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# APE1/Ref-1 regulates PTEN expression mediated by Egr-1

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**Abstract**
APE1/Ref-1, the mammalian ortholog of *E. coli* Xth, and a multifunctional protein possessing both DNA repair and transcriptional regulatory activities, has dual role in controlling cellular response to oxidative stress. It is rate-limiting in repair of oxidative DNA damage including strand breaks and also has co-transcriptional activity by modulating genes expression directly regulated by Egr-1 and p53 transcription factors. PTEN, a phosphoinositide phosphatase, acts as an 'off' switch in the PI-3 kinase/Akt signalling pathway and regulates cell growth and survival. It is shown here that transient alteration in the APE1 level in HeLa cells modulates PTEN expression and that acetylatable APE1 is required for the activation of the PTEN gene. Acetylation of APE1 enhances its binding to distinct trans-acting complexes involved in activation or repression. The acetylated protein is deacetylated *in vivo* by histone deacetylases. It was found that exposure of HeLa cells to $H_2O_2$ and to histone deacetylase inhibitors increases acetylation of APE1 and induction of PTEN. The absence of such induction in APE1-downregulated HeLa cells confirmed APE1's role in regulating inducible PTEN expression. That APE1-dependent PTEN expression is mediated by Egr-1 was supported by experiments with cells ectopically expressing Egr-1. Thus, the data open new perspectives in the comprehension of the many functions exerted by APE1 in controlling cell response to oxidative stress.

**Keywords:** *APE1/Ref-1, PTEN, Egr-1, siRNA, histone acetyltransferase inhibitors, oxidative stress response*

**Abbreviations:** *AP-1, activator protein-1; APE1/Ref-1, Apurinic/apyrimidinic (AP) endonuclease/redox effector factor-1; Egr-1, Early growth response protein-1; HATs, Histone acetyltransferases; HDACs, Histone deacetylases; NaBut, sodium butyrate; NF-κB, Nuclear Factor-κB; PTEN, protein phosphatase and tensin homologue; ROS, reactive oxygen species; TSA, trichostatin A.*

## Introduction

The APE1/Ref-1 protein (from now on simply indicated as APE1) plays a central role in the highly regulated process of cellular response to oxidative stress [1,2]. Its activation is part of a complex network of cellular events that determines the final outcome, namely cell growth arrest, death or survival of cells exposed to oxidative stress [1,3–6]. In addition to well-characterized toxic effects, mild oxidative stress activates survival/proliferative signalling [7]. Thus, tight

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temporal control and the extent of APE1 activation in response to oxidative stress could modulate cell growth and survival.

APE1 is a ubiquitous multifunctional protein possessing both DNA repair and transcriptional regulatory activities. APE1 enhances DNA binding of a number of transcription factors including p53, Egr-1 and NF-$\kappa$B [1,2,8–11] by acting as a transcriptional co-activator. Egr-1 is involved in the regulation of cell proliferation in response to extracellular signal such as mitogens and growth factors, as well as oxidative stress [12,13]. Studies of gene expression in human tumour cells and tissues support Egr-1's function as a tumour suppressor [14,15]. Egr-1 regulates expression of many genes such as p53 and the phosphoinositol phosphatase and tensin homologue (PTEN) that control cell-growth arrest and apoptosis [16,17]. PTEN negatively regulates the phosphoinositide 3-kinase/Akt signalling pathway, which was shown to be involved in regulation of cell growth and survival of several cell lines [18]. Thus, induction of PTEN activity could lead to inhibition of cell growth and ultimately to apoptosis. We previously observed that the PTEN promoter could be regulated by Egr-1 in an APE1-dependent manner [11], suggesting a functional relationship among these three proteins for growth suppressive effects in response to oxidative stress. We have also shown that PTEN expression is activated by oxidative stress [19]. The regulatory functions of the different APE1 activities could be implemented via three different mechanisms: (a) APE1's relocalization from the cytoplasm to the nucleus; (b) increase in APE1's level after transcriptional activation [1,5,11,20]; and (c) APE1's post-translational modifications (such as acetylation and phosphorylation). As recently demonstrated [6,21], acetylation appears to have a fine-tuning role in affecting APE1's different activities.

While it is known that nuclear accumulation of APE1 triggers activation of several transcription factors, such as Egr-1 and p53, the functional role of acetylation is barely understood. Acetylation of both histones and regulatory proteins is commonly catalysed by the histone acetyltransferase (HAT) p300/CBP and can be reversed by histone deacetylases (HDACs), which thus control the acetylation level of transcription factors or co-activators [22–24]. We observed that the balance between the acetyltransferase activity of p300/CBP and the deacetylase activity of HDAC1 maintains APE1's acetylation at Lys residues 6 and 7 (K6, K7) in response to $Ca^{2+}$ levels, thus controlling expression of target genes [6]. Inhibition of HDACs by HDACs inhibitors causes proteins hyperacetylation [25]. A correlation has been established between hyperacetylated histones and transcriptionally active chromatin [26,27]. Moreover, recent studies indicate a potential role for HDACs inhibitors in promoting differentiation of some tumour cells, suggesting their possible use as anticancer agents [28,29].

The purpose of the present study was to investigate the relationship between APE1, its acetylated form and PTEN expression. We observed direct correlation between the levels of APE1 and PTEN suggesting a functional link. Furthermore, PTEN was upregulated by treatment with $H_2O_2$ and HDACs inhibitors concomitant with enhanced acetylation of APE1. APE1-mediated PTEN activation appears to involve the transcription factor Egr-1. Collectively, our studies have unravelled a novel, although indirect, regulatory function of APE1 on PTEN activation, opening new perspectives in the comprehension of the many functions exerted by this multifunctional protein.

