Novel Prognostic Markers and Therapeutic Targets for Glioblastoma

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Abstract

Glioblastoma is the most common and lethal malignant brain tumor with a survival rate of 14.6 months and a tumor recurrence rate of ninety percent. Two key causes for glioblastomas grim outcome derive from the lack of applicable prognostic markers and effective therapeutic targets. By employing a loss of function RNAi screen in glioblastoma cells we found a list of 20 kinases that can be considered glioblastoma survival kinases. These survival kinases which we term as survival kinase genes, (SKGs) were investigated to find prognostic markers as well as therapeutic targets for glioblastoma. Analyzing these survival kinases in The Cancer Genome Atlas patient database, we found that CDCP1, CDKL5, CSNK1ε, IRAK3, LATS2, PRKAA1, STK3, TBRG4, and ULK4 genes could be used as prognostic markers for glioblastoma with or without temozolomide chemotherapeutic treatment as a covariate. For the first time, we found that patients with increased levels of NEK9 and PIK3CB mRNA expression had a higher probability of recurrent tumors. We also discovered that expression of CDCP1, IGF2R, IRAK3, LATS2, PIK3CB, ULK4, or VRK1 in primary glioblastoma tumors was associated with tumor recurrence prognosis. To note, of these recurrent prognostic candidates, PIK3CB expression in recurrent tumor tissue had much higher expression compared to primary tissue.

Further investigation in the PI3K pathway showed a strong correlation with recurrence rate, days to recurrence and survival emphasizing the role of PIK3CB in tumor recurrence in glioblastoma. In efforts to find effective therapeutic targets for glioblastoma we used SKGs as potential candidates. We chose the serine/threonine kinase, Casein Kinase 1 Epsilon (CSNK1ε) as a target for glioblastoma because multiple shRNAs targeted this gene in our loss of function screen and multiple commercially available inhibitors of this gene are available. Casein kinase 1 epsilon protein and mRNA expression were investigated using computational tools. It was revealed that CSNK1ε expression has higher expression in glioblastoma than normal tissue. To further examine this gene we knocked down (KD) or inhibited CSNK1ε in glioblastoma cells lines and noticed a significant increase in cell death without any significant effect on normal cell lines. KD and inhibition of CSNK1ε in cancer stem cells, a culprit of tumor recurrence, also revealed limited self-renewal and proliferation in cancer stem cells and a significant decrease in cell survival without affecting normal stem cells. Further analysis of downstream effects of CSNK1ε knockdown and inhibition indicate a significant increase in the protein expression of β-catenin (CTNNB1). We found that CSNK1ε KD activated β-catenin, which increased GBM cell death, but can be rescued using CTNNB1 shRNA. Our survival kinase screen, computational analyses, patient database analyses and experimental methods contributed to the discovery of novel prognostic markers and therapeutic targets for glioblastoma.

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General Audience Abstract

Glioblastoma (GBM) is the most common and deadly primary brain cancer with an average patient survival of 14.6 months and tumor recurrence rate of approximately 90%. Two major reasons for this high tumor recurrence and low mortality are the absence of effective therapies and the lack of biological markers that can give insight into the outcome or prognosis of patient survival. To discover new therapies/ biological markers for GBM, we used a screen which examined the effects of a class of genes (kinases) on GBM cell viability. We individually knocked down the function of over 700 kinases in GBM cells and found that only 20 of these genes were essential for cell survival. Analyzing these survival kinase genes (SKGs) in a GBM patient database, The Cancer Genome Atlas, we found that genes CDCP1, CDKL5, CSNK1ε, IRAK3, LATS2, PRKAA1, STK3, TBRG4, and ULK4 could be used as prognostic markers for glioblastoma with or without standard chemotherapeutic treatment. For the first time, we found that patients with higher levels of NEK9 and PIK3CB mRNA expression had a higher probability of recurrent tumors. We also discovered that expression of genes CDCP1, IGF2R, IRAK3, LATS2, PIK3CB, ULK4, or VRK1 in primary glioblastoma tumors was associated with tumor recurrence. To discover effective therapeutic targets for glioblastoma we again explored our SKGs as potential candidates. We chose to investigate Casein Kinase 1

Epsilon (CSNK1ε) as a target for glioblastoma. We discovered that CSNK1ε expression has higher expression in glioblastoma than normal tissue. To further examine this gene we knocked down (KD) or inhibited the function of CSNK1ε in glioblastoma cells lines, GBM cancer stem cells, GBM patient cells and non-cancer cells and found that GBM cell viability decreased significantly in cancer cells but non-cancer cells were not affected. Our survival kinase screen, computational analyses, patient database analyses and experimental methods contributed to the discovery of novel prognostic markers and therapeutic targets for glioblastoma. These results are significant because of the impact these gene targets can have on GBM patient survival and quality of life.

DEDICATION

This dissertation is dedicated to my amazing parents, Thomas and Sosamma Varghese, whose unconditional love, abundant sacrifice, and endless support have led to all of my achievements. Though I do not express it enough, I love you dearly.

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I. Background

a. Chapter 1: Glioblastoma

i. General information regarding glioblastoma

Glioblastoma multiforme (GBM), which is more classically identified as glioblastoma, is a fatal primary brain tumor. GBM is the most common brain tumor and has a patient median survival of only 14.6 months. Glioblastoma is a heterogeneous cancer morphologically as well as molecularly. It is the most prevalent of all gliomas and represents more than 50% of the cases of astrocytoma [1]. The morphology as well as the rate of growth characterizes gliomas. In each stage of gliomagenesis there are different prognostic and therapeutic standards. Gliomas can be categorized as grade I, grade II, grade III (anaplastic astrocytoma) and grade IV astrocytomas (glioblastoma) as well as the less prevalent oligodendrogliomas and ependymomas. Glioblastoma itself can be categorized based on histopathology into conventional (93%), gliosarcoma (2%), or giant cell glioblastoma (5%) [2]. Glioblastoma is a highly invasive disease branded by area of necrotizing tissue with hypoxic regions and hyperplastic blood vessels. Glioblastomas are considered the highest-grade glioma with the worst prognosis.

Glioblastoma is a disease, which does not discriminate. It can occur at any age and to either gender but is more prevalent in men aged 45-65. Glioblastoma can occur either de novo or through progression of a lesser grade glioma [3]. De novo glioblastoma is called primary glioblastoma, which tends to grow very rapidly and is more common (~90%).

Glioblastoma that has progressed from a lesser grade tumor is called a secondary glioblastoma. Secondary glioblastoma have a higher incidence in younger patients (=< 45 years old) and does not occur as often as primary [4].

The etiology of glioblastoma has been thoroughly researched but direct causes have not been found. The cause of brain tumors in general are unknown and hereditary can only be linked to approximately five percent of patients. Common associations that are thought to contribute to brain cancer development are genetic, environmental, and occupational hazards [5].

Glioblastoma is a multifaceted fatal disease with an all-encompassing array of victims that has been meticulously investigated, unfortunately without much success in advancement of patient survival. Interdisciplinary research is needed to properly diagnose, foresee prognosis, and treat GBM patients to increase patient quality of life and survival in this grim disease.

ii. Difficulties in diagnosing GBM

Glioblastoma is diagnosed through histological examination but clinical symptoms can be used to help support diagnosis. Diagnosis founded solely on clinical symptoms may be incorrect because symptoms may vary between patients based on varying tumor location and size. Types of symptoms in glioblastoma include but are not limited to seizures, headaches, and cognitive dysfunction. Seizures may be seen in 20-40% of all patients. Approximately 50% of patients have headaches present upon diagnosis [6]. Cognitive dysfunction, language complications as well as focal signs impairment (e.g. gait imbalance, personality changes, visual disturbances, hemiparesis, and sensory loss) can also be seen in patients [7]. **These clinical symptoms can be misdiagnosed in elderly patients and careful consideration must be considered.** If a brain tumor is assumed then imaging will be the 1st step towards a diagnosis.

Magnetic resonance imaging (MRI) is the preferred imaging technique if a brain tumor is assumed. Computerized tomography can be used if patient is unable to undergo MRI. The MRI contrasting agent used in suspected glioblastoma patients is gadolinium for the reason that it can pass the blood brain barrier. When interpreting an x-ray image the glioma is enhanced using gadolinium (Gd) contrast with a central area of necrosis. The tumor is often surrounded by a white matter which represents edema. Tumors are often unifocal (single location) but multifocal tumors can be seen. Unfortunately Gd treated MRI results can often be misdiagnosed for pseudoprogression of the tumor [8]. **The MRI scan is not sufficient to diagnose GBM because the images are often**

indistinguishable from multiple sclerosis, stroke, or brain abscesses [9]. Biopsies must be taken to diagnose GBM. Histological characteristics that are seen in GBM tissue consist of but are not limited to cell hyperplasia, microvascular proliferation, anaplastic multi-nucleating cells, and pseudo-palisading necrosis**. GBM diagnosis is a complex process that entails identifying clinical symptoms, MRI, surgery and microscopy that comprehensively are physically demanding with exceedingly high opportunity for error**. Applicable biomarkers are urgently needed and would lessen the physical burden, expense, and misdiagnosis for GBM that would have a profound effect on patient quality of life and survival.

iii. Difficulties of GBM prognosis

Upon diagnosis of GBM, prognostic understanding is very limited and research is needed in terms of finding novel prognostic markers. Glioblastoma is the most common and deadly malignant brain tumor with an incidence of 0.59-3.69 per 100,000 worldwide. The standard of care consists of surgery, ionizing radiation and temozolomide (TMZ) chemotherapy. Irrespective of this aggressive multi modal therapy the outcome of patients is bleak [10]. The median survival with surgery alone is 3-4 months, with radiation added it is 12 months, and with further TMZ treatment the median survival only is increased to 14.6 months with a poor quality of life throughout the course of the disease [11]. In the United States the five-year overall survival of patients with glioblastoma is only 4.7% [12] and decreased in other parts of the world [13]. Even after surgery and aggressive treatment virtually all patients experience disease progression

after 6.9 months from date of first diagnosis [14]. There is an urgent need to find prognostic markers in glioblastoma.

Universal cancer biomarkers can have clinical therapeutic importance but cancer researchers have had very little progress in finding novel prognostic or predictive markers for GBM [15]. Recent advances in genome technology have contributed to new avenues to find novel prognostic markers. For example, the gene isocitrate dehydrogenase 1 or 2 (IDH1/2) has been indicated as a biomarker which distinguishes primary from secondary glioblastoma [16] . **Mutations in IDH1/2 have also shown to correlate with a better prognosis but advancements in terms of therapy have yet to be established.** An example of one of the most established biomarkers in GBM is O-6-methylguanine-DNA methyltransferase (MGMT). In many clinical trials epigenetic silencing/methylation of MGMT has been shown to increase responsiveness to alkylating therapies such as TMZ as well as display an increase in overall and progression free patient survival [17] . MGMT is an enzyme that repairs TMZ induced DNA damage. One **limitation** of this biomarker is the heterogeneity of glioblastoma. This heterogeneity of GBM includes variable MGMT expression which can lead to limited effects of TMZ. Another **limitation** is that alternate pathways are also used to repair DNA lesions as well as the discovery of other proteins involved in DNA repair of TMZ induced lesions [18] . Other genes/biomarkers such as EGFR and CD133 have sparked great interest but have not been very successful in terms of clinical effectiveness [19, 20]. **Currently the lack of effective biomarkers in GBM has limited the ability to accurately predict patient's clinical course of therapy, response to treatment, and overall survival.** There is a pressing need to find prognostic markers in glioblastoma.

iv. Difficulties in GBM treatment

Surgical resection

The standard of care for GBM consists of surgery, ionizing radiation and TMZ chemotherapy [2]. Following neuroimaging, patients that are suspected to have glioblastoma will undergo maximum safe surgical resection. Craniotomies/brain surgeries have become very advanced with the use of multiple tools such as functional MRI and brain mapping to assist the neurosurgeon in avoiding vital areas and removing as much tumor as possible. There are multiple goals to surgical resection but hope of completely curing glioblastoma is improbable. The first goal is to reduce the effect of the growing mass, which if left untouched may cause secondary medical issues by pressing on surrounding brain tissue. Secondly, debulking the tumor/cytoreduction can remove pressure on brain tissue and lower seizure activity. Another priority of surgeons is to obtain adequate tissue for histology for proper staging/grading of the tumor. It has been shown that gross maximal resection increases patient survival [21]. **Depending on the location of the tumor the surgeon will remove as much tissue as possible but due to the invasiveness of glioblastoma complete resection of the cancer is implausible**.

Radiotherapy

Radiation therapy is given following surgical resection and biopsy results. GBM is considered one of the most radiotherapy resistant cancers but is still shown to a certain

extent to be effective in treatment [22-25]. Conventional fractionated external beam radiation is the standard method which is usually administered at 60 gray (Gy) divided into 5 days a week for 6 weeks [14, 24, 26]. **One limitation of radiation is it not only negatively affects cancer cells but normal cells as well**. Radiation therapy usually targets the tumor and surrounding tissue and local radiation can be used which can lessen the effect of damaging healthy tissue. Types of local radiotherapy are as follows: Conformal photon radiation which uses 2-D and 3-D spatial data to match tumor shape and size. Image guided radiation therapy which uses images from each treatment to confirm exact position of tumor. Proton beam therapy which uses protons attracted to tumor as a method to target location. Interstitial radiation can be inserted into a tumor during surgery. Stereotactic radiosurgery is also conventional which uses high dose radiation in exact location for shorter periods of time. Photodynamic therapy uses drug and light combinations and boron neutron radiation that releases compounds in the tumor itself. Using radiation-sensitizing drugs, antibody delivery and higher levels of oxygen are currently being used to assist in radiation efficacy and toxicity [27-29]. **At this point there is no evidence based on overall survival for choosing one of these radiation therapies over the standard therapies and many clinical trials are still being conducted.**

Chemotherapy

Concurrent and adjuvant chemotherapy is administered with/after radiation therapy and can be effective in operable as well as inoperable tumors [30]. **Chemotherapy also**

has the limitation of affecting not only cancer cells but also damaging normal cells. First line chemotherapy of glioblastoma is temozolomide that was found to increase progression free survival and overall survival when added to radiation therapy as a standard of care. Temozolomide (8-Carbomoyl-3-methylimidazo(,1-d)-1, 2, 3, 5-tetrazin-4(3H)- one) is considered a prodrug. It is taken orally and is metabolized in the body to its reactive form which is 5-3-(methyl)-1-(triazen-1-y)imidazole-4-carboxamide (MTIC). In this reactive form the drug causes DNA damage. This damage consists of methylation of the O^6 position of guanine. This methylation causes a base mismatch of thymine. Base mismatch damage collects and replication is blocked or double strand breaks accrue with subsequent cell death. Temozolomide is well tolerated for long periods of time with surprisingly low occurrence of side effects. The most common side effect is nausea and vomiting which can be treated by anti-emetics. **One major downfall of temozolomide is that to have optimal effects the cells must have active mismatch repair to trigger apoptosis [26]**. Another obstacle facing temozolomide treatment is DNA repair mechanisms such as O6-alkylguanine DNA alkyltransferase (MGMT). MGMT methylation occurs in approximately 45% of glioblastoma patients. This means that the epigenetic silencing of this gene inhibits the MGMT protein from reversing the DNA damage caused from temozolomide. Patients lacking MGMT methylation, treated with temozolomide, where shown to live 12.7 months compared to the patients with MGMT hypermethylation whom were shown to live up to 21.7 months. The 2-year survival of temozolomide treated patients with MGMT methylation also increased from 13.8% to 46% [17, 31-35]. **Even though temozolomide is part of the standard of care, MGMT**

methylation should be considered when considering prognosis. As previously stated, using MGMT as a biomarker has its own complications.

Other groups of alkylating agents commonly used in glioblastoma are nitrosoureas and metal salts i.e. Carboplatin. Examples of nitrosoureas are carmustine (BCNU), nimustine (ACNU), and lomustine (CCNU). Of these drugs, the FDA approved a carmustine biodegradeable wafer, which could be inserted directly into the prior location of the tumor after surgical resection [36-38]. **Clinical studies for this application and other chemotherapies proposed survival benefit at the cost of excessive toxicity.**

Targeted therapies

Precision medicine has been a prevalent term in medicine recently but the establishment of therapeutics in GBM has not yet been feasible [39]. An exciting therapeutic target was IDH1/2 which has been indicated as a biomarker which distinguishes primary from secondary glioblastoma. Mutations in these genes have also shown to correlate with a better prognosis [16]. Inhibitors of these genes induce differentiation and impede glioblastoma growth [40]. Unfortunately IDH1 most recently has been discovered not to accurately predict long term survival in patients [41] and more experimentation needs to be done to further evaluate negative consequences and downstream effects of IDH1 inhibition. Epidermal growth factor receptor (EGFR), a member of the tyrosine kinase family, is a gene that has potential in GBM therapeutics. EGFR are hypothetical targets for GBM because it is mutated or overexpressed in 57.4%

of GBM [42]. EGFR activation stimulates proliferation in GBM cells through signaling cascades e.g. PI3K-AKT pathway. One EGFR inhibitor, Gefitinib has already been approved on non-small cell lung cancer and was promising in GBM. Unfortunately when Gefitinib was used in clinical trials for GBM it did not change progression free or overall patient survival. Erlotinib is another popular drug in cancer therapeutics but for GBM clinical trials they were shown to be unsafe and were stopped due to excessive toxicity Both EGFR inhibitors were encouraging but eventually were shown to be unsuccessful [20, 43]. Of the patients with EGFR mutations, 50% present an in frame deletion of exon 2-7, which is known as EGFRvIII [44]. EGFRvIII vaccines have been manufactured but have shown to be ineffective because of problems in patient selection and tumor heterogeneity as well as being very expensive [42, 45]. Another possible target is bevacizumab. Bevacizumab is an angiogenesis inhibitor that has been approved for GBM reoccurrence. Bevacizumab is a monoclonal antibody, which inhibits vascular endothelial growth factor (VEGF-A). GBM is characterized by increased angiogenesis and high expression of VEGF-A. This drug has shown promise but unfortunately has not been able to increase overall patient survival [46, 47]. There is an imperative need for targeted therapies. **The components of the standard of care only modestly increase patient survival while targeted therapies have shown false promise without significant benefit.** Novel effective therapeutic targets are immediately needed to increase patient survival.

b. Chapter 2: Complications in GBM tumor recurrence

Even after multimodal aggressive therapy, virtually all patients experience tumor recurrence and disease progression. Tumor recurrence nomenclature has much variability among researchers but it can be defined as a change from the loss of complete resection or tumor absence. Tumor recurrence is almost inevitable in glioblastoma patients. In 90% of the patients the tumor will recur in the original location and 5% of these patients will see multiple lesions. Patients with tumor recurrence are faced with many difficulties for recovery. The tumor recurrence tissue has already been shown to be resistant to radiotherapy as well as chemotherapy. The patient also may not be able to have another surgery as well as more chemotherapy and radiation due to their weaken state [48]. The prognosis in GBM patients with tumor recurrence is very grim. The etiology of tumor recurrence has been attributed to cancer stem cells, infiltrating cancer cells, and treatment induced tumorigenesis.

Traditionally it was believed that GBM occurred through multiple accumulating genetic events (i.e. mutations) in differentiated glial cells which brought rise to tumor progression [49]. **A more recent theory, the cancer stem cell model deems that a small subpopulation within the tumor is responsible or aid in driving tumor proliferation, drug resistance, and tumor heterogeneity [50].** This subpopulation consists of cells referred to as cancer stem cells and in the case of gliomas are called glioma stem cells (GSCs). In GBM, these cells have many markers in common with neural stem cells but no single biomarker has yet been identified. GSCs may be the major

culprit in recurrence and treatment failure in GBM and remarkably only represent a mere 1-2% of the tumor. Glioblastoma stem cells and there role in recurrence are a fairly new notion and it should be kept in mind that GSCs have only been isolated from GBM tumor tissues about a decade ago [51, 52]. Since then, substantial evidence has been collected and demonstrated that, in general, GBM tumor growth and development depend on GSCs. GSCs have many of the same features of neural stem cells i.e. self-renewal, multipotent differentiation and a quiescent nature [53, 54]. Unlike neural stem cells they are extremely tumorigenic and invasive. In suspension these cells generate spheres called neurospheres and may become quiescent unless cued by the microenvironment to differentiate or proliferate [55]. Recent research has revealed cellular heterogeneity as another obstacle in treating GBM [56]. The ability of GSCs to self-renew without limit and differentiate into various cell types contributes to the heterogeneity of glioblastoma, which make GBM hard to effectively treat. More importantly, these cells are refractory and intolerant to current radiation and chemotherapies. The quiescent nature of GSCs makes them non-responsive to chemotherapy and radiotherapy because these therapies target rapidly dividing cells. These activities of GSCs are symptomatic of the process of tumorigenesis and tumor recurrence. Evidence is accumulating of the pivotal role that GSCs play in tumorigenesis as well as recurrence [57]. **GSCs are an appealing drug targets and new therapies should eradicate these cells as well as primary cells to successfully treat patients**.

c. Chapter 3: Investigation of useful tools to examine GBM

i. RNAi functional screen of human kinome to expose GBM

The need for new therapies in cancer has driven many cancer biologists to use new techniques to evaluate and treat cancer. DNA sequencing technology can give some insight into how to treat GBM but it can be challenging, very expensive, and time consuming to obtain useful information [58] . **The RNAi screen is a very well known method used in cancer biology and can be used as a more targeted approach to treat GBM [59]**. RNAi has been widely used in cancer research since Gregory Hannon and colleagues used the technology to silence p53 expression [60]. An RNAi screen operates by knocking down specific genes either by siRNA (small interfering RNA) or shRNA (short hairpin RNA), followed by functional analysis.

