Targeting Atypical Protein Kinase C iota Reduces Viability in Glioblastoma Stem-like cells via a Notch Signaling Mechanism

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Short Title

PKCι regulates GSC viability via NOTCH signaling

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Abbreviations

aPKC Atypical Protein Kinase C

ATM Aurothiomalate

ELDA Extreme Limiting Dilution Assay

FDR False Discovery Rate

GBM Glioblastoma

GSC Glioblastoma Stem-like Cell

HFNSC Human Fetal Neural Stem Cell

HRP Horse Radish Peroxidase

NOD-SCID Non-obese Diabetic/Severe Combined Immunodeficient

NSCLC Non-small Cell Lung Cancer

PB1 Phox-bem1

PLA Proximity Ligation Assay

PRKCI Protein Kinase C Iota

PRKCZ Protein Kinase C Zeta

RNAi RNA interference

shRNA Short Hairpin RNA

TMZ Temozolomide

Article Category

Molecular Cancer Biology

Abstract

In a previous study, Protein Kinase C iota (*PRKCI*) emerged as an important candidate gene for glioblastoma stem-like cell (GSC) survival. Here we show that PKCι is overexpressed and activated in patient derived GSCs compared with normal neural stem cells and normal brain

lysate, and that silencing of *PRKCI* in GSCs causes apoptosis, along with loss of clonogenicity and reduced proliferation. Notably, *PRKCI* silencing reduces tumor growth *in vivo* in a xenograft mouse model. PKCι has been intensively studied as a therapeutic target in non-small cell lung cancer, resulting in the identification of an inhibitor, aurothiomalate (ATM), which disrupts the PKCι/ERK signaling axis. However, we show that, although sensitive to pharmacological inhibition via a pseudosubstrate peptide inhibitor, GSCs are much less sensitive to ATM, suggesting that PKCι acts along a different signaling axis in GSCs. Gene expression profiling of *PRKCI*-silenced GSCs revealed a novel role of the Notch signaling pathway in PKCι mediated GSC survival. A proximity ligation assay showed that Notch1 and PKCι are in close proximity in GSCs. Targeting PKCι in the context of Notch signaling could be an effective way of attacking the GSC population in glioblastoma.

Introduction

Glioblastoma (GBM) is the most common and aggressive primary brain tumor in adults. The median survival of patients on current treatment regimes, which most commonly consist of surgical resection with adjuvant temozolomide (TMZ) and radiotherapy, is only 14.6 months [1]. Traditionally, GBM has been investigated using immortalized, serum-cultured adherent cell lines, which represent the highly proliferative tumor bulk. However, more recently, it has been shown that GBM is initiated and maintained by a small sub-population of cells which is capable of self-renewal and recapitulation of the original tumor in non-obese diabetic/severe combined immunodeficient (NOD-SCID) mice [2, 3]. These so-called glioblastoma stem-like cells (GSCs) can be enriched from patient derived tumor material by culturing in serum free conditions supplemented with basal FGF and EGF. It has been suggested that GBM cells cultured in this way more closely mirror the phenotype and genotype of primary tumors than do serum-cultured

cell lines [4, 5]. Furthermore, it has been shown that GSCs are linked to tumor recurrence and are enriched in recurrent malignant gliomas. Indeed, GSCs are more resistant to radio- and chemotherapy than bulk tumor cells [6-8], suggesting that targeting GSCs could be tantamount to improving therapy for GBM.

In recent years, much progress has been made in understanding gene expression patterns in many tumor types, with GBM being no exception [9-11]. Although such data have proved invaluable for discovering potential target genes for novel GBM therapies, the fact remains that gene expression does not necessarily reflect gene function. This is an issue which can be resolved by phenotypic screening studies, for example, by systematically silencing different genes and assessing a particular phenotypic effect. We have previously carried out one such screen, whereby the kinome and phosphatome was silenced in GSCs using a short hairpin RNA (shRNA) library. This revealed the importance of various genes for the survival of GSCs, including Protein Kinase C iota (*PRKCI*) [12].

PKCι is a member of the atypical Protein Kinase C (PKC) family, along with Protein Kinase C Zeta (PKCζ). *PRKCI* has been classified as an oncogene in various forms of human cancer [13-18], and its overexpression has been found to correlate with poor prognosis in lung and pancreatic cancer [19, 20]. Atypical PKC has also recently been shown to be activated and linked to NF-κB activation in GBM [21].

Here we decipher the function of PKCι in the survival of GSCs, demonstrating a new role for PKCι in glioblastoma in the Notch signaling mediated survival of GSCs.

Materials and Methods

Cell Culture Conditions

The GSC lines NCH421k, NCH644 and NCH441 were derived from primary GBM patients who underwent surgical resection according to the research proposals approved by the Institutional Review Board at the Medical Faculty of Heidelberg. Tissues were enzymatically dissociated and cells were cultivated as floating neurospheres in serum-free DMEM/F-12 medium, supplemented with 20 % BIT-admixture (Pelo Biotech) and basal fibroblast growth factor (Biomol) and epidermal growth factor (Life Technologies) at 20 ng/ml each. Genotypic and phenotypic studies have been carried out in previous investigations [5, 22]. Passaging and seeding was carried out by dissociation with accutase (Sigma-Aldrich). Human fetal neural stem cells were a kind gift from Dr. C Watts at the Department of Clinical Neurosciences, University of Cambridge. They were established from 12–14 week old human post-mortem fetal tissue as described by Iovino et al [23] and were cultured in the same serum-free medium conditions as the GSCs. A549 cells were cultured adherently in DMEM medium supplemented with 10 % FCS and 1 % penicillin/streptomycin.

