EFFECTS OF ALTERING CELL PROLIFERATION ON HEMATOPOIETIC STEM AND PROGENITOR CELL FUNCTION

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DEDICATION

To Trent, and my family

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ABSTRACT

Sara Lynn Rohrabaugh

EFFECTS OF ALTERING CELL PROLIFERATION ON HEMATOPOIETIC STEM AND PROGENITOR CELL FUNCTION

Cell cycle checkpoints guarantee movement through the cell cycle in an appropriate manner. The spindle assembly checkpoint (SAC) ensures the proper segregation of chromosomes into daughter cells during mitosis. Mitotic arrest deficiency 2 (*Mad2*), a member of the mitotic checkpoint proteins, appears to be crucial for generating the wait anaphase signal to prevent onset of anaphase. We first studied the SAC in hematopoietic stem cells (HSC) to ensure that it was functional. Our previous studies found that prolonged SAC activation was uncoupled from apoptosis initiation in mouse and human embryonic stem cells (ESC). We found that upon treatment with a microtubule-destabilizing agent, HSC arrested in M-phase and subsequently initiated apoptosis. Thus unlike ESC, HSC exhibit coupling of prolonged SAC activation with apoptosis. We studied the effects of *Mad2*$^{+/-}$ on *in vivo* recovery of bone marrow HPC from cytotoxic effects and also effects of cytostatic agents on HPC growth *in vitro* using *Mad2*-haploinsufficient (*Mad2*$^{+/-}$) mice. We found that *Mad2*$^{+/-}$ HPCs were protected from the cytotoxic effects of cytarabine (Ara-C), a cycle specific agent, consistent with *Mad2*$^{+/-}$ HPCs being in a slow or non-cycling state. *Mad2* haploinsufficiency did not affect recovery of functional HPC after treatment with

cyclophosphamide or high sub-lethal dose irradiation, both non-cycle specific agents. There were no differences in immunophenotype defined HSCs in *Mad2*$^{+/-}$ and *Mad2*$^{+/+}$ mice, data confirmed by functional HSC competitive repopulation assays. To better understand the role of *Mad2* in HPC, E3330, a cytostatic agent, was used to assess the redox function of Ape1/Ref-1, and colony formation *in vitro* was examined under normoxic and lowered O_2 tension. *Mad2*$^{+/-}$ HPCs were less responsive to E3330 than *Mad2*$^{+/+}$ HPCs, and E3330 was more effective under lowered O_2 tension. *Mad2*$^{+/-}$ HPCs did not exhibit enhanced growth in lowered oxygen tension, in contrast to *Mad2*$^{+/+}$ HPCs. Our studies have unexpectedly found that *Mad2* haploinsufficiency is protective from the cytotoxic effects of a cycle specific DNA synthesis agent *in vivo*, and Ape1/Ref-1 inhibitor *in vitro*.

Hal E. Broxmeyer Ph.D., Chair

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LIST OF ABBREVIATIONS

5-FU	5-Fluorouracil
Ala	Alanine
AP-1	Activator protein-1
APC	Allophycocyanin
Ape1/Ref-1	Apurinic/apyrimidinic endonuclease/redox factor-1
Ara-C	Cytarabine
BER	Base excision repair
BFU-E	Burst forming unit-erythroid
BIR	Baculovirus IAP repeat
BM	Bone marrow
BSA	Bovine serum albumin
CENP-E	Centromere protein-E
CD34	Cluster of differentiation molecule 34
Cdc	Cell division cycle
CDK	Cyclin dependent kinase
CFU-GEMM	Colony forming unit-granulocyte, erythroid, macrophage, megakaryocyte
CFU-GM	Colony forming unit-granulocyte-macrophage
CIN	Chromosomal instability
c-kit	SCF receptor
CLP	Common lymphoid progenitor

CMP	Common myeloid progenitor
CPC	Chromosomal passenger complex
CRA	Competitive repopulation assay
Cys	Cysteine
DAM	Duo1- and Mps1 interacting complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EB	Embryoid body
Egr-1	Early growth response protein-1
EPO	Erythropoietin
ESC	Embryonic stem cell
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FcγR	Fc (fragment crystallization) gamma receptor
Flt3	FMS-like tyrosine kinase 3 ligand
GMP	Granulocyte/macrophage progenitor
GM-CSF	Granulocyte/macrophage colony stimulating factor
H&E	Hematoxylin and eosin stain
HEC-1	Highly expressed in cancer-1
HIF-1α	Hypoxia inducible factor-1α
HPC	Hematopoietic progenitor cell
HSC	Hematopoietic stem cell
IAP	Inhibitor of apoptosis protein

IMDM	Iscove's modified Dulbecco's medium
INCENP	Inner centromere protein
i.p.	Intraperitoneal
i.v.	Intravenous
KL	C-kit$^+$, Lin$^-$
KMN complex	KNL-1, Mis12 complex, and Ndc80/HEC1
KNL-1	Kinetochore NuL-1
LDMC	Low-density mononuclear cells
Lin	Lineage
LSK	Lin$^-$, Sca-1$^+$, c-kit$^+$
Mad2	Mitotic arrest deficiency 2
MCC	Mitotic checkpoint complex
MCK	Mitotic centromere-associated kinesin
MDR	Multi-drug resistance
MEN	Mitotic exit network
MEP	Megakaryocyte/erythroid progenitor
MPD	Myeloproliferative disorder
Mps1	Monopolar spindle 1
NAC	N-acetyl-L-cysteine
NF-κB	Nuclear factor-κB
O_2	Oxygen
PARP	Poly (ADP-ribose) polymerase

PB	Peripheral blood
PBS	Phosphate buffered saline
PE	Phycoerythrin
PWMSCM	Pokeweed mitogen spleen conditioned media
RBC	Red blood cell
rhu	Recombinant human
rmu	Recombinant mouse
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SAC	Spindle assembly checkpoint
s.c.	Subcutaneous
Sca-1	Stem cell antigen-1/Ly-6A/E
Scc	Sister chromatid cohesin
SCF	Stem cell factor
SDF-1α	Stromal-cell derived factor 1 alpha
SL	Sca-1^{+}, lin $^{-}$
SMC	Structural maintenance of chromosome
Spl	Spleen
Thr	Threonine
TLK-1	Tousled-like kinase-1
TPO	Thrombopoietin

INTRODUCTION

Mitosis and the Spindle Assembly Checkpoint.

During S-phase of the cell cycle, a single round of DNA replication results in two identical DNA copies known as sister chromatids. This is followed by G_2 phase, in which the cells grows and prepares for M (mitotic) phase, which results in chromosome segregation and ultimately the generation of two daughter cells. In order for chromosomes to be evenly distributed into the daughter cells, sister chromatids need to be held together until the appropriate time with cohesin. Cohesin is a highly conserved protein complex comprised of four subunits, structural maintenance of chromosomes SMC1 and SMC3, and sister chromatid cohesins (Scc) Scc3 and Scc1 (Nasmyth 2001). Sister chromatids attach to spindle microtubules via kinetochores, which assemble at the centromere on each of the sister chromatids of a chromosome (Cleveland, et al. 2003; Hauf and Watanabe 2004; Mazia 1961). Cohesin is essential for sister chromatids to become attached to opposing poles of the mitotic spindle, a configuration known as amphitelic attachment, and a pre-requisite for accurate segregation of the genome (Homer 2006).

After all sister chromatids have become amphitelically attached in metaphase, the cell can then initiate anaphase, the stage of mitosis when chromosomes separate; this requires that cohesin along both chromosome arms and centromeres be resolved (Figure 1) (Homer 2006). In vertebrates, the majority of

arm cohesins are removed during prometaphase by a cleavage-independent mechanism mediated by polo-like kinase and Aurora-B kinase (Gimenez-Abian, et al. 2004; Losada, et al. 2002; Sumara, et al. 2002). Complete resolution of cohesin and anaphase onset is ultimately dependent upon cleavage of the Scc1 subunit of the cohesin complex by a thiol protease known as separase (Hauf, et al. 2001; Waizenegger, et al. 2000). The activity of separase is negatively regulated by its association with securin, an inhibitory chaperone, and by phosphorylation induced by cyclin-dependent kinase 1 (Cdk1) (Stemmann, et al. 2001). The destruction of securin and cyclin B, the latter resulting in Cdk1 inactivation, removes the block on separase, which allows it to engage Scc1. The destruction of securin and cyclin B is brought about by the anaphase-promoting complex or cyclosome (APC/C), a ubiquitin ligase, along with its activating subunit, Cdc20 (Irniger, et al. 1995; King, et al. 1995; Sudakin, et al. 1995). APC/C^{Cdc20} ubiquitinates securin and cyclin B, which causes them to be recognized and destroyed by the 26S proteosome (Glotzer, et al. 1991). The activity of APC/C^{Cdc20} must be tightly regulated in order to ensure that cells do not prematurely progress from metaphase to anaphase causing changes in chromosome number in the daughter cells.

Figure 1

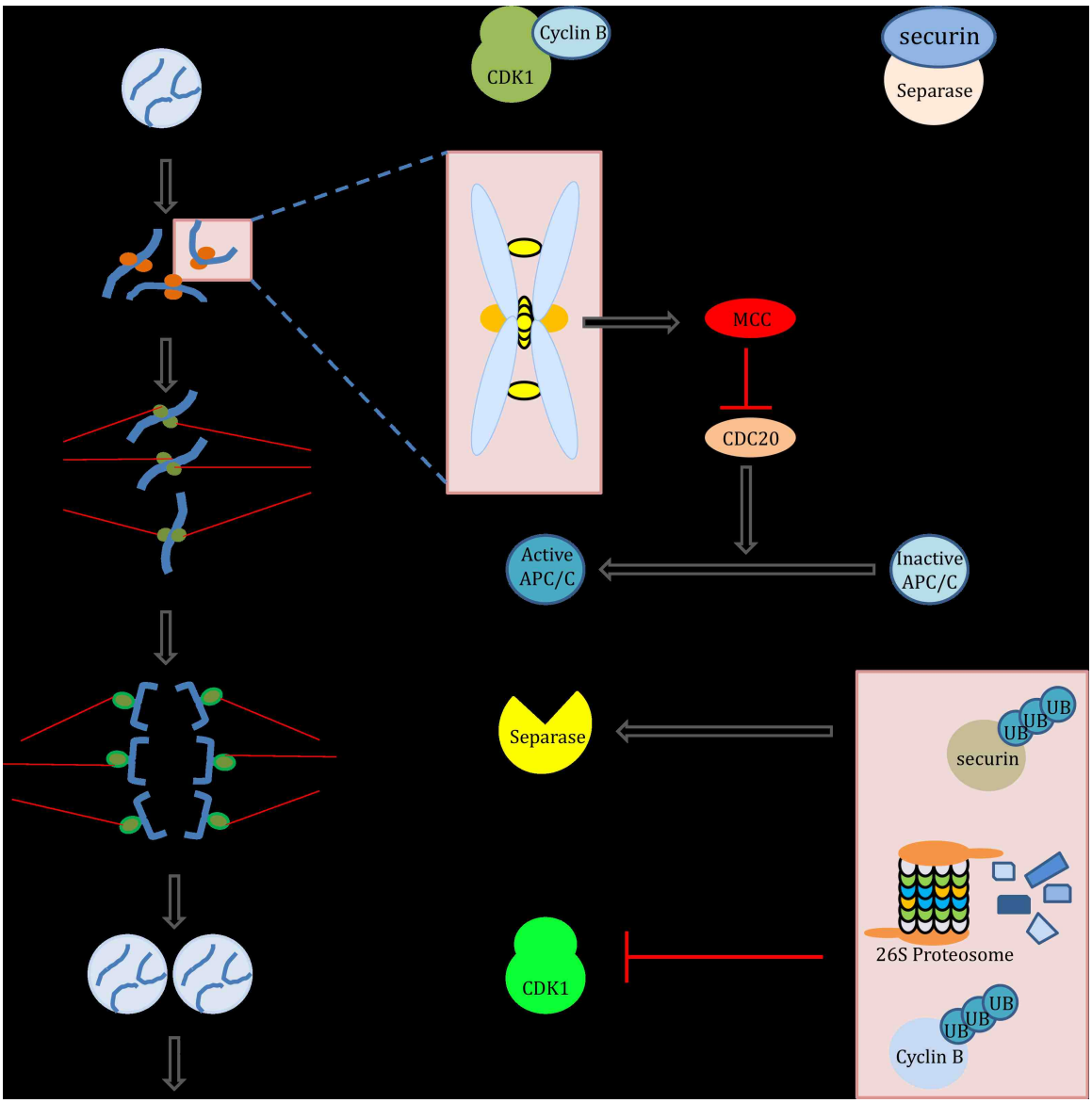

Cyclin B
CDK1
securin
Separase
MCC
CDC20
Active APC/C
Inactive APC/C
Separase
CDK1
UB
securin
26S Proteosome
Cyclin B

Figure 1. Summary of SAC Signaling During Mitosis.

Mitosis is subdivided into five consecutive phases: prophase, prometaphase, metaphase, anaphase, and telophase. To enter mitosis, the cell requires the activity of cyclin-dependent kinase 1 (CDK1), which depends on the binding of cyclin B to CDK1 (Peters 2006). The protease activity of separase is required to remove the cohesin binding together sister chromatids in order for the cell to transition from metaphase to anaphase. Prior to anaphase, separase is kept inactive by binding of the protein securin. Unattached kinetochores contribute to the formation of the mitotic checkpoint complex (MCC), which inhibits the activity of Cdc20 to activate the APC/C. Bipolar attachment of all sister chromatids inactivates the SAC, releasing Cdc20 to activate APC/C. APC/C activation results in polyubiquitylation and destruction of cyclin B and securin. Securin destruction results in the activation of separase, which cleaves the remaining cohesin holding together the sister chromatids. Cyclin B degradation inactivates CDK1, initiating cytokinesis and mitotic exit.

During eukaryotic cell division, the accurate transmission of the genome is essential for survival of the daughter cells and is ensured by intrinsic properties of the cell cycle machinery and by a series of checkpoints (Gillett and Sorger 2001). Checkpoint mechanisms monitor cell cycle progression and guarantee faithful duplication and precise segregation of the genome (Li, et al. 1997). The spindle assembly checkpoint (SAC), also known as the mitotic or spindle checkpoint, is a highly conserved signal transduction pathway that links the initiation of anaphase to spindle assembly and the completion of chromosome-microtubule attachment (Dobles, et al. 2000; Hoyt, et al. 1991; Li and Murray 1991; Li and Benezra 1996; Taylor and McKeon 1997). The presence of even a single unattached or misaligned chromosome is sufficient to activate the SAC, which blocks the activity of the APC/C and arrests the cells at the transition from metaphase to anaphase (Hwang, et al. 1998; Kim, et al. 1998; Li and Nicklas 1995; Li, et al. 1997; Rieder, et al. 1994). Arrest caused by an unattached chromosome is overcome by laser ablation of the kinetochore, the structure that mediates chromosome-microtubule attachment (Rieder, et al. 1995), indicating that the signal for checkpoint-dependent arrest arises from, or is transduced through, kinetochores (Li, et al. 1997).

Genes involved in the SAC were first identified in the budding yeast *S. cerevisiae* and include the mitotic arrest defective genes *MAD1-3* (Li and Murray 1991) and the budding uninhibited by benzimidazole genes *BUB1-3* (Hoyt, et al. 1991). All six of these genes are dispensable for normal growth, apparently due to the fact

that mitosis in *S. cerevisiae* lasts long enough for all chromosomes to attach to the spindle before anaphase begins, even in the absence of a checkpoint. The addition of anti-microtubule drugs causes the cells to proceed through mitosis without having established chromosome-microtubule attachment, resulting in extensive chromosome loss and cell death (Hoyt, et al. 1991; Li and Murray 1991). Homologues of these checkpoint proteins were later found in other organisms, including mammals (Table 1); these include *Mad1*, *Mad2*, *Mad3/BubR1*, *Bub1*, *Bub3*, and *Mps1* (Yu 2002). Upon isolation of the human homolog of *MAD2*, it was shown to be a necessary component of the mitotic checkpoint (Li and Benezra 1996). All of these proteins, with the exception of *Bub2*, act in a complex signaling network that regulates Cdc20-APC/C and chromosome segregation (Gardner and Burke 2000). In *S. cerevisiae*, *Bub2* appears to negatively regulate the mitotic exit network (MEN), which leads to the degradation of mitotic cyclins and other cell cycle regulators (Gardner and Burke 2000). The MEN is a signal transduction cascade, including several protein kinases, that ensures mitotic exit and cytokinesis is tightly coupled to nuclear division; this ensures that daughter cells inherit a complete set of chromosomes after cell division (Piatti, et al. 2006). In eukaryotes, mitotic exit takes place after mitotic cyclin-dependent kinases (CDKs) are inactivated, which is normally fulfilled by cyclin proteolysis (Piatti, et al. 2006). Inactivation of these CDKs in turn is necessary for spindle disassembly, licensing of replication origins, and cytokinesis (Piatti, et al. 2006).

Vertebrates	*S. cerevisiae*	Interactions & functions
Mad1	Mad1	Binds to Mad2 and recruits Mad2 to kinetochores; phosphorylated by Mps1 & Bub1 in vitro
Mad2	Mad2	Binds to Cdc20 & inhibits APC; binds to Mad1
BubR1	Mad3	Binds to Bub3 & Ccd20; inhibits APC in vitro; binds to the mitotic motor CENP-E
Bub1	Bub1	Binds to Bub3; binds to Mad1 & Cdc20 in *S. cerevisiae*; phosphorylates human Mad1 in vitro
--	Bub2	Negatively regulates Tem1 and the mitotic exit network in *S. cerevisiae*
Bub3	Bub3	Bind to Bub1 & BubR1 (Mad3)
Mps1	Mps1	Phosphorylates Mad1 in *S. cerevisiae*
Aurora B	Ipl	Binds to INCENP/Sli15/Pic1; promotes bi-orientation
Cdc20	Cdc20	Binds to Mad2, BubR1(Mad3), Emi1, APC & APC substrates; activates APC; ubiquitinated by APC-Cdh-1; phosphorylated by Cdk1 in mammals

Table 1. Components of the Spindle Assembly Checkpoint.

These proteins, along with others, have been found to play a role in the SAC in yeast and/or vertebrates.

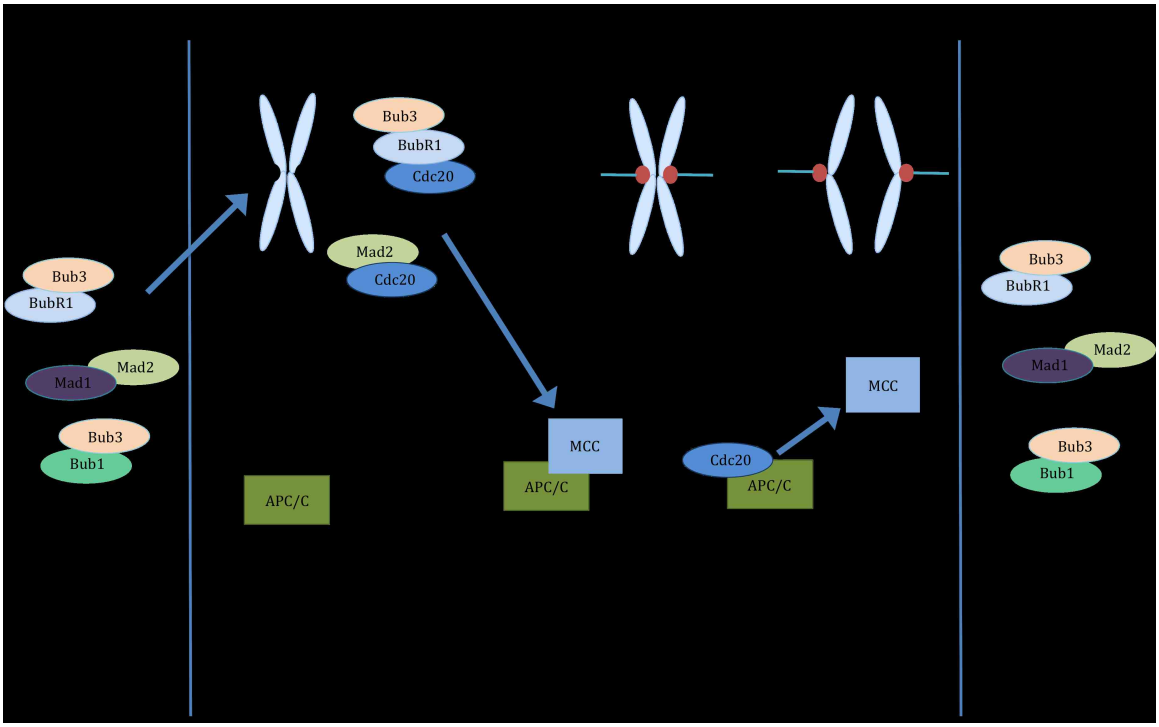
Recognizing the Lack of Occupancy at the Kinetochore.

The signaling process involved in the SAC, which is activated by the lack of tension and/or the lack of microtubule occupancy at the kinetochore, is quite complex. Distinguishing between the contributions of attachment and tension to SAC activation is difficult, since interfering with creation of tension probably also affects attachment (Pinsky and Biggins 2005; Zhou, et al. 2002). However, regardless of whether the SAC is regulated by tension independently of occupancy, kinetochore localization of checkpoint components is differentially regulated by occupancy and tension. A single unattached kinetochore is sufficient to activate the SAC, and generates a 'wait anaphase' signal that must produce a signal to block APC activity (Rieder, et al. 1995). This signal recruits SAC proteins to the unattached kinetochore (Vigneron, et al. 2004). Aurora-B/INCENP controls the localization of Mps1, Bub1, Bub3, and CENP-E at the kinetochores, and this localization, in turn, promotes the recruitment to the kinetochore of Mad1/Mad2, Cdc20, and the APC (Vigneron, et al. 2004). Both Mad1 and Mad2 accumulate at unattached kinetochores and are removed upon microtubule attachment, and their localization signifies lack of attachment (Skoufias, et al. 2001).

Recent studies have started to shed light on how a potential diffusible checkpoint signal inhibits APC. There are two related models on how the SAC regulates the interaction of Cdc20 and the APC. The APC is inhibited in both models through direct binding of the mitotic checkpoint complex (MCC), which contains BubR1,

Bub3, Mad2, and Cdc20 or the sub-complexes BubR1-Bub3-Cdc20 and Mad2-Cdc20 (Fang 2002; Fang, et al. 1998; Fraschini, et al. 2001; Hardwick, et al. 2000; Millband and Hardwick 2002; Sudakin, et al. 2001; Tang, et al. 2001); however, the two models differ in how the MCC assembles (Figure 2). In one model (Figure 2A), the unattached kinetochores catalyze the formation of the MCC or its sub-complexes, which then diffuse away from the kinetochore to inhibit the APC (Skoufias, et al. 2001; Taylor, et al. 2001). Both Mad2 and BubR1 appear to be able to bind Cdc20 directly, although they appear to have distinct binding sites on Cdc20 (Tang, et al. 2001; Wu, et al. 2000). In the other model (Figure 2B), the MCC is present through all of the cell cycle and its formation does not occur at the kinetochores (Sudakin, et al. 2001). Once the SAC is activated, an unknown diffusible signal turns over at the kinetochores and the APC is sensitized to inhibition by the MCC (Sudakin, et al. 2001).

