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The Genetic Basis of Susceptibility to Therapy-related Leukemia in Mice

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The Genetic Basis of Susceptibility to Therapy-related Leukemia in Mice

by
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ABSTRACT OF THE DISSERTATION

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Treatment-related acute myeloid leukemia (t-AML) arises as a result of treating primary malignancies with alkylator chemotherapy drugs and has a poor prognosis. Genetic background influences the risk of acquiring t-AML, yet little is known about susceptibility factors. To identify candidate risk factors, cohorts of twenty inbred mouse strains were treated with N-ethyl-N-nitrosourea (ENU), a potent alkylating agent in mice. Six of these mouse strains were susceptible to alkylator-induced leukemia. SWR/J mice were the most susceptible in this relatively small screen. We expanded on that study to characterize SWR/J mice as a susceptible strain to t-AML using 245 mice. Mice were treated with different permutations of steroid treatment to determine the effect they would have on leukemia numbers. In addition to the susceptible strains, quantitative trait loci mapping was used on the initial study to identify genetic components responsible for the leukemic phenotype. This analysis revealed two significant peaks of interest on chromosomes 3 and 14. The 1 Mb region on chromosome 3 contains six genes, one of which was myeloid leukemia factor 1 (*Mlf1*). In humans, *MLF1* was

previously identified for its involvement in a chromosomal translocation with nucleophosmin (*NPM*) that is restricted to AML and myelodysplastic (MDS) patients. We show that MLF1 is pro-apoptotic and decreases the viability of hematopoietic cells.

CHAPTER 1

INTRODUCTION

1.1.TREATMENT-RELATED ACUTE MYELOID LEUKEMIA

Treatment-related acute myeloid leukemia (T-AML) is a secondary disease that arises as a result of initial treatment of a variety of hematologic malignancies or solid tumors with chemotherapy agents. Alkylating agents represent a class of drugs that have been shown to contribute to this disease and are typically preceded by a therapy-related myelodysplastic syndrome (t-MDS) phase. The latency of disease is 5 to 7 years following chemotherapy [1]. Topoisomerase II inhibitors represent a second class of drugs contributing to t-AML. The latency is shorter with this class of drugs and develops within 2 to 3 years following treatment[1, 2]. Patients developing t-AML have a poorer outcome than those developing *de novo* AML and have a median survival of only 6-10 months [3]. T-AML comprises 10% of new AML patients, and this incidence continues to rise [4, 5]. Therefore, there is an urgent need to develop strategies aimed at prevention. We are interested in identifying susceptibility genetic (inherited) factors for t-AML that could lead to future prevention of this disease.

1.1.1. POLYMORPHISMS

There is some evidence that genetic polymorphisms influence t-AML susceptibility in humans, but the specific genetic factors that predispose one person to acquire t-AML over another is not yet known. Candidate genes that have been studied extensively encode factors involved in drug detoxification pathways and DNA repair pathways.

DETOXIFICATION PATHWAYS

There are two phases to drug metabolism. Phase I involves the activation of substrates into intermediates. Phase II proteins inactivate genotoxic substrates [6]. Many polymorphisms from these two phases have been studied in t-AML.

Phase I activation proteins include the cytochrome p450 (Cyp) enzymes. These enzymes function in the detoxification process. Their role is to create strong reactive intermediates that can cause major DNA damage unless further detoxified by Phase II enzymes[6].

Phase II conjunction proteins include glutathione S-transferase (GST) and NAD(P)H: quinone oxidoreductase NQO1. GST is from a family of isoenzymes that detoxify reactive electrophiles to prevent DNA damage. NADH and NADPH catalyze the electron reduction of its substrates. This prevents the formation of reactive oxygen species and free radicals that would lead to cellular damage [7].

DNA REPAIR PATHWAYS

Mismatch repair functions as a proofreader of the DNA to avoid propogation of mutations. When there is an error in which simple repetitive DNA sequences are added, stability is tested and microsatellite instability has formed. Loss of *MLH1* (via promoter methylation), a mismatch repair family member, causes microsatellite instability in t-AML patients [8, 9].

Double strand break repair is very damaging because of the amount of material that can be lost. These types of breaks can cause cell death or chromosomal abberations. This can occur after exposure to a chemotherapy drug [6]. Homologous recombination will use the intact chromosome as a template and will repair the damage caused by the double stranded break. Non-homologous end joining joins broken ends of DNA with little homology. This is another way to connect the broken ends.

The base excision repair pathway corrects one individual bases that are incorrect. Nucleotide excision repair removes structurally bulky damage. It also repairs DNA damage caused by chemotherapy drugs.

1.2. Genetic Basis of t-AML Susceptibility in Mice

Several years ago, the Graubert laboratory screened cohorts of 20 strains of inbred mice to determine if there was a genetic component to ENU-induced leukemia [10]. This was a small screen with 12 treated and 12 untreated mice. 6 strains were susceptible and 14 were resistant. This demonstrated that there was a genetic component to underlying susceptibility to ENU-induced myeloid leukemia. SWR/J mice were the most susceptible to disease, making them a target for further characterization, which will be discussed in Chapter 2.

1.3. MLF1 IS A CANDIDATE SUSCEPTIBILITY FACTOR for T-AML

Fenske also performed quantitative trait locus mapping to accompany his research. *MLF1* was found as a candidate by using quantitative trait locus mapping across the mouse genome that looked for peaks of the genome that associated with T-AML susceptibility. 6 genes were found within a peak on chromosome 3, and of those genes, *MLF1* was the strongest based on the QTL results and because it was discovered as a fusion protein with NPM1-MLF1 that associated with t-AML/MDS (myelodysplastic syndrome). Chapter 3 describes its function in hematopoietic cells.

The purpose of this research is to 1) further characterize SWR/J mice as a susceptible strain and 2) understand the basic biology of MLF1 in hematopoietic cells. This provides a genetic model to study t-MDS/AML. No models are complete, so having a background where the mice are susceptible to disease is useful. Breeding SWR/J mice to genetically-altered mice provides a powerful tool for studying t-MDS/AML. Understanding candidate genetic factors to susceptibility could lead to potential therapeutics in the future. We identified MLF1 as a

candidate susceptibility factor for t-AML. We then went on to show the function of this protein in hematopoietic cells.

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Chapter 2

SWR/J Mice are Susceptible to Alkylator-Induced Myeloid Leukemia

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2.1. Results and Discussion

Therapy-related acute myeloid leukemia (t-AML) is a late complication following chemotherapy and/or radiation therapy. Approximately 10% of AML cases are therapy-related and the incidence is rising[1]. Alkylator-associated t-AML has a latency of 5-7 years, is often preceded by a myelodysplastic phase (t-MDS) and is frequently associated with loss of material from chromosomes 5 and/or 7 in humans[2, 3]. The survival of these patients is poor, motivating efforts to improve treatment and prevention strategies.

It is not yet clear whether t-AML risk is influenced by host factors, or is solely a stochastic process. Previous work has shown that inherited polymorphisms in drug detoxification (e.g., p450 enzymes, phase II conjugation enzymes, and NAD(P)H:quinoneoxidoreductase) and DNA repair (including homologous recombination and nucleotide excision) pathways may contribute to t-MDS/AML susceptibility, but confer only modestly increased risk in humans[4-7].

Alkylators (e.g., cyclophosphamide, ethyl- N-nitrosourea) are often used to generate cooperating mutations in genetically-engineered mouse models of leukemia[8, 9]. In standard laboratory strains (e.g., C57BL/6J, 129Sv/J), alkylators promote thymic lymphomas efficiently, reducing the number of mice evaluable for myeloid neoplasms. We previously demonstrated that susceptibility to alkylator-induced cancer has a genetic component in mice[10]. For myeloid leukemia, six strains demonstrated variable degrees of susceptibility, while fourteen were resistant (including C57 and 129 substrains). Of the 20 strains tested, SWR/J was the most susceptible. In the current study, we evaluated a large cohort of SWR/J mice and characterized the phenotype of tumors induced by the prototypical alkylator, ethyl-N-nitrosourea (ENU).

SWR/J mice received either no treatment ('N' cohort, n=22), hydrocortisone (HC) alone ('HC' cohort, n=11), ENU alone ('E' cohort, n=27), ENU followed by HC ('EH' cohort, n=111), or HC followed by ENU ('HE' cohort, n=107) (see **Supplementary Information**). A spectrum of diseases was observed, including hematopoietic tumors of both myeloid and lymphoid origins, and invasive lung carcinoma (**Supplementary Table 1**). Lymphomas were characterized by infiltrating CD4+CD8+ cells (>10%) in the bone marrow or spleen, large mediastinal masses, and disruption of splenic architecture. Myeloid leukemias were identified by excess myeloblasts (>20% of myeloid precursors) in the bone marrow or fixed tissues. Lung cancers were grossly visible and were phenotyped by histologic examination.

Of the 245 mice treated with ENU, 205 were evaluable for cancer susceptibility (40 died immediately after ENU injection, or were found post-mortem). ENU treatment (with or without HC) resulted in 65 evaluable mice (31.7%) with lymphoma, 23 mice (11.2%) with myeloid leukemia, and 192 mice (92.7%) with lung carcinoma (**Supplementary Table 1**). These diagnoses were not mutually exclusive; 5 mice had both lymphoma and myeloid leukemia, 20 had both myeloid leukemia and lung cancer, and 61 had both lymphoma and lung cancer. The lung cancer, lymphoma, and leukemia incidences in this strain differ from our previously published results (60%, 0%, and 80%, respectively), likely due to small cohort size in the previous study (n=12 ENU-treated mice)[10]. Control mice developed rare lung cancers (1/22 and 1/11 evaluable mice from the untreated and HC only groups, respectively), but no spontaneous hematologic cancers.

Previous studies in mice have shown that co-administration of steroids increased the frequency of radiation-induced leukemias[11]. To test whether steroids could increase the incidence of ENU-induced leukemias and reduce the competing incidence of thymic lymphoma,

we treated mice with HC either before or after ENU treatment. Neither regimen decreased the proportion of lymphomas, nor increased the proportion of leukemias, compared to ENU alone (**Supplementary Table 1**). It is not clear why steroids increased the incidence of radiation-induced leukemia and had no impact on ENU-induced leukemia, but this may be attributable to differences in the dose, schedule of administration, or type of steroid used.

The overall survival was significantly shorter in ENU-treated mice, compared to control mice (N or HC; P=0.002) (**Fig. 1A**). Two mice in the control groups died with lung cancer and the remainder were electively sacrificed, while most of the ENU-treated mice were sacrificed when moribund. For the mice treated with ENU, the median survival was longer in the EH cohort (331 days), compared to E or HE (290 and 291 days, respectively; P<0.0001). The cumulative probability of developing lymphoma was higher than leukemia (48.4% vs. 23.9%), but the latency for these diseases was identical (134 days) following ENU exposure (**Fig. 1B**).

Lymphomas were characterized by splenomegaly, lymphadenopathy, leukocytosis, anemia, and mediastinal enlargement (**Supplementary Table 2**, and data not shown). The bone marrow was infiltrated by CD4+CD8+ cells (53.4% of cases), or single positive CD8+ cells (1.7% of cases) or CD4+ cells (19% of cases). Myeloid and erythroid precursors were reduced in frequency (**Supplementary Table 3**). Histologically, the lymphoblasts were characterized by open chromatin, numerous nucleoli, and vacuolization of the cytoplasm (**Supplementary Fig.1**).

Myeloid leukemias were characterized by splenomegaly, leukocytosis, and accumulation of immature myeloid precursors in the bone marrow (**Supplementary Tables 2 and 3**). The leukemias were uniformly Gr1+CD11b+ (**Supplementary Table 2**). The myeloblasts were characterized by abundant cytoplasmic granulation and frequent mitotic figures (**Supplementary**

Fig. 1). The bone marrow of 6 ENU-treated mice had significant dysplasia in the myeloid and/or erythroid lineages, of which 2 had myeloid leukemia, and all had significant anemia (mean Hb=11.3) (**Supplementary Table 4**). 27 additional mice (all with concurrent lung cancer) did not meet criteria for myeloid leukemia, but had splenomegaly and excess myeloblasts (10-15%) in the bone marrow. While we favor that these bone marrow proliferations are clonal in origin, a reactive proliferation cannot be excluded due to the co-occurrence of a non-hematopoietic tumor.

Tumors from two donors with myeloid leukemia were transplanted into sublethally-irradiated congenic recipients. All of the recipients died rapidly (3-8 weeks) after adoptive transfer (**Fig. 2A**). Phenotypic features of the primary mice (i.e., blood counts, spleen weight, immunophenotype) were recapitulated in the recipients, suggesting that the leukemias were cell-intrinsic, transplantable tumors (**Fig. 2B**).

SWR/J mice developed rare spontaneous lung cancers, but hematopoietic malignancies were not observed in mice that did not receive ENU. A study from 1973 reported a 37% incidence of lung tumors (both adenomas and adenocarcinomas) in untreated SWR/J mice[12]. Rare spontaneous lymphomas (3.6%) and myeloid leukemias (0.3%) were also observed in SWR/J mice followed up to 30 months in that study [12], supporting the notion that this strain has intrinsic susceptibility to hematopoietic malignancies. The mechanistic basis of cancer susceptibility in SWR/J is not known, but it is noteworthy that a quantitative analysis of myeloid progenitor activity in 10 inbred strains demonstrated that SWR/J had the lowest frequency[13], suggesting that this cellular compartment may be under proliferative stress in this strain.

Genetically-engineered mouse models containing a single lesion detected in humans with myeloid leukemia may develop leukemia only after long latency or not at all, suggesting that

additional cooperating mutations are required. Alkylator exposure is one strategy frequently used to induce secondary mutations, but this is fraught by a high incidence of competing thymic lymphomas, particularly in the most commonly used inbred strains (C57 and 129 substrains). The incidence of ENU-induced myeloid leukemia in SWR/J mice, while still modest, is higher than what we and others have observed in C57 and 129 substrains, suggesting that polymorphisms in the SWR/J genetic background predispose mice to myeloid leukemia. Susceptibility loci for a variety of cancers have been mapped in mice, including a *Cdkn2a* allele in lymphoid malignancies and a *Ptch* allele in skin cancer[14, 15]. We have mapped loci associated with t-AML susceptibility in mice[10], but the specific genes/polymorphisms responsible for this phenotype are not yet known.

Therapy-related acute myeloid leukemia (t-AML) is a lethal complication arising in patients treated for antecedent cancers or autoimmune disorders. Preventative strategies are needed, but robust predictors of susceptibility are not yet available. Here, we show that SWR/J mice develop lethal, transplantable myeloid leukemias with dysplastic features after ENU exposure, recapitulating features of the human disease. The SWR/J strain provides a suitable model for investigation of germline and somatic events that cooperate with alkylator exposure to cause myeloid leukemia.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

Supplementary information is available online.

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2.3. FIGURE LEGENDS

Figure 1. Kaplan-Meier analysis of overall survival in ENU-treated mice. (a) Overall survival was decreased in ENU-treated mice, compared to controls (P=0.002). Administration of HC prior to ENU extended median and overall survival slightly, compared to ENU alone or HC after ENU (P<0.0001). (b) The cumulative probability of lymphoma was higher than myeloid leukemia (48.4%vs.23.9%), but occurred with identical latency (134 days after ENU exposure).

Figure 2. ENU-induced myeloid leukemias are transplantable. (a) Adoptive transfer of splenocytes from two donors with ENU-induced myeloid leukemia causes lethality with short latency. (b) The spleen weight, (c) white blood cell count, and (d) hemoglobin were similar in donor and recipient mice.