Lentivirus Production and Transduction

HEK293T cells were seeded into 10 cm dishes and cotransfected with the plko.1 containing shRNA plasmids from The RNAi Consortium (TRC) and the packaging plasmids (pMD2.G, psPAX2) using Trans-IT transfection reagent (Mirus Bio). Virus containing medium was collected and filtered 72 hours after transfection and concentrated by ultracentrifugation for 1.5 hours at 25000 rpm. The titer was determined by FACS analysis using the plko.1-TurboGFP control plasmid. Non targeting shRNA was used as a negative control. *PRKCI*-sh1 and *PRKCI*-sh2 were used in functional studies. shRNA sequences are detailed in table S1. For transduction, GSCs were seeded at a density of 50000 cells per ml and transduced with a multiplicity of infection

(MOI) of 5. plko.1 TurboGFP containing lentivirus was used to estimate transduction efficiency via FACS analysis. Experiments were discarded if <70 % of cells were green.

Inhibitor Assay

Cells were seeded in 96 well plates at a density of 5000 cells per well. Sodium aurothiomalate (Sigma-Aldrich) or aPKC pseudosubrate (myristoylated) (Enzo Life Sciences) were applied at various concentrations and viability was measured after 3 days using a CellTiter-Glo$^®$ assay (Promega), according to the manufacturer's protocol.

Cell Death

Apoptosis of GSCs was assessed 4 days after transduction in 96 well plates using a Caspase Glo$^®$ 3/7 assay (Promega) according to the manufacturer's protocol. Additionally, apoptosis was measured 7 days after transduction in 6 well plates using 7-amino-actinomycin (7-AAD) and annexin V staining. GSCs were dissociated with accutase and incubated with annexin V binding buffer containing 10 % 7-AAD and 10 % annexin V-PE (BD Biosciences, Heidelberg, Germany). Annexin V and 7-AAD positive cells were measured using a FACSCanto II (BD Biosciences) and quantified with FACSDiva Software (BD Biosciences). Annexin V-positive cells (early apoptotic), 7AAD positive cells (necrotic) and doubly stained cells with both annexin V and 7-AAD (late apoptotic or necrotic) were deemed to be dead. For the rescue assay, NCH644-pLenti PGK V5-LUC Neo cells containing genes for the inducible expression of *PRKCI*-shRNA or NT-shRNA were transduced with pLVX-PRKCI at an MOI of 20. shRNA expression was induced by addition of 1 μg/ml doxycycline to the medium every two days. 5 days after doxycycline began to be applied, cell death was measured by propidium iodide staining and FACS analysis using a FACSCanto II.

Proliferation

Proliferation was measured using the Click-it EdU cell proliferation assay (Life Technologies) according to the manufacturer's protocol, whereby 10 µM EdU was added to the medium 72 hours after transduction. Incorporated EdU was detected 16 hours later by flow cytometry on a FACSCanto II after conjugation with Alexa Fluor 488. Data were analysed using the FACSDiva software.

Extreme Limiting Dilution Neurosphere Formation Assay

Two days after transduction with *PRKCI*-shRNA or NT-shRNA lentiviral particles, NCH421k cells were seeded into 96 well plates with densities of 1, 5, 10, 25 and 50 cells per well via a FACS sorter (FACSAria I, BD Biosciences). Spheres containing at least 5 cells were counted at day 10 after transduction the data analysed using the extreme limiting dilution assay (ELDA) software, available at http://bioinf.wehi.edu.au/software/elda/.

Western Blot Analysis

Cell lysates were quantified using a BCA assay, separated in 4-12 % polyacrylamide precast gels (Life Technologies, Darmstadt, Germany) under reducing conditions and transferred onto a PVDF membrane. Immunoreactive bands were detected using ECL or ECL Plus Substrate (Thermo Fischer Scientific). Band intensity was quantified using Image J software. Normal brain tissue lysate was obtained from BioChain. Primary antibodies PKCι (60175, BD Transduction Laboratories), Phospho-PKCι (07-881, Upstate Cell Signaling Solutions), PKCζ (9368, Cell Signaling Technologies), PathScan Multiplex Western Cocktail I (for P-ERK) (5301, Cell Signaling Technologies), Notch Intracellular Domain (4147, Cell Signaling Technologies), HES5 (ab194111, Abcam) were used at a 1:1000 dilution and α-tubulin (T6199, Sigma Aldrich) was used at a 1:5000 dilution. Horseradish peroxidase (HRP) coupled secondary antibodies (Abcam) were used at dilutions of 1:7500.

