Role of Diacylglycerol kinase alpha (DGKα) as a therapeutic target in Glioblastoma (GB)

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Summary

Glioblastoma (GB) is the most common high-grade fatal brain tumor. The standard of care treatment is surgery, followed by radiotherapy and chemotherapy. Despite decades of research, the median life expectancy of patients is still between 12 to 15 months. The activation of multiple receptor tyrosine kinases (RTKs) and/or downstream tumour-intrinsic mutations provide oncogenic stimuli to GB progression and accounts for resistance to current therapies. Identifying a target that is capable of simultaneously disabling of multiple, parallel oncogenic signals can represent an effective therapy.

Mounting reports indicate DGKα relevance as a therapeutic target across multiple cancers, given its role in different aspects of tumour biology. DGKα phosphorylates diacylglycerol (DG) resulting in the production of phosphatidic acid (PA). Both DG and PA are membrane bound secondary messengers that regulate signalling molecules involved in cancer. DGKs act simultaneously as both terminators and activators of DG- and PA-mediated signalling.

In order to exploit DGKα as a therapeutic target we investigated the role of DGKα in GB biology and signalling. Our results show that DGKα is required for GB stem-like cell long term viability and stemness maintenance and sensitize tumor cells to temozolomide.

Inhibition of DGKα strongly impairs NF-κB transcriptional activity and analysis of the TNFR signalling showed that DGKα is necessary for FAK and AKT activation downstream TNFa stimulation.

Taken together, the results of this study strongly suggest that DGKα plays a key role in stemness maintenance contributing FAK, Akt and NF-kB activation upon TNF stimulation and for this reason DGKα might represent a targetable oncogene that links inflammation and tumor growth and progression.

1. Introduction

1.1 Glioblastoma (GB) incidence and clinical presentation

Primary central nervous system (CNS) tumors are a histologically diverse group of neoplasms with over one hundred distinct entities identified in the current World Health Organization (WHO) classification system (Louis DN, 2016) (**Figure 1**).

GB is a grade IV astrocytoma and is the most common and fatal type of primary brain tumor in adults (Seymour T, 2015). GB may arise de novo in more than 90% of cases or secondary to the progression of lower-grade astrocytoma's in less than 10% of cases (Kleihues P, Ohgaki H, 1999; Ohgaki H, Kleihues P, 2013). Although genetic features suggest a different development of primary and secondary GB, these two tumor types show no morphological differences (Urbańska K, 2014; **Figure 1**).

GB has an incidence of two to three per 100,000 adults per year and accounts for about 50% of all primary brain tumors and 17% of all tumors of the brain (primary and metastatic) (Thakkar JP, 2014; Ostrom QT, 2016). The median age at diagnosis is 64 years. It is uncommon in young, accounting only approximately 3% of all brain tumors reported among 0–19 years old individuals (Thakkar JP, 2014), whereas the incidence continues to rise with increasing age, peaks at 75–84 years of age and drops after 85 years (Thakkar JP, 2014; Ostrom QT, 2016).

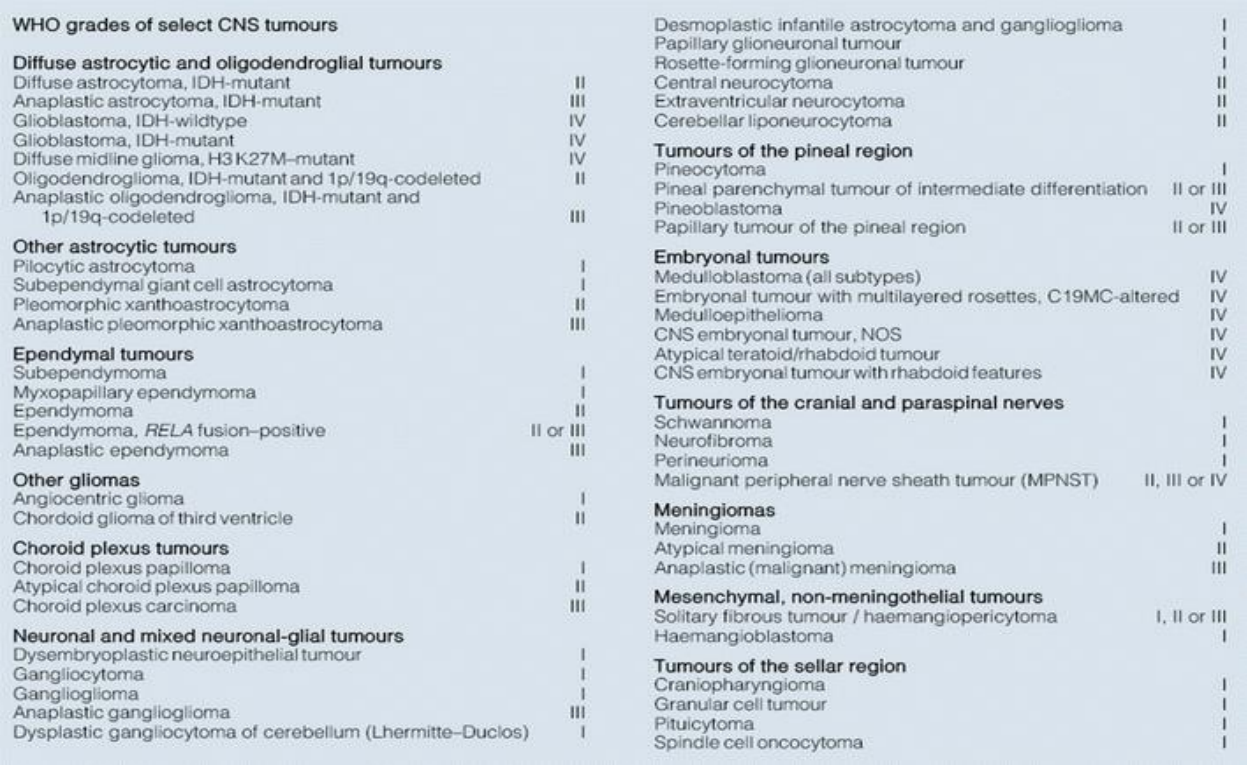
Differences in incidence and death rates based on race and ethnic groups as well as gender has been observed in GB (Thakkar JP, 2014). The incident rate (IR) of GB is 1.6 times higher in males as compared to females [3.97 versus 2.53] with a higher frequency of primary GB in men (male to female ratio, 1.33) and secondary GB in women (male to female ratio, 0.65). In the USA, whites have the highest incidence rates for GB, followed by blacks, Asian/Pacific Islanders and American Indian/Alaska Native (Thakkar JP, 2014).

The most frequent location for GB is cerebral hemispheres; with 95% of these tumors arising in supratentorial region, while only few percent of tumors occurring in cerebellum, brainstem and spinal cord (Nakada M, 2011). The clinical course of GB is determined by tumor location and dynamics of spread within the brain. Tissue destruction, edema, and epilepsy contribute to clinical symptoms, resulting in devastating neurological sequelae causing rapid deterioration in some patients (Alexander BM, Cloughesy TF, 2017).

Current clinical treatment for GB includes maximal surgical resection followed by postoperative radiotherapy and concomitant and adjuvant chemotherapy (Orringer D, 2012). Despite recent

advances in treating other solid tumors, treatment for GB remains palliative, with a very poor prognosis and a median survival rate of 12–15 months. Treatment failure is a result of several causes, including resistance to radiotherapy and chemotherapy (Seymour T, 2015; Stupp R, 2009). GB very seldom metastasizes outside the neuraxis; instead, local invasion/tumor recurrence is the leading cause of death (Lun M, 2011; Clarke J, 2010).

Recurrent tumors are frequently evolutionarily divergent from the original tumor, with distinct drivers and sensitivities, limiting the informative capacity of initial biopsies when treating recurrent disease (Kim J, 2015). Many of these features can be modeled through the lens of the cancer stem cell (CSCs) hypothesis. Functionally defined GB SCs (GSCs) have been identified in human brain tumors (Singh SK, 2004) and are considered to play a key role in contributing to tumor progression, treatment resistance, and tumor recurrence post-treatment and have become the focus of novel therapy strategies (Gimple RC, 2019; Seymour T, 2015).



WHO grades of select CNS tumours	
Diffuse astrocytic and oligodendroglial tumours	
Diffuse astrocytoma, IDH-mutant	II
Anaplastic astrocytoma, IDH-mutant	III
Glioblastoma, IDH-wildtype	IV
Glioblastoma, IDH-mutant	IV
Diffuse midline glioma, H3 K27M–mutant	IV
Oligodendroglioma, IDH-mutant and 1p/19q-codeleted	II
Anaplastic oligodendroglioma, IDH-mutant and 1p/19q-codeleted	III
Other astrocytic tumours	
Pilocytic astrocytoma	I
Subependymal giant cell astrocytoma	I
Pleomorphic xanthoastrocytoma	II
Anaplastic pleomorphic xanthoastrocytoma	III
Ependymal tumours	
Subependymoma	I
Myxopapillary ependymoma	I
Ependymoma	II
Ependymoma, *RELA* fusion–positive	II or III
Anaplastic ependymoma	III
Other gliomas	
Angiocentric glioma	I
Chordoid glioma of third ventricle	II
Choroid plexus tumours	
Choroid plexus papilloma	I
Atypical choroid plexus papilloma	II
Choroid plexus carcinoma	III
Neuronal and mixed neuronal-glial tumours	
Dysembryoplastic neuroepithelial tumour	I
Gangliocytoma	I
Ganglioglioma	I
Anaplastic ganglioglioma	III
Dysplastic gangliocytoma of cerebellum (Lhermitte–Duclos)	I
Desmoplastic infantile astrocytoma and ganglioglioma	I
Papillary glioneuronal tumour	I
Rosette-forming glioneuronal tumour	I
Central neurocytoma	II
Extraventricular neurocytoma	II
Cerebellar liponeurocytoma	II
Tumours of the pineal region	
Pineocytoma	I
Pineal parenchymal tumour of intermediate differentiation	II or III
Pineoblastoma	IV
Papillary tumour of the pineal region	II or III
Embryonal tumours	
Medulloblastoma (all subtypes)	IV
Embryonal tumour with multilayered rosettes, C19MC-altered	IV
Medulloepithelioma	IV
CNS embryonal tumour, NOS	IV
Atypical teratoid/rhabdoid tumour	IV
CNS embryonal tumour with rhabdoid features	IV
Tumours of the cranial and paraspinal nerves	
Schwannoma	I
Neurofibroma	I
Perineurioma	I
Malignant peripheral nerve sheath tumour (MPNST)	II, III or IV
Meningiomas	
Meningioma	I
Atypical meningioma	II
Anaplastic (malignant) meningioma	III
Mesenchymal, non-meningothelial tumours	
Solitary fibrous tumour / haemangiopericytoma	I, II or III
Haemangioblastoma	I
Tumours of the sellar region	
Craniopharyngioma	I
Granular cell tumour	I
Pituicytoma	I
Spindle cell oncocytoma	I

Figure 1. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary (adapted from Louis DN, 2016)

1.2 Molecular classification of GB

Before publication of the revised WHO Classification of Tumours of the CNS in 2016, gliomas were exclusively classified according to histological criteria defined in the 2007 WHO

classification (Louis DN, 2007). In addition to histological tumor typing, each tumor was assigned a histological grade based on the degree of anaplasia, from WHO grade I to IV. This WHO grading system reflects the degree of tumor malignancy and presumed natural disease course. Histological classification has for many decades served as the 'gold standard' for glioma diagnostics but is associated with considerable interobserver variability (van den Bent MJ. 2010). Moreover, several glioma types with identical histopathological classification, such as GB, encompass a spectrum of clinically and biologically distinct tumor groups associated with differences in age at onset, tumor location, and prognosis (Sottoriva A, 2013; Louis DN, 2016; Verhaak RG, 2010).

Molecular and translational studies over the past decade have revealed that the genetic factors underlying this variation could serve as clinically relevant biomarkers for more accurate classification, to improve prediction of patient outcomes, and to guide individualized treatment. For this reason, the 2016 WHO Classification of Tumours of the Central Nervous System revised glioma classification replacing traditional histology-based glioma diagnostics with an integrated histological and molecular classification system that enables more-precise tumor categorization. One of the major discoveries that markedly promoted molecular classification of gliomas was the identification of IDH1 gene mutations as a new hot spot alteration originally detected in a subset of GB from younger patients and secondary GB derived by progression from pre-existing lower-grade gliomas (Yan H, 2009; Parsons DW, 2008). The vast majority GB (>90%) are IDH wild-type and correspond to primary GB that preferentially occur in elderly patients and develop de novo, i.e., with usually short clinical history and without a pre-existing lower-grade precursor lesion. In contrast, IDH mutant GB (<10%) are typically seen in young adults and include the vast majority of secondary GB that developed by progression from a preexisting diffuse or anaplastic astrocytoma (Kleihues P, Ohgaki H. 1999; Ohgaki H, Kleihues P. 2013). The distinction of IDH wild-type and IDH mutant GB is important as these are not only biologically distinct entities but also associated with different clinical features as well as survival, with significantly longer OS seen in patients with IDH mutant GB (Sanson M, 2009; Weller M, 2009).

Progress in large-scale molecular analyses of cancer has enabled stratification of GB into distinct biologic groups beyond the mere distinction between IDH wild-type and mutant entities. Using an unsupervised hierarchical clustering analysis, Verhaak et al. (Verhaak RG, 2010) discovered four subtypes based on gene expression profiles, termed proneural, neural, mesenchymal, and classical. The clinical utility of stratifying patients according to these expression signatures is limited, however, as they can be heterogeneous within a given tumor and can change in response to external

stimuli, including therapy (Bhat KPL, 2013; Patel AP, 2014; Fedele, 2019). Nevertheless, the mesenchymal expression signature has been linked with radioresistance and unfavorable survival (Bhat KPL, 2013), whereas the proneural signature has been associated with longer survival outcomes compared to other subtypes and with a benefit from antiangiogenic treatment (Sandmann T, 2015).

In contrast to gene expression profiling, molecular classification based on DNA methylation and mutations appears to be more robust as it likely reflects the cell of origin and remains stable over tumor evolution. Large-scale genomic studies have revealed many mutations in tumor suppressor genes and oncogenes and significantly improved our understanding of the GB genomic landscape (Cancer Genome Atlas Research Network, 2008; Brennan CW, 2013; Aldape K, 2015; Reifenberger G, 2016). IDH-wild-type GB in adults are characterized by frequent gain of chromosome 7 (EGFR/MET/CDK6), monosomy of chromosome 10, mutation or homozygous deletion of PTEN, homozygous deletion of CDKN2A, CDKN2B and CDKN2C, and TERT-promoter mutations; other less-common alterations include mutations in TP53, PIK3CA, PIK3R1 and NF1. Gene amplifications are also commonly detected and involve the EGFR, PDGFRA and MET genes encoding mitogenic RTKs; the cyclin-dependent kinase genes CDK4 and CDK6, that mediate transition from G1 to S phase of the cell cycle; and MDM2 and MDM4, which encode proteins that inhibit the activity of p53.

Based on genetic and epigenetic features four major subgroups of adult GB have been identified (Sturm D, 2012; Brennan CW, 2013), including an IDH-mutant, Glioma CpG Island Methylator Phenotype (G-CIMP)-positive and typically MGMT-promoter-methylated subgroup with a proneural gene-expression profile, and three subgroups of IDH-wild-type GB (**Figure 2**).

Among the IDH-wild-type GB subgroups, ‘Receptor Tyrosine Kinase I’ (RTK I) GB predominantly occurs in adolescents and young adults and is characterized by PDGFRA amplification and a proneural gene-expression profile. The ‘Receptor Tyrosine Kinase II’ (RTK II) and the ‘mesenchymal’ IDH-wild-type GB subtypes predominate in patients >50 years of age, and are distinguished by different DNA-methylation profiles, as well as fewer copy-number aberrations and a mesenchymal gene-expression signature in mesenchymal GB (versus a ‘classic’ profile in RTK II GB) (Sturm D, 2012) (**Figure 2**).

Common somatic mutations and copy number deletion/amplification in GB genome can also be grouped into pathways and potential personalized treatment option can be selected according to these pathways alterations. Three pathways, the p53 signaling pathway, the RB signaling pathway,

and the RTK signaling pathway are frequently implicated in GB (Cancer Genome Atlas Research Network, 2008). In particular, p53 signaling pathway is affected by genomic alteration in >50% of cases through CDKN2A deletion, MDM2 and MDM4 amplification, or mutation and deletion of TP53. Also, most GB harbor at least one genetic mutation in the RTK pathways, including mutation or amplification in EGFR, mutation or deletion in NF1 and PTEN. Alterations in the RB signaling pathway occur in >50% samples, including deletion of CDKN2A/B on chromosome 9, amplification of CDK4, CDK6 and CCND2, and deletion or mutation of RB1.

There are drugs directly targeting these frequent genomic alterations including EGFR, PDGFRA, PTEN, PIK3CA, NF1, CDK4/6 and MDM2 (Haynes, Harry R, 2014; Touat, M, 2017). Multiple clinical trials based on targeted treatments are still on-going, and some have been completed. However, the efforts have not yet demonstrated consistent clinical activity in GB (Alexander BM, Cloughesy TF, 2017; Mooney J, 2019).

