Early inactivation of p53 tumor suppressor gene cooperating with NF1 loss induces malignant astrocytoma

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Summary

Malignant astrocytoma, the most prevalent primary brain tumor, is resistant to all known therapies and frequently harbors mutations that inactivate p53 and activate Ras signaling. We have generated mouse strains that lack p53 and harbor a conditional allele of the NF1 tumor suppressor that negatively regulates Ras signaling. The mice develop malignant astrocytomas with complete penetrance. The majority of tumors display characteristics of glioblastoma multiforme with concomitant alteration of signaling pathways previously described in the human counterparts of this neoplasm. We find that the sequence of tumor suppressor inactivation influences tumorigenicity and that earliest evidence of tumor formation localizes to regions of the brain that contain a multipotent stem cell population capable of in vivo differentiation into neurons and glia.

Introduction

Astrocytomas, composed predominantly of astrocyte-like cells, are the most common neoplasm in the central nervous system (CNS) (Kleihues and Cavenee, 2000). According to the World Health Organization (WHO) grading system, grade II–IV astrocytomas (collectively called malignant astrocytoma) are biologically malignant and diffusely infiltrate the brain. Grade IV astrocytoma, or glioblastoma multiforme (GBM), is one of the most aggressive human cancers, with a median survival of less than 1 year. Unfortunately, this prognosis has not changed significantly over the past two decades, despite advances in neurosurgery, radiation, and chemotherapy (Holland, 2001; Maher et al., 2001; Zhu and Parada, 2002).

There are two subtypes of GBMs. Primary GBMs arise rapidly or de novo without any evidence of prior clinical disease, whereas secondary GBMs derive from preexisting low-grade lesions after long latency ranging from 5 to 10 years (Kleihues and Cavenee, 2000; Maher et al., 2001). Human genetic studies indicate that two frequent molecular characteristics of low-grade astrocytomas are loss of p53 and elevated expression of platelet-derived growth factor (PDGF) and its receptor tyrosine kinase (RTK), PDGFR (Guha et al., 1995; Hermanson et al., 1992; Nister et al., 1988). These observations suggest that these pathways may participate in the initiation of astrocytoma. Individuals afflicted with neurofibromatosis type 1 (NF1) are predisposed to malignant astrocytoma in the brain with a greater than 5-fold increased incidence throughout their lives (Rasmussen et al., 2001). The NF1 tumor suppressor product neurofibromin is a functional Ras GTPase-activating protein (GAP) (Cichowski and Jacks, 2001; Zhu and Parada, 2001). Thus, loss of neurofibromin results in abnormal activation of Ras, a major mediator of RTK signaling.

Heterozygous mice carrying germline null mutations in both p53 and NF1 on the same chromosome (termed cisNF1/p53 mice) develop low- to intermediate-grade astrocytomas, with up to 75% penetrance depending on the genetic background (Reilly et al., 2000, 2004). We reasoned that the increased incidence of astrocytoma in NF1 patients might indicate a con-

SIGNIFICANCE

Recent studies indicate that brain tumors, including glioblastoma multiforme, contain a subpopulation of cancer cells that display stem cell characteristics, including self-renewal and multipotentiality, and are responsible for in vivo tumor growth. Whether these brain cancer stem cells are derived from neoplastic transformation of normal neural stem cells remains to be determined. Through analysis of mouse models with complete penetrance of malignant astrocytoma, we demonstrate that astrocytoma cells of all grades display stem cell characteristics and that early presymptomatic lesions reside within the subventricular zone (SVZ) of the lateral ventricle, one region of the CNS that contains neurogenic stem cells. These studies provide evidence that the SVZ cells may serve as a cell-of-origin for malignant astrocytoma.

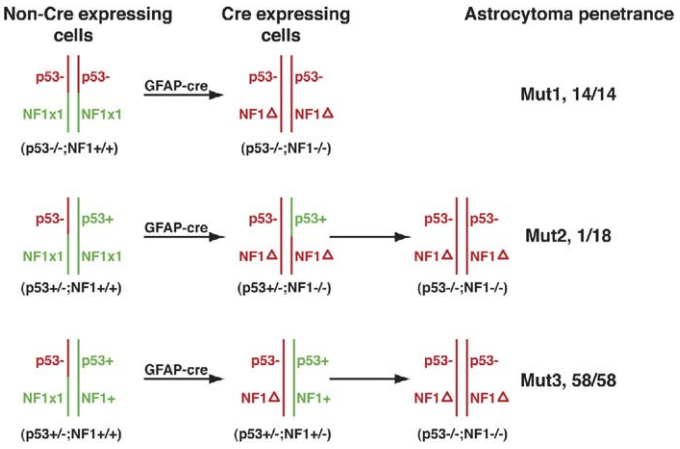

Figure 1. Schematic drawing of the genetic configurations of Mut1–3 mice

The green color is used to label the normal alleles, including the wild-type allele (+) and the floxed allele (x1); the red color is used to label the null alleles, including the recombined floxed allele (Δ) and the knockout allele (−). Genotypes for each cell type are indicated in parentheses.

vergence on the requirement for Ras signaling with sporadic astrocytomas via PDGFR activation. To refine our understanding of the target cell for p53 inactivation and Ras pathway activation in astrocytoma induction, we combined a germline p53 mutation (Jacks et al., 1994) with a neural-specific NF1 mutation (Zhu et al., 2001). The resultant mice develop malignant astrocytomas with 100% penetrance and provide evidence for the cell-of-origin in the subventricular zone (SVZ) of the adult brain and a requirement for p53 loss preceding or concomitant inactivation of NF1. The availability of an accurate mouse model for malignant astrocytoma initiation and progression will permit detailed investigation into the pathogenesis of, and novel therapeutic targets for, this incurable tumor.

Results

Mouse models for malignant astrocytoma

In mice, genetic p53 inactivation fails to induce astrocytomas. Instead, the predominant tumors are lymphomas or sarcomas (Donehower et al., 1992; Jacks et al., 1994). We generated three compound mutant mouse strains harboring mutations in the linked p53 and NF1 tumor suppressor genes (Mut1–3; Figure 1). Critical for this purpose was the genetic configuration of a floxed NF1 allele linked to a null p53 allele on mouse chromosome 11 (Figure 1) and the use of a Cre transgenic strain under the control of the human glial fibrillary acidic protein promoter (*hGFAP*-cre) (Malatesta et al., 2003; Zhu et al., submitted; Zhuo et al., 2001). Although GFAP is widely used as a mature glial-specific marker for normal and reactive astrocytes in the CNS (Ridet et al., 1997), more recent data indicate that the human GFAP promoter is also active in most embryonic radial glial cells that exhibit neural progenitor cell properties (Malatesta et al., 2000, 2003; Noctor et al., 2001; Zhuo et al., 2001). To determine the scope of *hGFAP*-cre transgene expression and activity, we crossed this mouse strain to the Rosa26-LacZ reporter strain (Soriano, 1999; Zhuo et al., 2001). Between embryonic day 10.5 (E10.5) and E12.5, Cre-mediated recombination, as revealed by LacZ expression, was evident in discrete neuroectodermal regions of the forebrain and hindbrain and in trigeminal ganglia (see Figures S1A–S1F in the Supplemental Data available with this article online). Consistent with previous reports (Malatesta et al., 2003; Zhuo et al., 2001), the consequence of early ventricular Cre expression in neural progenitor cells is that, at birth (P0.5), most cortical and hippocampal neurons express the LacZ reporter gene (Figures S1G–S1I). Thus, early onset of the human GFAP promoter in neural progenitor cells leads to Cre-mediated recombination in early CNS cells as well as their progeny, including adult neural stem/progenitor cells in the SVZ (Merkle et al., 2004; Tramontin et al., 2003) and neurons, astrocytes, and oligodendrocytes (Malatesta et al., 2003; Zhuo et al., 2001).