2.4. FIGURES

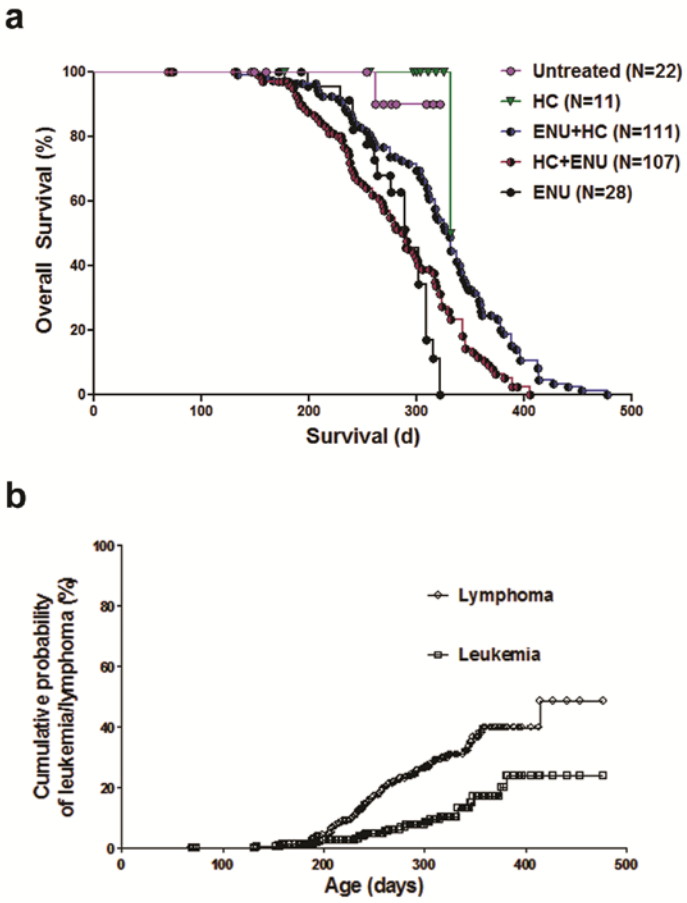

Figure 1

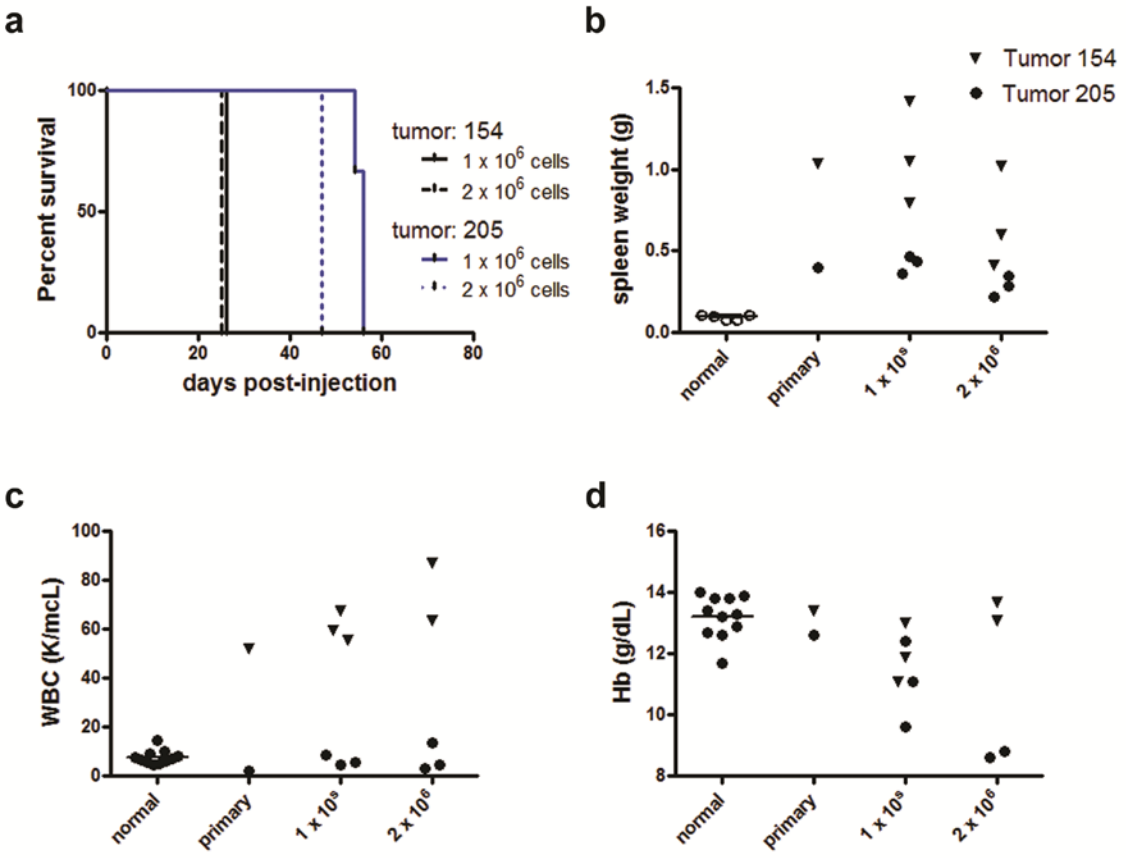

Figure 2

2.5. SUPPLEMENTARY INFORMATION

SWR/J Mice are Susceptible to Alkylator-Induced Myeloid Leukemia

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SUPPLEMENTARY METHODS

Mutagenesis protocol. SWR/J mice were obtained from Jackson Laboratory (Bar Harbor, ME) and housed in a pathogen-free facility. 100 mg/kg of ethyl- N-nitrosourea (ENU; Sigma-Aldrich, St. Louis, MO) was administered at 9 and 10 weeks of age intraperitoneally, as previously described[1]. Some mice were given 2.5 mg hydrocortisone (HC) intraperitoneally every other day for 3 days one week before ENU or beginning 24 hours after the second ENU treatment. All procedures were approved by the Washington University Animal Studies Committee.

Characterization of tumors. The mice were sacrificed and analyzed when moribund or at 15 months of age. The lungs, mediastinal lymph nodes, thymus, and spleen were removed, weighed, and preserved in10% formalin. Splenocytes were viably cryopreserved in 10% DMSO. Complete blood counts were obtained using a Forcyte veterinary cell counter (Oxford Sciences, Inc., Oxford, CT). Bone marrow and spleen cells were stained with conjugated antibodies to B220, CD3, CD45, c-kit, Gr-1, CD11b, CD34, ter119, CD4, or CD8 (BD

Biosciences, San Jose, CA) and analyzed by flow cytometry. Bone marrow smears were stained with modified Wright-Giemsa and 200 cell differentials were performed and independently reviewed by a board-certified anatomic pathologist with subspecialty training in hematopathology. Fixed tissues were imbedded in paraffin, sectioned, stained with H&E, then evaluated by a veterinary pathologist.

Adoptive transfer. Viably cryopreserved spleen cells from mice with myeloid leukemia were thawed on ice, washed in PBS, and counted. 1-2x10^6cells were injected via tail vein injection into sub-lethally irradiated (500cGy) sex-matched wildtype SWR/J recipients. Mice were sacrificed when moribund. Bone marrow, spleen, and peripheral blood cells were harvested for cell counts and flow cytometry.

Statistical analysis. Hematologic parameters were compared by one-way analyses of variance followed by Tukey-adjusted pairwise comparisons. Bone-marrow morphology variables were first rank-transformed and then analyzed by one-way analysis of variance and Tukey-adjusted pairwise comparisons. All analyses were done with SAS/STAT (ver9.3; SAS Institute, Cary, NC).

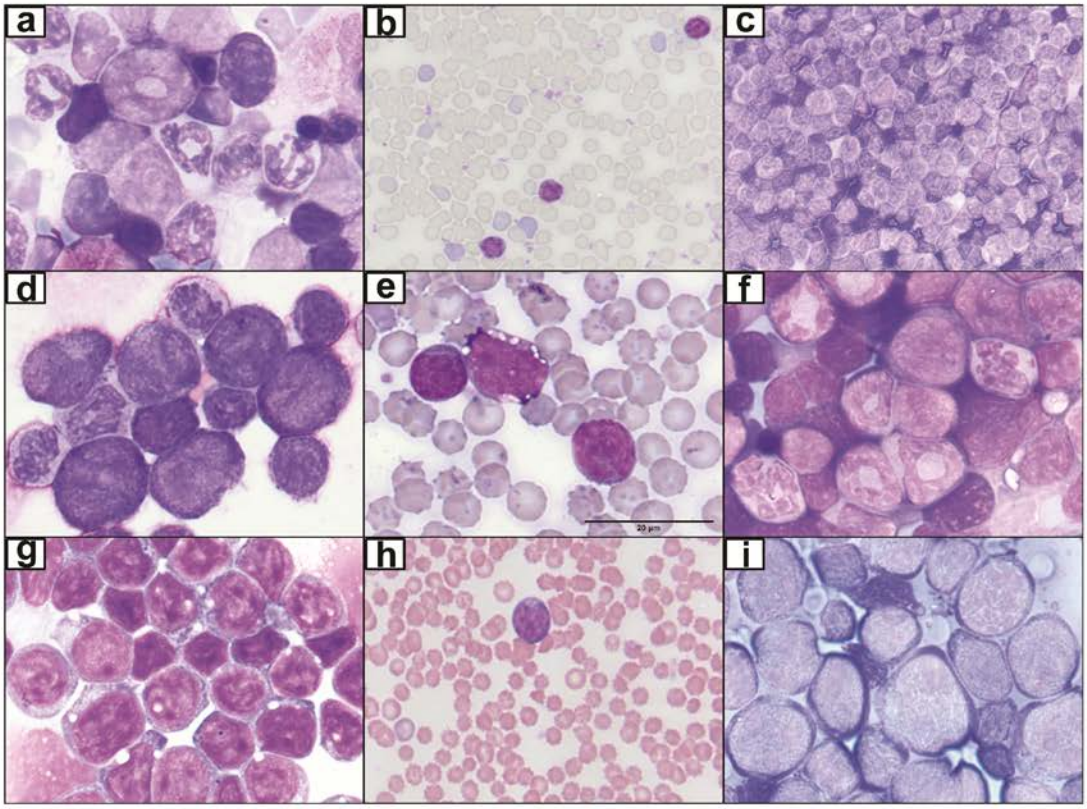
SUPPLEMENTARY REFERENCES

1. Fenske TS, McMahon C, Edwin D, Jarvis JC, Cheverud JM, Minn M*, et al.* Identification of candidate alkylator-induced cancer susceptibility genes by whole genome scanning in mice. *Cancer Research* 2006 May 15; **66**(10)**:** 5029-5038.

SUPPLEMENTARY FIGURE LEGEND

Supplementary Figure 1. Morphology of hematopoietic tumors in ENU-treated mice. (**a**,**d**,**g**) Bone marrow, (**b**,**e**,**h**) peripheral blood, and (**c**,**f**,**i**) spleen cells from (**a**-**c**) ENU-treated SWR/J mice with no pathology, (**d**-**f**) myeloid leukemia, and (**g**-**i**) lymphoma.

2.8. FIGURE



Supplementary Figure 1

Supplementary Table 1. Incidence of ENU-induced tumors in SWR/J mice

Treatment	n	No Disease	Lymphoma	Leukemia	Lung Cancer	Not Evaluable
Untreated	22	21 (95.5%)	0	0	1 (4.5%)	0
HC	11	9 (90.0%)	0	0	1 (10%)	1
ENU	27	1 (5.00%)	4 (20.0%)	3 (15.0%)	17(85.0%)	7
HC+ENU	107	3 (3.5%)	36(41.9%)	9 (10.5%)	81(94.2%)	21
ENU+HC	111	6 (6.1%)	25 (25.3%)	11 (11.1%)	92(92.9%)	12
Total	278					

HC, hydrocortisone; ENU, ethyl-N-nitrosourea;

Supplementary Table 2. Hematologic parameters in ENU-treated SWR/J mice

Disease	n[#]	spleen wt (g)	WBC (K/uL)	NE (%)	LY(%)	Hb (g/dL)	HCT (%)	MCV (fL)	PLT (K/uL)	Bone Marrow FACS^ CD11b+Gr1+ (%)	Bone Marrow FACS^ CD4+CD8+ (%)
No Disease	10	0.19	5.88	29.7	60.5	15.2	55.2	55.0	1068.9	100	0
Lymphoma	60	**0.62**	**63.22**	33.9	33.6	**10.7**	35.2	52.2	**498.0**	61.4	53.4
Leukemia	18	**0.86**	34.42	34.5	36.7	**10.2**	34.9	54.9	**549.2**	100	8.3
Lung Cancer	112	0.25	16.57	18.7	43.3	13.1	45.3	53.7	**670.7**	98.9	2.2

WBC, white blood cells; NE, neutrophils; LY, lymphocytes; Hb, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; PLT, platelets

[#]excluding cases of concurrent lymphoma or leukemia

^percent of cases positive (>10% of cells) for the marker shown.

Values in **bold** are significantly different, compared to mice with 'no disease' ($P<0.0001$ by Tukey-adjusted pairwise comparison).

Supplementary Table 3. Bone marrow morphology in ENU-treated SWR/J mice

Disease	n[#]	Myeloid			Erythroid (%)	Lymphoid (%)
		Early (%)	Mid (%)	Late (%)		
No Disease	10	16.9	26.8	27.6	25.1	3.7
Lymphoma	60	5.7	7.6	14.7	6.9	65.2
Leukemia	18	**51.0**	17.9	14.7	10.7	5.6
Lung Cancer	112	17.9	25.6	31.9	19.4	5.2

Data are the mean percentages per 200 cells
Early,myeloblasts + promyelocytes; mid, myelocytes; late, bands + neutrophils
[#]excluding cases of concurrent lymphoma and leukemia
Value in **bold** is significantly different, compared to mice with lymphoma or lung cancer (P<0.0001 by Tukey-adjusted pairwise comparison).

CHAPTER 3

MLF1 IS A CANDIDATE SUSCEPTIBILITY FACTOR FOR T-AML

3.1. INTRODUCTION

Several years ago, the Graubert laboratory used an inbred mouse screen to identify novel risk factors for t-AML[1]. This allowed us to control for environmental factors and test the effect of different genetic backgrounds. Cohorts of 20 inbred mouse strains were treated with ENU, a potent mutagen in mice, and observed for incidence of myeloid leukemia. Strains were classified as being susceptible or resistant to acquiring alkylator-induced t-AML. Quantitative trait locus mapping was used to identify candidate susceptibility factors.

Treating the mice with ENU resulted in 6 susceptible stains that had a myeloid cancer incidence which ranged between 18 and 80%. The other 14 strains were resistant. The difference in cancer incidence between the strains suggests that there is genetic component contributing to susceptibility.

A genome wide association study was used to identify any regions that contributed to myeloid leukemia. One peak on chromosome 3 exceeded the significant threshold at a genome wide level. The 1.07 Mb region associated with that peak contained six genes (Shox2, Rsrc1, Mlf1, Gfm1, Lxn, Rarres 1) (Figure 3.1). Of these genes, myeloid leukemia factor 1 (Mlf1) was chosen as the strongest susceptibility factor based on the results of the QTL mapping and previous findings of its involvement in a chromosomal translocation found in AML patients.

3.1.1. GENES IN PEAK ON CHROMOSOME 3

Shox2

Short stature homeobox 2 (Shox2) is a member of the homeobox family of genes. Shox2 has been shown to be hypermethylated in lung cancer patients. This correlated with gene amplification in lung cancer tissue [2].

