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Balance of Forces:

Regulation of protein tyrosine phosphorylation by PTPs and RTKs in glioma.

Annika van der Veen-Bourgonje
The research presented in this thesis was performed at the Department of Cell Biology, Radboud Institute for Molecular Life Sciences, Nijmegen in collaboration with the Department of Pathology at the Radboudumc, Nijmegen.


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Cover image: Artist impression of the delicate balance of phosphotyrosine-regulating enzymes, superimposed with cell stainings of glioblastoma cells with a membrane reporter (left-back), and EdU nuclear staining (bottom-right) and a spheroid with outgrowing cells (upper right).

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Balance of Forces:

Regulation of protein tyrosine phosphorylation by PTPs and RTKs in glioma.

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volgens besluit van het college van decanen
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Prof. Dr. A.H.M. Geurts van Kessel
Balance of Forces:

Regulation of protein tyrosine phosphorylation by PTPs and RTKs in glioma.

Doctoral thesis

to obtain the degree of doctor
from Radboud University Nijmegen
on the authority of the Rector Magnificus prof. dr. J.H.J.M. van Krieken,
according to the decision of the Council of Deans
to be defended in public on Monday, June 4, 2018 at 10.30 hours

by

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I have not failed.  
I've just found 10,000 ways that won't work.  
Just because something doesn't do what you planned it to do  
doesn't mean it's useless.

~Thomas Edison~
Abbreviations and Gene Nomenclature

α-KG  Alpha-ketoglutarate
ABC  Avidin biotin complex
ACTB  Actin-B
AEC  3-amino-9-ethyl-carbazole
AKT  Akt/PKB or AKT - serine/threonine protein kinase homologous to oncogene from AKT8 retrovirus, isolated from AKR mouse.
ANOVA  Analysis of variance
ARF  Alternate reading frame tumor suppressor
ATRX  ATP-dependent helicase
BrdU  Bromodeoxyuridine
C/S  Cysteine/serine
C2  Protein kinase C conserved region 2
CA  Carbonic anhydrase
CAH  Carbonic anhydrase- homology domain
CAMs  Cell adhesion molecules
CANX  Calnexin
CD34  Cluster of differentiation 34
cdc25  Cell division cycle 25
CDKN2A  Cyclin-dependent kinase inhibitor 2A gene
CDKs  Cyclin-dependent kinases
Cl  Class
c-MET  Tyrosine-protein kinase mesenchymal epithelial transition
CNS  Central nervous system
CS  Chondroitin sulfate attachment region
D1  Catalytic active PTP domain 1
D2  Catalytic inactive PTP domain 2
D-2-HG  D-2-hydroxyglutarate
DAB  3-3’-diaminobenzidine
DAPI  4’,6-diamidino-2-phenylindole
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DUSP26</td>
<td>Dual-specificity phosphatase 26</td>
</tr>
<tr>
<td>DUSP6</td>
<td>Dual-specificity phosphatase 6</td>
</tr>
<tr>
<td>DUSPs</td>
<td>Dual-specificity PTPs</td>
</tr>
<tr>
<td>E2F</td>
<td>E2F family of transcription factor proteins</td>
</tr>
<tr>
<td>EGF-BS</td>
<td>Epidermal growth factor-binding site</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EGFRvIII</td>
<td>Epidermal growth factor receptor variant III</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>ERBB2/3/4</td>
<td>Erb-B2 receptor tyrosine kinase 2/3/4</td>
</tr>
<tr>
<td>EdU</td>
<td>5-ethynyl-2’-deoxyuridine</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellular regulated kinase 1/2</td>
</tr>
<tr>
<td>EV</td>
<td>Empty vector</td>
</tr>
<tr>
<td>EYA</td>
<td>Eyes absent</td>
</tr>
<tr>
<td>FERM</td>
<td>4.1 protein-ezrin-radixin-moesin domain</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin fixed, paraffin-embedded</td>
</tr>
<tr>
<td>Fiji</td>
<td>Fiji is just image J</td>
</tr>
<tr>
<td>FNIII</td>
<td>Fibronectin type three domain</td>
</tr>
<tr>
<td>FYVE</td>
<td>Fab1/Yotb/Vac1p/early endosomal antigen-1 homology</td>
</tr>
<tr>
<td>G1</td>
<td>Gap 1 phase of cell cycle</td>
</tr>
<tr>
<td>G2</td>
<td>Gap 2 phase of cell cycle</td>
</tr>
<tr>
<td>Gab1</td>
<td>GRB2-associated-binding protein</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Gd-DTPA</td>
<td>Gadolinium with diethylenetriaminepentacetate</td>
</tr>
<tr>
<td>GLUT1</td>
<td>Glucose transporter 1</td>
</tr>
<tr>
<td>Grb2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HAD</td>
<td>Reductase, rhodanese, haloacid dehalogenase</td>
</tr>
<tr>
<td>HEK293FT</td>
<td>Human embryonic kidney cells, fast growing, large T antigen.</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HIER</td>
<td>Heat-induced epitope retrieval</td>
</tr>
<tr>
<td>HP</td>
<td>His phosphatase</td>
</tr>
<tr>
<td>HUVECs</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>IDH 1/2</td>
<td>Isocitrate dehydrogenase 1/2</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin-like domain</td>
</tr>
<tr>
<td>p16-INK4A</td>
<td>p16 inhibiting CDK4</td>
</tr>
<tr>
<td>IPT</td>
<td>Ig-like plexin and transcription factor domain</td>
</tr>
<tr>
<td>JAK/STAT</td>
<td>Janus kinase/signal transducer and activator of transcription</td>
</tr>
<tr>
<td>JMD</td>
<td>Juxta-membrane domain</td>
</tr>
</tbody>
</table>
List of Abbreviations

KD  Kinase domain
kDa  Kilodalton
KDM4C  Lysine-specific demethylase 4C
ki-67  Antigen identified by monoclonal antibody Ki-67
KIND  Kinase non-catalytic C-lobe domain
Lmw  Low-molecular weight PTP
LRRC4  Leucine rich repeat containing 4
M  Mitosis, M-phase of cell cycle
MAGI1/2/3  Membrane-associated guanylate kinase inverted 1/2/3
MAM  Meprin-A5-receptor protein tyrosine phosphatase mu homology domain
MAPK  RAS/RAF-initiated mitogen-activated protein kinase
MCT4  Monocarboxylate transporter 4
MDM2  Mouse double minute 2 homolog
MGMT  O6-alkylguanine DNA alkyltransferase
MKPs  MAPK phosphatases
MMAC  Mutated in multiple advanced cancers
MTMR4  Myotubularin-related phosphatase 4
MTMRs  Myotubularin-related phosphatases
Mupp1  Multi-PDZ domain protein 1
mut  Mutant
NADP+  Nicotinamide adenine dinucleotide phosphate
NADPH  Nicotinamide adenine dinucleotide phosphate (reduced)
NF1  Neurofibromin
NOS  Not otherwise specified
PD1  Phosphatase domain 1
PD2  Phosphatase domain 2
PDGFR (α/A/B)  Platelet-derived growth factor receptor (α/A/B)
PDK1  Phosphoinositide-dependent kinase-1
PDZ  Postsynaptic density-95/discs large/ZO1 homology domain
PH-G  Pleckstrin homology-“GRAM” domain
PHTS  PTEN hamartoma tumor syndrome
PI3K  Phosphatidylinositol-4,5-bisphosphate 3-kinase pathway
PIP3  Phosphatidylinositol-3,4,5-trisphosphate
PRL  Phosphatase of regenerating liver
PSD95  Postsynaptic density-95
PSI  Domain found in plexins, semaphorins and integrins
PSMB  Proteasome subunit beta
PTEN  Phosphatase and tensin homolog
PTENs  PTEN-related PTPs
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTN</td>
<td>Pleiotrophin</td>
</tr>
<tr>
<td>PTP</td>
<td>Catalytic active protein tyrosine phosphatase domain</td>
</tr>
<tr>
<td>PTP*</td>
<td>Protein tyrosine phosphatase catalytic inactive domain</td>
</tr>
<tr>
<td>PTPRM</td>
<td>Protein tyrosine phosphatase receptor Mu</td>
</tr>
<tr>
<td>PTPRN2</td>
<td>Protein tyrosine phosphatase receptor N2</td>
</tr>
<tr>
<td>PTPRT</td>
<td>Protein tyrosine phosphatase receptor rho</td>
</tr>
<tr>
<td>PTPRZ1</td>
<td>Protein tyrosine phosphatase receptor zeta</td>
</tr>
<tr>
<td>PTPRZ-A</td>
<td>Protein tyrosine phosphatase receptor zeta, A</td>
</tr>
<tr>
<td>PTPRZ-B</td>
<td>Protein tyrosine phosphatase receptor zeta, B</td>
</tr>
<tr>
<td>PTPRZ-S</td>
<td>Protein tyrosine phosphatase receptor zeta, S (secreted)</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>RAS</td>
<td>Retrovirus-associated dna sequences</td>
</tr>
<tr>
<td>RB</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>RET</td>
<td>Rearranged during transfection</td>
</tr>
<tr>
<td>RGDS</td>
<td>RGDS-adhesion recognition motif</td>
</tr>
<tr>
<td>RPTP</td>
<td>Receptor type protein tyrosine phosphatase</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinases</td>
</tr>
<tr>
<td>S</td>
<td>Synthesis phase of cell cycle</td>
</tr>
<tr>
<td>SCR</td>
<td>Scrambled</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>SLV</td>
<td>Serine/Leucine/Valine</td>
</tr>
<tr>
<td>SLV/TKV</td>
<td>Terminal amino acid residues of PDZ binding domains.</td>
</tr>
<tr>
<td>Snta1</td>
<td>Syntrophin alpha 1</td>
</tr>
<tr>
<td>Sntb1</td>
<td>Syntrophin beta 1</td>
</tr>
<tr>
<td>SRB</td>
<td>Sulforhodamine B</td>
</tr>
<tr>
<td>synj2bp</td>
<td>Synaptojanin 2 binding protein</td>
</tr>
<tr>
<td>tagRFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>TCGA</td>
<td>The cancer genome atlas</td>
</tr>
<tr>
<td>TET2</td>
<td>Methylcytosine dioxygenase 2</td>
</tr>
<tr>
<td>TKIs</td>
<td>Tyrosine kinases inhibitors</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane region</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue micro-array</td>
</tr>
<tr>
<td>TMZ</td>
<td>Temozolomide</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumor protein p53</td>
</tr>
<tr>
<td>TULA</td>
<td>T-cell ubiquitin ligand</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine residues</td>
</tr>
<tr>
<td>U1-70</td>
<td>Small nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>---------------</td>
<td>-----------------------------------------------------</td>
</tr>
<tr>
<td>VEGF (-A)</td>
<td>Vascular endothelial growth factor (-A)</td>
</tr>
<tr>
<td>VEGF-BS</td>
<td>Vascular endothelial growth factor-binding site</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>Vascular endothelial growth factor receptor 2</td>
</tr>
<tr>
<td>Veli-3</td>
<td>Vertebrate Lin-7 Homolog 3</td>
</tr>
<tr>
<td>VSV-G</td>
<td>Vesicular stomatitis Indiana virus G-protein</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction
Introduction
Currently, the second leading cause of death in the world is cancer. Both biomedical researchers as well as health care institutions and pharmaceutical companies are focusing on the development of more effective therapeutic options and improvement of the quality of life and disease outcome for cancer patients [95]. Many different types of cancer exist and each requires tailored treatment options and comes with its own specific prognostics. Central nervous system (CNS) tumors, including gliomas, are amongst the cancers with the highest number of lost life years per patient [13]. Gliomas represent a group of primary brain tumors that originate from (precursors of) glial cells. It is the most frequent primary tumor in the brain, with an incidence of 3.55 per 100,000 people each year [80].

Classification and histopathology of glioma.
According to the classification used by the World Health Organization (WHO), gliomas can be subdivided into four grades (I-IV). The most frequent WHO Grade I gliomas are named pilocytic astrocytomas, that are characterized by activating BRAF fusions or BRAF mutations, mostly affect children, generally show slow and relatively circumscribed growth and only very infrequently progress to a higher malignancy grade. Grade II-IV gliomas are so-called diffuse gliomas, reflecting their diffuse infiltrative migratory behavior in the brain parenchyma [63]. Diffuse gliomas are further classified by their histological resemblance to non-neoplastic glial cells (astrocytes and oligodendrocytes). Within the different diffuse glioma categories, the presence of necrosis and microvascular proliferations and mitotic activity are determinants for the grade assignment. Both grade II and grade III gliomas can present as oligodendrogliomas as well as astrocytomas. The vast majority of gliomas are more widely known as glioblastomas and display the most aggressive behavior. Primary glioblastomas originate de novo whereas secondary glioblastomas progress from lower-grade diffuse gliomas.

Nowadays the classification of gliomas involves more than just malignancy grade and is in fact based on a combination of histopathological and molecular diagnostic features of the tumors [63] (Fig. 1). Not only does this provide more information to build the proper diagnosis, it may also yield molecular entry points that aid in the design of treatment plans. Correct classification of diffuse gliomas is important because treatment efficacy differs greatly between grades, subtypes and molecular aberrations, and the outcome of clinical trials would benefit from more homogeneously defined groups (e.g [109] for glioblastomas, [49, 92] for lower-grade gliomas and [28, 87] for O6-methylguanin-DNA-methyltransferase (MGMT) status).

Some of the most important diagnostic and prognostic biomarkers are mutations in the isocitrate dehydrogenase 1 (IDH1) and IDH2 genes [63]. Mutations in these genes are associated with a unique DNA methylation status and are found in the vast majority of lower-grade diffuse gliomas and high-grade secondary glioblastomas, the most common being R132H in IDH1 [5, 99, 111] (for details see below). Presence of IDH mutations is a predictor of prolonged survival [8, 9]. Primary glioblastomas, which originate de novo, are wild type (WT) for IDH1 and IDH2 [81].
Introduction

<table>
<thead>
<tr>
<th>Diffuse Glioma</th>
<th>Lower-grade (II-III)</th>
<th>High-grade (IV)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histology</strong></td>
<td><strong>Histology</strong></td>
<td><strong>Histology</strong></td>
</tr>
<tr>
<td>Oligodendrocyte</td>
<td>Astrocyte</td>
<td>Astrocyte</td>
</tr>
<tr>
<td><em>IDH</em> mut</td>
<td><em>IDH</em> WT</td>
<td><em>IDH</em> mut</td>
</tr>
<tr>
<td>1p/19q co-deletion</td>
<td>often carry mutations in ATRX and/or TP53</td>
<td></td>
</tr>
<tr>
<td>(Anaplastic) Oligodendroglioma</td>
<td>Diffuse (Anaplastic) astrocytoma</td>
<td>Diffuse (Anaplastic) astrocytoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Primary glioblastoma (including giant cell glioblastoma and gliosarcoma)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Secondary glioblastoma</td>
</tr>
</tbody>
</table>

No genetic testing available or inconclusive: oligodendroglioma NOS; diffuse astrocytoma NOS; glioblastoma NOS.

Figure 1: Schematic overview of classification of diffuse glioma. According to the WHO 2016 classification of tumors of the CNS a more precise diagnosis of diffuse gliomas can be made based on presence/absence of particular molecular aberrations [63]. Abbreviations, 1p/19q, chromosomal arms 1p and 19q; ATRX, ATP-dependent helicase; TP53, Tumor protein 53; IDH, Isocitrate dehydrogenase; mut, mutant; WT, wild type; NOS, Not otherwise specified.

*IDH* WT glioblastomas have several known histological variants, including giant cell glioblastoma and gliosarcoma.

Another diagnostic classifier is the co-deletion of chromosome arms 1p and 19q present in oligodendrogliomas [63]. Patients with tumors carrying the co-deletion have a better prognosis compared to individuals with tumors of a similar grade without the co-deletion [43, 71]. 1p/19 co-deleted tumors are almost exclusively *IDH* mutated [49, 104]. Tumors without the co-deletion but with an oligodendroglioma component are similar to astrocytomas in both clinical outcome and genetic profile (reviewed in [92]).

**Expression signatures:**
Nowadays, glioblastomas, are being subdivided based on extensive genetic and transcriptional profiling, resulting in three distinct groups instead of only primary and secondary. Tumors in each of these subgroups have a particular transcriptional signature, distinct genetic aberrations and diverse clinical outcome and are now often referred to as being of the pro-neural, mesenchymal or classical type [11, 109]. The frequency of occurrence of these different subtypes is similar [109].
Classical glioblastomas show an amplification of chromosome 7 containing the Epidermal growth factor receptor (EGFR) gene in almost all cases. Deletion of chromosome 10 harboring the tumor suppressor gene phosphatase and tensin homolog (PTEN) is very common. Unexpectedly, inactivating mutations in tumor protein p53 (TP53) are hardly present even though it is the most common mutation found in glioblastomas overall. Classical glioblastomas show high expression of several stem cell markers and when looking at expression of mRNA, resemble primary astrocytes the most.

Mesenchymal glioblastomas often have a neurofibromin gene (NF1) deletion and high levels of the tyrosine-protein kinase c-MET. Also dysfunctional TP53 and/or PTEN are often encountered in these tumors, both separate as well as in combination. Mesenchymal tumors show EMT (epithelial to mesenchymal transition) and appear dedifferentiated or transdifferentiated. Also they show more necrosis and inflammatory responses than found in the other glioblastoma tumor types. The expression profiles of normal cultured astroglia cells are most similar to the profiles of this group of glioblastomas.

Proneural tumors frequently show a high amount of platelet-derived growth factor receptor alpha (PDGFRα) alterations and IDH1 or, less frequently, IDH2 mutations. Although PDGFRα mutations are mutually exclusive with IDH mutations they both only occur within this glioblastoma subtype. Furthermore proneural tumors are often TP53 mutated. They show a more oligodendrocyte-like expression profile and resemble the lower-grade gliomas in that respect. The mean age of diagnosis in the proneural group is also significantly lower than for the other three subtypes.

Treatment of diffuse glioma
The standard treatment for glioblastoma patients follows the so-called Stupp protocol which combines surgery with radiotherapy and temozolomide (TMZ) treatment [97, 98]. Application of this protocol helps to extend the median survival period to up to 15 months although response between subgroups of patients with primary glioblastoma (as mentioned above) varies greatly. Glioblastomas ideally are first excised to the maximum feasible extent. Next, radiotherapy is applied, with concomitant TMZ administration and irradiation to increase the DNA damage resulting from the radiotherapy. Subsequently six more rounds of TMZ treatment are given. Standard care for lower-grade gliomas is often more diverse. Some centers prefer a ‘wait and see’ policy whereas other centers perform a resection of the tumor followed by radiotherapy. Caution is advised as lower-grade gliomas can acquire additional mutations from radio- or chemotherapy and thus may progress to a high-grade gliomas faster [44]. However, all lower-grade diffuse gliomas will eventually progress to a higher grade, so intervention will ultimately be needed.

The migratory behavior of diffuse gliomas is a major reason why treatment is so difficult. Cells disseminate from the main mass of the tumor and extensively infiltrate into the neuropil creating complex networks deep in the brain [82]. Due to this diffuse nature, a complete resection of the tumor by surgery is impossible. Furthermore,
Introduction

The cellular composition of glioblastomas around the time of diagnosis can already be very diverse, with great intra- and inter-heterogeneity of tumors [94], associated with large variability in response to treatment and incidence of recurrence. Together this results in glioma being one of the most incurable malignancies (reviewed in [19]). Some cells have a high proliferative capacity and contribute significantly to the growth of the tumor. These are the cells that are most likely hit by the current treatment. There are also stem cell-like cells within the tumor which are relatively dormant. These cells are mostly unaffected by treatment and can increase their proliferative capacity after treatment has been terminated (reviewed in [96]). To be able to effectively treat glioblastoma, all these cells with different properties and different survival programs must be targeted simultaneously. For future improvement of glioblastoma therapy and the development of personalized therapy, strategies for comprehensive characterization of tumor composition for each individual patient and more extensive a priori knowledge about drug effects on the growth and survival capacity of different tumor cell types is therefore urgently needed.

Already now, many new treatment strategies involve therapy with combinations of drugs that are tailored to the individual patient, aimed to target specific genetic aberrations. For instance, tyrosine kinases inhibitors (TKIs) are used, that block the activity of hyperactive receptor tyrosine kinases (RTKs), like the EGFR that is frequently mutated in these tumors (specifics discussed below). Cancer cells displaying stem cell-like properties inside the tumor are often slow-cycling cells which are not susceptible to EGFR inhibitors due to increased genetic stability, decreased oxidative stress, or the expression of efflux pumps that cause drug resistance. A frequently observed problem with treatment with such drugs is that it can cause secondary point mutations in EGFR, upregulation of other receptor tyrosine kinases and upregulation of drug efflux pumps. For instance, when EGFR is inhibited, another RTK, c-MET can become upregulated. Additional inhibition of c-MET thus may help to increase treatment efficacy [46], and by targeting multiple RTKs at the same time resistance-generating compensation mechanisms may be prevented (reviewed in [102]).

Another example of where combination treatment may be beneficial, or maybe even necessary, is in combating angiogenesis. In tumorigenesis, neovascularization is important to supply the growing tumor with nutrients and to eliminate waste. Tumors secrete Vascular Endothelial Growth Factor (VEGF) which binds to the Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) on the surrounding endothelium of blood vessels. Activation of this transmembrane receptor tyrosine kinase facilitates neovascularization as endothelial cells will start to proliferate and migrate to create new blood vessels. Attempts have been made to improve survival of glioma patients suffering from VEGFR2-associated angiogenesis using bevacizumab, an anti VEGF-A blocking antibody, which reduces angiogenesis and leakiness of vessels in perinecrotic areas of the brain tumor. Although the quality of life was improved, no increase in survival was measured when patients were treated with bevacizumab [18, 37, 110]. To suppress associated problems caused by vessel normalization, drug insensitivity and increased invasiveness [24, 84] due to upregulation of c-MET [69], trials with combined inhibition of VEGFR2 and c-MET are now being initiated.
The above examples illustrate that reliable and detailed diagnosis is necessary in combatting gliomas. Genetic characterization can be informative and and provide clues about the nature of deranged signalling pathways and help in the design of targeted drug therapies.

**Molecular pathways affected in diffuse gliomas**

In the last few years large large-scale DNA and RNA databases resulting from consortia, such as the The Cancer Genome Atlas (TCGA), have provided insight into the core pathways affected in glioblastoma. Below – not necessarily in order of importance or the incidence of mutation - we will discuss the significance of these different pathways for fundamental understanding of glioma biology and for translational medicine.

**P53 pathway**

*TP53* is one of the most commonly mutated/deleted genes in human cancers. *TP53* encodes for the protein p53 which is a transcription factor that can inhibit cell cycle progression, promote senescence and induce cell death. Upon oncogenic stress such as DNA damage, ARF, an alternate reading frame tumor suppressor, is transcribed from the cyclin-dependent kinase inhibitor 2A gene (*CDKN2A*) and inhibits the E3 ubiquitin-ligase protein mouse double minute 2 homolog (MDM2). Under normal physiological conditions MDM2 targets p53 for degradation. Inhibition of MDM2 results in stabilization of p53, which in turn can initiate transcription programs to either induce cell cycle arrest or force cells into apoptosis, depending on the cell type and magnitude of stress (for review and overview of the pathways in cancer see [12]). Because of the high abundance of mutant p53 protein in cancer cells it was first thought that p53 functioned as an oncogene. It is now well-established that such mutant versions of p53 contain substitutions, mostly in the DNA binding domain, that stabilize and inactivate the protein. Mutations in one allele of p53 are often accompanied by loss of the other allele. Studies on TP53 null mice as well as humans having a germ-line mutations in *TP53* (Li-Fraumeni Syndrome) have revealed a dramatic increase in the formation of neoplasms. Collectively, these findings unveil the p53 protein as a hallmark tumor suppressor. Also proteins that regulate p53, such as MDM1, 2 and 4 and E3 ubiquitin ligase (reviewed in [32]), are often found mutated and (in)activated in cancers. In glioblastoma approximately 85% of tumors contain a mutation or deletion in the p53 pathway; 28% in TP53 itself, 15% in MDM1, 2, or 4, and 58% in CDKN2A [11].

**RB pathway**

Loss of the retinoblastoma protein (RB) by mutation in the *RB* gene was first discovered as being causative for retinoblastoma, a rare and highly malignant pediatric cancer that develops from immature retinal cells. As such it represents the first tumor suppressor gene found [50]. Since this discovery RB loss has also been implicated in many other cancers, including glioblastoma [63]. The RB protein and its partners control cell cycle progression by binding to members of the E2F family of transcription factor proteins. E2F proteins and their partner proteins form dimeric complexes that upon nuclear entry mediate the progression into S-phase of the cell cycle. RB
can disrupt the dimeric complex and bind E2F to arrest it in the cytosol, consequently preventing the cell from entering S-phase and thus stalling the cell in the G1 phase. Upon phosphorylation of RB by cyclin-dependent kinases (CDKs) in late G1 the association with E2F is lost, allowing nuclear entry and expression of products required for the cell’s progression into S-phase (reviewed in [56]). In glioblastoma mutations and deletions in RB and especially activating mutations in CDKs are common; 79% of glioblastomas contain a mutation or deletion in the RB pathway. The RB protein itself is affected in almost 8% of the cases whereas CDK4 and CDKN2A are affected in 15.5% and 57.8% of glioblastoma samples, respectively [11]. CDKN2A-derived alternative transcripts in fact encode different proteins. When translated into ARF it will influence the p53 pathway, when translated into p16 (INK4A) it will inhibit CDKs and in turn affect the RB pathway (for an overview of the interactions between p53 and RB pathways see [14]).

**Mutations in IDH1/2**

Specific mutations in types of genes that do not encode cell signaling proteins but for example have a metabolic role, are also a known hallmark of glioma. Prominent examples are mutations in IDH1 and IDH2, abnormalities which are rare in other pathologies, except in chondrosarcoma and acute myeloid leukemia. Nowadays, mutations in IDH1/2 are used as a biomolecular marker to differentiate between different glioma subtypes and patient outcomes. Mutations in IDH1 are most common; in particular the R132H missense mutation, although other amino acid changes (to C or G, for instance) can be found as well at that position. Occasionally mutations at other sites in IDH1 are reported, including the R314H inactivating mutation [60]. In IDH2, R172 and R140 are the hotspots [5, 118]. Normal IDH proteins form dimers that convert isocitrate to alpha-ketoglutarate (α-KG) while reducing NADP+ to NADPH. The mutant IDH1 R132H/C/G variants have lost this function but gained the ability to reduce α-KG to D-2-hydroxyglutarate (D-2-HG) while oxidizing NADPH to NADP+ [21]. D-2-HG then inhibits α-KG dependent enzymes via competitive inhibition [117]. This results not only in altered metabolism but importantly, also in changes in epigenetic regulation [10]. For instance TET2 and KDM4C, two demethylating enzymes, are both regulated in part by α-KG. In IDH mutated tumors these two enzymes are inhibited resulting in DNA hypermethylation profiles and histone demethylation, which in turn inhibits cell differentiation [64]. Because mutated IDH proteins consume α-KG as a substrate to create D-2-HG the tumor cell becomes dependent on α-KG provision. Tumors almost exclusively contain heterozygous, and rarely contain homozygous IDH mutations [5]. Three types of dimers may then form; homodimers consisting of two wildtype proteins or two mutant proteins, or a heterodimer consisting of a wildtype protein and a mutant protein. The wildtype protein converts isocitrate to α-KG and the mutant subunit converts it subsequently to D-2-HG. Next to converting α-KG, IDH mutant cells also have low levels of NADPH because the mutant proteins oxidizes NADPH back to NADP+, significantly altering the redox status of the cell [60]. Furthermore, D-2-HG inhibits succinate dehydrogenase which alters the succinylation levels of proteins [59]. Additionally, IDH mutants lack the ability to reduce α-KG back to isocitrate, which can function as a carbon source for fatty acid and lipid synthesis [74, 113].
In addition to these pathways in cell survival, growth and metabolism, pathways governed by kinases are often affected in glioma’s. More details of these pathways and mutations found in these proteins are discussed below.

**Pathways regulating proper phosphorylation balance in the cell**

RTKs and downstream pathways are affected in over 90% of glioblastomas [11]. RTKs are transmembrane receptors which upon binding of their cognate ligand will dimerize and phosphorylate one another. This phosphorylation leads to hyperactivation of their kinase activity. The consequent phosphorylation burst will affect the localization or activity of other proteins in the cell, ultimately leading to changes in the cell’s behavior. In cancer, amplification and auto-activation by mutations of these RTKs leads to increased downstream signaling. These signaling pathways may involve multiple downstream effectors and bifurcate signals via the RAS/RAF-initiated mitogen-activated protein kinase (MAPK) cascade or the Phosphatidylinositol-4,5-bisphosphate 3-kinase pathway (PI3K) that are involved in cell cycle progression and cell survival, respectively.

**EGFR – C-MET and PDGFR α**

EGFR amplification and mutations are highly frequent in glioblastomas with aberrations found in 57% of the cases. Next to EGFR, mutations and amplifications can also be found in other RTKs such as c-MET and PDGFRα. EGFR aberrations are almost exclusively found in IDH WT glioblastomas, indicating that EGFR mutations contribute to the formation of the de novo tumor. EGFR is part of a larger family of RTKs consisting of EGFR, ERBB2, ERBB3, and ERBB4. All members contain an extracellular ligand-binding domain with two cysteine-rich regions; a transmembrane domain and an intracellular cytoplasmic tyrosine kinase domain. Normally upon binding of EGF or TGFα to EGFR, the receptor homo-dimerizes or hetero-dimerizes (notably with ERBB2) and phosphorylates tyrosine residues which signal towards other pathways such as MAPK, RAS/RAF, proto-oncogene tyrosine-protein kinase Src, PI3K and JAK/STAT, regulating cell survival, growth and migration (Fig. 2). Heterogeneity of the tumor plays a major role where not all cells within the tumor suffer from EGFR amplifications or mutations leading to oncogene addiction [94].

The c-MET tyrosine kinase can also be mutated/amplified or overexpressed in glioblastoma [11, 54]. The c-MET protein consists of an alpha and a beta subunit. These two subunits originate from a precursor protein which is post-translationally cleaved. The two subunits are connected via a disulfide bond to form the mature receptor [103]. c-MET has only two known ligands, HGF and its splicing isoforms NK1/NK2. Upon ligand binding, c-MET dimerizes and is transphosphorylated on tyrosine residues Y1234/Y1235 in its kinase domain and additionally on tyrosine residues Y1349 and Y1356 [30, 61]. The latter function as docking sites for substrates such as Gab1, Grb2 and PI3K [90, 112]. Phosphorylation of c-MET can activate MAPK cascades as well as PI3K downstream signaling, leading to cell survival and invasion [39].
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Figure 2: Overlap in function between RTKs. Activation of RTKs by homo-dimerization and subsequent phosphorylation of tyrosine residues leads to downstream signaling via several key pathways that regulate oncogenic transformation and maintenance such as angiogenesis, proliferation, survival and migration. RTKs show a large overlap in the pathways they can activate, and inhibition of one could lead to activation of the other, complicating treatment. EGF-BS, Epidermal growth factor-binding site; IG, Immunoglobulin-like domain. IPT, Ig-like, plexin and transcription factor domain; KD, kinase domain; PSI, domain found in plexins, semaphorins and integrins; TM, transmembrane domain; Tyr, tyrosine residues (can consist of multiple tyrosine residues scattered over the C-terminal tail, number varies per RTK); VEGF-BS, vascular endothelial growth factor-binding site.
Next to the amplifications of RTK genes, resulting in highly activated RTK downstream effectors, most of the RTK genes mentioned above also have constitutively active variants as drivers of these tumors. The best-known one is EGFR variant III (EGFRvIII) where exons 2-7 are deleted in the gene, resulting in aberrant transcripts encoding a protein with a shorter extracellular domain that mediates ligand-independent auto-activation. Also mutant PDGFRα, with deletions in the PDGFRA gene in exons 8-9, and the newly discovered mutant c-MET with deletions of exons 7-8 (c-MET Δ7-8), are present in a subset of glioblastomas [11, 79]. PDGFRα Δ8-9 results in a deletion of an extracellular fragment responsible for receptor-receptor interactions, leaving the PDGFRα Δ8-9 constitutively active [83]. The deletion of exon 7 and 8 from the c-MET transcript results in a truncated protein that remains intracellular and is auto-active, leading to increased ligand-independent c-MET signaling [79].