Figure 2


Bub3
BubR1
Mad2
Mad1
Bub3
Bub1
APC/C
Bub3
BubR1
Cdc20
Mad2
Cdc20
MCC
APC/C
Cdc20
MCC
APC/C
Bub3
BubR1
Mad2
Mad1
Bub3
Bub1

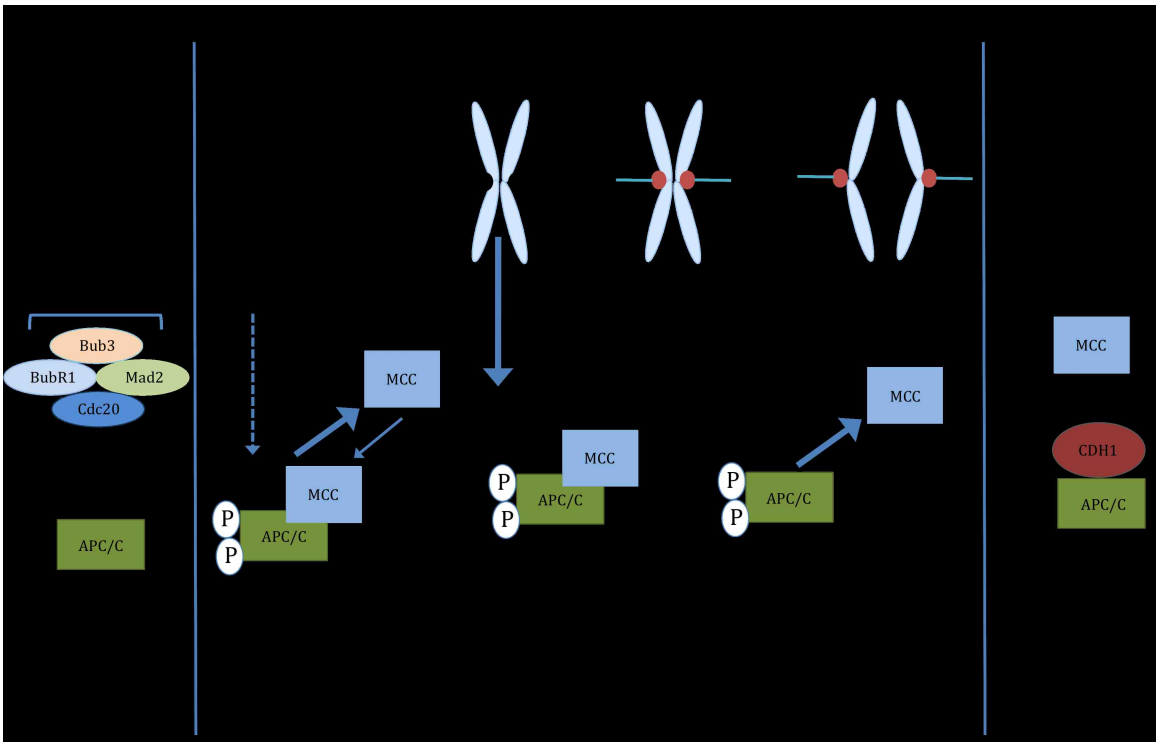

Bub3
BubR1
Mad2
Cdc20
APC/C
MCC
MCC
P
P
APC/C
MCC
P
P
APC/C
MCC
P
P
APC/C
MCC
CDH1
APC/C

Figure 2. Models of APC Inhibition Through Binding of the Mitotic Checkpoint Complex (MCC) in Response to Lack of Attachment at the Kinetochore.

Two related models exist on how the SAC regulates the interaction of Cdc20 and APC. The APC is inhibited in both models through direct binding of the mitotic checkpoint complex (MCC), which contains BubR1, Bub3, Mad2, and Cdc20 or the sub-complexes BubR1-Bub3-Cdc20 and Mad2-Cdc20; however, the two models differ in how the MCC assembles. (A) Numerous complexes of checkpoint proteins, including Mad1-Mad2, Bub1-Bub3, and Mad3/BubR1-Bub3, are detected throughout the cell cycle. Other checkpoint complexes, such as BubR1-Bub3-Cdc20 and Mad2-Cdc20, seem to be enriched in mitotic cells, possibly due to the higher concentrations of Cdc20 in mitosis. The unattached kinetochores catalyze the formation of the MCC or its sub-complexes, which then diffuse away from the kinetochore to inhibit the APC. (B) The MCC is present through all of the cell cycle but is incapable of inhibiting the APC until it has undergone mitotic modifications. Once the SAC is activated, an unknown diffusible signal turns over at the kinetochores and the APC is sensitized to inhibition by the MCC. In both models, binding of microtubules to kinetochores leads to dissociation of the MCC from the APC. This activates that APC, allowing the cell to progress through M-phase.

Regulation of Kinetochore-Microtubule Attachments in Lack of Tension. The lack of tension may activate specific subsets of checkpoint proteins, including Ipl1/Aurora-B (Biggins and Murray 2001). The chromosomal passenger complex (CPC) is composed of Aurora-B and three non-enzymatic subunits, INCENP, survivin, and borealin (Gassmann, et al. 2004; Honda, et al. 2003; Klein, et al. 2006; Sampath, et al. 2004). INCENP (inner centromere protein) appears to act as a scaffold, interacting with the other three members of the complex (Adams, et al. 2000; Gassmann, et al. 2004; Kim, et al. 1999; Wheatley, et al. 2001). The C terminus of INCENP is involved in the binding and regulation, more specifically the activation, of Aurora-B (Adams, et al. 2000; Kaitna, et al. 2000). Aurora-B/Ipl1 is a highly conserved Ser/Thr kinase (Terada, et al. 1998) and it phosphorylates Tousled-like kinase-1 (TLK-1) during prophase-prometaphase and this, in turn, increases Aurora-B activity in an INCENP dependent manner (Han, et al. 2005). The yeast kinase Ipl1 is required for establishing bi-orientation and tension of sister chromatids (Tanaka 2002; Tanaka, et al. 2002), and might also sense the lack of tension at kinetochores (Biggins and Murray 2001; Stern 2002). Aurora-B, the mammalian homologue of Ipl1, has been shown to be required for bipolar attachment of chromosomes and for proper function of the SAC in mammalian cells (Kallio, et al. 2002; Murata-Hori, et al. 2002; Murata-Hori and Wang 2002). Survivin is a conserved member of the inhibitor of apoptosis protein (IAP) family and contains a baculovirus IAP repeat (BIR) domain that is responsible for its dimerization (Chantalat, et al. 2000; Muchmore, et al. 2000; Verdecia, et al. 2000). Survivin can bind to the

other three members in the CPC complex and is phosphorylated by Aurora-B (Carvalho, et al. 2003; Wheatley, et al. 2004). Borealin was identified in a proteomic screen for new components of the mitotic chromosome scaffold and is phosphorylated by Aurora-B *in vitro* (Gassmann, et al. 2004). The CPC has various functions during mitosis and meiosis, ranging from monitoring chromosome-microtubule interactions to sister chromatid cohesion to cytokinesis, but its role in kinetochore attachment and chromosome segregation are of particular interest in further understanding the ability of the CPC to sense the lack of tension.

The CPC is required for proper chromosome bi-orientation at metaphase (Adams, et al. 2001; Gassmann, et al. 2004; Kaitna, et al. 2000), and is a key component in detecting aberrant kinetochore-microtubule attachments and, particularly, a lack of tension across the spindle (Kotwaliwale and Biggins 2006). This may involve the phosphorylation of key kinetochore-centromere components by Aurora-B, although INCENP and survivin may also be involved. In yeast, the kinetochore is composed of protein complexes that are classified as inner, central, or outer kinetochore complexes (Tan, et al. 2005). The DAM1 [Duo1 - and Mps1 (monopolar spindle 1) - interacting] complex, an outer kinetochore complex, plays a crucial role in mediating the kinetochore-microtubule connection, and is regulated through phosphorylation by the Ipl1/Aurora-B kinase (Tan, et al. 2005). Like Dam1, Ipl1 and Sli15/INCENP each bind microtubules *in vitro* (Tan, et al. 2005). Because of their association with both kinetochores and

microtubules, the Ipl1 and DAM1 complexes are believed to regulate various aspects of chromosome-spindle interaction (Kang, et al. 2001). The Ipl1 complex responds to the lack of tension in monotelic attachments, and acts to resolve these inappropriate attachments, probably through its substrates (Biggins, et al. 1999; Buvelot, et al. 2003; Cheeseman, et al. 2002). The ipl1 kinase phosphorylates Ndc10p *in vitro*, suggesting that Ipl1p regulates the kinetochore by phosphorylating Ndc10p (Biggins, et al. 1999). Ndc10p, along with Cep3p, Ctf13p, and Skp1, make up the CBF3 complex (Kaplan, et al. 1997; Lechner and Carbon 1991; Stemmann and Lechner 1996). The CBF3 complex binds to centromeric DNA and this interaction is essential for kinetochore function (Lechner and Carbon 1991; Sorger, et al. 1995). The CBF3 complex does not bind microtubules, but beads coated with centromeric DNA-CBF3 complexes can bind to microtubules after they have been incubated in yeast extracts (Sorger, et al. 1994), suggesting that CBF3 can recruit unknown microtubule-binding proteins to the kinetochore. These unknown binding proteins were later identified as an INCENP-survivin complex (Sandall, et al. 2006). It has been shown that the interaction between Ndc80 and DAM1 proteins is essential for the association of the DAM1 complex with kinetochores, and that the DAM1-Ndc80 binding affinity is weakened by Ipl1-mediated phosphorylation (Shang, et al. 2003). In the absence of tension, Ipl1 causes an increased turnover of kinetochore-microtubule connections, perhaps by influencing the Ndc80-DAM1 interaction, in this manner facilitating eventual bi-orientation of chromosomes (Cheeseman, et al. 2002; Shang, et al. 2003; Tanaka, et al. 2002). In addition, Ipl1 may also

regulate the assembly of the DAM1 complex itself (Cheeseman, et al. 2002). Recent data suggest that the MTW1 complex promotes kinetochore bi-orientation, which is monitored by the Ipl1 kinase (Pinsky, et al. 2003). The tension sensing mechanism might also involve the kinetochore-associated microtubule motor protein CENP-E, which binds to spindle microtubules and BubR1, allowing it to modulate signaling activity in a microtubule-dependent manner (Abrieu, et al. 2000). Stable association of BubR1 to kinetochores and sustained checkpoint signaling in response to lack of tension appears to depend on survivin (Lens, et al. 2003), further implicating the CPC in this process.

An important target of Aurora-B in the vertebrate kinetochore is HEC1 (highly expressed in cancer-1; Ndc80 in yeast), which is required for kinetochore-microtubule interactions and chromosome segregation (DeLuca, et al. 2003). HEC1, along with KNL-1 (Kinetochore NuL-1) and the Mis12 complex, appear to make up the KMN complex, which is essential for microtubule binding to the kinetochore (Cheeseman, et al. 2006). Aurora-B phosphorylation of HEC1/Ndc80 in the KMN complex appears to lead to microtubule release by the kinetochore in the presence of inappropriate attachments (Cheeseman, et al. 2006; DeLuca, et al. 2006). The kinesin 13 microtubule depolymerase MCAK (mitotic centromere-associated kinesin) is also involved in bipolar spindle assembly (Ohi, et al. 2004; Tulu, et al. 2006). Aurora-B phosphorylation of MCAK inhibits microtubule depolymerization activity (Andrews, et al. 2004; Lan, et al. 2004). The CPC is specifically enriched at non-attached and merotelically

(a single kinetochore binding to microtubules from both spindle poles) attached kinetochores (Knowlton, et al. 2006; Vagnarelli and Earnshaw 2004). Studies suggest that Aurora-B promotes MCAK accumulation and regulates its activity at there aberrant attachments (Andrews, et al. 2004; Lan, et al. 2004).

Overall, it appears that microtubule binding by kinetochores is very complex, involving a network of redundant weak interactions (Ruchaud, et al. 2007). HEC1/Ndc80 and the KMN network may be important for creating low-affinity links that can be released through the action of the CPC, in particular Aurora-B (Figure 3) (Cheeseman, et al. 2006). The low-affinity links that involve INCENP and survivin appear to enable kinetochores to sense tension within the mitotic spindle. MCAK uses its ability to destabilize microtubules to release undesirable kinetochore-microtubule attachments, even in the presence of normal local tension within the spindle. It is clear that the CPC, with Aurora-B as its kinase, is the regulator of kinetochore-microtubule attachments that control essential mechanisms of microtubule release. After the CPC and other proteins release improper kinetochore-microtubule attachments, other SAC signaling pathways can sense the lack of attachment at the kinetochore and arrest the cell before it transitions to anaphase.

Figure 3

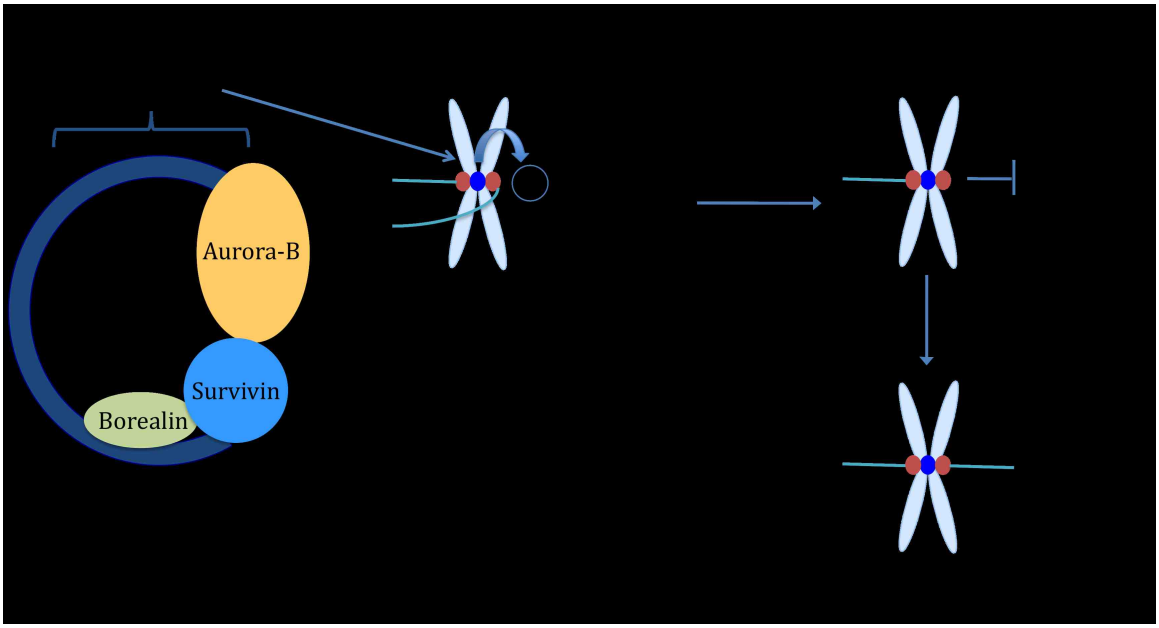


Figure 3. The Chromosomal Passenger Complex (CPC) Corrects Incorrect Microtubule Attachments and Indirectly Activates the SAC.

To obtain bipolar spindle attachment, improper attachments (syntelic and merotelic attachments) must be corrected to prevent chromosome missegregation. The CPC, mainly the kinase activity of Aurora-B, is required for this correction process. Aurora-B phosphorylates kinetochore proteins that bind to microtubules, modifying the affinity or stability of kinetochore-microtubule interactions. As a result, microtubules detach from the kinetochore allowing new microtubule attachments until bipolar attachments are obtained. During this process, unattached kinetochores are capable of activating the SAC and inhibiting the APC.

The Importance of Mad2 in the SAC.

The principal system for regulating the APC/C-Cdc20 interaction in response to the kinetochore-microtubule attachment status is the SAC (Homer 2006; Musacchio and Hardwick 2002; Taylor, et al. 2004). One model regarding SAC function hypothesizes that in response to kinetochores which lack microtubule attachment and/or tension, the primary downstream effect of SAC activation is the generation of a conformation of Mad2 capable of sequestering cellular Cdc20, thereby preventing APC/C activation and hence cell cycle progression (De Antoni, et al. 2005; Nasmyth 2005). This model takes into account that either lack occupancy or tension alone may be too simplistic to explain the signaling that occurs during SAC activation. Syntelic chromosomes, chromosomes with both sister chromatids attached to the same pole, often retain Mad2 at their kinetochores (Kapoor, et al. 2000), suggesting that attachment is not enough to silence the SAC. Furthermore, even though Mad2 is not found at kinetochores in taxol-treated cells, which lack tension across the mitotic spindle, it is still required to keep the cells arrested in M-phase (Waters, et al. 1998). In budding yeast, Mad2 was found to be required for cells to respond to lack of attachment or tension during mitosis (Shonn, et al. 2000) and meiosis (Stern and Murray 2001). Although the complete mechanism of SAC signaling in response to improperly attached kinetochores remains incompletely resolved, the net effect is indisputably APC/C-Cdc20 inhibition for which Mad2 is of central importance (Homer 2006).

Of the proteins involved in the spindle assembly checkpoint, Mad2 appears to be the most crucial protein for generating the wait anaphase signal that prevents the onset of anaphase in the presence of microtubule disruption (Amon 1999; Li and Benezra 1996; Wassmann and Benezra 2001). *Mad2* null (*Mad2*$^{-/-}$) embryos die *in utero* about 6.5-7.5 days after conception (E6.5-E7.5) (Dobles, et al. 2000). However, *Mad2*$^{-/-}$ embryos appear normal *in utero* and in culture until embryonic day E5.5 (Dobles, et al. 2000). These cells are unable to arrest in mitosis in response to drug induced spindle disruption, demonstrating they lack a functional spindle assembly checkpoint (Dobles, et al. 2000). *Mad2* null cells proceed to anaphase even when unattached chromosomes are present, leading to missegregation of chromosomes and resulting in apoptotic cell death (Dobles, et al. 2000). Deletion of one allele of the *Mad2* gene results in premature separation of sister chromatids and high frequency of chromosome missegregation in the presence of nocodazole, a microtubule-destabilizing agent and spindle inhibitor, in both primary murine embryonic fibroblasts and human cancer cells (Michel, et al. 2001). *Mad2*$^{+/-}$ mice exhibit increased germinal center formation in the spleen and a possible increase in tumor incidence when compared to age-matched *Mad2*$^{+/+}$ mice (Dobles, et al. 2000). Also, subtle differences in morbidity rates occur between the colonies of *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ mice, but overall the *Mad2*$^{+/-}$ animals appear largely normal (Dobles, et al. 2000). Even though the hematopoietic system of the *Mad2*$^{+/-}$ animals was not fully examined in these initial studies of *Mad2*, it was later shown to play a role in optimal hematopoiesis (Ito, et al. 2007).

Examining the Role of the SAC and Mad2 in Hematopoiesis.

The mammalian hematopoietic system is a complex tissue made up of various cell types capable of performing a wide range of functions, such as protection against infection and oxygen and nutrient delivery. All cells that make up the blood and immune system originate from hematopoietic stem cells (HSC) (Figure 4), which have the ability to renew themselves and differentiate, consequently providing a source of blood cells for the entire life of the organism. Understanding genome maintenance and possible causes of chromosomal instability (CIN) in hematopoietic cells during *ex vivo* expansion and transplantation is necessary to ensure their safe and effective use. Before further examining the specific role of Mad2 in hematopoiesis, the functionality of the SAC in HSCs needs to be determined. Both mouse and human embryonic stem cells (ESC) were found to exhibit checkpoint apoptosis uncoupling (Mantel, et al. 2007). When ESCs were treated with nocodazole to activate the SAC, they did not initiate apoptosis; instead, the ESCs exit mitosis and cycle in culture for a period of time as a polyploid population (Mantel, et al. 2007). These cells also appear to undergo tetraploid cell division, cycling between 4C and 8C DNA content, and upon differentiation, the polyploid ESCs initiate caspase dependent apoptosis (Mantel, et al. 2007). Understanding how the SAC functions in HSCs and if its activation leads to apoptosis initiation will be important to understand before examining the specific role Mad2 plays in this checkpoint.

Figure 4

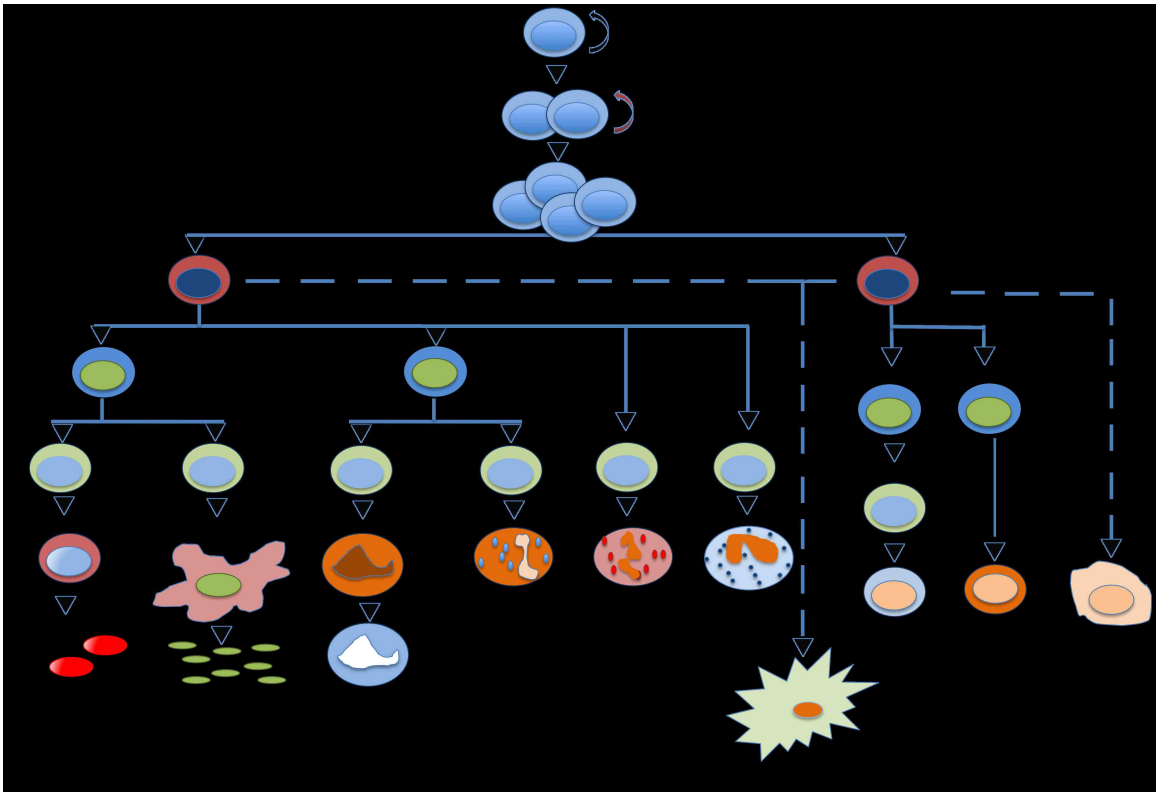


Figure 3. The Hierarchy of Hematopoietic Cells.

Hematopoietic stem cells are multipotent cells that can give rise to all the various lineages of blood cells, including myeloid and lymphoid lineages. The HSC population contains both long-term and short-term repopulating HSC; MPP, multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythroid progenitor; GMP, granulocyte/macrophage progenitor. The pluripotent population of HSC and MPP are Lin^-, $Sca\text{-}1^+$, $c\text{-}kit^+$.

Limited work has been done examining Mad2 and its role in hematopoiesis (Li, et al. 1997), and further examination of this protein could provide more important information on the function of Mad2 in hematopoiesis. Mad2 protein levels in bone marrow (BM) low-density mononuclear cells (LDMC) are reduced by half in *Mad2*$^{+/-}$ mice as compared to *Mad2*$^{+/+}$ mice (Ito, et al. 2007). When the colony forming ability of these cells was examined, *Mad2*$^{+/-}$ BM and spleen manifested decreased absolute numbers and cycling status of immature, but not mature hematopoietic progenitor cells (Ito, et al. 2007). In addition, *Mad2*$^{+/-}$ BM granulocyte-macrophage colony-forming units (CFU-GM) did not proliferate synergistically in response to stem cell factor (SCF) plus GM-CSF. Through the use of immunoprecipitation assays and confocal microscopy, Mad2 was found to associate with the c-kit receptor in the cytoplasm in the human growth factor-dependent cell line MO7e. The function of the hematopoietic progenitor cells (HPC) was looked at this study, but how *Mad2* haploinsufficiency affects the ability of HSCs to compete and self-renew was not examined.

Investigation of the Role Ape1/Ref-1 Plays in Hematopoiesis.

Apurinic/apyrimidinic endonuclease/redox factor 1, Ape1/Ref-1 (referred to here as APE1), is a multifunctional protein possessing both DNA repair and transcriptional regulatory activities. APE1 was first identified as a DNA repair enzyme by two independent groups (Demple, et al. 1991; Robson and Hickson 1991) and was subsequently found to also have redox function (Xanthoudakis and Curran 1992). APE1 acts as the major apurinic/apyrimidinic endonuclease

in the base excision repair (BER) pathway. APE1 is also a transcriptional regulator of gene expression by acting as a redox co-activator of transcription factors, including early growth factor response protein-1 (Egr-1), p53, hypoxia inducible factor-1α (HIF-1α), and activator protein-1 (AP-1) (Huang and Adamson 1993; Jayaraman, et al. 1997; Lando, et al. 2000; Xanthoudakis and Curran 1992). These two activities are located in two functionally distinct domains (Xanthoudakis, et al. 1994). The N-terminus is dedicated to redox function, through cysteine (Cys) 65, and the C-terminus is responsible for the enzymatic activity on abasic sites found in DNA. Although the endonuclease activity found in the C-terminus is highly conserved, the N-terminus containing the redox activity is not. APE1 is the human homologue of *E. coli* exonuclease III protein (Demple, et al. 1991; Robson and Hickson 1991); mammalian APE1 contains a 61-residue N-terminal domain essential for redox activity that is absent from the bacterial homologue (Walker, et al. 1993; Xanthoudakis, et al. 1994). It was hypothesized that the redox activity of APE1 could potentially involve a Cys residue (Xanthoudakis, et al. 1994). When each of the Cys residues in APE1 were individually substituted with alanine (Ala), only C65A hApe1 was redox inactive (Walker, et al. 1993), suggesting that Cys 65 is important for redox activity. In zebrafish Ape (zApe), the residue equivalent to mammalian Ape Cys 65 is threonine (Thr) 58; however, when wild-type zApe enzyme was tested for

redox activity, it was found to be inactive (Georgiadis, et al. 2008). The replacement of Thr 58 with Cys in zApe resulted in a redox active enzyme, further suggesting that a Cys residue at this position is critical for redox function (Georgiadis, et al. 2008).

