Using a preclinical mouse model of high-grade astrocytoma to optimize p53 restoration therapy

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Based on clinical presentation, glioblastoma (GBM) is stratified into primary and secondary types. The protein 53 (p53) pathway is functionally incapacitated in most GBMs by distinctive type-specific mechanisms. To model human gliomagenesis, we used a GFAP-HRasVT17 mouse model crossed into the p53ER Tam background, such that either one or both copies of endogenous p53 is replaced by a conditional p53ER Tam allele. The p53ER Tam protein can be toggled reversibly in vivo between wild-type and inactive conformations by administration or withdrawal of 4-hydroxytamoxifen (4-OHT), respectively. Surprisingly, gliomas that develop in GFAP-HRasVT17/p53ER Tam mice abrogate the p53 pathway by mutating p19ARF/MDM2 while retaining wild-type p53 allele. Consequently, such tumors are unaffected by restoration of their p53ER Tam allele. In contrast, gliomas arising in GFAP-HRasVT17/p53WT mice develop in the absence of functional p53. Such tumors retain a functional p19ARF/MDM2-signaling pathway, and restoration of p53ER Tam allele triggers p53 tumor-suppressor activity. Congruently, growth inhibition upon normalization of mutant p53 by a small molecule, Prima-1, in human GBM cultures also requires p14ARF/MDM2 functionality. Notably, the antitumoral efficacy of p53 restoration in tumor-bearing GFAP-HRasVT17/p53ER WT animals depends on the duration and frequency of p53 restoration. Thus, intermittent exposure to p53ER Tam activity mitigated the selective pressure to inactivate the p19ARF/MDM2/p53 pathway as a means of resistance, extending progression-free survival. Our results suggest that intermittent dosing regimes of drugs that restore wild-type tumor-suppressor function onto mutant, inactive p53 proteins will prove to be more efficacious than traditional chronic dosing by similarly reducing adaptive resistance.

preclinical model | Nutlin 3 | intermittent treatment

Glioblastoma (GBM) is the commonest and most lethal type of central nervous system neoplasm. Historically, GBMs are classified as primary and secondary glioblastomas, the latter developing from preexisting lower-grade astrocytic tumors. Despite their broadly similar tumor histopathologies, the genetics of human GBM is extremely diverse. Most GBMs appear to be driven by promiscuous activation of the rat sarcoma (Ras) signaling pathway, either through mutation/overexpression of receptor tyrosine kinases (1) or through inactivation of neurofibromatosis (NF1) (2). The protein 53 (p53) tumor-suppressor pathway is functionally inactivated in almost all types of human cancer and seems to be a necessary condition for oncogenic activation. Intriguingly, however, the mechanism by which p53-mediated tumor suppression is forestalled varies in differing tumor types. For example, in colorectal, breast, and lung carcinomas, p53 itself is inactivated, either by gene loss or through structural mutation (3–5). In contrast, p53 often remains functionally competent in other cancer types, but its activation is blocked by mutations that incapacitate transduction of its upstream activating signals. Thus, overexpression or amplification of mouse double minute (mdm2), the gene encoding the E3-ubiquitin ligase that targets p53 for degradation by the proteasome, is frequent in prostate cancer, whereas overexpression of the p53 transcriptional inhibitor MdmX is common in retinoblastoma (6, 7). In some breast, brain, and lung tumors, the upstream inhibitor of Mdm2 activity, p14ARF, is inactivated by gene loss, methylation, or repression (8–12), thus uncoupling p53 activation from oncogenic signaling (13, 14). Finally, in tumors associated with DNA tumor viruses such as HPV, simian vacuolating virus 40, and adenovirus, p53 typically is inactivated directly by viral oncoproteins.

The p53 pathway is functionally inactivated in almost all instances of GBM. However, direct inactivation of p53 itself is relatively rare in primary GBM (15); instead, the p53 pathway is compromised by deletion of the Ink4a/p14ARF locus or by amplification of mdm2. In contrast, mutations that directly inactivate or delete p53 itself are the norm in secondary GBM (16). More recent genome-wide systems analyses based on their transcriptome profiles have stratified gliomas into four molecular signatures: proneural, neural, classic, and mesenchymal (2). Although both oncogenic Ras signaling and inactivation of the p53 pathway are features common to GBMs of all four molecular genetic subgroups, the precise mechanism by which Ras is activated and p53 activation is curtailed varies among the four subtypes. Such differences presumably reflect the differing evolutionary ontogenies of each GBM subtype. These, in turn, intimate that therapeutic strategies may need to be tailored to each form of GBM (17). Indeed, O6-methylguanine-methyltransferase (MGMT) status (18, 19) and O6-ethylguanine-ethyltransferase (20).

Significance

Glioblastoma is the most common and aggressive form of brain cancer. GBM patients typically respond poorly to conventional therapies. The tumor-suppressor protein 53 pathway is disrupted in a majority of GBM cases. Using a mouse model that mimics the progression of human GBM, we evaluate and optimize the therapeutic efficacy of functional p53 restoration in gliomas. We show that the efficacy of p53 restoration therapy in the animal model as well as in human GBM cells is improved markedly by an episodic dosing regimen that circumvents the selective pressure for adaptive resistance when p53 function is chronically restored.


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that are histopathologically similar to human astrocytomas by age 12 wk, with a lifetime incidence of 95% (29). Although mutant V12-Ha-Ras is not prevalent in human GBMs, this well-established model exhibits MAPK pathway activation at a level comparable to human GBMs (29–32), suggesting that the levels of Ras pathway signaling in the GFAP-HRasV12 mouse model are not supra-physiological. To assess the contribution of a functional p53 pathway to the suppression of HRasV12-induced gliomagenesis, hemizygous GFAP-HRasV12 mice were crossed into the p53K1/K1 [knock-in (KI)] background in which the endogenous p53 gene has been replaced by one encoding the p53ERAM [estrogen receptor (ER)] fusion protein. p33ERAM is functional only in the presence of the synthetic steroid ligand 4-hydroxysterol (4-OHT). In the absence of 4-OHT, p33ERAM mice are functionally p53null (33) but are rapidly, systemically, and reversibly shifted to p53wt upon systemic administration of tamoxifen (Tam), which is metabolized in vivo to 4-OHT. GFAP-HRasV12:p53K1/K1, GFAP-HRasV12:p53+/KI, and GFAP-HRasV12:p53+/+ mice were monitored daily from birth for neurological deficits indicative of astrocytoma development, including abnormal movement and tone, hunching, and hydrocephalus. Affected animals were sacrificed, brain tissue was harvested, and the presence of astrocytoma was confirmed by HE staining and immunohistochemistry using the glial marker GFAP together with Ki67 as a marker of proliferation.

The mean latency of tumor formation in GFAP-HRasV12:p53+/+ animals was 17 wk, falling to 16 wk in GFAP-HRasV12:p53+/KI heterozygous mice and to 9 wk in GFAP-HRasV12:p53K1/K1 animals (Fig. 1A). Despite these significant differences in latency, however, tumors arising from each of the different p53 backgrounds exhibited very similar pathological features, all closely resembling high-grade gliomas in human patients (Fig. 1B). The high-grade gliomas arising in GFAP-HRasV12:p53+/+ and GFAP-HRasV12:p53K1/K1 mice exhibited increased cell density, nuclear polymorphism, infiltrating edges, regions of tissue necrosis, and a high Ki67-labeling index (Fig. 1B). Although the overall frequency of tumors among the differing p53 backgrounds was similar, p53-deficient animals exhibited accelerated formation of high-grade gliomas relative to p53 wild-type and p53 hemizygous backgrounds (Fig. 1C). Hence, a functional p53 pathway retards the evolution of HRasV12-driven gliomagenesis.

