Mitochondrial control by DRP1 in brain tumor initiating cells

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Brain tumor initiating cells (BTICs) co-opt the neuronal high affinity glucose transporter, GLUT3, to withstand metabolic stress. We investigated another mechanism critical to brain metabolism, mitochondrial morphology, in BTICs. BTIC mitochondria were fragmented relative to non-BTIC tumor cell mitochondria, suggesting that BTICs increase mitochondrial fission. The essential mediator of mitochondrial fission, dynamin-related protein 1 (DRP1), showed activating phosphorylation in BTICs and inhibitory phosphorylation in non-BTIC tumor cells. Targeting DRP1 using RNA interference or pharmacologic inhibition induced BTIC apoptosis and inhibited tumor growth. Downstream, DRP1 activity regulated the essential metabolic stress sensor, AMP-activated protein kinase (AMPK), and targeting AMPK rescued the effects of DRP1 disruption. Cyclin-dependent kinase 5 (CDK5) phosphorylated DRP1 to increase its activity in BTICs, whereas Ca2+-calmodulin-dependent protein kinase 2 (CAMK2) inhibited DRP1 in non-BTIC tumor cells, suggesting that tumor cell differentiation induces a regulatory switch in mitochondrial morphology. DRP1 activation correlated with poor prognosis in glioblastoma, suggesting that mitochondrial dynamics may represent a therapeutic target for BTICs.

Glioblastomas rank among the most lethal of human cancers, with current therapies offering only palliation1. Glioblastomas display striking intertumoral heterogeneity in transcriptional programs and genetic lesions2,3, but glioblastomas also phenocopy aberrant organ systems, with intratumoral heterogeneity within the neoplastic compartment derived from genetic and epigenetic forces, leading to cellular hierarchies with self-renewing BTICs at the apex4–6. Normal neural progenitor cells (NPCs) are functionally defined by self-renewal and differentiation into relevant lineages7. BTICs share these features but are distinguished by their frequency, proliferation, aberrant expression of differentiation markers, chromosomal abnormalities and tumor formation. While the nature of BTICs remain controversial because of unresolved issues over cell of origin and purification, they have generated substantial interest because of their resistance to conventional therapies, evasion of antitumor immune responses, promotion of tumor angiogenesis and invasion into normal tissues8–12.

Evolving models of cancer hallmarks have integrated metabolism as an essential feature of cellular transformation13. Metabolic changes are not simply a result of oncogenesis, as mutations in key enzymes are primary tumor-initiating lesions13. Isocitrate dehydrogenase 1 (IDH1) is mutated in most low-grade gliomas and secondary glioblastomas, leading to formation of an oncometabolite causing cellular dedifferentiation14,15. However, most glioblastomas express wild type IDH1, suggesting potential alternative regulation of metabolism14. Like most cancers, glioblastomas display derangement of metabolism to promote a shift toward glycolysis, known as the Warburg effect16. While all tumor cells display dysregulation of metabolic pathways, the differential growth patterns of BTICs suggest that these tumor subpopulations have metabolic features that distinguish them from the tumor bulk17–20. Recent studies suggest that the molecular machinery of nutrient sensation instructs the behavior of stem cells, particularly embryonic and hematopoietic stem cells21. As mitochondria represent the central metabolic organelle, mitochondria offer a potential link between cellular metabolism and differentiation state.

Mitochondria are highly dynamic organelles that synergize with the central cellular state22. To meet specific cellular demands of different cell types over time, cellular biogenesis is mediated through dynamic mitochondrial fusion and fission. Mitochondrial dynamics are tightly coordinated in association with the cell cycle and state, with complex structural and functional interactions leading to fusion and fission of mitochondria to alter the balance of oxidative phosphorylation, eliminate damaged mitochondrial components (for example, mitochondrial DNA) and regulate reactive oxygen species22. Embryonic stem cell maintenance and lineage commitment is regulated by mitochondrial dynamics23–25. Mitochondrial fission removes damaged mitochondrial components through mitophagy, but excessive fission may contribute to Parkinson’s and Huntington’s diseases22. Cancers, including glioblastomas, have increased rates of mitochondrial fission26–32. Thus, mitochondrial fission may be related to stem cell biology, beneficial for cancer and destructive in...
normal brain. Mitochondrial dynamic fusion and fission mediators have been closely linked to cell fate determination and development.33. Acquired alterations in these mitochondrial regulators occur in neurodegenerative diseases, vascular disorders and cancer. Inhibitors of mitochondrial fission, such as mitochondrial division inhibitor-1 (Mdivi-1), may ameliorate neurodegenerative diseases and reduce the cardiotoxicity of chemotherapy.34,35.

Here we interrogated the role of mitochondrial form and functional control in the cellular hierarchy of the most common primary intrinsic brain tumor, glioblastoma, using validated and well characterized models reflecting the tumor hierarchy.8,9,19,36,37. As metabolic control offers a potential node upon which diverse extrinsic and intrinsic signaling pathways converge, these studies may inform the development of anticancer therapies.

RESULTS

**BTICs display fragmented mitochondrial morphology**

To investigate mitochondrial control in BTICs, we isolated functionally validated matched BTIC and non-BTIC tumor cell populations from patient-derived xenografts. Tumorsphere culture is often used to enrich for BTICs, but this methodology prevents the prospective comparison of BTICs and non-BTIC tumor cells needed for these studies. Using cell surface markers immediately upon tumor collection prevents the loss of information as cell surface markers mediate interactions with the tumor microenvironment. Glioblastomas display substantial intertumoral heterogeneity, so it is not surprising that enrichment markers for BTICs are not universally informative. CD133 (PROMININ1) is the most widely used BTIC marker but is also controversial because of its technical challenges and variable expression patterns. However, we and others have repeatedly demonstrated in our models that CD133 used immediately on patient tumor specimens or xenografts is informative of functional BTICs as measured by stem cell marker expression and in vitro and in vivo limiting dilution, including the models included in this study.4,8,9,19,36,37.

