The p53–Mdm2 feedback loop protects against DNA damage by inhibiting p53 activity but is dispensable for p53 stability, development, and longevity

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The p53–Mdm2 feedback loop is perceived to be critical for regulating stress-induced p53 activity and levels. However, this has never been tested in vivo. Using a genetically engineered mouse with mutated p53 response elements in the Mdm2 P2 promoter, we show that feedback loop-deficient Mdm2P2/P2 mice are viable and aphenotypic and age normally. p53 degradation kinetics after DNA damage in radiosensitive tissues remains similar to wild-type controls. Nonetheless, DNA damage response is elevated in Mdm2P2/P2 mice. Enhanced p53-dependent apoptosis sensitizes hematopoietic stem cells (HSCs), causing drastic myeloablation and lethality. These results suggest that while basal Mdm2 levels are sufficient to regulate p53 in most tissues under homeostatic conditions, the p53–Mdm2 feedback loop is critical for regulating p53 activity and sustaining HSC function after DNA damage. Therefore, transient disruption of p53–Mdm2 interaction could be explored as a potential adjuvant/therapeutic strategy for targeting stem cells in hematological malignancies.

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The ubiquitously expressed p53 tumor suppressor is maintained normally in an inactive latent form but functions as the “guardian of the genome” in response to DNA damage (Lane 1992). In response to genotoxic stressors, p53 transactivates target genes involved in cell cycle arrest, senescence, or apoptosis pathways to halt progression of insults into heritable aberrations [Vousden and Lu 2002]. A range of inhibitors have been identified that regulate p53 activity under normal and stress conditions. Of these, Mdm2 is the major negative regulator of p53. Genetic deletion of Mdm2 in vivo results in embryonic lethality that is rescued by concomitant deletion of p53 [Jones et al. 1995; Montes de Oca Luna et al. 1995]. The prevailing view suggests that Mdm2 inhibits p53 by two different mechanisms. Mdm2 binds and masks the transactivation domain of p53 [Momand et al. 1992; Oliner et al. 1993]. Furthermore, Mdm2 is also an E3 ubiquitin ligase that promotes p53 degradation through the 26S proteasome machinery [Haupt et al. 1997; Honda et al. 1997; Kubbutat et al. 1997]. Interestingly, Mdm2 itself is a transcriptional target of p53, thus establishing a negative feedback loop. After DNA damage, stabilized/activated p53 binds to the P2 promoter of Mdm2 and promotes its transcription [Barak et al. 1993; Wu et al. 1993]. Mdm2 in turn inhibits p53 via one of the two mechanisms described above.

A wealth of correlative evidence suggests that the p53–Mdm2 autoregulatory loop functions as the principal mode of p53 regulation under normal and DNA damage conditions [Haupt et al. 1997; Saucedo et al. 1998; Mendrysa and Perry 2000, Marine et al. 2006]. After DNA damage, p53 levels increase, correlating with enhanced p53 binding to the P2 promoter of Mdm2 and promotes its transcription [Barak et al. 1993; Wu et al. 1993; Saucedo et al. 1998]. This acute response is soon followed by dampening of p53 back to baseline levels. As increased p53 levels are toxic for cell viability, it is generally believed that Mdm2 transactivated by p53 from the P2 promoter is central for down-modulation of p53. Interestingly, this cytoprotective feature of the p53–Mdm2 feedback loop is considered a major impediment in exploiting the potential of p53 reactivation as a therapeutic strategy in tumors with wild-type p53. However, in the absence of
an in vivo model, these hypotheses could not be directly evaluated.

To investigate the biological significance of the dual Mdm2 promoters and the p53–Mdm2 autoregulatory loop in vivo, we generated a knock-in mouse model with a defective p53–Mdm2 autoregulatory loop and analyzed the effects of the feedback deficiency during development and under normal and DNA damage conditions.

Results

Generation of Mdm2^{P2/P2} mice

To examine the in vivo significance of the p53–Mdm2 autoregulatory loop, we generated a knock-in mouse by mutating the critical C and G nucleotides in the two p53 response elements of the P2-Mdm2 promoter [Fig. 1A,B]. This in vivo approach allowed us to specifically abrogate p53-mediated up-regulation of Mdm2 while maintaining the normal stoichiometry and functionality of other p53 pathway components. The abrogation of P2 promoter function resulted in the normal stoichiometry and functionality of other p53 pathway components. The abrogation of P2 promoter function verified by in vitro luciferase reporter assay prior to cloning of the mutant promoter fragment into the targeting vector [data not shown]. The targeting construct [Fig. 1A] with a mutant Mdm2 P2 promoter was electroporated into TC1 mouse embryonic stem (ES) cells. Correctly targeted ES clones were identified by Southern blotting using 5' and 3' external probes [Fig. 1A] and injected into C57BL/6 blastocysts to generate Mdm2^{P2/P2} chimeras. Male chimera (>80%) were backcrossed to C57BL/6 mice to secure germline transmission of the mutant allele. The Neomycin selection cassette was subsequently deleted by crossing with Zp3-Cre deleter mice (Lewandoski et al. 1997). A PCR-based genotyping strategy on genomic DNA isolated from tail biopsies was used to follow the transmission of the mutant allele. Mice were backcrossed for a total of four generations to >90% C57BL/6 background for this study.

Mdm2^{P2/P2} mice are born in a normal Mendelian ratio

We intercrossed heterozygous Mdm2^{P2/P2} mice to generate Mdm2^{P2/P2} homozygous mice. Surprisingly, Mdm2^{P2/P2} mice were born at an appropriate Mendelian ratio with no phenotypic aberrations [Supplemental Fig. 1]. We sequenced the genomic DNA from an Mdm2^{P2/P2} homozygous mouse and confirmed the mutations in the germline [Fig. 1B]. We next generated Mdm2^{P2/−} mice with further reduced Mdm2 levels. Again, both Mdm2^{P2/P2} and Mdm2^{P2/−} mice survived to adulthood lacking any distinctive phenotype. These results demonstrate that reduction in the P2-Mdm2 (Mdm2 expressed from the P2 promoter) level does not lead to lethal activation of p53. Thus, the autoregulatory loop is dispensable for normal development.

