Forum Review Article

The Many Functions of APE1/Ref-1: Not Only a DNA Repair Enzyme

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Abstract

APE1/Ref-1 (APE1), the mammalian ortholog of *Escherichia coli* Xth, and a multifunctional protein possessing both DNA repair and transcriptional regulatory activities, has a pleiotropic role in controlling cellular response to oxidative stress. APE1 is the main apurinic/apyrimidinic endonuclease in eukaryotic cells, playing a central role in the DNA base excision repair pathway of all DNA lesions (uracil, alkylated and oxidized, and abasic sites), including single-strand breaks, and has also cotranscriptional activity by modulating genes expression directly regulated by either ubiquitous (*i.e.*, AP-1, Egr-1, NF-κB, p53, and HIF) and tissue specific (*i.e.*, PEBP-2, Pax-5 and -8, and TTF-1) transcription factors. In addition, it controls the intracellular redox state by inhibiting the reactive oxygen species (ROS) production. At present, information is still inadequate regarding the molecular mechanisms responsible for the coordinated control of its several activities. Both expression and/or subcellular localization are altered in several metabolic and proliferative disorders such as in tumors and aging. Here, we have attempted to coalesce the most relevant information concerning APE1's different functions in order to shed new light and to focus current and future studies to fully understand this unique molecule that is acquiring more and more interest and translational relevance in the field of molecular medicine. *Antioxid. Redox Signal.* 11, 601–619.

Introduction: An Overall View on APE1. General Considerations on APE1 Biological and Molecular Functions. Involvement of APE1 in Controlling Pro-Apoptotic and Pro-Survival Transcription Factors. Is This a Biological Paradox?

CELLULAR RESPONSE TO OXIDATIVE STRESS is a highly regulated and complex biological process (42). APE1/Ref-1 (also called HAP1 or APEX, and here referred to APE1), the mammalian ortholog of *Escherichia coli* Xth (exonuclease III), is a vital protein that acts as an essential master regulator of this response, highly contributing to the maintenance of the genome stability. After cloning by two independent groups in 1991 (21, 114) as a DNA repair enzyme first and as a redox protein the following year (144), APE1 has been described, in ~300 articles, as playing a role in several biological contexts. APE1 is a dual function protein involved both in the base excision repair (BER) pathways of DNA lesions, acting as the major apurinic/apyrimidinic endonuclease, and in eukaryotic transcriptional regulation of gene expression. This effect is obtained as a redox co-activator of different transcription factors such as the early growth response protein-1 (Egr-1), nuclear factor-κB (NF-κB), p53, hypoxia inducible factor-1α (HIF-1α), cAMP response element binding protein (CREB), activator protein-1 (AP-1), and paired box-containing proteins (Pax) in different cell systems (126). These two biological activities are located in two functionally distinct domains. The N-terminus, containing the nuclear localization signal (NLS) region, is principally devoted to the redox activity, through Cys65, while the C-terminus exerts the enzymatic activity on the abasic sites of DNA (146) (Fig. 1A). Homology with ExoIII starts from residue 62. A Blast homology search clearly displays the peculiar distribution of the primary structure conservation of APE1

