# INSIGHTS INTO DETERMINANTS OF CANCER SUSCEPTIBILITY, INITIATION, AND PROGRESSION: STUDIES ON MEDULLOBLASTOMA AND HISTIOCYTIC SARCOMA IN MOUSE MODELS

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## Dedication

This dissertation is dedicated to my mother, Shohreh Pirzad, who always encouraged me to be an educated, independent person.

## Abstract

This dissertation presents a discussion of both perigestational dietary influence on cancer predisposition as well as somatic genetic determinants of cancer development. Both projects used genetically engineered mouse models of cancer. The introductory chapter gives a brief historical introduction to cancer, background information on models of cancer, and a short description of our current understanding of cancer. Chapter two presents data on how maternal diet can affect the risk for medulloblastoma in offspring. Medulloblastoma presents a dismal prognosis even for the patients who are successfully treated. Prevention strategies are therefore of great interest in addressing this disease. Chapter three discusses a mouse model of Histiocytic Sarcoma (HS) that was developed to identify genes that can contribute to initiation and cause progression of disease. The genetics of HS are not well understood. Our model could provide important information on molecular targets that can be used to treat this dreadful disease. The final, fourth chapter, provides a brief and broad overview of some of the major future likely sources of cancer control success with a focus on new research.

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# Chapter 1) Introduction

## Cancer Defined

Cancer is a term that describes a variety of diseases defined by the uncontrolled growth of cells. The deadliest feature of cancer cells is their ability to not only proliferate uncontrollably, but also to spread or metastasize to other parts of the body. There are over 100 types of cancer and cancer can occur at any age. Cancer can be caused by both external factors that result in damage to DNA, cause epigenetic alterations, or influence cell behavior as well as inherited gene mutations. Indeed, cancer is likely to often result from interplay of the two. External factors can be linked to our behavior and can be modified by altering our exposure to environmental causes such as tobacco, chemicals and radiation. In addition, strong data support the notion that diet and exercise are major modifiers of cancer risk. However, most cancer is neither familial (or inherited) nor can it be ascribed to a clear external causal factor. Thus, cancer control remains an elusive goal.

Cancer is thought to arise by a stepwise accumulation, in a somatic clone of cells, of genetic and epigenetic changes. This genetic and epigenetic progression is characterized by the acquisition of twelve known hallmarks of cancer, that describe the biological capabilities that tumors possess [1]. It is thought that many genetic/epigenetic changes must occur, before cells acquire all twelve hallmarks are acquired, making cancer a complex disease. The twelve hallmarks of cancer include: (1) cancer cells ability to stimulate their own growth; (2) the ability to resist inhibitory signals; (3) the ability to resist apoptosis; (4) the ability to stimulate angiogenesis; (5) enablement of replicative

immortality; (6) the ability to metastasis; (7) sustaining proliferative signaling; (8) evading growth suppressors; (9) ability to evade immune destruction; (10) ability to promote tumor inflammation; (11) ability to deregulate cellular energetics; (12) increased genomic instability and mutation incidence [1]. The understanding of these hallmarks of cancer has greatly increased due to recent advances in cell biology, immunology, and molecular techniques that allow for higher genetic resolution of cancer cell genomes. The large number of genetic and epigenetic alterations in cancer is better understood than ever before. Yet despite the recent advancements made in the field of cancer genomics, the overall five-year survival for all cancers has not improved dramatically.

## History of Cancer Epidemiology

Cancer has existed for all of human history, with the earliest written account dating back to ancient Egypt in 3000 BC. Despite its early discovery, very little was known about cancer until the 17$^{th}$ century, due to cultural restrictions placed on performing autopsies. It was not until 1775 that a study showed an environmental factor could cause cancer. Dr. Percivall Pott associated environmental occupational exposure to ashes with a higher incidence of scrotum cancer among chimney sweeps [2].

In 1957, the U.S. Surgeon General's officially declared that smoking could cause cancer. Since then, many environmental factors, including some dietary factors, have been associated with cancer risk [3]. However, the extent to which dietary interventions could prevent cancer is unclear. The War on Cancer was initiated in 1972 by President Nixon to eliminate cancer as a major cause of death. The major initiative was to strengthen the National Cancer Institute through increased research to advance the

understanding of cancer biology and the advancement of new successful treatments [4]. It is often said that this "war on cancer" has been unsuccessful. Indeed, the statistics do not paint a rosy picture for most forms of cancer, with some notable exceptions such as the success in treatment of childhood leukemia, testicular cancer, and a few others.

## Cancer Surveillance and Prognosis

Cancer is the second most common cause of death in the US, accounting for nearly one of every 4 four deaths [5]. In 2012, the total number of cancer deaths for Americans is expected to be around 577,190 people. This amounts to more than 1,500 Americans a day. It as been estimated that by the time a cancer is diagnosed, even with the advanced techniques now available, more than 90% of the biologic life of the tumor is over [3]. This makes the best chance of controlling the malignant process inaccessible with current methods of cancer surveillance. Fewer than 50% of cancers are cured. Treatment effectiveness has been incremental and expensive, making early detection and prevention of cancer the best means to reduce cancer mortality [3]. Indeed, the subtle improvement in five year survival from the time of diagnosis, that has been observed in the last two decades, has been ascribed by some to simply the fact that cancer is diagnosed earlier than in the past, with no real improvement in preventing the inexorably development of aggressive, life-threatening cancer in those cases destined to develop that way. Systematic application of prevention strategies have been effective in reducing morbidity and mortality for many major diseases, including nutritional and infectious diseases and vehicular trauma. The incidence of cardiovascular disease has decreased as smoking has declined as a result of hard-hitting national antismoking campaigns. Encouraging

exercise has reduced cholesterol and blood pressure levels [3]. Indeed, it could be argued that in the coming decade, it is likely that simple prevention or early detection strategies will led to greater overall benefit to the health of the U.S. population, with regard to cancer, than the effects of the best cancer therapeutics. In general, physicians, researchers, and the National Cancer Institute have neglected the role of prevention strategies in the overall management of cancer [3, 5].

## Major Areas of Cancer Research

The standard way to treat cancer is surgical resection of the tumor followed by a regimen of chemotherapy and radiation, described as adjuvant therapies, which are designed to reduce the chance of cancer recurrence. There have been incremental advancements in treatment since the War on Cancer began with the primary mode of treatment still being surgical removal of the tumor mass with a scalpel. Further advancements in treatment modalities can only be made through expansion of our understanding of cancer biology through further progress in basic research.

The field of cancer research can be divided into three major areas: prevention, genetic etiology, and treatment and drug development. A comprehensive cancer control strategy would incorporate resource planning into each area commensurate with the likely public heath impact that could be achieved. Less than 5% of the NCI's 2011 budget was allocated to cancer prevention research. Some of the clearest evidence for success in cancer prevention involves reducing exposure to occupational or other exposures that have been found to cause cancer. Chief among these are the efforts to reduce lung, esophageal, and head and neck cancer by reducing the prevalence of

smoking. Indeed, this effort has now resulted in a downward trend in lung cancer prevalence among U.S. males in the last decade. Further reductions are expected. Other examples include the success in preventing radiation-induced leukemia and other cancers in health care workers using X-ray machines in hospital settings.

Studies of cancer epidemiology show that those who eat the most fruits and vegetables have the lowest cancer burden in general. The reasons for this trend are not entirely clear, and this work has not led to an easily implemented program of dietary supplementation that can influence cancer risk. Nearly half of the U.S. adult population takes nutritional supplementation, which is a 27 billion dollar industry, and the most common supplement is a multi-vitamin[6]. Surveys have shown that most people take these supplements to make up "for a poor diet" and hope to experience the benefits of a diet high in fruits and vegetables without actually consuming enough of them. Despite these facts, little data is available to prove definitively that any one regime of vitamin supplementation can reduce cancer risk. Indeed, a study of beta-carotene supplementation showed more lung cancer in high-risk patients than in the high-risk control, placebo group[7, 8]. The Physicians Health Study II showed no protection from prostate or other cancers from vitamin C or vitamin E supplementation[6, 9].

Chemoprevention using drug-like molecules has been successful in several cases. Taking a daily aspirin or other COX-2 inhibitor shows a reduced risk of colorectal cancer[10]. In women at high risk for breast cancer, the use of tamoxifen, a selective estrogen response modifier, or aromatase inhibitor shows clearly reduced risk of subsequent development of breast cancer[3].

Prevention measures such as identification of early detection of risk markers,

symptoms, and improved examination methods may have improved survival rate of cancers such as prostate, breast and colorectal cancers[3]. Indeed, regular screening could prevent up to 50% of colorectal cancer cases, as pre-cancerous polyps can be removed before they become malignant, if all U.S. citizens started a regular screening program at age 50[3-5]. Whether screening mammography, and use of prostate specific antigen screening, is of benefit for reducing breast cancer or prostate cancer mortality is controversial.

Advancements in the field of genetic etiology have been the result of better and cheaper sequencing methods, as well as major efforts by The Cancer Genome Atlas (TCGA) project, that have resulted in availability of an abundant amount of information about typical changes to the human cancer genome. Where this field's effort has perhaps fallen short, is in translating the data accumulated into significant progress in cancer treatment. One of the problems is the lack of functional knowledge and deficiency in understanding which alterations are causally involved in tumor initiation and which alterations have little functional impact. In order to properly use this information to impact overall cancer survival, we need to distinguish between alterations that play a causal role, so-called driver alterations as opposed to passenger alterations, so we may use the most effective treatment available to eradicate disease [11]. Nevertheless, the higher genetic resolution can have a profound impact in the field of cancer treatment with the development of targeted therapies.

Two targeted therapy success stories are the drug Gleevec for the treatment of Chronic Myelogenous Leukemia and the monoclonal antibody, Herceptin, for the treatment of Breast Cancer. Gleevec was the first successful targeted therapy that

attacked specific cancer cells rather than non-specifically killing cells[3]. Gleevec works by binding to the site of tyrosine kinase ABL in white blood cells and prevents its biochemical activity, thus initiating apoptosis [12]. Herceptin is used to treat breast cancers that are HER–2 positive and targets cancer cells that "overexpress," or make too much of the protein. HER-2 is a cell surface protein and a member of the epidermal growth factor receptor family that signals via its cytoplasmic tyrosine kinase domain[13]. Herceptin is a humanized monoclonal antibody that binds to HER-2, resulting in its down-regulation, and recruitment of cells and molecules of the innate immune system[13]. Both effects may be important for Herceptin effectiveness. Breast cancers that overexpress HER–2 account for about 20 to 30 percent of tumors and tend to be more problematic clinically due to their propensity to recur [13]. Women with breast cancer who receive Herceptin as a part of their treatment regimen live longer and have significantly less chance of the breast cancer coming back.

The pipelines from the bench to the clinic for Herceptin and Gleevec provide models for more targeted cancer therapies to follow. The human cancer genome is incredibly complicated with many genetic and epigenetic alterations that may serve as potential targets for advanced therapies. Progress in all three fields can influence one another in a continual feedback loop, with each advance working to improve cancer control.

## Experimental Models for Cancer Research

An important tool used in many areas of research as an experimental model is the genetically modified mouse (GMM). A GMM is a mouse whose genetic material has

been altered using genetic engineering techniques including transgenesis by pronuclear injection and gene modification by homologous recombination in mouse embryonic stem (ES) cells. GMM models exist to study at a wide range of cancers and other diseases such as diabetes, heart disease, addiction, asthma, and autoimmune diseases, just to name a few. There are two methods of creating GMMs, by overexpressing or knocking-out a gene of interest. The methods include pronuclear injection to insert transgenes of interest into a single cell of a mouse embryo, where it will randomly integrate into the mouse genome[14] or modifying embryonic stem cells with a DNA construct containing DNA sequences homologous to the target gene, and then injecting the modified ES cells into the mouse blastocyst resulting in the production of germline chimeras [15]. In cancer research, GMMs are generated to express oncogenes or eliminate expression of tumor suppressors to study cancer.

Two projects are presented in this thesis and each project focuses on a different area of cancer research. The first project focuses on cancer prevention. In this project we use a *Ptch* mutant mouse to study the incidence of the most common malignant early childhood brain tumor, medulloblastoma. *Ptch* functions as a tumor suppressor gene and is a receptor for sonic hedgehog; an important pathway implicated in the proper formation of embryonic structures, most notably the central nervous system [16]. The second project focuses on cancer etiology where we use a transposable element called *Sleeping Beauty* (SB) to identify cancer-initiating genes involved in Histiocytic Sarcoma (HS) development. The SB system is an unbiased method used to sort through the many alterations that occur to the genome to identify cancer causing genes.

## *Ptch Mouse*: Model for Medulloblastoma

### MEDULLOBLASTOMA

MB is a primitive neuroectodermal tumor (PNET) that arises from granule cell precursors in the cerebellum. It is the most common, malignant early childhood brain tumor, accounting for roughly 20% of all pediatric brain tumors affecting an estimated 450 U.S. children, <20 years of age, each year [17]. The peak incidence occurs at ~3 years of age with more affected males than females. Approximately 65% of children survive 5 years after diagnosis [18]. Unfortunately, many long-term survivors of brain tumors face significant life-altering consequences of the disease and its treatment including: second primary cancers, cardiovascular disease, performance limitations, reduced insurance coverage, and reduced employment rates [19-22]. Low survival rates together with the significant life challenges that childhood brain tumor survivors face emphasizes the need to identify preventable causes. Identification of causes is the first step in achieving the ultimate goal of primary prevention strategies that reduce the overall incidence of disease.

MB can appear sporadically or can be associated with a hereditary predisposition. Sporadic MBs have four distinct genetic signatures, based on gene expression patterns. Only two of the four have been characterized, altered WNT signaling and altered Sonic Hedgehog (SHH) signaling. Of the sporadic medulloblastoma, roughly 25% are associated with altered *Ptch1* signaling [23]. Genetic disease syndromes that increase susceptibility to medulloblastoma include: Li-Fraumeni (*TRP53* mutation), Nevoid basal cell carcinoma (Gorlin's syndrome; *PTCH1* mutation), and Turcot (*APC*, *MLH1*, or *PMS2* mutations) [16, 24]. The young age at which most cases of medulloblastoma are diagnosed suggests that prenatal exposures may be etiologically relevant.

