Generation of Mouse Models of Human Hematopoietic Disease and Their Use to Analyze Hematopoietic Development and Function

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Institute of Medical Science
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Abstract

Hematopoiesis is an intricately regulated homeostatic process that maintains all of the differentiated blood cell lineages. N-ethyl-N-nitrosurea (ENU) is a powerful mutagen that induces point mutations randomly in the genome. ENU was used in a dominant forward genetic screen to identify novel mutations in regulators of hematopoiesis and to create new mouse models of hematopoietic disease. The objectives of this thesis were to characterize two mutants that originated from the dominant screen (7192 and 7238) and to develop a pharmacologically sensitized screen that would detect a unique set of mutations undetectable in the dominant screen.

The 7192 mutant from the ENU dominant screen presented with elevated microcytic red blood cells (RBC) and increased polychromasia. The causative mutation was identified as a nonsense mutation in Ank1 (Q895X) that coded for a truncated ANK1 protein. Ank17192 is a novel mouse model of hereditary spherocytosis (HS), a human disease that results from increased
RBC fragility. We have demonstrated that \textit{Ank1}\textsuperscript{7192/+} mice model a mild HS and \textit{Ank1}\textsuperscript{7192/7192} mice model severe HS.

The 7238 mutant from the dominant ENU screen was macrothrombocytic and carried a missense mutation in \textit{Myh9} (Q1443L). The \textit{Myh9}\textsuperscript{7238/7238} mice are viable and have a more severe phenotype of macrothrombocytopenia. \textit{Myh9}\textsuperscript{7238} is the first mouse model for Myh9 related disorders that accurately models the genetic origins and the systemic manifestations of the disorder.

A pharmacologically sensitized screen using chemotherapeutic drugs was designed to induce stress hematopoiesis to detect mutations that alter cell cycle of hematopoietic progenitors or stress hematopoiesis. Analysis of both peripheral blood and progenitor recovery kinetics, determined that 5-fluorouracil (5FU) and phenylhydrazine were good candidates for a pharmacologically sensitized screen. 5FU was successfully incorporated into an ENU dominant screen, and 13 platelet recovery outliers were detected. From these outliers, three mutant lines were successfully established.
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# Table of Contents

Acknowledgments.......................................................................................................................... iv  
Table of Contents........................................................................................................................... vi  
List of Tables ................................................................................................................................. xi  
List of Figures ............................................................................................................................... xii  
List of Abbreviations and Acronyms............................................................................................. xv  
List of Appendices ..................................................................................................................... xviii  

Chapter 1: Introduction....................................................................................................................1  
  1.1 The Mouse as a Genetic Model for Human Disease............................................................2  
  1.2 Forward Mutagenesis Screens .............................................................................................2  
    1.2.1 History......................................................................................................................2  
    1.2.2 Forward vs Reverse Genetics...................................................................................4  
    1.2.3 Reverse Genetics......................................................................................................4  
    1.2.4 Forward Genetics .....................................................................................................6  
  1.3 Mechanisms of ENU Mutagenesis.....................................................................................12  
  1.4 ENU Mutagenesis Screen Design ......................................................................................17  
    1.4.1 Heritability Test G1 ...............................................................................................19  
    1.4.2 Genetic Mapping: Fine and Rough..........................................................................19  
    1.4.3 Finding the Causative Mutation.............................................................................22  
    1.4.4 Confirmation of Mutation......................................................................................23  
    1.4.5 Comparing Knockout to ENU Mutants .................................................................24  
  1.5 Hematopoiesis....................................................................................................................25  
    1.5.1 RBC ........................................................................................................................27  
    1.5.2 RBC Membrane Diseases ........................................................................................33
1.5.3 Platelets and Megakaryocytes ................................................................. 36

1.6 Pharmaceutical Induction of Cytopenia ....................................................... 43

1.7 Thesis Hypothesis and Objectives .............................................................. 50

2 Chapter 2: Ank1\textsuperscript{7192/7192}: A new mouse model for Hereditary Spherocytosis ........................................................................ 54

2.1 Abstract ........................................................................................................... 55

2.2 Introduction ..................................................................................................... 56

2.3 Materials and Methods .................................................................................. 59

2.3.1 Mice and ENU Mutagenesis ....................................................................... 59

2.3.2 Genetic Mapping and Sequencing ............................................................... 60

2.3.3 PCR Genotyping .......................................................................................... 60

2.3.4 Hematologic Analysis .................................................................................. 61

2.3.5 Histology ...................................................................................................... 61

2.3.6 \textit{In vitro} Progenitor Assays ...................................................................... 61

2.3.7 Flow Cytometry .......................................................................................... 62

2.3.8 RBC Ghost Protein Analysis and Immunoblotting ...................................... 62

2.4 Results ............................................................................................................ 64

2.4.1 Identification and Cloning of a New ENU-Derived HS Mutation .............. 64

2.4.2 Pathological Analysis .................................................................................. 67

2.4.3 Peripheral Blood Analysis .......................................................................... 72

2.4.4 Extramedullary Hematopoiesis .................................................................. 74

2.4.5 Protein Analysis ........................................................................................... 77

2.5 Discussion ...................................................................................................... 80

3 Chapter 3 Myh9\textsuperscript{72388}: A mouse model for Myh9-Related Disorders .......... 85

3.1 Abstract ........................................................................................................... 86

3.2 Introduction .................................................................................................... 87

3.3 Methods and Materials ................................................................................... 91
3.3.1 Mice and ENU Mutagenesis .................................................................91
3.3.2 Genetic Mapping ..................................................................................91
3.3.3 Hematologic Analysis .........................................................................91
3.3.4 Histology ...............................................................................................92
3.3.5 *In vitro* Progenitor Assays .................................................................92
3.3.6 Megakaryocyte Culture .......................................................................93
3.3.7 Ploidy Analysis .......................................................................................93
3.3.8 Bleeding Time .........................................................................................94
3.3.9 Aggregation .............................................................................................94
3.3.10 Perfusion Studies ................................................................................95
3.3.11 Sequencing ............................................................................................95
3.3.12 Genotyping ............................................................................................96
3.3.13 Transmission Electron Microscopy .......................................................97
3.3.14 Urine Analysis .......................................................................................97
3.3.15 Cataract Detection ...............................................................................98

3.4 Results .......................................................................................................98

3.4.1 Identification and Mapping of 7238 Mouse Line ...............................98
3.4.2 Redefining the 7238 Platelet Phenotype .............................................102
3.4.3 Normal Hematopoietic Progenitor Frequency in 7238 Mice .............105
3.4.4 7238 Megakaryocytes have Normal DNA Content, but Higher Nuclear to Cytoplasmic Ratio ..............................................................106
3.4.5 Reduced Plug and Platelet Aggregation in 7238/7238 mice ..............109
3.4.6 Identification of 7238 Mutation ...........................................................112
3.4.7 Neutrophil Inclusions ..........................................................................115
3.4.8 Urine Analysis .......................................................................................116
3.4.9 Cataracts ...............................................................................................117
3.4.10 Preputial Gland ....................................................................................................118

3.5 Discussion ........................................................................................................................120

4 Chapter 4: A Pharmacologically Sensitized Screen to Detect Defects in Developmental Hematopoiesis ....................................................................................................................126

4.1 Abstract ............................................................................................................................127

4.2 Introduction ......................................................................................................................128

4.3 Materials and Methods .....................................................................................................129

4.3.1 Mouse Strains .............................................................................................................129

4.3.2 Cytopenia Induction ....................................................................................................129

4.3.3 Hematologic Analysis ................................................................................................129

4.3.4 KTLS Analysis ..........................................................................................................130

4.3.5 In vitro Colony Assays ...............................................................................................131

4.3.6 Ploidy Analysis ...........................................................................................................131

4.3.7 ENU Mutagenesis and G1 Screen .............................................................................132

4.3.8 Genetic Mapping .......................................................................................................132

4.3.9 Statistical Analysis ....................................................................................................133

4.4 Results ..............................................................................................................................135

4.4.1 Effect of 5FU Dosage on Recovery Kinetics ...............................................................135

4.4.2 Strain-Specific Effects of 5FU administration ............................................................136

4.4.3 Strain-Specific Effects of PHZ Administration ..........................................................139

4.4.4 Determining an Effective HU Dosage - Low Dose HU ............................................139

4.4.5 Determining an effective HU dosage - high dose HU .............................................141

4.4.6 Strain-Specific Effects of HU Administration ............................................................141

4.4.7 Effects of Hematological Stress Agents on KTLS Cells in the Bone Marrow .......145

4.4.8 Effect of Hematological Stress Agents on BM Cellularity and CFU-C ...............147

4.4.9 The Effect of Hematological Stress Agents on Induction of Extramedullary Hematopoiesis .................................................................................................................................151
4.4.10 The Effect of Hematological Stress Agents on Megakaryocyte Ploidy ..............152

4.4.11 The Implementation of a 5FU Sensitized Screening Protocol as Part of a Dominant ENU Screen ........................................................................................................155

4.5 Discussion........................................................................................................................169

4.5.1 Drug Recovery Kinetics..............................................................................................169

4.5.2 Cytopenia Mode of Action - 5FU ........................................................................173

4.5.3 Cytopenia Mode of Action - PHZ........................................................................175

4.5.4 Cytopenia Mode of Action - HU .........................................................................175

4.5.5 Implementation of 5FU into an ENU Dominant Screen.......................................177

5 Conclusions and Future Directions .........................................................................................184

5.1 Overview.........................................................................................................................184

5.2 Identification and Characterization Ank17192 ..............................................................185

5.3 Identification and Characterization of Myh97238 .........................................................187

5.4 Pharmacologically Sensitized ENU Screen..................................................................192

References............................................................................................................................198

Appendices............................................................................................................................221

6 Appendices..........................................................................................................................221

6.1 Statistical Analysis from Chapter 4 .................................................................................221

6.2 Cytopenia Induction in Known Hematopoietic Mutants................................................229

6.2.1 Materials and Methods..............................................................................................230

6.2.2 Results.......................................................................................................................231

6.2.3 Discussion...................................................................................................................239

6.2.4 References..................................................................................................................242

6.3 Copy of Jak27254 paper submitted to Blood..................................................................244
List of Tables

Table 1-1. A Sampling of ENU Mutagenesis Screens Published.................................................. 16

Table 2-1. CBC performed on PB of *Ank*17192 mice on C3H and C3H/129 backgrounds. .......... 71

Table 2-2. Organ weight of *Ank*17192/17192, *Ank*17192/+ and wild-type mice. ................... 72

Table 3-1. CBC performed on peripheral blood of 7238/+ and 7238/7238 mice. ....................... 104

Table 4-1. Summary of G1 phenotypes and criteria for classification. ...................................... 157

Table 4-2. Summary of G1 screen ................................................................................................ 159

Table 4-3. G1 outlier’s phenotype summary .................................................................................. 162

Table 6-1. Statistical Analysis of 5FU Recovery Kinetics ............................................................. 221

Table 6-2. Statistical Analysis of PHZ Recovery Curves............................................................... 222

Table 6-3. Statistical Analysis of Low Dose HU Recovery Kinetics .............................................. 223

Table 6-4. Statistical Analysis of High Dose HU Recovery Curve .................................................. 224

Table 6-5. Statistical Analysis of Strain Comparison of HU Recovery Curves ......................... 225

Table 6-6. Summary of KTLS Population Over 2 Week Time Course Subsequent to Cytopenia Induction .................................................................................................................. 226

Table 6-7. Summary of c-kit high TLS Population 18hrs Post 5FU Treatment ....................... 226

Table 6-8. Statistical Analysis of Bone Marrow CFU-C Frequency .............................................. 227

Table 6-9. Statistical Analysis of Bone Marrow of CFU-C per Femur ......................................... 228

Table 6-10. Statistical Analysis of Splenic Extramedullary hematopoiesis ................................. 228
List of Figures

Figure 1-1. Breeding schemes for a dominant and recessive ENU screen. ................................. 8

Figure 1-2. Hematopoietic hierarchy. ...................................................................................................... 26

Figure 1-3. A schematic of the cross-section of a red blood cell................................................................. 32

Figure 2-1. Initial characterization and mapping of 7192 mice................................................................. 65

Figure 2-2. Identification of the *Ank1*<sup>7192</sup> allele........................................................................... 66

Figure 2-3. The survival of *Ank1*<sup>7192/7192</sup> mice at late embryo, neonatal and 3 weeks of Age. .... 69

Figure 2-4. The organ morphology and histology of *Ank1*<sup>7192/7192</sup> mice and littermate controls. 70

Figure 2-5. Peripheral blood analysis of *Ank1*<sup>7192/7192</sup> mice.............................................................. 73

Figure 2-6. Extramedullary hematopoiesis – erythroid precursors in *Ank1*<sup>7192/7192</sup> mice. .......... 75

Figure 2-7. Extramedullary hematopoiesis – hematopoietic and erythroid progenitors in *Ank1*<sup>7192/7192</sup> mice................................................................. 76

Figure 2-8. Identification and characterization of Ankl<sup>7192</sup> ANK1 protein. ................................. 78

Figure 2-9. Human HS mutations in Ankl. ................................................................................................. 79

Figure 3-1. Initial characterization and mapping of 7238 mice................................................................. 100

Figure 3-2. 7238/7238 survival and PLT phenotype. .................................................................................. 101

Figure 3-3. Redefining the 7238 PLT phenotype. ....................................................................................... 103

Figure 3-4. Characterization of bone marrow hematopoietic progenitors in 7238 mice. ........... 105

Figure 3-5. Measuring the DNA content of 7238 megakaryocytes. ..................................................... 107

Figure 3-6. Morphology of megakaryocytes *in vivo* and *in vitro*. ...................................................... 108

Figure 3-7. Increased bleeding time in 7238/7238 mice, demonstrates impaired PLT function. 110
Figure 3-8. 7238 PLT have reduced aggregation in vitro and reduced adherence and aggregation to a collagen matrix under physiological flow conditions.

Figure 3-9. Identification of the 7238 allele.

Figure 3-10. NMMIIA structure, mapping of the 7238 mutation and human MYH9RD mutations.

Figure 3-11. Neutrophil inclusions detected in Myh97238 mice.

Figure 3-12. Normal protein levels in urine of Myh97238 mice.

Figure 3-13. Increased incidence of cataracts in aged Myh97238 mice.

Figure 3-14. Gross morphology and histology of preputial gland in Myh97238 mice.

Figure 4-1. 5FU recovery curves

Figure 4-2. PHZ recovery curves

Figure 4-3. HU recovery curves

Figure 4-4. Time course study examining the alterations in KTLS population and profile post cytopenia induction

Figure 4-5. Time course study on bone marrow hematopoietic progenitors post cytopenia induction

Figure 4-6. Cytopenia induced extramedullary hematopoiesis

Figure 4-7. 5FU alters megakaryocyte ploidy and frequency

Figure 4-8. ENU breeding scheme and selection of optimized 5fu testing days

Figure 4-9. Analysis of G1 mice for defects in RBC, PLT and WBC recovery after 5FU administration

Figure 4-10. Analysis of G2 heritability in 5FU RBC mutants

Figure 4-11. Analysis of G2 heritability in 5FU platelet mutants

Figure 4-12. Analysis of G2 heritability multilineage mutants
Figure 6-1. Cytopenia induction in Kit$^{W+}$ mice. ................................................................. 233

Figure 6-2. Cytopenia induction in Sl heterozygous mice.................................................... 235

Figure 6-3. Cytopenia induction in EpoR heterozygous null mice......................................... 237

Figure 6-4 Cytopenia induction in Stat5a$^{+/+}$/b$^{+/+}$ mice......................................................... 239
List of Abbreviations and Acronyms

129 129S1/SVimJ
2N dipoid
A adenine
aa amino acid
ADP adenosine diphosphate
Ank ankyrin
ANOVA analysis of variance
B6 C57BL/6J
BFU-E burst-forming unit erythroid
BM bone marrow
bp base pair
BSS Bernard-Soulier syndrome
C cytosine
C3H C3H/HeJ
CBC complete blood count
CFU-C Colony forming unit in culture
CFU-E colony-forming unit erythroid
CFU-G colony forming unit granulocyte
CFU-GM colony forming unit granulocyte monocyte
CFU-MK colony forming unit megakaryocyte
CFU-S colony forming unit spleen
CMHD Centre for Modeling Human Disease
c-Myb myeloblastosis oncogene
COOH carboxy
Cre causes recombination
D aspartic acid
d day
DHS Dehydrated hereditary stomatocytosis
DNA deoxyribonucleic acid
E glutamic acid
EDTA ethylenediaminetetraacetic acid
ENU n-ethyl-n-nitrosurea
Epo erythropoietin
EpoR erythropoietin receptor
FBS fetal bovine serum
FITC Fluorescein isothiocyanate
FUTP fluorouridine triphosphate
G guanine
G1  first generation
G2  second generation
G3  third generation
ns  not significant
GSF  Gesellschaft fuer Strahlenforschung
HCT  hematocrit
HE  hereditary elliptocytosis
HET  heterozygous
Hgb  hemoglobin
HO  hereditary ovalcytosis
HOM  homozygous
HS  hereditary spherocytosis
HSC  hematopoietic stem cell
HU  hydroxyurea
IL  interleukin
IMDM  Iscove’s Modified Dulbecco’s Medium
IP  interperatineal
ITP  immune (idiopathic) thrombocytopenia
K/O  knockout
kb  kilobase
kDa  kilodaltons
kg  kilogram
KTLS  c-kit\(^+\), Thy-1\(^{lo}\), Lin\(^-\), Sca-1\(^+\)
1  liters
lin  lineage
Lox-P  locus of X-over P
M  molarity
Mb  megabases
MCH  mean corpuscle hemoglobin
MCHC  mean corpuscle hemoglobin concentration
MCV  mean corpuscle volume
mg  milligram
MgCl\(_2\)  magnesium chloride
min  minutes
MK  megakarocyte
MPV  mean platelet volume
Mpl  thrombopoietin receptor
mTPO  mouse recombinant TPO
Myh  myosin heavy chain
MYH9\(\Delta\)  Mk lineage restricted ablation of Myh-9
MYH9RD  Myh9-related disorder
N  asparagine
NA  not affected
nb  normoblastic anemia
NH₂  amino
NMMHC  non muscle myosin heavy chain
NMMII  Non-muscle myosin II
nt  nucleotide
°C  degrees Celsius
OHS  overhydrated hereditary stomatocytosis
PB  peripheral blood
PCR  polymerase chain reaction
PHZ  Phenylhydrazine
PLT  platelets
PRP  Platelet rich plasma
Q  Glutamine
R  arginine
RBC  red blood cell
rh  human recombinant
rm  mouse recombinant
RNA  Ribonucleic acid
RR  ribonucleotide reductase
s  seconds
SCF  stem cell factor
SD  standard deviation
SNP  single-nucleotide polymorphism
SSLP  simple sequence length polymorphisms
T  thymine
TBST  TBS + tween
TPO  thrombopoietin
TRAP  thrombin receptor activation peptide
Tris  tris (hydroxymethyl) aminomethane
TS  Thymidylate synthetase
U  unit
vs  versus
WT  wildtype
X  stop codon
List of Appendices

6.1 Statistical Analysis from Chapter 4 .................................................................221

6.2 Cytopenia Induction in Known Hematopoietic Mutants ..............................229
   6.2.1 Materials and Methods .............................................................................230
   6.2.2 Results .......................................................................................................231
   6.2.3 Discussion .................................................................................................239
   6.2.4 References ...............................................................................................242
Chapter 1: Introduction
1.1 The Mouse as a Genetic Model for Human Disease

The mouse is the most frequently used model organism in biomedical research. It is excellent for genetic studies because it is easy to maintain, has a well-characterized genome that is similar to humans (99%) and can model several human diseases. Mice have a short generation time [1], deliver large progenies and breed year round. Thus rapid genetic and pathophysiological characterization of large numbers of animals is possible [1, 2]. Studying all life stages in the mouse is possible because of its accelerated life span (1 mouse year = 30 human years). Another benefit to using the mouse is its ability to tolerate inbreeding well, compared to other mammalian species. Inbred mouse strains provide a defined genetic background reducing variability due to polymorphic variations[3]. The mouse genome was the first mammalian genome to be sequenced after the human genome in 2000 [4, 5]. The first mouse genome to be sequenced was a mix of different mouse strains (129S1/SvImJ, 129X1/SvJ, A/J, C57BL/6 and DBA/2J) [2], while in 2002, the C57Bl/6J mouse strain was fully sequenced [6]. Mice and humans share ninety-nine percent of genome identity and also retain gene functions [6]. The mouse genome is relatively easy to manipulate [2]. For example, mouse embryonic stem cells (ES cells) can be genetically engineered to cause lack of gene expression, conditional gene expression, reduced or increased gene expression or the expression of an altered protein product. The pathogenic consequences of genetic mutations in mice parallel those in humans [3]. Mouse models of human disease thus allow for in-depth biological studies which are not feasible in humans [1].

1.2 Forward Mutagenesis Screens

1.2.1 History

The house mouse was originally used in early genetic studies. The various visible phenotypes observed in the house mouse were found to be caused by spontaneous mutations [2]. By the
1970s, a large collection of spontaneous mutant mice were being analyzed[1]. However, the use of spontaneous mutations to examine the function of genes had its limitations, due to low spontaneous mutation rate in mouse germ cells (5x10^-6 per locus) [1]. In order to examine the function of all of the genes in the mouse genome more quickly, it soon became clear that physical or chemical means of inducing mutations must be found [2].

In 1927, Hermann Joseph Muller was the first to show that X-rays could be used to induce heritable mutations [2]. X-ray mutagenesis can induce mutations at a rate 20 times higher (13x10^-5 to 50x10^-5) than spontaneous mutations. The types of mutations that are elicited by X-rays include: chromosomal inversions, deletions and translocations. One of the drawbacks to using X-ray mutagenesis is that it causes large genetic lesions that affect multiple genes[7].

Since the discovery of X-ray mutagenesis, many chemicals have been tested for their ability to enhance the mutagenesis rate in germ cells in several model organisms. Spermatogenesis is a developmental process that results in the production of mature spermatozoa. It starts with spermatogonia that have self-renewal properties, but also can undergo meiosis to become spermatocytes, spermatids and finally spermatozoa. Various chemical mutagens have been categorized based on the stage of spermatogenesis affected. Chemicals affecting early spermatogenesis (spermatogonia stem cells) include: n-ethyl-n-nitrosurea (ENU), methylnitrosurea, procarbazine and triethylenemlamine. Chlorambucil and melaphalan were shown to affect mid-stage spermatogenesis (early spermatids). Late spermatogenesis (late spermatids and spermatozoa) is affected by ethylmethanesulfonate methyl methanesulfonate cyclophosphamide and diethylsulfate. Small genetic lesions are associated with chemicals affecting early spermatogenesis, while larger genetic lesions are made by chemicals affecting mid to late spermatogenesis[2]. In the 1970s, the Russell research group at the Oakridge National
Laboratory was the first to use ENU in the mouse. The group found that ENU is the most powerful mutagen of spermatagonia in mice [8], inducing mutations at a frequency of up to 5 $\times 10^{-3}$ [9-11]. ENU was shown to mainly induce point mutations in spermatagonia and ENU treated males were observed to produce mutant progeny for many months[12]. These findings, served as a milestone in mouse genetics, allowing the generation of new mouse mutants [1].

In the 1980s, geneticists began to embrace the technique of ENU mutagenesis, using ENU in a region-specific screen to dissect the mechanisms underlying the T complex [13, 14]. The first genome-wide recessive screens were conducted by Bode and by McDonald who studied the genes involved in the metabolism of phenylalanine that when mutated cause phenylketonuria [15, 16]. Brown at the Medical Research Council, UK and Rudi Balling at the Gesellschaftfueter Strahlenforschung (GSF) in Germany were the first to independently conduct large-scale mouse ENU mutagenesis projects [17, 18].

1.2.2 Forward vs Reverse Genetics

Two opposing, yet not mutually exclusive approaches exist for examining gene function in the genome, forward and reverse genetics. Reverse genetics is a gene-driven approach (gene to phenotype), where known genes are manipulated to produce genetically-modified mice. Forward genetics is a phenotype-driven approach, where mutagenized mice are examined for phenotypes of interest without previous knowledge of the genetic basis of mutation [19].

1.2.3 Reverse Genetics

Reverse genetics has traditionally been dominated by gene targeting. Gene targeting uses homologous recombination to delete or replace an endogenous gene [20] with another deoxiribonucleic acid (DNA) sequence. The genetically modified mice are examined for the resulting phenotypes: molecular, cellular and physiological. One of the pitfalls of reverse
genetics is that it requires some knowledge of the gene being targeted, and therefore, novel genes are seldom targeted. However, genome-wide association studies in humans have identified many genes with possible association to genetic disease, which provides potential candidates for gene targeting[19].

Reverse genetics can be combined with ENU mutagenesis as a successful approach to finding new mutations that alter the function of a gene of interest. ENU is attractive because it has the ability to give a full range of functional changes in any gene, such as hypomorphs, antimorphs and neomorphs, which go beyond the traditional nulls created by gene targeting. DNA archives from either the sperm of ENU-mutagenized mice or ENU-treated embryonic stem cells can be screened for mutations in candidate genes. Three options exist for mutation detection in such archives: temperature gradient capillary electrophoresis, denaturing high performance liquid chromatography and direct sequencing. Both temperature gradient capillary electrophoresis and denaturing high performance liquid chromatography provide high throughput and cost-effective methods for screening candidate genes for ENU induced single-nucleotide polymorphism (SNP) and then subsequent sequencing can be conducted on likely mutations[19].

Several groups have established parallel archives of genomic DNA and sperm, which were collected from G1 males used in ENU mutagenesis screens (e.g. Harwell, Riken, GSF, and Australian Phenomics Facility). Genomic DNA from G1 males is first screened for mutations within a specified region of a candidate gene and then potential mutations are confirmed by sequencing the corresponding sperm archive. Finally, the sperm archive is used to recover mutants of interest by using in vitro fertilization [19]. The first successful example of this was published in Nature Genetics in 2002 by Brown’s group at Harwell. In over 2000 DNA samples, four candidate genes were screened for silent, missense and stop mutations[21]. Similar studies
have been performed by other groups [22, 23]; however, one of the limiting steps of this approach is the production of mice from sperm archives. For example, Michaud et al. used intracytoplasmic insemination to recover mutants, because of problems faced using in vitro fertilization with sperm from a C57Bl/6 background[23]. In order for this approach to be effective, high quality sperm cryopreservation is needed [19].

ENU mutagenesis has been shown to be particularly advantageous in embryonic stem cells. The optimization of drug dosage can occur without the worry of animal viability or welfare, meaning that the mutational load can be quite high. The use of ENU-treated embryonic stem cells also eliminates the need to breed or house mice for the creation of the archive, which reduces costs and allows for the creation of large archives. Embryonic stem cells can also be directly phenotyped. In 2000, a number of groups showed that it was possible to mutagenize embryonic stem cells in culture and to create chimeric mice capable of germline transmission from these cells. A publically held archive is available in North Carolina containing over 3000 clones[19]. This approach was used successfully to create mice with mutations in Smad2/4 and c-Kit [24, 25].

1.2.4 Forward Genetics

For many years researchers have used forward genetics to uncover new mouse models of human disease. A phenotype-driven approach is unbiased, because it makes no assumptions with regards to the genetic basis or cause of a particular disorder. ENU has been a principle mutagenesis strategy in phenotype-driven screens of the mouse. A variety of considerations must be taken into account with ENU mutagenesis including mouse strain, ENU dose, breeding strategy and method of phenotypic detection. The success of any screen depends on the optimal selection of
these conditions, which are often dictated by the phenotype or disease that one wishes examine[19].

One of the first choices a researcher must make is whether to perform a dominant or recessive screen. In a dominant screen, a phenotype must be detected when only one copy of the mutant gene is present; whereas a recessive screen allows for the identification of genetic mutations that are inherited in a recessive fashion (i.e. both copies of the gene carry the mutation). The decision of whether to perform a dominant or recessive screen is frequently decided by the type of disease one wishes to examine, but the advantages and disadvantages of each type of screen, as discussed below, must also be considered [19].

**Dominant screen:** In a dominant screen, male mice are mutagenized with ENU (G0 male) and then bred to wild-type females to produce G1 (first generation) progeny, which consists of mutagenized offspring carrying many heterozygous mutations. The G1 progeny are then screened for a particular phenotype (Figure 1A). Dominant screens have a simple breeding scheme requiring a small number of animals, thereby reducing costs [19]. Dominant phenotypes can be difficult to find, because the mutation only occurs once (each G1 animal carries a unique set of mutations) and variations in expressivity can mean that subtle phenotypes are often missed. For example, the brachyury mutant T/+ phenotype varies from a few kinks in the tail to a tail that is completely absent; therefore, efficient phenotyping protocols are required. Mice carrying dominant mutations must also have near normal fertility and survival rates (i.e. no embryonic lethality) for the dominant screen to be successful. For example, Apc\(^{\text{Min}}\), a dominant mutant which causes multiple intestinal neoplasia, is so severe on the C57Bl/6 background that the mutation would have been lost upon breeding [26].
Figure 1-1. Breeding schemes for a dominant and recessive ENU screen.

A) Dominant Screen. Male mice are treated with ENU and after the male mice have recovered from a period of sterility they are breed to WT females. The ENU induced mutations in the spermatogonial stem cells in the ENU treated males are passed on to their offspring (G1). Screening for phenodeviant occurs on G1 mice.

B) Recessive breeding scheme commonly occurs in two versions (1 and 2). A recessive screen begins similarly to a dominant screen, ENU treated male mice are breed to WT female mice to produce the first generation of mice. G1 mice are then breed to WT mice to produce a second generation (G2). In version 1 (backcross), G2 female mice are breed to their G1 father to produce a third generation (G3). Alternatively, in version 2 (intercross), G2 females are crosses to their G2 brothers to produce G3 mice.

Regardless of the breeding scheme, screening for phenodeviant mice is performed on G3 mice.
**Recessive Screen:** A recessive screen involves a three-generation breeding scheme, which can be conducted in at least two ways. G0 males are bred to wildtype females to produce G1 progeny and then G1 males are crossed to wildtype females to produce G2 progeny. G2 females can either be crossed to other G2 males from the same pedigree (intercross) or they can be crossed to their fathers (backcross). In either case G3 progeny are produced. G3 animals are then screened for a particular phenotype [1, 7, 19]. In a recessive backcross, some litters will have 25% of pups affected, whereas other litters will have no affected animals [1, 7]. Alternatively, in a recessive intercross screen, 1/16 of pups from all litters are expected to show the mutant phenotype, which requires more breeding cages. A recessive backcross screen maintains 3/8 of the original mutagenized strain, whereas a recessive intercross screen maintains 1/4 of the original mutagenized strain, which means that a recessive intercross will have a reduced number of loci that can contribute to the phenotype making it easier to identify the causative mutation (Figure 1B). Research teams with more resources tend to use a recessive intercross screen and those with fewer resources use a recessive backcross [7].

In general, a recessive screen is advantageous because 90% of mutations are inherited in a recessive fashion, with mutations compensated for by the wild-type allele when it is present [19]. Thus, there is a greater chance of identifying mutations that contribute to a specific phenotype, and once detected, lines are easier to maintain [1]. Recessive screens, however, are complex, require intensive animal caretaking, large breeding facilities and more money when compared to dominant screens [1, 19]. The complex nature of recessive screens means that large scale genome-wide screens are not widely performed, although they have been performed in England at Harwell and in Germany at GSF. Recessive screens have been shown to be highly productive in focused screens (e.g. immune screening in Australia and a region-specific screen in USA)[27, 28].
Region-Specific Screen: Geneticists have been quite creative with the use of designer chromosomes to perform region-specific genetic screens. Designer chromosomes contain a rearrangement at a specified area in order to examine a specific region of the genome. Chromosomal rearrangements have been created by two strategies: irradiation and the Cre/LoxP system (causes recombination/ locus of X-over P) [1]. Rinchik was the first to perform a region-specific recessive screen using a mouse line that carried a visible deletion on chromosome 7. Rinchik used coat color to distinguish between mice that had no mutation, mice that carried a mutation outside the region of interest and mice that carried a mutation in the region within the chromosomal abnormality. A two-generation screen was used, where males homozygous for an albino coat color allele were treated with ENU (G0). G0 males were then crossed to wildtype females to create G1 progeny. Next the G1 males were crossed to females that were heterozygous for a deletion that spanned the albino coat color allele and also carried the chinchillia coat color allele, to produce G2 progeny. The G2 progeny were then easily divided into three separate groups: mice with normal coat color, which did not carry an ENU mutation within the region of interest, mice with a coat color that was intermediate between albino and chinchilla (light chinchilla), which were carriers for a mutation in the region of interest, and mice with albino coat color, which carried a recessive mutation in the specified region. Embryonic lethal mutations were also easily detected, because when breeding pairs that produced no pups with the albino coat color, this meant that a new lethal recessive allele was present [29].

A balancer chromosome is a chromosome with an inversion within a specific region and this inversion suppresses recombination within the particular area. Unlike mice with chromosomal deletions, mice with balancer chromosomes are viable and indistinguishable from normal mice. Justice was the first to perform a region-specific screen that used a balancer chromosome in mice. Specifically, a 34 Mb (megabases) inversion was created on chromosome
in order to study the specified region. Coat color, coat texture (curly vs. straight), and a mutation in Wnt3 were used to distinguish normal mice, mice that carried an ENU-induced mutation not within the inversion, and mice that carried an ENU-induced mutation within the inversion [28].

Sensitized Screen: A sensitized or challenge screen is a genetic screen that uses genetic background, environment or pharmaceuticals to exacerbate subtle phenotypes that are not normally detected by conventional genetic screens. For example, salt can be used to test for susceptibility to hypertension. A sensitized screen can be used to detect dominant mutations in genes that are linked to human disease, but often human diseases are caused by mutations in multiple interacting genes [7]. For example, mutations in BRCA1 and 2 predispose humans to breast cancer, but not all humans carrying mutations in BRCA1 or 2 get breast cancer, because of the influence of other genes and environmental factors [30]. Infection challenge has been successfully used to assess genes that influence host susceptibility to pathogens. For example, when infected with MCMV mutant mice were identified as having increased susceptibility to infection. Specifically, a mutant mouse was identified that carried a missense mutation in STAT-1, which resulted in a STAT-1 protein that could not be phosphorylated at a critical residue, and therefore, STAT-1 function was compromised [31].

Genetic Modifier Screen: A genetic modifier screen is a special type of sensitized screen that looks for genetic modifiers of known gene mutants. ENU is particularly useful as a mutagen in a screen of this type because it gives a full range of mutations including loss and gain of function, thereby, creating suppressors or enhancers of a particular phenotype. Suppressors of a disease phenotype reveal potential drug targets, while enhancers help to identify new disease genes or pathways that can also aid drug discovery. Genetic modifier screens can be performed as
dominant or as recessive screens. In a dominant genetic modifier screen, ENU-mutagenized males are mated with females heterozygous for a mutation in the gene of interest. Recessive genetic modifier screens are very labour intensive and costly because they require a three generation breeding scheme [19].

Carpinelli et al., conducted a large-scale dominant suppressor screen for thrombocytopenia utilizing thrombopoietin receptor knock-out mice (mpl−/−). Mpl−/− males were injected with ENU and then mated to mpl−/− females. The resulting G1 progeny were analyzed for PLT (platelets) levels three SD (standard deviation) or higher than untreated mpl−/− mice. Five outliers were detected and the founding mutation has been identified and published for four. Two have distinct mutations in c-myb (myeloblastosis oncogene), one has a mutation in p300 (E1A binding protein) and the other has a mutation in Suz12 (suppressor of zeste 12 homolog) [32-35].

1.3 Mechanisms of ENU Mutagenesis

N-ethyl-N-nitrosurea or ENU is currently the most potent agent for causing mutations in the mouse [8] and is relatively easy to handle compared to other mutagens[1]. ENU is an alkylating agent that can add ethyl groups to a number of nucleophilic nitrogen and oxygen groups on each of the four deoxyribonucleotides [36]. During DNA replication, the ethylated bases mispair and ultimately cause base pair substitution or loss, if not repaired [37]. Cells normally contain DNA repair mechanisms that fix such ethylated deoxyribonucleotides [38]. Three repair mechanisms used are: O6-alkylguanine-DNA alkyltransferase, nucleotide excision repair and mismatch repair. Animals deficient in any of these three repair mechanisms have increased rates of mutagenesis following ENU treatment [39, 40]. After DNA repair mechanisms are saturated, the ENU-induced mutation rate increases in a dose-dependent manner until health or fertility is affected [38].
The most common site for ENU generated point mutations is at A-T base pairs, where 70 to 80% of all mutations are either A-T to T-A or A-T to G-C (A adenine, C cytosine, G guanine, T thymine). G-C to C-G mutations are rarely seen. An A-T mutation bias means that some amino acid changes will be under-represented in ENU-induced mutagenesis. As well, an A-T mutation bias means that certain genes or domains will be mutated more often than others, because of differences in nucleotide make-up [41]. Other ENU mutagenesis biases exist and include strand location and residue context. For example, ENU preferentially mutagenizes thymine bases located on the non-transcribed DNA strand, and preferentially mutagenizes residues flanked by G or C in G/C rich regions [42, 43]. Despite these biases, ENU has been very effective in generating novel mutant models for more than 3 decades.

Approximately 70% of all ENU-induced mutations are non-synonymous and 30% result in silent or synonymous mutations [43]. In multiple surveys of non-synonymous ENU-induced mutants, approximately 63% were missense (amino acid change), 26% resulted in abnormal splicing, 10% were nonsense (introduced stop codon) and 1% were “makesense” (stop codon to amino acid) mutations. On a functional level, the most common form of mutation produces hypomorphs (partial loss of function), but one can also obtain hypermorphs (increased function), neomorphs (novel function), or antimorphs (dominant-negative function). Approximately 10% of all mutations are null [7].

A critical step in performing an ENU-mutagenesis screen is to determine the optimal dose of ENU [19]. The activity of ENU is determined by a balance between its alkylation efficiency and efficiency of the cellular repair mechanisms [1]. An optimal dose of ENU should provide the maximum mutational load, while having minimal effects on health and fertility [19]. Different mouse strains have varying levels of resistance to ENU, the most resistant strain is
BTBR, and the most sensitive is FVB. Balb/c and C3H are rather resistant to ENU, which makes them a good choice for use in mutagenesis screening, as demonstrated by the Medical Research Council in England and the GSF in Germany [17, 18]. Although C57Bl/6 mice are one of the most popular mouse strains for biomedical research, they often fail to regain fertility following ENU treatment, making them a poor choice for mutagenesis screening [44]. The mutation rate for a given dose of ENU can be assessed by a Specific Locus Test. The Specific Locus Test was designed to score for genetic damage in mammalian germ cells using visible markers. ENU-mutagenized males are mated to females from a tester strain that is homozygous for recessive alleles at 7 loci [45]. It has been found that an optimal dose of ENU will induce a mutation every 1 to 1.5Mb. Given this mutation rate, a loss-of-function mutation in a given gene should occur in 1 out of 700 gametes. This rate, however, is not a good indication of how often a particular disease will be observed. The rate at which one will observe a disease is determined by: the effectiveness of the screening technique, the number of genes that when mutated will cause a particular disease and the fact that not all mutations will cause disease. It is expected that any G1 animal in a mutagenesis screen will have between 25 to 50 mutations [46]. Multiple mutations in G1 animals can confound analysis, but this is rarely a problem. Every generation of breeding reduces the mutational load by one half, meaning that mutations unrelated to the phenotype under study are quickly eliminated. Once the mutational interval has been refined to 5Mb or less, it is statistically unlikely that more than one mutation will be found within the interval [19].

The primary site of action of ENU in the male mouse is on the pre-meiotic spermatogonia stem cells. Spermatogonia are repair-competent cells and are able to efficiently repair alkylated nitrogen atoms [37]. An ENU-treated male mouse will initially be sterile because of the depletion of partially and fully differentiated spermatozoa. After ENU-treatment the remaining spermatogonia repopulate the testis. Differentiation of spermatogonia into mature spermatozoa
takes approximately 10 weeks. The length of sterility is a sign of an effective ENU treatment, but sterility should not last longer than 10 weeks[9]. Male mice have between 150 to 200 spermatogonial stem cells and mutagenized males can produce 100 to 150 offspring. In order to avoid repeated identification of the same mutation, it is recommended that only 30 to 50 G1 mice are examined from any one G0 male [7]. It should be noted that female mice can also be treated with ENU and used as G0 breeders, but ENU-treated females can only be set up with one male at a time, while ENU-treated males can be set up with many females at once making breeding much more efficient [1].

While ENU is one of the most potent mutagens in mice and has successfully been used for many years, it has limitations. There is an upper limit to the mutation rate that can be achieved before ENU becomes harmful to the health of the animals or impedes their fertility. Not all kinds of mutations are produced by ENU. Most mutations caused by ENU that have been reported are located within the coding region or the splicing region of genes rather than within regulatory regions. It should be noted, however, that regulatory mutations are also harder to detect. Smaller genes are also less likely to be mutated by ENU because of their size. Mutations which produce a late onset phenotype are also unlikely to be detected in an ENU-mutagenesis screen, because most labs lack the resources to keep the mice for extended periods of time [1].
Table 1-1. A Sampling of ENU Mutagenesis Screens Published

<table>
<thead>
<tr>
<th>ENU Mutagenesis Centre</th>
<th>Genetic Approach</th>
<th>Mouse Region</th>
<th>Summary of Major Screen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammalian Genetics Unit, MRC, Harwell, United Kingdom</td>
<td>Dominant Recessive</td>
<td>Genome-wide Ch 13 36H Del</td>
<td>Basic neurobiology, hearing, vision, development, clinical chemistry</td>
</tr>
<tr>
<td>GSF, Neuherberg Germany</td>
<td>Dominant Recessive</td>
<td>Genome-wide Genome-wide</td>
<td>Dysmorphology, clinical chemistry, Immunology</td>
</tr>
<tr>
<td>Neuroscience Center and Heart, Lung, Blood and Sleep Disorders Centre, Jackson Laboratory, Bar Harbour United States of America (USA)</td>
<td>Dominant Recessive</td>
<td>Genome-wide Genome-wide, Ch 5</td>
<td>Neurobiology, heart, lung, blood and sleep disorders</td>
</tr>
<tr>
<td>RIKEN Genomic Sciences Center, Yokohama, Japan</td>
<td>Dominant Recessive</td>
<td>Genome-wide Genome-wide</td>
<td>Behavior, hematological, urinalysis, clinical biochemical analysis, X-ray imaging; late onset: vision, blood pressure, hearing, tumorigenesis</td>
</tr>
<tr>
<td>Oak Ridge National Laboratory, Oak Ridge, USA</td>
<td>Recessive</td>
<td>Ch 7, 10, 15 and X</td>
<td>Aging, auditory, drug and ethanol abuse, epilepsy, eye, general behavioral, neurohistology, social behavior</td>
</tr>
<tr>
<td>Case Western Reserve University, Cleveland, USA</td>
<td>Dominant Sensitized</td>
<td>Genome-wide</td>
<td>Circadian, general, behaviour, ataxia</td>
</tr>
<tr>
<td>Mouse Genome Center, Baylor College of Medicine Huston, USA</td>
<td>Recessive</td>
<td>Ch 11, Ch 4</td>
<td>Developmental phenotypes</td>
</tr>
<tr>
<td>Center for Modeling Human Disease, Toronto, Canada</td>
<td>Dominant Sensitized</td>
<td>Genome-wide</td>
<td>Development, bone, cardiac and kidney function, learning and memory, hematopoiesis</td>
</tr>
<tr>
<td>Medical Genome Center, Australia National University, Canberra, Australia</td>
<td>Recessive</td>
<td>Genome-wide</td>
<td>Immunology</td>
</tr>
<tr>
<td>University of Pennsylvania, Philadelphia, USA</td>
<td>Recessive</td>
<td>Ch 5</td>
<td>Circadian and general behavior</td>
</tr>
<tr>
<td>Northwestern University, Chicago, USA</td>
<td>Recessive</td>
<td>Genome-wide</td>
<td>Circadian rhythm, general behavior, learning and memory, stress and psychostimulant response, vision</td>
</tr>
<tr>
<td>McLaughlin Research Institute, Montana, USA</td>
<td>Recessive</td>
<td>Genome-wide</td>
<td>Behavior, neurodegeneration, prion disease, hearing, ear and kidney development, peripheral myelination</td>
</tr>
<tr>
<td>Brigham and Women’s Hospital, Boston, USA</td>
<td>Recessive</td>
<td>Genome-wide</td>
<td>Embryonic abnormalities</td>
</tr>
<tr>
<td>Memorial Sloan-Kettering Cancer Center, New York, USA</td>
<td>Recessive</td>
<td>Genome-wide</td>
<td>Embryonic, neural tube closure</td>
</tr>
<tr>
<td>Scripps Research Institute, La Jolla, USA</td>
<td>Modified Recessive</td>
<td>Genome-wide</td>
<td>Innate immune response</td>
</tr>
</tbody>
</table>
1.4 ENU Mutagenesis Screen Design

Choosing the right phenotyping method for an ENU-mutagenesis screen is essential for success. The phenotyping approach must be simple, reasonably rapid, standardized and should not involve sacrifice of the animal in the case of a dominant screen [7]. Simple phenotyping methods improve the chances of being able to efficiently following a phenotype in subsequent generations, therefore, requiring fewer mice for mapping [47]. Genetic mapping and eventual cloning of a causative mutation usually involves testing hundreds or thousands of mice, so the testing method must be reasonably rapid. In order to easily detect outliers, a phenotyping assay must have low variability within normal inbred or hybrid strains [7]. Also, the phenotype being tested must be stable from week to week to simplify analysis (i.e. the same cut off used throughout the period of screening). Viability and fertility of the animals that are being tested must also be maintained throughout the testing period, particularly in a dominant screen, because G1 animals are the only ones carrying the mutation and death or loss of fertility thus results in loss of the specific mutation [47].

