AMP-Activated Protein Kinase Induces a p53-Dependent Metabolic Checkpoint

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Summary

Repetitive cell division is an energetically demanding process that can be executed only if cells have sufficient metabolic resources to support a doubling of cell mass. Here we show that proliferating mammalian cells have a cell-cycle checkpoint that responds to glucose availability. The glucose-dependent checkpoint occurs at the G1/S boundary and is regulated by AMP-activated protein kinase (AMPK). This cell-cycle arrest occurs despite continued amino acid availability and active mTOR. AMPK activation induces phosphorylation of p53 on serine 15, and this phosphorylation is required to initiate AMPK-dependent cell-cycle arrest. AMPK-induced p53 activation promotes cellular survival in response to glucose deprivation, and cells that have undergone a p53-dependent metabolic arrest can rapidly reenter the cell cycle upon glucose restoration. However, persistent activation of AMPK leads to accelerated p53-dependent cellular senescence. Thus, AMPK is a cell-intrinsic regulator of the cell cycle that coordinates cellular proliferation with carbon source availability.

Introduction

Mammalian cells depend on extracellular cues for the regulation of growth, proliferation, differentiation, and survival. External signals provided by growth factors regulate mammalian cell homeostasis in part by activating signaling cascades that regulate nutrient uptake, thus matching cellular demand to the maintenance of bioenergetics. The insulin-like growth factor (IGF)/insulin receptor pathway in particular supports cellular growth by controlling the ability of a cell to take up nutrients, including glucose (for review, see Plas et al., 2002). Mutations in components of this pathway, notably activation of phosphatidylinositol 3'-kinase (PI3K) or Akt or loss of tumor suppressors PTEN, TSC1, or TSC2, renders cells able to take up nutrients in a cell-autonomous fashion, allowing cells access to the nutrients needed to support the bioenergetic demands of increased growth and proliferation (Shamji et al., 2003).

Deregulation of nutrient uptake may be a key step enabling transformation.

In addition to extracellular signals, intracellular nutrient levels may also regulate cell growth and proliferation by modulating the activity of nutrient-regulated kinases (Wilson and Roach, 2002). During times of nutrient or environmental stress, mammalian cells sense intrinsic energy levels and attempt to restore bioenergetic homeostasis through compensatory changes in the regulation of intermediate metabolism. In response to increases in the ratio of ADP to ATP, the enzymatic activity of adenylate kinase converts two molecules of ADP into ATP and AMP, thus restoring an effective ATP:ADP ratio sufficient to maintain energy-dependent cellular processes and generating AMP as a by-product. Since a rise in AMP is a consequence of a declining ATP:ADP ratio, the ratio of AMP to ATP is an indicator of overall energy charge. AMP stimulates a pathway of energy conservation by activating the AMP-activated protein kinase (AMPK) (for review, see Hardie et al., 1998). AMPK is regulated in vivo by the build-up of cellular AMP and phosphorylation by an upstream complex containing the tumor suppressor LKB1 (Hawley et al., 2003; Woods et al., 2003). Once triggered, AMPK activates catabolic pathways and inhibits anabolic pathways through direct phosphorylation of metabolic enzymes or by inducing alterations in gene expression. The net result of this AMP-induced signaling pathway is the engagement of ATP-generating pathways and inhibition of ATP-consuming processes (Carrling, 2004). Since AMPK activation is directly linked to the energy status of the cell, it is often considered to be a cellular fuel gauge. In addition to its metabolic effects, AMP accumulation has also been suggested, directly or indirectly, to have effects on cell growth and proliferation. Treatment of cells with adenine analogs that mimic AMP have been reported to induce cell-cycle arrest (Imamura et al., 2001). Recent work has uncovered a link between AMPK and TSC2 in the inhibition of translation during energetic stress (Inoki et al., 2003), indicating that AMPK may serve to link intracellular energy levels to the regulation of protein synthesis.

Limitation of low-molecular-weight nutrients was noted to inhibit cell proliferation over 30 years ago (Holley and Kiernan, 1974; Pardee, 1974), although the mechanisms underlying this process have remained unclear. The simplest model to explain this phenomenon is one of substrate limitation—cells simply cannot grow or proliferate when metabolic building blocks are in short supply. Alternatively, nutrient levels may tether cellular metabolism to endogenous pathways of cell-cycle control as a means to maintain cell viability when cells lack sufficient nutrients to support proliferation. To investigate whether specific forms of nutrient availability are coupled to cell-cycle control, we studied whether or not depletion of specific defined nutrients can induce cell-cycle arrest under conditions where the remaining nutrients are sufficient to maintain cell survival and cell growth. In this work, we demonstrate that glucose availability directly regulates cell proliferation and
Glucose Limitation Induces a G₁ Cell-Cycle Arrest

(A) Glucose limitation blocks cell proliferation. Growth curve of primary MEFs cultured for 6 days under conditions of 25 or 1 mM glucose. The data presented are the mean ± the standard deviation (SD) of three independent experiments.

