Mdm2 is critically and continuously required to suppress lethal p53 activity in vivo

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Summary

There is currently much interest in the idea of restoring p53 activity in tumor cells by inhibiting Hdm2/Mdm2. However, it has remained unclear whether this would also activate p53 in normal cells. Using a switchable endogenous p53 mouse model, which allows rapid and reversible toggling of p53 status between wild-type and null states, we show that p53 is spontaneously active in all tested tissues of *mdm2*-deficient mice, triggering fatal pathologies that include ablation of classically radiosensitive tissues. In apoptosis-resistant tissues, spontaneous unbuffered p53 activity triggers profound inhibition of cell proliferation. Such acute spontaneous p53 activity occurs in the absence of any detectable p53 posttranslational modification, DNA damage, or $p19^{ARF}$ signaling and triggers rapid p53 degradation.

Introduction

Either p53 or its attendant pathways is functionally inactivated in the majority of human cancers, attesting to p53's widespread role as a tumor suppressor. p53 is triggered by a variety of stresses that may arise in tumor cells, such as DNA damage, hypoxic and nutrient stress, and oncogene activation. Moreover, since many of these p53-activating signals can persist throughout tumor evolution, reengaging the p53 tumor suppressor pathway is a strategy for tumor cell-specific therapy. p53 activity is regulated in great part by Mdm2. Direct interaction of p53 with the N-terminal region of Mdm2 squelches p53 transcriptional activity (Momand et al., 1992; Thut et al., 1997), while the Mdm2 Ring finger E3-ubiquitin ligase maintains p53 at a low level in normal cells by targeting it for proteasomal degradation (Honda et al., 1997; Kubbutat et al., 1997). Mdm2 may also target p53 for nuclear exclusion (Roth et al., 1998). Since the gene encoding Mdm2 is induced by p53, the two short-lived proteins constitute an autoregulatory loop whereby p53 maintains expression of its own functional inhibitor (Deb, 2003).

For tumor cells that retain wild-type p53, one therapeutic strategy to reactivate p53 is to target Mdm2. Aside from direct inhibition of *mdm2* expression by antisense (Bianco et al., 2005; Tortora et al., 2000; Zhang et al., 2004), several other approaches have sought to interfere directly with the p53-Mdm2 interaction, including peptidomimetics (Bottger et al., 1997; Chene et al., 2000; Wasylyk et al., 1999) and, more recently, the cis-imidazoline derivatives dubbed Nutlins (Vassilev et al., 2004). An alternative approach is to inhibit the Mdm2 E3 ubiquitin ligase activity thought necessary to restrain accumulation of activated p53 (Yang et al., 2005). All such studies concur that inhibition of Mdm2 activates p53, triggering apoptosis in tumor cells harboring functional p53 (Bianco et al., 2005; Chene et al., 2002; Vassilev et al., 2004; Yang et al., 2005). Some also indicate that Mdm2 inhibition can augment the efficacy of cytotoxic therapies (Bianco et al., 2005). A recent in vivo study using human tumor xenografts in the mouse offered support for the idea that inhibition of Mdm2 function has a selectively toxic effect on tumor cells, and it was suggested that this therapeutic selectivity reflects either an intrinsic refractoriness of normal cells to the apoptotic influence of "unbuffered" p53 or, conversely, that activated oncogenes and endogenous DNA damage in tumor cells provide additional, p53-activating signals that tip the balance toward apoptosis over cell cycle arrest.

By contrast, the dramatic, p53-dependent preimplantation demise of *mdm2*-deficient/$p53^{WT}$ mouse embryos (Jones et al., 1995; Montes de Oca Luna et al., 1995) hints that Mdm2 must buffer against untoward p53 activity in at least some normal cells, at least during embryogenesis. Furthermore, reintroduction of p53 function in $mdm2^{-/-}$ MEFs triggers spontaneous p53 activation. While it is difficult to extrapolate from any in vitro experiment on a single, embryonic cell type to the diversity of adult tissues in vivo, elegant studies using mice carrying a hypomorphic *mdm2* mutation show that buffering against p53

SIGNIFICANCE

p53 or its attendant pathway is functionally inactivated in the great majority of human cancers, indicating strong negative selection against p53 tumor suppressor function. Consequently, there is great interest in the strategy of reactivating p53 in tumor cells as a cancer therapy. One strategy for doing this is to inhibit Mdm2, the endogenous buffer that keeps p53 function in check. However, this runs the risk of triggering p53 in normal tissues, with potentially deleterious consequences. Using a reversibly switchable endogenous p53 mouse model, we show that p53 is, indeed, spontaneously active in the absence of Mdm2, triggering a range of pathologies that resemble acute radiation exposure. Hence, Mdm2-inhibitory drugs will need to be used with great caution.

function is significantly reduced in many adult tissues, again intimating that complete absence of Mdm2 leads to promiscuous activation of p53 (Mendrysa et al., 2003). Yet other studies using conditional knockout Cre/lox strategies to excise *mdm2* from specific tissues concur that p53 is spontaneously active (Boesten et al., 2006; Francoz et al., 2006; Xiong et al., 2006).

The *p53ER^{TAM} KI* (*p53$^{KI/KI}$*) mouse expresses the 4-hydroxytamoxifen (4-OHT)-dependent variant of p53, p53ER^{TAM}, in place of wild-type p53 (Christophorou et al., 2005). Tissues and cells from *p53$^{KI/KI}$* mice can be rapidly and reversibly toggled between p53-deficient and p53-functional states by systemic administration or withdrawal of 4-OHT (Christophorou et al., 2005). Because *p53ER^{TAM} KI* mice can be raised to adulthood in the absence of functional p53, they can be crossed into an *mdm2*-deficient background, and the immediate and delayed consequences of acute p53 restoration in the absence of the Mdm2 buffer can be determined. Using such mice, we here show that p53 is spontaneously active in all tissues in *mdm2*-deficient mice.

Results

p53ER^{TAM} and wild-type p53 are similarly regulated by Mdm2

Previous in vitro and in vivo studies have indicated that, in the absence of 4-OHT, p53ER^{TAM} is functionally inactive but that all measurable nuclear and mitochondrial properties of wild-type p53 are faithfully restored upon provision of 4-OHT ligand (Chipuk et al., 2005; Christophorou et al., 2005; Vater et al., 1996). Nonetheless, given the critical role that Mdm2 plays in regulating wild-type p53 function, we first confirmed that regulation of p53ER^{TAM} by Mdm2 is similar to that of wild-type p53.

To ascertain that Mdm2 modulates the constitutive level of p53ER^{TAM}, whole protein lysates were derived from *mdm2$^{+/+}$*;*p53$^{-/-}$*, *mdm2$^{-/-}$*;*p53$^{KI/-}$* *mdm2$^{+/+}$*;*p53$^{KI/+}$*, and *mdm2$^{+/+}$*;*p53$^{+/+}$* MEFs (all in the presence of 4-OHT), and base-line levels of p53/p53ER^{TAM} and Mdm2 were ascertained by quantitative immunoblotting (Figure 1A). β-actin was used as a loading control. Steady-state levels of p53ER^{TAM} protein were significantly higher in *mdm2$^{-/-}$*;*p53$^{KI/-}$* MEFs compared with *mdm2$^{+/+}$*;*p53$^{KI/+}$* MEFs (Figure 1A, compare lanes 2 and 4), consistent with a significant role for Mdm2 in p53ER^{TAM} degradation. To compare accurately more subtle differences that might exist in basal expression of wild-type p53 versus p53ER^{TAM} in Mdm2-proficient cells, we used *p53$^{+/KI}$* MEFs, which coexpress both wild-type p53 and p53ER^{TAM} proteins, each driven from its own endogenous *p53* promoter. In this way, we could avoid the significant and uncontrollable variations that exist in p53 basal levels between different isolates, passage numbers, and strains of MEF. In such cells, steady-state levels of p53ER^{TAM} were around two to three times that of p53 (Figure 1A), consistent with the ~2- to 3-fold extended half-life of p53ER^{TAM} of ~50 min (Figure 1B), compared with 15–30 min for wild-type p53 (Alarcon et al., 1999; Figure S1 in the Supplemental Data available with this article online). In cells harboring two copies of the p53ER^{TAM} allele, steady-state p53ER^{TAM} levels were again higher than those of wild-type p53 in p53$^{+/+}$ MEFs. This increased level of p53ER^{TAM} was associated pro rata with more coimmunoprecipitated Mdm2. Importantly, the total amount of p53 protein (p53 + p53ER^{TAM}) in *p53$^{+/KI}$* cells did not exceed the total p53 protein level in *p53$^{+/+}$* cells (Figure 1A), indicating that the p53ER^{TAM}-Mdm2 feedback loop is functioning appropriately.

To assess the direct physical interaction between p53ER^{TAM} and Mdm2, we used an antibody specific to Mdm2 to coimmunoprecipitate p53ER^{TAM} from the *p53$^{KI/KI}$* MEF lysates used in Figure 1A. p53ER^{TAM} was clearly coimmunoprecipitated along with Mdm2 (Figure 1C). The reverse experiment, using an antibody against p53 (Pab246), gave the converse result. Thus, Mdm2 interacts physically and functionally with p53ER^{TAM}, despite the presence of the C-terminal ER^{TAM} domain.

To assay quantitatively the relative regulation by Mdm2 of p53ER^{TAM} versus wild-type p53, *p53$^{+/+}$* and *p53$^{KI/KI}$* MEFs were exposed to escalating doses of Nutlin-3, which blocks the p53-Mdm2 interaction, in the presence of 100 nM 4-OHT to maintain p53ER^{TAM} function. As a biological readout of p53 activation we monitored inhibition of proliferation by assaying BrdU incorporation (Figure 1D). As a direct readout of p53 transcriptional activity, we assayed expression of the p53 target gene *cdkn1a* by TaqMan (Figure 1E). By both criteria, p53ER^{TAM} and p53 exhibited extremely similar dose sensitivities to activation by Nutlin-3. We also compared time courses of p53 stabilization and p21^{cip1} protein induction, as well as induction of *cdkn1a* and *puma* message in *p53$^{+/+}$* and *p53$^{KI/KI}$* MEFs following acute exposure to Nutlin-3. In every way, the behaviors of p53 and p53ER^{TAM} appeared superimposable (Figure S2). Of note, Nutlin-3 failed to induce any measurable apoptosis in either *p53$^{+/+}$* or *p53$^{KI/KI}$* MEFs (Figures S2D and S2H), consistent with the refractoriness to cell death shown by mouse fibroblasts in high serum. We conclude that p53 and p53ER^{TAM} are regulated similarly within cells.

p53 is spontaneously active in all tissues of *mdm-2*-deficient mice

To assay the activity of p53 in the absence of Mdm-2 in normal tissues, homozygous *p53$^{KI/KI}$* and heterozygous *p53$^{KI/-}$* mice were bred into an *mdm-2*-deficient background. No pups carrying two copies of the *p53ER^{TAM}* allele were obtained in the absence of *mdm-2*. However, mice carrying a single *p53ER^{TAM}* allele over *mdm2$^{-/-}$* (*mdm2$^{-/-}$*;*p53$^{KI/-}$*) were born at Mendelian frequency, and adults appeared normal and viable into extended adult life. We guess that low-level leakiness from two, but not one, copies of the *p53ER^{TAM}* protein was sufficient to trigger early embryonic lethality in the absence of Mdm2. Consequently, *mdm2$^{-/-}$*;*p53$^{KI/-}$* mice were used thenceforth.

