Basic helix–loop–helix protein E47-mediated p21Waf1/Cip1 gene expression regulates apoptosis of intestinal epithelial cells

Sujoy BHATTACHARYA[1], Huazhang GUO, Ramesh M. RAY and Leonard R. JOHNSON

Department of Physiology, University of Tennessee Health Science Center, Memphis, TN 38163, U.S.A.

Inhibition of ornithine decarboxylase by DFMO (α-difluromethylornithine) and subsequent polyamine depletion increases p21Cip1 protein, induces cell cycle arrest and confers resistance to apoptosis on intestinal epithelial cells. However, the mechanism by which polyamines regulate p21Cip1 expression and apoptosis is unknown. On the basis of the involvement of p21Cip1 as an anti-apoptotic protein, we tested the role of p21Cip1 in providing protection from apoptosis. Simultaneously, we investigated the role of E47, a basic helix–loop–helix protein, in the regulation of p21Cip1 gene transcription. Gene-specific siRNA (small interfering RNA) decreased E47 protein levels, increased p21Cip1 promoter activity and protein levels and protected cells from TNFα (tumour necrosis factor α)-induced apoptosis. Knockdown of p21Cip1 protein by siRNA resulted in cells becoming more susceptible to apoptosis. In contrast, incubation with EGF (epidermal growth factor) stimulated p21Cip1 mRNA and protein levels and rescued cells from apoptosis. During apoptosis, the level of E47 mRNA increased, causing a concomitant decrease in p21Cip1 mRNA and protein levels. Polyamine depletion decreased E47 mRNA levels and cell survival. Caspase 3-mediated cleavage of p130Cas has been implicated in p21Cip1 transcription. The progression of apoptosis led to a caspase 3-dependent cleavage of p130Cas and generated a 31 kDa fragment, which translocated to the nucleus, associated with nuclear E47 and inhibited p21Cip1 transcription. Polyamine depletion inhibited all these effects. Transient expression of the 31 kDa fragment prevented the expression of p21Cip1 protein and increased apoptosis. These results implicate p21Cip1 as an anti-apoptotic protein and suggest a role for polyamines in the regulation of p21Cip1 via the transcription repressor E47. Caspase-mediated cleavage of p130Cas generates a 31 kDa fragment, inhibits p21Cip1 transcription and acts as an amplifier of apoptotic signalling.

Key words: α-difluromethylornithine (DFMO), epithelium, p130Cas, polyamine, Src, tumour necrosis factor α (TNFα).

INTRODUCTION

Apoptosis in the intestinal epithelium is an important regulator of mucosal homoeostasis. Detachment of epithelial cells as they approach the tips of the villi also induces apoptosis, termed anoikis, which leads to the shedding of cells into the lumen. Apoptosis occurs primarily at the stem cell position in the crypt area and is responsible for the balance in cell number between newly divided and shed cells. Although the polyamines spermidine, spermine and PUT (putrescine) are found in virtually all eukaryotic cells, and growing evidence implicates their diverse roles in proliferation and apoptosis of the intestinal epithelium, the molecular mechanism(s) involved are currently unclear. Extensive studies have examined the roles of polyamines in GI (gastrointestinal) mucosal homoeostasis, both in cultured non-transformed IEC-6s (intestinal epithelial cells) and in rat cells [1,2]. We have consistently shown that inhibition of ODC (ornithine decarboxylase) and the subsequent depletion of intracellular polyamines inhibits apoptosis induced by γ-radiation, camptothecin or TNFα (tumour necrosis factor α)/CHX (cycloheximide) [3–6].

Previous studies from our group have shown that polyamine depletion induces cell cycle arrest, increases the protein levels of p21Waf1/Cip1, p27Kip1, p53 and inhibits apoptosis in IEC-6 cells [3–4,6,7]. p21Cip1 is required and essential for the repression of cyclin/CDK (cyclin-dependent kinase) complexes and has a preference for the inhibition of CDK2 complexes [8,9]. p21Cip1 plays an important role in mediating growth arrest after DNA damage and is transiently expressed during G_1-, G_2- or S-phase cell cycle arrest [9]. Although the role of p21Cip1 in apoptosis is not well understood, recent evidence suggests that p21Cip1 is a major inhibitor of p53-dependent and -independent apoptosis [10]. Potential mechanisms related to the inhibition of apoptosis include interaction with pro-caspase 3, caspase 8, and ASK-1 [10–13], or the induction of cell cycle arrest by binding to cyclin A/cyclin E–CDK2 complexes [10,14]. E-box transcription factors with a basic HLH (helix–loop–helix) domain have been described as positive regulators of p21Cip1 transcription [15,16]. Separate classes of basic HLH proteins, often referred to as E proteins, such as E2-2, HEB and E2A, mediate homo- and hetero-dimerization and bind to a canonical DNA consensus sequence known as the E-box (CANNTG) [17,18]. E-box sites are present in the promoter regions of various genes, including the *ODC* gene [19]. Some studies suggest that overexpression of E47 (an E2A family transcription factor) and E12 promotes cell death [20,21], whereas others have shown that E2A suppresses activation of caspase 3 and protects cells from apoptosis [16,21,22].

Abbreviations used: BCA, bicinchoninic acid; CDK, cyclin-dependent kinase; CHX, cycloheximide; CIP, calf intestinal phosphatase; *Ct*, threshold cycle value; DFMO, α-difluromethylornithine; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular-signal-regulated kinase; FBS, foetal bovine serum; GI, gastrointestinal; HLH, helix–loop–helix; HRP, horseradish peroxidase; IEC-6, intestinal epithelial cell; JAK, Janus kinase; MEK, MAPK (mitogen-activated protein kinase)/ERK kinase; ODC, ornithine decarboxylase; PUT, putrescine; RLU, relative luciferase unit; siRNA, small interfering RNA; STAT, signal transducer and activator of transcription; TNFα, tumour necrosis factor α.

[1] To whom correspondence should be addressed (email sbhatta3@utmem.edu).

Kim et al. [23] showed that cleavage of p130Cas by an active caspase yielded an active 31 kDa fragment protein which binds E2A, contains a HLH domain and represses E2A-mediated p21Cip1 transcription and promotes apoptosis [23]. Overexpression of p130Cas protects cells from apoptosis [24], and inhibition of Cas phosphorylation decreases cell survival [25,26]. Caspase-mediated cleavage of Src [27], p130Cas [23] and p21Cip1 [28,29] is known to mediate apoptosis in various cell lines.

Given that polyamine depletion mediates Src-dependent Akt and JAK (Janus kinase)/STAT (signal transducer and activator of transcription)3 activation, which in turn prevents apoptosis [6] and increases the protein levels of p21Cip1, we sought to determine the role of polyamines in the regulation of p21Cip1 and its involvement in apoptosis.

Part of this work was presented at the FASEB Journal Advancing the Biomedical Frontier Experimental Biology meeting held in San Francisco, CA, U.S.A., on 1–5 April 2006.

EXPERIMENTAL

Reagents

Disposable cell culture items were purchased from Corning. Media and other cell culture reagents were obtained from Invitrogen and dialysed FBS (foetal bovine serum) was purchased from Sigma. The construct of p21Cip1-promoter–luciferase reporter (2.3 kb of the regulatory region of p21Cip1 gene fused to the luciferase reporter gene) was provided by Dr Jian-Ying Wang (Department of Surgery, University of Maryland, Baltimore, MD, U.S.A.). The 31 kDa-pcDNA3.0 HLH construct (31 kDa cleaved fragment of p130Cas with the HLH motif) was provided by Dr Woo Keun Song and Dr Kang YongSeok (Department of Life Science, Gwangju Institute of Science and Technology, Gwangju 500-712, Republic of Korea). Recombinant rat TNFα and EGF (epidermal growth factor) were obtained from BD Biosciences Pharmingen. ^{35}S-labelled methionine was purchased from Amersham Biosciences. The ECL (enhanced chemiluminescence) Western blot detection system was purchased from PerkinElmer. DFMO (α-difluoromethylorthinine) was a gift from ILEX Oncology. Rabbit anti-[p130Cas (phospho-Tyr^{410})] and rabbit anti-[cleaved active caspase 3 (Asp^{175})] antibodies were purchased from Cell Signaling. Mouse anti-p130Cas and mouse anti-p21Cip1 antibodies were obtained from BD Biosciences. PP2, an Src family tyrosine kinase inhibitor, rat recombinant active caspase 3, caspase 3 inhibitor II (z-DEVD-FMK) and caspase 3 inhibitor V (z-DQMD-FMK) were all purchased from Calbiochem. CIP (calf intestinal phosphatase) was from New England Biolabs. AG1478, an EGFR (EGF receptor) kinase inhibitor, was purchased from Biomol International. Rabbit anti-E47 antibody, mouse anti-actin antibody, Protein A–agarose and Protein G–agarose conjugates, p21Cip1 siRNA (small interfering RNA) (sc-29428) and E47-specific siRNA (sc-35252) were all obtained from Santa Cruz Biotechnology. TRIzol RNA extraction reagent was purchased from Invitrogen. Moloney murine leukaemia virus reverse transcriptase, PCR nucleotide mix, random primers and the luciferase assay system with reporter lysis buffer were bought from Promega. SYBR green PCR master mix was from Applied Biosystems. FuGENE^{TM}6 transfection reagent and Cell Death Detection ELISAPlus kit were purchased from Roche Diagnostics. The IEC-6 cell line (A.T.C.C. CRL 1592) was obtained from the A.T.C.C at passage 13. The cell line was derived from normal rat intestine and was developed and characterized by Quaroni et al [1]. IEC-6 cells originate from intestinal crypt cells, as judged by morphological and immunologic criteria. They are non-tumourigenic and retain the undifferentiated character of epithelial stem cells. Tests for mycoplasma were always negative. All chemicals were of the highest purity commercially available.

Cell culture

Cell stocks were maintained in a humidified 37 °C incubator in an atmosphere of 10 % CO_2. The medium consisted of DMEM (Dulbecco's modified Eagle's medium) supplemented with 5 % (v/v) heat-inactivated FBS, 10 μg/ml of insulin and 50 μg/ml of gentamicin sulfate. The stock flask was passaged weekly, fed three times per week, and passages 15–22 were used for experiments. The stock cells were harvested with 0.05 % (v/v) trypsin and 0.53 mM EDTA and counted using a Beckman Coulter Model Z1 Counter. For all experiments, cells were grown for 3 days in control medium (DMEM and 5 % (v/v) dialysed FBS, no DFMO), medium with 5 mM DFMO or medium with 5 mM DFMO plus 10 μM PUT. Cells were fed on day 2 and then serum-starved with control (DMEM without DFMO), DMEM with 5 mM DFMO or 5 mM DFMO plus 10 μM PUT for 24 h on day 3. On day 4, experimental treatments were carried out in serum-free medium (supplemented with DFMO or PUT as required), followed by harvesting. This 4 day pattern was used on the basis of our previous findings that maximal polyamine depletion occurs after 4 days of treatment with 5 mM DFMO [30]. Exogenous PUT added along with DFMO served as a control to indicate that all results were due to the depletion of polyamines and not to the presence of DFMO itself.

