Diacylglycerol Kinase α Is a Critical Signaling Node and Novel Therapeutic Target in Glioblastoma and Other Cancers

Charli L. Dominguez¹, Desiree H. Floyd¹, Aizhen Xiao¹, Garrett R. Mullins³, Benjamin A. Kefas¹, Wenjun Xin², Melissa N. Yacur¹, Roger Abounader⁴, Jae K. Lee², Gabriela Mustata Wilson⁵, Thurl E. Harris³, and Benjamin W. Purow¹
INTRODUCTION

High-grade gliomas are the most common brain tumors in adults and are universally fatal. These tumors partially resemble glial cells, but their cell of origin is unclear. Glioblastoma multiforme (GBM), grade 4 glioma, is the most common and aggressive variant. GBMs are primary cancers of the central nervous system (CNS) that appear de novo or arise from low-grade gliomas (1) and account for more than 51% of all gliomas diagnosed each year. GBMs are exceedingly treatment-resistant, even with combined surgical resection and radio- and chemotherapy, and always recur (1). These tumors are highly invasive and infiltrate the normal brain parenchyma in a diffuse fashion, which contributes to their resistance (2). The frequency and lethality of GBM, combined with resistance to treatment, present a critical need for novel therapeutic approaches.

Treatment resistance also arises in GBM and other cancers through their genetic diversity and complexity. It has been shown in cancer, perhaps most elegantly in GBM (3), that multiple signaling pathways are dysregulated in an individual cell. Thus, the inhibition of 1 or 2 pathways promotes the upregulation of other oncogenic pathways—in part through feedback loops—allowing the cancer cell to survive. It is therefore increasingly clear that more effective cancer treatment will require either cocktails of inhibitors or the discovery of critical signaling nodes that can be targeted to block numerous pathways simultaneously. Herein, we investigate a possible signaling node as a promising cancer target.

We previously showed Notch to be a potential therapeutic target in glioblastoma (4), and in subsequent efforts to determine its signaling role we have sought to better understand its cross-talk with other pathways. This led us to profile microRNAs (miRNA) regulated by Notch, as we have described previously (5). miRNA-297 was among the miRNAs found to be upregulated with Notch inhibition, and upon delivery to glioblastoma cells it was observed to be more toxic than any other miRNA tested in our laboratory. This led us to consider possible targets of miRNA-297. After an extensive search through online databases, we did not find any known oncogenes predicted to be strongly targeted by miRNA-297, but the gene diacylglycerol kinase α (DGKα) was among the top predicted targets.

Diacylglycerol (DAG) is a membrane lipid that is an established second messenger activating several signaling proteins, most of which have been implicated in cancer (6). DAG is typically metabolized through DGKs, resulting in the creation of phosphatidic acid (7). Phosphatidic acid is a phospholipid that is found at relatively low levels compared with other lipids, yet it has been implicated in regulating a number of signaling pathways and proteins (8). Although there are 10 known DGK enzymes, DGKα has been implicated in a variety of cellular functions apart from other DGKs. Through siRNA knockdown of DGKα, it was shown to play a positive role in the proliferation and migration of endothelial cells (9). DGKα also plays a role in the regulation of NF-κB in melanomas. Although DGKα is expressed in several melanoma lines, it is not expressed in noncancerous melanocytes (10). Of note, DGKα synthesis of phosphatidic acid can be attenuated by 2 established small-molecule inhibitors: R59022 (6-[2-{4-[(4-Fluorophenyl)phenylmethylene]-1-piperidinyl}ethyl]-7-methyl-5H-thiazolo-[3,2-a]-pyrimidin-5-one) and R59949.
stimulation and increasing DGKα RNA interference each inhibited VEGF-induced chemotaxis and a significant increase in caspase-3/7 activity in cells upon α-ketoglutarate (KGA) to increase hypoxic stress. The results indicate that R59949, a DGKα dominant-negative mutant, and specific RNA interference each inhibited VEGF-induced chemotaxis and DNA synthesis in human umbilical vein endothelial cells (HUVECs). The study also showed a correlation between VEGF-A stimulation and increasing DGKα levels. All of this taken together poses a mounting case for DGKα regulation of cell survival, proliferation, migration, and angiogenesis signaling in a variety of cellular environments. The need for further investigation of DGKα as a possible therapeutic target in cancer is evident, given its numerous connections to oncogenic pathways. Herein, we show in GBM and other cancers that DGKα is a critical signaling node essential for several oncogenic pathways and is a promising therapeutic target.

RESULTS

Attenuation of DGKα Causes Toxicity in Glioblastoma Cells

To assess the effect of this inhibition in established GBM cell lines, DGKα was silenced with siRNA and inhibited via small-molecule inhibitor R59022. Percentage cell death by Trypan blue was significantly increased in both U87 and U251 cell lines when compared with controls (Fig. 1A). In conjunction with the observed cell toxicity, cell viability by alamarBlue assay was significantly reduced with DGKα silencing in both GBM cell lines (Supplementary Fig. S1). To visualize cell toxicity changes after DGKα knockdown, U251 GBM cells were stained with Hoechst and propidium iodide, revealing a decrease in total cell number with an increase in membrane-compromised cells after DGKα knockdown (Fig. 1B). An immunoblot for DGKα was also done to verify transfection efficiency in both GBM cell lines as well (Fig. 1C). The toxicity seen in GBM cells was consistently observed with relative rapidity after treatment with either siRNA or small-molecule inhibitors targeting DGKα. We sought to confirm that cell death was being induced and by what mechanism. Typically, cell count assays were conducted between 72 and 96 hours posttreatment, showing a rapid effect on cell numbers given the typical time course for siRNA knockdown. A slowing in cell proliferation was considered unlikely, given no change in bromodeoxyuridine incorporation measured by ELISA assay data (not shown). Autophagy was also explored, but there was no difference in LC3-II levels by immunoblot (data not shown). To assess the possibility of cell death, Annexin V assay via fluorescence-activated cell sorting (FACS) analysis was conducted on the U87 and U251 GBM lines and the A-375 melanoma line (Fig. 1D), with the results indicating an increase in Annexin V–positive cells with DGKα knockdown. Next, caspase-mediated apoptosis was investigated at both 24- and 36-hour time points. After transfection, there was a significant increase in caspase-3/7 activity in cells upon silencing of DGKα in U87 and U251 GBM cells, as well as in melanoma cells (Fig. 1E). In addition, we observed an increase in cleaved PARP expression in cell lysates in which DGKα expression was silenced (Fig. 1F). These results suggest that DGKα inhibition causes cell toxicity in cancer cells through caspase-mediated apoptosis.

