AMPK and GCN2–ATF4 signal the repression of mitochondria in colon cancer cells

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INTRODUCTION

Mitochondria play a central role in the homeostasis of higher eukaryotic cells. The provision of metabolic energy by oxidative phosphorylation, the execution of cell death and intracellular signalling by Ca2+ and ROS (reactive oxygen species) are the main cellular functions of the organelle. A growing number of human diseases, including cancer, are associated with the molecular and/or functional alteration of mitochondria [1,2]. The biogenesis of mitochondria is a complex genetic programme that requires the concerted transcriptional response of nuclear and mitochondrial genes [3]. However, mechanisms that control the localization and translation of mRNAs also contribute to define the mitochondrial phenotype of the cell, offering the biogenesis of the H+–ATP synthase a paradigm in this regard [4]. Indeed, a master regulator in the provision of metabolic energy by oxidative phosphorylation is the mitochondrial H+–ATP synthase, a rotary engine that utilizes the proton electrochemical gradient generated by the respiratory chain as a driving force for the phosphorylation of ADP [5].

Cancer is a complex genetic disease in which the tumour microenvironment plays a fundamental role for disease progression [6,7]. A hallmark of cancer cells and tumours is their peculiar energy metabolism [1,8]. Carcinomas display increased glucose consumption rates due to a low activity of oxidative phosphorylation, concurrently showing higher rates of lactate production when compared with normal tissues [9]. It has been consistently reported that the relative expression of β-F1-ATPase (β catalytic subunit of the mitochondrial H+–ATP synthase), which is the catalytic subunit of the H+–ATP synthase, is significantly diminished in tumours when compared with its expression in normal tissues [1,10]. In a large number of different carcinomas, the down-regulation of β-F1-ATPase is accompanied by an increased expression of markers of the glycolytic pathway [1,10]. This proteomic feature of cancer defines a ‘bioenergetic signature’ of clinical relevance as an indicator of disease progression in colon, lung and breast cancer patients (for reviews see [1,4]) as well as a predictive marker of the cellular response to chemotherapy [1,11,12]. Remarkably, recent findings have demonstrated that the activity of the H+–ATP synthase itself is inhibited in human carcinomas by up-regulation of its physiological inhibitor FI1, which is a relevant regulator of energetic metabolism in cancer cells [13]. The expression of β-F1-ATPase in developing liver [14,15], brown adipose tissue [16], during progression through the cell cycle [17], and in rat [18] and human [19] carcinomas is elevated at the level of translation and mediated largely by the 3′UTR (untranslated region) of the mRNA [14,18,19]. Similar findings have been obtained in yeast, where deletion of the 3′UTR leads to reduced ATP synthesis and respiratory dysfunction [20]. In addition, oxidative phosphorylation is mimicked by the activation of AMPK and the silencing of ATF4 (activating transcription factor 4). These findings emphasize the relevance of translational control for normal mitochondrial function and for the progression of cancer. Moreover, they demonstrate that glycolysis and oxidative phosphorylation are controlled at different levels of gene expression, offering the cell a mechanistic safeguard strategy for metabolic adaptation under stressful conditions.

Key words: ATP synthase, cancer, cellular stress, glycolysis, mitochondrion, translational control.

Reprogramming of energetic metabolism is a phenotypic trait of cancer in which mitochondrial dysfunction represents a key event in tumour progression. In the present study, we show that the acquisition of the tumour-promoting phenotype in colon cancer HCT116 cells treated with oligomycin to inhibit ATP synthase is exerted by repression of the synthesis of nuclear-encoded mitochondrial proteins in a process that is regulated at the level of translation. Remarkably, the synthesis of glycolytic proteins is not affected in this situation. Changes in translational control of mitochondrial proteins are signalled by the activation of AMPK (AMP-activated protein kinase) and the GCN2 (general control non-derepressible 2) kinase, leading also to the activation of autophagy. Changes in the bioenergetic function of mitochondria are mimicked by the activation of AMPK and the silencing of ATF4 (activating transcription factor 4). These findings emphasize the relevance of translational control for normal mitochondrial function and for the progression of cancer. Moreover, they demonstrate that glycolysis and oxidative phosphorylation are controlled at different levels of gene expression, offering the cell a mechanistic safeguard strategy for metabolic adaptation under stressful conditions.

Abbreviations used: a.u., arbitrary units; ACC, acetyl-CoA carboxylase; AICAR, 5-amino-4-imidazolecarboxamide 1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; ATPase, ATP synthase; ATF, activating transcription factor; BRR, basal respiratory rate; COX, cytochrome c oxidase; eIF, eukaryotic initiation factor; DNP, 2,4-dinitrophenol; DNP, 2,4-dinitrophenol; eIF4E, eukaryotic translation initiation factor 4E; eIF2α, eukaryotic translation initiation factor 2α; eIF4E, eukaryotic translation initiation factor 4E; eIF4G, eukaryotic translation initiation factor 4G; eIF4F, eukaryotic translation initiation factor 4F; eIF4A, eukaryotic translation initiation factor 4A; eIF3, eukaryotic translation initiation factor 3; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation initiation factor 2α; eIF2α, eukaryotic translation init
RNABPs (RNA-binding proteins) of β-F1-ATPase mRNA (β-mRNA) have been shown to exert a relevant role in the control of translation of the mRNA both in development [14] and in carcinogenesis [18,21].