Rsrc1

Arginine/serine-rich coiled-coil 1 (Rsrc1) is a member of the arginine and serine rich-related protein family. Rsrc1 affects m-RNA splicing [3]. The protein typically functions early in sliceosome formation [4].

Gfm1

G elongation factor, mitochondrial 1 (Gfm1) encodes a mitochondrial elongation factor important for mitochondrial translation. Without normal translation of the respiratory system, there is a reduction of oxidative reduction that can underlie disease [5].

Lxn

Latexin (Lxn) influences size of hematopoietic stem cell populations [6]. Highest expression was found in lin- cells and overexpression assays showed a decrease in stem cell population size. Lxn is also the only known protein inhibitor of zinc-dependent metallocarboxypeptidases [7].

Rarres 1

Retinoic acid receptor responder (tazarotene induced) 1 is a retinoid acid (RA) receptor-responsive gene that encodes a type 1 membrane protein. Hypermethylation of the promoter downregulates RARRES1 expression in several cancers, including prostate cancer [8].

Mlf1

The nucleophosmin (NPM1)-MLF1 fusion protein is how MLF1 was first identified. The fusion protein is associated with MDS and AML patients, which in combination with the QTL results made *MLF1* the strongest risk factor to explore.

3.1.2. MLF1

MLF1 encodes 267 amino acids, corresponding with a 31 kDa protein [9]. Evidence suggests it is an oncoprotein [9]. There is no known homology with other characterized proteins to hint at the normal function of wildtype MLF1. Several groups, including ourselves, have sought to understand the normal biology of the protein.

3.1.3. FUNCTION

Williams *et al*. show that MLF1 is activated during during erythroid to myeloid switching [10]. They used J2E erythroleukemic cells to show that MLF1 expression could switch it to a monocytoid phenotype. Overall they show that MLF1 expression favors myeloid differentiation and impedes the erythropoietic pathway. Dysregulation could account for increased erythroleukemias. This is also true of MLF1 interacting protein (MLF1IP). MLF1IP may have its expression confined to the erythroid lineage.

One model for how Mlf1 might function involves Madm and Manp regulating the shuttling of Mlf1 between the nucleus and the cytoplasm [11]. Madm recruits a serine to Mlf1 so 14-3-3ζ will bind and sequester Mlf1 in the cytoplasm. When Mlf1 is not bound, it can shuttle into the nucleus where it binds Manp and DNA. Mlf1 acts as a gene expression regulator.

One gene that MLF1 regulates is p53 by suppressing COP1 via COP9 subunit 3 (CSN3). Both stress and overexpression of MLF1 lead to binding of CSN3 and a decreased level of COP1 [12]. COP1 is an E3 ubiquitin ligase for p53. Those signals lead to increased levels of p53. The final step leads to cell cycle arrest.

3.1.4. EXPRESSION

MLF1 is expressed in a variety of tissues. Two transcripts get expressed in tissues, the most commonly expressed is 1.7 kb and the other is 2.5 kb. Highest *MLF1* mRNA was found in testis and lowest in spleen, thymus and peripheral blood [9]. Normal human *MLF1* is expressed in CD34+ cells. In the Washington University *de novo* AML patient samples, *MLF1* expression was low in all patient samples, as well as in the CD34+ cells (Figure 3.2).

Matsumoto *et al.* used RNA from AML and MDS patient samples to show that *MLF1* is not expressed in peripheral blood, normal bone marrow, or in ALL patients, but that it is expressed in varying degrees across the subtypes of AML [13]. In addition, they show that patients with a prior MDS phase have increased MLF1 expression.

3.1.5. BINDING PARTNERS

Lim *et al.* identified the novel MLF1-adaptor molecule (Madm) and 14-3-3ζ as binding partners of wildtype MLF1 by performing yeast two-hybrids [14]. 14-3-3 (RSXSXP) motifs are important binding sites for many binding partners involved with important processes such as cell death, cell differentiation, and cell division [15-17]. The binding of 14-3-3ζ is thought to sequester Mlf1 in the cytoplasm [14]. Madm co-localizes with Mlf1. Madm recruits a serine kinase that activates the 14-3-3 binding motif of Mlf1. This is thought to keep Mlf1 in the cytoplasm and affects the ability of cells to differentiate.

MLF1-interacting protein (MLF1IP) is a novel protein identified by a yeast two-hybrid screen and confirmed by pulldown assays [18]. MLF1IP and MLF1 tend to colocalize and associate with one another. They have similar tissue distributions, MLF1IP having especially high expression in the hematopoietic organs. It is expressed in erythroblasts but not

differentiated erythrocytes. This suggests a potential role for deregulation of MLF1IP or MLF1 in erythroleukemias.

Yonedo-Kato *et al*. identified COP9 subunit 3 (CSN3) as a binding partner of MLF1 [12]. The binding of MLF1 and CSN3 leads to downregulation of COP1 which regulates p53. This is another important binding partner that gives a novel function of MLF1.

Mlf1-associated nuclear protein (Manp) is homologous to a heterogeneous nuclear ribonucleoprotein U-like molecule (hnRNP-U) and contains a DNA-binding domain [11]. Mlf1 and Manp colocalize in the nucleus, and Mlf1 is capable of binding DNA. Winteringham *et al*. also suggest that Mlf1 has a nuclear export signal.

3.1.6. FUSION PROTEINS

Nucleophosmin is a nucleolar phosphoprotein that is involved in ribosomal biogenesis, modulating tumor suppressors p53 and Arf, and is upregulted in response to DNA damage [19-21]. It shuttles between the nucleus and the cytoplasm [22]. Npm1 acts as a haploinsufficient tumor suppressor gene [23]. In addition, *NPMI* is the most frequently mutated gene in AML, with 35% being mutated in AML patients [24]. It is involved in translocations in which varying lengths of NPM1 fuse with MLF1, RARα, or ALK [25]. The fusion partner determines the disease and thus can be used as a diagnostic tool.

NPM-MLF1 is a rare chromosomal translocation t(3;5)(q25;q35) that occurs in less than 1% of AML cases and is associated with MDS [9]. 175 of 294 amino acids of NPM1 fuse with almost all of MLF1 (252 of 268 amino acids)(Figure 3.3). Importantly, NPM1 conserves more of its functional domains in the NPM1-MLF1 fusion than any of its other fusion partners [9]. One characteristic of this fusion is aberrant expression of MLF1 in the nucleus and NPM in the

cytoplasm. It also causes overexpression of MLF1 in hematopoietic cells where expression is generally low [25]. Ectopic expression of the fusion in cell lines induces apoptosis [26].

NPM-ALK forms the t(2;5)(p23;q35) translocation found in 85% of ALK^+ anaplastic large cell lymphoma (ALCL) [27]. Oligomerization of the oncogenic fusion protein leads to constitutive activation of the kinase ALK [28]. NPM-ALK, like NPM-MLF1, gets mislocalized to the nucleus, although this is not required for lymphomagenesis.

NPM- RARα is a rare t(5;17) chromosomal translocation involved with acute promyelocytic leukemia. The fusion protein interacts with wildtype NPM1 by mislocalizing it or altering its function in the nucleolus [29]. NPM- RARα disrupts the retinoid-responsive gene expression and arrests myeloid differentiation at the promyelocyte stage by regulating the retinoid-responsive gene expression [30].

3.2. RESULTS

MLF1 was first identified as a partner in a fusion protein (NPM-MLF1) associated with AML/MDS. We found *MLF1* using QTL mapping that identified regions in the mouse genome that associated with susceptibility to t-AML. The function of MLF1 on hematopoietic cells had never been explored. Expression of *MLF1* is low in hematopoietic cells, but is higher in AML patients and highest in patients with a prior MDS phase. In addition, *MLF1* is expressed higher in the more primitive CD34+ bone marrow cells. Together, this suggests that *MLF1* could affect leukemogenesis. That led us to hypothesize that the dysregulation of *MLF1* expression is relevant for the initiation of alkylator induced t-AML. The aims of this project were to: 1) determine the *in vitro* molecular consequences of *MLF1* overexpression on hematopoietic cells, 2) determine the functional consequences of MLF1 overexpression *in vivo*, and 3) determine the relevance of *Mlf1* on the initiation of alkylator induced leukemia.

3.2.1. OVEREXPRESSION *IN VITRO*

3.2.2. OVEREXPRESSION OF MLF1-*IRES*-YFP-MSCV IN Ba/F3 CELLS

To address how different levels of *Mlf1* expression might affect t-AML susceptibility, we used an *in vitro* overexpression assay in the pro-B cell Ba/F3 cell line. Ba/F3 cells are IL-3 dependent and thus can be sensitized to apoptosis by the removal of IL-3 from the medium. Approximately 13% of cells were transduced with the MLF1-*ires*-YFP-MSCV retrovirus, while 28% of the cells were transduced with the control vector (Figure 3.4). Cells overexpressing the YFP-MSCV control retrovirus expanded nearly 3-fold times more than those overexpressing the MLF1-*ires*-YFP-MSCV retrovirus at 48 and 72 hours following infection (Figure 3.5 b,c,d). To investigate whether this was a result of increased apoptosis, we stained cells with the apoptotic

markers Annexin V and 7-Amino-actinomycin D (7-AAD) and analyzed by flow cytometry. We found that there was 6-fold more death in cells overexpressing *MLF1* in comparison to the control empty vector (33% in MLF-*IRES*- YFP vs. 5% in control; $p<0.0001$) (Figure 3.6).

3.2.3. METHODS

Full length human *MLF1* cDNA was cloned into the multiple cloning site of the murine stem cell virus (MSCV) vector that contains an *ires*-YFP. 293T packaging cells were transfected with either MLF1- *ires*- YFP-MSCV or control YFP-MSCV vector to yield replication-incompetent retrovirus. Ba/F3 cells were transduced with either an MLF1-*ires*-YFP-MSCV retrovirus or control YFP-MSCV retrovirus with similar multiplicities of infection. The virus was removed after 3, 6, 12, or 24 hours of exposure and cells were replated with identical cell densities in RPMI medium containing different concentrations of IL-3 ranging from insufficient for cell growth (0ng/mL) to sufficient (5ng/mL). At 24, 48, and 72 hours post viral exposure, we analyzed cell growth and viability. Trypan blue staining was used to determine cell viability, while flow cytometry was used to determine the percentage of cells overexpressing each virus by gating on YFP+ cells.

3.2.4. OVEREXPRESSION OF MLF1-*IRES*-YFP-MSCV IN PRIMARY BONE MARROW CELLS

Initial studies were performed in Ba/F3 cells because they are hematopoietic, easily transduced, and provide an easily manipulated system in which to study the effects of *MLF1* overexpression. The next step was to analyze the effects of *MLF1* overexpression in primary bone marrow cells. This was more challenging than studies in cell lines because heterogeneity of the population and lower transduction efficiencies.

Results show that cells overexpressing *MLF1-ires*-YFP-MSCV have impaired growth in comparison to cells infected with the control virus (YFP-MSCV)(Figure 3.7). We show that this impaired growth is due to, at least in part, increased apoptosis. At 24 hours, cells overexpressing MLF1 had a significantly higher percentage of apoptosis than those overexpressing the control retrovirus ($p<0.001$) (Figure 3.7). At 48 hours there was no significant difference in apoptosis between the two groups. These results suggest that MLF1 is playing a role in inducing apoptosis in primary hematopoietic cells.

3.2.5. METHODS

We harvested bone marrow from 2 femurs and 2 tibias from a C57BL/6J mouse age 6-8 weeks and transduced bulk bone marrow cells with either MLF1-*IRES*-YFP-MSCV retrovirus or control YFP-MSCV with similar multiplicities of infection. Cell viability and apoptosis status was determined as described above.

3.2.6 TRANSGENIC

Vav was used as the promoter for making a HA-*hMLF1* transgenic mouse because of its exclusive pan-hematopoietic expression and modest expression in cells (Ogilvy 1999 Blood). Vav acts by transducing signals from surface receptors to Rho-like G proteins (Crespo, Nature 1997). The HA tag was to allow us to easily analyze a variety of tissues for the presence of the tagged protein. We cloned HA-tagged human *MLF1* into the HS321/45 *vav* vector (Figure 3.8). HA, MLF1, and GFP protein were all detected by Western blots when transduced into NIH3T3s (Figures 3.9). In addition to sequencing, this confirmed that the correct constructs were made. 3 founder lines were identified out of the 43 possible clones that expressed the transgene at high,

medium, and relatively low levels. The founder mice were all males and were bred extensively, but no transgene positive progeny were identified.

3.2.7 METHODS

Human *MLF1* was shuttled into vector topo2.1 (Invitrogen). The HA tag was added in frame to the 5' end of *hMLF1*. This was confirmed via sequencing. Next, HA-tagged human *MLF1* was cloned into the HS321/45 *vav* vector. This vector was confirmed by sequencing and by the expression of the correct proteins (HA, MLF1, GFP) when transduced into the NIH3T3 cell line.

To prepare the DNA for pronuclear microinjection, we first purified the plasmid (Qiagen Endo-Free Plasmid Maxi Kit). Then the vector backbone was removed in a restriction digest. The insert was separated from the vector on a 0.8% agarose gel and using a QIAquick PCR purification kit (Qiagen). Recovered DNA was at a concentration of 30ng/uL in microinjection buffer. The *vav-HA-hMLF1* construct was injected into FVB/N eggs to generate founder lines.

43 clones were tested by Southern blots to determine if they carried the transgene. Briefly, the clones were digested with an enzyme restriction and then run out on a gel. From there, the gel contents were transferred to a membrane where it was then probed with a radio-labelled single-stranded piece of DNA, hybridized, and analyzed by autoradiography. Then the probe was detected by putting the membrane through the X-Ray machine.

3.2.8 DISCUSSION

Overexpression of MLF1-*ires*-YFP in Ba/F3 cells and primary bone marrow cells led to decreased viability due to, at least in part, increased apoptosis. This is consistent with the

literature which suggested a pro-apoptotic role for MLF1 in NIH3T3s. We wanted to know the function of MLF1 in hematopoietic cells. We also wanted to know what overexpression of MLF1 would look like in a transgenic mouse. We chose to use the *vav* promoter which drives expression in hematopoietic cells. Clones were verified by southerns, but no pups were born from our founders. This is likely due to male infertility caused by the overexpression of a pro-apoptotic factor. Alternative methods such as a conditional mouse must be considered to make a viable mouse.

3.2.9 RELEVANCE OF MLF1 ON ENU-INDUCED LEUKEMIA AND STRESS

We wanted to determine if Mlf1 expression levels affected the response of bone marrow cells to treatment with the alkylating agent ENU. These sets of experiments were to understand whether Mlf1 was important following a potent mutagen treatment or stress condition. We hypothesized the dysregulation of Mlf1 would affect the cellular response to these stressors and contribute to disease.

3.2.10 RELEVANCE OF MLF1 FOLLOWING *IN VITRO* ENU TREATMENT

Bone marrow was harvested from wildtype C57BL/6J mice and from fully backcrossed Mlf1 knockout mice. 24 hours later the bone marrow cells were treated with ENU for 1 hour. After 16 hours the cells were analyzed by FACS using the myeloid lineage marker Gr-1 and Annexin V. There was a trend toward increase in the percentage of apoptotic cells in the wildtype mice between the untreated and treated cells ($p=0.101$), whereas there was no significant difference between untreated and treated Mlf1 -/- bone marrow cells (Figure 3.10a). In another experiment, the same phenotype was seen using bulk bone marrow (Figure 3.10b). This suggests that Mlf1 is required for an apoptotic response to ENU.

