Validation of MdmX as a therapeutic target for reactivating p53 in tumors

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MdmX, also known as Mdm4, is a critical negative regulator of p53, and its overexpression serves to block p53 tumor suppressor function in many cancers. Consequently, inhibiting MdmX has emerged as an attractive approach to restoring p53 function in those cancers that retain functional p53. However, the consequences of acute systemic MdmX inhibition in normal adult tissues remain unknown. To determine directly the effects of systemic MdmX inhibition in normal tissues and in tumors, we crossed *mdmX*$^{-/-}$ mice into the *p53ER*^{TAM} knockin background. In place of wild-type p53, *p53ER*^{TAM} knockin mice express a variant of p53, p53ER^{TAM}, that is completely dependent on 4-hydroxy-tamoxifen for its activity. MdmX inhibition was then modeled by restoring p53 function in these MdmX-deficient mice. We show that MdmX is continuously required to buffer p53 activity in adult normal tissues and their stem cells. Importantly, the effects of transient p53 restoration in the absence of MdmX are nonlethal and reversible, unlike transient p53 restoration in the absence of Mdm2, which is ineluctably lethal. We also show that the therapeutic impact of restoring p53 in a tumor model is enhanced in the absence of MdmX, affording a significant extension of life span over p53 restoration in the presence of MdmX. Hence, systemic inhibition of MdmX is both a feasible therapeutic strategy for restoring p53 function in tumors that retain wild-type p53 and likely to be significantly safer than inhibition of Mdm2.

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The p53 transcription factor coordinates responses in somatic cells to a variety of stresses, including DNA damage, hypoxia, and oncogene activation, by triggering replicative arrest or death of the damaged cell. The pivotal role that p53 plays in tumor suppression is evidenced by the fact that the p53 pathway is functionally inactivated in the majority of human cancers and that p53-deficient mice develop cancers at high frequencies (Kruse and Gu 2009; Vousden and Prives 2009). Intriguingly, many cancers retain expression of wild-type p53, inactivating instead upstream or downstream p53 pathway components, and intense research has focused on finding ways to reactivate p53 function selectively in such p53-competent tumors. Hitherto, most pharmacological strategies have sought to inhibit Mdm2, a critical negative regulator of p53 (Issaeva et al. 2004; Vassilev et al. 2004; Shangary et al. 2008; Sun et al. 2008; Vazquez et al. 2008; Canner et al. 2009; Hedstrom et al. 2009). While such studies confirm that inhibition of Mdm2 can activate latent p53 in tumor cells, it also triggers pathological p53 function in normal tissues (Boesten et al. 2006; Francoz et al. 2006; Grier et al. 2006; Ringshausen et al. 2006; Xiong et al. 2006; Maetens et al. 2007; Valentin-Vega et al. 2008), narrowing the therapeutic window available for Mdm2 inhibition in cancer therapy.

MdmX, also known as Mdm4, has recently emerged as a discrete critical negative regulator of p53 (Marine and Jochemsen 2005; Marine et al. 2007; Wade and Wahl 2009; Mancini et al. 2010). While MdmX is structurally related to Mdm2, it does not target p53 for degradation via ubiquitin ligation, but instead acts as a direct transcriptional squelcher of p53 (Marine and Jochemsen 2005; Marine et al. 2007; Kruse and Gu 2009; Wade and Wahl 2009). MdmX also modulates Mdm2 stability and function, thereby indirectly regulating p53 (Jackson et al. 2001; Uldrijan et al. 2007; Linke et al. 2008; Okamoto et al. 2009). Like Mdm2, MdmX is overexpressed in many cancers, especially those of breast, colon, and lung, as well as in glioma, lymphoma, and retinoblastoma (Danovi et al.

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2004; Laurie et al. 2006), where it is thought to promote tumorigenesis by suppressing p53 function. However, knockout (Parant et al. 2001; Migliorini et al. 2002) and conditional deletion (Francoz et al. 2006; Grier et al. 2006; Xiong et al. 2006) studies have shown that MdmX, like Mdm2, is required as a buffer against precocious and lethal p53 activity at various bottlenecks in embryonic development. Ascertaining whether acute MdmX inhibition also shares the same disquieting toxicity as does Mdm2 inhibition in p53-competent adult tissues has been confounded by this embryonic lethality in p53-competent *mdmX* knockout mice, as has evaluation of the utility of MdmX inhibition in cancer therapy.

To model directly the side effects of acute systemic MdmX inhibition in adult p53-competent animals, together with the therapeutic potential of MdmX inhibition in established tumors, we crossed $mdmX^{+/-}$ mice with $p53ER^{TAM}$ knockin ($p53^{KI}$) animals. $p53^{KI}$ mice express $p53ER^{TAM}$, a 4-hydroxy-tamoxifen (4-OHT)-dependent variant of endogenous p53, in place of wild-type p53 protein. $p53^{KI}$ mice are effectively p53-deficient in the absence of 4-OHT ligand and are p53 wild type in its presence (Christophorou et al. 2005), allowing for acute and reversible systemic functional restoration of wild-type p53 function in adult $mdmX^{-/-};p53^{KI}$ mice. We show that MdmX, like Mdm2, is indeed continuously required to suppress untoward p53 function in adult tissues and their stem cells. However, unlike even fleeting p53 restoration in $mdm2^{-/-}$ mice, which is rapidly and irrevocably fatal, transient p53 function is surprisingly well tolerated by $mdmX^{-/-}$ animals. We show that the milder impact of p53 restoration in $mdmX^{-/-}$ mice compared with restoration in $mdm2^{-/-}$ mice is likely a consequence of the fact that Mdm2 retains partial functionality in the absence of MdmX. Furthermore, we show that absence of MdmX potentiates the therapeutic benefit of p53 restoration in a lymphoma model. These data intimate that inhibition of MdmX would be an effective therapeutic strategy for restoring p53 function in cancer that is better tolerated than is inhibition of Mdm2.

Results

p53 is spontaneously active in MdmX-deficient adult tissues

To determine the extent to which MdmX continually restrains spontaneous p53 activity in normal adult mouse tissues, we crossed $p53^{KI/KI}$ mice with $mdmX^{+/-}$ mice. No pups harboring two copies of the $p53^{KI}$ allele were born in an $mdmX^{-/-}$ background, indicating that, as reported with $mdm2^{-/-};p53^{KI/KI}$ mice, even in the absence of 4-OHT there is some residual p53 activity in $mdmX^{-/-}$ mice from two copies of $p53ER^{TAM}$ that is sufficient to induce embryonic lethality. However, $mdmX^{-/-}$ mice harboring only a single $p53ER^{TAM}$ allele ($p53^{KI/-}$) were born at expected Mendelian ratios (as were $mdmX^{+/+};p53^{KI/-}$ and $mdmX^{+/-};p53^{KI/-}$ animals), indicating no biologically significant leakiness from a single copy of $p53ER^{TAM}$; consequently, $mdmX^{-/-};p53^{KI/-}$ animals were used in all subsequent experiments.

To ascertain the extent of spontaneous p53 activity in adult tissues in the absence of MdmX, p53 was restored in $mdmX^{-/-};p53^{KI/-}$ mice by systemic administration of tamoxifen and selected organs were harvested for analysis 6 h later. For direct evidence of p53 activity in the absence of MdmX, we assayed for the expression of known p53 target genes *cdkn1a* (encoding the Cdk inhibitor $p21^{cip1}$) and *puma* (encoding the proapoptotic BH3 protein PUMA). We noted rapid and dramatic induction of these two p53 target genes in tissues of tamoxifen-treated $mdmX^{-/-};p53^{KI/-}$, mice confirming that p53 is indeed spontaneously active in adult tissues of MdmX-deficient mice (Fig. 1A). However, quite unlike the situation following p53 functional restoration in the absence of Mdm2 (Ringshausen et al. 2006), where both *cdkn1a* and *puma* are profoundly induced in all tissues irrespective of whether or not that specific tissue then undergoes apoptosis, restoration of p53 function in $mdmX^{-/-};p53^{KI/-}$ tissues elicited more specific p53 target gene induction. While, with the exception of small intestine, p53 restoration potently induced *cdkn1a* in all tested tissues, the proapoptotic gene *puma* was induced only in classically radiosensitive tissues (bone marrow, spleen, thymus, and intestinal epithelium) (Fig. 1A).

