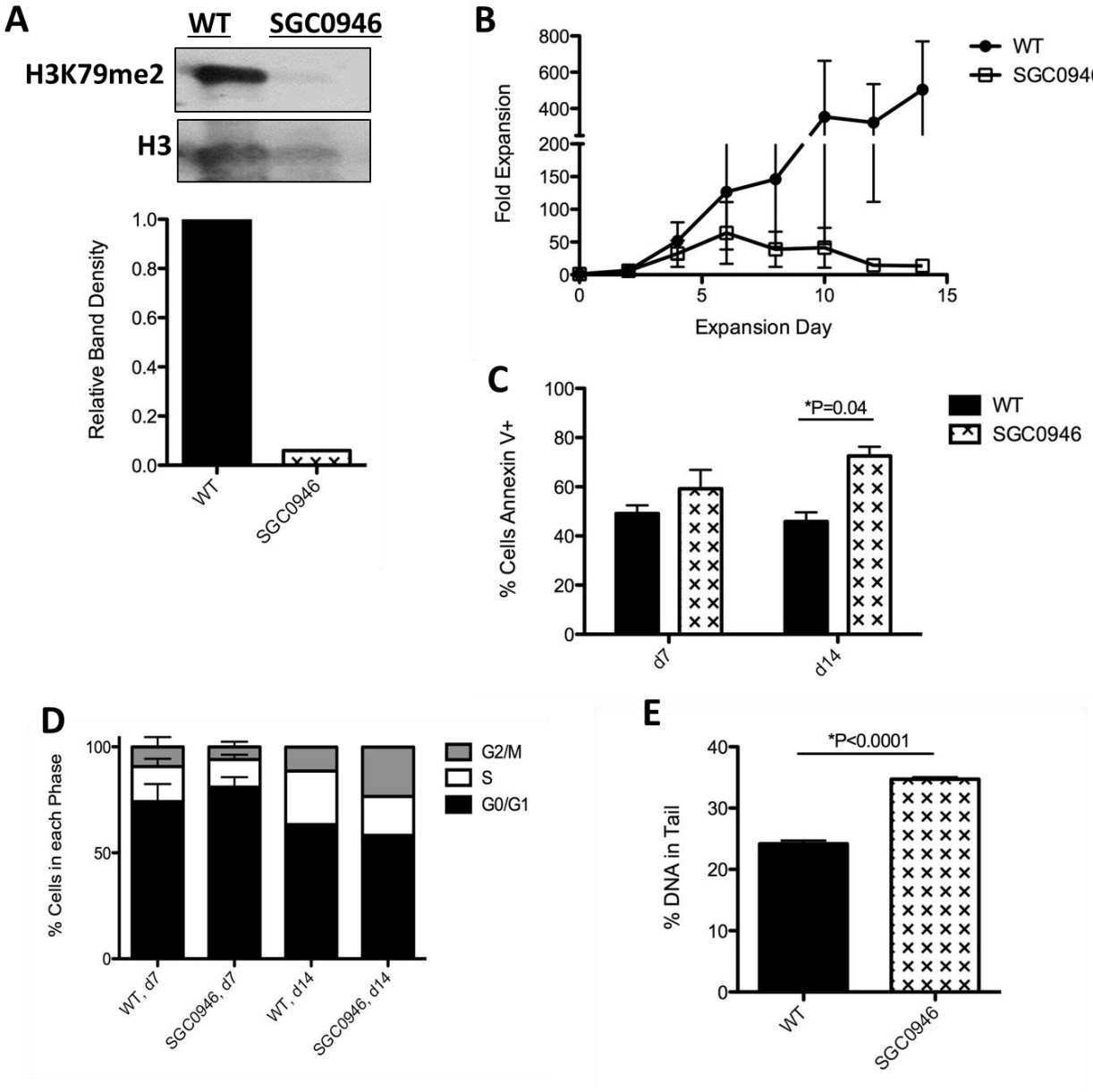

Figure 5.8: DOT1L inhibitor-treated wildtype extensively self-renewing erythroblasts (ESREs) display defective proliferation and survival.

E10.5 WT yolk sacs were cultured in expansion medium for ESREs with 250-400nM SGC0946, a DOT1L inhibitor drug. (A) Histones and were extracted from ESREs after 7days of inhibitor treatment and western blotted for histone H3 and histone H3 lysine 79 di-methylation (H3K79-me2). Band densities are shown relative to histone H3 and wildtype. (B) Cell number was counted via trypan blue exclusion every 2 days for 14 days, and fold expansion was calculated relative to day 0 of drug treatment (day 5 after isolation). On day 7 and 14 of counting, cells were labeled for Annexin V analyzed via flow cytometry for apoptosis (C) and cell cycle phase (D). (E) Alkaline Comet assays were performed on day 7 ESREs to assess DNA damage. DOT1L inhibitor-treated ESREs contained greater DNA damage than WT.