## Materials and methods

### *Chemicals*

Trichostatin A (TSA), sodium butyrate (NaBut) and other chemicals were purchased from Sigma (Milan, Italy), unless otherwise specified. HeLa cells were purchased from ATCC and HCT116 isogenic cell lines expressing p53 ($p53^{+/+}$) and non-expressing p53 ($p53^{-/-}$) were gifts of B. Vogelstein (Johns Hopkins University, Baltimore, MD). The *p53* gene was inactivated in HCT116 $p53^{-/-}$ cells by homologous recombination. Briefly, two promoterless targeting vectors containing either a geneticin or hygromycin resistance gene in place of genomic p53 sequences were sequentially transfected into HCT116 $p53^{+/+}$ cells to disrupt both *p53* alleles [30]. The phenotype of this cell line is stable as determined periodically by Western blot analysis.

### *Cell culture and analysis*

HeLa cells were grown in Dulbecco's Modified Eagle's Medium High Glucose (DMEMHG) supplemented with 10% (v/v) foetal bovine serum (FBS), 2 mM L-glutamine and 1% penicillin-streptomycin (100,000 Units/L penicillin and 100 mg/L streptomycin) at 37°C in 5% $CO_2$ and 95% $O_2$. HCT116$p53^{+/+}$ and HCT116$p53^{-/-}$ human colon carcinoma isogenic cell lines were cultured in McCoy's 5A medium supplemented with 10% FBS and penicillin/streptomycin, as previously reported [6].

Cellular extracts were typically prepared by lysing $3 \times 10^5$ cells in 50 μL of lysis buffer containing 50 mM Tris-HCl, (pH 7.5), 150 mM NaCl, 1% w/v Triton X-100, 1 mM EDTA, 0.5 mM PMSF, 0.1 mM DTT, 1 mM $NaVO_3$, 1 mM NaF, protease inhibitor cocktail (Sigma), then analysed for protein content [31] and stored in aliquots at −80°C.

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*Western blot analysis*

Cell extracts were analysed by SDS-PAGE (10% polyacrylamide) [20] and the separated proteins transferred to nitrocellulose membranes (Schleicher & Schuell). After pre-incubating with 5% non-fat dry milk in PBS/0.1% w/v Tween-20 for 1 h, the membranes were incubated with anti-APE1 monoclonal antibody [20] for 3 h at room temperature or with the polyclonal anti-PTEN antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) overnight at 4°C. The amount of acetylated APE1 (AcAPE1) was determined by using anti-acetylated APE1 peptide-specific polyclonal antibody, as previously described [32]. After incubation, the membranes were washed three times with PBS/0.1% w/v Tween-20 and incubated with appropriate secondary antibodies coupled to horseradish peroxidase (Sigma) for 1 h at room temperature followed by three washes with PBS/Tween-20 0.1% w/v. At the end, the blots were developed using the ECL chemiluminescence procedure (Amersham Pharmacia Biotech, Milan, Italy) and quantified using a Gel Doc 2000 videodensitometer (Bio-Rad, Hercules, CA, USA). APE1, AcAPE1 and PTEN signals were normalized to actin quantitated on the same blot with polyclonal anti-actin antibody (Sigma).

*Transfection studies*

HCT116 cells ($0.3 \times 10^6$ cells/100 mm dish) were cotransfected using Lipofectamine 2000 (Invitrogen, Milan, Italy) according to the manufacturer's protocol with various luciferase reporter plasmids. A $\beta$-galactosidase expression plasmid (CMV-$\beta$-Gal) was used as an internal control for normalizing transfection efficiency (Roche Diagnostic, Milan, Italy). Luciferase (Luc) activity was measured by using a chemiluminescence procedure [11] and normalized to $\beta$-galactosidase activity. In some cases, where the promoter activity of $\beta$-galactosidase expression plasmid was also affected, luciferase activity was normalized with the total protein in the extract. For studies with the PTEN promoter constructs, we used 0.5 μg of a plasmid containing 2 kb ($-1$ to $-1978$ bp) human PTEN promoter (*PTEN-Luc*) which was cloned into pGL3B-P10, 5′ to the Luc gene, as described earlier [16]. The pcDNA5.1-APE1-c-FLAG plasmid, encoding the C-terminal FLAG-tagged APE1 was used to over-express APE1; as control we used the pcDNA5.1-empty vector. The Egr-1-expressing plasmid pRSV-Egr-1 was described earlier [33].

*Transient and inducible expression of APE1 siRNA*

The oligonucleotides used for siRNA of APE1 using Mittal's empirical rule [34] (sense, 5′-CCTGCCA CACTCAAGATCTGC-3′; antisense, 5′-GCAGAT CTTGAGTGTGGCAGG-3′) were designed to bind APE1's 21 nt mRNA segment 175 nt downstream to the initiation codon. The sequences were cloned at the *BglII* and *HindIII* restriction sites of pSUPER and identity of the resulting plasmid pSUPER/Ref-1 was confirmed by direct sequencing. HeLa cells were transfected with pSUPER/Ref-1 in the presence of lipofectamine and, 48 h later, the cells were processed for further analysis.

For inducible down-regulation of APE1 expression, the same siRNA sequences were cloned in the pTER vector [35] with tetracycline- (doxycycline-) responsive promoter to generate the pTER/Ref-1 plasmid. HeLa cells were transfected with pcDNA6/TR, linearized with Bst1107I (Fermentas, St.Leon Rot, UK) and then incubated with blasticidin (Invitrogen) for 14 days to select resistant cells. After isolating individual clones using cloning cylinders (Sigma), the clones were expanded to $10^7$ cells. The TR5 clone with high level expression of the Tet-repressor was transfected with pTER/Ref-1 predigested with Bst1107I (Fermentas) and then selected for resistance to zeocine after 14–21 days' culture (Invitrogen). Thirty clones were then grown to $10^7$ cells, along with control, empty pTER-transfected cells. To induce APE1 siRNA in these cells, 1 μg/mL doxycycline (Sigma) was added to the medium and the cells were grown for 10 days and analysed for APE1 expression.

