Glioblastoma (GBM) remains an incurable disease, requiring more effective therapies. Through interrogation of publicly available CRISPR and RNAi library screens, we identified the alpha-ketoglutarate dehydrogenase (OGDH) gene, which encodes for an enzyme that is part of the tricarboxylic acid cycle (TCA cycle) as essential for GBM growth. Moreover, by combining a transcriptome and metabolite screening analyses we discovered that loss of function of OGDH by the clinically validated drug compound, CPI-613, was synthetically lethal with Bcl-xL inhibition (genetically and through the clinically validated BH3-mimetic, ABT263) in patient-derived xenograft as well neurosphere GBM cultures. CPI-613 mediated energy deprivation drove an integrated stress response with an up-regulation of the BH3-only domain protein, Noxa in an ATF4 dependent manner as demonstrated by genetic loss of function experiments. Consistently, silencing of Noxa attenuated cell death induced by CPI-613 in model systems of GBM. In patient-derived xenograft models of GBM in mice, the combination treatment of ABT263 and CPI-613 suppressed tumor growth and extended animal survival more potently than each compound on its own. Therefore, combined inhibition of Bcl-xL along with interference of the TCA-cycle might be a treatment strategy for GBM.
OGDH and Bcl-xL Loss Causes Synthetic Lethality in Glioblastoma

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Running title: OGDH is a critical driver of glioblastoma growth by blocking ISR signaling.

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Abstract

Glioblastoma (GBM) remains an incurable disease, requiring more effective therapies. Through interrogation of publicly available CRISPR and RNAi library screens, we identified the alpha-ketoglutarate dehydrogenase (OGDH) gene, which encodes for an enzyme that is part of the tricarboxylic acid cycle (TCA cycle) as essential for GBM growth. Moreover, by combining a transcriptome and metabolite screening analyses we discovered that loss of function of OGDH by the clinically validated drug compound, CPI-613, was synthetically lethal with Bcl-xL inhibition (genetically and through the clinically validated BH3-mimetic, ABT263) in patient-derived xenograft as well neurosphere GBM cultures. CPI-613 mediated energy deprivation drove an integrated stress response with an up-regulation of the BH3-only domain protein, Noxa in an ATF4 dependent manner as demonstrated by genetic loss of function experiments. Consistently, silencing of Noxa attenuated cell death induced by CPI-613 in model systems of GBM. In patient-derived xenograft models of GBM in mice, the combination treatment of ABT263 and CPI-613 suppressed tumor growth and extended animal survival more potently than each compound on its own. Therefore, combined inhibition of Bcl-xL along with interference of the TCA-cycle might be a treatment strategy for GBM.
Introduction

It remains unclear why glioblastoma IDH-wild-type, the most common primary brain tumor in adults, remains highly resistant to therapy. There are numerous explanations for this phenomenon, including the cellular heterogeneity, the blood brain barrier, the immunosuppressive micro-environment and altered metabolism (1-6).

While a role for metabolism in glioblastoma has been established more than a decade earlier (at the time of the discovery of IDH mutant gliomas) (1), we are still in progress to fully appreciate the impact of tumor metabolism on glioblastoma growth. In this context, it has been recently suggested that glioblastomas appear to utilize a range of different fuel sources rather than relying exclusively on glucose, which was the leading thought over the last century. In this regard, based on Otto Warburg’s theory, glucose is metabolized to lactate and in turn (to avoid glycolytic stasis), lactate is excreted into the micro-environment (7). However, we and other groups provided recent evidence that the tricarboxylic acid cycle (TCA-cycle) is pivotal for GBM growth, which involved the mitochondrial production of citrate that in turn is exported to the cytosol to be converted to acetyl-CoA, which serves as the initial substrate for fatty acid and cholesterol biosynthesis (8-12). The role of the oxidative portion of the TCA-cycle (starting from the isocitrate dehydrogenase reaction), which involves alpha-ketoglutarate dehydrogenase (OGDH) enzyme amongst others, remained unclear and its role in glioblastoma growth warrants investigation.

Intrinsic apoptosis is a critical component in response and resistance to therapy (13). At the molecular level, this process is carefully regulated by the pro- and anti-apoptotic Bcl-2 family of proteins. While Bcl-xL, Bcl-2 and Mcl-1 are anti-apoptotic, the Noxa, BIM, BAX and BAK are drivers of cell death. In this context, Noxa antagonizes the function of Mcl-1 and therefore high levels of Noxa are rendering cancers cell susceptible to BH3-mimetics, such as ABT263 or ABT199, also known as navitoclax and venetoclax, respectively (14-16).
Here, through interrogation of genome wide pooled CRISPR library screens performed in GBM models (https://depmap.org/portal/context/Glioblastoma) we unexpectedly found that OGDH is a critical driver gene for glioblastoma growth irrespective of their genetic background. The OGDH complex, which is comprised of OGDH, DLST and DLD, produces succinyl-CoA and NADH from 2-oxoglutarate in an irreversible chemical reaction (oxidative decarboxylation) (17, 18). Because OGDH is critical in the context of the TCA-cycle and because it was identified as a central dependency in CRISPR library screens in GBM cultures we focused on this enzyme in our studies. Moreover, a clinically validated inhibitor of OGDH is available as well enabling translational studies in GBM cultures and animal models. We demonstrated that loss of function of OGDH affects central carbon metabolism, activates the integrated stress response with up-regulation of pro-apoptotic Noxa, rendering GBM cells sensitive to Bcl-xL inhibition.
Results