The physiological relevance of APE1 was investigated by the creation of mice lacking a functional *ref-1* gene (Xanthoudakis, et al. 1996). Homozygous mutant mice die during early embryonic development; death occurs following blastocyst formation, shortly after the time of implantation (Xanthoudakis, et al. 1996). Degeneration of homozygous mutant embryos is evident by embryonic day 5.5, indicating that APE1 is essential for early embryonic development (Xanthoudakis, et al. 1996). In contrast, heterozygous mutant mice develop into adulthood with no apparent abnormalities (Xanthoudakis, et al. 1996). Attempts to isolate stable APE1-knockout cell lines have been unsuccessful to date, making the complete understanding of the molecular targets of APE1 very complicated (Tell, et al. 2009). While several conditional knockout and knockdown strategies have recently been developed (Fung and Demple 2005; Izumi, et al. 2005), which verify the importance of APE1 in cell survival, the understanding of the molecular targets it regulates is still not comprehensive. Initially, the importance of APE1 to cell viability appeared to be due to its DNA repair activity in the BER pathway (Fung and Demple 2005); however, this hypothesis may not be completely true. Attempts to restore APE1 activity to cells not expressing this protein by using the yeast homologue *Apn1* (Fung and Demple 2005), which lacks the redox-

transcriptional domain found in the mammalian protein, or an APE1 mutant lacking acetylation sites but not DNA repair activity (Izumi, et al. 2005), were not successful. These observations imply that both the redox functions and DNA repair activities of APE1 are essential to the role it plays in cell viability.

Several knockdown studies utilizing siRNA have been helpful in examining the role of APE1 in normal and cancer cells (Chen and Olkowski 1994; Evans, et al. 2000; Ono, et al. 1994; Walker, et al. 1994), but these studies do not reveal the individual role of the DNA repair or redox functions of APE1. Knockdown of APE1 removes not only its redox signaling function, but also its DNA repair function and protein-protein interactions with other DNA repair and other proteins independent of the redox or DNA repair functions. E3330, an anti-NF-κB drug, was found to target Ref-1 (Hiramoto, et al. 2002; Shimizu, et al. 2000). E3330 has been shown to be a highly specific inhibitor of Ape1/Ref-1 redox activity, without affecting the repair endonuclease activity (Luo, et al. 2008). E3330 does not increase the percentage of cells undergoing apoptosis, but rather blocks both normal and cancer cell growth by stopping cell proliferation (Fishel, et al. 2010; Luo, et al. 2008). While use of this specific small molecule inhibitor of APE1 redox is not directly comparable to siRNA knockdown studies, this specific inhibitor does allow the redox function of APE1 to be examined separately from its DNA repair activity.

The role that the redox function of APE1 plays in cellular functions has been examined in many settings, particularly in cancer cells as a therapeutic agent (Fishel, et al. 2010; Zou, et al. 2009; Zou and Maitra 2008); however little work has been done examining the role that APE1 redox function plays in normal hematopoiesis. A study in mouse embryonic stem (ES) cells and ES cell-derived embryoid body (EB) cells revealed that siRNA knockdown of APE1 expression induced G_1 arrest and decreased the percent of ES cells in S-phase, indicating APE1 regulates cell-cycle status in ES cells and also positively regulates the G_1/S transition of the cell cycle in EB cells (Zou, et al. 2007). Knockdown of APE1 in EB cells was also shown to decrease hemagioblast formation, as well as diminish formation of primitive and definitive hematopoietic colonies (Zou, et al. 2007). Through the use of E3330, it was found that the redox function, but not the DNA repair activity, of APE1 is required for normal embryonic hematopoiesis (Zou, et al. 2007). The redox function of this protein has not been thoroughly evaluated in normal adult hematopoiesis.

Research Goals.

We first wanted to determine if the SAC was functional in hematopoietic cells, and if its activation would lead to the initiation of apoptosis. Previous data shows that the SAC is uncoupled from apoptosis initiation in mouse and human ESCs (Mantel, et al. 2007). ESCs exit mitosis and become polyploid after prolonged activation of the SAC; ESCs accumulate in M-phase of the cell cycle after microtubule disruption with nocodazole, but exit mitotic delay and re-enter a

polyploid mitosis (Mantel, et al. 2007). This is in contrast to normal somatic cells and cell lines with normal p53 responses, which do not respond to prolonged activation of the SAC in this manner. These cells initiate apoptosis after exiting the cell cycle in a G_0/G_1-like state with 4C DNA content, often in two nuclei, or enter senescence (Lanni and Jacks 1998; Lanni, et al. 1997). These data, along with findings of increased pseudomitotic index in neural and hematopoietic progenitor cells (Damelin, et al. 2005), suggest that the SAC might also be uncoupled from apoptosis in HSCs. Proper function of the SAC is vital to prevent polyploidy/aneuploidy during *ex vivo* culture and expansion of HSC and their progenitors.

We were interested in further studying the function of Mad2 in mammalian hematopoiesis for two reasons. First, differentiated cells in many tissues have a relatively short life span and are continuously replaced by new cells generated from progenitor cells (Damelin, et al. 2005). In human bone marrow, approximately 200 billion erythrocytes and 70 billion neutrophilic leukocytes are produced and released into the bloodstream every day (Gunsilius, et al. 2001). Because HSCs and subsequently HPCs give rise to all the cells in the hematopoietic system, it is of special importance that these cells accurately transmit their genome to their many progeny and that their checkpoints function properly. Therefore, we were interested in studying HSCs to determine if *Mad2* haploinsufficiency adversely affects the stem cell's ability to self-renew and differentiate into multiple lineages. The process of stem cell self-renewal is not

completely understood. It is hypothesized that the stem cell must divide in a way that maintains pluripotency in at least one of the daughter cells. This type of division, an asymmetrical division, most likely occurs through the segregation of cell fate determinants, which is established by the spindle orientation during mitosis (Piatti, et al. 2006). Since positioning of the spindle is also part of the spindle assembly checkpoint (Mantel, et al. 2008), *Mad2* haploinsufficiency could affect this aspect of the cell cycle, and therefore, cause problems in stem cell self-renewal and differentiation. Second, chromosomal instability (CIN) leading to an aberrant chromosome number (aneuploidy) is a hallmark of many cancers (Bharadwaj and Yu 2004; Jallepalli and Lengauer 2001), but the mechanism and molecular determinants promoting CIN are not clearly understood (Jallepalli and Lengauer 2001). There is increasing evidence that suggests a role of the SAC in promoting aneuploidy and tumorigenesis (Taylor, et al. 2004). It is possible that *Mad2* haploinsufficiency could cause defects in both processes involved in the spindle assembly checkpoint: the first timer delaying the onset of anaphase until all chromosome are properly aligned, and the second timer regulating cytokinesis and mitotic exit. Deficiencies in either one or both of these timers could potentially lead to chromosome missegregation and aneuploidy in the *Mad2*$^{+/-}$ cells. *Mad2*$^{+/-}$ mice develop lung tumors at high rates after long latencies, implicating defects in the spindle assembly checkpoint in tumorigenesis (Michel, et al. 2001). Decreased expression of Mad2 in colon cancer cells, along with other spindle checkpoint proteins, causes deficiencies in SAC activation; this leads to mitotic aberrations including improper alignment of chromosomes at the

metaphase plate, lagging chromosomes during anaphase, anaphase/telophase chromatin bridges, multipolar divisions, and formation of polynucleated (Payne, et al. 2010).

Previous studies in our laboratory found a higher percentage of cell death in KL (c-kit^{+}, lin^{-}) BM of *Mad2*$^{+/-}$ mice compared to *Mad2*$^{+/+}$ BM, when cells were cultured in SCF and GM-CSF (Ito, et al. 2007). However, there was no significant difference between *Mad2*$^{+/-}$ and *Mad2*$^{+/+}$ mice in the percentage of cell death of BM cells before cell culture, suggesting that greater cell death may occur in *Mad2*$^{+/-}$ HPC in stress conditions but not in steady state conditions (Ito, et al. 2007). To determine if *Mad2*$^{+/-}$ cells are more sensitive than *Mad2*$^{+/+}$ cells, we treated *Mad2*$^{+/-}$ and *Mad2*$^{+/+}$ mice with various cytotoxic agents and examine the *in vivo* cytotoxic effects these agents have on HPC colony formation.

It was reported that the redox function, but not the DNA repair activity, of APE1 is required for normal embryonic hematopoiesis (Zou, et al. 2007); however, the redox function of this protein has not been thoroughly evaluated in normal adult hematopoiesis. Using E3330, we examined the effect that inhibiting the redox function of APE1 has on normal adult hematopoiesis and also on sub-optimal hematopoiesis.

MATERIALS AND METHODS

Mice.

Mad2$^{+/+}$ wild-type and *Mad2*$^{+/-}$ mutant mice were originally generated by interbreeding the wild-type and mutant mice kindly provided by Dr. Robert Benezra (Memorial Sloan-Kettering Cancer Center, New York, NY). These mice were backcrossed with C57Bl/6J (Bl/6; CD45.2) mice (Jackson Laboratories, Bar Harbor, ME, USA) for at least 8 generations in our animal housing facility before experimental use. All of the mice were used at 6 to 10 weeks of age. For competitive repopulation assay, C57/BoyJ F1 (F1; CD45.1/CD45.2) and B6.SJL-PtrcaPep3b/BoyJ (Boy J; CD45.1) mice were obtained from an on-site core breeding colony. The Institutional Animal Care and Use Committee of the Indiana University School of Medicine approved all experimental procedures.

Cytokines, Antibodies, and Other Agents.

Purified recombinant preparations of murine (rmu) granulocyte macrophage-colony stimulating factor (GM-CSF) and stem cell factor (SCF) were purchased from R&D Systems (Minneapolis, MN). Purified recombinant human (rhu) erythropoietin (Epo) was purchased from Amgen (Thousand Oaks, CA). Thrombopoietin (TPO) was purchased from Genentech (Oceanside, CA) and rhu FMS-like tyrosine kinase 3 ligand (Flt3) was purchased from Immunex (Seattle, WA). Pokeweed mitogen mouse spleen cell-conditioned medium (PWMSCM) was prepared as described (Cooper and Broxmeyer 1991).

For flow cytometric analysis, anti-mouse antibodies c-Kit, Sca-1, FcγR III/II, IL-7Rα, Lineage Cocktail (CD3e, CD11b, CD45R/B220, Erythroid Cells, and Ly-6G and Ly-6C), secondary antibodies, and isotype controls were purchased from BD Biosciences (San Diego, CA). Anti-mouse CD34 (cluster of differentiation molecule 34) antibody was purchased from eBioscience (San Diego, CA) and Sca-1 was also purchased from BioLegend (San Diego, CA). Hoechst 33342 used for staining DNA was obtained from Invitrogen (Carlsbad, CA). Antibodies to phospho-histone H3 (Ser10) and cleaved caspase 3 (Asp175) were purchased from Cell Signaling Technology (Danvers, MA). Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit was purchased from BD Biosciences (San Diego, CA).

The following drugs purchased from Sigma-Aldrich (St. Louis, MO) were used to stress mice and examine BM progenitor recovery: cyclophosphamide monohydrate (cytoxan) and cytarabine (Ara-C). To condition mice before BM transplantation or examine BM recovery, γ-radiation (^{137}Cs source, single dose) was used. E3330 was kindly provided by Dr. Mark R. Kelley; its synthesis has been previously described (Luo, et al. 2008).

Histological Analysis.

Spleen, liver, and other masses were collected from four wild-type mice and eleven *Mad2*$^{+/-}$ mice. Fresh tissue were prepared for histological analysis by freezing in peel away embedding molds (Electron Microscopy Sciences, Hatfield,

PA) with Tissue-Tek® O.C.T. Compound (Ted Pella, Inc., Redding, CA) on dry ice. The Clarian Pathology IHC Core Laboratory prepared slides of the tissue samples for histological analysis. Histology was performed on frozen tissue sections that were stained with hematoxylin and eosin (H&E) to show extramedullary hematopoiesis in spleen, liver, or other sites. Slides were kindly analyzed by Dr. Atillio Orazi (Weill Medical College of Cornell University and New York-Presbyterian Hospital, New York, NY).

Isolation and Culture of LDMCs From Murine BM.

Murine BM was isolated from both femurs of mice using aseptic procedures. Low-density mononuclear cells were isolated by Lympholyte®-M (Cedarlane Laboratories Ltd., Burlington, NC) separation. Cells were washed three times with phosphate buffered saline (PBS) and resuspended in Roswell Park Memorial Institute medium (RPMI) + 10% heat inactivated fetal bovine serum (FBS) + 100 ng/ml each of TPO, Flt3, and SCF and then incubated at 37ºC for 24 hours before treatment with nocodazole (Sigma-Aldrich, St. Louis, MO). The drug was dissolved and diluted in dimethyl sulfoxide (DMSO), and the concentration of nocodazole used in experiments was 1 ng/ml.

Multivariate Permeablized Flow Cytometry.

After harvesting and washing with PBS, single cell suspensions were stained with primary antibodies, followed by staining with secondary antibodies each for 30 minutes in the dark. Cells suspensions were washed three times with PBS +

1% bovine serum albumin (BSA) after primary and secondary staining. Cells were permeablized and fixed using Cytofix/Cytoperm™ Fixation/Permeabilization solution and then stained with various antibodies to phospho-histone H3 or cleaved caspase 3, washed with Perm/Wash Buffer, and counterstained with Hoechst 33342. Flow cytometric data was acquired using an LSR II (BD, San Diego, CA) analyzer. Data was analyzed with WinList program (Verity Software House, Topsham, ME).

Phenotypic Analysis.

The following hematopoietic stem and progenitor populations were assessed in *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ BM as previously described (Katsumoto, et al. 2006): lineage^{-} sca-1^{+} c-kit^{+} IL-7Rα^{-} cells (a population enriched for HSC), common lymphoid progenitor (CLP; Lin^{-} Sca-1^{+} c-kit^{+} IL-7Rα^{+}), megakaryocyte/erythroid progenitor (MEP; Lin^{-} Sca-1^{-} c-kit^{+} CD34^{-} FcγR$^{-/lo}$), common myeloid progenitor (CMP; Lin^{-} Sca-1^{-} c-kit^{+} CD34^{+} FcγR$^{-/lo}$), and granulocyte/macrophage progenitor (GMP; Lin^{-} Sca-1^{-} c-kit^{+} CD34^{+} FcγR$^{+/hi}$). Bone marrow from mice was collected and stained with antibodies to surface markers previously described. Data was collected from the samples using an LSR II (BD, San Diego, CA) and BD FACSDiva software (BD, San Diego, CA). Data was analyzed using WinList software (Verity Software House, Topsham, ME).

Clonogenic Progenitor Cell Assay.

Bone marrow from mice was assessed for colony-forming unit granulocyte-macrophage (CFU-GM), erythroid burst-forming unit (BFU-E), and multipotential granulocyte, erythroid, monocyte, megakaryocyte CFU (CFU-GEMM) progenitor cells, as described elsewhere (Broxmeyer, et al. 2005). In short, for methylcellulose assays, 5 x 10^4 BM cells or 5 x 10^5 spleen cells were plated and cultured in 0.9% methylcellulose culture medium with 30% non-heat inactivated FBS (Hyclone Laboratories, Logan, UT), 5% vol/vol PWMSCM, 50 ng/mL rmuSCF, 1 U/mL rhuEpo, 2 mM glutamine (Cambrex Bio Science, Walkersville, MD), 10^{-4} M 2-mercaptoethanol, and 0.1 mM hemin (Sigma-Aldrich, St. Louis, MO). For single cytokine assays, 5 x 10^4 cells were plated and cultured in 0.3% methylcellulose culture medium in the presence of 50 ng/mL rmuSCF, 10 ng/mL rmuGM-CSF, or a combination of the two. All test groups were plated in triplicate. Colonies were scored after 7 days of incubation at 37ºC, in 5% O_2 and 5% CO_2, unless otherwise noted. Absolute numbers of progenitors were calculated from the nucleated cellularity per femur and the number of colonies formed per number of cells plated. The percentage of progenitors in the S-phase of the cell cycle was estimated by use of the tritiated thymidine kill technique, as described previously (Cooper, et al. 1994). For assays examining the effect of E3330 on colony formation, 5 x 10^4 BM cells were incubated with varying concentrations of the inhibitor for 4 hours at 37ºC prior to plating in methylcellulose as described above. Colonies were scored after 7 days of incubation at 37ºC, in either 20% and 5% CO_2 or 5% O_2 and 5% CO_2.

Recovery of BM Progenitors From Cytotoxic Effects.

Mad2$^{+/+}$ and *Mad2*$^{+/-}$ mice were treated with cyclophosphamide/cytoxan (200 mg/kg, intraperitoneal (i.p.)), high sub-lethal levels of γ-irradiation (650 cGy whole body irradiation), or Ara-C (500 mg/kg, subcutaneously (s.c.)) on day zero of the experiment. For the mice treated with Ara-C, we analyzed mice at days 1, 3, and 5 after injection. Mice treated with cytoxan were analyzed on days 1, 3, 5, and 7 after injection. Mice treated with γ-irradiation were analyzed on days 7, 10, and 14 after irradiation. For each treatment, both control *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ mice were assessed at day zero without any treatment. For each treatment used, the days that we examined have been shown by our laboratory and others to be optimal for assessing rebound hematopoiesis (Broxmeyer, et al. 1976; Broxmeyer, et al. 2006; DeWys, et al. 1970; Hromas, et al. 2002; Radley and Scurfield 1979). For analysis, BM and spleen were aseptically harvested from the treated mice at the various time points stated above, and colony assays performed as previously described.

Competitive Repopulation Assay.

Estimation of stem cell function in lethally irradiated hosts was performed using a competitive repopulation assay (CRA) as adapted from Harrison and colleagues (Harrison 1980; Harrison, et al. 1993). In CRAs performed in ablated hosts, varying ratios of freshly isolated *Mad2*$^{+/+}$ or *Mad2*$^{+/-}$ C57/Bl/6 donor cells were mixed with freshly isolated Boy J competitor cells, and transplanted into 950 cGy conditioned (^{137}Cs source, single dose) F1 recipients as described previously

(Campbell, et al. 2009). Donor, competitor, and recipient peripheral blood chimerism was determined at 1, 2, 4, and 6 months posttransplantation. Peripheral blood was collected by tail-vein bleeding into heparinized microcapillary tubes (Fisher Scientific, Pittsburg, PA). Red blood cells (RBCs) in the PB were lysed by incubating with RBC lysis buffer (0.155 M NH_4Cl, 0.01 M $KHCO_3$, 0.1 mM EDTA in H_2O and filter sterilized) and samples were washed in PBS + 2% BSA. Cells were stain with antibodies to anti-CD45.1 PE (phycoerythrin) and anti-CD45.2 APC (allophycocyanin) (BD-Pharmingen) and analyzed using a FACSCalibur with APC (Campbell, et al. 2009). Peripheral blood from BoyJ, F1, and C57/Bl6 mice was used as controls. Data analysis was done using FCS Express V3 software (De Novo Software, Ontario, Canada).

Secondary Non-competitive Transplantation.

Six months after the primary competitive repopulation assay was started, BM cells were aseptically harvested from primary F1 recipient mice, resuspended in IMDM, and 2 x 10^6 cells were injected into secondary F1 recipient mice irradiated with 950 cGy total body irradiation. PB was collected from secondary animals at 1, 2, 4, and 6 months after transplantation and analyzed for CD45.1 and CD45.2 chimerism as described above.

Statistical Analysis.

Data were analyzed statistically using the Student's t-test. A P values less than 0.05 was considered statistically significant.

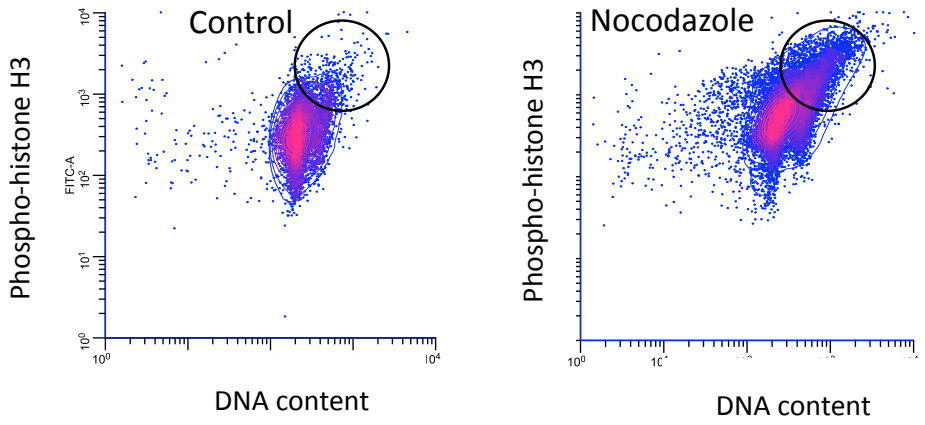
RESULTS

Nocodazole Treatment Causes Cell Cycle Arrest in HSCs.

To evaluate whether phenotypically defined mouse HSCs function like ESCs with respect to checkpoint-apoptosis coupling, we first examined the ability of HSCs to arrest in M-phase after microtubule disruption. Low-density mononuclear cells isolated from murine bone marrow were cultured in the cytokine cocktail of TPO, Flt3, and SCF for 24 hours before addition of nocodazole used to activate the SAC, in order to promote cycling in the normally quiescent HSC population. Leukocytes and platelets are the main constituents of the LDMC population from the BM, but this population also contains HPC and HSC. Cells were incubated for 48 hours after nocodazole treatment, then stained and analyzed by flow cytometry. Within the mononuclear fraction of cells, DNA content and levels of phospho-histone H3 were examined in Sca-1^{+}/c-kit^{+}/lin^{-} (LSK) cells. Phospho-histone H3 is tightly correlated with chromosome condensation during mitosis and therefore serves as a marker for mitosis (Gunsilius, et al. 2001). HSCs accumulated in M-phase of the cell cycle after microtubule disruption with nocodazole treatment (Figure 5A). This arrest is evident by the increase of phosphorylation at histone H3. This population of cells with higher levels of phospho-histone H3 also appears to have 4C DNA content, also indicative of cell cycle arrest in M-phase (Figure 5B).

Figure 5

A


Control
Nocodazole
Phospho-histone H3
Phospho-histone H3
FITC-A
DNA content
DNA content

B

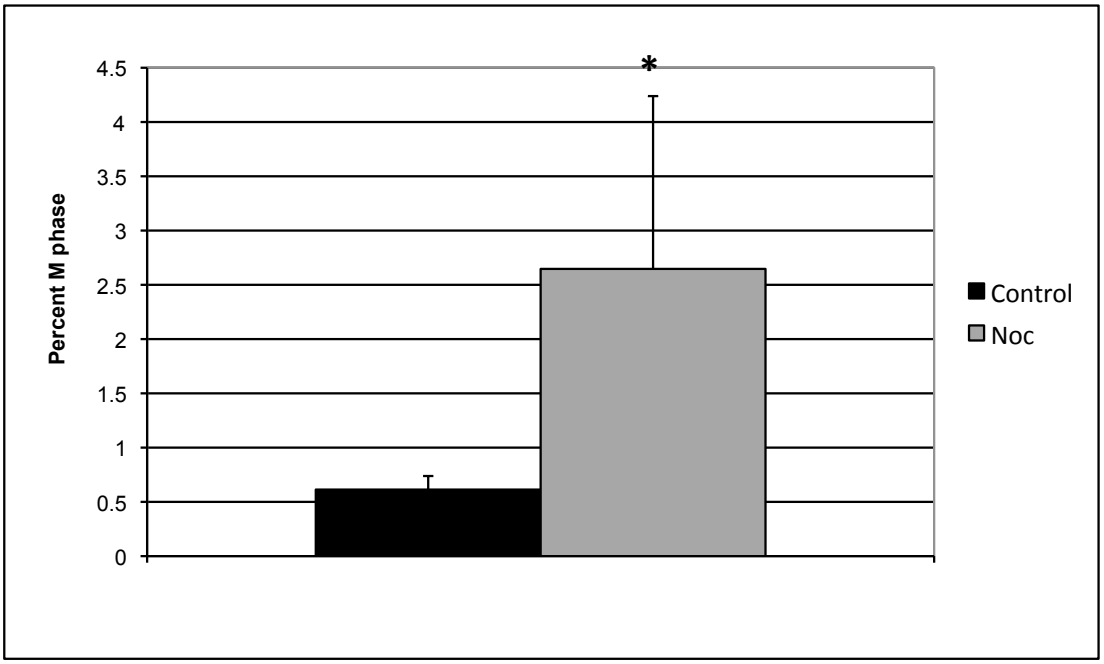

Percent M phase
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Control
Noc

Figure 5. HSCs Arrest in M-phase After Nocodazole Treatment.