(B) Glucose limitation inhibits cell-cycle entry. MEFs synchronized in G₀/G₁ by culture in 0.1% serum were induced to enter S phase by addition of media containing 10% serum and various concentrations of glucose. The percentage of cells in the S or G₁ phase was measured by BrdU/PI staining at 24 hr. The data presented are the mean ± SD for triplicate samples.

(C) Activation of AMPK in MEFs in response to low glucose. MEFs were treated for 2 hr in the presence of 25 or 1 mM glucose. Cell lysates were analyzed for phospho-T₁₇₂ AMPK, phospho-S₇₉ ACC, total AMPK, and actin levels by Western blot.

(D) Growth curve of MEFs in the presence of high-glucose media (25 mM) containing 0.5 mM AICAR. The data represent the mean ± SD for triplicate samples.

(E) AICAR induces proliferative arrest at the G₁/S cell-cycle checkpoint. MEFs were synchronized at G₀/G₁, then induced to enter S phase by addition of complete media (10% serum, 25 mM glucose) containing the indicated concentrations of AICAR. S or G₁ phase cells were determined at 24 hr with BrdU/PI staining. The data represent the mean ± SD.

that the metabolic sensor AMPK is a mediator of this process. Cells treated with low glucose or transduced with a constitutively active form of AMPK arrest in the G₁ phase of the cell cycle. Induction of this arrest is p53 dependent, and p53 activation is required to promote cell survival under conditions of glucose deprivation. The p53-dependent metabolic arrest in response to glucose deprivation is rapidly reversible upon readdition of glucose. In contrast, p53-deficient cells fail to arrest in response to AMPK activation and continue to proliferate under low-glucose conditions, leading to a rapid decline in cell viability. Activation of AMPK leads to phosphorylation of p53 at serine 15 (serine 18 of mouse p53), and this phosphorylation event is essential for mediating the effects of AMPK on p53-dependent cell-cycle arrest. These findings indicate that AMPK is a cell-intrinsic metabolic sensor that couples glucose availability to the cell-cycle machinery of a proliferating cell. This discovery highlights a previously unappreciated relationship between glucose metabolism and cell proliferation and implicates p53 as an essential component of metabolic cell-cycle control.

Results

Glucose Limitation Restricts Cell-Cycle Progression

Whether the availability and metabolism of specific nutrients contributes to control of the mammalian cell cycle is not known. Here we investigated the effects of glucose limitation on cell proliferation in otherwise complete medium. Primary mouse embryonic fibroblasts (MEFs) cultured in medium containing 1 mM glucose accumulated at a lower rate than cells incubated in full-glucose media (25 mM glucose) (Figure 1A).

When serum-deprived cells were induced to reenter the cell cycle, their ability to traverse the G₁/S boundary became progressively impaired as extracellular glucose was reduced (Figure 1B).

Proliferating mammalian cells use glucose as their primary fuel source for ATP production (Zong et al., 2004). Glucose availability is monitored in lower organisms by the bioenergetic sensor AMPK (Carlson et al., 1981). Decreases in extracellular glucose have also been shown to activate AMPK in mammalian cells (Salt et al., 1998). To test whether AMPK is activated at levels of extracellular glucose that impair mammalian cell proliferation, we measured the level of AMPK activation in MEFs cultured under low-glucose conditions (1 mM). Both the phosphorylation status and the activity of AMPK increased as cells were cultured in reduced glucose (Figure 1C). AMPK became phosphorylated on Thr-172, and increased Ser-79 phosphorylation of the AMPK substrate acetyl CoA carboxylase (ACC) was observed when cells were cultured at 1 mM glucose.

As an initial test of the role of AMPK activation in the cellular response to decreased glucose availability, we treated cells in glucose-replete medium with the AMPK activator 5-aminoimidazole-4-carboxamide riboside (AICAR). Even in 25 mM glucose, AICAR-treated cells failed to accumulate (Figure 1D). G₀/G₁-arrested MEFs also exhibited a dose-dependent block in the G₁ to S progression when stimulated with serum in the presence of AICAR (Figure 1E).

