AMPK Maintains Cellular Metabolic Homeostasis through Regulation of Mitochondrial Reactive Oxygen Species

Highlights
- Mitochondrial ROS (mROS) are a non-canonical activator of AMPK
- AMPK-deficient cells have elevated mROS and undergo premature senescence
- AMPK activation promotes a PGC-1α-dependent antioxidant response
- AMPK-PGC-1α control of mROS regulates Warburg metabolism

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In Brief
AMP-activated protein kinase (AMPK) regulates cellular metabolic balance in response to energy stress. This work by Rabinovitch et al. demonstrates that mitochondrial reactive oxygen species (mROS) are a physiological activator of AMPK and that AMPK couples mROS to an antioxidant program that regulates mitochondrial homeostasis and cellular metabolic balance.
AMPK Maintains Cellular Metabolic Homeostasis through Regulation of Mitochondrial Reactive Oxygen Species

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SUMMARY

Reactive oxygen species (ROS) are continuously produced as a by-product of mitochondrial metabolism and eliminated via antioxidant systems. Regulation of mitochondrially produced ROS is required for proper cellular function, adaptation to metabolic stress, and bypassing cellular senescence. Here, we report non-canonical regulation of the cellular energy sensor AMP-activated protein kinase (AMPK) by mitochondrial ROS (mROS) that functions to maintain cellular metabolic homeostasis. We demonstrate that mitochondrial ROS are a physiological activator of AMPK and that AMPK activation triggers a PGC-1α-dependent antioxidant response that limits mitochondrial ROS production. Cells lacking AMPK activity display increased mitochondrial ROS levels and undergo premature senescence. Finally, we show that AMPK-PGC-1α-dependent control of mitochondrial ROS regulates HIF-1α stabilization and that mitochondrial ROS promote the Warburg effect in cells lacking AMPK signaling. These data highlight a key function for AMPK in sensing and resolving mitochondrial ROS for stress resistance and maintaining cellular metabolic balance.

INTRODUCTION

Reactive oxygen species (ROS) are the byproducts of aerobic metabolism. Mitochondria are major producers of cellular ROS through their central role in energy metabolism. Incomplete reduction of molecular oxygen in the electron transport chain (ETC) results in mitochondrial ROS (mROS) production. Approximately 1%–3% of O2 consumed by cells is metabolized to ROS in the form of superoxides, hydrogen peroxide, and hydroxyl radicals (Valko et al., 2007). Cellular ROS levels increase with increased metabolic activity (Wellen and Thompson, 2010) but are also produced under conditions of metabolic stress, such as hypoxia (Bell et al., 2007). Mitochondrially derived ROS can act as secondary messengers for various signaling pathways and play an important role in the physiological regulation of proliferation and differentiation (Gough and Cotter, 2011; Reczek and Chandel, 2015). However, excessive mitochondrial ROS production can lead to oxidative damage to cellular components such as lipids, proteins, and mitochondrial DNA (Rubatti et al., 2014), promoting cytotoxicity and induction of cellular senescence.

Cellular ROS levels must be tightly regulated to prevent the production of ROS capable of inducing pathophysiological oxidative stress. To achieve this balance, cells coordinate adaptive responses to regulate ROS production or scavenge potentially dangerous levels of ROS using antioxidant systems. Superoxide dismutase (SOD) acts to convert superoxide (O2−) radicals produced during oxidative phosphorylation (OXPHOS) into H2O2, whereas H2O2 is reduced to water by catalase, glutathione peroxidases, and peroxiredoxins (Diebold and Chandel, 2016). ROS can stimulate the expression of antioxidant enzymes, triggering a feedback mechanism mediated by the transcriptional co-activator PGC-1α to attenuate ROS levels following oxidative stress (St-Pierre et al., 2006).

AMP-activated protein kinase (AMPK) is an evolutionarily conserved serine/threonine kinase that plays a central role in maintaining cellular metabolic balance. AMPK is regulated by the cellular adenylate charge and is activated in response to disruptions in cellular energy (i.e., decreases in cellular ATP) (Hardie et al., 2011; Oakhill et al., 2012). Under conditions of energetic stress, AMPK initiates biological changes aimed at restoring cellular energy balance, including induction of fatty acid oxidation and suppression of mTORC1 signaling (Shackelford and Shaw, 2009). Disruption of AMPK signaling, either though inhibition of AMPK itself or its upstream kinase LKB1, promotes a metabolic shift to the Warburg effect, marked by increased glycolysis and anabolic metabolism (Faubert et al., 2013, 2014; Kishton et al., 2016; Shackelford et al., 2009). AMPK also influences mitochondrial homeostasis by initiating mitochondrial fission and recycling of damaged mitochondria through direct phosphorylation of the mitochondrial fission factor (MFF) and ULK1 kinase, respectively (Egan et al., 2011; Toyama et al., 2016).
Despite the established relationship between mitochondrial dysfunction and AMPK activation, how AMPK couples changes in mitochondrial activity to downstream metabolic adaptations remains poorly understood. Here we report that mitochondrial ROS is a physiological activator of AMPK that triggers AMPK-dependent metabolic adaptations specific to mitochondrial homeostasis (ULK1 phosphorylation and upregulation of PGC-1α) and control of the Warburg effect. These results reveal AMPK as a sensor of mitochondrial activity that couples mitochondrial ROS to the control of cellular metabolic homeostasis.