Bone marrow was harvested from fully backcrossed Mlf1 wildtype, heterozygous, and knockout SWR/J mice and flow sorted for kit+lin- cells. After 24 hours the bone marrow was treated for one hour with ENU and then analyzed at 24 and 48 hours later. At 48 hours, there was a significant growth impairment between the wildtype and knockout cells ($p<0.0001$) (Figure 3.11a). There was increased cell death seen in all genotypes, especially in the wildtype cells, although this was not significantly more than the others (Figure 3.11b).

3.2.11 METHODS

Mlf1 null mice were generated and provided to us by the Steve Morris lab (unpublished). Briefly, a targeted strategy was utilized to disrupt *Mlf1* expression by replacing a portion of exon 2 with a *Neo* cassette which results in translational stops. We fully backcrossed *Mlf1* null mice to both C57BL/6J and SWR/J backgrounds which represent a strain that is resistant to myeloid leukemias (C57BL/6J) and susceptible (SWR/J). Speed congenics confirmed greater than 98% purity of the strains. Genotypes were identified by PCR. Bone marrow was flushed from 2 tibias and 2 femurs from mice wildtype, heterozygous, or null for the *Mlf1* allele (N=3/genotype). Unsorted bone marrow was cultured and treated with a range of doses of ENU or DMSO vehicle control. We analyzed the cells for apoptosis by flow cytometry at different time points up to 72 hours following treatment. Gr-1+ cells were marked to determine if there is a compartment specific response to ENU-induced apoptosis due to loss of *Mlf1*.

3.2.12 RESPONSE OF MLF1 GENOTYPES FOLLOWING STRESS

$Mlf1^{-/-,+/-,+/+}$ C57BL/6J mice were sub-lethally irradiated to determine if lacking Mlf1 protects those cells from undergoing cell death and thus recover quicker in response to stress. Complete blood counts were obtained through eye bleeds six times from day 0 to day 28. Each

genotype hit nadir around 6 days as expected. There was no significant change in genotype recovery in white blood cells, neutrophils, hemoglobin, lymphocytes, platelets, or monocytes (Figure 3.12).

3.2.13 METHODS

Mlf1$^{-/-,+/-,+/+}$ C57BL/6J (3 mice/genotype) ages 6-8 weeks first received eye bleeds and complete blood cell counts were conducted. The same day they were treated with a sub-lethal dose (500 RADs) of radiation using a gamma irradiator. Mice were kept in a pathogen-free facility for 28 days. They received an additional 4 more eye bleeds throughout the 28 days. This allowed for complete blood cell counts to monitor recovery following the stress. On the 28th day following irradiation, the mice were sacrificed and a heart stick was performed for the final time point.

3.2.14 DISCUSSION

MLF1 does influence how many cells undergo ENU-induced apoptosis. There was a significant difference between *MLF-/-* vs *MLF1+/+* mice when comparing ENU-induced cell death. There was no significant difference between knockout cells. This is the expected result. We have now shown that MLF1 is pro-apoptotic *in vitro* and *in vivo* under overexpression and stress conditions. There is still cell death occurring in the knockout cells. This tells us that MLF1 contributes to the apoptotic response, but is not essential.

When we observed recovery following sub-lethal irradiation, we did not see a difference between the *MLF1* genotypes in recovery time. We thought that the knockout might have an advantage gained from the protection it gets from surviving the initial stress. It could be that at the dose we gave the mice, it is too high and acts as an equalizer. You could play with doses

until you found a dose that shows a difference, but 500 RADS of irradiation. A more detailed analysis across a range of radiation doses may reveal a role for MLF1 in hematopoietic recovery following sub-lethal irradiation.

3.2.15 RELEVANCE OF MLF1 ON ENU-INDUCED LEUKEMIA

Loss or gain of pro-apoptotic *Mlf1* within the bone marrow may impact whether a cell undergoes apoptosis in response to ENU treatment, or if cells persist and acquire mutations that contribute to disease (Figure 3.13). We hypothesized that the dysregulation of *MLF1* expression contribute to disease. To test this, 10 wildtype, 18 heterozygous, and 11 knockout C57BL/6J mice were treated *in vivo* with ENU. They were sacrificed when moribund and analyzed for myeloid malignancies, lymphomas, and lung cancers. Each disease was identified as described in chapter 2.

Overall survival was not significantly different between the genotypes (Figure 3.14). The median survival for knockout mice was 307 days. Wildtype and heterozygous mice had nearly identical median survivals with 258 and 256 days, respectively.

Briefly, lymphomas were characterized by splenomegaly, enlarged mediastinal mass, leukocytosis, and anemia (Tables 2 and 3a,b,c). Knockout mice had 0% cases of greater than 10% CD4+CD8+ infiltration in the bone marrow, heterozygous mice had 11.8% cases double positive, and wildtype and 14.3% of cases. Wildtype mice had the greatest percentage of lymphomas with 57.1% being positive (Table 1). With that said, three of those cases involve concurrent lymphomas which were counted as both diseases.

Myeloid leukemias were characterized by splenomegaly, leukocytosis, and accumulation of immature myeloid precursors as called by a veterinary pathologist (Tables 2 and 3a,b,c). Only

3 wildtype mice developed leukemia, which were confirmed by a pathologist. This was in addition to the lymphoma and the lung cancer that they also developed. They were all Gr1+CD11b+.

3.2.16 METHODS

We used genetically defined mice for *Mlf1* loss on the C57BL/6J backgrounds described above. A cohort of at least 10 mice per genotype were intraperitoneally injected with 2 doses (100mg/kg) of ENU at 9 and 10 weeks of age. The mice were observed for up to 16 months and sacrificed when moribund. Mice were analyzed for the presence of myeloid malignancy, lymphomas, and lung cancers. Complete blood cell counts were determined by an automated cell counter. Bone marrow and peripheral blood were stained for flow cytometric analysis with antibodies conjugated to B220, CD3, CD45, c-Kit, CD11b, Gr-1, ter119, and Dx5. Tissues were fixed in 10% formalin for analysis by a veterinary pathologist. Survival curves were determined using the Kaplan-Meier method.

3.2.17 DISCUSSION

The only mice to get leukemia were the Mlf1 wildtype mice and these were concurrent with lymphomas. We had predicted that the knockout mice would be the ones to persist and acquire mutations that would lead to leukemia. Instead the knockout mice developed lymphomas and lung cancers at a high rate and died of that instead. Lymphomas do have a shortened latency compared to the other diseases. These experiments were performed in C57BL/6J background, which is predisposed to develop lymphomas because of retroviruses. You might see a different result if you tested a genetic background resistant to lymphomas, or if you bred in a transgene that sensitizes to development of leukemia (e.g. PML/RARA).

3.3. SUMMARY

Results from Ba/F3 cells indicate that YFP+ cells infected with the control virus expanded to nearly 3-fold greater levels than cells overexpressing *MLF1* when analyzed at 48 and 72 hours under sufficient IL-3 conditions (Figure 3.5). We show that the decreased accumulation of MLF1-expressing cells is due to, at least in part, a 6-fold increase in apoptosis (33% in MLF-*IRES*- YFP vs. 5% in control)(Figure 3.6).Overexpression of MLF1 in primary hematopoietic cells also led to decreased accumulation of those cells. It was partly due to the increase in apoptosis, especially at 24 hours. The MLF1 transgenic was not viable. Constitutive activation of MLF1 may cause male infertility issues, or cause developmental problems that cannot be overcome.

ENU induced a significant amount of apoptosis *in vitro* between C57BL/6J *MLF1* wildtype and knockout bone marrow cells. There was no significant response between knockout cells, suggesting that MLF1 is playing a role in apoptosis. SWR/J mice sorted for kit+/lin- cells before they were treated, and at 48 hours there was a significant difference between the wildtype and knockout cell growth and viability. When Mlf1 C57BL/6J mice of varying genotypes were sub-lethally irradiated to determine the effect on recovery post stress, we saw no significant difference between the genotypes. This was a little surprising given that Mlf1 had been shown to be significant when presented with the genotoxic stressor ENU.

Unexpectedly, Mlf1 wildtype mice developed the leukemias instead of the knockouts. This suggests that perhaps in this small study, the knockouts did persist when treated with ENU, but developed the fast growing lymphomas instead. Although, it is highly unlikely that 30% of mice get leukemia and we have shown that they do not with larger cohort sizes. Every cell in the

body is being affected by this treatment. Transplants might be the only way to get a true answer to what effect Mlf1 has on the mice *in vivo*.

3.4. FIGURE LEGENDS

3.1 Genomic locus associated with susceptibility to ENU-induced myeloid leukemia. A genome-wide association study was performed across the entire mouse genome to identify peaks that associated with the phenotype of susceptibility to ENU-induced myeloid leukemia. A peak on Chromosome 3 did exceed the threshold and within the 1 Mb region lies 6 genes. Horizontal line is a false discovery threshold of 10%.

3.2 MLF1 expression in Washington University *de novo* AML patient samples. 3 *MLF1* probes were used on *de novo* AML patients to determine expression in hematopoietic cells. 7 patients had very low expression of MLF1. Expression was also low in CD34+, Pros, and PMNs.

3.3 Structural and functional domains of the MLF1-NPM1 fusion protein and wildtype NPM1. NPM1-MLF1 fusion constitutes the N-terminus of NPM1 (amino acids 1-175) and almost the entire MLF1 protein, excluding the first 16 N-terminus amino acids. The fusion retains the NPM1 metal binding domain (MB), one complete acidic amino acid cluster (AC), and one of the nuclear localization signals (N). MLF1 retains a 14-3-3 binding site, two nuclear localization signals, and nuclear export signal.

3.4 IL-3 dependent Ba/F3 cells were transduced with either an MLF1 *ires* YFP-MSCV retrovirus or control YFP-MSCV retrovirus with similar multiplicities of infection. Cells were kept in RPMI and 5ng/mL IL-3. After 24 hours, cells were analyzed by flow cytometry to determine percent transduced using YFP as a marker. Cells transfected with the control MSCV-YFP retrovirus had 2-fold more YFP+ cells than those transduced with the MLF1-MSCV retrovirus.

3.5 MLF1-MSCV infected Ba/F3 cells have growth impairment. The MLF1-MSCV and empty retrovirus were removed after 12 hours and cells were replated in RPMI containing different concentrations of IL-3 ranging from insufficient for cell growth (0ng/mL) to sufficient (5ng/mL). **a)** Cells in IL-3 concentration insufficient for cell growth did not expand. **b,c,d)** YFP+ cells infected with the control virus expanded to nearly 3-fold greater levels than cells overexpressing *MLF1* when analyzed at 48 and 72 hours under sufficient conditions.

3.6 There is a decreased accumulation of *MLF1*-expressing cells is due to, at least in part, a 6-fold increase in apoptosis (33% in MLF *ires* YFP vs. 5% in control). Ba/F3 cells transduced by either MLF1-MSCV or YFP-MSCV control in different concentrations of IL-3 at **a)** 24 and **b)** 48 hours showed increased apoptosis. Cells overexpressing MLF1 at 24 hours in 0ng/mL IL-3 had a 6-fold increase in death. N=>5 times

3.7 Bone marrow cells transfected with MLF1-MSCV retrovirus have cell growth impairment. Wildtype C57BL/6J bone marrow was harvested and transfected with MLF1-MSCV retrovirus or the empty vector. **a)** At 24, 48, and 72 hours post-transfection, live and dead cells were counted using a hemocytometer and trypan blue staining to show decreased cell growth with cells transfected with MLF1-MSCV. **b)** Flow cytometry was used to determine percentage of transfected cells with MLF1-*ires*-YFP using YFP as the marker. Annexin V was used as the marker for cell death and showed that the growth impairment was due to a significant increase in apoptosis at 24 hours.

3.8 *vav*-HA-*hMLF1* Transgene. a) Schematic of the transgene showing the position of the promoter, HA, hMLF1, and the probe location used for southerns. **b)** Schematic of the HA-MLF1-*ires*-YFP retrovirus.

3.9 Plasmids *vav*-HA-*MLF1* and HA-*MLFI-IRES*-YFP-MSCV express both HA and overexpress MLF1. *Vav* as a promoter has the ability to drive a moderate level of overexpression. **a)** Untransfected and *vav*-HA-*MLF1* transfected 3T3 cells lack YFP therefore do not express protein detected by the GFP anibody as expected. This proves the backbone has been removed in the making of the *vav*-HA-*MLF1* plasmid. In contrast, HA-*MLFI-IRES*-YFP-MSCV still contains YFP and is detectable by the GFP protein. **b,c**) *vav*-HA-*MLF1* and HA-*MLFI-IRES*-YFP-MSCV both contain HA and MLF1.

3.10 ENU treatment results in significant death of wildtype C57BL/6J bone marrow cells. Bone marrow was harvested from C57BL/6J mice and analyzed by flow cytometry. **a)** There was a significant difference (p=0.0101) in Gr-1 wildtype C57BL/6J bone marrow cells that were apoptotic. There was no significant difference in the MLF1-/- mice. N/3 mice per genotype. **b)** The same phenotype was seen in bulk bone marrow cells. There was no significant difference between treated knockouts, but there was significance between wildtypes. N>5

3.11 SWR/J Mlf1$^{-/-}$ mice are significantly more viable than Mlf1$^{+/+}$ when treated with ENU. SWR/J bone marrow was harvested from genetically defined mice and sorted for kit+lin- cells. Cells were treated for 1 hour with ENU and left for 24 hours before being analyzed by flow cytometry **a)** Trypan blue staining was used to determine viablility of SWR/J Mlf1$^{-/-,+/-,+/+}$. **b)**

This was, in part, due to increased apoptosis. FACS marker Annexin V was used to determine cell death. N>2

3.12 There was no significant difference in recovery between C57BL/6J MLF1$^{+/+,+/-,-/-}$ mice following sublethal irradiation. 3 mice from each C57BL/6J MLF1$^{+/+,+/-,-/-}$ genotype were sublethally irradiated (500 RADs) using a gamma irradiator. They each received 6 eye bleeds for CBCs between days 0 and 28. Recovery was monitored to determine if MLF1 provided an advantage or disadvantage to the mice following a stress.

3.13 Model of MLF1 contribution to leukemia. We and others have shown that MLF1 is a pro-apoptotic protein, expressed in early myeloid progenitors, the cellular compartment relevant for leukemogenesis. We hypothesize that the expression levels of MLF1 may be one determinant that influences whether or not cells exposed to genotoxic chemotherapy agents undergo apoptosis. In this model, cells with relatively high levels of MLF1 are poised to undergo apoptosis, whereas cells with lower levels would be more likely to persist, accumulate mutations, and contribute to leukemias.

3.14 Kaplan–Meier analysis of overall survival in ENU-treated C57BL/6J Mlf1$^{-/-,+/-,+/+}$ mice. C57BL/6J mice of defined genetic backgrounds were treated with ENU and observed until moribund. Overall survival was not significantly different in the ENU-treated mice. The knockouts had a slightly shorter median than the heterozygous and the wildtypes (307 vs 356 and 358).