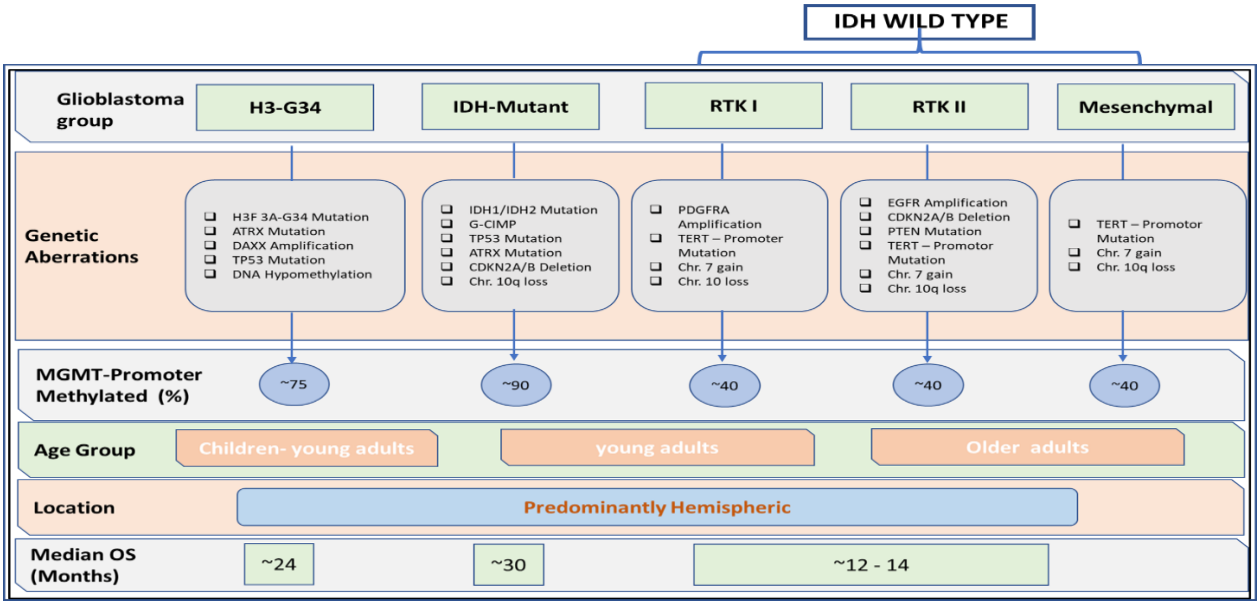


Figure 2. characterization of glioblastoma molecular subgruops, as identified by distinct genetic and epigenetic profiles Modified from Reifenberger G, 2016

1.3 Standard of care for GB

Despite tremendous advances in our understanding of glioma, unlike for the majority of other cancer types in which there have been treatment gains with the advent of targeted therapies, there have not been similar advances in GB treatment, where molecularly targeted therapies have, to date, failed in phase III trials (Alexander BM, Cloughesy TF, 2017; Mooney J, 2019). Patients in

ordinary clinical practice (outside of clinical trials) have now been treated with the same general protocol for more than 10 years, with classic treatment modalities of surgery, radiotherapy (RT), and/or chemotherapy (Weller M, 2014; Stupp R, 2005; Stupp R, 2009).

The BTSG (Brain Tumor Study Group) 6901 randomized trial showed that the addition of whole-brain radiotherapy (RT) more than doubled survival over supportive care alone after surgery and substantially improved survival compared with 1,3-bis(2-chloroethyl)-l-nitrosourea (BCNU). These results established RT as the backbone of adjuvant therapy after surgery (Walker MD, 1978), able to provide an overall survival benefit, but still with no long-term survivors (Seymour T, 2015).

Owing to rich tumor neovascularization, much hope was put into anti-angiogenic therapy (Cohen RB, 2012); however, negative results have been reported in GB clinical trials that used bevacizumab to target Vascular Endothelial Growth Factor (VEGF) receptor (Chinot OL, 2014; Gilbert MR, 2014). In contrast with negative results from recent GB trials focused on new pharmacotherapeutics, co-administration of RT with temozolomide (TMZ) had nearly tripled the 2-year survival of GB patients in the last decade from a dismal 10% with RT alone to 27% with the addition of TMZ and quintupled to 47% in patients with MGMT promoter methylation (Stupp R, 2009), representing an exciting advance after little progress in previous decades. For this reason, all patients currently receive TMZ, although this therapy remain unsuccessful in a significant fraction of cases.

More recently, a new therapeutic modality based on Tumor-Treating Fields (TTFs) has been shown to extend overall patient survival from 16 to 20.9 months when used in combination with radiotherapy and chemotherapy (Stupp R, 2017).

In any case, the multimodal regimen involving surgery, radiotherapy, and chemotherapy is only sufficient to temporarily eliminate the bulk of the tumor mass, since recurrence is inevitable and often poses major challenges for clinical management due to treatment resistance and failure to respond to targeted therapies (Stupp R, 2009; Vredenburgh JJ, 2007; Chen J, 2012).

GB relapse occurs essentially in all patients and is characterized by shorter survival rate (Damodaran O, 2014). A characteristic of the recurrent tumors is a high expression of VEGF, which has been associated with poor prognosis (Ferrara N, 2003). At recurrence, patients in good performance status are usually treated with cytotoxic chemotherapy following, if feasible, repeat surgery but there is no standard treatment in recurrent GB and OS ranges from 3 to 9 months

(Tanaka, Shota 2013; Weller, Michael, 2013). Bevacizumab, nitrosoureas, and TMZ represent the most commonly used systemic agents in recurrent high-grade gliomas (HGG), all of which are considered second line, with no single agent superior to another (Weller M, 2013).
The fact that current treatments for GB are only palliative highlights the need for a better understanding of the disease and the design of effective therapies (Seymour T, 2015).

1.4 Mechanisms of therapy resistance

The translational expectations of highly promising targeted agents have failed because of a combination of lack of selectivity with widely varying efficacies and side effects, and the presence of numerous compensatory pathways allowing long-term subversion of the antitumorigenic effects of TMZ (Burris HA 3rd, 2013; Messaoudi K, 2015; Singer E, 2015). Limitations secondary to drug delivery are also encountered because of the blood brain barrier, structured by endothelial cells with tight junctions and efflux pumps like active P-glycoprotein which can cause inferior/ inconsistent brain drug concentrations. Uneven drug distribution across different regions of tumor lead by invasive and densely packed nature of GB and hypoxia are other conditions limiting treatment (Harder BG, 2018).

Major resistance to current chemotherapy (TMZ treatment) is conferred by the expression of MGMT. MGMT is a DNA repair protein, which demethylates the major O_6-methylguanine (O6-meG) lesions produced by TMZ. If left unrepaired, O6-meG adducts act as miscoding bases leading to mispairing during the next round of DNA replication that eventually results in DNA strand breakage and cell death (Johannessen TC, 2012; Lee SY, 2016). Although the MGMT gene can be mutated or deleted, these events are uncommon and the predominant regulatory mechanism for expression is by epigenetic alteration of its promoter region (Esteller M, 2000). Repression of gene transcription is achieved by the methylation of CpG-rich islands located in the MGMT promoter region. Approximately 45% of newly diagnosed GB have MGMT gene silencing and this is one of the strongest prognostic-predictive biomarkers for OS (Hegi ME, 2005)
The suboptimal clinical response to TMZ even in methylated MGMT promoter GB indicates that additional mechanisms for chemoresistance exist. One such mechanism involves the DNA mismatch repair (MMR) system. In MGMT-deficient tumor cells, O6-meG persists and mispairs with thymine. In an attempt to restore this anomaly, the MMR system is activated and excises thymine from the daughter DNA strand leaving the O6-meG adduct on the template strand intact.

This process initiates repetitive cycles of futile repair involving thymine reinsertion and excision, leading to successively longer DNA resections, the accumulation of single- and double-strand breaks, and ultimately apoptotic tumor cell death (Zhang J, 2012).

In contrast to the genotoxic O6-meG adducts addressed by direct MGMT repair and the MMR system, the bulk of methyl-DNA base adducts (more than 90%) represented by either N7-meG or N3-meA, are substrates of the base excision repair (BER) system. The rapid efficiency of the BER system is the principal reason why TMZ-induced N7-meG and N3-meA lesions possess limited genotoxicity (Alexander BM, 2012). Whit a low BER activity, N3-meA, the relatively more toxic N-purine adduct, can trigger DNA replication fork collapse and double-strand breaks (Zhang Y, 2012). In support of the contribution of BER in TMZ resistance, high N-methylpurine DNA glycosylase protein levels were found to be negatively correlated with OS in a subgroup of patients with MGMT promoter-methylated GB (Agnihotri S, 2012), whereas inhibition of proteins involved in BER improves temozolomide cytotoxicity in vitro and in vivo (Johannessen TC, 2012; Messaoudi K, 2015; Miranda A, 2017).

Apart from DNA repair mechanisms, cell signaling pathways are also major contributors for resistance to TMZ and poor prognosis. Activated EGFR induces PI3K/AKT/mTOR or Ras/Raf/MAPK pathways, thus, promoting tumor progression and invasion, angiogenesis, and GSC maintenance and resistance to TMZ induced cell death (Westphal M, 2017). The strong activation of signaling downstream EGFR is a result of overexpression, amplification (observed in 60% of GB) and mutation. The most common EGFRvIII mutant is constitutively activated to stimulate glioma proliferation and survival (Huang PH, 2009). With respect to TMZ resistance, several preclinical studies have identified mechanisms verifying the role of EGFR signaling. Activation has been shown to abrogate TMZ-induced apoptosis by eliciting the expression of anti-apoptotic proteins such as Bcl-XL (Messaoudi K, 2015); EGFR-mediated MDR expression was also enhanced in response to TMZ exposure (Munoz JL, 2014). Finally, a high proportion of GSCs were discovered to have elevated EGFR activity, suggesting their reliance in exerting its trophic effects to induce chemoresistance (Liffers K, 2015). The EGFR is an appealing drug target due to its high degree of selectivity for GB. In comparison, most normal adult brain cells, except for neuroglial stem cells located at the hippocampus and subventricular zone, do not express the EGFR. Currently, several targeted therapies including small molecule inhibitors, monoclonal antibodies, and vaccines are being investigated in clinical trials with limited success (Azuaje F, 2015)

1.5 Advances in molecular therapeutic targeting of GB

Making TMZ more efficient could be an instant and effective strategy in counteracting GB. Pseudo substrates as O6-benzyl guanine (O6-BG) and O6-(4-bromothenyl) guanine have shown to be efficient in GB patients with high MGMT activity but were withdrawn from further consideration due to their high toxicity on normal cells (Rabik CA, 2006). PARP inhibitors enhanced TMZ cytotoxicity by counteracting BER system in GB (Muñoz-Gámez JA, 2015)). Targeting RTKs has been an effective treatment across wide range of cancers but failed to show any fruitful impact in treating GB. Currently EGFR-targeting agents such as small molecule inhibitor dacomitinib, monoclonal antibodies like nimotuzumab and immunotherapy by CART-EGFRvIII T cells, along with few other agents are in clinical studies (Rajaratnam V, 2020; Mooney J, 2019; Noch EK, 2018). The discovery of the programmed death-1(PD-1)/programmed death-ligand 1 (PD-L1) has opened new doors in immunotherapy concerning human cancers. PD-1 blocking antibodies have been approved by the FDA for the first time to treat melanoma in 2014, and for several other cancers during following years. Targeting immune check point protein PD-1 in GB preclinical models, increased $CD8^+$ cells and ongoing trials of these checkpoint inhibitors in GB (anti-PD-1 [nivolumab]) have proven to be safe (Mooney J, 2019; Noch EK, 2018).

1.6 Glioblastoma stem cells (GSC)

Major contributors to the poor prognosis of GB patients include a high degree of intratumoral cellular heterogeneity and plasticity, the infiltrative and migratory nature of GB cells, and a high rate of recurrence (Safa AR, 2015; Harder BG, 2018). Moreover, recurrent tumors are frequently evolutionarily divergent from the original tumor, with distinct drivers and sensitivities (Kim J, 2015). Many of these features can be modeled through the lens of the cancer stem cell (CSC) hypothesis. The CSC hypothesis represents one element of nongenetic complexity in cancer biology and holds that tumors mimic normal tissues with hierarchically arranged and dynamically regulated populations of cells, with stem-like cells at the apex that display regenerative potential and the capacity to recapitulate the entire functional diversity present within the original tumor (Clevers H, 2011; Reya T, 2001). The underpinnings of CSC modeling date back to early functional studies of cancer, which showed that the injection of a single leukemic cell into mice could produce a lethal leukemia in as little as 2 weeks (Furth J, 1937). More recently, the CSC model was revitalized by the identification of a subset of patient-derived leukemia cells able to

traffic to the bone marrow of immunodeficient mice, with sustained proliferation and maintenance of the original leukemic cell phenotypes (Lapidot T, 1994)

GB stem cells (GSCs) were first discovered as a population of CD133+ (neuronal stem cell marker) cells sorted from primary tumor capable of initiating highly invasive and angiogenic tumors in vivo (Singh SK, 2004). But, in spite of the evidence for the usefulness of CD133 as a marker for GB tumor initiating cells, later studies demonstrated the tumorigenic potential of CD133-neg cells and the derivation of CD133-pos cells from CD133-neg cells, thus invalidating the absolute value of CD133 as GSC marker (Beier D, 2007).

So far, no absolute GCS marker has been identified and GSCs can only be defined by a series of functional criteria, including tumor-initiating capacity following serial transplantation, self-renewal, and the ability to recapitulate tumor heterogeneity (**Figure 3**) (Singh SK, 2004; Lee J, 2006).

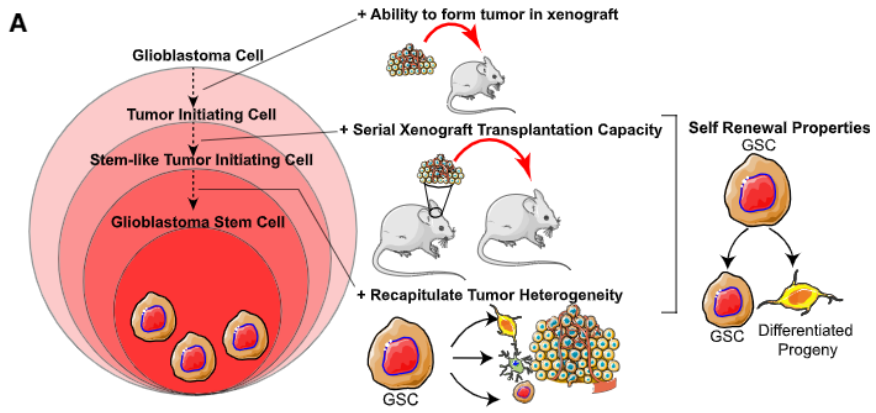


Figure 3. GSC definition and key features. (A) GSCs are defined by a series of functional criteria, including tumor-initiating capacity following serial transplantation, self-renewal, and the ability to recapitulate tumor heterogeneity (adapted from Gimple RC, 2019)

Functionally defined GSCs have been demonstrated to play an important role in mediating therapeutic resistance and recurrence (Chen J, 2012) through supporting radioresistance (Bao S, 2006), chemoresistance (Liu G, 2006; Chen J, 2012), angiogenesis (Bao S, 2006b; Cheng L, 2010) and invasiveness (Wakimoto H, 2009). Typical GSCs features including a low rate of replication, induction of quiescence and high efficiency in DNA repair may contribute to chemo- and radioresistance and explain tumor recurrence (Chen J, 2012). When most differentiated tumor cells die from treatment, GSCs are triggered to repopulate tumor mass. Following surgery, patients with GB are treated with adjuvant radiation and chemotherapy. While these treatments reduce the number of proliferative tumor cells, post-therapy inflammatory stimuli switch the residual

infiltrated GSCs into a mesenchymal phenotype to find the proper “soil” and eventually lead to tumor recurrence (Bao S, 2006; Liu G, 2006; Cheng L, 2010).

GSCs possess the ability to activate several checkpoint proteins, such as ATM, Rad17, Chk2, and Chk1, in response to DNA damage induced by radiation (Bao S, 2006). Hence, GSCs can efficiently repair damaged DNA, allowing better recovery than non-stem tumor cells (Cheng L, 2010). Thus, current clinical treatments probably enrich the GSCs subpopulation, which overtime recapitulates the tumor due to its self-renewal properties. Therefore, to achieve long-lasting remission or a cure for GB we need to target GSCs besides the highly proliferative more differentiated cells (Seymour T, 2015).

Recurrence of GB is shown to be largely associated with the regeneration of tumor from remaining GSCs after initial treatment. Thus, targeting GSCs is an extremely important aspect of the clinical treatment of GB. The functional aspects of CSC, such as cell proliferation and migration, are also important to consider, because they directly correlate with the invasive nature of GB. One proposed mechanism for targeting GSCs is to first induce differentiation, thus making the cells more amenable to other therapeutic agents. To make this therapeutic approach more complex, alongside a CSC hierarchical model, that proposes self-renewing CSCs dividing asymmetrically to form new CSCs and progenitor cells that in turn give rise to differentiated cancer cells that form the bulk of the tumor but are no more tumorigenic (Adams JM, Strasser A, 2008), experimental evidences demonstrate that GSCs represent a plastic state that most cancer cells can adopt. This phenotypic plasticity, induced by the reversible epigenetic modification, exists between different cellular states of the tumor, which allows for interconversion between GSC and non-GSC states depending on several factors (**Figure 4**) (Natsume A, 2013; Safa AR, 2015; Lee J, 2006). Microenvironmental exposures, including nutrient deprivation, hypoxia, radiation, and others, shift the dynamics of regulation of these interconversions, bringing about changes in the GSC and non-GSC pools, along with phenotypes such as proliferation or quiescence (Dirkse A, 2019).

Epigenetic plasticity and the tumor microenvironment inducing such dynamic phenotypic changes can be good therapeutic targets in the treatment of GB.

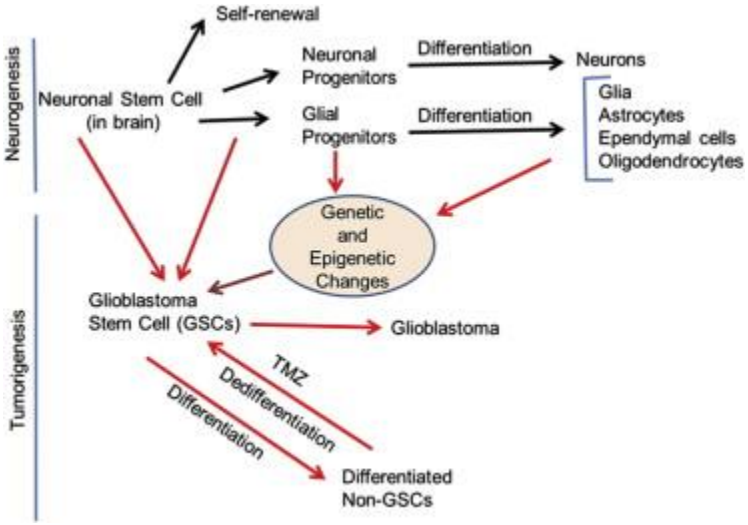


Figure 4. Relationship between neuronal stem cells (NSCs), differentiation, GSCs, cancer initiation, and dedifferentiation. NSCs are able to differentiate into neural progenitors. Neural progenitors differentiate into neurons and glial progenitors differentiate to oligodendrocytes, ependymal cells, and astrocytes. GB is initiated from the transformation of NSCs into GSCs. Similarly, glial progenitors are able to trigger tumor development following malignant transformation of normal progenitor cells. Astrocytes, neurons, oligodendrocytes, and ependymal cells also have the potential to initiate tumorigenesis (Adapted from *Safa AR, 2015)*

While the functional definition for GSCs is required in determining their relevance in disease and the mechanisms to selectively target these cells, it does not make any claims about tumor cell of origin and the cell responsible for initiating human GB have not yet been definitively identified. More recent studies have identified a slow-dividing quiescent population of stem-like cells in GB tumors (Patel AP, 2014) and have reinforced the hypothesis that the cell of origin of GB may be traced back to a sub-ventricular area-derived neural or astrocyte-like stem cell (Pollen AA, 2015; Lee JH, 2018).