To explore the spontaneous activity of functional p53 in *mdm2*-deficient tissues, p53 competence was restored in tissues of *mdm2$^{-/-}$*;*p53$^{KI/-}$* mice by daily systemic (intraperitoneal) administration of either Tamoxifen or 4-hydroxytamoxifen (4-OHT) (results were identical with each). Either single or repeated daily doses of Tamoxifen induced 100% fatality of mice within 5–6 days (Figure S3). By contrast, *mdm2$^{+/+}$*;*p53$^{KI/-}$* and *mdm2$^{-/-}$*;*p53$^{-/-}$* control animals remained healthy throughout 14 days of daily Tamoxifen administration, and thereafter.

Active p53 is a powerful trigger of both cell cycle arrest and apoptosis, two important arms of the p53 tumor suppressor response that can also induce pathologies in normal tissues. To investigate such potential p53-induced pathologies, multiple tissues were harvested from Tamoxifen-treated *mdm2$^{-/-}$*;*p53$^{KI/-}$* mice and analyzed histologically. All classically radiosensitive tissues (bone marrow, thymus, spleen white

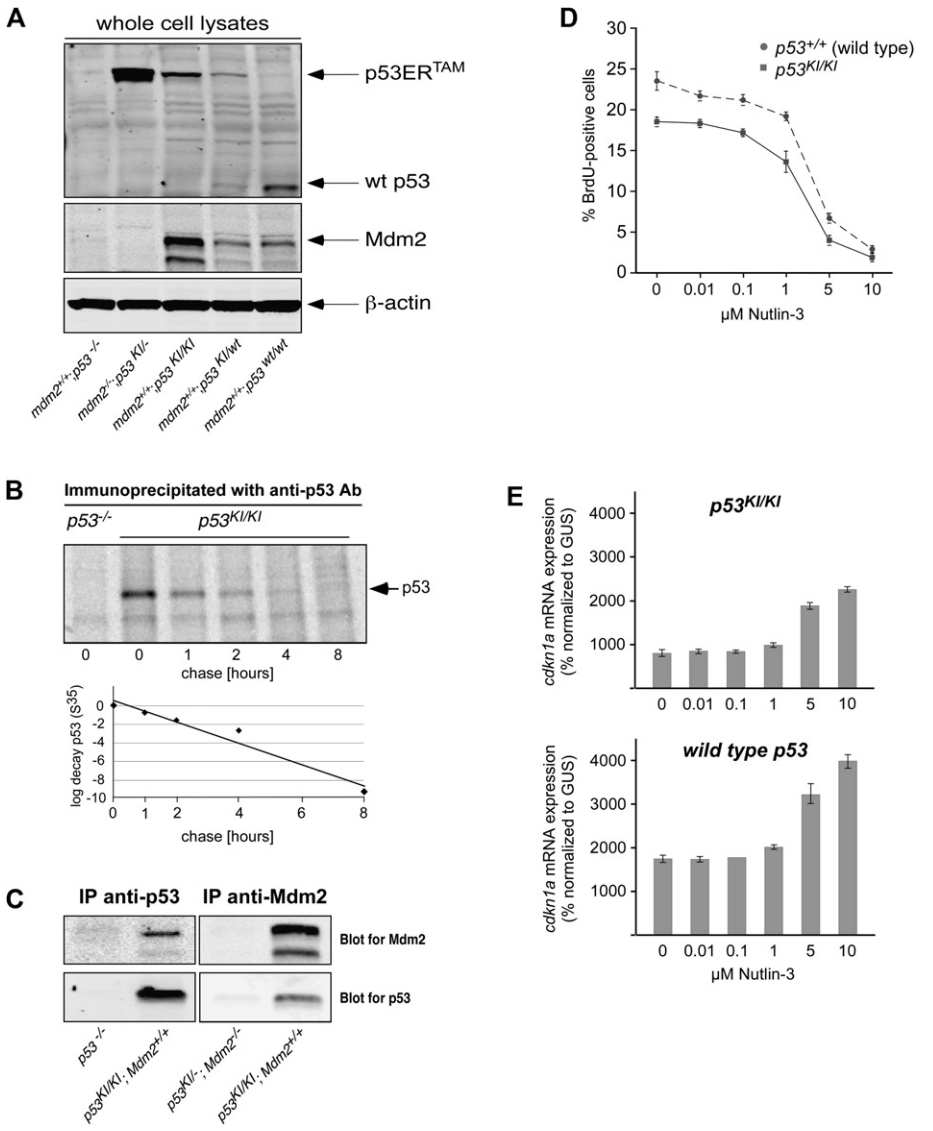

Figure 1. In the presence of 4-OHT, p53ER^{TAM} is regulated by Mdm2 similarly to wild-type p53

A: Quantitation of p53/p53ER^{TAM} and Mdm2 steady-state levels in mouse embryonic fibroblasts. Whole-cell lysates from passage-matched *p53*$^{-/-}$, *mdm2*$^{-/-}$;*p53*$^{KI/-}$, *p53*$^{KI/WT}$, and p53$^{+/+}$ MEFs were analyzed for the expression of p53 and Mdm2 in the presence of 4-OHT. Total protein concentrations were normalized prior immunoblotting as confirmed by equal expression of β-actin.

B: Measurement of p53ER^{TAM} half-life. Early-passage *p53*$^{KI/KI}$ MEFs were pulse labeled for 4 hr with 800 μCi/well ^{35}[S]-methionine and chased with cold methionine. Protein lysates were then harvested, normalized to total protein content, and immunoprecipitated with anti-p53 antibody. Immunoprecipitated material was fractionated by SDS PAGE, and the p53 band was quantitated by Phosphorimager.

C: p53ER^{TAM} interacts directly with *Mdm2*;*p53*$^{KI/KI}$. MEF extracts used in Figure 1A were immunoprecipitated with p53 pab246 monoclonal antibody, and the levels of coprecipitating p53/Mdm2 were detected by Western blotting. *p53*$^{-/-}$ MEFs were used as a negative control for specificity (left panel, left lane). The reverse immunoprecipitation was performed using the Mdm2-specific monoclonal antibody 2a10, and the levels of coprecipitating p53/Mdm2 were detected by Western blotting. *mdm2*$^{-/-}$; *p53*$^{KI/-}$ MEFs were used as a negative control for specificity (right panel, left lane).

D: Inactivation of Mdm2 by Nutlin-3 triggers similar cell cycle arrest, and with similar dose response, in early-passage wild-type *p53* versus *p53*$^{KI/KI}$ MEFs. Passage 5 *p53*$^{+/+}$ and *p53*$^{KI/KI}$ MEFs in 4-OHT were exposed to escalating doses of Nutlin-3 to activate p53, and cell proliferation was assayed by BrdU incorporation after 24 hr. *p53*$^{+/+}$ and *p53*$^{KI/KI}$ MEFs show essentially identical dose responses to Nutlin-3-induced cell cycle arrest. Error bars show mean ± SEM from triplicates derived from each MEF strain.

E: The p53 target gene *cdkn1a* is induced to comparable levels in early-passage wild-type *p53* versus *p53*$^{KI/KI}$ MEFs following inactivation of Mdm2 with Nutlin-3. Passage 5 *p53*$^{+/+}$ and *p53*$^{KI/KI}$ MEFs in 4-OHT were exposed to escalating doses of Nutlin-3 to activate p53 and *cdkn1a* expression assayed by TaqMan after 24 hr. Induction of *cdkn1a* in *p53*$^{+/+}$ and *p53*$^{KI/KI}$ MEFs exhibits dose dependence that is identical to that of Nutlin-3. Error bars show mean ± SEM from triplicates derived from each MEF strain.

pulp, small and large intestine) underwent dramatic atrophy, whereas the same tissues from relevant control mice (Tamoxifen-treated *mdm2*$^{-/-}$;*p53*$^{-/-}$ and *mdm-2*$^{+/+}$;*p53ER*$^{KI/-}$ mice, or oil-treated *mdm2*$^{-/-}$;*p53*$^{KI/-}$ mice) were unaffected (Figure 2A). After 5 days, Tamoxifen-treated *mdm2*$^{-/-}$;*p53*$^{KI/-}$ mice exhibited severe aplastic anemia: their bone marrow was essentially ablated, leaving only a few scattered plasma cells and macrophages, and accompanied by hemorrhage into the bone marrow lumen. Consistent with bone marrow ablation, analysis of peripheral blood indicated dramatic reduction in all hematopoetic lineages (leukocytes, lymphocytes, erythrocytes, and platelets) (data not shown). Thymi from the same animals appeared fibrotic with complete loss of lymphocytes, while spleens were totally devoid of white pulp (Figure 2A). Dramatic atrophy was also evident in the small intestine and colon, evidenced by severe shortening of villi and marked hypocellularity of crypts (Figure 2A). By contrast, classically radio-insensitive tissues like heart, lung, liver, and kidney (Rubin and Casarett, 1968) from the same *mdm2*$^{-/-}$;*p53*$^{KI/-}$ mice remained phenotypically normal following Tamoxifen treatment (Figure 2B). The dramatic atrophy induced by p53 restoration in classically radiosensitive tissues of Tamoxifen-treated *mdm2*$^{-/-}$;*p53*$^{KI/-}$ mice was preceded by, and presumably the result of, widespread apoptosis, clearly evident within 6 hr of p53 functional restoration (Figure 3). No discernible apoptosis was apparent at any time in radio-insensitive tissues such as lung, liver, brain, and kidney (data not shown). Recently, two independent studies using a conditional nestin-driven Cre recombinase conditional knockout strategy indicated that embryonic loss of Mdm2 function precipitates dramatic apoptosis in forebrain and the periventricular regions of the developing CNS (Francoz et al., 2006; Xiong et al., 2006). By contrast, restoration of p53 function