RESULTS

Non-Canonical Activation of AMPK by Mitochondrial ROS

Previous work has implicated ROS in the activation of AMPK under certain conditions (Emerling et al., 2009; Park et al., 2006; Shafique et al., 2013), although this has remained controversial (Shao et al., 2014; Zmijewski et al., 2010). To study the effect of physiological cellular ROS on AMPK activation, we cultured mouse embryonic fibroblasts (MEFs) with Trolox, a vitamin E analog and antioxidant that reduces cellular ROS levels (Figure S1A). MEFs cultured with Trolox displayed reduced basal AMPK activation, as determined by reduced phosphorylation of the AMPKα catalytic subunit at Thr-172 (Figure 1A).

AMPK is activated in cells in response to metabolic stresses such as glucose limitation (Jones et al., 2005; Salt et al., 1998), which is classically attributed to an imbalance in the AMP-to-ATP ratio (Hardie, 2011). Culturing MEFs with Trolox was sufficient to reduce the normal induction of AMPK phosphorylation by glucose withdrawal (Figure 1B). Interestingly, glucose withdrawal increased the AMP-ATP ratio (Figure 1C; Figure S1B) in both control and Trolox-treated cells despite reduced AMPK activation in the presence of Trolox (Figure 1B). Phosphorylation of acetyl-coenzyme A (CoA) carboxylase (ACC), a direct downstream target of AMPK, at Ser-79 was still induced following glucose withdrawal in Trolox-treated cells despite reduced levels of AMPKα phosphorylation (Figure 1B). In contrast, AMPK-dependent phosphorylation of the autophagy-inducing kinase ULK1 (on Ser-555) was inhibited by Trolox treatment (Figure 1B).

To assess the role of mitochondrially derived ROS on AMPK activation, we used RNAi to silence the Rieske iron-sulfur protein (RISP), a component of complex III of the ETC that regulates ROS production from the mitochondria (Tormos et al., 2011). Silencing RISP decreased mitochondrial respiration in cells (Figure S1C), leading to a compensatory increase in lactate production in MEFs regardless of AMPK expression (Figure S1D). Notably, silencing RISP increased mitochondrial ROS production both at baseline and following complex III inhibition (Figure S1E). RISP knockdown increased phosphorylation of both AMPK, as shown previously (Moiseeva et al., 2009), and the downstream effectors ULK1 and ACC in unstressed cells (Figure 1D). Moreover, silencing RISP promoted increased AMPKα activation in cells following treatment with the complex III inhibitor Antimycin A (AA; Figure 1D) or glucose withdrawal (Figure 1E). This difference in AMPK activation could not be attributed to differential effects on OXPHOS because RISP short hairpin RNA (shRNA) lowered oxygen consumption in both control and AMPKα-deficient MEFs (Figure S1C). In addition, there was no significant difference in the AMP-to-ATP ratio between control and RISP shRNA-expressing cells (Figure 1F; Figure S1F). AMPK-dependent phosphorylation of ULK1 was strongly dependent on the production of mitochondrial ROS because Trolox treatment reduced ULK1 phosphorylation in RISP shRNA-expressing cells (Figure 1E).

We next transfected cells with a construct expressing Sod2 and mitochondrion-targeted Catalase (Sod2-mCat) to reduce mitochondrial ROS in cells (Xiong et al., 2015; Figure 1G). Phosphorylation of AMPKα and ULK1 following glucose withdrawal (Figure 1H) or AA treatment (Figure 1I) was reduced in cells expressing Sod2-mCat compared with control cells. Collectively, these data indicate that mitochondrial ROS is a physiological activator of AMPK that affects distinct effectors downstream of AMPK signaling.

AMPK Regulates Mitochondrial ROS Production and Oxidative Stress Resistance

Previous work by several groups has demonstrated changes in ROS in LKB1-deficient cells (Faubert et al., 2014; Li et al., 2015). Using paired isogenic MEF cell lines with (wild-type [WT]) or lacking (knockout [KO]) AMPKα catalytic activity, we found that cells lacking AMPKα expression displayed approximately 50% higher basal levels of mitochondrial ROS, as determined by MitoSOX staining (Figure 2A). In addition, MEFs lacking AMPKα featured higher levels of mitochondrial ROS following AA treatment or glucose withdrawal compared with control cells (Figures 2B and 2C). Mitochondrial ROS levels were elevated rapidly in AMPKα-deficient MEFs following glucose deprivation and remained elevated over 24 hr of nutrient deprivation (Figure 2D).

