Abstract

Gliomas are the most common adult primary brain tumors, and the most malignant form, glioblastoma multiforme, is invariably fatal. The phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway is altered in most glioblastoma multiforme. PTEN, an important negative regulator of the PI3K-Akt pathway, is also commonly mutated in glioma, leading to constitutive activation of Akt. One ultimate consequence is phosphorylation and inactivation of FOXO forkhead transcription factors that regulate genes involved in apoptosis, cell cycle arrest, nutrient availability, DNA repair, stress, and angiogenesis. We tested the ability of a mutant FOXO1 factor that is not subject to Akt phosphorylation to overcome defects in tyrosine kinase receptor signaling through the phosphatidylinositol 3-kinase (PI3K)-Akt pathway.

PTEN is a major negative regulator of the PI3-Akt pathway. In cells that carry the PTEN mutation, the PI3K pathway is constitutively active, leading to inactivation of endogenous FOXO factors and possible initiation and progression of tumors (21). Indeed, several lines of evidence suggest that FOXO factors play a significant role in cancer. Tumorigenicity in nude mice induced by IKKβ or the receptor tyrosine kinase, HER-2 oncogene, can be overridden by an active form of FOXO3 or FOXO4, respectively (25). Cytoplasmic expression of FOXO3 in breast cancer also correlates highly with poor survival of patients (25).

In this study, we evaluated the potential of FOXO1 to correct the consequences of frequent genetic mutations in EGFR and PTEN in two representative cell lines of human glioblastoma multiforme, U87 and U251. Both cell lines are PTEN null and EGFR expression may be to target these transcription factors. For example, the effects of amplification of EGFR and loss of PTEN function can potentially be reversed through the activity of a single downstream transcription factor, such as FOXO1, to overcome defects in tyrosine kinase receptor signaling through the phosphatidylinositol 3-kinase (PI3K)-Akt pathway.

FOXO factors, characterized by a conserved DNA-binding domain termed the “forkhead box” (4), include FOXO1, FOXO3, and FOXO4 indicate that they transactivate many of the same genes involved in apoptosis, such as Bim (14, 15) and Fasl (16), and genes involved in cell cycle regulation such as p27 (17, 18), cyclin D1, and D2 (19, 20). As major direct substrates of Akt, FOXO factors are negatively regulated by Akt phosphorylation in the presence of growth factor signaling (21, 22), which results in their binding to 14-3-3 proteins, followed by their nuclear export (23). Once in the cytoplasm, FOXO factors are ubiquitinated and are subjected to degradation by the proteasome (21, 24).

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In this study, we evaluated the potential of FOXO1 to correct the consequences of frequent genetic mutations in EGFR and PTEN in two representative cell lines of human glioblastoma multiforme, U87 and U251. Both cell lines are PTEN null and EGFR expression has been associated with increased tumorigenicity in both (26–28). As malignant gliomas are characterized by intense microvascular proliferation, with tumor capillaries that supply hundreds of tumor cells, we hypothesized that FOXO1 would increase antitumor effects by interfering with neovascularization (13). The FOXO1 protein we tested is mutated in three of its Akt phosphorylation sites, T24, S256, and S319, which were converted into alanine residues (FOXO1:AAA). Thus, FOXO1:AAA is not subject to Akt phosphorylation events in the nuclear export of FOXO1. [Cancer Res 2009;69(13):5433–40]
phosphorylation control and should remain primarily nuclear (29, 30). We found that the recombinant adenovirus AdFOXO1;AAA could successfully restore cell cycle arrest and induce cell death in vitro and prolong survival in vivo in xenograft models of human glioma. However, U87 were much more resistant than U251 to AdFOXO1;AAA-induced death. Our results indicate this may be attributed to differences in cellular localization of FOXO1;AAA due to phosphorylation at non-Akt sites possibly through the activity of cyclin-dependent kinases (CDK).

