Supplemental materials and methods

Antibodies and reagents

ACC (#3676), AMPK α1/2 (#2532), AMPKβ1/2 (#4150), Phospho-4E-BP1<sup>Thr37/46</sup> (#9459), pAKT<sup>Ser473</sup> (#4051), pAMPK<sup>Thr172</sup> (#2535), pACC<sup>Ser79</sup> (#3661), pERK<sup>Thr202/Tyr204</sup> (#4370), Phospho-Histone H3<sup>Ser10</sup> (#9701), Ki-67 Clon MIB-1 (Dako) Phospho-mTOR<sup>Ser2448</sup> (#2971), pRb<sup>Ser807/811</sup> (#9308), Phospho-TSC2<sup>Ser939</sup> (#3615) from Cell Signaling. Ki-67 Clon MIB-1 from Dako. Anti BrdU (B2531), α–Tubulin (T5168) from Sigma. Lamin B (sc-6216) from Santa Cruz. Pten (#RB-072-P0) from NeoMarkers. Anti-Pan-Ras (OP40) from Oncogene. AICAR and Compound C were obtained from Calbiochem.

pACC antibodies may recognize the ACC1 phosphorylation site (Ser79) or equivalent phosphorylation site on ACC2 (Ser212, mouse), which is also an AMPK target. Here we designate both phosphorylated forms of mouse as pACC.

Apoptosis and senescence

Apoptotic cell death was assessed by fluorescent microscopic analysis of cell DNA staining patterns with Hoechst-33258 (Sigma). Cells were incubated with 20 μg/ml of Hoechst-33258 for 1 hour at 37 °C and examined under a fluorescence microscope. SA-β-galactosidase activity was assessed with the Senescence β-Galactosidase Staining Kit (Cell Signaling).

Western Blot Analysis and Subcellular Fractionation

Cells were rinsed in ice-cold PBS then scraped and lysed with lysis buffer (50mM Tris-HCL, pH7.5, 150mM NaCl, 1mM EGTA, 1mM EDTA, 1% TritonX-100, 5mM sodium
pyrophosphate, 1mM Na$_3$VO$_4$, 50mM NaF, 0,27M sucrose and protease inhibitor (Roche)). To obtain cell lysates from xenograft samples, human normal brain and human glioblastomas, pieces of the tissues were lysed with RIPA buffer containing protease inhibitor (Roche). Supernatants were collected by centrifugation at 14,000rpm, 20min, at 4°C. Protein content was quantified, and different protein levels were analysed by standard immunoblot analysis.

To isolate cytoplasmic and nuclear fractions, cells were incubated in lysis buffer [20mM HEPES, pH 7.9, 10mM NaCl, 1mM MgCl$_2$, 0,5M Sucrose, 0,2mM EDTA, 0,5mM DTT, 0,35% Triton X-100 1mM Na$_3$VO$_4$, 50mM NaF and a mixture of protease inhibitors (Roche)] for 10 min in ice. Cells were centrifuged 10 min at 800g and supernatant was frozen (cytoplasmic extract) at -20ºC. Pellets were resuspended in nuclear buffer [10mM HEPES, pH 7.4, 10mM KCl, 2mM MgCl$_2$, 1mM DTT, 1mM Na$_3$VO$_4$, 50mM NaF and a mixture of protease inhibitors (Roche)], centrifuged and resuspended in lysis buffer. Nuclear extracts were exposed to 3 cycles of frost/defrost. After 15min centrifugation at 13,000g, nuclear proteins were obtained in the supernatant. α-Tubulin was used as a marker of cytoplasm and β-Lamin of nucleus.

**Immunofluorescence and Immunohistochemistry**

Infected astrocytes were fixed with 4% paraformaldehyde, blocked with 1% BSA PBS containing 0.1% Triton-X100 and incubated with primary antibodies (pAMPK$^{Thr172}$ pRb$^{Ser807/811}$) at 4°C overnight. Microscopic visualization was obtained using appropriate Cy3-tagged (Jackson ImmunoResearch) secondary antibodies. Nuclei were counter-stained with Hoechst. For quantitative analysis of nuclear/cytoplasm pAMPK ratios nuclear and cytoplasm area was demarcated using the NIH Image J software in five independent microphotographs. Pixels occupying the nucleus or cytoplasm were
quantified and represented as percentage of OD nucleus/OD cytoplasm &gt; 2.5.

Tumour samples were obtained from U87MG xenografts 8 days after treatment with compound C. One hour before tumour dissection, mice were treated with BrdU 0.1 µg/gram. Samples were fixed immediately after removal in 10% buffered formalin solution for a maximum of 24 hours at room temperature before being dehydrated and paraffin embedded. Representative tumour areas, away from necrotic foci, were identified on a hematoxylin-eosin-stained section. Apoptosis, DNA replication and mitosis were visualized by using Cleavaged-Capase3, BrdU and phospho-Histone H3Ser10 antibodies, respectively.

