Ink4a/Arf loss promotes tumor recurrence following Ras inhibition


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Aberrant activation of rat sarcoma (Ras) signaling contributes to the development of a variety of human cancers, including gliomas. To determine the dependence of high-grade gliomas on continued Ras signaling, we developed a doxycycline-regulated Kirsten Ras (KRas) glioma mouse model. We previously demonstrated that KRas is required for the maintenance of glioblastoma multiforme tumors arising in the context of activated Akt signaling in vivo; inhibition of KRas expression resulted in apoptotic tumor regression and significantly increased survival. We utilized a well-established glioma mouse model to determine the reliance of gliomas on continued KRas signaling in the context of Ink4a/Arf deficiency, a common occurrence in human gliomas. Despite the dependency of primary gliomas on continued KRas signaling, a significant percentage of tumors progressed to a KRas-independent state in the absence of Ink4a/Arf expression, demonstrating that these tumor suppressors play a critical role in the suppression of glioma recurrence. While even advanced stages of gliomas may remain dependent upon KRas signaling for maintenance and growth, our findings demonstrate that loss of Ink4a/Arf facilitates the acquisition of oncogene independence and tumor recurrence. Furthermore, reactivation of the Ras mitogen-activated protein kinase pathway in the absence of virally delivered KRas expression is a common mechanism of recurrence in this context.

Keywords: glioma, Ink4a/Arf, mouse, Ras, tumor maintenance.

Mutations in RAS occur in approximately 30% of all human cancers. Because these signals result in deregulated cell growth and division, they can ultimately lead to oncogenesis; however, in several tumor types, aberrant rat sarcoma (Ras) activity may not be due to mutation in RAS itself but rather a consequence of its induction by upstream oncogenic signals. Alterations in receptor tyrosine kinase (RTK) growth factor receptors (EGFR, PDGFR, MET, and ERBB2), resulting in activation of Ras, are found in almost all World Health Organization grade II, III, and IV gliomas. A significant percentage of high-grade gliomas also have homozygous deletions that inactivate the cyclin-dependent kinase inhibitor 2A (CDKN2A) locus.1 This locus encodes 2 independent protein products, p16INK4a and p14ARF.2,3 By inhibiting the kinase activity of CDK4 and CDK6, p16INK4a blocks retinoblastoma protein (RB) phosphorylation and prevents progression from G1 to S in the cell cycle.3 The protein p14ARF (p19ARF in mice)4 has the physiological role of stabilizing levels of tumor protein (TP) 53 and cell cycle arrest in G1 and G2 in response to oncogenic stimuli.5,6 The mechanism by which p14ARF stabilizes TP53 levels is neutralization or elimination of human homologue of murine double minute 2 (HDM2).5 Disruption of the TP53 and RB pathways also occurs in glioblastoma multiforme (GBM) through direct mutation or deletion of TP53 and RB or amplification of HDM2 or CDK4, respectively.7

Most cancers contain multiple genetic alterations, making it difficult to determine which genes or combination of genes will be the most effective targets for therapy; whether any of these tumors are dependent on Ras for maintenance is unknown. Mouse models provide a tractable system to recapitulate the stepwise progression of human tumors. Models that permit regulated expression of oncogenes are especially useful for modeling the effects of targeted therapies because the abrogation of oncogene expression simulates pharmacological inhibition of the target. The use of a conditional genetic approach to inhibit oncogenic signaling allows the effects of targeted therapy to be modeled in vivo. To determine the effect of downregulating Ras signaling in tumors initiated by Ras activation, we utilized a mouse model based on the replication-competent avian sarcoma (RCAS)/tumor virus A (TVA) system and
Ink4a expression could be controlled using the tetracycline (tet)-regulated system postdelivery to somatic cells in vivo. Using this system, we have shown that downregulation of KRas in established tumors induced by activated KRas and Akt results in rapid tumor regression and increased survival. In this model, the TVA receptor is expressed from the nestin promoter (N-TVA), specifically in neural and glial stem and progenitor cells. This model can also be used to evaluate the loss of expression of specific genes using Cre/lox technology. KRas expression combined with deletion of Ink4a/Arf leads to the development of high-grade gliomas in mice. In this study, we examined the reliance of gliomas on continued KRas signaling in the context of Ink4a/Arf deficiency. Tumors lacking Ink4a/Arf underwent significant regression following abrogation of KRas expression, and the median survival for doxycycline-treated mice was significantly longer than that for untreated mice. Complete responses were observed in a fraction of the treated mice, but this response was not durable, as doxycycline withdrawal and subsequent re-expression of KRas induced relapse.