### *PTCH1* +/- MOUSE

To study risk factors of medulloblastoma we chose to use a heterozygous *Ptch1* (*Ptch1+/-)* transgenic mouse model because its phenotype has been well characterized and is similar to the human disease. The protein product of the *Ptch1* gene is part of the sonic hedgehog signaling pathway that plays a vital role during development and has been shown to be dysregulated in many human cancers [16]. Due to the importance of this pathway in embryonic development, *Ptch1* homozygous knockout mice are die before birth. However, heterozygotes are viable with slightly reduced survival to weaning and, similarly to humans, have an increased risk for medulloblastoma, basal cell carcinoma, and rhabdomyosarcoma. The incidence of medulloblastoma in these mice is incompletely penetrant varying from ~8%-~23% with tumors developing as early as 5 weeks depending on the strain background [25-27]. The strengths of this model that make it ideal for identification of environmental risk factors for medulloblastoma include: high phenotypic concordance between human and mouse medulloblastomas, a short latency, incomplete penetrance that has previously been shown to be modifiable by an environmental exposure, and a well-characterized signaling pathway for downstream mechanistic studies[16, 27].

## Folic Acid

Folic acid is the synthetic form of a naturally occurring nutrient, folate. Folate can be attained through the consumption of a wide variety of foods such as eggs, lentils, leafy vegetables that include spinach, asparagus, turnip greens, lettuce and other foods[28]. In the US and many other countries, processed grains and flour are fortified with folic acid [29]. Folic acid is part of the B complex vitamins and is water-soluble, therefore, easily

absorbed through the diet. Folic acid is not biologically active until it is converted to dihydrofolic acid in the liver[29, 30]. Folic acid is essential to many bodily functions, especially in periods of rapid cell division and growth. The human body needs folic acid to synthesize, repair and methylate DNA as well as to produce healthy red blood cells [29-32]. A folic acid deficiency is reported with very low incidence in the US because it takes months of a total lack of dietary folic acid before deficiency develops due to the body's ability to build a folic acid reservoir. If a deficiency occurs, it can lead to severe health problems, especially for a developing fetus in pregnant women[28, 29]. If a pregnant woman is folic acid deficient during her first trimester of pregnancy it can lead to a high likelihood of neural tube defects in the fetus.

Folic acid has a dual affect on cancer, thus the benefits of folic acid are contingent on who is taking it and when it is taken. Some studies have shown folic acid may be associated with a lower risk of some cancers such as esophageal, stomach, and ovarian cancers[29, 33]. On the other hand, some studies have demonstrated potential harmful affects that folic acid may have on an individual with a predisposition to cancer or to someone already suffering from cancer. This is likely related to the role folic acid plays in the synthesis of new cells, thus folic acid may essentially be feeding the growth of cancer cells [18, 34-45]. Low dietary folic acid may protect against the formation of preneoplastic lesions, and high folic acid may have the ability to promote carcinogenesis. The mechanism and role dietary folic acid holds in cancer development is incompletely understood, consequently, recommendations given to the public need to be considered carefully.

## Mouse Model of Histiocytic Sarcoma

### *SLEEPING BEAUTY* TRANSPOSON SYSTEM

The *Sleeping Beauty* (SB) transposon system has been used in the Largaespada laboratory to create a novel, unbiased method for cancer gene discovery. This system has been shown to work for many types of malignancies and be an effective tool to observe new phenotypes and understand the function of genes [11, 46]. This method relies on the use of transgenic mice in which the components of the SB transposable element, a member of the large Tc1/mariner family of transposons, have been introduced[46, 47]. SB transposase can be used to mobilize SB DNA transposon vectors, thus resulting in introduction of a specific sequence of DNA into the chromosomes of vertebrate cells/animals to introduce new traits via transgenesis or insertional mutagenesis[46, 47]. For cancer studies, an SB transposon vector, called T2/Onc, is designed to induce loss- or gain-of-function mutations in oncogenes or tumor suppressor genes, respectively, upon insertion into or near endogenous genes. In this way, the T2/Onc vector is "insertionally oncogenic" in mice[11, 46, 48-51]. A Cre/LoxP-regulated *Rosa26* "knockin" allele allows for tissue-specific expression of a highly active SB transposase gene, called SB11, when combined with tissue-specific Cre transgenes[46]. This, this allows for tissue-specific mutagenesis to be achieved. In many models, a cancer predisposed genetic background is also utilized in order to increase tumor penetrance and decrease latency. The models that have published to date show that SB can be used to model T cell leukemia, fibrosarcoma, hepatocellular carcinoma, gastro-intestinal tract adenocarcinomas, medulloblastoma, pancreatic cancer and melanoma. [11, 46, 48-50, 52]. Moreover, an analysis of the T2/Onc transposon insertion sites from these tumors revealed recurrently mutated loci, called common insertion sites (CIS), harboring genes,

many of which are also altered in the corresponding human version of the tumor[46]. This is a powerful system that can be used to study the genetics of cancer resulting in the identification of new cancer genes and genetic pathways.

### HISTIOCYTIC SARCOMA: THE HUMAN AND CANINE DISEASE

Histiocytic sarcoma (HS) in the dog, which includes both localized and disseminated histiocytic sarcoma, is a malignant disease most frequently observed in middle-aged Bernese mountain dogs (often with a familial association), Rottweilers, Flatcoated Retrievers, and Golden Retrievers [53]. For HS, there are no screening methods available and no effective treatments making HS extremely difficult to manage clinically. It is necessary to establish explicit definitions of HS that can only be done with better knowledge of the genetic etiology of the disease. This is especially true for Bernese Mountain dogs who are believed to carry a polygenic trait that predisposes a quarter of the population to this horrendous disease[53-55]. If no treatment is given, dogs with HS have an average lifespan, after diagnosis, of an estimated 49 days with a peak incidence of disease at 2-3 years of age. Even with aggressive systemic chemotherapy, HS is a rapidly progressive disease[53].

HS in humans is a rare, highly malignant disease derived from hematopoietic neoplasms, representing less than 1% of all non-Hodgkin's lymphomas [56, 57]. Primary lesions of HS appear in the spleen, lymph nodes, lung, bone marrow, skin and subcutaneous tissue. The phenotypic and biological definition is incomplete due to the rarity of HS. Since the genetic etiology of HS is largely unknown, HS is difficult to manage clinically and there is no standard recommended therapy for patients with HS.

There is no available statistical information compiled about the prevalence of HS with most of the literature published being case report studies. This is partly due to the rarity and partly due to the misdiagnosis of the disease. Before the immunohistochemistry of HS was understood, the diagnosis of HS was more common. It is now recognized that most cases of HS described in the past were, in fact, B-cell lymphoma or peripheral T-cell lymphoma [58]. The diagnosis of HS relies predominantly on the verification of histiocytic lineage and the exclusion of other malignancies such as lymphoma, carcinoma, and melanoma through extensive immunohistochemistry [57].

## The Cancer Genome, Model Systems and Progress in Cancer Control

There has been an extraordinary amount of progress made in the field of cancer genomics in the past two decades since the launch of large efforts such as the availability of The Cancer Genome Atlas and initiation of the Human Genome Project and the assembly of the International Cancer Genome Consortium, just to name a few. Oncogenomics as a field has also advanced in large part because of the improvement in research technologies such as high-throughput sequencing, array comparative genome hybridization, microarray analysis, and bioinformatics and functional analysis techniques[1]. With the evolution of the field, more scientific observation has resulted in more questions and has highlighted mechanistic concepts that were not initially considered to influence cancer development. Despite this ever expanding data explosion, an ongoing challenge of analyzing the data is being able to distinguish between genetic alterations that directly impact cancer initiation and alterations that do not play a causal role in disease development. The efforts that have been successful in addressing this issue have resulted in advanced, effective, targeted therapies for treating colorectal cancer, lung

cancer, breast cancer, and leukemia.

The challenge of the complexity of the cancer genome highlights the need for better models to accurately recapitulate molecular and phenotypic subtypes of cancer and tumor development. This can be achieved using genetically modified/engineered mouse models, due to the high degree of similarity between the mouse and human genomes. These models will not only allow true drivers, and linked cancer phenotypes, to be identified, but also serve as useful models for prevention and therapeutic testing. These studies will ultimately allow us to recognize clinically pertinent targets to treat human cancer. The recent successes made in the field illustrate how important it is to use all of the tools available to better understand cancer so we may treat and prevent it effectively.

**Statement of thesis**: I hypothesize that a mouse model of Nevoid Basal Cell Carcinoma Syndrome will show that perinatal dietary folate levels influence the likelihood of medulloblastoma formation and that low levels will increase risk. Further, I hypothesize mobilization of a Sleeping Beauty transposon vector in developing myeloid lineage cells in mice will cause histiocytic sarcoma and reveal recurrent genes and pathways that can drive this disease.

# Chapter 2) Maternal Dietary Folic Acid Deficiency Protects Against Medulloblastoma Formation in a Mouse Model of Nevoid Basal Cell Carcinoma Syndrome

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Nevoid basal cell carcinoma syndrome (NBCCS) is a hereditary condition caused by mutations in the *PTCH1* gene that result in a wide variety of neoplasms. Approximately 5–10% of NBCCS patients develop medulloblastoma (MB), the most common malignant childhood brain tumor in the United States. Epidemiological studies have found an inverse association between maternal intake of prenatal vitamins, which contain folic acid (FA), and childhood brain tumors in offspring. We hypothesized that low maternal intake of FA during the peri-gestational period would increase MB incidence in a transgenic mouse model of NBCCS that carries an autosomal dominant mutation in the *Ptch1* gene. A total of 126 female wild-type C57BL/6 mice were randomized to one of three diets containing 0.3 mg/kg (low), 2.0 mg/kg (control), or 8.0 mg/kg (high) of FA. Female mice were placed on the diet one month prior to mating with $Ptch1^{+/-}$ C57BL/6 males and maintained on the diet until weaning of their pups that were then aged for tumor development. Compared to the control FA group, the incidence of MB was significantly lower in low FA group offspring (Hazard Ratio=0.47; 95% confidence interval=0.27-0.80) at one year. There was no significant difference in offspring MB incidence between the control and high FA groups. Our results suggest that low maternal dietary FA levels during the peri-gestational period may decrease tumor incidence in mice genetically predisposed to tumor development. These findings may have implications for prenatal dietary FA intake recommendations for mothers in the presence of cancer syndromes.

## Introduction

Medulloblastoma (MB) is a cerebellar tumor representing the most common central nervous system (CNS) tumor in children aged 0–4 years [59, 60]. The peak incidence occurs at 6 years of age with a 1.7 fold higher incidence in males than females [54, 59]. Approximately 66% of children survive five years from diagnosis [18, 54] and many long-term survivors face adverse late effects as a result of the disease and its treatment [20-22]. These data underscore the need to understand the biology of MB and identify preventable causes.

Established risk factors for MB include prenatal radiation exposure (which accounts for very few cases currently) and certain genetic syndromes including Li-Fraumeni, tuberous sclerosis, and nevoid basal cell carcinoma syndrome (NBCCS) [61]. NBCCS, also known as Gorlin Syndrome, is an autosomal dominant disorder with near complete penetrance and extremely variable phenotype caused by a *PTCH1* mutation [24, 62]. NBCCS is estimated to affect one in 55,600 persons with symptom onset starting as early as age two years and a median onset of age 25 years [24, 62]. NBCCS is characterized by the presence of a variety of developmental anomalies and predisposition to several cancers especially basal cell carcinomas, rhabdomyosarcoma, and MB [63]. The risk of an NBCCS patient developing MB in the first years of life is 5-10% [24, 63].

Other risk factors for MB include high birth weight (increased risk), and prenatal vitamin supplementation (decreased risk), suggesting that factors related to maternal nutrition influence MB development in the child [64, 65]. In particular, since folic acid (FA) reduces the risk of neural tube defects [66], there is speculation that FA may be the

mediating nutrient in prenatal vitamins accounting for the protective effect observed for MB [36, 37].

Two main mechanisms have been proposed to explain how dietary FA could impact cancer risk. The first is through its role as a cofactor in the synthesis of purines and thymidylate that are necessary for nucleic acid synthesis. Deficiency in these molecules could increase cancer risk through higher rates of uracil incorporation into DNA resulting in breakage [34] that could subsequently lead to translocations and other chromosomal abnormalities [67]. The second mechanism is through its role as a necessary factor in the synthesis of S-adenosylmethionine (SAM), a donor of methyl groups for DNA methylation reactions [68, 69]. DNA methylation has been linked to gene expression levels [70, 71] and has been shown to be disturbed in nearly all tumors [72]. Furthermore, aberrant methylation of the imprinted gene insulin-like growth factor-2 (IGF-2) has been linked to an increased risk of childhood embryonal tumors and colon cancer in adults [72].

To clarify whether FA exposure during pregnancy can influence MB incidence, we modeled the association between maternal dietary FA intake and MB development in offspring using a mouse model of MB with intermediate penetrance. We used mice that were heterozygous for the *Ptch1* mutation [26]. Analogous to humans with NBCCS, mice with a mutant *Ptch1* allele have an increased risk for MB, rhabdomyosarcoma, and basal cell carcinoma. We found that low maternal dietary FA intake decreased MB incidence in offspring, suggesting that nascent tumors developing after loss of *Ptch1* function are highly sensitive to folate levels. Our results also imply that higher maternal dietary FA level may influence tumor development in genetically susceptible populations such as

NBCCS, and that optimal maternal dietary FA levels for people with cancer syndromes may differ from those of the general public.

## Materials and Methods

**ANIMALS AND DIETS.** The University of Minnesota Institutional Animal Care and Use Committee approved all protocols for this study. All mice were housed in plastic cages layered with shavings in a temperature and humidity-controlled room with 12-hour day/night cycles and had *ad libitum* access to food and water throughout the experiment. Our breeding colony was established with 3 heterozygous *Ptch1*$^{+/-}$ male mice obtained from Dr. James Waschecck that had been crossed onto the C57BL/6 background for at least six generations [26]. *Ptch1*$^{+/-}$ mice were bred for a minimum of three additional generations prior to being assigned to the experiment to ensure genetic homogeneity of experimental mice. Six to eight week old wild-type *(Ptch1*$^{+/+}$) female C57BL/6 female mice were purchased from Charles River Laboratory (Wilmington, MA) and assigned randomly to one of three amino acid defined diets that varied with respect to the FA content (described below). The female mice were fed the diets for one month prior to crossing them with *Ptch1*$^{+/-}$ male mice from the breeding colony. The dams were maintained on the diets until weaning at ~3 weeks at which time the offspring were removed from their mothers and placed on normal chow (Teklad Lab Animal Diets, Harlan Laboratories, Madison, WI) for the experiment's duration. Offspring were followed for up to twelve months for tumor development (Figure 1). Mice were monitored $\geq$ 3 times a week for signs of morbidity and neurodegenerative problems such as loss of balance, reduced motor function, and other behavioral abnormalities that are

indicative of a cerebellum tumor in this mouse model (www.jax.org) [16]. If any of these signs were present, the mouse was euthanized by $CO_2$ asphyxiation followed by necropsy. The age of death was defined as the number of days between birth and euthanization.

