AMPK activation by oncogenesis is required to maintain cancer cell proliferation in astrocytic tumors

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Abstract

AMPK is an energy sensor that controls cell metabolism, and it has been related with apoptosis and cell cycle arrest. Although its role in metabolic homeostasis is well documented, its function in cancer is much less clear. In this study, we examined the role of AMPK in a mouse model of astrocytoma driven by oncogenic H-RasV12 and/or with PTEN deletion, based upon the common constitutive activation of the Raf/MEK/ERK and PI3K/AKT cascades in human astrocytomas. We also evaluated the activity and role of AMPK in human glioblastoma cells and xenografts. AMPK was constitutively activated in astrocytes expressing oncogenic H-RasV12 in parallel with high cell division rates. Genetic deletion of AMPK or attenuation of its activity in these cells was sufficient to reduce cell proliferation. The levels of pAMK were always related to the levels of phosphorylated RB at Ser804, which might indicate an AMPK mediated phosphorylation of RB. We confirmed this AMPK-RB relationship in human glioblastoma cell lines and xenografts. In clinical specimens of human glioblastoma, elevated levels of activated AMPK appeared especially in areas of high proliferation surrounding the blood vessels. Together, our findings indicate that the initiation and progression of astrocytic tumours relies upon AMPK-dependent control of the cell cycle, thereby identifying AMPK as a candidate therapeutic target in this setting.
Introduction

In the 1920s a relationship between cellular metabolism and tumorigenesis was first proposed by Warburg, showing that cancer cells exhibited an increase in glycolysis even in conditions of high oxygen levels [1]. Since then many modifications in intermediary metabolism have been described in cancer cells, but the molecular mechanisms that interconnect the signalling pathways controlling metabolism and cell growth have only begun to be elucidated. One of the plausible links between cell metabolism and cancer is the fuel-sensing enzyme 5′-AMP-activated protein kinase (AMPK). This kinase has a major role in the regulation of glucose, lipid and protein metabolism in response to stimuli such as changes in fuel availability, oxidative stress, heat shock and hormones [2]. AMPK is a heterotrimer that contains one catalytic (α1 or α2), one regulatory (β1 or β2) and one AMP/ATP binding (γ1, γ2 or γ3) subunit [2]. Decreases in the cellular energy state, as reflected by an increase in the AMP/ATP ratio, induce conformational changes in AMPK that makes it susceptible to phosphorylation and activation by AMPK kinases [2]. Once activated, AMPK restores energetic balance stimulating catabolic pathways that enhance ATP generation and inhibiting others that consume ATP, but are not acutely necessary for survival [3]. Recent findings have also pointed to a link between AMPK and cell growth. First, it was demonstrated that the tumour suppressor LKB1 is a kinase that phosphorylates and activates AMPK, and that another tumour suppressor, tuberous sclerosis complex 2 (TSC2), is activated by AMPK [4, 5]. TSC2 activation by AMPK results in suppression of mTORC1 kinase activity. Second, AMPK was shown to modulate p53-dependent apoptosis by p53 phosphorylation [6]. Third, several studies indicated that the products of two p53 target genes, sestrin 1 and 2, increased AMPK activity inducing mTOR signaling inhibition [7]. Altogether, these data seemed to indicate a role of AMPK in cell growth arrest and cell death. Accordingly, activation of AMPK by energy depletion would function as a
metabolic check-point, in which normal cells with intact AMPK signalling undergo cell-cycle arrest, driving proliferation only when cell energy is sufficient to guarantee a successful cell division. It was shown that some cancer cells exhibit a defective AMPK activation (e.g., LKB1 deficiency) or alterations in key components of the AMPK pathway (e.g., TSC2 or p53 deficiency), which would uncouple fuel signals from growth signals, allowing tumour cells to divide under abnormal nutrient conditions. AMPK activation was then proposed as a therapeutic approach for cancer [2]. However, very recent reports have shown high pAMPK levels in some tumours and a role of AMPK in tumour cell growth and survival [8, 9]. Therefore, the function of AMPK in the biology of cancer is far from being understood.