*Statistical analysis*

Results are expressed as mean $\pm$ SD. Each data point represents the average of three measurements, each performed with at least three independent cell preparations. Statistical significance between means $\pm$ SD was analysed by Student's *t*-test or by a two-way analysis of variance (ANOVA) and differences were considered significant when $p < 0.05$.

## Results

*APE1-dependent regulation of PTEN expression*

We had previously shown by cotransfection experiments that the PTEN promoter activity was significantly enhanced in HeLa cells due to over-expression of APE1 [11], suggesting that the PTEN gene is regulated by APE1. In order to confirm direct correlation between the APE1 level and PTEN expression, we examined the effect of down-regulation of endogenous APE1 on the PTEN gene. APE1-specific small interfering RNA (siRNA), expressed from a plasmid, was used to reduce the level of endogenous APE1. HeLa cells were transiently transfected with the control siRNA or the APE1 specific siRNA-expressing plasmid (pSUPER/Ref-1) and the expression levels of APE1 and PTEN were measured by Western analysis at 48 h after

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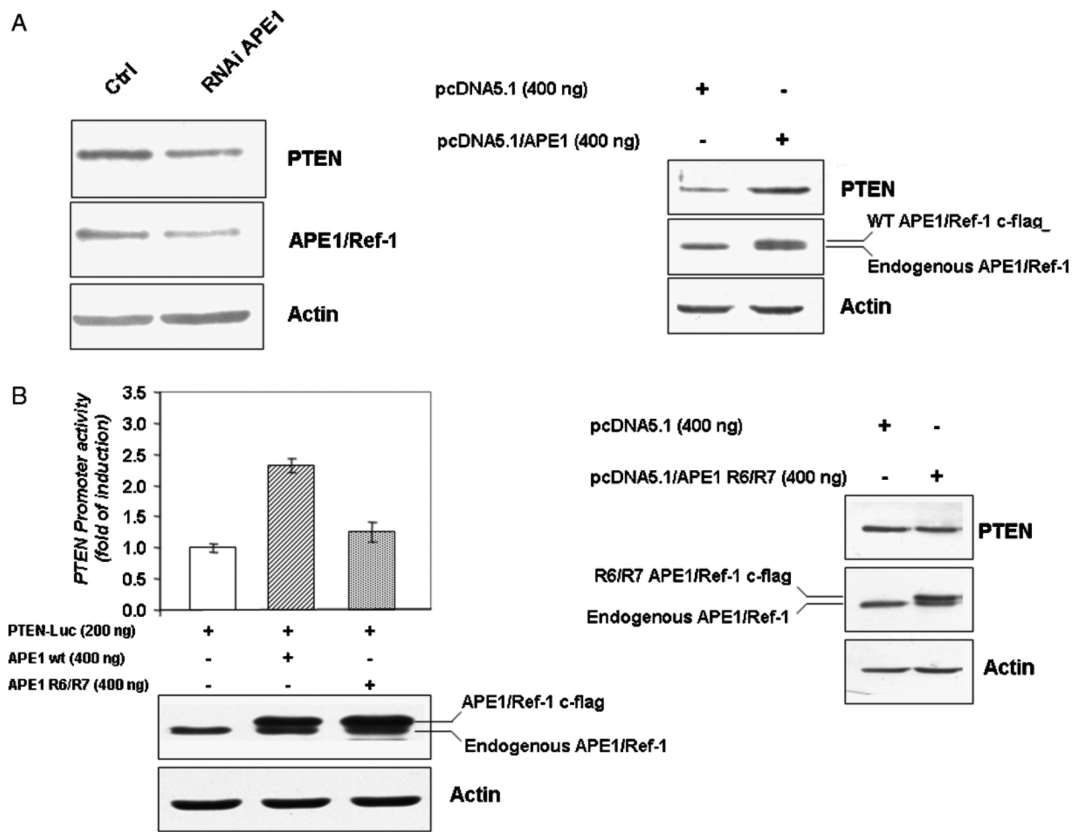


Figure 1. APE1-dependent regulation of PTEN expression. (A, Left) Down-regulation of PTEN due to siRNA-mediated transient reduction in APE1 expression. HeLa cells were transfected with 1.5 μg empty pSUPER (Ctrl) or pSUPER/Ref-1 plasmid; 48 h later, the cell lysates (15 μg) were used for Western analysis. (Right) Upregulation of endogenous PTEN due to transient over-expression of APE1/Ref-1. HeLa cells were transfected with 0.4 μg pcDNA5.1-empty or the pcDNA5.1-APE1-c-FLAG and, 48 h later, cell extracts (10 μg) were analysed as before. (B, Left) Effect of APE1 over-expression on PTEN promoter activity. HeLa cells were harvested and were cotransfected with PTEN luc plasmid and WT or R6/R7 APE1 mutant and 48 h later, luciferase and $\beta$-galactosidase activities were measured and represented in the histogram. The levels of ectopic APE1 expression in the same cell lysates were analysed by Western blotting. (Right) Transient over-expression of the R6/R7 APE1 mutant is unable to promote endogenous PTEN upregulation. HeLa cells were transfected with 0.4 μg pcDNA5.1-empty or the pcDNA5.1-R6/R7 APE1-c-FLAG and, 48 h later, cell extracts (10 μg) were analysed as before.

transfection. Expression of the APE1 siRNA resulted in a significant decrease ($\sim$80%) in the APE1 level, compared to that in control cells after transfection with the empty plasmid (Figure 1A, left). At the same time, the PTEN level was reduced by 50% in the APE1 knocked-down cells.

