Gliomagenesis Arising from Pten- and Ink4a/Arf-Deficient Neural Progenitor Cells Is Mediated by the p53-Fbxw7/Cdc4 Pathway, Which Controls c-Myc

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Abstract
Glioblastoma multiforme is the most common type of primary malignant brain tumor and may arise from a cell with neural stem-like properties. Deregelation of the retinoblastoma, phosphoinositide-3 kinase (PI3K), and p53 pathways are molecular hallmarks of this disease. Recent work has shown that p53<sup>−/−</sup> Pten<sup>−/−</sup> mice form gliomas in a c-Myc–dependent manner. To explore the role of the INK4A/ARF locus and Pten deletions in gliomagenesis, we generated Pten<sup>−/−</sup> Ink4a/Arf<sup>−/−</sup> mouse neural stem cells (mNSC) and such cells were highly proliferative, self-renewing, relatively refractory to differentiation, and induced both low- and high-grade glioma formation in vivo. In contrast to p53<sup>−/−</sup> Pten<sup>−/−</sup> mNSCs, however, Pten<sup>−/−</sup> Ink4a/Arf<sup>−/−</sup> mNSCs do not express appreciable levels of c-Myc in vitro, although glioma stem cells derived from these cells did. Sequencing of Pten<sup>−/−</sup> Ink4a/Arf<sup>−/−</sup> mNSC–derived tumors revealed spontaneous mutations in Tp53 in vivo with subsequent downregulation of Fbxw7. Expression of p53 mutants in Pten<sup>−/−</sup> Ink4a/Arf<sup>−/−</sup> mNSC or knockdown of Fbxw7 resulted in reexpression of c-Myc with enhanced Pten<sup>−/−</sup> Ink4a/Arf<sup>−/−</sup> mNSC tumorigenicity. We propose that p53 mutations contribute to gliomagenesis by both allowing the overexpression of c-Myc through downregulation of Fbxw7 and by protecting against c-Myc–induced apoptosis. Cancer Res; 72(22): 6065–75. ©2012 AACR.

Introduction
Glioblastoma multiforme (GBM) is the most common type of primary malignant brain tumor and one of the most lethal of all cancers and despite advances in neurosurgery, radiotherapy, and chemotherapy, still has an overall poor prognosis. Patients with GBM have a median survival of less than 18 months and there are few if any long-term survivors despite aggressive multidisciplinary therapy (1). The majority of cases (>90%) are primary GBMs, which are genetically characterized by loss of heterozygosity on chromosome 10q (70%), EGFR amplification (36%), p16<sup>INK4A</sup> deletion (31%), and Pten mutation (25%). TP53 mutations are also frequently found in primary GBM and even more frequently in low-grade gliomas that progress to secondary GBMs (2). These glioma-related genetic alterations deregulate growth factor receptor signaling (EGFR, PDGF, PTEN) disrupting both the cell cycle and normal regulation of apoptosis (INK4A, CDK4, RB, TP53; ref. 3).

The role of several key genes within the RB, p53, and PI3K pathways to gliomagenesis has been investigated using various genetic mouse models. Mice lacking Pten exhibited enlarged, histoarchitecturally abnormal brains resulting from increased cell proliferation, cell size, and reduced cell death in the Pten<sup>−/−</sup> stem/progenitor cell compartment (4–6). For example, the combination of Ink4a/Arf ablation and expression of constitutive active EGF receptor (EGFR) in mature astrocytes resulted in the formation of glial-like lesions after intracranial transplantation in mice (7). Trp53 and Nf1 double mutant mice generated gliomas of various stages ranging from low-grade astrocytoma to GBM (8). Mouse strains with germline or somatic heterozygous mutations of the Trp53, Nf1, and Pten developed high-grade astrocytomas (9, 10, 11). Despite these important studies, the precise role and contribution of each of these individual genetic aberrations leading to glioma initiation and progression remains imprecisely understood.

