Functional discovery of targetable dependencies in recurrent glioblastoma

Sheila Singh (ssingh@mcmaster.ca)
  McMaster University  https://orcid.org/0000-0003-1272-5300

Chirayu Chokshi
  McMaster University

David Tieu
  University of Toronto

Kevin Brown
  Donnelly Centre and Department of Molecular Genetics  https://orcid.org/0000-0002-5514-2538

Chitra Venugopal
  McMaster McMaster University, Stem Cell and Cancer Research Institute

Martin Rossotti
  National Research Council

Katherine Chan
  University of Toronto

Amy Tong
  University of Toronto

Laura Kuhlmann
  Princess Margaret Cancer Centre

Lina Liu
  McMaster University

Benjamin Brakel
  McMaster University

Vaseem Shaikh
  McMaster University

William Maich
  McMaster University

Yujin Suk
  McMaster University

Daniel Mobilio
  McMaster University

Neil Savage
  McMaster University

Nikoo Aghaei
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Abstract

Resistance to genotoxic therapies and tumor recurrence are hallmarks of glioblastoma (GBM), an aggressive brain tumor. Here, we explore the functional drivers of post-treatment recurrent GBM. By conducting genome-wide CRISPR-Cas9 screens in patient-derived GBM models, we uncover distinct genetic dependencies in recurrent tumor cells that were absent in their patient-matched primary predecessors, accompanied by increased mutational burden and differential transcript and protein expression. These analyses map a multilayered genetic response to drive tumor recurrence, identifying protein tyrosine phosphatase 4A2 (PTP4A2) as a novel modulator of self-renewal, proliferation and tumorigenicity at GBM recurrence. Mechanistically, genetic perturbation or small molecule inhibition of PTP4A2 represses axon guidance activity through a dephosphorylation axis with roundabout guidance receptor 1 (ROBO1), exploiting a functional dependency on ROBO signaling. Importantly, engineered anti-ROBO1 single-domain antibodies also mimic the effects of PTP4A2 inhibition. We conclude that functional reprogramming drives tumorigenicity and dependence on a multi-targetable PTP4A2-ROBO1 signaling axis at GBM recurrence.

Full Text

For decades, clinicians have administered radiation therapy and chemotherapy to treat cancer patients. In parallel, resistance to these genotoxic treatments and tumor recurrence have become an inevitable reality for aggressive tumors. However, despite the clinical relevance and applications, functional drivers of disease recurrence remain poorly understood. Glioblastoma (GBM) remains the most aggressive and prevalent malignant primary brain tumor in adults. Unchanged since 2005, standard of care (SoC) consists of surgical resection, followed by radiation therapy (RT) plus concurrent and adjuvant chemotherapy with temozolomide (TMZ). Despite these therapeutic efforts, patients inevitably succumb to recurrent disease with a median overall survival of 14.6 months and a five-year survival rate of 5.5-6.8%.

Unbiased genome-wide functional genomic screens have provided insights into genes and pathways regulating tumor cell survival, invasion, and sensitivity to TMZ in primary pre-treatment tumor cells. However, these studies do not examine changes at post-treatment tumor recurrence, and thus cannot explain treatment failure in ~70% of GBM patients. Here, we conduct a genome-scale comparison between patient-matched pre- and post-treatment GBM cells at the functional, transcriptomic, and proteomic levels. We uncover a therapeutic vulnerability for protein tyrosine phosphatase 4A2 (PTP4A2) at tumor recurrence, and introduce a modulatory role for PTP4A2 on axonal guidance proteins.

Comparing primary and recurrent GBM

We derived a pair of patient-matched GBM cell lines, one from a tumor specimen obtained at initial diagnosis prior to chemoradiotherapy (BT594, primary tumor cells), and a second specimen obtained at first disease recurrence post-therapy (BT972, recurrent tumor cells) (Figure 1A, Table S1). Consistent with previous observations, recurrent tumor cells showed a 25-fold increase in in vitro self-renewal capacity (P = 5.0e-09) and a 5-fold decrease in survival of xenografted mice (n = 5, P = 0.002), as
compared to patient-matched primary tumor cells (Figures 1B and 1C). To explain enhanced stemness and tumorigenicity, we profiled the genomic, transcriptomic and proteomic changes at recurrence. Of an average of 15,444 canonical genomic variants per model, 2,019 variants were predicted to have high impact on protein function (e.g. frameshift variants) or classified as deleterious to protein function, collectively henceforth referred to as mutations (Figures 1D and 1E; Table S2). Strikingly, tumor cells presented with 1,599 de novo mutations at recurrence, accounting for 79% of the overall mutational burden, including coding mutations in MSH6, ARID1A, PTEN, TP53, EGFR and RB1. These putative driver mutations emerging at recurrence likely represent subclonal therapy-driven or stochastic events, in keeping with a previous observation where 77% of subclones were exclusive to recurrent GBM specimens compared to their primary tumor predecessor\(^\text{13}\). In comparison, only 186 mutations were exclusive to primary tumor cells while 234 mutations were shared.

Recurrent tumor cells presented with de novo mutations impacting TP53 activity (including TP53 and CREBBP), chromatin organization (including ARID1A, KMT2C and KMT2D), and PI3K/AKT signaling (including PTEN, EGFR, MTOR, TSC2, IRS2, RICTOR and FOXO1), among other processes. In keeping with the largest longitudinal analysis that showed transcriptional changes in 63% of GBM patients at recurrence\(^\text{14}\), genomic events observed in recurrent tumor cells were accompanied by widespread differential expression at the transcript (n = 1,747 genes) and protein (n = 181 proteins) levels (|LFC| > 2, adjusted \(P < 0.05\)), most notably indicating a shift in subtype from predominantly classical in primary tumor cell populations to a mesenchymal subtype at recurrence (Figure 1F; Tables S3 and S4). These data are consistent with the view that the root cause and result of therapy failure in GBM is an accumulation of driver mutations at recurrence that drastically reconfigure cellular processes and increase tumorigenicity.

**Functional remodelling at recurrence**

To understand how mutational patterns relate to functional dependencies, we conducted pooled, genome-wide CRISPR-Cas9 screens in patient-matched primary (BT594) and recurrent (BT972) GBM models using the TKOv3 library\(^\text{15}\) (Figures S1A-S1C; Table S5). After excluding core essential genes (CEGs)\(^\text{15,16}\), 1,090 fitness genes were exclusive to primary tumor cells, 995 genes specific to recurrent tumor cells and 1,172 were required in both (FDR < 0.05) (Figure S1D; Table S6). Given that ~2/3 of the detectable genetic dependencies are different between patient-matched primary and recurrent models, we examined the extent to which in vitro treatment of primary tumor cells with SoC recapitulates dependencies observed at recurrence. To systematically identify genetic determinants of treatment resistance, we conducted CRISPR-Cas9 loss-of-function screens in treatment-naïve primary tumor cells treated with RT and/or TMZ. Applying the drugZ algorithm\(^\text{17}\), we measured differential effects in recurrent tumor cells or drug-treated primary tumor cells (RT and/or TMZ) compared to untreated primary tumor cells (Tables S5 and S7). Comparison of the normalized Z scores, with and without filtering for significant genes (FDR < 0.05 in at least one screen), revealed weak to moderate positive correlation among recurrent tumor cells as compared to drug-treated primary tumor cells (Figure 2A; \(R = 0.38–0.50\)). Therefore, treatment-specific conditional genetic interactions in drug-treated primary tumor cells do not completely recapitulate
differential dependencies at recurrence. Rather, these data suggest that treatment-evasive tumor cells continue to acquire novel dependencies post-treatment and into disease recurrence.

Focusing on the functional differences at recurrence, we identified 406 genes that confer increased (n = 229) or decreased (n = 177) fitness upon knockout in recurrent tumor cells as compared to primary tumor cells (FDR < 0.5; Figure 2B; Table S8). GO enrichment analysis of these fitness genes highlighted remodelling of cell cycle and signaling processes at recurrence (FDR < 0.026), including greater dependence on cyclin-dependent kinases (CDK2, CDK4, CCND1), loss of p53-dependent DNA damage response (TP53), and increased reliance on the 26S proteasome (PSMD11, PSMD13) (Figure 2C; Table S4). In addition, while primary tumor cells show strong dependence on Fanconi Anemia pathway genes, this dependence is lost at recurrence (FDR < 0.002; FANCA, FANCB, FANCC, FANCD2, FANCF). Combined with p53 loss, this likely sets the stage for increased accumulation of genomic mutations in recurrent tumors. Along with increased dependence on nucleotide metabolism and glycolysis, these findings are indicative of a shift towards a more proliferative state, with increased proteotoxic stress and loss of key DNA repair pathways in recurrent GBM.