Amino acid defined diets (TD.070056-TD.070058) were obtained from Harlan Laboratories (Madison, WI). The diets contained 0.3 (low), 2.0 (control), or 8.0 (high) mg/kg FA and 1% succinylsulfathiazole to prevent the synthesis of folate by intestinal bacteria [73]. Diets had identical formulation with the exception of FA and contained 15.4% protein, 63.9% carbohydrate (CHO), and 8% fat by weight. The low and control FA dosages where chosen based on the minimum dosage that has been shown to be compatible with a successful pregnancy outcome by other investigators, on recommendations of the National Research Council [74], and on results from our preliminary studies that showed dose dependent increases in RBC folate in association with dietary FA in both the dams and their offspring (data not shown).

**GENOTYPING.** Tails were clipped from pups at ~2 weeks of age and genomic DNA (gDNA) was purified using the DNeasy Blood & Tissue Kit (cat. # 69504, information available at http://www.qiagen.com/products/genomicdnastabilizationpurification/dneasytissuesystem/dneasybloodtissuekit.aspx) according to the manufacturer's instructions. Genotyping of animals for the *Ptch1* mutant allele was conducted with minor modifications using the Jackson Laboratory (Bar Harbor, ME) standard protocol available at http://jaxmice.jax.org/strain/003081.html. Briefly, PCR reactions containing 1 ml gDNA, 1X PCR Buffer (Carlsbad, CA), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 20 mM of each primer

below, 1 unit of Taq polymerase were carried out in an Eppendorf Mastercycler Gradient thermocycler at 94ºC for 1.5 minutes, 94ºC for 30 seconds, 69ºC for 1 minute, 72ºC for 45 seconds for 35 cycles, followed by 72ºC for 2 minutes. The PCR products were resolved on 1.5% agarose gels with an Invitrogen TrackIt 50 bp DNA ladder (cat # 10488-043, Carlsbad, CA) by running them at 100V for 1 hour. The primer sequences were as follows: *Ptch* 1 Forward (5'-GCCCTGAATGAACTGCAGGACG-3'); *Ptch* 2 Reverse (5'-CACGGGTAGCCAACGCTATGTC-3'); WT 1 Forward (5'-CTGCGGCAACTTTTTGGTTG-3'); and WT 2 Reverse (5'-AGGGCTTCTCGTTGGCTACAAG-3'). In mice that are heterozygous for the *Ptch1* mutant allele, two amplicons of 479 and 200 base pair fragments are observed that are indicative of the mutant and wild-type alleles respectively.

**RED BLOOD CELL FOLATE MEASUREMENT.** Samples of 25-50 μl of peripheral blood were obtained from the dams using glass capillary tubes coated with a non-coagulant, EDTA, through retro-orbital bleeding in accordance with NIH guidelines (http://oacu.od.nih.gov/ARAC/Bleeding.pdf) at weaning of their pups. Diluted aliquots (1:11) of the dam's blood in 1% ascorbic acid were frozen at -80ºC. RBC folate was measured using a modified version of the lactobacillus assay [75-77] as described briefly in supplementary methods.

**HISTOLOGY.** Mice were necropsied when they became moribund or at one year of age, whichever came first. Brains were removed and visually inspected for cerebellar tumors and/or hydrocephalus and then later confirmed by histopathology. Two separate board-

certified pathologists (American Board of Pathology) confirmed all tumors. If the pathologist disagreed, deeper sections of the tissues were made into slides and the pathologists re-examined the sample in question together to reach a diagnosis. Brain tumor samples, spleens and any abnormal tissues were archived by fixation in 10% buffered formalin and snap frozen in liquid nitrogen. Formalin fixed-paraffin embedded sections from brain tissues were sectioned at 5 microns using a standard microtome, mounted and heat-fixed onto glass slides. Slides were processed and stained with hematoxylin-eosin (HE) using standard protocols. The pathologist used the criteria of densely packed small, anaplastic cells with scant cytoplasm, and polygonal nuclei to verify that the tumor histology resembles that of human MBs in appearance.

**STATISTICAL ANALYSIS.** All statistical analyses were performed using SAS version 9.2 (Cary, NC). Significant differences in maternal RBC folate levels between dietary FA groups were assessed using one-way ANOVA with the dependent variable as RBC folate level and the independent variable as dietary group (low, control, high). Differences in the frequency of offspring *Ptch1* heterozygosity by maternal dietary group were assessed using chi-square tests. Cox proportional hazards regression analysis was used to model the hazard of tumor development in the offspring by maternal dietary FA group specifying a robust sandwich covariance matrix [78] to control for non-independence of litter mates. The effect of FA levels on overall survival was also examined using Kaplan-Meier analysis using Prism by Graph Pad Software with statistical differences in survival times between the groups calculated by the log rank test controlling for multiple comparisons through the Sidak method [79]. In both Cox proportional hazards regression

and Kaplan-Meier analysis, mice that had a microscopically confirmed cerebellar tumor were classified as events, while those who had no gross signs of tumor were classified as non-events. Survival time was defined as the interval from birth to tumor development, death from other causes, or one year of age, whichever came first. Differences in median survival time were assessed using the log rank test. Two tailed p-values of $\leq 0.05$ were considered statistically significant.

## Results

A total of 126 female mice were assigned to low, control, and high FA diets (42 mice/group) (Figure 1). There were no significant differences in the proportion of females with litters ($p=0.9$), litter size ($p=0.5$) or the percentage of heterozygote offspring ($p=0.95$) between the three diet groups (Table 1). Higher maternal dietary FA intake was significantly associated with RBC folate level at weaning ($p<0.001$) in a sample of dams assigned to each of the three dietary FA levels (low n=15; control n=21, high n=17) (Figure 2). RBC folate levels in the offspring also correlated with maternal dietary FA intake (Supplemental Figure 1). One hundred fifty sixty heterozygote offspring (low: n=53, control: n=50, high: n=53) that survived to weaning were observed for ~one year for MB development.

The overall MB incidence was 28% for the low FA diet, 50% for the normal diet and 45% for the high diet. The mean latency of tumor development varied significantly between groups with means of 28.5, 19.0, and 26.3 for the 0.3, 2.0, and 8.0 groups, respectively (ANOVA p-value 0.009) (Table 2). Cox proportional hazards regression results indicated a significantly reduced hazard for tumor development in the offspring of

mothers who were fed the low FA diet compared to those fed the control FA diet (HR=0.47, 95% CI 0.27-0.80). The offspring of mothers fed the high FA diet had a non-significant reduction in tumor incidence compared to control mothers (HR=0.73, 95% CI 0.43-1.23) (Table 3).

A Kaplan-Meier survival analysis indicated a more rapid rate of mortality for offspring of mothers assigned to the control FA group than those assigned to the low or high FA group. The overall difference in the survival groups was not statistically significant. The overall difference in survival between the low and control groups was also not significant with a log rank p value of 0.2325 (Figure 3A). The difference in survival of mice with MB at death was significant between the low and control groups with a log rank p value of 0.0031 (Figure 3B). At necropsy, a total of 64 cerebellar tumors were grossly visible and subsequently confirmed microscopically by two pathologists. The tumors collected at necropsy were found to recapitulate the classic subtype of MB. Neoplastic cells infiltrated a regionally extensive portion of the meninges and the molecular layer. These cells were polygonal to stellate, had multiple nucleoli, clumped chromatin, and displayed moderate anisocytosis /anisokaryosis. There was also mild dilation of lateral ventricles (Figure 4).

A portion of the offspring that became moribund did not have macroscopic tumors, even though they displayed signs similar to mice with macroscopic MB (enlarged head, ataxia). Upon histological analysis, these mice had intracranial signs indicative of hydrocephalus and/or observed structural defects in the ventricles leading to potential obstruction of the normal flow of cerebrospinal fluid [80]. The absence of cerebellar neoplastic lesions was subsequently confirmed microscopically. Among the total number

of mice without detectable MB (n=92), the frequency of presumed hydrocephalus varied non-significantly by maternal FA group with frequencies of 10%, (4/38), 4.0% (1/25) and 13.8% (4/29) in the low, control, and high FA groups respectively.

## Discussion

We examined whether maternal dietary FA level from conception through weaning affected MB tumor incidence in a genetically engineered mouse model that is strongly predisposed to MB development through a germline mutation in the *Ptch1* gene. Surprisingly, we observed a significantly lower incidence of MB at one year in the offspring of dams assigned to the low vs. control dietary FA. The *Ptch1* heterozygote percentage was similar between dietary FA groups and consistent with previous studies reporting weanling heterozygote frequency of ~40%, indicating that maternal dietary FA had no detectable influence on heterozygote survival [25]. *Ptch1*$^{+/-}$ mice are predisposed to structural defects in their ventricles, which can lead to the obstruction of proper flow of the cerebrospinal and thus lead to hydrocephalus [80]. This defect was macroscopically observed at necropsy in mice belonging to all FA diet groups. The developmental abnormalities observed in the ventricles *Ptch1*$^{+/-}$ brains could explain why we did not observe the same trend in overall survival between the diet groups as we observed in overall tumor burden.

Our results indicate that low dietary maternal FA decreases MB incidence in the *Ptch1*$^{+/-}$ mouse model; MB incidence in the offspring of mothers fed low dietary FA was >17% lower than in those fed the two higher dietary FA doses. However, we did not observe a dose response relationship between maternal dietary FA and offspring MB

incidence. There are at least two possible explanations for a lack of dose response. First, there could be a non-linear association between maternal dietary FA and offspring tumor development. Such a nonlinear effect of maternal dietary FA on offspring colon adenoma has been previously reported in the *Apc*$^{min/+}$ mouse model [44]. A significantly lower adenoma incidence was observed in the offspring of dams fed a FA deficient diet (0.3 mg/kg) compared to those of mothers who were fed the control FA diet (2 mg/kg) or high FA (20 mg/kg) at 10 weeks. Also similar to our results, no significant difference in tumor incidence was observed between the 2 and 20 mg/kg groups. The authors hypothesized that their results could be explained by a dual role of FA as both an initiator and a promoter of carcinogenesis suggesting that "any factor that limits folate availability to the intestine of the developing offspring" could impede tumor development in mice that have a strong genetic predisposition to early adenoma development. By analogy, the C57BL/6 *Ptch1*$^{+/-}$ mouse model has a high MB incidence ranging from 25-50% [81] with palpable tumors developing as early as 3 weeks of age. If FA is necessary for progression of preneoplastic lesions, then pre-weaning FA deficiency could impede tumor growth and result in a lower offspring tumor incidence. Alternatively, at high maternal dietary FA levels, FA may prevent tumor initiation. Our results provide evidence for this pattern.

A second explanation may be that it is difficult to model a high intake level of FA in mice. FA, also known as vitamin $B_9$, is water-soluble and is not biologically active until it is converted to tetrahydrofolate by dihydrofolate reductase in the liver [30]. Evidence indicates that this process may be slow in humans, making the risk for toxicity of FA very low since unprocessed vitamin is regularly removed from the body through urine [30]. There have been no adverse effects observed when 5.5 times the

recommended daily dose of FA for non-pregnant women is taken [30, 42]. The only adverse effects of FA are observed through antagonism with medications, disruption of zinc function, and masking of symptoms of anemia when FA is taken at roughly 27 times the recommended daily dose for non-pregnant women [30, 42]. The extremely low rate of conversion of FA suggests that the benefit in high doses will be limited by saturation of dihydrofolate reductase, limiting our ability to test high levels of FA in preclinical or clinical models [30, 42].

MBs are genetically heterogeneous embryonal tumors in which genetic alterations outline unique clinical subsets [82]. The largest clinical subset of MB involves altered function of Hedgehog pathways. Hedgehog signaling plays an important role in the development of neural cells and is altered in roughly 60% of MBs [82]. Mutations to components of the Hedgehog pathway such as *PTCH*, *SUFU*, and *SMO* only account for approximately 25% of MBs, suggesting additional signals influence activation [82]. Maternal dietary FA plays an important role fetal brain development long after the neural tube closes, which has been the focus of FA research for the past several decades [66]. After a period of rapid cell division and growth in which high levels of FA are important for neural tube closure in the first weeks of pregnancy, the level of FA still plays an essential role in the development of major parts of the brain such as the cerebellum, hippocampus, septum, cortex, the ventricular zone and the sub ventricular zone [66]. Maternal FA deficiency can lead to reduced proliferation of progenitor cells and increased apoptosis in regions of the brain [66], providing a mechanism by which low FA could protect against brain tumor development in those with a predisposition to brain tumor development [66] independent of component mutations in Hedgehog signaling.

We propose the following molecular mechanism to explain how low maternal dietary FA could reduce MB incidence. FA deficiency has been shown in both animal models and human studies to increase DNA damage including mutations, chromosomal aberrations, and single strand breaks [40, 45, 83, 84]. This DNA damage could either result in DNA repair and cell survival, or, alternatively, trigger an apoptotic response and cell death through the TP53 pathway [85]. Although our experiment was not designed to directly test this hypothesis, our data suggest that the cellular conditions produced by developmental FA deficiency in a highly predisposed tumor model favor cell death rather than cell survival, which results in suppression of growth of preneoplastic lesions.

Our results differ from human epidemiological studies of sporadic childhood brain tumors and maternal vitamin supplementation. Most childhood brain tumor case-control studies that included subjects diagnosed from the late 1950s-1990s reported protective effects in association with maternal vitamin supplementation with odds ratios ranging from 0.5 to 0.9 [64, 86]. Studies of PNETs, and MB, specifically, have shown similar results [36, 37]. The largest case-control study conducted to date showed a decreased early childhood brain tumor risk including PNETs, MBs, astroglial tumors, and other glial tumors in association with increased prenatal vitamin supplementation [87]. Since most mothers take prenatal supplementation in the form of multivitamins, the effects of individual micronutrients cannot be determined; it is possible a different nutrient protects from childhood brain tumors. One study that examined whether high vs. low FA intake through dietary sources reduced the risk of MB risk failed to show an association [38]. Unfortunately, these studies are hampered by surveys that have moderate accuracy [88] and difficulties with maternal recall of pregnancy diet [89].

