Published in final edited form as:

Cancer Res. 2017 September 15; 77(18): 4947–4960. doi:10.1158/0008-5472.CAN-17-0114.

MYC-regulated Mevalonate Metabolism Maintains Brain Tumor Initiating Cells

Xiuxing Wang¹, Zhi Huang¹, Qiulian Wu¹, Briana C. Prager^{1,2}, Stephen C. Mack¹, Kailin Yang^{1,2}, Leo J.Y. Kim¹, Ryan C. Gimple^{1,2}, Yu Shi¹, Sisi Lai¹, Qi Xie¹, Tyler E. Miller¹, Christopher G. Hubert¹, Anne Song^{1,2}, Zhen Dong¹, Wenchao Zhou¹, Xiaoguang Fang¹, Zhe Zhu¹, Vaidehi Mahadev¹, Shideng Bao^{1,2}, and Jeremy N. Rich^{1,2,*}

¹Department of Stem Cell Biology and Regenerative Medicine, Cleveland Clinic Lerner Research Institute, Cleveland, OH 44195, USA

²Department of Molecular Medicine, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University, Cleveland, OH 44195, USA

Abstract

Metabolic dysregulation drives tumor initiation in a subset of glioblastomas harboring isocitrate dehydrogenase (IDH) mutations, but metabolic alterations in glioblastomas with wildtype IDH are poorly understood. MYC promotes metabolic reprogramming in cancer, but targeting MYC has proven notoriously challenging. Here, we link metabolic dysregulation in patient-derived brain tumor initiating cells (BTICs) to a nexus between MYC and mevalonate signaling, which can be inhibited by statin or 6-fluoromevalonate treatment. BTICs preferentially express mevalonate pathway enzymes, which we find regulated by novel MYC binding sites, validating an additional transcriptional activation role of MYC in cancer metabolism. Targeting mevalonate activity attenuated RAS-ERK-dependent BTIC growth and self-renewal. In turn, mevalonate created a positive feed-forward loop to activate MYC signaling via induction of miR-33b. Collectively, our results argue that MYC mediates its oncogenic effects in part by altering mevalonate metabolism in glioma cells, suggesting a therapeutic strategy in this setting.

Keywords

cancer stem cell; glioblastoma; MYC; mevalonate; metabolism

INTRODUCTION

Glioblastoma is the most prevalent and lethal primary intrinsic brain tumor (1). Despite standard-of-care therapy consisting of maximal surgical resection followed by concurrent chemoradiation and adjuvant chemotherapy, glioblastomas remain universally fatal with

Corresponding should be addressed to: Jeremy N. Rich, MD, MHS, MBA, Department of Stem Cell Biology and Regenerative Medicine, Lerner Research Institute, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, drjeremyrich@gmail.com.

*Current affiliation: Department of Medicine, Division of Regenerative Medicine, University of California San Diego School of Medicine, San Diego, CA 92093, USA

Conflict of interest statement: The authors declare no potential conflicts of interest.

median survival of less than two years, even in patients enrolled in clinical trials (2). Glioblastoma represents one of the most extensively characterized solid cancers, yet the molecular understanding of these cancers has not translated into clinically meaningful therapeutic benefit. Among molecularly targeted therapies, only bevacizumab (Avastin) has been approved by the FDA for glioblastoma therapy, but bevacizumab does not extend overall survival in an unselected patient population (3). Glioblastomas can be segregated based on mutational and transcriptional profiles, but patient outcome and response to therapy has been minimally informed based on these molecular features, beyond a favorable outcome for patients harboring mutations in IDH1 or IDH2, representing fundamentally distinct cancers. Treatment failure in glioblastoma is derived from myriad causes, but prominently stem-like tumor cells, brain tumor initiating cells (BTICs) or glioma stem cells, promote tumor growth through sustained proliferation, self-renewal, therapeutic resistance, angiogenesis, and invasion of normal brain (4, 5). Although BTICs remain controversial due to unresolved questions of cell-of-origin and identification methods, glioblastoma remains one of the most reliable solid cancers for the study of cancer stem cells (6–8). Cancer stem cells, like normal stem cells, are defined by their functional characteristics, including stem cell marker expression, self-renewal, and recapitulation of the tissue from which they are derived (in this case, a heterogeneous tumor) (9). Due to their malignant behavior, therapies targeting cancer stem cells might have significant impact on patient outcome.

MYC is both a central regulator of stem cell biology and a contributor to the genesis of many human cancers, as one of the most common oncogenic events observed in cancer (10, 11). MYC mediates its effects in cancer through its roles as a transcription factor and chromatin regulator, with increasing evidence of a central role in metabolic reprogramming, the Warburg effect (10). Metabolic effects of MYC manifest in induction of glycolysis and glutaminolysis, as well as anabolic support of tumor growth (11). MYC promotes sustained metabolic changes with augmented mitochondrial and ribosome biogenesis (10). In glioma, MYC is over expressed in about 70% of tumors (12, 13). We previously showed that human BTICs preferentially express MYC and depend on its activity for self-renewal and tumor growth (14). Concurrently, the DePinho group showed that a genetically engineered mouse glioma model driven by mutation of Pten and p53 contained sphere-forming cells dependent on MYC (15). Thus, MYC appears to be an important target in BTIC biology. The role of MYC in glioblastoma biology is further supported by a role of TORC2 in controlling glycolytic metabolism through MYC upregulation (16). Despite its importance, or perhaps because of it, MYC has not proven amenable to effective therapeutic targeting, supporting the need for alternative targeting strategies.

The mevalonate metabolic pathway provides cells with bioactive molecules crucial for multiple cellular processes, including cell proliferation, differentiation, and survival (17). The mevalonate pathway produces cholesterol and coenzyme Q, as well molecules involved in signal transduction, including isoprenoid intermediates: geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) (18–20). Hundreds of proteins, including ras family members, undergo geranylgeranylation or farnesylation, permitting proper subcellular localization required for function. The rate-limiting step in mevalonate biology is the enzyme, 3-hydroxyl-methylglutaryl coenzyme A reductase (HMGCR), which is inhibited by statins (18). As would be expected by its central role in metabolism, mevalonate

has been implicated in numerous aspects of tumor development and progression (21). Some cancers have deficient feedback control of HMGCR or increased HMGCR expression and activity, leading to increased mevalonate metabolism (22). Epidemiology studies have suggested that patients prescribed statins for cholesterol control are at lower risk for cancer, and preclinical studies have shown that statins exhibit anti-tumor effects against various leukemia and solid tumor cells through HMGCR inhibition (19, 20). Thus, statins have been proposed as anti-neoplastic agents with acceptable toxicity based on millions of patients treated with these agents.

To date, metabolic crosstalk between the MYC and mevalonate pathways has been poorly understood. Based on the function of MYC in glioblastoma and the lack of effective therapies for this cancer, we interrogated the interplay between MYC and mevalonate in BTIC biology.

