Molecular and Cellular Pathobiology

Astrocyte Elevated Gene-1 Interacts with Akt Isoform 2 to Control Glioma Growth, Survival, and Pathogenesis


Abstract

The oncogene astrocyte elevated gene-1 (AEG-1; MTDH) is highly expressed in glioblastoma multiforme (GBM) and many other types of cancer, where it activates multiple signaling pathways that drive proliferation, invasion, angiogenesis, chemoresistance, radioresistance, and metastasis. AEG-1 activates the Akt signaling pathway and Akt and c-Myc are positive regulators of AEG-1 transcription, generating a positive feedback loop between AEG-1 and Akt in regulating tumorigenesis. Here, we describe in GBM cells a direct interaction between an internal domain of AEG-1 and the PH domain of Akt2, a major driver in GBM. Expression and interaction of AEG-1 and Akt2 are elevated in GBM and contribute to tumor cell survival, proliferation, and invasion. Clinically, in silico gene expression and immunohistochemical analyses of patient specimens showed that AEG-1 and Akt2 expression correlated with GBM progression and reduced patient survival. AEG-1–Akt2 interaction prolonged stabilization of Akt2 phosphorylation at S474, regulating downstream signaling cascades that enable cell proliferation and survival. Disrupting AEG-1–Akt2 interaction by competitive binding of the Akt2-PH domain led to reduced cell viability and invasion. When combined with AEG-1 silencing, conditional expression of Akt2-PH markedly increased survival in an orthotopic mouse model of human GBM. Our study uncovers a novel molecular mechanism by which AEG-1 augments glioma progression and offers a rationale to block AEG-1–Akt2 signaling function as a novel GBM treatment.

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Introduction

Glioma is the most common form of brain tumor (1). Approximately 34% of brain tumors are gliomas and 82% are malignant gliomas, graded as WHO 3 and 4 (1). Despite significant improvements in neurosurgical approaches and chemotherapy/radiotherapy, grade 4 glioblastoma (GBM) remains a cancer type with dire prognosis, primarily because GBM cells are highly invasive and are capable of infiltrating into surrounding normal brain tissue, resulting in failure to completely remove the tumor by surgery (2, 3). Consequently, glioma recurrence is a common event and recent research highlights a seminal role of radiation- and chemotherapy-resistant glioma stem cells as potential mediators of this recurrence (4, 5). Several studies have suggested that glioma cells are highly resistant to cytotoxic treatments and their dispersion may actually be triggered, in part, by these treatments (6, 7). In these contexts, defining appropriate targets for therapeutic intervention and approaches for delimiting invasion/spread and pathogenesis of GBM remain key priorities. Moreover, through comprehension of the mechanism(s) of glioma progression, an appropriate path may become evident for developing effective therapeutic reagents and strategies for malignant glioma treatment.

Astrocyte elevated gene-1 (AEG-1) was first identified as an upregulated transcript in primary human fetal astrocytes infected with HIV-1 or treated with TNFα and later characterized as a major oncogene highly upregulated in a broad array of cancers, including GBM (8–10). Subsequently, using in vivo phage screening, the mouse AEG-1 gene was cloned and named metadherin (MTDH) based on its prometastasis role in tumor cell homing to lung (11). AEG-1 is a multifunctional protein interacting with diverse partners in different cancer types and capable of promoting all hallmarks of cancer (9, 10, 12, 13). Through protein–protein interactions, AEG-1 functions as a key pathologic gene regulating a variety of diseases (12).

Akt is a nodal point in a variety of cancer signaling pathways that ultimately regulates tumor cell fates, including apoptosis, proliferation, survival, invasion, and cell

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cycling (14, 15). Akt signaling pathway is constitutively active in GBM (16, 17). Although Akt isoform distribution in gliomas has been investigated, limited functional studies were performed (18). Among the Akt isoforms, Akt2 is critical for glioma progression and resistance to chemotherapy and radiotherapy (19–21).