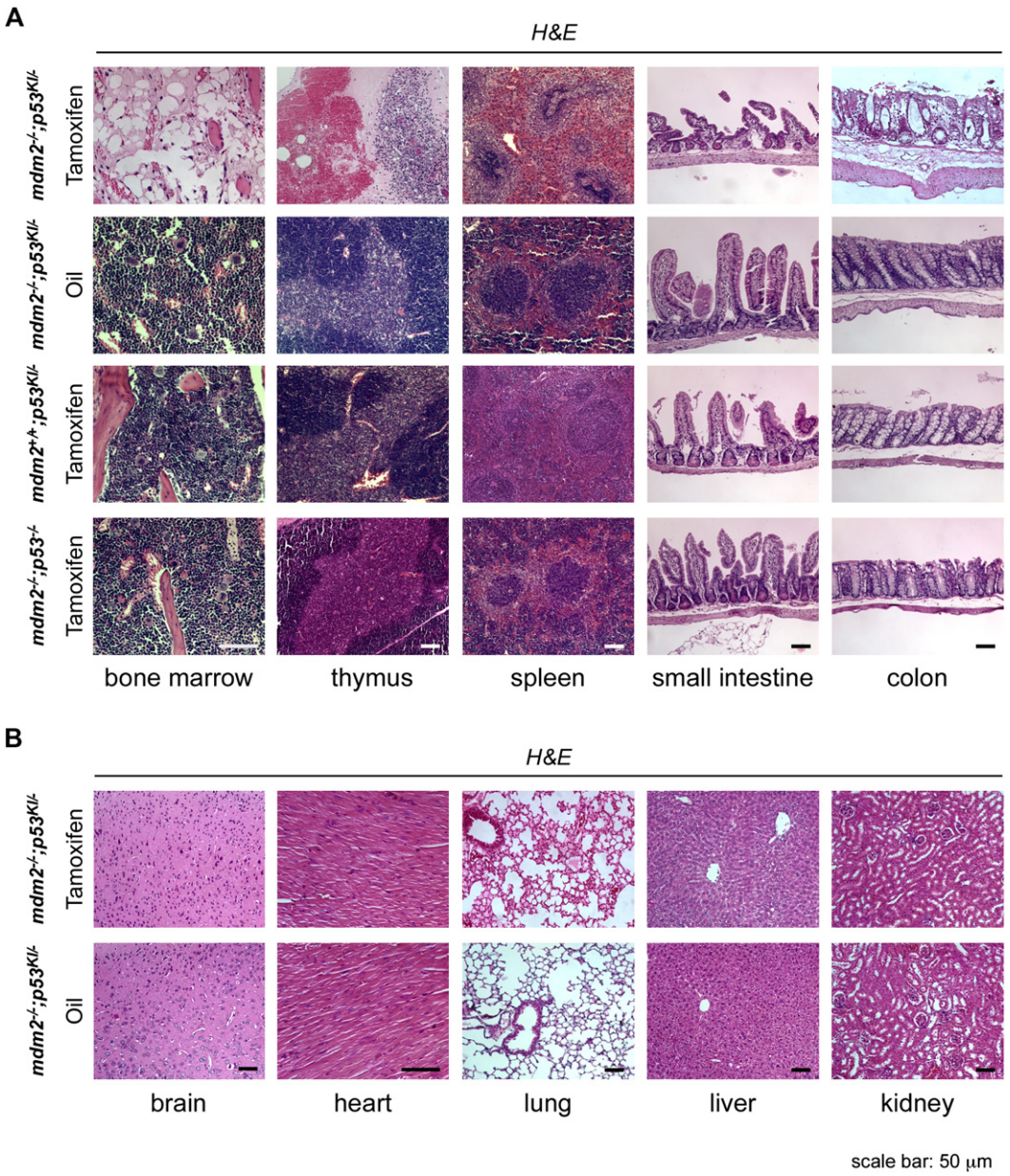

Figure 2. Restoration of p53 function is acutely lethal to adult mice in the absence of Mdm2 and ablates radiosensitive tissues

mdm2$^{-/-}$;*p53*$^{KI/-}$ mice were treated daily with Tamoxifen, and tissues from all organs were harvested from moribund mice at day 5 (prior to death). Control animals included *mdm2*$^{-/-}$;*p53*$^{KI/-}$ mice treated with oil and *mdm2*$^{+/+}$;*p53*$^{KI/-}$, *mdm2*$^{-/-}$;*p53*$^{-/-}$ mice treated daily with Tamoxifen. Tissue sections were stained with H&E. Restoration of p53 function effectively ablated bone marrow, thymus, and white pulp of the spleen and induced dramatic atrophy of both small and large intestines (**A**). In sharp contrast, classically radio-resistant tissues such as brain, lung, heart, liver, and kidney remained unaffected (**B**) and indistinguishable from those of control mice.

in adult *mdm2*$^{-/-}$;*p53*$^{KI/-}$ mice did not induce morphological changes in adult CNS or any detectable apoptosis in any regions of the brain, including the periventricular region, the forebrain cortex, and the cerebellum (Figure S4), consistent with the known radio insensitivity of adult CNS (Rubin and Casarett, 1968).

To assess directly p53 activity in various tissues of *mdm2*$^{-/-}$;*p53*$^{KI/-}$ mice after Tamoxifen-treatment in which p53 function had been restored, we used TaqMan to assay induction of the p53 target genes *cdkn1a* and *puma*. Expression was determined at both 6 and 24 hr in radio-insensitive tissues: in radiosensitive tissues, widespread apoptosis precluded meaningful analysis at the 24 hr time point. Both *cdkn1a* and *puma* were dramatically induced in all tested tissues, although both the absolute levels and the kinetics of *cdkn1a* and *puma* induction varied between tissue types (Figure 4). No significant induction of either gene was observed in any tissues derived from *mdm2*$^{+/+}$;*p53ER*$^{KI/-}$ mice. Importantly, profound induction of p53 target genes was observed in both radiosensitive and radio-insensitive tissues of Tamoxifen-treated *mdm2*$^{-/-}$;*p53*$^{KI/-}$ mice. Contrary to earlier reports (Fei et al., 2002), we discerned no correlation between relative expression of *puma* versus

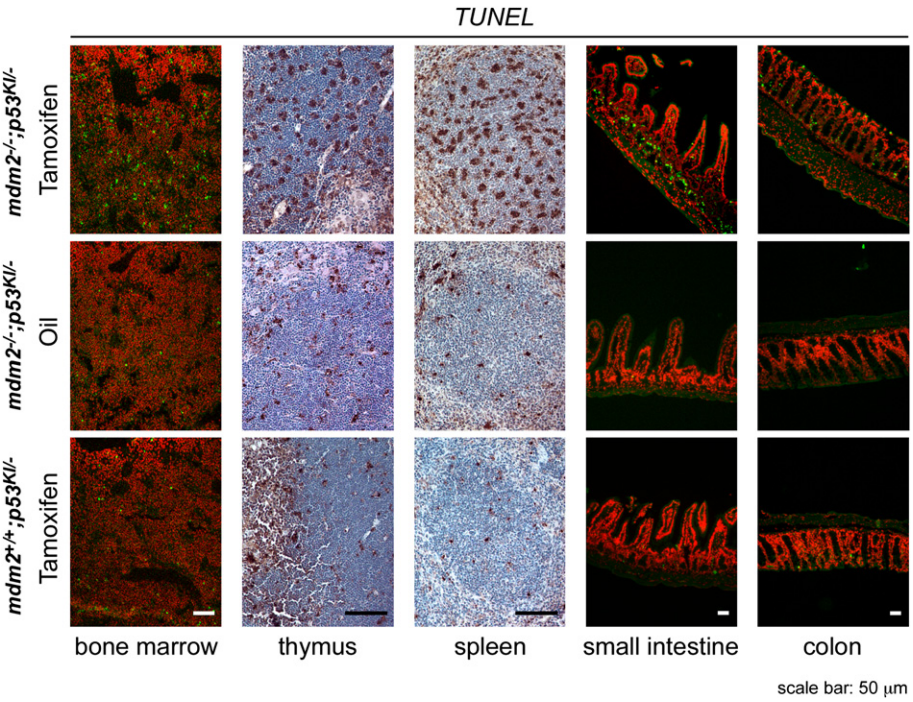

Figure 3. Atrophy of radiosensitive tissues induced by p53 restoration in $mdm2^{-/-}$ mice is caused by widespread apoptosis

Widespread apoptosis as indicated by TUNEL-positive staining is evident in $mdm2^{-/-};p53^{KI/-}$ mouse bone marrow, thymus, spleen, and small and large intestine harvested 6 hr after restoration of p53 function by i.p. injection of Tamoxifen. By contrast, no apoptosis is evident in tissues after p53 functional restoration in $mdm2^{+/+};p53^{KI/-}$ animals or in tissues of control oil-treated $mdm2^{-/-};p53^{KI/-}$ mice.

cdkn1a and the ultimate fate (apoptosis or no) of tissues (compare Figures 4A and 4B). Thus, in the absence of Mdm-2, functional p53 is spontaneously active in all tissues.

Although we observed no apoptosis in many $mdm2^{-/-};p53^{KI/-}$ tissues, the dramatic induction of *cdkn1a* and *puma* nonetheless indicated that p53 was profoundly active. We therefore asked whether such apoptosis-resistant tissues remained susceptible to p53-induced growth arrest. In the main, most radio-insensitive tissues have indolent proliferative rates, making any antiproliferative effect of p53 difficult to observe during the brief 5 day window prior to death of Tamoxifen-treated $mdm2^{-/-};p53^{KI/-}$ animals. However, testis underwent striking atrophy in Tamoxifen-treated $mdm2^{-/-};p53^{KI/-}$ mice, characterized by degradation of seminiferous tubules and near complete loss of proliferating spermatocytes, even though no apoptosis was evident. Cell proliferation analysis indicated that p53 restoration triggered profound growth arrest and that the atrophy arose from consequent failure to repopulate the tissue

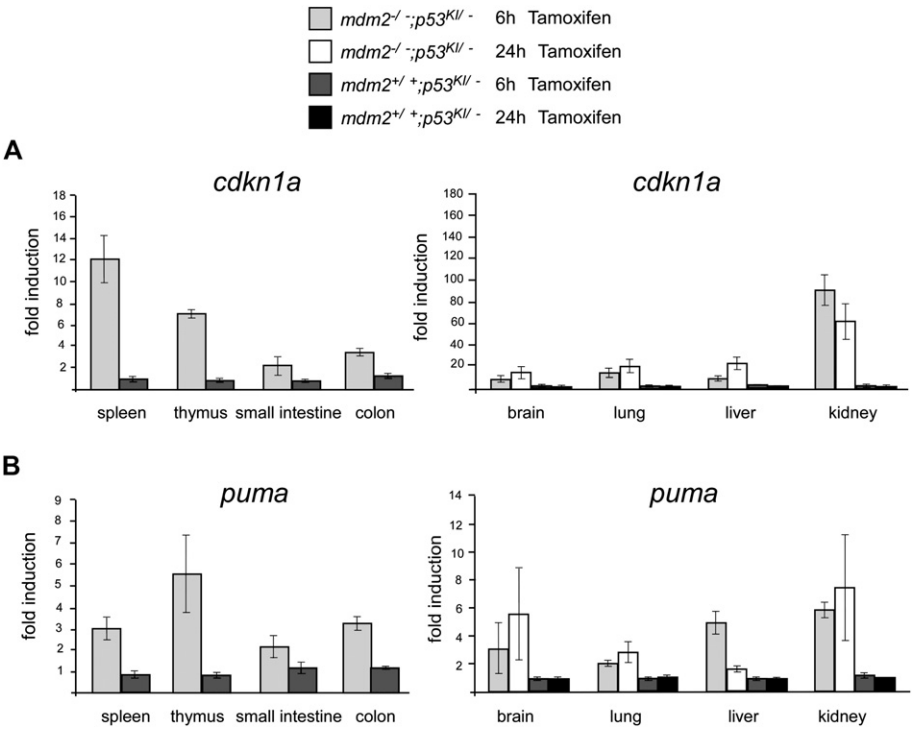

Figure 4. Acute induction of p53 target genes follows p53 functional restoration in tissues of $mdm2^{-/-}$ mice

p53 function was restored in either $mdm2^{-/-};p53^{KI/-}$ mice or $mdm2^{+/+};p53^{KI/-}$ littermates and tissues harvested after 6 hr (radiosensitive tissues) or 6 and 24 hr (radio-insensitive tissues). Widespread apoptosis precluded isolation of RNA from radiosensitive tissues at 24 hr. RNA was extracted from each tissue, and expression of the p53 target genes *cdkn1a* and *puma* was quantified by real-time PCR. Restoration of p53 function results in a marked upregulation of *cdkn1a* (**A**) and *puma* (**B**) in all tested tissues, although the kinetics and extent of induction vary between tissue types. No induction of *cdkn1a* or *puma* was evident in tissues from Tamoxifen-treated $mdm2^{+/+};p53^{KI/-}$. Fold induction is defined as compared to levels in untreated mice. Error bars show mean ± SEM from triplicates.

