Medium-chain acyl CoA dehydrogenase protects mitochondria from lipid peroxidation in glioblastoma

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Running Title: MCAD prevents toxic metabolite accumulation in glioma cells

Conflict of Interest:
C.A.L. has received consulting fees from Astellas Pharmaceuticals and is an inventor on patents pertaining to Kras regulated metabolic pathways, redox control pathways in pancreatic cancer, and targeting the GOT1-pathway as a therapeutic approach.
F.P., J.L., C.A.L., A.V., and G.F.D. was supported by NCI R01 CA218139 01 A1. G.F.D. was also supported by the Sewell Family Chairmanship in Genomic Medicine. C.A.L. was also supported by NCI R37CA237421, R01CA248160, R01CA244931 and UMCCC Core Grant P30CA046592. P.P.S. was supported by Cancer Prevention and Research Institute of Texas grants RP140672 and RP150519. F.P. and P.P. received support from the American-Italian Cancer Foundation. F.Y. was supported by the CPRIT Training Program RP170067. S. Srinivasan was supported by the CPRIT Graduate Scholar Fellowship. F.F.L. was supported by NCI 2P50CA127001, The University of Texas MD Anderson Cancer Center Moon Shots Program™, The Broach Foundation for Brain Cancer Research, and The Elias Family Fund to F.F.L. Metabolomics studies performed at the University of Michigan were supported by NIH grant DK097153, the Charles Woodson Research Fund, and the UM Pediatric Brain Tumor Initiative. This research was also supported by NCI Core Facility Grant CA16672 and the NCI Cancer Center Support Grant to MD Anderson P30CA16672.
ABSTRACT

Glioblastoma (GBM) is highly resistant to chemo- and immune-based therapies and targeted inhibitors. To identify novel drug targets, we screened orthotopically implanted, patient-derived glioblastoma sphere-forming cells (GSCs) using an RNAi library to probe essential tumor cell metabolic programs. This identified high dependence on mitochondrial fatty acid metabolism. We focused on medium-chain acyl-CoA dehydrogenase (MCAD), which oxidizes medium-chain fatty acids (MCFAs), due to its consistently high score and high expression among models and upregulation in GBM compared to normal brain. Beyond the expected energetics impairment, MCAD depletion in primary GBM models induced an irreversible cascade of detrimental metabolic effects characterized by accumulation of unmetabolized MCFAs, which induced lipid peroxidation and oxidative stress, irreversible mitochondrial damage, and apoptosis. Our data uncover a novel protective role for MCAD to clear lipid molecules that may cause lethal cell damage, suggesting that therapeutic targeting of MCFA catabolism could exploit a key metabolic feature of GBM.
STATEMENT OF SIGNIFICANCE

MCAD exerts a protective role to prevent accumulation of toxic metabolic byproducts in glioma cells, actively catabolizing lipid species that would otherwise affect mitochondrial integrity and induce cell death. This work represents a first demonstration of a non-energetic role for dependence on fatty acid metabolism in cancer.
INTRODUCTION

Glioblastoma (GBM) has a median survival of 15 to 19 months (1-3). Despite extensive efforts, studies of both signal transduction inhibitors and immune-targeted agents have failed to improve the prognosis for patients with these tumors (4-8). As with most cancers, altered metabolism is a defining characteristic of glioblastoma. Dependency on aerobic glycolysis (Warburg effect), as well as on glutaminolysis, are well documented (9-11), and recent landmark studies have identified mutations in GBM that directly impact mitochondrial metabolism (12,13). However, the molecular drivers underlying this metabolic reprogramming are only partly understood, and therapeutically actionable metabolic dependencies have not been fully determined.

In the present study, we applied an in vivo genetic screen platform (14) to orthotopically implanted, patient-derived 3D glioma tumor spheres and identified mitochondrial fatty acid metabolism as an essential pathway required for tumor growth. From among several enzymes in the β-oxidation pathway that emerged as hits, we selected medium-chain acyl CoA dehydrogenase (MCAD), a mitochondrial enzyme that catalyzes the first step of medium-chain fatty acid (MCFA) β-oxidation (FAO), for deep functional characterization. The uptake of MCFAs lacks any negative feedback regulation; therefore, in the absence of MCAD, MCFAs accumulate to toxic levels. Still, inherited MCAD deficiency, which is screened for perinatally, is a condition compatible with normal quality of life, and afflicted individuals require only dietary adjustment to thrive (15,16). These facts are suggestive of a therapeutic window to inhibit MCAD in lipid-addicted tumor types.

Both in vitro and in vivo, our studies showed that targeting MCAD in primary GBM models triggered massive accumulation of unmetabolized MCFAs in the mitochondria, which resulted in peroxidation of fatty acids byproducts. This overload of partially metabolized substrates generated oxidative stress that irreparably damaged the mitochondrial structure and caused tumor cell death. Thus, in GBM, the activity of MCAD does not solely support cellular energetics as part of the β-oxidation cycle, but our work demonstrates a previously unappreciated protective role for MCAD fatty acid catabolism to clear toxic accumulations of lipid molecules that may otherwise cause lethal damage to the cell.
RESULTS

ACADM is an essential gene in glioblastoma

To achieve a functional output of metabolic gene functions that might be essential for GBM cell survival, we generated a prioritized list of 330 metabolism genes and created a barcoded, deep-coverage (10 shRNAs/gene) shRNA library that encompasses a wide range of metabolic pathways and activities (Figure S1A). As a disease model, we selected low-passage, 3D-cultured, patient-derived glioma sphere-forming cells (GSCs) established at the MD Anderson Brain Tumor Center (17-19). Among over 70 genomically characterized GSCs available to us, for the genetic screen we selected two carrying distinct gene expression signatures classified as belonging to the proneural (GSC 8.11) or classical (GSC 6.27) (Figure S1B) subtypes. After shRNA library transduction, cells were implanted intracranially, and tumors were grown until mice showed tumor-related neurological symptoms (Figure S1C). At endpoint, tumors were excised and processed as previously described (14) (Figure 1A). Analysis of the most significantly depleted shRNAs in both GBM models uncovered the potential role of several mitochondrial enzymes involved in fatty acid metabolism in GBM growth (Figure 1B; Figure S1D-G). Given compelling evidence of its role in fatty acid metabolism in brain tissues, we focused our attention on MCAD, which oxidizes medium-chain (4- to 12-carbon) fatty acids (MCFAs). MCFAs such as octanoate are normally present in plasma at significant levels (20) and readily cross the blood-brain barrier (21). It has been shown that up to 20% of brain oxidative metabolism can be attributed to octanoate use in an intact rodent physiological system (22).

To explore a possible role for ACADM in GBM, we queried The Cancer Genome Atlas (TCGA) mRNA dataset and found that ACADM is highly expressed across GBM subtypes compared to normal brain (Figure 1C). Through immunohistochemistry analysis, we also determined that MCAD protein levels are elevated compared to normal brain tissue in a GBM tumor microarray series (Figure 1D, E). For in vitro studies, we selected normal neural stem cells (NSCs) and normal human astrocytes (NHAs) to represent normal brain cells, the latter based on previous reports of high ACADM mRNA levels relative to other brain-derived cells (23) (Figure S1H), and also given recent findings that astrocyte-like neural stem cells might be the GBM cell of origin (24). We confirmed lower MCAD levels in NSCs and NHAs compared to GSCs, with NSCs showing barely detectable MCAD levels (Figure S2A). A similar trend was observed in the relative expression levels of other enzymes involved in β-oxidation (Figure S2B). Consistent with expression levels and previous reports (25), analysis of mitochondrial bioenergetics demonstrated elevated oxidative metabolism in GSCs compared to NSCs and NHAs (Figure 1F). Further, we found that
GSCs produce ATP predominantly through mitochondrial metabolism (65 to 83%), whereas NSCs and NHAs generate ATP primarily via glycolysis (~75%) (Figure 1G), thus emphasizing the importance of oxidative metabolism in GBM. To functionally validate our findings, we performed metabolic flux analysis (MFA) using $^{13}$C-oleate as the substrate. Strikingly, these data demonstrated a significant enrichment of $^{13}$C in TCA cycle metabolites in GSCs compared to NSCs and NHAs (Figure 1H, Figure S2C), thus supporting that FAO strongly contributes to an enhanced oxidative metabolism in GBM.

**MCAD depletion impairs glioblastoma growth in vitro and in vivo**

To further investigate the relevance of MCAD in this context, we validated two independent ACADM-targeting RNA interference (shRNA) constructs for their ability to down-regulate MCAD protein expression (Figure S3A, B). We found that MCAD depletion dramatically impaired anchorage-independent growth in multiple GSCs representing different GBM subtypes (Figure 2A-B, Figure S3C), whereas MCAD overexpression rescued the viability (Figure S3B). Similar effects on GSC growth were obtained by ACADM deletion through CRISPR-Cas9 editing using three independent sgRNA constructs (Figure S3D-F). To better understand the effects of ACADM down-regulation on GSCs, we evaluated apoptosis in a time course experiment, which demonstrated that the growth arrest induced by ACADM ablation precedes a significant increase in apoptotic cell death (annexin V positivity) detected approximately 7 days after the end of selection in all GSCs tested (Figure 2C; Figure S4A,B). Indeed, no significant effect on cell viability was observed at earlier time points (72 hours) (Figure 2C, Figure S4A). Similar effects on cell growth and sphere-forming efficiency were observed upon treating GSCs with spiropentaneacetic acid (SPA), a compound known to specifically inhibit MCAD activity (26) (Figure 2D; Figure S4C,D).