RNA interference can occur naturally in eukaryotes. The process starts with double stranded ribonucleic acids that are transcribed and cleaved by an enzyme Dicer. Dicer cleaves double stranded RNA into fragments approximately 20 base pairs long called small interfering RNAs (siRNAs). These strands are then separated by helicase enzymes and modified by endonucleases. The antisense strand is incorporated into a complex termed the RNA induced silencing complex (RISC). This complex is composed of multiple proteins such as Argonaut, which is known as the catalytic unit of the RISC

complex and operates by cleaving target mRNAs. There are alternate pathways of siRNAs that are not as well studied but ultimately the same end point is reached [61].

Dropout viability screens/loss of function screens are a very valuable usage of RNAi. In a dropout viability screen, lentiviral packaged shRNA pools which target specific genes essential for survival are targeted [59]. Theoretically, if the shRNA is essential for survival of the cell line then inhibiting this gene would cause cell death. After infection of the lentiviral shRNA, the virus/shRNA package is integrated into the cell population and retained by the cells. In theory the entire population of cells will start off with all of the viruses/shRNA integrated and coded in their genome. When the shRNA is translated and if the target gene is essential for survival, then the cell will die. This gene/shRNA will "drop out" of the population. After sequencing the cell population at two time points you will see a "dropout" of the essential genes in the later time point. This dropout signifies the gene was essential for survival in that cell line. This can be a revealing method, which can give insight into gene function and survival in GBM.

The RNAi dropout screen can be employed to reveal potential biomarkers and therapeutics for GBM based on the knockdown of certain genes [59]. The gene of target should be able to affect the heterogeneous nature of glioblastoma. For a target gene to be effective in affecting survival in GBM it should affect multiple pathways. **Kinases are prevailing targets in cancer therapy because of their constitutive expression in cancer cells, involvement in signal transduction and tolerance of inhibition on normal cells [62].** Kinases have been extensively targeted for cancer since the discovery

of oncogenes. This is based on the fact that kinases are involved in mediating signal transduction pathways e.g. pathways involved in growth and survival. They are also known to have advantageous physical properties which aid in the creation of pharmacological inhibitors [63-65]. There are approximately 21 kinase inhibitors which are in clinical use for cancer therapy and there are many more in clinical trials [66]. Though these findings seem very promising, present-day kinase inhibitors have not been effective in treating GBM. **New targeted therapies and prognostic markers are urgently needed and RNAi screening of human kinases are an attractive manner in finding new effective targets in GBM.**

ii. Analyzing public human databases using survival analysis to discover novel prognostic markers and therapeutic targets

The Cancer Genome Atlas

Strides have been taken to decipher the complexities of GBM and to find prognostic and predictive biomarkers. With the advent of DNA sequencing technology enormous quantities of information have been generated. This data influx has been very complicated to store, decipher and annotate [15]. **The Tumor Cancer Genome Atlas (TCGA) project is a pivotal project in analyzing cancer genomes and extracting useful genomic data [67].** The Cancer Genome Atlas is a joint study from the National Institute of Health which was initially proposed to catalog all of the genomic alteration in patients of different types of cancers. This study began in 2006 and glioblastoma was the

first cancer analyzed. The outcome of the project was the characterization of 33 cancers from approximately 11,000 patients. The process in which TCGA data was collected and processed started with a tissue source site (TSS). This was where the clinical data and tissue were collected. The tissue was then sent to a designated biospecimen core resource (BCR) center. The data was organized and sent out to one of the data coordinating centers and sequencing centers where sequencing and analysis began. Many institutions worked together with different technologies and analytic tools to bring this project together. The first project, the TCGA GBM project had a total of 528 cases with extensive data which includes patient characteristics, gene expression, and clinical information. From these total 528 cases: 512 cases with exome data were collected, 523 cases of single nucleotide polymorphism (SNP) data, 524 cases of methylation data, 508 cases of mRNA data, 496 cases of miRNA data and clinical data on 523 patients.

TCGA GBM at present has added to the body of knowledge in this lethal disease. GBM was classified into 4 distinct genetic subgroups: classical, mesenchymal, proneural and neural. The classical and mesenchymal group showed aggressive treatment increased patient survival while no difference was shown with aggressive treatment in the proneural and neural groups in regards to lengthening survival [68]. More subtyping was also done in terms of methylation patterns in GBM. **In TCGA there are many cases of cross grouping of subtypes which unfortunately prevent using subtypes in terms of targeted treatment of glioblastoma.** Advantageously, survival analysis of TCGA public datasets can be effectively used and is of great value in terms of prognosis and therapeutics findings [69] .

Survival Analysis

The practice of linking TCGA datasets and statistical approaches that predict survival pattern associations with essential genes can be used to find prognostic markers [70]. Two well-established survival analyses tools to determine this association are the Kaplan Meier estimator and Cox Proportional Hazards. Kaplan Meier is a non- parametric test which is used often in medical/clinical research [71]. It can be used to measure the time for a certain event to take place given a certain situation. For example it may estimate the time a patient will survive after diagnosis of a disease or use of a treatment. Information that is required in this analysis is event occurrence (categorical/binary) as well as the length of time (numerical/continuous). If more than one set of data is being compared then a grouping name or assignment is required. One benefit of Kaplan Meier estimator is the function of censoring. It is possible that during clinical trials/experimentation an end- point (e.g. death) is not reached which could give inaccurate results if not calculated. The use of censoring allows these data points to be added into the analysis. The Kaplan Meier plot has either one or more lines depending on the number of groups analyzed. The percent/total of subjects in study is measured on the Y-axis and time to event in X-axis. If the sample size is large then horizontal steps in each line signify events that take place. If groups are to be compared then the log rank test can be used to test for significant differences between groups. The limitation of Kaplan Meier estimator is that only one covariate or factor can be used. To overcome this limitation Cox proportional hazards test can be used [72]. This survival model also allows for censoring and it provides a hazard ratio with either one or more covariates. A

hazard ratio is a term which signifies a value that can be used to estimate the likelihood of an event taking place at a certain time e.g. a patient having an accelerated rate of death because of a treatment compared to non-treatment group. Kaplan Meier and Cox proportional hazards analyses may both be used to determine prognostic markers from experimentally determined cancer survival genes. These analyses not only can determine the likelihood of certain genes to predict survival prognosis but also predict the likelihood of tumor recurrence.

GBM is a grim disease lacking prognostic markers that identify with the primary cancer or tumor recurrence. The identity of these prognostic biomarkers could play a vital role in finding new therapeutics and also understanding tumorigenesis of GBM and other cancers. New therapeutic are urgently needed for glioblastoma and recurrent GBM. It is a grim disease with a median survival of only 14.6 months despite the practice of surgery, radiation and chemotherapy treatment. The use of chemotherapy was the latest addition to the standard of care in treating GBM and it only modestly increased overall survival. Very little therapeutic advancements in terms of overall survival have been implicated in glioblastoma therapeutics in the last 30 years. New targeted therapies must be discovered [48, 73]. **The use of functional assays, computational tools and experimental techniques can be integrated to tackle this problem. To overcome the obstacles of GBM there are many hurdles to cross and important questions that need to be answered**.

d. Questions: Overcoming GBM obstacles

The lack of prognostic markers and therapeutic targets for GBM are of great concern. Certain question must be addressed to overcome the disease burden and increase the overall survival for GBM patients.

Effective ways to analyze essential GBM survival genes in patients and measure their association with survival must be found.

I. **Can we find effective prognostic markers in glioblastoma and glioblastoma recurrent tumors?**

Gene targets that efficiently treat GBM patients must be found. The integrated use of computational and cell biology techniques must be used to find an effective targeted therapy through the discovery of GBM survival genes, prognostic analysis, and molecular biology.

II. **Can we find a therapeutic target for glioblastoma, which is effective against the primary and cancer stem cells of glioblastoma?**

To answer these questions we employed the use of an RNAi loss of function screen of the human kinome in a GBM cell line to recover kinases that are responsible for GBM

survival. We then used the TCGA database and statistical survival analysis to uncover novel prognostic markers for GBM and GBM recurrence. Finally, we used data from both of these methods to investigate and find a therapeutic target for GBM which is effective against a heterogeneous population of GBM and GSCs.

II. Materials / Methods

a. General

i. Reagents/Solutions

The Cell-Titer 96® Aqueous One solution cell proliferation assay (MTS) was purchased from Promega. The SYBER green mix was purchased from Promega. The TRC kinase shRNA gene family library was purchased from GE Dharmacon. The Column-freeTM plasmid mini-prep kit was purchased from Lamda Biotech, Inc. and 2X LB were used. The QIAamp DNA extraction kit was purchased from QIAGEN. Puromycin was purchased from Thermo Fisher Scientific. Trizol and the SuperScript®III RT were purchased from Thermo Fisher Scientific. Poly-D Lysine (50µg/µL) for coating plates in virus production was purchased from Millipore. Polybrene for viral infection was purchased from Sigma. 10 X Lysis buffer (HEPES ph6.8 - 200 mM, NaCl -1400 mM, MgCl2 - 25 mM, CaCl2 - 25 mM), 10 X protein inhibitor (SIGMAFAST™ Protease Inhibitor Tablets(Sigma, #S8820), 1 tablet for 10ml 10 X protein inhibitor) , Lysis solution: 1ml(10 X Lysis buffer-100 µl, 10% NP40 -200 µl, 5% DOA -200 µl, protein inhibitor -200 µl, phosphatase inhibitor 2(#P0044) -10 µl phosphatase inhibitor 3 (#P5726) -10 µl, 80% glycerol -125 µl, ddH2O -155 µl) 2X SDS loading buffer-40ml (50% glycerol -10 ml, 10% SDS-16 ml , 0.5 M Tris, HCl, pH 6.8 -8 ml , 0.5 M EDTA - 0.16 ml, 1% Bromophenol Blue - 2 ml, ddH2O-3.84 ml)Gel Casting Reagents: 30% Acrylamide/Bis Solution (BIO-RAD#161-0156): (500 ml, 30% acrylamide and bis-acrylamide solution, 29:1) 0.5 M Tris-HCl, pH 6.8 (100 ml)(BIO-RAD:#161-0799) (Tris

base -6.06 g, diH2O ~60 ml pH adjusted to 6.8 with addition of HCl then diH20 added to 100ml) 1.5 M Tris-HCl, pH 8.8 (150 ml) (BIO-RAD:#161-0798)(Tris base -27.23 g, diH2O -80 ml,(pH adjusted to 8.8 with 6 N HCl, diH2O added total volume = 150 ml)) 10% (w/v) SDS (100 ml)(SDS -10.00 g, diH2O-90 ml(Dissolved with gentle stirring and diH2O added up to 100 ml)10% (w/v) APS (sigma# A3678) (used fresh daily, also can be store in 4°C, no more than 1 month)(Ammonium persulfate 0.10 g, diH2O -1 ml)) 10× SDS-PAGE running buffer (1 L)(250 mM Tris, 1.92 M glycine, 1% SDS pH 8.3, Tris base -30.30 g, Glycine -144.10 g, SDS -10.00 g, (diH2O added to 1 L))10× transfer buffer (1 L) 250 mM Tris, 1.92 M glycine, 0.1% SDS, Tris base -30.30 g, Glycine-144.10 g, SDS -1.00 g(Added diH2O to 1 L)1× transfer buffer (1 L)(1× transfer buffer-100ml, methanol -200ml, H2O -700ml)10× TBST Tris base -24.2 g, NaCl-80.06 g, Tween 20-20 ml, (Added diH2O to 1 L)) REAGENT: Bio-Rad Protein Assay Dye Reagent Concentrate: #500-0006, BSA: Fisher, #BP1600-100 Tris: Affymetrix, high purity grade, 22674, Glycine, MB grade 16407,SDS: SIGMA, #L3771, DTT: SIGMA, #D-0632, Glycine: SIGMA, #16407, Glycerol: SIGMA, #G5516, Dry milk: Nestle Carnation instant Non-fat dry milk.

Inhibitors: CSNK1ε inhibitors, IC261 (30mM in DMSO (Santa Cruz Biotech)) and PF 4800567 hydrochloride (10mM in DMSO (Santa Cruz Biotech)) were all prepared according to manufacturer provided directions. MELK inhibitor OTSSP167 hydrochloride (10mM in DMSO (Santa Cruz Biotech)) was prepared according to manufacturer provided directions.

Antibodies used for western immunoblotting: Antibodies were diluted as follows: anti–β–Catenin (Cell Signaling Technology®, 1:1000), anti-PIK3 Kinase p110α (Cell

Signaling Technology®, 1:1000), anti-PIK3 Kinase p110β (Cell Signaling Technology®, 1:1000), anti-MGMT (Cell Signaling Technology®, 1:300), anti-β-actin (Sigma-AldrichCo.LLC,1:10000), anti-phospho−β−Catenin(Cell Signaling Technology®, 1:1000), anti-Cleaved Caspase 3(Cell Signaling Technology®,1:1000), anti-LC3B(Cell Signaling Technology®,1:1000), anti-AKT (Cell Signaling Technology®, 1:1000), anti-CK1ε (Cell Signaling Technology®, 1:1000), anti−Active-β−Catenin (Millipore®, 1:1000), anti-GFAP (Cell Signaling Technology®, 1:500), antiNOTCH1 (Bio-Rad Laboratories Inc., 1:1000), and anti-GAPDH (Santa Cruz Biotechnology, Inc., 1:5000). Images were taken using a ChemiDoc™ MP System (Bio-Rad Laboratories Lnc). The intensity of the band was quantified using Image Lab software (Bio-Rad Laboratories Lnc.) or ImageJ as described earlier. Viability assays MTS or Trypan blue exclusion. In MTS assay, (as previously described in chapter 2),10 µl MTS (Promega) was added to each well then incubated at 37 °C for 1-4 h. The absorbance at 490 nm was measured using the FilterMax F3 microplate reader (Molecular Devices, LLC) according to manufacturer's instructions. Percent cell viability was obtained by dividing the absorbance of treatment groups to those of untreated groups after removal of background. In the Trypan blue viability assay, cells were trypsinized and stained with Trypan blue 1:1. Trypan blue cells were not considered viable. Trypan blue negative cells (lacking blue color) were counted using a hemocytometer. The percentage of viable cells was defined as the ratio of cell number in the treatment group to that of the control group. SDS-PAGE Gels were used in western blotting for protein quantification: 10% Separating gel (bottom- made 1st(2 hours to dry) (Water 2 mL, 30=% Acrylamide/Bis 1.65 mL, 1.5M Tris ph 8.8- 1.25 mL, 10% SDS 50µL, 10% Ammonium Persulfate 50µL,

TEMED 2μL (added last)) 10% Stacking gel (top resolving) - 30 minutes to dry(Water 1.1 mL, 30=% Acrylamide/Bis 335 μL, 0.5M Tris ph 6.8 -500 μL, 10% SDS 20μL, 10% Ammonium Persulfate 20μL, TEMED 2μL (added last))

ii. RNA interference screen plasmid/virus preparation

Cells were cultured in a 37°C incubator with 5% CO2. shRNA library preparation: 4,518 shRNA constructs (against 781 human kinases) were maintained as a single clone of bacteria in glycerol stock in 96-well plates. To prepare a mix of plasmids, each bacterial plate was replicated in another 96-well culture plate with 2X LB in each well. Plates were then incubated at 37°C with vigorous shaking for 24 hours. Plasmids were then prepared using a mini-prep kit adapted for 96-well plate. The concentration of each plasmid was determined using a nanodrop (Thermo Fisher Scientific). Equal amount of ~450 plasmids was mixed together as a single plasmid pool. The entire library was then divided into 10 pools.

The TRC shRNAs are built upon pLKO.1 vector that can be used to generate lentivirus. A pool of plasmids of the TRC kinase shRNA library was transfected with Effectene Transfection Reagent (Qiagen) into HEK293T cells together with packaging plasmids pMDG2.g and psPax2. On day 1: 500μL of Poly D Lysine was added to each well of a 6 well plate and placed in incubator for 1 hour. After incubation the Poly-D Lysine was removed and the well was washed with PBS. HEK293T cells were trypsinized and counted. 1 X 10^6 HEK 293T were plated into each well of 6 well plates. Day 2: 2ml micro centrifuge tube with 1ug of shRNA of interest, 1ug of psPAX2 and

0.5ug pMDG2 was placed in 100μL EC buffer and 3.2μL Enhancer, incubated at RT for 5 minutes. 10μL Effectene reagent was added to mixture and incubated at RT for 30 minutes. During incubation, HEK 293T cells were washed with PBS and 1600 μL of fresh media was added. 600μL of fresh media was added to transfection reaction after incubation. Transfection reaction was added dropwise to cells and placed into incubator. Day 3: Cells were refed with 30% FBS media. 48 hours after infection (Day 4) the culture media which contained lentiviruses was collected and spun down at 800G for 10 minutes. Supernatant was divided into small aliquots. Aliquots were stored at -80°C freezer. The virus titer was then determined using the serial dilution assay. This was performed by 1^{st} plating 1 x 10^5 HEK 293T cells in 12 well plates. Compare NS (non-silencing) plasmid with kinase shRNA. 12 tubes were arranged split into two rows. Each row was designated for each shRNA. 1 ml of DMEM media with polybrene (1ug/ml) in the 1^{st} tube (each row) followed with 900μL into each other tube. From the first tube 100μL was mixed thoroughly and aliquoted to the next tube. This was repeated in each tube (changing tips each time) excluding the last tube which would act as negative control. In the plates containing HEK293T cells were removed of media and 900μL of each virus mixture was added and incubated for 2 hours. This was followed by refeeding with 2ml of fresh media and 22 hour incubation at 37 degrees Celsius. The following day the cells were trypsinized and placed in 60 mm dishes for 2 hours. This was followed by removal of media and addition of 1μg/ml puromycin media. Selection took place for 7 days. Media is removed and dishes were washed with cold PBS. 0.5% crystal violet was added to each plate and incubated for 10 minutes RT. The crystal violet was carefully

washed off with water and colonies were counted. The calculation for rate of infection was or titer was: titer = colony # / volume of virus (μL) multiplied by 1000.

iii. Glioblastoma cell culture

GBM cell lines and patient primary samples were maintained in Dulbecco's modified Eagle medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) (Atlas Biologicals, Inc.), streptomycin (100μg/ml) and penicillin (100 IU/ml) (Thermo Fisher Scientific). Cells were cultured in a 37°C incubator with 5% CO2. Cancer stem cells were maintained in Dulbecco's modified Eagle medium (Thermo Fisher Scientific) supplemented with B27, EGF 20ng/ml, bFGF 20ng/ml, L-Glutamine, streptomycin (100 μg/ml) and penicillin (100 IU/ml) (Thermo Fisher Scientific). Cells were cultured in a 37°C incubator with 5% CO2. For adherent stem cells to be grown in spheres Poly 2-hydroxyethyl methacrylate (poly-HEMA, PH) coating was used. (Sigma #P3932) 1gram was dissolved in 50ml ethanol and rotated in 65 degrees Celsius for >3 hours. Cell culture dishes were coated until dry and UV light was used to sterilize. Normal brain cell lysate was purchased from Abcam. GBM cell lines Human GBM cell lines SF295, U87MG, A172, LN229, T98G, SF-268, U251, SNB-75, LN-18 were maintained in Dulbecco's modified Eagle medium (Life Technologies Corporation) supplemented with 10% fetal bovine serum (FBS) (Atlas Biologicals, Inc.), streptomycin (100 μg/ml) and penicillin (100 IU/ml). Human GBM stem cell (GSC) lines cells were maintained as spheres in Neuralbasal® media (Life Technologies Corporation) supplemented with Gibco® B-27® Supplements (Life Technologies Corporation), FGF

(ProSpec-Tany TechnoGene Ltd., 20 ng/ml), and EGF (ProSpec-Tany TechnoGene Ltd., 20 ng/ml). Primary cell culture: The isolation and all preparation of primary GBM cells and cancer stem cells from GBM Carilion patients and the use of these human specimens was approved by the Institutional Review Board at the Carilion Clinic in Roanoke Virginia. These freshly resected tissues were placed on ice in media immediately after surgery. Samples were rushed to our lab and tissue was minced into small pieces. Single cells were isolated using the Papain cell disassociation system (Worthington Biochemical Corporation) or Liberase (Roche Diagnostics) according the manufacturer's directions/instructions. Red blood cells were removed from the cell mixture using Red Blood Cell Lysis Solution (Miltenyi Biotec Inc). Tissue was placed in stem cell media, DMEM and frozen stocks were stored. Isolated cells were cultured in Dulbecco's modified Eagle medium (Life Technologies Corporation) supplemented with 10% fetal bovine serum (FBS) (Atlas Biologicals, Inc.), streptomycin (100 μg/ml), and 1 X Antibiotic-Antimycotic (Life Technologies Corporation). Primary GBM cells were maintained at low passages. GSCs were isolated and enriched using the sphere-formation assay followed by TrypLE disassociation and replating. Primary GBM cells were cultured in stem cell culture media (DMEM or neurobasal media (Life Technologies Corporation) supplemented with B-27 supplement (Life Technologies Corporation), 20ng/ml FGF-2 (Genescript), and 20ng/ml EGF (Genescript)). Isolated GSCs grew as spheres after 1-2 months of continuous culturing. GSCs were tested for their capability to self-renew using the sphere-formation assay described below. Immunoblotting and quantification of band intensity was determined for protein analysis and described below.

iv. Immunoblotting

Cell lysis: Cells were trypsinized and spun down 1200 rpm for 3 min and supernatant was removed. Cells were lysed on ice using lysis solution for 10 minutes. Lysis solution was spun down at 14000rpm in 4 degrees Celsius and supernatant collected. 1μL of supernatant was used I Bradford assay (Bio-Rad#500-006) 50μL of supernatant was mixed with 10uL DTT and 40μL 2X SDS loading buffer and incubated at 95 Degrees Celsius for 5 minutes.

