ISSN 0006-2979, Biochemistry (Moscow), 2012, Vol. 77, No. 3, pp. 261-269. © Pleiades Publishing, Ltd., 2012.
Published in Russian in Biokhimiya, 2012, Vol. 77, No. 3, pp. 330-341.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM11-224, January 22, 2012.

E2F1 Enhances 8-Chloro-adenosine-Induced G2/M Arrest and Apoptosis in A549 and H1299 Lung Cancer Cells

Hong-Ying Duan[1,2], Ji-Xiang Cao[1], Jun-Juan Qi[1], Guo-Sheng Wu[1], Shu-Yan Li[1], Guo-Shun An[1], Hong-Ti Jia[1,3], Wang-Wei Cai[2], and Ju-Hua Ni[1]*

[1]Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Peking University Health Science Center, Xue Yuan Road 38, Beijing 100191, PR China; fax: 86-10-82801434; E-mail: juhuani@bjmu.edu.cn
[2]Department of Biochemistry, Hainan Medical College, Haikou 571101, PR China
[3]Department of Biochemistry and Molecular Biology, Capital University of Medical Sciences, You An Men Xitoutiao 8, Beijing 100054, PR China

Received July 30, 2011
Revision received October 29, 2011

Abstract—The E2F1 transcription factor is a well known regulator of cell proliferation and apoptosis, but its role in response to DNA damage is less clear. 8-Chloro-adenosine (8-Cl-Ado), a nucleoside analog, can inhibit proliferation in a variety of human tumor cells. However, it is still elusive how the agent acts on tumors. Here we show that A549 and H1299 cells formed DNA double-strand breaks after 8-Cl-Ado exposure, accompanied by E2F1 upregulation at protein level. Overexpressed wild-type (E2F1-wt) colocalized with double-strand break marker γ-H2AX and promoted G2/M arrest in 8-Cl-Ado-exposed A549 and H1299, while expressed S31A mutant of E2F1 (E2F1-mu) significantly reduced ability to accumulate at sites of DNA damage and G2/M arrest, suggesting that E2F1 is required for activating G2/M checkpoint pathway upon DNA damage. Transfection of either E2F1-wt or E2F1-mu plasmid promoted apoptosis in 8-Cl-Ado-exposed cells, indicating that 8-Cl-Ado may induce apoptosis in E2F1-dependent and E2F1-independent ways. These findings demonstrate that E2F1 plays a crucial role in 8-Cl-Ado-induced G2/M arrest but is dispensable for 8-Cl-Ado-induced apoptosis. These data also suggest that the mechanism of 8-Cl-Ado action is complicated.

DOI: 10.1134/S0006297912030042

Key words: E2F1, 8-chloro-adenosine, DNA double-stranded breaks, G2/M arrest, apoptosis

8-Chloro-cAMP (8-Cl-cAMP) is a potential anticancer agent that exerts its cytotoxicity by converting into its metabolite, 8-chloro-adenosine (8-Cl-Ado) [1-4]. Cell cycle arrest and apoptosis are considered to be responsible for its effect. Metabolite analysis demonstrates that 8-Cl-Ado can be phosphorylated to 8-Cl-ATP in cells [3]. 8-Cl-Ado may inhibit cell growth by targeting cellular energy supply and RNA transcription [3, 5]. We previously found that 8-Cl-Ado induces G2/M arrest in human lung cancer cell lines A549 and H1299, in which the targeted cells are able to exit the G2 phase and enter the M phase due to loss of phosphorylated forms of protein kinase CHK2 and tyrosine phosphoprotein phosphatase CDC25C, followed by mitotic catastrophe [6]. Furthermore, we demonstrated that 8-Cl-Ado-induced G2/M arrest and mitotic division failure are correlated partially to the disruption of dynamic instability of microtubules and microfilaments [7]. Recently, we found that 8-Cl-Ado exposure can induce DNA double-strand breaks (DSBs) through inhibiting type II topoisomerases by 8-Cl-Ado-converted 8-Cl-ATP in human leukemia K562 cells [8]. We also demonstrated that 8-Cl-Ado-induced DNA damage activates G2/M phase checkpoint, which is associated with ATM-activated CHK1–CDC25C–CDC2 pathway in apoptosis-resistant human myelocytic leukemia K562 cells [9].

E2F1 belongs to the E2F transcription factor family, which comprises eight members from E2F1 to E2F8 in mammals – three activator and five repressor proteins [10, 11]. The E2F transcription factor family has a crucial role in the regulation of cell cycle progression [10, 12, 13]. E2F1 itself can exert its multiple functions through transcriptionally activating its downstream genes.

Abbreviations: ATM, ataxia telangiectasia mutated; ATR, ATM and Rad3-related kinase; BRCA1, breast cancer type 1 susceptibility protein; CHK2, checkpoint kinase 2; 8-Cl-Ado, 8-chloro-adenosine; 8-Cl-cAMP, 8-chloro-cAMP; DSB, double-strand break; NER, nucleotide excision repair.

* To whom correspondence should be addressed.

Physiologically, E2F1 promotes cell cycle progression from G1 to S phase through transactivating the cell cycle related genes [14]. In response to DNA damage or oncogene activation, E2F1 triggers apoptosis through p53-dependent and p53-independent mechanisms [15]. In addition, E2F1 induces autophagy by upregulating the expression of the autophagy genes LC3, Atg1, Atg5, DRAM, and Beclin 1 [16, 17].

E2F1 can be upregulated in response to DNA damage [18-22]. The signaling events leading to E2F1 induction upon DNA damage have also been delineated. ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related) phosphorylate E2F1 at Ser31 but do not phosphorylate E2F2 or E2F3, and this specificity accounts for the selective induction of E2F1 during DNA damage [21]. E2F1 is also phosphorylated by CHK2 [22]. Together, these phosphorylation events lead to stabilization and activation of E2F1. In addition, acetylation has also been recognized to be associated with the stabilization of E2F1 after DNA damage [23, 24]. Moreover, a recent study has show that histone-modifying enzymes Set9 and LSD1 (lysine-dependent demethylase 1) can regulate DNA damage-induced apoptosis via modulation of E2F1 stabilization. Set9 methylates E2F1 at Lys185, preventing E2F1 accumulation during DNA damage and activation of its proapoptotic target gene p73. This methyl mark is removed by LSD1, which is required for E2F1 stabilization and apoptotic function. All these covalent modifications of E2F1 mentioned above may have crosstalk with each other in regulating E2F1 stability. For instance, methylation at Lys185 inhibits acetylation and phosphorylation at distant positions and, in parallel, stimulates ubiquitination and degradation of the protein [25]. Based on these data, we formulated the conception that E2F1 is stabilized by genotoxic stresses through multiple types of posttranslational modifications without affecting its transcription. However, there is one exception, which reports that E2F1 transcription is induced by genotoxic stress through ATM/ATR activation [26]. Thus, there are multiple mechanisms that facilitate the accumulation of E2F1 after DNA damage in different contexts, and the role of E2F1 in 8-Cl-Ado-induced DNA DSBs response remains unclear.