To identify additional genetic vulnerabilities unique to recurrent GBM, we performed a CRISPR-Cas9 knockout screen in a second patient-derived recurrent tumor model, BT241 (Figure S1E-S1G; Tables S5 and S6). To identify genes functionally unique to recurrent tumor cells (FDR < 0.05 and Bayes Factor (BF) score > 5), we compared our screening results to the Cancer Dependency Map (DepMap) data\(^1\), and further refined our list to genes that are highly expressed in GBM specimens compared to normal brain tissue (The Cancer Genome Atlas (TCGA) GBM dataset\(^2\); adjusted \(P < 0.01\)). This analysis revealed a list of 13 candidate genes (Figure 3A), including the master stemness regulator SRY-Box transcription factor 2 (SOX2; FDR = 0)\(^22\)-\(^24\). The remaining genes are largely uninvestigated in GBM stemness and disease recurrence, such as protein tyrosine phosphatase 4A2 (PTP4A2), metabolic reprogramming gene karyopherin subunit alpha 2 (KPNA2) and mitochondrial gene oxidase (cytochrome C) assembly 1-like (OXA1L). Examination of these genes across our CRISPR-Cas9 screening data and those from previous studies\(^6\),\(^7\) indicates PTP4A2 (\(P_{\text{primary}} = 0.016, P_{\text{NSCs}} = 0.022\)), KPNA2 (\(P_{\text{primary}} = 1.3\text{e-}06; P_{\text{NSCs}} = 0.003\)), CCDC47 (\(P_{\text{primary}} = 2\text{e-}06; P_{\text{NSCs}} = 0.0049\)), DCAF13 (\(P_{\text{primary}} = 5.1\text{e-}05; P_{\text{NSCs}} = 0.013\)), NFIB (\(P_{\text{primary}} = 0.0059; P_{\text{NSCs}} = 0.0017\)) and SLC25A19 (\(P_{\text{primary}} = 0.03; P_{\text{NSCs}} = 0.051\)) are more functionally required in recurrent tumor cells relative to primary tumor cells or fetal neural stem cells (NSCs), respectively (pairwise unpaired t-test; Figure S2A). Of these genes, PTP4A2 has the highest mRNA expression in GBM samples and shows highly significant mRNA enrichment in tumor specimens compared to non-tumor tissue (adjusted \(P = 2.8\text{e-}30\), genome-scale moderated contrast t-test; TCGA GBM dataset\(^21\)).

**PTP4A2 vulnerability at recurrence**

PTP4A2 is a poorly characterized member of the 4A family of dual-specificity protein tyrosine phosphatases that dephosphorylate tyrosine, serine and threonine residues on target peptides\(^25\). Whereas invertebrates (i.e. *C. elegans*, *D. melanogaster*, *S. purpuratus* and *B. floridai*) express a single
phosphatase encoded by *PTP4A*, all vertebrate species have three genes (*PTP4A1*, *PTP4A2*, *PTP4A3*) with more than 80% amino acid sequence similarity\(^26\). Notably, *Ptp4a2* knockout leads to defects in spermatogenesis and self-renewal of hematopoietic stem cells in mice\(^27,28\).

In the context of gliomas, we find *PTP4A2* expression correlates with tumor grade, and higher expression is also associated with poor overall survival in patients (\(P < 0.0001\); Figures S2B and S2E). We validated the effects of *PTP4A2* perturbation in six additional patient-derived GBM models including three primary and three recurrent models. While there were no detectable changes to cell proliferation (PR) or secondary sphere formation (SF) (i.e. a measure of stemness) in primary tumor cells following perturbation of *PTP4A2* (Figures 3B and 3C; \(P > 0.05\), unpaired t-test), significant changes in PR or SF were seen in recurrent tumor cells (\(P < 0.05\), unpaired t-test) following *PTP4A2* knockout.

To further investigate the effects of *PTP4A2* perturbation, we created an inducible *PTP4A2* knockout construct using CRISPR-Cas9 in recurrent tumor cells, which were then engrafted orthotopically in immunocompromised mice. Induction with doxycycline led to a significant increase in survival following *PTP4A2* knockout (Figure 3D; \(n = 8\), \(P < 0.0001\)). Next, in order to determine which essential cellular processes are modulated by *PTP4A2* phosphatase activity in recurrent tumors, we leveraged a potent chemical inhibitor of the phosphatase family (*PTP4A1*, *PTP4A2* and *PTP4A3*)\(^29,30\). *In vitro* treatment of cells with the pan-PTP4A phosphatase inhibitor JMS-053 was found to be lethal in recurrent tumor cells, while patient-matched primary tumor cells and unmatched fetal NSCs (NSC201) showed little effect over a wide range of drug concentrations (Figure 3E). In a preliminary study, intraperitoneal treatment with 10 mg/kg JMS-053 for 14 days led to increased median overall survival in an orthotopic patient-derived xenograft (PDX) model of recurrent GBM (\(P = 0.00059\); Figure 3F). Additionally, recent toxicity studies indicate that JMS-053 is well tolerated up to 40 mg/kg for three weeks (data not shown).

**A PTP4A2-ROBO1 axis mediates**

Given that small molecule inhibition of *PTP4A2* phosphatase activity phenocopied genetic knockout, we examined the phosphorylation landscape of GBM cells to identify substrates that could be driving *PTP4A2* dependence. Patient-matched primary and recurrent tumor cells were treated with JMS-053 or control compound prior to phospho-proteomic profiling (Figure 4A). Phospho-proteomic profiling identified 11,182 phospho-peptides corresponding to 3,677 phospho-proteins, with short-term enrichment of 508 phospho-peptides (5 min; 431 proteins) and long-term enrichment of 1,154 phospho-peptides (30 min; 831 proteins) (treatment/control phospho-peptide intensity ratio > 1.5; Figures S3A and S3B). Moreover, only \(\sim 20\%\) of phospho-peptide and \(\sim 23\%\) of phospho-protein enrichments were shared between primary and recurrent tumor cells following short-term treatment with JMS-053 (Figures S3C and S3D; Table S9).

GO term analysis of phospho-proteins enriched in JMS-053-treated recurrent tumor cells reveals an over-abundance of proteins belonging to the axonogenesis and neurogenesis gene sets (\(P < 0.01\)), whereas this was not observed in treated primary tumor cells (Figures 4B and S3E). Furthermore, we profiled the
transcriptome of primary and recurrent tumor cells with knockout of *PTP4A2* (two gRNAs) or *AAVS1* control (Figure S4A; Table S10). A total of 1,283 differentially expressed genes (DEGs) were identified following *PTP4A2* knockout (|fold change| > 2 and adjusted \(P < 0.05\)), with 818 DEGs exclusive to recurrent tumor cells. In agreement with the phosphoproteomic analysis, gene set enrichment analysis (GSEA) revealed a depletion of axon guidance members at the transcriptomic level in recurrent tumor cells with *PTP4A2* knockout (adjusted \(P = 0.04\)); however, no such effect was seen in primary tumor cells (adjusted \(P = 1\)) (Figure S4B-S4C).

The largest differential phospho-protein enrichment post-PTP4A2 inhibition in recurrent tumor cells corresponded to the axon guidance member roundabout guidance receptor 1 (ROBO1) (Figures 4B). Given that neither *PTP4A1* nor *PTP4A3* are essential for survival of recurrent tumor cells (*PTP4A1* FDR = 0.213 and *PTP4A3* FDR = 0.412) or primary tumor cells (*PTP4A1* FDR = 0.390 and *PTP4A3* FDR = 0.426), we hypothesized that the effect of pan-PTP4A inhibition on axon guidance may be occurring via a PTP4A2-ROBO1 axis. In fact, PTP4A2 inhibition in recurrent tumor cells downregulates total ROBO1 levels and enriches phosphorylation of downstream ROBO1 signaling members (Figures 4C, 4D and S3F). These include SLIT-ROBO Rho GTPase-activating proteins 1 and 3 (SRGAP1, SRGAP3), cell division cycle 42 (CDC42) effector/kinase proteins (CDC42EP1, CDC42EP3, CDC42BPB), and RAP1 GTPase activating protein (RAP1GAP).

