STATEMENT OF TRANSLATIONAL RELEVANCE

We demonstrate that protein kinase CK2, indispensable for cell survival and a regulator of signaling cascades involved in GBM tumorigenesis, may be a novel therapeutic target for treatment of GBM. We demonstrate for the first time that CSNK2A1, the gene encoding CK2α, shows frequent gene dosage gains in human GBM tissues. We further determined that in classical GBM, these gene dosage gains occur in more than 50% of cases, highlighting patients with classical GBM 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 GBM-patient derived cells in vivo. Abnormal STAT-3 activity has been implicated in the resistance of GBM to temozolomide, and we show that CK2 expression/activity is critical for STAT-3 activation in GBMs, 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 GBM.
ABSTRACT

Purpose: Gliomas are the most frequently occurring primary malignancies in the brain, and glioblastoma (GBM) 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 neo-angiogenesis, 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 GBM progression. Since CK2 is involved in their activation, the expression and function of CK2 in GBM was evaluated.

Experimental Design and Results: Analysis of 537 GBMs from The Cancer Genome Atlas Project demonstrates the CSNK2A1 gene, encoding CK2α, has gene dosage gains in GBM (33.7%), and is significantly associated with the classical GBM 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 GBM xenografts. On a functional level, CX-4945 treatment decreases the adhesion and migration of GBM 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 GBM xenografts.

Conclusions: CK2 inhibitors may be considered for treatment of patients with GBM.
INTRODUCTION

Glioblastoma (GBM) is the most deadly primary malignant brain tumor. Genomic abnormalities in GBM 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 GBM patients is 12-15 months (2). Aberrant activation of signaling pathways has been implicated in GBM, particularly receptor tyrosine kinases (RTKs) such as EGFR and PDGFRA (3). However, clinical trials targeting these individual RTKs have been disappointing, due to concurrent activation of multiple RTKs in GBMs (3). Other pathways including JAK/STAT and NF-κB contribute to GBM progression (4-7). The JAK/STAT pathway transmits signals from IL-6 family cytokines by activation of STAT-3, which induces expression of genes that regulate anti-apoptotic behavior, angiogenesis and proliferation. Furthermore, STAT-3 is a master regulator of the mesenchymal GBM phenotype (4). We and others have demonstrated that levels of activated STAT-3 are elevated in GBM tissues (8, 9). JAK1 and JAK2 are also activated in human GBM xenografts (10). The NF-κB signaling pathway is also constitutively activated in GBM (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 pro-proliferative, anti-apoptotic and pro-angiogenic 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 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 GBM-specific protein interaction analysis identified CK2α as an important node connecting cell-cycle-associated proteins (21). Non-specific CK2 inhibitors such as TBB and DMAT exhibit cytotoxic potential towards GBM cells in vitro (22), Ellipticine and benzopyridoindole derivatives, which inhibit CK2 activity, display antitumor activity in a flank model of GBM (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 GBM cell line M059K (25). In this study, we analyzed expression of CK2 subunits and association with different subtypes of GBM, 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-κB and AKT signaling pathways in GBM, and downstream functional effects such as cell cycle progression, apoptosis, adhesion, migration and senescence. Finally, we tested the anti-tumor efficacy of CX-4945 in flank and intracranial human GBM xenograft models in vivo.
MATERIALS AND METHODS

**Gene Copy Number Variation and Genomic Analyses.** 537 GBM 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 pre-processed 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 normal 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 cgData 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 (Ab) to CK2β was from EMD Biosciences (San Diego, CA). CX-4945 was provided by Cylene Pharmaceuticals (San Diego, CA). Recombinant IL-6, sIL-6R, EGF, TNF-α and IL-1β were from R&D Systems (Minneapolis, MN). Abs to p-Y-STAT-3, STAT-3, p-Y-STAT-5, STAT-5, NF-κB p65S536, AKTS473 and AKT were from Cell Signaling (Danvers, MA). Abs to CK2α, CK2α’, p-Y-JAK2 and NF-κB p65 were from Santa Cruz Biotechnology (Santa Cruz, CA). Abs to Ki-67 and CK2α for immunohistochemistry, and JAK2 were from Millipore (Billerica, MA), NF-κB p65S529 Ab from Abcam (Cambridge, MA), AKTS129 Ab from Abnova (Taipei, Taiwan), and integrin β1 Ab from BD Biosciences (San Jose, CA). Normal human brain lysate was provided by Dr. Harald Sontheimer (UAB).