**PI3K/AKT pathway**

Mutations in PI3Ks and inhibitory enzymes in the PI3K pathway are found with high frequency in glioblastoma. The PI3K enzyme family is an important relay and balance system that transduces signaling from various growth factors such as the above mentioned RTKs. PI3Ks can phosphorylate the hydroxyl group at the 3’ position in the inositol ring of phosphatidylinositol. For instance, protein kinase B, also known as AKT, can bind to PtdIns(3,4,5)P3 and PtdIns(3,4)P2 that results from activated PI3K. AKT can then be effectively translocated to the membrane. In a similar manner phosphoinositide-dependent kinase-1 (PDK1) is translocated to the membrane where it then phosphorylates AKT. When finally also the mTORC2 complex phosphorylates AKT, AKT is fully activated and in the position to influence its downstream processes such as cell growth, proliferation, survival, metabolism and autophagy [108]. An intensely studied mutational event in the P13K-pathways is the inactivating deletion of the tumor suppressor protein PTEN found in 41% of the glioma cases. PTEN acts as an inhibitor on the AKT and PI3K pathway by dephosphorylating PtdIns(3,4,5)P3, hence counteracting PI3Ks specific activity (see further detailed explanation below). Furthermore, in 25% of glioblastomas mutations are found in genes encoding subunits and family members of PI3K3 (e.g. PIK3CA, PIK3R1, PIK3C2G).

The high incidence of RTK mutations in cancers, including gliomas, highlights the importance of the balance of phosphorylation of proteins in the cell. Deregulated phosphorylation may lead to increased signaling in survival and growth pathways (Fig. 2). Maintaining precise balance of phosphorylation and dephosphorylation of target tyrosine, serine and threonine residues in its proteins is thus of central importance for any cell.

**Protein tyrosine phosphatases**

Tight regulation of the specific phosphorylation status of proteins within the cell is in fact controlled by opposing kinase and phosphatase activities. Tyrosine kinases add a phosphate group to certain tyrosine residues on a protein and on the other hand the highly conserved protein tyrosine phosphatases (PTPs) dephosphorylate these tyrosine residues. Most phosphorylation events don’t target a single signaling pathway but have effects that span across a multitude of interacting networks.
Kinases are thought to control the amplitude of a response whereas phosphatases are more involved in controlling the rate and duration of a response in a signaling cascade [40, 42].

PTPs are evolutionary well preserved proteins, which have a conserved region in their catalytic site (HCX5R) with the essential cysteine residue that is responsible for removing the phosphate group from phosphorylated residues. PTPs can be classified into five classes (I-V). Class I PTPs represent the largest group in which a distinction can be made between cytosolic non-receptor PTPs, transmembrane receptor PTPs (RPTPs) and dual-specificity PTPs (DUSPs). The dual-specificity subgroup can be further divided into MAPK phosphatases (MKPs), PTEN-related (PTENs), phosphatase of regenerating liver (PRLs), myotubularin-related phosphatase (MTMRs) and atypical PTPs. The remaining four classes are much smaller and consist of low-molecular weight PTP (Lmw) (class II), cell division cycle (cdc25) (class III), eyes absent (EYA) (class IV) and the newly discovered T-cell ubiquitin ligand (TULA) (class V) enzymes, totaling 109 PTP genes found in the human genome [4, 105, 106]. There is molecular evolutionary evidence to also include the arsenate reductase, rhodanese, haloacid dehalogenase (HAD) and His phosphatase (HP) families that also dephosphorylate tyrosine residues. However instead of the critical cysteine they use aspartate or histidine, respectively [3], in their active site resulting in a PTP superfamily of 125 protein-encoding genes (Fig. 3).

**PTEN**

PTEN is one of the best studied tumor suppressor genes and, as mentioned above, found to be often mutated in glioblastoma. PTEN was originally also named MMAC (mutated in multiple advanced cancers) showing that early on PTEN was recognized as a tumor suppressor gene. PTEN is a dual-specificity phosphatase which can dephosphorylate serine/threonine and tyrosine residues. More important was the finding that PTEN can dephosphorylate phosphatidylinositol-3,4,5-trisphosphate (PIP3). Downregulation or deletion of PTEN results in increased phosphorylation of AKT, hence leading to increased survival signaling and stimulation of cell growth. Studies with mutant PTEN proteins deficient in key functions have shown that loss of phospholipid phosphatase activity results in the prevention of recruitment of AKT to the cell membrane (reviewed in [27]). Therefore AKT cannot be phosphorylated by its target kinases and downstream targets that are involved in cellular growth, proliferation and survival (for illustrations and review see [48]). PTEN, unlike many other phosphatases, has no closely related members which can compensate for loss of expression of PTEN even though there are several PTPs that show similarities to PTEN (legend to Fig. 3). Somatically, even a slight reduction in the expression of PTEN may have transforming effects [16]. Mutations and alterations in PTEN often suffice to induce somatic tumors in many different tissues. In PTEN knockout mice and patients with germline PTEN mutations who develop PTEN hamartoma tumor syndrome (PHTS) a wide variety of tumors is observed, but brain tumors do not develop. Nevertheless, Cowden Lhermitte-Duclos syndrome, caused by mutations in PTEN, does lead to formation of dysplastic cerebellar gangliocytoma, a WHO grade I CNS tumor [22]. Another important observation is that PTEN deletions have
been shown to occur later in tumor progression [94]. Therefore, although PTEN deletion may contribute to vulnerability in gliomagenesis, it is not enough to be the sole cause of glioma. PTEN mutations and deletions are rarely found in IDH mut glioblastoma or lower-grade gliomas (reviewed in [27]).

Next to its activity of dephosphorylating PIP3, recently other functions have been attributed to PTEN. One of those is PTEN’s involvement in regulating chromosomal integrity by increasing transcription of RAD51 and facilitating homologous recombination to repair double-stranded DNA breaks [93]. Mutations or deletions in PTEN therefore result in hampered double stranded break repair, leaving cancer cells more vulnerable to DNA damaging agents such as chemotherapy and radiotherapy [70]. TMZ treatment of glioblastomas results in mostly single-stranded DNA breaks [101]. One of the proteins that regulates single-stranded DNA break repair is PARP [72]. If not repaired, the single-stranded DNA breaks will develop into double-stranded ones in proliferating cells. Thus, by effectively hindering single-stranded DNA break repair in PTEN-deficient glioblastomas through PARP inhibition, a more effective irradiation and chemotherapy regime may be obtained. Strategies based on this principle have
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already been successfully exploited in the treatment of breast cancer [20].

Protein tyrosine phosphatase receptor z (PTPRZ) in cancer and glioma

Besides PTEN, other PTPs have been implicated in glioma [78] and in other cancers [45] and diseases [105]. One typical member of the family of PTPs with a prominent role in glioma pathobiology is PTPRZ. PTPRZ belongs to the class I transmembrane receptor PTPs (RPTPs), often resembling cell-adhesion molecules in their extracellular domains. In the intracellular section RPTPs usually have two phosphatase domains: D1 and D2, where D2 usually lacks the crucial cysteine and cannot dephosphorylate tyrosine residues (Fig. 3). However D2 is thought to be important in regulating enzyme activity, stability and specificity [105] (see below). PTPRZ's activity can be ligand controlled, for instance by pleiotrophin. Ligand binding leads to dimerization and this leads to blocking of an active site from one PTPRZ molecule by the other PTPRZ molecule [36, 67]. This is in contrast to RTKs, where ligand-induced dimerization leads to activation of the kinase molecules [57].

PTPRZ structure

The gene PTPRZ1 encodes three PTPRZ protein isoforms; PTPRZ-A, PTPRZ-B and phosphacan [52, 66, 68]. All isoforms share an N-terminal carbonic anhydrase-like domain (CA) and a fibronectin type III domain (FNIII). A spacer with sites for chondroitin sulfate proteoglycan attachment is only present in PTPRZ-A and phosphacan. Isoform PTPRZ-B contains a small extracellular domain that lacks this spacer and consequently only limited chondroitin sulfate chains can be added to this isoform. Phosphacan is secreted into the extracellular space because it contains neither the transmembrane nor the intracellular part present in the two other isoforms. [7, 52, 58]. The C-terminus of the intracellular part in PTPRZ-A and PTPRZ-B contains a PDZ binding motif ending in the amino acid sequence SLV.

PTPRZ function in normal brain

PTPRZ has known functions in development of the brain and in oligodendrocyte maturation. PTPRZ is expressed abundantly in both glial cells and in neurons in the central nervous system, especially in oligodendrocyte precursor cells, which reside in the subventricular zone during early development. The extracellular form of PTPRZ, phosphacan, is the most abundant proteoglycan in the brain [15, 29, 53, 55, 75]. PTPRZ can influence oligodendrocyte maturation and differentiation in several ways and plays a part in memory formation. Intracellularly the dephosphorylation of the p190RhoGAP protein by PTPRZ results in inhibition of differentiation of oligodendrocytes [53]. Furthermore, p190RhoGAP and its interaction with PTPRZ are involved in memory formation in the hippocampus in a Rho dependent manner [100]. PTPRZ can also modulate oligodendrocyte maturation extracellularly through binding to contactin-1. A complex between the two can inhibit proliferation of oligodendrocyte precursor cells and stimulate the differentiation into mature oligodendrocytes. This interaction is mostly facilitated by phosphacan, lacking the transmembrane and intracellular domain [55]. Thus, these two studies [53, 55] apparently contradict each other; in one PTPRZ is shown to inhibit differentiation and in the
other it rather promotes differentiation of oligodendrocytes. This work demonstrates that the configuration and balance of PTPRZ isoforms is important and that different parts of PTPRZ have different roles. To promote maturation in early development the extracellular domain is important in forming the complex with contactin-1 and to mature oligodendrocyte from oligodendrocyte precursor cells. In later development the full-length, intracellular domain-containing PTPRZ isoforms can dephosphorylate p190RhoGAP and inhibit differentiation of oligodendrocytes.

Another example of how delicate the balance of proteins can be is seen in neuronal outgrowth. PTPRZ on glial cells interacts with contactin-1 on neuronal cells. The short isoform (PTPRZ-B) promotes neuronal outgrowth while the proteoglycan versions (PTPRZ-A and Phosphacan) have inhibitory and repulsive effects. Contactin-1 can interact with cell adhesion molecules (CAMs), which bind to the spacer region that is lacking in the short form. Furthermore, phosphacan can function as a dominant-negative version against PTPRZ-B to sequester CAMs and ECM binding components necessary for neuronal outgrowth [66, 75, 91]. Most of the extracellular interaction partners of PTPRZ can also interact with other cell recognition molecules. For instance, tenascin can also bind to contactin-1 and Nr-CAM can bind both proteins as well (reviewed in [88]).

PTPRZ different modes of action
The extracellular domain of PTPRZ is large and can connect different ligands to different parts. An important ligand of PTPRZ is pleiotrophin (PTN) [66]. Furthermore, the heparin-binding growth factor midkine, which has a 45% sequence identity to PTN, binds to PTPRZ. Both ligands bind to the chondroitin sulfate region of PTPRZ [65]. Binding of PTN to PTPRZ leads to interaction with integrin receptor αvβ3 and subsequent activation of c-Src mediated signaling. Similar effects have been found when VEGFR2 receptor associates with integrin receptor αvβ3 upon binding of VEGF-A isoform VEGF165. VEGF165 is also known to bind PTPRZ and this interaction can be inhibited when PTN is overexpressed. Thus, PTN and VEGF165 compete for binding with PTPRZ to associate with αvβ3 and its subsequent signaling through c-Src [51]. Furthermore PTPRZ can associate with interleukin-34 at similar sites as PTN/midkine and VEGF165 [77]. PTPRZ and, more specifically, phosphacan are also known to bind FGF-2, another growth factor. However, chondroitinase treatment did not abolish the interaction between FGF-2 and PTPRZ, suggesting that not the chondroitin sulfate region but the CA and/or FNIII domain is involved [88]. This is in contrast to PTN and midkine, which bind to the CS region. Other binding partners of the CA and FNIII domain are N-CAM, Ng-CAM, contactin-1 and tenascin C and R [2, 55, 75, 88] (Fig. 4).

Intracellularly, PTPRZ has both a phosphatase and a scaffolding function. The former role involves direct interaction, whereby binding of its ligand PTN results in dimerization of the receptor and subsequent inactivation of its phosphatase domain. This leads to an increase of phosphorylated β-catenin [73], β-adducin [86], the Src-family member Fyn [85] and increased auto-activation of ALK [89]. Other direct substrates of PTPRZ are MAGI1 and ErbB4. ErbB4 phosphorylation is modulated by binding of
Figure 4: PTPRZ interacts with multiple partners, both intracellularly as well as extracellularly. Schematic representation of some of the known interactions of PTPRZ. Extracellularly, binding of domains of PTPRZ to growth factors and neuronal adhesion molecules is shown. Intracellularly, both PTPRZ’s dephosphorylating function as well as its scaffolding role are shown. Direct substrates of PTPRZ include fyn, paxillin, β-catenin, ALK and c-Src. PTPRZ’s scaffolding function is depicted in the interaction with MAGI1, where MAGI1 binds to the PTPRZ C-terminus and can subsequently be dephosphorylated. Furthermore, PTPRZ’s interaction with PSD95 and Erbb4 is shown, where PSD95 functions as a scaffolding protein to bring PTPRZ and Erbb4 in close enough proximity for Erbb4 dephosphorylation. Acronyms for PTPRZ domains are explained in the legends to Figure 3.
both PTPRZ and ErbB4 to protein PSD95. PSD95 functions as a scaffold, forming a bridge between PTPRZ and ErbB4, so PTPRZ can dephosphorylate ErbB4 [33]. PTPRZ binds to PDZ domain 2 in the scaffolding protein MAGI1 and subsequently dephosphorylates MAGI1 at tyrosine residues Y373 and Y858 [34]. Furthermore, PTPRZ can dephosphorylate the GTPase activating protein GIT1 [47] and the adapter protein paxillin, which localized to focal adhesions. Both proteins contain a similar substrate consensus sequence as found in MAGI1 [34].

The scaffolding function of PTPRZ partly serves in support of the enzymes’ dephosphorylation activity, for example as in the interaction with ErbB4 and PSD95 that was discussed above. But PTPRZ can also be part of scaffolding complexes that do not serve to dephosphorylate targets. For example, it is thought that Nr-CAM’s interaction with PTPRZ, contactin-1 and the contactin-interacting protein Caspr recruits proteins to the cell membrane [88]. PTPRZ contains a PDZ binding sequence ending in SLV whereas the Nr-CAM C-terminal sequence ends in SFV. Binding through these two PDZ binding motifs facilitates the formation of such a membrane-associated heteromeric complexes. Another example is the interaction of PTPRZ with MAGI3. Unlike MAGI1, MAGI3 is not dephosphorylated by PTPRZ but it can bind to its C-terminal PDZ binding sequence with one of its five PDZ domains [1], leaving plenty of anchoring possibilities for other proteins having PDZ binding tails.

**PTPRZ in glioma**

In diffuse gliomas (both with oligodendrocytic and astrocytic phenotype) PTPRZ is highly overexpressed [62, 76, 107]. The same holds true for many of its binding partners: pleiotrophin, contactin-1 and tenascin C [25, 26, 76]. PTPRZ is involved in glioma cell migration as knockdown of PTPRZ resulted in a reduction of the migratory potential of glioma cells [76, 107]. Overexpression of PTPRZ in glioblastoma cells increased migration of these cells further [62]. Knockdown of PTPRZ resulted in reduced tumor growth *in vivo* [76, 107]. PTPRZ related migration can be inhibited by expression of LRRC4, a leucine-rich repeat-containing synaptic adhesion protein, which in turn down-regulates PTPRZ expression [114]. In gliomas LRRC4 is known to be downregulated, further contributing to the oncogenic overexpression of PTPRZ [115, 119]. When LRRC4 is overexpressed in glioma it inhibits migration of glioma cells and normalizes RTK signaling [116]. Taken together, these findings put forward PTPRZ as an oncogenic contributor in glioma. Peptides against PTPRZ are now included in a clinical trial to immunize patients against glioblastoma [23]. Blocking antibodies against PTPRZ’s extracellular region have also been generated. The blocking antibody alone inhibited anchorage independent growth. When the antibody was paired with saporin, a ribosome-inactivating protein which inhibits protein synthesis, it reduced cell growth *in vitro* and *in vivo*. The antibody thus served in targeted delivery of the toxin [31]. Recently an inhibitor of the catalytic activity of PTPRZ has been constructed and it was found to reduce tumor growth, both *in vitro* and *in vivo* [35].

Many of PTPRZ’s binding partners are also involved in migration or adhesion. Tenascin C can, upon binding to the FNIII domain of PTPRZ, facilitate adhesion of glioma cells [2]. Blocking tenascin C binding using an antibody inhibited the migratory effect
of tenascin C through PTPRZ [26]. Furthermore, blocking binding of tenascin C to the transmembrane version of PTPRZ by adding Phosphacan showed a decrease in adhesion [38]. The overexpression of contactin-1 in glioma cells has a repellant effect on cells [25]. Both contactin-1 and tenascin C expression are elevated with increasing malignancy in diffuse gliomas [25, 26].

Finally, even fusion transcripts of PTPRZ have been found in glioma samples (2.5%) [6]. Fusion transcripts occur due to translocation events in the genome; the novel combination of components of existing genes results in hybrid transcripts and proteins that are aberrantly expressed. Translocations involving the genes PTPRZ1 and MET resulted in fusion transcripts that encode proteins which contain the entire c-MET sequence N-terminally preceded by pieces of varying length of PTPRZ’s extracellular domain [6, 17]. The fusion event correlated with a faster tumor progression and a shorter survival of patients compared to patients not carrying the translocation. Expression of the fusion transcript enhances cell migration and invasion in vitro [6]. Furthermore, expression of the transcripts upregulates expression of both PTPRZ and c-MET [17] and increases phosphorylation of c-MET. The fusion protein has similar processing and dimerization properties as c-MET and can enhance phosphorylation in both a HGF dependent and independent manner. It is noteworthy that, the larger the piece of PTPRZ in the fusion protein is, the less effect it has on phosphorylation of c-MET. The fusion protein only containing the tip of the N-terminus of PTPRZ shows the largest increase in phosphorylation of the wildtype c-MET [17].

Outline of this thesis.

It is clear that a correct balance of protein phosphorylation in the cell is important for maintaining normalcy in its growth and behavior. In this thesis, we present work in which we investigate the regulating role of PTPs and their counterparts, the RTKs, in diffuse glioma. Better understanding of the role that PTPs play in these tumors is important because they may function dually as oncogenic drivers and/or as tumor suppressors, thus forming an interesting class of targets for the design of novel therapeutic approaches. Improvement of treatment regimes is urgently needed because patients with glioblastoma have currently a median survival of only 15 months. Obtaining insight in the PTP- and RTK-associated pathways can thereby provide help. In chapter 2 we investigate new ways to reduce the excessive phosphorylation by tyrosine kinases VEGFR and c-MET. Next to chemical inhibitors of excess phosphorylation we examine the potential role of PTPs in regulating tumorigenic pathways by assessing their expression in glioma samples. In chapter 3 we also test the effect of potential candidates on tumorigenicity and explore the overall relation between PTP expression and the malignancy grade of glioma tumors. We next focus on PTPRZ as a possible oncogene in glioma and study its mechanism of action using a clinically relevant glioma cell model that recapitulates the diffuse infiltrative character of gliomas. In chapter 4 we investigate the functions of different domains of PTPRZ on glioma diffuse migration and proliferation and signaling using truncated proteins. In chapter 5 we continue this line of research and search for interactors of the intracellular domains of PTPRZ that regulates glioma proliferation. In chapter 6 we discuss the implications of the results presented in this thesis. We also provide
an outlook on future research strategies that will help in the further elucidation of the role of PTPs in glioma etiology and in the translation of new insight into novel treatment opportunities.

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ChIP-nexus


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Introduction


Chapter 2

Effects of dual targeting of tumor cells and stroma in human glioblastoma xenografts with a tyrosine kinase inhibitor against c-MET and VEGFR2.

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Abstract

Anti-angiogenic treatment of glioblastoma with Vascular Endothelial Growth Factor (VEGF)- or VEGF Receptor 2 (VEGFR2) inhibitors normalizes tumor vessels, resulting in a profound radiologic response and improved quality of life. This approach however does not halt tumor progression by diffuse infiltration, as this phenotype is less angiogenesis dependent. Combined inhibition of angiogenesis and diffuse infiltrative growth would therefore be a more effective treatment approach in these tumors.

The HGF/c-MET axis is important in both angiogenesis and cell migration in several tumor types including glioma. We therefore analyzed the effects of the c-MET- and VEGFR2 tyrosine kinase inhibitor cabozantinib (XL184, Exelixis) on c-MET positive orthotopic E98 glioblastoma xenografts, which routinely present with angiogenesis-dependent areas of tumor growth, as well as diffuse infiltrative growth. In in vitro cultures of E98 cells, cabozantinib effectively inhibited c-MET phosphorylation, concomitant with inhibitory effects on AKT and ERK1/2 phosphorylation, and cell proliferation and migration. VEGFR2 activation in endothelial cells was also effectively inhibited in vitro. Treatment of BALB/c nu/nu mice carrying orthotopic E98 xenografts resulted in a significant increase in overall survival. Cabozantinib effectively inhibited angiogenesis, resulting in increased hypoxia in angiogenesis-dependent tumor areas, and induced vessel normalization. Yet, tumors ultimately escaped cabozantinib therapy by diffuse infiltrative outgrowth via vessel co-option. Of importance, in contrast to the results from in vitro experiments, in vivo blockade of c-MET activation was incomplete, possibly due to multiple factors including restoration of the blood-brain barrier resulting from cabozantinib-induced VEGFR2 inhibition. In conclusion, cabozantinib is a promising therapy for c-MET positive glioma, but improving delivery of the drug to the tumor and/or the surrounding tissue may be needed for full activity.
Introduction

Glioblastoma is a highly aggressive primary brain tumor that is characterized by extensive areas in which tumor cells diffusely infiltrate the brain parenchyma. A well-known hallmark of this cancer type is the presence of a necrotic core, surrounded by a rim in which hypoxia-induced neovascularization occurs [1]. Angiogenesis in these areas is associated with vessel leakiness, which contributes to edema and high intracranial pressure, aggravating symptoms that by themselves can be lethal. Local vessel leakage is exploited to diagnose glioblastoma, as it results in extravasation of intravenously administered contrast agents like Gd-DTPA which can be readily visualized by MRI. Glioblastomas are generally operated upon to the maximum feasible extent, followed by radiotherapy and chemotherapy with temozolomide. Remnants of diffusely growing tumor cells will however inevitably result in tumor recurrence and median survival is currently still only 14.6 months [2].

It is well recognized now that inhibition of VEGF-A signaling pathways in neovascular endothelial cells, either by the neutralizing antibody bevacizumab or selective VEGFR2 tyrosine kinase inhibitors, induces a radiological response, significantly reduces edema and may substantially improve quality of life [3-6]. Bevacizumab is now approved by the FDA for treatment of recurrent glioma. However, it has also become clear from a number of preclinical but also clinical studies that the diffuse infiltrative phenotype of glioblastomas is not sensitive to angiogenesis inhibition [5,7-9]. We previously showed that different anti-angiogenic treatments of orthotopic E98 xenografts (displaying both angiogenesis and diffuse infiltration [10]) affect only the angiogenic tumor component [8,9,11]. Apparently, anti-angiogenic therapies drive tumor cells to adapt a resistant, angiogenesis-independent phenotype in which tumor cells obtain their blood supply entirely from pre-existent vasculature [12-16]. These therapies have even been suggested to increase tumor cell invasion in glioma and other tumor types [17,18] and this appears to be associated with induction of hypoxia [19]. It is therefore of major importance for effective glioma treatment that approaches become available that tackle diffuse infiltrative tumor growth.

The c-MET tyrosine kinase receptor has been linked to both tumor angiogenesis and the invasive phenotype of glial and other tumors [19,20]. Upon binding of its ligand hepatocyte growth factor (HGF, scatter factor), c-MET is phosphorylated on tyrosine residues Y1234/1235 (kinase domain) and Y1349 and Y1356, the latter two residues with their surrounding amino acids functioning as docking sites for substrates such as Gab1, Grb2 and phosphatidylinositol 3 kinase (PI3K) [21,22]. Downstream signalling of c-MET involves important pathways including RAS/PI3K and ERK/MAPK, which are associated with tumorigenesis and cancer progression [23].

Amplification of the c-MET gene (located on chromosome 7) is seen in glioblastomas [24] and both c-MET and HGF are frequently overexpressed in glioma specimens and cell lines. HGF is a strong stimulator of in vitro glioma cell migration [25-27] and c-MET expression has also been demonstrated in invasive glioma cells [25]. Simultaneous targeting of the VEGF and c-MET pathways may therefore be an interesting
therapeutic approach for c-MET-positive glioblastoma because it will reduce vessel leakage (resulting in edema reduction) and simultaneously may reduce tumor cell migration and thus tumor progression. Cabozantinib (XL-184, Exelixis, South San Francisco, CA) is a small compound tyrosine kinase inhibitor of VEGFR2, c-MET and RET and has been shown to block tumor development in the RipTAG2 mouse model of pancreatic carcinogenesis more effectively than blockade of c-MET or VEGFR2 alone [19,28,29]. Cabozantinib was recently FDA-approved for treatment of medullary thyroid cancer.

The aim of the current work was therefore to test the effects of cabozantinib in mice carrying highly aggressive orthotopic E98 glioma xenografts [10]. We show that cabozantinib blocks vascular leakage in this c-MET positive tumor model and gives a significant survival benefit which was not observed in previous experiments from our lab with bevacizumab or other VEGFR2 inhibitors [8,9]. Interestingly, whereas cabozantinib completely blocked c-MET tyrosine phosphorylation in vitro in E98 cell cultures, phosphorylated c-MET was still present in remaining diffuse infiltrative tumor areas in treated mice. We propose that the anti-VEGFR2 activity of cabozantinib results in a restoration of the blood-brain barrier, thereby precluding an efficient distribution of the compound to the tumor cells and reducing c-MET inhibitory activity.

Materials and methods

Cell culture
E98NT cells (cell line derived from E98 xenografts, described in [30]) were cultured in DMEM supplemented with 10% FCS and pen/strep in the presence of 5% CO2 at 37°C. HUVECs were cultured in EBM medium, supplemented with FCS, bFGF and VEGF (Lonza) according to the manufacturer’s instructions.

Protein expression analysis
E98NT cells or HUVECs were plated in 6 well dishes in appropriate media at a density of 5x10⁵ cells per well. The following day cabozantinib (0, 0.01, 0.1, 0.5, 1 and 10 μM in DMSO) was added. After a 30 min incubation, cells were washed with ice-cold PBS and protein extracts were generated using RIPA buffer supplemented with protease and phosphatase inhibitors (Roche Applied Science). Similarly, extracts were generated from E98NT cells treated for 24 h with 1 and 10 μM cabozantinib (for assaying apoptosis) and sections of snap frozen xenograft brains (approximately 20 mg of tissue). Protein lysates were cleared by centrifugation and protein concentrations were measured using the BCA Protein Assay Kit (Thermo Scientific) according to manufacturer’s instructions. Proteins (20-40 μg/lane) were subjected to SDS-PAGE and Western Blotting using antibodies directed against c-MET (clone EP1454Y, Epitomics and clone D1C2, Cell Signaling Technology (CST)), phospho-c-MET (Y1234/1235, clone D26, CST), phospho-AKT (S473, clone D9E, CST) phospho-ERK1/2 (Thr202/Tyr204, clone 20G11, CST), anti-U1-70 (to detect apoptosis, [31]) and α-tubulin (clone 236-10501, Molecular Probes) or γ-tubulin (clone C20, goat, Santa Cruz Biotechnology) as internal control. Antibodies were detected us-
ing appropriate secondary antibodies, labeled with IRDye700 or IRDye800 infrared dyes. Signals were visualized and quantified when appropriate, using the Odyssey Infrared Imaging System (LI-COR Biosciences Odyssey Application Software version 3.0.30).

Dose response analysis
IC50 of cabozantinib was determined as follows: cells were plated at a concentration of 2x104 cells per well in 96-wells plates. The next day increasing concentrations of cabozantinib (in DMSO) were added to the medium. Each condition was tested in quadruplicate in at least three independent experiments. Metabolic activity of the cells was determined 4 days following start of TKI treatment by incubation with 0.5 mg/ml MTT in PBS (Sigma-Aldrich, St. Louis, MO). After a 3.5 hr incubation at 37°C formazan crystals were dissolved in MTT solvent (0.1% NP40 and 3.4 mM HCl in isopropanol) and optical densities were measured at 560 nm. IC50 concentrations were determined using sigmoidal dose-response (variable slope) statistics and normalized in GraphPad Prism.

Cell migration assays
E98 spheroids were generated by the hanging drop method. In brief, 0.7 ml methylcellulose (Sigma M6385, final concentration 1.68 mg/ml) was added to 4.3 ml E98 cell suspension in normal culture medium (500,000 cells total) and drops of 25 µl containing approximately 2,500 E98 cells were seeded in a dry culture dish. The dish was then inverted and incubated overnight at 37°C in the presence of 5% CO2. The next day, individual spheroids were seeded in a matrigel-coated 96-well imaging culture dish (Matrigel: BD cat 356237, 96-well plates: BD falcon imaging plates cat 353219) and grown further at 37°C and 5% CO2. Individual spheroids were photographed at t=0 h in a culture system which allows for live cell imaging, after which they were incubated with 0, 1 or 10 µM cabozantinib. After 24 h, wells were washed with PBS and cells were fixed with 4% PFA in 0.1M phosphate buffer, followed by DAPI staining. Fluorescent and phase-contrast images were then taken and ImageJ software was used to quantify spheroid outgrowth. Briefly, cells which had migrated from the spheroids were selected automatically using a cell mask. Numbers of cells that had migrated out of the spheroids (n=at least 32 for each condition) were measured. Statistical analyses involved ANOVA and post-hoc Tukey’s Multiple Comparison Test.

Animals
Athymic female BALB/c nu/nu mice (18-25 gram, age 6-8 weeks) were kept under specified pathogen free conditions and received food and water ad libitum. The local Animal Experimental Committee of the Radboud University Nijmegen Medical Center approved all experiments. E98 glioblastoma cells derived from subcutaneous xenograft tumors were injected intracranially under isoflurane anesthesia as described previously [10]. All efforts were made to minimize suffering. Animals were closely monitored by visual inspection and weighed daily from start of treatment (see below) and sacrificed when evident signs of tumor burden (especially weight loss >20%, severe neurological dysfunction) were observed. Brain, liver and kidneys
were harvested, and parts were formalin fixed and paraffin embedded or snap-frozen in liquid nitrogen for further analysis.

**Therapy**

Animals carrying E98 tumors were randomly divided into placebo and cabozantinib treatment groups. Treatment was started at day 12, when signs of tumor growth became apparent, as evidenced by the presence of edema in T2 weighted MR imaging. Water-suspended cabozantinib (XL184, Exelixis, South San Francisco) was given daily by oral gavage in doses of either 60 mg/kg (n=3) or 100 mg/kg (n=10) in a volume of 100 μl. A control group (n=10) received water daily by oral gavage.

**Immunohistochemistry (IHC)**

Immunohistochemical stainings were performed as described before [9]. In short, after epitope retrieval by boiling in citrate buffer (pH 6.0), 4 μm tissue sections were incubated with primary antibodies against GLUT1 (Neomarkers), CD34 (clone MEC14.7, Hycult biotech), c-MET (clone EP1454Y, Epitomics), phospho-c-MET (Y1234/1235, clone D26, CST), MCT4 (clone H90, Santa Cruz), cleaved caspase 3A (clone C92-605, BD Pharmingen) and Ki-67 (clone Sp6, Thermo Fisher Scientific). Appropriate biotinylated secondary antibodies were used for detection using the Avidine Biotine Complex (ABC) method (Vector Laboratories). Specific signals were visualized by staining with 3-amino-9-ethyl-carbazole (AEC, Scytek Laboratories) or 3,3’-diaminobenzidine (Power-DAB, Immunologic) solution. All sections were counterstained with haematoxylin and mounted in Imsol Mounting medium (Klinipath B.V.).

**Image analysis**

Cell proliferation, hypoxia and vessel densities were quantified using KS400 software (Carl Zeiss AG, Germany) with a custom-written macro on images acquired on a Zeiss Axioskop II microscope coupled to a CCD-RGB camera. For proliferation, five random non-overlapping diffuse infiltrative or compact tumor containing microscopic fields (magnification x200) were analyzed per section. Compact and diffuse areas were recognized by gross histology, with compact areas being present in the ventricles or leptomeninges, and diffuse areas defined by the intermittent presence of white matter tracts in H&E staining. The proliferative fraction was defined as the number of proliferative cells (based on Ki-67 positivity) divided by the total number of nuclei in a tumor region. Ratios were determined and the average value for the different fields per slide was used in further calculations.

For hypoxia quantification, compact tumor areas, defined as sharply bordered regions lacking normal brain parenchyma in between the tumor cells, and hypoxic regions (based on MCT4 positivity) were selected interactively. The hypoxic fraction was defined as the total hypoxic area divided by the total tumor area.