This unexpected selectivity of p53 target gene induction in different $mdmX^{-/-}$ tissues was broadly mirrored by the biological outcomes of p53 restoration. Radiosensitive tissues (bone marrow, thymus, spleen, and intestinal epithelium) of tamoxifen-treated $mdmX^{-/-};p53^{KI/-}$ mice exhibited a marked increase in apoptosis compared with controls ($mdmX^{+/+};p53^{KI/-}$ and $mdmX^{-/-};p53^{-/-}$ mice treated with tamoxifen, or $mdmX^{-/-};p53^{KI/-}$ mice treated with oil) (Fig. 1B; data not shown). In contrast, no detectable apoptosis was observed in classically radioresistant tissues like liver, kidney, lung, and heart (Fig. 1B; data not shown). Of note, despite the crucial role played by MdmX inhibition of p53 during development of the cerebellum, especially with regard to suppression of p53-dependent apoptosis in post-mitotic cerebellar neurons in embryos (Francoz et al. 2006), we saw no apoptosis or any other evident impact of p53 restoration on histology or cell viability in the fully formed adult cerebellum of $mdmX^{-/-}$ mice, even though significant p53 activity was evident from the induction of *cdkn1a* (Supplemental Fig. S1). p53-induced apoptosis was evident only in the continuously proliferating subventricular zones of $mdmX^{-/-};p53^{KI/-}$ mouse brains (Supplemental Fig. S1).

$mdmX^{-/-}$ *mice survive transient p53 restoration*

Substantial evidence indicates that both Mdm2 and MdmX are crucial for reining in the toxic effects of untoward p53 activity. Indeed, we previously showed that even very transient restoration of p53 (i.e., a single tamoxifen injection) is lethal to adult Mdm2-deficient mice within 6 d (Ringshausen et al. 2006). In stark contrast, even 7 d of sustained (i.e., daily tamoxifen injections) p53 restoration was not lethal to MdmX-deficient mice (Fig. 2A), even

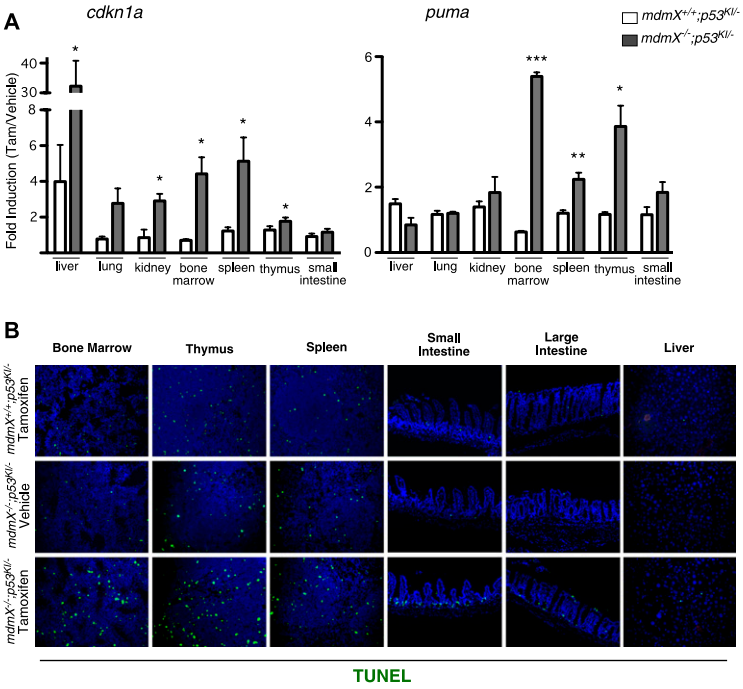


Figure 1. p53 is spontaneously active in tissues of $p53^{KI/-}$;$mdmX^{-/-}$ mice. (*A*) RNA was isolated from organs of mice treated with either vehicle (oil) or tamoxifen for 6 h, and *cdkn1a* and *puma* gene expression was quantified by TaqMan analysis. Fold induction (expression in tamoxifen-treated tissues over vehicle-treated tissues) is plotted. Fold inductions that were statistically significant are marked with an asterisk. Error bars show the SEM of triplicates. (*B*) A single bolus of either vehicle or tamoxifen was administered to mice of the indicated genotypes, and organs were collected 6 h later. Apoptosis was detected in tissue sections by TUNEL staining. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

though histological analysis indicated significant degenerative tissue-specific pathologies, including striking suppression of proliferation and decreased cellularity in the bone marrow and red pulp of the spleen together with loss of structural integrity in the thymus (Fig. 2B). Unexpectedly, given that intestines of tamoxifen-treated $mdm2^{-/-}$;$p53^{KI/-}$ mice rapidly succumb to severe and irrevocable damage (Supplemental Fig. S2; Ringshausen et al. 2006), intestinal epithelia of tamoxifen-treated $mdmX^{-/-}$;$p53^{KI/-}$ mice maintained normal architecture and barrier function throughout the 1 wk of p53 restoration, despite exhibiting significant ongoing levels of p53-dependent apoptosis (which, incidentally, is evidence that restored p53 function is indeed maintained throughout the 7 d of tamoxifen treatment) (Fig. 2C). One likely reason for the sustained structural integrity of $mdmX^{-/-}$ intestinal epithelia is that proliferation in $mdmX^{-/-}$ intestines continues throughout p53 restoration (Fig. 2C), a surprising observation that is nonetheless consistent with the failure of p53 to induce $p21^{cip1}$ in that tissue (Fig. 1A).

To ascertain whether $mdmX^{-/-}$ mice can survive permanent p53 restoration, $mdmX^{-/-}$;$p53^{KI/-}$ mice were repeatedly treated daily with tamoxifen and their health and viability were monitored. Long-term sustained p53 restoration was eventually lethal to $mdmX^{-/-}$ mice (median survival of 29 d) (Fig. 3A), albeit greatly delayed relative to the rapid demise (6 d) that succeeds p53 restoration in $mdm2^{-/-}$ mice. At the time of death, bone marrow, spleen, and thymus of tamoxifen-treated $mdmX^{-/-}$;$p53^{KI/-}$ mice exhibited severe attrition, with decreased cellularity and loss of architectural integrity, reminiscent of that accompanying the far more rapid deaths of tamoxifen-treated $mdm2^{-/-}$;$p53^{KI/-}$ mice (Fig. 3B). Once again, however, the dramatic loss of intestinal epithelial integrity that p53 restoration elicits in $mdm2^{-/-}$ animals (Supplemental Fig. S2; Ringshausen et al. 2006) was absent from $mdmX^{-/-}$ animals: Overall integrity of both the small and large intestines of $mdmX^{-/-}$;$p53^{KI/-}$ mice, as well as villus/crypt structure and ratio, were maintained even after long-term (26 d) sustained p53 restoration (Fig. 3C). Detailed histological analysis of such intestines indicated an abnormal expansion of Paneth cells in the crypts and the concurrence of both sustained intestinal crypt proliferation (again, consistent with the absence of $p21^{cip1}$ induction in intestines) and apoptosis (Fig. 3C).

Given this long-term maintenance of intestinal integrity in tamoxifen-treated $mdmX^{-/-}$;$p53^{KI/-}$ mice, the most plausible cause of their eventual demise is bone marrow failure: Indeed, 26-d tamoxifen-treated $mdmX^{-/-}$ mice were severely anemic (reduced red blood cell count, reduced hemoglobin and hematocrit) and exhibited overall reduction of blood counts (Supplemental Fig. S3).