**Materials and Methods**

**Mice and Genotyping**

Nestin-TVA/Ink4a/Arf\(^{\text{fox/lox}}\) mice and genotyping procedures have been described. The Nestin-TVA mice were on a mixed genetic background consisting of Friend leukemia virus B (FVB)/n, 129, and C57Bl/6. Ink4a/Arf\(^{\text{fox/lox}}\) mice were on an FVB/n background. PCR genotyping for the TVA transgene was performed as described, as was that for the Ink4a/Arf\(^{\text{fox}}\) and wild-type Ink4a/Arf alleles.

**Establishment of Glioma Cell Lines and Cell Culture Conditions**

Glioma cell lines were established following dissection of primary tumors by physical disruption into single cells using scalpels and trypsin. Glioma cultures and Doug Foster (DF)-1 cells were maintained in Roswell Park Memorial Institute media (RPMI) and Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Glioma cell lines were grown at 37°C, while DF-1 cells were grown at 39°C.

**Viral Constructs**

The retroviral vectors used in this study are replication-competent avian leukosis virus (ALV) terminal repeat (LTR), splice acceptor, and Bryan polymerase-containing vectors of envelope subgroup A (designated RCASBP(A)). RCANBP(A)/TRE-KRas, RCASBP(A)/et-off, and RCASBP(A)/Cre have been described. To propagate the RCAS viruses, the viral vectors were transfected into DF-1 cells using the calcium phosphate transfection method. RCAS vectors were replication-competent in the DF-1 cell line (an immortalized chicken fibroblast line), and high titer viral stocks can be obtained. Viral titers were determined as described. The supernatants were filtered through a 0.45-μm filter.

**Viral Infections in Vitro**

Astrocytes were seeded in 6-well plates at a density of 5 × 10⁴ cells/well and were maintained in RPMI with 5% FBS, 1% penicillin/streptomycin at 37°C. After the cells attached, 1 mL of filtered virus-containing medium was added in the presence of 8 μg/mL polybrene (Sigma) for 2 h at 37°C.

**In Vivo Infection**

Infected DF-1 cells from a confluent culture in a 10-cm dish were trypsinized, pelleted, resuspended in 50 μL phosphate buffered saline (PBS), and placed on ice. Newborn mice were injected intracranially 2 mm ventral from the bregma (intersection of the coronal and sagittal sutures) with 5 μL of infected DF-1 cells using a gas-tight Hamilton syringe.

**Histological Analysis**

Brain tissue from injected mice was fixed in formalin and paraffin embedded, and 5-μm sections were adhered to glass slides. Images were captured using a Zeiss Axioscope microscope equipped with an AxiosCam ICc3 camera (Zeiss).