Vessel densities were measured in GLUT-1 immunostainings and counted in 5 high power fields per section in both compact and diffuse areas. Since neovascularure in compact areas often lack blood-brain barrier characteristics (e.g. GLUT-1 expression), also CD34 stainings were performed.
Statistical comparisons were done by normality analysis, followed by a Student’s t-test (two-sided) using GraphPad Prism v4. For both control and 100 mg/kg cabozantinib treated brains, n=10. A p value of ≤0.05 was considered as statistically significant.

**Contrast-enhanced Magnetic Resonance Imaging**

CE-MRI was performed in a 7T MR system (ClinScan, Bruker BioSpin, Ettlingen, Germany) equipped with a clinical user interface (syngo MR, Siemens, Erlangen, Germany). When tumor-related symptoms became apparent, animals were anesthetized using 1-2% isoflurane in a 70% N2O and 30% O2 mixture and placed in a prone position in an MR cradle. Breathing was monitored throughout the MR experiment and the animals’ core temperature was maintained at 37.5°C using a continuous flow of warm air (SA Instruments, Inc., Sunny Brook, NY, USA). Anatomical references were acquired using a multi-slice localizer with slices in the three main orthogonal directions. A turbo spin echo sequence with following settings were used: repetition time (TR) 3880 ms, echo time (TE) 43 ms and a turbo factor of 7. The scans had a resolution of 98 μm in-plane and a thickness of 0.7 mm per slice. A bolus of 0.2 ml of Gd-DTPA (20 mM, Magnevist®, Schering, Germany) was injected intravenously via a pre-inserted tail vein catheter and additional sets of T1-weighted images were acquired 2-3 minutes after injection.

**Results**

Elevated expression of c-MET in glioma specimens has been documented by several groups. Whereas up to 35% of primary gliomas overexpress this RTK, this percentage increases to up to 75% in recurrent glioblastomas [32]. Often, heterogeneity in c-MET expression is found between tumor cells [33] and some papers have reported on the expression of c-MET on endothelial cells too [34]. This is especially interesting since multi-targeted tyrosine kinase inhibitors such as cabozantinib have been developed that have specificity against both VEGFR2 and c-MET.

In the past we have established a number of glioblastoma xenograft lines by either subcutaneous or direct intracerebral implantation of surgically derived tumor cell suspensions in immunodeficient mice, which are subsequently maintained by serial transplantation [10]. One xenograft line that has been extensively analyzed for its response to anti-angiogenic treatment is E98. This line routinely presents with diffuse tumor growth in the brain parenchyma, using co-opted blood vessels and white matter tracts as scaffold. The presence of additional areas of angiogenesis-dependent growth makes this model of high translational relevance for testing of targeted therapies. Western blot analysis using c-MET and phospho-c-MET specific antibodies (recognizing the phospho-tyrosines Y1234 and Y1235) revealed that only E98 xenografts express significant amounts of the activated receptor, consistent with our observation that chromosome 7 is amplified in this xenograft line (Figure 1A and [10]). IHC analysis confirmed c-MET expression in E98, showing expression in all tumor cells (note that the non-stained structures in Figure 1D correspond to white matter tracts, as is clear from H&E staining of the serial section in Figure 1B).
IHC using the phospho-specific anti-c-MET antibody also confirmed the presence of activated c-MET, although in a heterogeneous fashion, being present predominantly in diffuse infiltrating tumor cells in the corpus callosum and adjacent white matter (Figure 1E, arrow and 1F). Furthermore, activated c-MET was detected in a rim of non-infiltrative tumor cells (routinely present in this model in the leptominges and

Figure 1 – c-MET is activated in E98 xenografts. Panel A shows a Western blot containing protein extracts of different xenografts as indicated (40 µg/lane) and stained with a pan and an Y1234/1235 phosphorylated (P-) c-MET specific antibody. As a loading control, γ-tubulin was included. Immunohistochemical analysis reveals prominent c-MET expression and activation in orthotopic E98 xenografts (C-F). Gross appearances of an E98 tumor are shown in C and E, while D and F show magnifications of the boxed areas in C and E. The H&E section in B illustrates the diffuse nature of these tumors, arrows pointing at white matter tracts and comparison with D shows homogeneous expression of c-MET by tumor cells. Arrow in E points at diffuse infiltrative tumor cells in white matter with activated c-MET, while the arrowhead points at a more compact paraventricular tumor area. The inset in E represents an area with compact leptomeningeal growth partly positive for activated c-MET. The pictures shown are representative for this xenograft model. Size bars: B, D, F 200 µm; C 1 mm and E 500 µm.
ventricles) at the interface with normal brain parenchyma (inset in Figure 1E and not shown). In central regions of compact growing tumor areas c-MET was not activated (arrowhead in Figure 1E).

We reasoned that the partially angiogenic character of the E98 xenograft model, in combination with high c-MET expression and activation in diffuse infiltrative areas makes this model highly relevant to study simultaneous inhibition of VEGFR2 and c-MET signaling. First, we investigated whether cabozantinib therapy blocked c-MET activation in vitro. Treatment of the E98NT cell line, derived from the E98 xenograft model [30] resulted in an efficient and dose-dependent inhibition of c-MET phosphorylation after 30 minutes (Figure 2A). Downstream signaling via AKT was also significantly inhibited by cabozantinib (note the ~82% reduction of phosphorylated AKT and the accompanying decrease in phosphorylated ERK1/2 at concentrations higher than 0.5 µM). Consistently, cabozantinib caused a dose-dependent inhibition of proliferation in E98NT cells (Figure 2B, IC50 ~ 89 nM). Cabozantinib did not induce apoptosis in vitro as demonstrated by Western blot staining with anti-U1-70 antibody (Figure 2F). In an in vitro spheroid-based cell migration assay, we observed that cabozantinib significantly reduced the number of E98 cells that are able to migrate away from the spheroids (Figure 2C-D, p<0.001, Post-hoc Tukey’s Multiple Comparison Test). Thus, c-MET signals have bearing for E98 tumor cell migratory potential as well. These inhibitory effects can be attributed to c-MET inhibition since E98 cells do not express VEGFR2 (Figure 2E). The inhibitory activity of cabozantinib on VEGFR2 [28] was confirmed on cultures of HUVECs and was complete at concentrations of 10 µM (Figure 2E).

We next subjected mice carrying established orthotopic E98 xenografts, as determined by visibility of edema on T2-weighted MR imaging (see Figure 3A for an example) to treatment with cabozantinib. An initial pilot experiment with 60 mg/kg cabozantinib (n=3) resulted in a full radiologic response using Gd-DTPA enhanced MRI, similar to our previous observations with bevacizumab, vandetanib and sunitinib [7-9]. However, large invasive tumors with hypoxic compact regions (identified by MCT4 expression) remained present after treatment while no signs of hypoxia were seen in the diffuse tumor areas (not shown). There was a non-significant trend towards increased survival (mean survival of 19 days in control vs. 23 days in 60 mg/kg cabozantinib treated animals). A larger group of animals (n=10) was therefore treated with 100 mg/kg cabozantinib, which did result in significantly prolonged survival compared to control-treated mice (median survival of tumor-bearing control mice was 20 days vs. 32 days for the 100 mg/kg cabozantinib group, log rank test p<0.0001, Figure 3B). All further experiments refer to this group. Prior to sacrifice, mice were subjected to Gd-DTPA-enhanced MRI. Treatment with 100 mg/kg cabozantinib resulted, as expected, again in a complete absence of contrast enhancement (Figure 3C, lower panels) despite clear presence of extensive tumor (H&E staining in figure 3D, note that these sections correspond to the MR scans). Upon 100 mg/kg cabozantinib treatment, tumors generally had converted to a mainly diffuse infiltrative phenotype (see figure 3D, lower panels) similar to our previous findings with vandetanib and sunitinib [8,9]. In all animals limited areas of compact tumor
were present to varying extent and these areas were significantly more hypoxic than in control animals ($p=0.003$, see IHC for hypoxia induced monocarboxylate transporter-4 (MCT4, Figure 4A-C) confirming previous observations with other angiogenesis inhibitors [9]. Staining for GLUT-1, another marker for hypoxia, gave similar results (not shown).
Diffuse infiltrative tumor regions did not show signs of hypoxia in control and treated tumors and based on caspase stainings apoptotic cells were very infrequent, also in treated tumors (not shown). Both compact and more invasive tumor areas were analyzed for proliferation differences, based on Ki-67 positivity in IHC. The prolifera-
tive fraction did not differ between controls and treated tumors in diffuse infiltrative regions, but proliferation was significantly less in compact tumor regions after treatment (p=0.04, Figure 4D-F).

GLUT-1 is, except for hypoxic cells, also expressed on brain capillaries and can be utilized as a blood vessel marker in the CNS. Tumor vessel densities were quantified and did not differ in diffuse areas between treatment and placebo groups (Figure 4G-I). Vessel densities in compact areas were difficult to quantify based on GLUT-1

Figure 4 – In vivo effects of cabozantinib treatment in E98 xenografts. Panels A and B show representative examples of IHC for the hypoxia marker MCT4 in control and cabozantinib-treated tumor bearing animals. Hypoxia in compact tumor regions is significantly increased after treatment (Students t-test, p=0.003, panel C). D and E show examples of Ki67 stainings in compact tumor areas. Proliferation indices were significantly different in these regions (Students t-test p=0.04), but no difference was detected in diffuse tumor areas (panel F). Panels G and H show representative examples of GLUT-1 vessel staining. Automated quantification revealed no differences between vessel densities of diffuse tumor areas in control vs treated mice (I). Numbers of CD34-positive vessels were lower in cabozantinib treated mice (see panels J and K, arrows point at blood vessels), but these data were not quantified because vessels without CD34 expression were also observed in these mice. L: Western blot analysis of protein extracts (50 μg protein/lane), derived from cabozantinib-treated xenografts reveals a substantial, though not complete, reduction of c-MET phosphorylation. As a loading control, γ-tubulin was included. Immunohistochemistry for phospho-c-MET (Y1234/1235) also shows the presence of phosphorylated c-MET in treated animals, as visualized in panel K. Size bars: A-B 2 mm, D-E 100 μm, G, H, J, K 200 μm,
Automated quantification revealed no differences between vessel densities of diffuse tumor areas in control vs treated mice (I). Numbers of CD34-positive vessels were lower in cabozantinib treated mice (see panels J and K, arrows point at blood vessels), but these data were not quantified because vessels without CD34 expression were also observed in these mice. L: Western blot analysis of protein extracts (50 μg protein/lane), derived from cabozantinib-treated xenografts reveals a substantial, though not complete, reduction of c-MET phosphorylation. As a loading control, γ-tubulin was included. Immunohistochemistry for phospho-c-MET (Y1234/1235) also shows the presence of phosphorylated c-MET in treated animals, as visualized in panel K. Size bars: A-B 2 mm, D-E 100 μm, G, H, J, K 200 μm.

staining because neovasculature often does not express this differentiation marker. Based on CD34 stainings (a marker of activated (neo)vasculature), vessel densities appeared lower in cabozantinib treated animals (compare Figure 4J and K), but as this treatment may also result in a downregulation of CD34 expression on vessels, these data are difficult to interpret in terms of vascular densities. It is important to realize that our studies were not time-matched, making it impossible to compare tumor volumes in treated and control animals.

To investigate whether the remaining diffuse infiltrative tumor might be the result of incomplete c-MET inhibition, we prepared tumor extracts from treated and control animal brains and prepared Western blots. As opposed to our in vitro data, even dosing as high as 100 mg/kg cabozantinib (resulting in plasma levels of 30 μM [29]) was not able to fully prevent c-MET phosphorylation (Figure 4L) and this finding was confirmed with IHC for phospho-c-MET (Y1234/1235, Figure 4M).

Discussion:

The proposed functional role of c-MET in tumor cell migration makes this receptor an attractive therapeutic target in c-MET positive glioblastoma. The high expression and activation levels of c-MET in diffuse E98 tumor areas supports the notion that c-MET is actively involved in tumor cell migration [20]. Targeting of c-MET may further be beneficial as signaling from this receptor may induce and maintain the glioblastoma stem cell-like phenotype and therefore resistance to chemotherapy and radiotherapy [35].

Cabozantinib treatment of in vitro cultures of E98 cells greatly reduced phosphorylation of AKT and ERK. This was accompanied by a 50% inhibition of cell growth at concentrations as low as 89 nM, which is consistent with the involvement of these signal transducers in PI3K and MAPK signaling. In vitro migration assays showed potent effects of cabozantinib on single cell migration, further strengthening a role of c-MET in cell migration as well.

E98 tumors became largely invisible in CE-MRI under cabozantinib therapy, and similar phenomena have been observed in clinical studies, with a radiological response as early as one day after start of therapy [36]. This effect of vascular normalization, rendering capillaries in brain tumors impermeable to MR contrast agents by restoring the blood-brain barrier, is a well known phenomenon which is the result of VEGFR2 inhibition [7,15,37]. A reduced vessel density in compact tumor areas in
The high efficacy of cabozantinib to E98 cells in vitro contrasts with our in vivo results. Although the increased survival was unprecedented compared to previously used angiogenesis inhibitors, tumors could still escape therapy via diffuse growth. The exact contribution of c-MET during tumor progression in E98 xenografts is somewhat difficult to assess. In a recent paper an interesting explanation for increased invasiveness of glioblastoma in response to VEGF inhibition has been proposed [20]. These authors demonstrated that c-MET activation is inhibited in glioblastoma cells that also express VEGFR2 in the presence of VEGF. This was suggested to be the result of activation of the VEGFR2-associated protein tyrosine phosphatase PTP1B, resulting in dephosphorylation of its target c-MET. According to this hypothesis, inhibition of VEGF-A releases PTP1B from the multi-receptor complex, unleashing c-MET and resulting in increased diffuse infiltrative tumor growth. This might complicate the use of compounds like cabozantinib, as it may bear antagonistic activities in itself. However, it must be realized that this is only relevant for glioblastomas that are positive for both c-MET and VEGFR2. As E98 tumor cells do not express VEGFR2 this hypothesis is not applicable to this model and other explanations must be found for the escape from therapy.

One such alternative explanation comes from our finding that cabozantinib plasma concentrations of approximately 30 µM [29] did not entirely annihilate c-MET activation whereas in vitro, concentrations as low as 0.5 µM sufficed to achieve complete inhibition. Reduced penetration of the drug into the tumor and surrounding tissue, as well as reduced free concentrations of drug due to protein-binding in blood and tissue, may account for the apparent reduction in potency. Pharmacokinetic studies and measurement of steady-state levels in brain would be appropriate.

The difference between the apparent in vitro and in vivo potency of cabozantinib with respect to c-MET inhibition poses us with an interesting dilemma. We demonstrated in a previous study that the combination of vandetanib and the DNA-alkylating agent temozolomide was less effective than temozolomide alone [8]. This was attributed to
vessel normalization and concomitant ‘restoration’ of the blood-brain barrier, resulting in a hampered distribution of the chemotherapeutic agent to the tumor cells. To what extent cabozantinib can pass the blood-brain barrier is not exactly known. If passage would be inefficient, it may be envisioned that a short period of cabozantinib treatment, enough to restore the blood-brain barrier, will result in inhibition of distribution of the compound to tumor cells in later stages of treatment. According to this hypothesis, only during the initial cabozantinib administrations, the compound will reach tumor cells and have anti-migratory and anti-proliferative effects. In later stages of tumor growth, tumor access may be limited by the restored blood-brain barrier, although it is likely that this block is not 100%, given that phospho-c-MET is still significantly reduced in treated tumors. This may indeed explain the significant delay in tumor growth that we observed. In this respect it would be very worthwhile to investigate whether sequential targeting of c-MET and VEGFR2 with monotargeted compounds would be more effective. Such schemes may involve giving intermittent cycles of c-MET inhibitor, followed by VEGFR2 inhibitors, which would effectively result in locking up the c-MET inhibitors in the tumor cell compartment. Such studies are underway in our lab.

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Chapter 3

Comprehensive protein tyrosine phosphatase mRNA profiling identifies new regulators in the progression of glioma.

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Abstract

The infiltrative behavior of diffuse gliomas severely reduces therapeutic potential of surgical resection and radiotherapy, and urges for the identification of new drug-targets affecting glioma growth and migration. To address the potential role of protein tyrosine phosphatases (PTPs), we performed mRNA expression profiling for 91 of the 109 known human PTP genes on a series of clinical diffuse glioma samples of different grades and compared our findings with in silico knowledge from REMBRANDT and TCGA databases. Overall PTP family expression levels appeared independent of characteristic genetic aberrations associated with lower grade or high grade gliomas. Notably, seven PTP genes (DUSP26, MTMR4, PTEN, PTPRM, PTPRN2, PTPRT and PTPRZ1) were differentially expressed between grade II-III gliomas and (grade IV) glioblastomas. For DUSP26, PTEN, PTPRM and PTPRT, lower expression levels correlated with poor prognosis, and overexpression of DUSP26 or PTPRT in E98 glioblastoma cells reduced tumorigenicity. Our study represents the first in-depth analysis of PTP family expression in diffuse glioma subtypes and warrants further investigations into PTP-dependent signaling events as new entry points for improved therapy.
Introduction

Gliomas arise from glial (precursor) cells and represent the most frequent type of primary brain tumor. The vast majority is classified as diffuse gliomas, reflecting their infiltrative growth into the brain parenchyma along myelinated axon bundles and blood vessels [1, 2]. Diffuse gliomas are histopathologically graded into WHO grades II-IV, with grade IV astrocytoma (glioblastoma) being most malignant [3]. Their diffuse infiltrative character severely complicates glioma treatment. Whereas surgery and radiotherapy mostly address the central part of the tumor, cells that have migrated into the surrounding brain tissue remain relatively unaffected and give rise to recurrences. Although glioblastoma patient survival time has slightly improved over the past decades, the prospect with current treatment is only a median 15 months following diagnosis [4], urging for the development of novel drug modalities to combat these tumors.

Diffuse gliomas can also be subdivided into several classes based on molecular criteria. Lower grade (grade II-III) gliomas and secondary grade IV glioblastomas (arising from lower grade gliomas) display mutations in isocitrate dehydrogenase genes (IDH1/2) in 80% of the cases [5]. Primary glioblastomas (originating de novo) are wild type for IDH1 and IDH2 [6]. Presence of these mutations is now often used as an independent predictor of prolonged survival [7, 8]. Glioblastomas have been further subdivided on the basis of transcriptional profiles as cancers of the pro-neural, neural, mesenchymal and classical subtype [9, 10]. Pro-neural glioblastomas display IDH1/2 mutations, whereas classical and mesenchymal gliomas often show loss of chromosome 10 (containing the tumor suppressor gene PTEN at 10q23), and/or amplification of chromosome 7 (containing the proto-oncogene EGFR at 7p12) [3]. Constitutively active mutant versions of receptor tyrosine kinases (RTKs), e.g. EGFR (EGFRvIII), PDGFRA or MET (METΔ7-8 [11]), are commonly encountered in primary glioblastomas [12]. As a result, in about 90% of primary glioblastomas the RTK/RAS/PI3K signaling pathway is affected by mutations [10], underscoring deregulated phosphotyrosine-based signaling as a major driver in glioma etiology.

Protein tyrosine phosphorylation is controlled via balanced actions of protein tyrosine kinases and protein tyrosine phosphatases (PTPs) [13]. Tumor cell biological research has mostly focused on the druggable kinases, and many tyrosine kinase genes are listed as proto-oncogenes (e.g. EGFR, MET, ERBB2, and PDGFRA) [10]. Consequently, investigations on the countering, hence potentially tumor suppressive, PTPs [13, 14] lag behind. A well-known exception is PTEN [15, 16], the PTP family member that is mutated or deleted in 41% of primary glioblastomas [10] and whose tumor suppressive action is linked to its phospholipid phosphatase activity [17]. Although seemingly counter-intuitive, it has become clear that some PTPs (e.g. SHP2, DUSP6 and PTPRZ1) behave as oncogenes [18, 19]. Thus, the relevance of specific PTPs in certain cancer types is emerging [19, 20] but PTPome studies in diffuse glioma specimens are lacking so far.
We performed expression profiling of the PTP gene family in diffuse glioma samples of different grades and genetic backgrounds, and found that overall PTP expression levels decrease with increasing glioma grade and appear independent of characteristic genetic aberrations. Furthermore, for two of the PTPs whose expression levels differed consistently and significantly between lower grade (II-III) and high grade (IV, glioblastoma) gliomas and correlated with patient survival, we could demonstrate tumor suppressive activity. These findings highlight PTP impact on glioma tumorigenicity.

**Materials and methods**

*Tumor samples and cell lines*

Analyses of patient material were performed in accordance with the guidelines of the local medical ethical committee of the Radboud University Medical Center, Nijmegen, The Netherlands. Patient glioma samples (n=83), comprising 19 grade II, 15 grade III and 49 grade IV tumors, were obtained from the archives of the Radboud University Medical Center [see Additional file 1]. Histologically normal brain control tissue (temporal neocortex and white matter) was obtained from surgery on epilepsy patients at the VU University Medical Center, Amsterdam, The Netherlands.

HEK293FT cells were purchased from Invitrogen, LN-229 glioblastoma cells were from the American Type Culture Collection, and U-251 MG cells were kindly provided by Joost Schalkwijk (Radboudumc). Derivation and use of the xenograft-derived glioblastoma cell model E98 has been described elsewhere [21]. U-251 MG, LN-229, HEK293FT and E98 cells were cultured in high glucose Dulbecco’s Modified Eagle Medium (Life Technologies; cat.no.11960-044) supplemented with 10% Fetal Bovine Serum (PAA laboratories; Cat.no. A15-101), 4 mM glutamine and 1 mM pyruvate (Life Technologies), at 37 °C in a humidified incubator under 7.5% CO2. For some experiments 1 μM Gefitinib (Selleck Chemicals), 4 mM 2-hydroxyglutarate (Sigma H8378), or DMSO (solvent control) was added 48 hrs prior to isolation.

*RNA isolation*

Total RNA was isolated from frozen tumor material or from cell lines. Ten 4 μm cryosections were homogenized in 1.3 ml RNA-Bee (Tel-Test Inc., cs104B) through mechanical dissociation. Next, 260 μl chloroform was added and samples were vortexed and incubated on ice for 15 min before being centrifuged at maximum speed for 15 min at 4 °C. The top clear supernatant was transferred into a new tube and RNA was precipitated with 1 volume of isopropanol. The RNA pellet was dissolved in 400 μl NSE (50 mM NaAc, pH 5.2; 0.2 % SDS; 2 mM EDTA in RNase-free water) and re-precipitated with 1 ml ethanol and collected by centrifugation (30 min at 4 °C, 14,000 g). The RNA pellet was briefly air-dried and dissolved in 30 μl of RNase-free water. Concentrations were determined on a NanoVue plus spectrophotometer (GE Healthcare).
RT-reaction
Reverse transcriptase reactions were performed using the iScript™ cDNA synthesis kit (Bio-Rad). RNA concentrations that were used ranged from 20 ng/μl to 2 μg/μl, and applied RNA and RT-mix volumes were according to the manufacturers’ specifications. Synthesis of cDNA was performed by incubating for 5 min at 25 °C, followed by 30 min at 42 °C and 5 min at 85 °C. Resulting cDNA samples were diluted in distilled water (1:3 – 1:5) and stored at -80°C until further use.

qRT-PCR
Validated qPCR primer sets for mRNA transcripts from 91 PTPs and housekeeping genes ACTB, CANX and PSMB (described in [22]) were purchased from Qiagen and SABiosciences. Transcript abundance was determined real-time in 10 μl reactions using SYBR GREEN (Bio-Rad) and 3 μl of the diluted cDNA samples on a CFX96™ system using the C1000™ Thermal Cycler (Bio-Rad). PCR reactions were initialized at 95 °C for 15 minutes and followed by 40 cycles of 15 sec at 95 °C and 40 sec at 60 °C. To monitor primer specificity, at the end of the last cycle a melting curve with increments of 0.5 °C was recorded between 60 °C and 95 °C. PTP transcript amounts were normalized to housekeeping gene expression levels (ΔC¬t). Normalization to any of three housekeeping genes gave comparable results, and values normalized towards ACTB are used here. The ΔCt values are presented relative to the transcript levels in control brain tissue, according to the ΔΔCt method of Livak and Schmittgen [23]. The average expression level for a given PTP was considered to be meaningfully different between tumor grades when p<0.05 and │ΔΔCt│>1.5.

Tissue Micro-array staining
Immunohistochemical stainings were performed on 4-μm sections of tissue micro-arrays (TMAs) containing formalin-fixed paraffin-embedded (FFPE) glioma (lower grade, n=16; high grade, n=47). Slides were deparaffinised and rehydrated according to standard methods. Heat-induced epitope retrieval (HIER) was performed in TRIS/EDTA buffer, pH 9 (Klinipath), for 10 minutes in a microwave oven at 180 W. After cooling down, endogenous peroxidase activity was blocked using 3% H2O2 in PBS for 10 minutes at room temperature. After washing with PBS, primary antibodies against PTPRZ (BD Transduction Laboratories; #610179), DUSP26 (Atlas antibodies; HPA018221), MTMR4 (Pierce; #Pa5-13711), or PTPRT (Ab frontier; #LFMA0345 clone T20-3C7) were diluted in Normal Antibody Diluent (ImmunoLogic) and incubated for 1 hour at room temperature. Slides were then washed with PBS and incubated with Poly-HRP-GAM/R/R IgG (ImmunoLogic) as secondary antibody for 30 minutes at room temperature prior to detection with 3,3-diaminobenzidine staining (Power-DAB, ImmunoLogic). All sections were counter-stained with haematoxylin and mounted in QuickD Mounting Medium (Klinipath). Sections were scored by two observers (AB and KV), after instructions and partly supervised by a neuropathologist (PW), for intensity and percentage of cells stained. Samples were binned into three classes: high staining (high intensity staining, in the majority of tumor cells), medium staining (lower intensity staining, in a considerable amount of tumor cells) and low or no staining (low intensity or no staining, in a minor part of the tumor cells). For DUSP26 we defined a sample as low staining if there were positive cells...
in the sample and as no staining if not a single stained tumor cells was present.

*Generation of cells with aberrant EGFR expression*

Third generation lentiviral constructs that drive expression of wildtype (EGFR WT) or variant III (EGFRvIII) epidermal growth factor receptor were generated as follows. A Not I linker (self-annealed 5’- CTA GTC CGG GCC GCC CGG A -3’) was ligated into the cleaved XbaI site in PRK5-EGFR-WT (a mammalian expression vector harboring wild-type human EGFR cDNA, kindly provided by Reiner Lammers, Tübingen, Germany). The resulting plasmid was digested using Not I and HindIII and the EGFR WT-encoding fragment was ligated into NotI/HindIII-digested pENTR/Not-Xhol [21]. EGFRvIII cDNA was obtained using primers fw: 5’- GAT ATC ACC ATG CGA CCC TCC GGG -3’ and rev: 5’- CAG CGG CCG CTC ATG CTC CAA TAA ATT CAC TG -3’ in a PCR reaction with an EGFRvIII-positive glioblastoma cDNA pool as template. The amplicon was digested with Not I and resulting NotI/blunt-end EGFRvIII cDNA was ligated into HindIII/NotI-digested vector pENTR-1A (Invitrogen). Resulting pENTR plasmids were subjected to an LR reaction (Gateway® LR clonetm II Enzyme mix; Invitrogen # 11791-020) with pLenti6.2/V5-DEST as destination vector, and pENTR/Not-Xhol served to generate the empty vector control.

Lentiviruses were generated in HEK293FT cells as described earlier [21]. Small samples of virus-containing medium were diluted 1:2 with methanol to monitor viral titers by dot blot on nitrocellulose membranes (0.45 μM Schleicher&Schuell #401196). After blocking for 30 min with 3% non-fat dry milk in TBST (10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.1% Tween-20 (Sigma-Aldrich)) membranes were incubated with anti-VSV monoclonal antibody P5D4 [24] in blocking buffer for 1 hr. Membranes were then washed three times for 10 min in TBST and subsequently incubated with IRDye® 800CW-conjugated goat-anti-mouse IgG (Li-COR Biosciences, #926-32350) for 1 hr at room temperature in the dark. Blots were analyzed using the Odyssey imaging system (LI-COR Biosciences).

U-251 MG glioblastoma cells were transduced with lentiviral expression constructs for EGFR WT, EGFRvIII or empty vector control, and cultured in blasticidin-containing medium for 2 weeks. Resulting cell pools were cultured for 48 hrs in presence or absence of Gefitinib before being harvested for RNA and protein isolation. RNA was used for qRT-PCR as described above, to quantify transcript levels of three genes; *PTPRT, DUSP16* and *PTPRG*. Proteins from cell lysates were size-separated on 8% SDS-PAGE gels and immunoblotted as described previously [21] except that for detection of phosphorylation-dependent epitopes 5% Bovine Serum Albumin in TBST was used as blocking and incubation buffer. Primary antibodies were directed against EGFR (rabbit 1:2000 SC03; #1005), pTyr (mouse 1:1000 pY20; sc508), and GAPDH (rabbit 1:5000; Cell Signaling Technology #2118). Secondary antibodies were IRDye-680- or -800-conjugated goat-anti-mouse and goat-anti-rabbit antibodies. Detection was performed on the Odyssey imaging system.

*Generation of glioblastoma cells with PTEN deletion*
PTEN-deficient LN-229 cells were created using CRISPR/Cas9 technology and a previously described PTEN targeting sequence [25] aimed at the start of the coding sequence in exon 1 of PTEN. Oligonucleotides that contained the target sequence and overlapped with sequences in the receiving guide RNA expression plasmid (PTEN-CRISPR-1-Fw: 5'-TTT CTT GGC TTT ATA TAT CTT GTG GAA AGG ACG AAA CAC CGG ATC GTG AGC AGA AAG AAA -3'; PTEN-CRISPR-1-Rev: 5'-GAC TAG CCT TAT TTT AAC TTG CTA TTT CTA GCT CTA AAA CTT GGT TGC TAA CGA TCC -3') were annealed and extended using Klenow DNA Polymerase fragment. The PTEN CRISPR-1 heteroduplex was introduced into the Affli-linearized gRNA cloning vector (a gift from George Church; Addgene plasmid # 41824 [26]) using Gibson assembly (New England Biolabs) according to manufacturer’s instructions. PTEN wildtype LN-229 glioma cells were transfected with a 1:1:0.1 mixture of the PTEN-targeting gRNA plasmid, plasmid hCas (a gift from George Church; Addgene plasmid # 41815 [26]), and a GFP expression plasmid (pLenti6/shSCR-GFP [21]), respectively, using jetPRIME (Polyplus; Cat.no 114-07). Individual clones were derived via limiting dilution and tested for successful CRISPR/Cas9-mediated PTEN gene editing using a T7 endonuclease I assay. Briefly, cells were lysed (100 mM Tris-HCl, pH 7.5; 5 mM EDTA; 0.2 % SDS; 200 mM NaCl; 100 μg/mL Proteinase K) overnight at 50 °C. Subsequently, samples were cleared by centrifugation (5 min, 8,000 rpm) and DNA in the supernatant was precipitated using an equal volume of isopropanol, washed once with 70% ethanol, air-dried and finally dissolved in T10E0.1 (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA). Genomic DNA was then used in a PCR reaction with Q5 polymerase (NEB) containing Q5 GC enhancer according to manufacturer’s instructions. Primers used were: PTEN-T7-fw: 5’-GGC TGG GAA CGC CGG AGA GT-3’ and PTEN-T7-rev: 5’-CTA TCC TAC ATC ACC CCA TAG GG-3’. Amplicons were purified using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) and subjected to a T7 endonuclease I digestion assay (NEB, M0302S) for 15 min according to manufacturer’s instructions. Clones displaying edited PTEN alleles were further tested for PTEN status through qRT-PCR and immunoblot analyses as described above. Primary antibodies used were: rabbit anti-PTEN (1:2,000, CST#9559), mouse anti-tubulin monoclonal E7 (1:5,000; DSHB, University of Iowa), and rabbit anti-pAKT-Ser473 (1:2,000; CST #4058).

**PTPRT and DUSP26 expression constructs**

PTPRT cDNA encompassing the full-length coding sequence was isolated from plasmid pGW-PTPRT (a kind gift from Jae-Ran Lee, Daejeon, Korea) using BamHI and NotI, and ligated into the corresponding sites in vector pENTR/NotI-Xh0l [21]. Similarly, plasmid pEF-HA-DUSP26 (a kind gift from Rafael Pulido, Barakaldo, Spain) was digested using EcoRI and XbaI and the DUSP26 open reading frame was ligated into pENTR/NotI-Xh0l. An empty vector control (EV) was constructed as described above. An EGFP vector control was generated by PCR (Fw: 5’- TAT AGG ATC CAT GGT GAG CAA GGG CGA GG -3’; Rev: 5’- TAT ACT CGA GTT ACT TGT ACA GCT CGT CC -3’) using pEGFP as template, and the obtained amplicon was digested with BamHI and Xh0l and ligated into pENTR/NotI-Xh0l. All resulting plasmids were used in LR Gateway® reactions (Invitrogen) using pLenti6/ PGK-DEST-TagRFP [21] as destination vector. Obtained expression plasmids were...
used in HEK293FT cells to produce lentiviruses, as described above. E98 human glioblastoma cells [27] were lentivirally transduced twice, and three days later further processed for glioma cell behavior assays.