**Figure 1** BTICs and non-BTIC tumor cells display distinct mitochondrial morphologies. (a) Mitochondria of BTICs and non-BTIC tumor cells isolated from patient-derived xenografts (387, 3565 and IN528) were visualized with anti-TOM20 antibody. Scale bars, 5 μm. (b) Mitochondrial morphology was assessed from 150 cells on three different slides. Data are presented as mean ± s.e.m. (387, fragmented: P = 0.0052; 3565, fragmented: P = 0.0011, tubular: P = 0.0003; IN528, fragmented: P = 0.0040, tubular: P = 0.0014; Student’s t-test; n = 3 slides). (c) Transmission electron microscopy images of mitochondria in glioblastoma BTICs and non-BTIC tumor cells isolated from three xenografts (CW718, 3565 and 387). At least 30 mitochondria were analyzed per experiment. Scale bars, 1 μm. Data are presented as mean ± s.e.m. (CW718, P = 0.0023; 3565, P = 0.0095; 387, P = 0.0217; Student’s t-test; n = 3 slides).

Therefore, our claim of BTIC identity is based on functional criteria, not markers. As culture and xenograft conditions can induce drift, we used both xenografts and cultures at early passage (<5 passages). For maintenance, BTICs and non-BTIC tumor cells were cultured separately in optimal media, but for every experiment, all cells were cultured under identical conditions and media. The cellular hierarchy was revalidated functionally (data not shown).

Using this selection system, we compared the mitochondrial morphology of BTICs with non-BTIC tumor cells using a mitochondrial marker (translocase of outer mitochondrial membranes 20 kDa, TOM20) and three-dimensional Imaris image reconstruction of images obtained from confocal microscopy (Bitplane, South Winsor, CT). Mitochondria in BTICs were more fragmented and less tubular than in matched non-BTIC tumor cells (Fig. 1a,b). In line with these observations, electron microscopic examination confirmed shorter, rounded mitochondria in BTICs as compared with elongated, tubular structures in non-BTIC tumor cells (Fig. 1c,d). Taken together, these findings suggest that mitochondrial fragmentation may be a distinctive feature of BTICs.

**DRP1 phosphorylation controls BTIC mitochondrial morphology**

Mitochondrial length is determined by the competitive balance between central mediators of mitochondrial fission and fusion. A dynamin-like protein, DRP1 (dynamin-related protein 1), mediates...
mitochondrial fission, while MFN1 (mitofusin 1) and MFN2 are required for outer membrane fusion, and OPA1 (optic atrophy 1) is required for inner membrane fusion. On the basis of differences in mitochondrial length, we interrogated the expression levels of these proteins. We detected no consistent differences in whole cell protein levels of central mediators between matched BTICs and non-BTIC tumor cells directly derived from two primary human glioblastoma specimens (CCF3015 and CCF3038).

To determine whether DRP1 phosphorylation is critical for the mitochondrial morphological change between BTICs and non-BTIC tumor cells, we constructed a gain-of-function DRP1 containing both S616E (to mimic activating phosphorylation) and S637A (to block inhibitory phosphorylation) mutations. Overexpression of DRP1S616E,S637A in non-BTIC tumor cells potently induced remodeling of mitochondria (Fig. 3a–c). Mitochondria in non-BTIC tumor cells transduced by lentivirus expressing mutated DRP1S616E,S637A became more fragmented and less elongated than cells that expressed non-BTIC tumor cells directly isolated from primary glioblastoma clinical specimens without culture (Fig. 2d). To determine the relationship between cellular differentiation and DRP1 regulation, we induced differentiation in BTICs and found a marked switch in DRP1 phosphorylation from the activating modification (Ser616) to the inhibitory one (Ser637) (Fig. 2e), indicating that dynamic regulation of DRP1 by phosphorylation is important for BTIC self-renewal and differentiation. Collectively, these findings support the idea that DRP1 is hyperactivated in BTICs.

![Figure 2](https://example.com/image2.png) **Figure 2** DRP1 is hyperactivated in BTICs. (a) Immunoblot analysis of DRP1 total protein levels, activating phosphorylation (p-DRP1Ser616) and repressive phosphorylation (p-DRP1Ser637) in BTICs and non-BTIC tumor cells isolated from patient-derived xenografts (T387, T4302, T3565 and IN528). Images were cropped for presentation. Full-length blots are presented in Supplementary Figure 9. (b) Alternative enrichment of BTICs from patient-derived xenografts (387 and 3565) by SSEA1 confirmed differential activation on immunoblot of phospho-DRP1. (c) Immunofluorescent staining of activating phosphorylation of DRP1 (p-DRP1Ser616) with several BTIC markers, including SOX2 and OLIG2, in two primary human glioblastoma specimens (CW1617, CW1679). Scale bars, 10 μm. (d) Immunoblot analysis of DRP1 protein and its activating phosphorylation (phospho-DRP1Ser616) and repressive phosphorylation (phospho-DRP1Ser637) in BTICs and non-BTIC tumor cells directly derived from two primary human glioblastoma specimens (CCF3015 and CCF3038). (e) Immunoblot analysis of phospho-DRP1Ser616 and phospho-DRP1Ser637 during BTIC (4302 and 387) differentiation induced by 10% serum over time.
Figure 3 DRP1 phosphorylation regulates mitochondrial morphology and stem cell marker expression. (a) Immunofluorescent staining of mitochondria by TOM20 in 387 and 3565 non-BTIC tumor cells transduced by lentiviral control vector or vector carrying a DRP1S616E,S637A double mutant. Scale bars, 5 μm. Boxed regions are pictured in the enlarged images. (b) Mitochondrial morphology was assessed from 120 cells of three different experiments. Data are displayed as mean ± s.e.m. (387, fragmented: \( P = 0.0009 \), tubular: \( P = 0.0067 \); 3565, fragmented: \( P = 0.0030 \), tubular: \( P = 0.0072 \); Student’s t-test, \( n = 3 \) slides). (c) Immunoblot analysis of DRP1 protein in 387 and 3565 non-BTIC tumor cells transduced with control vector or vector encoding a DRP1S616E,S637A double mutant. Images were cropped for presentation.