One of the hallmarks of oxidative stress is the induction of cellular senescence, a state of irreversible growth arrest (Campisi and d’Adda di Fagagna, 2007). Consistent with increased levels of mitochondrial ROS in AMPKα-deficient cells, low-passage primary AMPKα-deficient MEFs rapidly underwent senescence (< 2–3 passages) compared with control cells, which typically underwent senescence after 7–8 passages (Figure 2E). This effect could not be rescued by culturing AMPKα-deficient cells under reduced O2 tension because AMPKα-deficient cells grown in 3% O2 still underwent senescence after 5–6 passages, similar to control cells grown under ambient (20%) O2 conditions (Figure 2F).

AMPK Activation Induces a PGC-1α-Dependent Antioxidant Response

We next examined whether direct AMPK activation could influence mitochondrial ROS levels. Treatment of MEFs with the AMPK activator A-769662 (A-76) resulted in lower mitochondrial ROS levels in unstressed cells and blocked the increase in mitochondrial ROS production induced by glucose withdrawal (Figure 3A; Figure S2A). To elucidate potential mechanisms by which AMPK activation influences mitochondrial ROS, we examined the expression of known antioxidant genes in control (WT) or AMPKα-deficient (KO) MEFs following A-76 treatment. A-76 treatment promoted an AMPK-dependent increase in several
Figure 1. Physiological Activation of AMPK by Mitochondrial ROS

(A) Immunoblot for AMPKα (total and phospho-T172) and actin in MEFs treated with vehicle (−) or Trolox (+, 1 mM) for 1 hr.

(B) Immunoblot for AMPKα activation (phospho-T172) and phosphorylation of the downstream AMPK effectors ACC (phospho-S79) and ULK1 (phospho-S555) in MEFs cultured with (+) or without (−) glucose and with or without Trolox (1 mM).

(C) AMP-to-ATP ratio in MEFs cultured with (+) or without (−) glucose for 1 hr in combination with (black) or without (white) 1 mM Trolox (mean ± SEM, n = 5).

(D) Immunoblot for AMPKα (total and phospho-T172), ACC (phospho-S79), ULK1 (phospho-S555), RISP, and actin in MEFs expressing control (−) or RISP-targeted (+) shRNA. Cells were cultured with or without Antimycin A (AA; 1 μM for 2 hr).

(E) Immunoblot for AMPKα (total and phospho-T172), ACC (phospho-S79), and ULK1 (phospho-S555) in shCtrl- or shRNA against RISP (shRISP)-expressing MEFs following 1 hr culture with (+) or without (−) glucose (25 mM) and/or Trolox (1 mM) as indicated.

(F) AMP-to-ATP ratio in MEFs expressing control (−) or RISP-targeting (+) shRNAs and cultured under standard growth conditions (mean ± SEM, n = 5).

(G) Histogram of mitochondrial ROS (mROS) levels in 293T cells expressing control (Ctrl, black) or Sod2-mCat (red) vectors, as measured by the mean fluorescence intensity (MFI) of MitoSOX staining. Cells were cultured without (−AA, open histograms) or with (+AA, shaded histograms) 0.5 μM AA for 2 hr.

(H) Immunoblot for AMPKα (total and phospho-T172), ACC (phospho-S79), and ULK1 (phospho-S555) in Ctrl or Sod2-mCat-expressing 293T cells following culture with (+) or without (−) glucose for 2 hr.

(I) Immunoblot for AMPKα (total and phospho-T172), ACC (phospho-S79), and ULK1 (phospho-S555) in Ctrl or Sod2-mCat-expressing 293T cells following culture with (+) or without (−) AA (0.1 mM) for 2 hr.
antioxidant genes, including Catalase, Sod1, Sod2, and Ucp2 (Figure 3B).

The transcriptional co-activator PGC-1α is a key regulator of mitochondrial biogenesis and antioxidant gene expression in response to oxidative stress (St-Pierre et al., 2006). Given that PGC-1α is a downstream effector of AMPK (Audet-Walsh et al., 2016; Jäger et al., 2007), we examined the involvement of PGC-1α in the AMPK-dependent control of mitochondrial ROS. At baseline, AMPKα-deficient cells exhibited lower levels of PGC-1α mRNA than WT cells (Figure S2B). Treating cells with A-76 increased PGC-1α but not PGC-1β mRNA levels in an AMPK-dependent manner (Figure 3C; Figure S2C). Similar to AMPKα-deficient cells, MEFs harboring a conditional mutation for Ppargc1a (PGC-1α KO) displayed elevated mitochondrial ROS levels under basal growth conditions (Figure 3D). We next stimulated control or PGC-1α-deficient MEFS with A-76 and found that the expression of several AMPK-dependent antioxidant genes, notably Catalase, Sod2, and Ucp2, was also dependent on PGC-1α expression (Figure 3E). Ectopic expression of PGC-1α restored antioxidant gene expression in AMPKα-deficient MEFS (Figure 3F; Figure S2D). Ectopic expression of PGC-1α in AMPKα-deficient MEFS also blunted the production of mitochondrial ROS induced by glucose deprivation (Figure 3G; Figure S2E), implicating PGC-1α as a downstream effector of AMPK in the control of mitochondrial ROS homeostasis.

