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The Intrinsic Apoptotic Cascade in Hematopoiesis and Susceptibility to Therapy-Related Leukemia

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by

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ABSTRACT OF THE DISSERTATION

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Apoptosis and the DNA damage response have been implicated in hematopoietic development and differentiation, as well as in the pathogenesis of myelodysplastic syndrome (MDS) and leukemia. However, the specific roles of direct mediators of apoptosis, such as caspases, in hematopoiesis and leukemogenesis have not been elucidated. In order to address this, we studied the effects of loss of *Caspase-9*, the initiator caspase of the intrinsic apoptotic cascade, and *Apaf1*, the key component of the apoptosome, on fetal and adult hematopoiesis. We first found that loss of these key regulators has significant effects on the hematopoietic stem and progenitor compartment, with decreases in erythroid and B-cell progenitor abundance and impaired function of hematopoietic stem cells after transplantation. Long term adult hematopoiesis is also altered in bone marrow chimeras lacking *Casp9* and *Apaf1*. Counter-intuitively, mice lacking these cell death components show low white blood cell counts, decreased B cell abundance and anemia. Ultimately, they die early due to bone marrow failure.

Defects in apoptosis have also been previously implicated in susceptibility to therapy-related leukemia, a disease caused by exposure to DNA-damaging chemotherapeutics used as treatment for other malignancies. Decreased apoptosis could allow cells to inappropriately survive exposure to DNA damaging agents, giving rise to a population of cells with increased DNA damage that was more prone to clonal outgrowth or malignant transformation. We examined whether loss of *Casp9* or *Apaf1* altered response to N-ethyl-nitrosurea (ENU), an alkylator similar to those used in chemotherapy, and found that loss of these genes did result in increased DNA damage in surviving cells after ENU-treatment. Furthermore, exome sequencing revealed that loss of Casp9 when combined with alkylator treatment gives rise to oligoclonal hematopoiesis, a precursor to diseases such as MDS and acute myeloid leukemia. Taken together these findings suggest that loss of apoptosis could be a key step in the pathogenesis of therapy-related disease.

CHAPTER 1

INTRODUCTION

1.1 Therapy-Related Acute Myeloid Leukemia

Therapy-related acute myeloid leukemia (tAML) is a hematological malignancy that develops following exposure to chemotherapeutic agents used as treatments for other tumors, most often breast cancer, Hodgkins disease (HD), non-Hodgkin Lymphoma (NHL) and acute lymphoblastic leukemia (ALL) [19]. It is the most common secondary malignancy and accounts for 10 to 20% of all AML cases in the US [45, 25, 19].

Two forms of tAML are recognized: tAML caused by exposure to alkylators such as cyclophosphamide, and tAML caused by exposure to topoisomerase II inhibitors such as etoposide. Approximately 75% of tAML cases are linked to alkylator exposure [41]. Alkylator-induced leukemia has an average latency of five years and is usually preceded by myelodysplastic syndrome (MDS), in which the patient develops peripheral cytopenias and trilineage dysplasia without frank leukemia [41]. Cytogenetically, this form of tAML is most often characterized by chromosome loss or deletion, particularly involving chromosomes 5 and 7 [25, 41, 22]. Topoisomerase IIinduced disease is less common but has a much more rapid course, with a latency of only 2 years and no MDS stage [45, 41]. In general, topoisomerase II inhibitors result in a disease characterized by chromosomal rearrangements, especially rearrangements involving the mixed lineage leukemia (MLL) locus at 11q23, which confer a more favorable prognosis and a better response to chemotherapy [41, 25].

Both classes of tAML carry a dire prognosis. This disease is resistant to treatment and has lower survival rates than *de novo* disease [25]. Various studies have placed the four or five year survival rate at anywhere from below 10% [2] to up to 24.5% in one German-Austrian study [15].

tAML patients are more likely to have unfavorable karyotypes such as monosomy 5 and are less likely to respond to chemotherapy than their *de novo* counterparts. Mean survival with an unfavorable karyotype is a dismal 5.6 months, while even with a favorable karyotype, patients survive only an average of 26.7 months after diagnosis [23].

Risk of tAML varies depending on the primary diagnosis and the types of chemotherapy used in primary treatment. A recent study found that risk for tAML is highest after treatment for Hodgkins Disease [34]. Other studies have reported the risk of tAML after HD or NHL as ranging from 0.8% to 10%, with risk increased by autologous hematopoietic stem cell

transplantation (aHCT) [13, 18, 10, 48, 26]. The risk of tAML after breast cancer is relatively low (0.2-1% in a recent study) [48], but because of the higher primary incidence of breast cancer and Non-Hodgkins Lymphoma, those two cancers actually accounted for about half of new tAML cases before 2008 [34]. Some reports suggest that the risk of tAML after certain primary malignancies may actually be declining due to changes in chemotherapy protocols. A study from Stanford University found that decreasing use of high doses of alkylating agents correlated with a decreasing risk of tAML after Hodgkins Disease from 1974 to 2003 [26]. The risk of tAML after breast cancer has also declined since the late 1970s, probably due to the decreasing use of the alkylating agent melphelan [34]. However, despite these few heartening examples, the overall rate of tAML is likely increasing. As the number of cancer survivors grows, so does the number of people at risk for tAML

Cancer survivors now make up 3.5% of the population, and that number is increasing by 2% per year [3, 28]. As survival rates following primary cancer increase and the use and dosage of chemotherapy escalates, these numbers will only increase [25]. Many physicians and scientists are now examining possible risk factors for tMDS/tAML in the hopes of being able to risk-stratify patients. As previously mentioned, risk for tAML does depends on the type of primary malignancy and the type and dosage of chemotherapy. Increasing age is also associated with increasing risk of tAML [15]. However, these are non-specific risk factors. Identification of more specific risk-factors for tAML could allow physicians to stratify patients by risk and modify primary cancer treatment accordingly, but few of these risk factors have yet been identified.

1.2 DNA Damage Response and Apoptosis

Both alkylators and topoisomerase II inhibitors act as anti-cancer drugs by inducing DNA damage in cells, leading to apoptosis. In theory, cancer cells should be more sensitive to these drugs because they replicate at a faster rate than healthy cells. However, all cells exposed to these drugs, healthy or otherwise, are susceptible to damage.

Alkylators such as n-ethyl-nitrosurea (ENU), the chemical used in this research, work by adding methyl or ethyl groups to DNA at the N7 and O6 atoms of guanine, the N1 and N3 atoms of adenine and the N3 atom of cytosine. N-alkylation is the most common effect of ENU, which belongs to the nitrogen mustard class of alkylators (similar to cyclophosphamide). However, the most common lesion induced by most alkylators used in chemotherapy (and the second most common lesion generated by ENU) is O6-methyl-guanine (O6MeG) [53, 19, 42]. Any of these lesions can be mutagenic. O6MeG lesions can be repaired directly by methyl-guanine methyltransferase (MGMT), which leads to point mutations, the most common being GC to AT transitions. N-methylation most often causes chromosomal aberrations, transversions, frameshifts and small deletions [19, 42, 53, 55].

While the mutagenic effects of these drugs are important, the key mechanism by which these drugs are thought to treat cancer is via induction of apoptosis. Any of the lesions listed above, if not repaired, can lead to apoptosis. Bone marrow cells express very low levels of MGMT, such that many O6MeG lesions in this compartment go unrepaired [3]. When these lesions are not repaired, mispairing occurs during DNA synthesis and this is recognized by the machinery of mismatch repair (MMR) [3]. The MMR machinery cannot excise the methylated base, and this leads to apoptosis either by direct activation of ATR and Chk1 by MMR proteins or by inappropriate processing of the lesion leading to secondary DNA

damage that blocks replication. Blocking replication causes S phase stress and/or DSBs. N-methylation is repaired by base excision repair (BER) or nucleotide excision repair (NER), which leads to depurination that can also cause replication blockage leading to S-phase stress and/or DSBs [42].

Regardless of the repair pathway initiated, most of these lesions eventually converge on DSBs and S phase stress, which induce apoptosis (see figure 1.1). DSBs initiate apoptosis by first activating ATM. ATM is recruited as an inactive dimer to the DSB by the MRN complex localized to phosphorylated H2Ax at the break point. ATM then autophosphorylates. Activated ATM has multiple targets that control cell cycle progression and apoptosis. ATM phosphorylates NBS1, which acts as an adaptor for ATM to phosphorylate Chk1 and/or Chk2, which in turn activates Cdc25, causing cell cycle arrest in S phase. ATM can also lead to arrest in G1 by phosphorylating p53, which activates p21 [42]. Induction of apoptosis is mediated by stabilization and phosphorylation of p53 and activity of Chk1 and Chk2. ATM phosphorylates the E2 ubiquitin ligase MDM2 to prevent it from inactivating p53. Activated p53 also upregulates Bax and PUMA, which cause mitochondrial outer membrane permeablization (MOMP). This releases cytochrome C and initiates the caspase cascade [6]. Activation of p53 is potentiated by this signaling cascade, as Chk1 and 2 also phosphorylate p53.

Chk1 and 2 activate E2F1, which stabilizes p53 and stimulates transcription of p73, APAF1 and Caspase-7 [5]. p73 and p63 are required for p53-mediated apoptosis, and p73 is also proapoptotic in its own right. p73, like p53, also upregulates PUMA, leading to Bax translocation to the mitochondria and cytochrome C release [6].

S phase stress activates apoptosis in a similar way to double strand breaks. Replication protein A (RPA) binds to single stranded DNA at the stalled replication fork and recruits

ATR. ATR then acts in much the same way as ATM, transmitting its signal to E2F1 and p53, leading to apoptosis [6]

Ultimately, all these pathways converge at the mitochondrial membrane. A key step in the intrinsic apoptotic cascade is the release of cytochrome C from the intermembrane space of the mitochondria. This release is accomplished by the formation of pores in the outer membrane of the mitochondria. Exactly how these pores form is still debated, but it is known to involve the Bcl-2 family proteins Bax and/or Bak. Bax transcription is upregulated by p53 in response to DNA damage, while p53 activates Bak by releasing it from its inhibitor Mcl-1. Bax and/or Bak then translocate to the mitochondria and hetero or homo-oligomerize in the outer mitochondrial membrane [6, 24]. These oligomers may act as channels on their own, allowing cytochrome C to enter the cytosol, or they may interact with VDAC to increase mitochondrial membrane permeability by opening the mitochondrial permeability transition pore [24].

Once cytochrome C is released, it binds to oligomers of APAF-1, which in turn bind procaspase-9, forming the apoptosome. Dimerization of procaspase-9 triggers autocatalytic activity, activating and releasing Caspase-9. Caspase-9 then cleaves the effector caspases 3 and/or 7, which cleave various target proteins, ultimately resulting in the characteristic sequelae of apoptosis such as DNA fragmentation, cell shrinkage, membrane blebbing and, finally, death (Tait and Green 2010 NRMCB).

1.3 MOMP and Caspase-Independent Cell Death

Many genetic alterations and novel chemical compounds have been shown to block apoptosis after the mitochondrial membrane has already been permeablized. Most often, this inhibition

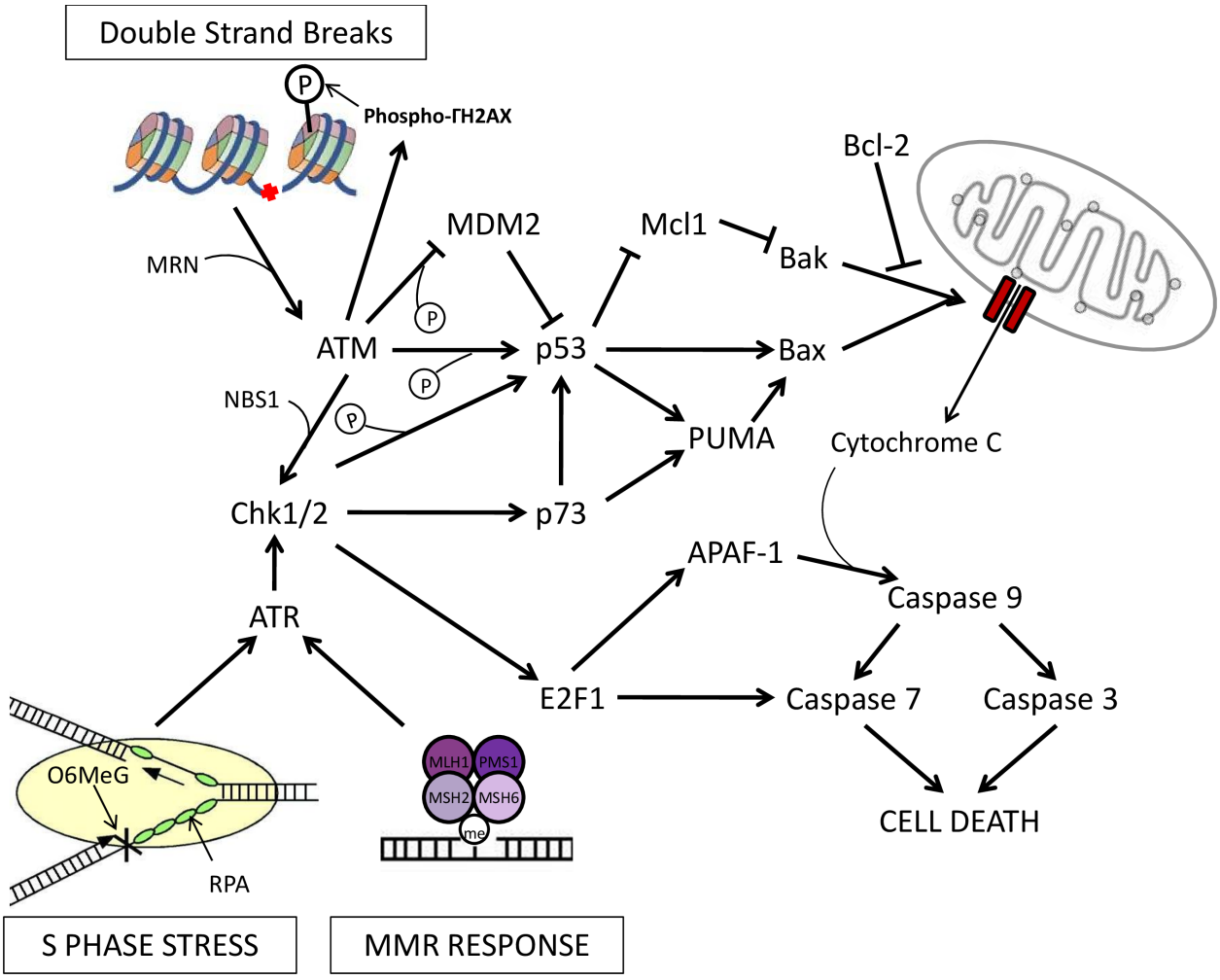

Figure 1.1: The Apoptotic Cascade in Response to DNA Damage

is accomplished by preventing activation of the caspase cascade. Mutations in caspase genes or in *Apaf1*, which activates Caspase-9, have been shown to decrease apoptosis in response to certain stimuli. Many caspase inhibitors have also been developed which show similar results. However, there have been conflicting reports concerning just how alive cells are after failing to undergo apoptosis due to caspase blockade. Cells that have permeablized their outer mitochondrial membrane but not activated the caspase cascade have been reported to die via Caspase Independent Cell Death (CICD) a slower but still effective method of cell death that does not require caspase activity [50]. Cells may divide up to two times after MOMP, but then they arrest and begin the process of CICD [27]. It has been suggested that CICD occurs due to the activities of pro-apoptotic factors, such as Apoptosis Inducing Factor, Smac or Omi, that are released from the mitochondria during MOMP. More recent evidence suggests that it is due mitochondrial dysfunction leading to progressive loss of oxidative phosphorylation and the ATP it provides [46, 14, 27].

However, there is also evidence to suggest that cells can avoid CICD and survive, albeit while manifesting severe dysfunction. Several groups have reported that cells deficient in Caspase-9 maintain coupled electron transport and respiration, even after cytochrome C release [9, 12, 44]. No evidence of CICD was found in mast cells deficient in Apaf1 or Caspase-9 or in cells lines either deficient in or expressing a dominant negative form of Caspase-9 [31, 9, 44]. When exposed to an apoptotic trigger these cells ceased proliferation but maintained metabolic activity and did not appear to die. This could be due to the fact that MOMP is perpetuated by caspase activity [27]. Activated caspases cleave Bid, Bcl2 and p75 (a component of complex I), increasing membrane permeablization after induction of apoptosis [52, 51]. As a result, cells deficient in caspase activity show relatively less of a decrease in mitochondrial membrane potential after apoptotic stimuli, indicating that MOMP is less

successful in these cells [12, 44, 14]. Thus, these cells may not undergo CICD after apoptotic stimuli, but can they recover from cell cycle arrest and proliferate?

Recent reports have suggested that incomplete MOMP may allow cells to recover from the initiation of apoptosis. Mitochondria that are largely intact can repopulate the cell with healthy mitochondria while the permeablized mitochondria are cleared away via mitophagy [51, 50, 14]. Provided that the cell can survive the ATP deprivation while the mitochondria repopulate, once this process is complete, it may go on to survive and proliferate.

1.4 DNA Damage Response and Apoptosis in Hematopoiesis

The regulation of the DNA damage response (DDR) and apoptosis is essential for normal growth and differentiation of all mammalian cells, but they have been shown to be of particular importance in the hematopoietic compartment. This importance was first demonstrated with the discovery that the process of hematopoiesis changes with age. This has been linked to the accumulation of DNA damage that occurs over time in aging cells, particularly in hematopoietic stem cells (HSCs). As humans age, the number of HSCs in the bone marrow increases, but the function of each individual stem cell decreases [49]. Lymphopoiesis also declines over time, possibly due to the exhaustion of lymphoid-competent stems cells, resulting in a myeloid-biased hematopoietic compartment [49, 30, 47]. The aging hematopoietic system is also more prone to developing myelogenous diseases such as MDS and AML [30].

While the studies of the aging bone marrow are largely correlative, much work in recent years has demonstrated that DNA damage can adversely affect HSCs. HSCs are a unique subset of hematopoietic cells; in the highly proliferative hematopoietic system, HSCs are

largely quiescent, maintaining themselves in the G0 phase and proliferating only rarely [36]. As such, they go through fewer cell cycle checkpoints when cellular machinery can detect and repair DNA damage [49]. HSCs can accumulate DNA damage more readily than more differentiated cells and the HSCs of aged mice show greater numbers of DNA breaks than progenitor cells from the same mice [35]. HSCs also respond differently to DNA damaging and apoptosis-inducing stimuli. When compared to progenitor cells, HSCs show less efficient or delayed repair of DNA lesions after irradiation or alkylator treatment, maintaining DNA lesions longer than more differentiated cells [35, 37, 7, 38, 33]. HSCs can also be induced to senesce or apoptose more easily in response to DNA damage, whether it be due to radiation or excessive endogenous reactive oxygen species (ROS) production [37, 33]. Ultimately, accumulated DNA damage is thought to induce HSCs to decrease self-renewal by death or differentiation, probably as a protective mechanism to prevent the propagation of the damage through the hematopoietic tree [49, 33, 54]

Since HSCs are so uniquely sensitive to the effects of DNA damage, it is not surprising that mutations in genes involved in DDR or apoptosis would have serious affects in these cells. Direct loss of mediators of DNA repair has a dramatic effect on hematopoiesis in vivo. Mutations in genes necessary for nucleotide excision repair (XPD), telomere maintenance (mTR and Terc), non-homologus end joining (Ku80, Lig4), mismatch repair (Msh2), and homologous recombination repair (Rad50, Brca2) all cause increases in detectable DNA damage in HSCs (and other cells) and are associated with impaired HSC function and premature exhaustion of stem cells. HSCs from these mice are more prone to spontaneously apoptose and also generally show lower rates of proliferation, although the absolute numbers of stem cells are not altered. [43, 35, 37, 1, 38].

Farther downstream in the DDR pathway sits p53, a key regulator that can direct damaged cells toward cell cycle arrest and repair or apoptosis depending on the severity of the lesions.

Expression of p53 is preferentially high in HSCs [36, 1], suggesting its importance in these cells. Loss of p53 allows hematopoietic cells to survive irradiation and enhances the ability of HSCs to engraft after such treatment [33]. P53 normally also promotes HSC quiescence and when it is lost, the cells more easily enter the cell cycle and self renewal in increased [36, 4]. These more proliferative HSCs have an initial advantage against wild type cells in competitive transplantation, but the HSCs are less effective than wild type in serial transplantation [36]. While p53-null mice do not suffer from the same stem cell exhaustion and bone marrow failure as DNA repair mutants, the failure of serial transplantation suggests that they do share a common, if less severe, defect in stem cell function in the long term.

At the end of the DDR pathway lies apoptosis, mediated by Bcl-2 family members and caspases. Interestingly, increasing expression of the proapoptotic Bcl2 has a proliferative phenotype. Mice overexpressing Bcl2 have enlarged HSC compartments and these stem cells have a competitive advantage in transplantation and in plating assays. HSCs from these mice actually cycle less frequently than wild type cells and do not show any exhaustion defect [17]. This would suggest that while accumulation of DNA damage in HSCs is ultimately damaging, evasion of the apoptosis that this damage might generate could be advantageous.