Full-length blots are presented in Supplementary Figure 10. (d,e) 387 non-BTIC tumor cells were transduced with vector encoding a DRP1S616E,S637A double mutant or control vector. Three days after infection, total RNA was isolated and cDNA was synthesized by reverse transcription. The mRNA levels of indicated genes were detected by real-time quantitative PCR. Data are displayed as mean ± s.e.m. 387: OLIG2, \( P = 0.0072 \); OCT4 (POUSF1), \( P = 0.0097 \); NANO2, \( P = 0.0087 \); NESTIN (NES), \( P = 0.0309 \); POU3F2, \( P = 0.0067 \); CD133 (PROM1), \( P = 0.0219 \); SSEA1 (FU14), \( P = 0.0041 \); GFAP, \( P = 0.0095 \); MAP2, \( P = 0.0001 \), 3565: OLIG2, \( P = 0.0334 \); OCT4, \( P = 0.0097 \); NANO2, \( P = 0.0074 \); POU3F2, \( P = 0.0022 \); CD133, \( P = 0.0093 \); SSEA1, \( P = 0.0251 \); GFAP, \( P = 0.0164 \); MAP2, \( P = 0.0020 \). Student’s t-test; \( n = 3 \) biologically independent experimental replicates).

DRP1 targeting decreases BTIC tumorigenicity

As induced differentiation of BTICs ablates the preferential hyperactivation of DRP1, suggesting a potential function for DRP1 in BTIC biology, we interrogated the requirement for DRP1 function in BTIC maintenance. We developed two independent, non-overlapping small hairpin RNA lentiviral constructs to knock down DRP1 (designated hereafter shDrp1#1 and shDrp1#2) and compared them to a control shRNA sequence that does not target mammalian mRNA, which serves to rule out off target effects (non-targeting control, NT shRNA). DRP1 shRNAs significantly reduced DRP1 protein expression on immunoblot (Fig. 4a).

We then examined the phenotypic consequences of shRNA-mediated reduction of DRP1 expression. Silencing DRP1 significantly decreased the growth of two BTIC models (Fig. 4a), whereas there was no effect on non-BTIC tumor cells or human NPCs (Supplementary Fig. 2), further supporting the preferential requirement for DRP1 in BTICs. To determine whether targeting DRP1 also influences tumorsphere formation, we performed an in vitro limiting dilution assay in BTICs expressing non-targeting control shRNA or DRP1-directed shRNAs. Targeting DRP1 resulted in a more than tenfold decrease in the frequency of tumorsphere formation and fourfold or greater decrease in the tumorsphere...
Figure 4 Targeting DRP1 by RNA interference decreases BTIC growth, self-renewal and tumor formation capacity. (a) Top: effects of DRP1 knockdown with two independent lentiviral shRNA constructs on cell proliferation in two BTIC models (387 and 3565). Data are displayed as mean ± s.e.m. (P < 0.0001 for both models; repeated measures ANOVA; n = 4 biologically independent samples per group). Bottom: Immunoblot of DRP1 following knockdown via shRNAs compared to nontargeting control shRNA sequence (NT shRNA) in two BTIC models. Images were cropped for presentation. Full-length blots are presented in Supplementary Figure 10. (b) In vitro extreme limiting dilution assays to single cells demonstrate that knockdown of DRP1 in two BTIC models (387 and 3565) decreases the frequency of tumoursphere formation (387, P = 9.05 × 10^{-38}; 3565, P = 2.9 × 10^{-38} by ANOVA). (c) Representative images from 100 images of tumourspheres derived from BTICs (387 and 3565) expressing NT control shRNA, shDrp1#1 or shDrp1#2. Scale bars, 100 μm. Quantification shows reduced tumoursphere size with DRP1 knockdown (387: shDrp1#1, P = 0.0019; shDrp1#2, P = 0.0021; 3565: shDrp1#1, P = 0.0002; shDrp1#2, P = 0.0002. ANOVA, n = 3 biologically independent experiments. Error bars, s.e.m. (d) Kaplan-Meier survival curves of immunocompromised mice bearing orthotopic BTICs (387 and 3565) expressing NT control shRNA, shDrp1#1 or shDrp1#2 (387, P = 0.0009; 3565, P = 0.0008 by log-rank analysis; n = 5 animals per group). (e) Representative images of 5 cross-sections (hematoxylin and eosin stained) of mouse brains collected on day 24 (left: 3565 BTICs) or day 22 (right: 387 BTICs) after transplantation of BTICs expressing NT control shRNA, shDrp1#1 or shDrp1#2. Scale bars, 2 mm.

size (Fig. 4b,c). BTIC assessment requires in vivo tumor growth, so we evaluated the potential antitumor effects of DRP1-directed interventions in vivo. BTICs transduced with either of two non-overlapping DRP1-targeting shRNAs or control NT shRNA were transplanted into the brains of immunocompromised mice. Animals bearing BTICs expressing shDrp1 displayed significantly reduced tumor formation and increased tumor latency and survival relative to those bearing BTICs expressing NT shRNA (Fig. 4d,e). Together, our data demonstrate that DRP1 is required to maintain the tumorigenic potential of BTICs and that attenuation of DRP1 expression results in a loss of BTIC phenotypes, including proliferation, self-renewal and tumor formation.