3.5. FIGURES

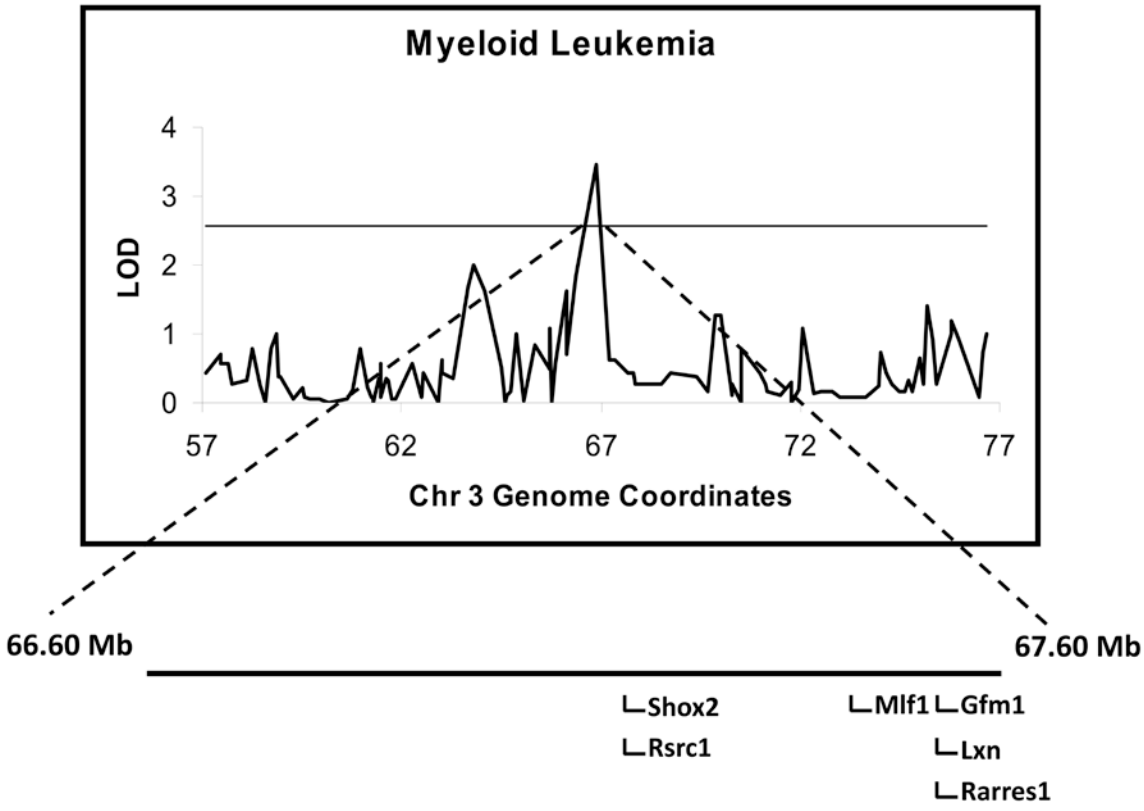


Figure 3.1

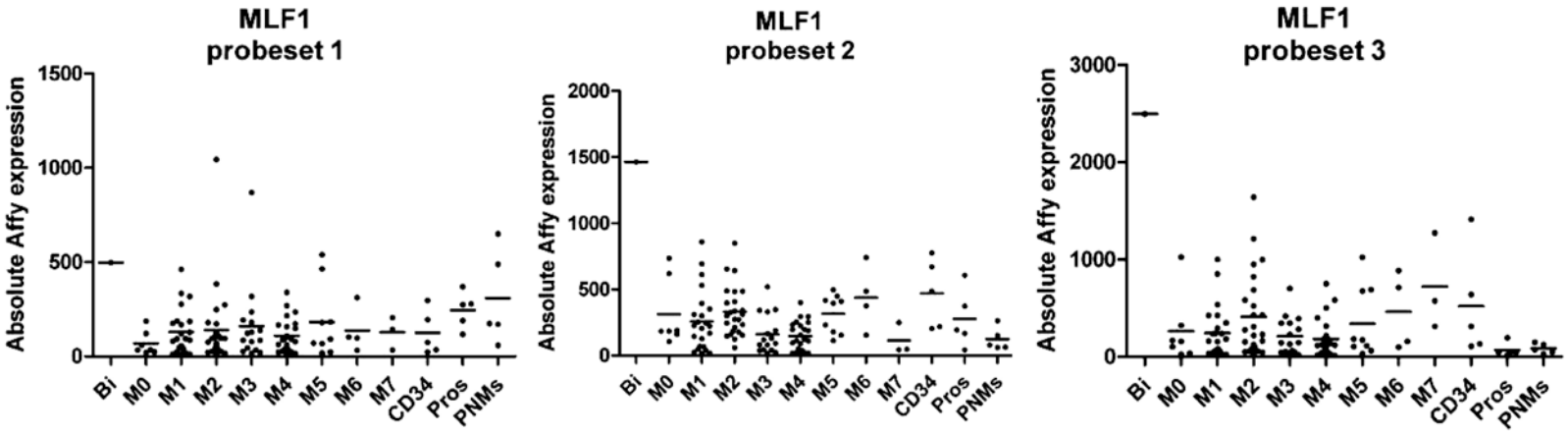


Figure 3.2

48

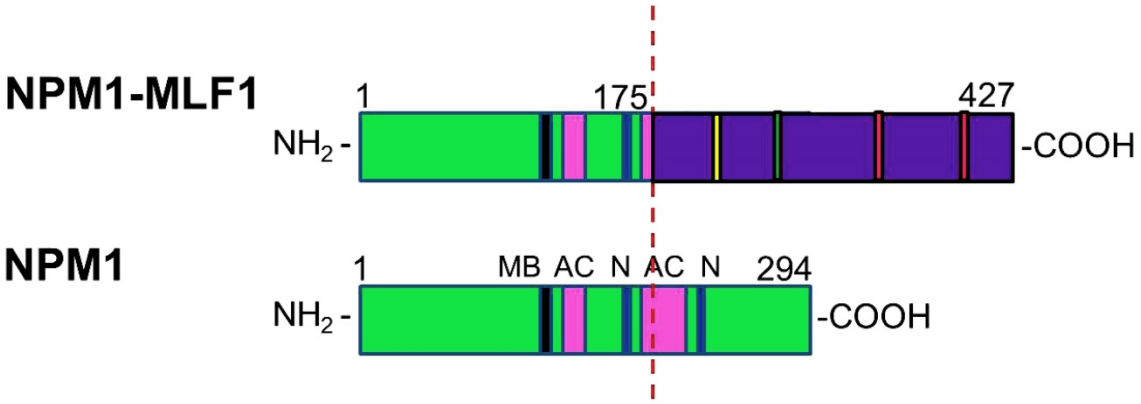


Figure 3.3

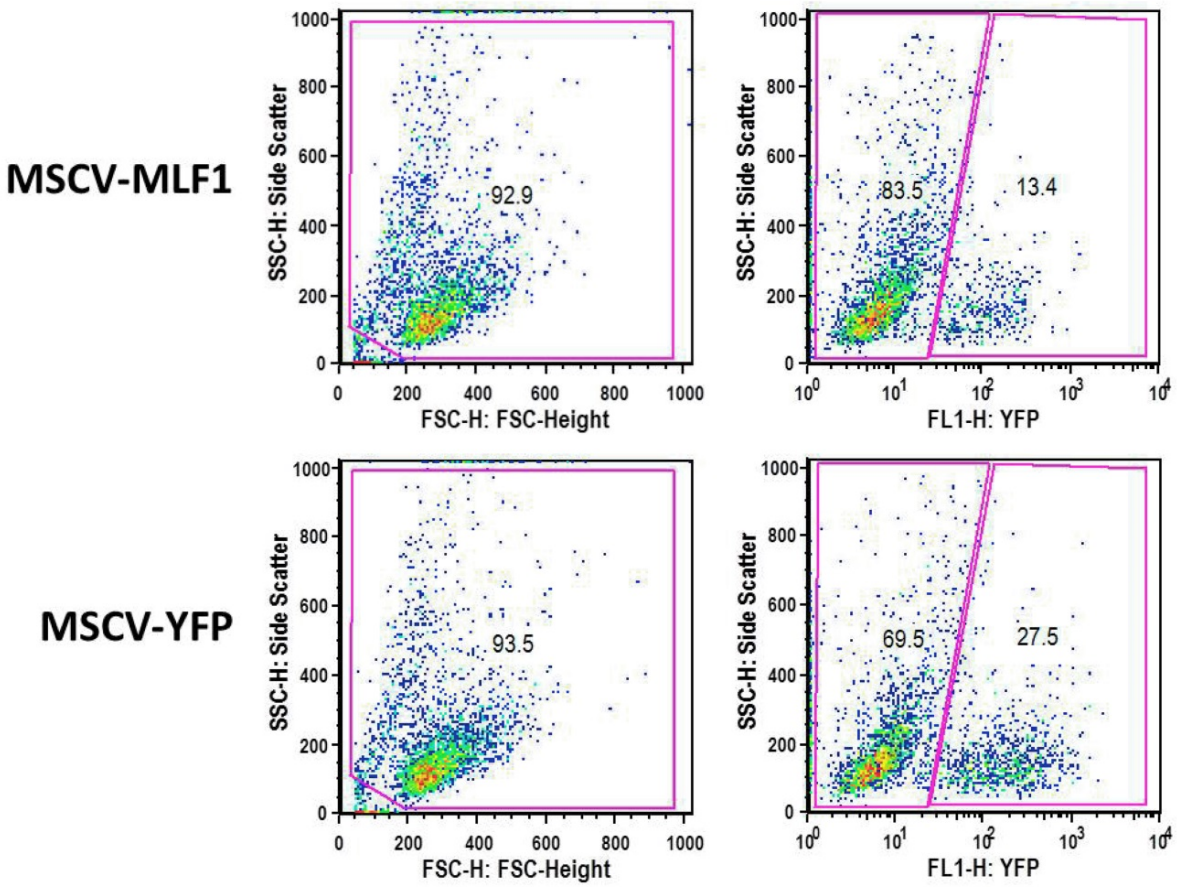
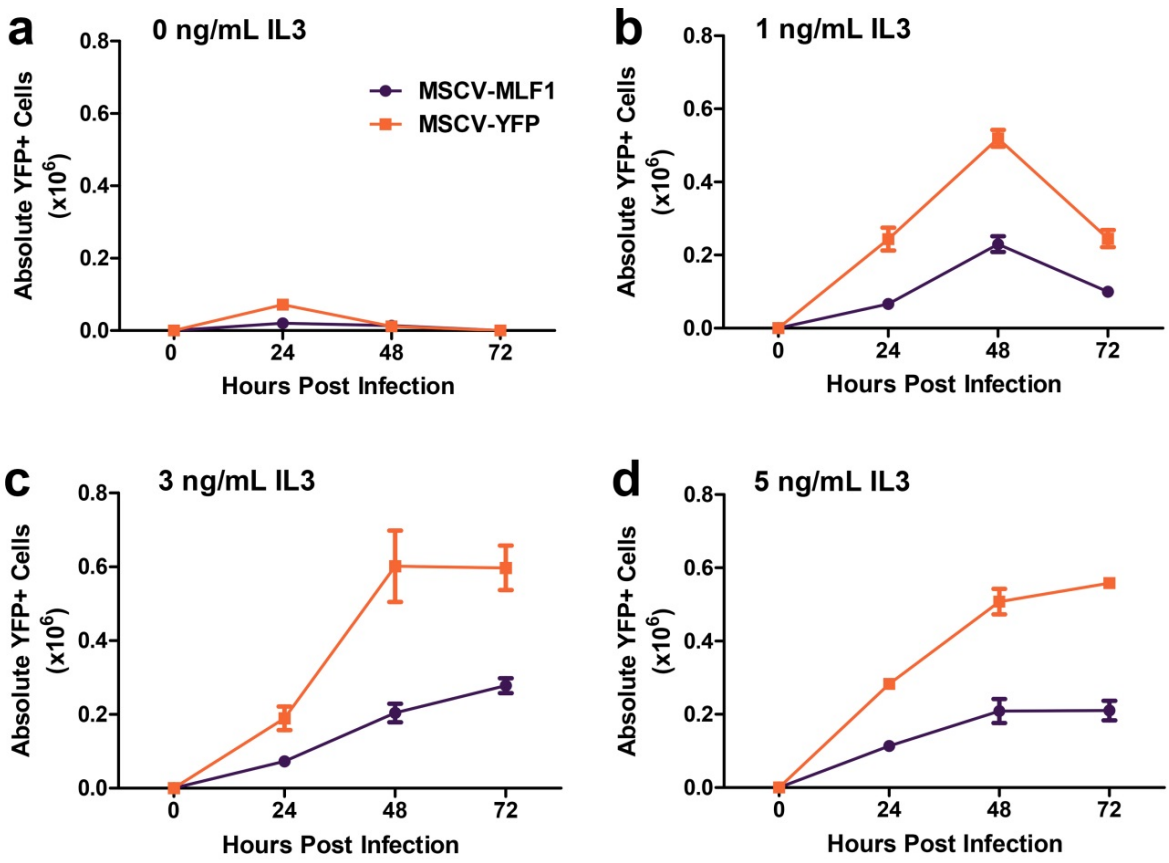


Figure 3.4



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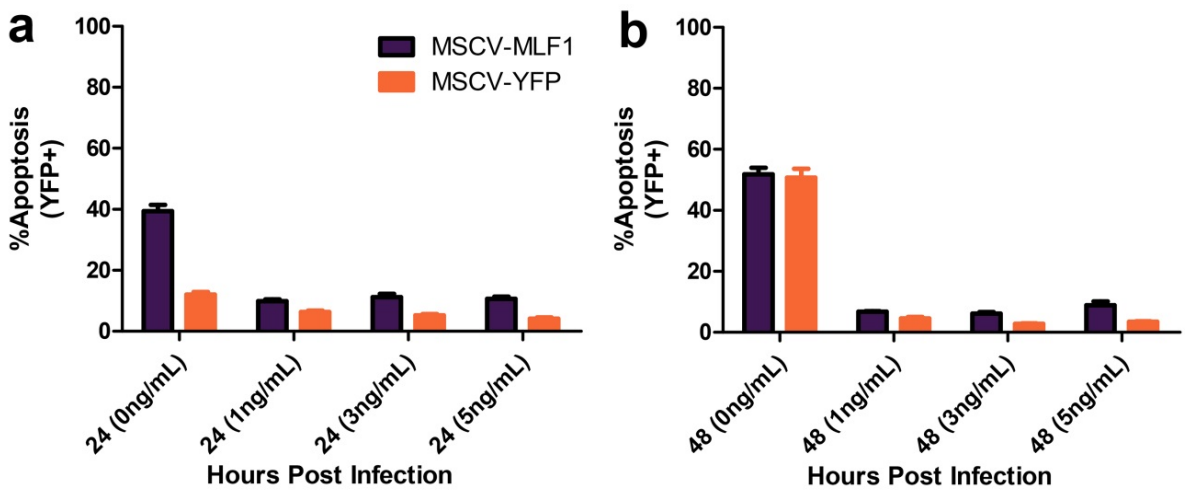