The pathologies induced in MdmX-deficient tissues by transient p53 functional restoration are fully reversible

To ascertain whether transient p53 restoration in $mdmX^{-/-}$ mice elicits irreversible damage in tissues, p53 was continuously restored in $mdmX^{-/-}$;$p53^{KI/-}$ mice for 7 d (daily injections), and tamoxifen was then withdrawn to assess the capacity of tissues to regenerate. All affected tissues (bone marrow, spleen, thymus, and intestine) quickly recovered, appearing largely normal after only 1 wk: Proliferation resumed in lymphoid organs (Fig. 4A), and apoptosis declined in intestinal epithelia (Fig. 4B). Thereafter, such mice lived long term and exhibited no adverse consequences of transient systemic

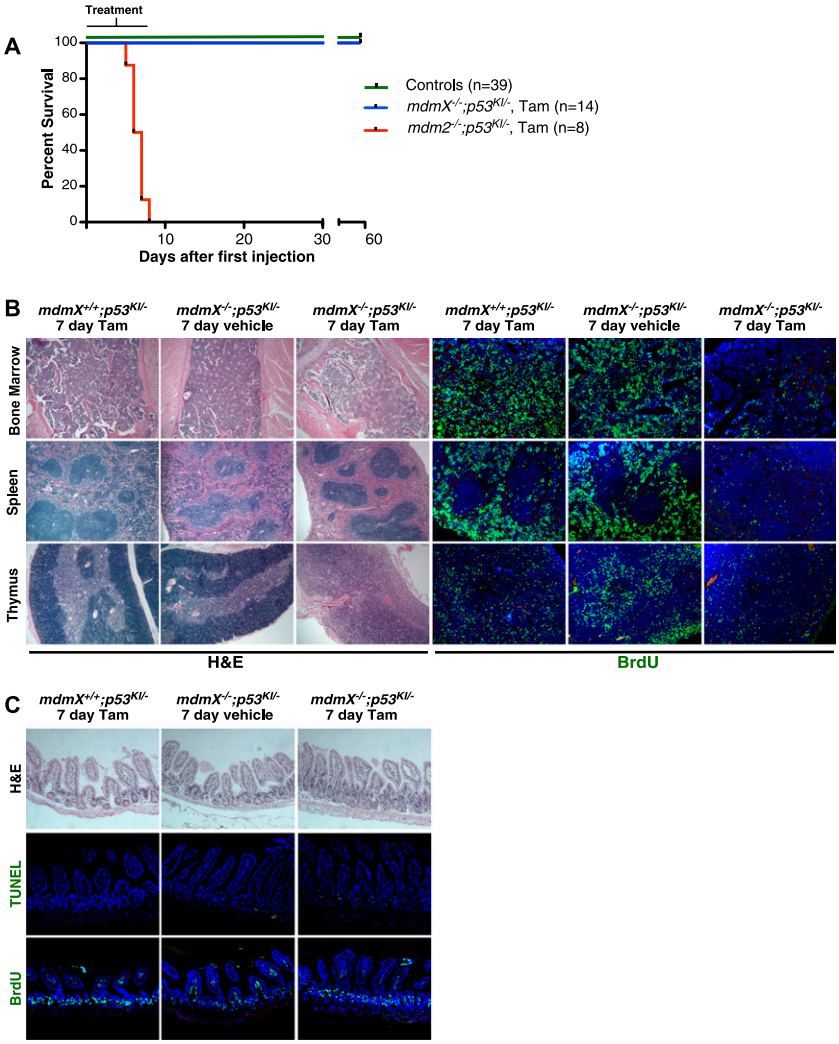


Figure 2. Transient p53 restoration in the absence of MdmX is well tolerated. (*A*) Kaplan-Meier curve showing survival of mice of the indicated genotypes given daily injections of vehicle or tamoxifen for 1 wk. Controls are $p53^{KI/-}$ mice treated with vehicle ($n = 8$), $p53^{KI/-}$ treated with tamoxifen ($n = 8$), $mdm2^{-/-};p53^{KI/-}$ mice treated with vehicle ($n = 6$), $mdmX^{-/-};p53^{KI/-}$ mice treated with vehicle ($n = 13$), and $mdmX^{-/-};p53^{-/-}$ mice treated with tamoxifen ($n = 4$). (*B*) Mice of the indicated genotypes were treated daily with either vehicle or tamoxifen for 7 d. The *left* panels show H&E staining of bone marrow, spleen, and thymus. The *right* panels show BrdU staining of the same organs (BrdU was administered 2 h prior to sacrifice). (*C*) H&E, TUNEL, and BrdU staining of sections of small intestine from mice treated as in *B*.

p53 activation. Hence, the extended survival of transiently tamoxifen-treated $mdmX^{-/-};p53^{KI/-}$ (relative to $mdm2^{-/-}$ mice) is accompanied by a greatly enhanced capacity to recover from the depredations of p53 restoration.

One possible explanation for both the maintenance of intestinal integrity and the expeditious recovery from p53 restoration in tamoxifen-treated $mdmX^{-/-};p53^{KI/-}$ compared with tamoxifen-treated $mdm2^{-/-};p53^{KI/-}$ animals is that the stem cell populations required to regenerate damaged tissues are less debilitated by the absence of MdmX than of Mdm2. To test this directly, we turned to the hematopoietic system in which the stem cell population is relatively well-characterized. Bone marrow cells were isolated from tamoxifen-treated $p53^{KI/-}$, $mdmX^{-/-};p53^{KI/-}$, and $mdm2^{-/-};p53^{KI/-}$ mice and immunostained, and the hematopoietic stem/progenitor cell compartment was quantitated by flow cytometry. While p53 restoration caused a slight reduction in whole bone marrow counts in $mdmX^{-/-};p53^{KI/-}$ mice, p53 restoration in $mdm2^{-/-};p53^{KI/-}$ mice triggered dramatic loss of total bone marrow cellularity within only 1 d: By 5 d, the bone marrow had been essentially annihilated—only a few hundred cells (1655.3 ± 558.2) were recoverable, making it impossible to conduct meaningful analysis of stem cells at this or later time points in these mice (Supplemental Fig. S4). Control $mdmX^{-/-};p53^{KI/-}$ mice treated with vehicle maintained similar cell counts to tamoxifen-treated $mdmX^{+/+};p53^{KI/-}$ mice (data not shown). Direct quantitation of stem/progenitor cells (Lin^-, $Sca1^+$, $CD150^+$, $CD48^-$) (Kiel et al. 2005) indicated that they are effectively ablated in $mdm2^{-/-};p53^{KI/-}$ mice after only 1 d of p53 restoration (Fig. 5). In comparison, although they are significantly decreased in number compared with wild-type controls, more stem/progenitor cells can be recovered from tamoxifen-treated $mdmX^{-/-};p53^{KI/-}$ mice even after 7 d of sustained p53 restoration than from 1-d-treated $mdm2^{-/-};p53^{KI/-}$ animals (Fig. 5). Furthermore, although the numbers of stem/progenitor cells remained low in $mdmX^{-/-};p53^{KI/-}$ bone marrow 1 wk after the end of tamoxifen treatment, their numbers were fully recovered when measured 1 wk later. Hence, hematopoietic stem/progenitor cells recover well from short-term p53 activity in the absence of MdmX but not of Mdm2. In conclusion, while both Mdm2 and MdmX are