**OGDH is an oncogene that promotes GBM growth and survival.**

Analysis of publicly available CRISPR and RNAi library screens of GBM cells from DepMAP database suggested dependency of GBM cells on the OGDH gene as well as on the TCA cycle (Figure 1, A and B). To confirm the involvement of OGDH in proliferation of GBM cells, we silenced the expression of this enzyme in KNS42, GBM22, and GBM12 cells through several shRNAs that specifically target OGDH. We observed a significant growth reduction of GBM cells with silenced OGDH (Figure 1, C and D). Following genetic interference with OGDH we also found an induction of cell death in GBM22 and GBM12 cells (Supplementary Figure 1A). Similar findings were observed in GBM cells treated with CPI-613, a drug that has reached phase III clinical testing, which is known to interfere with the OGDH enzyme (19-21) (Figure 1E). Notably, human astrocytes revealed only a marginal response to CPI-613, suggesting a favorable toxicity profile (Figure 1E). In order to assess the prognostic implications of OGDH we interrogated the TCGA database of GBM and found that high mRNA levels of OGDH correlated with a worse overall survival (Figure 1F). To evaluate the effects of this gene in vivo, GBM22 cells were transduced with non-targeting or OGDH specific shRNA followed by injection into nude mice. We found that mice harboring tumors derived from GBM22 cells transduced with OGDH shRNA revealed an extended overall survival as compared to the control animals (Figure 1, G and H and Supplementary Figure 1B). Overall, this finding suggested that OGDH acts as an oncogene that promotes GBM growth.

**Loss of function of OGDH affects intrinsic apoptosis by increasing pro-apoptotic Noxa protein levels.**

Next, we wanted to determine whether the induction of apoptosis by CPI-613 treatment is mediated by a change of expression of the Bcl-2 family of proteins. While the Mcl-1 protein levels were increased in the presence of CPI-613, we detected a reduction of Bcl-xL protein levels in
different GBM cells (GBM22, KNS42, GBM43, NCH644, and GBM12) (Figure 2A). The Bcl2 protein levels seemed to be unchanged in GBM cells treated with CPI-613 except in GBM43 and NCH644 cells (Figure 2A). Notably, we observed an up-regulation of the Noxa protein, a pro-apoptotic Bcl-2 family member, in GBM cells treated with increasing concentration of CPI-613 (Figure 2A). In addition, we also determined the mRNA expression levels of Mcl-1, Bcl-2, and Bcl-xL in GBM22, KNS42, and GBM43 cells. Overall, we observed a reduction of Bcl-xL in response to CPI-613 treatment (Supplementary Figure 2A). In addition, we determined the expression levels of Noxa, Mcl-1, Bcl-2 and Bcl-xL following genetic loss of function of OGDH. Akin to CPI-613 we found that shRNA mediated reduction of OGDH elicits an increase of pro-apoptotic Noxa in GBM22 and KNS42 cells, suggesting that Noxa might have a critical role (Supplementary Figure 2B).

To support the involvement of Bcl-xL in cell death elicited by CPI-613 treatment, we ectopically over-expressed Bcl-xL in GBM cells by using adenoviruses. First, we assessed the effect of Bcl-xL over-expression on CPI-613 treatment in a setting of a cellular viability assay. We observed a rescue of loss of cellular viability elicited by CPI-613 treatment in Bcl-xL over-expressing GBM cells (Figure 2B and Supplementary Figure 2C). Next, we extended our study to analyze apoptosis elicited by CPI-613 in the presence or absence of Bcl-xL over-expression in KNS42 and GBM43 cells. As anticipated, Bcl-xL over-expression mitigated cell death induced by CPI-613 (Figures 2C). These data suggest an involvement of Bcl-xL in the cell death caused by CPI-613 treatment in GBM cells.

To assess whether the up-regulation of Noxa protein is required for the cell death induction mediated by CPI-613, we silenced the expression of Noxa in KNS42 and GBM12 (Figures 2, D and E and Supplementary Figure 2, D-H). The knockdown of Noxa was confirmed by western blot (Supplementary Figure 2, E and H). We found that silencing of Noxa by two different siRNAs
mitigated CPI-613 mediated loss of cellular viability as well as cell death in KNS42 and GBM12 (Figures 2, D and E and Supplementary Figure 2, D-H). Notably, the effect size on cell death was more pronounced since Noxa is a primary regulator of apoptosis (14-16).

Loss of function of OGDH or CPI-613 treatment impairs TCA cycle functionality coupled with a reduced oxygen consumption rate.

To evaluate how CPI-613 affects the transcriptome of GBM cells we performed RNA sequencing and gene set enrichment analysis (GSEA). The GSEA data revealed suppression of gene sets related to oxidative phosphorylation as well as to the respiratory chain complexes in GBM cells treated with CPI-613 (Figure 3A), in keeping with its predicted targets. We also performed 13C carbon tracing analysis to study the impact of CPI-613 on the TCA cycle as well as other metabolic pathways. As anticipated, we found that CPI-613 treatment led to a substantial reduction of labeling of metabolites in the TCA cycle from glucose carbons (Figure 3, B-F and Supplementary Figure 3, A-C). The Warburg effect includes other additional metabolic pathways such as glycolysis, the pentose phosphate pathway and serine/amino acid synthesis. We noted a decrease of 13C glucose labeling of metabolites associated with these pathways (Supplementary Figure 3, B and C). Next, we assessed how genetic loss of function of OGDH affects glucose related labeling of citrate. We found that shRNA mediated suppression of OGDH reduced citrate labeling from glucose and suppressed the fraction of labeling of the m+2 citrate isotopologue (Figure 3G and Supplementary Figure 3D). Other metabolites were affected as well but revealed increases in labeling, consistent with predominant “counter-clockwise” cycling via the pyruvate carboxylase reaction (Supplementary Figure 3D). Glycolytic intermediates displayed some minor alterations as well (Supplementary Figure 3D).

The extracellular flux analysis also showed a suppression of the oxygen consumption rate (OCR) and ATP production mediated by loss of function of OGDH and CPI-613 treatment in KNS42 and
GBM22 cells (Figure 3, H-K and Supplementary Figure 4, A-D). To confirm that loss of energy production mediated cell death elicited by loss of function of OGDH, we treated KNS42 and GBM22 cells with CPI-613 in the presence or absence of ATP. We detected a partial rescue from loss of cellular viability driven by CPI-613 treatment (Supplementary Figure 4E). These observations support the notion that loss of function of the OGDH enzyme blocks the TCA-cycle and results in energy deprivation that in turn mediates loss of cellular viability in GBM cells.