**Materials and Methods**

**Cell Culture**

The human glioma cell lines U87 and U251 were obtained from the American Type Culture Collection, whereas U343 and U373 were gifts of Dr. R. DelMaestro (Montreal Neurological Institute). A subclone of the U251 cell line, U251N, which can produce intracerebral tumors in athymic nude mice, was a gift from Dr. P.A. Forysth. Cells were grown in DMEM supplemented with 10% fetal bovine serum and 2 mmol/L L-glutamine.

**Recombinant Adenovirus**

AdFOXO1;AAA was described previously (19). In this adenovirus construct, FOXO1;AAA is fused to the green fluorescent protein (GFP). Viral particles were purified by CsCl gradients and quantified by spectrophotometry at 260 and 280 nm. Infectious titers were estimated by cytopathic effect on 293A cells.

**In vitro Experimentation**

**Adenovirus infection.** Adherent cells were washed with PBS, medium was replaced with Opti-MEM (Invitrogen), and cells were incubated with adenovirus for 3 h followed by culture in DMEM/10% fetal bovine serum. Control infections were carried out using either AdLacZ or AdGFP at the same multiplicity of infection (MOI) as for AdFOXO1;AAA. To evaluate transduction with AdFOXO1;AAA, GFP fluorescence was assessed by digital fluorescence microscopy (Leica). Quantitation of transduction efficiency was determined by flow cytometry of unfixed cells to detect GFP fluorescence using a FACSCalibur (Becton Dickinson) in conjunction with CellQuest software (Becton Dickinson).

**Cell cycle analysis.** DNA content was measured by flow cytometry of fixed unsynchronized propidium iodide (PI)-stained cells (Sigma). Flow cytometric analysis was done on at least 10,000 cells for each sample and cell cycle data were evaluated using ModFit LT software (Verity). The percent of cells with sub-G1 peak was determined using CellQuest software. The number of live and dead cells was assessed in a separate experiment by flow cytometry of unfixed cells stained with PI to detect membrane permeability to PI. All cell cycle and live/dead studies were done for at least two independent adenovirus infections.

**Annexin V-PE staining.** Annexin V-PE (BD Pharmingen) staining was done as indicated by the manufacturer’s instructions and PI was used to distinguish between viable and nonviable cells. Samples were analyzed by flow cytometry and the percentage of cells undergoing apoptosis was determined using WinMDI software.

**Western blot.** Samples (at a density of 1 × 10^5 cells) were cultured in 6-well plates for 1 day before transduction with AdFOXO1;AAA or AdLacZ (at a MOI of 50) or mock infected (no virus). Cells were further cultured for 24, 48, or 72 h. Cell lysates were analyzed by SDS-PAGE followed by Western blotting. The following antibodies were used: rabbit anti-FOXO1 (Cell Signaling), rabbit anti-p27 (Santa Cruz Biotechnology), mouse anti–cyclin D1 (Neomarkers), rabbit anti–cyclin D2 (Santa Cruz Biotechnology), rabbit anti-Bim (Affinity BioReagents), mouse anti–caspase-9 (Oncogene Research Products), or mouse anti–β-actin (Abcam). The rabbit or mouse secondary antibodies were coupled to horseradish peroxidase (DAKO). Signal was detected in the presence of SuperSignal West Femto substrate (Pierce) and chemiluminescent images were captured by the CCD camera of GeneGnome (Syngene).

**CDK2 inhibitor treatment.** Cells plated at a density of 5 × 10^3 in 96 wells were infected with adenovirus at a MOI of 50 and 24 h later treated with 25 μmol/L CDK2 inhibitor II (CDK2iII; Calbiochem), dissolved in DMSO. CDK2iII selectivity has been analyzed in previous studies [compound 3 in Davis and colleagues (31) and compound 16 in Bramson and colleagues (32)], which established an IC50 of 0.06 μmol/L for CDK2 and 0.750 μmol/L for CDK1 (32). At the concentrations used here, the activities of both CDK1 and CDK2 would be inhibited. After 24 h incubation with CDK2iII, cells were examined by digital fluorescence microscopy (Leica) and all photographs were analyzed using ImageJ software. The number of cells exhibiting nuclear or nuclear and cytoplasmic FOXO1;AAA localization was determined by manual count of photos from sets of three independent infections.