Down-regulation of β-F1-ATPase and the suppression of the bioenergetic activity of mitochondria is a required event to promote in vivo tumour progression in colon cancer [22]. In fact, we have recently demonstrated [22] that tumour xenografts derived from three isogenic HCT116 colon cancer cell lines differing in the expression level of β-F1-ATPase only develop when cells with the highly glycolytic phenotype are selected, i.e. tumour development requires the selection of cancer cells with a repressed biogenesis and functional activity of mitochondria. In other words, cancer cells with a functional activity of mitochondria are unable to develop tumours [22]. Importantly, such tumours displayed the transcriptomic, proteomic and mitochondrial ultrastructure of the cells treated with OL (oligomycin) [22]. These findings strongly emphasized the relevance of the cancer cell microenvironment for tumour progression, further suggesting that cells treated with OL [22] could provide a model to unveil the mechanisms involved in the acquisition of the malignant phenotype. In the present study, we have addressed the mechanisms and signalling pathways that control the down-regulation of β-F1-ATPase in colon cancer cells treated with OL with the purpose of advancing the knowledge of the phenotype that is competent for tumour promotion [22]. The results have uncovered that glycolysis and oxidative phosphorylation are regulated by different mechanisms of gene expression. Moreover, the results highlight the relevance of translational control mediated by the activation of the stress kinases AMPK (AMP-activated protein kinase) [23] and GCN2 [general control non-derepressible 2, also called eIF2α (eukaryotic initiation factor 2α) kinase] [24,25] to specifically repress the translation of β-mRNA and the promotion of the abnormal bioenergetics of mitochondria in the cancer cell. Overall, we stress that the activation of AMPK and GCN2–ATF4 (activating transcription factor 4) marks the onset of colon cancer progression.

**EXPERIMENTAL**

**Cellular treatments and siRNA (small interfering RNA) silencing**

Human colorectal carcinoma HCT116 cells were grown at 37°C and 7% CO2 in McCoy’s 5A medium (Sigma) supplemented with 10% (v/v) fetal bovine serum. Cells were left untreated (M-type cells) or treated with 6 μM OL for 48 h (G-type cells) [22]. Where indicated the cells were incubated with 0.1 mM AICAR (5-transfection and cells were analysed at 48 h post-transfection for as a positive control. The medium was changed at 24 h post-transfection. The siRNA Qiagen protein (1:500), anti-p53 (1:200), anti-HIF (hypoxia-inducible factor) 1α (1:150), anti-PGC1α (peroxisome-proliferator-activated receptor γ co-activator 1α) (1:250), anti-p70S6K (ribosomal protein S6 kinase, 70 kDa, polypeptide 1) (1:1000), anti-HuR [ELAV (embryonic lethal abnormal vision)-like protein] (1:200), anti-eIF2α (1:1000) and anti-ATF4 (1:500) from Santa Cruz Biotechnology; anti-Akt (1:1000), anti-p- (phospho-) Akt (1:1000), anti-AMPK (1:1000), anti-p-AMPK (1:1000), anti-p38MAPK (mitogen-activated protein kinase 14) (1:1000), anti-mTOR (mammalian target of rapamycin) (1:1000), anti-p-mTOR (1:1000), anti-4EBP1 (eIF4E-binding protein 1) (1:1000), anti-p-4EBP1 (1:1000), anti-p-p70S6K (1:1000), anti-p-GRK2 (G-protein-coupled receptor kinase 2) (1:5000) and anti-LC3 (microtubule-associated protein 1 light chain 3ε) (1:5000) from Sigma; anti-β-catenin (1:500) from BD Biosciences; anti-GRK2 (G-protein-coupled receptor kinase 2) (1:500), anti-p53 (1:200), anti-HIF (hypoxia-inducible factor) 1α (1:150), anti-p62 (sequestosome 1) (1:1000) from Enzo Life Sciences. Secondary goat anti-rabbit, rabbit anti-goat and rabbit anti-mouse antibodies (1:5000) were used for detection using a mouse antibodies (1:5000) were used for detection using a chemiluminescence detection method [ECL (enhanced chemiluminescence), Invitrogen]. Membranes were exposed to X-ray films. Quantification of the immunoreactive bands [a.u. (arbitrary units)] was accomplished using a Kodak DC120 Zoom digital camera and the Kodak 1D Image Analysis Software for Windows.