To confirm that PTEN is a regulatory target of $\sim$APE1, we over-expressed FLAG-tagged APE1 in HeLa cells. The PTEN level was significantly increased in APE1-over-expressing cells relative to the control (Figure 1A, right).

APE1's regulatory activity could be fine-tuned due to various mechanisms including post-translational modification, redox regulation of target trans-acting factor and nucleo-cytoplasmic trafficking of the protein itself [1,2]. We have recently demonstrated that acetylation of APE1 at two specific Lys residues (i.e. K6/K7) constitutes a fundamental mechanism to modulate its regulation of $Ca^{2+}$-dependent promoters containing negative $Ca^{2+}$ responsive elements (nCaRE) [6]. Therefore, we tested whether acetylatable K6/K7 residues of APE1 are involved in PTEN expression. We analysed activation of PTEN promoter-driven luciferase expression mediated by the wild type APE1 or by the non-acetylatable R6/R7 mutant [6]. Figure 1B (left panel) shows that while over-expression of wild type APE1 protein increased activity of the PTEN promoter by $\sim$2-fold, expression of the R6/R7 mutant caused marginal activation under conditions of comparable ectopic expression of wild type vs mutant APE1. As expected, transient over-expression of the non-acetylatable APE1 protein was unable to promote endogenous PTEN expression (Figure 1B, right panel). These data suggest that acetylated lysine residues of APE1 are involved in APE1-mediated PTEN expression.

### *PTEN activation and APE1 acetylation are induced by $H_2O_2$*

Expression of PTEN, an early regulator of cellular growth suppression, is enhanced in thyroid cells by oxidative stress [19]. Here, we tested whether $H_2O_2$ treatment could similarly induce PTEN in HeLa cells. After 1 h serum starvation, HeLa cells were

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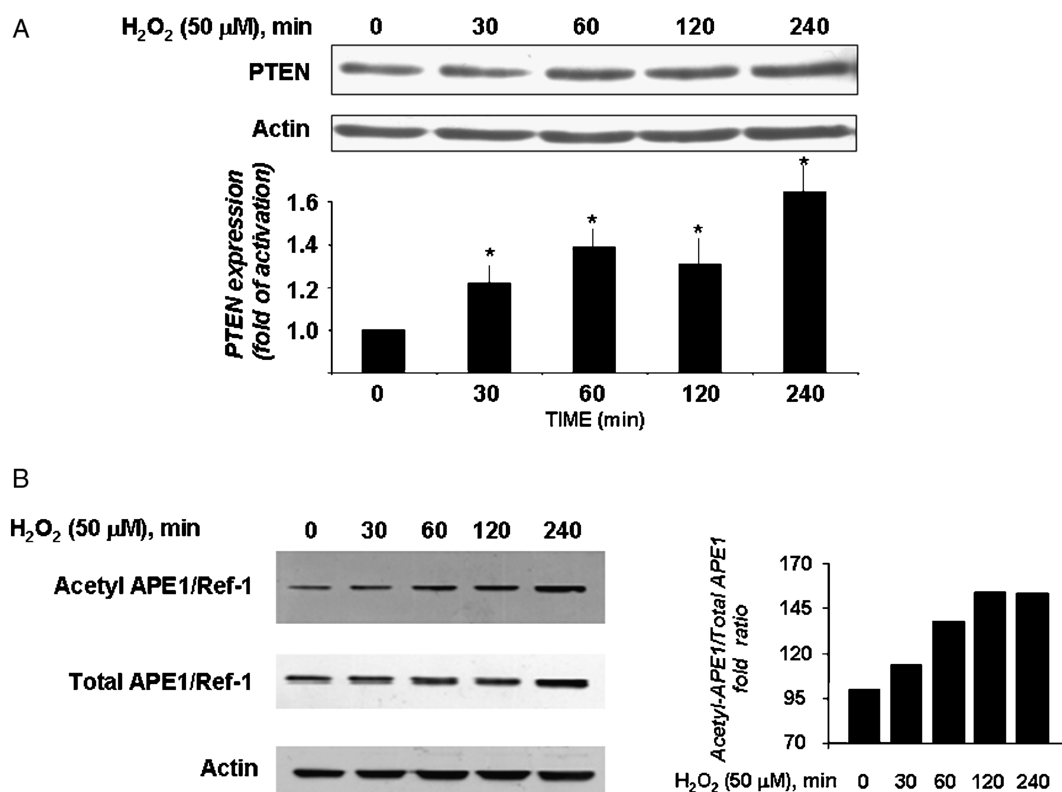


Figure 2. $H_2O_2$ treatment enhances PTEN expression and APE1 acetylation. (A) The levels of PTEN were determined after treatment with 50 μM $H_2O_2$ by Western analysis in HeLa cell extracts (10 μg). The data are also presented as histograms, representing the mean of three independent experiments (*$p < 0.05$ by Student's *t*-test, treated vs control). (B) Extracts of $H_2O_2$-treated HeLa cells were separately analysed for total and acetylated APE1 by Western blotting [6,20]. Actin was used as the loading control. Histograms, representing the mean of two independent experiments, show the relative amount in arbitrary scale of acetyl-APE1 vs total APE1 protein, after normalization for the actin level. Variation between the two independent experiments was less than 10%.

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treated for various times with 50 μM $H_2O_2$. As shown in Figure 2A, $H_2O_2$ treatment increased the endogenous PTEN protein level in a time-dependent manner.

Then, we tested the possibility that oxidative stress stimulates acetylation of APE1 which in turn activates the PTEN promoter. We could quantitate the amount of acetylated APE1 by using a polyclonal antibody that selectively recognizes AcAPE1. The antibody was raised against a peptide containing acetylated Lys residues corresponding to position 6 in APE1. This polyclonal rabbit antibody does not recognize at least 50-fold excess of unmodified APE1 (data not shown). Figure 2B shows that $H_2O_2$ treatment significantly increased the level of AcAPE1, which occurred early (30 min) after treatment. Estimation of the total APE1, by using a monoclonal antibody [20], indicated that its level was not significantly affected by $H_2O_2$ under these conditions.