During normal neurodevelopment, binding of secreted SLIT2 to ROBO1 leads to axon repulsion and inhibition of cell migration via SRGAP-mediated CDC42 inactivation\(^{31,32}\). Given that SLIT2 promoter methylation and inactivation is frequently observed in gliomas\(^{33}\), whereas ROBO1 is enriched at transcript and protein levels\(^{34}\), unhinged CDC42-driven cell motility may be modulated by the PTP4A2-ROBO1 axis. As measured by immunoprecipitation of GTP-bound CDC42, inhibition of PTP4A2 in recurrent tumor cells led to a stark decrease in active CDC42 and a modest decrease in total CDC42 (Figure 4E). In an effort to further validate the PTP4A2-ROBO1 axis, we developed a panel of camelid single-domain antibodies targeting human ROBO1 (Figure S5A). Of these antibodies, MKRo20 showed high affinity and specificity for human ROBO1 (\(K_D = 12.1 \pm 0.06\) nM) among other members of the ROBO family (ROBO2, ROBO3, and ROBO4) (Figures S5B-S5F). Strikingly, PTP4A2 inhibition and/or treatment with ROBO1-targeting MKRo20 led to robust decreases in recurrent tumor cell invasion and spheroid growth (Figures 4F, 4G and S5G), suggesting that treatment with a ROBO1-targeted antibody mimics PTP4A2 inhibition.

In addition to regulation of CDC42 activity during neurodevelopment, SLIT2 binding to ROBO1 weakens N-cadherin-mediated cell adhesion via phosphorylation of \(\beta\)-catenin by ABL\(^{35,36}\). Surprisingly, phosphoproteomic analysis of recurrent tumor cells post-PTP4A2 inhibition reveals enriched phosphorylation of \(\beta\)-catenin (S552), in addition to WNT signaling regulators APC and LBH (Figure S3G). In keeping with these observations, PTP4A2 inhibition in recurrent tumor cells increases phosphorylated \(\beta\)-catenin (S552) levels but decreases total \(\beta\)-catenin levels (Figure 4H).
Together, our observations reveal a context-specific vulnerability on axon guidance through a PTP4A2-ROBO axis in recurrent GBM (Figure 4I). In recurrent GBM, PTP4A2 may support ROBO1/CDC42-mediated tumor cell invasion via dephosphorylation of SRGAP1, SRGAP3 and CDC42 effector/kinase proteins. In addition, dephosphorylation of β-catenin (CTNNB1) may support WNT signaling-mediated tumor cell stemness and proliferation. Our identification of this axis is also supported by previous studies that link Ptpra1 expression with axon synaptogenesis in the central nervous system of *Drosophila*, the genome of which includes a single *Ptpra* gene. These results indicate that modulation of the PTP4A2-ROBO1 axis could be a viable and novel strategy to target recurrent GBM.

**Discussion**

We conducted the first set of unbiased functional genetic screens in patient-derived/matched GBM models to reveal functional modulators of disease recurrence. Not only do recurrent tumor cells rely on a distinct set of functional drivers when compared to their primary predecessors, therapeutic avenues to treat recurrent disease cannot be predicted without profiling tumors at recurrence. The surprising loss of ~30% of genetic dependencies in primary tumor cells at recurrence (e.g. *RUNX1*, *ZEB1* and *RHOA*), gain of an additional ~30% new functional dependencies (e.g. *FASN* and *CD151*), and further loss of crucial replicative checkpoints (e.g. *TP53*, *PTEN*, *NF1*, and *NF2*), highlight the dramatic remodelling from primary to recurrent disease. Therapy-driven events, along with continuous temporal evolution at the genetic and cellular levels, may select for a sub-clonal and treatment-resistant GSC population that redens the functional genetic landscape of the recurrent tumor.

Collectively, these results support a model in which therapy-driven and stochastic events lead to a functionally distinct tumor at recurrence. Our analysis of the genomic, transcriptomic, proteomic and functional genetic landscapes of patient-matched primary and recurrent tumor cells supports parallel tumor-intrinsic mechanisms of treatment resistance which rely on acquisition of immunosuppressive capacity. Not only are recurrent GBM cells burdened by greater stem-like properties and tumorigenic potential, presence of *de novo* driver mutations such as a defective mismatch repair (MMR) pathway (*MSH6 A1179V* and *T1219I*) may drive hypermutation and shield recurrent GBM cells from the host immune system and anti-PD-1 blockade. In stark contrast, anti-PD-1 blockade is a tractable therapeutic strategy in other aggressive cancers with hypermutation profiles (i.e. non-small cell lung cancer, colorectal cancer and melanoma) with a hypermutated profile. In addition, MMR-deficiency in recurrent GBM cells may predispose to a higher mutational burden but, unlike other cancers, these additional mutations may support an immunosuppressive microenvironment.

We observe that recurrent GBM cells with a defective MMR pathway member *MSH6 (A1179V and T1219I)* also harbour a deleterious mutation in *PTEN* (H123Y; Figure 1E; Table S2), previously shown to ablate phosphatase activity and prevent PTEN-mediated cell cycle arrest in breast cancer cells. In fact, MMR-deficiency and microsatellite instability (MSI) has been associated with truncal *PTEN* loss in gliomas, and likewise, *PTEN* loss occurs in 90% of human MSI endometrial carcinomas. In addition to MMR
deficiency, significant enrichment of PTEN mutations at recurrence has been associated with immunosuppressive expression signatures in GBM patients who were classified as non-responders to immune checkpoint blockade in clinical trials with nivolumab and pembrolizumab.

Altogether, our mutational, proteomic and functional characterization of the remodelled landscape in recurrent GBM reveals novel mechanisms of treatment resistance, warranting therapeutic approaches that exploit synthetic lethal vulnerabilities that emerge at recurrence. By performing the first genome-wide CRISPR-Cas9 gene knockout screens in patient-derived post-treatment recurrent tumor cells, we report an example of a context-specific vulnerability of PTP4A2. Pharmacological inhibition of PTP4A2 phosphatase activity revealed modulation of axon guidance via ROBO1 as a downstream effector of PTP4A2, further supported by a global enrichment and dependence on ROBO signaling. To develop a therapeutic approach targeting ROBO signaling, we present evidence supporting the use of an anti-ROBO1 single-domain antibody for treatment of recurrent tumor cells. These findings provide a template for studying recurrence and support development of therapeutic regimens that are informed by therapy-driven or longitudinal shifts in the functional genomic landscape of recurrent tumors.

Declarations

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Author contributions


Declaration of interests

The authors declare no competing interests.

Methods
Derivation and culture of patient-derived glioblastoma stem cell models

Primary glioblastoma (GBM) specimens and whole fetal brain samples were obtained from consenting patients and families as approved by the Hamilton Health Sciences and McMaster Health Sciences Research Ethics Board. After washing with PBS, specimens were mechanically dissociated followed by enzymatic dissociation in PBS containing 0.013 mg/mL Liberase Thermolysin Research Grade (Millipore Sigma no. 5401020001) at 37°C for 15 minutes. Dissociated cells were isolated by filtration through a 70 µm cell strainer (Millipore Sigma no. CLS431751-50EA), centrifuged and subjected to red blood cell lysis using 0.8% ammonium chloride solution (STEMCELL Technologies no. 07850). SB2b was a kind gift from Professor Bryan Day (Sid Faithfull Brain Cancer Laboratory) and Dr. Brett Stringer from Royal Brisbane Hospital in Herston, Australia. Tumor cells and fetal human neural stem cells (NSCs) were grown and maintained in NeuroCult NS-A proliferation kit (Human) (STEMCELL Technologies no. 05751), supplemented with 20 ng/mL EGF (STEMCELL Technologies no. 78006), 10 ng/mL FGF (STEMCELL Technologies no. 78003.2), 0.002% Heparin (w/v) (STEMCELL Technologies no. 07980), 1X antibiotic/antimycotic solution (Wisent Bio Products no. 450-115-EL). For the first two weeks of culture, patient-derived cells were treated for potential mycoplasma infection using 1X MycoZap Prophylactic (Lonza no. VZA-2031). Cells were cultured on tissue culture-treated dishes, cell-repellent dishes (Greiner Bio no. 628979), or tissue culture-treated dishes coated with Poly-L-ornithine (Millipore Sigma no. P4957) and mouse Laminin (Corning no. 354232). All cells were maintained at 37 °C and 5% CO₂. All cell lines were frequently tested for mycoplasma infection and, if needed, treated with additional 1X MycoZap for two weeks.