1.5 DNA Damage Response and Apoptosis in Leukemogenesis

Evasion of apoptosis has long been implicated as a critical step in tumorigenesis. In order for cancer cells to overgrow and become malignant, they must escape the machinery of programmed cell death designed to curtail such growth. The frequency of mutations in *p53* and other apoptosis pathway genes in solid tumors bears this out. However, the role of apoptosis

in the genesis of hematologic malignancies is not as well understood. Mutations in *p53* occur at a much lower rate in heme malignancies, specifically acute myeloid leukemia (AML), than in solid tumors. Various studies put the rate of *p53* mutations in de novo AML between 5 and 15% (compared to 50% in colon cancer). However, the low rate of mutation does not necessarily indicate that evasion of apoptosis is unimportant in leukemogenesis. *P53* mutations in AML are often associated with increased disease severity, complex karyotype, and chemoresistance [8, 39]. Also, alterations in many other components of the p53-apoptosis pathway have been identified in AML. Hypermethylation of the *p73* promoter, resulting in decreased p73 expression, was found in AML cell lines and primary samples [40]. Hypermethylation of the *Apaf1* promoter was similarly found in 25% of leukemia cell lines and 42% of primary AML cells studied [21]. Gene fusions associated with leukemia have also been shown to affect apoptosis. PML-RAR may impair p53 function, AML1-ETO represses transcription of *ARF*, a stabilizer of p53, and BCR-ABL inhibits the formation of the apoptosome [8, 39, 50].

Changes in apoptosis and DDR might be predicted to be particularly important in susceptibility to therapy-related AML, which develops after exposure to DNA damaging agents used as chemotherapeutics. Hematopoeitic stem or progenitor cells that were exposed to that initial DNA damaging agent and survived are the likely sources of the new tumor. This suggests that failure to undergo apoptosis could be key to susceptibility to this disease. There is some evidence in the literature to support this hypothesis. Somatic mutations of p53 are much more common in tAML than in *de novo* AML, and tAML case samples showed lower expression of p53 than control samples in a study of HD and NHL patients [48, 16]. Hypermethylation of the BRCA1 promoter is also much more common in tAML than in *de novo* AML [11]. There is also evidence to suggest that tAML is associated with an increased level of DNA damage. tAML blasts often show microsatellite instability, a sign of DNA damage

that is rare in *de novo* AML [11]. tAML is also associated with accelerated telomere shortening, which is linked to genomic instability [15, 13]. Both prospective and retrospective studies have been performed to try to isolate germ-line genetic variants that might predispose to tAML, and those found have often been associated with the DNA damage response or apoptosis. A functional polymorphism in the promoter of MLH1, which has been demonstrated to result in decreased expression of that key DNA repair gene, is associated with increased risk of tAML [56, 32]. The P72R SNP in p53 has also been reported to be associated with risk for tAML, although there are conflicting reports as to which allele is deleterious and whether cooperating SNPs are also required [16, 18]. Association studies such as these are difficult to perform in tAML because of the relative rarity of tAML cases, which necessitates the accumulation of cases over a long time period or the use of a very small sample size [20]. An interesting functional study, examining CD34+ cells destined for autologous stem cell transplantation in Hodgkins and Non-Hodgkins Lymphoma patients showed some promising results that might be missed by a gene association study. CD34+ cells from patients that eventually went on to develop tAML showed a unique gene expression signature that specifically showed decreased expression of genes involved in mitochondrial oxidative phosphorylation, cell cycle checkpoints and DNA repair. In functional assays, these cells also showed higher mitochondrial ROS production and sustained ROS elevation after treatment with DNA damaging agents as well as increased H2Ax foci after treatment with a nitrogen mustard compound. These findings suggest that even before development of tAML, stem cells from patients show functional defects in mitochondria and abnormal accrual of DNA damage [29].

1.6 Rationale

The DNA damage response and apoptotic cascade have already been proven important in hematopoietic homeostasis and in susceptibility to hematopoietic malignancy. However, the components of these pathways downstream of the mitochondria have not been well studied in normal hematopoiesis or in models of therapy-related disease. The work in this thesis addresses these gaps in our understanding. We specifically examine *Caspase-9*, the initiator caspase of the intrinsic apoptotic cascade. Caspase-9 activity is known to be essential for apoptosis in response to DNA damaging agents, cytokine withdrawal and intrinsic stimuli. However, it has not been thoroughly studied in the hematopoietic compartment and its role in leukemogenesis is unknown.

In the first section of this thesis, we examine the role of *Caspase-9* in a murine model of susceptibility to therapy-related leukemia. This allows us to determine if a correlation exists between expression of this key apoptotic effector and incidence of tAML in mice.

In the second section of this thesis, we examine how complete loss of *Caspase-9* in the hematopoietic compartment influences HSC abundance and function as well as long-term overall hematopoietic function. In this section we also establish how loss of *Caspase-9* affects the response to an alkylator and, though exome sequencing, show that loss of *Caspase-9* combined with alkylator treatment can lead to clonal hematopoiesis, an early sign of disease.

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CHAPTER 2

MOUSE STRAINS SUSCEPTIBLE TO ALKYLATOR-INDUCED LEUKEMIA EXPRESS NOVEL ISOFORMS OF CASPASE-9

2.1 Introduction

Therapy-related acute myeloid leukemia (tAML) is a late complication resulting from exposure to alkylating agents, radiation, and/or topoisomerase II inhibitors used as treatment

for primary malignancies and autoimmune disorders. tAML makes up 10 to 20% of new AML cases and the incidence is rising, but therapeutic options and disease outcome remain poor. Only a small percentage of patients exposed to alkylating agents and topoisomerase II inhibitors develop tAML and little is known about what predisposes these patients to tAML. While factors such as age and chemotherapy dose have some predictive value in tAML, current evidence suggests that predisposition to the disease is also genetic. Identification of genetic variants that predispose to tAML could be crucial to its prevention, since it would allow physicians to identify patients at risk for tAML and adjust chemotherapeutic regimens accordingly.

Our lab has used a murine model to study tAML and has found that susceptibility to alkylator-induced leukemia varies by strain. When mice are exposed to N-ethyl-nitrosurea (ENU), an alkylator similar to those used clinically, certain strains such as PL/J, SWR/J, and DBA/2J develop myeloid malignancies while others, such as C57Bl/6J and 129Sv/J do not [3]. Susceptibility was found to correlate with patterns of gene expression in kit+, lineage- hematopoietic cells (KL cells). [2] In this analysis, the single most differentially expressed gene was *Caspase-9* (*Casp9*). Caspase-9 is an aspartic acid-specific protease that functions as the initiator caspase of the intrinsic apoptotic cascade. It is activated following binding to APAF1 after cytochrome C release from the mitochondria and exerts most of its effects via cleavage and activation of effector caspases 3 and 7 [8] *Casp9* is particularly interesting as a candidate susceptibility factor in tAML because it has been previously implicated in the pathophysiology of various malignancies. Evasion of apoptosis is believed to be a key step in the etiology of many neoplasms, and decreased expression or loss of function in *Casp9* would accomplish this. Decreased expression of *Casp9* is associated with resistance to etoposide in melanoma cells [4]. Haplotypes of *Casp9* have been associated with changes in the risk of lung cancer and this correlated with functional data showing increased *Casp9* expression

with those haplotypes that correlated with lower susceptibility [7]. A similar study in non-Hodgkins lymphoma found one SNP in *Casp9* that was associated with decreased disease risk [6]. Most interestingly, a dominant negative isoform of Caspase-9 has been isolated from human gastric carcinoma cells and shown to suppress apoptosis *in vitro*.

Based on this evidence, we examined the expression of *Casp9* in a susceptible (DBA/2J) and a resistant (C57Bl/6) strain to determine how the observed differential *Casp9* expression might be contributing to susceptibility to ENU-induced leukemia. Our analysis revealed that susceptible strains express low levels of Casp9, but express two novel isoforms not seen in resistant strains. These isoforms are generated by alternative splicing but do not generate detectable protein. KL cells from susceptible strains show decreased apoptosis in response to an alkylating agent, likely due to the decreased expression of full length Casp9. This apoptotic defect could contribute to susceptibility to ENU-induced leukemia in these mice.

2.2 Materials And Methods

Mice. Male C57Bl/6J (stock number 000664) and DBA/2J (stock number 000671) inbred mice were obtained from The Jackson Laboratory at 8 weeks of age and analyzed within 2 weeks of arrival. Adult *Casp9*$^{+/-}$ mice were provided by Richard Flavell (Yale University, New Haven, CT). All mice were housed in a pathogen-free facility, and studies were performed in compliance with protocols approved by the Washington University Animal Studies Committee.

Isolation of c-kit positive, lineage negative (KL) cell population. Mice were sacrificed at 8 to 10 weeks of age. Bone marrow was removed from the femurs and tibias by centrifugation. For the C57Bl/6J strain, bone marrow from 4-5 males was pooled; for the

DBA/2J strains, bone marrow from 6-7 males was pooled to ensure stem/progenitor population cell counts similar to those of C57Bl/6J pools. Pooled bone marrow was subjected to red cell lysis, washed and stained for c-kit (BD Biosciences) and a panel of lineage markers (Gr-1, CD19, B220, CD3, CD4, CD8, Ter119, and IL-7R). After staining, c-kit+, lineage- (KL) cells were sorted (MoFlo, DAKO Cytomation) into cell culture media. RNA was isolated from sorted cells using the miRNA Easy kit (Qiagen). The remaining of cells were cultured overnight in complete media (RPMI containing 1%l-glutamine, 20%fetal bovine serum) supplemented with recombinant hematopoietic cytokines (100ng/ml stem cell factor, 6ng/ml IL-3, 50ng/ml Fms-related tyrosine kinase 3 ligand, 10ng/ml thrombopoietin; all from Peprotech, Rocky Hill, NJ, USA).

RNA expression analysis. cDNA was prepared from RNA isolated from sorted KL cells. Two sets of primers were designed for qRT-PCR, one targeting exons 2-3 and the other targeting exons 8-9 (see supplementary table 1). The sets were designed so that the products spanned two adjacent exons, thereby minimizing background fluorescence due to probe hybridization to amplified genomic DNA. Probes were dual-labeled and 6-careboxyfluorescein and 6-carboxytetramethyl-rhodamine. Strain-dependent *Casp9* expression was determined by qRT-PCR assays with Taqman detection chemistry and the ABI Prism 7300 Sequence Detection System. Each assay was performed in triplicate. Triplicates with standard deviations over 0.5 cycles were discarded. An independent primer/probe set targeting GAPDH was used as the positive control. Primer/probe calibration curves for the test and positive control primers were analyzed to ensure comparable primer/probe efficiency before running experimental assays. The three C^T values obtained from each triplicate were averaged and normalized against the positive control C^T values. Relative expression was calculated by the delta-delta C^T method. An undetermined C^T was imputed to be a C^T of 40, the maximum cycle number, for purposes of statistical analysis.

Qualitative PCR analysis and identification of isoforms. cDNA was amplified by PCR with a set of primers that encompassed the full *Casp9* cDNA sequence (see supplementary table 1). Casp9 isoforms were resolved by polyacrylamide gel electrophoresis and quantified by densitometry.

PCR products were cloned into the pCR2.1-TOPO Vector (Sigma-Aldrich). Clones of each of the three isoforms were sequenced using Sanger chemistry (see supplementary figure 1).

Retroviral transduction of fetal liver cells. Timed mating of *Casp9*$^{+/-}$ mice was performed and fetuses were obtained at embryonic day 15.5. Tail DNA was prepared (DNeasy, Qiagen) and used for genotyping. Fetal livers were homogenized to yield a single cell suspension and stored in freeze media (FBS with 10% DMSO) in liquid nitrogen until use.

A YFP cDNA (pEYFP-N1, Clonetech, Mountain View, CA, USA) was substituted for GFP in a recombinant murine stem cell proviral plasmid (MSCV2.2-*ires-GFP*, provided by Michael Tomasson, Washington University). *Caspase-9* isoforms were subcloned into MSCV2.2-ires-GFP and MSCV2.2-ires-YFP. Retroviral supernatants were generated by transient transfection of 293T cells with Ecopac (Cell Genesys, Foster City, CA, USA) and the MSCV constructs. Forty-eight hours after transfection, retroviral supernatants were harvested and titered on *Casp9*$^{+/-}$ fetal liver cells using flow cytometry.

Fetal liver cells were thawed and cultured in complete media (RPMI containing 1%l-glutamine, 20%fetal bovine serum) supplemented with recombinant hematopoietic cytokines (100ng/ml stem cell factor, 6ng/ml IL-3, 50ng/ml Fms-related tyrosine kinase 3 ligand, 10ng/ml thrombopoietin, and 10 ng/ml IL-6; all from Peprotech, Rocky Hill, NJ, USA) for 24hours. A total of 13 10^6cells were infected by centrifugation at 2500$g$for 90min in the presence of 10mcg/ml polybrene (Sigma, St Louis, MO, USA) and 33umHEPES with retroviral supernatants using an MOI of 1-2.

Induction and analysis of apoptosis. N-ethyl-nitrosurea (ENU; Sigma) was resuspended at a concentration of 100 ng/ul in dimethyl sulfoxide (DMSO) and stored at -20°C until use. KL cells or FLCs were treated with ENU 24 hours after sorting or viral infection, respectively. To ensure that all cells saw the same volume of DMSO even when exposed to varying dosages of ENU, ENU was serially diluted in DMSO and the same total volume of ENU in DMSO was added to each well of the culture dish. Cells were incubated with ENU/DMSO for 1 hour at 37°C. The cells were then washed in PBS and incubated overnight in fresh media. 24 hours after ENU treatment, apoptosis was measured by Annexin V staining via FACS analysis.

Western blot analysis. *Caspase-9* isoforms were also subcloned with an N-terminal myc tag into the p3xFlag-CMV-9 vector (Sigma-Aldrich, St Louis, MO). These isoform constructs were transfected into 293T cells using Lipofectamine 2000 (Invitrogen-Life Technologies, Grand Island, NY).

Protein was harvested from infected FLCs or transfected 293T cells 24 hours after infection/transfection by lysis in RIPA buffer with protease inhibitors. Protein was run on 10% bis-tris gels using the NuPAGE electrophoresis system (Invitrogen-Life Technologies, Grand Island, NY). Protein was then transferred to a nitrocellulose membrane and the membrane probed with Caspase-9 mouse monoclonal antibody, myc-tag mouse monoclonal antibody, and/or GFP rabbit antibody (all from Cell Signaling).

2.3 Results

Mouse strains susceptible to alkylator-induced myeloid malignancies express low levels of *Caspase-9*. We previously reported that susceptibility to alkylator-induced

leukemia varies by mouse strain, and that this variation correlates with differences in gene expression in c-kit positive, lineage negative (KL) cells [3]. The single most differentially expressed gene in that microarray expression analysis was *Caspase-9* (*Casp9*), the initiator caspase of the intrinsic apoptotic cascade. By microarray, mouse strains that are susceptible to alkylator-induced leukemia express much lower levels of *Casp9* than resistant strains (data not shown). To confirm these expression differences, we isolated *Casp9* mRNA from sorted KL bone marrow cells and performed qRT-PCR to quantify *Casp9* expression. Primers and probes targeting exons 2-3 and 8-9 were used. qRT-PCR with either set of primer/probes shows no detectable expression of *Casp9* in strains susceptible to alkylator-associated leukemia (DBA/2J, PL/J), while resistant strains (C57BL/6J, C3H/HeJ) show significant expression of *Casp9* (**Fig. 2.1a**).

DBA/2J KL cells express variant isoforms of Caspase-9. To further analyze *Casp9* mRNA expression in susceptible strains, we performed qualitative PCR using primers in exons 1 and 9 of *Casp9* to amplify the entire coding region. This PCR amplified full-length *Casp9* in both susceptible (DBA) and resistant (B6) KL cells, indicating that, even though expression of *Casp9* was undetectable by qRT-PCR, transcription of the *Casp9* gene is occurring in KL cells from susceptible strains. However, this PCR also amplified two variant isoforms of *Casp9* only in the DBA KL cells (**Fig. 2.1b**). Subsequent cloning and sequencing of these isoforms revealed them to be splice variants. The longer isoform (isoform B) is generated by insertion of a novel exon (exon 1a) between exons 1 and 2 in the transcript. The shorter isoform (isoform C) is generated by skipping of exon 2 (**Fig. 2.1d**). Both splice variants result in frameshifts and premature stop codons, predicted to cause early nonsense-mediated decay or production of a truncated protein product (**Fig. 2.2a**).

To assess whether these novel isoforms are translated or targeted for nonsense mediated decay, we cloned all three DBA/2J isoforms with an N-terminal Myc tag and expressed

these constructs in 293T cells. The epitope-tagged proteins were not detectable, although endogenous wild type Caspase-9 was detected (**Fig. 2.2b**). This indicates that the variant isoforms are either targeted for nonsense-mediated decay and therefore not translated, or that the translated protein is expressed below the level of detection.

KL cells from DBA/2J mice show decreased apoptosis in response to ENU. To determine if the variation in *Casp9* expression is associated with alterations in apoptosis in susceptible mouse strains, we exposed sorted KL cells from C57Bl/6J and DBA/2J mice to n-ethyl-nistrosurea (ENU), a prototypical alkylator, and measured apoptosis by Annexin V staining 24 hours after ENU treatment. KL cells from susceptible DBA/2J mice underwent approximately 20% less apoptosis than C57Bl/6J counterparts in response to high doses of ENU (**Fig. 2.3c**). This suggests that the variation observed in *Casp9* expression in DBA/2J KL cells is correlated with observable changes in the apoptotic response. However, this does not resolve whether these changes are due to the low overall expression of *Casp9* in these cells, or due to specific expression of the novel variants seen in DBA/2J cells.

DBA/2J variant Casp9 isoforms are hypomorphic. The novel isoforms, if expressed, are predicted to contain the first 44 amino acids of the Caspase Activation and Recruitment Domain (CARD), which is responsible for the binding of Caspase-9 to Apaf1 for activation and to other caspases for cleavage. A truncated form of Caspase-9 isolated in human gastric carcinoma, termed Caspase-9 beta, has been shown to act as a dominant negative suppressor of apoptosis [5]. Caspase-9 beta lacks 150 amino acids, including the enzymatic active site, but contains an in-frame CARD. The novel isoforms containing a partial CARD, could also act as dominant negatives via a similar mechanism.

To assess the functional status of the novel Casp9 isoforms, fetal liver cells (FLCs) lacking endogenous Casp9 were obtained from *Casp9*$^{-/-}$ E15.5 mouse embryos. *Casp9*$^{-/-}$ FLCs

undergo apoptosis in response to genotoxic stimuli at a much lower rate than *Casp9*$^{+/+}$ FLCs (**Fig. 2.3b**). Next, wild type *Casp9* from C57Bl/6J or DBA/2J was overexpressed in *Casp9*$^{-/-}$ FLCs with or without the two variant isoforms. The cells were exposed to ENU and apoptosis was assessed by Annexin V staining. Wild type *Casp9* from both strains of mice was able to rescue the *Casp9*$^{-/-}$ phenotype but the variant isoforms were not (**Fig. 2.3b**), suggesting that the novel isoforms do not have the pro-apoptotic function of wildtype Casp9.

To determine whether the isoforms have dominant negative function, each isoform was overexpressed in *Casp9*$^{+/+}$ FLCs followed by the same apoptosis assay. The rates of apoptosis in response to ENU were comparable in *Casp9*$^{+/+}$ FLCs infected with empty vector or MSCV containing either of the isoforms (**Fig. 2.3d**), indicating that the isoforms cannot suppress endogenous Casp9 function and thus do not have dominant negative function. Co-infection of wild type and variant Casp9 into *Casp9*$^{-/-}$ cells similarly showed no suppression of apoptosis (data not shown).

2.4 Discussion

We have previously reported that susceptibility to alkylator-induced leukemia varies between mouse strains. Here we demonstrate that this differential susceptibility correlates with expression of *Caspase-9*. Mouse strains that express very low levels of *Casp9* have increased susceptibility to alkylator-induced leukemia. These low levels of *Casp9* also correlate with decreased apoptosis in response to ENU, which likely contributes to the increased susceptibility. Interestingly, although *Casp9* was expressed only at very low levels in susceptible strains, we were able to identify variant isoforms of Casp9 that are unique to susceptible strains. These alternatively spliced isoforms cannot be detected by western blot and have

no detectable activity, suggesting that they do not contribute to the apoptotic phenotype observed in susceptible strains.

Novel isoforms of *Casp9* have been isolated previously and found to be functional. An isoform termed *Casp9b* or *Casp9* has been found in various human cell lines, including Jurkat cells and gastric carcinoma cell lines, and also detected in some normal tissues, especially skeletal muscle [5, 9]. This isoform is generated by alternative splicing and contains the Caspase Activation and Recruitment Domain (CARD) and the C-terminal small subunit caspase domain, but lacks the enzymatic active site. Casp9b acts as a dominant negative, probably by competing with full length Caspase-9 for binding to Apaf1. Casp9b can bind Apaf1 because it contains a CARD, but since it lacks the enzymatic active site it cannot be processed and, thus, cannot continue the caspase cascade. It has been estimated that a 1:1 ratio of Casp9b to full length Casp9 is sufficient to block 77% of apoptosis [9]. Another dominant negative isoform has also been isolated from normal rat tissue. This isoform, *Casp9-CTD*, contains an alternative C-terminal sequence that prevents cleavage of Caspase-9 after activation and inhibits its activity. This isoform, as well, is dominant negative, probably also via competition for Apaf1 binding [1]. The novel isoforms presented here contain only a partial CARD and, therefore, may not be able to bind to Apaf1. This would explain the failure of these isoforms to act in a dominant negative manner.