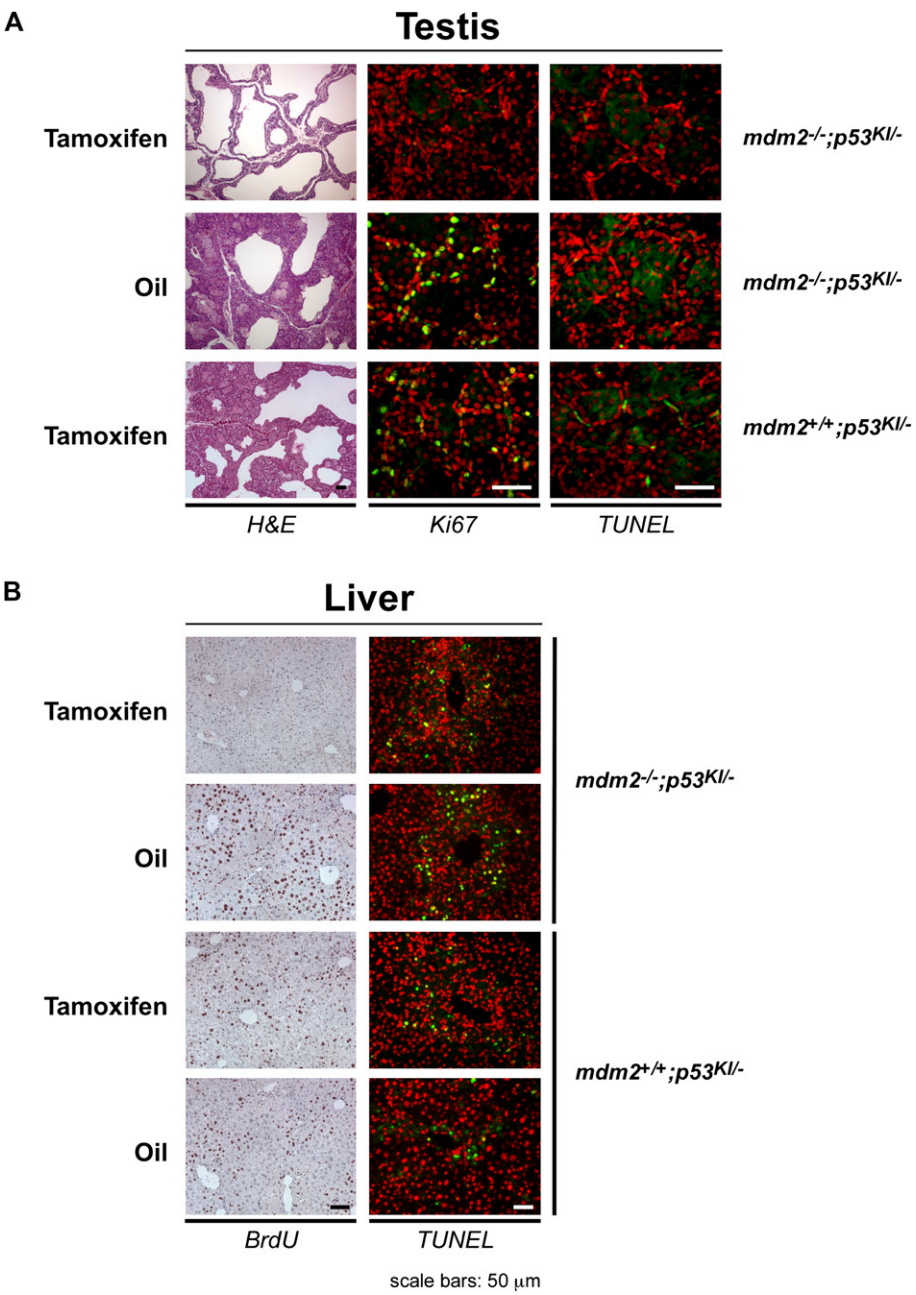

Figure 5. Restoration of p53 function in $mdm2^{-/-}$ mice induces profound proliferative arrest in apoptosis-resistant testis and liver

A: p53 was functionally restored in matched cohorts of male $mdm2^{-/-};p53^{KI/-}$ mice by daily injection of Tamoxifen. The animals were sacrificed after 5 days, revealing marked testicular atrophy. No testicular atrophy was evident in Tamoxifen-treated $mdm2^{+/+};p53^{KI/-}$ mice. Cell proliferation in testis was assayed immunohistochemically by staining with the proliferation marker Ki67. Apoptosis was assayed by TUNEL staining at 6 and 24 hr after p53 restoration. No apoptosis was evident in any cells at either 6 or 24 hr after p53 restoration (shown are tissues from animals treated for 6 hr). By contrast, p53 restoration induced complete inhibition of spermatocyte proliferation (a lower magnification is shown for the H&E staining to offer a better overview of overall tissue architecture).

B: Liver regeneration was induced in $mdm2^{-/-};p53^{KI/-}$ and $mdm2^{+/+};p53^{KI/-}$ mice by partial ablation with the hepatotoxin carbon tetrachloride. Thirty-six hours later, all animals were treated either with a single dose of Tamoxifen to restore p53 function, or with oil control. To assay hepatocyte proliferation, BrdU was administered systemically 21 hr after Tamoxifen treatment, and 3 hr later the animals were sacrificed and BrdU incorporation was assayed immunohistochemically. To assay for liver apoptosis, mice were sacrificed 6 hr after p53 restoration, and liver sections were stained by TUNEL. p53 restoration induces no significant apoptosis above the background induced by CCl_4 treatment alone but completely blocks hepatocyte proliferation.

(Figure 5A). While adult liver is quiescent, it can be induced to undergo rapid regenerative proliferation following chemical hepatectomy with CCl_4. Hepatocyte proliferation peaks at around 60 hr following CCl_4 injury, so a single dose of Tamoxifen was administered 36 hr after CCl_4 treatment, and mice were then sacrificed 24 hr later. Three hours prior to sacrifice, systemic BrdU was administered to label all hepatocytes in S phase. Analysis showed that hepatocyte proliferation was completely inhibited in Tamoxifen-treated $mdm2^{-/-};p53^{KI/-}$ animals (Figure 5B) compared with the widespread proliferation in regenerating livers of controls (oil- or Tamoxifen-treated $mdm2^{+/+};p53^{KI/-}$ and oil-treated $mdm2^{-/-};p53^{KI/-}$ mice). To ensure that the apparent lack of proliferating hepatocytes is not merely a consequence of p53 exacerbating CCl_4-induced hepatocyte death, CCl_4-treated mice were given a single dose of Tamoxifen 36 hr later and then sacrificed after 6 hr. This showed that, although CCl_4 itself induces hepatocyte apoptosis, its extent is unaffected by either *p53* or *mdm-2* status (Figure 5B). Taken together, our data in testis and liver are consistent with a profound antiproliferative action of spontaneously active p53 even in radio-resistant tissues of $mdm2^{-/-};p53^{KI/-}$ mice.

Spontaneous p53 function in Mdm-2-deficient tissues occurs without detectable upstream signals or serine 18 phosphorylation

p53 function in normal cells is activated by a variety of cellular stresses that give rise to a variety of posttranslational

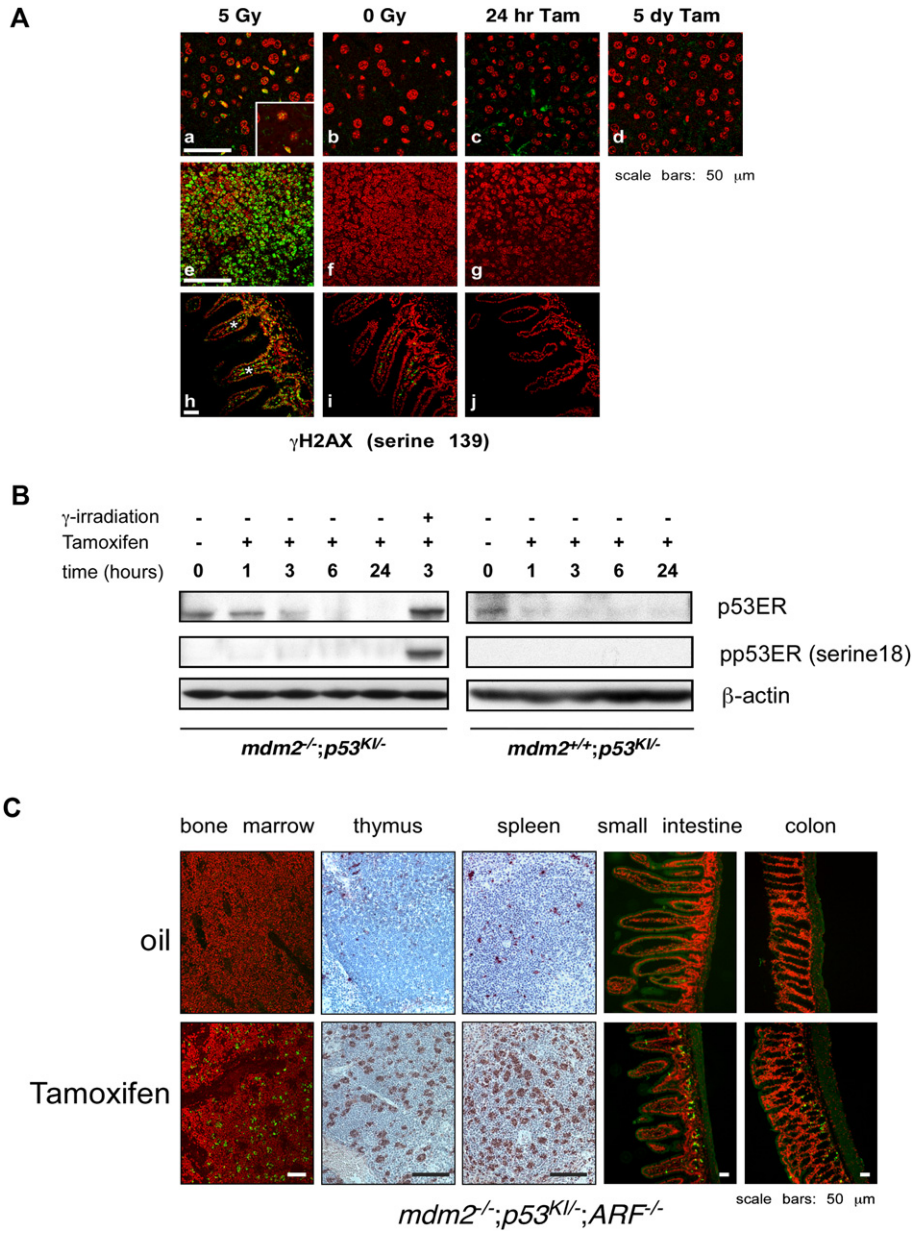

Figure 6. Spontaneous activity of p53 in *mdm2*-deficient mice occurs in the absence of detectable DNA damage and $p19^{ARF}$