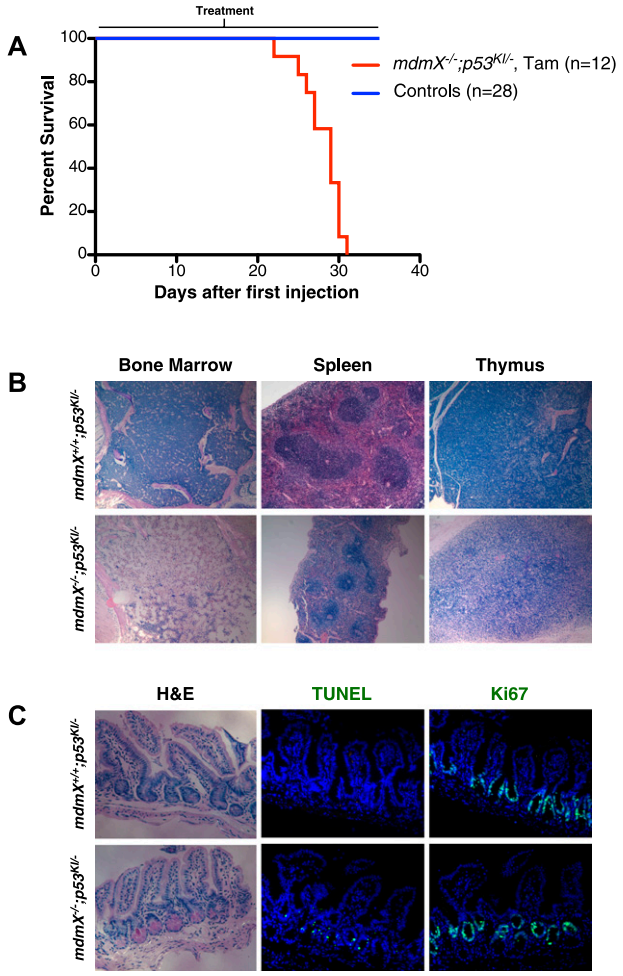


Figure 3. Permanent p53 restoration is lethal in the absence of MdmX. (*A*) Kaplan-Meier curve showing survival of mice of the indicated genotypes given continuous daily injections of vehicle or tamoxifen. Controls are $mdmX^{+/+};p53^{KI/-}$ mice treated with tamoxifen (n = 13), $mdmX^{-/-};p53^{KI/-}$ mice treated with vehicle (n = 9), and $mdmX^{-/-};p53^{-/-}$ mice treated with tamoxifen (n = 6). (*B*) H&E staining of organs harvested from $mdmX^{+/+};p53^{KI/-}$ and $mdmX^{-/-};p53^{KI/-}$ mice treated continuously with daily tamoxifen for 26 d. (*C*) H&E, TUNEL, and Ki67 staining of small intestine harvested from $mdmX^{+/+};p53^{KI/-}$ and $mdmX^{-/-};p53^{KI/-}$ mice treated continuously with daily tamoxifen for 26 d.

required for the long-term maintenance of hematopoietic stem/progenitor cells in the presence of functional p53, it is clear that Mdm2 is the most exigent restraint to p53 toxicity.

Mdm2 restrains p53 activity even when MdmX is absent, blunting the pathological impact of MdmX deficiency in p53-competent tissues

Why should p53 be less toxic in the absence of MdmX than in the absence of Mdm2? It is well established that p53 and Mdm2 form a negative feedback loop whereby active p53 induces expression of Mdm2 that in turn down-regulates p53 (for review, see Kruse and Gu 2009). We hypothesized that, even in the absence of functional MdmX, Mdm2 can still restrain excessive p53 activity, thus mitigating p53 toxicity. To test this, we took advantage of the fact that our acutely switchable $p53^{KI/KI}$ model allows us to follow engagement of the p53–Mdm2 negative feedback loop in real time. We previously showed that upon acute p53 restoration, p53 levels fall progressively—a down-regulation that is compromised in the absence of Mdm2 (Ringshausen et al. 2006). To determine whether p53 is also down-regulated by endogenous Mdm2 in the absence of MdmX, we isolated $mdmX^{+/+};p53^{KI/-}$ and $mdmX^{-/-};p53^{KI/-}$ mouse embryonic fibroblasts (MEFs) and treated them with 4-OHT in the presence or absence of the Mdm2 inhibitor Nutlin-3a (Vassilev et al. 2004). Upon acute restoration of p53 function in $mdmX^{+/+};p53^{KI/-}$ MEFs following 4-OHT addition, overt p53 activity was rapidly engaged by the known p53-activating stresses of in vitro culture, as evidenced by the induction of $p21^{cip1}$. Concurrently, $p53ER^{TAM}$ levels were down-regulated and Mdm2 levels rose, again confirming that p53, once restored, is activated. The decline in p53 level was partially abrogated

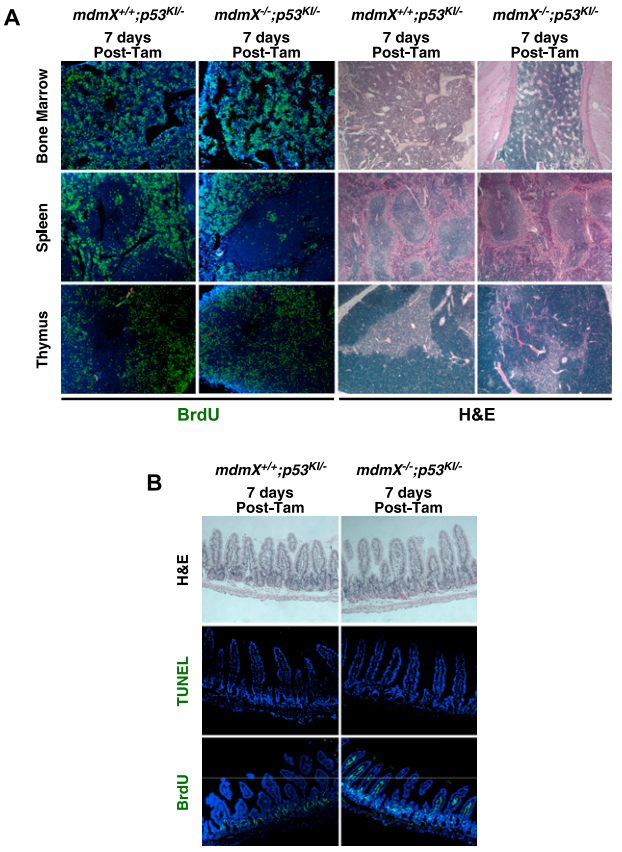


Figure 4. The pathological effects of transient p53 restoration in the absence of MdmX are reversible. (*A*) Mice of the indicated genotypes were treated daily with tamoxifen for 7 d and sacrificed 7 d after. The *left* panels show BrdU staining of sections of bone marrow, spleen, and thymus (BrdU was administered 2 h prior to sacrifice). The *right* panels show H&E staining of the same organs. (*B*) H&E, TUNEL, and BrdU staining of small intestine sections from mice treated as in *A*.

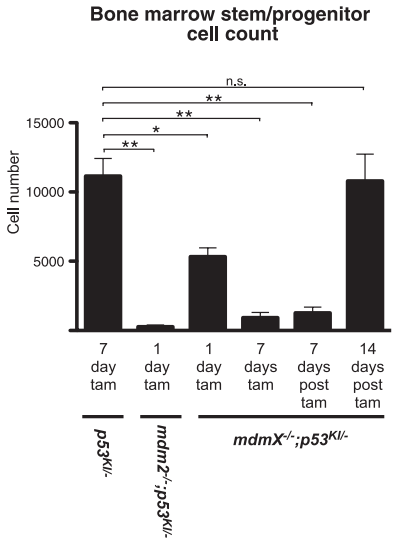


Figure 5. Stem/progenitor cells can recover from p53 restoration in the absence of MdmX. Hematopoietic stem/progenitor cells (Lin^-, $Sca1^+$, $CD150^+$, and $CD48^-$) were quantified in mice of the indicated genotypes, treated with tamoxifen as indicated. The last two columns show counts from $mdmX^{-/-};p53^{KI/-}$ mice 7 d and 14 d after a 7-d tamoxifen treatment, respectively. (*) $P < 0.05$; (**) $P < 0.01$; (n.s.) not significant.

when the $mdmX^{+/+};p53^{KI/-}$ MEFs were cotreated with Nutlin-3a, indicating that Mdm2 contributes to the reduction in p53 levels and the regulation of p53 activity in 4-OHT-treated $mdmX^{+/+}$ cells (Fig. 6A). Likewise, p53 levels dropped in $mdmX^{-/-};p53^{KI/-}$ MEFs after addition of 4-OHT, together with a concomitant increase in p53 activity and induction of Mdm2. This p53 down-regulation was also inhibited by Nutlin-3a, resulting in yet higher p53 activity (Fig. 6A), which demonstrates that endogenous Mdm2 still contributes significantly to the down-regulation of p53 levels and activity even in the absence of MdmX. Interestingly, p53 protein levels were higher in $mdmX^{-/-};p53^{KI/-}$ MEFs compared with wild-type controls, suggesting a role for MdmX in the regulation of p53 protein levels and consistent with previous studies that ascribe such a role to MdmX, principally through modulation of Mdm2 function and stability (Jackson et al. 2001; Gu et al. 2002; Okamoto et al. 2009).