Detecting protein expression of Casp9 in sorted kit+, lineage- cells proved challenging because of the small number of cells purified in sorting and because of the relatively low sensitivity of western blotting. The *Casp9* sequences detected in KL cells, however, can be expressed. We were able to clone sequences from KL cells and express them in plasmid vectors for exogenous expression in other cells, suggesting that our inability to detect Casp9 expression in KL cells was simply due to the difficulty of western blotting in a small number of cells.

Ultimately, the novel isoforms we report here do not appear to contribute to susceptibility to tAML. However, the variability in *Casp9* expression observed between strains and the correlation between low *Casp9* expression and decreased apoptosis could suggest a mechanism for susceptibility. Alkylators such as ENU are used as chemotherapeutics because they induce cell death. The DNA damage these agents cause is detected by the cellular machinery and, when the damage cannot be repaired, prompts the cell to undergo apoptosis. In mouse strains with low *Casp9* expression and, thus, lower levels of apoptosis, cells harboring DNA damage might be able to inappropriately survive after alkylator treatment. These cells might then be prone to malignant transformation and ultimately cause tAML. The fact that the variation in expression is detected in KL cells, a population enriched for the hematopoietic stem and progenitor cells thought most likely to give rise to leukemia, only makes this possible mechanism more interesting.

2.5 Figure Legends

Figure 2.1. Resistance to alkylator-induced AML in mice is related to levels of *Casp9* expression and expression of novel variant isoforms. **(a)** Mouse strains resistant to tAML show very low expression of the initiator caspase, *Casp9*, in kit+, lin- cells as determined by rtPCR using unique primer sets. To determine if any *Casp9* is expressed in resistant KL cells, RNA extracted from sorted kit+/lin- cells (KL) from C57Bl/6J and DBA/2J mice was reverse transcribed and the cDNA was subjected to qualitative PCR to amplify any *Casp9* transcripts (primer positions indicated by black arrows in panel B). **(b)** Three isoforms can be seen in DBA/2J cells: wild type (isoform A, blue), a novel long isoform (isoform B, red) and a novel short isoform (isoform C, green). These isoforms are not present in cDNA from C57Bl/6 mice. **(c)** The relative abundance of each isoform is

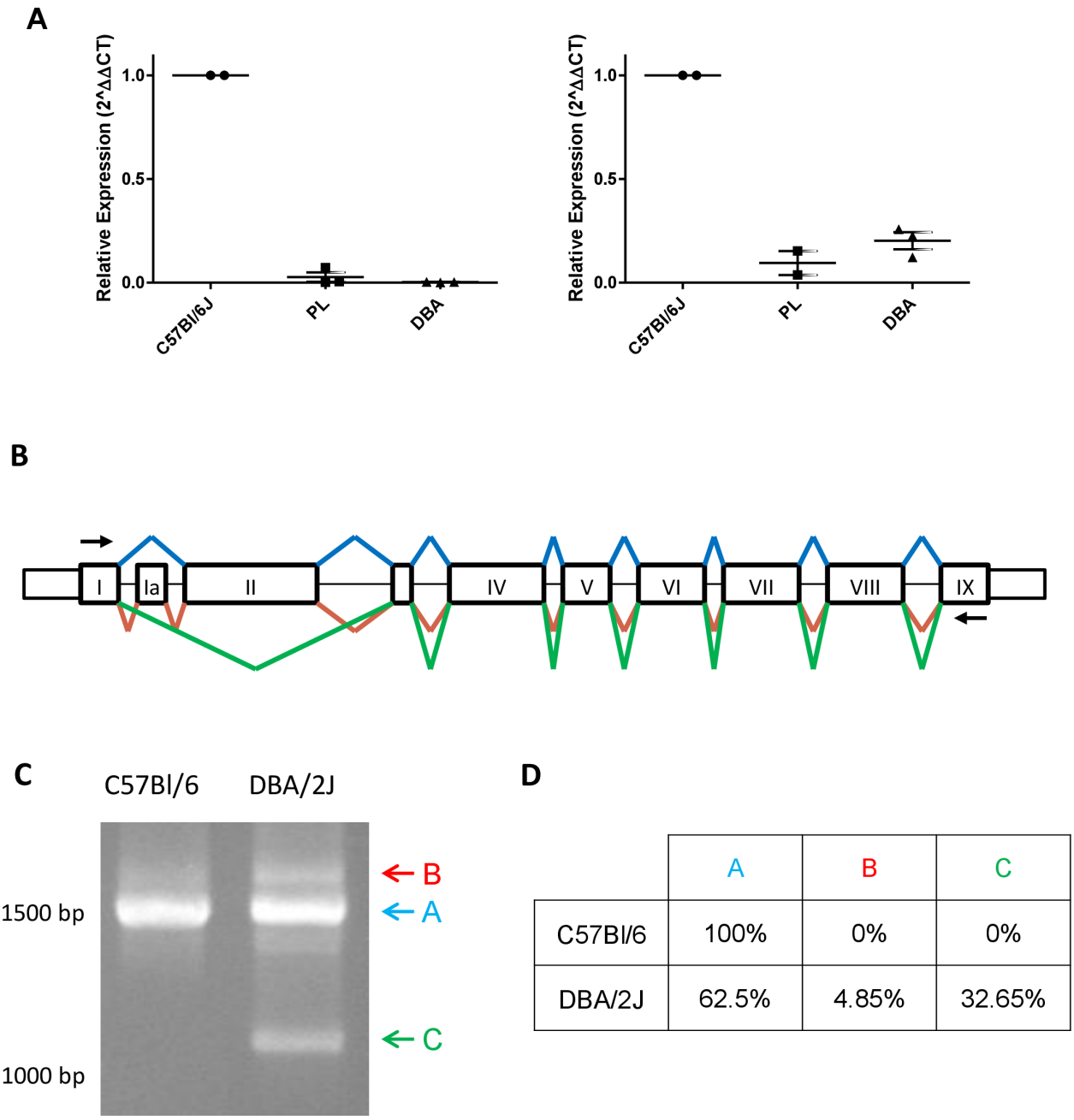
shown. This was calculated using band intensity from gel electrophoresis of qualitative PCR (n=4 biological replicates). **(d)** Sequencing of the PCR products revealed that both novel isoforms are generated by alternative splicing. Isoform B contains a novel exon (termed 1a) and Isoform C is generated by skipping of exon 2.

Figure 2.2. Variant Casp9 isoforms are not detectable by western blot. (a) The alternative splicing that generates isoforms B and C results in frameshifts which lead to premature stop codons. Both isoforms would contain only a partial CARD domain and have drastically reduced molecular weights (numbers in parentheses give weight with a Myc tag). Each isoform was cloned with a 5 Myc tag and transfected into 293T cells. **(b)** Protein expression was determined by western blot 24 hours after transfection. Only the wild type isoform can be detected, suggesting that if the alternative isoforms are expressed, it is at a level beneath the detection of this assay. Expected positions of isoforms B and C on the blot are shown with green and red arrows, respectively.

Figure 2.3. Variant *Casp9* isoforms are hypomorphic. The three detected DBA/2J *Casp9* isoforms were cloned into MSCV-IRES-GFP for expression in mammalian cells. (**a**) To assay functionality of the *Casp9* isoforms, *Casp9*$^{-/-}$ or *Casp9*$^{+/+}$ fetal liver cells (FLCs) were infected with MSCV containing each individual isoform. Levels of expression measured by western blot 24 hours after infection showed successful expression of wild type Caspase-9 from both C57Bl/6 and DBA/2J, with protein levels higher than endogenous levels in wild type cells. (**b**) 24 hours after infection, cells were treated with ENU for one hour. 24 hours after ENU treatment, cells were stained for AnnexinV (AnnV) and analyzed by FACS. Expression of wild type *Casp9* from either strain results in increased apoptosis relative to empty vector control in *Casp9*$^{-/-}$ cells (phenotypic rescue). However, expression of either variant isoform results in no increase in apoptosis, suggesting that they do not have caspase activity. (**c**) Expression of the isoforms is associated with decreased apoptosis. KL cells were

sorted from C57Bl/6 or DBA/2J bone marrow and cultured in cytokine-enriched media for 24 hours. Cells were then treated with varying doses of N-ethyl-nitrosurea (ENU), an alkylator, or DMSO (vehicle) for one hour. 24 hours after treatment, cells were analyzed for apoptosis by Annexin V staining. KL cells from DBA/2J mice showed a 20% decrease in apoptosis relative to C57Bl/6 counterparts. Mean of technical replicates (n=3) is shown for 1 of 4 biological replicate samples tested with similar results. (**d**) This association with decreased apoptosis suggests that the novel isoforms could be acting as dominant negatives, inhibiting endogenous Casp9 activity. To determine if the variant isoforms have dominant negative activity, *Casp9*$^{+/+}$ FLCs were infected with MSCV expressing each of the DBA/2J isoforms and then exposed to ENU. Expression of the variant isoforms does not suppress apoptosis induced by endogenous *Casp9*, thus they are not dominant negative. *$p<0.05$, **$p<0.005$

2.6 Figures



	A	B	C
C57Bl/6	100%	0%	0%
DBA/2J	62.5%	4.85%	32.65%

Figure 2.1: Resistance to alkylator-induced AML in mice is related to levels of *Casp9* expression and expression of novel variant isoforms.

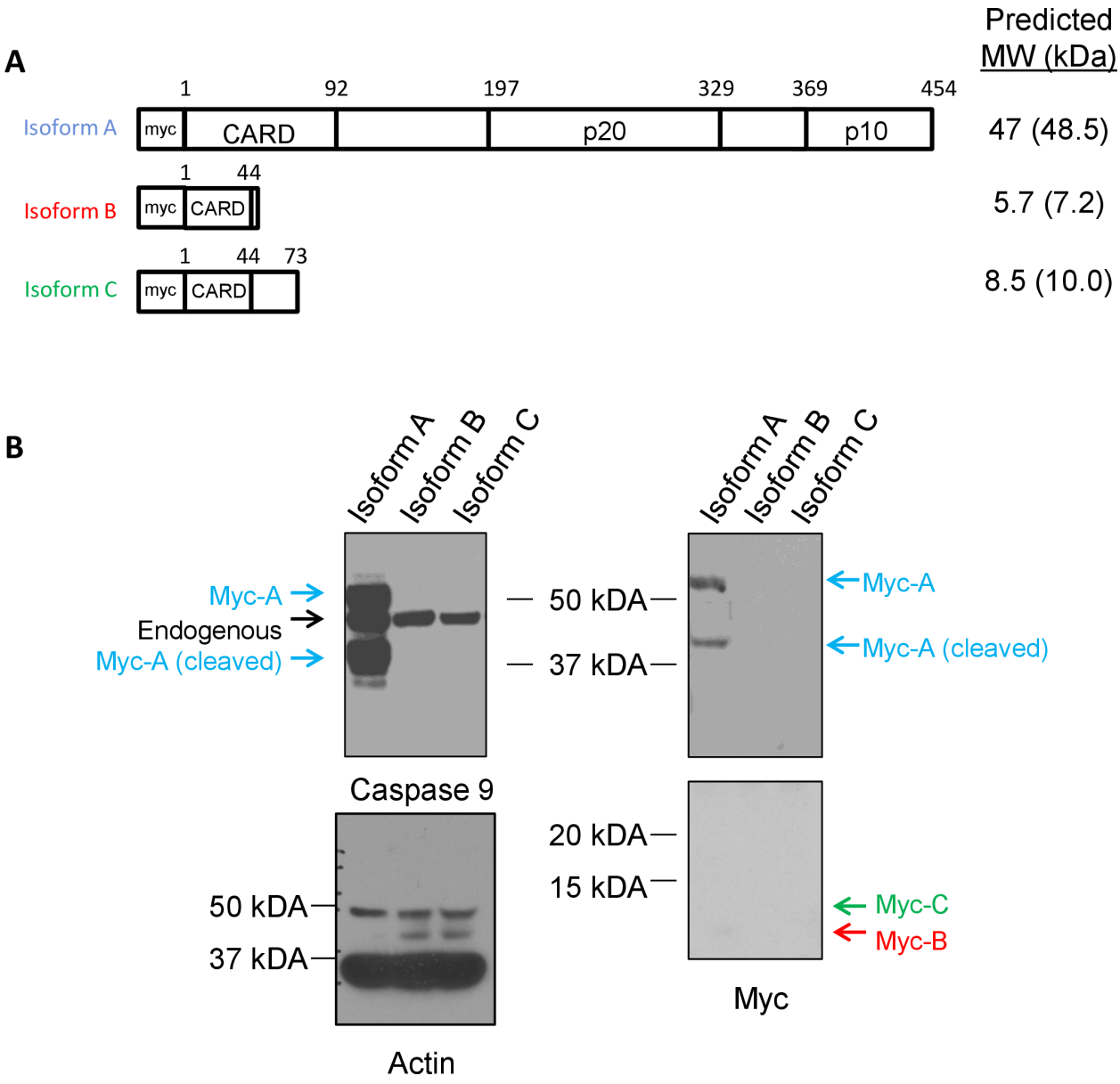


Figure 2.2: Variant Casp9 isoforms are not detectable by western blot.

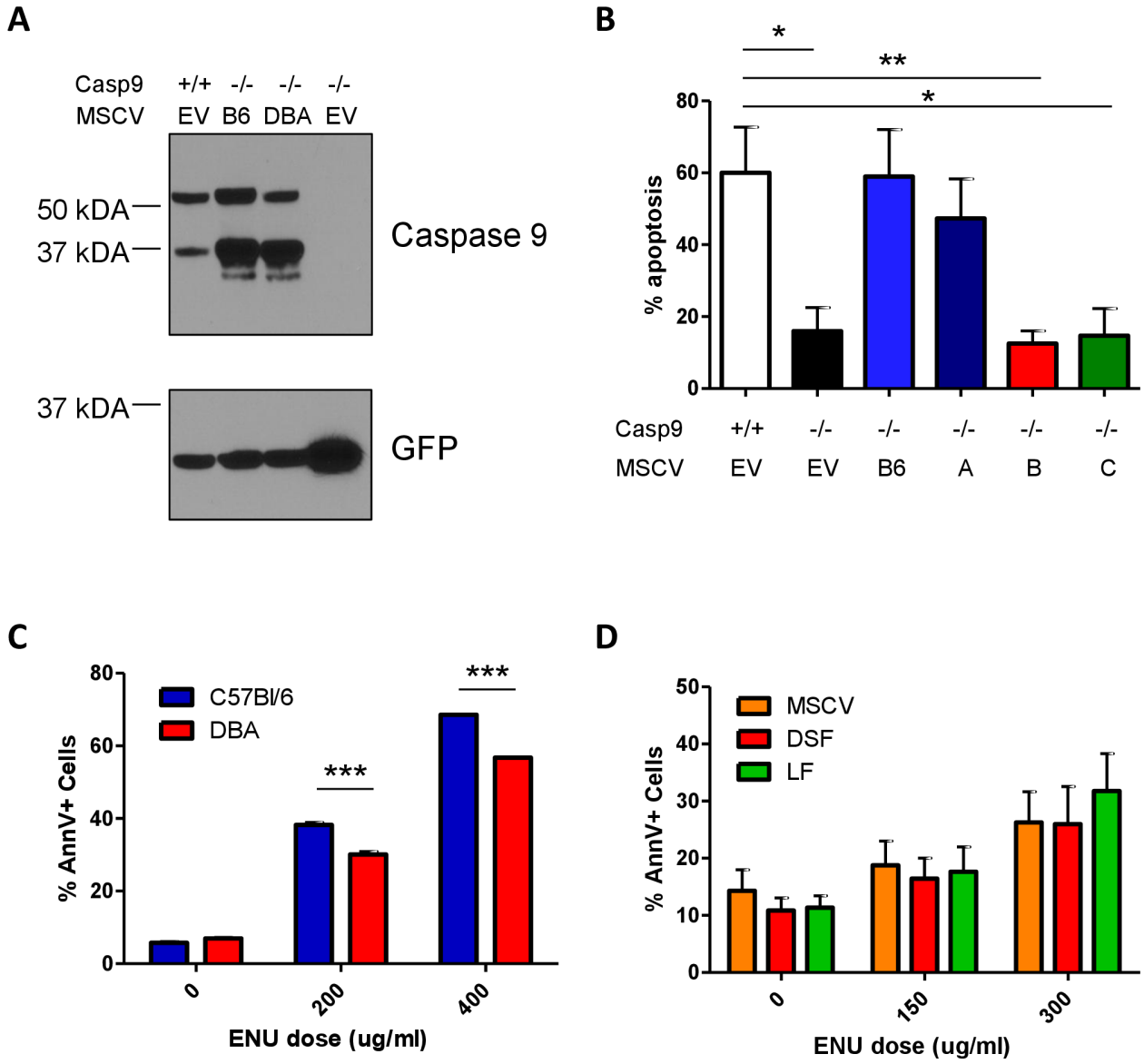


Figure 2.3: Variant *Casp9* isoforms are hypomorphic.

2.7 Supplementary Materials

Supplemental Table 1: Primer sequences for qualitative PCR and qRT-PCR.

	Qualitative PCR	qRT-PCR Exons2-3	qRT-PCR Exons 8-9
forward	CAGGGTGCGCCTAGTGAG	GGCCCGTGGACATTGGT	GAAGACCTGCAGTCCCTCCTT
reverse	TGCCCCAGTTCAAAATCCTA	TCTGCATGTCCCCTGATCTTC	GGAATCTGCTTGTAAGTCCCTTTC
probe		[6FAM]CTGGCGGAGCTCATGATGTCTGTGTTC[TAM]	[6FAM]TCAGGGTTGCCAATGC[TAM]

Table 2.1: Primer sequences for qualitative PCR and qRT-PCR.

