SIRT1 gene expression upon genotoxic damage is regulated by APE1 through nCaRE-promoter elements

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Running Title: APE1 regulation of biological functions

Abbreviations: **8-oxoG**; 8-hydroxyguanine; **APE1**, Apurinic apyrimidinic endonuclease/Redox effector factor 1; **BER**, base excision repair; **hAPE1**, human APE1; **GO**, Gene Ontology; **hnRNPL**, heterogeneous ribonucleoprotein L; **LC-ESI-MS**, liquid chromatography-electrospray ionization-mass spectrometry; **MMS**, Methyl methanesulfonate; **nCaRE**, negative calcium responsive element; **PTH**, parathyroid hormone; **SIRT1**, sirtuin-1; **SPR**, Surface Plasmon Resonance; **THF**, Tetrahydrofuran; **zAPE1**, zebrafish APE1.

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ABSTRACT

The Apurinic/apyrimidinic endonuclease 1 (APE1) is a multifunctional protein contributing to genome stability, through the repair of DNA lesions via BER pathway, also playing a role in gene expression regulation and in RNA metabolism. Another poorly characterized function is its ability to bind to negative calcium responsive elements (nCaRE) of some gene promoters. The occurrence of many functional nCaRE sequences, regulating gene transcription, could be envisioned since their conservation within ALU repeats. In order to look for functional nCaRE sequences within the human genome, we performed bioinformatic analyses and identified a list of 57 genes potentially regulated by APE1. We focused on the SIRT1 deacetylase due to its involvement in cell stress, including senescence, apoptosis and tumorigenesis, and its role in the deacetylation of APE1 after genotoxic stress. The human SIRT1 promoter presents two nCaRE elements stably bound by APE1 through its N-terminus. We demonstrate that APE1 is part of a multi-protein complex including hOGG1, Ku70 and RNA Pol II, which is recruited on SIRT1 promoter to regulate SIRT1 gene functions during early response to oxidative stress. These findings provide new insights in the comprehension of the role of nCaRE sequences in the transcriptional regulation of mammalian genes.

INTRODUCTION

The Apurinic/apyrimidinic endonuclease 1 (APE1), also known as Redox effector factor-1 (Ref-1), is a multifunctional and essential protein in mammals. It plays a vital role during the cellular response to oxidative stress (Fung *et al.*, 2005) and contributes to the maintenance of genome integrity (Tell *et al.*, 2005; Tell *et al.*, 2009; Tell *et al.*, 2010a). As AP-endonuclease, APE1

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is involved in the base excision repair (BER) pathway that copes with DNA damages induced by oxidative and alkylating agents, including chemotherapeutics (Chen *et al.*, 2005). APE1 has also a transcriptional regulatory activity, modulating gene expression through a redox-based co-activating function on several transcription factors involved in cancer promotion and progression (Huang *et al.*, 1993; Tell *et al.*, 1998; Gaiddon *et al.*, 1999). These two major APE1 activities are independent and located in distinct protein domains. The N-terminal portion of the protein is devoted to the transcriptional co-activating function, while the C-terminal domain exerts the endonuclease activity on DNA abasic sites (Xanthoudakis *et al.*, 1996; Tell *et al.*, 2005). The latter domain is highly conserved; conversely, the N-terminal region presents wider variability among different organisms, being more conserved in mammals, thus suggesting a recent acquisition during evolution (Georgiadis *et al.*, 2008; Fantini *et al.*, 2010; Poletto *et al.*, 2013). Through its N-terminal portion, APE1 also interacts with different proteins involved in ribosome biogenesis, pre-mRNA maturation/splicing and ribonucleotide catabolism. These observations highlighted an unexpected role of APE1 in RNA metabolism (Vascotto *et al.*, 2009b; Tell *et al.*, 2010b), as also shown by two independent studies that demonstrated the ability of APE1 to cleave abasic RNA *in vitro* and *in vivo* (Berquist *et al.*, 2008; Barnes *et al.*, 2009). Accordingly, APE1 has been proposed to act as a main candidate factor in the abasic RNA cleansing process, thus explaining that some of the activities exerted by APE1 on gene expression may involve post-transcriptional mechanisms (Tell *et al.*, 2010b).

Another interesting, yet poorly characterized aspect of APE1 transcriptional activity is represented by its role as a component of a *trans*-acting complex, which acts as a Ca^{2+}-dependent repressor of the parathyroid hormone (PTH) gene by binding the negative calcium responsive elements (nCaRE) in its promoter region (Okazaki *et al.*, 1991). In particular, an increase in extracellular Ca^{2+} concentration has been shown to inhibit PTH expression through a mechanism involving APE1 binding to two nCaRE elements, nCaRE-A and nCaRE-B (Yamamoto *et al.*, 1989). This observation was further extended to the promoter region of renin (Fuchs *et al.*, 2003), Bax (Bhattacharyya *et al.*, 2009) and APE1 itself (Izumi *et al.*, 1996); the latter case represents the first example of such a negative regulatory mechanism for a DNA repair enzyme. Subsequently, further experiments have demonstrated that APE1 requires additional factors, such as heterogeneous ribonucleoprotein L (hnRNPL) (Kuninger *et al.*, 2002), Ku antigen (KuAg) (Chung *et al.*, 1996) and PARP-1 (Bhattacharyya *et al.*, 2009) to stably bind to nCaRE elements.

nCaRE-B sequences are located within ALU repeats (McHaffie *et al.*, 1995; Shankar *et al.*, 2004); therefore, since ALU elements are transposable elements that occupy at least one tenth of the expressed human genome, many other functional nCaRE-B sequences could exist and play a role in the transcriptional regulation of genes. However, at present, specific information concerning: i) an accurate numbering; ii) the identity of genes containing these sequences within their own promoter, and iii) how these elements play an active biological function are still evanescent. Thus, the quest for functional nCaRE-B sequences on human genome would identify new potential genes whose expression may be regulated by APE1 through nCaRE binding.

The present work is devoted to address this issue and is focused on the characterization of the molecular mechanisms responsible for APE1 binding to nCaRE-B sequences on SIRT1 promoter. Bioinformatic analyses of human gene expression data obtained upon APE1 knock-down in cells (Vascotto *et al.*, 2009a) revealed the presence of multiple nCaRE-B sequences in genes deregulated upon APE1 silencing and conserved in mouse genome. Among these, we focused our studies on the two nCaRE sequences present within the promoter region of the human deacetylase sitruin 1 (SIRT1) gene, and their role in regulating the corresponding gene transcription. SIRT1 is a deacetylase participating in cell growth, adaptation to caloric restriction, apoptosis, tumorigenesis (Gorospe *et al.*, 2008; Kim *et al.*, 2008), and playing also a role in cell response to genotoxic agents through the deacetylation of APE1 (Yamamori *et al.*, 2010). Altogether, our data underline the importance of APE1 during the transcriptional initiation process, in positively promoting transcription of genes under genotoxic conditions.

RESULTS

Bioinformatic searching for nCaRE sequence-containing genes reveals SIRT1 gene as a novel candidate target of APE1 regulation

Bioinformatic analysis for the systematic retrieval of functional nCaRE-B sequences in the human genome was carried out by filtering through biological data obtained from gene expression profile of HeLa cells knocked-down for APE1 (Vascotto *et al.*, 2009a). Here, we developed a method that integrates different approaches devoted to address the problem on a whole genome scale, while minimizing the number of false positives. To this purpose, classical DNA pattern matching studies were integrated with independent information on gene regulation. We used three main sources for data filtering: i) functional annotation data collected in GO (Gene Ontology); ii) gene expression data derived from the microarray profile of APE1 knock-down HeLa cells (Vascotto *et al.*, 2009a); iii) human-mouse gene sequence comparisons. In fact, both expression data and functional annotation database are known to provide a wealth of information about co-regulation. This is of particular interest, since co-regulated genes likely share similar transcriptional regulatory mechanisms. At the same time, comparison with orthologous gene promoters highlighted sequences retained during evolution, whose conservation suggests their potential functionality. As a final result, we obtained a set of genes that passed the previously mentioned filters and might be considered as *bona fide* candidates co-regulated through nCaRE-B sequences (Supplemental Figure S1).

Practically, we collected the 6000 bp upstream regions of all human and mouse protein coding genes, which were then analysed for the presence of nCaRE-B elements using Glsearch as program for local alignment (Pearson, 2000). 8724 human genes were found to contain one or more nCaRE-B matches within their promoters; similarly, 2173 matches were retrieved in the mouse genome. We then cross-checked candidate genes with gene expression data obtained from APE1 knock-down cells (Vascotto *et al.*, 2009a), thus verifying some evidences of co-expression between genes carrying nCaRE-B elements. Through this analysis, we identified 384 common genes in the two datasets (Figure 1A). Then, we considered the GO annotations of these 384 genes, searching for statistically significant common annotations. We observed a strong over-representation of terms related to RNA processing and metabolism, in accordance to our previous studies (Vascotto *et al.*, 2009a). All the significant associations between genes and GO terms are reported in Supplemental Table S1. Finally, we applied the "phylogenetic footprinting filter" that evaluates whether a significant fraction of the selected genes, as obtained through the GO filter, shares homologous genes containing nCaRE-B-related sequences in the upstream region, with respect to the mouse gene dataset. As a final result, we extracted 57 genes that may be considered *bona fide* candidates bearing the putative nCaRE-B sequences within their regulatory elements (Supplemental Table S2). We performed a functional enrichment analysis to unveil if the 57 genes found are involved in common biological processes. This examination showed that candidate genes were associated with processes related to gene expression, activation or increment of the extent of transcription from an RNA polymerase II-driven promoter (Figure 1B and Supplemental Table S3). Among these 57 genes, several are involved with DNA repair process and cellular response to external stimuli and DNA damage, e.g. SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a member 4 (SMARCA4), sirtuin 1 (SIRT1), valosin-containing protein (VCP), multiple endocrine neoplasia I (MEN1), structural maintenance of chromosomes protein 6 (SMC6), early growth response 1 (EGR-1) and APE1 itself. Particular attention was paid on SIRT1, a NAD-dependent histone deacetylase belonging to the class III of the sirtuin family, based on the recent demonstration of a functional involvement of this enzyme in the deacetylation of some K residues in the N-terminal region of APE1 (Yamamori *et al.*, 2010; Lirussi *et al.*, 2012). The latter information and the data derived from our bioinformatic analysis led us to hypothesize the existence of an auto-regulatory loop between APE1 and SIRT1.

