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## THE CONSEQUENCES OF DISRUPTING THE MDM2-P53 BALANCE IN HEMATOPOIESIS

Hussein A. Abbas

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**THE CONSEQUENCES OF DISRUPTING THE MDM2-P53 BALANCE IN  
HEMATOPOIESIS**

**A**

**DISSERTATION**

Presented to the Faculty of  
The University of Texas  
Health Science Center at Houston  
Graduate School of Biomedical Sciences  
and  
The University of Texas  
M. D. Anderson Cancer Center  
Graduate School of Biomedical Sciences  
in Partial Fulfillment

of the Requirements

for the Degree of

**DOCTOR OF PHILOSOPHY**

By

Hussein A. Abbas, B.S.

May, 2010

## **DEDICATION**

I dedicate this dissertation to my parents Ali and Hanan, and my siblings Tania, Lara, Katia, Mohamad and Aya for their unconditional love.

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# **ABSTRACT**

## **THE CONSEQUENCES OF DISRUPTING THE MDM2-P53 BALANCE IN HEMATOPOIESIS**

**Publication No.\_\_\_\_\_**

**Hussein A. Abbas**

***Supervisory Professor: Guillermina Lozano, Ph.D.***

The bone marrow accommodates hematopoietic stem cells and progenitors. These cells provide an indispensable resource for replenishing the blood constituents throughout an organism's life. A tissue with such a high turn-over rate mandates intact cycling checkpoint and apoptotic pathways to avoid inappropriate cell proliferation and ultimately the development of leukemias. p53, a major tumor suppressor, is a transcription factor that regulates cell cycle, and induces apoptosis and senescence. Mice inheriting a hypomorphic *p53* allele in the absence of Mdm2, a p53 inhibitor, have elevated p53 cell cycle activity and die by postnatal day 13 due to hematopoietic failure. Hematopoiesis progresses normally during embryogenesis until it moves to the bone marrow in late development. Increased oxidative stress in the bone marrow compartment postnatally is the impediment for normal hematopoiesis via activation of p53. p53 in turn stimulates the generation of more reactive oxygen species and depletes bone marrow cellularity. Also, p53 exerts various defects on the hematopoietic niche by increasing mesenchymal lineage populations and their differentiation. Hematopoietic defects are rescued with

antioxidants or when cells are cultured at low oxygen levels. Deletion of *p16* partially rescues bone marrow cellularity and progenitors via a p53-independent pathway. Thus, although p53 is required to inhibit tumorigenesis, Mdm2 is required to control ROS-induced p53 levels for sustainable hematopoiesis and survival during homeostasis.



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# INTRODUCTION

## **p53: the tumor suppressor gene**

### *Discovery of p53*

p53 is one of the most studied genes in the genetics of cancer due to its high mutation rate in familial and somatic cancers, and its association with viral oncogenes. Discovered in 1979, p53 was identified as a protein with an approximate weight of 53,000 Daltons in extracts of simian virus 40 (SV40) transformed cell lines (DeLeo, Jay et al. 1979; Kress, May et al. 1979; Lane and Crawford 1979; Linzer and Levine 1979). Specifically, p53 protein formed a complex with the SV40 large T antigen. Within two years of its discovery, a plethora of human and rodent transformed cell lines were tested for p53 expression and many showed elevated levels of this protein (regardless of mode of transformation), contrary to little or no expression in normal cells (Crawford, Pim et al. 1981). Although these studies collectively correlated p53 expression with transforming events, p53's direct role in transformation remained unclear.

In 1984, the *p53* cDNA was cloned, and the predicted amino acid sequence showed homology to a tyrosine kinase acceptor site and, to a lesser extent, to the *myc* oncogene (Bienz, Zakut-Houri et al. 1984; Czosnek, Bienz et al. 1984; Jenkins, Rudge et al. 1984). Hence, molecular cancer biologists at that time became perplexed by the uncertainty of p53's characteristics. Several labs overexpressed the cloned cDNA and independently showed that p53 can immortalize and transform normal cell lines (Eliyahu, Raz et al. 1984; Jenkins, Rudge et al. 1984; Lane 1984). Varda Rotter's work in late 1984 confirmed that p53 can "play a causal

role in the conversion of normal fibroblasts into tumorigenic cells” upon cooperation with other known oncogenes such as *ras* (Parada, Land et al. 1984). It took several years for the field to realize that mutations in the cDNAs used for these transformation studies had concealed the actual role of p53.

### *Rectifying p53's role in tumorigenesis*

Skepticism about p53's transforming role culminated when Levine's lab showed for the first time that overexpressing a wildtype clone of p53 can actually suppress, rather than promote transformation of rodent cell lines (Finlay, Hinds et al. 1989). Concurrently, analysis of the *p53* gene status in human tumor samples evidently supported the Knudson's two-hit hypothesis of a tumor suppressor model (Knudson 1971; Vogelstein 1990). Specifically, colorectal cancer patients had one mutant allele of *p53* while the other allele was lost (Baker, Fearon et al. 1989; Vogelstein, Fearon et al. 1989). These experiments instigated a paradigm shift in describing the bona fide role of p53 in transforming events. Thus, by the late 1980's, p53 was finally recognized as a tumor suppressor and not as an oncogene (Finlay, Hinds et al. 1989).

### *Early p53 mouse models*

In 1989, with the use of mouse transgenic models, mutant p53 was overexpressed for the first time *in vivo* (Lavigne, Maltby et al. 1989). 20% of the transgenic-mutant p53 mice had high rate of tumors providing direct evidence that mutations in p53 may promote oncogenesis (Lavigne, Maltby et al. 1989). During that same time frame, *p53* germline mutations were detected in several families with

Li-Fraumeni syndrome causing an interest in analyzing p53 status in other sporadic tumors as well (Nigro, Baker et al. 1989; Malkin, Li et al. 1990). Another compelling piece of evidence for the role of p53 as a tumor suppressor came from generation of a *p53*-knock out mouse (Donehower, Harvey et al. 1992). *p53*-null mice were viable and with no apparent developmental defects, although a small fraction exhibited exencephaly (Donehower, Harvey et al. 1992; Sah, Attardi et al. 1995). The loss of *p53*, on the other hand, predisposed the mice to lymphomas and sarcomas and all *p53*-null mice died by 8 months of age due to tumor burden (Donehower, Harvey et al. 1992). Interestingly, loss of a single p53-allele still predisposed mice to tumorigenesis, but with a delayed onset and with higher frequency of sarcomas rather than lymphomas as compared to *p53*-null mice (Harvey, McArthur et al. 1993).

These *p53* mouse models showed that although p53 was not required for embryonic development, it was indispensable to suppress tumorigenesis in mice. Analysis of human tumor samples provided compelling evidence for the requirement of most tumors to disrupt the p53 pathway to initiate or progress. Additional studies were performed to delineate how p53 confers its tumor suppressor activities.

## **Tumor suppressor functions of p53**

### *DNA damage activates p53*

p53 is a stress response gene that binds DNA as a tetramer in a sequence specific manner and transactivates a plethora of genes (Bargonetti, Friedman et al. 1991; Kern, Kinzler et al. 1991; el-Deiry, Kern et al. 1992). Oncogenic, hypoxic,

and metabolic stresses instigate p53's activity (Junttila and Evan 2009). Cellular insults such as reactive oxygen species (ROS) can induce DNA damage and subsequently activate p53 (Ko and Prives 1996). The first evidence for a p53-dependent response to DNA damage came from studies on UV-treated non-transformed cells (Maltzman and Czyzyk 1984). Specifically, post-translational modifications stabilized p53 protein levels in these cells. p53 activation maintains the genomic integrity via induction of apoptosis, cell cycle arrest, differentiation or senescence (Ko and Prives 1996).

### *Apoptosis*

The role of p53 in induction of apoptosis was first observed in myeloid leukemic cells that overexpressed wildtype p53 (Yonish-Rouach, Resnitzky et al. 1991). Furthermore, exposure of thymocytes to ionizing radiation induces p53-dependent apoptosis as loss of *p53* protects against radiation-induced cell death (Lee and Bernstein 1993; Lowe, Schmitt et al. 1993). Apoptosis confers a major tumor suppressor activity to eradicate defective cells and avoid propagation of mutations in cells. Mechanistically, p53 binds the promoters of apoptotic genes such as *Noxa*, *Bax* and *Puma* (Miyashita, Krajewski et al. 1994; Oda, Ohki et al. 2000; Nakano and Vousden 2001). These proteins promote activation of the intrinsic apoptotic pathway initiated in the mitochondria through cytochrome c release and caspase proteins cleavage (Reviewed in (Kroemer and Reed 2000). Apoptotic activity of p53 can also be mediated by the extrinsic apoptotic pathway via regulation of some of its elements (Fridman and Lowe 2003). For instance, *Fas*, a death receptor, and its ligand are both direct targets of p53 and are important

players in the extrinsic pathway of apoptosis (Owen-Schaub, Zhang et al. 1995; Maecker, Koumenis et al. 2000).

### *Cell cycle arrest*

Treatment of leukemic cells harboring wildtype *p53* alleles with DNA damaging agents induces a G1 arrest and indicates that inhibiting cell cycle progression is another mechanism for the tumor suppressor activity of *p53* (Kastan, Onyekwere et al. 1991). The major cell cycle arrest target of *p53* is p21, a cyclin-dependent kinase (Cdk) inhibitor (el-Deiry, Tokino et al. 1993). p21 inhibits G1 to S transition of cell cycle by disrupting the activation of Cyclin E- and Cyclin A- Cdk complexes (Dulic, Kaufmann et al. 1994). *p53* can also arrest cells in G2 via transactivation of GADD45 and 14-3-3 $\sigma$  (Hermeking, Lengauer et al. 1997; Wang, Zhan et al. 1999). The importance of cell cycle arrest in tumor suppressor activity of *p53* is supported by the generation of *p53*<sup>515C/515C</sup> mice (Liu, Parant et al. 2004). *p53*<sup>515C</sup> allele mimics a rare *p53* mutation (Arginine to Proline) in human *p53* at amino acid 175 that lacks apoptotic activity but still retains the cell cycle arrest activity (Ludwig, Bates et al. 1996; Rowan, Ludwig et al. 1996). *p53*<sup>515C/515C</sup> mice are tumor prone but with longer latency than *p53*<sup>-/-</sup> mice (Liu, Parant et al. 2004). These experiments provide compelling evidence that the cell cycle arrest activity of *p53* is indeed tumor-suppressive but it is not sufficient to completely inhibit tumorigenesis.

## *Differentiation and senescence*

p53 also has a role in differentiation which is exemplified in the lymphoid system (Shaulsky, Goldfinger et al. 1991). Pre-B cells can be induced to a more mature state by introduction of wildtype p53 (Shaulsky, Goldfinger et al. 1991). This is indeed a remarkable observation given the high rate of DNA rearrangements and DNA breaks occurring during the process of B cell development (Ko and Prives 1996). In addition to the previously mentioned p53 tumor suppressor activities, p53 can alternatively induce senescence. Although the mechanisms underlying p53 induction of senescence remains unclear, most studies suggest that it is mediated via p53's transactivation of *p21*, followed by induction of *p16* (el-Deiry, Tokino et al. 1993; Beausejour, Krtolica et al. 2003). In the absence of *p53*, cells can still undergo senescence but it is generally delayed compared to cells with wildtype p53 (Bond, Wyllie et al. 1994).

The molecular events that dictate which pathway p53 activates following DNA damage remain unclear for the most part, although cell type and tissue specific modifiers seem to be of high importance (Midgley, Owens et al. 1995; Haupt, Barak et al. 1996; Prives and Hall 1999). Additionally, the interaction of p53 with its negative regulators may influence p53 binding to different promoters (Kruse and Gu 2009). Another speculation is that minor changes in p53 levels may alter the cell fate to either activate cell cycle arrest or apoptosis. Specifically, as p53 levels increase in the cell, there will be greater propensity for p53 to activate pro-apoptotic rather than cell cycle arrest genes (Vousden 2000; Barboza, Iwakuma et al. 2008).



### *p53: “guardian of the genome”*

Retrospectively, the aforementioned activities specify the tumor suppressor activities of p53 to maintaining the genomic stability of the cell. Hence, it is not surprising that p53 was dubbed as “guardian of the genome” (Lane 1992). This moniker was used due to prevalence of aberrant genetic amplifications and aneuploidy in cells lacking *p53* (Livingstone, White et al. 1992). *p53* loss confers checkpoint defects and disruption of genetic integrity, allowing uninhibited proliferative capacity and cancer initiation (Fridman and Lowe 2003). Upon DNA damage, cell cycle arrest creates a brake and presumably provides sufficient time for repair. Alternatively, the damaged cell undergo apoptosis or enter irreversible arrest or senescence. The role of differentiation in tumor suppression remains unclear and could have more of a developmental significance. Taken as a whole, the versatile activities of p53 converge towards maintaining the genomic stability and prevention of error propagation.

### **The structural biology of p53**

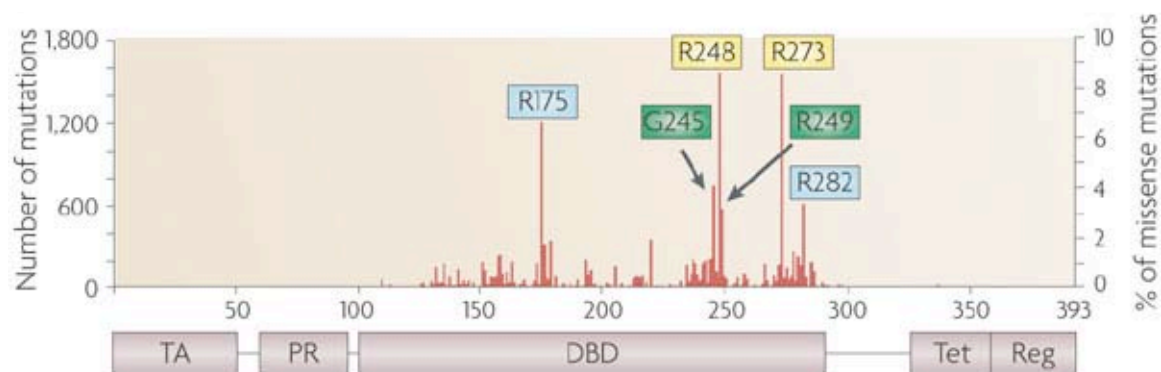
#### *N-terminus of p53*

Given the remarkable role of p53 in tumor suppression, understanding the structure of this protein is essential (Figure 1). The human p53 protein contains 393 amino acid residues and is longer by 2 residues than the mouse homolog (Zakut-Houri, Bienz-Tadmor et al. 1985). Five major domains have been identified in the p53 protein that spread among three distinct regions: the acidic N-terminus, the central domain and the carboxyl domain. The N-terminus contains the transactivation (TA) domain and a proline-rich (PR) domain. The TA domain

interacts with the chromatin remodeling proteins and the transcriptional machinery (Thut, Chen et al. 1995). Additionally, this region is important for the stability of p53 via interactions with p53's negative regulators and for postranslational modifications, such as phosphorylation (Meek 1994; Haupt, Maya et al. 1997; Kubbutat, Jones et al. 1997). The second domain in the N-terminus, the PR domain, is required for p53's apoptotic activity (Venot, Maratrat et al. 1998). The PR domain also acts as a spacer region connecting the TA domain with the next domain: the DNA-Binding Domain (DBD) (Joerger and Fersht 2008).

#### *Central region: DNA binding domain*

The DBD is the central portion of p53's protein and mediates p53 binding to DNA in a sequence specific manner (el-Deiry, Kern et al. 1992; Zambetti, Bargonetti et al. 1992). SV40 binds p53 at its DBD, suggesting that this binding renders p53 transcriptionally nonfunctional (Jenkins, Chumakov et al. 1988). Also, missense mutations are common in the DBD and inactivate p53's ability to bind DNA and suppress tumorigenesis. These mutations confer gain-of-function phenotypes as p53 mutant cells have increased metastasis (Raycroft, Wu et al. 1990; Brosh and Rotter 2009). Hence, it is not surprising that the majority of p53 mutations occur in its DBD with highest mutation frequency rate at codons R248 and R273, followed by R175 in humans (Figure 1) (Hollstein, Shomer et al. 1996); <http://www-p53.iarc.fr/>).



**\*Figure 1. p53 domains and hot spot mutations.** TA = transactivation domain; PR= Proline Rich domain; DBD = DNA binding domain; Tet = Tetramerization domain; Reg= carboxy-terminal Regulatory domain. Most p53 mutations occur in its DBD and their frequencies and number of mutations reported are noted.

\*Reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Cancer* 9, 701-713 (October 2009) doi:10.1038/nrc2693. Figure 2a.

### *C-terminus*

The third region of p53 is comprised of many basic amino acid residues and contains the tetramerization (TET) domain and the carboxy-terminal regulatory (Reg) domain. This region is subject to post-translational modifications such as acetylation and phosphorylation following DNA damage (Meek 1994; Prives and Hall 1999; McKinney, Mattia et al. 2004; Krummel, Lee et al. 2005; Brosh and Rotter 2009). It also regulates sequence-specific DNA binding by a mechanism that is still not well understood (Prives and Hall 1999). The TET domain mediates tetramer formation, subsequently providing optimal transcriptional activity (Jeffrey, Gorina et al. 1995). Truncations of the Reg domain result in inefficient transactivation of genes (McKinney, Mattia et al. 2004).

## **Regulating p53 levels**

### *Modes of regulation*

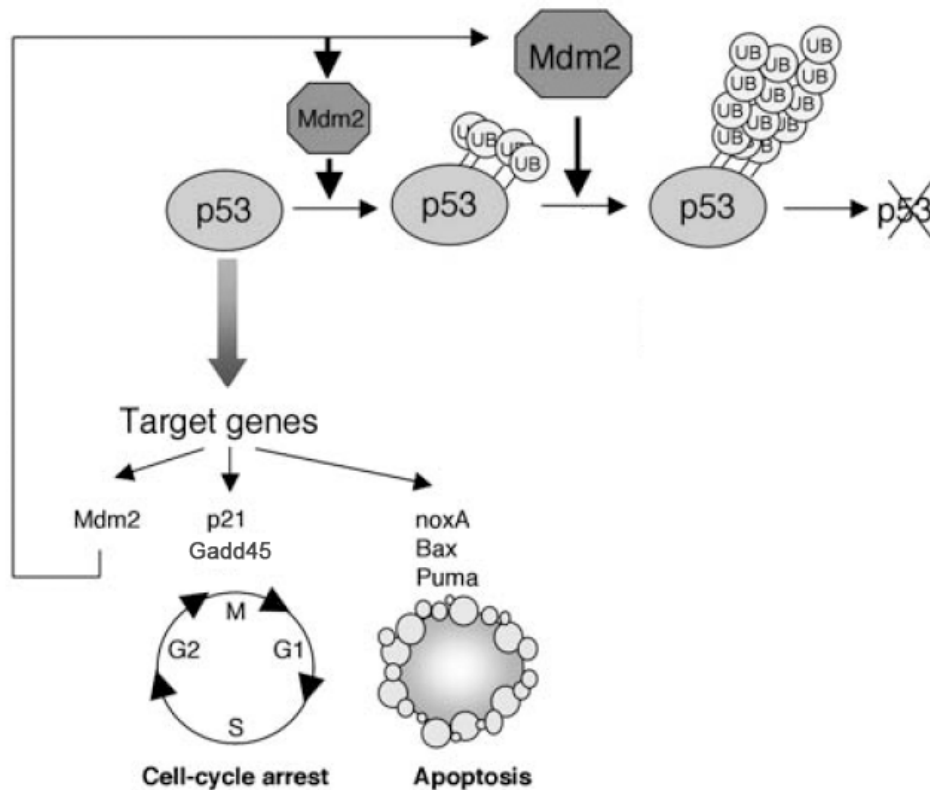
During unstressed conditions, cells have extremely low p53 levels due in part to its short half-life (Levine 1997; Marine, Francoz et al. 2006). However, following DNA damage, there exists an abundance of mechanisms and networks that are responsible for controlling p53 levels. Except for few studies, there is little evidence suggesting the control of p53 expression is at the mRNA level (Mosner, Mummenbrauer et al. 1995). One study for example suggests that *Wig-1*, a p53 target gene, binds 3' UTR of the p53 mRNA and stabilizes it (Vilborg, Glahder et al. 2009). However, most data demonstrate that p53 levels are regulated at the protein level via direct interactions with other negative regulators or indirectly, via post-translational modifications. For instance, Cop1, Pirh2, Trim24, Mdm2 and Mdm4 are

all reported to attenuate p53 levels. Stress-induced activation of p53 primarily aims at disrupting p53 interactions with Mdm2 and Mdm4 (Perry 2010).

### *Regulation by Mdm2 and Mdm4*

Mdm2 was first discovered as a protein overexpressed in murine tumor cell lines (Fakharzadeh, Trusko et al. 1991). Its exact role in oncogenesis remained elusive until Mdm2 was coimmunoprecipitated in a complex with p53 and showed it can abrogate p53's transcriptional activity. Mdm2 functions as an E3 ubiquitin ligase inhibiting p53 by binding to its TAD and targeting it for proteosomal degradation (Figure 2) (Momand, Zambetti et al. 1992; Honda, Tanaka et al. 1997). Mdm2 is itself a transcriptional target of p53 creating a negative feedback loop to maintain tight p53 levels in the cell (Barak, Juven et al. 1993; Wu, Bayle et al. 1993).

Mdm4 is another negative regulator of p53 that interacts with Mdm2 to inhibit p53. Binding of Mdm4 to Mdm2 stabilizes the later and subsequently leads to lower p53 levels in the cell (Gu, Kawai et al. 2002). In addition to p53, Mdm2 ubiquitylates Mdm4, and targets it to the nucleus where it is degraded (de Graaf, Little et al. 2003). Other negative regulators of p53 include Cop1 and Pirh2. Like Mdm2, these proteins target p53 for proteosomal degradation, and inhibit p53's transcriptional activity *in vitro* (Toledo and Wahl 2006). Most recently, Trim24 has been found to downregulate p53 levels via E3 ubiquitin ligase activity (Allton, Jain et al. 2009). The significance of Cop1, Pirh2 and Trim24 in regulation of p53 in mammalian systems is yet to be determined.



**\*Figure 2. Regulation of p53 by Mdm2.** Mdm2 ubiquitinates p53 and targets it for proteosomal degradation. Uninhibited p53 can transactivate cell cycle arrest and apoptosis target genes. P53 can transactivate Mdm2 creating a negative feedback loop.

\*Reprinted by permission from Macmillan Publishers Ltd: *Cell Death and Differentiation* (2010) 17, 93–102; doi:10.1038/cdd.2009.68; published online 5 June 2009. Figure 1.

### *Regulation by posttranslational modifications*

In addition to regulation by ubiquitylation, p53 levels can be regulated via phosphorylation (Kruse and Gu 2009). Serine 18 and Serine 23 of mouse p53 are phosphorylated after DNA damage, or other stresses, by various kinases such as ATM, ATR, Chk1 and Chk2 (Appella and Anderson 2001; Kruse and Gu 2009). Such phosphorylation of the N-terminus of p53 inhibits Mdm2-p53 and Mdm4-p53 interactions resulting in stabilization of p53. Moreover, ATM phosphorylates Mdm2 and Mdm4 at their C-termini leading to their rapid degradation (Khosravi, Maya et al. 1999; Chen, Gilkes et al. 2005). This insures rapid accumulation of p53 in the cell and induction of cell cycle arrest or apoptosis. Although modifications of p53's Reg domain are thought to be essential, a p53(7KR) mouse with C-terminal lysines changed to arginine is phenotypically normal but sensitizes thymocytes to DNA-damage easier than wildtype p53 (Krummel, Lee et al. 2005).

These studies collectively show that regulation of p53 is not dependent on one mechanism or pathway. Instead, various damaging signals can activate either of the aforementioned pathways. Negative regulators of p53 can form complexes and negatively or positively regulate each other as in the case with Mdm2 and Mdm4. The ultimate goal is to activate p53 when the cell detects abnormalities such as aberrant proliferation, and at the same time, to maintain low levels of p53 in a cell for its survival.

## Deleterious effects of uninhibited p53 in normal tissues

Mdm2 and Mdm4 are both indispensable for development and survival of the cell and organism. This is supported by the embryonic lethality of deleting *Mdm2* or *Mdm4* in mice (Jones, Roe et al. 1995; Montes de Oca Luna, Wagner et al. 1995; Parant, Chavez-Reyes et al. 2001). This lethality is p53-dependent as genetic ablation of *p53* rescues the *Mdm2*<sup>-/-</sup> and *Mdm4*<sup>-/-</sup> mice (Jones, Roe et al. 1995; Montes de Oca Luna, Wagner et al. 1995; Parant, Chavez-Reyes et al. 2001). Notably in these studies, while *Mdm2*<sup>-/-</sup> embryos die because of massive apoptosis before implantation, *Mdm4*<sup>-/-</sup> die at postimplantation at E7.5-E8.5 due to both loss of proliferative capacity and apoptosis. Although Mdm2 and Mdm4 are required for survival, excessive levels of these proteins are oncogenic and detrimental (Ringshausen, O'Shea et al. 2006; Toledo and Wahl 2006; Xiong, Van Pelt et al. 2007).

Because of the embryonic lethality of *Mdm2*<sup>-/-</sup> mice, understanding the physiological role p53 by Mdm2 is not possible (Marine and Lozano 2009). Therefore, several studies bypassed this impediment by manipulating the p53-Mdm2 balance using different genetic strains in mice (Marine, Francoz et al. 2006). For instance, Gerard Evan's laboratory used mice harboring a switchable endogenous p53 in an *Mdm2*<sup>-/-</sup> background and showed that p53 was stabilized and induced either apoptosis or cell cycle arrest (Ringshausen, O'Shea et al. 2006). Interestingly, p53 activation was independent of any post-translational modifications of p53 or any DNA damage indicating that the major role of Mdm2 is to dampen p53 levels even during homeostasis to maintain survival of mice (Marine, Francoz et al. 2006; Marine and Lozano 2009). In other work, p53 elevation due to a hypomorphic



*Mdm2* allele led to severe lymphoid tissue defects and susceptibility to sublethal irradiation (Mendrysa, McElwee et al. 2003).

Although *Mdm2*<sup>+/-</sup> and *Mdm4*<sup>+/-</sup> mice are normal and viable, their hematopoietic tissues are highly sensitive to sublethal irradiation (Terzian, Wang et al. 2007). Specifically, haploinsufficiency of *Mdm2* or *Mdm4* maintains elevated p53 levels after irradiation which depletes bone marrow cellularity (Terzian, Wang et al. 2007). Also, *Mdm2*<sup>+/-</sup> *Mdm4*<sup>+/-</sup> mice suffer from severe p53-dependent hematopoietic defects and die shortly after birth (Terzian, Wang et al. 2007). Thus, it is important to understand the effect of deregulated Mdm2-p53 balance in the bone marrow that is continuously active and cycling throughout the life of an organism.

## **Founding hematopoiesis**

### *Identification of hematopoietic tissues*

The aftermath of the atomic bombs in Hiroshima and Nagasaki in 1945 led to the observation that the hematopoietic system was very sensitive to radiation (Weissman and Shizuru 2008). Particularly, while patients exposed to high radiation dosages died shortly afterwards due to toxicity, those exposed to lower levels succumbed a few years later to infections or diminishing platelets counts (Domen, Wagers et al. 2006). In order to address how radiation could have instigated such severe hematopoietic defects, Leon Jacobson and his colleagues started a series of radiation experiments on mice in the late 1940's. The team then rescued the irradiation-induced mortality in rodents via shielding the spleen in adult mice and

showed that the spleen harbored hematopoietic activity (Jacobson, Marks et al. 1949). The same group also identified the liver, the leg and the head as other organs with hematopoietic potential, and that shielding any of these organs increased the survival of mice exposed to lethal irradiation (Jacobson, Simmons et al. 1951). Most notably, injecting unirradiated bone marrow or spleen cells into lethally irradiated rodents rescues these animals from death (Jacobson, Simmons et al. 1951; Nowell, Cole et al. 1956). These studies founded the concept of hematopoietic organs and their sensitivity to irradiation.

#### *Spleen: one habitat for hematopoiesis*

Based on the previously mentioned discoveries, Till and McCulloch (1961) examined the homing of the injected bone marrow cells into the lethally irradiated mice. The report, published in Radiation Research in 1961, identified the spleen as an environment where injected bone marrow cells home to and proliferate (Till and McCulloch 1961). Even more, the number of cells injected correlated with the number of visible hematopoietic nodules (Till and McCulloch 1961). Within these nodules, erythrocytic, granulocytic and megakaryocytic cells were identified (Till and McCulloch 1961). A few years later, the group also identified lymphoid cells in these colonies concluding that intact bone marrows can give rise to all lineages of the blood system (Wu, Siminovitch et al. 1968; Wu, Till et al. 1968).

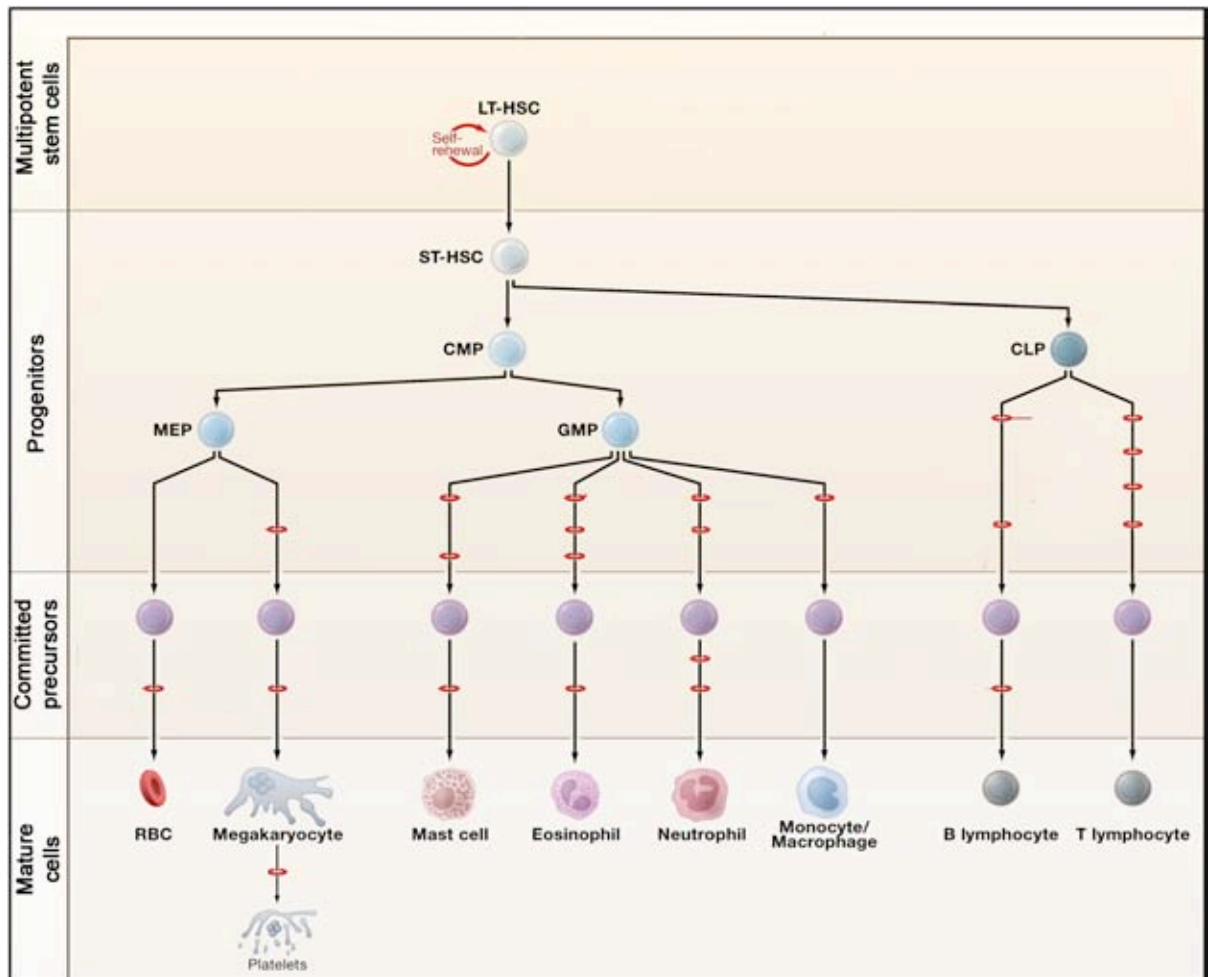
### **Isolation and characterizing of hematopoietic stem cells**

#### *Dissecting the hierarchy*

The current view of the hematopoietic system is its hierarchal structure with hematopoietic stem cells at its apex (Figure 3) (Orkin and Zon 2008). Hematopoietic stem cells give rise to short-term progenitors that produce more committed progenitors, namely common myeloid progenitors (CMP) and common lymphoid progenitors (CLP) (Orkin 2000; Orkin and Zon 2008). The latter cells then provide the committed precursors, which subsequently differentiate into mature hematopoietic cells which are relatively short-lived. Hence, hematopoietic stem cells repeat this cycle throughout their lifetime in order to replenish the blood and bone marrow with its constituents, while maintaining their ability to self-renew (Orkin and Zon 2002; Mikkola and Orkin 2006).

#### *First conception of a hematopoietic stem cell*

In 1956, Dr. E Donnall Thomas performed the first human bone marrow transplantation and rescued a 59-year-old leukemia patient (Thomas, Lochte et al. 1959). This work earned him the Noble Prize in 1990. While the cell of origin responsible for saving the patient was obscure, the idea of existence of a cell that was responsible to reconstitute all the blood was supported by observations from other groups via transplantation experiments in rodents (Barnes and Loutit 1953). Then, using a chromosomal marker, a team led by Till discovered that the nodules seen in the spleens of injected mice were clones from a single cell (Becker, McCulloch et al. 1963). Barnes, in a letter to Lancet's editor, described these cells as "haemopoietic stem-cells" and coined this term (Barnes and Loutit 1967). The concept that hematopoietic stem cells have the ability to produce all blood cell lineages and self-renew is now widely supported (Weissman 2000).



**\*Figure 3. Hematopoietic hierarchy.** Long term hematopoietic stem cells (LT-HSC) sit at the top of the hierarchy giving rise to progenitors that their progeny differentiate and are short lived.

\*Modified and reprinted by permission from Elsevier Ltd: *Cell* 132, 631-644 (February 2008) DOI 10.1016/j.cell.2008.01.025. Figure 5.

### *Negative selection markers*

Clinically, bone marrow transplantation became more popular by the late 1970s (Weissman and Shizuru 2008). Studies then emerged for prospective physical isolation of hematopoietic stem cells to aid in better transplantation efficiencies. The sole criteria for considering a population enriched for hematopoietic stem cells is whether it can more efficiently repopulate all the blood cell lineages in transplanted recipients (Weissman and Shizuru 2008). To that end, several groups devised a screen for cell-surface markers using fluoro-chrome-conjugated monoclonal antibodies that select for stem cells or exclude differentiated cells. The first monoclonal antibody generated against blood cells recognized murine differentiated B cells and their precursors (Coffman and Weissman 1981; Coffman and Weissman 1981). With the advancement of fluorescence-activated cell sorting (FACS) at that time, cells expressing the differentiated B-lineage marker, B220, were sorted and monitored for their potential to form colonies *in vitro* and *in vivo*. Studies concluded that cells expressing B220 were incapable of forming colonies or reconstituting hematopoiesis, in contrast to cells lacking B220 (Coffman and Weissman 1981; Muller-Sieburg, Whitlock et al. 1986). With the identification of more differentiation lineage markers (Ter119, CD11b/Mac1, CD4, CD8a, Gr-1), it became clear that cells expressing lineage committed markers cannot reconstitute hematopoiesis and hence should be used for negative selection of hematopoietic stem cells from the bone marrow (Muller-Sieburg, Whitlock et al. 1986; Morrison and Weissman 1994). Collectively, these markers of negative selection became known as Lin<sup>−</sup>, for “lineage negative”.

### *Positive selection markers*

After the identification of negative selection markers to enrich for hematopoietic stem cells, the search for markers that are expressed on hematopoietic stem cells took off. Mouse hematopoietic precursor cells were found to have low expression of the cell surface differentiation antigen Thy-1 (Berman and Basch 1985). A breakthrough in the enrichment of hematopoietic stem cells, however, came from a monoclonal-antibody screen for a putative pre-T hybridoma marker (Aihara, Buhring et al. 1986). One antibody, E13 161-7, bound lymphoid cells of bone marrow progenitors and thirty Lin<sup>−</sup> Thy-1<sup>lo</sup> cells that were also positive for the E13 161-7 antibody rescued 50 percent of lethally irradiated mice (Spangrude, Heimfeld et al. 1988; Spangrude, Klein et al. 1989). The antigen recognized by E13 161-7 antibody was dubbed “stem cell antigen-1” or Sca-1 (Spangrude, Heimfeld et al. 1988; Spangrude, Klein et al. 1989). The Lin<sup>−</sup> Thy-1<sup>lo</sup> Sca1<sup>+</sup> purified hematopoietic stem cell can differentiate into all lineages (Spangrude, Heimfeld et al. 1988). At the same time, bone marrow cells expressing c-kit, a known oncogene that encoded a tyrosine kinase receptor, were shown to harbor hematopoietic stem cell characteristics by rescuing lethally irradiated mice (Okada, Nakauchi et al. 1991; Ikuta and Weissman 1992).