1.7 Role of TNF alpha in GB

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) proteins are a family of transcription factors that mediate immune and inflammatory responses (Bassères DS, 2006; Hoffmann A, 2006; Karin M, 2006). In GB and many other cancers, NF-kB is constitutively activated and is an important driver of the malignant phenotype that confers a negative prognosis (Atkinson GP, 2010; Bhat KPL, 2013; Korkolopoulou P, 2008), in particular, the mesenchymal subclass of GB is characterized by elevated levels of NF-kB signaling components (TRADD, RELB, TNFRSF1A), enhanced chemo- and radiation resistance and an overall poorer prognosis than patients with other types of GB (Verhaak RG, 2010; Bhat KPL, 2013; Teng J, 2017).

In GB, NF-kB pathway can be trigger by both extracellular and intracellular cues. NF-kB may also be activated by numerous growth factors or signaling pathways that are dysregulated in gliomas (Nogueira L, 2011; Kenneth NS, 2014). Specifically, NF-kB is activated by EGF, and/or its

receptor, EGFR, the latter of which is frequently mutated and constitutively activated (Puliyappadamba VT, 2014; Bonavia R, 2012; Yang W, 2012). Genome-wide analysis study of 790 clinical GB samples showed a 23.4% rate of deletion of the NFKBIA gene that encodes IkBa (Bredel M, 2011). Loss of this key inhibitor of NF-kB activation results in constitutive NF-kB activation, has an effect that is similar to the effect of EGFR amplification, and is associated with comparatively short survival (Bredel M, 2011). Since EGFR gene amplification and mutation are detected in the classical subtype of GB, this suggests a pattern of mutual exclusivity between these 2 major mechanisms of NF-kB activation.

As mentioned previously, GB often fails to express PTEN, a tumor suppressor and negative regulator of the AKT pathway (Cancer Genome Atlas Research Network, 2013; Tohma Y, 1998). In the absence of PTEN, AKT is constitutively active and can, in turn, activate NF-κB (Atkinson GP, 2010; Dan HC, 2008; Madrid LV, 2001; Ozes ON, 1999).

NF-kB activation is also a hallmark of inflammation and has been a focus of intense interest in inflammation-induced cancer (Karin M, 2002). Signs of inflammation in GB can be detected in the form of infiltration by macrophages/microglia and lymphocytes, production of inflammatory cytokines, and activation of NF-kB, suggesting that inflammation may play a role in gliomagenesis (Roggendorf W, 1996; Nagai S, 2002). Cytokines such as TNFα and TRAIL can induce NF-kB by this canonical pathway. TNFα, a proinflammatory molecule secreted in the CNS by microglia, astrocytes and some neurons is one of the most potent activators of NF-kB (Grivennikov SI, 2011). TNFα signals through two receptors, TNF receptor 1 (TNFR1) and TNFR2 (Grivennikov SI, 2011) (**Figure 5**). TNFR2 is usually expressed on cells of the immune system (such as microglia) and oligodendrocytes, while TNFR1 is expressed on most cell types. The levels of TNFR1 expression in GB and GB-associated endothelial cells are elevated when compared with low-grade gliomas or normal brain tissues (Wang H, 2004; Hayashi S, 2001). TNFα, upon binding to TNFR1/TNFRII induces the formation of intracellular complexes that is either cytoprotective or cytotoxic (Figure 5; van Horssen R, 2006). The p55/TNFRI receptor plays a major role in activating the canonical NF-κB pathway that results in a strongly increased proliferation of neural stem cells (Widera D, 2006). In glioma, TNFα signaling through TNFR1 promotes NF-κB activation and subsequent anti-apoptotic responses (Conti A, 2005). As such, TNFα and TNFR1, via NF-κB activation, promote tumor growth and development. Interaction of GB tumor cells with tumor-infiltrating myeloid cells leads to paracrine production of TNFα, which contributes to tumor growth and spread and renders EGFR kinase inhibitors less effective (Kusne Y, 2014).

A growing body of experimental evidence suggests a key role of NF-κB in GSC. One study showed that COX2, a gene that is regulated by NF-κB signaling, shows higher levels of expression in U87-derived CD133+ cells than in CD133- cells from the same cell line (Atkinson GP, 2010). Blockade on NF-kB can differentiate glioma stem cells via a reduction in CD133, SOX2, and NAC1 expression (Zhang L, 2014). More recently, NF-kB activation downstream to TGFβ/TAK1 axis in $CD133^{+}$ GB cells has been shown to be necessary for GSC maintenance (Rinkenbaugh AL, 2016). Because of the relative newness of the field of CSCs, further studies are needed to define the function of TNFα/NF-κB signaling in controlling CSC properties in the context of GB.

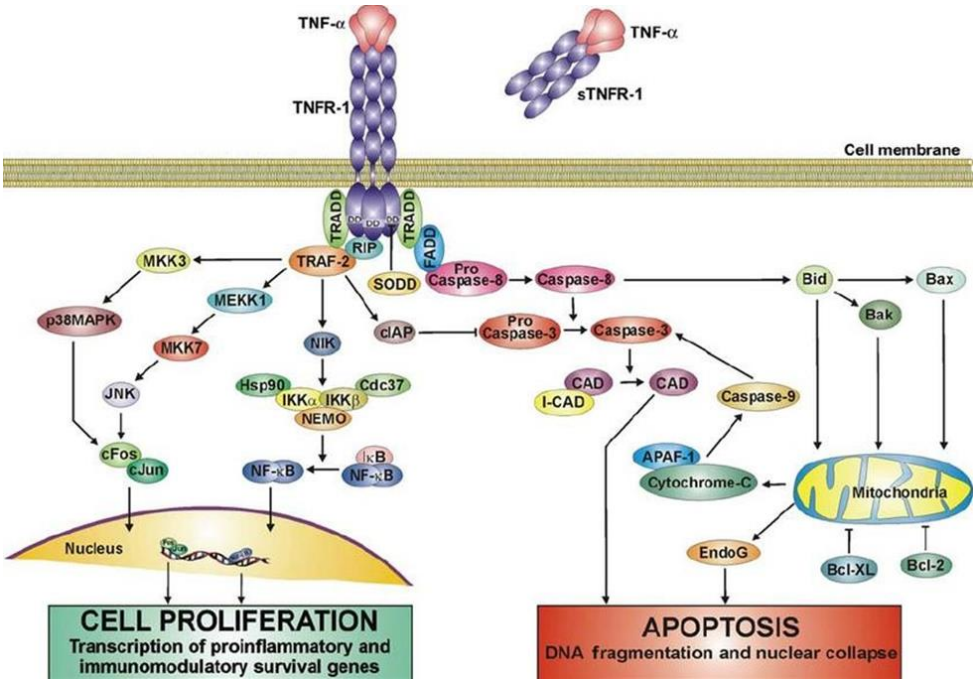


Figure 5. Tumor necrosis factor receptor 1 (TNFRI) signaling pathway. Tumor necrosis factor-alpha (TNFα) activates both survival and proliferation pathways along with apoptotic pathways via TNFR1. The separate pathways are well defined, while the survival-death balance regulation remains unclear. Abbreviations: APAF-1, apoptosis protein activating factor 1; Bcl-2, B-cell lymphoma 2; Bid, Bak, Bax, and Bcl-XL, mitochondrial proteins of the Bcl-2 family; CAD, caspase-activated DNAse; Caspase- 3/8/9, cysteine aspartase (apoptotic protease) 3/8/9; Cdc37, co-chaperon of HSP90; cIAP, cytoplasmic inhibitor of apoptosis; cFos/ cJun, transcription factors; DD, death domain; EndoG, mitochondrial DNAse; FADD, Fas-associated DD; HSP90, heat shock protein 90; I-CAD, inhibitor of CAD; I κ B, inhibitor of NF- κ B; IKK α / β , I κ B kinase; JNK, cJun n-terminal kinase; MEKK1, mitogen-activated protein kinase/extracellular signal–related kinase kinase 1; MKK3/7, MAPK kinase 3/7; NEMO, NF- κ B essential modulator; NF- κ B, nuclear factor kappa B transcription factor; NIK, NF- κ B inducing kinase; p38MAPK, p38 mitogen-activated protein kinase; RIP, receptor interacting protein; SODD, silencer of DD; sTNFR- 1, soluble TNFR-1; TNF- α , tumour necrosis factor alpha; TNFR-1, TNF receptor 1; TRADD, TNF receptor- associated DD; TRAF-2, TNF receptor-associated factor-2. ***(***Adapted from van Horssen R, 2006***)***

1.8 The diacylglycerol kinases

Diacylglycerol kinases (DGKs) phosphorylate diacylglycerol (DG) resulting in the production of phosphatidic acid (PA) (Mérida I, 2008; Shirai Y, 2014). Mammalian DGKs comprise an extended family, currently with ten members classified into five different subtypes based on the presence of different regulatory domains in their primary sequences (Figure 6). DGKs diversity is increased further by alternative splicing, which produces several isoforms with distinct domain structures. Mammalian type I DGKs (α, β and γ) display characteristic Ca^{2+}-binding EF-hands and a recoverin-like motif in the N-terminus. Type II isoforms are characterized by a pleckstrin homology (PH) domain. Members of the type IV group contain C-terminal ankyrin repeats and a PDZ-domain-binding sequence, as well as myristoylated alanine-rich C-kinase substrate (MARCKS) homology region upstream of the catalytic site. The single type V member has a Rho-binding domain, and the only type III member has the simplest structure, with no known regulatory regions (Mérida I, 2008).

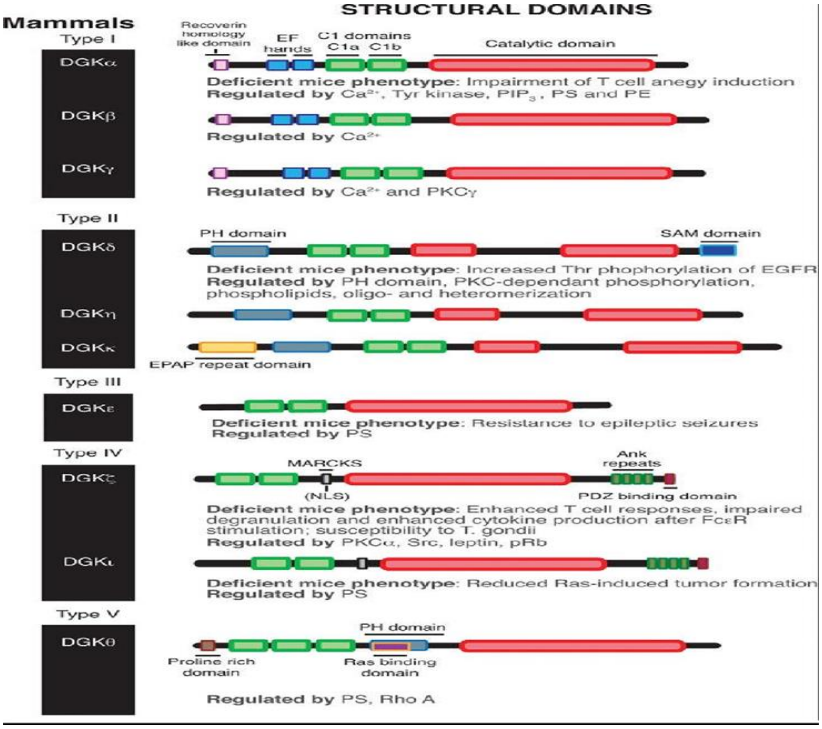


Figure 6. The DGK family (Adapted from Mérida I, 2008).

Both DG and PA are very important signaling molecules and, by regulating DG/PA balance in a reciprocal and highly compartmentalized manner, DGKs act simultaneously as both terminators and activators of DG- and PA-mediated signaling (Sakane F, 2007; Mérida I, 2008; Mérida I, 2009). DG regulates the activity and localization of several proteins, including classical and novel

protein kinase C (PKC), chimerins, Unc-13, and Ras guanyl nucleotide-releasing protein (RasGRP). PA also activates several enzymes, including phosphatidylinositol 4-phosphate 5-kinase, mTOR, and atypical isoforms of PKC (aPKC).

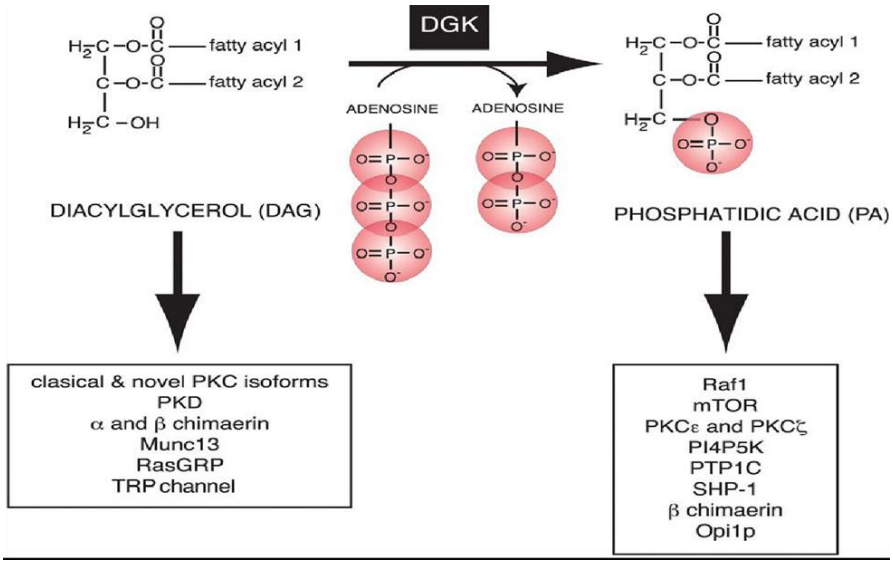


Figure 7. DGK enzymatic activity regulates levels of DG and PA at the membrane (Adapted from Mérida I, 2008).

1.9 Role of diacylglycerol kinase alpha (DGKα) in cancer

DGKα is part of the signaling network by which microenvironmental cues, including growth factors and extracellular matrix proteins, regulate cell behavior. DGKα is activated, in endothelial and epithelial cells, following phosphorylation by SRC downstream HGF/MET and VEGF/VEGFR (Baldanzi G, 2004) and, in HEC-1A endometrial adenocarcinoma, after treatment with 17-β-estradiol (Filigheddu N, 2011).

The N-terminal region plays an important role in the negative regulation of both catalytic activity and membrane translocation of DGKα. The negative regulation is mediated by the interaction of this region with the active site of the protein. Phosphorylation, the presence of calcium or particular lipids (phosphatidylethanolamine or cholesterol) induce the detachment of the N-terminal region from the catalytic site, resulting in the elimination of the negative control and allowing the activation of the protein resulting in translocation from the cytosol to the plasma membrane (Mérida I, 2008).

Different studies show that DGKα plays an important role in different cellular events involved in tumor development. Experimental evidence shows that DGKα can support the proliferation of

Kaposi's sarcoma cells (Baldanzi G, 2011) endometrial adenocarcinoma (Filigheddu N, 2011) and, more recently, of GB (Dominguez CL, 2013). It was also demonstrated that DGKα is involved in angiogenesis induced by VEGF, a process that is crucial in the development of malignancies (Baldanzi G, 2004).

The formation of metastases involves the acquisition by the tumor cells of migratory and invasive capacity, the base of which is the formation of membrane protrusions. DGKα participates in the formation of these protrusions and is necessary for the phenomenon to occur properly, because, upon activation in response to HGF, mediated by SRC, DGKα produces PA in the membrane which recruits and activates aPKC. aPKC allows the separation of Rac-GDP from RhoGDI and its activation in Rac-GTP (Chianale F, 2007; Chianale F, 2010). Rac activation induces the formation of ruffles and protrusions that allow the cell to migrate and invade the matrix.

In the breast carcinoma cell line MDA-MB-231, DGKα locates on the migration front and contributes to release of metalloproteinase 9 in response to SDF-1α (Rainero E, 2014). The stimulation with HGF, through the activation of DGKα, increases the invasiveness of MDA-MB-231, which is lost as a result of pharmacological kinase inhibition. Moreover, in these cells, DGKα promotes increased growth capacity in the absence of anchor (Filigheddu N, 2007; Rainero E, 2014). In SW480 cells DGKα increased expression shows a direct correlation with cell 3D culture survival and treatment resistance via DGKα and SRC functional interaction (Torres-Ayuso P, 2014).

Inhibition of αvβ3 integrin or expression of mutants of p53 promotes invasive cell migration by enhancing endosomal recycling of α5β1 integrin under control of the Rab11 effector Rab-coupling protein (RCP). Generation of PA downstream of DGKα is necessary for this process. In particular, DGKα-derived PA is required for RCP to be mobilized to and tethered at the tips of invasive pseudopods and to allow RCP-dependent α5β1 recycling and the resulting invasiveness of tumor cells (Rainero E, 2012).

The importance of DGKα in tumor development has not been determined only in vitro but also in animal models and humans. By xenotransplantation, Takeishi and colleagues demonstrated that hepatocellular carcinoma (HCC) cells over-expressing DGKα can generate larger tumors compared with the control by increasing proliferation, as evidenced by higher levels of Ki67 expression in cells over-expressing DGKα (Takeishi K, 2012). On the other hand, silencing of DGKα reduces the proliferative capacity and invasiveness of HCC cells. In human HCC, DGKα not only turns out to be over-expressed but also represents a poor prognosis marker in that

correlates with an increased likelihood of post-treatment relapse and with increased cell proliferation and invasion. On this basis, DGKα has been proposed as a possible therapeutic target for the treatment of HCC (Takeishi K, 2012). In melanoma, reduction of DGKα protein levels inhibits TNFα-regulated NF-KB activation (Yanagisawa K, 2007).