Mut1 (*p53$^{-/-}$;NF1$^{flox/flox}$;hGFAP-cre*+) mice lack p53 in the germline and NF1 function in CNS cells as a consequence of *hGFAP*-cre-mediated recombination in developing neural progenitor cells and mature astrocytes (Malatesta et al., 2003; Zhuo et al., 2001). As in Mut1, Mut2 (*p53$^{+/-}$;NF1$^{flox/flox}$;hGFAP-cre*+) mice lack NF1 in CNS cells but are heterozygous for p53. Mut3 (*cisp53$^{+/-}$;NF1$^{+/flox}$;hGFAP-cre*+) mice are compound heterozygotes for p53 and NF1 in CNS cells (Figure 1). All Mut1 (14/14; Figure 1) mice developed GFAP-positive astrocytomas ranging from infiltrative low-grade lesions (grade II; Figures 2A, 2D, and 2G); to higher-grade anaplastic astrocytomas (grade III; Figure 2B, 2E, and 2H); to GBMs (grade IV; Figures 2C, 2F, and 2I). As a consequence of germline p53 nullizygosity, these mice also developed lymphomas and sarcomas causing early mortality, likely explaining the reduced development of GBM (2/14) (Figures 2J and 2K). All Mut3 mice also developed CNS tumors (58/58; Figures 1 and 2K), and among the subset allowed to survive to end-stage of symptoms, approximately 70% had GBMs (14/21; Figure 2K) exhibiting every discernible feature of the human counterpart, including the presence of pseudopalisading tumor cells (Figures 3A and 3B), necrosis (Figures 3C and 3D), microvascular proliferation (Figures 3E and 3F), and secondary structures of Scherer (Figures 3G–3J). All symptomatic Mut3 mice had anaplastic astrocytomas or GBMs, while asymptomatic Mut3 mice had either low-grade tumors or no tumors (Figure 2K and Figure S2). Given the 100% incidence of tumor formation, these findings are most consistent with a model in which tumors arise as low-grade lesions that progress to higher-grade tumors with characteristics of GBM over time.

In contrast to the two genetic configurations described above, the Mut2 strain rarely developed CNS tumors (Figures 1 and 2K; see below). Immunohistochemical analysis showed that all Mut1 astrocytomas expressed Cre recombinase demonstrating NF1 deficiency (Figure 4A), while at the molecular level, astrocytomas from the Mut3 mice exhibited inactivation of both p53 and NF1 genes (Figures 4B and 4C). These data indicate that loss of p53 and NF1 is sufficient to initiate the development of a full spectrum of malignant astrocytomas in mice.

Molecular validation

Analysis of tumor tissues from human astrocytomas has demonstrated accrual of specific genetic lesions during progression from low-grade to high-grade astrocytoma (Holland, 2001; Maher et al., 2001; Zhu and Parada, 2002). These include

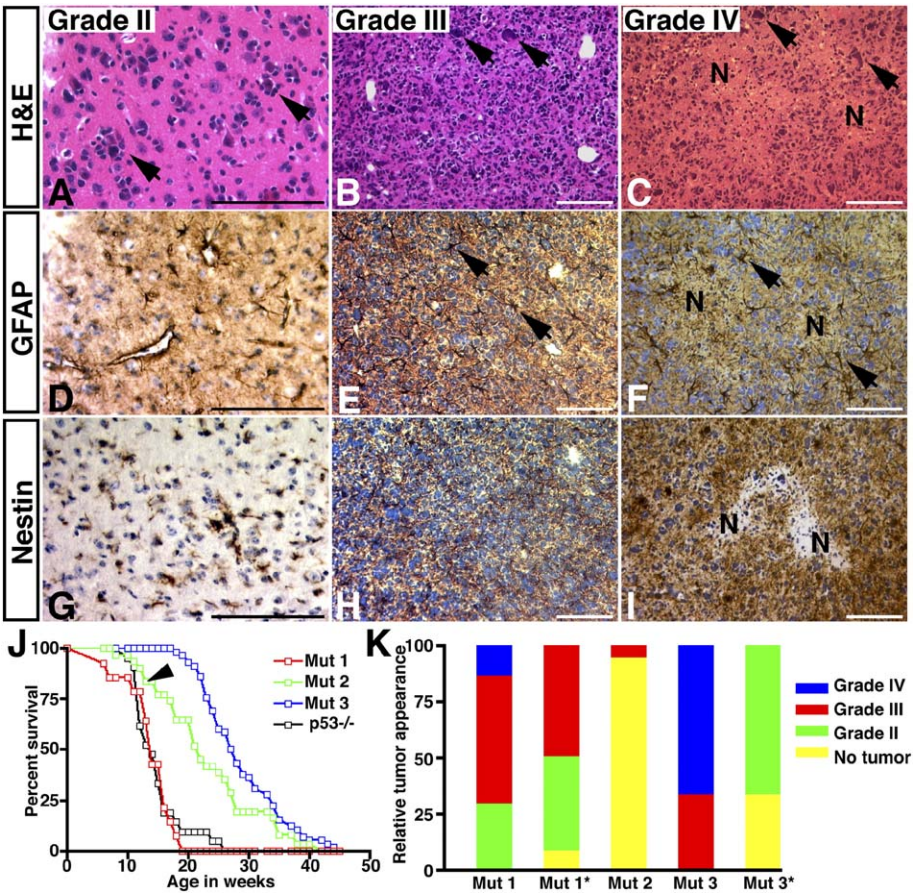

Figure 2. Mut1 mice develop a full spectrum of malignant astrocytoma

Sections from grade II, grade III, and grade IV astrocytomas of Mut1 brains were stained with hematoxylin and eosin (H&E) (**A–C**), an anti-GFAP antibody (**D–F**), and an anti-nestin antibody (**G–I**). Arrows in **A** point to abnormal tumor cells surrounding neurons (perineuronal satellitosis); arrows in **B** and **C** point to multinucleated giant cells; and arrows in **E** and **F** indicate reactive astrocytes. **J:** Survival curves of Mut1, Mut2, Mut3, and p53 null ($p53^{-/-}$) mice. The following numbers of mice were used for each group of mutant mice: Mut1, n = 14; Mut2, n = 21; Mut3, n = 58; $p53^{-/-}$, n = 20. The incidence of non-CNS tumors observed in Mut1–3 mice is as follows: Mut1, 9/14; Mut2, 4/21; and Mut3, 11/58. **K:** The graph shows astrocytoma frequency and grade observed in end-stage Mut1 mice (n = 14), asymptomatic Mut1* mice (n = 12; 6 to 10 weeks of age), end-stage Mut2 mice (n = 18), end-stage Mut3 mice (n = 21), and asymptomatic Mut3* mice (n = 3; 18 weeks of age). "N," necrosis. Scale bar, 50 μm.

deregulation of the Rb-mediated pathway and the PI-3K/AKT/PTEN pathway. We examined expression of two components of the Rb pathway, Cdk4 and cyclin D1, by immunohistochemistry (Figures 5A–5F; n = 5) and found that these high-grade tumors had intense nuclear expression of both Cdk4 (Figures 5A and 5D) and cyclin D1 proteins (Figures 5B and 5E), whereas normal CNS cells did not (Figure S3). To examine the status of the Ras pathway, activation of MAP kinase (MAPK) was assessed with phospho-specific antibodies. Consistent with loss of NF1 function, both low-grade (Figure 5G) and high-grade astrocytomas (Figures 5H and 5I) contain high levels of activated MAPK. In contrast, as in human tumors, activation of AKT was consistently found in higher-grade tumors (grade II, 0/5, Figure 5J; grade III, 1/5, Figures 5K and 5L; grade IV, 5/5, Figures 5M and 5P).