We reported previously that AEG-1 can activate the Akt signaling pathway in immortalized human astrocytes and Akt and c-Myc positively regulate AEG-1 transcription, thereby establishing a positive feedback loop between AEG-1 and Akt in promoting tumorigenesis (22, 23). AEG-1–Akt signaling promotes cell proliferation, survival, angiogenesis, and chemoresistance (24–26). However, the mechanism by which AEG-1 activates Akt remains unclear. We now demonstrate a novel interaction between AEG-1 and Akt2, in glioma, and document that this interaction is critical for downstream Akt signaling, which regulates tumor cell survival, proliferation, and invasion. In addition, the interacting regions in both molecules have now been mapped, which provides a defined target site for developing small-molecule drugs capable of disrupting this interaction that might provide potential novel therapeutics for GBM.

Materials and Methods

Cell cultures
Human glioma cell lines, U87 and U251, were obtained from ATCC and cultured with DMEM containing 10% fetal bovine serum. VG2, 4 and 6 primary glioma neurospheres were isolated from GBM tumor samples provided by VCU TDAAC. The tumor cells were isolated following the described protocol (27) and cultured with glioma stem cell media for no more than 5 passages. All cell lines were authenticated within 6 months using the “CellCheck” service provided by the Research Animal Diagnostic Laboratory (IDEXX BioResearch).

Coimmunoprecipitation
Pierce Protein G conjugated Agrose (Thermo Scientific) was used in coimmunoprecipitation (co-IP) experiments. Corresponding mouse or rabbit IgG (Jackson Immuno) were used as controls. Horseradish peroxidase–conjugated secondary antibody with light-chain IgG (mouse/rabbit) was used to eliminate potential overlapping heavy-chain background in all immunoprecipitation experiments.

Analysis of The Cancer Genome Atlas glioma genomewide expression datasets
Genome-wide expression datasets for glioma were downloaded from The Cancer Genome Atlas (TCGA; ref. 28). The level 3 (processed) datasets, along with accompanying clinical information, were obtained through the UCSC Cancer Genomics Browser (29). Data processing steps, using the Gene-E program (Broad Institute) started with inverse log (base 2) transformation, and quantile normalization of expression values, then annotation of samples with matching clinical records. Further statistical analyses were conducted using the JMP Pro 10 statistical program (SAS).

Tissue microarray, immunohistochemistry, immunofluorescence, and confocal imaging
GL801 glioma tissue microarray (TMA) slides from US Biomax were used for immunohistochemistry with standard protocol as described (30). The sample information, including sample number and grade, is listed in detail in Supplementary Table S2. Images (×20) were taken with a Nikon microscope system. AEG-1 and Akt2 staining was scored blindly by four independent investigators. Correlations between staining for both proteins were analyzed with the Prism 5 software. All 63 oil images were taken with Zeiss LSM 700 confocal laser scanning microscope and analyzed.

Quantitative Akt phosphorylation assay
U87 glioma cells were seeded (300,000 cells per dish) in 35-mm culture dishes and infected with control, AEG-1, or AEG-1-siRNA adenoviruses (50 MOI). LY294002 (4 μmol/L) was added 48 hours after infection, time 0. The samples were collected by flash freezing the dishes at 15 minutes, 30 minutes, 1 hour, 2 hours, and 3 hours. Total lysates were prepared on ice with phosphatase inhibitor cocktail (Roche). Western blots were analyzed by GelPro to measure total density of defined bands. Graph results were normalized using tAkt2 and Actin.

Intracranial implant of VG2 cells in mice and survival experiments
VG2 cells were established with lentivirus transduction to express AEG-1-shRNA and Akt2-PH domain (with a lentivirus Tet-on expression system) in four different combinations: Ctrl-shRNA/Ctrl (C/C), AEG-1-shRNA/Ctrl (A/C), Ctrl-shRNA/Akt2-PH (C/A), and AEG-1-shRNA/Akt2-PH (A/A). Using stereotaxic frame, these VG2 cells (10,000 cells in 2 μL PBS) were intracranially injected into athymic nude mouse brains with six animals in each group. The coordination of injection is 2.5-mm lateral, 0.4-mm frontal of bregma, and 3.5-mm deep from skull. The animals were continuously fed with doxycycline water (1 mg/mL) starting from the fourth day after tumor cell implants to induce Akt2-PH expression. Animals of each group were monitored until they reached the point of euthanization according to the VCU IACUC–approved protocol. Survival data were collected and analyzed with Prism 5.

