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GAMT, a p53-Inducible Modulator of Apoptosis, Is Critical for the Adaptive Response to Nutrient Stress

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SUMMARY

The p53 tumor suppressor protein has a well-established role in cell-fate decision-making processes. However, recent discoveries indicate that p53 has a non-tumor-suppressive role. Here we identify guanidinoacetate methyltransferase (GAMT), an enzyme involved in creatine synthesis, as a p53 target gene and a key downstream effector of adaptive response to nutrient stress. We show that GAMT is not only involved in p53-dependent apoptosis in response to genotoxic stress but is important for apoptosis induced by glucose deprivation. Additionally, p53→GAMT upregulates fatty acid oxidation (FAO) induced by glucose starvation, utilizing this pathway as an alternate ATP-generating energy source. These results highlight that p53-dependent regulation of GAMT allows cells to maintain energy levels sufficient to undergo apoptosis or survival under conditions of nutrient stress. The p53→GAMT pathway represents a new link between cellular stress responses and processes of creatine synthesis and FAO, demonstrating a further role of p53 in cellular metabolism.

INTRODUCTION

p53 is the most frequently inactivated tumor suppressor identified in human cancer and is activated in response to various cellular stresses (Vousden and Prives, 2009). Activation of p53 can induce cell responses such as cell-cycle arrest, senescence, and apoptosis that contribute to tumor suppression, either by maintaining genomic integrity or through the elimination of potentially oncogenic cells by apoptosis (Aylon and Oren, 2007). To date, emerging evidence indicates that p53 is capable of much broader cellular functions, including the regulation of energy metabolism and autophagy (Bensaad and Vousden, 2007; Crighton et al., 2006; Feng et al., 2005; Jones and Thompson, 2009). In response to nutrient stress, p53 is activated by AMP-activated protein kinase (AMPK), which promotes cell survival through the induction of a reversible cell-cycle checkpoint (Jones et al., 2005; Jones and Thompson, 2009). In addition, recent studies reveal that p53 can modulate the balance between glycolytic and respiratory pathways through the actions of TP53-induced glycolysis and apoptosis regulator (TIGAR) (Bensaad et al., 2006) or phosphoglycerate mutase (PGM) (Kondoh et al., 2005), and through the expression of synthesis of cytochrome *c* oxidase 2 (SCO2) (Matoba et al., 2006). Cells that lack functional p53 have enhanced glycolysis and show lower oxygen consumption by mitochondrial respiration, indicating a shift to glycolysis for the production of energy, thereby contributing to the metabolic change known as Warburg effect, which is characteristic of virtually all cancers (Bensaad and Vousden, 2007; Vander Heiden et al., 2009).

Creatine and phosphocreatine metabolism is involved in energy generating pathways that play an essential role in the regulation of ATP homeostasis (Wyss and Kaddurah-Daouk, 2000). Creatine is synthesized mainly in the liver and pancreas by two-step mechanism: (1) arginine:glycine amidinotransferase (AGAT) first forms ornithine and guanidinoacetate (GAA) from arginine and glycine, and (2) guanidinoacetate methyltransferase (GAMT) catalyzes S-adenosyl-L-methionine-dependent methylation of GAA to yield creatine and S-adenosyl-L-homocysteine. Creatine is then transported through the blood and taken up by the creatine transporter; thereafter, reversible phosphorylation of creatine by creatine kinase provides a high-energy ADP to ATP phosphate buffering system (Wyss and Kaddurah-Daouk, 2000). Due to the spontaneous conversion of creatine to creatinine (excreted in urine), the creatine pool must be maintained by daily nutritional intake and de novo synthesis. A GAMT deficiency syndrome has recently been described, which results from an inborn error of creatine biosynthesis. Manifestations of the disease include neurological and motor dysfunction, likely from abnormally high levels of GAA in the brain, highlighting the importance of creatine metabolism for normal psychomotor development and cognitive function in humans (Item et al., 2001;

Salomons et al., 2001; Stockler et al., 1994). Patients benefit temporarily from dietary creatine supplementation and arginine restriction, although these treatments do not return patients to normal health (Schulze et al., 2001; Stockler et al., 1996). With respect to cancer, previous studies reveal that brain-type creatine kinase is overexpressed in a wide range of solid tumors such as neuroblastoma, cervical cancer, and hepatocellular carcinoma (Choi et al., 2001; Meffert et al., 2005; Shatton et al., 1979) and that brain-type creatine kinase is negatively regulated by p53 (Zhao et al., 1994). Although these reports imply a connection between p53 and creatine metabolism, the relevance of this relationship is not yet fully understood. We anticipate that an increased understanding of the role of p53 in energy metabolism might provide critical clues toward creating new therapeutic targets for the treatment of cancer and metabolic disease.

In this study, we identify GAMT as a p53 target gene that functions as an effector of the adaptive response to nutrient stress. GAMT is required for p53-dependent apoptosis in response to genotoxic stress as well as glucose deprivation, which occurs via the intrinsic mitochondrial pathway. Of note, in response to glucose starvation, we demonstrate that p53 → GAMT regulates two cellular metabolic processes, creatine biosynthesis and fatty acid oxidation (FAO). The use of these pathways as energy sources suggests that p53 → GAMT may function to maintain ATP levels sufficient for survival or apoptosis when energy production by glycolysis is impaired, either due to the mitochondrial disruption caused by apoptosis via the intrinsic pathway (i.e., in response to DNA damage) or due to a lack of glucose altogether. Overall, our findings suggest that p53 can communicate with creatine biosynthetic and FAO pathways to coordinately regulate energy metabolism under conditions of nutrient stress.

RESULTS

Identification of GAMT as a p53-Inducible Gene

We identified GAMT as a p53-responsive gene through a cDNA expression array that compared gene expression in the presence or absence of p53. To confirm these data, we examined GAMT mRNA and protein expression in response to different types of stresses, in several cell lines, and in different p53 backgrounds. GAMT was induced under various stress conditions including DNA damage, oxidative stress, and γ-irradiation, with kinetics similar to those seen for established direct targets of p53 such as p21 (Figures 1A–1C and see Figure S1 available online). GAMT was also in the list for p53-inducible genes (PIGs) (Polyak et al., 1997) identified by SAGE analysis. Inhibition of p53 activation in response to DNA-damaging agents by siRNA targeting abrogated GAMT induction, demonstrating that GAMT induction in response to DNA damage is dependent upon p53 (Figure 1B, right panel). Murine GAMT also showed p53-dependent induction in response to exogenously expressed p53 and genotoxic stress (Figure 1D), which supports the concept that the regulation of GAMT by p53 is conserved between different species.

GAMT contains three potential p53-binding sites within an approximately 4.2 kb genomic region, 5′ of the GAMT translational start site (Figure 2A, top panel). We first investigated, using a reporter assay, whether GAMT is a direct p53 transcriptional target. Several deletions or mutations of this promoter sequence were generated and linked to the luciferase reporter gene of the pGL4 vector (Figure 2A). Cotransfection of the constructs together with a wild-type p53 (WT-p53) expression vector into p53 null Saos2 cells significantly increased luciferase activity, whereas cotransfection with mutant p53 (V143A) or pcDNA3.1 empty vector failed to do so (Figure 2A, left panel). Deletion of BS1, the most 5′ potential p53-binding site (P2), significantly reduced GAMT activity in response to WT-p53 (Figure 2A, left panel). In addition, the reporter construct mutated at the −4150 position (M1, GCT**C**AT**GCCT** → GCT**T**AT**AAAG**) showed a similar decrease of WT-p53-dependent transcriptional activity (Figure 2A, left panel). Similar results were obtained in reporter construct-transfected U2OS cells after endogenous p53 was activated by treatment with etoposide (ETO) (Figure 2A, right panel). We next performed an electrophoretic mobility shift assay (EMSA) to determine whether p53 can bind to the BS1 site of the human GAMT gene. With nuclear extracts from p53-transfected U2OS cells, we observed a shift with BS1, which was outcompeted by WT-BS1 oligonucleotides, but not by BS1 oligonucleotides containing mutations (Figure 2B and Figure S2). Furthermore, p53-binding activity to the BS1 was super shifted by anti-p53 antibody (Figure 2B). Using the same nuclear extracts, we also carried out EMSA experiments with p53 consensus (CS) BS oligonucleotides and observed similar shift with p53-CS BS (Figure 2B, right panel). In addition, we performed EMSA to show that purified p53 bound to BS1 oligonucleotides, but not to mutant BS1 (Figure S2). To further verify whether p53 could bind to this candidate p53-binding site (BS1) in vivo, we carried out chromatin immunoprecipitation (ChIP) in Saos2 cells infected with Ad-p53 or Ad-GFP. We also performed ChIP assay in ETO-treated U2OS cells to examine whether endogenous p53 could bind to the GAMT promoter in vivo after DNA damage. The GAMT genomic fragment containing the −4150 p53-binding site (BS1) was specifically immunoprecipitated as a p53 protein-DNA complex with a p53 antibody, but not with an HA antibody (Figure 2C). Although the fragments were present in both DMSO- and ETO-treated U2OS cells, there was a significant increase in the amount of DNA amplified from the complexes of the DNA-damaged cells (Figure 2C, upper panel). Quantitative real-time PCR (qPCR) amplification of a region containing a p53-binding site in the p21 promoter served as a positive control (Figure 2C). However, ChIP assays performed with another potential binding site, BS2 or BS3, in the GAMT promoter demonstrated no significant binding (Figure 2C, lower panel). These results indicate that GAMT is a transcriptional target of p53 and that the consensus p53-binding site located at −4150 of the GAMT promoter is responsible for p53-dependent GAMT transcriptional expression.