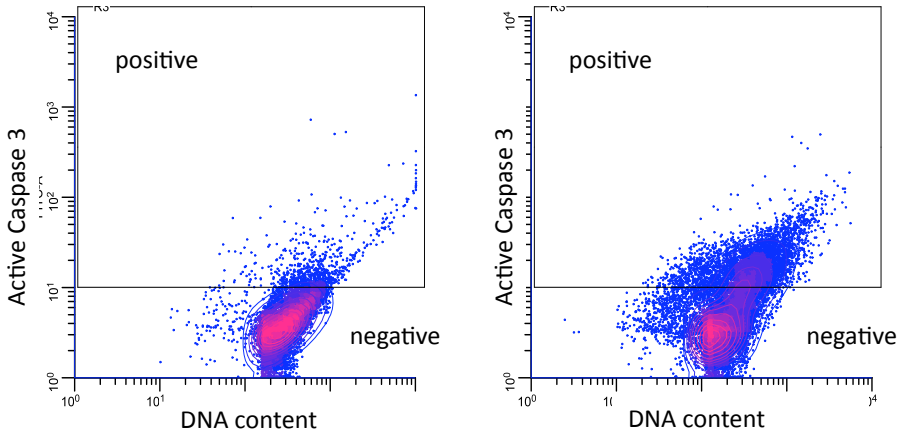
(A) Murine low-density mononuclear cells (LDMCs) were treated with nocodazole for microtubule depolymerization or control solvent for 48 hours in media containing RPMI + 10% FBS + 100 ng/ml each of TPO, Flt3, and SCF as described in "Materials and Methods" after a 24 hour incubation in media and cytokines alone. Cells were harvested and analyzed by multivariate permeablized flow cytometry for simultaneous phospho-histone (ser10) H3 and DNA content. Results for phospho-histone H3 and DNA content are those gated on the Sca-1^+/c-kit^+/lin^- (LSK) population of cells. The circular regions indicate the cells that are in M-phase, as indicated by high levels of phospho-histone H3 and 4C DNA content (N=3). (B) Percentage of cells in M-phase as indicated by cells with 4C DNA content (N=3). Data were analyzed using the Student's t-test. *$p < 0.05$.

Phenotypically Defined HSCs Initiate Apoptosis After SAC Activation.

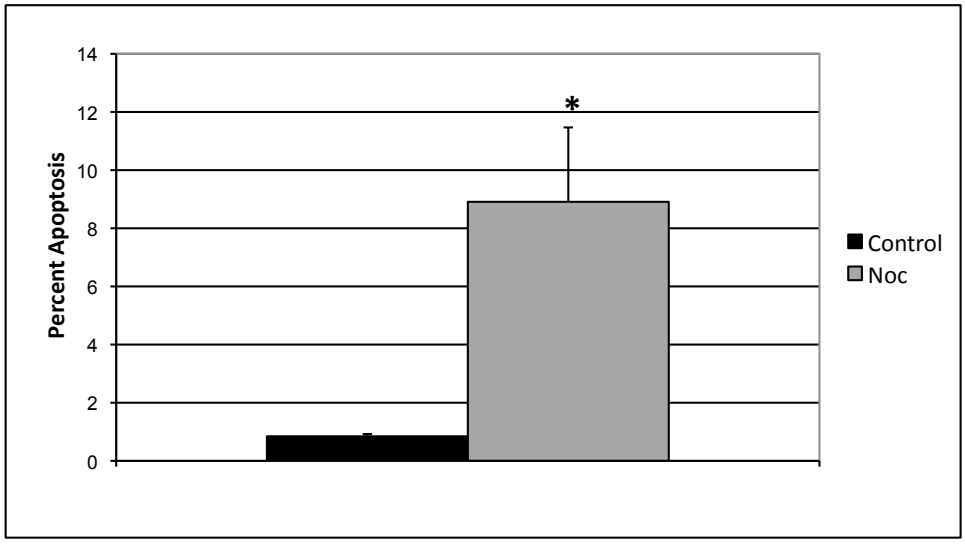
To further examine if HSCs function like ESCs with respect to checkpoint-apoptosis coupling, we investigated apoptotic responses in HSCs after SAC activation. Upon visual inspection, fewer nocodazole treated cells were observed when compared to the control non-treated cells although equal numbers of cells were initially present in both groups. However, the percentage of LSK cells present in the control and nocodazole treated population after culturing LDMC for the specified period of time was not significantly different, indicating that the percentage of LSK cells present was not altered by the presence of nocodazole. After SAC activation via nocodazole treatment, increased levels of activated caspase 3 (Figure 6A, B) and an increase in hypodiploidy (Figure 6C) were observed in the treated phenotypically defined HSCs compared to untreated HSCs. Caspase 3 is a crucial executioner of apoptosis; it is either partially or entirely responsible for the proteolytic cleavage of many key proteins, such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP) (Fernandes-Alnemri, et al. 1994). Increased levels of activated caspase 3 are indicative of apoptosis activation in this population of cells. These data suggest that HSC can efficiently initiate apoptosis after activation of the SAC, indicating that checkpoint activation is coupled with apoptosis in this population of cells.

Figure 6

A


positive
negative
Active Caspase 3
DNA content
positive
negative
Active Caspase 3
DNA content

B


Percent Apoptosis
14
12
10
8
6
4
2
0
*
Control
Noc

C

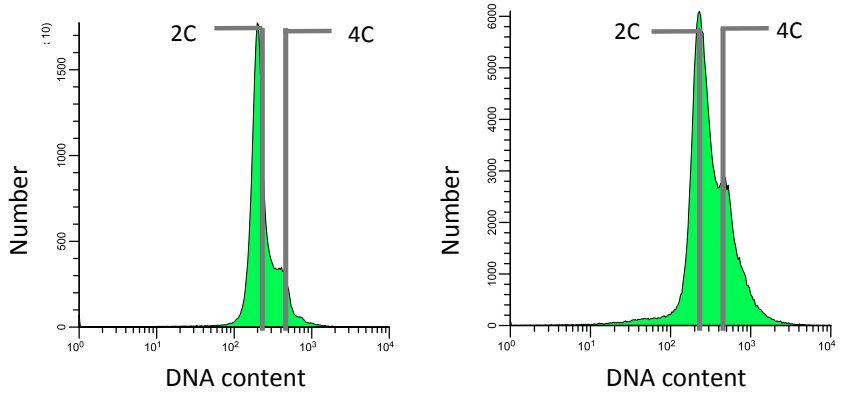

2C
4C
Number
DNA content
2C
4C
Number
DNA content

Figure 6. After Arresting in M-phase, HSCs Initiate Apoptosis.

Murine LDMC were treated as in Figure 5A and analyzed by flow cytometry for activated caspase 3 and DNA content. Results for activated caspase 3 and DNA content are those gated on the Sca-1^+/c-kit^+/lin^- (LSK) population of cells within murine LDMC. (A) Representative dot plots from flow cytometric analysis show elevated levels of activated caspase 3 and DNA content. (B) The percentage of apoptotic cells is indicated by cells with elevated levels of activated caspase 3 (N=3). (C) Cells treated with nocodazole exhibit high levels of sub-G_1 DNA content when compared to control cells, which is indicative of apoptosis. Data was analyzed using the Student's t-test. *$p<0.05$.

SAC Activation Results in Mitotic Arrest and Apoptosis in Phenotypically Defined HSCs From Both *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ Mice.

After determining that the SAC is functional in HSCs, we next examined if *Mad2* haploinsufficiency affected the ability of phenotypically defined HSCs to arrest in mitosis compared to wild-type HSCs. Low-density mononuclear cells from *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ mice were collected and treated with nocodazole, as described in the previous section. Within the mononuclear fraction of cells, DNA content and levels of phospho-histone H3 were examined in LSK cells. LSK cells from both *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ mice exhibited a significant increase in phospho-histone H3 levels, indicating that these cells arrest in M-phase of the cell cycle after nocodazole treatment for 24 hours (Figure 7A). Along with the arrest in M-phase, we noted that the 24 hour nocodazole treated *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ LSK cells have significantly increased levels of sub-G_1 DNA content when compared to the control treated cells (Figure 7B). No significant differences existed between the 24 and 48 hour treatment time points for each group examined, and also no differences were found between *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ control or nocodazole-treated groups. These data are in agreement with data examining mitotic arrest in cells from *Mad2*$^{+/+}$, *Mad2*$^{+/-}$, and *Mad2*$^{-/-}$ embryos in response to spindle disruption (Dobles, et al. 2000). Cells from *Mad2*$^{-/-}$ embryos failed to arrest in mitosis, but cells from *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ embryos exhibited similar levels of mitotic arrest in response to nocodazole treatment (Dobles, et al. 2000).

Figure 7

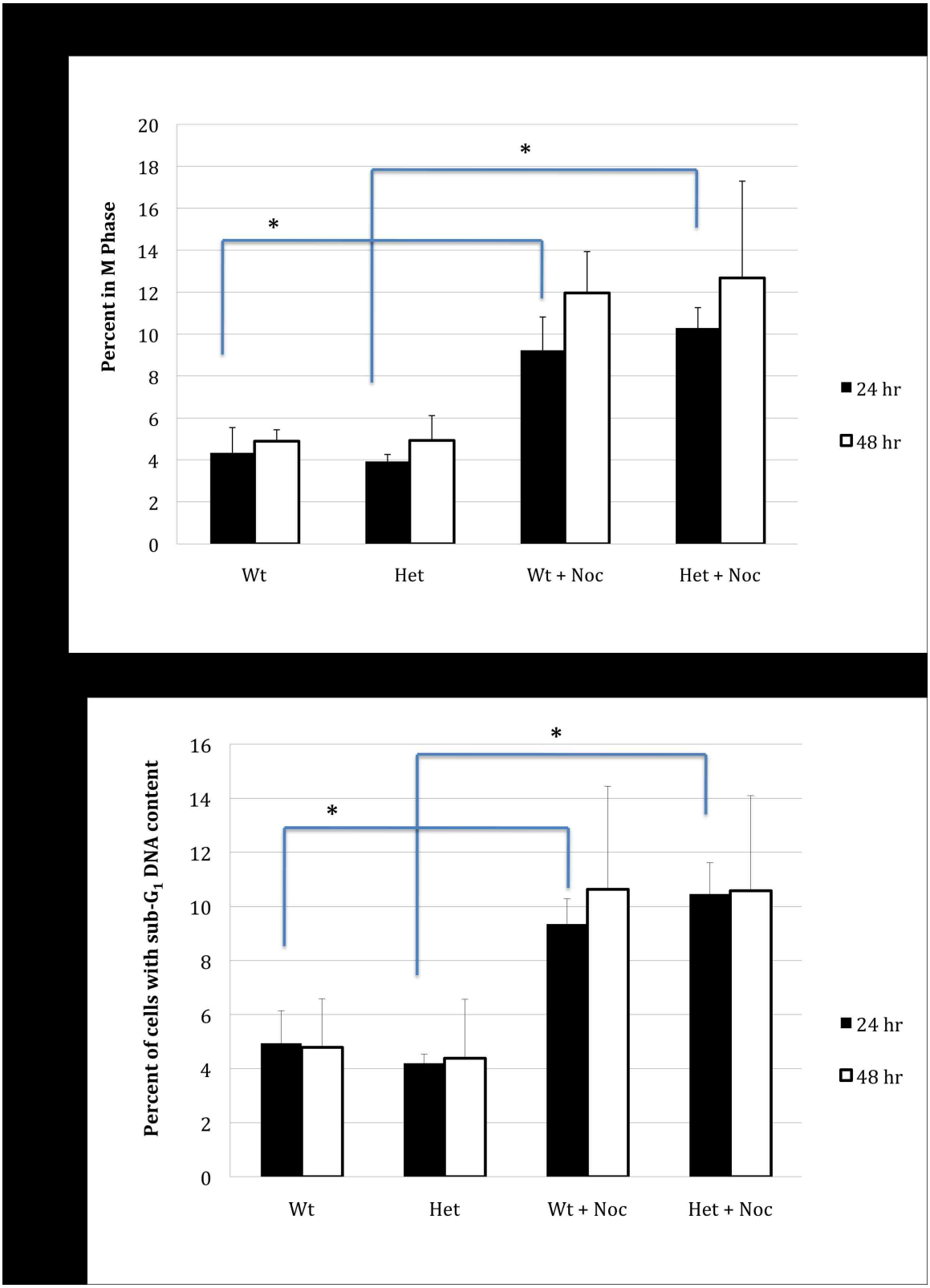

Percent in M Phase
20
18
16
14
12
10
8
6
4
2
0
*
*
Wt
Het
Wt + Noc
Het + Noc
24 hr
48 hr
Percent of cells with sub-G1 DNA content
16
14
12
10
8
6
4
2
0
*
*
Wt
Het
Wt + Noc
Het + Noc
24 hr
48 hr

Figure 7. *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ HSCs Arrest in M-phase After Nocodazole Treatment.

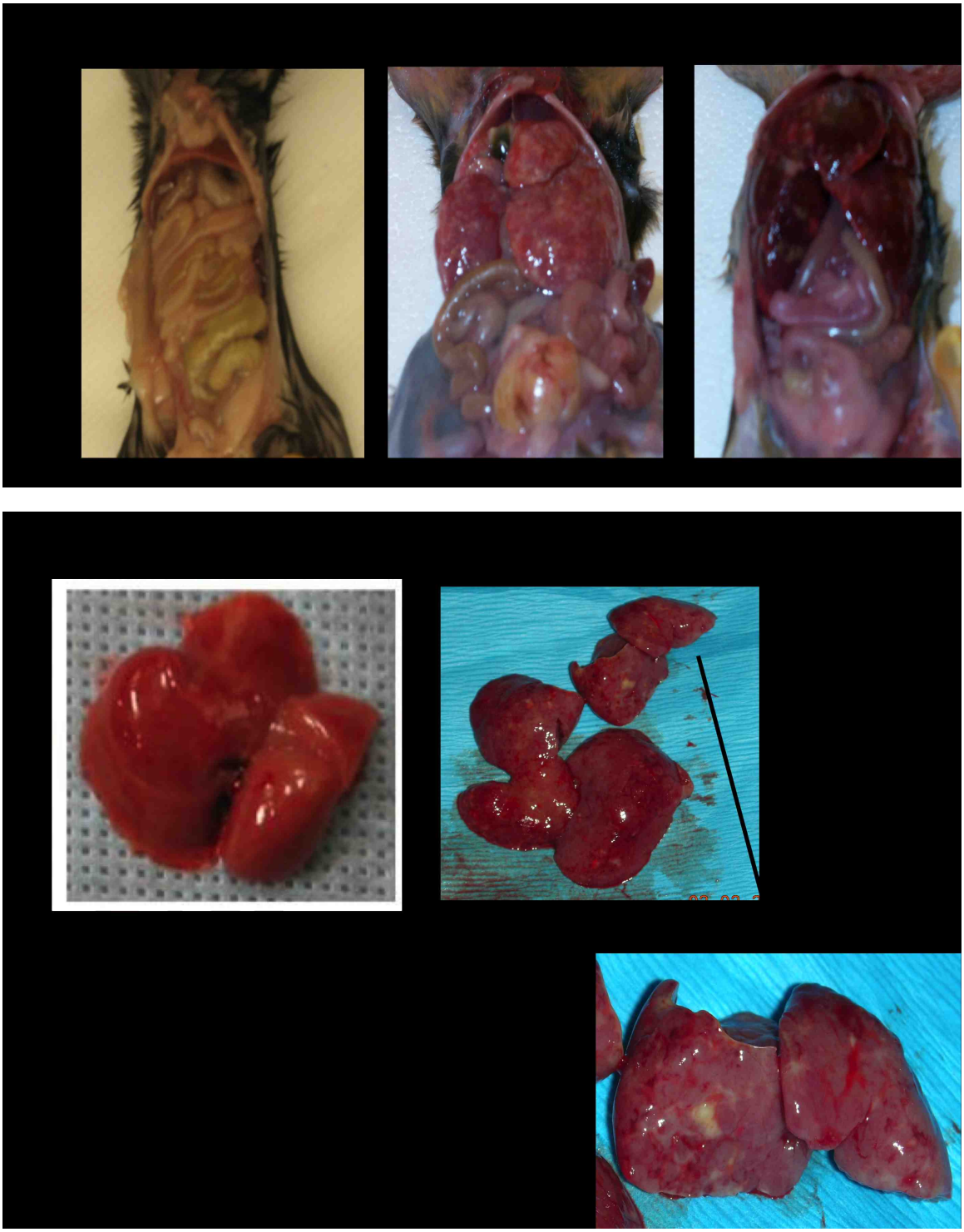
Low-density mononuclear cells from the BM of wild-type *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ mice were treated with nocodazole for microtubule depolymerization or control solvent for 24 or 48 hours in media containing RPMI + 10% FBS + 100 ng/ml each of TPO, Flt3, and SCF. Cells were harvested and analyzed by multivariate permeablized flow cytometry for phospho-histone (ser10) H3. Results for phospho-histone H3 are those gated on LSK population of cells. (A) Percentage of cells in M-phase as indicated by cells with 4C DNA content and increased phospho-histone H3 levels (N=3). (B) Both *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ cells treated with nocodazole exhibit higher levels of sub-G_1 DNA content when compared to untreated cells, which is indicative of apoptosis (N=3). Data were analyzed using the Student's t-test. *$p < 0.05$ when comparing control to nocodazole treatment *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ HSC.

Aged *Mad2*$^{+/-}$ Mice Exhibit Characteristics of a Myeloproliferative-like Disorder.

Previous studies noted that *Mad2*$^{+/-}$ mice develop normally, and blastocysts from *Mad2*$^{+/-}$ animals display no evidence of an impaired checkpoint activation, but premature sister-chromatid separation was not examined in these experiments (Dobles, et al. 2000). Subsequent studies found increased levels of premature sister-chromatid separation, as well as increase in aneuploidy, in *Mad2*$^{+/-}$ human cancer cells and primary murine embryonic fibroblasts when compared to wild-type (Michel, et al. 2001). Importantly, a high frequency of papillary lung adenocarcinomas, an extremely rare tumor in most wild-type mouse strains, is found in *Mad2*$^{+/-}$ mice killed between 18 and 19 months of age, suggesting a role for defects in the mitotic checkpoint in tumorigenesis (Tuveson and Jacks 1999). Defects in the mitotic checkpoint have been identified in colon, lung, and breast cancer cell lines (Li and Benezra 1996). While *MAD2* mutations have not been identified in cancer cell lines with mitotic checkpoint defects (Cahill, et al. 1999; Takahashi, et al. 1999), subtle differences in *MAD2* protein levels appear to be sufficient to markedly alter checkpoint function (Michel, et al. 2001). As a result, inactivation of one *MAD2* allele could be enough to create a haploinsufficient effect and lead to loss of mitotic checkpoint control. Therefore, we examined aged *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ mice, ranging in age between 11 and 39 months old, to see if any irregularities existed. The mice were dissected, and various organs were examined for the presence of abnormalities. Several of the *Mad2*$^{+/-}$ mice examined exhibited marked hepatomegaly (Figure 8A, B), and in most instances

splenomegaly was also observed. Some of these mice also had large masses in their lower abdomens, possibly originating from the small intestine. All observations in aged *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ mice are listed in Table 2. Organs from aged *Mad2*$^{+/+}$ (1 out of 4) and *Mad2*$^{+/-}$ (8 out of 11) mice exhibited an extremely high infiltration of hematopoietic cells, which is characteristic of a myeloproliferative disorder (MPD); representative slides of liver sections with H&E staining are shown in Figure 8C. MPD is characterized by the clonal proliferation of one or more hematopoietic cell lineages, predominantly in the bone marrow, but sometimes in the liver and spleen (Talarico 1998). A more complete analysis of these mice would be required in order to definitively diagnose them with MPD, including examination of multiple organs, particularly the bone marrow, as well as having age matched controls.

Figure 8



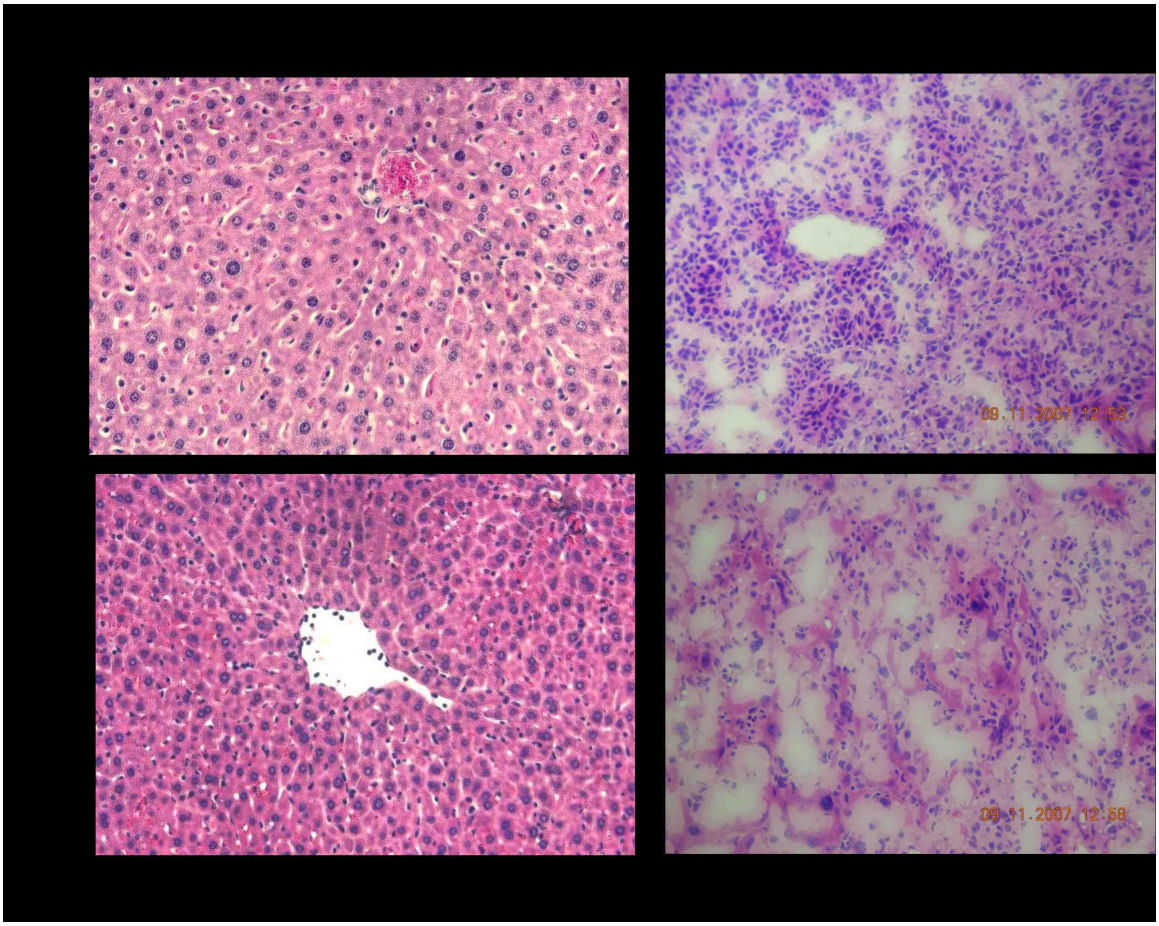

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Figure 8. Aged *Mad2*$^{+/-}$ Mice Exhibit Characteristics of Myeloproliferative Disorder.

(A, B) The mice were dissected, and organs were examined for the presence of abnormalities. Representative mice with liver abnormalities, along with wild-type mice are shown. (C) Frozen sections were prepared and stained with H&E to show extramedullary hematopoiesis in spleen, liver, or other sites. Representative slides from liver sections are shown.

Mouse #	Strain	Sex	Age	Observations
1	*Mad2*$^{+/-}$	Female	11 months	Extremely enlarged liver with white spots; large mass in abdomen of unknown tissue origin
2	*Mad2*$^{+/-}$	Female	39 months	Extremely enlarged liver
3	*Mad2*$^{+/-}$	Male	30 months	Slightly enlarged spleen; extremely enlarged liver with white masses
4	*Mad2*$^{+/-}$	Male	24 months	No abnormalities
5	*Mad2*$^{+/-}$	Female	26 months	Slightly enlarged spleen & liver; mass in lower abdomen
6	*Mad2*$^{+/-}$	Female	26 months	Slightly enlarged spleen & liver; mass in lower abdomen
7	*Mad2*$^{+/-}$	Male	24 months	Slightly enlarged liver
8	*Mad2*$^{+/-}$	Female	38 months	No abnormalities
9	*Mad2*$^{+/-}$	Female	20 months	No abnormalities
10	*Mad2*$^{+/-}$	Male	20 months	Extremely enlarged spleen & liver
11	*Mad2*$^{+/-}$	Male	33 months	Slightly enlarged spleen & liver
12	*Mad2*$^{+/+}$	Male	24 months	Enlarged liver; increased WBCs in PB
13	*Mad2*$^{+/+}$	Female	16 months	No abnormalities
14	*Mad2*$^{+/+}$	Female	13 months	No abnormalities
15	*Mad2*$^{+/+}$	Male	18 months	No abnormalities

Table 2. Observations in Aged *Mad2*$^{+/-}$ and *Mad2*$^{+/+}$ Mice.