Mutations abrogate p53 binding at the P2-Mdm2 promoter

We next tested the specificity of P2-Mdm2 promoter mutations by performing in vivo chromatin immunoprecipitation (ChIP) assays. We isolated spleens from Mdm2^{P2/P2} and Mdm2^{P2/−} mice after irradiation. Unirradiated Mdm2^{P2/−} and Mdm2^{P2/P2} mouse spleens were obtained as controls at the same time. In vivo ChIP with a p53 antibody followed by real-time PCR analyses confirmed abrogation of p53 binding at the mutant Mdm2 promoter [Fig. 1C], while p53 still bound to the promoters of canonical targets p21 and Puma. Notably, p53 binding to the p21 promoter was significantly enhanced in irradiated Mdm2^{P2/P2} mouse spleens compared with Mdm2^{P2/−} spleens for unknown reasons.

Degradation profile of p53 after ionizing radiation (IR) is not altered in Mdm2^{P2/P2} mice

According to current dogma, degradation of the accumulated p53 after DNA damage is attributed to its ability to transactivate Mdm2, which encodes the major E3 ubiquitin ligase for p53 (Wu et al. 1993; Barak et al. 1994; Zauberman et al. 1995, Haupt et al. 1997; Honda et al. 1997, Kubbutat et al. 1997). Therefore, we first investigated the role of Mdm2 in p53 degradation after IR in feedback-deficient Mdm2^{P2/P2} mice. We isolated spleens from irradiated Mdm2^{P2/−} and Mdm2^{P2/P2} mice at different time points. Immunoblotting of protein lysate revealed the anticipated post-IR induction of p53 in both genotypes [Fig. 2A]. A slight increase in p53 induction at the 4-h post-IR time point in Mdm2^{P2/P2} spleens in comparison with Mdm2^{P2/−} spleens was noticeable [Fig. 2A]. Nonetheless, the pattern of p53 degradation in Mdm2^{P2/P2} spleens remained similar to Mdm2^{P2/−} spleens. In both genotypes, p53 was stabilized 2 h after IR, peaked by 4 h, and subsequently returned to baseline levels by 8 h. As expected, Mdm2 induction was visible only in Mdm2^{P2/−} spleens in response to IR. To evaluate the role of the p53–Mdm2 feedback loop in p53 stability in other tissues, we further examined post-IR induction and degradation profiles of p53 in the skin, thymus, lungs, and kidneys of Mdm2^{P2/−} and Mdm2^{P2/P2} mice [Fig. 2B]. Again, a similar pattern of p53 induction and degradation was observed in the tissues of mice from either genotype [Fig. 2B]. Of note, a slight delay in p53 decay was observed only in the Mdm2^{P2/P2} mouse skin. As expected, no induction of p53 was observed in the liver.

We also examined the impact of further reduced Mdm2 levels on p53 levels by analyzing p53 degradation in spleens from irradiated Mdm2^{P2/−} mice. p53 induction was comparatively higher in Mdm2^{P2/−} mouse spleens after IR due to minimal Mdm2 expression from a single allele containing only the P1 promoter [Fig. 2C]. Nonetheless, the degradation profiles of p53 in Mdm2^{P2/−} spleens were similar to that of Mdm2^{P2/P2} spleens [Fig. 2C]. A dramatic decrease of p53 levels was observed 6 h after IR in both genotypes. However, p53 levels were not quite back to baseline at the 8-h time point in Mdm2^{P2/P2} spleens. Of note, while loss of Mdm2 stabilizes p53 in vivo (Francoz et al. 2006; Ringshausen et al. 2006; Xiong et al. 2006; Terzian et al. 2008), we did not observe any overt p53 stabilization in Mdm2^{P2/P2} or Mdm2^{P2/−} tissues in the absence of DNA damage (Fig. 2), indicating that basal levels of Mdm2 expressed from the P1 promoter are
sufficient to maintain normal p53 levels. The viability of these mice further supports this conclusion.

**p53 degradation profile after ultraviolet radiation (UV) damage is not altered in Mdm2**\(^{P2/P2}\) mice

We also tested whether another type of DNA damage could induce the feedback loop and alter p53 degradation. We used UV, which creates pyrimidine dimers to activate p53 (Saucedo et al. 1998). Mouse embryonic fibroblasts (MEFs) from \(Mdm2^{+/+}\) and \(Mdm2^{P2/P2}\) mice were exposed to 50 J/m\(^2\) UV and harvested at different time points. Protein lysates were analyzed by immunoblotting (Fig. 2D). Notably, p53 was stabilized in both sets after UV exposure, while an enhanced Mdm2 induction was restricted to \(Mdm2^{+/+}\) lysates. Moreover, the overall pattern of p53 induction and down-regulation remained similar in both MEF cell lines (Fig. 2D). These data emphasize the importance of basal Mdm2 levels in regulating p53 levels in response to DNA damage.