To determine the effect of MCAD loss in vivo, GSC 8.11 and GSC 6.27 cells harboring ACADM- or non-targeting shRNA constructs were implanted into the mouse forebrain, and tumor growth was monitored by luciferase and magnetic resonance imaging (MRI). MCAD depletion dramatically attenuated tumor growth and significantly extended survival time (Figure 2E-G; Figure S5A-C). Similarly, MCAD depletion in tumors established through intracranial injection of GSC 8.11 cells carrying doxycycline-inducible shRNA constructs resulted in significant retardation of tumor growth (Figure S5D). We next generated ACADM-deleted NHAs and NSCs using our validated sgRNA guides. Interestingly, neither the cytotoxic nor the anti-proliferative effects observed in GSCs were observed in NHAs and NSCs (Figure 2H; Figure S5E, F). Likewise, pharmacological MCAD inhibition with SPA did not result in significant toxicity in NHAs (Figure 2I). Given the very low levels of MCAD in NSCs (Figure S2A), we considered NHAs more suitable to
represent the normal brain counterpart for further validation experiments. Altogether, these data suggest that MCAD dependency may be a metabolic vulnerability unique to malignant cells.

**ACADM silencing impairs mitochondrial function in GSCs**

Given that GSC energetics seem to be heavily reliant on oxidative metabolism (Figure 1F,G), we characterized the early effects of MCAD downregulation on mitochondria between 48 and 72 hours post-MCAD depletion (shRNA/sgRNA ablation). First, we acquired transmission electron microscopy (TEM) images revealing that MCAD depletion resulted in mitochondria with darker matrices and swollen cristae surrounded by vacuolar structures (Figure 3A; Figure S6A). Next, a bioluminescence assay uncovered an overall decrease in ATP content in MCAD-depleted GSCs versus controls (Figure 3B). Analysis of oxygen consumption rate (OCR) in GSCs and NHAs revealed significant decreases in basal respiration and reserve respiratory capacity in **ACADM**-deleted GSCs (Figure 3C; Figure S6B), whereas **ACADM** deletion did not affect OCR in NHAs (Figure 3D). The depletion of the acetyl-CoA pool and TCA intermediates upon **ACADM** down-regulation (Figure 3E,F; Figure S6C), together with an increased contribution of carbon skeletons derived from fatty acids to central carbon metabolism in GBM (Figure 1H), confirm the critical dependence of GBM cells on oxidative metabolism, largely fueled by FAO, and the role of MCAD to support mitochondrial function in this context.

To distinguish whether the observed toxicity of MCAD inactivation is triggered by mitochondrial dysfunction or by the energy deprivation resulting from the inability of GSCs to oxidize fatty acids, we grew **ACADM**-silenced GSCs in medium supplemented with acetate, which is a source of carbon molecules that can bypass MCAD activity by directly fueling the TCA cycle (acetyl-CoA), or with long-chain fatty acids (LCFAs; e.g. linoleic acid) that are not metabolized by MCAD. Neither acetate nor LCFA supplementation rescued proliferation in MCAD-depleted GSCs (Figure S6D, E), which strongly suggests that the depletion of energy substrates following MCAD ablation, may be the consequence of a compromised mitochondrial function. In support of this hypothesis, we observed significant impairment of mitochondrial Complex III and Complex V (ATP synthase) activity in MCAD-depleted versus -proficient cells (Figure 3G, H).

The physiological role of MCAD is to degrade MCFAs that freely diffuse into cells (27). Thus, we hypothesized that decreased MCAD function may result in a toxic accumulation of lipids that are normally diverted to energy production, triggering mitochondrial failure. Consistent with this hypothesis, quantification of lipids in GSCs using Oil Red O lipid staining revealed massive accumulation of lipid droplets *in vitro* as early as 48 hours after puromycin selection in **ACADM**-silenced cells, as well
as in GSCs pharmacologically treated with SPA (Figure 4A; Figure S7A). By contrast, we did not observe lipid accumulation in NHAs (Figure S7A), likely because NHAs divert fatty acids to biosynthetic rather than oxidative pathways. Similarly, MCAD deficiency increased overall free fatty acid content, as assessed by a colorimetric assay (Figure 4B). Lipid accumulation was also confirmed in vivo in tumor remnants after 30 days of doxycycline-induced ACADM silencing (Figure 4C; as in Figure S5D). To test whether the accumulation of fatty acids is causally correlated with the MCAD-deficient phenotype, we ablated MCAD in GSC 8.11 cells grown in medium supplemented with normal or fatty acid-free (FAs-free). In FAs-free conditions, MCAD ablation did not result in accumulation of lipid droplets. Moreover, cell proliferation, as well as viability, albeit reduced, did not appear significantly impacted (Figure 4D, E; Figure S7B).

**MCAD depletion results in acutely toxic MCFA accumulation**

Our data strongly suggest that lipid accumulation contributes directly to the proliferation defect observed in MCAD-deficient GSCs. To understand mechanistically how lipid accumulation may exert toxicity, we investigated the effects of MCAD depletion on coenzyme A (CoA) pools in GSCs, as conjugation of fatty acids to CoA as acyl-CoA is required for fatty acids to migrate into mitochondria and undergo oxidation. Compared with wild type cells, MCAD-depleted cells had increased acyl-CoA and decreased free-CoA pools (Figure 4F). Further, peroxidation of accumulated lipids was increased in the absence of MCAD (Figure 4G), which would also be expected to impact cellular structures and functions. This important finding suggests that activated fatty acids may enter and accumulate in the mitochondria.

To characterize the type and size of lipids accumulated in MCAD-depleted cells, we compared free fatty acid profiles of ACADM-silenced versus control GSCs by gas chromatography mass spectrometry (GC-MS). Here, we observed an accumulation of MCFAs and a decrease in LCFAs (Figure 4H), consistent with a block in the metabolic pathway at the level of MCFA degradation. Next, we profiled acyl-carnitines by liquid chromatography mass spectrometry (LC-MS) in GSCs grown in medium supplemented with unmodified or uniformly carbon-13 (U\textsuperscript{13}C\textsubscript{18})-labeled oleate. This experiment revealed an increase in medium-chain acyl carnitine species that would be expected to accumulate in the mitochondria (Figure S7C). Thus, similar to observations in patients affected by MCAD deficiency (28), the accumulation of MCFAs is observed both as free fatty acid and acyl-carnitine species, suggesting that GSCs, to some extent, continue to engage in FAO (e.g., oleate), even upon attenuated MCAD activity.
MCFAs have been shown to induce apoptosis in some cancer models (29,30) and, based on the dramatic accumulation of MCFAs in GSCs upon MCAD depletion, we speculated that MCFAs may be directly causing toxicity. Indeed, acute treatment of GSCs with varied-length fatty acid species (between 4 and 16 carbons) revealed that C10 and C12 MCFAs negatively impacted cell viability, whereas treatment with the shorter or longer fatty acids tested did not change viability (Figure S7D). Interestingly, acute MCFA exposure induced an increase in ROS levels that was partially rescued when cells were concomitantly grown in the presence of antioxidants, such as of a cell-permeable form of glutathione (GSH-ethyl ester, GSH-EE) (Figure S7E, F). Because lipid peroxidation products have been associated with ferroptosis (31), an iron-dependent form of programmed cell death, we silenced ACADM in GSCs in the presence of ferrostatin-1, an iron chelating agent. Propidium iodide staining did not reveal any improvement in viability in ACADM-silenced GSCs upon ferrostatin-1 treatment (Figure S7G), which suggests that the toxicity exerted by fatty acid accumulation is iron independent. Taken together, our data support a model wherein MCAD inhibition negatively affects cell viability through the accumulation of toxic species as a consequence of impaired fatty acid degradation.