Library virus production and determination of multiplicity of infection

HEK293T (ATCC CRL-3216) cells were seeded (8 million per 15 cm dish) in DMEM containing glucose, pyruvate and 10% FBS. Twenty-four hours after seeding, a mix of 8µg of the Toronto KnockOut version 3.0 (TKOv3) guide RNA (gRNA) library (Addgene no. 90294), viral packaging/envelope vectors (4.8 µg psPAX2 and 3.2 µg pMD2.G), 48 µL X-treme gene transfection reagent (Roche) and 1.4 mL Opti-MEM medium was transfected into cells. Twenty-four hours post transfection, the medium was replaced with DMEM high glucose supplemented with 0.011 g/mL BSA, 1% penicillin-streptomycin. Forty-eight hours post transfection, virus-containing media was separated by centrifugation, aliquoted and frozen at -80°C.

To determine multiplicity of infection, tumor cells were plated on 15 cm tissue culture-treated dishes with or without poly-L-ornithine/laminin coating at ~25-50% density depending on cell line-specific doubling time, in a total of 25-30 mL of media. Twenty-four hours after plating, cells were infected with different dilutions of the TKOv3 library lentivirus. Twenty-four hours after infection, the virus-containing media was replaced with 25-30 mL of fresh medium containing puromycin (1-3 µg/mL) and cells were incubated for an additional 24-72 hours. Multiplicity of infection (MOI) was determined by calculating the fraction of infected cells that survived puromycin selection as compared to infected cells that were not selected.

Genome-wide CRISPR-Cas9 screens in GBM models
Tumor cells were plated on 15 cm tissue culture-treated dishes with or without poly-L-ornithine/laminin coating at ~25-50% density depending on cell line-specific doubling time, in a total of 25-30 mL of media. Twenty-four hours after plating, cells were infected with the TKOv3 library lentivirus at an MOI of ~0.3, ensuring that each gRNA was present in 200-500 cells. Twenty-four hours after infection, the virus-containing media was replaced with 25-30 mL of fresh medium containing puromycin (1-3 µg/mL) and cells were incubated for an additional 24-72 hours. Cells that survived puromycin selection were pooled together, and 30 million cells were collected as an initial reference timepoint (T0).

For dropout screens (BT241 and BT972), infected and puromycin-selected cells were split into three experimental replicates, with each replicate containing 15+ million cells (>200-fold library coverage). Cells were passaged every 2-3 doublings and maintained at >200-fold library coverage for 15-18 doublings. Cell pellets were collected during passaging from each replicate for downstream analysis. For screens involving drug treatment (BT594 and BT935), infected and puromycin-selected cells were split into one of four treatment arms: control DMSO treatment, sublethal IC_{20-30} radiotherapy (RT), sublethal IC_{20-30} temozolomide (TMZ), or sublethal IC_{20-30} RT and TMZ. For combined RT and TMZ therapy, each plate was treated with TMZ (Millipore Sigma no. T2577) one hour prior to RT (Faxitron RX-650). For the entire duration of the screen, treatments were administered in cycles of 5 days on and 2 days off to allow for recovery and passaging. Passaging and collection of cell pellets was performed as described above.

**Library preparation and Illumina sequencing**

Genomic DNA (gDNA) was extracted from cell pellets collected at the beginning (T0) and end of each screen (Tn) using the Wizard Genomic DNA Purification Kit (Promega). Following resuspension in TE buffer, gDNA concentration was determined by Qubit using double-stranded DNA (dsDNA) Broad Range Assay reagents (Invitrogen). For each sample, 50 µg of extracted DNA was subjected to two PCR reactions, the first of which enriched gRNA-containing regions and the second step amplified gRNAs and attached Illumina TruSeq adapters with i5 and i7 indices, as described previously Barcoded libraries were gel purified and concentrations were determined by qRT-PCR. Sequencing of libraries was performed on an Illumina HiSeq2500 using standard primers for dual indexing with HiSeq SBS Kit v4 reagents. The single-end sequencing run consisted of 21 dark cycles (base additions without imaging), followed by 36-base reads containing 2 index reads, first with i7 and the second with i5 sequences. The beginning (T0) and endpoints (Tn) were sequenced at 400- and 200-fold coverage, respectively.

**Mapping and quantification of gRNAs**

Following completion of Illumina sequencing, 20-bp gRNA sequences were extracted from FASTQ files by trimming reads according to constant sequence anchors. Trimmed reads were aligned to a FASTA file of gRNA sequences from TKOv3 using Bowtie (v0.12.8) 47. Two mismatches and 1 exact alignment were allowed during alignment (specific parameters: -v2 -m1 -p4 –sam-nohead). Processed reads were tallied for each sample and merged into a matrix.

**Quality control analysis and scoring of gRNAs**

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To begin quantifying the gene fitness in each screen, LFC of each gRNA was quantified by comparing read-depth-normalized gRNA counts at the beginning of each screen (T0) to the end of each screen (Tn). To perform quality control analysis, the LFC for all gRNAs targeting a single gene were averaged, with four gRNAs targeting each gene in TKOv3. Established gene sets of core essential genes (reference) and non-essential genes (background) were compared for dropout using LFC values and by computing precision over true positive statistics.

For all pooled CRISPR-Cas9 screens, LFC values of core essential genes and non-essential genes were used to apply the Bayesian Analysis of Gene Essentiality (BAGEL) algorithm to determine fitness of all genes. A Bayes Factor (BF) score of 5 and FDR<0.05 were used to identify genes essential for cell survival. For drug-treated CRISPR-Cas9 screens (BT594 and BT935), LFC values for gRNAs targeting each gene were compared between a drug-treated arm (treatment) and the DMSO-treated arm (control) for each cell line by applying the DrugZ algorithm. For each drug-treated arm (RT, TMZ, or combined RT and TMZ), positive and negative conditional genetic interactions (GIs) were identified using normalized Z scores combined with synthetic/suppressor FDR values.

Gene set enrichment analysis of conditional gRNA distributions

For all primary GBM CRISPR-Cas9 screens, normalized gene-level LFC values from each arm (DMSO, RT, TMZ, or combined RT and TMZ) were used to conduct gene set enrichment analysis (GSEA) using fgsea (v1.14.0). For each screen, GSEA of Reactome canonical pathways (c2.cp.reactome.v7.1) was conducted with a minimum of 1 and maximum of 1000 genes in each gene set, a total of 10,000 permutations, and an adjusted P<0.05. GSEA results were collapsed into parent gene sets using the collapsePathways() function from fgsea. Normalized enrichment scores (NESs) from all screens were compiled in a matrix (Supplementary Table 4).

Functional gene enrichment analysis

**Shared essential genes in primary and recurrent tumor cells.** A set of shared essential genes with BF scores greater than in primary BT594 (DMSO-treated) and recurrent BT972 tumor cells was determined. Genes belonging to core essential gene sets were filtered out and the remaining shared essential genes were subjected to functional enrichment analysis using gProfiler2 (v0.1.9). Using all genes targeted by TKOv3 as background, the gost() function was applied to determine enriched gene sets from Reactome, Gene Ontology (GO) biological processes and GO molecular functions with FDR < 0.05. Using Cytoscape (v3.8.0), enriched gene sets with a similarity of >50% were collapsed using the Enrichment Map function (v3.3.0), and the AutoAnnotate function (v1.3.3) was used to determine themes of overlapping gene sets.