DGKα is required for tumor growth even in GB models. Murine xenograft models of GB undergo an increase in their survival when treated with a lentiviral vector containing a direct shRNAmir against DGKα. In these models also, the treatment with the pharmacological inhibitor of DGKα R59022, introduced by daily intraperitoneal injection, increases survival with a reduction of tumor mass due to less tumor vascularization and increased apoptosis (Dominguez, 2013).

More recently, Olmez et al reported that inhibition of DGKα with the small-molecule inhibitor, ritanserin, or RNA interference preferentially targets the mesenchymal subtype of GB as well as mesenchymal models of lung and pancreatic carcinoma. This enhanced sensitivity of mesenchymal cancer cells to ritanserin is through inhibition of geranylgeranyltransferase I activity and downstream mediators previously associated with the mesenchymal cancer phenotype, including RhoA and NF-kB (Olmez I, 2018).

In response to the increased interest for DGKα as a therapeutic target, in addition to the two commercially available DGKα inhibitors (R59922 and R59949) (Jiang Y, 2000) several new small molecule inhibitors specifically targeting DGKα have been investigated for their activity against cancer cells. Ritanserin, a compound structurally similar to R59022 and antagonist of serotonin demonstrated safe for human use, has been identified as a DGKα inhibitor suitable for repurposing in oncologic therapies (Boroda S, 2017; Purow B, 2016). By a in silico approach followed by biochemical screening several molecules specifically targeting DGKα without affecting serotonin signaling have been identified (Liu K, 2016; Yamaki A, 2019; Velnati S, 2019). Treatment with these DGKα inhibitors reduce migration of cancer cells (Velnati S, 2019) or enhance caspase 3/7 activity reducing viability of hepatocellular carcinoma, GB, and pancreatic cancer cells (Yamaki A, 2019; Liu K, 2016).

2. Materials and methods

2.1 Cell culture

U87MG and T98G glioma cell lines were cultured in Minimal Essential Medium (MEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Lonza),1x antibiotics and antimitotic (Sigma Aldrich). U251 glioma cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Lonza), 1x antibiotics and antimitotic (Sigma Aldrich). hGSC#1, hGSC#2 and hGSC#3primary human GB stem-like cells (GSC)-derived cell lines (a kind gift from Prof. Giuliana Pelicci) were cultured and maintained in ultralow attachment plate or flask (Corning) as suspension using a special medium for stem cells consisting of 50% DMEM-50% F12 (Gibco Life Technologies), 1x antibiotics and antimitotic (Sigma Aldrich), 1% B27 Serum-Free Supplement (Gibco Life Technologies), βFGF (10 ng/ml, Peprotech), EGF (5 ng/ml, Peprotech). HEK 293T cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (Sigma-Aldrich). All cell lines were maintained in humid atmosphere at 37°C with 5% CO_2.

2.2 MTT viability assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2Htetrazolium bromide) from Sigma–Aldrich, was dissolved in distilled water (5 mg/ml) and filtered through a 0.22 µm Millipore filter. Cells were plated at number of 1000 cell/well in 50 µL of volume in a 96 wells plate and treated with different concentration of the DGKα inhibitors R59022, R59949, ritanserin, AMB639752 and CU3 and incubated for different time points in a final volume of 100 µL. For each concentration of drug, the same concentration of DMSO was used as control.

After incubation, MTT was added to each well and incubated from 4 hours to overnight at 37°C and 5% CO_2. Then, the formazan crystals were dissolved using acidic isopropanol (4 mmol/L HCl, 0,1% NP40 in isopropanol) and the absorbance was read at 570nm and 630 nm with a spectrophotometer plate reader (Perkin Elmer).

2.3 Lentivirus production and cell transduction

Recombinant lentiviruses were produced by transfecting HEK 293T cells with the transfer vector (PLKO.1-shNT; PLKO.1-shDGKα) and 3rd generation packaging plasmids using Lipofectamine 3000 (Thermo Fisher Scientific). $2x10^6$ HEK 293T cells were seeded in 15 cm plates and

transfected after 24 hours with a total of 10 ug of packaging plasmids and 10 ug of the vector plasmid. The conditioned medium was collected after 36 hours, cleared by centrifugation at 1300 RPM, filtered through 0.22 um pore-size filters, aliquoted and stored at -80°C.

For transduction, 1 x 10^5 GB adherent cells were seeded in 10 cm dish and incubated overnight with 1:20 dilutions of the viral suspension in the presence of polybrene 6 μg/ml. The supernatant was then replaced with culture media containing puromycin (1 μg/ml) to select only transduced cells. For GSCs, 0.5 x 10^6 cells were resuspended in 1:10 dilutions of the viral suspension in the presence of polybrene 6 μg/ml and centrifuged at 1600 rpm for 1 hour. The supernatant was removed, and pellets were then resuspended in stem cells culture media and puromycin 1 μg/ml was added after overnight culture.

2.4 RNA interference

GB cell lines were transfected with validated DGKα silencer or silencer negative control siRNA (Ambion). siRNA was mixed with lipofectamine 3000 (Thermo Scientific) and serum-free MEM. The above mix was incubated at room temperature for 15 minutes to form siRNA lipid complexes and added to the cells. Following overnight incubation in a humidified incubator at 37°C with 5% CO2, MEM supplemented with 10% FBS was added by replacing existing media.

2.5 Evaluation of NF-κB transcriptional activity

NF-κB transcriptional activity was evaluated using the pGL4.32[luc2P/NF-κB-RE/Hygro] Vector (Promega), containing five copies of an NF-κB response element (NF-κB-RE) that drives transcription of the luciferase reporter gene luc2P (Photinus Pyralis). Luc2P is a synthetically derived luciferase sequence with humanized codon optimization that is designed for high expression and reduced anomalous transcription. Cells were co-transfected with 200 ng of pGL4.32[luc2P/NF-κB-RE/Hygro] luciferase reporter construct and 5 ng of Renilla. Twenty-four hours after transfection, cells were serum-starved and stimulated overnight with TNFα. Luciferase reporter activity was detected using a dual-luciferase reporter assay kit (Promega) and the Victor spectrophotometer (PerkinElmer) detection system. Results of luciferase reporter were normalized by Renilla. Transfection was done using lipofectamine 3000 (Invitrogen) following the manufacturer's instructions.

2.6 Clonogenic assay in MethoCult™

Working concentrations of MethoCult™ (MC) were prepared by diluting with GSCs culture media (1:3) in sterile tubes. Due to the high viscosity of the medium, MC diluted solution was handled with sterile syringes. Cells were added to MC-containing media and mixed well. Air bubbles were removed, and cell suspensions were gently transferred to the bottom of ultra-low attachment 24 well-flat bottom plate (corning). To reduce evaporation, the edge rows of the plate were filled with sterile PBS to maintain the proper concentration of MC throughout the experiment, as the MC might thicken upon water loss. Any further addition of medium was avoided, and plates were placed in incubators and cultured for 12-15 days. Colony formation and growth was followed using a Zeiss Axiovert 40 CFL microscope. Colonies were stained with MTT and quantified using the OpenCFU software.

2.7 Clonogenic assay in soft agar

GSC's cells were collected carefully for each experimental condition and counted using a hemocytometer. 600 cells for each experimental condition were mixed with 0.3% semi-viscous agar medium and plated over 0.6% hard agar base in a 6-well plate. Plates were placed in a humidified incubator at 37°C with 5% CO_2 and cultured for 12-14 days. Colonies were stained with MTT and quantified using the OpenCFU software.

2.8 Extreme limiting dilution assay (ELDA)

T98G and U87MG cells were transduced with ShLuc and ShDGKα as described before. For extreme limiting dilution assay cells were plated at number of 50, 25, 12, 6, 3, 1 cells/well in an ultra-low attachment 96 wells plate using complete stem cell medium. Tumor sphere growth was monitored using a phase contrast microscope and pictures was taken by a camera linked to the microscope. The number and the dimension of the spheres was assessed 12 days after plating and data were analyzed using the ELDA software (http://bioinf.wehi.edu.au/software/elda/).

2.9 Immunoblotting

After treatments, whole cell lysates were prepared using RIPA lysis buffer (25 mmol/L Hepes pH 8, 135 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L ZnCl2, 50 mmol/L NaF, 1%

Nonidet P40, 10% glycerol) with protease inhibitors (AEBSF, aprotinin, bestatin, E-64, EDTA, leupeptin, Sigma-Aldrich) and orthovanadate. Lysates were then kept on a wheel for 20 minutes at 4°C and after centrifuged at 12,500 g for 15 minutes. Proteins contained in the samples were collected and quantified using Pierce BCA protein assay kit (Thermo Fisher Scientific). Successively, proteins were denatured at 95°C for 5 minutes in presence of 2% Sodium Dodecyl Sulfate (SDS), 150 mmol/L dithiothreitol (DTT) and 0,01% bromophenol blue. Electrophoresis of the samples was performed using 8 %, 10 % or 15% polyacrylamide gels and proteins were transferred from the gel to a PolyVinylidene DiFluoride membrane (PVDF, Amersham). Lastly, the membrane was saturated using 3% Bovine Serum Albumin (BSA, Sigma) in TBS/Tween-20 0.1% [Tris Buffered Saline 1X containing Trizma base 50 mmol/L, NaCl 120 mmol/L, 0,1% Polyethylene glycol sorbitan monolaurate (Tween-20)] for 1 hour and incubated with primary antibody dissolved in the same buffer with sodium azide 0,01%. Then membranes were washed with TBS 1X-Tween 0.1 % (TBS-T) for 3 times with a time gap of 15 minutes between each wash and incubated with secondary antibody for 60 minutes at room temperature. After extensive wash using TBS-T immunocomplexes were detected using ECL (Perkin Elmer) chemical reagents on Chemidoc Touch (BioRad).

2.10 Antibodies

Phospho AKT (Ser 473) rabbit mAb, Cell Signaling; Phospho AKT (Ser308) rabbit mAb, Cell Signaling; AKT mouse mAb, cell signaling; DGKα, Proteintech, Rabbit Polyclonal antibody; NFκB rabbit polyclonal antibody, Santa Cruz: phospho NFκB (ser 536) rabbit mAb, Cell Signalling; phospho FAK (tyr 397) and (tyr 925) rabbit mAb, Cell Signalling; FAK rabbit polyclonal antibody, cell signaling; phospho aPKC (Thr 410) rabbit mAb, Cell Signalling; α Tubulin, rabbit mAb, Sigma; GFAP Mouse mAb Cell Signaling**;** β3-Tubulin Rabbit mAb, Cell Signaling; Purified Mouse Anti-Human HIF-1α, BD; TNFR1 Mouse Monoclonal Antibody, proteintech. Secondary antibodies were goat anti-mouse IgG or donkey anti-rabbit IgG horseradish peroxidase (HRP).

2.11 RNA isolation and qRT-PCR

Total RNA was isolated by phenol-chloroform extraction using RNAzol RT (Sigma Aldrich) and following the manufacturer's instructions. Concentration of isolated total RNA was quantified using NanoDrop (Thermo Scientific). cDNA from total RNA (1μg) was synthesized, using the

iScript™ cDNA Synthesis Kit (Bio Rad) accordingly to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was carried out in a CFX96 real-time system (BioRad), using with custom primers and the SsoAdvanced universal SYBR Green supermix (BioRad) followed manufacturers instruction. Expression levels of mRNA were evaluated by using the ΔΔCt Method and GUSB as reference gene. Primers are shown in Table 1

Table 1. Primers used for qRT-PCR

GENE	NCBI REFERENCE SEQUENCE	PRIMER SEQUENCE	PRODUCT LENGTH
CD133	NM_001371406.1	F - ACT CCC ATA AAG CTG GAC CC R - TCA ATT TTG GAT TCA TAT GCC TT	133
NESTIN	NM_006617.2	F - GGT GGC CAC GTA CAG GAC CC R - TGG GGT CCT AGG GAA TTG C	124
SOX2	NM_003106.4	F – ACCGGCGGCAACCAGAAGAACAG R – GCGCGCGGCCGGTATTTAT	254
SOX8	NM_014587.5	F - AGC TGT GGC GCT TGC T R- TAC TTG TAG TCG GGG TGG TCC	100
OLIG2	NM_005806.4	F - CTC CTC AAA TCG CAT CCA GA R- AGA AAA AGG TCA TCG GGC TC	147
TUBB3	NM_001197181.2	F - AGTGATGAGCATGGCATCG R - ACGTACTTGTGAGAAGAGGCC	107
GFAP	NM_001363846.2	F - GGTACCGCTCCAAGTTTGC R - AGGTCAAGGACTGCAACTGG	114
GUSB	NM_000181.4	F - ATC GCC ATC AAC AAC ACA R- CTT GGG ATA CTT GGA GGT G	84
SOX 4		#qHsaCED0007041; BIORAD	
POU5F1		# qHsaCED 0038334; BIORAD	
SMAD7		#Hs00998193_m1; THERMO SCIENTIFIC	

2.12 Cell Proliferation Assay

Cell proliferation was evaluated on transduced hGSC#1 and hGSC#2 cells with the fluorescent dye CellTraceTM CFSE following the manufacturer's protocol (Thermo Fisher). 1 x 10^6 transduced cells after 48 hours of puromycin selection were incubated with 5 uM CellTraceTM in 1 ml of PBS for 20 minutes at 37°C. After incubation, cells were washed with 3 volumes of DMEM, and read immediately by flow cytometer as time zero. The remaining distributed in 6 wells plates and left to grow for 3 days. The proliferation rate was assessed quantifying CFSE fluorescence by FACS analysis.

2.13 Cell cycle analyses

hGSC#1 & hGSC#2 cells were transduced with shC/shDGKα. After overnight transduction, cells were selected with puromycin. At day 4 and day 7 post transduction cells were collected, washed and fixed with 70% alcohol solution for 15 min in ice. The fixed cells were treated with RNase A and stained with Vybrant™ DyeCycle™ Stain (Thermo Fisher Scientific) at 37°C for 30 min in dark. At least 100.000 events per sample were acquired by flow cytometer and the percentage of cells distributed in different phases of cell cycle was calculated using Flow Jo software.

3. Results

3.1 DGKα protein is required for GB proliferation and GSCs self-renewal. Several experimental evidences suggest DGKα as a promising new target for cancer therapy, in particular for GB (Purow B, 2015; Merida I, 2017). DGKα mediates various aspects of cancer cell survival and aggressiveness and contributes to modulate signaling pathways involved in stemness maintenance (Chen J, 2018; Kai M, 2009). We therefore questioned whether DGKα was required for sustaining the growth of GSCs, to provide further support for targeting this kinase in patients. We knocked down DGKα protein in GSCs derived from human primary samples (hGSCs) transducing cells with an shDGKA-expressing lentivirus and evaluated viability and sphere forming capacity. The lentiviral construct (shDGKA) strongly downregulated DGKα expression when compared with a non-targeting control sequence (shNT, **Figure 8A**). DGKα targeting resulted in a significant impact on long-term growth kinetic in the patient-derived GB neurospheres analyzed, as demonstrated by a 30 and 50% reduction of cell viability at day 8 post-transduction in hGSC#1 and hGSC#2, respectively (**Figure 8B**).

DGKA silencing caused no acute toxicity but had a detrimental effect on long-term growth of neurospheres, suggesting that DGKα protein is necessary for GSCs population maintenance. We therefore assessed the role of DGKα in GSCs self-renewal performing a methylcellulose-based clonogenic assay. Transduced single cells were plated in semi-solid medium, the single clones were counted after 14 days and the clonogenic cells were calculated as percentage of the total number of seeded cells. DGKα silenced GSCs formed a significantly lower number of colonies (**Figure 8C**). When spheres generated at the first plating were dissociated and single cells were seeded on methylcellulose, control cells formed spheres with high efficiency, whereas DGKα silenced cells generated significantly fewer spheres, confirming reduced self-renewal capacity (**Figure 8C, D**), although DGKα expression has been partially recovered (**Figure 8E**).