Shown are the strain, age, sex, and abnormalities observed in aged *Mad2*$^{+/-}$ and *Mad2*$^{+/+}$ mice. All mice examined were on a mixed strain background.

***Mad2* Haploinsufficiency Protects HPC From the Cytotoxic Effects of Cell-Cycle Specific Chemotherapeutic Agents.**

Because of the importance of Mad2 in mitosis, we hypothesized that the hematopoietic system of *Mad2*$^{+/-}$ mice might be more sensitive than *Mad2*$^{+/+}$ mice to the cytotoxic effects of irradiation and chemotherapeutic agents. We therefore evaluated the *in vivo* cytotoxic effects of irradiation and chemotherapeutic agents on BM HPC from *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ mice. Mice were treated with either cyclophosphamide (200 mg/kg, i.p.), a high sub-lethal dose (650 cGy) of γ-radiation, or cytarabine (Ara-C) (500 mg/kg, s.c.) and then assessed for recovery of the HPC, expressed as absolute numbers per femur. The recovery of HPC was evaluated through examination of colony-forming ability at lowered (5%) oxygen (O_2) tension. Lowered O_2 tension much more closely mimics the O_2 tension in bone marrow, and is more physiological. Bone marrow HPC colonies grow better at 5% O_2 tension compared to colonies grown at 20% O_2 tension (Bradley, et al. 1978). Hence the reason all of our studies assessing HPC were done at 5% O_2 tension, unless stated otherwise. No significant differences were observed in the recovery of functional BM or spleen HPC after treatment with cyclophosphamide (Figure 9A, B) or γ-radiation (Figure 10A, B); however, we found that *Mad2* haploinsufficiency protected the BM HPCs from the cytotoxic affects of Ara-C (Figure 11). The number of *Mad2*$^{+/-}$ HPCs, in contrast to the number of *Mad2*$^{+/+}$ littermate control HPCs, does not decrease during the nadir period of recovery from Ara-C treatment, and thus these cells appear to be protected from the cytotoxic effects of Ara-C. Thus, our studies have

unexpectedly found that *Mad2* haploinsufficiency does not render the hematopoietic system more susceptible than control mice to the suppressive effects of cytotoxic treatment.

Figure 9

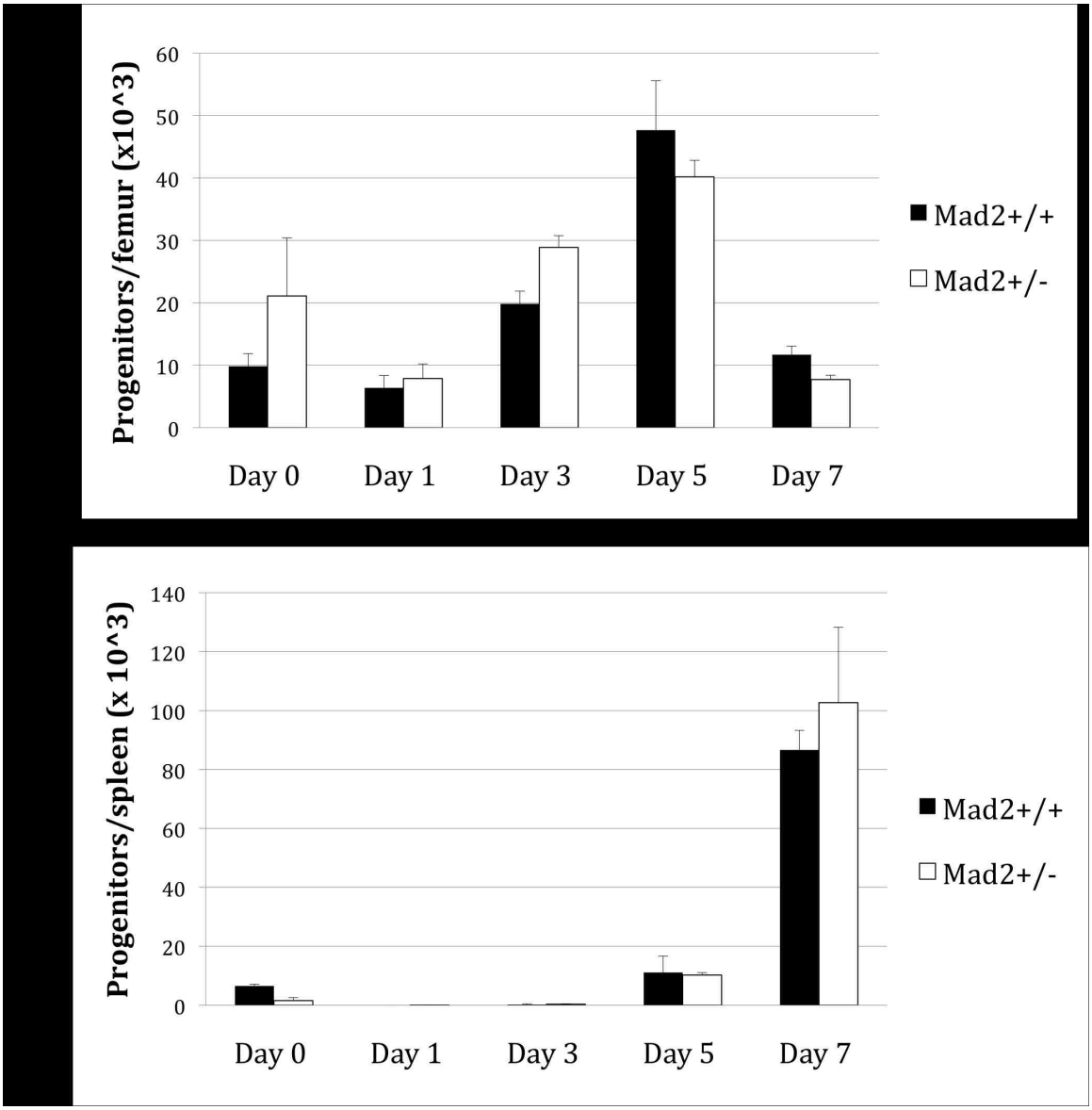

Progenitors/femur (x10^3)
60
50
40
30
20
10
0
Day 0
Day 1
Day 3
Day 5
Day 7
Mad2+/+
Mad2+/-
Progenitors/spleen (x 10^3)
140
120
100
80
60
40
20
0
Day 0
Day 1
Day 3
Day 5
Day 7
Mad2+/+
Mad2+/-

Figure 9. No Difference in the Recovery of *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ BM and Spl HPC After Cyclophosphamide Treatment.

Bone marrow (A) and spleen (B) cells collected from *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ mice treated with cyclophosphamide (200 mg/kg, i.p.) were assessed for total progenitor cells (stimulated *in vitro* with Epo, PWMSCM, and SCF) at days 0, 1, 3, 5, and 7 after treatment. In short, for the methylcellulose assay, 5 x 10^4 cells were plated and cultured in 0.9% methylcellulose culture medium with 30% FBS, 5% vol/vol PWMSCM, 50 ng/mL rmuSCF, 1 U/mL rhuEpo, 2 mM glutamine, 10^{-4} M 2-mercaptoethanol, and 0.1 mM hemin. Colonies were scored after 7 days of incubation at 37ºC, in 5% O_2 and 5% CO_2. Absolute numbers of progenitors were calculated from the nucleated cellularity per femur and the number of colonies formed per number of cells plated. Five mice of each genotype were used for each time point (N=1).

Figure 10

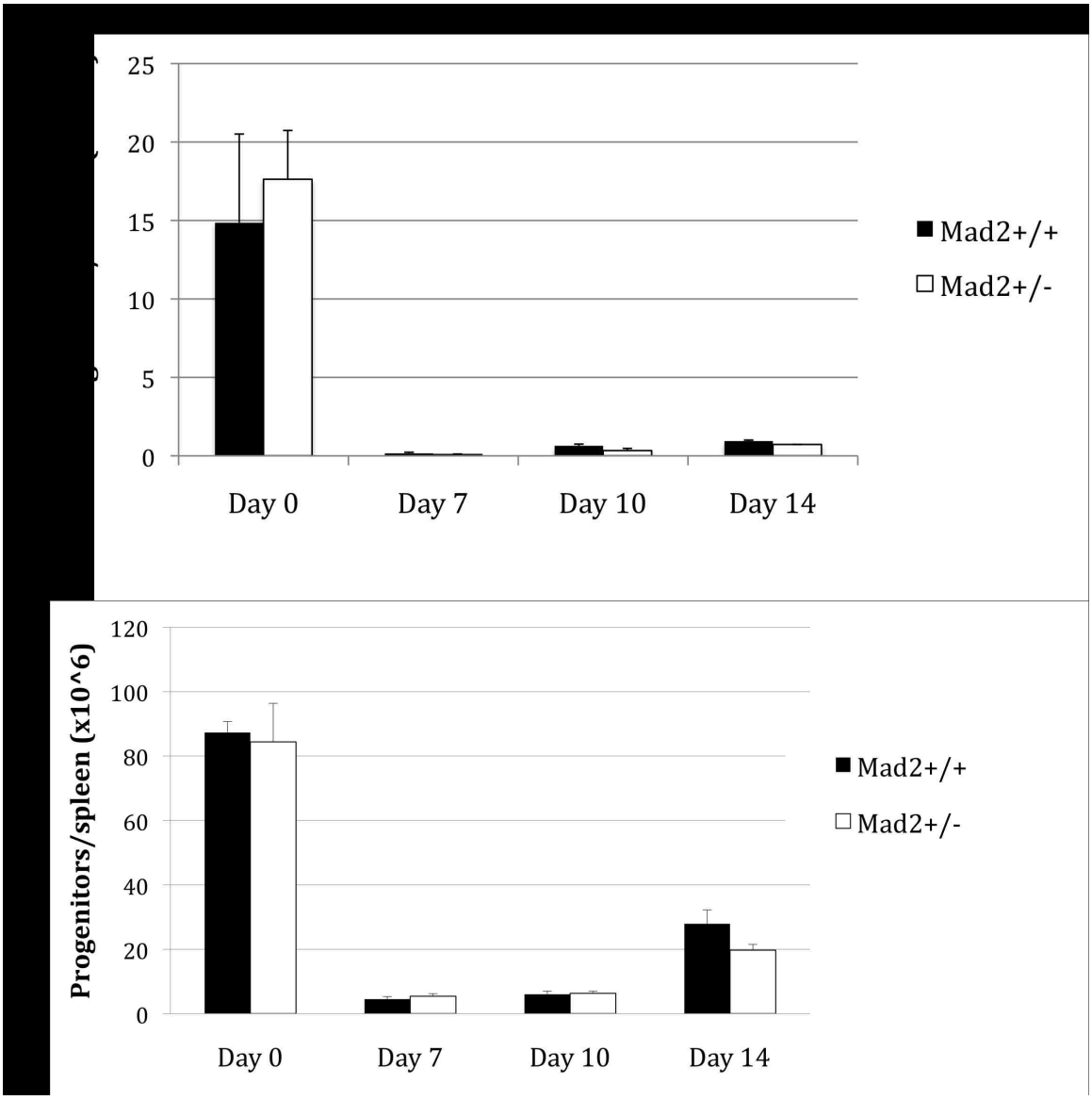

25
20
15
10
5
0
Day 0
Day 7
Day 10
Day 14
Mad2+/+
Mad2+/-
Progenitors/spleen (x10^6)
120
100
80
60
40
20
0
Day 0
Day 7
Day 10
Day 14
Mad2+/+
Mad2+/-

Figure 10. No Difference in the Recovery of *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ BM and Spl HPC After Treatment with γ-Irradiation.

Bone marrow (A) and spleen (B) cells collected from *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ mice treated with sub-lethal dose of γ-irradiation (650 cGy) were assessed for total progenitor cells (stimulated *in vitro* with Epo, PWMSCM, and SCF) at days 0, 7, 10, and 14 after treatment. In short, for the methylcellulose assay, 5 x 10^4 cells were plated and cultured in 0.9% methylcellulose culture medium with 30% FBS, 5% vol/vol PWMSCM, 50 ng/mL rmuSCF, 1 U/mL rhuEpo, 2 mM glutamine, 10^{-4} M 2-mercaptoethanol, and 0.1 mM hemin. Colonies were scored after 7 days of incubation at 37ºC, in 5% O_2 and 5% CO_2. Absolute numbers of progenitors were calculated from the nucleated cellularity per femur and the number of colonies formed per number of cells plated. Five mice of each genotype were used for each time point (N=1).

Figure 11

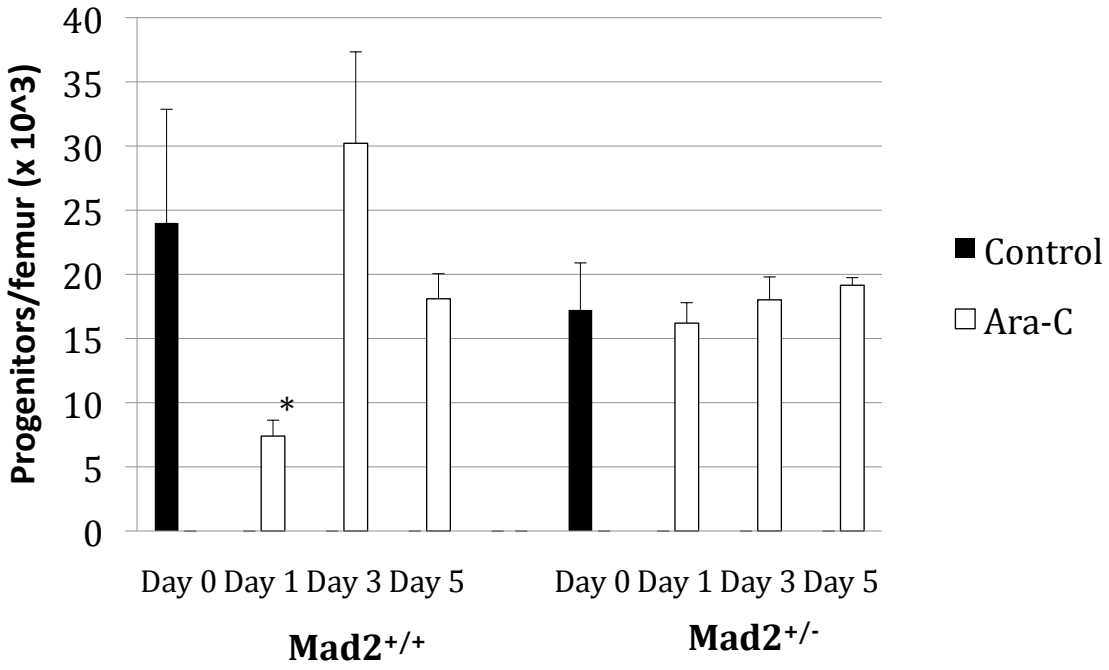

Progenitors/femur (x 10^3)
40
35
30
25
20
15
10
5
0
*
Day 0 Day 1 Day 3 Day 5
Day 0 Day 1 Day 3 Day 5
Mad2+/+
Mad2+/-
Control
Ara-C

Figure 11. *Mad2*$^{+/-}$ BM Cells, Unlike *Mad2*$^{+/+}$ BM Cells, are Protected From the Cytotoxic Effects of Ara-C.

Bone marrow cells collected from *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ mice treated with Ara-C (500 mg/kg, s.c.) were assessed for total progenitor cells (stimulated *in vitro* with Epo, PWMSCM, and SCF) at days 0, 1, 3, and 5 after treatment. In short, for the methylcellulose assay, 5 x 10^4 cells were plated and cultured in 0.9% methylcellulose culture medium with 30% FBS, 5% vol/vol PWMSCM, 50 ng/mL rmuSCF, 1 U/mL rhuEpo, 2 mM glutamine, 10^{-4} M 2-mercaptoethanol, and 0.1 mM hemin. Colonies were scored after 7 days of incubation at 37ºC, in 5% O_2 and 5% CO_2. Absolute numbers of progenitors were calculated from the nucleated cellularity per femur and the number of colonies formed per number of cells plated. Five mice of each genotype were used for each time point (N=1). *$p<0.05$ in comparison to day 0 untreated control.

Decreases in Progenitor Numbers and Cycling Status are Observed in BM HPC From *Mad2*$^{+/-}$ mice.

We had previously reported that *Mad2*$^{+/-}$ BM HPCs were in a slow or non-cycling state and the absolute numbers of these progenitors were decreased in comparison to *Mad2*$^{+/+}$ BM. This could potentially protect *Mad2*$^{+/-}$ HPC after *in vivo* treatment of mice with Ara-C. The mice used in our previously published studies (Ito, et al. 2007) were on a mixed background; however, the mice used in the present studies were on a C57Bl/6 background. To determine if functional differences are present in the *Mad2*$^{+/-}$ and *Mad2*$^{+/+}$ HPC from mice on a C57Bl/6 background, we set up clonogenic progenitor assays to assess absolute numbers and cycling status. As shown in Figure 12A, significantly decreased numbers of immature progenitors, CFU-GEMM, CFU-GM, and BFU-E (responsive to stimulation by multiple growth factors) were observed in *Mad2*$^{+/-}$ BM compared with *Mad2*$^{+/+}$ BM. Using the high-specific activity tritiated thymidine kill assay to analyze the cell-cycling status of progenitors, we found that the percentage of immature progenitor cells in the S-phase of the cell cycle was significantly decreased in *Mad2*$^{+/-}$ BM (Figure 12B). These results are in agreement with previous findings (Ito, et al. 2007), demonstrating that Mad2 plays a role in HPC proliferation in mice on a C57Bl/6 background. However, these changes in numbers of immature progenitors subsets of progenitors did not translate into changes in blood counts or in cellularity of BM and spleen between *Mad2*$^{+/-}$ and *Mad2*$^{+/+}$ mice (Ito, et al. 2007).

Figure 12

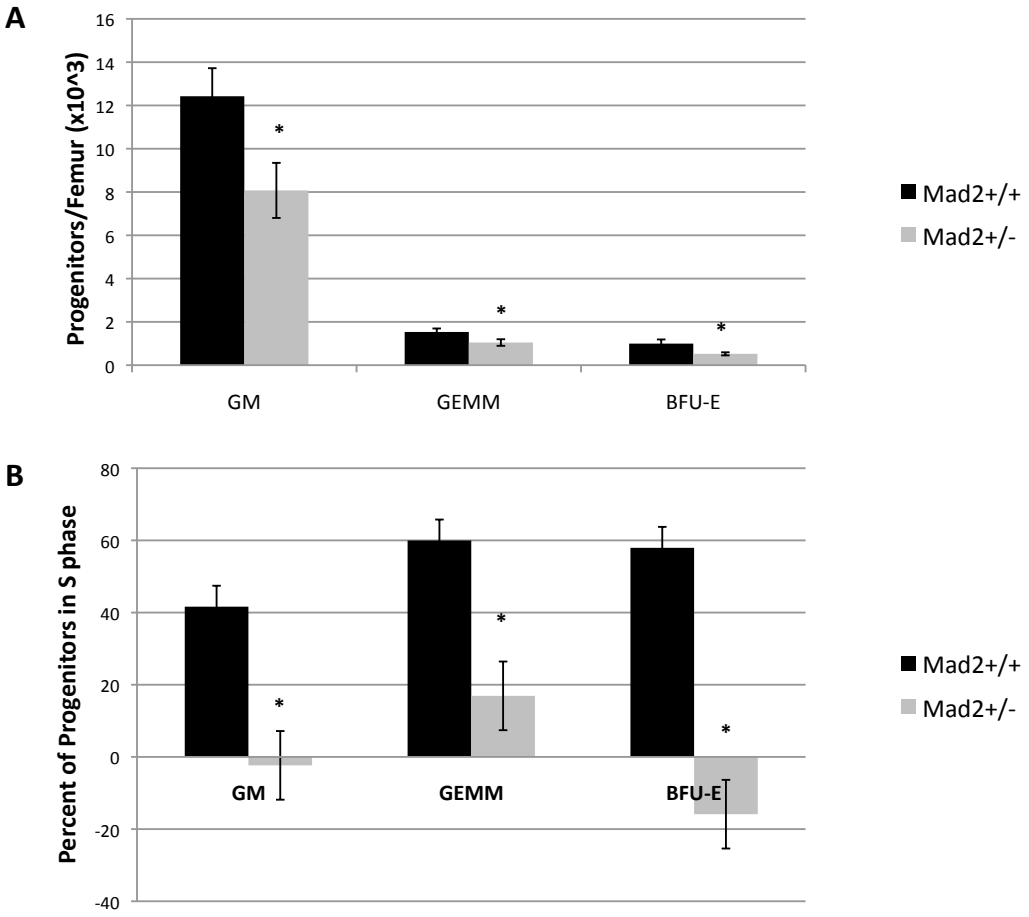

A
Progenitors/Femur (x10^3)
16
14
12
10
8
6
4
2
0
*
*
*
GM
GEMM
BFU-E
Mad2+/+
Mad2+/-
B
Percent of Progenitors in S phase
80
60
40
20
0
-20
-40
*
*
*
GM
GEMM
BFU-E
Mad2+/+
Mad2+/-

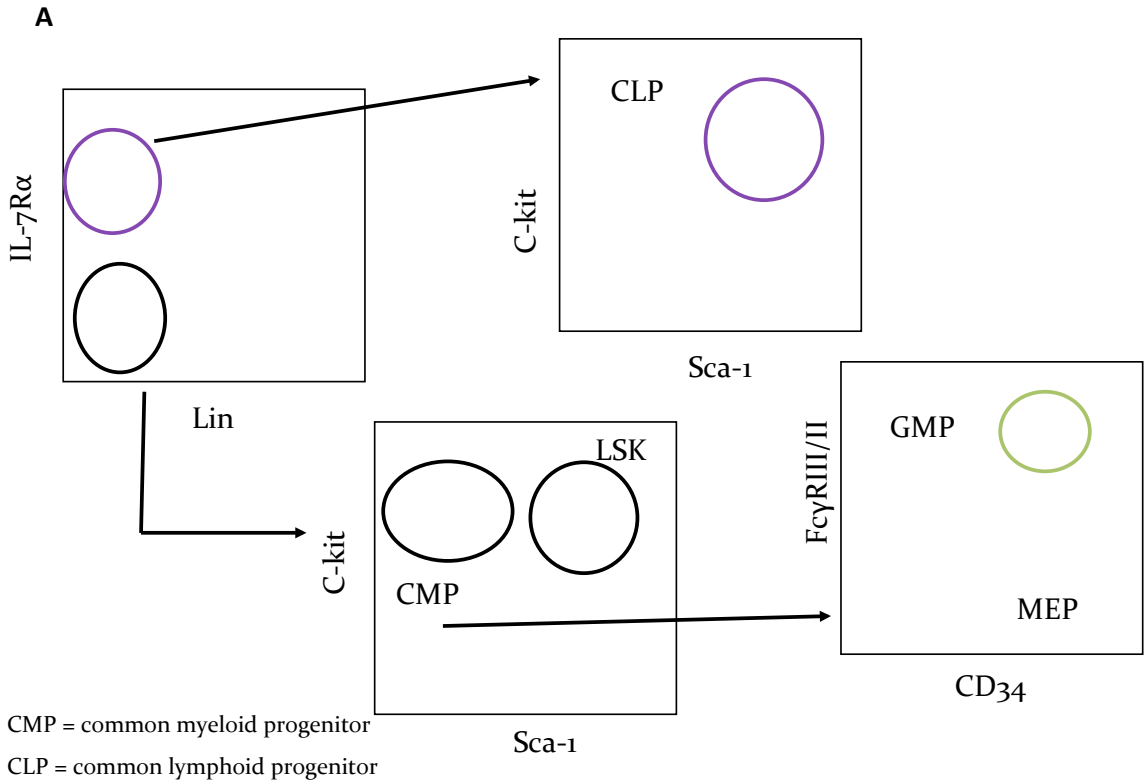
Figure 12. Functional Analysis of BM HPC From Untreated Mice Demonstrates Decreased Numbers and Cycling of *Mad2*$^{+/-}$ HPC.