RNA extraction, reverse transcription and real-time PCR

Cells were grown and serum-starved as described above, followed by treatment with EGF (10 ng/ml) in the presence and absence of AG1478 (an EGFR inhibitor), U0126 {a MEK [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase] 1/2 inhibitor}, PP2 (a Src kinase inhibitor) or TNFα and CHX in serum-free medium. Total RNA was extracted from the cells using TRIzol following the manufacturer's instructions and quantified by measuring A_{260} and A_{280}. The protocol for real-time PCR was performed using the method of Guo et al. [31]. Briefly, total RNA (1 μg) and random primers (0.5 μg) were used for the reverse transcription reaction, along with Moloney murine leukaemia virus reverse transcriptase, following the manufacturer's instructions. The resulting cDNA was used as a template for real-time PCR. The primers used for p21Cip1 (forward 5′-GCTGTCTTGCACTCTGGTGT-3′, reverse 5′-TTTCTCTTGCAGAAGACCAATC-3′), E47 (forward 5′-CAGACACAGTCTCAGCAGCA-3′, reverse 5′-GGGCTATCACAAGGCTTCTC-3′) and actin (forward 5′-TCTACAATGAGCTGCGTGTG-3′, reverse 5′-GGGGTGTTGAAGGTCTCAAA-3′) were designed with a melting temperature of 59–61 °C and an amplicon size of 50–150 bp. The PCR reaction contained SYBR reagent, diluted cDNA, and 300–900 nM primers in a 50 μl volume. PCR reactions were performed using an ABI Prism 7700 sequence detection system (Applied Biosystems). The conditions for PCR were as follows: enzyme activation at 95 °C for 10 min, denaturing at 95 °C for 15 s, annealing and extension at 60 °C for 1 min, with the denaturation and annealing steps being repeated for 40 cycles. Each sample was normalized on the basis of the transcript numbers of rat β-actin content. Results were finally expressed as N-fold variation in gene expression relative to β-actin as follows:

$$N = 2^{-(\Delta Ct\ \text{sample} - \Delta Ct\ \text{calibrator})}$$

where the *Ct* (threshold cycle value) of sample and calibrator were calculated by subtracting the average *Ct* value of a target gene from the corresponding *Ct* value of β-actin.

Transfection

The protocol for siRNA transfection in IEC-6 cells has been previously described [32,33]. Briefly, 20–80 pmols of E47 siRNA or p21Cip1 siRNA, or 2 μg of 31 kDa pcDNA3.0 plasmid were diluted in 100 μl serum-free medium and incubated with FuGENE™6 for 45 min at room temperature (22–25 °C). IEC-6 cells at passage number 16–17 were grown to 70–80 % confluence in 6-well and 24-well plates. For transfection, the cell monolayer was rinsed with serum-free medium and the DNA– or siRNA–transfection reagent mixture was added drop-wise on to the cell monolayer and incubated for a further 12 h at 37 °C. The medium was replaced with fresh serum-containing medium for 18–24 h, followed by serum starvation for a further 24 h. The cells were either harvested or used for apoptosis studies with TNFα and CHX. In some cases, total RNA or cell lysates were prepared and used for real-time PCR or Western blotting respectively.

Luciferase assay

Transient transfection with full-length p21Cip-1 promoter–luciferase construct (2.3 kb regulatory region upstream of the p21Cip1 gene cloned into the luciferase vector) was performed using the procedure described above. IEC-6 cells were grown in control medium (without DFMO) or 5 mM DFMO for 3 days in 24-well plates, followed by transfection with p21Cip1 plasmid alone or co-transfected with E47 siRNA and the 31 kDa fragment of p130Cas (31 kDa Cas) construct. Following transfection, the cells were incubated for an additional 24 h in control or DFMO-containing medium supplemented with serum. Cells were lysed in the reporter lysis buffer [25 mM Tris/phosphate, pH 7.8, 2 mM DTT (dithiothreitol), 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10 % (v/v) glycerol and 1 % (v/v) Triton X-100] and the luciferase assay was performed using the luciferase assay system, following the manufacturer's instructions. The luciferase reporter activity was expressed as RLU (relative luciferase units) per mg of protein (RLU/min per mg of protein).

Preparation of cytosolic and nuclear extract

Cell fractionation was performed using a nuclear extract kit from Active Motif (Carlsbad, CA, U.S.A.) as described previously [32]. Cells were grown to confluence for 3 days and serum-starved for 24 h, followed by treatment with TNFα and CHX for 3 h. Cells were washed with 5 ml of ice-cold PBS with phosphatase inhibitors and scraped in PBS followed by centrifugation at 700 ***g*** for 5 min at 4 °C. The cell pellet was resuspended in hypotonic buffer and incubated on ice for 15 min. Detergent (25 μl) was added and the sample was vortex-mixed for 10 s. The resulting suspension was centrifuged at 14000 ***g*** for 1 min at 4 °C and the supernatant was removed and retained (cytosolic fraction). The remaining nuclear pellet was lysed in 50 μl of lysis buffer containing 1 mM DTT and protease inhibitor cocktails (1:100 dilution, provided in kit), and incubated for 30 min on ice on a rocking platform. After vortex-mixing for 30 s, the lysate was centrifuged at 14000 ***g*** for 10 min at 4 °C. The resulting supernatant was saved as the nuclear extract. The protein concentration was determined by the BCA (bicinchoninic acid) method and subjected to SDS/PAGE.

Apoptosis studies

The quantitative DNA fragmentation assay was carried out using a cell death detection ELISA kit as described previously [32,33]. Apoptosis was induced by treatment with TNFα (20 ng/ml) and CHX (25 μg/ml) as described earlier [4,6]. Results are expressed as A_{405}/min per mg of protein.

Western blot analysis

Western blots were performed using the method of Bhattacharya et al. [32,33]. Cell lysates were centrifuged at 14000 ***g*** for 10 min at 4 °C, followed by SDS/PAGE. Proteins were transferred overnight on to Immobilon-P membranes (Millipore) and probed using the indicated primary antibodies at 1:1000 dilution overnight at 4 °C in TBS buffer containing 0.1 % (v/v) Tween 20 and 5 % (w/v) non-fat dry milk (Biorad). Membranes were subsequently incubated with either HRP (horseradish peroxidase)-conjugated goat anti-(rabbit IgG) antibody (1:20000 dilution, Sigma) or HRP-conjugated goat anti-(mouse IgG) (1:8000 dilution, Sigma) antibody at room temperature for 1 h and the immunocomplexes were visualized using an ECL detection system (PerkinElmer). The membranes were stripped and re-probed using an anti-actin antibody to determine equal protein loading and for normalization of the blots.

[^{35}S]Methionine incorporation

Incorporation of radiolabelled methionine was performed as described previously [34]. Briefly, cells were grown in control and DFMO-containing medium as required and serum-starved for 24 h. The medium was replaced with serum-free, methionine-free DMEM containing 0.2mCi [^{35}S]methionine and incubated in the presence and absence of TNFα (20 ng/ml) or CHX (25 μg/ml) in a humidified atmosphere of 90 % air and 10 % CO_2. Cells were washed with Dulbecco's PBS to remove radioactive methionine that had not been incorporated, and cells were then lysed in cell lysis buffer [20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1 % (v/v) Triton-X 100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 0.3 μM aprotinin). Cell lysates were clarified by centrifugation at 10000 ***g*** for 10 min at 4 °C and protein content was determined using the BCA method. Equal amounts of protein were precipitated by 2 % (v/v) TCA (tricholoroacetic acid) and radioactivity was counted using a Beckman scintillation counter, with the d.p.m. (disintegration per min) values expressed as a percentage of the control values.

Statistical analysis

All results are means ± S.E.M. Experiments were repeated three times, with triplicate samples for each experiment. ANOVA and appropriate post-hoc testing determined the significance of the differences between means. Values of $P < 0.05$ were regarded as significant.

RESULTS

p21Cip1 protein expression and apoptosis

Increased levels of p21Cip1 were detected in whole-cell extracts and cytoplasmic extracts from polyamine-depleted cells, and this increase in the expression level of p21Cip1 could be prevented by

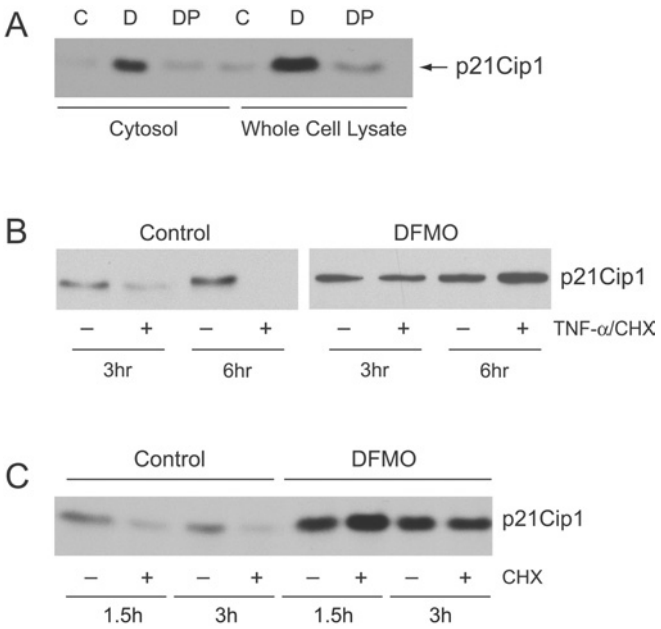


Figure 1 p21Cip1 expression in response to polyamine depletion and TNFα and CHX treatment

IEC-6 cells were grown in control medium (C), medium containing 5 mM DFMO (D) or medium containing 5 mM DFMO plus 10 μM PUT (DP) for 4 days and serum-starved during the final 24 h. Blots are representative of 3 experiments in each case. (**A**) Expression of p21Cip1 protein in whole-cell lysates and cytosolic fractions was determined by Western blot analysis. (**B**) Expression of p21Cip1 protein in whole-cell lysates from IEC-6 cells grown in control medium or DFMO-containing medium after 3 h and 6 h of treatment with TNFα and CHX (TNF-α/CHX). Expression was determined by Western blot analysis. (**C**) Expression of p21Cip1 protein in whole-cell lysates from IEC-6 cells grown in control medium or medium containing DFMO after 1.5 h and 3 h of treatment with 25 μg/ml CHX and expression was determined by Western blot analysis.

the addition of PUT into medium containing DFMO (Figure 1A). The level of actin detected did not change in all three groups (results not shown). Treatment of control cells with TNFα and CHX for 3–6 h resulted in a decrease in p21Cip1 protein levels, but the protein level of p21Cip1 in cells grown in DFMO was unaffected after 3 h of treatment, and had increased after treatment with TNFα and CHX for 6 h (Figure 1B). Since p21Cip1 protein levels in polyamine-depleted cells increased after 6 h of treatment with CHX, we investigated the effect of CHX on [^{35}S]methionine incorporation, which served as a measure of *de novo* translation. CHX blocked [^{35}S]methionine incorporation in control cells from a basal level of 100 ± 2.86 to 10.21 ± 0.40 % (means $\pm$ S.E.M., $n = 3$, $P < 0.05$). Basal level *de novo* protein synthesis was higher in polyamine-depleted cells (122.51 ± 6.93 %), and CHX inhibited [^{35}S]methionine incorporation to a lesser extent than observed in control cells (61.15 ± 7.06 %), indicating decreased efficacy of CHX in protein synthesis. TNFα alone significantly increased [^{35}S]methionine incorporation in both control cells and in polyamine-depleted cells (results not shown). Thus the increase in p21Cip1 protein levels after TNFα and CHX treatment observed in polyamine-depleted cells may be a result of the combined effects of an increase in protein levels by TNFα and a decrease in the effectiveness of CHX.

