Targeting Protein Kinase CK2 Suppresses Prosurvival Signaling Pathways and Growth of Glioblastoma

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Abstract

Purpose: Gliomas are the most frequently occurring primary malignancies in the brain, and glioblastoma is the most aggressive of these tumors. Protein kinase CK2 is composed of two catalytic subunits (α and/or α') and two β regulatory subunits. CK2 suppresses apoptosis, promotes neangiogenesis, and enhances activation of the JAK/STAT, NF-κB, PI3K/AKT, Hsp90, Wnt, and Hedgehog pathways. Aberrant activation of the NF-κB, PI3K/AKT, and JAK/STAT-3 pathways is implicated in glioblastoma progression. As CK2 is involved in their activation, the expression and function of CK2 in glioblastoma was evaluated.

Experimental Design and Results: Analysis of 537 glioblastomas from The Cancer Genome Atlas Project demonstrates the CSNK2A1 gene, encoding CK2α, has gene dosage gains in glioblastoma (33.7%), and is significantly associated with the classical glioblastoma subtype. Inhibition of CK2 activity by CX-4945, a selective CK2 inhibitor, or CK2 knockdown by siRNA suppresses activation of the JAK/STAT, NF-κB, and AKT pathways and downstream gene expression in human glioblastoma xenografts. On a functional level, CX-4945 treatment decreases the adhesion and migration of glioblastoma cells, in part through inhibition of integrin β1 and α4 expression. In vivo, CX-4945 inhibits activation of STAT-3, NF-κB p65, and AKT, and promotes survival of mice with intracranial human glioblastoma xenografts.

Conclusions: CK2 inhibitors may be considered for treatment of patients with glioblastoma. Clin Cancer Res; 19(23); 1–11. ©2013 AACR.

Introduction

Glioblastoma is the most deadly primary malignant brain tumor. Genomic abnormalities in glioblastoma have allowed molecular classification into four subtypes: classical, proneural, neural, and mesenchymal (1). Despite the combination of surgery followed by radiotherapy and chemotherapy, median survival of glioblastoma patients is 12 to 15 months (2). Aberrant activation of signaling pathways has been implicated in glioblastoma, particularly receptor tyrosine kinases (RTK) such as EGFR and PDGFRα (3). However, clinical trials targeting these individual RTKs have been disappointing, due to concurrent activation of multiple RTKs in glioblastomas (3). Other pathways including JAK/STAT and NF-κB contribute to glioblastoma progression (4–7). The JAK/STAT pathway transmits signals from interleukin (IL)-6 family cytokines by activation of STAT-3, which induces expression of genes that regulate antiapoptotic behavior, angiogenesis, and proliferation. Furthermore, STAT-3 is a master regulator of the mesenchymal glioblastoma phenotype (4). We and others have demonstrated that levels of activated STAT-3 are elevated in glioblastoma tissues (8, 9). JAK1 and JAK2 are also activated in human glioblastoma xenografts (10). The NF-κB signaling pathway is also constitutively activated in glioblastoma (11, 12), partly due to deletion of IκBα, an inhibitor of NF-κB (5).

Protein kinase CK2 is composed of two catalytic (α and/or α') and two β regulatory subunits. CK2 is a constitutively active serine-threonine kinase that plays a fundamental role in maintaining cell survival through proproliferative, antiapoptotic, and proangiogenic signaling (13, 14). Elevated CK2 expression and activity has been demonstrated in blood tumors and solid tumors (14). CK2 is a novel interaction partner of JAK1/2, potentiates JAK and STAT-3 activation (15), and regulates expression of IL-6 (16). The NF-κB and PI3K/AKT pathways are also positively regulated by CK2, promoting cell survival and inhibiting apoptosis (13). These characteristics identify CK2 as an attractive therapeutic target. CX-4945 is the first selective orally bioavailable CK2 inhibitor to advance into human clinical trials (17, 18). CX-4945
Translational Relevance

