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# Modeling the Therapeutic Efficacy of p53 Restoration in Tumors

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## SUMMARY

Although restoration of p53 function is an attractive tumor-specific therapeutic strategy, it remains unclear whether p53 loss is required only for transition through early bottlenecks in tumorigenesis or also for maintenance of established tumors. To explore the efficacy of p53 reinstatement as a tumor therapy, we used a reversibly switchable p53 knockin (KI) mouse model that permits modulation of p53 status from wild-type to knockout, at will. Using the well-characterized *Eμ-myc* lymphoma model, we show that p53 is spontaneously activated when restored in established *Eμ-myc* lymphomas in vivo, triggering rapid apoptosis and conferring a significant increase in survival. Nonetheless, reimposition of p53 function potently selects for emergence of p53-resistant tumors through inactivation of $p19^{ARF}$ or p53. Our study provides important insights into the nature and timing of p53-activating signals in established tumors and how resistance to p53 evolves, which will aid in the optimization of p53-based tumor therapies.

## INTRODUCTION

p53 is a transcription factor that triggers growth inhibitory and apoptotic responses to a wide range of insults, including DNA damage, stress, and oncogene activation. Inactivation of p53 function, or its attendant pathway, is a common feature of human tumors that often correlates with increased malignancy, poor patient survival, and resistance to treatment (Munro et al., 2005; Vogelstein et al., 2000; Vousden and Lu, 2002). Nonetheless, while strong selection against p53 function during tumorigenesis underscores the critical role that the p53 pathway plays in suppressing the emergence of incipient tumors, it tells us nothing about when and why such selection occurred during the protracted process of tumor evolution. The widely held ''Guardian of the genome'' (Lane, 1992) idea is that p53 suppresses tumorigenesis principally by mediating cellular responses to DNA damage, forestalling clonal accumulation of mutations by inducing death or arrest of damaged cells. If true, then absence of p53 is likely a transient requirement of early tumor initiation: once a tumor cell has acquired its requisite ensemble of mutations, p53 status may thenceforth be irrelevant. On the other hand, it may be that tumors harbor persistent and obligate p53-activating signals throughout their evolution, in which case absence of p53 is a continuous requirement for the maintenance of established tumors. The practical difference between these two ideas is profound: reinstatement of p53 function is an attractive tumor-specific therapeutic strategy (Bykov et al., 2003; Vassilev, 2005), but it will only work if tumors harbor persistent p53-activating signals that engage growth inhibition or death.

The p53-deficient mouse has proven invaluable in causally implicating p53 loss in spontaneous, oncogene- and mutagen-induced tumorigenesis and in resistance to anticancer drugs (Donehower et al., 1992; Harvey et al., 1993; Kemp et al., 1994; Schmitt et al., 1999). However, such models shed no light on when p53 inactivation is critical for the genesis and evolution of tumors nor on the likely impact of restoring p53 function in established tumors. Recently we described a unique, switchable p53 knockin (KI) mouse model ($p53^{KI/KI}$) (Christophorou et al., 2005) in which both copies of the *endogenous* p53 gene have been modified to encode the 4-hydroxytamoxifen (4-OHT) dependent $p53ER^{TAM}$ protein, a fusion between p53 and a modified form of the estrogen receptor (Vater et al., 1996). $p53^{KI/KI}$ mice can be reversibly and rapidly toggled between p53 wild-type (wt) and knockout states by, respectively, administration or withdrawal of 4-OHT (Christophorou et al., 2005). Our previous studies have shown that $p53ER^{TAM}$ is rendered functionally competent within ~1–2 hr of systemic 4-OHT administration, reverting back to a null state after ~30 hr unless 4-OHT is readministered. Importantly, provision of 4-OHT to either $p53^{KI/KI}$ cells in vitro or tissues of $p53^{KI/KI}$ mice in vivo does not itself activate $p53ER^{TAM}$ but rather renders $p53ER^{TAM}$ competent to become activated should appropriate signals arise in such cells. Furthermore, by all tested criteria 4-OHT-ligated $p53ER^{TAM}$ is functionally equivalent to wt p53 (Ringshausen et al., 2006).

In the well-characterized *Eμ-myc* mouse model, transgenic expression of the Myc oncoprotein driven from the immunoglobulin heavy chain enhancer leads to sporadic eruption of clonal B cell lineage lymphomas that closely resemble non-Hodgkin lymphoma in humans (Adams et al., 1985; Harris et al., 1988). The sporadic incidence of such tumors confirms their dependence upon the aleatory acquisition of secondary cooperating mutations. Indeed, as in many human and mouse cancers, the $p19^{ARF}$-Mdm2-p53 pathway is almost invariably inactivated in *Eμ-myc* lymphomas (Eischen et al., 1999; Schmitt et al., 1999), emphasizing the critical role of p53 in the intrinsic tumor suppressor response to deregulated Myc, as well as that of $p19^{ARF}$ as a mediator of that response (Zindy et al., 1998). The relevance of p53 inactivation for Myc-driven lymphomagenesis is confirmed by the greatly increased susceptibility of heterozygous $p53^{+/-}$ mice to *Eμ-myc*-induced lymphoma, 90% of which involve loss of the remaining p53 allele (Eischen et al., 1999; Schmitt et al., 1999). However, none of this speaks to whether inactivation of the p53 pathway is required early versus late or transiently versus continuously in Myc-driven lymphoma.

To ascertain the impact of restoring p53 function in tumors that have sporadically lost p53 function during tumor evolution, we crossed the *Eμ-myc* transgene into $p53^{KI/+}$ mice that carry one wt copy of p53 and one that is 4-OHT dependent. Tumors arise in such animals through sporadic inactivation of wt p53 function, recapitulating the sporadic loss of p53 in human tumors. However, the remaining switchable $p53ER^{TAM}$ allele can be functionally reinstated at any time, allowing us to ascertain the short- and long-term therapeutic impact of p53 restoration on established tumors.

## RESULTS

### Status of *p53* and $p53ER^{TAM}$ Alleles in Lymphomas Arising in *Eμ-myc*;$p53^{KI/+}$ Mice

To explore the consequences of restoring p53 function in tumors that had evolved via sporadic p53 loss, we used a variation on the p53 heterozygous model in which Myc-driven tumorigenesis arises following sporadic inactivation of a single extant wt *p53* allele. Lymphoma-prone *Eμ-myc* transgenic mice (Adams et al., 1985) were crossed into the $p53^{KI/+}$ background, which has one copy of wt p53 and one copy of the conditional, 4-OHT-dependent $p53ER^{TAM}$ allele (Christophorou et al., 2005). Incidence of spontaneous lymphomagenesis in such *Eμ-Myc*;$p53^{KI/+}$ animals in the absence of 4-OHT treatment was then monitored. Survival of *Eμ-myc*;$p53^{KI/+}$ was greatly reduced relative to that of *Eμ-myc*;$p53^{+/+}$ mice (*Eμ-myc*) (Figure 1; $p < 0.0001$) and similar to that reported for *Eμ-myc*;$p53^{+/-}$ mice (Schmitt et al., 1999). Thus, the $p53ER^{TAM}$ allele is nonfunctional in the absence of 4-OHT.

The increased incidence of lymphomas in *Eμ-myc*;$p53^{+/-}$ animals is accompanied by frequent LOH (loss-of-heterozygosity) of the remaining wt *p53* allele in tumors (Hsu et al., 1995; Schmitt et al., 1999). We therefore assessed whether the wt *p53* allele is similarly lost in lymphomas arising in *Eμ-Myc*;$p53^{KI/+}$ mice. Indeed, Southern blot analysis confirmed that 45% of such tumors had wt *p53* deletions (Figure 2A). Moreover, sequence analysis (Table S1) revealed that 7/7 tested tumors that ostensibly retain the p53 wt allele harbor point mutations in the DNA binding domain consistent with functional inactivation (http://www.umd.be:2072) (Vousden and Lu, 2002). Levels of mutant p53 protein were assayed in two of this latter class of tumors and found to be significantly elevated (Figure S1), as is typical of inactive p53 mutants in human cancers.