By the mid 1990's, the hematopoiesis field reached a consensus to use the Lin<sup>−</sup> ckit<sup>+</sup> Sca-1<sup>+</sup> (hereafter referred to as LKS) population of the adult bone marrow as a highly enriched population for hematopoietic stem cells. The LKS population contains a subset of long-term hematopoietic stem cells (LT-HSCs), with self-renewal capacity, and short-term hematopoietic stem cells (ST-HSCs), or

multipotent progenitors. LT-HSCs are capable of long-term (>5 months) reconstitution of hematopoiesis when transplanted into irradiated mice, while ST-HSCs can reconstitute recipients for <2 month as they have limited self-renewal capacity (Miller, Dykstra et al. 2008). The journey to decipher markers that distinguish LT-HSCs from ST-HSCs began.

### *Recent advancement in purification of hematopoietic stem cells*

CD34 and flk2/flt3 can be used to discriminate between LT-HSCs and ST-HSCs (Papathanasiou, Attema et al. 2009). Specifically, LKS CD34<sup>−</sup> flk2<sup>−</sup> and LKS CD34<sup>+</sup> flk2<sup>+</sup> represent LT-HSC and ST-HSC populations, respectively (Osawa, Hanada et al. 1996; Christensen and Weissman 2001). More recently, using gene expression arrays Morrison's group identified signaling lymphocyte activation molecules family, or SLAM, which regulates the proliferation and activation of lymphocytes, to select for LT-HSCs (Howie, Okamoto et al. 2002; Kiel, Yilmaz et al. 2005). The authors showed that 48% of LKS CD150<sup>+</sup> CD48<sup>−</sup> (LKS-SLAM) cells in adult bone marrows were LT-HSCs (Kiel, Yilmaz et al. 2005). Noteworthy, the accuracy of using LKS-SLAM for 48% enrichment of LT-HSCs is still subject to controversy when compared to side-population/dye exclusion analysis for enrichment of hematopoietic stem cells (Weksberg, Chambers et al. 2008). However, it is now generally accepted that LKS-SLAM population enriches more for LT-HSCs than just using LKS, and constitutes a more rigorous approach for hematopoietic stem cell analysis (Wagers 2005; Papathanasiou, Attema et al. 2009).

## **Developmental journey of hematopoietic stem cells**

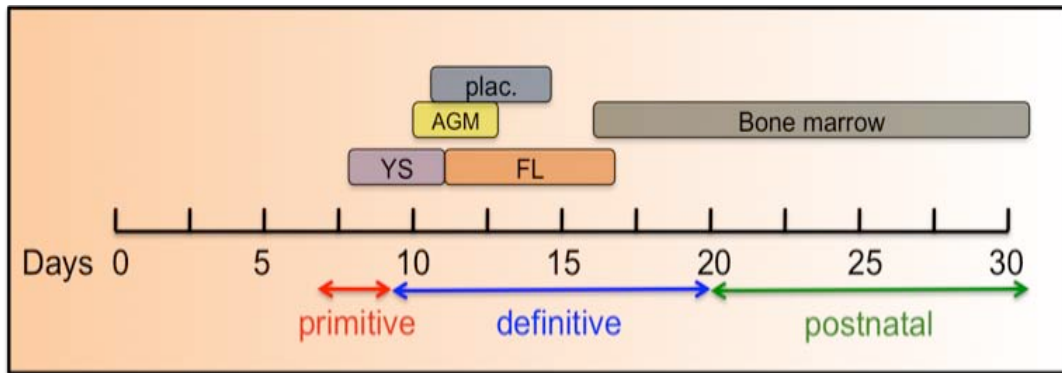
### *Conserved hematopoiesis map*

The developmental origins and the processes of hematopoiesis are generally conserved among vertebrates (Orkin and Zon 2008). Most of our understanding of these processes is based on studies that utilize the mouse as a genetic system, and to a lesser, but notable, extent from zebrafish studies (Orkin and Zon 2008). Since the anatomy of the embryo changes continuously during organogenesis, hematopoiesis progresses to different sites (Mikkola and Orkin 2006). The anatomical map and timeline of hematopoiesis undertaken from embryogenesis to adulthood have been successfully drawn (Figure 4). However, the molecular and signaling pathways that regulate the trafficking have yet to be determined.

### *Primitive hematopoiesis*

The ultimate purpose of hematopoiesis during early embryogenesis is to produce red blood cells to transfer oxygen to embryonic tissues for rapid growth (Orkin and Zon 2008). This phase of hematopoiesis is termed “primitive” and takes place in the yolk sac at around embryonic day (E) 7.0 until E8.25 in the mouse embryo (Palis, McGrath et al. 1995; Palis, Robertson et al. 1999). Red blood cells from primitive hematopoiesis retain their nucleus and are relatively large cells (Palis, Robertson et al. 1999; Ferkowicz, Starr et al. 2003). The origin of red blood cells precursors of primitive hematopoiesis has been a matter of controversy in the field since the early 1900's. In 1917, Florence Sabin suggested that the hemangioblast, a mesodermal precursor in the yolk sac, can give rise to blood and endothelial cells





**Figure 4. Anatomical distribution of hematopoiesis in the mouse.** Primitive hematopoiesis starts in the yolk sac (YS) at about E7.5 followed by definitive hematopoiesis. The aorta-gonad-mesonephros accommodate hematopoiesis at about E10. Placenta (plac.) and fetal liver (FL) are hematopoietic tissues as early as E11. FL becomes major organ of expansion of HSC until bone marrow can accommodate hematopoiesis at about E16 and throughout adulthood. Day 0 designates first day of pregnancy. Mice gestation period is approximately 20 days.

and is the origin of primitive hematopoiesis. Interestingly, some studies indicate that although primitive hematopoiesis is in the yolk sac, red blood cell precursors develop before they migrate to the yolk sac (Palis, Chan et al. 2001; Palis and Yoder 2001). This refutes the postulation that the hemangioblast is in the yolk sac, but not that a hemangioblast actually exists (Mikkola and Orkin 2006).

Hematopoietic cells are reported to be “budding” from endothelial tissues proposing that endothelial cells give rise to primitive hematopoiesis (Garcia-Porrero, Godin et al. 1995; Zovein, Hofmann et al. 2008). With the advancement of live imaging, endothelial cells are captured in *bona fide* time giving rise to blood cells (Eilken, Nishikawa et al. 2009). Although these data support the concept that endothelial cells give rise to blood cells, it does not prove that endothelial and blood cells have a common precursor, *i.e.* the hemangioblast. Also, the live imaging studies do not eliminate the possibility that other cells may still give rise to hematopoietic precursors (Eilken, Nishikawa et al. 2009).

### *Definitive hematopoiesis*

The short primitive hematopoiesis period is followed by what is known as “definitive” hematopoiesis. In this process, all lineages (erythroid, myeloid and lymphoid) are generated (Lancrin, Sroczynska et al. 2009). After it initiates in the yolk sac at E8.25, definitive hematopoiesis proceeds in the aorta-gonad mesonephros (AGM) at around E10, where the activity of hematopoietic stem cells increases by E11 (Medvinsky, Samoylina et al. 1993). Although the placenta acts as a hematopoietic organ as early as E9 in mice, mature hematopoietic stem cells are not found in the placenta until E10.5-E11 (Alvarez-Silva, Belo-Diabangouaya et al.

2003; Ottersbach and Dzierzak 2005). Hematopoietic stem cells in the placenta expand rapidly, and without differentiation, becoming 15 fold more than hematopoietic stem cells counts in the yolk sac or AGM (Gekas, Dieterlen-Lievre et al. 2005). The origin of placental hematopoietic stem cells is still controversial. Some thoughts indicate that these cells are generated *in situ*, while others indicate that these cells could have colonized in the placenta via circulation (Orkin and Zon 2002; Mikkola and Orkin 2006). At some point between E11 and E12, hematopoietic stem cells start expressing CD45 and Sca-1 markers, a hallmark of a more mature state (North, de Bruijn et al. 2002; Matsubara, Iwama et al. 2005).

#### *Hematopoiesis in the fetal liver*

As early as E10, hematopoietic activity becomes apparent in the fetal liver and hematopoietic stem cells start populating the liver at E11.5 (Jordan, McKearn et al. 1990; Orkin and Zon 2002). At E12.5, the fetal liver becomes the major organ for expansion and differentiation of hematopoietic stem cells, and at approximately E16, this activity starts to decline (Morrison, Hemmati et al. 1995; Ema and Nakauchi 2000). Unlike the yolk sac, AGM and placenta, the fetal liver hematopoietic compartment is quite rich with hematopoietic progenitors and lineage positive cells which significantly contributed to establishing the hierarchal structure of the hematopoietic system (Figure 3). The robust expansion of hematopoietic stem cells in the fetal liver is linked to signals from the fetal liver microenvironment that promote symmetric self-renewing division. However, identity of key cell types of the fetal liver microenvironment remains obscure and is under investigation.

### *Establishment of the bone marrow*

During the expansion of the hematopoietic compartment in the fetal liver at E12.5, the skeletal system is being established to accommodate hematopoiesis in the bone during late gestation and throughout the life of the organism (Collins, Olsen et al. 2005). Hematopoietic stem cells start seeding the bone at around E17.5 although other hematopoietic cells start migrating to the bone as early as E15.5 (Christensen, Wright et al. 2004). Little is known about the signals that the fetal hematopoietic stem cells receive to initiate their migration to the bone, however the chemokine stromal cell-derived factor-1 (Sdf1) is a key element (Ara, Tokoyoda et al. 2003). While in the bone, hematopoietic stem cells establish the marrow and finalize the hierarchical structure of the hematopoietic compartment.

### *The switch to adult-state hematopoietic stem cells*

While embryonic hematopoietic stem cells are highly cycling and expanding, adult hematopoietic stem cells are more dormant (Bowie, McKnight et al. 2006). This is concomitant with changes in expression of cell surface markers. For instance, embryonic hematopoietic stem cells express the lineage commitment markers Mac-1 (monocytic/macrophage marker) and Aa4.1 (B-cell marker), while adult hematopoietic stem cells do not (Jordan, McKearn et al. 1990; Morrison, Hemmati et al. 1995; Rebel, Miller et al. 1996). This switch from embryonic to adult hematopoietic stem cells occur at or around three weeks postnatally in mice and is marked by decreased cycling and loss of expression of Mac-1 and Aa4.1 (Bowie, McKnight et al. 2006; Bowie, Kent et al. 2007). The bone marrow then leads as the

major niche for sustaining hematopoiesis and accommodating hematopoietic stem cells throughout adulthood.

## **The hematopoietic niche**

### *The niche hypothesis*

A team led by Wolf (1967) transplanted bone marrows and spleens into irradiated mice and counted the various colonies that formed in anatomically distinct organs (Curry, Trentin et al. 1967). They later observed erythropoietic colonies in the spleen, while the bone marrow colonies were predominantly myelomonocytic (Wolf and Trentin 1970). Their experiments correlated the environment where hematopoietic cells were growing with the identity, or fate, of their progeny. A few years later, Schofield coined the term “niche” (to nest in French) to describe the environment where hematopoietic stem cells home to and reside (Schofield 1978). Schofield (1978) was the first to propose that the role of the bone marrow niche was to maintain the proliferative capacity of hematopoietic stem cells and inhibit their maturation.

### *Bone marrow endosteal niche*

As previously mentioned, the embryonic niche for hematopoietic stem cells is anatomically distinct. But very little is known about the cellular compartments in these tissues that govern the hematopoietic stem cell communication with the niche during embryogenesis. On the other hand, more is known about the underlying regions involved in maintenance of hematopoietic stem cells in the adult bone marrow. Despite various discrepancies in the literature due to technical issues, two

distinct environments have been identified: the endosteal and the vascular bone marrow niche (Wilson and Trumpp 2006).

The endosteum is the cellular lining that separates the bone from the bone marrow, and is constituted of osteoblasts, osteoclasts and stromal fibroblasts (Wilson and Trumpp 2006). Hematopoietic stem cells are found in close association with the endosteum, while more differentiated cells are in the central region of the bone marrow (Zhang, Niu et al. 2003; Kiel, Yilmaz et al. 2005). Direct evidence supporting the endosteal origin of the hematopoietic niche came from David Scadden's and Linhend Li's laboratories. Their studies unequivocally demonstrate that altering the abundance of osteoblasts via modulating levels of parathyroid hormone (PTH) or bone morphogenetic protein (BMP), regulators of bone formation and resorption, dictate the hematopoietic stem cell pool number and activity (Calvi, Adams et al. 2003; Zhang, Niu et al. 2003).

### *The vascular niche*

Since hematopoietic stem cells may also originate from endothelial cells, it is not surprising that the vasculature constitutes another niche for hematopoietic stem cells (Wilson and Trumpp 2006). The vasculature niche of the bone marrow, which is formed by the bone marrow sinusoidal endothelial cells (BMECs), is functionally and phenotypically different from the microvasculature endothelial cells of other organs (Kopp, Avecilla et al. 2005; Wilson and Trumpp 2006). BMECs secrete cytokines and express adhesion molecules that are required for hematopoietic stem cell engraftment, homing and mobilization (Sipkins, Wei et al. 2005).

### *Collaboration of endosteal and vascular niche*

The endosteal and vascular bone marrow niche are not two independent environments. These two niches cooperate to support self-renewal capabilities and inhibit differentiation of hematopoietic stem cells (Wilson and Trumpp 2006). In fact, it is estimated that quiescent hematopoietic stem cells reside in close proximity with osteoblasts of the endosteal niche (Calvi, Adams et al. 2003; Scadden 2006). As soon as these cells are required to proliferate and reconstitute hematopoiesis, they detach from the osteoblastic lining and migrate towards the vasculature zone which is located in the center of the bone marrow (Kopp, Avecilla et al. 2005). Since ablation of osteoblasts activates extramedullary hematopoiesis (Visnjic, Kalajzic et al. 2004), the vascular niche is not sufficient to support hematopoiesis in bone marrows and is considered a “secondary niche, requiring an influx of hematopoietic stem cells from the primary endosteal niches” (Wilson and Trumpp 2006).

### **Oxidative nature of the hematopoietic compartment**

#### *Hematopoietic stem cell and Reactive Oxygen Species distribution*

ROS are generated by intrinsic or extrinsic sources, such as metabolism and ionizing radiation, respectively, and can cause DNA damage (Lombard, Chua et al. 2005; Naka, Muraguchi et al. 2008). Hence, the oxidative nature of the hematopoietic niche may dictate the level of protection it can provide to hematopoietic stem cells. The osteoblastic lining of the bone is considered hypoxic and hence provides a protective environment to harbor hematopoietic stem cells (Naka, Muraguchi et al. 2008). In humans, bone marrows are thought to be in hypoxic conditions, but not enough to initiate a hypoxic molecular response

(Harrison, Rameshwar et al. 2002; Eliasson and Jonsson 2009). Also, slow cycling hematopoietic stem cells are associated with hypoxic regions of capillaries suggesting that low ROS maintain quiescence and self-renewal (Suda, Arai et al. 2005; Kubota, Takubo et al. 2008). Collectively, these studies suggest that hematopoietic stem cells are located in a low-oxygen milieu of the bone marrow – specifically associated with osteoblastic niche – supporting their long-term hematopoietic activity and protecting the cells from ROS-related oxidative stress.

### *ROS in hematopoietic stem cells*

The earliest correlative studies of ROS and hematopoietic stem cell maintenance came from the *Atm*<sup>-/-</sup> mouse model (Ito, Hirao et al. 2004). Briefly, *Atm*<sup>-/-</sup> mice suffered from hematopoietic defects that were manifested late in life (Ito, Hirao et al. 2004). *Atm* loss led to significantly elevated ROS levels in the hematopoietic compartment, ultimately leading to loss of hematopoietic stem cell self-renewal capability (Ito, Hirao et al. 2004). Other studies used ROS levels to distinguish among various HSC self-renewal capacities (Jang and Sharkis 2007). Specifically, HSCs with lower ROS content can engraft irradiated recipients better than HSCs with higher ROS (Jang and Sharkis 2007). Other pathways such as Bmi-1, p38-Mapk and FoxO can regulate ROS levels in hematopoietic stem cells and influence their activity (Ito, Hirao et al. 2006; Tothova, Kollipara et al. 2007; Liu, Cao et al. 2009). Conclusively then, disrupting ROS levels leads to hematopoietic stem cell exhaustion and depletion. Yet, the downstream targets of ROS and how these targets could affect hematopoiesis remain unknown.



## **Mdm2-p53 pathway in hematopoietic stem cells**

### *p53 loss increases hematopoietic stem cell cycling*

Since hematopoietic stem cells are subject to ROS-induced DNA damage, and since p53 is activated due to stress, it is important to delineate the role of the p53 pathway in hematopoietic stem cells and their maintenance. Loss of *p53* in hematopoietic stem cells increases their cycling and expands this population (Liu, Elf et al. 2009). This may ultimately lead to mutations and genetic aberrations, and consequently leukemias (Akala, Park et al. 2008; Liu, Elf et al. 2009)

### *Mdm2<sup>-/-</sup> p53<sup>515C/515C</sup>: a good model to address p53 role in hematopoiesis*

As previously discussed, the early embryonic lethality of *Mdm2*-null mice impedes the study of the effects of disrupting the Mdm2-p53 balance on hematopoiesis. Fortunately, *Mdm2*<sup>-/-</sup> mice can be rescued using a hypomorphic allele of *p53* (*p53*<sup>515C</sup>) (Liu, Terzian et al. 2007). However, *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice suffer from acellular bone marrows and die shortly after birth (Liu, Terzian et al. 2007). Intriguingly, *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> pups are born morphologically indistinguishable from control *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> littermates despite nullizygosity of *Mdm2*. Based on this observation, we asked several questions. First, why did hematopoiesis proceed during embryogenesis compared to neonatal development in absence of *Mdm2*? Also, why did *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice exhibit a severe phenotype after birth? Is p53R172P (the protein encoded by *p53*<sup>515C</sup>) stable and active during embryogenesis? How does the disruption of Mdm2-p53 pathway influence the hematopoietic stem cell and progenitor pools?

In this dissertation we provide evidence that hematopoietic stem/progenitor cells are highly sensitive to elevated ROS-induced p53R172P levels. p53R172P stimulates ROS production via activation of set of redox-related genes collectively known as p53-induced-genes. In the absence of Mdm2, this creates a positive feedback loop to maintain elevated p53 levels. Growing *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> hematopoietic stem cells at low oxygen conditions allowed normal expansion of this population. Moreover, genetic ablation of *p16* increases cycling in the hematopoietic compartment, expands the hematopoietic stem cell population and their progenitors, and increases survival. On the other hand, *p21* deletion did not affect the hematopoietic compartment. p53R172P stability exerted several effects on the hematopoietic niche leading to increased osteoblastic activity. We conclude that although p53 is required to inhibit tumorigenesis, uncontrolled p53 levels can be detrimental to the hematopoietic compartment and thus to the organism. Most importantly, Mdm2 is constantly and evolutionarily required to control p53 levels in bone marrows to insure the survival of the organism.

## RESULTS

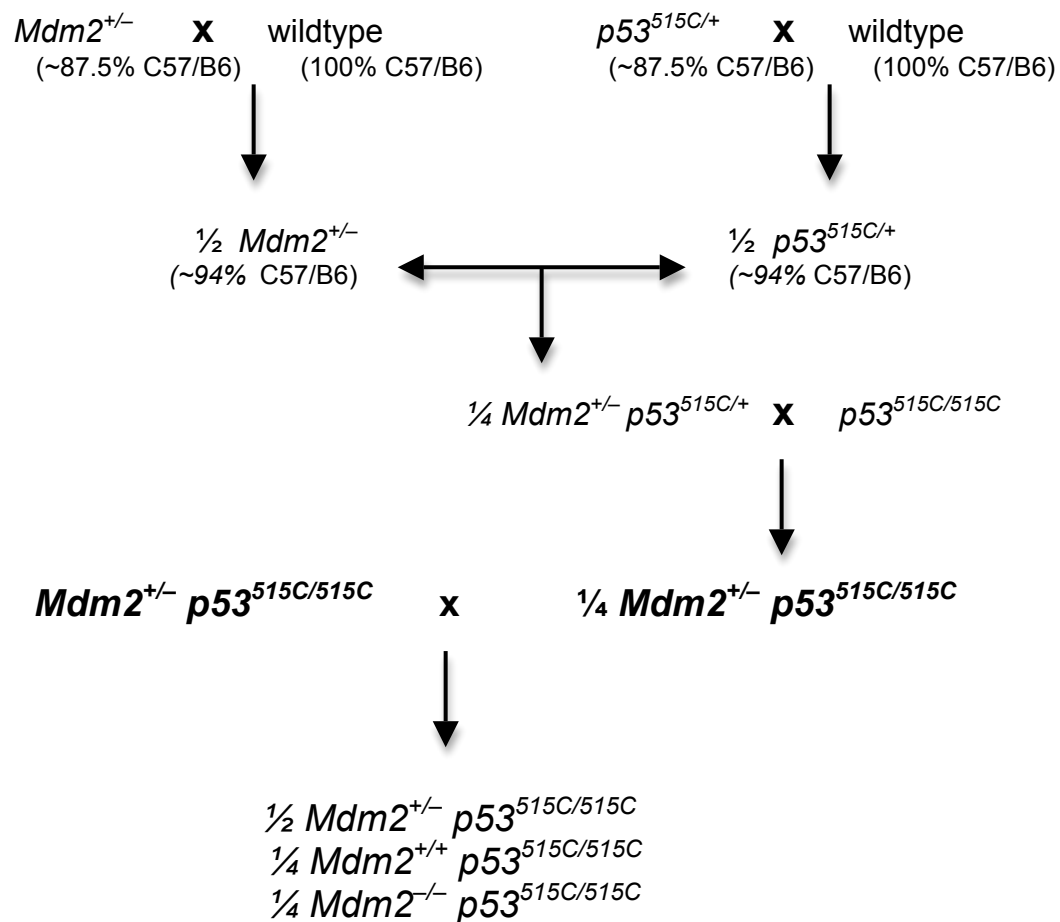
### Establishing and characterizing the cohort

#### *Genetic crosses*

In order to make  $Mdm2^{-/-} p53^{515C/515C}$  mice, we followed the genetic scheme shown in Figure 5. Briefly, we used previously generated  $Mdm2^{+/-}$  and  $p53^{515C/+}$  mice (Montes de Oca Luna, Wagner et al. 1995; Liu, Parant et al. 2004) that were 87.5% C57BL/6 and already housed in our animal facility. The first step was to backcross these mice into a higher C57BL/6 background to avoid immune rejections in case transplantation experiments were warranted during our studies. We performed Polymerase Chain Reaction (PCR) using previously published primers in order to distinguish the different  $Mdm2$  and  $p53^{515C}$  alleles (Montes de Oca Luna, Wagner et al. 1995; Liu, Parant et al. 2004).  $Mdm2^{+/-} p53^{515C/+}$  were generated by breeding  $Mdm2^{+/-}$  and  $p53^{515C/+}$  mice to each other.  $Mdm2^{+/-} p53^{515C/+}$  were bred to  $p53^{515C/515C}$  to get the  $Mdm2^{+/-} p53^{515C/515C}$  mouse colony. Finally,  $Mdm2^{+/-} p53^{515C/515C}$  mice were mated to each other to generate the  $Mdm2^{-/-} p53^{515C/515C}$  study group, and  $Mdm2^{+/-} p53^{515C/515C}$  and  $Mdm2^{+/+} p53^{515C/515C}$  control groups all from one cross in a 1:2:1 ratio, respectively.

#### *Survival curve*

Out of 106 pups born from intercrossing  $Mdm2^{+/-} p53^{515C/515C}$  mice, 26, 27 and 53 were  $Mdm2^{-/-} p53^{515C/515C}$ ,  $Mdm2^{+/+} p53^{515C/515C}$  and  $Mdm2^{+/-} p53^{515C/515C}$ , respectively (Table 1). Hence,  $Mdm2^{-/-} p53^{515C/515C}$  mice were born at normal Mendelian ratios and confirmed previously published work (Liu, Terzian et al. 2007).



**Figure 5. Genetic scheme** Backcrossing of  $Mdm2^{+/-}$  and  $p53^{515C/+}$  into a C57/B6 background followed by generation of  $Mdm2^{+/-} p53^{515C/515C}$ . Expected ratios are noted next to genotypes.

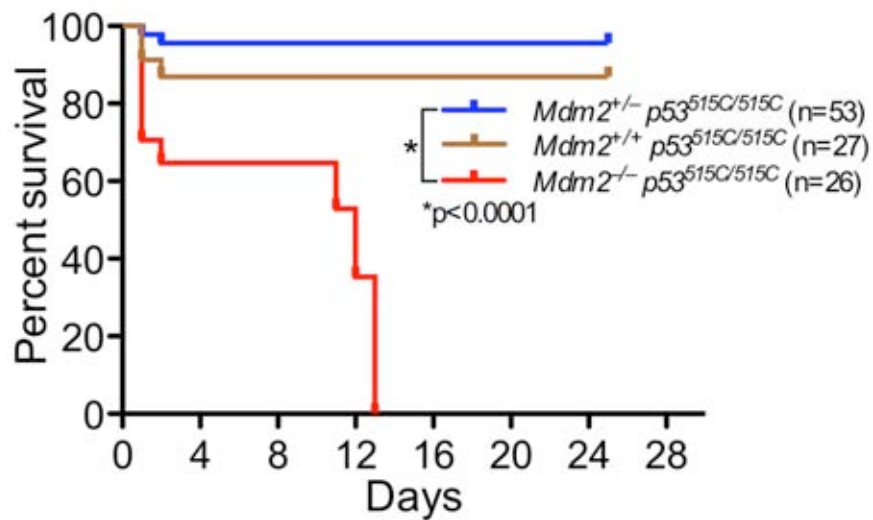
**Table 1.** Expected ratios and number of mice born of each genotype after crossing  $Mdm2^{+/-} p53^{515C/515C}$  mice to each other.

<b>Genotype</b>	<b><math>Mdm2^{+/+}</math> <math>p53^{515C/515C}</math></b>	<b><math>Mdm2^{+/-}</math> <math>p53^{515C/515C}</math></b>	<b><math>Mdm2^{-/-}</math> <math>p53^{515C/515C}</math></b>	<b><i>Total</i></b>
<b>Ratio Expected</b>	1/4	1/2	1/4	1/1
<b>Number expected</b>	26.5	53	26.5	106
<b>At birth</b>	<b>27</b>	<b>53</b>	<b>26</b>	<b>106</b>

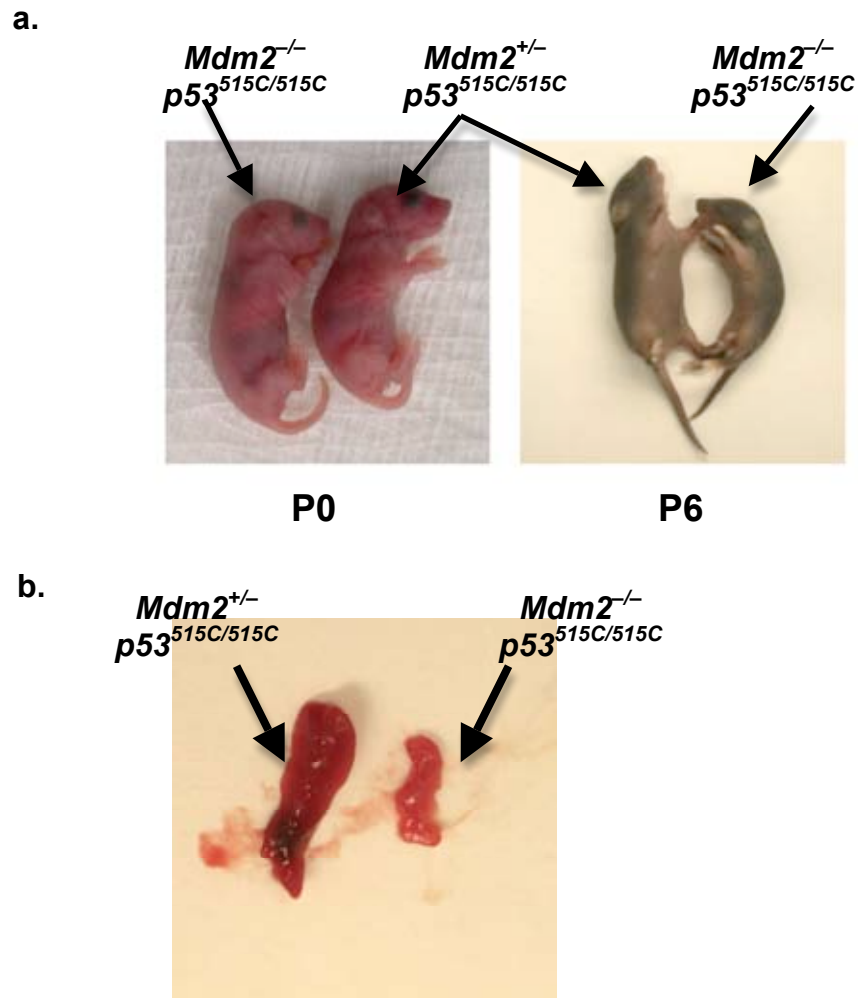
Although it was previously reported that all  $Mdm2^{-/-} p53^{515C/515C}$  die within two weeks after birth (Liu, Terzian et al. 2007), a detailed survival curve was still lacking. To that end, we monitored litters from pregnant females at least twice on daily basis. The day a litter was born was noted as postnatal day (P) 0. The number of pups at birth was counted twice a day, and tissues of dead pups were collected for genotyping. 95% of  $Mdm2^{+/-} p53^{515C/515C}$  and 87% of  $Mdm2^{+/+} p53^{515C/515C}$  pups survived until weaning age and were used for future breeding experiments (Figure 6). On the other hand, 65% of  $Mdm2^{-/-} p53^{515C/515C}$  pups were dead by P2 (Figure 6). Of the  $Mdm2^{-/-} p53^{515C/515C}$  pups surviving until P2, none died between P3 and P10. Between P11 and P13, all  $Mdm2^{-/-} p53^{515C/515C}$  pups died (Figure 6).

### *Growth retardation*

$Mdm2^{-/-} p53^{515C/515C}$  pups were indistinguishable from their control littermates at birth (Figure 7a). We then monitored the mice for any obvious changes in size or morphology. At approximately P6,  $Mdm2^{-/-} p53^{515C/515C}$  mice started showing signs of growth retardation (Figure 7a). By P10, all  $Mdm2^{-/-} p53^{515C/515C}$  pups were easily discernable from their control littermates by their smaller size (Liu, Terzian et al. 2007). No notable differences were observed between  $Mdm2^{+/-} p53^{515C/515C}$  and  $Mdm2^{+/+} p53^{515C/515C}$  littermates. Necropsy on 6 day old pups showed significantly smaller and pale spleens in  $Mdm2^{-/-} p53^{515C/515C}$  pups as compared to spleens of control  $Mdm2^{+/-} p53^{515C/515C}$  pups (Figure 7b). Although  $Mdm2^{-/-} p53^{515C/515C}$  pups were previously reported to have hematopoietic defects (Liu, Terzian et al. 2007), detailed analysis of this phenotype and the molecular changes at various time points remained to be elucidated.



**Figure 6. Kaplan-Meier survival curves of *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> (n=26), *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> (n=53) and *Mdm2*<sup>+/+</sup> *p53*<sup>515C/515C</sup> (n=27) mice.** *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> pups had a significantly ( $p<0.0001$ ) shorter survival curve compared to control *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> and *Mdm2*<sup>+/+</sup> *p53*<sup>515C/515C</sup> littermates. Notably, a high percentage of *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> pups died within the first two days after birth.



**Figure 7. Snapshots of P0 and P6 pups, and P6 spleens. (a)** *Mdm2*<sup>-/-</sup>*p53*<sup>515C/515C</sup> pups are indistinguishable at P0 but start showing signs of growth retardation at P6. **(b)** Spleens of *Mdm2*<sup>-/-</sup>*p53*<sup>515C/515C</sup> mice at P6 are smaller and pale.



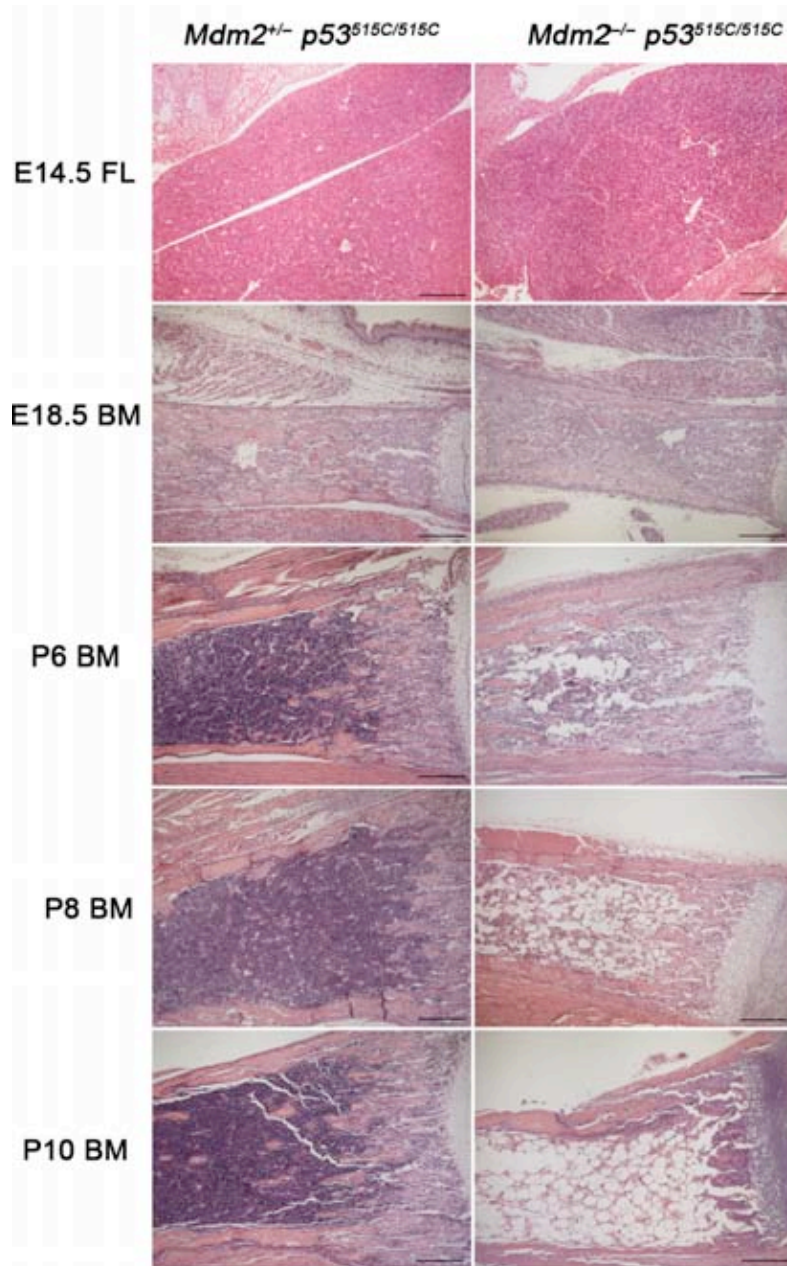
## Hematopoiesis during embryogenesis and postnatally

### *Normal histology of fetal livers and embryonic bone marrows*

Since  $Mdm2^{-/-} p53^{515C/515C}$  pups had no observable abnormalities at P0, we anticipated hematopoiesis was normal during embryogenesis. As previously mentioned, the fetal liver is the major hematopoietic organ between E12.5 and E15.5 until hematopoiesis migrates to the bone marrow at late embryogenesis (Mikkola and Orkin 2006). Hence, we sacrificed pregnant females at E14.5 and E18.5 to examine these two processes. Embryonic tissues were stained with hematoxylin and eosin (H&E) for histological analysis.  $Mdm2^{-/-} p53^{515C/515C}$  fetal livers at E14.5, and bone marrows at E18.5 had normal cellularity and no histologically detectable hematopoietic abnormalities (Figure 8).