Discussion

DOT1L H3K79 methyltransferase activity is necessary for embryonic hematopoiesis

The only documented activity of the DOT1L protein is as a H3K79 methyltransferase. The catalytic domain of DOT1L is located within amino acids 1-332 of the 1540 (in mice) [27]. The remaining portions of the protein are known to house binding domains for some transcriptional elongation factors, such as AF17 and AF9 [27]. Given its large size and involvement in an array of cellular activities, a *Dot1L* methyltransferase mutant mouse line was created to determine whether embryonic blood development was facilitated by the H3K79 methyltransferase activity, downstream binding domains, or other unidentified domain(s) of the protein. The methyltransferase mutant mouse line described here is the first of its kind; no other *Dot1L* methyltransferase domain null mouse lines have been reported.

Mutant mice displayed an embryonic lethality between E11 and E13.5. Mutants were also slightly smaller in size and slightly anemic compared to littermate controls, and their yolk sacs contained less prominent blood vessels. Hematopoietic differentiation assays of mutant E10.5 YS showed defects in erythroid, myeloid, and multipotential-mixed hematopoietic progenitor growth and survival. Expansion cultures of extensively self-renewing erythroblasts, obtained from E10.5 YS, also showed defects in proliferation and survival, as well as DNA damage in mutant cells. These effects were also replicated by addition of the DOT1L inhibitor drug SGC0946. The above results demonstrate that H3K79 methyltransferase activity plays a predominant role in the protein as a whole and is necessary for murine embryonic hematopoiesis.

The necessity of H3K79 methylation for DOT1L activity has also been shown in MLL-AF9 leukemia cell lines. Constructs containing wildtype DOT1L or methyltransferase inactive DOT1L (contained full-length DOT1L with a point mutation in the SAM-binding domain), were transfected into DOT1L-deficient MLL-AF9 cells. Cells transfected with wildtype construct

regained leukemic transformation, while cells transfected with the methyltransferase inactive construct did not [32]. Thus, in order for MLL leukemia to emerge, DOT1L H3K79 enzymatic activity is needed, not simply the binding domains for MLL translocation partners and transcriptional elongation machinery. Studies investigating the effects of methyltransferase-inactive DOT1L in other systems will be interesting and may reveal other functions of DOT1L outside of its role as a histone methyltransferase. The methyltransferase mutant mice would be a great tool in which to do *in vivo* work and explore the role of DOT1L protein domains (outside of its methyltransferase domain) in other developing organ systems, not just hematopoiesis.

Dot1L methyltransferase mutant erythro-myeloid progenitors display flawed self-renewal

Hematopoietic differentiation assays were performed on E10.5 YS to assess how well methyltransferase mutant mice could form definitive erythroid, myeloid, and multipotent, mixed progenitors. Although the mutants displayed a slight impairment in erythropoiesis and myelopoiesis, what was most striking was the near absence of mixed progenitor colonies (Figure 5.3A). EMPs form in the yolk sac beginning around E8.25 and are capable of forming multiple progenitors including macrophages, mast cells, basophils, neutrophils, and definitive erythroid progenitors [9,10]. This progenitor is an early blood stem cell that arises before HSCs, and they leave the YS to colonize the fetal liver, where they proliferate exponentially and differentiate into definitive erythroid and myeloid progenitors [7]. The mixed colonies in the methylcellulose assays represent a good assessment of their presence and ability to proliferate in the murine YS.

Given the lack of mixed colonies in the mutant YS, there clearly is an impairment in either EMP formation or the ability of the EMPs to proliferate/survive. If EMPs were not forming in the YS, then one could assume that there would not be any definitive erythroid or myeloid colonies present, since both definitive erythroid and granulocyte/macrophage colonies in the YS originate from EMPs, not primitive erythropoiesis. However, since definitive erythroid

colonies grew from mutant YS cells in similar numbers as wildtype, and the fact that myeloid colonies do still grow (albeit at reduced numbers), EMPs are forming in the methyltransferase mutant YS. In the mutant, it appears that there is an initial burst of EMP formation, but these stem cells then quickly differentiate into definitive erythroid and myeloid progenitors. Therefore, it is hypothesized that there is impairment in the ability of *Dot1L* methyltransferase mutant EMPs to self-renew.

