AMP-activated Protein Kinase α2 and E2F1 Transcription Factor Mediate Doxorubicin-induced Cytotoxicity by Forming a Positive Signal Loop in Mouse Embryonic Fibroblasts and Non-carcinoma Cells*

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Background: Despite the central position in chemotherapy, the clinical application of doxorubicin is compromised by severe adverse effect in different organs.

Results: Doxorubicin induces AMPKα2 transcription, and AMPKα2 in turn stabilizes E2F1 in non-carcinoma cells.

Conclusion: AMPKα2 and E2F1 mediate cytotoxicity of doxorubicin.

Significance: AMPKα2 might serve as a novel target for alleviating the cytotoxicity of doxorubicin.

Doxorubicin is one of the most widely used anti-cancer drugs, but its clinical application is compromised by severe adverse effects in different organs including cardiotoxicity. In the present study we explored mechanisms of doxorubicin-induced cytotoxicity by revealing a novel role for the AMP-activated protein kinase α2 (AMPKα2) in mouse embryonic fibroblasts (MEFs). Doxorubicin robustly induced the expression of AMPKα2 in MEFs but slightly reduced AMPKα1 expression. Our data support the previous notion that AMPKα1 harbors survival properties under doxorubicin treatment. In contrast, analyses of Ampkα2−/− MEFs, gene knockdown of AMPKα2 by shRNA, and inhibition of AMPKα2 activity with an AMPK inhibitor indicated that AMPKα2 functions as a pro-apoptotic molecule under doxorubicin treatment. Doxorubicin induced AMPKα2 at the transcription level via E2F1, a transcription factor that regulates apoptosis in response to DNA damage. E2F1 directly transactivated the Ampkα2 gene promoter. In turn, AMPKα2 significantly contributed to stabilization and activation of E2F1 by doxorubicin, forming a positive signal amplification loop. AMPKα2 directly interacted with and phosphorylated E2F1. This signal loop was also detected in H9c2, C2C12, and ECV (human epithelial cells) cells as well as mouse liver under doxorubicin treatment. Resveratrol, which has been suggested to attenuate doxorubicin-induced cytotoxicity, significantly blocked induction of AMPKα2 and E2F1 by doxorubicin, leading to protection of these cells. This signal loop appears to be non-carcinoma-specific because AMPKα2 was not induced by doxorubicin in five different tested cancer cell lines. These results suggest that AMPKα2 may serve as a novel target for alleviating the cytotoxicity of doxorubicin.

Doxorubicin is one of the most effective anti-cancer drugs and is widely used to treat solid tumors and hematological malignancies. However, the clinical use of doxorubicin has been regarded as a double-edged sword (1). Despite its central position in chemotherapy, severe adverse effects in different organs including the heart, brain, kidney, and liver have impeded clinical application of the drug. The most common side effects of doxorubicin are cumulative cytotoxicity, neurological disturbances, liver injury, and bone marrow aplasia (1–4). Although multiple mechanisms including oxidative stress, DNA damage, mitochondrial impairment, and induction of the apoptotic pathway have been implicated in doxorubicin cytotoxicity (5), the principal mechanisms are not fully understood. Therefore, elucidating the mechanisms underlying doxorubicin cytotoxicity is critical to efforts to increase the utility of doxorubicin for treating cancers.

AMP-activated protein kinase (AMPK)2 is a heterotrimeric protein kinase that consists of a catalytic subunit (α) and two regulatory subunits (β and γ), each with multiple isoforms, and plays a central role in cellular energy homeostasis (6). In response to various metabolic stressors, AMPK is activated by the accumulation of AMP as a result of ATP depletion and, in turn, regulates a multitude of metabolic pathways to balance cellular energy (6–9). In addition to balancing cellular energy, AMPK also induces apoptosis through several tumor suppres-

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The abbreviations used are: AMPK, AMP-activated protein kinase; MEF, mouse embryonic fibroblast; DKO, double knock-out; PLA, proximity ligation assay; CREB, cAMP-response element-binding protein; DKO, double knock-out; DN, dominant negative.
AMPKα2 and E2F1 Mediate Doxorubicin Cytotoxicity

sors including LKB1, TSC2, and p53. LKB1 is an upstream activating kinase of AMPK (10), and TSC2 and p53 are the direct substrates of AMPK (11–13). Therefore, AMPK seemingly has dual roles, such as protecting cells from energy depletion and inducing cellular apoptosis, but the underlying mechanisms by which AMPK determines cellular fate remain an enigma.

In contrast to the well-established role of AMPK in metabolic regulation, there is no consensus on its role or the underlying mechanism under genotoxic stress. Some reports have demonstrated that AMPK is activated in H9c2 (14) and cancer cell lines (15) by doxorubicin. In contrast, AMPK is inhibited by long-term treatment of doxorubicin in mouse embryonic fibroblasts (MEFs) (16). Moreover, there is a sharp contrast regarding the role of AMPK in the regulation of cell death under doxorubicin treatment (14, 17–19). In general, the aforementioned reports have been conducted without distinguishing the potentially differential roles of the AMPK isoforms. Two isoforms, AMPKα1 and AMPKα2, are encoded by two distinct genes (20). AMPKα1 is ubiquitously expressed, whereas AMPKα2 is highly expressed in metabolically active tissues including heart, skeletal muscle, and liver (21). In fact, AMPKα isoforms may have differential roles in the regulation of metabolism and/or blood vessel contraction (6, 22–26). Therefore, we determined whether each AMPK α subunit isoform was differentially associated with cell fate under doxorubicin treatment to reveal novel mechanisms for doxorubicin-induced cytotoxicity.