### *Acellular postnatal bone marrows*

$Mdm2^{-/-} p53^{515C/515C}$  pups started showing signs of growth retardation at approximately P6. We therefore sacrificed neonates at P6, P8, and P10 for careful examination of their bone marrows. By P6, bone marrow cellularity of  $Mdm2^{-/-} p53^{515C/515C}$  mice dropped to approximately 70% and by P10, these mice had less than 10% cellularity compared to control littermates (Figure 8). No notable defects were observed for bone marrow cellularity of control  $Mdm2^{+/-} p53^{515C/515C}$  mice at all time points examined. Previously, our lab also showed that the peripheral blood cellularity of  $Mdm2^{-/-} p53^{515C/515C}$  mice is severely low at P11 (Liu, Terzian et al. 2007). Hence, loss of  $Mdm2$  depletes the hematopoietic compartment in the bone marrows and in the peripheral blood postnatally. In order to address whether this is a p53- dependent phenotype, we generated  $Mdm2^{-/-} p53^{515C/-}$  mice. These mice



**Figure 8. *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice die postnatally due to hematopoietic failure.** Representative H&E staining of fetal livers (FL) at E14.5 and bone marrows (BM) at E18.5 P6, P8 and P10 from *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> (left) and *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice (right). Scale bar is 200μm.

were viable and had no obvious defects as previously reported (Liu, Terzian et al. 2007).  $Mdm2^{-/-} p53^{515C/-}$  males suffered only from atrophic testis and males were infertile with no notable hematopoietic defects (Liu, Terzian et al. 2007). These experiments confirmed that the hematopoietic defect observed in  $Mdm2^{-/-} p53^{515C/515C}$  was p53-dependent and was manifested at postnatal time points.

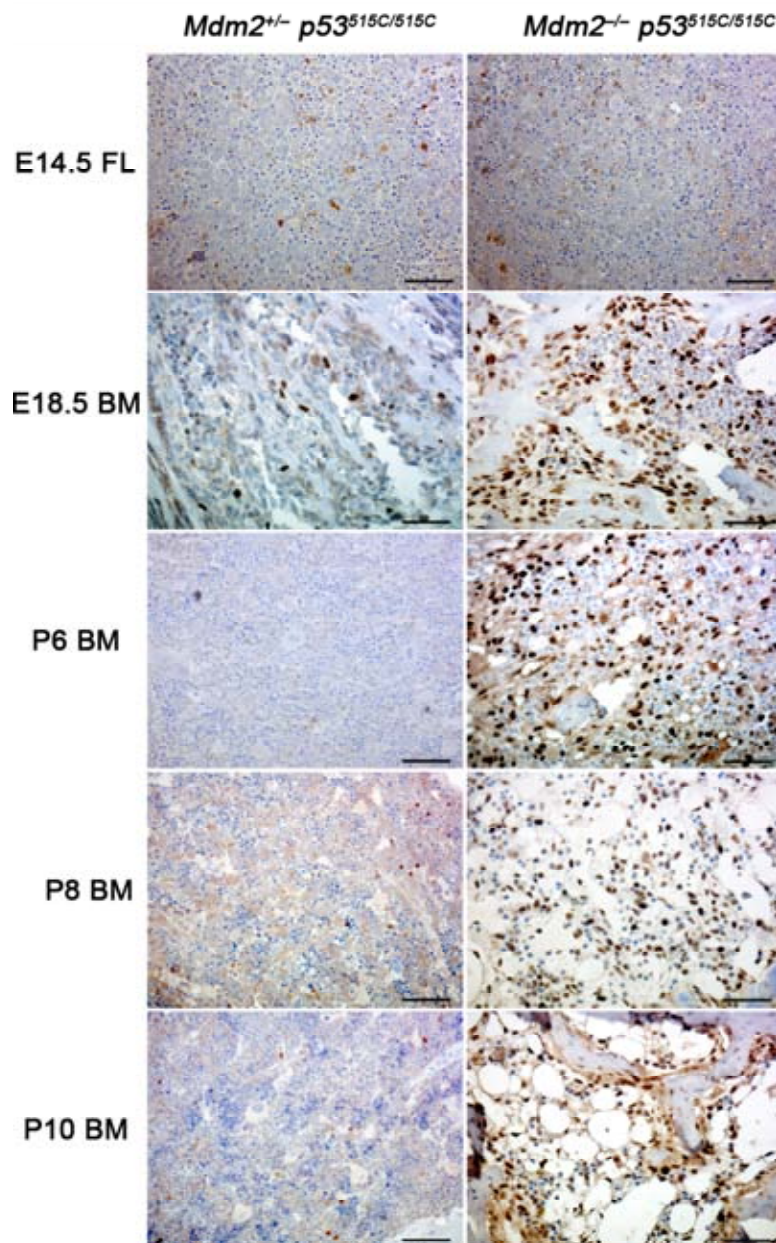
### **p53R172P stability and activity in the hematopoietic compartment**

#### *Elevated p53R172P in bone marrows, but not fetal livers*

$p53^{515C}$  encodes p53R172P protein. Since Mdm2 targets p53 for degradation (Haupt, Maya et al. 1997; Honda, Tanaka et al. 1997), we performed immunohistochemistry (IHC) on hematopoietic tissues at various time points to investigate p53R172P levels in moribund and control mice. E14.5 fetal livers of  $Mdm2^{+/-} p53^{515C/515C}$  (control) and  $Mdm2^{-/-} p53^{515C/515C}$  mice had very low levels of p53R172P staining and were indistinguishable (Figure 9). On the other hand, bone marrow cells of  $Mdm2^{-/-} p53^{515C/515C}$  mice had elevated p53R172P staining at E18.5, P6, P8 and P10. Control  $Mdm2^{+/-} p53^{515C/515C}$  littermates were negative for p53R172P at all time points except for a few p53R172P positively stained cells (Figure 9). Hence, high p53R172P levels correlated with decreased bone marrow cellularity in  $Mdm2^{-/-} p53^{515C/515C}$  mice postnatally.

#### *Increased p53R172P transcriptional activity postnatally*

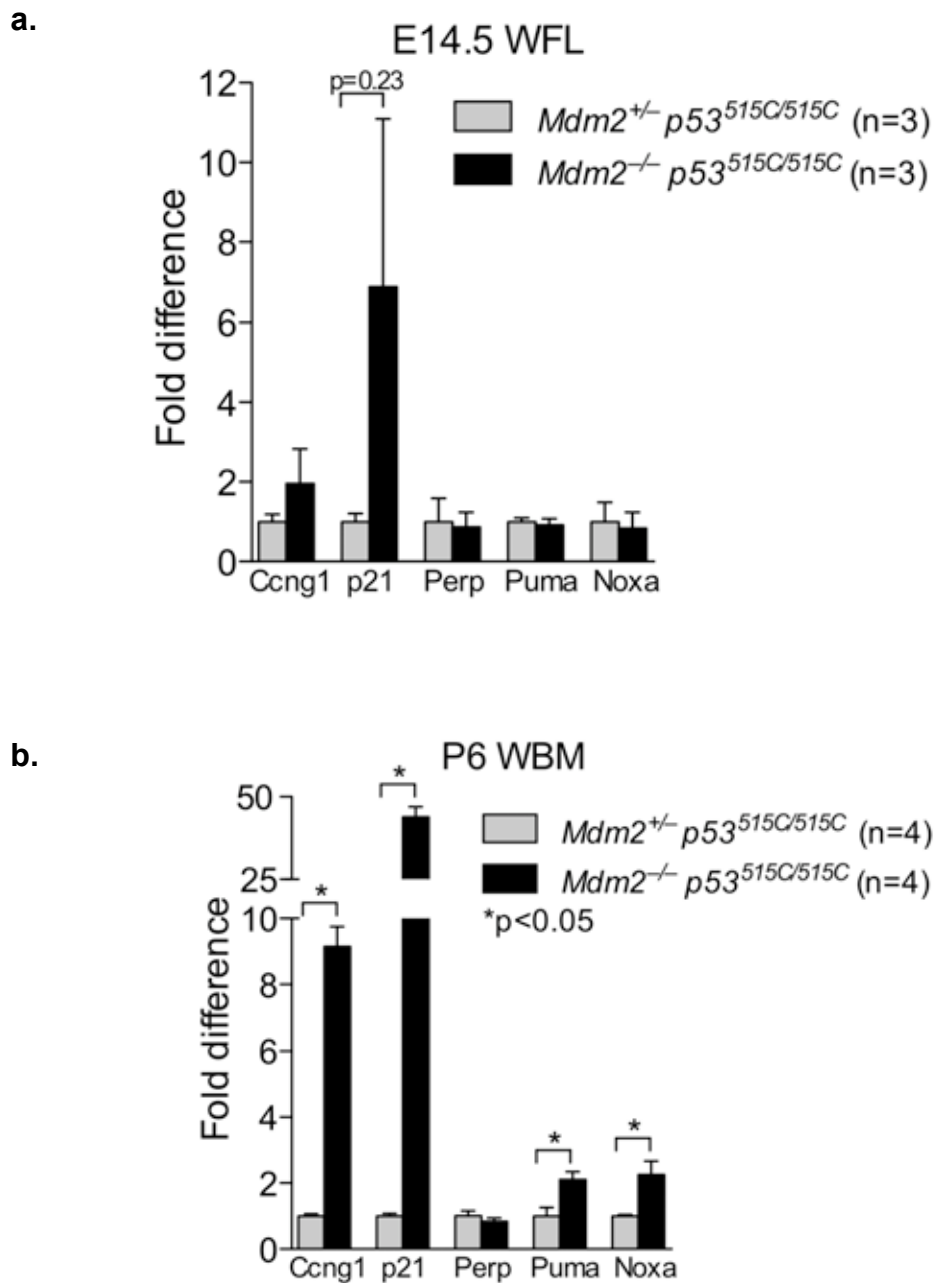
E14.5 fetal livers showed normal histology and no p53R172P staining (Figures 8 and 9). Also, P6 moribund mice were visually discernable from control



**Figure 9. p53R172P Immunohistochemical staining.** Paraffin-embedded sections of E14.5 fetal livers, and E18.5, P6, P8 and P10 bone marrows from *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> (left) and *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> (right) were stained for p53R172P and counterstained with hematoxylin. Scale bar is 50μm.

mice, and had stable p53R172P levels (Figure 7a). Most importantly, P6 bone marrows still retained 60-70% bone marrow cellularity (Figure 8) providing us with enough cells for mechanistic studies. Hence, we chose E14.5 fetal livers and P6 bone marrows as representative time points for detailed molecular characterization of the embryonic and neonatal phenotypes, respectively. We used Real Time reverse transcriptase-PCR (RT-PCR) to look for changes in mRNA levels of known p53 target genes.

We first isolated RNA from whole fetal livers at E14.5 and examined cell cycle arrest targets of p53: *p21* and *Ccng*. In fetal livers, differences in *p21* ( $p=0.23$ ) and *Ccng* (0.34) levels were statistically insignificant between  $Mdm2^{-/-} p53^{515C/515C}$  and control  $Mdm2^{+/-} p53^{515C/515C}$  (Figure 10a). One  $Mdm2^{-/-} p53^{515C/515C}$  fetal liver was 15 fold higher but this outlier did not affect the statistical significance (Figure 10a). We also examined the apoptosis targets of p53 and found no differences in induction of *Noxa* ( $p=0.81$ ), *Perp* ( $p=0.86$ ) or *Puma* ( $p=0.67$ ) in  $Mdm2^{+/-} p53^{515C/515C}$  compared to  $Mdm2^{-/-} p53^{515C/515C}$  mice (Figure 10a). On the other hand, all  $Mdm2^{-/-} p53^{515C/515C}$  whole bone marrows at P6 had dramatic and significant induction of p53 cell cycle targets *Ccng* (9 fold) and *p21* (43 fold) compared to control  $Mdm2^{+/-} p53^{515C/515C}$  littermates (Figure 10b). As for apoptotic targets, *Perp* levels were the same in all mice (Figure 10b). However, *Puma* and *Noxa* levels were consistently two fold higher in  $Mdm2^{-/-} p53^{515C/515C}$  moribund mice than in  $Mdm2^{+/-} p53^{515C/515C}$  mice ( $p<0.02$ ) (Figure 10b).



**Figure 10. p53R172P transcriptional activity induces cell cycle arrest genes and to a lesser extent apoptosis genes in postnatal bone marrows.** Real-Time PCR results from E14.5 whole fetal liver (WFL) cells (**a**) and P6 whole bone marrow (WBM) cells (**b**) on p53 cell cycle arrest (*Ccng1* and *p21*) and apoptosis (*Perp*, *Puma* and *Noxa*) targets. All levels were normalized to *Gapdh*. Error bars represent standard error of the mean.

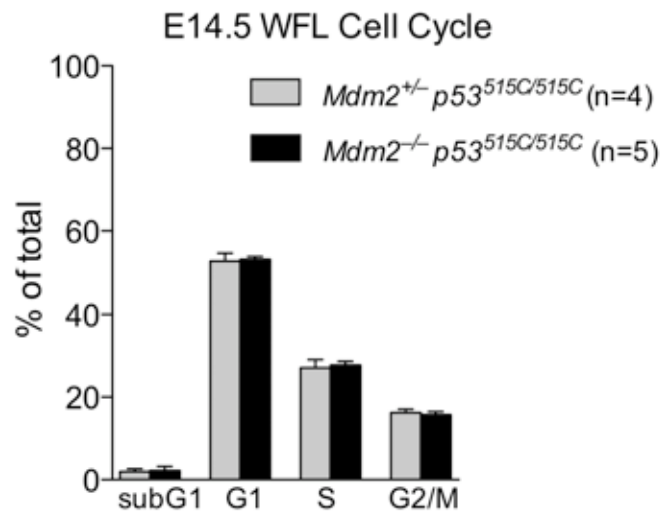
## Examining cycling, senescence and apoptosis

### *Decreased cell cycling in postnatal bone marrows, but not fetal livers*

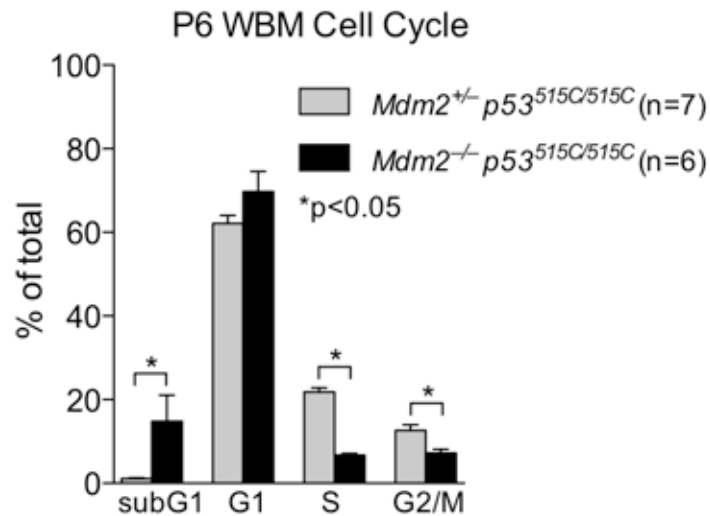
To investigate the cell cycle pattern in fetal livers and postnatal bone marrows, we fixed cells from these tissues and analyzed their DNA content with propidium iodide staining. As expected based on normal histological analysis and negative p53R172P staining, cell cycle patterns of E14.5 fetal livers between  $Mdm2^{-/-} p53^{515C/515C}$  and  $Mdm2^{+/-} p53^{515C/515C}$  littermates were indistinguishable (Figure 11a). Specifically, 53.1%, 27.7% and 15.7% of whole fetal liver cells in  $Mdm2^{-/-} p53^{515C/515C}$  embryos were in G1, S, G2/M phase, respectively, similar to 52.7%, 27.0% and 16.2% in control  $Mdm2^{+/-} p53^{515C/515C}$  littermates (Figure 11a). The fraction of cells in subG1 was less than 2% in both genotypes (Figure 11a).

P6 bone marrows, however, showed significant differences between the two genotypes. Less than 6.5% of  $Mdm2^{-/-} p53^{515C/515C}$  bone marrow cells were in S phase compared to 22% for control littermates (Figure 11b).  $Mdm2^{-/-} p53^{515C/515C}$  neonates had 7.1% of cells in the G2/M phase compared to 12.6% in  $Mdm2^{+/-} p53^{515C/515C}$  littermates (Figure 11b). A significant percentage (14.8%) of  $Mdm2^{-/-} p53^{515C/515C}$  bone marrow cells as compared to <1.1% of control bone marrow cells were in subG1 fraction suggesting cell death as another mechanism of depleting the cellular compartment ( $p=0.03$ ) (Figure 11b). Among the 6  $Mdm2^{-/-} p53^{515C/515C}$  analyzed at P6, three pups had subG1 fractions >14% and the other three had subG1 populations between 2% and 4%. Since all  $Mdm2^{+/-} p53^{515C/515C}$  mice had subG1 levels of less than 1.6%, we attained statistical significance.

a.



b.



**Figure 11. Induction of cell cycle arrest and cell death in postnatal bone marrows.** Cell cycle analysis of E14.5 whole fetal livers (WFL) (a) and P6 whole bone marrow (WBM) (b) using propidium iodide on fixed cells of these tissues. Error bars represent standard error of the mean.



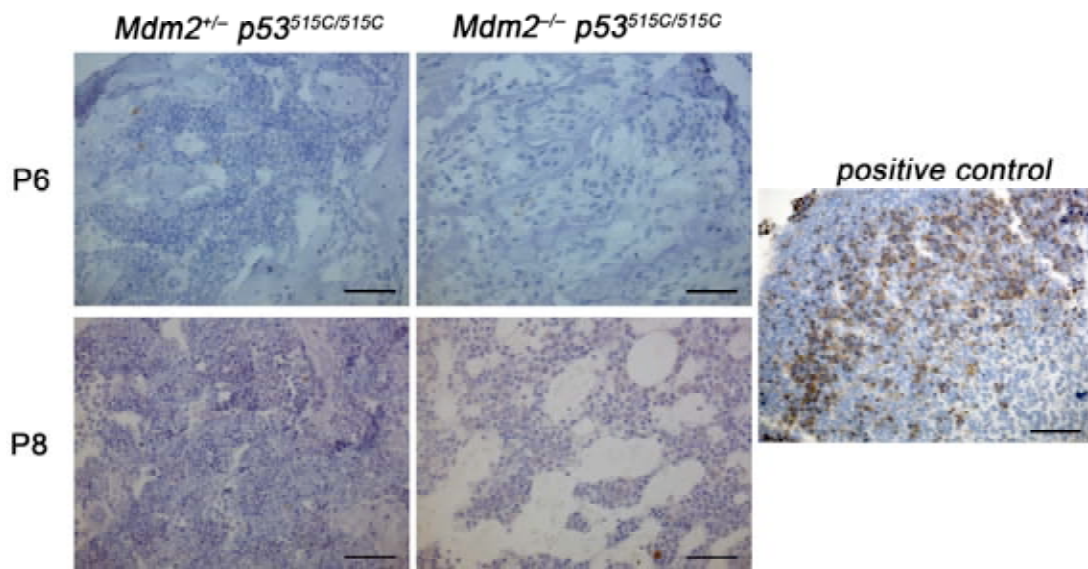
### *Caspase-3 and AnnexinV are not expressed in $Mdm2^{-/-}$ $p53^{515C/515C}$ bone marrows*

Since *Puma* and *Noxa* were elevated in  $Mdm2^{-/-}$   $p53^{515C/515C}$  P6 bone marrows, and since 14% of these cells were in subG1, we investigated apoptosis as a mechanism for depleting hematopoiesis. The caspase-3 pathway is usually activated in response to p53 dependent apoptosis (Fridman and Lowe 2003). We performed IHC in order to assay for cleaved caspase-3 in postnatal bone marrows at P6 and P8 (Figure 12). Except for a few positive cells, caspase-3 staining was negative in all bone marrows at these time points (Figure 12).

We then used AnnexinV staining as an alternative method to look for apoptosis with flow cytometry. E14.5 whole fetal livers of both  $Mdm2^{-/-}$   $p53^{515C/515C}$  and  $Mdm2^{+/-}$   $p53^{515C/515C}$  had less than 1.6% ( $p=0.71$ ) of cells expressing AnnexinV (Figure 13a). Also for P6 whole bone marrows there was no statistical difference between pups of the two genotypes for AnnexinV staining (Figure 13b). Hence, although cell death is occurring, the standard markers for apoptosis are not positive suggesting an alternative mechanism of cell death.

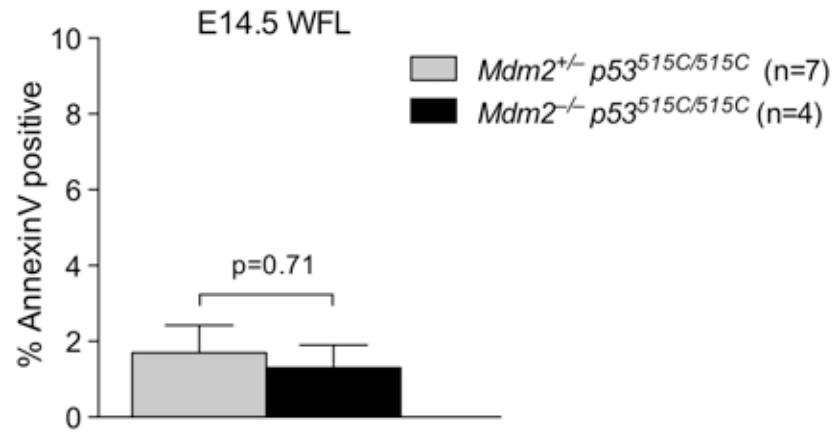
### *Increased senescence markers in $Mdm2^{-/-}$ $p53^{515C/515C}$ P6 bone marrows.*

p53R172P activates p21 which can induce senescence (Noda, Ning et al. 1994; Cosme-Blanco, Shen et al. 2007; Post, Quintas-Cardama et al. 2009). We hypothesized that  $Mdm2^{-/-}$   $p53^{515C/515C}$  bone marrows in early postnatal development may also exhibit a senescent phenotype. Accordingly, in addition to p21 which was 43 fold higher than controls (Figure 10b), we examined expression levels of known senescent markers: *Dcr2*, *p15*, *p16*, *Dec1* and *Pml* using RT-PCR

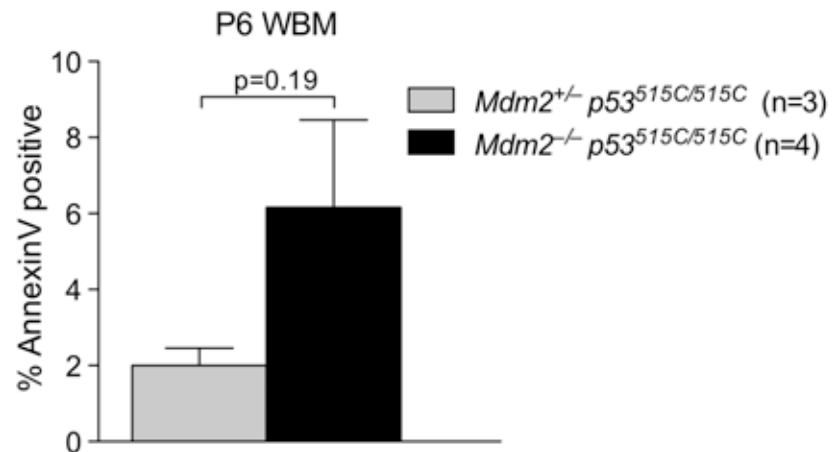


**Figure 12. Absence of cleaved caspase-3 staining in P6 and P8 *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> bone marrows.** Cleaved caspase-3 immunohistochemical staining on paraffin embedded bone marrows of *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> (left) and *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> (right) at P6 and P8. Irradiated wildtype thymus was used as a positive control for cleaved caspase-3 staining. Scale bar is 50µm.

a.



b.



**Figure 13. No statistically significant difference in AnnexinV expression in fetal livers and postnatal bone marrows.** (a) E14.5 Whole fetal liver (WFL) cells and (b) P6 whole bone marrow (WBM) cells were stained with AnnexinV and analyzed with flow cytometry. Error bars represent standard error of the mean. n= number of mice examined.

in E14.5 fetal livers and P6 bone marrows (Collado, Gil et al. 2005; Collado and Serrano 2006). Expression levels of these genes in E14.5 whole fetal liver cells were the same between  $Mdm2^{-/-}$   $p53^{515C/515C}$  embryos and controls (Figure 14a). However, in addition to  $p21$ ,  $Dcr2$  (17 fold) and  $p15$  (11 fold) were highly elevated in postnatal bone marrows of  $Mdm2^{-/-}$   $p53^{515C/515C}$  mice as compared to controls, while  $Dec1$ ,  $p16$  and  $Pml$  mRNA levels did not change (Figure 14b). This indicates that senescence could be another pathway activated by p53R172P to inhibit hematopoiesis.

#### *p21 deletion extends lifespan of $Mdm2^{-/-}$ $p53^{515C/515C}$ mice*

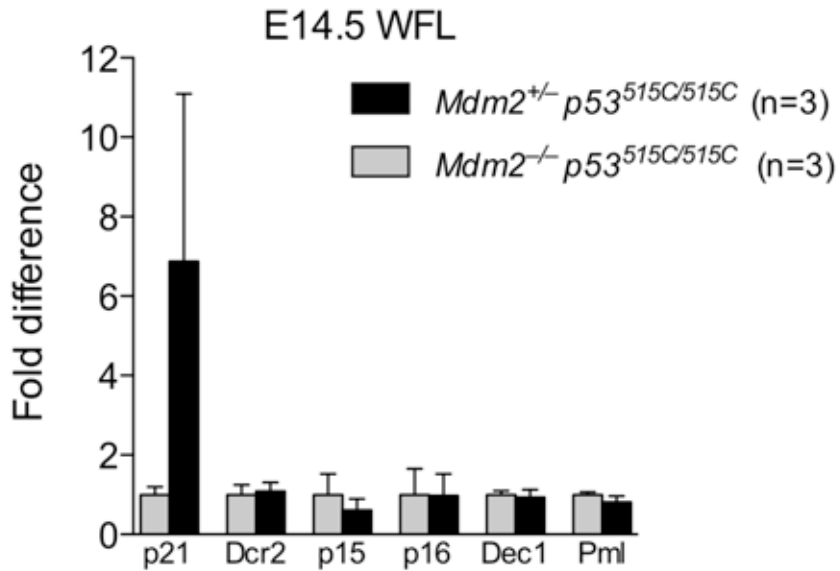
$p21$  expression levels were the highest in  $Mdm2^{-/-}$   $p53^{515C/515C}$  bone marrows among all genes tested at P6 (Figure 10b). Also,  $p21$  maintains quiescence in HSCs (Cheng, Rodrigues et al. 2000). Hence, we investigated whether  $p21$  deletion might rescue hematopoiesis by increasing proliferation and alleviating senescence of bone marrows. Contrary to our expectations,  $p21$  deletion did not rescue bone marrow cellularity (Figure 15b). However, the median age and life expectancy were longer for these mice indicating a partial rescue of postnatal lethality (Figure 15a). Increased survival may be due to effects on other tissues as  $Mdm2^{-/-}$   $p53^{515C/515C}$  mice also suffer from multiple other defects (Liu, Terzian et al. 2007).

### **Characterization of hematopoietic stem cells and progenitors**

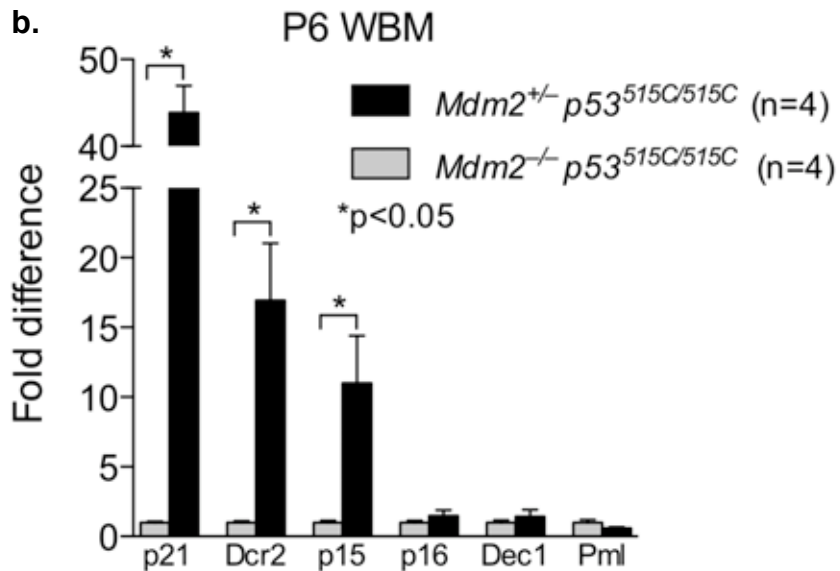
#### *A major defect in HSC and CLP/CMP populations.*

HSCs are responsible for replenishing the cellular compartment of the blood (Weissman 2000). Since  $Mdm2^{-/-}$   $p53^{515C/515C}$  bone marrows reflect absence of

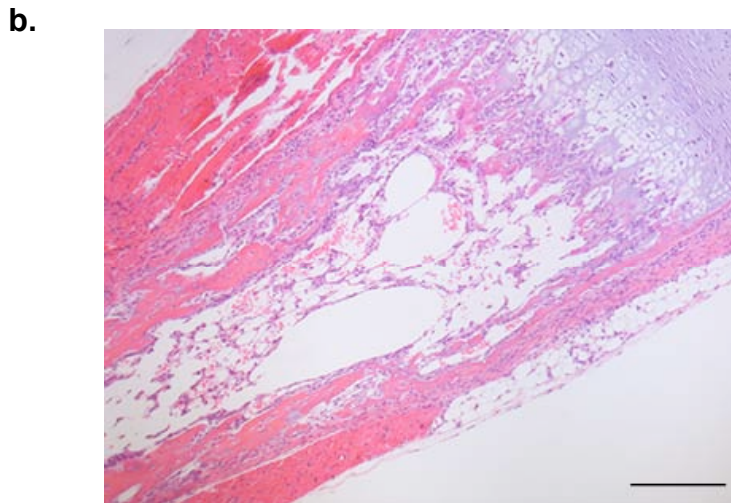
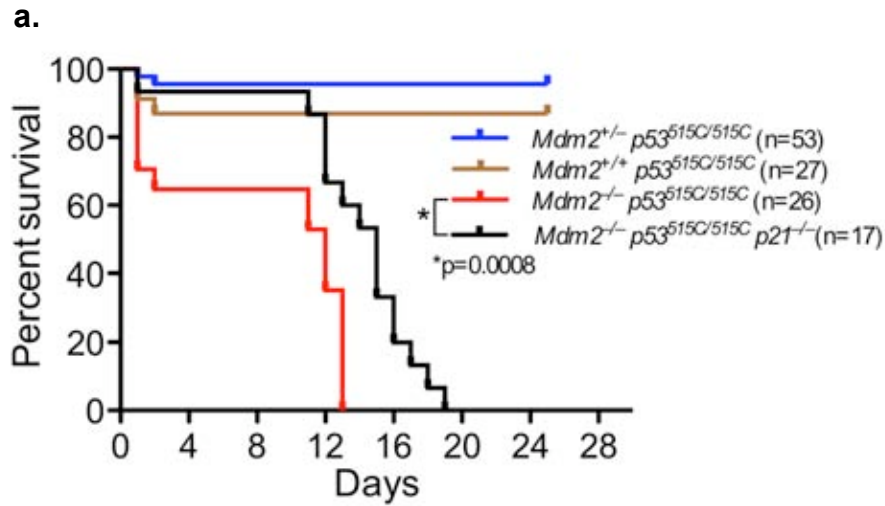
a.



b.



**Figure 14. Increased senescence markers in bone marrows but not fetal livers of *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice.** Expression levels of senescence markers at (a) E14.5 of whole fetal liver (WFL) and (b) P6 of whole bone marrow (WBM) RNA was measured by Real Time RT-PCR and was normalized to *Gapdh* levels. Error bars represent standard error of the mean.

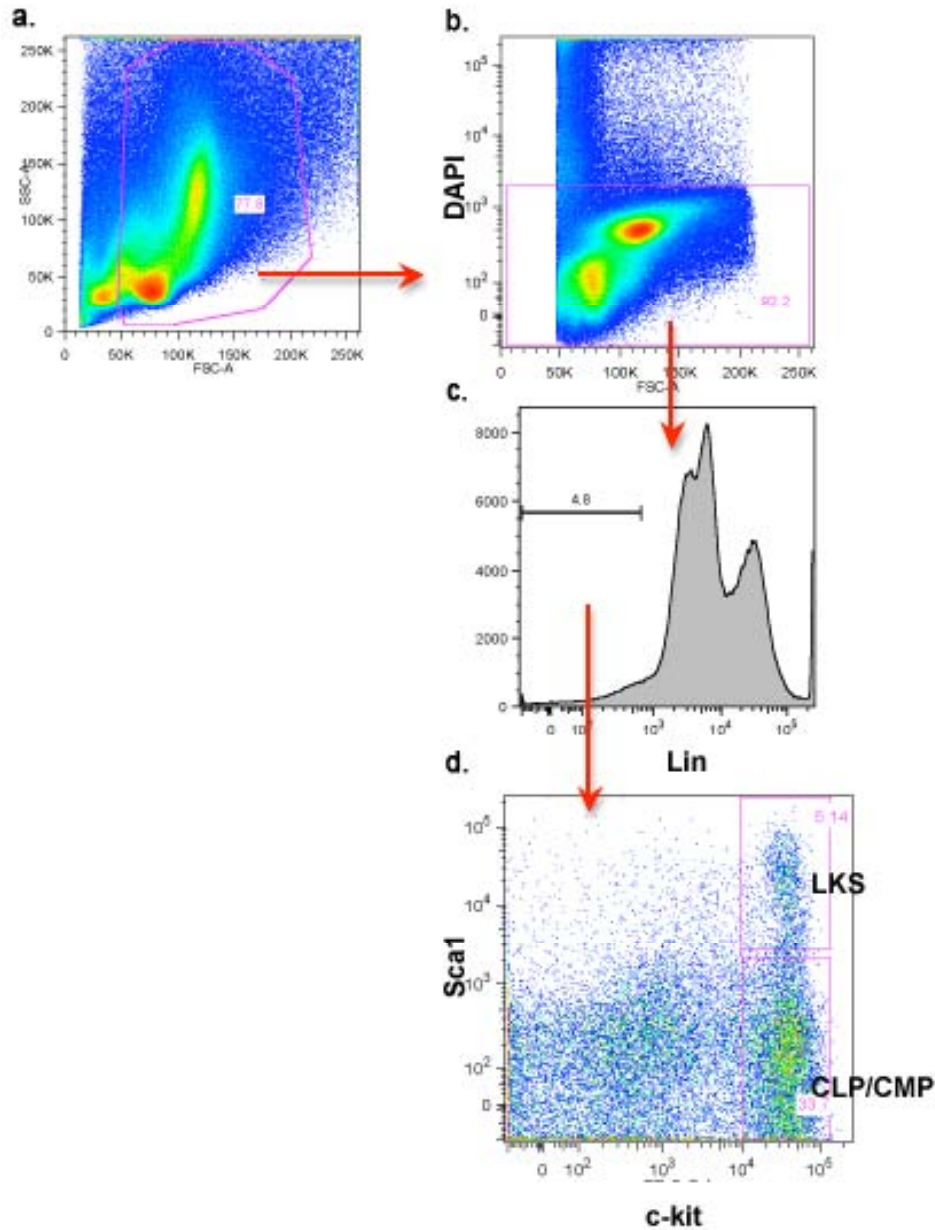


**Figure 15.  $p21$  deletion extends survival of  $Mdm2^{-/-} p53^{515C/515C}$  mice without rescuing bone marrow cellularity. (a)** Kaplan-Meier survival curve of  $Mdm2^{-/-} p53^{515C/515C} p21^{-/-}$  mice compared to other genotypes. Number of mice indicated between parenthesis. **(b)** H&E of P12 bone marrow of  $Mdm2^{-/-} p53^{515C/515C} p21^{-/-}$  mouse. Scale bar is 200 $\mu$ m.

sustainable hematopoiesis, we hypothesized a defect in the HSC and progenitor populations. We therefore examined Lin<sup>-</sup> ckit<sup>+</sup> Sca1<sup>+</sup> (LKS) and Lin<sup>-</sup> ckit<sup>+</sup> Sca1<sup>-</sup> /low cell populations that represent HSCs and common lymphoid and myeloid progenitors (CLP/CMP), respectively, at different time points throughout development (Morrison and Weissman 1994; Weissman and Shizuru 2008). The gating strategy for analyzing these populations is depicted in Figure 16.

