University of Massachusetts
UMASS Medical School

Institutional Animal Care and Use Committee (IACUC)
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Approval Letter

April 30, 2019

Docket #: A-1586-19

Investigator: Merav Socolovsky, Ph.D.
Molecular, Cell and Cancer Biology

Title: Flow Cytometric Analysis of Erythropoiesis

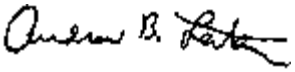
This is to certify that the project identified above has been reviewed by the Institutional Animal Care and Use Committee (IACUC), which has considered specifically the compliance with applicable requirements of the Animal Welfare Act and pertinent state and local laws, regulations, and adherence to the U.S. Public Health Service Policy and the NIH Guide.

The proposed study has been approved by the IACUC and complies with the institutional assurance certification of the University of Massachusetts Medical School. The University of Massachusetts Medical School is fully accredited by AAALAC and has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW). The Assurance number is: D16-00196 (A-3306-01), effective 1/4/2017.

Total number of animals approved for this study.

Animals/Species	Category (B)	Category (C)	Category (D)	Category (E)
Mouse	0	4974	150	0

PROJECT APPROVAL PERIOD : 04/30/2019 to 04/29/2022



Andrew B. Leiter, M.D., Ph.D.
Chair, Institutional Animal Care and Use Committee

To the Investigator:

Please note the enclosed copy of your protocol is the final, approved protocol by the IACUC, and overrides all previous versions.
Annual review will be required for protocols in accordance with the Animal Welfare Act Regulations and Department of Defense (DoD) instruction for use of animals.

Investigators will not be allowed to exceed the approved number of animals without IACUC approval.

Modifications of any type (additional species; new procedures that may cause discomfort, stress or pain; use of new biohazardous materials or methods; and addition of new personnel or investigators) must be reviewed and approved by the IACUC before being initiated.

For your information see the link for "A Quick Reference to the Responsibilities and Functions of the Principle Investigator in Research Facilities under the Animal Welfare Act" at http://www.nal.usda.gov/awic/pubs/Legislat/awabrief.shtml#Q14 and “What Investigator Need to Know About the Use of Animals” at http://grants.nih.gov/grants/olaw/InvestigatorsNeed2Know.PDF.

C(pdf): Animal Medicine

IBC reference:

UNIVERSITY OF MASSACHUSETTS MEDICAL SCHOOL

ANIMAL CARE AND USE COMMITTEE

Animal Study Protocol

PI Name: Merav Socolovsky	**Docket Number: A-**1586-19
PART 1: ADMINISTRATIVE QUESTIONS	
1.0 Study Type*	☐ New ☒ 3-Year ☐ Amendment (summarize below)
1.1 Docket #	A-1586-19
1.2 PI Name *	Merav Socolovsky
1.3 Faculty Title * If *"other" describe:*	Professor
1.4 Email Address *	merav.socolovsky@umassmed.edu
1.5 Phone Number *	
Telephone type	☐ OFFICE 508-856-3743 ☐ CELL 617-797-1633 ☐ OTHER
1.6 Academic Degree *	MBBS PhD
1.7 Department/Division or Company Name *	MCCB Molecular Cell and Cancer Biology
1.8 Emergency Phone Number *	617-797-1633
1.9 Faculty Sponsor (if required)	
1.10 Co-investigator	
1.11 Primary Contact Person *	Daniel Hidalgo
1.12 Primary Contact Email *	daniel.hidalgo@umassmed.edu
1.13 Primary Contact Phone Number *	860-597-7200
1.14 Project Title *	Flow Cytometric Analysis of Erythropoiesis

1.15 Grants Associated with this Animal Protocol

Grant Sponsor	Grant ID	Grant PI
NIH	*1R01 HL141402-01*	Merav Socolovsky, Allon Klein
NIH	*5R01 DK100915-05*	Merav Socolovsky
NIH	*R01 DK099281*	Merav Socolovsky
Choose an item.		

1.16 Has the study undergone peer-review? *	☐ X YES ☐ NO

PART 2: BACKGROUND QUESTIONS

2.1 Lay summary: Briefly summarize the background and goals of your animal studies using non-technical terms that could be understood by individuals with high school level education. *

Background: The proposed research aims to understand what regulates the rate at which red blood cells are made and the underlying molecular mechanisms. The principal role of red blood cells is to carry oxygen from the lungs to the tissues. Red cells in the circulation survive for only a short period (approximately 30 to 40 days in the mouse, 120 days in human), and so new red blood cells must be continuously made throughout life. Red cells arise as a result of differentiation of red-cell progenitor cells, found in blood-forming tissue: fetal liver, bone marrow or spleen. The rate of red cell production in both mouse and man varies through a remarkably wide range. In a healthy individual at sea level, red cell production rate is equivalent to the steady-state red cell loss through cell aging. However, in a variety of stress conditions the supply of oxygen to the tissues is threatened, including high altitude, heart or lung disease, anemia or bleeding. In an attempt to remedy this, the body responds by increasing the number of oxygen-carrying red cells. This ability to rapidly increase the rate of red-cell production is critical to the body's ability to cope with these types of stress.

It is well established that the principal regulator of red cell production rate is the hormone Erythropoietin (Epo). However, the precise identity of the progenitor cells on which it acts, as well as its molecular mechanisms of action, are largely unknown. The goal of the proposed research is to uncover the molecular mechanisms that operate in red blood cell progenitors to regulate the rate of red blood cell formation (erythropoiesis).

Red cells arise in blood-forming tissue, from more primitive cells known as progenitor cells. These red cell progenitors are present in only small numbers in the blood forming tissue. In order to understand what regulates the rate at which red cells are made, it is necessary to be able to identify these specialized progenitor cells and study their properties. Our laboratory has developed new methodology that makes it possible for us to identify these cells directly within the blood-forming tissue. This is done by the use of antibodies that can bind unique markers that are present only on the surface of these progenitor cells, and on no other cell. The binding of the antibodies to these cells is detected using a technique known as flow-cytometry, which can identify and isolate any cell that binds a fluorescent antibody (Socolovsky et al., Blood 98:3261-3273 2001; Liu et al., Blood 108:123-133 2006). This method therefore now allows the direct molecular study of red-cell progenitors under conditions of basal, as well as accelerated, red cell formation.

Our objectives are to understand how the biology of red blood cell progenitors is regulated by erythropoietin and by genes implicated in cancer, using novel flow-cytometry techniques. We will examine blood-forming tissue from mice that are subject to low-oxygen and/or high Epo conditions, or in which genes deleted in cancer are deleted, and study the biological and molecular consequences on the biology of red cell progenitors. We recently identified a new, unusual process that takes place during red cell formation: DNA of most of the cells in the body contains small 'tags' made of methyl groups, that have the effect of silencing genes in their vicinity. We found that these methyl tags are lost in red cell progenitors (Shearstone et al., Science 334:799, 2011). The work we are currently undertaking addresses the mechanism of this unusual phenomenon, and how it might regulate red cell formation rate and/or predispose red cell progenitors to cancer.

2.2 Scientific Summary: Briefly describe the specific aims and scientific approaches to achieving these aims. Detailed procedures should not be entered here. This section will help the committee understand the rationale and goals of your study. The IACUC does not use this section to conduct a scientific merit review. Limit to 5,000 characters. *

We are interested in the processes that regulate erythroid progenitors during normal physiology, during hypoxic stress, and during malignant transformation. We pioneered the study of erythroid progenitors directly within freshly harvested murine hematopoietic tissue, using cell-surface markers and flow-cytometry to identify and study differentiation stage-specific erythroid progenitors. We are in the process of expanding this approach to new cell surface markers and novel erythroid progenitor subpopulations.

The biological questions/ specific aims are:

1. Delineate the events early in erythropoiesis that regulate the onset of erythroid differentiation.

Our recent work (Pop et al., PLoS Biology 2010; Shearstone et al., Science 2011) identified an S phase-dependent commitment step in CFUe erythroid progenitors, which activates the onset of Epo dependence and the erythroid transcriptional program. This commitment step initiates a genome-wide wave of DNA demethylation, a process that was previously thought to be limited to early embryonic development. Our current work focuses on identifying regulators of this S phase-dependent commitment step and the consequent DNA demethylation, including the use of mice deleted for candidate regulators of these events: the transcriptional repressor PU.1, the cyclin-dependent kinase inhibitor (CDKI) $p57^{KIP2}$, Rb family proteins and the methylase DNMT1.

2. Determine the parameters within erythroid progenitors that regulate the rate of red cell production. We are investigating how high levels of Epo in blood generate high rates of red cell formation, as opposed to the basal rates seen in response to basal Epo levels. Our previous work focused on apoptotic mechanisms in erythroid progenitors and precursors. Given our recent work implicating S phase and DNA methylation in the activation of erythroid transcription, we will focus on the role of these processes in erythropoietic stress. We will test the hypothesis that glucocorticoids, in conjunction with high Epo levels, increase the rate of production of red cells by altering the DNA methylation and cell cycle status of CFUe progenitors. These experiments will make use of adult bone-marrow, spleen and of fetal liver, in wild-type mice as well as in a number of models of hypoxic stress, and also make use of mice deleted for potential regulators of S phase and of DNA methylation including mice deleted for p57KIP2, for the glucocorticoid receptor and for DNMT1.

3. Determine the mechanism of activation of the transcription factor Stat5 by Epo receptor (EpoR) signaling. We recently showed that degree of maturation of erythroid precursors determines the dynamics of Stat5 activation (Porpiglia et al., PLoS Biology 2012). We will use freshly harvested fetal liver cell erythroid progenitors from wild-type mice as part of our investigation of the mechanism regulating Stat5 activation.