RNA Extraction and Quantitative Real-time PCR Analysis

1 µg RNA was pretreated with DNAse I (Life Technologies) and reverse transcribed by Superscript II (Life Technologies) according to the manufacturer's protocol. Complementary cDNA was measured using Absolute SYBR Green ROX Mix (ABgene) in technical triplicate using an ABI PRISM 7900HT thermal cycler (Applied Biosystems). cDNA levels were quantified based on the standard curves produced from a serial dilution of cDNA transcribed from NCH421k RNA and normalization to three housekeeper genes (*ARF1, DCTN2* and *HPRT*). Oligonucleotide sequences are detailed in table S2.

Microarray Analysis

NCH421k and NCH644 cells were transduced in three independent experiments with shNT and *PRKCI*-sh2. After 4 days, RNA was extracted from cells using the RNAeasy Mini Kit (Qiagen) according to the manufacturer's protocol and examined for integrity using an Agilent 2100 Bioanalyzer (Agilent Technologies). Gene expression profiling was performed using the 4x44K Whole Human Genome Oligo Microarray (Agilent Technologies). Microarray slides were scanned on an Agilent Microarray Scanner G25505B and data extracted using the Agilent Feature Extraction software version 9.1. Data processing followed using ChipYard (developed in-house, www.dkfz.de/genetics/ChipYard). Statistical significance of differential gene expression was determined by applying a linear model and an empirical Bayes moderated t-test statistic to each gene tested, followed by Benjamini-Hochberg correction for multiple testing [24]. Significantly deregulated pathways were identified using Ingenuity software (Ingenuity® Pathway Analysis (Qiagen) www.qiagen.com/ingenuity).

Gene Set Enrichment Analysis

Gene set enrichment analysis was performed at www.broadinstitute.org/gsea. Genes were pre-ranked based on the mean fold change and analyzed for the enrichment of the

signaling_by_Notch signature from Reactome, as listed in the Molecular Signatures Database (http://www.broadinstitute.org/gsea/msigdb). Quantification of enrichment of the signature genes near the top of the ordered list of genes followed using a running-sum statistic, resulting in an enrichment score. The false discovery rate (FDR) q-value was used to set a significant threshold.

Proximity Ligation Assay (PLA)

NCH421k cells were grown adherently on poly-D-lysine/laminin coated coverslips. Cells were fixed with 4 % PFA and permeabilized with 0.1 % Triton–X100. From here, the Duo Link PLA Starter Kit (Sigma Aldrich) was used according to the manufacturer's protocol. Briefly, the cells were blocked for 30 min at 37ºC and PKCι (sc-727, Santa Cruz) and/or Notch1 (sc-6014, Santa Cruz Biotechnology) antibodies were incubated at 4ºC overnight at 1:100 dilutions. Single antibodies were used to control for background signal. After washing, PLA probes were incubated for 1 hour at 37ºC, followed by ligation and amplification reactions for 30 min and 100 min respectively. A DAPI containing mounting medium was applied, and the cells were imaged using a Zeiss Axioplan 2 microscope. 5 images per condition were made at 20 x magnification and the mean number of PLA signals per cell determined. PLA signals were only counted if they were in a cell, as estimated by the locations of the DAPI stained nuclei.

***In vivo* Tumor Propagation**

NCH644 GSCs were transduced with lentiviral particles carrying the pLenti PGK V5-LUC Neo plasmid (MOI 1), and transduced cells were selected under 400 μg/ml G418. shNT and *PRKCI*-sh2 shRNA was cloned into the Tet-pLKO-puro vector and lentiviral particles produced as described above. NCH644-pLenti PGK V5-LUC Neo cells were transduced (MOI 1) and selected under 2 μg/ml puromycin. Six week old NOD-SCIDγ mice were obtained from the animal facility at the DKFZ. All animal experiments were performed according to animal welfare regulations and were approved by the responsible authorities (Regierungspräsidium Karlsruhe, approval

number G64-14). NCH644-pLenti PGK V5-LUC Neo cells containing genes for the inducible expression of *PRKCI*-shRNA or NT-shRNA were treated with accutase and 100000 cells were injected into the striatum of the mice. Tumor development was measured twice weekly and 2 mg/l doxycycline was administered *ad libitum* after tumors had reached radiances of at least 220000 but less than 1300000 flux p/s. Tumor growth was monitored using an IVIS Lumina Pre-clinical in vivo Imaging System (Perkin Elmar) until the development of neuropathological symptoms, which were confirmed blindly by two independent scientists.

Immunohistochemistry

After perfusion with 10 % (w/v) formaldehyde, mouse brains were sectioned coronally, dehydrated in a STP 120 spin tissue processor (Thermo Fischer Scientific) and embedded in paraffin. Sections (4 µm thickness) were cut and mounted onto glass slides, deparaffinized in xylene and rehydrated in a descending series of alcohols. Slides were treated with PKCι (60175, BD Transduction Laboratories) or HES5 (ab194111, Abcam) primary antibodies overnight at 4°C and incubated with a HRP conjugated secondary antibody for 1 h at room temperature. Staining was visualized using the avidin-biotin peroxidase system (Vector Laboratories) and freshly prepared diaminobenzidine as a chromogen (Dako Deutschland). Slides were counterstained with haematoxylin, dehydrated and mounted. Images were made using a Zeiss Axioplan 2 microscope.