GBM Xenograft Tumors. Human GBM xenograft tumors X1016, X1046 and X1066 were maintained in the flank of nude mice with approval (APN #100908862) of the UAB IACUC (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 (i.p) twice a day at 75 mg/kg from days 3-5, and then orally twice a day from days 6-40. I.p. 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 X [1 - (Treated Day 40 – Treated Day 1)/(Vehicle Day 40 – Vehicle Day 1)]. For intracranial implantation, 0.5 x 10⁶ 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 RIPA buffer for 30 min, and ~20-50 µg of total protein used for immunoblotting (10).

**RNA Interference.** U251-MG cells were transfected with 100 nM of CK2α, CK2α', CK2β or non-target siRNA for 48 h using Dharmacon transfection reagent 1 (15).

**Realtime 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 Ct 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 DAPI, and analyzed by immunofluorescence microscopy.

**Colorimetric Cell Adhesion Assay.** Xenograft X1046 was treated with CX-4945 for 16 h, and cells seeded on fibronectin-coated plates (15 µg/ml) in serum-free medium for 1 h. 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 h. The cells were then scratched using a p200 pipette tip, and imaged at 0 and 16 h 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 x 10^4 cells/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 h, 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 h, 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’s 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. Odds ratios 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 GBM.** CNV analysis in 537 GBMs from the TCGA database indicates that CSNK2A1, the gene encoding CK2α and mapping to chromosome 20p13||C, shows low-level amplifications (i.e., gene dosage gains) in 33.7% of tumors (Figure 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 GBMs with CSNK2A1 gene dosage gains (Figure 1B). Among 490 GBM samples with molecular subtype information, CSNK2A1 gene dosage gain is more common (50.7%) in classical GBM than in non-classical GBM (21.3%) (Figure 1C). CNV analysis of CSNK2A2, the gene encoding CK2α’ at 16q21||C, 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||C (not shown).

**CK2 is required for JAK/STAT activation in GBM cells.** Activation of the JAK/STAT-3 pathway is implicated in GBM progression and propagation of GBM stem cells (27-30). We tested whether inhibition of CK2 affects STAT activation. Three human GBM 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 GBM xenografts and cell lines, as well as normal human brain lysate (Figure 2A). Expression of CK2α (Supplemental Figure 1A and 1B) and CK2α’ (Supplemental Figure 1B) 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). Down-regulation 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 (Figure 2B). CK2α siRNA causes decreased CK2β levels (Figure 2B, lanes 2 and 6), whereas CK2β siRNA resulted in decreased CK2α’ levels (Figure 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 (Figure 2C), X1046 (Supplemental Figure 2A) and U251-MG cells (Supplemental Figure 2B). IL-6-induced JAK2 activation was inhibited by CX-4945 in X1066 (Figure 2D). Total JAK2 levels were reduced after CX-4945 treatment (Figure 2D), suggesting CX-4945 may affect JAK2 stability. Oncostatin M (OSM), another IL-6 family member, is elevated in GBM tumors, and activates STAT-3 (34). Expression of OSM-induced STAT-3 target genes was inhibited by CX-4945 (Figure 2E). A bioinformatics analysis in glioma suggested CK2 could be downstream of EGFRvIII (35), the mutated oncogenic form of EGFR common in GBM (36). EGF can activate the STAT pathway (37); thus, we examined CK2 involvement in this system. EGF-induced STAT-5 and STAT-3 activation was inhibited by CX-4945 (Figure 2F), as was expression of EGF-induced c-Myc, a STAT-3 target gene (Figure 2G). EGF-induced STAT-3 activation was inhibited to a greater extent by CX-4945 than STAT-5.

**CK2 is required for NF-κB p65 activation in GBM cells.** CK2 positively regulates the NF-κB pathway by promoting IκB 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 knock-down of CK2α, CK2β or
CK2α’ (Figure 3A). TNF-α induced p65 phosphorylation (Figure 3B) and expression of downstream targets, IκBα and IL-8 (Figure 3C), was suppressed by CX-4945 in human GBM xenografts, as was IL-1β-induced p65 phosphorylation (Figure 3D).