Statistical analysis
All in vitro experiments were repeated at least in triplicate with three independent replicates. Animal number N = 5 was used in survival tests. Statistical analyses were calculated using one-way and two-way ANOVA for grouped samples, followed by the Bonferroni post hoc test; and two-tailed Student t test, which was used for comparison between control and treatment groups. *, P < 0.05; **, P < 0.01; and ***, P < 0.001 with mean ± SD are shown in the figures.

Results
AEG-1 interacts with Akt2 and expression correlates in glioma specimens
Because AEG-1 activates Akt signaling, we determined whether AEG-1 directly interacts with Akt. Co-IP analysis...
using anti-AEG-1 antibody effectively pulled down Akt in U87 cells (Fig. 1A, left). We overexpressed HA-tagged AEG-1 in U87, U251, and in a primary glioma (VG2) neurosphere (Fig. 1A). Immunoprecipitation with anti-HA antibody successfully pulled down AEG-1 and endogenous Akt in all of these cells, indicating an AEG-1–Akt protein complex in glioma cells.

Akt has three isoforms, which play different functional roles in tumor cells (18, 31, 32). Akt isoform expression was analyzed in VG2, VG4, and VG6 flash frozen clinical glioma samples to compare with a normal brain sample. C, protein levels of AEG-1 and Akt isoforms in glioma cells were compared with primary human fetal astrocytes by Western blotting. D, co-IP analysis of endogenous AEG-1 and Akt isoforms in U87 and VG2 total cell lysate with light-chain IgG rabbit secondary antibody used for detection. E, immunofluorescence followed by confocal microscopy imaging of AEG-1 and Akt2 in multiple glioma cells. Bar, 10 μm.

Figure 1. AEG-1 interacts with Akt in multiple glioma cells. A, left, endogenous AEG-1 and Akt were immunoprecipitated in U87 cells with AEG-1 antibody. Akt was detected with rabbit pan-Akt antibody and rabbit IgG was used as control. Right, HA-tagged AEG-1 was overexpressed in U87, U251, and in a primary GBM neurosphere VG2. Immunoprecipitation was performed with anti-HA antibody and detected with anti-HA and anti-Akt antibodies. Bottom, input Akt proteins were detected with pan-Akt antibody, which represents 10% of total loading. B, AEG-1 and Akt2 are upregulated in human glioma samples. Total lysates of VG2, VG4, and VG6 flash frozen clinical glioma samples were used to compare with a normal brain sample. C, protein levels of AEG-1 and Akt isoforms in glioma cells were compared with primary human fetal astrocytes by Western blotting. D, co-IP analysis of endogenous AEG-1 and Akt isoforms in U87 and VG2 total cell lysate with light-chain IgG rabbit secondary antibody used for detection. E, immunofluorescence followed by confocal microscopy imaging of AEG-1 and Akt2 in multiple glioma cells. Bar, 10 μm.

AEG-1–Akt2 Interaction in Malignant Gliomas

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(yellow in merged image) predominantly in the perinuclear region (Fig. 1E). Both AEG-1 and Akt2 are capable of translocating to different cellular compartments and play distinct functional roles (33–35). Further detailed subcellular analysis of AEG-1 and Akt2 interaction is warranted.