### *Elevated level of acetylated APE1 and PTEN upregulation after HDACs inhibition*

We have recently demonstrated that APE1 acetylation is mediated by p300/CBP histone acetyltransferase and that HDACs inhibition moderately enhanced the level of AcAPE1 in HCT116 cells [6]. Thus, we tested whether HDACs inhibition would affect AcAPE1 levels also in HeLa cells. We treated HeLa cells with two HDACs inhibitors, namely, NaBut and TSA. The longer time-frame used for this treatment, with respect to the previous experiment in Figure 2, was chosen on the basis of the activation kinetics of PTEN induction by HDACs inhibitors treatment (data not shown). Figure 3A shows that both inhibitors significantly increased the amount of acetylated APE1, albeit to a different extent, without affecting the total amount of APE1. The increase in AcAPE1 levels upon TSA treatment was visible even at earlier time points (data not shown).

Then, we tested whether HDACs inhibitors upregulate PTEN expression. HeLa cells were treated with the HDACs inhibitors for 30 h and the PTEN level was quantitated by Western blotting. Figure 3B shows that both NaBut and TSA significantly increased the PTEN protein level.

### *HDACs inhibitor-induced PTEN upregulation requires APE1 expression*

The causal involvement of APE1 in upregulation of PTEN expression was further tested by using HeLa cells in which expression of endogenous APE1 had

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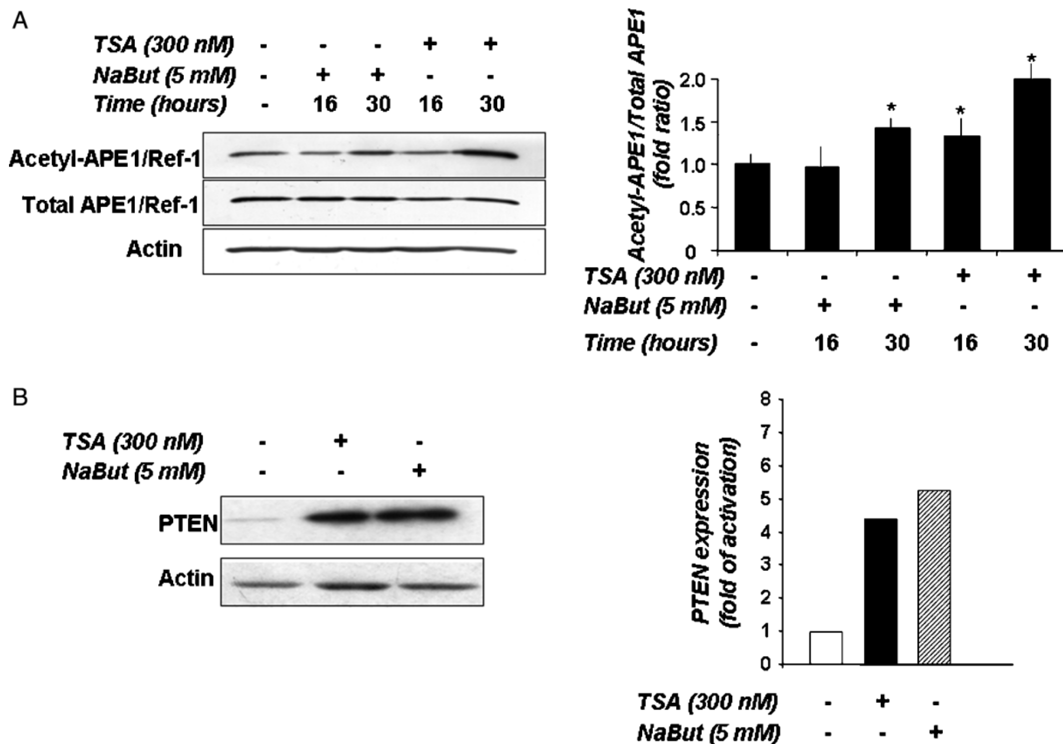


Figure 3. PTEN upregulation and APE1 acetylation due to inhibition of HDACs. (A) After treatment of HeLa cells with TSA or NaBut as indicated, the cell lysates were analysed for total and acetylated APE1 level as before. Histograms represent the relative amount of acetyl-APE1 vs total APE1 protein, after normalization for actin. The mean value of three independent experiments is shown with bars indicating the mean value $\pm$SD (*$p<0.05$ by Student's *t*-test, treated vs control). (B) Western analysis of PTEN in extracts of HeLa cells after treatment with TSA or NaBut, for 30 h. The mean value of two independent experiments is shown, whose variation is less than 10%.

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been conditionally knocked-down by stable transfection with inducible APE1-specific siRNA expression [35]. After 10 days' treatment with doxycycline, APE1 expression reached an almost undetectable level in HeLa cells expressing the APE1 specific siRNA; the control cells, transfected with the siRNA empty vector (Control) were unaffected (Figure 4A, inset). To test the effect of HDACs inhibition on PTEN gene expression, we measured luciferase activity under control of the 2-kb PTEN promoter [16]. Figure 4A shows that TSA treatment caused ~5-fold increase in Luc activity in control cells and NaBut increased the activity by ~11-fold relative to the untreated cells. In APE1 siRNA-expressing cells, TSA treatment caused only a 2-fold and NaBut only a 3.5-fold increase in the PTEN promoter activity.

These results, showing that silencing of APE1 prevents activation of the PTEN promoter caused by HDACs inhibition, support a major role of acetylated APE1 in transcriptional activation of the PTEN gene.