We demonstrate that protein kinase CK2, indispensable for cell survival and a regulator of signaling cascades involved in glioblastoma tumorigenesis, may be a novel therapeutic target for treatment of glioblastoma. We demonstrate for the first time that CSNK2A1, the gene encoding CK2α, shows frequent gene dosage gains in human glioblastoma tissues. We further determined that in classical glioblastoma, these gene dosage gains occur in more than 50% of cases, highlighting patients with classical glioblastoma as a population that may be responsive to CK2-modulating therapeutics. Utilizing CX-4945, the first selective CK2 inhibitor to advance into human clinical trials, we demonstrate the therapeutic effects of inhibiting CK2 activity in flank and intracranial models of glioblastoma-patient derived cells in vivo. Abnormal STAT-3 activity has been implicated in the resistance of glioblastoma to temozolomide, and we show that CK2 expression/activity is critical for STAT-3 activation in glioblastomas, highlighting the potential of CK2 inhibitors being used in combination with standards of care. Given that safety of CK2 inhibition in humans has been established, targeting this kinase may provide a new therapeutic venue for treatment of glioblastoma.

inhibits the activity of CK2α/CK2α', suppresses cell-cycle progression, angiogenesis, and PI3K/AKT signaling, and exhibits antitumor activity in breast, pancreatic, and prostate xenograft models (17, 19). In the clinical setting, CX-4945 inhibits CK2, leading to durable disease stabilization (≥16 weeks) in 20% of treated patients while being well tolerated (20).

A glioblastoma-specific protein interaction analysis identified CK2α as an important node connecting cell-cycle-associated proteins (21). Nonspecific CK2 inhibitors such as TBB and DMAT exhibit cytotoxic potential towards glioblastoma cells in vitro (22), Ellipticine and benzopyridoindole derivatives, which inhibit CK2 activity, display antitumor activity in a flank model of glioblastoma (23), and azonaphthalene derivatives, allosteric inhibitors of CK2, inhibit growth of U373-MG cells in the flank (24). Downregulation of CK2 by siRNA induces death of the glioblastoma cell line M059K (25). In this study, we analyzed expression of CK2 subunits and association with different subtypes of glioblastoma, using The Cancer Genome Atlas (TCGA) database. We evaluated the effect of inhibiting CK2α/CK2α' activity or silencing CK2α, CK2α', and CK2β expression on the JAK/STAT, NF-kB, and AKT signaling pathways in glioblastoma, and downstream functional effects such as cell-cycle progression, apoptosis, adhesion, migration, and senescence. Finally, we tested the antitumor efficacy of CX-4945 in flank and intracranial human glioblastoma xenograft models in vivo.

Materials and Methods

Gene copy number variation and genomic analyses

Five-hundred and thirty seven glioblastoma samples from the TCGA (http://cancergenome.nih.gov/) were used as a genomic discovery set. Raw Affymetrix Genome-Wide Human SNP Array 6.0 and Agilent Human Genome CGH Microarray 244A gene dosage data, Affymetrix Human Genome U133 Plus 2.0 Array and Agilent 244K Custom Gene Expression data, and clinical data were retrieved from the Open-Access and Controlled-Access Data Tiers Portal (https://tcga-data.nci.nih.gov/tcga/) of TCGA and preprocessed for downstream analyses. Gene-level copy number variation (CNV) was estimated using the circular binary segmentation algorithm from the “snapCGH” package using an R code, as described (5). Gene dosage segments were classified as chromosomal ‘gain’ or ‘loss’ if the absolute value of the predicted dosage was more than 0.75 times the interquartile range of the difference between observed and predicted values for each region. CNV data processed using the Genomic Identification of Significant Targets in Cancer (GISTIC2) algorithm were retrieved from the Broad Institute at http://gdac.broadinstitute.org/runs/analyses_2012_03_21/data/GBM/20120321/. The GISTIC2 algorithm (26) thresholded estimated CNV values to −2, −1, 0, 1, and 2 representing homozygous deletion, heterozygous (single copy) deletion, diploid nominal copy, low-level copy number amplification (i.e., gene dosage gains), or high-level copy number amplification, respectively. Genes mapped onto the human genome coordinates using the University of California, Santa Cruz cgdData HUGO probeMap were visualized using the Cancer Genomics Browser (https://genome-cancer.ucsc.edu/).