In this study, we demonstrate that 8-Cl-Ado exposure induces DNA DSBs, accompanied by the increase of E2F1 stabilization and accumulation in A549 and H1299 cells. The induction of E2F1 is functionally important for 8-Cl-Ado-induced G2/M arrest but not for apoptosis. These data suggest that the mechanism of the action of 8-Cl-Ado is complicated.

MATERIALS AND METHODS

Cell culture and chemical treatment. Human lung cancer cell lines A549 and H1299 were purchased from the ATCC (USA). The cells were cultured in RPMI medium 1640 (Life Technologies, Inc., USA) supplemented with 10% fetal bovine serum (GIBCO BRL, USA), 100 U/ml of penicillin, and 100 μg/ml streptomycin, and grown in a 37°C incubator with 5% CO_2.

8-Cl-Ado (State Laboratory for Natural and Biomimetic Drugs, Peking University HSC, China) was dissolved in sterilized 0.85% NaCl solution and added to cultures at the concentration of 10 μM for the indicated times.

Immunocytochemical labeling. Immunocytochemical labeling was performed as described with modifications [11]. Briefly, cells were fixed with 4% formaldehyde in PBS at 37°C for 30 min, washed in PBS, and then permeabilized with 0.5% Triton X-100 in PBS for 20 min at room temperature. Cells were washed in a blocking solution consisting of 5% BSA and 0.2% Triton X-100 in PBS and stored in the blocking solution at 4°C before labeling. For labeling, fixed cells were incubated for 2 h at 37°C with specific antibodies against to γ-H2AX (1 : 2000) (R&D Systems Inc., USA) and E2F1 (1 : 100) (Santa Cruz Biotechnology, USA) in the blocking solution, respectively, followed by three washes in blocking solution. Cells were incubated with rhodamine-conjugated goat anti-rabbit IgG (1 : 100) or fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1 : 100) (Santa Cruz Biotechnology) in the blocking solution at 37°C for 1 h. After three washes, cells were incubated for 10 min at room temperature with 5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes, USA) or 5 μg/ml Hoechst 33342 (Molecular Probes). After three washes in PBS, cells were mounted in a 90% glycerol–PBS mixture. Laser confocal microscopy was performed at room temperature using a Leica TCS SP2 instrument (Leica Microsystems Heidelberg GmbH, Germany).

Western blot. Cells were harvested with a plastic scraper and then washed twice with cold PBS. The cells were then lysed in lysis buffer (50 mM Tris-HCl, pH 7.0-7.2, 250 mM NaCl, 5 mM EDTA, 50 mM NaF). The proteins of the lysates were quantified with BCA™ Protein Assay Kit (Pierce, USA). Fifty micrograms of total proteins were subjected to 12% SDS-PAGE and transferred onto nitrocellulose membranes, blocked with 5% nonfat milk in TBS-T (20 mM Tris, pH 7.0-7.2, 500 mM NaCl, and 0.1% Tween 20) at room temperature for 2 h with rocking. The membranes were probed with specific antibodies overnight at 4°C. After washing with 5% nonfat milk/TBS-T three times for 15 min each, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) in 5% nonfat milk/TBS-T at room temperature for 1 h. After washing three times in TBS-T for 15 min, the protein–antibody complex was detected by enhanced chemiluminescence (Santa Cruz Biotechnology). Equal protein loading was verified by rehybridization of membranes and reprobing with anti-β-actin antibody. Specific antibody against E2F1 was from Cell

Signaling Technology. Anti-γ-H2AX antibody was from R&D Systems Inc. Anti-β-actin antibody was from Santa Cruz Biotechnology.

RT-PCR and real-time quantitative PCR. Total RNA was isolated with TRIzol (Invitrogen, USA) and reverse transcribed with M-MLV reverse transcriptase (Promega, USA). RT-PCR was performed using Taq DNA polymerase (Promega), and primers as follows: for GAPDH, 5′-CAG CCT CGT CCC GTA GAC A-3′ and 5′-CGC TCC TGG AAG ATG GTG AT-3′; for E2F1, 5′-CGG TGT CGT CGA CCT GAA CT-3′ and 5′-AGG ACG TTG GTG ATG TCA TAG ATG-3′. The cycle number was chosen for each primer pair that maintained approximately exponential amplification with the enriched sample. For real-time thermal cycling, triplicate aliquots of serially diluted amplicon or RT sample were used in a reaction mixture that contained 250 nM of each primer in a reaction volume of 20 μl using the SYBR Green Real-time PCR Master Mix (Toyobo, Japan). The ABI Prism 7300 SDS software was used for these experiments. The same primer sets used in RT-PCR were also used in the real-time PCR. The thermal cycling was started with an initial 5 min at 95°C, followed by 40 cycles of 95°C for 30 sec and 60°C for 2 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as an endogenous control. The expression of mRNA is represented as fold increase ($2^{-\Delta\Delta Ct}$).

Plasmid transfection. Vectors used for transfections in this study include wild-type HA-E2F1 and mutant HA-E2F1 (S31A). Twenty-four hours prior to transfection, A549 and H1299 cells were seeded into 6-well tissue culture plates. On the following day, these cells were transfected with wild-type HA-E2F1 or mutant HA-E2F1 (S31A) using QIAGEN Effectene^{R} Transfection Reagent as recommended by the manufacturer. Cells were then treated with 10 μM 8-Cl-Ado for 24 h after transfection and harvested for subsequent analysis.

Cell cycle analysis was performed as described with modifications [6]. A549 and H1299 cells were transfected with wild-type HA-E2F1 or mutant HA-E2F1 (S31A) and the cells were then treated with 8-Cl-Ado at 10 μM for 24 h. Aliquots of cells were pelleted and washed twice in cold PBS and then fixed in ice-cold 70% ethanol overnight at 4°C. Then the cells were washed in PBS and digested with DNase-free RNase A (10 μg/ml) at 37°C for 30 min. Before flow cytometric analysis, cells were resuspended in 1 ml of propidium iodide (PI) (10 μg/ml) (Sigma, USA) for DNA staining. Three independent experiments were performed, and cellular DNA contents were analyzed by FACScan (Becton Dickinson, USA). Computer programs CELL Quest and ModFit LT 2.0 for Power were used for cell-cycle analysis.

Annexin V apoptosis assay. Annexin V apoptosis assays were performed using Annexin V-FITC Apoptosis Assay Kit (Biosea, China). The A549 and H1299 cells were collected by trypsinization. Then the cells were centrifuged at 1000 rpm for 10 min at 4°C and washed with ice-cold PBS twice. After that, the cells were resuspended at a concentration of $5\cdot10^5$-$1\cdot10^6$ cells/ml in 1× binding buffer. Then, aliquots of cells (100 μl) were supplemented with 5 μl of Annexin V-FITC (1 μg/ml) and 5 μl of propidium iodide (2.5 μg/ml). The mixture was incubated at room temperature for 15 min in the dark, and then 400 μl of 1× binding buffer was added to each tube and the cells were analyzed by flow cytometry within 30 min of staining. Cells in early apoptosis (annexin$^+$/PI$^-$) were in the lower right quadrant of the plots in Fig. 6. Live cells (annexin$^-$/PI$^-$) were in the lower left quadrant. Cells in late apoptosis/necrosis (annexin$^+$/PI$^+$) were in the upper right quadrant.