Members of our group conducted an analysis of Surveillance, Epidemiology and End Results (SEER) Program data and found that increases in FA intake, as a result of mandatory FA fortification of enriched grains and cereal products between 1996-1998, did not impact MB incidence [90] for children between 0-4 years in the pre vs. post-fortification period (Incidence Rate Ratio= 1.10, 95%CI=0.81-1.49) [90]. These results make it seem unlikely that maternal FA levels play a major role in MB development in sporadic cases in humans. However, to our knowledge, no similar research has been conducted in genetically susceptible populations.

In conclusion, our study provides evidence that low maternal FA during the perigestational period decreases the incidence of MB in a mouse model that is predisposed to MB development. Our results offer significant information on the conceivable importance of altering levels FA during pregnancy, which subsequently raise questions about the use of FA for pregnant women in the presence of cancer syndromes. We caution, however, that these results must be repeated in other mouse models and in human epidemiological studies before any new dietary recommendations can be considered for people with a genetic predisposition to cancer. Other possible experiments include modeling the effect of FA on different genetic subtypes of MB (i.e. *WNT*), exploring the influence of timing of maternal dietary FA intake, as well as investigating other components of multivitamins.

Acknowledgements: The authors would like to thank Dr. Timothy K. Starr for review of this manuscript and assistance with the data analysis.

## Figure Legends

**TABLE 1. REPRODUCTIVE CHARACTERISTICS SIMILAR IN ALL DIET GROUPS.** The mean litter size was similar between groups and shows FA does not influence reproduction.

**TABLE 2. FOLATE DEFICIENT GROUP DEVELOPED THE LOWEST NUMBER OF TUMORS.** The 0.3 mg/kg FA diet group had the lowest number (15/53) of medulloblastomas and oldest average age for tumor detection and morbidity.

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**TABLE 3. COX PROPORTIONAL HAZARDS REGRESSION ANALYSIS** of offspring medulloblastoma development by dietary FA group. The low FA diet group had statistically significantly fewer medulloblastomas than the control.

**FIGURE 1. EXPERIMENTAL DESIGN**. One hundred and twenty six female mice were assigned in equal numbers to one of three maternal dietary FA groups (0.3 mg/kg (low), 2.0 mg/kg (control), or 8 mg/kg FA) one month prior to mating with *Ptch1*$^{+/-}$ heterozygote male mice. Dams were kept on their respective diets until weaning of their litters at three weeks of age. Offspring were genotyped at 2 weeks of age and RBC/plasma folate measurements were performed on blood samples from dams taken at the time of weaning of their offspring. Upon weaning offspring were switched to the standard mouse chow and followed for tumor development for 12 months.

**FIGURE 2. MEAN RBC FOLATE MEASUREMENTS IN DAMS AT WEANING.** RBC folate was measured using a modified lactobacillus assay [76]in dams at the weaning of their pups. RBC folate increased significantly with increasing dietary FA dose ($p<0.0001$).

**FIGURE 3. KAPLAN-MEIER SURVIVAL CURVES. A. OVERALL SURVIVAL COMPARING PTCH1+/- OFFSPRING OF MOTHERS RANDOMIZED TO THREE DIFFERENT DOSES OF FOLIC ACID PRIOR TO CONCEPTION THROUGH WEANING.** The difference in overall survival between offspring of different diet groups is not statistically significant. **B. Survival of mice with MB at death.** When mice with no evidence of MB at death are censored there is a significant difference in survival between the low and control groups. Log rank statistic =0.0031.

**FIGURE 4. PATHOLOGIC ANALYSES OF TUMOR TISSUE.** Photomicrographs of hematoxylin and eosin stained representative tissue showing *Ptch1*$^{+/-}$ brain sectioned in half with MB. The images were captured using the 4X and 20X objectives showing a Classic subtype of MB.

**Tables and Figures**

**Table 1:** Reproductive characteristics.

| Diet (mg/kg) | Number of assigned dams | Number of litters (%) | Number of weanling offspring | Mean litter size (SD) | Number of Ptch1$^{+/-}$ offspring (%) |
|---|---|---|---|---|---|
| 0.3 (low) | 42 | 23 (55) | 133 | 6.0 (1.6) | 53 (40) |
| 2.0 (control) | 42 | 23 (55) | 129 | 5.7 (2.0) | 52 (40) |
| 8.0 (high) | 42 | 21 (50) | 132 | 6.3 (1.7) | 55 (42) |
| Total | 126 | 67 (53) | 394 | 6.0 (1.8) | 160 (41) |

**Table 2:** Tumor characteristics

| Folic Acid Diet (mg/kg) | Number of Ptch1$^{+/-}$ offspring | % MB (N) | Median age | Mean Age (std. dev.) |
|---|---|---|---|---|
| 0.3 | 53 | 28 (15) | 29.7 | 28.5 (11.4) |
| 2.0 | 52 | 50 (25) | 16.4 | 19.0 (8.3) |
| 8.0 | 55 | 45 (24) | 27.3 | 26.1 (10.9) |
| Total | 160 | 41 (64) | 21.4 | 23.9 (10.7) |

**Table 3. Cox proportional hazards regression models for the association between maternal FA diet and MB incidence.**

| Diet (mg/kg) | Hazard Ratio | 95% CI | p-value[a] |
|---|---|---|---|
| Low (0.3) | 0.47 | 0.27-0.80 | 0.006 |
| Control (2.0) | 1.0 | Ref. | - |
| High (8.0) | 0.73 | 0.43-1.23 | 0.24 |

[a]p-value for statistical difference between test group and control group
Likelihood ratio test p-value 0.06

**Figure 1.**

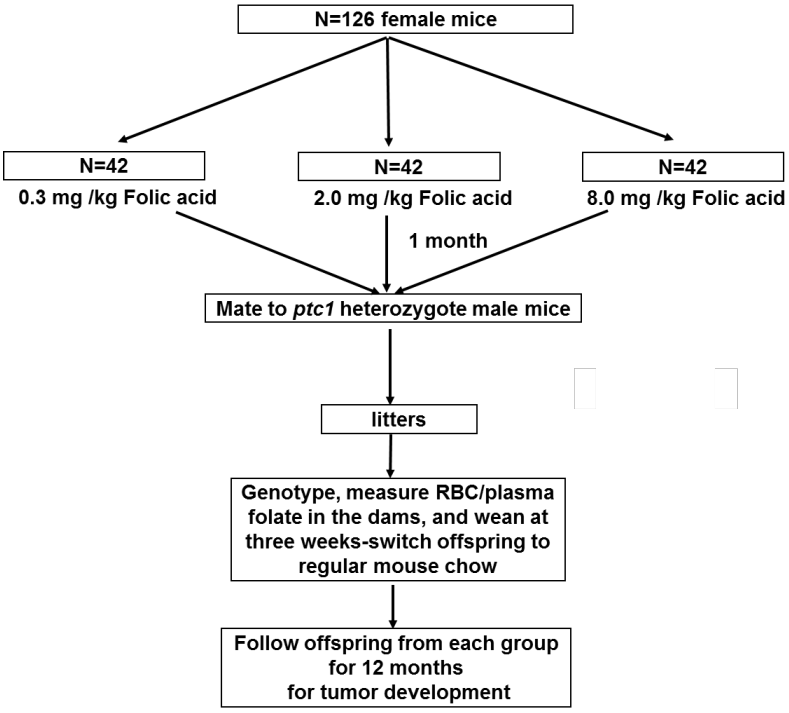



**Figure 2.**

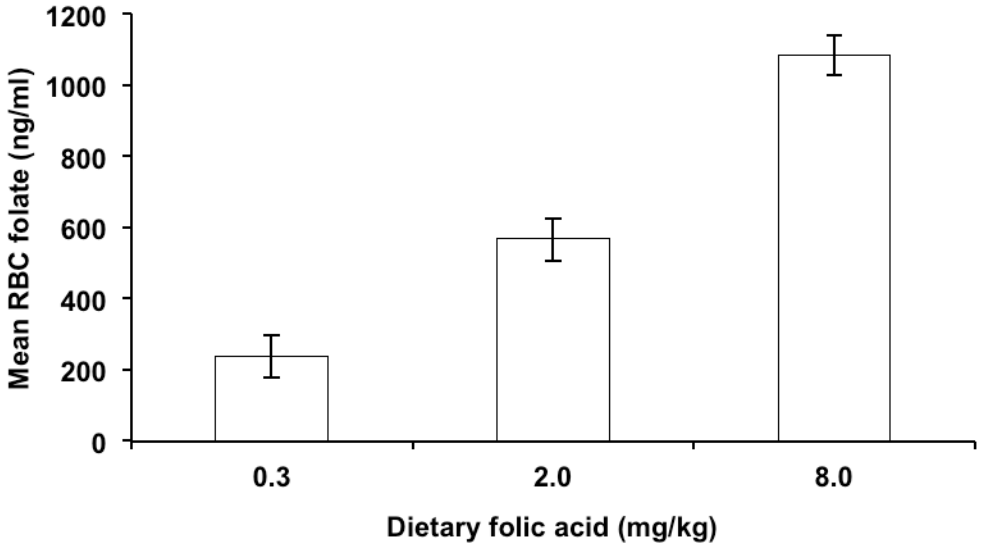
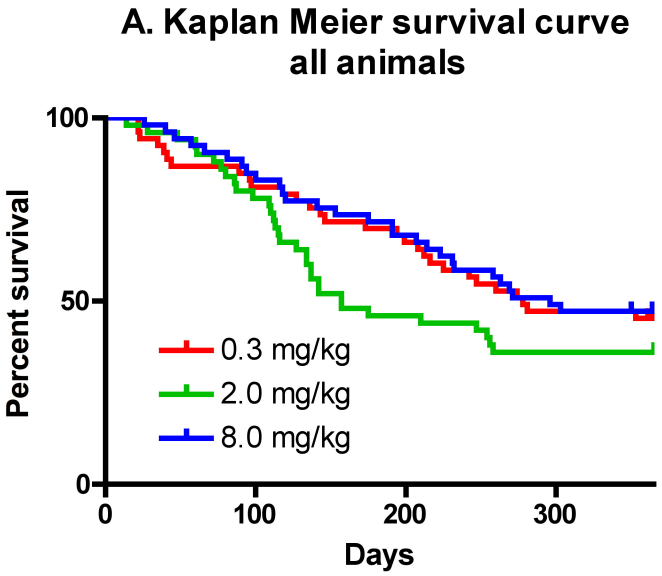



**Figure 3.**


A. Kaplan Meier survival curve
all animals
Percent survival
100
50
0
0
100
200
300
Days
0.3 mg/kg
2.0 mg/kg
8.0 mg/kg

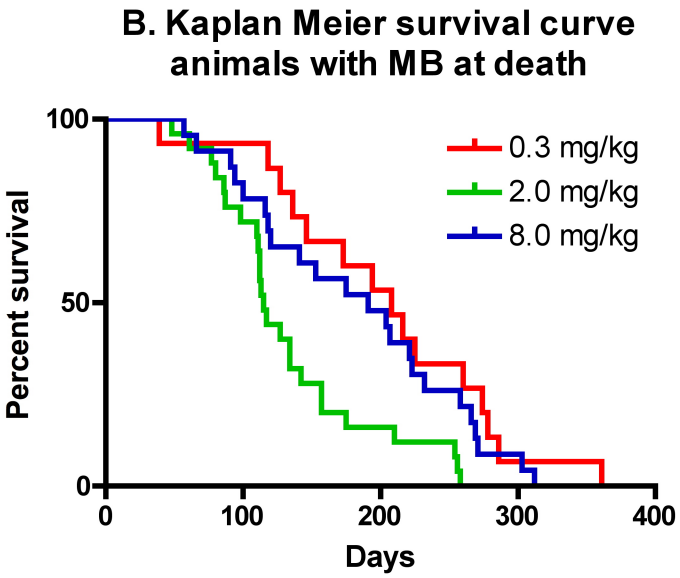
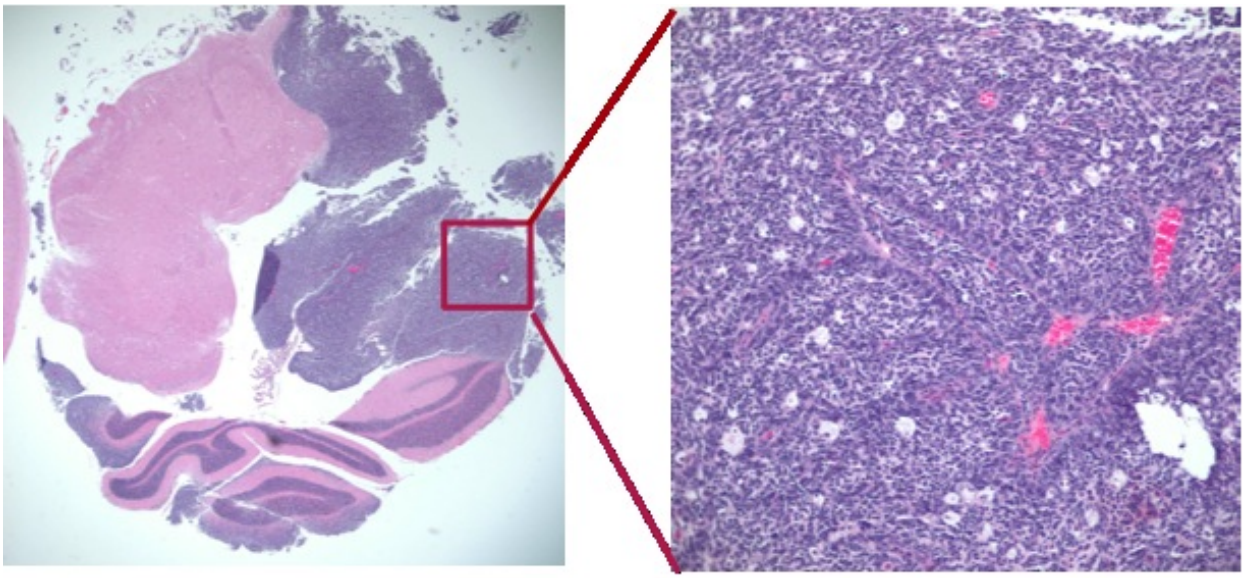

B. Kaplan Meier survival curve
animals with MB at death
Percent survival
100
50
0
0
100
200
300
400
Days
0.3 mg/kg
2.0 mg/kg
8.0 mg/kg