APE1 binds the nCaRE-B sequences present in the human SIRT1 promoter

In order to prove the functional relevance of the nCaRE-B sequences identified in the human SIRT1 promoter, we first tested the ability of APE1 to specifically bind these elements *in vitro*. Thus, we performed EMSA analyses using different APE1 recombinant protein forms and two double stranded (ds) oligonucleotides containing the SIRT1 nCaRE-B elements corresponding to the sequences present at -2701 (SIRT1-A) and -1754 bp (SIRT1-B) from the transcription start site (TSS), respectively (Figure 2A). Full length human wild type APE1 ($APE1^{WT}$), the N-terminal APE1 deletion mutant ($APE1^{N\Delta33}$) and the orthologous APE1 from zebrafish (zAPE1) expressed in *E. coli* were used to this purpose. As clearly demonstrated by the EMSA analyses, only the $APE1^{WT}$ protein was able to stably bind to both the SIRT1 nCaRE-B sequences (Figure 2B, lanes 2 and 6), while a complete absence of retarded complex was observed in the case of the truncated $APE1^{N\Delta33}$ form (Figure 2B, lanes 3 and 7). These findings underlined the importance of the first 33 amino acids present at APE1 N-terminus for a proper binding of the protein to nCaRE-B sequences. A similar poor DNA binding activity was also apparent in the case of zAPE1 (Figure 2B, lanes 4 and 8), which bears a non-related N-terminal domain (Fantini *et al.*, 2010). Altogether, these results suggest that the phylogenetically-evolved N-terminal domain of the protein is essential for a stable interaction between APE1 and the nCaRE-B sequences; in this context, it is conceivable that K residues present within this region, and acquired during phylogenetic evolution in mammals (Georgiadis *et al.*, 2008; Fantini *et al.*, 2010; Poletto *et al.*, 2013), may play a major role in protein binding to these DNA elements.

We then estimated the affinity of APE1 for SIRT1 nCaRE-B sequences through SPR analysis (Figure 2C). Biotinylated versions of the nCaRE-B (SIRT1-B) or polyT sequences were immobilized onto a streptavidin chip to be used as ligands in SPR experiments. $APE1^{WT}$ and $APE1^{N\Delta33}$ were then analyzed for their DNA binding properties. When testing $APE1^{WT}$ as analyte, a K_D value of 3.90±0.08 µM was measured (Poletto *et al.*, 2013); corresponding kinetic parameters are shown in Table 1. Conversely, when using $APE1^{N\Delta33}$, we did not observe any SPR signal variation (data not shown), in accordance with EMSA experiments (Figure 2B). This result confirmed that the protein region 1-33 is essential for a stable interaction of APE1 with these DNA elements. As a DNA-repair enzyme, APE1 has an intrinsic ability to bind to DNA in a sequence-independent manner; moreover, independent observations clearly pointed to a role of the nucleic acid secondary structure in positively modulating this activity (Poletto *et al.*, 2013). Here, we evaluated the protein capacity to bind to a single-stranded 24-mer oligo-dT (here called polyT). A K_D value of 308±3 µM was measured in the case of $APE1^{WT}$, while no binding was observed for $APE1^{N\Delta33}$ (Table 1 and Supplemental Figure S2), in agreement with EMSA analysis. These results indicated that APE1 poorly recognizes a non-structured oligonucleotide formed by a stretch of thymidines, confirming that this protein may bind to DNA with different affinities and the oligo-dT sequence may be used in EMSA analysis as a non-specific competitor.

Our EMSA and SPR analyses, on APE1 binding activity to the SIRT1 nCaRE-B sequences, showed a low affinity for recombinant APE1 alone; therefore, we tested whether additional factors, present in nuclear extracts of the cells, may increase the protein binding affinity to its oligonucleotide target. To this aim, further EMSA analyses, performed using HeLa cells nuclear fractions, confirmed that a nuclear activity able to specifically bind nCaRE-B sequences was indeed present (Figure 2D). The high affinity complex measured, even using much lower amounts of APE1 (0.63 pmol, as estimated from (Lirussi *et al.*, 2012)) with respect to that obtained with the recombinant purified protein alone (10 pmol) (Figure 2B), suggested that additional factors are required for an efficient APE1 binding to SIRT1 nCaRE-B sequences. To demonstrate the presence of APE1 in the retarded complex observed in Figure 2D, we used nuclear extracts obtained from a HeLa line (CL.3), where endogenous APE1 protein expression has been previously knocked-down through stable shRNA transfection (Vascotto *et al.*, 2009a). As apparent from lane 3 in Figure 2D, an overall reduction of the intensity (almost 70% with respect to lane 2) of the retarded nCaRE-

bound complex was observed, similarly to what occurred also for the clone expressing $APE1^{N\Delta33}$ (lane 5) (almost 50% with respect to lane 2). Nuclear extracts, obtained from HeLa cells reconstituted with ectopic $APE1^{WT}$ (lane 4), showed the same amount of bound complex as the control cell clone expressing a scrambled shRNA vector (indicated as SCR-1) (recovery of almost 80% with respect to lane 2). Pre-incubation of the nuclear extract from the SCR-1 clone with an anti-APE1 antibody resulted in the formation of a super-shifted complex (Figure 2E, lane 3). This complex was absent when a non-related antibody was used (lane 6), clearly demonstrating that APE1, present in the nuclear cell extract, is involved in the recognition of the nCaRE-B elements of the SIRT1 promoter. The reduced intensity of the retarded complex band upon APE1 silencing or in the reconstituted cells expressing $APE1^{N\Delta33}$ protein (Figure 2D), as well as the lower apparent electrophoretic mobility of the protein-DNA retarded complex when using nuclear extracts in place of the recombinant purified protein (Figure 2B), suggest that APE1 may be part of a multi-protein complex. Since Ku70 antigen protein was already demonstrated to bind nCaRE-A sequences in complex with APE1 (Chung *et al.*, 1996), we incubated the HeLa nuclear extract with an antibody recognizing the Ku70 antigen. The reaction was then subjected to EMSA analysis showing the formation of a super-shifted complex (Figure 2E, lane 4). Concurrent presence of APE1 and Ku70 in the same retarded complex was confirmed by performing a simultaneous pre-incubation with antibodies specific for these proteins (Figure 2E, lane 5). The presence of Ku70 in the complex with APE1 was also corroborated by additional EMSA analysis performed with the purified recombinant protein attesting that, when present alone, Ku70 is not able to stably bind to the SIRT1 nCaRE-B sequence *per se.* On the contrary, when concomitantly incubated with APE1, Ku70 significantly enhances APE1 DNA-binding activity to SIRT1 nCaRE-B element (Supplemental Figure S3 compare lanes 2-5 and 8-10). Data not shown demonstrated that this stimulatory activity on APE1 binding required the interaction through the APE1 33N-terminal domain. Overall, these data demonstrated that APE1 must be part of a multi-protein complex containing Ku70 to elicit its high-affinity binding potential to the nCaRE-B sequences present on the SIRT1 promoter.

Topology of the APE1-nCaRE complex

nCaRE-B element consists of a palindromic sequence that can possibly fold into self-complementary hairpins (Figure 3A, left panel). *In silico* analyses performed with mFold program were suggestive of the capacity of these elements to fold into cruciform-like structures. To evaluate whether SIRT1 nCaRE-B sequences can fold into cruciform duplexes and to specifically assess if APE1 binding to this element may depend on such secondary structures, we performed footprinting analyses by using the T7 endonuclease I (Figure 3A, right panel). This protein is a structure-sensitive enzyme that specifically recognizes conformationally-branched DNA and Holliday structures or junctions (Parkinson *et al.*, 1997; Déclais *et al.*, 2003; Fan *et al.*, 2006). Footprinting data supported the hypothesis of the existence of a secondary structure for the nCaRE-B sequences (Figure 3A and Supplemental Figure S4). In particular, our experiments showed the predominant formation of two bands (10 and 19 nt in length) in the digested samples, which corresponded to a cutting site close to the predicted loop or immediately adjacent to the predicted stem (Figure 3A, arrows). Interestingly, pre-incubation of the SIRT1 nCaRE-B oligonucleotide with APE1 impairs T7 endonuclease digestion (Figure 3A, lane 4). Consistent with these observations, EMSA analysis demonstrated that the digestion of the SIRT1 nCaRE-B oligonucleotide with T7 endonuclease affects APE1 binding to this element but conversely, when APE1 was first incubated with the nCaRE-B probe and then digested with T7 endonuclease, the binding was not affected. These data were suggestive for a protective role of APE1 to T7 endonuclease action, strongly supporting the hypothesis that these proteins may compete for the same binding site on the nCaRE-B sequences (Figure 3B).

The first 33 N-terminal amino acids of APE1 are required for protein binding to the nCaRE sequences (Figure 2, A and D). In order to further investigate the role of this protein portion on APE1 ability to bind to DNA elements, combined limited proteolysis-mass spectrometry

experiments were performed on recombinant APE1 protein either in the absence or the presence of its target nCaRE oligonucleotide (Figure 3C, left). In particular, parallel experiments with a specific proteolytic enzyme (used at a defined APE1 to protease w/w ratio) were carried out on a time-course basis on: i) isolated APE1; ii) APE1 complexed with SIRT1 nCaRE-B oligonucleotide; iii) APE1 complexed with PTH nCaRE-B oligonucleotide (Okazaki *et al.*, 1992) (see Supplemental Information Figure S5 and Supplemental Table S4). The latter was used as control. Resulting differential peptide maps provided information on amino acid(s) eventually protected from proteolytic attack as result of their occurrence at the protein-DNA complex interface (Figure 3C, left). In this context, all data were interpreted according to the concept of "molecular shielding" of amino acids from proteolytic attack (Scaloni *et al.*, 1998; Scaloni *et al.*, 1999; Renzone *et al.*, 2007) and were rationalized on the basis of the X-ray crystallographic APE1 structures reported so far (Gorman *et al.*, 1997; Beernink *et al.*, 2001). In order to maximize information resulting from limited proteolysis experiments, different proteolytic enzymes were used in separate assays.

Figure 3C, right summarizes the overall results of the limited proteolysis experiments, as obtained by using different proteases on recombinant APE1 alone (Figure 3C, top) or after its complex formation with the SIRT1 nCaRE substrate (Figure 3C, bottom) (see also Supplemental Figure S5 and Supplemental Table S4 for experimental details). General considerations can be driven concerning the native protein, as follows. Preferential hydrolyzed peptide bonds on isolated APE1 gathered into a specific region of the protein, namely the most exposed segment being the unstructured N-terminal domain, which contained 6 proteolytic sites (K6, K7, A9, A11, D15 and L17). An additional site occurred within the globular APE1 domain (L111). Interestingly, no other cleavage sites were detected in other protein regions, although exposed on the molecular surface (Gorman *et al.*, 1997; Beernink *et al.*, 2001). After complex formation with nCaRE-B oligonucleotides, a marked protective effect was observed, as demonstrated by the large decrease in the number of proteolytic sites present in the N-terminal region (from 6 to 1), thus confirming the involvement of this APE1 portion in binding to these DNA elements.

Transcriptional regulation of SIRT1 expression by APE1 protein

To determine whether the observations obtained *in vitro*, regarding APE1 binding to SIRT1 nCaRE-B sequences, have any relevance *in vivo*, we examined the APE1 occupancy of the nCaRE-B sequence in the SIRT1 promoter through ChIP analyses. To this purpose, we used HeLa cells co-transfected with a human SIRT1 promoter-containing plasmid (Yamamori *et al.*, 2010) and FLAG-tagged $APE1^{WT}$- or $APE1^{N\Delta33}$-expressing plasmids. The amount of immunoprecipitated SIRT1 promoter was significantly enriched in $APE1^{WT}$-transfected cells, when compared with that obtained from control cells transfected with the empty vector alone (Figure 4A). As expected, $APE1^{N\Delta33}$-transfected cells had a remarkable reduction, even though not complete, in the amount of immunoprecipitated SIRT1 promoter. A similar degree of reduction was also observed when ChIP analysis was performed by using a SIRT1 promoter bearing a mutated sequence within the nCaRE-B motif (Figure 4B), whose reduced specificity was previously assessed through SPR analysis (Table 1 and Supplemental Figure S2). These experiments revealed that the mutation introduced in the nCaRE-B sequence significantly reduced the APE1 binding to DNA, lowering the affinity of the complex by a 30-fold factor (K_D = 119 ± 3 μM). Competitive EMSA analyses were in agreement with these findings. In fact, addition of the unlabeled nCaRE ds oligonucleotide resulted in an almost complete elimination of bound complex formation. On the other hand, competition with the unlabeled mutant nCaRE oligo (nCaRE-mut) caused only a slight reduction of the nCaRE-binding complex, in agreement with ChIP and SPR data, and supports the notion of a sequence-dependent binding specificity (data not shown). These data were also confirmed with the endogenous APE1 promoter through Chip-Seq analyses (data not shown) further demonstrating the physiological relevance of these findings. Altogether, these results confirmed our *in vitro* observations (Figure 2) and support the hypothesis that, under basal conditions, APE1 is associated with the nCaRE-B sequence within the SIRT1 promoter also *in vivo*, possibly as part of a multiprotein complex.

