Discrete mechanisms of mTOR and cell cycle regulation by AMPK agonists independent of AMPK

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Abstract

AMP-activated protein kinase (AMPK) is a molecular hub for cellular metabolic control (1–4). It is a heterotrimer of catalytic α, regulatory β, and γ subunits. The rising AMP:ATP ratio during energy stress leads to AMP-dependent phosphorylation of the catalytic α subunits. This activates AMPK which then phosphorylates numerous substrates to restore energy homeostasis. It phosphorylates acetyl CoA carboxylase (ACCα) to inhibit fatty acid (FA) synthesis (5) and TSC2 and RAPTOR (6, 7) to inhibit mammalian target of rapamycin (mTOR)1. Because fatty acid synthesis and mTORC1 activity are essential for cell proliferation and growth (8), AMPK activation with two indirect AMPK agonists AICAR and metformin (now in over 50 clinical trials on cancer) has been correlated with reduced cancer cell proliferation and viability. Surprisingly, we found that compared with normal tissue, AMPK is constitutively activated in both human and mouse gliomas. Therefore, we questioned whether the antiproliferative actions of AICAR and metformin are AMPK independent. Both AMPK agonists inhibited proliferation, but through unique AMPK-independent mechanisms and both reduced tumor growth in vivo independent of AMPK. Importantly, A769662, a direct AMPK activator, had no effect on proliferation, uncoupling high AMPK activity from inhibition of proliferation. Metformin directly inhibited mTOR by enhancing PRAS40 as association with RAPTOR, whereas AICAR blocked the cell cycle through proteasomal degradation of the G2M phosphatase cd25c. Together, our results suggest that although AICAR and metformin are potent AMPK-independent antiproliferative agents, physiological AMPK activation in glioma may be a response mechanism to metabolic stress and anticancer agents.

Significance

Cancer cells reprogram their metabolism for optimal growth and survival. AMPK-activated protein kinase (AMPK) is a key energy sensor that controls many metabolic pathways including metabolic reprogramming. However, its role in cancer is poorly understood. Some studies claim that it has a tumor suppressor role while others show its protumor role. Two AMPK-activating compounds (including metformin, now in many clinical trials) are widely used to suppress cancer cell proliferation. We found that AMPK is abundantly expressed in high-grade gliomas and, in contrast to popular belief, these two AMPK activators suppressed glioma cell proliferation through unique AMPK-independent mechanisms.


The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1311121111/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1311121111

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PNAS | Published online January 13, 2014 | E435–E444

Molecular Biology

The multifunctional AMPK-activated protein kinase (AMPK) is an evolutionarily conserved energy sensor that plays an important role in cell proliferation, growth, and survival. It remains unclear whether AMPK functions as a tumor suppressor or a contextual oncogene. This is because although on one hand active AMPK inhibits mammalian target of rapamycin (mTOR) and lipogenesis—two crucial arms of cancer growth—AMPK also ensures viability by metabolic reprogramming in cancer cells. AMPK activation by two indirect AMPK agonists AICAR and metformin (now in over 50 clinical trials on cancer) has been correlated with reduced cancer cell proliferation and viability. Surprisingly, we found that compared with normal tissue, AMPK is constitutively activated in both human and mouse gliomas. Therefore, we questioned whether the antiproliferative actions of AICAR and metformin are AMPK independent. Both AMPK agonists inhibited proliferation, but through unique AMPK-independent mechanisms and both reduced tumor growth in vivo independent of AMPK. Importantly, A769662, a direct AMPK activator, had no effect on proliferation, uncoupling high AMPK activity from inhibition of proliferation. Metformin directly inhibited mTOR by enhancing PRAS40 as association with RAPTOR, whereas AICAR blocked the cell cycle through proteasomal degradation of the G2M phosphatase cd25c. Together, our results suggest that although AICAR and metformin are potent AMPK-independent antiproliferative agents, physiological AMPK activation in glioma may be a response mechanism to metabolic stress and anticancer agents.