Cells and reagents

U87-MG and U251-MG lines were authenticated as described (10). Antibody to CK2β was from EMD Biosciences. CX-4945 was provided by Cylene Pharmaceuticals. Recombinant IL-6, sIL-6R, EGF, TNF-α, and IL-1β were from R&D Systems. Antibodies to p-Y-STAT-3, STAT-3, p-Y-STAT-5, STAT-5, NF-kB p65S536, AKTS473, and AKT were from Cell Signaling Technology. Antibodies to CK2α, CK2α', p-Y-JAK2, and NF-kB p65 were from Santa Cruz Biotechnology. Antibodies to Ki-67 and CK2α for immunohistochemistry and JAK2 were from Millipore, NF-kB p65S529 antibody from Abcam, AKTS129 antibody from Abnova, and integrin β1 antibody from BD Biosciences. Normal human brain lysate was provided by Dr. Harald Sontheimer [University of Alabama at Birmingham (UAB), Birmingham, AL].

Glioblastoma xenograft tumors

Human glioblastoma xenograft tumors X1016, X1046, and X1066 were maintained in the flank of nude mice with approval (APN #100908862) of the UAB Institutional Animal Care and Use Committee (10). For subcutaneous implantation, 200 μL of X1046 tumor slurry was injected into flanks of female nude mice and tumor size measured by calipers and calculated as described (10). Mice were
randomized to vehicle or CX-4945 treatment, which was administered intraperitoneally twice a day at 75 mg/kg from days 3 to 5, and then orally twice a day from days 6 to 40. Intraperitoneal injections were discontinued because of irritation at the injection site. At day 40, mice were euthanized and tumors excised and snap frozen. Percent tumor growth inhibition (TGI) value was calculated as $100 \times \frac{[1 - (\text{Treated } \text{Day } 40 - \text{Treated } \text{Day } 1)]}{(\text{Vehicle } \text{Day } 40 - \text{Vehicle } \text{Day } 1)}$. For intracranial implantation, $0.5 \times 10^6$ X1046 or X1016 cells in 5 μL of methylcellulose were injected 2 mm anterior and 1 mm lateral to the bregma at a depth of 2 mm, as described (10). Vehicle or CX-4945 (75 mg/kg) was administered orally twice a day from day 5 for 4 consecutive weeks. Survival of mice was monitored.

**Immunoblotting**

Samples were lysed in radioimmunoprecipitation buffer for 30 minutes, and approximately 20–50 μg of total protein used for immunoblotting (10).

**RNA interference**

U251-MG cells were transfected with 100 nmol/L of CK2α, CK2α′, CK2β, or nontarget siRNA for 48 hours using Dharmacon transfection reagent 1 (15).

**Real-time RT-PCR and TaqMan gene expression assays**

RNA was extracted using TRIzol (Invitrogen). TaqMan primers were used, qRT-PCR performed, and data analyzed using the comparative $C_\text{t}$ method (10).

**Immunofluorescence and phalloidin staining**

X1016 or U251-MG cells were fixed with 2% paraformaldehyde, stained with antibodies to CK2α or CK2α′, or with Alexa Fluor 488 phalloidin, and 4′, 6-diamidino-2-phenylindole (DAPI), and analyzed by immunofluorescence microscopy.

**Colorimetric cell adhesion assay**

Xenograft X1046 was treated with CX-4945 for 16 hours, and cells seeded on fibronectin-coated plates (15 μg/mL) in serum-free medium for 1 hour. The cells were then fixed, stained with crystal violet, and absorbance measured at 540 nm.

**Scratch assay**

X1066 cells were treated with CX-4945 in serum-free medium for 4 hours. The cells were then scratched using a 200 μL pipette tip, and imaged at 0 and 16 hours with a Leica MZ16FA stereoscope equipped with a Leica DFC 490 CCD camera. The unhealed area was quantified using the ImageJ 1.41o program.

**WST-1 proliferation assay**

Cells were seeded in triplicate at 1.5 × 10⁴ cells per well in the absence or presence of CX-4945. WST-1 reagent was added and absorbance measured at 450 nm against 655 nm (15).

**Apoptosis assay**

Cells were treated with CX-4945 for 24 hours, trypsinized, stained with Annexin V and propidium iodide, and examined by flow cytometry using FlowJo 7.5.5 software (15).

**Cell-cycle analysis**

Cells were treated with CX-4945 for 24 hours, fixed with 70% ethanol overnight, stained with propidium iodide, and the percentage of cells in different cell-cycle stages was determined by FlowJo 7.5.5 software (15).