Results

PKCι is Overexpressed and Activated in GSCs

Using a kinome-wide RNA interference (RNAi) screen, we identified *PRKCI* as a gene whose silencing induces apoptosis in GSCs [12]. In order to investigate the specificity of *PRKCI* expression to GSCs, PKCι protein levels were measured in three different GSCs derived from 3 different patients (NCH421k, NCH644 and NCH441) and compared with human fetal neural

stem cells (HFNSCs) and normal brain extract. Protein levels of PKCι and especially phospho-PKCι (T555) were much lower in the HFNSCs and normal total brain extract (figure 1a). However, interestingly, there were no significant differences in mRNA levels between the GSC lines, HFNSCs and a pool of normal brain mRNA (Figure 1b). Nor are there significant differences in expression to be found in publically available expression datasets comparing glioma of different grades and controls (figure 1c).

PRKCI silencing Induces Apoptosis and Reduces Proliferation and Clonogenicity in GSCs

In order to investigate phenotypic changes upon *PRKCI* silencing, GSCs were transduced with two different shRNAs targeting *PRKCI* (sh1 and sh2) or non-targeting shRNA (shNT) (figures 2a and S1a-c). As demonstrated in a Caspase Glo® apoptosis assay 4 days after transduction of GSC lines NCH421k, NCH644 and NCH441, apoptosis was induced upon *PRKCI* silencing (figure 2b). This was confirmed in GSC lines NCH421k and NCH644 using an annexin V/7AAD apoptosis assay 7 days after transduction, which revealed that the percentage of annexin V/7AAD negaitive (i.e. living) cells was significantly decreased upon *PRKCI* silencing in these GSC lines (figure S1 d and e). In order to assess the specificity of this phenomenon, NCH421k, NCH644 and NCH441 GSCs and and HFNSCs were transduced with shNT, sh1 or sh2 and cell viability was assessed 5 days later using a CellTiter-Glo® assay (figure 2c). Notably, in sh2 transduced cells, where the knockdown is the strongest, only the viability of the GSCs is significantly reduced, whereas sh1 causes a significant reduction in the viability of both GSCs and HFNSCs. A possible explanation for this is that sh1 could have off-target effects. As PKCι has a similar sequence (72 % homology) to the other atypical protein kinase C, Protein Kinase C Zeta (PKCζ), it was determined whether the shRNAs against *PRKCI* also cause a reduction in protein levels of PKCζ. Western blot analysis showed that sh1 causes a reduction of PKCζ by about 50 % whereas sh2 does not affect the levels at all (figures S2a and b). Interestingly, although PKCζ protein levels seem to be very low in HFNSCs, levels are higher in normal brain

extract than in the GSC lines NCH421k and NCH441 (figure S2c). Furthermore, microarray data has shown that mRNA levels of PKCζ are significantly higher in normal brain than in GBM samples (figure S2d) [25].

Proliferation of living cells was measured 4 days after transduction by FACS assessment of EdU incorporation into NCH421k, NCH644 and NCH441 GSCs (figure 2d). Proliferation was significantly reduced upon *PRKCI* silencing in all GSC lines with both sh1 and sh2, suggesting that *PRKCI* knockdown causes cells to stop proliferating before they undergo apoptosis. An extreme limiting dilution assay showed that *PRKCI* silencing decreases the capacity of NCH421k cells to form neurospheres, which could suggest a loss of clonogenicity (figure S1f).

Effects of Pharmacological Inhibition of PKCι on GSC Viability

The effect of pharmacological inhibition of PKCι on GSC viability was assessed using a myristoylated atypical PKC pseudosubstrate peptide (aPKC-PSP), which blocks phosphorylation of PKCι at Thr555, and hence prevents the kinase from attaining its active conformation [26, 27]. NCH421k, NCH644 and NCH441 GSCs were treated with increasing concentrations of aPKC-PSP for 3 days and a reduction of viability was observed in all 3 cell lines (with an IC_{50} of around 20 µM for NCH421k and NCH441, and around 100 µM for NCH644) (figure 3a, upper panel). A reduction of the activity of PKCι upon incubation with the compound was confirmed by Western blotting with the P-PKCι (Thr555) antibody (figure 3a, lower panel).

A further inhibitor of PKCι, Aurothiomalate (ATM), was also tested for its activity against GSCs. In non small cell lung cancer (NSCLC) cells, it has been shown that ATM disrupts the interaction between PKCι and one of its binding partners, Par6 [28]. In order to test whether PKCι can be targeted in this way in GSCs, NCH421k, NCH644 and NCH441 GSCs were treated with increasing concentrations of ATM for 3 days. Lung cancer cell line A549 was used as a positive control, although it should be noted that other lung cancer cell lines have been reported to be

even more sensitive to ATM [29]. It was found that ATM reduced the viability of A549 cells with an IC_{50} of around 30 μM, whereas GSCs were unaffected at this concentration (figure 3b, upper panel). Treatment of A549 with ATM resulted in a dose dependent reduction in phospho-ERK levels, which indicates that the compound does indeed disrupt the interaction of PKCι with Par6, as ERK is a downstream target of this signaling axis. P-ERK levels were not reduced in NCH421k GSCs upon treatment with ATM (figure 3 b, lower panel). Taken together, this indicates that PKCι facilitated GSC survival is likely to be mediated differently in GSCs than in NSCLC cells.