Bone marrow from *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ mice was assessed for (A) absolute numbers of immature progenitor cells and (B) the percentage of these BM progenitors in S-phase. In short, for the methylcellulose assay, 5 x 10^4 cells were plated and cultured in 0.9% methylcellulose culture medium with the combination of 30% FBS, 5% vol/vol PWMSCM, 50 ng/mL rmuSCF, 1 U/mL rhuEpo, 2 mM glutamine, 10^{-4} M 2-mercaptoethanol, and 0.1 mM hemin. Colonies were scored after 7 days of incubation at 37ºC, in 5% O_2 and 5% CO_2. Absolute numbers of progenitors were calculated from the nucleated cellularity per femur and the number of colonies formed per number of cells plated (N=3; 3 mice of each genotype were used for each experiment). *$p<0.05$.

Phenotypic Characterization of HSC and HPC Populations in *Mad2*$^{+/-}$ and *Mad2*$^{+/+}$ Mice.

To further characterize the HSC and HPC populations in the BM, we did a phenotypic analysis of these cell populations. The markers used to define each population and the gating strategy used for flow cytometry are shown in Figure 13A, and a representative dot plot using this gating strategy to analyze mouse BM is shown in Figure 13B. Examination of the LSK cells revealed no significant differences in this population in the *Mad2*$^{+/-}$ and *Mad2*$^{+/+}$ mice. We examined the CLP, MEP, and GMP populations in both types of mice, and also found that no significant differences exist in these populations in both mice. We did find that the absolute numbers of CMP were slightly, but significantly increased in the *Mad2*$^{+/-}$ mice (Figure 13C). The fact that the numbers of phenotyped populations of CMP and GMP were respectively slightly increased or not different in *Mad2*$^{+/-}$ and *Mad2*$^{+/+}$ mice, yet there were significant decreases in the numbers and cycling status of *Mad2*$^{+/-}$ CFU-GM, BFU-E, and CFU-GEMM suggests that phenotypes of cells in this case do not provide telling information about the functional proliferative capacity of these cells after cytokine stimulation *in vitro*.

Figure 13

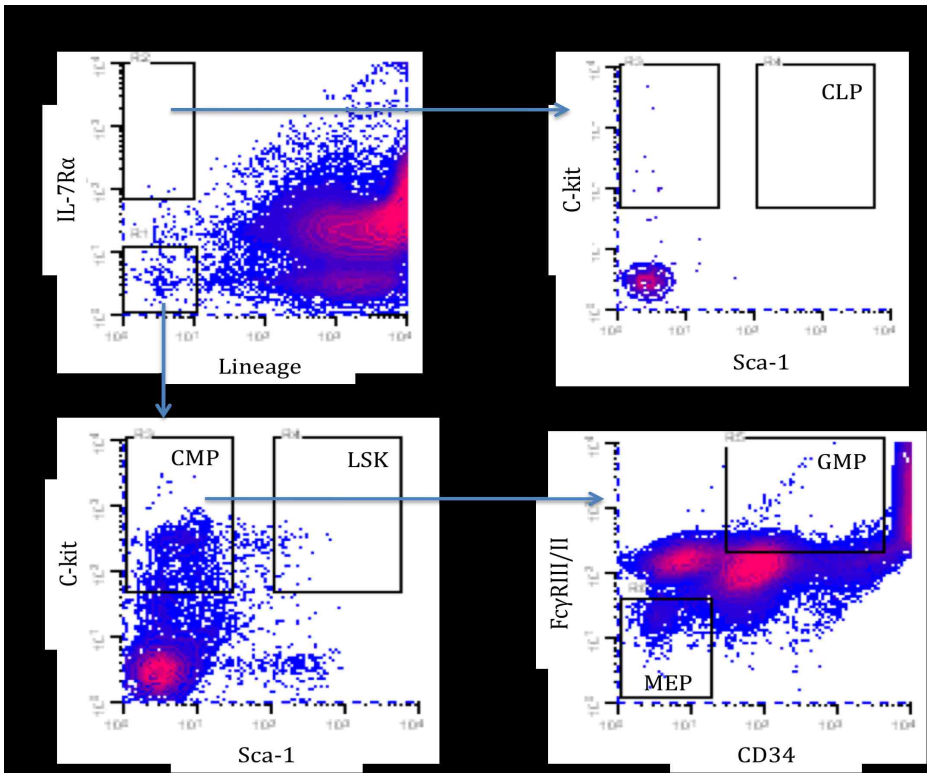


CMP = common myeloid progenitor

CLP = common lymphoid progenitor

MEP = megakaryocyte/erythroid progenitor

GMP = granulocyte/macrophage progenitor


IL-7Rα
Lineage
C-kit
CLP
Sca-1
C-kit
CMP
LSK
Sca-1
FcγRIII/II
GMP
MEP
CD34

C

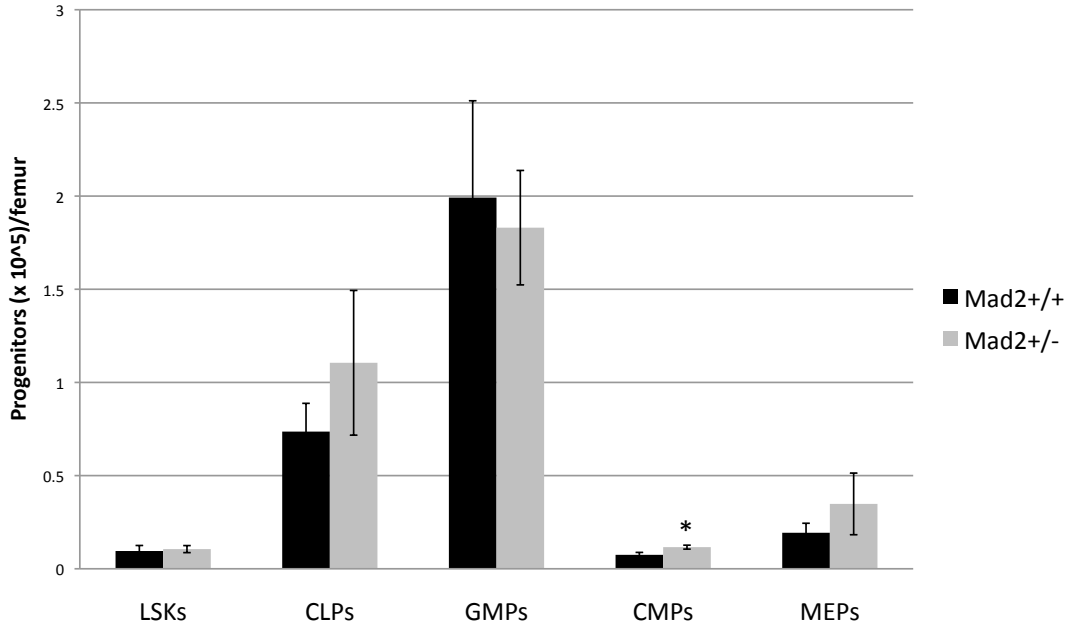

Progenitors (x 10^5)/femur
3
2.5
2
1.5
1
0.5
0
*
LSKs
CLPs
GMPs
CMPs
MEPs
Mad2+/+
Mad2+/-

Figure 13. Phenotypic Analysis of HSC and HPC Populations Reveals No Changes in LSK, CLP, GMP, and MEP, But a Slight Increase in CMPs in *Mad2*$^{+/-}$ Mice.

Bone marrow from *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ mice was assessed for the number of phenotypically defined progenitors per femur. (A) Bone marrow was collected and stained with fluorescent antibodies to various surface markers; cells were then analyzed by flow cytometry following the gating strategy shown. (B) A representative dot plot showing the different stem and progenitor populations in the BM. (C) The following stem and progenitor populations were examined: LSK - Lin$^-$ Sca-1$^+$ c-kit$^+$ IL-7Rα$^-$; CMP (common myeloid progenitor) - Lin$^-$ Sca-1$^-$ c-kit$^+$ CD34$^+$ FcγR$^{-/lo}$; GMP (granulocyte/macrophage progenitor) - Lin$^-$ Sca-1$^-$ c-kit$^+$ CD34$^+$ FcγR$^{+/hi}$; MEP (megakaryocyte/erythroid progenitor) - Lin$^-$ Sca-1$^-$ c-kit$^+$ CD34$^-$ FcγR$^{-/lo}$; CLP (common lymphoid progenitor) - Lin$^-$ Sca-1$^+$ c-kit$^+$ IL-7Rα$^+$. Results shown are the average of 3 independent experiments using 3 mice of each genotype per experiment. *$p<0.01$.

***Mad2* Haploinsufficiency Does Not Affect the Long-Term Repopulating Ability of HSC.**

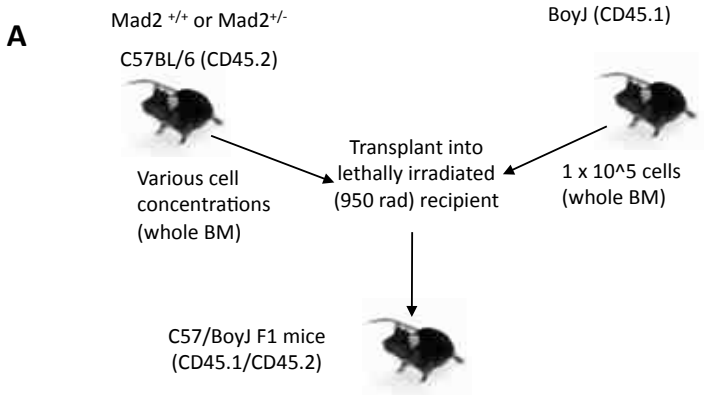
The discrepancy between phenotype and function of *Mad2*$^{+/-}$ HPCs made it clear that a functional assessment of *Mad2*$^{+/-}$ HSC was necessary. In order to determine if *Mad2* haploinsufficiency affects the function of HSC, we performed competitive repopulations assays (CRA) to examine the short- and long-term repopulating ability of *Mad2*$^{+/-}$ HSC compared to *Mad2*$^{+/+}$ HSC. Various concentrations of freshly isolated *Mad2*$^{+/+}$ or *Mad2*$^{+/-}$ Bl/6 BM (CD45.2 donor) cells were mixed with 1 x 10^5 freshly isolated Boy J BM (CD45.1 competitor) cells, and these mixtures were transplanted into the 950 cGy-conditioned F1 (CD45.1/CD45.2) recipients (Figure 14A). Peripheral blood donor cell chimerism was determined at 1, 2, 4, and 6 months posttransplant. Representative flow cytometry analyses from controls and recipients at 4 months posttransplant are shown in Figure 14B. The percentages in each quadrant indicate the percentage of the population that expresses either CD45.1 (PE single positive) or CD45.2 (APC single positive) or for both CD45.1 and CD45.2 (PE/APC double positive). The left panel shows the relative engraftment of fresh *Mad2*$^{+/+}$ donor cells, competitor cells, and residual recipient cells. In the right panel, recipients were transplanted with a mixture of competitor cells and *Mad2*$^{+/-}$ donor cells. The composite data for the donor cell chimerism (CD45.2) from the complete experiment is shown in Figure 14C. For the various different donor to competitor ratios used, no significant difference in the percent CD45.2 chimerism in the

peripheral blood was observed between *Mad2*$^{+/+}$ or *Mad2*$^{+/-}$ cells. This indicates that *Mad2* haploinsufficiency does not affect the ability of HSC to engraft and compete in a transplant setting.

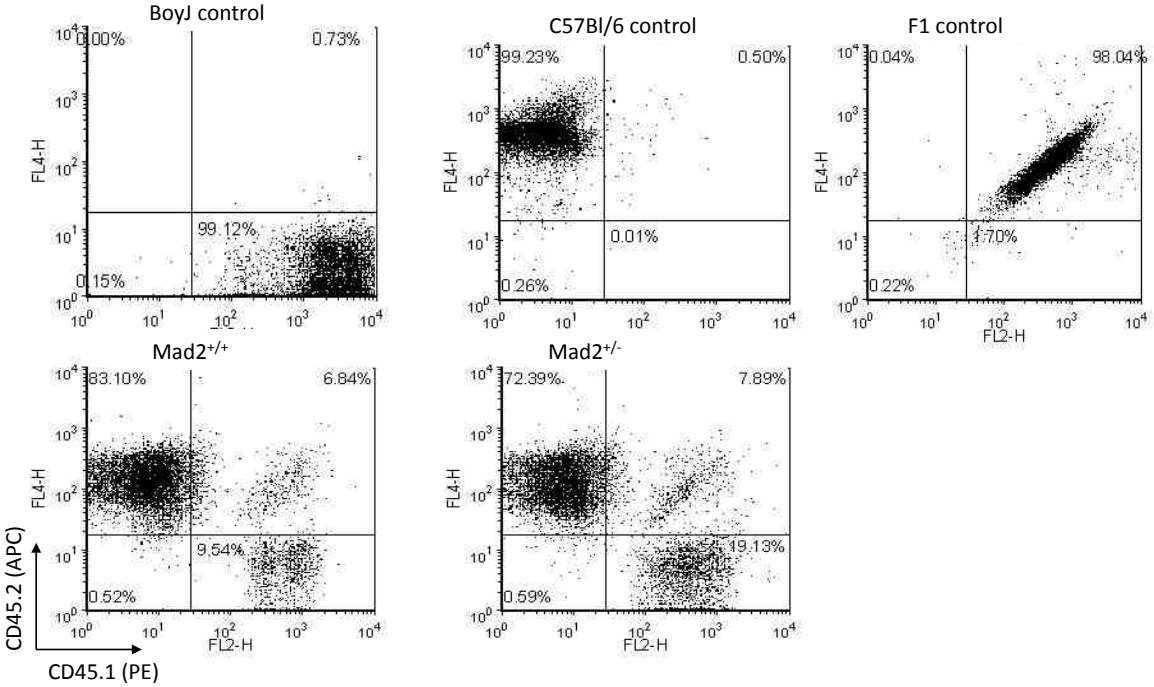
After 6 month posttransplant, the recipient F1 mice were sacrificed and BM was collected to perform a secondary non-competitive transplant into new 950 cGy-conditioned F1 recipients. Peripheral blood donor cell chimerism was determined at 1, 2, 4, and 6 months posttransplant. Data from the secondary transplant shows no consistent significant difference in the percent peripheral blood chimerism between *Mad2*$^{+/+}$ or *Mad2*$^{+/-}$ cells (Figure 14D). This suggests that *Mad2* haploinsufficiency does not affect either engrafting capability, numbers of functional HSC, or self-renewal of HSCs.

Figure 14

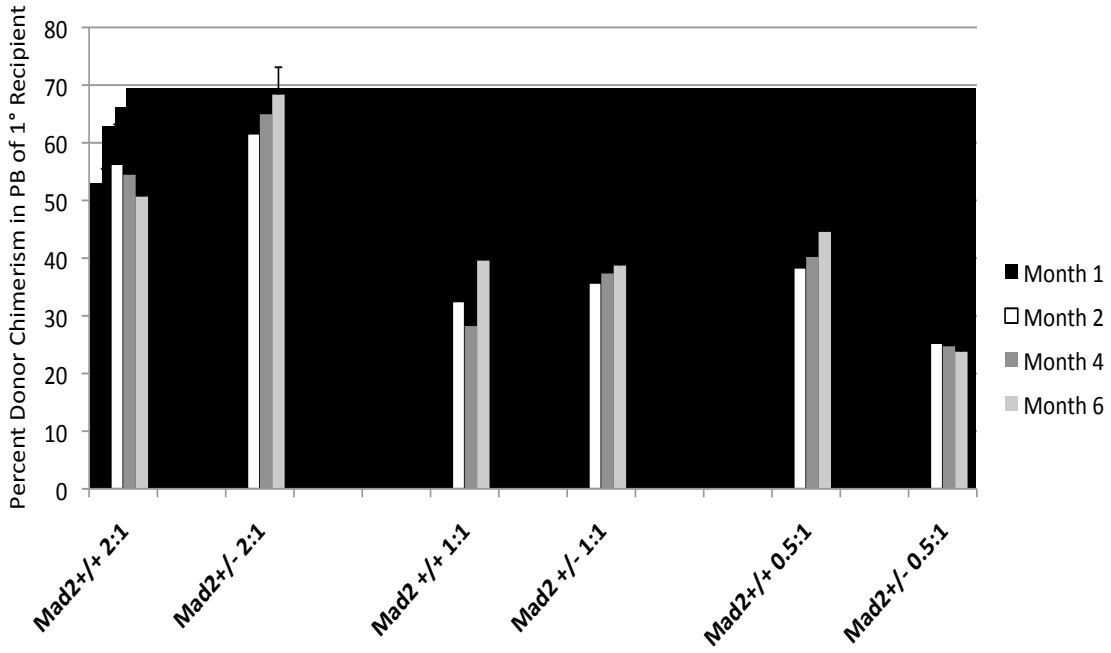
A


Mad2 +/+ or Mad2+/-
C57BL/6 (CD45.2)
BoyJ (CD45.1)
Various cell concentrations (whole BM)
Transplant into lethally irradiated (950 rad) recipient
1 x 10^5 cells (whole BM)
C57/BoyJ F1 mice (CD45.1/CD45.2)

B


BoyJ control
0.00%
0.73%
99.12%
0.15%
C57Bl/6 control
99.23%
0.50%
0.01%
0.26%
F1 control
0.04%
98.04%
1.70%
0.22%
Mad2+/+
83.10%
6.84%
9.54%
0.52%
Mad2+/-
72.39%
7.89%
19.13%
0.59%
FL4-H
FL2-H
CD45.2 (APC)
CD45.1 (PE)

C


Percent Donor Chimerism in PB of 1° Recipient
80
70
60
50
40
30
20
10
0
Mad2+/+ 2:1
Mad2+/- 2:1
Mad2 +/+ 1:1
Mad2 +/- 1:1
Mad2+/+ 0.5:1
Mad2+/- 0.5:1
Month 1
Month 2
Month 4
Month 6

D

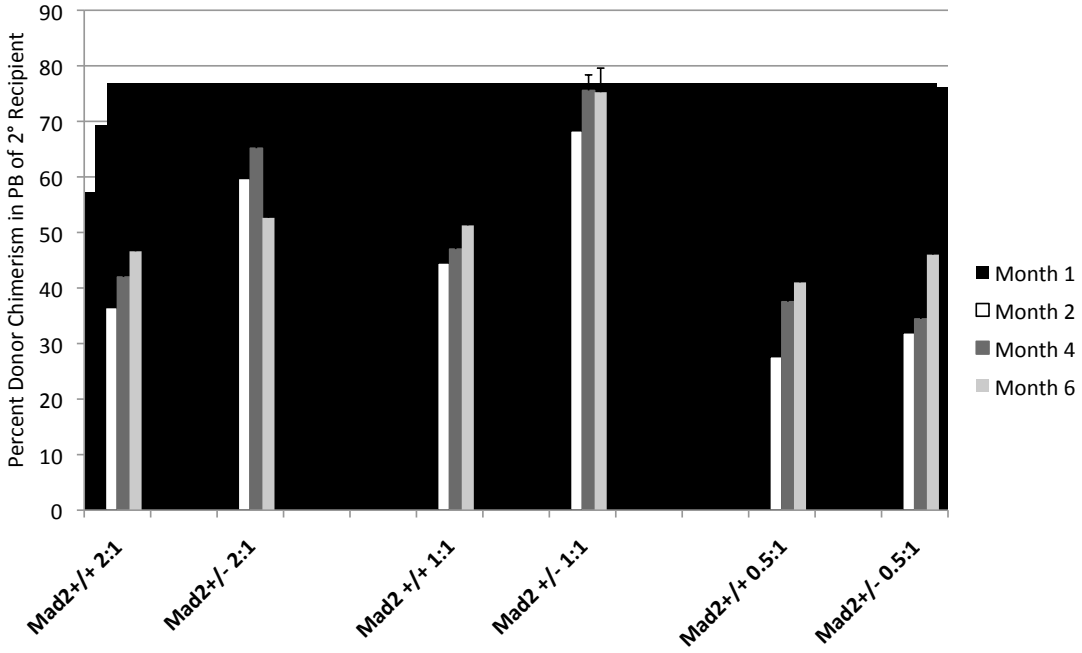

Percent Donor Chimerism in PB of 2° Recipient
90
80
70
60
50
40
30
20
10
0
Mad2+/+ 2:1
Mad2+/- 2:1
Mad2 +/+ 1:1
Mad2 +/- 1:1
Mad2+/+ 0.5:1
Mad2+/- 0.5:1
Month 1
Month 2
Month 4
Month 6

Figure 14. *Mad2* Haploinsufficiency Does Not Affect the Long-Term Repopulating Ability of HSC Compared to Wild-Type HSC.

(A) Donor (CD45.2) and competitor (CD45.1) BM cells were transplanted into a lethally irradiated recipient (CD45.1/45.2) mice at the ratios indicated. (B) Representative flow cytometry analyses from the recipients of either *Mad2*$^{+/+}$ or *Mad2*$^{+/-}$ donor cells 6 months post-transplant are shown along with controls. Peripheral blood (PB) was collected at months 1, 2, 4, and 6 after primary transplant (C); 6 months after the primary transplants, secondary transplants were performed and PB was collected at months 1, 2, 4, and 6 (D). Primary transplant data represents 3 separate experiments using 5 mice per transplant group. Secondary transplant data represents 2 separate experiments using 5 mice per transplant group. Data were analyzed using the Student's t-test. *$p<0.05$.

$Mad2^{+/-}$ BM HPCs are Less Sensitive to the Cytostatic Effects Caused by Blocking the Redox Function of Ape1/Ref-1 and Do Not Respond Well to the Effects of Lowered O_2 Tension.

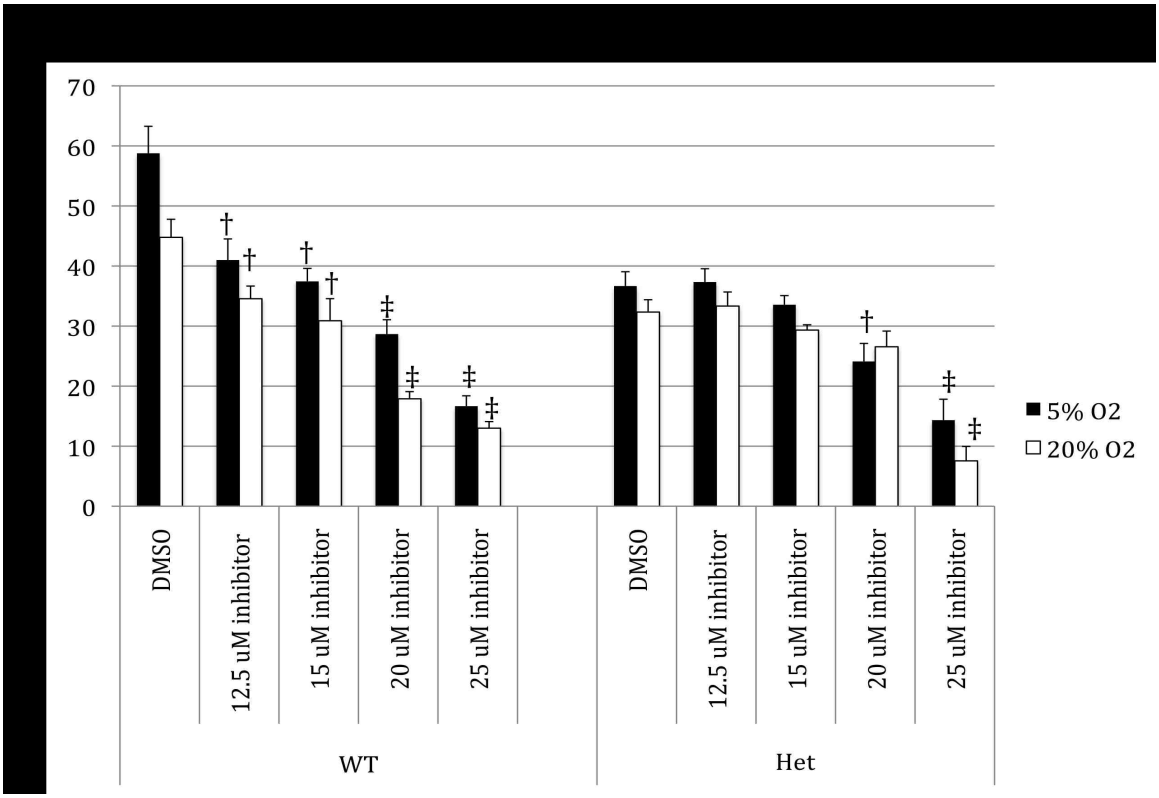
After finding that functional differences exist between $Mad2^{+/-}$ and $Mad2^{+/+}$ HPC, but not HSC, we decided to further examine HPC function in these mice. To examine how $Mad2^{+/-}$ BM HPCs respond to cytostatic effects of drugs in comparison to $Mad2^{+/+}$ HPC, we treated these cells with E3330. E3330 is a highly specific inhibitor of Ape1/Ref-1 (APE1) redox activity. It has been shown that E3330 does not increase the percentage of cells undergoing apoptosis, but rather stops them from proliferating (Fishel, et al. 2010; Luo, et al. 2008). APE1 is a multifunctional protein involved in both the base excision repair pathway and redox regulation of various transcription factors, including AP-1, HIF-1α, and p53 (Jayaraman, et al. 1997; Lando, et al. 2000; Xanthoudakis, et al. 1992). A previous study showed that the redox function, but not the DNA repair activity of APE1 is required for normal embryonic hematopoiesis (Zou, et al. 2007). However, the function of this protein has not been thoroughly invested in normal adult hematopoiesis. Using E3330, we examined colony-forming ability of both $Mad2^{+/+}$ and $Mad2^{+/-}$ HPC *in vitro* at normoxic (~20%) and at lowered (5%) oxygen (O_2) tension.