In untreated control cells, p21Cip1 protein levels increased in a time-dependent manner, whereas they remained unchanged in the DFMO group relative to actin levels. Changes in protein levels which were observed in the DFMO plus PUT-treated cells were similar to those in control cells (results not shown). The increased p21Cip1 protein levels in untreated control cells may be the result of prolonged serum starvation. A time-dependent increase in the amount of p21Cip1 protein was observed in control cells which had been serum-starved for a further 27 or 30 h and then treated with TNFα and CHX compared with cells which were serum-starved for only 24 h before treatment. DFMO-treated cells had higher basal protein levels of p21Cip1, and a marginal increase in p21Cip1 protein levels was detected after prolonged serum starvation (results not shown). Thus the maintenance of higher levels of p21Cip1 protein in polyamine-depleted cells treated with TNFα and CHX may also be the result of decreased protein degradation. To address this, we determined p21Cip1 protein stability using CHX. A sharp decrease in the levels of p21Cip1 was seen within 1.5 h of CHX treatment. The protein was barely detectable at 3 h in control cells when compared with the non-CHX-treated group, whereas in the polyamine-depleted cells, a decrease in p21Cip1 protein levels was less evident after CHX treatment (Figure 1C), indicating an increase in protein stability in the polyamine-depleted cells compared with the control cells.

As determined by real-time PCR, p21Cip1 mRNA levels were increased significantly in cells grown in the presence of DFMO (2.14 ± 0.036, means $\pm$ S.E.M., $n = 3$, $P < 0.05$) compared with control cells (1.0 ± 0.081), after mRNA levels were normalized to actin mRNA levels. Treatment for 3 h with CHX did not alter p21Cip1 mRNA levels compared with the respective untreated groups. TNFα and CHX significantly increased p21Cip1 mRNA levels within 1.5 h in cells which were cultured in control conditions (3.87 ± 0.11) compared with a significant decrease after 3 h (2.24 ± 0.08, $P < 0.05$). Cells grown in the presence of DFMO showed a significant increase in p21Cip1 mRNA levels when treated with TNFα and CHX for 1.5 h (3.33 ± 0.033, $P < 0.05$) and the mRNA level remained elevated after 3 h of treatment (3.43 ± 0.055).

We used siRNA to knockdown p21Cip1 expression and examined the effect on DNA fragmentation. p21Cip1-specific siRNA, but not control siRNA, reduced the levels of p21Cip1 protein (Figure 2A, inset). Actin was used as a loading control and was not affected (results not shown). Basal levels of apoptosis in cells transfected with control siRNA did not change when compared with cells transfected with p21Cip1 siRNA. Treatment of cells with p21Cip1 siRNA resulted in the down-regulation of protein expression and significantly increased TNFα and CHX-induced apoptosis compared with control siRNA-transfected cells (Figure 2A). These results suggest that p21Cip1 regulates TNFα- and CHX-induced apoptosis, and indicate that increased levels of p21Cip1 in response to polyamine depletion might result in decreased sensitivity of these cells to apoptosis.

Caspase 3-mediated cleavage of p21Cip1 has been established as a requisite for apoptotic cell death in various cell lines [28,29]. It has been previously demonstrated that there is a robust increase in caspase 3 activation and apoptosis after treatment of cells with TNFα and CHX for 3–6 h, which does not occur in polyamine-depleted cells [4]. We predicted that caspase 3-mediated cleavage of p21Cip1 might influence the rate of apoptosis in these cell lines. We first examined whether purified active caspase 3 can cleave p21Cip1 *in vitro*. p21Cip1 was immunoprecipitated from both control and DFMO-treated cells, incubated with either 1 unit or 2 units of purified active caspase 3 for 30 min at 37 °C and the level of intact p21Cip1 protein was determined. Intact p21Cip1 from control cells was barely detectable in samples treated with 1 unit of active caspase 3, and was completely cleaved after treatment with 2 units of active caspase 3 (Figure 2B). The cleavage of p21Cip1 protein from polyamine-depleted cells was less evident in samples incubated with 1 unit of the active caspase 3. However, a 2-fold increase in the concentration was sufficient to cleave p21Cip1

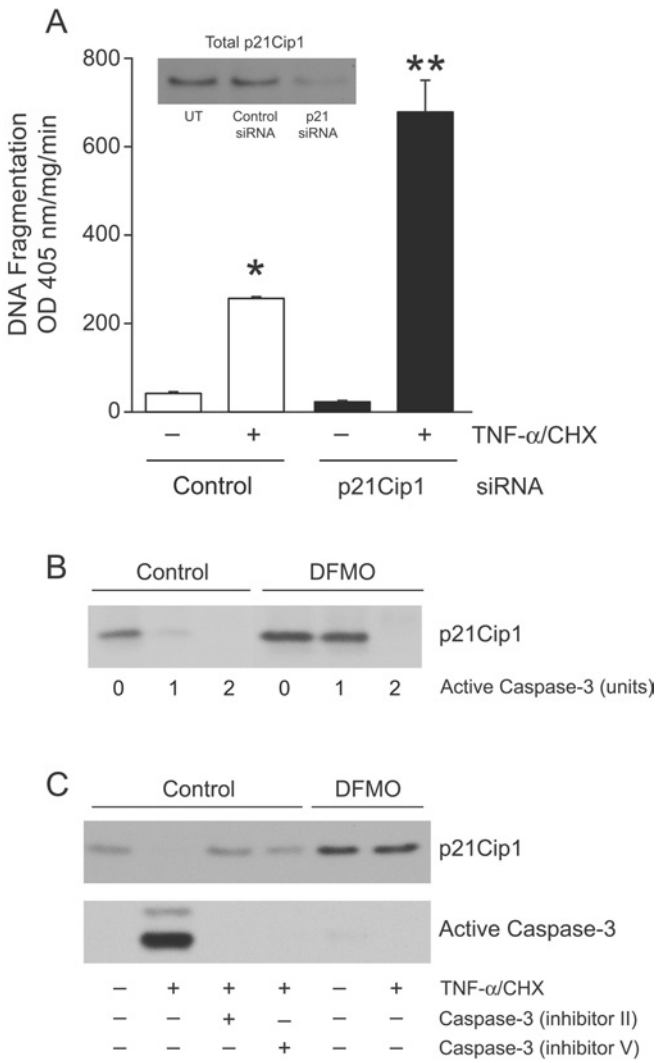


Figure 2 Expression and cleavage of p21Cip1 during apoptosis

(**A**) IEC-6 cells transfected with control or p21Cip1-specific siRNA were serum-starved for 24 h followed by treatment with TNFα and CHX for 3 h. DNA fragmentation was measured by ELISA. Results are means $\pm$ S.E.M., $n = 3$. *, significantly different ($P < 0.05$) from untreated cells transfected with control siRNA and **, significantly different ($P < 0.05$) from control cells transfected with control siRNA and treated with TNFα and CHX (TNF-α/CHX). Inset shows expression of p21Cip1 protein in whole-cell lysates from cells transfected with control siRNA or p21Cip1 siRNA. Presence of protein was determined by Western blot analysis and compared with untransfected controls (UT). (**B**) IEC-6 cells were grown in control medium or in medium containing 5 mM DFMO for 4 days and serum-starved during the final 24 h. Whole-cell lysates were subjected to immunoprecipitation using an anti-p21Cip1 antibody and immunoprecipitates were incubated with 1 and 2 units of recombinant active caspase 3 for 30 min at 30 °C, washed three times with lysis buffer, subjected to SDS/PAGE and the membrane probed with an anti-p21Cip1 antibody. (**C**) IEC-6 cells were grown in control medium or medium containing 5 mM DFMO for 4 days and serum-starved during the final 24 h. Controls were treated with TNFα and CHX (TNF-α/CHX) for 3 h in the presence and absence of caspase 3 inhibitor II (25 μM z-DEVD-FMK) and caspase 3 inhibitor V (25 μM z-DQMD-FMK). DFMO-treated cells were simultaneously treated with TNFα and CHX or left untreated. Expression of p21Cip1 and active caspase 3 proteins was determined by Western blot analysis.

from DFMO-treated cells comparable to that from control cells (Figure 2B).