The main objective of the present study was to give further insight into the regulatory role of AMPK in cell growth and proliferation in tumours. To accomplish this objective we evaluated the function of AMPK in both mice and human astrocytic tumours. In order to dissect out the mechanisms underlying the effect of AMPK in astrocytoma cell growth we first reproduced different molecular events that lead from tumour initiation to progression in the mouse. For this purpose we used astrocytes expressing HRas\textsuperscript{v12} and/or with \textit{Pten} deletion because a constitutive activation of Raf/MEK/ERK and PI3K/AKT cascades are common alterations in human astrocytomas [10] and both signalling pathways regulate cell metabolism [11-13]. We then confirmed the role of AMPK in human glioblastoma cell lines, as well as in human glioblastoma xenografts in SCID mice. Finally we examined the level of AMPK activation and activity in human glioblastoma tissue samples.

Here we showed that human glioblastomas exhibit high levels of AMPK activity, and we demonstrated that AMPK activation is essential for the proliferation of astrocytic tumour cells by promoting cell cycle progression in both mice and human.
Materials and Methods

Cell culture, transfection and retroviral infection

Cortical astrocytes were obtained from cerebral cortices of 3-day-old PtenloxP/loxP or AMPKαloxP/loxP mice and maintained in culture for 4-6 days. AMPKαloxP/loxP mice were generated as previously described by crossing AMPKα1loxP/loxP and AMPKα2loxP/loxP [14, 15]. The care and use of experimental animals was in accordance with institutional guidelines. Cell were cultured in DMEM supplemented with 10% FBS and to express HRasv12 and/or Cre-recombinase ecotropic retrovirus were used as previously published [16].

U87MG and U373MG human glioma cell lines were obtained from J. Seoane, VHIO, Barcelona in 2011. Both cell lines were periodically authenticated by morphologic inspection and tested negative for Mycoplasma contamination by PCR tests. Cells were cultured in DMEM supplemented with 10% FBS. For RNAi experiments, three different commercial AMPKα (a1, a2) siRNAs (sc-45312A, sc-45312B and sc-45312C) and control siRNA (sc-37007) were obtained from Santa Cruz Biotechnology. Transfection of cell lines with 10 and/or 20nM of siRNAs was performed with jetPRIME (Polyplus transfection) following the manufacturer’s recommendations. Cells were lysed after 48h post-transfection and used for subsequent experiments.

For AMPK activation/inhibition, astrocytes and cell lines were cultured in the presence of 0.2mM AICAR or 10μM compound C for the indicated times.

All treatments were performed in astrocytes cultured for 7 days after the infection in complete medium (DMEM supplemented with 10% FBS) except specifically indicated in the text.
Tumour growth in SCID mice

SCID mice were fed ad libitum and maintained on a 12h light-dark cycle at a controlled temperature. To estimate the effect of compound C in a xenograft tumour model, tumours were induced by subcutaneous (s.c.) injection of 2,5x10^6 U87MG cells, resuspended in 100µL of PBS into the right flank of 6 to 8 weeks old SCID mice (20–25g). When s.c. tumours had reached a detectable size of 30mm^3 mice were randomized into 2 groups: one group was treated with compound C, administered as a single dose of 10 mg/kg (intraperitoneal); the second group received one injection of the vehicle (100µL PBS/DMSO 10%). Mice were monitored daily for tumour size (using a calliper) and health status. Tumour volume was calculated by the formula: ((length^2 x width)/2) until the end of the experiment [17]. When the tumour exceeded 2000mm^3 animals were sacrificed, and this date was used to plot the Kaplan–Meier survival curve.

Growth curves and BrdU incorporation

We determined cell number in an indirect way using crystal violet as it has been previously reported [18]. In order to assess relative cell numbers, cells were grown in 24 multi-well plates and treated in each case as indicated in the text. At 0, 2, 4 and 7 days, cells were washed with PBS, fixed with methanol/acetic acid (3/1) for 20 min, stained with crystal violet for an additional 20 min, washed 5 times with PBS, and air dried. Dye was eluted from cells at room temperature by the addition of 1 mL of 10% acetic acid and absorbances were measured at 595 nm.

DNA replication was determined by detecting BrdU incorporation. Cells grown over 1,7cm^2 coverslips were treated in each case as indicated in the text and then labelled 5 hours with 10µM BrdU for astrocytes and 1 hour for cell lines. Cells were fixed with 75% EtOH, denaturalised 30min in 4N HCl and blocked with 1% BSA. Then, cells were incubated 1 hour at room temperature with anti-BrdU antibody and microscopic
visualization was possible using a Cy3-tagged secondary antibody (Jackson ImmunoResearch). Mounting medium containing DAPI was used and cells were visualized by immunofluorescence.