**Immunohistochemistry**

Tissue sections were deparaffinized, and antigen retrieval was performed in Diva Decloaking buffer (Biocare Medical) by boiling for 10 min. Sections were treated with 3% hydrogen peroxide and blocked in Background Sniper (Biocare Medical) for 10 min. Primary antibodies were diluted in Renaissance background reducing diluent (Biocare Medical). Sections were incubated overnight at 4°C and probed with Mach 4 rabbit polymer reagent (Biocare Medical) or Mach 4 mouse probe for 15 min followed by Mach 4 polymer for 15 min for mouse monoclonal antibodies. Visualization was carried out with 3,3′-diaminobenzidine (DAB) (Biocare Medical). Sections were counterstained with hematoxylin. KRas expression was detected using an antibody to the FLAG epitope (F7425, diluted 1:200; Sigma). Akt expression was detected using an antibody to the hemagglutinin (HA) epitope (HA.11, diluted 1:200; Abcam). p19ARF (ab80, diluted 1:200; Abcam). Cell proliferation was detected using an antibody to Ki67 (M7246, diluted 1:50; Dako). Apoptosis was detected by staining via terminal deoxynucleotidyl transferase dUTP (2′-deoxyuridine 5′-triphosphate) nick end labeling (TUNEL) using an in situ cell death detection kit (Roche) per the
manufacturer’s specifications. Expression of phosphatase and tensin homolog (Pten) was detected using a rabbit monoclonal antibody (9188, diluted 1:150; Cell Signaling). Assessment of neurofibromatosis 1 (NF1) expression was performed using a rabbit polyclonal antibody (sc67, diluted 1:1000; Santa Cruz Biotechnology); an NF1-specific blocking peptide was used as a control (sc67-p, Santa Cruz Biotechnology). Detection of MAPK activation was performed using a 1:200 dilution of a phospho–extracellular signal-regulated kinase (Erk) antibody (4370; Cell Signaling). Phosphorylation of S6 ribosomal protein at Ser235/236 was detected using a 1:150 dilution of a rabbit monoclonal antibody (4858; Cell Signaling). Stained cells were quantitated in triplicate using Image J software.

Western Blotting

Protein concentrations were determined using the DC Protein Assay (Bio-Rad). The proteins were separated on a 16% Tris-glycine polyacrylamide gel, transferred to nitrocellulose, and incubated for 1 h at room temperature in blocking solution (0.05% Tween 20 in Tris-buffered saline with 5% nonfat dried milk). Blots were immunostained with the primary antibody following the manufacturer’s instructions. Blots were immunostained for Ras expression using a pan-Ras antibody (05-516; Millipore) diluted 1:250; for phosphorylation of Erk at Thr202/Tyr 204 using a rabbit monoclonal antibody (4370; Cell Signaling) diluted 1:1000; for total Erk using an anti-ERK1/2 rabbit monoclonal antibody (9102, Cell Signaling) diluted 1:1000; and for anti-α-tubulin using a mouse monoclonal antibody (T9026, Sigma) diluted 1:5000. The blots were then incubated with an anti-mouse or anti-rabbit immunoglobulin G–horseradish peroxidase secondary antibody and incubated with enhanced chemiluminescence solution (Amersham) and exposed to film.

Statistical Analysis

Censored survival data were analyzed using a log-rank test of the Kaplan–Meier estimate of survival. Density analysis was performed using Image J, and the data are represented as mean ± SEM.

Magnetic Resonance Imaging

Images were acquired on a 7T Bruker MRI using a T1-weighted multi-spin–multi-echo (MSME) or axial T2-weighted TurboRARE sequences. Details are available upon request.

Mutation Screening

Paired tumor/normal tissue samples were collected from 10 μm formalin-fixed, paraffin-embedded tissue sections using a fine needle and placed in a 1.5-mL tube. Following de-waxing in Xylene, the tissue was rehydrated using graded alcohols (100%–50% ethanol) and digested with proteinase K overnight at 55°C (95 μL of 1 × Qiagen PCR buffer and 5 μL of 20 mg/mL Qiagen proteinase K solution). PCR was then performed with 2 μL of the resulting solution using the Qiagen Hotstart master mix kit. PCR cleanup was carried out using the QIAquick PCR purification kit (Qiagen), and 10 ng of PCR product was sequenced using the forward and reverse PCR primers by the Nevada Genomics Center (primer sequences available on request).