AMP-activated protein kinase (AMPK) is a molecular hub for cellular metabolic control (1–4). It is a heterotrimer of catalytic α, regulatory β, and γ subunits. The rising AMP:ATP ratio during energy stress leads to AMP-dependent phosphorylation of the catalytic α subunits. This activates AMPK which then phosphorylates numerous substrates to restore energy homeostasis. It phosphorylates acetyl CoA carboxylase (ACCα) to inhibit fatty acid (FA) synthesis (5) and TSC2 and RAPTOR (6, 7) to inhibit mammalian target of rapamycin (mTOR)1. Because fatty acid synthesis and mTORC1 activity are essential for cell proliferation and growth (8), AMPK activation with two indirect AMPK agonists AICAR and metformin have been correlated with suppression of cell proliferation and growth (9–11). AICAR is metabolized to an AMP mimetic, ZMP that activates AMPK, only AICAR inhibited proliferation of trisomic mouse fibroblasts (11). Moreover, although AICAR strongly increases glucose uptake through AMPK activation in muscle cells, it reduced fluorodeoxyglucose-PET signals and inhibited glioma growth in vivo (9), suggesting that reduced PET signals could be due to its AMPK-independent antiglioma action. The antiproliferative mechanisms of metformin also remain unclear. It is argued that because metformin inhibits mitochondrial respiration (19), it induces an energy crisis (metabolic stress), leading to AMPK activation, mTOR inhibition, and suppression of proliferation (20). However, Dykens et al. (21) showed that net cellular ATP is not affected by metformin. Other suggested mechanisms include disruption of cross-talk between GPCRs and insulin receptors (22), inhibition of the ErbB2/IGF1 receptor (23), and mTOR inhibition by blocking RAG function (24). In vivo, metformin and the direct AMPK agonist A769662 delayed onset but not progression of lymphoma in Pten+/−;LKB1+/− mice (25) (LKB1 is the upstream kinase that activates AMPK). Moreover, these experiments were not conducted on AMPK-deficient animals, making it unclear whether the drug effects were AMPK dependent. Contrary to these results, metformin prevented tumorigenesis without activating AMPK in lung tumors (26), and in fact, LKB1-deficient lung tumors were actually more responsive to the metformin analog phenformin (27). The latter results suggest that the LKB1–AMPK metabolism | glioma

PNAS PLUS

Biomedical Science

Edited by Gregg L. Semenza, The Johns Hopkins University School of Medicine, Baltimore, MD, and approved December 4, 2013 (received for review June 13, 2013)
pathway protects cancer cells from antiproliferative agents and may support tumorigenesis.

In line with the above idea, genetic studies showed a procancer role of AMPK in the in vivo growth of H-RAS-transformed fibroblasts and astrocytic tumors, in pancreatic cancer, and in a subtype of renal cell carcinoma (28–31). Additional genetic studies also underscore the requirement of AMPK in cancer cell metabolic programming (32, 33); cell division (34–37); migration (38); protection against stress; and anticancer therapy (39–41). However, in Myc-driven mouse lymphoma, AMPK was shown to function as a tumor suppressor (42), suggesting a context-dependent role of AMPK in cancer.

To definitively determine whether AMPK is necessary for the antiproliferative actions of AICAR and metformin, we conducted a comprehensive pharmacogenetic study in glioma. First, we found that gliomas express constitutively active AMPK, and that AICAR and metformin inhibit proliferation by distinct AMPK-independent and unique mechanisms. Second, A769662, a direct AMPK activator (43) showed no antiproliferative effects. Therefore, many agents that inhibit proliferation with concomitant AMPK activation may not require AMPK for their action. Instead, AMPK activation could be a response mechanism to counter stress induced by anticancer agents.

**Results**

**Active AMPK Is Abundantly Expressed in High-Grade Gliomas.** Metabolic stress in solid tumors like gliomas poses a formidable challenge for tumor cell survival. Because metabolic stress activates AMPK, we examined AMPK’s activation state in gliomas. Immunohistochemistry (IHC) of grade IV human gliomas (called glioblastoma or GBM) showed abundant active (phosphorylated) AMPK in all GBM tissues (Fig. 1A; IHC of 6 of 12 GBMs shown). Compared with normal astrocytes, human GBM cell lines also expressed significantly higher levels of phosphorylated AMPK and ACC (an AMPK substrate) (Fig. 1B). Furthermore, in a genetically engineered mouse model of high-grade glioma (Fig. 1C; ref. 44), we observed high pAMPK and pACC levels within the tumor compared with normal cortical tissue (Fig. 1D and E).