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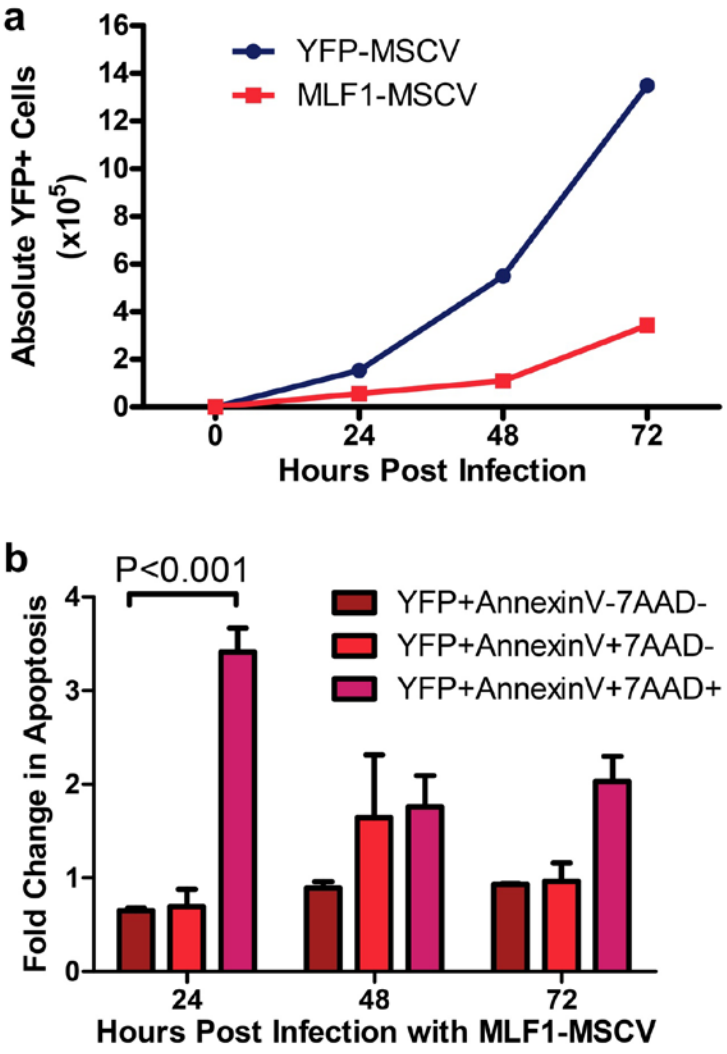

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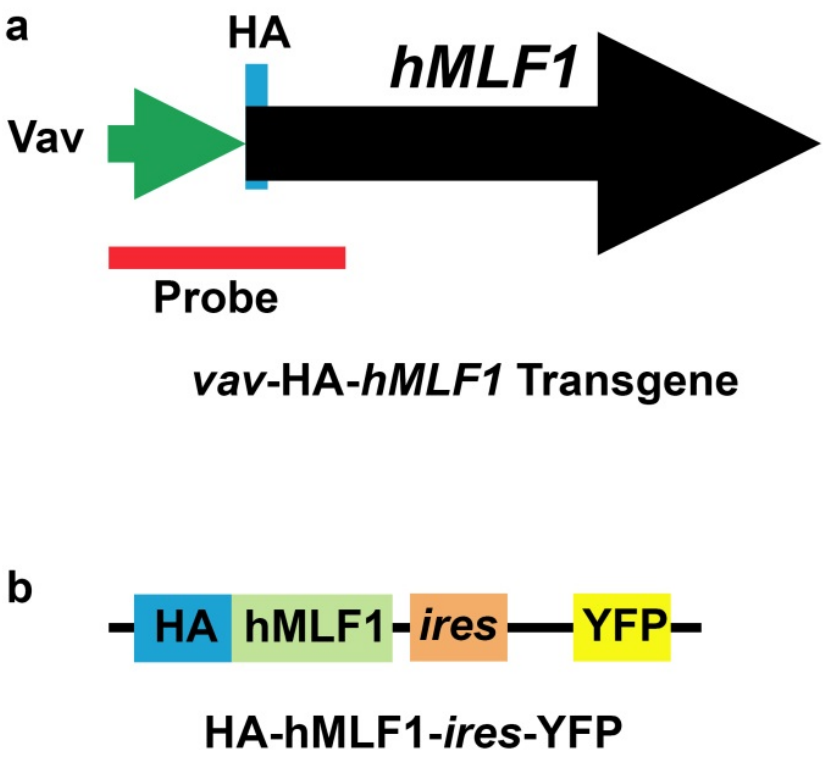
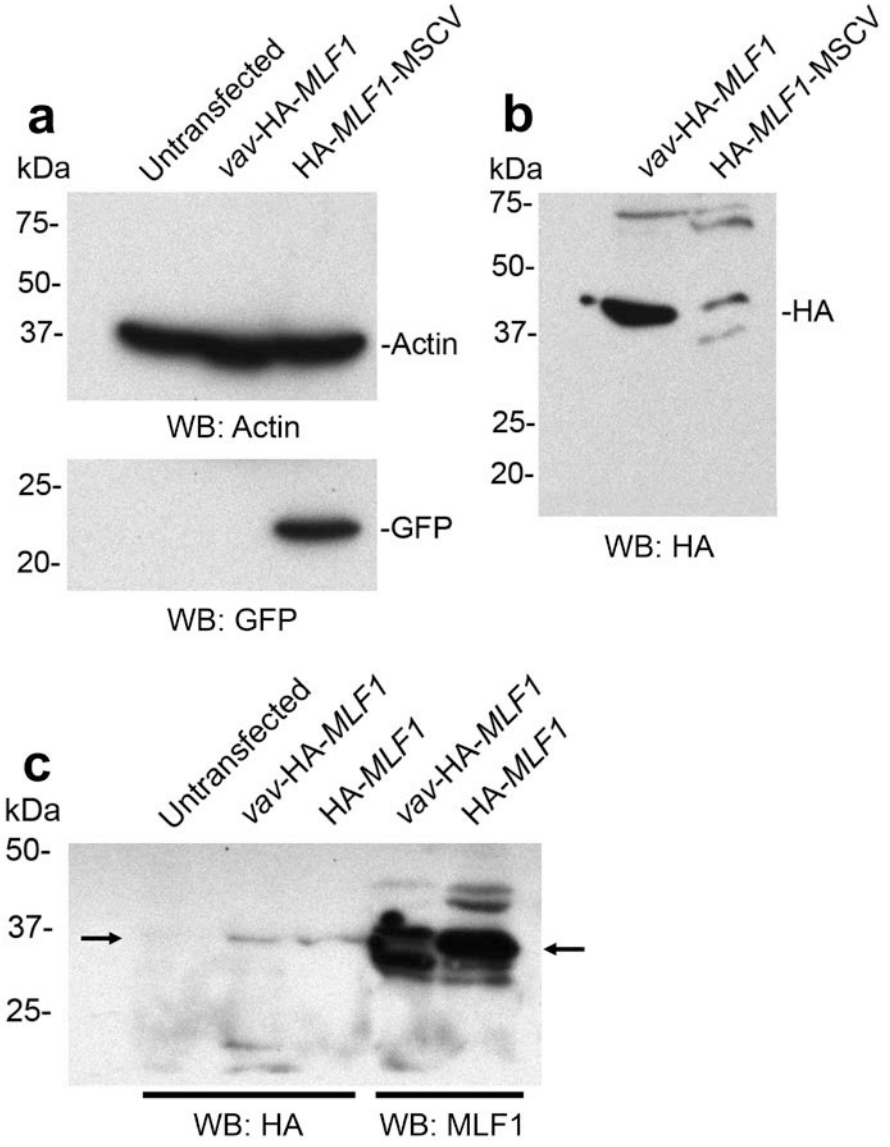


Figure 3.8



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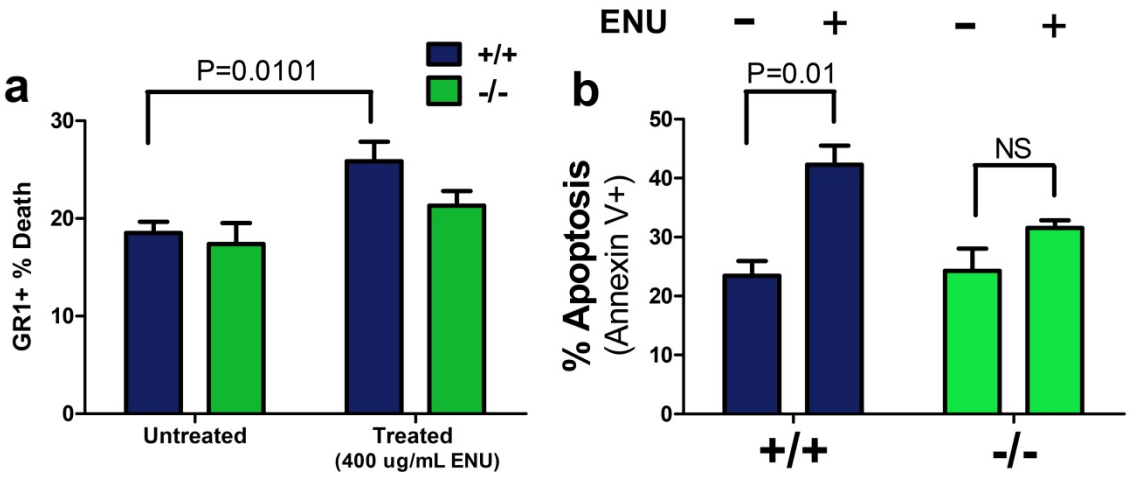