MATERIALS AND METHODS

Materials

Simvastatin (S6196; Sigma-Aldrich), 6-Fluoromevalonate (F2929; Sigma-Aldrich), Geranylgeranyl transferase I inhibitor (GGTI-298; Sigma-Aldrich), and U0126 (Cell Signaling Technology) were prepared in DMSO. A farnesyl transferase inhibitor (FTI-277; Sigma-Aldrich) was diluted in PBS. BMP4 (B2680, Sigma-Aldrich) was diluted in PBS. For in vivo studies, simvastatin (50 mg/kg) or vehicle control (DMSO) was administered intraperitoneally one time per day. Antibodies used for immunoblotting and chromatin immunoprecipitation (ChIP) included HMGCR (Abcam, ab174830), FDPS (Abcam, ab38854), MYC (N-262, Santa Cruz #sc-764), total ERK (Cell Signaling #9102), phospho-ERK (Cell Signaling #9101), OLIG2 (Milipore, MABN50), Autophagy Antibody Sampler Kit (Cell Signaling #4445), and α -TUBULIN (Sigma #T6074). ChIP was performed using the EZ-ChIP™ chromatin immunoprecipitation kit (EMD Millipore #17-371). The apoptosis assay was performed using an Apoptosis / Necrosis Detection Kit (blue, green, red) (Abcam, ab176749). Senescence was analyzed by the Senescence β -Galactosidase Staining Kit (CST, 9860). In separate experiments to study the effects of miR-33b, BTICs were either transduced with MISSION® microRNA Mimic hsa-miR-33b (Sigma-Aldrich, HMI0500) or, upon simvastatin treatment, MISSION® synthetic human microRNA inhibitor has-miR-33b (Sigma-Aldrich, HSTUD0500), and the cells were collected after 48 hours for additional assays. The hsa-miR-33b primer set (Thermo Fisher Scientific) was used to detect miR-33b expression.

Isolation and culture of cells

Glioblastoma tissues were obtained from excess surgical resection samples from patients at the Cleveland Clinic after neuropathology review with appropriate consent, in accordance with an IRB-approved protocol (CCF 2259). The patient studies were conducted in accordance with the Declaration of Helsinki. The Brain Tumor Initiating Cells were derived from human specimens at Duke University around 2005 to 2006. The normal neural cell lines were derived from human specimens at Cleveland Clinic around 2015 to 2016. To prevent culture-induced drift, patient-derived xenografts were generated and maintained as a

recurrent source of tumor cells for study. Immediately upon xenograft removal, a papain dissociation system (Worthington Biochemical) was used to dissociate tumors according to the manufacturer's instructions. Cells were then cultured in Neurobasal medium (Life Technologies) supplemented with B27, L-glutamine, sodium pyruvate, 1% penicillin/streptomycin, 10 ng/ml basic fibroblast growth factor (bFGF), and 10 ng/ml epidermal growth factor (EGF) (R&D Systems) for at least 6 hours to recover expression of surface antigens. As no marker is uniformly informative for BTICs, we employed a combination of functional criteria to validate BTICs. Both BTICs and differentiated glioma cells (DGCs) were derived immediately after dissociation or after transient xenograft passage in immunocompromised mice using prospective sorting followed by assays to confirm stem cell marker expression, sphere formation, and secondary tumor initiation. For experiments using matched BTIC and DGC cultures, we segregated AC133 marker-positive and marker-negative populations using CD133/1 antibody conjugated magnetic beads (Miltenyi Biotech, Auburn, CA), as previously described (5, 23). The BTIC phenotype was validated using stem cell marker expression (OLIG2 and SOX2), functional assays of self-renewal (serial neurosphere passage), and tumor propagation by in vivo limiting dilution. Cells were tested for Mycoplasma once every month. Cells were grown at 75% confluency for at least 48 hours. Media was then collected and PCR amplified using the following primers: MP1s-ACACCATGGGAGCTGGTAAT; MP1a- GTTCATCGACTTTCAGACCCAAGGCAT. Cells testing positive for mycoplasma contamination were either discarded or treated with Plasmocin (1:1000; Invivogen, ant-mpp) for two weeks as described by the manufacturer, after which successful eradication of mycoplasma was again confirmed by PCR amplification. STR analyses were performed on each tumor model used in this manuscript every six months for authentication. All cells were thawed within one month of these experimental procedures.

Plasmids and lentiviral transduction

Lentiviral clones expressing two, non-overlapping shRNAs directed against human c-Myc (TRCN0000039640, TRCN0000039642), human HMGCR (TRCN0000233114, TRCN0000233115) or a non-targeting control shRNA that has no targets in human genomes (shCONT: SHC002) were obtained from Sigma-Aldrich (St. Louis, MO). shRNAs with non-overlapping sequences that had the best relative knockdown efficiency were used for all experiments. A lentiviral overexpression plasmid for MYC was generated by cloning the ORF with the N-terminal Flag into the pCDH-MCS-T2A-Puro-MSCV vector (System Biosciences) (24). Lentiviral particles were generated in 293FT cells in Neurobasal complete medium (Life Technologies) with co-transfection of the packaging vectors pCMV-dR8.2 dvpr and pCI-VSVG (Addgene) using a standard calcium phosphate transfection method.

Proliferation and neurosphere formation assay

Cell proliferation was measured using CellTiter-Glo (Promega, Madison, WI). All data were normalized to day 0 and presented as mean $\pm$ SEM. Neurosphere formation was measured by in vitro limiting dilution, as previously described (23, 25). Briefly, decreasing numbers of cells per well (50, 20, 10, 5, and 1) were plated into 96-well plates. Seven days after plating, the presence and number of neurospheres in each well were recorded. Extreme limiting

dilution analysis was performed using software available at <http://bioinf.wehi.edu.au/software/elda>, as previously described (23, 25).

Quantitative RT-PCR

Total cellular RNA was isolated using Trizol reagent (Sigma Aldrich), followed by reverse transcription into cDNA using the qScript cDNA Synthesis Kit (Quanta BioSciences). Real-time PCR was performed using Applied Biosystems 7900HT cycler using SYBR-Green PCR Master Mix (Thermo Fisher Scientific). Sequences for gene-specific primer sets were: human HMGR forward 5'-TGATTGACCTTTCCAGAGCAAG-3' and reverse 5'-CTAAAATTGCCATTCCACGAGC-3'; human PMVK forward 5'-CCTTTCGGAAGGACATGATCC-3' and reverse 5'-TCTCCGTGTGTCACCTACCA-3'; human MVK forward 5'-CATGGCAAGGTAGCACTGG-3' and reverse 5'-GATACCAATGTTGGGTAAGCTGA-3'; human MVD forward 5'-CTCCCTGAGCGTCACTCTG-3' and reverse 5'-GGTCCTCGGTGAAGTCCTTG-3'; human IDI1 forward 5'-AACACTAACCACCTCGACAAGC-3' and reverse 5'-AGACACTAAAAGCTCGATGCAA-3'; human FDPS forward 5'-TGTGACCGGCAAAATTGGC-3' and reverse 5'-GCCCCGTTGCAGACACTGAA-3'; c-Myc forward 5'-GGCTCCTGGCAAAAGGTCA-3' and reverse 5'-CTGCGTAGTTGTGCTGATGT3'; and 18S RNA forward 5'-AACCCGTTGAACCCATT-3' and reverse 5'-CCATCCAATCGGTAGTAGCG-3'. For miR-33b detection, total cellular RNA was isolated using Trizol reagent (Sigma Aldrich), followed by TaqMan® MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, 4366596). Real-time PCR was performed using Applied Biosystems 7500HT cycler using TaqMan® Universal PCR Master Mix, no AmpErase® UNG (Thermo Fisher Scientific). Sequences for the miR-33b primer set are to be found with hsa-miR-33b (Thermo Fisher Scientific).