DGKα Is Upregulated and Increases Cell Numbers in Human Glioblastoma

Next, we sought to determine if DGKα might have oncogenic properties, given the substantial effect that silencing has on GBM cell proliferation. In U87, U251, and A-375 cells, forced overexpression of DGKα by transient transfection resulted in a significant increase in cell proliferation (Fig. 2A). Next, to establish long-term overexpression of DGKα, we infected U87, U251, and A-375 cells with a lentiviral DGKα vector. DGKα overexpression significantly increased tumor cell proliferation (Fig. 2B) in vitro. Upon quantification of DGKα protein by immunoblot in both normal brain and GBM human tissue samples, we found there to be modest but significant increases in levels of DGKα protein in GBMs (Fig. 2C). Also, both normal and GBM tissue samples were analyzed to determine mRNA levels of DGKα (Fig. 2D). Although the difference in mean DGKα mRNA levels was not significant, some GBM samples had markedly increased DGKα mRNA. Finally, other data available online from The Cancer Genome Atlas (TCGA; ref. 13) indicated amplification of DGKα in 1% to 4% of GBM and several other cancers (Supplementary Table S1). Nonetheless, the moderate overexpression of DGKα in GBM cells seems inconsistent with the apparent addiction to its expression, suggesting this may be an example of “nononcogene addiction”—in which cancer cells have a disproportionate dependency on a gene that is not overexpressed (14).

Glioblastoma Toxicity Is a Specific Effect of Decreased DGKα Activity

DGKα produces phosphatidic acid through the phosphorylation of diacylglycerol. To verify that the cellular toxicity observed in GBM cells is a specific consequence of the attenuation of DGKα activity, we investigated the role of phosphatidic acid. Knockdown of DGKα was conducted in GBM cells as above and exogenous phosphatidic acid added. Notably, the substantial cytotoxicity in GBM (Fig. 3A and B) and melanoma (Fig. 3C) cells upon DGKα knockdown was rescued with exogenous phosphatidic acid. Similarly, phosphatidic acid administration also rescued the phenotype observed upon treatment with small-molecule inhibitor R59022 (Fig. 3D and E) in GBM cells. Finally, to confirm that phosphatidic acid levels were decreased with DGKα knockdown, phosphatidic acid levels were measured through mass spectrometry. Total phosphatidic acid levels were significantly decreased in U251 GBM cells after transfection with DGKA siRNA (Fig. 3F). These results establish a role for DGKα production of phosphatidic acid in cancer cell viability.

Attenuation of DGKα Causes Toxicity through Regulation of Key Oncogenic Pathways

DGKα and its product phosphatidic acid have been linked to several established oncogenic pathways, including mTOR (15), HIF-1α (12), and AKT (16). To evaluate the effects that silencing DGKα has on these possible mediators, immunobLOTS
DGKα Is a Critical Signaling Node and Novel Target in Cancer

Figure 1. DGKα inhibition causes toxicity in glioblastoma and melanoma cells. A, DGKα knockdown was assessed in GBM cells U87 and U251 via transfection with either control or DGKα siRNA and inhibition via treatment with DMSO (v:v) or R59022 at 10 μmol/L. Percentage cell death was evaluated after 4 days. Fresh drug/vehicle was added daily for all inhibitor experiments. B, to visualize cell death changes after DGKα knockdown, U251 GBM cells were stained with Hoechst and propidium iodide. C, an immunoblot was used to verify transfection efficiency with siRNA in U87 and U251 cell lysates at 72 hours with α-tubulin as control. D, FACS analysis was conducted on both U87, U251, and A-375 cell lines showing an increase in Annexin V–stained cells after DGKα knockdown. E, caspase-3/7 activity was measured 36 to 72 hours after DGKα knockdown in U87, U251, and A-375 melanoma cells. F, protein levels of cleaved PARP were also increased in U251, A-375, and U87 cells after DGKα silencing (*, P < 0.05 and **, P < 0.01; Student t test). DMSO, dimethyl sulfoxide; PE, phycoerythrin; 7-AAD, 7-amino-actinomycin D.

were done in GBM cells with DGKα knockdown. There was a significant decrease in total mTOR and phos-mTOR_{ser2448} in GBM cells (Fig. 4A and B) and melanoma cells with attenuation of DGKα activity (Fig. 4C). HIF-1α and phos-mTOR_{ser2448} were decreased by DGKα knockdown in GBM cells (Fig. 4A) as well. In addition, we found that DGKα knockdown decreases c-Myc levels and phosphorylation of AKT_{ser473} (Supplementary Fig. S2). We were also prompted to assess whether DGKα inhibition influences the SREBP (sterol regulatory element-binding protein) cholesterol synthetic pathway by 2 recent reports, the first linking mTOR and the phosphatidic acid modulator lipin to SREBP activity (17), and the second establishing SREBP as oncogenic and a therapeutic target in GBM (18). Following DGKα knockdown
in glioblastoma cells, we determined mRNA levels of the SREBP targets farnesyl diphosphate synthase (FDPS), HMG-CoA reductase (HMGCR), and stearoyl CoA-desaturase (SCD; ref. 17). After normalization, each of the genes tested had significantly reduced mRNA levels when compared with control (Supplementary Fig. S3A). To assess whether any of these DGKα mediators might be central for the cytotoxicity of DGKα knockdown/inhibition in GBM cells, we carried our “rescue” experiments with overexpression of wild-type mTOR and constitutively active HIF-1α. Overexpression of mTOR and HIF-1α alone partially rescued the toxicity from DGKα knockdown and inhibition, and when combined the phenotypic rescue was slightly stronger in both GBM and melanoma cells (Fig. 4D–F). However, a similar overexpression of c-Myc failed to rescue the toxicity (Supplementary Fig. S3B). Taken together, these data suggest that decreased expression of mTOR and HIF-1α plays a substantial role in the cytotoxicity observed with DGKα knockdown and inhibition in cancer.