Results

High Penetration of Glioma Formation Initiated by Conditional Expression of KRas in the Context of Ink4a/Arf Deficiency

We previously generated a viral vector that allows expression of KRas to be regulated postdelivery using the tet-regulated system. A tet-responsive element (TRE) was inserted upstream of the KRas gene in the replication-competent avian retrovirus vector RCAN BP(A) such that KRas expression was driven from the TRE and not the viral LTR. Expression from the TRE requires the presence of a tetracycline transcriptional activator (tTA) such as tet-off or a reverse tTA (rtTA) such as tet-on. In the context of tet-on, the tet-responsive gene is expressed only in the presence of doxycycline (Dox); in the context of tet-off, the tet-responsive gene is repressed in the presence of Dox. Using this approach in the context of tet-off, we demonstrated that genetic suppression of KRas expression by Dox administration causes apoptotic regression of KRas- and Akt-induced gliomas in Nestin-TVA mice; however, it is unclear whether tumors that develop in the absence of Ink4a/Arf also remain dependent on KRas to maintain the neoplastic phenotype. This is relevant because deletion of the Ink4a/Arf locus occurs in ~50% of GBM. To determine the reliance of these tumors on continued KRas signaling in the context of Ink4a/Arf deficiency, Nestin-TVA mice were intercrossed to mice carrying a conditional Ink4a/Arf allele, Ink4a/Arflox/lox, to generate Nestin-TVA;Ink4a/Arflox/loy mice. In the absence of Cre, homozygous Ink4a/Arflox/lox mice express normal levels of p16Ink4a and p19Arf. We have previously demonstrated high-grade glioma formation in a high percentage of these mice by injection with KRas- and Cre-containing retroviruses. Likewise, intracranial injection of TRE-KRas, tet-off, and Cre containing retroviruses into newborn Nestin-TVA;Ink4a/Arflox/lox mice resulted in tumor formation in all injected mice. Importantly, TVA-negative;Ink4a/Arflox/lox mice infected with the same viruses remained tumor free (P = 4.8 × 10−7; Fig. 1A). Infection with KRas or Cre viruses alone is insufficient for tumorigenesis in these mice. In addition, there is no significant incidence of brain tumors in Ink4a/Arf-null mice within 100 days of age. Thus, delivery of these viruses in combination results in highly penetrant gliomagenesis in this model.
tumors express the tet-regulated KRas, and therefore, this model provides the opportunity to study whether expression of KRas is required for tumor maintenance in various contexts.

**Suppression of KRas Expression Results in Tumor Regression and Increased Survival**

To determine whether downregulation of KRas expression in KRas- and Cre-initiated gliomas results in tumor regression, tumors were induced in Nestin-TVA;Ink4a/Arflox/lox mice by intracranial injection of TRE-KRas-tet-off, and Cre-containing viruses at birth. MRI was used to identify tumor-bearing mice at weaning and to monitor tumor growth or regression at multiple time points throughout the study. Dox was fed to one cohort of tumor-bearing mice beginning at 21 days of age to suppress KRas expression, while a separate cohort of tumor-bearing mice received standard feed. The average tumor size prior to Dox treatment was $24 \pm 7.3$ mm$^3$. Survival rates were compared between untreated mice and mice given Dox to determine whether the administration of Dox, and subsequent loss of KRas expression, increased survival. While the median survival for the untreated mice was 34 days, the median survival for the treated mice was 104 days ($P = 2.2 \times 10^{-5}$) (Fig. 1B). All of the untreated mice succumbed to disease within $\sim 50$ days of age. Upon treatment with Dox, rapid tumor regression was observed by MRI in 14/21 animals; the remaining 7 died from disease progression prior to complete suppression of KRas expression.