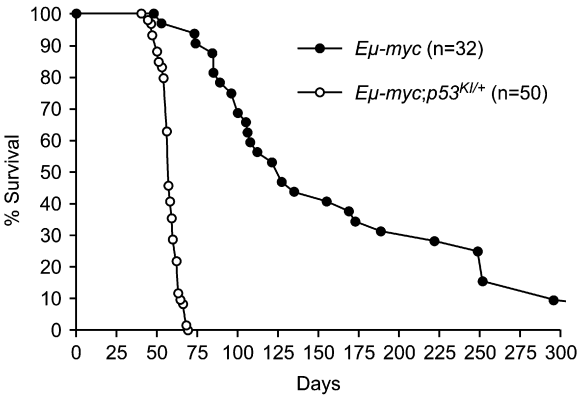


**Figure 1. Survival of *Eμ-myc*;$p53^{KI/+}$ Mice Is Decreased Compared to *Eμ-myc* Animals**

The survival percentage of untreated *Eμ-myc* and *Eμ-myc*;$p53^{KI/+}$ animals is plotted against their age.

To confirm directly that p53 function is absent from all lymphomas arising in *Eμ-Myc*;$p53^{KI/+}$ mice, and that function of the $p53ER^{TAM}$ protein encoded by the residual $p53^{KI}$ allele can be restored, cells from both p53-retaining (no Δp53) and p53-deleted (Δp53) lymphomas were explanted onto feeder cells in vitro (Schmitt et al., 1999) and 24 hr later exposed to either 4-OHT, to restore $p53ER^{TAM}$ function, or vehicle (ethanol). p53 activity in each of the two groups of tumors was assayed before and 2 hr after 4-OHT treatment by Taqman analysis of diagnostic p53 target genes (Vousden and Lu, 2002). Figure 2B shows the relative expression of *cdkn1a* in three independent lymphomas representing each of the two lymphoma groups (Δp53 and no Δp53). In the absence of 4-OHT (0 hr 4-OHT), all tumors exhibited similar, baseline expression of *cdkn1a*. By contrast, restoration of p53 function (2 hr 4-OHT) dramatically induced *cdkn1a* to a similar degree in each tumor group. Equivalent induction was seen for both *mdm2* and *puma* (Figure S2). Vehicle-treated cells showed no significant induction of p53 target genes relative to untreated controls (data not shown). Thus, all lymphomas from *Eμ-Myc*;$p53^{KI/+}$ mice have inactivated wt *p53* function, via deletion or mutation of the p53 wt allele but retain a functional, switchable $p53ER^{TAM}$ allele. Moreover, since $p53ER^{TAM}$ is spontaneously active when its function is restored, each of the lymphomas harbors constitutive p53-activating signals.

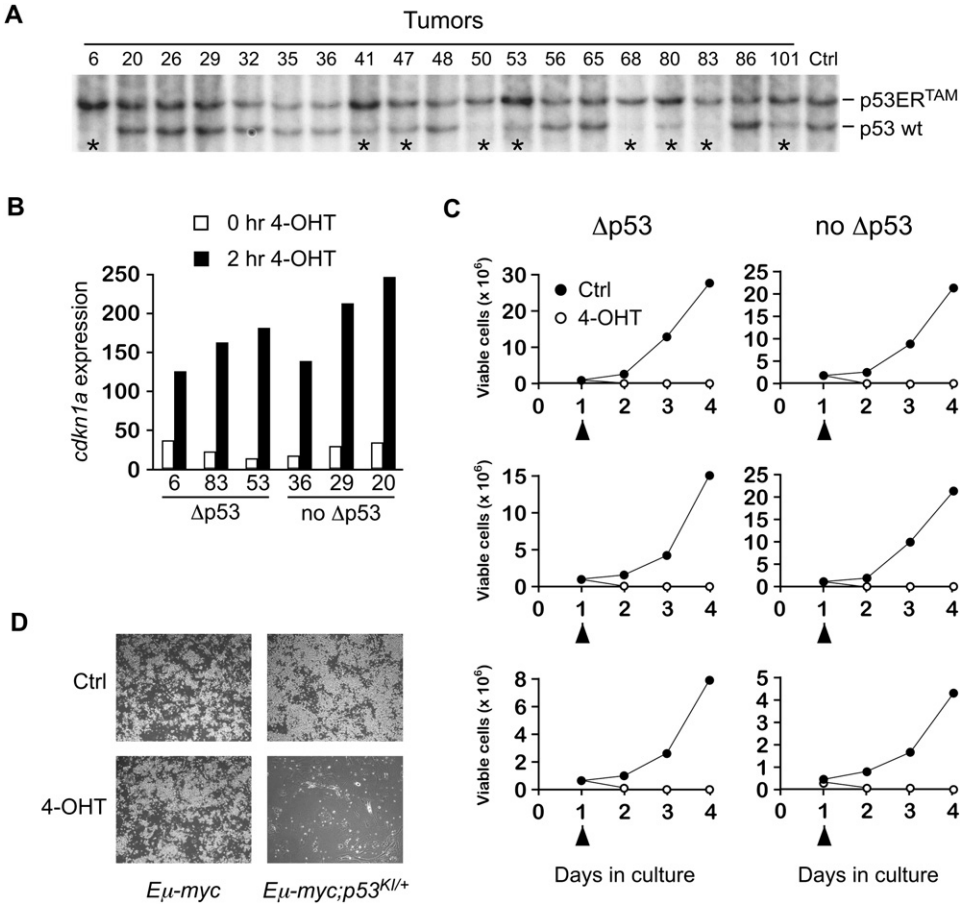


**Figure 2. Lymphomas from *Eμ-myc;p53*$^{KI/+}$ Animals Are Functionally Null for p53 in the Absence of 4-OHT**

(A) Southern blot analysis of lymphomas derived from *Eμ-myc;p53*$^{KI/+}$ mice (Tumors) and *p53*$^{KI/+}$ thymus (control: Ctrl). DNA was annealed with a *p53* probe that recognizes both the wt (*p53* wt, 5.0 kb) and the *p53ER*$^{TAM}$ (5.9 kb) alleles. Complete or partial p53 LOH is indicated (*).

(B) Taqman analysis of six independent lymphomas that either lost (Δp53: 06, 83, 53) or retained (no Δp53: 36, 29, 20) the *p53* wt allele. Relative levels of *cdkn1a* immediately before (0 hr) and 2 hr after 4-OHT (100 nM) treatment are depicted for each of the lymphomas analyzed. The *y* axis values indicate the percent expression of *cdkn1a* normalized to *gus*.

(C) Representative quantitative data for Trypan Blue exclusion of lymphoma cells from *Eμ-myc;p53*$^{KI/+}$ mice cultured in the absence (Ctrl, solid circles) or presence of 4-OHT (open circles). 4-OHT or vehicle was added to the cultures at day 1, indicated by arrowhead. Growth curves are presented for six independent tumors that either deleted (Δp53: 06, 47, 83) or retained (no Δp53: 26, 20, 29) the *p53* wt allele.

(D) Phase contrast images from day 4 cultures of tumor cells from *Eμ-myc* (wt *p53*) and *Eμ-myc;p53*$^{KI/+}$ mice treated with vehicle (Ctrl) or 4-OHT at day 1. Tumor cells (round white) were grown in suspension over feeder (fibroblastic) cells.

To ascertain the consequences of restoring p53 function for proliferation and survival of lymphomas, cells derived from 14 independent tumors arising in *Eμ-myc;p53*$^{KI/+}$ mice (five Δp53; nine no Δp53) were tested in a short-term in vitro proliferation/survival assay. As before, lymphoma cells were plated and 24 hr later either 4-OHT or vehicle added. All the lymphomas rapidly proliferated in the absence of 4-OHT, albeit with not unexpected differences in rate (Figure 2C; Ctrl). Furthermore, all shared a high index of viability (≥80%) prior to high confluence (typically ~day 3; data not shown). In contrast, 13/14 (93%) of the tested lymphoma cells underwent massive apoptosis upon restoration of p53 function with 4-OHT (Figure 2C; 4-OHT). Importantly, 4-OHT treatment of control *Eμ-myc* tumor cells arising in *p53*$^{+/+}$ mice had no effect on either lymphoma cell proliferation or survival, indicating that none of the effects observed is due to 4-OHT itself (Figure 2D).