Pharmacologic blockade of DRP1 inhibits BTIC growth

Mdivi-1, a selective, cell-permeable small molecule inhibitor of the DRP1 GTPase activity, has emerged as a promising proof-of-concept therapeutic agent for stroke, myocardial infarction and neurodegenerative disease. Mdivi-1 has activity against established cancer cell lines in vitro27–31, suggesting that inhibition of mitochondrial fission may be effective against tumor cells. In accord with the results of DRP1 knockdown experiments, blocking DRP1 activity with Mdivi-1 significantly decreased the growth of BTICs (Fig. 5a). Furthermore, Mdivi-1 treatment dramatically induced apoptosis measured by both Annexin V staining and PARP cleavage in BTICs but not non-BTIC tumor cells or NPCs (Fig. 5b,c and data not shown). We validated the effects of Mdivi-1 treatment on mitochondrial morphology both in vitro (Supplementary Fig. 3a) and in vivo (Supplementary Fig. 3b).

Next, we sought to evaluate the potential antitumor effects of Mdivi-1 in vivo. We orthotopically implanted BTICs into the brains of immunocompromised mice. Three days after implantation, mice were treated for 5 d by tail vein injection with Mdivi-1 or vehicle control (DMSO). Mdivi-1 treatment increased tumor latency and survival, with no evidence of toxicity (Fig. 5d and data not shown). Taken together, our findings support the efficacy of Mdivi-1 against BTICs growth and tumor formation, offering a new model for targeting stem-like brain tumor cells.

DRP1 inhibition induces AMPK activation in BTICs

On the basis of the function of DRP1 in controlling mitochondrial fission, we investigated BTIC metabolism in the context of disrupting DRP1 expression using a cell mitochondrial stress test kit (Seahorse Bioscience) that uses pharmacologic inhibitors and activators of mitochondrial function to measure mitochondrial respiration and proton excretion in real time. Initial basal respiration is disrupted by oligomycin treatment to determine ATP production (and proton leak), followed by trifluorocarbonylcyanide phenylhydrazone (FCCP) to stimulate maximal respiration, followed by antimycin A and rotenone to inhibit all mitochondrial respiration. FCCP stimulates mitochondrial respiration by uncoupling ATP synthesis from electron transport, while oligomycin and antimycin A inhibit respiration by inhibiting ATP synthase and oxidation of ubiquinol in the electron transport chain, respectively. DRP1 depletion dramatically decreased BTIC oxygen consumption rate, leading to mitochondrial dysfunction (Supplementary Fig. 4a).

AMP-activated protein kinase (AMPK) is a central cellular sensor of energy stress, suggesting a potentially critical function in determining the survival of cells under metabolic stress. Using a pharmacologic
activator of AMPK, we found that AMPK activation decreased BTIC growth and induced caspase activation (Supplementary Fig. 4b,c), a result concordant with studies in glioma cell lines31, phenocopying DRP1 targeting. We therefore interrogated a potential link to AMPK activation under DRP1 inhibition in BTICs. DRP1 inhibition by either shRNA knockdown (Fig. 6a) or treatment with Mdivi-1 (Fig. 6b) led to upregulation of AMPK activation, measured by increased phosphorylation of AMPKα. To determine whether AMPK regulation may serve as a critical downstream mediator of DRP1, we interrogated the potential for rescuing the phenotype caused by DRP1 targeting. While knocking down AMPKα by itself had minimal effect on BTIC growth or tumorsphere formation, targeting AMPKα or shDrp1#2 were immunoblotted with the indicated antibodies, including AMPKα/Thr172 and β-actin. shRNA-mediated knockdown of DRP1 induced AMPK activity. Images were cropped for presentation. Full-length blots are presented in Supplementary Figure 10. (b) Lysates of 387 and 3565 BTICs treated with Mdivi-1 or DMSO were immunoblotted with the indicated antibodies, including AMPKα/Thr172 and β-actin. shRNA-mediated knockdown of DRP1 induced AMPK activity. Images were cropped for presentation. Full-length blots are presented in Supplementary Figure 10.  

Figure 5 DRP1 inhibitor Mdivi-1 reduces brain tumor initiating cell growth and induces apoptosis. (a) Effects of Mdivi-1 treatment on cell proliferation in two BTIC models (387 and 3565). Plotted data are mean ± s.e.m. (387: 4 d, P < 0.0001; 3565: 4 d, P < 0.0001. Student’s t-test; n = 4 biologically independent samples per group). (b) Apoptosis measured by Annexin V staining in BTICs and non-BTIC tumor cells with Mdivi-1 or DMSO vehicle treatment. Data are presented as mean ± s.e.m. (387: BTICs, P < 0.0001; non-BTIC tumor cells, P = 0.066. 3565: BTICs, P = 0.0005; non-BTIC tumor cells, P = 0.5814 by Student’s t-test; n = 3 biologically independent samples per group). (c) Tumorsphere formation assay in two human neural progenitor cell (NPC) lines (ENSA and 15167) with Mdivi-1 or DMSO treatment. Data are presented as mean ± s.e.m. (ENSA: P = 0.2571; 15167: P = 0.1413 by Student’s t-test; n = 3 biologically independent samples per group). (d) Kaplan–Meier survival curves of immunocompromised mice bearing orthotopic 387 BTICs (3 × 10^5 cells per animal). Three days after tumor implantation, mice were treated with Mdivi-1 (2.5 mg/kg) or DMSO vehicle control for 5 d (P = 0.0134 by log-rank analysis; n = 7 mice per group).