**Figure 4**.



## Supplemental Methods

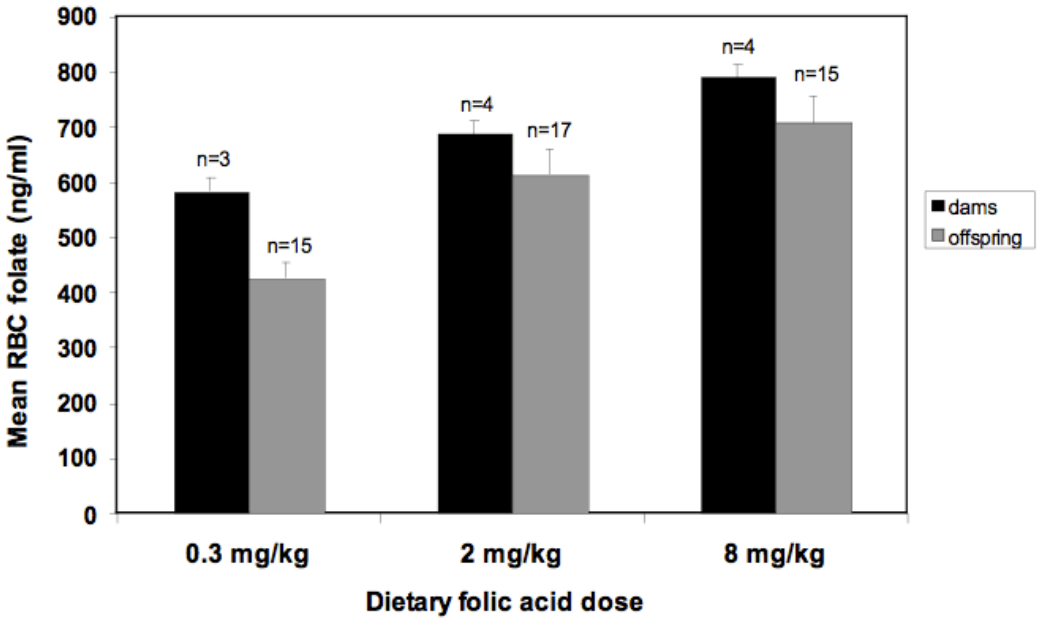
**Red blood cell folate measurement.** All reagents and supplies are as described previously [75]. Briefly, L. casei bacteria (obtained from ATCC; NCIB strain 10463, catalog number 27773 ) was grown from a lyophilized pellet according to the methods of Molloy et al [76]and aliquoted into tubes that were frozen at -80 degrees C until assayed. Upon thawing, additional dilutions were made at 1:140 or 1:70 in 0.5% sodium ascorbate. The assay was performed in 96-well microtiter plates with 200 ml of Folic Acid Casei assay medium (Catalog number 282210, Becton Dickinson, Maryland, USA) per well. For the assay calibrator plate, 5-methyltetrahydrafolic acid (5-MeTHF) (catalog number M1032-5, Sigma-Aldrich, Missouri, USA) was used as the folate standard and eight replicates at each of the eleven standard concentrations were plated from 0 to 1 nmol/L. Sodium ascorbate at 0.5% was used to bring wells up to a total volume of 300ul. For sample plates, 200 ml FA Casei assay medium was plated per well. Four well replicates per sample were done adding 50 ml of diluted sample to two wells and 100 ml to the other two wells. For RBC samples that were diluted 1:140 this made for a total sample dilution of 3080 for 50 ml samples or 1540 for 100 ml sample wells. If the RBC sample was diluted 1:70, the dilutions were 1540 and 770 respectively. An additional two wells were done for RBC folate to account for the color contribution from the blood. To these two wells, sodium azide was added at a concentration of 3 g/dL to eliminate bacterial growth. For 50 ml sample wells, 50 ml of 0.5% sodium ascorbate was added to make up to the 300 ml total volume. Lab personnel quality control samples and blank wells were also included on each plate. Plates were sealed with adhesive covers and inverted multiple times to mix thoroughly. Plates were then incubated 42-45 hours at 37 degrees

C followed by room temperature equilibration at which time they were inverted multiple times to mix followed by OD measurement on a VersaMax microplate reader (Molecular Devices, California, USA) at 590 nm. Results were exported to Microsoft Excel (v. 2003) and analyzed in nmol/L and adjusted for hematocrit level (a measure of the quantity of RBC in the blood sample) that was measured as described below.

Hematocrit was measured within 1 hour of sample collection in accordance with Hemavet recommended instructions (Drew Scientific, Inc., Waterbury, CT). A Hemavet automated cell counter was used to obtain hematocrit level in peripheral blood cells needed to calculate the concentration of RBC folate from dams. Accuracy was achieved by using whole blood calibration of instruments using manufacture recommended controls and procedures. The hematocrit is considered to be functioning properly if at least 95% results obtained with controls are within manufacturer's listed acceptable ranges. The RBC folate concentration was calculated in ng/ml according to the following formula: (RBC folate nmol/L/2.26)/(%Hematocrit /100) where 2.26 is the conversion factor for folate from SI to conventional units (http://www.unc.edu/~rowlett/units/scales/clinical_data.html ).

## Supplemental Data

**Supplemental Figure 1**. Dose dependent increases in RBC folate in association with dietary folic acid in dams and offspring.



## Chapter 3) Genetic Signature of Histiocytic Sarcoma Revealed by an Unbiased Genetic Screen in Mice

Histiocytic Sarcomas (HS) are rare neoplasms where the definitive cell of origin, genetic etiology, and critical drug targets for HS therapy are unknown. We conducted a forward genetic screen in mice that can identify mutations capable of causing cancer initiation and progression. The forward genetic screen uses the *Sleeping Beauty* (SB) DNA transposon as a random somatic mutagen, capable of both activating proto-oncogenes and inactivating tumor suppressor genes. Tumors generated in these mice are analyzed for recurrent transposon common insertion sites (CISs) using ligation-mediated PCR in combination with high-throughput sequencing. We hypothesized that SB-generated mutations in myeloid lineage cells would lead to myeloid leukemia or histiocytic sarcoma. We discovered that activation of SB mutation in cells expressing *LysozymeM* (*LyzM*) caused HS. Triple transgenic mice became moribund much faster than controls (Figure 2). Histopathologic analysis of tumor tissue from these mice indicates 30% of the experimental mice developed HS. We mapped 1,177 non-redundant insertions and identified 23 CISs. The candidate HS genes within these CISs included known cancer genes as well as novel candidate genes. Our top three candidate genes are *Raf1*, *Fli1*, and *Mitf*. *Raf1* and *Fli1* are known oncogenes associated with hematological neoplasms, and *Mitf* is a transcription factor associated with melanoma. Our list also includes known tumor suppressors *Trp53* and *Nf1*. Based on our study we conclude that: 1) A mature myeloid lineage cell can be the cell of origin for HS; 2) ETS and basic helix-

loop-helix (hHLH)-leucine zipper transcription factor networks can induce HS with Raf-activated pathways; and 3) RAF-MEK-ERK signaling is a possible target for HS treatment.

## Introduction

HS is a disease that affects both humans and dogs with a similar presentation [55, 56]. In humans, HS represents less than 1% of all non-Hodgkin's lymphomas [56, 57]. HS, although rare in humans, is very common in certain dog breeds, including Bernese mountain dogs, Rottweilers, golden retrievers, Labrador retrievers, flat-coated retrievers and occurs sporadically in other breeds. HS in dogs is very aggressive with a reported overall median survival time of 49-106 days after diagnosis [55]. The high prevalence of rapid disease progression means that many new HS treatments could be attempted in canine patients to advance knowledge of the human disease. Testing therapeutics in canine patients will markedly improve the chances that a useful therapy will be tested in human patients.

Primary lesions of HS appear in the spleen, liver, lymph nodes, lung, bone marrow, skin and hypodermis. The phenotypic and biological definition of HS is incomplete. HS can occur in isolation or in the context of other hematological malignancies to which the HS is sometimes clonally related [58]. Since the genetic etiology of HS is largely unknown, HS is difficult to manage clinically and there is no standard, recommended therapy for patients with HS. There is no available statistical information compiled about the prevalence of HS with most of the literature published in in the form of case report studies. This is partly due to the rarity and partly due to the misdiagnosis of the disease. Many cases of HS described in the past were, in fact, B-cell lymphoma or peripheral T-cell lymphoma [58].

The diagnosis of HS relies predominantly on the verification of histiocytic lineage and the exclusion of other malignancies such as lymphoma, carcinoma, and melanoma through extensive immunohistochemistry [57]. The complicated process of diagnosing HS is confusing for clinicians and drawn out for their patients because the definitive cell of origin, genetic etiology, and critical drug targets for HS therapy are unknown. HS is a horrifying disease where patients have to endure a grueling process of treatment, which is not standardized and is therefore conducted through trial and error, exemplifying the lack of knowledge about the cell of origin and the genetic drivers of HS. This research aims to improve the chances for the survival of canine and human HS patients, by uncovering commonly altered pathways using an unbiased approach. HS currently carries a dismal prognosis. Hopefully, a detailed understanding of the precise mechanisms that allow HS to develop and grow out of control will help in addressing the current dismal prognosis.

Through higher genetic resolution, and advances in understanding the molecular mechanisms of cancer, there has been extraordinary progress in the development of innovative, successful targeted therapies. Targeted therapeutics have been developed for the treatment of solid tumors such as breast cancer, non-small cell lung cancer, and renal cancer [91]. According to the National Institute of Cancer, the 5-year survival rate among all cancers has improved from 50% in 1977 to 68% in 2006. This improvement is largely due to improved chemotherapeutics, earlier diagnosis and targeted therapies. For example, the breast cancer 5-year survival rate improved to a remarkable 90% in 2006 from 75% in 1997[5]. Despite these accomplishments, there is still a significant amount of work to be done to further understand the tremendous genetic complexities of cancer. There are still a number of cancers that have no effective treatment available to patients

due to the lack of knowledge about the definitive cell of origin and genetic etiology. HS is one such example. HS is a highly malignant disease with an apparent origin in developing blood cells.

To help discover novel genes in HS that could be potential drug targets and learn more about the cell of origin, we performed an unbiased forward genetic screen in mice by using technology that was created at the University of Minnesota. The forward genetic screen uses the *Sleeping Beauty* (SB) DNA transposon as a random somatic mutagen, capable of both activating proto-oncogenes and inactivating tumor suppressor genes[46]. The SB transposon can be used to "tag" cancer genes in mouse somatic cells. The SB transposon was created at the University of Minnesota in the laboratory of Dr. Perry Hackett [47]. The DNA transposon uses a cut-and-paste method that relies on a transposase enzyme that can recognize a specific DNA sequence. It cuts the DNA and pastes the DNA into a different site of the genome[46]. SB is an effective tool for mutagenesis. Furthermore, we have created a conditional SB allele that can be activated in a single tissue by crossing to a tissue specific Cre recombinase expressing mouse line. We used a mouse line expressing *Cre recombinase* under control of the *LyzM* promoter to direct SB expression to myeloid cells[92, 93]. In our model SB is expressed in myeloid cells after the Cre recombinase has excised a stop cassette that is located between the constitutive ROSA26 promoter [11, 46, 48-50, 94] and the SB11 transgene (R26-Lox-stop-lox-SB11). This system will effectively confine SB mutagenesis to tissues expressing the Cre recombinase and avoid the extremely penetrant, fast lymphomagenesis that would otherwise happen if the transposase was expressed ubiquitously[94]. SB mediated mutagenesis has the ability to generate multiple mutations

in somatic cells through high rates of transposition [11, 46, 48-50, 94]. As in human cancer, our results often reveal a small number of frequently mutated genes that cooperate with a greater number of rare mutant genes that drive formation of tumors [11, 46, 50]. In this study we found that 30% of experimental mice developed HS and by mapping transposon insertions in these tumors we could identify13 candidate genes that we believe are causative for HS.

## Materials and Methods

### Generation of Transgenic Mice

In order to produce experimental mice we crossed three transgenic lines of mice, a tissue-specific *Lysozyme-Cre (Lyz-Cre)* transgenic line, a transgenic line carrying a concatamer of oncogenic transposons (*T2/Onc)* and a knock-in mouse line where a lox-stop-lox-SB11 vector was knocked into the Rosa26 promoter *(Rosa-26-lsl-SB11*). Two different T2/Onc founder lines were used, one where the concatamer of transposons was located on chromosome 1, and one where the concatamer was located on chromosome 15. *LyzM-Cre* mice (Jackson Laboratory #004781) [92] were bred to both T2/Onc lines(chromosome 15 or 1 concatomers, approximately 25 copies[48], or T2/Onc2 (TG6057, chromosome 4 concatomer, approximately 148 copies, [49]to generate *Lyz-Cre* x T2/Onc double transgenic mice. *R26-LsL-SB11[11, 49, 50]* mice were then bred to *Lyz-Cre* x T2/Onc double transgenic mice to generate mice with and without active transposition (Figure 1). All mice were bred and cared for according to the guidelines provided by the University of Minnesota Institutional Animal Care and Use Committee.

### GENOTYPING AND EXCISION PCR

We isolated tail biopsy DNA using a standard phenol:chloroform extraction method and diluted to a final concentration of 100 ng/μl in TE. PCR primer sequences for each transgene are as follows: LyzM-Cre: primer oIMR3066 5'- CCC AGA AAT GCC AGA TTA CG- 3', primer oIMR3067 5'- CTT GGG CTG CCA GAA TTT CTC-3', primer oIMR3068 5'- TTA CAG TCG GCC AGG CTG AC-3'; T2Onc or T2Onc2: Forward 5'- CGCTTCTCGCTTCTGTTCGC-3', Reverse 5'- CCACCCCCAGCATTCTAGTT-3'; LsL-SB11: Wild-type Forward 5'- GGAGGGGAGTGTTGCAATACCTTT-3'; Wild-type Reverse 5'- AACTCGGGTGAGCATGTCTTTAATCTAC-3'; Transgenic Forward 5'- GGCATTGGGGGTGGTGATATAAACT-3'; and T2Onc Excision PCR was performed as previously described by Collier et. al., 2005 using primer sequences: Forward 5'- GGGATGTGCTGCAAGGCGAT-3'; Reverse 5'- CAAGCTATGCATCCAACGCGTT-3'. PCR products were observed on a 1–1.5% Agarose gel containing 6 μl ethidium bromide (10 mg/ml) per 100 ml of TAE buffer.