Inhibition of GAMT Impairs p53-Mediated Apoptosis

In order to investigate the role of GAMT in p53-mediated apoptosis, we used the pBabe-U6-shRNA retroviral vector system to create GAMT knockdown constructs and assessed the effect of inhibiting endogenous GAMT expression on apoptosis induced by genotoxic stress. GAMT shRNA-transfected U2OS cells did not upregulate GAMT expression in

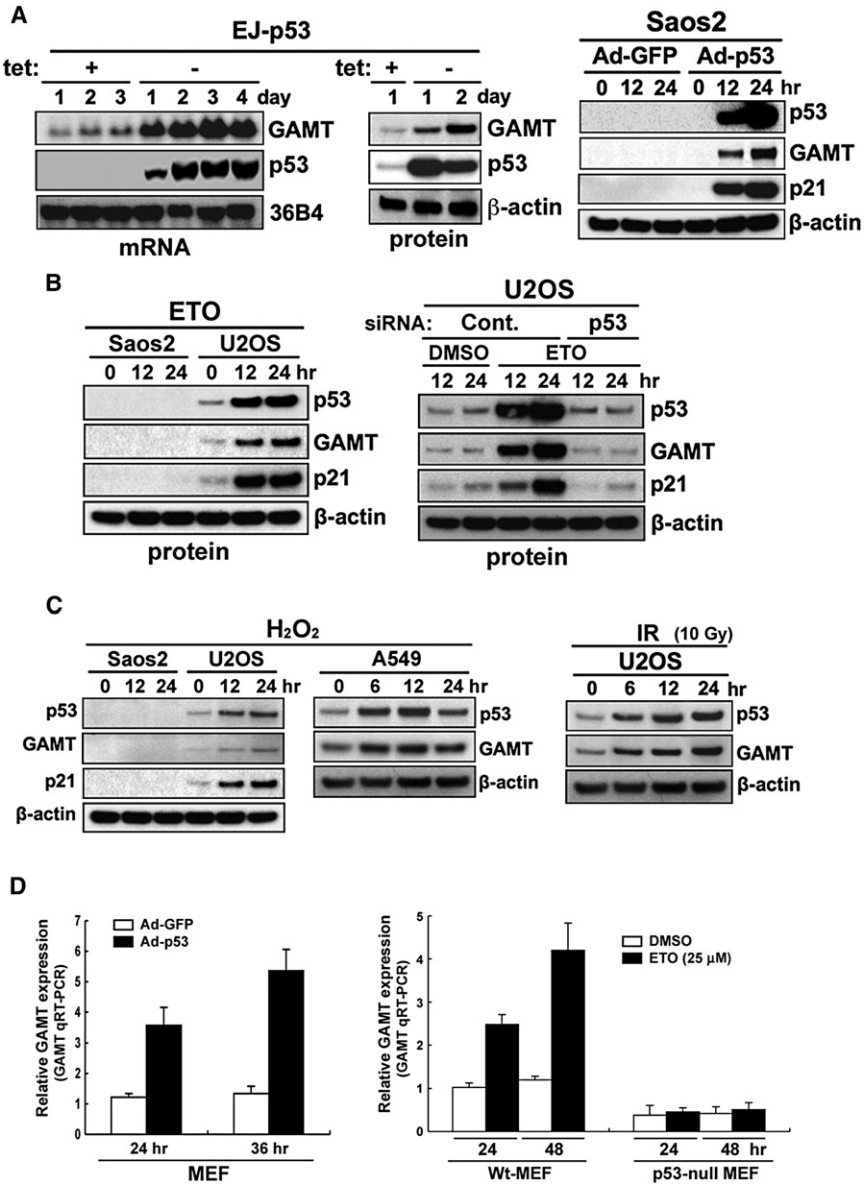

Figure 1. Identification of Guanidinoacetate N-Methyltransferase as a p53-Inducible Gene

(A) Guanidinoacetate N-methyltransferase (GAMT) mRNA and protein were measured after tetracycline (tet) removal in EJ-p53 cells (tet-off). Saos2 cells were infected with adenovirus expressing WT-p53 (Ad-p53) or GFP (Ad-GFP) for 24 hr.

(B) Saos2 and U2OS cells were treated with etoposide (ETO, 25 μM) or its solvent (DMSO) for 12 or 24 hr. In addition, U2OS cells were transfected with either siRNA oligos targeting p53 or control siRNA (luciferase), followed by exposure to ETO for 12 or 24 hr.

(C) Saos2 (p53 null), U2OS (WT-p53), and A549 (WT-p53) cells were treated with H_2O_2 (1 mM), and U2OS cells were exposed to ionizing radiation (IR, 10 Gy) for the indicated time.

(D) MEFs (mouse embryonic fibroblasts) were infected with adenovirus expressing WT-p53 (Ad-p53) or GFP (Ad-GFP). $p53^{+/+}$ and $p53^{-/-}$ MEF cells were treated with DMSO or ETO (25 μM). mRNA levels were normalized to 36B4 expression and presented as mean ± SEM (n = 3).

response to ETO treatment, validating our GAMT shRNA constructs (Figure 3A, left panel). Conversely, ETO-induced GAMT expression was observed in control (luciferase) shRNA-transfected U2OS cells (Figure 3A). GAMT or p53 shRNA-transfected U2OS cells showed a reduction in the percentage of dead cells compared to cells transfected with control shRNA 24 and 48 hr after exposure to ETO (Figure 3A, right panel; and Figure S3). In addition, inhibition of GAMT or p53 expression by shRNA targeting resulted in a decrease in the sub-G1 population of ETO-treated U2OS cells, compared to control-treated cells (Figure S3). Moreover, cleavage of PARP, an indicator of caspase activation, was reduced in GAMT knockdown cells as compared to the control cells upon ETO treatment (Figure 3A). sh*GAMT*-transfected U2OS cells showed a significant reduction in the percentage of TUNEL-positive cells compared to cells transfected with control shRNA 24 and 36 hr after exposure to ETO (Figure 3A). Thus, these cell death assays indicated that depletion of GAMT expression by shRNA effectively inhibited apoptosis induced by DNA damage in cell lines containing endogenous WT-p53. Next we examined the effect of GAMT depletion on a p53-dependent apoptotic response. p53 null Saos2 cells were transfected with GAMT shRNA or control shRNA, and the ability of exogenous p53 expression (by Ad-p53 infection) to induce apoptosis was evaluated. GAMT shRNA was effective in blocking Ad-p53-induced GAMT expression in Saos2 cells (Figure 3B, left panel). Inhibition of GAMT induction by Ad-p53 compromised the ability of these cells to undergo p53-mediated apoptosis (Figure 3B).

We also assessed whether GAMT overexpression could induce apoptosis when expressed alone. Overexpression of GAMT (either alone or in combination with p53) by transient transfection in Saos2 cells produced only a marginal increase in apoptosis in contrast to DNA damage- and p53-mediated apoptotic responses (Figure S4), indicating that GAMT itself is not sufficient for cell death. Together these results suggest that GAMT is an important component of the p53-mediated apoptosis, although GAMT acting alone is not sufficient for cell death.

p53 Regulates Creatine Synthesis

The connection between p53 and GAMT suggested a role for p53 in creatine metabolism. To investigate this possibility, the guanidino compounds creatine, creatinine, and guanidinoacetate