A: p53 function was restored in $mdm2^{-/-};p53^{KI/-}$ mice by i.p. administration of Tamoxifen. Zero and twenty-four hours later, tissues were harvested and stained for γH2AX foci. In addition, liver was harvested after 5 days daily Tamoxifen treatment to assess long-term genotoxicity of Tamoxifen in that organ. As a positive control for DNA damage, $mdm2^{-/-};p53^{KI/-}$ mice were exposed to 5 Gy γ-radiation and sacrificed 1 hr later. Representative sections from radio-resistant (liver, **Aa**–**Ad**) and radio-sensitive tissues (spleen, **Ae**–**Ag**; small intestine, **Ah**–**Aj**) are shown. Irradiated mice show widespread and strong staining for γH2AX in all tested tissues, whereas γH2AX foci are absent from nonirradiated and Tamoxifen-treated tissue. Note: the positive staining in the intestinal stroma (*) is due to a nonspecific binding of the secondary antibody.

B: $p53ER^{TAM}$ stability and serine 18 phosphorylation status were assayed using p53-specific and p53 serine 18-specific antibodies in extracts derived from $mdm2^{-/-};p53^{KI/-}$ and $mdm2^{+/+}$ $p53^{KI/-}$ spleens at various times after i.p. injection of Tamoxifen. Absolute levels in the two *mdm2* genotypes are not directly comparable: rather, the exposure of $p53ER^{TAM}$ intensities at the t = 0 time point was adjusted to be similar to aid in a direct half-life comparison ± Mdm2. Functional restoration of p53 in Mdm2-deficient spleen triggers rapid downregulation of p53 protein: neither p53 stabilization nor serine 18 phosphorylation is evident at any time. In sharp contrast, $p53ER^{TAM}$ stabilization and phosphorylation are clearly evident in γ-irradiated mice.

C: To ascertain whether ARF contributes to the precocious activity of p53 in $mdm2^{-/-}$ tissues, Tamoxifen was administered i.p. to $mdm2^{-/-}$; $p53^{KI/-}$;$ARF^{-/-}$ mice, and tissues were harvested 6 hr later for apoptotic cells by TUNEL staining. Control animals received an equivalent i.p. dose of oil vehicle. Absence of $p19^{ARF}$ had no inhibitory effect on induction of the same dramatic apoptotic phenotype as seen in $ARF^{+/+}$ tissues (Figure 3), indicating that ARF is dispensable for spontaneous p53 activity in the absence of Mdm2.

modifications of p53 that are implicated in its activation. However, there remains substantial controversy as to the extent to which such posttranslational modification is required for p53 activity versus its role in disabling Mdm2 buffering. Our analyses indicate that p53 is spontaneously active in Mdm-2-deficient tissue. However, there remained the possibility that such activity might be dependent upon DNA damage or other stress signals peculiar to tissues of $mdm2^{-/-};p53^{KI/-}$ mice. To investigate this possibility, we first examined representative radiosensitive and radio-resistant tissues from $p53^{KI/-};mdm2^{-/-}$ mice for the presence of γH2AX and phospho-ATM, two well-characterized indicators of acute DNA damage. Positive control tissues were derived from sibling $mdm2^{-/-};p53^{KI/-}$ mice exposed to 5 Gy γ-irradiation 1 hr prior to sacrifice. As shown in Figure 6A and Figure S5, we found no detectable evidence of widespread or sporadic DNA damage in tissues of unirradiated $mdm2^{-/-};p53^{KI/-}$ mice. Importantly, systemic application of Tamoxifen alone induced no measurable DNA damage in these tissues at any time, even over an extended 5 day period.

To help exclude the formal possibility that some other, undefined activating signal might be responsible for the spontaneous activity of p53 in $mdm2^{-/-};p53^{KI/-}$ mice, we used immunoblotting to assess posttranslational modification and stabilization of $p53ER^{TAM}$ in spleen from $mdm2^{-/-};p53^{KI/-}$ mice. Extracts from $mdm2^{+/+};p53^{KI/-}$ spleen served as negative control, while spleen extracts from $mdm2^{-/-};p53^{KI/-}$ mice irradiated with 5 Gy γ-radiation acted as positive controls. In the absence of 4-OHT, $p53ER^{TAM}$ was clearly detectable by immunoblotting in both $mdm2^{+/+};p53^{KI/-}$ and $mdm2^{-/-};p53^{KI/-}$. As expected, γ-radiation induced phosphorylation of the critical p53 serine 18 residue (equivalent to human serine 15) (Sluss et al., 2004) as well as profound stabilization of p53 (Figure 6B). Upon functional restoration, $p53ER^{TAM}$ levels fell rapidly, demonstrating that a negative feedback loop still operates even in the absence of

Mdm2 (Figure 6B), perhaps through the action of other p53-induced E3 ligases/proteases (Dornan et al., 2004; Leng et al., 2003). However, the delay in degradation of p53 in the absence of Mdm2 indicates the significant contribution Mdm2 makes to expeditious p53 degradation (compare $mdm2^{-/-}$ and $mdm2^{+/+}$ cells). Of note, by 6 hr after Tamoxifen treatment of $mdm2^{-/-}$;$p53^{KI/-}$ mice, the level of $p53ER^{TAM}$ protein had fallen so low as to be essentially undetectable by immunoblotting. Nonetheless, the sustained expression of the short-lived *cdkn1a* mRNA at this time (Figure 4) suggests that $p53ER^{TAM}$ remains functionally active. Importantly, the spontaneous $p53ER^{TAM}$ activity we observe in Mdm2-deficient tissues occurs in the absence of any detectable phosphorylation at serine 18. Together, these data suggest that both stabilization of p53 and phosphorylation at serine 18 are dispensable for its spontaneous activity in tissues of unperturbed $mdm2^{-/-}$;$p53^{KI/-}$ mice and, furthermore, intimate that no upstream activating signal is required for such p53 activity.

The tumor suppressor $p19^{ARF}$ activates p53 in a non-DNA-damage-dependent manner, in part through inhibition of Mdm-2 function. However, the protein ARF-BP1/ Mule (Chen et al., 2005a; Zhong et al., 2005) has recently been identified as an Mdm2-independent mediator of $p19^{ARF}$ activation of p53 (Chen et al., 2005a). Thus, $p19^{ARF}$ has the capacity to activate p53 even in *mdm2*-deficient cells. $p19^{ARF}$ is normally induced only by activated oncogenes and aberrant cell proliferation. However, p53 exerts an important negative feedback on *ARF* transcription (Robertson and Jones, 1998; Weber et al., 2000), as evidenced by the constitutively high basal levels of *ARF* mRNA and protein expression in p53-deficient MEFs in culture (Weber et al., 2000). This raises the possibility that aberrantly elevated basal levels of $p19^{ARF}$ might be responsible for the precocious activity of p53 in tissues of Tamoxifen-treated $mdm2^{-/-}$;$p53^{KI/-}$ mice. However, TaqMan analysis showed that *ARF* expression is negligible in all tested tissues of $p53^{-/-}$ and non-Tamoxifen-treated $mdm2^{-/-}$;$p53^{KI/-}$ mice in vivo, although high in all tumors arising in both $p53^{-/-}$ and $p53^{KI/KI}$ strains (data not shown). Thus, normal control of *ARF* expression is intact in p53-deficient cells in vivo. To confirm directly whether $p19^{ARF}$ contributes to the spontaneous activity of $p53ER^{TAM}$ in Tamoxifen-treated $mdm2^{-/-}$;$p53^{KI/-}$ mice, we crossed them into an $ARF^{-/-}$ background. Interestingly, as with *ARF*-competent mice, we were unable to generate $mdm2^{-/-}$ pups in a homozygous $p53^{KI/KI}$ background in the absence of *ARF*, further supporting the notion that $p19^{ARF}$ plays no role in the spontaneous activity of $p53ER^{TAM}$. Systemic treatment of $mdm2^{-/-}$;$p53^{KI/-}$;$ARF^{-/-}$ mice with Tamoxifen triggered an identical apoptotic phenotype in radiosensitive tissues with kinetics similar to that in $mdm2^{-/-}$;$p53^{KI/-}$;$ARF^{+/+}$ mice (Figure 6C), unequivocally excluding any role for $p19^{ARF}$ in the spontaneous activity of p53 in $mdm2^{-/-}$ tissues.

DNA damage signals synergize with absence of *mdm2* to activate p53

While Mdm2 is a critical buffer against untoward p53 activity, the rapid degradation of $p53ER^{TAM}$ we see in *mdm2*-deficient tissues upon restoration of p53 function (Figure 6B) indicates that other mechanisms exist that contribute to restraining p53 function. It is known, for example, that p53 activity is also regulatable by an abundance of other modulators, including MdmX/4, ARF-BP1/Mule, other E3-ligases like Cop-1 and Pirh2, HAUSP, PIAS-1, NADH quinone oxidoreductase 1, and PML, as well as multiple kinases, acetylases, and transcriptional cofactors (Liu and Chen, 2006; Resnick-Silverman and Manfredi, 2006) (Dornan et al., 2006). Thus, it is possible that overt p53-activating signals could yet further enhance p53 activity in Mdm2-deficient tissues by antagonizing these other negative regulators of p53, potentially engaging apoptosis even in radioresistant tissues. We therefore asked whether combining absence of Mdm2 with an overt p53-activating DNA damage signal converts the merely cytostatic effect of p53 in many tissues to a therapeutically more useful apoptotic one. First, we turned to MEFs, a prototypical cell type in which p53 activation in vitro induces arrest rather than apoptosis. Restoration of p53 function in early-passage *mdm2* wild-type $p53^{KI/KI}$ MEFs had little effect on either cell cycle progression or viability (Christophorou et al., 2005). In contrast, restoring p53 function in $mdm2^{-/-}$;$p53^{KI/-}$ MEFs induced dramatic cell cycle arrest (Figure 7A). Coexposure of 4-OHT-treated MEFs with doxorubicin induced a profound cell cycle arrest in all MEFs, irrespective of *mdm2* status, significantly augmenting the effect of 4-OHT alone in $mdm2^{+/-}$;$p53^{KI/-}$ MEFs and, to a degree, also in $mdm2^{-/-}$;$p53^{KI/-}$ cells (Figure 7A). Interestingly, such replicative arrest in $mdm2^{-/-}$;$p53^{KI/-}$ MEFs was not reversed upon withdrawal of 4-OHT (data not shown), unlike p53-dependent Ras-induced arrest (Christophorou et al., 2005). However, even the combination of p53 restoration and DNA damage failed to induce apoptosis.