**MCFA accumulation leads to lipid peroxidation and structural damage to the mitochondria in MCAD-depleted GSCs**

Next, to investigate the effects of MCAD depletion on cellular lipids more broadly, we conducted LC-MS-based analysis of lipid classes on whole-cell extracts and on mitochondria isolated from GSCs. Triacylglycerols (TAG) and some phospholipid (PL) classes, such as lysophosphatidylcholine (LysoPC), were significantly increased in whole-cell extracts from MCAD-depleted cells compared with MCAD-proficient controls (Figure 4I). This indicates that profound perturbations in the cellular content of complex lipids may reflect an adaptation by which MCAD-inhibited GSCs attempt to accommodate the accumulating MCFA species. Indeed, phosphatidylserine and sphingomyelin levels were approximately 20- and 10-fold higher, respectively, in MCAD-depleted versus control GSCs analyzed by LC-MS lipidomics (Figure 4J). The most dramatic change detected in mitochondria was in cardiolipin (CL), which decreased over 50-fold in MCAD-deficient cells compared to controls (Figure 4J). CL is located in the inner mitochondrial membrane, where it plays a crucial role in mitochondrial bioenergetics and regulates the efficiency of the electron transport chain (ETC). Interestingly, it is well established that CL is the phospholipid most susceptible to oxidative stress due to its composition, which is enriched in unsaturated fatty acids (32).
To evaluate whether these changes in mitochondrial lipids may relate to mitochondrial dysfunction, we measured ROS levels in MCAD-deficient GSCs. Similarly to MCFA treatment (Figure S7D-F), MCAD depletion using ACADM-targeting shRNA or SPA treatment altered the redox state in GSCs, as evidenced by increased ROS levels as well as decreased ROS scavengers and NADPH levels in all GSCs tested, but did not induce a similar increase in ROS in NHAs. (Figure 5A-E; Figure S8A-D). This correlates with our previous finding of high levels of lipid peroxidation in this context (Figure 4G). Notably, as evidenced by staining with Mitosox, the ROS identified upon MCAD ablation were specifically generated in mitochondria (Figure 5B). We also found high levels of 8-oxoguanine and 4-HNE-lysine, two indicators of oxidative damage and lipid peroxidation, which supports that MCAD depletion similarly affects redox balance in tumors in vivo (Figure 5F; Figure S8E). To investigate the impact of redox state on viability, we grew ACADM-silenced GSCs in suspension culture in the presence of a cell-permeable form of glutathione (GSH-ethyl ester, GSH-EE). GSH-EE exposure transiently rescued the growth inhibition observed upon MCAD depletion; however, it did not enable MCAD-depleted spheres to expand to the same extent as control spheres at later time points (Figure 5G, H; Figure S8F, G). Thus, despite the central role of ROS in driving the observed phenotype, our data convincingly demonstrate that oxidative damage is only one aspect of how deficient MCAD function affects GSCs. In strong support of this, culturing MCAD-depleted GSCs in LPDS medium preserved the proliferative phenotype (Figure 4E) and eliminated the increase in ROS induced by ACADM silencing (Figure S8H). To determine whether the oxidative stress induced by fatty acids overload in the mitochondria might be directly damaging the organelle structure, we first looked for oxidative damage of CL, as this phospholipid was determined to be significantly depleted upon MCAD inhibition (Figure 4J). As mentioned, the localization and the particular composition of CL make it a prime target for oxidative damage (33-35). We focused our attention on the most abundant (C18:2)-containing CL species and searched for products of linoleic acid with molecular weight increased by 48 Da, i.e. + 48 amu (atomic mass unit) adducts that would result from the addition of three oxygen atoms during the mitochondria-driven peroxidation of linoleic acid. Indeed, the formation of + 48 amu compounds has been largely reported as the result of the mitochondria-mediated peroxidation of linoleic acid (36,37). Interestingly, the proportion of oxidized CL markedly increased in GSCs following MCAD knockdown compared to control cells (Figure 6A). We identified 6 abundant CL species that underwent oxidation, resulting in signals with m/z of 723.54 (m/z 699.6 + 8 +16 = m/z 723.6, CL 68:4), 722.60 (m/z 698.6, CL 68:5), 750.60 (m/z 726.6, CL 72:5), 749.58 (m/z 725.6, CL 72:6), 748.60 (m/z 724.6, CL 72:7), 761.59 (m/z 737.59, CL 74:8). These data confirm previous findings (38) and further support the hypothesis that peroxidation of fatty acids byproducts
trapped in mitochondria following MCAD ablation leads to severe damage of important structures, such as CL, which ultimately results in mitochondrial functional impairment.

To investigate whether CL oxidation and its consequent decreased representation in mitochondria may be responsible for the observed mitochondrial failure and cell death, we grew GSCs in medium supplemented with 20 mM elamipretide, a small tetrapeptide designed to specifically target mitochondria by binding CL, thus protecting its structure from oxidative stress (39). Strikingly, sparing CL from peroxidation restored the overall CL content (Figure 6B) and significantly decreased the amount of mitochondrial ROS (Figure 6C), likely due both to the preservation of mitochondrial structures and to the antioxidant properties of elamipretide. As expected, the effect of MCAD inhibition on GSC growth in the presence of elamipretide was significantly attenuated (Figure 6D-F), which is likely driven by the protective effect of elamipretide on CL, although some contribution of the drug’s ROS-quenching activity cannot be excluded. Together with our demonstration that growing MCAD-depleted GSCs in medium deprived of lipids preserves cell growth and restores ROS to control levels (Figure 4D, E, Figure S7B, 8H), this observation further supports that the critical physiological consequence of targeting MCAD is the overload of MCFAs trapped in mitochondria as partially metabolized substrates, where they generate oxidative stress and trigger cell death.

Protecting mitochondria from fatty acids accumulation prevents oxidative damage and restores viability in MCAD-depleted GSCs

To delineate the role of the accumulation of mitochondrial FAs byproducts in the cytotoxic effects triggered by MCAD ablation, we grew GSCs 8.11 and 6.27 in medium containing 2.5 μM etomoxir, which is known to block at low concentration the transport of LCFAs into mitochondria by inhibiting CPT1. Cells were exposed to etomoxir beginning 48 hours before ACADM silencing through the end of the experiment. Etomoxir robustly rescued GSCs from the effects of MCAD ablation, as evidenced by no increase in mitochondrial ROS and restored cell viability (Figure 6G-K). Conversely, targeting diacylglycerol O-acyltransferase 1 (DGAT1), a key enzyme in lipid droplet formation that catalyzes the terminal and only committed step in triacylglycerol synthesis by using diacylglycerol and fatty acyl CoA as substrates, with a commercially available inhibitor (A-922500) decreased GSC 8.11 growth by an additional 20% upon ACADM silencing (Figure 6L, Figure S8I), thus accelerating the detrimental effects described above. It is well known that one of the main roles of lipid droplets is to buffer cellular levels of potentially toxic lipids to prevent lipotoxicity and oxidative stress (40), and the importance of DGAT1 in the context of fatty acid metabolism in GBM has been previously reported (41). As expected, A-922500
(DGAT1i) treatment effectively inhibited lipid droplet formation in MCAD-depleted GSCs (Figure S8J), suggesting that inhibition of the machinery that enables GSCs to counteract lipotoxicity induced by MCAD ablation further worsens the observed phenotype.

Taken together, our functional characterization of MCAD in glioblastoma models uncovers a novel, protective role wherein MCAD function is required for continuous degradation of molecular species that would otherwise accumulate and cause mitochondrial dysfunction, oxidative stress and, eventually, cell death.

**DISCUSSION**

In the present study, we interrogated metabolic dependencies in *in vivo* GBM tumors using our previously described PILOT platform (14). This screen and our subsequent studies confirmed MCAD, a mitochondrial enzyme that catalyzes the first step of medium-chain fatty acid (MCFA) β-oxidation (FAO), as a node of metabolic vulnerability for GBM tumor growth and maintenance. Specifically, MCAD depletion in primary GBM models triggered massive accumulation of unmetabolized MCFAs, lipid peroxidation, and oxidative stress, which resulted in irreversible mitochondrial damage and apoptosis. Altogether, this work uncovered an irreversible cascade of detrimental metabolic effects induced by MCAD depletion, and it represents a first demonstration of a non-energetic role for dependence on fatty acid metabolism in cancer.

MCAD deficiency is a human inherited autosomal recessive disorder caused by inactivation of the *ACADM* gene, and the presence of *ACADM* mutations is screened perinatally. Because the uptake of MCFAs lack any negative feedback regulation, affected individuals must follow appropriate dietary recommendations, and in doing so, can lead normal lives (15,16). The role of *ACADM* in the pathogenesis of MCAD deficiency has been confirmed by the generation of an *Acadm*-deficient mouse model that shows similar clinical manifestations and histopathological characteristics (42). Given these facts and our demonstration that depleting MCAD leads to toxic accumulation of MCFAs and cell death specifically in tumor cells, we expect that an actionable therapeutic window may exist to target MCAD in GBM patients.

Previous reports have indicated that the upregulation of FAO confers a survival advantage to GBM cells, at least in part by fueling oxidative metabolism to sustain cellular energetic requirements (43). The significance of FAO to fuel cancer cell survival beyond brain cancer is rapidly gaining recognition (44). Several studies have reported that blocking LCFA oxidation, primarily through CPT1 inhibition, exerts...
robust anti-tumor effects in pre-clinical models, but CPT1-targeted therapies have not yet proven to be clinically viable due to significant toxicity (45-49).

This work demonstrates that the sustained influx of fatty acids in GBM cells can become toxic when lipids are not properly metabolized. We show that accumulated MCFAs are particularly damaging to GSCs compared to fatty acids of different chain lengths. Thus, our data support that targeting MCAD may have advantages over inhibitors of CPT1, because partial oxidation of LCFA to MCFA may contribute to the overall accumulation of toxic lipid species in contexts where MCFA metabolism is inefficient. Owing to the toxicity associated with C10- and C12-lipids accumulation and the lack of a feedback mechanism to prevent continued MCFA accumulation, we posit that, in a lipid-rich environment such as the brain, overexpression of MCAD or enzymes capable of diverting potentially harmful lipid pools to energy production may confer a significant proliferation advantage to cancer cells. This may explain the high reliance of GSCs on sustained fatty acid metabolism, and it suggests that targeting MCAD in fatty acid-embedded tumor types may be especially effective.

We describe a novel role for MCAD to protect GSCs from accumulating toxic levels of lipids in the mitochondria, which can induce apoptosis. These data offer a new therapeutic concept for cancer cell metabolism, which has proven difficult to exploit with targeted drugs. While tumor cells have a documented, remarkable adaptability to shunt substrates to different metabolism pathways to survive metabolic perturbations, impairing MCFA catabolism in GSCs irreparably damages mitochondria and is acutely toxic. These findings, in combination with our data in normal human brain models and the known physiology of congenital MCAD deficiency, prompt further studies to translate this cancer cell-specific dependency into a clinical opportunity for patients with GBM.
METHODS

Human samples, primary cells and cell lines.