Phenotyping assays are often miniature versions of what would be used in human clinics, such as measuring blood glucose, hematological and immunological parameters, bone mineralization, cardiovascular and renal function, and behaviour. For example, blood pressure is assessed with a miniaturized blood pressure cuff on the tail of the mouse. In a quantitative screen, animals are considered affected if they are 2 to 3 SD outside the norm (outliers) and only 0.1% to 2% of G1 animals (in dominant screen) are expected to show scorable defects [7].

A critical step in designing a successful ENU screen is choosing an appropriate mouse strain for mutagenesis and for backcrossing [7]. More than 3000 mouse strains exist and include: inbred, outbred, hybrid, congenic, recombinant inbred and transgenic [2, 48]. Inbred strains of
mice are typically chosen for ENU screens because of their genetic uniformity, which equates to phenotypic uniformity. Phenotypic uniformity allows one to clearly distinguish mutant mice from their wild type counterparts. Typically, common inbred mouse strains (e.g. C57Bl/6, Balb/c, C3H and 129) are used for ENU mutagenesis, because of the large amount of pre-existing knowledge that exists for each strain [2]. The mutagenesis strain must be fairly resistant to ENU treatment in order to ensure that the mouse can carry a high mutational load while remaining healthy and fertile (e.g. Balb/c and C3H) [17, 18]. The mutagenesis and backcross strains must be significantly different from each other in order to effectively map the mutated locus, as differences between the two strains are exploited in genetic mapping. Conversely, the two strains must also be similar enough in order to follow a phenotype through several generations of backcrossing. This is because different strains of mice have different strain-dependent modifiers that can interfere with the mutated allele resulting in false-positive or false-negative phenotyping results. False positives can negatively affect genetic mapping and false negatives can result in the loss of the mutant [2]. Therefore, a careful balance of genetic similarities and differences must be attained between the mutagenesis strain and backcross strain.

In order to efficiently detect phenotypes of interest, the environment which mice live in must be highly controlled. Disease prevalence between studies is often difficult to compare, because differences in diet, light, time of year, infection and social behaviour all can influence phenotypic outcome. For example, Balb/c mice are prone to develop light-associated retinal degeneration and proximity to the light source will vary even in the same mouse room [49]. Variation in vision can greatly influence behavioural phenotypes that require visual cues; therefore, Balb/c mice may display differences in behavioural phenotypes based solely on their proximity to the light source in the room and not because of the influence of a genetic mutation. Infection may also alter a given phenotype. Infection with *Helicobacteri* species for example,
while relatively harmless can cause “phenotypes” that disappear if the bacteria are eliminated [50, 51]. The dynamics of a mouse deme within a cage can vary widely and alter phenotypes. For example, the pugilistic behaviour amongst Balb/c males is particularly stressful and can influence a phenotype that is being examined [48].

1.4.1 Heritability Test G1

After the ENU screen has been designed and implemented, mice that are outliers for a particular phenotype must be bred to determine if the phenotype is heritable. For example, in a dominant screen, G1 outliers are bred to the backcross strain and their offspring (G2 mice) are phenotyped to determine if any of the G2 offspring display the initial phenotype observed in the G1 mouse. In a dominant screen, 50% of the offspring are expected to have the founding G1 phenotype. When 50% of G2 animals have the founding G1 phenotype, the mouse line is classified as heritable with full penetrance. If no G2 animals exhibit the initial G1 phenotype, the founding phenotype is classified as non-heritable. A phenotype can be non-heritable if the initial phenotype is altered by differences in the environment or if the outlying phenotype overlaps the phenotype observed in wild type mice. Phenotypes can be heritable, but not fully penetrant, if the expressivity of the gene is variable.

1.4.2 Genetic Mapping: Fine and Rough

Once a heritable phenotype has been identified, the next step is to perform genetic mapping to identify the locus of the causative mutation. Genetic mapping correlates the inheritance of polymorphic markers of known chromosomal and the phenotype of interest to determine the location of the causative ENU-induced mutation. Polymorphic markers used in genetic mapping include: simple sequence length polymorphisms (SSLP) and SNP. SSLPs, or more specifically microsatellite markers, are a specific-type of CA repeats that occurs on average every 500kb
(kilobase), although not all microsatellite markers are polymorphic (informative) [2]. SNPs have now replaced microsatellites as the marker of choice because SNP coverage in the genome is one-thousand-times greater than microsatellite coverage [19]. Over 10 million SNP can be found in the Mouse Genome Informatics database, and individual SNP have been described in the order of 300bp (basepair) apart, although not all SNP are polymorphic [52]. Large SNP databases exist, providing information on millions of SNP in the genomes of many different inbred strains [19].

A rough genetic map is created to determine the general chromosomal location of the mutation. A rough map usually narrows the mapping interval to approximately a 20Mb region on a specific chromosome. After the general location of mutation is determined, a fine genetic map is performed to narrow the interval to 1Mb, if possible. Genetic mapping tests for the segregation of the mutagenized parental allele with the phenotypic trait. In a dominant screen, initial genetic mapping is performed on G2 animals, as G1 mice are uninformative because they are heterozygous at all loci. However, in a recessive screen, the G3 progeny is used for both phenotyping and genetic mapping [53].

Interval haplotype analysis is a method of chromosomal exclusion that can be conducted with a minimum of two markers at opposite ends of each chromosome. Traditionally this type of analysis was conducted with 40 to 100 SSLP. In a dominant screen, chromosomes that are inherited as non-recombinants (i.e. carry markers from a single strain) for the unmutagenized strain are excluded as candidates for carrying the causative mutation. The chromosome where the causative mutation is located will be heterozygous somewhere along its length in all phenotypically affected mice. False negatives can be produced in interval haplotype analysis if a double cross-over has occurred on a particular chromosome. Interval haplotype analysis is most
effective if conducted on a G2 backcross generation, because after this the parental alleles become fixed. Additional generations become informative in genetic mapping when assessing a candidate interval, because each successive backcross decreases the regions that do not carry the mutation but are heterozygous for the mutagenesis strain [53].

It is critical to determine the genetic map position as quickly as possible, because it allows the determination of whether the locus is novel or not. A defined mapping position can indicate a candidate gene for further examination either by sequencing or expression analysis. A defined recombinant interval will also allow for the detection of heterozygous carriers of the mutation by genotyping without the need for phenotyping, thereby, reducing cost and labour [53]. SSLP mapping provides a genetic map of low to moderate resolution and is quite-labour intensive. An additional downside to SSLP analysis is that no one panel of SSLP will work for all strain combinations. SNP mapping has become the common way to genetically map ENU mutants, because it provides dense genome coverage, polymorphisms have been described for many strains and high-throughput methods to detect SNP are available. Genome scans can be conducted using whole genome SNP panels that can screen multiple inbreed strain crosses. For example, Illumina has developed a panel with as few as 377 SNP that can be used to analyze several mouse strains [54].

A trait is easy to map if it has a binary distribution, where unaffected mice are easy to distinguish from affected mice. Mapping can be problematic if the distribution of a trait in the affected and unaffected mice overlaps or if a phenotype has low penetrance. The phenotype to genotype correlation is crucial in mice with recombination’s, especially if an overlap in the phenotype of affected and unaffected individuals exist, therefore, it becomes essential to breed any animals with critical chromosomal recombination’s.
1.4.3 Finding the Causative Mutation

Once the candidate interval has been narrowed to a feasible size (approximately, 5Mb), multiple options exist for finding the causative mutation: one can sequence candidate genes, sequence genes in the interval at random, or sequence the entire interval. Determining when it is time to begin sequencing depends upon several factors: the size of the interval, the likelihood of getting further recombinations and the quality of candidates within the region. Before sequencing genes, one must determine what regions to sequence. Usually the coding region and intron-exon borders are sequenced because almost every ENU-induced mutation has been found in the coding region. Structurally and functionally conserved regions of genes are good candidates for sequencing because they are likely to be critical to gene function. If no mutation is found in these regions, 5’- and 3’-untranslated regions can be sequenced. Candidate genes can be ranked on the following criteria: mutations in the gene itself or genes in the same biochemical pathway which are known to yield similar phenotypes; the expression of the gene in a tissue that correlates with the phenotype of interest; the expression level of gene is altered in affected mice; or whether a real transcript exists for the gene. A candidate gene approach will involve sequencing the highest ranked genes first followed by lower-ranked genes. If an interval has no obvious candidate genes, then genes can be sequenced at random or all at once. An alternative to sequencing genes at random or if no mutations have been found at the intron-exon borders or within exons is to sequence the entire region [47].

Next-generation sequencing technologies have been developed that will allow for the resequencing of the entire mapping interval, and even the entire genome. In these new technologies massive parallel sequencing is conducted by several methods including: 454 sequencing, Solexa sequencing and Solid sequencing. Although each sequencing method is unique, all of them use arrays in which a single DNA molecule is clonally amplified in a
spatially distinct location in order to provide template DNA for sequencing. Complex genomes, like those of mouse and human, can be entirely sequenced but require a huge amount of computational power. Therefore in order to simplify computational analysis of large scale sequencing, sequence capture technology allows for specific regions of DNA to be isolated from whole genomic DNA, using a microarray based technology. The custom array contains probes (>60bp) that are specifically designed to select for discrete regions of the genome. The selected DNA can then be amplified and sequenced. Sequence capture technology and next generation sequencing have been combined successfully for the re-sequencing of both the human and mouse genomes [55-57].

1.4.4 Confirmation of Mutation

Once the mutation has been found, how does one confirm that the mutation is causative? Confirmation of ENU-induced mutations can occur directly or indirectly. Indirect methods include determining whether mutations in these genes or in genes in the same functional pathway are found in lower organisms or humans, and whether these mutations cause a similar phenotype. A more direct method of mutational confirmation is to show that the mutation directly alters the expression or activity of the encoded protein. Methods to directly prove that the detected mutation is causative include further analysis of independent alleles of the gene, insertional mutagenesis (knockin mutation), small interfering RNAs or ameliorating the phenotype through expression of the WT (wildtype) allele [47]. Additionally, hypomorph or null mutations can be confirmed by complementation assays or by the use of transgenic rescue. Anti-morphic and neomorphic mutations or mutations in a novel gene can be difficult to confirm.
1.4.5 Comparing Knockout to ENU Mutants

Once the complete sequences of the mouse and human genomes were known (2001 and 2000, respectively), geneticists were anxious to understand the function of all genes within the genome. One strategy was to create knock-outs (K/O) of all genes in the mouse genome through homologous recombination. While many groups have been creating knockouts for several years, it wasn’t until recently that groups have developed a systematic approach. Large-scale international projects have begun with the aim to knock out all the genes in the genome including: EUCOMM (European Conditional Mouse Mutagenesis), NORCOMM (North American Conditional Mouse Mutagenesis project) and KOMP (Knockout Mouse Project). The functional consequence of each K/O must be studied and an international consortium, EUMODIC (The European Mouse Disease Clinic), has been formed in Europe to begin to analyze some of the mice created by EUCOMM [3].

ENU mutagenesis can be used to complement these large-scale knockout projects, because ENU provides a full range of mutations. The use of archives of sperm from ENU-treated mice or ENU-treated embryonic stem cells would allow for the generation of an allelic series of mutations in any given gene [19]. Using ENU-generated mutations to complement K/O projects will provide more information about the mutated genes because there are many examples where mice with a knockout in a given gene and those with an ENU-induced mutation in the same gene have very different phenotypes. For example, an ENU mutant (dominant negative) showed that the Clock gene is a master regulator of circadian rhythm [58], but K/O of the Clock gene resulted in only mild circadian defects [59]. In other cases, K/O and ENU mutants have displayed completely different phenotypes, for example, knockout of the Af4 gene affects lymphocyte development, but an ENU-induced mutation in Af4 causes cerebellar degeneration [60].
1.5 Hematopoiesis

The hematopoietic system performs numerous functions including the transport of oxygen, removal of carbon dioxide through the lungs, and immune surveillance. Hematopoiesis is an intricately regulated homeostatic process whereby all of the differentiated blood cell lineages are maintained by progenitors which originate from hematopoietic stem cells. The hematopoietic stem cell (HSC) is capable of self renewal and differentiation into the nine lineages that make up the hematopoietic system: erythroid, megakaryocytic, monocytic, granulocytic, basophilic, eosinophilic, B-lymphocytic, T-lymphocytic, and natural killer cells (Figure 2). At any one time, most hematopoietic stem cells are mitotically quiescent. The transition from the stem cell pool to the progenitor pool involves the loss of self-renewal capability, restriction in differentiation capacity and an increase in proliferation. The first restriction in differentiation capacity leads to the formation of a committed myeloid progenitor or a common lymphoid progenitor. The common myeloid progenitor is capable of forming all myeloid cells including erythrocytes, monocytes, neutrophils, megakaryocytes, eosinophils, and basophils. T cells, B cells, and natural killer cells are produced from the common lymphoid progenitor. Multipotential progenitors differentiate into bipotential and unipotential progenitors upon receipt of a series of signals from cytokines; these signals can work alone, in concert with, or in opposition to each other. The effect of cytokines is restricted by the expression of certain cytokine receptors on the plasma membrane of progenitor cells. Once commitment to a single lineage has occurred, the unilineage progenitor divides to produce immature cells, which upon receiving additional cytokine signals mature into fully differentiated cells. Genetic insults perturbing hematopoiesis can manifest in disease, including cytopenias (reduced blood cells), cythemias (increased blood cells), and leukemia [61].
Figure 1-2. Hematopoietic hierarchy.

A proposed model of hematopoietic maturation pathways that originate with a HSC. Abbreviations: Bas, basophil; burst forming unit; CFU, colony forming unit; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; E, erythrocyte; Eo, eosinophil; G, granulocyte; M, monocyte; MEP megakaryocyte erythroid progenitor; MK, megakaryocyte.
1.5.1 RBC

The production of red blood cells (RBC) is a highly dynamic and regulated process that is called erythropoiesis [62]. Erythropoiesis can be broken down into three distinct stages and begins with a committed-erythropoietic progenitor [63]. The most primitive committed erythroid progenitor is the BFU-E cell (burst forming unit erythroid), a proliferative and mobile progenitor that resides in the bone marrow, but can also be found in the peripheral blood (PB). The CFU-E (colony-forming unit erythroid) cell arises from the BFU-E (burst-forming unit erythroid) and is a more mature erythroid progenitor that is highly proliferative [62].

The second stage of erythropoiesis includes morphologically identifiable precursors (erythroblasts) that retain the ability to divide. Four distinct processes occur at this stage: the accumulation of hemoglobin, the expansion of erythroblast number, the reduction in cell size and the condensation of the nucleus and eventual e-nucleation [64]. Erythroblastic precursors undergo progressive differentiation, whereby precursors become smaller in size and the nucleus becomes denser. Pro-normoblasts are the most immature and largest precursor, becoming smaller in size and the nucleus denser in the basophilic normoblasts and polychromatophilic normoblasts. The orthochromatophilic normoblasts have the smallest and most dense nuclei and are the final cell type before the nucleus is extruded from the cell to become a reticulocyte [64]. The final stage of erythropoiesis is the maturation of the reticulocyte to become a mature circulating RBC [65]. Reticulocytes contain residual RNA (ribonucleic acid) and initially reside within the bone marrow, but soon move into the PB. Once in the PB, reticulocytes dismantle their ribosomal machinery, expel organelles and assume a biconcave discoid shape thereby becoming a mature RBC [65].
The majority of erythroid development (CFU-E to immature reticulocyte) occurs within the bone marrow at erythroblastic islands. An erythroblast island contains a central macrophage that is surrounded by a ring of developing erythroid cells [63]. Developing erythroid cells express adhesion molecules that prevent them from entering the PB. The time between the appearance of the pro-normoblast and the formation of the mature RBC is strictly regulated, but during periods of stress this time period is shortened either by decreasing the inter-mitotic interval or by skipping mitotic divisions. Hemoglobin (Hgb) concentration regulates cell division within erythroid precursors. When hemoglobin synthesis is delayed or reduced in erythroid precursors, additional cell divisions occur, which results in RBC that are microcytic. Alternatively, when Hgb concentrations are high, erythroid precursors skip a cell division and nuclear extrusion occurs earlier, resulting in macrocytic RBCs (as in megablastic anemias) [66].

Erythropoietin (Epo) is the primary humoral regulator of erythropoiesis and promotes proliferation, differentiation, maturation and survival of erythroid precursors. The majority of Epo is produced in the kidney by renal peritubular interstitial cells which are sensitive to the local oxygen content. Hypoxia inducible factor is a transcription factor that is activated when oxygen levels are low and promotes the production of erythropoietin. The cognate receptor of erythropoietin is the Erytropoietin receptor (EpoR). EpoR is first expressed on BFU-E at low levels. The expression of EpoR is highest on CFU-E and pronormoblasts and then declines in more mature erythropoietic cells. Epo is not required for the production of BFU-E or CFU-E progenitors, but does influence their proliferation and differentiation. The effects of Epo are greatest on CFU-E: increased levels of Epo cause CFU-E to proliferate and differentiate and decreased levels of Epo cause CFU-E to undergo apoptosis. Excess levels of Epo influence the differentiation of pronormoblasts and basophilic normoblasts, but the presence of Epo is not critical after the polychromatophilic normoblast stage [66].
Diseases associated with defects in erythroid production include anemia (low hematocrit, HCT) and erythrocytosis (high HCT). Anemia is caused either by decreased RBC production or increased RBC destruction. Erythrocytosis or polycythemia are diseases associated with increased erythroid production and fall into three categories: those in which high levels of Epo are detected, those in which normal or low levels of Epo are observed, and myeloproliferative diseases [66].

Function: The principle function of RBC is to deliver oxygen to and remove carbon dioxide from all the tissues of the body. Oxygen transport within the body is made possible by haemoglobin, a molecule that consists of four polypeptide chains each carrying a heme group. Iron combines with protoporphyrin to form heme. Most iron used in the formation of hemoglobin does not come from dietary iron, but is liberated from old phagocytized RBC. RBC have a unique biconcave discoid shape, which allows RBC to readily distort their form and makes it possible for RBC to pass through narrow capillaries. Additionally, the biconcave shape of RBC increases the surface area of the cell and therefore increases the capacity for oxygen transport. The distinctive RBC shape is maintained by strict membrane architecture. To traverse capillaries, RBC must be able to undergo repetitive and reversible deformations [67]. In part, because of these repetitive deformations, RBC have an average life-span of 48 days in the mouse (90 days in human), after which old or damaged RBCs are removed from circulation by the spleen, where they are destroyed [68].

Membrane: RBC are dynamic anuclear cells that rely heavily upon their membrane to perform many functions. The membrane is critical for responding to extracellular signals, iron uptake, carbon dioxide diffusion, cation and water content regulation, cellular metabolism and for resistance to vascular adhesion [68]. The unique structural organization of the red cell membrane
enables the cell to undergo large reversible deformation, while still maintaining its structural integrity. High levels of elasticity in the RBC membrane endow it with the capacity to form long membrane extensions and withstand high levels of shear stress without undergoing fragmentation [67]. The red cell can deform with linear extensions up to 250% of its radius, yet only a 3 or 4% loss in surface area results in cell lysis, which demonstrates both the robust and delicate nature of RBCs [69].

The RBC membrane is composed a lipid bilayer, which consists of equal amounts of cholesterol and phospholipids and is studded with transmembrane proteins. Five types of phospholipids are located within the lipid bilayer and they are unevenly distributed in the outer and inner lipid monolayers. Phosphatidylcholine and sphingomyelin mostly reside in the outer monolayer, whereas phosphatidylethanolamine, phosphatidlyserine and phosphoinositides are mainly located within the inner monolayer [67].

Transmembrane proteins perform many functions in the RBC membrane; they are involved in cell identity, transmembrane transport, cell adhesion, and cell signaling and cell structural integrity. With over 50 transmembrane proteins known, half of the transmembrane proteins are blood group antigens. Band 3, glycophorin C and Rh-associated glycoprotein play critical roles in membrane structural integrity, by linking the lipid bilayer to the cytoskeleton [67].

Cytoskeleton: The RBC cytoskeleton is a multi-protein complex that lies beneath and is attached to the plasma membrane [68]. The cytoskeleton forms a lattice at the inner surface of the plasma membrane [69], where it provides strength, therefore, increasing the ability of RBC to deform. Additionally, the cytoskeleton is important for the proper distribution of intra-membrane particles and glycoproteins within the plasma membrane. The RBC cytoskeleton is mainly
composed of $\alpha$ and $\beta$ spectrin, but it also contains actin, protein 4.1, adducin, dematin, tropomyosin, and tropomodulin [69]. Many of the erythroid cytoskeleton proteins are also expressed in non-erythroid cells [68].

Spectrins are the main component of the red cell cytoskeleton and play an important role in determining the viscoelastic properties of the red cell [68]. All spectrins have a conserved domain of triple helical repeats, 21 repeats in $\alpha$ spectrin and 15 repeats in $\beta$-spectrin[70]. $\alpha$ and $\beta$ spectrin form an antiparallel heterodimer, with the N-terminus of $\alpha$-spectrin interacting with the C-terminus of $\beta$-spectrin[71]. A spectrin tetramer is then formed from two spectrin heterodimers which then assemble to form a flexible spectrin rod [72]. Spectrin rods unite via a junctional complex to form a hexagonal lattice that makes up the red cell cytoskeleton. The spectrin lattice attaches to the plasma membrane at 2 sites via ankyrin (Ank) interacting with band3 and via protein 4.1 interacting with glycophorin C [73-76].

The folding and unfolding of spectrin repeats confers elastic properties to spectrin rods and hence to RBC [77]. Alterations in spectrin expression directly correlate with changes in the viscoelastic properties of red cells. For example, a decrease in the amount of spectrin or the expression of defective spectrin causes loss of elasticity. Decreased RBC elasticity increases red cell fragmentation and fragility and results in hemolytic anemia [68].

The spectrin junctional complex is composed of actin, protein 4.1, demantin, adducing, tropomodulin, tropomyosin, and p 55 [78-83]. Actin weakly interacts with the N-terminus of spectrin to connect spectrin rods, and protein 4.1 stabilizes the interaction [84]. Tropomyosin strictly regulates the length of actin filaments to 12 to 14 monomers and at opposite ends tropomodulin and adducin caps the actin filaments. The role of dematin is not well understood [69].
The attachment of the cytoskeleton to the plasma membrane in RBC is principally mediated by Ank and protein 4.1R [69]. Ank binds to the cytoskeleton by interacting with β-spectrin through its spectrin-binding domain and binds to the plasma membrane by interacting with Band 3 [85]. Protein 4.2 strengthens this linkage [86, 87]. Protein 4.1 links the plasma membrane to the cytoskeleton through its interaction with glycophorin C (plasma membrane) [88, 89] and the spectrin junctional complex (actin and β-spectrin) in the cytoskeleton [90].

Figure 1-3. A schematic of the cross-section of a red blood cell.

The lipid bilayer lies across the schematic, with the polar heads facing out and hydrophobic tails facing towards each other. Protein 4.2 (4.2), Rhesus polypeptide (Rh), Rh-associated glycoprotein (RhAG), Landsteiner-Wiener glycoprotein (LW) and protein 4.1R (4.1R). This diagram was published by Perrotta et al 2008 [91].
1.5.2 RBC Membrane Diseases

Hemolytic anemia is the most commonly inherited RBC membrane disease and is caused by defects or deficiencies in one or more cytoskeletal proteins that associate with the plasma membrane. There are two main types of defects observed in inherited hemolytic anemia: disruption of membrane organization and altered membrane transport. Three inherited diseases associated with defects in red cell membrane organization are hereditary spherocytosis (HS), hereditary elliptocytosis (HE) and hereditary ovalcytosis (HO). The molecular bases of HS, HE and HO are well known (see below). Dehydrated hereditary stomatocytosis (DHS) and overhydrated hereditary stomatocytosis (OHS) are inherited diseases whose molecular bases are not unknown, but appear to be caused by defects in membrane transport function[92].

Hereditary spherocytosis: The highest incidence of HS is seen in people of northern European and Japanese descent, while the disease is rare in people of sub-Saharan descent. It is the most common hereditary hemolytic anemia in Caucasians. HS is typically inherited dominantly, but can also be inherited non-dominantly and recessively. The manifestations of HS include: hemolysis of RBCs, anemia, spherical RBCs, jaundice, reticulocytosis, gallstones and splenomegaly. The severity of HS is highly varied and ranges from mild (hematologically normal) to severe (life-threatening) anemia. Common to all classes of the disease is a loss in RBC membrane surface area, which results in misshapen RBC (discocytes, stomatocytes, and spherocytes). Distorted RBC become sequestered in the spleen and are eliminated from circulation. Splenectomy can improve hemolytic anemia in humans that are symptomatic, because the splenic destruction of abnormally shaped RBC is the main cause of hemolysis. The loss of RBC membrane surface area is caused by defective anchoring of the cytoskeleton to the membrane (defects in the vertical axis )[92]. More than a 100 genetic defects are associated with HS, and they principally affect the production of the following proteins: ANK [93], α-Spectrin,
β- Spectrin [94], Band 3 [95, 96], and Protein 4.2 [97, 98]. In dominantly inherited HS, Ank mutations are most frequently functionally null and result from frameshift, splicing or nonsense mutations, which can result in an unstable ankyrin transcript or truncated peptide. Ankyrin mutations occur throughout the gene (figure 2.9) and principally result in decreased expression of full-length or normal ANK. Alternatively in cases of recessively inherited Ank associated HS, promoter mutations and compound heterozygous mutations (missense) are common. In Band 3 related HS, missense and framshift mutations have been identified and result in either altered function or decreased Band3 expression. Japanese patients typically have reduced or no protein 4.2 expression and may result from either homozygous mutations that destabilizes protein 4.2 transcript or compound heterozygous mutations that destabilize the protein 4.2 transcript and a missense or splice variant. α-spectrin mutations are often inherited recessively, where as β-spectrin mutations are inherited dominantly, because α-spectrin is expressed 3 times higher than β-spectrin. A patient with an α-spectrin defects is typically compound heterozygous for missense and low-expression mutation, where as a patient with a β-spectrin defect carries a null mutation (including frameshift, nonsense and splicing)[94].

Hereditary Eliptocytosis: HE is a relatively common disease that is both clinically and genetically heterogeneous. Cases of HE are seen worldwide, but the disease is most common in areas in which malaria is present. While the majority of people afflicted with HE are asymptomatic, 10% of individuals with HE are severely anemic. In these individuals, splenectomy often helps increase RBC number. It is likely that most individuals are asymptomatic because the eliptocytic shape of the RBC does not block the flow of blood or cause RBCs to get trapped in the spleen [92]. The majority of mutations associated with HE affect α-spectrin[99], but mutations have also been shown to affect β-spectrin and protein 4.1.
Mutations in $\alpha$-spectrin, $\beta$-spectrin and protein 4.1 interrupt protein/protein binding and are believed to result in destabilization of the spectrin cytoskeleton assembly. For example, mutations altering the N-terminus of $\alpha$-spectrin or the C-terminus $\beta$-spectrin alter the two proteins ability to oligomerize [100]. The molecular consequence of mutations in HE is a loss of horizontal linkage within the RBC cytoskeleton [92].

*Hereditary Ovalocytosis:* HO causes RBC to be oval in shape with a transverse ridge. It is most common in Southeast Asia where the incidence of malaria is high. In contrast to HS, the RBC membranes in people with HO are rigid with a loss of elasticity. This results in RBCs that are more stable; thus, hemolysis is uncommon. To date, a single mutation in Band 3 has been shown to be responsible for all known cases of HO, a deletion of nine amino acids (400 to 408). HO has autosomal dominance inheritance, and no humans homozygous for mutations in Band 3 have been detected. The mechanism by which a mutation in Band 3 reduces elasticity is not well understood [101].

*Hereditary Stomatocytoses:* Two types of hereditary stomatocytoses are known: DHS and OHS. Both DHS and OHS are rare genetic conditions that are caused by defects in RBC membrane transport, which causes defects in cell volume homeostasis. The molecular mechanisms of DHS and OHS are unknown, but both conditions are inherited in an autosomal dominance pattern. RBC in patients with DHS are dehydrated, because the intercellular potassium content is low, which causes water to exit the cell. RBC survival is only slightly affected in DHS, therefore, anemia is usually well-compensated and there is only a mild enlargement of the spleen. Patients with DHS are also noted for having high MCHC (mean corpuscle hemoglobin concentration) because water content in cells is low. In contrast to DHS, patients with OHS have RBC with increased volume, caused by increased intracellular sodium levels. Patients with OHS have
stomatocytes in PB smears and have moderate-severe to severe anemia. Stomatocytes with increased sphericity are trapped in the spleen, which results in decreased RBC survival [102]. While splenectomy is highly effective in patients with HS and HE to reduce anemia, it can be dangerous in patients with OHS because circulating stomaocytes can cause venous thromboembolisms [103, 104].

1.5.3 Platelets and Megakaryocytes

Megakaryocytes (MK) are specialized hematopoietic precursor cells that give rise to platelets. Cells from the MK lineage arise from a bipotenital progenitor that can develop down the erythroid and MK lineages. A MK burst forming unit (BFU-Mk) is the first detectable unipotential MK progenitor that can be observed in vitro. The BFU-Mk is a primitive MK progenitor with high proliferative capacity and can produce between 40 and 500 MK within one week when grown in culture[105]. The CFU-Mk is a more mature MK progenitor with limited proliferative potential. When CFU-Mk are grown in culture, they can produce colonies containing 3 to 50 MK [106, 107].

Three morphologically distinct MK precursors exist in the bone marrow and include: promegakaryoblasts, megakaryoblasts and promegakaryocytes. The transition between MK precursor to mature MK capable of producing platelets requires two main steps: endomitosis and cytoplasmic maturation[108].

MK are unique within the hematopoietic system as they are the only cells to undergo endomitosis. Endomitosis occurs when cells undergo repeated cycles of DNA replication without cell division and results in polypoid cells in comparison to normal cells that are diploid (2N) [109, 110]. The cell cycle of endomitotic cells is characterized by a short G1 phase, a normal S phase, a short G2 phase, followed by premature termination of mitosis[109]. Normal mitosis is
blocked after anaphase A in endomitotic cells, and cells are then prevented from undergoing anaphase B, telophase and cytokinesis. Premature termination of mitosis is followed by entry of cells into G1[111, 112]. Typically, a mature megakaryocyte undergoes three cycles of DNA replication (16N), but megakaryocytes can undergo two to six cycles of DNA replication (8N, 16N, 32N, 64N and 128N). The polyploidization of megakaryocytes results in gene amplification, which is believed to be necessary for increased protein synthesis that must keep pace with rapid increases in cell size[113].

The cytoplasm of a mature megakaryocyte is filled with platelet-specific proteins, organelles and membrane systems that become subdivided in platelets. Mature megakaryocytes develop a demarcation membrane system, which is an extensive network of membrane channels composed of flattened cisternae and tubules[114]. The demarcation membrane system is first detectable in promegakaryocytes, but is most prominent in mature MK. Invaginations of the plasma membrane form the demarcation membrane system and become a reserve for proplatelet formation and extension [115]. The demarcation membrane system is in contact with the extracellular environment and can be labelled with the extracellular markers [116].

Megakaryocytes also contain a dense tubular system that is not in contact with external milieu and is responsible for prostaglandin synthesis for platelets [117, 118]. Secretory granules are synthesized and packaged during MK development. \(\alpha\)-granules are the most abundant granules and contain proteins that are necessary for platelet adhesion and vascular repair[119].

PLT are produced from MK through the production of proplatelets [18, 120-124]. Proplatelets are long thin cytoplasmic processes that contain multiple PLT sized swellings that emanate from MK. Within four to ten hours after the start of proplatelet formation, the MK is completely broken down into a naked nucleus surrounded by proplatelets [125]. MK can move
into the PB and proplatelet formation has been documented in the bone marrow, lung and blood [114].

Three cytokines, SCF (stem cell factor), IL-3 (interleukin) and TPO (thrombopoietin) are important in the regulation of MK development and PLT formation. SCF regulates primitive hematopoiesis [126-128]. The early stages of megakaryocyte development up to endomitosis are regulated by the cytokine IL-3[129]. TPO regulates all stages of MK development, but is not required for proplatelet formation[130]. GATA-1 and NF-E2 are two transcription factors critical for the production of megakaryocytes and platelets. GATA-1 is important for the regulation of early and late megakaryocyte differentiation [131, 132] and controls the expression of Mk-specific proteins including NF-E2. NF-E2 is a heterodimeric transcription factor that regulates the maturation of megakaryocytes and biogenesis of PLTs [133].

TPO is the primary regulator of PLT production, and c-Mpl is its cognate receptor. TPO supports megakaryopoiesis beginning at the HSC, by promoting HSC survival and expansion (TPO) [134]. While TPO does not increase the proportion of progenitors formed by HSC, it does influence the commitment of HSC to the megakaryocyte lineage [135, 136]. Once progenitors are committed to the megakaryocyte lineage, TPO supports MK progenitor survival and proliferation, and finally, TPO promotes the differentiation of MK progenitors into megakaryocytes [137]. The terminal stages of MK maturation, including proplatelet production are independent of TPO [138]. Although TPO does not directly activate PLT (except at high concentrations)[139], it can make PLT more susceptible to a second agonist, such as adenosine diphosphate (ADP) [140]. The loss of TPO or c-Mpl expression in mice results in a drastic reduction in MK numbers and PLT numbers are reduced to 10% of normal [141-143]. Under steady state conditions, TPO is predominantly produced by the liver and kidney[144, 145], but in
times of stress it is also produced by bone marrow stromal cells [144]. TPO levels are inversely related to the number of circulating PLT and MK in the bone marrow [146]. Circulating PLT can act directly to reduce TPO levels, via binding of TPO to c-Mpl on platelets, subsequent complex internalization and proteolytic digestion [147].

The mechanisms that control both the number and size of platelets produced by an individual megakaryocyte are largely unknown, but dysregulation of this process can result in the formation giant platelets. Giant platelets are the hallmark of a group disorders associated with abnormally large PLT and thrombocytopenia (macrothrombocypopenia). Macrothrombocytopenia is most commonly an acquired disorder, caused either by immune (idiopathic) thrombocytopenia purpura (ITP) or myelodysplastic syndrome, but are can also be inherited in a small subset of patients. The three main causes of inherited macrothrombocytopenia are due to defects in the PLT cytoskeleton, the GPIb/IX/V complex and transcription factors[148].

Myh9-related disorders (MYH9RD), describes a group of four macrothrombocytopenias: May-Hegglin, Epstein syndrome, Fetchner syndrome and Sebastian syndrome. May-Hegglin syndrome was first described in 1909, as an autosomal-dominant macrothrombocytopenia with Dohle body-like neutrophils inclusions [149, 150]. In 1972, Epstein syndrome defined through the study of a group of patients with macrothrombocytopenia, hearing loss, and nephritis, but without neutrophil inclusions [151]. Fetchner syndrome was described in a group of patients with macrothrombocytopenia, neutrophil inclusions, cataracts, hearing loss and nephritis [152]. Sebastian syndrome was not reported until 1990, and describes patients with mild macrothrombocytopenia and small neutrophil inclusions [153]. The underlying mutations for each of these syndromes all mapped to overlapping intervals on chromosome 22, which included
MYH9 [154, 155]. Mutations in MYH9 (myosin heavy chain 9) were found in patients with May-Hegglin, Epstein syndrome, Fetchner syndrome, and Sebastian syndrome [156, 157]. Subsequently, it was decided that instead of 4 distinct syndromes, May-Hegglin, Epstein syndrome, Fetchner syndrome and Sebastian syndrome should be grouped together as a single entity and be referred to as MYH9RD [156, 158, 159]. Further evidence supporting MYH9RD as one disorder came when very small neutrophil inclusions were detected by immunofluorescence in Epstein syndrome patients. In addition, patients with Sebastian syndrome were found to also have cataracts and high frequency hearing loss [160]. MYH9RD now included patients with macrothrombocytes and neutrophil inclusions, who may also present a varying combination of cataracts, hearing loss and nephritis[148]. The clinical manifestations of MYH9RD are heterogeneous even among a family with the same mutation(s) and include the degree of bleeding and renal impairment and age of onset for cataracts and hearing loss [160].

Bernard-Soulier syndrome (BSS) is an autosomal-recessive macrothrombocytopenic disorder [161, 162]. Patients with BSS have a severe bleeding tendency and their PLT do not aggregate in response to ristocetin. The platelets from BSS patients are giant (similar to MYH9RD) and have a normal ultra structure [162]. BSS is caused by reduced or absent expression of the GPIb-IX-V receptor complex, which results in defective adhesion of the platelet to the vessel wall [162]. Patients with BSS have homozygous or compound heterozygous mutations in the GPIbα, GPIbβ or GPIX genes [163]. Most mutations in BSS patients are point mutations that result in premature stop codons, which lead to the production of unstable polypeptides, although missense mutations can also occur [163]. All four peptides (GPIbα, GPIbβ, GPV and GPIX) associate together early and each peptide in the GPIb-IX-V complex is required for the stability and maintenance and maintenance of the complex [164-166].
Heterozygous carriers of BSS mutations have a moderate bleeding tendency, mild macrothrombocytopenia and reduced expression of platelet GPIb/IX/V complex [163].

Mediterranean macrothrombocytopenia is an autosomal-dominant disorder [167, 168]. Patients with Mediterranean macrothrombocytopenia have no or mild clinical manifestations and present with normal bone marrow MK counts, normal PLT survival and normal in vitro PLT function[169]. Most cases of Mediterranean macrothrombocytopenia have been associated with mutations in the genes encoding the GPIbβ, GPIbα, or GPIX, and have therefore been classified as heterogeneous BSS [170, 171]. The genetic cause of the remaining cases of Mediterranean macrothrombocytopenia remains unknown [158].

Velocardiofacial syndrome is a congenital disorder associated with a microdeletion on chromosome 22. The small deletion on chromosome 22 contains GPIbβ [172]. Velocardiofacial syndrome is characterized by cleft palate, cardiac anomalies, typical facies and learning disabilities, as well as macrothrombocytopenia [173]. Platelet function is normal in most patients with velocardiofacial syndrome, although a few patients do not aggregate normally when stimulated with ristocetin [174, 175].

Paris-Trousseau syndrome and Jacobson syndrome are two macrothrombocytopenia disorders that are caused by deletions on similar regions of chromosome 11 [176, 177]. Two transcription factors are located within this region, Fli1 and Ets-1, which are both known to have overlapping roles in angiogenesis and hematopoiesis. Only ten percent of all platelets in Paris-Trousseau syndrome and Jacobson syndrome patients are enlarged, and fifteen percent of all PLT have enlarged α-granules (seen as a red granule in PLT in PB stained with Giemsa) [178]. Patients with Paris-Trousseau syndrome and Jacobson syndrome have normal PLT aggregation and also have normal PLT survival. Although PLT production is significantly reduced in patients with
Paris-Trousseau syndrome/Jacobson syndrome, megakaryocytes in the bone marrow are increased 3 fold and micromegakaryocytes are numerous [176]. In addition to macrothrombocytopenia, patients with Jacobson syndrome have heart defects, trigonocephaly, mental retardation and multiple organ malfunctions [179].

Gray platelet syndrome is a rare inherited macrothrombocytopenia, characterized by large pale gray PLT on PB smears (Wright’s stained) [180]. Platelets in patients with Gray platelet syndrome have empty α-granules[181]. Genetic transmission in Gray platelet syndrome patients occurs by a variety of modes, which include autosomal dominant and autosomal recessive transmission, although, rare sporadic cases have been reported [181-183]. The genetic lesion responsible for Gray platelet syndrome is unknown, but the pathogenesis appears to be due to a defect in the packaging secretory proteins in megakaryocytes [181].

X-linked macrothrombocytopenia is associated with missense mutations in GATA-1, a transcription factor important in megakaryopoiesis and erythropoiesis. In addition to macrothrombocytopenia, patients with X-linked macrothrombocytopenia have mild to moderate dyserythropoiesis. Missense mutations in GATA-1, lead to reduced transcription of target genes important in platelet production including GPIbα, GPIbβ, GPIX, and GPV [158, 184, 185].

Immune Thrombocytopenic Purpura (ITP) is an autoimmune disorder. Patients with ITP have low PLT counts and high MPV, but have normal a hemoglobin level, WBC count, differential count and blood smear. A diagnosis of ITP requires that other causes of thrombocytopenia must be eliminated. Chronic ITP is associated with macrothrombocytes and less than 10% of PLT are giant (larger then RBC). The bleeding tendency in patients with ITP is varied; however, severe bleeding is rare. The incidence of ITP is high in comparison to inherited forms of macrothrombocytopenia, it is estimated that 1 in 66,000 to 100,000 people with get ITP.
Infection plays an important role in the initiation of ITP and has been linked to almost any virus [186]. While, infection is required for initiation of ITP, it is not required for persistence of the disease. An abnormally high level of PLT destruction is noted in patients with ITP[187, 188]. The exact etiology of ITP is unknown, but is associated with the production of anti-platelet autoantibodies. It is believed that the production of anti-platelet autoantibodies, could be explained by the production of antibodies made against a virus that cross-react with antigens on the surface of PLT [189].

1.6 Pharmaceutical Induction of Cytopenia

5-fluorouracil (5FU) is a pyrimidine analog that disrupts DNA and RNA synthesis in cycling cells leading to cell death. 5FU has been used for the treatment of aggressive human neoplasms, but has severe side effects, including bone marrow toxicity, which have limited its use [190]. The bone marrow toxicity of 5FU has been exploited by researchers for many decades for the study of hematopoiesis in normal and genetically altered mice. The S-phase of the cell cycle is the primary target for 5FU, although 5FU can also affect cells in G1 because of the accumulation of 5FU metabolites become toxic to the cell during this stage of the cell cycle[191].

5FU enters the cell by means of a pH-dependent transport system in a manner similar to uracil and adenine. Once within the cell, 5FU is metabolized to its active form. 5FU is activated by three enzymes: orotate phosphoribosyltransferase and uridine phosphorylase for entry into the RNA synthesis pathway, and thymidine phosphorylase for entry into the DNA synthesis pathway. Orotate phosphoribosyltransferase converts 5FU directly into fluorouridine monophosphate, whereas uridine phosphorylase first converts 5FU to 5-fluorouridine, and then uridine kinase converts 5-fluorouridine into fluorouridine monophosphate [192].
5FU analogs become incorporated into both nuclear and cytoplasmic fractions of RNA, thereby, inhibiting both RNA processing and function. The affect of 5FU on RNA production and it has been shown that 5FU can incorporate into all species of RNA. The toxic effects of 5FU on RNA depend greatly on cell type, dose and timing of administration. Overall RNA synthesis can be decreased by 5FU treatment. FUTP (fluorouridine triphosphate) has also been shown to integrate into low molecular weight RNA (4S) at lethal doses, forming a stable and persistent complex that can lasts for many days. At low concentrations, 5FU inhibits the polyadenylation of messenger RNA [192].

Thymidylate synthetase (TS) is a critical enzyme in the biosynthesis of pyrimidines and acts by converting 2′-deoxyuridine-5′monophosphate to thymidine 5′-monophosphate [193]. FdUMP (a 5FU metabolite) competes with 2′-deoxyuridine-5′monophosphate (the natural substrate) for the catalytic site on TS [192]. When FdUMP binds to the catalytic site of TS, it forms a slowly reversible complex, which leads to a reduction in thymidine formation [192, 193]. The depletion of thymidine monophosphate and thymidine triphosphate causes a deoxyribonucleotide imbalance and inhibits DNA synthesis, which is toxic to rapidly dividing cells and eventually results in cell death [192].

The effects of 5FU on the hematopoietic system are severe and include depletion of both the myeloid and lymphoid compartments because actively dividing progenitors are killed. Within 5 days of 5FU treatment, a substantial drop occurs in the following cell types and measures: hematocrit, platelets, leukocytes, bone marrow and spleen cellularity, myeloid progenitors (bone marrow and spleen), pre B-lymphocytes and CFU-S [191, 194].

5FU spares primitive non-cycling CFU-S and long term repopulating stem cells, but kills cycling CFU-S [195, 196]. While long term repopulating stem cells are resistant to a single dose
of 5FU, 5FU causes long term repopulating stem cells to enter into cycle, which makes them sensitive to a second dose of 5FU[197]. 5FU treatment can increase the repopulating ability of bone marrow, and thus, 5FU-treated bone marrow can outgrow untreated bone marrow in competitive repopulation assays[193].