We also tested whether HDACs inhibition causes upregulation of the endogenous PTEN protein via mediation by APE1. We measured the PTEN protein levels in the control or APE1 knock-down cells after treatment with NaBut and TSA (Figure 4B). It is evident that the PTEN levels increased in a time-dependent fashion when HDAC was inhibited and that this response was completely blocked in APE1 knock-down cells (Figure 4B). Taken together, these data unequivocally demonstrate the essential role of APE1 in PTEN expression.

The unexpected observation that HeLa APE1 knocked-down cells present a higher basal level of PTEN protein could be explained due to the complex transcriptional mechanisms controlling PTEN expression, that may involve also other transcription factors such as p53 [36], CBF-1 [37], NF-$\kappa$B [38] or PPAR$\gamma$ [39], not excluding a possible role on protein stabilization. Thus, our data clearly point to a role for APE1 in controlling inducible rather than constitutive PTEN expression.

### *APE1-mediated PTEN activation requires Egr-1 but not p53*

Transcriptional activation of PTEN is under the control of several transcription factors, such as p53 and Egr-1 which are known to be co-activated by APE1 [8,10,16,18,36]. While p53 is responsible for basal PTEN expression, Egr-1 primarily activates PTEN expression in response to cellular stress [16]. We tested the involvement of p53 or Egr-1 in APE1-mediated PTEN expression, by using a nearly isogenic pair of human colon carcinoma cell line HCT116 expressing wild type or no p53. The PTEN-Luc promoter contains a 117 bp GC-rich sequence ($-1031$ to $-779$ bp), the minimal Egr-1

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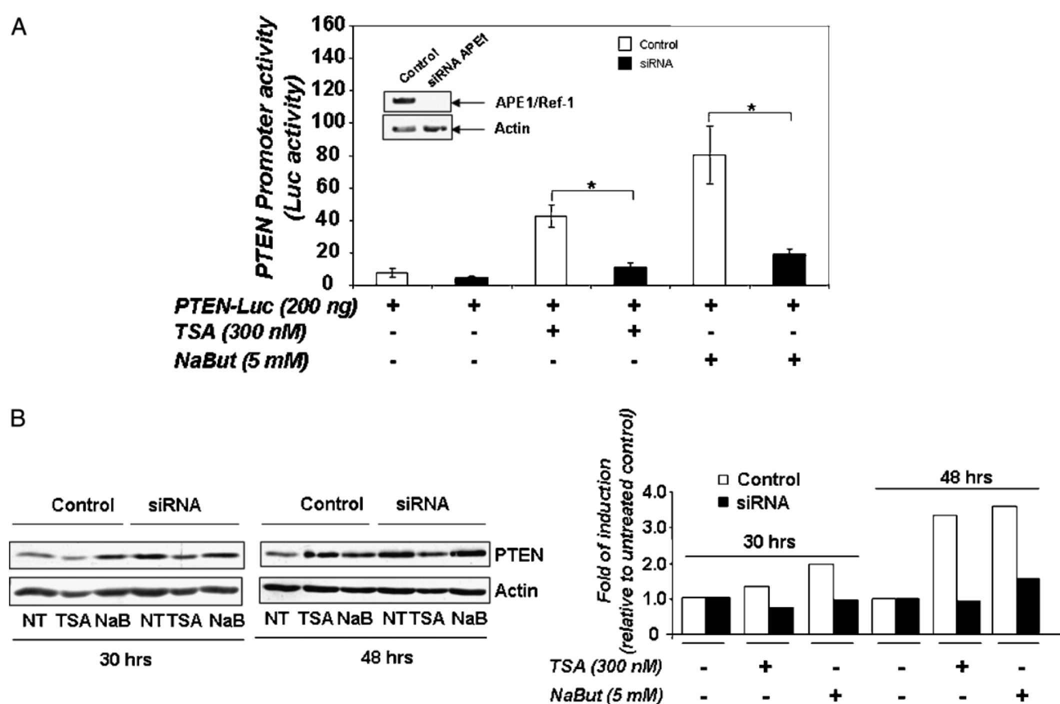


Figure 4. PTEN upregulation due to HDAC inhibition requires APE1. (A) Induction of PTEN promoter activity by HDACs inhibitors is abrogated in APE1 knocked-down cells. After 10 days of doxycycline treatment, the APE1 siRNA-expressing HeLa cells were transfected with PTEN promoter-Luc plasmid, followed by treatment with TSA and NaBut for 30 h. The cells were harvested 24 h later for measuring Luc and $\beta$-Gal activities. Luciferase activity was normalized with co-expressed $\beta$-galactosidase. Bars indicate the mean value $\pm$ SD of three independent experiments performed in duplicate ($*p < 0.05$ by Student's *t*-test). The inset shows the endogenous APE1 protein levels, in control and APE1 siRNA-expressing cells. (B) Prevention of HDACs inhibitor-dependent PTEN activation in APE1 knocked-down cells. APE1 siRNA-expressing HeLa cells were treated with TSA and NaBut for various times and PTEN level was determined. Other details are as in Figure 3.

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responsive element, that includes three Egr-1 binding sites [16]. This promoter also contains the p53 response element upstream to the Egr-1 binding sites (Figure 5A). HCT116$^{-/-}$ and HCT116$^{+/+}$ cells were transfected with plasmids encoding wild type Egr-1 and p53 and their effects on the PTEN promoter were analysed in cells ectopically expressing APE1 (Figure 5B). It is evident that APE1 over-expression caused significant ($\sim$2-fold) and similar activation of the PTEN promoter in both cell lines, indicating that the stimulatory activity of APE1 is independent of the p53 status but depends on Egr-1. To further confirm the lack of involvement of p53 in acetylated APE1-dependent PTEN upregulation, the effect of HDACs inhibitors on endogenous PTEN expression in HCT116$^{-/-}$ and HCT116$^{+/+}$ cells was examined (Figure 5C). Western blot analysis on total cell extracts showed that the increase in PTEN expression was dependent on HDACs and independent of the p53 status of the cell.