**Loss of function of OGDH activates the endoplasmic reticulum stress response, which facilitates Noxa up-regulation in a manner dependent on ATF4.**

Our transcriptome and gene set enrichment analysis pointed towards an increase of energy deprivation and an endoplasmic reticulum stress response (Figure 4A), consistent with the findings above (Figure 3). We hypothesized that the loss of energy will lead to an activation of the integrated stress response with up-regulation of ATF4 and ATF3 (bona fide stress response transcription factors) that in turn would affect the expression of pro-apoptotic Noxa. To this end, we determined the ATF3 and ATF4 protein levels following CPI-613 treatment. Our data showed an increased expression of ATF3 and ATF4 elicited by CPI-613 in KNS42, GBM12, GBM22, GBM43 and NCH644 (Figure 4B and Supplementary Figure 5A). We also observed an increase of ATF3 and ATF4 mRNA following treatment with CPI-613 treatment (Figure 4C). Consistently, we detected an up regulation of Noxa mRNA and protein levels that suggests an involvement of ATF4 in regulating Noxa levels following CPI-613 treatment (Figure 4, B and C). To demonstrate that the increase of both ATF4 and Noxa is due to the loss of function of OGDH we determined the expression levels of these proteins in GBM cells with silenced OGDH. We found that shRNA mediated reduction of OGDH led to a consistent increase of ATF4 and OGDH, consistent with the findings observed following CPI-613 treatment (Supplementary Figure 5B).
Next, we hypothesized that the loss of energy elicited by loss of function of OGDH led to an activation of the integrated stress response with activation of ATF4. To this purpose, we analyzed the expression of AMPK and phosphorylated AMPK (threonine 172) (Supplementary Figure 5C) and found an enhancement of AMPK phosphorylation, indicative of a decline of ATP levels. To prove that energy deprivation (mediated by loss of function of OGDH) is responsible for the up regulation of ATF4, GBM cells were treated with CPI-613 in the presence or absence of ATP. Consistently, we observed that ATP suppressed the CPI-613 mediated increase of ATF4 protein levels (Supplementary Figure 5D).

To elucidate how CPI-613 treatment led to an increase of Noxa protein and mRNA levels, we hypothesize that this is most likely related to the activation of the integrated stress response. To test this hypothesis, we analyzed the Noxa protein levels in GBM cells that were transfected with non-targeting or ATF4 specific siRNA and treated with increasing concentrations of CPI-613. The silencing of ATF4 abrogated the increase of Noxa protein level in the presence of CPI-613 treatment in both KNS42 and GBM22 cells (Figure 4D and Supplementary Figure 5E). To confirm that binding of ATF4 to the Noxa promoter is enhanced upon treatment with CPI-613, we performed chromatin immunoprecipitation of ATF4 and H3K27ac and amplified the Noxa promoter region. Notably, we found increased binding of ATF4 on the Noxa promoter following the CPI-613 treatment (Figure 4, E and F). Consistent with the up-regulation of Noxa, we detected an increased deposition of H3K27ac within the Noxa promoter as well (Figure 4F). These findings support the notion that CPI-613 activates the integrated stress response resulting in an increase of ATF4, which in turn binds to the Noxa promoter to up-regulate Noxa mRNA and protein levels.
Dual inhibition of Bcl-xL and OGDH elicits a synergistic reduction in cellular viability of GBM cells.

Our data showed an increase of Noxa protein levels mediated by CPI-613 treatment (Figure 2A). This observation led us to hypothesize that CPI-613 and BH3-mimetics might induce synergistic reduction of cellular viability in GBM cells. To this purpose, GBM12, GBM43, GBM22, and KNS42 cells were treated with ABT263, CPI-613, and the combination of both. ABT263 is known to inhibit both Bcl2 and Bcl-xL (15). Notably, we detected a stronger reduction of cellular viability following the drug combination treatment compared to the single treatments (Figure 5, A and B and Supplementary Figure 6A). The GBM12 cells displayed the most synergistic growth reduction at low nano-molar dosages for ABT263. The GBM22, GBM43 as well as the pediatric KNS42 also revealed a synergistic growth reduction following the combination treatment of CPI-613 and ABT263 (Figure 5, A and B). Next, we assessed whether the reduction of cellular viability by the combination treatment of BH3-mimetics and CPI-613 is due to the activation of apoptotic signaling pathway by performing Annexin V/ PI staining. We detected a higher rate of cell death in the drug combination of ABT263 and CPI-613 in GBM12, GBM43, GBM22, and NCH644 cells (Figure 5C and Supplementary Figure 6, B and C). With regards to non-neoplastic cells (astrocytes) we found that they demonstrated a reduced susceptibility to the combination treatment as compared to the GBM cells. We also tested the combination treatment of CPI-613 with other BH3-mimetics (ABT199, a Bcl2 inhibitor and A1210477, a Mcl1 inhibitor) (22, 23). We also detected an enhanced cell death following exposure to the various drug combinations of CPI-613 and ABT199/ A1210477 (Supplementary Figure 6, D-G).

To prove that indeed the loss of function of OGDH is critical for the susceptibility of GBM cells to BH3-mimetics, we specifically silenced OGDH. Silencing of OGDH by siRNA or shRNA led to an enhanced cell death in the presence of ABT263 treatment as compared to non-targeting siRNA or shRNA (Figure 5D and Supplementary Figure 6, H-K). Similarly, silencing of Bcl-xL or siMcl1
mediated an enhanced cell death in the presence of CPI-613 treatment (Supplementary Figure 7, A-F). All in all, these observations suggest a synergistic growth reduction by the combination treatment and that these effects are specific to the loss of function of the relevant targets (e.g. OGDH and Bcl-xL).