Gene Expression Profiling After *PRKCI* Silencing Implicates the Notch Signaling Pathway in *PRKCI* Mediated GSC Survival

In order to investigate the crucial role of *PRKCI* in GSC survival, gene expression microarray-based transcriptome analysis was conducted on GSCs transduced for 4 days with *PRKCI*-sh2 compared with GSCs transduced with shNT. *PRKCI*-sh2 was selected due to its high efficiency of silencing and its specificity for *PRKCI* (rather than *PRKCZ*). Three biological replicates were performed, twice in NCH421k and once in NCH644 GSCs. Empirical Bayes Moderated t-testing revealed 3367 differentially regulated genes (adj. p value < 0.05). The full dataset can be found at the Gene Expression Omnibus public functional genomics data repository with the GEO number GSE77030. Using a threshold of a fold change of 1.5, the 395 most deregulated genes were analysed using the Ingenuity® Pathway Analysis software (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity). The most significantly deregulated pathway was the Notch signaling pathway, known to be involved in stemness and self renewal (figure 4a).

In addition, gene set enrichment analysis, comparing the expression data to a Notch signaling signature of 103 genes involved in the pathway from Reactome (http://www.broadinstitute.org/gsea/msigdb), revealed that the downregulated genes upon *PRKCI* silencing are significantly enriched in genes from this signature (figure 4b).

The results of the gene expression profiling were validated by quantitative real-time polymerase chain reaction (qRT-PCR) analysis of the top 4 downregulated Notch pathway genes (*HES5*, *NOTCH2*, *NOTCH1* and *RBPJ*) upon silencing of *PRKCI* for 4 days in GSCs cells using sh1 and sh2. Levels of all 4 Notch genes were significantly downregulated in NCH421k cells when *PRKCI* is silenced using either shRNA (figure 4c). Figure S3a also shows that *HES5* mRNA levels were also reduced upon *PRKCI* silencing in NCH644 and NCH441 GSCs. Furthermore, a dramatic reduction in Notch intracellular domain (NICD) protein levels, which is a cleavage product released when Notch signaling is active, upon *PRKCI* silencing could be observed via Western blot analysis (figure 4d and S3b). Notably, pharmacological inhibition of PKCι in NCH421k GSCs using the aPKC-PSP, which prevents the activation of PKCι by blocking the phosphorylation of the activating threonine residue (Thr555), also caused a reduction in Notch signaling, as demonstrated by a reduction in NICD levels (figure 4e). Conversely, PKCι inhibition by disruption of the interaction of the kinase with Par6 by ATM in NCH42k1 GSCs did not lead to a reduction of Notch signaling (figure S3c).

In order to determine whether PKCι is in close spatial proximity to Notch1 in GSCs, which would suggest an interaction between the two proteins, an *in situ* proximity ligation assay was carried out. An enrichment of PLA signals could be detected when both PKCι and Notch1 antibodies were incubated with GSCs, compared with when only one of the antibodies was bound (figure 4e and S3c).

Finally, a rescue study was carried out, whereby stable NCH644 cells lines expressing doxycycline inducible shNT or sh2 were additionally transduced with a plasmid constitutively expressing *PRKCI* (figure 5a). Whereas cell death was induced in cells which do not overexpress PKCι 5 days after treatment with doxycycline, the overexpressing cells were not affected (figure 5b, upper panel). Furthermore, the reduction in the protein level of Notch signaling target gene *HES5* induced by *PRKCI*-sh2 was rescued in the cells in which *PRKCI* was

overexpressed (figure 5b, lower panel). This indicates that cell death and the reduction of Notch signaling are specifically caused by the silencing of *PRKCI* and not due to off-target effects.

Silencing of *PRKCI in vivo* Reduces Notch Signaling and Slows Tumor Growth

With the aim of investigating whether *PRKCI* silencing can affect GBM growth *in vivo,* GSCs stable for doxycycline inducible expression of shNT- or *PRKCI*-sh2 were injected into the striatum of NOD-SCID mice. The cells also expressed luciferase, allowing tumor growth to be monitored via bioluminescence detection. Sh2 was chosen as it results in the highest and most specific knockdown, and cell line NCH644 was selected as it has been shown to grow highly aggressively and invasively, making it an appropriate model of GBM [30]. Silencing of *PRKCI* via *ad libitum* administration of doxycycline resulted in the tumors growing more slowly (figure 6a), and increased survival of the mice (figure 6b), compared with those whose tumors expressed shNT or where the sh*PRKCI* expression was not induced. However, all mice eventually succumbed to the tumor, indicating that *PRKCI* silencing alone is not enough to cause tumor regression in this case. Immunohistological staining of the tumors confirmed that doxycycline administration indeed reduced PKCι levels in the tumor, although there appears to be some residual protein expression (6c, upper panel). Crucially, protein levels of the Notch signaling target protein HES5 are also markedly reduced in the tumors in which PKCι expression was depleted, suggesting that the slowed tumor growth was mediated via a reduction in Notch signaling (figure 6c, lower panel).