To test whether endogenous Mdm2 also restrains p53 level and activity in $mdmX^{-/-}$ tissues in vivo, we treated $p53^{KI/-}$, $mdmX^{-/-};p53^{KI/-}$, and $mdm2^{-/-};p53^{KI/-}$ mice for 1 d with either vehicle or tamoxifen to restore p53 and then monitored p53 levels over time in the liver. As predicted, p53 levels dropped in the livers of $p53^{KI/-}$ mice after p53 restoration, with little increase in p53 activity (measured by induction of *cdkn1a* mRNA) (Fig. 6B). p53 levels also dropped after restoration in livers of tamoxifen-treated $mdmX^{-/-};p53^{KI/-}$ mice, albeit not as efficiently, consistent again with a previously reported contribution of MdmX to the regulation of p53 protein levels (Fig. 6B). Nonetheless, despite this fall in overall p53 levels, p53 activity rose in the absence of MdmX, as evidenced by a clear increase in *cdkn1a* (Fig. 6B). Of note, *Mdm2* was also induced in $mdmX^{-/-};p53^{KI/-}$ livers after tamoxifen administration, where it presumably contributes to p53 down-regulation (Fig. 6B). In contrast, after p53 restoration in $mdm2^{-/-};p53^{KI/-}$ livers, p53 levels fail to drop and, indeed, increase, resulting in even higher p53 activity (evidenced by robust induction of *cdkn1a*) (Fig. 6B). Similar dynamics were observed in the spleen and thymus (Supplemental Fig. S5). Quantification of p53 protein levels in tamoxifen-treated $p53^{KI/-}$, $mdmX^{-/-};p53^{KI/-}$, and $mdm2^{-/-};p53^{KI/-}$ tissues showed that MdmX plays at least some role in the regulation of p53 protein level also in vivo. Nonetheless, it remains clear that Mdm2 is the principal regulator of p53 protein level in vivo, since Mdm2-null tissues exhibit the highest levels of p53 protein after restoration (Fig. 6B; Supplemental Fig. S4). Overall, the stark differences in down-regulation of $p53ER^{TAM}$ after its functional restoration in organs of $mdmX^{-/-}$ versus $mdm2^{-/-}$ mice affirms that, even in the absence of MdmX, endogenous Mdm2 retains some capacity to mitigate p53 levels and activity in vivo. Taken together, the persistence of the p53–Mdm2 negative feedback loop in $mdmX^{-/-}$ cells and tissues offers the most plausible explanation for the unexpectedly mild and recoverable impact of p53 in the absence of MdmX.

The therapeutic impact of restoring p53 in tumors is augmented by inactivity of MdmX

Several studies have indicated that restoring p53 function in tumors may be therapeutically beneficial (Martins et al. 2006; Ventura et al. 2007; Xue et al. 2007; Junttila et al. 2010). Since our data establish that transient p53 restoration in MdmX-deficient mice has only limited and delayed toxicity, we asked whether the absence of MdmX offers a useful therapeutic window by which to enhance the therapeutic benefit of restoring p53 in tumors in vivo. We crossed $mdmX^{-/-};p53^{KI/-}$ mice to the lymphoma-prone *Eμ-Myc* transgenic strain (Adams et al. 1985). Tumors from $mdmX^{+/+};p53^{KI/-};E\mu Myc$ and $mdmX^{-/-};p53^{KI/-};E\mu Myc$ mice were then isolated and transplanted into multiple wild-type recipients. Ten days after transplantation, recipient mice were treated for 1 wk with either vehicle or tamoxifen. Consistent with our previous study (Martins et al. 2006), tamoxifen treatment significantly extended the life span of mice harboring $mdmX^{+/+};p53^{KI/-};E\mu Myc$ tumors compared with sham-treated controls (Fig. 7). Vehicle-treated mice bearing $mdmX^{-/-};p53^{KI/-};E\mu Myc$ tumors showed no increase in overall survival relative to vehicle-treated MdmX-proficient controls, demonstrating that absence of MdmX alone does not affect tumor growth per se. However, p53 restoration in the absence of MdmX significantly increased overall survival relative to tamoxifen-treated $mdmX^{+/+};p53^{KI/-};E\mu Myc$ controls (Fig. 7). Hence, p53 restoration in the absence of MdmX confers a significant therapeutic benefit over p53 restoration in its presence.

p53 restoration therapy in *Eμ-Myc* tumors is eventually compromised by the emergence of resistant tumor clones harboring additional mutations in the p53 pathway (Martins et al. 2006). To investigate whether this remains a signif-

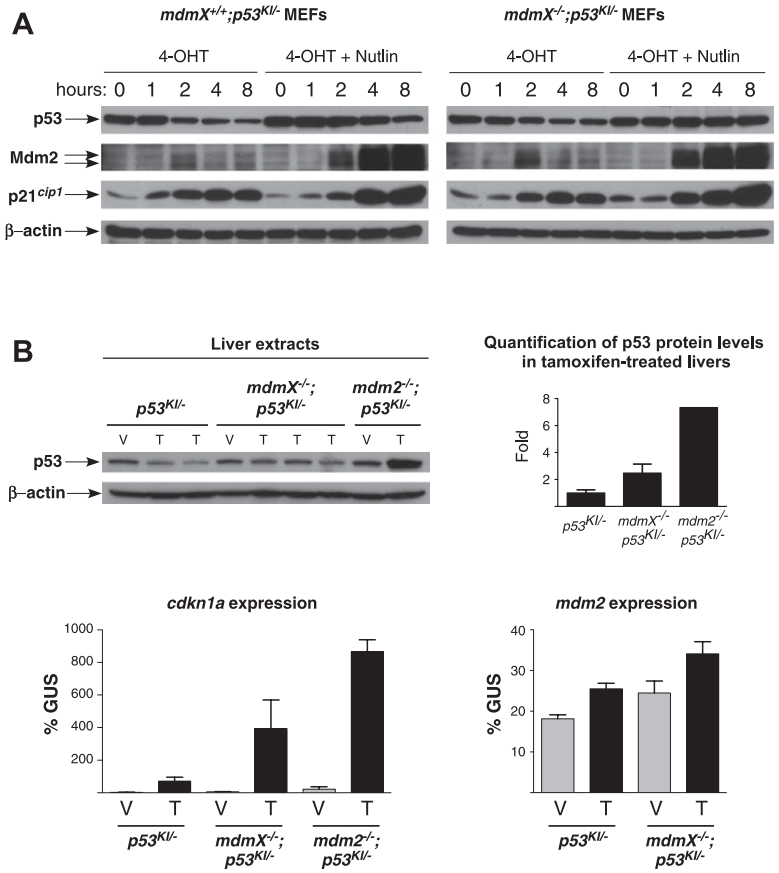


Figure 6. Mdm2 is functional and contributes to p53 down-regulation in the absence of MdmX. (*A*) $mdmX^{+/+};p53^{KI/-}$ and $mdmX^{-/-};p53^{KI/-}$ MEFs were treated with either 4-OHT or 4-OHT plus 10 μM Nutlin-3a for the indicated times. Immunoblotting was used to estimate levels of p53, Mdm2, and $p21^{cip1}$ at each time point. (*B*) Mice of the indicated genotypes were treated with either vehicle (V) or tamoxifen (T) for 1 d. The levels of p53 in the livers of these mice are presented (immunoblot; each lane is a separate mouse), together with a quantification of the p53 levels in tamoxifen-treated mice (from the blot shown). The relative induction of p53 target genes *cdkn1a* and *mdm2* (by TaqMan; *gus* is the control gene) in these mice is also shown. Error bars show the SEM from triplicates.

icant limitation, even when p53 is restored in the absence of MdmX, we harvested tumors from treated host mice and cultured them in vitro in the presence or absence of 4-OHT. Tumor cells derived from vehicle-treated mice were rapidly killed when exposed to 4-OHT, regardless of MdmX genotype, indicating that the absence of MdmX alone does not promote outgrowth of resistant clones (Fig. 8A). In contrast, $mdmX^{+/+}$ and $mdmX^{-/-}$ tumor cells isolated from relapsed tamoxifen-treated mice survived and grew in the presence of 4-OHT, confirming that p53 restoration does indeed still select for resistant clones irrespective of MdmX status (Fig. 8A).