### KAPLAN-MEIER ANALYSIS

Overall survival was examined using a Kaplan-Meier curve generated by Prism by Software (Graph Pad). Statistical differences in survival times were determined by the logrank test controlling for multiple comparisons through the Sidak method (Figure 2) [63].

### HISTOPATHOLOGY AND IMMUNOHISTOCHEMISTRY

Mice were necropsied when they became moribund or at two years of age, whichever came first. The lungs, heart, lymph nodes, spleen, pancreas, sternum and all abnormal tissues were removed and visually inspected for macroscopic tumors( Figure

3A-C). All abnormal tissues, tumors, as well as some normal tissues were viewed and classified by a board-certified pathologists (American Board of Pathology). All samples were archived by fixation in 10% phosphate buffered formalin and/or snap frozen in liquid nitrogen. Formalin-fixed samples were paraffin-embedded and were sectioned at 5 microns using a standard microtome, mounted and heat-fixed onto glass slides. Slides were processed and stained with hematoxylin-eosin (HE) using standard protocols (Figure 3D, H-J). Immunohistochemistry was conducted using standard methods with citrate-based antigen retrieval and was conducted by Comparative Pathology (CP) Shared Resource at the University of Minnesota Masonic Cancer Center. The CP core stained HS tumors with Mac-2, for identification of red pulp and white pulp macrophages, F4/80 antibody for increasing expression during monocyte to red pulp macrophage differentiation and PAX5 to rule out that the cells were of B cell lineage (Figure 3E-G).

#### LIGATION MEDIATED PCR AND AMPLICON SEQUENCING

Genomic DNA was isolated from HS tumors using standard proteinase K digestion and phenol-chloroform extraction and ethanol precipitation protocols. We then performed linker-mediated PCR on DNA from these 134 harvested tumors from 42 triple transgenic mice to generate PCR amplicons as previously described in Starr et. al.,2009. Amplicons were subjected to high-throughput sequencing using the Illumina GAIIx platform, which produced 39,793,201 sequence reads.

#### CIS ANALYSIS

Transposon-genomic DNA sequences were loaded into a database to identify and remove barcodes denoting the library of origin. Transposon sequences and linker fragments were trimmed off, leaving genomic sequence. Identical sequences derived

from the same library were condensed to a single sequence entry, retaining the total count observations. Potential genomic sequences were mapped to the mouse genome and common insertions sites (CIS) were determined using a bioinformatics pipeline called TAPDANCE (Figure 4). The pipeline is described in[51, 95]. Insertions were mapped to the genome using BOWTIE. Genomic windows were tested for significance using a Poisson distribution. The Poisson distribution demonstrated the probability of observing a given number of events within a region assuming that these events occur in an unbiased manor. Windows with a p-value $< 0.05$ were considered CISs.

## Results

### SB Insertional Mutagenesis Promotes Histiocytic Sarcoma Formation

The objective of this project was to induce HS in mice using a myeloid lineage-specific Cre recombinase to activate a conditional Sleeping Beauty transposase, which will then mobilize the T2/Onc transposon to induce random mutations. To create random somatic mutations in myeloid lineage cells we generated mice containing three elements: 1) a concatamer of oncogenic transposons, 2) a conditional SB11 transposase that requires Cre recombinase for activation, 3) a Cre recombinase transgene driven by the LyzM promoter (Figure1A). The SB transposon system is capable of generating high rates of transposition in mice [11, 48-50]. The T2/Onc transposon is an integral part of this unbiased system due to its ability to overexpress proto-oncogenes and inactivate tumor suppressor genes. T2/Onc can overexpress a nearby, downstream proto-oncogene through its murine stem cell virus long terminal repeat and splice donor site. T2/Onc inactivates expression of a tumor suppressor gene when it lands in an intron because it contains splice acceptor sites in both DNA strands and a bidirectional poly(A) signal[11,

50]. The Lyz-Cre transgene is expressed in granulocytes, macrophages, and splenic dendritic cells [92, 93], limiting activation of the conditional SB11 transposase allele to these cell types.

In an effort to evaluate tissue-specific SB mutagenesis in myeloid lineage cells, we created a cohort of 190 experimental and control mice (Figure 1B and C). We aged and monitored daily all the experimental and control mice for two years. The mice were necropsied at two years of age or when they became moribund, whichever came first. The triple transgenic mice became moribund at a faster rate than controls, beginning around one year of age (Figure 2). Of the 73 mice with active transposition, 22 of them developed HS (Table 1). In all cases the HS was visible at gross necropsy and infiltrated multiple tissues within the mouse (Figure 3A-C). HS was macroscopically and histologically observed in a wide variety of tissues including mouse spleen, pancreas fat pads, liver, peritoneal cavity, chest cavity (Table 2). We confirmed that 30% of our experimental mice died as a result of HS by combining data from gross observations at necropsy with pathology based on H&E stained tissue and with immunohistochemistry by using markers known to be associated with mouse HS (Figure 3).

Antibodies recognizing PAX5, F4/80, and Mac-2 were used to stain a subset of 17 HS samples. The subset was selected based on macroscopic observations recorded at necropsy. The HS phenotype, in our model, much like human and canine HS, infiltrated a variety of different tissues displaying varying phenotypes between mice. To be sure that all the observed phenotypes were in fact HS, we carefully selected tissues from many animals to conduct IHC on from a different mice with a variety of distinctive phenotypes. The antibody for PAX5 was chosen for its lineage-specific identification of the spectrum

of B cells ranging from bone marrow B cells up to, but not including, plasma cells[96]. This was done to make sure the tumors in the mice were not B-cell lymphoma or B-cell chronic lymphocytic leukemia. All of the samples stained with PAX5, stained negative, confirming that the HS samples were not derived from B cells (Figure 3G). We chose to use F4/80 to identify increasing expression during monocyte to red pulp macrophage differentiation. F4/80 is exclusively expressed by macrophages in the red pulp and is immunoreactive on the cell membrane. The population of round–to oval–shaped histiocytes stained less intensely with F4/80. All of our HS samples stained positive for F4/80 (Figure 3F) [96]. We used the Mac-2 antibody for identification of red pulp and white pulp macrophages (Figure 3E)[96]. Mac–2 was expressed by macrophages in both compartments of macrophages in the red pulp and the white pulp and shows predominantly cytoplasmic staining but some nuclear staining [96]. All of our HS samples stained positive for Mac–2. Through combining the histopathologic data with the IHC, our pathologist confirmed that the tumors were HS.

#### SB-INDUCED HISTIOCYTIC SARCOMA FORMATION IS DUE TO RECURRENT TRANSPOSON ALTERATIONS IN SPECIFIC GENES

To confirm that HS tumors collected were the result of SB mutagenesis, we performed a transposon excision PCR assay. This assay revealed that the T2/Onc and T2Onc2 transposons had mobilization from their initial concatomer locations in tumor samples analyzed (Figure 6A and B). We performed analysis on DNA isolated from 134 tumors to map transposon insertions to the mouse genome. Transposon insertion sites were identified by linker-mediated PCR using barcoded primers distinct for each tumor followed by sequencing using the Illumina Genome Alayzer IIx. We generated over 39,793,201 sequence reads, from which we were able to identify 23 CISs (Figure 4)

(Table 2). CISs are locations in the genome that contain transposons from multiple tumors at a rate higher than expected by chance, with a P-value less than 0.05, to identify candidate cancer genes that drive HS (Figure 4) [51, 95]. From our original list of 23, we generated a list of 13 top genes and compared it to human genes listed in the Catalog of Somatic Mutations in Cancer database (Table 2). We picked out top genes based on the number of unique insertions and the locations of insertions. From the 13 top genes, 12 were in the COSMIC database and known to be mutated in cancer. We had one novel alteration on our top CIS list, a ribosomal RNA, 5S_rRNA. 5S_rRNA has no known role in cancer but is involved with stabilization and transcription of the s5SRNA gene [97]. On the list of 13 tops genes there are three tumor suppressor genes that when altered are known to play a major in cancer development. These genes are *Nf1*, *P53*, and *Pten*.

Our top three hits, *Fli1*, *Raf1, and Mitf* are known to have oncogenic capabilities and play a role in cancer development. *Raf1* is a kinase in the Ras family functioning as a MAP3K. *Raf1* is important for cell fate decisions including proliferation, differentiation, apoptosis, survival and oncogenic transformation [98]. The cellular *RAF1* protein can phosphorylate and activate the dual specificity protein kinases *MEK1* and *MEK2*, which in turn phosphorylate and activate the serine/threonine specific protein kinases, *ERK1* and *ERK2*[98, 99]. Altered *RAF1* is associated with the development of Noonan and LEOPARD syndrome, prostate, lung, and breast cancer, and leukemia[98, 100-102]. *Fli1* is a known proto-oncogene and a member of the ETS transcription factor family. It is a sequence-specific transcriptional activator that recognizes the DNA sequence 5'-C[CA]GGAAGT-3'[103]. Defects in *FLI1* are a can result in the formation of Ewing sarcoma or acute myelogenous leukemia (AML)[103, 104]. Ewing sarcoma is a highly

malignant, metastatic, primitive small round cell tumor of bone and soft tissue that affects children and adolescents [105, 106]. *Mitf* plays a critical role in the differentiation of various cell types as neural crest-derived melanocytes, mast cells, osteoclasts and optic cup-derived retinal pigment epithelium [107-112]. Dysregulated *Mitf* has been associated with development of clear cell sarcoma (CCS) and melanoma [111, 113, 114].

## Discussion

HS are difficult to distinguish histologically due to the heterogeneity of the cell type involved in tumor formation. Therefore, to confirm that we are in fact using a mouse model for HS, we combined strict microscopic observations of morphology of HE stained slides and immunoreactivity on IHC stained slides with known markers for HS. For proper characterization we used antibodies recognizing PAX5, F4/80, and Mac-2 to stain HS tissues. A board certified pathologist analyzed the tissue to definitively determine the tumor type to be HS. Through IHC, our samples stained positive for F4/80 and Mac-2 but not PAX5. HS was shown to include cases with a predominance of round or spindle cells or mixed populations of both cell types. Our phenotypic analyses provided strong evidence that the round cells represent a more mature population of histiocytes given the higher levels of F4/80 expression. This perspective is based on the prior demonstration that expression of F4/80 increases with progressive maturation in the monocyte/macrophage lineage[96]. Expression of F4/80, is a clear indication that the tumor types were HS. Analyses of the markers Mac–2 confirmed a shared myeloid origin for the cytologically distinct populations of HS. The features of murine HS described in this study have many similarities to those of HS in humans. In humans, HS is a rare

neoplasm that is sometimes associated with lymphomas and thus is difficult to distinguish.

Importantly, we found overlap between the candidate driver mutations from our screen and known human cancer genes, which suggests that our screen is relevant to human HS. Of our top hits, *Raf1* and *Fli1*, are oncogenes associated with hematological neoplasms (Figure 5A and B). We believe *Raf1* and *Fli1* also act as oncogenes in our model based of the directions and location in the gene of their DNA insertion. The insertions in *Raf1* were all located in the first intron, suggesting a truncated form of Raf1 may be oncogenic (Figure 5a), while in *Fli1* all of the transposons inserted in the same direction, with the internal promoter of the transposon positioned to drive overexpression of Fli1 (Figure 5B). There is very little published information about what genes are involved in HS. The etiology of HS in humans is largely unknown, although recent studies indicate that genetic or epigenetic inactivation of *PTEN* may play a causal role in disease development. Our study also identified *Pten* as one of our top CISs, which is an internal validation of our CIS list demonstrating we were able to identify genes known to be involved in human HS in our model. This novel mouse model and gene list are important steps forward in understanding the molecular biology of a disease we know so little about.

In summary, our transposon-mediated forward genetic screen in mice identified genetic mutations that lead to the development of HS. The discovery of a significant overlap of mouse candidate genes and human genes that are altered in various cancers suggests that this mouse model will be useful for distinguishing between driver and passenger mutations. These genes can serve as potential novel drug targets. These results

show that relatively mature myeloid lineage cells can be targets for HS, and that the *Mitf* transcription factor and RAF-MEK-ERK signaling may be a useful target for HS treatment in humans and canines.

We have created a novel mouse model for HS and have started to unravel key information about the genetic drivers of the disease. With the dismal prognosis HS currently carries, any breakthroughs in our understanding of the mechanisms that allows HS to develop and progress is a success story and allows us to get one step closer to successfully treating the disease.

## Figure Legends

**FIGURE 1A.** Oncogenic Transposon (T2/Onc): Splice acceptors (SA-pA) in both orientations disrupt TSG expression while the MSCV promoter-splice donor can cause overexpression of an oncogene. The IR/DRs are required for sleeping beauty transposition. Rosa26 Lox-stop-Lox Sleeping Beauty : SB11 transposase knocked-in to the Rosa26 locus, but expression is blocked by an intervening floxed stop cassette. LyzM-Cre: Cre Recombinase driven by the myeloid lineage specific promoter LyzM.

**FIGURE 1B.** Demonstrated the breeding scheme used to generate control and experimental cohorts. Lyz-Cre transgenic line was initially first crossed with a T2/Onc transgenic mouse. Then the double transgenic mouse of Lyz-Cre: T2/Onc was crossed with a LsL-SB11 transgene mouse. The mice that possessed all three transgenic elements made up the experimental cohort. The controls were any combination of two out of the three transgenes.

**FIGURE1C.** Shows the total number of mice generated for each control group and the experimental group. For controls, we generated 34 *Lyz-Cre: LsL-SB11* mice, 52 *Lyz-Cre: T2/Onc* mice, and 31 *LsL-SB11:T2/Onc* mice. For experimental, we generated 72 *T2/Onc: LyzM-Cre:LsL-SB11*.

**FIGURE 2.** Is a Kaplan-Meier Survival Curve that shows the triple transgenic mice (T2/Onc, LyzM-Cre, Rosa-Lox-stop-Lox-SB11) (pink line) became moribund at a faster rate than double transgenic mice (purple line). This difference was statistically significant with a P-value less than 0.0001.