4. We will elucidate the mechanism and function of global DNA demethylation in erythropoiesis. Specifically, we will examine the role of the Tet family of proteins in DNA demethylation and hydroxymethylation. Tet proteins are epigenetic regulators that mediate oxidation of 5-methyl cytosine in DNA. Tet2, in particular, is one of the most frequently mutated gene in hematological malignancies, but the precise mechanism thorugh which Tet2 mutation contributes to malignancy is unknown. We are investigating how acute deletion of either Tet2, Tet3, or both these genes, impacts erythroid progenitors, including their potential malignant transformation.

5. We are exploring novel cell –surface markers to identify early erythroid progenitor subsets prospectively within the bone-marrow of adult mice and within the fetal liver.
2.3 Describe the potential scientific benefit of the proposed study to human or animal health, the advancement of scientific knowledge, or the good of society. *
The proposed research aims to uncover the molecular mechanisms that regulate the rate of red blood cell formation (erythropoiesis), particularly during the response to the stress of low tissue oxygen ('stress erythropoiesis). An efficient stress erythropoiesis is crucial to survival and recovery from clinical conditions that interfere with oxygen uptake or delivery to tissues, including cardiac or pulmonary syndromes, anemia of multiple etiologies, blood loss, and therapeutic procedures such as chemotherapy and stem-cell transplantation, used in the treatment of hematological malignancies. Our work may lead to the discovery of novel 'erythropoiesis stimulating agents' (ESAs) that could be used either in vivo, to treat anemia of various etiologies, or in vitro, in the production of red cells for transfusion. While erythropoietin is an extremely effective therapeutic agent, it is not useful in a class of anemias that are erythropoietin-resistant (e.g. Diamond Blackfan anemia), where patients are therefore transfusion-dependent. Further, some patients develop anti-erythropoietin antibodies, again preventing its use. Therefore, novel ESAs are continuously sought by the research community. There is growing evidence that inappropriate activation of stress erythropoiesis pathways may predispose to leukemic transformation. Therefore, a mechanistic understanding of stress erythropoiesis could impact multiple clinical settings. Our recent finding of global DNA demethylation in erythroid differentiation may be relevant to the pathogenesis of some hematological malignancies, particularly myelodysplastic syndrome (MDS), which frequently presents as refractory anemia and in which DNA methylation patterns are dysregulated.

2.4 Dates of Searches: Years covered by the search (From - To) *	From: January 1, 1985	To: March 8, 2019

2.5 Keywords searched: Keywords must include procedures that may generate more than momentary pain or distress. If not enter N/A. *
Erythropoiesis, anemia, EpoR signaling, erythroid differentiation; cell cycle progression; DNA methylation; animal use alternatives, Tet2, Tet3, Tet1

2.6 Databases searched to determine: • That you are not unnecessarily duplicating previous experiments, • that alternatives to animal use are either not available or not appropriate, • that procedures involving animals will avoid or minimize discomfort, distress and pain, AND • that alternatives to procedures that may cause more than momentary or slight pain or distress have been considered.	*☒PubMed/Medline *☒Scopus *☒Web of Science *☐ Agricola *☐ AltWeb *☐ Animal Welfare Information Center *☒UMMS Veterinarians *☐ Other Sources of Information (explain below)

2.7 Did the search identify less painful alternatives, previous studies that would be unnecessarily duplicated or alternative approaches that would not require animals? *	☐ YES ☒NO

2.8 If you answered YES to the above question, please explain why the proposed studies are required.

2.9 Why are vertebrate animals required for your study? *

*☒ The complexity of the processes being studied cannot be duplicated or modeled in simpler systems

*☒ The studies cannot be done by computer simulation

*☒ Preclinical studies in living animals are necessary prior to human testing.

*☒ This is a behavioral, learning or developmental study

*☒ Other. Please explain:

The complex regulatory feedback mechanisms that control the number and properties of erythroid progenitors in the body and that generate elevated erythropoietic rates are largely unknown, and cannot therefore be easily modeled in vitro. The flow-cytometric study of red cell progenitors from freshly isolated mouse hematopoietic tissue can reveal the changes that occurred in that tissue in vivo during elevated rates of erythropoiesis. There is no in-vitro equivalent model that can reproduce this type of system. However, specific aspects of erythropoiesis can be studied in vitro, and this will be done wherever possible. Our work in vitro requires freshly harvested erythroid progenitor cells from bone-marrow, spleen and the fetal liver, since these have the capacity to differentiate into red cells, allowing us to study the various aspects of the differentiation process. There is no cell line that undergoes an equivalent process that could replace the need for fresh tissue. Further, our investigation of a number of regulators of this process requires their genetic deletion or manipulation, which cannot be done in human tissue and is best studied in genetically engineered mouse models.

The validity of this approach was demonstrated in the study of the anemic Stat5a-/-5b-/- mouse (Socolovsky et al., Blood 98:3261-3273 2001) and in our more recent studies of death receptors in erythroid progenitors during stress (Liu et al., Blood 108:123-133 2006; Socolovsky et al., PLoS Biol. 2007, Koulnis et al., PLoS One 2011, Koulnis et al., Blood 2012). We used the flow-cytometric technique on freshly explanted spleen and bone marrow from a number of mouse models of erythropoietic stress, and found a previously unknown mechanism through which the number of erythroid progenitors in spleen and bone-marrow are regulated. This analysis revealed quantitative and biochemical features of red cell regulation which were not apparent from in-vitro culture models.

Many of the in-vitro studies we perform are on primary erythroid progenitors, derived from fetal liver. The experiments in vitro are invaluable since they provide much biochemical data on the response of these cells to many different drugs and hormones. The fetal liver is a rich source for primary erythroid progenitors. The properties of

these cells are unique, and are lost upon immortalization: there is no alternative cell line at the present time with bona fide progenitor properties that will undergo normal differentiation into red cells *in vitro*.

2.10 Why are the proposed species the most appropriate for your studies? Check all that apply. *

*☒ The anatomy, genetics, physiology or behavior of this species is uniquely suited to the study.

*☒ This is the phylogentically lowest species that provides adequate size, tissue, or anatomy.

*☒ Preliminary data supporting this project used this species

*☒ The mouse genome is similar to the humans in complexity and may be manipulated to mimic human disease

*☐ The rat is a widely used species for experimental neurological and psychological processes that model similar processes in humans

*☐ Other. Please explain:

2.11. Briefly explain your selection(s) from 2.10.*

The mouse regulates erythropoietic rate through a similar range (up to ten-fold the basal rate) to the human. Similar hormones operate to increase erythropoietic rates in the mouse and the human. However, non-mammals (e.g. fish or birds) are less suitable for this study since the type of red cells they possess, as well as the factors that regulate them, are quite different to those of mammals.

The molecules that make up the signaling pathways downstream of the receptor for erythropoietin in the mouse bear very close similarity to those in the human. Furthermore the mouse is the only available animal model having the genetic modifications that are required by our research, including modification of the erythropoietin receptor (either deletions or mutations) and a number of genetic models of anemia (e.g. beta-thalassemia).

PART 3: EXPERIMENTAL DESIGN

3.1 List the number of animals you will use over the duration (to a maximum of 3 years) of this protocol. Include mouse and rat fetuses ≥17 days of gestation. The total should include all animals bred for use in experiments AND all animals generated by breeding that are discarded due to the wrong genotype and/or sex.

Pain/distress level indicates maximum pain or distress level to be experienced by animals:

- B=USDA covered species only being bred but not used in testing, teaching or experiments (do not include mice/rats)
- C=negligible pain/distress
- D=pain/distress relieved by appropriate drug use
- E=pain/distress not relieved by appropriate drug use

Species	Category B	Category C	Category D	Category E	Total
Mice	0	4974	150		5124
Choose species.					
Choose species.					
Choose species.					
Choose species.					
Choose species.					

Note: This table will not automatically total columns. Please enter the total for each row.

3.2 Category E Justification: If you have animals in category E, briefly refer to the procedures producing pain or distress and/or a timeline of disease progression, and list the reasons why pain-relieving drugs cannot or will not be used to relieve pain or distress. If pain/distress relief would interfere with test results, justify why that is true.

3.3 Are you planning to reuse animals that were previously used for other experiments or procedures under a different protocol? *	☐ YES ☒NO

3.4 If the answer is "yes" to question 3.3 please provide more details (number of animals, what previous procedures done in these animals)

3.5 If there are federal guidelines or regulations that require the use of laboratory animals then please cite the agency, CFR title, number and specific section (e.g., Food and Drug Administration, 21:CFR 1030.110).

3.6 The number of animals requested for this protocol is based on the following. Guidance on animal number justification can be found on the UMMS IACUC web site. (select all that apply) *	*☒ A statistical estimate of the number required to achieve statistical significance *☒ The estimated minimum number necessary to achieve the goals of the study in the absence of a statistical estimate

	*☒ The number necessary to obtain sufficient tissue or other material for testing or analysis *☒ The number required to provide sufficient technical training or practice for the number of trainees expected *☒ The expected or established mortality associated with this procedure *☒ The number of animals generated from breeding that cannot be used due to the wrong genotype or gender *☐ Other (*explain below*)

3.6.a. Please provide details of the number of animals requested based on experimental design, including the experimental groups and the number of animals per group. Be sure that the total number of animals based on experimental design match the total # of animals in table 3.1.

The details of each experimental procedure is given below. Please see attached worksheets for full accounting of mouse numbers.