Supplemental Figure 1: Sequences of DBA/2J *Caspase-9* Isoforms

DBA/2J Isoform A:

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  1 ATGGACGAGG CGGACCGGCA GCTCCTGCGG CGATGCAGGG TGCGCCTAGT GAGCGAGCTG
 61 CAAGTCGCGG AGCTCTGGGA CGCTCTGCTG AGTCGAGAGC TCTTCACGCG CGACATGATC
121 GAGGATATTC AGCAGGCAGG CTCTGGGTCT CGGCGGGATC AGGCCAGGCA GCTGGTCACA
181 GACCTTGAGA CCCGAGGGAG GCAGGCCCTT CCTCTCTTCA TCTCCTGCTT AGAGGACACA
241 GGCCAAAGGA CCCTGGCTTC ACTCTTGCAA AGCGGTCGGC AAGCAGCCAA GCAGGATCCA
301 GAGGCTGTTA AACCCCTAGA CCACCTGGTG CCTGTGGTCC TGGGACCAAT GGGACTCACA
361 GCAAAGGAGC AGAGAGTAGT GAAGCTGGAC CCGTCACAGC CTGCCGTGGG AAACCTCACC
421 CCAGTGGTGC TGGGGCCAGA AGAGCTCTGG CCTGCTCGGC TCAAGCCAGA GGTTCTCAGA
481 CCAGAAACAC CCAGGCCAGT AGACATTGGT TCTGGCAGAG CTCATGATGT CTGTGCTCCA
541 GGGAAGATCA GGGGACATGC AGATATGGCA TACACCCTGG ATTCGGATCC CTGTGGCCAC
601 TGCCTCATCA TCAACAATGT GAACTTCTGC CCTTCCTCGG GGCTCGGCAC ACGCACGGGC
661 TCCAACTTGG ACCGTGACAA ACTTGAGCAC CGATTCCGCT GGCTGCGCTT CATGGTGGAG
721 GTGAAGAACG ACCTGACTGC CAAGAAAATG GTCACGGCTT TGATGGAGAT GGCGCACCGG
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 781 AACCACCGTG CCCTGGACTG CTTTGTGGTG GTCATCCTCT CTCATGGCTG CCAGGCCAGC
 841 CACCTCCAGT TCCCGGGTGC TGTCTATGGG ACAGATGGAT GCTCCGTGTC CATTGAGAAA
 901 ATTGTGAATA TCTTCAACGG GAGCGGCTGC CCCAGCCTGG GAGGGAAGCC CAAGCTCTTC
 961 TTCATCCAGG CCTGCGGTGG TGAGCAGAAA GACCATGGCT TTGAGGTGGC CTGCACTTCC
1021 TCTCAAGGCA GGACCTTGGA CAGTGACTCT GAGCCAGATG CTGTCCCATA TCAGGAAGGT
1081 CCAAGGCCCT TGGACCAGCT GGATGCTGTG TCAAGTTTGC CTACCCCCAG TGACATCCTT
1141 GTGTCCTACT CCACCTTCCC AGGTTTTGTC TCCTGGAGGG ACAAGAAAAG TGGCTCCTGG
1201 TACATCGAGA CCTTGGATGG CATTCTGGAG CAGTGGGCTC GCTCTGAAGA CCTGCAGTCC
1261 CTCCTTCTCA GGGTCGCCAA TGCTGTTTCT GCGAAAGGGA CTTACAAGCA GATTCCTGGC
1321 TGTTTTAACT TTCTCCGGAA AAAGCTGTTT TTTAAAACTT CATGA
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DBA/2J Isoform B:

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   1 ATGGACGAGG CGGACCGGCA GCTCCTGCGG CGATGCAGGG TGCGCCTAGT GAGCGAGCTG
  61 CAAGTCGCGG AGCTCTGGGA CGCTCTGCTG AGTCGAGAGC TCTTCACGCG CGACATGATC
 121 GAGGATATTC AGTTTCTAGG GCTGTGAAAT GGAACGGTGG TCATCCTGTC CTAGGACAAG
 181 GACATAGAAT CAGGATCTGT CTTTTTTGGC TGGGCCTTTA TCAAGTGCAT GTTTTATAAA
 241 GGAGTTCAGA AAAGAAGGAA TTTGATGCAG CAGGCAGGCT CTGGGTCTCG GCGGGATCAG
 301 GCCAGGCAGC TGGTCACAGA CCTTGAGACC CGAGGGAGGC AGGCCCTTCC TCTCTTCATC
 361 TCCTGCTTAG AGGACACAGG CCAAAGGACC CTGGCTTCAC TCTTGCAAAG CGGTCGGCAA
 421 GCAGCCAAGC AGGATCCAGA GGCTGTTAAA CCCCTAGACC ACCTGGTGCC TGTGGTCCTG
 481 GGACCAATGG GACTCACAGC AAAGGAGCAG AGAGTAGTGA AGCTGGACCC GTCACAGCCT
 541 GCCGTGGGAA ACCTCACCCC AGTGGTGCTG GGGCCAGAAG AGCTCTGGCC TGCTCGGCTC
 601 AAGCCAGAGG TTCTCAGACC AGAAACACCC AGGCCAGTAG ACATTGGTTT CTGGCAGAGC
 661 TCATGATGTC TGTGCTCCGG GGAAGATCAG GGGACATGCA GATATGGCAT ACACCCTGGA
 721 TTCGGATCCC TGTGGCCACT GCCTCATCAT CAACAATGTG AACTTCTGCC CTTCCTCGGG
 781 GCTCGGCACA CGCACGGGCT CCAACTTGGA CCGTGACAAA CTTGAGCACC GATTCCGCTG
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 841 GCTGCGCTTC ATGGTGGAGG TGAAGAACGA CCTGACTGCC AAGAAAATGG TCACGGCTTT
 901 GATGGAGATG GCACACCGGA ACCACCGTGC CCTGGACTGC TTTGTGGTGG TCATCCTCTC
 961 TCATGGCTGC CAGGCCAGCC ACCTCCAGTT CCCGGGTGCT GTCTATGGGA CGGATGGATG
1021 CTCCGTGTCC ATTGAGAAAA TTGTGAATAT CTTCAACGGG AGCGGCTGCC CCAGCCTGGG
1081 AGGGAAGCCC AAGCTCTTCT TCATCCAGGC CTGCGGTGGT GAGCAGAAAG ACCATGGCTT
1141 TGAGGTGGCC TGCACTTCCT CCCAAGGCAG GACCTTGGAC AGTGACTCTG AGCCAGATGC
1201 TGTCCCATAT CAGGAAGGTC CAAGGCCCTT GGACCAGCTG GATGCTGTGT CAAGTTTGCC
1261 TACCCCCAGT GACATCCTTG TGTCCTACTC CACCTTCCCA GGTTTTGTCT CCTGGAGGGA
1321 CAAGAAAAGT GGCTCCTGGT ACATCGAGAC CTTGGATGGC ATTCTGGAGC AGTGGGCTCG
1381 CTCTGAAGAC CTGCAGTCCC TCCTTCTCAG GGTCGCCAAT GCTGTTTCTG CGAAAGGGAC
1441 TTACAAGCAG ATTCCTGGCT GTTTTAACTT TCTCCGGAAA AAGCTGTTTT TTAAAACTTC
1501 ATGC
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DBA/2J Isoform C:

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   1 ATGGACGAGG CGGACCGGCA GCTCCTGCGG CGATGCAGGG TGCGCCTAGT GAGCGAGCTG
  61 CAAGTCGCGG AGCTCTGGGA CGCTCTGCTG AGTCGAGAGC TCTTCACGCG CGACATGATC
 121 GAGGATATTC AGGTGCTCCA GGGAAGATCA GGGGACATGC AGATATGGCA TACACCCTGG
 181 ATTCGGATCC CTGTGGCCAC TGCCTCATCA TCAACAATGT GAACTTCTGC CCTTCCTCGG
 241 GGCTCGGCAC ACGCACGGGC TCCAACTTGG ACCGTGACAA ACTTGAGCAC CGATTCCGCT
 301 GGCTGCGCTT CATGGTGGAG GTGAAGAACG ACCTGACTGC CAAGAAAATG GTCACGGCTT
 361 TGATGGAGAT GGCACACCGG AACCACCGTG CCCTGGACTG CTTTGTGGTG GTCATCCTCT
 421 CTCATGGCTG CCAGGCCAGC CACCTCCAGT TCCCGGGTGC TGTCTATGGG ACAGATGGAT
 481 GCTCCGTGTC CATTGAGAAA ATTGTGAATA TCTTCAACGG GAGCGGCTGC CCCAGCCTGG
 541 GAGGGAAGCC CAAGCTCTTC TTCATCCAGG CCTGCGGTGG TGAGCAGAAA GACCATGGCT
 601 TTGAGGTGGC CTGCACTTCC TCTCAAGGCA GGACCTTGGA CAGTGACTCT GAGCCAGATG
 661 CTGTCCCATA TCAGGAAGGT CCAAGGCCCT TGGACCAGCT GGATGCTGTG TCAAGTTTGC
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721 CTACCCCCAG TGACATCCTT GTGTCCTACT CCACCTTCCC AGGTTTTGTC TCCTGGAGGG

781 ACAAGAAAAG TGGCTCCTGG TACATCGAGA CCTTGGATGG CATTCTGGAG CAGTGGGCTC

841 GCTCTGAAGA CCTGCAGTCC CTCCTTCTCA GGGTCGCCAA TGCTGTTTCT GCGAAAGGGA

901 CTTACAAGCA GATTCCTGGC TGTTTTAACT TTCTCCGGAA AAAGCTGTTT TTTAAAACTT

961 CATGA
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CHAPTER 3

LOSS OF CASPASE-9 RESULTS IN DECREASED STEM CELL FITNESS AND CLONAL HEMATOPOIESIS AFTER ALKYLATOR EXPOSURE

3.1 Introduction:

DNA damage, due to UV irradiation, genotoxic exposure or simply metabolism and DNA replication is a constant process in mammalian cells. Various strategies (here termed DNA

damage response or DDR) have evolved to deal with this stress. In cases where the DDR response cannot correct the DNA damage, the cell can commit suicide via apoptosis. In the highly proliferative hematopoietic compartment, appropriate regulation of DDR and apoptosis are essential. Age-related changes in hematopoiesis, including decreased stem cell function and a myeloid bias, are thought to be largely due to the aberrant accumulation of DNA damage over time [31, 19, 29]. Much work in recent years has demonstrated that DNA damage can adversely affect hematopoieitic stem cells (HSCs), specifically. HSCs are a unique subset of hematopoietic cells; in the highly proliferative hematopoietic system, HSCs are largely quiescent, maintaining themselves in the G0 phase and proliferating only rarely [23]. As such, they go through fewer cell cycle checkpoints when cellular machinery can detect and repair DNA damage [31]. When compared to more differentiated cells, HSCs show less efficient or delayed repair of DNA lesions after irradiation or alkylator treatment, maintaining DNA lesions longer than more differentiated cells [22, 24, 3, 25, 21]. HSCs can also be induced to senesce or apoptose more easily in response to DNA damage, whether it be due to radiation or excessive endogenous reactive oxygen species (ROS) production [24, 21]. Ultimately, accumulated DNA damage is thought to induce HSCs to decrease self-renewal by death or differentiation, probably as a protective mechanism to prevent the propagaton of the damage through the hematopoietic tree [31, 21, 35]

Since HSCs are so uniquely sensitive to the effects of DNA damage, it is not surprising that mutations in genes involved in DDR or apoptosis would have serious affects in these cells. Mutations in genes necessary for nucleotide excision repair (XPD), telomere maintenance (mTR and Terc), Non-Homologus End Joining (Ku80, Lig4), mismatch repair (Msh2), and homologous recombination repair (Rad50, Brca2) all cause increases in detectable DNA damage in HSCs (and other cells) and are associated with impaired HSC function and premature exhaustion of stem cells. HSCs from these mice are more prone to spontaneous apoptosis

and also generally show lower rates of proliferation, although the absolute numbers of stem cells are not altered. [26, 22, 1, 24, 25]. Loss of p53, a critical mediator of the cell cycle and apoptosis, allows HSCs to more easily enter the cell cycle, increasing self renewal [23, 2]. These more proliferative HSCs have an initial advantage in competitive transplantation, but the HSCs are less effective than wild type in serial transplantation [23]. However, unlike DNA repair mutants, mice deficient in p53 do not suffer stem cell exhaustion.

The ultimate end of the response to DNA damage is apoptosis, but few direct mediators of apoptosis have been studied in depth in hematopoiesis. Increasing expression of the proapoptotic Bcl2 results in a proliferative phenotype, with an enlarged HSC compartment. HSCs overexpressing Bcl2 have a competitive advantage in transplantation and in plating assays, though they actually cycle less frequently than wild type cells [10]. This would suggest that while accumulation of DNA damage in HSCs is ultimately damaging, evasion of the apoptosis that this damage might generate could be advantageous.

Therapy-related acute myeloid leukemia (tAML) is a severe form of AML that develops after exposure to DNA damaging agents used as chemotherapeutic treatments for other tumors [12]. Changes in apoptosis and DDR might be predicted to be particularly important in susceptibility to this disease since hematopoeitic stem or progenitor cells that were exposed to that initial DNA damaging agent and survived are the likely sources of the new tumor. There is some evidence in the literature to support this hypothesis. Somatic mutations of p53 are much more common in tAML than in *de novo* AML, and tAML case samples showed lower expression of p53 than control samples in a study of HD and NHL patients [30, 9]. Hypermethylation of the BRCA1 promoter is also much more common in tAML than in *de novo* AML [5]. There is also evidence to suggest that tAML is associated with an increased level of DNA damage. tAML blasts often show microsatellite instability, a sign of DNA damage that is rare in *de novo* AML [5]. tAML is also associated with accelerated

telomere shortening, which is linked to genomic instability [8, 6]. Both prospective and retrospective studies have been performed to try to isolate germ-line genetic variants that might predispose to tAML, and those found have often been associated with the DNA damage response or apoptosis. A functional polymorphism in the promoter of MLH1, which has been demonstrated to result in decreased expression of that key DNA repair gene, is associated with increased risk of tAML [36, 20]. The P72R SNP in p53 has also been reported to be associated with risk for tAML, although there are conflicting reports as to which allele is deleterious and whether cooperating SNPs are also required [9, 11]. An interesting functional study, examining CD34+ cells destined for autologous stem cell transplantation in Hodgkins and Non-Hodgkins Lymphoma patients, showed some promising results that might be missed by a gene association study. In functional assays, cells from patients who went on to develop tAML showed higher mitochondrial ROS production and sustained ROS elevation after treatment with DNA damaging agents as well as increased H2Ax foci after treatment with a nitrogen mustard compound. These findings suggest that even before development of tAML, stem cells from patients show functional defects in mitochondria and abnormal accrual of DNA damage [18].

Much of the previous work on DDR and apoptosis in hematopoiesis and tAML has focused on studying components of this pathway well upstream of the actual apoptotic event. Mutations in the sensing and repair of DNA damage, as well as p53 and similar upstream regulators have been studied. However, Bcl2 is one of the only direct mediators of apoptosis that has been examined in depth in the hematopoietic system. Bcl2 is upstream of the mitochondria in the apoptotic cascade, meaning that at the point at which apoptosis is blocked by Bcl2, cytochrome C has not been released from the mitochondria. Cytochrome C release leads directly to formation of the apoptosome, a holoenzyme complex of cytochrome C, Apaf1 and Caspase-9 that activates Caspase-9. As the initiator caspase, Caspase-9 then activates

executioner caspases that go on to mediate all the familiar sequelae of apoptosis, including DNA fragmentation and membrane permeablization. Effectors of apoptosis downstream of the mitochondria have not been well studied in hematopoiesis or leukemogenesis, but given the importance of upstream mediators, it is likely that such effectors would play important roles in hematopoiesis and that defects in these effectors might increase the risk of tAML. Therefore, we have chosen to examine the effects of loss of a key downstream effector gene, *Casp9*, in hematopoiesis. In order to validate our findings, we also study the effects of loss of *Apaf1*, which is essential for activating Caspase-9 and should phenocopy its loss. We found that loss of these key effectors caused a stem cell defect in fetal hematopoiesis, which led to bone marrow failure in adult bone marrow chimeras. Loss of *Caspase-9* also altered the response to alkylator exposure, resulting in increased DNA damage after exposure and ultimately causing clonal hematopoiesis which could be indicative of progression to tAML.

3.2 Materials And Methods

Harvest and culture of fetal liver cells. *Casp9*$^{+/-}$ [15] (backcrossed >10 generations) or *Apaf1*$^{+/-}$ [13] (backcrossed 6 generations) mice were intercrossed and fetuses were obtained at embryonic day 15.5. Tail DNA was prepared (DNeasy, Qiagen) and used for genotyping (see supplemental methods). Fetal livers were homogenized to yield a single cell suspension and viably cryopreserved (FBS with 10% DMSO).

Flow Cytometric analysis. Fetal liver cells (FLCs) were thawed and cultured overnight in complete media (RPMI containing 1%l-glutamine, 20%fetal bovine serum) supplemented with recombinant hematopoietic cytokines (100ng/ml stem cell factor, 6ng/ml IL-3, 50ng/ml Fms-related tyrosine kinase 3 ligand, 10ng/ml thrombopoietin, and 10 ng/ml IL-6; all from Peprotech, Rocky Hill, NJ, USA). Kit+, lin-, sca+ (KLS) cells were defined as staining

negatively for lineage markers B220, CD3, Gr1 and Ter119, staining negatively for CD41, and staining positively for c-kit and sca markers. Long-term stem cells (LT-HSCs) were defined as KLS cells that stained positive for CD150 and negatively for CD48. All antibodies from eBioscience (San Diego, CA, USA).

Hematopoietic progenitor assays. FLCs were cultured in methylcellulose media (MethoCult m3434 or m3630, Stemcell Technologies, Vancouver, BC, Canada) at 37°C for 7 days. Progenitors were counted and, in the case of myeloid colonies, sub-classified (CFU-G, CFU-GM, or CFU-M). For erythroid progenitors (BFU-E or CFU-E), plates were stained with benzidine (0.43% benzidine in 13% acetic acid and 3% H^2O^2) for 3 minutes then immediately counted.

Induction and analysis of apoptosis and DNA damage. N-ethyl-nitrosurea (ENU; Sigma) was resuspended and serially diluted in dimethyl sulfoxide (DMSO). For in vitro assays, FLCs were treated with ENU 24 hours after thawing. Cells were incubated with ENU (or an identical concentration of DMSO) for 1 hour at 37°C. The cells were then washed in PBS and incubated overnight in fresh cytokine-enriched media. 24 hours after ENU treatment cells were stained with a 1:40 dilution of Annexin V (BD Pharmingen, San Jose, CA, USA) in Annexin V binding buffer (140 mM NaCl, 4 mM KCl, 0.75 mM $MgCl^2$and 10 mM HEPES) and analyzed by FACS. Cells were collected at 24 hour intervals and resuspended in PBS at a concentration of $5x10^5$ per mL, then embedded in agarose and subjected to the comet assay using the CometAssay Kit (Trevigen, Gaithersburg, MD). Comets were photographed under 10x magnification and analyzed using CometScore software (TriTek Corp, Sumerduck, VA). For *in vivo* DNA damage assays, bone marrow chimeras at 12 weeks post-transplant were injected IP with 200 mg/kg ENU or DMSO diluted 1:10 in phosphate-citrate buffer as previously described [12]. Mice were sacrificed at 72 hours post-treatment and bone marrow was harvested by centrifugation. Comet assays were performed

as described above. For in vivo determination of clonal hematopoeisis by exome sequencing, bone marrow chimeras at 6 weeks post-transplant were injected IP with 100 mg/kg ENU or DMSO diluted 1:10 in phosphate-citrate buffer as previously described [12]. They were injected again at 7 weeks post-transplant, and ultimately sacrificed 11 weeks later.

Generation of bone marrow chimeras. Hosts (Ly5.1+ or Ly5.1+/Ly5.2+ in a C57BL/6J background, 9-12 weeks of age) were irradiated (950 cGy) and transplanted 24 hours later (RO) with FLCs or bone marrow cells. Mice were treated with trimethoprim-sulfamethoxazole (40mg-200 mg per 5mL; HiTech Pharmacal, Amityville, NY, USA) in drinking water for two weeks following transplant and peripheral blood was sampled at one month post-transplant for donor chimerism by flow cytometry. For single genotype transplants, FLCs were thawed and cultured for 24 hours prior to transplantation. For competitive transplants, a 1:1 mixture of *Casp9*$^{+/+}$ or *Casp9*$^{-/-}$ (Ly-5.2+) and wildtype competitor (congenic Ly-5.1+) FLCs was made immediately after thawing, with no interim culture, and the exact ratio determined by flow cytometry before transplantation. For sequential transplants, primary transplant recipients were sacrificed at 12 weeks post-transplant and bone marrow was harvested by centrifugation.

Analysis of bone marrow chimeras. Peripheral blood was collected from transplant recipients at monthly intervals after transplant. Peripheral blood counts (WBC, Hb, etc) were determined using a veterinary Coulter counter (HemaVet 950 - Drew Scientific Group, Dallas, TX, USA). Chimerism and lineage composition were determined by staining with antibodies against chimeric markers Ly5.1 and Ly5.2 and lineage markers B220, CD3 and Gr1 followed by analysis by FACS. Mice were sacrificed when moribund or at 12 or 18 months post-transplant. At sacrifice, spleen weight was measured, blood counts determined, and FACS analysis of bone marrow and spleen performed to determine chimerism and lineage composition. Slides of bone marrow and peripheral blood were made and stained with

hematoxylin and eosin. Bone marrow counts could then be determined by microscopic analysis.

Exome sequencing. Bone marrow cells were harvested from transplanted mice at 12 weeks after ENU or DMSO treated and stained with directly conjugated Ly5.2 and Ly5.1 antibodies (vendor). Donor-derived cells (Ly-5.2+Ly-5.1-) were sorted (MoFlo, DAKO Cytomation) into cell culture media, genomic DNA was prepared (DNeasy kit, Qiagen), and quantified by fluorimetry (Qubit, Life Technologies, Carlsbad, CA). DNA had previously been prepared from limb tissue from the original donors. Multiplexed paired-end Illumina libraries were prepared according to the manufacturer's recommendations (Illumina Inc., San Diego, CA). Three libraries for were generated from each bone marrow sample (tumor) and a single library was generated from each donor (normal). Sequencing libraries were hybridized in solution to capture oligonucleotide probes targeting the C57BL/6J mouse exome (54.3 Mb of target sequence), according to the manufacturers protocol (SeqCap EZ, Roche NimbleGen, Madison, WI). qPCR was used to calibrate flow cell loading concentration and cluster density. Libraries were pooled and run on a single lane of an Illumina HiSeq2000 according the manufacturer's recommendations (Illumina Inc., San Diego, CA). Illumina reads were demultiplexed and aligned to the NCBI 37/mm9 reference sequence using BWA v0.5.9 [17]. Binary alignment/map (BAM) files were merged and duplicates marked using Picard v1.46 (http://picard.sourceforge.net). Read pileups were generated with the SAMtools v0.1.18 mpileup command using default settings. Read counts were extracted from pileup files with VarScan v2.3.5 using default parameters [14]. For each tumor sample, single nucleotide variants (SNVs) were called if the variant allele frequency (VAF) in the matched normal sample was 0% and at least 30 reads were obtained at the site in both tumor and normal.

3.3 Results

Loss of *Casp9* alters hematopoietic stem/progenitor compartment. Hematopoietic stem cells have been shown to be uniquely sensitive to the consequences of DNA damage and defects in the DNA damage response and apoptotic pathway. To determine how loss of the post-mitochondrial apoptotic pathway, specifically loss of the initiator caspase *Casp9*, would affect the abundance of HSPCs, we examined fetal hematopoiesis in *Casp9* deficient mice. Livers were harvested from fetuses at E15.5 and cultured in methylcellulose media containing cytokines and examined for colonies after seven days. $Casp9^{-/-}$ and $Casp9^{+/+}$ FLCs showed similar numbers of myeloid colonies, suggesting that myeloid progenitor numbers are not altered. However, both B cell and erythroid colonies were reduced in $Casp9^{-/-}$ FLCs (**fig 3.1a**). To assess the stem cell population in fetal liver, FLCs were examined by flow cytometry for stem cell markers. $Casp9^{-/-}$ fetal liver cells (FLCs) showed a roughly three-fold increase in the abundance of kit+, lineage-, sca+ (KLS) cells. These cells are enriched for hematopoietic stem cells (HSCs), but also contain other cell types. To further parse the stem cell compartment in these mice, FLCs were also stained for SLAM markers (CD48, CD150). KLS, CD48+, CD150- cells are thought to be long term HSCs. The abundance of SLAM cells within the KLS compartment is greatly reduced in $Casp9^{-/-}$ mice, resulting in no change overall in the abundance of SLAM cells within the total hematopoietic compartment (**fig 3.1b**).