## Discussion

APE1 has two distinct functions: in DNA repair and in transcriptional regulation [1,2]. The essentiality of APE1 for cellular survival and its frequent over-expression in tumour cells strongly suggest APE1's role in preventing cell death and in controlling cellular proliferation [4,21]. However, its ability to activate transcription factors, such as p53 and Egr-1 [10,11,40,41], that are mainly involved in controlling cell-cycle arrest and apoptosis, raises the possibility of opposite regulatory roles of APE1 in different cellular contexts. Thus, APE1 could act as a transcriptional co-activator or as a co-repressor for distinct transcription factors. It is also possible that these different functions are organized in a hierarchical manner.

In the present study, we tested whether PTEN, an Egr-1 target gene [10,16], is regulated by APE1 as we had previously suggested [11]. The experiments involving ectopic APE1 expression as well as down-regulation of the endogenous gene indicated that inducible PTEN expression is indeed regulated by APE1. As post-translational acetylation of APE1 was shown to repress the PTH promoter by binding to the nCaRE [6], we checked whether acetylation of APE1 may also control PTEN inducible activation. While over-expression of the wild-type APE1 caused PTEN promoter activation, similar ectopic expression of the non-acetylatable R6/R7 mutant had a marginal effect, suggesting that acetylation of APE1is involved in PTEN activation. Interestingly, oxidative stress, known to induce APE1 expression, enhanced APE1 acetylation with a concomitant stimulation of PTEN expression. Complementarily,

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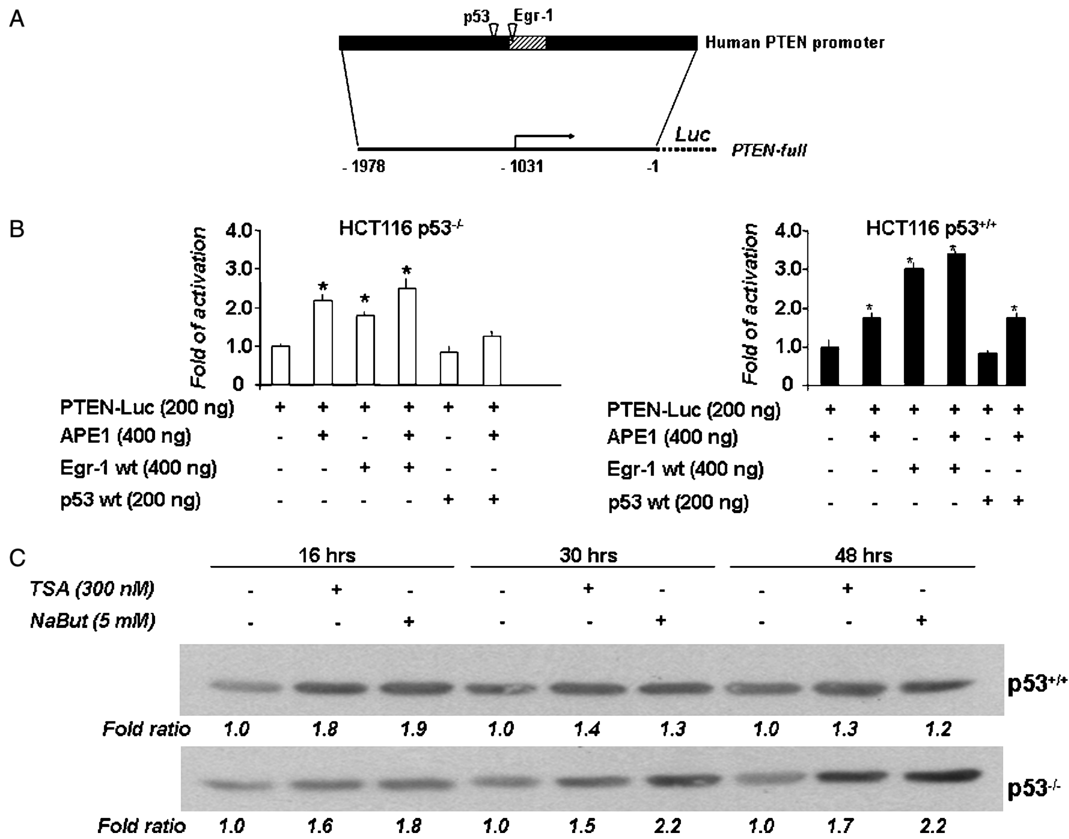


Figure 5. APE1-mediated PTEN upregulation is Egr-1-dependent and p53-independent. (A) Schematic representation of 2kb PTEN promoter, numbered relative to the translation start site (+1). The arrow represents the transcription start site. (B) HCT116 ($p53^{+/+}$) and HCT116 ($p53^{-/-}$) cells [30] were co-transfected with various plasmids as indicated and, 48 h later, the cells were harvested for measuring Luc and $\beta$-Gal activities. The effect of APE/Ref-1 alone or on the Egr-1-induced activity of PTEN promoter in p53-null $HCT116^{-/-}$ cells is shown on the left and in p53-expressing $HCT116^{+/+}$ cells is on the right. The bars indicate the mean value $\pm$SD of three independent experiments (*$p<0.05$ by ANOVA, with respect to control). (C) PTEN upregulation due to HDACs inhibition is independent of p53. $HCT116^{-/-}$ and $HCT116^{+/+}$ cell lines were treated with TSA or NaBut as before. Variation between two independent experiments is less than 15%.

treatment with HDACs inhibitors, TSA and NaBut, increased the acetylation level of APE1 together with enhanced PTEN expression, suggesting that induction of PTEN is mediated by APE1. These conclusions were confirmed with co-transfection studies where PTEN expression was examined in APE1 knocked-down cells. Experiments with the HDACs-inhibitors further confirmed that transcriptional activation is responsible for PTEN overexpression. It is known that oxidative stress promotes PTEN functional inactivation through cysteine oxidation [42]. Thus, the apparent paradox of the induction of PTEN expression by oxidative stress, that we observed in HeLa as well as in thyroid cells [19] and in osteoblasts (data not shown), can be explained in terms of cellular adaptive mechanisms aiming at the homeostatic regulation of endogenous PTEN functional levels. Further work is needed to address this issue.