1. Cell cycle status of erythroid progenitors will be measured in wild type mice, in mice subjected to hypoxia at 10% or 12% oxygen for up to 10 days, or in one of the mouse models deleted or mutated for the regulators we are currently investigating, including p57KIP2, the three Rb family proteins (pRb, p107, p130), PU.1, DNMT1, glucocorticoid receptor (GR). In those cases where gene deletion is lethal in utero we will measure the cell cycle status in fetal liver cells of the mid-gestation embryo. Adult female or male mice or timed-pregnant mice will be injected intraperitoneally with a thymidine analogue, either bromodeoxyuridine (BrdU), chlorodeoxyuridine (CldU) or iododeoxyuridine (IdU), at a single dose of 100 microliters, 10mg/ml. Mice will be sacrificed within 30 minutes to 72 hours after this single injection. In some experiments in which we aim to measure the length of S phase, we will inject two thymidine analogues within a few hours of each other (the second injection will be between 1 and 8 hours following the first). Mice will be sacrificed within 1 hour of the second injection. Each injection will be of 100 microliters, 10mg/ml of either CldU or IdU. Bone-marrow and spleen will be harvested. **6 mice per group** is likely to give statistically adequate significance levels to the biological differences we are investigating, based on our previous experience (see Liu et al., 108:123-133 Blood 2006).

2. RNA preparation for real-time quantitative PCR (qPCR) of erythroid gene induction during normal and stress erythropoiesis in bone-marrow and spleen. The following models of stress erythropoiesis will be used, each with an equal number of controls. Erythropoietic stress models:
(i) wild-type mice housed in 12% oxygen for up to 21 days (control: normoxia).

(ii) Other mouse strains that will also be tested for the erythropoietic stress response to hypoxia are: the DNMT1chip/chip mice, GRdim/dim, Tet2-null mice.

(iii) b-thalassemia mice (control: mice of the same colony that are wild-type)

(iv) mice injected with Epo (single dose subcutaneously, up to 300 units/25g mouse in a total injection volume of 150 microliters) for up to 14 days prior to being euthanized (controls: mice injected with equal volume of saline

(v) mice injected with dexamethasone, either alone or in combination with Epo. Dexamethasone is a synthetic glucocorticoid. Glucocorticoids are induced during erythroid stress and cooperate with Epo in its regulation. Co-injecting dexamethasone together with Epo will enable us to more closely mimic conditions of 'erythropoietic stress'. We plan to administer adult mice with dexamethasone at doses between 2mg/kg and 10mg/kg intraperitoneally, in a total volume of 150 microliters, dissolved in phosphoate-buffered saline (PBS). Injections will be carried out once per day for up to 5 consecutive days. Injection of dexamethasone in mice is frequently reported in the literature (e.g. Sadikot et al., *Am. J. Respir. Crit. Care Med. September 1, 2001 vol. 164 no. 5 873-878)* and has no adverse effects in the short term (days to weeks). Glucocorticoids such as dexamethasone are also widely used therapeutically in humans.

(vi) Epo and/or dexamethasone injections will be carried out in four mouse strains: EpoR-HM, EpoR-H, DNMT1chip/chip, GRdim/dim mice.

(vii) 'YAC' mice: these mice contain a transgenic yeast artificial chromosome (YAC) encoding the human b-globin locus. These mice will enable us to assess the effects of stress and cell cycle status on expression of the human b-globin genes. The human b-globin genes include the fetal gamma globin, which is absent in mice, the regulation of which is central in therapy for hemoglobinopathies such as b-thalassemia and sickle cell anemia.

For the above experiments, **6 mice per group,** either experimental or control, rationale as in '1' above. Where appropriate, mice will be euthanized at three time points after the onset of stress and bone-marrow and spleen will be harvested.

3. Genomic DNA will be prepared for analysis of DNA methylation, DNA hydroxymethylation, and for other epigenetic analysis including chromatin immunoprecipitation and 3C (chromosome conformation capture) analysis. The same experimental groups as in '2' above will be used. Wherever possible, we will extract both RNA and gDNA from the same cell sample, thereby minimizing the number of mice required.

4. Harvesting fetal livers for in-vitro erythroid differentiation and for gDNA and RNA analysis. The fetal liver is rich in erythroid progenitors and is a very convenient model for the study of erythropoiesis in vitro and in vivo. We routinely isolate differentiation-stage-specific erythroid subsets from the fetal liver for cell sorting. The sorted subsets are either analyzed immediately for gene expression (RNA isolation, as in '2' above) and genomic DNA methylation/hydroxymethylation marks, or alternatively, used for in vitro differentiation assays. (Tet2 and other strains).
To maximize the number of embryos and reduce total mouse numbers, we will use superovulation procedures prior to mating. Female mice will be injected with PMSG ('pregnant mare's serum gonadotropin', 5 IU intraperitoneally) 2 days prior to mating; on the day of mating, they will be injected with hCG (5 IU intraperitoneally). Plugs are examined the following morning.

5. **Transplantation of erythroid progenitors for lineage-fate analysis in vivo.** We are in the process of identifying and validating novel cell-surface markers that could be used to identify early erythroid progenitor populations in the adult mouse bone-marrow. We plan to isolate these cell populations using flow-cytometry from adult-bone-marrow of GFP transgenic mice donors (H2-BEX3, C57BL6 background, Gerstein lab). We will inject them into the tail-vein of recipient wild-type C57BL6 adult mice that are lethally irradiated, together with a radioprotective dose of adult bone-marrow cells. We will examine the appearance of GFP-positive erythroid progenitors and red blood cells in the peripheral blood of recipient mice. Each experiment will be carried out in triplicate. (2 donor mice per recipient mouse, so 9 mice -3 recipient, 6 donor-per erythroid subpopulation

tested; 5 different erythroid progenitor subpopulations, for a total of 45 donor and 15 recipient mice per experiment; we estimate we will carry out approximately 10 experiments, for a total of 150 recipient mice).

6. Where appropriate in all the above experimental approaches, we will start each experimental investigation with a pilot experiment as described in IIc above. If results look suggestive, we will continue experimentation, incorporating results from the pilot experiment into our overall statistical analysis. If a specific pilot experiment is not suggestive of a significant result, we will discontinue this particular experimental approach.

Calculation of mouse numbers is presented in the enclosed worksheet. Worksheet 1 was divided into 3 parts, A, B and C. Mouse strains are in rows, and experiment categories (up to 4: cell cycle, RNA, gDNA, in vitro differentiation of fetal liver cells) are in columns. An 'x' is entered in the appropriate row/column for each experiment category that we plan to carry out in a given mouse strain. The total number of mice required for a given strain is added up in the final, right-hand column labeled 'total mouse numbers per strain'.

Worksheet 1A refers to experiments in adult mice. Thirty mice are requested per experimental category per strain, calculated as follows: 5 groups each containing 6 mice, since in our experience 6 mice usually provides us with sufficient data for statistical significance. Of the 5 groups, one group will be control (or baseline or time zero) mice; the other 4 groups will be 4 distinct conditions or time points, for example, 2 different hypoxia conditions (10% and 12%) at two different time points in hypoxia (e.g. 12 hours and 48 hours). In some experiments we may choose to have only one set of hypoxia conditions (e.g. 12%) and examine 3 different time points (t= 12, 48, 96 hours) in addition to time zero.

We will attempt to obtain RNA and gDNA from the same cells. Therefore, the total number of mice per strain calculated in each row is the sum of all the experimental categories that will be required for that strain, multiplied by 30, with 30 subtracted in the case of overlapping gDNA and RNA. For all the listed strains, this equals 60 mice.

Worksheet 1B refers to experiments with embryos. We require 30 to 50 embryos at mid-gestation (E12.5 to E13.5) on a given experimental day, which are then used to sort early erythroid progenitors from the fetal liver. We project approximately 8 experimental days per experimental category per strain. This is because early erythroid progenitors comprise only 1 to 3% of the fetal liver. Forty embryos at E13 yield a total of 1-2 x 10^8 fetal liver cells. Of these, 11 approx.. 1-3 x 10^6 are the early erythroid progenitors of interest. This number of cells yields only 15-20% of the required total RNA or gDNA (3 to 5 micrograms) for many of our applications, including purifying RNA for gene expression, gDNA for genome-wide methylation studies, and downstream cell cycle analysis by flow cytometry and DNA combing. Further, in cases where the null phenotype is lethal late in pregnancy, as is true for several of our strains including EpoR-null, Rb-null and Stat5-null, we mate heterozygous mice. Only a quarter of the embryos are of the experimental null genotype, requiring even larger numbers of embryos and/or more experimental days. We use the rest of the littermate embryos (of heterozygous or wild-type genotypes) as controls.

To obtain 30 to 50 embryos per experimental day, we super-ovulate and mate 8 female mice with 8 males. Of these, approximately half become pregnant and yield 8 to 12 embryos per pregnancy (in the absence of super-ovulation, embryo yield is 30 to 50% of this). We monitor the progression of pregnancy by weighing the female mice daily. Mice that don't gain weight are not pregnant and are saved for future experiments. We therefore project approximately 50 adult mice will be required per experimental category (8 males used for mating, that will be replaced twice over

time, requiring 24 males altogether; and 4 females that will become pregnant per experiment, on 8 experimental days, giving a total of 32 females).

Of note, there are 4 experimental categories for fetal liver experiments, but only 150 mice are requested due to the overlap between gDNA and RNA experiments (see above).

Worksheet 1C adds the total number of experimental mice that will be required per strain, from the numbers projected for each strain in tables 1A and 1B.