We analyzed the clinical relevance of AEG-1 and Akt2 in GBM using the TCGA database. A total of 372 glioma patients were divided into Akt2_low or Akt2_high based on Akt2 levels below or above the median, respectively (Supplementary Table S1). The resulting Kaplan–Meier plot clearly indicated that high Akt2 level is associated with poor survival ($P < 0.0001$ in both log-rank and Wilcoxon statistical tests; Fig. 2A, left). However, when the patients were further classified according to AEG-1 expression (similar to Akt2 level), thus creating four patient subgroups, it is evident that patients belonging to the Akt2_low/AEG1_low subgroup had the best clinical outcome (followed by the Akt2_low/AEG1_high subgroup; Fig. 2A, right). In the same dataset, subgroups of Akt3 strikingly showed opposite results, whereas Akt1 subgroups only showed narrowed separation when compared with Akt2 (Supplementary Fig. S2). These results indicate a strong correlation between AEG-1 and Akt2 levels with survival of patients with GBM.

Next, we checked AEG-1 and Akt2 expressions in a glioma TMA by immunohistochemistry. Low levels of AEG-1 and Akt2 were detected in normal brain samples (Fig. 2B). Both AEG-1 and Akt2 expressions were progressively elevated in glioma samples from grade 1 to grade 4 versus normal brain and a high correlation between AEG-1 and Akt2 levels were observed in these samples ($r^2 = 0.9221$; Supplementary Fig. S3 and Supplementary Table S2). Collectively, these findings clearly suggest that interaction between AEG-1 and Akt2 is clinically relevant and further support our hypothesis that AEG-1–Akt interaction plays a germane role in glioma pathogenesis.

We mapped interaction regions of AEG-1 and Akt using HA-tagged deletion constructs (Fig. 3A) in co-IP analysis. Anti-HA antibody successfully pulled down endogenous AEG-1 upon cotransfection of Akt deletion mutants, D120-2 (Δ2), D120-3 (Δ3), and D120-4 (Δ4), but not ΔPH (Fig. 3B, left). In comparison, N2, N4, and C2 deletion mutants of AEG-1 interacted with endogenous Akt2, whereas C4 (a.a. 289–582 deleted) failed to interact with endogenous Akt2 (Fig. 3B, right). These results support the conclusion that relevant interaction domains between AEG-1 and Akt2 are located at 290 to 404 a.a. in AEG-1 and 1 to 118 a.a. in Akt (PH domain). To confirm
the specificity of AEG-1 to Akt2, we overexpressed PH domains from the three Akt isoforms with a Myc tag at the NH2-terminus and performed co-IP with anti-AEG-1 antibody. Only the Akt2 PH domain interacted with AEG-1 (Fig. 4A). Our domain mapping provides clues for potentially developing inhibitory compounds that may block the interaction between these two important molecules. However, defining precise local interaction motifs and structures is important and requires further investigation.

AEG-1–Akt2 interaction regulates Akt signaling and function

On the basis of domain mapping results, we hypothesized that the Akt2-PH domain might disrupt endogenous AEG-1–Akt2 interaction by competitively binding to AEG-1. To address this possibility, we performed competitive binding of Akt2-PH to AEG-1 by determining the amount of Akt2 immunoprecipitated with the anti-AEG-1 complex. When Akt2-PH was expressed at increasing concentrations, it dramatically reduced the magnitude of AEG-1–Akt2 interaction (Fig. 4B), which was associated with decreasing phosphorylation levels of pAkt2-S474 (Fig. 4C).