We wondered whether the cell death elicited by ABT263 and CPI-613 is mediated by the activation of caspases. To this end, we performed Annexin V/PI analysis of the drug combination treatment in the presence or absence of the pan-caspase inhibitor, zVAD-fmk. We found that zVAD-fmk partially rescued from the cell death mediated by the combination treatment of CPI-613 and ABT263 in GBM12 and GBM22 cells (Supplementary Figure 8, A and B). The western blot analysis of GBM cells also showed an enhanced cleavage of initiator caspase-9, effector capase-3, and cleavage of PARP upon exposure to the combination treatment (Supplementary Figure 8C).

Next, we determined whether Noxa-upregulation plays a pivotal role for the cell death induction by the drug combination treatment of ABT263 and CPI-613 by performing Annexin V/ PI staining. Our data indicated that silencing of Noxa substantially mitigated the effect of drug combination treatment to induce cell death in KNS42 and GBM12 cells (Figure 5E and Supplementary Figure 8D).

**Dual inhibition of Bcl-xL and OGDH extends animal survival in orthotopic patient-derived xenograft models of human GBM.**

Since CPI-613 and ABT263 have reached clinical testing (19-21), it was tempting to evaluate whether these two compounds would elicit a synergistic growth reduction in vivo as well. We employed dosages of compounds that showed no weight loss following treatment in mice (Supplementary Figure 9A). Next, we tested the efficacy of the drug combination treatment in a
PDX xenograft model (GBM12). We found that the combination treatment of ABT263 and CPI-613 reduced the tumor growth compared to single treatments or vehicle treatment (Figure 6, A and B). Due to the promise of the drug combination treatment in the subcutaneous GBM xenograft model, we decided to assess the efficacy of this drug combination treatment in PDX orthotopic mouse model systems. Animals that received the combination treatment of ABT263 and CPI-613 had a significantly longer overall survival compared to the vehicle or single drug treatment groups in two GBM PDX models, which implies potential clinical efficacy (Figure 6, C-F). The H&E staining (Supplementary Figure 9B) and the MRI imaging (Figure 6F) demonstrated a reduction in tumor growth following exposure to the combination treatment. With regards to the molecular alterations, we found that CPI-613 increased the number of TUNEL positive cells in orthotopic tumors of animals receiving the combination treatment of ABT263 and CPI-613 (Figure 6, G and I). Moreover, CPI-613 elicited an increase in the protein expression levels of Noxa in animals receiving either the single treatment (CPI-613) or the combination treatment (ABT263 + CPI-613) (Figure 6, H and J). Another critical feature of therapeutic interventions designed for brain tumors is their impact on neurons. To this end, we evaluated neuronal toxicity in vivo following the different drug treatments. We did not detect any form of neuronal cell death in the treatment groups (representative neurons from the hippocampal region are shown) (Supplementary Figure 9, C and D).
**Discussion**

Metabolism and the micro-environment of glioblastoma are currently key aspects that are being studied in GBM (24, 25) because these two features of glioblastoma may be critical drivers of GBM growth and treatment resistance (26-28). The tumor micro-environment is centered on two main components, which is either the interaction of immune-cells with tumor cells or neurons with GBM cells (29). It may even be conceivable that the two components are tightly interlinked with each other. By far, myeloid-derived suppressor cells, regulatory-T-cells and M2-macrophages are the main causes of an immune-suppressive environment in GBM that ultimately may allow GBM cells to hide from being killed by cytotoxic T-cells.

Metabolism is probably the most critical component to dictate response and resistance since it affects both tumor cells and the micro-environment and it has been recently suggested that metabolic reprogramming may be the driver of genetic alterations that are responsible for tumor growth and ultimately treatment resistance (30). Here, through genetic screening, we have identified the OGDH gene as a critical driver of glioblastoma growth. This was a surprising finding in that one would anticipate that the more proximal enzymes, such as citrate synthase, would be far more critical for GBM growth than the OGDH enzyme, especially in light of the fact that glutamine metabolism was found not to be as critical as glucose and acetate for glioblastoma growth based on prior C13 tracing studies in brain tumor patients (31). In our model systems of human PDX GBM lines we found that loss of OGDH was accompanied by a reduction of the oxygen consumption rate. This finding is anticipated since the OGDH enzyme is a key driver of the TCA-cycle that along with other enzymes in the cycle generates NADH, which critically fuels the respiratory chain. In terms of translatability, we utilized an inhibitor of OGDH that has reached phase III clinical testing in patients, called CPI-613 (19-21). For the most part, this compound phenocopied our findings obtained in genetic loss of function experiments.
Loss of function of OGDH led to an activation of the integrated stress response with an increase of ATF4 (Figure 4), which was preceded by a loss of ATP. Consequently, rescue experiments suggested that the activation of the ISR was related to loss of energy levels (Supplementary Figures 4E). While ATP did not completely restore viability upon inhibition of OGDH, the rescue was nevertheless statistically significant. However, these findings also suggest that alternate metabolites may be involved. In this regard, it is possible that certain amino acids may be involved as well, e.g. aspartate. Another explanation may be that the metabolite L-2 hydroxyglutarate may mediate parts of the anti-glioma effects of CPI-613 or loss of function of OGDH. For instance, upon loss of OGDH in hematopoietic stem cells L-2-HG accumulated and exerted the inhibitory effects on oxidative energy metabolism (32).