Glioblastoma sphere-forming cells (GSCs) were isolated from specimens from patients with glioblastoma, who had undergone surgery at the University of Texas MD Anderson Cancer Center (UTMDACC). The models used in this research are abbreviated GSC.811, GSC 6.27, GSC 7.11, and GSC 272, but elsewhere are published using the nomenclature MDA-GSC 8-11, MDA-GSC 6-27, MDA-GSC 7-11, and MDA-GSC 272, respectively. The diagnosis of glioblastoma was established by histological examination by the WHO (World Health Organization) classification. Samples derived from patients were obtained with the written informed consent of patients to an ethically approved Institutional Review Board protocol that is compliant with the principles of the Belmont Report (LAB04-0001, chaired by F. F. Lang (MDACC)), and are registered in the MDACC Cell Line Registry. Tumor specimens were dissociated and resuspended in Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12, Gibco) supplemented with B27 (×1, Invitrogen), bFGF (20 ng ml−1 Peprotech), and EGF (20 ng ml−1, Peprotech). Cells were cultured as neurospheres and passaged every 5–7 days on the basis of sphere size.

GSCs authentication and mycoplasma testing was performed by the MDACC Cell Line Authentication Core every 2 to 3 months. Authentication was performed by Short Tandem Repeat (STR) DNA profiling, also known as DNA fingerprinting, which is based on screening regions of microsatellite instability with defined tri- or tetrad-nucleotide repeats located throughout the chromosomes. PCR reactions using primers on non-repetitive sequences flanking those regions will generate PCR products of different sizes based on the number of repeats in the region; the size of these PCR products is determined by capillary electrophoresis. Between 8 and 16 STR loci, such as D5S818, D13S317, D7S820, D16S539, vWA, TH01, TPOX, and CSF1PO, are combined to uniquely identify a sample. The Core assay screens 16 loci using the Promega Powerplex 16 HS kit. The test also includes matching the STR profiles against an internal database comprised of public profiles and profiles that are unique to cell lines developed by MDACC investigators. MDACC current database has over 4000 profiles. The Lonza Mycoalert Mycoplasma Detection kit was used for mycoplasma testing. The length of time or number of passages between collection or thawing and use is described in the described experiments. All GSCs were used within 20 to 30 passages.

Normal Human Astrocytes (NHAs) were obtained from Sciencell Research Laboratories (NC0273812)and grown in Astrocyte Medium (#1801) according to instructions. Neural stem cells 1 (NSC-1, also referred as neural progenitor cells, NHNP) were purchased from Lonza (PT-2599). Neural stem cells 2 (NSC-2 also
referred as iPSC-derived NSCs) were obtained from iXCells Biotechnologies (NC1831429). T98G cells were obtained through ATCC and grown under standard tissue-culture conditions. NHAs, NSC-1, NSC-2, and T98G were authenticated by the manufacturer by using specific markers. NHAs, NSC-1, NSC-2, and T98G cells tested negative for mycoplasma per manufacturer’s certificate of analysis provided for every cell lot, and these cells were used within 8 passages from thawing.

**GSCs treatments, proliferation and sphere formation assays.**

Sodium butyrate (156-54-7), sodium hexanoate (C4026), sodium octanoate (C5038), sodium decanoate (C4151), and sodium dodecanoate (L9755) were obtained from Sigma-Aldrich. BSA-Palmitate (102720100) was purchased from Agilent Technologies. Spiropentaneacetic acid (SPA) was synthesized by the Institute of Applied Cancer Science Chemistry Core Facility at MD Anderson. For each treatment, GSCs were disaggregated and 50000 cells were plated in 6-well plates. Cells were disaggregated at different time points (as indicated in Figures); cell number and viability were determined by Trypan Blue exclusion assay. For sphere formation assays, approximately \( 2 \times 10^4 \) cells from disaggregated GSC spheres were resuspended in a 0.8% methylcellulose semisolid DMEM/F12 and seeded in 6-well plates. At different time points, the spheres were counted in at least 4 fields per well.

**Library Design and Construction**

A custom library was composed by 338 genes specifically belonging to key metabolic pathways (KEGG, see Figure S2A). This library was constructed by using chip-based oligonucleotide synthesis and cloned into the pRSI16 lentiviral vector (Cellecta) as a pool. The shRNA library is constituted by 338 genes with a coverage of 10 shRNAs/gene. The shRNA includes two G/U mismatches in the passenger strand, a 7-nt loop and a 21-nt targeting sequence. Targeting sequences were designed using a proprietary algorithm (Cellecta). The oligo corresponding to each shRNA was synthesized with a unique molecular barcode (18 nucleotides) for measuring representation by next generation sequencing.

**In Vivo shRNA Screens**

In vivo shRNA screens were performed using adapted procedures previously described in (14). In brief, concentrated lentiviral particles (TU, transducing units) from libraries or single plasmids were either purchased by Cellecta or produced by transfecting 293T cells according to the protocol in the Cellecta User Manual. Precisely calculated lentiviral particles, together with 2 μg/mL polybrene (Millipore), were added to single-cell dissociated GSCs to achieve a multiplicity of infection (MOI) = 0.3. Forty-eight hours after infection, the medium was replaced and puromycin (2 μg/mL) was added for 96 hours. For in

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*Authors have been peer reviewed and accepted for publication but have not yet been edited.*
vitro/in vivo validation studies, GSCs were infected at MOI = 3, with single shRNA knocking down specific target genes. Transduction efficiency was determined sample by sample as the percentage of GFP positive cells 2 days after infection as measured by FACS analysis. For the in vivo experiments, each intracranial injection was performed with $1 \times 10^6$ cells to ensure a coverage of ~300 cells/barcode. Upon brain collection, tumors displaying green fluorescence signal were precisely dissected, weighted and quickly snap frozen. Genomic DNA extraction, barcode amplification and next-generation sequencing were performed according to Cellecta User Manual for shRNA libraries processing (details about PCR primers, PCR conditions and reads counting in (14).

Bioinformatics analysis - Hit identification

Multiple replicates (N=3) were sequenced for in vitro samples for GSC 627 (N Ref = 2) and GSC811 (N Ref = 1) using Illumina HiSeq2500 and processed using CASAVA (v.1.8.2) to derive FASTQ files. The sequencing read consists of two 18-bp barcodes separated by a 4-bp spacer (CGAA) between the two barcodes. Reads were filtered for the 4-bp spacer and aligned with the 23- to 40-bp barcode using Bowtie (2.0.2) and counted using SAMtools.

Read counts were normalized for the amount of sequencing reads retrieved for each sample by using library size normalization (to 1 million reads). For each sample, (log2) fold-change was calculated compared to the reference pellet before injection. A summary measure per condition was derived using the median of quantile-transformed log2 FC across replicates. Thereafter, a modified version of the RSA algorithm was used to derive the gene-level summary measure per condition. Specifically, we controlled for the factor that at least 2 hairpins were used when calculating the minimum p-value (in RSA). In addition, hairpins that ranked above luciferase controls were not used in choosing the minimum p-value. Quantile rank of luciferase control barcodes was evaluated across all experiments. On average, luciferase barcodes ranked >0.6 (on the quantile transformed log2fc scale). Therefore, hairpins with quantile transformed log2fc > 0.6 were not used for gene-level RSA scores (log value) (50).

Bioinformatics analysis – TCGA data

Level 3 TCGA glioblastoma (GBM) and lower grade glioma (LGG) data for gene expression and mutations were downloaded from GDAC Firehose (https://gdac.broadinstitute.org). Data analysis using the TCGA cohort was performed using R.

Log2 RSEM values were calculated to compare expression levels of ACADM and other genes involved in the fatty acid synthesis pathway. Using the GBM dataset (n=167), analysis was performed comparing
expression between normal tissue (n=5), classical (n=68), proneural (n=46) and mesenchymal (n=48) subtypes (The Cancer Genome Atlas Network, 2015). ANOVA was performed across all groups to determine statistical significance, with pairwise Wilcoxon tests performed to identify which subtypes showed a difference. This was conducted using the “ggpubr” R package (R Core Team, 2017; Kassambara A, 2020). TukeyHSD was also calculated in R to interrogate pairwise differences driving significance with ANOVA.

**Animal Studies**

Male athymic nude mice (nu/nu) were purchased from The Jackson Laboratories. All procedures performed in this study were approved by the University of Texas MD Anderson Cancer Center. All animal manipulations were performed in the veterinary facilities in accordance with institutional, state, and federal laws and ethics guidelines under an approved protocol. Intraperitoneal (IP) injections of ketamine (100mg/kg)/xylazine (10mg/kg) were used to anesthetize animals in all experiments. For intracranial xenografts of GSC 8.11 and 6.27, 10^6 cells expressing a PLX304-mCherry-LUC vector (5 µl cell suspension) were implanted using a guide screw and a multiport microinfusion syringe pump (Harvard Apparatus, Holliston, MA) (51,52). For subcutaneous experiments, GSCs were injected subcutaneously (100 µl cell suspension) into the left flank. For all bioluminescence imaging, d-luciferin (150 mg kg^-1) was administered by subcutaneous injection to mice 10 min before imaging. In all experiments, mice were monitored daily for signs of illness and were euthanized when they reached endpoints. For in vivo studies of tumor maintenance, inducible shRNAs TRCN0000028530 and TRCN0000028509 cloned in a Tet-pLKO-puro vector were obtained from Sigma Aldrich. Doxycycline (2 mg/mL) was administered in drinking water to mice. The starting point for doxycycline administration correspond to the first day tumors were detected (approximately 20 days post injection) and was maintained until study completion.