**Densitometric and statistical analyses**

Level of significance was determined by Student $t$ test distribution. Kaplan–Meier survival curves were drawn using SigmaPlot 11.2 software, and Log-Rank significance test was performed, and 95% confidence intervals for median survival were computed. $P < 0.05$ was considered statistically significant. Linear regression analyses and graphical model validation were executed using R software. Scatterplots and locally weighted least squares smooths were used to confirm the suitability of linear regression analyses, and statistical significance of these relationships was assessed according to the $P$ value for the estimated slope of the regression line. Two-way contingency table analysis, unpaired $t$ test, and Wilcoxon rank-sum test were used as appropriate. ORs in the two-way contingency table analysis and 95% confidence intervals were computed using Woolf’s method for variance estimation.

**Results**

**CSNK2A1 shows frequent gene dosage gains in glioblastoma**

CNV analysis in 537 glioblastomas from the TCGA database indicates that CSNK2A1, the gene encoding CK2α and mapping to chromosome 20p13\[1\], shows low-level amplifications (i.e., gene dosage gains) in 33.7% of tumors (Fig. 1A). In most cases, these gene dosage gains are broadly spanning and involve whole chromosome 20, suggesting CSNK2A1 as one of potentially multiple target genes driving these aberrations. Significantly higher CSNK2A1 mRNA levels were detected in glioblastomas with CSNK2A1 gene dosage gains (Fig. 1B). Among 490 glioblastoma samples with molecular subtype information, CSNK2A1 gene dosage gain is more common (50.7%) in classical glioblastoma than in non-classical glioblastoma (21.3%) (Fig. 1C). CNV analysis of CSNK2A2, the gene encoding CK2α′ at 16q21\[1\], revealed only sporadic gene dosage gains, and analysis of the CSNK2B gene, encoding CK2β, revealed a modest percentage of deletion (7.3%) at 6p21.3\[1\] (not shown).

**CK2 is required for JAK/STAT activation in glioblastoma cells**

Activation of the JAK/STAT-3 pathway is implicated in glioblastoma progression and propagation of glioblastoma stem cells (27–30). We tested whether inhibition of CK2 affects STAT activation. Three human glioblastoma xenografts, X1016, X1046, and X1066, which have detectable...
basal STAT-3 activation (10) and two human glioma lines, U251-MG and U87-MG, were used. Expression of CK2α, CK2α', and CK2β was detected in the glioblastoma xenografts and cell lines as well as normal human brain lysate (Fig. 2A). Expression of CK2α (Supplementary Fig. S1A and S1B) and CK2α' (Supplementary Fig. S1B) is detected in both the cytoplasm and nucleus. U251-MG cells transfected with CK2α, CK2β, or CK2α' siRNAs were stimulated with IL-6 and sIL-6R, and examined for phospho-tyrosine STAT-3 levels. The combination of IL-6 and sIL-6R was used to promote optimal STAT-3 activation (31). Downregulation of CK2α, CK2β or CK2α' expression led to reduced IL-6–induced STAT-3 activation, with CK2α and CK2α' siRNA having the most pronounced effect (Fig. 2B). CK2α siRNA causes decreased CK2β levels (Fig. 2B, lanes 2 and 6), whereas CK2β siRNA resulted in decreased CK2α' levels (Fig. 2B, lanes 3 and 7); both phenomena have been previously observed (25, 32, 33). Basal and IL-6–induced
STAT-3 activation was inhibited by the selective CK2 inhibitor CX-4945 in a dose-dependent manner in X1066 (Fig. 2C), X1046 (Supplementary Fig. S2A) and U251-MG cells (Supplementary Fig. S2B). IL-6–induced JAK2 activation was inhibited by CX-4945 in X1066 (Fig. 2D), suggesting CX-4945 may affect JAK2 stability. Oncostatin M (OSM), another IL-6 family member, is elevated in glioblastoma tumors and activates STAT-3 (34). Expression of OSM-induced STAT-3 target genes was inhibited by CX-4945 in serum-free medium for 4 hours, and then stimulated with OSM (5 ng/mL) for 1 hour. mRNA was analyzed by qRT-PCR. *, P < 0.05. F, U251-MG cells were pretreated with CX-4945 in serum-free medium for 4 hours, and then stimulated with EGF for 1 hour. mRNA was analyzed by qRT-PCR. *, P < 0.05.