We have found that following blockage of OGDH the transcription factor ATF4 was enriched in the promoter region of the PMAIP1 gene, which encodes for the pro-apoptotic Bcl-2 family member, Noxa. In turn, ATF4 increased Noxa levels. Through rescue experiments we found that the increase of Noxa was involved in the reduction of cellular viability of GBM cells mediated by interference with OGDH, suggesting activation of classical intrinsic apoptosis (Figure 2). Because Noxa binds to Mcl-1 to facilitate the release of BAK from Mcl-1 high Noxa levels sensitize cancer cells to the cytotoxic effects of ABT263 and other BH-3-mimetics (33). Indeed, we were able to demonstrate inhibition of OGDH enhanced the efficacy of ABT263 to kill glioblastoma cells (Figure 5). Based on silencing studies these effects appeared to be mediated predominantly by loss of Bcl-xL. As anticipated, we noted that silencing of Noxa protected from ABT263 mediated reduction of cellular viability in GBM cells (Figure 5E). Our study establishes a functional link between loss of function of OGDH, ISR signaling and intrinsic apoptosis. These results position OGDH as a treatment target for GBM. Our findings are distinct from previous results obtained in breast and colonic carcinoma models, which showed an increased dependency on OGDH only in the presence of mutated PIK3CA (17). While we cannot completely exclude the possibility that
certain genetic alterations may render GBM cells even more dependent on OGDH for survival it seems more likely that OGDH is a key driver in a broad range of IDH-wild type glioblastoma likely to be independent of specific genetic mutations, such as PIK3CA. We performed two orthotopic GBM models to demonstrate that the combination treatment of ABT263 and CPI-613 is efficacious in vivo. In both model systems, the combination treatment led to an increase in animal survival, which was more pronounced in the GBM12. The difference in response to therapy between the GBM12 and GBM22 may be explained potentially by a different behavior of these cells when exposed to the brain parenchyma. However, other explanations may be possible as well. We also have not formally determined the extent of blood brain barrier penetration of both ABT263 and CPI613. While we see an anti-glioma effect in our models (including orthotopic models) it is possible that the efficacy of the drug combination may be mitigated due to the potential limited delivery of the compounds. A recent study suggested that some GBMs may be characterized as a mitochondrial subtype since these tumors appear to be particularly reliant on cellular respiration and oxidative phosphorylation. In agreement, classical inhibitors of either cellular respiration, e.g. metformin or the more specific complex I inhibitor, IACS-010759, or blockers of mitochondrial protein translation (tigecycline) reduced the viability of “mitochondrial subtype” GBM cells more potently (34). Based on these findings, it appears likely that CPI-613 may be efficacious in this GBM subtype as well.

While our studies focused exclusively on tumor cell metabolism it remains to be determined how OGDH affects the immune micro-environment in GBM. This is of relevance since immune suppressive cell types rely on oxidative energy metabolism. Therefore, it may be conceivable that myeloid derived suppressor cells display a dependency on OGDH to exert their immune suppressive effects in GBM. All in all, we have established OGDH as a target for GBM, which significantly extends our current understanding about GBM metabolism and how OGDH affects ISR signaling as well as intrinsic apoptosis.
Materials and Method

Sex as a biological variant

Sex was not regarded as a biological variable in this study. Our assessment focused solely on female mice, examining the effects of vehicle, single, and combination drug treatments.

Cell cultures

KNS42, GBM22, GBM43, GBM12 GBM cells were cultured in DMEM (Fisher Scientific, MT10013CV), 10% FBS (Gemini) and 100 µg/ml of Primocin (Invivogen, ant-pm-1). KNS42 cells were purchased from the Japanese Collection of Research Bioresources Cell Bank (JCRB, IFO50356). GBM22, GBM43, GBM12 cells were obtained from Dr. Jann Sarkaria (Mayo Clinic, Rochester, MN) between 2020 and 2023. For the treatment experiments, cells were cultured in DMEM containing 1.5% FBS and 100 µg/ml of Primocin (Invivogen, ant-pm-1). NCH644 stem cell-like glioma cells were purchased from Cell Line Services (Cell Line Services, 820403) and were cultured in StemPro NSC SFM (Thermo Fisher, A1050901) with 100 µg/mL primocin for maintenance and for drug treatment. All cells were incubated and maintained at 37°C and 5% CO₂.

Reagents

Devimistat (CPI-613) (HY-15453), Navitoclax (ABT-263) (HY-10087), A-1210477 (HY-12468), and Venetoclax (ABT-199) (HY-15531) were purchased from MedChemExpress. Z-VAD-FMK (S7023) and ATP disodium (S1985) were purchased from Selleckchem. Puromycin dihydrochloride (P9620) was purchased from Sigma. A 10 mM working solution in dimethylsulfoxide (DMSO) was prepared for all reagents with a final concentration below 0.1% (v/v).
**Cell viability assays**

Cells were seeded with a density of 3,000 (KNS42, GBM22, and GBM43) or 8,000 cells (GBM12 and NCH644) in a 96-well plate and allowed to attach overnight. The next day, cells were treated with targeted drugs for 72 hours and were analyzed for cell viability by using the CellTiter-Glo assays (Promega, G7571). For the ATP rescue experiments, cell viability was determined by using CyQUANT Cell Proliferation Assay (Thermo Fisher, C7026). To evaluate drug synergy, the median effect equation (Chou-Talalay) was performed. Following calculations, this approach yields normalized isobolograms and the combination index (CI), respectively (35).

**Flow cytometry**

Cells were seeded with a density of 30,000 (KNS42, GBM22, and GBM43) or 80,000 cells (GBM12 and NCH644) in a 12-well plate and allowed to attach overnight. On the day of the experiment, the cells were stained with FITC Annexin V/propidium iodide (BD Biosciences, 556420) to detect apoptosis and necrosis following the company’s instruction. Samples were detected by using LSRII flow cytometry (BD) and the data were analyzed with FlowJo software (version 8.7.1; Tree Star).

**RNA-seq and subsequent gene-set enrichment analysis**

KNS42 cells were treated with DMSO or 100 μM CPI-613 for 24h. Cells were extracted for total RNA by using miRNAeasy Mini Kit from QIAGEN (217004). 100 ng of RNA with RIN > 8 is used to mix with the RNA library SIRV-Set 1 (Iso Mix E0, E1, E2) - RNA-seq (Lexogene, SKU: 025.03) according to the company instruction. The RNA sequencing was performed at the Columbia Genome Center and the gene set enrichment analysis was performed by using Scidap.com. The experiments used in this study were deposited at GEO: GSE223297.