The PTEN promoter is controlled not only by Egr-1 but by p53 as well [36]. However, Egr-1 is involved primarily in the stress-mediated induction of the PTEN gene [16]. Our results, using HCT116 cell lines nearly isogenic for p53, demonstrated that APE1-mediated PTEN expression was dependent on the trans-acting function of Egr-1 rather than on that of p53. While this work was in progress, Pan et al. [43] demonstrated that PTEN expression was induced by histone deacetylase inhibitor TSA treatment via Egr-1 activation. Our data provide a novel molecular basis for such an activation, suggesting an upstream regulatory role of acetylated APE1 in Egr-1 activation. The precise mechanism of APE1's action in Egr-1-driven PTEN expression is unknown. Our attempts to demonstrate stable interaction between acetylated APE1 and Egr-1 were unsuccessful, similarly to the case observed for p53 [8]. However, the notion that APE1 may redox-regulate Egr-1 [10,11] supports the existence of a transient and weak interaction between these factors. We are currently investigating the role of APE1 acetylation in modulating interaction with Egr-1.

The double-function of APE1 in gene regulation is an example of an apparent biological paradox. In fact, while a number of papers clearly demonstrated APE1's anti-apoptotic roles as well as its positive effect on cell proliferation [1,2,4,21], there is some evidence for its potential role in controlling

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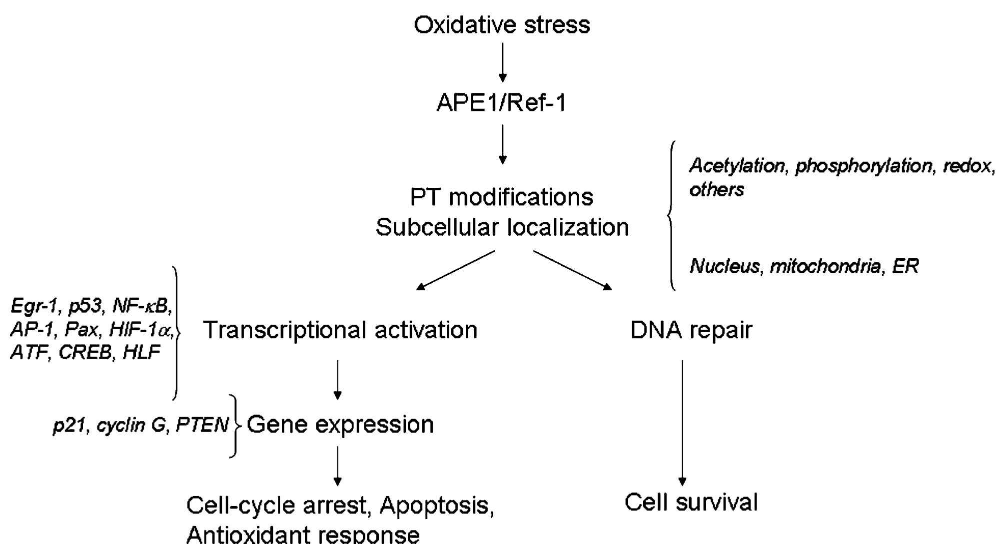


Figure 6. Model of APE1 multifunctional roles in coordinating cell response to oxidative stress. APE1 has dual function in cellular response to oxidative stress by acting as an AP-endonuclease in DNA-repair and as a transcriptional regulator of various transcription factors leading to cell-cycle arrest or to cell survival. APE1's post-translational modifications and regulation of its intracellular trafficking may be critical in *in vivo* fine-tuning of its activities.

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proapoptotic functions through p53-mediated activation [8,40,41] of p21, leading to cell cycle arrest by inhibiting cyclin-dependent kinase [44] and cyclin G [45]. It is also evident that the anti-apoptotic roles of APE1 are ascribable to its DNA-repair functions [4], rather than to its activities as a transcriptional co-activator (Figure 6). Thus, it is tempting to speculate that the latter function, the activation of transcription factors p53 and Egr-1, which ensures efficient cell-cycle arrest, may act in concert with repair of DNA damage to protect cells from accumulation of oxidative damage. Obviously, for proper modulation of these two interconnected functions fine-tuning is needed for regulating APE1 activities. Thus, an in-depth understanding of the processes controlling: (i) APE1's subcellular distribution; (ii) its post-translational modifications; (iii) its turnover and (iv) the recruitment of its diverse interacting partners in response to various endogenous or external signals, is required to unravel the complex picture.

Interestingly, the dual role of proteins in transcriptional regulation and DNA repair via diverse pathways may be a common phenomenon, as observed in the cases of BRCA1, ATM and p53. These regulatory proteins are involved in homologous recombinational repair (HR), non-homologous end joining (NHEJ), nucleotide excision repair (NER), base excision repair (BER) and mismatch repair (MMR) [46]. The existence and the precise regulation of a switching mechanism shifting cells from the DNA repair mode to apoptosis is central to avoidance of cancer progression, by preventing clonal expansion of cells in which unrepaired damage would lead to mutation and transformations. It would be interesting to see whether a cross-regulation, in terms of expression, does exist among partners of different pathways.

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