Bradford Assay was utilized to determine protein concentration: BSA prepared at 1μg/μL. Bradford assay mix diluted from 5X with DI water (200μL/sample). In a 96 well plate 200μL per well was added. In 1st row 0, 1,2,4,8 μL of BSA were added which indicate the known values. In the following wells 1uL of cell lysate was added and mixed well. The plate reader was set to an optical density of 595 nm wavelengths. A standard curve was calculated for the known values (BSA). All cell lysate samples concentrations were extrapolated from standard curve calculations and 25μg of total protein was used equally in SDS-PAGE gels.

SDS-PAGE gels to PVDF membrane blot transfer: Sequentially protein is suspended in SDS-PAGE and transferred into PVDF membrane. This was done by immersing PVDF membrane into methanol followed by cutting edges of SDS-PAGE gel was cut out to fit PVDF membrane. Mesh, filter paper, PVDF membrane and gel were packaged together using a western blot apparatus. The apparatus was submerged in transfer buffer and placed in gel tank using 0.2A for 2 hours to complete transfer. Blots were blocked in 1X TBST + 5% milk at RT overnight. Primary Antibodies were prepared in 1X TBST + 5%

milk and incubated 4 degrees Celsius overnight followed by 15 minutes 1X TBST wash. Secondary antibodies were incubated for 1 hour in room temperature followed by 1X TBST wash. To visualize bands 1ml of each Pico reagent (Thermofisher #34080) was added to each blot and incubated on shakers for 5 minutes RT. When bands were faint, 500 ml of each Femto (Thermo#34095) reagent was added and incubated for 5 minutes room temperature. Blots were examined using ChemiDoc™ MP System.

b. Computational Approach

i. Glioblastoma patient survival analyses

Glioblastoma patient survival analysis was performed using TCGA GBM database (https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp). Level 3 (normalized) clinical variables of glioblastoma patients (age, days to death, temozolomide treatment, radiation treatment, Karnofsky score, histology, progression, gender, barcodes, recurrence, status…) was downloaded from the TCGA GBM data portal (https://tcga-data.nci.nih.gov/tcga/tcgaCancerDetails.jsp?diseaseType=GBM&diseaseName=Glioblastoma%20multiforme). Gene expression data, including for glioblastoma patients (AgilentG4502A071; AgilentG4502A072), was downloaded from the Pan-cancer project (syn1461183) from the online database Synapse (http://www.synapse.org). Gene expression sets were combined together. Tumor histology was confirmed and duplicate patient samples were excluded. One gene from our survival kinase gene list was not found in the expression data and was excluded from analysis (CDK11B). Clinical

variable and gene expression files were merged using patient and aliquot barcodes. Gene expression values were sorted individually and statistical information was collected e.g. median mean, quartiles, distribution, and sample size (N). GBM patients were divided into high level expression (top 25%) and low expression groups (bottom 25%) for each gene based on mRNA levels. A master file was created with all data and information for all patients with grouping, gene expression and clinical information which we called the GBM mRNA master file. GBM mRNA master file was imported into JMP software. File processing was performed using Python Pandas, Microsoft Excel and JMP. Using the master file, Kaplan Meier analysis (survival/reliability) was performed for each gene independently and recorded. Cox proportional hazard model (Fit Proportional Hazards) was performed with gene group (continuous and nominal) and age or temozolomide cofactors and recorded. Other cofactors were also analyzed with negative results i.e. radiation, Karnofsky score, gender, genetic subtype. False discovery rate was also calculated using the Benjamini and Hochberg (1995) which is a way to reduce false positives. The add-in program "Benjamini-Hochberg False Discovery Rate PValue adjustment" had to be installed into JMP 11. P values of each analysis from Kaplan Meier or Cox analysis were combined into a JMP data sheet and ranked in order from smallest to largest value. FDR p values were calculated using the JMP software.

ii. Reverse phase protein analyses

The TCGA database has a wealth of information which includes RNA, epigenetic, clinical, and protein expression data. We examined microarray data from the TCGA GBM database as previously described. In brief, we explored over 200 patients and calculated prognostic significance based on of CSNK1ε expression using survival analysis. The TCGA protein data we examined in our experiments was downloaded from reverse phase protein analysis (RPPA). RPPA is a high throughput protein expression microarray which is used as a functional proteomic tools and is becoming more widely used for analyzing signaling and protein function. This RPPA analysis was completed at MD Anderson Cancer Center. This analysis can provide insight of signaling pathways in GBM patients through the use of antibodies and differential expression analysis. The TCGA analysis of CSNK1ε and affiliated genes have shown to be very informative and give support to further investigate CSNK1ε. RPPA analysis was obtained via TCGA database (https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp). Level 3 (normalized) clinical variables of glioblastoma patients such as days to death, was downloaded from the TCGA GBM data portal (https://tcgadata.nci.nih.gov/tcga/tcgaCancerDetails.jsp?diseaseType=GBM&diseaseName=Glioblastoma%20multiforme). Protein expression was downloaded from the Pan-cancer project (syn1461183) from the online database Synapse (http://www.synapse.org). Files were consolidated from the TCGA data portal and synapse. Tumor histology was confirmed and duplicate patient samples were checked. Clinical variable and protein expression files were merged using patient and aliquot barcodes. Protein expression

values were sorted individually and statistical information was collected e.g. median mean, quartiles, distribution, and sample size (N). GBM patients were divided into high level expression (top 25%) and low expression groups (bottom 25%) for each protein of interest based on protein levels for 1st round of analysis(Cox and Kaplan Meier) and all data values were used in 2nd round of analysis(Cox-continuous). Data files were imported into JMP software. File processing was performed using Python Pandas, Microsoft Excel and JMP.

iii. GBM Discovery Bio Portal and Gliovis software tools

GBM Discovery Bio Portal: To look at survival kinase genes in groups, we used the online software GBM Discovery Bio Portal. This program also retrieves data from the TCGA GBM project which we used in finding prognostic markers. The specific datasets we used are (1) AgilentG4502A_07 from University of North Carolina, (2) HT_HG-U133A from Broad Institute and (3) HuEx-1_0-st-v2 from the Berkeley Lab. Algorithms that are used on TCGA GBM patients include (1) The optimal number of clusters (NbClust); (2) Expression levels in GBM subgroups; (3) Prognostic Index, obtained by computing weighted averages of expression values with regression coefficients of a multi-gene Cox proportional hazards model

Gliovis: To examine the differences in expression of PIK3CA and PIK3CB in both primary and recurrent tissue we used the Gliovis software tool (http://gliovis.bioinfo.cnio.es/). We used these software to validate our results.

iv. Statistical analyses

Distribution, maximum value, quartiles, median, minimum value, mean, standard deviation, stand error of mean, upper and lower 95% mean, sample size (N), Kaplan Meier survival, Cox proportional hazards, Contingency analysis, Analysis of Means for Proportions, Two Sample Test for Proportions and Fisher's Exact Test (recurrence rates) for SKGs and isoforms was performed using the JMP Pro 11 software (SAS Institute Inc.).

c. Molecular Methods

i. Loss-of-function screen for survival kinase genes

U87MG (1×10^6) cells were transduced with 10 pools of lentiviruses that harbor the TRC kinase shRNAs. One day after viral infection, half of the infected cells (5 x 105) was collected as P0 (initial time point) and subjected to genomic DNA isolation described below. The other half of infected cells was cultured for another 7 days in the media containing 0.5 μg/ml puromycin and collected as P7 (end time point). Genomic DNA of cells at P0 and P7 was isolated using the QIAamp DNA extraction kit. shRNAs were then amplified by PCR (MF18 (5'-tacgatacaaggctgttagagag-3') and MF19 (5'-cgaaccgcaaggaaccttc-3')) and sequenced using the Solexa deep sequencing. The sequencing read number of each shRNA at P0 was divided by that at P7. The shRNAs with a 2-fold less of this number were considered as candidate SKGs.

ii. Cell viability assay

To validate the primary candidates identified from the loss of function screen, U87MG cells were transduced with viruses of NS or individual candidate shRNAs. Cells were then selected with puromycin (0.5 μg/ml) for a week. Cell viability was determined using the MTS assay. Media was removed and 100μL fresh media was used. 10μl of

MTS reagent was added to 100μL cell suspension. Cells were left in incubator at 37 degrees Celsius and measured at 2 and 4 hours. MTS colorimetric detection was quantified using the Software Max Pro software 6.2.1 on Filter Max F3 multi-mode plate reader (Molecular Devices). Settings were set to read absorbance at 490nm filter and orbital shaking was performed before each read to obtain more accurate results.

Cell viability of GSCs: Assay was performed by plating 2000 cells per well of 96 well plate. GBM 10 xenograft GBM cells, VTC 056, and VTC 061 GBM patient cancer stem cells were used in three different experiments. VTC 61 required PH coated plates to prevent from adherence. Half of the plate was treated with 1uM of IC261 and the other half was administered the vehicle control DMSO. One week after incubation the cells were retreated to avoid changes from evaporation. After 2 weeks the MTS reagent was added and absorbance was measured using Software Max Pro software 6.2.1 on Filter Max F3 multi-mode plate reader (Molecular Devices).

iii. Normal glial cell isolation

C57 black pup mice were sacrificed. Whole brains were minced with razors and ground and filtered through 70uM filters. Primary cells were placed in either Dulbecco's modified Eagle medium (Life Technologies Corporation) supplemented with 10% fetal bovine serum (FBS) (Atlas Biologicals, Inc.), streptomycin (100 μg/ml), and 1 X Antibiotic-Antimycotic. Stem cells were maintained in Dulbecco's modified Eagle medium (Thermo Fisher Scientific) supplemented with B27, EGF 20ng/ml, bFGF 20ng/ml, L-Glutamine, streptomycin (100μg/ml) and penicillin (100 IU/ml) (Thermo

Fisher Scientific). Stem cells were left in stem cell media for over 1 month before experimentation. All cells were cultured in a 37°C incubator with 5% CO2.

iv. Lentivirus preparation of CSNK1ε/β-Catenin

The CSNK1ε shRNAs and β-Catenin shRNA (shCTNNB1 #2, #42544) are built upon pLKO.1 vectors that can be used to generate lentivirus. The shRNAs were individually transfected with Effectene Transfection Reagent (Qiagen) into HEK293T cells together with packaging plasmids pMDG2.g and psPax2. This process is further elaborated on: Day 1: 500μL of Poly D Lysine was added to each well of a 6 well plate and placed in incubator for 1 hour. After incubation the Poly-D Lysine was removed and the well was washed with PBS. HEK293T cells were trypsinized and counted. 1 X 10^6 HEK 293T were plated into each well of 6 well plates. Day 2: 2ml micro centrifuge tube with 1ug of shRNA of CSNK1 ε or Non Silencing plasmids, 1ug of psPAX2 and 0.5ug pMDG2 was placed in 100μL EC buffer and 3.2μL Enhancer, incubated at RT for 5 minutes. 10uL Effectene reagent was added to mixture and incubated at RT for 30 minutes. During incubation, HEK 293T cells were washed with PBS and 1600 μL of fresh media was added. 600μL of fresh media was added to transfection reaction after incubation. Transfection reaction was added dropwise to cells and placed into incubator. Day 3: Cells were refed with 30% FBS media. 48 hours after infection (Day 4) the culture media which contained lentiviruses was collected and spun down at 800G for 10 minutes. Supernatant was divided into small aliquots. Aliquots were stored at -80°C freezer. The

virus titer was then determined using the serial dilution assay. This was performed by 1st plating 1 x 10^5 HEK 293T cells in 12 well plates. Compare NS (non-silencing) plasmid with kinase shRNA. 12 tubes were arranged split into two rows. Each row was designated for each shRNA. 1 ml of DMEM media with polybrene (1ug/ml) in the 1st tube (each row) followed with 900μL into each other tube. From the first tube 100μL was mixed thoroughly and aliquoted to the next tube. This was repeated in each tube (changing tips each time) excluding the last tube which would act as negative control. In the plates containing HEK293T cells were removed of media and 900μL of each virus mixture was added and incubated for 2 hours. This was followed by refeeding 2ml of fresh media and 22 hour incubation at 37 degrees Celsius. The following day the cells were trypsinized and placed in 60 mm dishes for 2 hours. This was followed by removal of media and addition of 1ug/ml puromycin media. Selection took place for 7 days. Media is removed and dishes were washed with cold PBS. 0.5% crystal violet was added to each plate and incubated for 10 minutes RT. The crystal violet was carefully washed off with water and colonies were counted. The calculation for rate of infection was or titer was: titer = colony # / volume of virus (μL) multiplied by 1000.

v. Glioma stem cell sphere formation assay

Sphere formation assay was performed by plating 50 cells per well of 96 well plate. GBM 10 xenografts GBM cells, VTC 056, and VTC 061 GBM patient cancer stem cells were used in three different experiments. VTC 61 required PH coated plates to prevent from adherence. Half of the plate was treated with 1uM of IC261 and the other half was

administered the vehicle control DMSO. One week after incubation the cells were retreated to avoid changes from evaporation. After 2 weeks the number of spheres was counted in each well. Spheres consisted of 4 or more cells together. The percentage of wells with spheres over the number of total wells with cells plated defined the ratio of sphere formation. Microscopy was performed using Carl Zeiss Axio Vert A1 Inverted Microscope.

vi. β-catenin downstream gene transcription analysis

Dual Luciferase Assay (Promega): After infection of 12 well plate with CSNK1ε or NS lentivirus (as described above) an accepted luciferase assay, pTOPFLASH/pFOPFLASH was used to determine transcription of genes downstream of β-catenin. In each set (NS or CSNK1ε) of infected cells two additional plasmids were transfected using the Effectene transfection kit. In a 1.5 ml microcentrifuge tube 300ng of either pTOPFLASH (positive) or pFOPFLASH with 6ng of Renilla plasmid (50:1 ratio) were added. This was followed by 72 μL of EC buffer and 2.5μL of enhancer. This mixture was vortexed for 1 second and incubated at RT for 4 minutes. After incubation 6 μL of Effectene was added to this mixture and pipetted up and down 10 times and tube was incubated for 9 minutes at RT. During incubation the plate with infected cells were taken out of incubator, media aspirated and washed with PBS. After incubation 400μL of media was added to transfection microcentrifuge tube and mixed using pipette twice. This was followed by dropwise addition of plasmid to appropriate cells. 33 hours after transfection reagents for dual luciferase assay were prepared and cells were lysed.

Reagent Preparation: 1X PLB was produced by adding 1 part 5X Passive Lysis Buffer to 4 volumes of distilled water. LAR II was produced by resuspending Lyophilized Luciferase Assay substrate in 10 ml of Luciferase Assay Buffer II. Stop and Glo reagent was made by adding 2.1ml of Stop and Glo substrate to 105ml of Stop and Glo buffer. Cell Lysis: Medium was removed from cells and plates were washed with PBS. PBS was removed and 1mL of 1X PLB was added to each well. Cells were scraped and collected into a new tube.

Luminescence Reading: 100μL of LAR II was added into wells of 96 well opaque plates. 20μL of PLB/Cell Lysate was mixed and added into LAR II solution. Luminescence of firefly luciferase (positive) activity was immediately read. 100μL of Stop and Glo reagent was added to lysate/PLB/LARII solution and renilla luciferase (internal control) activity was immediately recorded. Background noise was subtracted using PLB/LAR II/Stop and go mixture without cell lysate. Luminescence reading was recorded using Software Max Pro software 6.2.1 on Filter Max F3 multi-mode plate reader (Molecular Devices).

vii. CSNK1ε knock down cell viability rescue experiment

To determine the downstream survival effect of Casein Kinase 1 Epsilon we knocked down downstream protein CTTB1 to rescue cell survival. We used two sets of different populations of cells to conduct this experiment. Set 1: In a 96 well plate 2500 cells were plated per cell. Four well per NS, CSNK1ε shRNA, CTNNB1 shRNA, CSNK1ε shRNA

+ CTNNB1 shRNA, no shRNA trt (negative). After 24 hours cells were infected with lentiviral shRNAs as described. Puromycin selection was administered for 4 and 6 days. MTS analysis was analyzed after 2 and 3 hours incubation with and without plate shaking on device. All results were recorded.

Set 2: In a 96 well plate 50000 cells were plated per cell. 6 well per NS, CSNK1ε shRNA, CTNNB1 shRNA, CSNK1ε shRNA + CTNNB1 shRNA, no shRNA trt (negative). After 24 hours cells were infected with lentiviral shRNAs as described. Puromycin selection was administered for 4 and 6 days. MTS analysis was analyzed after 2 and 3 hours incubation with and without plate shaking on device. All results were recorded and analyzed.

III. Results

a. Chapter 1: Novel prognostic markers for glioblastoma

Identification of novel prognostic markers in GBM

In search of finding a method to test if genes that control multiple pathways are functionally vital for glioblastoma survival we employed the use of an RNAi screen of the human kinome to discover genes essential for GBM survival.

RNAi screen revealed kinases essential for U87MG cell survival

We hypothesized that knock down of kinases would reveal essential genes for survival in GBM. Kinases can involve and affect multiple signaling pathways in cells and also inhibitors have been shown to be designed proficiently which makes them ideal targets for cancer therapeutics. We employed an RNAi screen targeting the human kinome and infected U87MG GBM cell line. U87MG cells were infected with 4,518 shRNAs (781 kinase genes). The population of U87 MG cells was transduced with lentiviruses packaged with the 4,518 shRNAs and separated into two groups. Lentiviruses are known to integrate into the genome of cells, which allow stable transfections of these shRNAs as well as the ability to monitor which genes are present in the population. One group was sequenced immediately (p0) while the next group was sequenced after 7 days of culturing. (p7). After collecting cells populations, genomic DNA was isolated and

shRNAs libraries were PCR amplified. Both sets of DNA were deep sequenced using Solexa sequencing and analyzed for depletions in p7 compared to p0 (decrease in

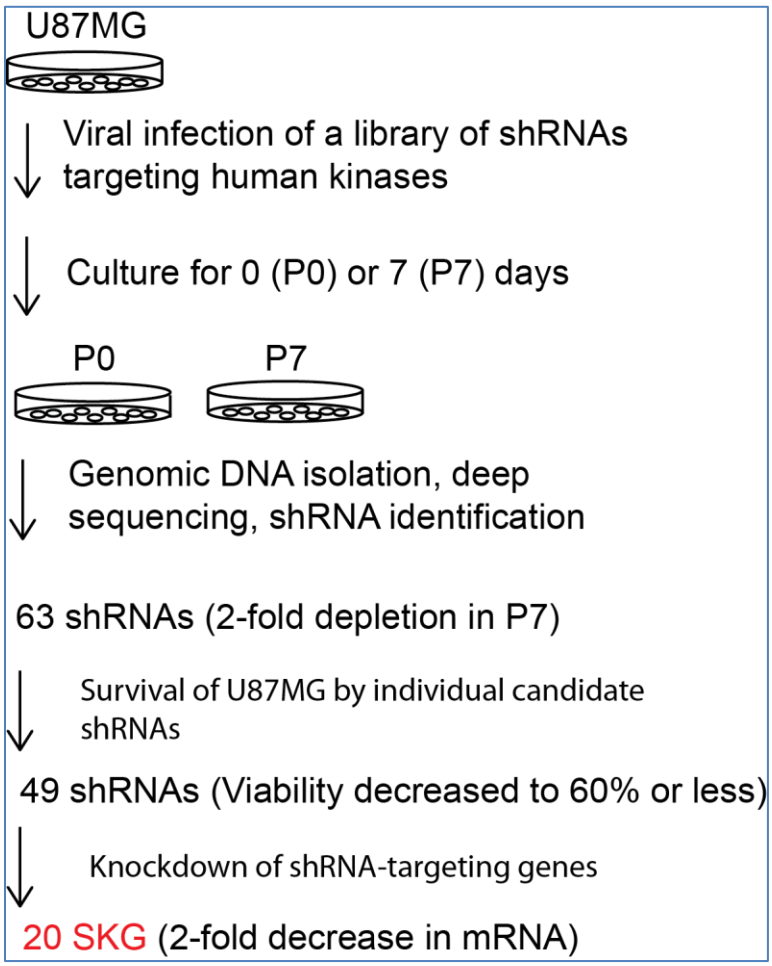


Figure 1: Schematic diagram of RNAi loss-of-function kinase screen in U87MG GBM cells.

sequencing read in p7). In theory all of the shRNAs would be represented in p0 because they were all integrated into the genome of these cells and the peak activity of shRNA transcription would not have occurred. After 7 days of culturing the shRNAs would have had the ability to knock down (KD) the effects of the targeted kinases. If essential genes were "dropped out" (killed) from the population on the 7th day then that gene would be

considered essential for survival. From the sequencing analysis we found that only 63 shRNAs showed a 2-fold depletion from p0 to p7. Each of the 63shRNAs was individually tested with a control (NS, non-silencing plasmid) on U87MG cells utilizing a cell viability assay. Only 49 of these shRNAs were validated with a decrease in cell viability of >60% compared to NS. To confirm the cell death resulted from the knockdown of the gene of interest, qRT-PCR was conducted. 23 shRNAs of these shRNAs caused a 2 fold depletion of mRNA compared to the NS confirming knockdown. On that list of kinases we noticed 2 shRNAs targeted MELK (maternal embryonic leucine zipper kinase) and 3 shRNAs targeting CSNK1ε (Casein Kinase 1 Epsilon). MELK has been previously cited in the literature as affecting cell viability in glioblastoma which can be used as positive control for our RNAi screen. **From our RNAi screen we have discovered a list of 20 genes (Table 1) that are essential for glioblastoma survival which we termed as survival kinase genes (SKGs)**.