Glucose Restriction Induces Activation of a Reversible Cell-Cycle Checkpoint

One mechanism that can limit cell accumulation in proliferating cultures is the induction of apoptotic cell death. Glucose depletion has been reported to induce apoptosis through activation of the proapoptotic molecule Bax (Rathmell et al., 2003). However, significant levels of apoptosis were not observed at concentrations of extracellular glucose that induce a near com-
AMPK Regulates p53-Dependent Cell-Cycle Arrest

Figure 2. Glucose Limitation Invokes a Reversible Cell-Cycle Checkpoint

(A) Cell viability is maintained under low-glucose conditions. G_{0}/G_{1}-synchronized MEFs were induced to proliferate by addition of media containing 10% serum and various concentrations of glucose. Cell viability was measured 24 hr following addition of serum by PI exclusion. The data presented are the mean ± SD.

(B) Low glucose promotes an mTOR-dependent increase in cell size. MEFs were cultured in the presence of low glucose (0.5 mM) for 24 hr, and cell size was measured by forward scatter (FSC) of G_{1} phase cells. Cell size of MEFs grown in full glucose (25 mM) is indicated in gray and test conditions in white. mTOR-specific growth was measured by culturing cells in the presence of 20 nM rapamycin (RAPA). A representative experiment is shown. Cell proliferation was measured by BrdU incorporation, and the percentage of BrdU+ cells (mean ± SD) was determined by flow cytometry.

(C) AICAR elicits dose-dependent effects on cell size. MEFs were cultured in the presence of low (0.5 mM) or high (2.0 mM) AICAR in medium containing full glucose (25 mM) for 24 hr, and cell size was determined by forward scatter (FSC) of G_{1} phase cells. Cell size of MEFs grown in the absence of AICAR (control, 25 mM glucose) is indicated in gray and test conditions in white. mTOR-specific growth was measured by culturing cells in the presence of 20 nM rapamycin (RAPA).

(D) Proliferative arrest induced by glucose limitation is reversible. MEFs were grown for 24 hr in media containing 25 or 1 mM glucose, washed with glucose-free media, then cultured in complete media containing 25 mM glucose. Cells undergoing active DNA synthesis before and after the switch to 25 mM glucose conditions were measured by BrdU incorporation. The percentage of BrdU+ cells is quantified in the upper right corner of each panel.

Recent evidence has demonstrated that ATP depletion can suppress cell growth by inhibiting protein translation, a process initiated by an AMPK-TSC1-TSC2-dependent pathway (Inoki et al., 2003). A reduced translation rate could lead to prolongation of the G_{1} phase of the cell cycle by reducing the rate of cell growth that normally accompanies proliferation. To assess whether glucose depletion alone affects cell growth, we measured the cell size of MEFs cultured...
under low-glucose conditions. Surprisingly, when MEFs were switched into medium containing low glucose for 24 hr, they exhibited a larger cell size than cells maintained in standard medium, even though they underwent a cell-cycle arrest (Figure 2B).

To test whether the size increase induced by glucose limitation was regulated by the activity of mTOR, we analyzed the growth of glucose-restricted cells in the presence of the TOR inhibitor rapamycin. Treatment with rapamycin completely suppressed the size increase observed when cells were cultured in low glucose in the presence of a full complement of amino acids (Figure 2B). Similar results were observed when cells were treated with 0.5 mM AICAR in the presence of complete medium (Figure 2C). At this level of AICAR, a G1/S arrest was observed while rapamycin-sensitive cell growth was maintained. However, at higher doses of AICAR, the cell-cycle arrest was associated with a progressive inhibition of protein translation and a loss of the rapamycin-sensitive increase in cell size in the p53−/− arrested cells (Figure 2C). Together, these results demonstrate that low extracellular glucose can induce a cell-cycle arrest under conditions where TOR remains active and continues to stimulate cell growth.

Mammalian cells can also respond to cellular stresses by initiating specific cell-cycle checkpoints that serve to maintain viability and functional integrity until the stress is corrected or removed. To assess whether the proliferative checkpoint triggered by low-glucose stress was reversible, MEFs arrested by glucose restriction were assessed for their ability to initiate proliferation upon the readdition of glucose. Within 24 hr of increasing glucose levels from 1 mM to 25 mM, cells were able to fully recover and maintain rates of DNA synthesis comparable to cells grown continuously under full-glucose conditions (Figure 2D).

AMPK Activity Is Sufficient to Induce Cell-Cycle Arrest

To address whether AMPK activity is sufficient to mediate cell-cycle arrest under normal growth conditions, we expressed a constitutively active mutant of the AMPKα2 catalytic subunit (CA-AMPK) in primary MEFs and assessed their proliferative capacity. Similar to cells exposed to AICAR or low glucose, MEFs expressing CA-AMPK were impaired in their ability to proliferate as judged by growth curves (Figure 3A). This impairment correlated with a reduced percentage of cells in S phase as measured by BrdU incorporation (Figure 3B).