**Mitochondrial ROS Regulate the Warburg Effect through an AMPK-PGC-1α-Dependent Circuit**

AMPK is required to maintain cellular metabolic homeostasis because both non-transformed and tumor cells display changes in cellular metabolism characteristic of the Warburg effect when LKB1 or AMPK signaling is disrupted (Faubert et al., 2013, 2014; Kishton et al., 2016). We previously demonstrated that this metabolic shift is due to elevated HIF-1α protein expression in cells lacking AMPK (Faubert et al., 2013). Given the links between mitochondrial ROS production and HIF-1α stabilization (Brunelle et al., 2005; Chandel et al., 2000), we tested whether increased mitochondrial ROS production because of defective AMPK-PGC-1α signaling could be a mechanistic driver of the Warburg effect. Consistent with this, treatment with Trolox or the antioxidants ascorbate (Asc) or N-acetyl cysteine (NAC), which lower cellular ROS levels (Figures S3A and S3B), reduced HIF-1α protein levels in AMPKα-deficient MEFs (Figures 4A and 4B). Trolox treatment also reduced the expression of several glycolytic genes transcriptionally induced by HIF-1α in AMPKα-deficient MEFS, including Aldolase, Ldha, and Pdk1 (Figure S3C).
PGC-1α-deficient MEFs, which show elevated levels of mitochondrial ROS (Figure 3D), also displayed characteristics of the Warburg effect, including increased HIF-1α protein expression (Figure 4C), increased glucose consumption and lactate production (Figure 4D), and increased glycolysis (Figure S3D) compared with control cells. Similar to AMPKα-deficient MEFs (Figure 4A), Trolox treatment ablated the elevated HIF-1α protein expression observed in PGC-1α-deficient cells (Figure 4E). Ectopic expression of PGC-1α in AMPKα-deficient cells lowered HIF-1α protein levels as well as protein levels for HIF-1α target genes (Aldolase, Ldhα, and Pdk1), similar to that observed following Trolox treatment (Figure 4F).

Finally, we assessed the contribution of mitochondrial ROS to the glycolytic phenotype of AMPKα-deficient cells. Two of the PGC-1α-dependent antioxidant genes induced by AMPK activation are Catalase and Sod2 (Figure 3B). Ectopic expression of Sod2 and mitochondrion-targeted Catalase was sufficient to reduce the elevated extracellular acidification rate (ECAR) of AMPKα-deficient MEFs (Figure 4G). Together, these data indicate that mitochondrial ROS are a physiological trigger for glycolysis that is regulated by AMPK-PGC-1α signaling.

DISCUSSION

AMPK regulates cellular adaptation to energetic stress by engaging appropriate cellular responses to cope with metabolic perturbations. Metabolic stresses that affect mitochondrial function cause an energetic imbalance but also promote increased...
mitochondrial ROS production. Here we report a role for mitochondrial ROS in promoting non-canonical AMPK activation and shaping AMPK-dependent metabolic reprogramming. Our data demonstrate that mitochondrial ROS are required for AMPK activation by various metabolic stressors and promote the activation of AMPK-dependent downstream effectors that influence mitochondrial homeostasis (i.e., ULK1 and PGC-1α). AMPK activation triggers a PGC-1α-dependent anti-oxidant response. Consequently, cells lacking either AMPKα or PGC-1α display increased mitochondrial ROS levels. Our data indicate that this AMPK-dependent circuit is essential for cellular metabolic homeostasis because loss of AMPK-α signaling leads to ROS-dependent activation of HIF-1α and stimulation of the Warburg effect. These data highlight a physiological role for mitochondrial ROS in coupling mitochondrial fitness to AMPK-dependent programs that help maintain cellular metabolic balance.

Proper mitochondrial function is essential for cell proliferation and adaptation to metabolic stress (Hamanaka et al., 2016; Martinez-Reyes et al., 2016). How cells monitor mitochondrial fitness has remained unclear, although mitochondrial products including metabolic intermediates (i.e., acetyl-CoA, tricarboxylic acid [TCA] cycle intermediates) and ROS have been suggested as candidates in this process (Chandel, 2015). Our data provide evidence for oxidative stress as a trigger for AMPK activity and identify mitochondria as a key source of these ROS signals. Enhancing mitochondrial ROS production promoted AMPK activation at baseline and under metabolic stress conditions such as glucose withdrawal, which has been previously attributed to changes in cellular adenylate levels (Gowans et al., 2013; Mayer et al., 2011; Oakhill et al., 2011). Quenching ROS reduces AMPK activation induced by metabolic stress despite changes in the AMP-to-ATP ratio predicted to trigger AMPK activation. Similarly, elevated mitochondrial ROS production induces AMPK activation independent of cellular adenylate levels. Our data indicate that mitochondrial ROS functions as a non-canonical activator of AMPK during the early phases of AMPK activation (< 2 hr) in response to metabolic stresses such as glucose limitation. It is unclear whether direct oxidation of AMPKα by mitochondrial ROS is sufficient to increase AMPK activity independent of AMP (Zmijewski et al., 2010), and this will remain an active area of study.