Resistance to p53 restoration arises in *Eμ-Myc* tumors by either loss of p53 itself or loss of $p19^{ARF}$, the essential upstream conduit for oncogenic activation of p53 (Zindy et al. 2003; Martins et al. 2006). To establish the mechanism of acquired resistance to p53 restoration in MdmX-deficient tumors, we determined the status of p53 and $p19^{ARF}$ expression in transplanted $mdmX^{+/+};p53^{KI/-}$; *EμMyc* and $mdmX^{-/-};p53^{KI/-}$;*EμMyc* tumors. Tumors from vehicle-treated recipients all exhibited high $p19^{ARF}$ and readily detectable levels of p53, regardless of MdmX genotype. In contrast, $mdmX^{+/+};p53^{KI/-}$;*EμMyc* tumors from tamoxifen-treated recipients had high $p19^{ARF}$ levels but substantially decreased p53 (Fig. 8B). $mdmX^{-/-}$; $p53^{KI/-}$;*EμMyc* tumors from tamoxifen-treated recipients also exhibited high $p19^{ARF}$ levels but had yet lower levels of p53 protein than even their $mdmX^{+/+}$ counterparts (Fig. 8B). Southern blot analysis revealed that the loss of p53 expression arises through wholesale deletion of the $p53^{KI}$ locus (Fig. 8C). Hence, even though p53 restoration therapy in the absence of MdmX confers a significant extension of survival in lymphoma-bearing mice, such therapeutic benefit is still limited by the emergence of resistant clones in which the restored p53 pathway has been incapacitated.

Discussion

MdmX is a critical negative regulator of p53 (Marine and Jochemsen 2005; Marine et al. 2007; Wade and Wahl 2009; Mancini et al. 2010), necessary to restrain the lethal consequences of unbridled p53 activity at various points in normal development (Parant et al. 2001; Migliorini et al. 2002; Francoz et al. 2006; Grier et al. 2006; Xiong et al. 2006). However, the embryonic lethality of p53-competent MdmX-null mice has confounded genetic analysis of any role that MdmX might play in governing p53 activity in adult tissues. Here, we used a switchable p53 mouse model, in which endogenous p53 function can be systemically and reversibly enabled or disabled at will (Christophorou et al. 2005), to establish the acute and long-term consequences for adult tissues of p53 function in the absence of MdmX. Our data show that p53 is spontaneously active in unperturbed $mdmX^{-/-}$ adult mouse tissues, in the absence of any overt or detectable upstream p53-activating signal, inducing p53 target genes in all tested organs and

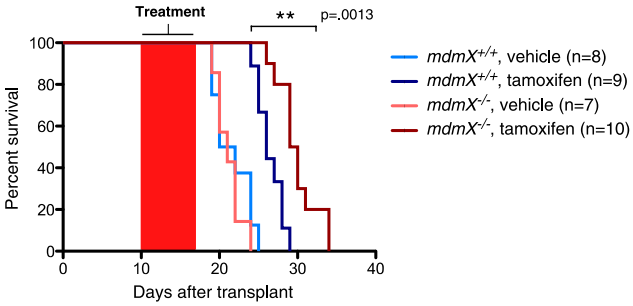


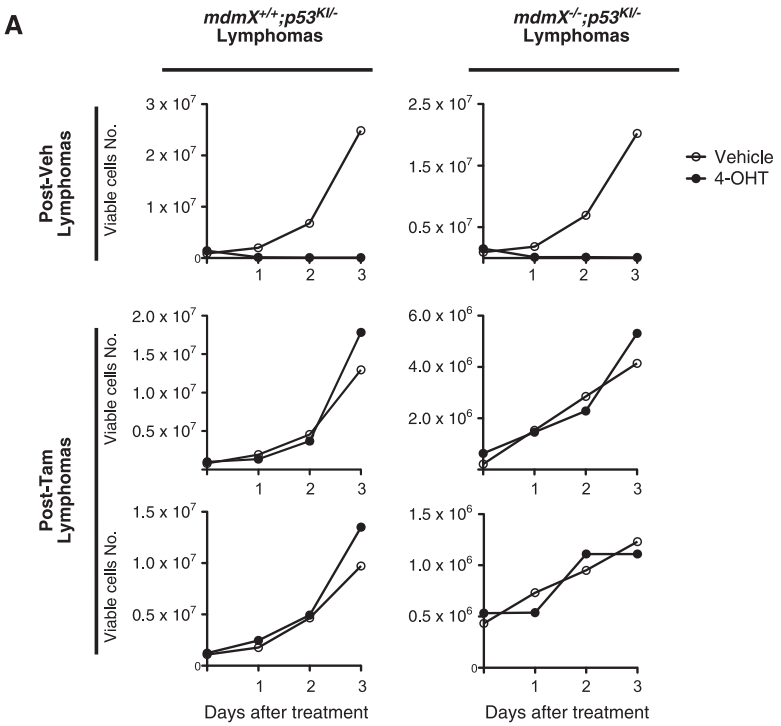
Figure 7. Restoration of p53 in the absence of MdmX extends overall survival in lymphoma-bearing mice. $mdmX^{+/+}$;$p53^{KI/-}$; *EμMyc* and $mdmX^{-/-}$;$p53^{KI/-}$;*EμMyc* tumors (the graph legend indicates the *mdmX* genotype only) were transplanted into wild-type recipients. Ten days after transplant, recipient mice were treated with either vehicle or tamoxifen for 1 wk. Their survival is plotted as a Kaplan-Meier curve.

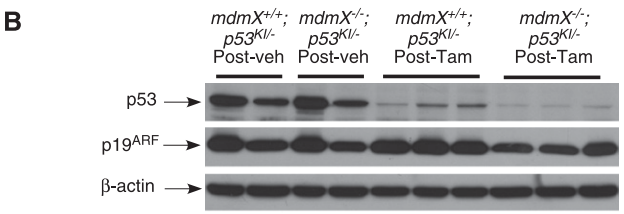
widespread apoptosis in radiosensitive tissues. Moreover, sustained systemic p53 function in such $mdmX^{-/-}$ mice is ultimately lethal. This unequivocally establishes MdmX, like Mdm2 (Ringshausen et al. 2006), as critically and continuously required to rein in precocious p53 activity in normal adult tissues.