The cancer stem cell hypothesis posits that tumorigenic potential is largely restricted to a subset of self-renewing tumor cells with stem cell-like properties designated as tumor stem cells (TSC) or tumor-initiating cells (TIC) (12, 13, 14, 15). The relatively recent identification of neural stem/progenitor cells (NSC) within the brains of adult mammals presents an attractive theoretical and experimental target cells for acquisition of glioma-causing mutations. Consistent with such a possibility, investigation of tumor development in mouse models suggests...
that the tumors often arise in the subventricular zone where adult NSCs reside (9, 10, 11, 16). Despite deletions and mutations in the Pten and Ink4a/Arf being 2 of the most frequent findings in human GBMs, combined knockout model mice have not been established and thus the mechanistic outcome of such combined deletions within the context of a NSC remains unclear. To examine whether mNSCs derived from PtenloxP/loxP Ink4a/Arf−/− mice facilitate gliomagenesis, we established cells derived from the forebrain of PtenloxP/loxP Ink4a/Arf−/− embryos. Here, we characterize Pten−/− Ink4a/Arf−/− mNSCs and show that these cells possess increased self-renewal potential and maintain an undifferentiated state compared with wild-type (WT) mNSCs counterparts, yet do not overexpress c-Myc in vitro. 

Despite the lack of c-Myc activity in vitro, Pten−/− Ink4a/Arf−/− mNSCs give rise to both low- and high-grade astrocytomas, including GBM, when orthotopically implanted in mice. Finally, we show that expression of Fbxw7 in the Pten−/− Ink4a/Arf−/− mNSCs is responsible for the posttranslational downregulation of c-Myc in vitro. These cells, however, ultimately accumulate c-Myc protein and become tumorigenic glioma stem cells (GSC) in vivo through spontaneously occurring mutations in TP53 with subsequent downregulation of Fbxw7. These studies show that inactivation of p53 and/or Ink4a/Arf results in increased phosphorylation of AKT and S6 (Fig. 1B). In addition, mNSCs displayed proliferation and self-renewal activities similar to WT mNSCs (Supplementary Fig. S1B). These results demonstrate that these cells possess increased self-renewal potential and maintain an undifferentiated state compared with wild-type (WT) mNSCs counterparts, yet do not overexpress c-Myc in vitro. 

Intracranial mNSCs and GSCs injection into SCID mice

An intracranial orthotopic model was used for the evaluation of mNSCs and GSCs tumorigenicity according to an approved animal study proposal by NIH-ACUC. mNSCs and GSCs were resuspended in 2 μL of Hank’s balanced salt solution and injected stereotactically into the striatum of female SCID mice (6- to 8-weeks of age) using a stereotactic device (coordinates, 2 mm anterior, 2 mm lateral, 2.5 mm depth from the dura). After injection, animals were followed up daily for the development of neurologic deficits.

Statistical analysis

Student t test was used for data analysis and a P value ≤0.05 was considered significant. All values are shown as mean ± SD. Kaplan–Meier survival analysis was conducted in Prism 4.0 software.

Results

Generation and characterization of Pten−/− Ink4a/Arf−/− mNSCs

PTEN negatively regulates neural stem cell proliferation by controlling cell cycle and apoptosis, whereas Ink4a/Arf deficiency is associated with an increased incidence of spontaneous and carcinogen-induced cancers in mice (6, 17, 18). To evaluate the effects of Pten and/or Ink4a/Arf deficiency specifically within mNSCs, we generated PtenloxP/loxIInk4a/Arf−/− mice by crossing the PtenloxP/loxP and Ink4a/Arf−/− mouse strains. mNSCs were isolated from the forebrain of E14 embryos and cultured in NBE media (13, 19). PtenloxP/loxIInk4a/Arf−/− and PtenloxP/loxP Ink4a/Arf−/− mouse-derived mNSCs were transduced with a Cre-adenovirus to delete the Pten locus. Ablation of Pten and Ink4a/Arf genes was confirmed by genomic PCR (Fig. 1A; Supplementary Fig. S1). Pten−/− and Pten−/− Ink4a/Arf−/− mNSCs showed increased phosphorylation of AKT and S6 (Fig. 1B). In addition, Pten−/− and Pten−/− Ink4a/Arf−/− mNSCs displayed increased proliferative activity (Fig. 1C) and self-renewal capability (Fig. 1D; Supplementary Fig. S1B). In contrast, the Ink4a/Arf−/− mNSCs displayed proliferation and self-renewal activities similar to WT mNSCs (Supplementary Fig. S1B). These results reveal that Pten deletion expands mNSCs proliferative and self-renewal capacity in vitro in either a WT or Ink4a/Arf−/− background, but that the Ink4a/Arf deletion alone does not, highlighting the crucial gatekeeper role of Pten in mNSCs.