Western blotting

Cells were collected and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.5% NP-40; 50 mM NaF with protease inhibitors), and incubated on ice for 30 minutes. Lysates were centrifuged at 14,000 rpm at 4°C for 10 minutes, and supernatant was collected. Protein concentration was determined using the Bradford assay (Bio-Rad Laboratories). Equal amounts of protein samples were mixed with SDS Laemmli loading buffer, boiled and electrophoresed using NuPAGE Bis-Tris Gels (Life Technologies), then transferred onto PVDF membranes (Millipore). Blocking was performed for 45 minutes using TBST supplemented with 5% non-fat dry milk and blotting performed with primary antibodies at 4°C for 16 hours.

Chromatin immunoprecipitation (ChIP) assay

4×10^6 cells per condition were collected, 5 µg MYC antibody (N-262, Santa Cruz #sc-764) or rabbit IgG was used for the immunoprecipitation of the DNA-protein immunocomplexes. Crosslinking was reversed by heating for 6 hours at 65°C, followed by digestion with proteinase K. The purified DNA was subjected to quantitative PCR using the following primer sets: HMGR forward 5'-AGATGGTGCGGTGCCTGTT-3' and reverse 5'-AGGAGCCCTCACCTTACGC-3'; PMVK forward 5'-ACATGTGCCACAGCAAATC-3'

and reverse 5'-GCGGCTAGAAAAGCAATCA-3'; MVK forward 5'-CGTGTACCTCTCTGCGTTC-3' and reverse 5'-CCTGGGACAACTGCGTAAC-3'; MVD forward 5'-ACCTGTCTGCTGGTGCTGT-3' and reverse 5'-TCACACAGCCCTTCGCACAG-3'; IDI1 forward 5'-CCTTGAGTGTGTTGCTGTCA-3' and reverse 5'-CGCAGGAGACTAAGAGTTGC-3'; FDPS forward 5'-CGCGACCTCTAGACTTCAGC-3' and reverse 5'-AGTAGTTCCCGCTCTCCCCA-3'.

In Vivo Tumorigenesis

Right cerebral cortex injection at a depth of 3.5 mm of NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ, The Jackson Laboratory) mice was performed using BTICs derived from human glioblastoma specimens. Treatment of mice with simvastatin was started on the seventh day after intracranial implantation of BTICs. Mice were treated with simvastatin at the dose of 50 mg/kg for body weight daily using intraperitoneal injection throughout the period of tumor growth. The control group subjects were treated with the same volume of vehicle control (DMSO) per day. All murine experiments were performed under an animal protocol approved the Cleveland Clinic Institutional Animal Care and Use Committee. Animals were maintained until neurological signs were apparent, at which point they were sacrificed. Brains were harvested and fixed in 4% formaldehyde, cryopreserved in 30% sucrose, and then cryosectioned. Sections were stained with hematoxylin and eosin for histological analysis. In parallel survival experiments, animals were monitored until they developed neurological signs.

Statistical Analysis

No statistical methods were used to predetermine sample sizes, but the choice of sample sizes was consistent with those reported in previous publications (23, 25). All grouped data were presented as mean $\pm$ SEM. Two-sided Student's t-test was used to assess differences between two groups. Kaplan-Meier survival curves were generated using Prism software and log-rank test was performed to assess statistical significance between groups. Correlation between gene expression and patient survival were performed through analysis of The Cancer Genome Atlas (TCGA) and brain tumor data sets downloaded from the TCGA data portal or NCBI GEO database. Raw data from enhancer profiling of primary glioma tissues were deposited at GSE101148. Chip seq data from Suva and colleagues were accessed from NCBI GEO database at GSE54792 (26) and GSE17312 (27).

RESULTS

Brain tumor initiating cells (BTICs) overexpress mevalonate pathway genes

The central role of mevalonate in core cellular metabolism suggested that pathway function may be linked to cellular differentiation state. We, therefore, interrogated mRNA expression levels of several mevalonate pathway genes (*HMGCR*, *PMVK*, *MVK*, *MVD*, *IDI1* and *FDPS*) in multiple BTIC models that we previously functionally validated as having a cellular hierarchy of self-renewal, stem cell marker expression, and tumor propagation (23, 25, 28–30). Each BTIC model expressed high baseline mRNA levels of all six mevalonate genes with substantial reduction of levels upon induction of differentiation (Fig. 1A–F). As serum could contain mevalonate that would possibly modulate mevalonate enzyme

expression levels, we confirmed these data by inducing differentiation in the absence of serum. We treated T387, T3691 and T4121 BTICs with 100 ng/ml of BMP4 in the absence of EGF and FGF for 1 week, then examined six mevalonate genes mRNA levels using real time PCR. Mevalonate genes mRNA levels were reduced upon BMP4-induced differentiation (Supplementary Fig. S1). To determine the potential upstream regulation of mevalonate pathway enzymes, we interrogated the comparative regulation of each gene promoter in normal brain, glioblastoma patient specimens, and three pairs of BTICs and matched DGCs derived from three independent glioblastoma patients (26). Consistent with a potential role for mevalonate metabolism in glioblastoma, the promoter of each key mevalonate gene showed increased levels of active promoters marked by histone 3 lysine 27 acetylation (H3K27ac) in tumors relative to normal brain (Fig. 1G–L). Further, BTICs showed increased activating promoter marks relative to DGCs (Fig. 1G–L). Co-regulation of mevalonate enzymes was additionally supported by substantial co-expression in glioblastomas, as determined by pairwise correlations (Supplementary Fig. S2). Confirming these transcriptional differences translated into differences in protein levels, elevated protein levels of mevalonate enzymes in BTICs and reduction upon differentiation were analyzed by immunoblot (Fig. 1M–O). Collectively, these results support the preferential upregulation of the mevalonate pathway in BTICs.

Mevalonate pathway inhibition preferentially targets brain tumor initiating cells

HMGCR catalyzes the conversion of HMG-CoA to mevalonic acid. As BTICs express elevated *HMGCR* mRNA (Fig. 1A) and protein levels (Fig. 1M–O), we hypothesized that HMGCR promotes BTIC maintenance. We, therefore, interrogated HMGCR function in BTICs using two non-overlapping shRNAs to knock down *HMGCR*, designated shHMGCR-1 and shHMGCR-2. The efficiency of *HMGCR* knockdown was confirmed at the mRNA level by real time PCR (Fig. 2A). Targeting *HMGCR* decreased BTICs growth and cell viability (Fig. 2B and C). Sphere formation represents a surrogate of self-renewal, albeit imperfect. Using an in vitro limiting dilution assay with BTICs expressing shCONT, shHMGCR-1 or shHMGCR-2, knockdown of HMGCR impaired self-renewal capacity, as measured by neurosphere formation (Fig. 2D and E). Sphere size, considered a surrogate of proliferation, was also reduced in BTICs expressing shRNAs targeting *HMGCR* compared to control shRNA (Fig. 2F). As in vivo tumor growth represents the most important BTIC phenotype, BTICs expressing shHMGCR or non-targeting shCONT were implanted into the brains of immunocompromised mice to interrogate the effect of HMGCR in tumor initiation in vivo. Mice bearing BTICs expressing shHMGCR demonstrated increased survival relative to shCONT mice, establishing the role of HMGCR in maintaining tumorigenic capacity of BTICs in vivo (Fig. 2G and H). These results establish that the HMGCR is essential to maintain BTICs proliferation, self-renewal, and tumorigenicity.