To understand the functional roles of AEG-1–Akt interaction on cell survival under stress, we subjected U87-MG (p53wt) and U251 (p53mut) cells, expressing Akt2-PH, AEG-1 or both, to serum starvation or TMZ treatment. In 1% FBS, AEG-1 overexpression enhanced colony formation ability in soft agar (Fig. 4D). Significantly fewer colonies developed in cells expressing Akt2-PH. Cells expressing both AEG-1 and Akt2-PH, but not Akt1-PH, showed significant reductions in colony number compared with AEG-1 alone (Fig. 4D). AEG-1 overexpression significantly improved overall cell viability in U87 and U251 cells upon TMZ treatment (Fig. 4E). In contrast, Akt2-PH, but not Akt1-PH, significantly decreased cell viability by itself, and also reduced the prosurvival effects of AEG-1 when treated with TMZ (Fig. 4E). These results indicate that AEG-1/Akt2 interaction is important for glioma cell survival under stress, which might be blocked by Akt2-PH, and this
Figure 4. Disruption of AEG-1–Akt2 interaction reduces glioma invasion and survival. A, co-IP of AEG-1 and PH domains of Akt1, 2, and 3 in U87 cell lysates. U87 cells were transfected with constructs expressing Myc-tagged PH domains of Akt1, 2, and 3. Immunoprecipitation was performed with anti-AEG-1 antibody and immunoblotting was performed with anti-AEG-1 and anti-Myc antibodies. Rabbit IgG was used as control. Input protein detected with anti-Myc represents 10% of total. B, U87 cells were transfected with 0, 3, and 6 μg of Akt2-PH-Myc plasmid DNA along with AEG-1-HA. Immunoprecipitation was performed with anti-HA antibody. Pull down of endogenous Akt2 was detected by immunoblotting with anti-Akt2 antibody. (Continued on the following page.)
effect is p53 independent. To distinguish between Akt1 and Akt2, which are frequently overexpressed in GBM, we tested cell invasion and proliferation assays using either overexpression of the relevant PH domain or by knocking down of Akt1 or Akt2 (Supplementary Fig. S4A and S4B). Interestingly, overexpression of PH domain or Akt isoform–specific RNAi abrogated cell proliferation, but AEG-1 failed to reverse the inhibitory effect (Supplementary Fig. S5). However, Akt2-PH, but not Akt1-PH, abrogated the proinvasive function of AEG-1 (Fig. 4F). Similar findings were observed with Akt2-siRNA, but not Akt1-siRNA (Fig. 4G). These results strongly suggest that the AEG-1–Akt2 interaction plays an important functional role in glioma invasion.

Classic Akt activation requires PI3K-dependent phosphorylation at the S473/474 site phosphorylated by mTORC2. Blocking of PI3K–Akt signaling by LY294002 has been shown to reduce Akt phosphorylation at both S473 and T308 sites (36). To define how AEG-1 regulates Akt activation, we overexpressed or knocked down AEG-1 and checked the phosphorylation status of Akt2-S474 in a time-dependent manner. We first measured phosphorylation of Akt at S473 and T308 sites (Supplementary Fig. S6). Overexpression of AEG-1 prolonged, whereas knockdown (KD) of AEG-1 reduced phosphorylation of Akt2-S474 versus control (Supplementary Fig. S6). No difference in total p-Akt-308 was observed among control, AEG-1 OE, and AEG-1 KD groups (Supplementary Fig. S6). We further studied the dynamics of p-Akt2-S474. Semi-quantitative analysis showed that overexpression of AEG-1 prolonged phosphorylation of Akt2-S474, and knockdown of AEG-1 reduced initial Akt phosphorylation and resulted in a shorter half-life of p-Akt2-S474 compared with control (Fig. 5A). A linear fit analysis of Western blot gel quantification indicated a greater effect of AEG-1 on phospho-S474 measured with 1/slope (smaller number equals slower decay and vice versa; Fig. 5B). In contrast, the dephosphorylation dynamics of p-Akt2-S473 was minimally changed when using the specific phospho-Akt1 antibody (Supplementary Fig. S9).

Because AEG-1 is required to stabilize phosphorylation of Akt at S473/474, we investigated the effects of disrupting AEG-1–Akt interaction by Akt2-PH on Akt and its downstream effectors. Disruption of the interaction downregulated phosphorylation of S474, but not T308, on Akt. Phospho-GSK3β and phospho-BAD, substrates of Akt, and cyclin D1 levels were increased upon AEG-1 overexpression, which was inhibited upon coexpression of Akt2-PH (Fig. 5C). Increased upon AEG-1 overexpression, which was inhibited phosphorylation of S474, but not T308, on Akt. Phospho-GSK3β and cyclin D1 levels also changed corresponding to phosphorylation of Akt2-S474 (Fig. 5C–E). Moreover, AEG-1 overexpression significantly rescued growth inhibition caused by LY294002 (Fig. 5F). Collectively, these data demonstrate that AEG-1–Akt2 interaction is important for glioma proliferation, survival, and invasion. Overexpression of AEG-1 or loss of AEG-1 in glioma can lead to changes in phosphorylation of Akt2-S474, thus resulting in downstream signaling activation or inhibition, respectively.