### Recurrent Tumors Develop After a Long Latency in the Absence of KRas and Ink4aArf Expression

Tumors recurred in 7 of the 14 responding mice in the absence of KRas expression. Interestingly, tumors grew back nearly simultaneously in 5 of the mice after a mean latency of 81 $\pm$ 1.6 days. The 6th mouse experienced tumor recurrence after 211 days, and the 7th mouse experienced tumor recurrence after a latency of 271 days (Fig. 1B). All of these tumors recurred while on Dox treatment in the absence of virally delivered KRas expression. The MRI data from a mouse that developed recurrent disease while on Dox treatment is shown in Figure 2. A small tumor is visible pre–Dox treatment. No tumor was detected at 97 days of Dox treatment; however, a large tumor was visible at 271 days of Dox treatment. Immunohistochemical (IHC) analysis of brain tissue confirmed KRas expression in untreated cohorts and demonstrated that KRas expression was reduced as expected in the Dox-treated mice (Fig. 3B, G, and L). Sections were stained with hematoxylin and eosin (H&E) to provide structural comparison (Fig. 3A, F, and K). Cre activity was also confirmed by loss of p19 Arf expression in tissue sections (Fig. 3C, H, and M). Assessment of cellular proliferation using IHC for the proliferation marker Ki67 demonstrated that the untreated tumors were significantly more proliferative than tumors in animals treated with Dox for 3 days ($P = .003$) or 86 days ($P = .0004$; Fig. 3D, I, and N). The average number of Ki67-positive cells in the untreated tumors with KRas expression was $733 \pm 52.4$, whereas the number of Ki67-positive cells in the tumors treated with Dox for 3 days or 86 days was $221 \pm 26.9$ or $147 \pm 15.3$, respectively. Because the tumors from Dox-treated mice initially showed signs of regression, TUNEL assays were performed to determine whether apoptotic cells could be detected. The average number of TUNEL-positive cells in the tumors treated with Dox for 3 days was $127 \pm 20$, whereas the number of TUNEL-positive cells in untreated tumors and recurrent tumors was $5.3 \pm 0.88$ and $4.6 \pm 1.3$, respectively.

To begin to delineate the mechanism of resistance for tumor recurrence in the absence of virally delivered KRas, we assessed activity of phosphatidylinositol 3-kinases (PI3K)/Akt/mammalian target of rapamycin (mTOR) by IHC for phosphorylated(p)-S6 in primary and recurrent tumors, but no differences were detected. Both primary and recurrent tumors were positive for p-S6 (data not shown). We also performed IHC for
Pten, and expression was detected in all samples analyzed (data not shown), suggesting that Pten loss is not the mechanism of resistance in this context. Interestingly, the majority of recurrent tumors appeared to have reactivated the Ras pathway in the absence of virally delivered KRas expression, as assessed by IHC of phosphorylated Erk (Fig. 3E, J, and O). To determine the cause of MAPK pathway reactivation, 5 large recurrent tumors were screened for the presence of de novo mutations in exons 2 and 3 of KRas, neuroblastoma RAS, and Harvey Ras, exons 3 and 6 of Mek, and exon 16 of BRaf by sequencing DNA extracted from microdissected tumor sections. No mutations were detected in any of these exons. We also performed IHC for Nf1, and expression was detected in all samples analyzed (data not shown), suggesting that Nf1 loss is not the mechanism of resistance in this context.

To further assess the function of the tet-regulatory module, western blot analysis was performed on cell lines established from recurrent gliomas (Fig. 4). Mouse astrocytes containing KRas constitutively expressed from the viral LTR were included as a control to demonstrate that Dox affects expression of KRas only when its expression is driven from the TRE. This analysis confirmed that virally delivered TRE-KRas expression in the recurrent tumors remained tet-responsive and demonstrated that the mechanism of resistance via pathway activation did not involve disruption of the tet-regulatory elements. Sustained levels of phosphorylated Erk in the absence of virally delivered KRas expression were observed in one of the cell lines analyzed by western blot, further confirming the IHC findings (Fig. 4).