**Fig. 1.** Phosphorylated (active) AMPK is abundantly expressed in gliomas and the direct AMPK activator A769662 does not inhibit glioma growth. (A) IHC of active AMPK (pAMPK) in six human GBMs (12 tumors were analyzed). (Inset) High magnification of A. (B) Immunoblot shows pAMPK in glioma cell lines and normal astrocytes. Histology (C), IHC (D), and immunoblot (E) of mouse high-grade gliomas (HGGs). Magnification: A, 20×; A, inset, 60×; C, 10×; D, 40×. A total of 12 tumors were analyzed. N, contralateral normal brain; T, tumor tissue. (F) Immunoblot analysis of glioma cells treated with AMPK agonists and (G) the effect AICAR, metformin, and A769662 on the proliferation of glioma cells. *P < 0.005. Data shown is representative of three to six independent experiments. Error bars represent mean ± SD.
E), akin to another report in a rat model of glioma (45). These data demonstrate that active AMPK is common in GBMs.

**Glioma Cell Proliferation Is Suppressed by AICAR and Metformin but Not by the Direct AMPK Activator A769662.** To examine whether the extent of AMPK activation correlates with the antiproliferative effects of AMPK activators in glioma, we conducted a dose- and time-dependent immunoblot analysis using AMPK and ACC antibodies and parallel cell proliferation assays. AICAR, metformin, and A769662 activated AMPK similarly at all doses (AICAR: 1 and 2 mM; metformin: 1, 2.5, 5, and 10 mM; and A769662: 100 and 200 μM; Fig. S1 A and B). Whereas AICAR inhibited proliferation similarly at all doses, metformin’s effect was significant only at 10 mM (Fig. S1C). Based on these results, we chose 1 mM AICAR, 10 mM metformin, and 100 μM A769662 to conduct the time-course analysis. All three agonists activated AMPK leading to ACC phosphorylation, with maximal activation occurring at 48 and 72 h (Fig. 1 F). The earliest time point AICAR and metformin suppressed proliferation was at 48 h which was maintained until 72 h (Fig. 1G). This reduction in viable cell numbers was not due to increased cell death (measured by Trypan blue exclusion at each time point; Fig. S1D) but largely due to the cytostatic effects of these agents. Surprisingly, despite robustly activating AMPK (Fig. 1F and Fig. S1B), A769662 did not suppress proliferation (Fig. 1G and Fig. S1E). Akt activation can oppose AMPK action; however, A769662 did not increase Akt phosphorylation (Fig. S1F). Our results suggest that the indirect AMPK activators AICAR and metformin inhibit proliferation possibly through pleiotropic actions, whereas the direct activator A769662 does not suppress cell proliferation.

**Inhibition of Lipogenesis Is Not a Likely Mechanism of AICAR and Metformin’s Antiproliferative Action.** AMPK inhibits ACCα and HMG-CoA reductase, to suppress FA and cholesterol synthesis, respectively. ACCα and FA synthase (FASN) work in conjunction to form palmitate. To test whether endogenous FASN is required for optimal proliferation of glioma cells and whether AICAR and metformin suppresses proliferation by inhibiting this pathway, we silenced FASN and ACC and examined whether silencing this pathway confers cellular resistance to these agents. shRNA-mediated silencing of FASN (Fig. 2 A, Inset) did not reduce proliferation in normal growth medium containing 10% serum (Fig. 2 A and Fig. S2A). Remarkably, regardless of the serum content, metformin and AICAR’s effects were essentially similar in control and FASN shRNA-expressing cells (Fig. 2 A and Fig. S2 A and B). Silencing of ACC (Fig. 2 B, Inset) or expression of a phosphorylation-incapable S79A mutant of ACC1 (AMPK target site) also did not suppress proliferation and did not confer resistance to AICAR or metformin’s action (Fig. 2 B and Fig. S2B). Addition of lipogenesis end products (palmitate and mevalonate) also did not reverse AICAR and metformin’s antiproliferative effects (Fig. S2 D–F). Furthermore, the FASN

![Fig. 2. Inhibition of lipogenesis is not a mechanism of AICAR and metformin’s antiproliferative action. Proliferation of FASN-silenced (A) and ACC-silenced (B) T98G and U87EGFRvIII glioma cells treated with AICAR and metformin. nt, nontarget. Immunoblot with FASN (A, Inset) and ACC (B, Inset) antibodies. nt, nontarget shRNA. *P ≤ 0.005. (C) Proliferation of glioma cells in the presence of lipogenesis inhibitors [C75 (10 μg/mL) and atorvastatin (1 μM)]. Data shown is representative of two to four independent experiments. Error bars in A–C represent mean ± SD.](image_url)
inhibitor had little effect on glioma cell proliferation (Fig. 2C). Collectively, these results indicate that AICAR and metformin suppresses proliferation through other mechanisms.