**FIGURE 3.** (A) Photo of a 16-month-old mouse euthanized due to morbidity. The mouse is showing diseased organs that have been infiltrated with HS demonstrating the destructive nature of this disease. The picture shows the macroscopic view of HS tumors of the liver, fat pads, and a blood filled chest variety along with other abnormalities. (B) A photo demonstrating the hemothorax, blood in the chest cavity, at a closer view. (C) A photo highlighting the abnormal, HS soft tissue mass at the heart base and surrounding the lungs. (D) An HE stained HS. Note Composed of sheets of spindeloid to polygonal cells; Nucleus round to oval; Atypical mitotic figures; Highly cellular; Lymphocytes scattered throughout. (E) Positive staining with Mac-2, for identification of red pulp and white pulp macrophages. (F) Positive staining with F4/80 antibody, for increasing expression during monocyte to red pulp macrophage differentiation. (G) Slide examined with PAX5 for identification of B cells, stained negative. (H) HE stained spleen and pancreas believe to be an HS. (I) HE stained pancreas believe to be an HS. (J) HE stained liver believe to be an HS.

**FIGURE 4.** Demonstrated how transposon insertion sites from multiple tumors are mapped to the mouse genome (colored bars). If the same locus is hit by a transposon insertion in several tumors, at a rate much higher than would be expected by chance, that locus is likely a driver of carcinogenesis due to a mutation caused by the transposon insertion. The gene(s) within that locus are identified as candidate cancer genes.

**FIGURE 5.** This demonstrates the location and directions of common insertions sites for Raf1 and Fli1, transposon insertions are indicated by the arrowheads. The insertions in Raf1 and Fli1 predict an overexpression role for the candidate oncogenes.

**FIGURE 6.** This is an excision PCR showing that HS tumors are undergoing transposition. Where there is a 225bp band present demonstrates that the transposon, t2/Onc has been mobilized. For negative controls, we used various tissues from control animals and WT animals including sleeping, liver, and pancreas.

## Tables and Figures

**TABLE 1. VARIOUS TISSUES AFFECTED IN MICE THAT DEVELOPED HISTIOCYTIC SARCOMA**

| **Tissue/location** | Spl | Thy | Panc | Liv | Chest Cavity | Peritoneum | Fat Pads |
|---|---|---|---|---|---|---|---|
| **% of Total Affected** | 30% | 13% | 29% | 33% | 27% | 27% | 11% |

Spl, spleen; Thy, thymus; Panc, pancreas; Liv, liver. The percent of all mice that were diseased showing macroscopic tumor infiltration in each tissue is shown.

**TABLE 2. CIS LIST**

| Gene | Chr | # of unique inserts | Function |
|---|---|---|---|
| Raf1 | 6 | 7 | Transduction of mitogenic signals, part of RAS-dependent signaling |
| Fli1 | 9 | 6 | Sequence-specific transcriptional activator |
| Mitf | 6 | 5 | Transcription factor, differentiation of melanocytes, mast cells, osteoclast |
| Sin3a | 9 | 3 | Transcriptional repressor, essential for embryonic development |
| Orai1 | 5 | 3 | Calcium release-activated calcium modulator |
| Nf1 | 11 | 3 | Stimulates the GTPase activity of Ras |
| Chuk | 19 | 3 | Acts in NF-kappa-B activation and phosphorylates inhibitors of NF-kappa-B |
| Trp53 | 11 | 2 | Tumor suppressor Induces growth arrest or apoptosis depending |
| 5S_rRNA | 5 | 2 | Physical transducer of information, communication between functional centers |
| Kdm6a | X | 3 | Histone demethylase, chromatic remodeling |
| Pten | 19 | 2 | Tumor suppressor, lipid phosphatase |
| Slit3 | 11 | 2 | Cell migration |
| Arih1 | 9 | 2 | E3 ubiquitin-protein ligase part of the E3 complex |

Mouse chromosome (Chr) of each common insertion site (CIS) associated gene is shown. The number of unique insertions recovered from different tumors is shown as well as the function, in brief, of each CIS-associated gene.