Patients and tissue sampling

Human glioblastoma and normal human brain tissue samples were obtained from patients of the Neurosurgery Service of the University Hospital of Santiago de Compostela. The study was performed after approval of the Ethics committee of the Xunta de Galicia. A small piece of the tumour and of human brain samples from epilepsy surgery sent to the Pathological Service was immediately frozen and used for western blot. The immunohistochemistry was performed in formalin-fixed paraffin-embedded samples from human glioblastomas and human normal brain from the files of the Pathological Service and Biobank of the University Hospital of Santiago de Compostela, Spain.

Data presentation and statistical analysis

Data represent mean ± SEM. Before all statistical analyses, data were examined for assumptions of normality of variance using the Kolmogorov-Smirnov test. t-test, one-way or two-way ANOVA were used to calculate statistical significance. Survival curves were estimated by the Kaplan-Meier method.
Results

AMPK is activated by oncogenic events

We used a cellular model of mice astrocytoma with astrocytes expressing HRas$^{V12}$ and/or with an activated PI3K/AKT pathway by Pten loss. These signalling pathways are frequently activated in human astrocytomas [13]. Although Ras mutations are not found in human astrocytic tumours, a hyperactivation of FGF and EGF receptors is frequently observed, consequently increasing Ras signalling.

As expected, astrocytes with HRas$^{V12}$ enhanced ERK1/2 phosphorylation, and Pten deletion induced AKT phosphorylation (Supplemental Figure 1A and 1B). Ras pathway activation was related with a high proliferation level, however no significant changes in cell growth rate were observed in Pten-null cells (Figure 1A). Furthermore, we were unable to detect significant levels of apoptosis and senescence in any of the experimental groups, as it has already been shown [16].

Interestingly an increased AMPK activation, determined by AMPK phosphorylation at the residue Thr172 (required for the mammalian AMPK activation), and a higher AMPK activity, assessed by ACC phosphorylation at the residue Ser79 (a well-established AMPK target) was found in astrocytes expressing HRas$^{V12}$ and/or Pten deletion. A greater effect was shown in the cells with oncogenic Ras (Fig. 1B). Total levels of AMPK and ACC were unchanged (Supplemental Figure 1C and 1D). This AMPK activation was likely dependent on the oncogenic events and not due to nutrient depletion caused by a high proliferation rate because phosphorylation levels of both AMPK and ACC were increased similarly in cells kept in culture medium for 7 days, or 24 hours after fresh complete medium was added to the cells (Figure 1C). Furthermore, no differences were found in the level of activation of AMPK in astrocytes with oncogenic Ras or Pten loss cultured with either 5 or 25 mM glucose. (Figure 1D).
AMPK is a mediator of cell proliferation

Previous reports have shown that AMPK activation reduces cell proliferation by stimulation of TSC2, consequently inhibiting mTORC1 pathway and by phosphorylation of p53. However, we show here that astrocytes with oncogenic Ras, with or without AKT activation maintained high proliferation rates in the presence of an elevated AMPK activity. HRasV12-expressing astrocytes and cells without Pten exhibited levels of pmtOR^{Ser2448} similar to those observed in control cells (Supplemental Figure 2A). These results probably reflect that AMPK was counteracting the effect of Ras and AKT on TSC2, inhibiting the anabolic effects of both signalling pathways.

To determine possible roles of oncogenic AMPK activation, we first analysed pAMPK cell localization. Immunofluorescence analysis showed that pAMPK was mainly localized in the cytoplasm of control cells, whereas oncogenic events induced an increase in pAMPK nuclear levels (Figures 2A and 2B). As it has previously been shown that AMPK nuclear localization was related to a higher expression of AMPKβ1 [19], we determined the levels of both AMPKβ1 and AMPKβ2. Interestingly, we found increased AMPKβ1 levels in HRasV12 and Pten^-/- cells and a reduced content of AMPKβ2 in HRasV12 astrocytes (Figures 2C and Supplemental 2C). Furthermore, subcellular fractionation data confirmed a nuclear increase in pAMPK and pRB^{Ser800/804} levels, a recently described AMPK target [19], in these groups of cells (Figure 2D). Nuclear to cytoplasmic ratio (Nuc/cyt) of pAMPK was significantly increased in oncogenic Ras-expressing cells (Supplemental Figure 2D). On the other hand, total AMPKα levels were also increased in the nuclear fraction in the astrocytes with the oncogenic events (Supplemental Figure 2B).