**Combination of Akt2-PH overexpression and AEG-1 KD decreases glioma cell proliferation and prolongs animal survival**

To examine the effect of inhibition of AEG-1–Akt2 interaction in *vitro* and *in vivo*, we combined knockdown of AEG-1 with conditional overexpression of Akt2-PH, in VG2 primary glioma neurospheres. We chose this low-passage primary GBM cell line because it is more closely associated with the clinical state. To disrupt AEG-1–Akt2 interactions, we stably knocked down AEG-1 and conditionally overexpressed Akt2-PH with a lentivirus Tet-on expressing system. Four different subgroups of cells in combinations were used: Ctrl-shRNA/Ctrl, AEG-1-shRNA/Ctrl, Ctrl-shRNA/Akt2-PH, AEG-1-shRNA/Akt2-PH. Overexpression of Akt2-PH domain was induced by doxycycline in the culture media. Western blotting analysis showed that phospho-Akt2-S474 level was dramatically reduced in the presence of AEG-1-shRNA and/or Akt2-PH (Fig. 6A). A significant inhibition of cell growth was observed when AEG-1 was knocked down or Akt2-PH was overexpressed following induction by doxycycline, and the combination resulted in a more profound inhibitory effect (Fig. 6B). Exclusion of doxycycline did not cause any inhibition in cell proliferation.

We performed orthotopic injection of aforementioned four subgroups of VG2 cells into athymic nude mice brains and subsequently induced Akt2-PH expression through doxycycline-containing water (Fig. 6C). The control group of animals started to die 15 days after intracranial tumor implant. AEG-1 KD accrued a 5-day improvement in median survival. In comparison, AEG-1 KD plus Akt2-PH expression significantly extended median survival to 42.5 days (***, P < 0.0001). This
Figure 5. AEG-1 enhances the half-life of phospho-Akt2-S474. A, U87 with either AEG-1 overexpression (OE) or knockdown (KD) was treated with 4 μmol/L LY294002. Cell lysates were collected over time. B, phospho-Akt2-S474 at each time point was quantified with GelPro Analyzer software and normalized with Akt2 and actin. Linear regression analysis was performed and displayed as different lines with shapes. C–E, AEG-1 augments downstream Akt signaling. C, Western blotting of the indicated proteins was performed in U87 overexpressing Akt2-PH-Myc, AEG-1, or both proteins. D, AEG-1 expression affects insulin-stimulated and PTEN-inhibited Akt signaling. AEG-1 was knocked down with RNAi in U87 and treated with 100 ng/mL insulin for 30 minutes. In another set, U87 were transfected with vector control or AEG-1, with and without PTEN. Phospho-Akt, phospho-GSK3β, and cyclin D1 levels were analyzed. E, LY294002 induces a significant reduction in the level of p-Akt2-S474, and its downstream effectors p-GSK3β and cyclin D1, which were significantly reverted by overexpression of AEG-1. F, overexpression of AEG-1 rescues U87 from growth inhibitory effects of LY294002. LY294002 was added at day 1 and day 3 at 2 μmol/L final concentration. Two-way ANOVA analysis showed significance at ***, P < 0.001 with mean ± SD.
finding strengthens our hypothesis that targeting AEG-1 and inhibiting AEG-1–Akt2 interaction might be an effective therapeutic approach for GBM.