Discussion

We have previously identified *PRKCI* as an important gene for GSC survival in a kinome and phosphatome wide shRNA screen [12]. In this study, we have built on this finding and show a cancer specific expression and activation of PKCι in GSCs compared with normal neural stem cells and normal brain lysate. We investigate the function of *PRKCI* in GSCs and a gene

expression profiling approach has identified a link between *PRKCI* and Notch signaling. Most notably, silencing *PRKCI* in a xenograft glioblastoma mouse model slowed tumor growth and prolonged survival, suggesting that PKCι would be a good therapeutic target in GBM.

PKCι, along with PKCζ, is a member of the atypical protein kinase C (aPKC) family of proteins, and has been shown to be overexpressed and overactivated in a range of cancer entities. Although mRNA levels of *PRKCI* are not increased in GBM according to our experiments and publicly available microarray data, we here demonstrate overexpression and activation of PKCι on the protein level in GSCs compared with normal neural stem cells and normal brain extract. Supportive of this, in a recent study, Kusne et al. demonstrated that aPKC abundance inversely correlates with glioblastoma survival by staining human brain tissue and comparing aPKC expression to patient survival data [21]. The discrepancy we observe between PKCι mRNA and protein levels in GSCs has been observed in other studies of PKCι in glioblastoma [21, 25, 31], and raises the question of how PKCι protein levels are regulated. Further experiments would be required to determine whether the kinase is regulated on the translational or post-translational level. However, as we have shown that PKCι is more highly phosphorylated in GSCs compared with normal neural stem cells and normal brain extract, it could be conceivable that the kinase is stabilized by cancer-dependent phosphorylation or perhaps yet unknown post-translational modifications.

We have shown that pharmacological inhibition of PKCι using a myristoylated pseudosubstrate peptide reduced the viability of GSCs *in vitro*. The pseudosubstrate peptide inhibited the phosphorylation of PKCι at Thr555, and is thought to render the kinase inactive in this way [26, 27]. It should be noted that this compound also inhibits PKCζ, meaning its effects on GSCs cannot be specifically attributed to PKCι. As we have shown that shRNA-mediated silencing of PKCι alone is sufficient to reduce the viability of GSCs, It would be interesting to test the effects of an inhibitor which is specific to PKCι only. PKCι has been very well studied in the context of

NSCLC, and the aPKC inhibitor ATM, which is already in use in the clinic for rheumatoid arthritis, has recently been tested in a phase I dose escalation clinical trial in patients with advanced NSCLC, ovarian cancer and pancreatic cancer [32]. This inhibitor forms a thio-gold adduct with a cysteine residue in the Phox-bem1 (PB1) domain of PKCι, which prevents the interaction with Par6 and thereby inhibits the pro-oncogenic ERK signaling pathway [28]. However, we have shown that GSCs are less sensitive to ATM treatment than the lung cancer cell line A549, and that the compound does not cause a reduction of ERK signaling in GSCs. Although it cannot be ruled out that ATM is not taken up as readily by GSCs as A549 cells, this suggests that GSCs are not solely dependent on the ERK signaling pathway for survival.

Therefore, we set out to investigate other possible roles of PKCι in GSCs, comparing gene expression of GSCs from two different patients transduced with lentiviral particles containing NT or *PRKCI*-targeting shRNA. This revealed the Notch signaling pathway as the most deregulated pathway in GSCs upon *PRKCI* silencing. Notch signaling is important in development and is associated with self-renewal, i.e. "stemness." In fact, it has been shown that Notch activation contributes to glioma growth and survival [33], and that Notch signaling inhibition depletes GSCs and inhibits growth of tumor neurospheres and xenografts [34]. We have shown that the silencing of *PRKCI* and pharmacological inhibition of PKCι using the aPKC-PSP leads to reduced Notch signaling and increased cell death in GSCs, and that Notch1 and PKCι are in close proximity in GSCs, indicating that they may directly interact. Supportive of this, it has been shown in the developing chick central nervous system that both PKCι and PKCζ bind to Notch1 and phosphorylate it at a distinct residue, leading to increased Notch signaling activity [35]. Furthermore, we have shown that the aPKC-PSP inhibitor, which inactivates PKCι by preventing its activation via the Thr555 phosphorylation, reduces Notch signaling, whereas ATM, rather functioning by blocking the interaction with PKCι and Par6, does not. This could also be indicative that the kinase activity of PKCι is important for its role in Notch signaling. Taken together, these data provide an attractive mechanism for how PKCι could modulate Notch

signaling in GSCs, although further investigation would be required to confirm this. Interestingly, although the aforementioned study by Sjoqvist et al. shows that aPKCs and NUMB likely act on the Notch pathway at distinct steps, it has recently been suggested that PKCι knockout in embryonic mouse cells leads to enhanced generation of stem cells via inhibition of NUMB, and hence activation of the Notch pathway [36]. These data highlight that PKCι could affect Notch signaling differently in distinct cellular contexts.