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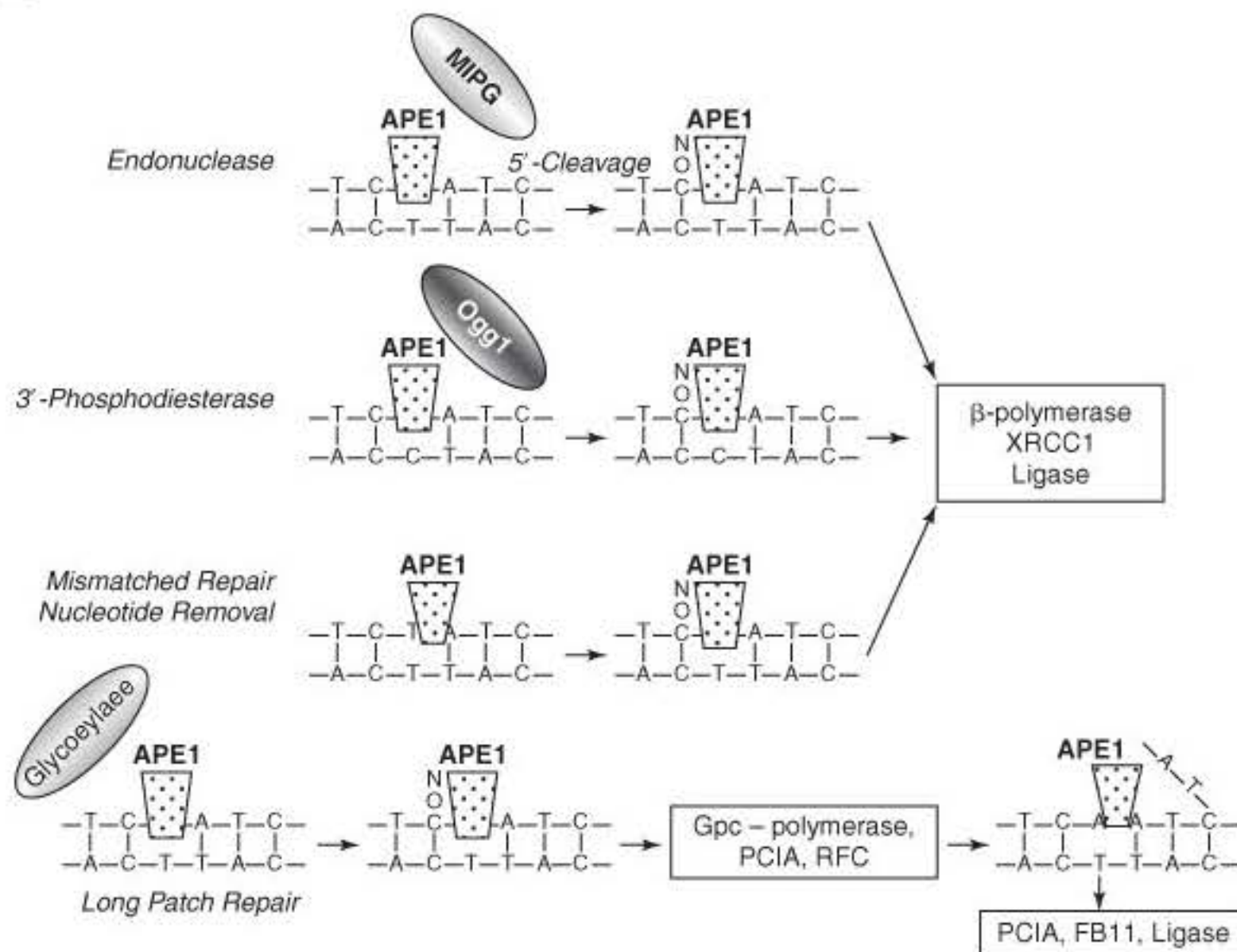[3]Department of Pediatrics, Herman B Wells Center for Pediatric Research, and the Departments of Biochemistry and Molecular Biology, and Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, Indiana.
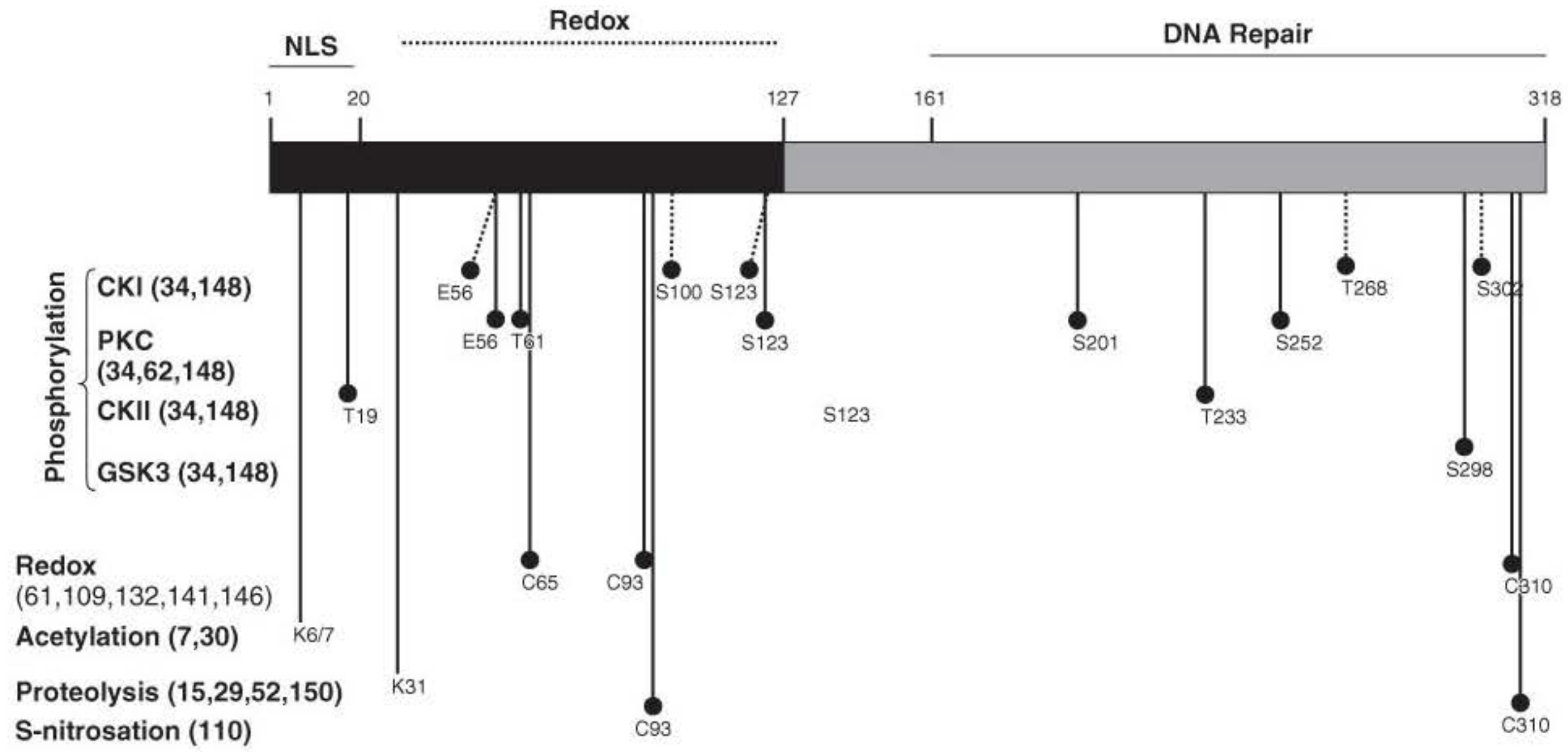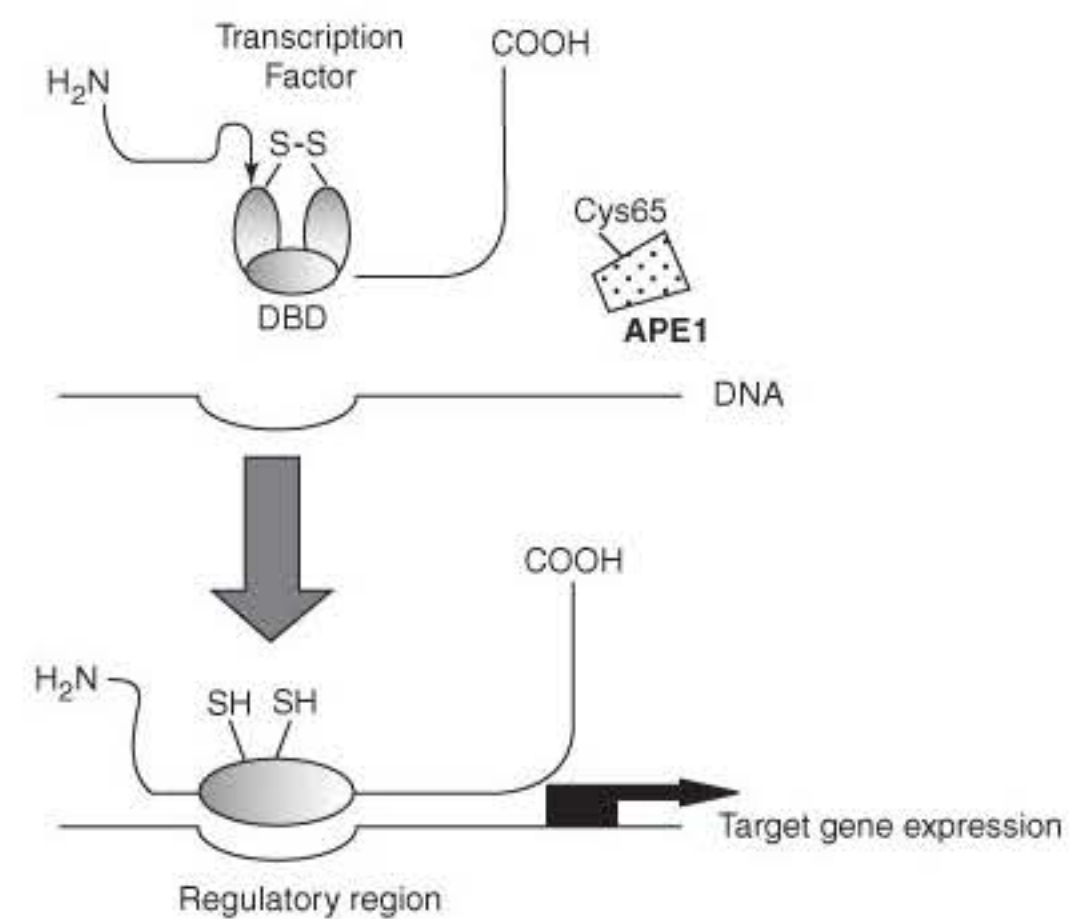
FIG. 1. Representations of APE1 domains structure and functions. (A) Schematic structure, based on functional studies (66, 144) of APE1 structure with critical residues. Schematic diagram of putative or experimental determined post-translational modifications of APE1/Ref-1. CKI, casein kinase I; CKII, casein kinase II; GSK3, glycogen synthase kinase 3; NLS, nuclear localization signal; PKC, protein kinase C. (B) APE1 functions in base excision repair. FEN1, flap endonuclease 1; MPG, methylpurine DNA glycosylase; Ogg1, 8-oxoguanine DNA glycosylase, PCNA, proliferating cell nuclear antigen; RFC, replication factor C; XRCC1, X-ray cross-species complimenting 1. (C) Theoretical molecular model of the redox function of APE1 as a transcriptional coactivator of several transcription factors. Thioredoxin (Trx) is responsible for completing the redox cycle by which APE1 reduced form is restored. DBD, DNA binding domain.