5FU affects erythropoiesis by killing actively cycling erythroid progenitors (BFU-E and CFU-E) causing a deficit in newly formed RBC and leading to transient anemia. A drop in immature erythrocytes or reticulocytes is also observed within days of 5FU treatment and is followed by reticulocytosis within 10 days of 5FU treatment. Reticulocytosis corresponds to increases in plasma erythropoietin. Erythropoietic recovery in mice post-5FU treatment is achieved, in part, by massive extramedullary erythropoiesis in the spleen. Near baseline values for hematocrit and RBC concentration have been observed within 10 to 12 days of 5FU treatment [198].

Platelets have a unique recovery post 5FU treatment in comparison to other PB parameters. 5FU leads to a mild reduction in circulating platelet numbers (nadir D7), which is followed by a dramatic and prolonged thrombocytosis (PLT overshoot) from D11 to 17. In order to understand the unique 5FU PLT recovery, it is important to note that 5FU interferes with DNA and RNA synthesis, meaning that megakaryocyte progenitors and megakaryocytes and not PLT are the potential target for 5FU. Bone marrow CFU-Mk drop within one day of 5FU treatment, but return to near baseline levels 5 days after treatment. One week after 5FU treatment, megakaryocytes become the predominant cell in the bone marrow. Splenic CFU-Mk, on the other hand overshoot baseline values within 11 days of treatment, and this is coupled with increased splenic megakaryocyte frequency. The elevated levels of splenic megakaryopoiesis cannot explain the PLT overshoot on its own, because the CFU-MK overshoot in the spleen
occurs after the start of the observed PLT overshoot [194]. Interestingly, CFU-GM (colony forming unit granulocyte monocyte) show similar recovery kinetics both the bone marrow and spleen, but profound leukocytosis does not occur.

Following 5FU treatment, the number of megakaryocytes in the bone marrow does not decrease to the same degree as the number of platelets; leading to the hypothesis that 5FU-induced thrombocytopenia causes a defect in platelet formation and not in megakaryocyte production. The endomitosis rate in megakaryocytes recovers quite quickly, and therefore, it is believed that a block in endomitosis does not explain thrombocytopenia post- 5FU treatment. Consequently, it is postulated that the inhibition in RNA synthesis caused by 5FU reduces the production of PLT, and this in turn is the cause of the observed thrombocytopenia [190].

Three genetically altered mouse lines have contributed to the understanding of 5FU PLT recovery. The cellular receptor c-mpl is the cognate receptor for TPO, the primary humoral regulator of megakaryocyte and platelet production. c-mpl<sup>−/−</sup> mice are severely thrombocytopenic, and when treated with 5FU, c-mpl<sup>−/−</sup> PLT overshoot baseline values, indicating that Tpo signaling is not required for the PLT overshoot [199]. SCF, however, plays a key role in the observed PLT overshoot. Mutations to SCF (c-Kit<sup>Sl/Sld</sup>) and the SCF receptor, c-Kit<sup>W/Wv</sup>, result in the loss of the PLT overshoot when these mice are treated with 5FU [200, 201].

Phenylhydrazine (PHZ) was first characterized by Hermann Emil Fischer in 1875. PHZ was first used as an antipyretic, but detrimental effects on RBC were observed following treatment. For many years PHZ has been used experimentally to induce anemia [202].

PHZ acts directly on RBCs without requiring conversion to metabolites for activation. PHZ interacts with oxygenated hemoglobin to produce H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub>· (reactive oxygen species),
phenyldiazene and phenyl radicals. Increased concentrations of reactive oxygen species lead to the oxidization of hemoglobin: the globin molecule is first oxidized into methaemoglobin and then into hemicrome [202]. PHZ also causes the depletion of glutathione, a cellular reactive oxygen species scavenger [202, 203]. The hemoglobin molecule is further destabilized by the replacement of the heme group with phenyl-substituted protoporphyrins. Denatured hemoglobin accumulates at the plasma membrane and precipitates into Heinz bodies [202, 203], which decrease RBC deformability [202]. Damaged RBCs are quickly removed from circulation by the spleen [202, 204, 205].

Various PHZ dosing regimens have been described, and generally consist of 1, 2, or 3 doses of PHZ in the range of 100 µg/g to 180 µg/g. Different PHZ dosing regimens produce unique RBC and reticulocyte recovery kinetics [204, 206-209]. PHZ treatment leads to a rapid drop in hemoglobin, RBC concentration and hematocrit usually within a day [202, 204, 205]. The replenishment of RBC also occurs quickly since RBC values can return to baseline levels within a week of PHZ treatment [204]. Marked reticulocytosis is observed within days of PHZ treatment, but the timing varies greatly depending on the dosing regimen [202, 204, 205]. The rapid RBC recovery can be primarily attributed to extramedullary hematopoiesis in the spleen. In steady state conditions, the spleen is primarily composed of lymphocytes, but the spleen is converted transiently into an erythroid organ after PHZ treatment[210, 211]. PHZ induces multi-fold increases in CFU-S, BFU-E and CFU-E in the spleen, but CFU-E are the predominant progenitors that is increased (100-fold increase from basal levels) [204, 207]. PHZ does not change the cycling status of bone marrow progenitors, but splenic CFU-S cycle more rapidly than bone marrow CFU-S [212]. Bone marrow erythropoiesis is also significantly altered after PHZ treatment. CFU-E increase in the bone marrow, where as BFU-E are decreased. The decrease in bone marrow BFU-E is partially explained by the presence of increased BFU-E in
the PB [204, 213]. The PB BFU-E and CFU-S are believed to seed the spleen and help establish the extramedullary hematopoiesis there[212].

SCF plays a critical role in erythroid expansion following PHZ treatment in mice. Specifically, SCF is needed for the homing of hematopoietic progenitors to the spleen after the induction of hemolytic anemia. Disrupting SCF signaling, either by mutations to its receptor (c-Kit<sup>W/W<sub>v</sub></sup>) or with the use of an antibody that blocks the receptor, significantly slows RBC recovery after PHZ treatment, and in particular, delays splenic erythropoiesis.

Hydroxyurea (HU) was first synthesized in 1860, and one hundred years later it was shown to be a potent myelosuppressive agent with few side effects. More recently, HU has been used as an inhibitor of lineage proliferation in chronic myelogenous leukemia, polycythemia vera and essential thrombocytosis. HU treatment does not cause remission, and therefore, has been replaced by more modern drugs, such as Gleevec for chronic myelogenous leukemia, or HU is used in combination with other drugs. Today, the major medical use for HU is in the treatment of sickle cell anemia. HU improves the symptoms and complications associated with sickle cell anemia, by promoting the expression of fetal hemoglobin, and also by suppressing myelopoiesis and erythropoiesis [192, 214].

HU inhibits ribonucleotide reductase (RR) causing death of actively dividing cells. RR converts ribonucleotide diphosphates into deoxyribonucleotides for use in <i>de novo</i> DNA synthesis or DNA repair. RR consists of two subunits, M1 and M2. M1 exists as a dimer, which contains a substrate binding site, and M1 is also needed for allosteric regulation. M2 also exists as a dimer, and contains the catalytic subunit of RR. M2 concentration peaks in S-phase of the cell cycle, whereas M1 concentration is consistent throughout the cell cycle (except G0), and therefore M2 concentration controls the functional operation of RR. RR is a component of a
bigger complex called replitase. Replitase is critical during the S-phase of the cell cycle and consists of several enzymes involved in DNA replication including: DNA polymerase, dihydrofolate reductase, nucleoside-5’ phosphate kinase, thymidylate synthase and thymidine kinase [214].

HU enters the cell by passive diffusion. Once inside the cell, HU inactivates the tyrosyl free radical on the M2 subunit of RR, disrupting the iron binding center of RR. The exact mechanism used by HU to inhibit the iron binding site of RR is not well understood, but may include the conversion of HU into nitric oxide, a known inhibitor of RR. Inhibition of RR results in the disruption of other enzymes in the replicase complex including DNA polymerase and thymidylate synthase. After HU treatment, cells continue to progress through the cell cycle, but are blocked at the G1-S interface and accumulate in S-phase. Cells arrested in S-phase undergo apoptosis by both p53 and non-p53 mediated pathways [214].

HU has not been as widely used in the study of murine stress hematopoiesis in comparison to PHZ and 5FU. A uniform dosing regimen of HU does not exist. HU has been administered in a single dose [215-218], as multiple doses [219-221], continuously (BD) and ultimately has different effects depending on the dosing regimen. Distinct dosing regimens lead to varied effects on the hematopoietic system. A double dose of HU causes no change in hematocrit, but decreases reticulocyte and granulocyte numbers, which is followed by an overshoot from baseline levels for each cell type [219]. Bone marrow cellularity decreased to 50% of baseline values with a variety of HU dosing regimens [215, 216, 219, 221, 222]. Primitive multilineage hematopoietic progenitors (CFU-S or KTLS cells (c-kit⁺, Thy-1⁻, Lin⁻, Sca-1⁺) have been observed to immediately decrease after HU treatment [215, 216, 219] and then overshoot baseline values, but the exact kinetics of recovery are dependent on the dose regimen.
used. HU treatment has been used to synchronize the cell cycle of early hematopoietic progenitors. Mature hematopoietic progenitors in the bone marrow (CFU-C and CFU-E) are actively dividing and hence decrease rapidly after HU treatment, but they recover quickly (e.g. CFU-C overshoot by 48hrs) [217-219]. Splenic extramedullary hematopoiesis is altered after HU treatment, as CFU-S, CFU-C and CFU-E decrease rapidly and then CFU-C and CFU-S overshoot their baseline values before returning to normal levels [218, 219].

The cytotoxicity of HU is dependent on the dose and duration of HU exposure. The primary action of HU is to inhibit the production of thymidine, which prevents the synthesis of DNA. HU does not directly alter RNA or protein synthesis[199]. Hematopoietic parameters recover rapidly after HU treatment and this may be because HU is metabolized quickly, and it only affects DNA synthesis [215].

1.7 Thesis Hypothesis and Objectives

The principle objective of this thesis was to use ENU mutagenesis to generate new mouse models of human hematopoietic disease that could be used to dissect the molecular and cellular causes of hematopoietic diseases. A two-fold approach was used to accomplish this objective: an established dominant screen was used to detect mutations that had defective steady-state hematopoiesis and the implementation of a sensitized screen to detect mutations that result in altered stress hematopoiesis.

**Hypothesis 1:** ENU mutagenesis combined with conventional hematopoietic assays will identify novel mutations that alter steady-state hematopoiesis and new mouse models of human hematopoietic disease, which can be used to determine the underlying molecular defects of such diseases, along with providing further insight into the process of hematopoiesis.
Hypothesis 2: A pharmacologically sensitized screen that induces transient cytopenias will aid in the identification of a unique set of mutations that affect cell cycle and or stress hematopoiesis.

The dominant ENU screen in which more than 3000 G1 mice were examined for defects in steady state hematopoiesis identified 88 G1 outliers. Of the 88 outliers, 30 were outliers for RBC size or hemoglobin content, which are often attributed to defects in the RBC cytoskeleton. Seventeen G1 mice were outliers for RBC and PLT levels, 3 of these G1 were selected to establish mutant lines that could be subsequently used to characterize the pathogenesis of the primary phenotype and to identify the underlying causative mutation ($Ank^{7192}$, $Myh9^{7238}$ and $Jak2^{7254}$). This thesis describes the characterization of two of the mutant lines: $Ank^{7192}$ and $Myh9^{7238}$.

The first objective of this thesis was to establish, characterize and identify the mutation for a G1 outlier in the ENU dominant screen that presented with high RBC, low MCV and MCV and increased polychromasia that was subsequently be referred to as $Ank^{7192}$. A novel mutation in Ank1 was found to be responsible for the microcystic RBC phenotype, and it was subsequently determined that $Ank^{7192}$ is a mouse model for hereditary spherocytosis (HS) (see Chapter 2). Specifically, $Ank^{7192/+}$ mice model a mild form of HS, with a normal hematocrit, spherical RBC and increased osmotic fragility of RBC. $Ank^{7192/7192}$ mice model a severe form of HS that included severe anemia, jaundice and extensive extramedullary hematopoiesis.

The second objective, described in Chapter 3, was to establish, characterize the pathogenesis and identify the mutation for a G1 outlier with low PLT levels that would subsequently be referred to as $Myh9^{7238}$. During the analysis, the true phenotype for $Myh9^{7238/+}$ mice was determined to be macrothrombocytes, not thrombocytopenia. $Myh9^{7238/7238}$ mice are
viable and presented with a more severe PLT phenotype of macrothrombocytopenia. The production of other hematopoietic lineages was unaffected by the Myh9\textsuperscript{7238} mutation, as CBC with WBC differentials were unaffected in Myh9\textsuperscript{7238} mice and CFU-C levels were normal. Massive parallel sequencing was used to identify a novel missense mutation in the tail region of MYH9 (Q1443L). Myh9\textsuperscript{7238} mice are a mouse model for the human disorder, MYH9RD, as they have the hallmarks of the disease including: giant PLT, neutrophil inclusions and a mutation in Myh9. The existence and incidence of the Alport-like manifestations of MYH9RD in MYH9\textsuperscript{7238} mice will require further study.

The third objective was to identify a chemotherapeutic drug that could successfully be used in a sensitized screen, where it would induce cytopenia in order to challenge the hematopoietic system, and therefore help to identify mutations that alter cell cycle or stress hematopoiesis (Chapter 4). 5FU, PHZ and HU were assessed by directly comparing the ability each drug to induce cytopenia and the recovery kinetics of mature blood cells, hematopoietic precursors and progenitors. 5FU and PHZ were strong inducers of cytopenia, and therefore, both were candidates for a pharmacologically sensitized ENU screen. HU was a poor candidate for a pharmacologically sensitized screen, because it did not induce a strong cytopenia, and there were large differences in inter-strain recovery kinetics. 5FU has the capacity to induce multi-lineage cytopenia and PHZ has the capacity to introduce anemia.

The fourth objective was to implement a 5FU-sensitized screening protocol into a dominant ENU screen to identify mutants that have altered cell cycle or stress hematopoiesis (Chapter 4). 5FU treatment was combined with the dominant ENU screen, and shown to most effectively aid in identifying mutants with altered PLT recovery (13 PLT recovery outliers). Three mutant lines were established from this screen and continued to be studied. The line
designations and phenotypes are: 7304, delayed RBC recovery; 7323, high PLT and normal 5FU recovery; and 7325, increased PLT overshoot. Although only two of the 13 PLT recovery outliers have been examined, it is possible to recover 6 more PLT lines from the sperm collected from each G1 male mouse.
Chapter 2: *Ank1^{7192/7192}*: A New Mouse Model for Hereditary Spherocytosis

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2.1 Abstract

During a dominant ENU screen, a G1 founder mouse designated 7192 presented with high RBC, low MCV and MCH on a complete blood count (CBC) and increased polychromoasia on peripheral blood smear. Genetic mapping of affected 7192 mice narrowed the mapping interval to a 7.2Mb region on chromosome 8 and this interval contained RBC structural candidate gene Ank1. Sequencing of Ank1 revealed an in-frame non-sense mutation arising from a single base transversion (G>T) in exon 26 generating a stop codon (X) at amino acid 895 (glutamic acid (E)). Phenotypic analysis of 7192/+ (heterozygotes for 7192 mutation) demonstrated that they exhibit diagnostic characteristics of mild hereditary spherocytosis in humans including, normal hematocrit, spherical RBC and increased osmotic fragility of RBC. 7192 homozygotes demonstrate symptoms similar to humans with severe HS that includes severe anemia, jaundice and extramedullary hematopoiesis. The 7192 mutation induces a premature stop codon and therefore we hypothesized that 7192/7192 mice should produce a truncated ANK1 protein 100 kilodaltons (kDa) and no full length protein. Coomassie blue staining of RBC ghost and immunobloting revealed an absence of full length ANK1 and a 100kDa protein as well as additional anti-Ank1 immunoreactive species. Ank17192/7192 mice produce no full length Ank1 and a putative partially-functional truncated Ank1 (neomorphic), but do produce a rare isoform (alternative splicing exon 41/42) of Ank1 that is expected to be functional and may contribute to 7192/7192 survival.
2.2 Introduction

Hereditary Spherocytosis (HS) is a heterogeneous group of inherited hemolytic anemias that are primarily characterized by spherically shaped RBC. The principal symptoms of HS are anemia, jaundice and splenomegaly. While cases of HS are seen worldwide, the highest incidence of HS is observed in people of northern European and Japanese decent [91]. HS is a clinically diverse disease ranging from asymptomatic to severe, and is often classified into four categories: mild, moderate, moderately severe and severe [223]. Mild cases of HS represent 20% of all HS cases and are primarily asymptomatic due to compensatory replacement of the RBC lost to hemolysis. A patient with mild HS can present with minor splenomegaly, mild reticulocytosis, minimum spherocytosis, increased osmotic fragility, and the disease often escapes detection until adulthood when gallstones occur [224]. The majority of patients with HS are classified as having moderate HS and present in childhood with anemia, jaundice and splenomegaly [91]. Ten percent of all HS patients are classified as having moderately severe HS, and these patients present with low hemoglobin, profound reticulocytosis and bilirubinaemia. Only three to five percent of all cases of HS are classified as severe and can cause life threatening anemia, which requires regular blood transfusions [223]. Almost all cases of severe HS are inherited non-dominantly ([225] and [226]).

The primary cellular defect in HS is the loss of RBC surface area, which compromised the ability of RBC to deform leading to an irregular shape [227]. Abnormally shaped RBCs are trapped and destroyed in the spleen, which is the underlying cause of hemolysis in HS patients [91, 228]. The molecular cause responsible for membrane surface loss in RBC is loss of vertical linkages between the plasma membrane and the cytoskeleton. Vertical linkage in RBC occurs at two multi-protein complexes: the ankyrin-1, band 3 and protein 4.2 complex; and the protein 4.1, p55 and glycoporphin C complex [91, 227]. Genetic lesions causing defects in the expression,
stability or function of ANK1, Band3, α-Spectrin, β-Spectrin or protein 4.2 are the main cause of HS [91, 226, 229]. The most common mutation found in people of northern European descent who have HS is in ankyrin-1 (40 to 65%)[67, 226] followed by band 3 (20-35%)[230, 231], β-spectrin (15-30%) and α-spectrin or protein 4.2 (together account for less than 5%) [67]. In contrast, people of Japanese decent with HS most commonly have mutations in protein 4.2 (40-50%) [91, 232]. While most cases of HS are inherited dominantly, the most severe cases of HS are usually caused by autosomal recessive mutations in α-spectrin, yet severe recessive mutations in ANK1 and Band3 have also been observed [226, 233, 234]. Typically HS cases associated with mutations in ANK1 are dominantly inherited, but de novo mutations and non-dominant or recessive inheritance have also been reported [235]. Mutations have been detected within the entire ANK1 locus, including the promoter, 5′ and 3′ untranslated region (UTR), splice acceptor and donors, and a variety of nonsense and missense substitutions and insertions in several coding exons [235]. The mutations in ANK1 have been shown to affect the expression or stability of other proteins such as α and β spectrin and protein 4.1, requiring Ankyrin-1 for appropriate function or assembly of RBC membranous cytoskeletal complexes [226, 236, 237].

The Ankyrin family of proteins consists of 3 family members: Ank1, 2, and 3. Ankyrins are multipotential adaptor proteins that link membrane proteins to spectrin and provide structural stability to the cell [238]. Ankyrin 1 expression is restricted to the erythrocyte, brain and muscle [239-241]. In contrast, Ank2 and 3 are expressed widely throughout most tissues of the body, but are critical in neural or epithelial tissues (Ank2 and 3, respectively) [242-244]. The expression of Ank1/ANK1 in mice and humans shows extensive transcriptional variation with many transcripts possible. In mice, there are at least two transcriptional start sites and several alternative splice sites within 5′ exons, internal exons and 3′untranslated regions [245]. Two near full-length
transcripts predominate in erythrocytes (7.5kb and 9kb) and code for two proteins with differences at the C-terminus. The 7.5kb transcript is predominant in reticulocytes (late erythropoiesis), whereas the 9.0kb transcript is predominant in the spleen (early erythropoiesis) indicating that alternatively spliced Ankyrin is required at different times of erythroid development providing different Ank isoforms with alternative binding affinities depending on the stage of erythropoietic development. Similar transcripts are also expressed in human reticulocytes (7.2kb) and bone marrow (9kb) [232, 246, 247]. After the initial description of the gene, a new exon was discovered within intron 39 and leads to the production of three novel mouse transcripts: 3.5, 2.0 and 1.5kb. These new transcripts are muscle specific and are thought to encode a protein that acts as a membrane anchor [246].

Full-length ankyrin protein is composed of 3 domains: an amino (NH2)-terminal membrane-binding domain, a central spectrin-binding domain and a carboxy-(COOH) terminal regulatory domain [248, 249], which contains a death domain homologous motif [250]. The membrane binding domain consists of 33 amino acid repeats that allow binding of Band 3 [240, 251, 252]. At the NH2-terminus of ankyrin, 24 ANK repeats are stacked to form a solenoid, which acts as a spring and contributes to the elastic nature of RBC [253]. The membrane-binding domains and the spectrin-binding domain of ANK1 is highly conserved among species (including between mouse and human) and among the ANK family of proteins. The regulatory domain of ANK1 is conserved (79% identity) between mice and humans, but not conserved among other Ank family members (39% identity) [254].

Two mouse models for HS have been described to date, nb (normoblastic anemia) [255] and RBC2 [256]. A spontaneous point mutation is responsible for the nb phenotype, and involves the deletion of a guanine residue at nucleotide 4367 within exon 36 of Ank1. The deletion of the
nucleotide in exon 36 causes a frame shift and results in a premature stop codon, 13 codons downstream of the mutation [257]. Initially, Ank1ⁿᵇ/ⁿᵇ mice were believed to be null for ankyrin-1, but further analysis revealed that a 157kD truncated ANK1 protein is translated. The truncated protein contains the membrane-binding domain and a spectrin-binding domain, but lacks a full-length regulatory domain [257]. The RBC2 phenotype is caused by an ENU-induced point mutations at the splice acceptor for exon 41, causing use of an alternative splice acceptor site 22 nucleotides downstream (3’) of mutation. The predicted protein product for RBC2 is a truncated ANK1 (194kDa); however, the truncated protein has not been detected suggesting that the mutant transcript is destroyed by nonsense-mediated decay leading to a null allele [256].

An ENU mutagenesis screen was conducted in order to identify mouse models of hematopoietic disease. Automated peripheral blood counts were combined with a dominant ENU screen to detect mice with altered peripheral blood counts. Heterozygous mice of strain 7192 presented with mild HS; however, homozygous 7192 mice presented with severe HS.

2.3 Materials and Methods

2.3.1 Mice and ENU Mutagenesis

Three intraperitoneal injections of ENU (85mg/kg) were given one week apart to male C57Bl/6J (B6) mice, as previously described (Justice et al., 2000). A F1 generation (C3:B6) was produced by breeding ENU-mutagenized males to C3H/HeJ (C3H) females, and the offspring were called generation 1 (G1). G1 mice were screened for traits of interest including haematological cell parameters as determined by an automated haematological analyzer (see below) and outliers where bred to C3H mice, producing G2 mice, which were used to test heritability and for genetic mapping. For genetic mapping and strain maintenance, affected mice were bred to C3H mice to produce G3 to G8. Once the mutation was identified, mice where bred to 129S1/SVimJ (129)
mice to improve breeding efficiency. B6 males, C3H males/females and 129 males/females were purchased from the Jackson Laboratory.

2.3.2 Genetic Mapping and Sequencing
Tail DNA was isolated at weaning according to established protocols. A microsatellite-based genome scan was performed on DNA from affected mice and non-affected mice to determine the chromosome location of the mutation. The initial chromosomal location was narrowed by fine mapping via analysis of successive backcrossed generations of affected mice (G3-8). Once the mapping interval was narrowed down to 10Mb or less, candidate genes within the interval were selected for sequencing. Candidate genes were analyzed by polymerase chain reaction (PCR) amplifying exons (including at least 30 bp of introns at intron/exon borders) and amplified DNA was then sequenced using ABI Big-Dye and run on an ABI 3730XL.

2.3.3 PCR Genotyping
Approximately 100ng of genomic tail DNA was amplified per 25 µl (micro liters) reaction using 1.25 units (U) of DNA polymerase in PCR buffer with a final concentration of 0.2mM deoxyribonucleotide triphosphate mix and 3mM MgCl₂ (magnesium chloride). Multiplex PCR was performed to genotype WT, Ank²¹⁹²/+ and Ank²¹⁹²²¹⁹² animals using four primers. Two outer primers were designed to flank the area of the mutation Ank-1 FO (5’-GACTTTCCGGGATAGCATTTGAATGTAA-3’) at 0.3µM and Ank-1 RO (5’-AACCTAGAGGCACAGGAAGATCATAACAC-3’) at 0.3µM. Additionally, two nested primers were designed to amplified the wild-type allele and the mutant allele: Ank-1 FI (5’-CCTTTCTCTTCTGCAGGCATCTAGAG-3’) at 0.6µM amplifies the wildtype allele (G) and Ank1 RI (5’-GGGAATGAGGGAATCCTCATCATAACAC-3’) at 1.2µM amplifies the mutant allele (T). The PCR reaction can produce up to three bands depending on the genotype: 290bp
(control), 202bp (WT) and 141bp (Ank 7192) band. The PCR cycle profile was as follows: 1 cycle at 94 degrees celcius (°C) for 5 minutes (min), followed by 35 cycles at 94°C for 30 seconds (s), 67°C for 30s, and 72°C for 30s and a final extension cycle at 72°C for 10 min.

2.3.4 Hematologic Analysis
At 6 and 8 weeks of age, CBC (RBC, HCT, Hgb, MCV (mean corpuscle volume), MCH (mean corpuscle hemoglobin), MCHC, PLT and WBC (white blood cell)) were performed on the peripheral blood of all animals. Peripheral blood (20 to 30 µl) from the saphenous vein was collected into ethylenediaminetetraacetic acid (EDTA)–coated capillary pipettes (Drummond) and then transferred to 0.6ml Eppendorf tubes. CBC was performed on a Coulter Ac-T Differential Hematology Analyzer with veterinary software (Beckman-Coulter).

2.3.5 Histology
Tissues were collected and fixed in 10% neutral buffered formalin or Bouin’s fixative. Tissue sectioning and staining was performed by Centre for Modeling Human Disease (CMHD) pathology core (http://www.cmhd.ca/enu_mutagenesis/pathology.html). Tissues sections (4 mm) were prepared and stained with Hematoxylin and Eosin. Photographs of sections were taken with an Olympus BX51 microscope and an Olympus DP71 Camera.

2.3.6 In vitro Progenitor Assays
Single-cell suspensions of bone marrow (BM), spleen and liver were prepared in Iscove’s Modified Dulbecco’s Medium (IMDM) (Gibco) with 5% fetal bovine serum (FBS). A RBC lysis was performed using a RBC lysis buffer (0.14M ammonium chloride and 0.017M Tris) on BM, splenocytes, liver and PB. Cell counts and viability were assessed on a ViCell automated cell counter (Beckman-Coulter). For each assay, 3 aliquots of 2x10^4 (BM) or 1x10^5 (splenocytes, liver and PB) cells were mixed with the appropriate methylcellulose media, plated, and grown in
humidified chambers at 37°C and 5% carbon dioxide. Cells for CFU-C assays were grown in methylcellulose M3434 and cells for CFU-E assays were grown in methylcellulose M3334 (Stem Cell Technologies). CFU-E colonies were counted based on benzidine staining and morphology two days after plating. After 8 to 10 days initial plating, CFU-C colonies were counted based on colony morphology.

2.3.7 Flow Cytometry

RBC-lysed, single-cell suspensions of BM, splenocytes, liver and PB were stained with the following conjugated antibodies: Fluorescein isothiocyanate- (FITC) conjugated anti-CD71 (R17317), phycoerythrin-conjugated anti-Ter119 (TER-119), phycoerythrin-conjugated anti-IgG2b, κ and FITC-conjugated anti-IgG2a, κ. All antibodies were purchased from eBioscience (San Diego, USA). Cells suspensions were stained with specified antibodies in phosphate buffered saline (PBS) (2% FBS) for 25min at 4°C and then washed twice with PBS. Stained cells were run on a Beckman Coulter FC 500 FACS and analyzed using Flowjo software.

2.3.8 RBC Ghost Protein Analysis and Immunoblotting

Antibodies raised against ankyrin-1 domain-specific peptides were generously provided by Dr. Connie L. Birkenmeier (The Jackson Laboratory, Bar Harbor, ME) and have been previously described [257]. All other primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), unless indicated, including specific antibodies to detect ankyrin-1 (8C3). The anti-actin antibody (AC40) used for immunoblotting was purchased from Abcam (Cambridge, MA, USA). Secondary antibodies for Western detection, anti-goat IgG 800CW and anti-rabbit IgG 700, were purchased from LICOR Biosciences (Lincoln, NE, USA).

Blood was collected by saphenous vein puncture into EDTA-coated capillary tubes or by cardiac puncture immediately after sacrifice (carbon dioxide asphyxiation). Collected blood was
immediately transferred into 5 volumes PBS containing 2 mM EDTA, cells were then pelleted, washed once with PBS, re-pelleted and then resuspended in 10 volumes ice cold RBC ghost preparation buffer (5 mM sodium phosphate (pH 8) containing 1 mM EDTA and 100 µM phenylmethylsulfonyl fluoride (PMSF)). The cell suspension was then incubated on ice 30 – 60 min with intermittent mixing by inversion. This suspension (1 ml aliquots) was transferred into microfuge tubes and ghosts were then pelleted at maximum speed for 5 min at 4°C. The supernatant was aspirated and the pelleted ghosts were washed 5 times with 1 mL RBC ghost preparation buffer, filtered through a filter-top FACS tube sieve (BD Bioscience, On Canada), pelleted and then resuspended in 200 µl RBC ghost preparation buffer. Total protein concentration was determined by BCA assay. Equal concentrations of total protein were boiled for 1 min in sodium dodecyl sulfate sample buffer containing 2-mercaptoethanol and then fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8% or 10%). Gels were either stained directly with Coomassie Blue or transferred to Polyvinylidene fluoride membrane for immunoblotting. Membranes were blocked in 5% BSA (in tris buffered saline) or TBST (tris buffered saline + 0.02% Tween 20)) for at least 1 hr at room temperature or overnight at 4°C. Primary antibodies were used to probe blocked membranes (typically overnight at 4°C) using the following antibody dilutions (1:500) prepared in 2% BSA in TBST, except for p89, p65, pA, pB, and pC. After thorough washing with TBST, secondary antibodies for western detection were used at 1:15,000 dilutions (in TBST) at room temperature for 45 min, after which the membranes were thoroughly washed again and reactive bands detected using the Odyssey system (LICOR).
2.4 Results

2.4.1 Identification and Cloning of a New ENU-Derived HS Mutation

We performed a large-scale ENU mutagenesis screen to identify dominant mutations affecting hematopoiesis. B6 male mice were injected with ENU and then bred to untreated C3H female mice to produce G1 progeny. G1 mice were screened for hematopoietic lineage defects by performing a full CBC at 6 and 8 weeks of age.

Strain 7192 was established from a G1 male (222-8-2), which presented with elevated RBC (greater than 2 standard deviations from the norm), low MCV, low MCH (4 to 5 standard deviations from the norm) and high polychromasia on a peripheral blood smear at 6 and 8 weeks of age. The G1 male was then breed to C3H females in order to test heritability of the red cell phenotype. A high proportion of the G2 progeny (11/32) presented with the same phenotype as the G1 founder (Figure 2-1A and B), which demonstrated that the phenotype was highly penetrant (69%). Affected mice were bred again to the C3H background in order to genetically map the mutation, by performing a genome scan. A genome scan using microsatellite markers indicated that the phenotype mapped to an 83Mb region on chromosome 8 (Figure 2-1C). Successive backcrossing of affected 7192 mice to C3H background was performed in order to narrow the mapping interval to 7.2Mb region (18.6Mb to 25.8) on chromosome 8. The 7.2 Mb region contained 103 genes. A likely candidate was Ank1: therefore, we sequenced exon and exon/intron boundaries of Ank1. We identified a point mutation in exon 26, specifically a G to T transversion at 2683bp (Figure 2-2A) in the 7192 strain, which causes an in-frame premature stop codon (E to X) at 895 amino acid (aa) (Figure 2-2B). Sequencing of exon 26 confirmed the presence of the mutation in homozygous animals and demonstrated that no mutation was present in either of the founding strains, B6 and C3H (Figure 2-2A). A PCR genotyping strategy confirmed the presence of an ENU-induced point mutation in affected mice (Figure 2-2C).
Figure 2-1. Initial characterization and mapping of 7192 mice.

(A) Peripheral blood smears of wildtype (WT) mice and affected mice (7192/+) stained with Giemsa-Wright at 400x magnification. (B) Correlation between the high RBC and low MCV phenotype of 7192 in G2 and G3 male mice. The green box represents G2 and G3 mice that were classified as affected and had RBC values 2 SD or higher and MCV values 2 SD lower than G2-control mice. (C) Schematic representing the mapping of the 7192 phenotype to an 83Mb region on chromosome 8. Ensemble release 56 was used to designate the location of microsatellites used on chromosome 8. White boxes indicate markers homozygous for C3H genotype and grey boxes indicated markers are heterozygous (one B6 and one C3H). The candidate interval on chromosome 8 is represented by a red box and is located between the centromere and D8Mit106.
Figure 2-2. Identification of the *Ank1*<sup>7192</sup> allele.

(A) DNA sequence chromatograph shows a G to T transversion at nucleotide 2693 located within exon 26 of *Ank1*<sup>7192/7192</sup> mice. (B) Schematic of the ankyrin1 transcript structure, location of the 7192 mutation and the amino acid change resulting from the mutation. (C) PCR genotyping of WT, *Ank1*<sup>7192/+</sup> and *Ank1*<sup>7192/7192</sup> mice. The PCR genotyping results: WT mice have two bands 290bp and 202bp; *Ank1*<sup>7192/+</sup> (heterozygous (HET)) mice will have three bands 290bp, 202bp and 141bp; and *Ank1*<sup>7192/7192</sup> (homozygous (HOM) mice will have two bands 290bp and 141bp.
2.4.2 Pathological Analysis

To determine the viability of \( AnkI^{7192/7192} \) animals, \( AnkI^{7192/+} \) animals were intercrossed. The \( AnkI^{7192/+} \) animals existed on a number of backgrounds including: C3H (G7 and G8 original backcross strain) and 129 (5 generation backcross), as well as a C3H/129 hybrid. The \( AnkI^{7192/+} \) were initially bred to 129 mice to improve sluggish breeding on the C3H background. No \( AnkI^{7192/7192} \) pups were detected on a C3H background at weaning (0/64) and no pale (anemic) or jaundiced pups were observed immediately after birth. On the 129 background, however, jaundiced pups were detected after birth (immediately or within one day, 4/16). Most of the \( AnkI^{7192/7192} \) mice on the 129 background died between 4 to 7 days after birth, although one homozygote mouse survived to seven weeks of age. Timed matings were set up with \( AnkI^{7192/+} \) mice on the 129 background, and embryos were harvested on D17.5. Seventeen percent (6/23) of D17.5 embryos were pale and jaundiced, and all of these embryos were determined to be \( AnkI^{7192/7192} \) by genotyping. Additionally, \( AnkI^{7192/7192} \) embryos were anemic (HCT--\( AnkI^{7192/7192} \): 0.202 +/-0.030 versus WT or \( AnkI^{7192/+} \):0.332 +/- 0.015) and runted (Body weight (g)--\( AnkI^{7192/7192} \):0.735+/- 0.042 vs. WT and \( AnkI^{7192/+} \): 0.890 +/- 0.042), suggesting that embryonic erythropoiesis is altered in \( AnkI^{7192/7192} \).

When \( AnkI^{7192/+} \) with the C3H/129 hybrid background were intercrossed, \( AnkI^{7192/7192} \) viable pups were observed at weaning (7/42), but at a frequency (11%) lower than Mendelian inheritance (25%) (Figure 2-3E). \( AnkI^{7192/7192} \) pups that did survive to weaning, could then survive up to 4 months of age, but others died between 3 weeks to 4 months of age (no animals were kept beyond 4 months of age). \( AnkI^{7192/7192} \) pups displayed mild to severe jaundice (Figure 2-3D) and severely jaundiced pups often died before weaning (as documented by the loss of jaundiced pups that were tattooed at birth). At birth, \( AnkI^{7192/7192} \) pups were runted and this lowered body mass continued throughout life (Table 2-2). By 8 weeks of age, \( AnkI^{7192/7192} \) mice
presented with severely macrocytic anemia (HCT--WT: 0.516 +/- 0.03 versus \textit{Ank}^{\text{7192/7192}}: 0.223 +/- 0.02, p<0.00001) and displayed profound leukocytosis (WBC--WT: 11.1 +/-3.11 vs \textit{Ank}^{\text{7192/7192}}: 169 +/-17.5, p<0.00001) (Figure 5A and Table 1).

Overall, homozygous mice displayed gross anatomical and histological changes at 12-14 weeks of age (males). For example, \textit{Ank}^{\text{7192/7192}} mice were 60% smaller than WT or \textit{Ank}^{\text{7192/+}} littermate controls (Body weight (g)—WT: 34.6 +/- 0.6 vs. \textit{Ank}^{\text{7192/7192}}: 21.6 +/- 3.6).

Histological examination of femoral BM showed no marked change in \textit{Ank}^{\text{7192/7192}} bone marrow architecture of homozygous mutant marrow (Figure 2-4Di WT and Figure 4Di\textsuperscript{v} \textit{Ank}^{\text{7192/7192}}).

Splenomegaly was evident in \textit{Ank}^{\text{7192/7192}} mice (Figure 2-4Ai) as demonstrated by a 19-fold enlargement of the spleen in homozygous mice (splenic weight (g)—WT: 0.129g +/- 0.023 vs. \textit{Ank}^{\text{7192/7192}}: 2.51 +/- 0.70). Histological examination of \textit{Ank}^{\text{7192/7192}} spleens showed a complete disruption of normal splenic architecture and evidence of marked diffuse extramedullary hematopoiesis and increased erythropoiesis (Figure 2-4Bii WT and Figure 2-4Bvi \textit{Ank}^{\text{7192/7192}}).

Liver/body weight index ((g liver/g total) x100) was 80% higher in \textit{Ank}^{\text{7192/7192}} mice when compared to littermate controls (Figure 2-4Aii), indicating that for their size \textit{Ank}^{\text{7192/7192}} have an enlarged liver. Pockets of extramedullary hematopoiesis were observed within the livers of \textit{Ank}^{\text{7192/7192}} in contrast to no evidence of extramedullary hematopoiesis was observed in WT livers (Figure 2-4Aiii WT and Figure 2-4Avii \textit{Ank}^{\text{7192/7192}}). 40% of \textit{Ank}^{\text{7192/7192}} males had pigmented gallstones by 12-13 weeks of age, but no gallstones were detected in littermate WT or \textit{Ank}^{\text{+/7192}} mice (Figure 2-4Aiai \textit{Ank}^{\text{7192/7192}} and Figure 2-4Aiib WT). The hearts of \textit{Ank}^{\text{7192/7192}} mice were also enlarged by 30% compared to WT mice (heart weight (g)—WT: 0.160g +/- 0.024 versus \textit{Ank}^{\text{7192/7192}}: 0.207g +/- 0.045) (Figure 2-4Aii and Table 2-2). Iron deposits were evident in the histological sections of kidneys from \textit{Ank}^{\text{7192/7192}} mice, but are absent from WT and heterozygous littermates (Figure 2-4Biv WT and 2-4Bviii \textit{Ank}^{\text{7192/7192}}).
Figure 2-3. The survival of \( \text{Ank}^{7192/7192} \) mice at late embryo, neonatal and 3 weeks of Age.

Figures A-C represent the results of timed matings between two \( \text{Ank}^{7192/+} \) mice on a 129 background, all embryos were harvested at D17.5 pc. (A) An anemic embryo (\( \text{Ank}^{7192/7192} \)) and a control embryo (\( \text{Ank}^{7192/+} \)) at D17.5 pc. (B) The genotype distribution of WT, \( \text{Ank}^{7192/+} \) and \( \text{Ank}^{7192/7192} \) embryos at D17.5 p.c. (C) The weight and hematocrit (left and right) of WT or \( \text{Ank}^{7192/+} \) (white bars) and \( \text{Ank}^{7192/7192} \) (black bars) embryos at D17.5. The weight of \( \text{Ank}^{7192/7192} \) embryos was significantly reduced compared to pooled values for WT and \( \text{Ank}^{7192/+} \) embryos (\( p<0.0009 \)). A significant reduction in the hematocrit of \( \text{Ank}^{7192/7192} \) embryos was observed when compared to WT or \( \text{Ank}^{7192/+} \) (\( p<0.0006 \)). Each column represents the mean of each genotype and error bars represent the standard error of the mean. Panels D and E represent the results of the \( \text{Ank}^{7192/+} \) intercross on a hybrid background (C3H/129). (D) Day 2 neonatal pups with the following genotypes from left to right: WT, \( \text{Ank}^{7192/+} \), WT and \( \text{Ank}^{7192/7192} \) (*). The * represents a homozygous pup that was both runted and jaundiced. (E) The genotype distribution of WT, \( \text{Ank}^{7192/+} \) and \( \text{Ank}^{7192/7192} \) pups surviving to 3 weeks of age.
Figure 2-4. The organ morphology and histology of $Ank^{7192/7192}$ mice and littermate controls.

All animals used were male mice at 12-13 weeks of age on a hybrid background. (A) Gross morphology of spleen, liver and hearts (i-iii) from WT and $Ank^{7192/7192}$ mice. The gall bladder of $Ank^{7192/7192}$ (ii a) appears abnormal with gallstones present and no evidence of gallstones are seen in WT littermates (ii b). Images were acquired with Olympus C-5060 digital camera. (C) Histological sections (hematoxylin and eosin stained) of femur (i & v), spleen (ii & vi), liver (iii & vii) and kidney (iv & viii). No abnormalities are observed in the bone marrow of $Ank^{7192/7192}$ mice (iv). Deposits of hematopoietic cells are observed in the liver from $Ank^{7192/7192}$ mice and are indicated by black arrows (vii). Iron deposits in the kidneys of $Ank^{7192/7192}$ mice are indicated by black arrows (viii).
Table 2-1. CBC performed on PB of Ank1\textsuperscript{7192} mice on C3H and C3H/129 backgrounds.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>C3H (G6/7)</th>
<th>Hybrid</th>
</tr>
</thead>
<tbody>
<tr>
<td># Mice</td>
<td>19</td>
<td>26</td>
</tr>
<tr>
<td>RBC</td>
<td>9.56 ± 0.48</td>
<td>11.06 ± 0.81*</td>
</tr>
<tr>
<td>Hgb</td>
<td>165 ± 7.88</td>
<td>169 ± 10.6</td>
</tr>
<tr>
<td>HCT</td>
<td>0.517 ± 0.03</td>
<td>0.541 ± 0.04*</td>
</tr>
<tr>
<td>MCV</td>
<td>54.0 ± 1.36</td>
<td>48.9 ± 2.12*</td>
</tr>
<tr>
<td>MCH</td>
<td>17.3 ± 0.37</td>
<td>15.2 ± 0.73*</td>
</tr>
<tr>
<td>MCHC</td>
<td>320 ± 8.73</td>
<td>310 ± 11.6*</td>
</tr>
<tr>
<td>PLT</td>
<td>1225 ± 210</td>
<td>1256 ± 241</td>
</tr>
<tr>
<td>WBC</td>
<td>10.6 ± 2.02</td>
<td>11.9 ± 2.38</td>
</tr>
</tbody>
</table>

The average values for each parameter are presented with the standard deviation after the ±. t-tests were performed to compare WT with Ank1\textsuperscript{7192/+} (C3H and Hybrid), and WT with Ank1\textsuperscript{7192/7192} (hybrid only), * indicates values that were significantly different (p < 0.05).
Table 2-2. Organ weight of $Ank1^{7192/7192}$, $Ank1^{7192/+}$ and wild-type mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT</th>
<th>$Ank1^{7192/+}$</th>
<th>$Ank1^{7192/7192}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>34.6 ± 6.0</td>
<td>32.3 ± 5.5</td>
<td>21.6 ± 3.6*</td>
</tr>
<tr>
<td>Spleen (g)</td>
<td>0.129 ± 0.023</td>
<td>0.140 ± 0.049</td>
<td>2.51 ± 0.70*</td>
</tr>
<tr>
<td>% of body weight</td>
<td>0.38 ± 0.097</td>
<td>0.44 ± 0.15</td>
<td>11.6 ± 2.7*</td>
</tr>
<tr>
<td>Kidney (g)</td>
<td>0.270 ± 0.039</td>
<td>0.250 ± 0.038</td>
<td>0.254 ± 0.057</td>
</tr>
<tr>
<td>% of body weight</td>
<td>1.44 ± 0.31</td>
<td>1.45 ± 0.39</td>
<td>1.17 ± 0.15</td>
</tr>
<tr>
<td>Heart (g)</td>
<td>0.160 ± 0.024</td>
<td>0.149 ± 0.017</td>
<td>0.207 ± 0.045*</td>
</tr>
<tr>
<td>% of body weight</td>
<td>0.478 ± 0.039</td>
<td>0.464 ± 0.038</td>
<td>0.956 ± 0.12*</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>1.34 ± 0.19</td>
<td>1.41 ± 0.24</td>
<td>1.61 ± 0.40</td>
</tr>
<tr>
<td>% of body weight</td>
<td>3.94 ± 0.66</td>
<td>4.37 ± 0.38</td>
<td>7.45 ± 1.3*</td>
</tr>
</tbody>
</table>

(WT n=7, $Ank1^{7192/+}$ n=7, $Ank1^{7192/7192}$ n=8). The average values for each parameter are presented with the standard deviation after the ±). t-tests were performed to compare WT with $Ank1^{7192/+}$ (C3H and Hybrid), and WT with $Ank1^{7192/7192}$ (hybrid only), * indicates values that were significantly different (p < 0.05).