AMPK Requires p53 to Induce Cell-Cycle Arrest

The results described to this point suggest that activation of AMPK, either through pharmacological or genetic means, can induce a reversible cell-cycle arrest. To assess whether AMPK activation is required to induce cell-cycle arrest under low-glucose conditions, AMPK activity was blocked using a dominant-negative form of AMPK (DN-AMPK) (Mu et al., 2001). MEFs infected with a retroviral vector expressing DN-AMPK displayed a 3-fold higher rate of S phase entry when grown under low-glucose conditions than control-infected cells (Figure 4A). A similar result was observed when MEFs expressing DN-AMPK were treated with AICAR (data not shown). The effect of AMPK was specific to low-glucose stress, as DN-AMPK-expressing cells arrested to the same extent as controls when treated with γ radiation (Figure 4A). MEFs deficient for the tumor suppressor p53 were included in these experiments as a negative control for γ radiation-induced arrest. As expected, p53−/− deficient MEFs failed to arrest in response to γ radiation. However, p53−/− deficient MEFs also displayed resistance to arrest under low-glucose conditions (Figure 4A).

p53 is a central cell-cycle checkpoint protein that can initiate cell-cycle arrest at either the G1/S or G2/M checkpoint in response to cellular stress. The continued ability of p53−/− deficient MEFs to proliferate under low-glucose conditions suggested that p53 might mediate the checkpoint response induced by glucose limitation. To assess the role of p53 in the glucose-sensitive checkpoint response, we examined the ability of p53−/− deficient MEFs to bypass the G1/S checkpoint triggered by glucose restriction. Unlike their wild-type counterparts, p53−/− MEFs were unable to initiate the cell-cycle arrest normally induced by low extracellular glucose and readily traversed the G1/S boundary under these conditions (Figure 4B). Proliferation of p53−/− deficient MEFs was suppressed to levels comparable to wild-type cells only under conditions of complete glucose withdrawal.

The observation that p53−/− MEFs have a defect in their ability to undergo cell-cycle arrest in response to glucose restriction raised the possibility that p53 was required for the G1 arrest induced by AMPK activation. To address this possibility, MEFs lacking p53 or their wild-type counterparts were infected with a control retrovirus (Vec) or a retrovirus encoding constitutively active AMPK (CA-AMPK), and their proliferation in culture was measured. Wild-type cells displayed a proliferative disadvantage when expressing active AMPK, while p53−/− MEFs expressing AMPK accumulated at a rate comparable to cells infected with the control vector (Figure 4C). p53−/− MEFs infected with CA-AMPK...
AMPK Regulates p53-Dependent Cell-Cycle Arrest

Figure 4. AMPK Requires p53 to Induce Cell-Cycle Arrest

(A) DN-AMPK prevents cell-cycle arrest induced by low glucose but not DNA damage. MEFs infected with control vector (Vec) or vector expressing dominant-negative AMPK (DN-AMPK) were incubated in low glucose (0.5 mM), and BrdU incorporation at 24 hr was measured. For arrest induced by DNA damage, MEFs were treated with 5.5 Gy of γ radiation (γ-Rad), and BrdU incorporation was measured after 16 hr. Uninfected p53−/− MEFs were included to control for stress-induced cell-cycle arrest. All values are calculated as a percentage of untreated controls and presented as mean ± SD.

(B) Loss of p53 disrupts the G1 arrest checkpoint induced by glucose restriction. Wild-type or p53−/− MEFs synchronized in G0/G1 by culture in 0.1% serum were induced to enter S phase by addition of media containing 10% serum, 65 μM BrdU, and various concentrations of glucose. Cells that have progressed into S phase after 24 hr were determined by BrdU incorporation (mean ± SD).

(C) Growth curves of p53+/+ and p53−/− MEFs infected with control retrovirus (Vec) or active AMPK (CA-AMPK). The data represent the mean ± SD for triplicate samples.

(D) p53−/− MEFs are resistant to AICAR-induced arrest. G0/G1-synchronized wild-type or p53−/− MEFs were induced to enter S phase by addition of complete medium in the presence (+) or absence (−) of 0.5 mM AICAR. Cells were treated continuously with 65 μM BrdU, and the percentage of BrdU+ cells (mean ± SD) was measured after 24 hr.

p53 Promotes Cell Viability in Response to Glucose Deprivation

An essential feature of an adaptive cell-cycle checkpoint is to enhance the ability of cells to survive an imposed insult. However, previous studies have suggested that p53 activation induces apoptosis. To investigate whether p53 activation affects the ability of cells to survive glucose deprivation, the viability of p53+/+ and p53−/− MEFs cultured in the absence of glucose was determined. Surprisingly, cells expressing wild-type p53 displayed a survival advantage over p53-deficient cells when deprived of glucose (Figure 5, closed bars). Moreover, p53+/+ MEFs cultured first under low-glucose conditions displayed a greater resistance to apoptosis upon removal of glucose, while p53−/− MEFs exposed first to low-glucose conditions were not protected from apoptosis induced by complete depletion of extracellular glucose (Figure 5, open bars). Together, these data indicate that glucose restriction promotes an adaptive response that enhances the ability of cells to survive further glucose deprivation. p53 is required to mediate this adaptive response.