Our data demonstrate that, similarly to the situation in tumors arising in *Eμ-myc;p53*$^{+/-}$ mice (Schmitt et al., 1999), lymphomas arising in *Eμ-myc;p53*$^{KI/+}$ mice are functionally null for p53 in the absence of 4-OHT. Importantly, in the great majority (93%) of *Eμ-myc;p53*$^{KI/null}$ tumors, all components of the p53 pathway save p53, including constitutive upstream p53-activating signals and downstream apoptotic p53 effector pathways, remain intact. Since we observed no consistent differences between the two (Δp53 and no Δp53) lymphoma groups in their sensitivities to p53 restoration, we can exclude the possibility of dominant-negative p53 mutations in

those no Δp53 tumors tested in vitro. Both groups of tumors were therefore considered appropriate for use in subsequent analyses and are hereafter referred to as *Eμ-myc;p53*$^{KI/null}$.

## Restoration of p53 Function in *Eμ-Myc;p53*$^{KI/null}$ Lymphomas Delays Tumorigenesis

Our in vitro data showed that *Eμ-myc;p53*$^{KI/null}$ lymphomas retain a functional, switchable *p53ER*$^{TAM}$ allele, which is lethal to lymphoma cells once its function is restored. We next addressed the therapeutic impact of p53 restoration in established lymphomas in vivo. This cannot be easily addressed in autocthonous tumors in *Eμ-Myc* mice, since the response of a primary tumor to treatment can be obscured by the outgrowth of a secondary malignancy. Fortunately, *Eμ-Myc* lymphomas can be serially transplanted into syngeneic mice, where they expand into tumors that closely resemble their primary forebear (Harris et al., 1988; Schmitt et al., 2000). To exploit the obvious advantages in experimental tractability and reproducibility of this approach, we separately transplanted *Eμ-Myc;p53*$^{KI/null}$ lymphoma cells derived from three independent primary tumors (6, 29, 68) into wt recipient mice by intravenous injection (i.v.) and allowed tumors to develop until palpable (typically 13–20 days posttransplantation). At this stage, animals were randomized into two groups and injected intraperitoneally (i.p.) with either Tamoxifen (Tam) or control vehicle (Oil) and sacrificed 6 hr later. Lymphomas cells were then analyzed by flow cytometry for DNA content (Propidium iodide, PI) and viability (AnnexinV/PI) to determine the immediate in vivo impact of p53 restoration on tumor maintenance. Representative data from Oil- and Tam-treated lymphomas are shown in Figure 3A. Control-treated tumors (6 hr Oil) show a DNA profile typical of rapidly proliferating cells together with a modest sub-$G_1$ population, the size of which varies between different primary tumors (not shown). By contrast, the DNA profile from Tam-treated tumors in which p53 function had been restored for 6 hr (6 hr Tam) shows significant depletion of S and $G_2$ populations, together with a substantial sub-$G_1$ population indicative of loss of viability (Figure 3A; left panels). Annexin V/PI staining of the same cells (Figure 3A; middle panels) confirmed widespread apoptosis in tumors from Tam-treated mice, with the percentage of viable cells falling to 6.5% within only 6 hr of p53 restoration (lower left quadrants). We also assayed directly for apoptosis in tumor tissue by TUNEL staining. This confirmed the dramatic extent of cell death that rapidly follows restoration of p53 function to *Eμ-myc;p53*$^{KI/null}$ lymphoma cells in vivo (Figure 3A, right panels; Figure S3). Systematic analysis of the impact of p53 restoration on lymphoma viability at later (>6 hr) time points was confounded by frequent morbidity of Tam-treated animals, most probably due to tumor lysis syndrome (Del Toro et al., 2005). Nonetheless, we can conclude that, as observed in vitro, p53 is spontaneously active in vivo upon functional restoration in *Eμ-myc;p53*$^{KI/null}$ lymphomas, triggering rapid and widespread tumor cell apoptosis.

The robust tumor suppressive effect we observe in vivo upon reinstatement of p53 function in established *Eμ-myc;p53*$^{KI/null}$ lymphomas suggests that p53 restoration should have a profound therapeutic impact on long-term survival of affected animals. To test this directly, we again transplanted *Eμ-myc;p53*$^{KI/null}$ primary lymphomas into wt recipients and randomly assigned them into control and Tam-treatment groups (see scheme in Figure 3B). To accommodate likely variability in responses between different primary lymphomas, we independently tested four *Eμ-Myc;p53*$^{KI/null}$ primary lymphomas (Δp53: 6, 68; no Δp53: 26, 29). To decrease the mortality from catastrophic tumor lysis, p53 function was restored 10 days posttransplantation, at which stage lymphomas are not yet palpable. Daily treatments (Tam or Oil) were then maintained for a total of 7 days. Essentially identical responses were obtained with each of the four primary tumors tested, allowing us to combine the data obtained from all transplanted mice into a single survival curve (Figure 3C). Control mice succumb to lethal tumors between days 14 and 25 after transplantation, with a mean survival of 18.6 days. By contrast, Tam-treated mice die of lymphomas between days 23 and 40 posttransplantation, a 28 day mean survival. Thus, 7 day in vivo restoration of p53 in *Eμ-myc;p53*$^{KI/null}$ lymphomas has a significant therapeutic impact ($p < 0.0001$), markedly delaying tumor onset and conferring a 50% (9.4 days) increase in mean survival.

## Transient Restoration of p53 Function In Vivo Potently Selects for p53 Resistance in Lymphomas

The recurrence of lymphomas in Tam-treated mice indicates that, despite the dramatic proapoptotic impact of p53 restoration, sufficient tumor cells escape to regenerate a tumor. Escape of tumor cells could arise either through chance or due to the presence of a constitutively p53-resistant subpopulation, perhaps refractory tumor stem cells or preexisting p53-resistant clones. To distinguish between these possibilities, we assayed the sensitivity of the recurring, secondary tumors to p53 restoration. Established secondary lymphomas harvested both from control and Tam-treated mice (hereafter termed Post-Oil and Post-Tam tumors, respectively) were cultured in vitro in the presence or absence of 4-OHT. To accommodate any intertumor variation, we assayed the growth and viability of 10 Post-Oil and 16 Post-Tam lymphomas, derived from all four of the primary *Eμ-myc;p53*$^{KI/null}$ lymphomas used in our in vivo study. Representative data is shown in Figure 4. Similarly to the primary tumors from which they were derived (Figure 2C), all Post-Oil tumors were rapidly killed upon exposure to 4-OHT (Figures 4A and 4B; Post-Oil lymphoma). By contrast, all 16 Post-Tam tumors continued to survive and proliferate in the presence of 4-OHT (Figures 4A and 4B; Post-Tam lymphomas), each growing at a rate at least that of its vehicle-treated Post-Oil lymphomas counterpart. Of note, we observed some variation in resistance of Post-Tam tumors to 4-OHT amongst the tumors: whereas some Post-Tam lymphomas were completely refractory to 4-OHT (Figure 4A; right