Interestingly, in inducible *Dot1L* knockout mouse models, *Dot1L*-deficiency leads to a particularly large decrease in the numbers of HSCs [32]. Other lineage-specific progenitors (common lymphoid, megakaryocyte/erythroid, granulocyte/macrophage, and common myeloid) are also significantly decreased [32,33]. In conditional mouse models of *Dot1L* deletion within the hematopoietic and endothelial compartments only, the earlier, more multipotential hematopoietic compartments were more prominently affected, as well [31]. *Dot1L*-mutants were able to form myeloid progenitors nearly as well as wildtype, but bi-potential granulocyte/macrophage, LSK HSCs, and common myeloid progenitors were significantly decreased [31]. Megakaryocyte/erythroid progenitors were not significantly decreased [31].

Additional support for this hypothesis is shown in the phenotype of the embryos themselves. E10.5 embryos contain blood and are only slightly more anemic than wildtype mice, but they still die by E13.5 (the majority by E12.5). Since EMPs colonize the fetal liver between E10.5 and E11.5 [10] and proliferate exponentially there, the ability of EMPs to self-renew is necessary at this stage of development. It is conceivable that methyltransferase mutant mice are dying by E12.5-13.5 because their EMPs cannot self-renew in sufficient numbers to seed the fetal liver.

Studies in MLL leukemia cell lines add support to this apparent phenotype of rapid differentiation versus self-renewal in the absence of DOT1L-mediated H3K79 methyltransferase

activity. When *Dot1L* was deleted from MLL-AF9 leukemia cells growing in methylcellulose culture, colonies were fewer in number, smaller, and displayed terminal differentiation in morphology, compared to the blast-like morphology seen in wildtype [31]. Likewise, similar changes were seen in DNMT3A-mutant AML cell lines. OCI AML2- and OCI AML3-DNMT3A-mutant cells treated with the DOT1L inhibitors SYC-522 and EPZ004777 seemed to undergo differentiation, as they had increased CD14 expression, a mature monocyte marker [36]. Also, EPZ004777-treated OCI AML2 and OCI AML3 cells underwent RNA-seq analysis. Ingenuity Pathway Analysis showed increased expression of genes involved in cell-cycle arrest, cell death, and differentiation [36].

The mechanisms dictating the lack of self-renewal are still being investigated. As DOT1L is known for involvement in transcriptional elongation [26], it could be that transcription factors necessary for self-renewal are not being activated. Possible factors include Leukemia inhibitory factor (LIF), interleukin-6, Sox17, or BMP4, all of which play roles in HSC self-renewal and expansion [11,18]. Previous studies found that *Dot1L* knockout E10.5 c-kit-positive YS progenitors had decreased GATA2 expression [3]. GATA2 is important for definitive erythroid progenitor (such as HSC) proliferation and survival in response to growth factors [18,16]. Thus, this decrease in GATA2 expression could prevent YS EMPs from self-renewing. It is also possible that these EMPs are more prone to cellular damage, such as DNA damage, cannot repair their genomes effectively, and differentiate as a result. Interestingly, several recent studies have documented a role for the DNA damage response in inducing differentiation of leukemic cells [46,47]. It is likely that accumulated DNA damage in mutant EMPs induces them to differentiate. *Dot1L* methyltransferase mutant YS cells plated in hematopoietic differentiation medium displayed G0/G1 cell cycle arrest and increased apoptosis compared to wildtype (Figure 5.6). Therefore, these progenitor cells are not growing or surviving either. Mutant ESREs also

displayed increased DNA damage in comet assays compared to wildtype, in addition to increased apoptosis and G0/G1 cell cycle arrest, further suggesting that DOT1L methyltransferase activity plays a role in hematopoietic progenitor cell genome stability. Transcription and DNA damage repair abnormalities could both be at play in the methyltransferase mutant mice and cause the observed defects in hematopoiesis (Figure 5.9).