**Shared essential genes in recurrent tumor cells.** A set of shared essential genes with BF scores greater than five in recurrent BT241 and BT972 tumor cells was determined. Enrichments and visualization were performed as above.
**In vitro assays for self-renewal and proliferation**

**Cell preparation.** Tumor cells, with or without genetic knockout, were dissociated into a single-cell suspension using TrypLE (ThermoFisher Scientific no. 12605028) for adherent cultures, or a combination of Liberase Thermolysin Research Grade (Millipore Sigma no. 5401020001) and DNase I (Worthington Biochemical Corporation no. 9003-98-9) for suspension spherical cultures. Cells were incubated with dissociation agent for 5 minutes at 37°C. After washing dissociated cells twice with PBS, the cell suspension was filtered through a 35µm cell strainer prior to plating for assays. All assays were performed using NeuroCult NS-A proliferation kit (Human), supplemented as described above. All experiments were conducted with five experimental replicates per sample.

**Limiting dilution assay.** Tumor cells were plated at varying densities (1, 5, 10, 50, 100, 200 cells/well) in cell-repellent 96-well, flat-bottom microplate (Greiner Bio no. 655970). Cells were cultured in 200 µL of media and incubated for 3-5 days at 37°C and 5% CO\(_2\). Following incubation, cell spheres were counted by microscopy. For each sample, the frequency of sphere-forming cells in each cell line was determined using the elda() function from statmod (v.1.4.34). A single-hit model with a log-log binomial regression was applied to determine confidence intervals for sphere-forming cell frequency. A chi-squared likelihood ratio test was used to assess differences between samples.

**Secondary sphere formation assay.** Tumor cells were plated at 200 or 300 cells/well in cell-repellent 96-well, flat-bottom microplate (Greiner Bio no. 655970). Cells were cultured in 200 µL of media and incubated for 3-5 days at 37°C and 5% CO\(_2\). Following incubation, cell spheres were counted by microscopy. An unpaired t-test was used to compare between samples.

**Proliferation assay.** Tumor cells were plated at 1000 cells/well in flat-bottom tissue culture-treated 96-well microplates. Cells were cultured in 200 µL of media and incubated for 3-5 days at 37°C and 5% CO\(_2\). To quantify proliferation, 20 µL of PrestoBlue Cell Viability Reagent was added to each well followed by incubation for four hours at 37°C and 5% CO\(_2\). Fluorescence was measured on a FLUOstar Omega Fluorescence 566 Microplate reader (BMG LABTECH) (excitation 540 nm and emission 570 nm) and analyzed using Omega software. An unpaired t-test was used to compare between samples.

**In vivo engraftment of tumor cells**

All animal experimental protocols were approved by the McMaster University Animal Research Ethics Board. Tumor cells were injected orthotopically into immunocompromised mice as described previously\(^54\). In brief, 10-12 week old NSG mice were anaesthetized using isoflurane gas (5% induction, 2.5% maintenance). A total of 250000 tumor cells were injected per mouse into the right frontal lobes, suspended in 5 µL PBS. All mice were euthanized at endpoint and survival analysis was conducted in R using the survfit() and surv() functions from the survival package (v.3.2-3), and plotted using the survminer package (v.0.4.8).

**In vivo inducible knockout of PTP4A2**
Recurrent BT241 tumor cells were infected with lentivirus containing adoxycycline-inducible Cas9 (iCas9) construct. Validated BT241-iCas9 cells were treated with doxycycline (DOX; 1.5µg/mL, Sigma) for 48 hours to induce Cas9 and BFP2 expression. DOX-induced and uninduced samples were run on a MoFlo XDP Cell Sorter (Beckman Coulter). After excluding dead cells using 7AAD (1/100, Beckman Coulter), BFP2+ cells were sorted into tubes containing NeuroCult NS-A proliferation kit (Human), supplemented as described above.

BT241-iCas9 cells were infected with a modified pLCKO vector (no Cas9) containing gRNA sequences targeting AAVS1 or PTP4A2, obtained from TKOv3 and cloned as described previously. Following selection with puromycin (3 µg/mL for 72 hours), 250000 gRNA-expressing BT241-iCas9 were injected in immunocompromised NSG mice as described above. Two weeks after injection, mice were treated with DOX in their water (2 mg/mL DOX with 1% sucrose) and feed (625 mg/kg DOX-supplemented Envigo no. 7004 feed) for two weeks. Following DOX treatment, mice were put back on normal water and feed. All mice were euthanized at endpoint and survival analysis was conducted as described above.

RNA sequencing

Sample preparation. Five million tumor cells (BT594 or BT972) were plated in cell-repellent dishes (Grenier Bio no. 628979) and infected with lentivirus containing single-gRNA lentiCRISPRv2 constructs targeting AAVS1 or PTP4A2 (2 gRNAs), with gRNA sequences obtained from TKOv3. Twenty-four hours post-infection, virus-containing media was replaced with fresh media containing puromycin (1-2 µg/mL) for 48-72 hours. Each cell line was cultured and processed in three experimental replicates. Following antibiotic selection, RNA was extracted using the RNeasy Kit (Qiagen), according to manufacturer's instructions. All samples were treated with DNase using RNase-free DNase Set (Qiagen no. 79254). Samples were submitted for mRNA-Seq at the Donnelly Sequencing Centre at the University of Toronto, Toronto, Canada (http://ccbr.utoronto.ca/donnelly-sequencing-centre). 300ng per sample was processed according to NEBNext Ultra II Directional RNA Library Prep sample preparation protocol (Part # E7760L) with the NEBNext Poly(A) mRNA Magnetic Isolation Module (Part # E7490). 1µL top stock of each purified final library was run on an Agilent TapeStation HS D1000 tape (Agilent). The libraries were quantified using Quant-iT dsDNA HS Assay Kit (ThermoFisher Scientific) and were pooled at equimolar ratios after size adjustment. The final pool was run on an Agilent Bioanalyzer dsDNA High Sensitivity chip and quantified using NEBNext Library Quant Kit for Illumina (NEB). The quantified pool was hybridized at a final concentration of 2.75pM and sequenced paired-end 2x50 bp on the Illumina NovaSeq6000 platform using an S2 flowcell to obtain an average of 28M reads per sample.

Data processing. Read pairs shorter than 36bp on either read1 or read2 were filtered out prior to mapping. Reads were aligned to reference genome hg38 and Gencode V25 gene models using the STAR short-read aligner (v2.6.1b). An average of 86.9% of the filtered reads mapped uniquely. Gene-level read counts, determined by STAR, were merged along with gene annotations into a single matrix using a bespoke R script.
**Differential expression.** Differentially expressed genes (DEGs) were identified using the Bioconductor packages *limma* (v3.44.3) and *edgeR* (v3.30.3). The read count matrix was filtered to remove low-expressed genes using the function `filterByExpr()` using default parameters. Principal component analysis (PCA) allowed for visualization of treatment effects and adjustment for batch-to-batch differences. Normalization was performed using `calcNormFactors` (method = 'TMM') and log2-transformed using `voom()`. A linear model was fit to account for differences between BT594 and BT972 cells (main effect) as well as among knockouts for *AAVS1*, *PTP4A2* (first gRNA) and *PTP4A2* (second gRNA). DEGs were extracted using `treat()` (*limma*) and determined using log2(fold change) > 1 and adjusted \( P < 0.05 \).

**Variant calling.** Genomic variants affecting protein-coding genes were identified from the RNAseq data using the Genome Analysis Toolkit (GATK) version 3.1 following the recommended “Best Practices”. Briefly, beginning with the BAM output file from STAR, the steps included AddOrReplaceReadGroups, MarkDuplicates, ReorderSam, SplitNCigarReads, HaplotypeCaller, and finally VariantFiltration. Variant consequences were classified using the Variant Effect Predictor (VEP) release 89, which includes predictions from SIFT, PolyPhen and Condel.

**Whole-cell proteomics.**

**Sample preparation.** Five million patient-derived tumor cells (BT594 and BT972) were cultured in cell-repellent 10 cm dishes (Greiner Bio no. 628979) with 10 mL of NeuroCult NS-A proliferation kit (Human) (STEMCELL Technologies no. 05751), supplemented as described above. Cells were incubated for 96 hours at 37°C and 5% CO\(_2\) to enrich for sphere formation. Following incubation, cells were washed thrice with cold PBS, centrifuged into pellets and snap frozen using liquid nitrogen. Cell pellets were stored at -80°C until further processing.