RESULTS

8-Cl-Ado exposure induces DNA DSBs in A549 and H1299 cells. DSBs lead to the rapid phosphorylation of histone H2AX yielding a modified phosphorylated histone H2AX (γ-H2AX), providing a reliable marker for DSB production and the means to spatially localize DNA DSBs within the nuclei of cells [27]. To detect 8-Cl-Ado-induced DNA DSBs, we examined γ-H2AX nuclear foci in exposed A549 and H1299 cells by immunocytochemical labeling (Fig. 1a; see color insert). The numbers of γ-H2AX foci increased significantly within 24-48 h after 8-Cl-Ado exposure. Consistent with the increase of γ-H2AX foci, the levels of γ-H2AX protein were globally increased after exposure (Fig. 1b; see color insert). These results are consistent with our recent observation of 8-Cl-Ado-induced DNA DSBs in K562 cells [8, 9], indicating that 8-Cl-Ado exposure may induce DNA damage in many types of tumor cells.

Induction of E2F1 accumulation in lung cancer cells exposed to 8-Cl-Ado. Several studies have shown that the E2F1 protein is upregulated following exposure of cells to a variety of DNA-damaging agents including ultraviolet (UV) radiation, ionizing radiation, and chemotherapeutic drugs [18-22]. To investigate whether 8-Cl-Ado exposure of lung cancer cells induces E2F1 accumulation, we examined the accumulation of E2F1 protein in A549 and H1299 cells exposed to 10 μM 8-Cl-Ado. We found that E2F1 was upregulated in detected cells (Fig. 2a). In addition, the induction of E2F1 was independent of p53 status as seen by the elevated E2F1 in A549 (p53 wild-type) and H1299 (p53 null) cells exposed to 8-Cl-Ado.

In response to genotoxic stresses, E2F1 is stabilized by phosphorylation and acetylation. ATM/ATR can phosphorylate E2F1 at Ser31 [21], and CHK2 can phosphorylate E2F1 at Ser364 [22]. Acetylation is associated with E2F1 Lys117, 120, and 125 [23, 24]. To establish the mechanism of E2F1 accumulation upon DNA DSBs, we examined the levels of E2F1 mRNA in A549 and H1299 cells exposed to 10 μM 8-Cl-Ado for indicated times by RT-PCR and real-time PCR. The results showed that

compared with unexposed cells, E2F1 mRNA levels remained unchanged in both A549 and H1299 cells after 8-Cl-Ado exposure (Figs. 2b and 2c), suggesting that accumulation of E2F1 at protein level is independent of transcriptional activation when DNA DSBs are induced.

Ser31 of E2F1 is irreplaceable for the maintenance of its stability in response to 8-Cl-Ado-induced DNA DSBs. Enhancement of E2F1 protein stability upon DNA damage correlates partly with the phosphorylation of E2F1 at Ser31 by the ATM or ATR kinase [21]. Our previous work has revealed that treatment of tumor cells with 8-Cl-Ado leads to DSBs and induction of ATM kinase activity [8, 9]. To explore the relationship between the Ser31 of E2F1 and its stability in response to 8-Cl-Ado-induced DNA DSBs, we transiently transfected A549 and H1299 cells with either wild-type HA-E2F1 (E2F1-wt) or mutant HA-E2F1 (S31A) (E2F1-mu) and then treated the cells with 8-Cl-Ado for 24 h. Following treatment, cells were harvested and assayed for the E2F1 protein by Western blot. As shown in Fig. 3b, E2F1 protein could be induced in E2F1-wt transfected A549 and H1299 cells, followed by 8-Cl-Ado exposure. However, the E2F1 protein levels were downregulated in E2F1-mu transfected A549 and H1299. Based on these results we conclude that the Ser31 is irreplaceable for the maintenance of E2F1 protein stability in response to 8-Cl-Ado-induced DNA DSBs. Since ATM can be activated in response to 8-Cl-Ado-induced DNA DSBs [8, 9], we suggest that E2F1 Ser31 may be phosphorylated in an ATM-dependent manner in the response to 8-Cl-Ado.

Accumulated E2F1 colocalizes with γ-H2AX in response to 8-Cl-Ado-induced DNA DSBs. The response