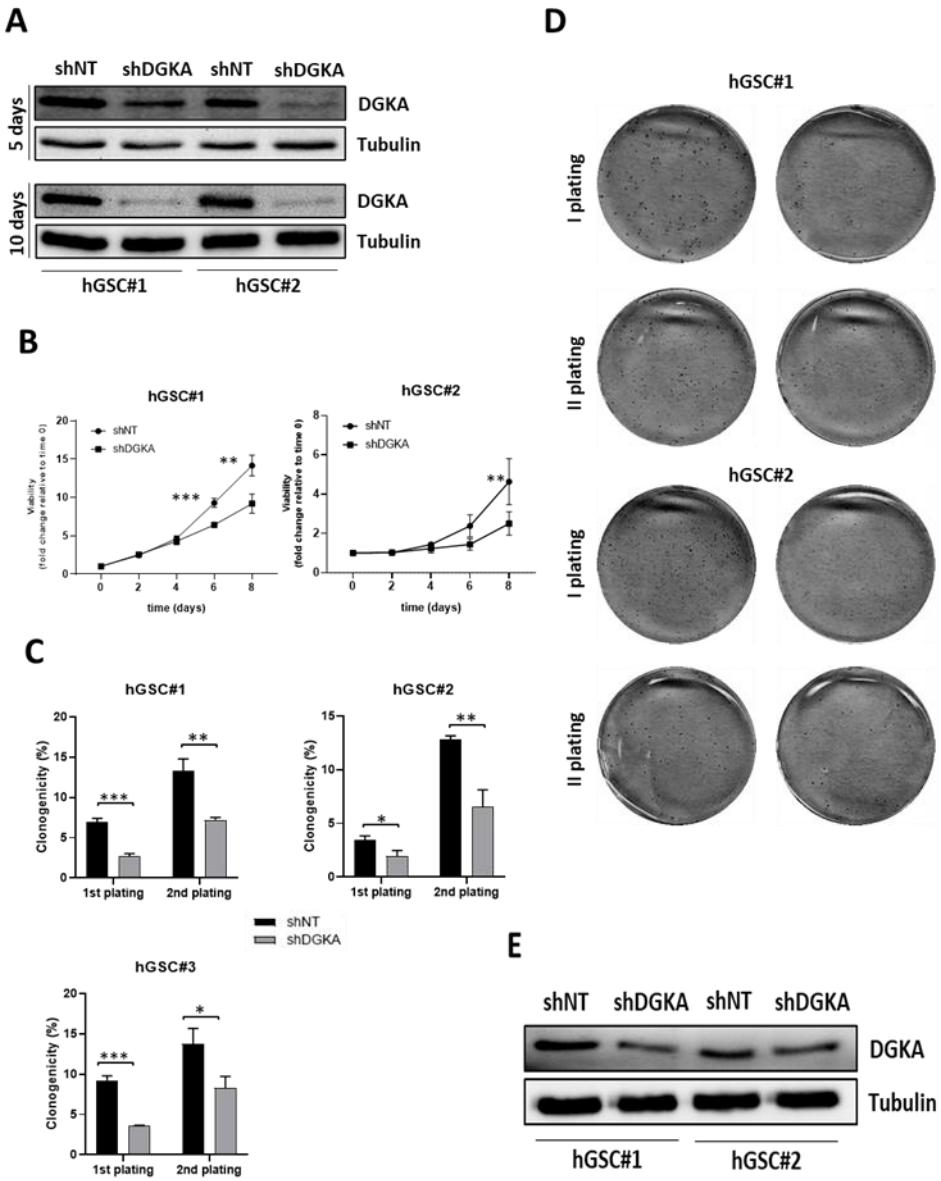


Figure 8. **DGKα silencing impairs GSCs viability and stemness. A)** DGKα silencing in GSCs. hGSC#1 and hGSC#2 were transduced with lentiviral vectors expressing non-targeting shRNA (shNT) or shRNA specific for DGKα (shDGKA) and lysed at days 5 and 10 after transduction. DGKα expression was evaluated by western blot. **B)** Effect of DGKα silencing on GSCs viability assayed by MTT. Two different GSCs lines (hGSC#1 and hGSC#2) were transduced with shNT or shDGKA and cell viability was assessed at days 2, 4, 6, 8. Results are presented as fold change relative to time zero. Data show mean ± SD of one representative experiment out of three independent experiments performed in quintuplicate. DGKα silencing significantly reduced GSCs viability at days 6 and 8. **, Student's T-test $p < 0.01$; ***, Student's T-test $p < 0.001$. **C)** Clonogenic assay performed on DGKα-silenced GSCs. Three different GSCs lines (hGSC#1, hGSC#2, and hGSC#3) were transduced with shNT or shDGKA and grown in methocult for 14 days. Cells were extracted, plated again in methocult and grown for 14 days. The histograms show clonogenicity calculated as percentage of the total number of seeded cells. Data are expressed as the mean % of colony formed ± SD of a representative experiment. DGKA silencing strongly reduced the clonogenic potential of the 3 CSCs tested at both I and II plating, indicating a depletion of the cancer stem cells compartment. *, Student's T-test $p < 0.05$; **, Student's T-test $p < 0.01$; ***, Student's T-test $p < 0.001$. **D)** Representative images of GSCs grown in methocult. Colonies were stained with MTT. **E)** hGSC#1and hGSC#2 extracted from the II plating in methocult were lysed and DGKα expression was evaluated by western blot.

Consistent with cell viability data, DGKα silencing slightly reduced GSCs proliferation rate, as demonstrated by measuring the reduction of CFSE fluorescence at day 5 post-transduction (*p*-value < 0.05 in all cases analyzed; **Figure 9A, B).** Analysis of DNA content by flow cytometry revealed at day 4 post-transduction that depletion of DGKα caused the early accumulation of cells in S phase accompanied by the decrease in the percentage of cells in G0/G1 phase followed, at day 7 post-transduction of a significant increase of cells in subG0 (**Figure 9C, D**).

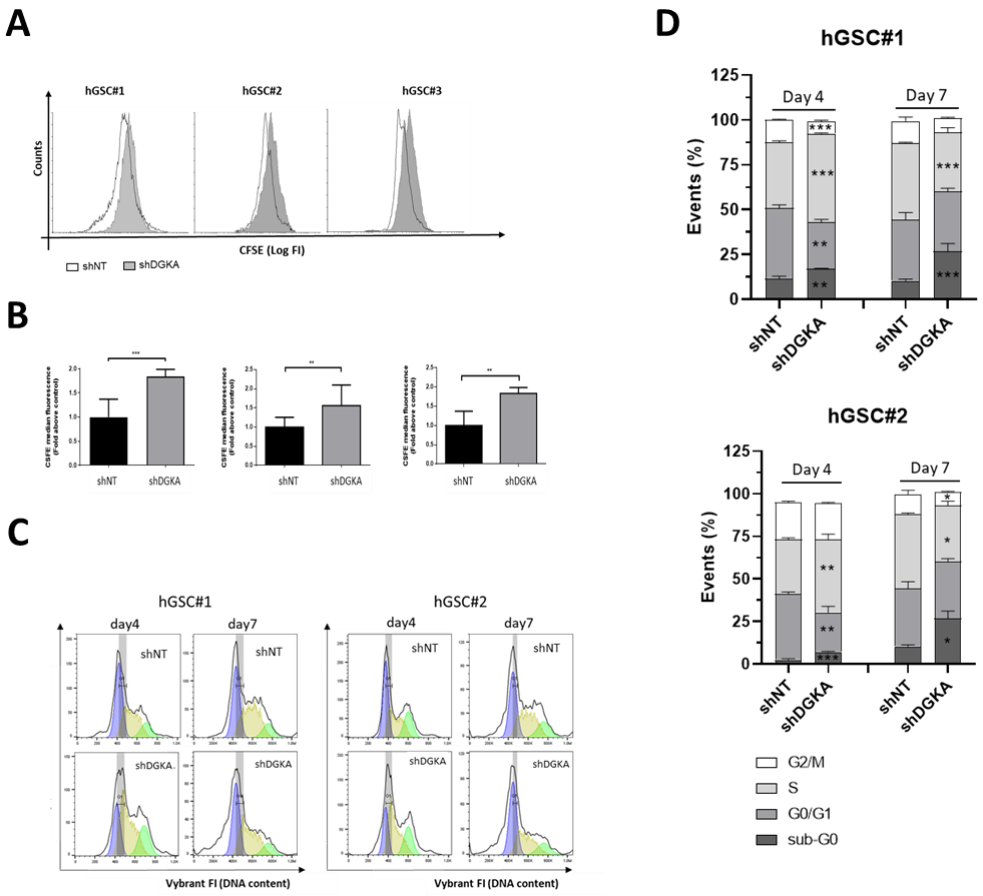


Figure 9. Cell cycle alteration in DGKA silenced cells. A) Evaluation of proliferation of GSCs by CSFE. Three different GSCs lines (hGSC#1, hGSC#2, hGSC#3) were transduced with shNT or shDGKA and labeled with CSFE. Cell proliferation was measured as CSFE dilution by FACS at day 5 post-staining. **B**) Cell proliferation was assessed on the basis of the median fluorescence intensity of CSFE. Results are presented as fold change relative to control (shNT). Data are expressed as the mean ± SD of 3 independent experiments performed in triplicate. DGKA silencing strongly reduced GSCs proliferation, as shown by the higher fluorescence signal in shDGKA-transduced GSCs respect to shNT-transduced control GSCs. **, Student's T-test $p < 0.01$; ***, Student's T-test $p < 0.001$. **C**) Cell cycle analysis in GSCs. Two different GSCs lines (hGSC#1 and hGSC#2) were transduced with shNT or shDGKA. DNA content was evaluated by Vybrant staining at days 4 and 7 after transduction. Representative FACS plot depicting sub-G0, G0/G1, S, and G2/M phases of cell cycle are shown. **D**) The % of events in each phase of cell cycle was evaluated by the FlowJo software "cell cycle analysis tool". Data show mean ± SD of one representative experiment out of three independent experiments performed in triplicate. *Student's T-test $p < 0.05$; **, Student's T-test $p < 0.01$; ***, Student's T-test $p < 0.001$

Silencing of DGKα reduced long-term viability also of differentiated GB cell lines (**Figure 10A**) and their anchorage independent growth capacity as shown by the reduced number of colonies grown in soft agar **Figure 10B, C**).

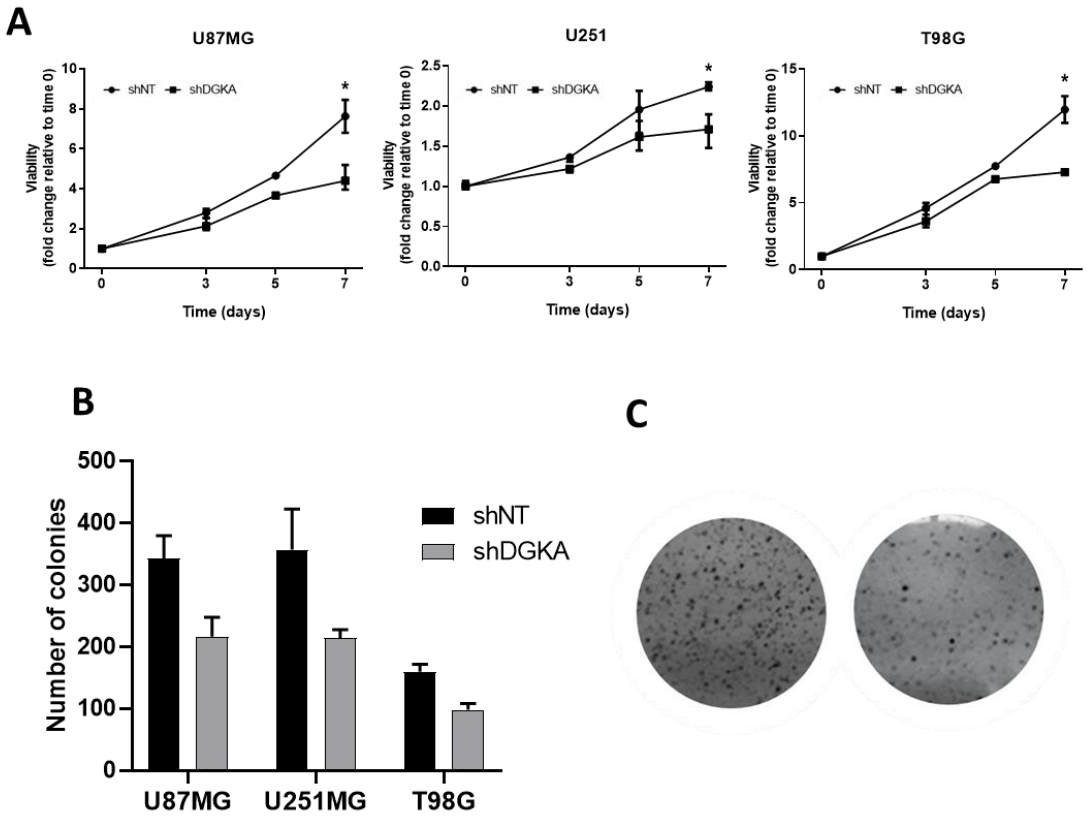


Figure 10. Silencing of DGKA reduced long-term viability and anchorage independent growth of differentiated GB cell lines. A) Effect of DGKα silencing on traditional GB cell lines viability assayed by MTT. Three different GB lines (U87MG, T98G, U251) were transduced with shNT or shDGKA and cell viability was assessed at days 3, 5, 7. Data are expressed as the mean ± SD of 1 representative experiment out of three independent experiments performed in quintuplicate. DGKα silencing reduces the viability of all the GB cell lines tested at day 7. *, Student's T-test $p < 0.05$. **B)** Soft agar assay performed on DGKA-silenced GB cell lines. Three different GB cell lines (T98G, U251, U87MG) were transduced with shNT or shDGKA and grown in soft agar for 14 days. The histograms show the number of grown colonies. Data are expressed as the mean number of colonies formed ± SD of a representative experiment out of three independent experiments performed in triplicate. A reduction in the number of colonies grown in soft-agar was observed in DGKα-silenced GB cell lines. **C)** Representative images of U87MG MTT-stained colonies are shown.

3.2 Short-term exposure to DGKs inhibitors is cytotoxic only at very high concentrations. Pharmacological inhibition of DGK always results in complicated interpretations since there drugs so far used were no isoform specific inhibitors, mainly if used at high micromolar concentrations (Sato M, 2013; Jiang Y, 2000). Here, we evaluated the acute cytotoxic effect of various DGKs inhibitors including the most characterized, namely, DGK inhibitor I (R59022) and DGK inhibitor II (R59949); ritanserin, a 5-HT2R antagonist structurally related to R59022 that was demonstrated to inhibit also DGKα activity (Boroda S, 2017); CU3 (Liu K, 2016) and AMB639752, a novel DGK inhibitor recently identified by Baldanzi group via virtual screening (Velnati S, 2019). Differentiated GB cell lines and GSCs were exposes for 72 hours to scalar concentration of drugs and cell viability was assessed by MTT assay (**Figure 11**).

None of the tested DGKα inhibitors caused acute toxicity at concentrations lower than 15 mM. Overall, DGKs inhibitors and, in particular, ritanserin, R59949 and R59022 showed a higher toxicity towards GSCs compared to other compounds. Ritanserin was the most effective drug, with an IC_{50} lower than 15 mM in 3 out of 4 GSCs tested. AMB639752 and CU3 reduced cell viability by less than 50% even at the highest drug concentration (60 mM).

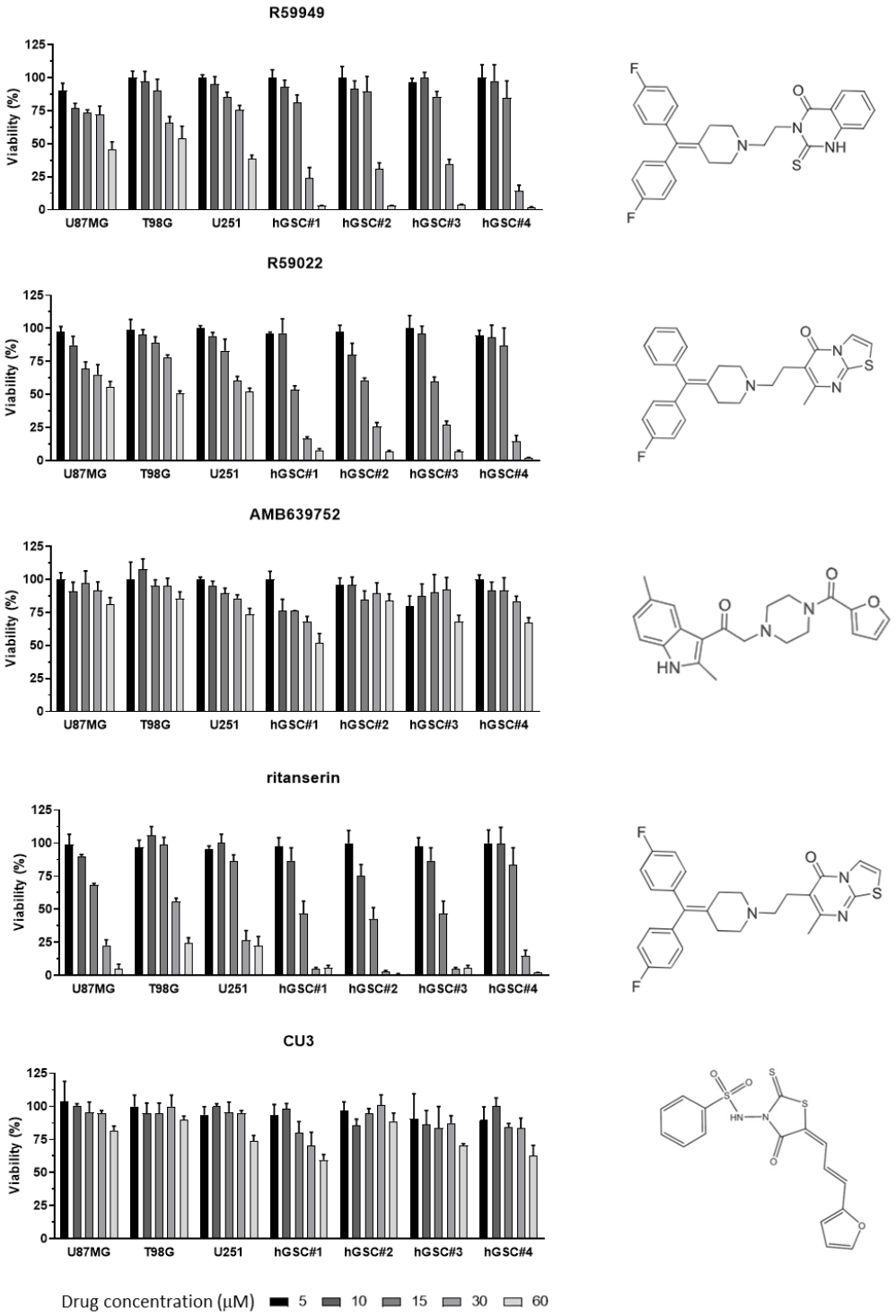


Figure 11. Effect of DGKα pharmacological inhibition on GB cells viability. Five different DGKα pharmacological inhibitors (R59949, R59022, AMB639752, ritanserin, and CU3) were tested on both differentiated GB cell lines (U87MG, T98G, U251) and hGSCs (hGSC#1, hGSC#2, hGSC#3, hGSC#4). The drugs were added at various concentrations (5, 10, 15, 30, 60 μM) and cell viability was assessed at day 3 by MTT.

Then we investigated the effect on GSCs of long-term exposure to R59949, R59022 and AMB639752. hGSC#1 viability was significantly reduced after 7 days of treatment only with drugs concentration >10 μM (**Figure 12**). Overall, our results show that these compounds are significantly toxic for GB cells only at very high doses whereas, at therapeutically significant concentrations, they moderately affect cells viability only after long-term exposure.