Two additional noteworthy molecular features of human astrocytoma are present in these mice. VEGF expression increases in the transition from lower-grade tumors to GBMs (Figures 5N and 5Q). In addition, although morphologically similar, high-grade tumors are molecularly heterogeneous, as evidenced by a regional downregulation of MAPK (Figure 5O) within tumors that retain activated AKT throughout (Figure 5R). These data are consistent with a causal role for loss of p53 and activation of Ras-MAPK in astrocytoma initiation followed by deregulation of the Rb-mediated pathway and the PI-3K/AKT/PTEN pathway in progression to GBM. These results provide experimental evidence that our tumor models resemble human malignant astrocytoma both at the pathologic and molecular levels.

Site of tumor origin and stem cell characteristics

The cell-of-origin of astrocytoma is a much investigated but unresolved question. While there has been increasing evidence that human astrocytoma cells resemble neural stem/progenitor cells (Hemmati et al., 2003; Ignatova et al., 2002; Singh et al., 2003), the existing evidence also supports the model that has evoked initial astrocyte dedifferentiation followed by malignant transformation (Bachoo et al., 2002). Consistent with the cancer stem cell model (Hemmati et al., 2003; Ignatova et al., 2002; Singh et al., 2003, 2004), astrocytomas of all grades (Mut1, n = 14; Mut3, n = 21) expressed a marker for neural stem cells, nestin (Figures 2G–2I; Figures S2E and S2F). In the adult brain, two areas have been identified as primary sources of multipotent neural stem cells (Gage, 2000). One is the SVZ of the lateral ventricle, and the other is the subgranular layer (SGL) in the dentate gyrus of the hippocampus (Alvarez-Buylla et al., 2001; Gage, 2000). Histological analysis of end-stage Mut1 and Mut3 brains showed that, as in human malignant astrocytoma, high-grade astrocytomas were dispersed throughout the brain (Figure S4A), and magnetic resonance imaging (MRI) provided radiographic images characteristic of human malignant astrocytoma (Figure S4B). To investigate whether tu-

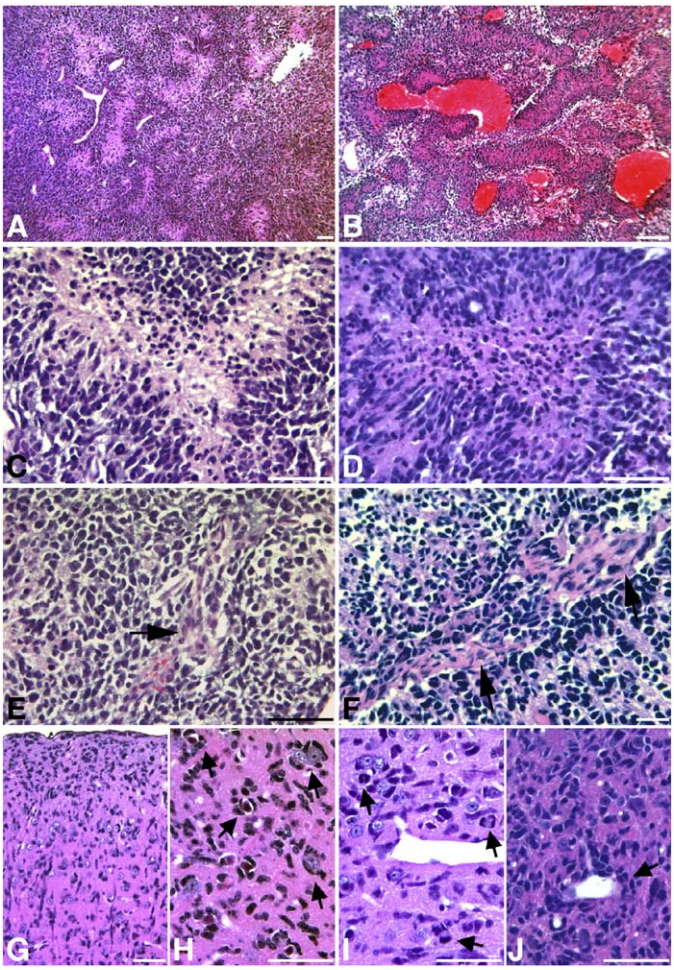

Figure 3. Histological hallmarks of GBM in Mut3 brains

Brain sections from the Mut3 brains were stained with H&E. GBMs from two independent Mut3 brains were characterized by the presence of multifocal pseudopalisading tumor cells (**A and B**), necrosis (**C and D**), microvascular proliferation (arrows in **E** and **F**), and secondary structures of Scherer, including accumulation of tumor cells in the subpial zone of the cerebral cortex (**G**), perineuronal satellitosis characterized by tumor cells surrounding neurons (arrows in **H**), and perivascular satellitosis characterized by tumor cells surrounding blood vessels (arrow in **J**). Arrows in **I** indicate mitotic figures amid the tumor cells. Scale bar, 50 μm.

mor origin was dispersed or confined to specific regions, we followed a cohort of seven asymptomatic 8-week-old Mut1 mice over a 3 week period (the last image was taken when mice were 11 weeks old). Detectable tumor growth occurred in five of these mice (Figure 6), always arising from the forebrain in association with the SVZ (Figures 6A–6C). However, tumor lesions that were barely detectable on the MRI scan (Figures 6D and 6E and Figures 6G and 6H) were of substantial size, precluding unambiguous identification of the specific site of tumor origin (Figures 6F and 6I), despite the fact that these tumors were also associated with the SVZ (Figure 6I, inset). We therefore next examined a cohort of 12 asymptomatic Mut1 mice at earlier time points between 6 and 10 weeks of age. Seven of the mutant mice showed one or two early lesions per brain. Among these seven mutant mice, five showed lesions

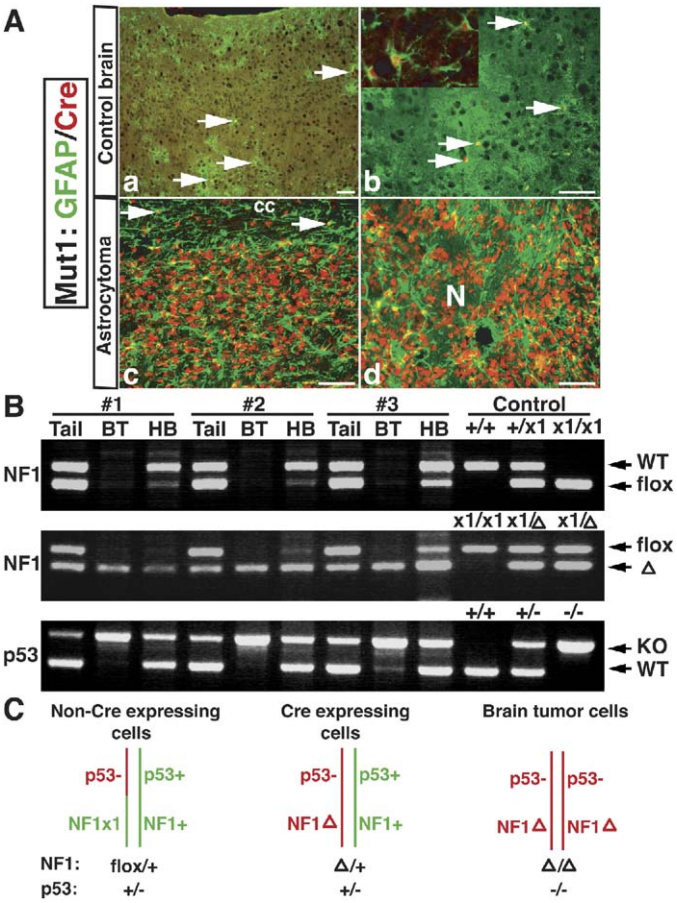

Figure 4. Inactivation of p53 and NF1 tumor suppressors in Mut1 and Mut3 astrocytomas

A: Sections from normal cerebral cortex (**Aa and Ab**), a grade III astrocytoma (**Ac**), and a GBM (**Ad**) of Mut1 brains were subjected to double-labeling immunofluorescence with anti-GFAP (green) and anti-Cre (red). In the normal adult brain, every Cre-positive astrocyte also expressed GFAP (arrows in **Aa** and **Ab**), indicating the specificity of the human GFAP promoter. The inset in **Ab** shows the morphology of the Cre-expressing GFAP-positive astrocytes. In astrocytomas and GBMs, every tumor cell expressed Cre recombinase indicative of NF1 deficiency in the Mut1 strain (**Ac and Ad**). Notably, a significant number of tumor cells downregulate GFAP expression in GBM (**Ad**). Arrows in **Ac** point to normal astrocytes adjacent to tumor tissues expressing both Cre and GFAP. CC, corpus callosum; N, necrosis. Scale bar, 50 μm.