Phosphorylation levels of FOXO1 protein were determined in AdFOXO1;AAA-infected cells, which had been treated with CDK2iII or vehicle (DMSO). CDK2iII (25 μmol/L) was replenished at 24 h after infection and cells were harvested for Western blot 48 h later. Blots were first incubated with a rabbit antibody specific for phospho-FOXO1 at S249 (Cell Signaling). The intensity of chemiluminescent bands captured by the CCD camera of GeneGnome was quantitated by densitometry using GeneTools (Syngene).

**Figure 1.** Effects of intratumoral delivery of AdFOXO1;AAA. Survival of mice implanted with U87LacZ (A and B) or U251N (C) cells followed 10 d later by intratumoral injection with AdFOXO1;AAA (3 × 10^5 plaque-forming units) or AdGFP (3 × 10^5 plaque-forming units) or left untreated. B, tumor volume was determined 35 d later (P = 0.042, unpaired t test). C, mice bearing U251N tumors and treated with AdFOXO1;AAA and AdGFP had significantly different median survivals by Kaplan-Meier analysis (P < 0.0001).
The same blots were stripped and incubated with rabbit anti-FOXO1 (Cell Signaling) and subjected to densitometry analysis to determine total FOXO1 expression. The level of phosphorylation was expressed as the ratio of phospho-S249FOXO1/totalFOXO1. For comparison between cell lines, the signal from U87 cells infected with AdFOXO1;AAA was assigned a value of 1. Quantitation was done on at least two independent adenovirus infections/inhibitor treatments.

To evaluate cell death following CDK inhibition, unpermeabilized cells were stained with PI at various time points after AdFOXO1;AAA infection and CDK2iII treatment. CDK2iII was replenished daily. Quantitation of PI-positive cells from three independent adenovirus infections/inhibitor treatments was done using ImageJ software on digital photographs acquired by fluorescence microscopy.

**In vivo Experimentation**

For intracerebral stereotactic injections into the caudate, U87LacZ and U251N human glioma cells (1 x 10^6 in 3 μL HBSS) were implanted as outlined previously (33). Ten days later, mice were injected intratumorally with AdFOXO1;AAA (1 x 10^9 plaque-forming units/mL) or AdGFP (1 x 10^9 plaque-forming units/mL) in a volume of 3 μL. During long-term survival studies, animals were monitored daily. All experiments were carried out according to the guidelines of the Institutional Animal Care Committee. Survival was assessed with Kaplan-Meier analysis.

**Statistical analyses.** All statistical analyses were done using GraphPad Prism software with the critical significance level (P) set to 0.05.