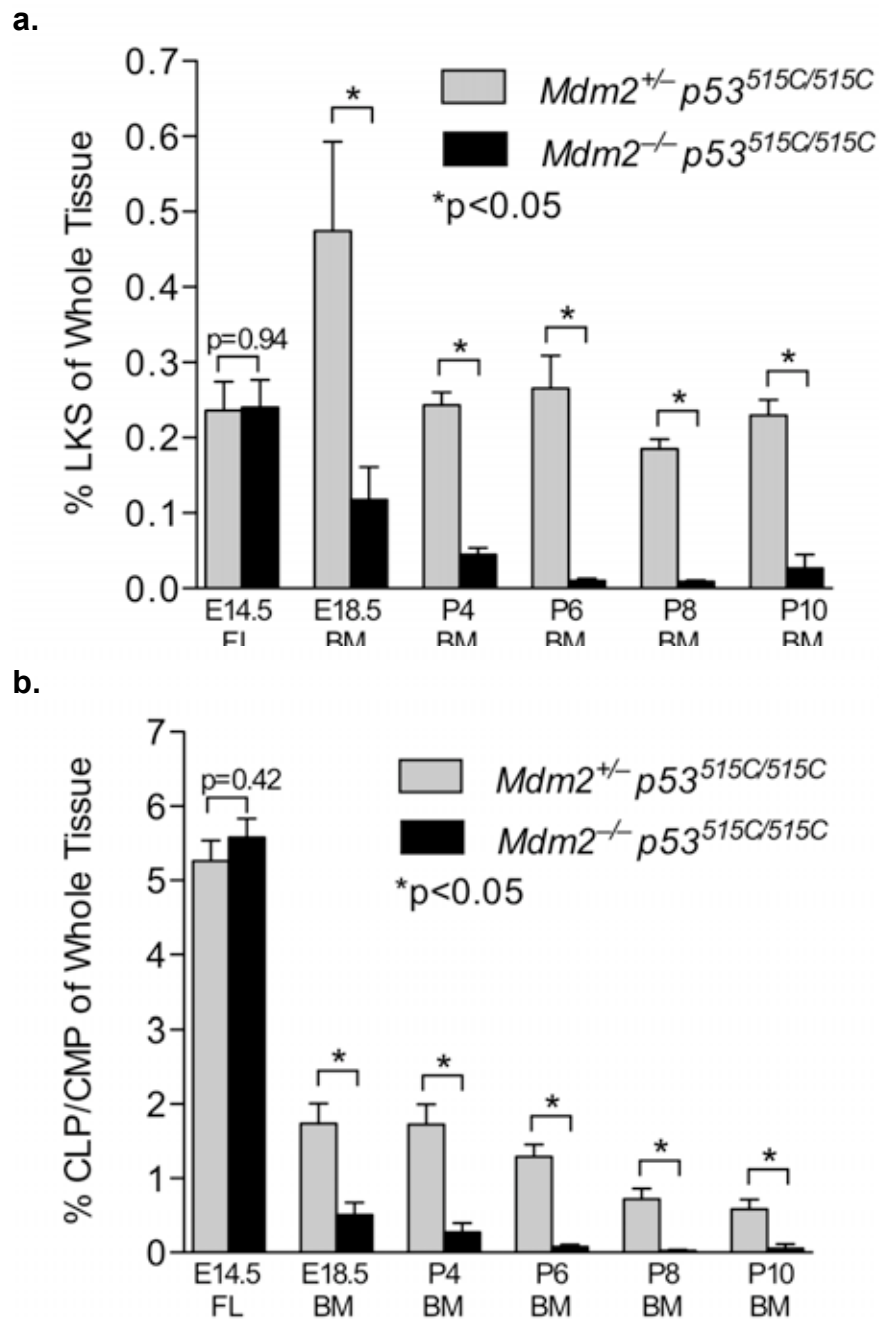
E14.5 fetal livers of *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> and *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> mice had normal LKS cell populations (0.236% and 0.24%) for *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> and *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> embryos, respectively, and normal CLP/CMP populations (5.26% and 5.58%) for *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> and *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> embryos, respectively (Figures 17a-b). This was expected given the normal histology, absence of p53R172P stability, and normal cell cycle pattern of fetal livers at E14.5. Unexpectedly, E18.5 bone marrows of *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> embryos showed significant decreases in LKS and CLP/CMP populations (Figures 17a-b). Specifically, 0.117% and 0.508% of whole bone marrow cells are LKS and CLP/CMP populations, respectively, in *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> compared to 0.474% and 1.74%, respectively, in control *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> littermates at E18.5 (Figures 17a-b).

Postnatally, the differences were even more pronounced. Specifically, starting at P4, LKS (0.044% in *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> vs. 0.243% in control) and CLP/CMP (0.273% in *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> vs. 1.726% in control) populations were dramatically low in bone marrows of *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice (Figures 17a-b). At P6, LKS and CLP/CMP levels decreased further to <0.0103% and <0.075% of whole bone marrows, respectively, while control *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> consistently had significant higher levels (0.265% for LKS and 1.292% for CLP/CMP of whole



**Figure 16. Gating strategy to analyze LKS and CLP/CMP populations.** (a) We first gated for normal Forward (size) and Side (granulation) scatter population. (b) Then dead cells are excluded using DAPI as positive marker for dead cells. (c) Live cells are next gated for Lineage-negative markers. (d) Finally the Lin<sup>-</sup>c-kit<sup>+</sup> Sca1<sup>+</sup> population that represents HSC and Lin<sup>-</sup>c-kit<sup>+</sup>Sca1<sup>-</sup>/low population that represents CLP/CMP are purified. Percentage of live cells is calculated. All data analyzed on FlowJo.





**Figure 17. HSC and progenitors are not affected in fetal livers but ebb gradually after birth *in vivo*.** (a) Percentage of LKS population of whole tissues from E14.5 fetal livers (FL), and E18.5, P4, P6, P8 and P10 bone marrows (BM). (b) Percentages of CLP/CMP population of whole tissues from same timepoints as in (a). Error bars represent standard error of the mean.

bone marrow) (Figures 17a-b). These populations became almost extinct by P8 in *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice (Figures 17a-b).

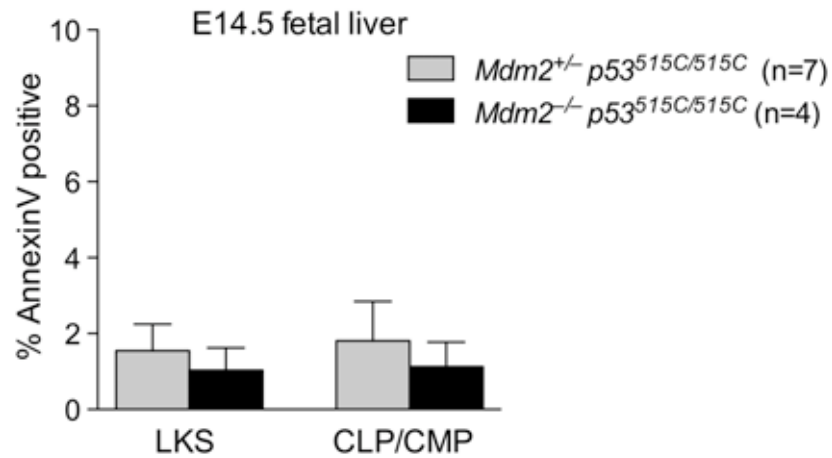
#### *AnnexinV levels in LKS and CLMP population*

Although we could not detect increased AnnexinV staining in whole bone marrows at P6 (Figure 13b), HSCs and progenitors could still be undergoing apoptosis. This is also suggested by the rapid ebbing of LKS and CLMP populations after birth. So, we analyzed apoptosis levels of LKS and CLP/CMP in E14.5 fetal livers and P6 bone marrows using AnnexinV staining. LKS and CLP/CMP populations from E14.5 fetal livers had less than 2% positive cells for AnnexinV in either genotype (Figures 18a-b). We also could not detect any increase of apoptotic activity in the LKS and CLMP cells of bone marrows that are still surviving at P6 in *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> pups (Figure 18a-b). Hence, like in the remaining hematopoietic compartment, apoptotic activity is not the major factor for depletion of HSCs and progenitors.

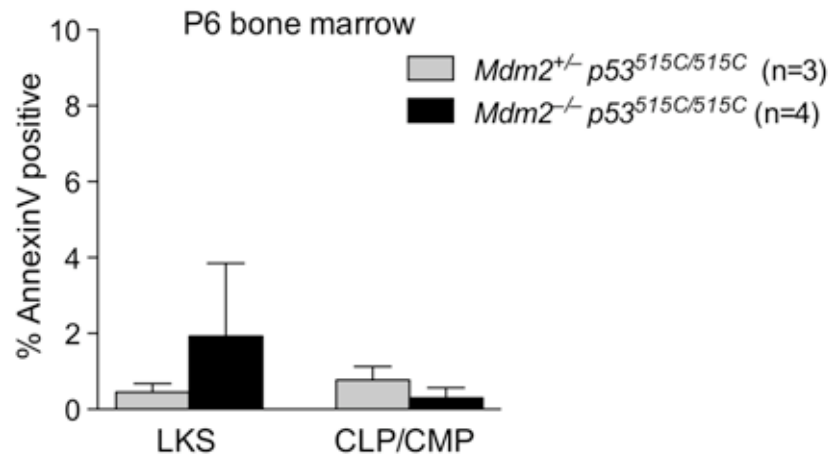
#### *Enriched HSC population is still defective in postnatal bone marrows.*

The LKS markers select for HSCs but not exclusively (Kiel, Yilmaz et al. 2005). In order to further enrich for HSCs, we used two additional markers (CD48 and CD150) previously identified by Morrison's group to be highly enriched in HSCs in fetal livers and bone marrows (Kiel et al.; Blood paper). Specifically, 48% of LKS CD48<sup>-</sup> CD150<sup>+</sup> cells, hereafter referred to as SLAM-LKS, are LT-HSCs. Hence, we included CD48 in our lineage depletion selection, and selected the ckit<sup>+</sup> Sca1<sup>+</sup> population for expression of CD150 (Figure 19).

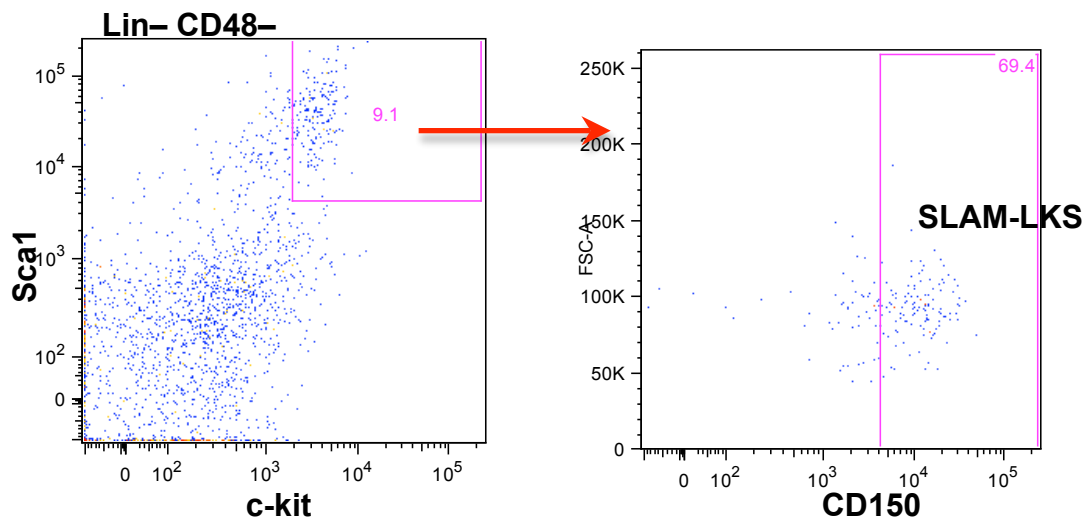
a.



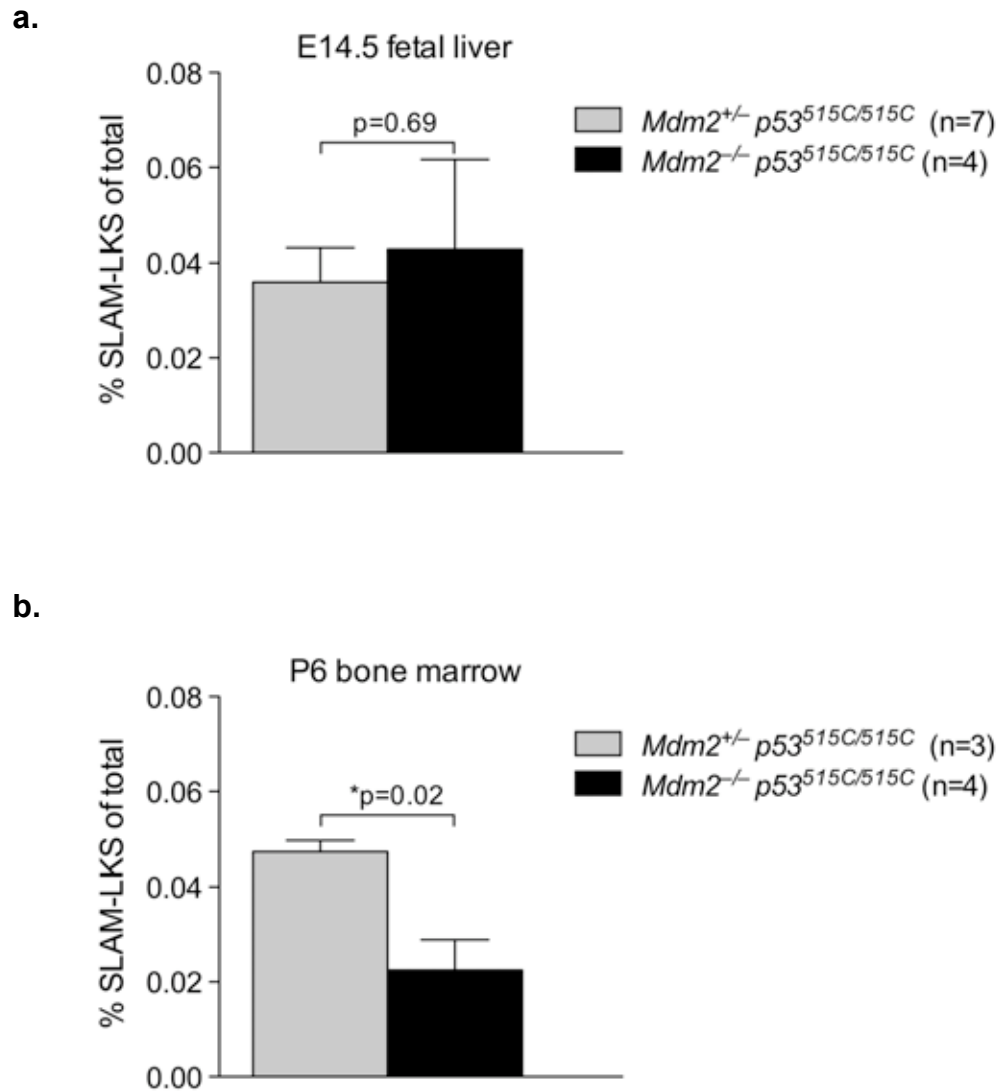
b.



**Figure 18. Absence of apoptotic activity in LKS and CLP/CMP populations.** Percentage of LKS (left) and CLP/CMP (right) populations that are AnnexinV positive in E14.5 fetal livers (a) and P6 bone marrows (b). Error bars represent standard error of the mean.



**Figure 19. Strategy to gate for SLAM-LKS.** Lineage and CD48 positive cells were gated out. Of the Lin<sup>-</sup> CD48<sup>-</sup> cells, we gated for c-kit and Sca1 double positive cells. Then Lin<sup>-</sup>CD48<sup>-</sup>Sca1<sup>+</sup>c-kit<sup>+</sup> cells was gated for CD150 expression versus forward scatter (FSC-A).



**Figure 20. SLAM-LKS population is the same in FL but significantly less in bone marrows.** Percentage of SLAM-LKS populations from E14.5 fetal liver (a) and P6 bone marrow (b). Number of mice used in each experiment is indicated next to genotype. Error bars represent standard error of the mean.

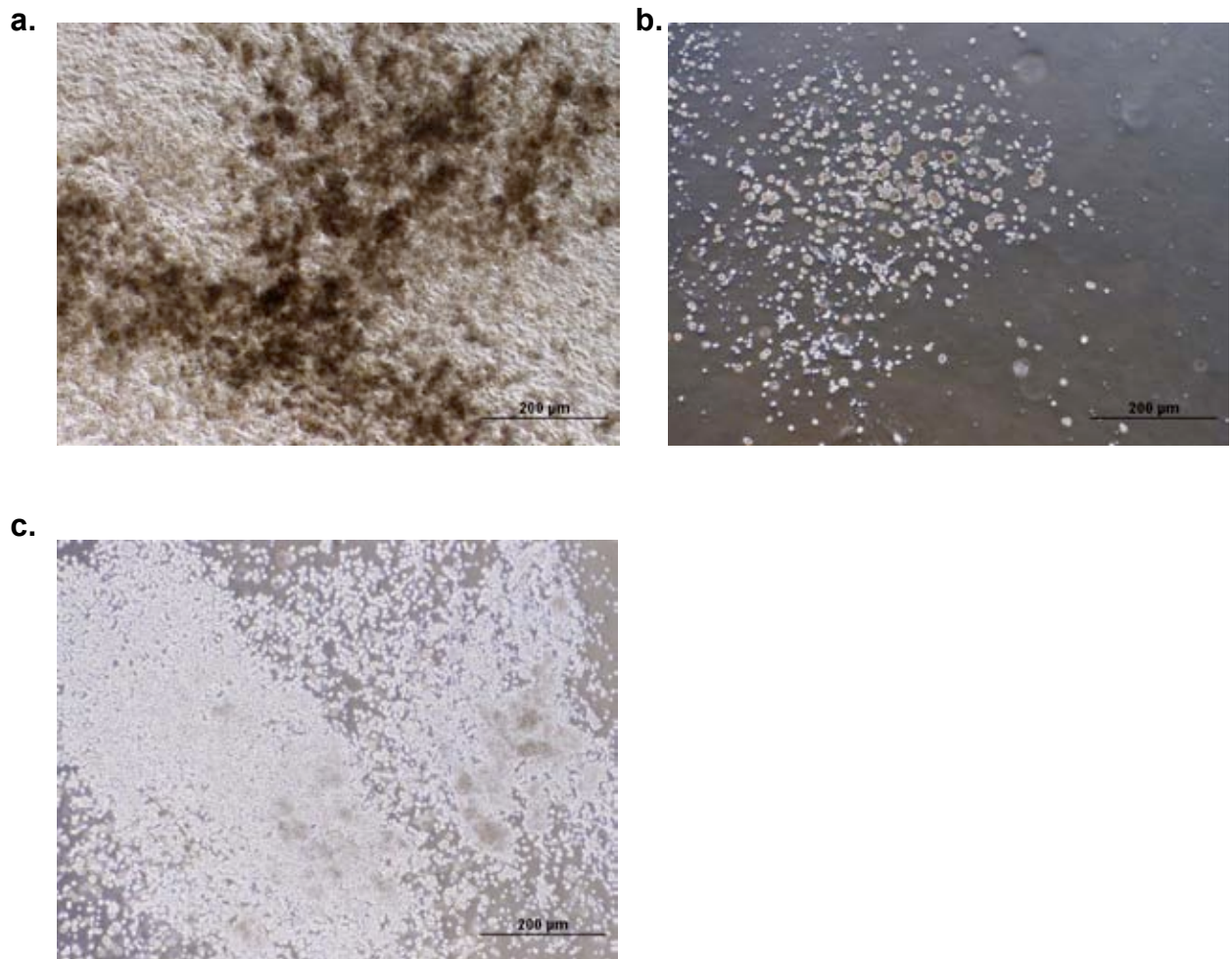
In E14.5 fetal livers, there was no statistical difference between SLAM-LKS populations ( $p=0.69$ ) (Figure 20a). 0.042% of  $Mdm2^{-/-} p53^{515C/515C}$  fetal liver cells were SLAM-LKS while 0.036% for  $Mdm2^{+/-} p53^{515C/515C}$  controls. As for P6 whole bone marrows, there was more than a 2 fold decrease in the SLAM-LKS population in  $Mdm2^{-/-} p53^{515C/515C}$  bone marrows as compared to control littermates ( $p=0.02$ ) (Figure 20b). Specifically, 0.047% and 0.022% of  $Mdm2^{+/-} p53^{515C/515C}$  and  $Mdm2^{-/-} p53^{515C/515C}$  bone marrow cells, respectively, were SLAM-LKS. Accordingly, regardless of mode of assaying or enriching for HSCs, this population was decreased in bone marrows of  $Mdm2^{-/-} p53^{515C/515C}$  pups but not in E14.5 fetal livers compared to control  $Mdm2^{+/-} p53^{515C/515C}$  littermates.

## **Ex vivo hematopoietic activity**

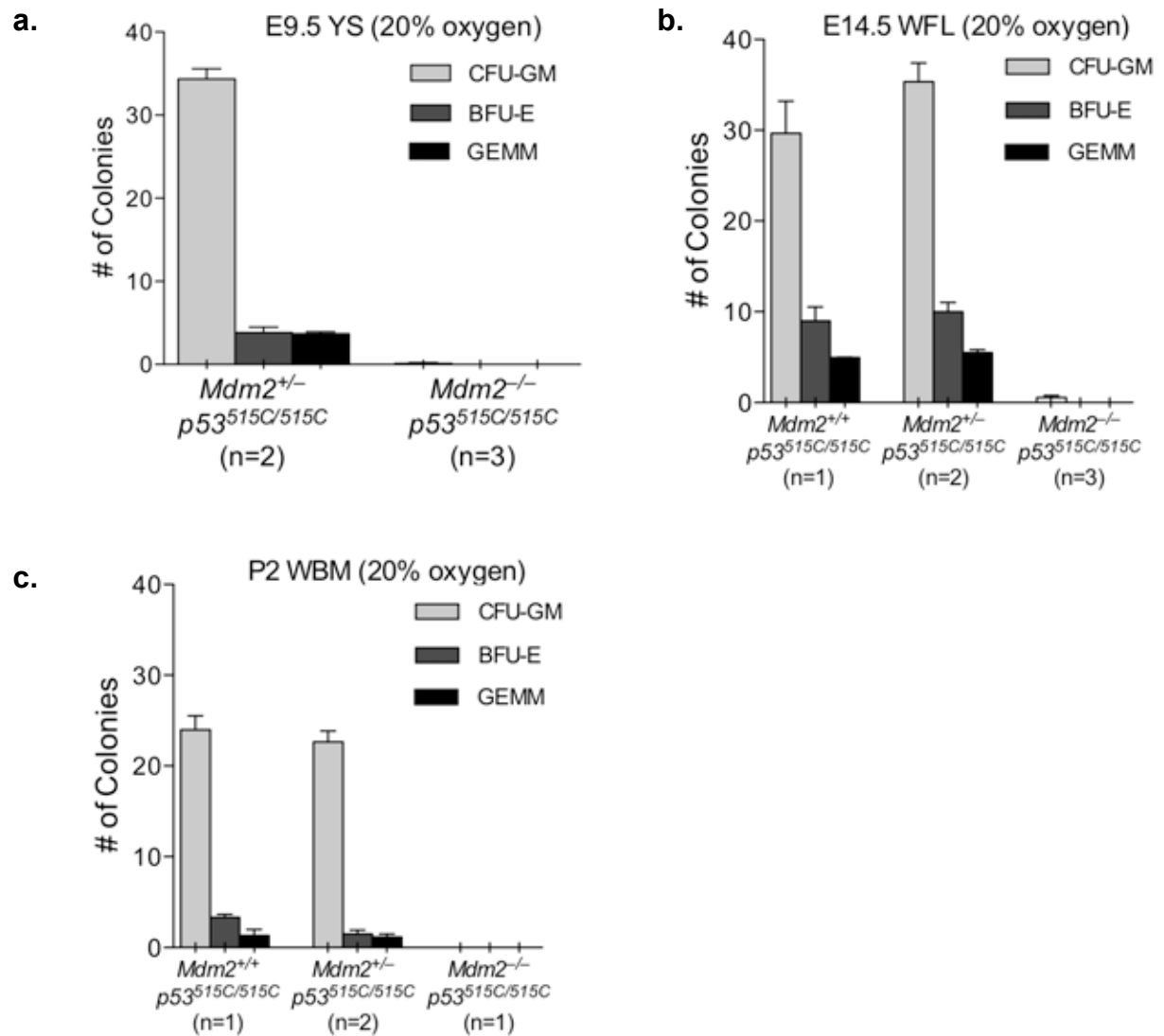
### *Hematopoiesis ex vivo*

In order to evaluate the potential of HSCs and CLP/CMP *ex vivo*, we used M3434 methylcellulose cultures (STEMCELL Technologies), a common assay that measures differentiation capacity of hematopoietic stem cells and progenitors. Colonies are identified based on morphology. Specifically, we looked for burst-forming unit erythroid (BFU-E) (Figure 21a), colony-forming unit granulocyte macrophage (CFU-GM) (Figure 21b) and colony-forming unit granulocyte erythroid monocyte and megakaryocyte (GEMM) (Figure 21c) colonies. We isolated whole tissues from yolk sac at E9.5, fetal livers at E14.5 and bone marrow at P2 and plated 20,000 cells in triplicate.

$Mdm2^{-/-} p53^{515C/515C}$  E9.5 yolk sacs (Figure 22a), E14.5 fetal livers (Figure 22b) and P2 bone marrows (Figure 22c) reflected absence of any hematopoietic



**Figure 21. Representative colonies for methocult.** Showing GEMM/mixed colony (a) characterized by all cell lineages (granulocytic, erythroid and megakaryocytes) with a dense core; BFU-E (b) colonies characterized by grape-like structures; CFU-GM (c) colony characterized by clear round cells which may also harbor a dense center but distinct from that of GEMM colony. Scale bar is 200 μm.



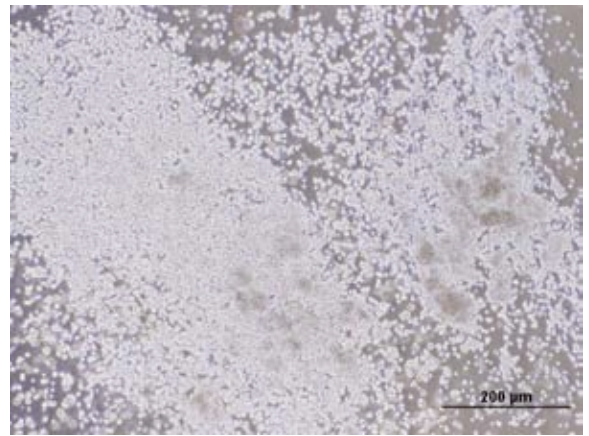
**Figure 22. Methocult assays reflects absence of hematopoietic activity *ex vivo* of all tissues tested.** Quantification of CFU-GM, BFU-E and GEMM colonies from methocult cultures of 20,000  $Mdm2^{+/+} p53^{515C/515C}$ ,  $Mdm2^{+/-} p53^{515C/515C}$  and  $Mdm2^{-/-} p53^{515C/515C}$  yolk sac (YS) (a), whole fetal liver (WFL) cells (b) and whole bone marrow (WBM) (c) cells at E9.5, E14.5 and P2, respectively. Error bars represent standard error of the mean.



**a.**



**b.**



**Figure 23. Less dense CFU-GM colony in  $Mdm2^{-/-} p53^{515C/515C}$  (a) compared to  $Mdm2^{+/-} p53^{515C/515C}$  (b).**

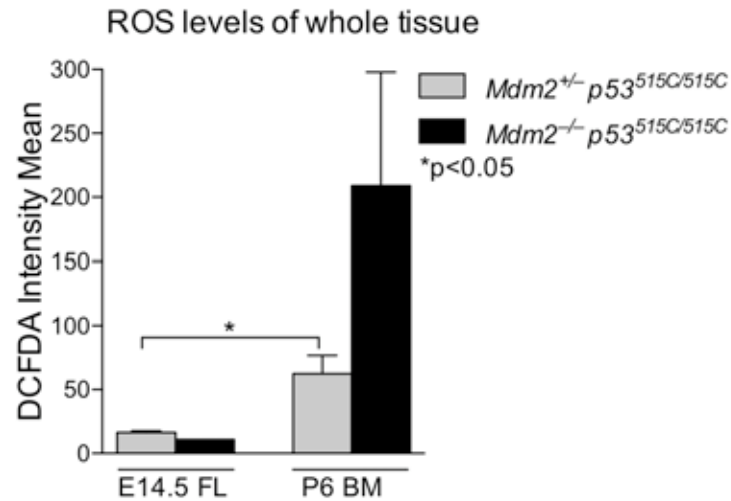
activity *ex vivo* except for few CFU-GM colonies, while the same tissues from control  $Mdm2^{+/-} p53^{515C/515C}$  littermates grew in culture with no impediments (Figures 22a-c.). Notably, the few CFU-GM colonies of  $Mdm2^{-/-} p53^{515C/515C}$  tissues that were visible were significantly smaller and uncondensed (Figure 23a) as compared to colonies from control genotypes (Figure 23b). In  $Mdm2^{+/-} p53^{515C/515C}$  cultures, CFU-GM colonies were the most predominant, followed by BFU-E and GEMM colonies for all tissues. Noteworthy, E14.5 fetal liver and P2 bone marrow of  $Mdm2^{+/-} p53^{515C/515C}$  hematopoietic activity in methocult showed no significant differences for all lineages compared to  $Mdm2^{+/-} p53^{515C/515C}$  (Figure 22b-c). Thus, the methocult *ex vivo* assays did not reproduce our *in vivo* results for fetal livers that showed normal LKS, LKS-SLAM and CLP/CMP populations. These differences suggested culture conditions were an impediment for growth in methocult. The major difference in culture versus *in vivo* is oxidative stress.

### **Oxidative stress in hematopoietic tissues.**

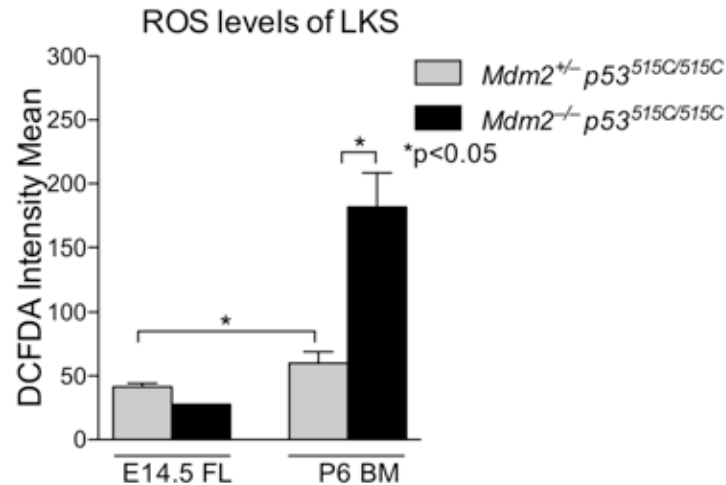
*Reactive oxygen species levels are elevated in  $Mdm2^{-/-} p53^{515C/515C}$  bone marrows*

As discussed in the introduction, ROS are major sensitizers of the hematopoietic system (Naka, Muraguchi et al. 2008). Whether ROS effects are mediated via the p53 pathway in the hematopoietic compartment is unknown. To examine ROS levels in fetal livers and postnatal bone marrows, we used 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate (DCFDA), a commonly used reagent to measure ROS levels in cells (Ito, Hirao et al. 2004; Liu, Cao et al. 2009). We chose E14.5 fetal liver and P6 bone marrow as representative embryonic and postnatal

a.



b.



**Figure 24. Increased ROS levels in postnatal bone marrows of *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice.** (a) ROS levels were measured in whole tissues (fetal livers (FL) and bone marrows (BM)) at E14.5 and P6 using DCFDA intensity as a readout. (b) Measurement of ROS levels with DCFDA in LKS populations of E14.5 fetal liver (FL) and P6 bone marrow (BM). Error bars represent standard error of the mean.

time points, respectively. ROS levels of whole fetal livers and LKS populations at E14.5 between  $Mdm2^{+/-} p53^{515C/515C}$  and  $Mdm2^{-/-} p53^{515C/515C}$  mice were not statistically different (Figures 24a-b).

Postnatally, mice are exposed to atmospheric oxygen levels and hence we expected higher ROS levels in postnatal bone marrows as compared to E14.5 fetal livers. Indeed,  $Mdm2^{+/-} p53^{515C/515C}$  whole bone marrows and LKS populations had 3.8 and 1.5 times more ROS, respectively, compared to the same populations in E14.5 fetal livers (Figure 24a-b). This indicates that ROS levels within the hematopoietic compartment is higher in bone marrows than fetal livers in normal  $Mdm2^{+/-} p53^{515C/515C}$  mice. Since all pups are littermates, we expected that ROS levels of  $Mdm2^{+/-} p53^{515C/515C}$  and  $Mdm2^{-/-} p53^{515C/515C}$  littermates to be the same at P6. To our surprise,  $Mdm2^{-/-} p53^{515C/515C}$  pups had 3.3 and 3 times higher ROS levels in whole bone marrow and LKS populations, respectively, than  $Mdm2^{+/-} p53^{515C/515C}$  littermates at P6 (Figure 24a-b). These data showed that  $Mdm2^{-/-} p53^{515C/515C}$  bone marrows intrinsically generated more ROS in whole bone marrows and LKS populations.

#### *Activation of PIGs in postnatal bone marrows*

To address what instigated the higher rates of ROS and apoptosis in P6 bone marrows of  $Mdm2^{-/-} p53^{515C/515C}$  mice compared to control littermates, we examined p53's role in ROS. Interestingly, p53 can transactivate a set of ROS-inducing genes dubbed p53-induced-genes (PIG) which lead to cell death detected by using a DNA binding dye (Polyak, Xia et al. 1997). Specifically, we examined mRNA levels of *Pig1*, *Pig8* and *Pig12* that are amongst the most highly induced p53

genes (Polyak, Xia et al. 1997). *Pig1* is a member of galectin family that can stimulate superoxide production (Yamaoka, Kuwabara et al. 1995). *Pig8* is also known as *Ei24* and its expression is induced following etoposide treatment that is known to generate ROS (Lehar, Nacht et al. 1996). *Pig12* also known as microsomal glutathione transferase homologue is involved in redox reactions (Lee and DeJong 1999).

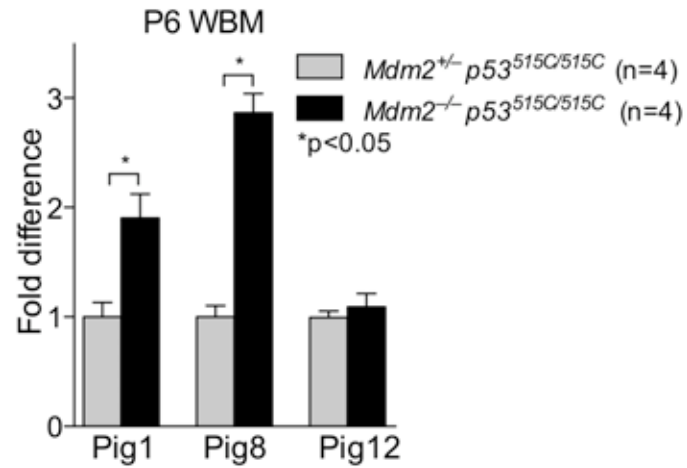
At P6, *Pig1* and *Pig8* levels were 2 (p=0.01) and 3 (p<0.0001) fold higher in *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> bone marrows as compared to control littermates (Figure 25a). *Pig12* levels were the same at P6. Since Polyak et al. (1997) reported that p53 activates *Pig* genes in a cascading manner, we examined expression of these genes in P10 whole bone marrows as well. Interestingly, *Pig12* levels at P10 were 2.8 fold higher (p=0.01) in moribund *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice as compared to control littermates (Figure 25b). *Pig1* (p=0.49) and *Pig8* (p=0.07) levels were not statistically different at P10 (Figure 25b). Hence, p53-dependent activation of *PIG* genes is contributing to elevated ROS levels in the hematopoietic compartment.