B: Genomic DNAs isolated from tail tissues, brain tumors (BT), and hindbrain (HB) of three independent Mut3 mice (#1–#3) were subjected to PCR-based assays for genotyping the NF1 and p53 gene. Upper panel: a PCR assay that identifies the wild-type (WT) and the floxed (flox) NF1 allele showed that the tail tissues of the Mut3 mice contained one wild-type NF1 allele and one floxed NF1 allele; Cre-expressing tissues (e.g., hindbrain) retained the wild-type NF1 allele but lost most of the floxed allele as a result of Cre-mediated recombination; and brain tumors lost both the wild-type and the floxed NF1 alleles indicative of NF1 deficiency. Middle panel: a PCR assay identifying the floxed NF1 allele and recombined floxed allele (Δ) confirmed that the floxed NF1 allele in both hindbrain and brain tumors transformed into the recombined allele. Of note, a subpopulation of cells in tail tissues underwent recombination as well. Bottom panel: PCR assay identifying the wild-type and the null allele (KO) of the p53 gene showing that tail tissues and hindbrain of Mut3 mice are heterozygous for the p53 gene, while brain tumors lost the wild-type p53 allele.

C: Schematic drawing of allelic loss of NF1 and p53 in astrocytoma formation as described in **B**. The green color is used to label the normal alleles, including the wild-type allele (+) and the floxed allele (flox); the red color is used to label the null alleles, including the recombined floxed allele (Δ) and the knockout allele (−).

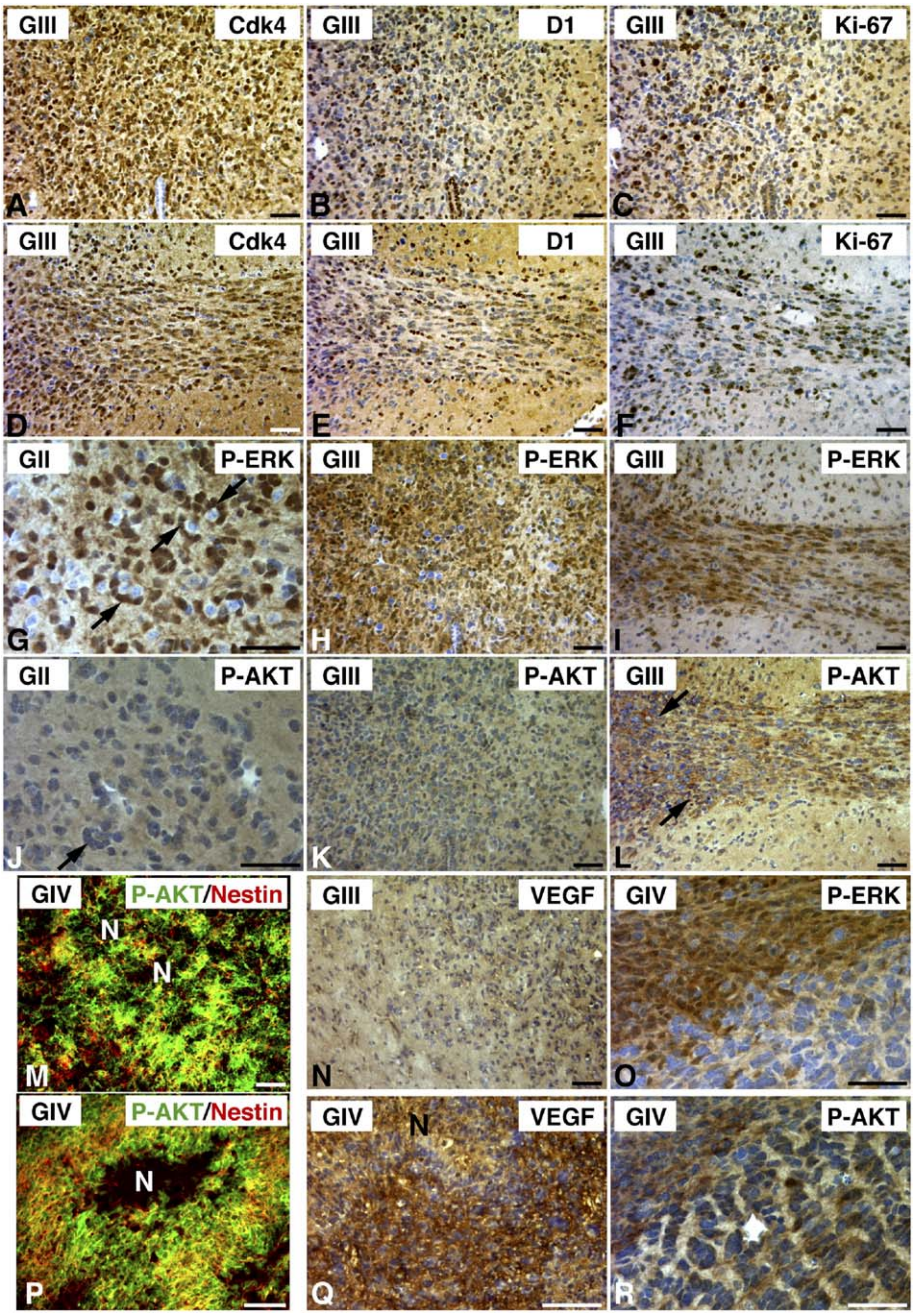

Figure 5. Molecular analysis of high-grade astrocytomas and GBMs

Adjacent sections from two grade III astrocytomas (GIII) were stained with anti-Cdk4 (**A and D**), anti-cyclin D1 (**B and E**), and a marker of proliferation, anti-Ki-67, to identify proliferating cells (**C and F**). In contrast to normal CNS cells that have little Cdk4 and cyclin D1 expression, high-grade tumors showed strong nuclear staining of Cdk4 and cyclin D1. Adjacent sections from a low-grade astrocytoma (GII) (**G and J**) and two grade III astrocytomas (GIII) (**H and K; I and L**) were stained with anti-phospho-erk (P-ERK) and anti-phospho-AKT (P-AKT), respectively. Arrows in **G** and **J** point to abnormal tumor cells surrounding neurons; arrows in **L** indicate P-AKT-positive tumor cells. Sections from a GBM were subjected to double-labeling immunofluorescence with anti-P-AKT (green) and anti-nestin (red) (**M and P**). "N," necrosis. Sections from a grade III astrocytoma (**N**) and a GBM (**Q**) were stained with anti-VEGF. Adjacent sections from a GBM were stained with anti-P-ERK (**O**) and anti-P-AKT (**R**). Scale bar, 50 μm.

directly associated with the SVZ (Figure 7), and the two others had a lesion in the hippocampus and the thalamus, respectively, but both contained abnormal cells within the SVZ (data not shown). Normally, adult neural stem cells are tightly organized in the SVZ (Figures 7A, 7D, and 7G). In contrast, early tumor cells were highly infiltrative. Early tumors showed infiltration into the white matter (Figures 7B, 7E, and 7H) and penetration into both the white matter and gray matter (Figures 7C, 7F, and 7I). Similar to normal neural stem cells (Figures 7D and 7G, arrows) (Doetsch et al., 1999), early tumor cells also expressed both nestin and GFAP (Figures 7E and 7F) and proliferated (Figures 7H and 7I). These early tumor cells were morphologically different from nestin/GFAP double-positive reactive astrocytes (Figure 7F, inset) that were identified within the high-grade tumors. The majority of Ki-67-positive cells expressed nestin (Figures 7H and 7I; 95% ± 2%; n = 96). These observations are most consistent with the interpretation that SVZ cells are most susceptible to p53/NF1-mediated astrocytoma formation and that incipient tumor cells share phenotypic characteristics with normal neural stem cells.