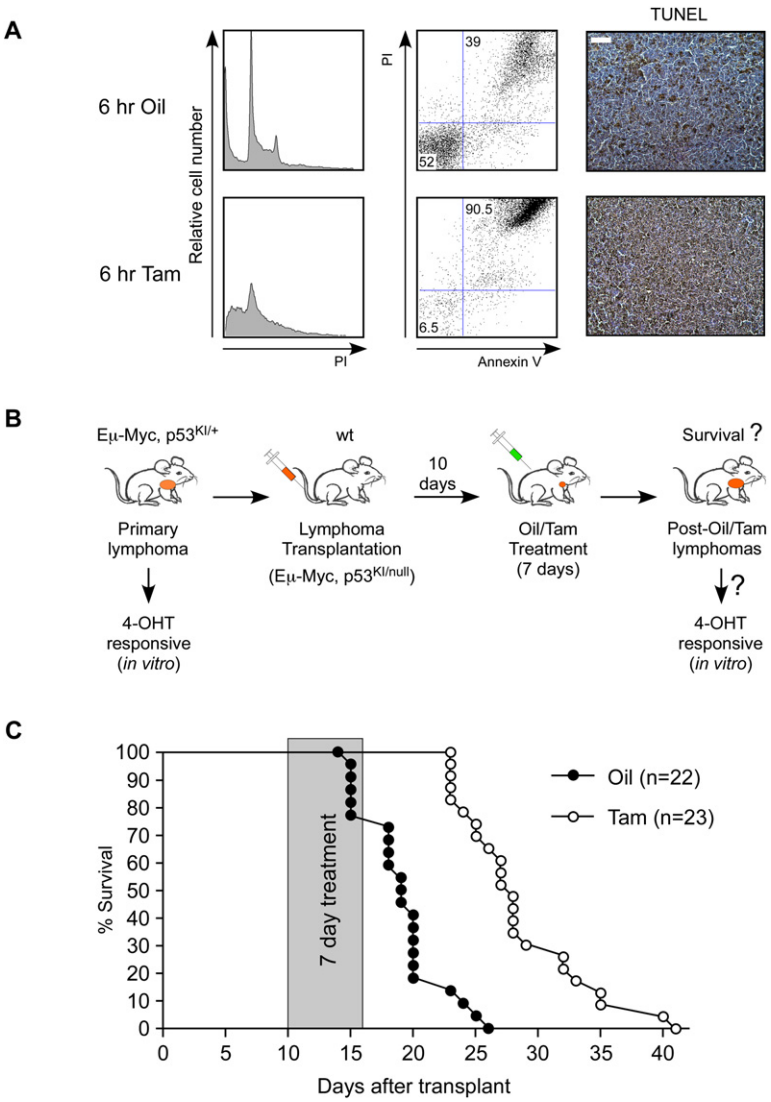


**Figure 3. p53 Restoration in *Eμ-myc*; *p53*$^{KI/null}$ Lymphomas Prolongs Host Survival**

(A) Representative data from *Eμ-myc;p53*$^{KI/null}$ lymphomas treated (i.p.) with Tam or carrier (Oil) and harvested 6 hr later. Flow cytometric analysis for DNA content (PI; left panels) and Annexin/PI (middle panels) staining of the same control and Tam-treated tumor cells is shown. Note that DNA content and Annexin/PI are assessed in fixed and fresh cells, respectively. TUNEL staining (brown) of lymphoma tissue (lymph nodes) is presented (right panels). Scale bar = 50 μm.

(B) Schematic representation of long-term survival study. *Eμ-myc;p53*$^{KI/null}$ primary tumors (4-OHT responsive in vitro) were transplanted into wt mice, which were then treated daily for 7 days with Tam or vehicle (Oil), starting at day 10 after transplantation. Assessment of the therapeutic effect of p53 restoration in lymphoma-bearing animals was based on tumor survival and response of recurring lymphomas to a second round of treatment (in vitro).

(C) The survival percentage of control (Oil) and Tam-treated mice is plotted over time after transplantation.

panels), others showed a slightly decreased propagation rate in its presence—most probably due to decreased viability (Figure 4A; middle panels). We can nonetheless conclude that transient exposure to p53 function efficiently selects for outgrowth of 4-OHT-resistant *Eμ-myc;p53*$^{KI/null}$ lymphoma clones.

*Eμ-myc;p53*$^{KI/null}$ Post-Tam tumors could in principle acquire resistance to 4-OHT by one of several mechanisms. First, p53ER$^{TAM}$ itself might be inactivated. Second, the pathway linking Myc with p53 might be severed, most plausibly through loss/inactivation of p19$^{ARF}$. Third, critical, nonredundant downstream p53 apoptotic/arrest effector pathways might become corrupted. Fourth, it is formally possible that tumor cells uptake of 4-OHT could be compromised by multidrug resistance. To discriminate between these possibilities, we first assessed by immunoblotting the status of the Myc/p19$^{ARF}$/p53 axis in Post-Oil and Post-Tam secondary lymphomas derived from all four primary tumors used in our study. As shown in Figure 5A, levels of Myc were comparable in all tumors analyzed, regardless of treatment, and consistent with a continuous requirement for Myc in lymphoma maintenance. Furthermore, all Post-Oil (O) lymphomas expressed high levels of p19$^{ARF}$ as well as detectable levels of p53ER$^{TAM}$ protein. By contrast, Post-Tam lymphomas exhibited one of two distinct mechanisms of resistance to p53 restoration. In one group of Post-Tam tumors (derived from primary lymphomas 6 and 47), p19$^{ARF}$ protein was absent, while p53ER$^{TAM}$ remained detectable. The majority of these tumors showed complete (5/8) or partial (2/8) deletion of the p19$^{ARF}$ alleles (Figure 5B), and in all cases, *ARF* mRNA expression was also lost (Figure 5C and not shown). In the second group (derived from primary lymphomas 26 and 29), p19$^{ARF}$ levels remained high but p53ER$^{TAM}$ protein was absent. Of note, this latter group corresponded to the lymphomas that showed complete refractoriness to 4-OHT restoration in the short-term survival studies, whereas the p19$^{ARF}$-deficient tumors often

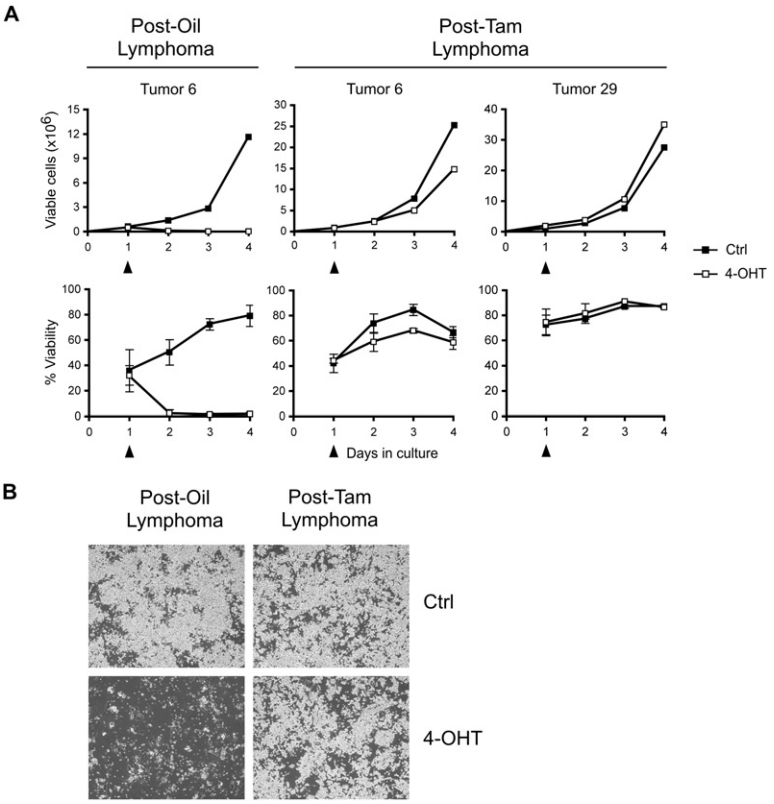


**Figure 4. p53 Restoration In Vivo Selects for Tumors Resistant to 4-OHT**

(A) Post-Tam and Post-Oil tumors were cultured in the absence (Ctrl) or presence of 4-OHT. The proliferative rate (upper panels, representative data) and viability (lower panels) of tumor cells was determined by Trypan Blue exclusion. 4-OHT or vehicle was added to the cultures at day 1, indicated by arrowhead. The secondary ($2^{ary}$) lymphomas shown were derived from *Eμ-myc;p53*$^{KI/null}$ primary tumors 6 (left and middle panels) and 29 (right panels). Error bars show the standard deviation between three independent tumors.