3.7 Write a brief description of experimental design using a chronological list of activities to be carried out on living animals from initial contact to euthanasia *

One of the following experimental procedures will apply:

1. Mice will be injected with Epo, Dexamethasone, or thymidine analogues, hours or days (up to 19) prior to euthanasia. Tissue (bone marrow and spleen) will be collected following euthanasia
2. Reduced oxygen chamber: mice will be placed in this chamber for up to 21 days. At the end of this period mice will be euthanized and tissue will be collected (bone marrow and spleen).
3. Timed pregnant mice will be euthanized, and fetal livers will be collected from embryos.
4. For acute deletion of Tet2, Tet3 or Tet2 & Tet3 in vivo: mice will be injected with Tamoxifen for up to 5 consecutive injections, every other day. Mice will be euthanized and tissue will be collected (fetal liver, bone-marrow or spleen) 4 days to 4 weeks following tamoxifen injections.
5. For bone-marrow transplantation experiments:

Donor mice:

i) Euthanasia for bone-marrow harvest

Recipient mice:

i) Irradiation
ii) Tail vein injection with donor bone-marrow
iii) Weekly facial or tail vein bleed (4 total)

iv) Euthanasia for tissue harvest.

3.8 If using an approved standard procedure from the UMMS IACUC website without modification please check the appropriate check box (s) below. Deviations to standard procedures should be described in section 3.10.	*☐ Facial Vein Blood Collection *☐ Lateral Tail Vein Blood Collection *☐ Retro-Orbital Bleeding *☐ Use of Freund's Complete Adjuvant (FCA) *☐ Ascites Production for Monoclonal Antibodies *☐ Tail Snipping of Mice up to 21 days of age *☒ Tail Snipping of Mice between 22-28 days of age *☒ Tail Snipping of Mice > 28 days of age *☐ Ear Punching as Alternative to Tail Snipping for Isolating DNA *☐ Intramuscular injections in rabbit

	*☐ Pearl Imaging *☐ IVIS Imaging

3.9 If using an approved procedure from a UMASS Research Core Facility please check the appropriate check box(s) below.

If procedures and/or species are not covered under the core protocol(s) please provide a detailed description of the procedures for each species in narrative form in section 3.10.

*☐ Advanced MRI Core

*☐ Transgenic Animal Modeling Core

*☐ Metabolism Core

*☐ Micro CT

*☐ Radio Labeling Small Animal Translational Imaging Core

3.10 Please provide a detailed description of the procedures for each species in narrative form. Do not include procedures done post mortem. Do not include survival surgery. Please use Form D to describe details of survival surgical procedures.

Adult tissue collection: mice are euthanized via CO_2 inhalation followed by cervical dislocation, and tissues are collected post-mortem.

Fetal liver cell collection: pregnant mice are euthanized via CO_2 inhalation, followed by cervical dislocation, and embryos (E11 to E16) are removed. The embryos are placed immediately in ice-cold solution of either phosphate-buffered saline or IMDM (Iscove's Modified Dulbecco's Medium, in an ice-bucket; fetal livers are then harvested.

Pulse thymidine analogue (BrdU, CldU or IdU) labeling in-vivo: mice are injected intra-peritoneally with a single injection of BrdU, CldU or IdU. A second injection may be delivered within 1 to 8 hours (all single injections of 10mg.ml in a total volume of 100 microliters of sterile saline). Mice are euthanized by CO_2 inhalation and cervical translocation 30 minutes after the last injection and tissues are collected post mortem. All thymidine analogues will be prepared and stored in accordance with IACUC polices: http://inside.umassmed.edu/IACUC/Policies/Converted-pdfs/304_USE-OF-5-BROMO-2-DEOXYURIDINE-BrdU-IN-MICE/

http://inside.umassmed.edu/IACUC/Policies/Converted-pdfs/305_USE-OF-5-ETHYNYL-2-DEOXYURIDINE-EdU-IN-MICE/

Longer bromodeoxyuridine (BrdU) labeling in-vivo: Adult mice (6 to 16 weeks) are injected intra-peritoneally with BrdU, once a day for up to 3 days. The volume of injected solution will be 100 microliters. Mice are euthanized as described under 'Adult tissue collection' at the end of this procedure and tissues will be collected post mortem.

Cell cycle experiments with H2B-GFP: Adaptive transfer of erythroid progenitors: We will sort erythroid progenitors at the pre-CFU-e stage (Lin^- Kit^+ $Sca1^-$ $CD150^+$ $CD105^+$) [12] from doxycyline-treated mice that conditionally-express H2B-GFP, and inject 10^4 of these cells, resuspended in sterile saline in a total volume not exceeding 150 microliters, into non-ablated wild-type recipients of the same background strain. We will sacrifice the recipients within the ensuing 7 days. Injection will be via the tail vein.

If necessary (in case the H2B-GFP label does not provide sufficient strength of signal), we will carry out a similar experiment, in which we use pre-CFU-e cells sorted from wild type mice. These cells will be labeled prior to injection into recipients in vitro with the vital dye CFSE (carboxyfluorescein diacetate, 5mM stock in DMSO will be diluted to 10 micromolar final concentration in sterile culture medium). This technique is used routinely to measure consecutive cell divisions in vivo (Lyons, A.B. (2000). Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution. J Immunol Methods *243*, 147-154.).

Induction of gene expression in conditional mouse models:

1. Mice expressing histone 2B-GFP will require doxycycline in their drinking water at 2mg/ml starting at 4 to 8 weeks for up to 6 weeks, prior ot their use in experimentation. Appropriate cage cards will flag cages with medicated water per IACUC guidelines
2. To induce Cre-mediated recombination *in utero* around E12.5 to E14.5 of the conditional triple knockout pRb-/-p130-/-p107-/- mouse, or in 'floxed' Tet2 and Tet3 mice: we will inject timed-pregnant female mice between 9.5 and 13.5 days post-coitus with a daily injection of tamoxifen intraperitoneally (IP) for two consecutive days.

We will use between 3 mg of tamoxifen in 0.1 ml volume, following a protocol described by Danielian et al., Current Biology 8:1323 1998 and in Nakamura et al., Developmental Dynamics 235:2603–2612, 2006.

3. To induce Cre-mediated recombination in adult 'floxed' Tet2 and Tet3 mice: adult mice age 8 to 12 weeks will be injected with tamoxifen as described in '2' above, for up to a total 5 consecutive injections, administered every other day. Mice will be euthanized for tissue harvest 4 days to 4 weeks following the first injection.
4. Tamoxifen (Sigma, T-5648) will be prepared as described in https://inside.umassmed.edu/globalassets/iacuc/documents/3.02.02_tamoxifen_10.22.18.pdf

Ingredients

Tamoxifen is light sensitive, and should be made and stored in a light blocking vessel (amber, or foil wrapped)

1. Tamoxifen (e.g. Sigma, T5648), the highest-grade equivalent chemical reagent
2. USP-grade Corn oil is available and can will be used

Directions

1. Prepare and store using sterile technique with sterile supplies (e.g. vials, tubes, tops, needles, and syringes)
2. Weigh tamoxifen into a clear glass vial, add oil to make the solution 10 mg/ml or 20 mg/ml depending on the required volume
3. Dissolve tamoxifen in oil by 1) heating to 50 °C and stirring for a few hours or 2) shaking overnight at 37 °C
4. Filter sterilize through an oil-resistant 0.2 micron filter (e.g. Pall Acrodisc PN 4602) into a sterile vial (required for parenterals)

*Use a Luer Lok Syringe to screw on to the filter as the oil-coated filter does not stay on the syringe very well.

Erythropoietin (Epo) injection: Adult mice are injected subcutaneously or intravenously through the tail vein with recombinant erythropoietin (Amgen corporation; we use human erythropoietin sold for intravenous injection in patients). Mice are injected with up to 12 units per gram, in a total volume of 150 microliters. The Epo stock is obtain from Amgen, pharmaceutical grade erythropoietin ('epogen' or 'procrit'). It is kept in its original sterile vial, and arrives pre-dissolved in an aqueous preparation made by the manufacturer that is used for human injections. Control mice are injected with the same volume of saline. Mice are euthanized as described under 'Adult tissue collection', between 15 minutes to 14 days post injection.

Reduced atmospheric oxygen Adult mice (6 to 16 weeks) in their own cage will be placed in a reduced-atmospheric oxygen (12% or 10% oxygen) chamber for up to 21 days. Mice are monitored visually every 24 hours for the duration of the experiment for signs of reduced activity or tiredness. Objective signs for well -being of mice that we will be looking for are: breathing rate; activity within the cage; food and water consumption. We find that with our current procedure of placing the mice directly at 12% oxygen, mice usually explore the new environment immediately and always appear to have made a nest out of the nestlet square within the first 24 hours. Food supplies will be adjusted and bedding changed as required. Mice placed in 10% oxygen are monitored more closely, every 6 hours for the initial 12 hours and every 12 hours for the following 48 hours. Mice tolerate 10-12% oxygen well, with no adverse signs. Erythropoietic response at 10-12% is significant but mild: the hormone erythropoietin (Epo) increases in serum 5 fold, while its known published range in serum in response to hypoxia or anemia in mice and humans is an increase of up to 1000 fold (Abbrecht et al., Journal of Applied Physiology 32:54-58). A protocol of 10% oxygen for 21 days has been used by others, e.g. Yu et al., *J. Clin. Invest*. **103**:691–696 (1999). The literature suggests that brain odema, a cause of stress in hypoxia, does not occur at 10% oxygen (though it begins to be seen at 8%, see Schoch et al., Brain (2002), 125, 2549±2557). If the mice show marked reduced activity and marked increased

respiratory rate they will be euthanized immediately. Mice will be euthanized as described under 'Adult tissue collection' at the end of this procedure and tissues will be collected post mortem

Description of chamber: We use a 'controlled atmosphere animal chamber' designed by BiosPherix (Redfield, NY 13437). The chamber dimensions are 30"(76cm)W x 20"(51cm)D x 20"(51cm)H (=' A-30274 Medium "A" Chamber'). It allows the placement of two mouse cages (of the routine size used at the LRB). The chamber is made of transparent plastic making it possible to monitor mice visually without interference with the chamber operation. It is housed in our mouse room at the LRB. The chamber is normobaric. It provides adjustable control of the partial pressure of oxygen by infusion of inert nitrogen gas to displace total air in the chamber. The normal barometric pressure is maintained via air ventillation holes. The chamber consists of an enclosed space with adjustable ventillation holes, oxygen sensor, carbon dioxide sensor, humidity and temperature sensor, regulator box for nitrogen infusion, and a nitrogen infusion port. We use microisolator cages that are placed within the chamber. The cages are altered in 2 ways: we add water bottles, and change the cover to a static cover that allows better airflow. In experiments that take longer than 48 hours the cages are changed in a hood every 3 days. The BiosPherix chamber itself is maintained cleaned by simple wiping with a cloth (no direct contact between the mice and the chamber, since the mice are placed within cages).