2.4.3 Peripheral Blood Analysis

$Ank1^{7192/7192}$ mice exhibited profound hemolytic anemia as evident by 50% decrease in hematocrit (Figure 2-5A). Peripheral-blood smears from homozygous mice showed spherical red cells, red cell fragments, increased polychromasia and nucleated immature red cells (Figure 2-5B). $Ank1^{7192/7192}$ mice had a 10-fold increase in Ter119/CD71 co-expression in peripheral blood (Figure 2-5C), suggesting an increase in red cell precursors (WT: 2.65+/− 1.91 vs. $Ank1^{7192/7192}$:37.5+/−4.0, p-value <0.02). There was also an increase in circulating hematopoietic progenitors in the peripheral blood of homozygous mice, as evident by a 6-fold increase in CFU-C progenitors (WT: 4.35 +/- 9.15 vs. $Ank1^{7192/7192}$: 56.2 +/- 51.8, p-value < 0.04) (Figure 2-5D).
Figure 2-5. Peripheral blood analysis of *AnkI*<sup>7192/7192</sup> mice.

(A) Altered PB parameters in WT, *AnkI*<sup>7192/</sup> and *AnkI*<sup>7192/7192</sup> mice including: RBC, MCV, HCT and WBC. An asterisk (*) indicates statistical significant difference values from WT mice (WT vs. *AnkI*<sup>7192/</sup> and WT vs. *AnkI*<sup>7192/7192</sup>) (p < 0.05). (B) Peripheral blood smears stained with Wright-geimsa at 400X magnification from WT, *AnkI*<sup>7192/</sup> and *AnkI*<sup>7192/7192</sup> 8-week-old males. (C) Flow cytometric analysis of the Ter119/CD71 expression in peripheral blood of WT, *AnkI*<sup>7192/</sup> and *AnkI*<sup>7192/7192</sup> mice. The black box represents cells that stained positive for both Ter119 and CD71 and the number inside the box represents the percentage of cells staining positive for representative individual mice. (D) Hematopoietic and erythroid progenitors were analyzed by colony assays (CFU-C) of the peripheral blood of WT, *AnkI*<sup>7192/</sup> and *AnkI*<sup>7192/7192</sup> mice. The average CFU-C frequency is represented by the entire bar and stacked within each bar is 5 sub-bars that represent the 5 colony types detected in a CFU-C assay.
2.4.4 Extramedullary Hematopoiesis

The 2.5-fold increase in Ter119/CD71 co-expression within bone marrow observed in \( AnkI^{7192/7192} \) mice (WT 20.3 +/- 6.6 versus (vs.) \( AnkI^{7192/7192} \) 50.6 +/- 9.3, p-value <0.006; Figure 2-6i WT and Figure 2-6iii \( AnkI^{7192/7192} \)) suggested that there was an increase in red cell precursors in the BM. A four-fold increase in erythroid-specific CFU-E frequency (WT: 22.8 +/- 3.3 vs. \( AnkI^{7192/7192} \): 102.4 +/- 29, p-value<0.004) was observed in the bone marrow of \( AnkI^{7192/7192} \) mice (figure 2-7iv), while the total CFU-C frequency was decreased by 35% (WT: 63.8 +/- 14.9 vs. \( AnkI^{7192/7192} \): 40.3 +/- 14.8), including BFU-E (WT: 5.6 +/- 4.4 vs. \( AnkI^{7192/7192} \): 2.63 +/- 2.0 p-value<0.07). The spleens of homozygous mice showed increased extramedullary hematopoiesis as evident by eight-fold increase in total CFU-C (WT: 5.8 +/- 2.0 vs. \( AnkI^{7192/7192} \): 50.0 +/- 10.3, p-value <0.0005; Figures 2-7ii and 2-7v), as well as a 125-fold increase in CFU-E (WT: 0.93 +/- 1.43 vs. \( AnkI^{7192/7192} \): 119 +/- 83, p-value=0.005). Red cell precursors were also increased in the spleen of \( AnkI^{7192/7192} \) mice as evidenced by a 25-fold increase in Ter119/CD71 co-expressing cells of the spleen (WT: 2.1 +/- 1.9 vs. \( AnkI^{7192/7192} \): 54.3 +/- 13.8, p-value <0.001). Hematopoietic infiltration of the liver was demonstrated by a 25-fold increase in CD71 expression in the liver (WT: 2.34 +/- 1.21 vs. \( AnkI^{7192/7192} \): 58.7 +/- 12.7, p-value <0.00005) and a 50-fold increase in CFU-C progenitors in the liver (WT 0.35 +/- 1.0 vs. \( AnkI^{7192/7192} \) 17.8 +/- 12.4, p-value <0.01).
Figure 2-6. Extramedullary hematopoiesis – erythroid precursors in Ank17192/7192 mice.

Erythroid precursors in bone marrow, spleen and liver were analyzed by flow cytometry. Freshly isolated bone marrow and spleen cells were stained with FITC-conjugated anti-CD71 and a phycoerythrin-conjugated anti-TER119, while liver cells were only stained with anti-CD71. Panels i, iv, ii and v represent density plots of BM cells from WT/Ank17192/+ and Ank17192/7192 and Sp cells from WT/Ank17192/+ and Ank17192/7192, respectively. The black boxes in panels i, ii, iv and v indicate cells that co-express CD71 and Ter119. The black line indicates the proportion of liver cells that express CD71. Ank17192/7192 (left panel) mice show a 2.5 fold increase in Ter119/CD71 co-expression in bone marrow (p-value <0.006). Co-expression of CD71/Ter119 was increased 25-fold in the spleen of Ank17192/7192 (p-value <0.001). The liver cells from Ank17192/7192 mice displayed a 25-fold increase in CD71 expression (p-value <0.00005).
Figure 2-7. Extramedullary hematopoiesis – hematopoietic and erythroid progenitors in Ank1<sup>17192/17192</sup> mice.

Hematopoietic and erythroid progenitors were analyzed by colony assays (CFU-C and CFU-E). In the top three panels, the average CFU-C frequency is represented by the entire bar and stacked within each bar is 5 sub-bars that represent the 5 colony types detected in a CFU-C assay. CFU-C assays were performed on bone marrow (left), spleen (center), and liver (right). Ank1<sup>17192/17192</sup> showed a 35% decreased in CFU-C colonies in the bone marrow (WT 63.8 +/- 14.9 vs. Ank1<sup>17192/17192</sup> 40.3 +/- 14.8; p-value <0.003). Within the spleen and liver large increases in CFU-C were seen in Ank1<sup>17192/17192</sup> mice, specifically an 8-fold (p-value <0.0005), a 6-fold (p-value<0.04) and a 50-fold increase (p-value <0.01), respectively. The bottom two panels (iv and v) represent the CFU-E of WT, Ank1<sup>17192/+</sup> and Ank1<sup>17192/17192</sup> generated from BM and spleen cells. A four-fold increase in CFU-E frequency was observed in the BM of Ank1<sup>17192/17192</sup> mice (p-value<0.004). The spleens of Ank1<sup>17192/17192</sup> showed a 125-fold increase in CFU-E colonies (p-value<0.005). All experiments were repeated 3 times with an n of 2-3 for each genotype within each experiment. All experiments used male mice at 12-13 weeks of age.
2.4.5 Protein Analysis

Analysis of protein expression in \textit{Ank1}\textsuperscript{7192} mice was performed by collaborator Michael Hughes. The predicted protein product for \textit{Ank1}\textsuperscript{7192} is a truncated Ankyrin1 protein that lacks the majority of the regulatory domain and the majority of the spectrin-binding domain, but has an intact band 3 binding domain. Coomassie Blue staining of membrane proteins extracted from RBC ghosts prepared from peripheral blood of WT, \textit{Ank1}\textsuperscript{7192/+} and \textit{Ank1}\textsuperscript{7192/7192} mice revealed an altered pattern of RBC membrane proteins in homozygous mice, in comparison to WT or heterozygous mice (Figure 2-8A). When the proteins were analyzed by immunoblot, no full-length Ank1 was detected with antibody targeting the NH\textsubscript{2}-terminus of Ankyrin, but an intense band was observed at 100kDa in \textit{Ank1}\textsuperscript{7192/7192}. The 100kDa band also was also observed in protein extracts from WT mice, but the band was more intense in homozygous mice; therefore, it is difficult to know whether the more intense band seen in \textit{Ank1}\textsuperscript{7192/7192} protein extracts is the predicted truncated protein, an Ank isoform or another protein with overlapping immunoreactivity (Figure 2-8B). An additional faint band was observed around 60 kDa in protein extracts from \textit{Ank1}\textsuperscript{7192/7192} mice, which was completely absent in WT mice. It is possible that this band is the truncated ANK1 protein, but it would be running much smaller than predicted (Figure 2-8B). Truncated ANK1 in \textit{Ank1}\textsuperscript{7192} mice should lack a spectrin-binding domain, but contain a band 3 binding domain, and this is confirmed by immunoblotting with domain-specific antibodies for Ank (Figures 2-8Ci and 2-8Cii). An immunoblot with antibodies raised to the protein product of alternatively spliced Ank1 (altered exon 41 and 42 splicing), revealed that \textit{Ank1}\textsuperscript{7192/7192} and WT mice express a near full-length ANK1 isoform but at low concentrations (Figure 2-8Ciii).
Figure 2-8. Identification and characterization of Ank1\textsuperscript{7192} ANK1 protein.

(A) Coomassie blue staining of RBC ghosts protein preparations from WT, Ank1\textsuperscript{7192/+} and Ank1\textsuperscript{7192/7192} mice. (B) Immunoblot RBC ghost proteins from WT, Ank1\textsuperscript{7192/+} and Ank1\textsuperscript{7192/7192} mice stained with anti-NH\textsubscript{2}-terminal ankyrin antibody. (C) Immunoblot of RBC ghost proteins from WT and Ank1\textsuperscript{7192/7192} mice with Ank-domain-specific antibodies (i and ii) and alternatively spliced Ank1 antibodies (iii and iv). (D) Schematic diagram representing the alternative splicing that occurs at the COOH-terminus of Ank1. (E) Schematic diagram of the domains within the ANK1 and the predicted ANK1 for nb, RBC2, and 7192 mice. Each domain within ANK1 is represented by the following colour: red, membrane-binding domain; green, spectrin-binding domain; blue, regulatory domain; and light blue, predicted-regulatory domain translated out of frame.
Figure 2-9. Human HS mutations in Ank1.

Schematic of human ANK1 associated HS mutations and the relative location of the $Ank^{7192}$ mutation. This diagram was developed from a table summarizing human mutations in ANK1 [235].
2.5 Discussion

A new mouse model for HS (7192 strain) was identified from a dominant ENU hematology screen. The mutation responsible for the 7192 phenotype, induces a premature stop codon in exon 26 (G to T at 2683 nucleotide (nt)), which is predicted to produce an Ankyrin protein 100kDa or 895aa in length. The truncated Ankyrin protein in 7192 mice contains an intact membrane-binding domain and a small portion of a spectrin-binding domain, but completely lacks a regulatory domain.

The AnkI^{7192/} mice exhibit mild HS. Heterozygous mice have unaltered hematocrit values, similar to patients with mild HS. AnkI^{7192/} mice have altered RBC morphology (low MCV and spherical RBC by scanning electron microscopy, data not shown) and increased polychromasia. Similar to humans with mild HS, AnkI^{7192/} mice have increased osmotic fragility (data completed by MH, data not shown). No evidence of splenomegaly or gallstones is observed in AnkI^{7192/} (hybrid) at 12-14 weeks of age in contrast to patients with mild HS, but this may be due to the age of the mice.

Late gestation AnkI^{7192/7192} embryos show near normal Mendelian distribution on a single genetic background (129), but have severe phenotypes in utero including anemia and runting. Genetic background and hybrid-vigour appear to play a vital role in the survival of neonatal 7192 homozygotes as no live homozygotes were detected on the C3H background and most AnkI^{7192/7192} on the 129 background die neonatally, but hybrid homozygous pups can survive with diminished numbers to weaning. Providing AnkI^{7192/7192} hybrid mice survive up to weaning, they can live to at least 4 months of age. In utero anemia and runting in AnkI^{7192/7192} embryos, as well as decreased viability of AnkI^{7192/7192} neonates, demonstrates the importance of Ank expression in the embryo. Genetic background and hybrid vigor also play an important role in
Embryonic phenotypes in Ank1<sup>7192/7192</sup> mice are shared with RBC2/RBC mutants [256], but are compensated for in nb/nb embryos [258]. Ank1<sup>7192/7192</sup> adults (12-13wks old) share many phenotypes of severe HS in humans with spherical RBCs, profound reticulocytosis and marked hemolysis. Bone marrow hematopoiesis in Ank1<sup>7192/7192</sup> shows a shift to the production of RBC with increased CFU-E and erythroblast production and a decrease in the formation of other CFU-C colony types. Extensive extramedullary hematopoiesis is observed in 7192 homozygous adults in the PB (increased reticulocytes, erythroblasts and hematopoietic progenitors), spleen (splenomegaly and increased erythroblast, CFU-E, CFU-C), and liver (increased erythroblast and CFU-C). Ank1<sup>7192/7192</sup> mice show evidence of iron overload, including iron deposits in the kidney and pigmented gallstones, which is caused by increased RBC processing. Many of the features of severe HS observed in Ank1<sup>7192/7192</sup> are shared by RBC2/RBC2 and nb/nb mice.

The predicted protein product is a 100kD protein that should lack a regulatory domain and most of the spectrin binding domain, but contain a full-length Band3 binding domain. This truncated protein is predicted to have partial functionality (neomorphic), because it is unable to bind to spectrin. No full-length Ank1 was detected in Coomassie blue stained RBC ghosts, nor was it detected by three different Ank1 antibodies. Although standard immunological reagents were unable to detect full-length Ank1 in 7192 homozygotes, two possible truncated proteins were detected at approximately 60 and 100kD. However, a 100kD band was also observed in protein extracts from WT mice with all antibodies capable of detecting the truncated ANK<sup>7192</sup>, making the identification of the Ank1<sup>7192</sup> protein difficult. The 100kD band in Ank1<sup>7192/7192</sup> mice is more intense than the band in WT mice, which suggests that the 100kD band in Ank1<sup>7192/7192</sup> mice represents a truncated Ank1 protein. A smaller band, of unknown transcriptional origin in
*Ank1*<sup>7192/7192</sup> mice was also detected that is 62kD, with the N-terminal and Band-3 domain Ankryin1 antibody. Domain-specific antibodies confirm the presence of truncated Ank1 proteins in 7192 homozygotes, which contain a Band 3-binding domain, but lack a spectrin-binding domain. Alternative splicing at the C-terminus of Ank occurs naturally at low levels in WT mice and is seen in *Ank*<sup>7192/7192</sup> mice, suggesting that a small amount of functional Ank1 is produced in our homozygous mice. The alternatively-spliced-functional Ank1 should not be formed in 7192 mice because the premature stop codon cause by the ENU mutation should terminate translation before exon 41. The production of an exon 41 alternative-spliced Ank1 in 7192 mice would either require a corrective read through exon 26 or additional exon skipping involving exon 26. Ank transcript analysis was performed by Kamal Garcha has been unable to detect a transcript that skips exon 26 in WT or 7192 mice (data not shown).

The *nb* mutation is a hypomorphic allele of Ank1 encoding a truncated protein product (157kD) that contains a band3- and spectrin-binding domains, but lacks the majority of the regulatory domain. The truncated protein in *nb/nb* mice is predicted to have reduced functional activity, but maintains normal membrane ultrastructure [257, 259]. Unlike 7192, no alternatively spliced Ank1 protein (exon41 and 42) has been detected in *nb/nb* mice [257]. The *RBC2* mutation occurs at the splice acceptor for exon 41 and is predicted to use an alternative splice acceptor within exon 41, which causes a frame shift leading to a premature stop codon and should result in a truncated protein (194kD). Increased RNA message and lack of truncated or full-length Ank1 protein was used to support the hypothesis that *RBC2/RBC2* mice are null for Ank1. No protein analysis of an exon 41/42 alternatively-spliced Ank protein was published for RBC2 mice, which is particularly interesting given the location of the mutation in RBC2 (splice acceptor of exon41) [256].
Functionally, 7192 and RBC2 homozygotes share many similarities and are more severe than nb homozygotes because 7192/7192 and RBC2/RBC2 mice present with in utero phenotypes and produce no full-length Ank1 or functional protein translated from the major transcriptional product [256]. All three mutations display diminished viability on inbred backgrounds and 7192 and nb mutations are more viable on a hybrid strains (RBC2 has only been studied on a Balb/c background) [255]. Phenotypically and molecularly, 7192 and RBC2 are quite similar, but differ because 7192 produce an alternatively spliced Ank protein at low concentrations that may be functional [256].

7192, RBC2 and nb homozygotes are all models of severe HS demonstrating active hemolysis, extensive extramedullary hematopoiesis and iron overload (jaundice and/or gallstones) [255, 256, 260]. Direct comparison of the three mouse models of HS is difficult because each strain was maintained on a different genetic background (or combinations of genetics backgrounds). A comparison of the three mouse models of HS is possible by comparing ankyrin proteins. 7192/7192 and RBC2/RBC2 homozygotes are likely to represent more severe models of HS compared to nb/nb, because nb/nb mice produce a truncated but functional Ank-1 while 7192/7192 and RBC2/RBC2 do not [256, 257]. Extensive data on the phenotype for RBC2 and nb heterozygotes is not available. Heterozygotes for the 7192 and RBC2 mutation would be good models for mild HS because they both have altered RBC shape (low mcv) and increased osmotic fragility, but have not been aged long enough to study the incidence of gallstones [256]. Similar phenotypes have not been reported in nb/+ mice.

In conclusion, Anki7192 mutant mouse represents a new model for studying HS and may play an important role in the investigation of ANK1 in RBC membrane architecture and HS. The Anki7192 mutation models a gamut of HS disease, because homozygous mice model severe HS
and heterozygous model mild HS. The three mouse models of HS nb, RBC2 and Ank7192; have distinct genetic causes and the resulting molecular consequences (respectively, hypomorph, null and neomorph) and therefore have the ability to complement to each other in the study of Ank1, RBC architecture and HS. Additionally, the three mouse models of HS represent the spectrum of Ank mutations in humans (figure 2.9).
Chapter 3: *MYH9*<sup>7238</sup>: A MOUSE MODEL FOR MYH9-RELATED DISORDERS

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3.1 Abstract

An ENU dominant screen for mutations affecting steady-state hematopoiesis yielded a mutant designated 7238/+ that presented with macrothrombocytes. Genetic mapping refined the mutation to a 3.0 Mb region on chromosome 15, which contained the candidate gene Myh9. Massive parallel sequencing of sequence captured DNA identified a missense mutation at amino acid 1443 (glutamine to leucine, Q1443L) that resulted from a single-base pair mutation at nucleotide 4566 (A → T transversion) in exon 30 of Myh9. Subsequent analysis revealed that Myh97238/7283 mice are viable and display a more severe phenotype than Myh97238/+, specifically macrothrombocytopenia. The Myh97238 mutation affects only the megakaryocyte lineage, as the number and proportion of other blood cells, along with CFU-C were unaltered. The pathogenesis of macrothrombocytopenia, is due to a defect in thrombopoiesis, not megakaryopoiesis, as CFU-MK and MK number were normal in Myh97238 mice. Myh9 (Myosin heavy chain 9) is a negative regulator of proplatelet formation, and therefore the defect in thrombopoiesis is in platelet shedding, consistent with DNA content of megakaryocytes being formed normally in Myh97238 mice. The combination of macrothrombocytes, neutrophil inclusions and a missense mutation in Myh9, make Myh97238 mice a mouse model of human Myh9-related disorders (MYH9RD). In addition to giant PLT and neutrophils inclusions, human patients with MYH9RD have Alport-like symptoms at varying degrees of incidence, which include kidney defects, hearing loss and cataracts. The incidence of cataracts in year old Myh97238 mice is increased, but the incidence of kidney defects and hearing loss have yet to be determined in Myh97238 mice and therefore will require further study. Myh97238 mice represent the first mouse model of MYH9RD to both accurately model the genetic origins of the disorder and have the capacity to model all of the systemic manifestations of the disorder.
3.2 Introduction

MYH9RD is a hereditary macrothrombocytopenia with neutrophil inclusions accompanied by, in a subset of patients with MYH9RD a variable penetrance of nephritis, hearing loss and presenile cataracts[160]. Once believed to be four distinct macrothrombocytopenic disorders MYH9RD, May-Hegglin disease, Fechtner syndrome, Epstein syndrome and Sebastian syndrome all have been grouped together because they are caused by mutations in MYH9 [156, 157, 261]. Also, the additional clinical manifestations that made the disorders distinct actually represent a continuous spectrum [262]. MYH9RD is the most commonly inherited macrothrombocytopenia, but is a relatively rare, with only 101 unrelated families reported [156, 157, 262-272]. The actual number of people with MYH9RD is thought to be much higher due to underreporting and misdiagnosis, because of the benign nature of the disease[160]. The clinical manifestations of MYH9RD, including bleeding tendency and renal impairment, as well as the age of onset for hearing loss and cataracts, are heterogeneous, even among a single family with the same mutation [160]. Although the reasons for clinical heterogeneity within MYH9RD are unknown, it is likely explained by unidentified genetic, epigenetic factors or environmental factors[148]. A major risk for patients with MYHRD is misdiagnosis as chronic autoimmune thrombocytopenia (ITP), as patients misdiagnosed with ITP undergo unnecessary immunosuppressive treatments and unnecessary splenectomies. The possible misdiagnosis of patients with MYH9RD underlines the requirement for a careful and rigorous diagnosis[160].

The MYH9 gene encodes non-muscle myosin heavy-chain IIA protein (NMMHCIIA). MYH9 is composed of 40 exons and is 1960 amino acids in length (220kD) [273, 274]. Non-muscle myosin II (NMMII) is a member of the myosin superfamily, and 3 isoforms are
expressed in mice and humans: IIA, IIB and IIC [275]. NMM IIA exists as a hexamer composed of two non-muscle myosin heavy chains (NMMHC) and 4 myosin light chains [274]. NMMHC have 3 functional and structurally distinct domains; the head, neck and tail [274]. The head domain is the motor domain for myosin and its binding to actin exposes an adenosine triphosphate binding site on the myosin head. The neck contains IQ motifs that allow the myosin light chain to non-covalently bind to NMMHC. The tail is the most diverse domain in myosin superfamily and is responsible for specific myosin functions including: myosin dimerization and filamentation, binding to cell membrane and binding to membrane proteins [276]. In NMMIIA the tail is composed of 2 alpha helices that allow for filament assembly and binding to the cell membrane [277, 278]. In NMMHC IIA, exons 1 to 18 encode the head, exon 19 encodes the neck domain and exons 20 to 40 encode the tail [274, 279]. Most cells express NMMHC IIA including PLT and leukocytes and cells of the kidney, cochlea and lens [265, 280]. Myosin light chain phosphorylation regulates the activity myosin heavy chains. The phosphorylation of myosin light chains can be accomplished by three kinases: Rho associated kinase, myosin light chain kinase and p21-activated kinase[281].

Ablation of myosin IIA leads to significant defects in cell contractility, focal adhesions and actin stress-fiber organization. Surprisingly cells deficient in myosin IIA have increased cell migration, demonstrating that myosin IIA is a negative regulatory of microtubule assembly as its absence stabilizes microtubule formation [282-287]. All three myosin II’s are expressed in mammalian cells, but are expressed in different proportions [288]. The three myosin II isoforms share considerable homology and some overlapping function, but have significant differences in enzymatic properties, sub-cellular localization, molecular interaction and tissue distribution [289]. For example, it has been shown that both A and B co-localize to zonula adherens, but have both have distinct and necessary roles in the zonula adherens. Within the zonula adheren it has
been shown that myosin II A and B are regulated by different mechanisms: myosin IIA is regulated by E cadherin adhesion, Rho/ROCK and myosin light chain kinase and myosin B is dependent on Rap [288].

MYH9RD is inherited by autosomal dominance, and to date, more than 30 mutations in MYH9 have been found throughout the gene. No patients have been found to be homozygous for mutations to MYH9. Mutations in MYH9 are have been described for eleven of its 40 exons (1, 10, 16, 24-26, 30, 31, 37-38 and 40), and are mainly point mutations, although deletions, duplications occur, but are rare [156, 157, 262-272, 290-299]. The most common mutations only affect a few residues and occur at amino acid N93, N96 (asparagine: exon 1, head), R702 (arginine: exon 16 head), R1165 (exon 26, tail), D1424 (aspartic acid: exon 30, tail), E1841 (exon 38, tail) and R1933 (exon 40, tail). Mutations in MYH9RD are mainly missense mutations (80% of families), but nonsense, frame shift and small deletions do occur (20% of families, primarily in exon 40) [300]. Mutations in the head domain are expected to cause more severe phenotypes, whereas mutations in the tail are expected to cause mild phenotypes. The existence of a genotype-phenotype correlation is a highly debated in the field, although it is generally accepted that individuals with mutations in exon 16 (head) are at a higher risk for bleeding, nephritis and hearing impairment [160, 301, 302].

Platelets are released from megakaryocytes in the form of proplatelets, a process which requires profound cytoskeletal reorganization. NMMIIA must to be down-regulated during microtubule expansion and then up-regulated during of proplatelet fragmentation as NMMIIA is needed to bifurcate the proplatelet shaft [303, 304]. NMMIIA acts as a negative regulator of
megakaryocyte proplatelet formation [305]. Deficiency in NMMIIA results in large misshapen platelets.

The role of NMMIIA in the non-platelet clinical manifestations of MYH9RD is uncertain. NMMIIA is expressed in epithelial structures (e.g. ear, lens and kidney) and is critical for cell-cell adhesion in epithelial tissues. Ablation of NMMIIA in vitro or mouse models does not result in the non-hematological phenotypes of MYH9RD, suggesting that dominant negative effects of the human mutation are required for these phenotypes [306]. Recently, MYH9 has been associated with non-diabetes-related end-stage renal disease in African-Americans, indicating the importance of MYH9 in kidney disease beyond MYH9RD [307, 308].

A targeted knock-out and several gene traps in mice exist for Myh9, but are embryonic lethal with embryos dying before E7.5. Ablation of NMMIIA in mice leads to a failure to develop a normally patterned embryo, and the embryo formed does not implant into the uterus [287]. Mice heterozygous for deletion allele of Myh9 are viable, fertile and have no gross anatomic, hematologic or renal abnormalities. In particular, Myh9+/− mice have normal PLT counts and sizes and have normal neutrophil morphology. A subset of Myh9+/− mice has hearing loss [306]. Megakaryocyte-restricted inactivation of Myh9 (MYH9Δ) results in a severe reduction in PLT count, a wide variation in PLT size and shape and increased bleeding time [309]. Megakaryocytes in MYH9Δ mice are irregularly shaped, have an abnormally organized and developed demarcation membrane, deficient peripheral zone and have increased proplatelet formation, indicating that NMMIIA acts as a negative regulator of thrombopoiesis [305]. To date, no Myh9 knock-in mutations in mice have been reported corresponding to the MYH9 mutations observed in the human.
An ENU dominant screen that was conducted to identify mouse models of hematopoietic diseases. During the course of the screen, a mouse with low PLT counts was detected and identified as 7238. Subsequent analysis revealed that 7238/+ were macrothrombocytic and 7238/7238 mice were macrothrombocytopenic. We determined that the 7238 mice recapitulate the primary clinical features of MYH9RD.

3.3 Methods and Materials

3.3.1 Mice and ENU Mutagenesis
One intraperitoneal injection of ENU (150mg/kg) was given to male 129 mice. An F1 generation was produced by breeding ENU-mutagenized males to B6 females, known as generation 1 (G1). G1 mice were screened for traits of interest and outliers were bred to C57Bl/6J mice, producing G2 mice used to test heritability and for genetic mapping. To continue genetic mapping and maintain the strain, affected mice were bred to B6 mice producing G3 to G10. B6 males/females and 129 males were purchased from the Jackson Laboratory.

3.3.2 Genetic Mapping
The tail DNA was isolated at weaning according to established protocols. A microsatellite based genome scan was performed on DNA from affected animals and non-affected animals to determine the region of chromosome where mutation is located. The initial chromosomal location was narrowed by fine mapping performed on successive backcrossed generations of affected mice, using a combination of microsatellites and SNP.

3.3.3 Hematologic Analysis
At 6 and 8 weeks of age, complete blood counts were performed on the peripheral blood of all animals. Peripheral blood (20 to 30 µl) from the saphenous vein was collected into EDTA (ethylenediaminetetraacetic acid)–coated capillary pipettes (Drummond) and transferred to
(Eppendorf) tubes. CBC was performed by means of a Coulter Ac-T Differential Hematology Analyzer with veterinary software (Beckman-Coulter). After March 2007, hematopoietic analysis was also performed using a HEMAVET 950 (Drew Scientific Inc., Oxford CT USA) hematological analyzer. The HEMAVET could perform a CBC and WBC differential with additional parameters (RDW: RBC distribution width, MPV: mean platelet volume, NE: neutrophils, LY: lymphocytes, MO: monocytes, EO: eosinophils, BA: basophil). Peripheral blood smears were performed at 8 weeks of age and were stained with Giemsa-Wright. Stained peripheral blood smears were examined with 100x oil emersion objective lens on a Leitz Aristoplan microscope and photographs were taken with a Leica DC300 camera.

3.3.4 Histology

Tissues were collected and fixed in 10% neutral buffered formalin. Tissue sectioning and staining was performed by CMHD pathology core (http://www.cmhd.ca/enu_mutagenesis/pathology.html). Tissues sections (4 mm) were prepared and stained with Hematoxylin and Eosin. Photographs of sections were taken with an Olympus BX51 microscope and an Olympus DP71 Camera.

3.3.5 In vitro Progenitor Assays

Single cell suspensions of bone marrow (BM) were prepared in IMDM (Gibco) with 5% FBS. A red blood cell lysis was performed on BM using a red blood cell lysis buffer (0.14M ammonium chloride and 0.017M Tris). Cell counts and viability were assessed on a ViCell automated cell counter (Beckman-Coulter). For each assay, 3 aliquots of 2x10^3 BM cells were mixed with the appropriate methylcellulose media, plated, and grown in humidified chambers at 37°C, 5% carbon dioxide. All CFU-C assays were grown in methylcellulose M3434 and CFU-E assays were grown in methylcellulose M3334 (Stem Cell Technologies). CFU-E colonies were counted.
based on benzidine staining and morphology two days after plating. After 8 to 10 days of growth, CFU-C were counted based on colony morphology. To assay megakaryocyte CFU precursors (CFU-Mk), cells were grown in Megacult-C/collagen medium) containing 50ng/ml of rhTPO, 10ng/ml of rmIL-3, 20ng/ml of rhIL-6, and 50ng/ml rh IL-11 (Stem Cell Technologies) and visualized by acetylcholinesterase activity after 6 to 8 days of culture.

3.3.6 Megakaryocyte Culture

Bone marrow was isolated from tibia, femur and iliac crest and then RBC were lysed and counted as described above. Bone marrow was resuspended to 1x10^6/ml of IMDM supplemented with 1% Nutridoma-SP (Roche Molecular Biochemicals, USA), 2mM L-glutamine, mTPO (mouse recombinant TPO) (37.5ng/ml) (Peprotech, USA) and then plated in a flask at a density of 1x10^6/ml. After 4 days of growth, cells were harvested and centrifuged at 300g for 10 minutes and then resuspended in 2ml of CATCH buffer. Dead cells were removed with Ficoll-Paque™ Plus treatment (Stem Cell Technologies Vancouver, Canada). 3ml of Ficoll was added to each tube and then 2ml of megakaryocyte sample was placed over the Ficoll and spun at 400g for 30 minutes. The cells at the media-Ficoll interface were removed and then washed twice with CATCH buffer.

3.3.7 Ploidy Analysis

Bone marrow was harvested from the iliac crest, femur and tibia and placed directly into CATCH buffer (Hanks balanced salt solution (without: phenol red, Ca^{2+}, Mg^{2+}) with 3% bovine serum albumin, 0.38% trisodium citrate, 1mM adenosine, 2mM theophylline). Bone marrow cells were enumerated with a ViCell automated cell counter (Beckman-Coulter), and a minimum of 2.5x10^7 cells were used from each animal. Bone marrow cells were incubated with anti-CD41 FITC or rat immunoglobulin G1 (IgG1) for 1 hour in the dark on ice (or 4°C). Cells are then
washed twice with CATCH buffer. The cells are then centrifuged to pellet cells and the supernatant is removed (300G for 5 min). Next, 3ml of propidium iodide solution (0.05mg/mL of propidium iodide in 0.1% trisodium citrate) was added to the cell pellet and incubated at 4°C in the dark overnight. Cells were then washed in CATCH buffer twice passed through a 100mm filter to remove cell aggregates and then cells are incubated with 50mg/ml of RNaseA for 30 minutes prior to analysis. The samples were analyzed on Beckman Coulter FC500 (Beckman Coulter) and data was analyzed using Flowjo software. Megakaryocytes were identified based on CD41 positive expression and propidium iodide staining intensity was used as a measure of DNA ploidy.

3.3.8 Bleeding Time

10- to 12-week-old male mice were anesthetized by inhalation isoflurane and then placed on warming plate at 37°C. The distal tip of the tail (5mm) is cut with a scalpel blade, and immediately after cutting, the remaining distal end of the tail was immersed into pre-warmed saline (37°C, 0.9% isotonic saline). Bleeding time was defined as the time required for bleeding to arrest, and if necessary, bleeding was stopped with cauterizer 10 minutes after tail was initially cut.

3.3.9 Aggregation

The mice (8-10 weeks old) were anesthetized with 2.5% tribromoethanol (0.015mL/g of mouse) and whole blood was collected via retro orbital bleeding using Heparin coated glass capillary tubes. The blood will be collected into a tube containing 3.8% sodium citrate (1/9, V/V (volume to volume)). Platelet rich plasma (PRP) was obtained by centrifugation at 300 g for 7 minutes. Platelet poor plasma will be prepared by centrifugation at 1500g for 25 minutes. Platelet aggregation will be performed at 37°C, 1000 rpm using a computerized Chrono-log
aggregometer (Chrono-Log corporation, Havertown, PA). 250µL of platelet rich plasma (3x10^8/mL) was stimulated by 5 to 20µM ADP (Sigma, St Louis, MO, USA) to induce platelet aggregation. For gel filtered aggregation analysis, platelets will be isolated from the platelet rich plasma using a Sepharose 2B column in piperazine-N,N′-bis (2-ethanesulfonic acid) buffer (5mM piperazine-N,N′-bis(2-ethanesulfonic acid), 1.37mM NaCl, 4mM KCl, 0.1% Glucose, pH 7.0). Platelet aggregation will be induced by 0.5 to 1U/ml thrombin, or 500µM thrombin receptor activation peptide (TRAP, AYPGKF-NH2, Sigma).

3.3.10 Perfusion Studies

Rectangular glass microcapillary tubes (dimensions 0.1 × 1.0 × 100 mm, height, width, length) (microslides; Vitro Dynamics, Rockaway, NJ, USA) were coated with 100µg/mL type I collagen fibrils (equine collagen Horm; Nycomed, Germany) overnight at 4°C. Whole blood was collected via retro orbital bleeding using Heparin (25 IU/ml) as anticoagulant. Anticoagulated whole blood was fluorescently labeled using DiOC6 (1µM; Sigma) for 10 minutes at 37°C. Fluorescently labeled whole blood was perfused through the collagen coated microcapillary tubes for 4 minutes. Platelet adhesion, aggregation, and thrombus formation was recorded in real-time over the course of perfusion under a Zeiss Axiovert 135-inverted fluorescent microscope by a computer (IBM IntelliStation Z Pro) using the Slidebook program and analyzed using the Slidebook program.

3.3.11 Sequencing

DNA from two heterozygous and two homozygous mice were used to perform Massive parallel sequencing. Briefly, long oligonucleotide probes were designed to the genomic interval chromosome 15:77,500,000-81,200,000 of the July 2007 (NCBI37/mm9) assembly. The probe design was done by Nimblegen Inc (Roche) using proprietary algorithms to normalize
oligonucleotide Tm and avoid repetitive regions. The probes were synthesized on a solid support (385k array) representing 4,159 regions across the interval representing 3,167,722 bases. Mouse DNA was sheared to ~500bp, end-repaired and adapters ligated for sequencing following the manufacturers protocol (Illumina, Inc.). The library was amplified for 9 cycles using adapter-specific primers and PCR. The amplified library was mixed with ten-fold mass excess of Cot-1 sheared mouse DNA, denatured and hybridized to the array for 70h following the manufacturers protocol (Roche). The array was washed twice and captured material eluted using 2 washes of 95C water. Eluted material was sequenced using the Illumina GAII platform.

The resulting sequence was aligned to the NCBI37 mouse reference and variants identified using the maq software package. 1,177 putative variants were identified. Coding nonsynonymous SNP were identified for evaluation. All known variants (SNP) were eliminated from examination using SNP databases on Mouse Genome Informatics and Ensembl, consequently, 274 potential variants remained. Eighteen variants were within exons or within 18bp of exons and the remaining were intronic or intragenic. A new cross over at 77.78 on Ch. 15 removed all 18 variants that were coding.

3.3.12 Genotyping

All Myh97238 genotyping was performed at The Centre for Applied Genomics, using a custom designed TaqMan®SNP genotyping assay. The custom TaqMan® assay uses to allelic discrimination to differentiate between the WT allele (A 4566nt) and the Myh97238 allele (T 4566nt). The Taqman assay combines PCR amplification of the region surrounding the mutation and two dye labelled probes each designed to recognize the two different alleles.
3.3.13 Transmission Electron Microscopy

Whole blood was fixed in 2% glutaraldehyde and 1% Osmium Tetroxide. The fixed sample was dehydrated through graded alcohols. Next, the samples were embedded, by treatment with propylene oxide and placement in Spurr resin, and were polymerized occurred overnight at 68°C. The block was then sectioned to a thickness of 70 nanometers with a diamond knife, mounted on copper grids and stained with uranyl acetate and lead citrate. The sections were viewed on a 100 kV FEI Transmission Electron Microscope.

3.3.14 Urine Analysis

Urine was collected directly from mice, either after handling the mouse or applying soft pressure to the abdomen over the bladder. Protein:creatinine concentrations were used to analyze protein levels in urine. The protein concentration was determined by a Bradford assay (Bio-rad Protein Assay). A standard protein curve was developed using known concentrations of BSA. Bradford dye was diluted 1 in 4 with PBS and then 200µl was added to 10µl of control or sample (diluted 1/10). Next the samples were incubated for 5 to 10 minutes at room temperature and then run on microplate photometer at 595nm (Multiskan Ascent, Thermo Labysystems). The creatinine concentrations were determined by a Creatinine Assay, which uses alkaline picrate solution as reagent. A working reagent was made with 37 mM picric acid and 0.3M sodium hydroxide in a 1:1 ratio. A creatinine standard curve was made using known concentrations of creatinine (Sigma). The creatinine reagent was added to control or sample (diluted 1/10) in a 2:1 ratio (reagent to sample). Samples were run on a plate reader at 485nm. The protein and creatinine standard curves were used to calculate the concentration of protein and creatinine (respectively) in the urine samples and then expressed as a ratio (protein:creatinine).
3.3.15 Cataract Detection

Mice were placed in a dark room for 45 min to 1 hour to allow their pupils to dilate. A visual inspection of both eyes was performed with the aid of a pen light to detect cloudiness or white patches in the lens of the eye (cataracts). Mice were examined for cataracts and while being blinded for genotype.

3.4 Results

3.4.1 Identification and Mapping of 7238 Mouse Line

We established a large scale ENU mutagenesis screen in mice to identify dominant mutations that affected hematopoiesis. 129 male mice were treated with ENU and then bred to untreated B6 females to create a G1 progeny. G1 mice were screened by analysis of complete blood counts at 6 and 8 weeks of age for defects in hematopoiesis. A G1 animal (s333-1-22) was identified for having low PLT counts (greater than 2 standard deviations from the norm) at 6 and 8 weeks of age. The G1 female was bred to a B6 male to produce generation 2 (G2) and the strain was designated as 7238. G2 mice were used to test heritability of the low PLT phenotype. Fifteen out of forty-two G2 mice exhibited the same phenotype as G1 founder, demonstrating that the phenotype was heritable and highly penetrant (71%) (Figure 3-1A). Affected mice (demonstrating the 7238 phenotype) were bred to mice of the B6 background in order to perform a genome scan to genetically map the mutation. The genome scan indicated that the 7238 phenotype was co-inherited with microsatellites that mapped the causative mutation to a 28.4 Mb region on chromosome 15 (Figure 3-1B). Further backcrossing of affected mice to the B6 background successfully mapped the 7238 causative mutation a 3.0Mb region on chromosome 15 (77.5Mb rs 31919996 and 80.5 D15Mit158).
To determine the viability of 7238/7238 mice, 7238/+ mice were intercrossed (G7 and G8 from original backcross to B6). Homozygous pups were observed at weaning (7238/7238 n=29, total pups n=153 pups and total litters n=26), but at a frequency (19%) slightly lower than expected by Mendelian inheritance (25%) (Figure 3-2 A). 7238/7238 mice that survive to weaning were viable up to and above one year of age. Platelet counts were significantly decreased in 7238/7238 in comparison to 7238/+ and WT mice (Figure 3-2B) (PLT: WT 1029 +/- 150, 7238/+ 609 +/- 110, 7238/7238 322 +/- 56).
Figure 3-1. Initial characterization and mapping of 7238 mice.

A) PLT levels in peripheral blood of wildtype control males (M) and 7238 M. The red box indicates mice that had PLT levels 2 SD below that of WT controls. B) Schematic representing the mapping of the 7238 phenotype to a 28.4Mb region on chromosome 15. White boxes indicate markers homozygous for B6 genotype and grey boxes indicate markers that are heterozygous (one B6 and one 129). The candidate interval on chromosome 8 is represented by a red box and is located between the D15Mit158 and D15Mit171.
Figure 3-2. 7238/7238 survival and PLT phenotype.

A) Genotypic distribution of pups surviving to weaning at 3 weeks of age (WT n= 39, 7238/+ n=85 and 7238/7238 n=29). B) PLT counts in peripheral blood as measured by a Beckman Coulter AcTDiff (7 WT n=25, 7238/+ n=23 and 7238/7238 n=6). Both 7238/+ and 7238/7238 mice had reduced PLT counts in comparison to WT littermate controls, PLT counts of 7238/+ mice were 41% lower than WT controls and PLT counts of 7238/7238 mice were 69% lower than of WT controls (WT vs. 7238/+ and WT vs. 7238/7238: p<0.0001). PLT values of 7238/7238 mice were significantly reduced (47%) compared to 7238/+ mice (7238/7238 vs. 7238/+: p<0.0001). Red lines represent the average PLT counts of each genotype and black lines represent statistically significant differences between genotypes.
3.4.2 Redefining the 7238 Platelet Phenotype

Initially, the primary 7238 phenotype was identified on the Beckman Coulter Ac-t Diff as low PLT (Figure 3-2B), but subsequent analysis on a new hematological analyzer (HEMAVET 950) revealed that 7238/+ and 7238/7238 mice were not thrombocytopenic, but rather they were macrothrombocytic for 7238/+ mice (WT: PLT 895 +/- 95 and MPV 4.6 +/-0.2; 7238/+: PLT 875 +/-136 and MPV 6.3 +/- 0.2) and macrothrombocytopenic in 7238/7238 mice (WT: PLT 895 +/- 95 and MPV 4.6 +/-0.2; 7238/7238: PLT 536 +/- 140 and MPV 7.5 +/- 0.1) (Figure 3-3A and Table 3-1). The phenotype of macrothrombocytes in 7238/7238 mice was confirmed by a peripheral blood smear (Figure 3-3B) and flow cytometry analysis (data not shown). PLT size in 7238/7238 mice is heterogeneous and ranged in size from normal to the size of a RBC.