(B) Phase contrast images from day 4 cultures of tumor 6 derived $2^{ary}$ lymphomas. Tumor cells (round white) were grown in suspension over feeder (fibroblastic) cells.

retained some sensitivity (Figure 4A; right and middle panels respectively). Southern blot analysis confirmed that the *p53ER*$^{TAM}$ allele had, in fact, been deleted in this second group (Figure 5D). Interestingly, all Post-Tam secondary tumors that originate from a common primary *Eμ-myc;p53*$^{KI/null}$ lymphoma evolve 4-OHT resistance by the same route—one class losing p19$^{ARF}$ and the other p53ER$^{TAM}$ (Figures 5B and 5D and data not shown). Thus, some preexisting constraint dictates the route by which resistance evolves.

The above data illustrate three important features of the selective pressure that p53 restoration imposes on *Eμ-myc*-induced lymphomas. First, even short-term restoration of p53 function in established tumors imposes powerful selection for inactivation of the p53 pathway. Second, while it is formally possible that the p53-resistant lymphoma variants could have evolved de novo after p53 was deactivated at the end of treatment, the efficiency and rapidity with which resistant disease appears suggests that a significant reservoir of p53-resistant cells preexists prior to imposition of selection. Third, the comparable likelihoods with which p19$^{ARF}$ and p53ER$^{TAM}$ are lost indicate that counter-selection targets the whole p19$^{ARF}$-p53 pathway rather than favouring p53 preferentially. This is intriguing, given that p19$^{ARF}$ loss incapacitates only one of multiple p53-activating pathways that plausibly exist in an established tumor cell.

## Acquired Resistance to p53 Restoration Can Be Bypassed In Vitro

Our data indicate that inactivation of p19$^{ARF}$ is a frequent mechanism by which established lymphomas acquire resistance to the therapeutic impact of p53 functional restoration. However, while inactivation of p19$^{ARF}$ uncouples p53 from the constitutively activating oncogenic signal generated by Myc, it leaves p53 function intact and, potentially, accessible for therapeutic activation by other signals such as DNA damage (Kamijo et al., 1997; Stott et al., 1998). We therefore tested whether p53 can still be activated to therapeutic effect in p19$^{ARF}$-deficient Post-Tam lymphomas. Post-Oil and Post-Tam pairs derived from primary lymphomas 6, 47, 68 (all of which evolve p53 resistance through p19$^{ARF}$ loss), and 29 (that becomes resistant through loss of p53ER$^{TAM}$) were treated with either 4-OHT or vehicle, irradiated 1 hr later, and cell viability measured 4 hr after irradiation by Annexin/PI flow cytometry. As expected, mere restoration of p53 function by addition of 4-OHT proved sufficient to induce widespread cell death in Post-Oil lymphoma cells (Figure 6A; lower left panel, viable cells < 2%) but not in the Post-Tam secondary variants (Figure 6A, second left panel; Table S2). Exposure of the same cells to γ-radiation without p53 restoration had a more muted, and variable, impact on the viability of Post-Oil and Post-Tam tumors (viable cells: 12%–56% and 17%–66%, respectively; Table S2). However, the

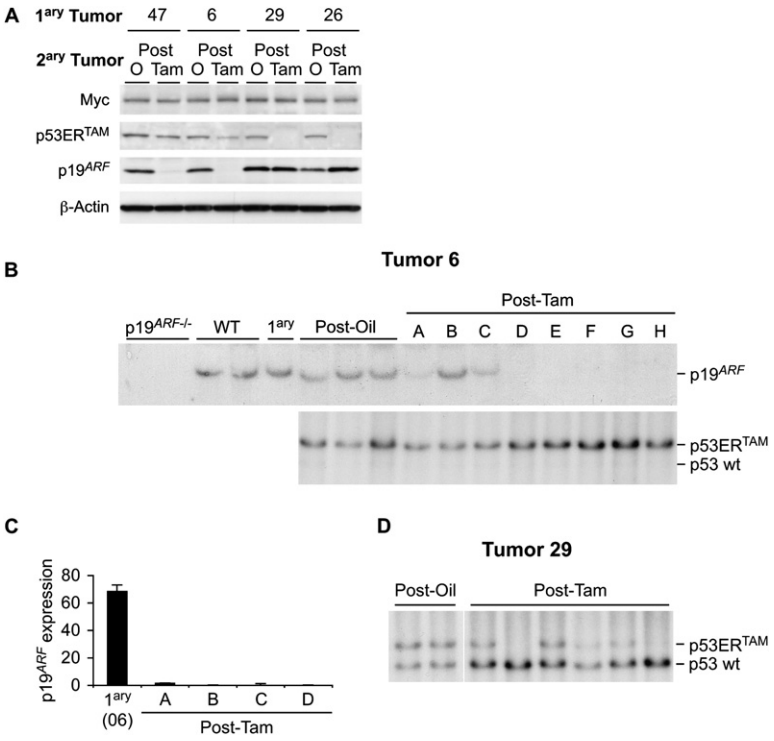


**Figure 5. Tumor Resistance to p53 Restoration Arises through Inactivation of the p19$^{ARF}$-p53ER$^{TAM}$ Axis**

(A) Protein lysates from recurrent secondary tumors (2$^{ary}$) derived from four (6, 26, 29, 47) independent *Eμ-myc;p53$^{KI/null}$* primary (1$^{ary}$) lymphomas were probed for Myc, p53, p19$^{ARF}$ and β-actin (loading control) expression by immunoblotting. Representative vehicle-(O) and Tam-treated transplants are shown.

(B) Southern blot analysis of Post-Oil and Post-Tam tumors derived from primary lymphoma 6 (1$^{ary}$). An exon1β probe was hybridized with tumor DNA to assess the integrity of the p19$^{ARF}$ locus. wt lymph nodes and *ARF*$^{-/-}$ MEFs were used as controls (upper panel). The *p53* wt and *p53ER$^{TAM}$* loci (lower panel) were analyzed as in Figure 2A. The upper (p19$^{ARF}$) and lower (*p53*) panels show the same Post-Oil and Post-Tam lymphomas.

(C) Taqman analysis of *ARF* expression in primary (1$^{ary}$) tumor 6 and its Post-Tam derivative tumors A, B, C, and D shown in (B). The *y* axis indicates the percent expression of *ARF* normalized to *gus*. Error bars show the standard deviation between three independent measurements.

(D) Southern blot analysis of Post-Oil and Post-Tam tumors derived from primary lymphoma 29. The *p53* wt and *p53ER$^{TAM}$* loci were analyzed as in Figure 2A.

combination of p53 restoration and irradiation efficiently triggered apoptosis in Post-Tam p19$^{ARF}$-deficient tumors (third column, lower panel 4-OHT+ γ-IR: viable cells ≤ 3%). By contrast, Post-Tam tumors lacking p53ER$^{TAM}$ were completely resistant to this combined treatment (right column, lower panel and Table S2).

We next asked whether the discrete Myc-p19$^{ARF}$ and DNA damage p53-activating signals can together cooperate to potentiate the activation of p53 in *Eμ-myc;p53$^{KI/null}$* lymphomas. To do this, we assayed induction of p53 target genes following p53 restoration and/or γ-irradiation in both the p19$^{ARF}$-proficient (Post-Oil) and p19$^{ARF}$-deficient (Post-Tam) secondary lymphomas. The proapoptotic p53 target gene *puma* (Figure 6B; left panel) was significantly induced (8–11-fold) in Post-Oil tumors upon 4-OHT treatment, presumably due to persistent p53 activation via the Myc-p19$^{ARF}$ axis. By contrast, radiation alone induced *puma* to a significantly lesser degree, presumably via p53-independent DNA-damage pathways. Importantly, combination of p53 functional restoration and irradiation synergized to enhance dramatically *puma* induction. Neither p53 restoration nor irradiation alone appreciably induced *puma* in p19$^{ARF}$-deficient Post-Tam lymphomas. However, *puma* was significantly induced, although to only ~50% of the level induced in p19$^{ARF}$-proficient tumors, when p53 restoration and γ-irradiation were combined. This is consistent with our FACS data showing that significant apoptosis in p19$^{ARF}$-deficient Post-Tam tumors occurs only in cells cotreated with 4-OHT and irradiation (Figure 6A). Similar synergy was evident in the induction of p53 target genes *cdkn1a* and *mdm2* (Figure 6B; right panels). Taken together, we can conclude the following three points. First, in the absence of p19$^{ARF}$, p53ER$^{TAM}$ remains competent to induce apoptosis when activated by DNA damage; hence, apoptotic effector pathways downstream of p53 remain intact. Second, p19$^{ARF}$ is required for the spontaneous activation of p53 in established *Eμ-myc* lymphoma cells, since p19$^{ARF}$ null cells harbor no measurable p53-activating signals. Third, the p19$^{ARF}$ and DNA damage pathways exhibit significant p53-activating synergy within the tumor cell population when coactivated.