**Proliferation and migration assays**
E98 cells were grown on collagen I-coated (10 µg/cm²; Invitrogen) coverslips to 60-80% confluency over 48 hours, and incubated for 1 hr with culture medium containing 10 µM EdU (5-ethynyl-2'-deoxyuridine). EdU incorporation was visualized using the click-iT® EdU Imaging kit (Thermo Fisher Scientific, #C10086) via the manufacturers’ instruction. Coverslips were mounted on microscope slides in DAPI-containing Mowiol (Sigma-Aldrich) and images were collected on a Leica DMRA Fluorescence microscope, equipped with a DFC340 FX CCD camera, using 40x and 63x objectives. DAPI- and EdU-positive nuclei were counted automatically using FIJI software [28].

Migration of E98 cells was assessed in spheroid outgrowth assays as follows. E98 spheroids were generated in hanging drops using methylcellulose (12 mg/mL; Sigma, M6385) in DMEM supplemented with 10% FCS (2500 cells per spheroid). The next day, individual spheroids were seeded in a 96-well imaging culture dish (BD Falcon, #353219) on top of a confluent mouse astrocyte layer in Matrigel-coated (30 µg/mL PBS; BD Biosciences, #356237) culture wells. 24 hrs later, cells were fixed and fluorescent (tagRFP) images were collected. Average migration distance of cells from spheroids (n>37), calculated as change in radius of the spheroid over 24 hours, were analyzed semi-automatically using FIJI software.

**In silico analyses**
REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT) was used to determine Kaplan-Meier curves based on PTP transcript levels (as determined via micro-array analyses) using the online Project Betastasis representation tool (http://betastasis.com/glioma/rembrandt/). Threshold was set at median expression for the respective PTP transcript. RNA-seq data from two different TCGA datasets (Brain Lower Grade Glioma [29] and Glioblastoma Multiforme; Provisional) were downloaded using cbioPortal (http://www.cbioportal.org/index.do) [30, 31]. These represented RNA-seq data of 166 *IDH1* WT glioblastoma samples, 9 *IDH1* mutant glioblastoma samples, 221 *IDH1* mutant lower grade glioma samples and 309 *IDH1* WT lower grade glioma samples.

**Statistics**
Statistical significance was tested using non-paired two-tailed Student’s *t*-test or non-parametric Kruskal-Wallis one-way analysis of variance with post-hoc Dunn’s Multiple Comparison Test. Patient survival data was automatically calculated by Project Betastasis using a log-rank test. Heat map was generated in R (version 3.0.1).
Results

Differential expression of PTP transcripts between lower grade and high grade glioma samples

To investigate which members of the family of PTPs play roles in the biology of gliomas of different grades, we exploited a previously established and validated set of qPCR primers for 91 human PTP transcripts [22] to screen glioma specimens. Following cDNA synthesis and qPCR, PTP transcript levels were determined relative to those in control brain tissue. Strikingly, a pattern emerged showing significantly lower overall PTP transcript levels in the glioblastoma samples as compared to lower grade gliomas (Figure 1a-b). In the first cohort of samples, expression levels of 36 PTPs differed significantly between lower grade (WHO grade II-III; n>11) and glioblastoma (WHO grade IV; n>15) samples [see Additional file 2]. To validate these findings, a second, independent cohort was tested with these 36 primer sets [see Additional file 3], resulting in the identification of seven PTP genes (DUSP26, MTMR4, PTEN, PTPRM, PTPRN2, PTPRT and PTPRZ1) that consistently and significantly differed in expression between lower and high grade gliomas (Figure 1c).

To investigate whether differences in transcript levels are reflected at the protein level, we performed immunohistochemical (IHC) staining on tissue micro-arrays containing 47 glioblastoma, 16 lower grade glioma samples and normal brain tissue. IHC could not be performed for PTPRN2-encoded IA-2β protein due to lack of appropriate antibodies. PTPRT protein levels were below detection limits in both normal brain tissue and in glioma tumor samples [see Additional file 4], in line with the high qPCR Ct values obtained for this PTP. Reduced PTEN and PTPRM protein levels in glioma specimens have been well documented (e.g. [19, 32]). The other PTPs were amenable for analysis by IHC. PTPRZ1 immunostainings revealed a clear membrane staining that was higher in lower grade specimens than in high grade counterparts (Figure 2a), substantiating our RNA data and earlier reports [19]. Immunodetection of MTMR4 resulted in high signals in normal brain tissue in structures resembling astrocytes (Figure 2b). In all samples (tumor and normal) also strong staining of the neuropil could be observed, with some samples additionally showing strong cytoplasmic staining in tumor cells (Figure 2biii). DUSP26 was detected in neurons and neuropil of normal brain tissue but staining was usually low or absent in tumor areas (Figure 2c). Low-level DUSP26 stainings were observed in 21% and 12% of lower grade and glioblastoma samples, respectively (Figure 2d), in concordance with our qPCR findings (Figure 1c). Also MTMR4 expression levels were higher in grade II-III gliomas than in glioblastoma samples (Figure 2d), in line with our RNA expression data (Figure 1c).

PTP expression patterns correlate with disease outcome

To corroborate our data, we performed in silico analysis of RNA-seq data from two TCGA glioma datasets and of micro-array data and survival probabilities as documented in the REMBRANDT brain tumor database. Out of the seven PTPs, all but PTPRZ1 had significantly lower expression levels in glioblastoma tumors as compared to lower grade glioma samples (Figure 3), in agreement with our qPCR findings. Especially DUSP26 and PTPRT show a very strong difference between grades.
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Figure 1: Seven PTP genes display significantly different expression levels in lower grade and high grade gliomas. a) Graph showing differential expression between lower grade (II-III) and high grade (IV) glioma samples for seven PTPs. PTP mRNA expression levels were determined by qRT-PCR and were normalized to β-actin transcript levels and depicted relative to expression levels in histologically normal brain tissue. Data depicted correspond to data obtained in the first cohort of tumor samples (i.e. supplementary Table S1). Error bars indicate standard error of the mean (SEM). Asterisks indicate significance levels (** p<0.001 ***p<0.001) as determined by two-tailed Student’s t-test. b-d) Representative images of formalin-fixed paraffin embedded specimens stained with antisera against PTPRZ1 (b), MTMR4 (c) or DUSP26 (d). For each protein an example is given of staining of (i) from normal brain tissue, (ii) negative or low intensity staining tumor area and (ii) high intensity staining. For DUPS26 a medium staining is shown in (iii). e) Trichotome analysis of PTP immunopositivity in lower grade and high grade specimens.
PTPRZ1 is in fact overexpressed in both glioma subgroups as evident by the high RSEM RNA expression values.

Using the Project Betastasis online representation tool, we subdivided all available tumors represented in the REMBRANDT dataset into low- and high-expressing types on the basis of available RNA micro-array data for our set of PTPs. Kaplan-Meier curves revealed a strong and significant correlation between low DUSP26, PTPRT, PTEN and PTPRM expression and disease progression (p<0.001; Figure 4a,f,c,d). MTMR4, PTPRN2 and PTPRZ1 did not correlate with survival (Figure 4b,e,g). Also within the individual histological types (oligodendroglioma (grade II-III), astrocytoma (grade II-III) and glioblastoma (grade IV)) we observed expression-dependent survival probabilities for DUSP26 and PTPRT (data not shown). PTEN expression correlated with survival probability in glioblastoma and astrocytoma samples, while for PTPRM such an expression-dependent effect only holds for glioblastoma specimens (data not shown).

Characteristic genetic aberrations in gliomas do not drive PTP expression

Strikingly, for 80 out of the 91 PTP genes a pattern emerged showing lower transcript levels in the glioblastoma samples as compared to lower grade gliomas (Figure 1a-b). This raises the question whether general PTP profiles are influenced by characteristic genetic alterations associated with lower grade glioma (frequently IDH mutant) or glioblastoma (e.g. EGFR amplification; PTEN deletion) or rather reflect the tumor pathogenesis, including cell of origin. To test this, U-251 MG glioblastoma cells were lentivirally transduced to over-express EGFR or EGFRvIII. Phosphorylation of EGFR and EGFRvIII was readily detected in the transduced cells and was effectively blocked by treatment with the EGFR inhibitor Gefitinib (Figure 5a). The increased EGFR signaling, however, did not influence expression levels of three representative PTPs (DUSP16, PTPRG and PTPRT; Figure 5c) that displayed the grade-related expression pattern as observed for the majority of the PTP cohort (Figure 1a-b).

PTEN-inactivating deletions or mutations represent another common aberration in primary glioblastoma samples and could potentially explain the pattern observed on the PTP transcripts. To genocopy PTEN loss, the PTEN WT glioblastoma cell line LN-229 was subjected to CRISPR/Cas9-mediated genome editing and resulting clones were inspected for PTEN levels and activity, respectively, as witnessed by PTEN and phospho-AKT levels under low-serum conditions (Figure 5b). Although we successfully generated PTEN-deficient LN-229 derivates and appropriate controls, no significant changes in expression levels were observed for the three representative reporter PTPs that were tested (Figure 5d).

We also monitored epigenetic effects due to the oncometabolite 2-hydroxyglutarate (2-HG) that is produced in lower grade gliomas and secondary glioblastomas as a result of the characteristic IDH1R132H mutation and inhibits DNA and histone demethylation. Tumor-relevant levels of 2-HG were added for 48 hrs to U-251 MG (IDH1 wild type) glioblastoma cells and potential changes in DUSP16, PTPRG and PTPRT...
expression levels were monitored. Addition of 2-HG significantly reduced the mRNA levels for two out of the three PTPs measured (Figure 5e). These results are in line with epigenetic regulation of DUSP16 and PTPRG but do not explain the generally higher PTP mRNA levels in IDH-mutant low-grade gliomas. Preliminary RNAseq data from WT or IDH1 R132H-containing glioblastoma xenografts also do not point to a difference in amount of transcripts reads for DUSP16, PTPRG and PTPRT depending on the IDH status (WPJL, unpublished data). Collectively, these data make it rather unlikely that genetic alterations characterizing lower grade glioma and glioblastoma subgroups are major determinants of the general PTP expression pattern observed (Figure 1a,b).
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Figure 3: RNA-seq reveals PTP expression differences in lower grade and high grade glioblastoma. a-g) Dot plots showing the mean estimated fraction of transcripts per million as calculated in RSEM; (horizontal black bars) and standard deviation for the indicated PTP in lower grade glioma (triangles) or high grade glioblastoma (dots) tumor samples as extracted from two RNA-seq datasets in the TCGA database. Asterisks indicate significance levels (***p<0.001) as determined by two-tailed Student’s t-test.
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Figure 4: Transcript levels of several PTPs correlate with patient survival probability. a-g) Kaplan-Meier analysis of glioma patient survival data according to low (red) and high (blue) PTP mRNA expression, obtained from microarray analyses on 524 glioma samples as present in the REMBRANDT repository. Insets show histograms of PTP expression distribution with the vertical black line indicating the cutoff point (taken at the median expression level) between low and high expressing samples. Asterisks indicate significance levels (**p<0.001) calculated using the log-rank test in the Project Betastasis online representation tool.
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Overexpression of DUSP26 or PTPRT in E98 glioblastoma cells results in reduced tumorigenicity

DUSP26 and PTPRT are the most down-regulated PTPs in gliomas with highest grade malignancy and both show a pronounced correlation with survival probability (Figures 1,3,4). To test direct effects of expression on cell behavior we overexpressed these PTPs in the E98 glioblastoma model [11, 21, 27, 33] and monitored effects on cell growth and migration (Figure 6). To facilitate tracking of successfully transduced E98 cells, lentiviral expression vectors were used that also carry a tagRFP fluorescent reporter. DUSP26 and PTPRT Protein expression of virus producing HEK293FT was visualized using immunoblotting [see Additional file 5]. Successful overexpression of DUSP26 and PTPRT in E98 cells was confirmed by qPCR (Figure 6a,b) and E98 cell proliferation capacity was investigated using EdU labeling of S-phase cells. Increased DUSP26 or PTPRT levels led to reduced EdU incorporation as compared to empty-vector controls (Figure 6c-d). This suppression

Figure 5: Characteristic mutations for lower/high grade gliomas exert no overt effect on PTP expression patterns. a) Immunoblot of lysates from U-251 MG glioblastoma cells expressing wild-type EGFR, EGFRvIII or empty vector (EV) control. Cells were treated with the EGFR inhibitor Gefitinib (or DMSO as solvent control) for 48h before being lysed. Immunostaining (IB) was with antibodies against EGFR, phosphotyrosine (pY) or GAPDH (as loading control). b) Immunoblot analysis for PTEN and Ser473-phosphorylated AKT levels in parental and (PTEN-targeted) CRISPR/Cas9-treated LN-229 cells. Lysates were prepared following 8 hrs of serum starvation (0.5%FCS), and Tubulin immunostaining served as loading control. c-e) Analysis of (ACTB-normalized) DUSP16, PTPRG and PTPRT transcript levels, as determined by qPCR, in cell models mimicking EGFR hyperactivity (c), PTEN deficiency (d) or mutant IDH1-mediated 2-HG exposure (e). Bars indicate mean with SEM, asterisk represents significance level (* p<0.05).
of proliferation in the E98 high grade glioma model following overexpression of these two PTPs is in line with the observed positive correlation with patient survival data. To also monitor potential DUSP26 or PTPRT effects on glioblastoma cell migration, we turned to a previously established spheroid outgrowth assay [4, 28]. Homogeneously-sized spheroids of lentivirally transduced E98 cells, generated using the hanging-drop method, were placed on a layer of mouse astrocytes and the migratory performance of outgrowing fluorescent E98 cells was calculated as the change in radius of the spheroid over 24 hrs. As compared to GFP-expressing control cells, the expression of either PTPRT or DUSP26 significantly reduced migration of E98 cells from the spheroid edge (Figure 6e-f). DUSP26 and PTPRT thus impinge on both growth and motility of glioblastoma cells. Collectively, these data underscore their involvement as tumor suppressors in glioma, in line with the mRNA profiling data (Figures 1, 3, 4).

**Discussion:**

Diffuse gliomas are invariably fatal tumors, with most glioblastoma patients surviving only up to 15 months after diagnosis [3], stressing the need for more effective treatment modalities. In this study we focused on the protein tyrosine phosphatase gene family as alternative entry points for treatment strategies. Seven out of ninety-one investigated PTPs displayed significantly different mRNA expression levels when comparing lower and high grade gliomas. Furthermore, for four out of these seven, expression levels correlated with patient survival probability, underscoring their role in tumor behavior. Importantly, overexpression of the two PTPs that showed the largest expression difference in our qPCR and in silico analyses, DUSP26 and PTPRT, resulted in reduced glioblastoma cell proliferation and migration, supportive of a tumor suppressive role.

In general, PTP expression levels in lower grade gliomas resembled those in normal brain tissue whereas levels in glioblastomas were generally lower. This overall reduction in PTP expression with increasing grade may relate to differences in the cell type of origin. Alternatively, it may be imposed by characteristic genetic alterations acquired during glioma development. We found that experimental introduction of EGFR hyperactivity, or of PTEN inactivation, in a glioblastoma cell line had no effect on overall PTP expression patterns. Furthermore, the transcriptional impact of 2-HG on PTP expression in IDH1 wildtype glioblastoma cells was at best opposite to the expected, and not able to tone down PTP mRNA levels to those in lower grade gliomas or normal brain tissue. These findings suggest that other differences in tumor pathogenesis between lower and high grade tumors is the major determinant for the PTP expression pattern. The overall reduced PTP levels in glioblastomas as compared to histologically normal brain and lower grade tumor levels, points to PTP agonists as a potential option for glioblastoma treatment.

In our screening rationale, stringent criteria led to a selection of seven PTPs that differed significantly in their expression between glioblastomas and lower grade gliomas. Our data revealing that PTEN expression was low in high grade gliomas con-
Comprehensive PTP mRNA profiling identifies new regulators in the progression of glioma.

Figure 6: DUSP26 and PTPRT suppress glioblastoma cell growth and motility. a) DUSP26 and b) PTPRT mRNA levels in lentivirally transduced E98 cells were determined by qRT-PCR. Expression, normalized to ACT-B, is given as fold change of that in GFP control cells. c) Transduced E98 cells were grown on cover-slips for 48 hrs, pulse-labeled with EdU for 1 hr, fixed, and EdU incorporation was visualized. Representative images of DAPI, EdU and tagRFP positivity are depicted. d) Percentage of EdU-positive nuclei among DAPI-stained cells was determined (n>3). Error bars indicate SD and asterisks reflect confidence levels (ANOVA; ** p<0.01; *** p<0.001). e) Spheroids of transduced E98 cells on a confluent mouse astrocyte layer were incubated for 24h before being fixed. Representative images of tagRFP-expressing spheroids with outgrowing cells are shown for the respective constructs. f) The average radius increase (in pixels) due to migrating cells was calculated per spheroid from the data in e), and plotted as gray data points on top of the box plot for the mean with standard deviation. Asterisks indicate significance (*** p<0.001; Kruskal-Wallis one way analysis of variance p<0.0001).
Chords with literature data on PTEN inactivation or absence in over one-third of high grade gliomas, resulting in increased proliferation and survival through activation of the PI3K/AKT pathway [10, 34]. PTPRM has also been proposed as a glial tumor suppressor (e.g. [19, 32]) and is consistently down-regulated in high grade samples in our screen. Together with its subfamily members PTPRU and PTPRK, they are amongst the most frequently mutated PTPs in a number of cancer types [16, 19, 20, 32, 35]. Some PTPs that had previously been implicated in primary glioblastoma etiology [19] were not selected by our screen, although some only just failed to reach our criteria (e.g. PTPN11, encoding SHP2). This may reflect that such PTPs could govern processes equally important to lower grade and high grade tumors.

We found that *PTPRZ1* expression is higher in lower grade tumors than in high grade tumors, but in online RNA-seq data the inverse was observed. This apparent discrepancy may result from histopathological differences in the tumor collections. In the RNA-seq dataset, a third of the lower grade samples represent astrocytomas while in our sample cohort lower grade tumors consisted mostly of oligodendrogial neoplasms with 1p/19q co-deletion. *PTPRZ1* is highly expressed in oligodendrocyte precursor cells and is involved in normal oligodendrocyte development [36, 37], thus providing rationale for the high *PTPRZ1* expression in our sample cohort. Irrespective, *PTPRZ1* expression is consistently up-regulated in all glioma specimens. We and others have shown that the encoded transmembrane PTP acts as an oncogene in gliomas [21, 38, 39] and, interestingly, two HLA-presented peptides derived from *PTPRZ*’s extracellular part are included in an experimental glioma vaccine that is currently explored in phase II clinical trials [40].

We additionally identified PTPRT, DUSP26, PTPRN2 and MTMR4 in our screen for glioma-relevant PTPs. PTPRT belongs to the same cell adhesion molecule-like PTP subfamily as PTPRM, PTPRU and PTPRK. Although compelling evidence has put forward *PTPRT* as a colon cancer susceptibility gene [20, 41–43], thus far no reports have linked PTPRT to gliomas. PTPRT mRNA levels are quite low in normal brain tissue and lower grade gliomas but are still on average 40-fold higher than those in high grade glioblastomas. In line with a tumor suppressive role, we found that PTPRT overexpression significantly suppressed glioblastoma cell proliferation and migration. The finding that the closely related PTPRT and PTPRM show distinct expression patterns in lower versus high grade gliomas (PTPRU just failed to reach the criteria in the first cohort) and correlate with patient survival, warrants further studies on their role in glioma-associated signaling pathways.

DUSP26 represents a dual-specificity phosphatase, i.e. it is able to dephosphorylate not only phosphotyrosines but also phospho-serine and -threonine residues in substrate proteins. In neuroblastoma cells DUSP26 dephosphorylates and inhibits the tumor suppressor p53 [44]. Furthermore, *DUSP26* amplification has been found in thyroid cancer, and DUSP26 knockdown impeded growth of anaplastic thyroid cancer cells [45]. Collectively, this indicates that *DUSP26* acts as an oncogene, and contrasts with our observation of low DUSP26 levels in glioblastomas in comparison with lower grade glioma samples and normal brain tissue. Importantly, we found that
DUSP26 overexpression in E98 glioblastoma cells resulted in decreased growth and motility. Other investigators also reported on down-regulated DUSP26 levels in glioblastoma [46] and Tanuma and colleagues additionally provided evidence that DUSP26 facilitates catenin/cadherin delivery to cell-cell junction sites, hence cell-cell adhesiveness [47]. It is becoming increasingly clear that diffuse glioma cells form functional networks via cell-cell contacts [48] and in such a context DUSP26 protein may indeed have tumor suppressive activity. Together with PTPRM and PTPRT, DUSP26 therefore holds promise for therapeutic intervention.

Intriguingly, PTPRN2 and MTMR4 both encode PTPs that, like PTEN, have phosphoinositides as substrates. PTPRN2 preferentially dephosphorylates phosphatidylinositol 4,5-bisphosphate (PI(4,5)P) [49] and MTMR4 prefers phosphatidylinositol 3-phosphate (PI(3)P) [50]. Whereas the tumor suppressive impact of PTEN’s phospholipid phosphatase activity is beyond doubt [51], only limited and contradicting data exist regarding PTPRN2 involvement in cancer. The hypermethylation of the PTPRN2 promoter region in glioblastomas and lung adenocarcinomas [52, 53] suggests tumor suppressor roles. In contrast, high PTPRN2 expression has been reported to correlate with poor clinical outcomes in breast cancer cases [54]. Furthermore, experimental down-regulation of PTPRN2 in metastatic breast cancer cells inhibited migratory potential and yielded smaller tumors in vivo [55], rather pointing to oncogenic potential. The tumor biological impact of PTPRN2 and MTMR4 may thus be context-dependent, and how these PTPs feed into glioma biology needs further investigation.

Taken together, our study – to our knowledge the first comprehensive PTP mRNA profiling in diffuse gliomas – highlights seven PTPs (DUSP26, MTMR4, PTEN, PTPRM, PTPRN2, PTPRT and PTPRZ1) of which expression decreases with increasing malignancy. Largest expression differences between lower grade and high grade diffuse gliomas were found for DUSP26 and PTPRT. Their expression correlated with patient survival probability, and overexpression inhibited glioblastoma cell growth and motility. Together with PTPs that impinge upon cellular contacts (DUSP26, \PTPRZ1) and phospholipid signaling (DMTMR4, PTEN and PTPRN2) they provide novel cues to explore and design glioma treatment options.

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Disclosure of Conflicts of Interest:
No potential conflicts of interest were disclosed.

Authors’ contributions
Conception and design: P. Wesseling, W.P.J. Leenders, W.J.A.J. Hendriks
Development of methodology: A.M. Bourgonje, J.T.G. Schepens, K. Verrijp, M. van den Eijnden
Analysis and interpretation of data: A.M. Bourgonje, W.P.J. Leenders, W.J.A.J. Hendriks
Writing, review and/or revision of the manuscript: A.M. Bourgonje, R. Hooft van Huijsduijnen, P. Wesseling, W.P.J. Leenders, W.J.A.J. Hendriks
Study supervision: W.P.J. Leenders, W.J.A.J. Hendriks

References:


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Supplementary material

Online resource 1: Molecular diagnosis data of glioma tumors samples.

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Comprehensive PTP mRNA profiling identifies new regulators in the progression of glioma.

Molecular diagnosis data as it is known for gliomas tumors samples used in the qPCR screen. 1p19q, EGFR, EGFRvIII and PTEN status were judged by MLPA data and IDH status was reviewed using specific antibody staining and subsequent sequencing by the diagnostic department of the Radboudumc. ND=not determined, HCA= high copy amplification and HD= homozygous deletion.

**Online Resource 2: ΔΔCt values of 1st cohort of diffuse glioma samples run on 91 validated primer sets.**

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Comprehensive PTP mRNA profiling identifies new regulators in the progression of glioma.

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The relative expression of PTPs compared to histologically normal brain tumor in different malignancy grades lower (grade II-III) vs high grade (grade IV) diffuse gliomas. PTPs are arranged alphabetically. Avg (average ΔΔCT across samples for a single PTP). sem (standard error of the mean of the samples in avg), n (number of samples tested in the first cohort per group). This varies per PTP because expression of some PTPs is so low that only a high amount of RNA and cDNA will show a reliable ΔΔCT value. P-value (statistically tested using Student t-test, calculated between lower-grade and high-grade glioma samples from the previous columns). diff (absolute difference in ΔΔCT between lower grade and high grade glioma samples). A candidate is defined as p<0.05 and |ΔΔCT|> 1.5.

Online Resource 3: ΔΔCt values of 2nd cohort of diffuse glioma samples run on 36 validated primer sets judged as candidates in the 1st cohort.

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Online Resource 3: ΔΔCt values of 2nd cohort of diffuse glioma samples run on 36 validated primer sets judged as candidates in the 1st cohort.
Comprehensive PTP mRNA profiling identifies new regulators in the progression of glioma.

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The relative expression of PTPs compared to histologically normal brain tumor in different malignancy grades lower (grade II-III) vs high grade (grade IV) diffuse gliomas. PTPs are arranged alphabetically. Avg (average ΔΔCt across samples for a single PTP). sem (standard error of the mean of the samples in avg, n (number of samples tested in the second cohort per group. P-value (statistically tested using Student t-test, calculated between lower grade and high grade glioma samples from the previous columns). diff (absolute difference in ΔΔCt between lower-grade and high-grade glioma samples). A candidate is defined as p<0.05 and |ΔΔCt| > 1.5.
Online resource 4
Immunohistochemical staining for PTPRT on formalin-fixed paraffin-embedded materials. Three different samples stained with antiserum against PTPRT: i) histologically normal brain tissue; ii) glioma sample; iii) HEK293FT cells transfected with a PTPRT expression construct.
Online resource 5
HEK293FT cells expressing lentiviral constructs for DUSP26 and PTPRT. a) Fluorescent images of HEK293FT cells expressing DUSP26, PTPRT, or GFP as a control, under a PGK promoter. Expression constructs also contained a CMV promoter-driven cassette for tagRFP, allowing assessment of transfection efficiency. b) Immunoblot of lysates from HEK293FT cells transfected with lentiviral expression constructs for DUSP26, PTPRT or GFP control, stained with antisera against DUSP26, PTPRT and Tubulin.
Chapter 4

Intracellular and extracellular domains of protein tyrosine phosphatase PTPRZ-B differentially regulate glioma cell growth and motility

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Abstract

Gliomas are primary brain tumors for which surgical resection and radiotherapy is difficult because of the diffuse infiltrative growth of the tumor into the brain parenchyma. For development of alternative, drug-based, therapies more insight in the molecular processes that steer this typical growth and morphodynamic behavior of glioma cells is needed. Protein tyrosine phosphatase PTPRZ-B is a transmembrane signaling molecule that is found to be strongly up-regulated in glioma specimens. We assessed the contribution of PTPRZ-B protein domains to tumor cell growth and migration, via lentiviral knock-down and over-expression using clinically relevant glioma xenografts and their derived cell models. PTPRZ-B knock-down resulted in reduced migration and proliferation of glioma cells in vitro and also inhibited tumor growth in vivo. Interestingly, expression of only the PTPRZ-B extracellular segment was sufficient to rescue the in vitro migratory phenotype that resulted from PTPRZ-B knock-down. In contrast, PTPRZ-B knock-down effects on proliferation could be reverted only after re-expression of PTPRZ-B variants that contained its C-terminal PDZ binding domain. Thus, distinct domains of PTPRZ-B are differentially required for migration and proliferation of glioma cells, respectively. PTPRZ-B signaling pathways therefore represent attractive therapeutic entry points to combat these tumors.
Introduction

Diffuse gliomas comprise a heterogeneous group of glial brain tumors that share high migratory potential, as evidenced by diffuse infiltration in the surrounding brain parenchyma along white matter tracts and blood vessels [1]. This phenotype severely reduces efficacy of conventional therapeutic strategies, i.e. surgery and radiotherapy [2]. A hallmark of the highly malignant WHO-grade-IV gliomas, with a very dismal prognosis of less than 15 months [3], is the additional local occurrence of angiogenesis. This angiogenesis has been combated using specific inhibitors in phase III trials, but thus far therapeutic survival benefit is lacking because the approach leaves the diffuse infiltrative component unaffected or even increases diffuse growth [4-6]. Additional treatment modalities aiming at diffuse tumor cells that escape surgery and radio-chemotherapy, hence knowledge about the molecular mechanisms that underpin glioma cell migration and proliferation in the brain parenchyma, are therefore urgently needed.

Aberrant phosphotyrosine-based signaling is a hallmark of cancer, and gliomas are no exception; tyrosine kinase membrane receptors like EGFR, ERBB2, PDGFRA, MET and VEGFR2 have been implicated in glioma growth, angiogenesis and cell motility [7]. A role for the counter-acting protein tyrosine phosphatase (PTP) enzyme family [8, 9] is much less studied. There is compelling evidence that PTPs influence cell migration [10], especially during neuronal development [8, 9]. Several PTPs have been linked to carcinoma development [11, 12] and their involvement in glioma biology is gradually being uncovered [13]. PTEN, for example, is inactivated or absent in one-third of high-grade gliomas, resulting in increased PI3K-mediated cell proliferation and survival [7, 14]. For multiple additional PTPs the contribution to glioma etiology, especially the diffuse infiltration in the neuropil, requires further study.

Within the receptor-type PTP (RPTP) subfamily there is a number of transmembrane enzymes that resemble cell adhesion molecules, having extracellular domains that might engage in interactions with neighboring cells or extracellular matrix components [8-10]. These characteristics make RPTP genes, including PTPRZ1, potentially important regulators of cell motility and growth. PTPRZ1 encodes three isoforms (PTPRZ-A, PTPRZ-B and phosphacan) that share a carbonic anhydrase-like (CAH) and a fibronectin type III (FNIII) domain at the protein’s N-terminus [15]. Furthermore, a spacer with chondroitin sulfate proteoglycan attachment sites is present in isoforms PTPRZ-A and phosphacan. PTPRZ-B lacks most of this spacer, resulting in a smaller extracellular part. PTPRZ-A and PTPRZ-B have identical intracellular parts consisting of a catalytically active membrane-proximal and an inactive membrane-distal PTP domain. The phosphacan isoform lacks these PTP domains and represents a secreted protein [15]. Several PTPRZ-interacting proteins have been identified. For instance, the extracellular ligand pleiotrophin binds to and inactivates PTPRZ, thereby increasing the phosphorylation of intracellular substrates β-catenin [16], Fyn [17], β-adducin [18] and Alk [19]. Additional interaction partners include contactin-1, which binds to the CAH domain [20], and tenascin-C and -R that bind to the FNIII domain [21]. It is thought that these proteins form complexes with the
extracellular matrix [22] to induce and facilitate migration.

PTPRZ expression, in particular PTPRZ-B [23], is up-regulated in glioma tumor specimens [24-26]. PTPRZ knock-down in glioblastoma cell lines reduced cell migration [25] and tumor growth [27], and PTPRZ overexpression enhanced cell migration [24]. However, these cell models produce circumscribed tumors that lack the highly invasive phenotype when grown orthotopically [28]. Furthermore, PTPRZ protein domains that steer glioma cell behavior still need to be uncovered. Here we investigated the role of PTPRZ and its protein domains, exploiting glioma models that faithfully recapitulate diffuse infiltrative growth in vivo [28-30]. Lentivirus-mediated knock-down and subsequent rescue experiments revealed that PTPRZ-mediated effects on migration rely exclusively on its extracellular domain, whereas impact on proliferation depends on the intracellular carboxyl-terminal PDZ domain binding site. These findings identify PTPRZ as a dual entry point for glioma therapy development.

**Materials and methods**

Experimental details on cell lines and antibodies used, plasmid construction, lentiviral transduction, immuno-blotting, -precipitation and -histochemical procedures, peptide microarray analysis, and statistical analyses are provided as supplementary material.

**Cell proliferation and viability assays**

E98 cells were grown on collagen I-coated (10 μg/cm²; Invitrogen) coverslips to 60-80% confluency, and incubated for 1hr with culture medium containing BrdU (50 μM; Life Technologies, #B23151). Cells were then washed three times with PBS and fixed in 2% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB: 46 mM NaH2 PO4; 354 mM Na2HPO4, pH 7.4). Subsequently, coverslips were washed with PBS and quenched with 50 mM NH4Cl in PBS. Cells were blocked and permeabilized for 1hr in blocking buffer (5% Normal Goat Serum and 0.1% Triton X-100 in PBS) and then incubated for 2.5hrs with anti-BrdU, anti-Ki67 or anti-cleaved Caspase 3 antibodies in blocking buffer with added DNAseI (100 ng/mL; Roche) and MgCl2 (2.5 mM) at 37 °C. Bound antibodies were detected with goat-anti-mouse Alexa 488 and goat-anti-rabbit Alexa 647 secondary antibodies in PBS. Coverslips were mounted on microscope slides in DAPI-containing Mowiol (Sigma-Aldrich) and images were collected on a Leica DMRA Fluorescence microscope, equipped with a DFC340 FX CCD camera, using 40x and 63x objectives. DAPI- and BrdU-positive nuclei and cleaved Caspase 3-positive cells were counted using FIJI software [44].