CDK5 activates DRP1 in BTICs

To determine the molecular mechanism activating DRP1 in BTICs, we investigated potential kinases regulating the phosphorylation status of DRP1. Cyclin-dependent kinase 1 (CDK1) has been reported to phosphorylate DRP1 on Ser616 (ref. 33). CDK family kinases—especially CDK1, CDK2 and CDK5—often share substrates. To determine whether the CDKs regulate BTIC DRP1 Ser616 phosphorylation, we treated BTICs with roscovitine, a pan-CDK1/2/5 inhibitor, and found that DRP1Ser616 phosphorylation was significantly reduced (Fig. 7a), with associated loss of fragmented mitochondrial morphology (Supplementary Fig. 5). In contrast, treatment with BMS-265246, a CDK inhibitor more specific for CDK1/2, did not alter DRP1Ser616 phosphorylation (Fig. 7b) or mitochondrial morphology (Supplementary Fig. 5), indicating that CDK5 may be the dominant regulating kinase responsible for DRP1Ser616 phosphorylation in BTICs. We screened the expression of the CDKs in matched BTICs and non-BTIC tumor cells and found that both CDK1 and CDK5 were preferentially expressed by BTICs in three different tumors (Fig. 7c).

To determine whether CDK5 can directly phosphorylate DRP1, we performed an in vitro kinase assay with CDK5, its regulatory partner cyclin-dependent kinase 5, regulatory subunit 1 (p25, CDK5R1) and glutathione S-transferase (GST)-tagged DRP1 (wild type or S616A mutant) and found that CDK5 directly phosphorylated DRP1 on the Ser616 site (Fig. 7d). RNA interference against CDK5, but not CDK1, resulted in specific diminished DRP1Ser616 phosphorylation (Fig. 7e). We next interrogated the functional importance of CDK5 in the regulation of mitochondrial morphology using the mitochondria marker...
CDK5 activates DRP1 in BTICs. (a) Lysates of 387 and 3565 BTICs treated with the CDK1/2 inhibitor roscovitine or DMSO vehicle control were subjected to immunoblot analysis with the indicated antibodies. Images were cropped for presentation. Full-length blots are presented in Supplementary Figure 5. (b) Lysates of 387 and 3565 BTICs treated with the CDK1/2 inhibitor BMS-265246 or DMSO were immunoblotted with the indicated antibodies. (c) Immunoblot analysis of CDK1, CDK5, and stem and differentiation markers in BTICs and non-BTIC tumor cells isolated from patient-derived glioma xenografts (4302, 387 and 3565). (d) In vitro kinase assays were performed with or without CDK5-CDK5R1 (p25) and either wild type or mutant (S616A) GST-tagged C-terminal fragment of DRP1 (amino acids 518–736), GST-DRP1CT. Knockdown CDK1, CDK5, or DMSO were immunoblotted with the indicated antibodies. Knockdown CDK5 but not CDK1 decreased phospho-DRP1Ser616 levels. (e) Lysates of 387 BTICs expressing NT control shRNA or shCDK5. Data are presented as mean ± s.e.m. (387: NT, P = 0.0088; shRNA versus shNT; tubular, P = 0.0011. 3565: NT shRNA, P = 0.0098; tubular, P = 0.0018; Student’s t-test; n = 3 slides). Scale bars, 5 μm.

CAMK2 inhibits DRP1 in non-BTIC tumor cells

To determine the inhibitory regulation of DRP1 in non-BTIC tumor cells, we screened potential upstream regulators of the inhibitory phosphorylation event (phospho-DRP1Ser637) in non-BTIC tumor cells. Calcium/calmodulin-dependent protein kinase type 1 (CAMK1) has been previously reported to phosphorylate DRP1Ser637 (ref. 42), so we targeted global CAMK function by a pan-CAMK pharmacologic inhibitor, KN93. We found downregulation of DRP1Ser637 phosphorylation in non-BTICs upon KN93 treatment (Supplementary Fig. 7a), but phospho-DRP1Ser637 phosphorylation was also compromised by treatment with a CAMK2-specific inhibitor, autocamtide-2-related inhibitor peptide (AIP) (Supplementary Fig. 7b). These results are consistent with the observation that expression of CAMK2 is higher in non-BTIC tumor cells, while CAMK1 does not display preferential expression between BTICs and non-BTIC tumor cells (Supplementary Fig. 7c).

Next we interrogated the functional importance of CAMK2 in the regulation of mitochondrial morphology. AIP treatment, inhibiting CAMK2, in non-BTIC tumor cells induced a shift toward fragmented mitochondria (Supplementary Fig. 7d), supporting CAMK2 as a selective antagonist of DRP1 function in non-BTIC tumor cells (Supplementary Fig. 7e). Collectively, we propose that CAMK5 activates DRP1 through phosphorylating the Ser616 site in BTICs while CAMK2 inhibits DRP1 activity through phosphorylation of Ser637 in non-BTIC tumor cells, creating a competitive yoked control of metabolism in the tumor hierarchy.

Mitochondrial morphological control informs prognosis

Our findings support a model in which DRP1-mediated mitochondrial fission distinguishes BTICs. To determine the clinical relevance of these findings, we performed a combination of tissue analysis and in silico studies. DRP1 can be regulated at both the expression and activity levels, so we performed immunohistochemistry of DRP1 using a new tissue microarray with normal brain and glioblastoma tissues. Total DRP1 levels were similar in normal and neoplastic brain tissues, but activating phosphorylation of DRP1 (DRP1Ser616) was selectively increased in glioblastomas (Fig. 8a). The clinical significance of these findings was supported by a strong inverse correlation between phosphorylation of DRP1 on Ser616 and poor survival of patients with glioblastoma (Fig. 8b).