We then evaluated if APE1 binding to SIRT1 promoter may play a role in SIRT1 transcriptional regulation by performing promoter-reporter assays. HeLa cells were co-transfected with a luciferase reporter vector bearing the SIRT1 promoter and $APE1^{WT}$ FLAG-tagged vector (Figure 4C). SIRT1 promoter-reporter assays showed that there was a significant increase in the luciferase signal detected in the presence of APE1, compared to that of the promoter alone. We evaluated the effect of APE1 silencing on endogenous SIRT1 mRNA expression levels through an inducible shRNA knock-down strategy (Vascotto *et al.*, 2009a,b). Endogenous APE1 knockdown (CL.3) caused a significant reduction in the SIRT1 endogenous expression levels (Figure 4D), which was rescued in cells reconstituted with a siRNA resistant APE1 cDNA expression plasmid (WT). These data demonstrated a positive effect of APE1 on SIRT1 transcriptional activation; although unexpected, since previous published data reported a transcriptional repressive role for APE1 through nCaRE sequence binding (Okazaki *et al.*, 1991; Fuchs *et al.*, 2003), these data are in agreement with our previous observations on gene expression profiling analysis (Vascotto *et al.*, 2009a), in which a reduced expression of SIRT1 in APE1 knocked-down HeLa cells was apparent.

Oxidative stress induces SIRT1 transcription via recruitment of BER enzymes

In order to better understand, at the molecular level, the transcriptional function exerted by APE1 through the binding to nCaRE-B sequences, we investigated if APE1's positive role on SIRT1 transcription could rely on its enzymatic activity on DNA. First, we observed that under basal conditions APE1 has no endonuclease activity on the SIRT1 nCaRE-B sequence, as assessed through an oligonucleotide cleavage assay (Figure 5A). APE1 cleavage, occurring at any site on cruciform nCaRE-B sequence, should lead to a site-specific single-stranded break that can be detected by the appearance of an extra fragment in the cleavage assay. Even using increasing amount of purified recombinant APE1 protein, we did not detect any nuclease activity that, on the contrary, was readily visible when using a radiolabeled 26-mer ds oligonucleotide containing a tetrahydrofuran mimicking an AP site (here referred as THF) (Berquist *et al.*, 2008). Substitution of the guanine residue at position 12, within the predicted loop of the nCaRE-B sequence, with a THF residue, resulted instead in an efficient APE1 cleavage activity on the nCaRE-B sequence. This activity depends on the APE1 catalytic function, since the catalytically inactive $APE1^{E96A}$ mutant (Izumi *et al.*, 1999) was unable to efficiently cleave the same substrate (Figure 5B).

Prompted by these findings, we hypothesized that the APE1 positive function, that we observed on SIRT1 promoter, may be ascribed to the APE1 catalytic activity on nCaRE-B sequences; this phenomenon can be exerted after specific stimuli, such as oxidative stress, which can lead to abasic site formation on nCaRE-B sequences (Amente *et al.*, 2010; Francia *et al.*, 2012). It is well known that SIRT1 expression and function are regulated by external stressors, including the exposure to genotoxic agents (Kim *et al.*, 2008; Yamamori *et al.*, 2010; Cohen *et al.*, 2004). We therefore measured the SIRT1 transcription after an oxidative stress condition, such as that generated by H_2O_2 exposure. First, we demonstrated that SIRT1 promoter activation was increased by H_2O_2 treatment in a concentration-dependent manner (Figure 5C). Next, we examined the effect of APE1 silencing or re-expression on SIRT1 transcriptional activation in HeLa cell clones upon H_2O_2-treatment (Figure 5D and Supplemental Figure S6). We treated the control (SCR-1), the APE1-silenced (CL.3) and the $APE1^{WT}$ cell clones with 1 mM H_2O_2 for 1 h. SIRT1 mRNA levels were then evaluated by Q-PCR analysis and compared with untreated clones. Upon oxidative treatment, SIRT1 mRNA resulted significantly increased and, notably, this response was higher in the presence of $APE1^{WT}$ protein, whereas it was lower in the case of APE1 knocked-down expressing cells. The residual activation of SIRT1 mRNA expression, which was apparent also in APE1 knocked-down cells, might be ascribable to the presence of residual endogenous APE1 protein and/or to the existence of further limiting factors, as already speculated (Chung *et al.*, 1996; Kuninger *et al.*, 2002; Bhattacharyya *et al.*, 2009). The observed induction of SIRT1 mRNA transcription in HeLa cells upon oxidative stress correlates with a parallel increase in its protein levels. Confirmation of the biological relevance of our findings was definitely demonstrated by the

observation of concomitant increased deacetylation activity of SIRT1 protein toward its substrate, i.e. K382 of p53 (Vaziri *et al.*, 2001) (Supplemental Figure S6).

We also confirmed the general relevance of our model by testing other hypothetical APE1 target genes containing an nCaRE-B element in their promoters and resulted dysregulated in APE1-kd cell model (Figure 1 and Supplemental Table S2) (Vascotto *et al.*, 2009a). To this aim, the expression levels, upon H_2O_2-treatment, of the Early growth response protein 1 (EGR-1) and Eukaryotic translation initiation factor 4E-binding protein 1 (EIF4EBP1), in the HeLa cell inducible-kd (CL.3) clone and in the reconstituted cell clone (WT) used here, were evaluated by Q-PCR analysis (Figure 5E). Similar results were also obtained using another genotoxic agent such as Methyl methanesulfonate (MMS) (Supplemental Figure S9). These data, showing inducible expression of these genes dependent on APE1 expression to a similar extent of that observed in the case of SIRT1, were suggestive of a general mechanism of gene activation upon DNA damage that involves APE1 binding to nCaRE-B elements.

To find a relationship between SIRT1 transcription induced by oxidative stress and the APE1 regulatory activity on the nCaRE-B sequences located within the promoter, we studied the dynamics of oxidative repair enzymes recruitment on the SIRT1 nCaRE-B sequence at early time upon H_2O_2 treatment. DNA base oxidation determines the formation of 8-oxodG, which is recognized by the DNA glycosylase OGG1. This enzyme initiates the BER pathway by removing the 8-oxodG lesion, which is further processed by APE1 that cleaves the apurinic site. To assess the dynamics of occupation of the nCaRE-B sequence on the SIRT1 promoter by these enzymes upon oxidative stress, we performed a time-course ChIP analysis on the SIRT1 nCaRE-B sequence after 1 mM H_2O_2 treatment (Figure 5F and Supplemental Figure S7). We immunoprecipitated SIRT1 nCaRE-B sequence with antibodies against 8-oxodG, OGG1 and APE1. We observed that the signal of 8-oxodG promptly reached its plateau, 10 min upon H_2O_2 treatment and subsequently decreased, concomitantly with the accumulation of OGG1, that is recruited immediately later (15 minutes), in accordance with the BER processes. The occupancy by APE1 follows the OGG1 recruitment, consequently. To demonstrate a direct link between DNA repair and SIRT1 transcriptional initiation, we checked the assembly of RNA polymerase II (RNPII) on SIRT1 nCaRE-B sequence. Under basal condition, we observed that RNPII was found with relatively low abundance on the SIRT1 nCaRE-B sequence; conversely, the polymerase was progressively recruited as soon as the oxidative stress began (15 min after H_2O_2 treatment). Afterwards (at 40 min after H_2O_2 addition), RNAPII was again recruited on the SIRT1 promoter showing a waiving behaviour. In concert with this observation, we also noticed an augmented interaction between APE1 and RNPII in accordance with the kinetics observed during ChIP analysis (Supplemental Figure S8). This oxidatively-induced recruitment of RNPII to the SIRT1 promoter may therefore suggest that oxidative stress could trigger SIRT1 transcriptional activation. It could be envisioned a mechanism in which H_2O_2, causing oxidation of the guanine at the SIRT1 nCaRE-B sequence, may promote the recruitment of components of the base excision repair system: OGG1 and APE1, together with proteins involved in nCaRE-B binding such as Ku70. When recruited to the SIRT1 promoter, APE1 (through its endonuclease activity) produces nicks on the SIRT1 nCaRE-B sequence, thus possibly favouring the DNA relaxation necessary for the formation of chromatin loops that bring RNAPII at the transcription start site (Figure 6); the latter enzyme in turn can initiate transcription.

Discussion

After its cloning by independent groups, at first as a DNA repair enzyme (Demple *et al.*, 1991; Robson *et al.*, 1991) and then as a redox co-activator protein (Xanthoudakis *et al.*, 1992), a number of articles have described the different APE1 functions, elucidating its involvement in several biological contexts. As the main apurinic/apyrimidinic endonuclease in mammalian cells, APE1 is classically renowned for its essential function as a DNA repair enzyme in the BER pathway. Beside this crucial role in the maintenance of the genome stability, APE1 was recently demonstrated to be also involved in redox signaling and in the regulation of gene expression (Tell *et*

al., 2010a; Wilson *et al.*, 2010), supporting the notion that it is a multifunctional protein, with features that go beyond the classical activities of a DNA-repair enzyme. Notably, its multifunctional nature ascribes APE1 as an ideal candidate protein that links together DNA damage sensing/repair and transcriptional regulation of genes during cell response to genotoxic damage. Among these non-canonical activities, another interesting APE1 function is represented by its ability to bind the nCaRE sequence of some gene promoters, thus acting as a transcriptional regulator. Okazaki's group was the first that identified two nCaRE sequences within the PTH gene promoter (i.e nCaRE-A and nCaRE-B) (Okazaki *et al.*, 1991). The presence of these elements was also described in the regulatory region of few other genes, such as the human APE1 (Izumi *et al.*, 1996), the rat atrial natriuretic polypeptide (Okazaki *et al.*, 1992), the human renin (Fuchs *et al.*, 2003) and the bax ones (Bhattacharyya *et al.*, 2009). Beside these few genes, no further evidences have been provided so far. However, since nCaRE-B elements are present within ALU repeats, that are widely distributed throughout the expressed genome, it is expected that APE1 could potentially regulate the expression of a large number of genes. Here, we performed an unbiased investigation on the whole human genome, searching for putative genes whose transcription may be mediated through APE1 ability to bind nCaRE-B elements. In particular, although it has been described that nCaRE elements seem to be active also at downstream regions (Izumi *et al.*, 1996), here, we specifically focused on the nCaRE-B elements present only on the upstream sequence of human genes; in a near future, we plan to extend this approach also to downstream and intron regions. Bioinformatic analyses revealed a number of genes potentially regulated by APE1, which are involved in several pathways related to gene expression (Figure 1). Among the 57 candidate genes retrieved from our bioinformatic analysis, we chose to study the human deacetylase Sirtuin1 (SIRT1) that bears two nCaRE-B elements in its promoter. Remarkable interest in SIRT1 was due to recent articles showing that this deacetylase controls the acetylation status of APE1 Lysine residues K6-7 (Yamamori *et al.*, 2010) and K27-35, thus modulating the subnuclear distribution of this protein and coordinating its enzymatic functions in BER pathway (Lirussi *et al.*, 2012). Therefore, we hypothesized the existence of a possible autoregulatory loop that can be established between the two proteins: APE1 should modulate SIRT1 expression that, in turn, may regulate APE1 functions through deacetylation.

