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# AMPK $\alpha$ 1 deficiency promotes cellular proliferation and DNA damage via p21 reduction in mouse embryonic fibroblasts



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## ABSTRACT

Emerging evidence suggests that activation of adenosine monophosphate-activated protein kinase (AMPK), an energy gauge and redox sensor, controls the cell cycle and protects against DNA damage. However, the molecular mechanisms by which AMPK $\alpha$  isoform regulates DNA damage remain largely unknown. The aim of this study was to determine if AMPK $\alpha$  deletion contributes to cellular hyperproliferation by reducing p21<sup>WAF1/Cip1</sup> (p21) expression thereby leading to accumulated DNA damage. The markers for DNA damage, cell cycle proteins, and apoptosis were monitored in cultured mouse embryonic fibroblasts (MEFs) isolated from wild type (WT, C57BL/6J), AMPK $\alpha$ 1, or AMPK $\alpha$ 2 homozygous deficient (AMPK $\alpha$ 1<sup>-/-</sup>, AMPK $\alpha$ 2<sup>-/-</sup>) mice by Western blot, flow cytometry, and cellular immunofluorescence staining. Deletion of AMPK $\alpha$ 1, the predominant AMPK $\alpha$  isoform, but not AMPK $\alpha$ 2 in immortalized MEFs led to spontaneous DNA double-strand breaks (DSB) which corresponded to repair protein p53-binding protein 1 (53BP1) foci formation and subsequent apoptosis. Furthermore, AMPK $\alpha$ 1 localizes to chromatin and AMPK $\alpha$ 1 deletion down-regulates cyclin-dependent kinase inhibitor, p21, an important protein that plays a role in decreasing the incidence of spontaneous DSB via inhibition of cell proliferation. In addition, AMPK $\alpha$ 1 null cells exhibited enhanced cell proliferation. Finally, p21 overexpression partially blocked the cellular hyperproliferation of AMPK $\alpha$ 1-deleted MEFs via the inhibition of cyclin-dependent kinase 2 (CDK2). Taken together, our results suggest that AMPK $\alpha$ 1 plays a fundamental role in controlling the cell cycle thereby affecting DNA damage and cellular apoptosis.

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## 1. Introduction

Several types of DNA damage, including oxidative damages, depurinations, single-strand breaks (SSB), and DNA double-strand breaks (DSBs), occur naturally during DNA replication [1]. DSBs are among the most deleterious lesions in the genome, as it can cause genomic rearrangements, chromosome breaks and translocations, leading to apoptosis, senescence, or tumorigenesis [2]. Most DNA damage can undergo DNA repair. For example, DSBs elicit a cascade of protein recruitment to the chromatin surrounding DNA lesions that regulate DNA damage response signaling and repair [3]. Cells repair DSB by initiating either DNA nonhomologous end-joining (NHEJ),

a mutation-prone pathway [4,5], or homologous recombination (HR) [6]. DSB repair by HR is largely error-free, as it employs undamaged homologous sister chromatid DNA sequences as templates for repair [2]. NHEJ is the prevalent DSB repair pathway in higher eukaryotes [7]. Several molecules have been reported to be involved in DSB response and repair. For example, p53-binding protein 1 (53BP1, also known as TP53BP1) is a key effector of this DSB response [8,9], as it promotes DNA damage repair by NHEJ [10–13].

Cyclin-dependent kinases (Cdks) play a pivotal role in the cell cycle. Among them, Cdk2 is essential for G1/S phase transition and S phase progression [14]. On the other hand, Cdk1 (formerly known as Cdc2) associated with Cyclin B is essential for regulating cell cycle entry and exits from mitosis [15,16]. In response to DNA damage, numerous cell cycle signals are activated, which causes arrest in G1, thereby controlling progression through S phase and blocking entry into mitosis with damaged DNA. For example, p21<sup>WAF1/Cip1</sup> (p21) binds to and suppresses Cdk2/cyclin E (CycE) complexes, thereby arresting cells at the G1/S checkpoint [17]. In addition, p21 plays a fundamental role in DNA damage response through inhibiting DNA synthesis via association and inhibition of proliferating cell nuclear antigen (PCNA) [18]. Recently, it was

**Abbreviations:** 53BP1, p53-binding protein 1; AMPK, adenosine monophosphate-activated protein kinase; DSB, double-strand breaks; MEFs, mouse embryonic fibroblasts; NHEJ, nonhomologous end-joining

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reported that p21 is critical in preventing excessive DNA damage accumulation in leukemia stem cells [19].

The well-known energy sensor, adenosine monophosphate-activated protein kinase (AMPK), consisting of a catalytic  $\alpha$  subunit ( $\alpha 1$  or  $\alpha 2$ ) and regulatory  $\beta$  ( $\beta 1$  or  $\beta 2$ ) and  $\gamma$  ( $\gamma 1$ ,  $\gamma 2$ , or  $\gamma 3$ ) units, also has a critical role in cell mitosis [20–22] and anti-oxidative stress [23]. Emerging data indicate that AMPK plays an important role in tumor suppression [24,25]. It was recently reported that AMPK regulates UVB-induced DNA damage repair in skin tumor cells [26], as well as playing a role in NHEJ via the LKB1-AMPK signaling pathway [27]. However, the exact role of the two AMPK $\alpha$  isoforms and the mechanism by which AMPK might control the cell cycle and DNA damage remain elusive. In this study, we address the involvement of AMPK $\alpha 1$  in cellular hyperproliferation, DNA damage, and apoptosis by analyzing cell cycle proteins and DNA damage markers in AMPK $\alpha 1^{-/-}$  mouse embryo fibroblasts (MEFs). We demonstrate here, for the first time, that AMPK $\alpha 1^{-/-}$  MEFs exhibit hyperproliferation, high levels of DNA DSB markers, and consequent apoptosis, partially due to the p21 reduction. Importantly, p21 overexpression decreased the foci formation of DSB repair protein 53BP1 in AMPK $\alpha 1^{-/-}$  MEFs. These findings establish a new role for AMPK $\alpha 1$  in cell cycle and DNA damage, providing novel insights into the mechanism of tumor suppression mediated by AMPK.

## 2. Materials and methods

### 2.1. Materials and reagents

The following antibodies were obtained from Cell Signaling Technology (Beverly, MA): rabbit anti-AMPK $\alpha$  (2532), rabbit anti-phospho-AMPK $\alpha$  (Thr172) (2535), rabbit anti-phospho-histone H3 (Ser10) (9701), rabbit anti-phospho-p53 (Ser18) (9284), mouse anti-p53 (2524), rabbit anti- $\gamma$ -H2AX (2577), anti-H2AX (2595), anti-phospho-Chk1 (Ser345) (2348), rabbit anti-53BP1 (4937), rabbit anti-cleaved caspase-3 (Asp175) (9664), rabbit anti-PARP (9542), rabbit anti-phospho-Cdk2 (Thr160) (2561), rabbit anti-Cdk2 (2546), rabbit anti-phospho-Cdk1(Thr161) (9114), and mouse anti-cyclin B1 (V152) (4135). The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): goat anti-AMPK $\alpha 1$  (sc-19128), goat anti-AMPK $\alpha 2$  (sc-19129), mouse anti-XRCC4 (sc-365118), mouse anti-p21 (sc-6246), mouse anti-GAPDH (sc-137179) and mouse anti- $\beta$ -actin (sc-47778). Caspase-3 inhibitor Z-DEVD-FMK (Cat. # FMK004) and Caspase-9 inhibitor Z-LEHD-FMK (Cat. # FMK008) were purchased from R&D Systems, Inc. Other chemicals and organic solvents of the highest available grade were obtained from Sigma-Aldrich. *Ampk $\alpha 1^{-/-}$*  and *Ampk $\alpha 2^{-/-}$*  mice were described elsewhere [28,29]. Mice were handled in accordance with study protocols approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center (Oklahoma City, OK).