The infiltrative characteristics of malignant astrocytoma typically place the tumor cells within a milieu of nontumor endogenous neurons and glia. Among a total of 14 GBMs, we analyzed pure noninfiltrative tumor tissues in six GBMs. Two GBMs

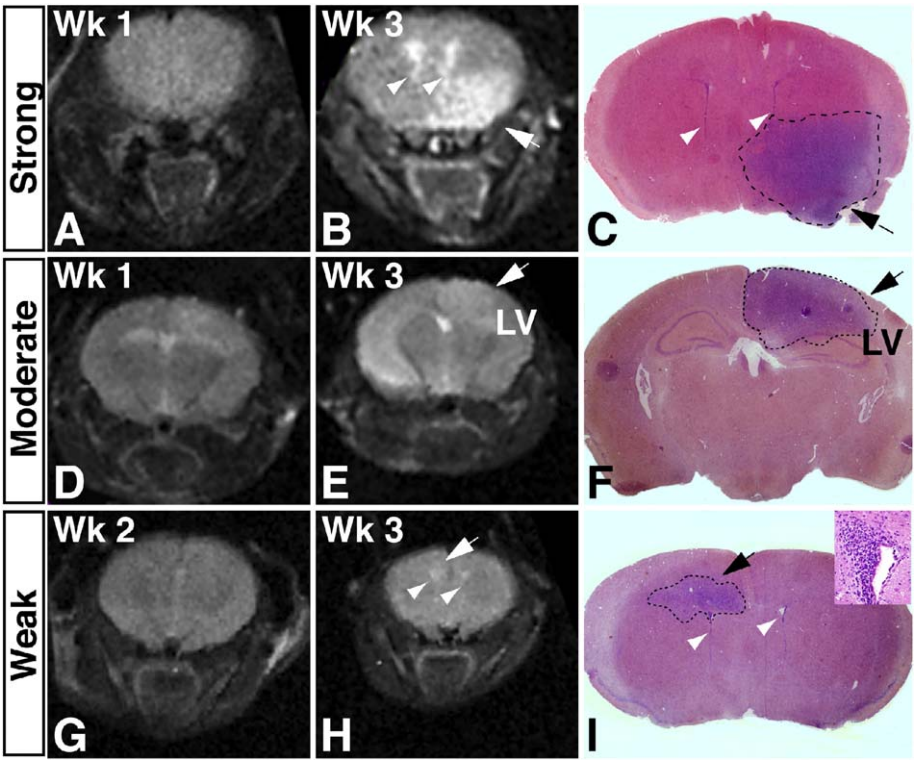

Figure 6. In vivo growth pattern of astrocytomas on the MRI scan

A cohort of asymptomatic Mut1 mice was subjected to MRI scan once a week over a 3 week period. Representative T2-weighted images of three Mut1 mice were scanned by MRI at week 1 (Wk1) (**A and D**), week 2 (**G**), and week 3 (Wk3) (**B, E, and H**). Following MRI, mice were subjected to histological analysis. Sections at similar levels of MRI images (**B, E, and H**) were stained with H&E (**C, F, and I**). No overt lesions were identified on the images scanned at early weeks (**A, D, and G**). Hyperintense T2 signals ranging from strong to moderate to weak were identified on the images at week 3 (**B, E, and H**). Arrows point to tumors, and arrowheads indicate the SVZ. The dashed lines in **C**, **F**, and **I** mark the tumor margin. The inset in **I** shows the abnormal tumor cells associated with the SVZ. LV, lateral ventricle.

contained tumor cells spreading on the surface of the brain (Figure 8A). We also obtained two GBM tumor samples that grew into the ventricular zone from the SVZ (Figure 8B). This rare direction of growth resulted in pure noninfiltrative tumor tissue. In addition, we analyzed two GBMs that formed multifocal pseudopalisading tumor cells (Figure 8C). The identity of these pure noninfiltrative tumor tissues was further confirmed by presence of a series of classic neuropathological characteristics including atypical nuclei. Immunohistochemical analysis revealed that these high-grade tumors contained cells from all neural lineages. Cells in the pure tumor tissues stained positive for the following: an astrocytic marker, GFAP (Figures 8D, 8E, and 8F); an oligodendrocytic marker, proteolipid protein (PLP), that is the most abundant myelin protein in the mammalian CNS (Figures 8G–8I) (Griffiths et al., 1998; Lu et al., 2000; Zhou et al., 2000); and neuronal markers including Tuj1 (Figures 8M–8O) and MAP2 (Figures 8P–8R). Of note, the expression of neuronal markers Tuj1 (Katsetos et al., 2001) and MAP2 (Singh et al., 2004) has previously been observed in human GBMs. Tumor cells expressed minimal or no myelin basic protein (MBP) (Figures 8J–8L), a marker for mature oligodendrocytes. The absence of MBP staining further confirmed that these tumor tissues contained minimal or no endogenous neurons or glia, as MBP is normally highly expressed in myelinating oligodendrocytes (Figure 8J, arrows). These data support the model that, in vivo, glioblastoma cells have the stem cell capacity to undergo multilineage differentiation (Singh et al., 2004).

Early p53 inactivation is critical for malignant astrocytoma formation

Genetic mutations in the p53 tumor suppressor gene are common hallmarks of progression in many solid tumors (Vogelstein et al., 2000; Vousden and Lu, 2002). In contrast, p53 mutations are among the earliest genetic lesions identified in malignant astrocytomas (Louis et al., 1993; Rasheed et al., 1994; van Meyel et al., 1994; von Deimling et al., 1992). The availability of the three Mut strains permitted us to examine whether the early timing of p53 loss affected tumorigenicity (Figure 1). Whereas the Mut1 strain harbors germline homozygous null p53 (resulting in p53 loss preceding NF1 loss); the Mut2 strain harbors a heterozygous germline p53 mutation such that, in the context of *hGFAP*-cre, NF1 loss is forced by recombination and precedes p53 loss of heterozygosity (LOH) in neural progenitors and derivatives. Finally, the Mut3 strain harbors a cisNF1flox/p53 mutant chromosome and a wild-type chromosome in CNS cells. LOH in this strain occurs by loss of the wild-type chromosome such that loss of both tumor suppressors occurs simultaneously (a process referred to as co-LOH) (Figure 1) (Cichowski et al., 1999; Reilly et al., 2000; Vogel et al., 1999). In contrast to the Mut1 and Mut3 strains, the majority of Mut2 mice analyzed (17/18) did not develop astrocytomas (Figures 1 and 2K). About 20% of Mut2 mice survived to the same age as tumorigenic Mut3 mice but did not develop astrocytomas (Figure 2J). Approximately 80% of Mut2 mice died at a significantly earlier age compared to Mut3 mice. Since the incidence of non-CNS tumors observed in Mut2 (4/21, 19%) and Mut3 (11/58, 19%) was almost identical, we cannot attribute the death of Mut2 mice to p53-associated tumors. We speculate that premature death of Mut2 mice may result from NF1 deficiency in developing CNS cells leading to abnormal CNS development (Zhu et al., submitted). To rule out the possibility that the premature death may preclude these Mut2 mice from developing astrocytomas, we studied three