Bone-marrow transplantation experiments:

Donor mice will be euthanized prior to tissue harvest (either bone-marrow or fetal liver).
Recipient mice will receive a split dose of 525 rads (LRB 1st floor gamma irradiator) x 2 (separated by a minimum of 3 hours) to minimize or eliminate non-hematopoietic toxicity then will receive approx $1x10^6$ donor cells plus 2x10^5 radioprotective wildtype marrow cells in 50-100 µl by tail vein injection. To visualize the tail veins, mice will be warmed under a heat lamp for 1-2min. If the animal appears agitated they will immediately be removed from heat source. Sterile gauze and pressure will be applied to site of injection to stop bleeding. Mice will be placed in clean cages and monitored daily for recovery. They will be maintained on acidified water (pH2.5-3.0 adjusted with HCl and supplied by Animal Medicine) for the first month. The split radiation dose given with radioprotective doses of rescue bone marrow immediately afterward do not result in apparent distress to the recipient animals. Mice will be followed closely after transplant and sacrificed if in distress, although no disease is anticipated. Bi-weekly blood sampling will be performed from the tail vein. No more than 2-3 drops (100-150 µl) of blood will be collected then bleeding will be stopped with gentle pressure to the puncture site. Mice will be euthanized for tissue harvesting at the end of the experiment, 2 to 8 weeks following transplantation.

Tail Snipping procedure

Genotyping on mice is usually performed between days 17 and 21. In this case, 0.2 cm tail biopsy will be obtained. If for any reason we need to perform this procedure between days 22 and 28, we will use a single dose of injectable analgesic 10 – 30 minutes before the procedures (usually, Buprenorphine, SC). Very occasionally and quite rarely, we may need to re-establish the genotype of a mouse if some uncertainty arises with respect to the first genotyping procedure at a younger age (e.g. a technical difficulty). In this case, if the mouse is > 28 days, we will use a single dose of an analgesic, and in addition, general anesthesia with isoflurane.

For all these cases, the procedure is as follows:

Disposable sterile gloves are worn throughout the procedure. The mouse is restrained gently, but securely; the state of anesthesia, if used, is tested with a toe pinch. The tail is swabbed with antiseptic, e.g. 70% ETOH, betadine or chlorhexidine. 0.2 cm (and not more than 0.5 cm) are snipped with a sterile razor or scalpel blade. A new blade will

be used for each animal. If for any reason blades need to be re-used, they will be pre-soaked in 70% ETOH for 30 minutes.

The incision site will be monitored for bleeding. Pressure with a dry gauze will be applied to aid hemostasis. The mice will be re-examined for bleeding or signs of pain after recovery.

The administration of any analgesics or anesthetics, as indicated above, will be documented. Any animals that were anesthetized will be monitored until they are awake and start to be mobile.

Between animal cages, the working surface, gloves and all equipment will be disinfected with 70% ETOH or chlorhexidine.

Breeding Scheme

Cages with continuous breeding have one male and one female per cage. However, we also employ harem breeding, particularly when setting up timed pregnancies (which do not result in birth, we usually sacrifice the pregnant female at mid-gestation. In harem breeding, we place 2 females and 1 male per cage. The pregnant female will be separated if it approaches term to avoid litters born into cages with harem breeding.

Mice will be weaned before 28 days. In continuous breeding cages, mice will be weaned at 21 days.

3.11 Indicate how you will identify animals. *	*☐ Animal identification is not necessary for this protocol. Cage cards will be used. *☒ Ear tagging/notching *☐ Tattoo *☐ Dye or ink marking *☐ SQ radio *☐ Tag (microchip) *☐ Vendor-placed tag or tattoo *☐ Other

[If "Other" is selected in 3.11, please explain the method you will use]

3.12 List any special housing, handling, husbandry or care that animals may need. Check all that apply.

Please provide a description and justification for each box checked above:

*☐ Single housing for social species. Provide a description and justification below.

*☐ Cage density exceeding limit. Provide a description and justification below.

*☐ Modified Cage change frequency exception. Provide a description and justification below.

*☐ Animal husbandry done by research staff. Provide a description and justification below.
*☒ Deviation from standard housing condition (cold exposure, light/dark cycle, standard enrichment plan). Provide a description and justification below.
We will use a reduced oxygen chamber to place the mice in their original cages for up to 10 days. This is a model of accelerated erythropoiesis, which we study, as detailed under 'background' (see page 3). The technical details of the reduced chamber are detailed under section 3.17.
*☐ Food/water manipulation (e.g. Medicated food and/or water, increase/decrease food, special diet)
*☐ Other: Please describe and provide a justification below.

3.13 Is death used as an endpoint in this study? *	☐ YES ☒NO
3.14 If you answered "YES" to 3.13, explain why an earlier end point is not acceptable. (Studies using death as an endpoint are Category E)	
3.15 Will there be the presence of pain, discomfort, or other adverse events caused by disease models, surgery, or other procedures used in your studies? *	☐ YES ☒NO
3.16 If you answered "YES" to 3.15, please indicate what will be monitored. Check all that apply.	
*☐ Activity *☐ Appearance *☐ Appetite *☐ Behavior *☐ Excreta *☐ Grooming *☐ Guarding *☐ Heart rate	*☐ Licking, biting *☐ Posture *☐ Respiratory rate *☐ Temperature *☐ Vocalizing *☐ Weight loss *☐ Wound site *☐ Other (describe below)

3.17 Animal monitoring is required for progressive disease models and painful/distressful procedures. Please describe frequency of monitoring during disease progression or procedures causing pain or distress (written documentation of ongoing monitoring will be required once protocol is approved). Do not include post-operative monitoring in this section.

Mice in the reduced oxygen chamber will be monitored once every 24 hours.

In the initial period of acclimatization to the chamber, mice in 10% oxygen will be monitored every 6 hours for the first 12 hours, and every 12 hours for the next 48 hours. In the initial pilot experiments for 10% oxygen we will be monitoring mice more frequently, see 'methods' above. We have done these experiments routinely (including in published work, e.g. Koulnis et al. Blood 2012) and in our experience mice tolerate these conditions well.

Following bone-marrow transplantation, mice will be monitored daily for the first 2 weeks, and bi-weekly in subsequent weeks. Diseased animals will be identified by ruffled fur, decreased activity, increased respirations, hunched posture, guarding or circling behavior will be promptly euthanized.

3.18 What criteria will be used to perform euthanasia earlier than planned? Please include conditions and complications that would lead to removal of an animal from the study.

Marked increase in respiratory rate or marked decreased activity and loss of appetite (loss of weight > 10%) will suggest that mice in the reduced oxygen chamber are in discomfort and will be euthanized. Respiratory rate would be excessive if it is increased to the point that the mice will be observed to refrain from any other physical activity other than very fast respiration that greatly (>50%) exceeds the normal respiratory rate and that persists for more than 6 hours.

3.19 Describe the method(s) of euthanasia for each species or procedure. *

Unless otherwise specified by the principal investigator, Department of Animal Medicine personnel who are euthanizing animals on behalf of the principal investigator will do so using any method listed as acceptable for the species in the most recent edition of the euthanasia guidelines of the American Veterinary Medical Association. Neonatal and pregnant rats and mice will be euthanized using methods approved by the UMMS IACUC.

Species	Primary Method	Dose	Route	Secondary Method	Comments
Mouse	CO2 asphyxiation	To effect	Inhalation	Cervical dislocation	
Mouse Embryos	Embryos are removed once the dam is dead				All embryos are harvested between E12.5 and E14.5. As per IACUC procedures, they do not require further action to ensure they are dead.
Select Species	Select Primary			Select Secondary	

Select Species	Select Primary			Select Secondary	
Select Species	Select Primary			Select Secondary	
Select Species	Select Primary			Select Secondary	
Select Species	Select Primary			Select Secondary	
Select Species	Select Primary			Select Secondary	
Select Species	Select Primary			Select Secondary	
Select Species	Select Primary			Select Secondary	
Select Species	Select Primary			Select Secondary	

FORM A: TRANSPORTING ANIMALS FOR USE OUTSIDE OF OR TO ANOTHER AREA OF THE ANIMAL FACILITIES

A1 Will animals be used in areas, e.g. laboratories, outside one of the general animal facilities? *	☐ YES ☒ NO

A2 If you answered yes to A1, list the building and room number(s) where animals will be housed or used outside the animal facility. Please check 'yes' for extended housing if animals will be kept in this area for greater than 24 hours (mice and rats) or 12 hours (all other species).