### 2.2. Cell culture and transfection

Mouse embryonic fibroblasts (MEFs) were isolated from AMPK $\alpha 1^{-/-}$ , AMPK $\alpha 2^{-/-}$ , and WT embryos at 13.5-days post-coitus and cells were immortalized by the 3T3 protocol as described previously [30,31]. Briefly, 13.5-day mouse embryo was decapitated, thoroughly minced, and trypsinized. The dissociated cells were re-suspended. To immortalize MEFs, cells were passaged continuously according to the 3T3 protocol ( $3 \times 10^5$  cells were plated per 60-mm dish every 3 days) until growth rates in culture stabilized. Cells were then cultured for an additional 15 passages (to about passage 35) and at that point were considered immortalized and used for experiments. MEFs were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, L-Glutamine (2 mM) (Lonza, Walkersville, MD), penicillin (100  $\mu$ g/ml) (Life Technologies, Grand Island, NY). For cell synchronization in G0/G1 phase, MEFs were serum starved, and then re-incubated in complete medium for various times. MEFs were

transiently infected with LacZ, p21, or AMPK $\alpha 1$  adenovirus (MOI = 50) for 24 or 48 h as previously reported [32].

### 2.3. Indirect immunofluorescence and microscopy

Cells were grown to exponential phase on poly-L-lysine-coated glass coverslips. Cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% TritonX-100 and blocked with image-IT Fix or BSA (Invitrogen). Primary antibodies used were: mouse anti- $\gamma$ -H2AX (1:100 v/v) or rabbit anti- $\gamma$ -H2AX (1:100 v/v), rabbit anti-53BP1, and mouse anti-p21. DNA was stained with antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA). For indirect immunofluorescence, Alexa Fluor® 488 and 555 were used for detection of the protein. Confocal microscopy was performed using a Zeiss 710 confocal microscope (Oberkochen, Germany), with a 63 $\times$  oil immersion lens. Image editing was performed in Adobe Systems Incorporated, San Jose, CA.

### 2.4. RNA extraction, cDNA synthesis, and real time PCR

Total mRNA was isolated and purified using the RNeasy mini kit from Qiagen (Valencia, CA) according to the manufacturer's instructions. cDNA was synthesized from isolated mRNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA), as described previously [29] and by the manufacturer's instructions. Real-time PCR was performed on a ABI PRISM 7700 sequence detection system (Applied Biosystems) with SYBR green PCR master mix (Applied Biosystems) and 1  $\mu$ l of first-strand cDNA as template with specific primers for p21 (5'-CCTGGTGATGTCCGACCTGTT-3', 5'-CCCCTTAGAAGTCCGGCGAG-3') [33]. The levels of gene expression were determined relative to that of  $\beta$ -actin (5'-TGGGCCGCTCTAGGCACCA-3', 5'-ACCGGAATCCCAAGTCCCC-3').

### 2.5. Comet assay

Single cell DNA damage (double-stranded breaks) was analyzed by neutral comet assay using the Trevigen's Comet Assay kit (4250-050-K, Gaithersburg, MD) according to the manufacturer's instruction. Briefly, cells were suspended in 0.7% low melting point agarose and spread on glass slides precoated with 1% agarose. Slides were overlaid with coverslips that were removed after the gel solidified. The gel was treated with lysis solution (Trevigen) for 60 min at 4 °C in the dark and electrophoresed at 1 V/cm for 20 min. Comet tails were stained and slides were captured by fluorescent microscope. Quantitative measurements of DNA damage were performed by using Comet Assay IV software (Perceptive Instruments).

### 2.6. Annexin V binding assay

Annexin V-FITC (Cat. # K101-100, BioVision, Milpitas, CA) was used to detect the phosphatidylserine exposure to the outer surface of the cell membrane by following the manufacturer's protocol. Briefly, cells were grown on cover slip in a 12 well plate with the desired treatment. Cells were washed with PBS and  $1 \times$  binding buffer, then incubated with Annexin V-FITC (1:70 dilution) diluted in  $1 \times$  binding buffer for 10 min. Then cells were washed twice with  $1 \times$  binding buffer and the coverslips were mounted on microscope glass slides with Dako fluorescent mounting medium. Randomly selected fields were counted using a fluorescence microscope and quantitated using NIH Image software.

### 2.7. Flow cytometry analysis

Cells were serum starved for 24 h, then cultured in regular culture medium for the indicated time. The treated cells were fixed in 80% ethanol and stained with 50  $\mu$ g/ml propidium iodide (PI) in the presence of 10  $\mu$ g of DNase-free RNase per ml [34]. Cell cycle profiles were

determined by FACSDiVa (BD Bioscience, San Jose, CA) and data were analyzed using FCS Express V3 software. LacZ or p21 adenovirus-infected WT or AMPK $\alpha 1^{-/-}$  MEFs were serum-starved for 16 h, then incubated with regular culture medium plus 10  $\mu$ M BrdU for 8 h. The cells were harvested and flow cytometric assay was performed by following the manufacturer's protocol in BrdU Flow Kit (Cat. # 559619, BD Biosciences, San Jose, CA). In addition, exponentially growing WT or AMPK $\alpha 1^{-/-}$  MEFs were pulsed with BrdU (10  $\mu$ M) for 40 min followed by washes with warmed serum-free medium twice and re-feeding with warmed and pre-gassed whole medium. Cells were fixed and stained using the FITC BrdU Flow Kit after 0, 40 min, 2, 4, and 6 h post pulse. The S and G2 + M phase durations were calculated as previously report [35].

## 2.8. Protein extraction, immunoprecipitation, and immunoblotting

Whole cell extracts were prepared using cell lysis buffer (9803) from Cell Signaling Technology with protease and phosphatase inhibitor cocktails I and II (Cat. # BP-479 and BP-480, Boston BioProducts, MA). Protein samples (30–50  $\mu$ g) were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and probed with different antibodies as previously described [36,37]. Following incubation with the appropriate horseradish peroxidase-associated secondary antibodies (Cell Signaling Technology), signals were visualized with an enhanced chemiluminescence detection system (GE Healthcare) and quantified by densitometry. Equal loading of protein was verified by immunoblotting with anti- $\beta$ -actin or -GAPDH antibody.

## 2.9. Subcellular fractionation

Subcellular protein fractionation of cultured cells was performed as described in the instructions for the commercial kit (Cat. # 78840, Thermo Scientific, Rockford, IL).