Tissues from \(Mdm2^{P2/P2}\) mice show higher levels of p53 activity after DNA damage

In vivo, loss of Mdm2 alone results in spontaneous p53 activation (Jones et al. 1995; Montes de Oca Luna et al. 1995; Mendrysa et al. 2003; Francoz et al. 2006; Ringshausen et al. 2006; Xiong et al. 2006). Therefore, we next examined whether p53 activity was altered in \(Mdm2^{P2/P2}\) mice. We isolated total RNA from unirradiated and irradiated \(Mdm2^{+/+}\) and \(Mdm2^{P2/P2}\) thymi and performed RT-qPCR analyses for p53 targets. A significant increase (\(P < 0.01\)) in \(p21\) and \(Puma\) transcript levels was observed in \(Mdm2^{P2/P2}\) mice compared with \(Mdm2^{+/+}\) mice (Fig. 3A). Analogous analyses of p53 targets in RNA from spleens also showed modest increase in transcript levels of p53 target genes \(CyclinG1\), \(Noxa\), \(Puma\), \(p21\), and \(Bax\) in \(Mdm2^{P2/P2}\) mice (Fig. 3B). Notably, \(p21\) levels were significantly higher in \(Mdm2^{P2/P2}\) mice, in agreement with the ChIP data (Fig. 1C) and Western blot analysis (Fig. 2A). No differences in basal transcript levels of these p53 target genes were observed between unirradiated \(Mdm2^{+/+}\)
Enhanced p53 functions in Mdm2P2/P2 mice

p53 functions to maintain genomic integrity by inducing apoptosis, cell cycle arrest, or senescence in damaged cells [Vousden and Lu 2002]. To examine the acute impact of DNA damage on p53 function, we performed Annexin-V FITC flow cytometry analyses on thymocytes isolated from irradiated Mdm2P2/P2 and Mdm2P2/P2 mice. An increase in apoptotic response was observed in Mdm2P2/P2 thymocytes, although this was not statistically significant ($P = 0.15$) [Fig. 3C; Supplemental Fig. 2]. To further examine the effect on cell cycle, we next used MEFs that preferentially undergo p53-dependent cell cycle arrest after DNA damage. We irradiated Mdm2P2/P2 and Mdm2P2/P2 MEFs and, after confirming p53 up-regulation [Supplemental Figure 3], analyzed them by flow cytometry. We observed a statistically significant decrease ($P < 0.001$) in the S-phase population of Mdm2P2/P2 MEFs compared with Mdm2P2/P2 in response to IR [Fig. 3D,E]. Irradiated Mdm2P2/P2 MEFs also had significantly higher ($P < 0.05$) p21 mRNA levels as compared with Mdm2P2/P2 MEFs [Fig. 3F]. Furthermore, as cell culture is itself a stressed system, we analyzed MEF growth using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT] assay [Fig. 4A]. Again, in confirmation with high p53 activity, the cell growth rate of Mdm2P2/P2 MEFs was lower than Mdm2P2/P2 MEFS. These data suggest that a defective autoregulatory loop augments p53-dependent activities after stress.

Mdm2P2/P2 mice are extremely radiosensitive

Changes in Mdm2 levels impact radiation response and survival in mice [Mendrysa et al. 2003, 2006; Ringshausen et al. 2006; Terzian et al. 2007]. To specifically investigate the importance of the p53–Mdm2 feedback loop long term, we irradiated mice with a sublethal dose of 6 Gy IR. While 100% of irradiated Mdm2P2/P2 mice survived 50 d [the duration of the experiment] with no significant pathology, 80% of irradiated Mdm2P2/P2 mutant mice died within 25 d [Fig. 4A]. This is similar to the post-IR lethality of Mdm2P2/P2 and Mdm2 hypomorphic mice [Mendrysa et al. 2003; Terzian et al. 2007]. However, in contrast to these published studies, Mdm2P2/P2 mice have normal levels of basal Mdm2 from P1 promoter and only lack expression of p53-induced P2-Mdm2.

We next assessed the impact of gene dosage of Mdm2 and its homolog, Mdm4, in determining radiation sensitivity of Mdm2P2/P2 mice. We compared the survival of Mdm2P2/P2, Mdm2P2/P2, and Mdm4P2/P2/Mdm4P2/P2 mice with Mdm2P2/P2 and Mdm4P2/P2 mice after 6 Gy IR [Fig. 4B]. As anticipated, the reduction in Mdm2 and Mdm4 levels further enhanced the radiosensitivity of Mdm2P2/P2 mice. We also

and Mdm2P2/P2 tissues [Fig. 3A,B]. Additionally, no increase in Mdm2 transcripts was observed in either tissue from irradiated Mdm2P2/P2 mice, in conformity with the ChIP data [Fig. 3A,B]. These results corroborate that the autoregulatory loop is engaged primarily under stress conditions [Mendrysa and Perry 2000] and also imply that in the absence of exogenous stress stimuli, basal levels of Mdm2 from the P1 promoter are sufficient for regulating p53 activity.

Figure 2. The Mdm2 generated from the P2 promoter is not involved in DNA damage-induced p53 degradation. (A) Western blot analysis for post-IR p53 levels in 6 Gy irradiated Mdm2P2/P2 and Mdm2P2/P2 mouse spleens at different time points. (--) Negative controls for p53/Mdm2 expression; (+) positive controls for p53/Mdm2 expression, (arrowhead) Mdm2 band; (*) non-specific band. The bottom panels show dynamics of Mdm2, p21, and S18-p53 induction. (B) Western blot analysis for p53 levels in 6 Gy irradiated Mdm2P2/P2 and Mdm2P2/P2 mouse skin, thymus, kidney, and liver tissues. (C) Western blot analysis for p53 kinetics in Mdm2P2/P2 and Mdm2P2/P2 mouse spleens. (D) Time course for p53 induction and degradation in MEF cells exposed to 50 J/m2 UV. Vinculin was used as loading control in these experiments. Blots are representative of three independent biological replicates. Numbers at the bottom denote p53 fold induction normalized to vinculin controls and relative to untreated Mdm2P2/P2 controls.
explored potential gender differences in determining radiosensitivity in Mdm2+/− and Mdm2−/− mice. However, no such difference was evident (Supplemental Fig. 4). These data suggest that the feedback loop is important for Mdm2-mediated inhibition of p53 activity and survival after DNA damage.