The Hemavet analysis also detected a slight increase (3%) in RBC distribution width in both heterozygote and homozygote 7238 mice, which is a measure of the variation in RBC diameter and is likely explained by the hematological analyzer mistakenly reading extremely large 7238 PLT as RBC. Additionally, a new phenotype of high WBC (22% increase) was detected by the hemavet analysis in homozygotes only.
Figure 3-3. Redefining the 7238 PLT phenotype.

A) PLT counts and MPV in peripheral blood of 7238 male mice at 6 weeks of age (n=11 to 15 per genotype), as measured with a Hemavet 950. The PLT counts were only significantly reduced in 7238/7238 mice (7238/7238 vs. WT: p<.0001; 7238/7238 vs. 7238/+ p<.01; 7238/+ vs. WT p>.05). MPV was significantly increased in 7238/+ mice and more so 7238/7238 mice compared to WT littermate controls (7238/+ vs. WT, 7238/7238 vs. WT, and 7238/+ vs. 7238/7238: p<.0001). Red lines represent the average PLT counts for each genotype and black lines represent statistically significant differences between two genotypes. B) Peripheral blood smear from WT (+/+) and 7238/7238 mice stained with Giemsa-Wright at 400x magnification.
Table 3-1. CBC performed on peripheral blood of 7238/+ and 7238/7238 mice.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>7238/+</th>
<th>7238/7238</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC</td>
<td>10.4 ± 0.60</td>
<td>10.4 ± 0.38</td>
<td>10.28 ± 0.60</td>
</tr>
<tr>
<td>Hgb</td>
<td>150 ± 7.1</td>
<td>148 ± 6.0</td>
<td>149 ± 6.3</td>
</tr>
<tr>
<td>HCT</td>
<td>0.510 ± 0.022</td>
<td>0.500 ± 0.022</td>
<td>0.498 ± 0.021</td>
</tr>
<tr>
<td>MCV</td>
<td>49.1 ± 1.2</td>
<td>47.9 ± 1.2</td>
<td>48.5 ± 1.5</td>
</tr>
<tr>
<td>MCH</td>
<td>14.4 ± 0.44</td>
<td>14.2 ± 0.47</td>
<td>14.5 ± 0.47</td>
</tr>
<tr>
<td>MCHC</td>
<td>294 ± 7.3</td>
<td>297 ± 9.3</td>
<td>300 ± 8.6</td>
</tr>
<tr>
<td>RDW</td>
<td>17.0 ± 0.52</td>
<td>17.6 ± 0.66 *</td>
<td>17.6 ± 0.72 *</td>
</tr>
<tr>
<td>PLT</td>
<td>957 ± 119</td>
<td>1070 ± 99.9 *</td>
<td>628 ± 113 *</td>
</tr>
<tr>
<td>MPV</td>
<td>4.78 ± 0.17</td>
<td>6.47 ± 0.29 *</td>
<td>7.56 ± 0.15 *</td>
</tr>
<tr>
<td>WBC</td>
<td>13.3 ± 3.2</td>
<td>13.6 ± 2.8</td>
<td>16.3 ± 4.7 *</td>
</tr>
<tr>
<td>NE</td>
<td>2.17 ± 0.81</td>
<td>2.33 ± 0.85</td>
<td>3.00 ± 0.72 *</td>
</tr>
<tr>
<td>LY</td>
<td>9.88 ± 2.4</td>
<td>9.87 ± 1.8</td>
<td>11.9 ± 4.0 *</td>
</tr>
<tr>
<td>MO</td>
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<td>1.22 ± 0.39</td>
<td>1.29 ± 0.59</td>
</tr>
<tr>
<td>EO</td>
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<td>0.157 ± 0.16</td>
<td>0.113 ± 0.15</td>
</tr>
<tr>
<td>BA</td>
<td>0.0071 ± 0.02</td>
<td>0.0045 ± 0.006</td>
<td>0.0037 ± 0.005</td>
</tr>
</tbody>
</table>

CBC were measured with a Hemavet 950 using saphenous blood taken from male mice at 6 weeks of age (WT n=17, 7238/+ n=20, and 7238/7238 n=19). The average values for each parameter are presented with the standard deviation. t-tests were performed to compare WT to 7238/+, and WT to 7238/7238, * indicates values that were significantly different (p < 0.05).
3.4.3 Normal Hematopoietic Progenitor Frequency in 7238 Mice

CFU-C frequency was unaltered in 7238/+ and 7238/7238 mice, including all CFU-C colony types (Figure 3-4A). Megakaryocyte progenitors analyzed by CFU-Mk were increased by 23% in 7238/+ mice (WT: 74.3 +/- 5.5; 7238/+: 83.4 +/- 4.9), but 7238/7238 mice did not show a statistically significant increase (Figure 3-4B).

Figure 3-4. Characterization of bone marrow hematopoietic progenitors in 7238 mice.

A) Bone marrow CFU-C frequency: number of CFU-C colonies per 2x10⁴ cells plated. No statistical differences in any colony type were observed between WT and 7238/+ or 7238/7238.

B) Bone marrow CFU-Mk frequency: number of CFU-MK per 2x10⁵ cells plated. 7238/+ mice displayed a 27% increase in CFU-Mk (p-value <.02). One experimental cohort was examined and consisted of littermate controls with 4 male mice of each genotype at 12 weeks of age. Each bar represents a genotype: WT in red, 7238/+ in blue and 7238/7238 in green. Data plotted represents the average ± SD for each genotype. Statistically significant differences from WT (WT vs. 7238/+; WT vs. 7238/7238) are indicated by a black line over each genotype, represented by a black histogram, whereas the results from the treated mice are represented by the red histogram.
3.4.4  7238 Megakaryocytes have Normal DNA Content, but Higher Nuclear to Cytoplasmic Ratio

The DNA content (ploidy) of megakaryocytes was analyzed by propidium iodide intensity. Megakaryocytes have different DNA content depending on their stage of maturation and range from 2N, 4N, 8N, 16N, 32N to 64N. Megakaryocytes were identified by CD41 expression and then examined for ploidy (excluding 2N and 4N from examination). *In vivo* bone marrow megakaryocyte ploidy was not significantly altered in either heterozygous or homozygous mice (Figure 3-5A). Histological examination of *in vivo* megakaryocytes revealed higher nuclear:cytoplasmic ratio in 7238/7238 mice (Figure 3-6A).

Megakaryocytes grown from bone marrow cells grown in media supplemented with TPO and Nutridoma were harvested on day four of culture. Ploidy analysis of megakaryocytes after four days of culture revealed no statistically significant differences in ploidy profile of 7238/+ mice (Figure 3-5B). Morphologically, *in vitro* megakaryocytes from 7238/+ mice showed increased nuclear to cytoplasmic ratio (Figure 3-6B).
Figure 3-5. Measuring the DNA content of 7238 megakaryocytes.

A) *In vivo* bone marrow megakaryocyte ploidy analysis. B) *In vitro* megakaryocyte ploidy analysis 4 days after bone marrow cells cultured with Tpo and Nutridoma. Data in each FACS plot (A and B) is representative from one animal from each genotype: WT mice are represented by a red histogram, 7238/+ mice are represented by a blue histogram and 7238/7238 mice are represented by a green histogram. Propidium iodide intensity represents DNA ploidy of bone marrow cells that are positive for CD41 expression. Propidium iodide intensity doubling was set at 2N, 4N, 8N, 16N, 32N and 64N on CD41+ cells.
Figure 3-6. Morphology of megakaryocytes *in vivo* and *in vitro*.

A) Histological sections of femur stained with hematoxylin and eosin from WT (i) 7238/7238 mice (ii) at 1000x magnification. Red asterisks indicate megakaryocytes within bone marrow. Megakaryocytes in 7238/7238 femoral sections show decreased nuclear:cytoplasmic ratio. B) Cytospins of day 4 megakaryocyte cultures from WT (i) and 7238/+ (ii) bone marrow were stained with May-Grunwald and Giemsa and examined at 200x magnification. 7238/+ MKs on the cytopsins appear smaller than WT, and display a lower nuclear:cytoplasmic ratio than WT mice.
3.4.5 Reduced Plug and Platelet Aggregation in 7238/7238 mice

Bleeding time is an *in vivo* assessment of PLT function, where the ability of PLT to participate in the formation a plug in a mouse after an injury was assessed. 7238/+ mice did not have significantly altered bleed times in comparison to WT controls. 7238/7238 mice demonstrate a statistically significant increase in bleeding times, with very heterogeneous results (Figure 3-7). Half of the homozygous mice (6/10) tested have bleeding times comparable to WT mice and the other half homozygous mice (4/10) tested had a bleeding time of at least 10 minutes, as bleeding was arrested artificially after 10 minutes of bleeding.

In vitro aggregation of platelets from 7238 PRP aggregated in response to stimulation with ADP (Figure 3-8 A), thrombin (Figure 3-8B) and TRAP (data not shown), but the degree of aggregation was reduced in 7238/+ and 7238/7238 PRP. While ADP was effective at activating platelets and inducing aggregation in 7238 PRP, maximum aggregation was decreased slightly in 7238/+ PRP and further decreased in 7238/7238 PRP when compared to PRP from wild type mice. Additionally the PLT aggregates were less stable after stimulation with ADP, as aggregates disappeared more quickly in homozygous PRP in comparison to aggregates in wild type PRP. The degree of aggregation was mildly reduced in 7238/+ PRP and moderately reduced in 7238/7238 PRP, after stimulation with thrombin. Stimulation with TRAP, demonstrated a similar reduction in the degree of aggregation in PRP from 7238/+ and 7238/7238 mice (data not shown).

An *ex vivo* assessment of PLT function was conducted using laminar flow chambers coated with collagen under different degrees of shear stress. Whole blood, treated with heparin as an anticoagulant, was perfused over the collagen coated surfaces of flow chamber at two shear rates, 350/s (low (Figure 3-8C)) and 1800/s (high (Figure 3-8D)). The amount of adhesion and
aggregation of PLT on a collagen surface was assessed by the degree of fluorescence observed over time. The number and size of thrombi appear reduced when 7238/7238 whole blood was perfused under both high and low shear stress conditions in comparison to WT whole blood.

**Figure 3-7. Increased bleeding time in 7238/7238 mice, demonstrates impaired PLT function.**

Only 7238/7238 mice had increased bleeding times when compared to WT littermate controls (p-value <0.003). Data points indicate the time required for bleeding to arrest, and when necessary, bleeding was stopped manually after 10 minutes of bleeding. Ten male mice of each genotype at 10 to 12 weeks of age were analyzed for bleeding time. Each WT mouse is represented by closed circles, each 7238/+ mouse is represented by open squares and each 7238/7238 mouse is represented by closed triangles. The average of each genotype is represented by a red horizontal line with error bars in red representing the SD. Statistically significant differences are represented by a line over each genotype.
Platelet aggregation was measured when stimulated by either ADP (A) or thrombin (B) and then measured by a Chrono-log aggregometer. Maximum platelet aggregation was reduced in 7238/+ and further reduced in 7238/7238 mice when PRP was activated by either ADP (A) or thrombin (B). Whole blood was collected from male mice (3-6) at 8-10 weeks of age. The whole blood was pooled for each genotype and then PRP was collected from whole blood samples and PLT concentrations were equalized to 3x10⁸/ml. The aggregation data for each genotype is indicated by: Red dots for WT, Blue dots for 7238/+ and Black dots for 7238/7238. C and D) PLT perfusion studies show that fewer 7238/7238 PLT adhere and aggregate to collagen matrices under physiological flow conditions. Heparinized whole blood from WT and 7238/7238 mice was perfused over collagen type I at a shear rate of 350/s (C) and 1800/s (D). Representative pictures of platelet adhesion/aggregation in whole blood. Platelet adhesion, aggregation, and thrombus formation were monitored under Zeiss Axiovert 135 inverted 1810 fluorescent microscope (32_/0.4 NA; Zeiss), and pictures were taken using the DP70 digital camera (Olympus).
3.4.6 Identification of 7238 Mutation

The entire mapping interval was sequenced, as well as all of \textit{Myh9}, by sequence capture technology followed by massive parallel sequencing in collaboration with the McPherson Lab. A point mutation in exon 30 of \textit{Myh9} was identified at nucleotide 4566 A\textrightarrow{}T (Figure 3-9), resulting in missense mutation at amino acid Q1443L (Figure 3-10). Subsequent sequencing revealed that the mutation was found exclusively in 7238/+ and 7238/7238 mice and not in either of the founder strains (B6 and 129). Glutamine (Q) 1443 is a highly conserved amino acid in mammalian species (including mouse, human, chimp and dog), and is located within the tail domain of NMMIIA. The replacement of the polar amino acid glutamine with the non-polar amino acid leucine is likely to cause a conformational change in the tail domain of NMMIIA. Myh9 protein levels were unaltered in the PLT isolated from \textit{Myh9}^{7238/+} and \textit{Myh9}^{7238/7238} as analyzed by western blot analysis (data not shown).
Figure 3-9. Identification of the 7238 allele.

DNA sequence chromatograph of 7238/+ and 7238/7238 mice as well as background strains C57Bl6/J and 129S1/SvImJ, indicates an A to T transversion at nucleotide 4566 located within exon 30 of 7238/+ (W= mix of A and T) and 7283/7238 mice (nt=4566 T) and result in a missense mutation Q1443L (glutamine to leucine).
Figure 3-10. NMMIIA structure, mapping of the 7238 mutation and human MYH9RD mutations.

A schematic of the NMMIIA protein showing the two heavy chains and 4 light chains. The heavy chain is composed of the head domain (blue oval), neck domain (yellow rectangle) and the tail domain (green rectangle). The four light chains are bound to the neck domain of the heavy chain and are indicated by red dots. B) Myh9 schematic color coded for the domains of NMMIIA that it encodes. The human mutations associated with MYH9RD that have been reported to date at the specific locations of the gene are indicated in black print. The $Myh9^{7238}$ mutation is represented in red.
3.4.7 Neutrophil Inclusions

Neutrophil inclusions are associated with most Myh9 mutations in humans. Analysis of peripheral blood smears from Myh9<sup>7238/7238</sup> mice indicates the presence of presumptive inclusions as indicated by a darkened blue area in the cytoplasm of some neutrophils (Figure 3-11 A-B). Preliminary data from transmission electron microscopy on whole peripheral blood from WT and Myh9<sup>7238/7238</sup>, indicates possible inclusions in neutrophils (Figure 3-11 C-E).

Figure 3-11. Neutrophil inclusions detected in Myh9<sup>7238</sup> mice.
Peripheral blood smears of wildtype (WT) mice (A) and Myh9<sup>7238/7238</sup> (B) at 400x magnification. Neutrophils are indicated by a red asterisk and speculative inclusions are indicated by a green arrow. (C-D) Preliminary neutrophils transmission electron microscopy data WT (C) and Myh9<sup>7238/7238</sup> (D). Red asterisks indicate potential inclusions.
3.4.8 Urine Analysis

To analyze kidney function in Myh9\textsuperscript{2238} mice, the protein levels in urine was analyzed by the measurement protein and creatinine concentration in urine and then expressing concentrations as a ratio (protein:creatinine). The protein:creatinine ratio was unaltered in the urine of Myh9\textsuperscript{2238/+} and Myh9\textsuperscript{2238/2238} mice measured at 31-37 weeks (Figure 3-12).

![Urine Analysis at 31 to 37 wks of Age](image)

**Figure 3-12. Normal protein levels in urine of Myh9\textsuperscript{2238} mice.**

The protein levels in the urine of Myh9\textsuperscript{2238/+} and Myh9\textsuperscript{2238/2238} were unaltered when compared to WT controls at 31 to 37 weeks of age. Protein levels in urine of 31 to 37 week old female: WT (n=8), Myh9\textsuperscript{2238/+} (n=6) and Myh9\textsuperscript{2238/2238} (n=9). The protein levels in Myh9\textsuperscript{2238} mice was not significantly altered (WT versus Myh9\textsuperscript{2238/+} and WT versus Myh9\textsuperscript{2238/2238}: p>0.05 or NS). Protein levels in urine were determined by detecting the concentration of both protein and creatinine and then expressing it as a ratio protein:creatinine.
3.4.9 Cataracts

The incidence of cataracts was 77% to 89% higher both $Myh9^{7238/+}$ and $Myh9^{7238/7238}$ females at 48 to 50 weeks of age, in comparison to WT controls (WT, 45%; $Myh9^{7238/+}$, 80%; and $Myh9^{7238/7238}$, 85%) (Figure 3-13). Mice were considered to carry a cataract if one or both of their eyes had general cloudiness, small focal white patches, or if the lens looked completely white.

![Figure 3-13. Increased incidence of cataracts in aged $Myh9^{7238}$ mice.](image)

All animals used were female mice between 48 and 50 weeks of age (WT (n=9), $Myh9^{7238/+}$ (n=6), $Myh9^{7238/7238}$ (n=7)). Mice were considered have cataracts when focal white patches, general cloudiness or if completely opaqueness was observed in one or both eyes.
3.4.10 Preputial Gland

The preputial gland is a specialized exocrine gland in male mice and produces pheromones [2]. On gross morphological examination the preputial glands of \( Myh9^{7238} \) mice are altered. Heterozygous mice had hypertrophic preputial glands that appear enlarged and opaque. Homozygous mice had atrophic preputial glands, which are small and opaque in comparison to preputial glands of littermate controls (Figure 3-14 A). Histological examination of the preputial glands \( Myh9^{7238/+} \) and \( Myh9^{7238/7238} \) mice, indicated that the overall structure of the gland was within normal limits (Figure 3-14 B). Synchronized maturation of glandular structures in \( Myh9^{7238} \) preputial glands is maintained and although ductal structures varied in size and number they were within normal limits.
Figure 3-14. Gross morphology and histology of preputial gland in Myh97238 mice.

All animals used were male mice at 12 weeks of age. A) Gross morphology of preputial gland from WT, Myh9\textsuperscript{7238/+} and Myh9\textsuperscript{7238/7238}. B) Histological sections of the preputial gland stained with hematoxylin and eosin from WT (i and iv), Myh9\textsuperscript{7238/+} (ii and v) and Myh9\textsuperscript{7238/7238} (iii and vi) at 10x magnification (i-iii) and 100x magnification (iv-vi).
3.5 Discussion

During the course of an ENU dominant screen, a mouse with macrothrombocytes was identified (7238 strain). Mice homozygous for the 7238 mutation were found to be macrothrombocytopenic. The causative mutation for the 7238 phenotype was identified as a missense mutation (Q1443L) located in exon 30 of Myh9 or the tail domain of NMMAII. Myh97238 is the first mouse strain to model the human disease MYH9RD. Although, two other mouse mutants have been created to understand the function of Myh9, total ablation of Myh9 [306] and conditional ablation of Myh9 (restricted to megakaryocyte lineage) [309], both ablation mutants are ineffective mouse models for MYH9RD. Total ablation of Myh9 leads to early embryonic lethality [287, 306] and while heterozygous mice are viable, they do not have the primary manifestations of MYH9RD including macrothrombocytopenia and neutrophil inclusions, although some hearing loss has been noted[306]. A megakaryocyte lineage restricted ablation of Myh9 can only examine the role of Myh9 in megakaryocyte and platelet production and does not address the role of Myh9 the neutrophil inclusions, nephritis, hearing loss and cataracts. An important distinction between Myh9 ablation mouse strains and patients with MYH9RD, is that ablation models look at the impact of a lack of Myh9 expression and hence the loss of NMMIIIA protein, whereas patients with MYH9RD produce a dysfunctional NMMIIA. Myh97238 is a good model for MYH9RD because it can be expressed in all tissues and results in a dysfunctional NMMIIA.

The Myh97238 mutation occurs at residue 1443, which is located within exon 30 of Myh9. Although no mutations at amino acid 1443 have been reported in humans with MYH9RD, exon 30 is the most commonly mutated exon in patients with MYH9RD, indicating that this region of NMMIIIA tail is important in the MYH9RD phenotype [160]. The Myh97238 mutation results glutamine to leucine substitution at residue 1443. The glutamine residue at 1443 is a highly
conserved amino acid of NMMIIA within mammals (human, rat, chimp and dog). Little is known about the exact structure of NMMIIA or the amino acids critical for tail-to-tail filamentation, as the crystal structure of NMMIIA has not been solved and the high level of diversity that exists in the tail domain of the myosin superfamily [310]. Glutamine is a polar amino acid and its replacement with the non-polar amino acid leucine (ref), is likely to affect NMMIIA function either directly or indirectly. The amino acid substitution in 7238 NMMIIA may alter function directly replacing a residue important in tail-to-tail interaction required for filamentation or indirectly by causing improper folding which would impact the tail-to-tail interactions.

The defect in Myh97238 hematopoiesis was limited to the megakaryocyte lineage, as the other hematopoietic lineages were unaltered (CBC and CFU-C). Thrombocytopenia in Myh97238/7238 mice cannot be explained by a defect in megakaryopoiesis, as CFU-Mk and megakaryocytes (femoral section) frequency was normal or slightly elevated in Myh97238 mice. The maturation of megakaryocytes can be divided into two categories: endomitosis (ploidy) and an expansion of the cytoplasm (demarcation membrane). 7238 megakaryocytes have normal ploidy and therefore megakaryocyte endomitosis is undisturbed in 7238 mice. The nuclear to cytoplasmic ratio is high in 7238 megakaryocytes compared to WT littermate controls (in vivo and in vitro) and indicates that the cytoplasm volume is reduced in 7238 megakaryocytes. A reduction in megakaryocyte cytoplasm may indicate a potential defect in cytoplasmic maturation or an accumulation of immature megakaryocytes due to reactive thrombopoiesis. Myh9Δ mice have both reactive thrombopoiesis and defective megakaryocyte cytoplasmic maturation (demarcation membrane and peripheral zone), indicating that either explanation could explain the 7238 megakaryocytes with a high nuclear to cytoplasmic [305].
In vitro of PLT function was mildly altered in $\text{Myh9}^{7238/+}$ PLT and moderately altered in $\text{Myh9}^{7238/7238}$ PLT. Specifically, all agonists tested (ADP, Thrombin, TRAP) induced aggregation of PLT in $\text{Myh9}^{7238/+}$ and $\text{Myh9}^{7238/7238}$ PRP, but the strength of the aggregation response was weakened in $\text{Myh9}^{7238/+}$ PRP and further reduced in $\text{Myh9}^{7238/7238}$ PRP. Ex vivo studies of PLT function, perfused whole blood from $\text{Myh9}^{7238/7238}$ mice over a collagen surface under high and low shear forces and demonstrated reduced adhesion to the collagen surface and decreased aggregation when compared to whole blood from wild type controls under similar conditions.

Although $\text{Myh9}^{7238/+}$ PLT aggregation was mildly decreased in vitro, the defects in aggregation did not correspond to altered PLT function in vivo, as the bleeding times of $\text{Myh9}^{7238/+}$ mice were similar to WT mice. Alternatively, the defects in both in vitro and ex vivo PLT function, corresponded to significantly increased bleeding times in $\text{Myh9}^{7238/7238}$ mice. A considerable heterogeneity in the bleeding times of $\text{Myh9}^{7238/7238}$ mice was noted, as half of the bleeding times for $\text{Myh9}^{7238/7238}$ mice were equivalent to WT controls and the other half of homozygous mice having bleeding times near 10 minutes. The heterogeneity in bleeding time for $\text{Myh9}^{7238/7238}$ mice indicates incomplete penetrance in bleeding time phenotype, suggesting the $\text{Myh9}^{7238}$ protein may have limited functionality to allow for normal clotting in some $\text{Myh9}^{7238/7238}$ mice.

To date we only have preliminary data on additional phenotypes associated with MYH9RD. Neutrophil inclusions have been observed on peripheral blood smears of $\text{Myh9}^{7238/7238}$ mice, but the incidence and type of the inclusion associated with the 7238 mutation has not been determined. $\text{Myh9}^{7238/+}$ and $\text{Myh9}^{7238/7238}$ female mice have not developed proteinuria by 31-37 weeks of age. It is uncertain whether the 7238 mutation will lead to a kidney phenotype (nephritis), as a thorough analysis of kidney dysfunction has yet to be performed. Additionally, $\text{Myh97238}$ mice have been maintained on a C57Bl/6J background (G9-10), which is protective against kidney injury, and may conceal the role of the 7238 mutation in
kidney function. The incidence of cataracts is higher in $\text{Myh9}^{7238/+}$ and $\text{Myh9}^{7238/7238}$ female mice at 48-50 weeks of age and indicates that the $\text{Myh9}^{7238}$ protein may play a role in cataract formation. Hearing phenotype in 7238 mice is currently being characterized by auditory brain response. To date a clear phenotype/genotype correlation has yet to be described in patients with MYH9RD, with the exception of mutations at amino acid 702, therefore it is hard to predict the age of onset or incidence of MYH9RD additional phenotypes that would be associated with the $\text{Myh9}^{7238}$ mutation and will require careful examination of the $\text{Myh9}^{7238}$ mutatants [160].

The preputial gland is a modified sebaceous gland located in front of the genitalia of male mice and produces pheromones. Pheromones are molecules secreted by an animal to convey sexual and social cues. The preputial gland secretes pheromones that accelerate the onset of puberty and induce estrous in females, whereas it induces aggression in other males[2, 311]. Hypotrophic preputial glands were observed in $\text{Myh9}^{7238/7238}$ male mice, whereas $\text{Myh9}^{7238/+}$ mice have normal or hypertrophic preputial glands. Histological examination of the preputial gland revealed that $\text{Myh9}^{7238/7238}$ glands have normal structural architecture. The smaller preputial gland observed in the $\text{Myh9}^{7238/7238}$ male mice maybe explained by a defect in the growth of the preputial gland (intrinsic or extrinsic) or a defect in secretion. It is currently unknown if $\text{Myh9}$ is expressed in the preputial gland, therefore it is unclear if $\text{Myh9}$ may play a role in intrinsic development of the preputial gland. The growth of the preputial gland is strongly influenced by the presence of androgens, removing androgens causes the gland to be hypotrophic and administration of excess androgens causes the hypertrophic preputial glands [312] Therefore, a defect in androgen production or signaling, could explain the phenotype observed in $\text{Myh9}^{7238}$ male mice.
Myh9\textsuperscript{7238} is inherited in an autosomal dominant manner, like humans with MYH9RD. Mice homozygous for the 7238 mutation are viable and have a more severe defect in thrombopoiesis, yet no humans homozygous for mutations in MYH9 have been identified. The rarity of the MYH9RD disorder likely explains why no patients homozygous MYH9 mutations have been identified, rather than impaired viability in humans homozygous for MYH9 mutations. Mice heterozygous for the 7238 mutation have normal PLT levels, elevated MPV, normal bleeding tendency, no proteinuria and increased incidence of cataracts and therefore model a mild form of MYH9RD. Whereas mice homozygous for the 7238 mutation have reduced PLT levels, elevated MPV, increased bleeding times, normal proteinuria and increased incidence of cataracts and hence model a moderate form of MYH9RD. Mice heterozygous for the 7238 mutation should most accurately mimic MYH9RD in humans, yet have a relatively mild PLT phenotype which may be indicative of the location of the mutation or the difference in biology between mice and humans.

The 7238 mouse strain represents the first mouse model of MYH9RD and has mild (Myh9\textsuperscript{7238/+}) to moderate (Myh9\textsuperscript{7238/7238}) platelet phenotypes of the disease. Myh9\textsuperscript{7238} mice have the capacity to provide additional insight into the role of Myh9 function in thrombopoiesis and platelet function. Potentially, Myh9\textsuperscript{7238} mice can provide insight into the pathogenesis of the additional clinical manifestations of MYH9RD, which include: cataracts, renal impairment and hearing impairment. Although the MYH9 is known to be involved in cellular contractility, focal adhesions, actin stress fiber organization and tail retraction, little is known about its normal function in a tissue specific manner. Most obviously, Myh9\textsuperscript{7238} model can provide insight into the role of MYH9 in MYH9RD tissues of the eye, kidney and ear. An understanding of the disrupted cellular processes in these tissues will provide information on the normal function of MYH9. Also, it will be important to understand if closely related family members NMMHCIIIB and C
can compensate for a defective MYH9 or whether the family members have distinct roles in a particular cell type or tissue. Additionally, a thorough characterization of the *Myh9<sup>7238</sup>* mouse may uncover novel functions of MYH9 in non-MYH9RD organs. A thorough characterization of the Myh97238 mouse model will provide new information of gene function and increase our understanding of the MYH9RD.
Chapter 4: A Pharmacologically Sensitized Screen to Detect Defects in Developmental Hematopoiesis

Acknowledgements

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4.1 Abstract

We conducted a pharmacologically sensitized dominant ENU screen to identify mutations affecting hematopoiesis. A sensitized ENU screen was used to exacerbate subtle phenotypes that may not be detected in a traditional dominant ENU screen because of hemizygous wild type gene expression or genetic redundancy. We evaluated three known chemotherapeutic drugs: 5FU, PHZ and HU. Each challenges the hematopoietic system to rebuild itself either by the elimination of mature erythroid cells (PHZ) or by targeting hematopoietic progenitor cells (HU and 5FU). Although 5FU and PHZ have been used previously to induce stress hematopoiesis, new protocols were required for all three drugs to ensure animal viability, simplify the procedure, and ensure robustness with little inter-strain variability. 5FU has the capacity to detect the largest set of mutations, because it affects a broad range of hematopoietic lineages. However, the careful selection of mutagenesis and backcross strains is required because of inter-strain variation. There was less inter-strain variation with PHZ treatment, but this drug could only be used to detect mutations affecting the erythroid lineage. HU was not a strong inducer of anemia or thrombocytopenia, but was effective at inducing leukopenia. Strong inter-strain variation was observed when B6, Balb/cJ and 129 mice were treated with HU, not only in the severity or timing of cytopenia, but also in the overall effect of HU treatment. 5FU was implemented as part of a dominant screen and it was very effective at detecting defects in platelet (PLT) recovery. Over 500 G1 mice were screened with 5FU and 10 PLT phenodeviants were detected: 8 with increased PLT overshoot and 3 with delayed PLT recovery.
4.2 Introduction

A sensitized ENU screen was designed and undertaken to reveal subtle phenotypes that might not have been detected in a traditional dominant ENU screen because of hemizygous wild-type gene expression or genetic redundancy. Three different types of pharmacological agents were selected to induce hematopoietic stress: 5FU and hydroxyurea, which affect the entire hematopoietic system and PHZ, which specifically affects erythropoiesis. Both 5FU and HU target dividing cells and because blood cells have a high rate of turnover compared to other cell types, the hematopoietic system is strongly affected by the administration of these drugs. 5FU and HU act through different mechanisms. 5FU inhibits thymidine synthase, affecting DNA synthesis and possibly RNA synthesis, killing cells in various stages of the cell cycle [192]. On the other hand, HU specifically inhibits ribonucleotide reductase and kills cells in the S-phase of the cell cycle, exclusively by inhibiting DNA synthesis [214]. Phenylhydrazine lyses RBC inducing hemolytic anemia leading to erythropoietic stress. The steep decline in RBC mass leads to high demand for the replenishment of RBC. In response to the loss of RBC, there is a surge in reticulocyte numbers and an increase of erythrocyte progenitors in both the spleen and bone marrow [204].

The first stage of the current study involved determining the optimal concentrations of each of these hematopoietic stressors for commonly utilized mouse strains.

The second stage of this study was to directly compare the effect of each drug on the hematopoietic system. The concentration of HSC, progenitors and megakaryocytes were monitored in parallel over a two week period after the administration of each drug to directly compare the affects of the three hematopoietic stressors.
A sensitized ENU screen utilizing 5FU was performed in order to detect novel genes involved in hematopoiesis using a protocol that had been optimized based on results from the experiments described above.

4.3 Materials and Methods

4.3.1 Mouse Strains

Inbred mouse strains $129S1/SvImJ$ (129), BALB/CJ (Balb/c) and $C57BL/6J$ (B6) were used to assess the hematopoietic recovery from the pharmacological stressors. Based on the results, B6 were chosen for the ENU mutagenesis experiment to detect novel hematopoietic genes. All mice in these experiments were 8-10 weeks of age. Mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in a conventional mouse facility at the Mount Sinai Hospital (Toronto, ON, Canada).

4.3.2 Cytopenia Induction

5FU (Sigma) was dissolved in PBS without Ca or Mg (Gibco) at 65°C for 15 min at a concentration of 15 mg/ml and then injected interperationally (IP) at a concentration of 120 µg/g. PHZ (Sigma) was dissolved in PBS without Ca and Mg at a concentration of 20 mg/ml and then subcutaneously injected with 100 µg/g. HU (Sigma) was dissolved in PBS without Ca and Mg at a concentration of 133 µg/ml, vortexed until completely dissolved and then injected IP at 4µg/g once and then a second time four hours later.

4.3.3 Hematologic Analysis

Peripheral blood (20 to 30 µl) from the saphenous vein was collected into EDTA (ethylenediaminetetraacetic acid)–coated capillary pipettes (Drummond) and transferred to (Eppendorf) tubes. Complete blood counts were performed by means of a Coulter Ac-T
Differential Hematology Analyzer with veterinary software (Beckman-Coulter). All mice were bled on day 0 (D0) to obtain a baseline and then every 4 days, unless otherwise stated.

4.3.4 KTLS Analysis

Single cell suspensions in Iscove modified Dulbecco medium (IMDM) (Gibco) with 5% FBS were prepared from bone marrow (femoral). A red blood cell lysis was performed, using a red blood cell lysis buffer (0.14M ammonium chloride and 0.017M Tris). Cell counts and viability was performed on a ViCell automated cell counter (Beckman-Coulter). Twelve million cells were stained with unconjugated rat antibodies specific for lineage (lin) markers (3µl of each) anti-CD3, anti-CD5, anti-CD11b, anti-B220, anti-Gr-1, anti-Ter119 obtained from eBioscience Inc.) for 30 min and washed twice with PBS with 2% FBS. Lin+ cells were partially removed using sheep anti-rat IgG-conjugated immunomagnetic beads (Dynabeads®, Invitrogen). Specifically beads were washed 3 times with PBS (without Mg and Ca). Washed beads were mixed in a 1:1 ratio of beads to cells. Cells and beads are incubated at 4°C on a rocker for 25 min and lin+ cells were removed by Dynabead magnet. The remaining lin− cells were stained by phycoerythrin Texas red goat anti-rat IgG polyclonal antibodies (Invitrogen) and the cells also stained with allophycocyanin-conjugated anti-c-Kit, phycoerythrin-conjugated anti-Sca-1 and FITC-conjugated anti-Thy1.2. Cells were next stained to assess viability with violet LIVE/DEAD Fixable Dead Cell Stain (Invitrogen). Cells were then analyzed using a modified BD LSR (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Lin-depleted cells were used for to stain single-color controls and were also used for fluorescence minus one control. Unstained controls were used to set voltages and single-colour controls were used for compensation. Fluorescence minus one controls were used to set gates.
4.3.5 In vitro Colony Assays

Single-cell suspensions in IMDM with 5% FBS were prepared from bone marrow (femoral). A red blood cell lysis was performed, using a red blood cell lysis buffer (0.14M ammonium chloride and 0.017M Tris). Cell counts and viability was performed on a ViCell automated cell counter (Beckman-Coulter). For each assay, 3 aliquots of 2x10^4 cells were mixed with the appropriate methylcellulose media, plated (6 well plates CFU-C and 24 well plates CFU-E), and grown in humidified chambers at 37°C, 5% carbon dioxide. All CFU-C colonies were grown in methylcellulose M3434 (Stem Cell Technologies) and CFU-E colonies were grown in methylcellulose M3334. CFU-E precursors were assayed after 2 days by staining in situ with benzidine (Sigma, St Louis, MO) to detect hemoglobin. After 7 to 10 days of incubation, BFU-E and CFU-C were counted based on colony morphology. All colonies were counted using an inverted microscope.

4.3.6 Ploidy Analysis

Bone marrow was flushed strain into CATCH Buffer (phenol red–free, Ca-free, Mg-free Hanks balanced salt solution containing 3% BSA, 0.38% trisodium citrate, 1 mM adenosine, 2 mM theophylline). The bone marrow cells were stained with FITC-conjugated anti-CD41 or rat IgG1 isotype control for 1 h on ice and then washed twice with CATCH buffer. Next cells were incubated in 3 ml of 0.05 mg/ml propidium iodide in 0.1% trisodium citrate at 4°C overnight. Cells were then washed with CATCH buffer, passed through a 100 μm cell strainer to remove cell aggregates and incubated with 50μg/ml of RNase A for 30 min before analysis. Samples were analyzed on a Beckman Coulter FC500 (BC FC500). Megakaryocytes were identified as CD41-positive cells and propidium iodide staining intensity was used as a measure of DNA ploidy.
4.3.7  ENU Mutagenesis and G1 Screen

ENU treatment of 129 male mice consisted of either one IP injection of ENU at a dose of 150 mg/kg or two IP injections of ENU at a dose of 100 mg/kg, given 1 week apart. ENU-injected males were designated G0 males. An F1 generation (B6;129S) was produced by breeding G0 males to B6 females, known as generation 1 (G1). G1 mice were screened for traits of interest (e.g. size, behaviour and appearance, 5FU sensitivity, electrocardiogram, bone density and learning and memory). G1 outliers were breed to B6 mice, which produced G2 mice used for testing heritability and genetic mapping (genome scan). An ENU line was considered heritable if 35-50% of G2 showed original phenotype of G1 parent and was considered heritable-low penetrance if 10-35% of G2 animals showed original phenotype of the G1 parent. In order to identify the mutation and maintain the line, affected mice were bred to C57Bl/6J mice producing G3 to G8.

Five-week-old G1 animals underwent a screen to identify defects in 5FU recovery. A peripheral blood sample of 20-30µl was collected from the saphenous vein on D0 to establish a hematological baseline. Next, the G1 mice were injected with 100µg/g of 5FU IP and then bled on days 6, 8 and 13 post-injection. The recovery kinetics of RBC, PLT and WBC were examined for defective recovery kinetics and mice were designated as outliers if they fit the criteria outlined in Table 4-1. Affected G1 mice underwent heritability testing, and lines designated as heritable or heritable-low penetrance were sent for a genome scan.

4.3.8  Genetic Mapping

A genome scan was performed using a whole genome SNP panel by the Illumina GoldenGate Platform at the The Center for Applied Genomics to give the general chromosomal region the mutated gene. Genomic DNA from five to ten affected G2 (G3 and G4) was sent to The Centre
for Applied Genomics for a genome scan. If an initial chromosomal region of interest was identified, further mapping was required to narrow the chromosomal interval and thereby reduce the number of candidate genes. A higher density map of the specific chromosomal region was created by using additional polymorphic SNPs between the 129 and B6 strains. The information was gathered from the following databases: Jackson Labs Mouse Genome Informatics (http://www.informatics.jax.org/), NCBI (http://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org/). Additional backcrosses were set up and all affected animals were tested with the higher density SNP panels in order to identify informative crossovers. When possible, animals with new informative crossovers were bred to confirm crossovers in affected offspring and eliminate the possibility of animal being a false positive (i.e. when new crossover mouse were bred and none of the offspring were affected).

4.3.9 Statistical Analysis

We made the assumption that the data for hematological recovery data for dose, strain and gender could be combined for two or more groups of animals (each group tested on a different days) to represent a single animal’s response and then used to construct a single recovery curve. Blood samples containing a clot or samples that led to sampling errors on a hematological analyzer were removed from consideration. Animals for which there were missing data points were removed from analysis, because one or two-way repeat measures analysis of variance (ANOVA) required all time points be present for analysis.

Hematological recovery curves following 5FU, PHZ and HU treatment were analyzed using one-way repeated measures ANOVA in order to test the null hypothesis that the particular drug being tested was ineffective in causing change to RBC, PLT and WBC. After a one-way repeat measures ANOVA, a Dennett’s post test was performed to determine at what time point
the parameters significantly changed from baseline values. Each group (dose, strain or gender) was analyzed by two-way repeated measures ANOVA in order to test the null hypothesis that dose (strain or gender) had no effect in altering the recovery patterns of RBC, PLT or WBC. A pair-wise two-way repeated measures ANOVA compared all two-group combinations to determine if significant differences exist between a pair of groups (e.g. strain Balb/c vs. B6). The pair-wise comparison was followed with a Bonferroni post test to determine when during the two week time course differences existed. In order to compare nadir or peaks that occur at different time points between two groups, a t-test with two-tailed distribution assuming unequal variance (Welch’s correction) was performed, while three or more groups were analyzed with a one-way ANOVA.

Statistical analysis was performed on KTLS populations to test the null hypothesis that each drug led to no change in KTLS population when compared PBS treated controls. We used a t-test with two-tailed distribution assuming unequal variance (Welch’s correction). Bone marrow and spleen colony assays were analyzed with a one way ANOVA to test the null hypothesis that a particular drug (5FU, PHZ or HU) caused no change colony number (e.g. CFU-G) from baseline. The one-way ANOVA was followed by a Bonferroni’s post test to detect differences between two time-points.

All statistical analysis was performed using GraphPad Prism software (version 5, GraphPad Software Inc. La Jolla CA). A p-value less than or equal to 0.05 was accepted as significant in all statistical tests including t-tests, ANOVAs, and post tests.
4.4 Results

4.4.1 Effect of 5FU Dosage on Recovery Kinetics.

In an effort to identify an optimal dose of 5FU, the recovery kinetics of RBC, PLT and WBC was analyzed following IP injections of mice at 100, 120 and 150 µg/g (Figure 4-1). While all doses of the drug are effective in inducing anemia (p<0.0001), significant differences were observed between the RBC recovery following each dose. The recovery kinetics for the 5FU doses of 120 and 150µg/g were similar, except that the 120µg/g curve was shifted to the right. Both 120 and 150µg/g of 5FU induced comparable reductions in RBC number and decreased to similar levels 6.5x10^{12}/L and 6.1x10^{12}/l (t-test, p=0.412), respectively, but a 5FU dose of 150µg/g caused RBC to drop more quickly to nadir on day ten, whereas 120 µg/g nadirs at day twelve. A 5FU dose of 100µg/g induced a much milder anemia (8.6x10^{12}/l; t-test p < 0.0005) in comparison to the two higher doses of 5FU, and the nadir occurred earlier on day 8.

All doses of 5FU caused a decrease in PLT numbers (thrombocytopenia) followed by a multi-fold overshoot in PLT numbers in comparison to baseline levels (all p<0.0001) (Figure 4-1). PLT recovery curves for 5FU doses of 100µg/g and 120µg/g were very similar with similar drops in PLT number occurring on D6, and overshoot values that were not significantly different (t-test, p=0.75). The only significant difference was that the PLT overshoot was maintained longer when animals are treated with 100µg/g of 5FU. However, the PLT recovery pattern following 150µg/g 5FU was significantly different from both the 100 and 120µg/g pattern. While the larger dose caused PLT to nadir to significant levels on D6 (Dunnett Multiple comparison test p<0.05), the PLT overshoot was higher and more prolonged in comparison to the lower 5FU doses.
All doses of 5FU led to significant alterations in WBC over the two-week time course (p<0.0001) (Figure 4-1). Initially WBC numbers remained constant, but an overshoot was observed during the second week (Dunnett Multiple comparison test p<0.05, 150: D12&14, 120:D10, 100: D12). There were no significant differences in the overall WBC recovery kinetics between the 100 and 120µg/g doses of 5FU (p>0.05). Both lower doses of 5FU induced a similar level of WBC overshoot (t-test p=0.56: 100µg/g D12 to 17.6x10^9/l versus 120µg/g D10 18.8x10^9/l). The highest dose of 5FU (150µg/g) induced significantly different overall WBC recovery kinetics in comparison to both of the lower doses (p<0.05). Specifically, the overshoot was significantly higher in comparison to the lower doses of 5FU. However, the increase in WBC observed on day 14 could be attributed to the increase in proerythroblasts observed in response to anemia, which would be falsely detected as WBC in this assay.

4.4.2 Strain-Specific Effects of 5FU administration

It is known that the hematological properties of mouse strains are distinct [313]. Therefore, we tested recovery kinetics in the commonly used laboratory strains: B6, Balb/c, C3H and 129.

The RBC baseline values of the four mouse strains were significantly different (one-way ANOVA, p<0.0001), with C3H females having the lowest baseline values in comparison to the other strains (Figure 4-1B). 5FU was effective at inducing anemia in the mouse strains, but the severity of anemia or timing the RBC nadir was different (one way ANOVA, p<0.0001). No significant differences were observed in the RBC recovery kinetics of B6 and Balb/c female mice. The RBC recovery pattern of 129 mice was similar to B6 and Balb/c mice, but 129 mice did not become as severely anemic and recovered more quickly compared to B6 or Balb/c mice. C3H mice had a much flatter recovery curve compared to the other strains.
Strain is an important factor in the overall PLT recovery kinetics (V=2.5%, p<0.0001). An overshoot in PLT numbers was observed in all strains tested, but significant levels of thrombocytopenia to were only observed in C3H and 129 strains. PLT numbers dropped to the lowest levels on D4 in Balb/c, C3H and 129 mice, but do not nadir until on D8 in B6 mice. All strains had a peak PLT overshoot between D10 and D14, but the precise timing and height of PLT overshoot differed among the strains (B6 PLT, 2730 x10^9/l on D10; Balb/c PLT, 2467 x10^9/L on D10; C3H PLT, 1789 x10^9/l on D10; 129 PLT, 2325 x10^9/l on D12). The PLT recovery kinetics of Balb/c and 129 mice were highly similar and were not significantly different overall (p>0.05, not significant (NS)), but Balb/c mice maintained the overshoot for a longer period of time (Balb/c, D10-16 vs. 129, D10-12). The overall pattern of PLT recovery for B6 mouse strain was similar to Balb/c and 129, but PLT values at specific time points were significantly different. C3H mice had a unique and significantly different PLT recovery pattern in comparison to the other mouse strains, with a reduced PLT overshoot.