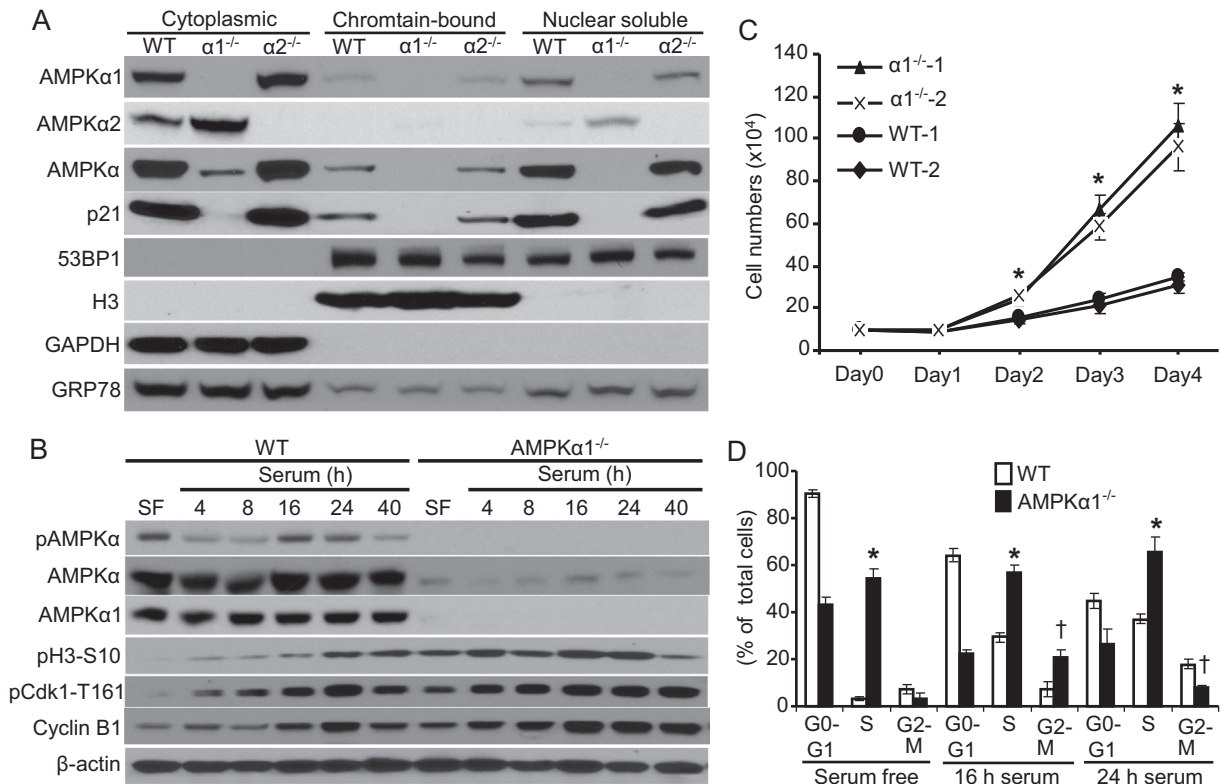
## 2.10. Statistical analysis

Unless otherwise stated, data are presented as mean  $\pm$  S.D. Differences between multiple means were evaluated by two-tailed Student's *t* test or analysis of variance with post hoc Bonferroni corrections. A *p* value < 0.05 was considered statistically significant.

## 3. Results

### 3.1. Subcellular localization of AMPK $\alpha$ isoforms and dynamic activation of AMPK $\alpha$ in MEFs

We examined the relative contribution of AMPK $\alpha 1$  and AMPK $\alpha 2$  isoform to total AMPK $\alpha$  in cytoplasmic and nuclear fractions. We performed subcellular fractionation followed by immunoblotting to assess the protein levels of AMPK $\alpha 1$ , AMPK $\alpha 2$  and AMPK $\alpha$  in the cytoplasmic and nuclear fractions. As expected, AMPK $\alpha 1$  and AMPK $\alpha 2$  were not detected in either cytoplasmic or nuclear fractions of AMPK $\alpha 1^{-/-}$  or AMPK $\alpha 2^{-/-}$  MEFs, respectively (Fig. 1A). Since AMPK $\alpha 1$  is the predominant AMPK $\alpha$  isoform in MEFs [30], AMPK $\alpha 1$  deletion dramatically decreased the cytoplasmic, nuclear soluble, and chromatin-bound AMPK $\alpha$  (Fig. 1A), but increased AMPK $\alpha 2$  protein levels, which is in



**Fig. 1.** AMPK $\alpha 1$  localizes to chromatin and is implicated in regulating the cell cycle. (A) MEFs were subcellularly fractionated by a commercially available kit from Thermo Scientific. AMPK $\alpha 1$ , AMPK $\alpha 2$ , AMPK $\alpha$ , p21, and 53BP1 were detected by Western blots. Histone H3 serves as a marker for chromatin. GAPDH serves as a cytoplasmic marker. GRP78 serves as a loading control for each fraction. Representative data from three independent experiments are shown. (B) AMPK $\alpha$  is activated during mitosis. WT and AMPK $\alpha 1^{-/-}$  MEFs were first serum-deprived for 24 h, then cultured in regular culture medium for the indicated times. The cells were lysed and analyzed by Western blot using anti-pAMPK $\alpha$ -T172, -AMPK $\alpha$ , -AMPK $\alpha 1$ , -Cyclin B1, -pCdk1-T161, or pH3-S10 antibody. Representative data from three independent experiments are shown. (C) WT and AMPK $\alpha 1^{-/-}$ -immortalized MEFs (two independent cell lines for each) were plated, and cells were counted at the indicated times. *n* = 8, \**p* < 0.05 vs WT. (D) Flow cytometric analysis of cell cycle progression in WT and AMPK $\alpha 1^{-/-}$  MEFs serum starved for 24 h or treated with serum for the indicated times. *n* = 3, \**p* < 0.01 vs WT in S phase; †*p* < 0.01 vs WT in G2-M phase.



agreement with the result from Ras-transformed AMPK $\alpha 1^{-/-}$  MEFs [38]. AMPK $\alpha 1$  and AMPK $\alpha 2$  were predominantly found in the cytoplasmic region; however a small fraction of both were also located in the nuclear region (Fig. 1A). Both AMPK $\alpha 1$  and total AMPK $\alpha$  were almost undetectable in the chromatin-bound and nuclear soluble fraction of AMPK $\alpha 1^{-/-}$  MEFs (Fig. 1A). Interestingly, AMPK $\alpha 1$ , but not AMPK $\alpha 2$  was chromatin-bound (Fig. 1A).