**DGKα Regulates mTOR Transcription through Modulation of Cyclic AMP Levels**

A similar degree of phenotypic rescue from DGKα knockdown occurred with a wild-type mTOR expression vector as with a constitutively active mTOR vector (data not shown). This, combined with a strong correlation of DGKA and mTOR mRNA expression in the TCGA GBM data (Fig. 4G and Supplementary Table S2), suggested that DGKα might regulate mTOR expression. Given prior reports that phosphatidic acid promotes activity of phosphodiesterases, decreasing cyclic AMP, and that a cAMP-modulated transcription factor could drive mTOR transcription (19, 20), we hypothesized that DGKα was diminishing cAMP levels to prompt an increase in mTOR transcription. To initially evaluate this, we first assessed the effects on mTOR expression of DGKA knockdown with a lentiviral hairpin RNA (shRNA). We observed a significant decrease in mTOR mRNA levels with prolonged DGKα knockdown versus control (Fig. 4H). Given this result, we used an mTOR promoter reporter luciferase assay to determine if DGKα transcriptionally regulates mTOR. With attenuation of DGKα activity via siRNA or small-molecule inhibitor, there was a significant decrease in mTOR promoter activity in GBM and melanoma cells (Fig. 4I and J). To evaluate whether DGKα was significantly affecting cAMP levels in GBM cells, ELISA was conducted after DGKα activity was attenuated via siRNA or small-molecule inhibitor. This revealed significant increases in cAMP levels with DGKα knockdown and inhibition in GBM and melanoma cells (Fig. 4K and L). To determine if cAMP regulates mTOR transcription in GBM, cells were treated with exogenous cAMP and mTOR promoter activity assessed by luciferase assay; we observed a significant decrease in mTOR transcription (Fig. 4M). In addition, cells treated with the phosphodiesterase-4D (PDE4) inhibitor rolipram also showed a significant decrease in mTOR transcription (Fig. 4N), further supporting the role of cAMP in the hypothesized pathway (Fig. 4O). These data indicate for the first time that DGKα regulates mTOR transcription, likely via modulation of cyclic AMP levels.
DGKα is a Critical Signaling Node and Novel Target in Cancer

**Figure 3.** DGKα inhibition and phosphatidic acid (PA) synthesis. **A**, U87 cells, and **B**, U251 GBM cells, and **C**, A-375 melanoma cells were transfected with control or DGKα siRNA with simultaneous administration of exogenous PA at 33 μmol/L or vehicle (1 MeOH:2 CHCl₃, v:v). Full phenotypic rescue of decreased cell viability was observed upon delivery of PA to DGKα siRNA transfected cells. Phenotypic rescue was achieved in **D**, U87 cells and **E**, U251 GBM cells treated with R59022 at 5 μmol/L. **F**, after transfection with siRNA, mass spectrometry was used to show a decrease in total PA levels in lipid lysates from U251 cells. *, P < 0.05; **, P < 0.01; Student t test. DMSO, dimethyl sulfoxide.

of cAMP levels. This novel pathway regulating mTOR expression may have implications not only for the role of DGKα in GBM, but also for studies of the role of cAMP and of mTOR regulation in cancer.

**Relative Lack of Cytotoxicity of Targeting DGKα in Noncancerous Cells**

Classically, one of the disadvantages of therapeutic treatments for cancer is the negative side effects due to nonspecific effects on noncancerous cells. To assess the effect of DGKα inhibition on noncancerous cells, we used normal human astrocytes and fibroblasts. First, we silenced DGKα expression in astrocytes (Fig. 5A) with siRNA and confirmed transfection efficiency with an immunoblot (Fig. 5B), with no significant effect on cell numbers. We then attempted to assess toxicity of the small-molecule inhibitors on these noncancerous cells, and similarly did not observe any significant decrease in cell viability at concentrations toxic in GBM cell lines.
**Figure 4.** DGKα induces glioblastoma cell death through regulation of oncogenic pathways. **A,** immunoblot analysis of HIF-1α, total mTOR, and phospho-mTOR<sub>ser2448</sub> in U251 cells transfected with DGKa or control siRNA. **B,** total mTOR and phospho-mTOR<sub>ser2448</sub> were decreased in U87 cell lysates, as well as in (C) A-375 cell lysates. To verify the role HIF-1α and mTOR play in the observed cell toxicity, each was overexpressed through plasmid transfection, and cell proliferation was assayed in (D) U87 cells, (E) U251 cells, and (F) A-375 cells transfected with siRNA. **G,** inhibition of DGKα mRNA levels in 576 human GBM samples (13). Spearman correlation test conducted with a P value of 6.159e-16 and a Pearson correlation test P value of less than 2.2e-16. **H,** in U87, mRNA levels of HIF-1α, mTOR, and p-mTOR<sub>ser2448</sub> were quantified by qRT-PCR in response to 1 μM, 10 μM, and 100 μM of 1HET-1, 5 μM, and 10 μM of rolipram, respectively. **I,** mRNA fold change of HIF-1α, mTOR, and phospho-mTOR<sub>ser2448</sub> in U87, A-375, and U251 cell lines. **J,** cAMP concentration (μmol/L) in A-375 cell lines. **K,** cAMP concentration (μmol/L) in U251 and U87 cell lines. **L,** cAMP concentration (μmol/L) in A-375 cell lines. **M,** cAMP concentration (μmol/L) in U251 and U87 cell lines. **N,** mTOR reporter luciferase activity assay. **O,** a schematic of the proposed pathway of DGKα regulation of mTOR transcription.
DGKα Attenuation Is Safe in Normal Cells

A, normal human astrocytes were transfected with either control or DGKα siRNA, and cell number was assessed at 3 days posttransfection. B, an immunoblot was done on cell lysates posttransfection to check for transfection efficiency. C and D, normal human astrocytes and fibroblasts were treated with 10 μmol/L R59022, R59949, or DMSO (v:v) control, and cell proliferation was assessed at 3 days posttreatment, with no significant decrease observed in cell number. E, levels of cAMP were also evaluated via ELISA 5 days after knockdown or inhibition of DGKα in astrocytes. F, effects of exogenous cAMP on MTOR transcription in astrocytes were assessed through MTOR promoter luciferase assay 5 days after treatment. G, rolipram was administered at 40 μmol/L to further test the effect of phosphodiesterase inhibition/cAMP levels on MTOR transcription in astrocytes, with promoter activity assayed at 6 days posttreatment. *, P < 0.05; **, P < 0.01; Student t test. DMSO, dimethyl sulfoxide.