We also assessed HSC abundance in adult bone marrow chimeras. $2x10^6$ $Casp9^{-/-}$ or $Casp9^{+/+}$ FLCs were transplanted into a lethally irradiated recipient. Recipient mice were sacrificed at three months post-transplant and bone marrow analyzed by FACS in the same way as FLCs. As with FLCs, $Casp9^{-/-}$ bone marrow showed approximately a three-fold increase in KLS cells, but no overall change in the abundance of LT-HSCs (KLS-SLAM cells; **fig 3.1c**).

***Casp9* deficient stem cells are functionally impaired**. The changes in stem and progenitor cell abundance observed in *Casp9* deficient fetal liver could indicate merely a change in number, but they could also correlate with changes in stem and progenitor cell function. To determine if *Casp9* deficient stem and progenitor cells have altered function, we first assayed for competitive fitness of HSPCs. $Casp9^{+/+}$ or $Casp9^{-/-}$ FLCs were transplanted in a 1:1 ratio with competitor wild type Ly5.1 FLCs. If there is no difference in function between $Casp9^{-/-}$ and $Casp9^{+/+}$ stem cells, then both should remain at approximately the same 1:1 ratio with competitor cells. However, we observed a decline in the relative abundance of $Casp9^{-/-}$ cells over time in transplant recipients, while no such decline, and in fact a slight increase, was observed in $Casp9^{+/+}$ cells (**fig 3.2a**). This indicates that loss of *Casp9* confers a competitive disadvantage in the setting of primary transplantation, suggesting a defect in the stem cells responsible for repopulating the hematopoietic compartment in transplant recipients. Stem cell function was further assayed by assessing the ability of $Casp9^{-/-}$ cells to sequentially repopulate the hematopoietic compartment of serial recipients. $Casp9^{-/-}$ or $Casp9^{+/+}$ FLCs were first transplanted into lethally irradiated primary recipients. Twelve weeks after this initial transplant, bone marrow was harvested and transplanted into secondary recipients. $Casp9^{+/+}$ cells were able to reconstitute the hematopoietic compartment of these secondary recipients, but $Casp9^{-/-}$ cells were not (**fig 3.2b**). Although $Casp9^{-/-}$ stem cells are capable of generating a functional hematopoietic compartment in a first transplant (**fig 3.2c**), they exhaust after this initial transplant, providing further evidence that the immunophenotypic changes observed previously correlate with a functional defect in $Casp9^{-/-}$ stem cells.

While $Casp9^{-/-}$ FLCs successfully engraft in primary transplant recipients, this does not necessarily mean that $Casp9^{-/-}$ stem cells can reconstitute normal hematopoiesis. To assess baseline hematopoiesis, $Casp9^{+/+}$ and $Casp9^{-/-}$ FLCs were transplanted into lethally

irradiated recipients and peripheral blood from these recipients was examined at monthly intervals for blood counts, chimerism and lineage markers. *Casp9*$^{-/-}$ transplant recipients have lower white blood cell counts and lower hemoglobin than *Casp9*$^{+/+}$ counterparts (**fig 3.2d**). *Casp9*$^{-/-}$ blood also has significantly lower numbers of B cells and slightly increased numbers of myeloid cells (data not shown). These data are consistent with the progenitor cell analysis in fetal liver, which showed decreased B cell and erythroid progenitor numbers. These data suggest that loss of *Casp9* in some way biases away from the B cell and erythroid lineages, thus resulting in disproportionate myeloid development.

To ensure that this phenotype was the result of the loss of apoptosis associated with loss of *Casp9* and not a defect unique to this particular mouse model, we performed similar experiments using a mouse deficient in *Apaf1*. *Apaf1* is the key component of the apoptosome and is essential for activation of Caspase-9 in response to apoptotic stimuli, and loss of *Apaf1* results decreased apoptosis similar to loss of *Casp9*. Mice receiving *Apaf1*$^{-/-}$ FLC transplants showed similar defects in hematopoiesis as observed in our *Casp9*$^{-/-}$ model: decreased WBC, Hb, and B cells with increased myeloid cell numbers (supplemental figure 3.1). This phenocopy of our *Casp9* results supports our conclusion that loss of apoptosis is responsible for the changes observed in hematopoiesis.

Loss of *Casp9* or *Apaf1* leads to bone marrow failure *in vivo*. The changes in stem cell function and hematopoiesis observed in *Casp9*$^{-/-}$ and *Apaf1*$^{-/-}$ transplant recipients could have varying long-term effects in the recipient mice. To assess whether these changes were deleterious in the long term, we followed transplant recipients, sacrificing mice when moribund or at 12 or 18 months post-transplant. Mice receiving *Casp9*$^{-/-}$ or *Apaf*$^{-/-}$ transplants showed significantly decreased survival relative to their wild type counterparts (**fig 3.3a-b**). One *Casp9*$^{-/-}$ mouse died of recipient-derived T-cell lymphoma, a fairly common sequelae of the irradiation necessary for bone marrow transplantation. Several mice, both *Casp9*$^{-/-}$

and *Apaf1*$^{-/-}$, also died before necropsy could be performed. At necropsy, mice from both cohorts demonstrated a consistent phenotype, consisting of leukopenia, slightly decreased spleen size, and mild anemia (**Table 3.1**). Bone marrow histology reveals a decrease in erythropoiesis and an increase in myeloid-committed cells. Within the myeloid lineage, there is also a shift to less differentiated forms. Most interestingly, there are signs of dysplasia in the erythroid lineage, specifically nuclear blebbing, and in the myeloid lineage, most prominently in the increase in hyper-lobulated mature neutrophils. These changes can be quantified and shown to be unique to *Casp9*$^{-/-}$ and *Apaf1*$^{-/-}$ transplant recipients (**fig 3.3c-e**). Based on these results, we conclude that loss of *Casp9* or *Apaf1* in the hematopoietic compartment ultimately leads to a bone marrow failure syndrome with dysplastic features.

Loss of apoptosis results in increased DNA damage after alkylator treatment. Defects in DDR and apoptosis have also been implicated in predisposition to therapy-related acute myeloid leukemia (tAML), a late complication of exposure to DNA damaging chemotherapeutics. We hypothesize that loss of apoptosis could lead to increased risk of tAML by allowing cells to inappropriately survive exposure to DNA damaging agents, thereby giving rise to a population of cells carrying significant DNA damage, making them more prone to malignant transformation.

To determine if *Casp9* deficiency allows hematopoietic cells to escape death while carrying DNA damage, we used N-ethyl-N-nitrosourea (ENU), a prototypical alkylator similar to drugs used in chemotherapy, to induce DNA damage. In vitro, we treated FLCs with varying doses of ENU and measured apoptosis by Annexin V staining at 24 hours post-treatment. DNA damage was assessed at various time points after treatment using the comet assay system, which detects single and double strand breaks as well as abasic sites. FLCs deficient in *Casp9* or *Apaf1* had significantly lower levels of apoptosis in response to ENU, as previously reported (**fig 3.4a**). This decreased apoptosis in vitro correlated with an increased level of

DNA damage that is dose dependent and becomes apparent at 48 hours post-treatment and endures beyond 120 hours post-treatment (**fig 3.4b-c**). Apoptosis peaks between 12 and 24 hours post-treatment (supplemental figure 3.2a). Thus the DNA damage apparent at later time points is present in the surviving cell population, consistent with our hypothesis.

To determine if *Casp9* or *Apaf1* deficiency results in excessive DNA damage that can be detected in vivo, we injected *Casp9* or *Apaf1* transplant recipients with 200 mg/kg ENU or equivalent volume of DMSO and sacrificed mice at 72 hours post-treatment. Bone marrow from these mice was then analyzed by comet assay. As with our in vitro assay, *Casp9* or *Apaf1* deficient bone marrow showed increased levels of DNA damage after ENU treatment (**fig 3.4d**). This DNA damage is not simply a marker of late stage apoptosis: the proportion of cells carrying a subdiploid complement of DNA, indicative of DNA fragmentation in late stage apoptosis, is very low regardless of genotype (supplemental figure 3.2b). Thus, loss of apoptosis in combination with a genotoxic insult results in the accumulation of cells with high levels of DNA damage *in vivo*.

Interestingly, *Casp9*$^{-/-}$ mice also showed higher levels of DNA damage even when only treated with DMSO (**fig 3.4d**). This could indicate that endogenous DNA damage occurring due to normal cellular processes also accumulates in apoptosis-deficient cells.

Loss of *Casp9* leads to clonal hematopoiesis after alkylator treatment. Loss of apoptosis combined with genotoxin exposure gives rise to a population of damaged cells in the short term. To determine if this could give rise to disease in the long term, a whole exome sequencing approach was employed.

Healthy hematopoiesis is not clonal: many different stem and progenitor cells give rise to the various cells that make up the bone marrow and no single cell has an advantage over its neighbors. Clonal hematopoiesis occurs when one or several stem or progenitor

cells accumulate mutations or other changes that give them a competitive advantage such that their descendants are over-represented in the bone marrow. Previous reports have demonstrated that clonal hematopoiesis occurs early in the progression of diseases such as Myelodysplastic Syndrome and AML. If loss of apoptosis predisposes to therapy-related MDS or AML, it should give rise to clonal hematopoiesis even before other signs of disease manifest.

Bone marrow chimeras were generated by FLC transplantation as discussed above. FLCs from three *Casp9*$^{-/-}$ and three *Casp9*$^{+/+}$ pups were each transplanted into four lethally irradiated recipients (24 recipients total). At 6 and 7 weeks post-transplant, two recipients of each cohort were injected with 100 mg/kg ENU and two were injected with an equivalent amount of DMSO. At 12 weeks post-transplant, two recipient mice (both *Casp9*$^{-/-}$ injected with ENU) showed signs of illness. At that time, two mice from each cohort (one ENU-treated and one DMSO-treated) were sacrificed. Bone marrow was harvested and sorted for donor cells. DNA was prepared from these sorted cells and also from tissue taken from the original donor pup. This DNA was subjected to whole exome sequencing and post-transplant DNA sequence was compared to donor DNA to identify somatic single nucleotide variants (SNVs) (supplemental figures 3.3 and 3.4). Variant allele frequencies were determined for these novel SNVs. If hematopoiesis is not clonal, all variants should be at or below the limit of detection, indicating that these mutations are present in only a small number of cells. Clonality is demonstrated by SNVs with varying VAFs, indicating certain mutations that are present in populations of cells with a competitive advantage.

The number of SNVs varied greatly across cohorts. Very few SNVs were found in *Casp9*$^{+/+}$ transplants and addition of ENU did not significantly impact their abundance. *Casp9*$^{-/-}$ transplants, on the other hand, showed very high numbers of mutations. Addition of ENU

also drastically increased the number of SNVs and altered the relative abundance of transitions and transversions (supplemental figure 3.5). This suggests that loss of apoptosis, even without exogenous genotoxic stress, increases the mutation burden. The additional SNVs associated with ENU are consistent with our comet assay data showing high levels of DNA damage in surviving cells after ENU treatment, both in vitro and in vivo.

VAFs in *Casp9*$^{+/+}$ transplants and *Casp9*$^{-/-}$ transplants treated with DMSO are low, suggesting that no population of cells has an advantage and indicating non-clonal hematopoiesis. However, *Casp9*$^{-/-}$ transplants treated with ENU showed VAFs ranging from <1% to >40%, in all three biological replicates (**fig 3.5**). This is indicative of oligoclonal hematopoiesis and, therefore, probable progression to tMDS or tAML.

3.4 Discussion

Inhibiting apoptotic cell death would conventionally be expected to result in increased cell numbers and a proliferative phenotype. However, our studies examining loss of post-mitochondrial pro-apoptotic genes suggest that cell death is not so simple. Loss of *Casp9* or *Apaf1* in the hematopoietic compartment does not lead to expansion of that compartment, but rather the opposite. Mice lacking these key apoptotic regulators die of bone marrow failure, not a proliferative or malignant disease. This is likely due to the effect of these alterations in stem cells. Like defects in DNA repair, defects in post-mitochondrial apoptosis decrease stem cell fitness and cause cells to exhaust, although LT-HSC numbers are not affected and KLS cells are increased (**fig 3.6**). The development of the HSC pool early in fetal development requires rapid proliferation and DNA replication, which brings with it the chance for DNA damage. When these damaged cells cannot undergo complete apoptosis, a population of only semi-functional cells forms within the HSC compartment. There have

been many conflicting reports concerning just how alive cells are after failing to undergo apoptosis due to caspase blockade. Cells that have permeablized their outer mitochondrial membrane (a process termed MOMP) but not activated the caspase cascade are thought to still die via Caspase Independent Cell Death (CICD) a slower but still effective method of cell death that does not require caspase activity [32]. These cells may divide up to two times after MOMP, but then they arrest and begin the process of CICD [16]. It has been suggested that CICD occurs due to the activities of pro-apoptotic factors, such as Apoptosis Inducing Factor, Smac or Omi released from the mitochondria during MOMP. More recent evidence suggests that it is due mitochondrial dysfunction leading to loss of oxidative phosphorylation and the ATP it provides [28, 7, 16]. However, if cells could simply undergo CICD in response to death signals in the absence of *Casp9* and *Apaf1*, one would expect that loss of these effectors would have very little effect. Our studies and the fact that loss of either gene is perinatal lethal contradict that idea. While CICD probably does occur in our fetal liver cells and transplanted mice, there is also evidence from recent work to suggest that cells can avoid CICD and survive, albeit manifesting severe dysfunction. When cells are triggered to die, not all of their mitochondria undergo MOMP. Those mitochondria that are intact can repopulate the cell with healthy mitochondria while the permeablized mitochondria are cleared away via mitophagy [33, 32, 7]. Provided that the cell in question can survive the ATP deprivation while the mitochondria repopulate, once this process is complete, it may go on to survive and proliferate. The fact that caspases act in a feedback loop to perpetuate MOMP by cleaving Bid, Bcl2 and p75 (a component of complex I), among others, suggests that cells lacking *Casp9* or *Apaf1* would be particularly prone to incomplete MOMP and subsequent recovery [34, 33]. The fact that cells lacking *Casp9* or *Apaf1* show less of a decrease in mitochondrial membrane potential than wild type cells bears this out [7, 16, 27]. Therefore, in cases where *Casp9* or *Apaf1*-deficient cells are triggered to die, some cells might die via CICD while others could undergo incomplete MOMP followed by

mitochondrial rescue. These surviving cells would have higher levels of DNA damage from the initial apoptotic stimulus and probably also due to the elevated levels of reactive oxygen species (ROS) released by the mitochondria after MOMP. Our data showing elevated levels of DNA damage and increased mutation burden in DMSO-treated *Casp9*$^{-/-}$ mice support this. The fact that some cells could still die via CICD might explain why we do not see the accumulation of many undead cells, while the defects in stem cells would explain the bone marrow failure observed as mice age.

We initially predicted that defects in apoptosis were most likely to play a role in susceptibility to therapy-related leukemia. We hypothesized that decreased apoptosis would allow cells to inappropriately survive exposure to DNA damaging agents, giving rise to a population of cells with excess DNA damage. These cells would then be more prone to dysplasia and/or malignant transformation. Indeed, we observed increased DNA damage in *Casp9* and *Apaf1*-deficient cells in vitro and in vivo after ENU treatment. With exome sequencing of *Casp9*$^{-/-}$ transplant recipients we were able to demonstrate that this increased DNA damage corresponds to a significantly increased mutation load. These mutations occurred above the level of detection of our assay, suggesting that they were present in clonal populations of cells within the bone marrow. While it is possible that this clonal hematopoiesis is the result of a smaller number of input stem cells in the original transplant, we believe this to be unlikely. The very slight decrease in LT-HSCs observed in transplant recipients at 12 weeks post-transplant is hardly sufficient to account for the clonality observed via sequencing. Furthermore, if it were simply a case of fewer stem cells giving the appearance of oligoclonal hematopoiesis, we would expect to see such clonality in the DMSO-treated *Casp9*$^{-/-}$ mice as well, which we do not. Therefore, we believe that the observed oligoclonal hematopoiesis is a result of the combined effects of the alkyaltor ENU and the defective apoptosis conferred by the loss of *Casp9* (**fig 3.6**). This oligoclonal hematopoiesis appears as a biomarker of

hematopoietic disease and could be a sign that these mice are developing therapy-related myelodysplastic syndrome or leukemia. With these experiments we have developed an assay that could be used in many forms to define clonality in mouse models.

Defects in apoptosis are not thought to be as important in leukemias as they are in many solid tumors. However, this work suggests that loss of function in the apoptotic cascade has dire consequences for the hematopoietic compartment and could play a role in susceptibility to tMDS or tAML. *Caspase-9* and *Apaf1* are not differentially expressed in AML versus CD34 cells [4]. However, apoptosis is a very complex cascade and even without obvious expression differences in single genes it is possible that variation in the apoptotic pathway exists in AML. Also, therapy-related AML has not been examined for variation in the expression of late apoptotic pathway genes and, given our results, it is possible that part of susceptibility to tMDS and tAML could be explained by inter-individual variation in the apoptotic cascade.

3.5 Acknowledgments

We are grateful to Dr. Richard Flavell for providing us with the *Casp9*$^{+/-}$ mouse model and to Dr. Anthony Rongvaux for assistance with that model. We thank Mieke Hoock and Deborah Leflamme for their assistance in maintaining our mouse colonies. We also thank Masayo Izumi for technical assistance.

Genotype	Phenotype	N	spleen weight (g)	WBC (K/ul)	Hb (g/dl)	MCV (fL)
Apaf1$^{+/+}$	elective	9	0.073 +/- 0.028	13.2 +/- 4.891	11.21 +/- 2.26	46.87 +/- 3.82
	BMF	1	0.107	NA	NA	NA
Apaf1$^{-/-}$	elective	3	0.107 +/- 0.019	12.97 +/- 2.26	9.2 +/- 3.29	46.47 +/- 2.32
	BMF	7	0.089 +/- 0.029	8.02 +/- 6.32	11.51 +/- 6.45	45.46 +/- 7.58
Casp9$^{+/+}$	elective	10	0.092 +/- 0.061	18.34 +/- 12.16	10.37 +/- 3.82	47.5 +/- 4.75
	BMF	0	NA	NA	NA	NA
Casp9$^{-/-}$	elective	8	0.087 +/- 0.024	11.68 +/- 3.14	10.86 +/- 1.18	46.94 +/- 3.27
	BMF	4	0.077 +/- 0.011	7.88 +/- 5.79	8.23 +/- 1.69	51.05 +/- 13.28

WBC, white blood cell; Hb, hemoglobin; MCV, mean corpuscular volume; NA, not available. BMF, bone marrow failure.

9 Apaf1+/+, 3 Apaf1-/-, 3 Casp9+/+, and 9 Casp9-/- transplants remain on tumor watch

3.6 Figure Legends

Figure 3.1: Loss of *Casp9* alters hematopoietic progenitor cell frequency. (**a**) The frequency of B cell and erythroid progenitors measured in methylcellulose assays was

decreased in *Casp9*$^{-/-}$ fetal liver cells (FLCs), although the frequency and type of myeloid progenitors was not significantly different. (**b**) The frequency of Kit^{+} Lineage^{-} Sca+ (KLS) cells was increased 3-fold in *Casp9*$^{-/-}$ FLCs, compared to wildtype or heterozygous littermates. However, the frequency of long-term stem cells (Kit^{+}Lineage^{-}Sca^{+}CD48^{-}CD150^{+}; SLAM) within the KLS compartment was significantly lower in *Casp9*$^{-/-}$ FLCs, resulting in no net alteration in long-term hematopoietic stem cell (LT-HSC) abundance. (**c**) Similar to these *in vitro* findings, bone marrow harvested from lethally-irradiated wildtype congenic mice 12 weeks after transplantation with *Casp9*$^{-/-}$ FLCs had an increase in KLS and a decrease in SLAM cells within the KLS compartment, compared to recipients of *Casp9*$^{+/+}$ FLCs, resulting in a modest decrease in the frequency of LT-HSCs overall. ns, not significant; *, $P<0.05$; **, $P<0.005$;***, $P<0.0005$; ns, not significant.