**AMPK-Dependent mTOR Inhibition Is Not Required by AICAR and Metformin to Suppress Glioma Cell Proliferation.** mTORC1 promotes cell proliferation and growth through the phosphorylation of its effectors 4EBP1 and S6. AMPK inhibits mTOR through phosphorylation of two mTORC1 regulators—TSC2 and RAPTOR. We first examined the importance of mTORC1 in glioma by using the mTORC1 inhibitor rapamycin. Low nanomolar concentrations of rapamycin completely suppressed S6 but had little effect on 4EBP1 phosphorylation or proliferation (Fig. S3A and B). This suggests that complete inhibition of 4EBP1 but not S6 phosphorylation may be necessary to halt proliferation. To examine whether AMPK agonists suppresses proliferation by AMPK-dependent mTORC1 inhibition, we first examined their effect on phosphorylation of S6 and 4EBP1 in glioma cells. As expected, all three AMPK agonists activated AMPK leading to RAPTOR phosphorylation (Fig. 3A and Fig. S3C). However, despite similar RAPTOR phosphorylation, metformin but not AICAR or A769662 significantly inhibited mTOR (suppressing both 4EBP1 and S6 phosphorylation; Fig. 3A and Fig. S3C). Inhibition of mTORC1 by AICAR and A769662, if any, was incomplete (Fig. 3A and Fig. S3C). Dephosphorylated 4EBP1 sequesters the translation initiation factor eIF4E (a CAP-binding protein) to inhibit CAP-dependent translation. Predictably, high amounts of 4EBP1 were bound to immunoprecipitated eIF4E.

![Fig. 3.](#) AMPK-dependent mTOR inhibition is not required by AICAR and metformin to suppress glioma cell proliferation. (A) Immunoblots showing the effects of AICAR, metformin, and A769662 on phosphorylation of AMPK substrates (ACC and RAPTOR) and mTOR effectors (S6 and 4EBP1) in T98G glioma cells. In B, eIF4E was immunoprecipitated with m7GDP-Sepharose and bound 4EBP1 was detected with 4EBP1 antibody. (C, Upper) Proliferation of control (nt shRNA) or AMPK β1 shRNA cells treated with AMPK agonists. nt, nontarget. *P ≤ 0.005. (C, Lower) Immunoblots show AMPK, ACC, and RAPTOR phosphorylation by AMPK agonists. Densitometry of pAMPK levels is also shown. Error bars represent mean ± SD. (D) Immunoblots demonstrate the effects of AMPK agonists on mTOR effectors (S6 and 4EBP1) in control (nt) and AMPK β1 shRNA T98G glioma cells. (E) The CAP-binding assay was done as in B in control or AMPK β1 shRNA T98G cells. Data shown is representative of two to five independent experiments.
in metformin-treated cells but not in A769662-treated cells (Fig. 3B). The high sensitivity of this assay showed that AICAR inhibits 4EBP1 phosphorylation only to a significantly lesser extent than metformin. Based on these results, we reasoned that because both AICAR and rapamycin partially inhibited mTOR but only AICAR suppressed proliferation, AICAR’s suppressive actions are likely mTOR independent. Metformin on the other hand, completely suppressed mTORC1 and proliferation, suggesting that mTOR inhibition could be necessary for its antiproliferative actions.

To examine whether mTOR inhibition by metformin is dependent on AMPK, we knocked down the AMPKβ1 subunit to repress AMPK function. We targeted this subunit because first, the β-subunits regulate the stability of the catalytic α-subunits of AMPK and second, glioma cells expressed significantly more β1 than β2 (Fig. S3D). Indeed, by silencing β1 we achieved an 80–90% reduction of active AMPK levels (Fig. S3 E–G). AICAR and metformin could not sufficiently activate AMPK in β1-silenced cells (Fig. 3C), yet they inhibited proliferation similar to (in fact more than) control cells (Fig. 3C). This clearly indicates that AICAR and metformin suppress proliferation regardless of AMPK. To rule out the possibility that these agents use the remaining AMPK β2 subunit or directly the AMPK α-subunits, we silenced both β1/β2 or α/α2 subunits in T98G cells (Fig. S3G) and also used mouse fibroblasts derived from β1/β2 double knockout (DKO) animals. AMPK agonist-dependent phosphorylation of ACC and RAPTOR was considerably reduced in β1/β2 and α/α2 silenced cells compared with NT cells (Fig. S3 H and I). Remarkably, both AICAR and metformin but not A769662 inhibited glioma cell proliferation in the double-silenced cells that was comparable to NT cells (Fig. S3 J and K).