Kusne et al. demonstrated that targeting aPKC pharmacologically reduces tumor progression in mouse models of GBM using a GBM cell line and patient derived xenografts [21]. As it has recently been suggested, at least in the context of pancreatic cancer, that PKCι and PKCζ have non-redundant roles, it could be important to consider the aPKCs separately in cancer therapy [37]. Here, we showed that silencing PKCι (and not PKCζ) is sufficient to reduce tumor growth and increase survival in a highly aggressive mouse model using patient derived GSCs, and that the slowed growth is likely to be mediated via a depletion of Notch signaling. However, the tumors did not decrease in size, and the mice eventually succumbed to the disease. One reason for this could be that the doxycycline induced expression of *PRKCI*-sh2 did not achieve a sufficient knock-down of PKCι in the tumor. In addition, it is not surprising that tumors in which PKCι is knocked-down can eventually circumvent the effects of reduced levels of the protein, as PKCι has been shown to be involved in many different signaling axes, including MAP kinase, NF-κB and apoptosis [31]. Therefore targeting PKCι as a therapeutic option for GBM must be considered in combination with other targeted therapies, for example, Notch inhibitors and MAP kinase signaling inhibitors. Furthermore, traditional therapies to deplete the bulk of the tumor may also be necessary, as targeting PKCι may only diminish the GSC compartment of the tumor.

In conclusion, we have shown that PKCι overexpression and -activation is cancer specific in a GSC context, and plays an important role for PKCι in GBM: namely the Notch signaling

mediated survival of GSCs. The insensitivity of GSCs to the PB1 domain interaction-disrupting PKCι inhibitor ATM highlights the need for a different strategy for targeting PKCι in glioblastoma therapy, for example by preventing the activation of the kinase by blocking the phosphorylation of Thr555.

Conflict of Interest

The authors declare no conflict of interest.

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Author Contributions

E.P. designed experiments, performed experiments, analyzed data and wrote the manuscript.

V.L. and J.B. performed experiments and analyzed data.

F.B and L.P. performed experiments.

D.T. and T.H. analyzed data.

C. H-M. provided samples and wrote the manuscript.

P.L. wrote the manuscript

V.G. designed experiments, analyzed data and wrote the manuscript.

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Figure Legends

Figure 1: PKCι is overexpressed and activated in GSCs on the protein level.

a)   Representative Western blot showing PKCι or phospho- PKCι (Thr555) (active) levels in GSCs from three different patients (NCH421k, NCH644 and NCH441), human fetal neural stem cells (HFNSCs) and brain tissue lysate. α-Tubulin is shown as a loading control.

b)   *PRKCI* mRNA levels in GSCs from three different patients (NCH421k, NCH644 and NCH441), HFNSCs and brain tissue lysate, normalized to three different housekeeper genes (*ARF1*, *DCTN2* and *HPRT*). (Experiment performed in technical triplicate).

c)   Levels of *PRKCI* 159 GBM cases compared with 8 normal controls [25]. Data was obtained from R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl). (***p<0.001, ns = not significant, one way analysis of variance [ANOVA]).

Figure 2: *PRKCI* silencing induces apoptosis and reduces proliferation in GSCs.

a)   Protein levels in NCH421k 4 days after transduction with shNT or two different shRNAs against *PRKCI*, normalized to α-tubulin levels and to shNT PKCι levels (error bars indicate standard deviation [SD] of biological triplicates, *p<0.05, ***p<0.001, student's t-

test). Inset: Representative Western blot showing PKCι protein levels with α-tubulin displayed as a loading control.

b) Caspase assay showing caspase 3/7 activity in NCH421k, NCH644 and NCH441 GSCs 4 days after transduction with shNT or two different shRNAs against *PRKCI* (error bars indicate SD of technical triplicates).

c) Cell viability assay depicting percentage of living cells normalized to cells transduced with shNT. This was determined by a CellTiter-Glo® viability assay 5 days after transduction of NCH421k, NCH644 or NCH441 GSCs (black and gray bars) or HFNSCs (red bars) with shNT or two different shRNAs against *PRKCI* (error bars indicate SD of biological triplicates, *$p < 0.05$, ns = not significant, student's t-test).

d) Cell proliferation assay showing percentage of NCH421k, NCH644 and NCH441 GSCs with incorporated EdU 4 days after transduction with shNT or two different shRNAs against *PRKCI* as determined by FACS analysis (error bars indicate SD of biological triplicates, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, student's t-test).

Figure 3: Effects of pharmacological inhibition of PKCι on GSC viability

a) Upper panel: Effect of aPKC pseudosubstrate peptide (aPKC-PSP) inhibitor on GSC NCH421k, NCH644 and NCH441 viability after incubation at the indicated concentrations for 3 days. Viability was normalized to cells incubated with the vehicle (H_2O) (experiment was performed in technical duplicate). Lower panel: Levels of active PKCι were assessed by Western blot with an antibody against phospho-PKCι (Thr555). Levels of total PKCι are also shown and α-tubulin as a loading control.

b) Upper panel: Effect of PKCι inhibitor ATM on GSC NCH421k, NCH644 and NCH441 growth compared with lung cancer cell line A549 5 days after treatment at the indicated concentrations. Cell viability was normalized to cells incubated with vehicle (DMSO) (NCH421k and A549 were performed in biological triplicate, with error bars representing

SD, *p<0.05, **p<0.01, student's t-test, and NCH644 and NCH441 were performed in technical triplicate). Lower panel: ERK signaling activity was assessed at the indicated concentrations in NCH421k and A549 by Western blotting using the PathScan Multiplex Western Cocktail I, which contains anti phospho-ERK1/2 and Rab11 as a loading control.