Cells were lysed in PBS:2,2,2-trifluoroethanol (TFE) (1:1 v:v)) as previously described (Cogger et al (PMID: 28835709)). Protein concentration was determined using the BCA kit (Thermo) according to the manufacturer’s instruction. Cysteins were reduced with dithiothreitol (DTT, 5mM final concentration) at 60°C for 30 minutes and subsequently alkylated using iodoacetamide (25mM final concentration) in the dark at room temperature for 30 minutes. Samples were diluted with four volumes 100 mM ammonium bicarbonate (pH 8.0), supplemented with calcium chloride (2mM final concentration) and digested for 16 h at 37°C with Trypsin:LysC (Thermo) added at a 1:50 ratio. The digestion was quenched using 0.5% formic acid (FA) and tryptic peptides were fractionated using a basic C18 fractionation method. Briefly, tryptic peptides were loaded on four 45 mm C18 extraction disks (Empore™, Fisher Scientific) and eluted in five fractions using increasing acetonitrile (ACN) (5%, 7.5%, 10%, 12.5%, 15%, 17.5%, 20%, 50%) concentration solutions prepared in 0.1% ammonium hydroxide. Fractions were pooled as follows: fractions 3+6; 4+7; 5+8. Fractions 1 and 2 were not pooled. The five resulting fractions were lyophilised and reconstituted in 21μL 3% ACN 0.1% FA. Peptide concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo) and were loaded on a 50 cm ES803 column (Thermo). Peptides were separated using a 4h gradient, at 250 nL/min flow, using the Thermo Scientific EasyLC1000 nano-liquid-chromatography system. The chromatography system was coupled to an Orbitrap Fusion Mass
Spectrometer (Thermo) and MS/MS data were acquired in a data dependent mode with full scans (350-1800 m/z) acquired using the ion trap mass analyser at a mass resolution of 120000 m/z. Fifteen most intense precursors from a survey scan were selected for MS/MS from each cycle and detected at a mass resolution of 15000 m/z. Tandem mass spectra were produced by high energy dissociation (HCD) method. Dynamic exclusion was set for 60 s. The automatic gain control for full MS was set to 1e5 ions and 1e2 ions for MS/MS with maximum ion injection times of 55 ms and 50 ms respectively.

Data analysis. The acquired raw data were analysed using the Max Quant software (version 1.6.1.0) using the complete human proteome (version 2016.07.13 containing 42,041 sequences). Search parameters were defined as follow: a maximum of two missed cleavages; carbamido-methylation of cysteine was specified as fixed modification and oxidation of methionine as variable modifications. Peptide length was specified to be 8-25 amino acids. The mass error was set to 10 p.p.m. for precursor ions and 0.5 Da for fragment ions. The false discovery rate (controlled using a target-decoy approach based on reversed sequences) was defined as 1% at peptide and protein levels. The Maxquant output was compiled into a matrix for further analysis.

Recurrent GBM-enriched gene essentiality and expression

Bayes factor (BF) scores for a total of 666 genome-wide CRISPR-Cas9 screens in human cancer cell lines were acquired from the PICKLES database, which included the Behan 2019 and Avana 2018 datasets. For each data set, BF scores were averaged by sample collection site and merged into a single matrix and merged with BF scores from recurrent GBM screens (BT241 and BT972). To incorporate differentially expressed genes (DEGs) in GBM, the GEPIA2 interface was utilized to compare genome-scale gene expression between The Cancer Genome Atlas (TCGA) GBM specimens and GTEX normal brain specimens, as described previously. To determine a set of recurrent GBM-specific essential genes, a stringent cut-off of BF score greater than five was used to classify a gene as essential. By filtering for genes with BF greater than five in recurrent tumor cell lines and BF less than or equal to five in all 666 screens from the PICKLES database, we determined a set of recurrent GBM-specific essential genes. This subset of genes was further filtered to include those genes that are enriched for expression in GBM tissues (TCGA) as compared to normal brain tissue (GTEX), with a log₂ fold change > one and an adjusted \( P < 0.01 \).

Gene expression and survival analysis from TCGA and CGGA

Gene expression and patient survival data was obtained for all low-grade glioma and GBM patients profiled by TCGA (GBMLGG RNA-seq dataset from Gliovis, www.gliovis.bioinfo.cnio.es) and the Chinese Glioma Genome Atlas (CGGA; mRNAseq_693 dataset from www.cgga.org.cn). To profile PTP4A2 gene expression in each dataset, \( PTP4A2 \) gene expression values were log₂-transformed and stratified according to WHO (World Health Organization) grading. Pairwise comparisons among WHO grades were conducted using the Wilcoxon rank sum test. To determine the effect of \( PTP4A2 \) expression on patient survival, patient specimen expression values were stratified into ‘high \( PTP4A2 \)’ and ‘low \( PTP4A2 \)’ groups.
according to the median expression value in each dataset. Data was plotted using the `survminer` package (v.0.4.8).

**In vitro** treatment of cells with pan-PTP4A inhibitor

Briefly, the pan-PTP4A inhibitor JMS-053 and an inactive control compound (JMS-038) were synthesized as described previously \(^2^9\) and resuspended at 5 mM in 100% DMSO. To test for cytotoxicity, cells (tumor cells or fetal NSCs) were plated at 1000 cells/well in flat-bottom tissue culture-treated 96-well microplates. Cells were treated once with various concentrations (20 nM to 20 µM) of JMS-038 or JMS-053. Cells were cultured in 200 µL of media and incubated for seven days at 37°C and 5% CO\(_2\). To quantify proliferation, 20 µL of PrestoBlue Cell Viability Reagent was added to each well followed by incubation for four hours at 37°C and 5% CO\(_2\). Fluorescence was measured on a FLUOstar Omega Fluorescence 566 Microplate reader (BMG LABTECH) (excitation 540 nm and emission 570 nm) and analyzed using Omega software.

**Phospho-proteomic profiling of PTP4A-inhibited tumor cells**

*Sample preparation.* 2.5 million tumor cells (BT594 and BT972) were plated at a density of 0.5 million cells/mL of NeuroCult NS-A proliferation kit (Human), supplemented as described above. Cells were either treated with 1 µM JMS-053 (active pan-PTP4A inhibitor) or 1µM JMS-038 (inactive control compound) for five minutes or 30 minutes at 37°C and 5% CO\(_2\). Following incubation, samples were immediately washed with cold PBS containing 1X Halt Protease and Phosphatase Inhibitor (Thermo Fisher Scientific no. 78442). Following three cycles of centrifugation and washing with HALT-containing PBS, samples were pelleted. Except for BT594 tumor cells treated with JMS-053 for 30 minutes, all samples were cultured, treated and processed in experimental duplicates. Cell pellets were suspended in 200 µL of lysis buffer comprising 8M urea (Sigma-Aldrich) and 100mM ammonium bicarbonate (Sigma-Aldrich) each. Cells were then vortexed at 2800rpm using Mini S-2 Vortex Mixer (Fisher Scientific) for ten seconds, followed by ten seconds of incubation on ice. This procedure was repeated six times. The lysate was then centrifuged at 21000xg for five minutes at 4°C. Protein reduction was conducted using 5mM of tris (2-carboxyethyl) phosphine (Sigma-Aldrich) for 45 minutes at 37°C. Subsequently, 10mM of iodoacetamide (Sigma-Aldrich) was added for protein alkylation for 45 minutes at room temperature (dark). Following alkylation, cell lysate was diluted five-fold with 100mM of ammonium bicarbonate to lower urea concentration. Based on protein amount, Sequencing Grade Modified Trypsin (Promega) was then added in (trypsin: protein(w:w) at 1:50) for overnight digestion at 37°C. Trifluoroacetic acid (Thermo Scientific) was added to reduce pH, and desalting was conducted with SOLA Solid Phase Extraction 10mg 96-well plates (Thermo Scientific). Peptides were eluted using 400mL 80% Acetonitrile - 0.1% trifluoroacetic acid. Eluted peptides were speed-vacuum dried using Labconco CentriVap Benchtop Vacuum Concentrator (Kansas City, MO).