**Inhibition of CK2 suppresses constitutive AKT activation in GBM cells.** Mutations or deletions in PI3K and PTEN have defined the PI3K/AKT pathway as one of the core pathways dysregulated in GBM (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 GBM cells, which was inhibited by CX-4945 (Supplemental Figure 3).

**CK2 inhibition decreases adhesion and migration of GBM cells.** CK2 regulates cell morphology and the cytoskeleton (42, 43). When GBM cells were treated with CX-4945, they became retracted and rounded after treatment for 12-16 h, suggesting reduced cell adhesion (Figure 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 (Figure 4B). U251-MG cells transfected with CK2α and/or CK2α’ siRNAs also became retracted and rounded, and displayed disorganized actin (Figure 4C). A small-scale PCR array was performed to identify genes involved in adhesion that may be affected by CX-4945. Among the genes identified, integrin α4 (*ITGA4*) and integrin β1 (*ITGB1*) were downregulated by CX-4945 (Figure 4D), as was ITGB1 protein expression (Figure 4E). U251-MG cells transfected with CK2α or CK2α’ siRNAs have decreased ITGB1 protein expression (Figure 4F). After GBM cells were treated with CX-4945 for 12 h to inhibit ITGB1 expression (Supplemental Figure 4A),
cells were washed and incubated without CX-4945 for 12 h. Expression of ITGB1 recovered compared to untreated control (Supplemental Figure 4B), and cell shape was restored (Supplemental Figure 4C), suggesting the effect of CX-4945 on GBM cell morphology and integrin expression is reversible. ITGA4/ITGB1 function as receptors for fibronectin (44), and CX-4945 inhibited GBM cell adhesion to fibronectin (Figure 4G). Furthermore, CX-4945 inhibited GBM cell migration (Figures 4H and 4I).

**Effects of inhibition of CK2 on GBM cell function.** Treatment with CX-4945 suppressed xenograft cell growth (Figures 5A, 5B and Supplemental Figure 5). CK2α or CK2α plus CK2α’ siRNAs also decreased GBM cell growth (Figure 5C). CX-4945 induced apoptosis (Figure 5D), and increased the percentage of GBM cells in G2/M phase, and decreased that in G1 and S phases (Figure 5E). CX-4945 treatment caused senescence in GBM cells after 72 h (Supplemental Figure 6), and 10 µM of CX-4945 abrogated GBM cell survival after 5 days (Supplemental Figure 7). Thus, CK2 inhibition abrogates many GBM biological functions that are critical to tumor growth and survival.

**CX-4945 inhibits GBM 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 (Figure 6A). At day 40, both groups of mice were euthanized 3 h after the last administration of CX-4945 or vehicle. Constitutively active STAT-3, NF-κB p65 and AKT was detected in tumors of the vehicle-treated group, while activation of these pathways was barely detectable in 3 of 4 mice of the CX-4945-treated group (Figure 6B). The one tumor from the CX-
CX-4945 group that had high levels of STAT-3, NF-κB p65 and AKT activation happened to be the largest tumor, thus having a poor response to CX-4945. CX-4945 treatment did not affect body weight (Figure 6C) or the number of white blood cells, red blood cells, or levels of hemoglobin (Figure 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) (Figure 6E). The intracranial model was repeated utilizing another xenograft, X1016 (classical subtype), in which mice were sacrificed during treatment to evaluate the anti-tumor 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 (Figure 6F). The percentage of Ki-67 positive cells in intracranial X1016 tumors was lower in CX-4945-treated mice compared to vehicle-treated mice (Figure 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 GBM, 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 GBM patients. Moreover, we identify a particular subset of the disease, classical GBM, that has more than 50% frequency of this alteration, highlighting patients with classical GBM as a population that may be responsive to CK2-modulating therapeutics. Genome-wide CNV analysis in GBM 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 for activation of pro-survival pathways, including the JAK/STAT, NF-κB and PI3K/AKT pathways in GBMs, 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 GBMs. 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 EGF-induced STAT-3 and STAT-5 activation is novel and requires separate investigation. Abnormal STAT-3 activity has been implicated in the resistance of GBM to TMZ (47), highlighting the potential of CK2 inhibitors being used in combination with standards of care. CK2 inhibition also resulted in a decrease in total JAK2 levels. HSP90 stabilizes numerous proteins including JAK2, and CK2 is essential for the chaperone function of HSP90 (48). Thus,
inhibiting CK2 will in turn inhibit HSP90 function, thereby promoting JAK2 degradation, which may be another novel mechanism by which STAT activation is inhibited.