**qRT-PCR (quantitative real-time PCR) analysis**

RNA samples were extracted from the cells using the RNAeasy Mini Kit (QIAGEN). Total RNA samples were quantified using a Nanodrop ND-1000 spectrophotometer. RNA integrity was assessed with an Agilent 2100 Bioanalyzer. RT (reverse transcription) reactions were performed using 1 μg of total RNA and the High Capacity Reverse Transcription Kit (Applied Biosystems) with random primers. Primers were designed with Probe Finder Software (Roche Applied Science). The primers used were: β-F1-ATPase-F (forward): 5′-CACGCAATTTTGAGGCAGTT-3′, β-F1-ATPase-R (reverse): 5′-CTTCAATGGGGTTCACCTA-3′; Hsp60-F: 5′-TGCTATGGCTGTTAGATTT-3′, Hsp60-R: 5′-CAGCAGCAGTAAAACATTTG-3′; GAPDH-F: 5′-AGCCA-ACATGGCTCGACAC-3′, GAPDH-R: 5′-GGCCAATACGACC-AAATCC-3′; G3BP1 (GTPase-activating protein-binding protein 1-F): 5′-CCTGGTGTTGTTGTACGTC-3′, G3BP1-R: 5′-TGCTCTTCTTCCAGTGGC-3′; LC3-F: 5′-GCCACC-TTGGAAACAAGAG-3′, LC3-R: 5′-TCACCCCTTGTTGATCCATCTTATATCA-3′; 18S-F: 5′-GCAATTTACCATCGGACAG-3′, 18S-R: 5′-GGGACCTAACTCAACGAGC-3′, ATF4-F: 5′-GGCTAATGGGTTTGTTGTCATGG-3′, 5 μg/ml leupeptin). Extracts were centrifuged at 11 000 g for 15 min at 4°C. Protein concentration in the supernatants was determined with the Bradford reagent. Cellular proteins (7–20 μg) were fractionated by SDS/PAGE (9% or 15% gels) and then transferred on to PVDF membranes. The primary antibodies were used (dilutions in parentheses): anti-β-F1-ATPase (1:50000) [10]; anti-Hsp60 (heat-shock protein 60) (SPA-807, Stressgen; 1:2000); anti-GAPDH (1:20000) and anti-PK (pyruvate kinase) (1:1000) from Abcam; anti-SDH (succinate dehydrogenase) (1:500), anti-PDH (pyruvate dehydrogenase) (1:500) and anti-(COX IV) [COX (cytochrome c oxidase) subunit IV] (1:250) from Invitrogen; anti-(COX I) (1:150) and anti-α-F1-ATPase (α catalytic subunit of the mitochondrial H+ ATP synthase) (1:1000) from Molecular Probes; anti-α-tubulin (1:5000) and anti-LC3 (microtubule-associated protein 1 light chain 3ε) (1:5000) from Sigma; anti-β-catenin (1:500) from BD Biosciences; anti-GRK2 (G-protein-coupled receptor kinase 2) (1:500), anti-p53 (1:200), anti-HIF (hypoxia-inducible factor) 1α (1:150), anti-PGC1α (peroxisome-proliferator-activated receptor γ co-activator 1α) (1:250), anti-p70S6K (ribosomal protein S6 kinase, 70 kDa, polypeptide 1) (1:1000), anti-HuR [ELAV (embryonic lethal abnormal vision)-like protein] (1:200), anti-eIF2α (1:1000) and anti-ATF4 (1:500) from Santa Cruz Biotechnology; anti-Akt (1:1000), anti-p- (phospho-) Akt (1:1000), anti-AMPK (1:1000), anti-p-AMPK (1:1000), anti-p38MAPK (mitogen-activated protein kinase 14) (1:1000), anti-mTOR (mammalian target of rapamycin) (1:1000), anti-p-mTOR (1:1000), anti-4EBP1 (eIF4E-binding protein 1) (1:1000), anti-p-4EBP1 (1:1000), anti-p-p70S6K (1:1000), anti-p-eIF2α (1:1000), anti-p-GCN2 (1:2000), anti-p-raptor (regulatory-associated protein of mTOR) (1:1000), anti-p-S6 (1:1000), anti-p-ACC (acetyl-CoA carboxylase) (1:1000), anti-ULK1 (unc-51 like kinase 1) Ser754 (1:1000 dilution) and anti-ULK1 Ser678 (1:1000) from Cell Signaling Technology; anti-GCN2 (1:2000) [27] and anti-c-e-Myc (1:400) from Roche Applied Science; and anti-p62 (sequestosome 1) (1:1000) from Enzo Life Sciences. Secondary goat anti-rabbit, rabbit anti-goat and rabbit anti-mouse antibodies (1:5000) were used for detection using a chemiluminescence detection method [ECL (enhanced chemiluminescence), Invitrogen]. Membranes were exposed to X-ray films. Quantification of the immunoreactive bands [a.u. (arbitrary units)] was accomplished using a Kodak DC120 Zoom digital camera and the Kodak 1D Image Analysis Software for Windows.
5′-TCTCCAGGCAAGGCTAA-3′, 3′-ATGATCCCTGATTTCCTGCGGATC-5′; GAPDH, 5′-GCCAAGTGAACGGTATCTTCT-3′; β-actin, 5′-GGACATCAGGGTCTTCT-3′. qRT-PCR was performed using Power SYBR® Green PCR Master Mix (Applied Biosystems) and a LightCycler® 2.0 Real-Time PCR System (Roche Applied Science). The relative expression of the mRNAs was determined using the comparative ΔΔCt method with GAPDH, G3BP1 or 18S (18S rRNA) as controls.

**Immunofluorescence microscopy**

Cells were fixed with 4% (w/v) paraformaldehyde for 20 min and processed for immunofluorescence. The primary antibody used was rabbit anti-LC3 antibody from Sigma (1:100 dilution). After three PBS rinses, the cells were incubated for 1 h in the dark with anti-(rabbit IgG) conjugated to Alexa Fluor® 594 (1:1000 dilution). Cellular fluorescence was analysed by confocal microscopy using a ×63 1.4 NA (numerical aperture) Plan Apochromat oil objective.

**Glycolysis and cellular respiration**

Lactate concentrations in the culture medium were enzymatically determined as an index of glycolysis [22]. Oxygen consumption rates were determined in an XF24 Extracellular Flux Analyser (Seahorse Bioscience). Cells were seeded in the microplates, treated as indicated and incubated at 37°C and 7% CO2 for 48 h. The final concentration and order of injected substances was 6 μM OL, 0.75 mM DNP (2,4-dinitrophenol), 1 μM rotenone and 1 μM antimycin [13].

**Statistical analysis**

Statistical analyses were performed using a two-tailed Student’s t test. Results shown are means ± S.E.M. The number of experiments is indicated. P < 0.05 was considered statistically significant.