We next investigated whether AMPK activation or inhibition had any effect on the proliferative capacity of our cells. To accomplish this we used AICAR (an adenosine
analog that is easily taken up by cells and which rapidly phosphorylated mimics the activating effects of AMP on AMPK) or compound C (a compound that acts as a potent, selective, reversible, and ATP-competitive inhibitor of AMPK). Accordingly, astrocytes after 6 days in culture were incubated with AICAR or with compound C for 24 hours and bromodeoxyuridine (BrdU) incorporation was evaluated. In agreement with previous reports AICAR induced AMPK activation, determined as higher levels of pACC, but concentrations higher than 0.2 mM reduced normal astrocytes viability (Supplemental Figures 2E and 2F). We treated then the cells with AICAR at a concentration of 0.2mM. Expression of oncogenic Ras induced a significant increase in BrdU incorporation, but treatment with 0.2 mM AICAR for 24 hours did not change the levels of BrdU incorporation exhibited by non-treated cells (Figure 2E). Interestingly, cells treated with compound C at concentrations that allowed cell viability in control astrocytes and previously described to reduce AMPK activation [20-23], (10µM, Supplemental Figure 2G) significantly reduced BrdU incorporation in the cells expressing HRasV12 (Figure 2F). Decreased levels of pRB800/804 accompanied this reduction, while no effect on the phosphorylation of RB was demonstrated after treatment with AICAR (Figure 2G and Supplemental Figure 2H). These results suggest a mechanism by which AMPK regulates proliferation in astrocytoma cells with a hyperactivated Ras pathway.

**AMPK is necessary to maintain cell proliferation in mice astrocytes expressing oncogenic Ras and in human glioblastoma cell lines.**

To further demonstrate the relationship between AMPK, RB and proliferation we used primary cultures of AMPKα-deficient mice astrocytes. We studied the effect of AMPK deletion only in normal and in astrocytes expressing HRasV12 because Pten loss showed a very moderate effect on the parameters evaluated.
Astrocytes from mice with both AMPK catalytic subunits floxed were infected with HRasV12 and/or Cre recombinase. As expected, AMPKα deletion reduced AMPKα levels and AMPK activity, as shown by ACC phosphorylation; the infected astrocytes with HRasV12 enhanced HRas levels (Supplemental Figure 3A).

Cell growth rate, and foci formation in astrocytes expressing HRasV12 were reduced by AMPK deletion, whereas no effect was shown in control cells. (Figure 3A and 3B). Furthermore, growth inhibition in cells with oncogenic Ras expression and AMPK deletion was not further increased when compound C (10 µM) was added to the culture medium for 7 days (Supplemental Figure 3B).

Proliferation, senescence and apoptosis were determined in these cells. BrdU incorporation was determined in cells after 7 days in culture. Astrocytes with HRasV12 and AMPK deletion showed a remarkable reduction in proliferation, with levels of BrdU incorporation within the range of control cells. Curiously, AMPK-null cells continued to proliferate as control astrocytes (Figure 3C). On the other hand, only a very low senescence increase was observed in cells with AMPK deletion and HRasV12 expression (Supplemental Figure 3C), whereas no change in the levels of apoptosis was detected among the different groups (data not shown).

Finally we determined pRBSer800/804 levels in the four experimental groups. In agreement with our results with compound C, we found a reduction in pRBSer800/804 in AMPK-deficient cells, in parallel to a decrease in ACC phosphorylation (Figure 3D). This might explain the reduced rate of proliferation observed in AMPK-deleted astrocytes, and confirm the important role of AMPK in maintaining the proliferation induced by HRasV12.

Due to the importance of AMPK in the proliferation of mice astrocytes expressing HRasV12 and/or Pten deletion, a plausible role of this kinase in human glioblastoma cells growth and proliferation was also investigated. We used U87MG and U373MG cell
lines, as previous reports had shown that these astrocytoma/glioblastoma cell lines did not have mutations in the RB gene [24, 25]. We first inhibited AMPK activity with compound C (10µM, 7 days). Interestingly, this treatment blocked cell growth even in the U87MG cell line, which exhibits a more aggressive phenotype than the U373MG (Figure 3E-G). Consistent with our previous results in mice astrocytes, compound C significantly reduced BrdU incorporation in both glioblastoma cell lines (Figure 3H), with no effect on senescence or apoptosis (data not shown). Moreover, we found lower levels of RB phosphorylation in Ser\textsuperscript{807/811} (corresponding to Ser\textsuperscript{800/804} in mouse), in parallel to a pACC-reduced content in the cells treated with compound C (Figure 3I). This effect of compound C suggested a similar role of AMPK on cell cycle regulation in mice astrocytes expressing HRas\textsuperscript{V12} and/or Pten loss and in human glioblastoma cell lines.