(http://www.expasy.ch/cgi-bin/blast.pl, accession number: P27695). While the C-terminal part of the protein is highly conserved during phylogeny, the N-terminus is not. Besides in the mammalian proteins, in which the N-terminus is highly conserved (>90%), this region is almost always absent in other organisms, with the exception of *Zebrafish, Drosophila, Xenopus*,

and *Dictyostelium*, where homology is <40%, suggesting that it may be a recent acquisition of evolution.

The redox function of APE1 is found only in mammals and not in other vertebrates, as demonstrated by the lack of redox function of the *Zebrafish* APE1 (zAPE1). The acquisition of the redox function in APE1 proteins is discussed in

a recent publication (39). The two major functions of APE1, redox and repair, are completely independent in their actions, as shown by the observation that a mutation of the Cys at position 65 (C65) abolishes the redox function but does not affect the repair function (86), whereas mutation of a variety of amino acids required for DNA repair activity, such as Histidine 309 (H309) and others (87, Vascotto et al., unpublished observations), do not affect the redox function. While the DNA repair active site of APE1 has been clearly delineated (44, 90), the redox domain is much less defined. The only Cys residue required for full redox function is C65, which is buried within the APE1 protein (44, 90), as recently confirmed by Georgiadis who mutated in the zAPE1 a Thr (T58) to a Cys located at the same position of the Cys in the mammalian APE1, resulting in the acquisition of redox activity of the mutated protein (39).