Irradiated Mdm2−/− mice die due to p53-dependent bone marrow (BM) ablation

To identify the particular tissue type severely affected by radiation in the absence of the feedback loop and instigating the demise of irradiated Mdm2−/− mice, we next performed a comparative histopathological examination of all major radiosensitive tissues from Mdm2−/− and Mdm2+/− mice. We observed mild to moderate hypoplasia of the spleen and thymus with atrophy of both the splenic white pulp and the thymic cortex (Supplemental Fig. 5). Areas of mild atrophy were also observed in the mucosa of the stomach, duodenum, and small/large intestine. A noted decline in cellularity was observed in BM sections from both genotypes, culminating in profound aplasia in Mdm2−/− mice by day 12, while Mdm2+/− mice recovered [Fig. 4C]. Peripheral blood cell counts at this time point revealed severe thrombocytopenia and neutropenia as well as moderate anemia in Mdm2−/− mice (Supplemental Fig. 6A). Additionally, BM differential analysis showed significant depletion of less differentiated elements of the white cell lineage in Mdm2−/− mice [Supplemental Fig. 6B]. Immunohistochemical analyses of BM sections with phospho-histone H3 and Ki-67 antibodies also revealed the absence of mitosis/proliferation in Mdm2−/− compared with Mdm2+/− irradiated mice [Supplemental Fig. 7A,B].

Overall, these results suggested that the death of Mdm2−/− mice was likely a consequence of BM failure, a tissue overtly sensitive to radiation-induced p53 ac-
activity (Komarova et al. 2004; Ringshausen et al. 2006; Terzian et al. 2007; Wang et al. 2011). To further confirm that the BM aplasia in Mdm2<sup>P2/P2</sup> mice was indeed a p53-dependent phenotype, we generated Mdm2<sup>P2/P2 p53<sup>+/−</sup></sup> and Mdm2<sup>P2/P2 p53<sup>/−/−</sup></sup> mice and exposed them to 6 Gy IR. Both genotypes completely averted BM failure and survived the radiation dosage (Supplemental Fig. 8A,B).

Irradiated Mdm2<sup>P2/P2</sup> mice exhibit hematopoietic stem cell (HSC) defects

Next, to characterize the particular cell types sensitized by loss of the p53–Mdm2 feedback loop in irradiated Mdm2<sup>P2/P2</sup> mice, we performed flow cytometry analyses of BM constituent cells from Mdm2<sup>P2/P2</sup> and Mdm2<sup>+/+</sup> mice. Flow cytometry analysis revealed similar cell numbers in unirradiated mice of both genotypes but an acute and statistically significant (<i>P < 0.001</i>) depletion of common lymphoid progenitor cells (CLPs)/common myeloid progenitor cells (CMP) (Lin<sup>−/−</sup>Sca-1<sup>−/−</sup>Sca-1<sup>−/low</sup>C-Kit<sup>++</sup>) and HSCs (Lin<sup>−/−</sup>Sca-1<sup>−/−</sup>C-Kit<sup>++</sup>) in irradiated Mdm2<sup>P2/P2</sup> mice (Fig. 4D).

p53 is important for maintaining HSC quiescence (Liu et al. 2009). To further confirm and characterize the role of the p53–Mdm2 feedback loop in HSC function, we examined the long-term engraftment potential of Mdm2<sup>P2/P2</sup> HSCs by competitive and noncompetitive BM transplantation. We mixed BM cells from unirradiated Mdm2<sup>P2/P2</sup> (expressing the CD45.2 leukocyte marker) and wild-type (expressing the CD45.1 leukocyte marker) mice in a 1:1 ratio and transplanted them into lethally irradiated wild-type recipient mice. Peripheral blood analysis after 16 wk confirmed normal engraftment and contribution to hematopoietic lineage by the Mdm2<sup>P2/P2</sup> HSCs (Fig. 4E). However, in a similar transplantation experiment in parallel, a mix of irradiated Mdm2<sup>P2/P2</sup> BM cells with unirradiated wild-type cells in a 4:1 ratio failed to engraft.
and contribute to hematopoiesis. This suggested that Mdm2P2/P2 HSCs are functionally normal in the absence of DNA damage. Furthermore, we also rescued the lethality of irradiated Mdm2P2/P2 mice by transplanting BM cells from unirradiated wild-type mice (Fig. 4F). Additionally, lethally irradiated wild-type mice transplanted with Mdm2P2/P2 BM cells in a noncompetitive assay acquired radiosensitivity (Supplemental Fig. 9). These results confirmed that the p53-dependent post-IR sensitivity in Mdm2P2/P2 mice was associated with stem cells per se and not the niche. Thus, the p53–Mdm2 feedback loop is critical for attenuating p53 response in the HSCs after genotoxic insults.

**p53-mediated apoptosis causes HSC depletion in Mdm2P2/P2 mice**

The p53 damage response predominantly initiates either cell cycle arrest or apoptosis. To identify the preferential p53 downstream pathway involved in the post-IR attrition of BM cells in feedback loop-deficient Mdm2P2/P2 mice, we first examined expression of two p53 transcriptional targets: p21, which encodes a cell cycle inhibitor, and Puma, a proapoptotic gene in BM cells of Mdm2P2/P2 mice. These genes have been previously implicated in radiosensitivity and HSC regulation (Cheng et al. 2000; van Os et al. 2007; Abbas et al. 2010; Shao et al. 2010; Yu et al. 2010). While the baseline levels were similar between Mdm2+//+ and Mdm2P2/P2 mice, we observed a significant and prolonged induction of these genes in irradiated Mdm2P2/P2 BM cells compared with irradiated Mdm2+/+ BM cells (Fig. 5A). As expected, Mdm2 mRNA levels were only induced in irradiated Mdm2+/+ BM cells (Supplemental Fig. 10).