To investigate the APE1 transcriptional regulatory function on SIRT1 expression, we first examined APE1 ability to bind the nCaRE-B sequences found in the SIRT1 promoter. Through different *in vitro* approaches, we showed that APE1 is able to bind SIRT1 nCaRE-B sequences (Figures 2 and 3). In particular, through combination of EMSA and SPR analyses with limited proteolysis experiments, we originally demonstrated that the APE1 N-terminal domain is essential for the proper binding of these elements. The essential role of this protein domain in DNA-binding is remarkable, particularly if we consider the phylogenesis of the nCaRE-B elements and that of the APE1 N-terminal region. nCaRE-B sequences are present within ALU repeats, which belong to SINE (short interspersed nucleotide elements) family of repetitive sequences that originally derived from the reverse transposition of 7SL RNA. This event took place in the genome of an ancestor of Supraprimates (Kriegs *et al.*, 2007); therefore, these repetitive elements have been found exclusively in primates (Deininger *et al.*, 1981), scandentians (Nishihara *et al.*, 2002) and rodents (Krayev *et al.*, 1980), all members of the placental mammalian clade Supraprimates (Euarchontoglires) (Murphy *et al.*, 2001). Similarly, current information regarding the sequence homology of the APE1 N-terminal domain across species, have pointed out the recent phylogenetic acquisition of this region. Sequence conservation of this domain is very high in mammals but almost absent in other organisms with the exception of *Danio rerio*, *Dyctostelium* and *Drosophila*. Accordingly, it could be envisioned that once ALU elements appeared in primates and were stabilized in their genomes, progressively losing their transcriptional potential, these organisms needed to evolve novel mechanisms to cope with the acquired RNA pol II regulatory sites present within ALU region. The concomitant acquisition of the APE1 N-terminal domain in mammals could explain new modulatory functions towards these DNA elements. The observation that

specific K residues (i.e. K24-27) within this reduced APE1 portion seem to be required for the correct binding of nCaRE-B (data not shown) nicely fits with this hypothesis. The zebrafish homologous of APE1, which shares less than 40% of the N-terminal aminoacidic sequence with the human protein and that lacks two out of five K residues in this region, is indeed no longer able to stably bind these nCaRE-B sequences (Figure 2B) (Poletto *et al.*, 2013). Similar results were also obtained when using an human recombinant APE1 mutant protein bearing specific K to A multiple substitution at K27/K31/K32/K35, in which the positive charges at amino acid side chain has been removed to mimic a condition similar to that exerted by K acetylation (data not shown).

The APE1 N-terminal domain seems required for the stable binding to the SIRT1 nCaRE-B elements, even though it is not sufficient (Poletto *et al.*, 2013). EMSA analysis, performed with the nuclear extracts of HeLa cells expressing the deletion form of APE1, indeed demonstrated that APE1 is certainly part of a multiprotein complex, not resulting the limiting factor in the binding reaction. As already speculated by Okazaki and also reported in later works, the binding affinity observed for the multiprotein complex is higher with respect to that detected when using purified APE1 protein alone (Figure 2). This suggested that other factors are necessary and cooperate with APE1 to fully exert this function, confirming previous observations (Chung *et al.*, 1996; Kuninger *et al.*, 2002). The two subunits of Ku antigen (Ku70 and Ku80) were among the protein factors already described by Chung *et al*. to be involved in the specific binding of nCaRE-A sequences (Chung *et al.*, 1996). Here, we demonstrated that Ku70 binding is not exclusively limited to the nCaRE-A elements, since we identified this protein in the complex that binds to the nCaRE-B sequence of SIRT1. The Ku heterodimer is a main component of the non-homologous end-joining (NHEJ) pathway repairing DNA double-strand breaks (DSBs), which are generally produced upon extensive oxidative and IR damage to DNA (Lieber, 2010). The peculiar structure of Ku allows recognition and tight binding to DSBs, together with the recruitment of DNA-PKcs and other factors to form the active protein kinase complex DNA-PK that facilitates processing and ligation of the broken ends (Walker *et al.*, 2001; Postow, 2011). Its involvement in the nCaRE binding is not clear, but emerging evidences underline the biological role of its non-canonical functions (Adelmant *et al.*, 2012). We envisage that Ku70 association with nCaRE elements could further facilitate APE1 binding, especially after DNA damage, since we observed an increased interaction between the two proteins upon an oxidative stress condition (Supplemental Figure S8).

We also better characterized the topology of the APE1-nCaRE complex. The palindromic nature of nCaRE-B sequences was already described by Okazaki *et al*. The authors suggested the possible involvement of a dimeric nuclear protein in this process (Okazaki *et al.*, 1991). Here, we suggested that the SIRT1 nCaRE-B, due to its palindromic sequence, can potentially fold into a cruciform-like structure and APE1 binding activity toward these elements strongly relies on the secondary conformation adopted by the oligonucleotide, as already established for other DNA and RNA substrates (Figure 3B and Supplemental Figure S4) (Poletto *et al.*, 2013). Formation of similar palindromic sequences forming cruciform-like structures has been already described in eukaryotic cells and their biological consequences has been related to different processes, including regulation of transcriptional events when present in close proximity of gene promoters (Pearson *et al.*, 1994; Shlyakhtenko *et al.*, 2000; Alvarez *et al.*, 2002; Cunningham *et al.*, 2003; Kurahashi *et al.*, 2004). We therefore hypothesize a similar mechanism for SIRT1 transcriptional regulation. However, the requirement of a specific recognition motif cannot be excluded since mutations in the SIRT1 nCaRE-B sequence, determining only a partial disruption of the oligonucleotide secondary structure, did not completely affect the APE1 binding activity *in vivo* (Figure 4B).

We further deepened into APE1 transcriptional function on SIRT1 promoter. We found that APE1 is able to bind nCaRE-B sequence of SIRT1 promoter *in vivo* and we also confirmed this data through ChIP-seq analysis (data not shown). In particular we observed that APE1 positively affects SIRT1 gene transcription. Although APE1 was implicated in the repression of PTH gene transcription in a Ca^{2+}-dependent fashion, we observed that APE1 overexpression activated the SIRT1 promoter (Figure 4C) apparently through a Ca^{2+}-independent mechanism. This unexpected

positive function on the transcription of a nCaRE-containing promoter was also reported in other works where the authors suggested that the role of the nCaRE sequences in the context of different promoters and cells conditions could affect nCaRE activity (Bhakat *et al.*, 2003; Fuchs *et al.*, 2003). Interestingly, we noticed that the positive effect exerted by APE1 was particularly pronounced during oxidative stress, through its binding to SIRT1 nCaRE-B sequences (Figure 5D). Treatment with H_2O_2 leads to an activation of the SIRT1 promoter that determines an increase of the corresponding transcriptional expression in an APE1-dependent manner. This positive transcriptional effect was also observed when we looked at the expression of other genes that present nCaRE-B elements in their promoters (Figure 5E), thus corroborating the hypothesis of a general mechanism of *SIRT1* gene activation upon DNA damage that involves functional activation of APE1. These findings are in line with previous data from Yamamori *et al*. demonstrating that the genotoxic insult stimulates SIRT1 expression and, therefore, its deacetylase activity on APE1 K6/7, favoring APE1 binding to XRCC1 (Yamamori *et al.*, 2010). Interestingly, these authors evidenced that a decrease of APE1 acetylation at later times after oxidative treatment is usually accompanied by SIRT1 up-regulation. Altogether, these findings are in accordance with a model of a positive autoregulatory loop between the two proteins. Thus, SIRT1 seems to be involved in a feedback mechanism that shuts off the cellular response mediated by APE1 acetylation (Yamamori *et al.*, 2010). Furthermore, another prove of APE1 and SIRT1 concert collaboration has been recently highlighted, demonstrating that APE1 stimulates SIRT1 activity in endothelial cells by reducing thiol moieties of cysteine residues in SIRT1 and thus protecting endothelium from oxidative stress (Jung *et al.*, 2013).

It has been suggested that DNA oxidation could trigger positive transcription in the context of Myc-mediated transcription through the involvement of BER enzymes, including APE1 (Perillo *et al.*, 2008; Gillespie *et al.*, 2010). Similarly, we speculated that the APE1 positive effect observed on SIRT1 transcription might depend on APE1 endonuclease activity over the nCaRE-B elements present within SIRT1 promoter. We here propose a model where oxidative-mediated DNA repair and gene transcription are linked together (Figure 6). During oxidative stress conditions, DNA oxidation determines the formation of 8-oxodeoxyguanine (8-oxodG) lesions, which are recognized and processed by enzymes of the BER pathway, including APE1. In our model, the oxidative burst is an early event, essential for the formation of a productive transcription initiation complex, which relies on the initial recruitment of BER enzymes. The nicks introduced at the chromatin level by APE1, during 8-oxodG removal might promote the local relaxation required for the formation of chromatin loops moving closer the active form of RNA polymerase II to the TSS of the gene, as previously recruited by APE1 on nCaRE-B sequence, turning on the transcription. Our data, therefore, can be generalized into a regulatory model for all those genes that contain nCaRE-B elements. Accordingly, a new hypothesis can be proposed for the molecular activation of specific genes during early response to DNA damage. This model links together DNA-repair enzymes and transcriptional regulation effectors and may constitute a general model for explaining the adaptive cell response to oxidative stress involving gene regulation and DNA damage in which APE1 plays a central role.