**RESULTS**

**OL treatment represses the bioenergetic phenotype of mitochondria**

Comparative transcriptomic analysis of HCT116 cells treated (G) or non-treated (M) with OL suggested a global repression of the metabolic and bioenergetic function of mitochondria in G-cells [22]. The results in Figure 1(a) indicate that G-cells have a diminished complement of PDH-E1α, SDH-A, subunits I (mtDNA-encoded) and IV (nDNA-encoded) of COX, and subunits α and β of the H+-ATP synthase when compared with non-treated M-cells. These changes occurred in the absence of relevant changes in the cellular content of the structural mitochondrial protein Hsp60 and in the abundance of the glycolytic markers GAPDH and PK (Figure 1a). Therefore both the mitochondrial activity (β-F/ Hsp60 ratio) and overall mitochondrial potential of OL-treated cells as assessed by its bioenergetic signature (β-F/GAPDH ratio) [4], were significantly diminished when compared with controls (Figure 1b). Interestingly, these changes also occurred in the absence of relevant changes in mtDNA copy number (Figure 1b). Consistent with the enzymatic phenotype displayed, G-cells had a lower activity of oxidative phosphorylation [OSR (OL-sensitive respiratory rate) in Figure 1c] and an increased flux of lactate production (Figure 1d).

**Inhibition of protein synthesis hampers the bioenergetic differentiation of mitochondria**

Quantification by qRT-PCR of the cellular content of the mRNAs encoding β-F-1-ATPase and GAPDH illustrated the lack of relevant differences in the abundance of these transcripts between M- and G-cells (Figure 2a). A slight, but significant, 15% reduction in Hsp60 mRNA levels was observed in G-cells (Figure 2a) although this difference had no impact on protein expression (Figure 1a).

**Metabolic labelling and immunoprecipitation**

For pulse experiments, cells (~10⁷) were incubated for 20 min in cysteine/methionine-free medium. Metabolic labelling was initiated by addition of an aliquot (0.7 μl) of culture medium supplemented with 0.65 μCi of [35S]methionine/ml. After 30–45 min, the radioactive medium was removed and the cells were washed three times with PBS containing 5 mM methionine and cysteine. Cellular pellets were resuspended in RIPA buffer (0.5 M NaCl, 2 mM EDTA, 50 mM Tris/HCl, pH 8.0, 0.2% SDS and 1% sodium deoxycholate) supplemented with protease inhibitor cocktail (Roche) and freeze–thawed three times. Protein concentration was determined using the Bradford reagent. The radioactivity incorporated into TCA (trichloroacetic acid)-precipitable protein was determined [15]. Immunoprecipitations were carried out from 100 μg of protein obtained from cellular extracts using Protein G–Sepharose pre-coated with 6–7.5 μg of the IgGs of the following monoclonal antibodies: anti-Hsp60 (clone 17/9-15 G1), anti-β-F-1-ATPase (clone 11/21-7 A8), anti-PK (clone 1415-21/24) or anti-GAPDH (clone 273A-E5) [28]. Pre-coating of Protein G–Sepharose was carried out overnight at 4°C in 1 ml of RIPA buffer supplemented with 5 mg of BSA. After formation of the immune complexes, the resin was washed [21] and the immunoprecipitated material was resuspended in 1 x SDS/PAGE sample buffer and loaded on to an SDS/9% PAGE gel. Gels were processed further for fluorography and exposed to X-ray films.

**Determination of adenine nucleotides**

Approximately 10⁷ cells were scraped off the plates into PBS. Cells were precipitated and extracted with 500 μl of a 6% perchloric acid solution. After centrifugation at 15000 g to remove cell debris, the resulting supernatants were neutralized with 2% KOH and freeze-dried. Determination of the cellular content of ATP, ADP and AMP was carried out by standard enzymatic procedures.

**Determination of mtDNA (mitochondrial DNA) copy number**

Cellular DNA [nDNA (nuclear DNA) + mtDNA] was extracted from cells (4 x 10⁷) using the High Pure PCR Template Preparation Kit from Roche. Quantification of mtDNA (mtDNA/nDNA) was performed by qRT-PCR using the LightCycler® 2.0 Real-Time PCR System (Roche Applied Science). The nuclear ATP5B and the mitochondrial 12S rRNA-encoded genes were chosen to determine the ratio of mtDNA to nDNA. The primers used were: 12S-F, 5′-CCACAGGTGTCAGTCTTTC-3′; 12S-R, 5′-CTTACGCGCTTACATTC-3′; hβ-F, 5′-CAGCAGATTTGCGAGTGC-3′; hβ-R, 5′-CTTCAATGGTGTCCTCCATCA-3′. The relative mtDNA copy number was calculated using the ΔΔCt method and the 12S/hβ ratio was used to compare the samples.

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These findings indicate that down-regulation of β-F1-ATPase in G-cells (Figure 1a) could not be ascribed to a limited activity of the transcription of the gene encoding ATP5B or to a regulatory event affecting the abundance of its mRNA. Rather, the results suggest that β-F1-ATPase expression is being controlled at the level of mRNA translation.