Next, we crossed Mdm2P2/P2 mice to p53515C/515C mice and generated Mdm2P2/P2, p53515C/515C mice. Previously, our laboratory had shown that p53515C/515C mice express a mutant form of p53 (p53R172P) that activates only cell cycle arrest (partially) but not apoptotic programs (Liu et al. 2004). Interestingly, Mdm2P2/P2, p53515C/515C mice survived exposure to 6 Gy IR [see Supplemental Fig. 8B]. This suggested a predominance of the p53 apoptotic pathway in radiosensitization of Mdm2P2/P2 BM cells.

Next, to directly evaluate the role of apoptosis in post-IR BM attrition, we performed Annexin-V FITC flow cytometry analysis on BM cells from irradiated Mdm2+/+ and Mdm2P2/P2 mice [Supplemental Fig. 11]. We could not detect a significant difference in apoptosis between the two genotypes using various doses of IR analyzed at different time points. It is possible that the rate of apoptosis essentially remains the same between the two genotypes but is prolonged in the case of Mdm2P2/P2 mice, causing increased loss of BM cellularity. A sustained induction of Puma [Fig. 5A] also supports this notion.

Finally, to segregate the in vivo role of apoptosis and cell cycle arrest pathways in radiation-induced BM aplasia, we crossed Mdm2P2/P2 mice with p21-null or Puma-null mice. Notably, Puma deficiency but not lack of p21 completely rescued the radiosensitivity [Fig. 5B, C]. Furthermore, Puma heterozygosity also rescued the phenotype [Fig. 5C], thereby confirming that in the absence of the feedback loop, p53-mediated apoptosis is the principal pathway involved in HSC depletion.

**Mdm2P2/P2 mice have a normal life span**

Increase in p53 activity is also linked with increased genomic aberrations, stem cell depletion, and aging phenotypes (Tyner et al. 2002; Liu et al. 2010). To rule out the possibility that reduced levels of stress-induced Mdm2 in the feedback-defective Mdm2P2/P2 mouse could modulate stem cell function throughout life and impact survival, we monitored a cohort of Mdm2P2/P2, Mdm2P2/-, and Mdm2+/+ mice long term. No difference in survival was evident between the genotypes [Fig. 6A]. Furthermore, the Mdm2P2/P2 and Mdm2P2/- mice reproduced and aged normally under standard nonstress laboratory conditions. This further confirms that the p53–Mdm2 feedback loop is dispensable for development and that its mere absence is not detrimental for normal functions. Moreover, basal Mdm2 levels from a single promoter (P1) are sufficient to regulate p53 and sustain life.

Finally, we examined whether exposure to a minor genotoxic insult capable of inducing p53 and initiating the feedback loop could alter the survival profile of these mice (Christophorou et al. 2006; Post et al. 2010; Gannon et al. 2012). To that end, we exposed Mdm2P2/P2 and Mdm2+/+ mice to 3 Gy IR and monitored them for survival [Fig. 6B]. No difference in survival was evident in either group. In addition, the post-IR survival of Mdm2P2/P2 and Mdm2P2/- mice [Fig. 6B] was quite similar to the unirradiated mouse cohort [Fig. 6A]. This suggests that a minor transient increase in p53 activity even in the absence of the p53–Mdm2 feedback loop is well tolerated and does not alter long-term stem cell functionality and overall survival.

**Discussion**

In the present study, using a mouse model with mutations at the p53-binding site in the Mdm2 P2 promoter, we provide the first in vivo characterization of the p53–Mdm2 feedback loop. In contrast to the prevailing paradigm, our results show that the p53–Mdm2 feedback loop is not essential for development, homeostasis, and longevity. Thus, constitutive Mdm2 levels expressed from the P1 promoter are sufficient for maintaining normal p53 protein levels. A second surprise is that after DNA damage, even in the absence of induced Mdm2 expression, the p53 degradation profile does not change. p53 levels appear at ~2 h after radiation, peak at 4 h, and are barely detectable by 8 h in the radiosensitive spleen in normal mice and mice that lack the feedback loop. A similar pattern of p53 degradation is also evidenced in other tissues, such as the skin, thymus, lungs, and kidneys of these mice.

Still, p53 degradation is impeded in a Mdm2 mutant/null background (Ringshausen et al. 2006; Itahana et al. 2007). This suggests that additional factors may be involved in signaling p53 degradation. One possibility is
that Mdm2 monoubiquitinates p53 and primes it for subsequent polyubiquitination and degradation in collaboration with other proteins (Grossman et al. 2003; Li et al. 2003). It is also possible that some as yet unknown E3 ligase is involved in p53 degradation after DNA damage in a p53-dependent fashion. Feedback-deficient Mdm2P2/P2 mice provide an excellent system to test these hypotheses in the future.

Regardless, the activity of p53 is compromised in Mdm2P2/P2 mice after DNA damage. While the absence of stress-induced Mdm2 does not significantly alter p53 protein levels, it does result in a modest increase in p53 activity that is well tolerated by most Mdm2P2/P2 mouse tissues. However, this increase in p53 activity turns catastrophic for the integrity of the hematopoietic system. Eighty percent of the Mdm2P2/P2 mice die due to hematopoietic failure, while all wild-type mice survive. The post-IR death of Mdm2P2/P2 mice could be simply averted by BM transplantation with wild-type donor cells. The hematopoietic system is in fact the most radiosensitive tissue and functions as a readout for small increases in p53 activity (Komarova et al. 2000; Liu et al. 2007; Terzian et al. 2007; Abbas et al. 2010; Wang et al. 2011). The importance of Mdm2 in inhibition of IR-induced p53 activity has been observed in heterozygous or hypomorphic Mdm2 mice (Mendrysa et al. 2003; Terzian et al. 2007). However, these mice have low levels of basal Mdm2 and correspondingly higher basal p53 activity, which is easily enhanced to lethal limits by IR. In contrast, Mdm2P2/P2 mice have normal levels of Mdm2 from the P1 promoter and normal p53 activity. Thus, the specific role of p53-induced Mdm2 could be clearly evaluated in these mice.