Gene Symbol	Gene full name
CDCP1	CUB Domain Containing Protein 1
CDK11B	Cyclin-Dependent Kinase 11B
CDKL5	Cyclin-Dependent Kinase-Like 5
CSNK1E	Casein Kinase 1, Epsilon
IGF2R	Insulin-Like Growth Factor 2 Receptor
IQCD	IQ Motif Containing D
IRAK3	Interleukin-1 Receptor-Associated Kinase 3
LATS2	Large Tumor Suppressor Kinase 2
MAP4K3	Mitogen-Activated Protein Kinase Kinase Kinase Kinase 3
MELK	Maternal Embryonic Leucine Zipper Kinase
NEK9	NIMA-Related Kinase 9
PFKP	Phosphofructokinase, Platelet
PIK3CB	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase, Catalytic Subunit Beta
PRKAA1	Protein Kinase, AMP-Activated, Alpha 1 Catalytic Subunit
PRPSAP1	Phosphoribosyl Pyrophosphate Synthetase-Associated Protein 1
ROR2	Receptor Tyrosine Kinase-Like Orphan Receptor 2
STK3	Serine/Threonine Kinase 3
TBRG4	Transforming Growth Factor Beta Regulator 4
ULK4	Unc-51 Like Kinase 4
VRK1	Vaccinia Related Kinase 1

Table 1: Survival kinase genes (SKGs) discovered from RNA interference screen of human kinome.

SKGs are enriched in glioblastoma compared to normal cells

We hypothesized that the knockdown of SKGs affecting cell viability was the results of higher gene expression of these genes in glioblastoma cells. If SKGs were more highly expressed in GBM tissue compared to non-diseased tissue than this expression would reflect the importance of this gene in GBM survival. Also if gene expression is higher in GBM tissue, knock down or inhibition of these genes could be used as a therapeutic to deplete GBM cell viability [74]. To test this hypothesis we turned to online tools and gene expression

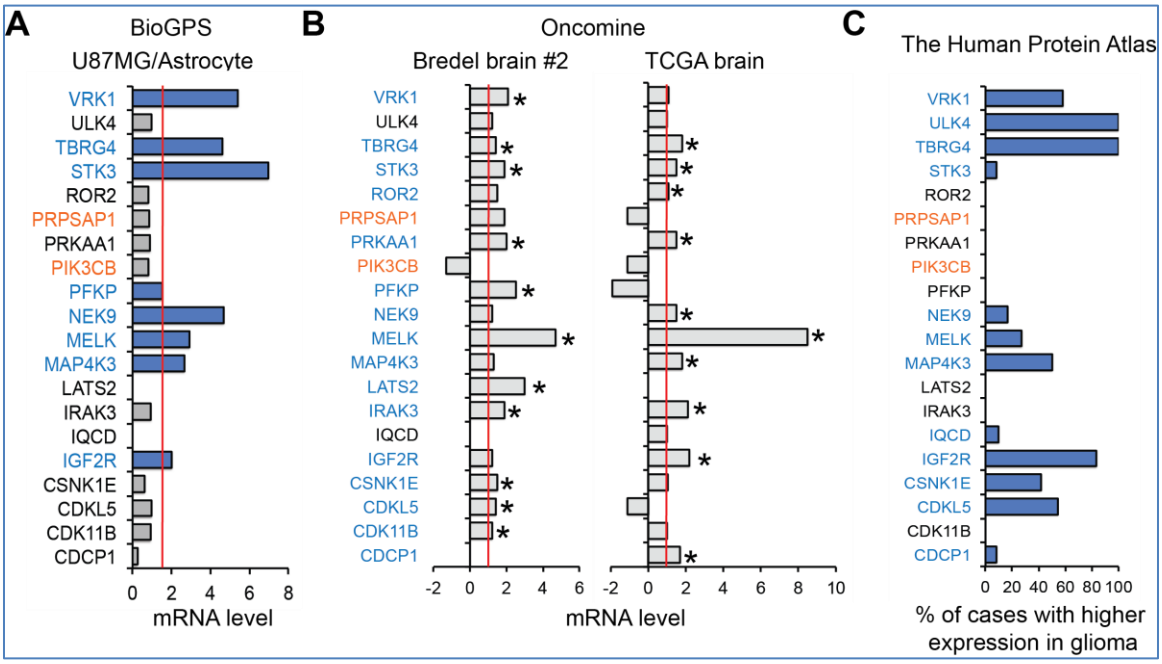


Figure 2: **SKG gene expression profiles. (**A) BioGPS: SKG mRNA expression levels in U87MG cells compared to normal astrocytes. Blue bars indicate genes that are at minimum 1.5 fold higher expression. (B) Oncomine: mRNA levels of 2 human GBM datasets (Bredel Brain #2 and TCGA). Genes that have a significantly higher expression in GBM cells compared to normal tissue is represented with an asterisk (*) (C) The Human Protein Atlas: Protein levels of glioma tissue compared to normal tissue. Blue bars indicate SKGs with a minimum 8 % of cases with higher expression in glioma tissue.

databases. We searched BioGPS, Oncomine (Thermo Fisher Scientific) for mRNA expression and The Human Protein Atlas for protein expression in normal and diseased brain (Figure 2). Using BioGPS, we found that 8 of our SKGs (IGF2R, MAP4K3, MELK, NEK9, PFKP, STK3, TBRG4, and VRK1) had a 1.5 fold higher expression in U87MG cells than in normal astrocytes. Next we looked at two different human datasets in Oncomine which was the Bredel brain #2 and TCGA brain dataset. In these two datasets we found that all but 3 SKGs (IQCD, PIK3CB, and PRPSAP1) showed a statistically significant ($P < 0.5$) higher mRNA expression in glioblastoma diseased tissue compared to normal brain tissue. We next looked at protein expression in The Human

Protein Atlas database which has analyzed cases (human sample sets) of normal tissue protein expression compared to glioma tissue expression. It was shown that 12 SKGs were more highly expressed in glioma of 8% or more of the cases investigated compared to normal tissue. **With the data we obtained through online sources we conclude from mRNA and protein expression levels that the SKGs are enriched in glioblastoma**.

Literature search confirms novelty of SKGs in GBM prognosis

We hypothesized that the SKGs from our RNAi screen (Figure 1) and computational analysis (Figure 2) would be novel prognostic markers in GBM because of the lack of prognostic markers in GBM and the interdisciplinary technique we used. Upon a thorough investigation of the literature we found that our SKGs had prognostic capability in different cancers but not in GBM. **We established that our SKGs as prognostic markers in GBM would be of novel finding (Table 2)**.

Gene Symbol	Effect of SKGs on prognosis	Cancer type (Reference*)
CDCP1	High level → poor prognosis.	Ovarian cancer (101); Breast cancer (102); Colorectal cancer (103)
	Low level → poor prognosis.	Esophageal cancer (104)
CDK11B	No reports	
CDKL5	No reports	
CSNK1E	Low level → poor prognosis.	Colerectal cancer (105); Oral cancer (106)
IGF2R	Low level → poor prognosis.	Lung cancer (107); Hepatocellular carcinoma (107); Head and neck cancer (108)
IQCD	No reports	
IRAK3	Low level → poor prognosis.	Hepatocellular carcinooma (109)
LATS2	High level → poor prognosis.	Nasopharyngeal carcinoma (110)
MAP4K3	No reports	
MELK	High level → poor prognosis.	Lung cancer (111); Breast cancer (112, 113); Prostate cancer (114)
NEK9	No reports	
PFKP	No reports	
PIK3CB	High level → poor prognosis.	Rectal carcinoma (115); Colorectal cancer (116); Diffuse large B cell lymphoma (117); Breast cancer (118)
PRKAA1	Low level → poor prognosis.	Colorectal cancer (119); Melanoma (120); Non-Hodgkin lymphoma (121); Ovarian cance (122)
PRPSAP1	No reports	
ROR2	High level → poor prognosis.	Cervical cancer (123); Colorectal cancer (124); Breast cancer (125); Gastrointestinal stromal tumor (126); Osteosarcoma (127)
STK3	No reports	
TBRG4	No reports	
ULK4	No reports	
VRK1	High level → poor prognosis.	Breast cancer (128)

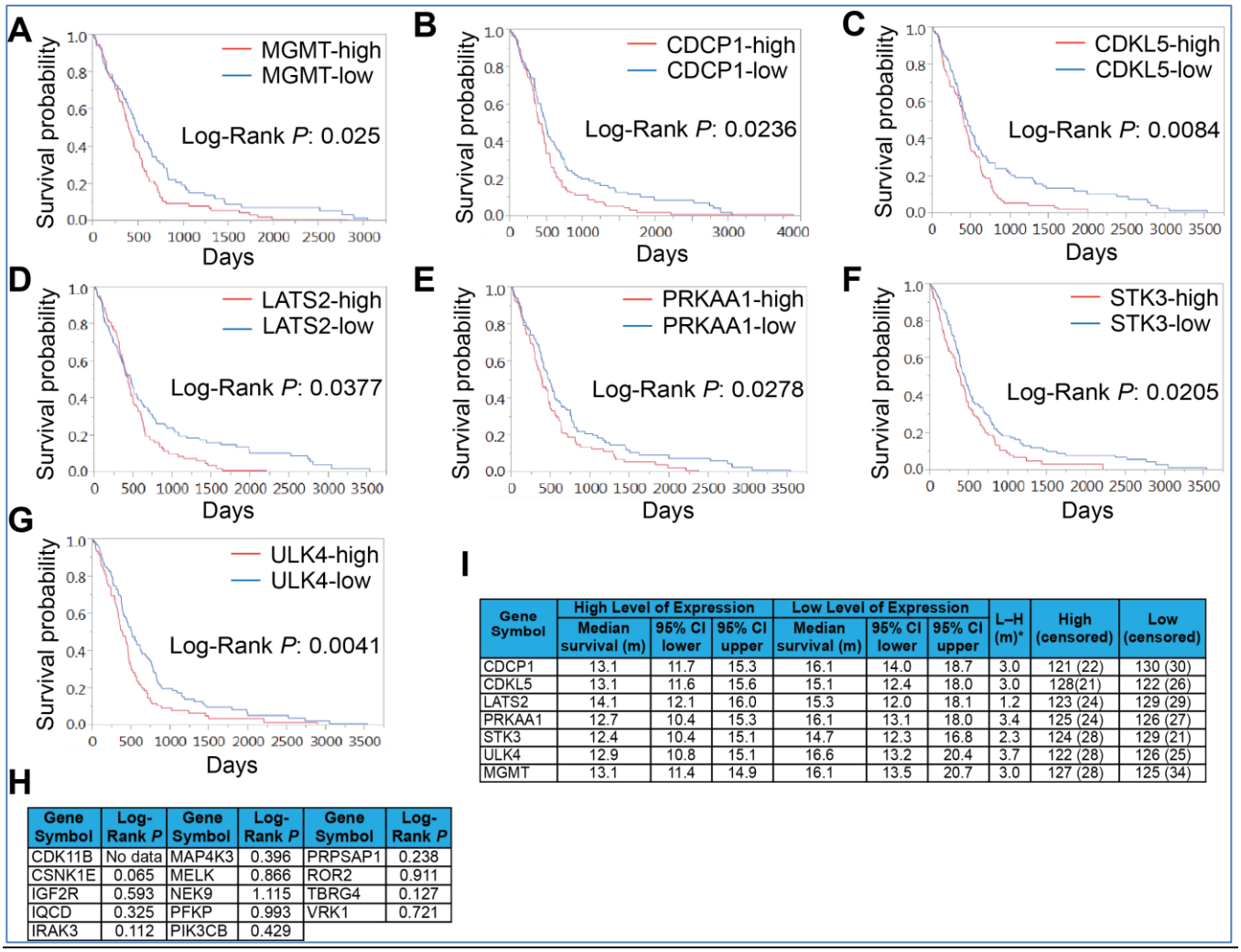
Table 2: **Prognostic capability of SKGs in various cancers. SKGs** discovered in our analyses have been found to be prognostic markers in various types of cancers but not in GBM

Kaplan Meier analyses of SKGs reveal novel prognostic markers in GBM

We postulated that our previous findings from the RNAi screen and computational analysis revealed a list of 20 SKGs which are prognostic markers for GBM. These SKGs may be used as biomarkers which suggest patient prognosis and can be used to aid in therapy as well as clinical regimen. To investigate the prognostic value of these markers

we tested the correlation of gene expression and glioblastoma patient survival [69]. We retrieved the gene expression data of GBM patients and their clinical information from TCGA (http://cancergenome.nih.gov/). The MGMT mRNA level (used as a positive control) showed an inverse correlation with the overall survival (OS) of GBM in agreement with the literature. We then analyzed 20 SKGs using the Kaplan Meier (KM) survival analysis and found that the expression of CDCP1, CDKL5, LATS2, PRKAA1, STK3, and ULK4 was inversely associated with overall survival of GBM. The analysis for these genes showed statistical significance and their Log-Rank *P* values were less than 0.05 or 0.01 (Figure 3). One gene did not have gene expression data (CDK11B) and the other genes in our survival kinase gene list did not show statistical significance in analysis ($p < .05$). When investigating months of survival we found that patients considered low gene expression groups (low level SKGs) lived from 1.2 to 3.7 months longer. **Hereafter, the six SKGs (CDCP1, CDKL5, LATS2, PRKAA1, STK3, and ULK4) will be considered prognostic markers for glioblastoma**.

SKGs that were found to be prognostic using Kaplan Meier estimator



H

Gene Symbol	Log-Rank P	Gene Symbol	Log-Rank P	Gene Symbol	Log-Rank P
CDK11B	No data	MAP4K3	0.396	PRPSAP1	0.238
CSNK1E	0.065	MELK	0.866	ROR2	0.911
IGF2R	0.593	NEK9	1.115	TBRG4	0.127
IQCD	0.325	PFKP	0.993	VRK1	0.721
IRAK3	0.112	PIK3CB	0.429		

I

Gene Symbol	High Level of Expression			Low Level of Expression			L–H (m)*	High (censored)	Low (censored)
	Median survival (m)	95% CI lower	95% CI upper	Median survival (m)	95% CI lower	95% CI upper			
CDCP1	13.1	11.7	15.3	16.1	14.0	18.7	3.0	121 (22)	130 (30)
CDKL5	13.1	11.6	15.6	15.1	12.4	18.0	3.0	128(21)	122 (26)
LATS2	14.1	12.1	16.0	15.3	12.0	18.1	1.2	123 (24)	129 (29)
PRKAA1	12.7	10.4	15.3	16.1	13.1	18.0	3.4	125 (24)	126 (27)
STK3	12.4	10.4	15.1	14.7	12.3	16.8	2.3	124 (28)	129 (21)
ULK4	12.9	10.8	15.1	16.6	13.2	20.4	3.7	122 (28)	126 (25)
MGMT	13.1	11.4	14.9	16.1	13.5	20.7	3.0	127 (28)	125 (34)

Figure 3: Expression of 6 SKGs correlates with GBM prognosis. The Kaplan Meier estimator was performed using the TCGA GBM datasets. The survival curves for positive control (A) MGMT and significant candidates (B) CDCP1 (C) CDKL5 (D) LATS2 (E) PRKAA1 (F) STK3 (G) ULK4 and (H) table of Log Rank P values of non-significant candidates, (I) The sample size, confidence intervals and median intervals are shown, The difference between the two expression groups in terms of survival is represented with an asterisk (*). Kaplan Meier curve red lines indicate the higher gene expression patients while the blue line represents the lower gene expression groups.

SKGs that were **NOT** found to be prognostic using Kaplan Meier estimator

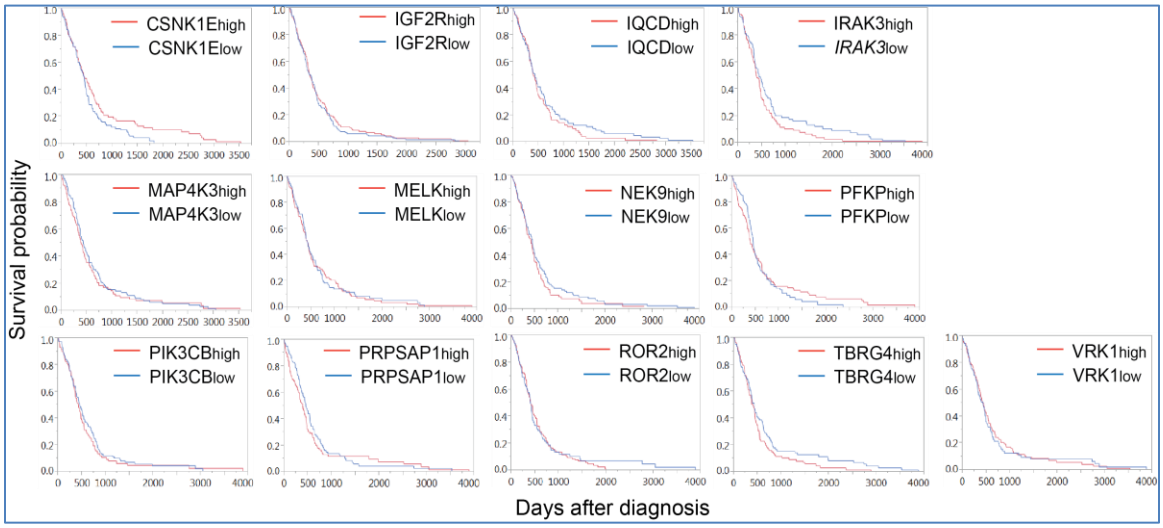


Figure 4: Kaplan Meier survival analysis of SKGs *without* statistical significance. The survival analysis results of 13 SKGs which were not found to have a statistically significant difference between expression groups.