**Western blot and protein capillary electrophoresis**
All samples were lysed in 1X Laemmli buffer (Bio-Rad) containing protease and phosphatase inhibitor cocktail (Thermo Fisher, 78440). For standard western blot, samples were run on a 4%–12% SDS PAGE gel (Invitrogen, NP0321BOX) and were transferred to Immun-Blot PVDF membrane (Bio-Rad, 1620177). The blots were captured by using the Azure (C300) imaging system (Azure Biosystems). For the protein capillary electrophoresis, samples were detected by using the Wes instrument (ProteinSimple) following the company instruction.

The following primary antibodies were used in the standard western blot: Mcl-1 (D35A5) (CST 5453; 1:500), Bcl-xL (54H6) (CST 2764; 1:500), Bcl-2 (D55G8) (CST 4223; 1:500), Noxa (Calbiochem OP180, clone 114C307; 1:500), β-actin (Sigma Aldrich A1978, clone AC15; 1:3,000), ATF3 (D2Y5W) (CST 33593; 1:500), ATF4 (D4B8) (CST 11815; 1:500), PARP (46D11) (CST 9532; 1:500), Cleaved Caspase-9 (Asp330) (D2D4) (CST 7237; 1:500), Caspase-3 (8G10) (CST 9665; 1:500). The indicated secondary antibodies were used in the standard western blot: anti-rabbit IgG (H+L), HRP (Thermo Fisher, 31460) and anti-mouse IgG (H+L), HRP (Thermo Fisher, 31430).

The primary antibodies were used in the protein capillary electrophoresis: Vinculin (Abcam ab129002; 1:500), AMPK (D5A2) (CST 5831; 1:25), pAMPK (Thr172) (CST 2531; 1:25), ATF4 (D4B8) (CST 11815; 1:25). The secondary antibodies were used: Anti-Rabbit Secondary HRP Antibody (ProteinSimple, 042-206) and Anti-Mouse Secondary HRP Antibody (ProteinSimple, 042-205).

**Real-time PCR analysis**

Cells were extracted for total RNA by using the miRNAeasy Mini Kit from QIAGEN (217004). The RNA was synthesized to cDNA by using the qScript™ cDNA SuperMix, QuantaBio kit (101414–106). The RT-PCR was performed by using PerfeCTa® SYBR® Green FastMix® Reaction Mixes, Quantabio (101414–276) with the condition 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, on a qPCR Instrument
(Quantabio). All RT-PCR was performed in quadruplicate and the average fold changes were calculated based on 18S in the threshold cycle (Cq). Primer sequences are in the supplementary Table S1.
siRNA transfection and lentivirus transduction

The siRNAs were purchased from Dharamacon: non-targeting siRNA-pool (scramble; D-001810–10–20), ATF4 siRNA-pool (L-005125-00-0005), ATF4-10 siRNA-10 (J-005125-10-0002), ATF4-12 siRNA-12 (J-005125-12-0002), ATF3 siRNA-pool (L-008663-00-0005), OGDH siRNA-pool (L-009679-00-0005), Mcl1-16 siRNA (J-004501-16-0002), Mcl1-17 siRNA (J-004501-17-0002). PMAIP1-1 siRNA (s10708) and PMAIP1-2 siRNA (s10709) were purchased from Thermo Fisher. Bcl-xL-1 (CST, 6362) and Bcl-xL-2 (CST, 6363) siRNA were purchased from Cell Signaling Technology. Cells were transfected with Lipofectamine RNAiMAX (Invitrogen, 13778075) according to the manufacturers’ instructions.

OGDH shRNAs (TRCN0000028618, TRCN0000028643, and TRCN0000028580) were purchased from Sigma. Adeno CMV Null Adenovirus (Ad-CMV-Null 1300) and human BCL2L1 Adenovirus (ADV-202038) were purchased from Vector Biolabs. Lentivirus was generated by transfection of pMD2.G, psPAX2, and the relevant lentivirus plasmid into 293T cells for 72 hours. The viral supernatant was collected, filtered with 0.45 μm SFCA (surfactant free cellulose acetate) syringe filter (Fisher Scientific, 09-754-21), and concentrated with an Amicon Ultra-15 Centrifugal filter unit (Sigma, UFC910024) before they were transduced into GBM cells. GBM cells were transduced with lentivirus for 48 hours and were selected with puromycin (2μg/ mL) for a week.

Isotope tracing and LC/MS

Cells were seeded with a density of 1 × 10⁶ cells per 10 cm dish and allowed to attach overnight. Next day, cells were exposed to DMEM no glucose, no glutamine, and no phenol red (Thermo Fisher, A1443001), containing 25 mM (U-13C6) D-Glucose (Cambridge Isotope Laboratories, Inc, CLM-1396–2), 4 mmol/L Glutamine (Fisher Scientific, 15410314), and 1.5% dialyzed FBS (Thermo Fisher, A3382001) for 24 hours. Cells were collected and analyzed for the polar metabolites by Metabolomics Core Facility at Weill Cornell.
**Extracellular flux analysis**

Cells were seeded with a density of $3 \times 10^4$ cells per XFe24 cell culture microplate (Agilent) and allowed to attach overnight. Cells were treated with target drugs for 24 hours. The oxygen consumption rate (OCR) was measured with a Seahorse XFe24 Analyzer (Agilent) using the mito stress assay kit (Agilent, 103015–100) in the Seahorse XF base medium (Agilent, 102353–100) containing 10 mM glucose, 2 mM glutamine, and 1 mM pyruvate. The following compounds were injected in a sequential order: 2 µM oligomycin (OM), 2 µM Carbonyl cyanide–4 (trifluoromethoxy) phenylhydrazone (FCCP), and 0.5 µM rotenone/antimycin (R/A) following the company’s instruction.

**Subcutaneous xenograft and orthotopic glioblastoma PDX mouse model**

Sex was not regarded as a biological variable in this study. Our assessment focused solely on female mice, examining the effects of vehicle, single, and combination drug treatments.