**Results**

**Differential cytotoxic capacity in vivo.** We tested the effect of AdFOXO1;AAA in preimplanted intracerebral tumors of two established and extensively used human glioma cell lines, U87 and U251. These tumors were allowed to grow for 10 days before intratumoral injection of 3 x 10^9 particles of AdFOXO1;AAA or AdGFP control vector. In short-term studies with the U87 cell line, when tumor volumes were determined 35 days following intratumoral injection, the AdFOXO1;AAA tumors were significantly smaller (Fig. 1B). However, in long-term survival studies, there was no significant difference between mice implanted with U87 and treated with AdFOXO1;AAA or AdGFP (Fig. 1A; Supplementary Table S1). In contrast, mice implanted with U251N and treated with AdFOXO1;AAA had a significantly longer median survival than AdGFP-treated and nontreated animals (P < 0.0001; Fig. 1C). In fact, when the experiment was stopped at the end of 1 year, 33% of the tumor-bearing mice that had received AdFOXO1;AAA were still alive, whereas all control animals had been euthanized by day 115 at the latest (Fig. 1C; Supplementary Table S1). As both cell lines can be transduced by Ad5 vectors to a similar degree (>90% transduction at MOI of 50), these results indicate that they are differentially sensitive to the effects of AdFOXO1;AAA.
Evaluation of cytotoxic activity in vitro. To determine whether response to AdFOXO1;AAA was also discordant in vitro, we investigated the degree of cell death triggered by AdFOXO1;AAA infection of U87 and U251 glioma cells. In adenovirus-transduced cells, we examined by flow cytometry the sub-G1 peak (indicative of DNA loss) following PI staining of fixed cells. There was a dose-dependent increase in the sub-G1 peak for both U87 and U251 cells infected with AdFOXO1;AAA over a period of 72 h; the higher the MOI used, the more cell death was observed in both cell lines (Fig. 2A). Of interest, AdFOXO1;AAA-induced death was much more pronounced in U251 cells. Similar data were obtained with flow cytometry of unfixed PI-stained cells, which provided a count of live and dead cells (Supplementary Fig. S1). To confirm whether cell death was attributable to apoptosis, flow cytometric analysis of Annexin V-PE-stained cells was done at 48 h postinfection. Cells infected with AdFOXO1;AAA had a much larger Annexin V-positive population, indicating that these were actively undergoing apoptosis (Fig. 2B). Consistent with the sub-G1 data, the apoptotic proportion was greater in U251 cells than in U87 cells (59.0 ± 11.5% versus 13.1 ± 0.7%; Fig. 2B). We tested the sensitivity of two additional human glioma cell lines, U343 and U373. As seen in Fig. 2C, all glioma cell lines expressed equivalent levels of exogenous FOX1 when transduced with AdFOXO1;AAA at a MOI of 50. However, U251 cells were the only ones particularly susceptible
to cell death as ascertained by quantitation of sub-G1 peak at 72 h postinfection (Fig. 2D). Taken together, these data suggest that AdFOXO1;AAA mediates a dose-dependent increase in cell death through induction of apoptosis and that the cell death occurs in a larger proportion of AdFOXO1;AAA-transduced U251 cells than U87, U343, or U373 cells.

Cell cycle analysis. As FOXO1 can also regulate genes involved in cell cycle arrest (19), we analyzed the cell cycle profile of glioma cells by flow cytometry (Fig. 3). At 24 h, AdFOXO1;AAA-infected U87 cells exhibited a dose-dependent decrease in the S-phase proportion (Fig. 3B) and an increase in the G2-M phase. Interestingly, the S-phase decrease was transient in the U87 cells, with the S-phase proportion increasing at 72 h, whereas there was a drop in the G2-M proportion without a further increase in the G2-M fraction (compared with 24 h). This suggests that the AdFOXO1;AAA-infected U87 cells were once again proceeding through the cell cycle. U251 cells exhibited a slightly different cell cycle profile (Fig. 3B). At 24 h postinfection with AdFOXO1;AAA, U251 cells had a higher proportion of cells in the G2-M phase compared with uninfected control. U251 cells also showed a decrease in the S-phase proportion compared with control, but unlike the case with U87 cells, the proportion of cells in the S phase did not increase with time. After 48 h, the majority of U251 cells infected with AdFOXO1;AAA were sub-G1 (and were not plotted). These data show that AdFOXO1;AAA induces cell cycle arrest in both U87 and U251, but arrest is followed by extensive apoptotic death only in U251 cells.