## **Antioxidants treatment.**

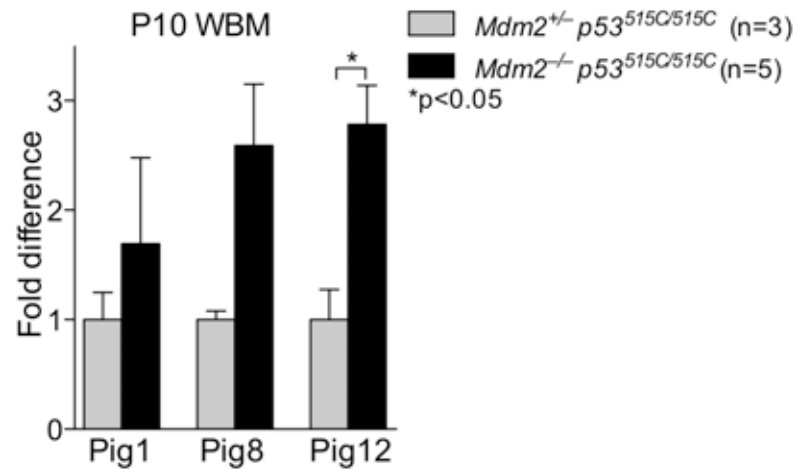
### *NAC injections partially rescue HSC and CLP/CMP at P6*

N-acetyl Cysteine (NAC) is a commonly used anti-oxidant usually delivered in drinking water. Since *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice begin to lose their HSCs and progenitors a few days after birth, we injected pups at P3 and P5 with 1g NAC per 1 kilogram body weight (equivalent to the dosage in drinking water for adult mice). Unfortunately, this technique was highly invasive and most of the pups from all genotypes injected died after the first injection.

a.

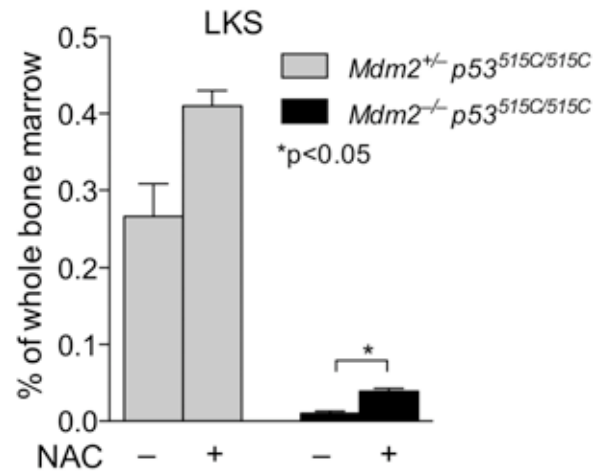


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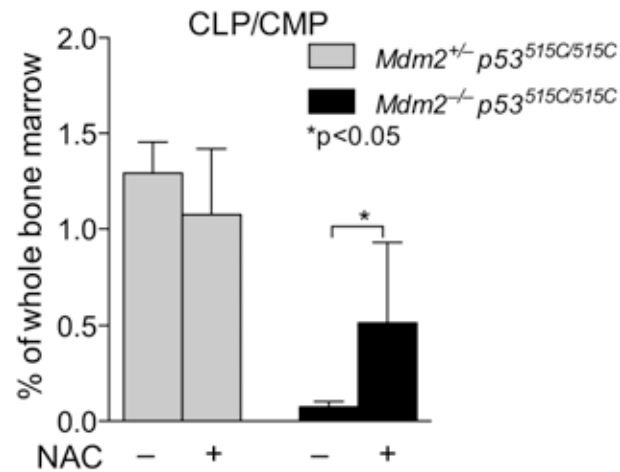


**Figure 25. Increase of PIGs in *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> compared to *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> mice.** Measurement of transcript levels of *Pig1*, *Pig8* and *Pig12* from whole bone marrow (WBM) cells at P6 (a) and P10 (b) from *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> compared to *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> measured by Real Time RT-PCR and normalized to *Gapdh* levels. Error bars represent standard error of the mean.

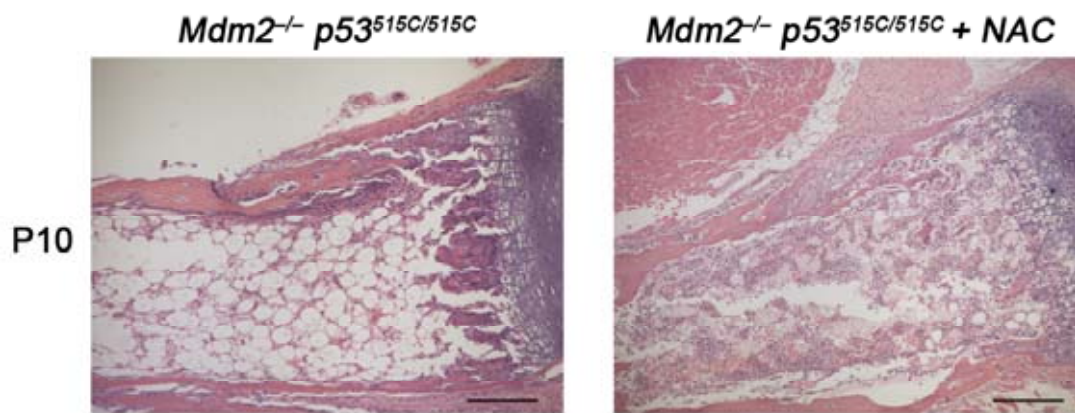
a.



b.



**Figure 26. Partial rescue of LKS and CLP/CMP populations upon treatment with antioxidant.** (a) LKS populations of whole bone marrow at P6 for NAC treated and untreated mice. (B) CLP/CMP levels of whole bone marrow at P6 for NAC treated and untreated mice. Error bars represent standard error of the mean.



**Figure 27. Rescue of bone marrow cellularity after NAC treatment.** Representative H&E staining for P10 bone marrow of a *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mouse treated with NAC (right) compared to untreated (left). Scale bar is 200 $\mu$ m.



Two  $Mdm2^{+/-} p53^{515C/515C}$  and two  $Mdm2^{-/-} p53^{515C/515C}$  pups survived and were sacrificed at P6 to look for changes in HSC and CLP/CMP populations. Interestingly, a modest but significant increase in both of these populations was prominent in  $Mdm2^{-/-} p53^{515C/515C}$  pups injected with NAC compared to untreated mice of same genotype (Figure 26a-b). Specifically, there was 4 fold ( $p=0.001$ ) increase in LKS population of whole bone marrow (0.041% for treated compared to 0.01% for untreated) and 7 fold ( $p=0.01$ ) increase in CLP/CMP population of whole bone marrow (0.5 % for treated compared to 0.07% for untreated) (Figure 26a-b). The two  $Mdm2^{+/-} p53^{515C/515C}$  pups treated with NAC that survived had no significant differences in LKS and CLP/CMP populations compared to untreated genotype-matched mice (Figure 26a-b).

Notably, a third  $Mdm2^{-/-} p53^{515C/515C}$  mouse survived NAC injections at P3 and P5 and received a third injection at P7 then sacrificed at P10 to look for changes in bone marrow cellularity. Interestingly, bone marrow cellularity was ~80% restored in the treated mouse compared to  $Mdm2^{-/-} p53^{515C/515C}$  untreated control (Figure 27).

### *Rescue of ex vivo hematopoiesis at 3% oxygen*

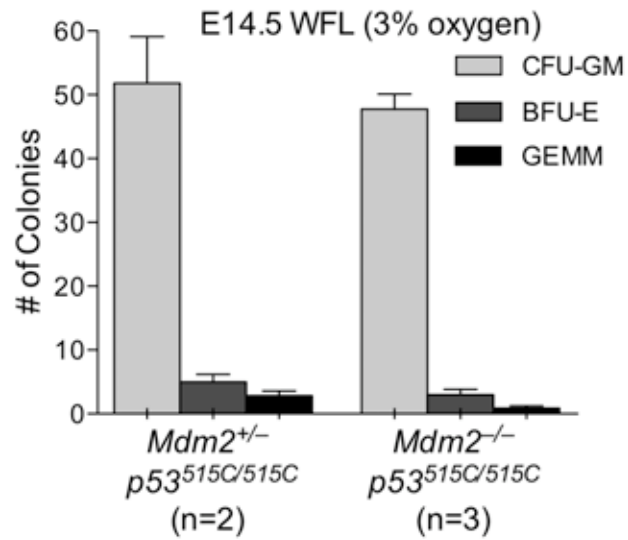
These data suggest that ROS contributes to the hematopoietic defect in  $Mdm2^{-/-} p53^{515C/515C}$  mice. Our initial *ex vivo* assays were performed at 20% oxygen. We therefore examined hematopoiesis *ex vivo* at 3% oxygen levels. Specifically, 20,000 cells from E14.5 fetal livers and P2 bone marrows of the pups used in (Figures 22b-c) were plated in triplicate onto methycellulose and allowed to grow. Contrary to culturing in 20% oxygen, 3% oxygen levels allowed

hematopoiesis to proceed unimpeded and all  $Mdm2^{-/-} p53^{515C/515C}$  tissues formed CFU-GM, BFU-E and GEMM (Figure 28a-b). Hence, all  $Mdm2^{-/-} p53^{515C/515C}$  fetal livers and P2 bone marrows examined showed complete rescue of hematopoiesis in methylcellulose cultures at 3% oxygen. Notably, these colonies were large, condensed and indicative of normal progression of hematopoietic activity. These data indicate that oxidative stress was the major impediment for sustainable hematopoiesis in the absence of *Mdm2* due to activation of p53R172P.

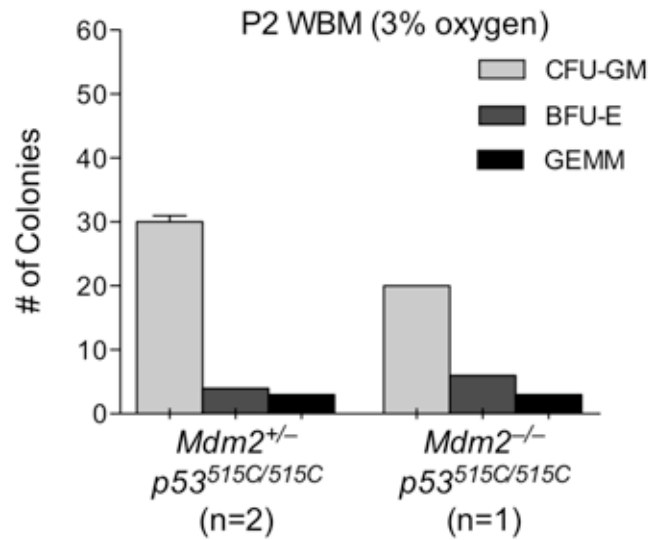
#### *NAC treatment in methocult does not rescue hematopoiesis*

In order to address whether NAC can rescue *ex vivo* hematopoiesis, we added 1 mM NAC to E14.5 fetal liver methocult plates cultured at 20% oxygen every three days. Cells from the same fetal livers were cultured at 20% oxygen with or without NAC, and at 3% oxygen level in methocult. Supplementing NAC to methocult was not sufficient to rescue hematopoiesis and we saw no differences between plates with and without NAC of  $Mdm2^{-/-} p53^{515C/515C}$  fetal livers (Figures 29a-b). Specifically, only few CFU-GM colonies grew out from  $Mdm2^{-/-} p53^{515C/515C}$  fetal livers after 10 days in culture. NAC did not affect cultures of  $Mdm2^{+/-} p53^{515C/515C}$  plates. On the other hand, 3% oxygen level, as already shown, was permissive for growth of  $Mdm2^{-/-} p53^{515C/515C}$  fetal livers *ex vivo* (Figure 29c).

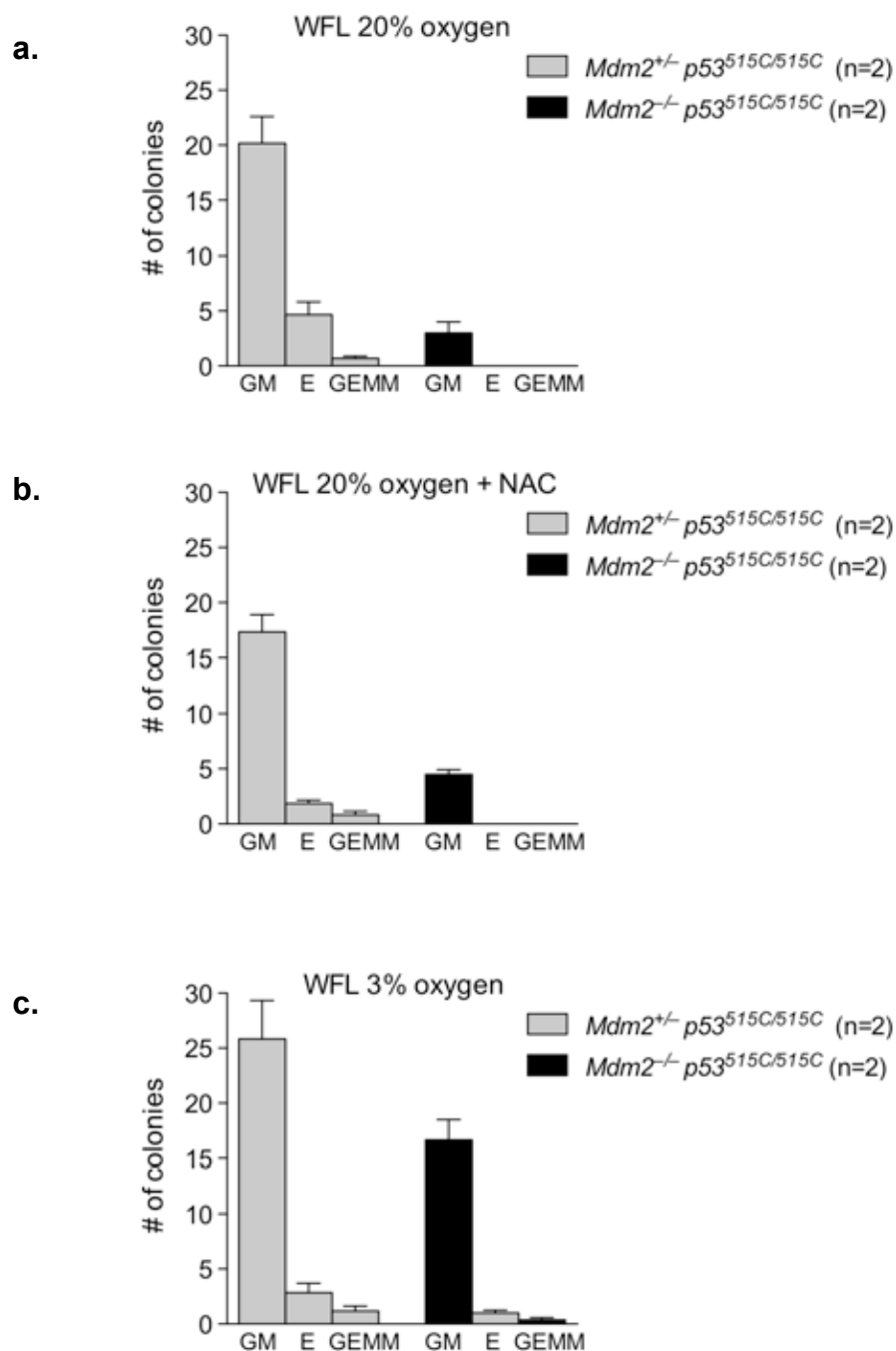
a.



b.



**Figure 28. Rescue of ex vivo hematopoiesis at 3% oxygen.** 20,000 cells of *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> and *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> whole fetal livers (WFL) (a) and whole bone marrow (WBM) (b) cells were cultured at 3% oxygen and the number of CFU-GM, BFU-E and GEMM colonies were quantified. Error bars represent standard error of the mean.



**Figure 29. NAC treatment does not rescue *ex vivo* hematopoiesis.** 20,000 whole fetal liver cells were cultured from each mouse in triplicates at 20% oxygen without (a) and with (b) NAC, and at 3% oxygen (c).

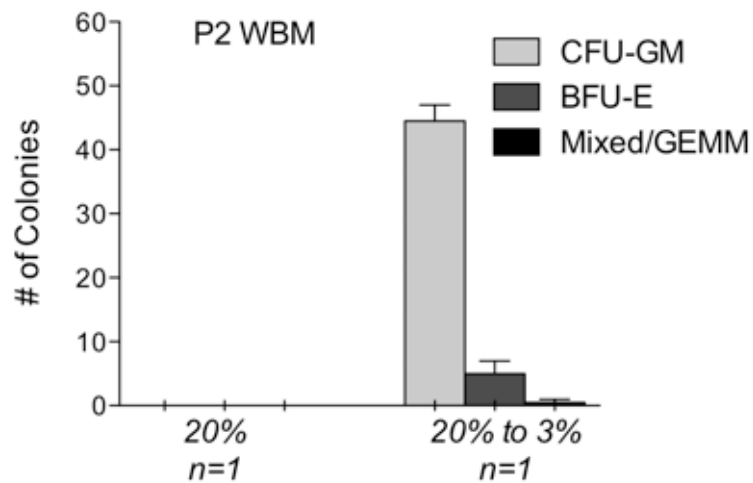
### *Reestablishing hematopoiesis at 3% after 10 days of culturing at 20% oxygen*

After 10 days of culturing  $Mdm2^{-/-} p53^{515C/515C}$  bone marrow cells at 20% oxygen and showing no growth, we transferred 2 plates to 3% oxygen incubators. Surprisingly,  $Mdm2^{-/-} p53^{515C/515C}$  cultures started growing again and within 10 days we had noticeable large colonies (Figure 30). As a control we kept  $Mdm2^{-/-} p53^{515C/515C}$  plates at 20% and even after 20 days of culture we could not detect any obvious increase in colony formation. Hence, although the cells at 20% oxygen did not grow, apparently they were in a dormant state until culture conditions allowed their growth.

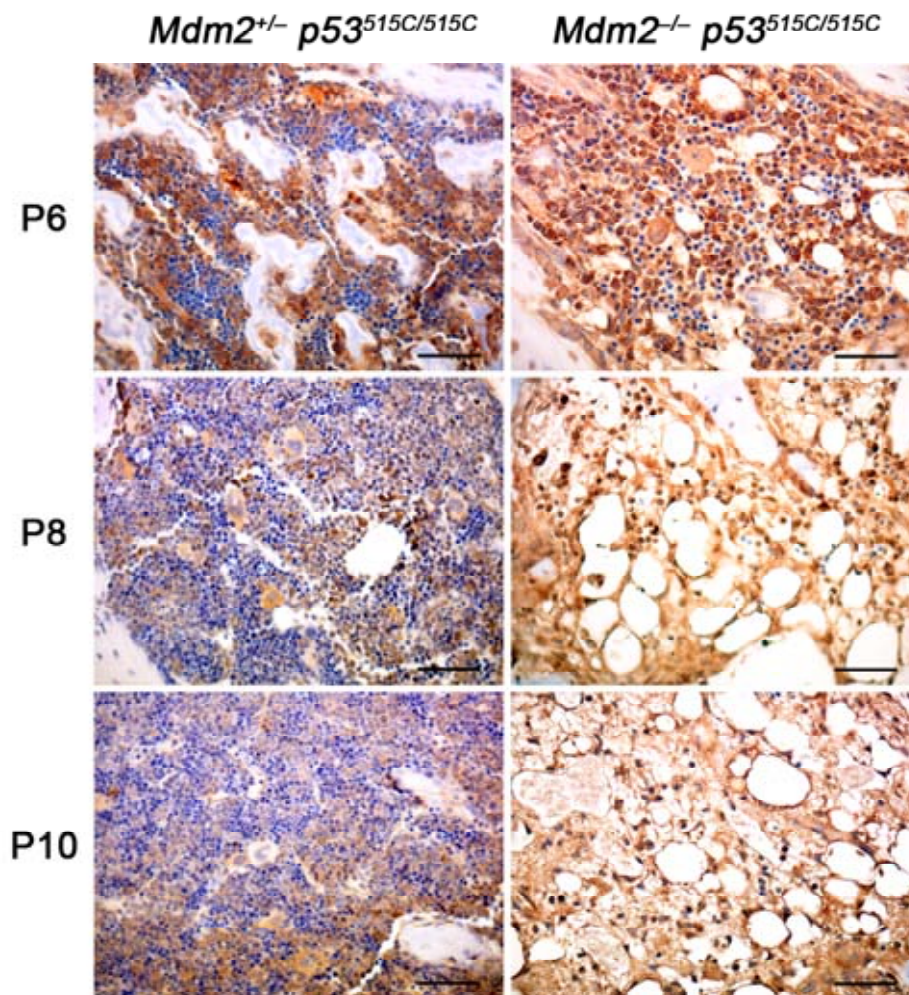
### **Analyzing p16 status and generating $Mdm2^{-/-} p53^{515C/515C} p16^{-/-}$ mice**

#### *p16 is stable in $Mdm2^{-/-} p53^{515C/515C}$ bone marrows*

p16 is a cyclin-dependent kinase inhibitor and can induce cell cycle arrest and its overexpression in HSCs inhibit their proliferation (Park, Qian et al. 2003; Akala, Park et al. 2008). p16 is also implicated in ROS regulatory pathways of HSCs. Specifically, loss of *Atm* confers severe hematopoietic stem cell defect characterized by elevated ROS levels (Ito, Hirao et al. 2004). ROS in this system activates p16 whose levels are dampened upon antioxidant treatment (Ito, Hirao et al. 2004). Moreover, restoring p16 expression in progenitors inhibits their expansion (Lewis, Chinswangwatanakul et al. 2001). Since ROS levels were elevated in  $Mdm2^{-/-} p53^{515C/515C}$  bone marrows, we examined p16 status using IHC. Although p16 mRNA levels were indistinguishable between  $Mdm2^{-/-} p53^{515C/515C}$  and control littermates at P6 (Figure 14b), we detected significantly elevated p16 protein levels at P6, P8 and P10 in  $Mdm2^{-/-} p53^{515C/515C}$  bone marrows (Figure 31). Bone



**Figure 30. Transfer of methocult to 3% oxygen restores hematopoiesis in P2 WBM.** 20,000 whole fetal liver cells were cultured from *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> P2 whole bone marrow (WBM) and were incubated at 20% oxygen for 3 weeks (left). After 10 days at 20%, 2 plates from same mouse were transferred to 3% oxygen incubators and number of colonies was counted 10 days after (right).



**Figure 31. p16 levels are higher in *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> bone marrows.** a) Immunohistochemical staining of cytoplasmic p16 in P6, P8 and P10 bone marrows of *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> (left) and *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> (right) mice. Scale bar is 50μm.

marrows of control  $Mdm2^{+/-} p53^{515C/515C}$  littermates had some positive cells for p16 staining but much less than  $Mdm2^{-/-} p53^{515C/515C}$  mice (Figure 31). These data suggest that ROS in the hematopoietic compartment of  $Mdm2^{-/-} p53^{515C/515C}$  mice can induce p16 levels.

#### *p16 deletion rescues bone marrow cellularity and extends survival of $Mdm2^{-/-} p53^{515C/515C}$ mice*

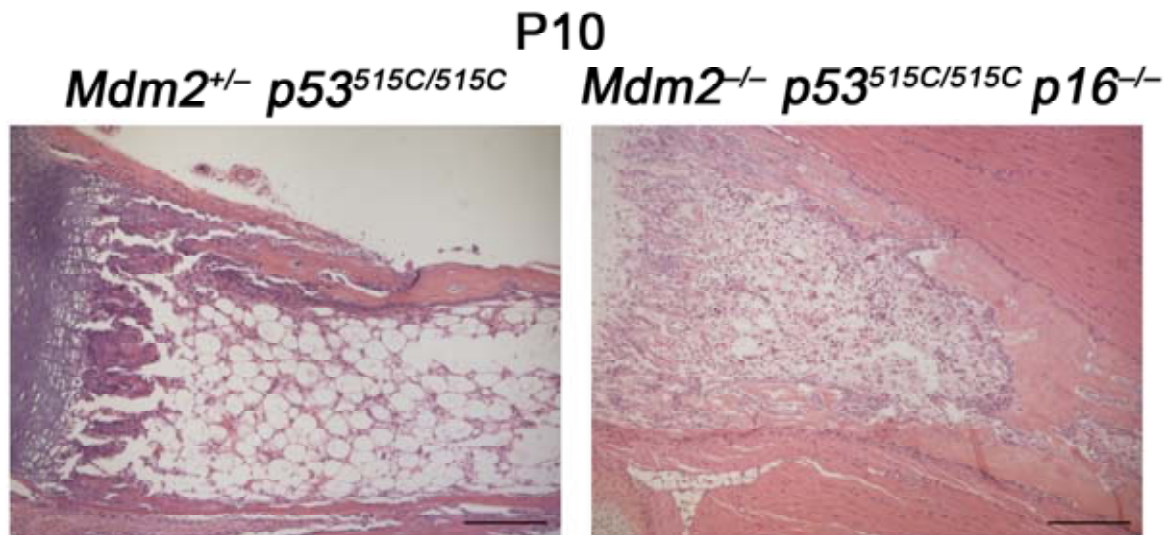
In order to address whether *p16* deletion can restore hematopoiesis, we generated  $Mdm2^{+/-} p53^{515C/515C} p16^{-/-}$  mice and crossed them to each other. Although, the size of these mice was smaller than normal and their pregnancy rate was low, they were still able to generate pups.  $Mdm2^{-/-} p53^{515C/515C} p16^{-/-}$  mice still suffered from growth retardation. Three  $Mdm2^{-/-} p53^{515C/515C} p16^{-/-}$  mice from two independent crosses were sacrificed at P10 (where bone marrow acellularity is most dramatic in  $Mdm2^{-/-} p53^{515C/515C}$  mice) and their bone marrows were stained with H&E for histological analysis. Interestingly, *p16* deletion restores bone marrow cellularity by at least 70% (Figure 32). Also,  $Mdm2^{-/-} p53^{515C/515C} p16^{-/-}$  mice lived significantly ( $p < 0.0001$ ) longer than  $Mdm2^{-/-} p53^{515C/515C}$  mice (Figure 33).

#### **Characterization of $Mdm2^{-/-} p53^{515C/515C} p16^{-/-}$ bone marrows**

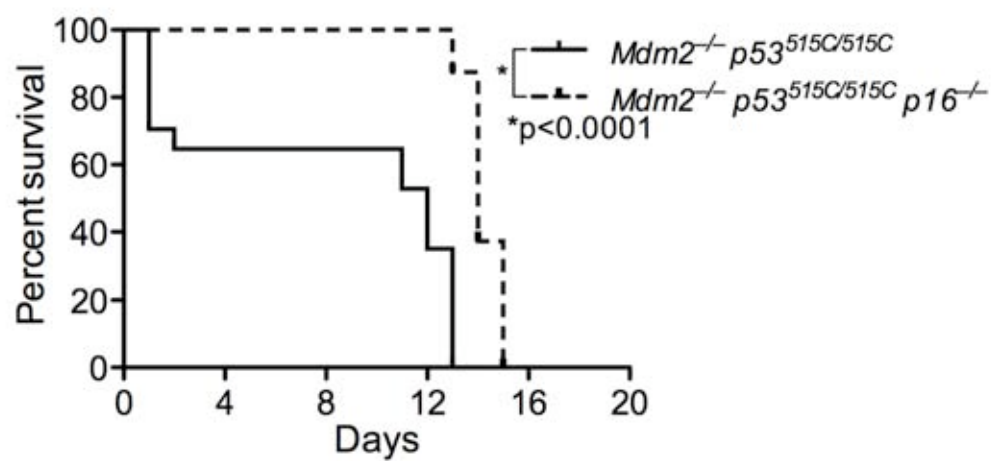
##### *P53R172P levels are elevated in $Mdm2^{-/-} p53^{515C/515C} p16^{-/-}$ bone marrows*

In order to examine the mechanism behind rescue of bone marrow cellularity upon genetic deletion of *p16*, we first examined p53R172P levels in  $Mdm2^{-/-} p53^{515C/515C} p16^{-/-}$  bone marrows by IHC at P10. p53R172P was still highly detectable in  $Mdm2^{-/-} p53^{515C/515C} p16^{-/-}$  bone marrows indicating that *p16* loss





**Figure 32. *p16* deletion partially restores cellularity of *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> bone marrow.** H&E staining of P10 bone marrows from *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> (left) and *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> *p16*<sup>-/-</sup> (right) mice. Scale bar is 200 $\mu$ m.

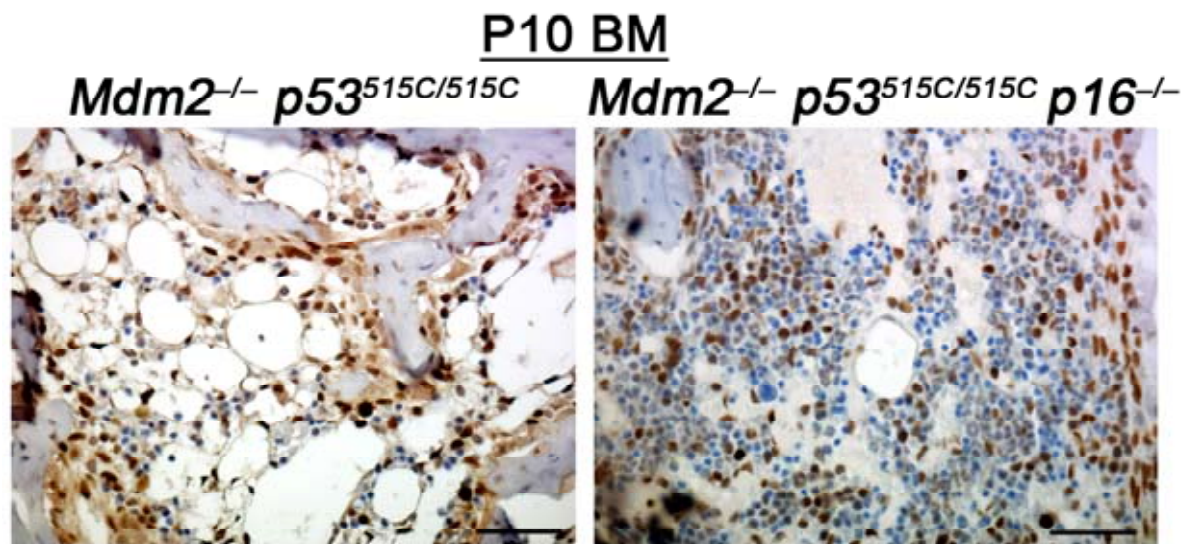


**Figure 33. *p16* deletion extends survival.** Kaplan-Meier survival curve of  $Mdm2^{-/-} p53^{515C/515C} p16^{-/-}$  mice (n=8) compared to  $Mdm2^{-/-} p53^{515C/515C}$  mice (n=26).

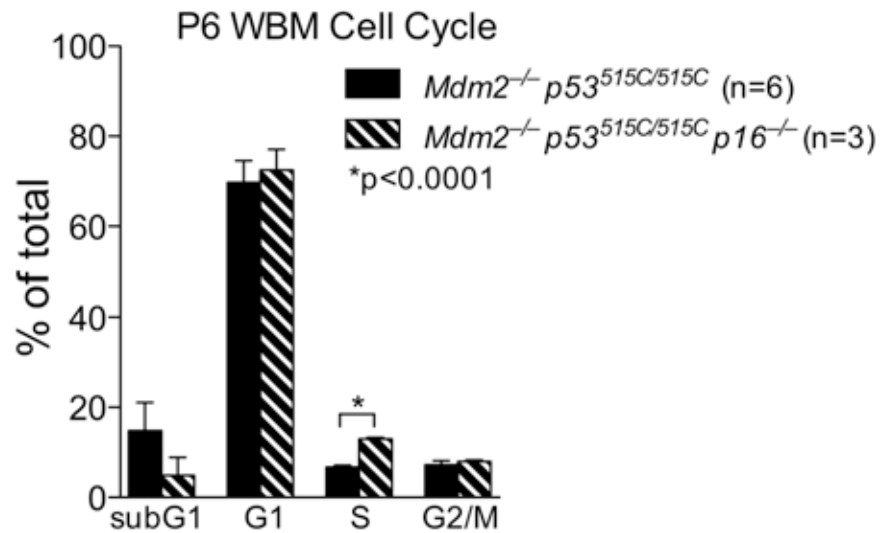
alleviates p53-independent pathways (Figure 34). We then examined the cell cycle status of  $Mdm2^{-/-} p53^{515C/515C} p16^{-/-}$  bone marrows at P6. We expected that deletion of p16 should increase bone marrow cycling. Indeed, The fraction of cells in  $Mdm2^{-/-} p53^{515C/515C} p16^{-/-}$  bone marrows that were in S phase was two fold higher (12.9%) than bone marrows of  $Mdm2^{-/-} p53^{515C/515C}$  mice (6.5%) (Figure 35). This indicates that increased bone marrow cycling contributed to rescue of bone marrow cellularity.

*$Mdm2^{-/-} p53^{515C/515C} p16^{-/-}$  bone marrows have increased LKS and CLP/CMP populations compared to  $Mdm2^{-/-} p53^{515C/515C}$  bone marrows*

$p16$  deletion partially restored the bone marrow cellularity. Since LKS and CLP/CMP are responsible for replenishing the bone marrows, we asked whether  $p16$  loss could contribute to rescuing these populations as well. To that end, we analyzed the LKS and CLP/CMP populations of  $Mdm2^{-/-} p53^{515C/515C} p16^{-/-}$  mice compared to  $Mdm2^{-/-} p53^{515C/515C}$  bone marrows at P6. Indeed, there was a significant increase in both of these populations. Specifically, LKS populations increased by 4 fold ( $p=0.0002$ ) to 0.041% in whole bone marrow upon deletion of  $p16$  compared to 0.01% in  $Mdm2^{-/-} p53^{515C/515C}$  mice (Figure 36a). CLP/CMP levels increased 9 fold ( $p=0.0036$ ) upon deletion of  $p16$  and reached 0.68% of whole bone marrow (Figure 36b). Although LKS and progenitor levels did not return back to normal levels as in  $Mdm2^{+/-} p53^{515C/515C}$  mice, this increase was consistent and can explain the replenishment of bone marrow cellularity at P10.  $Mdm2^{+/-} p53^{515C/515C} p16^{-/-}$  bone marrows had no significant changes in LKS or CLP/CMP populations compared to  $Mdm2^{+/-} p53^{515C/515C}$  bone marrows (Figure 36a-b).

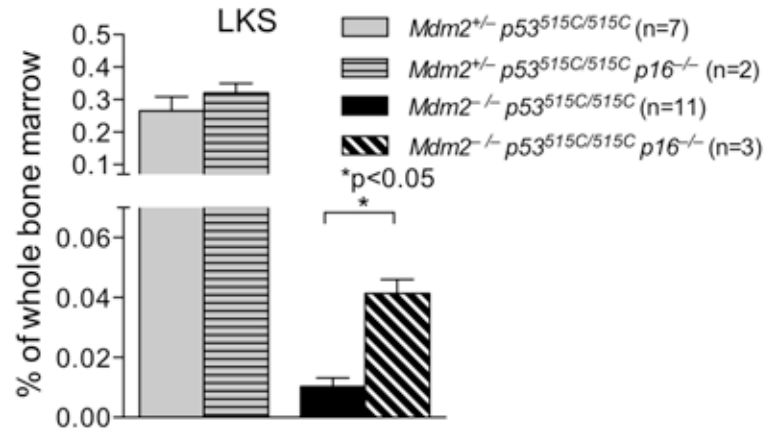


**Figure 34. p53R172P is high in *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> *p16*<sup>-/-</sup> bone marrows.** p53R172P immunohistochemical analysis on P10 bone marrows (BM) of *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> (left) and *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> *p16*<sup>-/-</sup> (right) mice. Scale bar is 50μm.

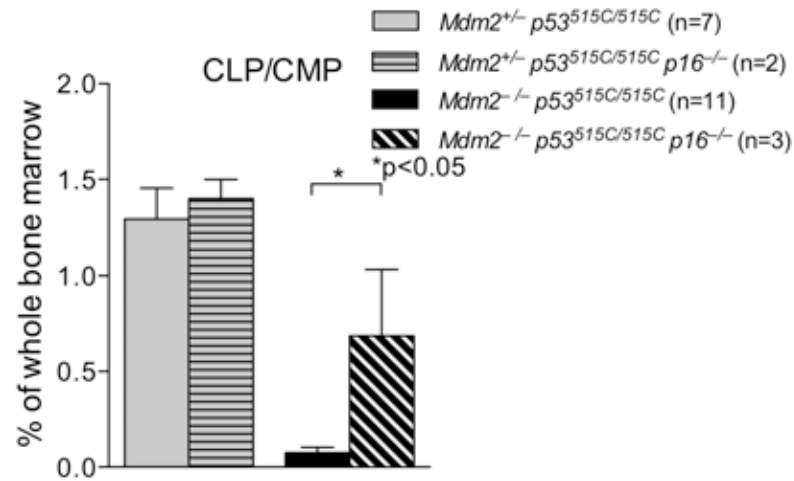


**Figure 35. Increase S phase in *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> *p16*<sup>-/-</sup> bone marrows.** Cell cycle status of P6 whole bone marrow cells (WBM) of *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> with and without *p16*. Error bars represent standard error of the mean.

a.



b.



**Figure 36. Partial rescue of LKS and CLP/CMP populations upon deletion of *p16* from *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice.** LKS (a) and CLP/CMP (b) populations of P6 whole bone marrow of *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> *p16*<sup>-/-</sup> mice compared to *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice. Error bars represent standard error of the mean.