**Results**

**p53 Deficiency Accelerates Initiation of Harvey RasV12-Driven Gliomagenesis.** We modeled gliomagenesis in vivo using GFAP-Harvey Ras (HRas)V12 animals, 50% of which develop tumors that are histopathologically similar to human astrocytomas by age 12 wk, with a lifetime incidence of 95% (29). Although mutant V12-Ha-Ras is not prevalent in human GBMs, this well-established model exhibits MAPK pathway activation at a level comparable to human GBMs (29–32), suggesting that the levels of Ras pathway signaling in the GFAP-HRasV12 mouse model are not supra-physiological. To assess the contribution of a functional p53 pathway to the suppression of HRasV12-induced gliomagenesis, hemizygous GFAP-HRasV12 mice were crossed into the p53K1/K1 [knock-in (KI)] background in which the endogenous p53 gene has been replaced by one encoding the p53ERAM [estrogen receptor (ER)] fusion protein. p33ERAM is functional only in the presence of the synthetic steroid ligand 4-hydroxysterol (4-OHT). In the absence of 4-OHT, p33ERAM mice are functionally p53null (33) but are rapidly, systemically, and reversibly shifted to p53wt upon systemic administration of tamoxifen (Tam), which is metabolized in vivo to 4-OHT. GFAP-HRasV12:p53K1/K1, GFAP-HRasV12:p53+/KI, and GFAP-HRasV12:p53+/+ mice were monitored daily from birth for neurological deficits indicative of astrocytoma development, including abnormal movement and tone, hunching, and hydrocephalus. Affected animals were sacrificed, brain tissue was harvested, and the presence of astrocytoma was confirmed by HE staining and immunohistochemistry using the glial marker GFAP together with Ki67 as a marker of proliferation.

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**Fig. 1.** Loss of p53 accelerates HRasV12-induced gliomagenesis. (A) Survival plot (in weeks after birth) of GFAP-HRasV12 (G-Ras) animals from various p53 backgrounds. The size of cohorts is indicated. Statistical analysis was performed using a Mantel–Cox test, *p < 0.01 for GFAP-HRasV12:p53+/+ vs. GFAP-HRasV12:p53K1/K1; p53+/+ vs. GFAP-HRasV12:p53+/KI; GFAP-HRasV12:p53+/+ vs. GFAP-HRasV12:p53K1/K1; and GFAP-HRasV12:p53+/+ vs. GFAP-HRasV12:p53K1/K1. (B) Examples of H&E images of high-grade tumors arising in GFAP-HRasV12:p53+/+, GFAP-HRasV12:p53+/KI, and GFAP-HRasV12:p53K1/K1 genetic backgrounds. Tumors exhibit increased cell density (I, II), nuclear polymorphism (III, IV), infiltrative edges (V, VI), areas of tissue necrosis (VII, VIII), and high Ki67-labeling index (IX, X). (C) A schematic representation of the onset and classification of tumors that developed in GFAP-HRasV12 animals of different p53 genotypes.
HRas\(^{V12}\)-Induced Gliomas Arising in p53-Competent Mice Retain Functional p53 but Inactivate the p53 Pathway Upstream. GFAP-\(HRas^{V12/p53^{+/Ki}}\) heterozygous mice harbor one wild-type and one 4-OHT–dependent copy of p53. Hence, in the absence of 4-OHT, such mice have only a single copy of p53. In other tumor models loss of the remaining functional p53 allele in both p53\(^{+/–}\) and p53\(^{+/Ki}\) animals is by far the most common mechanism of p53 pathway inactivation (34–37). For example, \(Eμ\)-myc–driven lymphomas arising in p53\(^{+/Ki}\) mice invariably inactivate the wild-type copy of p53, and subsequent restoration of the second, 4-OHT–dependent p53ERTAM allele triggers dramatic p53-dependent apoptosis and tumor regression and significantly extends overall survival (37).

We reasoned that if the single wild-type p53 allele is inactivated during GFAP-\(HRas^{V12/p53^{+/Ki}}\) tumor progression, then restoration of the remaining conditional p53ERTAM allele to wild-type function should impair tumor maintenance and subsequent progression. To address this notion, TAM, which is metabolized to function should impact tumor maintenance and subsequent progression. To address this notion, TAM, which is metabolized to function should impact tumor maintenance and subsequent progression.

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GFAP-HRas\(^{V12/p53^{+/Ki}}\) derived from 3 (Fig. 2A) is representative of p53ERTAM once it has been functionally restored by TAM. Functional p53 but Inactivate the p53 Pathway Upstream. Functional p53 but Inactivate the p53 Pathway Upstream. Functional p53 but Inactivate the p53 Pathway Upstream.

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other signal, for example, DNA damage (Fig. 2B and C) despite expression of the p53ERTAM fusion protein in tumor tissue (Fig. S1A). Consistent with this finding, the restoration of p53 activity in explanted tumors in vitro by 4-OHT did not affect the proliferation nor viability of tumor cells (Fig. 2F) or the expression of bona fide p53 target genes (Fig. S1B).

\[C\]

Although one possible explanation for the lack of impact of TAM in GFAP-\(HRas^{V12/p53^{+/Ki}}\) tumors is that the 4-OHT–dependent p53ERTAM allele had been inactivated in some way, it also is possible that the glioma lack a requisite signal to activate p53ERTAM once it has been functionally restored by TAM. In the latter scenario, functionally restored p53ERTAM still should cause growth arrest and/or apoptosis in response to some other signal, for example, DNA damage (Fig. 2D). To determine the status of both p53 and p53ERTAM in tumors arising in GFAP-\(HRas^{V12/p53^{+/Ki}}\) mice, tumor-bearing animals were exposed to 7 Gy of \(γ\)-radiation to activate p53 directly. As a comparison, we also irradiated tumor-bearing p53\(^{Ki/Ki}\) mice, which are totally deficient for p53 activity in the absence of TAM. Tumors from all irradiated animals then were analyzed for DNA damage-induced apoptosis by immunohistochemical staining for activated caspase 3 (Fig. 2E). Radiation-induced apoptosis was absent from tumors derived from GFAP-\(HRas^{V12/p53^{+/Ki}}\) mice treated with vehicle control but was evident once p53ERTAM had been functionally restored by administration of TAM, thus confirming that such apoptosis is p53 dependent (Fig. S1C). Radiation-induced apoptosis (3.75% of total tumor cells) was evident in the gliomas arising in GFAP-\(HRas^{V12/p53^{+/Ki}}\) mice irrespective of whether TAM was administered, indicating that the wild-type p53 allele was still functional (Fig. 2E). Similarly, radiation-induced apoptosis and the induction of p53 target genes was apparent in cultured glioma cells derived from p53\(^{+/Ki}\) mice irrespective of 4-OHT (Fig. 2G) but not in the wild-type p53-deficient tumor cells from p53\(^{Ki/Ki}\) mice. In the latter case, both apoptosis and post-irradiation induction of the p53 target genes \(puma\) and cyclin-dependent kinase inhibitor 1a \(CDKN1A\) were evident only when 4-OHT was added to the medium (Fig. S1 D and E). Thus, the p53-dependent, DNA damage-induced apoptotic pathway remains intact in GFAP-\(HRas^{V12/p53^{+/Ki}}\) tumors. Furthermore, DNA sequence analysis confirmed that the wild-type p53 allele in such tumors harbored no detectable mutations. Hence, gliomas arising in GFAP-\(HRas^{V12/p53^{+/Ki}}\) retain their functional wild-type p53 allele.