AMPK Promotes p53 Activity through Phosphorylation at Ser-15

Given the dependence of AMPK on p53 to induce cell-cycle arrest, the possibility that AMPK may modulate
Figure 6. AMPK Promotes p53 Phosphorylation at Ser-15

(A) Wild-type MEFs infected with control vector (Vec) or vector expressing active AMPK (CA-AMPK) were analyzed by Western blot for p53 protein expression. Expression of myc-tagged CA-AMPK was detected using anti-myc antibody.

(B) AMPK activates p53-responsive promoters. Wild-type (p53+/+) or p53 null (p53−−) MEFs were transfected with empty vector or active AMPK (CA-AMPK) along with a luciferase reporter containing tandem p53 consensus binding sites (pG13) or the human p21 reporter (pWWP). Promoter activity is expressed as the ratio of luciferase to renilla activity (RLU, relative light units) 48 hr posttransfection. The data represent the mean ± SD for triplicate samples.

(C) The AMPK phosphorylation site in rat ACCα and potential sites found in human and mouse p53.

(D) AICAR induces p53 phosphorylation. Human 293T cells were treated with various concentrations of AICAR for 24 hr, and the level of phospho-Ser-15 p53 was examined by Western blot.

(E) AMPK activity induces Ser-18 phosphorylation of p53. H1299 cells were cotransfected with mouse p53 and active AMPK (CA-AMPK) or control vector. Levels of phospho-Ser-18 p53, p53, and p21 were determined 48 hr posttransfection by Western blot.

(F) AMPK kinase activity promotes phosphorylation of p53 at Ser-18. Wild-type (myc-AMPK) or dominant-negative (myc-DN-AMPK) AMPK
p53 activity was investigated. Levels of endogenous p53 protein were elevated in cells expressing active AMPK (Figure 6A). In addition, p53-dependent transcription was strongly induced by active AMPK in wild-type MEFs (p53+/+)

Finally, to assess whether AMPK-dependent p53 phosphorylation is required for AMPK-induced cell-cycle arrest, the ability of AMPK to induce cell-cycle arrest in p53S18A/S18A MEFs was examined. In contrast to wild-type MEFs in which CA-AMPK induces a proliferative arrest, p53S18A/S18A mutant MEFs expressing CA-AMPK failed to undergo cell-cycle arrest and proliferated at a similar rate to controls (Figure 6I).

p53 Controls AMPK-Induced Cellular Senescence

The induction of a persistent G1 arrest in the face of continued ability to undertake translation and cell growth is a hallmark of cellular senescence. Recent evidence has associated a progressive bioenergetic compromise with this process (Zwerschke et al., 2003). To assess the influence of AMPK activity on replicative senescence, constitutively active (CA-AMPK) and dominant-negative (DN-AMPK) mutants of AMPK were introduced into low-glucose primary MEFs by retroviral-mediated gene transfer, and the infected cells were subjected to serial passage following a modified 3T3 protocol. Growth curves of the infected MEFs demonstrated that elevated AMPK activity induced a premature proliferative arrest in primary MEFs, while suppression of AMPK activity extended replicative life span (Figure 7A).

p53 has been implicated as a critical component of the senescence program (Itahana et al., 2001). Given the link established between AMPK and p53, the involvement of AMPK in the replicative senescence of primary MEFs lacking p53 was examined. Constitutively active AMPK was introduced into p53−/− or p53+/− MEFs, and growth curves for each genotype were established.
Figure 7. AMPK Induces p53-Dependent Premature Senescence in MEFs

(A) Serial passage of primary MEFs expressing AMPK mutants. Early passage MEFs from p53+/+ embryos infected with vector expressing dominant-negative AMPK (DN-AMPK), constitutively active AMPK (CA-AMPK), or control vector (Vec) were subjected to serial passage by a modified 3T3 protocol. Population doublings (PDL) at each time point are expressed as the mean ± SD for triplicate cultures.

(B) AMPK cannot induce senescence in p53-deficient MEFs. Early-passage MEFs from p53+/− or p53−/− embryos infected with control vector (Vec) or vector expressing active AMPK (CA-AMPK) were subjected to serial passage following selection. Population doublings (PDL) are plotted as a function of time.