To firmly establish a direct relationship between AMPK inhibition, RB phosphorylation and cell growth in these cells lines, we knocked down AMPK with siRNAs targeting the catalytic subunits of AMPK (α1 and α2). We studied the efficacy of three different siRNAs in reducing AMPK\textsubscript{α} levels and activity, by transfecting U87MG glioblastoma cells with the siRNAs (20 nM, 48 hours), separately or in combinations. All siRNAs tested reduced AMPK levels and activity, as determined by pACC levels, although with different degrees of efficiency. The combination of the 3 pooled siRNAs reduced AMPK\textsubscript{α} and pACC levels further (Supplemental Figure 3D and E). Likewise, individual and pooled siRNAs reduced pRB\textsuperscript{807/811} levels and BrdU incorporation. The decrease was greater with the 3 siRNAs together (Supplemental Figure 3D, E and F). Finally, we transfected U87MG and U373MG cells with the 3 pooled AMPK siRNAs (10 and 20nM; 48 hours), and we demonstrated a significant reduction in BrdU incorporation and pRB\textsuperscript{807/811} levels in both cell lines (Figure 3J and 3K).
All these data demonstrated the relevance of AMPK in human glioblastoma cell proliferation.

**AMPK inhibition decreases the in vivo growth of xenografts of human glioblastoma cell lines.**

We have shown that AMPK inhibition reduced cell growth in human and murine astrocytic tumour models *in vitro*. However, stress conditions characteristic of a solid tumour microenvironment (hypoxia and metabolic stress) might affect the role of AMPK. To determine the effect of compound C on the growth of human glioblastoma cells *in vivo*, we injected U87MG tumour cells, subcutaneously, in immunodeficient SCID mice. We chose these cells because they are very tumorigenic *in vivo* in contrast to U373MG cells, which has been shown not to develop tumours in animals [26]. Consistent with our *in vitro* findings, one single dose of compound C (10 mg/Kg, i.p) was able to reduce significantly the growth rate of U87MG tumours (Figure 4A) and this treatment increased the survival of tumour-bearing mice (Figure 4B). We were unable to detect a higher effect when we treated the mice with several doses of compound C (Supplemental Figure 4), which suggests that AMPK inhibition with compound C might produce a selection against growth of tumor cells with this pathway activation. In any case, our data reflect the important role of AMPK during *in vivo* glioblastoma tumour progression.

To further analyse the molecular mechanisms underlying this effect of AMPK we determined apoptosis as well as cell proliferation levels in the tumour samples. Immunohistochemistry of cleaved caspase-3 did not show significant changes between tumours in animals with or without treatment (Figure 4C). However, tumours in mice treated with compound C showed a reduction in BrdU incorporation and in the levels of p-histone H3 (Figure 4C and 4D), demonstrating once again the relevant effect of
AMPK in the control of cell proliferation. Finally we studied the plausible AMPK-pRB signalling pathway in the tumours of animals with and without compound C treatment. Interestingly, RB phosphorylation was decreased in parallel with a reduced AMPK activity (estimated by pACC Ser79 levels) in U87MG tumours treated with the AMPK antagonist (Figure 4E). These results further illustrate that AMPK inhibition reduces in vivo tumorigenesis.