For proliferation assays, stably transduced E98 cells were seeded in triplicate in a 96-well microtiter plate (10,000 cells/well), and cultured in 10% FCS-containing DMEM. At various time points, cells were washed with PBS and fixed using 10% trichloroacetic acid for 1hr at 4 °C, washed with water and afterwards frozen until all time points were collected. Cells were then stained with 1% Sulforhodamine B (SRB; Sigma-Aldrich) in 1% acetic acid for 20 minutes. Plates were subsequently washed
with 1% acetic acid and dried at 60 °C for 3 hrs. Protein-bound SRB was then dissolved in 10 mM Tris (pH 10.5) and absorption was measured at 510 nm using a micro-plate reader (Bio-Rad). Lentivirally transduced E434 spheroids were seeded in a 96-well imaging plate (BD Biosciences, #353219) and allowed to settle overnight. The next day, images were taken using a BD Pathway 855 high-content bio-imager (BD Biosciences). Fluorescent images (TagRFP or EGFP-based) were taken longitudinally at days 4, 6 and 8 and the average increase in spheroid area was calculated automatically using FIJI software. After images had been taken on the fourth day, half of the medium was replenished and spheroid growth was continued.

2D and 3D migration assays
E98 spheroids were generated by the hanging-drop method. In brief, 0.7 mL methylcellulose (12 mg/mL; Sigma, M6385) was added to 4.3 mL E98 cell suspension in DMEM supplemented with 10% FCS (500,000 cells total) and 25 µL drops were seeded in a dry culture dish. The dish was then inverted and incubated overnight at 37 °C in the presence of 5% CO2. The next day, individual spheroids were seeded in a 96-well imaging culture dish (BD Falcon, #353219) coated with Matrigel (30 µg/mL PBS; BD Biosciences, #356237) and further incubated in DMEM with 10% FCS, at 37 °C and 5% CO2. For E434, cultured spheroids were directly plated onto Matrigel-coated wells containing B27-supplemented neurobasal medium. After 24 hrs, wells were washed with PBS and cells were fixed with 2% PFA in 0.1 M PB, followed by DAPI staining. Fluorescent images were collected on a high-content microscope (Leica DMI6000B) extended with a motorized x-y scanning stage (Leica EL6000 illumination source), and FIJI software [44] was used to automatically quantify spheroid outgrowth. Briefly, cells which migrated from the spheroids were selected, coordinates of each individual cell were determined, and distance from the spheroid border was calculated using the coordinates and radius of the spheroid. For each experiment the average cell migration distance across all spheroids (n>31) was calculated.

Cell cultures in solidified Matrigel/Agarose matrices were prepared as follows. 10 µL Matrigel and 5 µL 2% agarose (Seaplaque Agarose; Lonza #50101 in PBS) were mixed with 35 µL 10% FCS-containing DMEM and 50 µL of Matrigel/agarose mix was added to the wells of a 96-well microtiter plate which was placed on ice. One minute later, 50 µL cell suspension (50,000 cells) was mixed resulting in a 3D environment containing cells. Cells were imaged overnight with 10-min intervals on a time lapse system (Nikon Diaphot 300 with Hamamatsu C8484-05G CCD Camera, Okolab 4 well CO2 stage incubator and Okolab 2D time lapse software) at 37 °C and 5% CO2 with a 10x objective. Cells were manually tracked for at least 2 hrs using the FIJI plug-in [44].

Intracerebral injection of spheroid-derived cells
All animal experiments were approved by the local Animal Experimental Committee of the Radboud University Medical Center. Athymic female BALB/c nu/nu mice (18–25 gram, age 6–8 weeks) were kept under specified pathogen-free conditions and received food and water ad libitum. E98 and E434 cells were grown as spheroids in supplemented neurobasal medium for at least two weeks prior to orthotopic
injection \[29\]. A 20 μL cell suspension (107 cells/mL in PBS) containing a mixture of shSCR/GFP and shPTPRZ1/TagRFP labeled cells was injected per animal (n=5 and 4 for E98 and E434 cells, respectively). Prior to injection, the ratio of GFP- and TagRFP-positive cells was determined using an EVOS fluorescence imaging system (AMG) and FIJI Software. Animals were closely monitored and sacrificed when signs of tumor burden (especially weight loss and neurological dysfunction) were observed. Brains were harvested, and parts were either formalin-fixed and paraffin-embedded (FFPE) or snap-frozen in liquid nitrogen, and stored for (immuno)histochemical analysis.

**Results**

**Modulation of PTPRZ-B expression levels in glioblastoma cells**

In line with previous reports \[24-26\], high *PTPRZ1* expression levels are detectable in glioma tumors (data not shown) and in human xenograft-derived cells in culture (Fig. 1). The two well-characterized glioma xenograft lines E98 and E434 \[28\] differ in their *in vitro* culture regimen; anaplastic oligodendroglioma-derived E434 cells only propagate under neurosphere growth conditions, using serum-free neurobasal medium \[31\], whereas glioblastoma-derived E98 cells additionally grow in standard DMEM/10%FCS as an adherent monolayer (Fig 1A). To assess PTPRZ influence on glioma growth and migration, lentiviral vectors for PTPRZ-B expression and *PTPRZ1* shRNA-mediated knock-down (targeting all three isoforms) were generated (supplementary Fig. S1). We introduced a silent mutation in the PTPRZ-B open reading frame to create an shRNA-insensitive lentiviral PTPRZ-B expression construct and used this throughout for validation and rescue purposes. Following lentiviral transduction of E98 and E434 cells with *PTPRZ1* shRNA, a five- to twenty-fold reduction of *PTPRZ1* transcript levels (Fig. 1B,C) and a five- to ten-fold drop in PTPRZ-B protein content (Fig. 1D,E) was obtained. As for C6 glioma cells \[23\], it is the short transmembrane variant PTPRZ-B that was detected in E98 and E434 lysates (Fig. 1D,E). Use of the lentiviral PTPRZ-B expression vector resulted in PTPRZ-B protein levels that were one to three times that of the endogenous protein, also in presence of *PTPRZ1* shRNA (Fig. 1D).

**PTPRZ-B stimulates glioma cell growth in vitro**

PTPRZ-B over-expression and knock-down effects on E98 and E434 cell proliferation was assessed via direct measurement of cell content and via BrdU incorporation. Sulforhodamine B (SRB) proliferation assays revealed that *PTPRZ1* knock-down significantly inhibited growth of adherent E98 cells, as illustrated by an increased cell doubling time (Fig. 2A). Accordingly, BrdU pulse-labeling showed a significant reduction of the percentage of S-phase cells in shPTPRZ1-transduced samples (p<0.05). PTPRZ-B over-expression did not significantly affect E98 proliferation, in agreement with the modest increase in PTPRZ-B levels on immunoblot. Nevertheless, *PTPRZ1* knock-down effects were fully rescued by PTPRZ-B re-expression (Fig. 2B).

To investigate whether observed *PTPRZ1* knock-down effects are on the level of cell viability, apoptosis or cell cycle progression, BrdU pulse-labeled cells were im-
Intracellular and extracellular domains of PTPRZ-B differentially regulate glioma cell growth and motility.

munostained for BrdU, for the G1-S-G2-M marker Ki-67 and for cleaved Caspase-3. The percentage of apoptotic cells was less than 1% for all conditions (data not shown) and, with the exception of PTPRZ1 knock-down cells, approximately 20% of cells ended up BrdU-positive. Intriguingly, always half of the cells stained positive for Ki-67, even among shPTPRZ1-tranduced cells of which 10% is in S-phase (Fig. 2B-C). This suggests that the growth impairment upon PTPRZ1 knock-down reflects increased duration of cell cycle time.

Knock-down of PTPRZ1 also resulted in significantly delayed growth in E434 cells. Since these cells can only be propagated as spheroids, we measured proliferation capacity by means of the spheroid diameter (Fig 2D-E). Fluorescent protein signals in the lentivirally transduced cells were used to image spheroid size over time. Whereas shSCR-transduced E434 spheroids showed clear signs of growth, shPTPRZ1-transduced spheroids only increased a little in size.

Figure 1: PTPRZ1 expression or knock-down in E98 and E434 cells. A) Fluorescent images of glioma cells containing shSCR or shPTPRZ1 knock-down constructs carrying GFP or TagRFP fluorescent reporters, respectively. E98 cells were DAPI counterstained. B) PTPRZ1 mRNA levels in lentivirally transduced E98 cells were determined by qRT-PCR. Expression, normalized to β-actin, is given as percentage of that in control shRNA-expressing (shSCR) cells. EV; empty vector control. C) Normalized PTPRZ1 mRNA levels in lentivirally transduced E434 spheroids, determined by qRT-PCR. D) E98 cells were lentivirally transduced with expression constructs for PTPRZ-B protein or EV and/or PTPRZ1 or control (SCR) shRNAs. After three days, lysates were analyzed on immunoblots using PTPRZ-B (upper) and GAPDH (lower) antibodies. Normalized PTPRZ-B levels, relative to that in shSCR control lysates, are depicted in between the blot images. Molecular weight indications (in kDa) are on the left. E) Immunoblot analysis (as in D) of E434 lysates 72 hrs after lentiviral transduction with the indicated constructs.
We next investigated whether PTPRZ1 influences on cell migration [24, 25] is represented in our glioma models. Following PTPRZ1 knock-down, migration of individual E98 cells in a 3D Matrigel/agarose environment was significantly reduced (p<0.001), an effect that was rescued by expression of the shRNA-insensitive PTPRZ-B transcript in knock-down cells (Fig. 3A-B). To confirm this in a different setting, we generated transduced E434 spheroids using the hanging-drop method. Resulting homogeneously sized E98 spheroids were subsequently placed on a thin Matrigel layer for 24 hours and migratory performance of outgrowing cells, expressed as distance travelled from the edge of the spheroid [32], was calculated semi-automatically. Again, PTPRZ1 knock-down cells migrated significantly less (p<0.05) than scrambled controls, and restoration of PTPRZ-B levels rescued this phenotype (Fig. 3C-D). Of note, PTPRZ-B over-expression by itself did not alter E98 motility. In a similar fashion, outgrowth of lentivirally transduced E434 spheroids was inhibited by PTPRZ-B.
Intracellular and extracellular domains of PTPRZ-B differentially regulate glioma cell growth and motility.

Figure 3: PTPRZ1 knock-down reduces glioma cell migration. A-B) E98 cells were lentivirally transduced with indicated protein (PTPRZ-B), EV control or shRNA (shPTPRZ1 or shSCR control) expression vectors, and seeded in an agarose/Matrigel 3D matrix. A) Migration paths were recorded overnight and analyzed by single-cell tracking. B) Averaged migration speeds (arbitrary units, AU) of individual cells, normalized to shSCR (n>30), are shown (gray points). Box-plot whiskers represent minimum and maximum. Asterisks indicate significance (*** p<0.001; n.s., not significant; Kruskal-Wallis p<0.0001). C) E98 spheroids were seeded on a Matrigel layer and fixed and DAPI-stained 24hrs later. Two representative images are shown. D) Pixel distance from the spheroid border was calculate for individual cells and per spheroid (n>32) average outgrowth was plotted (grey data points). Box-plot whiskers represent minimum and maximum. Asterisks indicate significance (*** p<0.001; n.s., not significant; non-parametric ANOVA: Kruskal-Wallis p<0.0001). E) Representative pictures of transduced E434 spheroids, fixed and DAPI-stained, after 24hrs on Matrigel. F) Mean pixel distance that cells traveled from the spheroid border was calculated, and averages per spheroid (n=53) are plotted (grey data points). Box-plot whiskers represent minimum and maximum, and asterisks indicate significance (***p<0.001).
knock-down (p<0.05; Figure 3E-F). Interestingly, whereas outgrowing E98 cells presented as scattered, amoeboid-like migrating cells, the migrating E434 cells largely remained interconnected, suggestive of collective migration [33]. Taken together, the reduced migration in vitro due to PTPRZ1 knock-down, which was rescued following PTPRZ-B re-expression, corroborates a stimulatory role for PTPRZ-B in cell motility.

PTPRZ1 knock-down impairs glioma cell growth in mouse brain
To investigate how these PTPRZ-B knock-down effects translate to in vivo tumor behavior, we co-injected intracerebrally shPTPRZ1- and shSCR-expressing E98 cells that were tagged with TagRFP and EGFP fluorescent marker proteins, respectively. When cultured under serum conditions, E98 cells grow to compact non-diffuse tumors upon orthotopic injection, but when cultured as spheroids they display diffuse infiltrative properties (our unpublished results). Stably transduced E98 cells were therefore grown as spheroids in serum-free neurobasal medium for at least two weeks prior to injection. E434 cells exclusively grow as spheroids in neurobasal medium and were directly injected after dissociation. ShPTPRZ1/TagRFP and shSCR/EGFP spheroids were processed to single cell suspensions and a 1:1 mixture of both cell types was injected intracerebrally in immunodeficient mice. Animals were sacrificed when symptoms of tumor burden appeared, and brains were examined for the distribution of EGFP- and TagRFP-containing cells (Fig. 4). EGFP-positive control cells greatly outnumbered the TagRFP-expressing PTPRZ1 knock-down cells in the tumors (p<0.01), in accordance with the low in vitro proliferation rate of the PTPRZ1 knock-down. The ratio of knock-down versus control cells did not significantly differ between compact and diffuse infiltrative tumor regions (Fig4A). Collectively, in vitro and in vivo data demonstrate a significant reduction of tumor growth upon PTPRZ1 knock-down, prompting us to address the molecular mechanisms by which PTPRZ-B impacts on glioma cell behavior.

The PTPRZ-B PDZ binding domain mediates growth stimulatory signals
PTPRZ-B may exert its stimulatory effect on E98 glioma proliferation in multiple ways, potentially involving extracellular CAH, FNIII and chondroitin sulfate-containing domains or intracellularly the two PTP domains and the C-terminal PDZ domain target site (Fig. 5A). Because the PTPRZ-B knock-down phenotype in E98-shPTPRZ1 cells could be rescued with an shRNA-insensitive full-length expression construct (Figs. 2-3), we tested the contribution of the individual PTPRZ-B protein domains using this assay. Three different shRNA-resistant PTPRZ-B cDNA versions were generated (Fig. 5A). In PTPRZ-B ecto-VSV the transmembrane and intracellular PTPRZ-B segments were replaced by a VSV-G epitope tag, effectively leading to the secretion of a C-terminally tagged PTPRZ-B extracellular domain (Fig. 5B). PTPRZ-B C/S represents a cysteine-to-serine catalytically inactive mutant, and in PTPRZ-B-VSV the ‘SLVCOOH’ PDZ domain target site is blocked by a C-terminal VSV-G tag. Expression of all constructs was readily detected in E98-shPTPRZ1 cells (Fig 5B-C).
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BrdU pulse-labeling experiments revealed that reduction in percentage of cells in S-phase, due to knock-down of endogenous PTPRZ, was fully rescued by full-length PTPRZ-B and the inactive PTPRZ-B C/S mutant. Expression of PTPRZ-B ecto-VSV or PTPRZ-B-VSV did not rescue the knock-down phenotype (Fig. 5D-E). Thus, PTPRZ-B impacts on cell cycle progression through mechanisms that are independent of PTPRZ-B phosphatase activity and require interactions with the PTPRZ PDZ domain target site.

Extracellular PTPRZ-B interactions impact on glioma cell migration
Rescuing abilities of PTPRZ-B variants were also assessed in the spheroid migration assay. Migratory impairment due to PTPRZ1 knock-down in E98-shPTPRZ1 cells was effectively rescued upon re-expression of full-length PTPRZ-B, PTPRZ-B C/S and, importantly, also by PTPRZ-B ecto-VSV (Fig. 6A-B). Moreover, purified VSV-tagged PTPRZ-B ectodomains, isolated from conditioned medium of transfected HEK-293FT cells, also rescued the PTPRZ knock-down effect on E98 cell migration (Fig. 6C). Contactin-1 has been put forward as a membrane-anchored PTPRZ
Figure 5: The PTPRZ-B C-terminus is required to rescue the proliferation phenotype in E98 PTPRZ1 knock-down cells. 

A) Schematic representation of PTPRZ-B variants used in the study. CAH, carbonic anhydrase-like; FNIII, fibronectin type-III; CS, chondroitin sulfate chain; TM, transmembrane region; PD1, phosphatase domain 1; PD2, inactive phosphatase domain 2; VSV, C-terminal VSV-G epitope tag. C/S indicates the mutation of the catalytic site cysteine to serine that renders PD1 inactive.

B) Conditioned medium from E98 cells expressing PTPRZ-B ecto-VSV or from empty vector (EV) control cells was subjected to immunoprecipitation using anti-VSV monoclonal antibody and subsequent immunoblot analysis using rabbit antiserum against VSV. Size markers (in kDa) are indicated on the left. Arrowhead on the right indicates the chondroitin sulfated PTPRZ-B extracellular part.

C) E98 shPTPRZ1 cells were transduced with the indicated expression constructs and 72 hrs later lysates were prepared and analyzed on Western blots using PTPRZ-B antiserum. GAPDH staining served as loading control and E98 shSCR lysate was included for comparison.

D) E98 shPTPRZ1 cells were seeded on collagen-coated glass coverslips and hours later, transduced with the indicated expression constructs. Three days after transduction cells were labeled with BrdU, fixed and stained. Fluorescent images were taken and the percentage of BrdU-positive nuclei among DAPI-stained cells was determined (n>3). Error bars indicate SD. Confidence levels, as determined by ANOVA, are represented by asterisks (* p<0.05; n.s., not significant).

E) Percentage of BrdU-positive nuclei in DAPI-stained E98 or E98 shPTPRZ1 cells following transduction with the indicated constructs (n=3). Error bars indicate SD. Confidence levels, as determined by ANOVA, are represented by an asterisk (* p<0.05; n.s., not significant).
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Our results did not disclose PTPRZ phosphatase activity as a regulator of glioma cell migration and proliferation. It is reasonable to assume, however, that the phosphotyrosine-controlled activity of cellular kinases is affected following PTPRZ1 knockdown in E98 cells. A comparison of E98-SCR and E98-shPTPRZ1 cell extracts in a tyrosine kinase microarray activity assay [34] revealed that PTPRZ1 knock-down cells displayed reduced activity, most notably towards PDGFRB- and MET-derived peptides (Supplementary Fig. S2). We previously reported on the importance of MET for E98 cell migration [32]. Immunoblot analyses confirmed that in shPTPRZ1-expressing E98 cell lysates MET tyrosine phosphorylation was reduced four-fold (Fig. 6E). Additionally, PTPRZ1 knock-down also resulted in lower MET protein levels, suggesting that PTPRZ-B regulates MET transcription, synthesis or degradation. Interestingly, PTPRZ1 knock-down effects on MET are partly alleviated by PTPRZ-B ecto-VSV expression (Fig. 6F). Taken together, results demonstrate a dual oncogenic role for PTPRZ1 in glioma cells. The PTPRZ-B intracellular PDZ domain binding site is required to orchestrate a submembranous complex that boosts cell proliferation, while the extracellular portion binds receptors and adhesion molecules on nearby cells and stimulates migration.

Discussion:
Effective treatment of glioma patients requires that also tumor cells that diffusely spread out into the brain parenchyma are therapeutically addressed. In the current study we assessed the contributions of different PTPRZ-B domains to glioma cell migration and proliferation using models that faithfully recapitulate infiltrative tumor growth. We show that PTPRZ1 knock-down in E98 glioblastoma and E434 oligodendroglioma cells results in impaired growth and motility in vitro and reduced tumor growth in vivo, also in diffuse infiltrative tumor areas. Furthermore, our rescue experiments disclose a dyad functionality for this RPTP. The intracellular PTPRZ-B C-terminal PDZ domain binding site, and not its enzymatic PTP activity, turned out essential for effects on cell proliferation. Conversely, the PTPRZ-B extracellular moiety, which in part resembles the naturally occurring phosphacan isoform, impacted on cell migration.

PTPRZ1 is upregulated in gliomas, which likely contributes to enhanced tumor cell migration [24-26]. A role for PTPRZ in proliferation was less clear. Reduction of tumor growth as well as absence of growth effects have been reported for PTPRZ1 knock-down experiments [25, 27]. Administration of soluble PTPRZ ectodomain is known to inhibit proliferation of oligodendroglial precursor cells [35]. In our study, the PTPRZ-B ectodomain did not affect the reduction of E98 cells in S-phase that resulted from PTPRZ1 knock-down. Addition of wild-type or enzymatically inactive PTPRZ-B did suffice to rescue proliferation impairment, but a C-terminally tagged version did not. If PTPRZ phosphatase activity would have been crucial for glioma
Figure 6: The PTPRZ-B ectodomain stimulates glioma migration. A) E98 or E98 shPTPRZ1 cells were lentivirally transduced with indicated constructs. Three days later spheroids were generated and the next day seeded on a thin Matrigel layer. After being cultured for another 24 hrs cells were fixed and stained with DAPI. Pictures were collected and two representative images are shown. B) Using these images the migration, in arbitrary units, was calculated per spheroid (light grey data points) as the average distance cells traveled from the spheroid border using FIJI-based software. Box-plot whiskers represent minimum and maximum values. Significance levels are indicated by asterisks (* p<0.05; ** p<0.01; *** p<0.001; Kruskal-Wallis p<0.0001). C) Using the above set-up, spheroid migration was also determined following addition of immunopurified PTPRZ-B ecto-VSV to E98 shPTPRZ1 cells (shPTPRZ1 + PTPRZ-B ecto-VSV protein) during the 24 hrs of culturing on Matrigel. Kruskal-Wallis p<0.003. D) PTPRZ-B ecto-VSV was immunopurified, using mouse anti-VSV monoclonal antibody-coupled beads, from conditioned medium (CM) of HEK-293FT cells transfected with PTPRZ-B ecto-VSV (ecto-VSV) or empty vector (EV) expression plasmids (left panel). Subsequently, beads were incubated with E98 whole cell lysates and co-purifying proteins were analyzed on western blots using Contactin-1 antiserum (right panel). E) Lysates of E98 cells, that were transduced with indicated shRNA constructs and blasticidin-selected, were prepared and analyzed on immunoblots. Size markers (kDa) are indicated on the left. Upper panels: PTPRZ-B immunostaining, using GAPDH as loading control. Middle and lower panels: blots were probed with an-
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cell proliferation, expression of an inactive ‘substrate protection’ mutant in PTPRZ1 knock-down cells might act dominant-negative and worsen the effect. On the contrary, proliferation rescue by the catalytically dead PTPRZ-B mutant and not by the C-terminally tagged version rather implicates PTPRZ-B’s C-terminal protein interaction potential as important for signaling complexes that steer glioma proliferation.

Multiple PDZ-containing proteins have been reported to bind to the PTPRZ C-terminus; PSD95 [36], MAGI-3 [37], MAGI-1, GOPC, Mupp1, Synj2bp, Snta1, Sntb1 and Veli-3 [38]. This opens up several mechanisms by which PTPRZ may influence proliferation. For instance, tyrosine kinase ErbB4 auto-activation is suppressed by PSD95 through PDZ domain-mediated interactions with both the enzyme PTPRZ and the substrate ErbB4 [39]. Likewise, PTPRZ and its substrate β-catenin form a complex through MAGI-1 [38, 40]. However, current findings argue against an enzymatic role for PTPRZ in such PDZ-based complexes and rather point to a scaffolding role. By clustering proteins submembranously in glioma cells, PTPRZ might contribute to efficient funneling of growth stimulatory signals towards the cell’s interior.

In both our glioblastoma and anaplastic oligodendroglioma model, the PTPRZ ectodomain did not impinge on proliferation but rather was instrumental for migration. Also in U87-MG glioblastoma cells, PTPRZ positively influenced adherence and migration [24] and an antibody targeting the PTPRZ extracellular region delayed U87-MG compact tumor formation in vivo [41]. PTPRZ ectodomain binding partners include extracellular matrix components like pleiotropin, tenascin-C and tenascin-R, and cell surface molecules [35] like contactin-1 [20, 21]. Extracellular binding of PTPRZ and contactin-1 is hypothesized to recruit additional proteins, such as tenascins, to the complex [22]. In oligodendroglial precursors this inhibits proliferation and triggers differentiation towards myelinating cells. In our glioma models, PTPRZ-contactin-1 interactions apparently lead to migratory responses. This difference may result from separate PTPRZ-mediated interactions in either system or, alternatively, by absence or presence of facilitator proteins involved. Contactin-1, tenascin-C and tenascin-R have adhesion and migration effects in glioma cells [21, 24, 25, 41, 42].

Recently we showed that MET inhibition by cabozantinib effectively stops E98 cell migration [32]. Here, migration impairment in E98-shPTPRZ1 cells co-incided with reduced MET activity. For a candidate substrate, rather increased phosphorylation levels are expected upon knock-down of PTPRZ, and thus far MET does not meet PTPRZ substrate criteria [43]. Together with ErbB and PDGFR family members,
MET represents one of the oncogenic drivers in glioma tumor biology [7]. Whereas E98 cells displayed a scattered, single-cell migration pattern in spheroid outgrowth experiments, E434 cells showed a more collective migratory behavior. This may reflect MET signaling differences in the two models, bearing in mind ‘scatter factor receptor’ as alternative name for MET. In our in vivo experiments we were unable to assess PTPRZ-B’s migratory role on tumor formation because this was blurred by effects on cell proliferation. Current findings now provide a basis to experimentally separate both type of effects through independent targeting of PTPRZ-B intracellular and extracellular binding potential. Further knowledge on PTPRZ intracellular and extracellular signaling involvement will strengthen its candidacy as a therapeutic target in gliomas.

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**Disclosure of Potential Conflicts of Interest**

L. Hovestad and R. Hilhorst are employed in PamGene International BV. No other potential conflicts of interest were disclosed.

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Supplementary material

Cell lines and antibodies
Generation and maintenance of orthotopic glioma xenograft models E98 (glioblastoma) and E434 (anaplastic Oligodendroglioma) have been described previously (28). E98 and E434 orthotopic xenograft-derived cells were cultured as spheroids in neurobasal medium supplemented with B27 Supplement, 2 mM L-glutamin, 2 μg/mL Heparin, 1% penicillin/streptomycin (all Gibco), 20 ng/mL EGF and basic-FGF (both PromoCell) (32). Regular passaging of spheroids was done by gentle mechanical dissociation. In addition, E98 cells were grown as adherent cultures in DMEM supplemented with 10% FCS, and passaged using trypsinisation (30). E434 cells cannot be propagated under these conditions.

Antibodies used were as follows: for Western Blotting: mouse anti-PTPRZ (1:1000; BD Biosciences, #610179), mouse anti-VSV (IP: 5μl; P5D4 (45)), mouse anti-tubulin (1:5000; DSHB, University of Iowa, E7), rabbit anti-MET (1:2000; Cell Signaling Technology, #8198), rabbit anti-pMET (1:2000; Cell Signaling Technology, #3027), rabbit anti-GAPDH (1:5000; Cell Signaling Technology #2118), rabbit anti-Contactin-1 (1:2000) (22), for immunohistochemistry mouse anti-BrdU (1:50; Sigma-ALDRICH, #Rm-9106) rabbit-cleaved caspase 3 (1:200; Cell Signaling Technology, #9661) rabbit anti-GFP (1:1000) (46), and rabbit anti-TagRFP (1:500; Evrogen, AB233). As secondary antibodies, IRDye 680- or 800-conjugated goat anti-mouse and goat anti-rabbit antibodies (1:20.000; LI-COR Bioscience), Alexa Fluor 488 goat-anti-mouse and Alexa Fluor 647 goat-anti-rabbit antibodies (1:300; Alexa) or biotinylated goat-anti-rabbit IgGs (1:200; Dako) were used.

Plasmid constructs
The plasmid pLenti6/BLOCK-iT-DEST (Invitrogen) was adapted to also contain CMV promoter-driven TagRFP or EGFP expression cassettes. Briefly, the CMV-TagRFP cassette was PCR-amplified from pTagRFP-C (Evrogen) using oligonucleotide set 1 (all oligonucleotide sequences are listed in Supplementary Table 1), digested with Xbal and Xhol, and ligated into Xbal/Xhol-digested pLenti6/BLOCK-iT-DEST. Likewise, the CMV-driven EGFP expression cassette was amplified from pEGFP-N2 (Clontech) using oligonucleotide set 2, digested with Xbal and ligated in the Xbal-linearized pLenti6/BLOCK-iT-DEST. For over-expression purposes, the mouse phosphoglycerate kinase (PGK) promoter was inserted upstream of the attR1 recombination site in these pLenti6/Block-it-DEST-TagRFP and -EGFP variants. Briefly, oligonucleotide set 3 was used to amplify the pLenti6.2/V5-DEST (Invitrogen) PGK fragment and the ClaI digested amplicon was ligated to produce plasmids pLenti6/PKG-DEST-TagRFP and pLenti6/PKG-DEST-EGFP. To create knock-down constructs, shPTPRZ1 (set 4) and shSCR (set 5) oligonucleotide heteroduplexes were ligated in pENTR/U6 vector and subsequently Gateway-cloned into pLenti6/Block-it-DEST-TagRFP (for shPTPRZ1) and pLenti6/Block-it-DEST-EGFP (for shSCR) according to the manufacturer’s protocols (Invitrogen).

A pENTR/U6 derivative was customized by inserting oligonucleotide heteroduplex
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PTPRZ-B cDNA was subsequently adjusted via site-directed mutagenesis to encode an enzymatically inactive PTPRZ-B C/S mutant (GC to CG, at positions 3613-3614) using oligonucleotide set 10 and the afore-mentioned protocol. Furthermore, a C-terminally VSV-tagged full-length PTPRZ-B variant was generated by first generating a KpnI site (AGTTTAA to GGTA, pos. 4757-4763) using oligonucleotide set 11, and subsequently introducing oligonucleotide heteroduplex set 12, encoding an in-frame C-terminal VSV-G epitope tag (flanked by KpnI sites). To enable expression of the PTPRZ-B ecto-domain only, first a KpnI site was created at the codon preceding the PTPRZ-B transmembrane-encoding region (AGTTATA to GGTA, pos. 2726-2732) using oligonucleotide set 13 in the mutagenesis protocol. Subsequent CiaI digestion and re-ligation resulted in removal of residues 776-1448 comprising the PTPRZ intracellular domains. Finally, the new KpnI site was used to insert the VSV-G epitope tag-encoding heteroduplex 12. All resulting pENTR-PTPRZ-B plasmid variants were sequence-verified before being used in Gateway LR cloning reactions with pLenti6/PGK-DEST-TagRFP as destination vector. Also using Gateway cloning, the empty pENTR/NotI-XhoI vector served to generate pLenti6/PGK-EV-TagRFP as empty vector control.

**Lentiviral transduction of glioblastoma cells and spheroids**

Lentiviruses were produced using HEK-293FT cells according to the manufacturer’s instructions (Invitrogen). Briefly, 95% confluent 10cm culture dishes with HEK-293FT cells were transfected overnight, using JetPRIME reagent (Westburg) and the appropriate plasmid cocktail. The next day, medium was refreshed and 48-72 hrs later virus-containing medium was harvested, passed through a 0.45 μm pore size filter and stored at -80 °C. E98 Glioma cells or E434 spheroids were transduced by adding virus-containing medium to the cultures, at a 1:2 to 1:5 virus to medium ratio. After an overnight incubation, cells were superinfected with virus to increase the percentage of transduced cells. Routinely, this led to 80-100% transduction efficiency for E98 cells and 40-80% for the spheroid E434 cultures. Stably transduced E98 cells were selected by adding Blasticidin (2 μg/mL; Invitrogen). For rescue experiments, cells were first transduced twice with shPTPRZ1-expressing lentiviruses. Several days later, two or three rounds of transduction with PGK promoter-driven rescue constructs were performed and cells were subjected to the proliferation and migration assays 72 hrs later.
**Real-time quantitative RT-PCR**

Total RNA was isolated using RNA-Bee (Tel-Test Inc. cs104B) using standard trizol-chloroform extraction methods, and concentrations were measured spectrophotometrically. Reverse transcriptase reactions were performed using Iscript™ cDNA synthesis kit (Bio-Rad) according to the supplier’s specifications. Specificity and efficacy of real-time quantitative PCR primer pairs for *PTPRZ1* and β-actin (Qiagen) have been verified previously by Schmidt et al. (47). Reactions, containing 3 μL of cDNA, 1 μL of the pre-mixed primer pair, 5 μL of SYBR Green PCR master mix (Bio-Rad) and 1 μL MQ, were run on a CFX96™ Real Time system using the C1000™ Thermal Cycler (Bio-rad). Reactions were initialized at 95 °C for 15 minutes and then cycled 40 times at 95 °C for 15 s and 60 °C for 40 s. After the last cycle, a dissociation curve was recorded between 60 °C and 95 °C with and increment of 0.5 °C. The amount of *PTPRZ1* RNA was determined relative to ACTB levels using the delta Ct method (48).