AMPK has emerged as an important regulator of mitochondrial function, coupling stress signals to processes that regulate mitochondrial homeostasis (Egan et al., 2011; Jaeger et al., 2007; Kim et al., 2011; Toyama et al., 2016). Interestingly, AMPK-dependent phosphorylation of ACC, which mediates the effects of AMPK on lipid metabolism, was largely unaffected by blocking mitochondrial ROS production. Rather, AMPK-dependent pathways associated with mitochondrial quality...
control (ULK1 phosphorylation and upregulation of PGC-1α) were highly dependent on mitochondrial ROS. We speculate that mitochondrial ROS may affect a pool of cellular AMPK associated with mitochondria, effectively promoting the bifurcation of AMPK signaling to favor pathways that affect mitochondrial function. In such a scenario, mitochondrially localized AMPK could sense local regions of mitochondrial dysfunction, coupling increased ROS production by the ETC in these regions to a coordinated program of mitochondrial fission and ULK1-mediated mitophagy. Consistent with this, artificially targeting AMPK to mitochondria is sufficient to induce mitophagy and cell survival (Liang et al., 2015). We speculate that non-canonical methods of AMPK activation may function to tether AMPK activity to specific subcellular locations to exert distinct biological effects at these locations. Recent work by Zhang et al. (2017) demonstrated that non-canonical activation of AMPK via recruitment to the lysosome was induced by loss of fructose-1,6-bisphosphate (FBP) binding to aldolase. Mitochondrial ROS may contribute to this process through HIF-1α-dependent control of aldolase expression, thereby allowing greater sensitivity of AMPK to changing FBP levels.

One consequence of chronically increased mitochondrial ROS is the stabilization of HIF-1α, which acts as a survival mechanism by re-directing energy production away from the mitochondria to glycolysis. This increase in glycolysis can also support additional nicotinamide adenine dinucleotide phosphate (NADPH) production to fuel the glutathione antioxidant system to deal with elevated ROS levels. AMPK has previously been shown to affect cellular ROS by maintaining cellular NADPH levels (Jeon et al., 2012). In cells with normal mitochondrial function, disruption of LKB1-AMPK signaling (Faubert et al., 2013, 2014; Kishton et al., 2016; Shackelford et al., 2009) or PGC-1α (as shown here) promotes HIF-1α-dependent reprogramming of glucose metabolism characteristic of the Warburg effect. Reducing mitochondrial ROS production abolished the elevated HIF-1α expression characteristic of AMPKα2-deficient cells, implicating elevated ROS production as the driver of this metabolic phenotype. Interestingly, both low and high levels of AMPK signaling are associated with increased ROS production and Warburg metabolism. Loss of FLCN, a negative regulator of AMPK, promotes constitutive AMPK activation, leading to increased PGC-1α-dependent mitochondrial biogenesis and a ROS-induced Warburg phenotype (Yan et al., 2014). These data position AMPK-PGC-1α signaling as a regulator of ROS-dependent metabolic balance under both low- and high-energy conditions.

Non-canonical regulation of AMPK by mitochondrial ROS may explain the beneficial effects of AMPK activation on longevity and aging (Burkewitz et al., 2014). Treating cells with A-76, which stimulates AMPK activation in the absence of cellular energetic stress (Vincent et al., 2015), blocked mitochondrial ROS production induced by glucose withdrawal. Cells lacking AMPK signaling cannot respond to physiological mitochondrial ROS signals, leading to disrupted mitochondrial ROS homeostasis and induction of cellular senescence. We speculate that mitochondrial ROS triggers AMPK-PGC-1α signaling as a feedback loop to limit potentially damaging levels of ROS, thereby averting ROS-induced cellular damage and senescence (Sena and Chandel, 2012). ROS-dependent regulation of AMPK may also explain the complex role of AMPK in cancer. Many cancer cells exhibit elevated ROS levels, which may enhance tumor cell growth through increased signaling but also damage cellular components (Chandel and Tuverson, 2014; Szatrowski and Nathan, 1991). AMPK-dependent stimulation of antioxidant genes may benefit cancer cells when ROS levels are sufficient to promote cell death; for example, during radiotherapy. However, mitochondrial ROS can also be pro-oncogenic by promoting genomic instability (Samper et al., 2003). In this context, AMPK-dependent regulation of ROS homeostasis could exert tumor suppressor functions. This corresponds to observations that AMPK may be pro- or anti-tumorigenic, depending on context (Faubert et al., 2015). Overall, our data reveal a dynamic relationship between mitochondrial ROS and AMPK that influences overall cellular metabolic balance.