In order to determine whether caspase 3 activation has a possible consequence for p21Cip1 protein levels *in vivo*, we treated control and polyamine-depleted cells with TNFα and CHX for 3 h and compared their responses to treatment with two different caspase 3 inhibitors. TNFα and CHX treatment increased caspase

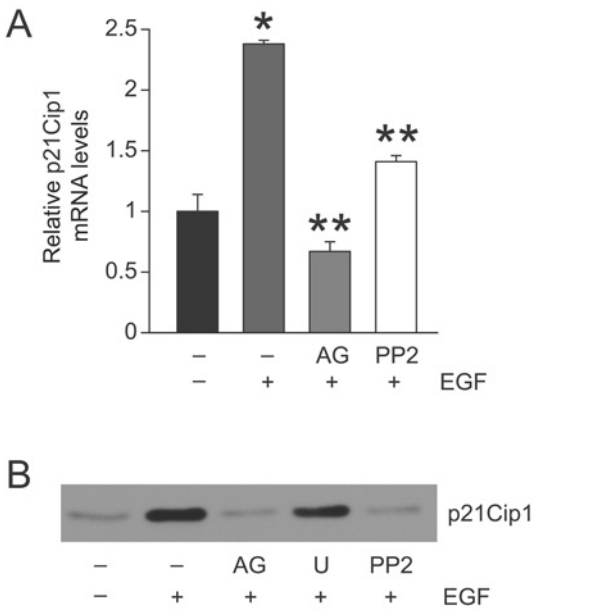


Figure 3 EGF-mediated p21Cip1 expression rescues cells from apoptosis

(**A**) IEC-6 cells were grown for 4 days and serum-starved during the final 24 h. Cells then were incubated at 37 °C in serum-free medium with and without 10 μM AG1478 (AG) or 20 μM PP2 for 2 h, followed by the addition of EGF (10 ng/ml) for 1 h. RNA was prepared and real-time PCR for p21Cip1 mRNA was performed. Results are means $\pm$ S.E.M., $n = 3$. *, significantly different ($P < 0.05$) from untreated cells and **, significantly different ($P < 0.05$) from EGF-treated cells. (**B**) IEC-6 cells were grown for 4 days and serum-starved during the final 24 h. Cells were then incubated at 37 °C in serum-free medium with or without 10 μM AG1478 (AG), 20 μM PP2 or 10 μM U0126 (U) for 2 h, followed by the addition of EGF (10 ng/ml) for 1 h. Expression of p21Cip1 protein in whole-cell lysates was determined by Western blot analysis.

3 activation in control cells with a concomitant decrease in p21Cip1 protein levels (Figure 2C). Consistent with our previous observations, polyamine depletion inhibited caspase 3 activation, and sustained levels of p21Cip1 were observed before and during TNFα- and CHX-induced apoptosis (Figure 2C). Cell-permeable and irreversible caspase 3 inhibitors, inhibitors II (z-DEVD-FMK) and V (z-DQMD-FMK), both blocked caspase 3 activation and prevented p21Cip1 protein cleavage in control cells, indicating a role for caspase 3 in decreasing p21Cip1 protein levels during apoptosis (Figure 2C).

EGF-mediated p21Cip1 expression and apoptosis

Polyamine depletion protects cells against apoptosis via the Src and MEK/ERK pathways [2,6]. EGF (10 ng/ml) increased p21Cip1 mRNA and protein levels in confluent serum-starved cells (Figure 3). EGF treatment also significantly decreased TNFα- and CHX-induced apoptosis (as determined by DNA fragmentation) from 158.8 ± 16.3 to 63.1 ± 3.8 (means $\pm$ S.E.M., $n = 3$, $P < 0.05$), and caused a similar decrease in the basal level of apoptosis from 13 ± 0.7 to 3.0 ± 0.7 ($P < 0.05$) within 3 h. Treatment of cells with 10 μM AG1478, a potent EGFR-kinase inhibitor for 30 min prior to TNFα and CHX treatment and for 3 h during TNFα and CHX treatment completely abolished the EGF-mediated increase in p21Cip1 mRNA and protein levels (Figure 3) and significantly increased basal levels of apoptosis from 13 ± 0.7 to 106.5 ± 8.6 ($P < 0.05$) and TNFα- and CHX-induced apoptosis from 158.8 ± 16.3 to 220 ± 20.4 ($P < 0.05$). PP2 (20 μM), a pharmacological inhibitor of Src kinase, blocked the increase in p21Cip1 mRNA and

protein levels mediated by EGF (Figure 3), and significantly increased DNA fragmentation from 13 ± 0.7 to 154 ± 10.4 ($P < 0.05$) in untreated cells and in TNFα- and CHX-treated cells from 158.8 ± 16.3 to 268.6 ± 30.6 ($P < 0.05$). However, inhibition of MEK1/2 by U0126 decreased the EGF-mediated increase in p21Cip1 protein levels to a lesser extent than AG1478 or PP2 (Figure 3B). Actin was used as a loading control and was unaffected (results not shown). Our results indicate a role for EGFR and Src kinase in the regulation of p21Cip1 expression in IEC-6 cells, and a partial role for MEK1 in this regulation is also suggested. An anti-apoptotic role for p21Cip1 is implied from these results.

E47 and p21Cip1 gene expression

Promoter sequences of p21Cip1, p15INK4B and p16INK4BE2A contain specific E-box sequences [16], and E47 is known to stimulate endogenous p21Cip1 expression in HEK 293T cells [human embryonic kidney cells expressing the large T-antigen of SV40 (simian virus 40)] [15]. To determine whether p21Cip1 could be a potential effector target of E2A transcription factors, we transfected IEC-6 cells with control and E47-specific siRNA and studied the effects on the expression of p21Cip1 mRNA and protein. E47-specific siRNA significantly down-regulated E47 protein levels compared with control siRNA (Figure 4A, inset), which was accompanied by a 3-fold increase in p21Cip1 mRNA levels (Figure 4A). Down-regulation of E47 increased p21Cip1 protein expression compared with untransfected and control siRNA-transfected cells (Figure 4B, inset). Actin was used as an internal loading control and did not change (results not shown). Since the increase in p21Cip1 protein levels correlated with a decrease in apoptosis, we investigated whether E47-siRNA transfection conferred protection from apoptosis. TNFα and CHX-induced DNA fragmentation was significantly increased in cells transfected with control siRNA (Figure 4B), whereas E47 siRNA greatly reduced TNFα- and CHX-induced and basal levels of DNA fragmentation (Figure 4B), providing further confirmation of the role of E47-mediated p21Cip1 expression in apoptosis.

Since E47 has been shown to negatively regulate p21Cip1 transcription, and polyamine depletion increased p21Cip1 transcription, we determined the E47 mRNA level in DFMO-treated cells. Following 4 days of DFMO treatment, E47 mRNA levels were significantly decreased when compared with control cells (Figure 4C). TNFα and CHX treatment increased E47 mRNA in control cells, but had no significant effect in polyamine-depleted cells (Figure 4C). These results provide evidence that polyamine depletion inhibits E47 transcription, which contributes to a sustained increase in p21Cip1 at both the mRNA transcript and protein levels. Conversely, an increase in the transcription of E47 may be involved in the inhibition of p21Cip1 transcription during apoptosis in control cells.

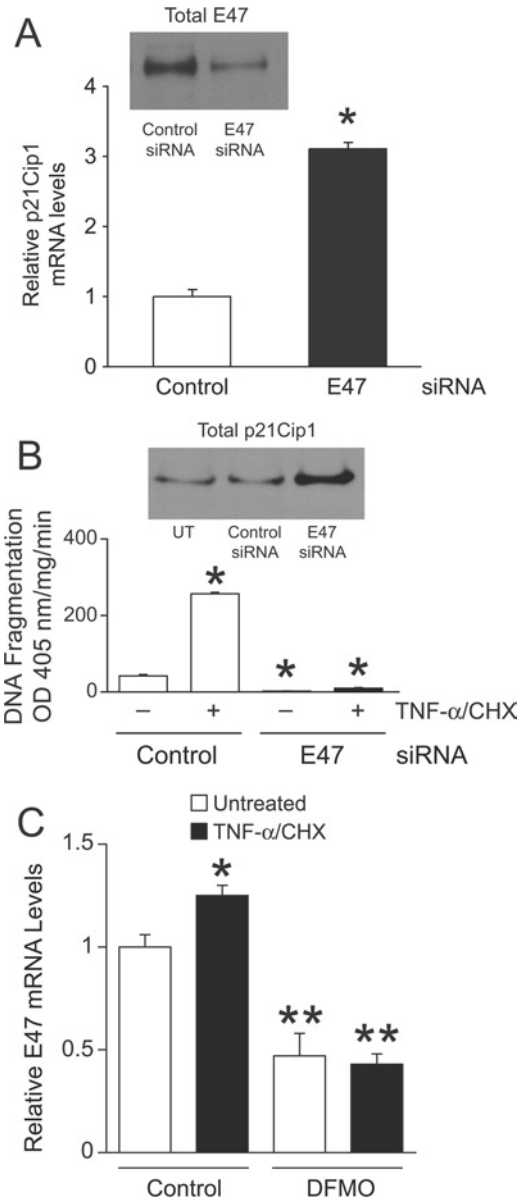


Figure 4 Knockdown of E47 increases p21Cip1 expression and protects cells from apoptosis

(**A**) IEC-6 cells were transfected with control or E47-specific siRNA, RNA was isolated and real-time PCR for p21Cip1 mRNA was performed. Results are means $\pm$ S.E.M., $n = 6$. *, significantly different ($P < 0.05$) from control siRNA-treated cells. Inset shows expression of E47 protein in whole-cell lysates as determined by Western blot analysis. Whole-cell lysates were separated by SDS/PAGE and immunoblotted with an anti-E47 antibody. (**B**) IEC-6 cells transfected with control or E47-specific siRNA were serum-starved for 24 h followed by treatment with TNFα and CHX (TNF-α/CHX) for 3 h. DNA fragmentation was measured by ELISA and results are means $\pm$ S.E.M., $n = 3$. *, significantly different ($P < 0.05$) from untreated cells transfected with control siRNA. Inset shows expression of p21Cip1 protein in whole-cell lysates from cells transfected with control siRNA or E47 siRNA. Expression was determined by Western blot analysis and compared with untransfected controls (UT). (**C**) IEC-6 cells were grown in control medium or 5 mM DFMO-containing medium for 4 days and serum-starved during the final 24 h, followed by treatment with TNFα and CHX (TNF-α/CHX) for 3 h. RNA was then isolated and real-time PCR for E47mRNA was performed. Results are means $\pm$ S.E.M., $n = 3$. *, significantly different ($P < 0.05$) from untreated control and **, significantly different ($P < 0.05$) from untreated and TNFα- and CHX-treated control.