Expression of FOXO1 target genes in AdFOXO1;AAA-transduced glioma cells. To determine what mechanisms may be contributing to AdFOXO1;AAA-induced apoptosis and cell cycle arrest, immunoblot analysis of FOXO1 and its target gene products was done. Infection with AdFOXO1;AAA at a MOI of 50 led to expression of FOXO1 at ~80 kDa in both U87 and U251 cells 24 h postinfection (Fig. 4A). There were undetectable levels of endogenous FOXO1 expression in uninfected or AdLacZ-infected U87 and U251 glioma cells. Noticeably, in U87 cells, there was a decline in FOXO1 expression over 72 h, which may only be partly related to increased cell death because only 30% undergo cell death at this time point (Supplementary Fig. S1) and >80% of the population is still transduced (fluoresced green) at 72 h at a MOI of 50 (Fig. 3A). In U251 cells, maximum expression was seen at 48 h, and most cells were dead by 72 h, at which point FOXO1 protein was no longer detected. These analyses revealed a marked difference in FOXO1 levels in the two cell lines following transduction with AdFOXO1;AAA.

We further examined protein levels of selected FOXO target genes implicated in cell cycle control, p27 and cyclins D1 and D2. The CDK inhibitor p27 is transcriptionally regulated by FOXO factors (17), causing both G1 and G2 arrest (34), whereas D-type cyclins promote cell cycle progression from G1 to S and are down-regulated by FOXO factors in a manner that does not require binding to DNA (19). At 24 h postinfection in both U87 and U251 cells, p27 was up-regulated in response to AdFOXO1;AAA, whereas cyclins D1 and D2 were down-regulated (Fig. 4B), a result consistent with the literature (19). Interestingly, in U87 cells, there were changes in expression profile during the course of the 72 h infection: cyclin D1 and D2 levels in response to AdFOXO1;AAA returned to control levels, whereas the expression of p27 declined slightly. In contrast, neither p27 nor cyclins D1 and D2 in U251 cells changed from 24 to 48 h. Therefore, the cell cycle arrest seen at 24 h postinfection with AdFOXO1;AAA may be mediated by a down-regulation of D-type cyclins and an increase in p27. Importantly, the transient arrest in U87 cells may be the result of declining FOXO1;AAA expression along with a return of D-type cyclins and a fall in p27 levels.

FOXO1 also transcriptionally up-regulates the proapoptotic protein Bim (14, 15), a member of the Bel-2 family, as well as caspase-9, an initiator caspase of the mitochondrial apoptotic signaling pathway, which must be cleaved for activation. In U87 cells at 48 h, Bim was modestly up-regulated, but not cleaved caspase-9, although pro-caspase-9 was increased (Fig. 4B). At 72 h, pro-caspase-9 and Bim levels fell, consistent with our previous observations that FOXO1;AAA expression is transient in U87 cells. In contrast, in U251 cells, the expression of Bim and cleaved caspase-9 increased, suggesting that susceptibility to apoptotic death may be attributed to Bim activation and consequent death mediated via caspase-9.

Cellular localization of FOXO1;AAA and modulation of FOXO1;AAA activity by phosphorylation at S249. Because nuclear localization of FOXO proteins is required for their transcriptional regulatory functions, a comparison of the subcellular localization of FOXO1;AAA (fused to GFP) was done to ascertain whether this may partially explain the varying sensitivity of glioma cells to AdFOXO1;AAA-induced apoptosis. At 24 and 48 h after infection, FOXO1;AAA expression in U251 cells remained primarily nuclear (Fig. 5A); in contrast, at 48 h in U87 cells,
FOXO1:AAA was almost evenly distributed between the cytoplasm and the nucleus (Fig. 5A). Thus, in U87 cells, FOXO1:AAA may be regulated in an Akt phosphorylation-independent manner, leading to its nuclear export and attenuation of transcriptional activity. This greater propensity for cytoplasmic localization in U87 cells may offer an explanation for the transient nature of the expression profile of FOXO1 target genes and the lower percentage of cell death in U87 cells compared with U251 cells.