Here, we report that doxorubicin robustly induced AMPKα2 expression, whereas it slightly reduced the level of AMPKα1 in MEFs. In accordance with a previous report (16), our data support that AMPKα1 harbors survival properties. Moreover, we provide novel evidence that AMPKα2 and E2F1 mediate doxorubicin cytotoxicity by forming a positive signal loop, revealing the differential role of AMPKα isoforms. Doxorubicin induced AMPKα2 transcription via E2F1, and AMPKα2 in turn stabilized E2F1 in MEFs. The transcription factor E2F1 is stabilized and activated by DNA damage (27) and transactivates a number of genes including p27kip1, Apaf-1, and Bim in the apoptotic pathway (28–30). This signal loop significantly contributed to cell death in several non-carcinomas including H9c2, C2C12, and ECV cells as well as mouse liver under doxorubicin treatment. Notably, AMPKα2 was not induced by doxorubicin in cancer cells. Collectively, our results suggest that AMPKα2 may serve as a novel target for alleviating the cytotoxicity of doxorubicin.

EXPERIMENTAL PROCEDURES

Materials and DNA Constructs—DMEM and F-12 (1:1), RPMI 1640 medium, and fetal bovine serum (FBS) were purchased from Lonza (Walkersville, MD). M199 medium was obtained from Welgene (Daegu, Korea). Doxorubicin, etoposide, resveratrol, piceatannol, and fisetin were purchased from Sigma. Antibodies to phospho-acetyl-CoA carboxylase α-Ser79, phosphor-H2A.X-Ser139, caspase-3, p27kip1, Bim, acetyl-CoA carboxylase α, Apaf-1, Sirt1, and α-tubulin were from Cell Signaling Technology (Danvers, MA). Antibodies to AMPKα1 and AMPKα2 were from R&D Systems (Minneapolis, MN). Antibodies to E2F1 (c-20), poly (ADP-ribose) polymerase, c-Myc (9E10), FLAG, and green fluorescent protein (GFP) and the antibody that recognizes both AMPKα1/α2 (sc-25792) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). E2F1 wild type and E2F1 ΔTA were generated by polymerase chain reaction (PCR) and cloned into the pCMV vector. pGIPZ non-silencing control (RHS4346), pGIPZ sh-AMPKα1 (RHS4430-101519639), pGIPZ sh-AMPKα2 (RMM4431-99954844), and pLKO.1 sh-E2F1 (RMM3981-9609832) were purchased from Open Biosystems (Waltham, MA). The [E2F]4–Luc reporter construct was generously provided by Dr. Young-Chae Chang (The Catholic University, School of Medicine, Daegu, Korea). The promoter region of the human AMPKα2 gene (~2.7kb) was cloned by PCR from HEK293 genomic DNA and cloned into the pGL3-basic reporter vector.

Cell Culture and Treatment—AMPK WT, AMPKα2+/−, AMPKα1−/−, and AMPKα1/2 double knock-out (DKO) MEFs were generated and immortalized as described previously elsewhere (23, 31). Sirt1 WT and Sirt1−/− MEFs were gifts from Chu-Xia Deng (National Institutes of Health, MD). HEK293 (human embryonic kidney cells) and C2C12 (mouse myoblast cells) cells were maintained in DMEM supplemented with 10% FBS. H9c2 (rat cardiomyoblasts) cells were maintained in DMEM and F-12 (1:1) supplemented with 10% bovine serum. ECV cells were maintained in M199 supplemented with 10% FBS, 3.5 mM glutamine. HCT116 (human colorectal carcinoma), A549 (human lung adenocarcinoma), AGS (human gastric adenocarcinoma), DU145 (human prostate cancer), and HeLa (human cervical carcinoma) cells were maintained in RPMI 1640 supplemented with 10% FBS. All cells were supplemented with antibiotics and cultured at 37 °C in 95% air and 5% CO2.