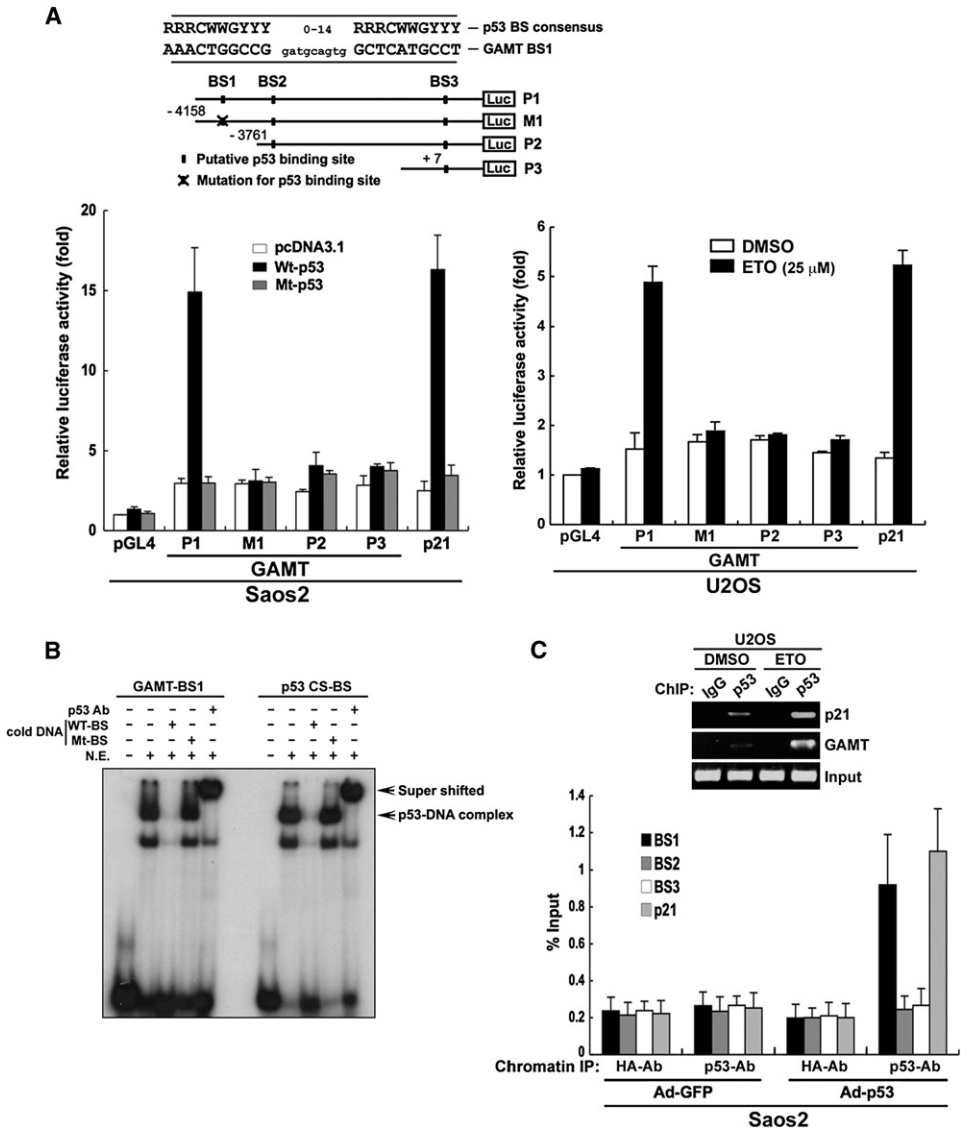

Figure 2. p53 Regulates GAMT Transcription

(A) Luciferase assay reporter gene constructs containing the putative p53 recognition sequences within the GAMT promoter were cotransfected into Saos2 cells with either WT-p53 or mutant p53 (V143A) expression construct as well as a control pcDNA3.1 empty vector. The same reporter constructs were also transfected into U2OS cells followed by ETO treatment. The reporter plasmids used were the following: empty pGL4 vector, the full-length (P1) promoter in pGL4, and three reporter constructs with p53-binding site deletions or specific mutations (P2, P3, and M1); the p21 promoter was used as a control. The upper panel shows putative p53-recognition sequences with the GAMT promoter and the p53-consensus binding sequences (BS). Error bars represent ± SD (n = 3).

(B) For EMSA, nuclear extracts (N.E.) from U2OS cells infected with Ad-p53 were incubated with radiolabeled WT-GAMT BS1 oligonucleotides in the presence of cold-specific or nonspecific mutant competitor and, where indicated, pAb421 antibody. The migrating positions of bound tetramer and super shifted antibody-p53-DNA complex are indicated as arrows.

(C) U2OS cells were treated with ETO (25 μM) for 24 hr. Saos2 cells were infected with Ad-p53 or Ad-GFP for 24 hr (lower panel). ChIP assay was then performed with either a p53 (DO-1 Ab) antibody or an HA-tag antibody (negative control). The percent inputs of coprecipitating DNAs were calculated by qPCR and presented as mean ± SEM (n = 3). The GAMT p53 BS1 amplification product from ETO-treated U2OS cells is shown in the agarose gel, and p21 was used as a positive control.

(GAA) were measured in a p53-inducible EJ cell line. An increase in creatine (output) and a decrease in the ratio of GAA (precursor) to creatine plus creatinine (GAA/Cr + Crn) are routinely used as indicators of GAMT activity. After tetracycline removal in EJ-p53 cells, creatine levels were dramatically increased and the GAA/Cr + Crn ratio was decreased, demonstrating that p53 promotes the conversion from GAA to creatine and creatinine (Figure 4A). Because GAMT is an essential creatine synthetic enzyme, inhibition of GAMT induction (Figure 4A) in response to p53 expression (tetracycline removal) by shRNA targeting

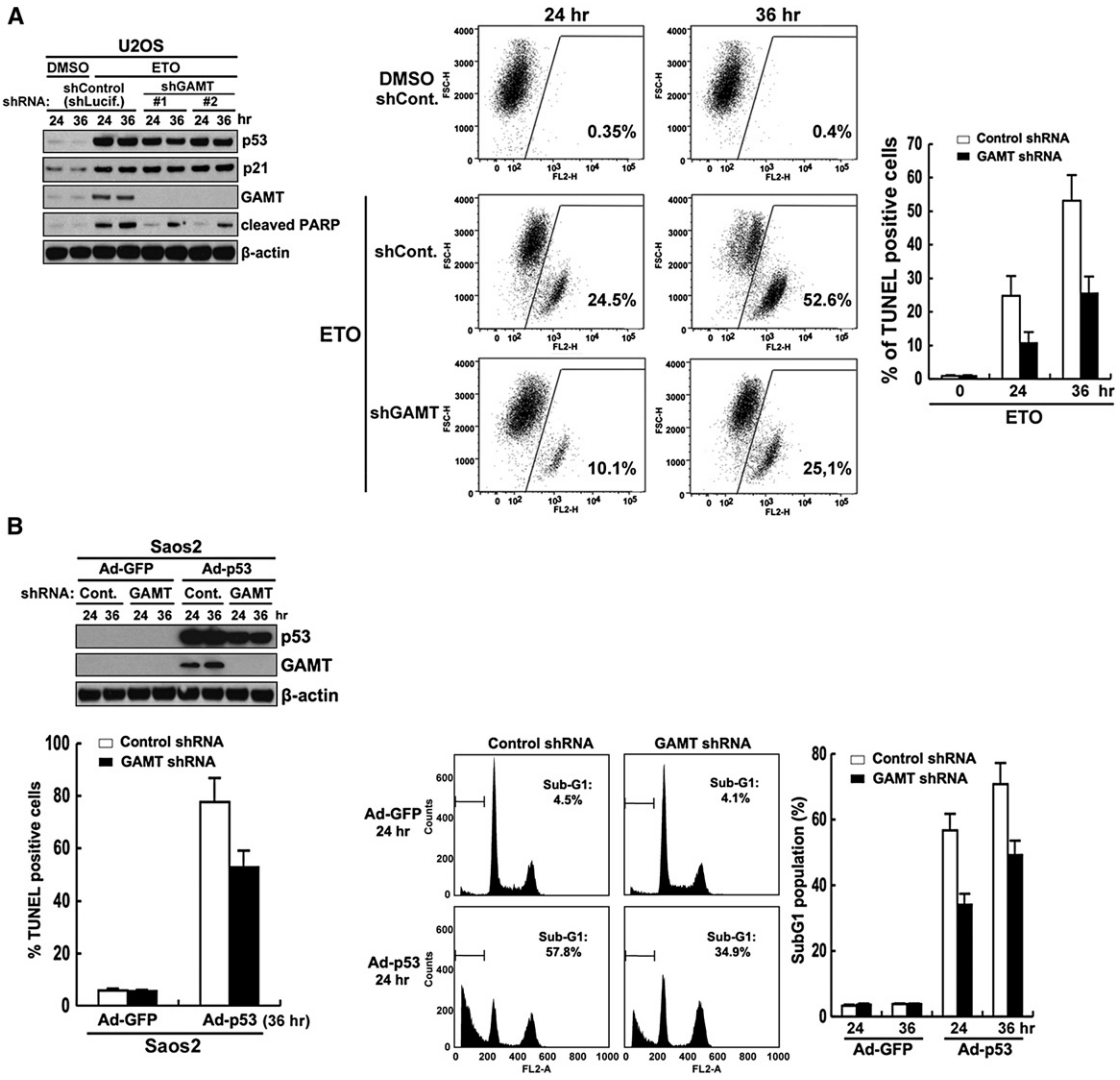

Figure 3. Inhibition of GAMT Induction Impairs p53-Dependent Genotoxic Stress-Mediated Apoptosis

(A) U2OS cells were transfected with either GAMT shRNA (sh*GAMT*#1 and #2, respectively) or control luciferase shRNA (sh*Cont.*) and then treated with ETO (25μM) for the indicated times. Protein extracts were immunoblotted, and cells were also assessed for apoptosis by TUNEL assay (the middle panel). In the right panel, the percentage of TUNEL-positive cells is shown as the mean ± SEM (n = 3).