**Immunoblotting and immunoprecipitation**

Cells were washed with cold PBS and scraped in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% Triton X-100 (Serva); 1 mM PMSF; 100 mM NaF; 2 mM Na3VO4; 20 mM Na4P2O7; complete protease inhibitor cocktail, Roche, #13006200 (1 tablet in 1.5 mL MQ, 20 μl solution per mL lysis buffer)). Samples were either further processed for immunoprecipitation (see below) or added to 2× SDS sample buffer (60 mM Tris-HCl, pH 6.8; 2% SDS; 100 mM dithiothreitol; 0.001% bromophenol blue; 10% glycerol) and heated at 95 °C for 5 min. Protein samples were size-separated using SDS-PAGE on 8% gels and electro-blotted onto PVDF membrane (Immobilon-FL, #IPFL00010) according to standard protocols. Membranes were blocked using 1% BSA in TBST (10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.1% Tween-20 (Sigma-Aldrich)) for 1 hr at RT, and subsequently incubated with primary antibodies in 1% BSA in TBST, overnight at 4 °C. Membranes were then washed three times for 10 min in TBST and subsequently incubated with the appropriate secondary antibodies for 1 hr at RT in the dark. Blots were analyzed using the Odyssey imaging system (LI-COR Biosciences). Immunoblot images were analyzed quantitatively using standard FIJI software.

To visualize PTPRZ-B ecto-VSV in E98 conditioned medium, immunoprecipitation was performed. 30 μl ProtG sepharose beads (GE Healthcare, #17-0618-01) were incubated with mouse-VSV antibody overnight at 4 °C. Beads were washed 5 times with TBS and incubated with E98 conditioned medium overnight at 4 °C. Beads were washed 5 times with TBS and suspended in SDS sample buffer. After centrifugation, the supernatant was used for gel loading and blotted as described above. For purification purposes PTPRZ-B ecto-VSV was produced in HEK-293FT cells transfected with pLenti6/PGK-PTPRZ-B ecto-VSV-TagRFP, using JetPRIME according to the manufacturer instructions. The secreted PTPRZ-B ectodomain was purified from conditioned medium via immunoprecipitation using mouse anti-VSV bound to ProtG sepharose beads, as described above. VSV-tagged proteins were eluted from the beads using excess VSV peptide (49).
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For co-immunoprecipitation, mouse anti-VSV was coupled to ProtG sepharose beads by overnight rotation at 4 °C in TBS. After 5 subsequent washes with TBS, conditioned medium from HEK293FT cells transfected with either pLenti6/PGK-EV-TagRFP or pLenti6/PGK-PTPRZ-B ecto-VSV-TagRFP was added to the beads allowing coupling to VSV overnight at 4 °C. After 5 washes, E98 cell lysates (prepared as described above) were added to the beads and were incubated overnight at 4 °C. The next day, beads were washed 5 times with TBS before being taken up in 2x SDS sample buffer. Samples were processed for immunoblotting as described above.

**Immunohistochemistry**

FFPE sections of 4 μm were subjected to immunohistochemical stainings according to standard procedures (29). In brief, sections were de-paraffinized in xylene and rehydrated in PBS. Endogenous peroxidases were blocked in 3% H2O2 in PBS, followed by epitope retrieval (10 min boiling in 10 mM sodium citrate, pH 6.0). Slides were then washed twice in PBS, blocked in 20% normal serum (from the species in which the secondary antibody was raised) in PBA (1% BSA in PBS) for 20 min, and incubated overnight at 4 °C with primary antibodies in PBA. After 3 PBS washes, sections were incubated with biotinylated secondary antibodies in PBA for 1 hr at room temperature. Slides were washed 3x with PBS and signals were then enhanced by incubation with Avidin/Biotin complexes (Vector Laboratories, #PK-6100), both 1:100 in PBA for 45 min, followed by 3 PBS washes and visualization of specific signals using 3,3′-diaminobenzidine (Bright-DAB, ImmunoLogic, #B04). All sections were counterstained with haematoxylin and mounted in Quick-D Mounting medium (Klinipath BV, #7280).

Quantification of immunodetected EGFP and TagRFP signals was done using KS400 software (Carl Zeiss AG) and a custom-written macro. Sections of FFPE brains with orthotopic glioma xenografts were included in the analysis (n=3 and 2 for E98 and E434, respectively) and at least five non-overlapping microscopic fields (magnification x200) were measured per immunostaining for each animal. The TagRFP- or GFP-positive area per tumor field was divided by the total tumor area as determined via nuclear DAPI staining, and average values per animal were determined and used to calculate TagRFP/GFP ratios. Ratios were compared to those prior to injection using the one-sample Student’s t-test.

**Peptide microarray analysis**

E98 cells stably expressing shSCR/GFP and shPTPRZ1/TagRFP constructs were grown to 80% confluency in 6-well plates (4 wells per sample). Cells were washed twice with ice-cold PBS prior to lysis with M-PER Mammalian Extraction Reagent supplemented with protease and phosphatase inhibitor cocktails (Thermo Scientific) for 30 min at 4 °C. Lysates were centrifuged (15 min, 14,000 rpm, 4 °C), and supernatants were snap frozen in liquid nitrogen and stored at -80 °C. Protein concentrations were determined using the BCA protein assay (Thermo Scientific). Kinase activity measurements were performed in quadruplicate on Tyrosine kinase PamChip arrays on a PamStation 12 instrument (PamGene International BV) essentially as described (35). Sample input was 5 µg per array. A Student’s t-Test was used to
identify the peptides that are significantly (p<0.05) different between the treatments.

**Statistical analysis:**
Statistical analysis was performed using GraphPad Prism 5 or PamGene’s proprietary BioNavigator software. For two conditions the Student t-test was applied. For other conditions ANOVA, repeated measure ANOVA or non-parametric Kruskal-Wallis with post-hoc tests were performed. When all experimental conditions were included, Tukey’s multiple comparison test was used. In contrast, when comparisons were made to a single “control” experimental condition the Dunn’s post-hoc test was applied.

Supplementary Figure S1: Plasmid maps of constructs used to create lentiviral vectors for glioma cell transduction. A) Schematic overview of the expression construct that is generated in an LR Gateway cloning reaction, using pLenti6/PGK-DEST-tagRFP and the appropriate cDNA-containing pENTR plasmid (in this case full-length PTPRZ-B). B) Schematic overview of the scrambled control knock-down construct that resulted from an LR Gateway reaction involving the pLenti6/Blocki-iT-DEST variant (with added fluorescent protein expression cassette) and the pENTR/U6 plasmid with inserted oligonucleotide heteroduplex for shSCR production. Long terminal repeat (LTR) regions, attR1 and attR2 Gateway recombination sites, and the CMV promoter-driven TagRFP and EGFP expression cassettes as well as the SV40 early promoter-driven Blasticidin selection cassette are indicated.
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Supplementary Figure S2: Kinase activity profiling of E98 cells stably expressing shSCR/GFP and shPTPRZ1/TagRFP constructs. A) Heatmap representation of the mean signals (n=4) for 144 different peptides on the Tyrosine Kinase PamChip® Array upon incubation with lysates from shSCR or shPTPRZ1-expressing E98 cells. The order of peptide signal depiction (top, highest signal in red; bottom, lowest signal in blue) was determined by that in the shSCR sample. B) List of 37 peptides that were significantly differentially phosphorylated by shSCR- and shPTPRZ1-expressing E98 lysates. The ID column contains the protein name and first and last amino acid position of the peptide. Corresponding UniProt accession numbers are shown in the second column, and column ‘Tyr’ lists the positions of the phosphorylatable tyrosines in the peptide. The final column is a color-coded representation of the lower signals obtained with shPTPRZ1 E98 extracts as compared to the scrambled control.
Supplementary Table S1: Oligonucleotide sequences used in generating plasmid constructs.

<table>
<thead>
<tr>
<th>Oligonucleotide (set)</th>
<th>Sequence</th>
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</table>
| **1: CMV-TagRFP**     | **Fw** 5'-GGCTCGAGTAGTTATTAATAGTAATC -3'  
|                       | **Rv** 5'-CCCTCTAGCAATTAAAGTTTGTGCC -3'  |
| **2: CMV-EFGP**       | **Fw** 5'-GGCGCTCTAGAAAGTTTGTGCCC -3'  
|                       | **Rv** 5'-GGCGCTCTAGATTACTGAGTCAGCTCCATG -3'  |
| **3: PGK promoter**   | **Fw** 5'-GGCGATGATCCGGCTGGGATGGGAGAAGC -3'  
|                       | **Rv** 5'-GGCGATGATCCGGCTGGGATGGGAGAAGC -3'  |
| **4: shPTPRZ1**       | **Fw** 5'-GAAGAAATTTTCTGAGGCTGATTCAGGTG -3'  
|                       | **Rv** 5'-GAAGAAATTTTCTGAGGCTGATTCAGGTG -3'  |
| **5: shSCR**          | **Fw** 5'-GAAGAAATTTTCTGAGGCTGATTCAGGTG -3'  
|                       | **Rv** 5'-GAAGAAATTTTCTGAGGCTGATTCAGGTG -3'  |
| **6: NotI-Xhol adapter** | **Fw** 5'-TCGACGCGGCCGCGGTTTCTGAGGCTGATTTGAC -3'  
|                       | **Rv** 5'-TCGACGCGGCCGCGGTTTCTGAGGCTGATTTGAC -3'  |
| **7: SstI linker**    | **Fw** 5'-AGCTGACCGGGTCG -3'  
|                       | **Rv** 5'-AGCTGACCGGGTCG -3'  |
| **8: PTPRZ-B cDNA**   | **Fw** 5'-GCGCCCGGGCTGGGTTTCTGAGGCTGATTTGAC -3'  
|                       | **Rv** 5'-GCGCCCGGGCTGGGTTTCTGAGGCTGATTTGAC -3'  |
| **9: PPRZ-B rescue mut.** | **Fw** 5'-CATCACCCTTCCAGAAATTCATTCTTCTTGG -3'  
|                       | **Rv** 5'-CATCACCCTTCCAGAAATTCATTCTTCTTGG -3'  |
| **10: PTPRZ-B C/S, mut** | **Fw** 5'-CTTATACGAGTAAAGCTTCTGAGGCTGATTTGAC -3'  
|                       | **Rv** 5'-CTTATACGAGTAAAGCTTCTGAGGCTGATTTGAC -3'  |
| **11: PTPRZ-B, mut**  | **Fw** 5'-GCTGACACGATCATGACGTGTGTGACGCTGAGGCTGATTTGAC -3'  
|                       | **Rv** 5'-GCTGACACGATCATGACGTGTGTGACGCTGAGGCTGATTTGAC -3'  |
| **12: KpnI VSV-stop, mut** | **Fw** 5'-GCTGACACGATCATGACGTGTGTGACGCTGAGGCTGATTTGAC -3'  
|                       | **Rv** 5'-GCTGACACGATCATGACGTGTGTGACGCTGAGGCTGATTTGAC -3'  |
| **13: PTPRZ-B ectodomain, mut** | **Fw** 5'-GCTGACACGATCATGACGTGTGTGACGCTGAGGCTGATTTGAC -3'  
|                       | **Rv** 5'-GCTGACACGATCATGACGTGTGTGACGCTGAGGCTGATTTGAC -3'  |
Intracellular and extracellular domains of PTPRZ-B differentially regulate glioma cell growth and motility.
Chapter 5

PTPN13 AS A CANDIDATE ANCHORING PROTEIN IN THE PTPRZ-B INTERACTOME IN GLIOMA

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Abstract

Glioblastoma cells rapidly proliferate and migrate diffusely into the healthy brain parenchyma, complicating surgical resection and radiation therapy. The prognosis for glioma patients following treatment is generally poor, with a median survival time of less than 15 months. Glioblastoma cells express high levels of the receptor-type protein tyrosine phosphatase PTPRZ-B and we previously demonstrated that the intracellular domain of PTPRZ-B is facilitating their proliferation. Here, we set out to study the proteins that interact with the intracellular segment (RZi) of PTPRZ-B using immune pull-down experiments and proximity labeling strategies. We found that PTPN13, a PDZ domain-containing submembranous protein tyrosine phosphatase, interacts with the C-terminus of PTPRZ-B. Knockdown of PTP13 resulted in reduced glioblastoma cell proliferation, and in addition triggered cell death and conspicuous cell fusion events resulting in multinuclear cells reminiscent of giant cell glioblastomas. Further studies regarding the PTPRZ-B and PTPN13 interactome in glioblastoma may ultimately provide novel therapeutic targets for these tumors.
Introduction

Diffuse gliomas represent the most common primary brain tumors among humans, with an incidence of 3-5 per 100,000 people each year. Arising from glial cells or their precursors, the tumors are notorious for their highly infiltrative nature, explaining their devastating effect and the relatively short life expectancy of patients after diagnosis. Gliomas are classified into several distinct subtypes based on WHO criteria [37], of which high-grade astrocytomas (named glioblastomas) are the most malignant. Although research over the last decades has helped to reveal many molecular etiological features of gliomas, a significant improvement in patient survival is still lacking [56]. Current efforts in the field are therefore strongly concentrated on finding new druggable targets and the development of alternative treatment strategies.

As in other cancer types, glioblastoma cell growth relies on the activation of oncogenic signaling pathways and loss of tumor-suppressive mechanisms due to genetic mutations [8, 26, 30, 43]. In the vast majority of glioblastomas this involves aberrancies in growth factor signaling pathways (notably receptor tyrosine kinases PDGFRA, MET, EGFR and the autoactive deletion variant EGFRvIII) leading to high levels of phosphotyrosine-mediated signaling [8, 44, 46]. Phosphotyrosine levels are, however, not controlled by protein tyrosine kinases only. The protein tyrosine phosphatase (PTP) class of enzymes play an equally important role (e.g. [43]). Targeted anti-tumor strategies nowadays mostly focus on protein tyrosine kinases because of their direct role in signaling and their better druggability. Almost no attention is currently spent on the role of counteracting PTPs. This situation may need change, as evidence for PTP relevance for tumor biology is growing. This is perhaps best illustrated by the family member PTEN, a tumor suppressor that is lost in about one-third of primary (IDH-WT) glioblastomas [8]. PTEN’s activity directly impinges on PI3K signaling [11]. Earlier, in our in-depth analysis of PTP family member expression in diffuse glioma subtypes, we obtained evidence that the levels of three additional PTPs - DUSP26, PTPRM and PTPRT – have prognostic value for patient outcome. Also, multiple studies revealed that gene PTPRZ1 is highly up-regulated in all glioma types [7, 42, 57] and PTPRZ1-targeted knock-down or PTPRZ blocking antibodies demonstrated that this PTP regulates glioma cell migration and proliferation [18, 23, 42, 57].

Here we will focus on the pathobiological relevance of this latter PTP family member. Gene PTPRZ1 is mainly expressed in brain and is important for neuronal development [29, 35]. PTPRZ1 encodes three protein isoforms that all consist of an extracellular N-terminal carbonic anhydrase domain followed by a fibronectin type III repeat and a chondroitin sulfate attachment region [45]. Isoform PTPRZ-S is a secreted extracellular protein, whereas the other two isoforms (PTPRZ-A and PTPRZ-B) are single-pass transmembrane proteins that differ in the size of their chondroitin sulfate attachment region but further have identical intracellular parts, which essentially consist of two phosphatase domains and a C-terminal tail that ends in the amino acid sequence –SLVCOOH and represents a PSD95/Dlg1/ZO-1 (PDZ)-binding motif [3, 34, 38]. PTPRZ isoforms interact extracellularly with a wide range of ligands and substrates. For example, binding of the ligand pleiotrophin induces
homo-dimerization of the transmembrane PTPRZ-B isoforms, and resulting steric hindrance keeps the PTP from dephosphorylating its downstream targets that include β-catenin, β-adducin, FYN and ALK [41, 47–49]. Protein interaction screens yielded two membrane-associated guanylate kinases, PSD95 and MAGI1, that bind to the PTPRZ-B C-terminus by means of their PDZ domains [22, 25, 31].

PDZ domains are globular (80–90 amino acids) protein-protein interaction modules that play roles in protein targeting and complex assembly [36]. In the canonical binding mode, PDZ domains interact with C-terminal binding motifs. Specificity is hereby mainly determined by the ultimate and antepenultimate amino acid residues of the target proteins. In the case of PTPRZ this entails the three C-terminal amino acids SLV. Also examples of internal PDZ-binding motif recognition exist. PSD95 contains three such PDZ domains of which the second one binds PTPRZ-B [31]. It is thought that this scaffolding mediates clustering of PTPRZ-B and by simultaneous binding to Erbb4 is instrumental in dephosphorylation of this growth factor receptor [21]. Another known substrate of PTPRZ-B is MAGI1 [41]. MAGI1 is a submembranous anchoring protein for β-catenin [16] with six PDZ-domains, of which domain two binds the PTPRZ-B C-terminus [22].

In previous experiments we have shown that the PTPRZ-B extracellular part is involved in migratory behavior of glioma cells whereas its intracellular moiety supports proliferation [6]. Here, we used the entire intracellular segment of PTPRZ-B in glioma cells for non-biased partner protein identification methodology (Bio-ID) as well as for testing of candidate interactors, in order to unravel how PTPRZ-B impacts on cell cycle behavior. We identified its family member PTPN13 as a new PTPRZ-B interactor that is important for glioblastoma cell survival.

**Materials and methods**

*Construction of plasmids:*
The plasmids pENTR-EGFP [7], pENTR-NsiI/XhoI [6], pENTR-PTPRZ-B and pENTR-PTPRZ-B-VSV (with coding sequence modifications that render them resistant to shRNA mediated knockdown [6]), PSD-95-pTagRFP (Addgene plasmid # 52671; a gift from Johannes Hell), pCDNA3-PTP-BL [14] and pCMV5-HA-PTPN13 [27] have all been described elsewhere.

For the construction of the pCDNA3-GFP-RZi-(VSV) expression vectors the following 3-point ligation strategy was used. The GFP-encoding fragment was amplified using forward (5’-CGGGATCCATGGTGAGCAAGGGCGAGGAGCTG-3’) and reverse (5’-CGCGGATCCCTTGTACAGCTCGTCCATGCCG-3’) primers that contained *EcoR*I and *BamH*I sites (underlined), respectively, using pENTR-EGFP as template. The cDNA fragments encoding the intracellular PTPRZ-B part, with (RZi-VSV) or without (RZi) the VSV-G epitope tag, were PCR-amplified from pENTR-PTPRZ-B and pENTR-PTPRZ-B-VSV, respectively, with use of primers that introduced 5’ *BamH*I and 3’ *XhoI* sites (underlined): 5’-CGGGATCCAGGAAATGCTTCC-
CACACTGCACACT-3’ as forward primer and 5’-GCCCGGCACGCTCGAGTTAAC-TAAAGACTCTGA-3’ or 5’-CGCTGAGCTACATTCTCAGGTTTCACTCTCT-3’ as reverse primer. Three-point ligation of EcoRI-Xhol-digested vector pENTR-NotI/Xhol with the EcoRI-BamHI GFP-encoding and BamHI-Xhol RZi(-VSV)-encoding segments yielded pENTR-GFP-RZi and pENTR-GFP-RZi(-VSV), respectively. Next, inserts in these entry clones were subsequently transferred into pcDNA-DEST by use of Gateway recombination reactions with Clonase II to produce expression plasmids pCDNA3-GFP-RZi(-VSV), per manufacturer’s instructions (Invitrogen).

For construction of an expression vector encoding the biotinylating enzyme biotin ligase (BirA*), alone or as fusion product with PTPRZ’s intracellular domains, the pcDNA3.1 mycBioID plasmid a gift from Kyle Roux (Addgene plasmid # 35700) [51], was used as a template. BirA*-encoding cDNA was generated using PCR with primers Fw: 5’-GGCCGCGGCAGTACGAGGAAACACTCAT-3’ and rev: 5’-GGCCGATCTACTGATCGAGAAGAGATTT-3’. The resulting fragment was NotI and BamHI digested (sites are underlined in primer sequences) and ligated into NotI/BamHI digested pENTR, resulting in pENTR-BirA*. Similarly the amplified NotI/BamHI BirA* fragment was introduced in pENTR-GFP-RZi(VSV), effectively replacing the GFP cDNA.

pDONR223-MAGI1 was a gift from William Hahn & David Root (Addgene plasmid # 23523) [28]. MAGI1 was amplified using PCR to introduce flanking EcoRI and KpnI sites (fw 5’-GCCGGAATTCACCATGCGATCC-3’: rev 5’-AAGGTACCTGATCTTCTCTGCGCTTCTCAGGGAGATTT-3’). GFP-RZi in pENTR-GFP-RZi-VSV was substituted by MAGI1 by exchanging Eco R1/KpnI inserts, creating a MAGI1-VSV reading frame. Resulting pENTR clones were used in Gateway recombination reactions with pcDNA3.1-DEST or pLenti6.2/V5-DEST (both Invitrogen) as recipients, using Clonase II (Invitrogen #11791) essentially as described above.

For shRNA-mediated knockdown of PTPN13 in glioma cells, we converted the shRNA-expression cassette from retroviral vector pSuperRetro into a Gateway-compatible plasmid to facilitate Lentiviral transduction. First, forward (5’-GCGGTCGACGAATTCACCATGCTCAAGATGATC-3’) and reverse (5’-GGCTGACCATCGATCGACGATACTTTAGATCTAC-3’) primers were used to PCR-amplify nucleotide region 1429-1673 from plasmid pRS (retroviral pRETSUPER RNA interference vector [9]) and to introduce flanking SalI and XbaI restriction sites (underlined). The resulting amplicon, containing the H1 promoter-based shRNA-expression cassette, was inserted into the SalI-XbaI-digested pENTR/U6 (invitrogen) plasmid backbone in between the att recombination sites, yielding vector pENTR/H1. To generate pENTR/H1shPTPN13-1 and pENTR/H1shPTPN13-2 appropriate oligonucleotide heteroduplexes (of 5’-gatcccagccagcaaacactgtttaatcagagatttaacagtggttttgcttggg-3’ with 5’-agcttttccaaaaaagacccaaacactgtttaatcagagatttaacagtggttttgcttggg-3’) were ligated into the BglII and HindIII digested pENTR/H1 vector. Subsequently, Gateway-type re-
combination reactions into pLenti6/BLOCK-it-DEST-TagRFP [6] were used to generate plasmids for production of lentiviruses containing shRNA as well as fluorescent marker protein expression cassettes. PTPRZ1-directed shRNA constructs and the non-targeting knockdown control (shSCR) have been described previously [6].

**Cell lines, culturing, transfection and lentivirus production:**

HEK 293FT cells were purchased from Invitrogen. The xenograft-derived glioblastoma cell model E98 has been described elsewhere [13]. Cells were cultured in high glucose Dulbecco’s Modified Eagle Medium (Life Technologies; cat.no.11960-044) supplemented with 10% Fetal Bovine Serum (PAA laboratories; Cat.no. A15-101), 4 mM glutamine and 1 mM pyruvate (Life Technologies), at 37 °C in a humidified incubator under 7.5% CO2. Expression plasmids were transfected into HEK293FT cells using JetPRIME reagent (Westburg), according to the manufacturers’ instructions. Generation of lentiviruses has been described previously [6]. Briefly, lentiviral plasmids with supporting packaging plasmids were transfected into HEK293FT cells using JetPRIME reagent and medium was replaced after 8 hours. After 48 hours, medium containing lentivirus was harvested for infection of E98 cells.

For BioID experiments, cells were incubated for 16 hours with 50 μM Biotin (Sigma #B4501) in the culture medium. Afterwards, excess biotin was removed by washing cells three times for five minutes with with biotin-free medium.

**Affinity-purification and immunoblotting:**

Cell lysates for GFP- or HA-tagged immunoprecipitation were prepared using Sucrose buffer (10 mM Tris-HCl, pH 7.5; 2 mM MgCl2; 3 mM CaCl2; 300 mM Sucrose; 1 mM PMSF; 2 mM Na3VO4; 10 mM NaP2O7; complete protease inhibitor cocktail, Roche, #13006200 (1 tablet in 1.5 mL MQ, 20 μl solution per mL lysis buffer)) or MPER (30 mM Tris-HCl, pH 7.5; 25 mM Bicine buffer, pH 7.5; 50 mM NaCl; and protease and phosphatase inhibitors as described above and 0.1% CHAPS). Cells were scraped in ice-cold buffer and passed through a 0.5 μm needle to shear the cells. Lysates were incubated on ice for 30 min with regular mixing, after which they were centrifuged at 20,000 g at 4°C for 10 min. Supernatant was collected and stored at -20 oC until use. For immunoprecipitation of GFP-tagged fusion protein-containing complexes, anti-GFP antibody-coated magnetic beads were used (chromotek GFP-Trap®_MA). For immunoprecipitation of HA-tagged fusion proteins and their associated proteins, 30 μl ProtG sepharose beads (GE Healthcare, #17-0618-01) that were pre-loaded with anti-HA antibody (12CA5) [17] were used. All beads were incubated with cleared lysates either overnight (sepharose beads) at 4°C, or at RT for one hour (GFP magnetic beads). Beads with bound proteins were washed five times with lysis buffer and finally transferred into a new tube.

For BioID experiments the cells were scraped in Triton buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% Triton X-100 (Serva); and protease- and phosphatase inhibitors as described above). Lysates were incubated on ice for 30 min and mixed regularly, after which they were centrifuged at 20,000 g at 4oC for 10 min. Supernatant was collected and the pellet discarded. For pulldown of biotinylated proteins,
MyOne Streptavidin T1 Dynabeads (Thermo scientific) were incubated with lysate at RT for one hour and further processed as described above. Affinity purification was performed as described [50]. Biotinylated protein-containing beads were washed five times with PBS. Subsequent on-bead digestion, in-gel digestion and mass-spectrometric analysis was performed as described [55].

**Immunoblotting:**

Protein lysates and immunoprecipitates were processed by adding 2x SDS sample buffer (60 mM Tris-HCl, pH 6.8; 2% SDS; 100 mM dithiothreitol; 0.001% bromophenol blue; 10% glycerol) and heating the lysates at 95 °C for 5 min. Proteins were size-separated using SDS-PAGE on 8% PAA gels according to standard protocols. In case of BioID experiments, commercial 4-12% gradient gels (BOLT Thermo fisher scientific #NW04122) were used according to manufacturer’s instruction.

Gels were electro-blotted onto PVDF membrane (Immobilon-FL, #IPFL00010). Membranes were blocked using 5% BSA in PBST (PBS; 0.1% Tween-20 (Sigma-Aldrich)) for 1 hr at RT, and subsequently incubated with primary antibodies or streptavidin-IR-Dye800 in 5% BSA-containing PBST, overnight at 4 °C. Membranes were then washed three times for 10 min in PBST and either stored until imaged (in the case of conjugated streptavidin-IR-Dye800) or subsequently incubated with the appropriate secondary antibodies for 1 hr at RT in the dark. Immuno-fluorescent signals were visualized using the Odyssey imaging system (LI-COR Biosciences) and images were analyzed using Image Studio Light (LI-COR Biosciences).

Antibodies used were as follows: mouse anti-PTPRZ (1:1000; BD Biosciences, #610179), rabbit anti-VSV (1:20.000; LifeSpan Biosciences), mouse anti-tubulin (1:5000; DSHB, University of Iowa, E7), rabbit anti-GAPDH (1:5000; Cell Signaling Technology #2118), mouse anti-GFP (1:1000; Santa Cruz, J051), rabbit anti-TagRFP (1:500; Evrogen, AB233), mouse anti-PSD95 (1:5000; NeuroMab), rabbit anti-PTPN13 (1:1000; Santa Cruz, H300), rabbit anti-Myc (1:1000; Santa-Cruz #sc789), rabbit anti-HA (12CA5) [17], and rabbit anti-LYAR (1:1000; Invitrogen #PA5-44683) antibodies. As secondary antibodies, IRDye 680- or 800-conjugated goat anti-mouse or goat anti-rabbit antibodies (1:20,000; LI-COR Bioscience) were used. IRDye 800-conjugated streptavidin (1:1000; LI-COR Biosciences) were used to visualize biotinylated proteins.

**Proliferation assay:**

E98 cells were transduced with shRNA-expressing lentiviruses according to the protocol described in [6] and grown to 60-80% confluency on collagen I-coated (10 µg/cm²; Invitrogen) coverslips. Cells were then incubated for 1 hr in culture medium containing 10 µM EdU (5-ethyl-2’-deoxyuridine), incorporation of which was visualized on fixed cells using the click-iT® EdU Imaging kit (Thermo Fisher Scientific, #C10086) following the manufacturers’ instruction. Coverslips were mounted on microscope slides in DAPI-containing Mowiol (Sigma-Aldrich) and images were collected using a 20x objective on a Leica DMi6000B Fluorescence microscope that was equipped with a motorized x-y scanning stage and Leica EL6000 illumination.
source. DAPI- and EdU-positive nuclei were counted automatically using FIJI software [53].

Results

Association of the PTPRZ-B C-terminus with PDZ domain-containing proteins

Our previous work revealed a signaling role for the C-terminal PDZ binding motif in PTPRZ-B in the regulation of glioma cell proliferation. Disrupting this SLV sequence motif by fusion with a VSV-G epitope sequence eliminated its effects on cell proliferation [6]. Here we set out to study how the structure and domain topology of the entire PTPRZ-B intracellular segment (further referred to as RZi) is involved in regulating proliferation. Therefore, to identify proteins that are capable of interacting and thus might have a role in the observed signaling, we constructed a series of vectors encoding tagged variants of the RZi segment (Fig. 1A) and used these in subsequent affinity purification experiments. Addition of an N-terminal GFP tag to RZi would enable purification of stable protein complexes on GFP-Trap beads, whereas use of an N-terminal Myc-BirA* tag that catalyzes the promiscuous biotinylation of vicinal proteins in the cell (a method known as Bio-ID [50]) should enable their purification over streptavidin beads. Both types of RZi fusion proteins were properly expressed in HEK 293FT cells, as revealed by immunoblot analyses, also when PTPRZ-Bs PDZ binding motif was altered by C-terminally adding the VSV-G epitope tag (Fig. 1B,C).

First, to verify that addition of the VSV tag to the SLV sequence motif in RZi indeed prohibits interactions with PDZ domain proteins, we co-transfected HEK 293FT cells with pcDNA3-type expression plasmids encoding the two GFP-RZi variants and RFP-tagged PSD95 (PSD95-tagRFP) or VSV-tagged MAGI1 (MAGI1-VSV), two known PTPRZ-B interactors [22, 31]. Using anti-GFP-coated magnetic beads, these interactors were both efficiently co-purified with RZi. When GFP-RZi-VSV was used as a bait no interaction was detectable (Fig 2A and B), corroborating previous observations.

Screening for interactors of the PTPRZ-B intracellular part using BioID

The mutant biotin ligase BirA* biotinylates lysines in any protein within a radius of 10nm [32]. To identify RZi-mediated interactions in a proper physiological setting, we chose to explore this BioID approach in glioma cells. Prior to the in-cell vicinity labeling approach we verified whether expression of N-terminal BirA* fusions of RZi indeed results in biotinylation of known interactors. To this end, HEK 293FT cells were co-transfected with expression plasmids for PSD95 or MAGI1, and either BirA*, BirA*-RZi or BirA*-RZi-VSV. After addition of biotin to the cells and subsequent lysis, protein biotinylation was analyzed following SDS-PAGE and blotting. Indeed PSD95 and MAGI1 were found to be preferentially biotinylated by BirA*-RZi as compared to BirA*-RZi-VSV (Fig 3A,B). This demonstrates that BirA* addition renders a functional bait which can even biotinylate interactors that bind at the PDZ-binding motif at the other end of RZi, but only when this C-terminal motif is freely available and not blocked by the VSV-tag. Biotinylation of RZi partners that bind elsewhere, not at sites located an entire length span of the protein away from the BirA domain, should thus also be possible.
As a next step, E98 glioblastoma cells were lentivirally transduced with expression constructs for BirA*, BirA*-RZi or BirA*-RZi-VSV. Stably transduced cell lines were exposed overnight to biotin, lysed and proteins in the lysates were subjected to affinity purification using streptavidin magnetic beads. Captured proteins were then sub-
mass spectrometric (Supplementary Fig. S1 and Table S1) and immunoblot (Fig. 3C,D) analyses. The different BirA* variants were all properly expressed and active, as witnessed by the biotin incorporation in most notably the recombinant protein itself and by visualization of the Myc-tag on the myc-BirA*-tagged proteins (Fig. 3C). Mass spectrometric analysis, however, revealed overall low peptide counts. Only a single protein, called LYAR, was found enriched in the fraction that originated from BirA*-RZi expressing cells, compared to that from BirA*-RZi-VSV transduced cells (Supplementary Fig. S1). This zinc finger protein could, however, not be detected in the streptavidin-purified sample on immunoblot (Fig. 3D). Likewise, endogenous PSD95 – that might have served as appropriate control – was also not appreciably biotinylated (Fig. 3D), leaving the evidence for interaction inconclusive and the nature of glioblastoma partners for domains of the intracellular segment of PTPRZ-B as yet enigmatic.