Nonetheless, while both Mdm2 and MdmX serve as constitutive buffers against untoward p53 activity in adult tissues in vivo, their roles are quantitatively and qualitatively quite distinct. Both the anti-proliferative gene *cdkn1a* and the proapoptotic gene *puma* are indiscriminately induced in all $mdm2^{-/-}$ tissues following acute p53 restoration, irrespective of whether the cells within that tissue then undergo apoptosis or viable cell cycle arrest (Ringshausen et al. 2006). In contrast, p53 target gene induction is more selective in tamoxifen-treated $mdmX^{-/-}$;$p53^{KI/-}$ tissues: *cdkn1a* is still induced in most $mdmX^{-/-}$ tissues (with the notable exception of intestinal epithelium), but, in the main, *puma* up-regulation is restricted to those (classically radiosensitive) $mdmX^{-/-}$ tissues that undergo apoptosis. Given the evidence suggesting that *cdkn1a* and *puma* are induced by different threshold levels of p53 activity (Vousden and Prives 2009; Morachis et al. 2010), the most plausible explanation for such differential target gene induction is the markedly lower overall p53 activities elicited by p53 restoration in $mdmX^{-/-}$;$p53^{KI/-}$ tissues compared with those in their $mdm2^{-/-}$ counterparts. Presumably, it is this higher level of p53 activity in *mdm2*-deficient tissues that is responsible for the far more precipitous and deleterious effects of p53 restoration in $mdm2^{-/-}$ tissues. Of note, our observations are not consistent with a simple binary model whereby loss of Mdm2 directs p53 to induce apoptosis while loss of MdmX directs p53 to engage growth arrest (Barboza et al. 2008). For example, both the proapoptotic gene *puma* and overt apoptosis are potently induced in classically radiosensitive adult tissues of $p53^{KI/-}$;$mdmX^{-/-}$ mice after acute p53 restoration. Conversely, while *puma* is potently induced by p53 restoration even in radioresistant $mdm2^{-/-}$ tissues, no apoptosis occurs even if those tissues are forced to proliferate (Ringshausen et al. 2006). Hence, the proclivity of any adult tissue to undergo p53-induced apoptosis appears to be an intrinsic attribute of that tissue and not the purview of either Mdm2 or MdmX.

There is still debate as to the extent to which MdmX plays a role in controlling p53 protein stability. Some studies suggest that MdmX principally controls p53 activity but not its level, the latter being reserved for the E3-ubiquitin ligase activity of Mdm2 (Boesten et al. 2006; Francoz et al. 2006; Barboza et al. 2008). However, other studies implicate MdmX in the control of p53 protein levels, most likely through regulation of Mdm2 protein stability (Sharp et al. 1999; Stad et al. 2000; Jackson et al. 2001; Gu et al. 2002; Okamoto et al. 2009). In this study, we found that the levels of p53 protein are consistently higher in tamoxifen-treated MdmX-null tissues compared with *mdmX* wild-type controls. It is quite possible that such elevated p53 levels contribute to the higher overall p53 activity seen in MdmX-null tissues. The role of MdmX as a negative regulator of p53 activity being well established, our data also further support the notion that MdmX plays at least some role (most likely indirect) in the regulation of p53 levels in vivo. Consistent with many existing studies, however, our new data nonetheless confirm that Mdm2 is the principal regulator of p53 levels in vivo, as evidenced by our observations that Mdm2-null tissues exhibit the highest levels of p53 protein after restoration.

Of late, restoration of p53 function has been vigorously pursued as a therapeutic approach to treat cancers and, moreover, has recently been shown to be therapeutically beneficial in several preclinical mouse cancer models (Martins et al. 2006; Ventura et al. 2007; Xue et al. 2007). Accordingly, numerous efforts have focused on blocking endogenous inhibitors of p53, in the main Mdm2, as a strategy for reactivating p53 in tumors (Issaeva et al. 2004; Vassilev et al. 2004; Shangary et al. 2008; Sun et al. 2008; Canner et al. 2009; Hedstrom et al. 2009). Unfortunately, efficient inhibition of Mdm2 triggers p53 not only in tumor cells but also in all normal cells, raising the specter that injudicious pharmacological inhibition of Mdm2 inhibition would elicit rapid, irreversible, and eventually lethal side effects (Ringshausen et al. 2006). In contrast, our data indicate that even complete inhibition of MdmX, if relatively transient, is a far less intrinsically toxic and hazardous therapeutic strategy for reactivating p53: Although transient p53 activity in the absence of MdmX does some damage to lymphoid organs and bone marrow, it has unexpectedly mild effects on intestinal epithelium—arguably the most critical tissue limiting therapeutic toxicity, since its failure is rapidly and irreversibly fatal due to fluid loss and bacterial incursion. Our data indicate that, provided that intestinal integrity is preserved, all other sequelae arising from transient p53 activity in the absence of MdmX are reversible, with all mice recovering and thereafter living long term. Furthermore, we show that absence of MdmX does indeed augment the therapeutic impact of restoring p53 function in treating lymphoma. This, together with the muted toxicity of p53 in the absence of MdmX compared with the absence of





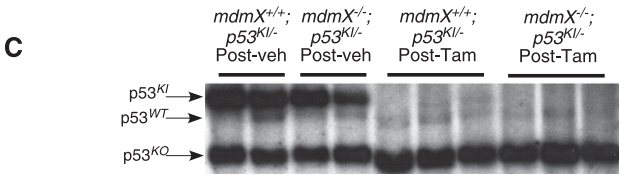


Figure 8. p53 restoration in the absence of MdmX does not prevent the emergence of resistant clones that deleted p53 in *Eμ-Myc* tumors. (*A*) *mdmX*$^{+/+}$;*p53*$^{KI/-}$;*Eμ-Myc* and *mdmX*$^{-/-}$;*p53*$^{KI/-}$;*Eμ-Myc* transplanted tumors isolated from recipient mice previously treated with either vehicle (designated as Post-veh) or tamoxifen (designated as Post-Tam) in vivo were cultured in the presence or absence of 4-OHT. In vitro growth of cells derived from representative tumors is presented. (*B*) Immunoblot analysis of p53 and p19^{ARF} levels in transplanted tumors isolated from recipient mice treated with vehicle (designated as Post-veh) or tamoxifen (designated as Post-Tam). Each lane represents an individual tumor. (*C*) Southern blot analysis of DNA isolated from the same tumors as in *B*. The p53 probe detects a 5.9-kb band from the *p53*^{KI} locus, a 5.0-kb band from the wild-type p53 locus (from the host tissue), and a 3.0-kb band from the p53 knockout locus.

Mdm2 and the complete reversibility of its pathological side effects, supports the notion of MdmX inhibition as a feasible strategy for restoring p53 function in tumors that retain expression of wild-type p53.

Increasing evidence now implicates p53 in the regulation of adult stem cell homeostasis. Most notably, chemical inhibition or genetic ablation of p53 elicits defects in stem cell self-renewal and increased proliferation rates that eventually lead to an expansion of both hematopoietic and neural stem cells (TeKippe et al. 2003; Meletis et al. 2006; Liu et al. 2009; Leonova et al. 2010). Our data now add to the emerging view that preternatural p53 activity causes attrition of hematopoietic stem cells (Liu et al. 2010; Abbas et al. 2010). However, consistent with the very different toxicities associated with p53 activity in the absence of MdmX versus Mdm2, the severity and rapidity of stem cell attrition were very different in each instance. Even fleeting p53 restoration in *mdm2*-deficient mice rapidly and irreversibly wiped out hematopoietic stem/progenitor cells, together with any regenerative capacity in the bone marrow once p53 function was subsequently deactivated. In contrast, absence of MdmX provoked a much milder and protracted erosion of stem/progenitor cells, which were able to recover once p53 was removed, thus allowing for complete bone marrow regeneration and recovery of animals even after extended periods of p53 restoration. This difference has important practical implications. Although our data indicate a previously unappreciated role for both Mdm2 and MdmX in buffering p53 activity in adult hematopoietic stem cells,

they also indicate that overly aggressive pharmacological inhibition of Mdm2 (even if transient) would rapidly and irreversibly deplete stem cell pools, whereas efficient MdmX inhibition would not. That this might also hold true for stem cells in other tissues is suggested by our observation that intestinal epithelia are relatively unaffected by p53 restoration in the absence of MdmX compared with restoration in the absence of Mdm2, indicating that intestinal stem cells continue to regenerate and maintain epithelial integrity in $mdmX^{-/-}$ mice after p53 restoration, but not in their $mdm2^{-/-}$ counterparts.