(C) Senescence-associated β-galactosidase activity in MEF cultures. p53+/− or p53−/− MEFs infected with vector control (Vec) or active AMPK (CA-AMPK) were replated after passage 4 and then stained for SA-β-gal activity. Representative images are shown.

(D) Quantitation of SA-β-gal-positive cells from (C). The data represent the mean ± SD for triplicate samples.

Discussion

Initiation of cell division commits cells to a metabolically demanding process. Recently, it has been suggested that mammalian cells require specific extracellular signals to license their ability to take up nutrients. If true, this suggests that there exist mechanisms by which cells coordinate proliferation relative to their ability to take up specific extracellular metabolites. To successfully undergo cell division, cells must have a sufficient supply of essential nutrients to support both ATP production and macromolecular synthesis. We report here that the G1/S transition in mammalian cells is tethered to glucose availability by AMPK. Glucose restriction can induce a reversible cell-cycle arrest in the presence of sufficient growth factors and amino acids to support TOR-dependent growth. Induction of AMPK activity results in cell-cycle arrest at levels of extracellular glucose that are still capable of supporting the proliferative expansion of cells. The ability of AMPK to induce cell-cycle arrest is dependent on p53. The coupling of glucose availability to cell-cycle progression has the hallmarks of a nutrient-sensing pathway that turns off cell proliferation before glucose availability drops to levels that cannot sustain cell viability. The existence of such a glucose-sensitive cell-cycle checkpoint may act as a buffer to prevent cells from accumulating to levels that are not ultimately sustainable. Disruption of this checkpoint through loss of p53 permits unchecked proliferation despite limiting nutrients but ultimately leads to decreased cell viability. By promoting senescence, AMPK-dependent activation of p53 may promote the conservation of the remaining available glucose to support the survival and physiologic function of the existing cell.
In addition to the cell-cycle effects demonstrated here, recent work indicates that AMPK activation can also regulate protein translation. AMPK activity suppresses translation by inhibiting the activities of elongation factor-2 (eEF2) or ribosomal S6-Kinase (S6K). In addition, AMPK can phosphorylate TSC2 in response to acute energy starvation, leading to stabilization of the TSC1-TSC2 complex and an inhibition of mTOR, S6K, and elf4E signaling (Inoki et al., 2003). The tumor suppressor LKB1 acts upstream of AMPK to mediate these effects (Corradetti et al., 2004; Shaw et al., 2004). These results have raised the possibility that an AMPK-dependent reduction in translation could prolong the G1 phase of the cell cycle and thus slow proliferation. However, our observation that glucose levels of 0.5–1 mM promote AMPK-dependent cell-cycle arrest associated with TOR-dependent increases in cell size suggest that AMPK can mediate its cell-cycle effects without significantly affecting translation. Together these observations suggest that cell cycle and cell size are differentially controlled by nutrient availability. Under the conditions reported here, proliferation is more sensitive to glucose limitation than is growth control. While complete depletion of glucose can inhibit translation by either AMPK-dependent inhibition of mTOR (Inoki et al., 2003) or by inducing an ER stress response (Kaufman et al., 2002), the cell-cycle arrest observed here occurs at glucose levels that are able to sustain proliferative expansion of MEFs either deficient for p53 or in which AMPK activity is suppressed.

The tumor suppressor p53 is one of the primary genes that regulates cellular commitment to DNA replication and cell division (Vogelstein et al., 2000). The present data indicate that p53 plays an essential role in the cell-cycle arrest triggered by glucose limitation. Like cells with impaired AMPK activity, p53 null MEFs fail to arrest in low-glucose conditions. The dependence of AMPK on p53 for its antiproliferative effects is supported by the resistance of p53-deficient cells to proliferative arrest induced by AMPK activation, either by low glucose, AICAR, or overexpression of active AMPK. Thus, AMPK appears to activate a nutrient-sensitive signaling pathway that initiates p53-dependent cell-cycle arrest during times of energy deficiency. Activation of this stress pathway appears to be specific to nutrient limitation; inhibition of AMPK activity does not antagonize other p53-dependent stress responses such as cell-cycle arrest induced by DNA damage. Thus, p53 can serve as a metabolite sensor and coordinate reversible cell-cycle arrest in response to glucose deprivation. Surprisingly, although the activation of p53 induced by DNA damage is associated with reduced survival, the metabolic activation of p53 is associated with the enhanced ability of cells to survive progressive glucose depletion. Based on these results, we propose that the ability of AMPK to induce p53 activity represents a key integration point between the cellular metabolic state and the cell cycle.