MATERIALS AND METHODS

Bioinformatic analysis

All human and mouse DNA sequences were retrieved from the Ensembl database (release 56) (http://www.ensembl.org/) by using a dedicated program written in Perl that collects entries from this archive. A sequence window that contains 5' genomic DNA of every gene coding for a protein was selected. This region extends from 6000 bp upstream and 1000 bp downstream of each transcriptional start site. Gene Ontology (GO) annotations were obtained from Ensembl database by using the data mining tool BioMart (http://www.ensembl.org/) (Spudich *et al.*, 2007). Human and mouse promoter regions were scanned for significant similarities to nCaRE-B by using Glsearch as program for local alignment (available in the Fasta3 program package) (Pearson, 2000). Glsearch

was chosen because it calculates an alignment that is global in the query and local in the library. The following nCaRE-B sequences were used as query (Izumi *et al.*, 1996):

Name	Sequence (5' to 3')
nCaRE-B (PTH)	TTTTTGAGACAGGGTCTCACTCTG
nCaRE-B1 (APE1)	TTTTGAGACAGTCTCAGCTCTG
nCaRE-B2 (APE1)	TTTTGAGACAGAGTTTCACTCTTG

Alignments were computed with Altschul and Gish's statistical estimates, which are more suitable for searching of short query sequences (-z 3 option) (Altschul, 1991). We selected only those promoter genes that showed one or more matches for nCaRE-B sequences, allowing up to two mismatches in the case of human genome and up to three mismatches in the case of mouse one; in fact, in the latter case, most alignments were found with three mismatches. From mouse promoter genes that contained nCaRE-B elements, we retrieved only orthologous man/mouse genes, as obtained from the BioMart Ensembl database. For the microarray filter, we cross-checked human genes selected from alignment search with microarray data obtained from the gene expression profile of HeLa cells silenced for APE1 by RNA interference (Vascotto *et al.*, 2009a). GO filter identified co-regulated human genes, as determined by microarray analysis, studying the prevalence of their GO annotation terms. This analysis was obtained by using a Perl program kindly provided by Caselle and coworkers (Corà *et al.*, 2004), which performs an exact Fisher's test based on hypergeometric distribution to determine whether the term appears in the set significantly more often than what expected by chance. This program uses four different entries: i) a file containing the whole GO database structure (OBO version 1.2, available at http://www.geneontology.org/); ii) the list of genes from whole human genome; iii) a list of all genes with all the GO terms associated with them (as obtained from Ensemble-BioMart); iv) the set of genes to be tested. In general, a GO annotation term was considered to be significantly overexpressed when the corresponding *p*-value (not corrected for multiple testing) was lower than 1E-4. Phylogenetic footprinting analysis consisted in the last selection from significant data obtained from GO filter of that genes also present in the mouse orthologous dataset.

Gene annotations co-occurrence analysis

Gene identifiers corresponding to the list of 57 putative genes regulated by APE1 were submitted to GeneCodis (http://genecodis.cnb.csic.es/), a web-based tool for the ontological analysis (Carmona-Saez *et al.*, 2007; Nogales-Cadenas *et al.*, 2009; Tabas-Madrid *et al.*, 2012), selecting *Homo Sapiens* as the source for annotations and 'Biological Process' as the Gene Ontology category to perform the gene annotation co-occurrence analysis.

Cell culture and transient transfection experiments

HeLa cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Euroclone, Milan, Italy), 100 U/ml penicillin, and 10 μg/ml streptomycin sulfate. One day before transfection, cells were seeded in 10-cm plates at a density of 3×10^6 cells/plate. Cells were then transiently transfected with plasmids of interest by using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions. Cells were harvested 48 h after transfection.

Inducible APE1 knock-down and generation of APE1 knock-in cell lines

Inducible silencing of endogenous APE1 and reconstitution with mutant proteins in HeLa cell clones was performed as already described (Vascotto *et al.*, 2009a,b). For inducible shRNA experiments, doxycycline (1 μg/ml) (Sigma) was added to the cell culture medium and cells were grown for 10 days.

Plasmids and expression of recombinant proteins

Plasmid containing the human SIRT1 promoter was kindly provided by Dr. Irani, University of Pittsburgh, USA. This plasmid consists of a fragment of the human SIRT1 promoter (–1266 to +137 relative to transcription start site) cloned into the pGL4.1 firefly luciferase reporter vector (Promega) (Yamamori *et al.*, 2010). The human SIRT1 promoter carrying the mutation at nCaRE-B

sequences was generated with a Site-Directed Mutagenesis Kit (Stratagene), using the following primers:
SIRT1-B mut for 5'-TCATCTAGGTTTTATTTATATATTTTTTTGCTAAGGAGCGTCGCTCTTGCTGCCCAGGCTGGTGTG-3' and SIRT1-B mut rev5'-CACACCAGCCTGGGCAGCAAGAGCGACGCTCCTTAGCAAAAAAATATATAAATAAAACCTAGATGA-3'.

Expression and purification of recombinant proteins from *E. coli* were performed as previously described (Vascotto *et al.*, 2009b; Fantini *et al.*, 2010).

Antibodies and Western blotting analysis

For Western blotting analyses, the indicated amounts of cell extracts were resolved in 10% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). Membranes were blocked with 5% w/v non-fat dry milk in PBS containing 0.1% v/v Tween 20 and probed with the monoclonal anti-FLAG antibody (Sigma), the monoclonal anti-APE1 antibody (Vascotto *et al.*, 2009a), the monoclonal anti-Ku70 (sc-12729, Santa Cruz Biotechnology, Inc.), the monoclonal anti-RNA polymerase II (Abcam), the monoclonal anti-SIRT1 (Abcam), the monoclonal anti-p32 (Santa Cruz Biotechnology, Inc.) and the polyclonal anti-p53(acetyl K382) (Abcam); blots were developed by using the ECL enhanced chemiluminescence procedure (GE Healthcare) or Western Lightning Ultra (Perkin Elmer). Data normalization was performed by using a monoclonal anti-tubulin antibody (Sigma). Blots were quantified by using a Chemidoc XRS video densitometer (Bio-Rad).

Alignments and secondary structure predictions

Multiple alignments were performed using the software CLUSTALW2, available at (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Potential secondary structures for SIRT1-B nCaRE-B oligonucleotide were determined by using the mFold Web Server program available at (http://mfold.rna.albany.edu/?q=mfold). Structure predictions were run by setting the program parameters as close as possible to the conditions used in binding assays (i.e. 37°C and 50 mM monovalent cation).

Chromatin immunoprecipitation (ChIP) Analysis

ChIP assay was performed by using a protocol described previously (Lirussi *et al.*, 2012) on HeLa cells co-transfected with hSIRT1 promoter and APE1-FLAG tagged expressing vectors.

Preparation of nuclear cell extracts

Nuclear protein extracts were prepared as described earlier (Ziel *et al.*, 2005).

Electrophoretic Mobility Shift Assay (EMSA) analysis

APE1 binding to nucleic acids was assessed as already described (Fantini *et al.*, 2010), with some modifications. Briefly, the indicated amount of recombinant proteins or 5 μg of the reported nuclear extract were incubated at 37°C for 15 minutes with 250 pmol of unlabeled poly(dT), or 250 ng of sonicated salmon sperm DNA (Sigma). 2.5 pmol of ^{32}P-labeled double-stranded (ds) oligonucleotides were then added and incubated for additional 15 min and further separated onto a native 6% w/v polyacrylamide gel at 150 V, for 4 h. When performing super-shift assays, 5 μl of monoclonal anti-APE1 (Vascotto *et al.*, 2009a), anti-Ku-70 (sc-12729, Santa Cruz Biotechnology, Inc.) or anti-P2Y6 (Alomone Labs) were pre-incubated with HeLa nuclear extract from APE1^{SCR-1} clone at 4°C, for 3 h.

Oligonucleotides used for EMSA were the following:

Name	Sequence (5' to 3')
nCaRE-B SIRT1-A	For TTTTTGAGACAGAGTTTCACTCTTG
	Rev CAAGAGTGAAACTCTGTCTCAAAAA
nCaRE-B SIRT1-B	For TTTTTGAGACGGAGTTTCGCTCTTG
	Rev CAAGAGCGAAACTCCGTCTCAAAAA

Poly(dT)	For TTTTTTTTTTTTTTTTTTTTTT

T7 endonuclease I footprinting

Footprinting analysis on SIRT1 nCaRE-B sequence were conducted using T7 endonuclease I. Briefly, 5'-^{32}P-end-labeled nCaRE-B SIRT1-B was digested with 5U of T7 endonuclease I at 37°C, for 1h and then incubated with 35 pmol of APE1 recombinant protein for 15 min at 37°C. The reaction mixtures were then loaded and separated for 2h onto a denaturing 8M urea sequencing gel. After separation, the gel was incubated for 30' in a 10% methanol and 10% acetic acid solution for 30' and then wrapped in Saran wrap and exposed to film for autoradiograpy.

Determination of AP endonuclease activity

Determination of APE1 AP endonuclease activity was performed using an oligonucleotide cleavage assay, as described previously (Vascotto *et al.*, 2009b). The indicated amount of recombinant APE1 protein was incubated with a 5'-^{32}P-end-labeled 26 mer ds oligonucleotide containing a single tetrahydrofuranyl (here called THF) artificial AP site at position 14, which is cleaved to a 14-mer in the presence of AP endonuclease activity. Alternatively, a 5'-^{32}P-end-labeled ds SIRT1-B nCaRE-B oligonucleotide or a 5'-^{32}P-end-labeled ds nCaRE SIRT1-B oligonucleotide, bearing a single tetrahydrofuranyl residue at position 12 (bolded) 5'-TTTTTGAGACG**G**AGTTTCGCTCTTG -3' (Integrated DNA Technologies) were used. The conversion of the radiolabeled THF-containing oligonucleotide substrate (S) to the shorter product (P) was evaluated on a denaturing 20% polyacrylamide gel.

Reporter assays

For reporter assay experiments, we used a human SIRT1 promoter plasmid allowing for promoter activity measurements upon luciferase assay, as already described (Yamamori *et al.*, 2010). To this purpose, $2.5x10^4$ HeLa cells were seeded in a 96-well plate and co-transfected with 15 ng of human SIRT1 promoter, 0.3 ng of a constitutive renilla reporter plasmid and 75 ng of a vector expressing FLAG-tagged APE1 protein. When performing luciferase assays upon H_2O_2, cells were challenged with increasing amounts of H_2O_2 in serum-free medium for 1h, at 37°C, and then firefly and renilla luciferase activities were measured 24 h after the treatment by using the Dual-Glo Luciferase assay system (Promega), according to manufacturer's recommendations. Firefly activity was normalized to renilla activity to correct for differences in transfection efficiency. Results are from triplicate experiments.

Q-PCR

Total RNA from cell lines was extracted with the SV Total RNA isolation System kit (Promega). One microgram of total RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad), according to the manufacturer's instructions. qRT-PCR was performed with a CFX96 Real-Time System (Bio-Rad) using iQ™ SYBR® Green Supermix (Bio-Rad). Primer sequences for human SIRT1 were those reported in (Yamamori *et al.*, 2010). Human GAPDH was used as internal control; sense, 5'- CCCTTCATTGACCTCAACTACATG -3'; antisense 5'-TGGGATTTCCATTGATGACAAGC -3'.

Surface Plasmon Resonance (SPR) analysis

Real time binding assays were performed on a Biacore 3000 SPR instrument (GE Healthcare). Biotinylated ds oligonucleotides were immobilized on a SA-chip at the desired level, as result of their injection at a concentration of 500 nM in HBS (20 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, and 0.005% v/v P20 surfactant, 0.1 mM tris(2-carboxyethyl)phosphine), at 10 µL/min as flow rate. Flow cell 2 contained 100 RU of poly(dT); flow cell 3 and 4 contained 77 and 60 RU of SIRT1-B nCaRE-B and SIRT1-B nCaRE-B mutated respectively, and flow cell 1 (with streptavidin) was left blank to be used as a reference surface. APE1 and its deletion mutant APE1$^{N\Delta 33}$ were serially diluted in running buffer to the indicated concentrations and injected at a flow rate of 20 µL/min for 4.5 min, at 20°C. Disruption of any complex that remained bound after a 3-min dissociation was achieved by using an injection of 1 M NaCl at 20 µL/min, for 1 min. BIAevaluation analysis package version 4.1 (GE Healthcare) was used to subtract blank signal, and to evaluate kinetic and

dissociation constants. Kinetic parameters were estimated assuming a 1:1 binding model and using version 7 4.1 Evaluation Software (GE Healthcare). An affinity steady state model was applied to fit the RUmax data versus proteins concentrations and fitting was performed with GraphPad Prism v4.00 (Fantini *et al.*, 2010).