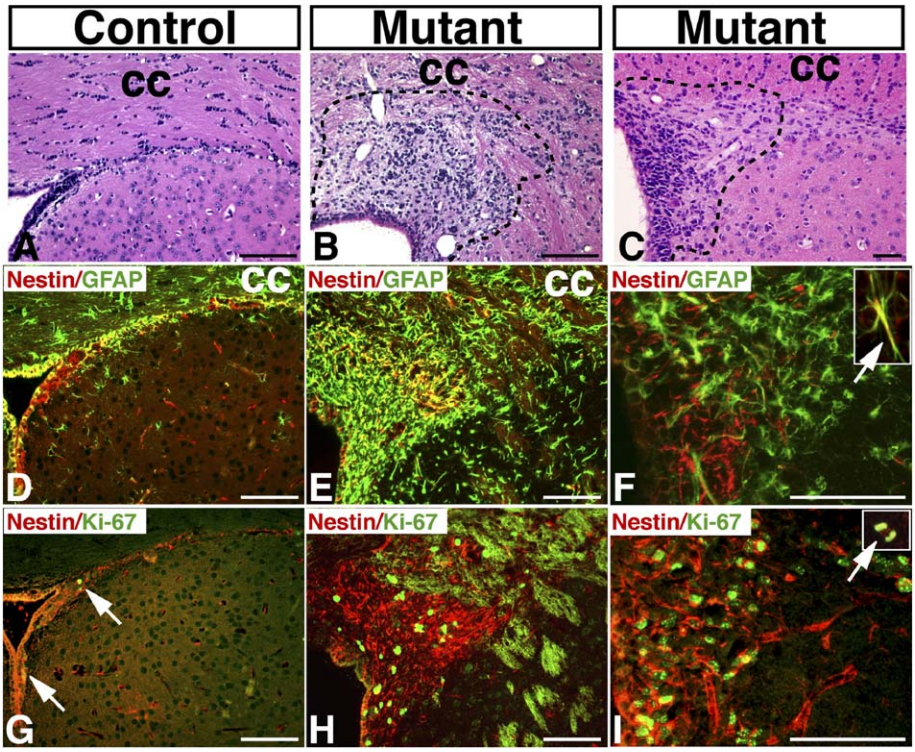

Figure 7. Astrocytomas are confined to the subventricular zone of the lateral ventricle

Adjacent sections from the SVZ of a control and two mutant brains were stained with H&E (**A–C**) and immunofluorescence by anti-nestin (red)/anti-GFAP (green) (**D–F**) and anti-nestin (red)/anti-Ki-67 (green) (**G–I**). Of note, mutant brains had nascent tumors associated with the SVZ. The tumor margin is marked by the dashed lines (**B and C**). The inset in **F** (arrow) shows a nestin/GFAP double-positive reactive astrocyte, and the inset in **I** (arrow) shows a nestin/Ki-67 double-positive (arrow) cell undergoing mitosis. CC, corpus callosum. Scale bar, 100 μm.

asymptomatic Mut3 mice at 18 weeks of age, a time point when over 85% of Mut2 mice were alive and tumor free (Figure 2J, arrowhead). Histological analysis revealed that two of three asymptomatic Mut3 mice already exhibited low-grade astrocytomas (Figure 2K and Figure S2). We therefore conclude that the target cells that give rise to malignant astrocytoma require p53 loss prior to or concomitant to NF1 loss (Ras activation). In the converse configuration, early loss of NF1 fails to provide selective advantage to the target cells for tumor initiation. These genetic data demonstrate that the early loss of p53 is essential for astrocytoma formation in these mouse models.

Discussion

Mouse models

Detailed molecular characterization of human astrocytoma has provided considerable insight into the many oncogenes, tumor suppressors, and signaling pathways that become altered in the processes of tumor initiation and progression (Holland, 2001; Maher et al., 2001; Zhu and Parada, 2002). The present study demonstrates that loss of p53 and activation of the Ras pathway via NF1 inactivation in CNS cells is sufficient to cause malignant astrocytoma formation with 100% penetrance. Although malignant astrocytomas of all grades were identified in both Mut1 and Mut3 models, exclusively low-grade lesions were found only in presymptomatic mice, except for two Mut1 mice with p53-associated lymphomas. As compared to well-differentiated low-grade astrocytomas in humans, tumors of similar grade in these models display greater degree of nuclear atypia. Along with these observations, rapid development of GBMs in both Mut1 (from 10 to 20 weeks) and Mut3 (from 20 to 45 weeks) mice suggests that these models may resemble primary GBM in humans. Consistently, a recent population-based study reported that p53 mutations were identified in about 28% of human primary GBMs, a subset of which (7.5%) also harbored amplification of epidermal growth factor receptor (EGFR) (Ohgaki et al., 2004).

The role of the NF1 tumor suppressor gene in malignant astrocytoma

Although benign astrocytoma in the optic pathway (optic pathway glioma) occurs in 15% to 20% of children with NF1, the NF1-associated risk of developing malignant astrocytomas has not been determined until recently. One study using U.S. death certificates from 1983 to 1997 reported that brain tumors occurred 5.5 times more frequently in NF1 patients than in the general population (Rasmussen et al., 2001). Particularly, the relative risk of brain tumors in NF1 patients older than 10 years was estimated to reach as much as 100 times greater than those without NF1. Many of the tumors from such patients were high-grade malignant astrocytomas (Gutmann et al., 2002). Furthermore, molecular analysis revealed that NF1-associated malignant astrocytomas harbored genetic alterations, including p53 mutations and p16INK4A/ARF deletions, that are commonly observed in sporadic counterparts (Gutmann et al., 2003). Together, these observations not only provide evidence that p53 inactivation can cooperate with NF1 loss in the development of human malignant astrocytomas, but also suggest that NF1-associated and sporadic malignant astrocytomas may share similar molecular mechanisms underlying tumor progression. Thus, our models may provide a useful tool to dissect the molecular basis of tumor progression