*Labeling, phoso-peptide enrichment and LC/MS/MS.* Tandem Mass Tag (TMT)11-plex Isobaric Label Reagent Set (Thermo Fisher) were used to label peptides from each condition following the
manufacturer's protocol. Briefly, dried peptide samples were resuspended in 100mM of triethylammonium bicarbonate (TEAB) (Sigma-Aldrich) to 1µg/µL. Then, 0.2mg TMT reagents were mixed with 50µg of peptide samples at 4:1 (wt/wt) ratio and incubated at room temperature for one hour. Following incubation, each TMT reaction was quenched with 2µL of 5% hydroxylamine (Sigma-Aldrich) for 15 minutes at room temperature. Labeled samples were pooled together at equal ratio and then speed-vacuum dried. Desalting was then performed to purify salt-free TMT-label peptide samples. Phosphopeptides-enrichment were conducted using Ni-NTA magnetic agarose beads (Qiagen). Briefly, 138µl of Ni-NTA magnetic agarose beads were washed three times with 400µL of ultrapure nuclease-free water, and then incubate with 400µL of 100nM EDTA for 30 minutes. After three washes with 400µL of ultrapure nuclease-free water, the beads were incubated with 100nM FeCl₃ for 30 minutes. Then beads were washed three times with 400µL of ultrapure nuclease-free water and one time with 400µL of 80% acetonitrile - 0.1% trifluoroacetic acid. 550µg TMT-labeled peptides were resuspended in 550µL of 80% acetonitrile - 0.1% trifluoroacetic acid and added to the Fe-activated beads, rotating for 30 minutes. After capture, the supernatant was saved for analysis of unphosphorylated peptides and phosphopeptides were eluted using 50µL of 1.4% of ammonia hydroxide solution followed by another 50 µL of ultrapure nuclease-free water.

Enriched phosphopeptides were separated into nine fractions by high pH reverse-phase liquid chromatography (RPLC) using a home-made C18 column (200 µm x 30cm bed volume, Waters BEH 130 5µm resin) from 11%-32% acetonitrile-20mM ammonium formate (pH10) at flow rate of 5µL/minute. Each fraction was then loaded onto a home-made trap column (200 µm x 5 cm bed volume) packed with POROS 10R2 10µm resin (Applied Biosystems), followed by a home-made analytical column (75µm x 25cm bed volume) packed with Reprosil-Pur 120 C18-AQ 1.9µm particles (Dr. Maisch) with integrated Picofrit nanospray emitter (New Objective). LC-MS experiments were performed on a Thermo Fisher Ultimate 3000 RSLCNano UPLC system that ran a 3hr gradient (11%-38% acetonitrile-0.1% formic acid) at 70nl/min, coupled to a Thermo QExactive HF quadrupole-Orbitrap mass spectrometer. A parent ion scan was performed using a resolving power of 120,000 and then up to the 30 most intense peaks were selected for MS/MS (minimum ion counts of 1000 for activation), using higher energy collision induced dissociation (HCD) fragmentation. Dynamic exclusion was activated such that MS/MS of the same m/z (within a range of 10ppm; exclusion list size=500) detected twice within 5s were excluded from analysis for 30s.

Data analysis. LC-MS data were searched against a UniProt human protein database (Ver 2017-06, 42,173 entries) for protein identification and quantification by Protein Discover software (Thermo). From 300970 MS/MS spectra acquired in all nine fractions, 84496 peptide-to-spectrum matches (PSMs), 20443 unique peptide groups (with Peptide FDR < 0.01), and 4732 unique proteins (Protein FDR < 0.01) were identified. Among the identified peptide groups, 17127 hits were phosphopeptides. The density of these phosphopeptides was normalized by total TMT reporter intensity values from a separate LC/MS/MS run of the phosphoenrichment flow-through. Phosphopeptide fold-change between JMS-053 and JMS-038 for both cell lines was calculated, and the mean value of TMT reporter intensity for each
phosphopeptide was calculated as well. These values were then uploaded to Perseus software to generate significance B values.

Functional enrichment analysis of enriched phospho-proteins

Phospho-peptide intensities for all samples were filtered for <50% coefficient of variation (Rfast v.1.9.9). Phospho-peptides enriched after short-term treatment with JMS-053, with a treatment/control phospho-peptide intensity fold change > 1.5, were determined and the corresponding phospho-proteins were subjected to functional enrichment analysis. Using the gProfiler2 package (v0.1.9), The gost() function was applied to determine enriched gene sets from GO cellular component processes with FDR < 0.05.

Development of anti-ROBO1 antibodies

Generation of anti-ROBO1 single-domain antibodies. Camelid single-domain antibodies were raised by llama immunization and phage display essentially as previously described \(^{59,60}\). A phage-displayed heavy chain (V\(_{H}\)) library was constructed from the peripheral blood B-cell repertoire of a llama immunized with recombinant human ROBO1-Fc (8975-RB-050, R&D Systems, Minneapolis, MN). Antigen-reactive V\(_{H}\)-phage were enriched by panning against ROBO1-Fc with subtraction on Fc alone.

Antibody expression, purification and validation. Monomeric single-domain antibodies bearing His\(_{6}\) and biotin acceptor peptide tags were expressed in the periplasm of Escherichia coli BL21 (DE3) cells, enzymatically biotinylated \textit{in vitro} using BirA\(^{61}\), and purified by immobilized metal affinity chromatography followed by size exclusion chromatography (Superdex 75 Increase 10/300 GL, GE Healthcare, Chicago, IL). ROBO1 extracellular domain (NP_598334.1 aa 1–858) and ROBO1 Ig1-Ig2 domains (NP_002932.1 aa 61–266) were produced by transient transfection of HEK293-6E cells with pTT3/pTT5 vectors and antibody binding to these proteins was assessed by indirect titration ELISA. The affinities and kinetics of the interactions between monomeric single domain antibodies and human ROBO1 (25°C, pH 7.4) were determined using surface plasmon resonance. ROBO1 and other proteins were immobilized on a sensor chip CM5 (GE Healthcare) by amine coupling and antibodies were flowed over the antigen surfaces on a Biacore T200 instrument (GE Healthcare). Data from multi-cycle kinetic analysis were fit to a 1:1 binding model.

Generation of human Fc-linked MKRo-20 (MKRo-20-hFc). The encoding sequence of MKRo20 was codon optimized (Gene Art, ThermoFisher Scientific) and cloned into pTT5-hIgG1. Bivalent V\(_{H}\)-human IgG1 Fc fusions were produced by transient transfection of HEK293-6E cells followed by protein A affinity chromatography as described previously \(^{62,63}\). The same procedure was applied for the generation of A20.1 (AXX-hFc), a V\(_{H}\) specific for clostridium difficile toxin A \(^{64}\) used as negative control \(^{62,63}\). The same procedure was applied for the generation of A20.1 (AXX-hFc), a V\(_{H}\) specific for clostridium difficile toxin A \(^{64}\) used as negative control.
Flow cytometry validation of MKRo-20-hFc binding to recurrent tumor cells. Cells were dissociated using Liberase (Millipore Sigma no. 5401127001) and DNase I (Worthington Biochemical Corporation no. 9003-98-9) into a single-cell suspension. After washing once with PBS, cells were resuspended in PBS. To quantify ROBO1 expression on the cell surface, 500000 live cells were stained with 0.002 mg/mL anti-ROBO1 mouse-anti-human IgG1-AF647 (R&D Systems no. FAB71181R) or various concentrations of MKRo-20-hFc (3.4e-5 to 1.1e-3 mg/mL) for 15 minutes at room temperature. Equivalent concentrations of the isotypic control (AXX-hFc) for MKRo-20-hFc were also used to stain cells. Following staining, cells were washed with PBS, followed by staining with 1/1000 dilution of anti-human IgG Fcγ-specific AF488 (Jackson ImmunoResearch no. 109-545-008) for 30 minutes on ice. After washing with PBS, cells were stained with 1/100 7-AAD viability dye (Beckman Coulter no. B88526). Samples were run on a CytoFLEX LX (Beckman Coulter), and data was analyzed using analysis software Kaluza 2.0 (Beckman Coulter). Forward scatter (FSC)-Area vs. side scatter (SSC)-Area is used as the initial gate to exclude debris. Viability gate is set to exclude non-viable cells by gating on the 7-AAD negative population. Isotype control AXX-hFc is used to set the gate for expression of ROBO1, where the gate is drawn to separate ROBO1-positive cells from non-specific binding of AXX-hFc.

In vivo validation of MKRo-20 in recurrent GBM

Immunocompromised mice were intracranially injected with 250000 live cells/mouse of recurrent BT241 tumor cells. Six days post-injection, mice were administered four intracranial treatments of 40 µg tetrameric MKRo-20 (n = 4) or 40 µg tetrameric control (n = 4) spaced evenly over two weeks. One week after the last treatment (27 days post-injection), mice were euthanized, brains were collected, fixed with formalin and subjected to paraffin-embedding for hematoxylin and eosin (H&E) staining.