Figure 5. DGKα attenuation is safe in normal cells. A, normal human astrocytes were transfected with either control or DGKα siRNA, and cell number was assessed at 3 days posttransfection. B, an immunoblot was done on cell lysates posttransfection to check for transfection efficiency. C and D, normal human astrocytes and fibroblasts were treated with 10 μmol/L R59022, R59949, or DMSO (v:v) control, and cell proliferation was assessed at 3 days posttreatment, with no significant decrease observed in cell number. E, levels of cAMP were also evaluated via ELISA 5 days after knockdown or inhibition of DGKα in astrocytes. F, effects of exogenous cAMP on MTOR transcription in astrocytes were assessed through MTOR promoter luciferase assay 5 days after treatment. G, rolipram was administered at 40 μmol/L to further test the effect of phosphodiesterase inhibition/cAMP levels on MTOR transcription in astrocytes, with promoter activity assayed at 6 days posttreatment. *, P < 0.05; **, P < 0.01; Student t test. DMSO, dimethyl sulfoxide.

DGKα Inhibition Is Cytotoxic in Multiple Cancer Lines

Given the toxicity observed in GBM cell lines, its impact on major oncogenic pathways, the previous report on DGKα overexpression in melanoma cells (10), and its amplification in subsets of several cancers (described above), we sought to determine if DGKα is a potential therapeutic target in other types of cancer as well. First, lysates from various cancer cell lines and normal human astrocytes and fibroblasts were evaluated by immunoblot to assess the basal DGKα levels in each cell line used in this work (Fig. 6A). Also, in melanoma (Fig. 6B), cervical cancer (Fig. 6C), and breast cancer (Fig. 6D) cell lines, the percentage cell death after knockdown of DGKα or inhibition via small-molecule inhibitor R59022 (10 μmol/L) was assessed by Trypan blue cell counts. Second, to evaluate the potential therapeutic window of the small-molecule inhibitor R59022, we conducted a dose–response assay in each cancer and normal cell line used above. Each cell line was treated with doses ranging from 5 to 100 μmol/L, with dimethyl sulfoxide (DMSO; v:v)
Figure 6. Attenuation of DGKα activity has preferential toxicity for cancer cell lines. A, basal DGKα levels were evaluated by immunoblot in normal human cell lines and various cancer cell lines, with α-tubulin shown as loading control. Cell toxicity was assessed by cell counts/Trypan blue in (B) A-375 (melanoma), (C) HeLa (cervical cancer), and (D) MDA-MB-231 (breast cancer) lines 4 days after DGKα knockdown or treatment with R59022 at 10 μmol/L or DMSO vehicle. E, dose–response curves were generated for astrocytes, fibroblasts, U251, U87, A-375, HeLa, MDA-MB-231, and 0308 (GSC) cells by cell counts and normalized for DMSO (v:v) at 5, 10, 20, 40, 60, 80, and 100 μmol/L R59022 after 4 days of treatment. *, P < 0.05; **, P < 0.01; Student t test.

cell controls at each dose. Percentage cell survival was evaluated at 4 days for each dose and dose–response curves plotted (Fig. 6E). Cancer cell lines were substantially more sensitive to R59022 than normal cells.

DGKα Knockdown and Inhibition Affect Tumor Growth, Angiogenesis, and Survival of Mice with Intracranial and Subcutaneous Tumors

To test DGKα knockdown as a potential therapy, we first used a GBM stem cell (GSC) xenograft treatment model in mice. DGKα knockdown via lentiviral vector was tested against a GSC line in vitro (Supplementary Fig. S4) and in vivo. First, 0308 GSCs were stereotactically injected into the brain of severe combined immunodeficient (SCID) mice and given a week to become established. Lentiviral particles containing control or DGKA shRNA were delivered via convection-enhanced delivery (CED), to increase delivery volume and to promote diffusion of the virus. The treatment group had significantly increased survival (P = 0.0073; Fig. 7A), and MRI images also showed significantly smaller tumor size in this group as well (Fig. 7B).

To predict whether one of the small-molecule DGKα inhibitors would penetrate the blood–brain barrier (BBB) sufficiently, we used an in silico algorithm based on the BBB.
DGKα Is a Critical Signaling Node and Novel Target in Cancer

**Figure 7.** DGKα knockdown and inhibition affect in vivo tumor growth, angiogenesis, and mouse survival. A, after in vivo implantation of 0308 GSCs and CED infusion of lentiviral particles with control or DGKα shRNA 1 week later, mouse survival was followed. A Kaplan-Meier curve and log-rank analysis exhibits a significant increase in survival of mice in the treatment group when compared with control mice (P = 0.007). B, MRIs were conducted at 40 days posttumor implantation and show characteristically smaller tumors in mice in the treatment group. C, plot of CNS activity presents several known CNS-penetrating (blue points) and peripherally acting (red points) drugs, with the green points denoting either DGKα inhibitor R59022 or R59949. D, mice were injected with U87 cells intracranially and then treated with daily intraperitoneal injections of R59022 or vehicle on days 8 through 19. Intraperitoneal injections of R59022 at 2 mg/kg significantly increased median survival (P < 0.01; log-rank analysis). E, tumor volume of subcutaneous U87 tumors in vivo was assessed after daily treatment with R59022 10 mg/kg or vehicle, with treatment beginning 37 days after tumor implantation. F, subcutaneous U87 tumors were resected and exhibited a visible difference in vascularity after treatment with R59022 versus vehicle, and (G) frozen sections were stained for CD34 to assess blood vessels after treatment (magnification of ×200). H, immunohistochemistry was done for cleaved caspase-3 to assess apoptosis in the resected tumors above (magnification of ×100). I, tumor volume of subcutaneous A-375 tumors in vivo was evaluated after daily treatment with DMSO or R59022 at 10 mg/kg starting 4 days after tumor implantation. J, to evaluate the pharmacokinetics of R59022 in vivo, after a single intraperitoneal dose of R59022, blood was collected at various time points via cardiac puncture, with the samples used for mass spectrometry for blood plasma levels of R59022. *, P < 0.05; **, P < 0.01; Student t test.
penetration of hundreds of diverse compounds in rodents. This algorithm predicted that R59022 would have adequate BBB penetration, whereas R59949 would not, despite their very similar structures (Fig. 7C; Supplementary Table S3; ref. 21).

Next, the DGKα small-molecule inhibitor R59022 was used in vivo to initially evaluate the therapeutic potential of systemic DGKα inhibition. SCID mice were implanted with U87 GBM cells by the techniques above. After tumor establishment, mice were given daily intraperitoneal injections of either DMSO or 2 mg/kg of R59022 for 12 consecutive days. The treatment group had significantly increased survival (P = 0.01; Fig. 7D). It is important to note that there was no decrease in mouse weights with R59022 treatment at doses of 2 or 10 mg/kg (data not shown).