Subcutaneous xenograft: $3 \times 10^6$ GBM12 cells were implanted subcutaneously into the flanks of 6-8-week-old female nude mice (Taconic Biosciences, NCRNU- F sp/sp, CrTac:NCr-Foxn1nu). ABT263 (75mg/kg) and CPI-613 (50 mg/kg) were dissolved in a mixture containing DMSO, cremophor EL (Sigma, 61791–12–6), Ethyl Alcohol (Pharmco-Aaper, 200 Proof), and PBS at the ratio: 10:32:8:50 (v/v/v/v). Intraperitoneal treatment was administered three times a week. Tumor sizes were measured with a caliper and were calculated as $(\text{length} \times \text{width}^2) / 2$.

Orthotopic PDX models: $5 \times 10^4$ GBM12 and GBM22 cells were intracranial injected at 3 mm lateral of the bregma and 3 mm down in 6-8-week-old female nude mice. Treatments were given until the animals became moribund or when neurologic deficits were observed (retardation, lethargy, seizures). The dosages for ABT263 and CPI-613 were based on our earlier published work (7, 22, 36). The survival curve was analyzed by Kaplan–Meier survival fractions and the log-rank test was employed to assess the statistical significance. Immunohistochemical analysis for TUNEL and Noxa were performed in a fashion as previously described (37). Regarding the
quantification Noxa IHC score was assigned by taking into account both the staining intensity (0: no staining, 1: weak staining intensity, 2: intermediate staining intensity, 3: strong staining intensity) and the percentage of positive cells (0: no cells labeled, 1: 1-25%: cells labeled, 2: 25-74%: cells labeled, 3: 75-100% cells labeled).

**Statistical analysis**

Statistical significance was determined by using Prism version 9 (GraphPad, La Jolla, CA). The two-tailed student’s t-test or ANOVA (for multiple comparisons) were used. A p < 0.05 was considered statistically significant.

**Study approval**

All procedures were done in accordance with Animal Welfare Regulations and approved by the Institutional Animal Care and Use Committee at the Columbia University Medical Center (AC-AABC6505 and AC-AABI3633).

**Data availability**

All data are included in the Supporting Data Values file. Any data that support the findings of this study are available from the corresponding authors upon reasonable request. The RNA-sequencing data used in this study were deposited at GEO: GSE223297 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE223297).
Author contributions