**PDZ-domain containing protein PTPN13 binds to the PTPRZ-B C-terminus**

The PTPRZ-B C-terminal SLV amino acid sequence represents a canonical class I PDZ domain binding site (-S/TxVCOOH)[4]. PSD95 and MAGI1 not only bind to PTPRZ-B but in fact to multiple proteins carrying class I C-terminal sequences, including the glioma tumor suppressor PTEN [54]. PTPRZ-Bs’ RZi segment may thus well represent a bait for other proteins that bind class I PDZ domain targets.

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**Figure 2: Verification of intracellular interaction between the C-terminal PDZ binding motif of PTPRZ and known partners PSD95 and MAGI1**

A. GFP-containing protein complexes were affinity-purified (IP) from lysates of HEK 293FT cells expressing tRFP-PSD95 and GFP-RZi variants using GFP-antibody-coupled magnetic beads and analyzed on Western blots (IB). Presence of co-purifying partner proteins in input cell lysates before and after immune-capture (IP) was probed by Western blot analysis using anti-PSD95. VSV serum was used to visualize GFP-RZi-VSV variant. Migration positions of full-length and cleaved PSD95 are indicated to the right of the upper pane in kDa. B. Similarly, whole cell lysates of HEK293FT cells expressing MAGI1-VSV in presence or absence of GFP-RZi and GFP-RZi-VSV fusion proteins were analyzed. Western blot pictures of products in the input lysates before, and purified proteins after capture on GFP-immune magnetic-beads stained by anti-VSV and anti-GFP antisera are shown. Migration positions of MAGI1-VSV and GFP-RZi-VSV (both stained with anti-VSV) are shown to the right of the upper panel in kDa.
PTPN13 as a candidate anchoring protein in the PTPRZ-B interactome in glioma

Figure 3: Identification of binding partners of intracellular PTPRZ using BioID

Biotinylated proteins in whole cell lysates of HEK293FT cells transfected with an expression vector encoding PSD95-tagRFP (panel A) or MAGI1-VSV (panel B) and different cDNA3 plasmids encoding BirA* and BirA* fusion proteins size-separated on a gradient gel and analyzed on Western blots by staining with conjugated streptavidin (Strept.). Migration positions of biotinylated input proteins are indicated to the left of both panels. C. E98 cells were transduced with lentiviruses encoding BirA* control or BirA-RZi (+ or -VSV) fusion proteins as indicated and exposed to medium with or without biotin before lysis. Proteins in these lysates were size-separated on a gradient gel and analyzed by Western blotting and staining with conjugated streptavidin or with (IB) anti-c-Myc, anti-PTPRZ or anti-VSV antibodies, respectively. D. Lysates of E98 cells used in C. were harvested in large quantities and used for Western blot and mass-spectrometry analyses. Proteins stained with anti-PSD95 and anti-LYAR sera in samples of the whole
ing this line of reasoning, we identified PTPN13, a submembranous PTP harboring five PDZ domains, as a protein that binds to class I PDZ binding motifs [4]. In particular PTPN13’s implication in glioblastoma cell survival [19] triggered our interest and made us investigate whether PTPN13 could also be a candidate PTPRZ-B interacting protein. Indeed, PTPN13 was efficiently co-precipitated using GFP-RZi as a bait in transfected HEK293FT lysates and this did not occur if GFP-RZi-VSV was used (Fig. 4A). Vice versa, upon pull-down of HA-tagged human PTPN13 using anti-HA-coated beads, GFP-RZi but not GFP-RZi-VSV was efficiently co-purified (Fig. 4B). Collectively, these findings put forward PTPN13 as a potential PTPRZ-B interactor.

Membrane localization is crucial for PTPRZ-B effects on glioblastoma proliferation
In a previous study, we found that the reduction of E98 cell proliferation due to PTPRZ1 knockdown could only be reversed by re-expression of shRNA-resistant full-length PTPRZ-B but not when its PDZ binding motif was blocked [6]. To test whether the truncated intracellular segment of the protein (RZi) is also capable of rescuing the proliferative impairment of E98 cells with reduced PTPRZ1 expression, cells were transduced with two different lentiviruses; one for shRNA mediating knockdown of endogenous PTPRZ-B production and the other for expressing the shRNA resistant GFP-tagged RZi variants (Fig. 5A). One to two days later, the proliferation capacity of the resulting E98 cells was determined using a 1h EdU incorporation as a readout (Fig. 5B). Knockdown of PTPRZ1 reduced E98 cell proliferation by a factor of two and this effect could not be rescued or partially reverted by expression of GFP-RZi, GFP-RZi-VSV, or by the GFP moiety as a control. Since expression of full length PTPRZ does rescue proliferation in PTPRZ-knock down cells [6], this shows that not only availability of a functional PDZ binding domain, but also proper membrane anchoring of the intracellular segment in PTPRZ-B is required to facilitate its effects on glioma proliferation. The above findings are in line with existence of a submembranous interaction niche for the PTPRZ-B C-terminus, a subcellular compartment that is also harboring PTPN13 (see e.g. [5, 15]).

PTPN13 knockdown impinges on proliferation and viability of glioblastoma cells
Based on the fact that transmembrane PTPRZ-B regulates E98 cell proliferation and that this role may possibly be exerted in conjunction with the submembranous protein PTPN13, we also wished to address the role of this latter PTP in these cells. To this end we lentivirally transduced the glioblastoma cells with shRNA expression constructs targeting PTPN13 (shPTPN13-1 and shPTPN13-2) or with a non-targeting control (shSCR). Both PTPN13-targeted shRNAs caused a firm reduction of PTPN13 protein levels and had profound effects on E98 cell survival (Fig. 6A-B). EdU incorporation revealed a significant reduction of the percentage of S-phase cells upon shPTPN13-2 expression (Fig. 6C). For shPTPN13-1 this was not evident but the outcome may have been obscured by the reduction in the amount of cells as
measured by nuclear coverage (Fig. 6B). Strikingly, upon PTPN13 knockdown with either shRNA the E98 glioblastoma cells tended to fuse, resulting in the production of giant multi-nucleated cells. Application of shPTPN13-2 resulted in a more modest phenotype, with cells containing up to 10 nuclei, whereas shPTPN13-1 expressing cells occasionally contained over 50 nuclei (Fig. 6D-E). Time-lapse recordings (data not shown) revealed that most multi-nucleated cells die shortly after fusion, thus explaining the dramatic reduction in cell numbers. The ones that did survive, however, formed giant multinucleated cells. Importantly, E98 cells that received the non-targeting control shRNA displayed no such cell fusion events (Fig. 6D-E).

**Discussion**

We have previously shown that the intracellular part of PTPRZ-B impinges on glioblastoma cell proliferation [6]. In the current study, we searched for relevant partner proteins that bind to PTPRZ-B intracellular domains using immunoprecipitation and proximity labeling strategies. PDZ domain-mediated binding to RZi was detected for known (PSD95 and MAGI1) as well as predicted (PTPN13) interaction partners when using ectopic expression systems and dedicated lysis buffers. BioID in-cell vicinity labeling of endogenous interaction partners in E98 glioblastoma cells did, however, not reveal additional potential proteins, presumably because the RZi moiety lacks the proper guidance signals for transmembrane localization, and proper embedding in the cell membrane is required for its functional recognition. Intriguingly, like for PTPRZ-B the shRNA-mediated knockdown of *PTPN13* in E98 cells caused a reduction in cell proliferation. Additionally, PTPN13 knockdown caused extensive cell fusion and cell death, phenomena that are not observed upon PTPRZ-B knockdown.
The observation that PTPRZ-B knockdown affects E98 glioblastoma cell proliferation and that the intracellular part of PTPRZ-B is essential for this effect [6] points to involvement of interacting proteins, including the known PTPRZ interactors PSD95 and MAGI1. The bridge function of PSD95 and MAGI1 between PTPRZ and its targets (Erbb4 and β-catenin [21] [17, 41], combined with those targets having links

Figure 5: A cytosolic intracellular PTPRZ-B fragment is unable to rescue PTPRZ1 knockdown effects.

A. E98 cells were transduced with a knockdown construct targeting PTPRZ-B (shPTPRZ) or a non-targeting control (shSCR) as well as with expression constructs for GFP or GFP-RZι fusion variants. Lysates were prepared and analyzed on immunoblot (IB). Knockdown efficacy (indicated below the GAPDH image) was determined by ratioing endogenous PTPRZ-B (upper) and GAPDH (lower) immunosignals and normalizing relative to the shSCR control. Ectopic expression of GFP variants was visualized on blot using GFP antibody. Difference in GFP levels between the shSCR- and the shPTPRZ-expressing samples are due to the CMV promoter-controlled GFP marker in the shSCR construct versus tagRFP as reporter in the shPTPRZ constructs. Amount of GFP fusion products relative to endogenous PTPRZ was assessed using VSV and PTPRZ antibodies and depicted below the lower PTPRZ image. Size markers (in kDa) are on the right. Bracket indicates different migration speeds of PTPRZ due to post-translational modifications. B. Transduced E98 cells were grown on coverslips and labeled with EdU for 1 hr. Percentage of EdU-positive nuclei among DAPI-stained cells was determined (n=3). Error bars indicate SD, n.s. means not significant, * indicates p-value < 0.05.
Figure 6: PTPN13 knockdown results in reduced proliferation, survival and increased cell fusion in glioblastoma cells.

A. E98 cells were lentivirally transduced with different shRNAs against PTPN13 or a scrambled control. After three days cell lysates were analyzed on immunoblots using PTPN13 (upper) and tubulin (lower) antibodies to determine knockdown efficiency relative to that in shSCR control (depicted below the images). Sizes in kDa are depicted on the right. B. E98 cells were transduced as described under A. One week later cells were fixed and the cumulative nuclear area was measured by DAPI staining (n=3). C. E98 glioblastoma cells were lentivirally transduced with the indicated constructs and grown on coverslips before being labeled with EdU for 1 hr. Percentage of EdU-positive nuclei among DAPI-stained cells was determined (n=3). Error bars indicate SD and * indicates p-value < 0.05. D. E98 cells expressing the indicated shRNA constructs (as well as the linked tagRFP reporter) were grown on coverslips and the number of nuclei within one cell membrane as defined by tagRFP and DAPI fluorescence was determined and binned. E. Fluorescent images depicting cell coverage and cell fusion. Representative images are shown. Note that shSCR cells co-express a GFP reporter whereas shPTPN13 constructs carry the tagRFP reporter cassette.
with cancer (eg. [17, 56]), depicts an appealing mechanistic framework for explaining an analogous proliferation-stimulatory role for PTPRZ-B. PDZ domain-mediated interactions are notoriously detergent-sensitive and may not be the only mechanism involved in partner binding. Therefore we explored the proximity-dependent BioID strategy to identify additional binding partners for the intracellular segment of PTPRZ-B with impact on glioma cell proliferation. One should bear in mind that the promiscuous BirA biotin ligase mutant (BirA*) used in BioID experiments works only optimally within a relatively small confined space with ~10 nm radius. Moreover, it is able to intra-molecularly biotinylate itself and may dimerize [12], tendencies that might complicate proper subcellular localization of the bait and labeling of associating proteins. Also GFP tagging may affect a protein’s tendency to oligomerize [14]. Full-length PTPRZ-B is a type-I transmembrane receptor that becomes enzymatically inactive following ligand-induced dimerization [24, 40]. Any superimposed multimerization effect caused by tagging with BirA or GFP as used here may thus evoke abnormal sterical hindrance effects and thereby prevent binding and dephosphorylation of interacting substrates. We thus must realize that our strategy to disclose protein interactions by introducing N-terminal BirA* or GFP tags to the RZi segment may have affected its binding to interactors. Nevertheless, both GFP- and BirA*-tagged RZi specifically bound to known interactors in transfected HEK 293FT cells. Of note, the over-expressed partner proteins also encounter BirA* by chance, explaining background signals in control experiments and underscoring the need for physiological protein levels to obtain meaningful results.

Our current functional rescue experiment suggests that not only accessibility of the intracellular PTPRZ-B C-terminus is essential in supporting glioblastoma cell proliferation but that also proper membrane anchoring is important. Earlier we demonstrated that expressing only the ectodomain of PTPRZ-B cannot rescue glioblastoma proliferation upon PTPRZ1 knockdown [6], and here we now show that a cytosolic version of the PTPRZ-B intracellular domain is not sufficient either. Also the possibility that relevant endogenous partner proteins are only present in sufficient amounts in the submembranous area of the cell needs further attention. Indeed, preferred localization of PSD95 and MAGI1 complexes containing Erbb4 and/or β-catenin [10] in the vicinity of the membrane has already been demonstrated. Finally, also additional measures may be needed to make cells fully devoid of competing endogenous PTPRZ-B. Thus, to reveal interactions shRNA inhibition strategies as used here, or even complete CRISPR-Cas9-mediated knockout of the PTPRZ-B gene may be necessary.

Intriguingly, also the newly identified PTPRZ-B interactor PTPN13 is a submembranous protein. This non-receptor-type PTP with five PDZ domains is capable of binding a multitude of proteins that are instrumental in cell growth and differentiation [1]. For example, PTPN13 downregulates the cell surface expression of the FAS receptor, and thus the cells’ sensitivity to FAS-induced cell death [20], by binding to the FAS C-terminus via its second PDZ domain [4]. Since PTPRZ-B ends in the exact same amino acid sequence as FAS (SLV) we expect the PDZ2 domain of PTPN13 to mediate the interaction with its enzyme family member PTPRZ-B as well.
A new striking finding in this study was that PTPN13 knockdown in glioblastoma cells not only impacted on cell viability but also triggered cell fusion. Massive multinucleated cell bodies were formed within a few hours. The phenomenon was not observed upon PTPRZ-B knockdown. This points at an interesting effects of PTPN13 in cell fusion events. In line with this, Sardina and coworkers [52] reported increased numbers of polyploid cells following PTPN13 silencing in megakaryocyte differentiation, a phenotype also observed following β-catenin knock-down. Whether β-catenin is also mediating the PTPN13 silencing effect in our study and whether or not the PTPN13-related cell fusion phenomenon is key in the development of giant cell glioblastoma, a distinct tumor variant characterized by multinucleated giant cells with abundant cytoplasm [33], requires further study.

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References

PTPN13 as a candidate anchoring protein in the PTPRZ-B interactome in glioma


Supplementary figure 1: Mass spectrometry results. Vulcano plot comparing mass spectrometry result from RZi and RZi-VSV. The green section indicates the protein that was significantly enriched in the RZi fraction compared to the RZi-VSV fraction. The pink section indicates proteins significantly enriched in the RZi-VSV fraction compared to the RZi fraction.
PTPN13 as a candidate anchoring protein in the PTPRZ-B interactome in glioma
Chapter 6

General Discussion
**General Discussion**

Glioblastoma, the most malignant form of glioma, is a devastating brain tumor arising from glial cells or their precursors. After diagnosis patients with this type of tumor face an average life expectancy of only 15 months with treatment [77]. Therefore, new insights that ultimately result in improved median survival or better quality of life are desperately needed. At the molecular level much progress has been made and key mutations that deregulate signaling and metabolic pathways in glioma have been mapped [13]. Cellular processes such as proliferation, differentiation, migration, and survival are normally tightly controlled by multiple—often opposing—forces that collectively determine whether the pertinent cellular response is activating or inhibiting. Cell fate in tissue homeostasis and growth is mostly regulated by signal cascades involving protein tyrosine kinases (TKs) and their enzymatic counterparts, the protein tyrosine phosphatases (PTPs). The balance between activities of these two enzyme classes is pivotal in maintaining cell and tissue homeostasis.

In the research described in this thesis we have investigated the expression levels of PTP family members in glioma and have studied the significance of the TK c-MET and the PTP PTPRZ-B in particular. Presence of a mutated and amplified MET gene in patient-derived E98 glioblastoma cells [58] allowed us to test the efficacy of a TK inhibitory compound for c-MET and VEGFR2, both *in vitro* and *in vivo* (Chapter 2). Using glioma patient samples and online databases we were able to show that in general PTPs display low expression levels in gliomas as compared to healthy tissue and that levels decrease with increasing malignancy (Chapter 3). An exception is formed by the *PTPRZ1* gene which, alike many other oncogenes in tumors, is highly expressed in gliomas. Our *in vitro* and *in vivo* experiments revealed that the PTPRZ-B extracellular part contributes to the migratory phenotype whereas its intracellular domains are responsible for proliferative signals in glioma cells (Chapter 4). We subsequently launched an attempt to uncover the PTPRZ-B binding partners involved in specifying glioma’s typical features and found that the PDZ domain-containing PTPN13 is a potential candidate that regulates glioma cell survival (Chapter 5). Here I will present some intriguing findings regarding the glioblastoma cell model E98 that I gathered over the years and that may have bearing for future studies. Additionally, I will elaborate on the potential role of PTPs in gliomas, and new insights and treatment strategies that build on the reversibility of phosphotyrosine growth signals will be discussed.

**Cancer stem cell-like properties of E98 glioblastoma cells**

For many experiments described in this thesis the human patient-derived E98 glioblastoma cell model was used. Genetic characteristics of E98 cells, including an amplification and auto-activating mutation of MET as well as a deletion of PTEN, have been documented [20, 58]. E98 cells that are cultured as adherent cells under standard serum-containing conditions will give rise to compact tumors upon orthotopic injection in mice. Importantly, when cultured in neurobasal medium (essentially serum-free, but with selective growth factors added) E98 cells grow as neurospheres *in vitro* and display the clinically relevant diffuse migratory phenotype *in vivo* [20]. In-
triguingly, E98 cells display doubling times of 35-40 hours in vitro but appear highly proliferative in vivo [12, 20]. Upon in vitro labeling of cells in S-phase for an hour, using either BrdU or EdU, around 20% of the cells were found positive (Chapter 4). Still, half of the E98 cells entered the cell cycle, as visualized by Ki67 immunostaining or by using the live cell cycle reporter Fucci [67]. The latter was introduced in the E98 cells by lentiviral transduction of a home-made bicistronic construct encoding the ubiquitination-based cell cycle indicator components Cdt1-Kusabira-Orange2 and Geminin-Azami-Green1 that differentially label G1 and S/G2/M cells with orange and green fluorescence, respectively (Krause et al., manuscript in preparation). The remaining 50% of non-cycling cells appear senescent, as judged from senescence-associated beta-galactosidase staining (Fig. 1A). Whether this bifurcation in cell fate specification is due to artificial in vitro cultivation conditions, is currently not known (see also discussion below).

As mentioned earlier (Chapter 1), tumors are composed of a related but heterogeneous population of cancer cells [72] and ample studies have shown that their cells are able to undergo switches in functional state (e.g. [42, 62, 76]). Therefore we separated the cycling and the apparently senescent fractions of the population of E98 cells, based on the fluorescent Fucci reporter-positivity of these cells, prior to monitoring their behavior in proliferation and migration assays. Using the spheroid migration assay [12] the non-cycling cells demonstrated a migratory potential that sharply contrasted with the hardly moving proliferative cells (Figure 1C). Furthermore, when cultured for several passages, the Fucci-positive subset gradually reverted back to an E98 cell population with only half of the cells in cycle. Conversely, within the highly migratory non-cycling population an increasing number of E98 cells re-entered the cell cycle over time (Figure 1D). Apparently, E98 cells in culture switch between cycling and dormant states and consequently the non-cycling cells are not senescent or permanently terminally differentiated. A similar plasticity may be envisioned for the E98 cells when a switch is made from adherent to neurosphere conditions; only the latter regime allows the reproducible generation of diffusely outgrowing tumors upon orthotopic injection in mouse brains [12, 20, 57].

The above phenomena are reminiscent of the situation for stem cells in physiological conditions in that the majority is dormant and only a small fraction of cells, once activated when needed, will start cycling [47]. Also for tumors in vivo, the concept of stem cell-like cancer cells is appealing as it can explain why some cells are resistant to therapy and why tumor recurrence is inevitable in case of incomplete resection (e.g [76]). Progeny of stem cell-like cancer cells accumulate independent mutations and undergo clonal selection, leaving some subclones to become dominant inside the tumor and/or its metastases. Selection due to radio- or pharmaco-therapy may eliminate some populations but other subclones may prove resistant to the therapy and will prevail [11]. Importantly, because of their semi-dormant state especially the stem-cell like cancer cells display a different, usually reduced, sensitivity towards conventional cancer treatments. Also in glioblastoma a small population of tumor cells displays stem cell-like properties as seen in adult neural stem cells. These so-called glioma stem cells, or glioma initiating cells, show expression of stemness
markers such as Nestin, Bmi-1, Olig2 and Sox2 [24]. The identification of such tumor-founding cells is important and discoveries of ways to target them specifically may hold great promise to reduce tumor recurrence and increase the overall patient survival.

Fig 1: E98 shows some characteristics of stem cell-like cancer cells.

A) E98 cells, grown as neurospheres in neurobasal medium (left) or adherent in serum-containing medium (right), were stained for senescence-associated β-Galactosidase (Promo Kine #PK-CA577-K320). B) E98 cells, lentivirally transduced with a bicistronic version of the Fucci cell cycle reporter, were FACS sorted to separate GFP- (green) or mOrange-positive (red), cycling (Fucci+ sorted) cells from non-cycling (Fucci-sorted) cells. Subsequently, the cells were grown as spheroids, using the hanging drop method, and seeded in matrigel-coated 96 wells plates. Spheroids were cultured for another 24 hours, after which the cells were fixed, counterstained with DAPI (purple) and microscopically examined to monitor migration from the spheroid. Representative images are shown. C) FACS-sorted Fucci- and Fucci+ populations were cultured on glass slides in serum-containing medium and imaged at day 1 and day 24 after sorting. Pictures represent an overlay of mOrange (red) and brightfield images taken at the indicated time points.
Stem cell-like properties of cells overexpressing c-MET

In glioblastoma, recurrent tumors often display a mesenchymal profile [78]. This could mean that the original tumor already contained a minority of cells with a mesenchymal signature. These cells may be inherently resistant to therapy and have the ability to expand and create a recurrence. Such a recurrence may happen even if the predominant clones before were classical, proneural or neural [11]. Interestingly, c-MET is a ‘signature gene’ for glioblastoma’s specifically those with a mesenchymal signature [78] and cells thereof with upregulated c-MET levels retain a stem cell-like phenotype upon isolation and culture [7, 41, 49]. Remaining low c-MET-expressing cells from the same tumor do not show these stem cell-like properties [41]. Furthermore, when ‘high c-MET’ cells are cultured in serum-containing differentiation medium, c-MET is downregulated [7], suggestive of a link between c-MET levels and stem cell characteristics in glioma cells. As mentioned above, cancer stem cells have been found to be more resistant to therapy because of their relative dormancy [19]. This bears clinical relevance since radiotherapy leads to upregulation of c-MET expression through the ATM/NF-κB pathway [6]. Furthermore, pharmacological inhibition of c-MET sensitizes cells to radiotherapy [41]. Thus, two possible models on how c-MET levels may correlate with radioresistance may be postulated. Either subclones that are already high in c-MET and better resistant to radiotherapy will become more dominant following radiotherapy and will give rise to the recurrence, or low-c-MET subclones upregulate c-MET in order to become resistant (reviewed in [11]).

Amplification or overexpression of c-MET is often associated with loss of the tumor suppressor PTEN [11]. PTEN-loss may contribute to the invasive phenotype observed with glioma cells overexpressing c-MET as both c-MET and PTEN have effects on the PI3K pathway (e.g. [17, 60]). Where c-MET has an activating role leading to cell survival, PTEN has an inhibitory role in the PI3K pathway. Overexpression of c-MET in a PTEN deleted/ mutated cell might further the damage done by over-activation of the PI3K pathway. The reverse is also true, c-MET amplified cells which undergo PTEN deletion might have a growth advantage because the PI3K pathway is further activated. Simultaneous inhibition of c-MET and inhibition of mTOR (which is normally inhibited by PTEN), resulted in greater reduction of migration and proliferation than either compound alone [48], indeed suggesting a link between PTEN and c-MET downstream effectors.

Interaction of c-MET and PTPRZ

Another PTP which c-MET may interact with is PTPRZ. In chapter 4 we show that PTPRZ has an effect on c-MET phosphorylation. We found that knock-down of PTPRZ-B levels in glioblastoma cells resulted in reduced cell migration and a reduction of phospho-MET levels. Reintroducing PTPRZ-B’s extracellular domain into these cells normalized migration behavior as well as increased MET phosphotyrosine levels [12]. How the two proteins interact is currently unclear. There is no evidence that c-MET is a direct substrate for PTPRZ-B and in fact the catalytic domain of PTPRZ was not required for restoration of phospho-MET levels in our rescue experiment. Furthermore, PTPRZ-B knock-down should rather result in an increase, and
not a decrease, in phosphotyrosine levels of its substrates. Thus it is likely that the connection involves other protein intermediates. Interestingly, PTPRZ has recently been implicated as a marker of stem cell-like glioma tumor cells, in a similar way as c-MET [32]. Authors found that paracrine stimulation of these cells with macrophage-derived-pleiotrophin via PTPRZ played a crucial role in their maintenance [70]. Furthermore, pharmacological inhibition of PTPRZ or its family member PTPRG, resulted in a reduction in stemness markers [32]. Thus there may be interplay between c-MET and PTPRZ in the poising of stemness. Studying their interaction in the context of stem-cell like tumor cells, may yield insight that will become beneficial in combatting these hard to target cells.

To determine whether PTPRZ and c-MET may participate in the same signaling complex on the cell surface one could turn to proximity ligation assays [26]. In this technique antibodies directed against two proteins of interest are exploited to bring conjugated oligonucleotides in close enough proximity to allow rolling circle amplification and subsequent detection. One may also probe via immune-pull down and complex purification whether the kinase-phosphatase pair is part of the same complex, but own experience (Chapter 5) showed that the success of such experiments is critically dependent on the composition of lysis and purification buffers. Ideally, one would like to also identify the unknown additional interactors in such a c-MET / PTPRZ-B assembly. Tandem affinity purification followed by mass spectrometry (TAP-MS) then comes to mind. In this technique bait proteins are tagged twice to enable sequential purification steps of prey interactors thereby reducing background signals [43]. Again, like with immunoprecipitation, the challenge remains to keep the protein complex intact during lysis and purification steps. Two recent methods now circumvent this problem by making use of the enzyme APEX2 or BirA*. Both are engineered enzymes that generate highly reactive biotin-phenoxyl and biotinoyl-5-AMP radicals, respectively [46, 66]. The covalent coupling of these radicals to vicinal proteins allows for later purification using high-affinity avidin-based beads. Both methods require the expression of a fusion product of the protein of interest with the APEX2 or BirA* enzyme. As is evident from the experiments in chapters 4 and 5, inclusion of such labeling tags in itself could hamper the binding of partner proteins to the complex. For example, our addition of the VSV epitope tag to the PTPRZ-B C-terminus, at that time for immunoprecipitation purposes, presumably blocked the binding of PDZ domain-containing proteins required to affect the proliferation of glioblastoma cells. Also the distance between the APEX2 or BirA tag and the actual site of interaction – and thus bait topology – may be a factor that determines success with this methodology for interactor protein identification.

The interaction between c-MET and PTPRZ-B does not only occur at the protein level. In about 10% of glioblastoma’s, due to translocation events involving introns 3 or 8 of PTPRZ1 and intron 1 of MET, there is even a covalent link between PTPRZ and c-MET. The resulting fusion transcripts encode primary proteins that consist of various lengths of the extracellular domain of PTPRZ-B followed by the entire c-MET open reading frame [9, 18]. The fusion proteins contain only a small part of the extracellular domain of PTPRZ-B and, due to proteolytical processing during intracellular
transport, the mature protein on the cell surface ends up being identical to c-MET [18]. Thus in view of the short stretch of PTP-derived amino acids sequence in the premature fusion protein, PTPRZ-B binding partners will have no bearing for ‘fused c-MET’ regulation. However, during their journey in the cell the various PTPRZ-c-MET fusions appear less well protected from HGF-independent activation and consequently display overall elevated phosphotyrosine levels. Furthermore, the promoter switch from MET’s own promoter to that of PTPRZ1 considerably upregulates the expression level of c-MET [18]. Indeed, this is compliant with our finding that, PTPRZ1 is strongly expressed in glial tumors as compared to normal tissue (Chapter 3). This combination of enhanced expression and increased ligand-independent phosphorylation of c-MET, induced by the fusion to PTPRZ1, may well explain the oncogenicity of the translocation event. It seems appealing, therefore, to specifically target the fusion transcripts using therapeutic oligonucleotides, as proposed for treatments of neurodegenerative diseases like Huntington’s Disease and Amyotrophic Lateral Sclerosis [23].

**Interactions of PTPRZ-B with PDZ-containing proteins.**

We studied the role of PTPRZ-B in glioblastoma proliferation and migration and noted that its extracellular part, possibly involving c-MET, is mediating migratory signals whereas the proliferative effects are provided by the intracellular part (Chapter 4). More specifically, the PTPRZ-B C-terminal PDZ binding motif appeared crucial for the latter process and thus a collection of potential binding partners and interacting proteins deserves further discussion (Fig.2)

Postsynaptic density protein 95 (PSD95) is part of the membrane-associated guanylate kinases family (MAGUK) and contains three PDZ domains, an SH3 domain and a guanylate kinase domain. First discovered in yeast two hybrid screens, it is the second PDZ domain of PSD95 that binds to the PTPRZ-B C-terminus [44]. Using either its first or second PDZ domain PSD95 can also bind to ErbB4. Thus, PSD95 can simultaneously bind both ErbB4 and PTPRZ-B, facilitating the dephosphorylation of ErbB4 by PTPRZ-B, but it can also facilitate ErbB4 homo-dimerization and subsequent auto-activation [29]. PDZ domain-containing proteins thus may regulate phosphotyrosine-mediated signals through the organization of RTK/RPTP clusters. It also implies that in cell systems, overexpression of components of such a regulatory cluster will not be without consequence. High amounts of PTPRZ-B, as in glioblastoma cells, could cause a decrease in ErbB4 phosphorylation in two ways. Both sequestering PSD95 and preventing PDZ-mediated ErbB4-ErbB4 homodimer formation as well as direct dephosphorylation of ErbB4 could be involved. Like its family member ErbB1 (EGFR), ErbB4 uses PI3K and Ras signaling pathways. However, whereas EGFR leads to increased proliferation and survival, the prolonged activation by ErbB4 leads to an inhibitory effect of these pathways on proliferation, survival and differentiation in brain maintenance and development (reviewed in [40]). It is tempting to speculate that the high PTPRZ-B levels in glioma hamper this ErbB4-mediated inhibition of proliferation as part of their tumor-promoting effect.
Both PTPRZ-B and ErbB4 can also bind to MAGI2, and possibly MAGI1 and MAGI3, in a similar manner as to PSD95 [16]. MAGI proteins also belong to the MAGUK family of PDZ domain-containing submembranous anchoring proteins, and a MAGI/ErbB4/PTPRZ-B complex may serve similar purposes as described above for PSD95. Alternatively, PSD95 and MAGI members could compete for binding to ErbB4 and PTPRZ-B, and direct them to specific subcellular niches to exert their signaling effects. In addition, MAGI1 represents a PTPRZ-B substrate as it is dephosphorylated on Y373 and Y858 following binding [30]. MAGI3 is not a substrate itself but being a
scaffolding protein it can provide PTPRZ-B access to substrates like the phosphoprotein p130 in glioma cells [2]. Likewise, MAGI1 might facilitate the dephosphorylation of β-catenin by PTPRZ-B [56] by using its fifth PDZ domain for β-catenin and PDZ domain 2 for PTPRZ-B anchoring [22, 33]. Of note, β-catenin levels are often upregulated in cancers and result in the transcription of proliferation-associated genes [52]. Moreover, tyrosine-phosphorylated β-catenin increases Wnt signaling and tumorigenesis. Since an increase in PTPRZ-B would be expected to result in more dephosphorylated β-catenin in the presence of abundant MAGI1, halting β-catenin nuclear activation and stabilizing cadherin-mediated cell adhesions, we reason that the MAGI-dependent link with β-catenin does not represent PTPRZ-B’s tumorigenic contribution in glioma.