Silencing CK2 expression or suppressing CK2 activity also abrogates NF-κB p65 activation, and expression of NF-κB target genes in GBMs. The IκBα gene is heterozygously deleted in GBM, leading to NF-κB activation (5). Analysis of TCGA data indicates that gene dosage gain of CSNK2A1 and heterozygous deletion of NFKBIA show a pattern of mutual exclusion (not shown). Deletion of the NFKBIA gene is associated with the non-classical subtypes of GBM (5), and CSNK2A1 gene dosage gains are significantly associated with the classical subtype (Figure 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 GBM, 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 GBMs. Expression of integrins including ITGB1 and ITGA4 depends on CK2 expression or activity in GBM cells, which is the first association of CK2 and integrin expression. Interestingly, ITGA4 and ITGB1 are expressed at higher levels in human GBM samples compared to non-tumor 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 \textit{ITGA4} and \textit{ITGB1} promoters contain binding sites for both STAT and NF-κB transcription factors.

Inhibition of CK2 activity by CX-4945 suppresses \textit{in vivo} GBM xenograft growth and promotes survival. In a GBM 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 GBM tumors and promotes the survival of mice bearing intracranial human GBM tumors. These findings describe the first use of any CK2 inhibitor in orthotopic models of GBM using primary human GBM xenografts. CK2 is a remarkably nodal kinase, and its upregulation in GBM has a strong impact on cellular processes indispensable for cancer cell survival. Targeting this pleiotropic kinase that influences multiple signaling cascades involved in GBM progression may prove more effective than strategies that target a single pathway.
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REFERENCES

FIGURE LEGENDS

Figure 1. Gene dosage gain of CSNK2A1 in GBM. (A) Gene-level CNV analysis across chromosome 20 in 537 GBMs. Gene dosages are mapped according to gene order on chromosome 20. CSNK2A1 shows gene dosage gains in 33.7% of tumors (green line, CSNK2A1 locus on 20p13||C). The RefSeq genes track shows coding gene locations. The proportions diagram shows the frequency of gene dosage gains along chromosome 20. Color intensity is proportional to the deviation from zero. Gene-dosage values indicate the log2 ratio of red (R, Cy5) to green (G, Cy3) intensity of the fluorescence dye (or log2R/G). (B) Gene dosage gain of CSNK2A1 is associated with significant gain of CSNK2A1 mRNA expression in 482 and 428 patients whose tumors were profiled on two different platforms at the Broad Institute (BI) and University of North Carolina at Chapel Hill (UNC), respectively. Values for gene dosage and gene expression are presented as log2R/G ratios. Locally weighted least squares (LOWESS) smooth fits (in red) confirmed the appropriateness of the linear regression models; p value indicates statistical significance according to estimated slope of the regression line. The corresponding box plots show the distribution of CSNK2A1 expression in tumors containing and lacking CSNK2A1 gains; p value calculated via Wilcoxon rank-sum test. (C) Gene-dosage profiles for CSNK2A1 across 490 GBMs along with its relationship to four molecular subtypes of GBM. A corresponding two-way contingency-table analysis reveals a significant association of CSNK2A1 gain with the classical subtype. CI denotes confidence interval.

Figure 2. Inhibition of CK2 Suppresses STAT Activation. (A) 20 µg of Xenografts X1016, X1046 and X1066, 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 nM of non-target, CK2α, CK2β or CK2α’ siRNAs for 48 h, then stimulated with 10 ng/ml of IL-6 and 25 ng/ml of sIL-6R for 10 min. (C-D) Xenograft X1066 was treated with CX-4945 in serum-free medium for 4 h, and then stimulated as above. Lysates were immunoblotted with indicated antibodies. (E) U251-MG cells were pretreated with CX-4945 in serum-free medium for 4 h, and then stimulated with OSM (5 ng/ml) for 1 h. mRNA was analyzed by qRT-PCR. *, p<0.05. (F) U251-MG cells were pretreated with CX-4945 in serum-free medium for 4 h, and then stimulated with EGF (50 ng/ml) for 10 min. (G) U251-MG cells were serum-starved overnight, treated with CX-4945 for 4 h, and stimulated with EGF for 1 h. mRNA was analyzed by qRT-PCR. *, p<0.05.