Similar results were observed in mouse embryonic fibroblasts (MEFs). As expected, the catalytic α-subunits of AMPK became completely unstable in the β1/β2 DKO MEFs and consequently AMPK substrate phosphorylation (ACC and RAPTOR) was completely inhibited (Fig. S4A). Even in these cells both AICAR and metformin robustly inhibited proliferation. In fact, AMPK-null MEFs were inhibited more than wild-type (WT) MEFs (Fig. S4B). Together, these results confirm that AMPK is not required for the antiproliferative actions of AICAR and metformin. Besides, the relatively greater sensitivity of both glioma cells and AMPK-null MEFs to AICAR and metformin shows that AMPK probably provides resistance to the detrimental actions of anticancer agents as shown by others (41).

Pharmacological AMPK activation by all three AMPK agonists caused RAPTOR phosphorylation. However, only AICAR and metformin inhibited proliferation. In any case, there were differential effects of these agonists on the two mTOR effectors (S6 and 4EBP1). Therefore, we examined whether the AMPK–mTOR axis is preserved during physiological AMPK activation. A time kinetics of glucose deprivation (GD) revealed that mTOR inhibition could be necessary for its antiproliferative actions. In fact, AMPK-completely inhibited (Fig. S4C) at 48 and 72 h, suggesting a resetting of the bioenergetic equilibrium. The high sensitivity of this assay showed that AMPK probably provides resistance to the detrimental actions of anticancer agents as shown by others (41).

Induction of Chronic Energy Stress Is Not a Mechanism by Which AICAR and Metformin Repress Glioma Cell Proliferation. It has been suggested that AICAR and metformin induce energy stress to activate AMPK and inhibit proliferation (10, 11). We therefore examined whether these reagents cause energy stress. HPLC analysis showed that surprisingly, neither AICAR nor metformin decreased ATP:AMP ratio acutely (4 h) or chronically (72 h). In fact, we observed an increase in ATP:AMP ratio following treatment with these agents (Fig. 4A). Total ATP content measured by luciferase assay was also increased (Fig. S5 C–E), suggesting that these agents directly stimulate ATP production or AMPK-dependent response mechanisms exist in these cells to counter energy stress caused by these agents.

To further validate these results, we examined energy-producing pathways by measuring the extracellular acidification rate (ECAR), a measure of glycolysis, and the O2 consumption rate (OCR) using the Seahorse XF-analyzer (Seahorse Bioscience). Metformin but not AICAR sharply reduced OCR (Fig. 4B and Fig. S5D), but metformin also increased compensatory ECAR (Fig. 4C and Fig. S5E). These effects of metformin on ECAR and OCR were AMPK independent. (Fig. 4 D and E). To provide additional evidence that metformin does not inhibit proliferation by suppressing mitochondrial OCR, we created mitochondria-deficient glioma (Rho-0) cells. Our results show that metformin’s growth inhibition does not require functional mitochondria as it inhibited proliferation of Rho-0 cells similar to mitochondria-proficient cells (Fig. 4F).

AICAR and Metformin Suppresses Glioma Growth in Vivo Independent of AMPK. We next determined whether the AMPK-independent growth inhibitory effects of AICAR and metformin are also true in vivo. To test this, we created flank xenografts using U87EGFRvIII glioma cells expressing NT or AMPKβ1 shRNA and treated mice with AICAR, metformin, or vehicle. Both AICAR and metformin significantly suppressed tumor growth at all time points (P < 0.03). Consistent with our in vitro results, both agents, particularly AICAR, caused a greater growth inhibition of AMPK-silenced tumors than control tumors (Fig. S4 A and B). We conclude that AMPK is not required by AICAR and metformin to inhibit glioma proliferation in vitro or growth in vivo.

AICAR Blocks Cell Cycle by AMPK-Independent Proteosomal Degradation of cdc25C. We next examined the mechanism of AICAR’s growth suppression by conducting cell cycle analysis. AICAR blocked the cell cycle at G2M consistently in all cell lines (Fig. 5C). This effect of AICAR was also AMPK independent (Fig. 5D). Additionally, cell proliferation experiments using the thymidine analog 5-ethyl-2′-deoxyuridine that incorporates into newly synthesized DNA showed no significant S-phase arrest by
AMPK activators (Fig. S6 A and B). To examine the mechanism of cell cycle arrest we studied several cell cycle regulators including Cyclin A, D1, D2, and E; CDK2; p16; p21; p27; p53; and Rb phosphorylation. AICAR had no significant effect on any of these proteins (Fig. S6C).