Building	Room #	Species	Survival Surgery	Terminal Surgery	Non-Surgical Procedure	Extended Housing
Choose location			☐ YES ☐ NO	☐ YES ☐ NO	☐ YES ☐ NO	☐ YES ☐ NO
Choose location			☐ YES ☐ NO	☐ YES ☐ NO	☐ YES ☐ NO	☐ YES ☐ NO
Choose location			☐ YES ☐ NO	☐ YES ☐ NO	☐ YES ☐ NO	☐ YES ☐ NO
Choose location			☐ YES ☐ NO	☐ YES ☐ NO	☐ YES ☐ NO	☐ YES ☐ NO
Choose location			☐ YES ☐ NO	☐ YES ☐ NO	☐ YES ☐ NO	☐ YES ☐ NO

A3 If animals will be held, housed and/or used in an area outside of the animal facility for an extended period of time, please describe the duration and reason.

A4 How will you transport animals from the animal facility to a study area outside of the animal facility? *

A5 When moving animals from one Department of Animal Medicine managed housing room to a different animal housing room, do you agree to use the Web Animal Ordering System. *	☒ YES ☐NO

FORM B: BREEDING	
B1 How many animals do you expect to breed for each species? *	
Species	Number of animals to be bred
mouse	5124
B2 Male / Female standard ratio: check all that apply *	*☐ a ratio of 1:1 will be used. No continuous breeding. *☐ a ratio of 1:1 with continuous breeding will be used. *☒ a ratio of 1:2 or 1:3 will be used (Harem breeding). *☐ Non-standard ratio
B3 Explain non-standard ratios:	
B4 I have read the IACUC Mouse Breeding Policy that prohibits more than one litter per cage. I agree to separate pregnant females to other cages prior to their giving birth in harem breeding and to wean continuously bred litters at day 21 if the female is pregnant. *	☒ YES ☐NO
B5 At what age will the offspring be weaned (if applicable)? *	
21 to 28 days	
B6 Please justify exceptions to the IACUC policies on rodent breeding and overcrowding.	

FORM C: ANESTHESIA AND ANALGESIA

C1 Pre-anesthetic Agents (e.g. tranquilizers, narcotics) and Anesthetic agents. Please refer to IACUC website for specific policies on the use of ketamine/xylazine, isofluorane or avertin.

Species	Agent	Dose	Route	Frequency & Duration	Procedure
Mouse	*Isoflurane*	*We will employ the "drop method" of anesthetic delivery with appropriate volumes of liquid isoflurane administered per the* ***Dosing Chart*** *on the IACUC intranet site*	inhalation	*Very rarely, if required for tailing of mice older than 28 days*	*Tail snipping*

C2 Please describe secondary anesthetic dosing, if applicable.	
C3 If anesthetizing animals with isoflurane using the open drop method, will IACUC policy 4.04 (hyperlink) Use of Isoflurane: drop method be followed? *	☒ YES ☐ NO
C4 List any non-pharmaceutical grade anesthetic or pre-anesthetic agents listed in C1.	☐ YES ☐ NO ☐ NA
C5 If using non-pharmaceutical grade anesthetics or pre-anesthetic agents, please justify	☐ YES ☐ NO ☐ NA
C6 I agree to follow IACUC policy 3.01 on handling and storage of drugs/chemicals to maintain sterility. *	☒ YES ☐ NO
C7 Monitoring of Anesthesia: Describe what will be monitored.	* ☐Palpebral reflex * ☐Heart rate * ☒Respiration * ☐Temperature * ☐Toe Pinch * ☐Other: describe below
C8 Describe how frequently each of the signs listed in C7 will be monitored.	
We will monitor breathing rate continuously, and at least every 2 minutes, allowing it to decrease down to 1 to 2 per second, at which point the animal will be considered anesthetized for a brief period and be removed into an isoflurane-free environment.	
C9 Describe the anesthetic gas scavenging system you will use to eliminate waste anesthetic gas.	* ☐Building vacuum system * ☒Non-recirculating fume hood or externally hard-ducted hood * ☐Activated charcoal canisters (e.g. F/air canister) * ☐Other: describe below
We use isoflurane in extremely low amounts (one or two drops at most). It is used in the procedure room flow hood, which is hard-ducted. We let the isoflurane-dabbed gauze dry while in the flow hood before disposing of it.	

C10 Please describe analgesic agents (must be USP grade).

Species	Agent	Dose	Route	Frequency & Duration	Procedure
Mouse	*Buprenorphine*	*1 mg/kg ,*	SC	once	*Only when tail snipping age >21 days*
Mouse	*isoflurane*	1-2 drops	inhalation	once	Only when tail snipping age >28 days

FORM D: SURVIVAL SURGERY INCLUDING LAPAROSCOPY, ENDOSCOPY	
(and any procedure that has a reasonable potential of causing a permanent physical or physiological handicap.)	
D1 Explain why it is necessary for the animals to recover from surgery/anesthesia. *	
D2 Describe pre-operative care (including physical examinations, lab tests, and any preconditioning apparatus) Note: All anesthetic agents and pre-operative medications should be listed in Form C. *	
D3 Animal pre-surgical preparation. If using small animals, will you follow pre-surgical animal preparation as described in IACUC policy 6.12 for Basic Survival Surgical Preparation? If no, please describe. *	☐ YES ☐ NO
D4 Instrument pre-surgical preparation. If using small animals, will you follow pre-surgical instrument preparation as described in IACUC policy 6.10 for Autoclave Sterilization of Surgical Instruments? If no, please describe. *	☐ YES ☐ NO
D5 Surgeon's pre-surgical preparation. If using small animals, will you follow pre-surgical surgeon preparation as described in IACUC policy 6.12 for Basic Survival Surgical Preparation? If no, please describe. *	☐ YES ☐ NO
D6 Describe each surgical procedure in detail. *	

D7 Describe post-operative monitoring until fully recovered. *	
D8 Describe any expected or potential post-operative complications. *	
D9 Will animals undergo more than a single survival surgical procedure? If yes, please explain. *	☐ YES ☐ NO

FORM E: ADMINISTRATION OF SUBSTANCES OTHER THAN ANESTHETICS/ANALGESICS

E1 Biologics: Cells or Cell/Tissue Extracts or Blood Products (do not include purified cell products here).

Agent	Species of Origin	Species	Dose	Volume	Route	Frequency & Duration
Bone marrow cells	*Mouse*	*Mouse*	*Up to 10^7 cells*	*<0.5ml*	*IV*	*1 dose*

E2 Agents/Drugs (excluding analgesics and anesthetics)						
Agent	**Species**	**Dose**	**Volume**	**Route**	**Frequency Duration**	**USP (Y/N)**
Bromodeoxyuridine (BrdU)	*Mouse*	*50mg/Kg*	*0.1 ml*	*Intra-peritoneal*	*Once a day for up to 3 days*	*Y*
Reduced atmospheric oxygen	*Mouse*	*10 to 15% oxygen*		*inhalation*	*A single exposure of up to 21 days*	*N/A*
Erythropoietin (Epo)	*Mouse*	*Up to 12 units/gram*	*0.15 ml*	*intravenous or subcutaneous*	*Once only*	*Y*
PMSG (pregnant mare serum gonadotrophin)	*Mouse*	*5 IU*	*0.1 ml*	*Intra-peritoneal*	*Once 2 days prior to mating*	*Y*
HCG (human chornionic gonadotrophin)	*Mouse*	*5 IU*	*0.1 ml*	*Intra-peritoneal*	*Once on the eve of mating*	*Y*
Doxycycline	*Mouse*	*2mg/ml*		*In drinking water*	*starting at 4 to 8 weeks for up to 6 weeks, prior ot their use in experimentation.*	*Y*
dexamethasone	*Mouse*	*2mg/kg to 10 mg/kg*	*0.15 ml of sterile saline*	*intraperitoneal*	*once per day for up to 5 consecutive days*	*Y*
tamoxifen	*Mouse*	*3mg*	*0.1ml in USP Grade Corn oil*	*intraperitoneal*	*Up to 5 times, every other day*	*Y*
Chlorodeoxyuridine (CldU)	*Mouse*	*50mg/Kg*	*0.1 ml of 10mg/ml stock*	*Intra-peritoneal*	*Once only*	*N (no USP grade in exists)*
Iododeoxyuridine (IdU)	*Mouse*	*50mg/Kg*	*0.1 ml of 10 mg/ml stock*	*Intra-peritoneal*	*Once only*	*N(no USP grade in exists)*
Ethynyldeoxyuridine (EdU)	Mouse	*50mg/Kg*	*0.1 ml of 10 mg/ml stock*	*Intra-peritoneal*	*Once only*	*N(no USP grade in exists)*

E3 Rodent-derived materials must be tested for pathogens prior to use according to UMMS policy. If using rodent-derived materials, do you agree to submit pathogen testing results prior to use? *	☐ YES ☐ NO ☒ N/A
E4 Please justify use of non-USP grade for agents listed:	* ☒ USP is not available in suitable formulation * ☒ Other: describe below
BrdU, CldU, EdU and IdU do not exist in pharmceutical grade. We obtain BrdU preparations for injection from BD Biosciences. We obtain CldU and IdU from Sigma. All three analogues are >99% pure and are prepared in a stock of 10mg/ml in sterile phosphate-buffered saline.	
E5 I agree to follow IACUC policy 3.01 on handling and storage of drugs/chemicals to maintain sterility. *	☒ YES ☐ NO

FORM F: PROLONGED PHYSICAL RESTRAINT OR STRESS OF CONSCIOUS ANIMALS	
F1 Will animals undergo: *	*☐ Prolonged physical restraint of conscious animals beyond routine handling *☐ Electric Shock *☐ Other: describe below
F2 Why are restraints or adverse conditions required? *	
F3 Describe condition(s) and/or device: *	
F4 For animals confined to a device, please describe the duration and frequency animals will be confined. Please include how the general well-being of the animals will be determined and observation interval during confinement.	
F5 For animals confined to a restraint device, describe the acclimation to device, if applicable.	