Limited proteolysis

Suitable experimental conditions were chosen by testing proteolysis with different enzyme/substrate values; no preventive removal of DNA was performed. Thus, limited proteolysis experiments on recombinant APE1 were conducted in 50 mM NH_4HCO_3, pH 7.5 (reaction buffer) at 37°C, by using an enzyme to substrate ratio ranging from 1:500 to 1:5000 w/w. Three identical aliquots of APE1 (500 pmol) were combined with reaction buffer or DNA nCaRE-B ds oligonucleotides (PTH or nCaRE SIRT1-B) (5:1 mol DNA/protein) dissolved in reaction buffer to generate samples (100 µl final volume each), which were incubated for 15 min at 37°C, before protease addition. After digestion starting, the extent of proteolysis was monitored on a time-course basis by sampling 10 µl of the mixture at time intervals ranging from 5 to 120 min. Reaction samples were immediately quenched with 5% formic acid and then frozen in dry-ice before LC-ESI-MS analysis.

LC-ESI-MS analysis

APE1 digests were analyzed with Q-TOF Premier mass spectrometer (Waters, Milford, MA) equipped with a nanospray source. Peptide mixtures were separated on an Atlantis C_{18} column (100 µm x 100 mm, 3 µm), using a linear gradient ranging from 30 to 60% acetonitrile in 1% formic acid, over a period of 50 min, at a flow rate of 800 nl/min. Spectra were acquired in the *m/z* 650-2500 range. Data were processed by using the MassLynx software (Waters). Mass calibration was performed by means of the multiply charged ions from horse heart myoglobin (Sigma). Depending on polypeptide size, mass values have been reported as monoisotopic or average values. Observed mass values were assigned to specific polypeptides by using the Paws software (Proteometrics Inc.), based on APE1 sequence and selectivity of the protease used for protein digestion.

Statistical analyses

Statistical analyses were performed by using the Microsoft Excel data analysis program for Student's t test analysis. $P < 0.05$ was considered as statistically significant.

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Table 1. Dissociation constant and kinetic parameter values as determined for APE1 by SPR analysis.

Ligand	Ka (Ms 10^5)	Kd (1/s)	K_D (µM)
SIRT1-B nCaRE	0.270	0.105	3.90±0.08
SIRT1-B mutated	0.0198	0.236	119±8
polyT	0.004	0.112	308±3

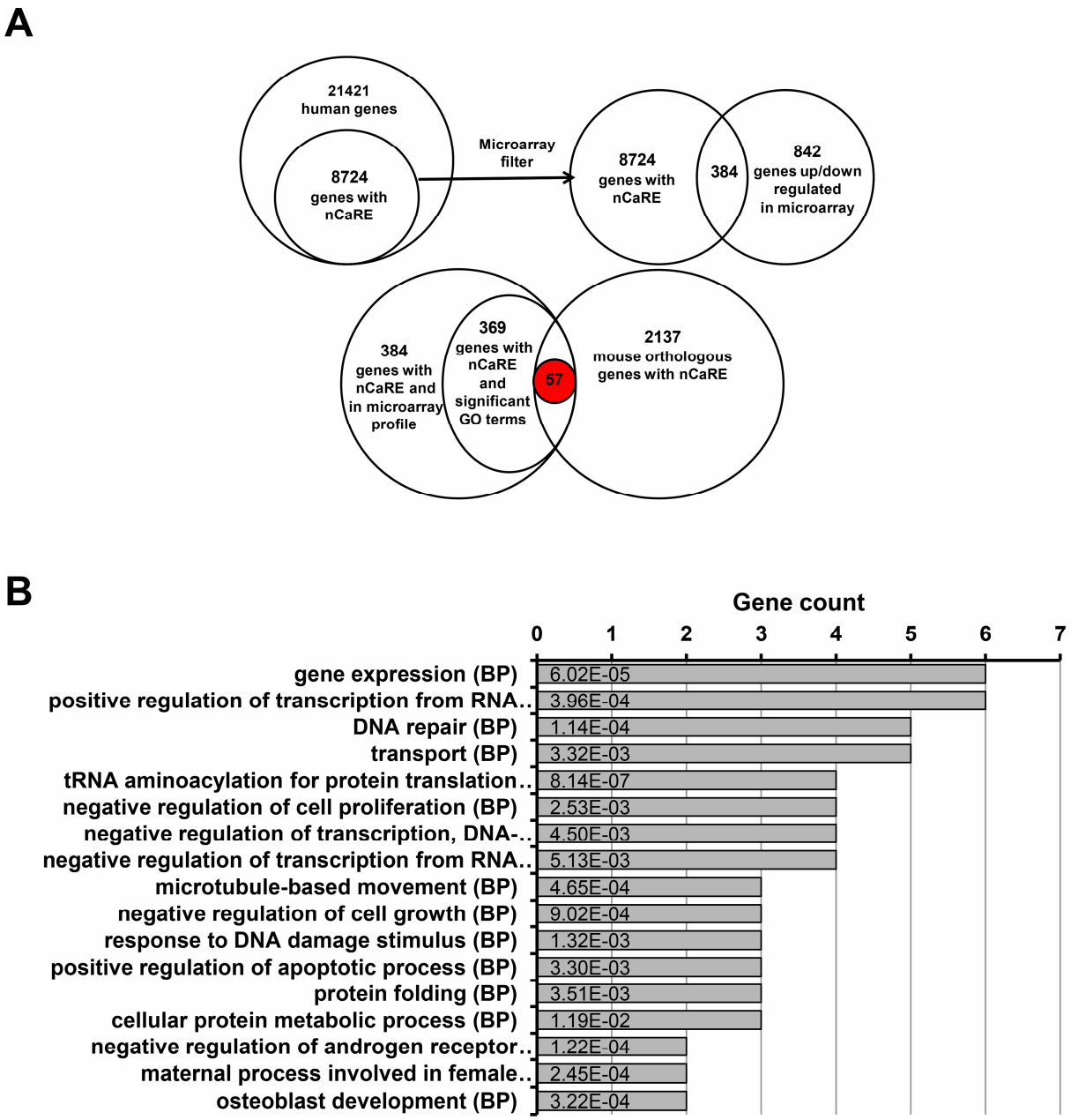

Figure 1. Bioinformatic research of nCaRE sequences. (A) Results obtained from the application of the different filters. *Upper panel*, data derived from alignment research of nCaRE sequences on human gene promoters and the subsequent cross-check with microarray data. *Bottom panel*, final results deriving from the combined Gene ontology and phylogenetic footprinting analyses. (B) Functional enrichment analysis of the 57 putative genes regulated by APE1 performed according to their biological process annotations. For simplicity, only the most representative functional categories are reported. The number of genes for each category is provided on the horizontal axis, together with the list of the first seventeen co-occurrence terms. Statistical significance belonging to each category is shown within each bar.

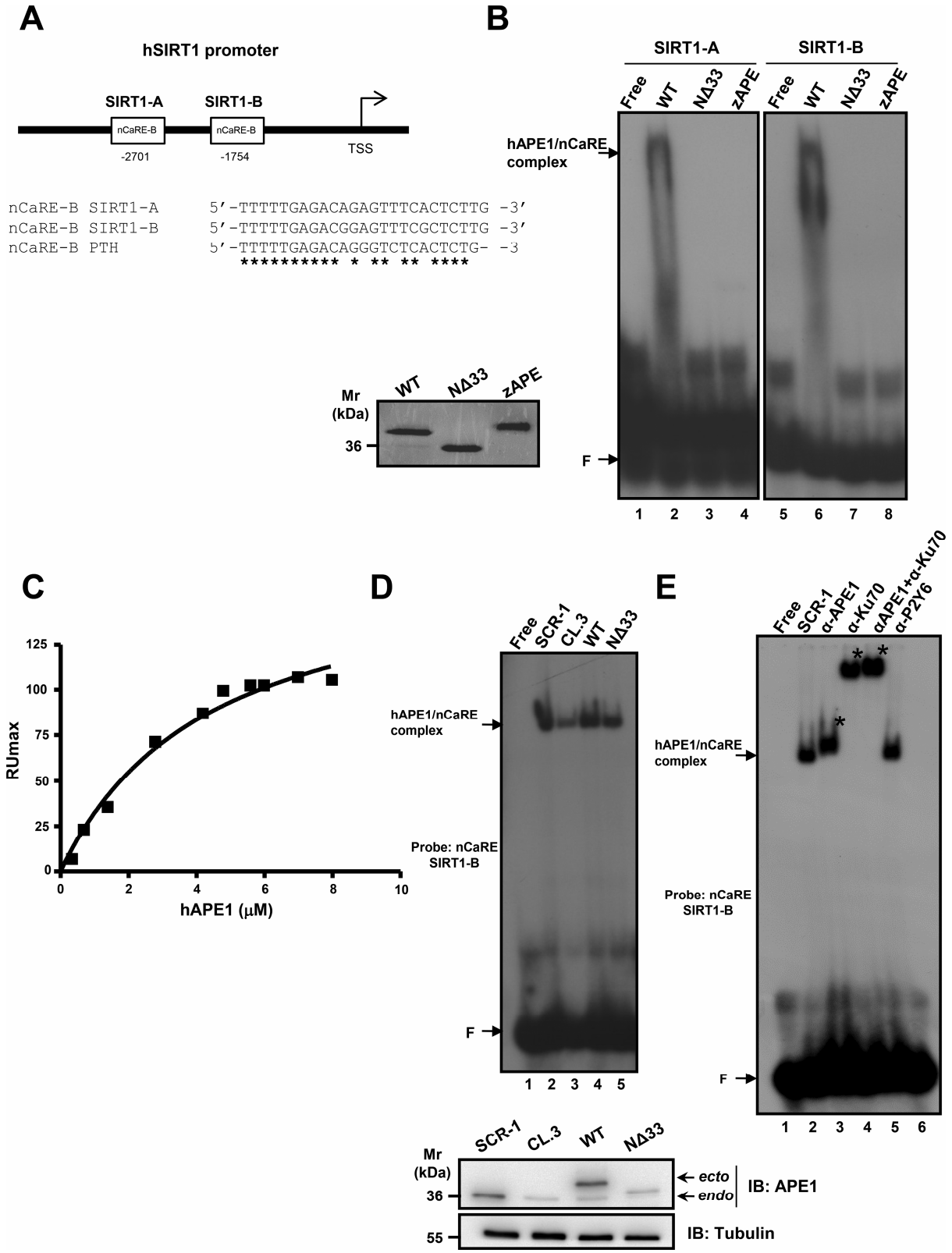

Figure 2. APE1 is part of a nuclear protein complex that binds to SIRT1 nCaRE sequence through its N-terminal domain. (A) Schematic representation (*upper panel*) and multiple sequence alignment (*bottom panel*) of two nCaRE-B sequences found on human SIRT1 gene promoter (SIRT1-A and SIRT1-B) with the nCaRE sequence found on the human PTH promoter (Okazaki *et al.*, 1991). (B). EMSA analysis of nCaRE SIRT1-A and SIRT1-B sequence challenged with 10

pmol of purified APE1^{WT}, APE1$^{N\Delta 33}$ and zAPE1 proteins. Coomassie staining of the purified recombinant proteins is shown on the left. (C) SPR analysis on the hAPE1–nCaRE interaction. Recombinant hAPE1 and biotinylated nCaRE SIRT1-B (Table 1) was used as analyte and ligand, respectively. Plot of RUmax from each binding vs. hAPE1 concentrations (0.5-8 μM); data were fitted by non-linear regression analysis. (D) *Upper panel*, EMSA analysis of nCaRE SIRT1-B incubated with HeLa nuclear extract of different clones: control clone, APE1^{SCR-1} (lane 2), clone silenced for APE1, APE1$^{CL.3}$ (lane 3), clones reconstituted with APE1^{WT} (lane 4) or with APE1$^{N\Delta 33}$ (lane 5). *Bottom panel*, Western blotting analysis of APE1 protein in HeLa nuclear cell extracts. (E) EMSA analysis of nCaRE SIRT1-B with HeLa nuclear extract from APE1^{SCR-1} clone alone (lane 2) or pre-incubated with monoclonal antibody against APE1 (lane 3) or/and with an antibody against Ku-70 (lanes 4 and 5). Lane 6 corresponds to APE1^{SCR-1} nuclear extract incubated with a non-specific antibody (α-P2Y6). Free indicates probe alone; F shows the position of the free oligonucleotide probe. Specific APE1/nCaRE interaction is indicated by the arrow. Asterisk indicates super-shift.