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Figure 1. Genetic or pharmacological inhibition of OGDH, a key enzyme of the TCA-cycle, reduces the growth of GBM cultures. (A and B) CRISPR and RNAi library screening (obtained and analyzed from the DepMAP database) points towards increased reliance of GBM cells on several TCA-cycle enzymes, especially the OGDH gene. (C) KNS42, GBM22, and GBM12 cells were transduced with lentiviral vectors, containing either non targeting shRNA or shRNAs against OGDH. Cellular viability analysis was performed for up to four days (n = 4 per group). (D) Western blots of KNS42, GBM22, and GBM12 cells transduced with lentiviral vectors, containing either non targeting shRNA or shRNAs against OGDH. Actin is used as a loading control. (E) GBM22, GBM12, KNS42, NCH644, and astrocyte cells were treated with increasing concentration of CPI-613 for 72 hours, labeled with Annexin/PI dye, and analyzed by flow cytometry for apoptosis induction (n = 3 per group). (F) Shown is the survival curve of patients (wild-type and mutated IDH) with high or low mRNA levels of OGDH from the TCGA database. Cutoff point (maximally selected rank statistics) was calculated through gliovis, which yielded a cutpoint of 11.3 for mRNA. High levels of OGDH correlate with a worse overall survival in patients. (G and H) GBM22 cells were transduced with lentiviral vectors, containing either non targeting shRNA or shRNAs against OGDH, and were implanted in the right striatum of nude mice. The representative MRI images of brain tumors from the experiment in G are shown (Bruker BioSpecTM, 9.4 Tesla). The log-rank test was used to assess statistical significance (n = 5 in shNT and n=7 in shODGH-80). Median survival in GBM22-shNT is 40 days and in GBM22-shODGH-80 is 56 days. Statistical significance was assessed ANOVA with Dunnett multiple comparison test in E and by two-tailed Student t test in H. Data are shown as mean ± SD. **** p < 0.001.
Figure 2. Treatment with CPI-613 increases the expression of pro-apoptotic Noxa and suppresses Bcl-xL levels to induce apoptosis. (A) GBM22, KNS42, GBM43, NCH644, and GBM12 cells were treated with increasing concentrations of CPI-613 for 24 hours and were analyzed for the Bcl-2 family members by western blotting. (B) GBM22 and KNS42 cells were transduced with an empty vector (EV) or a vector containing Bcl-xL cDNA (using adenoviruses), treated with increasing concentrations of CPI-613, and cellular viability analysis was performed (n = 4 per group). (C) GBM43 and KNS42 cells were transduced with an empty vector (EV) or a vector containing Bcl-xL cDNA, treated with increasing concentrations of CPI-613, labeled with Annexin/PI dye, and analyzed by flow cytometry (n = 2 per group). (D and E) KNS42 cells were transfected with non-targeting siRNA (siNT) or siRNAs against Noxa. Transfected cells were treated with CPI-613 and cellular viability analysis was performed in D (n = 4 per group) and flow cytometry following labeling with Annexin V/PI was performed in E (n = 3 per group). Statistical significance was assessed by two-tailed Student t test in B, D, and E. Data are shown as mean ± SD. *p < 0.05, **p < 0.01, **** p < 0.001.
Figure 3. CPI-613 treatment reduces the labeling of TCA-cycle metabolites from glucose and the oxygen consumption rate in GBM cells. (A) KNS42 cells were treated with CPI-613 for 24 hours, subjected to RNA sequencing and followed by gene set enrichment analysis. NES: normalized enrichment score. FDR: false discovery rate. (B and C) KNS42 cells were treated with CPI-613 for 24 hours and were processed for polar metabolite LC/MS analysis. The metabolite enrichment analysis was performed by using MetaboAnalyst. Shown is the enrichment pathway analysis and the citric acid cycle is highlighted in red. (D-F) KNS42 cells were treated with 100 μM CPI-613 in the DMEM media containing 25 mM U-13C glucose, 4 mM glutamine and 1.5% dialyzed FBS for 24 hours. Shown are fractions of the isotopologues for each metabolite (n = 3 per group). (G) GBM22 cells were transduced with either non targeting shRNA or shRNAs against OGDH. The transduced cells were cultured in the DMEM media containing 25 mM U-13C glucose, 4 mM glutamine and 10% dialyzed FBS for 24 hours (n = 3 per group). (H-K) KNS42 and GBM22 cells were treated with CPI-613 (CPI) for 24 hours and subjected to extracellular flux analysis to analyze maximal respiration and coupled respiration in J and K. OM, oligomycin; FCCP, carbonylcyanide-4 (trifluoromethoxy) phenylhydrazone; R/A, rotenone and antimycin (n = 3 per group). Statistical significance was assessed ANOVA with Dunnett multiple comparison test in G and by two-tailed Student t test in D-F, J and K. Data are shown as mean ± SD. **p < 0.01, ***/****p < 0.001.
Figure 4. CPI-613 treatment causes energy deprivation and activates endoplasmic reticulum stress signaling. (A) KNS42 cells were treated with CPI-613 for 24 hours, subjected to RNA-sequencing and followed by GSEA. NES: normalized enrichment score. FDR: false discovery rate. (B) Western blots of KNS42, GBM22, GBM43, NCH644, and GBM12 cells treated with increasing concentrations of CPI-613 for 24 hours. Actin is a loading control. (C) Real time PCR analysis of GBM22, KNS42 and GBM43 cells treated with increasing concentrations of CPI-613 for 24 hours. 18S is a housekeeping gene. (D) Standard western blot (KNS42) or protein capillary electrophoresis analyses (GBM22) of cells transfected with non-targeting siRNA (siNT) or with siRNA against ATF4 (total) followed by treatment with CPI-613 for 24 hours. Actin or Vinculin is a loading control. (E) KNS42 and GBM22 cells were treated with CPI-613 for 24 hours and were subjected to chromatin immunoprecipitation with either a control antibody (IgG; negative control) or an antibody against ATF4. The Noxa region was amplified by PCR. (F) KNS42 and GBM22 cells were treated with CPI-613 for 24 hours and were subjected to chromatin immunoprecipitation with either a control antibody (IgG; negative control) or an antibody against H3K27ac. The Noxa region was amplified by PCR (n = 4 per group). Statistical significance was assessed ANOVA with Dunnett multiple comparison test in C and by two-tailed Student t test in E and F. Data are shown as mean ± SD. *p < 0.05, **p < 0.01, **** p < 0.001.
Figure 5. Dual inhibition of ABT263 and CPI-613 elicits a synergistic reduction in cellular viability of GBM cells. (A and B) GBM12, GBM22, GBM43, and KNS42 cells were treated with ABT263, CPI-613, or the combination of both for 72 hours, and cellular viability was analyzed. Shown are isobolograms in A and the quantifications in B (n = 4 per group). (C) GBM12, GBM22, GBM43, and NCH644 cells were treated with ABT263, CPI-613, or the combination of both, labeled with Annexin V/PI dye, and analyzed by flow cytometry (n = 3 per group). (D) KNS42 cells were transduced with lentiviral vectors, containing either non targeting shRNA or shRNAs against OGDH, were treated with increasing concentrations of ABT263 followed by flow cytometry after Annexin V/PI labeling (n = 3 per group). (E) GBM12 and KNS42 cells were transfected with non-targeting siRNA (siNT) and siRNA against Noxa. Cells were treated as indicated and labeled with Annexin V/PI followed by flow cytometry (n = 3 per group). Statistical significance was assessed ANOVA with Dunnett multiple comparison test in B, C, and D and by two-tailed Student t test in E. Data are shown as mean ± SD. **** p < 0.001.
Figure 6. Dual inhibition of Bcl-xL and OGDH extends animal survival in orthotopic patient-derived xenograft models of GBM in mice. 

(A and B) GBM12 cells were implanted into the subcutis of immunocompromised Nu/Nu mice. Seven days later the mice were divided into four treatment groups: vehicle, CPI-613 (50 mg/kg), ABT263 (75 mg/kg), and the combination of both. The tumor volume over time is shown on the left and the tumor volume on the last day of the experiment is shown on the right (n = 9 each group). 

(C and D) GBM12 and GBM22 cells were implanted in the right striatum of nude mice. Four groups were randomly assigned: vehicle, CPI-613, ABT263, and the combination of both. Seven days after the implantation, mice were treated three times per week and animal survival is provided (Kaplan–Meier-curve). The log-rank test was used to assess statistical significance (GBM12: n = 5 each group and GBM22: n = 4 for vehicle, CPI-613, and ABT263 and n=6 for the CPI-613+ABT263). Median survival in GBM12: 20 days for vehicle and ABT263, 23 days for CPI-613, and 32 days for CPI-613+ABT263. Median survival in GBM22: 21.5 days for vehicle, 28 days for ABT263 and CPI-613, 31.5 days for CPI-613+ABT263. 

(E and F) The representative MRI images of brain tumors as well as their quantifications from the experiment in D are shown (Bruker BioSpecTM, 9.4 Tesla). 

(G and I) Shown are representative immunohistochemical stains of TUNEL as well as the related quantifications in I. 

(H and J) Shown are representative immunohistochemical stains of Noxa as well as the related quantifications in J. Scale bar: 20 µm. Statistical significance was assessed by using ANOVA with Dunnett multiple comparison test in B-E, I and J. Data are shown as mean ± SEM in A and SD in B-D, I and J. *p < 0.05, **p < 0.01.