The vital role of APE1 seems to be due to its fundamental activity in the base excision repair (BER) pathway of DNA lesions (35). However, the biological relevance in eukaryotic transcriptional regulation of gene expression as a redox coactivator of different transcription has yet to be fully elucidated (126).

Another interesting function is associated to its ability to indirectly bind to the negative calcium response elements (nCaRE) of some promoters (i.e., parathyroid hormone (PTH) and APE1 promoters) acting as a transcriptional repressor. In particular, binding to nCaRE-B, in the PTH gene promoter, requires Ku70 (Ku86) as an additional factor in the complex (17), while binding to nCaRE-B2 in the APE1 promoter involves hnRNPL interaction (77). This activity seems to be regulated by acetylation of APE1 through the CBP/p300 HAT action (7). A more recent finding has identified APE1's role in mediating production of single-strand DNA (ssDNA) breaks in gene promoters during repair of targeted base oxidation lesions caused by oxygen radicals generated during physiologic signaling (10, 153, 154). Thus, defects in the APE1-mediated step in BER pathway could be linked to altered gene expression besides altering transcription factor state. This is discussed later in this review.

APE1 subcellular distribution, in different mammalian cell types, is mainly nuclear and is critical in controlling cellular proliferative rate (35, 57, 67). Interestingly, the expression of APE1 is strictly connected to different tumorigenic processes (126). The necessity of APE1 for cellular survival and its frequent overexpression in tumor cells strongly suggest a fundamental role of this protein in preventing cell death and in controlling cellular proliferation. However, APE1 abilities to activate transcription factors, such as p53 and Egr-1 (14, 30, 38, 54, 64, 107, 117), mainly involved in controlling cell-cycle arrest and apoptotic programs, leave open the debate concerning the mechanisms responsible for controlling the different functions in several contexts. APE1 protein is also localized within mitochondria in different cell types but, at present, the role in this organelle has not been completely elucidated (15, 88, 122), as well as the molecular mechanism regulating its mitochondrial localization (15).

The fact that APE1 is essential for cell viability was originally demonstrated by genetic studies. Knockout of APE1 in mice causes postimplantation embryonic lethality on days E5 to E9 (85, 147), and attempts to isolate stable APE1-knockout cell lines were totally unsuccessful. For these reasons, a detailed comprehension of molecular targets of APE1 func-

tions has been very difficult. In the last 3 years, conditional knockout and knockdown strategies (35, 67, Vascotto et al., unpublished observations) confirmed the crucial role of this protein for cellular existence and allowed the establishment of cell models to better investigate and characterize the major functions of APE1. However, complete knowledge of molecular effectors regulated by APE1 in determining its biological essentiality is still scanty. The necessity of APE1 for mammalian cells seems to be mainly due to its DNA repair activity in BER pathway. Interestingly, attempts to restore the DNA repair activity in cells not expressing APE1, by using the yeast homologous Apn1 (35) which lacks the redox-transcriptional activation domain, or an APE1 mutant lacking the acetylation sites but not the DNA repair activity (67), or with the redox-defective mutant C65S (Vascotto et al., unpublished observations) did not completely restore the loss of cell viability. Moreover, by specifically blocking the APE1 redox but not DNA activity with an APE1-specific redox inhibitor, E3330, it has been shown that the cytokine-mediated hemangioblast development in vitro was significantly impaired (155). Collectively, these data demonstrate that the exact knowledge of the precise molecular mechanisms for APE1's vital role in mammalian cells is still inadequate. They do, however, indicate that the repair function is crucial for survival and the redox function for cell growth (Vascotto et al., unpublished observations).

DNA Repair Activity of APE1

It is already well established that a plethora of base lesions are induced in mammalian cell genomes by different physical and chemical agents, among which reactive oxygen species (i.e., O_2^-, H_2O_2 and ˙OH, collectively named ROS) play a dominant role. These lesions, if not adequately repaired, are at the basis of a variety of diseases (including cancer) and of aging.

The BER is the most used pathway to cope with the single base lesion. The BER pathway is also involved in repairing the DNA single-strand breaks (SSB) induced by free radical agents. One of the key enzymes of the BER pathway in mammals is APE1. The basic reactions of the BER pathway have been extensively reviewed (26, 58, 81, 88, 126). They require the coordinated activity of a number of enzymes including: (a) a DNA glycosylase capable of excising a specific modified base; (b) an AP endonuclease, such as APE1, which cleaves the 5' phosphodiester bond, generating 3'OH and 5'dRP termini; (c) an exonuclease activity (β-polymerase, FEN, APE1); (d) a DNA polymerase (β-polymerase, XRCC1 or δ/ε-polymerase with PCNA); and, finally (e) a ligation activity (DNA ligases I and III, XRCC1), (Fig. 1B). The three ways by which APE1 produces the 3'OH terminus for priming and the types of evolutionarily conserved amino acids necessary for these functions have been already reviewed (26, 58 and other articles in this issue of ARS).

All the steps of the BER pathway are finely orchestrated, both from the thermodynamic and the kinetic point of view, to provide an accurate repair of the damaged base and to avoid the generation of intermediate products that are toxic for the cell (58). This implies the coordinated interaction of the various players in the BER process and, when necessary, their further interaction with the DNA replication machinery, as demonstrated by the co-immunoprecipitation of the

BER repair proteins with cyclin A and DNA replication proteins (103). In this context, it is noteworthy that RPA proteins are able to suppress the APE1 endonuclease activity in ssDNA of a replicative fork but not in a transcription bubble or in dsDNA (27) and that Cockayne syndrome B protein potentiates the APE1 activity on fully paired AP-DNA but much more on bubble AP-DNA, suggesting a role for this protein in the transcription-repair pathway (143).