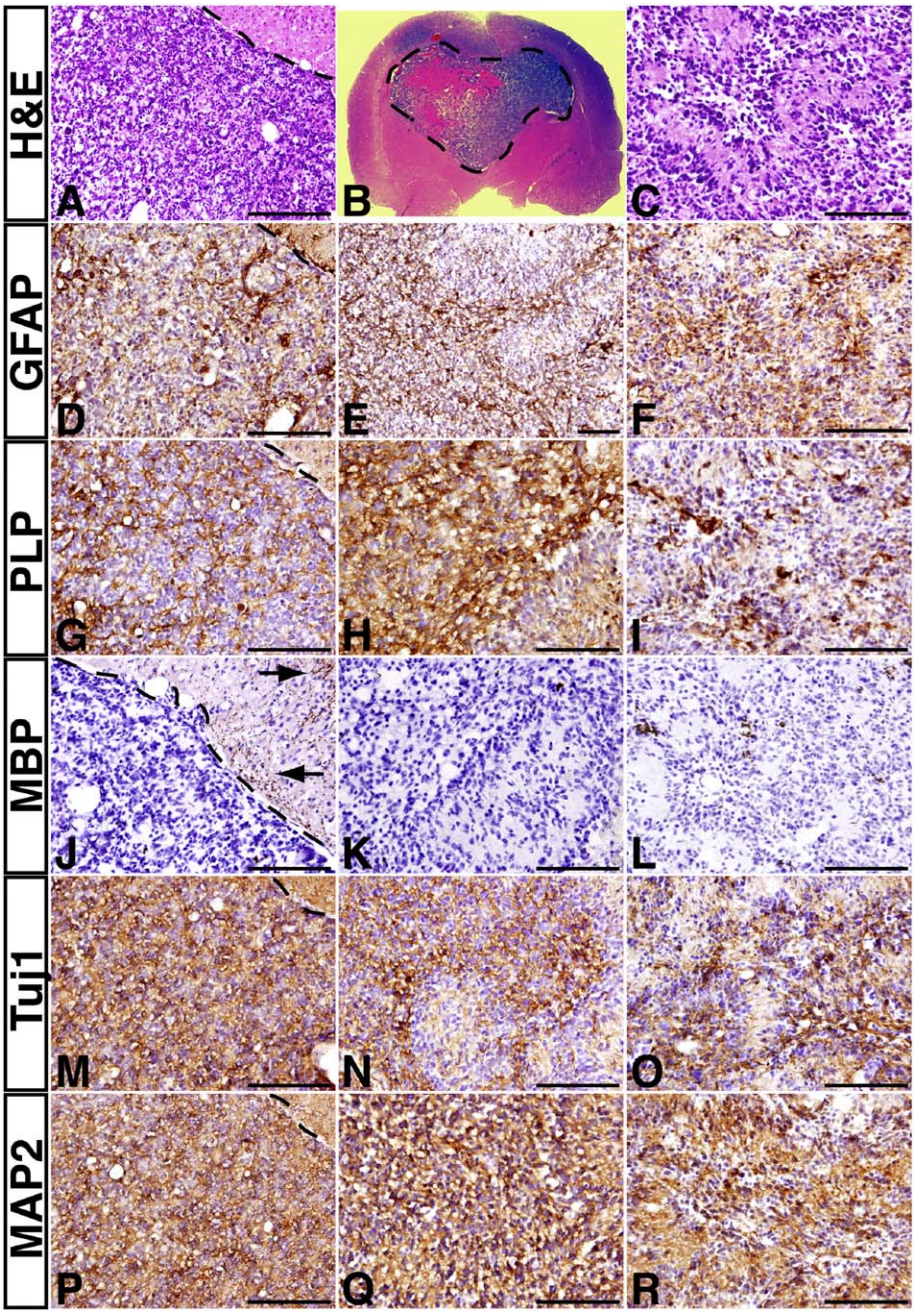

Figure 8. GBM cells have the stem cell capacity to undergo multilineage differentiation in vivo

A–C: Sections from three representative GBMs that contained pure noninfiltrative tumor tissues were stained with H&E. The dashed lines in **A** and **B** mark the border between the main tumor mass and surrounding brain tissues. Adjacent sections of **A**–**C** were stained with anti-GFAP (**D–F**), anti-PLP (**G–I**), anti-MBP (**J–L**), anti-Tuj1 (**M–O**), and MAP2 (**P–R**). Of note, in contrast to normal brain that contains extensive MBP staining (arrows in **J**), minimal or no staining was detected in these pure noninfiltrative tumor tissues (**J–L**). Scale bar, 100 μm.

underlying both NF1-associated and sporadic malignant astrocytomas.

Although one of the first characterized NF1 mutations in somatic cells was identified in a sporadic grade III malignant astrocytoma (Li et al., 1992), loss of NF1 mRNA or protein expression was not observed in sporadic astrocytomas in a subsequent study using a large number of human primary tumors and cell lines (Gutmann et al., 1996). Thus, it is generally believed that NF1 is not involved in sporadic malignant astrocytomas, which instead frequently activate Ras pathways via deregulated PDGFR or EGFR (Holland, 2001; Kleihues and Cavenee, 2000; Maher et al., 2001; Zhu and Parada, 2002). Particularly, p53 mutations and overexpression of PDGFR are often identified in the same tumors, suggesting a cooperative effect between these two pathways in the development of human malignant astrocytoma (Hermanson et al., 1996). Furthermore, the cooperative effect between p53 inactivation and PDGF overexpression has also been established in a mouse model using murine retroviral gene transfer (Hesselager et al., 2003). However, in a recent study using the replication-competent avian leukemia virus splice acceptor (RCAS)/tv-a system, targeted overexpression of a PDGF ligand (PDGF-B) into nestin- and GFAP-expressing cells in neonatal mouse brains induced oligodendrogliomas and oligoastrocytomas, respec-

tively (Dai et al., 2001). Furthermore, loss of p53 did not enhance the formation of these PDGF-induced tumors. Possible explanations for the discrepancy between experimentally induced tumors via p53/NF1 or PDGF alterations include potentially different cell-of-origin for these tumors, and differences in genetic backgrounds. The present studies demonstrate that the most likely location for the cell-of-origin for malignant astrocytoma in p53/NF1 mutant mice is in the SVZ of the adult brain (see below). The localization and origin of PDGF-induced tumors arising in neonatal brain cells remains unclear. The target cells in that model apparently have greater potential to undergo oligodendrocytic differentiation (Sauvageot and Stiles, 2002).

The site of tumor origin

Recent studies indicate that brain tumors, including GBM, contain a subpopulation of stem cell-like cells or cancer stem cells that display stem cell characteristics, including self-renewal and multipotentiality (Hemmati et al., 2003; Ignatova et al., 2002; Singh et al., 2003, 2004), and are responsible for in vivo tumor growth (Galli et al., 2004; Singh et al., 2004). Whether these brain cancer stem cells are derived from neoplastic transformation of normal neural stem cells remains to be determined. As compared to previously published mouse models for malignant astrocytoma (Ding et al., 2001; Hesselager et al., 2003; Holland et al., 1998, 2000; Reilly et al., 2000; Xiao et al., 2002), the complete penetrance of the tumor phenotype and rapid tumor development in Mut1 model allowed histological and noninvasive MRI analysis to investigate the site of tumor origin. The results indicate that, as in human GBM, mature tumors are dispersed throughout the CNS, and yet the earliest identifiable area of a tumor is confined to the SVZ. In the genetic setting of the Mut1 model, all GFAP-positive mature astrocytes or committed glial progenitor cells throughout the adult brain contain the same genetic mutations (inactivation of both p53 and NF1 tumor suppressors), as do the adult neural stem cells within the SVZ. Therefore, the observation that malignant astrocytomas specifically are located within the SVZ indicates that (1) there exists a specific cell type(s) in the SVZ that is more susceptible to p53/NF1-mediated astrocytoma formation as compared to those in other regions of the brain, or (2) the microenvironment in the SVZ provides a favorable niche for the growth of early tumor cells, which either arise within the SVZ or migrate there from the other regions of the brain. Based upon current knowledge, one fundamental difference between the SVZ and the other regions of the adult brain (except for the SGL of the hippocampal dentate gyrus) is the existence of specific GFAP-positive "astrocyte"-like cells, which possess the capacity of stem cells to undergo self-renewal and multilineage differentiation (Alvarez-Buylla et al., 2001; Gage, 2000; Temple and Alvarez-Buylla, 1999). It has been shown that these GFAP-positive neural stem cells are present in the SVZ of both human and rodent adult brains (Doetsch et al., 1999; Sanai et al., 2004). In light of these observations, our results suggest that the GFAP-positive neural stem cells in the SVZ of the adult brain are a likely candidate to be the cell-of-origin for malignant astrocytomas in our models.

A recent study demonstrated that neonatal astrocytes may serve as a cell-of-origin for malignant astrocytoma in response to deregulated EGFR signaling and INK4a/ARF deficiency. Thus, it is possible that different cell types in the CNS, including neural stem/progenitor cells, glial progenitor cells (e.g., NG2-positive glia), or even terminally differentiated astrocytes, may serve as a cell-of-origin for malignant astrocytoma. The combination of different glioma-associated genetic lesions may induce different CNS cell types to form malignant astrocytoma. Therefore, our models may mimic a subset of human malignant astrocytomas with p53 deficiency and activated Ras signaling, which may arise from the SVZ cells.