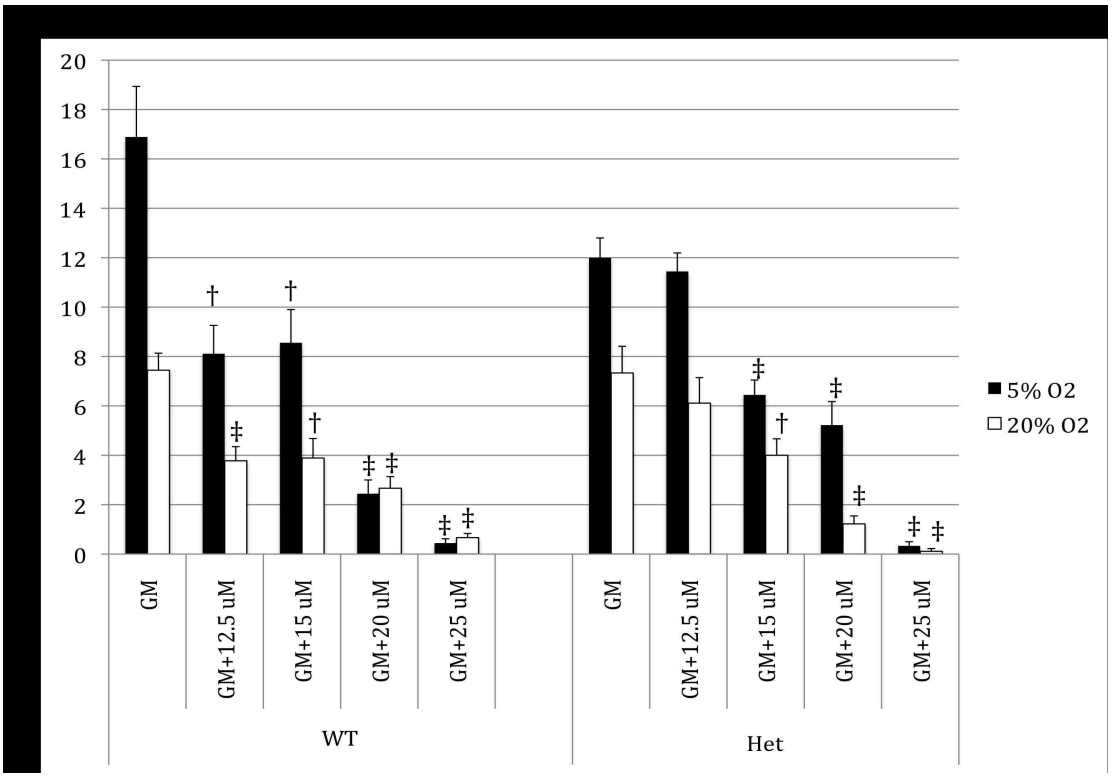
For wild-type $Mad2^{+/+}$ BM HPC, E3330 has a greater effect under 5% oxygen tension, causing not only a decrease in colony formation under maximum stimulation or with single growth factors (Figure 15A, B), but also blocking

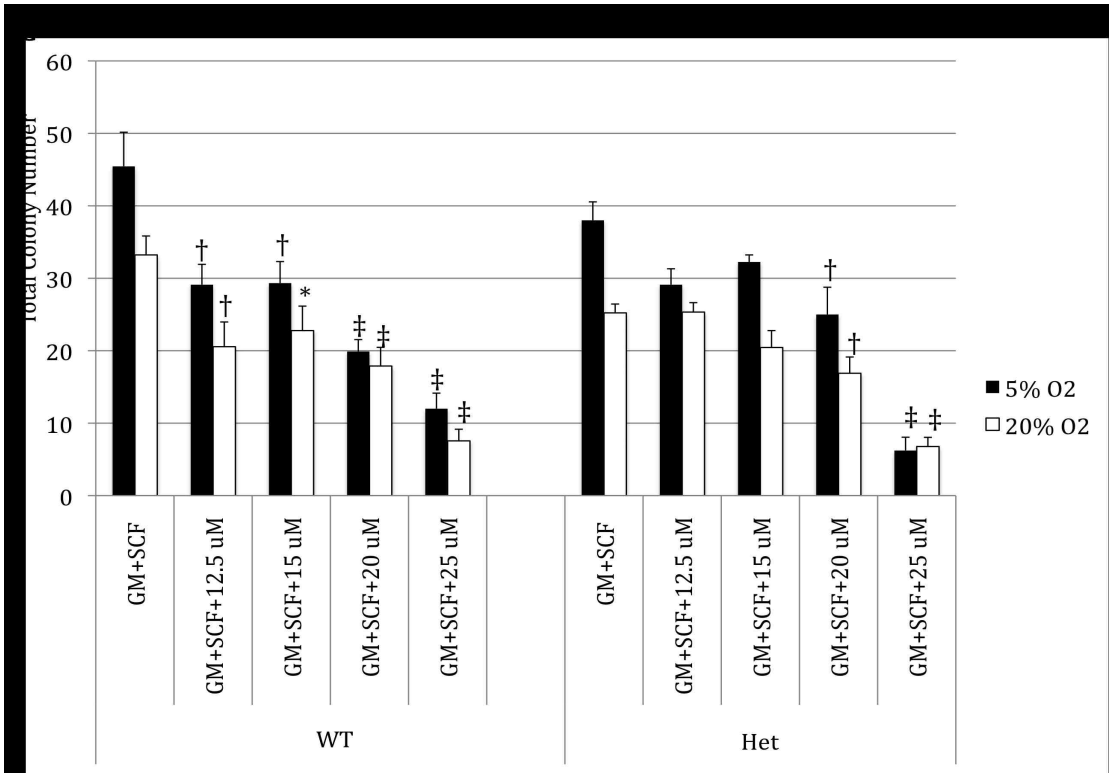
synergistic growth with defined growth factors (Figure 15C). Upon examination of colony formation from *Mad2*$^{+/-}$ HPC with the lowest concentration of E3330 used, we found no significant differences in colony formation between E3330 and vehicle control treated cells, while wild-type HPC colony formation was significantly inhibited. The percent decrease compared to DMSO control is significantly greater in the wild-type cells compared to *Mad2*$^{+/-}$ cells (Figure 15D-F). This is true at both 5% and 20% O_2 tension, and interestingly, *Mad2*$^{+/-}$ HPC do not grow better at lower O_2 tension as do wild-type HPC. However, by increasing the concentration of E3330 used, we were able to inhibit *Mad2*$^{+/-}$ HPC colony formation, but still not to the extent of *Mad2*$^{+/+}$ HPC.

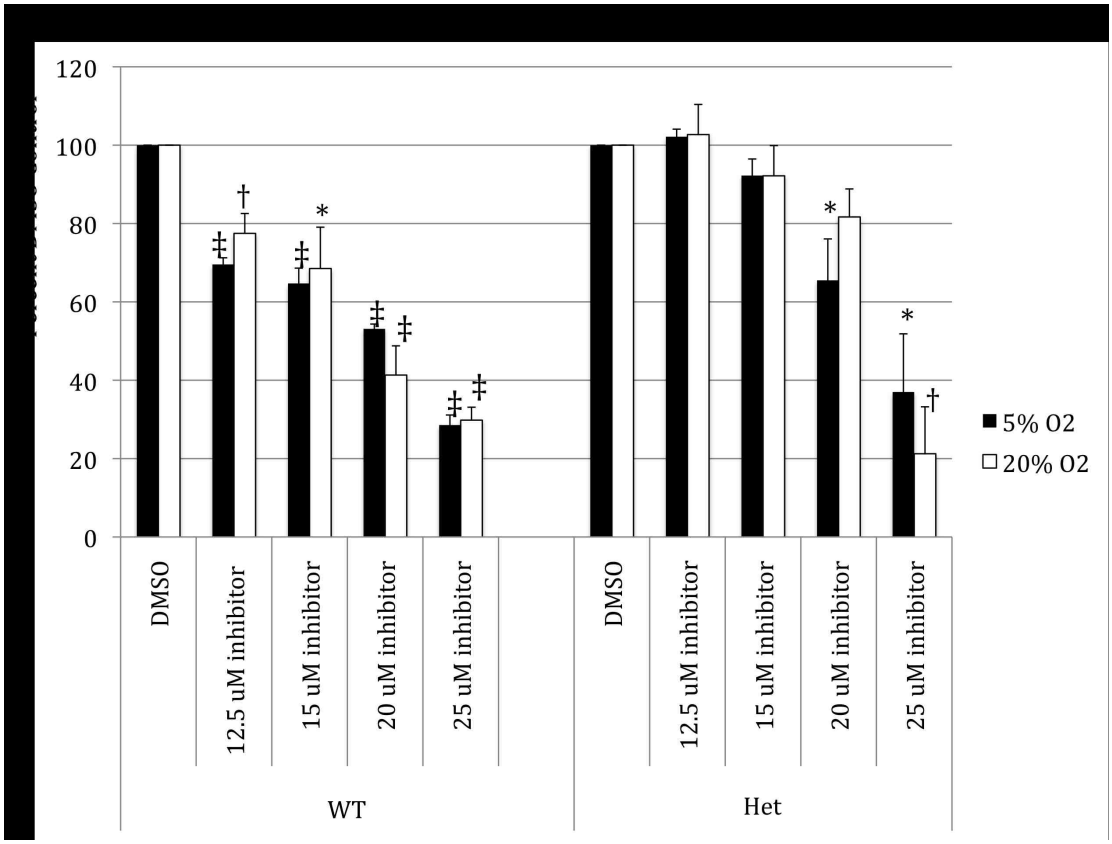
To ensure the differences in inhibition were not due to inherent changes in cell cycle between wild-type and *Mad2*$^{+/-}$ HPC and to ensure that E3330 is not cell cycle specific, a high specific activity tritiated thymidine kill assay was performed in the presence and absence of E3330 to estimate the percentage of cells in S-phase. The percentage of wild-type cells in S-phase is similar in the presence and the absence of E3330 (Figure 15G) and the same is true for *Mad2*$^{+/-}$ HPC; this indicates the inhibitor is not cell cycle specific. The inhibition of *Mad2*$^{+/-}$ HPC colony formation only under higher concentrations of E3330 suggests that *Mad2*$^{+/-}$ HPC are less sensitive to the cytostatic effects of the inhibitor compared to *Mad2*$^{+/+}$ HPC.

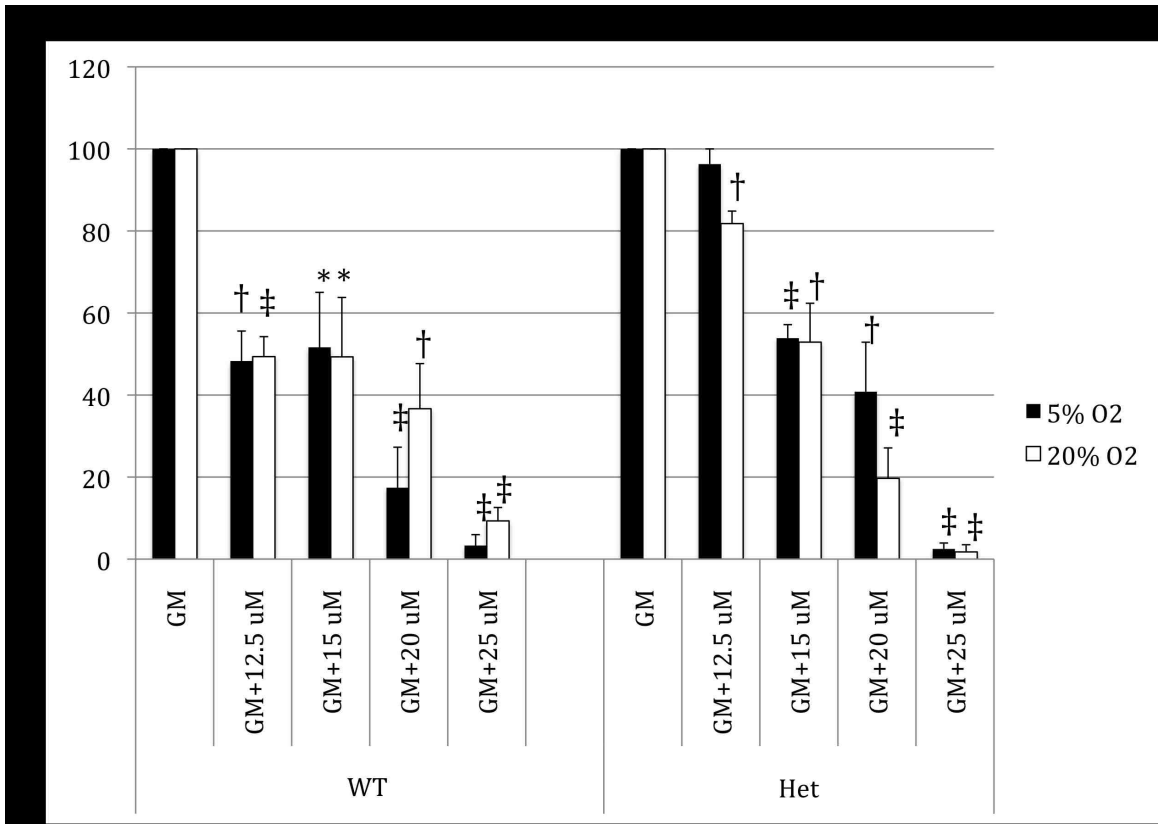
Figure 15

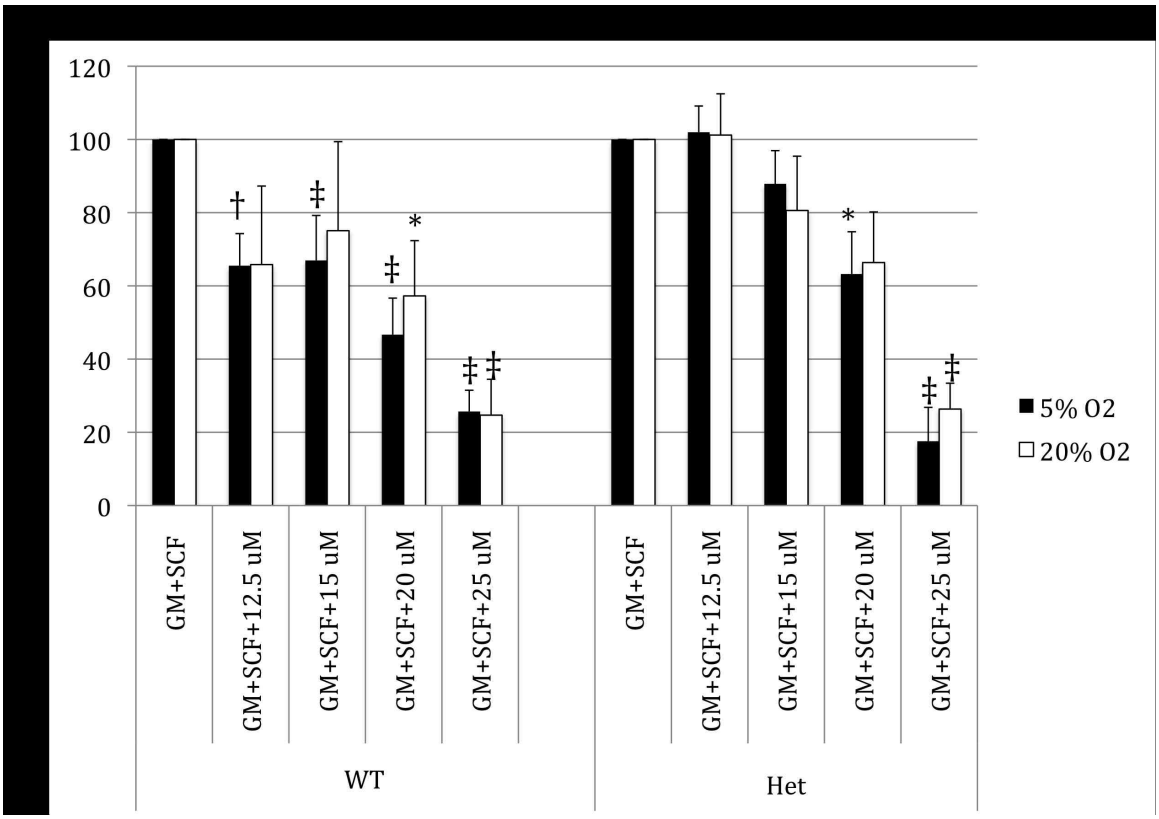

70
60
50
40
30
20
10
0
†
‡
DMSO
12.5 uM inhibitor
15 uM inhibitor
20 uM inhibitor
25 uM inhibitor
WT
Het
5% O2
20% O2


20
18
16
14
12
10
8
6
4
2
0
GM
GM+12.5 uM
GM+15 uM
GM+20 uM
GM+25 uM
WT
Het
5% O2
20% O2


60
50
40
30
20
10
0
GM+SCF
GM+SCF+12.5 uM
GM+SCF+15 uM
GM+SCF+20 uM
GM+SCF+25 uM
WT
Het
5% O2
20% O2


120
100
80
60
40
20
0
DMSO
12.5 uM inhibitor
15 uM inhibitor
20 uM inhibitor
25 uM inhibitor
WT
Het
5% O2
20% O2


120
100
80
60
40
20
0
GM
GM+12.5 uM
GM+15 uM
GM+20 uM
GM+25 uM
WT
Het
5% O2
20% O2


120
100
80
60
40
20
0
†
‡
‡
*
‡ ‡
*
‡ ‡
5% O2
20% O2
GM+SCF
GM+SCF+12.5 uM
GM+SCF+15 uM
GM+SCF+20 uM
GM+SCF+25 uM
GM+SCF
GM+SCF+12.5 uM
GM+SCF+15 uM
GM+SCF+20 uM
GM+SCF+25 uM
WT
Het

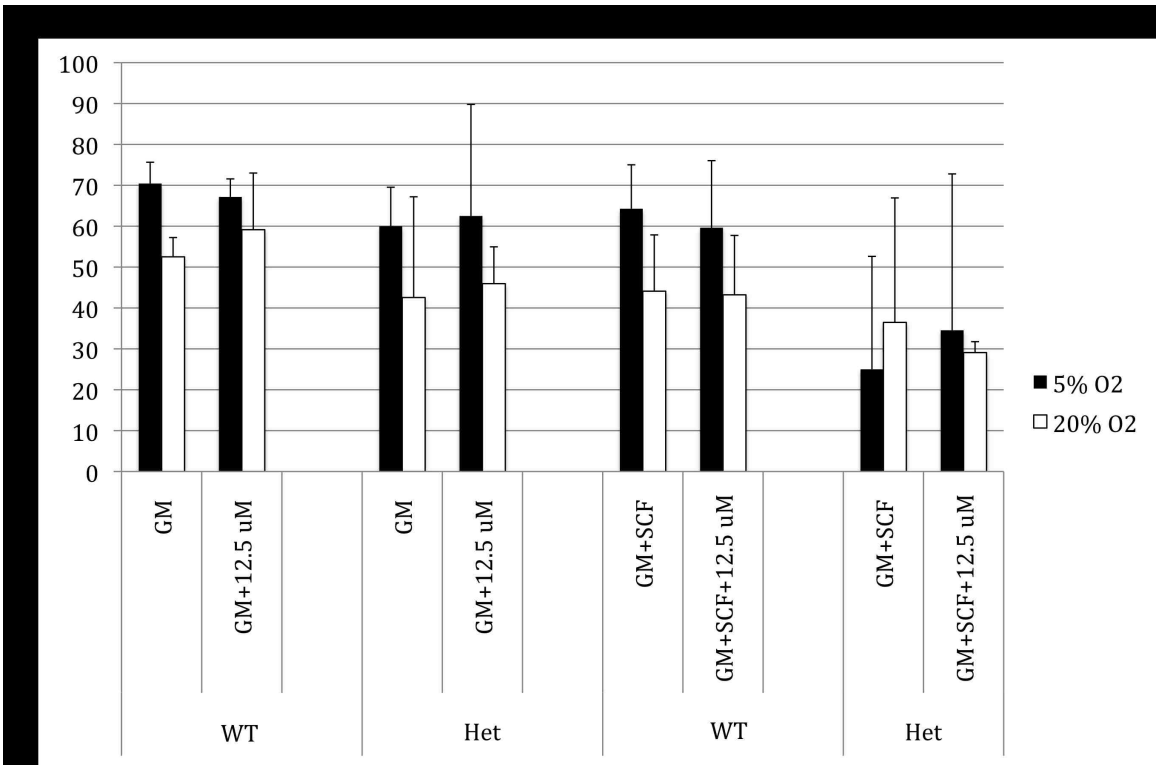

100
90
80
70
60
50
40
30
20
10
0
5% O2
20% O2
GM
GM+12.5 uM
GM
GM+12.5 uM
GM+SCF
GM+SCF+12.5 uM
GM+SCF
GM+SCF+12.5 uM
WT
Het
WT
Het

Figure 15. ***Mad2***$^{+/-}$ **BM Cells are Less Sensitive to the Cytostatic Effects Caused by Blocking the Redox Function of Ape1/Ref-1 Compared to** ***Mad2***$^{+/+}$ **BM Cells.**

Bone marrow cells collected from *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ mice were treated with various concentrations, ranging from 12.5 μM to 25 μM, of E3330 or DMSO control for 4 hours before placing cells into colony assays. (A) Cells plated in the presence of Epo, PWMSCM, and SCF; (B) cells plated with GM-CSF; (C) cell plated with GM-CSF and SCF. (D, E, F) The percent DMSO control in the presence of E3330 for the same conditions used in A, B, and C, respectively. (G) The percentage of these BM progenitors in S-phase as estimated by thymidine kill. Colonies were scored after 7 days of incubation at 37ºC, in 5% O_2 and 5% CO_2 or 20% O_2 and 5% CO_2 (at least 3 mice of each genotype were used in these experiments). Data were analyzed using the Student's t-test. A P value less than 0.05 was considered statistically significant. N ≥ 3 *$p<0.05$, †$p<0.01$, ‡$p<0.001$ compared to DMSO control.

DISCUSSION

We demonstrate that phenotypically defined HSCs show an arrest in the M-phase of the cell cycle in response to treatment with nocodazole, a microtubule-depolymerizing agent. This arrest, shown by an increase in phospho-histone H3 in nocodazole treated cells, implies that the SAC is functional in this population of cells. Also in response to nocodazole, HSCs initiate apoptosis, evident by increased levels of activated caspase 3, subdiploid levels of DNA, and decreases in cell number. These data lead to the conclusion that HSCs exhibit coupling of apoptosis to activation of the SAC, a condition observed in somatic cells. This is in contrast to ESCs, which accumulate in M-phase after nocodazole treatment, but then exit this mitotic delay and re-enter a polyploid mitosis. We also conclude that HSCs do not function like embryonic stem cells, which exhibit uncoupling of apoptosis from checkpoint activation and tolerance to tetraploidy/aneuploidy (Mantel, et al. 2007).

The efficiency of the SAC is particularly important in HSCs, since these cells give rise to many different types of progenitor cells populations, including the myeloid and lymphoid lineages of cells. Uncoupling of SAC activation from apoptosis could lead to chromosome instability not only in HSCs themselves, but also in all the progenitor populations descending from these cells. As previously described,

chromosome instability leading to aneuploidy characterizes many cancers (Bharadwaj and Yu 2004). Therefore, chromosome instability in HSCs and their progenitors could lead to cancers derived from these cell types, such as leukemia or lymphoma.

Ex vivo culture and expansion of hematopoietic stem and progenitor cells could prove problematic in a situation of checkpoint-apoptosis uncoupling. In numerous settings, *ex vivo* manipulation of HSC and HPC occurs before the cells are used in research or clinical applications. If these cells exhibit defects in checkpoints and/or apoptosis initiation as previously suggested (Damelin, et al. 2005; Mantel, et al. 2007), cells with genomic abnormalities could accumulate and negatively affect the outcome of experiments or treatments. However, the observation that HSCs efficiently initiate apoptosis after SAC activation indicates these cells will undergo programmed cell death if problems arise during mitosis, lessening concerns that the stress of *ex vivo* expansion of HSCs could cause instability in HSCs that might lead to disordered growth and survival after transplantation of the expanded cells.