While the differences in WBC baseline values are statistically significantly different between the four mouse strains (one-way ANOVA p<0.03), minimal variation was observed (WBC D0: B6 14.1x10^9/l, Balb/c 11.3x10^9/l, C3H 11.6x10^9/l, 129 14.2x10^9/l) (Figure 4-1B). Three of the mouse strains (B6, Balb/c and 129) lost 40-60% of their WBC mass between D8 and D10, but only B6 and Balb/c overshoot baseline values (Dunnett’s Post-test p<0.05: B6 D6-10 and 16; Balb/c D4-8 and 14; 129 D4-8). C3H mice had a unique WBC recovery pattern as WBCs reached the lowest value much earlier compared to the other mouse strains (D4) and overshoot earlier on D8 (Dunnett’s Post-test p<0.05: D2-4 and 8). While the WBC recovery patterns of B6, Balb/c and 129 mice are similar, statistically significant differences exist between the three strains (p<0.02). The differences between B6, Balb/c and 129 were most pronounced in the height and duration of WBC overshoot.
A) 5FU dose response (B6 females were injected IP with 100, 120 or 150µg/g of 5FU) and the recovery curves for RBC, PLT and WBC were determined (left, center and right). B) Effect of mouse strain on 5FU. Cytopenia and the subsequent recovery of RBC, PLT and WBC. Four commonly used inbred strains of mice in biomedical research: C57Bl/6J, Balb/cJ, 129S1/SVImJ and C3HJ were used for this comparison. All mice were females. Each group of mice consisted of two subgroups of 8-10 animals, one subgroup was tested at D0, 4, 8, 12, 16 and the second was tested at D0, 2, 6, 10, 14. The two subgroups were combined to create one group represented by a single recovery curve, which includes the baseline and data sampled every two days post-treatment. A summary of statistical analyses performed can be found in Appendix Table 6.1. Cytopenia was induced by IP administration of 120µg/g 5FU.
4.4.3 Strain-Specific Effects of PHZ Administration

A single injection of 100ug/g PHZ administered by subcutaneous injection was selected as an optimal dose in all experiments [206]. Slight differences in RBC baseline values were observed between B6, Balb/c and 129 mouse strains (one-way ANOVA p<0.001) (Figure 4-2). Overall, B6, Balb/c and 129 strains had very similar RBC recovery curves and no significant difference existed between the three mouse strains (Appendix Table 6.2: V=0.32% and p=0.415). A Dunnett’s post-test revealed that each mouse strain returns to near baseline levels by D9-11 (p<0.05: B6 D1-8, Balb/c D1-10 and 129 D1-10).

The baseline PLT values of B6, Balb/c and 129 female mice are significantly different (one-way ANOVA, p<0.05), as observed in the 5FU experiments. While PHZ was effective at inducing changes in PLT values in all three mouse strains over a two week time course (B6 p<0.02, Balb/c p<0.007 and 129 p<0.007), only 129 females had a clear pattern in PLT recovery kinetics (Figure 4-2). PLT in 129 females peaked on D3 and then return to baseline on D8. Overall, PLT recovery after PHZ administration was similar for all strains (p<0.07), but significant differences were observed at specific time points (Dunnett’s Post test p<0.05: B6 vs. 129 D2, 4, 6 and 11 and Balb/c vs. 129 D2 and 6).

Overall, mouse strain did not influence the WBC/Ret recovery kinetics (p<0.0001) (Figure 4-2). Differences were observed in the height of the WBC/Ret surge, as the reticulocyte overshoot was not as high in 129 mice compared to B6 and Balb/c mice.

4.4.4 Determining an Effective HU Dosage - Low Dose HU

The effect of HU on RBC, PLT and WBC recovery was examined. Doses of 0.5 mg/g, 0.9 mg/g and 0.5 mg/g x2 HU were administered twice with a 4 hour interval between injections, and CBC was performed D0, 2, 5, 8, 11. Only the dose of 0.5 mg/g x 2 of HU was
effective in inducing a slight anemia over the test period (p=0.04) (Figure 4-3A). This dose induced a 10% decrease in RBC two days after drug treatment in 6 of 10 mice (Dunnett’s Post-test p<0.05). Similarly, only the highest dose induced an significant increase (15%) in PLT numbers at 11 days post treatment (One-way repeat measures ANOVA p<0.03 and Dunnett’s Post-test D11 p<0.05), and 9/10 mice tested showed the increase (Figure 4-3B). Leukopenia was significantly induced with a dose of 0.9 mg/g of HU (p<0.007) (Figure 3C). At D5 there was a 25% reduction in WBC (Dunnett’s Post-test p<0.05), and only 7/10 mice showed cytopenia.

Figure 4-2. PHZ recovery curves

Each group of mice consisted of four subgroups of 8-10 animals: one subgroup was tested at D0, 1, 5, 9, 13; the second at D0, 2, 6, 10, 14; the third at D0, 3, 7, 11; and the fourth at D0, 4, 8, 12. The four subgroups were combined to create a single recovery curve that represents the recovery pattern for each group for at baseline (D0) and every day for two weeks post-treatment. The effects of cytopenia induction and subsequent recovery was examined for RBC, PLT and WBC number in all groups and is found in the left, middle and right panel. All animals were treated with PHZ (D0) at 5 weeks of age, except for the 129 strain, which was tested at 8 weeks. PHZ was administered s.c. to each animal a dose of 100μg/g. The summary of statistical analysis performed can be found in appendix table 6.3. B6, Balb/c and 129 female mice were used.
4.4.5 Determining an effective HU dosage - high dose HU

Three higher doses of HU, 3 mg/g or 4 mg/g, were administered either once or twice with a four hour interval in between (Figure 4-3D). None of these treatments induced anemia in B6 females over the time course (p>0.05) or at any specific time point (Dunnett’s Post Test p>0.05). Single doses, but not repetitive doses, of HU were effective at inducing changes in PLT levels over time (p<0.05). Single doses of 3mg/g or 4mg/g induced an increase in PLT 2 days post-treatment (Dunnett’s Post-test p<0.05). Even though the strain used was identical, the 4 groups of B6 mice that we used to compare higher doses of mice had significantly different baseline WBC values (one-way ANOVA p<0.002). All of the high doses of HU were effective at inducing leukopenia (p<0.001), and WBC values were shown to drop at least 50% six days after HU injection (Dunnett’s Post-test p<0.05: D0 vs. D6). No significant differences were observed in the WBC recovery kinetics for high doses of HU (0.6% and p=0.42).

A similar HU dose analysis was performed on Balb/c females (data not shown) and very similar results were observed.

In replicate experiments a HU dose of 3mg/g administered once or twice was not consistent at inducing leukopenia in B6 males and was only consistent at a higher dose of 4mg/gx2. Therefore, a higher dose of 4 mg/gx2 was used for all remaining experiments.

4.4.6 Strain-Specific Effects of HU Administration

RBC baseline values were similar for the B6, Balb/c and 129 female mice (one-way ANOVA p=0.06) (Figure 4-3E), but differences were observed in the RBC recovery kinetics (16% of variance and p<0.0001). Specifically, HU was effective at inducing a mild anemia (nadir: B6 D2 and 129 D4), which resolved quickly in B6 and 129 female mice (B6 p<0.02 and 129 p<0.007). On the other hand, the RBC values of Balb/c mice did not drop until D10 with HU
treatment, and RBC values did not return to near normal until D16 post-HU treatment (Dunnett’s Post-test p<0.05: D10 and D12). No statistically significant difference was observed in the level of the RBC between the three strains (one-way ANOVA p=0.23).

All three strains tested had significantly different PLT baseline values (one-way ANOVA p<0.0001) (PLT DO: B6 1226x10^9/L, Balb/c 1082x10^9/L and 129 934x10^9/L) (Figure 4-3E). The differences in PLT recovery kinetics were not only statistically significant (V=9%, p<0.0001), but are also visually striking and distinct. HU was effective at inducing a mild thrombocytopenia, which quickly reversed in B6 and Balb/c females (nadir D4) (effective Dose p=0.0001 and Dunnett’s Post-test p<0.05: D4 only), but the thrombocytopenia was more severe in Balb/c females (T-test p<0.004). Unlike B6 and Balb/c mice, HU induced thrombocythemia, but never induced thrombocytopenia in 129 females, where PLT values continually increased until they reach a peak on D12 (PLT increase 50% from baseline) (Dunnett’s Post-test p<0.05: D8-12). A statistically significant difference in PLT recovery kinetics of HU-treated mice was only observed between B6:129 mice and B6:Balb/c mice, but not between Balb/c and 129 mice.

WBC baseline values between the B6, Balb/c and 129 strains showed significant differences, as previously observed (one-way ANOVA p<0.03). The WBC recovery kinetics of HU-treated mouse strains were visually and statistically distinct (V=8%, p<0.02). HU induced a mild leukocytosis followed by a mild leucopenia in B6 females, which resolved quickly as WBC values have returned to near normal values by D12 (Dunnett’s Post test p<0.05: D4 and D8). HU also induced leukopenia in Balb/c females, which fell to a nadir on D6 and did not return to baseline values until D16 (Dunnett’s Post-test p<0.05: D2-D16). HU did not induce thrombocytopenia in 129 females, but induced leukocytosis, where WBC values consistently increased until D8, after which WBC values returned to near baseline values on D10 (Dunnett’s Post-test p<0.05: D6-8). A pair-wise factorial analysis, however, only revealed a significant
difference between B6 and Balb/c females. Although a two-way repeat measures ANOVA did not show significant differences between B6 or Balb/c and 129 strains, the recovery kinetics of these strains are clearly different, because HU in B6 and Balb/c induced leukopenia, while it induced leukocytosis in 129 females. A two-way repeat measures ANOVA most likely does not detect the difference, because 129 baseline WBC values were much lower.
Figure 4-3. HU recovery curves

A-C) Low HU doses induced significant changes in RBC, PLT and WBC recovery. Each group consisted of ten C57Bl6/J mice tested at D0, 2, 5, 8, and 11. D) shows the effect of high HU doses on RBC and PLT recovery. Each group consists of five C57Bl6/J females tested at D0, 2, 6, and 9. E) Effect of strain on recovery from high-dose HU treatment. Each group of mice consisted of two subgroups of 8-10 animals, one subgroup was tested at D0, 4, 8, 12, and 16 and second was tested at D0, 2, 6, 10, 14. The two subgroups were combined to create a single recovery curve that represented one treatment group and includes baseline data and data sampled every two days post-treatment. The effect of cytopenia induction and subsequent recovery of RBC, PLT and WBC number was examined in all groups and is found in the left, center and right panel (respectively). All animals were 5 weeks of age at HU treatment, unless otherwise stated. HU was administered IP once or twice with a 4 hr interval. The dose administered per injection in D and E was 4 mg/g. A summary of statistical analysis performed can be found in Appendix Tables 6.4 and 6.5.
4.4.7 Effects of Hematological Stress Agents on KTLS Cells in the Bone Marrow

KTLS are a heterogeneous group of hematopoietic cells that contain early hematopoietic progenitors and stem cells (HSC)[314]. HSC are predominantly quiescent, so it is particularly important to understand if 5FU or HU affects cells of this population and to compare the recovery of KTLS cells after hematopoietic stress.

The frequency of KTLS cells within the bone marrow was unaltered 18 hrs after 5FU injection (Figure 4-4). While the total frequency of KTLS cells was unchanged, a sub-population of c-kit$^+$ was greatly reduced (76-88%). A 3- to 5-fold increase in KTLS frequency is observed one week after 5FU treatment. Two weeks after 5FU treatment KTLS frequency had returned to control levels (data not shown).

PHZ did not alter the frequency of KTLS cells in the bone marrow 18 hrs after treatment (Figure 4-4). One week after PHZ treatment, the KTLS population increased 2- to 5-fold. The KTLS population had returned to control levels two weeks after PHZ treatment (data not shown).

Injections of 4mg/g x 2 of HU caused no statistically significant alteration in KTLS frequency at any time point examined.
Figure 4-4. Time course study examining the alterations in KTLS population and profile post cytopenia induction

Each experimental cohort is represented by four groups of three B6 female mice at treated at 5 weeks of age; each group was treated with either PBS only, HU, 5FU or PHZ. Cohorts were examined at 18 hours, 7 days and 14 days after treatment (Table 6.6 and 6.7). At each time point, bone marrow cells were analyzed by flow cytometry for KTLS expression. All animals were analyzed individually and a representative example is shown. Each flow plot shows a Lin\textsuperscript{−}Thy1\textsuperscript{lo} population on a c-kit and Sca-1 plot and the red box represents the KTLS gate, while the proportion of cells inside each box is shown as a percentage of total cell number and represents the average for a group in an individual experiment. 5FU-treated mice showed a loss of c-kit\textsuperscript{hi} expressing cells 18hrs post treatment. The c-kit\textsuperscript{hi} KTLS positive population is shown in the green box. The proportion of cells in this population is expressed as a percentage of the total cell number and is shown inside the green box.
4.4.8 Effect of Hematological Stress Agents on BM Cellularity and CFU-C

5FU treatment alters bone marrow (BM) cellularity two weeks following treatment (p<0.0001) (Figure 4-5A). Specifically, BM cellularity dropped 18hrs after 5FU treatment (p<0.05) and continued to decrease on until day 7 (40% of baseline values, p<0.05). Within two weeks of 5FU treatment, bone marrow cellularity had returned to near baseline values (p>0.05).

CFU-C frequency was also altered during by 5FU treatment (p<0.0001). Eighteen hours after 5FU administration, CFU-C frequency dropped by over 60%, and then overshot baseline values by more than 2-fold at two weeks post-treatment (Figure 4-5B). Changes in CFU-C number can be attributed to changes in all colony types (G, M, GEMM, GM or E) (p<0.0001, except BFU-E p<0.03). No specific colony type decreased significantly 18 hours after 5FU treatment (p>0.05, NS). All colony types (except BFU-E) showed a 2-4 fold increase in frequency seven days after 5FU treatment (p<0.05). The overshoot in CFU-C continued until day 14 and can be attributed to a significant overshoot of CFU-G and CFU-GM (p<0.05). All other colony types were not significantly different from baseline by two weeks (p>0.05, NS).

5FU administration significantly influences the frequency of CFU-E formation (p<0.05, Figure 4-5D). Bone marrow CFU-E frequency dropped by 97% at 18 hours post-5FU treatment (p<0.05) and returned to near baseline values within seven days. These values were maintained until the end of the observation period.

PHZ treatment did not alter bone marrow cellularity over the two-week period (p=0.1, NS), nor were there any significant differences from baseline at any time point (p>0.05, NS) (Figure 4-5A).
PHZ treatment did not induce significant changes in CFU-C frequency over the two week time course (p=0.08, NS), nor were any significant differences observed between baseline values and values at any time point tested (p>0.05, NS) (Figure 5B). Analysis of CFU-C differentials revealed that the only significant change was in CFU-GM (p<0.02) with an overshoot observed on D7 (p<.05).

CFU-E frequency changed over the two week period following PHZ treatment (p<0.02), with increased CFU-E within 18 hrs after treatment (p<0.05), but no statistically significant differences from baseline were observed (p>0.05, NS) (Figure 4-5D).

Overall, bone marrow cellularity was significantly altered by the treatment of HU (p<0.0001) (Figure 4-5A). Bone marrow cellularity decreased 45% within 18 hrs of HU treatment (p<0.0001) and began to return to normal one week after treatment.

HU treatment had no effect on total CFU-C frequency over the two-week period (p=0.25, NS), and no significant changes occurred at any time point (p>0.05, NS). However, when the changes in bone marrow cellularity were taken into account, CFU-C number per femur dropped by over 35% 18 hours after HU treatment (p<0.05), after which CFU-C increased to near baseline values on D7 and finally overshot baseline values on D14  (p<0.05). The differences in CFU-C number can be attributed to changes in CFU-G (colony forming unit- granulocyte) number over the two week period (p<0.0001). CFU-G dropped 45% within 18hrs of HU treatment (p<0.05) and began to return to baseline values seven days after treatment (Figure 4-5C).

HU administration had effects on CFU-E frequency (p<0.005) (Figure 5D). CFU-E frequency decreased by almost 40% (p=0.11, NS) within eighteen hours of HU treatment, CFU-
E colonies continued to decrease until D7 (p<0.05) and then rebound was observed on D14 (p>0.05, NS). When changes in cellularity were taken into account, HU still led to changes in CFU-E number per femur over time and to a near 80% loss in CFU-E number 18 hrs after HU treatment. This reduction continued for up to one week following treatment (p<0.05).
Figure 4-5. Time course study on bone marrow hematopoietic progenitors post cytopenia induction

A) Bone marrow cellularity was assessed by the total number of cells isolated per femur flushed with IMDM + FBS. B) CFU-C Frequency: the number of CFU-C colonies per $2 \times 10^4$ bone marrow cells plated. Each bar indicates total CFU-C number along with the frequency of CFU-G, CFU-M, CFU-GEMM, CFU-GM and BFU-E colonies. C) Number of CFU-C per femur was calculated from (CFU-E freq/cell plated)*total BM cells per femur. D) CFU-E Frequency: the number of CFU-E colonies that stained positive after benzidine staining per $2 \times 10^4$ bone marrow cells plated. E) Number of CFU-E per femur was calculated from (CFU-E freq/cell plated)*total BM cells per femur. Each experimental cohort was represented by 4 groups of 3 C57Bl/6J female mice treated at 5 weeks of age; each group was treated with either PBS only, HU, 5FU or PHZ. Cohorts were examined at each time point of 18 hours, 7 days and 14 days post-treatment. Data plotted represents the average + SEM for all 3 cohorts representing a total of 9 animals for each treatment at each time point. A statistical significance difference between two groups is indicated by a line over each group (summary in Appendix Table 6.8 and 6.9).
4.4.9 The Effect of Hematological Stress Agents on Induction of Extramedullary Hematopoiesis

5-fluorouracil causes changes in extramedullary hematopoiesis as assessed by examining CFU-C in the spleen over a two week period (p<0.0001) (figure 4.6). Splenic CFU-C values dropped 90% after 18hrs, increased to near baseline levels at D7, and then overshot 4.2-fold above baseline levels at D14 (p<0.05). The changes in extramedullary hematopoiesis caused by 5FU were due to changes in both red (BFU-E) and white colonies (CFU-G, CFU-M, CFU-GM, CFU-GEMM) (p<0.0001). 5FU treatment led to more than a 95% loss in red colonies 18hrs post injection, remained low on D7 and finally returned to near normal levels by D14 (p<0.05). White colonies also were reduced by 90%, 18hrs after treatment (t-test p=0.0006). One week after 5FU treatment, white colonies returned to baseline and then overshot 6.2-fold over baseline values two weeks after treatment (p<.05).

The treatment of B6 mice with PHZ led to no significant changes in total splenic CFU-C at any time point (p>0.05, NS). While no significant changes in red colony frequency was observed over the time course (p>.05, NS), white colonies significantly overshot baseline values on D7 by almost 3-fold (p<0.05). No other significant differences in white colonies compared to baseline values were observed (p<0.05).

HU increased extramedullary hematopoiesis over the time course of the experiment (p<0.02), where splenic CFU-C colonies increased 3.6-fold at one week post-treatment (p<0.05). This was primarily due to a 5.6-fold increase in white colonies on D7.
Each experimental cohort is represented by 4 groups of 2-3 C57Bl/6J female mice treated at 5 weeks of age; each group was treated with either PBS only, HU, 5FU or PHZ. 2-3 cohorts were examined at each time point of 18 hours, 7 days and 14 days post-treatment. Splenic CFU-C frequency: the number of CFU-C colonies per 1x10^5 spleen cells plated. Data plotted represents the average + SEM for 2-3 cohorts representing a total of 6-9 animals for each treatment at each time point. Each bar is shows both red colonies in black (BFU-E) and non-red colonies in white (all other CFU-C colony types: CFU-G, CFU-M, CFU-GEMM, and CFU-GM). Statistically significant differences from baseline (D0) are indicated by a line over each group (summary in appendix table 6.10).

4.4.10 The Effect of Hematological Stress Agents on Megakaryocyte Ploidy

Megakaryocyte ploidy was examined in the bone marrow of 5FU-, PHZ- and HU-treated B6 females. CD41 is a marker of megakaryocytes, and its total expression was analyzed, along with the ploidy of CD41^+ cells. Megakaryocyte ploidy was analyzed by correlation of propidium iodide intensity of CD41^+ cells with DNA content.

5FU induced changes in the frequency of CD41 expressing cells and ploidy profiles over the two-week time course (figure 4-7). Within 18hrs of 5FU administration, a left shift in ploidy was detected, with an increase in the percentage of 2N and 4N CD41^+ cells (40 and 30%, respectively) and a decrease in the percentage of 8N CD41^+ cells (25%) (T-test, p<0.05: 2N, 4N,
Four days after 5FU treatment, the left shift in ploidy continued and became more severe with the majority of cells in 8N. After 4 days, 4N and 8N CD41⁺ cells increased 3.6-fold and 3.2-fold, respectively, and 16N and 32N CD41⁺ cells decreased 56% and 57%, respectively (T-test p<0.05: 4N, 8N, 16N and 32N). The megakaryocyte ploidy profile returned to that of untreated animals one week after 5FU treatment, except that the frequency of CD41⁺ cells with a propidium iodide intensity of 32N increased by 51% (T-test p<0.05). Two weeks after 5FU treatment, the percentage of CD41 positive cells had returned to untreated levels, but the ploidy profile had not returned to normal, because slight, but significant, increases in 4N (p<0.05), 8N and 64N cells (0.1>p>0.05) were observed.

The frequency of bone marrow cells expressing CD41 was unchanged after PHZ treatment at all time points tested. Within 18hrs of PHZ treatment, the megakaryocyte ploidy profile had shifted to the left, with a decrease in percentage 4N and 8N cells by 24% and 38%, respectively, and a 2-fold increase in the percentage of 32N cells (T-test p<0.05 8N, 0.1>p>0.05 4N and 32N). The megakaryocyte ploidy profile returned to normal one week after PHZ treatment. A slight change in megakaryocyte profile was observed two weeks after PHZ treatment, where the frequency of cells in 32N had increased by 65% (p<0.05).

No significant changes in the frequency of cells expressing CD41 was observed after HU treatment (p>0.05, NS). HU treatment caused modest changes in megakaryocyte ploidy one and two weeks after administration. Specifically, there was a 50% increase in 2N cells and a 70% decrease in 8N cells one week after HU treatment. Two weeks after HU treatment, megakaryocyte ploidy was almost back to normal, except for a two-fold increase in 8N megakaryocytes.
Figure 4-7. 5FU alters megakaryocyte ploidy and frequency.

**A-B)** The DNA (ploidy) content of megakaryocytes was measured by propidium iodide intensity of bone marrow cells that have been gated for positive CD41 expression (which represents ploidy). Propidium iodide intensity doubling was set at 2N, 4N, 8N, 16N and 32N on CD41+ cells. The y-axis represents the % of CD41 positive cells that had a given DNA content. Results from control mice are represented by a black histogram, whereas the results from the treated mice are represented by the red histogram. **A)** Ploidy analysis 18hrs after drug treatment for the three treatment groups: 5FU (left), PHZ (centre), HU (right). **B)** Ploidy analysis 4 days after drug 5FU treatment. **C)** Histograms for CD41 expression for control (far left) or 5FU- (centre left), PHZ- (centre right) and HU-treatment (far right). The gate for CD41 positive cells is based on IgG stained control and the average for each group is located over the gate. An experimental cohort is represented by 4 groups of 3 C57Bl/6J female mice; each group was treated at 5wks old with PBS only, HU, 5FU or PHZ. One experimental cohort was used for each time point of 18hrs, 4 days, 7 days and 14 days post-treatment. Data in each FACS plot is representative from one animal from each group.
4.4.11 The Implementation of a 5FU Sensitized Screening Protocol as Part of a Dominant ENU Screen

The goals of the pharmacologically sensitized ENU screen were to challenge the hematopoietic system in order to elicit phenotypes associated with defects in developmental pathways that cannot be easily be detected in steady state hematopoiesis. Of the three chemotherapeutic drugs tested, 5FU was selected because it affects a broad range of hematopoietic lineages and therefore has the capacity to detect the most mutations. Specifically, we believed that 5FU would allow us to detect mutations affecting single and multi-lineage progenitors in the hematopoietic system.

A 5FU screen was integrated into an existing ENU dominant screen, where 129 male mice were mutagenized with ENU, and subsequently bred to B6 females, to produce the first generation (G1) of ENU-exposed mice (Figure 4-8A). Five-week-old G1 mice were challenged with 100 µg/g of 5FU and then the recovery kinetics of RBC, PLT and WBC were monitored. Reference 5FU recovery curves were generated for unmutagenized 129 x B6 hybrid mice (Figure 4-8B). To assess 4 distinct phenotypes in one cohort of mice (RBC, PLT and WBC delayed recovery and PLT overshoot; Table 4-1), we determined the optimal testing days were D0, D6, D8 and D13.
Figure 4-8. ENU breeding scheme and selection of optimized 5fu testing days.

A) The CMHD dominant ENU breeding scheme used for the 5FU screen. The mutagenized mouse strain was 129 and the backcross strain was B6. B) The recovery kinetics for G1 hybrid (B6:129) control males of RBC, PLT and WBC are shown in black circles. The testing days chosen for the 5FU screen to identify defects in multi-lineage and erythroid recovery in G1 hybrid males are indicated by the red squares. All error bars represent the SEM.
Table 4-1. Summary of G1 phenotypes and criteria for classification.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Phenotype</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC</td>
<td>Delayed RBC Recovery</td>
<td>RBC’s decrease at every time point and the D13 value is 2SD or greater below the norm</td>
</tr>
<tr>
<td>PLT</td>
<td>Delayed PLT Recovery</td>
<td>PLT decrease at every time point between D0 to D8 and the drop from D6 to D8 was 2 or more SD below the norm</td>
</tr>
<tr>
<td>PLT</td>
<td>Increased PLT Overshoot</td>
<td>PLT values on D13 are 2.75SD higher than the norm.</td>
</tr>
<tr>
<td>WBC</td>
<td>Delayed WBC Recovery</td>
<td>WBC values decrease at continuously at every time point and D13 values are 2SD or more below the norm</td>
</tr>
<tr>
<td>Multiple Lineages</td>
<td>Multilineage Recovery Mutant</td>
<td>A G1 animal is an outlier for two or more of the above parameters</td>
</tr>
</tbody>
</table>

To determine whether a 5FU dose of 100µg/g had been effective at inducing cytopenia and if the subsequent recovery had occurred uniformly within the G1 mice, the recovery of RBC, PLT and WBC was examined in G1 male mice (figure 4-9A). A period of recovery or overshoot is indicated by positive values (values above the red line). Alternatively, a period of time representing cytopenia or a parameter returning to baseline after overshoot is represented by negative values (values below the red line). The RBC recovery kinetics in G1 males was variable, particularly between D6 and D8, when 61% of G1 males showed a decrease in RBC and became more anemic, while 39% of G1 males were recovering from anemia. Overall, the PLT recovery kinetics post 5FU treatment occurred in unison. In particular, 5FU-induced thrombocytopenia occurred in unison between D0 and D6, as well PLT recovery and early overshoot occurred between D6 and D8. Discord was observed in PLT recovery kinetics, during the time from D8 to D13, as 62% of mice continued to demonstrate an increase in PLT overshoot, while the remaining mice returned to baseline. WBC baseline values were highly variable, for example, in G1 males the average D0 WBC was 12.6x10⁹/l with a large standard
deviation of 3.0. 5FU induction of leukopenia occurred relatively in parallel, as the WBC values for 93% G1 males dropped from D0 to D6. Leukopenia was resolved in the majority of G1 males (74%) by D8, and WBC values continued to increase between D8 and D13.

Once an outlier of interest was identified in the G1 screen, it was subsequently bred to a B6 mouse of the opposite gender to determine if the phenotype observed in the G1 was heritable. A phenotype was designated as heritable, if some of the offspring (up to 50%) displayed the same phenotype as the G1 parent. In a dominant screen, a phenotype is fully penetrant (100% penetrance), when 50% of the offspring have the same phenotype as the G1 parent. Heritable lines were then sent for a genome scan to map the chromosomal location of the causative mutation. In the genome scan, we used an Illumina SNP chip, which contained 750 informative SNP (between 129 and B6 mouse strains) all of which were distributed across the 19 autosomal chromosomes and as well as chromosome X. A mouse line was considered to map to a specific chromosomal location, if all mice analyzed were heterozygous for a certain set of SNP at a specific location.

Five hundred and forty-six G1 animals were tested in the 5FU screen. The 5FU screen was designed to uncover alterations in RBC, PLT and WBC recovery. A total of 25 outliers were identified and were classified into 5 categories: delayed RBC recovery, delayed PLT recovery, increased PLT overshoot, delayed WBC recovery and alterations in multiple parameters (multilineage outliers)(Table 4-2).
Table 4-2. Summary of G1 screen

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Of the 10 delayed RBC recovery outliers, 4 were chosen for heritability testing (S430-1-23, S430-5-2, S432-9-24, S450-1-1). The mice selected for heritability testing had z-scores between -2.7 and -5.7 (D13- D8) (Table 4-3). The RBC values on D0 for S430-1-23(7304) and S430-5-2(7307) were elevated (RBC D0 z-score: 1.3 and 1.6, respectively), but were not high enough to be picked out in the dominant screen with CBC testing alone (z-score not above 2). In addition to elevated baseline RBC values, S430-1-23(7304) and S430-5-2(7307), had the largest decrease from D8 to D13 RBC outliers of RBC outliers selected for further analysis (RBC D13-8 SD: -5.3 and -5.7, respectively). The baseline RBC values for S432-9-24(7310) and S450-1-1(7303) were near normal (Z-score greater than 0.0 and less than 0.2); however, the delayed recovery was minimal but significant compared to other outliers (Figure 4-9B).

Two categories of PLT recovery outliers were detected in the 5FU screen: delayed PLT recovery and increase in PLT overshoot. Three G1 mice were outliers for the delayed PLT recovery between D6 and D8, with significant z-scores (2.1 to 3.5) (Table 4-3). A single G1 with delayed PLT recovery was selected for subsequent analysis, S442-19-3 (7324). Increased PLT overshoot was detected in 10 G1 mice with z-scores between 2.9 and 5.8 (Table 4-3). Of the 10
increased PLT overshoot outliers, only one G1 was selected for further analysis, S469-3-1 (7325). The S469-3-1 (7325) mouse had near normal baseline PLT values and an elevated PLT overshoot (z-score 2.9) (Figure 4-9C). An additional G1 was selected for subsequent analysis, S443-5-1 / 7323, which had increased PLT on D0 (z-score 2.7) normal 5FU PLT recovery (Figure 4-9C).

During the 5FU screen, a single G1 with delayed WBC recovery was detected, S417-12-27/7300 (Table 4-3 and Figure 4-9D) and sent for subsequent analysis.

A single G1 (S449-9-2 7321) was detected in the 5FU screen that was an outlier in multiple parameters including: delayed RBC recovery and increased PLT overshoot, and increased WBC overshoot (Figure 4-9E and Table 4-3). The S449-9-2 7321 mouse also had high baseline values for RBC, PLT and WBC (z-score: RBC 1.7; PLT 2.6; WBC 3.2).

Of the 25 G1 outliers detected in the 5FU screen, 8 G1 mice were chosen for further analyses, which began by testing the heritability of a specific phenotype. Once the G1 mouse were bred, the subsequent line was referred to by its G2 ID (7000 number).
Figure 4-9. Analysis of G1 mice for defects in RBC, PLT and WBC recovery after 5FU administration

A) The RBC, PLT and WBC recovery for all G1 males tested in the 5FU screen. The horizontal red line in each graph indicates when no change occurred, and points above the line indicate that the values were increasing between two time points, while points below the line indicate that values were decreasing between the two time points. B) The G1 RBC recovery outliers that were chosen for heritability testing. C) All the G1 PLT recovery outliers that were chosen for heritability testing. D) The G1 WBC recovery outlier that was chosen for heritability testing. E) The G1 Multiple lineage recovery outlier that was chosen for heritability testing is shown.
Table 4-3. G1 outlier’s phenotype summary

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<th>Phenotype</th>
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<th>D8</th>
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<td>10.83</td>
<td>9.59</td>
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<td>9.56</td>
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<td>RBC</td>
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<td>8.4</td>
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All four G1 mice selected for the delayed RBC recovery phenotype were heritable with low penetrance: 7303 (S450-1-1) with 16% penetrance, 7304 (S430-1-23) with 18% penetrance (Figure 4-10 A), 7307 (S430-5-2) with 34% penetrance, and 7310 (S 432-9-24) with 22% penetrance. The 7303 line was ended because of very low penetrance. In addition to the delayed RBC phenotype, the 7310 line showed a secondary phenotype of low RBC at D0 (Figure 4-10B). Three out of the four heritable lines (7304, 7307 and 7310) were sent for a genome scan to map the causative mutation. A genome scan of 7304 (7 of 7 G2 and G3 mice) revealed that the causative mutation mapped primarily to a 23.3Mb location on chromosome 14 flanked by rs4230248 (32Mb) and rs13482191 (55.3) (7/7 mice). Additional mapping locations for 7304 were observed on chromosome 6, 18, and 19 (6/7 mice), but these chromosomal locations were eliminated as possible candidates through further analysis of subsequent generations. Fine mapping narrowed the interval to a 12 Mb region on chromosome 14.

Fifteen mice from the 7307 line (G2-4) were sent for a genome scan. The causative mutation for the 7307 line did not map to any chromosomal location. The 7307 line was terminated because of poor mapping.

Nine mice from the 7310 line (G2) with the primary phenotype were used for the genome scan, and revealed that the causative mutation mapped 2 locations: a 55Mb region at the telemetric end of chromosome 14 (rs13482265 (79.4Mb) to the telomere (125.2Mb)) (9/9 mice) and the centeromeric end of chromosome 7 (8/9 mice). The secondary phenotype for 7310, also mapped to the same regions on chromosome 14 and chromosome 7. In subsequent generations only the delayed RBC recovery phenotype was followed in the 7310 line. Chromosome 7 was eliminated as a possible map position through further analysis of subsequent generations. The
7310 line was eventually ended due to high rate of false positives (phenotypically outliers, but genotypically WT).

Figure 4-10. Analysis of G2 heritability in 5FU RBC mutants

A) The 7304/S430-1-23 phenotype is delayed RBC recovery, which was heritable with 19% penetrance. B) The 7310/S432-9-24 displayed two phenotypes: a primary phenotype of delayed RBC recovery is displayed in red and a secondary phenotype of low baseline RBC is displayed in orange. The primary phenotype was heritable with low (22%) penetrance and the secondary phenotype was heritable low (22%) penetrance. The mean values for the 129/B6 G1 controls are shown in back and error bars represent the standard deviation. Not affected (NA) G2 mice are shown in blue and affected G2 mice are shown in red. If a secondary phenotype was observed, mice with this phenotype are shown in orange.
The high PLT phenotype of 7323 was heritable with 30% penetrance (Figure 4-11A), interestingly only male mice from G2 and G3 had the high PLT phenotype. Eight mice were used for a genome scan, which mapped the causative mutation to a 24Mb region at the telomeric end of chromosome 11 (rs3661058 (98.8Mb) to the telomere (121.8Mb)). New crossovers seen in successive backcrosses of 7323 line has narrowed the mapping interval to a 10.6 Mb region on chromosome 11.

Heritability testing revealed that the 7324 phenotype was heritable with low penetrance (20%) (Figure 4-11B). A genome scan was performed on 8 mice (G2-4) and all 8 mice mapped to 5 separate chromosomal locations (chromosome1, 5, 7, 13 and 19). In subsequent analysis on the fourth generation of the 7324 line, the causative mutation no longer mapped to any of the previous locations. The 7324 line was discarded due to difficulty in mapping.

A heritability test was performed on the 7325 line, which proved that the phenotype of high PLT overshoot was heritable with low penetrance (19%) (Figure 4-11C). A genome scan was conducted on 9 mice from the second and third generation of the 7325 line, which mapped the causative mutation to a 45Mb region at the distal end of chromosome 11 (bound rs13481084 to the telomere). The mapping interval on chromosome 11 has been narrowed to a 25.7Mb region on chromosome 11.

When the 7321 line underwent heritability testing, no G2 had all of the three phenotypes originally seen in the G1 founder, but G2 mice did have single phenotypes (Figure 4-12). The delayed RBC phenotype was the most penetrant at 35%, the WBC overshoot phenotype was heritable at 25% penetrance and PLT overshoot phenotype heritable with low penetrance (15%).
The 7321 was not sent for mapping and the line was discontinued because of the complicated inheritance of the phenotype.
Figure 4-11. Analysis of G2 heritability in 5FU platelet mutants

The 7323/S443-5-1 phenotype was high platelets on D0, but normal PLT recovery. 7323 was heritable with a 30% penetrance. B) 7324/S442-19-3 phenotype had delayed PLT recovery. 7324 is heritable with 20% penetrance. C) 7325/S469-3-1 5/54 phenotype has PLT overshoot on D13. 7325 is heritable with 18.5% penetrance. Only female mice are displayed in graph because all affected G2 mice are females. 129/B6 G1 controls are shown in back and error bars represent standard deviation. NA G2 mice are shown in blue and affected G2 mice are shown in red.
Figure 4-12. Analysis of G2 heritability multilineage mutants

The 7321/S449-9-2 phenotype was delayed RBC recovery, PLT and WBC overshoot. 7321 was heritable for RBC phenotype with 35% penetrance. PLT overshoot was heritable with 15% penetrance. The WBC overshoot phenotype was heritable with 25% penetrance. No mice have all three phenotypes originally seen in the G1. 129/B6 G1 controls are shown in back and error bars represent standard deviation. NA G2 mice are shown in blue and affected G2 mice are shown in red.
4.5 Discussion

4.5.1 Drug Recovery Kinetics

5FU-induced anemia appears to have a threshold affect, since both 120µg/g and 150µg/g caused similar levels of anemia whereas the response was more modest at 100µg/g. In a dominant ENU screen, the need for low mortality is critical because the G1 animals must be viable for breeding, maintenance of the phenotype and genome analysis to discover the causative mutation. Because the G1 animals were shared among other researchers, it was essential that the mortality rate was as close to zero as possible. Also, rapid recovery after 5FU treatment was desirable as the cohort of mice underwent other tests including electrocardiogram at 8 wk of age. Therefore, 100µg/g of 5FU was chosen. A dose of 100µg/g of 5FU resulted in no mortality and recovery was more rapid than higher doses of 5FU.

Mouse strain plays a critical role in 5FU recovery kinetics, as no two strains tested had the same recovery kinetics in all parameters. Balb/c and B6 showed a high degree of similarity, with no significant difference in RBC and PLT recovery curves. The 129 strain was similar to the B6 and Balb/c strains in the overall timing of recovery kinetics (nadir and overshoot), but substantial differences exist. Anemia was much more severe and recovery occurred more slowly in Balb/c and B6 compared to 129 mice. Recovery from thrombocytosis occurred quickly in the 129 mouse strain, but thrombocytosis was more prolonged in the B6 and Balb/c mouse strains. The C3H mouse strain had completely different recovery kinetics in both timing and severity of cytopenia and overshoots. The nadir of each parameter occurs earlier in C3H mice in contrast to the B6, Balb/c and 129 mouse strains. Also, the scale of cytopenia and overshoot was much less in C3H mice. There are two main reasons why differences in recovery kinetics were observed among different mouse strains: differences in hematopoiesis and drug interactions. The
hematopoietic differences in mouse strains may be attributed to differences in the size of progenitor pools, the number of progenitors in cycle, or the expression of regulators in the cellular pathways that respond to hematopoietic stress. As well, 5FU may be absorbed, metabolized or excreted at different rates in unique strains. While the exact reason for the difference in recovery kinetics is not critical for the implementation of 5FU into an ENU screen, it is important to have knowledge of these differences. The ideal strain combination for an ENU screen is B6 and Balb/c, because the high level of similarity in the recovery curves observed in these mice minimizes false positives or negatives. One area that might be problematic with this strain combination is the PLT nadir and immediate recovery, since the nadir and recovery occurred 4 days apart, which might lead to false positives/false negatives. 129 mice could be combined with B6 or Balb/c strains, to become the mutagenized strain or the backcross strain, but this combination would not be ideal. For example, crossing 129 to Balb/c to assess PLT recovery might be quite advantageous, because of the high level of similarity between the two strains. On the other hand, the same cross to examine RBC recovery would likely cause false positives leading to problematic mapping or false negatives, and thus, the possibility of losing the line depending on which strain was chosen to be the mutagenized strain. C3H mice would not be a good strain to combine with any other strain tested, because it has dramatically different recovery kinetics.

Overall, 5FU caused changes in multiple hematopoietic lineages, and therefore, could be used to identify a large array of genes involved in hematopoiesis. Recovery from 5FU-induced cytopenia, however, takes longer in comparison to the other drugs tested, and every additional week of housing greatly increases the cost of initial G1 screening.
An optimized PHZ dosing protocol was developed by a collaborator, Robert Paulson, and involved a single subcutaneous injection of 100 µg/g of PHZ [206]. The optimized PHZ protocol caused no mortality in a PHZ ENU trial screen.

Mouse strain had very little influence on the PHZ recovery kinetics. No significant differences among B6, Balb/c and 129 mice were observed in RBC recovery kinetics. The height of WBC/RET overshoot in 129 mice was not as high as B6 and Balb/c, but the timing of the recovery was similar. Unlike 5FU, PHZ acts directly on RBCs and does not require metabolic activation, which may explain why the three strains had very similar recovery kinetics [202]. B6 and Balb/c were the ideal strain combination for a PHZ ENU screen. The high degree of similarity between B6 and Balb/c recovery curves would mean a very low rate of false positives/negatives. The 129 strain could be combined with either B6 or Balb/c for an effective ENU screen to examine RBC recovery alone. Along with the high similarity of responses to PHZ observed in the different strains of mice, PHZ also has other benefits for use in an ENU screen. Peripheral blood parameters returned to baseline within two weeks after treatment with PHZ and therefore the time required to keep G1 animals was shorter than with 5FU treatment, which would potentially reduce costs. One major limitation of PHZ is that it principally affects the erythroid lineage, and thus, reduces the number of potential mutations and genes that would be detected in an ENU screen.

Unlike, 5FU or PHZ, HU has not been used as a hematopoietic stressor and therefore, a uniform dosing regimen for cytopenia induction did not previously exist. Dosing regimens for other purposes have been reported and vary from continuous HU administration via a mini-pump implanted under the skin to single or multiple injections spaced hours or days apart depending on the intended use [217, 219, 220, 222]. Continuous administration of HU or more than 2
injections is not practical for an ENU mutagenesis screen. Since no dosing regimen that I tested was effective in inducing anemia or thrombocytopenia, I therefore focused on the effects HU had on inducing leukopenia. Originally, no difference was observed between doses of 3mg/g and 4mg/g of HU in inducing leukopenia, and thus, 3mg/g x2 was the dosing regimen initially chosen to induce cytopenia. When the dosing regimen of 3mg/g x2 of HU were tested on B6 male mice, it was found to be ineffective in inducing leukopenia (data not shown). However, 4mg/g x2 of HU was effective and this higher dose was used for all subsequent experiments.

Mouse strain plays a critical role in HU recovery kinetics, since each strain tested displayed distinct recovery patterns. HU induced only mild cytopenia in B6 females compared to females of other strains. While B6 and Balb/c females showed similar recovery patterns over time, HU induced more severe cytopenias in Balb/c females. HU treatment of Balb/c females induced a mild anemia 10-12 days later. HU was most effective in inducing moderate thrombocytopenia and leukopenia in Balb/c females compared to other strains. The difference in recovery kinetics could be a combination of greater HU absorption, differences in the cellular pathways involved in hematopoiesis following stress or in renal excretion. It is possible that leukopenia or thrombopenias were missed, because testing only occurred every two days, but it appears that 129 mice had a different stress response to HU. The recovery kinetics of 129 females was unlike other strains tested as it caused increases in PLT and WBC, leading to thrombocytosis and leukocytosis. Of the three mouse strains tested, Balb/c and B6 would make the best strain combination for a HU-sensitized ENU screen of the strains tested, but this combination is not ideal because the degree of cytopenia is different and would lead to false positive or negatives.
Whether there is a potential use for HU in an ENU screen is uncertain and requires further investigation into HU dose and recovery kinetics. A lower dose of HU may be effective in inducing leukopenia, because HU had the least effect on recovery in B6 females, which were used for analyzing HU dose. The current HU protocol did not identify an ideal strain combination, which is problematic. Also, detection of HU-associated phenotypes would be limited, as HU is ineffective at inducing anemia and only induces clear changes in WBC recovery.