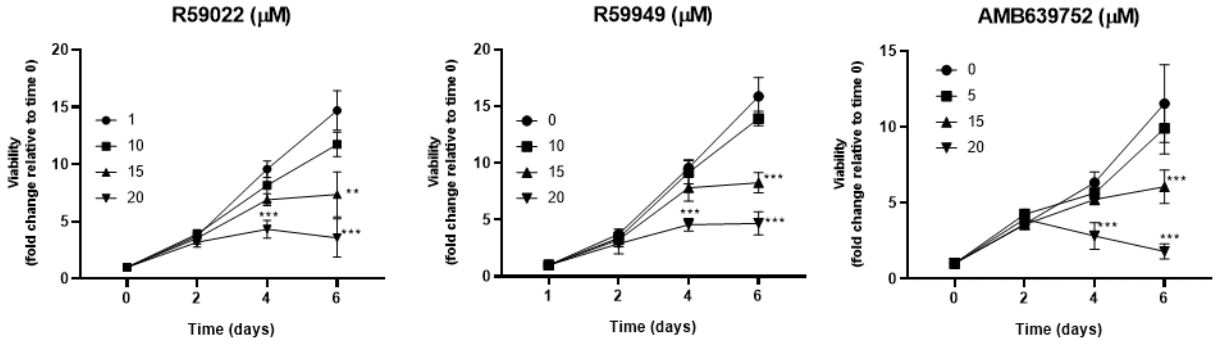


Figure 12. Inhibition of DGKα enzymatic activity reduced long term viability of GSCs. Effect of DGKα pharmacological inhibition on GSCs viability assayed by MTT. hGSC#1 were treated with various concentrations of 3 different DGKα pharmacological inhibitors (R59022, R59949, AMB639752). Cell viability was assessed at days 2, 4, 6. Results are presented as fold change relative to time zero. Data are expressed as the mean ± SD of 3 independent experiments performed in quintuplicate. A significant reduction of cell viability was detected only at concentrations of 15 μM and 20 μM for all the inhibitor tested. **, Student's T-test $p < 0.01$; ***, Student's T-test $p < 0.001$

3.3 Inhibition of DGKα enzymatic activity impairs self-renewal and clonogenic potential of GSC. Experimental evidence suggests that DGKα protein is necessary for GB tumor growth conceivably through its enzymatic activity. R59949 was reported to be efficient in blocking mainly DGKα among the Ca^{2+}-dependent type I DGK isoforms (de Chaffoy de Courcelles DC, 1985; de Chaffoy de Courcelles D, 1989, Sakane F, 1989; Jiang Y, 2000) and was demonstrated to reduce colony formation and anchorage independent growth of different cancer cell lines (Baldanzi G, 2011; Filigheddu N, 2011; Torres-Ayuso P, 2014). Since DGKα is the most expressed type I isoform in GB (data not shown), and R59949 reduces GSCs viability (**Figure 12**), we investigated the effect of R59949 on self-renewal and tumorigenic potential of GSCs by evaluating the consequences of long-term inhibition of DGKα. We grew GSCs for 5 days in the presence of R59949, then we removed the drug and plated cells in semisolid-medium without drug. After 15 days of culture we observed a significant lower number of neurospheres with reduced dimension derived from cells pre-treated with R59949 compared to control (**Figure 13**). These observations

strongly suggest that long term loss of DGKα enzymatic activity is sufficient to compromise stemness and tumorigenic potential of GSCs.

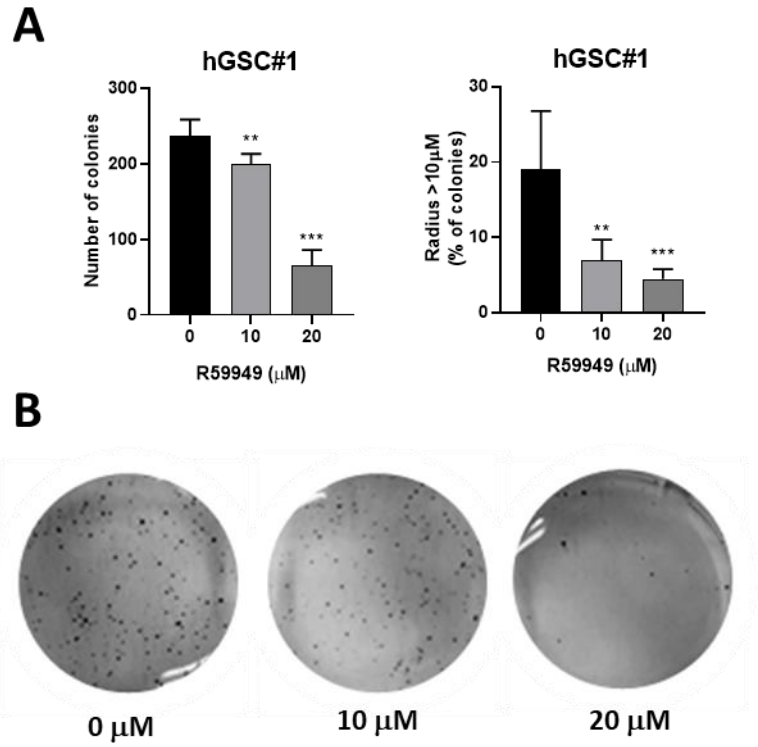


Figure 13. Inhibition of DGKα enzymatic activity reduced GSCs clonogenicity. Clonogenic assay performed on hGSC#1 pre-treated with DGKα inhibitor. hGSC1 cells were treated with R59949 (10-20 μM) for 5 days and then plated as single cells in methocult and let to grow for 14 days without drug. Cells were stained with MTT and counted. (**A**) The histograms show the number of colonies detected (left panel) ± SD and the % of colonies with radius >10 μM (right panel) ± SD of a representative experiment. Pretreatment of hGSC#1 with R59949 is sufficient to deplete the cancer stem cell compartment, as depicted by the reduced number of colonies detected. **, Student's T-test $p < 0.01$; ***, Student's T-test $p < 0.001$. (**B**) representative images of MTT stained colonies

3.4 Inhibition of DGKα activity increases sensitivity of GB to TMZ. Since our data suggest that DGKα is necessary to maintain GB stem-like features and GSCs are highly resistant to chemotherapy (Chen J, 2012; Eramo A, 2006), we aimed to investigate the possible employment of DGKα pharmacological inhibitors as adjuvants in GB therapy evaluating their effect on sensitivity of GB to TMZ. hGSC#1 were pre-treated for 72h with AMB639752 (10 μM) and then treated overnight with TZM (125 μM). TMZ was removed and cells were seeded in methylcellulose. Spheres were counted at day 14. Whereas hGSC#1 treated with AMB639752

alone showed a modest, although not significant, decrease in the number of colonies compared to control, GSC#1 treated with TMZ + AMB639752 demonstrated more than 50% reduction in sphere formation when compared to cells treated with TZM alone ($p<0.05$) (**Figure 14**).

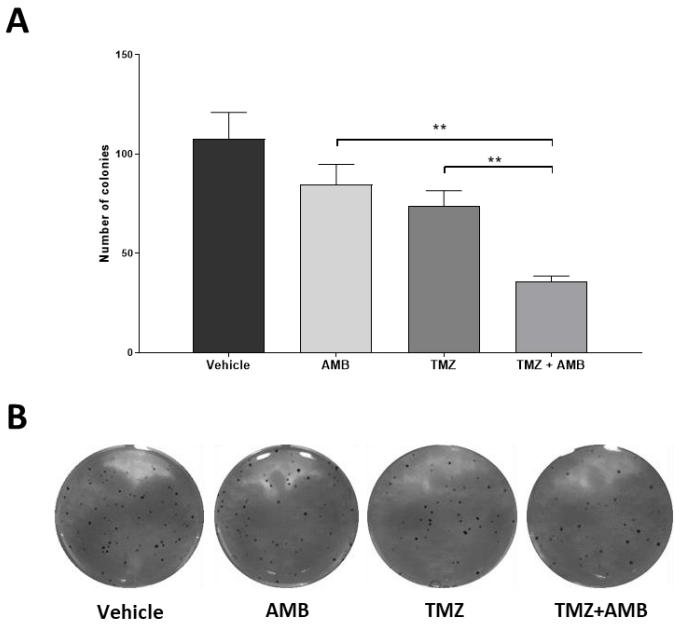


Figure 14. Pharmacological inhibition of DGKα sensitizes GSCs to TMZ. A) Colony formation assay in hGSCs#1 treated with AMB639752, TMZ or both. hGSC#1 were grown for 3 days in the presence of 10 μM AMB639752 (AMB) or DMSO (Vehicle) and then overnight with 125 μM TMZ (TMZ and TMZ + AMB). The drug was removed, and the cells were grown in methocult for 14 days. The histograms show the number of grown colonies. Data are expressed as the mean number of colonies formed ± SD of a representative experiment out of three independent experiments performed in triplicate. DGKα inhibition by AMB639752 treatment significantly increases the efficacy of TMZ on GSCs. **, Student's T-test $p < 0.01$. **B)** Representative images of MTT-stained colonies are shown.

We also evaluated the effect of combined treatment with DGKα inhibitor and TMZ on viability of both GSCs growth as neurospheres and differentiated adherent cell lines. GB cells were incubated in culture medium containing 10 μM AMB639752, TMZ(5-25-125μM) or a combination of 10 μM AMB639752 and TMZ (5-25-125μM) for 96 hours. Combined treatment with TMZ and DGKα inhibitor AMB639752 significantly enhanced cell sensitivity to TMZ, reducing IC_{50} values in all cell lines tested (**Table 2**; **Figure 15**). AMB639752 alone showed no effect on viability in all cell lines tested (**Figure 15**).

Table 2. Effect of DGKα inhibitor on TMZ sensitivity

	IC_{50} (μM) ± SD	
	TMZ	**TMZ+AMB**
T98G	260 + 124.5	178 + 17.42
U87MG	129 + 11.0	92.3 + 5.87
U251	192 + 36.2	66.2 + 3.56
hGSC#1	167 + 21.6	87.0 + 10.4
hGSC#2	147.4 + 12.2	82.4 + 8.39

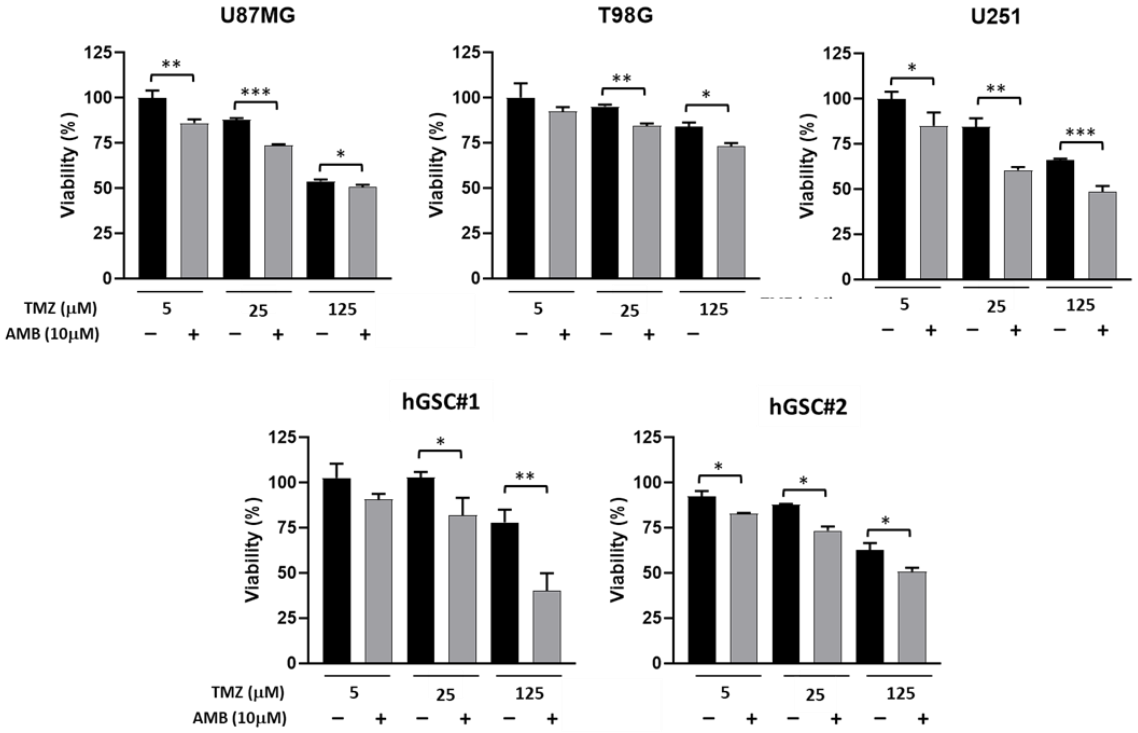


Figure 15. Efficacy of cotreatment with TMZ and AMB639752 on hGSCs and GB cell lines. Effect of AMB and TMZ treatment on GB cell lines and hGSCs lines. Viability was assayed by MTT. Differentiated GB cell lines (U87MG, T98G, U251) and hGSCs (hGSC#1 and hGSC#2) were treated with TMZ alone (5-25-125 μM) or in presence of the DGKα inhibitor AMB639752 (10 μM). Results are presented as % of viability relative to control cells treated with vehicle only. Data show mean ± SD of one representative experiment out of three independent experiments performed in quadruplicate. An enhanced sensitivity to TMZ treatment was observed when DGKα is pharmacologically inhibited. *Student's T-test $p < 0.05$; **Student's T-test $p < 0.01$; ***Student's T-test $p < 0.001$

To confirm the specific role of DGKα inhibition in inducing chemosensitization we treated hGSCs#1 transduced with shDGKA or shNT overnight with TMZ (125 μM). Cells were seeded in methylcellulose and spheres were counted at day 14. A significant reduction in the number of colonies was observed in DGKα silenced hGSC#1 treated with TMZ compared to both non-treated DGKα silenced cells and TMZ-treated shNT controls ($p<0.01$; **Figure 16A**). A significant reduction in colony area was also observed in TMZ-treated DGKα silenced hGSC#1 compared to TMZ-treated shNT hGSC#1 ($p<0.01$; **Figure 16B).**

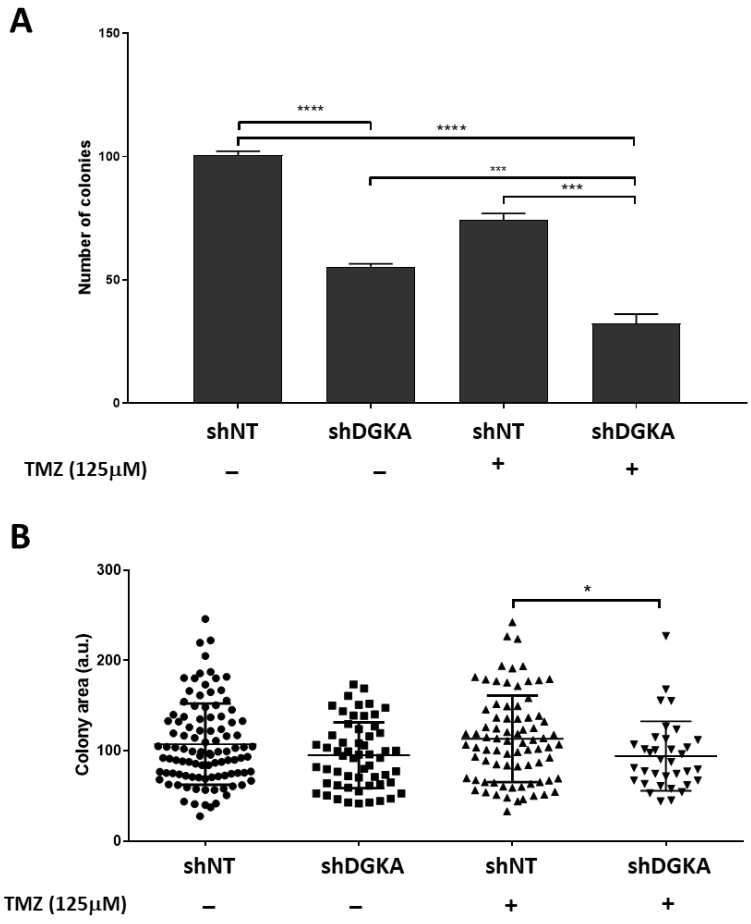


Figure 16. 14. Knock down of DGKα sensitizes GSCs to TMZ. A) Clonogenic assay performed on DGKα-silenced GSCs treated with TMZ. hGSC#1, were transduced with shNT or shDGKA and treated with TMZ overnight before plating in methocult for 14 days. The histograms show total number colonies of seeded cells. Data are expressed as the mean of triplicate ± SD. DGKα silencing strongly reduced the clonogenic potential of hGSC#1 and clonogenic potential further reduced in presence of TMZ. ***, Student's T-test $p < 0.001$; ****, Student's T-test $P \leq 0.000$. **B**) Scatter plot showing colony area of each individual colony.

3.5 DGKα silencing is associated to expression of aberrant differentiation markers in GSCs. To deeper investigate the role of DGKα in stemness maintenance of GSCs, we examined the expression of differentiation and EMT-related markers in hGSCs transduced with shDGKA and controls growth as neurospheres in CSC medium by real-time quantitative PCR (RTQ-PCR) and western blotting (**Figure 17**). In hGSC#1, the mRNA expression of CD133, SOX2, SOX4 and SOX8 was significantly reduced in DGKα silenced GSCs compared to control; on the contrary, the expression of the glial differentiation marker, glial fibrillary acidic protein (GFAP), and neural cell differentiation marker, β3-tubulin (TUBB3) was significantly higher in DGKα silenced cells (**Figure 17A**). Overexpression of TUBB3 and GFAP was further confirmed at the protein level by western blotting (**Figure 17B**). Similar results were obtained in hGSC#2 and hGSC#3 (data not shown).