**Figure 3. Inhibition of CK2 Suppresses NF-κB Activation.** (A) U251-MG cells were transfected as described in Figure 2B, then stimulated with TNF-α (1 ng/ml) for 10 min. (B) X1066 was treated with CX-4945 for 4 h in serum-free medium, and then stimulated as above. (C) X1066 was treated with CX-4945 (10 µM) for 4 h in serum-free medium, and then stimulated with TNF-α for 1 h. mRNA was analyzed by qRT-PCR. *, p<0.05. (D) X1046 was pretreated with CX-4945 (10 µM) for 4 h in serum-free medium, and then stimulated with IL-1β for 10 min.

**Figure 4. Inhibition of CK2 Suppresses Adhesion and Migration of GBM Cells.** (A) X1016 was treated with CX-4945 (10 µM) for 16 h (upper panel), and U87-MG (middle panel) and U251-MG cells (lower panel) were treated for 12 h. Scale bar is 2 µm. (B) X1016 was treated with CX-4945 (10 µM) for 16 h, and stained with Alexa Fluor 488 phalloidin and DAPI. (C)
U251-MG cells were transfected with 100 nM of non-target, CK2α or CK2α’ siRNAs, or CK2α (50 nM) plus CK2α’ (50 nM) siRNAs for 48 h, and stained with Alexa Fluor 488 phalloidin and DAPI. The scale bar is 14 µm. (D) X1016 cells were treated with CX-4945 (10 µM) for 16 h. mRNA was analyzed by qRT-PCR. *, p<0.05. (E) X1046 was treated with CX-4945, and lysates immunoblotted with indicated antibodies. (F) U251-MG cells were transfected with 100 nM of non-target, CK2α or CK2α’ siRNAs for 48 h, and lysates immunoblotted with indicated antibodies. (G) X1046 was treated with CX-4945 for 16 h, and seeded onto fibronectin-coated plates for 1 h. The cells were then fixed, stained with crystal violet, and absorbance measured at 540 nm. The normalized value of untreated cells was set to 1. *, p<0.001. (H) X1066 cells were incubated in the absence or presence of CX-4945 for 4 h, scratched with a p200 pipette tip, and images taken at 0 and 16 h later. The scale bar is 1 µm. (I) Images from the assay were quantified. Representative of three experiments (*, p<0.01; **, p<0.001).

Figure 5. Inhibition of CK2 Suppresses GBM Cell Survival. X1046 (A) and X1066 (B) cells were treated with CX-4945, and cell survival measured by the WST-1 assay. (C) U251-MG cells were transfected with 100 nM of non-target, CK2α or CK2α’ siRNAs, or CK2α (50 nM) plus CK2α’ (50 nM) siRNAs for 48 h, and survival 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 h. Cells were stained with Annexin V and propidium iodide and examined by flow cytometry. Triplicate experiments, and error bars show ± S.D.. *, p<0.05. (E) U251-MG cells were treated with CX-4945 for 24 h, 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.
Figure 6. CX-4945 Inhibits In Vivo Growth of Xenograft GBM 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 by i.p. from days 3-5, 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 HEMAVET®950. 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) Five x 10^5 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 LogRank analysis was performed. (F) Five x 10^5 cells/5 µl of Xenograft X1016 were injected intracranially. Cages were randomized, and vehicle (n=3) or 75 mg/kg of CX-4945 (n=3) was administered orally twice a day starting at day 3, and mice euthanized between days 13-15. Tumors were homogenized, and lysates immunoblotted with indicated antibodies. (G) Five x 10^5 cells/5 µl of X1016 were injected intracranially. Cages were randomized, and vehicle (n=2) or 75 mg/kg of CX-4945 (n=2) 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, embedded in paraffin and sections stained with anti-Ki-67 antibody and counter stained with
Hematoxylin. Representative images and Ki-67+ percentages (in bracket) are shown. The scale bar is 50 μm.
Zheng et al., Figure 2
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Targeting Protein Kinase CK2 Suppresses Pro-survival Signaling Pathways and Growth of Glioblastoma

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