Pten−/− Ink4a/Arf−/− mNSCs generate gliomas in vivo

To examine whether Pten and/or Ink4a/Arf deletion in mNSCs are sufficient to induce glioma formation in vivo, we injected Ink4a/Arf−/−, Pten−/−, and Pten−/− Ink4a/Arf−/− mNSCs (5 × 105 cells/mouse) into the SCID mice. To minimize any potential in vitro artifact, early passage (less than passage 5) mNSCs were injected. As both Pten−/− and Pten−/− Ink4a/Arf−/− mNSCs exhibit similar self-renewal capacity and altered
signaling pathways in vitro, we expected that both would be able to induce gliomas (Fig. 1C and D). Interestingly, however, only Pten\textsuperscript{−/−} Ink4a/Arf\textsuperscript{−/−} mNSCs were able to form tumors and did so with 100% penetrance and with a median survival time of 157 days (Fig. 2A). In contrast, Pten\textsuperscript{−/−} mNSCs, Ink4a/Arf\textsuperscript{−/−} mNSCs, and WT mNSCs failed to form tumors in vivo. Pathologic examination of the Pten\textsuperscript{−/−} Ink4a/Arf\textsuperscript{−/−} mNSC–generated tumors revealed gliomas of varied histologic grades including infiltrating astrocytomas (grade 2), anaplastic astrocytomas (grade 3), and GBMs (grade 4; Fig. 2B; Supplementary Table S1).

Hematoxylin and eosin staining of the high-grade tumors showed the salient features of human glioma, including nuclear atypia, vascular proliferation, and necrosis (Fig. 2C). All tumor cells showed high expression of markers associated with human gliomas (GFAP, EGFR, and PDGFRA), neural stem cells (Nestin), and proliferation (Ki-67). Consistent with the derivation of these tumors exclusively from Pten\textsuperscript{−/−} Ink4a/Arf\textsuperscript{−/−} mNSCs, all tumor cells were Pten negative by immunohistochemistry, whereas host physiologic astrocytes and vascular cells were Pten positive (Fig. 2D). This pattern indicates that tumors derived from Pten\textsuperscript{−/−} Ink4a/Arf\textsuperscript{−/−} mNSCs are highly proliferative and maintain features associated with both neural stem cells and glioma. Thus, Pten\textsuperscript{−/−} Ink4a/Arf\textsuperscript{−/−} mNSCs are capable of developing heterogeneous glioma xenografts of varying histologic grades showing key phenotypic features associated with spontaneous human gliomas. In addition, the restriction of tumorigenic capacity to Pten\textsuperscript{−/−} Ink4a/Arf\textsuperscript{−/−} mNSCs and not to Pten\textsuperscript{−/−} or Ink4a/Arf\textsuperscript{−/−} mNSCs further highlights the necessity of inactivating both, rather than any one, of these pathways for glioma formation.

Histologic grade influences heterogeneous GSC behavior in vitro

We next sought to determine the tumor forming capacity of GSCs derived from the different low- and high-grade xenograft tumors from the Pten\textsuperscript{−/−} Ink4a/Arf\textsuperscript{−/−} mNSCs. We isolated the GSCs from mice-bearing intracerebral xenograft tumors of grades 2, 3, and 4, and expanded them in NBE media. Hereafter, cells derived from grades 2, 3, and 4 gliomas are designated as T1, T2, and T3, respectively. We injected these xenograft-derived glioma cells (2.5 \times 10^5 cells/mouse) into the periventricular cortex of adult SCID mice. As expected, GSCs isolated from all tumor grades resulted in secondary tumors in 100% of
the mice injected. Interestingly, GSCs isolated from primary xenografts with varying histologic grades induced secondary xenograft gliomas with remarkably different survival dates (Fig. 3A). Kaplan–Meier survival data showed a stepwise progression in malignancy with median survival times of 131, 74.5, and 21 days for T1, T2, and T3 GSCs, respectively. Thus, although T1 GSCs formed slightly more aggressive tumors than the original Pten−/− Ink4a/Arf−/− mNSCs (131 days vs. 157 days), both T2 and T3 were significantly more aggressive than the primary high-grade xenografts they were derived from. All T3-injected mice developed tumors with phenotypic features characteristic of human GBM, including marked brain infiltration, large regions of necrosis, and hemorrhage (Supplementary Fig. S2A). These data suggest that the heterogeneity of histologic grades in xenograft tumors derived from Pten−/− Ink4a/Arf−/− mNSCs is reflected in biologically relevant and distinct populations of GSCs, with xenograft latency inversely related to histologic grade from the tumor they were derived from. Furthermore, serial GSC orthotopic transplantation selects for increasingly more malignant GSC subpopulations.