EXPERIMENTAL PROCEDURES

Cell Lines, DNA Constructs, and Cell Culture

MEFs conditional for prkaa1 and prkaa2 or prargc1a were generated by timed mating as described previously (Jones et al., 2005). 293T cells were obtained from the ATCC. Cells were grown in DMEM containing 10% fetal calf serum (FCS), L-glutamine, and penicillin/streptomycin unless stated otherwise. Knockdown of Uqcrfs1 (RISP) was achieved using lentiviral shRNA vectors from the RNAi Consortium (TRC) shRNA collection (Sigma-Aldrich, St. Louis MO; ID TRCN0000071089). Ectopic expression of PGC-1α was achieved using Ppargc1a (GFAP-tagged) lentivirus particles from OriGene (MR210711L2). SOD2-mCat (a gift from Connie Cepko, Addgene plasmid 67635) was subcloned into pcDNA3 using EcoRI and NcoI. Trolox, NAC, and ascorbate were obtained from Sigma.

Immunoblotting

Cells were lysed in modified AMPK lysis buffer or 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS) buffer (Faubert et al., 2013) and supplemented with the following additives: protease and phosphatase tablets (Roche), DTT (1 μM), and benzamidine (1 μM). Cleared lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and incubated with primary antibodies. Primary antibodies to AMPK (phospho-T172-α), PGC1α (Ser555), aldolase (Alkaline phosphatase (HRP))-conjugated anti-rabbit and anti-mouse secondary antibodies were obtained from Cell Signaling Technology (Danvers, MA). Anti-HIF-1α antibodies were from Cayman Chemical (Baton Rouge, LA). Anti-RISP antibodies were obtained from Abcam (Cambridge, UK).

ROS Measurements

Cellular and mitochondrial ROS were assessed by incubating cells with 2',7'-dichlorofluorescin diacetate (DCFDA) or MitoSOX red, respectively, followed by analysis by flow cytometry. ROS levels were quantified as the mean fluorescence intensity (MFI) for MitoSOX staining. All flow cytometry was conducted using BD FACSCalibur, BD LSRSortessa, BD FACSanto (BD Biosciences, San Diego, CA), or Gallios (Beckman Coulter, Fullerton, CA) flow cytometers and analyzed with FlowJo software (Tree Star, Ashland, OR).

Quantitative Real-Time PCR

Total mRNA was isolated from cells using Trizol (Invitrogen), and cDNA was synthesized from 100 ng of total RNA using the Superscript variable input, linear output (VLO) cDNA synthesis kit (Invitrogen). Quantitative PCR was performed using SYBR Green qPCR SuperMix (Invitrogen) and an Mx3005 qPCR machine (Agilent) using primers against ant, catalase, cytochrome c, gpx1, tdp, pgc1α, pgc1b, sod1, sod2, ucp2, ucp3, idhα, aldolase, hif1α, and pdk1. All samples were normalized to β-actin mRNA levels. Primer sequences have been described previously (Faubert et al., 2013; St-Pierre et al., 2006).
Metabolic Assays
The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of cells were measured using an XF96 extracellular flux analyzer (Seahorse Bioscience, Billerica, MA) as described previously (Faubert et al., 2014). Metabolites for liquid chromatography coupled to mass spectrometry (LC-MS) were extracted using ice-cold 50% methanol, bead-beaten, and then analyzed by LC-MS as described previously (Ma et al., 2017).

Statistical Analysis
Statistics were determined using paired Student’s t test, ANOVA, or log-rank (Mantel-Cox) test using Prism software (GraphPad). Data were calculated as the mean ± SEM unless otherwise indicated. Statistical significance is represented in figures as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.09.026.

AUTHOR CONTRIBUTIONS

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Supplemental Information

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Primary mouse embryonic fibroblasts (MEFs) conditional for *prkaa1* and *prkaa2* or *ppargc1a* were generated by timed mating as previously described (Jones et al., 2005). Unless otherwise stated, MEFs were immortalized with SV40 Large T Antigen. Serial passage of primary MEFs was conducted following a 3T3 passage protocol as previously described (Jones et al., 2005). 293T cells were obtained from the ATCC. Knockdown of *Uqcrfs1* (RISP) was achieved using lentiviral shRNA vectors from the TRC shRNA collection (Sigma-Aldrich, St. Louis MO, ID: TRCN0000070108-10). Ectopic expression of PGC-1α was achieved using *Ppargc1a* (GFP-tagged) lentivirus particles from OriGene (MR210710L2V). Viral supernatants were generated using standard methods (Huang et al., 2012), and transduction of cell lines was conducted as previously described (Jones et al., 2005). Retrovirus-infected cells were cultured in 2 μg/ml puromycin or sorted 7 days post-infection by flow cytometry (for hCD8-expressing cells). SOD2-mCat from the AAV-CMV-SOD-2A-Catalase-WMRE vector (a gift from Connie Cepko, Addgene plasmid #67635) was subcloned into pcDNA3 using EcoRI and NotI. For transient ectopic gene expression, 293Ts and MEFs were transfected using Lipofectamine 2000 or Lipofectamine 3000 (Thermo Fisher Scientific), respectively. Trolox, NAC, and ascorbate were obtained from Sigma.
Immunoblotting