To confirm this idea, we studied the initial rates of protein synthesis after a short time-pulse of [35S]methionine in M- and G-cells. We observed no significant differences in the overall rate of protein synthesis between M- and G-cells (Figure 2b). However, the results showed a significant 2–3-fold reduction in the relative rate of synthesis of β-F1-ATPase and Hsp60, the two nuclear-encoded mitochondrial proteins, in G-cells (Figure 2b). In contrast, the relative rates of synthesis of the two glycolytic proteins were no different when compared with M-cells (Figure 2b). These findings indicate that the switch from the M- to the G-phenotype of colon cancer HCT116 cells [22] involves the specific repression of the translation of the mRNAs encoding mitochondrial proteins. In this regard, reanalysis of the transcriptomic study carried out to compare M- and G-cells [22] further indicated a highly significant (Benjamini P value of 5.4×10\(^{-5}\)) repression of the expression of a large number of the mitochondrial aminoacyl-tRNA synthetases in G-cells. The lack of changes observed in Hsp60 expression in G-cells (Figure 1a) despite a significant reduction in its initial rate of synthesis (Figure 2b) further suggests the participation of mechanisms operating at the level of protein turnover.

**Cellular adaptive response to OL treatment**

Next, we monitored some of the relevant signalling pathways that are involved in the control of cell proliferation, survival and invasion and that might participate in the repression of the bioenergetic function of mitochondria. Interestingly, we found that HIF1α was not differentially expressed in G-cells when compared with M-cells (Figure 3), consistent with the observation

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**Figure 1 Oligomycin treatment represses the bioenergetic function of mitochondria**

HCT116 cells were incubated with OL (closed bars, G) or left untreated (open bars, M) to promote changes in the bioenergetic phenotype. (a) Representative Western blots of the expression of PDH-E1α, SDH-A, COX I, COX IV, α-F1-ATPase, β-F1-ATPase, Hsp60, GAPDH, PK and tubulin in three different preparations (lanes 1–3) of M- and G-cells. (b) Mitochondrial (β-F1-ATPase/Hsp60) and cellular (β-F1-ATPase/GAPDH) normalized expression of β-F1-ATPase relative mtDNA copy number (12S/β-F1 ratio). In (a) and (b), histograms show the quantification of the bands normalized to the mean value in control M-cells. (c and d) Rates of OL-sensitive respiration and of lactate production respectively. Results are means ± S.E.M. for at least three determinations. *P < 0.05 compared with M-cells by Student’s t test; ns, not significant. The molecular mass of each protein in kDa is indicated on the left-hand side of the blot.

**Figure 2 Translational control hampers the bioenergetic function of mitochondria**

HCT116 cells were incubated with OL (closed bars, G) or left untreated (open bars, M). (a) Cellular RNA was extracted and the relative mRNA abundance of β-F1-ATPase (β-F1), Hsp60 and GAPDH was determined by qRT-PCR. Results are means ± S.E.M. for three experiments. (b) Determination of the in vivo rate of β-F1-ATPase, Hsp60, GAPDH and PK synthesis in M- and G-cells after metabolic labelling with [35S]methionine followed by immunoprecipitation. The radioactivity incorporated into TCA-precipitable protein (Total) was determined. Histograms show the mean ± S.E.M. of the quantification of the bands normalized to the mean value in control cells for three to six different preparations. Pi, non-specific immunoglobulin. *P < 0.05 compared with M-cells by Student’s t test. The molecular mass of each protein in kDa is indicated on the left-hand side of the blot.
that OL blocks HIF1α expression [29]. Similar results were obtained for β-catenin, GRK2, p38MAPK and for both the total and phosphorylated forms of the serine/threonine kinase Akt (Figure 3). Notably, we found significant differences in the expression of the stress kinases AMPK and GCN2 supporting the activation of these pathways in cells with the G-phenotype (Figure 3). Indeed, both the total and phosphorylated forms of AMPK were augmented in G- when compared with M-cells (Figure 3). Consistent with the activation of AMPK, we observed an increased cellular AMP/ATP ratio after short-term (2 h) treatment of HCT116 cells with OL. Interestingly, the AMP/ATP ratio was not significantly different between M- and G-cells after 48 h of treatment (Figure 3). In the case of the GCN2 kinase, only the phosphorylated active form of the protein was augmented in cells with the G-phenotype (Figure 3).

**Downstream targets of AMPK and GCN2**

AMPK is a master protein kinase that regulates cell metabolism [23] and controls the activity of essential mediators of the cellular adaptive response, such as p53, HuR and mTOR. Consistent with the activation of AMPK in G-cells, we observed a large phosphorylation of raptor (Figure 4) together with the down-regulation of the cellular content of mTOR and HuR in the absence of relevant changes in p53 when compared with M-cells (Figure 4). Interestingly, ACC, a well-established target of AMPK that is phosphorylated and inhibited upon AMPK activation, was not affected in G-cells (Figure 4), indicating the relevance that the synthesis of lipids has as a pathway to allow cellular proliferation in colon cancer cells. The serine/threonine kinase mTOR controls translation and autophagy [30]. In agreement with the lesser signalling through mTOR, we observe an increase in the total cellular content of the cap-dependent translation repressor 4EBP1 [31] in G-cells (Figure 4). The content of the phosphorylated form of 4EBP1 was also diminished in G-cells (Figure 4), further supporting the sequestration of the rate-limiting eIF4E [32]. On the other hand, and to our surprise, other downstream targets of mTOR, such as p70S6K, its phosphorylated form (p-p70S6K) and that of its target the ribosomal protein S6 (p-S6), and of the transcription co-activator PGC1α, revealed no significant changes between both cellular phenotypes (Figure 4). Likewise, and consistent with the activation of GCN2 in G-cells (Figure 3), we observed a significant increase in the cellular content of the translation factor ATF4 when compared with M-cells (Figure 4). It is well established that GCN2 represses translation via phosphorylation of eIF2α and the subsequent up-regulation of the translation of ATF4 [38]. However, in the present study the content of both eIF2α and its phosphorylated form was significantly down-regulated (Figure 4), suggesting feedback inhibition of the phosphorylation of eIF2α after long-term (48 h) activation of GCN2. Overall, these results strongly suggest that a major point of regulation for promoting the repression of the bioenergetic function of mitochondria in G-cells is exerted at the level of translation by activation of the AMPK and GCN2 stress kinases.