Figure 3.10

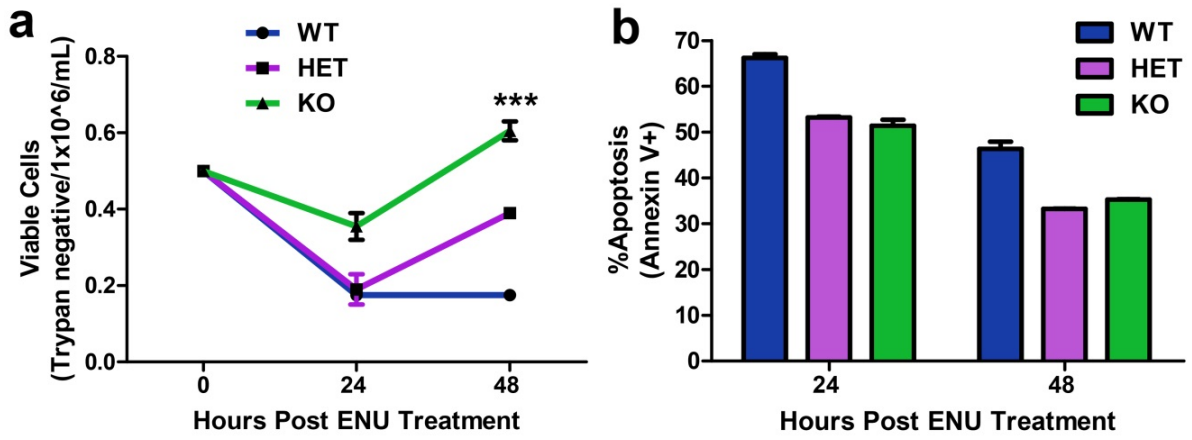

Figure 3.11

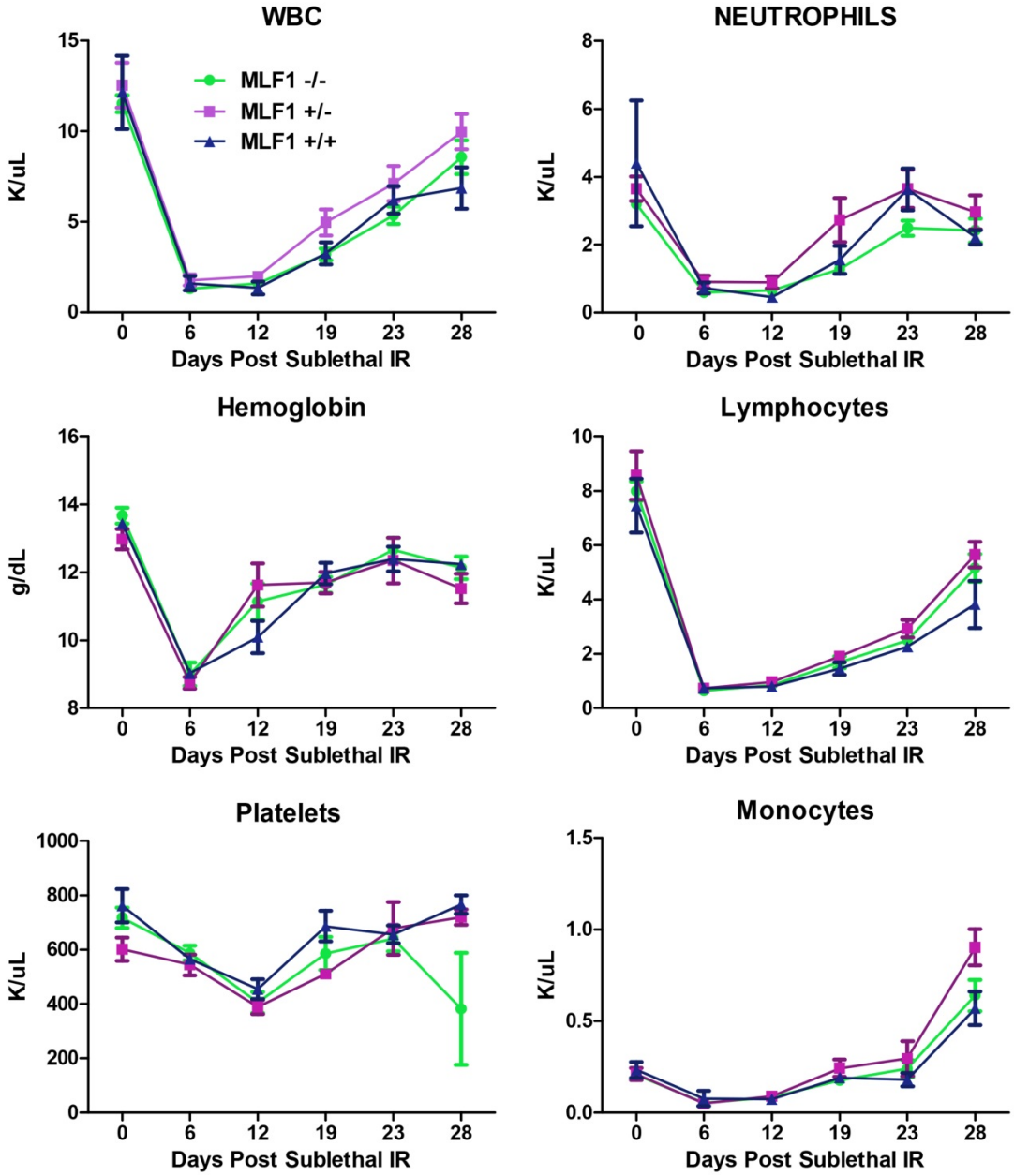

Figure 3.12

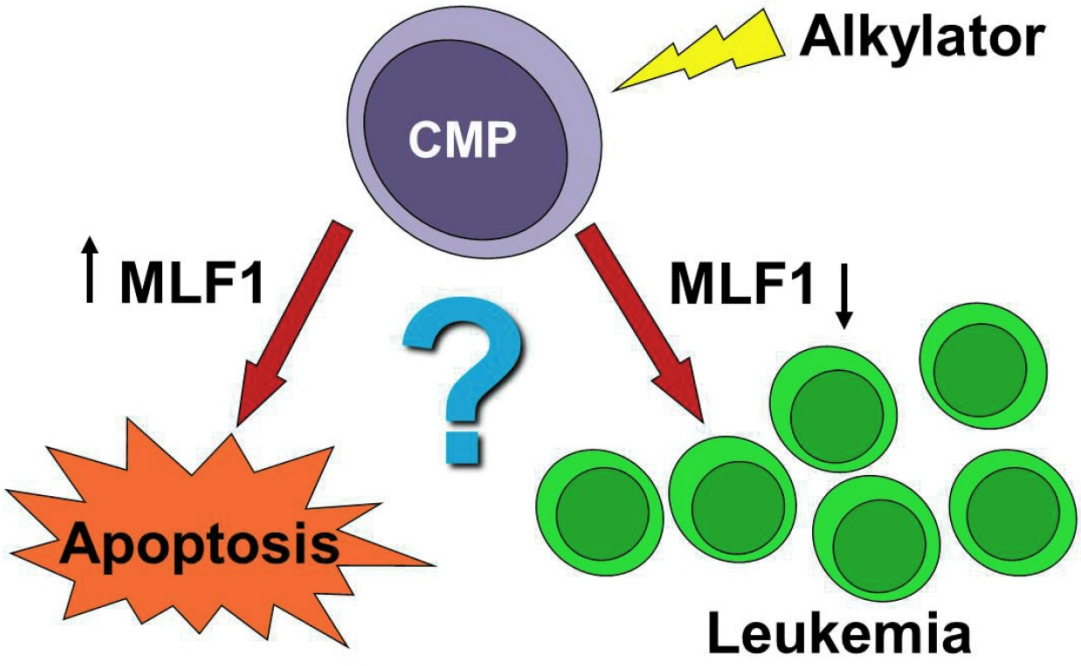

Figure 3.13

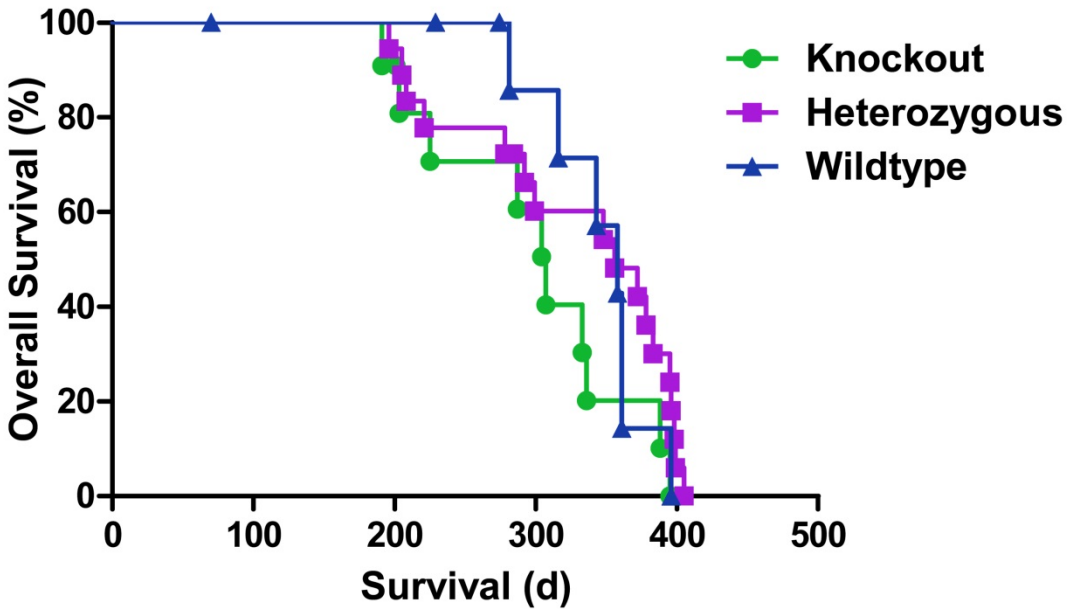

Figure 3.14

Table 1. Incidence of ENU-induced tumors in C57BL/6J mice with varying *Mlf1* genotypes

Treatment	n	No Disease	Lymphoma	Leukemia	Lung Cancer	Not Evaluable
ko (-/-)	11	1 (10.0%)	5 (50.0%)	0	4 (40.0%)	1
het (+/-)	18	3 (17.6%)	8 (47.1%)	0	6 (35.3%)	1
wt (+/+)	10	0	4 (57.1%)	3 (42.9%)	3 (42.9%)	3
Total	39					

ko, knockout; het, heterozygous; wt, wildtype

*3 concurrent leukemias and lymphomas did occur and are represented in both columns

Table 2. Hematologic parameters in ENU-treated C57BL/6J mice with different *Mlf1* genotypes

Genotype	Disease	n#	spleen wt (g)	WBC (K/uL)	NE (%)	LY(%)	Hb (g/dL)	HCT (%)	MCV (fL)	PLT (K/uL)	Bone Marrow FACS^	
											CD11b+Gr1+ (%)	CD4+CD8+ (%)
Knockout	No Disease	1	0.37	12.86	29.95	55.75	7.60	24.00	16.60	1146.00	55.29	1.49
	Lymphoma	5	0.26	8.76	31.43	56.38	11.68	36.54	13.70	791.20	74.95	3.90
	Leukemia	0										
	Lung Cancer	4	0.43	12.23	17.43	72.05	7.53	25.07	14.53	1330.67	63.75	1.12
Heterozygous	No Disease	3	0.41	8.62	23.76	63.27	7.97	27.17	14.23	712.33	59.64	0.70
	Lymphoma	8	0.28	14.36	28.65	53.63	6.65	20.30	22.36	347.63	56.79	12.44
	Leukemia	0										
	Lung Cancer	6	0.46	14.52	19.61	66.14	6.02	20.08	14.27	1196.17	64.62	1.47
Wildtype	No Disease	0										
	Lymphoma	4	0.72	9.73	32.81	49.33	7.48	26.23	13.78	625.25	64.68	5.53
	Leukemia	3	0.73	9.77	33.24	48.16	6.67	24.43	12.97	581.67	60.92	5.90
	Lung Cancer	3	0.35	18.74	23.39	55.76	7.47	24.97	14.93	968.67	58.57	1.48

WBC, white blood cells; NE, neutrophils; LY, lymphocytes; Hb, hemoglobin; HCT, hematocrit;
MCV, mean corpuscular volume; PLT, platelets

#including cases of concurrent lymphoma or leukemia

^percent of cases positive (>10% of cells) for the marker shown

3a: Blood Cell Counts of ENU treated Mlf1 C57Bl/6J mice

ID	STRAIN	Survival	Lung CA (0/1)	Lymphoma (0/1)	Leukemia (0/1)	Evaluable (0/1)	EMH (0/1)	Dysplasia (0/1)	spleen wt(g)	WBC (K/uL)	NE (K/uL)	NE (%)	LY (K/uL)	LY(%)	Hb (g/dL)	HCT (%)	MCH (pg)	PLT (K/uL)
7179	-/-	333	0	0	0	1	0	0	0.366	12.86	3.85	29.95	7.17	55.75	7.6	24	16.6	1146
7335	-/-	336	1	0	0	1	0	0	0.31	9.98	1.97	19.7	6.86	68.72	6.4	18.6	14.5	1536
7394	-/-	395	1	0	0	1	0	0	0.56	12.14	1.9	15.69	8.46	69.67	4.6	20	14.9	1591
7396	-/-	388	1	0	0	1	0	0	0.748									
7667	-/-	304	1	0	0	1	0	0	0.114	14.58	2.46	16.89	11.34	77.75	11.6	36.6	14.2	865
6930	-/-	203	0	1	0	1	1	0	0.145	5.88	2.54	43.24	2.59	44.09	12	34.8	14.9	1075
7169	-/-	201	0	1	0	0	0	0	0.194									
7324	-/-	191	0	1	0	1	0	0	0.138	10.26	4.23	41.19	5.32	51.9	18.2	58.7	14.1	817
7591	-/-	287	0	1	0	1	0	0	0.563	4.66	1.21	26.05	2.42	52.03	7.2	26.4	13.1	513
6870	-/-	307	1	1	0	1	0	0	0.19	9.82	2.21	22.51	6.27	63.86	8.9	27.3	14.1	422
6929	-/-	225	1	1	0	1	1	0	0.248	13.18	3.18	24.15	9.23	70.02	12.1	35.5	12.3	1129
7178	+/-	299	0	0	0	1	1	0	0.65	3.92	0.81	20.54	2.66	67.87	2.4	9	13.8	465
7323	+/-	196	0	0	0	1	0	0	0.087	15.5	2.5	16.1	11.67	75.3	12.9	41.6	12.6	876
7388	+/-	378	0	0	0	1	0	0	0.49	6.44	2.23	34.65	3	46.65	8.6	30.9	16.3	796
7666	+/-	284	0	0	0	0	0	0										
7180	+/-	292	1	0	0	1	0	0	0.165	20.34	5.91	29.06	10.74	52.81	8.9	29.7	13.5	963
7249	+/-	372	1	0	0	1	0	0	0.759	14.68	3.12	21.22	10.25	69.83	5.9	20.3	14.9	1608
7250	+/-	399	1	0	0	1	0	0	0.456	3.5	0.36	10.42	2.86	81.68	2.9	12	13.6	609
7334	+/-	348	1	0	0	1	1	0	0.927	19.44	4.77	24.55	9.62	49.49	2.5	7.1	16.9	1036
7395	+/-	396	1	0	0	1	0	0	0.3	10.18	1.47	14.4	7.25	71.25	6.4	23.3	12.5	1667
7590	+/-	405	1	0	0	1	0	0	0.127	18.96	3.41	18	13.61	71.79	9.5	28.1	14.2	1294
6871	+/-	356	1	1	0	1	0	0	0.105	10.82	2.98	27.55	7.33	67.77	10.9	33.9	15	831
7168	+/-	205	1	1	0	1	0	0	0.073	5.86	0.92	15.7	3.94	67.2	4.1	2	78.8	80
7172	+/-	383	1	1	0	1	0	0	0.1	24.02	7.93	33	9.88	41.15	12.3	38.6	15.3	252
7248	+/-	221	1	1	0	1	0	0	0.362	45.84	13.59	29.64	20.82	45.41	6	18.4	14.1	425
7332	+/-	278	1	1	0	1	0	0	0.568	13.98	6.57	46.96	3.23	23.08	4	14	17.9	79
7390	+/-	398	1	1	0	1	1	0	0.207	1.88	0.35	18.67	1.26	66.81	1.3	5.9	9.9	117
7393	+/-	208	1	1	0	1	0	0	0.057	3.86	1.03	26.66	2.37	61.29	6.3	21.6	12.1	395
7401	+/-	395	1	1	0	1	0	0	0.73	8.62	2.67	31.02	4.85	56.3	8.3	28	15.8	602
6869	+/+	70	0	0	0	0	0	0										
6872	+/+	229	0	0	0	0	0	0										
6928	+/+	281	1	0	0	1	0	0	0.505	14.58	3.79	25.98	7.63	52.36	6.4	20.5	14.9	1104
7171	+/+	358	1	0	0	1	0	0	0.27	22.48	5.37	23.91	13.16	58.53	8.7	29.1	14	864
7397	+/+	396	1	0	0	1	0	0	0.284	19.16	3.88	20.27	10.8	56.38	7.3	25.3	15.9	938
7247	+/+	274	1	1	0	0	0	0	0.067									
7333	+/+	361	1	1	1	1	1	0	1.225	9.72	2.97	30.55	5.59	57.46	6.1	21.4	13	536
7336	+/+	361	1	1	1	1	1	0	0.615	13.26	2.82	21.29	7.32	55.23	6.3	20.7	14.1	719
7389	+/+	343	1	1	0	1	0	0	0.69	9.58	3.02	31.52	5.06	52.84	9.9	31.6	16.2	756
7668	+/+	316	1	1	1	1	0	0	0.335	6.34	3.04	47.88	2.01	31.78	7.6	31.2	11.8	490

Table 3b: Bone Marrow FACS of ENU treated Mlf1 C57Bl/6J mice

ID	STRAIN	B220+ (%)	CD3+ (%)	CD45+ (%)	c-kit+ (%)	CD45+c-kit+ (%)	CD11b+ (%)	Gr1+ (%)	CD11b+ Gr1+ (%)	CD4+ (%)	CD8+ (%)	CD4+CD8+ (%)	ter119+ (%)
7179	-/-	8.4	3.69	33	1.05	40.73	1.48	7.43	55.29	3.93	2.84	1.49	10.47
7335	-/-	12.45	6.07	61.45	0.9	14.77	1.84	3.56	56.47	6.84	3.93	2.6	16.56
7394	-/-	4.8	1.27	79.86	0.2	6.96	2.57	3.93	71.91	2.26	1.94	0.45	3.38
7396	-/-	10.09	2.28	77.65	0.08	10.37	1.9	3.24	76.75	8.63	2.54	0.88	3.68
7667	-/-	16.86	5.08	53.65	0.08	26.13	3.49	3.6	49.86	3.01	4.19	0.53	2.52
6930	-/-	70.68	0.54	55.29	0.07	37.2	1.02	0.79	89.5	18.16	0.4	4.87	0.11
7169	-/-												
7324	-/-	8.67	4.66	52.62	0.25	40.75	0.36	14.33	70.79	1.65	3.24	0.97	1.4
7591	-/-	16.48	2.7	73.79	0.11	14.9	2.46	4.74	76.25	12.59	2.7	4.65	1.4
6870	-/-	5.3	6.72	45.72	1.59	27.84	3.67	4.35	56.74	7.52	2.47	4.71	9.56
6929	-/-	47.54	0.72	40.71	0.23	52.66	0.83	3.59	81.48	6.77	0.68	4.28	1.31
7178	+/-	2.12	5.74	41.05	2.03	44.14	0.19	25.38	55.26	1.34	4.54	0.3	8.84
7323	+/-	13.11	3.85	43.7	0.9	41.12	0.7	16.77	55.34	1.65	3.82	1.2	4.17
7388	+/-	5.34	2.63	74.93	0.05	4.39	2	2.5	68.33	3.62	1.46	0.6	1.17
7666	+/-												
7180	+/-	5.94	2.84	40.06	0.3	45.28	0.31	14.58	65.53	1.47	3.11	0.81	3.52
7249	+/-	4.67	2.63	71.33	0.68	12.32	0.98	6.83	73.26	4.36	1.55	2.03	3.83
7250	+/-	11.25	3.81	53.19	0.09	11.2	6.1	1.29	54	10.13	2.39	2.13	3.65
7334	+/-	6.34	4.35	71.95	0.27	13.18	4.37	1.59	69.2	5.77	2.27	1.67	3.87
7395	+/-	6.82	0.37	73.78	0.05	5.14	2.09	3.93	63.53	6.62	1.4	0.29	4.79
7590	+/-	15.19	6.75	44.15	0.88	41.86	1.9	6.81	62.21	4.18	4.07	1.91	6.68
6871	+/-	9.73	1.97	46.63	0.31	34.26	0.56	8.88	61.13	14.32	1.1	14.11	1.39
7168	+/-	14.99	3.37	50.31	0.56	34.88	1.33	5.99	64.54	8.72	1.4	2.63	5.25
7172	+/-	11.18	4.28	70.66	0.05	14.24	1.23	7.87	59.68	6.52	2.69	2.24	2.47
7248	+/-	1.94	3.05	36.55	0.86	49.23	1.49	11.93	65.59	0.79	4.81	2.89	7.02
7332	+/-	3.76	33.13	75.1	0.01	20.99	1.22	0.82	20.14	7.94	4.4	70.1	0.04
7390	+/-	7.59	7.94	69.72	0.07	11.78	2.6	6.05	56.63	8.17	5.92	2.48	5.73
7393	+/-	15.42	1.74	46.24	0.1	42.95	1.12	5.53	67.72	9.34	1.3	2.2	2.55
7401	+/-	2.62	8.14	79.61	0.06	6.38	2.21	6.92	58.87	8.25	8.71	2.83	0.98
6869	+/+												
6872	+/+												
6928	+/+	0.05	1.75	40.98	2.62	35.02	0.24	7.3	67.68	0.37	1.44	0.86	8.93
7171	+/+	14.61	2.61	32.73	1.59	43.6	2.37	7.54	54	9.97	2.7	3.28	9.92
7397	+/+	5.89	0.38	68.72	0.1	4.95	2.3	4.95	54.03	1.89	2.01	0.3	7.12
7247	+/+												
7333	+/+	6	16.35	62.13	0.05	14.46	5.58	1.37	59.48	10.53	8.51	15.69	0.98
7336	+/+	10.6	1.22	59.21	2.14	19.15	3.81	7.72	51.87	11.78	0.71	1.35	1.54
7389	+/+	2.87	6.13	75.44	0.17	12.39	0.78	5.78	75.94	8.01	2.37	4.42	2.42
7668	+/+	4.58	6.47	51.28	0.19	35.67	2.04	4.73	71.41	2.3	5.12	0.65	1.39