## **c-kit analysis in $Mdm2^{-/-}$ $p53^{515C/515C}$ mice**

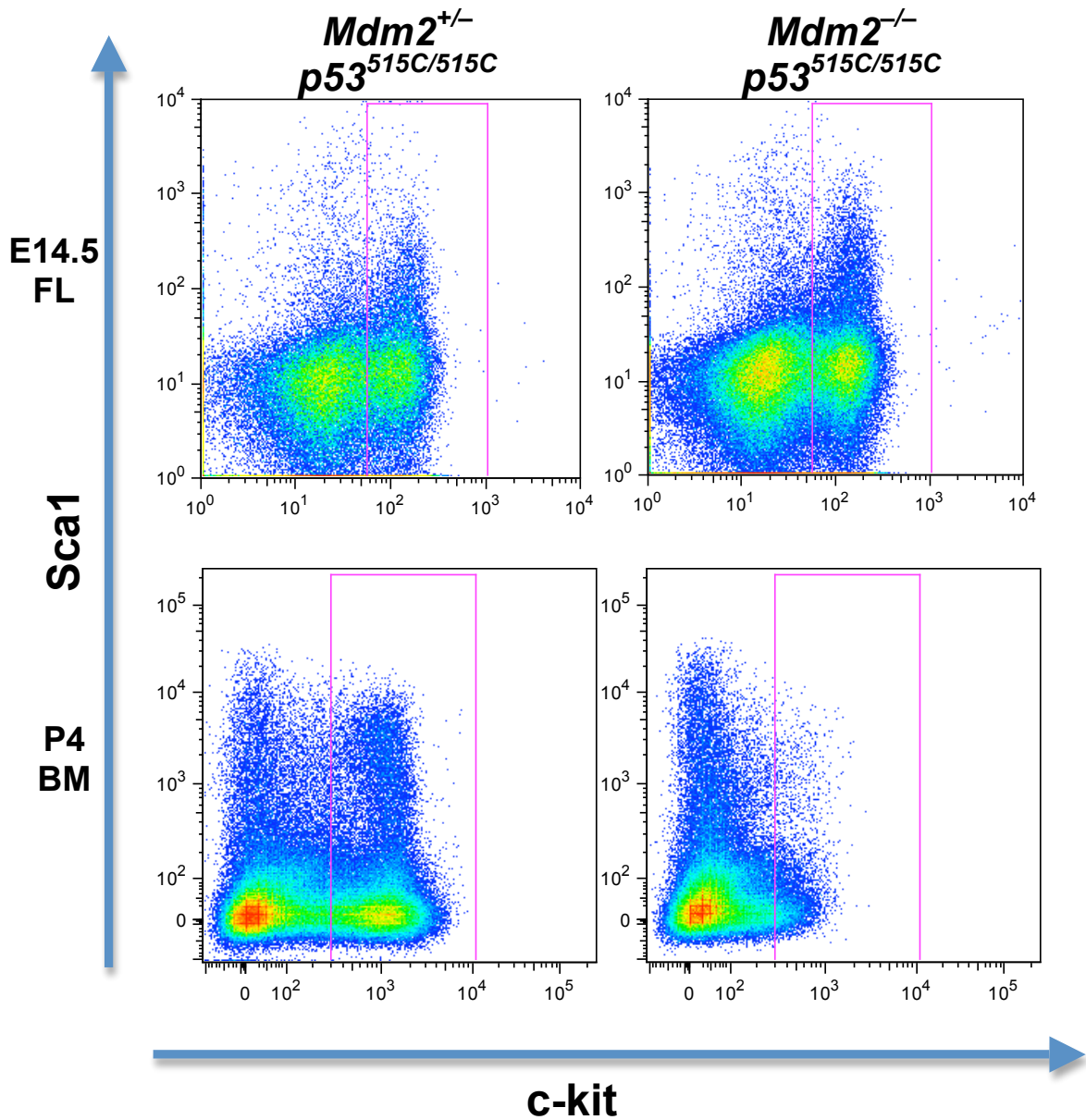
### *c-kit downregulation*

c-kit, also known as CD117, is a tyrosine kinase receptor that is expressed on hematopoietic stem cells and progenitors (Okada, Nakauchi et al. 1991). Binding of Steel factor (Sl), c-kit's ligand, activates a plethora of downstream targets that are involved in self-renewal, differentiation and expansion of hematopoietic stem cells and their progenitors (Kent, Copley et al. 2008). Mice mutant for c-kit or Sl have severe hematopoietic defects and die shortly after birth (McCulloch, Siminovitch et al. 1964; Trevisan, Yan et al. 1996). While loss of c-kit is detrimental, its overexpression is oncogenic (Masson and Ronnstrand 2009).

Using flow cytometric analysis, we noticed rapid depletion of c-kit positive population in postnatal bone marrows of  $Mdm2^{-/-}$   $p53^{515C/515C}$  but not in their fetal livers (Figure 37). At E14.5, the fetal liver cells had no obvious defects in c-kit expression using flow cytometry. We next measured *c-kit* transcript levels in whole bone marrows using RT-PCR. *c-kit* levels were 5 fold ( $p=0.001$ ) and 7 fold ( $p=0.0003$ ) less in  $Mdm2^{-/-}$   $p53^{515C/515C}$  mice compared to control  $Mdm2^{+/-}$   $p53^{515C/515C}$  (Figures 38a-b).

### *p53 does not bind c-kit promoter*

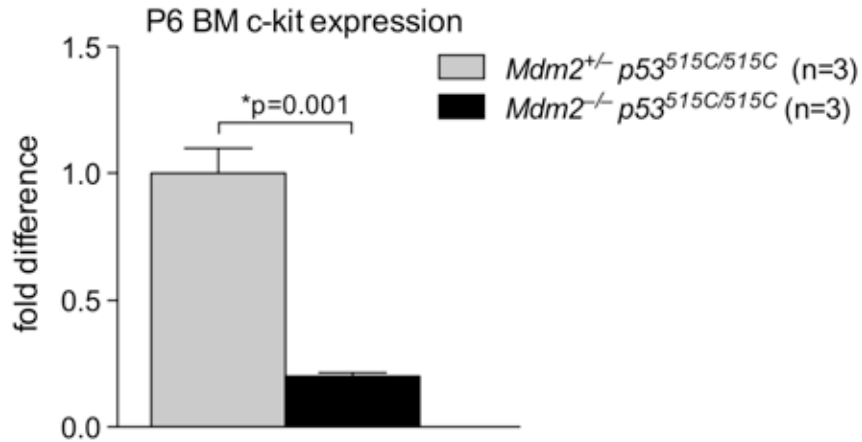
Since c-kit loss of expression correlated with p53R172P expression, and since p53 can bind and inhibit transcription of some genes (Rother, Kirschner et al. 2007), we investigated whether c-kit downregulation was p53-binding dependent using chromatin immunoprecipitation (ChIP). To that end, we used intestines from



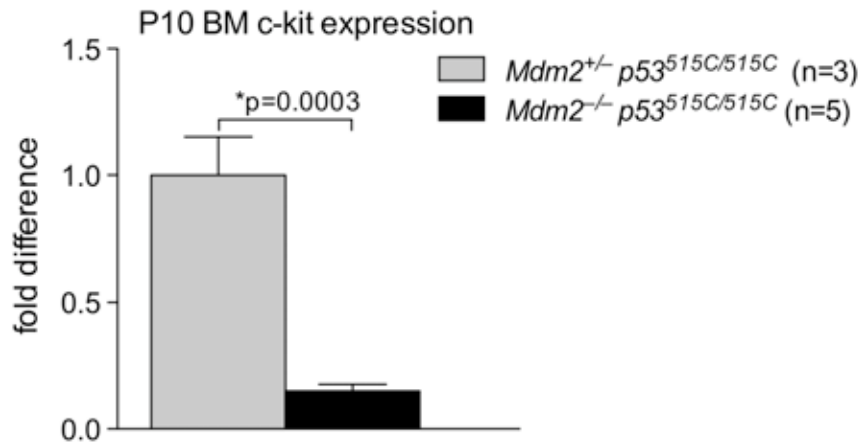
**Figure 37. Ebbing of c-kit expression in postnatal bone marrows but not fetal livers of *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice.** c-kit expression in *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> (left) and *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> (right) mice from fetal livers (FL) (upper two) and bone marrows (BM) (lower two). Pink gating designated the c-kit<sup>+</sup> population after Lin-depletion.



a.



b.



**Figure 38. c-kit mRNA levels are significantly lower at P6 and P10 in *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> bone marrows.** c-kit mRNA expression measured with RT-PCR at P6 (a) and P10 (b) of whole bone marrow (BM) cells from *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> and *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice. Error bars represent standard error of the mean.

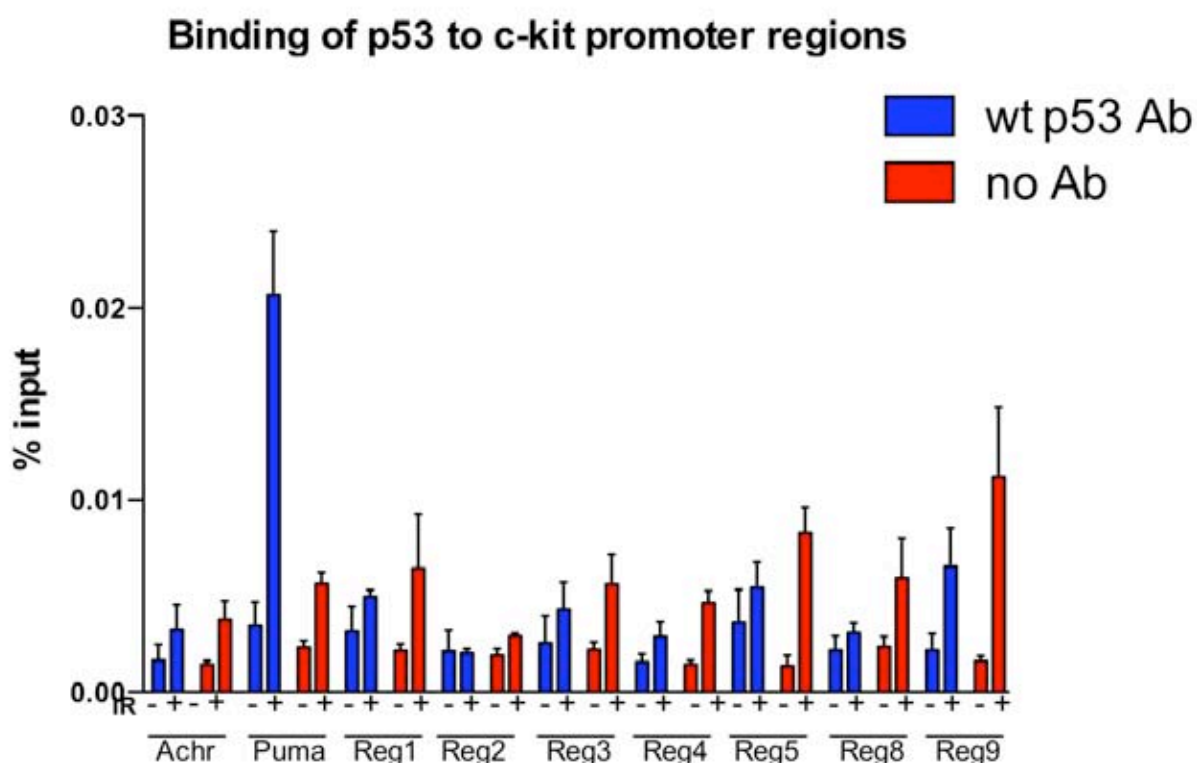
irradiated and non-irradiated wildtype mice, as the intestine is a highly responsive tissue for p53 activity. p53 binding on acetylcholine receptor (AChR) and Puma promoters were used for negative and positive controls, respectively. We screened the promoter region upstream of c-kit transcriptional start site with 7 different primer sets spaced by approximately 600 bases each and covering approximately 5000 bases. Unfortunately, we could not detect any significant binding of p53 on the c-kit promoter (Figure 39).

## **Characterizing the mesenchymal lineage**

### *Significant increase in Lin<sup>-</sup> Sca1<sup>+</sup> c-kit<sup>-</sup>/low population*

Multipotent mesenchymal strom cells (MSCs), also known as mesenchymal stem cells, constitute a rare population in the bone marrow that are responsible for forming the hematopoietic niche (Short, Brouard et al. 2001). Progeny of MSCs can form osteoblasts, chondrocytes or myoblasts which contribute to formation of muscle, fat, cartilage and bone (Dennis, Merriam et al. 1999; Pittenger, Mackay et al. 1999). The hematopoietic niche can dictate the capacity of HSCs to self-renew or differentiate (Walkley, Shea et al. 2007). Contrary to human MSCs, murine MSCs are ill-defined and hard to isolate due to dearth of information about their localization in the bone marrow and their very low numbers (Short, Brouard et al. 2001).

Although Lin<sup>-</sup> Sca1<sup>+</sup> c-kit<sup>-</sup>/low (hereafter referred to as MSC-like) population is a crude representative of MSCs, it can still delineate an understanding of the frequency of MSCs in *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice. We first noticed an obvious increase in this population when gating for c-kit and Sca1 after lineage depletion

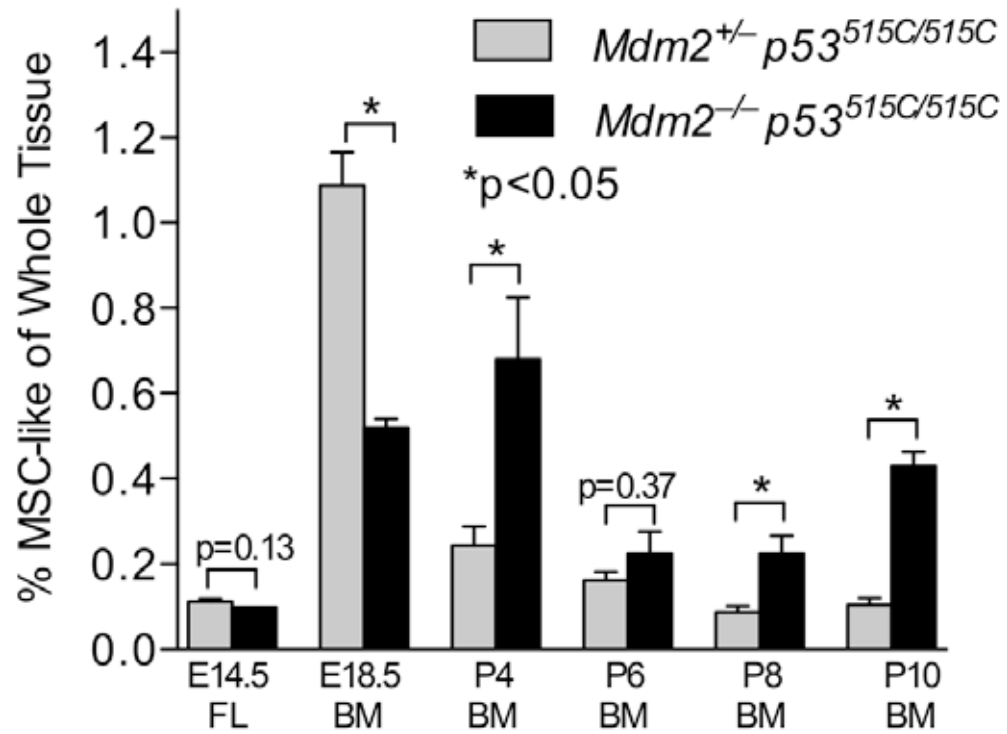


**Figure 39. Wildtype p53 does not bind the promoter region of c-kit.** AchR and Puma are used as negative and positive control for p53 binding, respectively. Primers designed to amplify 7 regions of c-kit promoter designated as Reg1 to Reg9. IR (ionizing radiation) was used to induced p53 activity. Error bars represent standard error of the mean.

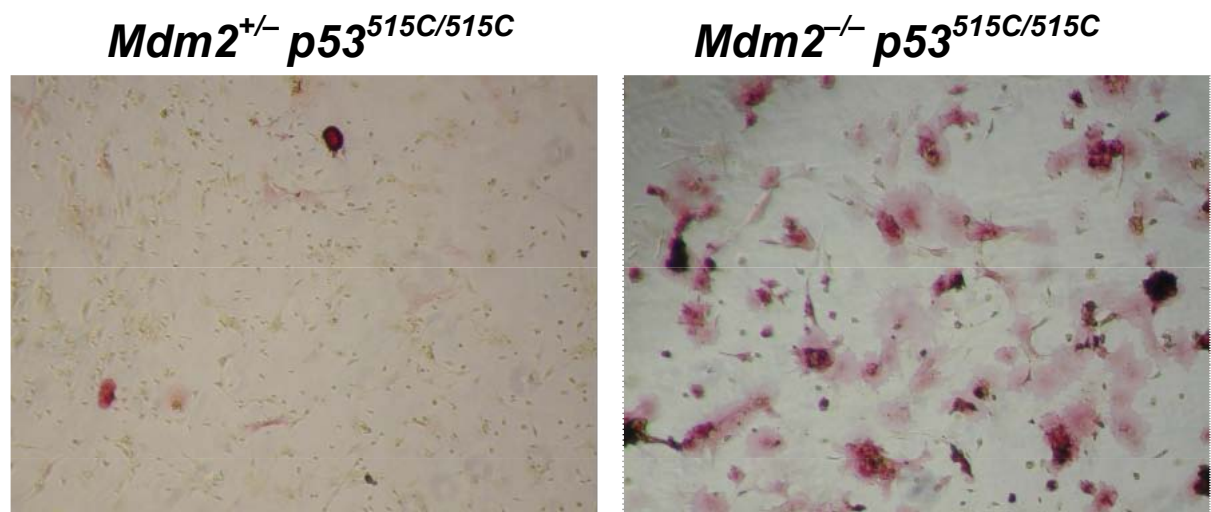
with flow cytometric analysis in postnatal bone marrows but not fetal livers of  $Mdm2^{-/-} p53^{515C/515C}$  mice compared to control  $Mdm2^{+/-} p53^{515C/515C}$  littermates (Figure 37). E14.5 fetal livers of either genotype had approximately 0.11% of cells as MSC-like (Figure 40). At E18.5 bone marrows of  $Mdm2^{-/-} p53^{515C/515C}$  mice had almost two fold decrease ( $p=0.0088$ ) in MSC-like cells. However, as early as P4,  $Mdm2^{-/-} p53^{515C/515C}$  bone marrows were three times more enriched for this population compared to control (Figure 40). This population then leveled off at P6 with no significant differences between the two genotypes ( $p=0.37$ ) (Figure 40). At P8 and P10, the MSC-like population differences started to become more pronounced in  $Mdm2^{-/-} p53^{515C/515C}$  mice as compared to control  $Mdm2^{+/-} p53^{515C/515C}$  mice (Figure 40).

#### *Increased osteoblastic and adipogenic activity of $Mdm2^{-/-} p53^{515C/515C}$ mice in vitro*

In order to evaluate the activity of MSC-like cells *in vitro*, we collected P6 whole bone marrows of either genotype and plated them in low well densities using mesenchymal-specific media. Typically, cells are plated for a few days until fibroblastic-like colonies form. These colonies are then induced to differentiate to certain lineages depending on factors added to the media. Surprisingly, cultures of  $Mdm2^{-/-} p53^{515C/515C}$  bone marrows differentiated into osteoblastic lineage without any induction as seen by increased alkaline phosphatase staining (Figure 41).



**Figure 40. Increase in MSC-like population in  $Mdm2^{-/-} p53^{515C/515C}$  bone marrows.** Fetal livers (FL) and bone marrows (BM) were collected from designated time points and analyzed with flow cytometry for frequency of MSC-like population (Lin<sup>-</sup> Sca1<sup>+</sup> ckit<sup>-</sup>). At least three mice were used for each time point. Error bars represent standard error of the mean.



**Figure 41. Increase osteoblastic differentiation of P6 bone marrows of *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice *in vitro*.** P6 whole bone marrows were allowed to grow for MSC colony formation. *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> (right) cells spontaneously differentiated into osteoblastic lineage as seen by red alkaline phosphatase activity while *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> (left) retained undifferentiated state.

## Transplantation experiments

### *P6 whole bone marrows of $Mdm2^{-/-}$ $p53^{515C/515C}$ rescue lethally irradiated mice*

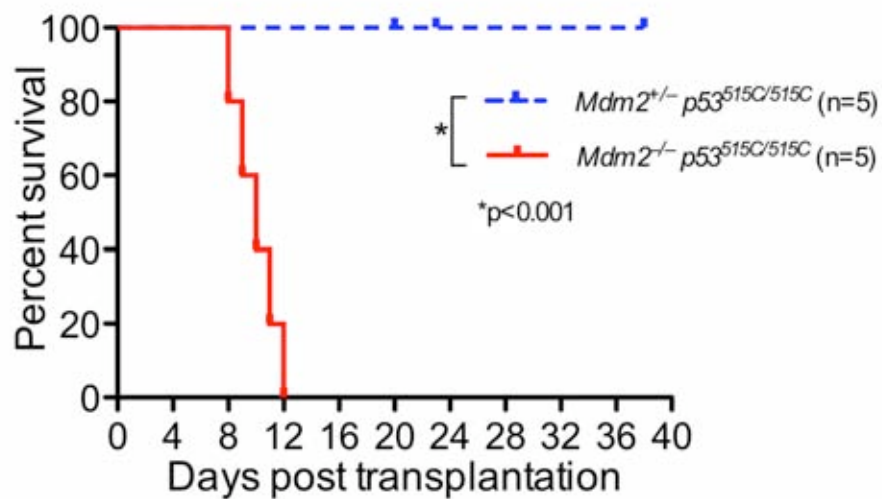
The potential of HSCs to engraft into irradiated recipients and repopulate the blood is a readout for functionality of these cells (Ema and Nakauchi 2004). If donor-derived cells reconstituted all lineages in the recipient mice, HSCs are said to be functional in the new niche. The generation of two congenic C57/B6 strains distinguished by a leukocyte common antigen (CD45.1 vs CD45.2) allows us to distinguish between donor and recipient derived hematopoietic cells in transplantation experiments (Saga, Tung et al. 1986; Saga, Lee et al. 1990).

In order to address whether the hematopoietic failure in  $Mdm2^{-/-}$   $p53^{515C/515C}$  was due to intrinsic HSC failure, niche defects or both, we performed transplantation assays. Since our donor  $Mdm2^{-/-}$   $p53^{515C/515C}$  and  $Mdm2^{+/-}$   $p53^{515C/515C}$  mice were in a C57/B6 CD45.2+ background, we used 8-12 weeks old wildtype C57/B6 CD45.1 mice as recipients. We first retroorbitally transplanted  $0.5 \times 10^6$  whole bone marrow cells of wildtype CD45.2 mice into lethally irradiated CD45.1 as a positive control for transplantation experiments. 100% (5 out of 5) of transplanted mice survived several weeks post-transplantation (Figure 42). To ensure that the irradiation was effective, we irradiated two mice but did not transplant them with donor cells. These mice died within two weeks post-lethal irradiation.

We then transplanted  $0.5 \times 10^6$  whole bone marrow cells of P6  $Mdm2^{+/-}$   $p53^{515C/515C}$  pups to first validate that bone marrow cells at this time point can reconstitute hematopoiesis. 100% (5 out of 5) transplanted mice with  $Mdm2^{+/-}$   $p53^{515C/515C}$  bone marrows survived until 4 weeks post-transplantation proving that

*Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> HSC population is normal and can reconstitute hematopoiesis in irradiated recipients (Figure 42). On the other hand, 100% (5 out of 5) lethally irradiated CD45.1 transplanted with 0.5x10<sup>6</sup> *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> whole bone marrow cells died within two weeks indicating that the HSC defect in *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice is intrinsic to HSCs and progenitors.





**Figure 42: Kaplan-Meier survival curve of recipient CD45.1 mice after transplantation.**  $0.5 \times 10^6$  bone marrow cells of *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> and *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> were injected into lethally irradiated CD45.1 recipients. n=number of mice injected per genotype

## DISCUSSION

### Circumventing *Mdm2*<sup>-/-</sup> lethality

Mdm2 is the major negative regulator of p53 and its loss is lethal at early stages of embryogenesis due to p53-dependent apoptosis (Jones, Roe et al. 1995; Montes de Oca Luna, Wagner et al. 1995). This phenotype impedes understanding the role of p53 regulation by Mdm2 in normal tissues and during homeostasis at later stages of development. With the availability of a *p53*<sup>515C</sup> hypomorphic allele, we were successful in generating *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice. Remarkably, *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice are indistinguishable from their control *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> littermates at birth despite the lack of *Mdm2*. Since p53R172P cannot transactivate apoptosis genes (Ludwig, Bates et al. 1996; Rowan, Ludwig et al. 1996), *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice bypass this early apoptotic barrier and are born at normal Mendelian ratios. *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> pups live up to two weeks after birth. However, they suffer from growth retardation during postnatal development due to lack of sufficient blood cells and adequate hematopoiesis to support growth. This provides us with a window of time to address several questions about the effect of *Mdm2* loss and the stability of p53R172P in the hematopoietic compartment during embryogenesis and postnatally. We could not detect p53R172P stability until E18.5 in bone marrows despite absence of Mdm2. One justification is the existence of other negative regulators such as Mdm4 or Cop1 that are controlling p53R172P levels in the absence of Mdm2 during embryogenesis.

## **p53 dependence**

The hematopoietic and postnatal growth retardation defects and survival are rescued upon deleting one *p53* allele confirming that this is a p53-dependent phenotype. *Mdm2*<sup>+/-</sup> *Mdm4*<sup>+/-</sup> mice, carrying wildtype *p53* alleles, suffer from similar hematopoietic defects to *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice (Terzian, Wang et al. 2007). Specifically, *Mdm2*<sup>+/-</sup> *Mdm4*<sup>+/-</sup> mice have growth retardation, acellular bone marrows, low cell counts in the blood and die shortly after birth (Terzian, Wang et al. 2007). Additionally, *Mdm2*<sup>+/-</sup> or *Mdmx*<sup>+/-</sup> mice subject to sublethal irradiation die shortly after irradiation due to wildtype p53-dependent hematopoietic phenotype (Terzian, Wang et al. 2007). This demonstrates that the phenotypes in *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> are not due to the mutant *p53* allele *per se* but due to p53 activity. This suggests that the bone marrow is a very sensitive tissue to elevated p53 levels. This could be due to the high turn-over rate in this tissue that requires adequate checkpoints to avoid error propagation. Therefore, uninhibited p53 is deleterious for bone marrows and Mdm2 is indispensable for regulating p53 levels during homeostasis.

## **p53R172P modes of action to abrogate hematopoiesis**

The rapid depletion of bone marrow cellularity is achieved by the combinatorial effect of several p53 activities. First, the elevation of p21 and Ccng levels in postnatal bone marrows indicates activation of cell cycle arrest and is supported by significantly lower percentage of bone marrow cells in S phase of cell cycle in *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> bone marrows compared to control littermates. Senescence genes are also upregulated, further exacerbating the phenotype. This further supports the senescence activity of p53R172P as seen in a lymphoma

mouse model (Post, Quintas-Cardama et al. 2009). Although Puma and Noxa levels were significantly higher, a 2 fold increase in these apoptosis-inducing genes is unlikely to cause the dramatic loss of cellularity. Because of absence of apoptotic markers (cleaved caspase-3 and AnnexinV) in *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> bone marrows, other types of cell death may be occurring. This is evident from the high subG1 peak in 3 out of 6 *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice and the rapid loss of cellularity of bone marrows within few days after birth. Unfortunately, the exact mode of cell death in bone marrow cells is still undetermined. Another mouse model that lacks the Mdm2-p53 feedback loop has less Mdm2 expression and also reveals high sensitivity of bone marrows to wildtype p53 activity post irradiation (Pant, Lozano, unpublished data). Notably, like in our mouse model the major cause of cell death in these mice is not apoptosis (Pant, Lozano, unpublished data). Since p53R172P levels are low or undetectable in fetal livers, cells have a normal cell cycle pattern and are histologically normal. Hence, the hematopoietic phenotype is the result of p53-dependent induction of cell cycle arrest, senescence and cell death, and the absence of apoptosis supports previous work (Ludwig, Bates et al. 1996; Rowan, Ludwig et al. 1996) showing no apoptotic activity of *p53*<sup>515C</sup> allele.

### **Sensitivity of HSCs and progenitors to p53R172P**

HSCs, progenitors and hematopoiesis are normal in E14.5 fetal livers. One potent effect of p53R172P is the depletion of LKS population (which includes LT-HSC and ST-HSC) and CLP/CMP populations postnatally. Remarkably, these cellular populations are unaffected up until E18.5 when hematopoiesis progresses in the bone marrows. Although bone marrows are supposed to be highly cycling at

postnatal time points and until three weeks after birth (Bowie, McKnight et al. 2006), in the absence of HSCs normal bone marrow cellularity cannot be attained. In other studies, an increase in p53 activity or levels due to *Bmi1* or *Cited2* nullizygosity depletes HSCs (Park, Qian et al. 2003; Akala, Park et al. 2008; Kranc, Schepers et al. 2009). In these studies, deletion of *p53* partially or totally rescues HSCs in *Bmi1* or *Cited2* null mice, respectively. Hence, the sensitivity of HSCs to elevated p53 levels in these mouse models is consistent with our data.

Upon further enriching for LT-HSCs with SLAM-LKS isolation, there are still significant differences between *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> and control *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> mice at P6. And consistent with histological analysis, the SLAM-LKS population is normal in the fetal liver. Tissue specific differences could contribute to these variations. For instance, there could be other negative regulators than Mdm2 that are regulating p53 in fetal livers, such as Cop1 and Mdm4, but are not potent enough in postnatal bone marrows. Regardless of the method of enrichment of HSCs and progenitors, all essays confirm the sensitivity of these populations to elevated p53 levels.

### **Elevated ROS levels in bone marrows compared to fetal livers: spatial versus temporal factors**

Since there were no detectable hematopoietic defects in fetal livers in *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> embryos, we speculated that fetal livers could provide us with a hematopoietic resource for mechanistic and molecular studies *ex vivo*. Hematopoiesis of the fetal liver *ex vivo* is abrogated similarly to the *in vivo* postnatal bone marrows. Specifically, fetal liver hematopoietic cells of *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup>

embryos at E14.5 did not grow in methocult. This is indeed a surprising result and is contrary to our expectations. One major difference between *in vivo* (embryogenesis) and *ex vivo* (methocult) processes is higher oxidative stress in the latter. Culturing fetal livers at 20% oxygen levels induced higher oxidative stress while 3% oxygen levels consistently permit growth.

*In vivo*, the elevated ROS levels in  $Mdm2^{+/-} p53^{515C/515C}$  bone marrows at P6 compared to fetal livers at E14.5 of same genotype suggest that hematopoiesis in postnatal bone marrows proceeds under higher oxidative stress. These variations of ROS levels *in vivo* are due to several factors attributed to spatial or temporal variations. The fetal liver could be a more potent hematopoietic niche in chelating and reducing ROS levels than the bone marrow. For instance, upregulating antioxidant genes could be one way of achieving this. The fetal liver could also be producing less free radicals compared to bone marrows due to less exposure to oxygen.

As for temporal effects, metabolic activity produces ROS that eventually may accumulate (Eriksson 2007). By the time hematopoiesis is established in the bone marrow, enough ROS could have been built up. Noteworthy, embryos receive oxygen through exchange between maternal and fetal blood. Postnatally, neonates are exposed to direct atmospheric oxygen, which can be a stronger source for ROS generation. In either case, ROS can damage DNA and certainly induce p53 activity (Ko and Prives 1996). The one *Mdm2* allele harbored in  $Mdm2^{+/-} p53^{515C/515C}$  mice is sufficient to dampen ROS-induced p53R172P levels. In the case of  $Mdm2^{-/-} p53^{515C/515C}$  mice, however, p53R172P goes uncontrolled in absence of *Mdm2* and its levels surge wiping out the bone marrow compartment. A critical role for *Mdm2* is to keep p53R172P in check in response to basal ROS levels during homeostasis.

### **p53: ROS-generator**

*Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> and *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice are housed in the same conditions. Yet, ROS levels in bone marrows of *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> pups are significantly higher than their control littermates. This suggests that *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> bone marrow cells are intrinsically generating more ROS. This is further validated by the significant upregulation of PIGs which are ROS-inducing genes and direct targets of p53 (Polyak, Xia et al. 1997). The surge of different PIGs at different time points (P6 versus P10) supports the temporal variation of their p53-dependent upregulation as previously shown by Bert Vogelstein's group (Polyak, Xia et al. 1997). ROS generation increases cell death and contributes to depletion of bone marrow cellularity. Maintaining elevated ROS could potentially create a positive feedback loop to keep p53R172P levels high and ultimately induce cell death. In the absence of Mdm2, this feedback loop cannot be broken. It would be interesting to address whether p53-induction of cell death in other tissues after stress, such as ionizing radiation, is also mediated through an increase in ROS production. This is a novel finding as it sheds light on an alternative mechanism of inducing cell death in the absence of p53's normal apoptotic activity in the hematopoietic compartment. To our knowledge, this is the first observation of p53-dependent production of ROS in an *in vivo* system in the hematopoietic compartment.

### **ROS generation in hematopoietic compartment of other mouse models**

Several studies correlated loss or gain-of-function of genes with elevated ROS in HSCs. For instance, *Atm*<sup>-/-</sup> mice accumulate ROS in their bone marrows

and HSCs in a mechanism that is still not clear (Ito, Hirao et al. 2004). On the other hand, mTOR overactivation due to targeted mutation of its negative regulator *Tsc1* stimulates mitochondrial ROS biogenesis (Chen, Liu et al. 2008; Chen, Liu et al. 2009). This is concomitant with increased cycling of HSCs and ultimately loss of their “stemness”. In another model, *Bmi1* loss, a Polycomb repressor, impairs mitochondrial function and instigates higher ROS levels, thus damaging HSC activity (Liu, Cao et al. 2009). Genetic deletion of *Chk2*, a DNA damage response pathway and activator of p53, alleviates the *Bmi1*-dependent phenotype (Liu, Cao et al. 2009). Based on our model, we imagine that the *Bmi1* effect is mediated through Chk2 activation of p53. This can be supported by the rescue of the effect of *Bmi1* loss on hematopoiesis by triple deletion of p53, *p16* and *p19* in a different study (Akala, Park et al. 2008). Hence, while one study correlated *Bmi1* with ROS and *Chk2*, and another correlated *Bmi1* with p53, our study links these studies by showing that regardless of the mode of activation of p53 (loss of *Bmi1* or Mdm2, or activation of *Chk2*), p53 activates a set of ROS-inducing genes that lead to cell death and depletion of HSCs and their progenitors.

### **Potential ROS effects on HSCs of *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice**

Collectively, the aforementioned studies provide enough evidence to support that high ROS levels can negatively influence HSC and progenitor activities. However, the exact mechanism remains an important question to explore. Elevated ROS levels in myeloid progenitors in *Drosophila* induces their differentiation via downregulation of Polycomb complexes and upregulation of JNK and FoXo signaling pathways (Owusu-Ansah and Banerjee 2009). Since p53 activates



differentiation pathways (discussed in introduction), this remains a plausible effect of p53R172P-dependent ROS production in *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> HSCs and progenitors. For instance, p53 can induce rapid differentiation of HSCs and progenitors into committed myeloid cells that are short-lived. Testing such a scenario would require lineage tracing of a SLAM-LKS sorted out population. The low abundance of this population in our mouse model remains an obstacle to address this question.

Alternatively, as in the *Atm*<sup>-/-</sup> mouse model (Ito, Hirao et al. 2004), elevated ROS could have abrogated self-renewal capacity of HSCs in *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice. And since HSCs are typically highly cycling within the first three weeks after birth (Bowie, McKnight et al. 2006), ROS-dependent depletion of bone marrow cellularity could have “exhausted” the HSC pool and impaired their self-renewal capacity while trying to regenerate bone marrows. Additionally, localization of HSCs in the bone marrow is dictated by variations of oxidative stress intensities at different sites of the niche (Suda, Arai et al. 2005). It is possible that changes in ROS levels in HSCs of *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice altered their localization in the niche. Since HSCs acquire signals from the environment that dictate their potential to proliferate and differentiate, changes in their localization would hinder such signaling pathways. Hence, a p53-dependent increase of ROS levels in HSCs may obstruct their normal function, although exactly how this is achieved is still not clear.