4.5.2 Cytopenia Mode of Action - 5FU

The role of 5FU in stress hematopoiesis has been studied for over 30 years. 5FU indiscriminately kills actively cycling hematopoietic progenitors leading to multi-lineage cytopenia [191, 194]. 5FU also causes strong PLT rebound, overshooting baseline values several fold [194].

A multi-fold expansion of KTLS and CFU-C population has been observed within one week of 5FU treatment and correlates with the beginning of PLT and WBC recovery. Unlike WBC and PLT, RBC do not start to recover until day twelve post 5FU injection. The delay in RBC recovery may indicate a requirement of splenic hematopoiesis, as CFU-C populations in the spleen are expanded two weeks after 5FU treatment, but the CFU-C population in the bone marrow is still increased at 2 weeks. CFU-E are actively cycling progenitors and within 18hrs of 5FU treatment were reduced to 5% of baseline values in the bone marrow. Bone marrow CFU-E recovered rapidly and returned to baseline values within one week of treatment, and are therefore not likely to contribute greatly to RBC recovery.

PLTs have a unique 5FU recovery pattern of in compared to RBC and WBC, where PLT substantially overshoot their baseline values. RBC and WBC display unique recovery following
5FU treatment, with no overshoot. The distinctive 5FU recovery kinetics of PLT, suggests that genetic control on the PLT production is either not as strong or that 5FU affects progenitors or precursors of the megakaryocyte lineage differently. A left shift in MK ploidy or DNA content occurs as early as 18hrs following 5FU treatment and persists till D4, indicating that a build-up of immature megakaryocytes and or loss of mature megakaryocytes has occurred. Within a week of 5FU treatment, megakaryocyte ploidy distribution returned to normal, but a multi-fold increase in total Mk was observed. Increased megakaryocytes corresponded to the beginning of PLT recovery. Previously, it has been shown that an expansion of extramedullary megakaryopoiesis occurs in the spleen and plays a role in PLT overshoot, but the spleen is not critical as splenectomised mice still have a strong PLT overshoot [199]. 5FU interferes with DNA replication, inhibiting increases in ploidy and megakaryocyte maturation. Interruption in RNA synthesis may also prevent cells from developing a fully mature demarcation membrane system and prevent PLT shedding. It is interesting that bone megakaryocyte numbers and ploidy had returned to baseline after two weeks, but the PLT overshoot was still present.

The present study has shown that peripheral blood parameters and hematopoietic progenitors do not return to baseline values two weeks following a 5FU dose of 120ug/g. Other studies have also indicated that the hematopoietic system has not returned to pre-treatment levels until at least 3 weeks after 5FU treatment [194, 199] or even 6 weeks for multi-lineage progenitors [193]. In competitive repopulation studies, bone marrow from 5FU-treated mice showed improved repopulation ability for up to 4 wks after 5FU treatment [193], indicating that 5FU enhances the hematopoietic system repopulation ability (likely alters HSC) and that the hematopoietic system has not returned to pre-treatment state one month after treatment. Taken together, this indicates administering 5FU again to confirm an original phenotype and hence
eliminate false positives could not effectively occur until at least one month after the first 5FU treatment.

4.5.3 Cytopenia Mode of Action - PHZ

The effect of PHZ on the hematopoietic system has been extensively studied in mice, but often with a different dosing schedule than was used in our study. Typically PHZ has been administered in 2 or 3 doses over several days [204, 207, 213], whereas we used a single dose of PHZ. Although each dosing regimen is likely to affect the same group of progenitors and precursors, the timing of the recovery kinetics will be different. For example, the reticulocyte surge that we observed one day after treatment with a single dose of PHZ was not observed until D7 in other studies in which a multiple dosing regimen was used. Given the lack of information about the effect of a single dose of PHZ, it was critical for us understanding when the hematopoietic system returns to baseline, particularly because retesting mice in an ENU screen is important as a means to confirming a phenotype and reducing false positives.

PHZ treatment caused no significant changes in bone marrow cellularity at any time point examined. Without an intermediate time point between 18 hrs and D7, the majority of the alterations bone marrow hematopoiesis and extramedullary hematopoiesis of the spleen were likely missed. All parameters measured in this study had returned to baseline two weeks after PHZ injection. Parameters returning to baseline at the end of two weeks indicate that it would likely be possible to perform a retest on G1 outliers within 2-3 weeks of initial PHZ administration without generating false negatives.

4.5.4 Cytopenia Mode of Action - HU

HU treatment caused a sharp 50% decrease in bone marrow cellularity within 18 hrs, which did not return to baseline levels until fourteen days post treatment. Similar or greater
decreases in bone marrow cellularity have been documented by others, even at lower doses of HU [215, 216, 219, 221, 222]. Although a double dosing regimen is likely to cause a massive loss in hematopoietic progenitors (first injection activate progenitors to cycle and second injection kills more), a loss of hematopoietic progenitors doesn’t explain the quick drop in bone marrow cellularity.

In the bone marrow KTLS cells and the CFU-C frequency were unchanged after HU administration over the two-week time course. However, since cellularity decreased, but the proportion of KTLS cells remained the same, the number of KTLS cells/femur decreased. The CFU-C/femur decreased by one-third 18hrs after treatment, returned to near baseline levels after one week and slightly overshot at two weeks. The majority of the changes in CFU-C/femur could be attributed to changes in CFU-G/femur, as CFU-G/femur was the only colony type to significantly decrease. CFU-E frequency changed over the time course, suggesting a higher percentage of CFU-E death. CFU-E are more susceptible to the cytocidal actions of HU, when compared to BFU-E or CFU-GEMM, because a higher fraction of later erythroid progenitors are in the S-phase of the cell cycle. Increased extramedullary hematopoiesis was seen after HU treatment and was evident as a three-fold increase in CFU-C colonies. The increase in CFU-C colonies can be attributed to an increase in non-erythroid progenitors. No alterations in megakaryocyte number or ploidy were detected during the experiment.

The effect of HU on PLT levels in B6 females appears to be minimal, as only a slight drop in PLT values was observed on day 4. As well, no changes in ploidy or megakaryocyte number were observed in B6 female mice at any time point. Balb/c females showed a more substantial drop in PLT levels when compared to B6 females and 129 mice become thrombocythemic in response to HU treatment. Although megakaryocyte number or ploidy in B6
mice did not change significantly, it is possible that changes in these parameters maybe present in Balb/c and 129 mice, since Balb/c and 129 mice have significant change in PLT recovery after HU treatment and HU treatments causes little change in PLT values. It is interesting that no substantial changes in PLT or megakaryocyte parameters occurred in B6 females treated with HU, given that 5FU caused substantial changes in PLT levels and that 5FU and HU work by similar mechanisms.

A double dose of HU in mice induced rapid changes in hematopoietic parameters, and almost all parameters had returned to baseline levels two weeks after treatment. Because the changes in hematopoiesis occur very rapidly, additional analysis of bone marrow and spleen colony assays, as well as megakaryocyte ploidy may be beneficial to understand HU-induced cytopenia.

Optimized drug dosage protocols and a comprehensive understanding of 5FU, PHZ and HU recovery kinetics allowed for the selection of a specific days to effectively interrogate areas of interest within the recovery curves, thereby enabling us to determine whether mouse strains with heterozygous mutations in known hematopoietic genes would have observable differences in the timing of cytopenias and the subsequent recovery following drug treatment. Therefore, we would be able to assess whether the chosen testing days would likely be effective in an ENU dominant screen. All the selected mutants had subtle or non-existent peripheral blood phenotypes, because they carried one normal copy of the hematopoietic gene, and therefore, it would be unlikely that they would be isolated from a non-sensitized dominant ENU screen.

4.5.5 Implementation of 5FU into an ENU Dominant Screen

Our main interest was to discover ENU mutations that affect developmental pathways in the hematopoietic system, in particular HSC and multilineage defects. 5FU was first chosen for
use in an ENU screen because it has the highest potential to expose defects in several types of hematopoietic genes. The 5FU screen was part of a larger dominant ENU mutagenesis screen conducted at the CMHD for one year. The strain combination, 129 for mutagenesis and B6 for the backcross, was selected by the CMHD. Although 129/B6 is not an ideal strain combination, both strains share similar timing in recovery kinetics. In order for a successful 5FU screen to be conducted, a thorough understanding the recovery kinetics of this strain combination was necessary, to minimize false positive detection in the G1 screen. A recovery curve for RBC, PLT and WBC following 5FU treatment of the B6:129 hybrids were determined because it most closely mimics the genotype of the G1 animals. The hematological analyzer used in the screen required a very low amount of blood (12µl), and allowed for repeated sampling from one animal. The recovery pattern for RBC, PLT and WBC did not display a similar timing, making it difficult to select days to detect delayed recovery in all three lineages, while minimizing the number of testing days. Four individual days (D0, D6, D8, and D13) were chosen for analysis of RBC, PLT and WBC 5FU-recovery kinetics.

A baseline value was collected on D0, and was critical for determining whether the G1 animals had a pre-existing hematological defect that contributed the 5FU phenotype. As platelet recovery occurred quickly, PLT nadir was analyzed on D6, recovery on D8 and potential overshoot on D13. RBC and WBC recovery was assessed on D8 and D13. The automated hematological analyzer used in this screen did not provide a WBC differential, because the analyzer discriminates cell type based on size, and therefore, the WBC parameter did not differentiate between neutrophils, lymphocytes and immature RBC, which are not likely to undergo the same recovery pattern. Therefore, it is possible that G1 mutants carrying relevant mutations were missed in the screen.
A post-screen analysis was performed on all G1 animals in the 5FU screen, to determine the effectiveness of selected testing days in assessing recovery of RBC, PLT and WBC. A great deal of variation in RBC recovery was observed, in particular between D6 to D8 and demonstrates a lack of synchronicity in RBC recovery kinetics. This may be attributed to a suboptimal 5FU dose, leaving some G1 animals recovering as early as D8 and others not recovering until D13. A higher than expected fraction of G1 animals were outliers with delayed RBC recovery, suggesting the testing days may not be effective in eliminating false positives (mice that exhibit delayed RBC recovery but the alteration in RBC recovery is not explained by a mutation and these mice likely within the normal range but are extreme). The platelet recovery pattern of most G1 mice was very similar. The testing days chosen to assess delayed PLT recovery appear to be highly effective with little or no false positives, as only 0.5% of G1 were outliers. As predicted with control data, WBC recovery of G1 animals was variable. Part of the variation is explained by the fact that no WBC differentials were available and the use of non-optimal testing days for WBC delayed recovery. False negatives are mice that carry a mutation, for example affecting WBC recovery, but are not detected because the phenotypic screen misses the altered phenotype. Therefore, the lack of WBC outliers (1 delayed WBC outlier) indicates that the WBC testing days likely miss true WBC outliers.

Delayed RBC recovery outliers were defined as G1 mice whose RBC values continually decreased throughout the time course and whose D13 RBC value were two or more standard deviations below 129/B6 hybrid controls (or negative two Z-scores). Ten (1.8%) G1 mice were outliers for delayed RBC recovery. A frequency of 1.8% of phenotypic positives is considered high, suggestive of the potential for false positives. Four out of ten G1 animals were chosen for heritability testing, and the remaining six where not examined further because of weakness in their phenotype, death of the G1 outlier or a limitation of resources. All four G1 mice had defects
that were heritable with low penetrance (16 to 34%), and the low penetrance may be due to inter-
strain quantitatively linked traits (QTL) or ineffectiveness of the lower 5FU dose in inducing
anemia. Although the mutation in the 7303 line was shown to be heritable, it was not sent for
mapping because of it had the lowest penetrance. 7307 was eliminated from further analysis after
a genome scan failed to reveal a map location, even though 7307 had the highest penetrance and
was the most robust of all delayed RBC- recovery outliers.

Both 7304 and 7310 passed heritability testing and traits mapped to two different (non-
overlapping) regions on chromosome 14, suggesting that two different mutations were
responsible for the similar phenotype. The 7304 line was followed for seven generations and
informative cross-overs were confirmed through identification of multiple cross-overs at the
same location and breeding informative cross-overs, which allowed location of the causative
mutation to be narrowed to a 12Mb region on chromosome 14. As predicted, false positives were
detected in 7304 and demonstrated as positive phenotypes, but did not map to the interval on
chromosome 14. The 7304 line was eventually ended because of difficulties in following
mapping caused by false positives. Interestingly, when the G1 founder for 7310 was bred; two
different yet related phenotypes arose: delayed RBC recovery and low baseline RBC. Both
phenotypes mapped to a 45 Mb location on chromosome 14 and an 11 Mb region on
chromosome 7. However, chromosome 7 was eventually ruled out as a possible mapping
location after further analysis of subsequent generations. Multiple phenotypes observed upon
breeding can be explained in two ways: either one mutation was causing the two related
phenotypes or two mutations where causing two separate phenotypes. It is conceivable that these
two phenotypes were caused by one mutation, whose functional consequence is different
depending on the background strain due to quantitatively link traits. One mutation causing two
related phenotypes is especially believable if delayed RBC is considered to be caused by a
smaller RBC progenitor pool or a block in differentiation that is caused by lower RBC baseline values on one strain, but is compensated for and leads to delayed recovery on another strain. The original phenotype was followed in case the secondary mutation was causing low baseline RBC. The 7310 phenotype was difficult to follow because of the high false-positive frequency, and therefore, the line was discarded after 5 generations.

Overall, delayed RBC recovery following anemia induction with 5FU was not a successful phenotype to follow in this ENU screen and was complicated by low penetrance and a high false positive rate. The lower drug dose used in this screen may have sacrificed robustness of the phenotype for lowered lethality of 5FU. It may not have been advantageous to continue to following lines with low penetrance, because false positives were likely according to control data, as mice that didn’t carry a mutation are likely to show up as affected given the testing days chosen. Instead, it may have been more useful to look for lines that had higher penetrance at the second generation. Also, our analysis of strain-dependent effects of 5FU suggests that an optimal strain combination for detection of RBC outliers would be B6 x Balb/c.

The similarity in PLT recovery between the 129 and B6 mouse strains made PLT recovery an ideal phenotype to follow in this sensitized ENU screen. Two classes of PLT recovery defects were detected with the selected testing days: delayed PLT recovery and PLT overshoot. Post-screen analysis show 3 delayed PLT recovery outliers and 10 PLT overshoot outliers. Because interest in PLT recovery didn’t occur until later in the screening process, only 3 outliers were chosen for further analysis: high resting PLT but normal PLT recovery (7323), delayed PLT recovery (7324), and increased PLT overshoot on D13 (7325).

The 7323 line showed high PLT baseline values, but had no detectable differences in PLT recovery following 5FU treatment. The high baseline PLT phenotype was heritable at low
penetrance (30%) and mapped to the distal end of chromosome 11 (23Mb). The high PLT phenotype was seen predominantly in males, but did not show linkage to the X chromosome. The chosen testing days were not optimized for observing the PLT overshoot, and it is therefore possible that a higher PLT overshoot occurred in the 7323 line. If 7323 has normal 5FU recovery, it suggests that the mutation does not affect thrombopoiesis and could be caused by an extended PLT half-life or be a secondary to the primary phenotype.

The 7324 line was one out of three outliers for delayed PLT recovery; unfortunately the G1 mice had the least robust phenotype. The PLT defect in 7324 was heritable and was mapped to multiple loci in the genome scan. Interestingly, the same percentage of G2 mice showed high PLT at D8 as low PLTs at D8, suggesting multiple mutations may have been affecting the same pathway in the original G1 mouse. In fact, mapping to a single loci or a group of loci failed to occur after the third generation, suggesting that that were identified indicated that the phenotype in 7324 may have been caused by more than one mutation. Continued backcrossing of the 7324 line could have resulted in segregation of loci that were unrelated to the original phenotype or could have suggested that the original phenotype was a false positive in the screen.

The 7325 line was an outlier for an increased PLT overshoot on D13 and was one of ten G1 mice to display a similar phenotype. This trait in the 7325 line was heritable with low penetrance and mapped to the distal 45Mb region on chromosome 11. Although low penetrance was seen in this line, the false positive rate was minimal. Mapping in subsequent generations revealed that the defect in 7325 maps to a 25.7 Mb region on chromosome 11 and suggests that the defects in 7323 (105.3 Mb and 115.8 Mb) and 7325 (73.1Mb to 98.7) are caused by mutations in the same loci. Increased PLT overshoot on D13 could indicate that the PLT recovery/overshoot is delayed or that PLT overshoot is higher overall, and therefore, the return to
baseline PLT levels takes longer. Since the mechanisms for PLT overshoot are not well understood, the causative mutations for 7325 may provide important insight into 5FU-induced PLT recovery kinetics and megakaryopoiesis.

The 7321 line was a multilineage outlier and was derived from the one that was G1 defective in multiple parameters. The phenotypes for the 7321 line included delayed RBC recovery, increased PLT overshoot and increased WBC overshoot. When 7321 was bred to test heritability, all of the phenotypes were heritable, but not together. The complex inheritance was the principal reason why this line was not examined further.

Overall, the current 5FU screening protocol within the 129/B6 ENU screen was successful at detecting PLT recovery mutants, as it identified 13 G1 outliers and therefore potentially identified 13 novel mutations. The PLT phenotypes were easy to follow through generations of backcrossing, because PLT recovery was consistent between mouse strains. These mutants have the potential to improve our understanding of MK development and PLT shedding and well as the mechanisms that control stress thrombopoiesis. 5FU was not effective at detecting multi-lineage and erythropoietic phenotypes. 5FU could be used to effectively detect altered RBC recovery or defects in multiple lineages recovery in an ENU, if the following changes where implemented: mouse strain (Balb/c and B6), 5FU dose, or the hematological analyzer (WBC differential).
Conclusions and Future Directions

5.1 Overview

The Centre for Modeling Human Disease is multidisciplinary collaboration of investigators whose goals are to functionally annotate the genome and to develop new mouse models of human disease. ENU-induced random mutagenesis was a method employed by the CMHD to perform a forward genetics screen in order to identify dominant mutations that cause defects in hematopoiesis, the cardiovascular system and bone mineralization. In the dominant ENU screen, G1 mice were screened with multiple phenotyping tools including CBC, electrocardiogram, and a bone mineral densitometer.

Our laboratory is part of the CMHD, and our objective was to generate new mouse models of human hematopoietic disease that could be used to dissect the molecular and cellular causes of hematopoietic diseases. In addition to examining CBC, which assesses steady state hematopoiesis (Dominant Screen), we sought to implement a pharmacologically sensitized screening protocol that would help to identify a unique group of mutations that result in defects in cycling of progenitors and or demonstrate defects in stress hematopoiesis (Sensitized Screen). The mutants could then be used to model human hematopoietic diseases or determine novel regulators of hematopoiesis. Potentially, these mouse models could also be used to discover new therapeutics.

This thesis summarizes three distinct studies that employed ENU mutagenesis to generate new mouse models of hematopoietic disease in order to identify novel regulators of hematopoiesis. The two mouse lines that were identified in the dominant screen presented with the initial phenotypes of high RBC, low MCV and low MCH (Ank17192 Chapter 2) and high
MPV (Myh9<sup>7238</sup> Chapter 3). In addition, the study summarized in Chapter 4, details the implementation and execution of a pharmacologically sensitized screen.

5.2 Identification and Characterization <i>Ank1<sup>7192</sup></i>

The <i>Ank1<sup>7192</sup></i> mutant mouse is a new model for studying HS and also for the investigation of ANK1 in RBC membrane architecture. The <i>Ank1<sup>7192</sup></i> mutation models a spectrum of HS disease, as homozygous mice have symptoms similar to severe HS and heterozygous mice have mild HS symptoms. The ENU-induced mutation in <i>Ank1<sup>7192</sup></i> mice, introduces a premature stop codon in exon 26, which results in the expression of a truncated protein that lacks the spectrin binding domain and the regulatory domain, and therefore is only partially functional. The <i>Ank1<sup>7192</sup></i> mouse complements two existing mouse models of HS, <i>nb</i> and <i>RBC2</i>. All three mouse lines have symptoms of severe forms of HS[255, 256]. The <i>nb/nb</i> mouse has existed for more than 40 years and has provided a wealth of information on the role of <i>Ank1</i> in RBC cytoskeletal structure and function, as well as insight into the pathogenesis of HS [255]. The <i>nb</i> mouse caries a spontaneous mutation that introduces a premature stop-codon within exon 36 of <i>Ank1</i>. This leads to the production of a truncated protein that lacks a regulatory domain, but is still functional [257]. The <i>RBC2</i> mouse was recently identified in an ENU screen and a point mutation was identified at the splice acceptor site for exon 41, which results in the destruction of Ank1 message by non-sense mediated decay. The <i>RBC2</i> mouse is an Ank1 null mouse line [256]. Of the two existing models, <i>Ank1<sup>7192</sup></i> most closely resembles the <i>RBC2</i> as both mutations result in Ank1 null mice, despite the differences in genetic lesions. Despite the different genetic backgrounds, both <i>RBC2</i> and <i>Ank1<sup>7192</sup></i> display: hemolysis, jaundice, extramedullary hematopoiesis and iron overload. Additionally, both <i>RBC2</i> and <i>Ank1<sup>7192</sup></i> embryos exhibit anemia <i>in utero</i> [256].
Previous studies have indicated the presence of a variety of ANK1 isoforms resulting from an array of alternatively spliced Ank1 transcripts, indicating a complex pattern of transcript modification is present. In particular, a rare set of alternatively spliced Ank1 transcripts have been identified, that are produced via alterative splicing between exons 41 and 42 [240, 315]. Our study has identified the presence of rare near-full length ANK1 isoforms in RBC from Ank17192/7192 mice (immunoblot), despite the presence of a premature stop that should terminate translation at exon 26, preventing its formation. The expression of these Ank1 isoforms in Ank17192/7192 mice requires a mechanism to circumvent the ENU-induced stop codon located in exon 26, which could be explained by either exon skipping or corrective read through. A preliminary transcript analysis used a PCR-based strategy to detect potential skipping of exon 26 in Ank17192 and WT mice, but was unsuccessful in detecting a transcript could explain the skipping of exon 26.

To investigate the mechanism that allowed the formation of this alternatively spliced Ank1 isoforms in Ank17192/7192 mice, a more rigorous transcript and protein analysis is required. A PCR-based strategy for the detection of alternatively spliced Ank1, is biased, because this most easily detects the predominant WT transcript and would not likely detect rare transcripts that encode the Ank1 isoforms. Instead, a northern blot analysis using probes that span the two proposed alternatively spliced exons, would be a non-biased approach and would more readily detect rare transcripts. To detect corrective read through, a PCR-based strategy could be used that would use primers designed to specifically amplify the exon 41/42 alternatively spliced transcripts and would then allow subsequent analysis of the sequence of exon 26. A question that remains unanswered is whether the Ank1 isoforms is produced in a quantity sufficient to enhance RBC survival. Confirmation of the Ank1 isoforms in Ank17192 mice would indicate that Ank17192 mice are in fact not Ank1 null mice and produce a near full length Ank1 isoforms that
may enhance RBC survival in Ank17192 mice. These ANK1 isoforms are not expressed, beyond a faint band on an immunoblot of RBC lysate from nb/nb mice. Production of a near full length ANK1 isoform in Ank17192 mice, would suggest a more complicated transcript splicing than previously known.

This study raises an interesting question as to whether one of these ANK1 isoforms is produced in RBC2 erythrocytes, particularly because the ENU mutation alters the splice acceptor of exon 41. No immunoblots were published that used the antibodies specific for ANK1 isoforms that would result from alternative splicing around exons 41 and 42.

5.3 Identification and Characterization of Myh97238

An ENU dominant screen identified the 7238 mouse line, which presented with macrothrombocytes and in homozygous mutants a more severe PLT phenotype macrothrombocytopenia. The causative mutation for Myh97238 mice was identified as a missense mutation in the tail domain (exon 30) of MYH9 (NMMIIA). Humans with a defect in this gene have a heterogeneous disorder called MYH9RD. The disorder is characterized by giant PLT, neutrophil inclusions and varied penetrance of Alport-like phenotypes (kidney defects, cataracts, hearing loss) [160]. Myh97238 is the first mouse model of MYH9RD. Myh97238 mice present with the hallmarks of MYH9RD, including giant PLT and neutrophil inclusions, but the manifestation of Alport phenotypes in Myh97238 mice has not been characterized.

Although MYH9RD was first described in the early twentieth century, little is known about its pathogenesis. Characterization of this disorder has been confounded by many factors including the low occurrence of the disorder, the benign nature of the disorder, the lack of biopsies of affected tissues and the lack of an appropriate animal model for the disease. Many questions regarding the pathogenesis of MYH9RD in humans remain unanswered and Myh97238
mouse provides a unique opportunity to study the development of MYH9RD. Ablation of MYH9 has demonstrated its importance in cell function, which includes: cellular contractility, focal adhesions, actin stress fiber organization and tail retraction [282-286, 289, 316, 317]. The role of Myh9 in pathogenesis of many of the phenotypes in MYH9RD, including neutrophil ultrastructure, kidney function, cataract formation and hearing loss, remains largely unknown.

Once the existence of the Alport -like phenotypes has been established in Myh97283 mice, this mouse model can be used to dissect the role of Myh9 in the pathogenesis many aspects of this disease. An important question that remains unanswered in MYH9RD is whether the location of the mutation (head, neck tail) influences the phenotypes, age of onset and severity of the clinical manifestations of MYH9RD, therefore determining the existence, age of onset and severity of Alport-like phenotypes of the tail mutation in Myh97238 mice could be informative on a phenotype genotype correlation.

Beyond MYH9RD, a more extensive study of Myh97238 will provide considerable insight into the function of MYH9 in tissues like the kidney, eye and ear. In particular, Myh97238 could be used to provide insight into the role of MYH9 in the kidney as it has recently been linked to another human disease non-diabetes-related chronic kidney disease in African Americans [307, 308, 318, 319]. It will be important to highlight any significant differences that exist between Myh97238/+ and Myh97238/7238 mice, as it has been hypothesized that the dominant-negative effects of heterozygous mutations in humans are responsible for the MYH9RD phenotype.

Myh9 has been identified as a negative regulator of proplatelet formation in a study of a lineage-restricted conditional knock down of Myh9 in the mouse [305]. Although the Myh9Δ mouse has provided insight into the role of MYH9 in thrombopoiesis, it may not appropriately model the defect in thrombopoiesis in MYH9RD, because it is a Myh9 amorphic allele (null) that
only produces NMMIIA in small quantities, and the genetic defect in MYH9RD is caused by a hypomorphic allele that codes for a dysfunctional protein [160]. Thrombopoeisis has not been well characterized in humans because bone marrow biopsies are rare MYH9RD patients, making the study of thrombopoiesis in MYH9RD difficult. Careful analysis of thrombopoiesis in \( MYH9^{7238} \) mice could provide considerable insight into the pathogenesis of the PLT defect in MYH9RD. Thorough characterization of MK ultrastructure by transmission electron microscopy, will allow us to determine if there are disturbances in the demarcation membrane system or peripheral zone, both of which are disrupted in \( Myh9^\Delta \) MK. Wild-type MYH9 is localized to the cytoplasm. Determination of the cellular localization of mutated MYH9 in megakaryocytes, by immunofluorecence, would indicate if the MYH9\(^{7238} \) is located in the proper location and therefore act as a dominant negative or hypomorph in \( Myh9^{7238/+} \) and \( Myh9^{7238/7238} \), or whether the dysfunctional protein is sequestered abnormally in the cell and therefore prevents the protein from performing its normal function, indicating that the \( Myh9^{7238} \) mouse would phenocopy the thrombopoiesis phenotype in \( Myh9^\Delta \) MK mice. Study of proplatelet formation in \( Myh9^{7238} \) megakaryocytes will help to characterize the impact of a dysfunctional MYH9 in megakaryocytes.

Analysis of PLT morphology is required to determine the ratio of giant to normal sized PLT in heterozygous and homozygous \( Myh9^{7238} \) mice. The distribution of MYH9 in the PLT of \( Myh9^{7238} \) mice is unknown. Although PLT dysfunction has been studied in \( MYH9^\Delta \) mice and in MYH9RD patients, documenting the severity of PLT dysfunction is an important component in the characterization of \( Myh9^{7238} \) MYH9RD mouse model. Our study has shown that PLT function in \( Myh9^{7238/7238} \) mice is significantly altered, whereas \( Myh9^{7238/+} \) mice have near normal PLT function as analyzed by plug formation, platelet aggregation and perfusion studies. Although we performed aggregation studies with multiple agonists (ADP, TRAP and thrombin),
we have not yet assessed collagen-induced aggregation of Myh\textsubscript{9}\textsuperscript{7238} PLT. The agonist collagen is the only agonist to show significant deficits aggregation in humans with MYH9RD. Major defects in PLT function are rare in MYH9RD patients; therefore, the milder aggregation phenotype seen in Myh\textsubscript{9}\textsuperscript{7238/+} and Myh\textsubscript{9}\textsuperscript{7238/7238} mice more closely resembles the human disease.

Myh9 is expressed in most cell types in the body, yet inclusions have only been documented in neutrophils and are not seen in PLT, lymphocytes or monocytes in patients with MYH9RD. Neutrophil inclusions are characterized by abnormally clustered NMMIIA in the cytoplasm (punctate), with fragments of rough endoplasmic reticulum and ribosomes. To understand the pathogenesis of neutrophil inclusions in Myh\textsubscript{9}\textsuperscript{7238} mice, it will be important to characterize the type and incidence of neutrophil inclusions through the use of immunofluorescence and transmission electron microscopy. Immunofluorescence will be performed on purified neutrophils, using an anti-Myh9 antibody to detect the number and size of inclusions per neutrophil and to determine the incidence neutrophils with inclusions. The ultrastructure of neutrophil inclusions will be characterized by transmission electron microscopy to determine the components and organization of the inclusion in Myh\textsubscript{9}\textsuperscript{7238} mice.

The role of MYH9 in the formation of presenile cataracts in MYH9RD patients is not known. The current study demonstrated that the incidence of cataracts was higher in an aged cohort of Myh\textsubscript{9}\textsuperscript{7238/+} and Myh\textsubscript{9}\textsuperscript{7238/7238} mice and no difference in the incidence of cataracts exist between the two groups. A more rigorous examination of cataract formation in Myh\textsubscript{9}\textsuperscript{7238} mice is required to characterize the role of MYH9 in cataract formation. A longitudinal study of Myh\textsubscript{9}\textsuperscript{7238} cataract formation would determine the age of onset and should include characterization of the cataracts by visual inspection and histological examination. Examination of cataracts in Myh\textsubscript{9}\textsuperscript{7238} mice should include a visual inspection of the lens of the eye with the
assistance of an opthalmoscope and histological examination to classify the type of cataracts detected in \textit{Myh}^{97238} mice.

Many myosins including Myosin I, Myosin IIIA, Myosin VI, Myosin VIIA, Myosin XVA, MYH9 and MYH14 are expressed in the inner ear and have been linked to hereditary hearing loss in humans[320]. Specifically, Myh9 is expressed embryonically within the cochlea and this expression continues until adulthood, where it is expressed in the stereocilia. The function of Myh9 expression in the ear is unknown. Hearing loss in MYH9RD patients with mutation in the tail region of MYH9 does not typically occur until older ages and is often subclinical. To establish whether Myh9^{97238} mice can serve as a model for hearing impairment a longitudinal assessment for hearing loss should be performed. Hearing loss can be assessed by an audiometer to determine auditory brain responses, which is a neurologic test of auditory brain stem function in response to auditory stimuli. It can be used to assess hearing at multiple sound frequencies. If a hearing deficit is detected in \textit{Myh}^{97238} mice, these mice could then be used to understand the pathogenesis of hearing loss in MYH9RD. For example, in depth examination of the cochlea could be performed, including morphology of cochlea (sectioned and stained with H and E) and localization of Myh9 in the mutants (immunostaining of cochlear sections).

Normally, MYH9 is widely expressed in the kidney with strongest expression within the podocytes of the glomerulus. MYH9 is believed to play an important actin-myosin contractile apparatus in the podocyte foot process. In MYH9RD, MYH9 is abnormally distributed within the mesangial cells, tubular cells, and podocytes [321]. It is not currently known whether Myh9^{97238} mice have defects in kidney function a more thorough examination is required. To address this, a longitudinal study of kidney function and morphology in the \textit{Myh}^{97238} mouse would be required. Kidney dysfunction could be assessed by measuring serum plasma levels,
proteinuria and or the hematuria. Morphologic analysis of the kidney could be conducted by H and E staining of kidney sections, along with a more detailed analysis of glomular structure by transmission electron microscopy. Additionally, immunohistochemistry could be performed on the kidney sections form WT, Myh\textsuperscript{7238/7238} and Myh\textsuperscript{7238/7238} to detect alterations in MYH9 distribution. Myh\textsuperscript{7238} mice are congenic on a B6 background, which is extremely resistant to kidney lesions, so examining mice on this background may require a stress test (endotoxemia by lipopolysaccharide treatment) to elicit a latent kidney phenotype in these mice. If no kidney abnormalities are observed in Myh\textsuperscript{7238} mice on the B6 background, Myh\textsuperscript{7238} could be bred onto a strain more susceptible to kidney phenotype such as 129, FVB and ICR.

5.4 Pharmacologically Senitized ENU Screen

The purpose of this study was to conduct a pharmacologically sensitized screen that would help in the identification of a unique group of ENU mutants that have defects in the cycling of progenitors and demonstrate defects in stress hematopoiesis. Specifically, we investigated three chemotherapeutic drugs 5FU, PHZ and HU, to determine their potential utility in the sensitized ENU-dominant screen and determined that both 5FU and PHZ would be effective. We also determined that HU was not a strong candidate for in an ENU screen, because it was a weak inducer of anemia and thrombocytopenia and exhibited strong inter-strain differences in leukopenia induction and subsequent recovery. 5FU was selected because it caused defects in multi- and single-lineage hematopoietic progenitors and therefore had the potential to reveal the greatest genes involved in hematopoiesis. When 5FU was combined with the ENU mutagenesis screen, it was the most powerful at detecting mutations related to thrombopoiesis (13 G1 PLT recovery outliers). PHZ is a strong candidate for a future ENU screen as it robustly induces stress erythropoiesis and exhibits little inter-strain variation.
The results from this study have provided the foundation for several subsequent studies. The first series of studies would be to fully characterize the three mouse lines that were established from the 5FU-sensitized screen: 7304, delayed RBC phenotype; 7323, high baseline PLT and normal 5FU recovery phenotype; and 7325, increased PLT overshoot phenotype. Each of these lines, were established from a G1 outlier that successfully underwent heritability testing, and a genome scan was performed to establish the chromosomal location for the causative mutation.

The causative mutations remain to be cloned for the following lines: 7304, 7323 and 7325. To identify the causative mutation, the mapping interval must be refined from the rough mapping position provided by the genome scan by fine mapping. Fine mapping will identify new informative chromosomal crossovers that occur in mice arising from mouse lines serially backcrossed to the C57Bl/6J background. Mice presenting with novel crossovers will be bred to the C57Bl6 background to confirm that the affected offspring carry the same cross-over detected in the parent. Once the mapping interval has been reduced to 5Mb or less, it will be possible to investigate individual genes for the causative mutation. A candidate gene approach can be employed, which examines all of the genes within the mapping interval for known relation to original phenotype, expression in hematopoietic tissue or link to human hematopoietic disease. The majority of ENU mutations have been identified in the coding regions of genes, which are highly conserved and therefore the exons and intron/exon boundaries of candidate genes will be sequenced to identify novel SNP. The transcript of strong candidate genes can be examined for alterations in splicing or expression. If the candidate gene approach has been exhausted, the subsequent strategy would greatly depend on the size of the interval and density of genes within the given mapping interval. For example, if the mapping interval only contained 10 genes, then gene-by-gene sequencing strategy would be the most cost effective strategy to find the causative
mutation. On the other hand, if the mapping interval is small (5Mb or less), but gene rich and no candidate genes remain, then the most cost effective strategy would be to sequencing the entire region by massive parallel sequencing.

The dominant screen detected mice heterozygous for the ENU-induced mutations (ENU/+) but has not investigated mice homozygous for ENU mutations (ENU/ENU). It will be important to determine in each of the 5FU lines whether ENU/ENU mice are viable and to determine whether these mice exhibit a more profound phenotype than ENU/+. To accomplish this, an intercross will be performed, by breeding two affected mice heterozygous for the mapping region. The offspring will then be genotyped at weaning, by SNP analysis to determine if any ENU/ENU pups survive to three weeks of age. If ENU/ENU mice are detected at weaning, an initial CBC will be performed at 5wks to determine whether ENU/ENU mice have a stronger baseline phenotype, and the mice will then be challenged with 5FU to determine whether stress hematopoiesis is affected more profoundly. If no ENU/ENU mice are detected at weaning, it is possible that they might be dying neonatally or in utero. Timed matings will be performed, pups will be sacrificed immediately after birth, and DNA will be isolated from the tissue samples. Neonates from the intercross heterozygous breeding will be genotyped to detect if homozygous pups survive to birth. If no ENU/ENU pups are detected at birth, timed matings will be performed and embryos will be collected at E9.5, E12.5 and E17.5.

Until the causative mutation for each of the 5FU lines is identified, a broad initial assessment of hematopoiesis can be performed under conditions of steady state and stress, to define the mechanism responsible for the altered hematopoietic recovery following 5FU. Further characterization of each 5FU outlier can further define the initial phenotype and identify new lineages altered by the mutation, and therefore aiding in identifying candidate genes. An initial
assessment of hematopoiesis in each of the mouse lines can also assist identifying candidate
genes for further analysis. The hematopoietic system will be examined for defects in steady state
hematopoiesis to determine if the underlining cause of the 5FU phenotype is the result of altered
HSC, progenitor or mature blood cell number. In addition, hematopoietic lineages not previously
assessed will be examined to determine if other hematopoietic lineages are affected by the
causative mutation. This analysis will be performed on both the bone marrow and spleen, as both
organs are critical in 5FU-induced stress hematopoiesis. Phenotypic characterization of these
mutants will begin with the assessment of stem cells by flow cytometry to analyze KTLS
frequency which represents stem cells and early hematopoietic progenitor frequency. Colony
assays (CFU-C, CFU-Mk, and CFU-E) will be performed to examine hematopoietic cells of
various lineages. The analysis of mature blood cells from multiple lineages will be examined by
flow cytometry. If steady state hematopoiesis is normal, then assessment of stress hematopoiesis
will be performed, by examining the changes in HSC and progenitor frequency after the
administration of 5FU.

Although a general assessment of hematopoiesis will be imperative to define the
mechanism responsible for the 5FU phenotype, specialized testing for defects in the lineage that
is altered in the 5FU test will be critical. The phenotype of delayed RBC recovery (7304) is most
likely the result of a defect in the erythroid lineage. Characterization of erythroid precursors,
beyond CFU-E and BFU-E will include analysis of the expression of erythroid markers CD71
and Ter119 by flow cytometry. Testing 7304 mice with PHZ will help to determine if the
causative mutation alters both stress-induced hematopoietic pathways.

The phenotypes in observed in the PLT mutants, 7323 and 7325, can likely be attributed
to defects in megakaryopoiesis or thrombopoiesis. A characterization of megakaryocytes will be
conducted at steady state to determine frequency, DNA content and morphology of megakaryocytes in the mutants. 7325 mice have a PLT stress phenotype; therefore an assessment of megakaryocytes will be conducted under stress conditions in addition to steady state. Altered PLT number is often accompanied with impaired PLT function; therefore, PLT function will be assessed *in vivo* (tail bleeding) and *in vitro* (PLT aggregation).

The 5FU screening protocol developed in this study, was the most effective at detecting delayed PLT recovery mutants and increased PLT overshoot mutants; therefore, it would be intriguing to investigate these PLT phenotypes further. As part of the CMHD ENU screening protocol, sperm from all G1 male mice was cryopreserved and archived making it possible to re-establish ENU lines by *in vitro* fertilization. In total, 13 G1 mice with altered 5FU PLT recovery were identified in our screen. It is possible to revive 6 new G1 outliers (1 delayed PLT recovery and 5 increased PLT overshoot), because 6 of the G1 outliers were male and therefore had sperm archived.

Our study has demonstrated that PHZ is a strong candidate to be used for a pharmacologically sensitized screen. A successful sensitized ENU screen requires a screening tool to maintain the viability, while consistently producing a robust phenotype that can be easily measured. Depending on the strains of mice selected to generate the G1 hybrid strain, a dose of PHZ will be selected that will preserve viability, while maintaining the maximal robust induction of anemia. Once an appropriate dose of PHZ has been selected, it will be used to characterize the PHZ RBC recovery curve created with G1 hybrid control mice, in order to select the optimal testing days to assess delayed RBC recovery. The optimized PHZ testing protocol can be implemented into ENU-dominant screen and a cohort of G1 mice will be tested.
This thesis describes a series of studies that utilized ENU mutagenesis to investigate hematopoiesis at steady state (Dominant Screen) and under stress (5FU screen) to identify novel hematopoietic mutations. The dominant screen employed a CBC to detect outliers in following parameters: RBC number, RBC parameters (Hgb, MCV, MCH and MCHC), PLT number and WBC number. Eighty-eight G1 outliers were identified in the G1 dominant screen, from a total of 3392 G1 tested. The dominant screen was most effective at detecting defects in RBC size and Hgb concentration (34% of outliers MCV and MCH). Of the 88 G1 outliers, three were kept for subsequent analysis: *Ank1*<sup>7192</sup>, 1 high RBC, low MCV and MCH; *Myh9*<sup>7238</sup>, Low PLT/ High MPV; and *Jak2*<sup>7254</sup> High PLT). The ENU dominant screen successfully produced two novel mouse models of human disease, *Ank1*<sup>7192</sup> for HS and *Myh9*<sup>7238</sup>, which is the first mouse model of MYH9RD. The *Jak2*<sup>7254</sup> mutation is a novel mutation in *Jak2* that results in a null allele and has identified a novel PLT phenotype in a loss of function *Jak2* allele.