RNA Isolation and Real-time PCR—Real-time PCR was performed to quantify messenger RNA expressions using the SYBR Green PCR Master Mix and the ABI PRISM 7300 Real-time PCR system (Applied Biosystems, Foster City, CA), according to the manufacturer’s instructions. Relative messenger RNA expression was quantified using the comparative Ct (ΔΔCt) method and expressed as 2−ΔΔCt, where ΔΔCt = ΔCt – ΔCt, ΔCt = Ct target – CtE GAPDH, and ΔC = Ctt target – Ctt GAPDH (E is the experimental result, and C is the control). The primers for the PCR analysis were as follows: for AMPKα1 forward (5’-GATCGGCCACTACATCCTGG-3’), AMPKα1 reverse (5’-GATGGGAAGGTCCCTGACA-3’), AMPKα2 forward (5’-TGACAGGCCCATAGTGCA-3’), AMPKα2 reverse (5’-TCTTCCACCCGCCCATGTTT-3’), AMPKα1/α2 forward (5’-TGTCCAGAGGCATATTGTC-3’), AMPKα1/α2 reverse (5’-ATACGATCATTTCACCTGACTACC-3’), E2F1 forward (5’-GACGATTTGCGGATG-3’), E2F1 reverse (5’-GATGCCCCGATTTGATA-3’), Sirt1 forward (5’-AGGAGAAGGTCAAAAGTCAGGCCA-3’), Sirt1 reverse (5’-GATCGGCTACATCCTGG-3’), GAPDH forward (5’-GGAGAGGCTTCATGACACCA-3’), GAPDH reverse (5’-GCAGTGACACACTTGTTT-3’), P27 forward (5’-AGGAGGAGAACGCGAG-3’), P27 reverse (5’-GCTCCGCTAACCCAGGCGGA-3’), Apaf-1 forward (5’-AGGAGAAGGTCAAAAGTCAGGCCA-3’), Apaf-1 reverse (5’-ACTTGGCGCAGCCTGCT-3’).

The level of each mRNA was normalized with that of GAPDH.

Cell Viability Assay—The Vi-CELL XR Cell Viability analyzer (Beckman Coulter, Fullerton, CA) that performs an automated trypan blue exclusion assay was used to measure cell count.
viability. The assay is based on uptake of trypan blue dye by dead cells due to loss of membrane integrity. A 1-ml aliquot of cell suspension in a plastic cuvette was aspirated and mixed with trypan blue and then pumped into the flow cell for imaging. The instrument collected 50 images of cells to compute viability. Dead cells appear darker than viable cells due to different contrast between live and dead cells.

**Reporter Gene Assay**—Cell were seeded onto 24-well culture plates at 4 × 10^4 cells/well and incubated for 24 h. Plasmids were transfected into cells using JetPEI (Polyplus Transfection, Illkirch, France) according to the manufacturer’s instructions. A 1:1 ratio between reporter gene constructs and expression vector was used for co-transfections. After 24 h of transfection, luciferase activity was determined by mixing 20 g of cell extract with 100 l of luciferase assay reagent, and relative light units were measured for 10 s in a luminometer (TD-20/20 luminometer, Turner Designs, Sunnyvale, CA).

**Establishment of Stable Cell Lines**—MEFs were transfected with the pLKO.1 vector expressing shRNA for E2F1 or the pGIPZ vector expressing shRNA for AMPKα1 and -α2. Stable cells were isolated in media 24 h after the transfection with 3 g/ml puromycin for 2 weeks. Additionally, MEFs were transfected with the pCMV vector expressing E2F1-WT or E2F1ΔTA. After a 24-h transfection, positive-expressing clones were selected with G418 (300 μg/ml) after about 2 weeks of selection.

**Annexin V-FITC Assay by Flow Cytometry**—The percentages of apoptotic cells were determined by flow cytometry using the annexin V-FITC Apoptosis Detection Kit Plus (BioVision, Inc) according to the manufacturer’s instructions. Briefly, MEF cells were harvested and resuspended in a binding buffer. Cells (10^6/ml) were mixed with 5 μl of annexin V-FITC. After incubation at room temperature for 15 min in the dark, the stained cells were analyzed using fluorescence-activated cell sorting (BD FACS Canto II, BD Biosciences) reading the emission selected by 530 nm (FITC).

**Coimmunoprecipitation**—Two milligrams of cell extract was incubated with 2 g of anti-E2F1 or 2 g of anti-AMPKα2 antibody at 4 °C for overnight. Twenty microliters of Protein G-agarose beads were added, and the incubation was continued at 4 °C for 4 h. After centrifugation, immuno-complexes were washed 4 times with 1 ml of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% (v/v) Nonidet P-40), resuspended in 30 l of SDS/PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 2 mM 2-mercaptoethanol, 12.5 mM EDTA, 0.02% (w/v) bromphenol blue), heated to 90 °C for 5 min, and analyzed via SDS-PAGE.

**In Situ Proximity Ligation Assay (in Situ PLA)**—The proximity ligation assays were performed using the Duolink® II detection kit (Olink Bioscience, Uppsala, Sweden). After transfection with the indicated expression vectors or exposure to doxorubicin, the cells were fixed in 4% paraformaldehyde for 5 min at room temperature. After washing with PBS, the cells were treated with 0.1% Triton X-100 in PBS for 3 min at room temperature. To reduce the nonspecific signals, the cells were incubated with a blocking solution for 30 min at 37 °C. Primary antibody diluents containing two primary antibodies (1:500 dilutions of anti-FLAG rabbit antibodies and anti-GFP mouse
AMPκ2 and E2F1 Mediate Doxorubicin Cytotoxicity

A. 

B. 

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D. 

E. 

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antibodies for transfection study or 1:250 dilutions of anti-E2F1 rabbit antibodies and anti-AMPKα2 goat antibodies for detecting endogenous proteins) were added to the cells and incubated overnight at 4 °C. Then the PLA Probes (Detection Reagents Green or Detection Reagents Red) were added and incubated for 2 h at 37 °C. The images of the cells were acquired using an LSM 700 Confocal microscope (Carl Zeiss, Jena, Germany).