Discussion

Our previous studies demonstrated a positive feedback loop involving AEG-1–Akt signaling that regulates cell proliferation, survival, angiogenesis, chemoresistance, and invasion (9, 10). However, the details of how AEG-1 signals to Akt remain unclear. Here, we report that AEG-1 specifically interacts with Akt2 and not with Akt1 or Akt3 isoforms in GBM. Moreover, this interaction plays a decisive role in glioma cell biology. Our results demonstrate a high correlation between expression of AEG-1 and Akt2 in clinical glioma samples and suggest that AEG-1–Akt2 signaling is more dominant in the high-grade glioma, GBM. Our results also suggest that various Akt isoforms may play distinct roles in gliomas. Akt3 is known to play a more significant role in triple-negative breast cancer (38) and in our study we found that VG4 low-grade glioma shows higher Akt3 expression. Interestingly, our results also demonstrate that disruption of AEG-1–Akt2 interaction reduced AEG-1 proinvasive function, which is consistent with a previous report that Akt2 promotes breast cancer cell migration and invasion, whereas Akt1 has an opposite effect (32). A recent study shows that Akt2, but not Akt1, is necessary for PTEN-deficient prostate tumor cell survival (39). AEG-1 is localized in different compartments of the cancer cell (40). When both Akt-S473/474 and T308 sites are phosphorylated, Akt is fully activated and translocates from the cytoplasm into the
nucleus (33, 34). Interestingly, AEG-1 and Akt2 interaction occurs in the perinuclear region of glioma cells. We show that AEG-1 prolongs Akt phosphorylation at the S473/474 site. Collectively, our findings suggest that AEG-1 might temporally regulate Akt activity and localization through phosphorylation of the S473/474 site. AEG-1 interacts with the Akt-PH domain, which is also the interaction domain that recruits Akt to cell membranes by PIP3 (15). Most likely, this interaction happens after release of PIP3 binding, because AEG-1 has no direct impact on phospho-T308, which is the initial step of Akt activation at the cell membrane.

Our experiments support the importance of AEG-1–Akt2 signaling in glioma survival, proliferation, and invasion. Akt2 is essential for glioma survival, whereas knocking down Akt1 did not affect cell survival or proliferation (18). Our domain mapping results confirm that Akt2-PH domain is responsible for interacting with AEG-1 in glioma. Although protein sequence alignment indicates an overall high homology among human Akt isoforms, it is striking that there is about 35% amino acid sequence difference among the PH domains of Akt isoforms (Supplementary Fig. S8). Results from functional assays indicate that AEG-1–Akt2 signaling is more important for glioma cell invasion. AEG-1 failed to rescue inhibition of cell proliferation by both Akt1-PH and Akt2-PH, indicating possible modulation through interruption of Akt-PH-PIP3 lipids interaction. As a regulator, AEG-1 might interact with multiple proteins, which allow it to bind and change dynamics of Akt2-PH protein–lipid and protein–protein interactions. Hence, these differences might explain the binding specificity of AEG-1 to Akt2 rather than to the other Akt isoforms. The discovery of direct interaction between AEG-1 and Akt2 reveal the mechanism of previously reported activation of AEG-1–Akt signaling. It also provides valuable information to potentially develop anticaner strategies to target malignant gliomas harboring overactive Akt2.

Akt isoforms are highly conserved domain structures with different sequence variations and deletions. They play widespread roles in cancer biology (18, 41). We demonstrate that Akt2, rather than the other two Akt isoforms, is more abundant in high-grade gliomas and interacts with AEG-1. Furthermore, disrupting AEG-1–Akt2 interaction reduced cell proliferation and reverted TMZ resistance (Hu et al; unpublished data), which are consistent with a previous report that indicated that knockdown of Akt2 decreased cell proliferation and increased chemosensitivity in glioma cells (19). Transgenic Akt2 knockout animals are viable but show metabolic defects with impaired insulin-stimulated glucose transport/uptake (42). Our finding that AEG-1–Akt2 interaction regulates GSK3β phosphorylation correlates with the phenotype of cellular deficiency in glucose uptake. Akt2 is also involved in cancer cell invasion and metastasis (41). Our results confirmed that disruption of the AEG-1–Akt2 interaction reduces cell invasion, which is consistent with a previous report indicating that Akt2 promotes cell migration and invasion in other cancers (43, 44). Cytoplasmic localization of AEG-1 is important for enhanced cancer cell survival (35). It is possible that AEG-1–Akt2 interaction in the cytoplasm may enforce pro-survival signaling in glioma cells.