**KRas Inhibition Elicits Regression of Advanced Gliomas but Resistance Ensues**

Because most human gliomas are detected at an advanced size, we also treated mice with larger tumors. The MRI data from one of these mice are...
Fig. 3. Immunohistochemical characterization of gliomas arising in N-TVA; Ink4a/Arf<sup>lox/lox</sup> mice injected with Cre, tet-off, and TRE-KRas containing viruses. Tumor tissue was assessed from an untreated mouse, a mouse treated with Dox for 3 days, and from a tumor that responded to treatment and subsequently recurred while on treatment for 86 days. (A, F, K) Sections were stained with H&E to provide structural comparison; (B, G, L) IHC for the FLAG epitope tag on KRas; (C, H, M) Loss of p19 expression indicates Cre activity; (D, I, N) IHC for the proliferation marker Ki67; (E, J, O–S) IHC for phosphorylated Erk (p-Erk) to assess canonical MAPK pathway activation. All slides were counterstained with hematoxylin (blue nuclei). Scale bars represent 200 μm.
shown in Figure 2. Within just 1 week of Dox treatment, the tumors had regressed significantly and continued to regress for an additional 5 weeks. At this point, 1 tumor was no longer detectable by MRI. The other 2 tumors remained detectable by MRI, but growth of these tumors was dormant for an additional 9 weeks, after which time the tumors began to grow back even in the presence of Dox treatment. Tumor volumes calculated at multiple time points are shown in Table 1.

**Table 1.** Tumor volumes calculated from MRI scans at multiple time points for Dox-treated mice with advanced tumors

<table>
<thead>
<tr>
<th>Week</th>
<th>Tumor 1 Volume in mm³</th>
<th>Tumor 2 Volume in mm³</th>
<th>Tumor 3 Volume in mm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment</td>
<td>186.54</td>
<td>447.10</td>
<td>170.84</td>
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<td>1 week</td>
<td>39.33</td>
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<tr>
<td>2 weeks</td>
<td>19.30</td>
<td>117.50</td>
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<td>3 weeks</td>
<td>6.90</td>
<td>70.01</td>
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<td>4 weeks</td>
<td>1.91</td>
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<tr>
<td>5 weeks</td>
<td>2.17</td>
<td>44.85</td>
<td>18.53</td>
</tr>
<tr>
<td>6 weeks</td>
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<td>42.63</td>
<td>16.11</td>
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<td>Not detectable</td>
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<td>21.63</td>
</tr>
<tr>
<td>23 weeks</td>
<td>—</td>
<td>—</td>
<td>508.85</td>
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Doxycycline Withdrawal and Subsequent Re-expression of KRas Induces Relapse

Complete responses were observed in 33% of the mice treated with Dox for 1 year. To determine whether KRas suppression induces durable responses in these mice, the mice were monitored for relapse after Dox withdrawal. Consistent with the presence of residual microscopic disease, 4 of these mice relapsed relatively rapidly, with a mean latency of 41 ± 9.8 days following Dox withdrawal. The 5th mouse relapsed after a latency of 126 days (Fig. 5). Two mice died of natural and unrelated causes. To determine if further rounds of KRas inhibition would induce tumor regression, 2 of the mice with relapsed disease were re-administered Dox. This resulted in the rapid regression of the tumors as visualized by MRI (Fig. 2). These mice have been maintained on Dox for an additional 7 months without detectable disease.