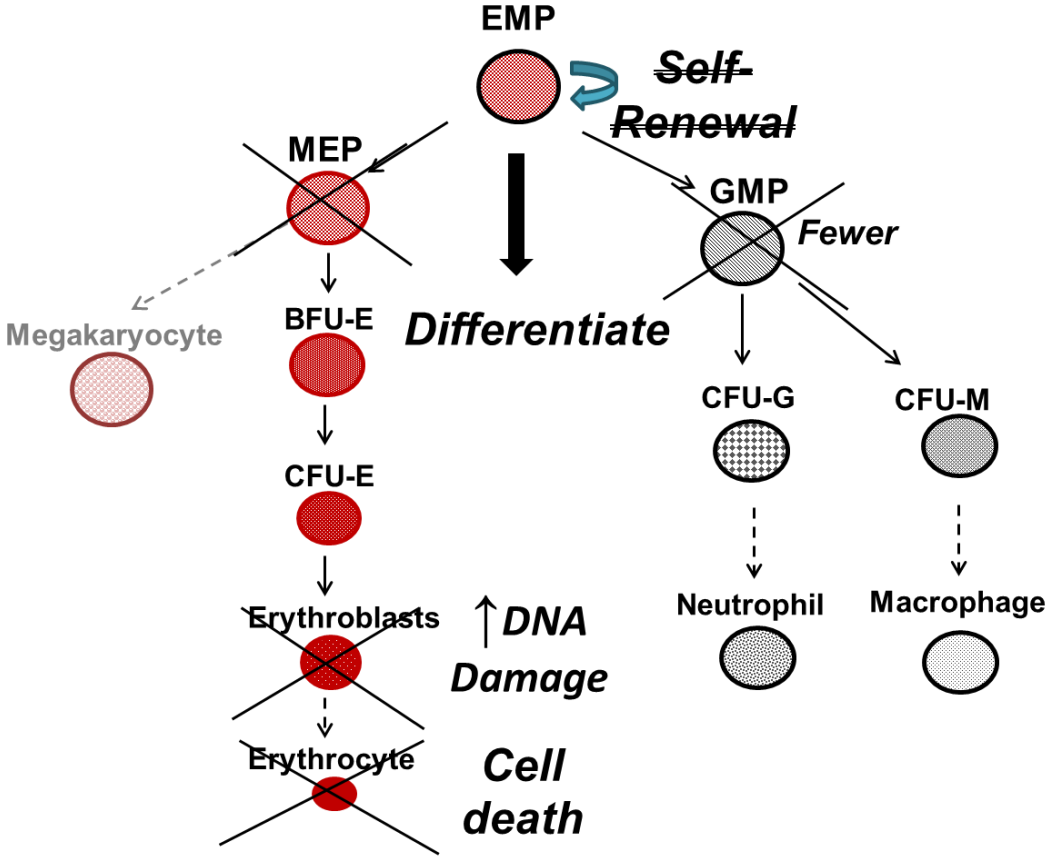

Figure 5.9: Possible mechanisms for the hematopoietic defects seen in *Dot1L* methyltransferase mutant mice

Dot1L methyltransferase mutant phenotype is not as severe as Dot1L knockout

Although *Dot1L* methyltransferase mutant mice displayed defects in hematopoiesis, both in the methylcellulose hematopoietic differentiation assays (Figure 5.3) and in the ESRE expansion cultures (Figure 5.7), the methyltransferase mutants were able to form more erythroid

progenitors than *Dot1L* knockouts. This increased ability to carry out erythropoiesis was also seen in the E10.5 embryos themselves. Methyltransferase mutant embryos contained visible blood in the developing heart and AGM region, and looked only slightly more anemic than their littermate controls. *Dot1L* knockout embryos, however, displayed a near absence of blood within the embryo and yolk sac (Figure 1.1, Figure 5.2).

In the E10.5 YS hematopoietic differentiation assays, methyltransferase mutant mice formed larger erythroid colonies than the knockouts (Figure 5.3B). Also, methyltransferase mutant YS cells plated in methylcellulose medium that promoted only mature erythroid differentiation (mature BFU-E and CFU-E colonies) grew at near equal levels as wildtype (Figure 5.5). The knockouts, on the other hand, displayed significant defects in colony number and also had increased apoptosis [3]. Furthermore, in the ESRE expansion cultures, methyltransferase mutant ESREs reached a peak expansion of about 20-fold, whereas the knockout only reached a peak of 5-fold. Also, methyltransferase mutant ESREs did not start to die off until around day 10, while the knockouts died off rapidly after day 6 (Figure 5.7A). Knockout ESREs also contained greater DNA damage than the methyltransferase mutants (Figure 5.7D). This increased ability to undergo erythropoiesis in methyltransferase mutants signifies that there may in fact be other domains of DOT1L outside of the methyltransferase domain that are involved in the regulation of erythropoiesis and/or DNA damage repair.