Previous studies suggest that AEG-1 regulates cancer cell proliferation and promotes glioma survival through cross-talk with different signaling pathways (40, 45). AEG-1 interacts with the p65 subunit of NF-κB, resulting in enhanced cancer cell invasion and migration by increasing MMPs (46). We explored the potential cross-talk between AEG-1–Akt2 and NF-κB signaling by using CAPE (Caffeic Acid Phenethylster), blocking IKK. CAPE weakly activated AEG-1–Akt2 signaling (Supplementary Fig. S7), suggesting that NF-κB activation by AEG-1 is independent of Akt activation. Akt plays a pivotal role in tumor cell survival through direct phosphorylation of the proapoptotic protein BAD. Phosphorylation levels of BAD were regulated by AEG-1–Akt2 interaction and affected glioma survival under different stress conditions (Figs. 4D and E and Fig. 5C). These results suggest that AEG-1–Akt2 complex protects glioma cells from classic intrinsic mitochondrial apoptosis pathway. In addition, tumor cell proliferation is controlled by AEG-1–Akt2 via GSK3β-cyclin D1 cascade. Consistent with earlier studies, the present study shows that a combination of AEG-1 KD with Akt2-PH overexpression dramatically inhibits survival, again confirming that AEG-1 plays a significant role in tumor cell proliferation and survival. AEG-1 was reported to have a functional role in chemoresistance through MDR in hepatocellular carcinoma (25) and our previous studies showed that AEG-1 could activate AMPK and induce protective autophagy in cancer cells (47, 48). Even though our in vivo experiments suggest better survival following combination of AEG-1 KD and Akt2-PH overexpression, we cannot rule out the possibility that AEG-1 might modulate other prosurvival signaling pathways, such as p38, EGFR, or Wnt signaling, to affect glioma cell survival. Nonetheless, we demonstrate here that AEG-1–Akt2 is a critical protein–protein signaling complex in glioma. This interaction may also occur in other cancer contexts in which both of these molecules are highly upregulated. Accordingly, it is worth exploring further this connection in other cancers.

On the basis of our interaction domain mapping, we hypothesize that it may be possible to synthesize small-molecule compounds to block AEG-1 and Akt2 interactions. Escape from conventional chemoradiotherapy and development of recurrence remain major hurdles for effectively treating gliomas in the clinic. It is unlikely that simply targeting AEG-1 or Akt2 alone will offer long-lasting efficacy in genetically diverse malignant gliomas. As such, small-molecule inhibitors designed to disrupt AEG-1–Akt2 interactions in combination with conventional treatment modalities might provide an optimal strategy to cure this dismal disease.

In summary, we document a direct interaction between AEG-1 and Akt2, two important cancer drivers, in malignant gliomas. The association between elevated expression of these two molecules and poor glioma patient survival has been confirmed using TCGA database information and this correlation in expression also exists when performing immunohistochemistry. We further elucidated the molecular mechanism of AEG-1–Akt2 signaling and demonstrate
that this interaction plays an important functional role in glioma biology (Fig. 6D). In these contexts, our results expand our understanding of the mechanism of AEG-1–Akt signaling in glioma and suggest a novel potential antitumor strategy that could use a combination of therapeutic drugs blocking the AEG-1–Akt2 association with conventional therapies to treat malignant gliomas. Considering the now documented important role of AEG-1 in many diverse cancers (49, 50), defining ways of inhibiting the functions of this oncogene holds significant potential to impact on cancer phenotype with broad therapeutic applications.

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

Authors’ Contributions
Conception and design: B. Hu, L. Emdad, S.K. Das, P.B. Fisher Development of methodology: B. Hu, M.D. Bacolod, X.-N. Shen Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Hu, X.-N. Shen

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