For Magnetic Resonance Imaging (MRI), a 7T Bruker Biospec (BrukerBioSpin), equipped with 35mm inner diameter volume coil and 12 cm inner-diameter gradients, was used. A fast acquisition with relaxation enhancement (RARE) sequence with TR/TE of 2,000/39 ms, matrix size 256x192, Resolution was 156µM, 0.75 mm slice thickness, 0.25 mm slice gap, 40 x 30 cm FOV, 101 kHz bandwidth, 4 NEX was used for acquired in coronal and axial geometries a multi-slice T2-weighted images. To reduce the respiratory motion the axial scan sequences were respiratory gated. The brain lesions’ volumes were quantified manually using Image J software. All animal imaging, preparation and maintenance was carried out in accordance with MD Anderson’s IACUC policies and procedures.
ETC activity

The Mitochondria Isolation Kit (Abcam ab110170) was used to isolate mitochondria from MCAD silenced and control GSCs, which were then used to test the activity of Complex III and V. Activity of mitochondrial complex III was analyzed using the Mitochondrial Complex III Activity Assay Kit (Biovision K520-100). Cytochrome C was added to the reaction and its reduction through the activity of Complex III was measured at 550 nm. ATP synthase activity was measured by using the ATP Synthase Specific Activity Microplate Assay Kit (Abcam ab109716). The antibody for ATP Synthase was precoated in the wells of the microplate, and samples containing 50 µg of mitochondrial extracts were added to wells. In this assay, the conversion of ATP to ADP by ATP synthase was coupled to the oxidative reaction of NADH to NAD+. The formation of NAD+ resulted in a decrease in absorbance at 340 nm. Subsequently, in these same wells, the quantity of ATP synthase was determined by adding an ATP synthase specific antibody conjugated with alkaline phosphatase. This phosphatase changed the substrate pNPP from colorless to yellow (OD 405 nm) in a time dependent manner proportional to the amount of protein captured in the wells. Absorbance was read by a Benchmark microplate reader (Bio-Rad). All tests were done in triplicate.

ATP quantification

ATP was quantified by using the luminescent ATP detection assay from Abcam (ab113849). The assay quantifies the amount of light emitted by luciferin when oxidized by luciferase in the presence of ATP and oxygen. Cells were plated on the same day into a 96-well plate in DMEM/F12 (100 µl) without glucose and supplemented with B27, EGF and bFGF (20 ng/ml), as well as galactose (10 mM) to obtain the ATP amount generated by mitochondrial activity. ATP standards were loaded on the same plate as references. The assay was performed according to the manufacturer’s instructions.

Oxygen consumption rate and extracellular acidification rate measurements

The functional status of mitochondria in MCAD deficient GSCs 8.11 and NHA was determined by analyzing multiple parameters of oxidative metabolism using the XF96 Extracellular Flux Analyzer (Agilent), which measures extracellular flux changes of oxygen and protons. Cells were plated in XF96-well microplates (30,000 cells per well) in a final volume of 80 µl of DMEM/F12 medium (17.5 mM glucose, 2.5 mM glutamine) supplemented with B27, EGF (20 ng/ml), bFGF (20 ng/ml). For the mitochondrial stress test, cells were incubated at 37 °C in the absence of CO₂ and in 180 µl DMEM-F12.
(17.5 mM glucose, 2.5 mM glutamine) supplemented with B27, EGF (20ng/ml), bFGF (20ng/ml of XF-Mito-MEM) per well for 1 hour before the assay. The ports of the sensor cartridge were sequentially loaded with 20 μl per well of the appropriate compound: the ATP coupler oligomycin (Sigma, O4876), the uncoupling agent carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, Sigma C2920) and the complex I inhibitor rotenone (Sigma, R8875).

Immunoblotting

Protein lysates were resolved on 4–15% gradient polyacrylamide SDS gels and transferred onto nitrocellulose membranes according to standard procedures. Membranes were incubated with indicated primary antibodies, washed, and probed with HRP-conjugated secondary antibodies. The detection of bands was carried out upon chemiluminescence reaction followed by film exposure. The following primary antibodies were used: Anti-ACADM antibody (ab92461), Anti-Oxoguanine 8 antibody (ab206461), Anti-4 Hydroxynonenal antibody [HNEJ-2] (ab48506), Anti-Vinculin antibody [VIN-54] (ab130007), Anti-GAPDH antibody [EPR6256] (ab128915), Anti-beta Actin antibody (ab8224)

Transmission electron microscopy (TEM)

TEM was performed at the MDACC High Resolution Electron Microscopy Facility. Samples were fixed with a solution containing 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.3, for 1 hour. After fixation, the samples were washed and treated with 0.1% millipore-filtered cacodylate buffered tannic acid, post-fixed with 1% buffered osmium tetroxide for 30 min, and stained in bloc with 1% millipore-filtered uranyl acetate. The samples were dehydrated in increasing concentrations of ethanol, then infiltrated and embedded in LX-112 medium. The samples were polymerized in a 60 °C oven for 2 days. Ultrathin sections were cut using a Leica Ultracut microtome, stained with uranyl acetate and lead citrate in a Leica EM Stainer, and examined in a JEM 1010 transmission electron microscope (JEOL) at an accelerating voltage of 80 kV. Digital images were obtained using an AMT Imaging System (Advanced Microscopy Techniques Corp).

Oxidative stress detection

Reduced and oxidized forms of glutathione (GSH and GSSG, respectively) were measured in GSC 8.11 and GSC 6.27 using a GSH/GSSG Ratio Detection Assay Kit (Abcam, ab138881) according to the manufacturer’s protocol. Data from 3 separate experiments were averaged for the results. NADP+ and NADPH levels were measured from GSC 8.11 and 6.27 using a NADP/NADPH Assay Kit (Abcam, ab65349)
according to the manufacturer’s instructions. NADP and NADPH levels in total lysate were calculated by comparison with the standard curve.

Flow Cytometry

Cellular apoptosis was detected using a FITC Annexin V Apoptosis Detection Kit I (BD Biosciences), according to the manufacturer's instructions. Following virus infection with sh-ACADM or sh-scr and 48 hours of puromycin selection, cells were harvested and re-suspended in cold PBS. Subsequent to centrifugation at 1000 rpm for 5 min at 4°C, the cells were resuspended with 500 µl binding buffer and mixed with 5 µl annexin V-FITC. The cells were subsequently incubated with 5 µl propidium iodide (PI) in the dark at room temperature for 5-15 min. Excitation was at 488 nm and the emission filters used were 515–545 BP (green, FITC) and 620 LP (red, PI). All assays were performed in triplicate.

To detect reactive oxygen species (ROS), 2x10^5 cells were stained with CellRox Green (100 nM) (Molecular Probes) for 20 min, washed twice and resuspended in PBS. Excitation was at 488 nm and the emission filters used were 515–545 BP (green, FITC). ROS were induced by 4-hydroxynonenal treatment (10 µM) for positive controls. For mitochondrial ROS, cells were stained with MitoSOX™ Red (2.5 µM) (Molecular Probes) for 10 min at 37 degrees, washed twice and resuspended in HBSS/Ca/Mg. Excitation was at 510 nm and the emission filters used were 580 BP (red). Gating strategies to exclude doublets and dead cells (DAPI) were always employed. After staining, samples were acquired using a BD FACSCantoll flow cytometer. Data were analyzed by BD FACSDiva or FlowJo (Tree Star).

Immunohistochemistry and immunocytochemistry

For immunohistochemistry (IHC) staining, tumor samples were fixed in 4% formaldehyde for 2 to 4 hours on ice, moved in 70% ethanol for 12 hours, and then embedded in paraffin (Leica ASP300S). After cutting (Leica RM2235), baking and deparaffinization, slides were treated with Citra-Plus Solution (BioGenex) according to specifications. Endogenous peroxidases were inactivated by 3% hydrogen peroxide. Non-specific signals were blocked using 3% BSA, 10% goat serum and 0.1% Triton. Tumor samples were stained with primary antibodies. ImmPress and ImmPress-AP (Vector Lab) were used as secondary antibodies; Nova RED, Vector BLUE and DAB were used for detection (Vector Lab). Images were captured with a Nikon DS-Fi1 digital camera using a wide-field Nikon EclipseCi microscope. For Oil Red O lipid staining of cells, GSCs were grown on coverslips and GSCs were attached to tissue slides by cytopsin. For Oil Red O lipid staining of tumors, tumor tissue was 4% PFA fixed, cryoprotected with 30%
sucrose, OCT embedded and sectioned (5 μm thick). Oil Red O Lipid staining was performed according to the manufacturer instructions (Abcam, ab150678).