**AMPK is activated in human glioblastomas**

To determine whether AMPK is activated in human glioblastomas, we performed IHC to detect pACCSer79 on tissue samples from glioblastomas of 10 patients (Table 1 and Figure 5A). Negative controls consisting of omission of primary antibody or substitution of primary antibody by normal rabbit serum (NRS) were performed (Supplemental Figure 5). High levels of pACC^Ser79 were observed in 100% of the tumours evaluated (Table 1). Normal brain tissue was either completely negative or showed only a weak staining for pACC^Ser79 (see representative photographs in Figure 5A). In contrast, all glioblastoma tissue samples showed a heterogeneous pattern of positive cells. The immunoreactivity was found predominantly, if not entirely, in the cytoplasm. The highest number of pACC^Ser79 immunoreactive cells was found in areas surrounding the blood vessels. We also determined Ki67 immunoreactivity as a proliferation index and we found a good correlation between Ki67 and pACC^Ser79 immunoreactive levels, especially around the blood vessels (Table 1 and Figure 5A). Very interestingly, high levels of pERK^Thr202/Tyr204 were found in all the tumours evaluated in this study, especially in perivascular regions and areas with high proliferative rate (Table 1 and Figure 5A). This localization of pACC cells demonstrates that, in human glioblastomas, oncogenic events but not hypoxia, energy depletion or low nutrients availability are responsible for AMPK activation. Finally, to further
determine the relationship between AMPK activation and proliferation in human glioblastomas, we determined pAMPK, pACC and pRB<sup>807/811</sup> by western blot in 4 tissue samples of human glioblastomas and human normal brain. We found higher levels of pAMPK<sup>Thr172</sup> and pACC<sup>Ser79</sup> in the 4 tumours in relation with normal tissues, in agreement with the results of immunohistochemistry (Figure 5B). Furthermore and consistent with our previous results in mice astrocytes and in human glioblastoma cell lines, AMPK activation in the human glioblastoma tissue samples studied here was accompanied by high levels of pRB<sup>807/811</sup>

In conclusion, our results demonstrate that AMPK has an important role in tumour growth, suggesting that an AMPK antagonist might potentially be used as a treatment in astrocytic tumours.
Discussion

While in various cancer cells a loss of appropriate AMPK signalling has been demonstrated and AMPK activation has been proposed as a therapeutic approach to reduce cancer cell growth [2], in some others an activation of AMPK has been found, and a role of AMPK as a prosurvival factor in tumour cells has been described [9, 27]. In the present study we evaluated the function and mechanism of action of AMPK in cell growth and proliferation in murine and human astrocytic tumours.

The novel and striking result of this study was the finding of AMPK as inductor of proliferation in these tumour cells. Specifically, we show here that oncogenic Ras and Pten deletion activates AMPK, and that this activation stimulates cell proliferation. Furthermore, human glioblastoma exhibits elevated levels of activated AMPK and inhibition of AMPK in astrocytes expressing HRas\textsuperscript{V12} or in human glioblastoma cells significantly reduces cell growth.

A recent report has shown high pAMPK levels in rat gliomas [8], and it was demonstrated that AMPK inhibition in prostate cancer cells or in MEF transformed by oncogenic Ras and SV40 large T-antigen reduced tumour growth [9, 28]. Nevertheless, in these reports it was hypothesized that AMPK activation was a consequence of hypoxic or ischemic microenvironments. Here, we found that AMPK was highly activated in astrocytes expressing oncogenic Ras and Pten deletion not only after 7 days in culture, but also 24 hours after fresh culture medium was added to the cells, or in the presence of high concentrations of glucose (25 mM), indicating that oncogenic signalling pathways, but not hypoxia or glucose depletion, were responsible for this activation. Moreover, the localization of cells with high levels of AMPK activity (determined as levels of pACC) in human glioblastomas, surrounding blood vessels in areas with a high Ki67 proliferative index, and high levels of pERK gives further support to this idea. In fact, it has been reported that AMPK activity may also be
elevated under non-stressed conditions, for example by hormones like leptin, adiponectin and interleukin-6 [29]. Curiously enough these adipokines have been implicated in the development and progression of some human cancers [30].

The activation of AMPK observed in astrocytes with oncogenic events and in the human glioblastomas probably indicates that AMPK provides metabolic support by directing cells to generate ATP to maintain energy homeostasis in these demanding conditions of high rate division. However, as our results suggest, the role of AMPK in cancer cells is not restricted to regulation of ATP levels, but is also involved in cell proliferation.