In Vitro AMPK Kinase Assay—E2F1 was overexpressed and purified as a fusion protein with glutathione S-transferase in Escherichia coli and used as a substrate for in vitro AMPK assay. The AMPK complex was overexpressed in HEK293 cells by cotransfection of HA-AMPKα2, Myc-AMPKβ1, and Myc-AMPKγ1, immunoprecipitated with anti-HA antibody, eluted with HA peptide, and further purified by desalting with desalting spin column. The recombinant GST-E2F1 and AMPKα2 complexes were incubated in the kinase assay buffer (62.5 mM HEPES, pH 7.0, 62.5 mM NaCl, 62.5 mM NaF, 6.25 mM sodium pyrophosphate, 1.25 mM EDTA, 1.25 mM EGTA, and 1 mM dithiothreitol containing 200 μM AMP) containing 10 μM cold ATP and 10 μCi of [γ-32P]ATP at 37 °C for 1 h. The reaction was terminated by adding SDS sample buffer, and the reaction mixtures were subjected to SDS-PAGE and autoradiography. GST-SAMS contains the AMPK phosphorylation peptide sequence of acetyl-CoA carboxylase (HMRSAMSGLHLVKRR) and used as a positive control for AMPK activity.

Statistical Analysis—Results are expressed as the means ± S.E. We used Student’s t test and Prism 5 software. Differences were considered significant at a p value of <0.01. **, p < 0.01; ***, p < 0.001.

RESULTS

AMPKα2 Expression Is Induced in Response to Doxorubicin in MEFs—MEFs were exposed to various DNA damaging agents or to glucose-free medium for 12 h, and then the expression levels of the AMPKα subunits were examined by Western blot analysis (Fig. 1A). AMPKα2 was hardly detectable in MEFs under basal conditions, but the DNA damaging agents including H2O2, doxorubicin, etoposide, and ionizing radiation dramatically induced expression of AMPKα2 as well as a DNA damage marker, phosphorylation of H2AX. Under these conditions, the level of AMPKα1 was unaffected or decreased slightly. In contrast to the DNA damaging agents, metabolic stress such as glucose starvation had no effect on AMPKα2 expression, indicating that AMPKα2 is specifically induced by DNA damage. Indeed, doxorubicin robustly induced AMPKα2 expression in a time- and dose-dependent manner, but the level of AMPKα1 decreased (Fig. 1, B and C). E2F1 was simultaneously induced by doxorubicin (Fig. 1, A–C). The AMPKα2 mRNA level increased ∼15-fold by doxorubicin treatment (Fig. 1D). AMPKα1 is a predominant form in wild type MEF under basal conditions. As a net sum of the decreased AMPKα1 and the increased AMPKα2 under doxorubicin treatment, the protein and mRNA amount of total AMPKα appears to be same or slightly increased (Fig. B–D). We examined the effect of doxorubicin in subsequent studies.

AMPKα2 Exerts Pro-apoptotic Properties under Doxorubicin Treatment—To investigate the function of each AMPKα subunit, we compared the cellular sensitivity of MEFs from Ampkα wild type, Ampkα1/−/− deletion, and Ampkα2/−/− deletion to doxorubicin. When cell viability was measured directly, Ampkα2/−/− MEFs were most resistant to doxorubicin-induced cytotoxicity, whereas Ampkα1/−/− MEFs showed the increased sensitivity to doxorubicin-induced cytotoxicity compared with wild type MEFs (Fig. 2A). Fluorescence-activated cell sorting analysis of AMPK deletion MEFs with annexin V staining (Fig. 2B) and the comparison of apoptotic markers including the cleaved form of poly(ADP-ribose) polymerase and caspase-3 among AMPKα isoform-specifically gene knocked-down MEFs (Fig. 2C) revealed similar results showing that AMPK isoforms exert opposing effects on doxorubicin-induced apoptosis. Our data suggest that AMPKα1 is anti-apoptotic, whereas AMPKα2 is pro-apoptotic in response to doxorubicin. In fact, the previous report demonstrated that AMPKα1 plays the protective role against doxorubicin-induced apoptosis in MEFs and H9c2 cells (16), but the role of AMPKα2 remains unknown. Therefore, we primarily focused on revealing a novel role of AMPKα2 and the underlying mechanisms throughout the present study. To unequivocally demonstrate the role of AMPKα2, we next examined the specific role of AMPKα2 in Ampkα1/−/− MEFs. AMPKα2 was still induced by doxorubicin in Ampkα1/−/− MEFs, and knock-down of AMPKα2 in Ampkα1/−/− MEFs diminished the apoptotic response to doxorubicin (Fig. 2D). Compound C, an AMPK inhibitor, effectively blocked the AMPKα2 activity in Ampkα1/−/− MEFs after doxorubicin treatment, as judged by the phosphorylation level of acetyl-CoA carboxylase (ACC) Ser79 (32), the most well characterized AMPK substrate (Fig. 2E), leading to attenuation of the Ampkα1/−/− MEF apoptotic response to doxorubicin (Fig. 2E) and alleviation of the Ampkα1/−/− MEF doxorubicin cytotoxicity (Fig. 2F). In conclusion, when the induction of AMPKα2 by doxorubicin was hampered by genetic, molecular, and pharmacological approaches, MEFs became resistant to doxorubicin-induced apoptosis and cytotoxicity, indicating that AMPKα2 exerts pro-apoptotic properties. The pro-apoptotic role of AMPKα2 was further demonstrated; Ampkα1/−/−α2/−/− DKO MEFs were transfected with vector expressing the wild type (WT) and dominant negative form (DN) of AMPKα2 and then treated with doxorubicin (Fig. 2G). The percentage of apoptotic cells under doxorubicin treatment, as
AMPKα2 and E2F1 Mediate Doxorubicin Cytotoxicity