FORM G: HAZARDOUS AGENT INFORMATION	
G1.a Will this project require the use of any of the following agents in live animals:	
G1.a Infectious biological agents (pathogenic to man or animal)? *	☐ YES ☒ NO
G1.b Recombinant nucleic acids (e.g., recombinant plasmids, lentivirus, adenovirus, AAV)? *	☐ YES ☒ NO
G1.c Synthetic nucleic acids? *	☐ YES ☒ NO
G1.d Materials of human origin (tissues, cells, etc.)? *	☐ YES ☒ NO
G1.e Materials of non-human primate origin (tissues, cells, etc.)? *	☐ YES ☒ NO
G1.f Biotoxins (e.g., LPS)? *	☐ YES ☒ NO
G2 List below the hazardous agents that require housing of animals in animal biocontainment ABSL-2 or ABSL-3 per the IBC-approved animal addendum. These agents must also be listed in form E.	
G3 Will this project require creation of new transgenic, knockout, knock-in or other mutations in the genome of animals in any UMMS laboratory or transgenic core facility? *	☐ YES ☒ NO
G4 Will this project require the breeding of transgenic animals that contain transgene encoding more that 50% of the genome of an exogenous eukaryotic virus from a single family or express transgene under the control of gammaretroviral long terminal repeat? *	☐ YES ☒ NO
G5 Will your research activities potentially expose humans, other than researchers and Animal Medicine personnel who are already trained, to zoonotic infections (e.g., Q fever from sheep)? *	☐ YES ☒ NO
G6 If checked yes to G3-G5, please describe briefly	
G7 Will your research involve working with nonhuman primates? *	☐ YES ☒ NO
G8 If you have IBC approval for the studies described in this protocol please fill in the requested information below. NOTE: Only individuals listed and approved in the IBC and IACUC protocols are authorized to work with hazardous biological agents in live animals.	
G8.a IBC protocol number	
G8.b Principle Investigator of the IBC protocol	
G9 Will this project require the use of ionizing radiation in live animals? If Yes, Radiation Safety approval is required*	☒ YES ☐ NO
G10 If checked "yes" for G9, identify the name of the agent(s) below	
<u>Bone-marrow transplantation experiments:</u> Recipient mice will receive a split dose of 525 rads (LRB 1st floor gamma irradiator) x 2 (separated by a minimum of 3 hours).	

G11 Will this project require the use of hazardous chemicals in live animals? *	☒ YES ☐ NO

G12 If checked "yes" for G11, identify the name of the agent(s) below

We will be using thymidine analogues, BrdU, ClDU, EdU or IdU, in cell cycle status studies. This will involve one or two intraperitoneal injections of 100 microliters of one of the analogs, at a concentration of 10mg/ml in sterile saline. The mice will be euthanized within 30 minutes to 8 hours of these injections.

Tamoxifen: this will be used for deletion of genes in vivo in mice with 'floxed' genes (tet2, Tet3, Rb family proteins). Mice will be injected intraperitoneally with 3mg of tamoxifen, dissolved in corn oil as described above, in a total volume of 200 microliters. Injections will be repeated every 48 hours for up to 5 total injections. Mice will be euthanized within 4 days to 4 weeks of the first injection.

FORM H: USE OF ANIMALS IN A TRAINING COURSE

This form is designed for PIs conducting training courses. Training participants are not permitted to work independently with live animals and must be under the supervision of a trained UMMS investigator during any contact with live animals. According to federal regulations, the IACUC must ensure the occupational health and safety of all individuals working with animals, and that all individuals working with animals receive appropriate training. Because the training participants may not be known at the time of protocol submission, the PI must provide assurance that Occupational Health and Safety and training requirements will be met before trainees participate in the course. Below outlines requirements for participants.

FOR PARTICIPANTS AFFILIATED WITH UMMS (students, residents, employees or volunteers must complete the following requirements before beginning the course):

1. ***Occupational Health and Safety Requirements:*** health clearance by Employee Health Services. An initial health clearance form must be submitted to Employee Health Services and approved before participating in the course.

2. ***Regulatory training requirements:*** Training through the AALAS Learning Library: Module 1, Module 2 and species-specific training. Information on accessing the AALAS Learning Library is available on the IACUC website.

3. Submission of an amendment to add the participant and an approval letter from the IACUC Office.

FOR PARTICIPANTS NOT AFFILIATED WITH UMMS:

1. ***Occupational Health and Safety Requirements:***
   - If the participant is NOT a UMMS student, resident, employee or volunteer, Initial Health Clearance and Visiting Researcher forms may be submitted to Employee Health Services.
   - If trainees are only attending a single training session, in lieu of obtaining health clearance, the participant may submit a signed Informed Consent form indicating they are aware of and understand the risks of working with animals and agreeing to participate at his/her own risk. The PI is required to submit the consent forms to the IACUC office along with the participant roster.
2. ***Regulatory training requirements:***
   - Training through the AALAS Learning Library: Module 1, Module 2 and species-specific training. Information on accessing the AALAS Learning Library is available on the IACUC website.
   - In lieu of the AALAS Learning Library, the PI or his designee can provide training in a lecture format (the lecture should include an overview of Federal Regulations, ethical guidelines for using animals, hazards and risks associated with the use of animals, species-specific information, humane techniques for animal procedures, and other appropriate information relevant to the experimental procedures being used)
3. Submission of the participant roster (below) to the IACUC office, as training sessions occur, including the name of participants and method of health clearance for each individual.

Participant Name	Date of Training Course	Health Clearance	Regulatory Training
		☐ Informed Consent ☐ Health Clearance from EHS	☐ Instructional lecture ☐ AALAS Learning Library
		☐ Informed Consent ☐ Health Clearance from EHS	☐ Instructional lecture ☐ AALAS Learning Library
		☐ Informed Consent ☐ Health Clearance from EHS	☐ Instructional lecture ☐ AALAS Learning Library
		☐ Informed Consent ☐ Health Clearance from EHS	☐ Instructional lecture ☐ AALAS Learning Library
		☐ Informed Consent ☐ Health Clearance from EHS	☐ Instructional lecture ☐ AALAS Learning Library
		☐ Informed Consent ☐ Health Clearance from EHS	☐ Instructional lecture ☐ AALAS Learning Library
		☐ Informed Consent ☐ Health Clearance from EHS	☐ Instructional lecture ☐ AALAS Learning Library
		☐ Informed Consent ☐ Health Clearance from EHS	☐ Instructional lecture ☐ AALAS Learning Library

PART 5: PARTICIPATING PERSONNEL

First	Last	Phone	Emergency Phone	Responsibilities	Years experience with technique	Years experience with species	First time user (Y/N)
Merav	*Socolovsky*		*617 7971633*	*PI* *Set up matings; Euthanasia for the purpose of colony management or experiments; Injection of agents*	*25*	*25*	N
Daniel	*Hidalgo*	*508 856 3704*		*Graduate Student* *Set up matings; Euthanasia for the purpose of colony management or experiments; Injection of agents Tail snipping*	*9*	*9*	N
Yung	*Hwang*	*508 856 1945*		*Graduate Student* *Set up matings; Euthanasia for the purpose of colony management or experiments; Injection of agents Tail snipping*	*5*	*5*	N
Aishwarya	*Swaminathan*	*508 856 6102*		*Postdoctoral fellow* *Set up matings; Euthanasia for the purpose of colony management or experiments; Injection of agents Tail snipping*	*5*	*5*	N

Briefly describe how the individuals with less than one year of experience with the technique(s) in species will be trained and/or supervised. Identify the individual(s) who would be responsible for training and/or supervising new or inexperienced personnel. Note that hands-on training can be given by an experienced personnel or designated Department of Animal Medicine staff. Those who perform survival surgery must receive aseptic Survival Surgery Training from the Department of Animal Medicine before approved as a surgeon. Please contact Van Gould (extension 66811) or Suzanne Wheeler (extension 62363) for training.

ADDITIONAL NOTES ON PARTICIPATING PERSONNEL:

IACUC training webpage IACUC new personnel webpage

- Before other new personnel perform any procedures, a written minor amendment request must be submitted to and approved by the IACUC. All personnel new to UMMS or those who never worked with animals will have to complete all the Training and Occupational Health and Safety Requirements before added on an IACUC protocol.
- The principal investigator is responsible for ensuring that all personnel adhere to the conditions approved by the IACUC.

APPLICANT'S CERTIFICATION

IACUC is charged with carrying out the rules and regulations of the Federal Government's Animal Welfare Act governing the care and use of animals in research and instruction. The Act stipulates that (a) Principal Investigators must give written assurance that the activities do not unnecessarily duplicate previous experiments; (b) procedures involving animals must avoid or minimize discomfort, distress, and pain to the animals; (c) Principal Investigators must consider alternatives to procedures that cause more than momentary or slight pain or distress to the animals and give a written description of methods used to determine that alternatives are not available; and (d) paralytic agents cannot be used in unanesthetized animals. Accordingly, the Applicant, who must be a member of the faculty holding Principal Investigator status, is required to read and sign the following certification:

BY SIGNING BELOW, I CERTIFY THE FOLLOWING:

1. I am thoroughly familiar with the literature in the field of research proposed in this application, and I have determined that the research does not unnecessarily duplicate experiments, that appropriate non-animal models are not available, and that the research must be conducted on living animals.