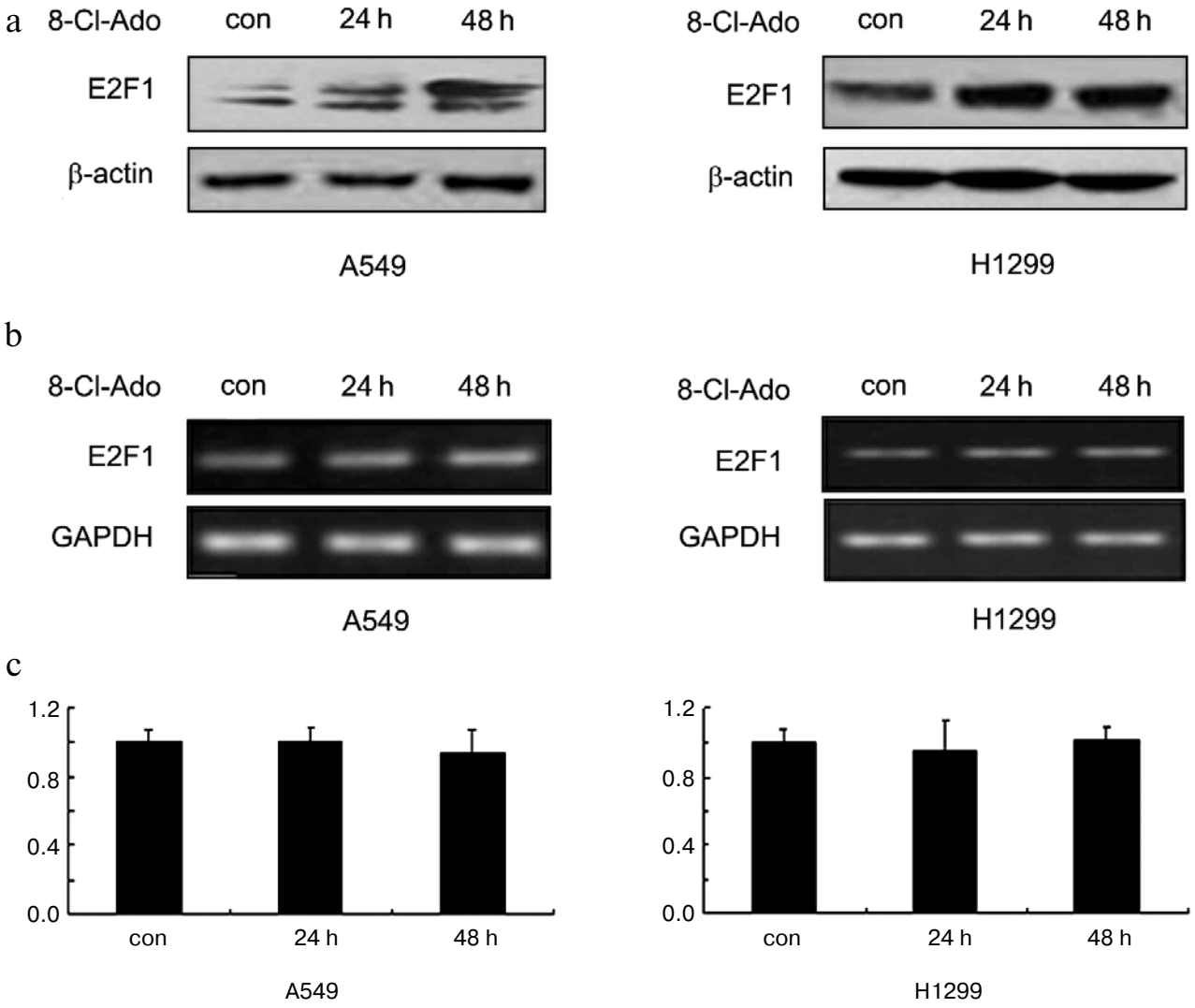

Fig. 2. Induction of E2F1 accumulation in A549 and H1299 cells exposed to 8-Cl-Ado. The cells were exposed to 10 μM 8-Cl-Ado for the indicated time periods. a) Western blot was performed to estimate E2F1 protein level. β-Actin was used as a loading control. b) RT-PCR was performed to investigate E2F1 mRNA level. GAPDH was used as a loading control. c) Real-time quantitative PCR (RT-qPCR) was performed to investigate E2F1 mRNA level. GAPDH was used as a loading control.

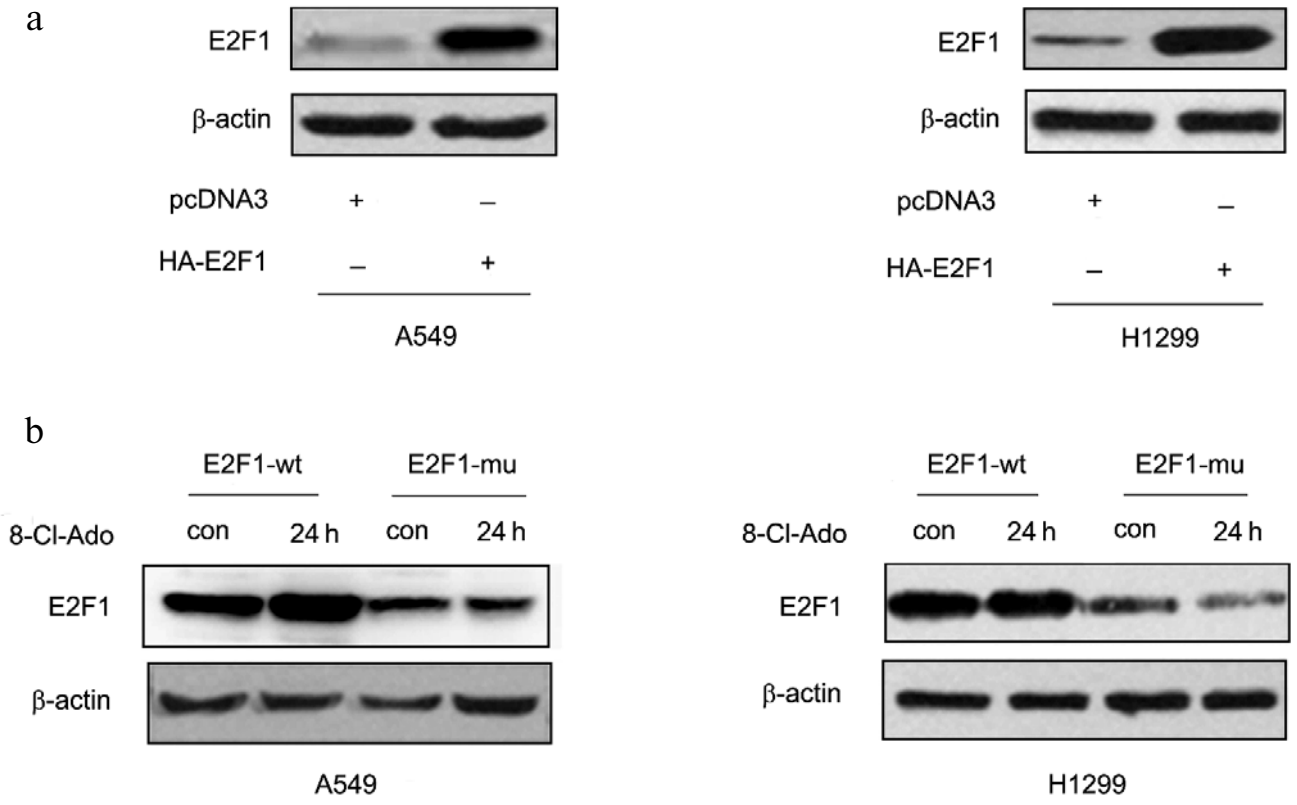

Fig. 3. Ser31 of E2F1 is irreplaceable for the maintenance of its stability in response to 8-Cl-Ado-induced DNA DSBs. a) A549 and H1299 cells were transiently transfected with 2 μg HA-E2F1 plasmid and the transfection efficiency rates were assessed through Western blot analysis. pcDNA3 empty vector was used as a negative control. b) Western blot analysis of E2F1 in A549 cells and H1299 cells transfected with E2F1-wt or E2F1-mu plasmid and treated with 10 μM 8-Cl-Ado respectively for the indicated time periods. β-Actin was used as a loading control.

of eukaryotic cells to DSBs in genomic DNA includes the sequestration of many factors into nuclear foci where many DNA repair related proteins, such as BRCA-1, RAD50, and NBS1 (nibrin), can colocalize with γ-H2AX in response to DNA DSBs [28-30]. Most recently, it was found that E2F1 could stimulate nucleotide excision repair (NER) by enhancing the recruitment of DNA repair factors to the sites of UV-induced DNA damage [31], and E2F1 associates with the GCN5 histone acetyltransferase in response to UV radiation and recruits GCN5 to sites of damage [32]. To demonstrate whether E2F1 colocalizes with γ-H2AX in response to 8-Cl-Ado-induced DNA DSBs in A549 and H1299 cells, immunocytochemical labeling was performed with anti-E2F1 and anti-γ-H2AX antibodies.