(B) Saos2 cells were transfected with GAMT shRNA or control shRNA, followed by infection with adenovirus expressing GFP (Ad-GFP) or p53 (Ad-p53) for the indicated times. The apoptotic response of Ad-infected Saos2 was assessed by TUNEL assay and PI staining. In all cases, error bars indicate ± SD (n = 3).

reduced creatine induction and increased the GAA ratio (Figure 4A, right panels). In addition, transfection with a WT-p53 or GAMT expression plasmid into p53 null Saos2 cells increased creatine levels, whereas transfection with mutant p53 or pcDNA3.1 empty vector failed to do so (Figure S5). Thus, it seems that p53 and GAMT operate in the same creatine biosynthetic pathway. If this were the case, then we would expect to see GAMT-mediated changes in creatine metabolism after p53 activation by genotoxic stress. Indeed, creatine levels increase and GAA/Cr+Crn ratios decrease after treatment with ETO in two independent cell lines, HCT116 and U2OS (Figure 4B, left panel). Since we have established that p53→GAMT contributes to apoptosis induced by ETO treatment (Figure 3 and Figure S3), these results point to a further role for creatine metabolism in genotoxic stress-induced apoptosis. We tested this by treating cells with cyclocreatine (cCR) to inhibit the creatine kinase/creatine phosphate circuit and then measured their apoptotic response after treatment with ETO. As a substrate analog of creatine, cCR is phosphorylated efficiently in vitro and in vivo by creatine kinase to generate cyclocreatine phosphophate (cCR-p). However, due to the stability of the phospho-nitrogen bond of cCR-p, its conversion back to cCR is less efficient compared with creatine, resulting in a pool of cCR-p and reduced ATP generation (Lillie et al., 1993). Creatine depletion produced a pronounced decrease in apoptosis induced by ETO in both HCT116 and U2OS cells (Figure 4C). As a putative mechanism

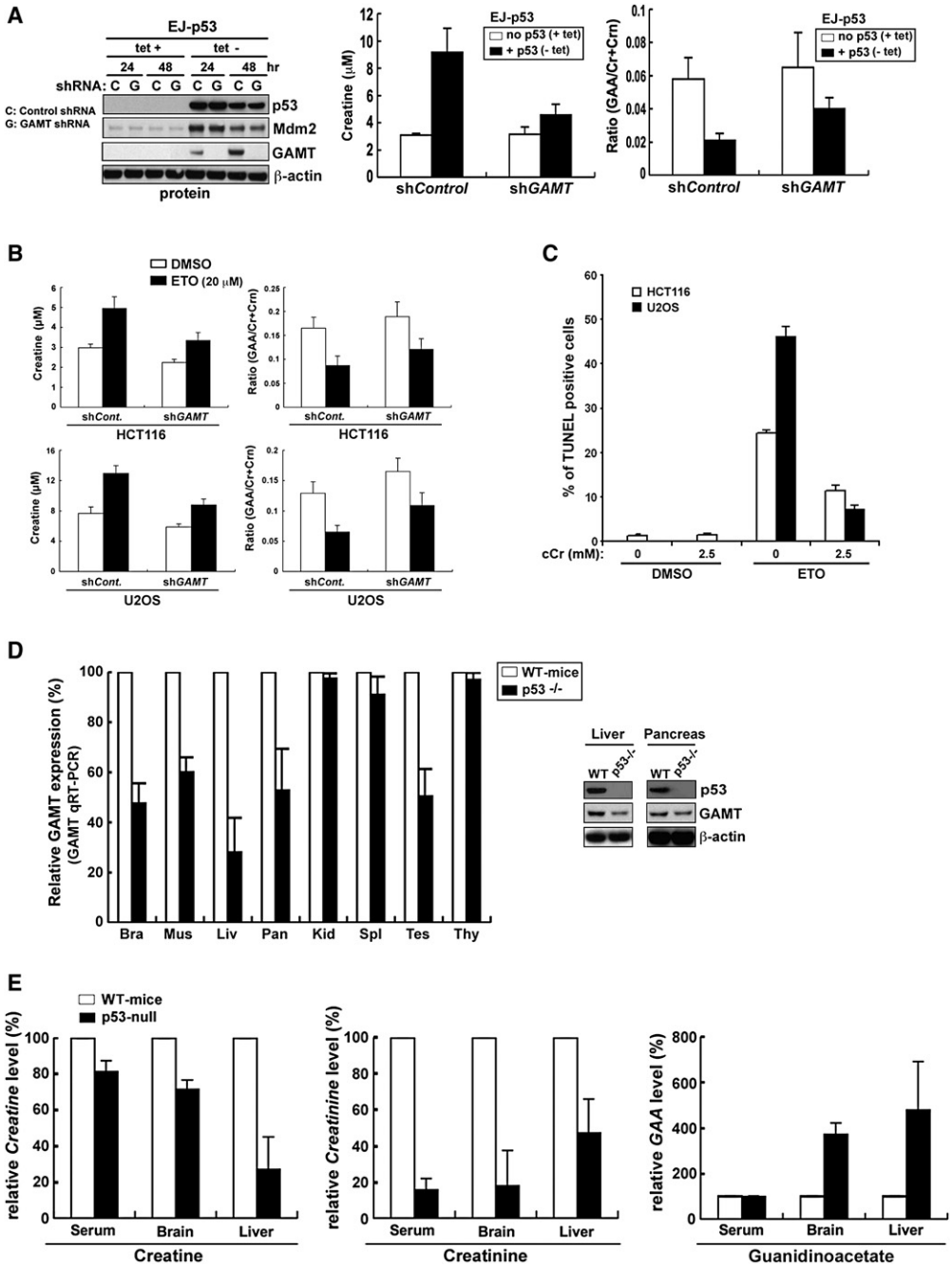

Figure 4. p53 Regulates Creatine Biosynthesis Pathway via GAMT

(A) EJ-p53 cells were transfected with shRNA against GAMT (sh*GAMT*) or luciferase as a control (sh*Control*), and p53 and its target Mdm2 were induced after tetracycline (tet) removal. At 48 hr, the levels of guanidino compounds creatine (Cr), creatinine (Crn), and guanidinoacetate (GAA) were measured in normalized cell lysates. The ratio between GAA and creatine + creatinine was calculated in p53-induced cells in comparison to the levels found in control cells (right panel). Error bars represent ± SD (n = 3).

(B) HCT116 and U2OS cells were transfected with either GAMT shRNA (sh*GAMT*) or control luciferase shRNA (sh*Cont.*) and then treated with ETO (25 μM). At 24 hr, the levels of guanidino compounds were measured. Error bars represent ± SD (n = 3).

(C) HCT116 and U2OS cells were treated with cyclocreatine (cCr, 2.5 mM) and then treated with ETO (25 μM) for 36 hr. Apoptosis was measured by TUNEL assay. Error bars represent ± SD (n = 3).

(D) The ratio between the mean of GAMT mRNA levels in WT-mice and the mean of $p53^{-/-}$ counterparts is described for the indicated tissues and is presented as mean ± SEM (left panel, n = 3). GAMT protein expression was also measured in liver and pancreas tissues.

(E) The value of each guanidino compound in several tissues was determined in $p53^{-/-}$ mice relative to WT mice, which was set equal to 100%, and shown by mean value ± SD (n = 3).

to account for the ability of GAMT and creatine to regulate apoptosis, we looked for changes in the levels of intracellular ROS following DNA damage and creatine depletion, since ROS is known to influence p53 cell-fate decisions (Vousden and Prives, 2009; Liu et al., 2008). ETO treatment of HCT116 cells resulted in an increase in intracellular ROS that was inhibited by cCR (Figure S6). Also, treatment of both HCT116 cells and U2OS cells with creatine produced an increase in intracellular ROS (Figure S6 and data not shown). Taken together, these results support a new role for GAMT and creatine metabolism in p53-dependent apoptosis.