Because p53 in GFAP-\(HRas^{V12/p53^{+/Ki}}\) tumors remains functional and is responsive to DNA damage, the likely explanation for its inactivity is the absence of an upstream signal to activate p53 in response to oncogenic signaling. The principal mediator of such oncogenic activation of p53 is the tumor suppressor p19\(^{ARF}\) (p14\(^{ARF}\) in humans), which is specifically induced by aberrantly elevated flux through oncogenes such as Myc and Ras (38, 39) and acts to antagonize the p53-suppressive action of Mdm2 (40, 41). This pathway may be incapacitated either through loss of p19\(^{ARF}\) itself or by overexpression of Mdm2 (Fig. 2D). To determine whether the p19\(^{ARF}\)/Mdm2 regulatory pathway is functionally compromised in GFAP-\(HRas^{V12/p53^{+/Ki}}\) gliomas, we used Nutlin 3, a pharmacological inhibitor of Mdm2, to probe its functionality. Nutlin 3 induced significant apoptosis in disaggregated tumor cells from two independent GFAP-\(HRas^{V12/p53^{+/Ki}}\) tumors irrespective of the presence of 4-OHT (Fig. 2H). This effect was completely p53 dependent (Fig. S1F). Likewise, systemic administration of Nutlin 3 in vivo triggered significant apoptosis (13.1% of tumor cells) in tumors arising in GFAP-\(HRas^{V12/p53^{+/Ki}}\) mice (Fig. 2J), although not in wild-type p53-deficient GFAP-\(HRas^{V12/p53^{−/−}}\) mice, without affecting the viability of normal astrocytes (Fig. 2J). Moreover, the level of Mdm2 protein (a target of Nutlin 3) was significantly higher in the disaggregated tumor cells from two independent GFAP-\(HRas^{V12/p53^{+/Ki}}\) tumors compared with the astrocytes isolated from their GFAP-\(HRas^{V12}\) transgene-negative littermates (Fig. 2F).

These observations indicate that the block in p53 activation in GFAP-\(HRas^{V12/p53^{+/Ki}}\) tumors lies upstream of p53 and most probably within the Ras oncogene-sensing p19\(^{ARF}/\)Mdm2 pathway.

HRas\(^{V12}\)-Induced Gliomas Arising in the Absence of Functional p53 Retain Persistent p53-Activating Signals. The studies described above all modeled the evolution of gliomas in which sporadic Ras pathway activation precedes p53 pathway inactivation, and they show that, when functional p53 itself is present, Ras activation drives selection that retains functional p53 in favor of other p53 pathway-inactivating mutations. To model the alternative evolutionary path, in which sporadic p53 loss precedes or coincides Ras activation, Ras-driven gliomas were allowed to form in GFAP-\(HRas^{V12/p53^{+/Ki}}\) transgenic animals. From their GFAP-\(HRas^{V12}\) transgene-negative littermates, we used Nutlin 3, a pharmacological inhibitor of Mdm2, to probe its functionality and assessed any effects of such restoration. Indeed, restoration of p53 triggered a dramatic drop in tumor cell proliferation—the proportion of actively proliferating BrdU-positive tumor cells fell from 13.3% before p53 restoration to 1.1% after p53 restoration (Fig. 3A)—and also induced widespread apoptosis in tumors (but not in normal tissue) (Fig. 3B), occasionally resulting in macroscopic destruction of the tumor mass (Fig. S2). This single transient restoration of p53, accompanied by marked induction of p53 target genes (Fig. 3C), significantly extended the survival of tumor-bearing GFAP-\(HRas^{V12/p53^{+/Ki}}\) mice (17 d vs. 1.8 d in the non–TAM-treated controls) (Fig. 3D). TAM treatment of mice (p53ER-Deleted) also rapidly led to a reduction in neurological deficits in animals and increased general health (Movies S1 and S2).

Because p19\(^{ARF}\) is a crucial upstream regulator of p53 activity, we assayed p19\(^{ARF}\) expression in the tumors before (p53ER OFF) and 24 h after p53 restoration by addition of TAM (p53ER-Deleted). The percentage of the p19\(^{ARF}\)-positive cells in tumors fell from 32.2 to 5.67% following restoration of the p53ER allele (Fig. 3E). We reasoned that GFAP-\(HRas^{V12/p53^{+/Ki}}\) arising in the absence of functional p53 harbor persistent p53-activating signals, such as elevated levels of p19\(^{ARF}\), which antagonize Mdm2. Upon restoration of functional p53, these p53-activating signals efficiently engage p53-mediated tumor-
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ties, i.e., induction of apoptosis and/or growth arrest. Loss of the

p53-activating signal (e.g., DNA damage) or by pharmacological inhibition of

settings, p53 activity might be restored by the induction of an alternative

GFAP-HRasV12;p53+/KI irradiated with 7 Gy(Gy). Arrows indicate apoptotic cells. (Scale bars: 20 μm.)

Gliomas arising in p53-competent mice lose upstream p53-activating pathways. (A) Life span (in days after treatment) of tumor-bearing GFAP-HRasV12;p53+/KI animals following treatment for 24 h with either vehicle (p53ER OFF; control) or TAM (p53ER Restored). Cohorts of seven animals per group were analyzed. Statistical analyses were performed using a two-tailed Student t test; ns, no statistical significance. (B) Immunohistochemical analysis of apoptosis in tumors derived from GFAP-HRasV12;p53+/KI mice after treatment for 24 h with either vehicle (p53ER OFF; control) or TAM (p53ER Restored). Tumor area was determined by increased GFAP staining and cell density and is outlined by the dashed line. Cell death was assayed by staining for activated caspase 3. The percentage of caspase 3-positive cells out of all tumor cells is indicated. The arrow indicates the appearance of an apoptotic cell in the tumor mass. (Scale bars: 50 μm.) (C) (Left) Immunohistochemical analysis of cell proliferation status by BrdU incorporation and Ki67 staining in the tumors described in B. The percentage of BrdU/Ki67-positive cells out of all tumor cells is indicated. Arrows indicate double-positive cells. (Scale bars: 20 μm.) (Right) A schematic representation of the regimen for p53ER+/−ARF−/− allele restoration is shown. A single dose of TAM was administered i.p. to symptomatic animals (p53ER Restored), and a single dose of vehicle was administered to control (Ctrl) animals (p53ER OFF). BrdU was administered 22 h later, and tissues were harvested 2 h after BrdU administration. (D) Schematic representation of the p53 tumor-suppressor pathway. Activated oncogene(s) (and other potential signals) induce expression of the p53 target genes p19ARF and p21WAF1, which stabilize and activate p53 by blocking the p53 inhibitor Mdm2. Activated p53 then executes its principal tumor-suppressive activities, i.e., induction of apoptosis and/or growth arrest. Loss of the ARF focus or up-regulation of Mdm2 inactivates the functional p53 pathway. In such settings, p53 activity might be restored by the induction of an alternative p53-activating signal (e.g., DNA damage) or by pharmacological inhibition of Mdm2 (e.g., by Nutlin 3). (E) Immunohistochemical analysis of cell death assayed by staining for activated caspase 3 in GFAP-HRasV12;p53+/KI and GFAP-HRasV12;p53+/KI tumors in nonirradiated animals (Ctrl) and in animals irradiated with 7 Gy(Gy). Arrows indicate apoptotic cells. (Scale bars: 20 μm.) (F) Cell death depicted as the percentage of total tumor cells in vitro tumor cell cultures derived from GFAP-HRasV12;p53+/KI and GFAP-HRasV12;p53+/KI mice after treatment with vehicle (Ctrl), 4-OHT to restore p53 function (4-OHT), and irradiation (7 Gy) in combination with either vehicle treatment (Gy/Ctrl) or p53 restoration (Gy/4-OHT). The data represent experiments on three independently derived tumors analyzed in triplicate. Cell viability was determined by trypan blue exclusion. ***P ≤ 0.001; **P ≤ 0.01; ns, no statistical significance (statistical analyses were performed using a two-tailed Student t test). (G) Quantitative RT-PCR (qRT-PCR) analysis of mRNA expression of the p53 target genes puma and p21WAF1 (CDKN1A) in cells cultured in vitro from tumors of GFAP-HRasV12;p53+/KI and p53+/+ mice and after exposure to 7-Gy γ-radiation in combination with 24-h exposure to 4-OHT (Gy/4-OHT) versus vehicle (Gy/Ctrl). The data are presented as fold induction relative to nonirradiated samples and represent experiments from three independently derived tumors, each assayed in triplicate. ns, no statistical significance (two-tailed Student t test). (H) Percent of tumor cells undergoing apoptosis (as determined by the trypan blue exclusion method) in vitro after treatment of either vehicle-treated (dark gray bars) or 4-OHT-treated (light gray bars) tumor cell cultures derived from GFAP-HRasV12;p53+/KI animals with the Mdm2 inhibitor Nutlin 3 at a concentration of 16 (16 μM Ntl) or 32 (32 μM Ntl), or with the vehicle for Nutlin treatment (DMSO). The graph represents experimental data from two tumor cell cultures independently originated from two different tumors derived from different animals [tumor-145 (T-145) and tumor-890 (T-890)], each analyzed in triplicate. ***P ≤ 0.0001; **P ≤ 0.001. (I) Immunoblotting analysis of Mdm2 protein expression in tumor-derived cell cultures from GFAP-HRasV12;p53+/+ and GFAP-HRasV12;p53−/− tumors. Two independent, derived primary tumor cultures from the same p53 genotype are presented. Astrocytes isolated from the GFAP-HRasV12−/−-negative littermates (p53−/+ and p53−/−, respectively) are used as controls. β-Actin was used as an equal loading control.