Of particular interest in the definition of new functions and targets of APE1 is the finding that, after UVA irradiation, APE1 co-localizes with Ogg1 in the nuclear speckles, organelles associated to transcription and RNA processing. This localization is abolished in the presence of antioxidants (12), suggesting that ROS are the driving force of this localization. Another interesting APE1 function, connected to its capacity to bind to SSB, is that of inhibiting the same binding by PARP1, with consequent inactivation of the poly-ADP-ribosylation and prevention of necrosis (121).

A good example of how APE1 may use its dual nature to produce a cut in the DNA strand and to activate other proteins is that of the VEGF gene which contains a hypoxic response element (HRE) target of HIF-1α. Oxygen deprivation induces formation of an abasic site in the response element followed by HIF-1α binding. The data suggest that the abasic site is the target of APE1 activity with consequent increased flexibility of DNA which causes HIF-1 binding (10) and transcriptional activation of the VEGF gene (46, 153).

Very recently, a role for APE1 (and for APE2) has been suggested also in the antibody class switch recombination, where the abasic site generated by uracil DNA glycosylase, following cytosine deamination, is converted in a single-stranded break by a standard AP-endonuclease procedure (51). Additionally, the recently found inhibitory crosstalk between the oncogene protein Bcl2 and the DNA repair activity of APE1 (152), suggests a novel mechanism which may promote genetic instability and tumorigenesis.

Redox Regulation of Transcription Factors Activities

The intracellular redox status reflects the balance between the activity of antioxidant enzymatic and nonenzymatic cell systems (including GSH/GSSG, superoxide dismutase, catalase, peroxidases, glutathione peroxidases, etc.) and the amount of ROS produced: (a) as byproducts of respiration; (b) as a consequence of external noxious agents such as ionizing radiation; (c) as 'second messengers' (23, 42); and (d) during pathological states in activated neutrophils (93). Of notice is the observation that variations in the redox state may result in alterations in gene expression profile asset. The molecular mechanism at the basis of this regulation is exerted through the modulation of transcription factors (TFs) activity. In particular, the redox status of reactive Cys residues, located within the DNA-binding domain of some TFs, may control the transcriptional activity of the TFs itself. APE1 has been identified as a protein capable of nuclear redox activity, inducing the DNA binding activity of several transcription factors, such as AP-1 (144), NF-κB (95), Myb (145), PEBP2 (1), HLF (25), NF-Y (94), Egr-1 (14, 64), HIF-1α (63), ATF/CREB family (145), p53 (38, 54, 117), and Pax proteins (13, 128, 129). In each case, this effect was accomplished

by maintaining the cysteine residues of the TFs in the reduced state (Fig. 1C), through a redox cycle in which Trx would restore the reduced form of APE1 (61, 109, 132, 141). By using 'in vitro' assays, it has been shown that Cys65 is the redox-active site of the protein (39, 139). Since Cys65 appears buried in the 3-D model structure of the protein (44, 90), the redox regulation may implicate the occurrence of unfolding or conformational change of APE1 to allow interaction with redox-sensitive TFs. However, Jayaraman et al. suggested that the stimulatory role played by APE1 on p53 activity may also occur in a redox-independent way (70). This was subsequently corroborated by Ordway et al. (101) who provided the first in vivo evidences that the Cys65 residue of APE1 is unexpectedly not essential for redox regulation of AP-1 DNA binding and, thus, challenging the previous hypotheses. However, the presence of compensatory mechanisms occurring in transgenic mice cannot be excluded, and recent publication by Georgiadis et al. has confirmed the original finding of Cys65 being required for APE1 redox function (39).

Regulation of APE1 at the Gene Expression Level, at the Subcellular Localization Level, and Role of Identified Posttranslational Modifications