To further evaluate the effects of R59022 administration, U87 GBM cells were injected into the flank of nude mice to establish subcutaneous tumors. Daily injections were given as above, and we noted that mean tumor volumes were significantly smaller after treatment with the DGKα inhibitor (Fig. 7E; Supplementary Table S4A). When we allowed some tumors from mice treated with R59022 time to catch up in size with tumors from DMSO-treated mice, the resected tumors displayed an obvious difference in vascularity (Fig. 7F). Given this change in vascularity, immunohistochemistry for CD34 was conducted to visualize blood vessels at the microscopic level. There was a sharp decrease in blood vessel density in the treated tumors (representative image in Fig. 7G). To assess for apoptosis in these resected tumors, immunohistochemistry for cleaved caspase-3 (Fig. 7H) was conducted, indicating clear signs of apoptosis in tumors after DGKα inhibition.

To determine if systemic DGKα inhibition might have therapeutic potential against other cancers, we also tested it with subcutaneous implantation of A-375 melanoma cells. After daily intraperitoneal injections with the inhibitor R59022, mean tumor volume of treated mice was significantly smaller in comparison with control mice (Fig. 7I and Supplementary Fig. S4). We have confirmed and extended the work of others to show that inhibition of DGKα decreases the expression and/or phosphorylation of mTOR, HIF-1α (12), AKT (16), and c-Myc (33). We hypothesized that regulation of these key oncogenic pathways underlies the cytotoxicity of DGKα inhibition. To assess their relative importance, we delivered mTOR, HIF-1α, and c-Myc plasmids to GBM cells treated with DGKα inhibition. Both mTOR and HIF-1α plasmids partially rescued cell toxicity, and even more so when combined. Although inhibition of mTOR and HIF-1α were critical in mediating DGKα inhibition in GBM cells, it is important to note that other pathways may be more central in other cancers; DGKα has been shown by others to mediate Ras/Raf (34), ALK (35), Met (36), and VEGF (9) signaling. Furthermore, we found a link between DGKα inhibition and the transcriptional regulator SREBP (37), which has recently been found to promote tumor growth in patients with GBM through the PI3K/AKT signaling pathway (18). After DGKα silencing, mRNA expression of several genes induced by SREBP was significantly decreased. It is notable that SREBP has also been linked to mTOR and phosphatidylinositol signaling, which suggests that DGKα may regulate SREBP via more than one pathway. Although

DISCUSSION

Although previous reports have linked DGKα to specific cellular pathways, this research has largely focused on cellular signaling (9, 10, 12, 22–24) or immunology (25, 26). This kinase has yet to receive significant attention for its impact on cancer cell viability and its potential as a cancer target. Notably, one recent report establishes a key role for DGKα in cancer cell migration (22), and, even more recently, it was noted that DGKα restrains the antitumor immune response (27). Our work establishes DGKα as a promising therapeutic target for the treatment of GBM, with potential for other cancers as well. The attenuation of DGKα through siRNA, shRNA, and small-molecule inhibitors all produced striking cellular toxicity in GBM cells, as well as in other cancers. Conversely, overexpression of this kinase promoted GBM cell proliferation in vitro, and expression levels were moderately increased in human GBM tissue samples. DGKα thus exhibits oncogene-like characteristics; however, the increase in DGKα expression is moderate, suggesting the possibility of a cancer-specific nononcogene addiction and not a classic oncogenic model. The nononcogene addiction model suggests that certain genes, while not significantly overexpressed in cancer, are nonetheless far more necessary for the survival of cancer cells than normal cells and can represent promising therapeutic targets (14, 28). That being said, profiling studies such as TCGA have indicated amplification of the DGKA locus in 1% to 4% of several cancers, as noted above. Further studies with higher sample numbers need to be done to assess expression levels of DGKα in patient samples of GBM and other cancers to help clarify its degree of overexpression.

The history of cancer research is rife with examples of promising therapeutic strategies that proved disappointing in patients. This is due in large part to the genetic instability and heterogeneity of cancer cells, which render them able to develop resistance to treatments and adapt cellular networks to maintain a malignant phenotype (29). This seems especially likely for therapies directed toward a single target or pathway, as in therapies specifically targeting angiogenesis (30) or tyrosine kinases (31). Targeting a signaling node such as DGKα, with critical roles in numerous key cancer pathways, represents one answer to these obstacles. We have confirmed and extended the work of others to show that inhibition of DGKα decreases the expression and/or phosphorylation of mTOR (32), HIF-1α (12), AKT (16), and c-Myc (33). We hypothesized that regulation of these key oncogenic pathways underlies the cytotoxicity of DGKα inhibition. To assess their relative importance, we delivered mTOR, HIF-1α, and c-Myc plasmids to GBM cells treated with DGKα inhibition. Both mTOR and HIF-1α plasmids partially rescued cell toxicity, and even more so when combined. Although inhibition of mTOR and HIF-1α were critical in mediating DGKα inhibition in GBM cells, it is important to note that other pathways may be more central in other cancers; DGKα has been shown by others to mediate Ras/Raf (34), ALK (35), Met (36), and VEGF (9) signaling. Furthermore, we found a link between DGKα inhibition and the transcriptional regulator SREBP (37), which has recently been found to promote tumor growth in patients with GBM through the PI3K/AKT signaling pathway (18). After DGKα silencing, mRNA expression of several genes induced by SREBP was significantly decreased. It is notable that SREBP has also been linked to mTOR and phosphatidylinositol signaling, which suggests that DGKα may regulate SREBP via more than one pathway. Although
the intricacies of the mechanisms through which DGKα inhibition exerts its toxic effects on different cancers need further investigation, we are optimistic that this approach may damage too many oncogenic pathways for cancer cells to adapt to treatment.