Future Directions

This study investigated yolk sac hematopoiesis, but it would be useful to study other areas of hematopoiesis in the developing mouse embryo, such as in the AGM region and fetal liver. Methyltransferase mutant mice appear only slightly more anemic and smaller than littermate controls at E10.5, yet most die by E12.5. Given that the yolk sac ceases being the primary location of hematopoiesis after E10.5, defects in the ability of the AGM region to form

HSCs or defects in the ability of EMPs from the yolk sac or HSCs to colonize and proliferate in the fetal liver could be responsible for these embryos' demise. Experiments specifically identifying HSC presence and fetal liver hematopoietic differentiation cultures could answer these questions.

Likewise, investigation of primitive erythropoiesis in methyltransferase mutant mice may add insight into why these mice appear only slightly more anemic at E10.5 yet still die by E12.5 and why their developmental defects are not as severe as those seen in the *Dot1L* knockout mice. Attempts were made to investigate primitive erythropoiesis in E8.5-E8.75 mouse embryos (since primitive erythropoiesis occurs in mice from E7.25-E9) but were unsuccessful due to technical difficulties related to very low embryo/yolk sac cell numbers. Other models, such as the mESC-based culture system described in this study, may be useful in studying primitive erythropoiesis in the methyltransferase mutant mice. This study used mESC-derived day 8 EBs to study definitive erythropoiesis, but previous studies have shown that day 6 EBs produce primitive erythroid colonies at much higher numbers than day 8 EBs when cultured in hematopoietic differentiation medium [10,44]. The same wildtype, methyltransferase mutant, and knockout1 mESC clones used in this study could be differentiated into EBs, collected at day 6, then plated into the secondary hematopoietic differentiation, methylcellulose medium. This assay would give a good estimate of how well primitive erythropoiesis ensues in *Dot1L* methyltransferase mutant mice relative to wildtype and knockout mice. Based on the fact that E10.5 *Dot1L* methyltransferase mutant mice appear only slightly anemic and contain more blood than E10.5 knockout embryos, it is expected that *ex vivo* and *in vitro* cultures of methyltransferase mutant yolk sacs and mESCs, respectively, will form greater numbers of and larger primitive erythroid colonies than knockout mice/mESCs.

Identification of DOT1L-regulated genes involved in stem cell self-renewal would also be an interesting area to investigate. This study hypothesizes a role for DOT1L H3K79 methyltransferase activity in EMP self-renewal. Identification of possible genes involved EMP self-renewal and how DOT1L interacts with/regulates them will not only add insight into murine yolk sac hematopoiesis but may also suggest new clinical targets to treat human leukemias and anemias.

Chapter 6 : Conclusions

Numerous studies have shown that absence of *Dot1L* causes significant defects in both embryonic and adult hematopoiesis [3,25,32,33]. Also, DOT1L inhibitor drugs are currently the frontrunners in the treatment of MLL-rearranged leukemia. These drugs work by inhibiting the H3K79 methyltransferase activity of DOT1L. However, it has not been determined whether H3K79 methyltransferase activity is responsible for normal hematopoiesis, or if other domains of the DOT1L protein are facilitating it. This study sought to answer this question by use of *Dot1L* methyltransferase domain mutant mESCs and mouse line.

Both *in vitro* and *ex vivo* cultures of mutant mESCs and mutant E10.5 yolk sacs showed strikingly similar results. Mutant cells formed significantly fewer myeloid and mixed colonies compared to wildtype. The decrease in the number of mixed colonies was the most striking, with a near 90% reduction. Both models also showed defects in mutant cells' ability to form definitive erythroid colonies. Colony numbers were lower on average than wildtype, and they were significantly smaller. These results show that mESC *in vitro* cultures are a useful model in which to study hematopoiesis. They also show that the H3K79 methyltransferase activity of DOT1L is necessary for murine embryonic hematopoiesis.

This finding of H3K79 methyltransferase activity being necessary for murine embryonic hematopoiesis not only adds to the field of developmental biology, but it has implications in clinical medicine. Pharmacological agents that induce DOT1L methyltransferase activity could be used to treat certain anemias. Likewise, drugs that inhibit DOT1L methyltransferase activity could be used to stop blood cell cancer proliferation. Indeed, small molecule competitive inhibitors of DOT1L's methyl-donating cofactor S-adenosyl methionine (SAM) are currently being used in clinical trials to treat MLL-rearranged leukemia [29]. Additionally, this study found that loss of DOT1L methyltransferase activity leads to increased DNA damage and red

blood cell death. Therefore, synergistically treating leukemia with DOT1L inhibitors and DNA-damage inducing drugs may be more effective than treating with DOT1L small molecule inhibitors alone. Since DOT1L is a histone methyltransferase that interacts with several proteins and DNA, further research into its specific interactions and activities is likely to spawn significantly more treatment options for leukemia and anemia patients.

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