Formalin-fixed paraffin-embedded samples from human glioblastomas (grade IV) and human normal brain were retrieved from the files of the Pathological Service and Biobank of the University Hospital of Santiago de (CHUS), Spain. Sections, 4 mm-thick, were mounted on FLEX IHC microscope slides (Dako, Glostrup, Denmark) and heated in an oven at 60°C for 1 hour. The immunohistochemical technique was automatically performed using an AutostainerLink 48 (Dako). After deparaffination and epitope retrieval for 20 min at 97°C (in Tris-EDTA pH 9 for pACCSer79 antibody immunostaining and in EnVision FLEX target retrieval solution (low pH) for pERK and Ki67 immunostaining), the slides were allowed to cool in PT Link to 65°C and then in Dako wash buffer for 5 min at room temperature. The immunostaining protocol includes incubation in: (1) EnVision FLEX peroxidase-blocking reagent (Dako) for 5 min; (2) pACCSer79 at a dilution of 1/200, pERK at a dilution of 1/400 and FLEX Anti-Human Ki-67, all for 30 min; (3) EnVision FLEX/HRP (dextran polymer conjugated with horseradish peroxidase and affinity-isolated goat anti-mouse and anti-rabbit immunoglobulins) for 20 min; (4) substrate working solution (mix) (3,3’-diaminobenzidine tetrahydrochloride chromogen solution) (Dako) for 10 min and (5)
EnVision FLEX hematoxylin (Dako) for 9 min. Negative controls in which the primary antibodies were omitted were included in each staining run. For pACC$\text{Ser}^{79}$ immunostaining, additional negative controls consisting of substitution of the primary antibody by normal rabbit serum (NRS) at the same concentration were included in parallel (Supplemental Figure 5).

**Supplemental figure legends**

**Supplemental Figure 1. Characterization of astrocytes with HRAS$^{V12}$ expression and/or Pten deletion.** Cells were maintained in culture for 7 days. Data were represented as mean ± SEM from at least 3 independent experiments. (A) Immunoblot analysis of whole cell lysates showing PTEN loss, HRAS$^{V12}$ expression and intracellular pathway activation using antibodies against PTEN, pAkt and α-Tubulin, or (B) H-Ras$^{V12}$, pERK1/2 and α-Tubulin. (C) Western blot showing AMPKα and α-Tubulin or (D) ACC and α-Tubulin levels.

**Supplemental Figure 2. AMPK maintains low levels of mTOR pathway activation and induces pRb phosphorylation.** Cells were maintained in culture for 7 days. Data were represented as mean ± SEM from at least 3 independent experiments. (A) Western blot showing pmTOR$^{\text{Ser2448}}$ levels. α-Tubulin was used as control loading. (B) Western blot showing total levels of AMPK in cytoplasm and nucleus. (C) Densitometry of western blots of five independents experiments. AMPK β1 and β2 levels were normalized by α-Tubulin. * p<0.05. (D) Densitometry of western blots of three independents experiments. pAMPK levels were normalized by α-Tubulin in cytoplasm and β-Lamina in nucleus and represented as a percentage of nucleus to cytoplasm (Nuc/cyt) ratio. (E) Pten$^{\text{loxP/loxP}}$ cells were maintained 24 hours with AICAR at the concentrations indicated and relative cell number determined by crystal violet was represented. (F) Western blot showing pACC$^{\text{Ser79}}$ and α-Tubulin levels in cells treated with vehicle or in the presence of AICAR (0.2 mM) for 24 hours or 7 days. (G) Pten$^{\text{loxP/loxP}}$ cells were maintained 24 hours with compound C at the concentrations indicated and relative cell number determined by crystal violet was represented. (H) Representative microphotographs showing pRb$^{800/804}$ immunoreactivity in cells treated with vehicle, in the presence of AICAR (0.2 mM) or compound C (10µM) for 24 hours. Scale bar represents 100µm.

**Supplemental Figure 3. Characterization of AMPK role in proliferation of astrocytes with HRAS$^{V12}$ expression.** Astrocytes of four different genotypes: AMPK$^{\text{loxP/loxP}}$, AMPK$^{\text{loxP/loxP}}$HRas$^{V12}$, AMPK$^{-/-}$ and AMPK$^{-/-}$HRas$^{V12}$ were maintained in culture for 7 days. Data were represented as mean ± SEM from at least 3 independent experiments. (A) Western blot showing AMPKα, pACC$^{\text{Ser79}}$, HRAS and α-Tubulin levels. (B) Relative cell number of cells treated with vehicle or compound C 10µM
determined by crystal violet. (C) Percentage of cell senescence quantified by β-galactosidase activity.
U87MG cells were transfected with 20nM of scrambled or AMPKα siRNA separately (siRNA1, 2 or 3) and with different combinations (siRNA 1-2, 1-3, 2-3 or 1-2-3), and analysed after 48 hours. siRNA1 is sc-45312A, siRNA2 is sc-45312B and siRNA3 is sc-45312C. (D) Densitometry of western blots. AMPKα, pACC^{Ser79} and pRb^{Ser807/811} levels were normalized by α-Tubulin. (E) Western blot showing AMPKα, pACC^{Ser79}, pRb^{Ser807/811} and α-Tubulin. (F) Percentage of BrdU positive cells.

**Supplemental 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 30mm³ in size, mice were treated i.p. with 10 mg/kg compound C or vehicle. Arrows indicate the days when mice were treated: 2 injections in (A) or 3 injections in (B).

**Supplemental Figure 5. Human glioblastomas present high levels of pACC.** Representative microphotographs of negative controls of pACC: omission of primary antibody and substitution of primary antibody by normal rabbit serum (NRS) in a human glioblastoma sample determined by IHC. Scale bars represent 100 µm.