To evaluate the clinical relevance of mevalonate pathway activation in BTICs, we interrogated the TCGA glioblastoma database for the rate-limiting mevalonate enzyme, HMGCR. Befitting a role in glioblastoma biology, HMGCR levels were associated with poor prognosis in glioblastoma patients in The Cancer Genome Atlas (TCGA) with a mesenchymal transcriptional profile, which has been associated with tumor progression after therapy (Ref. (31); Fig. 3A). Further in silico analyses of the Ivy Glioblastoma Atlas Project

(Ivy GAP) and TCGA revealed that the expression of mevalonate genes was enriched in areas of infiltrating tumor and in oligodendroglioma, respectively (Supplementary Fig. S3A and B). To leverage these results into clinically actionable findings, we treated BTICs with statins, which function predominantly through disruption of the mevalonate pathway, to examine pathway function in BTIC maintenance. Statin (simvastatin) treatment inhibited BTIC viability and cell proliferation (Fig. 3B and C). Cell cycle analysis of statin-treated BTICs revealed arrest in G₁ and reduction of the S phase, as measured by flow cytometry (Supplementary Fig. S4A–C). Using an *in vitro* limiting dilution assay, we demonstrated that statin treatment reduced the frequency of sphere formation (Fig. 3D–F). In contrast, statin treatment did not significantly alter cellular proliferation in matched DGCs (Supplementary Fig. S5A–C), supporting a dispensable role of mevalonate pathway in differentiated tumor cells. To determine the cellular mechanism by which statin therapy reduced BTIC cell numbers, we ruled out effects on autophagy (Supplementary Fig. S6) and senescence (Supplementary Fig. S7A–C), but we detected induction of apoptosis in statin-treated BTICs (Supplementary Fig. S8A and B). As all drugs have off-target effects, we investigated the effects of another mevalonate pathway inhibitor, 6-fluoromevalonate, which has a mechanism of action distinct from statins. Similar inhibitory effects on BTIC proliferation were detected with 6-fluoromevalonate treatment, supporting BTIC dependence on mevalonate metabolism (Fig. 3G–I). Collectively, these results demonstrate that the mevalonate pathway is essential specifically for BTIC growth.

Mevalonate inhibition prolongs survival of tumor-bearing mice associated with activation of the Ras-Erk pathway

Mevalonate generates isoprenoid intermediates, including GGPP and FPP (21), which bind to Ras family members and other guanidine triphosphate (GTP)-binding proteins to facilitate proper intracellular localization and function (32). We, therefore, investigated the roles of the prenylation transferases catalyzing geranylgeranylation and farnesylation in BTIC maintenance. Befitting the activation of key oncogenic pathways, including ras family members, by prenyl post-translational modifications, treatment with either a farnesyl transferase inhibitor (FTI) or geranylgeranyl transferase inhibitor (GGTI) impaired BTIC viability and sphere formation (Supplementary Fig. S9A–F).

Having elucidated mevalonate pathway function in BTICs *in vitro*, we examined mevalonate function *in vivo*. BTICs were implanted into the brains of immunocompromised mice, and tumor-bearing hosts were treated systemically with a mevalonate pathway inhibitor. In two BTIC models derived from different patients, statin treatment extended the survival of tumor-bearing mice relative to vehicle control (Fig. 4A and B). Reduced tumor size upon statin therapy was confirmed by histology (Fig. 4C and D). Although we expected inhibition of the mevalonate pathway to block prenylation and, thus, ras activation, tumors derived from statin-treated mice demonstrated a modest increase in activated ERK levels (Fig. 4E and F).

To more thoroughly elucidate the molecular mechanism involved in mevalonate metabolism, we examined the impact of targeting mevalonate pathway function on ras activation by treating BTICs with two inhibitors with distinct mechanisms, simvastatin and 6-

fluoromevalonate, and found that ERK activity was increased upon treatment, confirming the in vivo findings (Fig. 5A and B). In contrast, simvastatin treatment of normal primary neural cells failed to activate ERK, supporting a BTIC-specific link between mevalonate and ERK (Fig. 5C). ERK activity progressively increased in simvastatin-treated BTICs over a 48-hour time course (Fig. 5D). To determine if activation of ERK was involved in the mechanism of statin treatment, MEK inhibitor (U0126) treatment rescued the decreased cell viability caused by simvastatin therapy (Fig. 5E–G). These results suggest that targeting the mevalonate pathway in BTICs attenuates tumor initiation through RAS-ERK pathway activation.

MYC is an upstream regulator of the mevalonate pathway

Based on our prior studies demonstrating specific expression of MYC in BTICs and the role of MYC in BTIC metabolism, we hypothesized that MYC may regulate the expression of the enzymes in the mevalonate pathway. Analyzing the promoters of the six major mevalonate enzymes revealed that each contained MYC binding sites (data not shown). To determine the potential regulation of these genes by MYC, we targeted MYC expression in BTICs using lentiviral transduced shRNA (Fig. 6A), revealing marked reduction in the expression levels of all six mevalonate pathway genes at the mRNA (Fig. 6B–G) and protein levels (Fig. 6H), compared to control, non-targeting shRNA. As further support to these loss-of-function studies, we performed gain-of-function studies in differentiated tumor cell progeny; MYC overexpression in DGCs (Fig. 6I) induced increased expression of all six mevalonate pathway genes at the mRNA (Fig. 6J–O) and protein levels (Fig. 6P). To confirm direct binding of MYC to the relevant gene promoters, we performed chromatin immunoprecipitation followed by PCR (ChIP-PCR) for all six genes, revealing an enrichment of binding compared to the IgG control (Fig. 6Q and R). MYC regulates the expression of numerous molecular targets, so we interrogated the contribution of mevalonate metabolism in the effects of targeting MYC expression. The addition of FPP, one of the isoprenoid intermediates generated in mevalonate metabolism, partially rescued the defect in cell viability, sphere number and sphere size of BTICs induced by shMYC (Supplementary Fig. S10A–C). These results demonstrate that MYC directly regulates the transcription of mevalonate pathway genes through binding to the promoter regions of these genes to maintain BTIC growth.

Mevalonate regulates MYC in brain tumor initiating cells through miR-33b

Based on the regulation of oncogene activation by mevalonate, we investigated the co-expression of mevalonate genes and *MYC* mRNA levels in glioblastoma. In glioblastomas, *MYC* mRNA levels correlated with a mevalonate gene signature in the TCGA database (Fig. 7A). As mevalonate can activate oncogenes, we investigated the possibility of a feed-forward regulatory loop between mevalonate and MYC, as mevalonate may be both an upstream and downstream regulatory node of MYC. Indeed, targeting mevalonate pathway function through statin treatment of BTICs attenuated MYC expression at both the mRNA and protein levels (Fig. 7B and C). To validate these results as specific to mevalonate inhibition, treatment with 6-fluoromevalonate also reduced MYC mRNA and protein levels (Fig. 7D and E). To confirm these results in vivo, 3691 BTICs was implanted into the immunocompromised mice, and tumor-bearing mice were treated with simvastatin for 2

weeks. Statin treatment decreased MYC mRNA and protein levels in vivo (Supplementary Fig. S11A and B). As mevalonate inhibition activates ERK to mediate its biologic effects, we investigated the effects of the MEK inhibitor, U0126, on the statin effects on MYC expression in BTICs, rescuing MYC expression levels depleted by statin therapy (Fig. 7F and G). To further define the mechanistic link between mevalonate and MYC, we examined mevalonate regulation of a miR previously reported to regulate MYC levels, miR-33b (33, 34). After statin therapy, miR-33b expression levels increased in vitro (Fig. 7H) and in vivo (Supplementary Fig. S11C). Further, treatment with a MEK inhibitor (U0126) blocked statin-mediated increase in miR-33b expression, suggesting miR-33b regulation by MEK-ERK signaling (Fig. 7H). To determine the relative position of MYC and miR-33b downstream of mevalonate, we treated BTICs with a miR-33b mimic, finding decreased MYC mRNA and protein levels (Fig. 7I and J). Further supporting the linkage between miR-33b and MYC, transducing BTICs with a miR-33b inhibitor partially rescued simvastatin-mediated decrease in MYC (Supplementary Fig. S12A) and loss of proliferation in BTIC (Supplementary Fig. S12B). Collectively, these results demonstrate that the mevalonate pathway regulates MYC through a RAS-ERK-miR33b pathway, creating a feed-forward loop.