Shchors et al.
upstream p19ARF/MDM2 regulators of p53 function and not against the p53 gene itself.

Optimizing of p53 Restoration Therapy. Our data indicate that restoring p53 function can exert a profound initial therapeutic impact in gliomas that evolve in the absence of functional p53. However, that therapeutic impact is eroded rapidly by the emergence of secondarily p53-resistant tumor clades that outgrow in the face of the selective pressure imposed by p53 restoration. It is known that both the rate at which adapted species emerge and the evolutionary mechanism by which they do so can be influenced profoundly by whether selection is sustained or episodic (43). Given that sustained p53 restoration in GFAP-HRasV12;p53KI/KI gliomas drives such rapid emergence of lethal secondary p53 pathway mutants, we asked how altering the timing and duration of p53 restoration influences the emergence of resistance. To start, we transiently restored p53 function once in 21-d-old GFAP-HRasV12;p53KI/KI mice by administering a single dose of TAM. Surprisingly, even this single short period of TAM 4 h later to restore p53 transiently. Both naive tumors and tumors arising in animals after a single transient restoration of p53 at the age of 21 d (1x TAM). The tumor-bearing animals were injected with BrdU to label actively proliferating cells and were given a control vehicle or a single dose of TAM 4 h later to restore p53 transiently. Both naive tumors and tumors that reemerged after a single exposure to TAM at the age of 21 d contained p19ARF−/−;BrdU double-positive cells (36.4% and 30.2%, respectively) before transient restoration of p53 function (Fig. 5C). The percentage of p19ARF−/−;BrdU double-positive cells dropped significantly (8.6% and 6.98%, respectively) 20 h after restoration of p53 function (p53ER-Restored), suggesting that most of the actively proliferating p19ARF−/−;BrdU double-positive cells in both naive tumors and tumors arising in animals after transient p53 restoration (1x TAM) presumably are eliminated by apoptosis when p53 is restored for 24 h (p53ER Restored). Moreover, any remaining p19ARF−/−;BrdU double-positive cells still present in both the naive and 1x TAM tumors were all Ki67 negative (Fig. 5D), indicating their growth arrest.

Having established that tumors recurring after a single, transient restoration of p53 remain largely responsive to a second round of p53 restoration, we next asked whether repeated, in
termittent transient p53 restoration in GFP-\(\text{HRas}^{12/12}\;\text{p53}^{\text{KIKI}}\) mice might confer a therapeutic advantage over sustained p53 restoration. A cohort of 21-d-old GFP-\(\text{HRas}^{12/12}\;\text{p53}^{\text{KIKI}}\) animals was subjected to transient p53 restoration (a single TAM injection) once a week for 10 wk. Remarkably, more than 80% of such intermittently treated mice remained symptom-free, surviving beyond 100 d (Fig. 5E).

The significant survival benefit afforded by intermittent transient p53 restoration over sustained p53 restoration suggests that, as with tumors remerging after single transient p53 restoration at age 21 d (1× TAM), the tumors retain p53 sensitivity throughout subsequent rounds of p53 restoration. To confirm this possibility, we assayed induction of apoptosis after repeated rounds of p53 restoration. Seven-week-old asymptomatic animals previously subjected to transient p53 restoration were treated with the Mdm2 inhibitor Nutlin 3 or with vehicle (Control) as described in Materials and Methods. The tumor area and the percentage of caspase 3-positive cells in tumors are indicated. (Scale bars: 50 μm.) (C) Immunohistochemical analysis of p19ARF expression in previously untreated tumors (p53ER-Off) and in tumors that developed under sustained p53ER\(\text{Restored-Sustained}\) restoration (p53ER-Restored-Sustained). Analysis of p19ARF expression in tumors following 24-hour p53ER\(\text{Restored-24 hrs}\) restoration (p53ER-Restored-24 hrs) is provided as a control. (Scale bars: 50 μm.) (D) A graphical representation of the quantification analysis of p19ARF-positive cells exemplified in Fig. 4C is presented as the percentage of total tumor cells. At least four animals were analyzed for each treatment. Immunohistochemical analysis was performed in duplicate; 10 randomized fields per staining were considered. **P ≤ 0.001; ns, no statistical significance. Statistical analyses were performed using a two-tailed Student t test.

To model the therapeutic impact of p53 restoration in human gliomas of each class, we exposed human GBM cell lines (44) to 2, 2- bis(hydroxymethyl)-3- quinuclidinone (Prima-1), a small-molecular-weight compound that restores the defective conformation of mutant p53, rescuing competence for both DNA binding and activation of p53 target genes (45). Human GBM cell lines carrying either wild-type p53 (p53WT) or mutated copies of p53 (p53MT) in combination with either competent CDKN2A or CDKN2A- and CDKN2A-deleted GBM cell lines were exposed to Prima-1 as compared with control vehicle for a period of 1, 3, or 5 d, and effects on cell proliferation and p53 target gene induction were assayed. At the concentration used (20 μM), Prima-1 elicited only minor p53-independent effects (proliferation of p53WT tumors fell by 14% upon Prima-1 exposure) (Fig. 6B). In contrast, Prima-1 exposure induced significant growth inhibition (98% reduction in cell proliferation) in glioma cells harboring mutant p53, but only if they also retained CDKN2A (Fig. 6B). The antiproliferative effect of Prima-1 coincided with the induction of p53 target genes (CDKN1A (p21*) or p21**), mdm2, and growth arrest and DNA damage gene 45a (gadd45a) (Fig. 6C). This increased sensitivity of CDKN2A (ARF)-competent GBM cells to Prima-1 as compared with CDKN2A-deficient GBM cells was confirmed by analysis of an independent set of human primary GBM cultures (Fig. 7C).

Our data suggest that in human GBMs, as in gliomas arising in GFP-\(\text{HRas}^{12/12}\;\text{p53}^{\text{KIKI}}\) mice, the presence of functional p53-activating signals is crucial to reduce the p53 tumor-suppressor activities upon p53 restoration.