We then examined the downstream (AMPK) and upstream (CDK5 and CAMK2) regulatory nodes of DRP1 for clinical significance. Because we found that these regulators were all controlled at the expression level in BTICs, we interrogated available in silico glioblastoma databases. Using the large National Cancer Institute Repository for Molecular Brain Neoplasia Data (REMBRANDT) glioma data set, we found that upregulation of PRKAA2 (AMPKeα2) mRNA twofold or greater in all glioma samples (mixed grade) correlated with a significant increase in patient survival (Fig. 8c). CDK5 and CAMK2 regulate DRP1 in opposite ways, with high levels of CDK5 expected to associate with higher DRP1 activity and CAMK2 with lower DRP1 activity. The REMBRANDT database confirmed that higher CDK5 expression correlated with longer patient survival (Fig. 8d) while higher CAMK2A and CAMK2G expression was associated with shorter patient survival (Fig. 8e). Of note, mRNA gene expression signatures of putative BTIC markers (SOX2, OLIG2, POU5F1 and SALL2) did not
Figure 8. DRP1 regulation informs patient prognosis. (a) Immunohistochemical staining and scatter plot of DRP1 and phospho-DRP1Ser616 in human primary glioblastomas (GBM) and non-neoplastic brain tissues in a tissue microarray. Brown staining denotes binding of antibody to DRP1 or to phosphorylated DRP1. Blue represents hematoxylin staining. Scale bars, 50 µm. Phosphorylated DRPSer616, \( P < 0.0001 \); total DRP1: \( P = 0.681 \) by Student’s t-test.

Black center line represents mean and colored error bars represent s.e.m. (b) Kaplan-Meier plot of tissue microarray data indicates that higher Ser616 phosphorylation of DRP1 (phospho-DRP1Ser616/total DRP1) correlates with poor glioblastoma patient survival, \( P = 0.0125 \) by log-rank analysis. (c) Analysis of REMBRANDT data indicates that lower AMPKα2 mRNA expression correlates with poor glioma patient survival, \( P = 0.0059 \) by log-rank analysis. (d-f) Analysis of REMBRANDT data indicates that higher CDK5 \( (P = 0.0046 \) by log-rank analysis) and lower CAMK2 mRNA expression \( (P = 0.047 \) and 0.0023 by log-rank analysis for CAMK2A and CAMK2G, respectively) correlate with poor glioma patient survival.

DISCUSSION

Glioblastomas rank among the most lethal of cancers, with decades of research adding only a few months to the median survival of patients afflicted with these cancers1. The explanation for the failure of current therapy to extend patient survival has many causes, but one contributing force may be the presence of complex intratumoral heterogeneity derived from heterogeneous expression of oncogenic drivers as well as cellular hierarchies that phenocopy the normal brain hierarchy, albeit with aberrant control: that is, BTICs. Targeting BTICs has proven daunting because of the resistance of these cells to pathways that have been developed for the treatment of tumor cells. This functional difference was driven by an unbalanced ratio of the post-translational regulation of DRP1 phosphorylation toward activation and away from inhibition. Our data suggests that, beyond gene expression, protein modifications—including phosphorylation, acetylation, methylation, SUMOylation and ubiquitination—offer worthwhile avenues for further exploration in BTIC research.
Metabolism represents a domain for potential cancer therapeutic for BTICs. While IDH1 mutations promote chromatin alterations throughout a tumor to promote a loss of differentiation that can be targeted by small molecules, IDH1 mutations are infrequent in glioblastoma. Postmitotic neurons express CDK5, which can serve to control metabolism even in non-cycling neurons. Limited previous reports have suggested that CDK5 is expressed by glioma cell lines and may contribute to cell invasion and survival after radiation, phenotypes found in BTICs. Thus, it appears that BTICs have co-opted another neuronal metabolic control mechanism to augment cellular survival to promote tumor growth.

Aberrant metabolism is not solely a byproduct of mutations or altered transcriptional programs, but represents a driving force in the initial stages of tumorigenesis. Recently, studies of BTIC metabolism in glioblastoma and leukemia have yielded discordant responses in relative dependence on glycolysis versus oxidative phosphorylation and reactive oxygen species levels, but it is clear that the metabolic state of BTICs varies within the cellular hierarchy, as in the hierarchy of the normal hematopoietic system. As a highly specialized and dynamic organelle, the mitochondrion must be central to these distinctive features in energy metabolism. We found that inhibition of DRP1-mediated mitochondrial fission decreased cellular oxygen consumption rate and caused metabolic stress in BTICs. In addition, we detected AMPK activation in response to such energy homeostasis, supporting the idea that energy stress is present. The depletion of AMPK largely rescued the antiproliferative and proapoptotic effects of targeting DRP1-mediated mitochondrial fission decreased cellular oxygen consumption rate and caused metabolic stress in BTICs. In addition, we detected AMPK activation in response to such energy homeostasis, supporting the idea that energy stress is present. The depletion of AMPK largely rescued the antiproliferative and proapoptotic effects of DRP1 inhibition (for example, through Mdivi-1) and AMPK activation in response to such energy homeostasis, supporting the idea that energy stress is present. The depletion of AMPK largely rescued the antiproliferative and proapoptotic effects of DRP1 inhibition (for example, through Mdivi-1) and AMPK activation in response to such energy homeostasis, supporting the idea that energy stress is present. The depletion of AMPK largely rescued the antiproliferative and proapoptotic effects of DRP1 inhibition (for example, through Mdivi-1) and AMPK activation in response to such energy homeostasis.