DISCUSSION

Metabolic dysregulation represents a hallmark of many advanced cancers. In gliomas, mutations in the metabolic enzymes IDH1 or IDH2 are tumor initiating mutations that define distinct genetic tumor subgroups with favorable prognoses. While IDH wildtype tumors may not share such a central link to metabolism in tumor initiation, many of the common genetic lesions (e.g. Pten deletion, PIK3CA mutations, EGFR mutation and amplification, etc.) have been directly linked to cellular metabolism. In the current studies, we focused our efforts on the mevalonate metabolic pathway, which creates essential metabolites including those involved in the post-translational modification of signaling molecules, especially the RAS family members. MYC and RAS represent two of the most powerful canonical oncogenes. We and other previously reported that MYC is preferentially expressed in BTICs (14, 15). In our studies, we now connect MYC to RAS pathway regulation in BTICs through mevalonate metabolism.

Ras family members undergo prenylation to direct their localization to selected membrane compartments to permit participation in signaling. While ras mutations are rare in glioblastoma (35, 36), ras activity is commonly increased in glioblastomas, potentially through alterations of growth factor signaling or NF1 mutations. Some pediatric brain tumors, notably pilocytic astrocytomas, harbor mutations or fusion in downstream effectors of ras, specifically BRAF (37) (38). Mutant H-ras has been a frequent tool in the generation of genetically engineered glioma models. Collectively, these results have stimulated the translation of ras-targeting approaches into clinical trial for glioma patients, including farnesyl transferase inhibitors and raf inhibitors. Unfortunately, the results of these trials, to date, have been disappointing, although trials with novel antagonists, including dabrafenib and selumetinib, are underway.

Our results suggest that mevalonate inhibition, which can be disrupted with statins or genetic targeting of HMGCR, could have high therapeutic indices that may be used as monotherapy or in combination with other anti-neoplastics. The therapeutic use of statins is an example of drug repositioning, which involves the use of agents developed for other indications for the treatment of cancer (39). Previous preclinical studies also indicate that statins could be used as anticancer agents in glioblastoma (40, 41). Statins induce apoptosis in C6 rat glioma cells when GGPP biosynthesis is inhibited and consequently decrease the level of phosphorylated ERK1/2 and AKT (42). In malignant glioma cells, statins induce apoptosis by the activation of JNK1/2 or by increasing the expression of BIM (41, 43). However, micromolar concentrations of statins can cause induction of apoptosis in normal cells, such as primary cultures of neuronal brain cortices (44, 45). Our studies are distinguished from the prior studies, which were focused on established cell lines, like U87, which poorly represent BTICs, so may underestimate the value of these agents. Cohort studies have suggested contradictory conclusions with regard to correlation between use of preoperative statin use and decreased risk of glioma formation and mortality (46, 47). Due to the small number of patients, retrospective nature of these studies and more importantly, lack of sustained use of statins, further work is needed to understand the role of statins in glioblastoma therapy. The low toxicity of statins suggests that their addition to clinical paradigms may hold benefit with a large therapeutic index.

In our studies, the activation of ERK signaling with statin treatment appears counterintuitive initially, as ERK family members are downstream of the ras family members, so might be expected to be inactivated with mevalonate pathway inhibition. However, ERK function in central nervous system development is complex and context-dependent with genetic models demonstrating key roles in fate determination between neuronal and astrocyte lineages (48). Further, statin-induced apoptosis may force surviving cells to activate ERK to promote survival. The interplay between ras signaling and mevalonate also serves to feedforward onto MYC, with its myriad effects on cellular differentiation state and tumor growth through miR-33b, suggesting that miR-33b and ERK activation may represent early therapeutic response biomarkers to statin therapy.

While statin treatment and other agents targeting mevalonate appear somewhat promising, our studies and the limited clinical trial data would suggest that clinical efficacy will require combination with other agents. As radiation therapy is the most effective non-surgical therapy and we previously showed that BTICs are preferentially resistant to radiation (5), we expect that future studies will focus on the development of combinatorial efforts. In fact, statin use has been suggested in the context of other cancers in which cardiovascular effects of radiation may be partially ameliorated by statin use. As radionecrosis and radiation-induced stroke complicates brain tumor treatment, statins may represent agents that may have substantial efficacy against tumors, while reducing treatment toxicity. In conclusion, our results demonstrate that mevalonate metabolism may serve as an important therapeutic target against cancer stem cells with limited toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We appreciate flow cytometry assistance from C. Shemo and S. O'Bryant, and the glioblastoma tissue provided by M. McGraw and the Cleveland Clinic Tissue Procurement Service. We thank members of the Rich lab laboratory for critical reading of the manuscript and helpful discussions.

Financial support: The studies were funded by the National Institutes of Health grants CA197718, CA154130, CA169117, CA171652, NS087913, NS089272 (J.N.Rich), CA184090, NS091080, NS099175 (S.Bao).

References

1. Ostrom QT, Gittleman H, de Blank PM, Finlay JL, Gurney JG, McKean-Cowdin R, et al. American Brain Tumor Association Adolescent and Young Adult Primary Brain and Central Nervous System Tumors Diagnosed in the United States in 2008–2012. *Neuro Oncol.* 2016; 18(Suppl 1):i1–i50. [PubMed: 26705298]
2. Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol.* 2009; 10:459–66. [PubMed: 19269895]
3. Gilbert MR, Dignam JJ, Armstrong TS, Wefel JS, Blumenthal DT, Vogelbaum MA, et al. A randomized trial of bevacizumab for newly diagnosed glioblastoma. *N Engl J Med.* 2014; 370:699–708. [PubMed: 24552317]
4. Bao S, Wu Q, Sathornsumetee S, Hao Y, Li Z, Hjelmeland AB, et al. Stem cell-like glioma cells promote tumor angiogenesis through vascular endothelial growth factor. *Cancer Res.* 2006; 66:7843–8. [PubMed: 16912155]
5. Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature.* 2006; 444:756–60. [PubMed: 17051156]
6. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, et al. Identification of human brain tumour initiating cells. *Nature.* 2004; 432:396–401. [PubMed: 15549107]
7. Galli R, Binda E, Orfanelli U, Cipelletti B, Gritti A, De Vitis S, et al. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer Res.* 2004; 64:7011–21. [PubMed: 15466194]
8. Hemmati HD, Nakano I, Lazareff JA, Masterman-Smith M, Geschwind DH, Bronner-Fraser M, et al. Cancerous stem cells can arise from pediatric brain tumors. *Proc Natl Acad Sci U S A.* 2003; 100:15178–83. [PubMed: 14645703]
9. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature.* 2001; 414:105–11. [PubMed: 11689955]
10. Hsieh AL, Walton ZE, Altman BJ, Stine ZE, Dang CV. MYC and metabolism on the path to cancer. *Semin Cell Dev Biol.* 2015; 43:11–21. [PubMed: 26277543]
11. Dang CV. MYC on the path to cancer. *Cell.* 2012; 149:22–35. [PubMed: 22464321]
12. Herms JW, von Loewenich FD, Behnke J, Markakis E, Kretzschmar HA. c-myc oncogene family expression in glioblastoma and survival. *Surg Neurol.* 1999; 51:536–42. [PubMed: 10321885]
13. Annibali D, Whitfield JR, Favuzzi E, Jauset T, Serrano E, Cuartas I, et al. Myc inhibition is effective against glioma and reveals a role for Myc in proficient mitosis. *Nat Commun.* 2014; 5:4632. [PubMed: 25130259]
14. Wang J, Wang H, Li Z, Wu Q, Lathia JD, McLendon RE, et al. c-Myc is required for maintenance of glioma cancer stem cells. *PLoS One.* 2008; 3:e3769. [PubMed: 19020659]
15. Zheng H, Ying H, Yan H, Kimmelman AC, Hiller DJ, Chen AJ, et al. p53 and Pten control neural and glioma stem/progenitor cell renewal and differentiation. *Nature.* 2008; 455:1129–33. [PubMed: 18948956]
16. Masui K, Tanaka K, Akhavan D, Babic I, Gini B, Matsutani T, et al. mTOR complex 2 controls glycolytic metabolism in glioblastoma through FoxO acetylation and upregulation of c-Myc. *Cell Metab.* 2013; 18:726–39. [PubMed: 24140020]