A. HEK293 cells

C. WT MEFs

B. WT MEFs

D. WT MEFs

E. WT MEFs

F. HEK293 cell

G. WT MEFs

H. Mouse Liver tissue
ampkAlpha2 and E2F1 Mediate Doxorubicin Cytotoxicity

assessed by annexin V staining, significantly increased in the presence of AMPKalpha2-WT but not AMPKalpha2-DN.

E2F1 is a Transcriptional Regulator of AMPKalpha2 Gene Expression under Doxorubicin Treatment—E2F1 is a transcription factor that regulates expression of a subset of genes involved in cell proliferation and apoptosis (27–30, 33). A recent report demonstrated that AMPKalpha2 is downstream of E2F1 when the phosphatidylinositol 3-kinase signal is blocked; AMPKalpha2 is induced in a E2F1-dependent manner under serum-free conditions or with phosphatidylinositol 3-kinase inhibitor treatment (34). E2F1 is stabilized by DNA damage and induces a number of genes involved in apoptosis (28–30). As E2F1 was induced by doxorubicin in MEFs (Fig. 1), we examined whether AMPKalpha2 was induced by doxorubicin in a E2F1-dependent manner. First, we constructed wild-type functional E2F1 (FLAG-E2F1-WT) and non-functional E2F1 (FLAG-E2F1-DBD, in which DNA-binding domain was deleted). The activity of E2F1-WT and E2F1-DBD was determined by cotransfection with a luciferase reporter vector containing four-repeated E2F1-responsive element ((E2F1 × 4)-Luc) in HEK293 cells; E2F1-WT induced a 2-fold increase in luciferase activity, whereas E2F1-DBD showed no activity (Fig. 3A). Then, we established MEFs stably expressing E2F1-WT and E2F1-DBD. Overexpression of E2F1-WT, but not E2F1-DBD, resulted in a significant induction of AMPKalpha2, the protein (Fig. 3B) and mRNA level (Fig. 3C). The known target genes of E2F1 including p27kip1, Bim, and Apaf-1 was also induced by E2F1-WT (Fig. 3, B and C). Moreover, AMPKalpha2 and Bim expression was profoundly induced by doxorubicin when E2F1-WT, but not E2F1-DBD, was overexpressed (Fig. 3D). In contrast to overexpression, knockdown of E2F1 expression by shRNA distinctively blocked doxorubicin-induced expression of AMPKalpha2, Bim, and apoptosis biomarkers, indicating that E2F1 acts upstream of AMPKalpha2 with doxorubicin treatment (Fig. 3E). We next cloned a human AMPKalpha2 promoter (~2.7 kb) into the pGL3 luciferase reporter vector, and cotransfection with E2F1-WT in HEK293 cells resulted in a 14-fold induction of luciferase (Fig. 3F). Cotransfection with E2F1-WT in wild-type MEFs induced a ~3-fold increase in AMPKalpha2 promoter activity, and this induction was further increased by doxorubicin, but E2F1-DBD or E2F1-ΔDBD, in which DNA-binding domain was deleted, failed to induce AMPKalpha2 promoter activity (Fig. 3G). We also observed the induction of AMPKalpha2, E2F1, and its target genes including Bim and ULK1 and the pro-apoptotic markers such as Bax and cleavage of caspase-3 in mouse liver from animals injected with doxorubicin through the tail vein (Fig. 3H). Collectively, these results indicate that AMPKalpha2 gene expression is induced by E2F1 in response to doxorubicin and that both E2F1 and AMPKalpha2 show pro-apoptotic properties.

AMPKalpha2 Positively Regulates E2F1 Protein Stability—We next explored the possibility of feedback regulation between AMPKalpha2 and E2F1. Overexpression of wild-type AMPKalpha2, but not its dominant negative form, in wild-type MEFs (Fig. 4A) as well as in Ampkalpha2-/- MEFs and Ampkalpha2-/- E2F1 DOKO MEFs (Fig. 4B) induced endogenous E2F1 and its target gene expression. We compared the effect of AICAR, a pharmacological activator of AMPK, on the degree of endogenous E2F1 induction in Ampkalpha2-/- MEFs and Ampkalpha2-/- E2F1 DOKO MEFs to investigate the specific role of AMPKalpha2. AICAR induced phosphoacetyl-CoA carboxylase and E2F1 in Ampkalpha2-/- MEFs, but not in Ampk DKO MEFs, without altering E2F1 mRNA levels (Fig. 4C). Moreover, E2F1 induction and subsequent expression of its target genes by doxorubicin were markedly diminished in Ampk DKO MEFs compared with Ampkalpha2-/- MEFs, in which AMPKalpha2 was still induced by doxorubicin (Fig. 4D). The AMPKalpha2 isoform-specific regulation for E2F1 was further demonstrated by AMPKalpha1 gene knockdown approaches; AMPKalpha2 down-regulation by shRNA, but not AMPKalpha1 down-regulation, significantly blocked doxorubicin-induced E2F1 expression in wild-type MEFs (Fig. 4E). These data raised the possibility that AMPKalpha2 positively regulates E2F1 induction at the post-transcriptional level because the E2F1 mRNA level was not affected.