Figure 3.2: *Casp9*$^{-/-}$ hematopoietic stem cells are functionally impaired. (**a**) FLCs from *Casp9*$^{-/-}$ or *Casp9*$^{+/+}$ pups (Ly-5.2^{+}) were transplanted 1:1 in competition with wildtype congenic (Ly-5.1^{+}) FLCs into lethally-irradiated wildtype congenic (Ly-5.1^{+}Ly-5.2^{+}) recipients. Peripheral blood was analyzed for donor chimerism (%Ly-5.2^{+} %Ly-5.1^{+}Ly-5.2^{+}) at monthly intervals. *Casp9*$^{-/-}$ cells demonstrated a competitive disadvantage at 1-3 months, suggesting that their short-term repopulating cells are functionally impaired. Each curve represents the mean (+/- SEM) of 3-4 recipients from separate FLC donors (n=3 donors per genotype, 21 recipients total). Dotted line indicates no difference in competitive advantage (test competitor = 1). (**b**) In secondary transplants, bone marrow cells derived from *Casp9*$^{-/-}$ primary recipients failed to reconstitute the hematopoietic compartment of lethally-irradiated secondary recipients, although secondary transplants from donors engrafted with *Casp9*$^{+/+}$ cells were successful (*final cohort at 1 month on 8-25, sac on 9-25*). Each curve represents the mean (+/- SEM) of 4 recipients from separate donors (n=3 donors of each genotype, 24 recipients total). (**c**) Noncompetitive transplants with *Casp9*$^{-/-}$ or *Casp9*$^{+/+}$

FLCs had similar engraftment in lethally irradiated wildtype congenic (Ly-5.1$^+$) recipients (mean % donor cells at 1 month of 87.0% and 81.2% for *Casp9*$^{+/+}$ and *Casp9*$^{-/-}$, respectively; P=0.086). Each curve represents the mean (+/- SEM) of 1-5 recipients from *Casp9*$^{-/-}$ (n=7) or *Casp9*$^{+/+}$ (n=5) donors. (**d**) Recipients of *Casp9*$^{-/-}$ FLCs developed leukopenia, anemia, and lymphopenia, compared to recipients of *Casp9*$^{+/+}$ FLCs. ***, P<0.0005

Figure 3.3: Loss of *Casp9* or *Apaf1* leads to bone marrow failure after transplant. (**a**) FLCs from *Casp9*$^{+/+}$ or *Casp9*$^{-/-}$ pups were transplanted into lethally-irradiated wildtype C57BL/6J recipients. Mice were followed after transplant and sacrificed when moribund or electively (vertical ticks). Recipients of *Casp9*$^{-/-}$ FLCs had significantly decreased survival, compared to mice transplanted with *Casp9*$^{+/+}$ FLCs. (**b**) Similarly, recipients of *Apaf1*$^{-/-}$ FLCs had significantly reduced survival, compared to mice transplanted with *Apaf1*$^{+/+}$ FLCs. (**c**) Examination of bone marrow in moribund *Casp9*$^{-/-}$ or *Apaf1*$^{-/-}$ transplant recipients revealed signs of erythroid dysplasia (arrows: nuclear blebbing), and features of abnormal myeloid differentiation, including hyper-lobulated neutrophils and a shift to less differentiated forms (**d**). (**e**) The proportion of cells showing dysplastic changes in the erythroid and myeloid lineages were increased in recipients of *Casp9*$^{-/-}$ or *Apaf1*$^{-/-}$ FLCs. *, P<0.05; **, P<0.005; ***, P<0.0005

Figure 3.4: Loss of *Casp9* or *Apaf1* results in decreased apoptosis and increased DNA damage after ENU treatment. (**a**) FLCs from *Casp9*$^{-/-}$ or *Apaf1*$^{-/-}$ pups (or wildtype littermates) were treated with ENU or an equivalent concentration of vehicle only (0.1% DMSO) for one hour then allowed to recover for 24 hours. Cells were then stained with Annexin V to measure apoptosis. *Casp9*$^{-/-}$ and *Apaf1*$^{-/-}$ FLCs had reduced apoptosis in response to ENU. Each bar represents the mean (+/- SEM) for 3-9 mice. (**b**) To determine if the reduction in apoptosis detected in *Casp9*$^{-/-}$ and *Apaf1*$^{-/-}$ FLCs was associated with an increase in DNA damage in the surviving cells, the alkaline comet assay was used to measure

DNA damage in cells exposed to varying doses of ENU with varying lengths of recovery time after exposure. *Casp9*$^{-/-}$ cells showed increased DNA damage at all ENU doses tested at 72 hours post-ENU treatment (when apoptosis is complete). DNA damage decreased in ENU-treated *Casp9*$^{+/+}$ FLCs as apoptotic cells were cleared, but was sustained at high levels up to 120 hours after ENU exposure (300 ug/mL) in *Casp9*$^{-/-}$ FLCs. Each bar represents the mean (+/- SEM) of at least 140 nuclei from 2-3 (*Casp9*$^{+/-}$) or 3-5 (*Casp9*$^{-/-}$ and *Casp9*$^{+/+}$) FLC samples. (**c**) Comets from *Casp9*$^{-/-}$ and *Apaf1*$^{-/-}$ nuclei (300 ug/ml ENU, 72 hours after treatment) had significantly higher levels of DNA damage (% of DNA in tail), compared to other genotypes. Bars show the mean of at least 200 nuclei from at least 3 mice, with vertical lines extending to the 1st and 3rd quartiles. (**d**) Lethally-irradiated wildtype congenic mice were transplanted with *Casp9* $^{+/+}$, *Casp9*$^{-/-}$, *Apaf1*$^{+/+}$ or *Apaf1*$^{-/-}$ FLCs and treated with ENU (200 mg/kg IP) or vehicle only (10% DMSO) at 12 weeks post-transplant. 72 hours after injection, mice were sacrificed and bone marrow nuclei were analyzed for DNA damage by comet assay. Consistent with *in vitro* results, *Casp9*$^{-/-}$ and *Apaf1*$^{-/-}$ bone marrow nuclei showed increased levels of DNA damage, compared to wildtype nuclei after ENU treatment (n=3-4 mice, at least 270 nuclei from each). *, $P<0.05$; ***, $P<0.0005$

Figure 3.5: Loss of *Casp9* leads to oligoclonal hematopoiesis after alkylator treatment. *Casp9*$^{-/-}$ or *Casp9*$^{+/+}$ FLCs were transplanted into lethally-irradiated wildtype congenic recipients. At 6 and 7 weeks post-transplant, recipient mice were injected with ENU (100 mg/kg) or an equivalent volume of 10% DMSO. At 12 weeks after treatment, donor cells were sorted from the bone marrow, and exome sequencing was performed. Somatic single nucleotide variants (SNVs; present in the bone marrow and absent in matched donor fetal tissue) were identified. SNVs were more frequent and had higher variant allele frequencies (VAFs) in DMSO-treated *Casp9*$^{-/-}$ cells, compared to *Casp9*$^{+/+}$ cells (mean SNVs 386 vs. 10, P=0.025; mean VAF 4.38% vs. 5.591%, P=0.0002) and in ENU-treated *Casp9*$^{-/-}$ cells,

compared to *Casp9*$^{+/+}$ cells (mean SNVs 1306 vs. 28, P=0.0014; mean VAF 5.87% vs. 3.80%, P<0.0001).

Figure 3.6: Model for impact of *Casp9* deficiency on hematopoiesis and response to genotoxic stress. *Casp9*$^{-/-}$ fetal livers have increased numbers of KLS cells, but no change in the abundance of LT-HSCs. *Casp9*$^{-/-}$ HSCs have reduced repopulating ability and generate a lineage-biased hematopoietic compartment with reduced numbers of B-cell and erythroid progenitors resulting in lymphopenia and anemia. Exposure of *Casp9*$^{-/-}$ cells to genotoxic stress results in reduced apoptosis, accumulation of DNA damage, and outgrowth of clonal hematopoiesis.

3.7 Figures

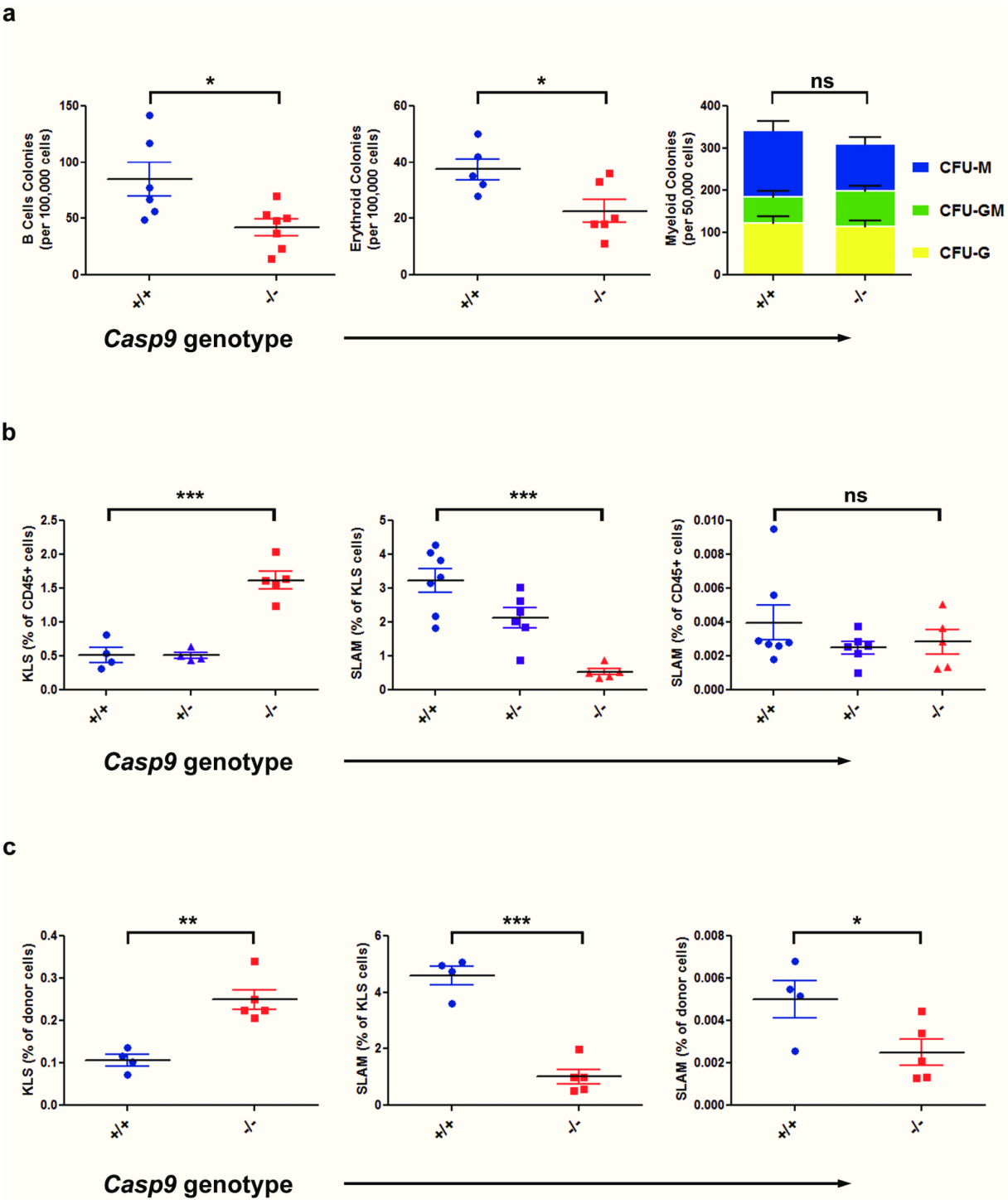

Figure 3.1: Loss of *Casp9* alters hematopoietic progenitor cell frequency.

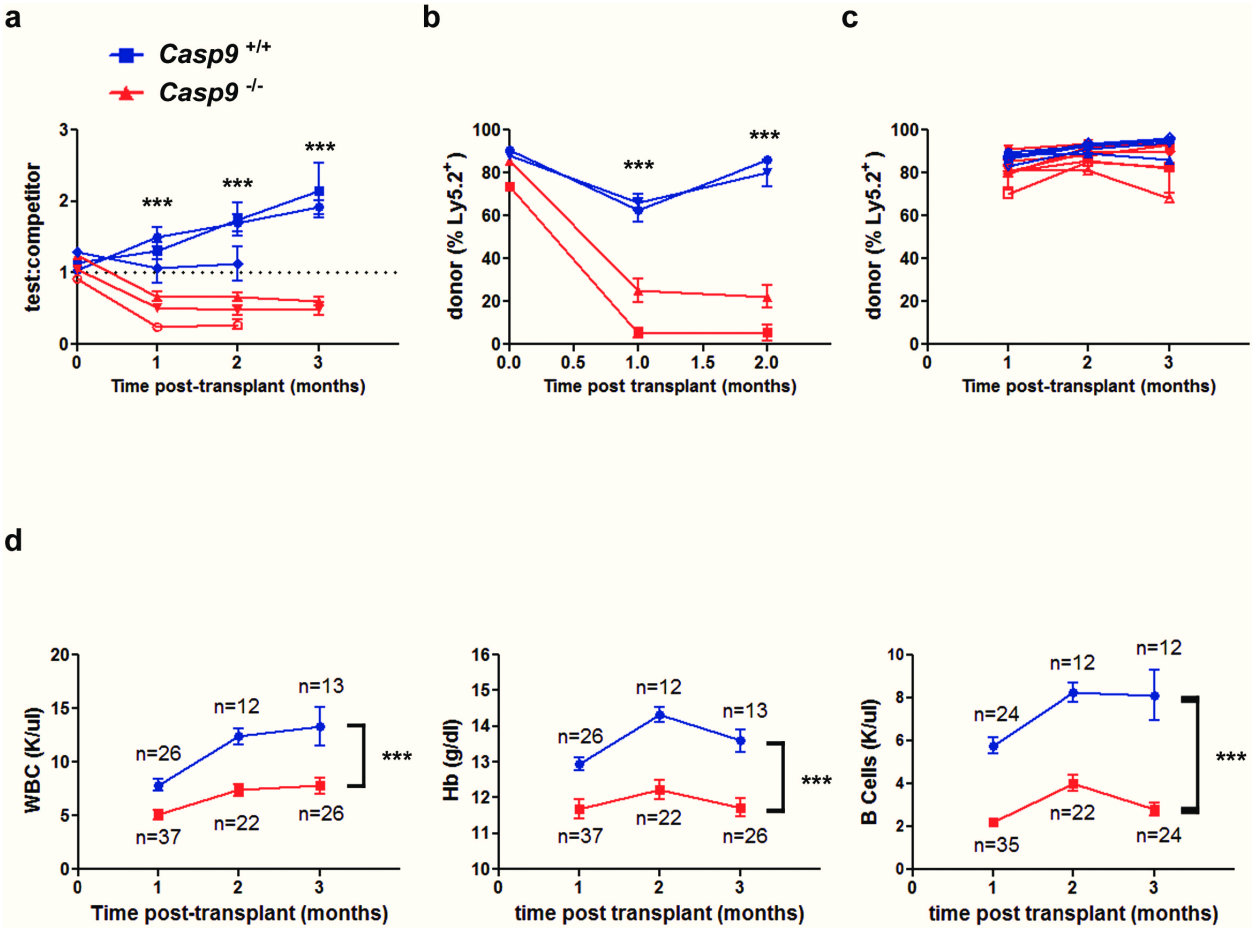

Figure 3.2: *Casp9*$^{-/-}$ hematopoietic stem cells are functionally impaired.

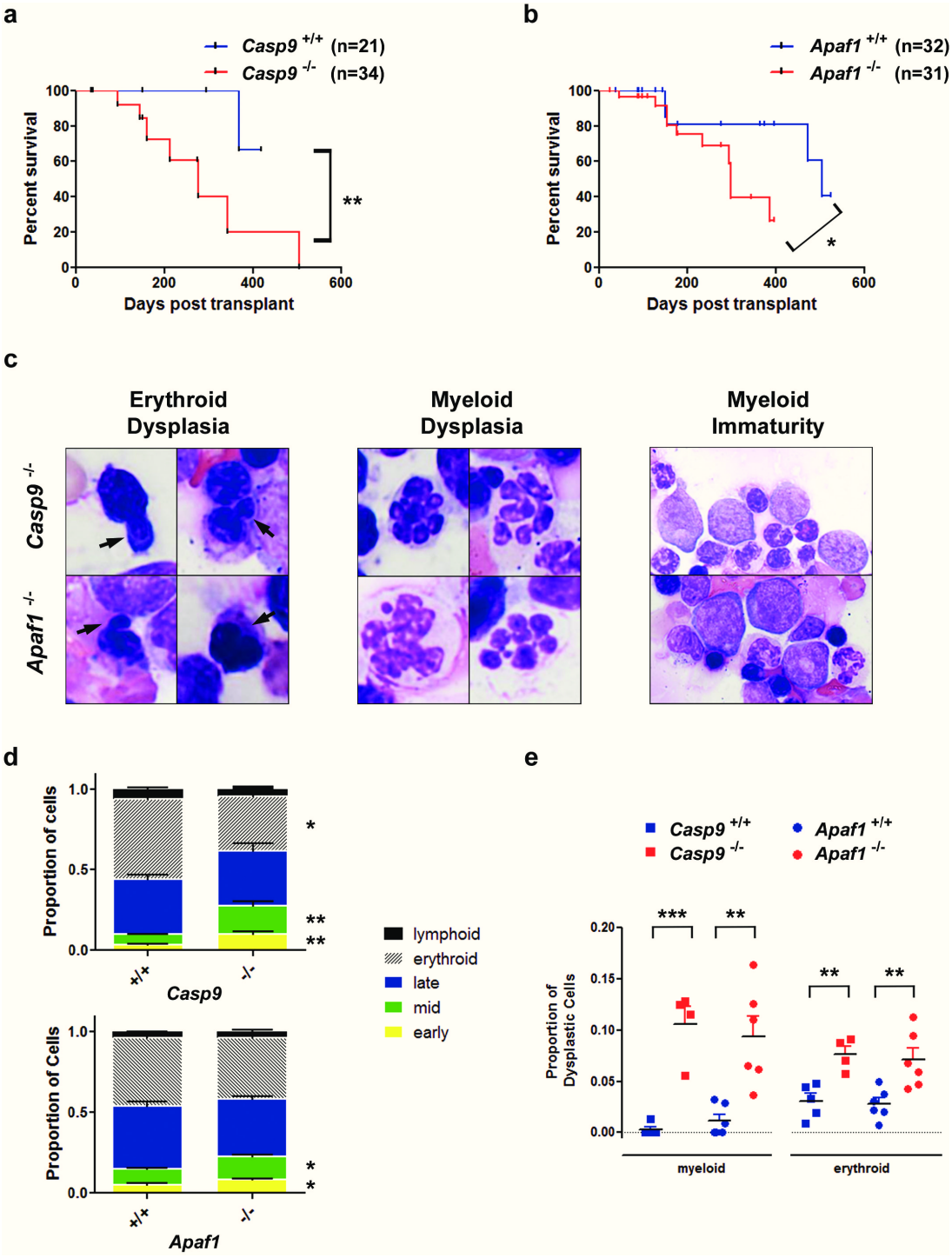

Figure 3.3: Loss of *Casp9* or *Apaf1* leads to bone marrow failure after transplant.

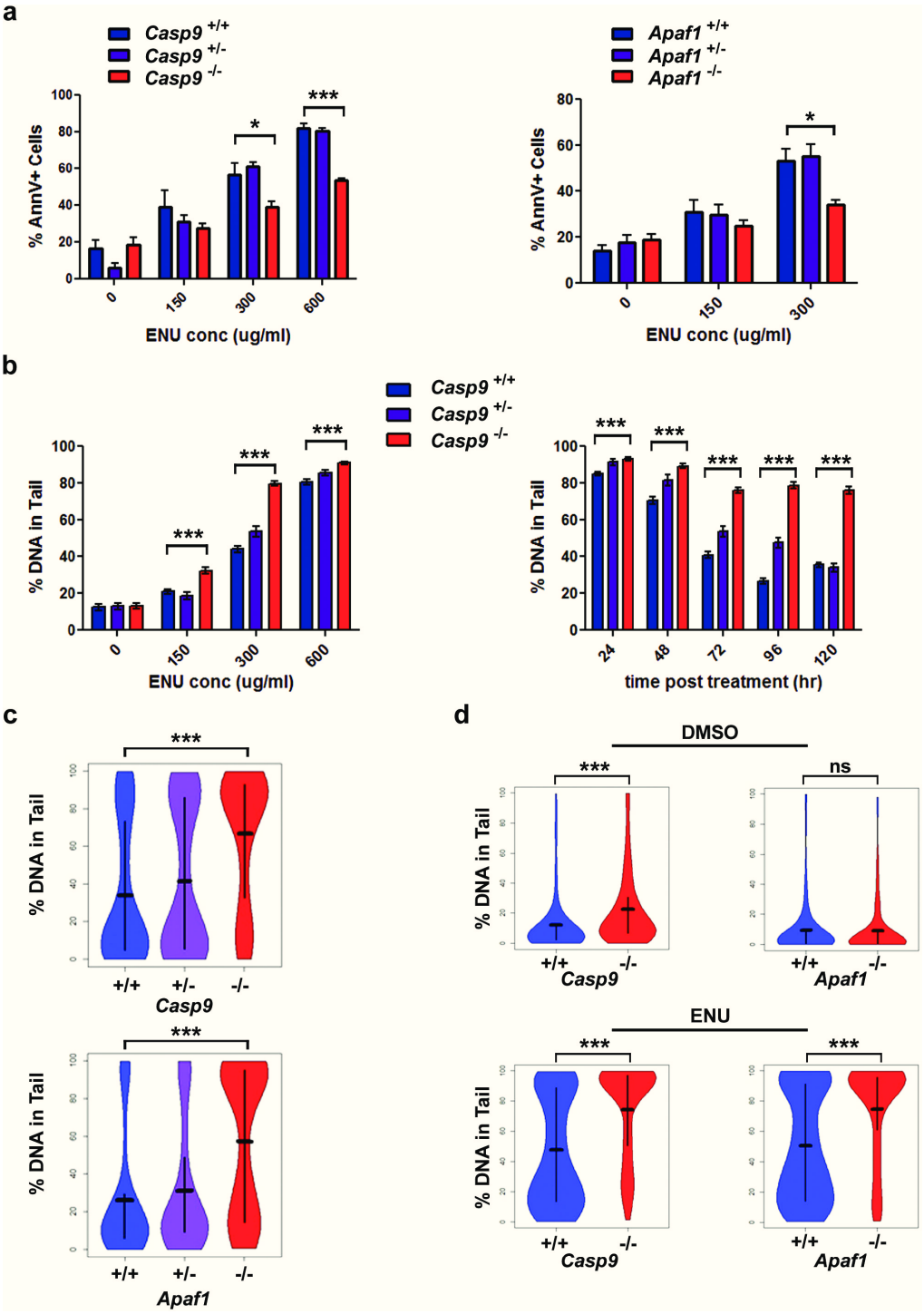

Figure 3.4: Loss of *Casp9* or *Apaf1* results in decreased apoptosis and increased DNA damage after ENU treatment.