As shown in Figs. 4a and 4b (see color insert), E2F1 was diffusely distributed within the nucleus in both types of unexposed cells. After 8-Cl-Ado exposure for 24 h, E2F1 relocalized to the sites of DNA damage to form distinct foci and colocalized with γ-H2AX, which could be visualized using immunofluorescence. When we transfected these two cells with E2F1-wt followed by 8-Cl-Ado exposure, the colocalization of E2F1 with γ-H2AX became more obvious. However, E2F1-mu transfection again led to a diffuse distribution of E2F1 in the nuclei. These results indicate that accumulated E2F1 relocalizes to the sites of DNA damage and colocalizes with γ-H2AX in response to 8-Cl-Ado-induced DNA DSBs, which is dependent on the Ser31 of E2F1. All these findings suggest that E2F1 may also play a critical role in recruiting other proteins at sites of DNA damage.

E2F1 plays a critical role in 8-Cl-Ado-induced G2/M arrest. To determine the cell-cycle checkpoint response to 8-Cl-Ado-induced DNA DSBs, flow cytometry experiments were performed to analyze the cell-cycle distribution. A typical flow cytometry showed that exposure of A549 cells to 8-Cl-Ado for 24 h caused an increase of G2/M subpopulation (Fig. 5a). This is consistent with our previous study [6]. To examine the role of E2F1 accumulation in 8-Cl-Ado-induced G2/M arrest, we further analyzed the changes in cell cycle distribution in A549 cells transfected with E2F1-wt or E2F1-mu (S31A) plasmid and treated with 8-Cl-Ado. By flow cytometric analysis, a more obvious G2/M arrest was observed in E2F1-wt transfected A549 cells, but such an arrest was ablated in E2F1-mu-transfected A549 in response to 8-

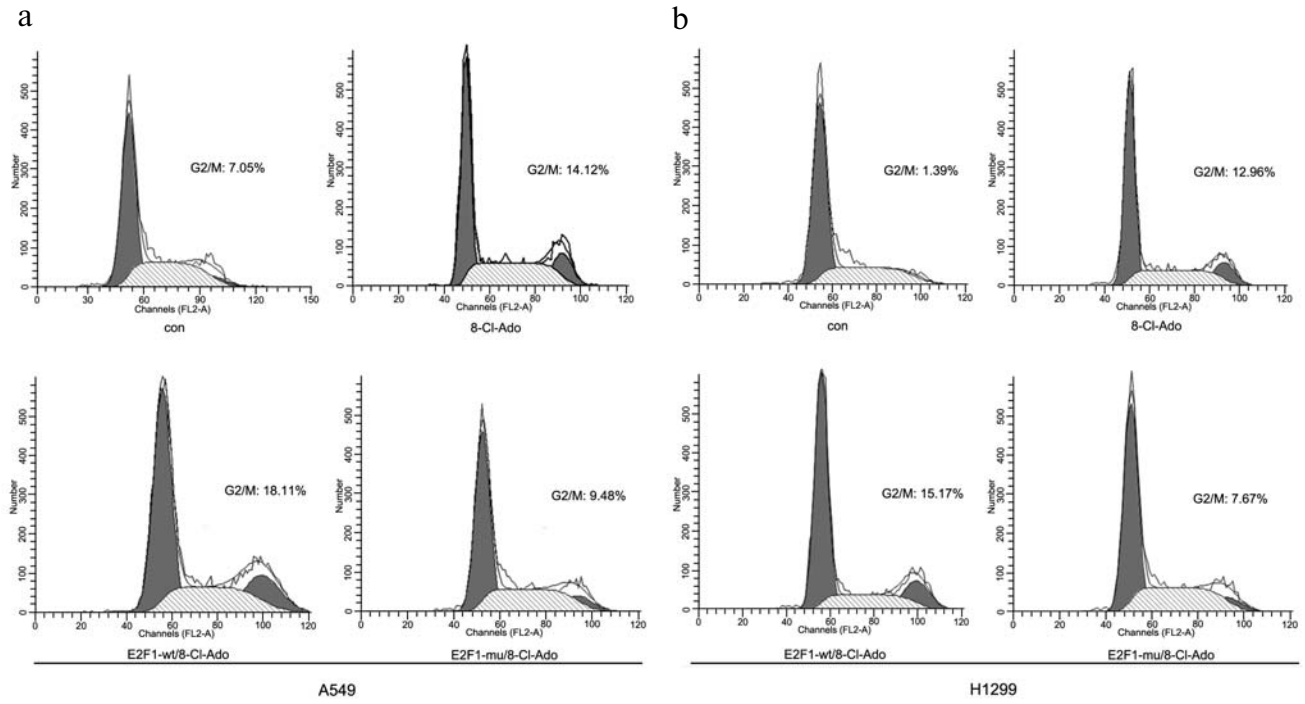

Fig. 5. E2F1 plays a critical role in 8-Cl-Ado-induced G2/M arrest. Cell cycle distribution analysis of A549 (a) and H1299 (b) cells transfected with E2F1-wt or E2F1-mu (S31A) plasmid and treated with 8-Cl-Ado (10 μM) for 24 h. Cell cycle distribution was determined by the propidium iodide staining method, and stained cells were analyzed by flow cytometry using the CELL Quest software. The data are representative from one of three independent experiments.

Cl-Ado exposure (Fig. 5a). In H1299 cells, similar results were observed (Fig. 5b). These data indicate that to a certain extent, 8-Cl-Ado-induced G2/M checkpoint is mediated by E2F1, for which Ser31 of E2F1 is required.

E2F1 overexpression induces apoptosis in A549 and H1299 cells exposed to 8-Cl-Ado. We have previously shown that apoptosis follows mitotic catastrophe only after long (48-96 h) exposure of A549 and H1299 cells to 8-Cl-Ado [6]. To explore the role of E2F1 in inducing apoptosis in lung cancer cells, we exposed A549 and H1299 cells, which were transfected with E2F1-wt or E2F1-mu expression plasmid, to 8-Cl-Ado for 24 h and measured the proportion of apoptotic cells by Annexin V apoptosis assay. Consistent with our previous observation [6], 8-Cl-Ado exposure for 24 h could not induce significant apoptosis in either cell type (control vs. 8-Cl-Ado, $p > 0.05$) (top and bottom panels in Fig. 6). However, 8-Cl-Ado exposure caused a marked increase of apoptotic proportion in E2F1-wt transfected A549 and H1299 (8-Cl-Ado vs. E2F1-wt/8-Cl-Ado, $p < 0.05$) (middle and bottom panels). Unexpectedly, this agent also led to significant apoptosis in E2F1-mu-transfected cells (8-Cl-Ado vs. E2F1-mu/8-Cl-Ado, $p < 0.01$). These results indicate that E2F1 can promote 8-Cl-Ado-induced apoptotic cell death and the S31A mutation of E2F1 may also stimulate apoptosis. In addition, we noticed that necrosis occurred to a certain extent (upper-right quadrant representing nonviable, late apoptotic/necrotic cells), suggesting that the mechanisms of 8-Cl-Ado action are complicated.