We next sought to determine whether in human GBM cell lines intermittent reactivation of p53 circumvents acquired resistance to p53-restoration therapy, similar to effect of intermittent p53 reactivation in the conditional mouse model. We propagated human GBM cell lines harboring mutant p53 but wild-type CDKN2A (ARF) in the presence of sustained or in-
terrestrial exposure to Prima-1 at two different concentrations (10 and 20 μM) for a duration of 7 wk. At the end of the treatment, the cells were allowed to expand in Prima-1-free medium for an additional week. The resulting tumors were injected with vehicle (p53ER-OFF) or with TAM (p53ER-Restored). The samples were collected 24 h later and were analyzed. The percentage of p19ARF-positive cells within the populations of BrdU- and Ki67-positive cells is indicated. The dotted lines demarcate the areas enlarged in the insets showing double-positive cells. (Scale bars: 20 μm.) (Right) A schematic representation of the treatment. (E) Comparative survival curves (in days after birth) of control GFAP-HRasV12−/−; p53K/O (CTRL, black lines and squares) versus a cohort of mice subjected to repeated p53 restoration once a week (Intermittent TAM, purple lines and circles). The arrowhead indicates the initiation of TAM treatment. *P < 0.01. The dotted line indicates the duration of treatment. One animal succumbed to thymic lymphoma during the experiment and was removed from the study. Statistical analysis was performed using the Mantel–Cox test.

Discussion

Emerging evidence suggests that primary and secondary GBMs exhibit different patterns of genetic alterations, reflecting their distinct etiologies and potentially influencing their responsiveness to certain therapies, in particular therapies targeting specific molecular pathways (22). Alterations that diminish or abrogate the functions of the p53 tumor-suppressor pathway are seen in both primary and secondary GBM. However, p53 pathway dysfunction arises by different mechanisms in each of the two GBM subtypes. Loss of p53 itself through inactivating mutations is an early event in the multistep development of about two thirds of secondary GBM (46). In contrast, in primary GBM the p53 tumor-suppressor pathway is incapacitated most frequently by the deletion of the Ink4/ARF locus or by overexpression of MDM2, rather than by loss or mutational inactivation of p53 itself (15, 16, 47).

Using mice in which the GFAP-HRasV12 transgene is combined with either hemi- or homozygosity for a conditional allele of p53 (p53K/O), we modeled the evolution of these two distinct subtypes of gliomas and ascertained the therapeutic potential of p53-based therapy in each. Although human GBMs rarely display HRasV12 mutations, the elevated activity of constitutively expressed MAPK pathway activation comparable to those observed in human GBMs with either EGFR or PDGF receptor A/B (PDGFR-A/B) amplification and/or activating mutations, which are common driver oncogenic mutations in human GBM (31, 48). GFAP-HRasV12−/−; p53K/O mice, in which p53 is inactive throughout Ras-induced gliomagenesis, mimic the evolution of human gliomas wherein p53 itself is inactivated at the outset of tumor progression. We observed that the absence of functional p53 significantly accelerated Ras-induced gliomagenesis, consistent with the recently demonstrated role played by p53 inactivation in the progression of astrocytomas in humans (49). Functional restoration of p53 in such tumors triggered immediate p53 activation, arrest of tumor cell growth, and apoptosis, indicating that both upstream p53-activating signals and downstream tumor-suppressor effectors for apoptosis and growth arrest remained intact when p53 function was missing throughout tumorigenesis. By analogy, we predict that p53-restoration therapies are likely to be efficacious in human GBM in which p53 is inactivated early in tumor evolution.

In contrast, malignant astrocytomas developing in GFAP-HRasV12−/−; p53K/O mice (in which one p53 allele is wild type)
model the evolution of human tumors, such as primary GBMs, in which Ras activation precedes the loss of a functional p53 pathway (13). In such tumors restoration of p53 function is therapeutically irrelevant, because selection against the p53 pathway preferentially elicits either loss of p19ARF or up-regulation of Mdm2, rather than direct inactivation of p53 protein itself as observed in the (cis)p53+/−;NF1(+/−) fl/fl;razor;GFP-cre−positive model (50). These results highlight the mutual complementarity of the two models. Indeed, human glioblastomas deficient for NF1 activity exhibit relatively low levels of total and phospho-proteins in the PI3K and MAPK pathways, indicating oncogene-signaling activity, as compared with GBMs carrying elevated expression of and/or mutations in EGFR or PDGFR A/B (48). Although restoration of functional p53 is inconsequential for p53-competent tumors, activation of p53 by Nutlin 3, an Mdm2 inhibitor, has been proven to induce cell death in these tumors (Fig. 2f). For gliomas with wild-type p53 status, either Nutlin 3 itself or other inhibitors of Mdm2 activity potentially can be translated into clinical settings. Unfortunately, it is impossible to predict whether in these tumors an intermittent regimen of Mdm2 inhibition in gliomas will be as efficacious as the intermittent restoration of p53 in the p53-deficient lesion. The resistance of tumor cells to treatments based on Mdm2 inhibition might be achieved by mechanisms other than resistance to p53 restoration (e.g., Mdm2 amplification).

We evaluated the potential impact of p53 restoration in human GBM cell lines that carry p53 gene mutations by using a small-molecular-weight molecule, Prima-1, that selectively restores sequence-specific DNA-binding activity to mutant forms of p53 protein. As in the GFAF-Hras12V−/−-driven gliomas, the restoration of p53 activity inhibited human GBM cell proliferation. However, as in the GFAF-Hras12V−/−-driven gliomas, the p53 tumor-suppressor activity and the induction of p53 transcriptional targets following exposure to Prima-1 were detected only in tumors in which the p53-activating branch (p14ARF/MDM2) remained intact (Fig. 6B and C and Fig. S7). Moreover, in human GBM cells the selective pressure against the p53 pathway imposed by sustained exposure to Prima-1 resulted in the loss of p14ARF expression and subsequent resistance to the therapeutic intervention (Fig. 6D–F). Around 11% of all human GBMs contain mutations in both the p53 gene and its activating tumor-suppressing branch, p14ARF/MDM2, as was established recently by the molecular profile of human GBMs (http://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp and ref. 2). Our data suggest that restoration of p53 activity by Prima-1 alone (or by other small molecules modulating the DNA-binding activity of mutant p53 (51)) is not sufficient to induce p53 tumor-suppressor activity in these doubly mutant patients, and alternative means of p53 activation (e.g., DNA damage) should be considered for therapeutic intervention based on p53 restoration.

Given the prospect of using such p53-restoration therapies in GBM patients, we went on to use the GFAF-Hras12V−/−;p53−/− model to assess potential regimens for p53 restoration in the p53-mutant fraction of gliomas. In GFAF-Hras12V−/−;p53−/− tu-
Tumors, sustained restoration of p53 function rapidly selects for the emergence of tumor cell populations resistant to p53 function in the context of abrogating p53-activating signals, i.e., loss of p19ARF or amplification of MDM2. Further analyses established that there is a significant difference between tumors subjected to sustained p53 restoration and the tumors that developed in the p53-competent fraction of gliomas. In GFAF-Hras12V−/−;p53+/+ tu-
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tumors from which it regenerates are, once again, susceptible to p53-induced apoptosis. Possibly such resistant glioma cells represent an innately apoptosis-resistant tumor stem cell population or, alternatively, cancer cells transiently residing in a protected somatic niche. The trivial possibility that some tumor cells survive p53 restoration through lack of exposure to 4-OHT seems unlikely, given the ubiquitous restoration of p53 that we have observed following systemic administration of 4-OHT to p53−/− mice, the facile capacity of 4-OHT to cross the blood–brain barrier, and the persistence of 4-OHT in plasma for up to 24 h following the administration of a single bolus (52, 53). In addition to these p53-sensitive populations, there evidently is a third population of tumor cells that harbors a preexisting secondary p53 pathway-inactivating mutation and that therefore is innately unresponsive to p53 restoration.