17. Buhaescu I, Izzedine H. Mevalonate pathway: a review of clinical and therapeutical implications. *Clin Biochem.* 2007; 40:575–84. [PubMed: 17467679]
18. Winter-Vann AM, Casey PJ. Opinion - Post-prenylation-processing enzymes as new targets in oncogenesis. *Nat Rev Cancer.* 2005; 5:405–12. [PubMed: 15864282]
19. Konstantinopoulos PA, Karamouzis MV, Papavassiliou AG. Post-translational modifications and regulation of the RAS superfamily of GTPases as anticancer targets. *Nat Rev Drug Discov.* 2007; 6:540–55.
20. Ahearn IM, Haigis K, Bar-Sagi D, Philips MR. Regulating the regulator: post-translational modification of RAS. *Nature Reviews Molecular Cell Biology.* 2012; 13:39–51.
21. Thurnher M, Nussbaumer O, Gruenbacher G. Novel aspects of mevalonate pathway inhibitors as antitumor agents. *Clin Cancer Res.* 2012; 18:3524–31. [PubMed: 22529099]
22. Bjarnadottir O, Romero Q, Bendahl PO, Jirstrom K, Ryden L, Loman N, et al. Targeting HMG-CoA reductase with statins in a window-of-opportunity breast cancer trial. *Breast Cancer Res Tr.* 2013; 138:499–508.
23. Flavahan WA, Wu Q, Hitomi M, Rahim N, Kim Y, Sloan AE, et al. Brain tumor initiating cells adapt to restricted nutrition through preferential glucose uptake. *Nat Neurosci.* 2013; 16:1373–82. [PubMed: 23995067]
24. Fang X, Huang Z, Zhou W, Wu Q, Sloan AE, Ouyang G, et al. The zinc finger transcription factor ZFX is required for maintaining the tumorigenic potential of glioblastoma stem cells. *Stem Cells.* 2014; 32:2033–47. [PubMed: 24831540]
25. Xie Q, Wu Q, Horbinski CM, Flavahan WA, Yang K, Zhou W, et al. Mitochondrial control by DRP1 in brain tumor initiating cells. *Nat Neurosci.* 2015; 18:501–10. [PubMed: 25730670]
26. Suva ML, Rheinbay E, Gillespie SM, Patel AP, Wakimoto H, Rabkin SD, et al. Reconstructing and reprogramming the tumor-propagating potential of glioblastoma stem-like cells. *Cell.* 2014; 157:580–94. [PubMed: 24726434]
27. Bernstein BE, Stamatoyannopoulos JA, Costello JF, Ren B, Milosavljevic A, Meissner A, et al. The NIH Roadmap Epigenomics Mapping Consortium. *Nat Biotechnol.* 2010; 28:1045–8. [PubMed: 20944595]
28. Schonberg DL, Miller TE, Wu Q, Flavahan WA, Das NK, Hale JS, et al. Preferential Iron Trafficking Characterizes Glioblastoma Stem-like Cells. *Cancer Cell.* 2015; 28:441–55. [PubMed: 26461092]
29. Xie Q, Wu Q, Kim L, Miller TE, Liao BB, Mack SC, et al. RBPI maintains brain tumor-initiating cells through CDK9-mediated transcriptional elongation. *J Clin Invest.* 2016; 126:2757–72. [PubMed: 27322055]
30. Yan K, Wu Q, Yan DH, Lee CH, Rahim N, Tritschler I, et al. Glioma cancer stem cells secrete Gremlin1 to promote their maintenance within the tumor hierarchy. *Genes Dev.* 2014; 28:1085–100. [PubMed: 24788093]
31. Bhat KP, Balasubramaniyan V, Vaillant B, Ezhilarasan R, Hummelink K, Hollingsworth F, et al. Mesenchymal differentiation mediated by NF-kappaB promotes radiation resistance in glioblastoma. *Cancer Cell.* 2013; 24:331–46. [PubMed: 23993863]
32. Konstantinopoulos PA, Karamouzis MV, Papavassiliou AG. Post-translational modifications and regulation of the RAS superfamily of GTPases as anticancer targets. *Nat Rev Drug Discov.* 2007; 6:541–55. [PubMed: 17585331]
33. Xu N, Li Z, Yu Z, Yan F, Liu Y, Lu X, et al. MicroRNA-33b suppresses migration and invasion by targeting c-Myc in osteosarcoma cells. *PLoS One.* 2014; 9:e115300. [PubMed: 25546234]
34. Takwi AA, Li Y, Becker Buscaglia LE, Zhang J, Choudhury S, Park AK, et al. A statin-regulated microRNA represses human c-Myc expression and function. *EMBO Mol Med.* 2012; 4:896–909. [PubMed: 22887866]
35. Knobbe CB, Reifemberger J, Reifemberger G. Mutation analysis of the Ras pathway genes NRAS, HRAS, KRAS and BRAF in glioblastomas. *Acta Neuropathol.* 2004; 108:467–70. [PubMed: 15517309]
36. Ceccarelli M, Barthel FP, Malta TM, Sabedot TS, Salama SR, Murray BA, et al. Molecular Profiling Reveals Biologically Discrete Subsets and Pathways of Progression in Diffuse Glioma. *Cell.* 2016; 164:550–63. [PubMed: 26824661]