We were initially surprised to see equally strong rescue in cancer cell toxicity from DGKα inhibition with expression vectors for mTOR and constitutively active mTOR (data not shown). This, along with a strong correlation between DGKA and MTOR expression levels in TCGA GBM samples, suggested that DGKα might be regulating mTOR expression as well as activity. Further experiments supported this, via a unique DGKα-PA-PDE-cAMP-mTOR transcription pathway. This seems to be a significant function for DGKα in cancer cells. These results have importance not only for neuro-oncology, but also for the study and targeting of cAMP and mTOR in cancer. It indicates a new approach to mTOR inhibition, using small-molecule DGKα inhibitors to decrease mTOR expression. Reducing mTOR expression may have advantages over mTOR inhibitors now in use that inhibit mTORC1 and/or mTORC2, given that mTOR has been hypothesized to participate in an mTORC3 complex and may have other pro-cancer functions as well. DGKα inhibition also seems to represent a novel cancer-specific means to elevate cAMP levels selectively in cancer cells, potentially avoiding side effects from nonselective cAMP-elevating agents such as phosphodiesterase inhibitors. In addition, these findings are likely to have implications for other signaling pathways in cancer that also regulate cAMP.

Most successful cancer treatments have a broad therapeutic window; that is, they affect cancer cells at much lower concentrations than they do normal cells. The ideal treatment would pose little risk to normal cells, while efficiently killing cancer cells. It is important to note that DGKα inhibition significantly affects cell viability in normal human cells only at very high concentrations, suggesting there may be a substantial therapeutic window in vivo. The minimal effect in noncancerous cells, combined with the marked toxicity in GBM cells, also points to the possibility of cancer cells having a dependence on DGKα that is not seen in normal cells. Although there have been 10 DGK enzymes discovered to date, there does not seem to be a significant redundancy and DGKα seems to be particularly relevant for cancer cells. For example, it has been shown that normal melanocytes do not express DGKα, whereas melanoma cells do express this isoform (10). It is notable that DGKA-knockout mice are generally healthy with a defect in T-cell anergy (38). Our in vivo experiments supported the potential safety of using DGKα small-molecule inhibitors, as no toxicity was observed and there was no decrease in mouse weights with R59022 treatments at doses of 2 or 10 mg/kg (data not shown). Though promising in these preliminary studies, the safety profile of DGKα inhibition needs to be evaluated with detailed animal studies.

These results also showed the use of a single injection of DGKα-targeted therapy in a challenging GBM treatment model, using highly invasive and resistant GSCs. CED was used for the infusion of lentiviral particles carrying DGKα shRNA, in a manner that could be clinically applicable. The shRNA-targeting DGKα was delivered in lentiviral particles to achieve long-term expression (39). The fact that DGKα knockdown through a single infusion had a significant effect on tumor growth in vivo is encouraging, and it is likely that repeated treatment with DGKα inhibition would result in improved efficacy.

The therapeutic potential of DGKα inhibition is facilitated by its being druggable, with 2 compounds already available. Although it is always possible with small-molecule agents such as R59022 and R59949 that nonspecific effects contributed to their toxicity in cancer cells, the phosphatidic acid-replacement rescue experiments shown here argue against this. Initial in vivo experiments supported the therapeutic potential of these DGKα small-molecule inhibitors. This was true in both orthotopic and subcutaneous GBM models, as well as in a subcutaneous melanoma model. Our experiments also suggested that this in vivo efficacy might have been due at least in part to potent antiangiogenic effects. Pharmacokinetic studies indicated that the positive in vivo results were obtained despite a short R59022 half-life in the mouse. It is possible that R59022 accumulates in the tumor with repeated dosing, or that tumor cells are sensitive to lower concentrations in vivo than they are in vitro. We show that R59022 exhibits a pronounced antiangiogenic effect in vivo, and this may occur at lower concentrations than what was used in vitro. Regardless of mechanism, the pharmacokinetic data suggest that better efficacy could be achieved with DGKα inhibitors optimized for in vivo usage.

We propose that this work sets the foundation for DGKα as a promising therapeutic target in cancer. These results shed light on the significant effects of DGKα inhibition on cancer cell viability, the possibility it is an oncogene or example of nononcogene addiction, and its safety for normal cells. Our results establish DGKα as a single therapeutic target linked to multiple oncogenic pathways, with relevance for GBM and other cancers as well. This work also indicates the importance of the DGKα product phosphatidic acid in cancer cell biology, and ongoing studies are evaluating this signaling phospholipid as a therapeutic target in itself.

**METHODS**

**Cell Lines and Patient Samples**

U87MG, MDA-MB-231, HeLa, and human fibroblast cell lines were obtained from American Type Culture Collection. U251MG was a gift of Howard Fine (New York University, New York, NY). A-375 was a gift of Daniel Gioeli (University of Virginia, Charlottesville, VA). Astrocytes were purchased from Lonza. The tumor stem cell line 0308 was obtained from the Tumor Bank at the University of Virginia under an approved Institutional Review Board protocol. The cancer lines U87, U251, A-375, MDA-MB-231, and HeLa were authenticated by short tandem repeat profiling in 2013 by Laragen, Inc. The GSC line 0308 is a primary line and therefore cannot be authenticated but is used at low-passage number (<15 passages from its initial stocks) in these experiments. The normal human fibroblasts and astrocytes are from a commercial vendor and, as they are primary cells, authentication does not apply.

| July 2013 | Cancer Discovery | 793 |
**Cell Transfection**

The effects of DGKα knockdown by siRNA were examined in U251MG (GBM), U87MG (GBM), 0308 (GSC), A-375 (melanoma), MDA-MB-231 (breast cancer), and HeLa (cervical cancer) cell lines. Cells were plated in 6-well tissue culture plates at a density of between 4.0 × 10⁴ and 6.0 × 10⁴ per well and transfected 24 hours later. Cells were transfected with Oligofectamine (Invitrogen) according to the manufacturer’s protocol, with a concentration of 10 nmol/L siRNA.

**ImmunobLOTS**

ImmunobLOTS were conducted as previously described (4). Primary antibodies included DGKα (ProteinTech), phospho-AKT, HIF-1α, phos-mTOR, c-Myc, and PARP. All antibodies were obtained from Cell Signaling Technology unless otherwise noted. Horseradish peroxidase-conjugated secondary antibodies to rabbit or mouse immunoglobulin G (IgG) were used (1:7,500; Jackson ImmunoResearch Laboratories). Conjugated secondary antibodies were obtained from Cell Signaling Technology unless otherwise noted. Horseradish peroxidase-conjugated secondary antibodies to rabbit or mouse immunoglobulin G (IgG) were used (1:7,500; Jackson ImmunoResearch Laboratories).

**Annexin V Staining**

Cells were harvested 4 days posttransfection, and Annexin V staining was conducted according to the manufacturer’s description (BD Biosciences).

**Caspase-3/7 Assay**

Cells were harvested with trypsin (Fisher MediaTech). Caspase-3/7 assay was conducted according to the manufacturer’s guide (Promega). After 1-hour incubation at 25°C, each sample was measured in a Promega Glomax 20/20 Luminometer.