Next, we characterized the T1, T2, and T3 GSCs in vitro. To compare the differentiation potential of all GSCs with their parental Pten−/− Ink4a/Arf−/− mNSCs and WT mNSCs, cells were cultured in FBS media (Fig. 3B). In NBE media, GSCs and mNSCs highly express markers associated with neural stem cells (Nestin and Sox2) and minimally express markers associated with differentiation of astrocyte (GFAP) and neuron (TuJ1). In contrast, culturing cells in the presence of FBS resulted in downregulation of nestin and sox2 along with upregulation of GFAP to a much greater extent in WT mNSCs than in either Pten−/− Ink4a/Arf−/− mNSCs or the GSCs. In addition, we analyzed the expression of the CD133 and CD15 surface markers that have been associated with the enrichment for GSC tumorigenic potential (15, 20). When compared with either WT mNSCs, Pten−/− Ink4a/Arf−/− mNSCs, or grade II–derived T1 GSCs, both T2 and T3 GSCs showed an increased CD15-positive (CD15+) population of cells (Fig. 3C), consistent with their accelerated tumorigenic potential. All GSCs and Pten−/− Ink4a/Arf−/− mNSCs expressed barely detectable levels of CD133-positive (CD133+) cells (less than 2%; Fig. 3D, Supplementary Fig. S2B). In a manner consistent with histologic
grade, T3 GSCs exhibited the highest level of proliferative activity, whereas T2 and T3 GSCs had significantly greater self-renewal capability than T1 GSCs (Fig. 3E and F). These results showed that GSCs derived from Pten\(^{-/-}\) Ink4a/Arf\(^{-/-}\) mNSCs represent a poorly differentiated, population of cells whose self-renewal and tumorigenic potential increases with the histologic grade of the tumor from which they were derived.

C-Myc protein is downregulated in Pten\(^{-/-}\) Ink4a/Arf\(^{-/-}\) mNSCs in vitro but upregulated in xenograft-derived GSCs

C-Myc is an important transcription factor for the induction and maintenance of iPSCs, embryonic stem cells (ES), neural stem cells, and cancer stem cells with its depletion resulting in a loss of self-renewal capacity and the induction of differentiation.
(16, 21, 22, 23). Furthermore, high c-Myc activity has been shown to be particularly important in glioma biology and was recently showed to be required for p53−/−Pten−/− mNSCs mediated gliomagenesis (16). Because Pten−/−Ink4a/Arf−/− mNSCs showed increased self-renewal, proliferation, and poor differentiation potential, similar to GSCs and p53−/−Pten−/− mNSCs, we investigated whether c-Myc was also increased in Pten−/−Ink4a/Arf−/− mNSCs. Surprisingly, Pten−/−Ink4a/Arf−/− mNSCs expressed slightly lower levels of c-Myc protein than WT, Pten−/−, and Ink4a/Arf−/− mNSCs (Supplementary Fig. S3A). More impressively, c-Myc expression level in Pten−/−Ink4a/Arf−/− mNSCs was dramatically lower than it was in the GSCs (Fig. 4A). To measure c-Myc activity, we transfected cells with a luciferase c-Myc reporter construct. Consistent with the protein expression, Pten−/−Ink4a/Arf−/− mNSCs expressed significantly decreased luciferase activity compared with WT mNSCs (0.28 vs. 1). In addition, GSCs had significantly increased luciferase activity (more than 15-fold) compared with WT mNSCs (Fig. 4B). Consistent with these data, Western blot analysis showed significantly decreased phospho-S62 c-Myc in Pten−/−Ink4a/Arf−/− mNSCs and increased phospho-S62 c-Myc in WT mNSCs and especially in GSCs (Supplementary Fig. S3B). In addition, ubiquitin pull-down assay revealed c-Myc ubiquitination was significantly higher in Pten−/−Ink4a/Arf−/− mNSCs than in either WT or GSCs, whereas c-myc total protein levels became similar in all cell types after treatment with ALLN (Fig. 4C, Supplementary Fig. S3C). Nevertheless, despite the significant difference in c-Myc protein expression and activity between Pten−/−Ink4a/Arf−/− mNSCs and GSCs, there was no significant difference in total c-Myc mRNA expression (Fig. 4D). These data suggested that the difference in c-Myc protein expression and functional activity between Pten−/−Ink4a/Arf−/− mNSCs, WT mNSC, and GSCs was likely due to posttranslational regulation.