**ACADM shRNAs and sgRNAs**

The hairpin RNA interference plasmid for human ACADM, pLKO.1 ACADM TRCN0000028530 (sh-AC1), TRCN0000028509 (sh-AC2) and the scramble control pLKO.1-Puro plasmid (sh-scr) were obtained from Sigma-Aldrich. The sequence of shRNAs are:

- **sh-AC1**, CCGGGCTGGCTGAAATGGCAATGAACTCGAGTTCATTGCCATTTCAGCCAGCTTTTTTTT
- **sh-AC2**, CCGGGTGCAGATACTTGGAGGCAATCTCGAGATTGCCTCCAAGTATCTGCACTTTTTTT

The inducible shRNA was obtained from Cellecta by cloning into a pRSIT16-U6Tet-sh-CMV-TetRep-2A-TagRFP-2A-Puro vector the following hairpin sequence:

ACCGGGAGTTCACTGAACAGTAGAAAGTTAATATTCATAGCTTTCTGCTGTTCGGTGAACTCTTTT

To generate ACADM sgRNAs three pairs of oligonucleotides were designed and used as follows:

- **Sg1**: SgACADM1F CACCGAAGATGTGGATAACCAACGG
  SgACADM1R AAACCCGTTGGTTATCCACATCTTC
- **Sg2**: SgACADM2F CACCGACACACATTCCAGAGAACTG
  SgACADM2R AAACCAGTTCTCTGGAATGTGTGTC
- **Sg3**: SgACADM3F CACCGATTGGCTTATGGATGTACAG
  SgACADM3R AAACCTGTACATCCATAAGCCAATC

All sgRNAs were validated for their KO efficiency. sgACADM1 was selected for further studies to knockout ACADM. To specifically amplify ACADM in genomic DNA, the following oligonucleotides were used:

- **ACADM1F** TATTCAAGGCTTATTGTGTAACAGAACC
- **ACADM1R** CTGAACTGTTTATAATCTCCTTTAGGCC
- **ACADM2F** AATAATTTTCCCTAGAGTTCACCGAAC
- **ACADM2R** GACTGAGTAGGCTTGCTCAGCGTAC
- **ACADM3F** AAACCCGTTGGTTATCCACATCTTC
- **ACADM3R** AAACCTGTACATCCATAAGCCAATC

ACCGGGAGTTCACTGAACAGTAGAAAGTTAATATTCATAGCTTTCTGCTGTTCGGTGAACTCTTTT
Lipidomics of total cell extracts and mitochondria

After adding equal volumes of dichloromethane/methanol/PBS (1:1:1), samples were centrifuged (2000 rpm; 5 min) to collect the organic phase. The extraction was repeated twice. After drying using a gentle stream of N\textsubscript{2} gas, samples were dissolved in 8 mM ammonium fluoride dichloromethane/isopropyl alcohol/methanol (2:1:1) and sonicated (5 min) before an internal standard was added. Lipids were measured using the shotgun lipidomics by directly infusing a modified Blight-Dyer extract into a SCIEX Triple TOF 5600+ mass spectrometer for MS/MS\textsuperscript{ALL} analysis as previously described (53). Acquisition was performed once in positive and once in negative ion mode. The phospholipid species were identified based on their characteristic \(m/z\) value, fragmentation analysis, and precursor ion or neutral loss scans. Using precursor ion scanning techniques, negative precursors of \(m/z\) 241 and 196 identified parents of phosphatidylinositol, phosphatidylethanolamine, and lysophosphatidylethanolamine. Positive precursors of \(m/z\) 184 yielded parents of sphingomyelin, phosphatidylcholine, and lysophosphatidylcholine. Neutral loss scans of 141 and 185 yielded parents of phosphatidylethanolamine and phosphatidylerine, respectively. Neutral lipids, including triacylglycerides, diacylglycerides, and cholesterylesters, were identified based on their fatty acid neutral loss in positive ion mode using the Lipid Maps database (http://www.lipidmaps.org/). The same extraction method was used for lipidomics on purified mitochondria. Cardiolipin peaks were identified as their [M-2H]\textsuperscript{2−} ions in the negative-ionization mode by PI scanning of \(m/z\) 153. The intensity of each peak was normalized to the total lipid signal and to the internal standard. The normalized data relative to each lipid species were summed to give the intensity of each class, which was reported as percentage of all lipids.

Lentivirus production

Lentivirus was produced by polyethyleneimine (PEI) transfection of 293T cells. Four million cells were transfected with 30 μg of transfer vector, 19 μg of packaging plasmid pCMV-Δ8.74 and 9.5 μg of envelope plasmid pMD2G-VSVG. After 16 hours medium was replaced and viral particles in the medium were collected 72 hours later by ultracentrifugation at 23,000 \(\times\) g for 3 hours.

Cell infection
Cells were infected with lentiviral particles expressing shRNAs, inducible shRNAs or sgRNAs as indicated for each experiment. 4 x 10^6 GSCs were infected with 4 x 10^6 lentiviral particles and selected for 48 hours with Puromycin (3 μg/ml). In the experiments with NHAs and T98G 1 x 10^6 cells were infected with 1 x 10^6 lentiviral particles.

**T7E1 assay**

72 hours post transfection or 1 week post lentiviral infection of guide and nuclease, cell genomic DNA was extracted with Qiagen DNeasy blood and tissue kit per manufacture’s protocol. Genomic DNA was used for the template of PCR with NEB Onetaq mastermix per manufacture’s protocol. The product was first denatured at 98°C for 5 minutes, then slowly annealed to 75°C at 1°C/s and eventually to 25°C at 0.1°C/s. 5U T7 Endonuclease I was used for digestion of less than 300 ng annealed product for 30 minutes. The digested product was subjected to 2% TAE gel electrophoresis.

**Colorimetric/Fluorometric Assays**

Free fatty acids levels (MAK044, Sigma Aldrich), acetyl CoA (ab87546, Abcam), coenzymeA/acyl CoA levels (MAK034, Sigma Aldrich) and MDA concentrations (MAK085, Sigma Aldrich) were determined according to manufacturer’s instructions. A PHERAstar FS (BMG Labtech) microplate reader was used for quantifications.

**Fatty Acid Analysis**

MCAD silenced or control GSCs were grown for 24 hours after Puromycin selection in complete DMEM-F12. After 24 hours cells were harvested and the pellet were placed at -80°C. Microtubes containing cell pellets were removed from -80°C storage and maintained on wet ice throughout the processing steps. 20 µL of 0.75 µg/µl D27 myristic acid was spiked in as internal standard (IS). 1mL of methanol was added to each sample, subject to sonication for 2 min, and centrifuged at 4000 rpm for 10 min at 4 °C. Supernatant was transferred into a new tube and dried under N2. For medium samples, 500 µl of sample was spiked with 20 µl D27 myristic acid and prepared as above. Stock solutions of hexanoic acid, octanoic acid and decanoic acid were prepared at 28 µg/µl, 11 µg/µl, 11 µg/µl, respectively, in methanol. The D27 myristic acid IS was spiked into standards at 0.75 µg/µl. Cell lysates, media samples, and standards were then derivatized, as follows. 100 µl of N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA, Pierce) with 1% trimethylchlorosilane (1% TMCS, Thermo Scientific) was added to react for 30 min in 60°C. 1 µl of sample was injected into GCMS for detection. GC/MS analysis was performed using an Agilent 7890A GC equipped with a 15m DB-5MS+DG capillary column and a Leap CTC PAL ALS as the
sample injector. The GC was connected to an Agilent 5975C quadrupole MS operating under positive electron impact ionization at 70eV. All tunings and data acquisition were done with an HP PC with Win 7 professional OS that included the ChemStation E.02.01, PAL Loader 1.1.1, Agilent Pal Control Software Rev A and Pal Object Manager updated firmware. MS tuning parameters are in default settings. GC injection port was set at 250 °C and GC oven temperature was held at 60 °C for 5 min and increased to 220 °C at 10 °C/min, then held for 10 min under constant flow with initial pressure of 10.91 psi. MS source and quadrupole were held at 230 and 159 °C, respectively, and the detector was run in scanning mode, recording ion abundance in the range of 26-600 m/z with solvent delay time of 2 min. The data set was translated into .D format. The extraction was done with Agilent MassHunter WorkStation Software GCMS Quantitative Analysis Version B.07. Identification was performed by searching NIST2011. A one-point calibration was performed in this study and data was normalized by IS to generate the final report.

$^{13}$C tracer metabolomics

Cells were grown in complete DMEM-F12 supplemented with $^{13}$C-oleate/$^{13}$C-glucose. After 6/12 hours cells were harvested and the pellet Cell pellets were dissolved in 50 μL water/methanol (50:50) and x μL were injected onto a Waters Acquity UPLC BEH TSS C18 column (2.1 x 100mm, 1.7µm) column on an into an Agilent 1260 UHPLC with mobile phase A) consisting of 0.5 mM NH4F and 0.1% formic acid in water; mobile phase (B) consisting of 0.1% formic acid in acetonitrile. Gradient program: mobile phase (B) was held at 1% for 1.5 min, increased to 80% in 15 min, then to 99% in 17 min and held for 2 min before going to initial condition and held for 10 min. The column was at 40 °C and 3 μl of sample was injected into an Agilent 6520 Accurate-Mass Q-TOF LC/MS. The LC-MS flow rate was 0.2 mL/min. Calibration of TOF MS was achieved through Agilent ESI-Low Concentration Tuning Mix. In negative acquisition mode, key parameters were: mass range 100-1200 da, gas temp 350 °C, fragmentor 150 v, skimmer 65 v, drying Gas 10 l/min, nebulizer flow at 20 psi and Vcap 3500 v, reference ions at 119.0363 and 980.01637 da, ref nebulizer at 20 psi. In positive acquisition mode, key parameters were: key parameters were: mass range 100-1200 da, gas temp 350 °C, fragmentor 150 v, skimmer 65 v, drying Gas 10 l/min, nebulizer flow at 20 psi and Vcap 3500 v, reference ions at 121.050873 and 922.009798 da, ref nebulizer at 20 psi. Agilent Mass Hunter Workstation Software LC/MS Data Acquisition for 6200 series TOF/6500 series Q-QTOF Version B.06.01 was used for calibration and data acquisition.