**Autophagy is enhanced in G-cells**

The phosphorylation of raptor (Figure 4) and of Ser555 of the autophagy initiator kinase ULK1 (Figure 5a) suggested the AMPK-mediated inhibition of the mTOR signalling pathway and the subsequent activation of autophagy in cells with the G-phenotype [30]. Consistent with the mTOR-mediated activation of autophagy, we also noted the dephosphorylation of Ser78 of ULK1 in G-cells (Figure 5a). Interestingly, a significant decrease in the cellular content of the p62 marker of autophagy was noted in G-cells (Figure 5a). Gene expression analysis using the Affymetrix platform also indicated the transcriptional activation of the autophagic LC3 gene (fold-change increase of 1.7 in G-cells, $P < 0.009$ [22]). Quantification of the expression of LC3...
mRNA by qRT-PCR showed a significant increase in its cellular content in G-cells when compared with M-cells (Figure 5b). Moreover, Western blot data confirmed that the ratio of the conjugated form of LC3II to LC3I, which is a marker of autophagosome formation, is significantly increased in the G-phenotype (Figure 5b). The higher abundance of autophagosomes in G-cells was further confirmed by immunofluorescence microscopy (Figure 5c). Overall, these findings support that the activation of autophagy is an additional pro-survival strategy of the cancer cell to mediate the phenotypic changes that accompany the acquisition of the tumour-promoting behaviour in response to a bioenergetic stressor.

**AMPK activation and ATF4 mediate the inhibition of mitochondrial ATP synthesis**

To assess the effect of the activation of the AMPK pathway in cellular bioenergetics, we first determined the oxygen consumption rates of HCT116 cells (Figure 6a). The results show a representative experiment of cellular O₂ consumption after the sequential addition of OL, DNP, rotenone and antimycin to the cells, illustrating a normal respiratory response to the inhibition of oxidative phosphorylation (OL), mitochondrial uncoupling (DNP) and the inhibition of respiration in HCT116 cells. Moreover, data in Figure 6(b) indicate the linear response of the MRR (maximum respiratory rate), BRR (basal respiratory rate) and OSR of the cells at increasing numbers. The activation of the AMPK pathway by treating the cells with AICAR, an analogue of AMP that is widely used as an activator of the pathway, promoted a significant decrease in OSR in HCT116 cells concurrently with the phosphorylation of AMPK (Figure 6c). AICAR was used at a concentration of 0.1 mM because titration experiments of its effect on the proliferation of HCT116 cells indicated a high toxicity of the compound above this concentration (results not shown). Moreover, the expression of a constitutively active AMPK mutant (α1CA, T172D), which is a truncated version that is not dependent on regulatory subunits to activate the pathway [26] as revealed by the phosphorylation of AMPK (Figure 6d), also promoted a large inhibition of oligomycin-sensitive respiration when compared with their respective controls (Figure 6d). These findings indicate that activation of AMPK partially hinders the activity of the mitochondrial ATP synthase. Interestingly, and consistent with our previous observation of the lack of changes in p-ACC in G- when compared with M-cells (Figure 4), we show that the overexpression of α1CA (Figure 6d) or AICAR treatment (Figure 6c) does not affect the phosphorylation of ACC, stressing the relevance of lipogenesis for proliferation of HCT116 colon cancer cells.

To confirm that activation of AMPK participates in controlling mitochondrial oxidative phosphorylation in colon cancer cells, we next studied the effect of AICAR on the rates of β-F1-ATPase synthesis (Figure 7). The results demonstrated that the activation of AMPK significantly reduced the initial rate of β-F1-ATPase synthesis in the absence of relevant changes on overall protein synthesis and on the synthesis of Hsp60, GAPDH and PK (Figure 7), indicating that the activation of AMPK participates in the repression of the bioenergetic function of colon cancer mitochondria.

To support the participation of the GCN2–ATF4 pathway in silencing the bioenergetic activity of mitochondria, we determined the effect of silencing ATF4 in cellular respiration of M-cells (Figure 8). The results obtained indicated that 40–60% silencing of ATF4 at both the mRNA and protein levels (Figures 8a and 8b) resulted in a significant increase in oxidative phosphorylation (Figure 8c), also supporting the participation of this pathway in promoting the repression of the bioenergetic activity of mitochondria in colon cancer.

**DISCUSSION**

We have recently documented that colon cancer progression requires the selection of cancer cells with a highly glycolytic phenotype due to the repression of the bioenergetic function of mitochondria [22]. However, the mechanisms and signalling...
pathways that trigger the down-regulation of β-F1-ATPase and hence of mitochondrial oxidative phosphorylation in colon cancer remained to be investigated. Colon cancer cells treated with oligomycin provide a model to decipher the mechanisms and signalling pathways that mediate the acquisition of the metabolic phenotype compatible with tumour progression [22,33].