**Quantitative Real-Time PCR**

Cells were lysed using Qiazol (Qiagen) and then transferred to Qiashredder columns (Qiagen) and centrifuged at 10,000 g for 3 minutes, then RNA isolated using the RNAeasy kit according to the manufacturer’s instructions (Qiagen). Real-time PCR (RT-PCR) was conducted on 100 ng of RNA using the miScript Reverse Transcription Kit (Qiagen), and 18S were conducted using their specific forward primer and reverse primers according to the manufacturer’s protocol (Qiagen). 18S was used as a control. Applied Biosystems (StepOnePlus) RT-PCR system was used to carry out the quantitative PCR, using hot start 95°C (15 minutes), then denaturation 95°C (15 seconds) with annealing at 58°C (30 seconds), extension 72°C (30 seconds) for 40 cycles, followed by a melt curve analysis. Data analysis for differences in gene expression between control and treated cells, or normal and GBM tissue, was carried out using Microsoft Excel: housekeeping gene primer C values were subtracted from test primer values to find the ΔCt, then ΔΔCt was found by subtracting the average ΔCt of the vehicle-treated sample from itself and the drug-treated samples. Fold change was calculated using the formula fold change = 2ΔΔCt.

**Phosphatidic Acid Rescue Experiments**

U251MG and U87MG were transfected or treated with small-molecule inhibitors as above. Simultaneously, exogenous phosphatidic acid (Avanti Polar Lipids) at either 33 or 50 μmol/L and vehicle (v/v) of 1:2 methanol and chloroform solution were administered. Treatment was repeated every 24 hours.

**Cellular Lipid Extraction and LPA, PA, and DAG Profile Assays**

Total lipid was extracted from GBM cells by methods described previously (40–43). The cellular content of DGα, lyso-phosphatidic acid (LPA), and phosphatidic acid profiles was analyzed by liquid chromatography/mass spectrometry (LC/MS) on a Shimadzu Prominence ultra fast liquid chromatography system equipped with a C8 column (Nucleodur 5 μm, 2 mm × 125 mm; Machery-Nagel), and detection was carried out using an Applied Biosystems 4000 Q Trap triple quadrupole liquid chromatography/tandem mass spectrometry (LC/MS-MS) system equipped with an electro-spray ionization system. For phosphatidic acid and LPA, multiple reaction monitoring protocols in negative mode were developed for each phosphatidic acid using commercial pure phosphatidic acids and the most intense product ions were selected for the analysis of biologic samples. For DAG, analyses were conducted by monitoring product ions generated by neutral loss (41) of the ammonium ions from DAG’s ammonium adducts [M+NH₄]⁺ as previously described (43). Quantitative methods for the measurement of glycerolipids were conducted using the chromatographic and spectrometric methods described above in conjunction with the use of 0.1 nmol C17 LPA and C17 ceramide as an internal standard, for LPA/PA and DAG respectively, to correct for recovery and the protein concentration of the cellular lysates. The amount of each species of glycerolipid in biologic samples was calculated from the peak areas obtained using the software that controls the LC/MS system (Analyst 1.5; Applied Biosystems). Raw peak areas were corrected for recovery and sample loading as described above and then transformed into amounts of analyte using standard curves made with commercial glycerolipids.

**cAMP Assay**

The cells were treated with DGKα inhibitor R59022 (Sigma-Aldrich) at 10 μmol/L for 5 days or transfected with DGKα siRNA. cAMP concentration was determined in cell lysates from cell culture of 3.5 × 10⁶ cells using a commercially available assay (cAMP Competitive ELISA Kit; Thermo Scientific) following the manufacturer’s instructions. The assay is based on the competition between cAMP in the standard or sample and alkaline phosphatase conjugate cAMP (cAMP-AP) for a limited amount of cAMP monoclonal antibody bound to an anti-rabbit IgG precoated 96-well plate. The assay is colorimetric, and absorbance is read at 405 nm.

**Luciferase Assay**

The cells were transfected with DGKα siRNA as for 4 hours using Oligofectamine or treated with DGKα inhibitor R59022 10 μmol/L, or exogenous cAMP 20 or 80 pmol for 3 days, and subsequently transfected with β-galactosidase (2 ng/μl), MTOR promoter luciferase reporter, or empty promoter vector (Switchgear Genomics Inc.), following the manufacturer’s instructions, for 48 hours. Luciferase assays for MTOR activity were conducted using the LightSwitch Assay System (Switchgear Genomics Inc.) and for β-galactosidase activity using Galecto-Light Plus β-Galactosidase Reporter Gene Assay System (Applied Biosystems). Luminescence was measured on a Promega GloMax 20/20 luminometer and normalized as described previously (5). MTOR luciferase activities were double-normalized by dividing each well by both β-galactosidase activity and the average luciferase/β-galactosidase value in a parallel set done with constitutively expressed luciferase expression vector.

**Pharmacologic Reagents**

The small-molecule inhibitors R59022 and R59949 were obtained from Sigma-Aldrich. Fresh drug/vehicle was added daily for cell culture experiments. siRNA duplexes were synthesized by Sigma-Aldrich. Oligofectamine (Invitrogen) was used for transfection of siRNA into cells per manufacturer’s instructions and as previously described (4).
DGKα Is a Critical Signaling Node and Novel Target in Cancer

Estimates of BBB Penetration of Small-Molecule Inhibitors

R59022 and R59949 were evaluated for their predicted ability to cross the BBB using the ACD/ADME software (ACD/Labs, Advanced Chemistry Development, Inc.). The module used for predictions is Pharma Algorithms (21), which provides a comprehensive evaluation of BBB penetration potential of compounds in rodents. Each compound of interest is given an estimate of whether it would be permeable enough to exhibit CNS activity. Qualitative classification is based on reliable and theoretically reasonable predictions of the rate and extent of BBB permeation (expressed as logPS and logBB constants, respectively) governed by passive diffusion.

The LogBB predictive model is based on a dataset containing more than 500 brain to plasma partitioning ratios (expressed as logBB constants) measured in mice and rats. Under the assumption of passive transport across the BBB, logBB is viewed as a cumulative effect of drug binding to plasma and brain constituents. Calculations therefore use octanol/water logP (main determinant of brain tissue binding) and unbound fraction in plasma (fu, plasma) as input parameters. The LogPS module provides more detailed output of the ionization-specific predictive model of BBB permeability in rats. The model was developed using in-vivo experimental data of rates of passive diffusion across BBB for more than 200 compounds, expressed as logPS constants. Calculations are carried out using essential physicochemical properties such as lipophilicity, ionization constants, hydrogen-bonding parameters, and molecular size (calculated or experimental if available) as inputs (21).