Future studies may show that combining DRP1 inhibition (for example, through Mdivi-1) and AMPK activation (for example, through 5-amino-1-β-d-ribofuranosyl-imidazole-4-carboxamide (AICAR)) may create additional synergy. As DRP1 expression and AMPK activity may be altered in response to cytotoxic ionizing radiation treatment, these therapeutic modalities may have even greater impact when used in combination. In sum, results implicate a close interaction between DRP1 controlled mitochondrial fission and AMPK-mediated energy stress response that awaits further detailed study of the molecular mechanism of regulation of AMPK activity by DRP1 and mitochondrial dynamics. Our discovery of a prosurvival function of DRP1 in BTICs is particularly exciting as targeting DRP1 may increase survival of normal brain cells and improve toxicity of chemotheraphy. We found that targeting DRP1 by RNA interference or a pharmacologic inhibitor retarded cell growth and induced apoptosis in BTICs, with limited toxicity against normal human NPCs. In contrast with its role in BTICs, DRP1 inhibition is advancing as a target in treatment to alleviate neuronal death in Alzheimer’s, Parkinson’s and Huntington’s diseases. For example, a recent report showed that treatment of a genetically engineered mouse model of Huntington’s disease with P110-TAT, another inhibitor of DRP1, strongly reduced neurological toxicity caused by radiation and chemotherapy treatments has not been well investigated, it is encouraging to think that these approaches may simultaneously treat the tumor and attenuate the negative effects of conventional therapy.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

Q.X. and J.N.R. designed the experiments, analyzed data and prepared the manuscript. Q.W., Q.W., K.Y.W., S.M.D., Z.H., X.F. and A.N.F performed the experiments. W.A.F. and Y.S. performed database analyses. C.M.H. performed the experiments. W.A.F. and Y.S. performed database analyses. C.M.H. performed pathologic analyses. S.B. and D.F.K. provided scientific input and helped to edit the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Isolation and culture of cells. Glioblastoma tissues were obtained from excess surgical materials from patients undergoing procedures with informed written consent at the Cleveland Clinic after review by a neuropathologist in accordance with an approved protocol by the Institutional Review Board. The models tested were wild type for ID1 (data not shown). To prevent culture-induced drift, patient-derived xenografts were generated and maintained as a source of tumor cells for study. As described previously, cultures enriched for or depleted of BTICs were derived from primary patient brain tumor specimens or specimens passed for seven or fewer passages as xenografts in immunocompromised mice. A papain dissociation system (Worthington Biochemical, Lakewood, NJ) was used to dissociate tumors according to the manufacturer’s instructions (http://www.worthington-biochem.com/PDS/default.html).

Western blotting. Cells were collected and lysed in hypotonic buffer with 10% serum. Immunofluorescence staining. DMEM with 10% serum.

Immunofluorescence staining. Cells or 10-µm-thick slices of xenografted brain tissue were fixed in 4% paraformaldehyde then permeabilized and blocked with 5% normal goat serum. Cells or slices were incubated with primary antibodies overnight at 4 °C. After washing three times with 0.1% Tween 20 in PBS, secondary antibodies were applied and observed with a Leica Titan confocal or DM4000 upright microscope.

Vectors and lentiviral transfection. Lentiviral clones to express either shRNA directed against DRP1 (TRCN0000019097, TRCN0000318425), CDK1 (TRCN0000196602, TRCN000005083, TRCN00000196603), CDK5 (TRCN0000195513, TRCN0000194974, TRCN0000199652) or a non-targeting (NT) shRNA (SHC002) were obtained from Sigma-Aldrich (St. Louis, MO). The NT vector contains an shRNA insert that does not target human or mouse genes, serving as a negative control for off-target effects in experiments. A lentiviral shRNA clone targeting AMPKα (sc-45132-SH) and a scrambled non-targeting control (sc-108060) were purchased from Santa Cruz Biotechnology. shRNAs with non-overlapping sequences that had the best relative knockdown efficiency were used for all experiments. Lentiviral particles were generated in 293FT cells in stem cell medium with cotransfection with the packaging vectors pCMV-dR8.2 dvpr and pCI-VSVG (Addgene) by Lipofectamine 2000 (Invitrogen). Efficiency of lentiviral shRNA clones was determined by immunoblot and real-time PCR. Viral stocks were concentrated via precipitation using PEG-8000 and then subsequently titered using the manufacturer’s protocol. A DRP1 shRNA clone targeting (TRCN0000195513, TRCN0000194974, TRCN0000199652) or a non-targeting clone was generated with the QuickChange Multi III Site-Directed Mutagenesis kit (Stratagene, Torrey Pines, CA). Western blotting. Cells were collected and lysed in hypotonic buffer with 10% serum. Immunofluorescence staining. DMEM with 10% serum.