AMPK can be found both in the nucleus and the cytoplasm [31], and in mammalian cells, leptin [32], heat shock, and different types of stress [33, 34] have been reported to regulate the AMPK nuclear translocation. In this work we show that nuclear localization of pAMPK was increased by HRasV12 expression or Pten loss. Previous data have related AMPKβ1 to nuclear localization of AMPK [19]. In our hands astrocytes with the oncogenic events exhibited higher levels of the AMPKβ1 subunits and a reduced content of the AMPKβ2, which reinforces the hypothesis of an important function of AMPK at the nuclear level in these cells. The increase of the nuclear localization of pAMPK would enhance the phosphorylation of its nuclear targets. Interestingly, it was recently demonstrated that AMPK is an important kinase of RB, specifically at Ser800/804, and that this phosphorylation has a key role during brain development by cell cycle regulation [19]. Our data showed that the increase of nuclear pAMPK by HRasV12 expression and/or Pten deletion was accompanied by higher levels of pRBSer800/804, and this correlation was also found in the human glioblastoma cells lines. Furthermore, AMPK inhibition in mice astrocytes with compound C or AMPK deletion reduced significantly HRasV12–induced cell growth by decreasing BrdU incorporation and pRBSer800/804 levels. This fundamental role of AMPK in regulating cell growth was also
demonstrated in U87MG and U373MG cell lines by blocking the expression of AMPK with siRNA or inhibiting AMPK activity by using compound C.

Finally, we could demonstrate that a single i.p. dose of compound C significantly decreased tumour growth of U87MG xenografts, increasing the survival of the tumour-bearing mice. Once more when we studied the molecular mechanisms underlying this inhibitory effect we found reduced levels of pRBSer807/811 and proliferation.

The evidence presented here that AMPK activation and activity is induced early and remains elevated in human glioblastoma contributes to our understanding of the role of AMPK in astroglial tumour development, a type of brain tumours with a very poor survival rate. A better knowledge of tumour biology is essential for more specific targeting and the development of pharmacological methods of treatment.

In conclusion, although it has been demonstrated that AMPK acts as a survival factor protecting cells from hypoxia and nutrient deprivation, limiting ATP utilization in these situations [28, 35-38], AMPK is not required for growth in normal cells with abundance of nutrients. By contrast in oncogenic situations (e.g. in the presence of oncogenic Ras signalling) AMPK activation is essential for cell growth and proliferation, by regulating RB phosphorylation and therefore cell cycle progression. By putting all these data together, we propose that the use of AMPK inhibitors might be a good therapeutic approach in tumour cells with high levels of pAMPK.
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Author contributions

RS and JAC conceived the study. RS, JAC and MR designed the experiments. RS, JAC, BV, ML and MR designed the AMPK<sub>loxP/loxP</sub> experiments. AP and MF selected the patients, obtained the human tissue and tumour samples and performed the pathological study and the immunohistochemistry of the tumour tissue samples. MR performed all experiments and analysis. RS, JAC, BV, ML and MR discussed and interpreted the data. RS, JAC, BV, ML and MR generated all figures and wrote the paper. RS and JAC, equally contributing authors.
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Table legends

Table 1. Human glioblastomas show high levels of AMPK activity and pRB Ser807/811 levels. Human glioblastoma tissue samples and human normal brain tissue analyzed by immunohistochemistry (IHC) or Western blot (WB). High levels (+) and low levels (-) of immunoreactivity. N, number of tissue samples from different patients evaluated. n/a, not available.

Figure legends

Figure 1. AMPK is activated by HRasV12 expression and Pten deletion in astrocytes. Neonatal mice astrocytes were infected with retroviral vectors encoding HRasV12 and/or Cre recombinase, generating four different genotypes: PtenloxP/loxP, PtenloxP/loxPHRasV12, Pten-/- and Pten-/-HRasV12. Cells were maintained in culture for 7 days. Data were represented as mean ± SEM from at least 3 independent experiments. (A) Relative cell number determined the 7th in culture by crystal violet. (* p<0.001 vs PtenloxP/loxP). (B) AMPK activation was examined by immunoblot analysis of pAMPK Thr172 and pACC Ser79. α-Tubulin was used as a loading control. (C) pAMPK Thr172, pACC Ser79 and α-Tubulin levels when cells were cultured during 7 days or when new medium was added the 6th day in culture, 24 hours before protein extraction. Western blot analysis (D) pACC Ser79 levels by western blot in astrocytes cultured in the presence of 5.5 mM or 25 mM D-glucose.