In the absence of the p53–Mdm2 feedback loop and in response to DNA damage, a modest increase in p53 activity promotes apoptosis of HSCs/CLPs/CMPs and impairs the normal dynamics of progenitor cell proliferation.

Figure 5. Absence of feedback loop promotes p53-dependent apoptosis in irradiated Mdm2P2/P2 mice. [A] Relative p21 and Puma mRNA induction in Mdm2+/- and Mdm2P2/P2 BM cells at different time points normalized to Rplp0 mRNA levels with −IR sample set to 1. n = 6, ±SEM; P-value was calculated by Student’s t-test. [B] Hematoxylin and eosin-stained BM sections of Mdm2P2/P2, Mdm2P2/P2p21-/-, and Mdm2P2/P2Puma-/- mice 12 d after 6 Gy IR [10x magnification]. [C] Kaplan-Meier survival curve for 6 Gy irradiated Mdm2P2/P2p21-/- and Mdm2P2/P2Puma-/- mice.

Figure 6. Lack of p53–Mdm2 feedback loop does not affect life span. [A] Kaplan-Meier survival curve for Mdm2+/-, Mdm2P2/P2, and Mdm2P2/p21-/- mice. [B] Kaplan-Meier survival curve for Mdm2+/- and Mdm2P2/P2 mice after 3 Gy IR. Curves are censored at 800 d.
Crosses with p53-null mice rescue the phenotype, suggesting p53 dependence. More importantly, crosses with Puma-null mice [an apoptotic gene that is a target of p53] but not p21-null mice [cell cycle arrest/senescent target of p53] completely rescue the stem cell phenotype. Thus, it is the p53-mediated apoptosis pathway that causes the demise of the animals after DNA damage. To our knowledge, this is the first study to simultaneously evaluate the role of p53-dependent apoptotic and cell cycle arrest pathways in determining radiation sensitivity in a mouse model.

Previously, mathematical modeling predicted that the autoregulatory loop controls the frequency and amplitude of p53 DNA damage response (Lahav et al. 2004). Our data suggest that it also regulates the duration of the p53 response in vivo. In particular, this is emphasized in the hematopoietic compartment after DNA damage, wherein the basal Mdm2 levels are likely insufficient. It will be interesting to test whether other tissue stem cells are also similarly sensitized in the Mdm2P2/P2 mice.

We also show that absence of the feedback loop does not promote aging or impede stem cell function under normal or low-dose IR (3 Gy) conditions. This suggests that it is dispensable for normal homeostasis and protection against minor stress conditions. Moreover, Mdm2 levels expressed from the single P1 promoter are sufficient in sustaining normal life. Overall, these results challenge our conventional view of the p53–Mdm2 feed-forward loop and question the accepted role of Mdm2 as the main executioner against minor stress conditions. Moreover, Mdm2 null mice were a gift from Dr. T. Jacks (Massachusetts Institute of Technology), and Puma-null mice were from Dr. G. Zambetti [St. Jude Children's Research Hospital; Mdm2+/− and Mdm2P2/P2+ mice have been described previously (Montes de Oca Luna et al. 1995; Xiong et al. 2006). Mice were maintained in >90% C57BL/6 background. All mouse studies were conducted in compliance with Institutional Animal Care and Use Committee protocols. Genotyping was carried out either as described earlier (Post et al. 2010) or by PCR amplification over the p53 response elements with primer sets Mdm2-F [5'-GGTCCAGG AGGTTGACAGGT-3'] and Mdm2-R [5'-AGCTTTTGGCAGTAGCTC-3'] followed by EcoRV digestion and resolution on agarose gel.

**Materials and methods**

**Targeting construct and generation of mice**

The Xho1–Xho1 DNA fragment [1 kb] from intron 2 of mouse Mdm2 covering the P2 promoter and the p53 response elements was cloned into pBluescript vector. Site-directed mutagenesis was carried out using QuickChange kit [Stratagene]. An EcoRV restriction enzyme site was created at the second p53 response element to enable us to distinguish wild-type and mutant alleles for genotyping. Mutated DNA was sequenced and cloned as part of the 5-kb 5′ homologous arm into pLG1 targeting vector backbone. A 1.2-kb homologous fragment was added as the 3′ arm. Lox-pGKNeo-Lox and Hsv-Tk1 cassettes were included for positive and negative selection, respectively [Fig. 1A]. The targeting construct was sequenced completely and electroporated into TC1 mouse E5 cells. G418-resistant clones were analyzed for correct homologous recombination by Southern blotting using 5′ and 3′ external probes [Fig. 1A]. Two independently targeted clones were expanded and injected into C57BL/6 blastocysts to generate Mdm2P2+/− chimeras. Male chimeras were backcrossed to C57BL/6 mice to secure germline transmission of the mutant allele.