To further confirm this possibility, wild-type MEFs were cotransfected with FLAG-tagged E2F1 and AMPKalpha2, and overexpression of wild-type AMPKalpha2 resulted in a significant induction of exogenous E2F1 (Fig. 4F) as well as the transcriptional activity (Fig. 4G). We next transfected FLAG-tagged E2F1 into Ampkalpha2-/- MEFs and Ampkalpha2 DKO MEFs, and the GFP expression vector was cotransfected as a control to assess transfection efficiency. The protein level of exogenously introduced E2F1 was ~8-fold higher in Ampkalpha1-/- MEFs than that in Ampkalpha DKO MEFs, whereas the GFP level was essentially the same under these conditions (Fig. 4H). We further determined the role of AMPKalpha2 in regulation of the half-life of the E2F1 protein. After transfection with FLAG-E2F1, Ampkalpha2-/- MEFs were treated with cycloheximide, an inhibitor of new protein synthesis, in the absence or presence of compound C, and the result indicated that E2F1 was rapidly degraded in the presence of the AMPK inhibitor (Fig. 4I). These data collectively suggest that AMPKalpha2 positively regulated E2F1 protein stability. As E2F1 induces AMPKalpha2 at the transcriptional level, these two molecules form a positive signal amplification loop in response to doxorubicin.

AMPKalpha2 Directly Interacts with and Phosphorylates E2F1—To examine whether AMPKalpha2 directly interacts with E2F1, we

FIGURE 3. E2F1 regulates gene expression of AMPKalpha2 in the presence of doxorubicin. HEK293 cells were cotransfected with FLAG-E2F1-WT or E2F1-ΔTA and with [E2F1 × 4]-Luc, and then luciferase activity was measured (A). Wild-type MEFs stably expressing FLAG-E2F1-WT and Flag-E2F1-ΔTA were established, and then the indicated protein (β) and mRNA levels (c) were measured. These cells were exposed to doxorubicin (1 μM, 6–12 h), and the expression of AMPKalpha2 and Bim were compared (D). Two clones of wild-type MEFs stably expressing E2F1 shRNA were isolated, and the indicated protein level was examined in the presence of doxorubicin (0.1 μM, 24 h (E)). A human AMPKalpha2 promoter (~2.7 kb) was cloned into the pGL3 luciferase reporter vector. This vector was cotransfected into HEK293 cells with FLAG-E2F1-WT or FLAG-E2F1-ΔTA, and then the luciferase assay was performed (F). After performing an identical cotransfection in wild-type MEFs, the cells were exposed to doxorubicin (DOX; 1 μM, 12 h), and the luciferase assay was performed (G). Data represented are the means ± S.E. n ≥ 3. Four-week-old male SlcICR mice were injected with doxorubicin (5 mg/kg) through the tail vein at 3-day intervals, whereas the control mice were injected with a comparable volume of water. The mice were sacrificed after 15 days, liver lysates were extracted, and Western blot analysis was performed (H).
cotransfected HEK293 cells with E2F1 and Myc-tagged AMPKα2 or GFP-tagged AMPKα2 expression vector and then performed a co-immunoprecipitation assay. In a complex precipitated with E2F1 antibody, the presence of Myc-tagged AMPKα2 or GFP-tagged AMPKα2 was confirmed (Fig. 5A). Likewise, E2F1 was also detected in a complex precipitated with AMPKα2 antibody (Fig. 5A). The proximity ligation assay (PLA) also visualized the in situ protein complex between AMPKα2 and E2F1 (Fig. 5B). PLA is a combination of immunohistochemistry and rolling cycle amplification between adjacent, oligonucleotide-coupled secondary antibodies (35). The protein complex formation between endogenous AMPKα2 and E2F1 was also detected in AMPK wild type MEFs, and doxorubicin treatment significantly enhanced the protein complex formation (Fig. 5, C and D). Moreover, AMPKα2 directly phosphorylated GST-E2F1 fusion protein in vitro but not GST alone (Fig. 5E). These data suggest that AMPKα2 forms a complex with and phosphorylates E2F1 in response to doxorubicin treatment.