## Combining p53 Restoration with Exogenous p53 Activation Increases the Therapeutic Impact of p53 in Lymphomas

Our in vitro data show that acquisition of resistance to p53 restoration through loss of the endogenous p19$^{ARF}$ pathway still leaves resistant cells vulnerable to p53 activation by exogenous DNA damage. Furthermore, the distinct but cooperative nature of the Myc-p19$^{ARF}$ and exogenous DNA damage p53-activating pathways raises the possibility that coactivation of both might offer a significantly enhanced therapeutic effect over either alone. To test this, wt mice were transplanted with lymphoma cells from primary tumor 6 and, 10 days later, treated as previously with either Tam or Oil for 7 days. Half of the mice in each cohort in addition received a single dose of γ-radiation

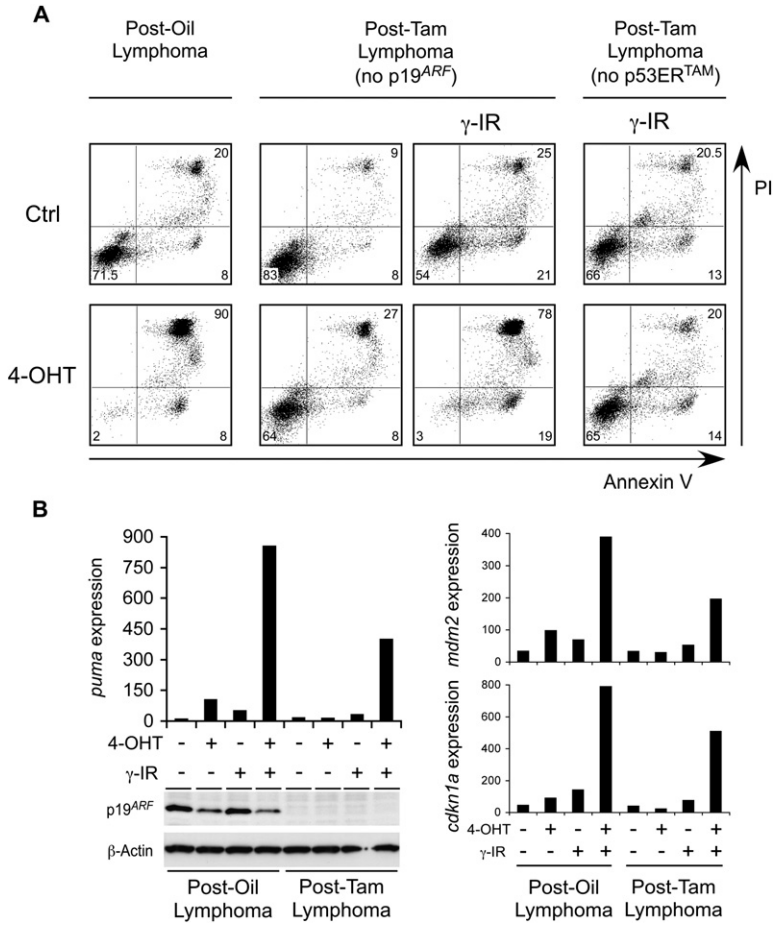


**Figure 6. DNA Damage Restores p53 Response in p19$^{ARF}$-Deficient Post-Tam *Eμ-myc*; *p53*$^{KI/null}$ Lymphomas**

(A) Flow analysis of AnnexinV/PI staining of Post-Oil and Post-Tam lymphomas, treated in vitro with 4-OHT or carrier (Ctrl) and exposed 1 hr later to 4 Gy of γ-irradiation (γ-IR). Tumor cells were collected 5 hr after Ctrl/4-OHT treatment. Viable cells occupy the lower left quadrant of each panel, while early apoptotic and late apoptotic/dead cells appear in the lower right and upper right quadrant, respectively. The tumors analyzed were derived from primary lymphoma 6 (Post-Oil and Post-Tam-no p19$^{ARF}$) and 29 (Post-Tam-no p53ER$^{TAM}$).

(B) Post-Oil and Post-Tam lymphomas derived from primary tumor 6 were treated as above, collected 4 hr after Ctrl/4-OHT treatment (3 hr after irradiation, γ-IR), and analyzed by Taqman for *puma, mdm2*, and *cdkn1* expression. Representative data of three Post-Oil and Post-Tam lymphomas is shown. The *y* axis indicates the percent expression of *puma, mdm2*, or *cdkn1* normalized to *gus*. These samples were also probed by immunoblotting for the expression of p19$^{ARF}$ and β-actin (loading control).

2 hr after the first injection of Tam (or Oil). As shown in Figure 7, radiation treatment (IR) alone offered only a small (11%) and statistically insignificant (p = 0.844) increase in mean survival compared with Oil-treated animals (mean survival: 20 versus 18 days for IR and Oil, respectively). Tam treatment extended mean survival to 26 days (equivalent to a 44% [8 day] increase) relative to Oil treatment. However, when γ-radiation and Tam treatment were combined, 50% of the mice survived more than 31.5 days, which corresponds to a significant (p = 0.002) increase in mean survival of 30% over Tam treatment alone and a 75% extension (13.5 days) in mean survival relative to control (Oil-treated) animals. Thus, combining p53 restoration with exogenous DNA damage significantly enhances the therapeutic impact of p53 restoration in established tumors, providing direct in vivo evidence of synergy between the intrinsic p19$^{ARF}$-p53 tumor suppressor and the exogenous DNA-damage-p53 pathways.

## DISCUSSION

The frequent inactivation of p53 in human tumors (Vogelstein et al., 2000; Vousden and Lu, 2002) suggests that restoring p53 function in tumor cells might be an attractive and tumor cell-specific strategy for treating cancers. However, it is axiomatic that p53 restoration will only work as a cancer therapy if p53-activating signals persist in established tumor cells. Whereas p53 inactivation in cancers unequivocally demonstrates the strong selection against p53 pathway activity at some point during tumor evolution, it does not tell us when such selection occurs, or why. Using a genetic model of p53 restoration we now establish, for the first time, that p53-activating signals are persistent features of an established tumor, what the nature of such signals is, and whether they are sufficient, either alone or in combination with other p53-activating signals, to achieve a therapeutic effect.

Our study shows that untreated *Eμ-myc*;*p53*$^{KI/+}$ mice resemble *Eμ-myc*;*p53*$^{+/-}$ animals (Schmitt et al., 1999), developing lymphoma at similar accelerated rates relative to *Eμ-myc* mice. All lymphomas arising in *Eμ-myc*;*p53*$^{KI/+}$ mice were functionally null for p53, due to sporadic deletion or mutation of the wt *p53* copy. This recapitulates the sporadic loss of p53 in lymphomas arising in *Eμ-myc*;*p53*$^{+/-}$ mice, where *p53* LOH frequency ranges from 55% (Hsu et al., 1995) to 94% (Schmitt et al., 1999). Interestingly, and in contrast to some human tumors and germline mutations of Li Fraumeni syndrome carriers (Royds and

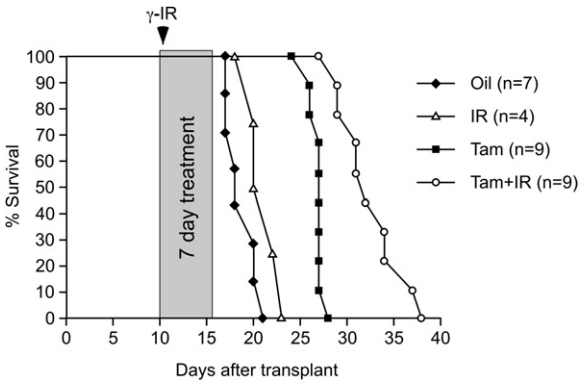


**Figure 7. Extrinsic DNA-Damage Signals Improve the Therapeutic Response of *Eμ-Myc;p53$^{KI/null}$* Lymphomas to p53 Restoration**

Wild-type mice were transplanted with untreated cells from primary tumor 6 and treated for 7 days with Tam or vehicle (Oil) as in Figure 3C. Half the mice from each group also received a single dose of γ-irradiation (4 Gy; IR) 2 hr after the first Tam/Oil treatment (day 10, arrow). The survival percentage for the four groups of mice is plotted against time after transplantation.