In WT MEFs, phosphorylated AMPK $\alpha$  on Thr-172 (pAMPK $\alpha$ -T172), an indicator of active AMPK $\alpha$  [39], was higher with serum deprivation, while AMPK activity was significantly inhibited during serum stimulation for 4–8 h (Fig. 1B). pAMPK $\alpha$  in WT MEFs was increased during serum stimulation for 16 or 24 h, then decreased, which was consistent with the upregulation of histone H3 phosphorylation at Ser10 (pH3-S10) (Fig. 1B), a typical marker of cell mitosis [40]. Our data is in line with previously published data demonstrating that AMPK activation is dynamic during the cell cycle of HeLa cancer cells [22] and HEK293 cells [41]. However, AMPK $\alpha 1$  deletion showed higher levels of pH3-S10 even under serum-free conditions, although pAMPK $\alpha$  was undetectable. Taken together, these results imply that AMPK $\alpha 1$  might act on chromatin and is required for normal cell cycle. Thus, loss of AMPK $\alpha 1$  might lead to a dysregulated cell cycle in MEFs.

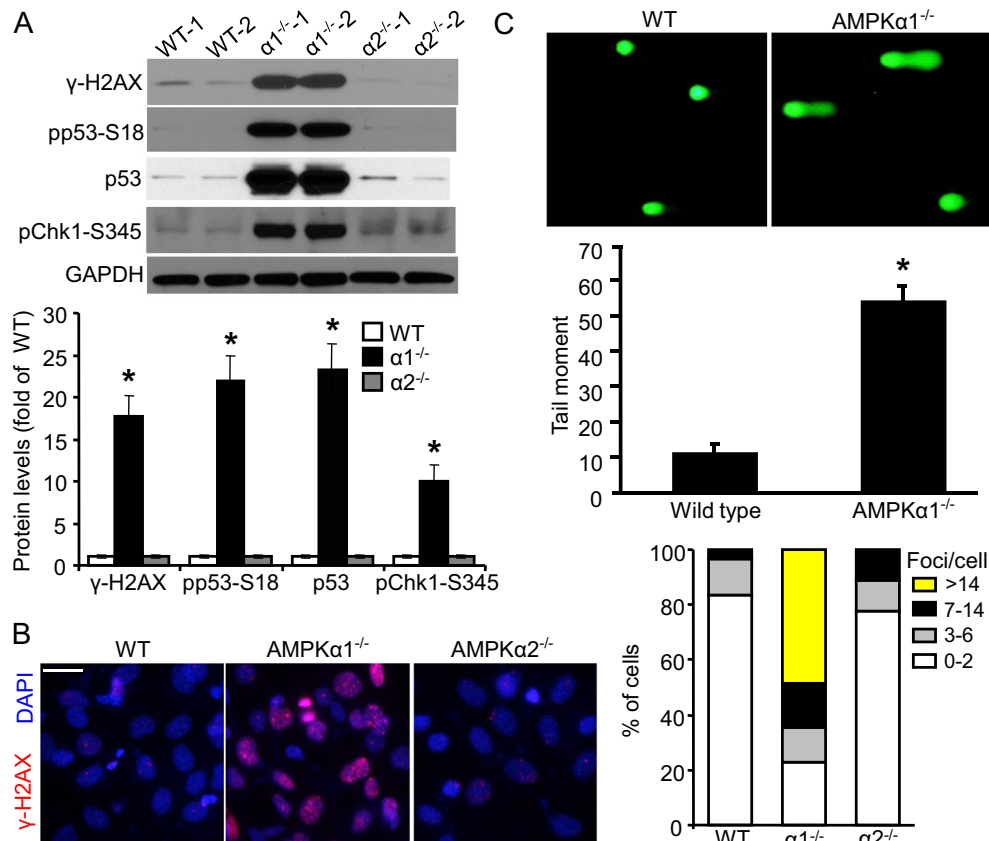
### 3.2. AMPK $\alpha 1$ deletion enhances cell division in MEFs

It is reported that AMPK activation by glucose limitation suppresses cell-cycle progression via arresting the cell cycle at G0–G1 phase [42]. As depicted in Fig. 1C, AMPK $\alpha 1$  deletion significantly accelerated cell proliferation beginning at day 2 in culture compared

with WT MEFs. As calculated, the mean population doubling time of AMPK $\alpha 1^{-/-}$  MEFs was around 18 h, while that of WT MEFs was about 35 h. Furthermore, flow cytometry data (Fig. 1D) demonstrated that AMPK $\alpha 1$  deletion had less cells in G0/G1 phase (about 40%) compared with WT MEFs (~92%) in response to serum starvation, however, AMPK $\alpha 1^{-/-}$  MEFs had more cells with S phase (about 55%) under either serum-free or serum-stimulated conditions. AMPK $\alpha 1$  deletion resulted in more cells in G2/M phase at 16 h after serum stimulation, however, fewer cells were in G2/M phase at 24 h of serum stimulation. Next, BrdU pulse-chase time course experiments demonstrated that AMPK $\alpha 1^{-/-}$  MEFs had shorter S traverse time ( $4.5 \pm 1.1$  h) than WT MEFs ( $11 \pm 1.2$  h). The G2/M phase traverse time ( $3.1 \pm 0.4$  h) in AMPK $\alpha 1^{-/-}$  MEFs was also shorter than that ( $8.8 \pm 0.9$  h) in WT MEFs. These results imply that AMPK $\alpha 1^{-/-}$  MEFs has reduced DNA replication time. AMPK $\alpha 1$  deletion enhances cell cycle progression from the G1 to S phase and G2 to M phase, which may be associated with the persistently high levels of Cyclin B1 and phosphorylated Cdk1 at T161 (Fig. 1B), an active form of Cdk1 [16,43].

### 3.3. AMPK $\alpha 1$ , not AMPK $\alpha 2$ , deletion leads to increased DNA damage in MEFs

Since AMPK activation regulates UVB-induced DNA damage repair in skin tumor cells [26], we sought to identify whether AMPK regulates DNA damage signaling in MEFs. As shown in Fig. 2A, AMPK $\alpha 1$ , not AMPK $\alpha 2$ , deletion dramatically increased the protein level of serine 139-phosphorylated H2AX ( $\gamma$ -H2AX), an important and widely used molecular marker for DNA DSB [6,44,45]. Furthermore, compared to



**Fig. 2.** AMPK $\alpha 1$ , not AMPK $\alpha 2$ , deficiency leads to elevated DNA damage in MEFs. (A) (Upper) DNA damage signals in two independent cell lines for each genetic background were evaluated by Western blots with the indicated antibody. (Bottom) Quantification data from upper panel.  $n = 6$ , \* $p < 0.05$  vs WT. (B) Immunofluorescent staining of MEFs with indicated genotypes using nuclear (DAPI-blue) and DSB markers ( $\gamma$ -H2AX-red) (left). Scale bar = 50  $\mu$ m. Quantitation for the percentages of cells with indicated number of  $\gamma$ -H2AX foci (right). WT MEFs ( $n = 81$ ); AMPK $\alpha 1^{-/-}$  MEFs ( $n = 112$ ); AMPK $\alpha 2^{-/-}$  MEFs ( $n = 94$ ). (C) Quantitation for comet tail assay measuring tail moment for WT and AMPK $\alpha 1^{-/-}$  MEFs.  $n = 20$ , \* $p < 0.05$  vs WT. Representative images are shown in the upper panel.