Recent reports indicate that CDK1 and CDK2 may influence the nuclear export of FOXO1 through phosphorylation of S249, a residue that is unmodified in FOXO1:AAA. In U87 cells, a 24 h treatment with the CDK2 inhibitor (CDK2iII) altered the subcellular localization of FOXO1:AAA, with a greater proportion of transduced cells manifesting a nuclear signal for the fusion protein than in DMSO-treated control cells (Fig. 5B and C). In support of these observations, 48 h after AdFOXO1:AAA transduction, U87 cells manifested higher levels of phospho-S249 compared with U251 cells (Fig. 6A and B). Treatment with CDK2iII greatly diminished the ratio of phospho-S249 to total FOXO1 in both cell lines (Fig. 6A and B). Next, we tested whether CDK inhibition would potentiate AdFOXO1:AAA-mediated cell death in the U87 cell line (Fig. 6C). As expected, incubation of cells with CDK2iII alone led to a low level of cell killing (~13%). However, in AdFOXO1:AAA-transduced U87 cells, treatment with CDK2iII restored their susceptibility to cell death, with 54.5 ± 8.3% cells undergoing death at 48 h compared with 20.1 ± 6.5% in untreated cells (P < 0.001; Fig. 6C). Taken together, these data suggest that the transient effect of AdFOXO1:AAA on the expression of cell cycle and apoptotic proteins in U87 may occur through regulation of S249 phosphorylation by CDK1 and CDK2.

Discussion

Molecular characterization of glioblastoma multiforme has led to development of therapies targeting specific defects. For example, activation of EGFR occurs in ~50% of primary glioblastoma multiforme (35) and thus provides an obvious target. However, only ~10% of patients show some modest response to EGFR kinase inhibitors (36). Glioblastoma multiforme is characterized by frequent, concurrent dysregulation of several growth factor receptor tyrosine kinase pathways (37), which may explain the poor efficacy of single treatments. Hyperactivation of PI3K-Akt signaling follows from dysregulation of receptor tyrosine kinase pathways and can lead to excessive cell proliferation, migration, and resistance to apoptosis and cell cycle arrest.

Recently, PI3K-Akt signaling was shown to be altered in 88% of analyzed glioblastoma multiforme tumor samples (35). We reasoned that a mutant FOXO1 transcription factor, which cannot be negatively regulated by Akt phosphorylation, may act distally to target multiple growth factor receptor tyrosine kinase pathways. Indeed, in an intracerebral mouse model with a preestablished glioma xenograft tumor, adenovirus-mediated gene transfer of FOXO1:AAA led to prolonged survival. As well, in vitro experiments showed dose-dependent restoration of cell cycle arrest and apoptosis. However, differential cytotoxicity of AdFOXO1:AAA was noted both in vivo and in vitro, with U251 cells being more sensitive to AdFOXO1:AAA-induced death than U87, U343, and U373 cells (Fig. 2). The relatively lower capacity for AdFOXO1:AAA to induce apoptosis in U87 cells, compared with U251 cells, resulted in failure to improve survival in U87 xenografts (Fig. 1). In contrast, tumor eradication and prolongation of survival was achieved in the U251N xenografts, in which 33% of tumor-implanted mice were still alive at the end of the 1 year observation period (Fig. 1; Supplementary Table S1). These data indicate that although U87 and U251 glioma cells both exhibit a mutant PTEN tumor suppressor, individual genetic changes likely affect outcome. Such discordance was also observed after infection with AdPTEN, which resulted in anoikis in U251 cells (38), whereas G1 cell cycle arrest occurred in U87 cells (39, 40).