To ascertain the combined impact of p53 restoration and genotoxic injury in *mdm2*-deficient tissues in vivo, $mdm2^{-/-}$;$p53^{KI/-}$ mice were treated with Tamoxifen to reinstate p53 and 2 hr later exposed to 5 Gy γ-radiation. A clear additive impact on induction of apoptosis was observed in radiosensitive tissues (Figure 7B), confirming the existence of *Mdm2*-independent mechanisms by which DNA damage signals can enhance p53 activity. However, even in combination, p53 restoration and DNA damage failed to induce any detectable apoptosis in refractory tissues such as brain, lung, liver, and kidney (Figure S6).

Our data clearly indicate that p53 restoration in Mdm2-deficient tissues triggers p53-dependent cell cycle arrest or apoptosis (depending upon cell type) in the absence of any overt p53-activating signal. By contrast, a recent in vivo study using the Mdm2 inhibitor Nutlin-3 concluded that long-term treatment with the drug has no adverse pathological impact on any mouse tissue, suggesting that pharmacological inhibition of Mdm2 may be qualitatively distinct from genetic ablation. We therefore undertook an analysis of the impact of Nutlin-3 on a variety of early-passage, normal primary human cells in vitro. Primary human epithelial breast cells (Figure 8A), primary small airway epithelial cells (Figure 8B), and primary human hepatocytes (Figure 8C) were all cultured in the presence of escalating doses of Nutlin-3. As a positive control, the DNA-damaging agent doxorubicin was added to the same cells. Twelve hours later, p53 activation was then assessed by assaying levels of p53 and $p21^{cip1}$ via quantitative immunoblotting, and at 24 hr cell proliferation was assayed by BrdU incorporation. All human cells exhibited striking sensitivity to spontaneous activation of p53 by Nutlin-3, stabilizing p53 and upregulating $p21^{cip1}$. In hepatocytes, basal proliferation rates were too low to evaluate the impact of p53. However, doses of Nutlin-3 as low as 1 μM were sufficient to induce a nearly complete arrest in both primary human breast epithelial and small airway epithelial cells (Figure 8). Strikingly, the

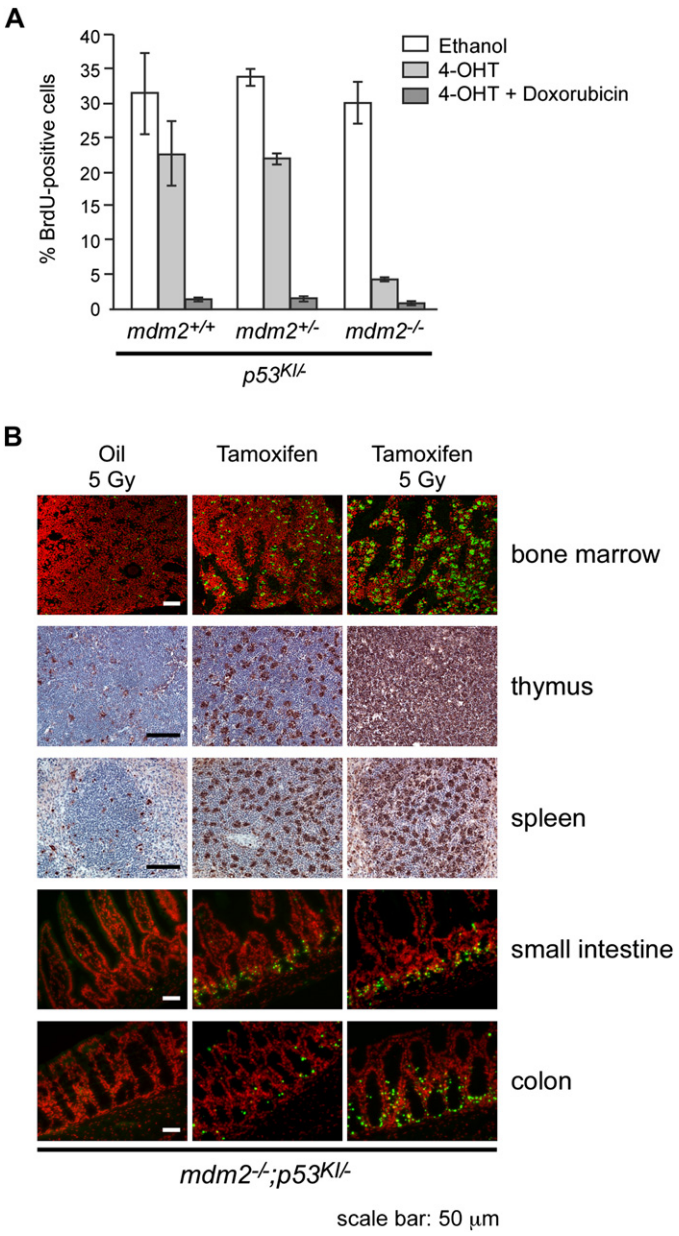

Figure 7. DNA damage and absence of *mdm2* synergize to activate p53 in vitro and in vivo

A: 4-OHT (100 nM) was added to cultures of early-passage MEFs (passage 4) derived from $mdm2^{-/-};p53^{KI/-}$, $mdm2^{+/-};p53^{KI/-}$, and $mdm2^{+/+};p53^{KI/-}$ mouse embryos. In identical parallel cultures, 500 ng/ml doxorubicin was also added. Twenty-four hours later, all cells were isolated, and both proliferation and apoptosis were assessed by BrdU incorporation and Annexin-V/propidium iodide staining, respectively. Addition of 4-OHT triggered profound cell cycle arrest in $mdm2^{-/-};p53^{KI/-}$ MEFs. In contrast, restoration of p53 activity induced only modest inhibition of proliferation in MEFs harboring either one or two copies of *mdm2*, consistent with the weak antiproliferative effect of restored p53 on early-passage, presenescent cultured MEFs (Christophorou et al., 2005). Coinsult of MEFS with doxorubicin activates p53 and triggered 4-OHT-dependent arrest in *mdm2*-positive MEFS. Cotreatment of *mdm2*-deficient MEFS with doxorubicin reproducibly exacerbated the growth-suppressive impact of 4-OHT alone.

B: p53 function was restored in $mdm2^{-/-};p53^{KI/-}$ mice 2 hr prior to 5 Gy total body γ-irradiation. Six hours after irradiation, radiosensitive tissues were harvested and apoptosis assayed by TUNEL staining. In one control group, p53 function was never restored; in the other, p53 function was restored in nonirradiated mice. Negligible apoptosis is evident in irradiated tissues from mice in which p53 function was never restored, as reported previously for $mdm2^{+/+}$ mice (Christophorou et al., 2005). By contrast, restoration of p53 function alone induces significant apoptosis in all radiosensitive tissues, and subsequent exposure to radiation significantly augments this.

extent of p53 activation by Nutlin-3 is similar to that induced by doxorubicin in epithelial cells. Moreover, in primary hepatocytes Nutlin-3 proved an even more potent inducer of $p21^{cip1}$ than doxorubicin (Figure 8C). Thus, the action of Nutlin-3 on these cells precisely phenocopies the potent cell cycle arrest triggered by p53 restoration in Mdm2-deficient epithelial tissues of Tamoxifen-treated $mdm2^{-/-};p53^{KI/-}$ mice.

Discussion

p53 is activated by a wide variety of stress, damage, oncogenic, and other signals, triggering cell death and/or replicative arrest. Tight regulation of p53 activity is therefore necessary to confine its toxic effects only to cells that sustain injury or oncogenic mutation. p53 is expressed constitutively in normal cells, but its functionality is regulated by a variety of posttranscriptional mechanisms that effectively buffer p53 activity. Of these, the autoregulatory feedback loop that intertwines p53 and Mdm2 is thought to be pivotal for limiting untoward p53 activity. The *mdm2* gene is a transcriptional target of p53, while its Mdm2 protein product suppresses p53 through direct interaction—squelching p53 transcriptional activity, promoting p53 nuclear export, and targeting p53 for proteasome degradation via its E3-ubiquitin ligase activity. However, the contribution made by each of these Mdm2 functions to restraining p53 function in vivo remains unclear. Furthermore, Mdm2 is but one of several known endogenous inhibitors of the p53 tumor suppressor that include the Mdm2 sibling MdmX/Mdm4, which binds p53 directly and squelches p53 transcriptional activity, and the ubiquitin ligases Pirh2, Cop1, and ARF-BP1/Mule ,which direct p53 degradation. In the case of MdmX, at least, germline knockout induces an embryonic lethality that, like that elicited by *mdm2* deletion, is rescued by codeletion of p53 (Parant et al., 2001). Thus, the extent to which Mdm2 versus these other regulators has a uniquely critical, nonredundant role in buffering p53 is also unclear.

To ascertain directly the role of Mdm2 in buffering against spontaneous p53 activity in adult tissues, we used the $p53^{KI/KI}$ mouse (Christophorou et al., 2005). Adult tissues of untreated $p53^{KI/KI}$ mice exhibit no measurable p53 activity. However, p53 function is rapidly and reversibly restored to all tissues by systemic administration of the synthetic ligand 4-OHT, reinstating responses to stress, DNA damage, and activated oncogenes (Christophorou et al., 2005). Thus, by maintaining $p53ER^{TAM}$ in the nonfunctional state, embryonic lethality due to absence of *mdm2* can be circumvented, allowing $p53^{KI/-}$; $mdm2^{-/-}$ mice to be raised viably to adulthood, whereupon p53 function can be acutely restored ab initio and the extent of its spontaneous activity in the absence of Mdm2 can be then assessed.