Dephosphorylation of the kinase cdc2 by the phosphatase cdc25c is crucial for G2M transition. We observed that AICAR-treated cells had significantly reduced cdc25c levels (Fig. 5 E). This effect of AICAR was independent of AMPK (Fig. 5F). Reduced cdc25c levels resulted in increased phosphorylation and inhibition of cdc2 (Fig. 5 E and F). Cyclin B1 (which associates with cdc2) was not affected. To examine how AICAR suppresses cdc25c, we studied ROS because cdc25c is downregulated by ROS (47) and AICAR was shown to cause ROS production (48). However, AICAR did not produce ROS in glioma cells (Fig. S6D). RT-PCR analysis confirmed that AICAR does not affect cdc25c transcript levels (Fig. 5G).

Because cdc25c is also regulated by the proteasome pathway (49), we examined whether AICAR degrades cdc25c through the proteasome. Indeed, the proteasome inhibitor MG132 (Sigma)
restored cdc25c levels in AICAR-treated T98G cells and U87 cells (Fig. 5H and Fig. S6F) and this effect was independent of AMPK (Fig. 5H). To validate that cdc25c down-regulation has a functional consequence on glioma cell proliferation, we silenced cdc25c (Fig. 5I, Inset). Three independent clones of cdc25c shRNA significantly arrested glioma cells at G2M and repressed glioma cell proliferation by about 35% (Fig. 5I). Together, our findings demonstrate that AICAR’s growth-suppressive effects are independent of AMPK and occur through multiple mechanisms, important among which is a previously unidentified mechanism that involves degradation of a key cell cycle protein by the proteasome.

Metformin Enhances PRAS40–RAPTOR Association to Inhibit mTOR and Suppress Glioma Proliferation, Independent of AMPK. Because 4EBP1 phosphorylation by mTOR is crucial for cancer cell proliferation and growth (50) and metformin strongly inhibited 4EBP1 phosphorylation in glioma cells, we tested whether mTOR and 4EBP1 are necessary for metformin’s suppressive action by knocking down these proteins (Fig. 6A and B and Fig. S7A and B). Predictably, mTOR silencing reduced and 4EBP1
silencing enhanced cell proliferation (Fig. 6C and Fig. S7C). Remarkably, the effect of metformin was significantly blunted in mTOR-silenced (>90% rescue) and 4EBP1-silenced (>70% rescue) cells (Fig. 6C and Fig. S7C), suggesting that metformin requires mTOR to suppress proliferation. Unlike metformin, AICAR’s inhibitory effects were maintained in mTOR and 4EBP1 knockdown cells (Fig. 6C and Fig. S7C).

Next, we explored the mechanism of metformin’s mTOR inhibition. In the in vitro kinase assay using recombinant 4EBP1 and mTOR, metformin did not inhibit mTOR kinase activity (Fig. 6D). The Akt substrate PRAS40 binds to RAPTOR to negatively regulate mTORC1 (51, 52). Because cell stressors (like 2DG, oligomycin) inhibit mTOR by increasing PRAS40 binding with RAPTOR (52), we questioned whether metformin inhibits mTOR by enhancing this association. Indeed, both metformin and its analog phenformin significantly enhanced PRAS40–RAPTOR association (Fig. 6D). To examine whether this is a crucial mechanism of metformin’s inhibitory action in glioma cells, we tested whether PRAS40-silenced cells resist metformin’s inhibitory action. Knocking down PRAS40 significantly rescued glioma cells from metformin’s inhibition (Fig. 6E), whereas a second shRNA that did not silence PRAS40 failed to rescue these cells.

Our overall findings provide compelling evidence that AMPK is not required for the antiproliferative actions of two widely used AMPK agonists. Based on our unique results, we propose a model (Fig. 6F) in which the direct AMPK activator A769662 has little effect on mTOR or proliferation; AICAR and metformin suppress proliferation independent of AMPK: AICAR by proteasomal degradation of cdc25c and metformin by increasing PRAS40-mediated mTOR inhibition.