Transduction efficiencies in U87 and U251 cells were relatively similar (Figs. 2A, 3A, and 4A), but subcellular localization of the FOXO1 protein was very different between the two cell lines. In U251 cells, FOXO1:AAA was almost exclusively nuclear (Fig. 5A), whereas it was present in both the nucleus and the cytoplasm in U87 cells at 48 h postinfection, suggesting that fewer FOXO1:AAA proteins were engaged in transcriptional activity than in U251 cells. Cytoplasmic localization of FOXO1:AAA was unexpected, as this mutant is considered by many to be constitutively nuclear (16, 19, 30). Subcellular localization of wild-type FOXO1 is controlled by its nuclear export and attenuation of transcriptional activity.
primarily by phosphorylation, leading to nuclear export and subsequent repression of transcriptional activity (21, 23, 41, 42). Akt phosphorylation in the DNA-binding domain (S256) results in disruption of the nuclear localization signal and DNA binding as well as exposing T24 and S319 to Akt phosphorylation, facilitating nuclear export. Wild-type FOXO1 is also phosphorylated by other protein kinases, such as SGK (43), CK1 (44), and DYRK1A (45). Activation of Akt, and/or SGK, CK1, and DYRK1A leads to phosphorylation of a stretch of four serine residues in FOXO1, which form an acidic patch inducing high-affinity binding of 14-3-3 proteins, thereby assisting nuclear export (41, 46). FOXO1;AAA, however, cannot be phosphorylated by Akt nor can it be phosphorylated by SGK (43) or CK1 (46). Therefore, control of FOXO1;AAA subcellular localization may be independent of phosphorylation at T24, S256, and S319.

CDK2 (47) and, more recently, CDK1 (48, 49) were reported to regulate FOXO1 by phosphorylation on S249, which is unaltered in our phosphosite mutant. CDK2 phosphorylation on S249 leads to cytoplasmic localization and decreased activity in both wild-type and FOXO1;AAA in a p53-independent fashion as measured by target gene expression (47). Indeed, in U87 cells treated with the CDK2 inhibitor, CDK2II, we observed increased nuclear localization of FOXO1 protein (Fig. 5B and C). Furthermore, the ratio of phospho-FOXO1 on S249 to total FOXO1 protein was much greater in U87 cells than in U251 cells (Fig. 6A). These data suggest that the decreased sensitivity of U87 cells to AdFOXO1;AAA-induced death may be related to increased levels of S249 phosphorylation of FOXO1 and subsequent decreased FOXO1 activity.

As predicted, treatment of U87 cells with CDK2II reduced the level of S249 phosphorylation and increased their susceptibility to cell death (Fig. 6). Thus, the transient effect of AdFOXO1;AAA in U87 cells on expression of cell cycle and apoptotic proteins and the increased cytoplasmic localization of FOXO1;AAA may be due to FOXO1 S249 phosphorylation.

Our results show that FOXO1 transcription factor, which acts at a convergence point of several growth factor receptor tyrosine kinase pathways, can effectively induce cell death and inhibit tumor growth of glioma cells. These studies also highlight the complexities of targeting the PI3K-Akt pathway, even distally. However, combination therapy in which both FOXO1 phosphorylation and nuclear export (50) are inhibited may provide a wider application and increase efficacy in a variety of tumor genotypes.

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

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Figure 6. FOXO1 phosphorylation. A, Western blot of AdFOXO1;AAA-infected U87 and U251 cells with a phosphospecific antibody for S249 of FOXO1 (top). Blots were stripped and reprobed with anti-FOXO1 antibody (bottom). B, quantification of signal of AdFOXO1;AAA-infected cells from A by densitometry indicating ratio of phospho-S249 FOXO1 to total FOXO1. Arbitrary units were assigned such that U87 cells infected with AdFOXO1;AAA were given a value of 100. Quantification from at least two separate infections. C, number of PI-positive dead U87 cells following infection with AdFOXO1;AAA at various MOI and/or treatment with CDK2II 48 h postinfection/treatment. Quantification from three independent infections (P < 0.001, ANOVA). Cultures treated with inhibitor and infected at MOI 30 and 50 were significantly different from untreated cultures, infected at the same MOI (*, P < 0.001; Bonferroni correction for multiple comparison).
References

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