The eventual outgrowth of this third population of p53-resistant tumor cells and the p53-resistant tumor they regenerate limits how long survival may be extended by periodic p53 restoration. Hence, factors that govern the rate of outgrowth of this population will be critical in determining the overall therapeutic efficacy of p53 restoration. Although sustained p53 restoration affords a selective advantage only to innately p53-resistant tumor cells, transient p53 restoration (once relaxed) permits the outgrowth of both innately p53 resistant tumor cells and those tumor cells that are only adventitiously refractory to p53-induced apoptosis. Perhaps competition between these two populations or the differential activity of Ras signaling in tumor cells governs the outgrowth of the resistant tumor cells (Fig. S8). We suspect that these mechanisms underlie the greater therapeutic efficacy of intermittent versus sustained p53 restoration. However, even though an intermittent regimen of a p53-reactivating treatment significantly lengthened the lifespan of tumor-bearing animals, we nonetheless detected tumors in all the intermittently treated animals surviving beyond 100 d, and all animals from the independent cohort left for surveillance following cessation of intermittent treatment eventually succumbed to disease. Hence, intermittent p53 restoration typically delays, rather than stops, disease progression. As is consistent with this effect, the human GBM cultures maintained under intermittent exposure to Prima-1 continue to proliferate once the drug is removed. These data suggest that the application of therapy based on p53 restoration in combination with other antiglioma strategies, for example conventional GBM therapies such as temozolomide, will be more beneficial for patients than temozolomide alone. In particular, patients with secondary GBMs exhibiting a high frequency of p53 mutations and displaying MGMT promoter silencing (46), which is crucial for the tumor sensitivity to temozolomide (18), could be regarded as ideal candidates. Nevertheless, our data predict a significant therapeutic advantage for intermittent versus sustained regimens of p53-restoration therapy, making intermittent therapy worthy of serious consideration in future clinical trial designs involving p53-reactivating agents.

Immunoblotting. Primary mouse cell culture was performed as described in SI Materials and Methods. Primary mouse tumors cultured cells and mouse astrocytes were frozen as a cell pellet at −80 °C, lysed in buffer (50 mM Tris, 150 mM NaCl, 20 mM EDTA, 0.5% Nonidet P-40) supplemented with protease and phosphatase inhibitors, and centrifuged at 20,000 × g for 15 min at 4 °C. Protein concentration was determined with the Bio-Rad protein assay. Protein lysates were run in 4–20% gradient gels (Invitrogen) and blotted onto PVDF membranes (Immobilon-P). Membranes were probed with anti-Mdm2 (SMP14; BD Pharmingen), p14ARF (AC64; Cell Signaling), and anti-β-actin (AC-15; Sigma).

Tagman Analysis and p53 Sequencing Analysis on the Mouse Tumors. Total RNA was isolated using TRIzol reagent (15956-018; Invitrogen) according to the manufacturer’s protocol and DNase treated (18068-015; Invitrogen) before reverse transcription (iScript; Bio-Rad). Tagman analysis was performed by the University of California, San Francisco Comprehensive Cancer Center Genome Analysis core facility. All data were normalized to β-glucuronidase (gus) expression. The following primers were used: for mouse p53 forward: 5′-GCA GAG GCA GTC AGT CTG AGT-3′ and reverse: 5′-GCA GCC AGC GTC AGT CAG AGT-3′ (Applied Biosystems); for mouse mdm2, Mm00487656_m1 (Applied Biosystems); for mouse PUMA, Mm00519268_m1 (Applied Biosystems); and for mouse p53, Mm00487656_m1 (Applied Biosystems). For p53 sequence analysis, cDNA was amplified with primers p53 forward: 5′-CCA TGG AGG AGT CAC AGT CG-3′ and p53 reverse: 5′-GCA GAG GCA GTC AGT CAC-3′ as described (37).

Human Glioma Cell Lines. Human glioma cell lines LN18 and LN215 were kindly provided by M. Hegi (University Hospital of Lausanne, Lausanne, Switzerland). U87MG was obtained from American Type Culture Collection. The p53 and p14ARF status in these cell lines was described previously (44). U87MG has wild-type p53, LN215 has a p53 deletion 191–192, and LN18 has a p53 mutation at C2385. U87MG is p14ARF null, LN215 is p14ARF wild type, and LN18 is p14ARF null. All cells were cultured in DMEM with 10% (vol/vol) FCS. For analysis of tumor cell proliferation, glioma cell lines were plated at 3,000 cells per well in 96-well BD Falcon white/clear plates (353377; BD Bioscience) and for mouse mdm2, Mm00487656_m1 (Applied Biosystems). Human spheroids were grown in suspension in serum-free medium (Chemical). Cell viability was analyzed by the Cell Titer GloR luminescent assay (G7570; Promega). Experiments were done in triplicate.

To generate sustained and intermittent Prima-1–treated cell populations of LN215 cell cultures, LN215 cells were cultured for 7 wk in the presence of 15 μM Prima-1. For weekly treatment, Prima-1 was added to the cells for 24 h once a week. Twenty-four hours later the cells were washed once in 1x PBS, and fresh drug-free medium was added. For daily treatment, the cells were exposed to Prima-1 continuously, and the medium containing
expression of p19

GraphPad Prism5. The statistical analysis of the survival curves was done

as described in

Kaplan–Meier survival curves were generated using

GraphPad Prism5. The statistical analysis of the survival curves was done

during the Mantel–Cox test. Tumor proliferation, apoptosis, and

expression of p19ARF were quantified by MetaMorph Imaging V7.01 and

ImageJ software (National Institutes of Health). The statistical analysis was

carried out using two-tailed Student t test. At least four animals were

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Supporting Information

Shchors et al. 10.1073/pnas.1219142110

SI Materials and Methods

Primary Mouse Cell Culture. Tumor tissues (identified based on high cell density, morphology, and abundance of blood vessels) were isolated using a stereoscope dissection microscope in ice cold 1× HBSS. Tissues were washed and treated with papain (Worthington Chemicals) for 30 min at 37 °C. Cells then were stained through a 70-μm grid and were seeded onto laminin-coated six-well plates for expansion. Primary coronal astrocytes were purified from control p6-p10 GFAP-HRasV12 transgene-negative mice by dissociation with papain, as described above, and were cultured in Neurobasal medium supplemented with 2 mM t-glutamine, B27 supplement, 20 ng/mL FGF-2, and 20 ng/mL EGF. p53ER-TAM was functionally restored in vitro by the addition of 100 nM 4-OHT to the culture for the indicated period. Cell number and viability were assessed by the trypan blue exclusion method. For the irradiation experiment, cells were treated with 100 nM 4-OHT or vehicle (ethanol) for 2 h and were exposed to 7 Gy γ-radiation using a Mark 1 Cs137 Cesium source (0.637 Gy/min). For in vitro MDM2 inhibition studies, cells were treated with the indicated concentrations of Nutlin-3 (Cayman Chemical) or control vehicle (DMSO) for 2 h before restoration of p53 with 100 nM 4-OHT. The percentage of apoptotic cells was determined by the trypan blue exclusion method. All experiments were performed on three independently derived tumor cell cultures and were performed in triplicate.