Initially, homozygous $p53^{KI/KI}$ mice were crossed into an $mdm2^{-/-}$ background: however, no $mdm2^{-/-};p53^{KI/KI}$ pups were born. Given the well-described toxicity of unfettered p53 during early mouse embryonic development of *mdm2*-deficient mice (Jones et al., 1995; Montes de Oca Luna et al., 1995) this

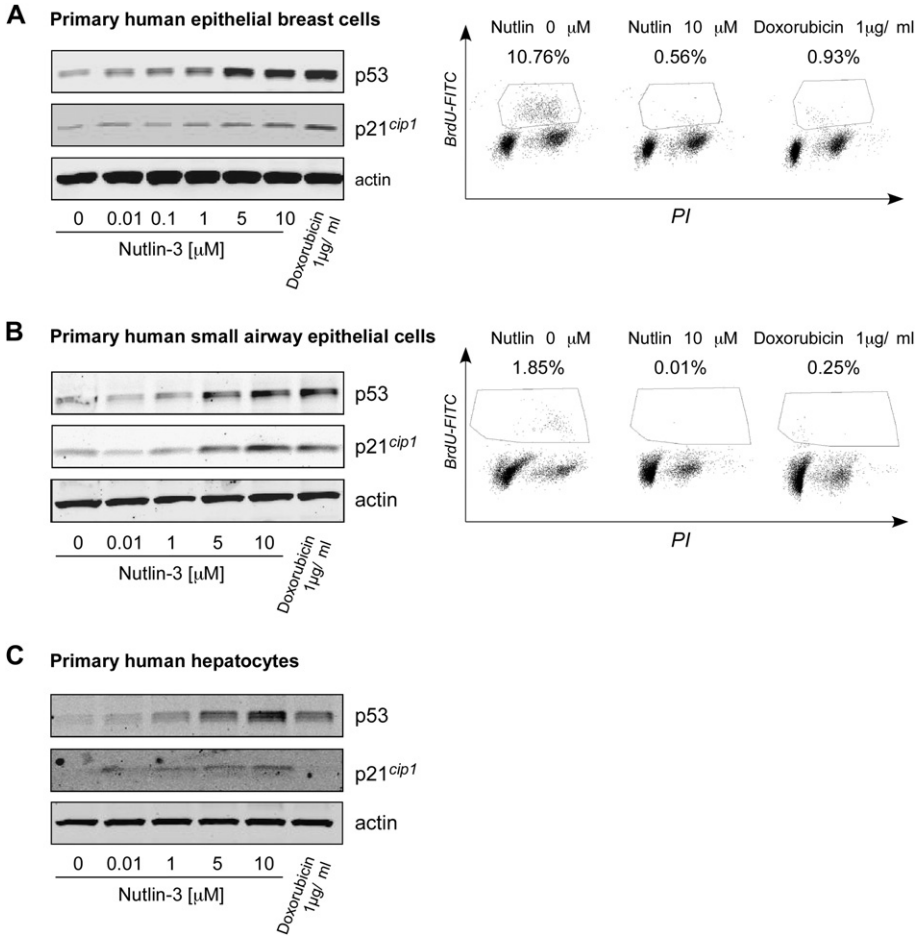

Figure 8. Nutlin treatment of primary human cells in vitro phenocopies the spontaneous activity of p53ER^{TAM} in *Mdm2*-deficient tissues in vivo

Primary human mammary epithelial cells (**A**), primary small airway epithelial cells (**B**), and primary human hepatocytes (**C**) were treated for 12 hr with a dose range of Nutlin-3 or 1 μg/ml doxorubicin. Protein lysates were harvested, and p53 and p21^{cip1} protein levels were assayed by immunoblotting. In addition, 20 hr after Nutlin-3/doxorubicin treatment, cells were pulsed with BrdU for 4–5 hr. Cells were harvested and stained, and BrdU/PI incorporation was analyzed by flow cytometry. Control untreated primary mammary epithelial cells and human small airway epithelial cells were analyzed similarly. Nutlin-3 treatment induces a cell cycle arrest in primary human cells as evidenced by the lack of Brdu/propidium iodide double-positive cells (gated region) compared to control untreated cells. The extent of this arrest is similar to that induced by the chemotherapeutic drug doxorubicin. As expected, there was no appreciable apoptosis in either Nutlin-3- or doxorubicin-treated human mammary epithelial cells, as assessed by Annexin V/PI staining, in the growth factor-rich culture conditions used (data not shown).

suggests that low-level leakiness of p53ER^{TAM} in the absence of 4-OHT, a leakiness undetectable in *mdm2*$^{+/+}$ animals (Christophorou et al., 2005), might be responsible for the prenatal death of *mdm2*$^{-/-}$;*p53*$^{KI/KI}$ embryos. This notion was supported by crossing *p53*$^{KI/-}$ heterozygous mice into an *mdm2*-deficient background: with only a single *p53ER*^{TAM} allele, *mdm2*$^{-/-}$;*p53*$^{KI/-}$ pups were born at Mendelian frequency. *mdm2*$^{-/-}$;*p53*$^{KI/-}$ mice remained viable through adult life, with all adult tissues appearing normal and of normal size and cellularity. This contrasts with the recently described *mdm2* hypomorphic mouse, in which inadequately buffered p53 activity causes reduced adult body mass, mild anemia, leucopenia, and decreased lymphoid cellularity (Mendrysa et al., 2003, 2006), and indicates negligible constitutive p53ER^{TAM} activity in *mdm2*$^{-/-}$;*p53*$^{KI/-}$ mice in the absence of 4-OHT ligand.

Acute restoration of systemic p53 function in *mdm2*$^{-/-}$;*p53*$^{KI/-}$ mice was invariably lethal within 5–6 days. Even a single dose of Tamoxifen, which, we infer from the known half-life of Tamoxifen, restores p53 function for only ~24 hr, killed all mice, indicating that even transient restoration of p53 function in the absence of Mdm2 initiates an ineluctable pathology. Analysis of expression of the p53 target genes *cdkn1a* and *puma* showed that systemic restoration of p53 function elicited spontaneous activation of p53 in all tested tissues. This is consistent with previous studies of the *mdm2* hypomorphic mutant that suggested preternatural activity of p53 in the absence of Mdm2 (Mendrysa et al., 2003). The extent and kinetics of both absolute and relative induction of *cdkn1a* versus *puma* varied significantly between differing tissues, but neither exhibited any correlation with the ultimate fate of that tissue with respect to apoptosis or survival. Thus, *puma*, which encodes a proapoptotic BH3 protein, was potently induced in liver and kidney, neither of which undergo apoptosis, but only to a lesser extent in spleen and intestine, which do. Similarly, p53-induced expression of the proapoptotic Bax protein is not restricted to radiosensitive tissues but also evident in lung, kidney, and heart following irradiation (Bouvard et al., 2000). Conversely, the Cdk inhibitor gene *cdkn1a*, which is reported to suppress p53-dependent apoptosis (Seoane et al., 2002), is induced not only in radioresistant tissues but also in those, like lymphoid organs and intestine, that die. The net consequence of such widespread p53 activity is widespread apoptosis in classically radiosensitive tissues—bone marrow, thymus, spleen white pulp, and small and large intestinal epithelium—and essentially ablating the entire bone marrow and lymphoid organs. Given the profound loss of body weight, diarrhea, and severe aplastic anemia we observe, it seems likely that it is this widespread apoptosis in bone marrow and intestine that underlies the rapid and irreversible commitment of affected animals to death. Nonetheless, many *mdm2*-deficient tissues appeared completely refractory to p53-induced apoptosis, consistent with the idea that the ultimate cellular response to p53 activation is determined by the

configuration of downstream p53 response pathways peculiar to each cell type and lineage.

Even in tissues that are spared apoptosis, restoration of p53 function triggers profound p53 activation and potent inhibition of cell proliferation. In some tissues, such as testis, where continuous cell proliferation is required to maintain tissue integrity, the consequences of p53-induced cell cycle arrest are clearly evident. However, most adult somatic tissues replicate only sporadically, so any effect of p53-mediated growth arrest would take a long time to be manifest. Nonetheless, by inducing acute liver regeneration we could demonstrate the potent antiproliferative action of p53 restoration in the absence of Mdm2 even in this classically radioresistant tissue. We predict that the same would occur in other radio-insensitive tissues if damaged.

In *mdm2*-proficient cells and tissues, p53 is typically activated by upstream damage or oncogenic signals. DNA damage activates the various transducer and effector kinases of the ATM/ATR/Chk kinase families, which phosphorylate downstream substrates, including p53. However, it has remained unclear whether such posttranslational modifications of p53 confer new functional properties on the p53 protein itself, such as increased DNA binding or selective gene targeting (Bode and Dong, 2004), or instead act solely to disable Mdm2 buffering. We show that restoring p53 function in the absence of Mdm2 leads to spontaneous activation of p53 in the absence of any measurable DNA damage signals, or any modification of the p53ER^{TAM} molecule on serine 18. This is consistent with studies using the p53-Mdm2 inhibitor Nutlin-3 where p53 activity is elicited without concomitant p53 modification (Thompson et al., 2004; Vassilev et al., 2004). There remains the remote possibility, however, that Tamoxifen itself is responsible for activation of *p53* in our p53ER^{TAM} model. Indeed, Tamoxifen can be α-hydroxylated by cytochrome P450 3A4 and then O-sulfonated by hydroxysteroid sulfotransferase to form α-(N2-deoxyguanosinyl)tamoxifen DNA adducts, so it is conceivable that such DNA damage could trigger activation of p53ER^{TAM}. However, this seems unlikely for several reasons. First, α-(N2-deoxyguanosinyl)tamoxifen adducts are largely confined to liver, the site of the cytochrome P450 3A4 monooxygenase responsible for Tamoxifen α-hydroxylation, so are unlikely to be responsible for dramatic effects in bone marrow, lymphoid, and intestinal tissues. Second, p53 activation is very rapid (<2 hr), whereas Tamoxifen-DNA adducts arise only slowly (~24 hr) after Tamoxifen administration. Furthermore, cells affected by adducts are relatively rare, whereas p53 activation and concomitant apoptosis are essentially universal in radiosensitive tissues. Third, as discussed above, the spontaneous p53 activity we observe in *mdm2*-deficient cells and tissues occurs without any detectable posttranslational modification of p53 at the bellwether residue serine 18, which would be expected in cells sustaining genotoxic injury. Fourth, administration of 4-OHT, the active ER^{TAM} ligand generated from Tamoxifen, induces effects identical to those of Tamoxifen in *mdm2*$^{-/-}$;*p53*$^{KI/-}$ mice even though 4-OHT and its metabolites have a negligible genotoxicity profile (Phillips, 2001). Finally, we directly show that Tamoxifen treatment induces no detectable γH2AX or pATM foci in *mdm2*$^{-/-}$;*p53*$^{KI/-}$ tissues (Figure 6A and Figure S5). Even in the liver, the principal site of Tamoxifen O-sulfonation and adduct formation, 5 day sustained exposure to Tamoxifen resulted in no accumulation of detectable DNA damage foci.