Figure 2. Inhibition of CK2 suppresses STAT activation. A, twenty micrograms of xenografts X1016, X1046, and X1066, glioblastoma (GBM) lines U251-MG and U87-MG, and normal brain (NB) lysate were immunoblotted with indicated antibodies. B, U251-MG cells were transfected with 100 nmol/L of non-target, CK2α, CK2β, or CK2α siRNAs for 48 hours, then stimulated with 10 ng/mL of IL-6 and 25 ng/mL of sIL-6R for 10 minutes. C and D, xenograft X1066 was treated with CX-4945 in serum-free medium for 4 hours, and then stimulated as above. Lysates were immunoblotted with indicated antibodies. E, U251-MG cells were serum-starved overnight, treated with CX-4945 for 4 hours, and stimulated with EGF for 1 hour. mRNA was analyzed by qRT-PCR. *, P < 0.05. F, U251-MG cells were pretreated with CX-4945 in serum-free medium for 4 hours, and then stimulated with EGF for 1 hour. mRNA was analyzed by qRT-PCR. *, P < 0.05.

CK2 is required for NF-κB p65 activation in glioblastoma cells

CK2 positively regulates the NF-κB pathway by promoting IkB degradation and enhancing DNA binding through p65 phosphorylation (13), and TNF-α induces phosphorylation of p65 serine 529 by CK2 (38). TNF-α induced p65 phosphorylation was partially inhibited by knockdown of CK2α, CK2β, or CK2αβ (Fig. 3A). TNF-α induced p65 phosphorylation (Fig. 3B) and expression of downstream targets, IκBα and IL-8 (Fig. 3C), was suppressed by CX-4945 in human glioblastoma xenografts, as was IL-1β-induced p65 phosphorylation (Fig. 3D).

Inhibition of CK2 suppresses constitutive AKT activation in glioblastoma cells

Mutations or deletions in PI3K and PTEN have defined the PI3K/AKT pathway as one of the core pathways dysregulated in glioblastoma (39). CK2 positively regulates the PI3K pathway by affecting PTEN stability (40) and phosphorylating serine 129 of AKT, which promotes its catalytic activity (41). Constitutive phosphorylation of serine 129 and 473 was detected in glioblastoma cells, which was inhibited by CX-4945 (Supplementary Fig. S3).

CK2 inhibition decreases adhesion and migration of glioblastoma cells

CK2 regulates cell morphology and the cytoskeleton (42, 43). When glioblastoma cells were treated with CX-4945, they became retracted and rounded after treatment for 12 to 16 hours, suggesting reduced cell adhesion (Fig. 4A).
Staining for Phalloidin showed that there were more actin bundles on the membrane, and actin stress fibers appeared to be disorganized and impaired after CX-4945 treatment (Fig. 4B). U251-MG cells transfected with CK2α and/or CK2α' siRNAs also became retracted and rounded, and displayed disorganized actin (Fig. 4C). A small-scale PCR array was performed to identify genes involved in adhesion (Fig. 4D). CK2α' siRNAs have decreased ITGB1 protein expression (Supplementary Fig. S5). Thus, CK2 inhibition abrogates many glioblastoma biologic functions that are critical to tumor growth and survival.

**Effects of inhibition of CK2 on glioblastoma cell function**

Treatment with CX-4945 suppressed xenograft cell growth (Fig. 5A and B and Supplementary Fig. S5). CK2α or CK2α' plus CK2α' siRNAs also decreased glioblastoma cell growth (Fig. 5C). CX-4945 induced apoptosis (Fig. 5D) and increased the percentage of glioblastoma cells in G2–M phase, and decreased that in G1 and S phases (Fig. 5E). CX-4945 treatment caused senescence in glioblastoma cells after 72 hours (Supplementary Fig. S6), and 10 μmol/L of CX-4945 abrogated glioblastoma cell survival after 5 days (Supplementary Fig. S7). Thus, CK2 inhibition abrogates many glioblastoma biologic functions that are critical to tumor growth and survival.