**P53–Fbxw7 pathway suppresses c-Myc expression in Pten−/−Ink4a/Arf−/− mNSCs and is inactivated in gliomas**

We have shown that Pten−/−Ink4a/Arf−/− mNSCs express decreased levels of c-Myc compared with WT mNSCs through posttranslational regulation (Fig. 4, Supplementary Fig. S3). These data led us to further investigate genes involved in c-Myc regulation. Fbxw7 has been shown to interact with c-Myc and
medicate its ubiquitination in a Thr\(^{96}\) phosphorylation–dependent manner. Fbxw7 is a p53-dependent tumor suppressor and its activation by p53 leads to ubiquitination-mediated suppression of several oncoproteins (24, 25, 26). Hence, we hypothesized that the p53–Fbxw7 pathway might be involved in the suppression of c-Myc expression in Pten\(^{−/−}\) Ink4a/Arf\(^{−/−}\) mNSCs and decreased in GSCs. Because Fbxw7 is a p53 target gene, we next asked whether the decreased Fbxw7 expression in our GSCs correlated with increased c-Myc expression in T3 GSCs. Eleven tumors had the C132W (C135W in mouse) mutation in human p53) mutation (Fig. 6C, Supplementary Table S2). These data suggest that p53 regulates c-Myc protein expression with little effect on mRNA levels (Fig. 6A and B). Knockdown of Fbxw7 resulted in increased c-Myc expression in T3 GSCs in vitro (Fig. 5C and D). We analyzed several substrates of Fbxw7 and found that c-Jun and Aurora A are increased in GSCs although cyclin E was not (Supplementary Fig. S4). These data suggest that Fbxw7 suppresses c-Myc in Pten\(^{−/−}\) Ink4a/Arf\(^{−/−}\) mNSCs in vitro, an effect that is relieved in GSCs in vivo.

Because Fbxw7 is a p53 target gene, we next asked whether the decreased Fbxw7 expression in our GSCs correlated with their p53 status. Interestingly, although WT p53 expression is low in Pten\(^{−/−}\) Ink4a/Arf\(^{−/−}\) mNSCs, GSCs expressed much higher levels of the p53 protein (Fig. 6A). Furthermore, Pten\(^{−/−}\) Ink4a/Arf\(^{−/−}\)–derived xenograft tumors showed high levels of p53 protein expression within the nuclei of all xenograft tumor cells (Fig. 6B). Because high basal levels of p53 protein are commonly seen when p53 is mutated (27), we sequenced the Tp53 gene in our Pten\(^{−/−}\) Ink4a/Arf\(^{−/−}\) mNSC–generated xenograft tumors. Although the p53 sequence was wild-type in Pten\(^{−/−}\) Ink4a/Arf\(^{−/−}\) mNSCs, we identified 1 of 2 nonsynonymous point mutations in the p53 gene in each of the xenograft tumors. Eleven tumors had the C132W (C135W in human p53) mutation and 3 tumors revealed the R270C (R273C in human p53) mutation (Fig. 6C, Supplementary Table S2). Each of these mutations has been previously found in primary human gliomas and both are located in the DNA binding domain of p53 (http://p53.free.fr/Database/p53_cancer/p53_brain.ed by html).

To better understand the relevance of p53 status to the expression of c-Myc, we carried out knockdown of p53 or overexpression of either the C132W or the R270C mutation in Pten\(^{−/−}\) Ink4a/Arf\(^{−/−}\) mNSCs. Both p53 knockdown and overexpression of the p53 mutants resulted in decreased Fbxw7 mRNA and protein expression in vitro while increasing c-Myc protein expression with little effect on c-Myc mRNA levels (Fig. 6D–G). These data suggest that p53 regulates c-Myc protein levels through Fbxw7 and WT p53 function needs to be deregulated to allow accumulation of c-Myc that is necessary for gliomagenesis.