Primary Human Glioblastoma Cultures. Serially xenografted glioblastoma (GBM) 28, 14, and 43 cultures, originally isolated from patients at Mayo Clinic, Minnesota, were kindly provided by David James (University of California, San Francisco). Protein 53 (p53) status had been characterized previously in these cultures (1). The p53 gene in GBM28 is mutated at M246T, GBM14 has wild-type p53, and GBM43 has a p53 mutation at F270C. p14ARF status also had been characterized previously in these cell lines: GBM28 is p14ARF wild type, whereas both GBM14 and GBM43 are p14ARF-null (2). Human GBM cells were dissociated using papain and were plated on ultra-low-adherent (Corning) or polynithine/laminin-coated plates and were cultured in Neurobasal medium (NBE) consisting of Neurobasal-A-glutamine, B27 supplement, and 2 mM t-glutamine, penicillin/streptavidin, 20 ng/mL FGF-2, and 20 ng/mL EGF (Sigma-Aldrich). Media were changed every 3 d, and cells were passaged using Accutase (Innovative Cell Technologies). Analysis of tumor cell proliferation was performed as follows: dissociated human GBM cells were plated at 3,000 cells per well in 96-well polynithine/laminin-coated plates in NBE medium and were cultured for 5 d with 0 or 5 μM Prima-1 (Cayman Chemical). Cell numbers were estimated using total DNA content as a guide by the Cyquant NF proliferation assay (Invitrogen) according to the manufacturer’s protocol. A standard curve was generated by plotting the number of plated cells (1,000–40,000) against corresponding fluorescent values, resulting in the equation y = x + 150, R² = 1. The number of cells in each sample was calculated using this equation. Experiments were done in triplicate.

To analyze mRNA expression of p53 target genes in human GBM cell lines, total RNA was isolated from the cell lines described in Materials and Methods and treated with 0 or 5 μM of Prima-1 for 24 h. To analyze mRNA expression of p53 target genes in the primary human glioblastoma cultures described above, the cultures were treated with 0 or 5 μM of Prima-1 for 5 d. The cDNA was synthesized with the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer’s protocol. The expression analysis was performed with the RotorGene SybrGreen PCR kit (Qiagen) on the RotorGeneQ and was analyzed with RotorGeneQ software (Qiagen). The following primers were used for the analysis: CDKN1A(p21) forward: 5′-TGT CCG TCA GAA CCC ATG C-3′, reverse: 5′-AAA GTC GAA GTT CCA TCG CTC-3′; mdm2 forward: 5′-TCG TCG GTG GAG GTT ACT G-3′, reverse: 5′-AAC CAC TTC TTG GAA CCA GGT-3′; gadd45a forward: 5′-GAG AGC AGA AGA CCG AAA GGA-3′, reverse: 5′-CAG TGA TCG TGC GCT GAC T-3′. All data were normalized to ribosomal protein L13a (RPL13A) and β-actin expression. RPL13A forward: 5′-GCC ATC GTG GCT AAA CAG GTA-3′, reverse: 5′-GTG GTT GTT CAT CCG CTG GC-3′; β-actin forward: 5′-CAT GTA CGT TGC TAT CCA GGC-3′, reverse: 5′-CTC CTT ATT GAC ACG CAC GAT-3′. All analyses were done in triplicate.

Tumor Cell Transplantation Experiment. Tumor cells were harvested from GFAP-Harvey Ras (HRas)V12;p53R172H [knock-in (KI)] animals and were cultured briefly in NBE medium [Neurobasal-A (Invitrogen), penicillin/streptavidin, 2 mM t-glutamine, B27 supplement (Invitrogen), 20 ng/mL FGF-2 (Peprotech), and 20 ng/mL EGF (Sigma-Aldrich)]. Tumor cells (8 × 10^4 in 10 μL NBE medium) then were engrafted intracranially into anesthetized transgene-negative p53R172H (p53R172H) littermates (1.5 mm lateral and 2 mm posterior to the bregma and 3 mm deep) using a Hamilton syringe and a stereotactic rig. Animals were monitored daily for symptoms of glioma. A single injection of either tamoxifen (TAM) or vehicle was administered i.p. to recipient animals 3 wk after transplantation. Each cohort consisted of seven animals, and the experiment was repeated twice with independently purified tumors.


Fig. S1. Gliomas arising in GFAP-HRasV12;p53+/-KI mice but not in GFAP-HRasV12;p53-/-KI mice lose upstream p53-activating pathways. (A) Immunohistochemical analysis of p53ER<sup>TAM</sup> (estrogen receptor (ER)) allele expression in the brain tissue from tumor-bearing GFAP-HRasV12;p53+/-KI animals. The normal brain tissue (N) and tumor area (T) were determined by the density of tissue cellularity. The dotted lines demarcate the areas enlarged at right showing comparable p53ER<sup>TAM</sup> allele expression in normal and tumor areas. Lateral ventricles (LV) are indicated. (Scale bars: 50 μm.) (B) Quantitative RT-PCR (qRT-PCR) analysis of mRNA expression of the p53 target genes cyclin-dependent kinase inhibitor 1a (CDKN1A) (p21<sup>cip1</sup>), puma, and mouse double minute (mdm2) in tumor-derived cell cultures from GFAP-HRasV12;p53+/-KI animals after 24-h exposure to 4-hydroxytamoxifen (4-OHT) in vitro. Data are presented as fold induction relative to control (vehicle-treated) samples. The data present experiments on three independently derived tumors, each analyzed in triplicate. (C) Immunohistochemical analysis of cell death in GFAP-HRasV12;p53-/-KI (Left) and GFAP-HRasV12;p53+/-KI (Right) tumors from animals treated with TAM for 2 h before exposure to 7-Gy whole-body γ-radiation. Cell death is indicated by staining for activated caspase 3. The tumor boundary is indicated in the left panel. (Scale bars: 50 μm.) (D) Percent of total tumor cell death in vitro of cultured glioma cells derived from GFAP-HRasV12;p53<sup>/-KI</sup> animals after treatment with vehicle (Ctrl), restoration of p53ER<sup>TAM</sup> allele for 24 h (4-OHT), and irradiation (7 Gy) in combination with vehicle treatment (Gy/CTRL) or p53 restoration (Gy/4-OHT). The data are derived from three independently derived tumors, each analyzed in triplicate. (E) qRT-PCR analysis of mRNA expression of p53 target genes puma and CDKN1A in cultured cancer cells derived from tumors from GFAP-HRasV12;p53<sup>/-KI</sup> animals. Cells were exposed in vitro to γ-radiation in combination with 24-h exposure to 4-OHT (Gy/4-OHT) or vehicle (Gy/CTRL). The data are presented as fold induction relative to nonirradiated samples. The data represent experiments on three independently derived tumors, each analyzed in triplicate. (F) Percent of total tumor cells undergoing apoptosis in control-treated (dark gray bars) or 4-OHT-treated (light gray bars) tumor cell cultures derived from GFAP-HRasV12;p53<sup>/-KI</sup> animals and after treatment in vitro with the MDM2 inhibitor Nutlin 3 at concentrations of 16 (16 μM Nut) or at 32 μM (32 μM Nut) or with the vehicle for Nutlin treatment, DMSO. The data represent experiments on two tumor-cell cultures independently derived from different tumor-bearing animals (T-288 and T-129, respectively), each analyzed in triplicate.

Fig. S2. Restoration of p53ER<sup>TAM</sup> function in GFAP-HRasV12;p53<sup>/-KI</sup> tumor-bearing animals partially ablates tumors and reestablishes animal well-being. Representative H&E analysis of tumors collected from GFAP-HRasV12;p53<sup>/-KI</sup> animals treated with vehicle (p53ER-OFF) or TAM (p53ER-Restored) for 24 h. (Scale bars: 20 μm.)
Fig. S3. Gliomas arising in the absence of functional p53 retain the p53-activating pathway. (A) Immunoblotting analysis of Mdm2 expression in tumor-derived cell cultures from GFAP-HRas^{V12};p53^{−/−} and GFAP-HRas^{V12};p53^{+/+} animals. Three independently derived GBM cultured cells of each genotype are presented. β-Actin was used as an equal loading control. (B) Immunohistochemical analysis of p19<sup>arf</sup> expression in GFAP-HRas^{V12};p53^{+/+}, GFAP-HRas^{V12};p53^{−/−}, and GFAP-HRas^{V12};p53^{+/−} tumors. The location of the lateral ventricle (LV) is indicated, and the percentage of p19<sup>arf</sup>-positive cells in tumors is shown below the images. (Scale bars: 20 μm.)