Caspase 3-mediated cleavage of p130Cas during TNFα- and CHX-induced apoptosis

Sustained phosphorylation of Src, FAK (focal adhesion kinase) and p130Cas is critical for the transmission of survival signals to downstream effectors. Dephosphorylation of p130Cas followed by proteolytic cleavage during etoposide-induced apoptosis in Rat-1 fibroblasts has been shown to disrupt survival signals [23]. Previous studies from our group showed that following TNFα and CHX treatment, decreased levels of phosphorylation of Src (at Tyr^{416}) and Akt (at Ser^{473}) was observed, along with concomitant increases in caspase 3 activation and apoptosis [6,32]. We compared p130Cas phosphorylation during apoptosis in control cells and in polyamine-depleted cells. In control cells, TNFα and CHX decreased the levels of p130Cas (phosphorylated at Tyr^{410}) and total p130Cas protein in a time-dependent manner (Figure 5). The 31 kDa Cas

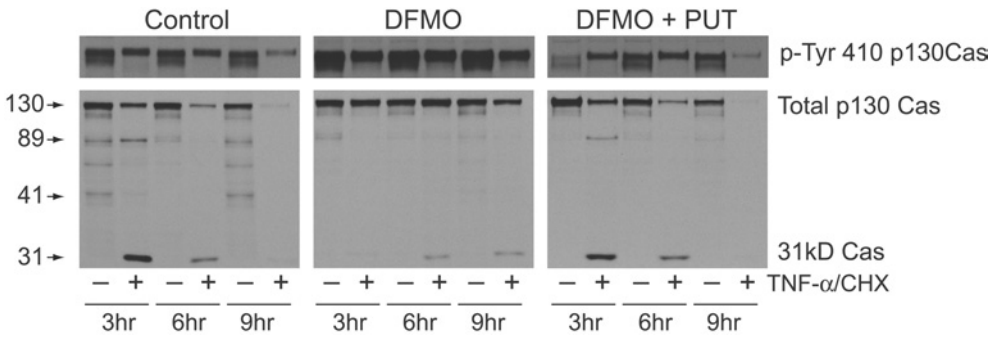


Figure 5 Polyamine depletion activates p130Cas phosphorylation and blocks cleavage during TNFα-induced apoptosis

IEC-6 cells were grown in control medium, medium containing 5 mM DFMO, or medium containing DFMO plus 10 μM PUT for 4 days and serum-starved during the final 24 h. Expression of phosphorylated p130Cas protein was determined in whole-cell lysates after 3, 6 and 9 h of treatment with TNFα and CHX (TNF-α/CHX). Expression was determined by Western blot analysis using an anti-[p130Cas (phospho-Tyr^{410})] antibody. Expression of total p130Cas protein was then determined by stripping the blots and reprobing with an anti-p130Cas antibody that recognized p130Cas protein and the proteolytically cleaved 31 kDa fragment (31 kD Cas).

form was abundant at 3 h and then gradually declined with time, which correlated with a decrease in the total p130Cas protein levels (Figure 5). Polyamine depletion increased p130Cas phosphorylation, which was maintained throughout the entire time-course following TNFα and CHX treatment. Unlike control cells, p130Cas protein levels did not change significantly in DFMO-treated cells during 3–6 h of treatment with TNFα and CHX, and the 31 kDa Cas form was undetectable at 3 h and barely detectable thereafter (Figure 5). Levels of p130Cas phosphorylation (at Tyr^{410}) and p130Cas protein in cells grown with DFMO plus PUT were similar to those in control cells (Figure 5). Actin was used as a loading control and was not affected (results not shown).

Inhibition of Src prevents p130Cas activation and p21Cip1 transcription

SFKs (Src family kinases) bind to the C-terminus of p130Cas and phosphorylate the substrate domain [35]. Our previous studies showed that integrin β3-mediated Src activation is essential for the survival of polyamine-depleted cells [6]. In the present study, TNFα and CHX increased caspase 3 activity which correlated with a decrease in p130Cas phosphorylation (at Tyr^{410}) and loss of p130Cas protein, and was associated with increased levels of the 31 kDa Cas form in both control and DFMO plus PUT-treated cells (Figure 6A). PP2 further decreased the amount of p130Cas phosphorylation and increased caspase 3 activation, leading to the accumulation of the 31 kDa Cas form in control cells (Figure 6A). We have previously shown that PP2 can significantly increase DNA fragmentation (a measure of apoptosis) in control and polyamine-depleted cells in response to TNFα and CHX treatment [6]. In control cells, PP2 alone induced caspase 3 activation, which in turn mediated p130Cas cleavage similarly to that observed for TNFα- and CHX-induced responses. Polyamine depletion prevented caspase 3 activation and the degradation of p130Cas protein in response to TNFα and CHX. Inhibition of Src completely blocked the p130Cas Tyr^{410} phosphorylation normally seen in polyamine-depleted cells, and induced its cleavage (Figure 6A). Cells grown in the presence of DFMO plus PUT had lower levels of p130Cas phosphorylation, and in these cells TNFα and CHX and PP2 produced responses that were identical with those in control cells.

Since inhibition of Src kinase increased p130Cas cleavage and sensitized polyamine-depleted cells to apoptosis, we measured p21Cip1 transcription by quantitative real-time PCR following

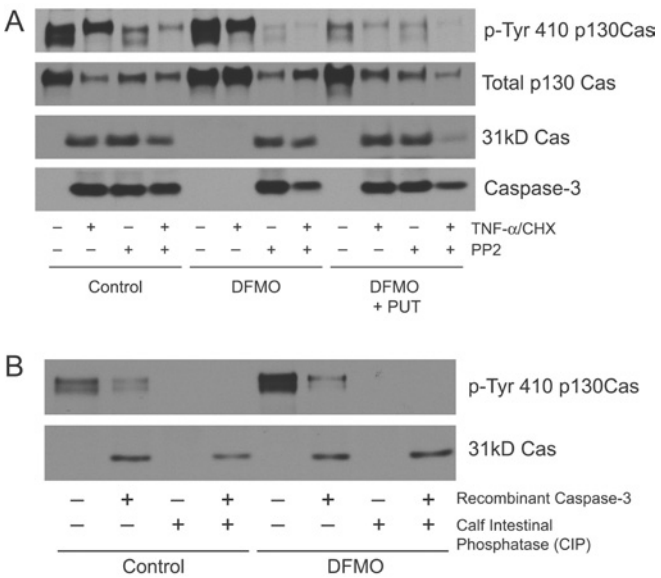


Figure 6 Inhibition of Src kinase with PP2 activates caspase 3, blocks p130Cas Tyr^{410} phosphorylation, generates 31 kDa Cas fragment and inhibits p21Cip1 transcription

(**A**) IEC-6 cells were grown in control medium, medium containing 5 mM DFMO, or medium containing DFMO plus 10 μM PUT for 4 days and serum-starved during the final 24 h. Cells were then pre-treated with 20 μM PP2 for 3 h, followed by 3 h of treatment with TNFα and CHX (TNF-α/CHX). Expression of phosphorylated p130Cas protein in whole-cell lysates was determined by Western blot analysis using p130Cas phospho-Tyr^{410} antibody. To determine the total p130Cas protein expressed, the blots were stripped and reprobed with an anti-p130Cas antibody that recognized p130Cas protein and the proteolytically cleaved 31 kDa fragment (31 kD Cas). Expression of active caspase 3 in these same lysates was also determined by Western blot analysis. (**B**) IEC-6 cells were grown in control medium or medium containing 5 mM DFMO for 4 days and serum-starved during the final 24 h. Whole-cell lysates were subjected to immunoprecipitation using an anti-p130Cas antibody, and immunoprecipitates were incubated with either 1 unit of recombinant caspase 3 for 30 min at 30 °C or treated with 5 units of CIP for 30 min, followed by incubation with active caspase 3. The immunoprecipitates were washed three times with lysis buffer, subjected to SDS/PAGE, and the membrane was probed with p130Cas phospho-Tyr^{410} antibody. Expression of cleaved 31 kDa Cas was then determined by stripping the blots and reprobing with an anti-p130Cas antibody that recognizes the proteolytically cleaved 31 kDa (31kD Cas) fragment.

PP2 treatment. Consistent with our observations in Figure 1, polyamine depletion increased p21Cip1 transcription (control: 1.0 ± 0.13; DFMO: 1.5 ± 0.06; means $\pm$ S.E.M., $n = 6$, $P < 0.05$)

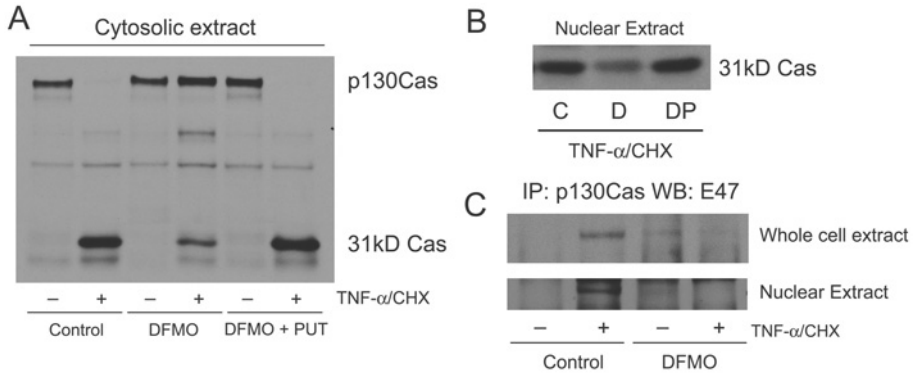


Figure 7 31 kDa Cas translocates to the nucleus and associates with E47

(**A**) IEC-6 cells were grown in control medium, medium with 5 mM DFMO, or medium with DFMO plus 10 μM PUT for 4 days and serum-starved during the final 24 h, followed by treatment with TNFα and CHX (TNF-α/CHX) for 3 h. Cells were fractionated, and cytosolic extracts were prepared as described in the Experimental section. Expression of total p130Cas protein in the cytosolic fraction was determined by Western blot analysis using an anti-p130Cas antibody that recognizes p130Cas protein and the proteolytically cleaved 31 kDa (31kD Cas) fragment. (**B**) IEC-6 cells were grown in control medium (C), medium containing 5 mM DFMO (D), or medium containing DFMO plus 10 μM PUT (DP) for 4 days and serum-starved during the final 24 h, followed by treatment with TNFα and CHX (TNF-α/CHX) for 3 h. Cells were fractionated and nuclear extracts were prepared as described in the Experimental section. Expression of total 31 kDa Cas protein in the nuclear fraction was determined by Western blot analysis using a p130Cas antibody that recognizes the proteolytically cleaved 31 kDa fragment. The blot is representative of 3 experiments. (**C**) IEC-6 cells were grown in control medium or medium containing 5 mM DFMO for 4 days and serum-starved during the final 24 h followed by treatment with TNFα and CHX (TNF-α/CHX) for 3 h. Whole-cell and nuclear extracts were immunoprecipitated with an anti-p130Cas antibody and the immunoprecipitates were subjected to SDS/PAGE. The membrane was probed with an anti-E47 antibody.

and PP2 significantly blocked p21Cip1 transcription in control and polyamine-depleted cells (control + PP2: 0.05 ± 0.01; DFMO + PP2: 0.07 ± 0.01; $P < 0.05$). These results indicate that Src-mediated signalling regulates p21Cip1 transcription and may involve p130Cas Tyr^{410} phosphorylation during apoptosis.