WT and AMPK $\alpha 2^{-/-}$ , AMPK $\alpha 1$  deletion significantly increased the basal levels of serine-18 phosphorylation of p53, an indicator of DNA damage associated with cellular apoptosis [30,46], as well as serine-345 phosphorylation of Chk1, a general DSB sensor and response effector [47]. The percentage of cells with greater than 7  $\gamma$ -H2AX foci per cell were markedly increased in AMPK $\alpha 1^{-/-}$  MEFs when compared with WT and AMPK $\alpha 2^{-/-}$  MEFs (Fig. 2B). To further verify the spontaneous accumulation of DNA-strand breaks in the absence of AMPK $\alpha 1$ , the neutral comet assay was performed in both WT and AMPK $\alpha 1^{-/-}$  MEFs. In agreement with the data above, the comet assay demonstrated that AMPK $\alpha 1$  deletion exerts longer comet tails (Fig. 2C), which is closely associated with DNA damage [48]. These results suggest that AMPK $\alpha 1$  plays an important role in regulating DNA damage.

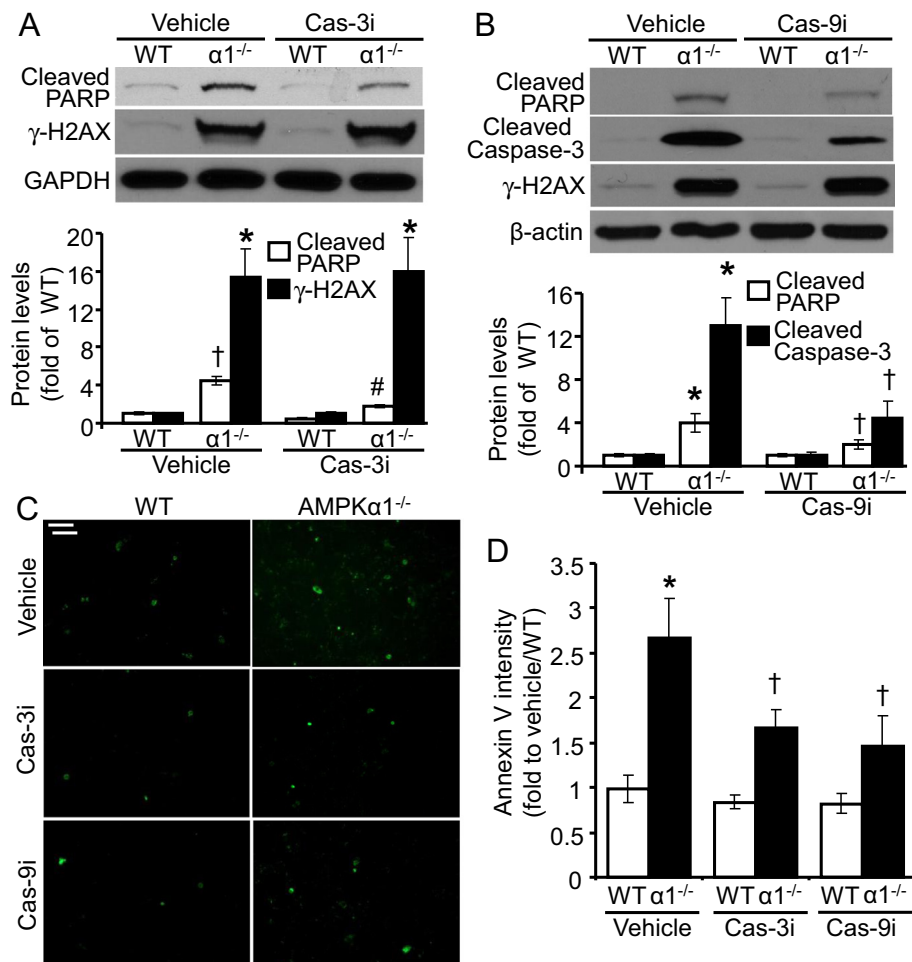
#### 3.4. AMPK $\alpha 1$ deletion-elevated DNA damage contributes to the increased apoptosis

Since  $\gamma$ -H2AX induction and phosphorylation of p53 at S18 also exist in apoptotic cells [49], it is important to validate whether the observed increased  $\gamma$ -H2AX signal in AMPK $\alpha 1$ -deleted MEFs is due to DNA damage or apoptosis. As shown in Fig. 3, AMPK $\alpha 1$  deletion dramatically enhanced cell apoptosis as demonstrated by increased poly(ADP-ribose) polymerase (PARP) cleavage (Fig. 3A and B) and Annexin V staining

(Fig. 3C and D), which is consistent with a previous report [30]. The treatment with Caspase-3 specific inhibitor Z-DEVD-FMK [50] significantly inhibited PARP cleavage and Annexin V staining (Fig. 3C and D), but did not alter the  $\gamma$ -H2AX signaling (Fig. 3A). Moreover, the Caspase-9 specific inhibitor Z-LEHD-FMK [51] clearly suppressed apoptosis in AMPK $\alpha 1^{-/-}$  MEFs, indicated by the reduction of cleaved PARP and Caspase-3 (Fig. 3B) and Annexin V staining (Fig. 3C and D), however, the treatment had no effect on the elevated  $\gamma$ -H2AX (Fig. 3B). These data indicate that  $\gamma$ -H2AX induction in AMPK $\alpha 1^{-/-}$  MEFs is due to the increased DNA damage, which leads to the enhanced apoptosis.

#### 3.5. Elevated DNA damage in AMPK $\alpha 1^{-/-}$ MEFs is partially due to p21 reduction

One possibility for the increased DNA damage seen in AMPK $\alpha 1^{-/-}$  MEFs is the inability for the cell to activate DNA damage repair mechanisms. Since 53BP1 plays critical roles in the repair of damaged DNA, we investigated whether AMPK $\alpha 1$  deletion alters the protein levels of 53BP1. As depicted in Fig. 4A, AMPK $\alpha 1$  deletion did not change 53BP1 protein levels. Additionally, the amount of XRCC4, a DNA repair protein [52], is similar between WT and AMPK $\alpha 1^{-/-}$  MEFs (data not shown). Previous studies have indicated that p21 is involved in DNA lesion repair



**Fig. 3.** Increased DNA damage contributes to the elevated apoptosis in AMPK $\alpha 1^{-/-}$  MEFs. (A) (Upper) Caspase-3 inhibitor (Cas-3i) Z-DEVD-FMK dramatically suppressed apoptosis in AMPK $\alpha 1^{-/-}$  MEFs, but had no effect on DNA damage signal as measured by  $\gamma$ -H2AX expression. (Bottom) Quantification of Western blot data.  $n = 4$ ,  $^{\dagger} p < 0.01$  vs WT/Vehicle;  $^{\#} p < 0.01$  vs  $\alpha 1^{-/-}$ /Vehicle;  $^* p < 0.001$  vs WT/Vehicle. (B) (Upper) Caspase-9 inhibitor (Cas-9i) Z-LEHD-FMK significantly inhibited apoptosis in AMPK $\alpha 1^{-/-}$  MEFs, but had no effect on DNA damage signal as measured by  $\gamma$ -H2AX expression. (Bottom) Quantification of Western blot data.  $n = 3$ ,  $^* p < 0.001$  vs WT/Vehicle;  $^{\dagger} p < 0.01$  vs  $\alpha 1^{-/-}$ /Vehicle. (C) Representative images showing phosphatidylserine externalization detected by Annexin V labeling in MEFs treated with Cas-3i and Cas-9i. Scale bar, 100  $\mu$ m. (D) Quantification of Annexin V intensity.  $n = 15$ ,  $^* p < 0.01$  vs WT/Vehicle;  $^{\dagger} p < 0.05$  vs  $\alpha 1^{-/-}$ /Vehicle.