Iacopetta, 2006; Sigal and Rotter, 2000), we found no evidence for dominant-negative p53 mutations in *Eμ-myc;p53$^{KI/null}$* lymphomas, perhaps reflecting the fact that in the absence of 4-OHT there is selective pressure against only a single *p53* allele in *Eμ-myc;p53$^{KI/+}$* tumorigenesis. Indeed, in none of the primary tumors did we observe loss of the *p53ER$^{TAM}$* allele, confirming the functional inactivity of p53ER$^{TAM}$ in the absence of 4-OHT (Christophorou et al., 2005).

In vitro restoration of p53 function to *Eμ-myc;p53$^{KI/null}$* lymphoma cells triggered dramatic and rapid induction of p53 target genes, as well as apoptosis, confirming the functional competence of the residual *p53ER$^{TAM}$* allele in the presence of 4-OHT. Importantly, since 4-OHT does not itself activate p53ER$^{TAM}$ but, instead, merely restores its functionality, such spontaneous p53ER$^{TAM}$ activity suggests that cultured *Eμ-myc* tumor cells harbor constitutive, persistent p53-activating signals. However, in vitro culture is known to expose cells to a variety of p53-activating insults (Sherr and DePinho, 2000). Therefore, to confirm that persistent p53-activating signals are inherent to established *Eμ-myc;p53$^{KI/null}$* lymphoma cells and not in vitro artifacts, we also assessed the effects of restoring p53 function in vivo following (i.v.) transplantation of primary tumors into multiple wt recipients. Restoration of p53 function in tumors triggered rapid and widespread apoptosis within 6 hr of Tam treatment, confirming the presence in *Eμ-myc;p53$^{KI/null}$* lymphoma cells in vivo of both constitutive upstream p53-activating signals and functional downstream effectors. Indeed, induction of tumor cell death was so potent that many animals with large tumors became moribund following Tam treatment (data not shown), consistent with the recognized phenomenon of tumor lysis syndrome (Del Toro et al., 2005). Thus, our data demonstrate for the first time that p53 inactivity is, indeed, essential for maintenance of established tumors. Transient (7 day) restoration of p53 in *Eμ-myc;p53$^{KI/null}$* lymphomas also exerted a significant therapeutic impact on survival, leading to a 50% increase in mean survival over that of controls. Importantly, this therapeutic effect was not limited to particular tumors but observed with similar efficacy in each of four distinct *Eμ-myc;p53$^{KI/null}$* primary lymphomas (increase in mean survival: 44%–57%). p53 restoration also extended the disease-free phase, delaying overt tumor onset from 14 to 23 days posttransplantation. Thus, p53 restoration in lymphomas is a credible tumor-specific therapy.

Nonetheless, despite the delay afforded by p53 restoration all treated animals eventually relapsed. Since we only restored p53 transiently, one possibility was that tumors recur because p53 fails to kill a small, innately apoptosis-resistant "tumor stem cell" population that regenerates the original (p53 sensitive) tumor when p53 function is switched back off. Such a p53-resistant population was recently identified in bone marrow progenitors (Wu et al., 2005). However, tumor stem cell regeneration seems an untenable explanation given that the entirety of cells within each of the recurring, Post-Tam-treated tumors exhibits p53 resistance. Indeed, we show that resistance to p53 restoration arises through somatic inactivation of either p53ER$^{TAM}$ or p19$^{ARF}$, most commonly through gene deletion. Thus, the resurgent secondary tumors are likely the product of a pre-existing, exapted, and p53-resistant subpopulation within the original tumor that expands under the selective pressure imposed by p53 restoration. Intriguingly, all 2$^{nd}$ generation Tam-resistant tumors arising from a common primary tumor evolved 4-OHT resistance via the same route (i.e., all inactivate *p19$^{ARF}$* or all inactivate *p53ER$^{TAM}$*). This supports the notion that rare clones harboring specific resistance-conferring mutations pre-exist prior to transplantation and p53 selection. Moreover, the evolutionary route adopted for p53 resistance appears constrained by the initial mechanism via which p53 function is inactivated. Thus, all (4/4) primary lymphomas that delete the wt *p53* gene gave rise to 4-OHT-resistant secondary tumors that lose p19$^{ARF}$ expression. Conversely, 2/2 primary lymphomas with *p53* wt allele mutations spawned second-generation 4-OHT-resistant tumors that lose *p53ER$^{TAM}$*. It will be doubtless informative to ascertain the evolutionary constraints at play.

Recent data show that the tumor suppressive function of p53 in incipient neoplasms is triggered by p19$^{ARF}$ rather than DNA damage (Christophorou et al., 2006; Efeyan et al., 2006). Here we asked whether p19$^{ARF}$ is also the principal conduit of p53 activation in established tumors. The comparable frequencies of p53ER$^{TAM}$ and p19$^{ARF}$ inactivation in p53-resistant tumors indicates that selective pressure operates against the whole Myc-p19$^{ARF}$-*p53ER$^{TAM}$* axis rather than preferentially against p53, implying that loss of p53 confers no significant oncogenic advantage over loss of p19$^{ARF}$. Indeed, loss of p19$^{ARF}$ or p53 seems to confer a broadly equivalent

selective advantage for tumor growth, since the survival of mice bearing p53ER$^{TAM}$ or p19$^{ARF}$-deficient lymphomas is very similar (not shown). This is surprising given that established *Eμ-myc;p53 null* lymphomas might be expected to harbor a variety of p19$^{ARF}$-independent p53-activating signals, such as DNA damage. Indeed, *Eμ-myc;p53 null* lymphomas are typically aneuploid due to underlying chromosomal instability (Schmitt et al., 1999). Moreover, Myc overexpression in vitro induces reactive oxygen species, which generates DNA double-strand breaks (Vafa et al., 2002), while Myc overexpression in the mouse epidermis in vivo can activate p53 via an ATM-mediated DNA-damage response (Pusapati et al., 2006). Finally, persistent DNA damage is reported to be a frequent feature in human cancers from their earliest stages, possibly due to deregulated DNA replication (Bartkova et al., 2005; Gorgoulis et al., 2005). For all these reasons, loss of p53 might have been expected to confer significant tumorigenic advantage over p19$^{ARF}$ loss. One possibility is that additional mutations might be responsible for the inability of p19$^{ARF}$-deficient *Eμ-myc;p53$^{KI/null}$* lymphomas to undergo p53-dependent apoptosis. However, this seems unlikely given that both the p53ER$^{TAM}$ present in p19$^{ARF}$-deficient *Eμ-myc;p53$^{KI/null}$* lymphomas, and its downstream effector pathways, all remain functionally competent and activatable by exogenous DNA damage, triggering widespread apoptosis. Alternatively, DNA damage may be infrequent and sporadic, triggering only an ephemeral p53-activating signal that rapidly attenuates after the immediate genomic injury has been resolved (Christophorou et al., 2006). Whatever the explanation, our data indicate that p19$^{ARF}$ is the only persistent determinant of p53 triggering in established *Eμ-myc* lymphoma.