**Mouse breeding, maintenance, and genotyping**

p53-null and CD45.1 mice were purchased from Jackson Laboratories. p21-null mice were a gift from Dr. T. Jacks [Massachusetts Institute of Technology], and Puma-null mice were from Dr. G. Zambetti [St. Jude Children's Research Hospital; Mdm2+/− and Mdm2P2/P2+ mice have been described previously (Montes de Oca Luna et al. 1995; Xiong et al. 2006). Mice were maintained in >90% C57BL/6 background. All mouse studies were conducted in compliance with Institutional Animal Care and Use Committee protocols. Genotyping was carried out either as described earlier (Post et al. 2010) or by PCR amplification over the p53 response elements with primer sets Mdm2-F [5'-GGTCCAGG AGGTTGACAGGT-3'] and Mdm2-R [5'-AGCTTTTGGCAGTAGCTC-3'] followed by EcoRV digestion and resolution on agarose gel.

**Protein analysis**

Protein lysates were prepared by lysing tissues or MEFs in NP-40 buffer. Protein extraction was carried out with BCA [Protein Assay kit, Pierce]. Fifty micrograms of lysate was resolved on 8% SDS-PAGE and immunoblotted with antibodies against Mdm2 [1:500; 2A10, Calbiochem], Mdm4 [1:500; MX82, Sigma], p53 [1:1000; CM5, Vector Laboratories], S18-p53 [1:1000; 9284, Cell Signaling], Vinculin [1:1000; V9131, Sigma], and p21 [1:1000; 556431, BD Pharmingen]. Western blots were repeated at least three times with biological replicates. p53 expression was quantitated using ImageJ software [National Institutes of Health [NIH]].

**ChIP assay**

Spleens harvested from irradiated and nonirradiated mice were washed with PBS and frozen-pulverized under liquid nitrogen. Chromatin was fixed with formaldehyde, and ChIP assay was carried out as described earlier [Jackson and Pereira-Smith 2006].

**Quantitative RT-PCR**

RNA isolation and quantitative RT–PCR were carried out as previously described in Pant et al. [2011].

**MEF preparation and cell culture**

MEFs prepared from 13.5 d post-coitum [dpc] embryos were maintained in Dulbecco's modified Eagle's medium (DMEM) [Invitrogen] supplemented with 10% FBS and penicillin [100 IU/mL] and streptomycin [100 µg/mL]. Early passage MEFs [P2–P3] were used for analysis.

**IR and UV studies**

Mice were irradiated at 6 Gy in a cesium-137 irradiator and killed at different time points. Tissues were harvested and lysed in NP-40 buffer for protein or TRizol for RNA analyses. MEFs cultured in a 100-mm tissue culture dish were irradiated at 1000 cGy.
Statistical analysis

P-value was calculated by Student’s t-test using Graphpad software, and values <0.05 were considered significant. (*) P < 0.05, (**) P < 0.01, and (***) P < 0.001.

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References

Abbas HA, Maccio DR, Coskun S, Jackson JG, Hazen AL, Sills TM, You MJ, Hirschi KK, Lozano G. 2010. Mdm2 is required for survival of hematopoietic stem cells/progenitors via dampening of ROS-induced p53 activity. Cell Stem Cell 7: 606–617.

Barak Y, Juven T, Haffner R, Oren M. 1993. mdm2 expression is induced by wild type p53 activity. EMBO J 12: 461–468.

Barak Y, Gottlieb E, Juven-Gershon T, Oren M. 1994. Regulation of mdm2 expression by p53: Alternative promoters produce transcripts with nonidentical translation potential. Genes Dev 8: 1739–1749.

Cheng T, Rodrigues N, Shen H, Yang Y, Dombkowski D, Sykes M, Scadden DT. 2000. Hematopoietic stem cell quiescence maintained by p21cip1/waf1. Science 287: 1804–1808.

Cheok CF, Verma CS, Baselga J, Lane DP. 2011. Translating p53 into the clinic. Nat Rev Clin Oncol 8: 25–37.

Christophorou MA, Ringshausen I, Finch AJ, Swigart LB, Evan GI. 2006. The pathological response to DNA damage does not contribute to p53-mediated tumour suppression. Nature 443: 214–217.

Francoz S, Froment P, Bogarts S, De Clercq S, Maetsens T, Doumont G, Bellefroid E, Marine JC. 2006. Mdm4 and Mdm2 cooperate to inhibit p53 activity in proliferating and quiescent cells in vivo. Proc Natl Acad Sci 103: 3232–3237.

Gannon HS, Woda BA, Jones SN. 2012. ATM phosphorylation of Mdm2 Ser394 regulates the amplitude and duration of the DNA damage response in mice. Cancer Cell 21: 668–679.

Grossman SR, Deato ME, Brignone C, Chan HM, Kung AL, Tagami H, Nakatani Y, Livingston DM. 2003. Polyubiquitination of p53 by a ubiquitin ligase activity of p300. Science 300: 342–344.

Haupt Y, Maya R, Kazaz A, Oren M. 1997. Mdm2 promotes the rapid degradation of p53. Nature 387: 296–299.

Honda R, Tanaka H, Yasuda H. 1997. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Lett 420: 25–27.

Ithana K, Mao H, Jin A, Ithana Y, Clegg HV, Lindstrom MS, Bhat KP, Godfrey VL, Evan GI, Zhang Y. 2007. Targeted inactivation of Mdm2 RING finger E3 ubiquitin ligase activity in the mouse reveals mechanistic insights into p53 regulation. Cancer Cell 12: 355–366.

Jackson JG, Pereira-Smith OM. 2006. Primary and compensatory roles for RB family members at cell cycle gene promoters that are deacetylated and downregulated in doxorubicin-induced senescence of breast cancer cells. Mol Cell Biol 26: 2501–2510.

Jones SN, Roe AE, Donehower LA, Bradley A. 1995. Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. Nature 378: 206–208.

Kamara EA, Christov K, Faerman AI, Gudkov AV. 2000. Different impact of p53 and p21 on the radiation response of mouse tissues. Oncogene 19: 3791–3798.