In Vivo Treatment Models

Mouse protocols were approved by the Institutional Animal Care and Use Committee at the University of Virginia. Eight-week-old male SCID/NCr Balb/C mice (from the National Cancer Institute, Bethesda, MD) were stereotactically implanted with 25,000 U87 GSCs in 10 μL of neurobasal media. The surgical procedure was done as described previously (5). CED of lentiviral particles (Sigma-Aldrich) containing shRNA was done at 7 days postimplantation. Animals were randomly divided into 2 groups: control group (7 mice) receiving control shRNA and treatment group (6 mice) receiving DGKA shRNA. CED was done using the same coordinates as for the tumor implantation. The CED volume was 10 μL as well, at a speed of 300 nL/min. The solution also contained 1:2,000 polybrene and 7.5% mannitol to provide spread of infusate. General appearance, neurologic status, and body weight were monitored daily, and mice were euthanized when they showed signs of illness, pain, or 20% weight loss.

Alternatively, following the same protocol mentioned above, 100,000 U87 GBM cells were implanted in 10 μL of Dulbecco’s Modified Eagle Medium. Beginning at 7 days postimplantation of tumor cells, mice were given daily intraperitoneal injections with either DMSO (v:v), 2 mg/kg or 10 mg/kg of R59022 dissolved in DMSO in 50 μL volume.

Immunohistochemistry

Immunohistochemical staining with anti-CD34 antibody (EMD Millipore) and anticleaved caspase-3 antibody (EMD Millipore), with horseradish peroxidase-conjugated secondary antibody, was done on frozen-mounted slices by the University of Virginia Biorepository and Tissue Research Facility using standard techniques.

Plasma R59022 Extraction and Quantitation

R59022 was extracted from mouse plasma using a modified Bligh-Dyer extraction method. To a 5 mL polypropylene tube containing 100 μL of sample, 500 μL methanol (MeOH), 250 μL chloroform (CHCl₃), and 100 μL distilled water (dH₂O) were added, mixed, and incubated on ice for 30 minutes. To extract, 250 μL CHCl₃, and 200 μL 0.2 mol/L sodium chloride (NaCl) were added. The organic phase was dried and suspended in 100 μL of a mixture of CHCl₃:MeOH (1:1), and 50 μL was injected into a Shimadzu LC-20AD LC system equipped with a Discovery (Supelco) C18 column (50 mm × 2.1 mm, 5-μm bead size). The LC was coupled to a triple quadrupole mass spectrometer (Applied Biosystems 4000 Q-Trap). R59022 was measured in positive mode using the following transition: 460.3 → 193.1. Mass spectrometer settings, obtained by direct infusion of a 1 μmol/L solution in solvent B, were as follows: DP, 66; EP, 10; CE, 43; CXP, 14; ion spray voltage, 5,500; temperature, 500; curtain gas, 40. Chromatography was carried out using a mobile phase A consisting of 79% H₂O, 20% MeOH, 1% formic acid, and a mobile phase B consisting of 99% MeOH, 1% formic acid. The solvent gradient was as follows: 0.5 minutes 100% solvent A, a linear gradient to reach 100% solvent B at 5.6 minutes, 4.3 minutes 100% solvent B, 1 minute 100% solvent A. Total flow was 1 mL/min. Retention time was 3.9 minutes. Quantification was carried out by measuring peak areas using commercial software (Analyst 1.5.1).

Statistical Analysis

In vitro experimental results were analyzed by two-tailed Student t-test and plotted with Microsoft Excel (Microsoft Corporation). The in vivo experimental results were analyzed using the Kaplan–Meier function using both the log-rank (Mantel–Cox) test and the Gehan–Breslow–Wilcoxon test in GraphPad Prism 5 (GraphPad Software, Inc.). Refutation of the null hypothesis was accepted for P values of less than 0.05. Error bars indicate SD from the mean in all figures.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: C.L. Dominguez, B.A. Kefas, R. Abounader, T.E. Harris, B.W. Purow


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.L. Dominguez, D.H. Floyd, A. Xiao, G.R. Mullins, M.N. Yacur, T.E. Harris, B.W. Purow

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.L. Dominguez, D.H. Floyd, A. Xiao, G.R. Mullins, B.A. Kefas, W. Xin, J.K. Lee, G.M. Wilson, B.W. Purow

Writing, review, and/or revision of the manuscript: C.L. Dominguez, D.H. Floyd, G.R. Mullins, B.A. Kefas, R. Abounader, J.K. Lee, G.M. Wilson, T.E. Harris, B.W. Purow

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.L. Dominguez, B.W. Purow

Study supervision: C.L. Dominguez, B.W. Purow

Acknowledgments

The authors thank Dr. Gregg Semenza for the constitutively active HIF-1α plasmid and Drs. Fadila Guessous and Ying Zhang for assistance with in vivo techniques.

Grant Support

This work was supported in part by NIH R01 CA136803 (to B.W. Purow).
Received May 11, 2012; revised March 29, 2013; accepted April 1, 2013; published OnlineFirst April 4, 2013.

REFERENCES


DGKα Is a Critical Signaling Node and Novel Target in Cancer


Diacylglycerol Kinase α Is a Critical Signaling Node and Novel Therapeutic Target in Glioblastoma and Other Cancers


Updated version  Access the most recent version of this article at: doi:10.1158/2159-8290.CD-12-0215
Supplementary Material Access the most recent supplemental material at: http://cancerdiscovery.aacrjournals.org/content/suppl/2013/04/04/2159-8290.CD-12-0215.DC1 http://cancerdiscovery.aacrjournals.org/content/suppl/2021/03/01/2159-8290.CD-12-0215.DC2

Cited articles This article cites 43 articles, 22 of which you can access for free at: http://cancerdiscovery.aacrjournals.org/content/3/7/782.full#ref-list-1
Citing articles This article has been cited by 13 HighWire-hosted articles. Access the articles at: http://cancerdiscovery.aacrjournals.org/content/3/7/782.full#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions To request permission to re-use all or part of this article, use this link http://cancerdiscovery.aacrjournals.org/content/3/7/782. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.