[18,19]. Therefore, we tested whether AMPK $\alpha$ 1 could modulate p21 expression. Indeed, AMPK $\alpha$ 1 deletion dramatically reduced total, cytoplasmic, chromatin-bound, and nuclear soluble p21 protein levels compared with WT and AMPK $\alpha$ 2 deletion (Figs. 4A and 1A). To examine whether p21 protein reduction is due to increased protein degradation, we treated cells with MG132, a potent inhibitor of the 26S proteasome [53]. MG132 treatment did not reverse the p21 reduction in AMPK $\alpha$ 1 $^{-/-}$  MEFs, whereas p21 protein levels in both WT and AMPK $\alpha$ 2 $^{-/-}$  MEFs were significantly increased (Fig. 4B), indicating that the ubiquitin-proteasome system is not responsible for the p21 reduction in AMPK $\alpha$ 1 $^{-/-}$  MEFs. However, qRT-PCR assay demonstrated that p21 mRNA levels were markedly down-regulated by AMPK $\alpha$ 1 deletion compared with WT and AMPK $\alpha$ 2 deletion (Fig. 4C). Overexpression of p21 for 48 h partially, but significantly reduced  $\gamma$ -H2AX signal in AMPK $\alpha$ 1 $^{-/-}$  MEFs (Fig. 4D). These results suggest that AMPK $\alpha$ 1 deletion impairs p21 transcription hence leading to increased DNA damage which is partially rescued upon p21 overexpression.

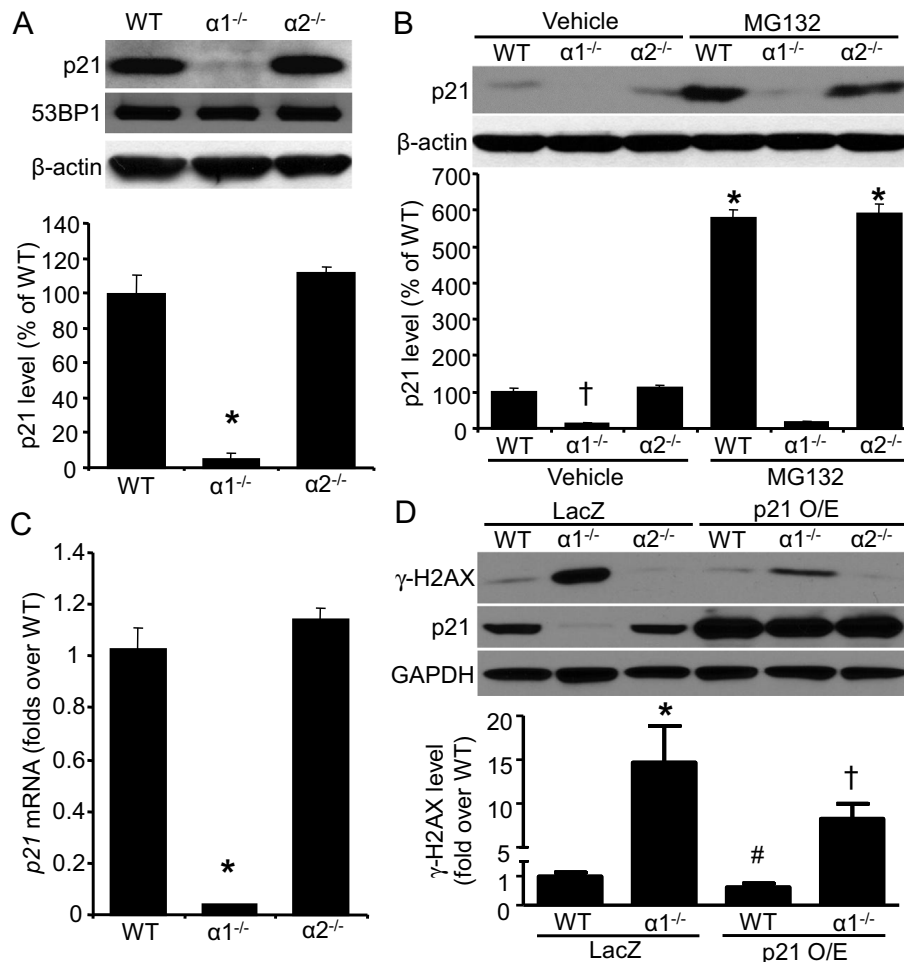
### 3.6. p21 inhibits 53BP1 foci formation

Next, we investigated whether p21 overexpression affects the foci formation of repair protein 53BP1. The overall 53BP1 protein level was similar in WT and AMPK $\alpha$ 1 $^{-/-}$  MEFs (Figs. 1A and 4A). Initial