p53 activity is maintained at low levels in normal cells through the activity of Mdm2, which decreases the function of tetrameric p53 and targets it for degradation by the proteasome (Haupt et al., 1997; Kubbutat et al., 1997). Posttranslational modifications triggered following cellular insult, including phosphorylation at serine 15 (serine 18 in mouse), allow p53 to escape negative regulation. Phosphorylation of p53 at Ser-15 in the N-terminus promotes p53 stabilization in part by disrupting the p53-Mdm2 interaction (Shieh et al., 1997). Moreover, phosphorylation at this site promotes the recruitment of cofactors such as p300 (Lambert et al., 1998), which acetylate p53 and further augment its ability to induce cell-cycle arrest (Barlev et al., 2001). Phosphorylation of mouse p53 at Ser-18 is a critical event in the p53 response to DNA damage (Chao et al., 2000). This process is mediated by the ataxia telangiectasia-mutated (ATM) kinase, which targets p53 phosphorylation at this residue in response to DNA damage (Banin et al., 1998; Canman et al., 1998). AMPK activation results in phosphorylation of this same serine residue in response to energetic stress. In the absence of bioenergetic stress, treatment of cells with AICAR can lead to p53 phosphorylation (Imamura et al., 2001). Consistent with a role for AMPK in p53 phosphorylation, we find that this AICAR-dependent p53 phosphorylation is not blocked by caffeine, an inhibitor of ATM kinase activity, suggesting that the effect of AICAR on Ser-15 phosphorylation is ATM independent (data not shown). The inability of AMPK to induce cell-cycle arrest when Ser-18 of mouse p53 is mutated confirms that this phosphorylation event is critical for control of proliferation when glucose levels are low. This response is likely modified by further changes in the metabolic state: SIRT1 has been shown to regulate p53 action through deacetylation in an NAD-regulated fashion (Luo et al., 2001; Vaziri et al., 2001).

Replicative senescence is proposed to be a tumor suppressor mechanism in response to bioenergetic stress (Ben-Porath and Weinberg, 2004). Environmental factors that place oxidative stress on cells promote the early onset of senescence (Parrinello et al., 2003). Such stresses cause cells to shuttle available glucose from the glycolytic pathway to the pentose phosphate shunt to increase the production of reducing equivalents. Such a change would reduce the available intracellular glucose required to support ATP production. Consistent with this, the AMP:ATP ratio increases by approximately 30-fold in senescent cells (Zwerschke et al., 2003). This increased level of AMP could lead to AMPK-dependent activation of p53 as documented here. The tumor suppressor LKB1 is likely involved in this pathway, as Lkb1−/− MEFs, which are impaired in their ability to activate AMPK (Hawley et al., 2003), do not senesce (Bardeesy et al., 2002). Activation of a p53-dependent metabolic cell-cycle checkpoint by AMPK enables cells to survive unfavorable growth conditions. To this end, the involvement of AMPK in the regulation of senescence may at least partially explain its role as a tumor suppressor (Kato et al., 2002).

Experimental Procedures

Materials and DNA Constructs

Myc-tagged AMPKα2 cDNA constructs (wild-type and dominant-negative K45R mutant) have been described previously (Mu et al., 2001). Active AMPK (CA-AMPK) was generated by introducing a T172D mutation into AMPKα2 truncated at residue 312. All AMPK cDNAs were subcloned into pcDNA3 or the MSCV-based murine retroviral vector MlgC8. A S18A mutation was introduced into the
mouse p53 sequence by PCR. The PCR products encoding GST fusion proteins GST-SAMS (SAMS AMPK plasmid (wild-type AMPK or DN-AMPK) or control plasmid, and GST-p53 S18A) were generated by PCR. 5-aminoimidazole-4-carboxamide ribo-
side (AICAR) was obtained from Toronto Research Chemicals.

**Cell Lines, Transfection, and Retroviral Transduction**

Primary MEFS (p53+/+, p53−/−, and p53−/−) were prepared from day E13 embryos and passaged as previously described (Zong et al., 2004). p53STAG104/104 MEFS were generated as previously described (Chao et al., 2003). Transfections were conducted using Lipofec-
tamine 2000 (Invitrogen) or Fugene6 (Roche). High-titre retrovirus was generated by transfecting retroviral vector (20 μg) and packag-
ing virus DNA (10 μg) into 293T cells using standard calcium phos-
phate transfection methods. Virus was collected 48 and 72 hr post-
transfection and cleared prior to use. MEFS (7.5 × 10^5/10 cm dish) were infected four times over 2 days using 4 ml of retroviral superna-
tant and 8 μg/ml polybrene. CD8+ cells were sorted 4 days post-
fection and grown for 2 days following sorting prior to use in proliferation assays. The level of infection was measured by flow cytometry using anti-human CD8 antibodies (eBioscience).