Cells were lysed in modified AMPK lysis buffer (Faubert et al., 2013) or CHAPS buffer (10mM Tris-HCl, 1mM MgCl₂, 1mM EGTA, 0.5mM CHAPS, 10% glycerol, 5mM NaF), and supplemented with the following additives: protease and phosphatase tablets (Roche), DTT (1 μg/ml), and benzamidine (1 μg/ml). Cleared lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and incubated with primary antibodies. Primary antibodies to AMPK (phospho-T172-specific and total), phospho-Acetyl-CoA-carboxylase (pS79), phospho-ULK (pS555), aldolase, LDHA, PDK1, and actin, as well as HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were obtained from Cell Signaling Technology (Danvers, MA). Anti-HIF-1α antibodies were from Cayman Chemical (Baton Rouge, LO). Anti-RISP antibodies were obtained from Abcam (Cambridge, UK).

ROS Measurements

Measurement of total cellular ROS and mitochondrial ROS was assessed by incubating cells with DCFDA or MitoSOX red for 15-30 minutes, respectively, followed by analysis by flow cytometry. Mitochondrial ROS was quantified as the mean florescence intensity (MFI) for MitoSOX staining. All flow cytometry was conducted using BD FACSCalibur, BD LSRFortessa, BD FACSCanto (BD Biosciences, San Diego, CA), or Gallios (Beckman Coulter, Fullerton, CA) flow cytometers, and analyzed with FlowJo software (Tree Star, Ashland, OR).
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Total mRNA was isolated from cells using Trizol (Invitrogen), and cDNA synthesized from 100 ng of total RNA using the Superscript® VILO™ cDNA Synthesis Kit (Invitrogen). Quantitative PCR was performed using SYBR Green qPCR SuperMix (Invitrogen) and an Mx3005 qPCR machine (Agilent) using primers against \( \text{ant, catalase, cytochrome c, gpx1, ibp, pgc1a, pgc1b,} \) \( \text{sod1, sod2, ucp2, ucp3, ldha, aldolase, hif1a,} \) and \( \text{pdk1} \). All samples were normalized to \( \beta \)-actin mRNA levels. Primer sequences have been previously described (Faubert et al., 2013; St-Pierre et al., 2006).

Metabolic Assays

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of cells were measured using an XF96 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA) as previously described (Faubert et al., 2014a). In brief, cells were plated at \( 3 \times 10^4 \)/well in 100 \( \mu \)l non-buffered DMEM containing 25mM glucose and 2mM glutamine. Cells were incubated in a CO\(_2\)-free incubator at 37°C for 1 hour for temperature and pH equilibration prior to loading into the XF96 apparatus. XF assays consisted of sequential mix (3 min) measurement (5 min) cycles, allowing for determination of OCR/ECAR every 8 minutes. For measurement of extracellular metabolites, media was removed from cells and analyzed for glucose, lactate, and glutamine using a BioProfile 400 Analyzer (NOVA Biomedical, Waltham, MA), and metabolite concentration expressed relative to cell number. Cells were grown under standard growth conditions for 2-3 days prior to metabolic analysis.
Liquid chromatography coupled to mass spectrometry (LC-MS) was performed on metabolite extracts from MEFs using previously described methods (Ma et al., 2017). In brief, 2x10^6 MEFs were seeded in 10cm plates and cultured under standard growth conditions overnight prior to experimental manipulation. Metabolites were extracted using ice cold 50% methanol, bead beaten, then analyzed by LC-MS as previously described (Ma et al., 2017). Metabolite concentrations were determined per million cells.

**Statistical Analysis**

Statistics were determined using paired Student’s t-test, ANOVA, or Log-rank (Mantel-Cox) test using Prism software (GraphPad). Data are calculated as the mean ± SEM unless otherwise indicated. Statistical significance is represented in figures by: *, p<0.05; **, p<0.01; ***, p<0.001.
Supplementary Figures
Figure S1, Related to Figure 1. Regulation of ROS and adenylate levels in AMPKα-deficient cells.

A) Total ROS

B) mROS

C) Lactate production

D) $\text{mROS}^{\text{shRISP}}$

E) $\text{mROS}^{\text{shCtrl}}$

F) $\text{mROS}^{\text{shRISP}}$

G) $\text{mROS}^{\text{shRISP}}$

H) $\text{mROS}^{\text{shRISP}}$

Rabinovitch et al., Figure S1
Figure S1, Related to Figure 1. Regulation of ROS and adenylate levels in AMPKα-deficient cells.

A) Total cellular ROS levels in MEFs following treatment with Trolox. MEFs were incubated with Trolox (1 mM) for 1h, and total cellular ROS levels were measured by DCFDA staining and flow cytometry. Shown is the mean fluorescence intensity (MFI) for DCFDA staining for triplicate samples.

B) Concentration of adenylates (AMP, ADP, and ATP) in MEFs. Cells were cultured with (empty bars) or without (filled bars) glucose (25 mM) for 1 h, in combination with (red) or without (black) Trolox (1 mM). Adenylate levels (nM per million cells) were determined by LC-MS, and data expressed as mean ± SEM (n=5). These data were used to calculate the AMP to ATP ratios in Figure 1C.