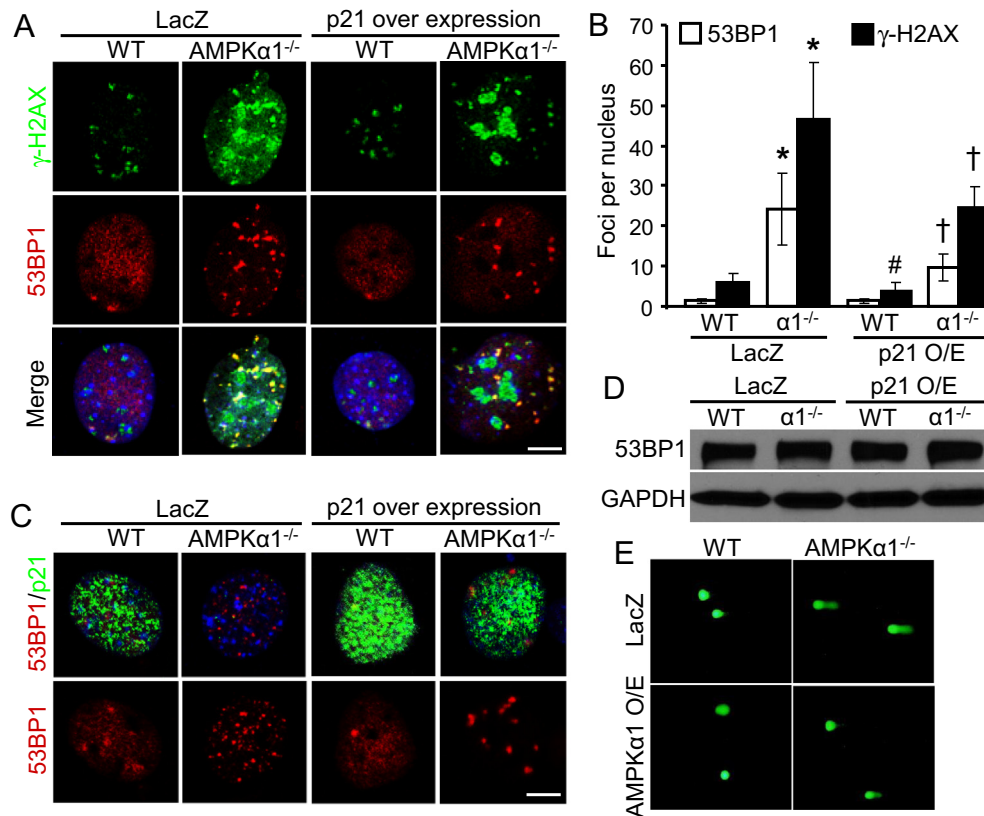
qualitative assessment indicated that there was a stronger  $\gamma$ -H2AX signal in AMPK $\alpha$ 1 $^{-/-}$  MEFs compared to WT MEFs (Fig. 2A) which was confirmed by immunofluorescence (Fig. 5A). No 53BP1 foci was observed in about 85% of WT MEFs, which may be due to less DNA damage (Figs. 5B and 2B). In contrast, 68% of AMPK $\alpha$ 1 $^{-/-}$  MEFs exhibited 53BP1 foci formation. p21 overexpression significantly decreased 53BP1 foci number in AMPK $\alpha$ 1 $^{-/-}$  MEFs, which is in line with the decreased foci number of  $\gamma$ -H2AX. p21 did not show colocalization with 53BP1. Furthermore, p21 overexpression did not alter the total 53BP1 protein levels (Fig. 5D). In addition, AMPK $\alpha$ 1 overexpression decreased DNA damage in AMPK $\alpha$ 1 $^{-/-}$  MEFs as demonstrated by comet assay. These results imply that p21 overexpression inhibits 53BP1 foci formation highly associated with DNA damage [9] in AMPK $\alpha$ 1-deleted MEFs.

### 3.7. p21 overexpression partially abrogates the hyperproliferation and apoptosis in AMPK $\alpha$ 1 $^{-/-}$ MEFs

Given the role of p21 in DNA repair and cell cycle regulation, we analyzed whether p21 overexpression can normalize the phenotype of AMPK $\alpha$ 1 $^{-/-}$  MEFs by evaluating cellular proliferation. As depicted in Fig. 6A, p21 overexpression significantly attenuated cell proliferation of AMPK $\alpha$ 1 $^{-/-}$  MEFs, while only mildly inhibiting the proliferation of WT MEFs. After synchronizing the cells with serum-free medium for



**Fig. 4.** Increased DNA damage is partially due to p21 reduction in AMPK $\alpha$ 1 $^{-/-}$  MEFs. (A) (Upper) AMPK $\alpha$ 1 deletion significantly down-regulated p21, but not 53BP1. (Bottom) Quantification of Western blot data.  $n = 8$ ,  $*p < 0.001$  vs WT. (B) Proteasome inhibitor, MG132 (20  $\mu$ M, 8 h) did not reverse p21 reduction in AMPK $\alpha$ 1 $^{-/-}$  MEFs.  $n = 4$ ,  $\dagger p < 0.001$  vs WT,  $*p < 0.001$  vs WT/vehicle or  $\alpha$ 2 $^{-/-}$ /vehicle, respectively. (C) Quantitative RT-PCR analysis of p21 expression in MEFs.  $\beta$ -actin was used as endogenous control. Values are mean  $\pm$  SEM of four independent experiments,  $*p < 0.001$  vs WT. (D) p21 overexpression partially reduced DNA damage in AMPK $\alpha$ 1 $^{-/-}$  MEFs.  $n = 4$ ,  $*p < 0.01$  vs WT/LacZ;  $\#p < 0.05$  vs WT/LacZ;  $\dagger p < 0.01$  vs  $\alpha$ 1 $^{-/-}$ /LacZ.



**Fig. 5.** 53BP1 foci formation is inhibited by p21 expression in AMPK $\alpha$ 1<sup>-/-</sup> MEFs. (A) p21 overexpression decreases 53BP1 foci formation in AMPK $\alpha$ 1<sup>-/-</sup> MEFs. Representative images showing 53BP1 and  $\gamma$ -H2AX foci formation. Scale bar = 10  $\mu$ m. (B) Quantitative analysis of 53BP1 and  $\gamma$ -H2AX foci per nucleus. n = 20, \* $p$  < 0.01 vs WT/LacZ; † $p$  < 0.01 vs  $\alpha$ 1<sup>-/-</sup>/LacZ for 53BP1 or  $\gamma$ -H2AX, respectively; # $p$  < 0.05 vs WT/LacZ. (C) Representative images showing 53BP1 foci formation and p21 expression. Scale bar = 10  $\mu$ m. (D) p21 overexpression does not alter total 53BP1 protein. (E) AMPK $\alpha$ 1 overexpression attenuates DNA damage as determined by comet assay.

16 h, LacZ or p21 adenovirus-infected MEFs were labeled with BrdU for 8 h. The flow cytometric results indicated that deletion of AMPK $\alpha$ 1 increased the BrdU-positive cells by 6 times as compared with WT (Fig. 6B), implying that there are more cells in S-phase for AMPK $\alpha$ 1<sup>-/-</sup> MEFs. Importantly, p21 overexpression significantly decreased BrdU-positive cells in AMPK $\alpha$ 1<sup>-/-</sup> MEFs by 46%, as compared to LacZ infection (Fig. 6B). Furthermore, the CDK2 phosphorylation at T160, an active form of CDK2 [54], in AMPK $\alpha$ 1<sup>-/-</sup> MEFs, was markedly ablated by p21 overexpression (Fig. 6C). In addition, the apoptotic signal in AMPK $\alpha$ 1<sup>-/-</sup> MEFs was profoundly blunted by p21 overexpression (Fig. 6D).