Figure 4: Gene expression profiling reveals Notch signaling to be downregulated in *PRKCI*-silenced GSCs

a) *PRKCI* was silenced using *PRKCI*-sh2 in GSCs from two different patients. Biological replicates were performed: twice in NCH421k and once in NCH644. Gene expression profiling was carried out 4 days after transduction. Pathway analysis of the most significant deregulated genes was carried out using Ingenuity Pathway Analysis Software. Bars represent the –log p-value of the significance of the pathway and the red line shows the ratio of genes deregulated in the pathway (*Benjamini-Hochberg adj. p<0.05, **B-H adj. p<0.01, ***B-H adj. p<0.001, right-tailed Fischer Exact test).

b) Gene Set Enrichment Analysis (GSEA) revealed an overall downregulation of Notch signaling associated genes in *PRKCI*-silenced GSCs. The analysis was performed with the Notch Signaling Signature from Reactome as listed in the Molecular Signatures Database (http://www.broadinstitute.org/gsea/msigdb) in a rank order based on the mean linear fold change of the genes. Genes showing an upregulation after *PRKCI* knockdown are depicted in red and genes showing a downregulation depicted in blue. The green curve corresponds to the running sum of enrichment score which reflects the degree to which the Notch signature is overrepresented at the bottom of the list. Enrichment score (ES) = -0.48, normalized enrichment score (NES) = -1.67, false discovery rate (FDR) = 0.001).

c) mRNA levels of *PRKCI* and most down-regulated genes involved in Notch signaling (*NOTCH1, NOTCH2, RBPJ and HES5*) in NCH421k 4 days after transduction with shNT or two different shRNAs against *PRKCI*, normalized to 3 different housekeeper genes (*ARF1*, *DCTN2* and *HRPT*) and to shNT expression levels (error bars indicate SD of biological triplicates, *p<0.05, *p<0.01, *p<0.001, student's t-test).

d) Protein levels of PKCι and the Notch Intracellular Domain (NICD) in NCH421k GSCs 4 days after transduction with shNT or two different shRNAs against *PRKCI*, with α-tubulin displayed as a loading control.

e) Left: Protein levels of PKCι phopsho-PKCι (Thr555) and the Notch Intracellular Domain (NICD) in NCH421k after 4 days of treatment with the aPKC pseudosubstrate peptide inhibitor (aPKC-PSP) at 0, 10 and 20 µM, with α-tubulin displayed as a loading control. Right: Quantification of the Western blot, normalized to α-tubulin and 0 µM sample.

f) *In situ* proximity ligation assay (PLA) showing an increase of PLA signals in GSCs incubated with antibodies against PKCι and Notch1, compared with the single antibody controls (Representative experiment from biological triplicates, scale bar indicates 10 µm, error bars indicate SD of 5 images).

Figure 5: Doxycycline inducible sh*PRKCI* GSC cell line overexpressing *PRKCI* enables apoptosis phenotype and Notch signaling reduction to be rescued

a) Western blot analysis showing protein levels of PKCι in NCH644 GSCs with doxycycline inducible shNT or *PRKCI*-sh2 5 days after addition of doxycycline or PBS. α-Tubulin is shown as a loading control.

b) Upper panel: Phenotype rescue. % PI positive cells were measured by FACS analysis 5 days after doxycycline addition to NCH644 cells stably transduced with doxycycline

inducible shNT or *PRKCI*-sh2 and constitutively expressing PKCι (or not) (error bars indicate SD of biological triplicates, *p<0.05, ns=not significant, student's t-test). Lower panel: Notch signaling rescue: Western blot analysis showing PKCι (short and long exposure) and HES5 protein levels in the cells described in the left panel, with α-tubulin as a loading control.

Figure 6: *in vivo* silencing of *PRKCI* slows tumour growth but does not cause tumor regression

a) Tumor progression of mice after injection of NCH644 GSCs with doxycycline inducible shNT or *PRKCI*-sh2 based on luminescence flux. Two representative mice are shown. Nd = not determined as shNT mouse was sacrificed on day 24.

b) Kaplan Meier graph showing the survival of mice after tumors had formed and doxycycline administered (or not in the case of the *PRKCI*-sh2-dox mice). Mice were given doxycycline via the water supply when the luminescence flux from the tumor reached 2.2×10^5 – 1.3×10^6. Mice were sacrificed when they began to display neuropathological symptoms (p*<0.05, p**<0.01, log rank (Mantel-Cox) test).

c) Immunohistological staining of the tumors from the shNT+dox and *PRKCI*-sh2+dox mice shown in a) and a representative mouse from the *PRKCI*-sh2-dox group with antibodies against PKCι (upper panel) and HES5 (lower panel). Scale bar = 50 μm.