P53–Fbxw7 pathway regulates c-Myc–induced apoptosis and gliomagenesis

It has been previously shown that ectopic expression of c-Myc induces apoptosis in fibroblasts in the absence of growth factors (28), in mouse embryonic fibroblasts (29) and in U87MG glioma cell lines following glycogen synthase kinase-3 (GSK) inhibition (30). We therefore hypothesized that elevated c-Myc induces p53-dependent apoptosis in Pten\(^{−/−}\) Ink4a/Arf\(^{−/−}\) mNSCs, an effect that is suppressed in GSCs...
with p53 mutations. To test this hypothesis, we overexpressed c-Myc or knocked down Fbxw7 (see Fig. 5C), both of which resulted in elevated c-Myc followed by increased cellular apoptosis as shown by elevation of cleaved caspase-3 (Fig. 7A, B, and D). In contrast, overexpression of the GSC-derived C132W and R270C p53 mutants in Pten−/−Ink4a/Arf−/− mNSCs resulted in increased c-Myc but little apoptosis (Fig. 7C and D). These data suggest that elevated c-Myc fails to induce p53-dependent apoptosis in GSCs by inactivation of the p53–Fbxw7 pathway.

Finally, to evaluate the effects of Fbxw7 expression on p53 mutant GSCs, we stably overexpressed Fbxw7 in T3 GSCs and injected them (1 × 10³/mouse) into the periventricular cortex of adult female SCID mice. As can be seen in Fig. 7E, mice inoculated with Fbxw7-overexpressing T3 GSCs lived significantly longer than control T3 GSC–inoculated animals (Median survival is 39 days for Fbxw7 and 23 days for control; Fig. 7E). These data show that the p53–Fbxw7 pathway is a key regulatory mechanism for controlling c-Myc–induced apoptosis and gliomagenesis.

Discussion

In this study, we show that Pten−/−Ink4a/Arf−/− mNSCs exhibit GSC features and induce gliomas in adult SCID mice with 100% penetrance. Like human GSCs, Pten−/−Ink4a/Arf−/− mNSCs display poor differentiation relative to WT mNSCs, possess increased self-renewal and proliferative properties, and are capable of forming a heterogeneous range of glial tumors including both low- and high-grade astrocytomas. In addition, we found inactivation of the p53–Fbxw7 pathway during gliomagenesis results in elevated c-Myc expression necessary for gliomagenesis.

High-throughput genomic data have shown a wide array of genetic and epigenetic alterations within the genomic landscape of GBM. These alterations can differ significantly from one tumor to another; however, many of these alterations ultimately converge to deregulate 3 signaling pathways, the PI3K, RB, and p53 pathways. Disruption of any single pathway has not proven to be sufficient to generate gliomas in the mouse system. For instance, although Ink4a/Arf−/− and p53−/− mice
null mice each are at higher risk of tumor formation, gliomas are rarely seen and Pten null mice fail to generate gliomas. In contrast to single pathway disruption, perturbations of combinations of pathways have generated gliomagenic phenotypes. For example, mice with mutations in Trp53 and Nf1 as well as mice strains with germline or somatic heterozygous mutations of Trp53, Nf1, and Pten develop high-grade astrocytomas (8, 9, 10, 11). Similarly, Zheng and colleagues showed combinations of pathways have generated gliomagenic phenotypes. For example, mice with mutations in Trp53 and Nf1 as well as mice strains with germline or somatic heterozygous mutations of Trp53, Nf1, and Pten develop high-grade astrocytomas (8, 9, 10, 11). Similarly, Zheng and colleagues showed that p53−/−Pten−/− mNSCs are capable of forming gliomas dependent on c-Myc expression (16). These observations are consistent with previous data showing that although the c-Myc gene is rarely amplified or mutated in gliomas, high c-Myc activity is commonly observed. In human gliomas, c-Myc is expressed in more than 75% of gliomas and the level of expression correlates with the grade of malignancy (31).