The 5FU screen represents the first pharmacologically sensitized screen designed to detect hematopoietic phenotypes. This study has successfully identified 29 G1 outliers for defects in 5FU recovery including: RBC delayed recovery, PLT delayed recovery, increased PLT overshoot, WBC delayed recovery and multi-lineage recovery defects. We have demonstrated that 5FU is the most successful at identifying PLT recovery mutants. Three 5FU lines have been established from the G1 screen (7304, 7323 and 7325) and potentially represent three novel hematopoietic mutations. The 5FU screen has also identified an additional six 5FU PLT recovery G1 mice could be used to establish up to six more 5FU lines. Additionally, we have shown that PHZ is an excellent candidate for a new pharmacologically sensitized screen.
References


300. Nyitray, L., E.B. Goodwin, and A.G. Szent-Gyorgyi, Complete primary structure of a scallop striated muscle myosin heavy chain. Sequence comparison with other heavy


### 6.1 Statistical Analysis from Chapter 4

#### Table 6-1. Statistical Analysis of 5FU Recovery Kinetics

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Dose Parameter</th>
<th>RBC</th>
<th>PLT</th>
<th>WBC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Two-way repeat measures ANOVA</td>
<td>p&lt;0.0001</td>
<td>P&lt;0.0001</td>
<td>p=0.001</td>
<td></td>
</tr>
<tr>
<td>Variance</td>
<td></td>
<td>12.9%</td>
<td>5.5%</td>
<td>1.9%</td>
</tr>
<tr>
<td>Significant?</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Two-way repeat measures ANOVA pairwise analysis (p&lt;.05): Bonferroni Post-test (p&lt;.05)</td>
<td>150 vs 120 Yes: (D6, 8, 16), 150 vs 100 Yes:(D6-14), 120 vs 100 Yes:(D10-16)</td>
<td>150 vs 120 Yes: (D8, 12-16, 150 vs 100 Yes:(D8-14), 150 vs 100 Yes:(D12-14)</td>
<td>150 vs 120 Yes: (D8-10)</td>
<td></td>
</tr>
<tr>
<td>Effective Dose - One way ANOVA 5FU Dose ug/g (p-value)</td>
<td>150(p&lt;0.0001), 120 (p&lt;0.0001), 100 (p&lt;0.0001)</td>
<td>150(p&lt;0.0001), 120 (p&lt;0.0001), 100 (p&lt;0.0001)</td>
<td>150(p&lt;0.0001), 120 (p&lt;0.0001), 100 (p&lt;0.0001)</td>
<td></td>
</tr>
<tr>
<td>Sample Size</td>
<td>150 n=5, 120 n=6, 100 n=10</td>
<td>150 n=5, 120 n=7, 100 n=10</td>
<td>150 n=5, 120 n=7, 100 n=10</td>
<td></td>
</tr>
<tr>
<td>Days Included in Analysis</td>
<td>D0, 2, 4, 6, 8, 10, 12, 14, 16</td>
<td>D0, 2, 4, 6, 8, 10, 12, 14, 16</td>
<td>D0, 2, 4, 6, 8, 10, 12, 14, 16</td>
<td></td>
</tr>
</tbody>
</table>
Table 6-2. Statistical Analysis of PHZ Recovery Curves

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Parameter</th>
<th>RBC</th>
<th>PLT</th>
<th>WBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two-way repeat measures ANOVA</td>
<td>p=0.415</td>
<td>p=0.069</td>
<td>p&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Variance</td>
<td>0.32%</td>
<td>2.10%</td>
<td>0.60%</td>
<td></td>
</tr>
<tr>
<td>Significant ?</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Two-way repeat measures Anova</td>
<td>B6 vs Balb/c</td>
<td>No:(D6), B6 vs 129</td>
<td>B6 vs Balb/c No:(All -NS), B6 vs 129</td>
<td></td>
</tr>
<tr>
<td>Pairwise Analysis</td>
<td>No:(D2), Balb/c vs 129</td>
<td>No:(D2, 4, 6, 11), Balb/c vs 129</td>
<td>Yes:(D1-4), Balb/c vs 129 vs 129 Yes(D1, 3-6)</td>
<td></td>
</tr>
<tr>
<td>Post-test (p&lt;.05)</td>
<td>129 No:(D2,6)</td>
<td>No(D2,6)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Effective Dose - One way anova</td>
<td>B6 (p&lt;0.0001), Balb/c (p&lt;0.0001), 129 (p&lt;0.0001)</td>
<td>B6 (p&lt;0.02), Balb/c (p&lt;0.007), 129 (p&lt;0.007)</td>
<td>B6 (p&lt;0.0001), Balb/c (p&lt;0.0001), 129 (p&lt;0.0001)</td>
<td></td>
</tr>
<tr>
<td>Strain (p-value)</td>
<td>B6 n=5, Balb/c n=5, 129 n=5</td>
<td>B6 n=5, Balb/c n=5, 129 n=6</td>
<td>B6 n=5, Balb/c n=5, 129 n=7</td>
<td></td>
</tr>
<tr>
<td>Sample Size</td>
<td>D0-14, except D5</td>
<td>D0-14, except D6</td>
<td>D0-14, except D7</td>
<td></td>
</tr>
</tbody>
</table>
Table 6-3. Statistical Analysis of Low Dose HU Recovery Kinetics

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>HU Low Dose</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>HU</td>
<td>RBC</td>
<td>PLT</td>
<td>WBC</td>
</tr>
<tr>
<td>Effective Dose - One way anova</td>
<td>0.5(p=0.28),</td>
<td>0.5(p&lt;0.06),</td>
<td>0.5(p=0.51),</td>
</tr>
<tr>
<td></td>
<td>0.5x2 (p=0.04),</td>
<td>0.5x2 (p&lt;0.03),</td>
<td>0.5x2 (p=0.1),</td>
</tr>
<tr>
<td></td>
<td>0.9(p=0.56)</td>
<td>0.9(p=0.61)</td>
<td>0.9(p&lt;0.007)</td>
</tr>
<tr>
<td>Sample Size</td>
<td>n=10</td>
<td>n=10</td>
<td>n=10</td>
</tr>
<tr>
<td></td>
<td>n=10</td>
<td>n=9</td>
<td>n=10</td>
</tr>
<tr>
<td>Days Included in Analysis</td>
<td>D0, 2, 5, 8, 11</td>
<td>D0, 2, 5, 8, 11</td>
<td>D0, 2, 5, 8, 11</td>
</tr>
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</table>
Table 6-4. Statistical Analysis of High Dose HU Recovery Curve

<table>
<thead>
<tr>
<th>Factor</th>
<th>High Dose HU</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RBC</td>
</tr>
<tr>
<td>Two-way repeat measures ANOVA</td>
<td>p=0.87</td>
</tr>
<tr>
<td>Variance</td>
<td>0.90%</td>
</tr>
<tr>
<td>Significant?</td>
<td>No</td>
</tr>
<tr>
<td>Two-way repeat measures ANOVA</td>
<td>All NS: All time points</td>
</tr>
<tr>
<td>Pairwise analysis (p&lt;.05): Bonferroni Post-test (p&lt;.05)</td>
<td>3x1 (p=0.50), 3x2 (p=0.66), 4x1 (p=0.62), 4x2 (p=0.41)</td>
</tr>
<tr>
<td>Effective Dose - One way anova</td>
<td>3x1 n=5, 3x2 n=5, 4x1 n=5, 4x2 n=5</td>
</tr>
<tr>
<td>Sample Size</td>
<td>D0, 2, 6, 9</td>
</tr>
<tr>
<td>Days Included in Analysis</td>
<td>D0, 2, 6, 9</td>
</tr>
</tbody>
</table>
Table 6-5. Statistical Analysis of Strain Comparison of HU Recovery Curves

<table>
<thead>
<tr>
<th>Factor</th>
<th>RBC</th>
<th>PLT</th>
<th>WBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Two-way repeat measures ANOVA</td>
<td>p&lt;0.0001</td>
<td>p&lt;0.0001</td>
<td>p&lt;0.02</td>
</tr>
<tr>
<td>Variance</td>
<td>16%</td>
<td>9.2%</td>
<td>8.0%</td>
</tr>
<tr>
<td>Significant ?</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Two-way repeat measures Anova</td>
<td>B6 vs Balb/c</td>
<td>Yes.(D2,10),</td>
<td>B6 vs Balb/c</td>
</tr>
<tr>
<td>Pairwise analysis (p&lt;.05): Bonferroni</td>
<td>Yes.(D10,12), B6 vs 129</td>
<td>Yes.(D0,2,6,12), vs 129 No:(D4-12),</td>
<td></td>
</tr>
<tr>
<td>Post-test (p&lt;.05)</td>
<td>Yes:(D2,10,12)</td>
<td>No:(D6,12),</td>
<td>No:(D0,6,8)</td>
</tr>
<tr>
<td>Effective Dose - One way anova</td>
<td>B6 (p&lt;0.02), Balb/c</td>
<td>B6 (p=0.0001), Balb/c (p&lt;0.0001),</td>
<td>B6 (p&lt;0.0001), Balb/c (p&lt;0.0001),</td>
</tr>
<tr>
<td>Sample Size</td>
<td>B6 n=10, Balb/c</td>
<td>B6 n=10, Balb/c</td>
<td>B6 n=10, Balb/c</td>
</tr>
<tr>
<td>Days Included in Analysis</td>
<td>D0,2,4,6,8,10,12</td>
<td>D0,2,4,6,8,10,12</td>
<td>D0,2,4,6,8,10,12</td>
</tr>
</tbody>
</table>
Table 6-6. Summary of KTLS Population Over 2 Week Time Course Subsequent to Cytopenia Induction

<table>
<thead>
<tr>
<th>Cohort 1 18hrs</th>
<th>PBS</th>
<th>SD</th>
<th>5FU</th>
<th>SD</th>
<th>PHZ</th>
<th>SD</th>
<th>HU</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>D7</td>
<td>1.46</td>
<td>0.05</td>
<td>7.61</td>
<td>3.55</td>
<td>8.37</td>
<td>3.48</td>
<td>3.23</td>
<td>0.80</td>
</tr>
<tr>
<td>D14</td>
<td>2.06</td>
<td>0.19</td>
<td>2.48</td>
<td>0.66</td>
<td>2.02</td>
<td>0.26</td>
<td>1.41</td>
<td>0.12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cohort 2 18hrs</th>
<th>PBS</th>
<th>SD</th>
<th>5FU</th>
<th>SD</th>
<th>PHZ</th>
<th>SD</th>
<th>HU</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>D7</td>
<td>1.14</td>
<td>0.21</td>
<td>3.80</td>
<td>0.45</td>
<td>2.23</td>
<td>0.61</td>
<td>1.05</td>
<td>0.22</td>
</tr>
<tr>
<td>D14</td>
<td>0.52</td>
<td>0.22</td>
<td>0.28</td>
<td>0.10</td>
<td>0.55</td>
<td>0.27</td>
<td>0.19</td>
<td>0.19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cohort 3 18hrs</th>
<th>PBS</th>
<th>SD</th>
<th>5FU</th>
<th>SD</th>
<th>PHZ</th>
<th>SD</th>
<th>HU</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>D7</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>D14</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

The values represent the mean for each cohort. A t-test was performed comparing treated group (5FU, PHZ or HU) versus control group (PBS) and trends (0.1< p <0.05) are represented by ~ and statistically significant values (p<0.05) are signified by *.

Table 6-7. Summary of c-kit high TLS Population 18hrs Post 5FU Treatment

<table>
<thead>
<tr>
<th>Cohort 18hrs</th>
<th>PBS</th>
<th>SD</th>
<th>5FU</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.043</td>
<td>0.011</td>
<td>0.011*</td>
<td>0.011</td>
<td></td>
</tr>
<tr>
<td>Cohort 18hrs</td>
<td>PBS</td>
<td>SD</td>
<td>5FU</td>
<td>SD</td>
</tr>
<tr>
<td>0.049</td>
<td>0.004</td>
<td>0.006*</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>Cohort 18hrs</td>
<td>PBS</td>
<td>SD</td>
<td>5FU</td>
<td>SD</td>
</tr>
<tr>
<td>0.099</td>
<td>0.015</td>
<td>0.028~</td>
<td>0.040</td>
<td></td>
</tr>
</tbody>
</table>

The values represent the mean for each cohort. A t-test was performed comparing treated group (5FU) versus control group (PBS) and trends (0.1< p <0.05) are represented by ~ and statistically significant values (p<0.05) are signified by *.
Table 6-8. Statistical Analysis of Bone Marrow CFU-C Frequency

<table>
<thead>
<tr>
<th>Drug</th>
<th>Frequency</th>
<th>One-way Anova</th>
<th>Significant?</th>
<th>Bonferroni’s Post-Test p&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>HU</td>
<td>CFU-E</td>
<td>p&lt;0.005</td>
<td>Yes</td>
<td>D0 vs D7</td>
</tr>
<tr>
<td></td>
<td>CFU-C</td>
<td>p=0.25</td>
<td>No</td>
<td>D0 vs D14, 18hrs vs D7, 18hrs vs D14</td>
</tr>
<tr>
<td></td>
<td>CFU-G</td>
<td>p&lt;0.02</td>
<td>Yes</td>
<td>D0 vs D14, 18hrs vs D14</td>
</tr>
<tr>
<td></td>
<td>CFU-M</td>
<td>p=0.55</td>
<td>No</td>
<td>All NS</td>
</tr>
<tr>
<td></td>
<td>CFU-GEMM</td>
<td>p=0.30</td>
<td>No</td>
<td>All NS</td>
</tr>
<tr>
<td></td>
<td>CFU-GM</td>
<td>p=0.34</td>
<td>No</td>
<td>All NS</td>
</tr>
<tr>
<td></td>
<td>BFU-E</td>
<td>p=0.18</td>
<td>No</td>
<td>All NS</td>
</tr>
<tr>
<td>5FU</td>
<td>CFU-E</td>
<td>p=0.0001</td>
<td>Yes</td>
<td>D0 vs18hrs, 18hrs vs D7, 18hrs vs D14</td>
</tr>
<tr>
<td></td>
<td>CFU-C</td>
<td>p&lt;0.0001</td>
<td>Yes</td>
<td>D0 vs18hrs, D0 vs D7, D0 vs D14, 18hrs vs D7, 18hrs vs D14, D7 vs D14</td>
</tr>
<tr>
<td></td>
<td>CFU-G</td>
<td>p&lt;0.0001</td>
<td>Yes</td>
<td>D7, 18hrs vs D14</td>
</tr>
<tr>
<td></td>
<td>CFU-M</td>
<td>p&lt;0.0001</td>
<td>Yes</td>
<td>D14, D7 vs D14</td>
</tr>
<tr>
<td></td>
<td>CFU-GEMM</td>
<td>p&lt;0.0001</td>
<td>Yes</td>
<td>D14, D7 vs D14</td>
</tr>
<tr>
<td></td>
<td>CFU-GM</td>
<td>p&lt;0.0001</td>
<td>Yes</td>
<td>D0 vs D7, D0 vs D14, 18hrs vs D7, 18hrs vs D14, D7 vs D14</td>
</tr>
<tr>
<td></td>
<td>BFU-E</td>
<td>p&lt;0.03</td>
<td>Yes</td>
<td>All NS</td>
</tr>
<tr>
<td>PHZ</td>
<td>CFU-E</td>
<td>p&lt;0.002</td>
<td>Yes</td>
<td>D0 vs 18hrs, 18hrs vs D7, 18hrs vs D14</td>
</tr>
<tr>
<td></td>
<td>CFU-C</td>
<td>p=0.08</td>
<td>No</td>
<td>All NS</td>
</tr>
<tr>
<td></td>
<td>CFU-G</td>
<td>p=0.08</td>
<td>No</td>
<td>All NS</td>
</tr>
<tr>
<td></td>
<td>CFU-M</td>
<td>p=0.10</td>
<td>No</td>
<td>All NS</td>
</tr>
<tr>
<td></td>
<td>CFU-GEMM</td>
<td>p=0.49</td>
<td>No</td>
<td>All NS</td>
</tr>
<tr>
<td></td>
<td>CFU-GM</td>
<td>p&lt;0.02</td>
<td>Yes</td>
<td>D0 vs D7</td>
</tr>
<tr>
<td></td>
<td>BFU-E</td>
<td>p=0.82</td>
<td>No</td>
<td>All NS</td>
</tr>
</tbody>
</table>
Table 6-9. Statistical Analysis of Bone Marrow of CFU-C per Femur.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Per Femur</th>
<th>One-way Anova</th>
<th>Significant?</th>
<th>Bonferroni’s Post-Test p&lt;0.05</th>
<th>Yes, p&gt;0.05</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>HU</td>
<td>Cellularity</td>
<td>p&lt;0.0001</td>
<td>Yes</td>
<td>D0 vs 18 hrs, 18 hrs vs D7, 18 hrs vs D14, D7 vs D14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFU-E</td>
<td>p&lt;0.0001</td>
<td>Yes</td>
<td>D0 vs 18 hrs, D0 vs D7, 18 hrs vs D14, D7 vs D14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFU-C</td>
<td>p&lt;0.0001</td>
<td>Yes</td>
<td>D0 vs 18 hrs, D0 vs D14, 18 hrs vs D7, 18 hrs vs D14, D7 vs D14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFU-G</td>
<td>p&lt;0.0001</td>
<td>Yes</td>
<td>D0 vs 18 hrs, D0 vs D14, 18 hrs vs D7, 18 hrs vs D14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFU-M</td>
<td>p=0.01</td>
<td>Yes</td>
<td>18 hrs vs D7, 18 hrs vs D14</td>
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<tr>
<td>CFU-GEMM</td>
<td>p&lt;0.04</td>
<td>Yes</td>
<td>18 hrs vs D14</td>
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<tr>
<td>CFU-GM</td>
<td>p=0.0003</td>
<td>Yes</td>
<td>18 hrs vs D7, 18 hrs vs D14</td>
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<tr>
<td>BFU-E</td>
<td>p&lt;0.02</td>
<td>Yes</td>
<td>18 hrs vs D14</td>
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Table 6-10. Statistical Analysis of Splenic Extramedullary hematopoiesis

<table>
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<tr>
<th>Drug</th>
<th>Frequency</th>
<th>One-way Anova</th>
<th>Significant?</th>
<th>Bonferroni’s Post-Test p&lt;0.05</th>
<th>Yes, p&gt;0.05</th>
<th>No</th>
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<tr>
<td>HU</td>
<td>CFU-C</td>
<td>p&lt;0.02</td>
<td>Yes</td>
<td>D0 vs D7, D7 vs D14</td>
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<tr>
<td>Red</td>
<td>p=0.38</td>
<td>No</td>
<td>All NS</td>
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<tr>
<td>Non-Red</td>
<td>p&lt;0.006</td>
<td>Yes</td>
<td>D0 vs D7, 18 hrs vs D7, D7 vs D14</td>
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<tr>
<td>SFU</td>
<td>CFU-C</td>
<td>p&lt;0.0001</td>
<td>Yes</td>
<td>D0 vs D14, D18 vs D14, D7 vs D14</td>
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</tr>
<tr>
<td>Red</td>
<td>p&lt;0.0001</td>
<td>Yes</td>
<td>D0 vs 18 hrs, D0 vs D7, D18 vs D14, D7 vs D14</td>
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<tr>
<td>Non-Red</td>
<td>p&lt;0.0001</td>
<td>Yes</td>
<td>D0 vs D14, D18 vs D14, D7 vs D14</td>
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<tr>
<td>PHZ</td>
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<td>No</td>
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6.2 Cytopenia Induction in Known Hematopoietic Mutants

**Objective:** Compare and contrast the effectiveness of three chemotherapeutic drugs (5FU, PHZ and HU) on detecting stress hematopoiesis phenotypes in mice with known mutations (heterozygous) and to determine if each drug would be effective in a dominant ENU screen.

**Abstract:** Three known chemotherapeutic drugs 5FU, PHZ and HU (protocols developed in chapter 4) were used to induce stress hematopoiesis in mice carrying mutations in genes (c-kit, c-kitl, EpoR and Stat5a/b) known to be important hematopoiesis. In order to assess the potential of each drug to detect altered stress hematopoiesis phenotypes in a dominant ENU screen, only mice with heterozygous mutations were tested. 5FU was the most effective at eliciting stress-induced phenotypes in these mutants; in particular EpoR+/- mice had delayed RBC in compare to WT controls. PHZ induces stress erythropoiesis specifically, but could only detected mild alterations in stress erythropoiesis in the mutants tested. Finally, HU could only inconsistently induce slight alterations in WBC recovery in Kitl++/ mice. Interestingly, despite the baseline anemia in Kitl++/ mice, none of the drugs revealed an alteration in stress erythropoiesis in Kitl++/ mice.

**Introduction:** Erythropoietin (Epo) is the primary cytokine regulator of erythropoiesis, and its receptor EpoR is expressed on erythroid progenitors [322]. Epo and EpoR nulls die embryonically at D13 demonstrating that Epo signaling is critical for definitive erythropoiesis [322, 323]. No erythroid defects have been reported for EpoR heterozygous nulls. It has been revealed that Stat5 binding motif on EpoR is critical for mediating erythroid stress response in response to PHZ and 5FU treatment, as mice that lack this motif show delayed RBC recovery [324].
The transcription factor Stat5 belongs to the signal transducers and activators of transcription family and is activated by many hematopoietic cytokines including: Epo, Tpo, IL-3 and GM-CSF [325]. Stat5a/b nulls are viable and have normal RBC, hemoglobin, hematocrit and PLT levels [326], with lowered WBC counts attributed to a decreased peripheral T lymphocytes [327]. While adult erythropoiesis is normal in Stat5a/b nulls, Stat5 is critical in fetal erythropoiesis [328]. Stat5 signaling is also important in bone marrow repopulation in transplant studies [325, 326].

The tyrosine receptor kinase, c-kit, and its ligand, stem cell factor (SCF or c-Kitl), are critical in hematopoiesis, and a lack in expression of either leads to in utero or neonatal death. A large number of naturally occurring mutants in both SCF and c-Kit have been identified, and are typically easy to identify because of coat color defects. Homozygous mutants display complete loss of skin pigmentation, profound mast cell deficiency and hypoplastic microcytic anemia, and the most severe mutants die in utero or neonatally from anemia. On the other hand, heterozygous mutants typically have only have minor erythroid or mast cell defects [329, 330]. SCF and c-Kit are critical in recovery after hemolytic anemia induced by PHZ treatment [209, 331], and in particular in splenic hematopoiesis [209]. Also, SCF and c-Kit are critical in the PLT overshoot seen after 5FU treatment [200, 201].

6.2.1 Materials and Methods

Mouse Strains All mice in these experiments were 8-10 weeks of age. WB/ReJ Kit\textsuperscript{W/J} (Kit\textsuperscript{W}) and WC/ReJ Kit\textsuperscript{Sl/J} (Kit\textsuperscript{sl}) mice and age-matched controls were used at 8-20 weeks of age. Stat5a\textsuperscript{+/-}B\textsuperscript{+/-} and EpoR\textsuperscript{+/-} mice where bred and maintained in the animal care facility at Ontario Cancer Institute (Toronto, On, Canada). Stat5 and EpoR mice and C57BL/6J age matched controls were used at 12-30 wks of age. Except for Stat5 and EpoR mice, all mice were obtained
from The Jackson Laboratory (Bar Harbor, ME) and maintained in a conventional mouse facility at the Mount Sinai Hospital (Toronto, ON, Canada).

**Cytopenia Induction** 5-Fluorouracil (Sigma) was dissolved in PBS without Ca or Mg (Gibco) at 65°C for 15 min at a concentration of 15 mg/mL and then injected interperationally (IP) at a concentration of 120 µg/g. Phenylhydrazine (Sigma) was dissolved in PBS without Ca and Mg at a concentration of 20 mg/mL and then subcutaneously injected with 100 µg/g. Hydroxyurea (Sigma) was dissolved in PBS without Ca and Mg at a concentration of 133 µg/mL, vortexed until completely dissolved and then injected IP at 4µg/g once and then a second time four hours later.

**Hematologic Analysis** Peripheral blood (20 to 30 µL) from the saphenous vein was collected into EDTA (ethylenediaminetetraacetic acid)–coated capillary pipettes (Drummond) and transferred to (Eppendorf) tubes. Complete blood counts were performed by means of a Coulter Ac-T Differential Hematology Analyzer with veterinary software (Beckman-Coulter). All mice were bled on day 0 (D0) to obtain a baseline and then every 4 days, unless otherwise stated.

**6.2.2 Results**

Mice heterozygous for null mutations in genes involved in hematopoiesis and wild-type controls were both treated with pharmacological agents that induce cytopenia (HU, 5FU, and PHZ) and then the hematological recovery patterns were compared to test whether the mutant mice were more sensitive to the agents. The purpose of this series of experiments was to test whether this approach would likely allow for the detection of novel hemotopoietic genes in a subsequent ENU dominant screen.
Kit<sup+w/+</sup> mice had normal RBC recovery post 5FU treatment (p=0.16), despite the fact that RBC baseline values were lower in mutant mice (p<.05) (Figure 6.1A). The PLT recovery kinetics of Kit<sup+w/+</sup> mice were also not significantly different (p<.06), but Kit<sup+w/+</sup> mice did have an increased PLT overshoot on D10 (p<0.05), which returned to control levels on D14. Although the WBC recovery pattern of Kit<sup+w/+</sup> mice was not significantly different from controls (p<0.09), the WBC overshoot peaked earlier in Kit<sup+w/+</sup> mice (D10) and then returned to near baseline values on D14, whereas the overshoot in control mice increased until D14.

Kit<sup+w/+</sup> mice had a slight, yet statistically significantly, altered RBC recovery pattern (p<0.03) following PHZ treatment, although no specific day of the time course showed significant difference from the control mice (p>0.05, NS) (Figure 6.1B). The overall recovery kinetics for reticulocyte overshoot was significantly altered in Kit<sup+w/+</sup> mice (p<0.003), and the reticulocyte overshoot was not as high in Kit<sup+w/+</sup> mice (p<.05: D2 & 4).

Kit<sup+w/+</sup> mice had no consistent defect in RBC, PLT, or WBC recovery kinetics following HU treatment (data not shown).
KitlSl/+ mice

RBC recovery after 5FU treatment was significantly different in KitlSl/+ mice when compared to controls (p<0.0001), but the timing and degree of anemia of observed in the KitlSl/+ mice was highly similar to controls. (Figure 6.2A). No significant difference was observed in the PLT recovery in KitlSl/+ mice (p=0.35). The WBC recovery pattern in KitlSl/+ mice was significantly different from controls (p<0.02). The PLT recovery pattern of KitlSl/+ mice was similar to controls (p=0.35).

Although RBC recovery curves of PHZ-treated KitlSl/+ mice was significantly different from WT controls (p<0.0001), the pattern of recovery (timing and degree of anemia induced) is
similar in both Kit\textsuperscript{sl/+} and WT mice and the differences observed can be attributed to the fact that Kit\textsuperscript{sl/+} are anemic pre-PHZ treatment (Figure 6.2B). No statistical differences were observed in the overshoot in reticulocyte numbers found in of PHZ-treated Kit\textsuperscript{sl/+} mice when compared to control mice (data not shown).

RBC recovery after HU treatment was significantly different Kit\textsuperscript{sl/+} when compared to controls (A p<0.0001 and B p<0.005), but again the RBC recovery patterns of Kit\textsuperscript{sl/+} and controls were the same, and the RBC values for Kit\textsuperscript{sl/+} mice were lower at every time point (Figure 6.2C). Overall WBC recovery was significantly different or a trend in Kit\textsuperscript{sl/+} compared to controls (p<0.02, p=0.26). No significant difference was seen in PLT recovery (data not shown).
Figure 6-2. Cytopenia induction in Sl heterozygous mice.

A) The 5FU recovery kinetics of RBC and WBC (right and left panel). B) The PHZ recovery kinetics for RBC. C) The HU recovery kinetics for RBC and WBC (right and left).

EpoR^{+/-} mice

No significant differences in RBC baseline values were observed in EpoR^{+/-} and control mice, but the overall RBC recovery after 5FU-induced cytopenia was significantly different. (p=0.04) (Figure 6.3A). In control mice, RBC values began to return to baseline values on D11 after 5FU
treatment, but RBC values of EpoR\(^{+/−}\) mice continued to drop on D11 and do not begin to recover until D13. 5FU treatment of EpoR\(^{+/−}\) and WT mice led to significantly different PLT recovery patterns compared to WT mice (p<0.02). In addition, the PLT overshoot in EpoR\(^{+/−}\) mice was 25% higher than WT controls on D11 and D13 (p<0.05). As well, the WBC recovery pattern of EpoR\(^{+/−}\) mice was distinct from controls (p<0.05) with the WBC overshoot in EpoR\(^{+/−}\) mice being 30% higher than WT controls (p<0.05). Two weeks after 5FU treatment CFU-Es from EpoR\(^{+/−}\) mice were 67% lower than those from WT mice (T-test p<0.0005) (Figure 6.3B).

PHZ treatment did not consistently lead to significant changes in overall RBC recovery kinetics (p<0.02, p=0.22) (Figure 6.3C), but the RBC values on D11 were 10% lower in EpoR\(^{+/−}\) mice (t-test p<0.0005). The reticulocyte overshoot in EpoR\(^{+/−}\) mice was the same as controls (p=0.66).

Following HU treatment, there were no significant differences in RBC, PLT or WBC recovery kinetics between the two groups of mice (data not shown).
Figure 6-3. Cytopenia induction in EpoR heterozygous null mice

A) The 5FU recovery kinetics for RBC, PLT and WBC (left, centre and right panel). B) CFU-E assay performed after completion of 5FU time course experiment. C) RBC recovery pattern in EpoR\(^{+/−}\) mice (red squares) and WT controls (black circles) after PHZ-induced cytopenia.

**Stat5a\(^{+/−}\)b\(^{+/−}\) mice**

RBC recovery after 5FU treatment was unaltered in comparison to WT in Stat5a\(^{+/−}\)b\(^{+/−}\) mice (p=0.32) (Figure 6.4A). Although the overall PLT recovery kinetics following 5FU treatment was not significantly different in Stat5a\(^{+/−}\)b\(^{+/−}\) mice (p=0.15), there was a 9% greater overshoot in PLT at D13 in the Stat5a\(^{+/−}\)b\(^{+/−}\) mice (p<0.05). Additionally the WBC recovery kinetics were also altered in Stat a\(^{+/−}\)b\(^{+/−}\) mice (p<.0001), as WBC values for Stat5a\(^{+/−}\)b\(^{+/−}\) mice were consistently lower than WT controls, but the values were statistically significant only on D11 and D13. Two
weeks after 5FU treatment, the CFU-E frequency was 53% lower in Stat5a<sup>+/−</sup>b<sup>+/−</sup> mice compared to WT controls (t-test p<0.03) (Figure 6.4 B).

No differences in RBC recovery patterns were seen in the Stat5a<sup>+/−</sup>b<sup>+/−</sup> mice when compared to WT control mice after PHZ treatment (p=0.28) (Figure 6.4C). While the overall recovery kinetics for the reticulocyte overshoot was not significantly different (p=0.76), the reticulocyte numbers remained high on D7 in Stat5a<sup>+/−</sup>b<sup>+/−</sup> mice and did not return to baseline as quickly as the reticulocyte numbers in WT mice (p<0.05).

The recovery kinetics in RBC, PLT, or WBC after HU treatment was the same for Stat5a<sup>+/−</sup>b<sup>+/−</sup> mice and controls (data not shown).
Figure 6-4 Cytopenia induction in Stat5a\textsuperscript{−/−}/b\textsuperscript{+/+} mice.
A) The 5FU recovery kinetics for PLT and WBC (right and left panel). B) Results from the CFU-E assay that was performed at end of 5FU time course. C) The PHZ recovery kinetics for WBC/Ret.

6.2.3 Discussion

SCF (Kitl) is a known hematopoietic cytokine that binds to its receptor c-kit. Many naturally occurring mutations of both SCF and c-kit genes are known to exist, and the most severe mutations (homozygous) Sl/Sl and W/W lead to \textit{in utero} or perinatal death because of severe anemia. Viable mutants, such as Sl/Sld or W/Wv, have severe macrocytic anemia and defects in mast cells [330] and heterozygous mutations, Kit\textsuperscript{W} and Kitl\textsuperscript{Sl}, exhibit only minor RBC defects. Inhibition of SCF signaling, either with an antibody or by mutation (W/Wv or Sl/Sld), has demonstrated that SCF is critical in thrombocytosis following 5FU treatment and in splenic hematopoiesis following PHZ treatment [200, 201, 209].
Kit\textsuperscript{W} mutants when treated with the optimized 5FU protocol demonstrated no defects in RBC recovery, but did have a reduced WBC overshoot. The automated hemocytologic analyzer used does not allow for the grouping together of neutrophils or lymphocytes and immature RBC. Since neutrophils and lymphocytes do not overshoot baseline values in wild-type animals \cite{194}, but reticulocytes do overshoot, \cite{198} a decreased erythroid response in reticulocyte overshoot, may explain the WBC overshoot. The predicted PLT phenotype for Kit\textsuperscript{W/+} mice is a decreased PLT overshoot, given that SCF signaling is required for PLT overshoot. Opposite to what we predicted, Kit\textsuperscript{W/+} mice displayed a more rapid PLT recovery or an increased PLT overshoot. One possible explanation is that the loss of one allele of c-kit, led to increased SCF production or that the cells become more sensitive because of up regulation of other signaling proteins. The predicted phenotype for PHZ-treated Kit\textsuperscript{W} mice, is decreased a reticulocyte overshoot. When Kit\textsuperscript{W} mice were tested with the optimized PHZ protocol, a slight decrease in overall RBC and reticulocyte recovery were observed, which confirms the predicted phenotype.

Despite the importance of SCF in erythropoiesis, none of the optimized drug protocols altered RBC recovery in kit\textsuperscript{ld} mice compared to wild-type controls. It is also interesting that PLT recovery was unaltered in Kit\textsuperscript{ld} mice, as SCF signaling has been shown to be critical in PLT overshoot. One possible explanation for the normal recovery patterns of Kit\textsuperscript{ld} is gene dosage. The single wild-type allele may be able to compensate and make enough SCF for normal recovery to occur. Although SCF signaling was shown to be critical for PLT overshoot, the mechanism by which SCF works is unclear (ref). SCF signaling is important in hematopoietic cell homing and migration, in particular, for the movement of cells from the bone marrow to the spleen. The PLT overshoot is associated with increased megakaryopoiesis in the spleen, but is not essential as splenectomised mice still have a robust overshoot \cite{199}.
EpoR is the receptor for erythropoietin (Epo), the primary cytokine regulator of erythropoiesis. EpoR null mice die *in utero* at mid-gestation of severe anemia [322]. EpoR<sup>+/−</sup> mice have normal RBC and HCT levels and no hematopoietic defects have been published in these mice. EpoR<sup>+/−</sup> mice had delayed RBC recovery after 5FU and PHZ treatment, but the effect of 5FU treatment was more pronounced. Specifically, RBC values in EpoR<sup>+/−</sup> mice continued to fall after 5FU treatment even though RBC values had begun to recovery in wild-type controls. As well as delayed RBC recovery, EpoR<sup>+/−</sup> mice treated with 5FU had increased WBC and PLT overshoots. This was unexpected and may be due to homeostatic balance. One possible explanation for the prolonged decline in RBC numbers in R<sup>+/−</sup> mice is that more progenitors (CFU-E’s) are normally in cycle, and therefore, more progenitors are killed by 5FU. This may also explain why EpoR<sup>+/−</sup> mice don’t show a deficit in RBC number. Fourteen days after initial 5FU treatment, CFU-E colonies were significantly reduced in EpoR<sup>+/−</sup>, suggesting that even though RBC values of EpoR<sup>+/−</sup> mice are almost equivalent to WT, a severe deficit in erythroid progenitors still exists. The delay in RBC recovery following PHZ treatment was minor, yet significant, and no differences in reticulocyte recovery were detected with our PHZ recovery protocol. Not surprisingly treatment with HU did not elicit a defect in recovery of EpoR<sup>+/−</sup> animals, because HU is not an efficient inducer of anemia, although it does kill CFU-E efficiently.

Stat5 is a transcription factor downstream from several hematopoietic cytokine receptors including: erythropoietin, thrombopoietin, granulocyte macrophage colony-stimulating factor and granulocyte colony stimulating factor[327]. Stat5 is encoded by two highly related genes Stat5a and Stat5b. Stat5a/b compound nulls have near a normal hematocrit, but have been shown to have abnormal PHZ-induced stress erythropoiesis, demonstrating the critical role of Stat5a/b in early erythroblast survival [328, 330]. It is expected that Stat5a/b compound heterozygous nulls would have a decreased reticulocyte overshoot following drug treatment. No evidence
exists for such a defect in our experiments. In fact, reticulocyte recovery in Stat5a<sup>+</sup>/b<sup>+</sup> mice was sluggish in its return to baseline. Reticulocytes were not individually counted by our hematological analyzer and are detected in the WBC parameter, therefore, it is possible that differences in reticulocyte numbers or overshoot may exist that cannot be detected by our screening method. No 5FU recovery defect has previously been reported for Stat5a<sup>-/-</sup>/b<sup>-/-</sup> mice. The decreased WBC overshoot and decreased CFU-E in Stat5a<sup>+</sup>-/b<sup>+</sup>- mice after 5FU treatment that we observed, suggests a role for Stat5a/b in 5FU-related stress erythropoiesis. The defects in PLT overshoot, while minor, provide the first evidence that STAT5 may be important in stress thrombopoiesis. Taken together, the minor 5FU recovery defects seen in Stat5a<sup>+</sup>-/b<sup>+</sup>- mice, suggest a role for Stat5 in recovery from 5FU-induced stress hematopoiesis. Because of known redundant roles for the two isoforms of Stat5, it would be interesting to treat Stat5a/b null mice with 5FU to fully elucidate the role of Stat5 in stress hematopoiesis.

6.2.4 References


6.3 Copy of Jak2⁷²⁵⁴ paper submitted to Blood.

ENU MUTAGENESIS IDENTIFIES A NOVEL PLATELET PHENOTYPE IN A LOSS-OF-FUNCTION JAK2 ALLELE

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KEYWORDS: ENU, Dominant screen, JAK2

RUNNING TITLE: A Novel Platelet Phenotype in a LOF JAK2 Mutant

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ABSTRACT

Utilizing ENU mutagenesis, we identified a mutant mouse with elevated platelets. Genetic mapping localized the mutation to an interval on Chromosome 19 that encodes the Jak2 tyrosine kinase. We identified an A3056T mutation resulting in a premature stop codon within exon 19 of Jak2 (Jak2^{R915X}), resulting in a protein truncation and functionally inactive enzyme. This novel platelet phenotype was also observed in mice bearing a hemizygous targeted disruption of the Jak2 locus (Jak2^{−/+}). Timed pregnancy experiments revealed that Jak2^{R915X/R915X} and Jak2^{−/+} displayed embryonic lethality, however, viable embryos were observed at E14.5 in Jak2^{R915X/R915X}. Our data suggest that altered Jak2 expression may have unexpected consequences and correspondingly, important implications for treatment of hematological disorders with constitutive Jak2 activity.

(119 words)
INTRODUCTION

Cytokines play an integral role in hematopoiesis by providing growth signals to progenitor and committed cells that promote mitogenesis, survival and differentiation. Once bound to their cognate receptors, cytokines mediate downstream signaling through activation of components of the Jak-Stat signaling pathway. Thrombopoietin (Tpo) is the principal cytokine regulator of megakaryopoiesis, through binding to the Tpo receptor (Mpl). Tpo activates the Jak2 and Tyk2 tyrosine kinases \(^1\) and the Stat3 and Stat5 transcription factors \(^2\). The importance of Tpo, its receptor and proximal signaling pathways in platelet function, is illustrated by gain-of-function mutations in \(TPO\) \(^3\), \(MPL\) \(^4,5\) and \(JAK2\) \(^6,9\) resulting in Essential Thrombocythemia (ET). Similarly, loss-of-function mutations in \(MPL\) have been documented in Congenital Amegakaryocytic Thrombocytopenia \(^10\). Jak2 is critical for murine embryogenesis since mice lacking Jak2 expression die of anemia at E12.5 \(^11,12\).

While screening ENU mutagenized mice for dominant hematopoietic defects, we identified a mouse with thrombocytopenia and determined that the mutation resulted in a truncated allele of Jak2 that lacked tyrosine kinase activity. Analysis of this mutation has uncovered a novel function of Jak2 in the megakaryocyte/platelet lineage.
MATERIALS AND METHODS

Mice, *ENU mutagenesis, hematologic analysis, genetic mapping and sequencing*

C57BL/6J (B6) and 129S1/SvImJ (129J) mice were purchased from The Jackson Laboratory. B6 Jak2+/− mice were provided by Dr. James Ihle, Memphis, TN. Mice were maintained in specific-pathogen free facilities at Toronto Centre for Phenogenomics or Ontario Cancer Institute. One intraperitoneal injection of 150mg/kg ENU was administered to male 129 mice (mutagenized strain) to induce random mutations. The F1 generation (129;B6) was produced by out-crossing ENU-mutagenized males to B6 (mapping strain) females – pups from this breeding were designated generation 1 (G1). G1 mice were screened to detect dominant traits deviating from normal homeostatic venous blood parameters by at least two standard deviations from ‘normal’ G1 parameters. Peripheral blood from 6-8 week old mice was collected. Complete blood counts (CBC) were performed. Affected mice were sequentially bred to B6 to confirm heritability and to genetically map the mutation using microsatellite base genome scan and single-nucleotide polymorphism markers differentiating 129 and B6 alleles. Once the mutation was mapped to a 6.7Mb region of chromosome 19, candidate gene analysis was used to select genes for exon sequencing. The *Jak2<sup>F915X</sup>* allele was maintained on a B6 background by intercrossing heterozygous or wild type (WT) mice.

Genotyping

Multiplex PCR was used to genotype *Jak2<sup>F915X+</sup> and Jak2<sup>+</sup> mice using genomic DNA prepared from tail or biopsy tissue.
**Clonogenic assays**

CFU-C, CFU-E and CFU-Mk assays were performed as previously described\(^{14}\).

**Antibodies**

Anti-Jak2 and β-tubulin antibodies were purchased from Cell Signaling Technology (Beverley, MA) and Millipore (Billerica, MA). The HA antibody was from Covance (Laval, QC).
RESULTS AND DISCUSSION

We identified a G1 mouse, strain 7254, with elevated platelets. Back-crossing on to the B6 strain and SNP-based mapping resulted in the identification of a 6.7 Mb heritable region on chromosome 19 as the interval carrying the responsible mutation. We noted that Jak2 was within this interval and hypothesized that the mutation underlying the 7254 phenotype may be in Jak2. We performed genomic DNA sequencing, which identified an A3056T mutation in exon 19 of the Jak2 locus. This mutation leads to a K915X premature stop codon in the coding sequence of the functional JH1 tyrosine kinase domain of Jak2 (Figure 1A).

Western blotting of splenocytes isolated from Jak2<sup>K915X<sup>−/−</sup></sup>, Jak2<sup>+/−</sup> and Jak2<sup>+/+</sup> mice revealed a novel 95 kDa protein in Jak2<sup>K915X<sup>−/−</sup></sup> mice (Figure 1B, lanes 4 and 5), that co-migrated with the expressed Jak2<sup>K915X</sup> protein in 293T cells (lanes 8 and 9). Overexpression of HA-tagged Jak2 and HA-Jak2<sup>K915X</sup> in 293T cells revealed selective tyrosine phosphorylation of wild-type Jak2 (Figure 1C, lanes 2 and 3). The possibility that nonsense-mediated decay occurs was examined by expression of Jak2 cDNAs that lacked or contained the Jak2 3’ untranslated region (UTR). The presence of the Jak2 3’UTR resulted in comparable protein expression (lanes 4 and 5). While Jak2 and Jak2<sup>K915X</sup> protein was reduced compared to the cDNA lacking the 3’ UTR (lanes 2 and 3), both forms of Jak2 were readily detected when the 3’ UTR was present.

To determine whether the Jak2<sup>K915X</sup> allele phenocopied a Jak2 null allele or represented a neomorphic allele, we compared mice bearing homo- and heterozygous mutations at this locus. Elevated platelets were found in male and female mice at both 6 and 8 weeks of age in Jak2<sup>K915X<sup>−/−</sup></sup> mice (Figure 2A). Interestingly, Jak2<sup>+/−</sup> mice showed an identical phenotype, although this phenotype has not previously been reported. In contrast, other hematological
parameters (data not shown) were comparable in both Jak2\textsuperscript{K915X+} and Jak2\textsuperscript{+/−} mice, with the exception of decreased RBC in Jak2\textsuperscript{K915X+} mice at 6 wk (Figure 2B).

Clonogenic assays were performed on bone marrow and spleen cells from Jak2\textsuperscript{K915X+} and Jak2\textsuperscript{+/−} mice (Supplementary Figure 1). There were no significant differences in hematopoietic progenitor number or morphology between Jak2\textsuperscript{+/−} and Jak2\textsuperscript{K915X+}. Interestingly, no change was observed in CFU-Megakaryocyte assays from bone marrow isolated from Jak2\textsuperscript{+/−} and Jak2\textsuperscript{K915X+} mice (Supplementary Figure 1G).

Jak2\textsuperscript{K915X+} and Jak2\textsuperscript{+/−} mice were challenged with 150 μg/g 5-fluorouracil or 100 μg/g Phenylhydrazine. Recovery curves were similar for all genotypes in response to hematopoietic stress induced by 5-fluorouracil (Supplementary Figure 2) or Phenylhydrazine (Supplementary Figure 3).

Jak2\textsuperscript{+/−} embryos die at E12.5 due to a block in fetal hematopoiesis. Timed matings were conducted to generate Jak2\textsuperscript{K915XX915X}, Jak2\textsuperscript{K915X−}, and Jak2\textsuperscript{−/−} embryos to determine whether embryo lethality is similar between the Jak2 alleles (Figure 2C). Embryos were dissected at E12.5, E13.5 and E14.5 and segregated into healthy (red) or anemic (white or re-absorbing) categories. Reabsorbing and white embryos were assumed to be incapable of producing viable pups. No Jak2\textsuperscript{+/−} embryos were observed at E13.5 or E14.5. However, red Jak2\textsuperscript{K915X−} and Jak2\textsuperscript{K915XX915X} embryos were present at E13.5. The increased viability of embryos expressing one allele of K915X may be due to the presence of a truncated Jak2 protein product capable of scaffolding Jak2 interacting proteins, but that is catalytically inactive. No Jak2\textsuperscript{K915XXK915X} or Jak2\textsuperscript{K915X−} embryos were identified later than E14.5.

The initial characterization of the Jak2 knockout mouse revealed that Jak2 played a critical role in erythropoiesis and thrombopoiesis, with embryonic lethality observed at E12.5.
Since the EPO and EPO-R null mice die at E13.5, the slightly earlier death observed in Jak2-/- embryos was attributed to the recruitment of Jak2 to other cytokine receptors including the TPO-R. Beyond this initial evaluation of the homozygote mice, little characterization of Jak2+/- mice has been performed.

Utilizing random mutagenesis, we have demonstrated a critical, yet subtle, role for Jak2 in the regulation of platelet biogenesis. Loss of one functional allele of Jak2, either through truncation in Jak2<sup>Y915X</sup>-/- or deletion in Jak2<sup>+/</sup>, leads to thrombocythemia. Mutation of JAK2 is observed in several hematological disorders including ET, Polycythemia Vera, Primary Myelofibrosis and Acute Lymphoid Leukemia (ALL). The JAK2 signaling pathway also participates in disease mediated by MPL mutations in ET and CRLF2 mutations in T cell ALL. As Jak2 inhibitors including CEP-701, TG101348, INCB018424 and AZD1480 are currently in clinical trials to treat these disorders, this study suggests that altering JAK2 expression may lead to unexpected clinical outcomes.

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AUTHORSHIP CONTRIBUTIONS

NA, MJJ, EB, MLB, KH and ZB performed experiments; LRO participated in mapping and mutation detection; AB, AF, SLA and JR were involved in ENU mutagenesis; CW, KMM,
RFP, MDM, WLS and DLB planned experiments and the manuscript was written by NA, MJJ, EB, MLB, WLS and DLB.

**CONFLICT OF INTEREST**

The authors have no relevant conflicts to disclose.
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