Resveratrol Alleviates Doxorubicin Cytotoxicity by Suppressing the AMPK-E2F1 Signal Loop in MEFs and Non-carcinoma Cells—A number of reports have suggested that resveratrol attenuates doxorubicin-induced cytotoxicity, but precise mechanisms have not been substantiated (36–39). We next examined the effect of resveratrol on the AMPK-E2F1 signal loop. Wild type MEFs were exposed to doxorubicin in the presence of resveratrol and structurally similar flavonoids including piceatannol and fisetin. These flavonoids significantly blocked doxorubicin-induced expression of E2F1, AMPKα2, and poly(ADP-ribose) polymerase cleavage (Fig. 6A). Accordingly, these flavonoids blocked the cytotoxicity induced by doxorubicin (Fig. 6B). A recent report demonstrated that E2F1 activity is down-regulated by Sirt1 deacetylase (40). Because resveratrol can activate Sirt1 (39, 41), we tested whether Sirt1 is involved in the resveratrol action on E2F1 and AMPKα2. Wild type MEFs and Sirt1−/− MEFs were exposed to doxorubicin in the absence or presence of resveratrol, and the results showed that resveratrol continued to block doxorubicin-induced AMPKα2 and E2F1 in Sirt1−/− MEFs (Fig. 6C). Sirt1−/− MEFs were more sensitive to doxorubicin-induced apoptosis than that of the wild type, as assessed by the level of cleaved poly(ADP-ribose) polymerase and caspase-3 (Fig. 6C). However, resveratrol decreased the apoptotic markers to a similar degree in both cells, when comparing a long exposure of wild type with short exposure of Sirt1−/− MEFs (Fig. 6C). These data suggest that the AMPK-E2F1 signal loop is a novel target for resveratrol action, but Sirt1 is not likely to be involved in this regulation.

In addition to MEFs, doxorubicin also induced the expression of AMPKα2 and E2F1 in rat cardiomyoblast H9c2 cells, mouse myoblast C2C12 cells, and human vascular endothelial cells (Fig. 7A). Furthermore, resveratrol suppressed doxorubicin-induced AMPK-E2F1 signal axis and apoptosis (Fig. 7B), thereby protecting these cells from doxorubicin-induced cytotoxicity (Fig. 7C).

AMPKα2 Is Not Induced by Doxorubicin in Cancer Cells—We finally examined the effect of doxorubicin on AMPKα2 and E2F1 in cancer cells. Five different cancer cell types were exposed to the indicated concentrations of doxorubicin for 12 h. Interestingly, E2F1 was still induced by doxorubicin in these cancer cells, but no AMPKα2 induction was observed (Fig. 8A). Moreover, doxorubicin killed cancer cells regardless of the AMPKα2 level; AGS cells did not express AMPKα2, but doxorubicin decreased AGS cell viability as effectively as that in other cancer cells (Fig. 8B). Transfection of wild type AMPKα2 into AGS cells did not alter the E2F1 expression level or the apoptotic response to doxorubicin (Fig. 8C). Collectively, our data suggest that AMPKα2 does not form a positive signal loop with E2F1 in cancer cells, further suggesting that a signal loop between AMPKα2 and E2F1 quite specifically operates in non-carcinoma cells.

**DISCUSSION**

Multiple mechanisms have been implicated in the development of doxorubicin toxicity including redox damage, but none have been fully substantiated. Administration of compounds harboring anti-oxidant or anti-apoptotic activity has been attempted to reduce doxorubicin cytotoxicity, but some of these attempts failed in clinically relevant animal models and clinical trials (4, 42, 43). Moreover, the idea of redox damage was challenged by a recent study showing that electron transport chain damage is a major mechanism for doxorubicin cytotoxicity (44). Indeed, despite 40 years of research, there is no effective approach to alleviate doxorubicin cytotoxicity. In the present study we revealed a novel signal pathway that potentially serves as a molecular target for alleviating doxorubicin cytotoxicity in non-carcinoma cells; AMPKα2 formed a positive signal amplification loop with the E2F1 transcription factor after doxorubicin treatment and mediated doxorubicin cytotoxicity in MEFs.

In the present study we first observed that the AMPKα2 transcript was dramatically induced by DNA damage including doxorubicin in MEFs (Fig. 1). The major regulatory mechanism for AMPK is short term regulation. AMPK enzyme activity is allosterically regulated by the AMP and ATP cellular ratio (7, 8). In addition, AMPKα2 is phosphorylated at Thr172 and activated by upstream kinases including LKB1, CaMKK (CaM kinase), and TAK (6, 9, 45–47). These two mechanisms have been intensively investigated and largely explain the rapid change in AMPK activity in response to a number of metabolic stressors. However, little information is available on the
AMPKα2 and E2F1 Mediate Doxorubicin Cytotoxicity

A. HEK293 cell

|   | vector | E2F1 | Myc-AMPKα2 | GFP-AMPKα2 |
|---|---|---|---|---|
| IP; E2F1 WB; AMPKα2 | + | + | - | - |
| IP; AMPKα2 WB; E2F1 | + | + | - | - |
| AMPKα2 whole cell lysate | - | - | + | + |
| α-tubulin | - | - | - | - |

B. In situ PLA assay

Transfection:
- DAPI
- Red PLA signal
- Merge

Transfection:
- GFP-AMPKα2 + Flag E2F1

Flag-E2F1

C. WT MEFs

|  | E2F1 | IgG | Input |
|---|---|---|---|
| IP; DOX | - | + | - |
| WB; AMPKα2 | - | + | - |

D. WT MEFs

|  | 0 hr | DOX 6 hr | 12 hr |
|---|---|---|---|
| DAPI | green PLA signal | Merge | DAPI | green PLA signal | Merge |