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Table 3c: Spleen FACS of ENU treated Mlf1 C57Bl/6J mice

ID	STRAIN	B220+ (%)	CD3+ (%)	CD45+ (%)	c-kit+ (%)	CD45+c-kit+ (%)	CD11b+ (%)	Gr1+ (%)	CD11b+ Gr1+ (%)	CD4+ (%)	CD8+ (%)	CD4+CD8+ (%)	ter119+ (%)
7179	-/-	29.65	13.06	44.46	1.06	16.62	2.6	3.86	11.17	10.13	8.23	5.6	15.48
7335	-/-	40.38	18.8	66.12	0.95	4.75	1.95	2	6.88	3.61	8.02	13.99	23.12
7394	-/-	8	2.64	41.86	0.89	4.85	2.21	2.17	29.22	5.36	2.22	3.06	26.58
7396	-/-	24.68	6.1	60.58	0.27	7.25	1.7	6.59	52.46	15.85	5.59	5.3	7.41
7667	-/-	52.66	21.59	77.98	0.22	1.99	4.73	1.78	2.12	16.17	9.99	1.11	0.3
6930	-/-	44.11	11.53	52.25	0.85	10.96	9.59	3.63	25.9	12.44	3.76	17.04	2.1
7169	-/-												
7324	-/-	37.17	26.65	69.22	0.83	14.85	1.36	12.01	9.21	15.97	14.82	3.58	8.22
7591	-/-	23.87	13.47	56.74	1.03	10.93	3.34	7.51	30.09	14.09	7.54	7.71	5.61
6870	-/-	48.18	14.34	59.39	2.65	8.73	8.69	7.76	9.86	8.31	10.12	13.09	8.14
6929	-/-	13.36	9.59	51.36	0.66	32.06	7.72	12.49	25.13	8.27	6.39	12.92	0.85
7178	+/-	16.77	13.79	36.48	2.09	15.73	1.1	12.23	10.34	6.52	9.73	3.58	30.55
7323	+/-	56.03	18.88	80.29	0.46	9.71	2.43	7.87	4.94	18.06	12.05	2.73	0.28
7388	+/-	21	3.86	61.88	0.44	4.2	2.81	3.67	26.03	9.2	3.86	3.2	1.96
7666	+/-												
7180	+/-	41.26	18.08	33.03	4.69	29.36	2.35	16.59	13.32	14.43	13.76	5.99	2.93
7249	+/-	12.6	7.76	37.17	1.02	9.04	2.46	4.39	24.26	5.13	3.36	8.65	38.2
7250	+/-	15.41	8.31	26.95	0.41	3.88	2.02	0.42	7.61	3	3.35	6.81	7.1
7334	+/-	5.17	4.22	17.17	1.96	3.7	1.3	0.48	9.76	2.5	1.53	3.18	30.6
7395	+/-	36.01	2.85	71.79	0.1	1.66	4.07	3.57	11.68	14.38	6.36	1.17	5.65
7590	+/-	42.52	25.58	67.18	1.75	5.91	5.62	13.19	5.88	16.63	17.2	4.51	1.62
6871	+/-	48.46	15.15	74.94	0.67	9.24	3.31	9.26	8.64	17.57	3.36	2.86	1.7
7168	+/-	58.4	14.87	83.15	0.12	7.99	10.15	4.15	7.32	7.12	8.65	13.95	0.42
7172	+/-	42.61	22.75	74.83	0.5	4.92	2.83	8.69	10.3	7.44	10.36	17.12	1.52
7248	+/-	17.36	13.9	23.31	6.43	40.17	6.28	12.32	13.22	5.83	32.45	19.29	14.17
7332	+/-	4.45	42.04	66.3	1.4	18.03	2.11	0.51	3.42	0.84	28.5	53.65	4.56
7390	+/-	34.83	29.14	77.2	0.14	1.8	3.78	4.18	4.5	27.28	10.19	4.2	4.35
7393	+/-	56.67	18.63	87.82	0.03	2.33	3.34	5.8	7.3	6.75	9.02	16.18	0.26
7401	+/-	13.84	10.77	66.6	0.1	2.79	2.66	6.44	28.52	13.2	11.67	2.33	7.16
6869	+/+												
6872	+/+												
6928	+/+	9.37	13.02	35.15	2.79	14.57	1.02	14.27	14.71	1.37	5.9	7.21	35.88
7171	+/+	23.53	4.86	34.43	2.41	23.69	2.35	6.04	21.4	5.85	3.5	11.49	15.33
7397	+/+	42.23	1.66	70.72	0.31	2.51	5.65	3.65	9.65	10.12	5.7	1.68	4.77
7247	+/+												
7333	+/+	3.82	11.98	47.43	0.89	9.1	5.16	1.67	35.65	7.45	6.28	12.12	5.86
7336	+/+	28.97	10.73	55.16	1.88	10.38	6.62	0.69	23.94	11.12	4.1	14.94	5.03
7389	+/+	22.34	4.51	65.08	0.21	7.88	4.44	4.59	30.95	3.74	5.52	12.42	4.5
7668	+/+	21.23	26.64	52.3	4.66	7.16	3.29	6.67	14.46	5.18	24.21	4.22	3.95

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CHAPTER 4

SUMMARY AND FUTURE DIRECTIONS

4.1. SUMMARY

4.1.1. SWR/J MICE ARE SUSCEPTIBLE TO ENU-INDUCED LEUKEMIA

We treated a cohort of 245 SWR/J mice with the potent mutagen ENU. This was to generate tumors in the mice of the hematopoietic lineages. We used different permutations of steroid to try to reduce the onset of lymphomas. We were able to determine that SWR/J mice are susceptible to ENU-induced leukemia (11.2%). The steroids did not have any effect on reducing the number of lymphomas that occurred. Overall, this strain will provide an important background for studying t-AML and for crossing with other mice that have been genetically altered to mimic some aspect of the disease. This will lead to models that will more closely recapitulate the disease and will bring us closer to a therapeutic solution.

4.1.2. MLF1 INDUCES APOPTOSIS IN HEMATOPOIETIC CELLS

The work here shows that MLF1 is pro-apoptotic in hematopoietic cells. When we overexpressed *MLF1* using the MLF1-*ires*-YFP retrovirus we were able to show both in the Ba/F3 cell line and primary bone marrow cells that MLF1 induced apoptosis and decreased the viability of cells. When we tested the effect of MLF1 using genetically defined mice and treated those cells with ENU, again we saw that MLF1 status was important for the apoptotic phenotype and for the viability of cells. C57BL/6J mice of different *MLF1* genotypes that were challenged by the additional stressor of sub-lethal irradiation showed no difference in recovery rates. C57BL/6J mice treated *in vivo* resulted in wildtype obtaining 3 leukemias in a small cohort compared to no leukemias in knockout mice. This difference in frequency of leukemias may not be meaningful, given the small sample size. This result contradicts our model because instead of inducing apoptosis and evading leukemia, the wildtype mice still went on to develop disease.

Mice lacking MLF1 went on to develop the rapidly developing lymphomas, which may be due to the fact that they could not induce apoptosis at as high of rate and thus carried more mutations that led to lymphoma and lung cancer development.

4.2. ONGOING WORK AND FUTURE DIRECTION

What is the molecular pathway that MLF1 works through?

It has been shown that MLF1 has multiple, novel binding partners (MLF1IP, Manp, Manp, CS3N, 14-3-3ζ). One group shows that MLF1 interacts with CS3N which decreases levels of COP1 [1]. This leads to increased levels of p53, and eventually cell cycle arrest in NIH3T3 cells. One work in progress was to ask whether MLF1 induced apoptosis was p53-dependent or independent. We were in the process of shuttling HA-MLF1 over to pcDNA3.1+ vector (Invitrogen). From there, we were going to transduce HCT116-/- and HCT116+/+ cells with the vector. HCT116-/- cells are p53 knockouts. Our prediction was that if MLF1 apoptosis was dependent on p53, you would not see cell death in the HCT116-/- cells but would still see death in the wildtype cells.

What portion or sequence variant is required for apoptosis?

To fully understand MLF1, any functional domains need to be closely mapped out. We started to perform fine-mapping to define the region. The idea was to transduce cells with the mutants and see if any or all of them still induced apoptosis. If the results were all of them, we would continue our fine-mapping strategy in new areas until we found the region responsible for apoptosis. In addition to this, there are point mutations in *MLF1* that may influence how much apoptosis gets induced. One of these point mutations is $MLF1^{P226T}$. The P variant is found at a higher ratio than the T variant. It is possible that these single point mutations do matter even if

they do not change expression levels of the protein they may slightly change the function by changing binding partners, altering subcellular localization, etc.

Alternative strategies to generate Mlf1 transgenic mice?

We speculate that *MLF1* overexpression in male germ cells was toxic, resulting in inability to transmit the transgene through the germline. That is what we suspect happened. It is also possible that the constitutive overexpression of *MLF1* is too potent in hematopoietic cells. We have already shown that MLF1 increases cell death and decreases viability in Ba/F3 cells. Another potential caveat is that a *MLF1* transgenic mouse was not viable due to bone marrow failure that prevents a mouse from developing. Future directions would need to address ways to circumvent this issue.

One alternative approach would be to make a conditional mouse using the tetracycline responsive system reviewed by Ryding *et al*. [2]. This system relies on the control tetracycline repressor has over the gene of interest. This happens by binding to the tetracycline operator sequences within the promoter. This results in repressed transcriptional activation. Adding tetracycline binds up all the repressor and turns the gene "on". We would need to have two transgenics. One would be the tTA expressing line to cross with the *tetO*-vav-HA-hMLF1 mouse. Tetracycline would be added to turn the gene on once the mouse was fully developed.

Another approach would be to overexpress *MLF1* using a MLF1-*ires*- YFP-MSCV retrovirus. Bone marrow from C57BL/6J CD45.2 donor mice will be harvested and infected by the retrovirus. Cells will be sorted to enrich for the YFP+ population and $1x10^6$ cells will be transplanted into lethally irradiated C57BL/6J CD45.1 recipient mice. Peripheral blood will be obtained via retro orbital eye bleeds one month following transplantation. FACS will be used to

determine percent engraftment (CD45.1 vs CD45.2) and the percentage of YFP+ cells. Mice will be analyzed as described above.

Another caveat could be that we do not see a phenotype in the transgenic mouse. This would suggest that *MLF1* overexpression alone is not sufficient to disrupt normal bone marrow function *in vivo*. This mouse model would still provide a useful tool for mutagenesis and for breeding to other mouse models with genetic disruptions that may cooperate with *MLF1* to contribute to disease.

How is SWR/J mice susceptibility to ENU-induced leukemia affected by knocking out Mlf1?

We fully backcrossed the MLF1 null allele into the SWR/J (sensitive) genetic background. Future experiments could test whether MLF1 deficiency converts this sensitive strain to a resistant one. Based on our initial results attempting to convert a resistant strain (C57BL/6J) to a sensitive one, large cohort sizes and/or additional sensitizing transgenes (e.g., PML/RARA) may be required to test this definitively.

Is the pro-apoptotic effect of MLF1 dependent on stage of differentiation?

Here we hint that in C57BL/6J mice, the Gr-1+ cells are susceptible to MLF1 induced cell death. In data not shown, we have seen that macrophages are resistant and monocytes are the most susceptible to MLF1 induced apoptosis. On the SWR/J background we show that kit+lin- cells are susceptible. A careful study of both backgrounds needs to be conducted where bone marrow will be stained for flow cytometric analysis with antibodies conjugated to B220, CD3, CD45, c-Kit, CD11b, Gr-1, ter119, and Dx5. Cells will also be kit+lin- flow sorted

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Professional Profile

Ph.D. molecular biologist with experience in the field of cancer biology. Designed gain-of-function and loss-of-function experiments using mouse models and primary and cell line cultures to determine if myeloid leukemia factor 1 (*MLF1*) is relevant for the initiation of treatment-related acute myeloid leukemia. Radiation safety trained and certified to obtain increased control access to irradiator. Annually passed the Environmental Health and Safety Laboratory Safety Training. Proficient in the use of drug-induced apoptotic assays, retroviral transductions, and Microsoft Office Suite. Presented research orally at an internationally attended scientific meeting. Recipient of a Montana Space Grant Consortium award.

Education

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Ph.D. Molecular Cell Biology
Washington University, St. Louis, Missouri

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The University of Montana-Western, Dillon, Montana

Experience

Graduate Research Assistant *2006-1/2014*

Ph.D. Thesis. "Myeloid Leukemia Factor 1 is a Candidate Susceptibility Factor for Treatment-related Acute Myeloid Leukemia." Mentor: Timothy A. Graubert

- Janke, MR *et al.* SWR/J Mice are Susceptible to Alkylator-Induced Myeloid Leukemia *Blood Cancer Journal* **3** (2013), *published*
- Developed mouse models to study the effect of MLF1 on leukemogenesis
  -Designed and constructed transgenic mouse
- Radiation safety trained and certified to obtain increased control access to irradiator
- Mentored a rotation student and performed duties as a teaching assistant
- Annually passed the Environmental Health and Safety Laboratory Safety Training

Undergraduate Research *2003-2006*

Thesis. "The initial characterization of the *Candida albicans SEC61*, *SEC63*, and *SSS1* genes using *in vivo* and *in vitro* experiments." Mentor: Michael W. Morrow

- Designed and mutagenized plasmids used in yeast plasmid rescue experiments and tetrad dissections
- Performed initial characterization on 3 *C. albicans* genes
- Discovered that 2 of 3 genes can at least partially functionally replace their *S. cerevisiae* homologues during translocation of pre-secretory proteins into the ER

Summer Research Fellowship, American Society for Microbiology *2005*
The University of Montana-Western, Montana
- Determined that the *C. albicans SEC63* gene, at least partially, functionally replaces its *S. cerevisiae* homologue during the translocation process

Honors and Awards

American Society of Hematology Travel Award
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Summa cum laude
Montana Space Grant Consortium Scholarship
Best Poster, Montana Academy of Science
Faculty Association Scholarship, University of Montana-Western (awarded to one student each year)

Memberships

American Society of Hematology
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Publications and Presentations

- **Janke MR**, Baty J, Graubert TA. SWR/J Mice are Susceptible to Alkylator-Induced Myeloid Leukemia. *Blood Cancer Journal* **3** (2013), *published*

- **Janke MR**, Babatunde F, Cahan P, Cheverud JM, Graubert TA. *MLF1* is a Candidate Susceptibility Factor for t-AML that Induces Apoptosis in Normal Hematopoietic Cells. December 2008. American Society of Hematology Annual Meeting. San Francisco, CA., oral presentation

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References

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