The timing of genetic mutations

We also provide genetic evidence that not only is inactivation of both p53 and NF1 sufficient for initiation of astrocytoma formation, but the timing of inactivation is critical. For astrocytoma induction, p53 inactivation must either precede or coincide with Ras activation via NF1 loss. This is consistent with the observation that mutations in the p53 gene are among the earliest genetic lesions identified in human malignant astrocytoma (Holland, 2001; Maher et al., 2001; Zhu and Parada, 2002). In many other solid tumors, including colon cancer and malignant peripheral sheath tumors (Kinzler and Vogelstein, 1996; Zhu and Parada, 2002), p53 inactivation occurs in later stages of tumor development when tumor cells already display invasive phenotypes. Thus, either early p53 inactivation provides a selective advantage for astrocytoma cells to acquire early onset invasive phenotype or activation of Ras signaling via NF1 loss leads to apoptosis or senescence of target cells, which can be attenuated by p53 inactivation.

Although activated Ras mutations have not been identified in human malignant astrocytoma, in mice, overexpression of an activated H-Ras transgene under the control of the hGFAP promoter leads to the development of malignant astrocytomas with high penetrance (Ding et al., 2001). This is in striking contrast to the observations that loss of NF1 alone (e.g., Mut2 mice in this study) is not sufficient to cause astrocytoma formation in the brain (Bajenaru et al., 2002). Apparently, oncogenic Ras signaling is not equivalent to NF1 inactivation. Given that human malignant astrocytoma typically arises during adulthood, it is unlikely that human patients contain a significant number of developing CNS cells with high levels of Ras signaling as the Ras transgenic model does. In contrast, mutant cells with p53/NF1 deficiency via co-LOH in the Mut3 model most likely arise during adulthood, as these mutant mice had normal development and did not develop tumors until 5 months of age. In this regard, the Mut3 mutant mice may provide a better model that mimics human malignant astrocytoma as an adult disease compared to the Ras transgenic mice.

Clinical implication

Our study cannot rule out alternative models for astrocytoma initiation such as cellular dedifferentiation and transformation (Bachoo et al., 2002; Galli et al., 2004; Singh et al., 2004). The expression of nestin and other lineage-specific markers in tumor cells can simply reflect deregulated gene expression. Whether these nestin-expressing cells within tumors represent the cancer stem cells remains to be determined. Nevertheless, identification of the SVZ as the site of tumor origin in our models may have implications on therapeutic strategies for preventing and treating a subset of human malignant astrocytomas (Recht et al., 2003). These observations challenge models of gliomagenesis, which assume tumor development from at large transformed glia or committed glial progenitors. As a con-

sequence, for a tumor that arises from the SVZ, current treatment focusing merely on local lesions will not be sufficient to eradicate tumor cells, as these cells may be eventually replenished from the cell pool within the SVZ. This may offer one explanation of why the current treatment for malignant astrocytoma ultimately fails.

Experimental procedures

Generation of Mut1–3 mutant mice

The mutant mice harboring the linked p53 null mutation and the floxed NF1 allele on the same chromosome (*cis* mice) were generated by crossing $NF1^{flox/+}$ mice to $p53^{+/-}$ mice to generate $p53^{+/-};NF1^{flox/+}$ *trans* mice that were then crossed to wild-type F1 129Svj/C57Bl6 mice (Jacks et al., 1994; Zhu et al., 2001). As for $NF1^{flox/+}$ mice and $p53^{+/-}$ mice, $p53^{+/-};NF1^{+/flox}$ *cis* mice were maintained on 129Svj/C57Bl6 hybrid background. The Mut3 strain was generated by crossing $p53^{+/-};NF1^{+/flox}$ *cis* mice to *hGFAP*-cre transgenic mice that were generated on the FVB background (Zhuo et al., 2001). The resultant Mut3 strain was crossed five generations onto 129Svj/C57Bl6 hybrid background. Mut1 and Mut2 strains were generated by crossing the Mut3 mice to $p53^{+/-};NF1^{flox/flox}$ *cis* mice. Therefore, Mut1–3 mice analyzed in this study were maintained on approximately 99% 129Svj/C57Bl6 (49.5%/49.5%) and 1% FVB hybrid background. Many Mut1–3 mice analyzed in this study were littermates. Mice with the floxed NF1 allele, p53 null allele, and Cre transgene were genotyped as described previously (Jacks et al., 1994; Zhu et al., 2001). All mice in this study were cared for according to the guidelines that were approved by the Animal Care and Use Committees of the University of Texas Southwestern Medical Center at Dallas and University of Michigan at Ann Arbor.

Histology and tumor analysis

Mice were aged until signs of distress appeared. Then, mice were perfused with 4% paraformaldehyde (PFA), and brains were dissected, followed by overnight postfixation in 4% PFA at 4°C. Brains were divided into three pieces along the rostral to caudal axis, and each piece was processed for either paraffin-embedded or cryostat sections. Serial sections were prepared at 5 μm for paraffin sections or 14 μm for cryostat sections. Every tenth slide was stained by H&E. Stained sections were examined under light microscope by Y.Z. and D.K.B. independently. Tumor grading was determined by Y.Z. and D.K.B. based upon the WHO grading system for malignant astrocytoma (Kleihues and Cavenee, 2000). Adjacent sections were subjected to immunohistochemistry (see below). For PCR analysis, tumor tissues were dissected in ice-cold PBS and digested with proteinase K as described (Zhu et al., 2002).

Immunohistochemistry

Paraffin sections were deparaffinized and rehydrated. Sections were subjected to immunohistochemistry as described previously (Zhu et al., 1998, 2001). The visualization of primary antibodies was performed with either a horseradish peroxidase system (Vectastain ABC kit, Vector) or immunofluorescence by using Cy3-conjugated anti-rabbit/mouse and Cy2-conjugated anti-mouse/rabbit secondary antibodies (Jackson lab) at 1:200 dilution. The dilutions of primary antibodies used in this study were as follows: GFAP (rabbit, 1:2000, DAKO), nestin (mouse, 1:200, Chemicon), Cre (mouse, 1:1000, BABCO), P-ERK (rabbit, 1:200, Cell Signaling), P-AKT (rabbit, 1:100, Cell Signaling), Cdk4 (rabbit, 1:200, Santa Cruz), cyclin D1 (mouse, 1:200, Zymed), Ki-67 (rabbit, 1:1000, Novocastra), VEGF (mouse, 1:50, Upstate Biotech), Olg2 (rabbit, 1:300, a gift of Dr. R. Lu), NeuN (mouse, 1:500, Chemicon), Tuj1 (mouse, 1:200, Covance), NF200 (rabbit, 1:200, Sigma). Sections were examined under either a light or a fluorescence microscope (Olympus). The colocalization of two antigens was further confirmed by confocal microscopy (Zeiss).

MRI analysis

Mutant mice were scanned when they exhibited signs of distress. In preparation for MRI, a mouse was given Avertin (0.4 ml, 20 mg/ml; i.p.) and maintained under general gaseous anesthesia (1 dm^3/min air and 0.8% isoflurane). A mouse tail vein was catheterized using a 27G butterfly (Abbott Laboratories, Abbott Park, IL) for contrast agent administration. MRI experiments were performed using a 4.7 T horizontal magnet equipped with a Varian Inova imaging system. The mouse was placed in a home-built eight-element birdcage coil (~2 cm in diameter). Fast scout images in a sagittal orientation indicated pertinent slices for high-resolution images. T1-weighted (TR = 250 ms; TE = 15 ms) and T2-weighted (TR = 1500 ms; TE = 80 ms) spin echo multislice (SEMS) axial images were acquired, and contrast-enhanced T1-weighted images were acquired immediately after i.v. bolus injection of Gd-DTPA-BMA (0.1 mmol/kg body weight; Omniscan). MRI parameters included a field of view (FOV) of 3–4 cm, a matrix size of 128 × 128, a slice thickness of 1 mm, and two acquisitions. Following MRI, the mouse was sacrificed, and a whole mouse brain was dissected and was subjected to histological analysis, as described above. Follow-up study examined asymptomatic mutant mice once a week over a 3 week period to detect tumor development.

Supplemental data

The Supplemental Data include four figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/8/2/119/DC1/.

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