Amino acid analysis of p130Cas has identified several caspase cleavage sites [23,35]. In order to examine the phosphorylation dependent cleavage of p130Cas by caspase 3, we immunoprecipitated p130Cas from control and polyamine-depleted cells and treated the immunoprecipitates with purified recombinant active caspase 3 for 30 min at 37 °C. Simultaneously, p130Cas immunoprecipitates were subjected to treatment with CIP, followed by incubation with active caspase 3 to determine p130Cas cleavage. *In vitro* cleavage by active caspase 3 generated the 31 kDa Cas form in both control cells and those grown in DFMO (Figure 6B). Pre-treatment with CIP completely dephosphorylated p130Cas, but its susceptibility to proteolytic degradation by active caspase 3 was largely unaltered (Figure 6B). Our results indicate that polyamine depletion inhibits caspase 3 activation and prevents degradation of p130Cas protein, which in turn blocks generation of the 31 kDa Cas and apoptosis.

31 kDa cleaved fragment of p130Cas associates with nuclear E47 during apoptosis

Previous studies have shown that p130Cas is cleaved by active caspase 3 during apoptosis in various cell lines [23,36]. In order to determine the subcellular localization of 31 kDa Cas during apoptosis, we analysed cytosolic and nuclear extracts using an anti-p130Cas antibody that specifically recognizes the cleaved fragment. During apoptosis, cytosolic p130Cas protein was degraded and generated the 31 kDa Cas fragment in control cells (Figure 7A). Cleavage of p130Cas was significantly reduced in polyamine-depleted cells. Exogenous addition of PUT with DFMO restored cleavage of p130Cas. Figure 7(B) showed that the nuclear extract from TNFα- and CHX-treated cells grown in the presence of DFMO had lower amounts of 31 kDa Cas when compared with either control cells or DFMO + PUT-treated cells.

Since the 31 kDa Cas fragment generated in the cytosol during apoptosis is eventually localized to the nucleus, this fragement was tested to see if it could interact with E47. Cell fractionation studies indicated that E47 was localized to the nucleus in both control and polyamine-depleted cells (results not shown). Whole-cell lysates and nuclear extracts obtained from TNFα- and CHX-treated control and polyamine-depleted cells were subjected to immunoprecipitation using an anti-p130Cas antibody and analysed for the presence of E47. Bound E47 was undetectable in immune complexes from untreated control and polyamine-depleted cells in whole-cell lysates, as well as in immunocomplexes derived from nuclear extracts. However, E47 was found to associate with p130Cas in control cells treated with TNFα and CHX (Figure 7C) in both whole-cell and nuclear extracts, indicating their association during apoptosis, which was not observed in polyamine-depleted cells.

Ectopic expression of 31 kDa Cas blocks p21Cip1 expression and sensitizes cells to apoptosis

Since E47 was found to interact with the 31 kDa Cas fragment during apoptosis, we examined p21Cip1 protein levels following the transient expression of a recombinant 31 kDa-Cas fragment in IEC-6 cells. E47 siRNA and 31 kDa Cas were transfected separately or were co-transfected, and p21Cip1 protein levels were determined. The inset in Figure 8(A) shows that inhibition of E47 protein with a specific siRNA increased p21Cip1 protein levels, which is consistent with the result that p21Cip1 mRNA levels are increased in the presence of E47 siRNA (Figure 4A). Expression of the 31 kDa Cas fragment alone and co-transfection of E47 siRNA plus the 31 kDa Cas fragment completely prevented the expression of p21Cip1 (Figure 8A). These results indicate that 31 kDa Cas can also interact with nuclear E47 and inhibit p21Cip1 expression.

To further define the role of E47 and 31 kDa Cas in p21Cip1 gene transcription, we used a p21Cip1 promoter–luciferase construct. Transfection of E47 siRNA into cells expressing the p21Cip1 promoter–luciferase construct increased p21Cip1

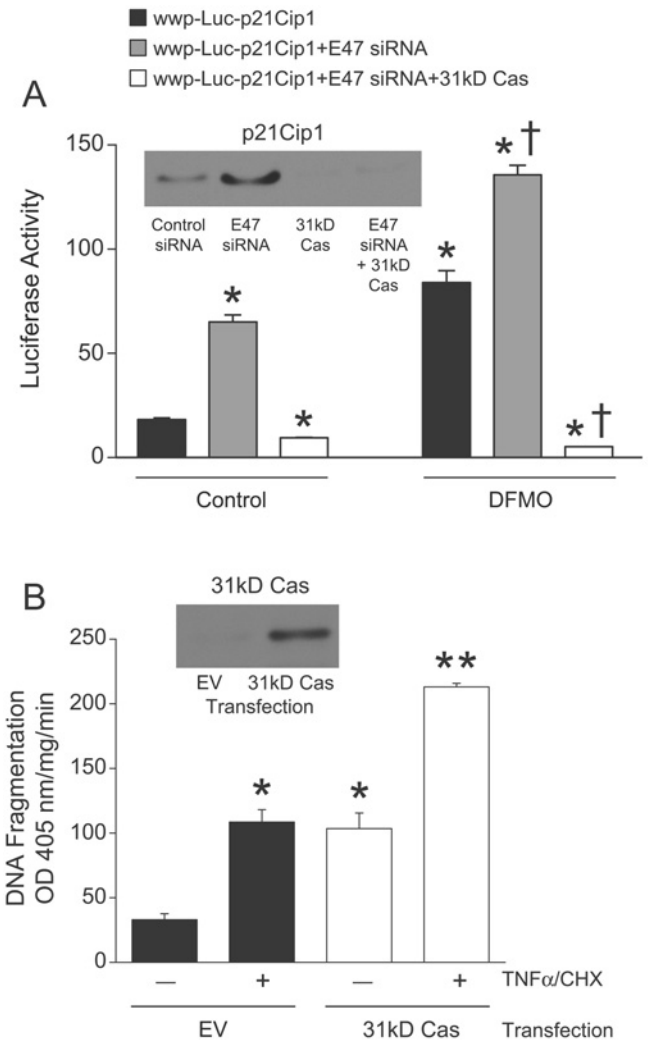
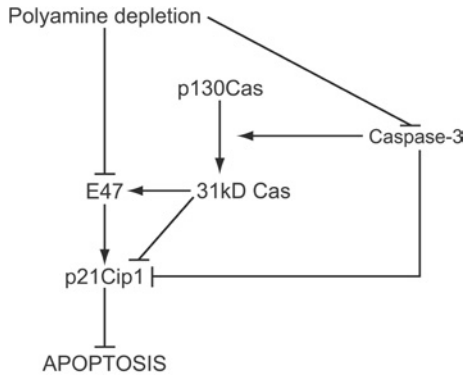


Figure 8 31 kDa Cas inhibits p21Cip1 expression

(**A**) IEC-6 cells grown in control medium or DFMO-containing medium were transfected with full-length p21Cip1 promoter (wwp-Luc-p21Cip1) alone or co-transfected with E47 siRNA or 31 kDa-Cas-pcDNA3.0 construct (31kD Cas). Cells were harvested and assayed for luciferase activity using the Promega luciferase assay kit. Values are expressed as RLU/min per mg of protein. Results are means ± S.E.M., $n = 6$. *, significantly different ($P < 0.05$) from untreated control wwp-Luc-p21Cip1 and †, significantly different ($P < 0.05$) from DFMO and wwp-Luc-p21Cip1. Inset shows expression of p21Cip1 protein in whole-cell lysates from transfected cells as determined by Western blot analysis. (**B**) IEC-6 cells were transiently transfected with empty vector (EV) or 31 kDa Cas (31kD Cas) and treated with TNFα and CHX (TNF-α/CHX) for 3 h. DNA fragmentation was assayed by using the ELISA kit from Roche. Results are means ± S.E.M., $n = 3$. *, significantly different ($P < 0.05$) from untreated EV and **, significantly different ($P < 0.05$) from cells expressing EV and treated with TNFα and CHX. Inset shows expression of 31 kDa Cas (31kD Cas) in whole-cell lysates from transfected cells, as determined by Western blot analysis.

promoter-activation in control cells, demonstrated by an increase in promoter–luciferase reporter activity. Transfection of the 31 kDa Cas form also blocked p21Cip1 reporter gene expression in the absence of E47 protein (Figure 8A). Polyamine depletion increased the basal p21Cip1 promoter–luciferase activation compared with control cells. E47 siRNA transfection further increased p21Cip1 promoter activitation in polyamine-depleted cells, whereas 31 kDa Cas transfection completely repressed p21Cip1 promoter activation (Figure 8A) in cells transfected with E47 siRNA. Thus our results suggest that the 31 kDa Cas form



Scheme 1 Schematic representation of p21Cip1 gene expression and its control in the apoptotic signalling pathway in polyamine-depleted IEC-6 cells

Polyamine depletion inhibits E47 transcription which in turn increases p21Cip1 gene expression and protects IEC-6 cells from TNFα- and CHX-mediated apoptosis. Polyamine depletion also decreases caspase 3 activity, sustaining the phosphorylation of p130Cas and preventing the cleavage of p130Cas, which generates 31 kDa Cas. The 31 kDa Cas inhibits p21Cip1 expression and sensitizes cells to apoptosis. (↓ and ⊥ refer to activation and inhibition respectively).

can repress p21Cip1 promoter activation, both in the presence and absence of E47.

Since generation of the 31 kDa Cas form through caspase 3-mediated cleavage of p130Cas leads to the progression of apoptosis via inhibition of p21Cip1 transcription, we examined whether expression of recombinant 31 kDa Cas protein is sufficient to trigger basal apoptosis. Transient transfection showed a robust expression of 31 kDa Cas protein in these cells (Figure 8B, inset). Actin was used as a loading control and did not change (results not shown). Expression of 31 kDa Cas increased both basal apoptosis and TNFα- and CHX-induced apoptosis (Figure 8B). These results suggest that cleavage of p130Cas is required for the execution of apoptosis in IEC-6 cells, and that the cleaved fragment promotes cell death through the inhibition of p21Cip1 gene transcription (Scheme 1).

DISCUSSION

Apoptosis is an essential process in the regulation of cell numbers in proliferating tissues. Our results and reports from other laboratories have shown that polyamine-depleted cells are resistant to apoptosis [3–6,37]. We have previously shown that polyamine depletion increases p21Cip1 levels and arrests cells in the G_1 phase of the cell cycle by inhibiting CDK2 activity, and these changes could be prevented by the addition of exogenous PUT. Increasing evidence indicates that p21Cip1 is a major regulator of apoptosis, apart from being a master regulator of cell proliferation and the cell cycle [10]. Interestingly, increased p21Cip1 protein levels were observed in the cytoplasmic fraction of polyamine-depleted cells (Figure 1A). p21Cip1 interacts with the N-terminus of caspase 3 and suppresses its activation by masking the serine proteinase cleavage site [10–13]. Akt phosphorylates p21Cip1 at Thr^{145}, which causes relocalization of p21Cip1 to the cytoplasm where it complexes with apoptosis signal-regulating kinase 1 and inhibits cell death induced by TNFα [10,38]. The CDK inhibitor p21Cip1 behaves as a cell cycle inhibitor when localized to the nucleus, and cytoplasmic localization of the protein increases resistance to apoptosis [38,39]. These reports suggest that p21Cip1 performs an anti-apoptotic role in the cytoplasm. Cytoplasmic localization of p27 and p21Cip1 has been documented in breast cancer cells

and shown to be correlated with drug resistance and decreased sensitivity to TNFα-induced apoptosis [39].