Cox proportional hazards analyses of SKGs with and without covariates reveal novel prognostic markers for GBM

Following our Kaplan Meier analysis we utilized Cox proportional hazard model to test univariate as well as multivariate cofactors contributing to or affecting survival. We proposed that Cox proportional hazards would reveal similar results and TMZ treatment or age as covariates would increase our biomarkers list. TMZ treatment is a standard of care in GBM and should be considered when examining patient survival and age is a common covariate tested in prognostic analysis [14, 75]. The univariate analysis using gene expression as the only variable in the model we found that the same survival kinase genes discovered in Kaplan Meier analyses were significant and found no change in either negative or positive results (Table 3). When utilizing the multivariate cox proportional hazards test of the survival kinase genes we discovered that TMZ treatment

or age could be used as a covariate with SKGs to better measure GBM prognosis. TMZ and age both have been associated with GBM clinical outcomes and TMZ is a standard of care for GBM treatment. The hazard ratios (HRs) in our list of prognostic SKGs were equivalent to or higher than that of MGMT, whereas non-prognostic SKGs had a lower HRs verifying the results of KM survival analysis and inferring that higher expression of these genes may be associated with a higher probability of death at any time point. When using multivariate cox proportional hazards and using TMZ but not age as a covariate we found that the HRs of all prognostic SKGs except for CSNK1ε increased. Interestingly, IRAK3 and TBRG4 were also shown to be significant indicating to be

Gene Symbol	Cox Univariate				Cox Multivariate with TMZ				Cox Multivariate with Age			
	HR (H vs L)	95% CI lower	95% CI upper	Log-Rank *P*	HR (H vs L)	95% CI lower	95% CI upper	Log-Rank *P*	HR (H vs L)	95% CI lower	95% CI upper	Log-Rank *P*
CDCP1	1.382	1.042	1.832	***0.025***	1.384	1.040	1.842	***0.0257***	1.332	1.006	1.764	***0.0451***
CDK11B	No Data											
CDKL5	1.467	1.102	1.957	***0.009***	1.441	1.074	1.935	***0.0146***	1.368	1.027	1.827	***0.0322***
CSNK1E	0.762	0.569	1.017	0.065	0.684	0.507	0.921	***0.0123***	0.954	0.708	1.282	0.755
IGF2R	0.927	0.702	1.224	0.594	0.804	0.598	1.081	0.150	1.025	0.774	1.356	0.865
IQCD	1.152	0.868	1.527	0.326	1.226	0.918	1.637	0.167	1.077	0.810	1.432	0.607
IRAK3	1.255	0.947	1.661	0.113	1.416	1.056	1.898	***0.0202***	1.050	0.790	1.396	0.735
LATS2	1.356	1.016	1.810	***0.039***	1.411	1.048	1.903	***0.0232***	1.057	0.790	1.417	0.710
MAP4K3	1.127	0.854	1.483	0.397	1.274	0.960	1.686	0.093	1.042	0.789	1.373	0.772
MELK	1.024	0.775	1.354	0.866	1.125	0.843	1.503	0.425	0.989	0.748	1.309	0.941
NEK9	1.166	0.875	1.552	0.293	1.344	0.991	1.821	0.057	1.056	0.792	1.407	0.708
PFKP	0.999	0.749	1.333	0.993	0.854	0.633	1.150	0.298	1.191	0.887	1.600	0.245
PIK3CB	1.119	0.845	1.478	0.431	1.160	0.869	1.543	0.313	0.918	0.690	1.219	0.555
PRKAA1	1.372	1.034	1.822	***0.029***	1.499	1.120	2.006	***0.0064***	1.227	0.923	1.633	0.159
PRPSAP1	1.185	0.893	1.573	0.239	1.229	0.916	1.648	0.169	1.174	0.884	1.558	0.266
ROR2	1.016	0.766	1.353	0.911	1.078	0.805	1.449	0.616	1.138	0.854	1.522	0.379
STK3	1.393	1.050	1.848	***0.022***	1.558	1.166	2.078	***0.0027***	1.096	0.817	1.471	0.540
TBRG4	1.245	0.939	1.650	0.128	1.399	1.042	1.880	***0.0255***	1.179	0.889	1.565	0.254
ULK4	1.518	1.139	2.023	***0.005***	1.550	1.153	2.080	***0.0037***	1.244	0.926	1.671	0.147
VRK1	0.950	0.717	1.260	0.722	1.050	0.785	1.407	0.743	0.976	0.737	1.295	0.864
MGMT	1.390	1.040	1.858	***0.026***	1.406	1.045	1.893	***0.0241***	1.369	1.023	1.833	***0.0344***

Table 3: Survival analysis of SKGs using the Cox proportional hazards model. Data was retrieved from the TCGA GBM project. H: High expression of SKG and L: Low expression of SKG. HR: Hazard ratio. CI: Confidence interval. *($p<0.0$) and **($p<0.01$). HR (H vs. L) denotes the hazard ratio of the high gene expression group compared to the low level group. Highlighting: Red indicates statistical significance. Blue indicates statistical significance when TMZ was added as a covariate. Yellow indicates statistical significance in terms of high gene expression group correlates with longer survival.

possible predictive biomarker of TMZ treatment in glioblastoma patients. Both IRAK3 and TBRG4 gene expression showed a statistical significant inverse correlation of $P<0.05$ when factored with TMZ. When age and gene expression are covariates in this model we find that only CDCP1 and CDKL5 showed statistical significance. Therefore these results suggest that gene expression of SKGs and temozolomide provide a better prognostic correlation than with age and SKGs. **Analyzing SKGs using cox proportional hazards with and without TMZ, we found 9 (CDKL5, LATS2, IRAK3, TBRG4, CSNK1ε, CDCP1, PRKAA1, ULK4, STK3) prognostic markers for GBM**.

False discovery rate strengthen finding of prognostic markers

We hypothesized that false discovery rate will strengthen the findings of our prognostic candidates. False discovery rate (FDR) is a method to lower the rate of type I errors in statistical hypothesis testing when conducting multiple comparisons [76]. Type I errors are defined as the incorrect rejection of the null hypothesis which are also called false positives. FDR is often used in multiple testing of samples in genomics studies e.g. differential expression analysis. **Using an adjusted P value for FDR of 0.10 we found congruent results to our previous multivariate analysis, 9 genes (CDKL5, LATS2, IRAK3, TBRG4, CSNK1ε, CDCP1, PRKAA1, ULK4, and STK3) which can be used as GBM prognostic markers (Table 4).**

Survival Kinases	Univariate Cox-Continous		Kaplan Meier Univariate		Univariate Cox Proportional Hazard		Multivariate Cox Proportional Hazard (+TMZ)	
Gene Symbol	PValue	FDR Adj PValue	PValue	FDR Adj PValue	PValue	FDR Adj PValue	PValue	FDR Adj PValue
ULK4	0.0065	0.0627	0.0041	0.0738	0.0045	0.0817	0.0037	0.03515
LATS2	0.0066	0.0627	0.0377	0.1131	0.0385	0.12192	0.0232	0.05426
CDKL5	0.013	0.08233	0.0084	0.0756	0.0086	0.0817	0.0146	0.05426
CSNK1E	0.0527	0.21318	0.0652	0.16766	0.0654	0.17751	0.0123	0.05426
CDCP1	0.0561	0.21318	0.0236	0.10008	0.0246	0.10868	0.0257	0.05426
IRAK3	0.08	0.25333	0.1115	0.25088	0.1132	0.26885	0.0202	0.05426
PIK3CB	0.1406	0.33749	0.4294	0.59455	0.4313	0.58534	0.3128	0.37145
STK3	0.1421	0.33749	0.0205	0.10008	0.0216	0.10868	0.0027	0.03515
PRKAA1	0.2243	0.47352	0.0278	0.10008	0.0286	0.10868	0.0064	0.04053
TBRG4	0.3047	0.56335	0.1268	0.2536	0.1279	0.27001	0.0255	0.05426
PRPSAP1	0.3404	0.56335	0.2375	0.4275	0.239	0.4541	0.1688	0.22909
VRK1	0.3558	0.56335	0.721	0.8652	0.7215	0.85678	0.7426	0.7426
IQCD	0.4293	0.62744	0.3249	0.53165	0.3263	0.51664	0.1674	0.22909
NEK9	0.5109	0.69336	NA	NA	0.2933	0.50661	0.0569	0.10811
IGF2R	0.7268	0.92061	0.5932	0.76269	0.5938	0.75215	0.1496	0.22909
MAP4K3	0.8149	0.96769	0.3955	0.59325	0.3973	0.58067	0.0934	0.16133
PFKP	0.908	0.98642	0.9932	0.9932	0.9932	0.9932	0.2984	0.37145
MELK	0.9345	0.98642	0.8657	0.96438	0.8658	0.96151	0.4247	0.47466
ROR2	0.9957	0.9957	0.9108	0.96438	0.9109	0.96151	0.6156	0.6498

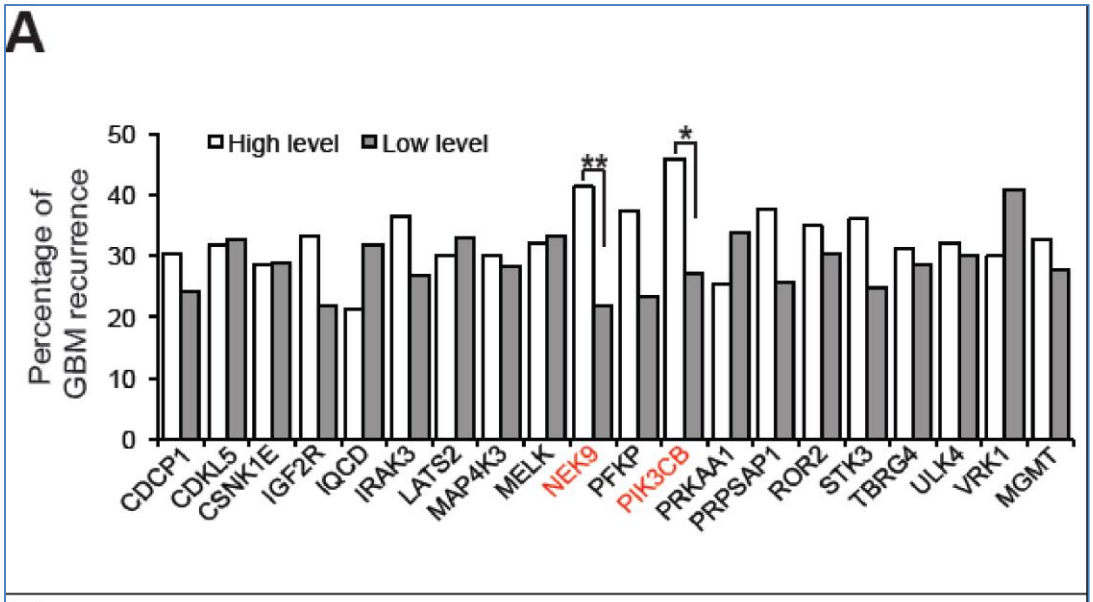
Table 4: False Discovery Rate Analyses. False discovery analyses performed by JMP software indicate congruent results (Table 2) when using TMZ as a covariate and adjusted FDR p values. Red highlighting indicates FDR p value below 0.05 and green highlighting indicates p value below 0.10.

Recurrence rate and prognosis of recurrent

GBM of SKGs reveals prognostic markers for tumor recurrence

We hypothesized that TCGA data and survival analysis of SKGs could reveal novel prognostic markers in GBM recurrence. Roughly 90% of glioblastoma patients will present with a recurrent tumor in two years after surgery and treatment [48]. These recurrent tumors have been shown to be resistant to radiation and chemotherapy. Because of the toll that neurosurgery has on a patient's health, they may not be physically able to

undergo another neurosurgery or intensive treatment [48]. These complications leave glioblastoma recurrent patients without many viable options. The discovery of new prognostic markers for tumor recurrence could aid in early detection, diagnosis, and therapeutics for recurrent patients which can increase overall survival. To discover new prognostic markers for glioblastoma we investigated the role of SKGs in tumor recurrence. This was done by reanalyzing and parsing the TCGA clinical data for disease progression status of patients. This narrowed down our sample size (17 to 33) because of the limited information regarding tumor recurrence. We compiled the new data set consisting of tumor recurrence and disease progression and analyzed the recurrent rate of patients with high gene expression level (top 25%) or low gene expression levels (bottom 25%). Surprisingly the higher gene expression levels of 14 SKGs were associated with a greater incidence of recurrence (Table 5A). **Out of 14 SKGs associated with a greater incidence of recurrence, NEK9 ($P<0.01$) and PIK3CB ($P<0.05$) presented a statistically significant difference**.



B

Gene Symbol	KM Log-Rank *P*	Median Survival (m)						
		High level	95% CI lower	95% CI upper	Low level	95% CI lower	95% CI upper	L-H
CDCP1	0.0004**	15.4	11.7	18.3	24.8	16.4	58.7	9.4
CDKL5	0.168	14.3	9.4	20.6	20.6	15.0	27.8	6.3
CSNK1E	0.414	16.5	9.1	21.9	15.1	10.8	17.9	-1.4
IGF2R	0.048*	14.9	11.7	16.9	24.2	17.9	31.1	9.4
IQCD	0.823	16.9	10.8	29.2	18.5	10.3	27.2	1.5
IRAK3	0.005**	15.4	10.8	19.3	20.6	16.5	30.2	5.2
LATS2	0.015*	17.7	12.2	20.6	29.2	16.5	53.1	11.6
MAP4K3	0.817	17.8	10.3	23.1	17.6	12.2	23.5	-0.2
MELK	0.275	14.9	10.3	20.6	19.3	15.1	23.5	4.4
NEK9	0.126	17.8	12.2	19.7	18.3	9.6	58.7	0.6
PFKP	0.963	16.4	12.2	23.0	19.3	15.1	26.8	2.9
PIK3CB	0.011**	16.9	11.7	21.9	23.5	18.3	31.1	6.6
PRKAA1	0.116	15.4	9.6	26.3	21.9	16.4	27.2	6.5
PRPSAP1	0.891	15.1	10.8	26.8	18.3	15.4	20.6	3.2
ROR2	0.363	17.7	12.2	22.3	21.9	12.2	27.2	4.2
STK3	0.413	17.9	12.2	26.8	18.5	15.1	29.2	0.6
TBRG4	0.072	17.8	11.7	21.9	20.6	15.0	29.0	2.8
ULK4	0.024*	15.4	9.3	19.3	23.5	16.4	29.2	8.1
VRK1	0.049*	18.3	12.7	26.8	15.1	11.0	17.9	-3.2
MGMT	0.131	17.7	9.4	22.7	23.0	16.5	33.2	5.4

Figure 5: Expression of SKGs correlates with the incidence rate and prognosis of recurrent GBM. (A) Recurrence rate: GBM recurrence rates in patients with high level (white bars) or low level of SKGs (grey bars) were shown. The statistical difference between the two groups was determined by the Fishers exact test. Statistically significant SKGs were highlighted in red. (B) Kaplan Meier survival analysis. The relationships of SKGs with recurrent tumors were analyzed. The KM Log-rank P values were shown. The Log-rank P values less than 0.05 were highlighted in grey. VRK1 (highlighted in blue) differed from other SKGs in terms of prognosis do to the fact that higher expression indicates longer survival. 95% confidence intervals (CI) and median survival times of high or low level groups was listed. *P < 0.05; **P < 0.01.

The significance of these two genes highlights the possibility of NEK9 and PIK3CB as a possible diagnostic marker for glioblastoma recurrence (Table 5A). Next we used Kaplan Meier survival estimator to examine the association of SKG expression and survival in patients with tumor recurrence (Table 5B).. **The survival analysis of SKGs in patients with recurrence**

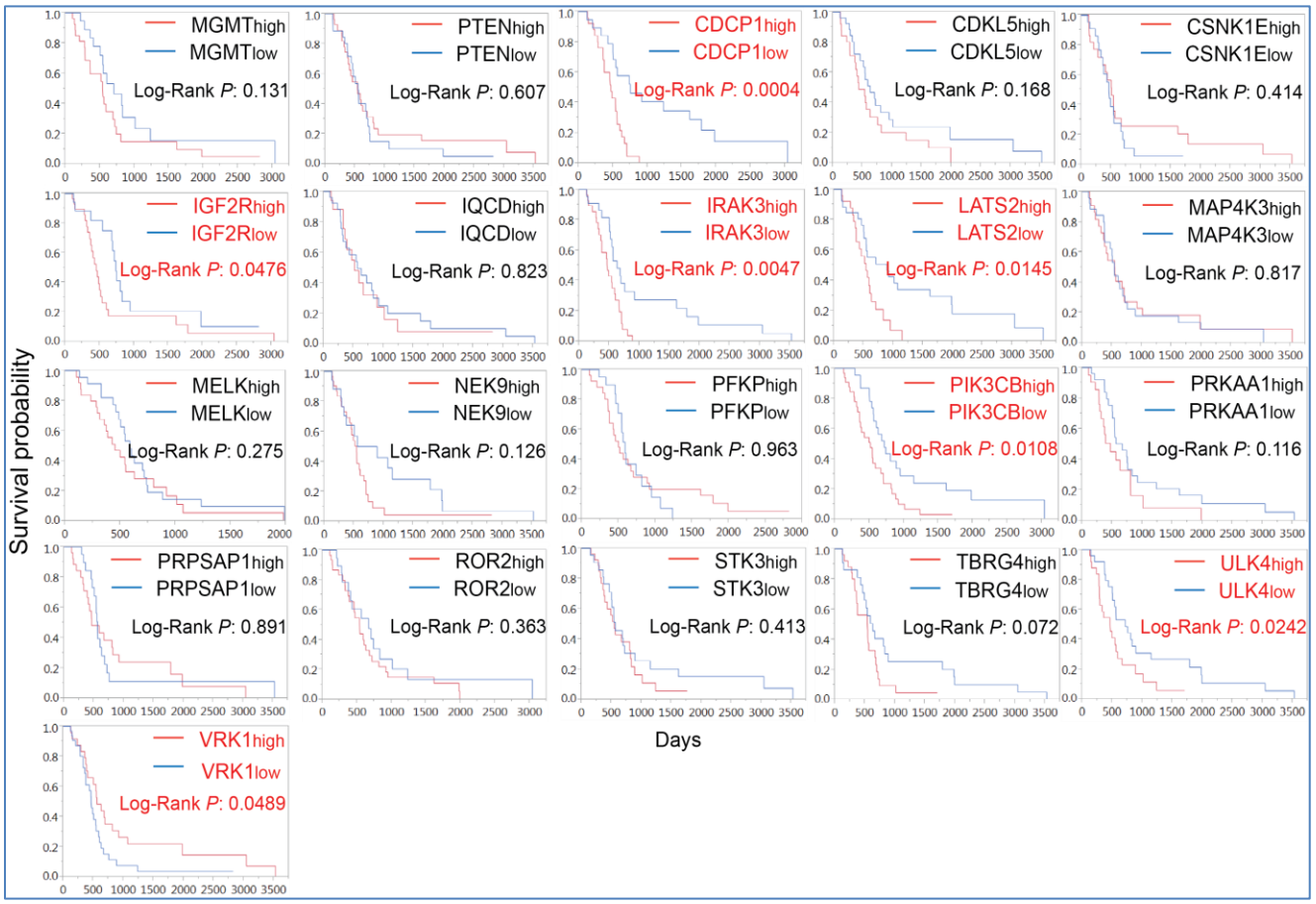


Figure 6: Kaplan Meier analysis of SKGs in recurrent GBM. The survival analysis results in patients with GBM recurrence. SKGs which were statistically significant were highlighted in red.

revealed that expression of CDCP1, IGF2R, IRAK3, LATS2, PIK3CB, ULK4 and VRK1 were statistically significant (Log-Rank: P<0.05). These Log-Rank p values suggest that expression of these survival kinase genes in newly diagnosed GBM has a

statistically significant correlation with the patient associated with recurrence. Interestingly our previous analysis discovered CDCP1, IRAK3, LATS2, and ULK4 as well, to also be prognostic SKG for newly diagnosed glioblastoma. **In patients who experience recurrent tumors, in the prognostic markers discovered (CDCP1, IGF2R, IRAK3, LATS2, PIK3CB, ULK4 and VRK1), the difference in survival ranged from 5.2 to 11.6 months.** This difference greatly contrasted significantly from the difference in survival of prognostic SKGs (previous analysis of glioblastoma patients) which had a maximum of 3.7 month survival. The drastic differences in survival demonstrate the critical role of SKGs in glioblastoma recurrence. Low expression of CDCP1, IGF2R, IRAK3, LATS2, PIK3CB, and ULK4 were all associated with longer survival but low expression of VRK1 was associated with a shorter life span. For comparison we also measured the expression of glioblastoma known prognostic markers PTEN and MGMT and found that these genes failed to show a correlation with recurrence rate or survival. **All information considered, we have discovered new biomarkers for the diagnosis and prognosis of glioblastoma patients with tumor recurrence.**

<u>PIK3CB was found as a diagnostic and prognostic marker for GBM recurrence.</u>

We hypothesized that PIK3CB was not only an indicator of recurrence and survival but also associated with accelerated rate of GBM recurrence. Our previous analysis examined the role of SKGs in GBM recurrence (Figure 5). We looked at how these genes

affect the rate of recurrence and also how they correlate with survival. PIK3CB was the only SKGs that was statistically significant and showed a strong correlation with both recurrence rate and survival in recurrent patients. We next were interested to examine the influence of PIK3CB expression on the length of days to recurrence. Using the TCGA database we found that PIK3CB expression negatively correlated with days to recurrent tumor (Figure 7). **Using Kaplan Meier estimator we found that high PIK3CB expression was significantly associated with accelerated recurrence rate**.

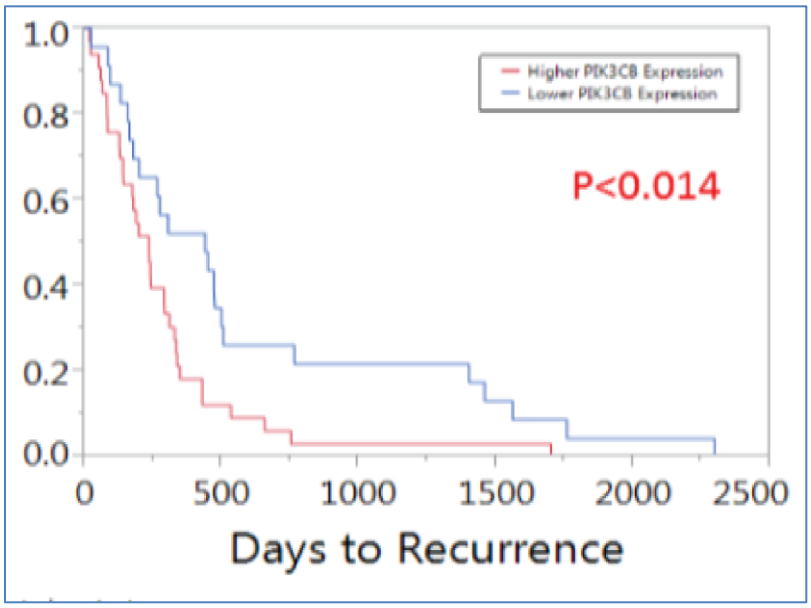


Figure 7: PIK3CB was shown to be a predictive and prognostic indicator for GBM recurrence. PIK3CB was shown to have prognostic significance (Figure 5B) as well as indicate likelihood of recurrence (Figure 5A). We found that PIK3CB expression has also been shown to predict days to recurrence. Kaplan Meier estimator revealed PIK3CB higher expression group (red line) had a significantly shorter time span in days to GBM recurrence compared to lower PIK3CB patients (blue line).