Nonetheless, the slight growth inhibitory effect of p53 restoration on p19$^{ARF}$-deficient tumor cells (Figure 4A) indicates that, although weak, p19$^{ARF}$-independent, p53-activating signals remain present in *Eμ-myc;p53$^{KI/null}$* lymphoma cells. This raises the possibility that p19$^{ARF}$ might cooperate with such signals to activate p53. Indeed, we show that the presence of p19$^{ARF}$ significantly potentiates activation of p53 by DNA damage in lymphoma cells, consistent with the notion that Myc-p19$^{ARF}$ and DNA-damage signaling pathways act as independent conduits to p53 activation that can be combined to augment p53 activity within the tumor population. However, whether such cooperation arises through potentiation of p53 activity within each individual cell or by each pathway co-opting p53 in discrete tumor cell populations remains unclear.

The p53-activating synergy between p19$^{ARF}$ and DNA-damage signals offers an accommodation for apparent contradictions in the role p19$^{ARF}$ plays in the p53-mediated DNA-damage response. While several investigators find no requirement for p19$^{ARF}$ in p53-mediated DNA-damage responses (Christophorou et al., 2006; Kamijo et al., 1997; Stott et al., 1998), others do. For example, *ARF$^{-/-}$* mouse embryonic fibroblasts (MEFs) show reduced p53-dependent responses to ionizing radiation (Khan et al., 2000), ATM and p19$^{ARF}$ collaborate to induce p53 Ser15 phosphorylation in cell lines (Li et al., 2004), and p19$^{ARF}$ deficiency can reverse the premature senescence of *ATM$^{-/-}$* MEFs (Kamijo et al., 1999). Our guess is that while p19$^{ARF}$ does not directly mediate DNA-damage activation of p53, in cells where p19$^{ARF}$ is constitutively expressed, such as tumor cells and cells cultured in vitro, it elevates basal p53 activity, so lowering the threshold for its activation by DNA damage.

In turn, the cooperation between the Myc-p19$^{ARF}$ and DNA-damage axes in p53 activation suggests that the therapeutic efficacy of harnessing endogenous, oncogene-induced p19$^{ARF}$ signals in tumors might be enhanced by coexposing tumors to exogenous DNA damage. Indeed, the combination of p53 restoration with irradiation significantly delayed lymphomagenesis compared with either treatment alone. Moreover, the combination was synergistic rather than merely additive. Although such combined treatment still failed to prevent ultimate relapse, it offered a 75% increase in mean survival compared with that of control-treated mice. Thus, the synergy between p53 restoration and DNA damage extends not only to the extent of p53 activation but also to the therapeutic outcome of such activation. Of note, lymphomas recurring after combined treatment all lost p19$^{ARF}$ but retained expression of p53ER$^{TAM}$ and, consequently, retained sensitivity to the subsequent combination of p53 restoration and DNA damage (Figures S4A and S4B). These recurring tumors may thus arise from cells that escaped the initial DNA-damage insult, most probably due to an insufficient dose of irradiation. This suggests that the therapeutic outcome of p53 restoration may be improved by coexposure to more aggressive levels of irradiation or systemic treatment with a chemotherapeutic. By demonstrating how restoration of p53 to p53 null tumors can enhance the therapeutic response to irradiation in tumors, our data also underscore the pivotal role that p53 functionality plays in response to cancer therapies (Brown and Attardi, 2005; Gudkov and Komarova, 2003).

In summary, our data strongly support the principle that p53 restoration has potent therapeutic potential against established tumors. However, it is not just the status of p53 itself that determines therapeutic efficacy of p53 restoration but also the status of p53-activating signals that pre-exist, or can be induced, in the tumor cells. Moreover, while the subsequent evolution of p53-resistant clones remains a major stumbling block to long-term eradication of disease, by enlisting both endogenous and exogenous p53-activating pathways in tumor cells, it is possible to maximize both the potency and extent of p53 activation within the tumor cell population and so optimize the therapeutic benefit that p53 restoration affords.

## EXPERIMENTAL PROCEDURES

### Mice and Lymphoma Cultures

Animals were kept under SPF conditions and maintained under approved UCSF IACUC protocols. *Eμ-myc* mice (Adams et al., 1985)

(C57BL/6, Jackson Laboratories) were crossbred to $p53^{KI/+}$ (Christophorou et al., 2005) (C57BL/6-129/Ola) to obtain $E\mu$-*myc* and $E\mu$-*myc*; $p53^{KI/+}$ progeny. Mice were sacrificed when tumor diameter $\geq$ 1.5 cm or terminally ill (survival curves) or when indicated. Statistical analysis of survival was performed with the Kaplan-Meier log-rank test. Lymphomas were collected and fixed (histopathology), frozen (Southern analysis) or processed to single-cell suspensions, aliquoted, and frozen. Lymphomas were cultured as described (Schmitt et al., 1999). Twenty-four hours after plating, 100 nM 4-OHT or vehicle (ethanol) was added to the cultures, which were followed for 3 additional days. Cell number and viability was assessed daily by Trypan Blue exclusion. Where appropriate, cells were irradiated (4 Gy) 1 hr after 4-OHT/ethanol treatment. Mice and cells were irradiated using a Mark 1-68 $^{137}$Cesium source (0.637 Gy/min).

### Lymphoma Transplantation and In Vivo Treatment

Prior to transplantation, independent aliquots of all primary lymphomas used were tested in vitro to confirm response to 4-OHT treatment. For transplants, lymphoma cells were thawed immediately before use, washed, counted, and injected intravenously ($10^6$ cells/mouse in 200 μl phosphate-buffered saline [PBS]) into multiple genetically matched wt mice (immunosuppressed with 4 Gy 3–6 hr prior to transplantation). For short-term p53 restoration studies and survival curves, 4–6 and 8–14 recipients were used per primary tumor, respectively. For practicality and economy in in vivo studies, we substituted 4-OHT with Tam, which is rapidly and efficiently converted to 4-OHT by the liver and has identical pharmacological efficacy in vivo to 4-OHT, as an activator of $ER^{TAM}$ (unpublished data). Tam (1 mg/100 μl of peanut oil/mouse) or carrier was administered once, when tumors were palpable (~0.5 cm diameter: short-term studies) or daily for 7 days, from day 10 posttransplantation.

### Southern Analysis

For Southern analysis, 10 μg of genomic DNA/sample were digested with *EcoRV* (p53) or *AflII* ($p19^{ARF}$), separated in a 0.7% agarose gel, transferred to a nylon membrane, and hybridized with $^{32}$[P] labeled probes (Rediprime II RPN1633, Amersham).

### Cell Cycle and Viability

For cell-cycle analysis, 1 × $10^6$ freshly isolated lymphoma cells were ethanol-fixed, incubated with propidium iodide (PI) (50 μg/ml PI, 0.5 mg/ml RNase A in PBS) for 30 min at room temperature, and analyzed. For viability studies, 1 × $10^6$ cells were washed in PBS, resuspended in Ca/HEPES buffer (10 mM HEPES, pH 7.4; 140 mM NaCl; 2.5 mM $CaCl_2$) and incubated with Annexin V (FITC: 556419 BD-Pharmingen) for 30 min. PI was added prior to analysis. Samples were analyzed using a Becton-Dickinson FACSCalibur flow cytometer. TUNEL staining of formalin-fixed, paraffin-embedded tissue sections was performed using the ApopTag Peroxidase in situ system (Chemicon International).

### Immunoblotting

Lymphoma cells were analyzed for protein expression essentially as described (Martins and Berns, 2002). Protein lysates (50 μg) were run in 4%–20% gradient gels (Invitrogen) and blotted on to PVDF membranes (Immobilon-P). The antibodies used were pan-Myc (Moore et al., 1987), anti-p53 (CM5, Vector), anti-$p19^{ARF}$ (C3, Novus), and anti-β-Actin (AC-15, Sigma).

### Taqman Analysis

Total RNA was isolated with RNeasy™ kit (Qiagen) and DNase treated (Invitrogen 18068-015) prior to reverse transcription (iScript, BioRad). Taqman analysis was performed as previously described (Christophorou et al., 2005). All data were normalized to *gus* expression.

### Supplemental Data

Supplemental data include four figures and two tables and can be found with this article online at http://www.cell.com/cgi/content/full/127/7/1323/DC1/.

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