APE1 functional activation is a consequence of different stimuli that may generate both physiological and toxic oxidative stress conditions or increase the intracellular cAMP levels leading to different outcomes (Fig. 2).
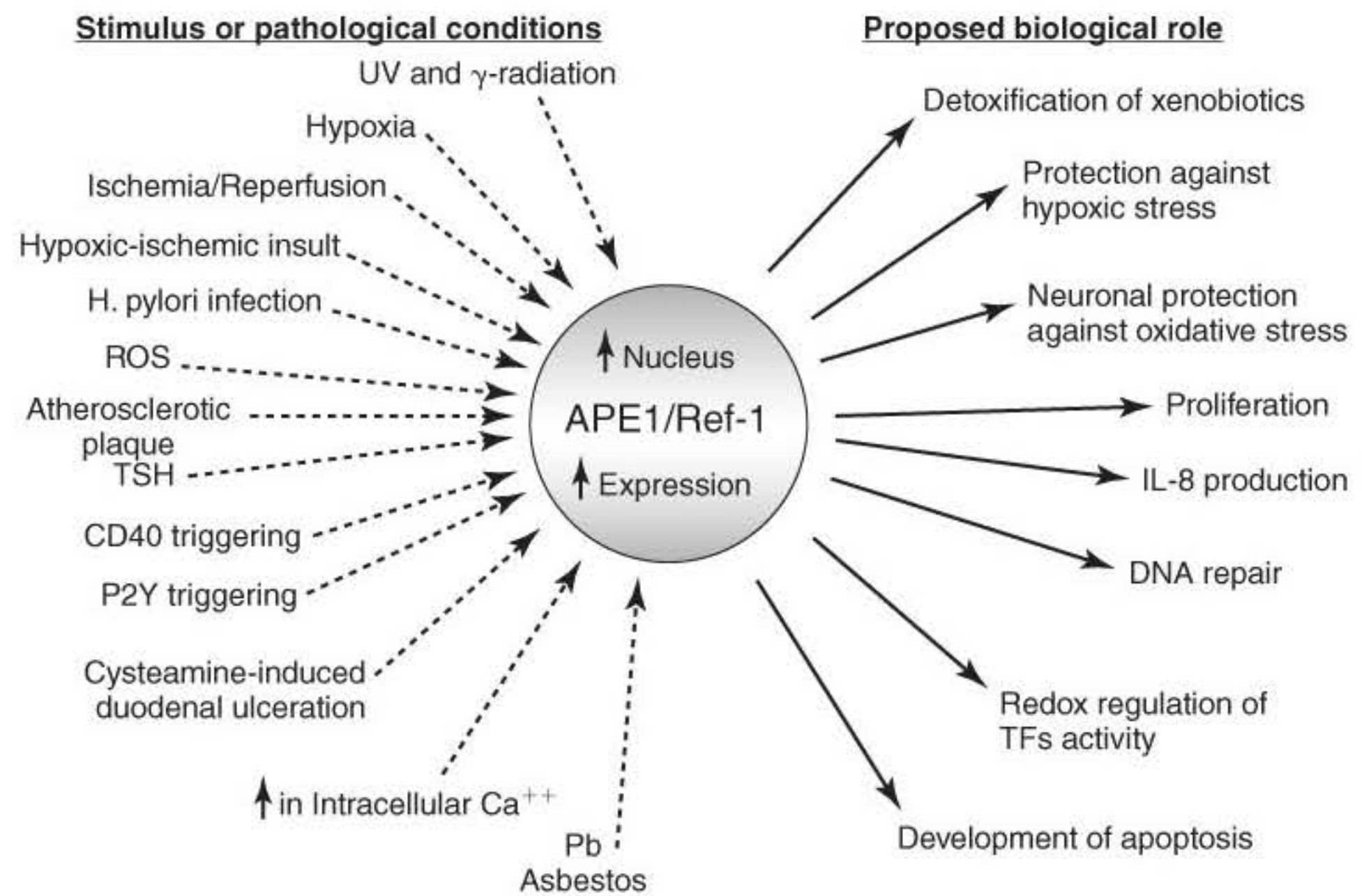
The regulatory functions of the different APE1 activities can be fine-tuned and implemented via three different mechanisms: (a) increase in APE1's level after transcriptional activation (107, 108, 111, 126); (b) relocalization of APE1 from the cytoplasm to the nucleus (126); and (c) modulation of APE1's post-translational modifications (PTM), such as acetylation and phosphorylation. As recently demonstrated, in addition to redox regulation, acetylation appears to have a fine-tuning role in affecting APE1's different activities (7, 30, 67).

Both in vivo and in vitro studies demonstrated that different oxidative or toxic agents and/or intracellular produced ROS efficiently and rapidly (within minutes to hours, depending on the specific ROS-generating stimuli) promote a transient increase in APE1 protein levels, which is inhibited by cycloheximide (111, 129). Different transcription factors, including Sp-1 (36), Egr-1 (107), STAT3 (53), CREB (48), and Jun/ATF4 (37) are involved in the inducible expression of APE1. APE1 itself may inhibit its own expression through the binding to nCa-RE sequences within the APE1 distal promoter, thus constituting an autoregulatory functional loop (68). In addition, APE1 expression is linked in a positive autoregulatory loop with Egr-1 (107), and in a negative inhibitory loop with p53 (151). Interestingly, protein upregulation is always associated with an increase in both redox and AP endonuclease activity, followed by an increase in cell resistance toward oxidative stress and DNA damaging agents (49, 87, 88, 111, 126), strengthening the conclusion that an upregulation of APE1 protein levels has profound biological consequences.

The activation of APE1 is also obtained by a process independent from de novo synthesis and involves cytoplasm to nucleus translocation after exposure of cells to oxidative

FIG. 2. Schematic representation of some of the stimuli known to activate APE1/Ref-1 expression and/or function. APE1 functional activation, which is associated to nuclear accumulation and upregulation of protein expression, is a consequence of different conditions and implies different outcomes. Thus, APE1 is a central actor in the adaptive cellular response to oxidative stress.

Stimulus or pathological conditions

UV and γ-radiation
Hypoxia
Ischemia/Reperfusion
Hypoxic-ischemic insult
H. pylori infection
ROS
Atherosclerotic plaque
TSH
CD40 triggering
P2Y triggering
Cysteamine-induced duodenal ulceration
↑ in Intracellular Ca^{++}
Pb
Asbestos

↑ Nucleus
APE1/Ref.-1
↑ Expression

Proposed biological role

Detoxification of xenobiotics
Protection against hypoxic stress
Neuronal protection against oxidative stress
Proliferation
IL-8 production
DNA repair
Redox regulation of TFs activity
Development of apoptosis

stress conditions (111, 126, 127) or upon physiologic increase in intracellular ROS production (108). Nuclear localization of APE1 is controlled by the first 20 amino acids at the N-terminal sequence, as determined by Jackson *et al.* (69) and nuclear import is controlled through a bipartite NLS comprising residues 1–7 and 8–13 with the involvement of an importin system. In fact, the first 20 residues directly bind to karyopherin α1 and α2. Data obtained by treatment of cells with the nuclear export inhibitor leptomycin B suggested the presence of a nuclear export signal (NES) that may reside in a Leu-rich region (L291, L292, L295 residues, which are exposed in the 3-D structure) (69). Recently, it has been shown that the region comprising amino acids 64–80 contains a NES (110). Thus, both nuclear import and export may control subcellular distribution of APE1. In addition, the interaction with specific nuclear proteins could be a means to maintain APE1 within the nucleus. This hypothesis, recently proposed by Jung *et al.* based on their data showing that nuclear localization of APE1 was dependent on GADD45a nuclear protein expression (71), deserves further experimental support.