PIK3CB expression is higher in recurrent tissue than primary tissue

We posited that PIK3CB is vital to GBM recurrence and has more influence on GBM recurrence than PIK3CA. PIK3CA is a commonly mutated gene in GBM and is associated with many cancers. To determine the role of PIK3CB opposed to PIK3CA in recurrence we employed the use of TCGA data using the Gliovis software. Using the Gliovis software we find that PIK3CA has very little mRNA expression variance between primary, secondary and recurrent tissue expression (Figure 8). **PIK3C**B **mean expression is higher in recurrent tumors when compared to primary and secondary GBM tumors**.

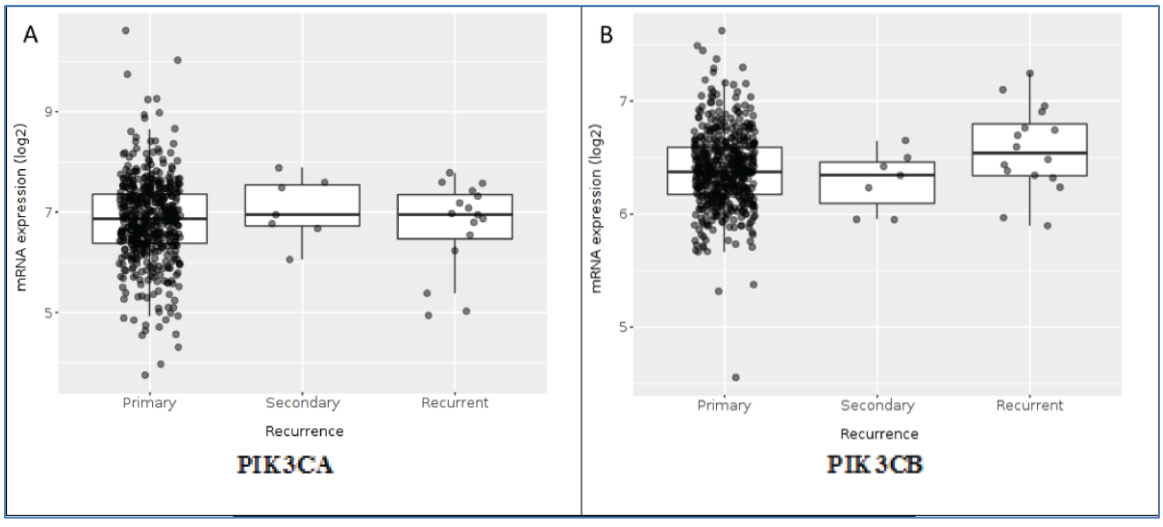
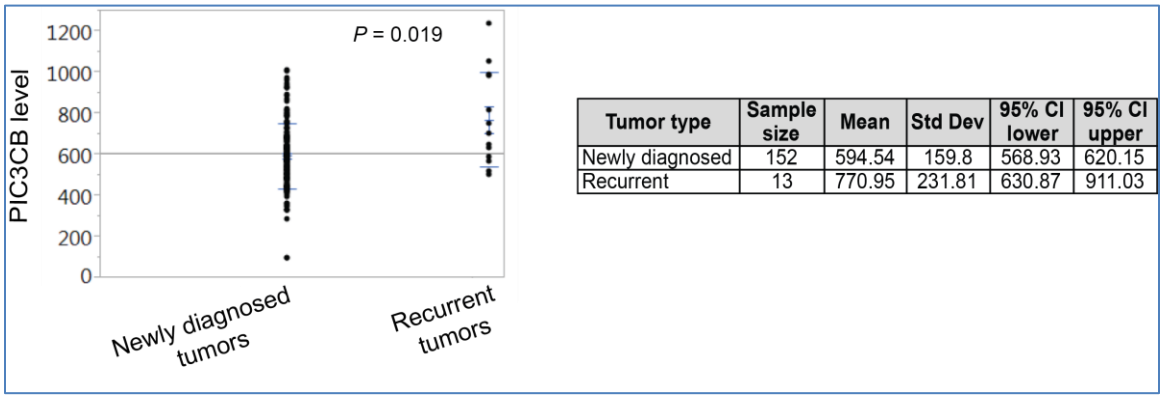


Figure 8: The expression of PIK3CA and PIK3CB in recurrent and primary tissue. We employed the Gliovis software to determine the role of PIK3CB in GBM recurrence. (A) PIK3CA mRNA expression reveals no significant difference between primary, secondary, and recurrent tissue. (B) PIK3CB mRNA expression is higher in recurrent tissue compared to primary and secondary tissue.



Tumor type	Sample size	Mean	Std Dev	95% CI lower	95% CI upper
Newly diagnosed	152	594.54	159.8	568.93	620.15
Recurrent	13	770.95	231.81	630.87	911.03

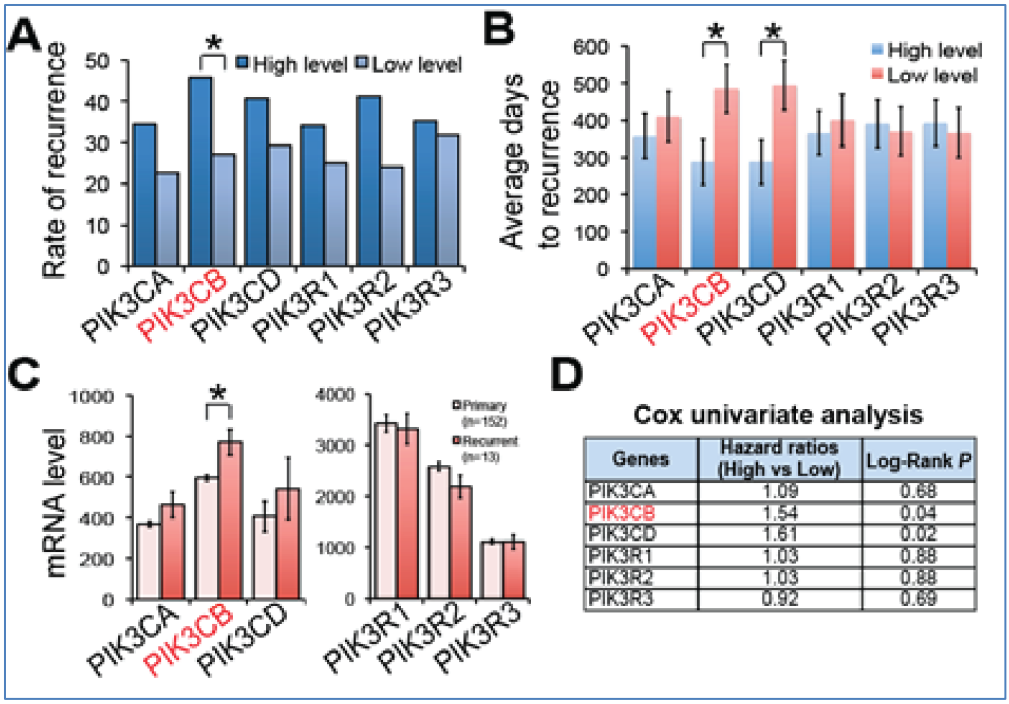
Figure 9: PIK3CB mRNA expression in newly diagnosed tissue presented significantly lower expression compared to recurrent GBM tissue. Graphical representation of TCGA GBM RNA-Seq data was shown with significant Log-rank P value of 0.019. Statistical summary calculated using JMP software. Std Dev: standard deviation; CI: confidence interval.

Next we used the TCGA GBM database to retrieve RNA-seq expression data to compare the gene expression of PIK3CB in recurrent tissue opposed to GBM newly diagnosed primary tissue (Figure 9). **We found that recurrent tissue PIK3C**B **gene expression was significantly higher (P = 0.019) compared to newly diagnosed GBM tissue.** Taken together we find PIK3CB to be more vital in GBM recurrent tumorigenesis.

PI3K/AKT pathway is implicated in GBM recurrence

PIK3CB is a catalytic subunit of the PI3K/AKT pathway [77]. The PI3K/AKT pathway has been implicated in many cancers as well as in GBM recurrence [78, 79]. We proposed that the PI3K pathway is implicated in GBM tumor recurrence and that certain kinases are more vital than others. The PI3K pathway has been targeted recently in GBM recurrence. The phosphatidylinositol 3 Kinase (PIK3) family is a group of lipid kinases that regulate multiple signaling pathways. This includes cell proliferation, movement, growth and metabolism which are why dysfunctions in the family are linked to tumorigenesis. The PIK3 family is divided into 3 classes, which are based on the structural homology and specificity of substrates. Class I is the best studied of all of the classes, little is known of class II. We concentrated on class I, which are also subdivided into 2 subclasses 1A and 1B. Class 1A contains heterodimers of p110 (alpha, beta, delta) catalytic subunit and a p85- type (5 isoforms) regulatory units. Class 1B are encoded by catalytic PIK3CG (gamma) and regulatory isoform p101 or p87. P110 alpha and p110 beta are ubiquitously expressed but p110 delta and p110 gamma are usually limited to leukocytes. The PIK3 pathway mechanistically functions by an activation signal. Without activation the regulatory unit binds to the catalytic subunits and inactivates the function of the enzyme. When a signal activates either receptor tyrosine kinase (RTK) or G protein coupled receptor (GPCR) then this heterodimeric molecule is recruited to the plasma membrane and the regulatory unit is removed. P110 phosphorylates PtdIns 4, 5-biphospate (PtdIns(4,5)P2)to create PtdIns (3,4,5) P3. PtdIns (3, 4, 5) P3 activates AKT is involved in many cellular processes such as growth and survival. Known signal activators

are EGF (epidermal growth factor), shh (sonic hedgehog), IGF-1 (insulin-like growth factor 1) and insulin. A popular negative regulator of this pathway is PTEN (phosphatase and tensin homolog) which is a phosphatase involved in dephosphorylating PtdIns (3, 4, 5) P3 into PtdIns(4,5) P2. The involvement of PI3K in cell motility, survival and growth are strongly associated with the hallmarks of cancer. A fine balance of PIK3 pathway is of utmost importance. Deficiencies in this pathway have been linked to type II diabetes while hyper activation has been linked to cancer [77, 80]. Interestingly PIK3CA is a common mutation in glioblastoma, PIK3R1 mutation has also been reported but mutations in PIK3CB have not been recorded in the literature [67, 81, 82]. Using the TCGA database we found that PIK3CB higher expression has a significant rate of recurrence compared to other components (Figure 10A). We next found that PIK3CB and PIK3CD both were significant in terms of days to recurrence (Figure 10B). Upon investigation of primary and recurrent tumors we found that PIK3CB expressed significantly higher RNA in recurrent tumor compared to primary (Figure 10C). When analyzing survival in 99 patients with recurrent tumors PIK3CB and PIKCD both had a significant difference between high and low expression groups and higher hazard rates for higher expression (Figure 10D). **Taken together, the PI3K pathway is important in GBM recurrence and PIK3CB plays a vital role.**

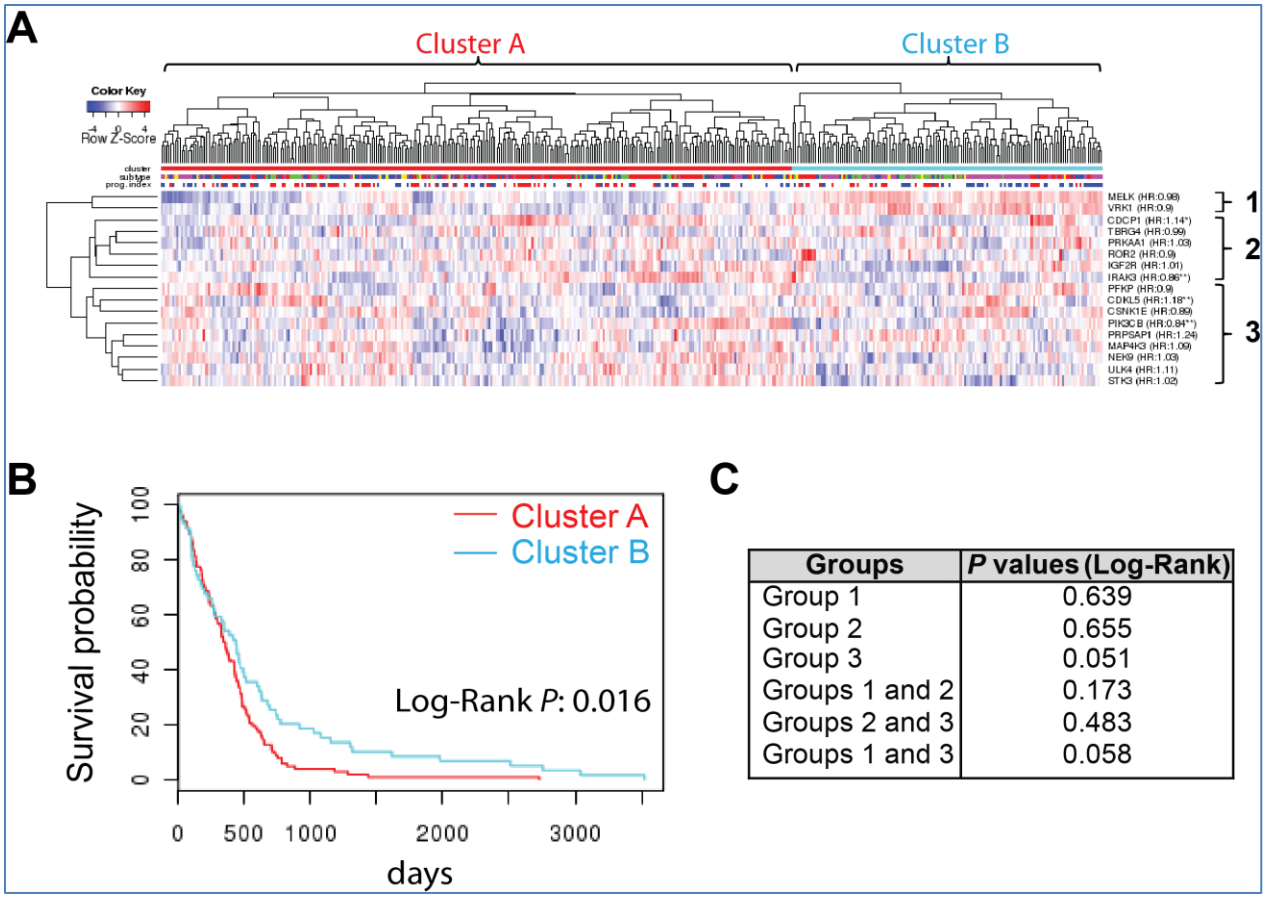


Genes	Hazard ratios (High vs Low)	Log-Rank *P*
PIK3CA	1.09	0.68
PIK3CB	1.54	0.04
PIK3CD	1.61	0.02
PIK3R1	1.03	0.88
PIK3R2	1.03	0.88
PIK3R3	0.92	0.69

Figure 10: PI3K subunits and recurrence using TGCA. (A) All subunits show an increased likelihood of recurrence with higher expression. PIK3CB indicates a statistically significant difference. Dark blue bars represent higher expression groups and light blue are low level. (B) PI3K subunits and average days to recurrence. PIK3CB and PIK3CD show a significant difference between high and low expression groups in days to recurrence. Both subunits indicate higher gene expression groups have shorter time in days for tumor recurrence. Red bar represent low level group and blue bars are high level. (C) mRNA expression levels of PI3K subunits comparing primary and recurrent tumors show us PIK3CB has a significantly higher expression in recurrent tissue. Primary tissue is represented in peach bars and recurrent tissue in red bars. (D) Cox univariate analysis revealed that PIK3CB and PIK3CD were both statistically significant with increased hazard ratios as expression increases. Hazards ratios more than 1 indicate a higher likelihood or greater chance of accelerated death at any given point.

SKGs serve as a novel prognostic gene signature in GBM

We posited that our SKG list was not only individually important in prognosis in GBM patients but also serve as a novel gene signature for GBM. Gene signatures can be very beneficial as diagnostic markers as well as prognostic markers in cancer patients [83]. To investigate the role of SKGs as a novel prognostic gene signature in GBM we used the online program Glioblastoma Bio Discovery Portal (GBM-BioDP) to examine the role of gene sets. GBM-BioDP is an online tool which uses different algorithms to cluster genes together based on gene expression retrieved from TCGA GBM datasets [84]. We selected mRNA expression of the 20 SKGs but gene expression data was not found for 3 genes (CDK11B, IQCD, and PRKAA1) allowing us to analyze 17 SKGs(Figure 11A). The software classified the gene set of the remaining 17 SKGs into 2 clusters (Cluster A and Cluster B). Cluster A gene expression when compared to Cluster B showed a statistically significant shorter life duration with a log rank P value of 0.016 (Figure 11B). The software further sub grouped the SKGs into 3 different classifications but did not show any statistical significance in gene expression and survival (Figure 11C). **Altogether, our findings strongly suggest that a group of 17 SKGs present a novel gene signature for GBM prognosis.**



Groups	*P* values (Log-Rank)
Group 1	0.639
Group 2	0.655
Group 3	0.051
Groups 1 and 2	0.173
Groups 2 and 3	0.483
Groups 1 and 3	0.058

Figure 11: SKGs represent a GBM novel prognostic signature. (A) TCGA GBM patients were clustered into two main groups based on prognostic capability. (B) Kaplan Meier (KM) survival analysis depicts significant difference in survival between clusters. (C) The Clusters were further subdivided into subgroups. Subgroups and combinations of subgroups were analyzed using KM estimator which resulted in no significant results. Analysis was done using GBM Bio Discovery Portal.

b. Chapter 2: Novel therapeutic targets for glioblastoma

Results

A kinase RNAi screen revealed CSNK1ε as a survival kinase gene in glioblastoma

From our lentiviral shRNA kinase screen (Figure 1) we discovered 23 shRNAs were essential for glioblastoma survival. These shRNAs were validated and confirmed through repeating assays and qRT-PCR knock down validation. These 23 shRNAs consisted of 20 genes which we termed survival kinase genes (SKGs). Of the 23 shRNAs, we noticed 2 shRNAs targeted MELK (maternal embryonic leucine zipper kinase) and 3 shRNAs targeted CSNK1ε (Casein Kinase 1 Epsilon). We became interested in the SKGs CSNK1ε and MELK because multiple shRNAs targeted the same genes. When investigating these SKGs we discovered that MELK has been previously cited in the literature as affecting cell viability in glioblastoma, which can be used as positive control in our future experiments [85-87]. CSNK1ε was not previously found to be involved in glioblastoma survival (Table 2). **From our previous analysis in our RNAi screen we discovered that CSNK1ε is essential for glioblastoma.**

Casein Kinase 1 Epsilon is involved in cell signaling and cancer

CSNK1ε is a serine threonine kinase and a member of the casein kinase (CK) family. The CK family consists of 7 gene isoforms α, β, γ1, γ2, γ3, δ, and ε, which all except CSNK1β are expressed in humans. The CK family is involved in apoptosis, circadian rhythms, immune response, DNA damage response, and growth. Dysfunctions in this family are linked to neurological disorders and cancer [88]. CSNK1ε is associated as a prognostic marker for different types of cancers but the correlation depends on the tissue [89-93]. The uncovering of this involvement of CSNK1ε has led to the production of many commercially available inhibitors [94-96]. CSNK1ε is also involved in β-catenin signaling which if activated is often associated with poor prognosis in many cancers [97]. **The involvement in β-catenin signaling, DNA damage response and cell growth indicates the complex influence that CSNK1ε may have in cancer which has never been studied in GBM**.