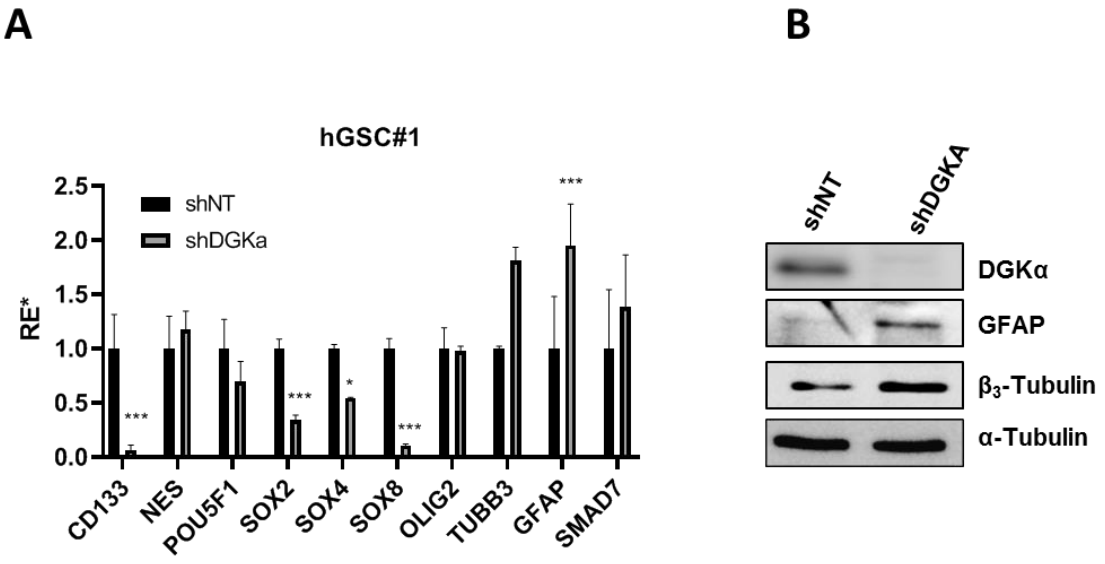


Figure 17. Aberrant expression of markers of differentiation in GSCs. A) mRNA expression was evaluated by the ΔΔCt method, using GUSB as a control gene. RE*, fold change expression relative to control (neurospheres after 7 days from transduction with shNT). *Student's T-test $p < 0.05$; **, Student's T-test $p < 0.01$; ***, Student's T-test $p < 0.001$. **B)** Western blot analysis showed an increase in the expression of GFAP and β_3-tubulin in DGKα silenced hGSC#1

3.6 DGKα is required for dedifferentiation of GB cells into GSCs. Environmental and therapeutic stress can promote cellular plasticity, enhancing the conversion of non-stem GB cells to highly aggressive, tumor initiating GSCs which contribute to the high invasiveness and resultant poor outcome in GB patients (Safa AR, 2015; Gimple RC, 2019).

Since DGKα silencing severely affect genes involved in stemness and differentiation in GSCs, we explored the capacity of DGKα silenced GB cells to revert back to a stem cell state. Toward this aim we examined the neurospheres formation rate of U87MG in stem cell medium (**Figure 18**). DGKα silenced U87MG showed a reduction of approximately 50% in neurospheres forming capacity compared to control after 7 day from plating (**Figure 18A**). Extreme limiting dilution assays (ELDA) demonstrated a significant reduction in neurospheres formation potential of DGKα silenced T98G and U87MG cells in comparison to the controls. The estimated stem cell frequency decreased from 1/22.5 to 1/145.6 for T98G and from 1/20.8 to 1/48 for U87MG (**Figure 18B**).

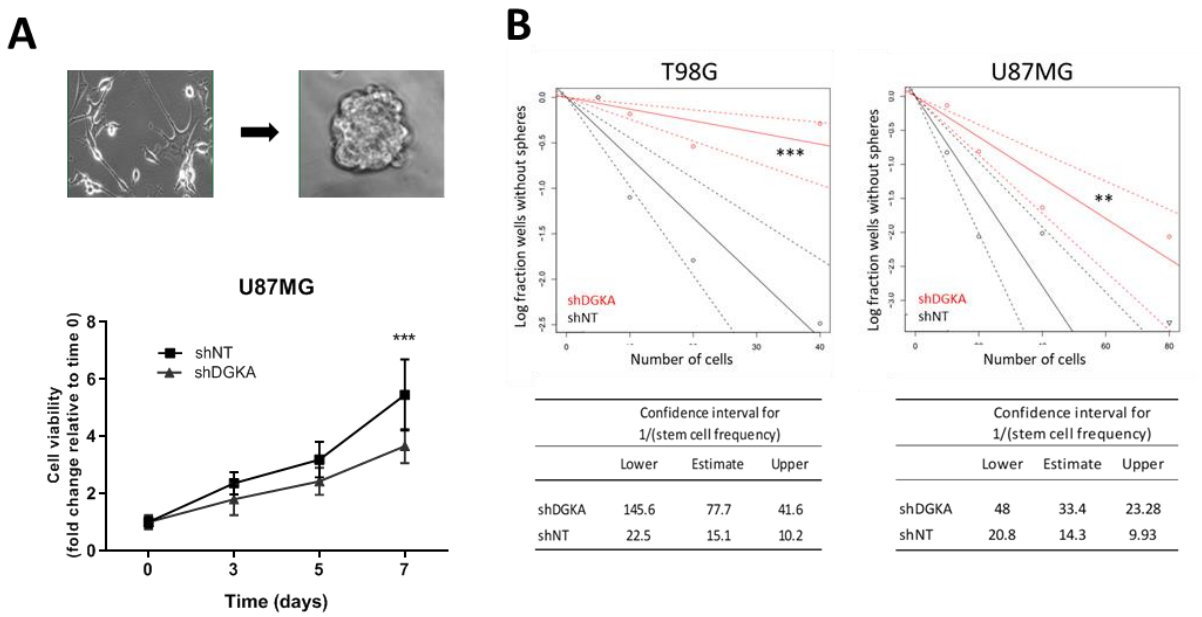


Figure 18. DGKα silencing affected GB cell lines dedifferentiation. A) Sphere formation assay performed on DGKα-silenced U87MG. U87MG cells were transduced with shNT or shDGKA and grown as neurospheres. Cell viability was assessed at days 3, 5, 7 by MTT. Results are presented as fold change relative to time zero. Data are expressed as the mean ± SD of 2 independent experiments performed in quintuplicate. DGKα silencing compromises dedifferentiation, as shown by the decreased in cell viability. ***Student's T-test $p < 0.001$. **B)** Extreme limiting dilution (ELDA) on DGKα-silenced GMB cell lines. T98G and U87MG cells were transduced with shNT or shDGKA and grown as neurospheres for 12 days. Estimation of stem cell frequency was performed using the ELDA software (http://bioinf.wehi.edu.au/software/elda/). DGKα silencing compromises dedifferentiation and self-renewal, as depicted by decreased stem cell frequencies. ** Chi-squared p≤0.01; *** Chi-squared p≤0.00001

3.7 DGKα is required for NF-kB transcriptional activity. Our data show that DGKα is involved in TZM chemoresistance and is required for maintenance of stem-like properties of GB cells. Activation of transcription factors by extrinsic cues such as the tumor microenvironment or therapeutic stimuli, can transform the genetic landscape of GB tumors, which can be reflected by an increased in invasion, proliferation, as well as therapeutic resistance (Mani SA, 2008; Bhat KPL, 2013). NF-kB is one of the major drivers of CSCs in several cancer and in GB (Rinkenbaugh AL, 2016; Soubannier V, Stifani S, 2017; Nougueria L, 2011), TNFα, one of the most potent activators of NF-kB, can be secreted in the tumour microenvironment by immune cells or tumour cells (Hayden MS, Ghosh S, 2014). TNFα can drive tumour plasticity and increase CSCs maintenance, as observed in numerous malignancies including GB (Bhat KPL, 2013; da Hora CC, 2019). Several evidences suggest a relationship between TNFR, DGKα and NF-kB activation. In one study, DGKα has been demonstrated to be implicated in suppression of TNFα induced apoptosis of human melanoma cells via NF-kB (Kai M, 2009) and, more recently, DGKα has been reported to be involved in AKT/NF-kB signalling in oesophageal squamous cell carcinoma (Chen J, 2018).

These evidenced prompted us to validate the hypothesis that DGKα is required in signal transduction downstream TNFR to activate NF-kB and other pathways necessary for stemness maintenance and therapy resistance of GSCs.

Fist we investigated the role of DGKα in NF-κB transcriptional activity induction by TNFα stimulation by means of a reporter-gene assay expressing the response element for NF-kB (**Figure 19**). The expression of the reporter gene was assessed in U87MG cells after overnight simulation with TNFα. Treatment with DGKα enzymatic inhibitor R59949 significantly attenuated both basal and TNFα induced NF-kB activity (**Figure 19A**). To confirm specificity of R59949 for DGKα, we transfected the reporter gene pGL4.32-Luc into shDGKA/shNT U87MG. As shown in **Figure 19B**, DGKα silenced U87MG cells exhibited negligible NF-kB transcriptional activity both at basal conditions and after overnight stimulation with TNFα.

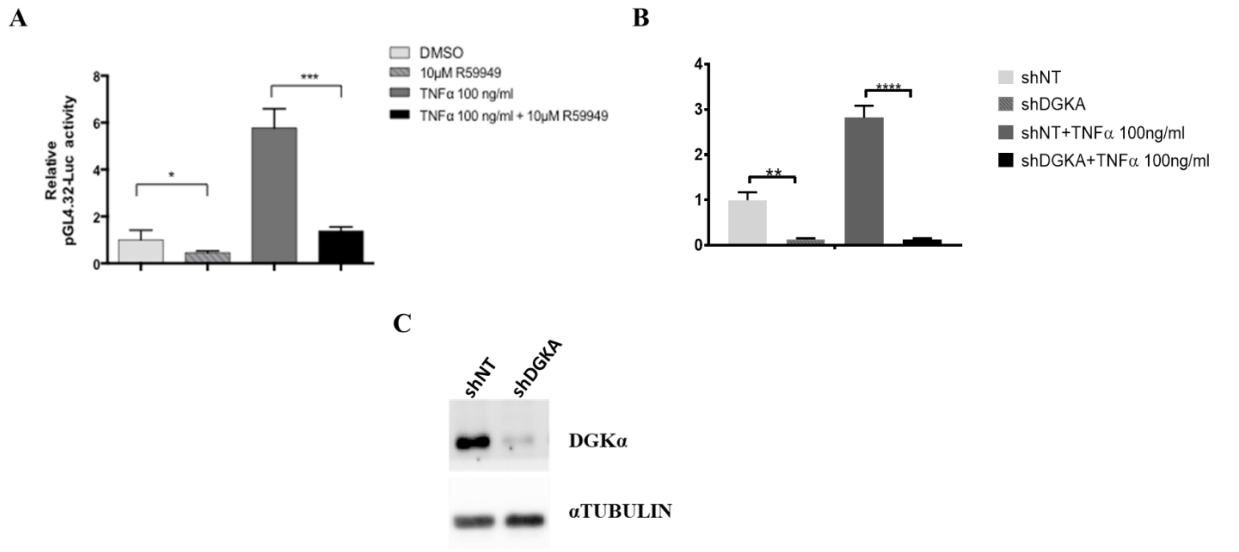


Figure 19. Evaluation of NF-kB transcriptional activity. NF-kB transcriptional activity was evaluated using the pGL4.32 [luc2P/NF-κB-RE/Hygro] Vector (Promega), containing five copies of an NFκB-RE that drives transcription of the luciferase reporter gene luc2P in U87MG cells treated with DGKα inhibitor (**A**) or transduced lentivirus expressing a DGKα specific shRNA (shDGKA) (**B**). The results are expressed as the relative luciferase activity (mean±SD of five determinations) compared with that obtained for the control cells). *, p<0.05; ***, p<0.001 relative luciferase activity (mean±sSD of five determinations). **(C)** western blot image showing DGKα knock down.

3.8 Loss of DGKα enzymatic activity impairs FAK and AKT activation downstream TNFα in U87MG cells

Since our data suggest that DGKα is required for NF-kB transcriptional activity upon TNFα stimulation, we investigated signaling downstream TNFα (**Figure 20**). After overnight stimulations with TNFα in starved conditions we can observe activation of AKT (phosphorylation in Ser473 and Ser308) and NF-kB (phosphorylation in Ser536) in control, but not in cells treated with R59949.

We further extended our analysis to FAK, a tyrosine kinase associated with focal adhesions, previously reported to be required for TNFα downstream signaling in several tumors (Schlaepfer DD, 2007; Mon NN, 2009) and, in particular, for NF-kB activation (Funakoshi-Tago M, 2003). Our data clearly show that TNFα activates FAK in vehicle treated cells but not in cells treated with R59949.

Since atypical PKCs (aPKCs) are required for paracrine TNFα-dependent activation of NF-kB (Estève PO 2002; Duran A, 2003; Raychaudhuri B, 2007; Kusne Y, 2014) and PA generated by DGKα recruits aPKCs making them available to PDK1-dependent activation at the plasma membrane (Chianale F, 2010), we investigated the activation status of aPKCζ. We found that, already under basal conditions, aPKCζ has a high phosphorylation status at the activation loop

threonine 410 that was not affected by treatment with TNFα and/or with DGKα inhibitors. NF-kB phosphorylation in Ser 311, specific target of aPKC (Estève PO 2002; Duran A, 2003; Raychaudhuri B, 2007; Kusne Y, 2014) was not affected by treatment with TNFα and/or with DGKα inhibitors.

We next investigated the expression of HIF1α, whose expression requires NF-kB activity downstream TNFα (Görlach A, Bonello S, 2008). We observed that R59949 treatment strongly reduces HIF1α expression in response to NF-κB.

TNFR levels in controls and in cells treated with DGKα inhibitors were comparable, suggesting that variations in downstream signaling was not caused by alterations in receptor exposure or degradation.

Notably, in cells treated with R59022 we observed a significant reduction of FAK Tyr 576/577 and NF-kB Ser 536 phosphorylation, and a very mild reduction of FAK Tyr 397 whereas all the other investigated markers were unaffected by this drug.

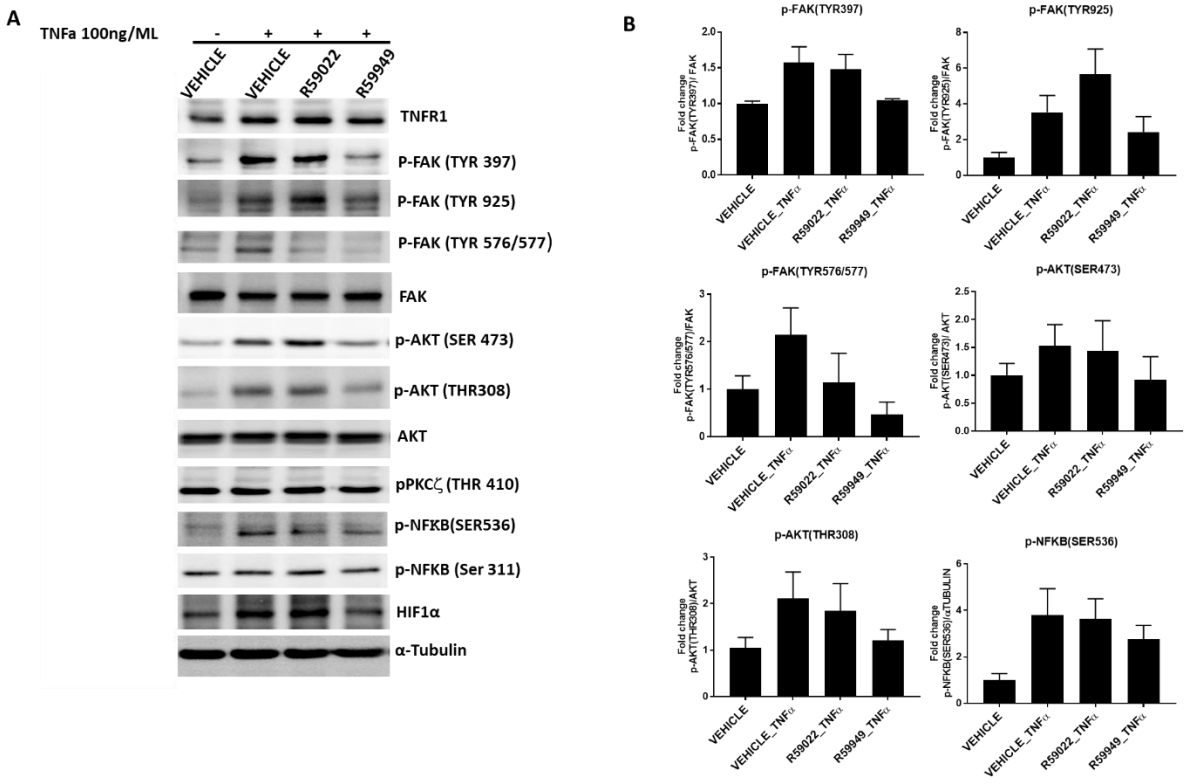


Figure 20. Pharmacological inhibition of DGKα enzyme by R59949 impairs TNFα signaling in U87MG GB cell line. A) Cells were treated with DGKα inhibitors R59949 and R59022 in absence of serum and stimulated with TNFa 100 ng/ml O/N and lysed. Proteins were analyzed by WB. **B)** densitometric analysis of protein activation normalized to total protein and expressed in fold change relative to vehicle (time 0) from two independent experiments.

Then we performed a time course analysis of U87MG cells treated with R59949 in response to TNFα (**Figure 21**). WB analysis shows the total lack of AKT activation in the cells treated with DGKα inhibitor and a strong downmodulation of NF-kB Ser 536 phosphorylation in response to TNFα.