During the last few years, several lines of evidence have been accumulating, demonstrating that functional triggering of membrane-bound receptors (such as those for TSH, CD40L, ATP, IL-2, *etc.*) can lead to APE1 functional activation through intracellular generation of sublethal doses of ROS (126). Noteworthy is the observation that APE1 is also directly responsible for the control of the intracellular ROS levels through its inhibitory effect on Rac1 (2, 52, 102, Vascotto *et al.*, unpublished observations), the regulatory subunit of a membrane nonphagocytic NADPH oxidase system. This enzyme, composed of multiple membrane-associated (Mox and p22^{phox}) and cytosolic components (p67^{phox}, p47^{phox}, and Rac1), catalyzes the transformation of the molecular oxygen to the superoxide anion by transferring an electron from the substrates NADH or NADPH (3). Since we have recently demonstrated that NADPH-mediated ROS production in-

duced by P2Y triggering was able to promote APE1 functional activation (108), we propose the existence of an autoregulatory loop between these two systems. This mechanism may be of therapeutic relevance for endothelial, fibroblastic, and smooth muscle cells, and should be analyzed in diseases of the vascular system where an overactivation of the NADPH oxidase system is involved (19), as well as in the angiogenesis process (133), where an additional autoregulatory loop between APE1 and VEGF may be inferred (10, 153). This observation could be therapeutically relevant in the treatment of tumor progression and cancer metastasis.

Based on the above-mentioned considerations, APE1 seems to act as an intracellular signaling tool involved both in modulating the cellular response to acute and chronic oxidative stress conditions, and also in controlling the endogenous ROS levels during the physiological generation of ROS as intracellular signaling molecules. Since the cell system must be able to discriminate between different ROS-generating stimuli, APE1 behaves as an integrating signaling molecule.

APE1 is an abundant protein ($\sim10^4$–10^5 copies/cell) within eukaryotic cells and with a relatively long half-life [$\sim$8 h (Vascotto *et al.*, unpublished observations)]. Therefore, the fine-tuning of the multiple functions of this pleiotropic protein may reside in the impact that PTMs have on the function of APE1 and on the modulation of the APE1-interactome under different conditions. Whereas for the former hypothesis some experimental evidences have been obtained [acetylation of K6/K7 residues (7, 30)], very little information is now available on the protein interacting partners of APE1. Pioneering *in silico* studies discovered that several different phosphorylation sites were scattered throughout the molecule. These potential phosphorylation sites included consensus sequences for casein kinase I and II (CKI and CKII), for protein kinase C (PKC), and for GSK3 (Fig. 1A) (34, 148). Initial *in vivo* studies confirmed a role for PKC in

phosphorylating APE1 in response to PMA or to alkylating agents (*i.e.*, MMS) leading to AP-1 activation (62). However, these studies have not been repeated nor followed up. Therefore, the role of phosphorylation on APE1 is still not clear.

APE1 is a site for redox regulation by the dithiol-reducing enzyme Trx (61, 109, 132, 141), through Cys35 and Cys32 in the catalytic center of Trx, and involving the Cys65 redox sensitive site of APE1 (139, 141, 146). The Trx-mediated redox regulation of APE1 is required for the functional activation of p53 (132) and AP-1 (61). Though the biological relevance of Cys65 residue seems determined, it is not currently known whether this Cys residue undergoes PTM *in vivo*.

Qu *et al.* (110) demonstrated that two (Cys93 and Cys310) of the seven Cys residues of APE1 can undergo *S*-nitrosation in response to nitric oxide stimulation, leading to nucleus to cytoplasm relocalization of the protein in a CRM1-independent process, possibly as a consequence of demasking a putative nuclear export signal (aa 64–80). *S*-nitrosation may therefore constitute a specific molecular switch to strictly control the intracellular distribution of APE1 between nucleus and cytoplasm, and provides a new working hypothesis for the cytoplasmic accumulation of APE1 observed in more aggressive tumors (126). Unfortunately, no detail is available about the functional implications of *S*-nitrosation on the different biological functions of APE1. Accordingly, since both NO and APE1 are associated with tumorigenesis and neurodegenerative diseases, future work is needed to address whether nitrosative stress leads to genomic instability, and may be the target for designing new therapeutic strategies.