Computational tools shows us that CSNK1ε expression is higher in diseased brain opposed to normal tissue

We hypothesized that CSNK1ε expression is higher in diseased brain compared to normal tissue. Higher expression can serve as a potential target for inhibition or knockdown in cancer cells. To explore this comparison we searched Oncomine (Thermo Fisher Scientific) for CSNK1ε mRNA expression and The Human Protein Atlas for CSNK1ε protein expression in normal and diseased brain. We looked at the "Liang

Brain" dataset in Oncomine which showed a significant increase in GBM brain tissue opposed to normal brain (Figure 12A). We next looked at CSNK1ε protein expression in The Human Protein Atlas database and found that had glioma brain tissue had higher protein expression when compared to normal brain (Figure 12B). **With the data we obtained through online sources we conclude that mRNA and protein expression levels of CSNK1ε are enriched in glioblastoma.**

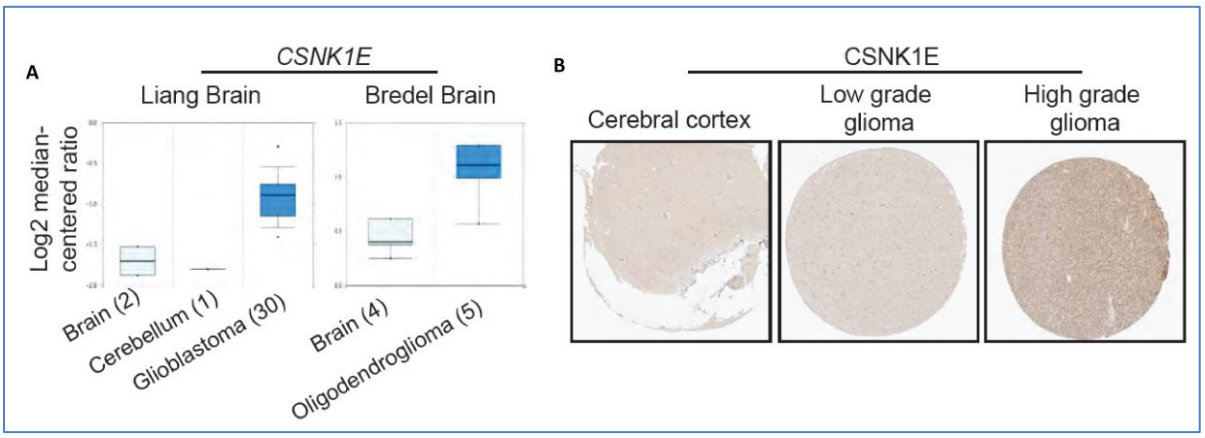


Figure 12: Expression of CSNK1ε in GBM compared to normal tissue. (A) Oncomine database revealed two separate datasets which show higher expression of CSNK1ε in GBM compared to normal brain tissue. (B) The Human Protein Atlas revealed that CSNK1ε protein expression is higher in glioma compared to normal tissue.

CSNK1ε knockdown affects GBM survival in 9 GBM cell lines and primary cells

We posited that CSNK1ε knock down has the capability of affecting cell survival in different GBM genotypes. To further analyze the role of CSNK1ε we knocked down CSNK1ε in GBM cell lines. Knock down of CSNK1ε was confirmed through western blot protein assay (Figure 13A). Lentiviral packaged CSNK1ε and non-silencing (NS) shRNAs were transduced in 9 GBM cell lines. The 9 GBM cell lines all had diverse

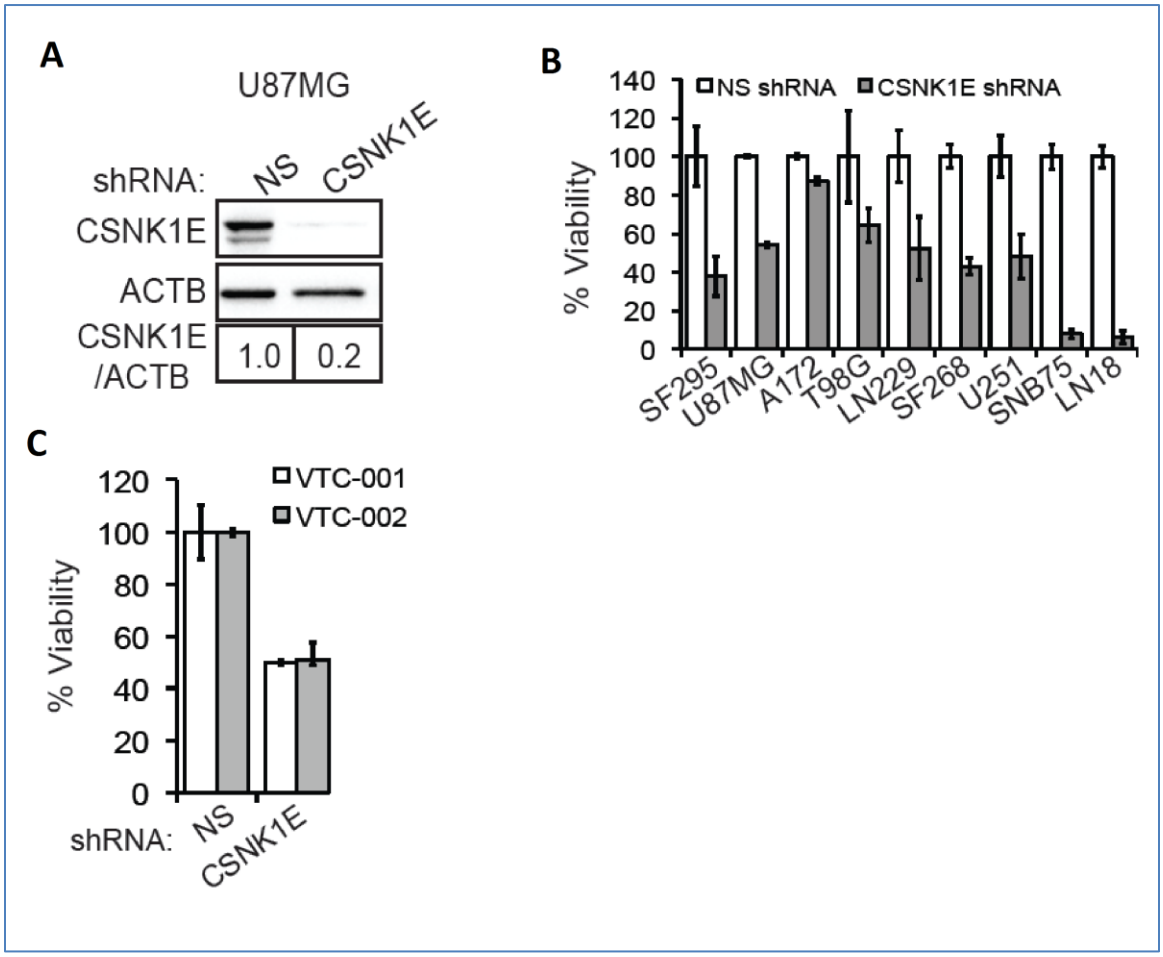
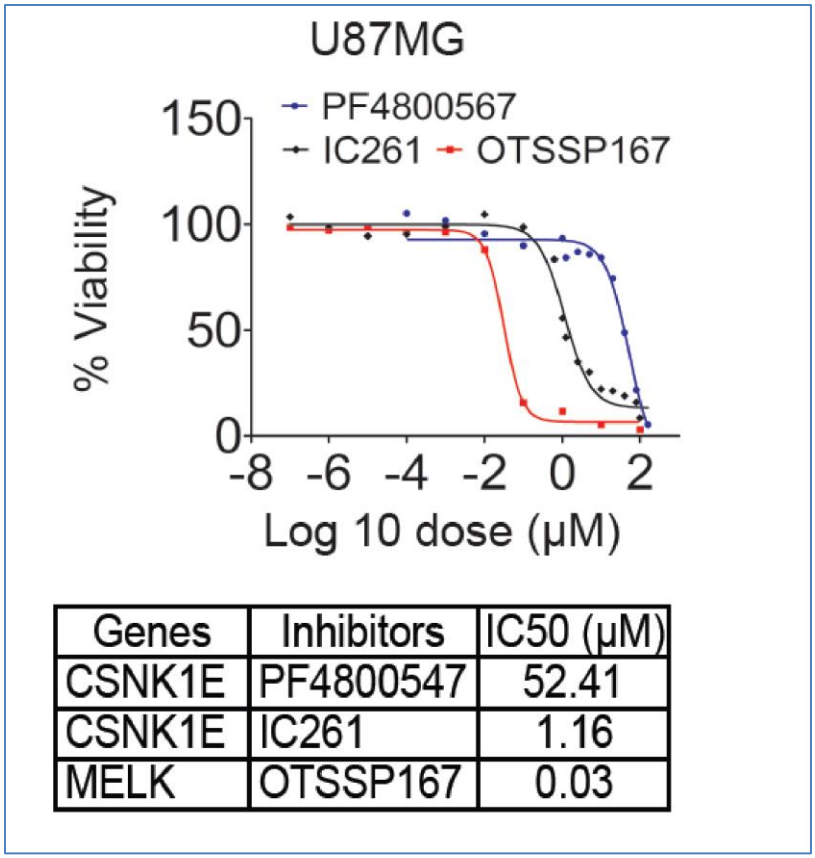


Figure 13: CSNK1e knockdown in cell lines and primary cells. (A) CSNK1ε knockdown was confirmed through the use of lentiviral packaged CSNK1ε shRNA. (B) CSNK1ε knockdown resulted in increased cell death in 9 different GBM cell lines. (C) CSNK1ε knockdown significantly reduced cell viability in primary cells.

genotypes. MTS cell viability assay was performed to reveal that all cell lines with different genotypes were affected by CSNK1ε knockdown. Eight out of the 9 GBM cell lines were significantly reduced (Figure 13B).We next compared the effect of CSNK1ε KD in two primary patients. We found that CSNK1ε KD significantly reduced survival in primary GBM patient cells (Figure 13C) compared to the control. **CSNK1ε KD was shown to be effective in different GBM genotypes in cell lines and primary patient cells**.

CSNK1ε inhibitors depress GBM survival

We postulated that CSNK1ε inhibition would have a significant effect on GBM survival. If knockdown of CSNK1ε caused cell death then we hypothesized that inhibition would cause more significant cell death. The use of commercially available inhibitors could speed up clinical trials for use in GBM and expedite the time to treat patients. Presently CSNK1ε inhibitors are commercially available that have been tested in clinical trials. To investigate the efficacy of CSNK1ε inhibition we used two CSNK1ε inhibitors (IC261 and PF8006567) which we administered to U87MG cells to determine dosage and to measure cell viability in GBM (Figure14). **It was discovered that U87MG cells were detrimentally affected by the CSNK1ε inhibition**.



Genes	Inhibitors	IC50 (μM)
CSNK1E	PF4800547	52.41
CSNK1E	IC261	1.16
MELK	OTSSP167	0.03

Figure 14: IC261 (CSNK1e inhibitor) inhibited the cell viability of U87MG cells.
IC50s of MELK (OTSSP167) and CSNK1e inhibitor (IC261, PF4800547) were extrapolated. Inhibitor indicated by color: OTSSP167 (red), IC261 (black), PF4800547 (blue).

CSNK1ε KD does not affect non cancer cell lines and non-diseased astrocytes

CSNK1ε expression is higher in diseased brain compared to normal brain tissue which lead us to hypothesize that CSNK1ε levels are more sensitive to manipulation in GBM cells compared to normal cells. A major concern of chemotherapeutic treatment is the effect on non-cancerous cells which can ultimately decrease quality

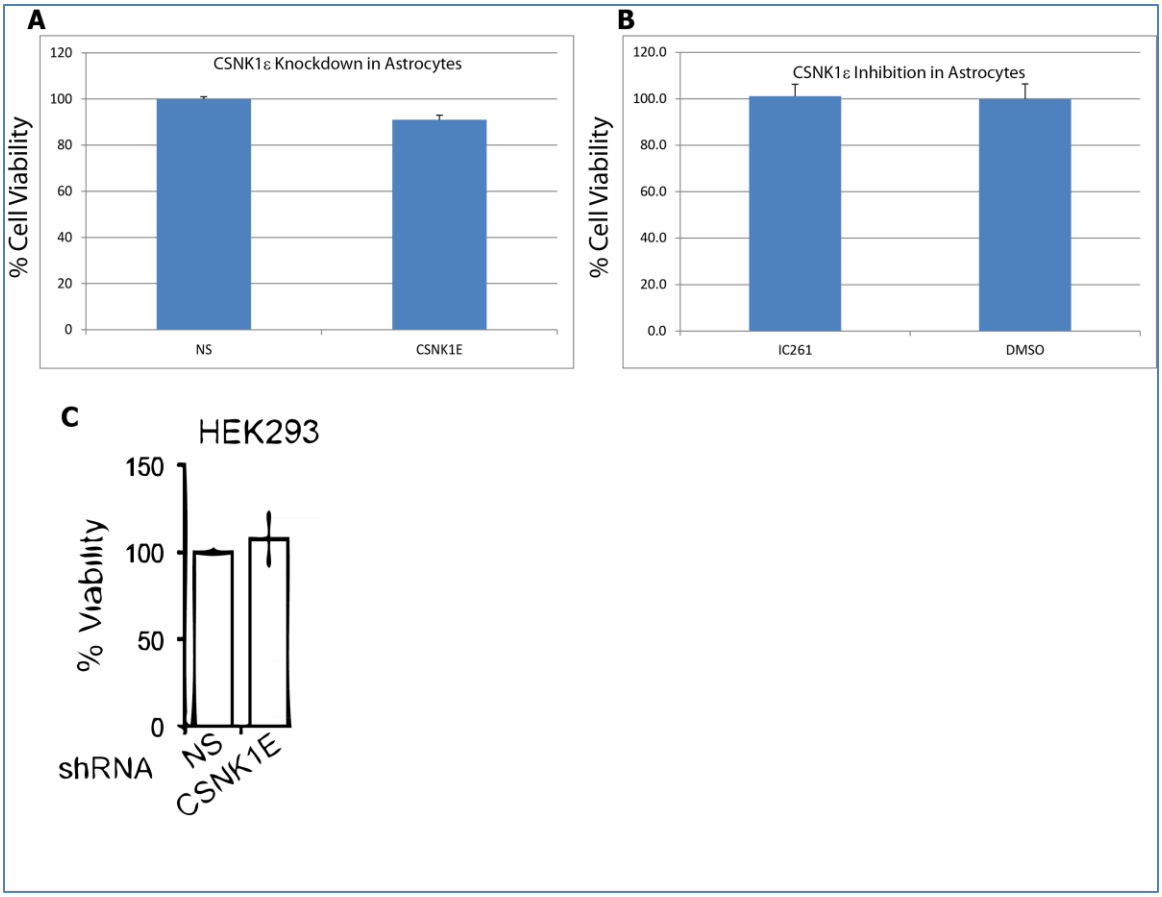


Figure 15: CSNK1e depletion does not impact non-cancerous cell line or primary astrocytes. (A) Cell viability of C57 primary astrocytes was not affected by CSNK1e knockdown. (B) Cell viability of C57 primary astrocytes was not affected by CSNK1e inhibition from IC261. (C) HEK293T cell viability was not affected by CSNK1e knockdown.

of life and overall survival [98]. To determine the effect of CSNK1ε on normal cells we knocked down a non-cancer cell line (HEK-293T) and found that they were not affected by CSNK1ε knockdown. To test the effect of CSNK1ε KD on brain cells, we isolated C57 non-diseased mouse astrocytes. CSNK1ε KD or inhibition on C57 astrocytes cells did not affect cell survival (Figure 15). **Taken together, CSNK1ε KD or inhibition does not affect normal cell survival.**

CSNK1ε KD/inhibition results in an increase in activated β-catenin with subsequent apoptotic cell death in GBM cells

We hypothesized that CSNK1ε is upstream in signaling pathway which leads to decline in GBM survival. CSNK1ε is involved in β-catenin signaling which has an established role in many cancers. To investigate the role of β-catenin in CSNK1ε KD we looked at two cell lines which presented conflicting effects when CSNK1ε KD was administrated. CSNK1ε KD of U87MG showed significant cell death while A172 presented the opposite effect Knock down of CSNK1ε was confirmed through western blot protein assay (Figure 16A). When measuring β-catenin expression, both total β-catenin and active β-catenin protein levels were measured. Active β-catenin represents the un-phosphorylated form of β-catenin. This form is associated with poor prognosis in breast and colon cancer. We found that in U87MG cells the amount of active β-catenin increased 2 fold but in A172 cells the protein levels of active β-catenin were not increased upon CSNK1ε KD (Figure 16B). We recapitulated these results in U87MG cells with the use of the CSNK1ε inhibitor IC261 (Figure 16C). Apoptosis was confirmed through cleaved caspase 3 protein immunoblotting. Apoptosis was verified for CSNK1ε KD in U87MG cells but no activity of apoptosis was reported in A172 cells (Figure 16D). **Taken together, we found that CSNK1ε KD or inhibition activates β-catenin which leads to apoptotic cell death in GBM cells.**

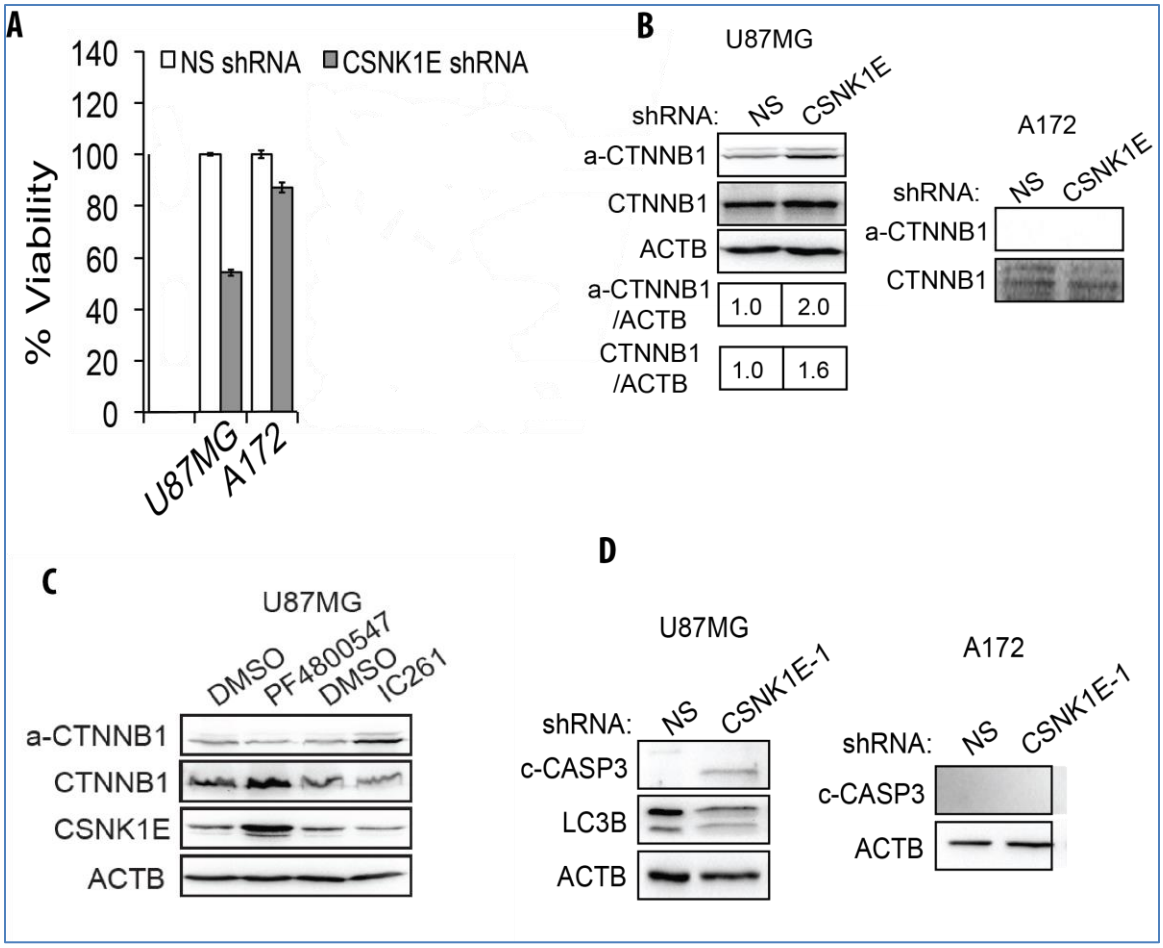


Figure 16: Immunoblotting of cell death pathways and β-catenin (CTNNB1) proteins were examined to test downstream effect of CSNK1ε knockdown (KD) and inhibition in two cell lines which differed in response to CSNK1e KD. (A) CSNK1ε KD caused U87MG cell viability to be significantly reduced but CSNK1ε KD in A172 cells did not significantly decrease cell viability. (B) CSNK1ε KD increased activated β -catenin levels in U87MG cells but no difference was found in A172 cells. (C) IC261 treatment resulted in increased activated b-catenin in U87MG cells. (D) Upon CSNK1ε KD, U87MG cells expressed high levels of cleaved Caspase 3 (apoptosis related protein) protein and a decrease in LC3B (autophagy related protein) protein levels.