We previously demonstrated that, despite the high levels of $p19^{ARF}$ present in *Eμ-Myc* tumors, p53 is nonetheless not maximally active when functionally restored in such lymphoma cells, since its activity can be yet further augmented via an overt p53-activating insult, such as irradiation (Martins et al. 2006). This suggests that factors in addition to Mdm2 are suppressing maximal p53 activity, an observation consistent with our data indicating that MdmX plays an additional and independent role in buffering unbridled p53 activity. We now show that absence of MdmX enhances p53 restoration therapy in a transplant model of lymphoma. In this context, it is intriguing that irradiation-induced activation of p53 in lymphoma tumors has recently been shown to depend greatly on effective down-regulation of MdmX in these tumor cells (Wang et al. 2009), which makes MdmX inhibition an even more attractive strategy for enhancing the therapeutic impact of p53 restoration in tumors.

Ultimately, however, the capacity of p53 restoration therapy to prolong overall survival is limited by the probability that further mutations will arise that again incapacitate the restored p53 pathway—mutations that either inactivate p53 itself or interrupt critical upstream or downstream components of the greater p53 tumor suppressor pathway. One notable example of this is inactivation of $p19^{ARF}$, the principal effector linking upstream oncogenic signals and p53 activation (Martins et al. 2006; Junttila et al. 2010; Young and Jacks 2010). Our data show that such resistant secondary tumors do indeed arise relatively rapidly following p53 restoration in MdmX-deficient tumors, in our case specifically by deletion of p53, which renders the absence of MdmX moot. However, such wholesale deletion of p53 may be less likely in human tumors, since it would necessarily entail loss of both copies of p53, in which case MdmX inhibition may still prove to be an effective therapeutic strategy in human tumors. Given that p53 pathway-defective cells presumably pre-exist in the *Eμ-Myc* tumors prior to the initial p53 restoration, and it is to these exapted clones that the mice eventually succumb, how might inactivation of MdmX enhance the therapeutic potential of p53 restoration? One possibility is suggested by our immunoblot analysis, which indicates that a low level of p53 remains in the resistant tumors. Since deletion of the *p53* gene is the mechanism of secondary resistance to tamoxifen in such recurring tumors, such low-level p53 presumably indicates the persistence of a minor remnant population of p53-positive cells. Of note, the residual level of p53 in recurring MdmX-deficient tumors is lower than that in their MdmX-competent counterparts, likely indicating that fewer p53-competent tumor cells survive the initial p53 restoration. A better initial "kill" of tumor cells in the absence of MdmX would be consistent with the extended survival of the tamoxifen-treated mice bearing MdmX-deficient tumors. It has also recently emerged that tumors can evade p53-mediated tumor suppression by failing to breach the signaling threshold required to trigger the p53 pathway (Sarkisian et al. 2007; Murphy et al. 2008; Junttila et al. 2010). In such cases, inhibiting MdmX has the potential to lower the p53-activating threshold, thus engaging the p53 pathway in a tumor-selective manner. The recent identification of the first small-molecule inhibitor of MdmX (Reed et al. 2010) may therefore be an important step forward in the genesis of novel therapies that are truly tumor-specific.

Materials and methods

Genetically engineered mice and in vivo studies

$p53ER^{TAM}KI$ ($p53^{KI/KI}$) mice (Christophorou et al. 2005) were crossed with $p53^{-/-}$ (Jacks et al. 1994), $mdmX^{-/-}$ (Parant et al. 2001), $mdm2^{-/-}$ (Montes de Oca Luna et al. 1995), and *Eμ-myc* mice (Adams et al. 1985) to generate the appropriate genotypes. All animals were kept under SPF conditions, and all experimental procedures adhered to approved University of California at San Francisco Institutional Animal Care and Use Committee protocols.

To restore p53, mice were injected intraperitoneally with 100 μL of 10 mg/mL tamoxifen (Sigma, T5648) in peanut oil at the indicated frequencies. To restore p53 in vitro, cultured cells were exposed to 100 nM 4-OHT (Sigma, H7904). Nutlin-3a was purchased from Cayman Chemical Company (catalog no. 18585). Where appropriate, mice were injected with a single bolus of 100 μL of 10 mg/mL BrdU (Sigma, B5002) 2 h prior to sacrifice.

Prior to lymphoma transplantation, 4 Gy of γ-radiation was administered to recipient wild-type mice for 4 h using a Mark 1-68 cesium-137 source (1.972 Gy/min) prior to tail vein transplantation of lymphomas. Lymphoma transplantation and in vivo treatment were performed as described (Martins et al. 2006). Lymphoma cell cultures were established and maintained as described (Martins et al. 2006). Statistical analysis of survival was performed with the Kaplan-Meier log-rank test.

TaqMan analysis

Total RNA was isolated with Trizol (Invitrogen, catalog no. 15596026) and DNase-treated (Invitrogen, catalog no. 18065-015) prior to reverse transcription (iScript, Bio-Rad, catalog no. 170-8891). TaqMan analysis was performed as previously described (Christophorou et al. 2005). All data were normalized to *gus* expression (control gene).

Immunoblotting

Proteins were extracted from organs and cells using standard RIPA buffer. Protein lysates (50–100 μg) were fractionated on SDS polyacrylamide gels and blotted onto PVDF membranes (Immobilon-P, Millipore, IPVH00010). The antibodies used were anti-ER (Santa Cruz Biotechnology, sc-542), anti-p21 (BD Pharmingen, catalog no. 556431), anti-$p19^{ARF}$ (Novus, clone C3), anti-Mdm2 (clone 2A10, Calbiochem OP115, and clone 4B2, Calbiochem OP145), and anti-β-actin (AC-15, Sigma). When indicated, blots were quantified using ImageJ software (NIH).

Immunohistochemistry

All harvested organs were fixed overnight, washed, processed, and embedded in paraffin. BrdU staining and TUNEL were performed using kits (Roche, reference 11299964001, and Millipore, catalog no. S1760, respectively), following the manufacturers' instructions. Anti-Ki-67 antibody was from NeoMarkers (SP6).

Flow cytometry

Leg, hip, and arm bones from individual mice were pooled and crushed to harvest bone marrow cells. Following lysis of erythrocytes with ACK, bone marrow cells were separated from bone residue and dead cells by FICOL gradient centrifugation. Bone marrow cells were then resuspended in Hank's buffered saline (HBSS) containing 2% heat-inactivated FBS (Hyclone), and total viable cells numbers were quantitated using a VI-CELL XR (Beckman-Coulter) imaging hemacytometer. For hematopoietic stem cell analysis, 10×10^6 bone marrow cells were incubated with rat unconjugated lineage antibodies (CD3, CD4, CD5, CD8, B220, Ter119, Mac1, and Gr1), stained with PE-Cy5-conjugated goat anti-rat IgG (Caltag), and blocked with rat IgG (Sigma). Cells were then stained with Sca1-PB, CD150-PE, and CD48-APC (BioLegend). For mature analysis, 1×10^6 cells were stained for B cells (B220-APC, CD19-PE, eBioscience) and myeloid cells (Mac1-PE-Cy7, Gr1-PB, eBioscience). After staining, cells were resuspended in HBSS + 2% FBS and 1 μg/mL propidium iodide for dead cell exclusion and analyzed on an LSRII (Becton Dickinson).

Southern blot analysis

For Southern analysis, 10 μg of genomic DNA per sample was digested with EcoRV, fractionated in a 0.7% agarose gel, transferred to a nylon membrane, and hybridized with ^{32}[P]-labeled probe (Rediprime II RPN 1633, Amersham). The p53 probe was generated by PCR with the following primers: 5′-GGTACCTTA TGAGCCACCCGAGG-3′ (forward) and 5′-CGAACCTCAAAG CTGTCCCGTCC-3′ (reverse).

Statistics

An unpaired *t*-test and Kaplan-Meier log-rank test were used to assess statistical difference. Two-tailed *P*-values of <0.05 were considered significant.

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