**CX-4945 inhibits glioblastoma tumor growth in vivo**

CX-4945 was tested in a subcutaneously implanted xenograft model, using Xenograft X1046 (classical subtype), as assessed using the CLANC algorithm (1). CX-4945 treatment significantly inhibited tumor growth and exhibited 76.4% TGI (Fig. 6A). At day 40, both groups of mice were euthanized 3 hours after the last administration of CX-4945 or vehicle. Constitutively active STAT-3, NF-κB p65 and AKT activation happened to be the smallest tumor, thus having a poor response to CX-4945. CX-4945 treatment did not affect body weight (Fig. 6C) or the number of white blood cells, red blood cells, or levels of hemoglobin (Fig. 6D). CX-4945 was then tested in the intracranial xenograft model. CX-4945 treatment significantly increased survival of X1046 tumor-bearing mice, with the median survival time after tumor implantation increasing from 38 (95% confidence interval: 35.6–40.4) to 59 days (95% confidence interval: 50.2–67.8; Fig. 6E). The intracranial model was repeated utilizing another xenograft, X1016 (classical subtype), in which mice were sacrificed...
during treatment to evaluate the antitumor effects of CX-4945. Constitutively active STAT-3, NF-κB p65 and AKT was detected in intracranial X1016 tumors of the vehicle-treated group, while activation of these pathways was diminished in mice treated with CX-4945 (Fig. 6F). The percentage of Ki-67 positive cells in intracranial X1016 tumors was lower in CX-4945–treated mice compared with vehicle-treated mice (Fig. 6G).

Discussion

The exact mechanism responsible for CK2 overexpression in cancer is not known. We report for the first time that CSNK2A1, the gene encoding CK2α, harbors frequent gene dosage gains in glioblastoma, and such gains correlate with increases in CK2α mRNA. This information suggests the genetic basis leading to overexpression of CK2α mRNA and protein expression in patients with glioblastoma. Moreover, we identify a particular subset of the disease, classical glioblastoma, that has more than 50% frequency of this alteration, highlighting patients with classical glioblastoma as a population that may be responsive to CK2-modulating therapeutics. Genome-wide CNV analysis in glioblastoma has been limited to evidence that chromosome 20 harbors frequent gains in gene dosage that may be driven by several oncogenic targets (45). Our novel finding that CK2 expression/activity is required
activation of prosurvival pathways, including the JAK/STAT, NF-κB, and PI3K/AKT pathways in glioblastomas, suggests that the CSNK2A1 gene could be one of the important oncogenic drivers for the selection of chromosome 20 gains during gliomagenesis.

Silencing CK2 expression with CK2 siRNA or inhibiting CK2 activity with CX-4945 abrogates JAK/STAT activation and target gene expression in glioblastomas. While the regulation of JAK2 and STAT-3 by CK2 has been previously described by us and others (15, 16, 46), our discovery that CK2 is involved in EGFR-induced STAT-3 and STAT-5 activation is novel and requires separate investigation. Abnormal STAT-3 activity has been implicated in the resistance of classical and non-classical tumors select for distinct genetic aberrations that may have a similar effect in the pathogenesis of glioblastoma, namely activation of NF-κB.

Our results indicate that inhibition of CK2 causes cell rounding and actin disorganization, decreases adhesion to extracellular matrix components such as fibronectin, and suppresses migration of glioblastomas. Expression of integrins including ITGB1 and ITGA4 depends on CK2 expression or activity in glioblastoma cells, which is the first association of CK2 and integrin expression. Interestingly, ITGA4 and ITGB1 are expressed at higher levels in human glioblastoma samples compared with nontumor controls (Rembrandt Database. Accessed 2013 January). In addition, activation of ITGB1 increases the invasiveness of malignant glioma (49). Therefore, inhibition of integrin expression is a novel mechanism by which CK2 inhibitors cause cell morphology changes and decrease cell adhesion and migration.

The molecular basis underlying how CK2 regulates integrin expression is of interest given that ITGB1 and ITGA4 are associated with the classical subtype (Fig. 1). Considering that both alterations can lead to constitutive activation of NF-κB, these data suggest that classical and non-classical tumors select for distinct genetic aberrations that may have a similar effect in the pathogenesis of glioblastoma, namely activation of NF-κB.

NFKBIA show a pattern of mutual exclusion (not shown). Deletion of the NFKBIA gene is associated with the non-classical subtypes of glioblastoma (5) and CSNK2A1 gene dosage gains are significantly associated with the classical subtype (Fig. 1). Considering that both alterations can lead to constitutive activation of NF-κB, these data suggest that classical and non-classical tumors select for distinct genetic aberrations that may have a similar effect in the pathogenesis of glioblastoma, namely activation of NF-κB.