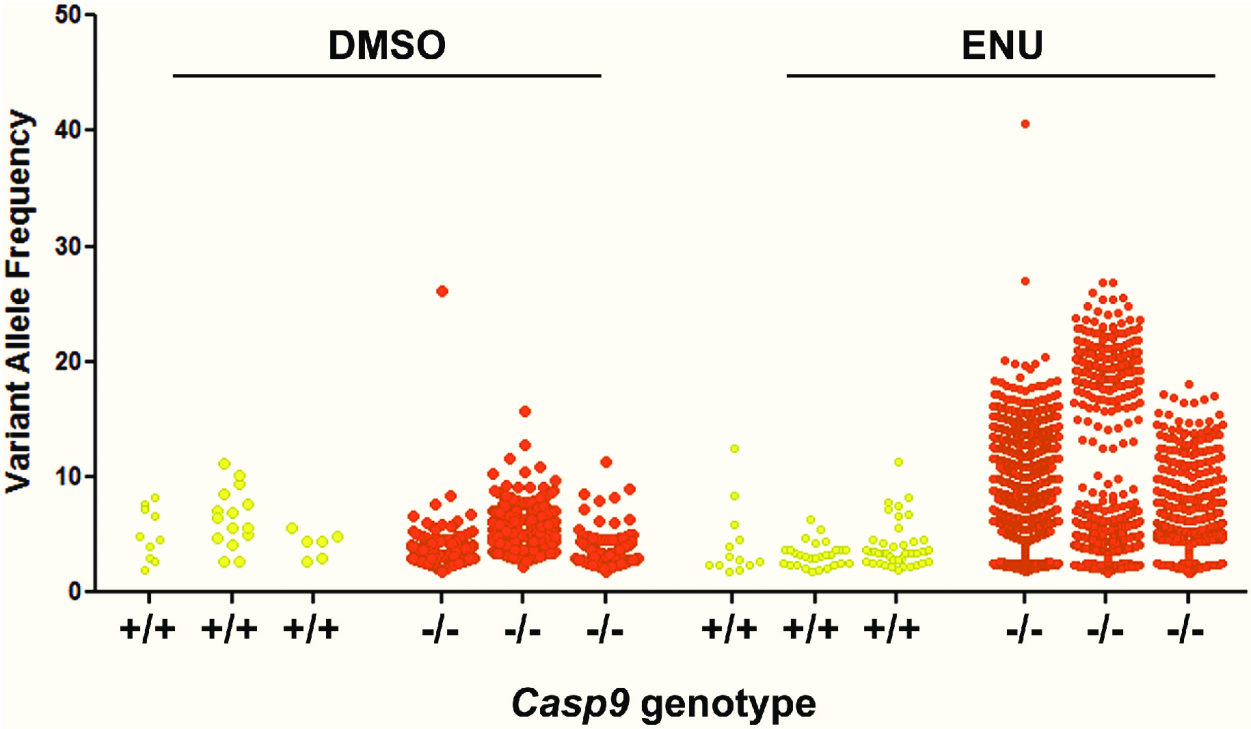

Figure 3.5: Loss of *Casp9* leads to oligoclonal hematopoiesis after alkylator treatment.

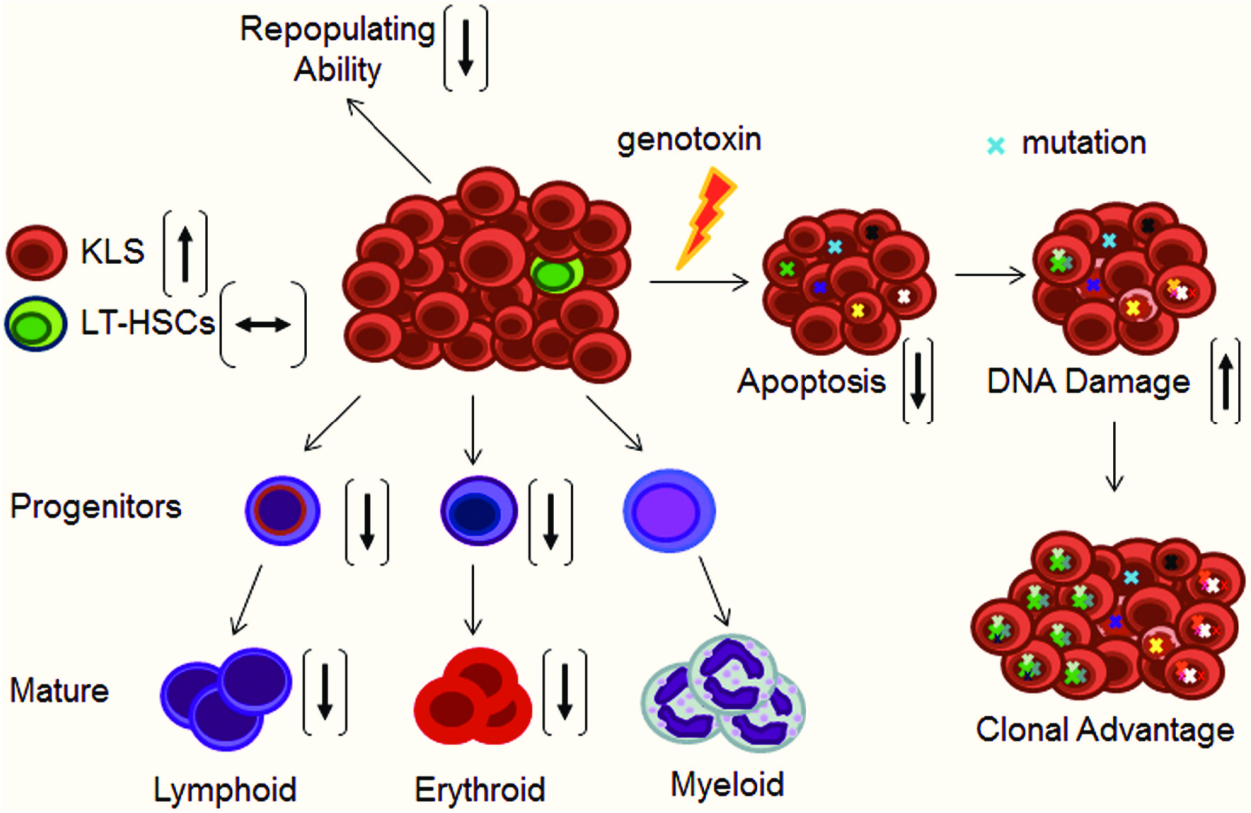

Figure 3.6: Model for impact of *Casp9* deficiency on hematopoiesis and response to genotoxic stress.

3.8 Supplementary Materials

METHODS

Genotyping. Genotyping for *Apaf1* mice was performed as described by Jackson Laboratory. Genotyping for *Casp9* mice was performed using the primers given in the table below. Two separate PCR reactions were performed using the following protocol: 94°C for 2 minutes, 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 2 minutes, 72°C for 5 minutes, with 35 cycles.

	Wild Type	Knock Out
Forward	AGGCCAGCCACCTCCAGTTCC	TGCTAAAGCGCATGCTCCAGACTG
Reverse	CAGAGATGTGTAGAGAAGCCCACT	ATCACCACATCTTGACCACACCCT
Expected Band Size	270 bp	350 bp

FIGURE LEGENDS

Supplemental Figure 3.1: Hematopoietic consequences of *Apaf1* deficiency phenocopies *Casp9* deficiency. (**a**) 2x10^6 FLCs from *Apaf1*$^{+/+}$ or *Apaf1*$^{-/-}$ mice (Ly-5.2$^+$) were transplanted into lethally-irradiated congenic wildtype C57Bl/6J (Ly-5.1$^+$) recipients. Cells from all genotypes successfully engrafted (mean donor chimerism of 89.9% and 86.9% for *Apaf1*$^{+/+}$ and *Apaf1*$^{-/-}$ respectively; P=0.12) and the graft was maintained beyond 3 months. Each curve represents mean (+/- SEM) of 4 recipients from *Apaf1*$^{-/-}$ (n=3) or *Apaf1*$^{+/+}$ (n=5) donors. (**b**) Recipients of *Apaf1*$^{-/-}$ donor cells developed modest leukopenia and anemia and (**c**) had decreased contribution to peripheral blood B220$^+$ and Gr1$^+$ cells. (**f**) Transplantation of *Apaf1*$^{-/-}$ bone marrow failed to durably reconstitute hematopoiesis in secondary recipients (n=3 donors per genotype, 1-4 recipients each). (*Data collection will be complete on 090513.*)

Supplemental Figure 3.2: Apoptosis peaks between 12 and 24 hours after ENU treatment. (a) FLCs from *Casp9*$^{+/-}$ pups were treated with ENU or an equivalent concentration of vehicle only (0.1% DMSO) for one hour then allowed to recover for 24 hours. Cells were then stained with Annexin V to measure apoptosis. The time course of treatment with 600 ug/ml ENU is shown. Zero hour time point was determined by averaging % Annexin V positivity in DMSO-treated samples (n=10). **(b)** To determine if the DNA damage observed by the comet assay could be due to delayed-onset apoptosis, FLCs and Bone marrow treated ENU and analyzed for DNA damage by comet assay were also fixed and permeablized and stained with DAPI. The percentages of cells with a subdiploid complement of DNA, a sign of late stage apoptosis, are shown.

Supplemental Figure 3.3: Experimental design to determine clonality by exome sequencing. FLCs from three *Casp9*$^{-/-}$ and three *Casp9*$^{+/+}$ pups (Ly-5.2+) were transplanted into four lethally-irradiated congenic (Ly-5.1) wildtype recipients each. At 6 and 7 weeks post-transplant, recipient mice were injected with ENU (100 mg/kg) or equivalent volume of 10% DMSO. At 12 weeks after treatment, one mouse from each donor/treatment group was sacrificed (12 mice total). Bone marrow was harvested and sorted for donor cells (Ly-5.2+). DNA was prepared from these sorted cells as well as from limb tissue gathered from the original FLC donor. Exome sequencing was performed using 3 replicate libraries per bone marrow (tumor) sample and a barcoded pool of matched normal libraries from each donor.

Supplemental Figure 3.4: Coverage statistics for exome sequencing. Exome sequencing generated 158-228X coverage, on average, for bone marrow (tumor) samples and 25-44X coverage, on average, for matched normal samples.

***Supplemental Figure* 3.5: ENU alters the mutation spectrum in mice transplanted with *Casp9*$^{-/-}$ cells.** (**a**) The proportion of C>A and A>T SNVs were higher and A>G and G>A SNVs were lower in ENU-treated *Casp9*$^{-/-}$ bone marrow cells, compared to DMSO-treated *Casp9*$^{-/-}$ bone marrow cells. (**b**) ENU-treated *Casp9*$^{-/-}$ bone marrow cells had disproportionately greater transversion and fewer transition SNVs, compared to SNVs in ENU-treated *Casp9*$^{+/+}$ or DMSO-treated bone marrow cells. *, $P<0.05$; **, $P<0.005$; ***, $P<0.0005$

3.9 Supplementary Figures

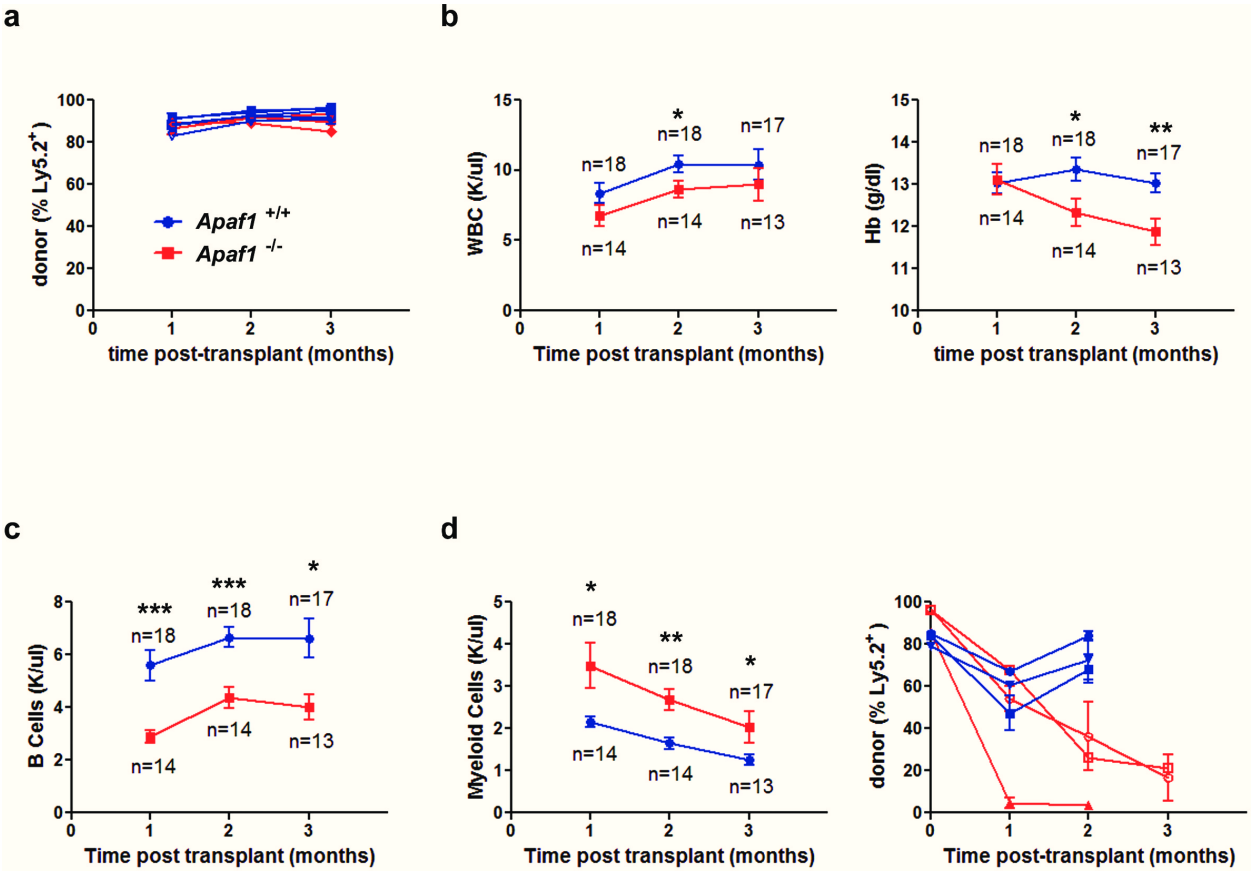

Figure S3.1: Hematopoietic consequences of *Apaf1* deficiency phenocopies *Casp9* deficiency.

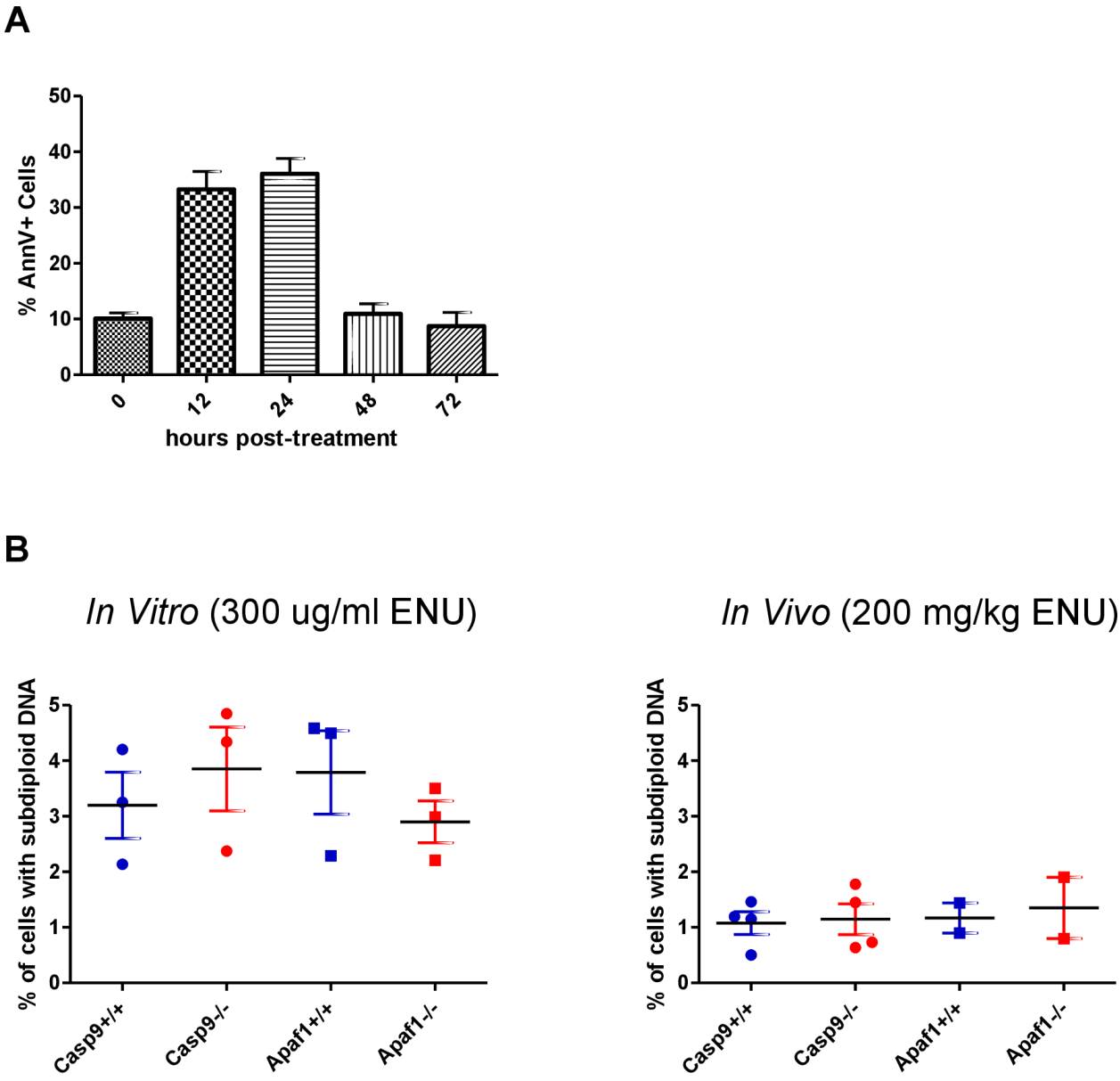

Figure S3.2: Apoptosis peaks between 12 and 24 hours after ENU treatment.