2. I will abide by all UMMS policies and procedures regulating use of animals in instruction and research, by the provisions of the PHS/NIH Guide for the Care and Use of Laboratory Animals, and by all other applicable laws, policies, and regulations governing the use of animals in instruction and research.

3. I will supervise all experiments involving live animals. Furthermore, I will ensure that all listed participants are qualified or will be trained in proper procedures, including animal handling, anesthesia, surgery, post-procedural management, and euthanasia. Also, I will ensure that individuals not listed in the application will not have responsibility in experiments involving animals.

4. All listed personnel will read the IACUC-approved Application to Use Vertebrate Animals in Research or Instruction before undertaking any procedures on laboratory animals.

5. Survival surgery will be performed using standard aseptic procedures.

6. Animal Medicine clinical veterinary staff will be consulted as needed to ensure satisfactory veterinary care.

7. In the event of an animal health emergency, my staff or I will contact the Department of Animal Medicine. We will not attempt animal treatment by ourselves, unless it is a life-threatening emergency.

8. If I cannot be contacted, and animals in this project show evidence of illness or pain, emergency care, including euthanasia, may be administered at the discretion of the Animal Medicine veterinary staff.

9. Significant changes in study objectives and procedures require IACUC approval.

10. Unanticipated adverse events will be reported to the IACUC as required by UMMS IACUC policy

11. This application meets all animal use and care requirements of the funding agencies that have been asked to support the research.

12. By signing below, I certify that all animal studies described in grant proposals using this protocol are described in this animal use application.

PI's Signature: Date:

Worksheet # 1

A **Experiments with adult mice will be done with the following strains:**

	cell cycle status (*1)	RNA (*2)	gDNA (methylation studies) (*3)	bone-marrow transplantation	Total mouse number per stain
Tet2 f/f Cre+ or Cre-	x	x	x		60
Tet3 f/f Cre+ or Cre-	x	x	x		60
Tet2 f/f Tet3 f/f Cre+ or Cre-	x	x	x		60
EpoR-HM mice	x	x	x		60
Balb/C	x	x	x		90
C57BL/6	x	x	x	x	90
$DNMT1^{Chip/Chip}$	x	x	x		90
$GR^{dim/dim}$ (EMMA ID # 02123)	x	x	x		90
conditional H2B-GFP	x	x	x		60
B6;129S-*Tet2*$^{tm1.1Iaai}$/J x Vav/Cre	x	x	x		90
b-globin YAC transgenic	x	x	x		90
H2-BEX3 (GFP transgenics)				x	45

*1. Cell cycle status experiments in adults: up to 30 mice per strain (this includes controls). Includes mice in normoxia and hypoxia and at different time points, up to 10 days, following onset of hypoxia; and mice injected with 2 thymidine analogues at different time intervals for the measurement of S phase length.

*2. RNA experiments: up to 30 mice per stain; erythroid progenitors at a frequency of <0.5% in bone-marrow and spleen will be sorted by flow cytometry.
*3. gDNA for methylation studies: mouse numbers overlapping with RNA experiments, no additional numbers necessary.

B **Experiments with mid-gestation embryos with the following strains (timed pregnancies):**

	cell cycle status (*1)	RNA (*2)	gDNA (methylation studies) (*3)	in vitro differentiation of fetal liver cells (*4)	mouse numbers for this procedures
B6;129S-*Tet2*$^{tm1.1Iaai}$/J x Vav/Cre	x	x	x	x	150
EpoR-null	x	x	x	x	150
Stat5-null	x	x	x	x	150
Balb/C	x	x	x	x	150
C57BL/6	x	x	x	x	150
Rb-/-	x	x	x	x	150
PU.1-/-	x	x	x	x	150
pRb, p107, p130 conditional triple K	x	x	x	x	150
p107-/-	x	x	x	x	150
p130-/-	x	x	x	x	150
$DNMT1^{Chip/Chip}$	x	x	x	x	150
$p57KIP2^{-/-}$	x	x	x	x	150
conditional H2B-GFP	x	x	x	x	150
b-globin YAC transgenic	x	x	x	x	150
$GR^{dim/dim}$ (EMMA ID # 02123)	x	x	x	x	150
Tet2 f/f Rosa 26-CreERT2	x	x	x	x	150
Tet3 f/f Rosa 26-CreERT2	x	x	x	x	150
Tet2 f/f Tet3 f/f Rosa 26-CreERT2	x	x	x	x	150
EpoR-HM mice	x	x	x	x	150

*1. Cell cycle status experiments in adults: up to 50 adult mice per strain. Includes male mice required for plugging, and female mice for timed pregnancies. We aim to isolate approx. 50 embryos per experiment, which requires setting up approximately 7 matings for timed-pregnant mice.
*2. RNA experiments: up to 50 adult mice per stain. Timed pregnancies will be set up as for the cell cycle experiments.
*3. gDNA for methylation studies: mouse numbers overlapping with RNA experiments, no additional numbers necessary.
*4. Erythroid differentiation in vitro experiments: up to 50 adult mice per stain. Timed pregnancies will be set up as for the cell cycle experiments.

C **Total Number of experimental mice needed (from A and B above):**

	Strain*	Adult expts	Embryo expts	Total
1	Tet2 f/f Rosa 26-CreERT2	60	150	210
2	Tet3 f/f Rosa 26-CreERT2	60	150	210
3	Tet2 f/f Tet3 f/f Rosa 26-CreERT2	60	150	210
4	EpoR-HM mice	60	150	210
5	Balb/C	90	150	240
6	C57BL/6	90	150	240
7	$DNMT1^{Chip/Chip}$	90	150	240
8	$GR^{dim/dim}$ (EMMA ID # 02123)	90	150	240
9	conditional H2B-GFP	60	150	210
10	B6;129S-*Tet2*$^{tm1.1Iaai}$/J x Vav/Cre	90	150	240
11	b-globin YAC transgenic	90	150	240
12	HRI-/-		150	150
13	EpoR+/- / HRI-/-		150	150
14	PU.1-/-		150	150
15	pRb, p107, p130 conditional triple KO		150	150
16	p107-/-		150	150
17	p130-/-		150	150
18	EpoR-null		150	150
19	Stat5-null		150	150
20	$p57KIP2^{-/-}$		150	150
21	$Rosa26\text{-}CreER^{T2}$	60	150	210
22	H2-BEX3 (GFP transgenics)	150		150
Total				**4200**

* Control mice for each strain are included in the cited numbers (for example, for EpoR-HM, control mice are Balb/C mice, but these are included in the numbers cited for EpoR-HM).

Worksheet # 2

D **Breeding and calculation of total mouse numbers: (required mouse numbers calculated in 'C' above are in column '6'):**

	1 mouse strain (including matched controls):	2 outside sources**	3 # of matings	4 litter size	5 total pups	6 pups required for expts (see 'C' above)	7 not used due to wrong genotype	8 total # of mice for breeding	total animals
1	Tet2 f/f Cre+ or Cre-	0	12	6	72	60	0	12	72
2	Tet3 f/f Cre+ or Cre-	0	12	6	72	60	0	12	72
3	Tet3 f/f Cre+ or Cre-	0	12	6	72	60	0	12	72
4	EpoR-HM mice	0	12	6	72	60	0	12	72
5	Balb/C	100	48	6	288	240	0	48	388
6	C57BL/6	100	48	6	288	240	0	48	388
7	$DNMT1^{Chip/Chip}$	4	48	6	288	240	0	48	292
8	$GR^{dim/dim}$ (EMMA ID # 02123)	4	48	6	288	240	0	48	292
9	conditional H2B-GFP	4	48	6	288	240	0	48	292
10	GFP reporter mice		48	6	288	240	0	48	288
11	b-globin YAC transgenic		48	6	288	240	0	48	288
12	PU.1-/- or PU.1+/-	4	40	6	240	150	45	45	244
13	HRI-/-	4	40	6	240	150	45	45	244
14	EpoR+/-/HRI-/-	4	40	6	240	150	45	45	244
15	pRb, p107, p130 conditional triple KO	4	40	6	240	150	45	45	244
16	p107-/- or p107+/-	4	40	6	240	150	45	45	244
17	p130-/- or p130+/-	4	40	6	240	150	45	45	244
18	$Rosa26\text{-}CreER^{T2}$	4	40	6	240	150		45	244
19	EpoR-null or EpoR+/-		40	6	240	150	45	45	240
20	Stat5-null or Stat5+/-		40	6	240	150	45	45	240
21	p57KIP2-/+ or $p57KIP2^{-/-}$		40	6	240	150	45	45	240
22	H2-BEX3 (GFP transgenics)	0	30	6	180	150	0	30	180
	Total Mouse number (including experimental and breeding mice)								**5124**
	Mice being bred, but not used in experiments:								924

** We are planning to obtain 2 breeding pairs, equivalent to 4 mice, for mouse strains that we have not yet acquired
*** The Rosa26-ER mice will be crossed with the p107 and p130 floxed mice and Rb +/- mice to generate the triple KO (line 14); they will not be used for experiments.

mouse strains that will not need to be genoty: Balb/C, C57BL/6, $Rosa26\text{-}CreER^{T2}$

Number of mice in pain level C:	1200	These mice will not need to be genotyped
Mice that will need to be genotyped	3774	These mice will be in pain level C because of genotyping at 21 to 28 days with analgesic cover
	150	Mice that will be irradiated (pain level D)
total mice in the protocol	5124	