**Discussion**

The function of AMPK in cancer is not fully understood. High AMPK activity is observed in colorectal, cervical, and brain cancers (refs. 30, 45, and 53–56 and this study), whereas low AMPK activity has been observed in others. Because LKB1, the kinase that activates AMPK is a tumor suppressor, AMPK activation by agonists is perceived to have therapeutic benefits. This belief remains despite the puzzling observation that in contrast to canonical tumor suppressors, LKB1 is required for cellular transformation (1) and LKB1- or AMPK-deficient cancer cells can be sensitive to chemotherapeutic agents (27, 31, 57). Ironically, these agents include the very AMPK agonists that are used to activate AMPK. To unravel this conundrum we examined whether these AMPK agonists work independent of AMPK. We found that two indirect AMPK agonists, AICAR and metformin (that do not activate AMPK in cell-free assays) (58), inhibit proliferation independent of AMPK and to our surprise, the direct AMPK activator A769662 has no such inhibitory effects in normal or glioma cells. We discover that although AICAR blocks the cell cycle by a unique mechanism that involves proteasomal degradation of cdc25c, metformin suppresses proliferation by inhibiting mTOR through enhanced PRAS40 binding to RAPTOR. We conclude that in line with other observations (12, 15), AICAR and metformin’s growth inhibition in vitro does not require AMPK.

The growth suppressive mechanisms of the AMPK agonists in vivo are as enigmatic as the role of AMPK itself in tumorigenesis. In PTEN+/−, LKB1 hypomorphic mice that develop lymphoma (25), A769662, metformin, and its analog phenformin delayed the onset but not progression of tumor growth. It is not known whether these agents sufficiently activated AMPK inside tumors. In fact, metformin prevented lung tumorigenesis without known whether these agents sufficiently activated AMPK inside tumors. In fact, metformin prevented lung tumorigenesis without activating AMPK inside tumors, likely due to its systemic effects on insulin signaling (26). It is possible that the growth-inhibiting effects in our GBM xenografts are a combination of the AMPK-independent mTOR-dependent cellular and systemic effects of metformin. Contrasting results about AMPK’s antitumor role in lymphoma (42) and protumor role in brain cancer (30) suggest...
that AMPK likely plays context-dependent diverse roles in tumorigenesis and, depending on the model, much of the inhibitory effects of the AMPK agonists in vivo are independent of AMPK.

Similar to our results, AICAR suppressed GBM xenograft growth (9). In that pharmacological study, AICAR was shown to inhibit lipogenesis in vitro and lipogenesis inhibitors reduced proliferation in vitro. Whether AICAR’s action required AMPK was not examined. In contrast, we found that in fact, AMPK-silenced GBM tumors are hypersensitive to both AICAR and metformin. Furthermore, knockdown of lipogenic genes (ACC and FASN) did not interfere with glioma proliferation, akin to that observed in human breast cancer cells (59). However, AICAR and metformin were still inhibitory on these ACC- or FASN-silenced cells indicating that they clearly work through other pathways. We speculate that regulation of FA utilization in cancer cells (de novo synthesis vs. import) is far more complex than that in normal lipogenic cells and AMPK activation is allowed at specific stages during the evolution of certain tumors to endure metabolic stress without significant inhibition of FA synthesis at the level of the AMPK substrate ACC.

Our study shows that mTOR regulation by AMPK activators in glioma cells (and probably in other cancer cells) is different from that in normal cells. By comparing the effects of rapamycin, AICAR, A769662, and metformin on mTOR, we conclude that inhibition of 4EBP1 but not S6 phosphorylation by metformin, independent of AMPK, is critical for the suppression of glioma cell proliferation. Unlike metformin, mTOR inhibition by AICAR and A769662 was at best partial or marginal. To unravel this discrepancy, we examined the extent of TSC2 and RAPTOR phosphorylation (the two AMPK substrates that inhibit mTOR) by the AMPK agonists. We found that although phospho-TSC2 antibodies yielded multiple nonspecific bands and were thus unreliable, the RAPTOR antibody was specific. All three AMPK agonists phosphorylated RAPTOR. It is unclear at this time why the signal from RAPTOR phosphorylation did not completely reach mTOR in glioma cells. Discrete localization of the various AMPK complexes and mTOR substrates in specific subcellular compartments, together with signal interference from the oncogenic pathways in cancer cells may explain some of these results. It is worth noting that AMPK silencing did not increase baseline mTOR signaling in glioma cells, suggesting that in certain contexts basally active AMPK may allow mTOR activation. It is also clear from our results that although the AMPK–mTOR axis is strongly preserved during physiological metabolic stress in normal fibroblasts, it is less so in glioma cells. Future studies will unravel this intriguing uncoupling of the AMPK–mTOR axis in glioma.