**Measurement of Cell Proliferation, Cell-Cycle Analysis, and Cell Viability**

Cell proliferation curves in all MEF lines were determined by cell counting using trypan blue exclusion. For low-glucose culture con-
ditions, cells were washed twice with PBS, then cultured in glu-
cose-free DMEM (Invitrogen) supplemented with 10% dialyzed FBS (dFBS) (Invitrogen) and concentrations of glucose as indicated. Ra-
diation-induced cell-cycle arrest was measured 16 hr following ex-
position of cells to 5.5 Gy of γ radiation. Actively proliferating cells were assayed by incorporation of BrdU. For cell-cycle analysis, MEFs (1 × 10^3/30 mm dish) were pulsed with BrdU, then trypsin-
ized, fixed in 70% ethanol, labeled with anti-BrdU antibodies and propidium iodide (Sigma), and analyzed by flow cytometry. Cell via-
bility was determined by flow cytometry using PI as a viability dye.

**Determination of Cell Size**

Cell size of viable MEFS was measured by flow cytometry. MEFS were trypsinized and resuspended in FACS buffer (phosphate-buf-
fered saline containing 1.5% BSA and 0.02% sodium azide) con-
taining 1 μg/ml Hoechst 33342, and the mean FSC intensity was measured for G1 phase cells using an LSR flow cytometer (Becton Dickinson).

**Western Blots**

Cells were lysed in modified RIPA buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-Cl (pH 8.0), 0.1% SDS) supplemented with the following protease and phosphatase inhibitors: Na3VO4 (100 μM), PMSF (1 mM), leupeptin (1 μg/ml), benzamidine (1 μg/ml). Cleared lysates were resolved by SDS-PAGE and transferred to nitrocellulose. Blots were probed as previously described (Rathmell et al., 2003) using the following antibodies: anti-p53 (Ab7) (Oncogene Research Products), anti-p53 (FL-393), anti-p21 (C-19), anti-actin (l-19) (Santa Cruz Biotechnology), anti-myc tag, anti-p53 (total and phospho-
Ser-15), anti-p21, and anti-His tag (Cell Signaling Technology). Rab-
bit polyclonal antibodies against total AMPK and phospho-Thr-172 AMPK have been described (Mu et al., 2001).

**Promoter Assays**

AMPK-dependent activation of p53-responsive promoters was as-
sessed by luciferase assay using a Dual Luciferase Assay kit (Pro-
mega). 3T3 MEFS (wild-type p53 or p53−/−) were plated on 2 × 10^6 cells/35 mm dish and transfected 12-16 hr later with 1 μg CA-
AMPK plasmid or vector control, 0.75 μg promoter construct (p53-
responsive promoter p03 or human p21 promoter pWWP [el-Deiry et al., 1993]), and 0.25 μg Renilla control. Luciferase activity was measured 48 hr posttransfection on a TRIT1 Luminometer (Tropix).

**AMPK Kinase Assay**

293T cells (5 × 10^5/10 cm dish) were transfected with 20 μg of AMPK plasmid (wild-type AMPK or DN-AMPK) or control plasmid, and cells were harvested 24 hr later. Cells were lysed in 50 mM Tris/HCl (pH 7.4), 250 mM mannitol, 50 mM NaF, 1 mM sodium pyrophosphate, 1 mM benzamidine, 1 mM PMSF, 5 μg/ml trypsin inhibitor, and 0.5% Triton X-100, and cleared by centrifugation, and the supernatant fraction was preclari-
ated with protein-G agarose beads (50:50 slurry) for 2 hr. The beads were washed and used in a kinase reaction in HEPES-
Brij buffer containing 5 mM MgCl2, 0.2 mM ATP with 0.5 μCi/μl [γ-32P]ATP, 0.3 mM AMP, and 1 μg of recombinant protein substrate (produced as described by Canman et al. [1998]). Recombinant GST-p53 fusion proteins containing the first 98 amino acids of mouse p53 (GST-p53[1-98]) or mutant GST-p53 S18A(1-98) were used as substrates. Recombinant GST-SAMS and GST-SAMSp fu-
sion proteins were used to control for AMPK kinase activity.

**Serial Passage and Detection of Senescent Cells**

Early-passage MEFS (4-passage MEFS) were infected with supernatants containing MigCD8, MigCD8-DN-AMPK, or MigCD8-CA-AMPK retrovirus, sorted 3 days postinfection, and subjected to serial pas-
sage according to a modified 3T3 protocol. Population doublings (PDL) per passage were calculated using the following formula: \[ \Delta \text{PDL} = \log_2(C_F/C_I) \] where \( C_I \) is the initial number of cells and \( C_F \) is the number of cells after 3 days of culture. Senescent cells were detected by measuring the percent β-galactosidase-positive cells in three separate fields.

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