DISCUSSION

When cells encounter DSBs, γ-H2AX is generated, forming discrete nuclear foci at the damaged sites [27]. We have previously demonstrated that DSBs are generated in 8-Cl-Ado exposed K562 cells [8, 9]. Here we found that the foci numbers and protein levels of γ-H2AX were significantly increased in 8-Cl-Ado exposed A549 and H1299 cells (Fig. 1). It has been reported that E2F1 can be upregulated upon DNA damage [18-22]. Consistent with this, we found that E2F1 protein levels were elevated in A549 and H1299 following exposure to 8-Cl-Ado within 48 h (Fig. 2a). However, the mRNA level of E2F1 remains unchanged in exposed cells (Figs. 2b and 2c). We therefore conclude that the accumulation of E2F1 by 8-Cl-Ado is associated with the increased stability of E2F1. Phosphorylation of E2F1 at Ser31 by ATM/ATR and/or CHK2 leads to stabilization and activation of E2F1 upon DNA damage [21, 22]. Supporting this notion, we found that E2F1 accumulation in response to 8-Cl-Ado was largely dependent on the Ser31, since the S31A mutant resulted in the decrease in E2F1 protein (Fig. 3). Our data suggest that the phosphorylation of Ser31 is involved in

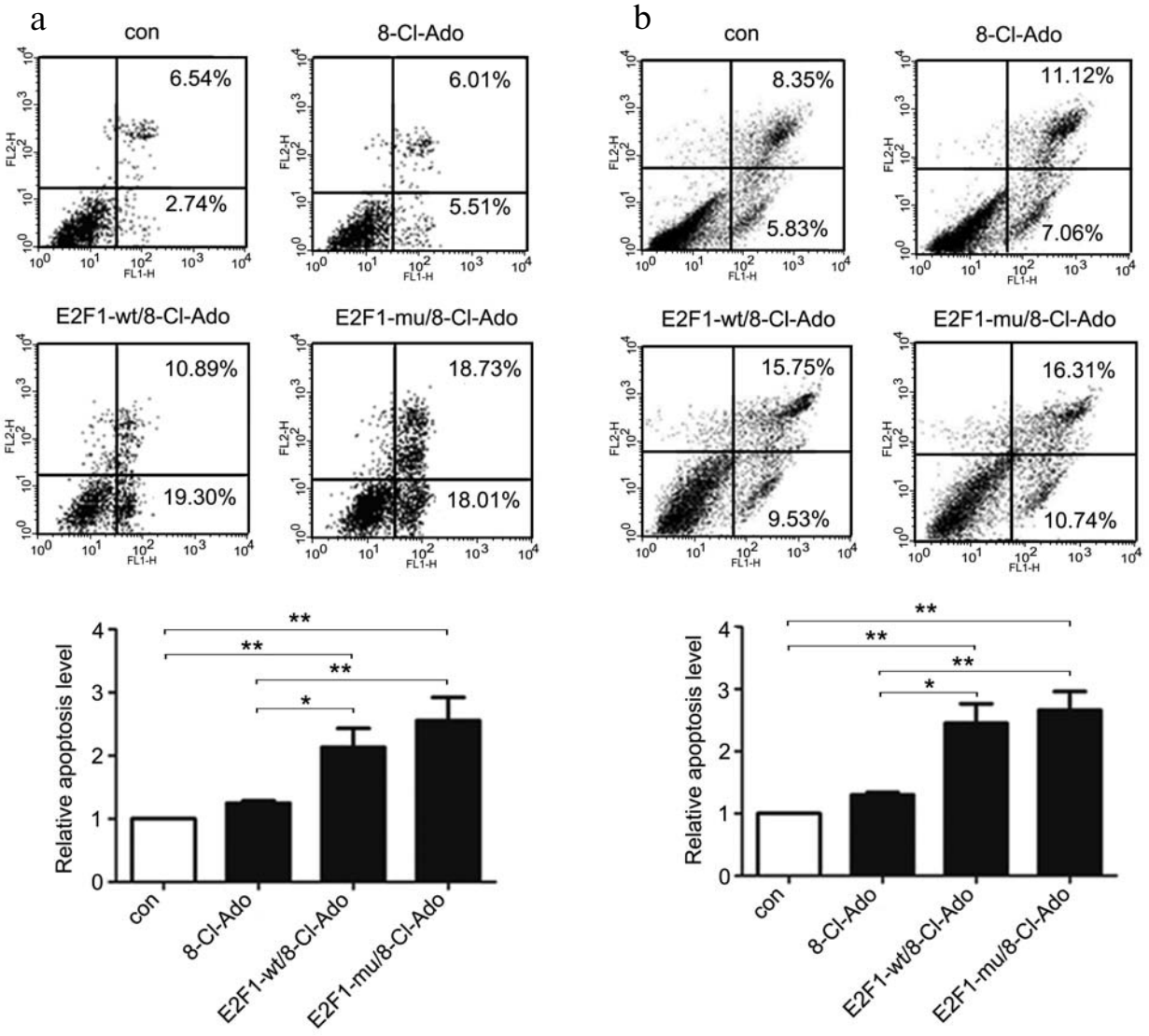

Fig. 6. E2F1 overexpression induces apoptosis of A549 and H1299 cells exposed to 8-Cl-Ado. The A549 and H1299 cells were transfected with E2F1-wt or E2F1-mu plasmid and treated with 10 μM 8-Cl-Ado for 24 h, respectively. The apoptotic proportion of A549 (a) and H1299 (b) cells were assessed by Annexin V/PI double staining according to the manufacturer's protocol. Bottom right quadrant, cells stained mainly by Annexin V (early apoptotic cells); top right quadrant, cells stained by both PI and Annexin V (late apoptotic/necrotic cells); top left quadrant, cells stained mainly by PI (dead cells); bottom left quadrant, cells negative for both Annexin V and PI (viable cells). The bottom histograms display the total apoptosis levels in specific contexts each corresponding to one of the four plots shown in A549 (a) and H1299 (b) cells, respectively. * $p < 0.05$, ** $p < 0.01$.

the accumulation of E2F1 in response to 8-Cl-Ado-induced DNA damage.

E2F1 was initially characterized as an activator of genes expressed at the G1/S transition to regulate cell cycle progression [33], but it is also known to regulate G2/M genes [33, 34]. However, it remains unknown how E2F1 affects human tumors. Several previous studies have revealed that E2F1 is highly expressed in lung, breast, thyroid, and pancreatic tumors, and sBL lymphoma specimens [35-37]. Other studies suggest that E2F1 may function as a tumor suppressor for human neoplasias such as large B-cell lymphomas, colon, prostate, lung, and invasive bladder cancer [36]. As to the effect of E2F1 on G2/M arrest, the reports are contrary. Transfection of adenovirus E2F1 expression vector into H1299 cells results in a dramatic increase in the G2/M population [38]. E2F1 is also involved in p53-induced G2/M arrest [39]. Moreover, expression of E2F1 in HSG cells prior to irradiation induces the cells to be maintained in S phase and delays entry into G2/M phase [40]. Similarly, reduction of E2F1 expression in sBL cells may lead to their accumulation in G2 [37]. The different effects of E2F1 on G2/M arrest may be attributed to the factors such as cell-types, stimulators, lesion degrees and properties, and

environments. Our previous study demonstrates that 8-Cl-Ado activates G2/M phase checkpoint in human leukemia K562 cells, which is associated with ATM-activated CHK1–CDC25C–CDC2 cascade joined by BRCA1–CHK1 [9]. 8-Cl-Ado can also induce G2/M arrest in A549 and H1299 cells [6].