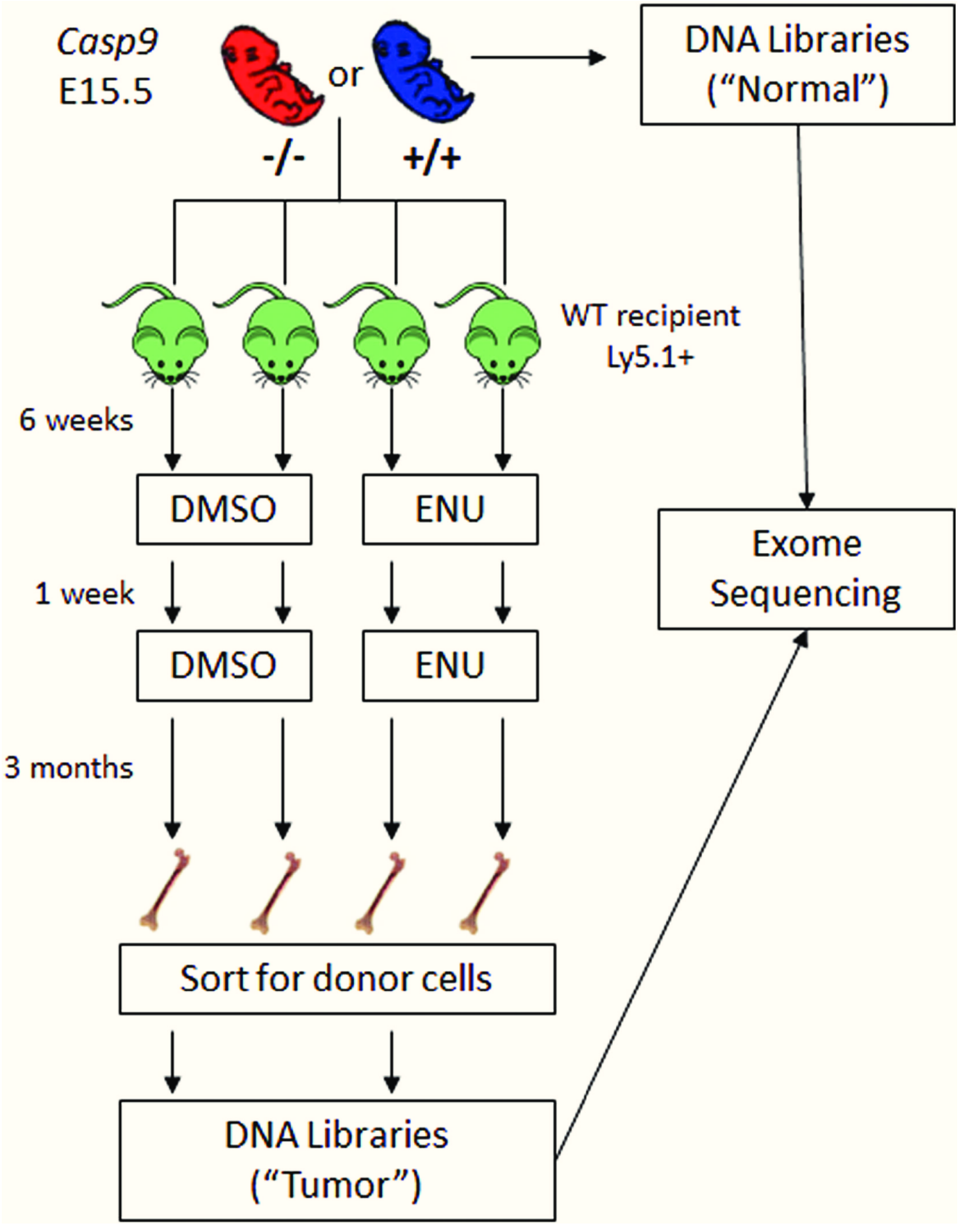

Figure S3.3: Experimental design to determine clonality by exome sequencing.

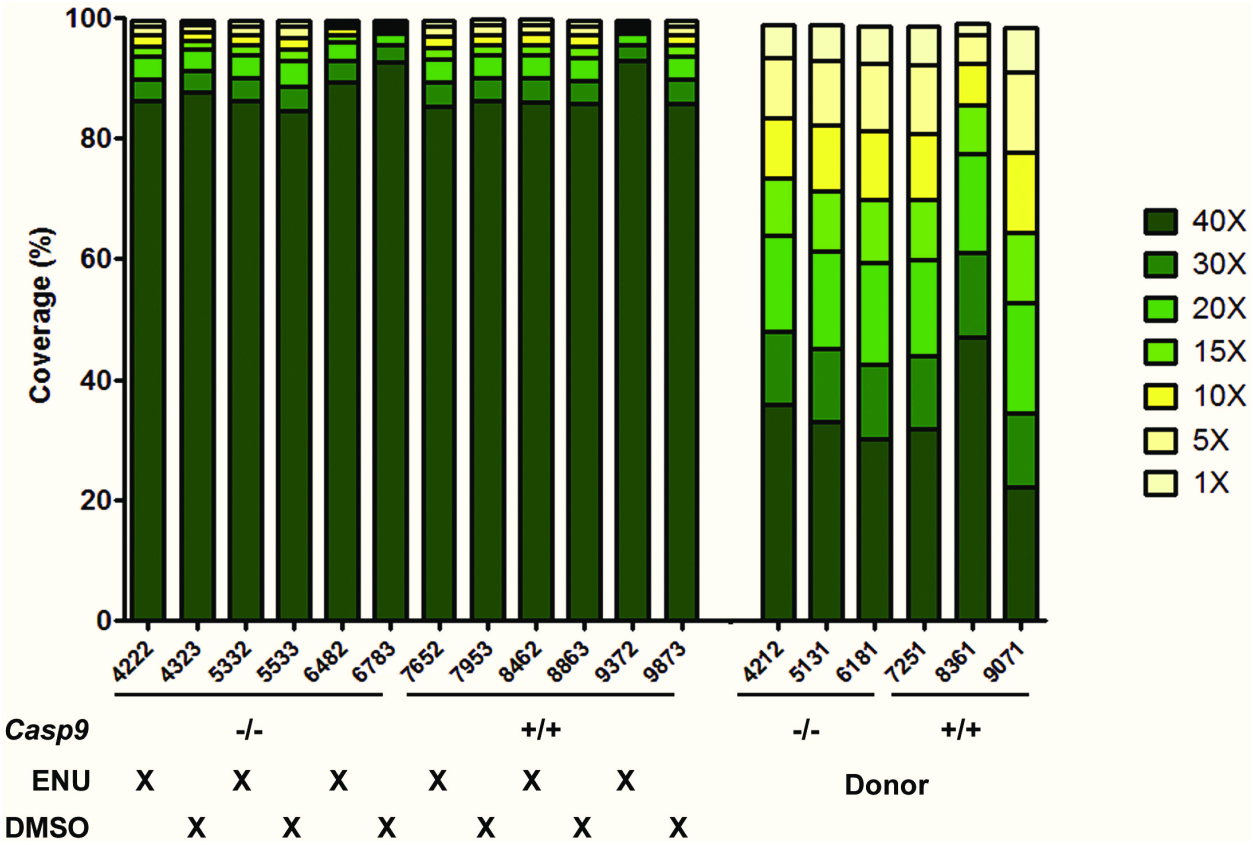

Figure S3.4: Coverage statistics for exome sequencing.

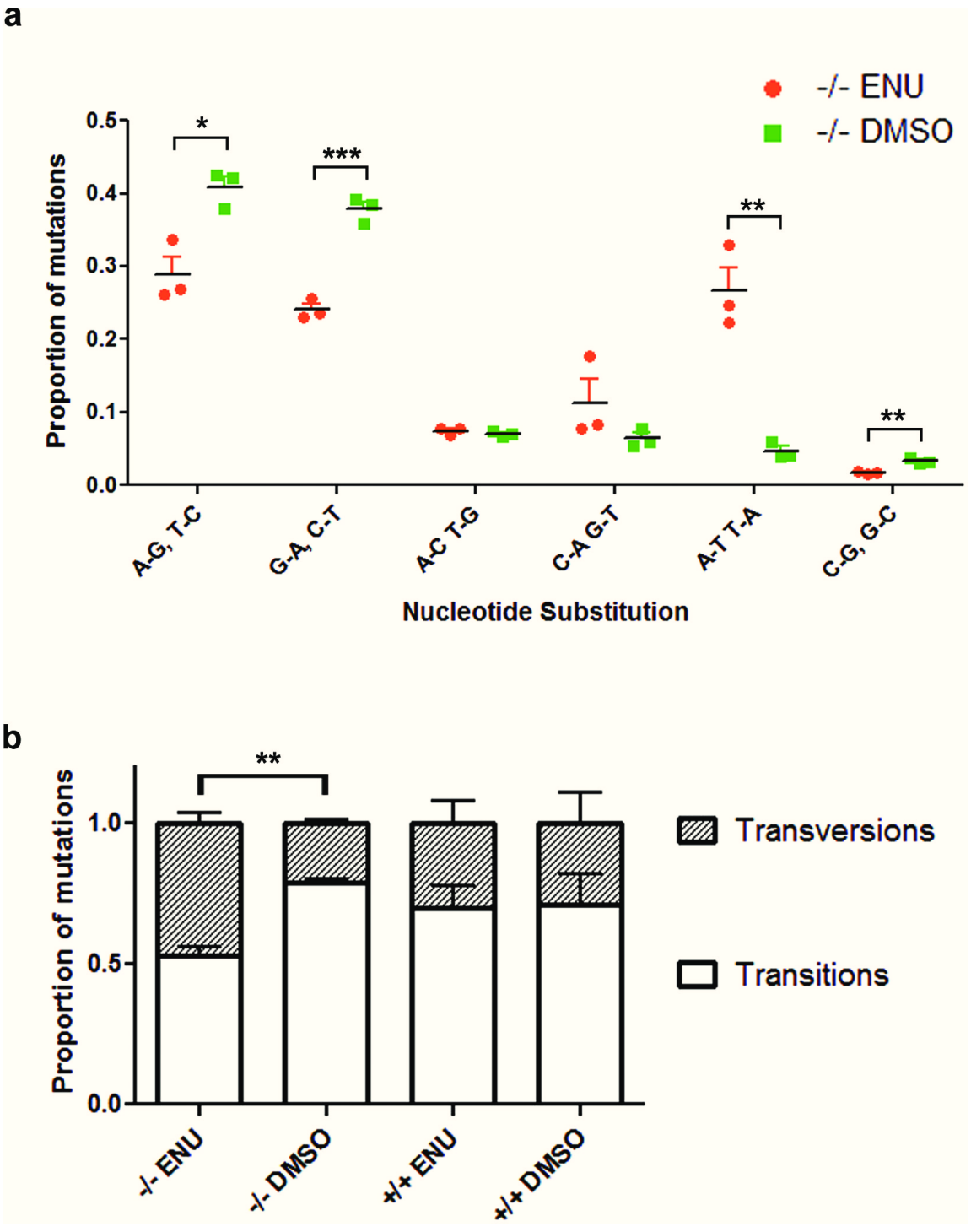

Figure S3.5: ENU alters the mutation spectrum in mice transplanted with *Casp9*$^{-/-}$ cells.

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CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

In the work presented in this thesis, we have attempted to elucidate how loss of late-stage apoptosis affects hematopoiesis and susceptibility to therapy-related leukemia. In chapter 2 we described the identification of novel splice variants of Caspase-9 in mice susceptible to alkylator-induced leukemia. Although we found these splice variants to be non-functional, we found a correlation between Caspase-9 expression level, apoptosis in HSPCs, and susceptibility to tAML. Given that decreased apoptosis was associated with increased likelihood of tAML, we hypothesized that decreased apoptosis in HSPCs could allow cells damaged by exposure to alkylators to inappropriately survive carrying DNA damage. These cells could then become dysplastic or give rise to leukemia via malignant transformation.

In chapter 3, we addressed this hypothesis by examining the effects of complete loss of Caspase-9 on hematopoieitic function both with and without alkylator exposure. Loss of Caspase-9 resulted in decreased hematopoietic function, particularly in the stem cell compartment. Caspase-9-deficient HSCs were at a competitive disadvantage in competitive repopulation assays and also could not reconstitute hematopoiesis in serial transplantation. A lineage bias also resulted from loss of Caspase-9, with Caspase-9 deficient mice showing decreased erythroid and B-cells and a possible increase in myeloid cell abundance in peripheral blood and bone marrow. Ultimately, most Caspase-9-deficient mice succumbed to bone marrow failure and died permaturely relative to wild type mice. This phenotype was replicated in Apaf-1 deficiency as well, indicating that this a true consequence of the apoptotic defect of Caspase-9 deficiency and is not unique to this single mouse model. This indicates that loss of late stage apoptosis results in a defect in stem cells both in self-renewal and repopulation and in lineage determination.

The mechanism behind this effect is not clear and future work should address this. Why does loss of late-stage apoptosis render HSCs less fit? Previous work studying overexpression of Bcl2, which prevents cells from undergoing apoptosis by preventing permeablization of the mitochondrial membrane, has no such effect. Mice overexpressing Bcl2 have increased numbers of HSCs and these HSCs have a competitive advantage in transplantation, quite the reverse of what we seen in Caspase-9 or Apaf1 deficient mice [5]. This suggests that the defects we see in our mice are not simply due to evasion of apoptosis. The stem cell defect in Caspase-9 and Apaf1-deficient mice is similar in many ways to defects seen in mice deficient in DNA damage-repair pathways or in p53. It is also reminiscent of the phenotype observed in aging mice and humans. This suggests that our stem cell defect could be related to accumulation of DNA damage. Cells deficient in Caspase-9 or Apaf1 can progress through apoptosis do the point of MOMP, but then can go no farther. If they were triggered to die

due to DNA damage, then they inappropriately survive carrying that original damage. But MOMP can exacerbate that further, since it has been reported that MOMP increases the level of ROS in the cell [9, 4]. ROS are known mutagens that could contribute to further DNA damage. ROS are also variously reported to cause HSC quiescence [8], limit serial transplantation efficiency [1], and even cause increased HSC cycling and loss of "stemness" by disrupting quiescence [7, 1]. Therefore, increase in ROS production in cells that have undergone MOMP could also contribute to the stem cell defect.

Because the HSPC pool is forming during fetal development, DNA replication is happening rapidly and DNA damage accumulation can occur much faster than during other stages hematopoiesis. Therefore, it is quite possible that fetal liver HSCs would have accrued sufficient DNA damage to be dysfunctional by E15.5, when we analyze them. Just such accumulation of DNA damage during fetal development has been reported in human cases of Fanconi Anemia [3], which is also associated with an HSC defect and progressive bone marrow failure.

In the future, we could address this question by sorting various HSPC sub-populations from fetal liver or adult bone marrow and measuring DNA damage and ROS levels. Although this would not prove that DNA damage causes the stem cell defect, if we do indeed see increased levels of DNA damage in HSCs it would at least provide correlative evidence.

It would also be of value to further immunophenotypically characterize the stem cell compartment in the absence of late-stage apoptosis. Complete characterization of stem cells in our studies was hampered by the unique difficulties of staining for stem cell markers in fetal liver cells. FLCs are studied less frequently and are, therefore, less well characterized than adult bone marrow in mice. Therefore, we could not determine the baseline abundance of multipotent progenitor cells or determine precisely what cell population was responsible

for the increase in the KLS compartment. The generation of conditional or lineage-specific knockout mice could address these difficulties. A mouse with no expression of Caspase-9 in the hematopoietic compartment would allow characterization of adult HSCs without the confounding factor of bone marrow transplantation. In these studies, we generated bone marrow chimeras by fetal liver cell transplantation. This transplantation step induces stress in the transplanted cells. Therefore, it would be interesting and informative to examine caspase-9-deficient adult hematopoiesis without the addition of stressors. We would predict that the phenotype would be very similar, but it is possible that the bone marrow failure phenotype we observed is, in part, induced by the stress of transplantation.

We next sought to determine how loss of Caspase-9 affects response to alkylators and, subsequently, how it might cause increased susceptibility to tAML. Treating Caspase-9 or Apaf1-deficient cells in vitro or in vivo with ENU resulted in decreased apoptosis and increased DNA damage in the surviving cells, validating our model. Assaying for tAML susceptibility was more complex because murine models of MDS and leukemia do not fully recapitulate human disease and alkylator exposure in C57Bl/6 mice often causes T-cell lymphoma. Therefore, instead of simply treating chimeric mice with ENU and trying to determine if they later succumbed to MDS or AML, we developed an approach based on exome sequencing. Clonal hematopoiesis is a hallmark of both MDS and AML, whether therapy-related or not, and it occurs early in disease progression. Therefore, we believed that we could use clonality as a marker of progression to tMDS or tAML in our mouse model. We treated wild type or Caspase-9-deficient bone marrow chimeras with ENU in vivo, then performed exome sequencing on the donors and post-treatment recipient mice. In wild type mice, the number of somatic SNVs was low, regardless of ENU-treatment. This suggests that the cells that acquired DNA damage from the ENU-treatment died and did not propagate their mutations, as our model predicted. However, DMSO-treated Caspase-9 deficient mice showed signficantly

higher numbers of SNVs than wild type mice. This suggests that even endogenous DNA damage accrues in apoptosis-deficient cells. ENU-treated Caspase-9 deficient mice showed the greatest mutation burden of all, and many SNVs occurred at VAFs >10%, suggesting that they were present in a clone of cells descended from a single founder that had a growth or survival advantage. Clonal hematopoiesis only in ENU-treated Caspase-9-deficient mice suggests that the combination of defective apoptosis and alkylator treatment could result in tMDS or tAML.

In light of these results, there are many future avenues to explore. First, how do cells deficient in late stage apoptosis survive exposure to ENU, avoid CICD, and go on to grow clonally? Preliminary data from in vitro ENU treatment of FLCs suggests that Caspase-9 and Apaf1-deficient cells disproportionately enter cell cycle arrest after ENU treatment. This is consistent with published reports of cell cycle arrest after exposure to DNA damaging agents, but this does not explain long term survival and eventual clonal outgrowth. Is incomplete MOMP and mitochondrial regeneration responsible? Examination of mitochondrial membrane potential and markers of oxidative phosphorylation after ENU treatment could determine if cells are recovering mitochondrial function. It is also possible that cells are becoming dependent on glyclolysis for energy generation, rather than regaining complete mitochondrial function, so this should also be explored.

Given that Caspase-9 deficient HSCs have decreased fitness, how does addition of a DNA damaging agent in these mice result in clonal outgrowth? If ENU simply added further damage to an already feeble hematopoieit system, it could be expected to simply worsen the original defect leading to a more rapid progression of bone marrow failure. However, addition of ENU results in oligoclonal hematopoiesis, suggesting that some of these "less fit" HSCs somehow gained an advantage and gave rise to a greater number of progeny cells than others.

As shown in our DNA damage assays, Caspase-9-deficient cells accumulate DNA damage after exposure to ENU. Therefore, it is possible that Caspase-9-deficient HSCs accumulated DNA damage, leading to mutations, some of which could be advantageous. These advantageous mutations then allowed these HSCs to proliferate, giving rise to clones of cells descended from a few founders. This clonal outgrowth would be more likely to happen in Caspase-9 deficient mice, despite their underlying stem cell defect, because their cells accrue DNA damage. Wild type HSCs carrying DNA damage simply undergo apoptosis and, therefore, cannot give rise to clones.

Another possibility is that ENU-induced apoptosis itself triggers Caspase-9 deficient HSCs to proliferate. There are some reports that high levels of ROS induce HSC replication and proliferation [1, 7]. If Caspase-9 deficient cells are triggered to undergo apoptosis, they undergo MOMP but do not complete the caspase cascade, which may result in increased ROS production in the undead cell. If the cell can survive this and avoid CICD or necrosis, then this increased ROS could induce it to cycle. If the cell in question is an HSC, this could result in the expansion of clone of HSCs which would then give rise to clonal hematopoiesis. This process occurring in multiple HSCs would give rise to oligoclonal hematopoieis.

These two possibilities are not mutually exclusive and are difficult to tease apart. As discussed previously, determining if ENU treatment actually results in an increase in ROS production would be a first step in determining if ROS-induced cycling is an important mediator of clonality. Further analysis of our exome sequencing data to identify any possible driver mutations that could confer clonal advantage would also help to elucidate this mechanism. However, absence of obvious driver mutations in the exome does not exclude mutation accumulation as a mechanism for clonal outgrowth. The exome comprises only a small fraction of the genome as a whole, and it is likely that mutations in regulatory sequences not covered by our sequencing assay could serve as driver mutations.

We are also working to determine if the clonal hematopoiesis observed in our ENU-treated, Caspase-9 deficient mice is indicative of a transplantable disease. We have taken spleen cells from ENU treated mice, both wild type and Caspase-9 deficient, and transplanted these into lethally irradiated recipients. If the sequenced mice had developed leukemia or were progressing towards leukemia, we would expect to see the transplants engraft successfully and cause disease in the host. Given that serial transplantation with Caspase-9 deficient cells fails under normal conditions, observing just successful engraftment of ENU-treated Caspase-9 deficient cells would indicate a change from baseline due to ENU treatment.

Ultimately, determining the role of apoptosis in susceptibility to tAML in humans is the most important next step. Expression data indicates that neither Caspase-9 nor Apaf1 are significantly differentially expressed in AML samples when compared to normal CD34 cells (Duckworth and Ley, unpublished data). However, we would be interested to know the expression patterns of these genes in tAML patients specifically. Also, because apoptosis is such a complex and often redundant pathway, it is reasonable to look at functional apoptotic capacity rather than only examining expression of a few specific genes. There is some work in the literature to support this approach. Inter-individual variation in the tendency to undergo apoptosis has been reported in various cell types, including AML myeloblasts, human embryonic stem cells and cord blood CD34+ cells [10, 6, 2]. Mitochondrial priming of myeloblasts and human embryonic stem cells has been correlated with apoptotic response to DNA damaging agents and, in the case of myeloblasts, the ultimate success of chemotherapy in AML patients. Mitochondrial priming indicates the general predilection of a cell to undergo apoptosis or not and is the result of the balance of pro- and anti-apoptotic factors around the mitochondrial membrane. Based on the work presented in this thesis, we would hypothesize that patients with a higher degree of mitochondrial priming in their HSCs would

be less likely to develop MDS or AML after chemotherapy, because those HSCs would be more likely to undergo apoptosis after treatment.

4.1 References

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