Despite the fact that deletions and mutations in the Pten and Ink4a/Arf genes are 2 of the most frequent findings in human GBMs, combined knockout mouse models have not been characterized and thus the mechanistic outcome of such combined deletions within the context of an NSC remains unclear. We had initially hypothesized that the phenotype and tumorigenic properties of Pten−/−Ink4a/Arf−/− mNSCs would be similar to those of the p53−/−Pten−/− mNSCs. We had assumed, however, that Pten−/−Ink4a/Arf−/− mNSCs would generate an even more aggressive phenotype than the p53−/−Pten−/− mNSCs, given the deregulation of all 3 GBM-associated signaling pathways with the loss of PTEN, deregulation of the RB pathway through loss of p16^INK4A, and deregulation of p53 through deletion of p14^ARF. To our surprise, however, the phenotype of the Pten−/−Ink4a/Arf−/− mNSCs was different than that of the p53−/−Pten−/− mNSCs in a couple of important and unexpected ways. First, in contrast to p53−/−Pten−/− mNSCs, the Pten−/−Ink4a/Arf−/− mNSCs expresses very little c-Myc protein with correspondingly low c-Myc activity. Despite previous data showing that the downregulation of c-Myc inhibits the tumorigenic capacity of p53−/−Pten−/− mNSCs, Pten−/−Ink4a/Arf−/− mNSCs still formed gliomas with 100% penetrance. Unlike Pten−/−Ink4a/Arf−/− mNSCs, however, GSCs cultured from all of the Pten−/−Ink4a/Arf−/− mNSCs-generated xenografts expressed very high levels of c-Myc.

Because c-Myc mRNA levels were not substantially different between WT mNSCs, Pten−/−Ink4a/Arf−/− mNSCs and GSCs, we reasoned that the difference in c-Myc protein and activity levels between the cells was primarily posttranslationally regulated. Our data suggest that the posttranslational regulation of c-Myc expression occurs spontaneously in vivo through downregulation of the Fbxw7. Fbxw7 is a p53-dependent tumor suppressor and its activation by p53 leads to ubiquitination-mediated suppression of several oncoproteins functioning in cellular growth and division pathways, including c-Myc, cyclin E, Notch, and c-Jun (24, 25). The dependency of Fbxw7 expression on p53 prompted us to look at the status of p53 in our GSCs compared with Pten−/−Ink4a/Arf−/− mNSCs. We
found that the c-Myc–dependent glioma formation in vivo required downregulation of Fbxw7 that occurred as a consequence of spontaneous mutation of p53. Consistent with the importance of p53 regulation of Fbxw7 in gliomagenesis was the fact that the C132W and R270C p53 mutations we saw in the glioma xenografts were the ones that resulted in defective Fbxw7 expression and upregulation of c-Myc when transfected into Pten–/−Ink4a/Arf−/− mNSCs (Figs. 6 and 7). Furthermore, restoration of Fbxw7 expression in the xenograft GSCs repressed their tumorigenic potential in vivo, confirming the role of Fbxw7 as a tumor suppressor within GSCs (Fig. 7E). Our data are consistent with the results from a conditional knockout Fbxw7 mouse model in which inactivation of Fbxw7 resulted in acute T-cell lymphoblastic leukemia/lymphomas that was accompanied by c-Myc accumulation (32, 33).

In addition to the inhibition of Fbxw7-mediated degradation of c-Myc, p53 mutations likely also contributed the ability of the Pten−/−Ink4a/Arf−/− mNSCs to tolerate high levels of c-Myc activity through inhibition of p53-dependent oncogene-induced stress-mediated apoptosis. Supportive of this idea is the fact that our numerous attempts to establish Pten−/−Ink4a/Arf−/− mNSCs that overexpress c-Myc failed secondary to significant apoptosis that could be inhibited by the C132W and R270C mutant p53 genes. These findings are consistent with our prior work showing that GSK3 inhibition-mediated hyperactivation of c-Myc activity causes c-Myc–induced apoptosis. Thus, it seems that there exists a range of c-Myc activity in gliomas, high enough to support gliomagenesis, but not so high to induce apoptosis. These data suggest that mutation of p53 contributes to gliomagenesis in part both through upregulation of c-Myc activity and through inhibition of c-Myc–mediated apoptosis. In summary, our Pten−/−Ink4a/Arf−/− mNSCs model supports the hypothesis that genomic alterations commonly occurring in human glioblastoma, and arising within a mammalian neural stem cell compartment, results in malignant transformation and the formation of glioma stem cells. Our characterization of these cells address novel mechanisms in gliomagenesis concerning the regulation of c-Myc accumulation during oncogenic stress, and the importance of the Pten, Ink4a/Arf, and p53–Fbxw7 pathway in regulating normal neural stem cell and gliomas. These data support efforts to identify and test novel therapeutic approaches targeting the p53–Fbxw7 and c-Myc–related pathways for the treatment of gliomas.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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