Basal levels of GAMT mRNA and protein expression were next examined using different tissues from WT and p53 null mice. For some organs, especially the liver and the pancreas, basal GAMT expression was significantly lower in p53 null mice compared to their WT littermates (Figure 4D and Table S1). Importantly, the levels of creatine and creatinine in serum, brain, and liver homogenates were lower in p53 null mice, compared with WT animals (Figure 4E and Table S1). In contrast, GAA levels in brain and liver homogenates were significantly increased in p53 null mice (Figure 4E and Table S1). These biochemical analyses of serum, brain, and liver tissues in p53 null mice are consistent with the tendency of GAA to accumulate with GAMT deficiency. Together, our findings closely resemble those previously reported for GAMT knockout mice (Schmidt et al., 2004) and further demonstrate a role for p53 in the creatine biosynthetic pathway.

p53-Dependent GAMT and Creatine Induction in Response to Metabolic Stress

Glucose is a major energy source for mammalian cells, and a decrease in the glucose levels represents a common cellular nutrient stress (Jones et al., 2005; Vander Heiden et al., 2009). In this type of cellular stress, p53 can communicate with the central metabolic regulators AMPK and mTOR (Feng et al., 2005; Jones and Thompson, 2009). To address the possible crosstalk between such an energy stress response and the creatine metabolic pathway, we first investigated whether glucose deprivation affects GAMT expression. Under conditions of glucose starvation, GAMT expression was induced in several cell lines of diverse tissue organs (Figure 5A). In HCT116 cells containing endogenous WT-p53, glucose deprivation led to an increased level of phosphorylated AMPKα, and an accumulation of p53, which coincided with GAMT induction (Figure 5B, upper left panels). In contrast, GAMT expression remained low and unchanged in p53-deficient HCT116 cells despite an increased level of AMPKα phosphorylation induced by glucose deprivation (Figure 5B, upper left panels). Additionally, inhibition of p53 activation in response to glucose deprivation by siRNA targeting of p53 attenuated GAMT upregulation, demonstrating that GAMT induction in response to glucose withdrawal is dependent upon p53 (Figure 5B, upper right panels). Interestingly, p53-deficient HCT116 cells showed a basal level of GAMT protein expression, implying the existence of p53-independent mechanisms to regulate GAMT expression under nonstressed conditions. The levels of guanidino compounds were also studied under conditions of nutrient stress. Glucose starvation led to an increase in creatine levels and a decrease in the GAA/(creatine + creatinine) ratio in p53 WT HCT116 cells, whereas p53-deficient HCT116 cells showed little change in these factors (Figure 5B, lower panels). To confirm these in vitro results, we compared GAMT expression and measured creatine metabolites in fed and starved WT versus p53 null mice. When animals were subjected to 24 hr fasting, GAMT mRNA and protein expression were increased in several tissues of WT mice (Figure 5C, top panel). GAMT induction upon starvation was more obvious in liver and pancreas. On the other hand, GAMT expression was unchanged in the same tissues of p53 null mice (Figure 5C, bottom panel). Moreover, starved WT animals showed an increase in creatine levels and a decrease in GAA levels, especially in liver tissues (Figure 5D and Table S2), consistent with the liver being the major organ of de novo creatine synthesis (Wyss and Kaddurah-Daouk, 2000). Taken together, these findings indicate that metabolic stress induces GAMT expression to regulate creatine levels in a p53-dependent manner.

GAMT Is Involved in Metabolic Stress-Induced Apoptosis

Apoptosis is a common cellular response to metabolic stress in mammalian cells (Jones et al., 2005; Okoshi et al., 2008). To investigate whether GAMT could participate in metabolic stress-induced cell death, we first carried out trypan blue exclusion analysis to detect cell death after glucose deprivation. Upon glucose deprivation cleavage of PARP, caspase-9 (associated with the intrinsic apoptotic pathway) and caspase-3 were reduced in GAMT knockdown cells as compared to the control cells (Figure 6A). p53 WT HCT116 cells transfected with GAMT shRNA also showed a reduction in the percentage of dead cells compared to cells transfected with control shRNA after glucose starvation (Figures 6A and 6B). Additionally, inhibition of GAMT expression by shRNA targeting resulted in a decrease in the percentage of TUNEL-positive cells and the sub-G1 population of glucose-starved HCT116 cells, in comparison to control-treated cells (Figure 6B). The effect of GAMT inhibition was specific for the stress-induced apoptotic response, since control- versus shGAMT-treated cells showed similar cell-cycle distributions after glucose starvation (Figure S7). Together, these assays suggested that depletion of GAMT expression by shRNA inhibits apoptosis induced by glucose starvation in cell lines containing endogenous WT-p53. On the other hand, p53-deficient HCT116 cells showed no GAMT induction in response to glucose deprivation (Figure 6C). Also, GAMT repression or overexpression did not affect glucose deprivation-induced cell death in p53-deficient cells (Figures 6C and 6D). We next examined the effect of GAMT depletion on the intrinsic cell death pathway by determining whether cytochrome *c* release from mitochondria could be affected upon glucose starvation. As shown in Figure 6E, in cell fractionation experiments, we observed that knockdown of GAMT resulted in the inhibition of the release of cytochrome *c* from mitochondria after glucose starvation. Apoptosis induced by glucose deprivation is known to have both p53-dependent and independent components (Fabre et al., 2007; Yeo et al., 2006); our results demonstrate that GAMT is necessary for the p53-dependent component of glucose deprivation-induced cell death involving the intrinsic apoptotic pathway.

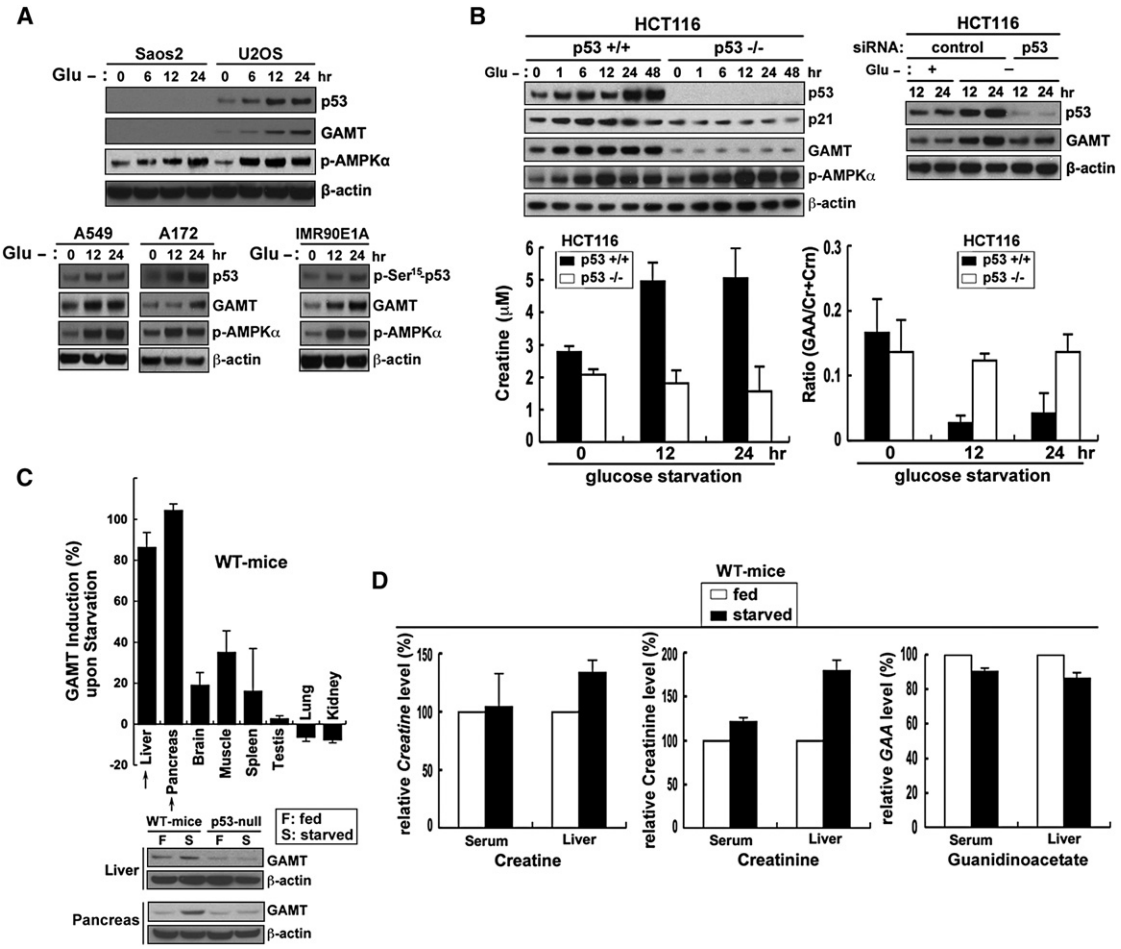

Figure 5. p53-Dependent Inductions of GAMT and Creatine in Response to Metabolic Stress

(A) Saos2 (p53 null), U2OS, A549, A172, and IMR90E1A cells were cultured in the absence of glucose for the indicated times, and protein extracts were immunoblotted with antibodies against phospho-AMPKα, p53, phospho-Ser^{15}p53, GAMT, and β-actin.

(B) HCT116 p53$^{+/+}$ and HCT116 p53$^{-/-}$ cells were cultured in the absence of glucose for the indicated times (0–48 hr), and the levels of p53, p21, GAMT, phospho-AMPKα, and β-actin were examined. HCT116 cells were transfected with either siRNA-oligos targeting p53, or control siRNA oligos, followed by glucose withdrawal for the indicated times. In addition, the levels of guanidino compounds were measured. Error bars indicate ± SD (n = 3).

(C) The calculated percent increases of GAMT mRNA upon starvation (24 hr) in WT mice of various tissues are presented as mean ± SEM (n = 3) (the top panel). The levels of GAMT proteins in liver and pancreas of WT mice and p53 null mice under fed or starved condition were determined (bottom panel).