37. Jones DT, Kocalkowski S, Liu L, Pearson DM, Backlund LM, Ichimura K, et al. Tandem duplication producing a novel oncogenic BRAF fusion gene defines the majority of pilocytic astrocytomas. *Cancer Res.* 2008; 68:8673–7. [PubMed: 18974108]
38. Pfister S, Janzarik WG, Remke M, Ernst A, Werft W, Becker N, et al. BRAF gene duplication constitutes a mechanism of MAPK pathway activation in low-grade astrocytomas. *J Clin Invest.* 2008; 118:1739–49. [PubMed: 18398503]
39. Shchors K, Massaras A, Hanahan D. Dual Targeting of the Autophagic Regulatory Circuitry in Gliomas with Repurposed Drugs Elicits Cell-Lethal Autophagy and Therapeutic Benefit. *Cancer Cell.* 2015; 28:456–71. [PubMed: 26412325]
40. Bouterfa HL, Sattelmeyer V, Czub S, Vordermark D, Roosen K, Tonn JC. Inhibition of Ras farnesylation by lovastatin leads to downregulation of proliferation and migration in primary cultured human glioblastoma cells. *Anticancer Res.* 2000; 20:2761–71. [PubMed: 10953355]
41. Jiang P, Mukthavaram R, Chao Y, Nomura N, Bharati IS, Fogal V, et al. In vitro and in vivo anticancer effects of mevalonate pathway modulation on human cancer cells. *Br J Cancer.* 2014; 111:1562–71. [PubMed: 25093497]
42. Yanae M, Tsubaki M, Satou T, Itoh T, Imano M, Yamazoe Y, et al. Statin-induced apoptosis via the suppression of ERK1/2 and Akt activation by inhibition of the geranylgeranyl-pyrophosphate biosynthesis in glioblastoma. *J Exp Clin Cancer Res.* 2011; 30:74. [PubMed: 21831290]
43. Koyuturk M, Ersoz M, Altioek N. Simvastatin induces proliferation inhibition and apoptosis in C6 glioma cells via c-jun N-terminal kinase. *Neurosci Lett.* 2004; 370:212–7. [PubMed: 15488325]
44. Michikawa M, Yanagisawa K. Inhibition of cholesterol production but not of nonsterol isoprenoid products induces neuronal cell death. *J Neurochem.* 1999; 72:2278–85. [PubMed: 10349836]
45. Schulz JG, Bosel J, Stoeckel M, Megow D, Dirnagl U, Endres M. HMG-CoA reductase inhibition causes neurite loss by interfering with geranylgeranylpyrophosphate synthesis. *J Neurochem.* 2004; 89:24–32. [PubMed: 15030386]
46. Gaist D, Hallas J, Friis S, Hansen S, Sorensen HT. Statin use and survival following glioblastoma multiforme. *Cancer Epidemiol.* 2014; 38:722–7. [PubMed: 25455652]
47. Bhavsar S, Hagan K, Arunkumar R, Potylchansky Y, Grasu R, Dang A, et al. Preoperative statin use is not associated with improvement in survival after glioblastoma surgery. *J Clin Neurosci.* 2016; 31:176–80. [PubMed: 27396375]
48. Samuels IS, Saitta SC, Landreth GE. MAP'ing CNS development and cognition: an ERKsome process. *Neuron.* 2009; 61:160–7. [PubMed: 19186160]

followed by deep sequencing (ChIP-seq) for five primary glioblastoma tumors, one normal brain tissue, and three matched pairs of BTICs and DGCs from patient-derived glioblastoma specimens. H3K27ac ChIP-seq data were downloaded from NCBI Gene Expression Omnibus (GEO) GSE54047. **M–O**, BTICs upregulate protein levels of mevalonate synthesis enzymes relative to DGCs. Protein levels for mevalonate enzymes (HMGCR and FDPS) and a BTIC marker (OLIG2) were assessed by immunoblotting in three matched pairs of BTICs and DGCs derived from three glioblastoma patient-derived xenografts (T3691, T387, T4121). TUBULIN was used as a loading control.

Figure 2. HMGR regulates BTIC growth and self-renewal

A, Real time PCR quantification of *HMGR* mRNA levels after transducing two BTIC models, T3691 and T387, with a control, non-targeting shRNA sequence (shCONT) or one of two independent shRNAs (designated as shHMGR-1 and shHMGR-2). Data are presented as mean $\pm$ SEM from three independent experiments. **, $p < 0.01$. **B**, Two independent shRNAs targeting HMGR decreased the growth of T387 (top) and T3691 (bottom) BTICs in comparison to shCONT, as measured by a CellTiter-Glo assay. Data are presented as mean $\pm$ SEM from six independent experiments. **, $p < 0.01$. **C**, Two independent shRNAs targeting HMGR decreased the growth of T387 (top) and T3691

(bottom) BTICs in comparison to a non-targeting control shRNA (shCONT), as measured by direct cell number count. Data are presented as mean $\pm$ SEM from four independent experiments. **, $p < 0.01$. **D**, In vitro extreme limiting dilution assays (ELDA) demonstrate that knockdown of HMGCRC in T387 (top) and T3691 (bottom) BTICs decreased the frequency of neurosphere formation. **E**, Knockdown of HMGCRC in T387 (top) and T3691 (bottom) BTICs decreased the number of spheres formed in ELDA (per 1,000 cells seeded). Data are presented as mean $\pm$ SEM from six independent experiments. **, $p < 0.01$. **F**, Representative images of neurospheres derived from T387 (left) and T3691 (right) BTICs expressing shCONT, shHMGCRC-1, or shHMGCRC-2. Scale bar, 400 μ m. **G**, Kaplan-Meier survival curves of immunocompromised mice bearing intracranial T387 (top) or T3691 (bottom) BTICs expressing shCONT and two independent HMGCRC shRNA. $p < 0.0001$. 5 mice were used in each group. **H**, Representative images of hematoxylin and eosin stained cross-sections of mouse brains harvested on day 18 after transplantation of T387 (top) or T3691 (bottom) BTICs expressing shCONT and two independent HMGCRC shRNA. Scale bar, 2 mm.

measured by CellTiter Assay. Data are presented as mean $\pm$ SEM from six independent experiments. **, $p < 0.01$. **D**, Representative images of neurospheres derived from T387, T4121 and T3691 BTICs following treatment with simvastatin (2 μ M, 5 days). Scale bar, 400 μ m. **E**, *In vitro* extreme limiting dilution assays (ELDA) demonstrated that simvastatin (2 μ M, 5 days) decreased the frequency of neurosphere formation in T387, T4121 and T3691 BTICs. **F**, Treatment with simvastatin (2 μ M, 5 days) decreased clonal growth of T387, T4121 and T3691 BTICs compared to vehicle control (DMSO), as measured by neurosphere formation (measured per 1,000 cells seeded). Data are presented as mean $\pm$ SEM from six independent experiments. **, $p < 0.01$. **G**, Treatment with a distinct mevalonate pathway inhibitor, 6-fluoromevalonate (6-Fluoro, 10 μ M for 1, 3, and 5 days), decreased the growth of T387, T4121 and T3691 BTICs in comparison to DMSO, as measured by direct cell number count. Data are presented as mean $\pm$ SEM from six independent experiments. **, $p < 0.01$. **H**, Treatment with a distinct mevalonate pathway inhibitor, 6-fluoromevalonate (6-Fluoro, 10 μ M for 1, 3, and 5 days), decreased the growth of T387, T4121 and T3691 BTICs in comparison to DMSO, as measured by cell-titer assay. Data are presented as mean $\pm$ SEM from six independent experiments. **, $p < 0.01$. **I**, Representative images of neurospheres derived from T387, T4121 and T3691 BTICs following treatment with 6-fluoromevalonate (10 μ M for 5 days). Scale bar, 400 μ m.

Figure 4. Simvastatin inhibits BTIC growth and prolonged survival of mice bearing intracranial tumors

A and **B**, Kaplan-Meier survival curves of immunocompromised mice bearing intracranial T387 (**A**) or T3691 (**B**) BTICs treated with simvastatin. Five mice were used in each group. **C** and **D**, Representative images of hematoxylin and eosin stained cross-sections of mouse brains harvested day 35 after transplantation of T387 (**C**) and T3691 (**D**) BTICs treated with simvastatin. Scale bar, 2 mm. **E** and **F**, BTICs upregulate RAS activity after simvastatin treatment. RAS activity was assessed by Ras Activation Assay Kit in BTICs derived from patient-derived xenografts (T3691 and T387).