In the present study, we provide evidence that up-regulation of p21Cip1, mediated by polyamine depletion, is responsible for protection against TNFα- and CHX-induced apoptosis. We show that polyamine depletion significantly increased transcription of the p21Cip1 gene, resulting in higher levels of protein (Figures 1A and 1C). Although TNFα and CHX increased p21Cip1 mRNA expression after incubation for 1.5 h, the translation of p21Cip1 mRNA was inhibited by CHX. Levels of p21Cip1 protein started to decline after 3 h of TNFα and CHX treatment. Kim et al. [23] have reported that p21Cip1 protein levels increased markedly at the onset of etoposide-induced apoptosis, but gradually declined within the following 24 h. TNFα- and CHX-induced apoptosis is accompanied by increases in the activation of caspases 6, 9 and 3 in control cells, and all of these effects are significantly delayed in polyamine-depleted cells [3,4]. Thus decreased p21Cip1 protein levels during TNFα and CHX treatment in control cells and sustained higher levels in DFMO-treated cells (Figure 1B) correlates with the induction of apoptosis as reported in other studies [3,4,6,32,33]. The observed increase in the levels of p21Cip1 in untreated control cells was the result of prolonged serum starvation. CHX treatment rapidly diminished levels of p21Cip1 within 3 h of TNFα and CHX treatment in control cells, but had no effect on the levels of p21Cip1 in polyamine-depleted cells (Figure 1C), indicating that polyamine depletion increases p21Cip1 protein stability. Moreover, p21Cip1 protein levels increased in polyamine-depleted cells between 3 h and 6 h incubation with CHX, owing to the combined effects of increased protein translation mediated by TNFα and decreased effectiveness of CHX-mediated inhibition of translation.

Since TNFα and CHX increased caspase 3 activity [3,4,6], and a wide variety of cellular proteins, including p21Cip1, are cleaved by active caspase 3 [28,29], we speculate that decreased p21Cip1 protein levels during TNFα and CHX treatment may be a result of caspase 3-mediated cleavage. Figures 2(B) and 2(C) clearly show that p21Cip1 is a substrate for active caspase 3, both *in vitro* and *in vivo*. These results suggest that increased levels of p21Cip1 in polyamine-depleted cells during TNFα- and CHX-induced apoptosis is caused by increased expression and stability of p21Cip1 and decreased caspase 3-mediated cleavage when compared with non-induced cells. These results demonstrate that a clear relationship exists between p21Cip1 levels and apoptosis.

We previously reported that DFMO significantly protects cells against TNFα- and CHX-induced apoptosis by activating MEK/ERK and Src/JAK/STAT/Akt pathways, which are also activated in response to growth factors [2,6]. Therefore EGF might modulate p21Cip1 levels in a similar manner to DFMO. In this study, EGF significantly increased p21Cip1 mRNA and protein levels, protected cells from TNFα- and CHX-induced apoptosis and also decreased basal apoptosis. Inhibition of EGFR and Src kinases completely decreased the expression of p21Cip1 mRNA and protein (Figure 3). However, inhibition of MEK1/2 had a partial effect on p21Cip1 expression (Figure 3B). Knockdown of p21Cip1 by siRNA significantly increased TNFα- and CHX-induced apoptosis (Figure 2A). This leads us to believe that EGFR-mediated activation of Src plays a crucial role in regulating p21Cip1 expression and provides protection against apoptosis. However, the mechanism by which polyamines regulate these processes is unknown.

The family of E2A transcription factors up-regulates p21Cip1 transcription through three conserved E-box elements in the p21Cip1 promoter [15,16]. Our results show that E47 acts as a repressor of p21Cip1 transcription. Down-regulation of E47 protein by E47 siRNA (Figure 4A, inset) increased p21Cip1 gene expression (Figure 4A) at the promoter level (Figure 8A), with a simultaneous increase in p21Cip1 protein in control cells (Figure 4B, inset). Polyamine depletion increased basal p21Cip1 promoter activity (Figure 8A), and transfection of E47 siRNA further increased its activity (Figure 8B). However, it is not clear which transcription factors interact with E47 to enable it to act as a repressor. Expression of p21Cip1 is known to be both p53-dependent and -independent, and there are several reports that ODC inhibition by DFMO induces p21Cip1 expression independent of p53. Hu et al. [40] found that DFMO increased p21Cip1 expression and caused a G_1–S phase block in the MDA-MB-435 human breast cancer cell line, but failed to increase p53 levels. Nemoto et al. [41] reported increased expression of p21Cip1 by DFMO in MKN45 gastric cancer cells by increased binding of STAT1 to the p21Cip1 promoter region independently of p53. In IEC-6 cells, Liu et al. [42] demonstrated that polyamine-induced c-Myc acts as a repressor of p21Cip1 transcription through Miz-1 and Sp-1 binding sites on the proximal p21Cip1 promoter. The same study showed that ectopic expression of ODC significantly increased cellular polyamine levels, and this was accompanied by an increase in c-Myc and complete loss of p21Cip1 expression [42].

However, our observation that depletion of cellular polyamines by DFMO decreased E47 transcription and thereby increased p21Cip1 protein (Figure 4C) is most significant. The level of E47 mRNA significantly increased during apoptosis in control cells (Figure 4C). Polyamine depletion prevented E47 transcription during apoptosis (Figure 4C), which in turn derepressed p21Cip1 transcription and increased p21Cip1 protein production. Thus there appears to be a complex network of various transcription factors that variably modulate transcription of p21Cip1 in IEC-6 cells. Polyamines are important effectors that allow the activation or inhibition of various transcription factors, thereby influencing epithelial cell proliferation and apoptosis. Interestingly, E2A family proteins have been extensively correlated with cell survival. E2A proteins are required for the survival of T-cells and pro-B-lymphocytes [20–21,43]. Thymocytes isolated from $E47^{-/-}$ mice have higher rates of apoptosis and caspase 3 activation than their wild-type littermates [21]. On the other hand, studies have shown that E2A proteins suppress caspase 3 activation, blocking apoptosis in T-lineage cells and pro-B-lymphocytes and rescuing rat embryonic fibroblasts from Id3-induced apoptosis [21]. In this study, knockdown of E47 protein increased p21Cip1 expression, which protected cells from apoptosis (Figure 4B, inset), demonstrating an anti-apoptotic role for E47 in IEC-6 cells.

Heterodimerization of E2A family transcription factors with the 31 kDa Cas fragment generated by caspase 3-mediated cleavage during apoptosis leads to the inhibition of p21 Cip1 transcription [23]. A central role for p130Cas has been documented in celecoxib-induced anoikis in multiple colon carcinoma cell lines [36], apoptosis in Rat-1 fibroblasts (induced by etoposide, nocodazole and staurosporine) [23] and during anoikis in epithelial cells [24,44]. In all these studies, p130Cas was proteolytically degraded by active caspase 3, generating a 31 kDa Cas fragment during apoptosis. Consistent with these observations, our current study showed that TNFα- and CHX-induced apoptosis resulted in a gradual time-dependent decrease in the level of total and Tyr^{410}-phosphorylated p130Cas and the generation of 31 kDa Cas (Figure 5). Recombinant active caspase 3 cleaved p130Cas *in vitro*, generating a 31 kDa fragment irrespective of whether p130Cas was immunoprecipitated from control cells or those grown in DFMO (Figure 6B). These results also indicate that caspase 3-mediated cleavage of p130 Cas is independent of its phosphorylation status, as inhibition of Src kinase prevented p130Cas phosphorylation and induced caspase 3 activation,

leading to apoptosis. During apoptosis, active caspase 3 cleaves p130Cas protein and generates a 31 kDa cleaved fragment, which inhibits p21Cip1 mRNA expression and positively modulates cell death.

Our results clearly show that the 31 kDa Cas fragment is generated in the cytoplasm during apoptosis, translocates to the nucleus and forms a complex with E47 (Figure 7). Additionally, our findings indicate that polyamine depletion significantly blocks caspase 3-mediated proteolysis of p130Cas and the generation of the 31 kDa Cas fragment (Figures 5–7). Nuclear localization of this fragment and its association with E47 was significantly reduced in polyamine-depleted cells compared with control cells (Figure 7). However, our results also indicate that forced expression of the 31 kDa Cas protein decreases p21Cip1 promoter activity and protein expression (Figure 8A), which may eventually facilitate apoptosis. Indeed, transient overexpression of the 31 kDa Cas protein increased both spontaneous and TNFα-induced apoptosis (Figure 8B). Interestingly, expression of 31 kDa Cas in cells transfected with E47 siRNA decreased p21Cip1 promoter activation and protein expression (Figure 8A). These results suggest that 31 kDa Cas can repress p21Cip1 expression independently of E47.

The mucosa of the GI tract is one of the fastest growing tissues in the body, where the rate of proliferation is balanced by cell loss through apoptosis. Intracellular polyamines are critical for the expression of p21Cip1, which inhibits apoptosis (Scheme 1). Our current study shows that polyamine depletion represses E47 expression, prevents caspase 3-mediated cleavage of p130Cas (to form 31 kDa Cas) and increases expression of p21Cip1. Furthermore, polyamine depletion activates Src, leading to inhibition of caspase 3 via AKT/JAK/STAT3 [2,6] and prevents degradation of p21Cip1.

This work was supported by the NIDDK (National Institute of Diabetes and Digestive and Kidney Disease; grant DK-16505), and by the Thomas A. Gerwin Endowment. We thank Dr Woo Keun Song and Dr Kang YongSeok for the 31 kDa-pcDNA3.0 HLH construct and Dr Jian-Ying Wang for the p21Cip1-promoter luciferase reporter construct. We thank Mr Gregg Short and Mr Danny Morse for help in preparing the Figures and acknowledge Ms Mary Jane Viar and Ms Rebecca West for valuable suggestions.