(D) Guanidino compounds in serum and liver was determined in starved WT mice relative to fed mice, which was set equal to 100%, and shown by mean value ± SD (n = 3).

GAMT Regulates Metabolic Stress-Induced Fatty Acid Oxidation in a p53-Dependent Manner

Recent studies reveal p53 has a key role in energy metabolism, such as regulating glucose metabolism and mitochondrial respiration (Bensaad et al., 2006; Matoba et al., 2006; Jones and Thompson, 2009). However, little is known about how p53 coordinates changes in cellular metabolism to maintain energy and nutrient homeostasis. First, we evaluated the effect of inhibition of endogenous p53 or GAMT expression on cellular ATP levels in the presence or absence of glucose. p53 WT or p53-deficient HCT116 cells stably expressing control and GAMT shRNA did not show any change in ATP level under normal growth conditions (Figure S8A), supported by previous studies that report that the total amount of ATP is similar in both WT and p53-deficient cells (Matoba et al., 2006). Under conditions of glucose starvation, p53 WT HCT116 cells, stably transfected with control shRNA, were able to maintain ATP levels, whereas GAMT knockdown or p53-deficient HCT116 cells showed a reduced ATP level in comparison to control-treated HCT116 p53$^{+/+}$ cells (Figure S8A). Moreover, transfection with GAMT expression plasmid into both WT-p53 and p53-deficient HCT116 cells as well as p53 null Saos2 cells increased cellular ATP levels in the absence of glucose, compared to control transfection (Figures S8B and S8C), indicating that GAMT acts to generate ATP downstream of p53 under conditions of nutrient stress. These findings prompted us to speculate that alternative energy-generating pathways can be activated in response to glucose deprivation and might be required for p53→GAMT-induced responses. To

address this hypothesis, we sought to identify the alternate source of energy besides the creatine biosynthesis pathway. FAO is reported to be the first alternate pathway used by most tissues when glucose is not available (Wolfe, 1998). In HCT116 cells, inhibition of FAO resulted in the suppression of ATP levels, in particular under conditions of glucose deprivation, demonstrating that FAO sustains cellular ATP level in the absence of glucose (Figure 7A). In addition, inhibition of creatine metabolism by cCR showed a marked decrease in cellular ATP levels under both normal and starvation conditions (Figure 7A). Moreover, cotreatment of cCR and etomoxir further decreased ATP levels upon glucose starvation (Figure 7A). Therefore, these results suggest that FAO contributes to the maintenance of ATP levels acting as an alternative energy source in the absence of glucose.

Next, we investigated whether p53 or GAMT could be involved in FAO in response to glucose deprivation. Inhibition of p53 or GAMT by shRNA targeting abolished glucose deprivation-induced FAO, compared with control-treated cells (Figure 7B). In addition, transfection with a WT-p53 or GAMT expression plasmid into Saos2 cells increased FAO, whereas transfection with mutant p53 or pcDNA3.1 empty vector failed to do so (Figure 7C). p53- or GAMT-mediated FAO increase was confirmed by a tet-regulated p53 expression system (EJ-p53). p53 induction by tet removal enhanced FAO and knockdown of GAMT induction through p53 inhibited p53→GAMT-mediated FAO (Figure S9). We also examined whether endogenous p53→GAMT activation upregulates FAO and whether GAMT is essential for FAO increase in response to genotoxic stress in ETO-treated HCT116 and U2OS cells. ETO treatment increased FAO in both HCT116 and U2OS cells, and knockdown of GAMT induction inhibited ETO-mediated FAO increase (Figure S10). However, FAO inhibition did not influence ETO-mediated apoptosis (Figure S11), suggesting that FAO is mainly involved in energy metabolism rather than apoptosis. Although p53-deficient HCT116 cells could not augment FAO under conditions of glucose starvation, exogenous GAMT rescued this effect, and these cells showed increased FAO in comparison to control-treated cells (Figure 7D). These results demonstrate that GAMT plays an important role in glucose deprivation-induced FAO and suggested that creatine metabolism is connected to FAO, which is supported by other studies (Ceddia and Sweeney, 2004). Since AMPK is known to regulate FAO through ACC (acetyl-CoA carboxylase) (Hardie and Pan, 2002), we investigated whether creatine (GAMT metabolite) itself could increase FAO by AMPK activation and ACC inhibition (phosphorylation of ACC). Western blot analysis of HCT116 cells showed that creatine increased phospho-AMPK and phospho-ACC, which were similar to that observed with AICAR, an activator of AMPK. The effects of creatine treatment translated into an increase in FAO (Figure 7E). Lastly, we confirmed these results in vivo by investigating FAO in the liver tissues of fed or starved WT-p53 and p53 null mice. In p53 WT mice, starvation induced liver FAO that was higher than that of fed mice (Figure 7F). Conversely, p53 null mice showed much lower overall levels of liver FAO compared to WT animals, which did not change with either fed or starved conditions (Figure 7F), suggesting that p53 contributes generally to FAO and that this contribution is most marked in response to nutrient stress. Together, our findings demonstrate that p53 regulates FAO, particularly under conditions of glucose deprivation, and that this regulation is further dependent upon GAMT. This is the first evidence to implicate p53 in energy maintenance by the alternative FAO pathway. Thus, we propose a model (Figure 7G) that, under conditions of metabolic stress, p53 activates energy metabolism via GAMT induction, influencing creatine biosynthesis and FAO.

DISCUSSION

Here we have provided additional insight into p53 function by demonstrating that p53 plays a role in creatine and fatty acid metabolism through the upregulation of GAMT, a creatine synthesis enzyme, which we identify as a p53-transcriptional target gene. GAMT induction in response to DNA damage and glucose deprivation is shown to be p53 dependent, and activated p53 binds to the most 5′ of three p53 consensus binding sites within GAMT promoter, indicating that GAMT is likely a direct p53 target. GAMT expression correlates with p53 expression and activation by both genotoxic and nutrient stress, which induce p53-mediated cell death via the intrinsic apoptotic pathway. Importantly, inhibition of GAMT by shRNA targeting abrogates p53-dependent apoptosis in response to these conditions of cellular stress. Despite the requirement of GAMT for these apoptotic responses, GAMT itself induces little cell death, demonstrating that it is necessary but not sufficient to induce apoptosis in response to p53 activation. Although further studies are required to elucidate precisely how GAMT cooperates with other apoptosis-related genes, and whether they act in a cell- or stimulus- specific manner, our findings suggest that GAMT is an important component of the p53-mediated death signal.

GAMT is also induced in response to metabolic stress such as glucose deprivation and fasting in mice, which is followed by an increase in creatine and creatinine levels. In both tumor cell lines and mice, this increase in creatine biosynthesis was shown to be p53 dependent. Glucose levels are known to regulate AMPK activation in response to nutrient availability (Hardie, 2004), and recent studies reveal that AMPK-dependent p53 activation acts as a metabolic sensor in response to glucose limitation (Jones and Thompson, 2009; Jones et al., 2005). In this pathway, p53 accumulation occurs through posttranslational modifications such as phosphorylation of p53 at Ser-15 and Ser-46, and also through the transcriptional upregulation of p53 (Feng et al., 2005; Okoshi et al., 2008). These findings support our results demonstrating that glucose deprivation leads to the phosphorylation of AMPKα and p53(Ser-15), leading to p53 accumulation in HCT116 cells. In addition, several studies demonstrate that p53 activation in response to glucose deprivation causes cell-cycle arrest and apoptosis; thus, there is an established role for p53 in cellular responses to nutrient stress (Jones et al., 2005; Rathmell et al., 2003). Our study supports and expands this precedent by demonstrating that inhibition of GAMT attenuates glucose starvation-induced apoptosis in WT-p53 cells, but not in p53-deficient cells. Furthermore, cleavage of PARP, caspase-9, and caspase-3 were much reduced in GAMT knockdown cells, compared to control cells after glucose deprivation. Taken together, these data support