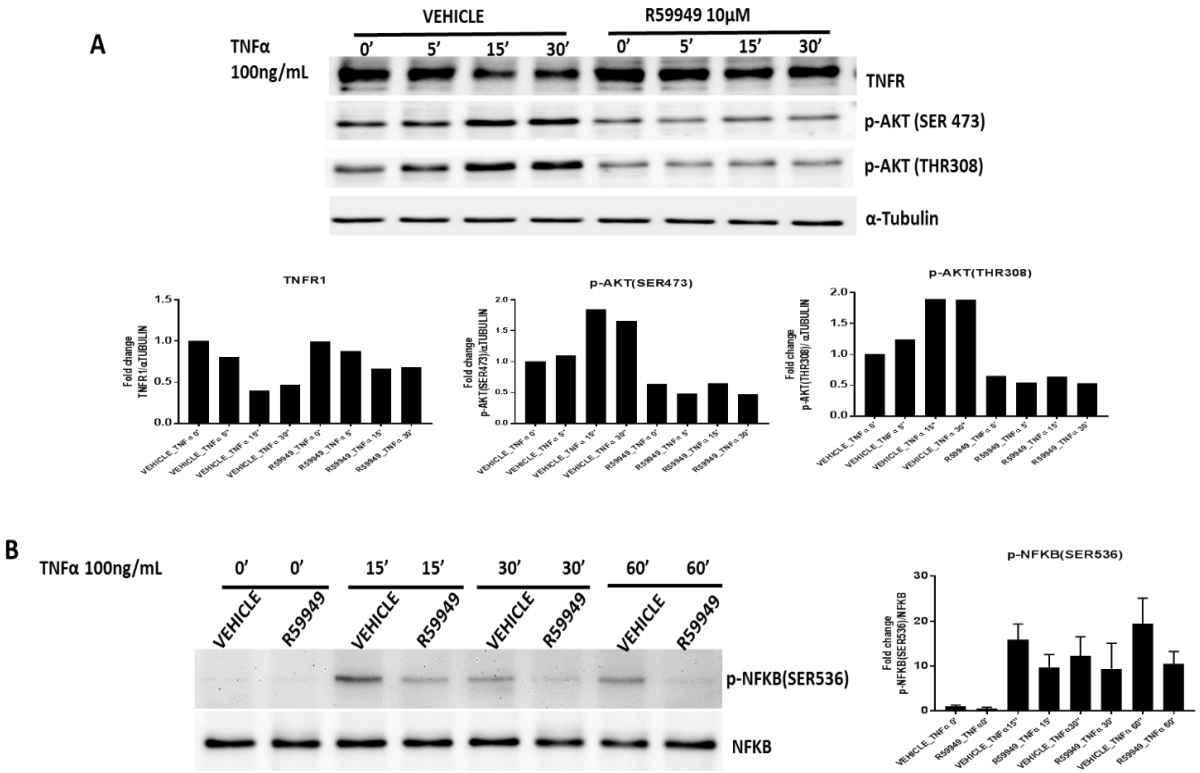


Figure 21. **Inhibition of DGKα enzymatic activity impairs TNFα signaling in U87MG GB cell line.** Cells were serum-starved and treated with 10μM R59949 overnight. Cells were then stimulated with TNFα 100 ng/ml and lysed at 0, 5, 15 and 30 minutes after stimulation and analyzed by Western Blot. (**A**), Western blot results indicate that R59949 down modulate the activation of p-AKT(SER473) and p-AKT(THR308). Lower panel shows densitometric analysis of protein activation normalized to total protein and expressed in fold change relative to control (time 0). Results from a representative experiment are shown (**B)** Representative western blot results indicate that R59949 down modulate the activation of p-NF-kB (SER 536). Lower panel shows densitometric analysis of protein activation normalized to total protein and expressed in fold change relative to vehicle (time 0) from two independent experiments.

In order to validate the role of DGKα in TNFα signaling and NF-κB activation, U87MG cells were transduced with shRNAs specific for DGKα (shDGKA) or with shRNA control (shC) and then stimulated with TNFα (**Figure 22**).

A reduction of phosphorylation of both p-NF-kB (Ser 536), p-FAK (Tyr 925) p-FAK (Tyr 397) was confirmed in U87MG after silencing of DGKα gene. Notably, although also in this experimental model, PKCζ Thr 410 was already phosphorylated at basal conditions and was not affected by treatment with TNFα, its phosphorylation was significantly reduced in DGKα-silenced

cells, suggesting that DGKα protein is required for aPKCζ activation by phosphorylation in the activation loop.

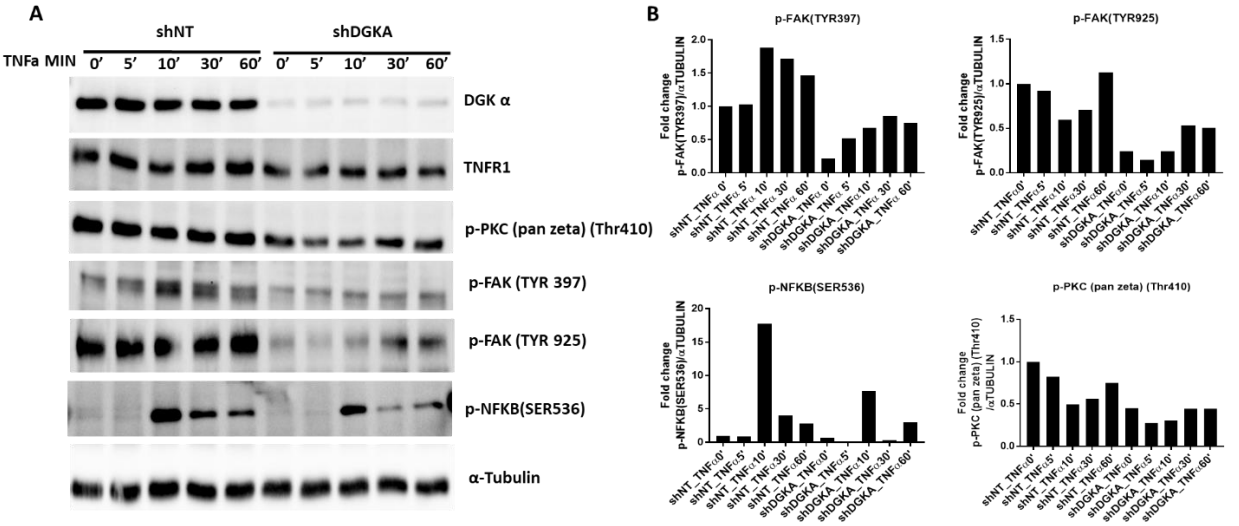


Figure 22. DGKα is required for TNFα signaling in U87MG GB cell line. A) Cells were starved ON. Cells were then stimulated with TNFα (100 ng/ml) and lysed at 0, 5, 10, 30, and 60 minutes after stimulation and proteins were analysed by WB. Results from a representative experiments are shown **B**) densitometric analysis normalized to total protein and expressed in fold change relative to control (time 0).

4. Discussion

GBM is a devastating and still intractable disease. The current standard of care includes maximal safe surgical resection, followed by a combination of radiation and chemotherapy with temozolomide (Stupp R, 2009; Weller M, 2014). Despite that, remnant tumor cells undergo active biological progression and recurrence is inevitable (Kim J, 2015; Chen J, 2012), and so continue search for more effective treatments both for initial therapy and at the time of recurrence is mandatory.

Personalized therapies against molecular targets that drive the growth of the bulk of primary tumors have so far been unsuccessful in clinical trials, warranting new approaches (Lau D, 2014; Bastien JI, 2015; Mooney J, 2019). The activation of multiple receptor tyrosine kinases and/or downstream pathways can account for tumor progression and therapy resistance. Identifying and targeting such pathways can improve therapeutic efficacy, although such efforts may require simultaneously disabling multiple, parallel oncogenic signals.

DGKα, by modulating PA and DG levels contribute to regulate multiple signaling pathways. Recent evidence suggests this enzyme as a promising new target in the fight against cancer, with DGKα inhibition exhibiting multiple anticancer mechanisms of action (Purow B, 2015; Merida I, 2017). Downstream effects of DGKα in cancer may be due largely to modulation of total PA, or specific PA molecules, or PA in specific cellular locations, as well as to reduction of DG (Sakane F, 2007; Kai M, 2009; Baldanzi G, 2014).

In this study we show that DGKα is required for GSCs growth and stemness maintenance. Loss of DGKα activity progressively reduces GSCs growth and their *in vitro* clonogenic potential. The requirement of DGKα for stemness maintenance and tumorigenic potential has been further corroborated by our group by *in vivo* limiting dilution assays in xenograft models, demonstrating a reduction of the number of tumor initiating cells in GSCs silenced for DGKα (Pelicci G, personal communication).

Although DGKα inhibitors showed acute cytotoxicity only at high doses, long-term exposure to R59949 at therapeutically relevant concentrations was able to reduce both *in vitro* clonogenic potential (this study) and *in vivo* tumorigenic capacity (Pelicci G, personal communication) of GSCs, suggesting that long term loss of DGKα enzymatic activity is sufficient to shape tumor phenotype and definitively compromising stemness and tumorigenic potential of GSCs. R59949 was previously demonstrated to reduce colony formation and anchorage independent growth in

different cancer cell lines (Baldanzi G, 2011; Filigheddu N, 2011; Torres-Ayusoet P, 2014), whereas in vivo treatment with R59022 reduced tumor growth in GB and melanoma xenograft models (Dominguez CL, 2013). Though these observations support a future clinical use of DGKα pharmacological inhibitors as anticancer agents, their efficacy is limited by their rapid clearance (Purow B, 2015). Furthermore, these inhibitor are also able to target different isoforms of DGKs (Jiang Y, 2000; Sato M, 2013) and a study conducted by Boroda et al. recently demonstrated their strong antagonistic activity on 5-HT2 receptors (Boroda S, 2017). Notably, in our study, U87MG cells response to TNFα, in presence of R59022, was not entirely superimposable to that observed with R59949. For all these reasons, the identification of more efficient and selective DGKα inhibitors is mandatory for targeting this kinase in patients.

In our study neither DGKα silencing or treatment with enzymatic inhibitors at clinically relevant concentrations demonstrated short term cytotoxicity. Our data are in contrast with previous observations in melanoma (Yamaki A, 2019), HepG2 and Hela cell lines (Liu K, 2016) but also in GB (Dominguez CL, 2013), where DGKα inhibitors were reported to rapidly reduce cell viability with induction of apoptosis. The different tumor models and the exposure to high doses of drug utilized in those studies can explain the different observations. On the other hand, in other tumor models, i.e. Kaposi Sarcoma (Baldanzi G, 2011) or leukemia (Poli A, 2017) inhibition of DGKα activity was reported to reduce cell proliferation without inducing short term apoptosis, reflecting our observations in GSCs, whereas in colon cancer, DGKα was necessary for 3D tumor growth but was dispensable in a 2D context (Torres-Ayuso P, 2014).

Even if TMZ is the standard of care in the treatment of GB, its effect on overall survival remains modest (Stupp R, 2009). Moreover, TMZ resistance is inevitable, resulting in GB recurrence that now become untreatable (Osuka S, 2017). Recurrence initiating cancer cells emerge from the residual tumor cell population that survived therapy and have stem-like properties, because they can initiate a recurrent GB with a diversity of tumor cells; for this reason the higher the capacity to eradicate GSCs in primary tumor, the less likely it is to have relapse.

Since our data suggest that DGKα is necessary to maintain GB stem-like features and GSCs are strongly resistant to chemotherapy (Chen J, 2012), we tested DGKα specific pharmacological inhibitor AMB639752 for its capacity to sensitize GB to TMZ. We observed that both co-treatment and pre-treatment of GSCs with DGKα inhibitor were able to enhance cell sensitivity to TMZ. The specific role of DGKα in chemoresistance was also confirmed by gene silencing.

A growing number of molecular pathways have been associated with therapeutic resistance in GSCs and should be particularly relevant to recurrence initiating cancer cells. Survival signaling pathways are activated through autocrine or paracrine secretion of growth factors/cytokines, as well as homotypic tumor cell contacts and heterotypic tumor-stroma interactions, involving tumor cells, endothelial cells, and immune cells (Schonberg DL, 2014; Lathia JD, 2015; Osuka S, 2017). In cancer, a host of molecular pathways involved in therapy resistance are altered by DGKs, including HIF1α (Temes E, 2004; Temes E, 2005), mTOR (Fang Y, 2001; Veverka V, 2008), c-Met/HGF (Filigheddu N, 2007; Baldanzi G, 2011), and VEGF (Baldanzi G, 2004).

The above signals are integrated through the activation of a limited number of transcription factors that control a variety of functions underlying GSC maintenance, including survival, self-renewal, proliferation, metabolism, and stemness state. Our data show that silencing of DGKα is associated with reduction in the mRNA expression of CD133, a distinctive markers of GSCs stemness (Glumac PM, LeBeau AM, 2018) and SOX2, SOX4 and SOX8 transcriptional factors that are typically increased in GSCs (Ikushima H, 2011), activate DNA damage repair pathways that contribute to the therapeutic resistance of GSCs (Huang Z, 2010; Lathia JD, 2015) and are associated with cancer poor prognosis (Grimm D, 2019).

Notably, DGKα silencing also induced the expression of the glial differentiation marker GFAP and neural marker TUBB3. GFAP is an intermediate filament which is expressed in mature astrocytes in the nervous system and in GB is used to determine glial differentiation, which is associated with a less malignant tumor (Middeldorp J, Hol EM, 2011). Its expression was also associated with astrocytic differentiation and senescence induced by metabolic reprogramming in GB (Xing F, 2017). Both GFAP and TUBB3 are upregulated in response to differentiating therapies (Chao CC, 2015; Ciechomska IA, 2016; Zhou D, 2018) and their expression in GB is associated with induction of autophagy, reduction of stem-like properties and tumor grown reduction (Guichet PO, 2013; Ciechomska IA, 2016; Arif T, 2017)

Our data suggest that induction of differentiation is a possible mechanism by with DGKα depletion reduces the tumorigenic potential of GSCs. This hypothesis is corroborated by recent data reported by Olmez et al showing that treatment of GSCs with ritanserin preferentially kills the mesenchymal subtype of GSCs and causes a switch from a mesenchymal to proneural subtype (Olmez I, 2018).

Moreover, our study also demonstrates that DGKα is necessary for successful dedifferentiation of adherent differentiated cells into neurospheres. This aspect is quite relevant for GB therapy since both environmental and therapeutic stress can promote cellular plasticity, enhancing the

conversion of non-stem GB cells to highly aggressive, tumor initiating GSCs which contribute to the high invasiveness and resultant poor outcome in GB patients (Safa AR, 2015; Gimple RC, 2019).

Activation of transcription factors by extrinsic factors such as the tumor microenvironment or therapeutic stimuli, can shape the phenotypic features of GB cells with acquisition of self-renewal capacity, therapeutic resistance and tumor progression (Mani SA, 2008).

Nf-kB is one of the major drivers of GSC, resistance to TMZ and negative prognosis in patients with GB (Cahill KE, 2016; Bhat KPL, 2013; Nogueira L, 2011), and is activated by growth factors, inflammatory mediators and DNA damaging agents (Cahill KE, 2016). Here we demonstrated that NF-κB transcriptional activity can be induced in U87MG cells by TNFα stimulation and that this activation requires DGKα enzymatic activity.

Several experimental evidences support a role for DGKα in TNFα and NF-kB signaling. In esophageal squamous cell carcinoma DGKα was upregulated by inflammatory stimulants, including TNFα (Chen J, 2018), whereas treatment with ritanserin downregulated NF-kB transcriptional activity in mesenchymal GSCs (Olmez I, 2018). One of the earliest studies on DGKα in cancer reported DGKα suppression of TNFα-induced apoptosis by promotion of NF-kB signaling in melanoma (Yanagisawa K, 2007). In this tumor model, DGKα activity was required for activation of NF-kB mediated by Ser311 phosphorylation of p65/RelA by aPKCζ (Duran A, 2003; Kai M, 2009). Although in U87MG cells we observed a basal phosphorylation of NF-kB Ser311, this phosphorylation did not change after TNFα stimulation and/or DGKα enzymatic inhibition. We also noticed that aPKC Thr 410, localized in the activation loop, was not affected by treatment with TNFα and/or with DGKα inhibitors whereas it was downregulated in U87MG shDGKA cells, suggesting a possible role of DGKα protein in aPKC activation independently from TNFα.

Both DGKα gene silencing and enzymatic treatment with R59949, caused a reduction in NF-kB Ser 536 phosphorylation, as well as the downmodulation of AKT Ser 473 and Thr 308 phosphorylation in response to TNFα.

NF-kB transcriptional activity is enhanced upon phosphorylation of Ser536, localized in the p65 transactivation domain, by several kinases, such as IKKs (Sakurai H, 1999), ribosomal subunit kinase 1 (RSK1), (Bohuslav J, 2004) or TBK1 (Buss H, 2004). Classically, activation of the IKKs downstream receptors involves the participation of a number of components including IKK

gamma/NEMO, RIPK1, TAK1, TRAF1/2, and cIAP1/2 (Napetschnig J, 2013). Notably NF-κB has been also reported to be activated downstream the PI3K/AKT pathway. For example, it has been demonstrated that AKT can promote NF-κB activity by directly phosphorylating IKK (Ozes ON, 1999; Madrid LV, 2001; Sizemore N, 2002). Moreover, in PTEN-null/inactive prostate cancer cells the AKT-dependent mTOR and IKK interaction stimulates IKK activity directed toward the phosphorylation of RelA/p65 (Dan HC, 2008). In our study, silencing of DGKα is associated to a reduced activation of AKT downstream TNFα stimulation. These data support the hypothesis that, in GB, the PI3K/AKT pathway can be involved in the activation of NF-κB in response to TNFα and requires DGKα.

Our data clearly show that, in GB, FAK is implicated in TNFα-mediated signal transduction pathway and that DGKα is required for FAK phosphorylation downstream TNFR, since phosphorylation of both FAK Tyr 397 and FAK Tyr 925 are reduced in GSCs silenced for DGKα. Several papers reported the role of FAK in activating NF-κB after TNFα stimulation. The physical association of FAK with TNFR1, RIP and TRAF2, necessary for NF-κB activation, has been previously demonstrated (Funakoshi-Tago M, 2003; Schlaepfer DD, 2007; Tseng WP, 2010) as well as FAK association with p65 after TNFα stimulation (Tseng WP, 2010). Moreover, phosphorylated FAK Tyr397 is the binding site for the SH2 domain of the p85 subunit of PI3K and is required for the activation of the p110 catalytic subunit of PI3K (Chen HC, 1996) and, more recently, Chen et al demonstrated in esophageal squamous cell carcinoma that the direct interaction of DGKα with the FERM domain of FAK is required to relieve the auto-inhibitory effect of FERM domain on FAK and PI3K activity (Chen J, 2018).

Taken together, the results of this study strongly suggest that DGKα plays a key role in stemness maintenance contributing FAK, Akt and NF-kB activation upon TNFα stimulation and for this reason DGKα might represent a targetable oncogene that links inflammation and tumor progression. Since FAK is one of the major components of focal adhesions and is activated by a variety of stimuli (McLean GW, 2005; Sieg DJ, 2000) further studies will clarify if DGKα is also involved in other FAK-dependent growth factor receptors and integrins signals governing fundamental processes in GB cells.

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