ROS activation of *p16* has also been implicated in the hematopoietic compartment (Ito, Hirao et al. 2004). Treatment with antioxidants decreases *p16* levels and ameliorates hematopoietic activity (Ito, Hirao et al. 2004). However, a direct genetic proof is still lacking. *p16* elevation in bone marrows of *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice further supports the ROS-mediated activation of *p16*. Here, we

provide the first genetic evidence that *p16* deletion from HSCs predisposed to cell death due to *Mdm2* deletion and p53 activation can mitigate cell death and partially expand this population, subsequently increasing bone marrow cellularity. Also, the same effect of *p16* deletion is exerted on the CLP/CMP population. One explanation for this partial rescue is that *p16* deletion increases the percent of cells in S phase, creating a reservoir of bone marrow cells. However, this is not sufficient to completely rescue the phenotype due to consistent elevation of p53R172P levels and persistence of other non-hematopoietic phenotypes. This indicates that *p16* deletion is affecting other pathways which are p53-independent. *p16* loss increases the regenerative and self-renewal capacity of HSCs in transplantation studies (Janzen, Forkert et al. 2006). In a more recent work, Tariq Enver's group clearly demonstrated that the deletion of both *p16* and *p19*<sup>ARF</sup> can rescue *Cited2*<sup>-/-</sup> HSCs numbers and activity (Kranc, Schepers et al. 2009). This further supports our data that deletion of *p16* can expand HSC and progenitor populations despite elevated p53R172P levels.

### **Antioxidants and rescue of hematopoiesis**

Pharmacological treatment of the antioxidant NAC rescues hematopoiesis in several mouse models with elevated ROS levels such as *Bmi1*<sup>-/-</sup> and *Atm*<sup>-/-</sup> (Ito, Hirao et al. 2004; Liu, Cao et al. 2009). However, in *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice NAC treatment produces only a partial rescue of progenitor and LKS populations. This could be attributed to several factors. For instance, the most common mode of administering NAC is generally in drinking water for adult mice. Since *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice are still lactating, we attempted to inject them with the drug

instead. However, most of the mice that were injected succumbed to toxic and invasive side effects regardless of their genotype. Also, we could not insure 100% delivery of the antioxidant in the two injections we administered. Of the *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice surviving the treatment, LKS and CLP/CMP populations are significantly higher. Ideally, treated and untreated samples should have been analyzed at the same day. However, we could not get sufficient pups at P6 at the same time for this experiment. Since a significant increase in bone marrow cellularity occurred at P10 following NAC treatment, we conclude that NAC can partially rescue hematopoiesis. The rescue of hematopoietic activity *ex vivo* when hematopoietic tissues are cultured at 3% rather than 20% oxygen provides additional evidence that oxidative stress impedes hematopoietic activity.

### **p53 and the supportive niche**

HSCs integrate intrinsic and extrinsic signals in order to maintain hematopoiesis (Walkley, Shea et al. 2007). Extrinsic signals are provided by the cells of the hematopoietic such as osteoblasts and fat cells which are progeny of MSCs. Surprisingly, a distinct MSC-like population is predominant in *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice although this population is supposed to be low in bone marrows. Studies suggest that an increase in fat cell deposition can be a barrier for hematopoietic activity (Naveiras, Nardi et al. 2009). Specifically, irradiated human and murine bone marrows showed increased fat cell deposition which delayed homing of transplanted HSCs and regeneration of bone marrows (Naveiras, Nardi et al. 2009). In *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> bone marrows we clearly detect an increase of fat cell deposition using histological analysis at P10 when hematopoietic cells are

wiped out. Hence, the increase of adipocytes could be another factor for inhibiting hematopoiesis. The autonomous differentiation of P6 cultured bone marrows into osteoblastic lineages further support the evidence that while p53R172P is depleting hematopoietic activity, it also increases mesenchymal lineage activity. This goes against other studies showing that p53 inhibits osteoblastogenesis (Wang, Kua et al. 2006; Molchadsky, Shats et al. 2008). These studies use mouse embryonic fibroblasts to address the role of p53 in mesenchymal lineage differentiations. However, we use bone marrow cells which provide a more accurate representation of mesenchymal activity. One interesting hypothesis is that HSC marked by Lin<sup>-</sup> ckit<sup>+</sup> Sca1<sup>+</sup> could have lost or downregulated their expression for c-kit and switched to MSC-like population (Lin<sup>-</sup> ckit<sup>-/low</sup> Sca1<sup>+</sup>). The RNA levels of c-kit support a p53R172P dependent downregulation of c-kit. Although we couldn't detect p53R172P binding to the promoter region of c-kit, p53R172P could be regulating c-kit levels indirectly. Unfortunately, a c-kit transgenic mouse is not available to address whether c-kit overexpression can aid in rescuing hematopoiesis and restoring the LKS population. Hence, p53 exert various effects not only on the hematopoietic cells but also their environment.

Transplantation experiments remain the ultimate assay to address hematopoietic reconstitution activity. The failure of transplanted *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> bone marrow cells to rescue lethally irradiated wildtype mice confirms the intrinsic defects that *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> HSCs harbor. *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> hematopoietic cells could not respond to the normal signaling from the wildtype niche of the recipient mice. Alternatively, *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> hematopoietic cells could have failed to engraft and died while in circulation. On the other hand, *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> hematopoietic cells from same age are successful in engrafting and

reconstituting hematopoiesis of lethally irradiated mice. This result rules out the possibility that alterations in *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> niche, although still occurring, is triggering the defect in hematopoietic activity.

## **Impact on Leukemia Stem Cells**

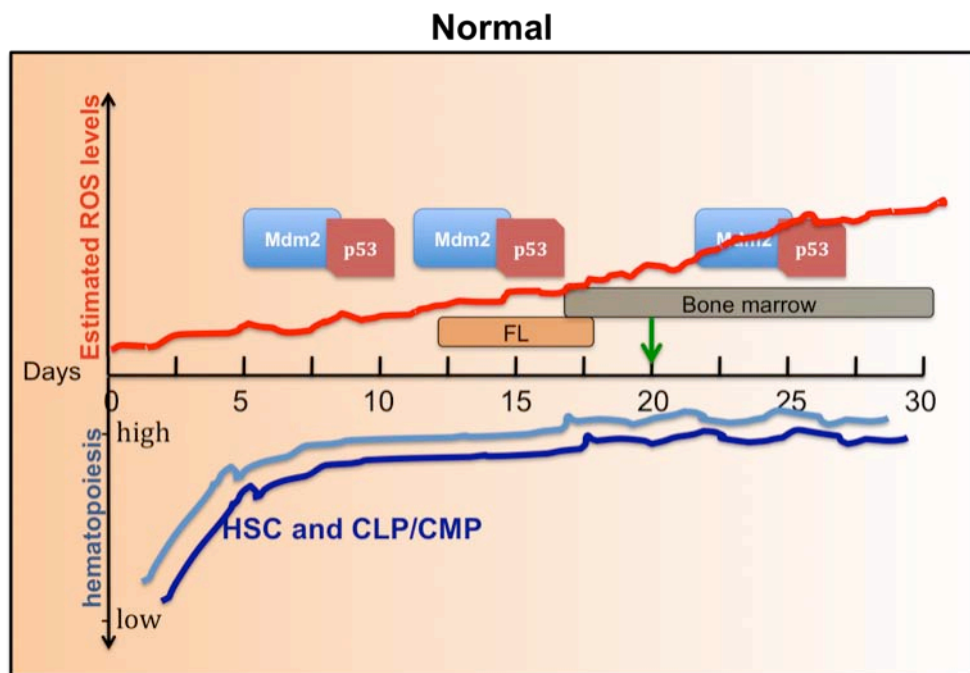
Until 1997, leukemia was perceived as a genetic or epigenetic disorder acquired by committed progenitors cells that allowed their unlimited proliferation and lack of differentiation (Passegue, Wagers et al. 2005). As discussed in the Introduction, p53 is a major regulator of both of these pathways. Using human acute myeloid leukemia (AML) cells John Dick was the first to show that the true cell of origin for leukemia could be a normal primitive cell (Bonnet and Dick 1997). More evidence for a normal HSC as origin of leukemia came from analyzing the AML1-ETO, a hallmark of AML, status in patients in remission. Specifically, these patients had the AML1-ETO fusion in their normal HSCs (Miyamoto, Weissman et al. 2000). In addition to the fundamental characteristic of self-renewal, normal HSCs and LSCs share other features (Passegue, Jamieson et al. 2003; Passegue, Wagers et al. 2005). For instance, both cell types express CD34, lose expression of CD38, are dormant and give rise to progeny that are more differentiated (Passegue, Jamieson et al. 2003). Accordingly, it became apparent that pathways that sensitize normal HSCs could have similar effect on leukemia stem cells.

We propose that p53 can be effective in inhibiting leukemiagenesis at various stages. Genetic aberrations or DNA damaging events in the HSC compartment can trigger p53 activity. Subsequently, p53 can halt cell cycle until damage is repaired or alternatively induce cell death. Our *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mouse model clearly

demonstrated the sensitivity of HSCs and their progenitors to disruptions of Mdm2-p53 levels. Given the similarities of HSCs and LSCs, it would be plausible that by exploiting the p53 pathway in leukemias, we can exert the same effect on LSCs and eradicate this population. The higher level of ROS by p53-dependent induction of *PIGs* in HSCs of *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice demonstrates a unique mode for induction of cell death in this population which can also be utilized in leukemia treatment.

### **Suggested model**

Increased ROS levels in bone marrows of *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice is a major finding of this study. In our model (Figure 43), we propose that hematopoiesis takes place under less oxidative stress while in the fetal liver compared to postnatal bone marrows. In a normal mouse, there would be sufficient Mdm2 to control p53 levels. However, in the absence of Mdm2, p53 goes unchecked and triggers cell death, cell cycle arrest, ROS generation and senescence. Hence, although p53 is required to inhibit tumorigenesis and maintain genetic integrity, uncontrolled p53 levels can be detrimental. The evolutionary existence of Mdm2 as a negative regulator of p53 provides protection against elevated p53 levels, although Mdm2 can itself be oncogenic. Disrupting the balance between these proteins is indeed destructive to the organism.



**Figure 43. Suggested model.** Hematopoiesis progresses at low ROS levels during embryogenesis starting day 0. In bone marrows, ROS levels increase due to accumulation of metabolic products or direct exposure to oxygen after birth (green arrow time point). In a normal case, Mdm2 inhibits ROS activation of p53 and hematopoiesis proceeds normally in the fetal liver (FL) and bone marrows throughout adulthood. In absence of Mdm2, p53 goes uninhibited due to ROS dependent activation in the bone marrows. Subsequently, p53 induces more ROS and depletes hematopoiesis, hematopoietic stem cells (HSCs) and common lymphoid and myeloid progenitors (CLP/CMP).

## Future directions and remaining questions

This dissertation clearly demonstrates the deleterious effects of p53 on hematopoietic stem cells. It also briefly addresses the effects of p53 stability on the bone marrow niche. However, it would be interesting to decipher whether the *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> hematopoietic niche can support wildtype HSCs. Specifically, we propose to transplant GFP-tagged wildtype HSCs into *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> neonates of age P3 via the jugular vein and check for engraftment and reconstitution of hematopoietic activity. With GFP, we can trace cell fates and investigate whether HSCs would home to other hematopoietic organs such as spleen or liver if bone marrow niche were defective. Additionally, if wildtype HSCs completely reconstituted hematopoiesis, we ask whether a complete rescue of hematopoiesis is enough to significantly prolong the life of *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice to weaning age and beyond in the midst of other defects.

*p21* deletion prolonged survival of mice without rescue of hematopoiesis, while *p16* rescued hematopoiesis. We propose to generate *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> *p16*<sup>-/-</sup> *p21*<sup>-/-</sup> mice and monitor hematopoiesis and survival. However, such a genetic cross is complicated and not necessarily achievable due to many allelic changes. If *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> *p16*<sup>-/-</sup> *p21*<sup>-/-</sup> mice survive, they can be monitored for tumor latency. Also, the reversible dormancy of *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> hematopoietic cells when cultured at 20% oxygen is a unique observation and requires further exploration. To that end, cells could be analyzed for senescence markers when cultured at 20% oxygen to confirm whether these cells are truly senescent and actually came out of senescence when cultured at lower oxygen levels. Investigating chromatin structure, senescent genes levels and b-



galactosidase activity can aid in addressing this question.

The *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mouse model provides a genetic system to address the effect of p53 on other stem cells. *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice also suffer from cerebellar defects (Liu, Terzian et al. 2007). Since p53 induced such a dramatic defect on blood stem cells, is the same effect induced in neural stem cells? Are all normal stem cells susceptible to increased p53 activity in the absence of Mdm2 or is it just blood stem cells? Careful examination of other tissues in *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice would help resolve such questions. This is a pertinent discussion in the field as p53 is at the crossroads of aging, regenerative medicine, and cancer, all of which are correlated to stem cells.

Finally, the obvious c-kit downregulation in the bone marrow is quite an intriguing observation and requires further research. We believe that some HSC population may still reside in the bone marrows of *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> mice. However, this population would lack c-kit expression and hence be incapable of undergoing hematopoietic activity. Loss of c-kit expression could be due to p53 transactivation of genes that can inhibit c-kit transcription, leading to the latter's downregulation. To address whether p53 is a major player in c-kit downregulation, c-kit<sup>+</sup> cells from adult wildtype bone marrows can be sorted out. Subsequently, we can transfect p53 plasmid into these cells and monitor c-kit expression (RNA and protein) in these cells at various time points. c-kit is an oncogene in several hematological cancers and such a correlation with p53 can shed more light on the role of p53 in leukemia formation and treatment.

## EXPERIMENTAL PROCEDURES

**Mice:** We used previously described *Mdm2*<sup>+/-</sup> (Montes de Oca Luna, Wagner et al. 1995) and *p53*<sup>515C/515C</sup> (Liu, Parant et al. 2004) mice to make our *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> controls. These mice were bred to each other to generate *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup>, *Mdm2*<sup>+/+</sup> *p53*<sup>515C/515C</sup> and *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> littermates. Breeding cages were checked at least twice daily to monitor survival of pups. The day a plug was detected in females was designated as E0.5 and the day a litter was born was designated as P0. All mice were at >90% C57BL/6 background as determined by marker analysis by Genetic Services at UT MD Anderson Cancer Center, Smithville. For genotyping, we extracted DNA from tail snips and PCR reaction was performed as previously described for *p53*<sup>515C</sup> (Liu, Parant et al. 2004), *Mdm2* (Montes de Oca Luna, Wagner et al. 1995), *p16* (Sharpless, Bardeesy et al. 2001) and *p21* (Brugarolas, Bronson et al. 1998). For antioxidant injections, NAC (A7250, Sigma-Aldrich, MO, USA) was prepared in phosphate buffered saline and injected at P3 and P5 at 1mg/g using a 28 gauge needle around the groin region. Pups were sacrificed at P6. All animal studies were approved by the Institutional Animal Care and Use Committee.

**Transplantation experiments:** C57/B6 CD45.1+ mice were irradiated with a split dose of 5.5 Gy 4 hours apart. Two hours after the second irradiation, 0.5x10<sup>6</sup> cells whole bone marrow cells from P6 *Mdm2*<sup>-/-</sup> *p53*<sup>515C/515C</sup> and *Mdm2*<sup>+/-</sup> *p53*<sup>515C/515C</sup> mice were injected retroorbitally into isofluorane-sedated mice. Mice were monitored for survival on daily basis. For transplantation control, we used 0.5x10<sup>6</sup> cells from adult C57/B6 CD45.2 wildtype bone marrows as donors. For irradiation

control, two mice were irradiated without transplantation for each experiment.

**Histology and immunostaining of hematopoietic tissues:** Pregnant females were sacrificed at designated time points. Neonates were decapitated. Tissues were either fixed in formalin for 48 hours then paraffin embedded for histological studies, or processed for RNA isolation and stem cell analysis as described below. For tissues, slides were sectioned at 6  $\mu$ m and stained with Hematoxylin & Eosin for histology by the Department of Veterinary Medicine and Surgery Histology at UT MD Anderson Cancer Center, Houston. For immunohistochemistry, we used an antibody to p53 (#CM5, Vector Laboratories, CA, USA) at 1:500, p16 (#M-156, Santa Cruz Biotechnology, CA, USA) at 1:50 and cleaved caspase-3 (#9661, Cell Signaling, MA, USA) at 1:200 as previously described procedure (Post, Quintas-Cardama et al. 2009). Hematoxylin (#H-3401, Vector Laboratories, Inc., CA, USA) was used as counterstain.

**RNA analysis:** E14.5 fetal livers, and P6 and P10 bone marrows were homogenized and RNA was extracted using TRIzol (#155-96-026, Invitrogen, MD, USA) and treated with DNase (#04716728001, Roche, IN, USA) as previously described (Jackson and Pereira-Smith 2006). First-strand DNA was generated by using First-strand cDNA synthesis kit from (#27-9261-01, GE Healthcare, NJ, USA). Bone marrows from two littermates of the same genotype were combined to provide an adequate amount of tissue to produce sufficient RNA for the experiment. Three combined pairs of littermates and one single mouse were used to generate an N of 4 some experiments. Primer sequences are in Supplementary Table 2. All levels were normalized to *Gapdh* levels.

**Tissue homogenization and flow cytometry:** After sacrificing the mice, tissues

were washed with Hanks' Balanced Salt Solution (HBSS) (#21-022-CV, Mediatech, Inc., VA, USA) supplemented with 2% Fetal Bovine Serum (#S11150, Atlanta Biologicals, GA, USA), 10mM Hepes Buffer (#25-060, Mediatech, Inc., VA, USA) and 1x Penicillin-streptomycin solution (#30-002, Mediatech, Inc., VA, USA), hence termed HBSS+. Then cells were kept in Dulbecco's Modified Eagle Medium (DMEM) (#10-107, Mediatech, Inc., VA, USA) with supplements as described for HBSS+. Fetal livers were homogenized with 181/2 G needle and passed through a 40 µm filter (#342340, BD Falcon, NJ, USA). Bones from four limbs were gently isolated by removing any muscle or cartilage. Then, bone marrows were finely chopped, passed through 18G/12 needle and finally filtered through a 40µm filter. All cellular suspensions were kept on ice until further processing. For flow cytometry, murine HSC and MPP were identified using eBioscience (CA, USA) fluorochrome conjugated antibodies against ckit (PE\_Cy7), Sca-1 (APC), and for lineage depletion we used CD4 (PE), CD8a (PE), B220 (PE), Ter119 (PE) and Gr-1 (PE) in HBSS+. Mac-1 was not included in the lineage depletion cocktail as it is expressed in HSCs during late embryogenesis and early postnatal time points (Bowie, McKnight et al. 2006). For SLAMF7, we used CD48 (PE, eBioscience), CD150 (PE\_Cy7, Biolegend) and c-kit (APC\_H7, BDbioscience) in combination with Sca1 (APC, ebioscience) and Lin- markers. Cells were stained for 20 minutes in the dark on ice then washed with HBSS+ before analysis on BD LSRII System or BD FACSARIA at UT MD Anderson Cancer Center Flow Cytometry Core Facility. Dead cells were excluded with 7AAD or DAPI staining (eBioscience, CA, USA).

**Cell cycle analysis:** For cell cycle, tissues were fixed with 70% ethanol and kept at 4 degrees until stained with propidium iodide (#P3566, Invitrogen, MD, USA).

Samples were analyzed on BD FACSCalibur System. Data were analyzed on FlowJo.

**AnnexinV staining:** E14.5 fetal livers and P6 bone marrows were stained with FITC-conjugated Annexin V using ApoAlert Annexin V Apoptosis Kit (#630109, Clontech, CA, USA) according to manufacturer's protocol. Briefly, cells were washed with the kit's Staining Buffer for 2 minutes on ice, then incubated with Annexin V-FITC for 20 minutes on ice before running on BD LSRII System. For Annexin V staining on LKS, cells were stained for LKS markers first and then for Annexin V. Data were analyzed on FlowJo.

**ROS levels:** For measurement of ROS levels, we used 2',7'-dichlorodihydrofluorescein diacetate (2',7'-dichlorofluorescein diacetate; H2DCFDA) (#D-399, Invitrogen, MD, USA). 1  $\mu$ M of DCFDA in DMSO was freshly prepared for each experiment. Then the stock was diluted in HBSS+ in 1:1000 factor for a final concentration of 1 nM DCFDA. After LKS staining, cells were stained with 1 nM DCFDA in HBSS+ at 37°C in the dark for 20 minutes. Cells were then washed and resuspended with HBSS+ at 37°C for recovery. Emission at 488nm wavelength was analyzed on BD FACSAria. Data were analyzed using FlowJo.

**Colony forming unit assay:** After homogenizing tissues, live cells were counted with trypan blue. 20,000 cells were plated from each tissue, grown in methylcellulose media (#MethoCult M3434, STEMCELL Technologies, BC, Canada) and maintained in a mini-humidity chamber in 20% or 3% oxygen tissue culture incubators. Colonies were counted within two weeks after plating and identified based on morphology, specifically for CFU-GM, BFU-E and GEMM/Mixed colonies. In some experiments, NAC was added every three days at a final concentration of 1 mM.

**Alkaline phosphatase staining:** Whole bone marrow cells from P6 mice were plated on 6 well plates in  $\alpha$ MEM media supplemented with 15% FBS. After 10 days, plates were washed and stained with Leukocyte Alkaline Phosphatase Kit (#86R, Sigma-Aldrich, MO, USA) as in manufacturer's manual. Red dye deposition indicated alkaline phosphatase activity.

**ChIP assay:** For ChIP, DNA was prepared as previously described (REFERENCE). Briefly, wildtype mice were irradiated at 6Gy then intestines were collected four hours later. CM5 antibody was used to pull-down p53-bound to DNA. Levels of DNA binding was measured using RT-PCR. Primer sequences are in Table 3.

**Statistics:** Two-way t-test and Kaplan-Meier analyses were performed to assess statistical difference. A factor was considered statistically significant if it had a P-value of <0.05.

**Table 2.** List of primers used for Real Time – PCR experiments.

<b>Gene</b>	<b><i>Forward primer</i></b>	<b><i>Reverse primer</i></b>
<i>Ccng1</i>	<i>gcgaagcatcttgggtgtgt</i>	<i>tcctttcctcttcagtcgcttt</i>
<i>Dcr2</i>	<i>agctaaccagcccataatcgtc</i>	<i>agttcccttctgacaggactggc</i>
<i>Dec1</i>	<i>ggagacctgtcagggatgga</i>	<i>cccgcctggactgtacact</i>
<i>Gapdh</i>	<i>tcaccacatggagaaggc</i>	<i>gctaagcagttggtggtgca</i>
<i>Noxa</i>	<i>tcgcaaaagagcaggatgag</i>	<i>cactttgtctccaatcctccg</i>
<i>p15</i>	<i>agatcccaacgccctgaac</i>	<i>cccatcatcatgacctggatt</i>
<i>p16</i>	<i>gccgcaccggaatcct</i>	<i>tgcaccgtagttgagcagaaga</i>
<i>p21</i>	<i>caggcaccatgtccaatcct</i>	<i>gagacaacggcacactttgct</i>
<i>Pig1</i>	<i>tggacacttccgaggttgtct</i>	<i>cctcacggccccatttg</i>
<i>Pig8</i>	<i>ggcatctgtaccatctcaaagct</i>	<i>tcgacgctgttcctctctcttc</i>
<i>Pig12</i>	<i>ggcttgggatccagagatgtc</i>	<i>gtggcagagctgcagaaagaa</i>
<i>Perp</i>	<i>gctgcagccacgcttttc</i>	<i>ggcgaagaacgagagaaatgaa</i>
<i>Pml</i>	<i>gcacccgctgcaaagg</i>	<i>tgagctgttcacactcgaaaca</i>
<i>Puma</i>	<i>gcggcggagacaagaaga</i>	<i>agtcccatgaagagattgtacatgac</i>

**Table 3.** List of primers used to span c-kit promoter. RE designates “Putative Response Element” region.

<b>Primer</b>	<b>Forward primer</b>	<b>Reverse primer</b>
<b>ckitRE 1</b>	CTCCAGGTGCGCTATGCA	TGGGTGCTTTGCCTGTTTCT
<b>ckitRE 2</b>	TGTAGCGCCAGCACTTGTG	AGCTGAGGATGGCTTTGAACTC
<b>ckitRE 3</b>	CCAACAGAGCAACACAAAGCA	GAATAGGTTTCCCCCTCCATCT
<b>ckitRE 4</b>	GCGCAGCGTTCAACCTGTA	CCTGAGACACCCACCTCACA
<b>ckitRE 5</b>	CAGGGCTCCCATCTCAGATC	AGCGAGGCACTGTTAGTAGATGT G
<b>ckitRE 8</b>	TGGAGAAACTGAGCATGAAAAA TT	GCACCCCTGACCTCAGTAAAAG
<b>ckitRE 9</b>	CCGGTGGTTGTCCTTTATTGTC	GCCACGAGCGCATTAGGTA
<b>Puma</b>	GGACGGTCGCCTTGCA	CACCTTAGTCCCAGTGATGAAA
<b>AchR</b>	FCCTCCCCCAACTCCACTTTT	GGAGGTTGGAGGGAGAAGGA



## BIBLIOGRAPHY

- Aihara, Y., H. J. Buhring, M. Aihara and J. Klein (1986). "An attempt to produce "pre-T" cell hybridomas and to identify their antigens." Eur J Immunol **16**(11): 1391-9.
- Akala, O. O., I. K. Park, D. Qian, M. Pihajla, M. W. Becker and M. F. Clarke (2008). "Long-term haematopoietic reconstitution by Trp53(-)/(-)p16(Ink4a)(-/-)p19(Arf)(-/-) multipotent progenitors." Nature **453**(7192): 228-232.
- Allton, K., A. K. Jain, H. M. Herz, W. W. Tsai, S. Y. Jung, J. Qin, A. Bergmann, R. L. Johnson and M. C. Barton (2009). "Trim24 targets endogenous p53 for degradation." Proc Natl Acad Sci U S A **106**(28): 11612-6.
- Appella, E. and C. W. Anderson (2001). "Post-translational modifications and activation of p53 by genotoxic stresses." Eur J Biochem **268**(10): 2764-72.
- Baker, S. J., E. R. Fearon, J. M. Nigro, S. R. Hamilton, A. C. Preisinger, J. M. Jessup, P. vanTuinen, D. H. Ledbetter, D. F. Barker, Y. Nakamura, R. White and B. Vogelstein (1989). "Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas." Science **244**(4901): 217-21.
- Barak, Y., T. Juven, R. Haffner and M. Oren (1993). "mdm2 expression is induced by wild type p53 activity." EMBO J **12**(2): 461-8.
- Barboza, J. A., T. Iwakuma, T. Terzian, A. K. El-Naggar and G. Lozano (2008). "Mdm2 and Mdm4 loss regulates distinct p53 activities." Mol Cancer Res **6**(6): 947-54.

- Bargonetti, J., P. N. Friedman, S. E. Kern, B. Vogelstein and C. Prives (1991). "Wild-type but not mutant p53 immunopurified proteins bind to sequences adjacent to the SV40 origin of replication." Cell **65**(6): 1083-91.
- Barnes, D. W. and J. F. Loutit (1953). "Protective effects of implants of splenic tissue." Proc R Soc Med **46**(4): 251-2.
- Barnes, D. W. and J. F. Loutit (1967). "Haemopoietic stem cells in the peripheral blood." Lancet **2**(7526): 1138-41.
- Beausejour, C. M., A. Krtolica, F. Galimi, M. Narita, S. W. Lowe, P. Yaswen and J. Campisi (2003). "Reversal of human cellular senescence: roles of the p53 and p16 pathways." EMBO J **22**(16): 4212-22.
- Becker, A. J., E. A. McCulloch and J. E. Till (1963). "Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells." Nature **197**: 452-4.
- Berman, J. W. and R. S. Basch (1985). "Thy-1 antigen expression by murine hematopoietic precursor cells." Exp Hematol **13**(11): 1152-6.
- Bienz, B., R. Zakut-Houri, D. Givol and M. Oren (1984). "Analysis of the gene coding for the murine cellular tumour antigen p53." EMBO J **3**(9): 2179-83.
- Bond, J. A., F. S. Wyllie and D. Wynford-Thomas (1994). "Escape from senescence in human diploid fibroblasts induced directly by mutant p53." Oncogene **9**(7): 1885-9.
- Bonnet, D. and J. E. Dick (1997). "Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell." Nat Med **3**(7): 730-7.
- Bowie, M. B., K. D. McKnight, D. G. Kent, L. McCaffrey, P. A. Hoodless and C. J. Eaves (2006). "Hematopoietic stem cells proliferate until after birth and show

- a reversible phase-specific engraftment defect." J Clin Invest **116**(10): 2808-16.
- Brosh, R. and V. Rotter (2009). "When mutants gain new powers: news from the mutant p53 field." Nat Rev Cancer **9**(10): 701-13.
- Chen, C., Y. Liu, R. Liu, T. Ikenoue, K. L. Guan and P. Zheng (2008). "TSC-mTOR maintains quiescence and function of hematopoietic stem cells by repressing mitochondrial biogenesis and reactive oxygen species." J Exp Med **205**(10): 2397-408.
- Chen, C., Y. Liu and P. Zheng (2009). "The axis of mTOR-mitochondria-ROS and stemness of the hematopoietic stem cells." Cell Cycle **8**(8): 1158-60.
- Chen, L., D. M. Gilkes, Y. Pan, W. S. Lane and J. Chen (2005). "ATM and Chk2-dependent phosphorylation of MDMX contribute to p53 activation after DNA damage." EMBO J **24**(19): 3411-22.
- Christensen, J. L., D. E. Wright, A. J. Wagers and I. L. Weissman (2004). "Circulation and chemotaxis of fetal hematopoietic stem cells." PLoS Biol **2**(3): E75.
- Crawford, L. V., D. C. Pim, E. G. Gurney, P. Goodfellow and J. Taylor-Papadimitriou (1981). "Detection of a common feature in several human tumor cell lines--a 53,000-dalton protein." Proc Natl Acad Sci U S A **78**(1): 41-5.
- Curry, J. L., J. J. Trentin and N. Wolf (1967). "Hemopoietic spleen colony studies. II. Erythropoiesis." J Exp Med **125**(4): 703-20.
- Czosnek, H. H., B. Bienz, D. Givol, R. Zakut-Houri, D. D. Pravtcheva, F. H. Ruddle and M. Oren (1984). "The gene and the pseudogene for mouse p53 cellular tumor antigen are located on different chromosomes." Mol Cell Biol **4**(8): 1638-40.

- de Graaf, P., N. A. Little, Y. F. Ramos, E. Meulmeester, S. J. Letteboer and A. G. Jochemsen (2003). "Hdmx protein stability is regulated by the ubiquitin ligase activity of Mdm2." J Biol Chem **278**(40): 38315-24.
- DeLeo, A. B., G. Jay, E. Appella, G. C. Dubois, L. W. Law and L. J. Old (1979). "Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse." Proc Natl Acad Sci U S A **76**(5): 2420-4.
- Dennis, J. E., A. Merriam, A. Awadallah, J. U. Yoo, B. Johnstone and A. I. Caplan (1999). "A quadripotential mesenchymal progenitor cell isolated from the marrow of an adult mouse." J Bone Miner Res **14**(5): 700-9.
- Domen, J., A. Wagers and I. L. Weissman (2006). "Regenerative Medicine." NIH: 13-35.
- Donehower, L. A., M. Harvey, B. L. Slagle, M. J. McArthur, C. A. Montgomery, Jr., J. S. Butel and A. Bradley (1992). "Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours." Nature **356**(6366): 215-21.
- Dulic, V., W. K. Kaufmann, S. J. Wilson, T. D. Tlsty, E. Lees, J. W. Harper, S. J. Elledge and S. I. Reed (1994). "p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest." Cell **76**(6): 1013-23.
- Eilken, H. M., S. Nishikawa and T. Schroeder (2009). "Continuous single-cell imaging of blood generation from haemogenic endothelium." Nature **457**(7231): 896-900.
- el-Deiry, W. S., S. E. Kern, J. A. Pietenpol, K. W. Kinzler and B. Vogelstein (1992). "Definition of a consensus binding site for p53." Nat Genet **1**(1): 45-9.

- el-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler and B. Vogelstein (1993). "WAF1, a potential mediator of p53 tumor suppression." Cell **75**(4): 817-25.
- Eliyahu, D., A. Raz, P. Gruss, D. Givol and M. Oren (1984). "Participation of p53 cellular tumour antigen in transformation of normal embryonic cells." Nature **312**(5995): 646-9.
- Ema, H. and H. Nakauchi (2004). "'Homing to Niche," a new criterion for hematopoietic stem cells?" Immunity **20**(1): 1-2.
- Eriksson, J. W. (2007). "Metabolic stress in insulin's target cells leads to ROS accumulation - a hypothetical common pathway causing insulin resistance." FEBS Lett **581**(19): 3734-42.
- Fakharzadeh, S. S., S. P. Trusko and D. L. George (1991). "Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line." EMBO J **10**(6): 1565-9.
- Farley, F. W., P. Soriano, L. S. Steffen and S. M. Dymecki (2000). "Widespread recombinase expression using FLPeR (flipper) mice." Genesis **28**(3-4): 106-10.
- Finlay, C. A., P. W. Hinds and A. J. Levine (1989). "The p53 proto-oncogene can act as a suppressor of transformation." Cell **57**(7): 1083-93.
- Fridman, J. S. and S. W. Lowe (2003). "Control of apoptosis by p53." Oncogene **22**(56): 9030-40.
- Gekas, C., F. Dieterlen-Lievre, S. H. Orkin and H. K. Mikkola (2005). "The placenta is a niche for hematopoietic stem cells." Dev Cell **8**(3): 365-75.

- Gu, J., H. Kawai, L. Nie, H. Kitao, D. Wiederschain, A. G. Jochemsen, J. Parant, G. Lozano and Z. M. Yuan (2002). "Mutual dependence of MDM2 and MDMX in their functional inactivation of p53." J Biol Chem **277**(22): 19251-4.
- Harvey, M., M. J. McArthur, C. A. Montgomery, Jr., J. S. Butel, A. Bradley and L. A. Donehower (1993). "Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice." Nat Genet **5**(3): 225-9.
- Haupt, Y., Y. Barak and M. Oren (1996). "Cell type-specific inhibition of p53-mediated apoptosis by mdm2." EMBO J **15**(7): 1596-606.
- Haupt, Y., R. Maya, A. Kazaz and M. Oren (1997). "Mdm2 promotes the rapid degradation of p53." Nature **387**(6630): 296-9.
- Hermeking, H., C. Lengauer, K. Polyak, T. C. He, L. Zhang, S. Thiagalingam, K. W. Kinzler and B. Vogelstein (1997). "14-3-3 sigma is a p53-regulated inhibitor of G2/M progression." Mol Cell **1**(1): 3-11.
- Hollstein, M., B. Shomer, M. Greenblatt, T. Soussi, E. Hovig, R. Montesano and C. C. Harris (1996). "Somatic point mutations in the p53 gene of human tumors and cell lines: updated compilation." Nucleic Acids Res **24**(1): 141-6.
- Honda, R., H. Tanaka and H. Yasuda (1997). "Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53." FEBS Lett **420**(1): 25-7.
- Ito, K., A. Hirao, F. Arai, S. Matsuoka, K. Takubo, I. Hamaguchi, K. Nomiyama, K. Hosokawa, K. Sakurada, N. Nakagata, Y. Ikeda, T. W. Mak and T. Suda (2004). "Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells." Nature **431**(7011): 997-1002.
- Jacobson, L. O., E. K. Marks and et al. (1949). "The role of the spleen in radiation injury." Proc Soc Exp Biol Med **70**(4): 740-2.

- Jacobson, L. O., E. L. Simmons, E. K. Marks and J. H. Eldredge (1951). "Recovery from radiation injury." Science **113**(2940): 510-11.
- Jain, A. K. and M. C. Barton (2009). "Regulation of p53: TRIM24 enters the RING." Cell Cycle **8**(22): 3668-74.
- Janzen, V., R. Forkert, H. E. Fleming, Y. Saito, M. T. Waring, D. M. Dombkowski, T. Cheng, R. A. DePinho, N. E. Sharpless and D. T. Scadden (2006). "Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a." Nature **443**(7110): 421-6.
- Jeffrey, P. D., S. Gorina and N. P. Pavletich (1995). "Crystal structure of the tetramerization domain of the p53 tumor suppressor at 1.7 angstroms." Science **267**(5203): 1498-502.
- Jenkins, J. R., P. Chumakov, C. Addison, H. W. Sturzbecher and A. Wade-Evans (1988). "Two distinct regions of the murine p53 primary amino acid sequence are implicated in stable complex formation with simian virus 40 T antigen." J Virol **62**(10): 3903-6.
- Jenkins, J. R., K. Rudge and G. A. Currie (1984). "Cellular immortalization by a cDNA clone encoding the transformation-associated phosphoprotein p53." Nature **312**(5995): 651-4.
- Jenkins, J. R., K. Rudge, S. Redmond and A. Wade-Evans (1984). "Cloning and expression analysis of full length mouse cDNA sequences encoding the transformation associated protein p53." Nucleic Acids Res **12**(14): 5609-26.
- Joerger, A. C. and A. R. Fersht (2008). "Structural biology of the tumor suppressor p53." Annu Rev Biochem **77**: 557-82.