We discovered two AMPK-independent mechanisms by which AICAR and metformin repress glioma proliferation. We found that AICAR blocks glioma cells at G2M by activating the proteasomal pathway through a yet unidentified mechanism. This leads to down-regulation of the critical G2M phosphatase cdc25c (and possibly other proteins). Indeed, akin to other studies (60), knockdown of cdc25c in glioma cells caused G2M arrest and suppressed proliferation. Additional studies, including the effects of AICAR on protein ubiquitination, will be required to determine how AICAR activates the proteasomal pathway in glioma cells. In contrast to AICAR, metformin clearly required mTOR to repress proliferation. mTOR is regulated by multiple mechanisms and the dominant mechanism might vary according to cell type and context. We found that metformin treatment enhanced binding of PRAS40 with RAPTOR, an association known to be enriched during stress to inhibit mTOR activity. Although metformin inhibits mitochondrial respiration, we did not find any chronic energy reduction in metformin-treated glioma cells. It is possible that metformin causes other types of cell stress (e.g., unfolded protein response, DNA damage response, and endoplasmic reticulum stress) that are transmitted through PRAS40 to mTOR inhibition. Finally, we surmise that AMPK plays context-specific roles in tumorigenesis that depend on tumor stage and energy state, as well-nourished vascular tumors undergo AMPK-dependent metabolic reprogramming (32) and evolve into substrate-limited, hypoxic, and invasive tumors. To delineate these processes and define the role of AMPK in cancer, genetic studies and use of specific AMPK modulators will be crucial. Metformin is used in several cancer clinical trials. Our study warrants careful interpretation of data on the anticancer mechanisms of this useful drug.

Experimental Procedures

Patient Samples. GBM samples were obtained under an institutional review board-approved protocol from The University of Cincinnati Brain Tumor Center. Informed consent was obtained from the patients whose samples were used in our research. For details, see SI Experimental Procedures.

Mouse Strains. All animal procedures were carried out in accordance with the Institutional Animal Care and Use Committee-approved protocol of Cincinnati Children’s Hospital Medical Center. AMPK1−/− and AMPK2−/− mice have been described (35, 45). Quadruple conditional knockout mice were derived by crossing triple knockout mice (GFAP-CreERT; PtenloxP/loxP; Trp53−/−; Tsc2−/−) with Rub110−/− mice. Filial-1 progeny were intercrossed to generate a quadruple homozygous targeted strain. Deletion of conditional alleles was induced by i.p. tamoxifen injections.

Cell Culture and Growth Analysis. Cell culture methods are described in SI Experimental Procedures. P1−/−; P12−/− MEFs were generated by crossing by (P1−/− and P12−/− mice. For proliferation and viability analysis, a fluorescence-based drug assay was used. Drugs were added 24 h postseeding and cell viability was determined at indicated times.

Immunoblot Analysis. Western blot analysis was carried out following standard methods (35). For details, see SI Experimental Procedures.

shRNA and Lentivirus. The following shRNA clones were purchased from the LentiSHRNA Library Core (Cincinnati Children’s Hospital Medical Center (CCHMC)); AMPK1 (TRCN00000004770, FASN (TRCN00000003127) or TRCN00000003128), mTOR (TRCN0000038674 or TRCN0000038677), ACCα (TRCN000004766, TRCN000004767 and TRCN000004769), PRAS40 (TRCN0000165347), cdc25c (TRCN000002432, TRCN000002432 and TRCN000002434), and pLKO.1-puro scrambled (NT); 4EBP1 shRNA (TRCN0000042023) was a gift from George Thomas (University of Cincinnati, Cincinnati). For details, see SI Experimental Procedures.

Metabolic Experiments. ATP and AMP were analyzed by HPLC as described (45). ATP was additionally measured by using the ApoSensor ATP Luminescence Assay Kit (BioVision). ECAR and OCR were analyzed by using the Seahorse XF-Analyzer. For details, see SI Experimental Procedures.

Cell Cycle Analysis. Cell cycle analysis was done in a FACScan analyzer (BD). Full method is in SI Experimental Procedures.

Statistical Analysis. Student t test was used to calculate statistical significance with P < 0.05 representing a statistically significant difference.

ACKNOWLEDGMENTS. We thank Nancy Ratner for manuscript review; George Thomas for the 4EBP1 shRNA plasmid, and PS3−/− and TSC2−/− MEFs; Paul Mischel for U87EGFR and U87EGFRvIII cells; Russell Jones for AMPKα1/2 shRNA plasmid; Carol Mercer for the control GST plasmid; Peter Vogt for the Rbplambda plasmids; and Nissim Hay for the ACC1 S79A plasmid. This work was supported by the CancerFreeKids, the Smith–Brinker Golf Foundation, a CCHMC Trustee Scholar grant, and National Institutes of Health Grant 1R01NS075291-01A1 (all to B.D.).