The results of the present study illustrate that metabolic stress triggered by OL treatment of the cells promotes the persistent activation of the stress kinases AMPK and GCN2 (Figure 9). AMPK is a serine/threonine kinase that regulates energetic metabolism and is activated under conditions that elevate the AMP/ATP ratio, such as glucose deprivation, hypoxia and muscle contraction [34]. AMPK activation triggers important changes in downstream targeted proteins with a wide functional relevance in homeostatic adaptation of the cell, including the regulation of mitochondrial biogenesis [35] and function [36], autophagy [30] and angiogenesis [37]. Consistent with the activation of AMPK in G-cells, we report the phosphorylation of raptor, a significant inhibition of mTOR, the subsequent inhibition of the synthesis of mitochondrial proteins and the activation of autophagy (Figure 9). The up-regulation of the expression of genes involved in angiogenesis and vascular development has already been described in G-cells [22]. GCN2 is also a stress kinase activated by nutrient deprivation and viral infection [24,25] that represses the translation of most mRNAs, but selectively increases translation of ATF4 [38]. Consistent with the activation of GCN2, we show that the content of ATF4 is significantly increased in G-cells (Figure 9). It is likely that the co-ordinated activation of AMPK and GCN2 mediate the repression of the translation of nuclear-encoded mRNAs of mitochondria through 4EBP1 and eIF2α respectively (Figure 9). These changes trigger a profound switch in cellular energetic metabolism to an enhanced glycolysis due to a diminished activity of oxidative phosphorylation (Figure 9). Remarkably, we report that, whereas the synthesis and expression of nuclear-encoded mitochondrial proteins is stringently dependent on the activity of cap-dependent translation, that of the enzymes of glycolysis is not affected by interference with this pathway, suggesting the existence of a specific pathway for translation of the mRNAs involved in glycolysis. Owing to the essential cellular function of glycolysis, it is possible that the translation of glycolytic mRNAs shares signalling pathways and mechanisms with the translation of the mRNAs involved in cellular adaptation to stressful conditions [27]. These findings provide the first indication that the mechanisms that control the expression of the enzymes of the two main pathways of energy metabolism (oxidative phosphorylation and glycolysis) are exerted independently and at a different level of regulation of gene expression. In addition, it is likely that metabolic adaptation also involves the participation of additional mechanisms acting at the level of protein turnover. The repression of the bioenergetic function of mitochondria, which is a reversible phenotypic trait of the cancer cell [22], is thus orchestrated by the activation of the stress sensors AMPK and GCN2. In agreement with our findings (Figure 9), the activation of AMPK in colorectal [39] and ovarian [40] carcinomas and the requirement of the GCN2–ATF4 pathway for maintaining metabolic homeostasis and tumour cell survival [38] have been described recently.

Interestingly, the repression of β-F1-ATPase expression observed in G-cells is not paralleled by changes in the cellular abundance of its mRNA. In fact, reduced β-F1-ATPase expression results from a translation-masking event of β-mRNA in agreement with similar findings observed in human [19] and rat [18] carcinomas. It is tempting to suggest that the diminished expression of other mitochondrial proteins observed in G-cells (Figure 1) could result by regulation of a common and presently unknown factor needed for translation of this particular set of nuclear-encoded mRNAs of mitochondria. Translation masking of β-mRNA during development [14] and in carcinogenesis [18,21,41] is due to the binding of specific RNABPs to the transcript. It has been suggested that regulated interactions within the ribonucleoprotein complex [42] hamper the efficient translation of β-mRNA [4]. Moreover, protein binding to β-mRNA was shown to depend on a low energy and oxidized state of the cell [43]. Consistent with these findings, it is possible that the increase in the AMP/ATP ratio observed in the short term after treatment of the cells with OL could mediate the rapid silencing of β-mRNA by favouring the binding of repressor proteins on to the transcript (Figure 9). In fact, AMPK activity has been correlated with the activity of some RNABPs such as HuR [44], a relevant RNABP involved in post-transcriptional regulation of gene expression that interacts with the 3′UTR of β-mRNA [41]. In agreement with recent findings [33], we observed that the activation of glycolysis in response to OL
Figure 6  AMPK activation promotes the inhibition of oxidative phosphorylation

HCT116 cells were seeded at various densities in microplates. (a) Oxygen consumption rates (OCR) after the sequential addition of O{	extsubscript}2 (a), DNP (b), rotenone (c) and antimycin (d) to the cells. (b) Linear response of the BRRs, OSRs and MRRs (DNP-induced) at various cell densities. (c) Incubation of M-cells with 0.1 mM AICAR promoted a significant decrease in OSR (closed bars) when compared with untreated cells (open bars). Results are means ± S.E.M. for three experiments. Representative Western blots of the expression of p-AMPK and p-ACC are shown. (d) Expression of a constitutively active AMPK mutant (α1CA), as revealed by Western blotting with either anti-(Myc tag) or anti-AMPK antibody, promotes a large inhibition of OSR (closed bar) when compared with control (CRL) GFP-transfected cells (open bar). Results are means ± S.E.M. for three experiments. *P < 0.05 compared with control cells by Student’s t test. Representative Western blots of the expression of p-AMPK and p-ACC are shown. The molecular mass of each protein in kDa is indicated on the left-hand side of the blot.

Figure 7  AMPK activation inhibits β-F1-ATPase synthesis

HCT116 cells were incubated with 0.1 mM AICAR (closed bars) or left untreated (open bars). Determination of the in vivo rate of β-F1, Hsp60, GAPDH and PK synthesis in AICAR-treated and untreated cells after metabolic labelling with [35S]methionine followed by immunoprecipitation. The radioactivity incorporated into TCA-precipitable protein (Total) was determined. Histograms show the mean ± S.E.M. of the quantification of the bands normalized to the mean value in control cells from three different preparations (lanes 1–3; pi, non-specific immunoglobulin. *P < 0.05 when compared with untreated cells by Student’s t-test. The molecular mass of each protein is indicated on the left hand side of the blot.