Figure 2. HRASV12 expression induces nuclear pAMPK localization and subsequently RB phosphorylation. Astrocytes of the four different genotypes: PtenloxP/loxP, PtenloxP/loxPHRasV12, Pten-/- and Pten-/-HRasV12 were maintained in culture for 7 days. Data were represented as mean ± SEM from at least 3 independent experiments. (A) Representative confocal microphotographs of pAMPK Thr172 immunofluorescence showing its nuclear and cytoplasmatic localization. Scale bar represents 100µm. (B) Nuclear and cytoplasmatic fluorescence was quantified for each cell and the percentage of cells with a ratio of nucleus/cytoplasm pAMPK Thr172 fluorescence >2.5 was represented. (* p<0.05 vs PtenloxP/loxP). (C) AMPKβ1, AMPKβ2 and α-Tubulin levels were determined by immunoblotting. (D) Cytoplasmic and nuclear extracts were used to determine pAMPK Thr172 and pRB Ser800/804. Levels of α-Tubulin and β-Lamina were used as control. (E and F) Cells were treated with 0.2mM AICAR or 10µM compound C for 24 hours the 7th day in culture. (E) Percentage of BrdU positive astrocytes. (F) pACC Ser79, pRB Ser800/804 and α-Tubulin levels determined by immunoblotting.

Figure 3. AMPK deletion, inhibition, or knocked-down reduces growth in astrocytes-expressing HRASV12 and human glioblastoma cell lines. Astrocytes of four different genotypes: AMPKloxP/loxP, AMPKloxP/loxPHRasV12, AMPK-/- and AMPK-/-HRasV12 were maintained in culture for 7 days. Data were represented as mean ± SEM from at least 3 independent experiments. (A) Representative microphotographs of cells stained with crystal violet. Scale bar represents 100µm. (B) Growth curve showing relative cell number of the four experimental groups. (C) Percentage of BrdU positive astrocytes (* p<0.001 vs AMPKloxP/loxP). (D) Levels of pAMPK Thr172, pACC Ser79, pRB Ser800/804 and α-Tubulin levels by western blot. U87MG and U373MG cells were treated with or without 10µM compound C for 7 days. (E) Representative microphotographs of crystal violet staining. (F, G) Growth curve
showing relative cell number. U87MG and U373MG cells were treated with 10µM compound C for 24 hours. (H) Percentage of BrdU positive cells. (I) pACC<sup>Ser79</sup>, pRB<sup>Ser800/804</sup> and α-Tubulin levels by immunoblotting. U87MG and U373MG cells were transfected with 10-20nM of scrambled or AMPKα siRNA (48 hours). (J) Percentage of BrdU positive cells. (K) pAMPK<sup>Thr172</sup>, pACC<sup>Ser79</sup>, pRB<sup>Ser807/811</sup> and α-Tubulin were determined by western blot. (* p<0.05 vs control conditions).

**Figure 4. AMPK inhibition reduces tumour growth ratio in a xenograft model.** U87MG cells were injected s.c. in SCID mice and when the tumours achieved 30mm<sup>3</sup> in size, mice were treated i.p. with 10 mg/kg compound C or vehicle. (A) Tumour volume in mice treated with vehicle (n=6) or compound C (n=7). (B) Kaplan–Meier curves of mice treated with vehicle (n=6) or compound C (n=7). (* p<0.0012 vs animals treated with vehicle). (C) Representative microphotographs of immunohistochemistry showing BrdU incorporation, pHistone H3<sup>Ser10</sup> and Cleavaged-Capase3 in tumours 8 days after vehicle or compound C treatment. Scale bar represents 100µm. (D) Percentage of BrdU positive cells in U87MG tumours 8 days after vehicle or compound C treatment. (* p<0.0001 vs non treated tumours). (E) ACC<sup>Ser79</sup>, pRB<sup>Ser807/811</sup> and α-Tubulin levels were determined by western blot in U87MG tumours 8 days after vehicle or compound C treatment.

**Figure 5. AMPK activity is increased in human glioblastomas especially in areas of high proliferation.** (A) Representative microphotographs of normal human brain and human glioblastoma tissue samples stained with hematoxylin and eosin (H&E) or processed by IHC to detect pACC<sup>Ser79</sup>, Ki67 and pERK<sup>Thr202/Tyr204</sup>. Scale bars 100 µm. (B) Lysates of normal brain and glioblastoma tissue samples were used to determine pAMPK<sup>Thr172</sup>, pACC<sup>Ser79</sup>, pRB<sup>Ser807/811</sup> and α-Tubulin levels by immunoblotting.
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Table 1
Figure 2
Figure 4
Figure 5
AMPK activation by oncogenesis is required to maintain cancer cell proliferation in astrocytic tumors

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