#### 4. Discussion

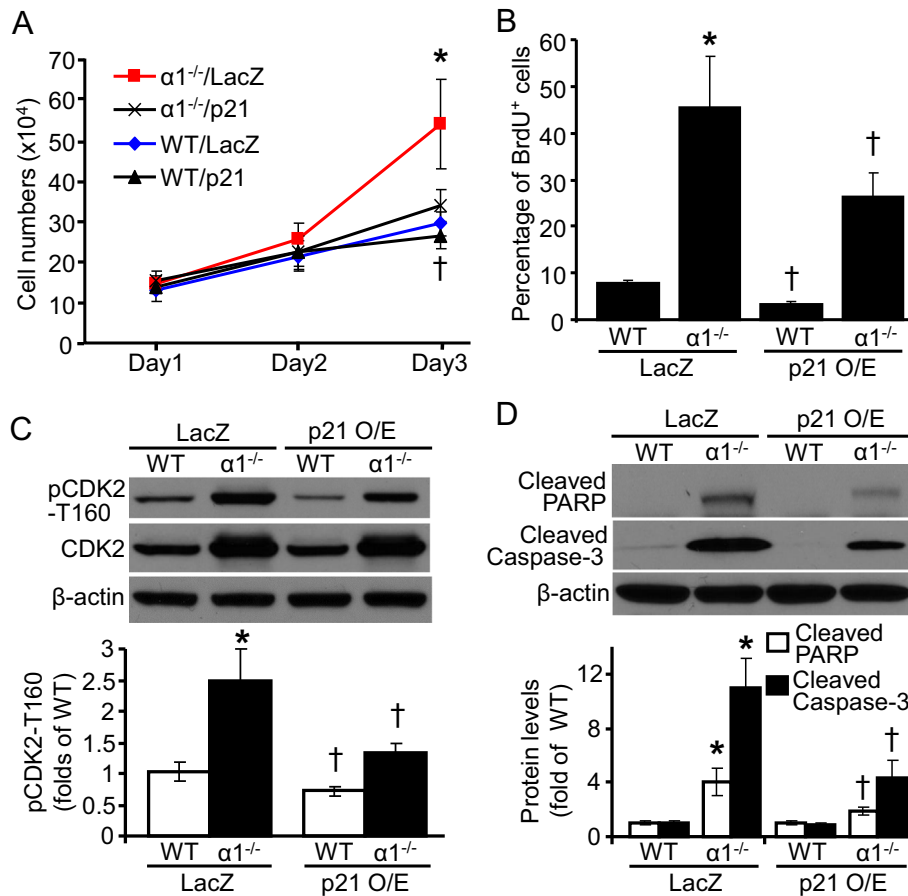
In the present study, we have demonstrated that AMPK $\alpha$ 1, but not AMPK $\alpha$ 2 deletion, mediates aberrant cell proliferation, spontaneous DNA DSB damage, and apoptosis in MEFs. The mechanism underlying this process is partly due to p21 reduction. The enhanced cell proliferation in AMPK $\alpha$ 1<sup>-/-</sup> MEFs is due to the G1 to S transition and impaired G2/M arrest resulting from activation of Cdk2 and Cdk1 by p21 reduction. p21 overexpression decreases 53BP1 foci formation in AMPK $\alpha$ 1-deleted MEFs. These findings indicate that AMPK $\alpha$ 1 is a pivotal regulator for cell cycle, DNA DSB and resultant apoptosis.

A previous report indicated that AMPK $\alpha$  is associated with the efficient repair of UVB-induced DNA damage in SKH-1 mouse skin, via the regulation of xeroderma pigmentosum group C (XPC) [26], a crucial initiator of global genome nucleotide excision repair [55]. Here, we have, for the first time, demonstrated that AMPK $\alpha$ 1 isoform deletion down-regulated p21 and transient overexpression of p21 partially reduced this DNA damage (Figs. 4D and 5A), suggesting that p21 may

play an integral part in DNA damage and repair. Recently, it is reported that p21 inhibits DNA damage through interaction with PCNA [18]. These results are consistent with the findings in leukemia stem cells [19]. Furthermore, a critical and previously unrecognized role of p21 as a gatekeeper of AMPK $\alpha$ 1 deletion-induced DNA damage was demonstrated. p21 overexpression decreased 53BP1 foci formation in AMPK $\alpha$ 1-deficient cells via an unknown mechanism. How 53BP1 is recruited to DSB sites has been recently studied. Histone modifications are involved in 53BP1 recruitment to DSB sites [10]. Our data imply that p21 may be an additional 53BP1 effector. In addition, p21 reduction in AMPK $\alpha$ 1-deleted MEFs is unlikely p53-dependent, a widely reported molecular mechanism [56–59], since p53 is up-regulated by AMPK $\alpha$ 1 deletion (Fig. 2A). Hence, the mechanism involved in p21 reduction by AMPK $\alpha$ 1 deletion or inhibition warrants further investigation.

Increasing evidence indicates that AMPK controls the cell cycle [29, 60] and mitosis [20–22] via distinct mechanisms. For example, constitutive expression of AMPK-related kinase NUA1 leads to gross aneuploidies in WI-38 human fibroblast [61]. Additionally, p21 is required for proper cell cycle control and consequent chromosome stability in *Trp53*<sup>515C/515C</sup> mice (encoding p53R172P, the corresponding murine p53 mutant) which is deficient for apoptosis but retains a partial cell cycle arrest function [62]. Unrepaired DNA damage is a trigger for cellular apoptosis and/or enhanced cell proliferation [63], and AMPK $\alpha$ 1 deletion enhances MEFs apoptotic signaling [30]. Here, AMPK $\alpha$ 1 deletion increased Cdk2 protein levels and decreased p21, as well as stimulated cell proliferation. p21 overexpression partially inhibited Cdk2 phosphorylation at T160 and consequently hindered cell hyperproliferation and the resultant DNA damage. In addition, p21 overexpression partially suppressed apoptosis in AMPK $\alpha$ 1<sup>-/-</sup> MEFs, which is due to the increased DNA damage.





**Fig. 6.** p21 overexpression attenuates cellular hyperproliferation and apoptosis in AMPK $\alpha 1^{-/-}$  MEFs. (A) p21 overexpression blocked the hyperproliferation of AMPK $\alpha 1^{-/-}$  MEFs.  $n = 10$ , \* $p < 0.01$  vs  $\alpha 1^{-/-}/\text{p21}$ ; † $p < 0.05$  vs WT/LacZ. (B) p21 overexpression decreased the percentage of BrdU positive cells in AMPK $\alpha 1^{-/-}$  and WT MEFs.  $n = 4$ , \* $p < 0.01$  vs WT/LacZ; † $p < 0.01$  vs WT/LacZ,  $\alpha 1^{-/-}/\text{LacZ}$ , respectively. (C) (Upper) p21 overexpression significantly inhibited phosphorylation of CDK2 at T160 (pCDK2-T160), while had no effect on total CDK2 protein level. (Bottom) Quantification of Western blot data.  $n = 3$ , \* $p < 0.001$  vs WT/LacZ; † $p < 0.01$  vs WT/LacZ, or  $\alpha 1^{-/-}/\text{LacZ}$ , respectively. (D) p21 overexpression suppressed apoptotic signal (PARP and Caspase-3 cleavage) in AMPK $\alpha 1^{-/-}$  MEFs.  $n = 3$ , \* $p < 0.001$  vs WT/LacZ; † $p < 0.01$  vs  $\alpha 1^{-/-}/\text{LacZ}$ .

Loss of AMPK $\alpha 1$  culminates in elevated DNA damage, which when prolonged can lead to accumulation of a lethal amount of damaged DNA or mutated DNA, thereby resulting in apoptosis or hyperproliferation associated with cancer development. As AMPK is an important modulator of cell metabolism, it will be interesting to examine whether AMPK $\alpha 1$  regulates DNA damage, in part via modulating fuel switching.

In summary, our studies reveal an important role for AMPK $\alpha 1$  in cell biology and connect two hallmarks of tumor cells: hyperproliferation and DNA damage [64–66], which may be due to p21 reduction. Given the importance of AMPK in the cell cycle, these findings hold profound implications for understanding the molecular mechanisms by which AMPK functions as a promising tumor suppressor or senescence blocker.

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