- Jones, S. N., A. E. Roe, L. A. Donehower and A. Bradley (1995). "Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53." Nature **378**(6553): 206-8.
- Junttila, M. R. and G. I. Evan (2009). "p53--a Jack of all trades but master of none." Nat Rev Cancer **9**(11): 821-9.
- Kastan, M. B., O. Onyekwere, D. Sidransky, B. Vogelstein and R. W. Craig (1991). "Participation of p53 protein in the cellular response to DNA damage." Cancer Res **51**(23 Pt 1): 6304-11.
- Kent, D., M. Copley, C. Benz, B. Dykstra, M. Bowie and C. Eaves (2008). "Regulation of hematopoietic stem cells by the steel factor/KIT signaling pathway." Clin Cancer Res **14**(7): 1926-30.
- Kern, S. E., K. W. Kinzler, A. Bruskin, D. Jarosz, P. Friedman, C. Prives and B. Vogelstein (1991). "Identification of p53 as a sequence-specific DNA-binding protein." Science **252**(5013): 1708-11.
- Khetchoumian, K., M. Teletin, J. Tisserand, M. Mark, B. Herquel, M. Ignat, J. Zucman-Rossi, F. Cammas, T. Lerouge, C. Thibault, D. Metzger, P. Chambon and R. Losson (2007). "Loss of Trim24 (Tif1alpha) gene function confers oncogenic activity to retinoic acid receptor alpha." Nat Genet **39**(12): 1500-6.
- Khosravi, R., R. Maya, T. Gottlieb, M. Oren, Y. Shiloh and D. Shkedy (1999). "Rapid ATM-dependent phosphorylation of MDM2 precedes p53 accumulation in response to DNA damage." Proc Natl Acad Sci U S A **96**(26): 14973-7.
- Kiel, M. J., O. H. Yilmaz, T. Iwashita, O. H. Yilmaz, C. Terhorst and S. J. Morrison (2005). "SLAM family receptors distinguish hematopoietic stem and



- progenitor cells and reveal endothelial niches for stem cells." Cell **121**(7): 1109-21.
- Knudson, A. G., Jr. (1971). "Mutation and cancer: statistical study of retinoblastoma." Proc Natl Acad Sci U S A **68**(4): 820-3.
- Ko, L. J. and C. Prives (1996). "p53: puzzle and paradigm." Genes Dev **10**(9): 1054-72.
- Kopp, H. G., S. T. Avecilla, A. T. Hooper and S. Rafii (2005). "The bone marrow vascular niche: home of HSC differentiation and mobilization." Physiology (Bethesda) **20**: 349-56.
- Kranc, K. R., H. Schepers, N. P. Rodrigues, S. Bamforth, E. Villadsen, H. Ferry, T. Bouriez-Jones, M. Sigvardsson, S. Bhattacharya, S. E. Jacobsen and T. Enver (2009). "Cited2 is an essential regulator of adult hematopoietic stem cells." Cell Stem Cell **5**(6): 659-65.
- Kress, M., E. May, R. Cassingena and P. May (1979). "Simian virus 40-transformed cells express new species of proteins precipitable by anti-simian virus 40 tumor serum." J Virol **31**(2): 472-83.
- Kroemer, G. and J. C. Reed (2000). "Mitochondrial control of cell death." Nat Med **6**(5): 513-9.
- Krummel, K. A., C. J. Lee, F. Toledo and G. M. Wahl (2005). "The C-terminal lysines fine-tune P53 stress responses in a mouse model but are not required for stability control or transactivation." Proc Natl Acad Sci U S A **102**(29): 10188-93.
- Kruse, J. P. and W. Gu (2009). "Modes of p53 regulation." Cell **137**(4): 609-22.
- Kubbutat, M. H., S. N. Jones and K. H. Vousden (1997). "Regulation of p53 stability by Mdm2." Nature **387**(6630): 299-303.

- Lancrin, C., P. Sroczyńska, C. Stephenson, T. Allen, V. Kouskoff and G. Lacaud (2009). "The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage." Nature **457**(7231): 892-5.
- Lane, D. P. (1984). "Cell immortalization and transformation by the p53 gene." Nature **312**(5995): 596-7.
- Lane, D. P. (1992). "Cancer. p53, guardian of the genome." Nature **358**(6381): 15-6.
- Lane, D. P. and L. V. Crawford (1979). "T antigen is bound to a host protein in SV40-transformed cells." Nature **278**(5701): 261-3.
- Lavigne, A., V. Maltby, D. Mock, J. Rossant, T. Pawson and A. Bernstein (1989). "High incidence of lung, bone, and lymphoid tumors in transgenic mice overexpressing mutant alleles of the p53 oncogene." Mol Cell Biol **9**(9): 3982-91.
- Lee, J. M. and A. Bernstein (1993). "p53 mutations increase resistance to ionizing radiation." Proc Natl Acad Sci U S A **90**(12): 5742-6.
- Lee, S. H. and J. DeJong (1999). "Microsomal GST-I: genomic organization, expression, and alternative splicing of the human gene." Biochim Biophys Acta **1446**(3): 389-96.
- Lehar, S. M., M. Nacht, T. Jacks, C. A. Vater, T. Chittenden and B. C. Guild (1996). "Identification and cloning of El24, a gene induced by p53 in etoposide-treated cells." Oncogene **12**(6): 1181-7.
- Levine, A. J. (1997). "p53, the cellular gatekeeper for growth and division." Cell **88**(3): 323-31.

- Lewandoski, M., K. M. Wassarman and G. R. Martin (1997). "Zp3-cre, a transgenic mouse line for the activation or inactivation of loxP-flanked target genes specifically in the female germ line." Curr Biol **7**(2): 148-51.
- Lewis, J. L., W. Chinswangwatanakul, B. Zheng, S. B. Marley, D. X. Nguyen, N. C. Cross, L. Banerji, J. Glassford, N. S. Thomas, J. M. Goldman, E. W. Lam and M. Y. Gordon (2001). "The influence of INK4 proteins on growth and self-renewal kinetics of hematopoietic progenitor cells." Blood **97**(9): 2604-10.
- Linzer, D. I. and A. J. Levine (1979). "Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells." Cell **17**(1): 43-52.
- Liu, G., J. M. Parant, G. Lang, P. Chau, A. Chavez-Reyes, A. K. El-Naggar, A. Multani, S. Chang and G. Lozano (2004). "Chromosome stability, in the absence of apoptosis, is critical for suppression of tumorigenesis in Trp53 mutant mice." Nat Genet **36**(1): 63-8.
- Liu, G., T. Terzian, S. Xiong, C. S. Van Pelt, A. Audiffred, N. F. Box and G. Lozano (2007). "The p53-Mdm2 network in progenitor cell expansion during mouse postnatal development." J Pathol **213**(4): 360-8.
- Liu, J., L. Cao, J. Chen, S. Song, I. H. Lee, C. Quijano, H. Liu, K. Keyvanfar, H. Chen, L. Y. Cao, B. H. Ahn, N. G. Kumar, Rovira, II, X. L. Xu, M. van Lohuizen, N. Motoyama, C. X. Deng and T. Finkel (2009). "Bmi1 regulates mitochondrial function and the DNA damage response pathway." Nature **459**(7245): 387-92.
- Liu, Y., S. E. Elf, Y. Miyata, G. Sashida, G. Huang, S. Di Giandomenico, J. M. Lee, A. Deblasio, S. Menendez, J. Antipin, B. Reva, A. Koff and S. D. Nimer

- (2009). "p53 regulates hematopoietic stem cell quiescence." Cell Stem Cell **4**(1): 37-48.
- Livingstone, L. R., A. White, J. Sprouse, E. Livanos, T. Jacks and T. D. Tlsty (1992). "Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53." Cell **70**(6): 923-35.
- Lowe, S. W., E. M. Schmitt, S. W. Smith, B. A. Osborne and T. Jacks (1993). "p53 is required for radiation-induced apoptosis in mouse thymocytes." Nature **362**(6423): 847-9.
- Ludwig, R. L., S. Bates and K. H. Vousden (1996). "Differential activation of target cellular promoters by p53 mutants with impaired apoptotic function." Mol Cell Biol **16**(9): 4952-60.
- Maecker, H. L., C. Koumenis and A. J. Giaccia (2000). "p53 promotes selection for Fas-mediated apoptotic resistance." Cancer Res **60**(16): 4638-44.
- Malkin, D., F. P. Li, L. C. Strong, J. F. Fraumeni, Jr., C. E. Nelson, D. H. Kim, J. Kassel, M. A. Gryka, F. Z. Bischoff, M. A. Tainsky and et al. (1990). "Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms." Science **250**(4985): 1233-8.
- Maltzman, W. and L. Czyzyk (1984). "UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells." Mol Cell Biol **4**(9): 1689-94.
- Marine, J. C., S. Francoz, M. Maetens, G. Wahl, F. Toledo and G. Lozano (2006). "Keeping p53 in check: essential and synergistic functions of Mdm2 and Mdm4." Cell Death Differ **13**(6): 927-34.
- Marine, J. C. and G. Lozano (2009). "Mdm2-mediated ubiquitylation: p53 and beyond." Cell Death Differ **17**(1): 93-102.

- Masson, K. and L. Ronnstrand (2009). "Oncogenic signaling from the hematopoietic growth factor receptors c-Kit and Flt3." Cell Signal **21**(12): 1717-26.
- McCulloch, E. A., L. Siminovitch and J. E. Till (1964). "Spleen-Colony Formation in Anemic Mice of Genotype Ww." Science **144**: 844-6.
- McKinney, K., M. Mattia, V. Gottifredi and C. Prives (2004). "p53 linear diffusion along DNA requires its C terminus." Mol Cell **16**(3): 413-24.
- Medvinsky, A. L., N. L. Samoylina, A. M. Muller and E. A. Dzierzak (1993). "An early pre-liver intraembryonic source of CFU-S in the developing mouse." Nature **364**(6432): 64-7.
- Meek, D. W. (1994). "Post-translational modification of p53." Semin Cancer Biol **5**(3): 203-10.
- Meroni, G. and G. Diez-Roux (2005). "TRIM/RBCC, a novel class of 'single protein RING finger' E3 ubiquitin ligases." Bioessays **27**(11): 1147-57.
- Midgley, C. A., B. Owens, C. V. Briscoe, D. B. Thomas, D. P. Lane and P. A. Hall (1995). "Coupling between gamma irradiation, p53 induction and the apoptotic response depends upon cell type in vivo." J Cell Sci **108 ( Pt 5)**: 1843-8.
- Mikkola, H. K. and S. H. Orkin (2006). "The journey of developing hematopoietic stem cells." Development **133**(19): 3733-44.
- Miller, C. L., B. Dykstra and C. J. Eaves (2008). "Characterization of mouse hematopoietic stem and progenitor cells." Curr Protoc Immunol **Chapter 22**: Unit 22B 2.
- Miyamoto, T., I. L. Weissman and K. Akashi (2000). "AML1/ETO-expressing nonleukemic stem cells in acute myelogenous leukemia with 8;21 chromosomal translocation." Proc Natl Acad Sci U S A **97**(13): 7521-6.

- Miyashita, T., S. Krajewski, M. Krajewska, H. G. Wang, H. K. Lin, D. A. Liebermann, B. Hoffman and J. C. Reed (1994). "Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo." Oncogene **9**(6): 1799-805.
- Molchadsky, A., I. Shats, N. Goldfinger, M. Pevsner-Fischer, M. Olson, A. Rinon, E. Tzahor, G. Lozano, D. Zipori, R. Sarig and V. Rotter (2008). "p53 plays a role in mesenchymal differentiation programs, in a cell fate dependent manner." PLoS One **3**(11): e3707.
- Momand, J., G. P. Zambetti, D. C. Olson, D. George and A. J. Levine (1992). "The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation." Cell **69**(7): 1237-45.
- Montes de Oca Luna, R., D. S. Wagner and G. Lozano (1995). "Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53." Nature **378**(6553): 203-6.
- Mosner, J., T. Mummenbrauer, C. Bauer, G. Sczakiel, F. Grosse and W. Deppert (1995). "Negative feedback regulation of wild-type p53 biosynthesis." EMBO J **14**(18): 4442-9.
- Naka, K., T. Muraguchi, T. Hoshii and A. Hirao (2008). "Regulation of reactive oxygen species and genomic stability in hematopoietic stem cells." Antioxid Redox Signal **10**(11): 1883-94.
- Nakano, K. and K. H. Vousden (2001). "PUMA, a novel proapoptotic gene, is induced by p53." Mol Cell **7**(3): 683-94.
- Naveiras, O., V. Nardi, P. L. Wenzel, P. V. Hauschka, F. Fahey and G. Q. Daley (2009). "Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment." Nature **460**(7252): 259-63.

- Nigro, J. M., S. J. Baker, A. C. Preisinger, J. M. Jessup, R. Hostetter, K. Cleary, S. H. Bigner, N. Davidson, S. Baylin, P. Devilee and et al. (1989). "Mutations in the p53 gene occur in diverse human tumour types." Nature **342**(6250): 705-8.
- Oda, E., R. Ohki, H. Murasawa, J. Nemoto, T. Shibue, T. Yamashita, T. Tokino, T. Taniguchi and N. Tanaka (2000). "Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis." Science **288**(5468): 1053-8.
- Okada, S., H. Nakauchi, K. Nagayoshi, S. Nishikawa, Y. Miura and T. Suda (1991). "Enrichment and characterization of murine hematopoietic stem cells that express c-kit molecule." Blood **78**(7): 1706-12.
- Orkin, S. H. and L. I. Zon (2008). "Hematopoiesis: an evolving paradigm for stem cell biology." Cell **132**(4): 631-44.
- Owen-Schaub, L. B., W. Zhang, J. C. Cusack, L. S. Angelo, S. M. Santee, T. Fujiwara, J. A. Roth, A. B. Deisseroth, W. W. Zhang, E. Kruzel and et al. (1995). "Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression." Mol Cell Biol **15**(6): 3032-40.
- Owusu-Ansah, E. and U. Banerjee (2009). "Reactive oxygen species prime *Drosophila* haematopoietic progenitors for differentiation." Nature **461**(7263): 537-41.
- Parada, L. F., H. Land, R. A. Weinberg, D. Wolf and V. Rotter (1984). "Cooperation between gene encoding p53 tumour antigen and ras in cellular transformation." Nature **312**(5995): 649-51.
- Parant, J., A. Chavez-Reyes, N. A. Little, W. Yan, V. Reinke, A. G. Jochemsen and G. Lozano (2001). "Rescue of embryonic lethality in Mdm4-null mice by loss

- of Trp53 suggests a nonoverlapping pathway with MDM2 to regulate p53." Nat Genet **29**(1): 92-5.
- Park, I. K., D. Qian, M. Kiel, M. W. Becker, M. Pihalja, I. L. Weissman, S. J. Morrison and M. F. Clarke (2003). "Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells." Nature **423**(6937): 302-5.
- Passegue, E., C. H. Jamieson, L. E. Ailles and I. L. Weissman (2003). "Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics?" Proc Natl Acad Sci U S A **100 Suppl 1**: 11842-9.
- Passegue, E., A. J. Wagers, S. Giuriato, W. C. Anderson and I. L. Weissman (2005). "Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates." J Exp Med **202**(11): 1599-611.
- Perry, M. E. (2010). "The Regulation of the p53-mediated Stress Response by MDM2 and MDM4." Cold Spring Harb Perspect Biol **2**(1): a000968.
- Pittenger, M. F., A. M. Mackay, S. C. Beck, R. K. Jaiswal, R. Douglas, J. D. Mosca, M. A. Moorman, D. W. Simonetti, S. Craig and D. R. Marshak (1999). "Multilineage potential of adult human mesenchymal stem cells." Science **284**(5411): 143-7.
- Polyak, K., Y. Xia, J. L. Zweier, K. W. Kinzler and B. Vogelstein (1997). "A model for p53-induced apoptosis." Nature **389**(6648): 300-5.
- Post, S. M., A. Quintas-Cardama, T. Terzian, C. Smith, C. M. Eischen and G. Lozano (2009). "p53-dependent senescence delays Emu-myc-induced B-cell lymphomagenesis." Oncogene.
- Prives, C. and P. A. Hall (1999). "The p53 pathway." J Pathol **187**(1): 112-26.



- Raycroft, L., H. Y. Wu and G. Lozano (1990). "Transcriptional activation by wild-type but not transforming mutants of the p53 anti-oncogene." Science **249**(4972): 1049-51.
- Ringshausen, I., C. C. O'Shea, A. J. Finch, L. B. Swigart and G. I. Evan (2006). "Mdm2 is critically and continuously required to suppress lethal p53 activity in vivo." Cancer Cell **10**(6): 501-14.
- Rother, K., R. Kirschner, K. Sanger, L. Bohlig, J. Mossner and K. Engeland (2007). "p53 downregulates expression of the G1/S cell cycle phosphatase Cdc25A." Oncogene **26**(13): 1949-53.
- Rowan, S., R. L. Ludwig, Y. Haupt, S. Bates, X. Lu, M. Oren and K. H. Vousden (1996). "Specific loss of apoptotic but not cell-cycle arrest function in a human tumor derived p53 mutant." EMBO J **15**(4): 827-38.
- Saga, Y., J. S. Lee, C. Saraiya and E. A. Boyse (1990). "Regulation of alternative splicing in the generation of isoforms of the mouse Ly-5 (CD45) glycoprotein." Proc Natl Acad Sci U S A **87**(10): 3728-32.
- Saga, Y., J. S. Tung, F. W. Shen and E. A. Boyse (1986). "Sequences of Ly-5 cDNA: isoform-related diversity of Ly-5 mRNA." Proc Natl Acad Sci U S A **83**(18): 6940-4.
- Sah, V. P., L. D. Attardi, G. J. Mulligan, B. O. Williams, R. T. Bronson and T. Jacks (1995). "A subset of p53-deficient embryos exhibit exencephaly." Nat Genet **10**(2): 175-80.
- Schofield, R. (1978). "The relationship between the spleen colony-forming cell and the haemopoietic stem cell." Blood Cells **4**(1-2): 7-25.

- Shaulsky, G., N. Goldfinger, A. Peled and V. Rotter (1991). "Involvement of wild-type p53 in pre-B-cell differentiation in vitro." Proc Natl Acad Sci U S A **88**(20): 8982-6.
- Short, B., N. Brouard, R. Driessen and P. J. Simmons (2001). "Prospective isolation of stromal progenitor cells from mouse BM." Cytotherapy **3**(5): 407-8.
- Spangrude, G. J., S. Heimfeld and I. L. Weissman (1988). "Purification and characterization of mouse hematopoietic stem cells." Science **241**(4861): 58-62.
- Suda, T., F. Arai and A. Hirao (2005). "Hematopoietic stem cells and their niche." Trends Immunol **26**(8): 426-33.
- Terzian, T., Y. Wang, C. S. Van Pelt, N. F. Box, E. L. Travis and G. Lozano (2007). "Haploinsufficiency of Mdm2 and Mdm4 in tumorigenesis and development." Mol Cell Biol **27**(15): 5479-85.
- Thomas, E. D., H. L. Lochte, Jr. and J. W. Ferrebee (1959). "Irradiation of the entire body and marrow transplantation: some observations and comments." Blood **14**(1): 1-23.
- Thut, C. J., J. L. Chen, R. Klemm and R. Tjian (1995). "p53 transcriptional activation mediated by coactivators TAFII40 and TAFII60." Science **267**(5194): 100-4.
- Till, J. E. and E. A. McCulloch (1961). "A direct measurement of the radiation sensitivity of normal mouse bone marrow cells." Radiat Res **14**: 213-22.
- Toledo, F. and G. M. Wahl (2006). "Regulating the p53 pathway: in vitro hypotheses, in vivo veritas." Nat Rev Cancer **6**(12): 909-23.
- Trevisan, M., X. Q. Yan and N. N. Iscove (1996). "Cycle initiation and colony formation in culture by murine marrow cells with long-term reconstituting potential in vivo." Blood **88**(11): 4149-58.

- Venot, C., M. Maratrat, C. Dureuil, E. Conseiller, L. Bracco and L. Debussche (1998). "The requirement for the p53 proline-rich functional domain for mediation of apoptosis is correlated with specific PIG3 gene transactivation and with transcriptional repression." EMBO J **17**(16): 4668-79.
- Vilborg, A., J. A. Glahder, M. T. Wilhelm, C. Bersani, M. Corcoran, S. Mahmoudi, M. Rosenstjerne, D. Grander, M. Farnebo, B. Norrild and K. G. Wiman (2009). "The p53 target Wig-1 regulates p53 mRNA stability through an AU-rich element." Proc Natl Acad Sci U S A **106**(37): 15756-61.
- Visnjic, D., Z. Kalajzic, D. W. Rowe, V. Katavic, J. Lorenzo and H. L. Aguilu (2004). "Hematopoiesis is severely altered in mice with an induced osteoblast deficiency." Blood **103**(9): 3258-64.
- Vogelstein, B. (1990). "Cancer. A deadly inheritance." Nature **348**(6303): 681-2.
- Vogelstein, B., E. R. Fearon, S. E. Kern, S. R. Hamilton, A. C. Preisinger, Y. Nakamura and R. White (1989). "Allelotype of colorectal carcinomas." Science **244**(4901): 207-11.
- Vousden, K. H. (2000). "p53: death star." Cell **103**(5): 691-4.
- Walkley, C. R., J. M. Shea, N. A. Sims, L. E. Purton and S. H. Orkin (2007). "Rb regulates interactions between hematopoietic stem cells and their bone marrow microenvironment." Cell **129**(6): 1081-95.
- Wang, X., H. Y. Kua, Y. Hu, K. Guo, Q. Zeng, Q. Wu, H. H. Ng, G. Karsenty, B. de Crombrughe, J. Yeh and B. Li (2006). "p53 functions as a negative regulator of osteoblastogenesis, osteoblast-dependent osteoclastogenesis, and bone remodeling." J Cell Biol **172**(1): 115-25.
- Wang, X. W., Q. Zhan, J. D. Coursen, M. A. Khan, H. U. Kontny, L. Yu, M. C. Hollander, P. M. O'Connor, A. J. Fornace, Jr. and C. C. Harris (1999).

- "GADD45 induction of a G2/M cell cycle checkpoint." Proc Natl Acad Sci U S A **96**(7): 3706-11.
- Weissman, I. L. (2000). "Stem cells: units of development, units of regeneration, and units in evolution." Cell **100**(1): 157-68.
- Weissman, I. L. and J. A. Shizuru (2008). "The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases." Blood **112**(9): 3543-53.
- Weksberg, D. C., S. M. Chambers, N. C. Boles and M. A. Goodell (2008). "CD150-side population cells represent a functionally distinct population of long-term hematopoietic stem cells." Blood **111**(4): 2444-51.
- Wilson, A. and A. Trumpp (2006). "Bone-marrow haematopoietic-stem-cell niches." Nat Rev Immunol **6**(2): 93-106.
- Wolf, N. S. and J. J. Trentin (1970). "Differential proliferation of erythroid and granuloid spleen colonies following sublethal irradiation of the bone marrow donor." J Cell Physiol **75**(2): 225-9.
- Wu, X., J. H. Bayle, D. Olson and A. J. Levine (1993). "The p53-mdm-2 autoregulatory feedback loop." Genes Dev **7**(7A): 1126-32.
- Xiong, S., C. S. Van Pelt, A. C. Elizondo-Fraire, B. Fernandez-Garcia and G. Lozano (2007). "Loss of Mdm4 results in p53-dependent dilated cardiomyopathy." Circulation **115**(23): 2925-30.
- Yamaoka, A., I. Kuwabara, L. G. Frigeri and F. T. Liu (1995). "A human lectin, galectin-3 (epsilon bp/Mac-2), stimulates superoxide production by neutrophils." J Immunol **154**(7): 3479-87.

- Yonish-Rouach, E., D. Resnitzky, J. Lotem, L. Sachs, A. Kimchi and M. Oren (1991). "Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6." Nature **352**(6333): 345-7.
- Zakut-Houri, R., B. Bienz-Tadmor, D. Givol and M. Oren (1985). "Human p53 cellular tumor antigen: cDNA sequence and expression in COS cells." EMBO J **4**(5): 1251-5.
- Zambetti, G. P., J. Bargonetti, K. Walker, C. Prives and A. J. Levine (1992). "Wild-type p53 mediates positive regulation of gene expression through a specific DNA sequence element." Genes Dev **6**(7): 1143-52.

## APPENDIX

### Generation of *Trim24* conditional knockout mouse

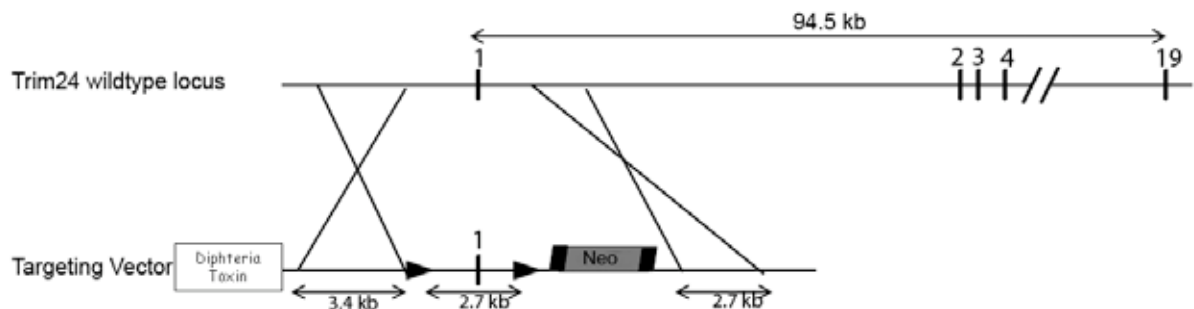
#### Background and rational

The levels of p53 in unstressed cells are consistently low and below a threshold that may induce apoptosis or cell cycle arrest (Jain and Barton 2009). The fine-tuning machinery responsible for controlling p53 levels is always expanding in the literature and includes but not limited to Mdm2 and Mdm4. In a screen for p53 interacting proteins using mass spectroscopy, Alton *et al.* identified Trim24 as a novel negative regulator of p53 (Allton, Jain et al. 2009). Trim24 belongs to the tripartite-motif family of proteins characterized by an N-terminus containing a RING domain, Zinc finger binding B box domain and a coiled-coil domain (Meroni and Diez-Roux 2005). Trim24 acts as an E3 ubiquitin ligase and targets p53 for proteosomal degradation in *in vitro* assays (Allton, Jain et al. 2009).

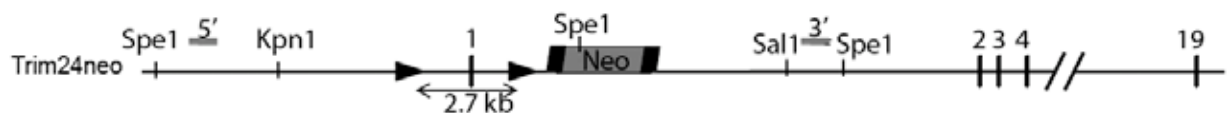
Cop1 and Pirh2 are also negative regulators of p53 and target it for proteosomal degradation (Marine, Francoz et al. 2006). However, Cop1- and Pirh2-null mice are not lethal (personal communication). This indicates that although certain proteins may play a role in regulating p53 levels *in vitro*, their biological significance remains controversial in mammalian systems. *Trim24*<sup>-/-</sup> mice have been recently generated and are tumor prone (Khetchoumian, Teletin et al. 2007). Specifically, *Trim24* deletion impedes the withdrawal from cell cycle during neonatal-to-adult transition in liver cells and these mice develop metastatic hepatocellular carcinomas. These data do not support the oncogenic role of Trim24 as a negative regulator of p53 as suggested by Alton *et al.*. On the contrary, they

show that Trim24 is a tumor suppressor. Noteworthy, the *Trim24*-null allele is generated by targeted deletion of its fourth exon. This leaves behind the first three exons which encode for RING domain. The E3 ubiquitin ligase activity of Trim24 is mediated via its RING domain

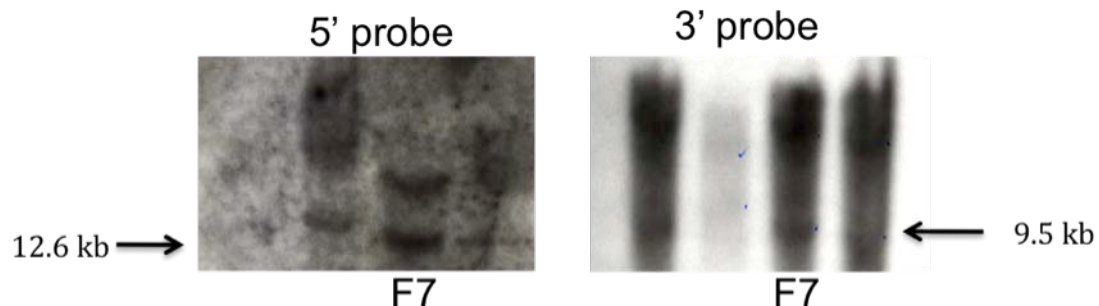
a.



b.



c.



**Figure 44. Targeting strategy for *Trim24*.** (a) Showing *Trim24* wildtype locus spanning a 94.5 kb region. Targeting vector contains diphtheria toxin and Neomycin (Neo) for negative positive selection, respectively. LoxP sites (triangles) inserted at each side of Exon 1. Neomycin cassette flanked by two Frt sites (tilted triangles). (b) Spe1 enzyme used to digest genomic DNA for southern blotting analysis with 5' probe (5') and 3' probe (3'). Neo contains an Spe1 site which helps in detecting of right band size. Kpn1 and Sal1 sites denote the end of arms of homology. (c) Successful targeting of clone #F7 at 5' end and 3' end as shown by expected band size (12.6 and 9.5 kb).

(Allton, Jain et al. 2009). Hence, an incomplete deletion of its RING domain could have concealed the actual role of Trim24 to regulate p53.

### Summary of results

To that end, we decided to generate a conditional knockout mouse of *Trim24* by genetic targeting of its first exon (Figure 44a). Using Southern Blotting, we were successful in identifying clone #F7 as correctly targeted for both 5' and 3' ends (Figure 44b-c). We then injected this clone into blastocysts of pseudopregnant females. Progeny with mixed coat color were bred to wildtype mice. The pups from this litter were analyzed for presence of *Trim24*<sup>Neo</sup> allele by PCR to determine if the allele was transmitted through germline (Figure 45).



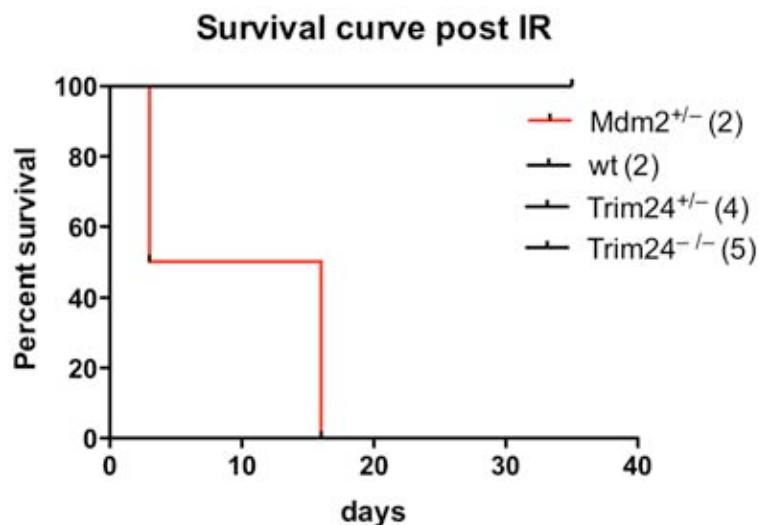
**Figure 45. Representative PCR result to detect germline transmission.** Lower band is wildtype while upper band represent *Trim24*<sup>Neo</sup> allele.

We then generated *Zp3-Cre Trim24*<sup>Neo/+</sup> mice by crossing *Trim24*<sup>Neo/+</sup> mice to *Zp3-Cre*. *Zp3-Cre Trim24*<sup>Neo/+</sup> females were mated to FLPeR males. FLPeR male has the FLP recombinase ubiquitously expressed from ROSA26-locus (Farley, Soriano et al. 2000). FLP excised out the Frt sites and thus the Neo cassette. Also, *Zp3* drives expression of Cre from the mouse zona pellucida 3 (*Zp3*) gene which is expressed in growing oocytes (Lewandoski, Wassarman et al. 1997). By Cre



recombinase activity, loxP sites are recombined and the first exon is excised leading to generation of a mouse with one of its *Trim24* loci lacking Exon 1 hereafter designated as *Trim24*<sup>+/-</sup>. *Trim24*<sup>+/-</sup> mice were crossed to each other to generate *Trim24*<sup>-/-</sup> mice. *Trim24*<sup>-/-</sup> mice were born with no obvious defects.

In order to investigate whether Trim24 is important for regulation of p53 post ionizing radiation (IR), we irradiated 8 weeks old *Trim24*<sup>-/-</sup> mice with 6Gy of IR. *Mdm2*<sup>+/-</sup> die within two to three weeks post sublethal irradiation (Terzian, Wang et al. 2007) and were used as positive control in this experiment. *Mdm2*<sup>+/-</sup> mice died as expected within 2-3 weeks post IR (Figure 46). However, all *Trim24*<sup>+/-</sup> and *Trim24*<sup>-/-</sup> mice survived 5 weeks post IR and had no obvious defects.



**Figure 46. Survival curve of mice post sublethal irradiation.** Mice of indicated genotypes were irradiated with 6Gy and then monitored for survival. Number of mice used is indicated next to each genotype. *Mdm2*<sup>+/-</sup> mice died as expected. None of *Trim24*<sup>+/-</sup> or *Trim24*<sup>-/-</sup> mice died after 35 days. Mice were sacrificed then. wt= wildtype.

## Discussion and future directions

The lack of lethal phenotype in *Trim24*<sup>-/-</sup> mice proves that the regulation of p53 by Trim24 is dispensable during embryogenesis. Since *Trim24*<sup>-/-</sup> mice do not die post sublethal irradiation, it is also not crucial for regulating p53 post radiation stress. These results indeed do not support the *in vitro* characterization of the role of Trim24 in regulation of p53. However, although Trim24 seems dispensable, it could be a minor player in the regulatory mechanisms of p53 *in vivo*. Its role in regulating p53 could be mediated through interactions with other proteins, such as Mdm2. To address this possibility, we will generate *Trim24*<sup>-/-</sup> *Mdm2*<sup>+/-</sup> mice and investigate whether the haplo-insufficiency of Mdm2 in the absence of Trim24 could exert any p53-dependent phenotypes. Additionally, we will monitor *Trim24*<sup>-/-</sup> mice for any tumor phenotypes that may arise. At the same time, we will perform whole mouse histological analysis on *Trim24*<sup>-/-</sup> mice and investigate whether there are any minor defects taking place. Trim24 regulation of p53 could be in certain cell types that their loss is not detrimental to the organism. Careful examination of all tissues should address this. Finally, we will examine p53 levels in *Trim24*<sup>-/-</sup> mice post irradiation. There could be a change in p53 levels compared to wildtype mice but not high enough to kill the mice.

Briefly, we were successful in generating *Trim24*<sup>-/-</sup> mice. These mice are viable and fertile. *Trim24*<sup>-/-</sup> mice are three months of age now and do not show any obvious defects. They are resistant to sublethal irradiation, unlike *Mdm2*<sup>+/-</sup> mice. Further investigation of the status of this allele is required to characterize these mice. It is still premature to conclude the significance of Trim24 regulation of p53 in

mammalian system *in vivo*. The survival of *Trim24*<sup>-/-</sup> mice suggests that Trim24-regulation of p53 is not important during embryogenesis like Mdm2 and Mdmx. However, Trim24 can be important in certain tissues or after certain stresses postnatally which requires further investigation.

## **VITA**

Hussein A. Abbas was born in Kuwait, Kuwait on December 22, 1985, the son of Ali Abbas and Hanan Hammoud. He completed his primary education in Beirut Baptist School, Beirut, Lebanon. In June 2006, he received his Bachelor of Science with a major in biology from Lebanese American University, Beirut, Lebanon. In September 2006, he matriculated in the Doctor of Philosophy program at The University of Texas Health Science Center in Houston. He had his predoctoral training in the laboratory of Guillermina Lozano at Department of Genetics in University of Texas M. D. Anderson Cancer Center. He is continuing his education as of August 2010 at Lebanese American University to pursue the degree of Doctor of Medicine.