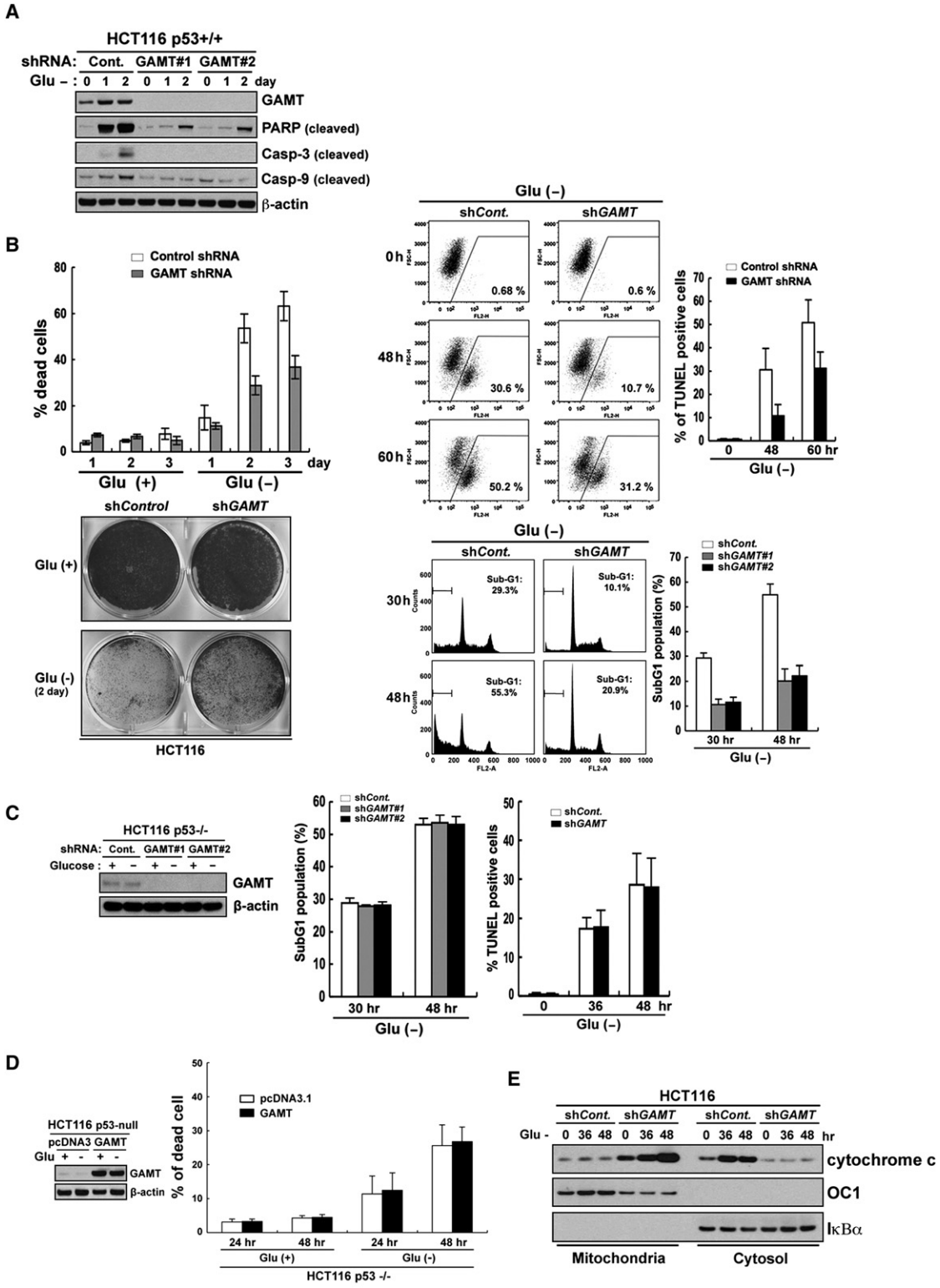

Figure 6. Modulation of GAMT Expression Influences Metabolic Stress-Mediated Cell Death/Apoptosis in a p53-Dependent Manner

(A) GAMT-depleted HCT116 $p53^{+/+}$ cells (pooled #1 and #2) were grown in the absence of glucose for the indicated times (0–2 days). Cell lysates were analyzed by immunoblotting with antibodies against GAMT, PARP (cleaved), caspase-3 (cleaved), caspase-9 (cleaved), and β-actin (loading control).

(B) HCT116 cells were transfected with either GAMT shRNA #1 or #2, or control (luciferase) shRNA, then cultured in the absence or presence of glucose for the indicated times. The percentage of dead cells was determined by trypan blue exclusion analysis, TUNEL assay, and PI staining-FACS analysis. Error bars indicate ± SD.

that a p53→GAMT-mediated response is an important component of the nutrient stress response under conditions of glucose deprivation. Since GAMT is an enzyme involved in creatine biosynthesis metabolism, we investigated and found a connection between p53 and creatine levels. Most compelling was the finding that creatine and GAA levels in serum and tissue homogenates are decreased in p53 null mice in comparison to their WT littermates. Although these findings are similar to those of GAMT knockout mice, GAMT knockout mice show a much more pronounced phenotype (i.e., neonatal mortality, muscular hypotonia, and reduced body fat mass) compared to p53 null mice, likely because GAMT is a direct regulator of creatine biosynthesis (Schmidt et al., 2004). Moreover, p53 null mice and human cell lines have detectable levels of uninduced GAMT protein, indicating that p53-independent pathways exist that are responsible for basal GAMT expression, as is the case for other p53 target genes (Bouvard et al., 2000). Interestingly, it has been previously reported that p53-deficient mice display some neurological problems (Torremans et al., 2005) and have decreased exercise activity (Matoba et al., 2006), which could be a milder manifestation of the GAMT deficiency syndrome (i.e., cognitive impairment and muscular hypotonia) (Amson et al., 2000). These findings are in agreement with our suggestion that p53 is involved in creatine biosynthesis through the regulation of GAMT expression.

Creatine is normally transported through the blood and taken up by the creatine transporter; thereafter, reversible phosphorylation of creatine by creatine kinase provides a high-energy ADP-to-ATP phosphate buffering system (Wyss and Kaddurah-Daouk, 2000). The participation of the creatine kinase/phosphate creatine/creatine system in energy metabolism mainly relies on the creatine kinase-mediated shuttling of ATP/ADP in and out of mitochondria. Since GAMT depletion resulted in a decrease of total cellular ATP, it is conceivable that GAMT could additionally affect intracellular ATP pools (i.e., mitochondria versus cytoplasmic) via creatine kinase-mediated ATP/ADP shuttling. Creatine biosynthesis is a pathway with an essential role in regulating ATP homeostasis (Wyss and Kaddurah-Daouk, 2000), and the connection between p53→GAMT and creatine biosynthesis metabolism prompted us to investigate whether these proteins play a role in other alternate energy-generating pathways that are utilized when glucose is scarce, primarily FAO. We demonstrate that p53-dependent GAMT upregulation is required to induce a metabolic program of FAO in response to glucose deprivation. In mammals, changes in nutrient availability trigger organism-wide changes in the utilization of energy and energy-generating pathways, resulting in a shift from glycolysis to FAO and to the delivery of gluconeogenic precursors such as lactate and alanine from the muscle to the liver (Storlien et al., 2004). Previous studies reveal that glucose withdrawal leads to FAO as the first alternate source of energy in several cell lines (Jelluma et al., 2006; Wolfe, 1998). Consistent with these reports, our study also showed that glucose deprivation induces FAO, leading to the maintenance of ATP levels in human colon cancer cell lines. Moreover, this ATP supply seems to rely preferentially on FAO rather than creatine biosynthesis, even though creatine is increased by GAMT induction in response to glucose deprivation. It is also possible that other energy-generating processes such as amino acid breakdown or gluconeogenesis might contribute to ATP production upon glucose deprivation. Importantly, p53-dependent GAMT upregulation is required for the induction of FAO after glucose deprivation, suggesting a role for p53 in fatty acid metabolism. The increase in glycolytic capacity is known to be a common feature of most cancers, known as the Warburg effect, and it is reported that increased FAO can inhibit glycolysis (Delarue and Magnan, 2007). In addition, recent studies reveal that carnitine palmitoyltransferase 1 (CPT1), a key regulator of FAO, is decreased in human colon cancer specimens (Mazzarelli et al., 2007). These findings suggest the interesting possibility that p53→GAMT regulation of FAO might be a means of inhibiting glycolysis, circumventing the Warburg effect, and thereby contributing to tumor suppression. Another intriguing possibility is that p53→GAMT acts to maintain ATP levels sufficient for apoptosis when energy production by glycolysis is impaired, either due to the mitochondrial disruption caused by apoptosis via the intrinsic pathway, or due to a lack of glucose altogether under conditions of starvation. Indeed, cellular stress-induced apoptosis is known to be an ATP-dependent process (Curtin et al., 2002; Liu et al., 1996). Overall, these findings might prove to be insightful for other diseases such as diabetes, in which there is a down-regulation of fatty acid utilization and mitochondrial oxidative genes (Mootha et al., 2003; Patti et al., 2003). Further examinations are required to address this idea. Recently Crighton et al. provide evidence for another unexpected function of p53 in metabolism: involvement of p53 in the autophagy pathway that is known as another type of cell death ("type 2" cell death) when nutrients are limiting. They show that p53 induction leads to activation of the autophagy pathway through DRAM, a lysosomal protein required for fusion of autophagosomes (Crighton et al., 2006). It will be interesting to test whether GAMT activation and resulting actions upon nutrient stress may promote autophagy.

In conclusion, we show here that GAMT is a p53-inducible modulator of apoptosis in response to genotoxic and nutrient stress, and that p53→GAMT is essential for responding to conditions of nutrient deprivation by increasing FAO. This metabolic adaptation might provide clues for the creation of new therapeutic interventions in the treatment of cancer and metabolism-related diseases.