Kamara EA, Kondrakov RV, Wang K, Christov K, Golovkina TV, Goldblum JR, Gudkov AV. 2004. Dual effect of p53 on...
radiation sensitivity in vivo: p53 promotes hematopoietic injury, but protects from gastro-intestinal syndrome in mice. Oncogene 21: 3265–3271.

Kubbhat MH, Jones SN, Vousden KH. 1997. Regulation of p53 stability by Mdm2. Nature 387: 299–303.

Lahav G, Rosenfeld N, Geva-Zatorsky N, Levine AJ, Elowitz MB, Alon U. 2004. Dynamics of the p53–Mdm2 feedback loop in individual cells. Nat Genet 36: 147–150.

Lane DP. 1992. Cancer. p53, guardian of the genome. Nature 358: 15–16.

Lewandoski M, Wassarman KM, Martin GR. 1997. Zp3-ere, a transgenic mouse line for the activation or inactivation of loxP-flanked target genes specifically in the female germ line. Curr Biol 7: 148–151.

Li M, Brooks CL, Wu-Baer F, Chen D, Baer R, Gu W. 2003. Mono-versus polyubiquitation: Differential control of p53 fate by Mdm2. Science 302: 1972–1975.

Liu G, Terzian T, Suh YA, Iwakuma T, Post SM, Neumann M, Lang GA, Van Pelt CS, Lozano G. 2008. The inherent instability of mutant p53 is alleviated by Mdm2 or p16INK4a loss. Genes Dev 22: 1337–1344.

Mendrysa SM. 2000. The p53 tumour suppressor protein does not regulate expression of its own inhibitor, MDM2, except under conditions of stress. Mol Cell Biol 20: 2023–2030.

Mendrysa SM, McElwee MK, Michalowski J, O’Leary KA, Young KM, Perry ME. 2003. mdm2 is critical for inhibition of p53 during lymphopoiesis and the response to ionizing irradiation. Mol Cell Biol 23: 462–472.

Mendrysa SM, O’Leary KA, McElwee MK, Michalowski J, Eisenman RN, Powell DA, Perry ME. 2006. Tumor suppression and normal aging in mice with constitutively high p53 activity. Genes Dev 20: 16–21.

Montes de Oca Luna R, Wagner DS, Lozano G. 1995. Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. Nature 378: 203–206.

Oliner JD, Pietenpol JA, Thiagalingam S, Gyuris J, Kinzler KW, Vogelstein B. 1993. Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. Nature 362: 857–860.

Pant V, Xiong S, Iwakuma T, Quintas-Cardama A, Lozano G. 2011. Heterodimerization of Mdm2 and Mdm4 is critical for regulating p53 activity during embryogenesis but dispensable for p53 and Mdm2 stability. Proc Natl Acad Sci 108: 11995–12000.

Post SM, Quintas-Cardama A, Pant V, Iwakuma T, Hamir A, Jackson JG, Maccio DR, Bond GL, Johnson DG, Levine AJ, et al. 2010. A high-frequency regulatory polymorphism in the p53 pathway accelerates tumor development. Cancer Cell 18: 220–230.

Ringshausen I, O’Shea CC, Finch AJ, Swigart LB, Evan GI. 2006. Mdm2 is critically and continuously required to suppress lethal p53 activity in vivo. Cancer Cell 10: 501–514.

Saucedo LJ, Carstens BP, Seavey SE, Albee LD, Perry ME. 1998. Regulation of transcriptional activation of mdm2 gene by p53 in response to UV radiation. Cell Growth Differ 9: 119–130.

Shao L, Sun Y, Zhang Z, Feng W, Gao Y, Cai Z, Wang ZZ, Look AT, Wu WS. 2010. Deletion of proapoptotic Puma selectively protects hematopoietic stem and progenitor cells against high-dose radiation. Blood 115: 4707–4714.

Terzian T, Wang Y, Van Pelt CS, Box NF, Travis EL, Lozano G. 2007. Haploinsufficiency of Mdm2 and Mdm4 in tumorigenesis and development. Mol Cell Biol 27: 5479–5485.

van Os R, Kammenga LM, Ausema E, Bystryk LV, Drijaer DP, van Pelt K, Donjte B, de Haan G. 2007. A Limited role for p21Cip1/Waf1 in maintaining normal hematopoietic stem cell functioning. Stem Cells 25: 836–843.

Vousden KH, Lu X. 2002. Live or let die: The cell's response to p53. Nat Rev Cancer 2: 594–604.

Wang YY, Leblanc M, Fox N, Mao JH, Tinkum KL, Krummel K, Engle D, Piwnica-Worms D, Piwnica-Worms H, Balmain A, et al. 2011. Fine-tuning p53 activity through C-terminal modification significantly contributes to HSC homeostasis and mouse radiosensitivity. Genes Dev 25: 1426–1438.

Wu X, Bayle HJ, Olson D, Levine AJ. 1993. The p53–mdm-2 autoregulatory feedback loop. Genes Dev 7: 1126–1132.

Xiong S, Van Pelt CS, Elizondo-Fraire AC, Liu G, Lozano G. 2006. Synergistic roles of Mdm2 and Mdm4 for p53 inhibition in central nervous system development. Proc Natl Acad Sci 103: 3226–3231.

Yu H, Shen H, Yuan Y, XuFeng R, Hu X, Garrison SP, Zhang L, Yu J, Zambetti GP, Cheng T. 2010. Deletion of Puma protects hematopoietic stem cells and confers long-term survival in response to high-dose γ-irradiation. Blood 115: 3472–3480.

Zauberman A, Flusberg D, Haupt Y, Barak Y, Oren M. 1995. A functional p53-responsive intronic promoter is contained within the human mdm2 gene. Nucleic Acids Res 23: 2584–2592.