Statistical analysis

All experimental data is accompanied by number of experimental experiments in the figure legends or text. Unless stated otherwise, statistical significance was assessed using an unpaired Student’s t-test. In all cases otherwise states, *** p<0.0001, ** p<0.001, *p<0.05. Statistical analyses were performed using the R language programming environment.

Data and code availability statement

All processed data is included in the manuscript and supplemental materials. Raw sequencing data (i.e. FASTQ files) will be uploaded to an appropriate online database prior to publication. All custom code will be made available via GitHub.

Biological material availability statement

All unique biological materials are available upon request from corresponding authors.

References


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**Figures**
Figure 1

Genome-scale genetic and proteomic comparison of patient-matched, pre-treatment primary and post-treatment recurrent tumor cells.

(a) Schematic of patient-matched primary (BT594) and recurrent (BT972) tumor cell derivation.
(b) Limiting dilution assay to assess *in vitro* sphere formation in patient-matched primary (blue) and recurrent (red) tumor cells. Data are presented as means with 95% confidence interval (CI); *P* value from chi-squared likelihood ratio test.

(c) Kaplan-Meier survival analysis of immunocompromised mice engrafted with tumor cells, indicating death when mice reach endpoint. Primary (blue), *n* = 5; recurrent (red), *n* = 5; *P* value from log-rank (Mantel-Cox) test.

(d) Tally of damaging mutations in primary and recurrent tumor cells, separated by mutation type. Mutations were called using the Genome Analysis Toolkit (GATK) and filtered to include canonical variants with predicted high impact on protein function and/or classified as deleterious by Condel, SIFT and PolyPhen.

(e) Circos plots of deleterious mutations in primary and recurrent tumor cells separated by mutation type (i.e. point, frameshift, transcriptional and splicing variants). Mutations are aligned to their chromosomal position. For each mutation, gene-level mutation frequency (% of total patients) was obtained from 10,000 patient tumor specimens profiled by Zehir, et al. Mutations present in >1% of patients are highlighted for primary and recurrent tumor cells.

(f) Fast gene set enrichment analysis (fGSEA) was performed on fold change-ranked transcripts (RNA sequencing) and proteins (whole cell proteomics) in recurrent tumor cells as compared to primary tumor cells (recurrent/primary). Integrated pathway gene sets (March 2021 version) were obtained from the Bader Lab (www.baderlab.org/Software). The top seven enriched gene sets in recurrent tumor cells (normalized enrichment score (NES) > 0) and primary tumor cells (NES < 0) are highlighted, with dot size and color representing gene set size and adjusted *P* value, respectively. Gene sets with common biological themes are indicated by text color.
Figure 2

Functional screens reveal relative fitness trends at GBM recurrence.

(a) Gene-level cGI Z scores from screens performed in a treated primary tumor cell line relative to patient-matched recurrent cells. Pearson correlation coefficients and $P$ values shown for each pairwise comparison with (black) and without (grey) filtering for genes that meet FDR < 0.05 in at least one
screen. Conditional scores compute normalized difference of Z-scores between the condition indicated on the axis and BT594 DMSO control cells.

(b) Differential fitness genes between patient-matched primary and recurrent tumor cells. Genes that confer significantly increased (blue/dark blue; LFC > 0) or decreased (yellow/dark yellow; LFC < 0) tumor cell fitness in recurrent tumor cells relative to primary tumor cells are shown (FDR < 0.5). Node size and shade correspond to mean LFC and FDR (recurrent/primary), respectively. (Inset) Density plot with all data points shown.

(c) Network map of Reactome gene sets enriched in genes that exhibit differential fitness in recurrent GBM (BT972), with genes responsible for gene set enrichment listed alphabetically for each cluster. Network is designed using the Enrichment Map plugin in Cytoscape, and nodes are clustered using Auto Annotate (MCL Cluster algorithm, cluster cut-off = 1.0). Nodes represent enriched pathways, while edges connect related pathways.
Figure 3

Functional interrogation of patient-derived and post-treatment recurrent tumor cells.

(a) BF scores of recurrent tumor-specific essential genes (BF score > 5) as compared to CRISPR-Cas9 screens in human cancer cell lines from the Cancer Dependency Map (DepMap)\(^{16,18}\). Bar plot on right
presents median mRNA level in primary GBM specimens, with all genes significantly upregulated in The Cancer Genome Atlas (TCGA) GBM specimens as compared to non-tumor tissue (FDR < 0.05).

(b,c) Evaluation of protein tyrosine phosphatase 4A2 (PTP4A2) knockout (2 gRNAs, A and B) on (b) secondary sphere formation and (c) proliferation of primary and recurrent tumor cells, compared with a gRNA targeting AAVS1 (control). All data are represented as mean +/- s.d.; n = 5 experimental replicates; P values are from unpaired Student’s t-tests.

(d) Survival analysis of immunocompromised mice engrafted with recurrent tumor cells with an inducible PTP4A2- or AAVS1- knockout (n = 8 per knockout).

(e) Cell viability assays of patient-matched primary (BT594) and recurrent (BT972) tumor cells, and an unmatched fetal neural stem cell line (NSC201FT), exposed to increasing doses of JMS-053 over the course of 168 hours. Data presented as mean +/- s.d., n = 5 experimental replicates.

(f) Kaplan-Meier survival analysis of JMS-053-treated orthotopic patient-derived xenograft model (PDX) of recurrent GBM. Mice were treated with JMS-053 or vehicle control from days 7 to 20 days post-injection (n = 8). Vehicle (black), n = 8; JMS-053 (red), n = 8; P value from log-rank (Mantel-Cox) test.
Figure 4

A PTP4A2-ROBO1/β-catenin dephosphorylation axis drives recurrent GBM

(a) Schematic of phospho-proteomic profiling of patient-matched primary and recurrent GBM cells treated with PTP4A phosphatase inhibitor JMS-053 (active compound) or JMS-038 (inactive control). Phosphorylated peptides were quantified following five or 30 min treatment of cells, followed by

(b) Fold change (recurrent/primary) of ‘axonogenesis and neurogenesis’ phospho-peptide levels following PTP4A inhibition (JMS-053/JMS-038) for five min or 30 min.

(c) Immunoblotting of recurrent GBM cultures for ROBO1 post-treatment with DMSO (control) or 10 µM JMS-053. Cultures were grown in spheroid (low binding) conditions, followed by treatment for 96 hours.

(d) Schematic of PTP4A2-mediated direct or indirect dephosphorylation of members belonging to ROBO1-SRGAP-CDC42 signaling axis. Identified dephosphorylation sites are indicated for each member.

(e) Immunoblotting of recurrent GBM cultures treated with DMSO (control) or 10 µM JMS-053 with immunoprecipitated active CDC42-GTP and total CDC42. Cultures were grown in invasive (with 2.5% Matrigel) conditions, followed by drug treatment for 24-96 hours.

(f-g) Recurrent GBM spheroid growth or invasiveness following treatment with DMSO (Control), JMS-053 (4µM or 8µM), control antibody (AXX), 100nM MKRo20 (anti-ROBO1), or combination (4µM JMS-053 and 100nM MKRo20). Representative images (f) and quantification over 120 hours post-treatment (g) are shown.

(h) Immunoblotting of phosphorylated β-catenin Ser552 or total β-catenin following JMS-053 or control treatment in recurrent GBM cultures. Cultures were grown in spheroid (low binding) conditions, followed by drug treatment for 24-96 hours.

(i) Schematic of ROBO1-mediated signaling in normal neurodevelopment (left) and proposed PTP4A2-modulation of tumor cell invasion, stemness and proliferation via a PTP4A2-ROBO1/β-catenin dephosphorylation axis (right). **Left:** SLIT2-mediated ROBO1 signaling inhibits CDC42-driven cell motility and reduces N-cadherin/β-catenin-mediated cell adhesion. **Right:** Proposed PTP4A2-mediated direct or indirect dephosphorylation of the ROBO1-SRGAP-CDC42 axis and β-catenin inhibit tumor cell invasion and stemness, respectively.

**Supplementary Files**

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- TableS1.docx
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- TableS3.xlsx
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