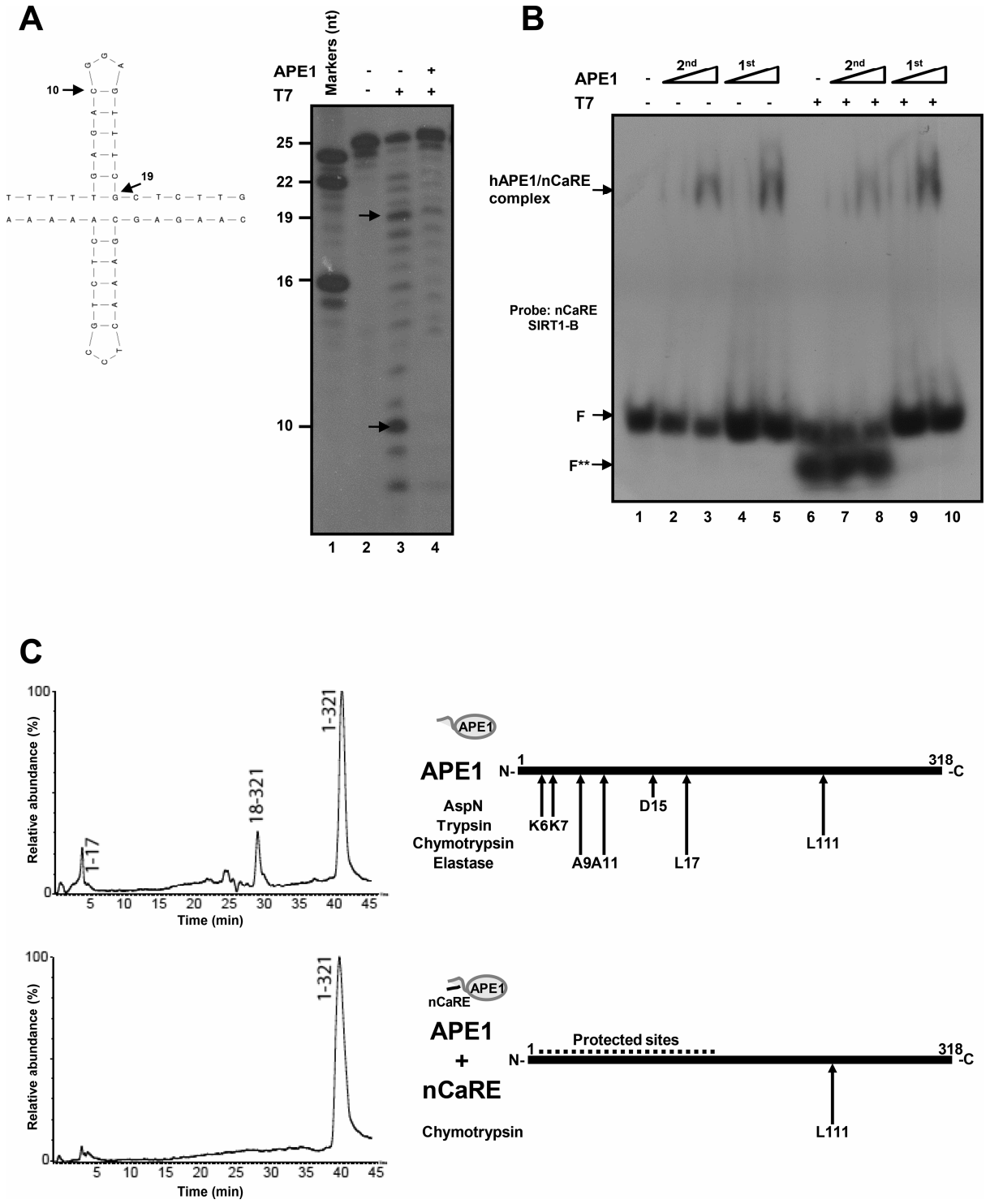

Figure 3. APE1 recognizes structured nCaRE sequences through its N-terminal domain. (A) *Left*, predicted cruciform structure of nCaRE SIRT1-B double-stranded oligonucleotide. Arrows indicate the cleavage site of T7 endonuclease I and the length of the products. *Right*, 5'-^{32}P-end-labeled nCaRE was pre-incubated (lane 4) or not (lane 3) with APE1 recombinant protein and then subject to T7 endonuclease digestion. (B) EMSA analysis of APE1 binding to nCaRE sequence after digestion with T7 endonuclease (lanes 7, 8) or upon pre-incubation with APE1 and subsequent digestion with T7 endonuclease (lanes 9, 10). Lanes 1 is the probe alone; 1st and 2nd indicate if APE1 incubation with the probe was performed temporally before (1st) or after (2nd) T7 digestion; F

shows the position of the free oligonucleotide probe. F** indicates the T7 endonuclease-digested probe. Specific APE1/nCaRE interaction is indicated by the arrow. (C) Schematic representation of the amino acids present within the N-terminal domain of APE1 and involved in the nCaRE oligonucleotide binding. (Left) Proteolytic maps obtained following incubation of recombinant APE1 alone (top) or recombinant APE1 complexed with SIRT1 nCaRE-B oligonucleotide (bottom) with endoprotease AspN. Experiments were performed on a recombinant APE1 form bearing three additional amino acids at the protein N-terminus with respect to the native counterpart. Peptides identified by mass spectrometry analysis are indicated at the top of the corresponding chromatographic peaks. (Right) Proteolytic sites identified in native APE1 alone (top) and in APE1 complexed with SIRT1 nCaRE-B oligonucleotide (bottom); this figure summarizes the results from independent experiments performed by using different proteases. See Supplemental Information for experimental details, and Supplemental Figure S4 and Supplemental Table S4 for complete data.

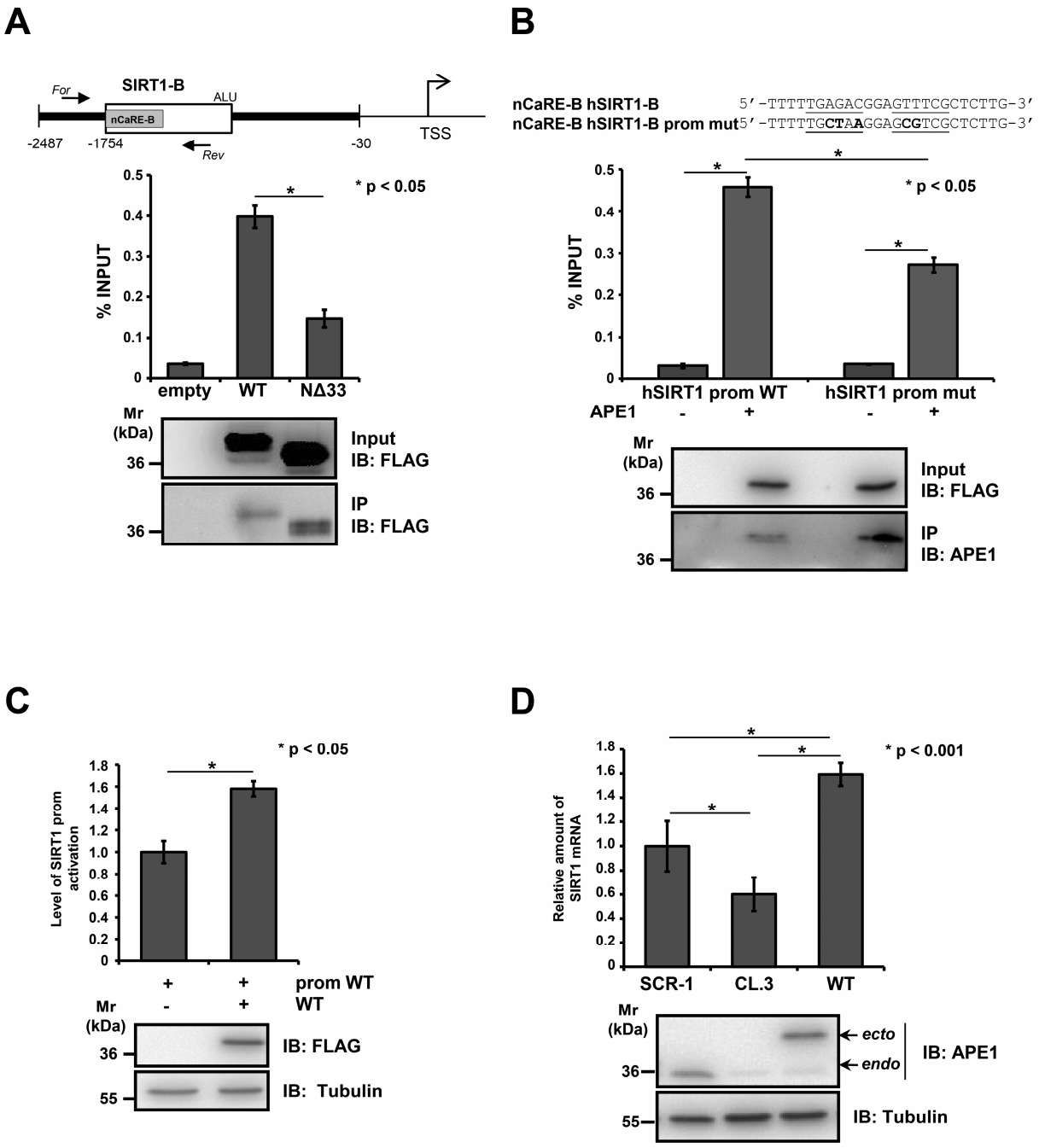

Figure 4. APE1 positively regulates SIRT1 expression at the promoter level. (A). *Upper panel*, schematic representation of the human SIRT1 promoter used for HeLa transfection. *For* and *Rev* arrows indicated the RT-PCR primer designed for the quantification of the human SIRT1 nCaRE sequence bound to APE1. *Bottom panel*, ChIP assay for APE1-nCaRE sequence association. The values report the percentage of immunoprecipitated nCaRE DNA relative to that present in total input chromatin. Data were further normalized to the amount of immunoprecipitated protein. Western blotting analysis was performed on total cell extracts (input) and on immunoprecipitated material (IP) with specific antibody for FLAG and APE1. IB: immunoblot. (B) ChIP analysis on mutated human SIRT1 promoter. *Upper panel*, base composition of the nCaRE SIRT1-B mutated sequence used for site-directed mutagenesis of SIRT1 promoter. Divergent sequences in the mutant nCaRE are bold. *Bottom panel*, HeLa cells were co-transfected with vector expressing $APE1^{WT}$ and, alternatively, with wild type hSIRT1 promoter or mutated hSIRT1 promoter. The histogram

represents the amount of hSIRT1 promoter sequence that was immunoprecipitated. Data are presented as percentage of input and were normalized to the amount of APE1 immunoprecipitated, as evaluated by Western blotting analysis. (C) hSIRT1 promoter is activated in presence of APE1, as shown in the reporter assay. Western blotting analysis showing the normalization of protein levels. (D) Analysis of SIRT1 mRNA level with Q-PCR in clones expressing $APE1^{WT}$ or APE1 silenced (CL.3) cells. Western blotting analysis on protein extract of clones showing the suppression of endogenous APE1 expression, upon ten days of treatment with doxycycline.