After exposing BM low-density mononuclear cells (LDMCs) from *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ mice to nocodazole for either 24 or 48 hours, both cells types arrest in M-phase and subsequently initiate apoptosis. These results are similar to observations in cells from *Mad2*$^{+/-}$ embryos; cells from *Mad2*$^{-/-}$ embryos failed to arrest in mitosis, but cells from *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ embryos exhibited similar

levels of mitotic arrest in response to nocodazole treatment (Dobles, et al. 2000). After exposure to nocodazole for over 48 hours, Hct-*MAD2*$^{+/-}$ cells (generated from Hct-116 human colon carcinoma cell line) become multinucleated with a polyploid DNA content; the checkpoint status of ten independent subclones derived from the Hct-116 parental line was found to be normal, which excludes clonal variation as the cause of the checkpoint defect in Hct-*MAD2*$^{+/-}$ clones (Michel, et al. 2001). Although we do not see any differences between *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ HSC in the ability to arrest in M-phase after microtubule disruption, we have not ruled out the possibility that changes in *Mad2* protein levels can lead to changes in ploidy. Murine embryonic fibroblasts (MEF) derived from *MAD2*$^{+/-}$ mice showed high frequencies of premature sister chromatid separate after SAC activation, which leads to aneuploidy in these cells. Also, we have not examined the levels of survivin in *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ HSC to see if they are altered. Survivin is not only a member of the chromosomal passenger complex, but also functions as an inhibitor of apoptosis

We observed marked hepatomegaly and in some instances splenomegaly in aged *Mad2*$^{+/-}$ mice. Histological analysis of their livers and spleens with H&E staining revealed these organs exhibited an extremely high infiltration of hematopoietic cells, which is characteristic of a myeloproliferative disorder (MPD). These observations suggest that *Mad2* haploinsufficiency might contribute to development of an MPD-like condition after a long latency period. Although previous studies noted that *Mad2*$^{+/-}$ mice develop normally, and

blastocysts from *Mad2*$^{+/-}$ mice display no evidence of an impaired checkpoint activation (Dobles, et al. 2000), examination of metaphase spreads from MEFs derived from a *Mad2*$^{+/-}$ mouse (Dobles, et al. 2000) revealed a significant increase in the number of aneuploid cells relative to the controls (Michel, et al. 2001). Papillary lung adenocarcinomas are found in 18-19 month old *Mad2*$^{+/-}$ mice (Tuveson and Jacks 1999). The long latency period essential for tumor formation in the *Mad2*$^{+/-}$ mice suggests that additional mutations at other oncogenic or tumor-suppressor loci are required for transformation in checkpoint-defective cells. Defects in the mitotic checkpoint have been identified in colon, lung and breast cancer cell lines (Li and Benezra 1996). A defect in one allele of Cdc20, in which the Mad2-binding sites were mutated so that Mad2 can no longer inhibit the protein, renders the SAC dysfunctional (Li, et al. 2009). Mice with this defect contain a high percentage of aneuploid cells and develop spontaneous neoplasms (13.8% were hepatomas and the rest were lymphomas) at an increased rate, indicating that the SAC-mediated inhibition of Cdc20 is an important tumor-suppressing mechanism. Further analysis of the mutant cells revealed that the timing of anaphase depended on Mad2-Cdc20 interaction (Li, et al. 2009). These data imply that defects in Mad2, as well as the SAC, may not only be a direct cause of chromosomal instability (CIN), but may also contribute to tumorigenesis.

Control of the SAC is compromised in virtually all CIN tumors (Li and Zhang 2009; Rajagopalan and Lengauer 2004). The best understood aspect of CIN is

aneuploidy, and most carcinomas show variations in chromosome number that arise from the loss and gain of entire chromosomes during mitosis (Jallepalli and Lengauer 2001). Aneuploidy is one of the first oncogenic events in mouse models of adenomatous polyposis coli (Alberici, et al. 2007), and mathematical models predict that aneuploidy is required for sporadic carcinogenesis (Nowak, et al. 2002). Tumor cells with defects in the spindle checkpoint, such as Hct-*Mad2*$^{+/-}$ cells, have been shown to become highly aneuploid and continue to cycle after prolonged exposure to spindle inhibitors (Michel, et al. 2001), suggesting checkpoint-defective tumors could potentially be resistant to anti-mitotic chemotherapeutics. Unexpectedly, Mad2, along with other proteins in the SAC, play a role in prolonging G_2/M arrest after recognition of an unrepaired double-strand break in the DNA (Dotiwala, et al. 2010); this further implies the importance of Mad2 in the cell and demonstrates how defects in Mad2 could lead to tumorigenesis. The development of an MPD-like condition in aged *Mad2*$^{+/-}$ mice suggests that compromised control of the SAC plays a role in the development of this condition. The classification MPD includes polycythemia vera (PV), chronic idiopathic myelofibrosis (CIMF), essential thrombocythemia (ET), chronic myelogenous leukemia (CML), and chronic myelomonocytic leukemia (CMML) (Talarico 1998). In some disorders, specific characteristic chromosomal changes are seen. CML is characterized by a specific chromosomal abnormality and specific molecular abnormality; the Philadelphia chromosome is a reciprocal translocation between the long arms of chromosomes 9 and 22, which creates the fusion gene *bcr/abl* (Linker Charles A

2011). Interestingly, down-regulation of BRCA1 has been observed in hematopoietic cells expressing the BCR-ABL fusion protein (Deutsch, et al. 2003). Recent findings showed that BRCA1, in addition to its role in DNA damage response, acts as an upstream regulator of genes involved in the mitotic checkpoint regulation, thus protecting against promotion of aberrant divisions and aneuploidy (Wolanin, et al. 2010). Bcr-Abl leads to decreased expression of genes involved in the mitotic checkpoint activation - *Mad2, Bub1, Bub3*, and *BubR1*, resulting in mitosis perturbances, weakened mitotic checkpoint function, and mitotic slippage after nocodazole treatment (Wolanin, et al. 2010). In the other subsets of MPD, however, various abnormalities or deletions of whole chromosomes or particular chromosome arms have been frequently observed (Linker Charles A). Genetic inactivation of a single allele of human *MAD2* confers CIN on diploid cells (Michel, et al. 2001), and therefore it is plausible that the inactivation of a single allele of murine *MAD2* could cause any of the cells in a *Mad2*$^{+/-}$ mouse to lose or gain whole or partial chromosomes. These alterations to the genome could be one of many changes in the hematopoietic cells that lead to the development of a myeloproliferative-like disorder observed in aged *Mad2*$^{+/-}$ mice.

***Mad2* Haploinsufficiency Affects HPC Function, But Not HSC Function.**

Using *Mad2*$^{+/-}$ mice on a C57Bl/6 background, we found decreases in progenitor numbers and cycling status in BM HPC, demonstrating that the previously described effects of *Mad2* haploinsufficiency of mice on a mixed strain

background on hematopoiesis (Ito, et al. 2007) were not strain dependent. However, our present functional data seem to be in disagreement with our phenotypic analysis showing that the GMP and MEP populations are not different and CMPs are slightly, but significantly increased in the BM of *Mad2*$^{+/-}$ mice compared to *Mad2*$^{+/+}$ mice. One explanation for this could be that although there are greater numbers of CMPs in *Mad2*$^{+/-}$ BM, these cells could in fact just be less functional than their wild-type counterparts due to decreases in *Mad2* protein levels. The functional differences may not be reflected in the overall phenotypically defined population because of the massive reserve of hematopoietic progenitor cells that normally exist in the BM of mice (Broxmeyer, et al. 2005).

To examine if functional differences exist between *Mad2*$^{+/-}$ HSC compared to *Mad2*$^{+/+}$ HSC, we set up a competitive repopulation assay. The CRA defines the competitive nature of the HSC, and secondary repopulation studies allow for the measurement of self-renewal capabilities of the HSC. We found no significant differences in the long-term engraftment of *Mad2*$^{+/-}$ HSC and *Mad2*$^{+/+}$ HSC in either the primary or secondary transplants. This indicates that *Mad2* haploinsufficiency does not have a negative effect on the ability of the HSC to compete, engraft, or self-renew. This may not be too surprising considering that *Mad2*$^{-/-}$ embryos appear normal *in utero* and in culture until embryonic day E5.5 while *Mad2*$^{-/-}$ embryos die *in utero* about 6.5-7.5 days after conception (E6.5-E7.5) (Dobles, et al. 2000). Subtle differences in morbidity rates occur between

the colonies of *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ mice, but overall the *Mad2*$^{+/-}$ animals appear largely normal (Dobles, et al. 2000). Also, *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ SL (Sca-1^{+}, lin $^{-}$) cells have been found to express similar levels of CXCR4, and both *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ BM HPC migrate toward SDF-1 in a chemotaxis assay (Broxmeyer, et al. 2007). Since these studies were done in BM HPC, it does not necessarily mean these results will carry over into HSC. However, since we do not observe significant differences in the competitive repopulating ability of *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ HSC, these chemotaxis assays may provide an accurate estimation of the ability of *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ HSC to home to the BM microenvironment. Another possible reason why we observe no difference in the competitive repopulating ability of *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ HSC is that the high turnover rate of mature hematopoietic cells might prevent us from observing any significant differences. Although we are ultimately examining HSC function, we examine the PB for percentage of hematopoietic cells from our donor populations to gauge what is happening in the BM. While this is method normally reflects what is occurring in the HSC population, it might not be in the case for *Mad2*$^{+/-}$ HSC. It is also possible that even at the lowest donor to competitor ratio used, the BM compartment is at capacity due to the fact that both donor and competitor HSC and HPC are transplanted into the recipient, which might still retain a small portion of its own hematopoietic compartment despite lethal irradiation. Since we do not see significant differences in competitive repopulating ability, using an even lower number of donor and competitor cells, doing quaternary or tertiary transplants, or even performing a non-competitive repopulation assay might allow

differences to be detected if they truly do exist. Much like the observance that papillary lung adenocarcinomas are found only in aged *Mad2*$^{+/-}$ mice (Tuveson and Jacks 1999), *Mad2*$^{+/-}$ HSC might also require this long latency period to exhibit differences in repopulating ability compared to *Mad2*$^{+/+}$ HSC.

Previous studies in our laboratory found a higher percentage of cell death in KL (c-kit^{+}, lin^{-}) BM of *Mad2*$^{+/-}$ mice compared *Mad2*$^{+/+}$ BM when cells were cultured in SCF and GM-CSF (Ito, et al. 2007). However, there was no significant difference between *Mad2*$^{+/-}$ and *Mad2*$^{+/+}$ mice in the percentage of cell death of BM cells before cell culture, suggesting that greater cell death may occur in *Mad2*$^{+/-}$ HPC in stress conditions but not in steady state conditions (Ito, et al. 2007). To examine this, we exposed *Mad2*$^{+/-}$ and *Mad2*$^{+/+}$ mice to various cytotoxic and cytostatic agents. No significant differences were observed in the recovery of functional BM or spleen HPC from *Mad2*$^{+/-}$ and *Mad2*$^{+/+}$ mice after treatment with cyclophosphamide or γ-irradiation. In contrast, we found that *Mad2*$^{+/-}$ HPCs were not affected by the cytotoxic affects of Ara-C even though *Mad2*$^{+/+}$ HPCs were. In fact, *Mad2* haploinsufficiency may be somewhat myeloprotective to a cell cycle specific chemotherapy. The decreased percentage of cycling *Mad2*$^{+/-}$ HPCs compared to *Mad2*$^{+/+}$ HPCs might in part account for the differences observed in the recovery of HPCs from treatment with Ara-C, which selectively inhibits DNA synthesis, mainly affecting rapidly dividing cells. The differences might also be due to the increased absolute numbers of CMP observed in *Mad2*$^{+/-}$ mice. Even though the *Mad2*$^{+/-}$ HPCs did not appear

to be protected from the effects of treatment with cyclophosphamide or γ-irradiation, they recovered in a similar manner to *Mad2*$^{+/+}$ HPC, indicating that they are not any more sensitive to these cytotoxic treatments than the *Mad2*$^{+/+}$ HPC.

After finding that functional differences exist in the BM HPC populations of *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ mice, we examined how these populations responded to blocking the redox function of APE1. It has previously been shown that E3330 stops cells from proliferating (Fishel, et al. 2010; Luo, et al. 2008); it does not increase the percentage of cells undergoing apoptosis. APE1 is a transcriptional regulator of gene expression by acting as a redox co-activator of many transcription factors (Huang and Adamson 1993; Jayaraman, et al. 1997; Lando, et al. 2000; Xanthoudakis, et al. 1992). Although the precise molecular mechanisms for APE1's vital role in mammalian cells is still not fully understood, data indicates that the redox function is crucial for cell growth (Vascotto, et al. 2009). When examining the cytostatic effects of E3330, the drug appears to have a greater effect in 5% O_2 tension than in 20% O_2 tension. At 5% O_2, we observed a significant decrease in colony formation of *Mad2*$^{+/+}$ HPC under maximum stimulation and also blocking of synergistic growth with defined growth factors in the presence of the lowest concentration of E3330. Significant changes were not observed under 20% O_2. These data suggest that the redox function of APE1 is important in normal adult hematopoiesis, particularly under lower oxygen tension. This may also suggest the transcription factors that are

normally upregulated under hypoxic conditions may be important for colony formation and growth. The growth inhibitory effects of E3330 have been shown to be accentuated by hypoxia in pancreatic cancer cells (Zou and Maitra 2008), also suggesting an enhance requirement for APE1 redox function in hypoxia.

When comparing colonies from *Mad2*$^{+/-}$ HPC to wild-type HPC colonies, we found no significant differences in colony formation between E3330 and vehicle control treated *Mad2*$^{+/-}$ cells with lower concentrations of E3330. In contrast, wild-type HPC colony formation was significantly inhibited using the same E3330 concentration. The percent decrease compared to DMSO control is always greater in the wild-type cells compared to *Mad2*$^{+/-}$ cells under either oxygen tension. Also, *Mad2*$^{+/-}$ HPCs do not grow better at lower O_2 tension as do wild-type HPC. We observed similar colony numbers formed from *Mad2*$^{+/-}$ HPCs with mixed growth factors at 5% and 20% O_2 tension. This has never been shown before with *Mad2*$^{+/-}$ HPC colony formation; earlier studies in our laboratory only examined *Mad2*$^{+/-}$ HPC growth under 5% O_2 tension (Ito, et al. 2007). However, when higher concentrations of E3330 were used, *Mad2*$^{+/-}$ HPC colony formation was inhibited, but not to the same extent as wild-type *Mad2*$^{+/+}$ HPC. Inhibition of *Mad2*$^{+/-}$ HPC colony formation only under higher concentrations of E3330, along with the lack of inhibition of *Mad2*$^{+/-}$ HPC by low concentrations of E3330, suggests that *Mad2*$^{+/-}$ HPC are less sensitive to the cytostatic effects of the inhibitor compared to *Mad2*$^{+/+}$ HPC. Initially we thought that the differences between *Mad2*$^{+/-}$ HPC and *Mad2*$^{+/+}$ HPC colony formation in the presence of

E3330 might be due to inherent differences in the cycling status of the progenitors. We found the percentage of wild-type HPC in S-phase is similar in the presence and the absence of E3330; the same is true for *Mad2*$^{+/-}$ HPC. This indicates the inhibitor is not cell cycle specific and therefore the cycle status of *Mad2*$^{+/-}$ HPC cannot account for the differences in sensitivity of E3330 observed in *Mad2*$^{+/-}$ and *Mad2*$^{+/+}$ HPC. However, a study in mouse ES cells and ES cell-derived embryoid body (EB) cells revealed that siRNA knockdown of APE1 expression induced a G_1 arrest and decreased the percent of cells in S-phase, indicating APE1 regulates cell-cycle status in ES cells and also positively regulates the G_1/S transition of the cell cycle in EB cells (Zou, et al. 2007). It is possible that *Mad2*$^{+/-}$ HPC are less sensitive the effects of E3330 because they already exhibit cell cycle defects. However, knockdown of APE1 removes not only its redox signaling function, but also its DNA repair function, along with protein-protein interactions with other DNA repair and other proteins independent of the redox or DNA repair functions. Therefore, inhibition of APE1 redox function with the small molecule inhibitor E3330 is not directly comparable to siRNA knockdown studies.

In conclusion, the data presented in this study provides evidence that the SAC is functional in phenotypically defined HSCs, and treatment with a microtubule-destabilizing agent results in no differences in mitotic arrest and apoptosis in *Mad2*$^{+/-}$ and *Mad2*$^{+/+}$ HSCs. It also suggests that *Mad2* haploinsufficiency affects the function of HPCs, but not the HSC population. Although *Mad2*$^{+/-}$ HPCs have

decreased colony numbers and cycling status compared to *Mad2*$^{+/+}$ HPCs, they appear to be protected from cytotoxic effects *in vivo* of a cell-cycle specific agent, as well as the cytostatic effects of an APE1 inhibitor. We also found that the redox function of APE1 positively regulates adult hematopoiesis, and control of the cell cycle also appears to be important in this regulation process, events associated with HPC growing at lowered O_2 tension. These data, along with previously published data (Ito, et al. 2007), would suggest that *Mad2* haploinsufficiency alters the function of functional HPC due to changes in signaling. However, the exact signaling pathways altered in *Mad2*$^{+/-}$ HPC need to be determined in order to fully understand the role *Mad2* plays in hematopoiesis.

FUTURE DIRECTIONS

Although we observed no differences in the ability of *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ phenotypically defined HSC to arrest in M-phase and initiate apoptosis after nocodazole treatment, we did not determine whether premature sister chromatid separation occurred in these experiments. Previously published data found that *Mad2*$^{+/-}$ MEF cultures exhibited high frequencies of premature sister chromatid separation in the presence of colcemid, a spindle inhibitor, along with increases in the overall number of aneuploid cells (Michel, et al. 2001). To determine if this occurs in *Mad2*$^{+/-}$ HSC, we would examine metaphase spreads from both *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ HSCs after treatment with a spindle inhibitor. This would allow us to observe differences in the ability of the HSCs to stop progression into anaphase while the checkpoint is activated.

To determine if aged *Mad2*$^{+/-}$ mice do in fact develop a MPD, further analysis can be performed on additional unstained slides prepared from the previously collected samples discussed in the Results section to determine which hematopoietic lineage is leading to clonal proliferation observed in the various organs. The disorders included in the classification of MPD [PV, CIMF, ET, CML, and CMML (Talarico 1998)], exhibit various changes in the hematopoietic compartment. Determining the composition of the infiltrating hematopoietic cells could point us toward one specific disorder, and potentially help us identify other diagnostic criteria to look for in later studies. Also, a study examining age and

sex matched *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ mice could be set up; at various time points, the peripheral blood could be examined for changes in the hematocrit, platelet count, white blood cell count, and red blood cell morphology (Linker Charles A). If abnormalities exist in these areas, the mice would be sacrificed and the bone marrow, spleen, liver and other organs could be examined for further indications of MPD.

In our studies examining the effect of *Mad2* haploinsufficiency on recovery of BM HPC from *in vivo* cytotoxic effects, we used two non-cycle specific agents, but only one cell-cycle specific agent, Ara-C. Since we observed that *Mad2*$^{+/-}$ HPC, but not *Mad2*$^{+/+}$ HPC, are protected from the cytotoxic effects of Ara-C, we wanted to determine if these observations were unique to treatment with Ara-C, or if we would obtain similar results with another cycle specific agent. Therefore, we would like to examine the recovery of *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ HPC after treatment with 5-Fluorouracil (5-FU). 5-FU inhibits the activity of thymidylate synthetase (Peters, et al. 2002), which affects pyrimidine synthesis and leads to depletion of intracellular TTP pools (Elstein, et al. 1997). We will inject *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ mice with 5-FU (150 mg/kg, intravenous (i.v.)) on day zero of the experiment. We will analyze the mice at days 5, 7, and 9 after treatment, and both control *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ mice will be assessed at day zero without any treatment. For analysis, BM and spleen will be aseptically harvested from the treated mice at the various time points stated above, and colony assays will be

set up as previously described. If our results with Ara-C are common to cycle specific inhibitors, then we would expect to see a protective effect in the *Mad2*$^{+/-}$ HPC after 5-FU treatment.

APE1 has been shown to regulate cell cycle status in EB-derived hematopoietic progenitors, and to positively regulate the G_1/S transition in EB cells (Zou, et al. 2007). We have found that inhibition of the redox function of APE1 in *Mad2*$^{+/-}$ HPC is less effective when compared to *Mad2*$^{+/+}$ HPC, possibly suggesting that *Mad2*$^{+/-}$ HPC are less sensitive the effects of E3330 because they already exhibit cell cycle defects. It is possible that the reduced sensitivity of *Mad2*$^{+/-}$ HPC to E3330 is due to the fact the E3330 blocks signaling in the cell cycle that is already defective in the *Mad2*$^{+/-}$ HPC. If this were true, this could explain why E3330 is more effective in the *Mad2*$^{+/+}$ HPC. Therefore we need to determine the role that APE1 plays in cell cycle regulation. We will examine both *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ HPC after treatment with E3330 or vehicle control for changes in important cell cycle proteins, such as cyclins, securin, CDKs, and active APC/C, to determine how both APE1 redox function and *Mad2* haploinsufficiency affect cell cycle transitions.

Our data demonstrating that the colony formation of *Mad2*$^{+/+}$ HPC is significantly inhibited by E3330 suggests that the redox function of APE1 is important in normal adult hematopoiesis, particularly for HPC growing under lower oxygen tension. This also suggests that transcription factors that are normally

upregulated under lowered oxygen conditions may be important for colony formation and growth. The growth inhibitory effects of E3330 are accentuated by hypoxia in pancreatic cancer cells (Zou and Maitra 2008), also suggesting an enhanced requirement for Ape1/Ref-1 redox function in hypoxia. We will culture wild-type HPC under both 5% and 20% O_2 tension, and in the presence and absence of E3330, to determine how oxygen tension effects the activation of transcription factors, such as Egr-1, HIF-1α, and AP-1, which are known to be activated by the redox function of APE1.

We will also examine these transcription factors in *Mad2*$^{+/-}$ HPC under the same conditions as *Mad2*$^{+/+}$ HPC. *Mad2*$^{+/-}$ HPCs are not only less sensitive to inhibition by E3330, but also are not responsive to changes in oxygen tension. To examine if reactive oxygen species (ROS) plays a role in the lack of response to changes in oxygen tension, we will compare *Mad2*$^{+/-}$ and *Mad2*$^{+/+}$ HPC growth in the presence and absence of the ROS scavenger NAC (N-acetyl-L-cysteine) in 5% and 20% O_2 tension to see if this will have an effect on colony number. Other potential factors that could lead to decreases in *Mad2*$^{+/-}$ HPC sensitivity to E3330 could be changes in APE1 levels, the binding affinity of E3330 to APE1, and transcription factor activation mediated by the redox function of APE1. It is also important to consider that possibility that *Mad2*$^{+/-}$ HPC might exhibit a multi-drug resistance (MDR) in response to E3330. MDR is a condition in which cells or organisms are able to resist drugs or chemicals targeted at eradicating them. MDR to a particular drug can be brought about by many different mechanisms,

including enzymatic deactivation, altered metabolic pathways, decreased cell permeability, and increased efflux. A study examining P-glycoprotein (Pgp) and multidrug resistance protein 1 (MRP1) in mouse HSC and HPC found that MRP1 activity was present among different subpopulations of precursor cells (Kyle-Cezar, et al. 2007). Pgp and MRP1 are members of the ATP-binding cassette (ABC) family of transporter proteins. Both molecules are membrane-associated, energy-dependent efflux pumps with different substrate selectivity, and they may play a role in the activation, differentiation and function of hematopoietic cells. Since MRP1 is present in both HSC and HPC from mice, this protein needs to be examined in *Mad2*$^{+/-}$ hematopoietic populations to determine if it is more active, which could lead to decreased sensitivity to E3330.

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Publications

Mantel C, Guo Y, Lee MR, Han MK, Rohrabaugh S, Kim KS, Broxmeyer HE. Cells enter a unique intermediate 4N stage, not 4N-G1, after aborted mitosis. Cell Cycle. 2008 Feb 15; 7(4): 484-92.

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Abstracts at Scientific Meetings

"*Mad2* Haploinsufficiency Affects the Function of Hematopoietic Progenitor Cells." IU Simon Cancer Center's Cancer Research Day, Indianapolis, IN, May 2010.

"Mad2 Haploinsufficiency Unexpectedly Protects Hematopoietic Progenitor Cells From Ara-C Mediated Cytotoxicity." American Society for Hematology Annual Meeting, New Orleans, LA, December 2009.

"*Mad2* Haploinsufficiency Protects Hematopoietic Progenitor Cells From Ara-C Mediated Cytotoxicity & Gives Hematopoietic Stem Cells A Competitive Advantage *In Vivo*." IU Simon Cancer Center's Cancer Research Day, Indianapolis, IN, May 2009.

"*Mad2* Haploinsufficiency Protects Hematopoietic Progenitor Cells from Ara-C Mediated Cytotoxicity." 2008 Cancer Biology Chair and Directors Retreat and CABTRAC Annual Meeting, Park City, UT, October 2008.

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