C) Oxygen consumption rate (OCR) of wild type (WT, white) or AMPKα-deficient (KO, black) MEFs expressing control (-) or RISP-specific (+) shRNAs as determined using a Seahorse XF96 extracellular flux analyzer. Data represent the mean ± SEM for biological replicates (n = 5).

D) Lactate production by control (WT) and AMPKα-deficient (AMPK KO) MEFs expressing control (shCtrl) or RISP-targeted (shRISP) shRNA. Cells were grown under standard culture conditions for 48 h prior to collection of media and metabolite measurements. Data are normalized to cell number and presented as mean ± SEM for triplicate cultures.

E) Mitochondrial ROS levels in MEFs expressing control (shCtrl) or RISP-targeting (shRISP) shRNAs. Cells were treated with or without 1μM antimycin A for 2 h, and mitochondrial ROS
determined by MitoSOX staining. Data are presented as the MFI for MitoSOX staining (± SEM) for triplicate samples.

F) Concentration of adenlyates (AMP, ADP, and ATP) in MEFs transduced with control (shCtrl) or RISP-targeting (shRISP) shRNAs. Cells were grown under standard conditions, and adenylate concentrations (nM per million cells) were measured as in (B). Data are represented as the mean ± SEM (n=5). These data were used to calculate the AMP to ATP ratios in Figure 1F.

G) Immunoblot for AMPKα (total and phospho-T172), ACC (phospho-S79), and ULK (phospho-S555) following transduction of control (WT) and AMPKα-deficient (KO) MEFs with control or RISP-targeted shRNAs. Cells were cultured with or without antimycin A (AA; 1μM for 2 h). RISP and actin protein levels following shRNA knockdown are shown.

H) Relative expression of Sod2 transcript in 293T cells transiently transfected with control (white) or Sod2-mCat-expressing (black) vectors. Transcript levels were determined by qPCR, expressed relative to actin mRNA levels for triplicate samples, and normalized relative to expression in control cells.
Figure S2, Related to Figure 3. Effect of AMPKα and PGC-1α expression on mitochondrial ROS and antioxidant gene expression.

A) Mitochondrial ROS levels in MEFs treated with the AMPK activator A-769662. MEFs were pre-treated overnight with or without A-769662 (A-76, 25μM), followed by culture in medium containing (+Glc) or lacking (-Glc) glucose (25 mM) for 1h. Mitochondrial ROS levels were quantified by MitoSOX staining and flow cytometry. Presented are representative flow cytometry plots for MitoSOX staining intensity.
B) *Ppargc1a* mRNA transcript expression in wild type (WT) or AMPKα-deficient (KO) MEFs. Transcript expression was determined by qPCR, expressed relative to *actin* mRNA levels for triplicate samples, and normalized relative to expression levels in control cells.

C) Relative expression of *Ppargc1b* mRNA transcript in wild type (WT, white) and AMPKα-deficient (KO, black) MEFs cultured with (+) or without (−) 25 μM A-76 for 16h. Data were normalized to untreated levels for both wild-type and AMPKα-deficient cells.

D) Relative expression of *Ppargc1a* mRNA transcript in AMPKα-deficient MEFs transduced with control (white) or PGC-1α-expressing (black) vectors. Transcript expression was determined by qPCR, expressed relative to *actin* mRNA levels for triplicate samples, and normalized relative to expression levels in AMPKα-deficient control cells.

E) Histograms of mitochondrial ROS levels in AMPKα-deficient MEFs expressing control or PGC-1α-expressing vectors. Cells were cultured with or without 25 mM glucose for 24 hours, and mitochondrial ROS levels were determined by MitoSOX staining. Histograms display representative flow cytometry plots for MitoSOX staining for cells treated as indicated.
Figure S3, Related to Figure 4. Effects of antioxidants and PGC-1α expression on cellular ROS levels and metabolism.

A-B) Effect of antioxidant treatment on cellular ROS levels. MEFs were incubated with ascorbate (A) or N-acetyl-cysteine (NAC, B) for 1h, and total cellular ROS levels were measured by DCFFDA staining and flow cytometry. Shown are the MFI of DCFDA staining for cells (n = 3) treated with ascorbate (A) or NAC (B).

Figure S3, Related to Figure 4. Effects of antioxidants and PGC-1α expression on cellular ROS levels and metabolism.

Rabinovitch et al., Figure S3
C) Relative mRNA expression of glycolytic genes in AMPKα-deficient MEFs cultured with (black) or without (white) Trolox (1 mM) for 1h. Transcripts are expressed relative to actin mRNA levels, and normalized relative to expression levels in AMPKα-deficient control cells.

D) Bioenergetic profile of PGC-1α-deficient MEFs. ECAR (left) and OCR (right) for wild type (WT) and PGC-1α-deficient (KO) MEFs grown under standard culture conditions. Data represent the mean ± SEM for biological replicates (n = 5).

*, p<0.05; **, p<0.01.