Here we found that the upregulation of E2F1 was concomitant with G2/M arrest in 8-Cl-Ado-exposed A549 and H1299 (Figs. 2 and 5). Furthermore, E2F1-wt transfection could promote 8-Cl-Ado-induced G2/M arrest, but S31A mutant transfection did not (Fig. 5). Since the wild-type E2F1 but not S31A mutant could effectively be accumulated in 8-Cl-Ado-exposed cells (Fig. 3b), our data suggest that E2F1 is required for 8-Cl-Ado-induced G2/M arrest. At present we have no direct evidence to explain how E2F1 induces G2/M arrest. A recent study shows that rodent p19ARF, an E2F1 target, can affect G2/M regulators to cause G2/M arrest [41].

We have previously demonstrated that p14ARF, the p19ARF homolog in humans, is activated by E2F1 in 8-Cl-Ado-exposed H1299 [42]. We suppose that E2F1 might mediate G2/M arrest through activating its target p14ARF gene in H1299 (p53-null) and A549 (p53-wt). In addition, E2F1 can activate p53, and E2F1-mediated G2/M arrest by p53 induction may involve the p53 targets GADD45 and 14-3-3-σ, which affect the activity of the CDC2/CDK1 kinase [39]. We therefore do not exclude the roles of p53 targets in E2F1-mediated G2/M arrest in 8-Cl-Ado exposed A549.

8-Cl-Ado can induce cell death by apoptosis in a variety of tumor cells [1-3, 43]. We have previously described that 8-Cl-Ado inhibits human lung cancer cell growth by inducing G2/M arrest and mitotic catastrophe [6], whereas apoptosis occurs only after long (>48 h) exposure [8, 9]. Late apoptosis/necrosis is also observed in 8-Cl-Ado-exposed thyroid cancer cells and lymphocytes [44]. In living cells, 8-Cl-Ado can be phosphorylated to 8-Cl-ATP that targets cellular energetics and inhibits RNA synthesis, leading to decrease of survival gene transcripts and apoptosis [3, 5]. However, this finding cannot explain the upregulation of cyclin B1, p53 [43], $p21^{WAF/CIP1}$ [9], E2F1, and p14ARF [42] in response to 8-Cl-cAMP/8-Cl-Ado exposure. Therefore, the mechanism of 8-Cl-Ado-induced apoptosis is uncertain.

Consistent with our previous observation [6, 7], exposure of A549 and H1299 to 8-Cl-Ado for 24 h could not cause significant apoptosis (upper and bottom panels in Fig. 6). However, a more obvious characteristic of apoptosis was observed in both cells transfected with either wild-type E2F1 or S31A mutant (middle and bottom panels in Fig. 6). Recently, we showed 8-Cl-Ado-induced E2F1 [42, 45], which leads to p14ARF-mediated apoptosis [45]. We have also detected p53 upregulation in 8-Cl-Ado-exposed A549 (unpublished data). Possibly, upregulation of E2F1 in response to 8-Cl-Ado induces apoptosis by activating p14ARF and/or p53.

The question to be answered is why transfection of S31A mutant can also promote 8-Cl-Ado-induced apoptosis. One possibility is that the S31A point mutation might affect the stability rather than the activity of E2F1, which needs to be determined. Previous study has shown that E2F1 accumulates at sites of UV-induced DNA damage, and phosphorylation of E2F1 at Ser31 by ATR stabilizes the interaction between E2F1 and TopBP1 at sites of damage. E2F1 then appears to be involved in a process that enhances damage recognition and nuclear excision repair (NER) globally throughout the genome [31]. It is possible that E2F1 accumulation at damage sites is reduced when Ser31 is mutated to alanine (Fig. 4), thereby interfering with DNA damage repair and initiating apoptosis. It is also possible that 8-Cl-Ado may induce apoptotic cell death in E2F1-dependent and E2F1-independent ways. We have demonstrated that 8-Cl-Ado-induced disorder of actin skeleton is responsible partly to its inhibitory effects on cell death including apoptosis and mitotic catastrophe in A549 and H1299 cells [7]. In addition, the p38 MAP kinase can mediate 8-Cl-Ado-induced apoptosis [4]. 8-Cl-Ado-converted 8-Cl-ATP may target cellular energetics [3] and inhibit RNA synthesis [3, 5]. Taking the previous finding and our data, we conclude that 8-Cl-Ado may induce apoptosis in E2F1-dependent and E2F1-independent ways, and the mechanism of 8-Cl-Ado action is complicated.

In summary, we have demonstrated that E2F1 can be stabilized and upregulated in response to 8-Cl-Ado-induced DNA DSBs in human lung cancer cells, which is dependent on the Ser31 of E2F1. The stabilized E2F1 plays a critical role in 8-Cl-Ado-induced G2/M arrest, but it is dispensable for 8-Cl-Ado-induced apoptosis due to the multiple ways of 8-Cl-Ado-induced cell death. These results indicate complicated mechanisms of 8-Cl-Ado action. Further investigation of the mechanisms of E2F1 accumulation and its subsequent functions in DNA repair, cell growth control, and apoptosis upon DNA damage may have valuable implications for understanding tumorigenesis and clinical therapeutics.

We thank Dr. Joseph R. Nevins (Duke University Medical Center, USA) for pcDNA3-HA-E2F1, pcDNA3-HA-E2F1 (S31A). We thank Professor Ran Xiao (China Academy of Medical Sciences, China) for reviewing of the manuscript.

This work was supported by National Natural Science Foundation of China grants 30200131, 30671062, 30770464 and 30971449, and Education Committee of Beijing Teaching Reinforcement Plan.

REFERENCES

1. Halgren, R. G., Traynor, A. E., Pillay, S., Zell, J. L., Heller, K. F., Krett, N. L., and Rosen, S. T. (1998) *Blood*, **92**, 2893-2898.