Figure 8  Silencing of ATF4 promotes the bioenergetic function of mitochondria

(a) Cellular lysates of control (c), GAPDH-silenced (siGAPDH, also used as control) and ATF4-silenced (siATF4) cells were analysed by Western blotting for the expression of β-F1-ATPase (β-F1), Hsp60, GAPDH and ATF4. Histograms show the quantification of the bands normalized to the mean value in control cells. (b) Cellular mRNA was extracted from control and ATF4-silenced (si) cells and ATF4 mRNA was determined by qRT-PCR. (c) Silencing of ATF4 mediates a large increase in OSR when compared with control cells. Results are means ± S.E.M. for three (b) or five (c) experiments. *P < 0.05 compared with control cells by Student’s t test. The molecular mass of each protein in kDa is indicated on the left-hand side of the blot.
treatment provides enough ATP to recover a normal AMP/ATP ratio at long-term. Hence it is likely that the persistent activation of AMPK observed in G-cells after 48 h of OL treatment is being mediated by additional regulators of the bioenergetically stressed cell.

AMPK activation is related to an increased mitochondrial biogenesis in muscle and endothelial cells through transcriptional regulation mediated by the PGC1α/NRF (nuclear respiratory factor)/Tfam (transcription factor A, mitochondrial) pathway [3,35]. However, we do not observe changes in the expression of PGC1α in colon cancer stressed cells. In fact, our results indicate that the persistent activation of AMPK in stressed cells results in the repression of the bioenergetic differentiation of mitochondria by mechanisms controlled at the level of translation. This repression is most probably mediated through 4EBP1 for transcripts encoded in nuclear genes and by a lower expression of the mitochondrial aminoacyl-tRNA synthetases for the transcripts encoded in mtDNA. A general belief is that the activation of AMPK and the inhibition of mTOR are cytotoxic to cancer cells [32,45]. However, we observed that persistent activation of AMPK and the inhibition of mTOR are required to establish the tumour-promoting phenotype of colon cancer cells by repression of the bioenergetic function of mitochondria [22]. In this regard, and consistent with our observations, it has been described that the inhibition of the mTOR pathway down-regulates mitochondrial oxygen consumption and the ATP synthetic capacity [36]. In agreement with this, we show that AICAR treatment or the overexpression of a constitutively active mutant of AMPK, resulted in diminished ATP synthetic capacity and the inhibition of β-F1-ATPase synthesis. These findings strongly support that the activity of the mTOR pathway is essential for maintaining the bioenergetic function of mitochondria [36] and specifically of the synthesis of β-F1-ATPase. Within this framework, and in contrast with the beneficial effects of targeting mTOR in cancer [32,45,46], our results raise a reasonable argument against therapies targeting the activation of AMPK and the inhibition of mTOR for the treatment of colon cancer. In agreement with our observation, it has been described that the inhibition of AMPK sensitizes cancer cells to chemotherapy [47]. It is likely that these differences in cell response might stem from cell-type-specific changes in the mechanisms that regulate the biogenesis and function of mitochondria [3].

AMPK and GCN2 are activated by stress signalling, such as glucose deprivation, hypoxia and starvation of amino acids [24,25,34]. Interestingly, none of these stimuli, including hypoxia, seem to operate in our model. However, it is remarkable that the energetic stress that activated AMPK in G-cells is very similar from a mechanistic viewpoint to the HIF-independent hypoxia-induced energy stress that activates mechanisms of energy conservation to promote survival under stressful conditions [48]. In this regard, we suggest that a product of mitochondrial metabolism, such as ROS, could act as a signalling metabolite to maintain the cellular stress and the persistent activation of AMPK [49] and GCN2. The scenario where small molecules define the cellular energy status offer a very attractive hypothesis to explain the mitochondrial phenotype and its short-term adaptive response upon changes in the metabolic condition of the cell. In this regard, we show that in response to energy stress the autophagic pathway is activated in G-cells, perhaps as a strategy to eliminate the damaged proteins produced during adaptation to stress in order to optimize the limited energy supplies [30]. We show that the activation of the GCN2/eIF2α/ATF4 pathway is involved in minimizing the bioenergetic function of mitochondria, allowing G-cells to conserve resources and the reconfiguration of gene expression to manage the energetic stress imposed, leading to the activation of glycolysis as alternative energy provision pathway (Figure 9). In this regard, our results provide the first demonstration illustrating the implication of this pathway to control the stress response when the mitochondrial bioenergetic function is impeded. Consistent with this, we show that the silencing of ATF4 restores the bioenergetic function of mitochondria.
Overall, we conclude that persistent activation of AMPK and GCN2 regulates the balance between glycolysis and aerobic metabolism, specifically repressing the translation of nuclear-encoded mitochondrial proteins. Activation of these signalling pathways is essential to implement a successful response that could allow cancer cells to survive under metabolic stress. Finally, our results open new insights in the pathways involved in the acquisition of a tumour promoter phenotype that implies a deregulated mitochondrial function, highlighting the importance that the organelle has as a tumour suppressor.

AUTHOR CONTRIBUTION

Inmaculada Martínez-Reyes and María Sánchez-Aragó carried out experiments. Inmaculada Martínez-Reyes, María Sánchez-Aragó and José Cuezva contributed to the analysis of the data. Inmaculada Martínez-Reyes and José Cuezva designed experiments and wrote the paper. All authors read and approved the final version of the paper.

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