**FIGURE 1A. THREE TRANSGENES USED TO DIRECT MUTAGENESIS TO MYELOID CELLS**

**T2/Onc:** Transposon concatomer of ~25 or ~125 copies, low and highy copies


**Conditional SB transposase:** LSL-SB11 transposase knocked-in to the Rosa26 locus


**Lyz-Cre:** Cre recombinase under the control of the myeloid lineage specific promoter LyzM


**FIGURE 1B. BREEDING SCHEME TO GENERATE CONTROL AND EXPERIMENTAL COHORTS.**

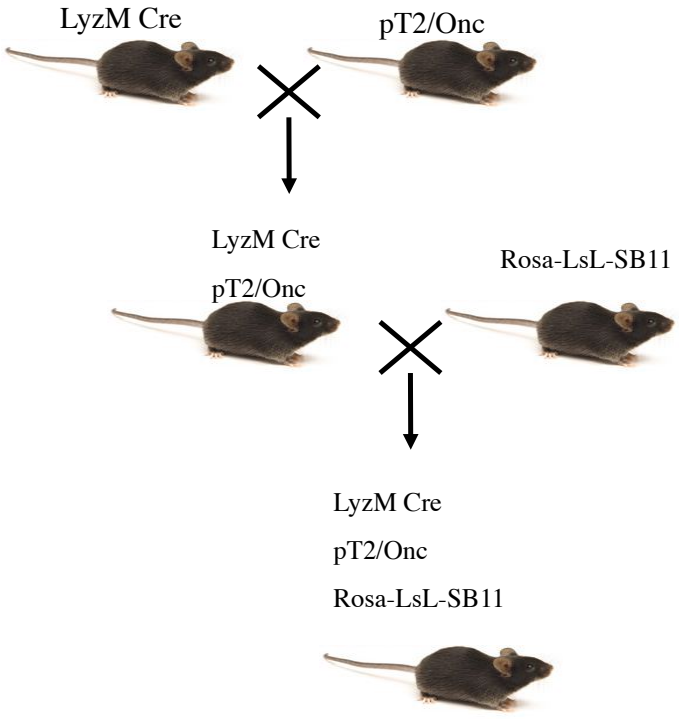


**FIGURE1C. TOTAL NUMBER OF MICE GENERATED.**

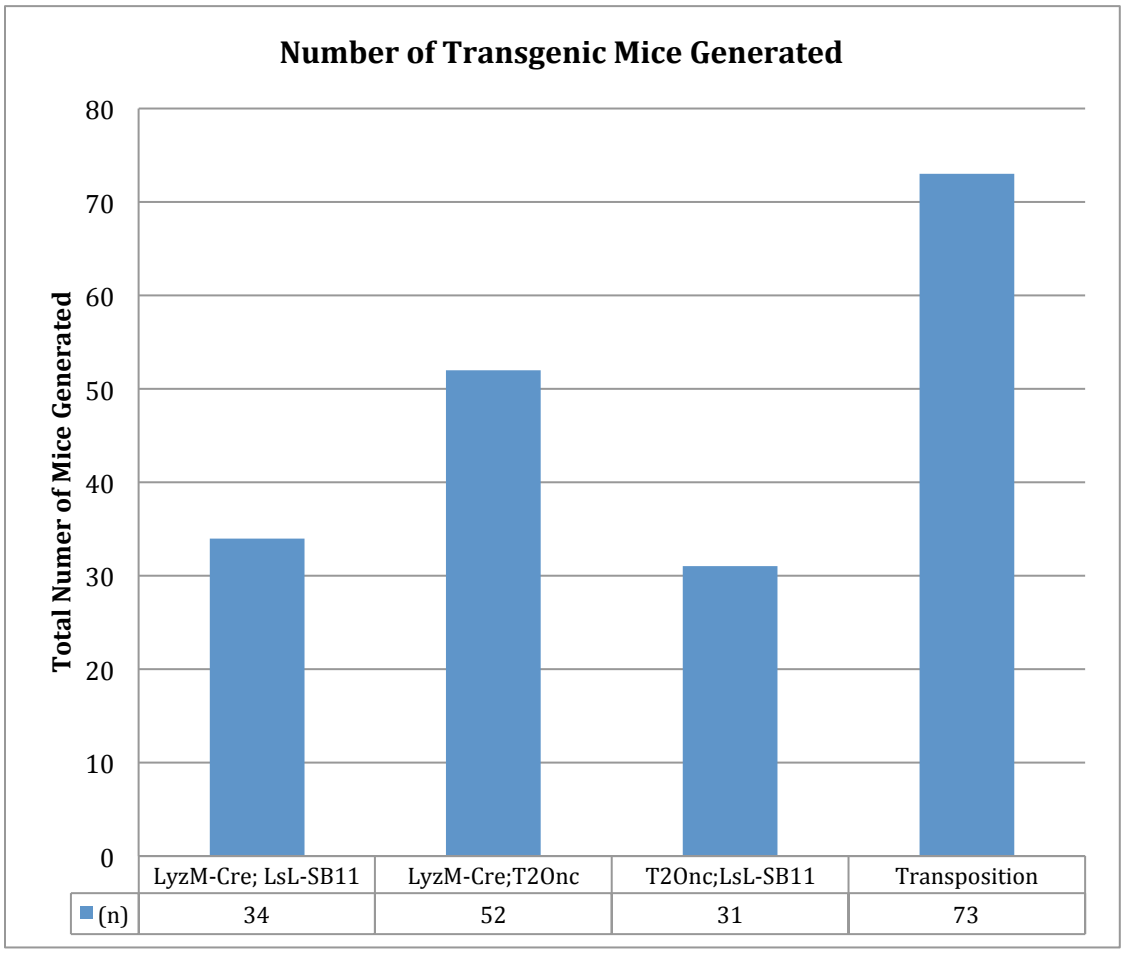


FIGURE 2. KAPLAN-MEIER SURVIVAL CURVE

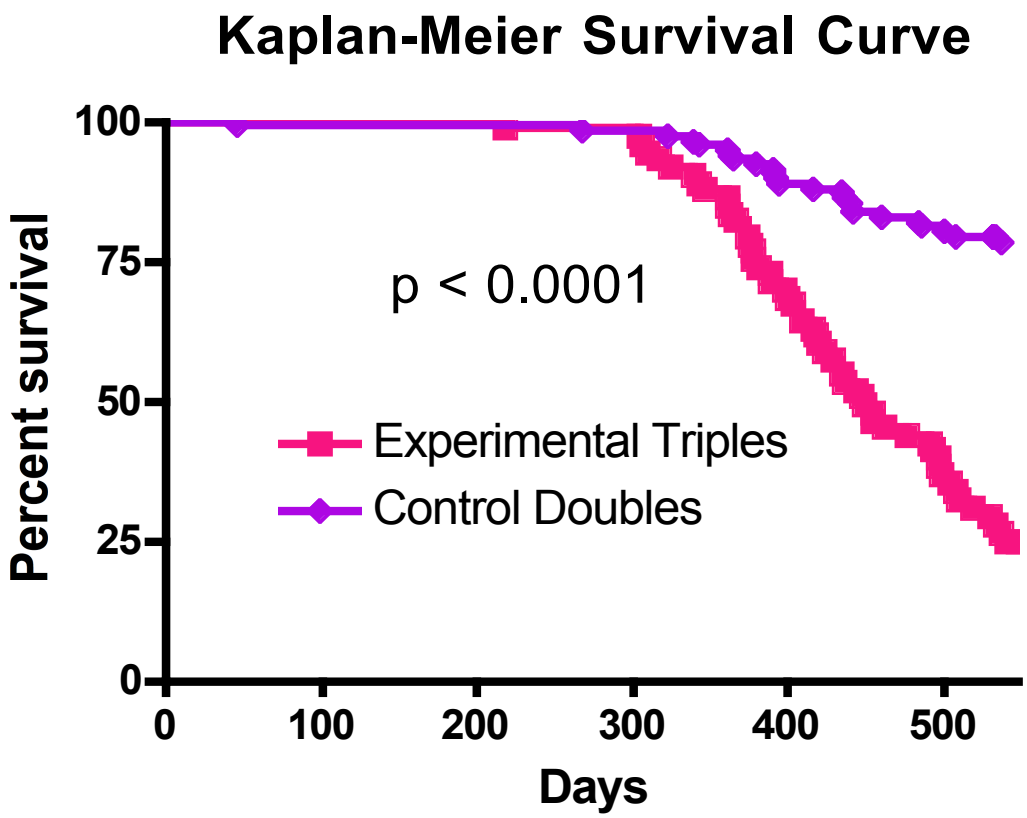


**FIGURE 3A-J. PATHOLOGY AND IHC OF VARIOUS TISSUE TYPES SUGGEST HISTIOCYTIC SARCOMA.**

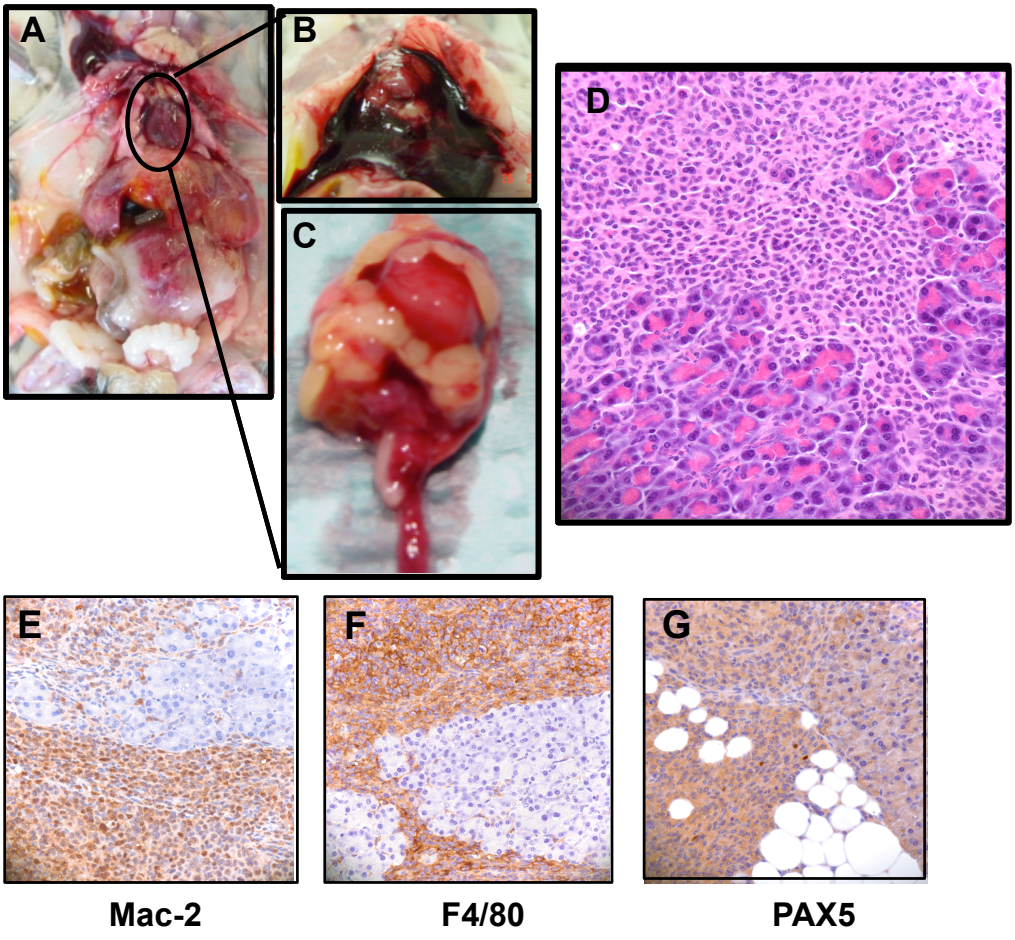


**FIGURE 4. COMMON INSERTION SITES IDENTIFY CANDIDATE CANCER GENES**

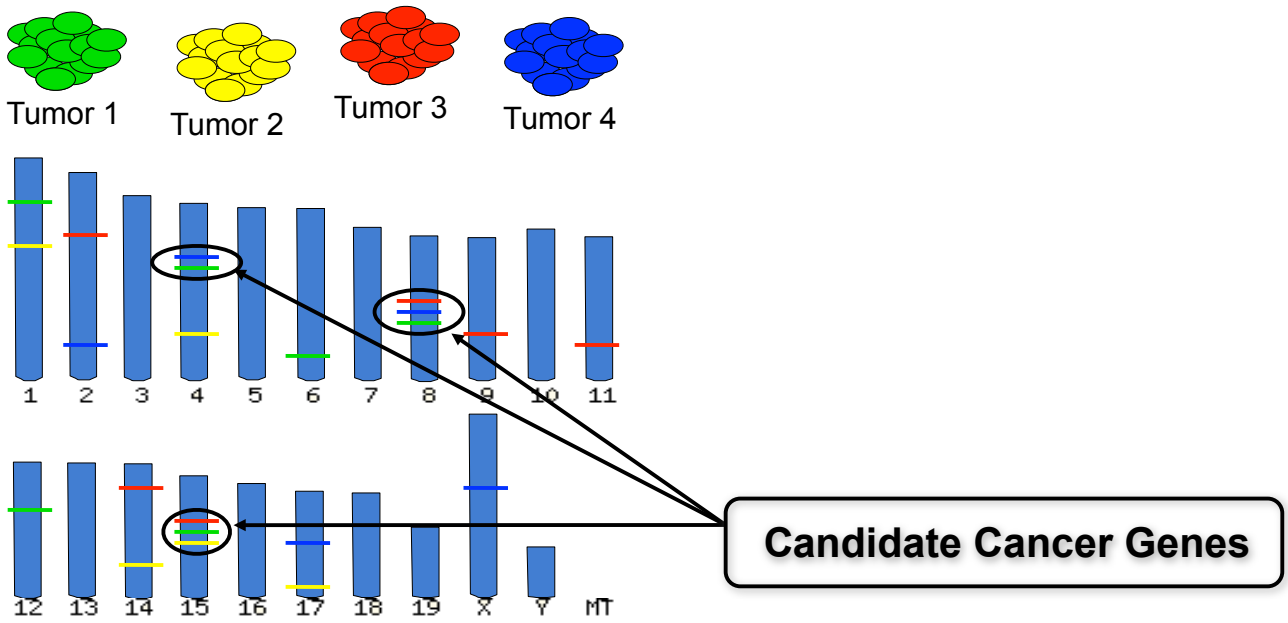
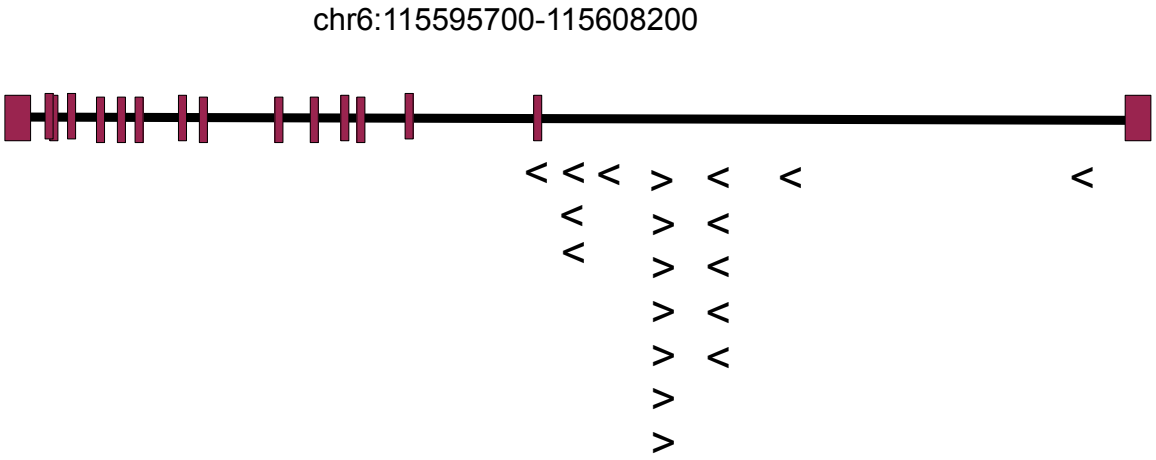


**FIGURE 5. EXAMPLE OF COMMON INSERTION SITE**

A. *Raf1* Insertions




B. *Fli1* Insertions

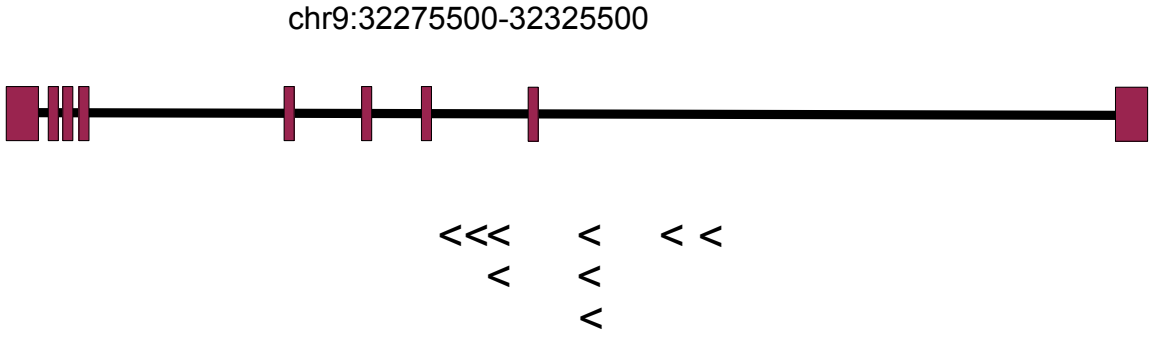
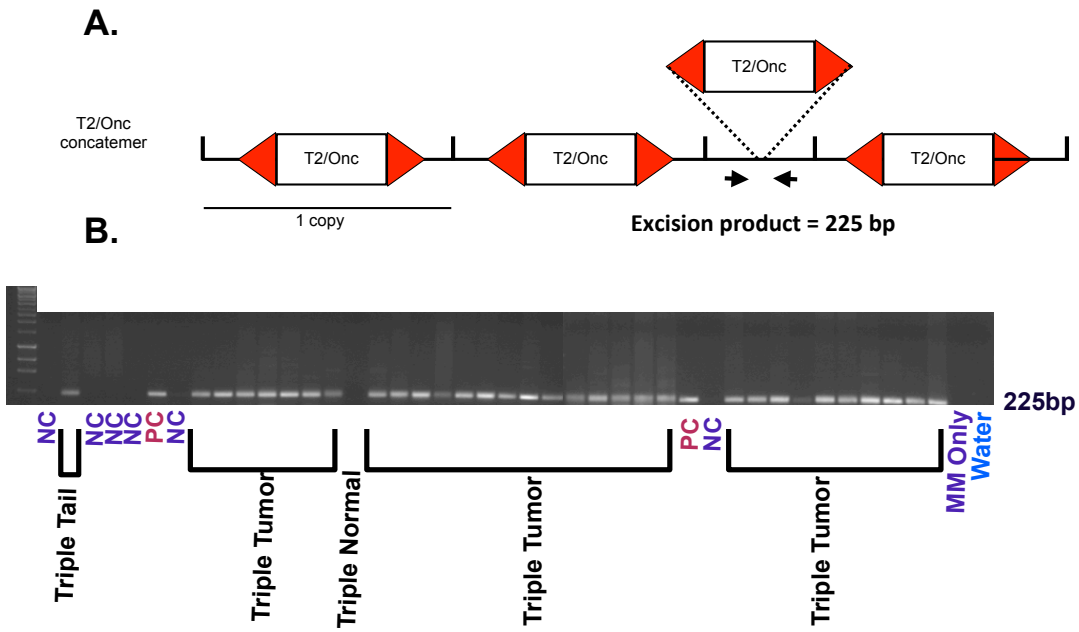


**FIGURE 6. EXCISION PCR SHOWS HS TUMORS ARE UNDERGOING TRANSPOSITION.**




Negative Control: NC  Posative Control: PC

## Chapter 5) Conclusions

The human body is composed of many different cell types, in many tissue environments, that can develop into cancer. The alterations causing cancer can happen on the genetic or the epigenetic level. Cancer genes are distinct alleles of normal genes that arise as a result of mutations. These mutations can happen in somatic cells or in germline cells. When a patient develops cancer, as a result of an alteration in their germline cells, the mutation can be passed on to future generations. This distinguishes familial, inherited cancer from sporadic cancer. Most of the mutations that must accumulate to cause a clinically evident tumor are thought to occur in somatic cells. When a mutation occurs in somatic cells, those mutations cannot be passed on. Despite popular belief, the majority of cancers are not inherited, but rather new mutations are caused by environmental factors in rare somatic cells. Indeed, most cancer is sporadic in nature and in the modern world most of the populations cancer risk is influenced by diet, exposure and lifestyle choices. The take home message is that most cancers are preventable [3-5]. Despite this fact, we can do better to reduce population cancer risk by identifying as many safe biological modifiers that could be employed. This fact is exemplified by the huge differences in the relative risk for certain cancers in different populations around the world. Identifying those critical factors that explain these differences and making selected changes to the western diet, perhaps in the form of supplementation, could be of enormous benefit in cancer control. Before attempted in large numbers of people, hypotheses must be supported by studies in model organisms and/or model human cell lines. The development of normal human cell culture technology, such as induced pluripotent stem (iPS) cell technology, could make this goal a reality. Perhaps mice that

have been “humanized” by infusion of such cells would provide ideal models for testing prevention strategies. The idea of using human epidemiological studies as the basis for testing biological plausibility in these model systems has great appeal for subsequently prioritizing these interventions in human populations.

Recent studies show that by the time a cancer is detected, more than 90% of the biologic life of the tumor is over [3]. Despite how far technology has progressed in the last decade, the best treatment of malignant disease remains prevention. Early detection markers and advanced chemotherapeutics have increased the overall five-year survival for all cancers from 49% in 2001 to a 67% 5-year survival [5]. Taking this improvement into account, fewer than 50% of cancers are cured [3]. Over a lifetime, the chances of men developing cancer is about ½ and the chances of women developing cancer is about 1/3 [3]. With so much of the population being affected, there has been a tremendous effort to find effective treatments for cancer through a better understanding of the biological alterations that occur when cancer forms through undertakings such as the human genome project and publication of the cancer genome atlas. Disappointingly, the result of billions of dollars spent through efforts of the private and public sectors has been little gains in treatment effectiveness. Progresses of current treatments have been slow, incremental and expensive. To date, prevention strategies remain the most effective way to fight cancer but are principally overlooked by the research community.

The three key areas of early detection and prevention of cancer that should be pursued aggressively are 1) etiology and pathogenesis, 2) screening and early detection, and 3) chemoprevention. To understand the etiology and pathogenesis we need to find better ways to identify high-risk individuals based on familial/genetic and environmental

influences[3, 5]. Smoking tobacco was identified as a causal factor in lung cancer development in the 1950's and to this day still remains the number one cause of cancer deaths, accounting for approximately 30% of the mortality [3]. There are effective screening and early detection techniques that have been applied systematically and proven successful. Some of the examples for cancer include visual examination (skin, oral), cytology (cervical cancer), mammography (breast cancer), and fecal occult blood, sigmoidoscopy, and colonoscopy (colorectal cancers) [3]. One more cancer we may soon add to the list is prostate cancer, the number two killer of men in the US in 2012. Hopefully this figure will change in the coming years due to proper use of prostate cancer screening by serum prostate-specific antigen (PSA). There is no method for lung cancer screening, but the incidence of lung cancer in the US pollution has been steadily going down since the early 1990s due to the implementation of a large, nationwide antismoking campaign [4, 5]. Chemoprevention can be extremely effective in the battle against caner in populations at high risk of developing the disease. The effectiveness of this chemoprevention has been established through the use of the antagonist tamoxifen and raloxifene for breast cancer, hepatocellular carcinoma vaccination against hepatitis virus B, and cervical cancer vaccination against human papillomavirus [3]. Many major diseases have been controlled via the methodical application of prevention strategies; many common cancers could be on this list, with a concerted research effort.

Despite our best prevention efforts, some cancer is likely to occur. The identification of all cancer drivers is likely in the coming decade. This will occur by appropriate combination of human cancer genome characterization and functional studies in mouse models, such as transposon based screening, and human cell lines. Much work

should be invested in building even more refined model systems to recapitulate all the molecular subtypes of each type of human cancer. It is likely that use of these models to test combination therapies for cancer will yield the most durable clinical responses. The enterprise of testing new agents for cancer therapy may need to change to make it easier to test new combinations so that this process is not too slow, however. This means new modes of clinical intervention and rapid testing of many new ideas in small Phase I clinical trials is perhaps the best future route for progress in cancer treatment.

In summary, my research experiences have given me perspective on the challenges of doing meaningful discovery research in cancer. Cancer is an enormous problem that can only be solved by a highly multidisciplinary, team based approach to science. My own work combined epidemiology, nutrition, genetics and mutagenesis studies. It highlighted the fact that the model systems chosen for research dictate the results, which one type of cancer consists of many molecular subtypes that may not behave in the same way, and that each must be considered separately. The work also shows that a dietary intervention could have both positive and negative results, depending on the individual. Thus, the overall theme is that of individualizing prevention measure, nutrition, and disease treatment for each person. Ultimately, maximizing health will require this perspective.

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