(C) HCT116 $p53^{-/-}$ cells stably expressing control and GAMT shRNA (#1 and #2) were cultured with or without glucose for 30 or 48 hr. The cells were then harvested for western blot analysis to confirm the knockdown of GAMT expression. Cells were further assessed for cell death/apoptosis by PI staining-FACS analysis and TUNEL assay for the indicated times. Error bars indicate ± SD (n = 3), with duplicate plates.
(D) HCT116 $p53^{-/-}$ cells transiently transfected with GAMT expression plasmid or pcDNA3.1 vector were cultured with or without glucose for 24 or 48 hr. Cells were assessed for GAMT expression and for cell death/apoptosis by TUNEL assay for the indicated times. Error bars indicate ± SD (n = 3), with duplicate plates.
(E) Control (luciferase) and GAMT (#1) knockdown HCT116 cells were cultured with or without glucose, harvested at 0, 36, and 48 hr. Fractionated lysates were assessed for the presence of cytochrome *c*, oxidative complex 1 (OC1, mitochondrial fraction), and IκBα (cytosolic fraction) by immunoblot analysis.

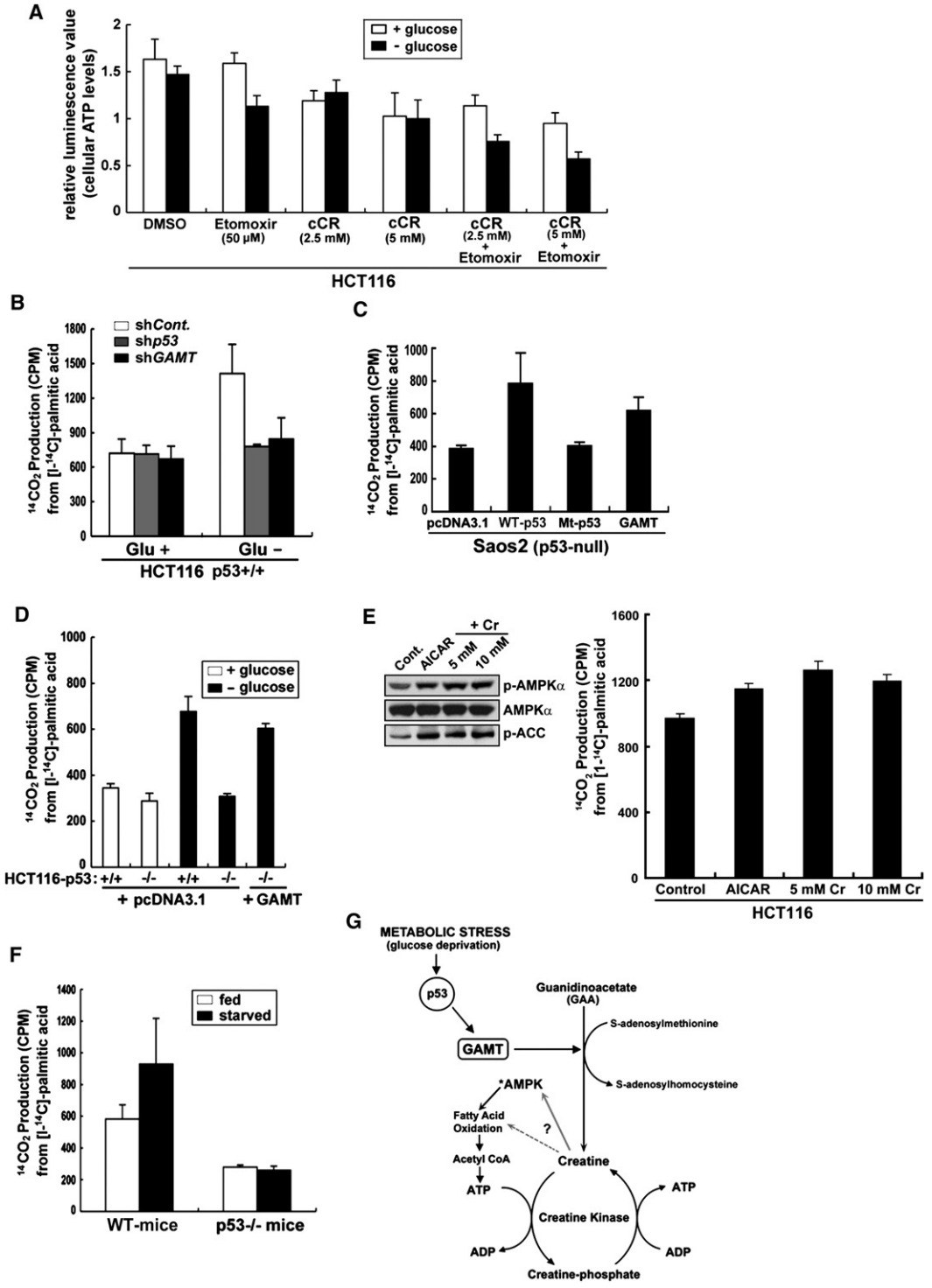

Figure 7. p53 Regulates Metabolic Stress-Induced Fatty Acid Oxidation through GAMT

(A) HCT116 cells were grown in complete medium containing glucose or no glucose, with or without FAO inhibitor etomoxir (50 μM), with or without a creatine kinase inhibitor, cyclocreatine (cCr, 2.5 mM and 5 mM, respectively) for 24 hr, and relative ATP levels were measured. Error bars indicate ± SD (n = 3).

(B) HCT116 cells were transfected with p53 shRNA, GAMT shRNA, or control shRNA and then incubated with or without glucose for 24 hr, followed by FAO measurement. Error bars indicate ± SD.

(C) Saos2 cells were transfected with WT-p53, mutant p53, GAMT, or empty vector pcDNA3.1 as a control, and the rate of FAO was measured. The values were shown by mean ± SD (n = 3), with duplicate plates.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6J background $p53^{+/-}$ mice were obtained from Jackson Laboratories (ME) to generate p53 null mice. (Genotyping strategy is detailed in the Supplemental Data.) Ten-week-old mice were sacrificed, and their blood and tissues were used for analyses. To study the effect of starvation, mice were deprived of food for 24 hr but received water ad libitum.

Creatine Metabolites

Samples used for creatine metabolites were prepared as followed. Serum samples were collected from blood centrifuged for 10 min (1000 × g at 4°C). For brain and liver samples, tissues were homogenized in 1 ml of PBX buffer (0.1% Triton X in PBS) followed by centrifugation, and the supernatant was used. For cell lines, total lysates normalized to protein concentration were used. The guanidine compounds were separated and quantified using a high-performance liquid chromatography (HPLC) system. Detection was performed by tandem mass spectrometry, as previously described in detail (Bodamer et al., 2001).

ATP Levels

Cells were plated in white opaque 96-well plates at 2.5×10^3 cells per well and allowed to adhere for 24 hr. Following specified treatments, ATP contents were determined using the ATPlite Luminescence Assay kit (PerkinElmer) according to the manufacturer's protocol. The luminescence was measured by a Victor^3 Multi Label plate reader (PerkinElmer). A luciferin-luciferase bioluminescence assay using the ATP determination kit (Molecular Probes) was also used to confirm the ATP levels.

Fatty Acid Oxidation

Palmitate oxidation was measured by the production of $^{14}CO_2$ from [1-^{14}C] palmitic acid and was adapted from a protocol described earlier (Suzuki et al., 2007). Briefly, cells were exposed to [1-^{14}C] palmitic acid (American Radiolabeled Chemicals) for 30 min after incubation with or without glucose. The culture supernatant was then transferred to a 50 ml tube (Falcon) and mixed with a 1/10 volume of 1M HCl. The $^{14}CO_2$ produced during incubation of the mixture for 1 hr at 30°C was trapped with a paper filter soaked with NaOH. The paper filter was then transferred to vials, and the radioactivity was measured with a scintillation counter. An alternate method is detailed in the Supplemental Data. To study liver FAO, liver tissues were homogenized in Ca^{2+}-free Krebs-Henseleit buffer (pH 7.4) followed by centrifugation, and the supernatant was used for measuring FAO.

SUPPLEMENTAL DATA

Supplemental Data include two tables, thirteen figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.cell.com/molecular-cell/supplemental/S1097-2765(09)00691-1.

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(D) HCT116 $p53^{+/+}$ or HCT116 $p53^{-/-}$ cells were transfected with either GAMT or pcDNA3.1 empty vector (control), then maintained in the absence or presence of glucose for 24 hr, followed by FAO measurement. Error bars indicate ± SD.

(E) FAO was measured in HCT116 cells that were treated with or without creatine (Cr, Sigma) at 5 and 10 mM for 24 hr. Cells were treated with AICAR (2 mM) for 2 hr as positive control. Values shown are mean ± SEM (n = 6). Cell lysates were subjected to immunoblotting with phospho-AMPKα and phospho-ACC antibodies.

(F) FAO was measured in liver tissues from fed or starved (24 hr) WT mice and p53 null mice. Error bars indicate ± SD (n = 3).

(G) Model for the role of p53→GAMT pathway in creatine biosynthesis and FAO pathway.

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