The spontaneous activity shown by functionally restored p53 in *mdm2*-deficient tissues, in the absence of p53 modification or detectable upstream, p53-activating signals, at first sight supports the notion that the posttranslational modifications that activate p53 serve solely to disable Mdm2 buffering. However, several lines of evidence suggest that inhibiting *Mdm2* is not the only pathway promoting p53 activation. MdmX/4, although lacking an E3-ligase function and unable to promote p53 degradation, nonetheless acts to squelch p53 transcriptional activity (reviewed in Marine and Jochemsen, 2005), and the relationship between Mdm2 and MdmX/4, as well as their interdependence, is under intense scrutiny (Gu et al., 2002; Toledo et al., 2006). However, our data clearly indicate that, whatever the role played by MdmX/4, in the absence of Mdm2 it is insufficient to buffer precocious p53 activity. It remains to be determined whether the reciprocal is also true—namely that Mdm2 is unable to rescue adult p53 deficiency in the absence of MdmX/4. To address this question, the appropriate crosses between *mdmX/4*$^{-/-}$ and our p53ER^{TAM} knockin mice are underway.

Importantly, coexposure of *mdm2*$^{-/-}$;*p53*$^{KI/-}$ cells and tissues to a combination of both Tamoxifen and DNA damage significantly further increases the level, activity, and pathological effect of restored p53 (Figures 6B and 7), indicating that DNA damage signals promote p53 activation even in the absence of Mdm2. This is consistent with the notion that, even in the absence of Mdm2, there remain additional buffers of p53 activity that are sensitive to inhibition by ancillary p53-activating signals. Plausible examples of such Mdm2-independent mechanisms of p53 inhibition include the p53 transcriptional squelcher Mdmx/Mdm4, other p53-specific proteases, and direct modulation of p53 function by diverse posttranslational modifications.

Recently, the p53-Mdm2 interaction has become a focus of pharmacological attention because of its potential to activate p53 directly in that significant proportion of human tumors that retain the wild-type protein, without resorting to indirect genotoxic triggers and their many iatrogenic complications. Several studies, variously using peptidomimetics, small cis-imidazoline derivatives (Nutlins), antisense, and E3-ligase inhibitors all validate the principle that interfering with Mdm2 function is an effective way of activating p53 (Bianco et al., 2005; Bottger et al., 1997; Buolamwini et al., 2005; Chen et al., 2005b; Chene et al., 2002; Espinoza-Fonseca, 2005; Klein and Vassilev, 2004; Vassilev et al., 2004; Yang et al., 2005). It has been suggested that blocking *Mdm2* action in normal cells, which lack the additional constitutive damage and stress signals in tumor cells, will induce only a viable and reversible p53-induced growth arrest, offering a possible therapeutic window for the use of Mdm2 inhibitors in triggering the selective death of tumor cells. However, our data indicate that acute ablation of Mdm2 buffering alone elicits profound p53 activation, rapidly and irrevocably triggering lethal pathologies in all normal ''radiosensitive'' tissues akin to those induced by high-dose whole body irradiation. This conclusion was presaged by the study of Mendrysa et al., which showed precocious activity of p53 in mice constitutively harboring a hypomorphic *mdm2* mutant (Mendrysa et al., 2003).

By contrast, a recent study using the Mdm2 small-molecule antagonist Nutlin-3 showed significant suppression in growth of human tumor subcutaneous xenografts without any apparent ill effects on the mouse host (Vassilev et al., 2004) Likewise, antisense inhibition of *mdm2* expression apparently exerted no

toxicity on normal tissues (Bianco et al., 2005; Tortora et al., 2000; Zhang et al., 2004). Given the dramatic pathological outcomes of spontaneous p53 function in radiosensitive tissues, we presume that the inhibition of Mdm2 achieved in such experiments was insufficient to trigger spontaneous p53 activity in normal tissues, yet adequate to lower the activation threshold for p53 in the engrafted human tumor cell lines harboring additional p53-activating signals. One potential mechanism for how Nutlin-3 might achieve only a partial inhibition of Mdm2 is suggested by recent work demonstrating that Mdm2 and p53 interact not only via their N termini but also via the DNA binding domain of p53 with the core acidic domain of Mdm2, an interaction required for p53 polyubiquitination (Kulikov et al., 2006; Ma et al., 2006). Wallace et al. have purposed a model in which binding of the N termini of Mdm2 and p53 induces a conformational change in Mdm2, allowing interaction at the second sites (Wallace et al., 2006). Interaction between p53 and Mdm2 at these second sites is essential for p53 polyubiquitination. Consequently, it is possible that pharmacological interference by Nutlin-3 at only the N terminus of Mdm2 is insufficient to activate p53 fully, possibly exposing a differential sensitivity between normal and tumor cells. Weighed against this, however, are our data that indicate that Nutlin-3 readily induces both *cdkn1a* and cell cycle arrest in normal, primary, early-passage mouse and human cells (Figures 1 and 8; Figure S2). However, such in vitro experiments need to be interpreted cautiously given that cells in vitro are known to sustain a variety of p53-activating stresses.

Importantly, our observation that DNA damage signals can further augment p53 activity over that induced by abrogation of Mdm2 does intimate that modest inhibition of Mdm2 may yet be a useful therapeutic strategy for sensitizing cells to endogenous or exogenous p53-activating signals. Indeed, the recent observation that endogenous DNA damage signals in a human breast cancer cell line can sensitize cells to Nutlin-3-mediated arrest (Brummelkamp et al., 2006) suggests that, at least in tumor cell lines in vitro, such sensitization might be usefully harnessed. Whether such sensitization harbors any therapeutically usable differential in specificity between normal and tumor cells, especially given the exquisite sensitivity of radiosensitive tissues to Mdm2 inhibition, remains to be determined. On the other hand, the potent and irreversibly lethal impact of p53 in the absence of its Mdm2 buffer indicate that very careful titration, dosage, and delivery of Mdm2-inhibitory drugs will be a prerequisite for their use in cancer therapy.

Experimental procedures

Genetically engineered mice

p53ER^{TAM} KI (*p53$^{KI/KI}$*) mice have been described previously (Christophorou et al., 2005). Homozygous *p53$^{KI/KI}$* and heterozygous *p53 $^{KI/-}$* and *mdm2$^{-/-}$;p53$^{KI/-}$* mice were all maintained under standard conditions. All studies involving animal subjects were approved by the USCF Institutional Animal Care and Use Committee and conducted strictly in accordance with the relevant protocol number (A10518-25856-02).

Reagents and antibodies

All primary antibodies were used at a concentration of 1:1000 for immunoblotting and 1:200 for immunohistochemistry and immunofluorescence. Sources of antibodies were as follows: anti-BrdU (Roche), anti-Ki67 (clone SP6, NeoMarker), anti-p19^{ARF} (clone ab80, Abcam), anti-(phospho-serine 139)-γH2AX (clone JBW103, Upstate), anti-(phospho-serine 1981)-ATM (Rockland), anti-(phospho-serine 15/18)-p53 (#9284, Cell Signaling), anti-mouse p21^{cip1} (clone F5, Santa Cruz Biotechnology), anti-mdm2 (H221, Santa Cruz Biotechnology), β-actin (clone AC-15, Sigma), anti-p21^{cip1} (mixed monoclonals, Upstate). Mouse and human p53 were immunoblotted with rabbit p53 CM5 (Vector) and DO-1 (Santa Cruz Biotechnology) antibodies, respectively. Immunoprecipitation of p53/p53-ER^{TAM} and Mdm2 were performed in 1% NP-40 buffer using anti-p53 (Pab246 Calbiochem) and anti-Mdm2 (4B11) antibodies, respectively.

TaqMan quantitation of p53 target-gene expression

Whole-cell RNA was isolated from tissues using an RNeasy kit (Qiagen). Total RNA concentration was determined spectrophotometrically (NanoDrop, Rockland, DE), and RNA quality was assessed with a Bioanalyser (Agilent Technologies, Palo Alto, CA). Total RNA was incubated with DNase (Ambion, Austin, TX) to remove contaminating DNA, and the DNase was then inactivated and removed according to the manufacturer's protocol. RNA was reverse transcribed into cDNA using iScript (BioRad, Hercules, CA), and quantitative PCR analysis was performed on an AB Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA). Quantitative detection of specific nucleotide sequences was based on the fluorogenic 5′ nuclease assay (Ginzinger, 2002), and relative expression was calculated as described previously (Livak and Schmittgen, 2001). Assays were designed (using Primer Express software v1.5, Applied Biosystems) to incorporate 5′ 6-FAM fluorophore the quencher BHQ1 at the 3′ end (at concentrations of 500 nM and 200 nM, respectively). cDNA equivalent to 3–5 ng RNA was measured in triplicate by real-time PCR using a QPCR master mix with final concentrations of 5.5 mM $MgCl_2$, 200 μM dNTPs, and 0.5 units HotStart AmpliTaq Gold (Applied Biosystems) in a 20 μl volume in 384-well plates. cDNA equivalent to 3–5 ng RNA input was measured for GUS, and all data were then normalized to GUS expression. *puma*/*bbc3* and *cdkn1a* messages were assayed using the Hs00248075_m1 and Mm00432448_m1 probe/primer sets (Applied Biosystems), respectively.

Determination of p53 and p53ER^{TAM} half-lives by ^{35}[S]-methionine pulse-chase labeling

MEFs were starved in cysteine/methionine-free media (in the presence of 100 nM 4-OHT) for 2 hr at 37°C/5% CO_2. MEFs were then labeled for 4 hr with ^{35}[S]-methionine (Translabel, NEN) (800 μCi per 10 cm dish in 5 ml of media), washed twice in PBS, and either harvested (t = 0), or chased with prewarmed/pregassed medium containing 5× nonradioactive cysteine and methionine for various times. Lysates were extracted in 1% NP-40 buffer, and protein concentrations were normalized. For immunoprecipitations, 800 μg of lysate was precleared with Zysorbin (Zymed) together with 40 μl of mouse serum for 2 hr. p53 was immunoprecipitated using 1 μg of Pab246 and Protein G-Sepharose. The precipitates were washed three times, resuspended in 1× SDS sample buffer, and run on 4%–20% Tris-glycine gels, which were fixed with Coomassie blue/acetic acid/methanol, washed and enhanced for 20 min in Amplify (Amersham), dried, and exposed to a phosphor screen. The gels were visualized and analyzed using a phosphorimager (Molecular Dynamics).

Supplemental data

The Supplemental Data include Supplemental Experimental Procedures and six supplemental figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/10/6/501/DC1/.

Acknowledgments

We thank Dr. Gigi Lozano for providing *Mdm2*-deficient mice and the UCSF Comprehensive Cancer Center Mouse Pathology Core for important facilities and services. We are indebted to Fanya Rostker for her expert assistance with animal studies, Jamie Smyth for assistance with primary human cells, and all members of the Evan Laboratory for advice and criticism. This work was supported by the Deutsche Krebshilfe/Dr. Mildred Scheel Stiftung (I.R.) and grants CA100193 (NCI) and AG025413 (NIA) to G.I.E.

Received: February 23, 2006
Revised: June 16, 2006
Accepted: October 6, 2006
Published: December 11, 2006

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