2. Carlson, C. C., Chinery, R., Burnham, L. L., and Dransfield, D. T. (2000) *Neoplasia*, **2**, 441-448.
3. Gandhi, V., Ayres, M., Halgren, R. G., Krett, N. L., Newman, R. A., and Rosen, S. T. (2001) *Cancer Res.*, **61**, 5474-5479.
4. Ahn, Y. H., Jung, J. M., and Hong, S. H. (2005) *Cancer Res.*, **65**, 4896-4901.
5. Chen, L. S., and Sheppard, T. L. (2004) *J. Biol. Chem.*, **279**, 40405-40411.
6. Zhang, H. Y., Gu, Y. Y., Li, Z. G., Jia, Y. H., Yuan, L., Li, S. Y., An, G. S., Ni, J. H., and Jia, H. T. (2004) *Neoplasia*, **6**, 802-812.
7. Gu, Y. Y., Zhang, H. Y., Zhang, H. J., Li, S. Y., Ni, J. H., and Jia, H. T. (2006) *Biochem. Pharmacol.*, **72**, 541-550.
8. Yang, S. Y., Jia, X. Z., Feng, L. Y., Li, S. Y., An, G. S., Ni, J. H., and Jia, H. T. (2009) *Biochem. Pharmacol.*, **77**, 433-443.
9. Jia, X. Z., Yang, S. Y., Zhou, J., Li, S. Y., Ni, J. H., An, G. S., and Jia, H. T. (2009) *Biochem. Pharmacol.*, **77**, 770-780.
10. Trimarchi, J. M., and Lees, J. A. (2002) *Nat. Rev. Mol. Cell. Biol.*, **3**, 11-20.
11. DeGregori, J., and Johnson, D. G. (2006) *Curr. Mol. Med.*, **6**, 739-748.
12. Stevaux, O., and Dyson, N. J. (2002) *Curr. Opin. Cell. Biol.*, **14**, 684-691.
13. Cam, H., and Dynlacht, B. D. (2003) *Cancer Cell*, **3**, 311-316.
14. DeGregori, J. (2002) *Biochim. Biophys. Acta*, **1602**, 131-150.
15. Iaquinta, P. J., and Lees, J. A. (2007) *Curr. Opin. Cell. Biol.*, **19**, 649-657.
16. Polager, S., Ofir, M., and Ginsberg, D. (2008) *Oncogene*, **27**, 4860-4864.
17. Wang, B., Ling, S. Y., and Lin, W. C. (2010) *PLoS One*, **5**, e10409.
18. Blattner, C., Sparks, A., and Lane, D. (1999) *Mol. Cell. Biol.*, **19**, 3704-3713.
19. Wei, Y. Y., Liu, D., Ge, Y. Q., Zhou, F. B., Xu, J. J., Chen, H., Gu, J. X., and Jiang, J. H. (2008) *J. Biochem.*, **144**, 539-546.
20. O'Connor, D. J., and Lu, X. (2000) *Oncogene*, **19**, 2369-2376.
21. Lin, W. C., Lin, F. T., and Nevins, J. R. (2001) *Genes Dev.*, **15**, 1833-1844.
22. Stevens, C., Smith, L., and La Thangue, N. B. (2003) *Nat. Cell. Biol.*, **5**, 401-409.
23. Ianari, A., Gallo, R., Palma, M., Alesse, E., and Gulino, A. (2004) *J. Biol. Chem.*, **279**, 30830-30835.
24. Pediconi, N., Ianari, A., Costanzo, A., Belloni, L., Gallo, R., Cimino, L., Porcellini, A., Screpanti, I., Balsano, C., Alesse, E., Gulino, A., and Levrero, M. (2003) *Nat. Cell. Biol.*, **5**, 552-558.
25. Haroula, K., and Iannis, T. (2010) *Mol. Cell*, **39**, 152-160.
26. Carcagno, A. L., Ogara, M. F., Sonzogni, S. V., Marazita, M. C., Sirkin, P. F., Ceruti, J. M., and Canepa, E. T. (2009) *IUBMB Life*, **61**, 537-543.
27. Rogakou, E. P., Boon, C., Redon, C., and Bonner, W. M. (1999) *J. Cell. Biol.*, **146**, 905-915.
28. Liu, K., Lin, F. T., Ruppert, J. L., and Lin, W. C. (2003) *Mol. Cell. Biol.*, **23**, 3287-3304.
29. Frame, F. M., Rogoff, H. A., Pickering, M. T., Cress, W. D., and Kowalik, T. F. (2006) *Oncogene*, **25**, 3258-3266.
30. Cerosaletti, K. M., and Concannon, R. (2003) *J. Biol. Chem.*, **278**, 21944-21951.
31. Guo, R., Chen, J., Zhu, F., Biswas, A. K., Berton, T. R., Mitchell, D. L., and Johnson, D. J. (2010) *J. Biol. Chem.*, **285**, 19308-19315.
32. Guo, R., Chen, J., Mitchell, D. L., and Johnson, D. G. (2011) *Nucleic Acids Res.*, **39**, 1390-1397.
33. Hallstrom, T. C., Mori, S., and Nevins, J. R. (2008) *Cancer Cell*, **13**, 11-22.
34. Zhu, W., Giangrande, P. H., and Nevins, J. R. (2004) *EMBO J.*, **23**, 4615-4626.
35. DeGregori, J. (2002) *Biochim. Biophys. Acta*, **1602**, 131-150.
36. Tsantoulis, P. K., and Gorgoulis, V. G. (2005) *Eur. J. Cancer*, **41**, 2403-2414.
37. Molina-Privado, I., Rodriguez-Martinez, M., Rebollo, P., Martin-Perez, D., Artiga, M.-J., Menarguez, J., Flemington, E. K., Piris, M. A., and Campanero, M. R. (2009) *Cancer Res.*, **69**, 4052-4058.
38. Kuhn, H., Liebers, U., Gessner, C., Schumacher, A., Witt, C., Schauer, J., Kovesdi, I., and Wolff, G. (2002) *Eur. Respir. J.*, **20**, 703-709.
39. Polager, S., and Ginsberg, D. (2009) *Nat. Rev. Cancer*, **9**, 738-748.
40. O'Connell, A. C., Lillibridge, C. D., Zheng, C., Baum, B. J., O'Connell, B. C., and Ambudkar, I. S. (1998) *J. Cell. Physiol.*, **177**, 264-273.
41. Liu, L., Li, J., Xu, Y. P., Qiao, W. T., Chen, Q. M., and Geng, Y. Q. (2009) *Prog. Biochem. Biophys.*, **36**, 541-548.
42. Zhang, H. J., Li, W. J., Yang, S. Y., Li, S. Y., Ni, J. H., and Jia, H. T. (2009) *J. Cell. Biochem.*, **106**, 464-472.
43. Grbovic, O., Jovic, V., Ruzdijic, S., Pejanovic, V., Rakic, L., and Kanazir, S. (2002) *Cancer Invest.*, **20**, 972-982.
44. Robinson-White, A. J., Bossis, I., Hsiao, H. P., Nesterova, M., Leitner, W. W., and Stratakis, C. A. (2009) *J. Clin. Endocrinol. Metab.*, **94**, 4061-4069.
45. Li, W. J., Gu, Y. Y., Zhang, H. J., Zhou, J., and Jia, H. T. (2009) *Chemotherapy*, **55**, 335-343.