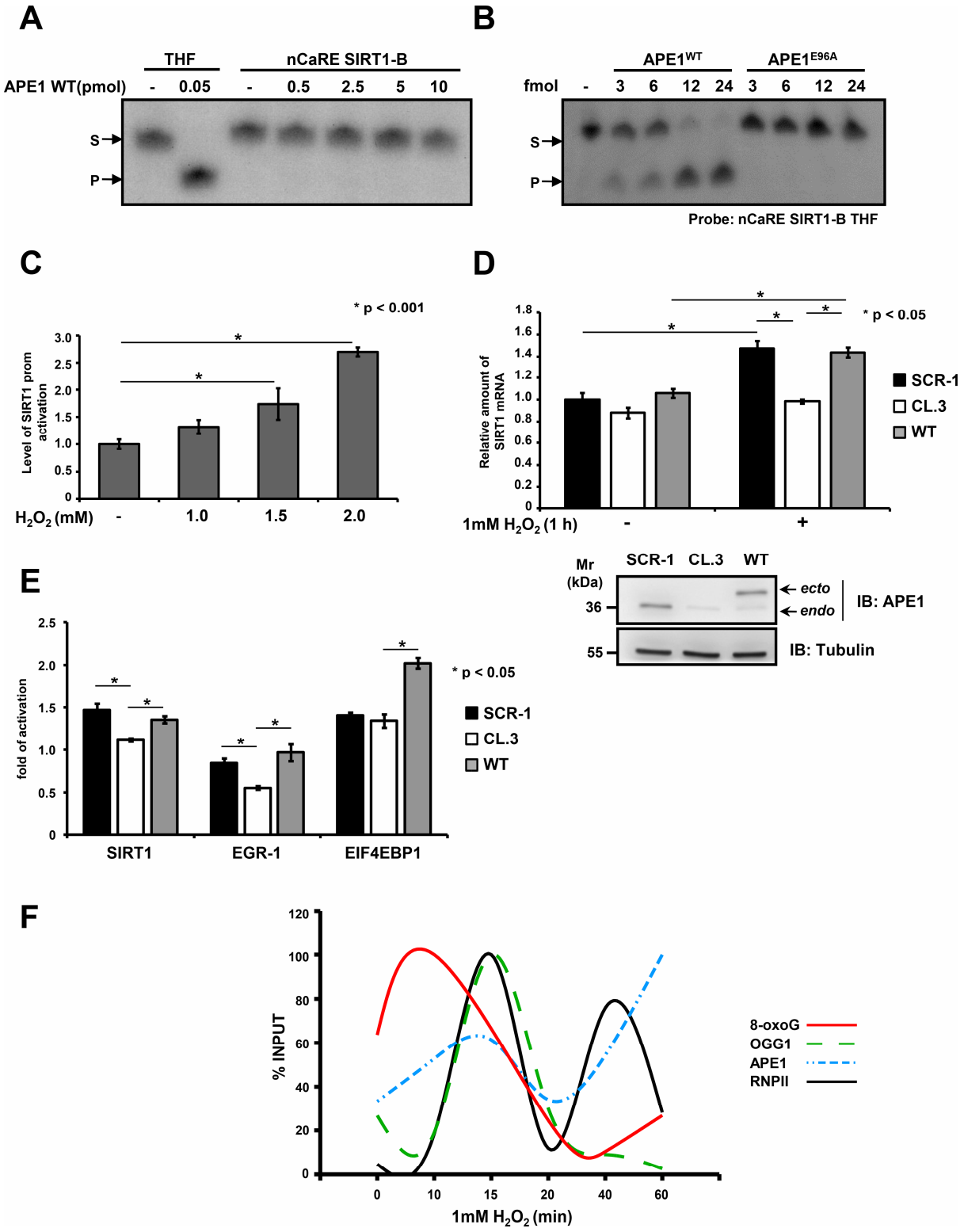

Figure 5. Recruitment of BER enzymes on the SIRT1 promoter. (A) APE1 endonuclease activity on ds nCaRE SIRT1-B radiolabeled oligonucleotide. A radiolabeled ds THF-containing deoxyoligonulceotide (THF) was used as control. Reactions were performed with increasing amounts (pmol) of recombinant APE1^{WT} protein. (B) APE1 AP endonuclease activity on nCaRE SIRT1-B THF-containing probe incubated with increasing amounts of recombinant APE1^{WT} protein or a catalytic inactive APE1 mutant (APE1^{E96A}). (C) Reporter assay with HeLa cells transfected

with hSIRT1 firefly reporter vector and challenged with increasing doses of H_2O_2, for 1h, as indicated. (D) Q-PCR analysis of SIRT1 mRNA levels in clones expressing $APE1^{WT}$ or APE1 silenced cells $APE1^{CL.3}$ after 1 mM H_2O_2 treatment, for 1 h. Western blotting analysis on the protein extracts. (E) Q-PCR analysis of SIRT1, EGR-1 and EIF4EBP mRNA levels in $APE1^{CL.3}$ or $APE1^{WT}$ clones after 1 mM H_2O_2 treatment, for 1 h. Data shown in the histogram are reported as fold of activation after H_2O_2 treatment. (F) Histogram reports the results of four independent ChIP analyses relative to the accumulation of 8-oxoGs, OGG1, APE1 and RNA polymerase II protein on the SIRT1 promoter after 1 mM H_2O_2 treatment, for different times (as reported). Data are presented as percentage of input and were normalized to the quantity of DNA immunoprecipitated by α-tubulin (α-tub). See Figure S4 for detailed information.

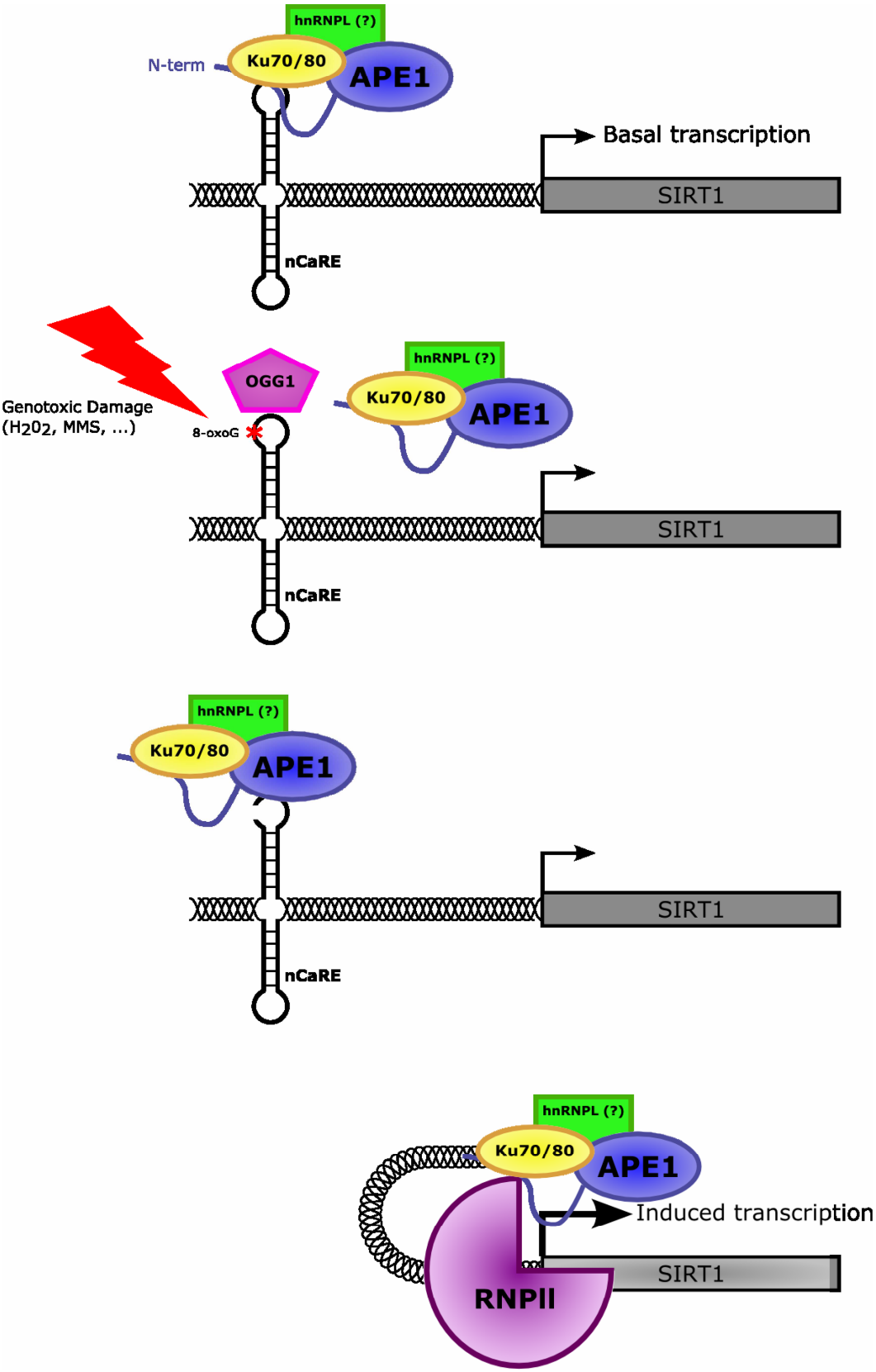

Figure 6. Mechanistic model for the role of APE1 in oxidatively mediated SIRT1 transcription. Under normal condition, APE1, together with other protein factors, is bound to nCaRE element present within SIRT1 promoter involved in the basal activation of SIRT1 transcription. Conversely,

upon oxidative stress conditions, DNA oxidation determines the formation of 8-oxodeoxyguanine (8-oxoG) lesions at nCaRE sequence present in SIRT1 promoter, which are recognized and processed by BER enzymes including APE1. The nicks introduced at the chromatin level by APE1, during 8-oxoG removal, might promote the formation of chromatin loops moving closer the active form of RNA polymerase II to the TSS of the gene turning on the transcription.