REFERENCES

1 Quaroni, A., Wands, J., Trelstad, R. L. and Isselbacher, K. J (1979) Epithelial cell cultures from rat small intestine. Characterization by morphologic and immunologic criteria. J. Cell Biol. **80**, 248–265

2 Johnson, L. R. and Ray, R. M. (2006) In Cellular Signaling and Polyamines in the Control of Apoptosis in Intestinal Epithelial Cells (Wang, J. Y. and Casero, Jr, R. A., eds), pp. 233–248, Humana Press Inc., Totowa

3 Ray, R. M., Viar, M. J., Yuan, Q. and Johnson, L. R. (2000) Polyamine depletion delays apoptosis of rat intestinal epithelial cells. Am. J. Physiol. Cell Physiol. **278**, C480–C489

4 Bhattacharya, S., Ray, R. M., Viar, M. J. and Johnson, L. R. (2003) Polyamines are required for activation of c-Jun NH2-terminal kinase and apoptosis in response to TNFα in IEC-6 cells. Am. J. Physiol. Gastrointest. Liver Physiol. **285**, G980–G991

5 Deng, W., Viar, M. J. and Johnson, L. R. (2005) Polyamine depletion inhibits irradiation-induced apoptosis in intestinal epithelia. Am. J. Physiol. Gastrointest. Liver Physiol. **289**, G599–G606

6 Bhattacharya, S., Ray, R. M. and Johnson, L. R. (2006) Integrin β3-mediated Src activation regulates apoptosis in IEC-6 cells via Akt and STAT3. Biochem. J. **397**, 437–447

7 Ray, R. M., Zimmerman, B. J., McCormack, S. A., Patel, T. B. and Johnson, L. R. (1999) Polyamine depletion arrests cell cycle and induces inhibitors $p21^{Waf1/Cip1}$, $p27^{Kip1}$, and p53 in IEC-6 cells. Am. J. Physiol. Cell Physiol. **276**, C684–C691

8 Harper, J. W., Elledge, S. J., Keyomarsi, K., Dynlacht, B., Tsai, L., Zhang, P., Dobtowolski, S., Bai, C., Connell-Crowley, L., Swindell, E. et al. (1995) Inhibition of cyclin dependent kinases by p21. Mol. Biol. Cell **6**, 387–400

9 Gartel, A. L. and Radhakrishnan, S. K. (2005) Lost in transcription: p21 repression, mechanisms and consequences. Cancer Res. **65**, 3980–3985

10 Gartel, A. L. and Tyner, A. L. (2002) The role of cyclin-dependent kinase inhibitor p21 in apoptosis. Mol. Cancer Therapeutics **1**, 639–649

11 Suzuki, A., Tsutomi, Y., Akahane, K., Araki, T. and Miura, M. (1998) Resistance to fas-mediated apoptosis: activation of caspase-3 is regulated by cell cycle regulator p21Waf1 and IAP gene family ILP. Oncogene **17**, 931–939

12 Xu, S. Q. and El-Deiry, W. S. (2000) p21(waf1/Cip1) inhibits initiator caspase-cleavage by TRAIL death receptor DR4. Biochem. Biophys. Res. Commun. **269**, 179–190

13 Asada, M., Yamada, T., Ichijo, H., Delia, D., Miyazono, K., Fukumuro, K. and Mizutani, S. (1999) Apoptosis inhibitory activity of cytoplasmic p21(Cip1/Waf1) in monocytic differentiation. EMBO J. **18**, 1223–1234

14 Raj, K., Ogston, P. and Beard, P. (2001) Virus-mediated killing of cells that lack p53 activity. Nature **412**, 914–917

15 Prabhu, S., Ignatova, A., Park, S. T. and Sun, X. H. (1997) Regulation of the expression of cyclin-dependent kinase inhibitor p21 by E2A and Id proteins. Mol. Cell Biol. **17**, 5888–5896

16 Pagliuca, A., Gallo, P., Luca, P. D. and Lania, L. (2000) Class A Helix-Loop-Helix proteins are positive regulators of several cyclin-dependent kinase inhibitors promoter activity and negatively regulate cell growth. Cancer Res. **60**, 1376–1382

17 Murre, C., McCaw, P. S. and Baltimore, D. (1989) A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. Cell **56**, 777–783

18 Lassar, A. B., Davis, R. L., Wright, W. E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D. and Weintraub, H. (1991) Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins *in vivo*. Cell **66**, 305–315

19 Pegg, A. E. (2006) Regulation of ornithine decarboxylase. J. Biol. Chem. **281**, 14529–14532

20 Engel, I. and Murre, C. (1999) Ectopic expression of E47 or E12 promotes the death of E2A-deficient lymphomas. Proc. Natl. Acad. Sci. U.S.A. **96**, 996–1001

21 Kee, B. L., Bain, G. and Murre, C. (2002) IL-7Rα and E47: independent pathways required for development of multipotent lymphoid progenitors. EMBO J. **21**, 103–113

22 Kee, B. L., Rivera, R. R. and Murre, C. (2001) Id3 inhibits B lymphocyte progenitor growth and survival in response to TGFβ. Nat. Immunol. **2**, 242–247

23 Kim, W., Kook, S., Kim, D. J., Teodorof, C. and Song, W. K. (2004) The 31-kDa caspase-generated cleavage product of p130Cas functions as a transcriptional repressor of E2A in apoptotic cells. J. Biol. Chem. **279**, 8333–8342

24 Wei, L., Yang, Y., Zhang, X. and Yu, Q. (2002) Anchorage-independent phosphorylation of p130(Cas) protects lung adenocarcinoma cells from anoikis. J. Cell. Biochem. **87**, 439–449

25 Almeida, E. A., Ilic, D., Han, Q., Hauck, C. R., Jin, F., Kawakatsu, H., Schlaepfer, D. D. and Damsky, C. H (2000) Matrix survival signaling: from fibronectin via focal adhesion kinase to c-Jun NH_2-terminal kinase. J. Cell. Biol. **149**, 741–754

26 Weng, L. P., Wang, X. and Yu, Q. (2002) Transmembrane tyrosine phosphatase LAR induces apoptosis by dephosphorylating and destabilizing p130Cas. Genes Cells **4**, 185–196

27 Widmann, C., Gibson, S. and Johnson, G. L. (1998) Caspase-dependent cleavage of signaling proteins during apoptosis. A turn-off mechanism for anti-apoptotic signals. J. Biol. Chem. **273**, 7141–7147

28 Jin, Y. H., Yoo, K. J., Lee, Y. H. and Lee, S. K. (2000) Caspase-3 mediated cleavage of p21Waf1/Cip1 associated with the cyclin A dependent kinase 2 complex is a prerequisite for apoptosis in SK-HEP-1 cells. J. Biol. Chem. **275**, 30256–30263

29 Gervais, J. L., Seth, P. and Zhang, H. (1998) Cleavage of CDK inhibitor p21(Cip1/Waf1) by caspases is an early event during DNA damage-induced apoptosis. J. Biol. Chem. **273**, 19207–19212

30 McCormack, S. A., Viar, M. J. and Johnson, L. R. (1993) Polyamines are necessary for migration by a small intestinal crypt cell line. Am. J. Physiol. Gastrointest. Liver Physiol. **27**, G367–G374

31 Guo, H., Ray, R. M. and Johnson, L. R. (2003) RhoA stimulates IEC-6 cell proliferation by increasing polyamine-dependent Cdk2 activity. Am. J. Physiol. Gastrointest. Liver Physiol. **285**, G704–G713

32 Bhattacharya, S., Ray, R. M. and Johnson, L. R. (2005) Decreased apoptosis in polyamine depleted IEC-6 cells depends on Akt-mediated NF-κB activation but not GSK3β activity. Apoptosis **10**, 759–776

33 Bhattacharya, S., Ray, R. M. and Johnson, L. R. (2005) STAT3-mediated transcription of Bcl-2, Mcl-1 and c-IAP2 prevents apoptosis in polyamine-depleted cells. Biochem. J. **392**, 335–344

34 Ray, R. M., Patel, A., Viar, M. J., McCormack, S. A., Zheng, Y., Tigyi, G. and Johnson, L. R. (2002) RhoA inactivation inhibits cell migration but does not mediate the effects of polyamine depletion. Gastroenterology **123**, 196–205

35 Defilippi, P., Di Stefano, P. and Cabodi, S. (2006) p130Cas: a versatile scaffold in signaling networks. Trends Cell Biol. **16**, 257–263

36 Casanova, I., Parreno, M., Farre, L., Guerrero, S., Cespedes, M. V., Pavon, M. A., Sancho, F. J., Marcuello, E., Trias, M. and Mangues, R. (2006) Celecoxib induced anoikis in human colon carcinoma cells associated with deregulation of focal adhesions and nuclear translocation of p130Cas. Int. J. Cancer **118**, 2381–2389

37 Schipper, R. G., Penning, L. C. and Verhofstad, A. A. J. (2000) Involvement of polyamines in apoptosis. Facts and controversies; effectors or protectors? Semin. Cancer Biol. **10**, 55–68

38 Zhou, B. P., Liao, Y., Xia, W., Spohn, B., Lee, M. H. and Hung, M. C. (2001) Cytoplasmic localization of p21Cip1/Waf1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. Nat. Cell Biol. **3**, 245–252

39 Wang, Z., Kishimoto, H., Bhat-Nakshatri, P., Crean, C. and Nakshatri, H. (2005) TNF-α resistance in MCF-7 breast cancer cells is associated with altered subcellular localization of $p21^{Cip1}$ and $p27^{Kip1}$. Cell Death and Differ. **12**, 98–100

40 Hu, X., Washington, S., Verderame, M. F. and Manni, A. (2005) Interaction between polyamines and the mitogen-activated protein kinase pathway in the regulation of cell cycle variables in breast cancer cells. Cancer Res. **65**, 11026–11033

41 Nemoto, T., Kamei, S., Seyama, Y. and Kubota, S. (2001) p53 independent G_1 arrest induced by DL-α-difluoromethylornithine. Biochem. Biophys. Res. Commun. **280**, 848–854

42 Liu, L., Guo, X., Rao, J. N., Zou, T., Marasa, B. S., Chen, J., Greenspon, J., Casero, Jr, R. A. and Wang, J.-Y. (2006) Polyamine-modulated c-myc expression in normal intestinal epithelial cells regulates p21Cip1 transcription through a proximal promoter region. Biochem. J. **398**, 257–267

43 Norton, J. D. and Atherton, G. T. (1998) Coupling of cell growth control and apoptosis functions of Id proteins. Mol. Cell. Biol. **18**, 2371–2381

44 Wei, L., Yang, Y., Zhang, X. and Yu, Q. (2004) Cleavage of p130Cas in anoikis. J. Cell. Biochem. **91**, 325–335

Received 28 February 2007/25 June 2007; accepted 9 July 2007
Published as BJ Immediate Publication 9 July 2007, doi:10.1042/BJ20070293