**Fig. 4.** Western blot analysis of Ras expression and pathway activation in glioma cell lines. (Lanes 1–2; KRas) N-TVA-positive immortalized astrocytes infected in culture with RCASBP(A)KRas; (Lanes 3–8; A, B, and C) 3 independent glioma cell lines derived from recurrent tumors originally induced with viruses containing Cre, tet-off, and TRE-KRas. Cells were either untreated (−) or treated with Dox for 72 h (+). Lysates were separated on a 16% Tris-glycine polyacrylamide gel, transferred to nitrocellulose, and probed with antibodies directed against Ras, phosphorylated Erk (p-Erk), or total Erk (Erk) as indicated. The membrane was reprobed with an antibody against α-tubulin (bottom) to ensure equal loading. The Ras blot shows endogenous Ras (lower band) and the virally delivered KRas (upper band). The virally delivered KRas is a higher molecular weight as a result of the FLAG epitope tag.

**Fig. 5.** Kaplan–Meier analysis of disease recurrence. All mice were injected with Cre, tet-off, and TRE-KRas containing viruses at birth, and only tumor-bearing mice were used for this analysis. Dox treatment was administered from 21 days of age continuously for 1 year and then withdrawn. All of the mice in this analysis were tumor-negative by MRI after Dox treatment. Dox was withdrawn after 1 year and mice were monitored by MRI for disease recurrence. The solid line indicates the duration of Dox treatment.

Discussion

We previously demonstrated the critical importance of the Ras pathway in glioblastoma maintenance in the context of tumors induced by activated Ras and Akt; continuous suppression of Ras signaling was necessary and sufficient to suppress the tumorigenic potential of the altered glial progenitor cells. This glioma model has provided insight into how activated Ras promotes tumor maintenance. In this context, histological characterization of regressing tumors at various stages after doxycycline administration showed rapid apoptosis of tumor cells. In this study, we observed that in the context of Ink4a/Arf loss, Ras inhibition was sufficient for tumor regression but KRas-independent tumors recurred in several mice. These results indicate that Ras-independent tumor recurrence is a relatively common event following Ink4a/Arf loss. Although regression took place in the majority of primary tumors, the largest of the tumors were observed to persist without complete regression. It is possible that the remaining areas of contrast observed by MRI in...
these mice were mostly dead tumor cells or scar tissue; however, subsequent recurrence in these mice suggests the presence of residual live tumor cells.

Notably, the KRas-independent tumors arose in the same area of the brain that had previously harbored a KRas-initiated tumor. The failure to observe secondary tumors, combined with the absence of tumors in mice injected with KRas or Cre alone, strongly suggests that these KRas-independent tumors represent true recurrences of KRas-initiated tumors rather than de novo tumor formation in the presence of Dox. Our findings suggest that a significant percentage of mice in which gliomas have regressed beyond detection by MRI following administration of Dox harbor residual neoplastic disease. Given the association of Ink4a/Arf loss with the progression of gliomas, it is not surprising that our data suggest that Ink4a/Arf deficiency results in decreased disease-free survival by promoting the emergence of KRas-independent clones from this putative population of residual neoplastic cells.

The latency of tumor recurrence suggests that additional alterations were required for growth of these cells in vivo. Resistance can emerge by escape of the TRE-KRas from the conditional regulation of the tetracycline system, activation of alternative signaling pathways, or selection for additional genetic changes that affect the Ras pathway. Expression of the virally delivered KRas was not detectable in recurrent tumors or in Dox-treated cell lines derived from these tumors, demonstrating continued responsiveness to Dox administration. In addition, no difference in activation of the PI3K/Akt/mTOR pathway was observed between primary and recurrent tumors. Interestingly, we observed activation of downstream targets of the canonical Ras pathway in recurrent tumors and cell lines derived from recurrent tumors in the absence of virally delivered KRas expression. This mechanism of resistance further highlights the importance of the Ras/MAPK pathway in glioma maintenance. Although no mutations were found in common hotspots in Ras, BRaf, or Mek1, there are a great many other candidate genes that may have become altered, leading to enhanced upstream activation or constitutive activation of the MAPK pathway. For example, overexpression and/or mutation of RTKs, with the ability to activate the canonical MAPK pathway (RAS/RAF/MEK/ERK), are frequently found in glioma. Dietary restriction and histology cores for assistance.

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