**Acyl-carnitine profiling**
GSC 8.11 were grown for 24 hours in complete DMEM-F12 supplemented with $^{13}$C-oleate. After 24 hours cells were harvested and the pellet were placed at -80°C. Microtubes containing cell pellets were removed from -80°C storage and maintained on wet ice throughout the processing steps. To initiate protein precipitation, 0.3 mL of a chilled mixture of methanol and chloroform (8:2) (EMD, Billerica, MA) was added to each sample, the mixture was vortexed briefly, and allowed to incubate on ice for 10 mins. Post-incubation, the vortex step was repeated, and samples centrifuged at 14,000 RPM, for 10 mins in 4°C. Post-centrifugation, 100 µL of supernatant was transferred to an autosampler vial for LC-MS analysis. From the remaining supernatant from each sample, a small aliquot was transferred to a new microtube to create a pooled sample for quality control purposes.

LC-MS analysis was performed on an Agilent system consisting of a 1290 UPLC module coupled with a 6490 QqQ mass spectrometer (Agilent Technologies, CA, USA.) A 1 µL injection of acyl carnitine metabolites were separated on an Acquity HSS-T3 1.8 µM, 2.1 x 50 mm column (Waters, Milford, MA) maintained at 40°C, using 10 mM ammonium acetate in water, adjusted to pH 9.9 with ammonium hydroxide, as mobile phase A, and acetonitrile as mobile phase B. The flow rate was 0.25 mL/min and the gradient was linear 0% to 80% A over 7 mins, then 80 to 100% over 1.5 mins, followed by isocratic elution at 100% A for 5 minutes. The system was returned to starting conditions for 3 mins to allow for column re-equilibration before injecting another sample. The mass spectrometer was operated in ESI-mode with the following instrument settings: Gas temp: 275°C, flow: 15 l/min, nebulizer: 35 psi, capillary 3500 V, sheath gas 250°C, and sheath gas flow 11 l/min. The ion funnel high/low pressure RF settings were: 150/60 V respectively. Acyl carnitine transitions were monitored for the 85 Da product ion that is common to each carnitine species. Data Analysis: Metabolites were identified by matching the retention time and mass (+/- 10 ppm) to authentic standards. Isotope peak areas were integrated using MassHunter Quantitative Analysis vB.07.00 (Agilent Technologies, Santa Clara, CA.) Peak areas were corrected for natural isotope abundance using an in-house written software package based on the method of (54), and the residual isotope signal was reported. Data were normalized to cell protein content prior to analysis of metabolite fluxes for Central Carbon, Acyl carnitine and Fatty acid metabolites.
ACKNOWLEDGMENTS

We thank Dr. Jason Huse from the MD Anderson department of Anatomical Pathology for advice regarding GBM classification; Jay Dunn at Seahorse Bioscience for support; Kenneth Dunner Jr. and the High Resolution Electron Microscopy Facility at MDACC for TEM (NCI CA16672); the MDACC Flow Cytometry and Cellular Imaging (FCCI) Core Facility (NCI P30CA16672) for flow-cytometers and FACS; Robert Nguyen for lab management; the MD Anderson veterinary medicine staff, Shan Jiang, and Ivonne Flores for support with in vivo experiments.
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10.1056/NEJMoa043330.


FIGURE LEGENDS

Figure 1. **ACADM emerges as a clinically relevant dependency of GBM.** **a.** Schematics of experimental design for intracranial metabolome shRNA screens in patient-derived glioblastoma sphere-forming cells (GSCs). The lentiviral library was transduced at a low multiplicity-of-infection (MOI) (less than one integrant/cell) **b.** Gene-rank analysis highlighting the behavior of genes involved in fatty acid metabolism (**ACADM, ACADL, PRKACB** and **ACSL6**) in in vivo screens executed in two independent GSC models: GSC 8.11 and GSC 6.27 (RSA = redundant shRNA activity, logP). **c.** **ACADM** mRNA levels in glioma subtypes vs normal brain (TCGA dataset). Log2 RSEM values were calculated to compare expression levels of **ACADM** and other genes involved in the fatty acid synthesis pathway. Using the GBM dataset (n=167), analysis was performed comparing expression between normal tissue (n=5), classical (n=68), proneural (n=46) and mesenchymal (n=48) subtypes. ANOVA was performed across all groups to determine statistical significance, with pairwise Wilcoxon tests to identify which subtypes showed a difference. **d.** IHC for MCAD on TMA derived from normal brain and GBM tissue. Scale bar, 100 μm for ×4 and 25 μm for ×20. **e.** GBM percentage distribution based on MCAD expression levels in 3 independent TMAs. The scores 1-3 were independently determined using the following scoring system to approximate the percentage of cells positive for staining with the MCAD antibody: 1=0% to 10%, 2=11% to 50%, 3=51% to 100%. Representative tissue scoring is presented in (d). Data represent the analysis of three independent TMAs. **P values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups.***

**f.** Bioenergetic profiling of NHA, NSC and GSC lines using Seahorse technology. Basal oxygen consumption rate (OCR) (pMoles/min) and extracellular acidification rate (ECAR) (mpH/min) were used for calculations. Values represent the mean ± SD of four independent experiments. **P values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups.***

**g.** Quantification of energy production in the indicated cell lines by Seahorse XF Real-Time ATP Rate Assay. MitoATP Production Rate and glycoATP Production Rate were calculated from OCR and ECAR measurements under basal conditions. Values are expressed as mean ± SD; **P values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups.***

**h.** Isotopologue patterns for incorporation of 13C-labeled oleate into TCA cycle intermediates, as measured by LC-MS in NHAs, NSCs and GSCs in basal conditions. Cells were cultured with 13C oleate for 6 hours prior to sample collection. N = 4 biological replicates, error = +/- SD. **P values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups.***
Figure 2. **MCAD is essential for in vitro and in vivo tumor growth.** a. Five-day growth curve of GSC 8.11 cells upon shRNA **ACADM** silencing. Day 0 was defined as 48 hours post-puromycin selection. Values represent the mean ± SD of three independent experiments. *P* values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups. *P* ≤0.02. b. Representative optical microscopy images of GSC tumor spheres 4 days after puromycin selection. Scale bar = 100 μm. c. Quantification of apoptosis in GSC 8.11 infected with anti-**ACADM** or scrambled shRNA by Annexin V-FITC/PI. Staining was evaluated by flow cytometry at 72 hours and on day 7 after puromycin selection. Values represent the mean ± SD of three or four independent experiments; *P* values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups. *P*< 0.04. d. (Top) Cell viability assessed by Trypan Blue exclusion of GSC 8.11 treated with indicated concentration of SPA. Data represent mean ± SEM of three biologically independent replicates. *P* values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups. **P = 0.0065.** (Bottom) Dot plot showing GSCs 8.11 sphere formation efficiency (SFE) upon SPA treatment at indicated concentrations. DMSO was used as control. Values represent the mean ± SD of four independent experiments; *P* values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups. **P = 0.0011.** e. MRI images of tumor progression after implantation of GSCs 8.11 or 6.27 at 4 and 8 weeks for GSCs 8.11 and at 4 and 12 weeks for GSC 6.27. f. Quantification of tumor progression after implantation of GSC 8.11 as measured by MR volumetry (n=8 mice per group). *P* values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups. *P= 0.04, **P = 0.0082.** g. Kaplan-Meier survival analysis after implantation of GSCs 8.11. For sh-ctrl, sh-**ACADM1**, sh-**ACADM2**, n=8 mice per group. *P* values were generated using log-rank test. **** *P < 0.0001.** h. Growth curve of NHAs infected with sgRNA targeting either **ACADM** or GFP. Values represent the mean ± SD of three independent experiments. i. Cell viability assessed by Trypan Blue exclusion of NHAs treated with indicated concentrations of SPA or DMSO for 6 days. Mean values ± SD of three biologically independent replicates.

Figure 3. **ACADM silencing causes mitochondrial failure in GSCs.** a. Transmission electron microscopy images of mitochondria in GSC 8.11 upon MCAD silencing (magnification x15,000 and 50,000; scale bar= 1 μm, 300 nm) b. Measurements of mitochondrial ATP levels using a bioluminescence assay. Values are expressed as mean ± SD of three independent experiments; *P* values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups. **P < 0.006.** c., d. Oxygen consumption rate (OCR) measured in GSC 8.11 (c) and in normal human astrocytes (NHA) (d) following MCAD silencing by
metabolic flux assay before and after the addition of oligomycin, FCCP and rotenone/antimycin to perturb mitochondrial respiration. Values represent the mean ± SD of one representative experiment with n = 4 technical replicates. Experiments were repeated three times with similar results (see also Figure S6B). e. Intracellular acetyl-CoA levels in GSC 8.11 after ACADM silencing as assessed by fluorometric assay. Values are expressed as mean ± SD of three independent experiments; P values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups. ****P = 0.0001. f. Relative abundance of TCA cycle metabolites in GSC 8.11, as detected by LC-MS analysis. Values are expressed as mean ± SD of three independent experiments. g. Mitochondrial electron transport chain complex III activity in GSC 8.11 as measured by colorimetric assay. Values are expressed as mean ± SD of six independent experiments. P values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups. *P = 0.03, **P = 0.0079.

h. Mitochondrial complex V (ATP synthase) activity in GSC 8.11 as measured by colorimetric assay. Values are expressed as mean ± SD of five independent experiments; P values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups. *P = 0.0313, **P = 0.0071.