Quantitative RT-PCR. Total cellular RNA was isolated using the RNeasy kit (Qiagen, Venlo, Netherlands) and reverse-transcribed into cDNA using qScript cDNA SuperMix (Quanta BioSciences, Gaithersburg, MD). Equal amounts of 1 µg RNA were measured using an ABI-7900 system (Applied Biosystems, Carlsbad, CA). Quantitative reverse transcription PCR was performed using the manufacturer’s protocol on an Applied Biosystems 7900HT cycler using SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA). Equal amounts of 1 µg RNA were measured using an ABI-7900 system (Applied Biosystems, Carlsbad, CA). Quantitative reverse transcription PCR was performed using the manufacturer’s protocol on an Applied Biosystems 7900HT cycler using SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA). Equal amounts of 1 µg RNA were measured using an ABI-7900 system (Applied Biosystems, Carlsbad, CA). Quantitative reverse transcription PCR was performed using the manufacturer’s protocol on an Applied Biosystems 7900HT cycler using SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA). Equal amounts of 1 µg RNA were measured using an ABI-7900 system (Applied Biosystems, Carlsbad, CA). Quantitative reverse transcription PCR was performed using the manufacturer’s protocol on an Applied Biosystems 7900HT cycler using SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA). Equal amounts of 1 µg RNA were measured using an ABI-7900 system (Applied Biosystems, Carlsbad, CA). Quantitative reverse transcription PCR was performed using the manufacturer’s protocol on an Applied Biosystems 7900HT cycler using SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA). Equal amounts of 1 µg RNA were measured using an ABI-7900 system (Applied Biosystems, Carlsbad, CA). Quantitative reverse transcription PCR was performed using the manufacturer’s protocol on an Applied Biosystems 7900HT cycler using SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA). Equal amounts of 1 µg RNA were measured using an ABI-7900 system (Applied Biosystems, Carlsbad, CA). Quantitative reverse transcription PCR was performed using the manufacturer’s protocol on an Applied Biosystems 7900HT cycler using SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA). Equal amounts of 1 µg RNA were measured using an ABI-7900 system (Applied Biosystems, Carlsbad, CA). Quantitative reverse transcription PCR was performed using the manufacturer’s protocol on an Applied Biosystems 7900HT cycler using SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA). Equal amounts of 1 µg RNA were measured using an ABI-7900 system (Applied Biosystems, Carlsbad, CA). Quantitative reverse transcription PCR was performed using the manufacturer’s protocol on an Applied Biosystems 7900HT cycler using SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA). Equal amounts of 1 µg RNA were measured using an ABI-7900 system (Applied Biosystems, Carlsbad, CA). Quantitative reverse transcription PCR was performed using the manufacturer’s protocol on an Applied Biosystems 7900HT cycler using SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA). Equal amounts of 1 µg RNA were measured using an ABI-7900 system (Applied Biosystems, Carlsbad, CA). Quantitative reverse transcription PCR was performed using the manufacturer’s protocol on an Applied Biosystems 7900HT cycler using SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA). Equal amounts of 1 µg RNA were measured using an ABI-7900 system (Applied Biosystems, Carlsbad, CA).
Three metabolic inhibitors were sequentially loaded to each well at specific time points: oligomycin (0.75 µM), followed by FCCP (0.75 µM), followed by a combination of rotenone (0.1 µM) and antimycin (0.1 µM).

**In vitro limiting dilution assay.** For in vitro limiting dilution assays, cells were sorted by fluorescence-activated cell sorting (FACS) with decreasing numbers of cells per well (20, 10, 5 and 1) plated in 96-well plates. Ten days after plating, the presence and number of tumorspheres in each well was quantified. Extreme limiting dilution analysis was performed using software available at http://bioinfo.wehi.edu.au/software/elda/, as previously described8,9,19,36,37.

**Intracranial tumor formation in vivo.** GICs were transduced with lentiviral vectors expressing Drp1 shRNA or a non-targeting (NT) control shRNA for the knockdown experiments. 36 h after infection, viable cells were counted and engrafted intracranially into NSG (NOD.Cg-PrkdBTICid Il2rgtm1Wjl/SzJ) mice under a protocol approved by the Cleveland Clinic Foundation Institutional Animal Care and Use Committee. Animals were then maintained until neurological signs were apparent, at which point they were sacrificed. The brains were removed and fixed in 4% formaldehyde, cryopreserved in 30% sucrose and then cryosectioned. Sections were stained with hematoxylin and eosin. In parallel survival experiments, animals were monitored until they developed neurological signs.

**Immunohistochemical quantification of glioma tissue microarrays.** Expression of total DRP1 and phosphorylated DRP1Ser616 in WHO grades II–IV gliomas was performed on tissue microarrays (TMAs) similar to that previously described50. Briefly, a TMA of deidentified, formalin-fixed, paraffin-embedded (FFPE) gliomas was immunostained for DRP1 (1:100, 611738, BD Transduction Laboratories, San Jose, CA) or phospho-DRP1Ser616 (1:200, CST-4494, Cell Signaling, Danvers, MA). Secondary antibodies used were EnVision-labeled polymer-HRP (horseradish peroxidase) anti-mouse (BA-9200, Vector Laboratories, Burlingame, CA) or anti-rabbit (BA-BA-1000, Vector Laboratories), as appropriate. Staining was visualized using the chromogen 3,3′-diaminobenzidine (DAB) (Dako, Carpinteria, CA). Each tumor was represented by three separate 2-mm cores on the TMA, with each core embedded in a separate TMA block. Each TMA core was semiquantified on a relative scale of intensity from 0 to 3, with 0 being negative and 3 being strongest. Results from the three cores were averaged together to produce a final score for a tumor. For survival analysis, ratios of phosphorylated to total DRP1 were calculated for each tumor (represented by three cores each) and a mean score was derived across all tumors. Dichotomized levels (high versus low) were assigned relative to the global mean value. Each case was annotated with clinical data from the Kentucky Cancer Registry.

**Bioinformatic analysis.** National Cancer Institute’s Repository for Molecular Brain Neoplasia Data (REMBRANDT, https://caintegrator.nci.nih.gov/rembrandt/) or The Cancer Genome Atlas (TCGA, https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp) microarray databases annotated with patient survival were used to correlate survival and multiple gene expression in malignant glioma biopsies.

**Statistical analysis.** No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications8,9,19,36,37. Data acquisition and analysis was not blinded. All grouped data are presented as mean ± s.e.m. Data distribution was assumed to be normal, but this was not formally tested. Differences between groups were assessed by Student’s t-test or ANOVA using GraphPadInStat software (GraphPad Software, La Jolla, CA). Kaplan-Meier curves were generated and log-rank analysis was performed using MedCalc software (Ostend, Belgium).

A Supplementary Methods Checklist is available.

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