E. Kinase:

|   | vector | AMPKα2 WT | AMPKα2 ΔN | AMPKα2 ΔN ΔY1 | AMPKα2 ΔN ΔY1 |
|---|---|---|---|---|---|
| gamma 32P | - | | | | |
| WB; GST | GST | GST-SAMS | GST-E2F1 | vector | | |
changes in AMPK transcription except that AMPK expression level is tissue-specific; AMPKα is present in every tissue, whereas AMPKβ is highly expressed in heart, skeletal muscle, and liver (21). Herein, we report that the AMPKβ mRNA level was induced dramatically by doxorubicin in MEFs (Fig. 1), and we further identified E2F1 as a transcription factor for AMPKβ during doxorubicin treatment. E2F1 directly increased AMPKβ promoter activity and mRNA levels, and gene knockdown of E2F1 under doxorubicin treatment significantly blocked AMPKβ induction (Fig. 3). Our data suggest that long term regulation such as transcriptional regulation may be critical for regulating AMPK under genotoxic stress in addition to short term regulation.

Our data also highlight the specific and differential function of AMPKα isoforms. Inhibition of AMPKα2 activity by genetic deletion, gene knockdown, or pharmacologically alleviated doxorubicin cytotoxicity suggests that AMPKα2 functions as a pro-apoptotic molecule, whereas AMPKα1 contributes to cell survival under this condition in accordance with a previous report (16) (Fig. 2). The role of each isoform may vary depending on the stress conditions. Indeed, gene knock-out animal studies suggest that the functions of these isoforms are quite different for regulating metabolism as well. When Ampkα2−/− mice are fed a high fat diet, derangements occur in the regulation of glucose tolerance, insulin sensitivity, and weight gain, whereas these changes were not detected in Ampkα1−/− mice (23–26). In contrast, endothelial nitric-oxide synthase activation and blood vessel relaxation are observed in Ampkα1−/− mice, but not in Ampkα2−/− mice, in response to the AMPK activator (6, 22). At present the precise mechanisms that confer a differential role for each isoform remain almost unknown, although differences in tissue distribution, cellular localization, and substrate specificity of AMPK isoforms have been reported (21, 48, 49). Therefore, we suggest that future studies should consider the potentially differential role of AMPKα isoforms, and such approaches will reveal the more
precise AMPK-involved cellular events under metabolic and genotoxic stressors.

Our results are summarized in a diagram (Fig. 9). In initial response to DNA damage, it is well known that E2F1 is stabilized by phosphorylation via ATM, ATR, or Chk2 (27, 50, 51) or by acetylation via p300/CREB-binding protein-associated factor (P/CAF) (52). Our data propose that the stabilized E2F1 after DNA damage acts as an upstream transcription factor for AMPKα2, promoting the transcription of AMPKα2 (Fig. 3), which further contributes to E2F1 stability (Fig. 4). The pro-
apoptotic role of E2F1 in response to DNA damaging agent has been well established. Our data suggest that AMPKα2 contributes to induction of apoptosis by stabilizing E2F1 by direct phosphorylation. Doxorubicin enhanced the direct protein interaction between AMPKα2 and E2F1, and furthermore AMPKα2 directly phosphorylated E2F1 in vitro (Fig. 5). Although we do not have direct evidence at this time, phosphorylation of E2F1 by AMPKα2 may be critical for E2F1 stability because inhibition of AMPKα2 activity resulted in rapid protein degradation of E2F1 (Fig. 4F). Characterizing the phosphorylation site(s) of E2F1 by AMPKα2 will provide us with more detailed information on the underlying mechanisms for E2F1 regulation by AMPKα2. The tight association between these two molecules was further demonstrated by pretreatment of flavonoids including resveratrol, piceatannol, and fisetin; these flavonoids dramatically blocked the induction of both AMPKα2 and E2F1 and protected MEFs in the presence of doxorubicin (Fig. 6). This signal loop was also observed in several other cell lines including H9c2, C2C12, and ECV, and resveratrol blocked the signal loop, resulting in reduced doxorubicin cytotoxicity in these cells as well (Fig. 7).

We also observed the induction of AMPKα2, E2F1, and its target genes, apoptotic markers in mouse liver from animal injected with doxorubicin (Fig. 3H).

Notably, doxorubicin did not induced AMPKα2 in five different cancer cell lines under our experimental conditions despite E2F1 induction (Fig. 8A). Moreover, it was difficult to identify a significant correlation between the expression level of AMPKα2 and the cellular response to doxorubicin in the tested cancer cells (Fig. 8, B and C). We currently do not understand how AMPK is differentially regulated in normal and cancer cells, but an intensive analysis of the AMPKα2 gene promoter including epigenetic difference or characterization of E2F1 response element in normal and cancer cells may provide a clue. Collectively, our data suggest that a signal amplification loop between AMPKα2 and E2F1 in the presence of doxorubicin appears to be non-carcinoma cell-specific. As this signal loop mediates doxorubicin cytotoxicity in non-carcinoma cells and serves as a novel target for resveratrol action, more studies on this signal pathway will provide a better understanding of the nature of doxorubicin cytotoxicity.

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