Figure 4. Accumulation of MCFAs induces toxic alterations of lipid metabolism in GSCs. a. Oil Red O staining in GSC 8.11 cells infected with anti-ACADM or non-targeting shRNA in vitro. Black arrows indicate sites of lipid accumulation. Scale bar = 20 μm. b. Colorimetric determination of free fatty acids from GSC whole-cell extracts. Values represent the mean ± SD of three independent experiments. P values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups. **P = 0.0061; ***P = 0.0003. c. Oil Red O staining in xenograft tumor tissues derived from GSC 8.11 infected with inducible shRNA constructs. Doxycycline was administered approximately 20 days after cell implantation. Oil Red O staining quantification of tumor tissues shown in (c) was obtained using ImageJ software analysis. Scale bar = 25 μm P values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups. *P = 0.033. d., e. Oil Red O staining (d) and growth curve (e) of ACADM wild type or null GSC 8.11 grown in normal or FAs-free medium. Cells were selected with puromycin for 48 h prior to starting the experiment. Oil Red O staining in cells after 48-h puromycin selection. Scale bar in d = 10 μm. Data in (e) represent the mean ± SD of three biologically independent replicates. P values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups. **P ≤ 0.007. f. Colorimetric analysis of acyl-CoA species in MCAD-deficient GSC 8.11 cells. Data represent the mean ± SEM of four biologically independent replicates. P values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups. *P <0.03. g. Quantification of lipid peroxidation.
determined by measuring the production of malondialdehyde (MDA) using the Colorimetric Microplate Assay for Lipid Peroxidation Kit. Data represent the mean ± SD of four biologically independent replicates. *P = 0.02, **P = 0.01. h. Quantitative LC-MS/MS lipid profiling of FFA content of ACADM wild type or null GSC 8.11 cells. Data represent mean ± SEM of three biologically independent replicates. *P < 0.03; **P < 0.004. i., j. Relative amount of total lipid classes measured by mass spectrometry of whole-cell extracts (i) or mitochondria (j) from GSC 8.11 cells infected with shRNA targeting ACADM. Data are reported as fold change over control cells infected with non-targeting shRNA. Mean values ± SD of three biologically independent replicates. *P values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups. ***P = 0.001; ****P = 0.0001. SM, sphingomyelin; LPE, lyso-phosphatidylethanolamine; LPC, lyso-phosphatidylcholine; HexCER, hexosylceramide; Hex2CER, dihexosylceramide; CER, ceramide; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PA, phosphatidic acid; TAG, triacylglycerol; DAG, diacylglycerol; CE, cholesteryl ester; FAHFA, fatty acid ester of hydroxyl fatty acids.

**Figure 5.** **MCAD knockdown triggers ROS-related damage in GSCs in vitro and in vivo.** a. ROS production as measured by CellROX Green in flow cytometry (left) and quantification of fluorescence intensity (right) in GSC 8.11 harboring anti-ACADM or non-targeting shRNA. Values represent the mean ± SD of four independent experiments. *P values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups. **P < 0.002. b. Mitochondrial ROS quantification by MitoSox staining intensity in GSC 8.11 cells harboring anti-ACADM shRNA or sgRNA 72 hours after gene silencing. Values represent the mean ± SD of four independent experiments. *P values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups. *P= 0.046, **P= 0.0033. c. ROS production quantification by CellRox Green staining intensity in GSC 8.11 and 6.27 upon 72-hour exposure to SPA (400 µM) or DMSO. Values represent the mean ± SD of three independent experiments. *P values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups. ****P= 0.0001. d. e. GSH/GSSG and NADP/NADPH ratios in GSC 8.11 measured by colorimetric assay. (d) *P ≤ 0.03; (e) *P ≤ 0.03. Values represent the mean ± SD of three independent experiments. *P values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups. f. Immunostaining for 8-oxoguanine (showing oxidized DNA, top panels) or HNE adducts (showing lipid peroxidation and oxidative protein
damage, bottom panels) and relative quantification of GSC 8.11-derived tumors from experiment shown in Figure S5D; scale bar 50 μm. Quantification was conducted with ImageJ software, six images per condition, where each condition was represented by three biological replicates. Values are expressed as mean ± SD; P values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups. *P ≤ 0.02. g. Representative images of the rescue effect of GSH-ethyl ester (GSH-EE) on GSC 8.11 tumor spheres with doxycycline-induced MCAD knockdown (scale bar 400 μm). h. (Top) Growth curve of GSC 8.11 cells infected with ACADM or non-targeting shRNA grown in the presence or absence of GSH-EE. Values are expressed as mean ± SD; P values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups. *P ≤ 0.03; (bottom) Quantification of the number of spheres formed by GSC 8.11 and GSC 6.27 harboring ACADM or non-targeting shRNAs grown for 72 hours or 7 days in the presence or absence of GSH-EE, as indicated. Values represent the number of spheres per field expressed as mean ± SD. P values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups. *P= 0.04.

Figure 6. Overloading of mitochondria with fatty acids triggers structural and functional alterations upon MCAD ablation and leads to GSCs death. a. Relative amount of (C18:2)-containing CL species with molecular weight increased by 48 Da (+ 48 amu adducts) measured by mass spectrometry in mitochondria from GSC 8.11 cells infected with shRNA targeting ACADM. Data are reported as fold change over native (non-oxidized) CL. Mean values ± SD of three biologically independent replicates. P values were generated using one-way ANOVA. *P <0.05 ****P = 0.001; ****P = 0.0001. b. Cardiolipin content per mg of protein as determined by a fluorometric assay 72 hours following ACADM silencing in GSC 8.11 mitochondria. Cells were grown in normal medium or supplemented with elamipretide as indicated. Values represent the mean ± SD of four independent experiments. P values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups * P <0.05 c. Quantification of mitochondrial ROS (mean of fluorescence intensity) as measured by MitoSox staining in GSC 8.11 cells harboring anti-ACADM shRNA or sgRNA 72 hours after gene silencing in the presence or in the absence of elamipretide. Values represent the mean±SD of four independent experiments. P values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups * P <0.05, *** P< 0.001. d. Representative optical microscopy images of GSC 8.11 tumor spheres 6 days after ACADM silencing in the presence or absence of elamipretide (20 μM). Scale bar = 40 μm. e. f. GSC 8.11 and 6.27 cells were grown with elamipretide (20 μM) for one week before genetic ACADM silencing. The number of viable cells (as percentage of the ctrl) was assessed at 96 hours after gene silencing by Trypan Blue
exclusion. Values represent the mean ± SD of three or four independent experiments. \( P \) values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups * \( P < 0.05; \) *** \( P < 0.001 \).

**g.** GSC 8.11 and 6.27 cells were pretreated with etomoxir (2.5 \( \mu \)M) for 48 hours before genetic \textit{ACADM} silencing.

**h.** The number of viable cells (as percentage of the ctrl) was assessed upon genetic \textit{ACADM} silencing by Trypan Blue exclusion. Values represent the mean ± SD of three or four independent experiments. \( P \) values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups * \( P < 0.05 \).

**i.** Representative optical microscopy images of GSC 8.11 tumor spheres 6 days after genetic \textit{ACADM} silencing in the presence or absence of etomoxir (2.5 \( \mu \)M). Scale bar = 40 \( \mu \)m.

**j.** ROS quantification (mean of fluorescence intensity) as measured by CellRox Green (j) and MitoSox (k) staining in GSC 8.11 cells harboring anti-ACADM shRNA or sgRNA 72 hours after genetic silencing. Values represent the mean ± SD of three or four independent experiments. \( P \) values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups * \( P < 0.05; \) ** \( P < 0.01 \).

**k.** The number of viable cells (as percentage of the ctrl) was assessed 96 hours after genetic \textit{ACADM} silencing by Trypan Blue exclusion. Values represent the mean ± SD of four independent experiments. \( P \) values were generated using Kruskal–Wallis ANOVA. Dunn’s test for comparison among groups. * \( P < 0.05; \) ** \( P < 0.01 \).
Fig. 1
**Figure 4**

**A**

Images showing cell morphology with labels: ctrl, sh-ACADM1, sh-ACADM2.

**B**

Graph showing Free Fatty Acids levels with comparison of sh-ctrl, sh-ACADM1, and sh-ACADM2.

**C**

Images of tissue sections with labels: -DOX, +DOX, ish-scr, ish-ACADM.

**D**

Images showing GSC.11 with controls and sg-ACADM1.

**E**

Line graph showing the number of cells over time (days) with different conditions: ctrl, sg-ACADM, ctrl FA free, sg-ACADM FA free.

**F**

Bar graphs showing Total Acyl-CoA levels with comparison of sg-ctrl, sg-ACADM1.

**G**

Graph showing Lipid peroxidation with MDA concentration compared to sh-ctrl.

**H**

Graph showing Relative Fatty Acid levels normalized to sh-ctrl.

**I**

Lipidomics graph for whole cells with comparison of sh-ACADM1, sh-ACADM2.

**J**

Lipidomics graph for mitochondria showing change over sh-ctrl normalized to total lipid signal.
Medium-chain acyl CoA dehydrogenase protects mitochondria from lipid peroxidation in glioblastoma

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Cancer Discov  Published OnlineFirst May 26, 2021.