CSNK1ε KD activation of CTNNB1 initiates transcription of β-catenin signaling genes

We hypothesized that CSNK1ε KD or inhibition increases active β-catenin which increases transcription of downstream β-catenin signaling genes. When CTNNB1 (β-catenin) is activated, β-catenin translocates to the nucleus and multiple genes are transcribed. To test whether active β-catenin was stimulating target gene transcription, we employed the use of a popular assay, the dual luciferase assay and the usage of pTOPFLASH/pFOPFLASH plasmids. pTOPFLASH is a plasmid that emits a luciferase signal when CTNNB1 is bound to TCF/LEF transcription factors. pFOPFLASH is a mutated version of this plasmid which acts as a negative control without the function of luciferase. When analyzing the effect of CSNK1ε KD we found that in U87MG cells had an accelerated rate of gene transcription while no activity was seen in the negative control cells (A172) (Figure 18). **Collectively, we found that CSNK1ε KD activates CTNNB1, which translocate into the nucleus to turns on transcription of β-catenin signaling genes.**

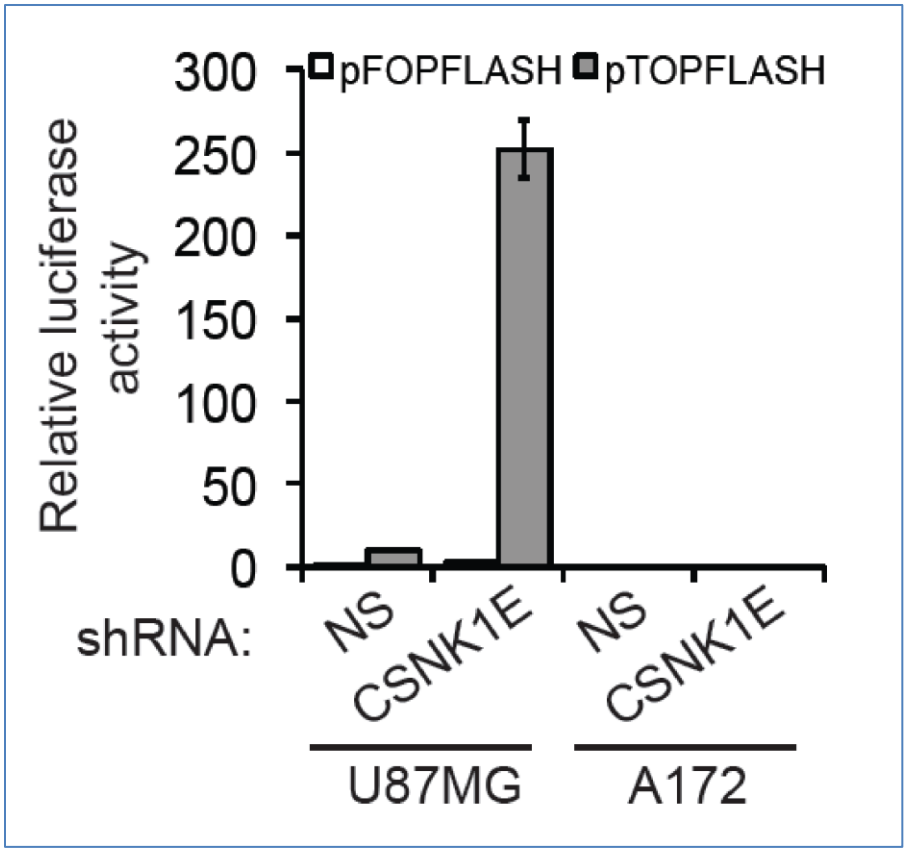


Figure 18: **CSNK1ε dependent GBM cells: CSNK1ε Knockdown (KD) activates downstream β-catenin gene transcription**. Dual luciferase assay by means of pTOPFLASH/pFOPFLASH in U87MG cells revealed significantly high transcription compared to A172 cells which revealed immeasurable low transcription. Transcription was evaluated from the binding of TCF/LEF to activated CTNNB1 which was measured using pTOP (shaded bar) luciferase activity.

CSNK1ε KD induced GBM cell death can be rescued by KD of CTNNB1

We hypothesized that CSNK1ε knock down initiates the cell death process in GBM cells which could be salvaged by CTNNB1 knockdown. If CSNK1ε knockdown activated b-catenin which was responsible for cell death then the reversal of this would validate the role that

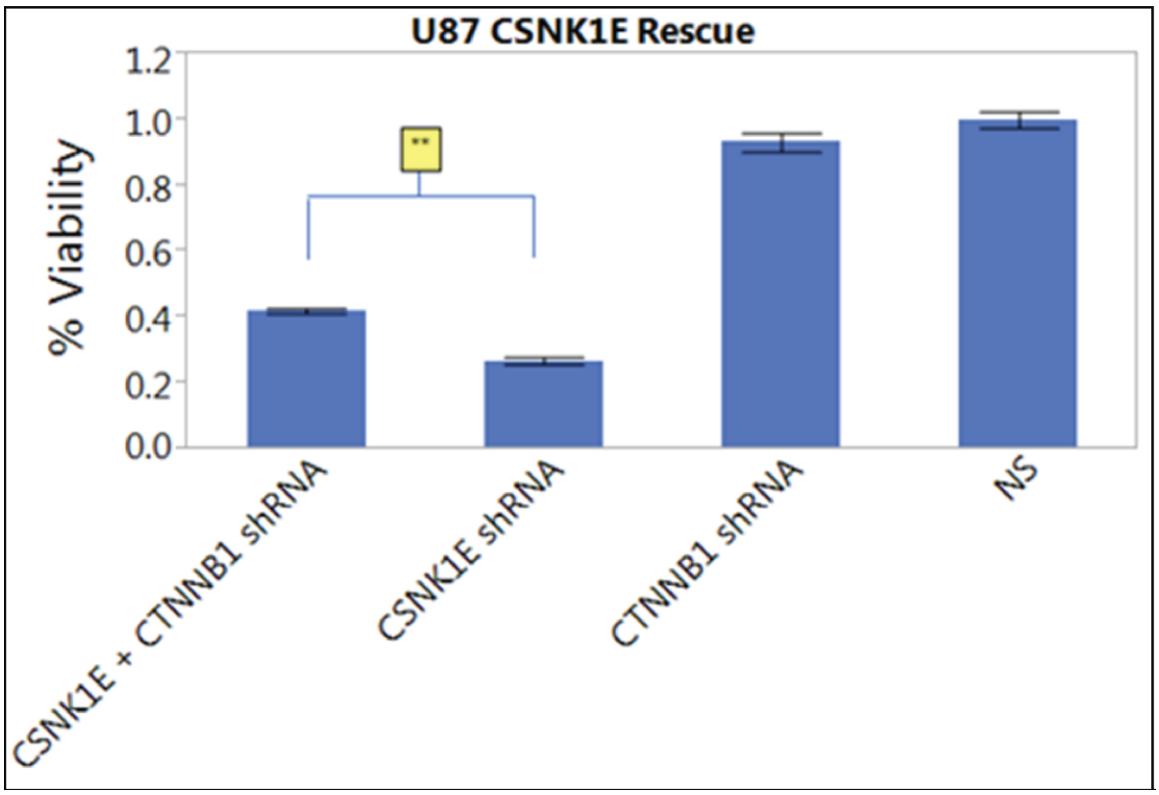


Figure 18: To test if CSNK1ε knockdown (KD) induced cell death can be rescued we employed the use of the MTS cell viability assay. We found that CSNK1ε KD caused substantial cell death in U87MG cells which can be significantly restored with the use of CTNNB1 KD. ** indicates p value < 0.001 (Student's-test).

β-catenin plays in GBM survival. To investigate the role of CTNNB1 and CSNK1ε in GBM survival we evaluated the KD of CTNNB1, CSNK1ε, NS, and CTNNB1 and

CSNK1ε shRNA in U87MG cells. We found that CSNK1ε caused significant cell death in GBM cells while CTNNB1 shRNA did not (figure 18)**. We also found that cell death from CSNK1ε KD in GBM cells can be rescued at a significant extent by CTNNB1 KD**.

CSNK1ε inhibition affects GSC survival and self-renewal

We posited that CSNK1ε inhibition would detrimentally affect GSC survival since it affects GBM primary and cell lines of various genotypes. GSC survival is very important to consider when targeting GBM because of its role in tumor recurrence, therapy resistance, and tumor heterogeneity [53, 99, 100]. To investigate the role of CSNK1ε in GSCs we measured the outcome of CSNK1ε inhibition on GSC cell viability of GBM patients and a GSC xenograft. We found that in all cases CSNK1ε inhibition significantly reduced cell survival opposed to DMSO (vehicle) treatment (Figure 19A).. We next measured the ability for GBM GSCs to self-renew. Self-renewal can allow cells to become resistance to standard therapy in GBM. We used a self-renewal assay to determine the effects of CSNK1ε inhibition on self-renewal. When comparing xenograft and patient GSCs we found that in both cases the self-renewal capability was significantly reduced (Figure 19B). We also noticed that the health (size and shape) of the cells were greatly dampened by the use of CSNK1ε inhibitor IC261 (Figure 19C). **CSNK1ε inhibition negatively affects GSC survival and self-renewal**.

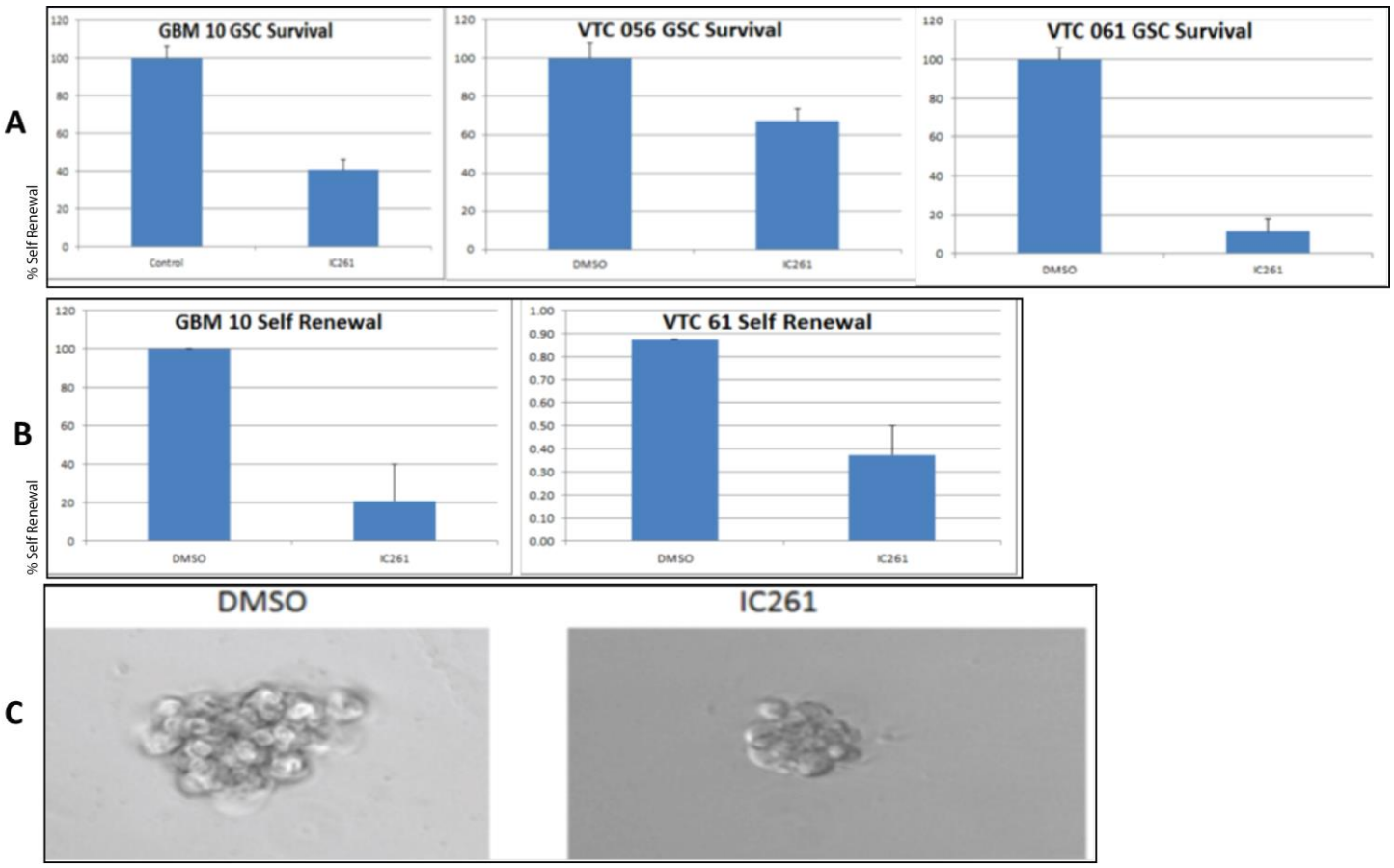


Figure 19: CSNK1ε inhibition depletes cell viability and the ability of self-renewal in glioma stem cells (GSCs). (A) To determine the effect of CSNK1ε inhibition on cell viability IC261 or DMSO (vehicle) was administered to GSCs. In all cases CSNK1ε inhibition caused a decrease in cell viability with depletion up to 89%. (B) To determine the effect of CSNK1ε inhibition on self-renewal IC261 or DMSO was administered to GSCs and neurosphere formation was measured. CSNK1ε inhibition caused a decrease in self renewal in both cases with a 79% reduction in GBM10 GSCs. (C) CSNK1ε inhibition deleteriously affected the cell morphology, size, and quantity of neurospheres which was not seen in the vehicle treatment.

IV. Discussion

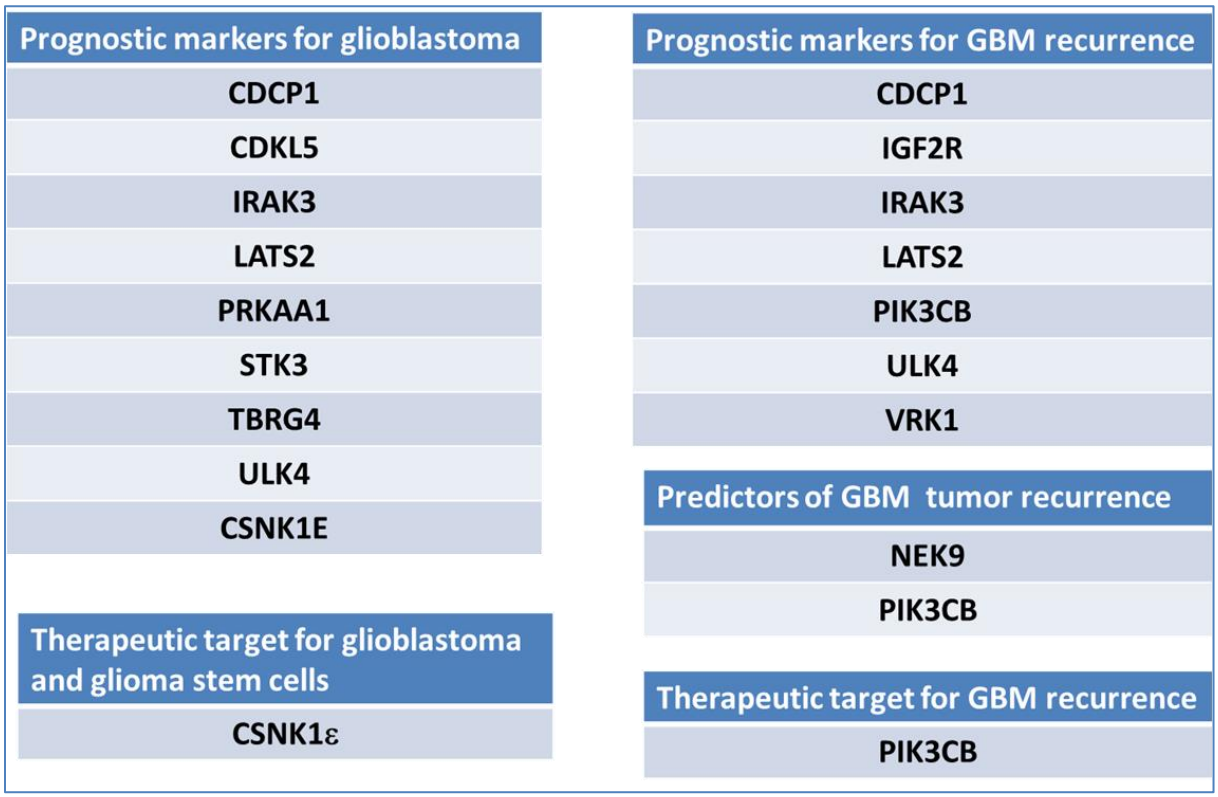


Figure 21: Summary of achieved results. Molecular methods and computational tools were used to discover novel prognostic markers and therapeutic targets for GBM.

Innovative and effective method for discovering genes essential in glioblastoma

Our study was important because we found a method to test if genes that control multiple pathways are essential for glioblastoma survival. The method used the knowledge obtained from a popular molecular technique to find novel targets for glioblastoma. The technology of sequencing and microarrays has been valuable but can often be misrepresented without experimental validation. The use of RNAi using a class of proteins that are vital for signal transduction shows a creative way to maneuver around

the obstacles in treating glioblastoma. We revealed 20 kinase genes that we termed survival kinase genes. These genes were validated using individual shRNAs and compared to a control (NS). The majority of these kinases also were shown to be overexpressed in diseased brain compared to normal brain. Next we found effective prognostic markers using the results from our GBM survival screen.

Novel prognostic and predictive biomarkers for glioblastoma and GBM recurrence

This work is of great importance because it presents biomarkers to measure prognosis not only in glioblastoma but also in tumor recurrence of GBM patients. Our finding of survival kinase genes as prognostic markers in glioblastoma is a novel finding. Each one of these prognostic markers are kinases which present as a possible therapeutics both in terms of effectiveness and drug design capability. We categorized these genes as novel prognostic markers for glioblastoma but they can possibly be used to predict therapeutic options for other cancers as well. 10 SKGs have been shown to be predictive or prognostic biomarkers for other types of cancers. In our study we found 9 prognostic markers for GBM overall survival with or without TMZ treatment (Figure 21). In the majority of our prognostic markers, high expression correlates with poor prognosis in GBM patients. This correlation indicates that targeting these SKGs by inhibition or knock down may be effective therapeutically in GBM patients. Our study also revealed 7 biomarkers for prognosis of patients with tumor recurrence. It should be noted that the CSNK1ε (GBM prognostic marker) and VRK1 (recurrence prognostic marker) expression and correlation to survival were opposite from other prognostic genes i.e.

CSNK1ε/VRK1 high expression levels associated with longer survival. This difference may indicate a downstream target of these genes responsible for survival and should be further investigated. Our discovery of 7 novel prognostic markers is an important finding because the only biomarker to date for tumor recurrence is MRI (Figure 21). MRI is a costly time consuming process which is confounded by radiation, chemotherapy and pseudoprogression. These factors can mimic tumor progression which may lead to misdiagnosis. Our study has also found two genes (NEK9 or PIK3CB) to be predictive of glioblastoma recurrence in newly diagnosed/primary GBM tumors and may be an important diagnostic tool (Figure 21). Measuring gene expression levels in tumor biopsies from the primary tumor provide an effective and less expensive method to gauge prognosis. Additional surgery will not be needed as biopsies of the primary tumor are customary for determining the disease in patients. Taken together, we have identified novel prognostic markers for glioblastoma and glioblastoma recurrent tumors. These SKGs can be used to predict response to therapies, early diagnosis of recurrence, and serve as prognostic markers for survival and recurrence. These genes can and should also be further investigated as effective therapeutic targets for this dismal disease. PIK3CB is a very intriguing prognostic marker from GBM recurrence. The PI3K pathway has been implicated in GBM and PIK3CA is a commonly mutated gene in GBM. Efforts have been made to test the PI3K pathway clinically but these efforts unfortunately showed limited advantage. One possible problem may arise from the targeting of PIK3CA or use of pan PI3K inhibitors. In our analyses we have identified PIK3CB to be a diagnostic and prognostic marker which should be specifically targeted (Figure 21). Clinical trials have been conducted which has shown that PIK3CB is less toxic to normal cells compared to

PIK3CA. Taken together, rather than targeting the PI3K pathway, PIK3CB should be specifically targeted to treat recurrent GBM tumors and PIK3CB expression should be measured after biopsy to prevent or slow down tumor recurrent GBM tissue. Excitingly, PIK3CB specific inhibitors are clinically available which could expedite trials on GBM and GBM recurrence.

Novel therapeutic target for glioblastoma and GSCs

Finally we found a CSNK1ε to be a therapeutic target for glioblastoma which is effective against primary and cancer stem cells of glioblastoma (Figure 21). These results we have revealed are of great importance because they present a novel targeted therapy for glioblastoma and glioma stem cells (GSCs). We showed that CSNK1ε KD/inhibition negatively affected GBM survival in 9 GBM cell lines of different genetic backgrounds. KD and inhibition were also effective in treating different GBM patient samples. An expansive genotypic range should be considered when targeting a very heterogeneous disease such as GBM. CSNK1ε KD and inhibition was also effective in primary and cell line derived GSCs. GSCs have many similarities to stem cells such as the ability to self-renew or differentiate as a multipotent cell. GSCs may be a major culprit in the initiation of tumor recurrence and therapeutic failure. We found that CSNK1ε KD/inhibition not only affected GSC survival but also limited self-renewal of GSCs. This finding has the capability to not only treat glioblastoma but also inhibit the formation and treat glioblastoma recurrence through the targeting of GSCs. One major flaw of modern chemotherapeutics is the fact that many of these drugs are poisonous or harmful to non-

cancer cells. We have discovered that CSNK1ε knockdown and inhibition is relatively nontoxic to different cell types. Our study also revealed the activation of CTNNB1 as being downstream of CSNK1ε KD/inhibition which is associated with survival. This mechanistic understanding may bring forth other targets for GBM as well as other cancers associated with β-catenin. Our finding of CSNK1ε involvement as an essential kinase and therapeutic target for glioblastoma and glioblastoma stem cells is a novel finding.

V. References

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