Fig. S4. Gliomas arising during sustained restoration of p53 lack p19<sup>arf</sup> expression in the actively proliferating cells. Immunohistochemical analysis of p19<sup>arf</sup> expression (green) in Ki67-positive cells (red) in control tumors (p53ER-OFF) and in tumors subjected to the sustained restoration of switchable p53 (p53ER-Restored-Sustained). The star marks the location of the area enlarged in the Inset. Arrowheads point to p19<sup>arf</sup>-positive cells in the p53ER-Restored-Sustained tumors. At least four animals were analyzed for each treatment. Immunohistochemical analysis was performed in duplicate; 10 randomized fields per staining were considered. The percentage of p19<sup>arf</sup>-positive cells is indicated. (Scale bars: 20 μm.)
Fig. S5. GFAP-Hras\textsuperscript{V12};p53\textsuperscript{KI/KI} tumors transplanted intracranially into recipient mice retain p53-activating signals. (A) Survival curves (shown as days after transplantation) of p53\textsuperscript{KI/KI} animals transplanted with tumors derived from TAM-untreated (naive) GFAP-Hras\textsuperscript{V12};p53\textsuperscript{KI/KI} mice. Because gliomas arise continuously in GFAP-Hras\textsuperscript{V12};p53\textsuperscript{KI/KI} mice, it is reasonable to assume that the tumors emerging after single, transient restoration of p53\textsubscript{ER}\textsubscript{TAM} allele (presented in Fig. 5B) are de novo tumors rather than recurring lesions. To address this point, primary tumors isolated from TAM-naive GFAP-Hras\textsuperscript{V12};p53\textsuperscript{KI/KI} mice were transplanted into p53\textsuperscript{KI/KI} congenic recipients. Three weeks after the transplantation, animals were subjected to a single TAM treatment to restore p53 function transiently (1x TAM, purple lines and circles) or to vehicle (CTRL, black lines and squares). (The arrowhead indicates the time point of the treatment after transplantation.) The p53\textsuperscript{KI/KI} animals transplanted with GFAP-Hras\textsuperscript{V12};p53\textsuperscript{KI/KI} cancer cells succumb to recurring tumors after a single transient activation of p53\textsubscript{ER}\textsubscript{TAM} allele. *P < 0.01. Statistical analyses were performed using the Mantel–Cox test. (B) Graph showing apoptotic tumor cells as a percentage of total tumor cells determined by the TUNEL assay. Restoration of p53 function induces apoptosis in transplanted tumors recurring after 24-h restoration of the p53\textsubscript{ER}\textsubscript{TAM} allele. The primary tumors (Primary) that develop in GFAP-Hras\textsuperscript{V12};p53\textsuperscript{KI/KI} animals undergo apoptosis in response to a transient restoration of the p53\textsubscript{ER}\textsubscript{TAM} allele by TAM (First Round; also see Fig. 3A). The tumors recurring after the first round of a transient p53\textsubscript{ER}\textsubscript{TAM} restoration retain p53-activating signals and die by apoptosis following a subsequent 24-h restoration of p53 (Second Round; also see Fig. 5B). The transplanted GFAP-Hras\textsuperscript{V12};p53\textsuperscript{KI/KI} tumors (Transplanted) that develop in recipient p53\textsuperscript{KI/KI} animals also retain the p53-activating signals and respond by apoptotic cell death to a 24-h restoration of p53\textsubscript{ER}\textsubscript{TAM} (First Round), and a subsequent 24-h restoration of p53 activity in recurring tumors described in Fig. S3A (Second Round). Five independent tumors were analyzed for each data point. Ten randomized fields were analyzed.

Fig. S6. GFAP-Hras\textsuperscript{V12};p53\textsuperscript{KI/KI} tumors recurring after a transient restoration of p53\textsubscript{ER}\textsubscript{TAM} retain p19\textsubscript{ARF} expression. Immunohistochemical analysis of p19\textsubscript{ARF} and Ki67 in GFAP-Hras\textsuperscript{V12};p53\textsuperscript{KI/KI} tumors that reemerge in animals after a single treatment with TAM (1x TAM) at age 3 wk compared with tumors from animals subjected to weekly rounds of 24-h-long p53\textsubscript{ER}\textsubscript{TAM} restoration starting at age 3 wk (Intermittent TAM). Dotted lines indicate areas enlarged in Insets. The percentage of total tumor cells positive for p19\textsubscript{ARF}/Ki67 is indicated. (Scale bars: 20 μm.)

Fig. S7. Restoration of p53 activity in human glioma cell lines. (A) Proliferation (presented as percent of control) of primary human cell lines (described in SI Materials and Methods) with differential p53 and CDKN2A (ARF) status, mock-treated (dark gray bars) or treated with 20 μM Prima-1 (light gray bars) for 5 d. MT, mutated. (B) The expression of p53 target genes CDKN1A (p21, mdm2), and growth arrest and DNA damage gene 45a (gadd45) in the primary human glioma cell cultures after Prima-1 treatment is presented as the fold expression relative to the vehicle-treated control samples. The expression of the target genes was normalized to ribosomal protein L13a (RLP13A) gene expression. The differential status of p53 and CDKN2A (ARF) is indicated. All analyses were performed in triplicate.
Fig. S8. Possible mechanisms behind the therapeutic advantage of intermittent versus sustained restoration of p53. (A) A schematic representation of the growth advantage hypothesis that p19ARF-positive tumor cells have higher proliferating capacity than tumor cells lacking the p19ARF expression. (I) In the presence of sustained activation of p53, p19ARF-negative tumor cells continue to proliferate and generate tumors resistant to subsequent p53 restoration. (II) Upon single transient p53 restoration, the majority of p19ARF-positive cells undergo apoptosis and reversible growth arrest. The surviving p19ARF-positive cells are capable of regenerating the tumor mass faster than their p19ARF-negative counterparts. (III) Further intermittent restoration of p53 in mostly p19ARF-positive tumors results in p53-induced apoptosis and promotes survival of the tumor-bearing animals. (B) A schematic representation of the oncogene dosage hypothesis that the tumor mass consists of cells expressing high and low levels of p19ARF depending on the level of rat sarcoma (Ras) oncogene activation. The proliferation status of the cells expressing low levels of p19ARF is independent of p53 functionality as described by Murphy et al. (1). (I) Upon sustained restoration of p53, the cells expressing high levels of p19ARF undergo apoptosis or growth arrest in response to functional p53, whereas cells expressing low levels of p19ARF continue to proliferate in a manner independent of p53 status. (II) Transient activation of p53 results in the elimination of the preexisting cells expressing high levels of p19ARF. However, some of the preexisting p19ARF-negative cells might be converted to cells expressing high levels of p19ARF because of an increase in Ras signaling or the accumulation of additional mutations. (III) The converted cells become sensitive to p53 restoration and can be targeted by additional rounds of p53 treatment.

**Movie S1.** Live images of a symptomatic GFAP-HRas\textsuperscript{V12};p53\textsuperscript{K12} animal (ID#33782) taken 10 min before exposure to TAM.

**Movie S2.** Live images of a symptomatic GFAP-HRas\textsuperscript{V12};p53\textsuperscript{K12} animal (ID#33782) taken 7 d after exposure to TAM.