Figure 5. Inhibition of CK2 suppresses glioblastoma (GBM) cell survival. X1046 (A) and X1066 (B) cells were treated with CX-4945, and cell survival was measured by the WST-1 assay. C, U251-MG cells were transfected with 100 nmol/L of non-target, CK2 α, or CK2α’ siRNAs, or CK2α (50 nmol/L) plus CK2α’ (50 nmol/L) siRNAs for 48 hours, and survival was determined by the WST-1 assay. Triplicate experiments and error bars show ± S.D., P < 0.05. D, U251-MG cells were treated with CX-4945 for 24 or 48 hours. Cells were stained with Annexin V and propidium iodide and examined by flow cytometry. Error bars show ± S.D., P < 0.05. E, U251-MG cells were treated with CX-4945 for 24 hours, fixed overnight, stained with propidium iodide, and digested with RNase. The percentage of cells in the sub-G1, G1, S, and G2–M phase was examined by flow cytometry.
Inhibition of CK2 activity by CX-4945 suppresses in vivo glioblastoma xenograft growth and promotes survival. In a glioblastoma xenograft flank model, administration of CX-4945 inhibits activation of STAT-3, NF-κB p65, and AKT, and suppresses tumor growth. Importantly, treatment with CX-4945 inhibits activation of STAT-3, NF-κB p65, and AKT in intracranial glioblastoma tumors and promotes the survival of mice bearing intracranial human glioblastoma tumors. These findings describe the first use of any CK2 inhibitor in orthotopic models of glioblastoma using primary human glioblastoma xenografts. CK2 is a remarkably nodal kinase, and its upregulation in glioblastoma has a strong impact on cellular processes indispensable for cancer cell survival. Targeting this pleiotropic kinase that influences multiple signaling cascades involved in glioblastoma progression may prove more effective than strategies that target a single pathway.

Disclosure of Potential Conflicts of Interest
D. Drygin is employed (other than primary affiliation; e.g., consulting) as a Vice President, Biology in Cyane Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: Y. Zheng, E.N. Benveniste Development of methodology: Y. Zheng, S.L. Bellis, M. Bredel Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Zheng, B.C. McFarland, H. Yu, M. Bredel Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Zheng, H. Yu, H. Kim, M. Bredel Writing, review, and/or revision of the manuscript: Y. Zheng, D. Drygin, H. Kim, M. Bredel, E.N. Benveniste Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Zheng, B.C. McFarland, H. Kim Study supervision: E.N. Benveniste

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Figure 6. CX-4945 inhibits in vivo growth of xenograft glioblastoma tumors. A–D, xenograft X1046 was injected subcutaneously into nude mice. Cages were randomized and vehicle (n = 4) or 75 mg/kg of CX-4945 (n = 4) was administered twice a day intraperitoneally from days 3 to 5, and by oral gavage twice a day from day 6. Tumor size (A) and body weight (C) were measured on the indicated days. On day 40, all mice were euthanized. Data represent mean ± SEM. *, P < 0.05; **, P < 0.005; ***, P < 0.001. B, tumors were homogenized and lysates immunoblotted with indicated antibodies. D, blood from vehicle and CX-4945-treated mice was obtained by cheek bleeding before euthanasia on day 40, and analyzed with HEMAVET950. The numbers of white blood cells, neutrophils, lymphocytes, monocytes, eosinophils, basophils, red blood cells, and hemoglobin are shown in arbitrary units, n.s., not significant. E, a total of 5 × 10⁵ cells/5 μL of xenograft X1046 were injected intracranially. Cages were randomized and vehicle (n = 14) or 75 mg/kg of CX-4945 (n = 15) was administered orally twice a day for 28 days starting at day 5. Survival was monitored and mice were euthanized upon moribund. Kaplan–Meier survival with Log-Rank analysis was performed. F, a total of 5 × 10⁵ cells/5 μL of xenograft X1016 were injected intracranially. Cages were randomized, vehicle (n = 3) or 75 mg/kg of CX-4945 (n = 3) was administered orally twice a day starting at day 3, and mice were euthanized at day 18. Brain tissue from vehicle and CX-4945-treated mice was fixed in formalin and embedded in paraffin, and sections were stained with anti-Ki-67 antibody and counterstained with hematoxylin. Representative images and Ki-67+ percentages (in brackets) are shown. The scale bar is 50 μm.
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