Figure 5. Simvastatin selectively inhibits BTIC growth through ERK pathway activation
A and **B**, BTICs (T3691, T387, and T4121) were treated with mevalonate pathway inhibitors, simvastatin (**A**, 2 μ M for 48 hours) or 6-fluoromevalonate (**B**, 10 μ M for 48 hours), at 2 μ M for 48 hours. Whole cell lysates were resolved by SDS-PAGE, and levels of phospho-ERK and total ERK were assessed by immunoblot. Tubulin was used as a loading control. **C**, Five normal neural cell cultures (NM32, NM53, NM55, NM97, and ENSA) were treated with simvastatin (2 μ M) for 48 hours. Whole cell lysates were resolved by SDS-PAGE, and levels of phospho-ERK and total ERK were assessed by immunoblot. Tubulin was used as a loading control. **D**, BTICs (T3691, T387, and T4121) were treated with

simvastatin (2 μ M) over a 48-hour time course. Whole cell lysates were resolved by SDS-PAGE, and levels of phospho-ERK and total ERK were assessed by immunoblot. **E**, MAPK-ERK pathway inhibitor, U0126, treatment partially rescued the cell growth defect of BTICs (T387 and T3691) treated with simvastatin, as measured by cell-titer assay. Data are presented as mean $\pm$ SEM from six independent experiments. **, $p < 0.01$. **F**, MAPK-ERK pathway inhibitor, U0126, treatment partially rescued the cell growth defect of BTICs (T387 and T3691) treated with simvastatin, as measured by direct cell number count. Data are presented as mean $\pm$ SEM from four independent experiments. **, $p < 0.01$. **G**, Representative images of neurospheres derived from T387 (left) and T3691 (right) BTICs treated with simvastatin alone, U0126 alone, and simvastatin combined with U0126. Scale bar, 400 μ m.

Figure 6. MYC regulates *de novo* mevalonate synthesis pathway enzymes in BTICs

A – G, mRNA levels of *MYC* (**A**) and mevalonate enzymes – *HMGCR* (**B**), *PMVK* (**C**), *MVK* (**D**), *MVD* (**E**), *IDI1* (**F**), and *FDPS* (**G**) -- were assayed by qRT-PCR in T387 and T3691 BTICs upon *MYC* knockdown using two independent shRNAs. Data are presented as mean ± SEM from three independent experiments. **, $p < 0.01$. **H**, Knockdown of *MYC* in BTICs (T387 and T3691) using two independent shRNAs decreased protein levels of *HMGCR* and *FDPS* assessed by immunoblot in T387 and T3691 BTICs expressing shCONT, shMYC-1, or shMYC-2. TUBULIN was used as a loading control. **I – N**, *MYC* overexpression (**I**) upregulates mRNA levels of enzymes involved in the mevalonate synthesis pathway – *HMGCR* (**J**), *PMVK* (**K**), *MVK* (**L**), *MVD* (**M**), *IDI1* (**N**) and *FDPS* (**O**). **P**, Immunoblot of *MYC*, *HMGCR*, *FDPS*, and Tubulin in T387 and T3691 BTICs expressing Vector or MYC. **Q**, Fold change of enzymes in T387 BTICs upon MYC overexpression. **R**, Fold change of enzymes in T3691 BTICs upon MYC overexpression. Data are presented as mean ± SEM from three independent experiments. **, $p < 0.01$.

(**O**) -- in differentiated glioma cells (T387 and T3691) transduced with either an empty vector (Vector) or a *MYC* overexpression vector (*MYC*), as measured by quantitative RT-PCR. Data are presented as mean $\pm$ SEM from three independent experiments. *, $p < 0.05$; **, $p < 0.01$. **P**, *MYC* overexpression upregulates protein levels of enzymes involved in the mevalonate synthesis pathway – HMGCR and FDPS -- in differentiated glioma cells (T387 and T3691) expressing an empty vector (Vector) or a *MYC* overexpression vector (*MYC*), as measured by immunoblot. TUBULIN was used as a loading control. **Q** and **R**, *MYC* binds directly to promoter regions of enzymes in Mevalonate synthesis pathway. Cross-linked chromatin was prepared from T387 (**Q**) and T3691 (**R**) BTICs, and then immunoprecipitated using an anti-*MYC* antibody or rabbit IgG control followed by real-time PCR using primers specific to promoter regions of *HMGCR*, *PMVK*, *MVK*, *MVD*, *IDII* and *FDPS*. Data are presented as mean $\pm$ SEM from three independent experiments. **, $p < 0.01$.

Figure 7. The mevalonate pathway regulates MYC expression through mir-33b

A, MYC activation significantly correlates with a transcriptional signature of mevalonate synthesis in TCGA glioblastoma samples. Single sample GSEA scores were calculated for TCGA RNA-seq data using a MYC activation signature derived from the Broad Institute Molecular Signatures Database and from a six-gene signature of mevalonate pathway genes. Shading indicates 95% confidence interval. **B**, MYC mRNA levels were measured in BTICs (T3691, T387, and T4121) by qRT-PCR upon treatment with vehicle control (DMSO) or simvastatin (2 μ M) for 48 hours. Data are presented as mean $\pm$ SEM from three independent experiments. **, $p < 0.01$. **C**, BTICs (T3691, T387, and T4121) were treated with simvastatin (2 μ M) for 48 hours. Whole cell lysates were resolved by SDS-PAGE, and MYC protein levels were assessed by immunoblot. Tubulin was used as a loading control. **D**, MYC mRNA levels were measured in BTICs (T3691, T387, and T4121) by qRT-PCR after treatment with vehicle control (DMSO) or 6-fluoromevalonate (2 μ M) for 48 hours. Data are presented as mean $\pm$ SEM from three independent experiments. **, $p < 0.01$. **E**, MYC protein levels were measured in BTICs (T3691, T387, and T4121) by immunoblot after treatment with vehicle control (DMSO) or 6-fluoromevalonate (2 μ M) for 48 hours. Whole cell lysates were resolved by SDS-PAGE, and levels of MYC was assessed by immunoblot.

TUBULIN was used as a loading control. **F**, *MYC* mRNA levels were measured in BTICs (T3691 and T387) by qRT-PCR upon treatment with vehicle control (DMSO), simvastatin (2 μ M), U0126, or simvastatin and U0126 for 48 hours. Data are presented as mean $\pm$ SEM from three independent experiments. **, $p < 0.01$. **G**, *MYC* protein levels were quantified in two BTIC models (T3691 and T387) treated with simvastatin, U0126, or U0126 plus simvastatin for 48 hours. **H**, miR-33b levels were measured by qRT-PCR in two BTIC lines (T3691 and T387) treated with simvastatin, U0126, or U0126 plus simvastatin for 48 hours. Data are presented as mean $\pm$ SEM from three independent experiments. **, $p < 0.01$. **I**, *MYC* mRNA levels were quantified by qRT-PCR in two BTIC lines (T3691 and T387) transduced with a miR-33b mimic for 48 hours. Data are presented as mean $\pm$ SEM from three independent experiments. **, $p < 0.01$. **J**, *MYC* protein levels were assayed by immunoblot in two BTICs (T3691 and T387) transduced with either an empty vector or a miR-33b expression vector for 48 hours. TUBULIN was used as a loading control.