**PTEN and PTPRZ-B share a large part of their PDZ-mediated interactome.**

Interestingly, the C-terminus of PTEN is bound by many of the same PDZ domain-containing proteins as those that bind to PTPRZ-B, including PSD95, MAGI1 and PTPN13 [71] (fig.2). Recently, a large bioinformatics screen using yeast and human interaction databases led to the suggestion of thousands of potential synthetic lethal gene pairs (meaning inactivation of one gene will not kill the cell because the other will compensate, but inhibiting both genes will kill the cell) and the combination PTPRZ1 - PTEN was present in their top 20% [74]. This triggered us to perform a pilot experiment using the LN229 PTEN wildtype and PTEN deficient isogenic cell lines that were generated using CRIPSR/Cas9 technology (Chapter 3). Upon PTPRZ-B knock-down (using the lentiviral shRNA constructs described in Chapter 4) the PTEN proficient cells demonstrated a mild reduction in survival and growth, but cells that lack PTEN showed a drastic decrease in proliferation and survival. This is in line with a synthetic lethal interaction of PTEN and PTPRZ1 in these glioblastoma cells. Determining which processes PTPRZ-B and PTEN have in common will be the next step in elucidating a potential therapeutic target. Use of information about shared interactions such as those between partners and at the above mentioned PDZ-containing proteins will form a solid basis for the start of such an endeavor. Furthermore, conditional knock-out of PTPRZ using CRISPR/Cas9 experiments will be needed to test this hypothesis accurately in E98 cells, as we have only achieved a relatively mild knockdown of PTPRZ (50-80%) in these cells so far.

Although PTEN and PTPRZ1 are cancer modulating genes in their own right, the above hypothesis sheds a new light on the occurrence of PTEN deletion and PTPRZ-B upregulation in glioma specimens. Since PTEN and PTPRZ-B at least in part share their PDZ domain-containing interactome, the altered expression of either one of them will modify the hardwiring of the other and thereby change underlying signaling pathways. Hence, the interaction with their PDZ domain-containing partners has a distinct consequence for the two PTPs. PTEN recruitment to the cell membrane by means of PDZ domain-mediated anchoring allows a more efficient dephosphorylation of phospholipids, both by localizing and stabilizing PTEN but also by increasing its enzymatic activity (reviewed in [55]). PTPRZ-B on the other hand already resides at the cell membrane, and it has no such impact on the PI3K path-
way, although it can affect AKT through FYN [70]. Determining which processes are affected by both proteins and which are not, could therefore provide new therapeutic insight and is worth investigating.

PTPN13 regulates PTPRZ-B dependent and independent pathways in glioblastoma. The list of protein interactors that bind PTPRZ-B [34] will undoubtedly grow in the coming years. Interactions listed in databases like BioGrid, String and InAct currently add up to 138 interactors. We found experimental evidence for a novel interaction with the large non-transmembrane protein PTPN13, also a protein tyrosine phosphatase. PTPN13 consists of a KIND domain, a FERM domain, five PDZ domains and finally the protein tyrosine phosphatase catalytic domain. This handful of PDZ domains enable PTPN13 to bind a wide range of protein C-terminus [27], among these the PDZ binding motif of FAS that ends with the sequence ‘SLV’ [68], the same as in PTPRZ-B (Chapter 5). PTPN13 is contributing to the endocytosis and degradation of FAS, also known as the death receptor. High cell surface levels of FAS makes cells sensitive to apoptosis signals mediated by the FAS ligand. Reduction of FAS levels, by PTPN13-triggered internalization, increases cell resistance to apoptosis [68]. Furthermore, PTPN13 is subject to autophagy. Cells within a population with high autophagy degrade PTPN13 which sensitizes cells to FAS induced apoptosis, thereby selectively determining cell-fate [35]. Interestingly, we observed high levels of cell death in glioblastoma cells upon PTPN13 shRNA-mediated knockdown (Chapter 5). Intriguingly, the PTPN13 knock-down in E98 glioblastoma cells had a similar effect as for PTPRZ-B knockdown, namely a reduction in proliferation, however, there were also distinct differences. For one, PTPRZ-B knockdown did impair migration whereas knockdown of PTPN13 showed a trend towards increased migration (data not shown). Moreover, PTPN13 knockdown resulted in massive cell death and cell fusion (Chapter 5), which is not seen in E98 cells upon depletion of PTPRZ-B (Chapter 4). Thus, PTPN13 impinges on glioma formation and growth also in PTPRZ-B-unrelated ways. Investigating these PTPRZ-coupled and PTPRZ-independent processes and determining the autophagic potential of the different cells within the E98 population might elucidate new pathways and entry points for treatment.

PTPN13 indeed has been recognized as a player in the cancer field, displaying both oncogenic as well as tumor suppressive functions, depending on the cellular context of its activity and on the available partners [27]. First I will describe evidence for a tumor suppressor function for PTPN13 and then turn to studies that highlight its tumor-promoting contribution. In breast cancer cells PTPN13 was found to dephosphorylate IRS-1. This leads to reduced phospho-Akt levels, hence to inhibition of the PI3K pathway, making cells more vulnerable for apoptosis (reviewed in [28]). Furthermore, lower PTPN13 levels, due to either increased protein degradation, promoter hypermethylation or reduced mRNA levels, have been associated with a more tumorigenic character of the specimens, implying PTPN13 as a tumor suppressor [36, 73, 82]. In line with this, mutations in PTPN13 have been found in several carcinomas [39, 65, 79]. PTPN13 down-regulation by miR-26a has also been implicated as a reason for EGFR inhibitor resistance.
Tumorigenic functions for PTPN13 have also been suggested. For instance, PTPN13 mediates the degradation of the PI3K p85β regulatory subunit, thereby preventing the inhibition of PI3K by excess free p85β through competition with p85-p110 dimers for IRS1 binding (reviewed in [28]). Furthermore, in Ewing’s sarcoma a chromosomal translocation results in expression of an aberrant transcription factor, EWS-FLI1, which strongly up-regulates PTPN13 protein levels, thereby boosting cell growth and motility [1]. Finally, as mentioned before, PTPN13 controls FAS cell surface levels in several cancer types and thus facilitates their resistant to apoptosis (reviewed [28]). Combined, these findings support the importance of PTPN13 in cancer [80].

We can conclude that the role of PTPN13 is complex. The specific localisation and function of PTPN13 will depend on its many binding partners (reviewed in [27]). There is one additional aspect I would like to address here: our reproducible finding that PTPN13 knockdown in E98 cells causes glioblastoma cell fusion (Chapter 5). The amount of cells that fuse varies within and between experiments. Small cells with only a few nuclei are observed but also cells that contain over a hundred nuclei were observed after PTPN13 knockdown. The speed at which the fusions occur rule out that the underlying mechanism reflects a defect in cytokinesis, reminiscent of the findings of Erdmann et al.[38]. Instead, we see intriguing parallels with a glioblastoma subtype that displays giant cells containing multiple nuclei [61]. In the WHO classification this giant cell glioblastoma is a variant of especially IDH WT glioblastoma [51]. This variant is rare, making extensive studies difficult, but it is worthwhile to have a close look at PTPN13 levels in the various glioblastoma subtypes. Formation of multinucleated giant cells also happens during osteoclast formation, where it is dependent on PI3K-activated proteins that drive the necessary cytoskeletal remodeling [59]. As mentioned above, PTPN13 has an effect on PI3K activity and PTPN13 knockdown could therefore affect PI3K-dependent cell fusion. Furthermore, PTPN13 has been linked to actin dynamics and thus could facilitate fusion via cytoskeletal adaptations (reviewed in [27]). E98 cells are PTEN-deficient, so it would be interesting to subject PTEN-wildtype cells to PTPN13 knockdown experiments and monitor fusion events. It also remains to be seen whether its role in cell fusion can be pinpointed to one of its interaction domains and/or requires the PTP’s catalytic activity.

**PTPs as treatment targets**

Based on the comparison of relative expression levels in normal and neoplastic brain tissue and expression and survival data from relevant databases, we identified a number of PTPs (Chapter 3) that could serve as possible entry points for glioma treatment. However, whereas many small compounds and biologicals exist that act on tyrosine kinases, only very few (ant)agonists have been established for PTPs [75]. We found that many PTPs were downregulated in glioma specimens (Chapter 3) and increasing either their activity or amount thus seems an appealing treatment strategy. Unfortunately, such an intervention is much more difficult than inhibiting enzyme activity. Knowledge on transcriptional regulatory mechanisms that act on PTP genes is not at an applicable level yet, and details on how to boost PTP enzymatic activity is sparse, hard to modulate or limited to a few members only [75].
For example, redox modulation, allosteric regulation, and protein oligomerization are among these regulatory mechanisms. The latter two strategies touch upon aspects that will be discussed below since these reflect PDZ domain-mediated interactions and receptor-type PTP dimerization, respectively. Also, the pharmaceutical inhibition of PTP activity is difficult to achieve, because of the highly conserved PTP catalytic domain structure [37].

PTPRT was found to be significantly down-regulated in glioma specimens and upon re-expression in vitro a reduction of proliferation was obtained (Chapter 3). PTPRT is part of the R2B receptor-type PTP family that also comprises PTPRM, PTPRU, and PTPRK. All four members are single-pass transmembrane proteins that are post-translationally cleaved, resulting in their extracellular domain being shed. This shed portion can then bind again to the rest of the molecule at a different site [21]. Also, PTPRM was significantly downregulated in glioma samples as compared to normal brain tissue [14] but for both PTPRU and PTPRK the trend towards down-regulation did not meet our criteria (Chapter 3). The involvement of the R2B RPTP subfamily as tumor suppressors in diverse cancer types is well documented [21]. For glioma, notably PTPRK mutations that associate with clinical outcome have been reported, and reconstitution of PTPRK in glioma cells resulted in reduced proliferation and migration and an improved response to therapy [3, 4]. Since β-catenin and EGFR are PTPRK substrates, PTPRK re-expression lowers EGFR and β-catenin phosphotyrosine content, hence improves sensitivity to EGFR inhibition and reduces β-catenin expression levels [3]. Assuming all R2B family members act in a similar way, our findings on PTPRT and PTPRM relevance in glioma suggest that a combined use of Temozolomide and EGFR inhibitors might prove fruitful when tumors carry dysfunctional R2B PTPs.

A cautionary note should be issued, however, as endogenous, smaller isoforms of PTPRM and PTPRU were found to act tumorigenic in glioma samples, even when full-length proteins were expressed at reduced levels [15] [83]. Similar results were found in gastric cancer [50]. Apparently, nuclear-localized isoforms act as dominant-negative by sequestering β-catenin and preventing its dephosphorylation by full-length PTPRM and PTPRU. In line, tumor-associated mutations in PTPRK not only affect its phosphatase activity but also lead to abnormal proteolytic processing [3]. Therefore it is important to understand in more detail how proteolytic processing affects the activity of R2B receptors before considering compounds that block their cleavage in tumor treatment.

**PTPRZ-B as a drug target**

Several attempts have been made to utilize PTPRZ-B, a major contributor to glioma tumorigenicity, as a therapeutic target. The most successful, at least from a development point of view, is a peptide derived from PTPRZ-B’s extracellular moiety that forms part of a peptide-based glioma vaccine called IMA950 [64]. Currently this vaccine is in phase I/II clinical trials. Historically, the first preclinical attempt to target overexpressed PTPRZ on glioma cells in vivo dates back to 2006 when a saporin-coupled antibody that targets the PTPRZ-B extracellular domain was found
to significantly delay human U87 glioma tumor growth in a subcutaneous mouse xenograft model [25]. The most recent endeavor involved the development and testing of SCB4830, a small inhibitory compound that specifically targets the PTPRZ-B catalytic site [31]. Here, C6 glioblastoma cells had been transplanted into rat brains and the inhibitor was applied as a liposome complex by means of daily intracerebroventricular injections to find a reduction in tumor growth.

We reported that the extracellular and intracellular domains of PTPRZ-B regulate different aspects of glioblastoma behavior (Chapter 4), indicating that combining a therapeutic antibody, which targets the extracellular domain, with an inhibitor for the intracellular catalytic domain may have synergistic effects. However, in our set-up we also found that it is not so much the catalytic activity of PTPRZ-B that is important for glioma cell proliferation but rather the protein interactions mediated by its C-terminus [12]. Perhaps we missed a contribution of PTPRZ-B enzyme activity in our knockdown rescue experiments because PTPRZ-B knockdown was incomplete. PTPRZ-B homodimerizes upon ligand binding, leading to inactivity of the catalytic domain due to sterical hindrance [33, 54]. It is also conceivable that PTPRZ-B forms inactive dimers when at high concentrations, as in glioma specimens. In such a scenario, PTPRZ-B knockdown would actually increase its activity. Likewise, re-expression of a catalytically inactive mutant may rescue by heterodimerizing with endogenous PTPRZ-B, as it still can bind ligands and can sterically inhibit the activity of the wildtype partner. In sharp contrast, a PTPRZ-B variant that was blocked in its C-terminal binding domain was not able to rescue E98 proliferation impairment that resulted from PTPRZ-B knockdown. But perhaps both enzymatic activity and C-terminal protein interactions govern the same processes. After all, PTPRZ-B will only dephosphorylate substrates when at high concentrations when at high concentrations, as in glioma specimens. In such a scenario, PTPRZ-B knockdown would actually increase its activity. Likewise, re-expression of a catalytically inactive mutant may rescue by heterodimerizing with endogenous PTPRZ-B, as it still can bind ligands and can sterically inhibit the activity of the wildtype partner. In sharp contrast, a PTPRZ-B variant that was blocked in its C-terminal binding domain was not able to rescue E98 proliferation impairment that resulted from PTPRZ-B knockdown. But perhaps both enzymatic activity and C-terminal protein interactions govern the same processes. After all, PTPRZ-B will only dephosphorylate substrates that come within close range and this may well require binding of the enzyme and the substrates for PSD95 or MAGI1. Therefore, compounds that also block PDZ domain binding sites may have added value in glioma treatment regimens as well.

**C-terminal binding motifs as a potential targeting mechanism for treatment.**

PTPRZ-B is not the only PTP, and certainly not the only transmembrane protein, that is bound by a specific C-terminal binding motif. Submembranous orchestration of protein complexes using C-terminal binding domains may also serve to regulate substrate specificity for the catalytically very efficient PTP family [10]. Especially the sequence conservation within the C-terminal tails among RPTP orthologs underscores their importance in regulating PTP function. Multiple PTPs whose expression was found to be significantly altered in gliomas and correlated with survival (PTPRZ, PTEN, PTPRT), contain a PDZ binding motif [10]. Further investigation is thus needed to map the relevant interactions with PDZ domain-containing proteins and to develop interaction-specific intervention molecules. Some compounds that specifically block PDZ interactions have been discovered, and these might prove beneficial in treating glioma. For instance, the peptide that blocks PDZ1 and PDZ2 of PSD95 [8] may disrupt the interaction with PTPRZ-B and affect glioblastoma cell proliferation. As mentioned before, PTPRZ-B and PSD95 can interact with many proteins in a...
PDZ-dependent way, including PTEN, posing an enormous puzzle on how to keep the actions of such protein-protein interaction-disrupting compounds both targeted and effective. Recently, a compound that prevents the syntenin PDZ domain from binding targets was shown to increase radiosensitivity and decrease growth of gliomas *in vivo* [45]. Because of the conservation of PDZ domains and their interacting PDZ binding motifs, this compound may block more than only syntenin-mediated interactions. Potentially it could even disrupt interactions that involve PTPRZ-B.

Further proof for the potential validity of PDZ domain-mediated interactions as therapeutic targets in glioma comes from study of two additional PTPs: PTPN13 and PTPN4. A small molecule that blocks the afore-mentioned association of PTPN13 and FAS is currently under development to treat idiopathic pulmonary fibrosis (patent pending: US Patent Application 20120148528; published international patent WO/2012/064763). Such a small molecule would sensitize cells to apoptosis and might have future applications in glioma as well. Furthermore, a peptide targeting PTPN4’s PDZ domain has shown to be effective in inducing cell death in a glioblastoma cell model *in vitro* [5]. Targeting the enzymatic activity of PTPN4 can be achieved using a PDZ-blocking compound [53], giving us additional options for targeting the enzymatic activity of PTP’s.

**Concluding remarks.**

In this thesis I presented evidence that many PTPs (with the exception of PTPRZ-B) are expressed at much lower levels in gliomas than in healthy tissue, in line with an anti-tumor role. PTPs, currently underrepresented as potential therapeutic targets, thus deserve further study despite their stigma of being difficult to block pharmacologically. We argue that, apart from initiatives to specifically target the catalytic PTP domains, strategies that aim at protein interactions mediated by these enzymes, notably PDZ domain-based interactions, provide appealing routes to modify PTP functioning. Currently little is known about the PTP targets that we have identified, such as MTMR7, DUSP26 and PTPRN2, in glioma (Chapter 3). The effect of DUSP26 on tumorigenicity is documented [63, 69, 81] but the underlying mechanism still needs to be disclosed. For PTPRT and PTPRM compounds that block proteolysis could prove beneficial, and drugs that block interactions with the C-terminus of PTPRZ-B likely affect glioblastoma proliferation. Interestingly, also PTPRT contains a C-terminal PDZ binding motif that, in the light of our PTPRZ-B findings, is worth investigating. Understanding the interplay between TK’s and PTP’s is crucial to design combination therapies with kinase inhibitors, PTP (ant)agonists and/or protein-protein interaction-blocking compounds that ultimately may combat diffuse gliomas effectively.

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Thesis appendices

Thesis Summary in English
Nederlandse samenvatting
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Thesis Summary in English

Patients diagnosed with glioblastoma that undergo standard treatment currently have a very low five-year survival rate of only 5%. Novel therapeutic modalities for this devastating type of brain cancer are therefore urgently needed. Unfortunately, our current knowledge about the development, growth and cellular homeostasis of brain tissue and the transformation of brain cells into cancer is still rather limited. It is therefore important to study the mechanisms that govern these processes in more detail before new avenues for treatment can be opened up.

In this thesis, I present new findings regarding the role of one of the key cellular regulatory mechanisms, protein tyrosine phosphorylation, in diffuse glioma. Phospho-tyrosine content of cellular proteins is determined by the balanced action of protein tyrosine kinases (TKs) on the one hand and – their counterparts – protein tyrosine phosphatases (PTPs) on the other. Thus far the role of PTPs in brain tumorigenesis has not received much attention in the literature despite the fact that these proteins can both function as oncogenic drivers as well as tumor suppressors, depending on the cellular context in which they exert their activity.

In chapter 2 we investigated new ways to reduce the abnormally high level of phosphorylation by tyrosine kinase c-MET in glioma cells. We used the new compound cabozantinib to inhibit the kinase activity of both VEGFR and c-MET and observed that this resulted in reduced migration and proliferation of glioblastoma cells in vitro and led to a reduction in tumor load and an increase in survival in mouse models for this brain tumor.

Apart from the effects of chemical phosphorylation inhibitors in specific growth regulatory pathways we also examined the potential role of PTPs in regulating tumorigenic pathways in a more broader sense, by assessing the expression levels of the majority of PTP family members in glioma samples. In chapter 3 we describe how we explored the overall relation between PTP expression and the malignancy grade of glioma tumors. We identified several PTPs which are downregulated in gliomas and whose expression level correlates with patient survival data. Two of the most noticeable candidates in the PTP family are DUSP26 and PTPRT. Low expression of these PTPs correlated with reduced survival. Upon increasing the expression of either one of these two PTPs in a glioblastoma cell model, we observed a reduction in proliferation and migration of these tumor cells, in line with a tumor suppressive role for these PTPs. Furthermore, we confirmed the results of other studies, and showed PTPRZ1 expression to be high in glioma, specifically in oligodendroglioma, tumor samples.

We next focused on PTPRZ1 as a possible oncogene in glioma and studied its mechanism of action using a clinically relevant glioma cell model that recapitulates the diffuse infiltrative character of the tumor. In chapter 4 we describe how different domains of PTPRZ-B have distinct roles in glioma diffuse migration and proliferation signaling, as inferred from studies with truncated proteins. We discovered that shRNA-mediated knockdown of PTPRZ-B resulted in a reduction of proliferation as well as migration of E98 tumor cells. Re-expression of the extracellular domain of PTPRZ-B was able to rescue the migratory phenotype, but not the effects that knockdown of endogenous PTPRZ-B has on proliferation. In contrast, re-expression of the full-length wild type protein or a catalytic dead PTPRZ-B variant fully rescued both knockdown effects. Only when the PDZ-binding domain at the PTPRZ-B C-terminus was blocked by a VSV-epitope tag, the full-length protein lost the ability to rescue the reduced proliferation phenotype. Thus, the intracellular moiety of PTPRZ-B, in particular the PDZ-binding target at its 3’ terminal end, is involved in the regulation of cell proliferation.
In chapter 5 we continued this line of research and searched for interactors of the intracellular domains of PTPRZ-B that regulate glioma proliferation. We describe how the entire PTPRZ-B intracellular domain was used in unbiased (i.e. with BIO-ID methodology) and targeted (i.e. with co-immunoprecipitation technology) binding studies. We uncovered a new PTPRZ-B interactor, PTPN13, also a member of the large family of PTPs. PTPN13 is highly expressed in glioblastomas and shRNA-mediated reduction of its expression strongly impaired survival of tumor cells \textit{in vitro}. Interestingly, knockdown of PTPN13 triggered the fusion of glioblastoma cells into giant multinucleated cells. We further obtained indirect evidence that transmembrane anchoring of the PTPRZ-B intracellular domain might be important for proper regulation of its biological activity, because the cytosolic intracellular domain did not rescue cellular proliferation in a similar manner as the full-length, membrane-anchored proteins.

Lastly, in chapter 6 I discuss the implications of the findings presented in this thesis and give an outlook on new research lines to further elucidate the role that PTPs have in the etiology of gliomas. Focus is on a few specific themes, i.e. on the links of PTPRZ1 and MET with stem cell-like characteristics, the role of the PDZ binding domain of PTPRZ-B, and on the function of PTEN as an central node in regulating PDZ-associated aspects of tumor cell behavior. Also possible ways in which PTPs may be used as druggable targets for combating glioblastoma are described. The new insights in the complex biology of phosphotyrosine signaling in glioblastoma growth and migration may ultimately lead to the development of new treatment opportunities for these devastating tumors.
Proefschrift samenvatting in het Nederlands

Patiënten met de diagnose glioblastoom die standaard behandeling krijgen hebben helaas nog steeds een zeer lage vijf-jaars overlevingskans (slechts zo’n 5%). Nieuwe therapieën voor deze verwoestende tumoren zijn daarom dringend nodig. Helaas is onze huidige kennis op het gebied van ontwikkelen, groei en cellulaire homeostase van hersenweefsel en het ontstaan van kanker niet toereikend. Om nieuwe, meer effectieve therapieën te ontwikkelen is het nodig om de mechanismen die deze processen aansturen in meer detail te bestuderen.

In dit proefschrift laat ik nieuwe bevindingen zien met betrekking tot de rol van één van de belangrijke cellulaire processen, fosforylering van eiwitten, in diffuse gliomen. Dit proces wordt bepaald door de balans tussen de activiteit van proteïne tyrosine kinases (TKs) enerzijds en de activiteit van proteïne tyrosine fosfatases (PTPs; protein tyrosine phosphatases) anderzijds. Tot op heden is de rol van PTPs in de tumorvorming in hersenen nog sterk onderbelicht gebleven in de wetenschappelijke literatuur. Begrijpen hoe deze eiwitten hun rol uitoefenen is belangrijk omdat ze zowel tumorgroei kunnen stimuleren als hinderen, afhankelijk van de context van de cel.

In hoofdstuk 2 beschrijven we een nieuwe aanpak om het abnormale niveau van fosforylering door de tyrosine kinase c-MET in glioblastoom-cellen te normaliseren. Een nieuw experimenteel medicijn, cabozantinib, werd gebruikt om zowel de kinase-activiteit van c-MET als van VEGFR te remmen. Dit resulteerde in een vermindering van migratie en groei van glioblastoom-cellen in vitro en een reductie van de grootte van tumoren in muizen, met een verbetering in hun overleving als gevolg.

Behalve naar het effect van chemische blokkering van fosforylering, hebben we ook gekeken naar de potentiële rol van PTPs in het reguleren van tumorigene processen in bredere zin. Hiervoor hebben we de expressie van het merendeel van de PTP-eiwitfamilieleden bepaald in gliombiopten. In hoofdstuk 3 beschrijven we hoe we hebben gekeken naar de globale relatie tussen PTP-expressie en glioom-gradatie. We hebben een aantal PTPs geïdentificeerd die een lagere expressie hebben in gliomen en wiens expressie correleert met de overleving van patiënten. Twee in het oog springende kandidaten uit de PTP-familie zijn DUSP26 en PTPRT. Lage expressie van deze twee PTPs correleerde met een vermindere patiënt-overleving. Toen we de expressie van deze twee PTPs afzonderlijk omhoog brachten in een celmodel voor glioblastoom, nam de groei en migratie van deze tumorcellen af. Dit wijst op een tumor-onderdrukkende werking van deze PTPs. Verder zagen wij ook de reeds eerder beschreven sterk verhoogde expressie van PTPRZ1 in gliomen, met name in oligodendrogliomen.

Vervolgens hebben we ons gericht op PTPRZ1 als mogelijk tumorgen in diffuse gliomen. Om het werkingsmechanisme van producten van dit gen te ontrafelen gebruikten we een glioma celmodel dat het typerende diffuse infiltratieve groeidrag van gliomas goed weerspiegelt. In hoofdstuk 4 beschrijven we dat verschillende domeinen uit het PTPRZ-B eiwit een eigen rol hebben in migratie en groeisignaling van E98 tumor cellen. Verlaging van de endogene PTPRZ-B niveaus in E98 cellen zorgde voor een vermindere migratie en afname van celgroei. Re-expressie van het extracellulaire deel van PTPRZ-B in deze cellen leidde tot een herstel van het migratie-fenotype, maar hielp niet om de effecten op proliferatie te compenseren. Her-expressie van het hele natieve eiwit of een katalytisch dode variant daarvan normaliseerde daargenevens zowel de effecten op migratie als ook op proliferatie. Alleen wanneer het zogenaamde PDZ-bindingsdomein aan de C-terminale kant van PTPRZ-B geblokkeerd was door plaatsing van een extra stukje eiwit, een VSV-epitoom, werd het positieve effect van intacte PTPRZ-B op proliferatie weer teniet gedaan. Wij concluderen hieruit dat het extracellulaire deel van PTPRZ-B, meer specifiek
het PDZ-bindingsmotief op het 3’ einde daarvan, een rol heeft in de regulering van celgroei.

In hoofdstuk 5 gaan we hierop verder en beschrijven we hoe gezocht is naar partner-eiwitten welke interactie vertonen met de intracellulaire domeinen uit PTPRZ-B, d.w.z. het deel van het eiwit dat de glioom proliferatie aanstuurt. We gebruikten daartoe nieuwe hypothesevrije methodes zoals BIO-ID of gerichte methodes zoals, immuunprecipitatie om bindende eiwitten te identificeren. Daarbij vonden we een nieuwe PTPRZ-interactiepartner, PTPN13, een eiwit dat ook zelf lid is van de grote familie van PTPs. PTPN13 komt in relatief hoge concentraties voor in glioblastomas en shRNA-remming van expressie van dit eiwit had een sterk remmend effect op overleving van tumorcellen in in vitro kweek. Een interessante waarneming was dat remming van expressie van PTPN13 leidde tot fusie van glioblastoom cellen, waarbij gigantisch grote multinucleaire cellen worden gevormd. Additioneel, verkregen we ook indirecte aanwijzingen dat membraanbinding van het intracellulaire domein van PTPRZ-B belangrijk is voor regulatie van de biologische activiteit. Wanneer het eiwit los in het cytosol voorkomt mist het het vermogen om de celproliferatie te stimuleren wat het volledige membraangebonden eiwit wel heeft.

Tot slot, in hoofdstuk 6, bediscussieer ik de vindingen die in dit proefschrift beschreven staan. Ook geef ik een vooruitblik op vervolgonderzoek dat nodig is om de rol van PTPs in de etiologie van gliomen nog beter te doorgronden. Ik richt me daarbij op een paar specifieke themas, zoals de band die er lijkt te bestaan tussen PTPRZ1 en MET genexpressie en karakteristieken van tumorstamcellen, de rol van het PDZ-domein van PTPRZ-B, en de centrale regulerende rol van PTEN in het door PDZ-domeinen gestuurde gedrag van tumorcellen. Ook worden opties beschreven voor het gebruik van PTPs als doel-eiwitten voor de behandeling van glioblastomen met medicijnen. Nieuwe inzicht in fosfotyrosinesignaleringsmechanismen en hun betekenis voor groei en migratie van glioblastoom tumorcellen zal uiteindelijk bijdragen tot de ontwikkeling van nieuwe vormen van therapie voor deze hoogst aggresieve vorm van kanker.
List of Publications


# RIMLS Portfolio

**Name PhD student:** AM Bourgonje  
**Department:** Cell Biology  
**Research School:** Radboud Institute for Molecular Life Sciences  
**PhD period:** 01-04-2012 – 11-09-2016  
**Promotor(s):** Prof. B. Wieringa, Prof P. Wesseling  
**Co-promotor(s):** Dr. WJAJ Hendriks, Dr. WP Leenders

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## TRAINING ACTIVITIES

### a) Courses & Workshops
- RIMLS Graduate Course: 2012, 2 ECTS  
- Artikel 9 course: 2012, 3.5 ECTS  
- Academic Writing: 2014, 3 ECTS  
- Fiji course: 2014, 1.75 ECTS  
- Meet the experts in Amsterdam: 2014, 0.2 ECTS

### b) Seminars & lectures
- RIMLS Technical Forums: 2012-2016, 1.3 ECTS  
- RIMLS in the Spotlight: 2012-2013, 1.2 ECTS  
- Seminars: 2012-2016, 5.3 ECTS  
- Lecture Series: 2012-2014, 0.5 ECTS  
- Radboud Research Rounds: 2015-2016, 0.4 ECTS  
- Minisymposium Glioma: 2015, 0.2 ECTS

### c) (Inter)national Symposia & congresses
- New Frontiers Symposium: 2012-2016, 4 ECTS  
- LWNO wetenschapsdag: 2012, 0.25 ECTS  
- RIMLS PhD retreat*: 2012-2015, 3 ECTS  
- FASEB protein phosphatase meeting*: 2014, 1.75 ECTS  
- 2nd international Symposium on Clinical and Basic Investigation in Glioblastoma*: 2015, 1.5 ECTS

### d) Other
- Organizing Technical Forum: Imaging: 2015, 0.5 ECTS

## TEACHING ACTIVITIES

### e) Lecturing

### f) Students
- PT01 supervising practicum: 2012-2015, 0.9 ECTS  
- Supervising Literature thesis: 2015, 0.5 ECTS  
- Supervising students: 2013-2015, 7.5 ECTS

**Total:** 39.25 ECTS

* poster presentation given at the events.
Curriculum Vitae

Anna Maria (Annika) Bourgonje was born on October 11th 1987 in Epe, where she also grew up. Ever since she saw Spiderman on cartoon network as a little girl, where he got his powers by accident using a neogenic recombinator, she was intrigued by DNA. Furthermore, Annika was always fascinated by human behavior and what influences this. She attended high school at the RSG-Noord-Oost Veluwe and graduated with the profile Nature and Health with advanced chemistry and physics. After high school she contemplated becoming a doctor and going into psychiatry to study human behavior, but fate brought her to the bachelor’s degree program in biology instead. At the University of Groningen she majored in Behavioral and Neuro-sciences. To further understand different aspects of behavior and the basis of thought, she minored in Artificial Intelligence. After getting her Bachelor degree she applied to the research master Behavioral and Cognitive Neurosciences major and was accepted into the track of Molecular and Clinical Neurosciences, where she combined her interest in DNA with her interest in the human brain in two internships. The first was at the department of Genetics where she studied the effect of genetic background on the aggregation behavior of huntingtin protein, causing Huntington’s disease. For her second internship she moved to Berkeley, California to investigate aspects of emotional and facial recognition. Her trip to Berkeley did confirm that she felt most comfortable in the lab working with cells and therefore - after graduation from her Masters at the University of Groningen in 2011- she applied for a PhD position in which she could integrate her passion for genes and brain in a lab setting. That is how she ended up at the department of Cell Biology at the Radboudumc, working on a research project under supervision of dr. Wiljan Hendriks. On the first of April 2012 she started her PhD project of which the results are presented in this thesis. Her work entailed many aspects of tumorigenicity of brain tumors and in particular she studied the effect of the protein tyrosine phosphatase class of enzymes on the tumorigenic behavior of glioblastoma cells. In pursuit of these results she learned many new techniques including RNA expression determinations, microscopic techniques, statistical methods and handling of large data sets. During the course of her Ph.D. trajectory she attended multiple courses given by the Graduate school associated with the Radboud institute for Molecular Life Sciences (RIMLS) as well as several symposia (details can be found in the portfolio provided in this thesis). Since June 2017 Annika is working as a data analyst at ValueCare, a company that analysis large data sets from mental health care institutions.
Dankwoord

'It takes a village to raise a child'. Dat gezegde kan ook zeker toegepast worden op een PhD. Dit manuscript is dan ook niet alleen door mij tot stand gekomen. Hieronder wil ik graag mijn ‘village’ bedanken voor het ondersteunen bij het werk wat in dit manuscript beschreven staat.

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