An interesting post-translational processing that has been recently described is proteolysis occurring at residue Lys31. This PT regulation of APE1 protein is responsible for enhanced cell death mediated by granzyme A (GzmA) (29) and granzyme K (GzmK) (52). APE1 is associated with the endoplasmic reticulum in a macromolecular complex of 270–420 kDa containing evolutionarily conserved proteins called SET, pp32, and HMG2. GzmA cleaves APE1 after Lys31, giving rise to a protein form called NΔ33APE1, and alters its ability to be actively accumulated within nuclei of cells (15, 69, and our unpublished observations) and to interact with XRCC1 (136). However, some authors claimed that truncated APE1 may loose its AP-endonuclease activity (29) and acquire a nonspecific DNAse function (150). This peculiar processing is not limited to immune cells but may constitute a general molecular device for redirecting APE1 to mitochondria (125), as suggested by Chattopadhyay *et al.* (15) and Mitra *et al.* (88), in spite of the intriguing finding of a proteolysis occurring at the level of Asn33 rather than Lys31. Again, if the removal of the terminal 31–33 amino acids is responsible for APE1 to move to the mitochondria to function in mitochondrial BER as an AP endonuclease, it is hard to understand this truncated protein having nonspecific DNAse activity (150) unless it is very cell-type specific. Accordingly, previous work by many investigators has never observed a nonspecific nuclease activity with the cleavage of the first 61 amino acids (66) and additional data clearly showed that the truncated APE1 protein has an unaltered AP-endonuclease activity (15, and our unpublished observations), at least *in vitro*.

While it is known that nuclear accumulation of APE1 triggers the activation of several transcription factors, the functional role of acetylation is barely understood. Acetylation of both histones and regulatory proteins is commonly catalyzed by the histone acetyltransferase (HAT) p300/CBP, and can be reversed by histone deacetylases (HDACs), which in turn control the acetylation level of transcription factors or co-activators (50, 74). Bhakat *et al.* have reported 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 (7). More recently, we found that exposure of HeLa cells to H_2O_2 and to histone deacetylase inhibitors increases acetylation of APE1 at residues Lys6/Lys7, leading to Egr-1-mediated induction of the tumor suppressor PTEN gene expression (30). Our data open new perspectives in the comprehension of the many functions exerted by APE1 in controlling cell response to oxidative stress and underline the double-face nature of APE1 which plays a role in both pro-survival and in cell cycle arrest mechanisms. Interestingly, despite the very low homology degree in the N-terminal region (<40%), K6 or K6/K7 are much more conserved, thus reinforcing their primary role during phylogenesis.

Altogether, these observations have raised the possibility that subtle PTMs provide a means for channeling the multifunctional APE1 to different activities and interactions and thus could act as a regulatory switch in performing different functions. APE1 subcellular localization is quite variable. Most cell types exhibit only nuclear, others display only cytoplasmic, while others show both nuclear and cytoplasmic localization (126). Such a complex distribution pattern suggests that localization is not random but, on the contrary, is controlled by a strictly regulated process. Though of fundamental interest for a full comprehension of the role of APE1 in different pathological conditions, the clear understanding of the biological relevance of APE1 subcellular compartmentalization still remains elusive. Whereas we can rather easily figure out the role for nuclear localization of APE1 based on its main DNA repair and co-transcriptional activity, a convincing explanation for the extranuclear roles of APE1 is still evanescent. Cytoplasmic localization of APE1, such as that reported for fibroblasts, spermatocytes, thyrocytes, lymphocytes, hepatocytes, and hippocampal cells (20, 22, 24, 72, 73, 112, 126, 129, 142), is associated with high metabolic or proliferative rates and may be related to a cell cycle-dependent expression (36). Possible explanatory hypotheses for cytoplasmic expression of APE1 may come from the mitochondrial role of the protein, as described above. A further functional explanation comes from its association with endoplasmic reticulum membranes, as evidenced by ultrastructural (125) and biochemical (28, 29, 47) analysis. It has been suggested that APE1 redox activity in the cytoplasm may be required to maintain newly synthesized transcription factors in a reduced state during their translocation to the nucleus (24). Therefore, future work is required to shed more light on the extranuclear role(s) of APE1, starting from the explanation of its cytoplasmic function.

Molecular Basis for the Vital Role of APE1 in Mammalian Cells: New Perspectives

To understand the vital role of APE1 in mammalian cells, we recently combined gene expression array and proteomics analysis to identify the genes directly or indirectly regulated

by APE1. By generating an inducible knockdown cell model, in which endogenous APE1 expression could be inhibited by siRNA technology (Vascotto *et al.*, unpublished observations), we built up the molecular networks in which APE1 is involved (see Fig. 3A, B, and C for models network). APE1 silencing induces apoptosis through mitochondrial pathway and a strong inhibitory effect on cell growth. There is evidence of p53 activation and perturbation of the glucocorticoid receptor signaling pathway that could explain an induction of apoptosis. Glucocorticoid receptor NR3C1 (GCR-alpha) itself is downregulated, upon APE1 silencing, whereas p53 is not. Instead, a large number of p53 controlled transcription factors are differentially expressed in our APE1 silencing experiment, further supporting an important role of APE1 in modulating p53 functions (38, 54, 117) (Fig. 3B and C). Differentially expressed genes in our model strongly indicate a significant perturbation of the glucocorticoid receptor signaling pathway. Proteins of the HSP70 complex (*i.e.*, HSPA1A, HSPA1B, and HSPA8) are significantly upregulated as a consequence of APE1 silencing, and it is known that HSP70 is required for the assembly of the glucocorticoid receptor–HSP90 complex (92). Moreover, other key components and targets of glucocorticoid receptor signaling pathway were also on our gene list (Egr-1, C/EBP-beta, c-Myc, SUMO-4, 14-3-3 proteins, and tubulin proteins).

One of the genes that emerged from our analysis and is involved in the control of cell growth processes, as well as in the antioxidant response, is Egr-1. This transcription factor resulted as a good candidate target of APE1, since its expression was significantly reduced in APE1 knocked-down cells both basally and upon serum treatment after starvation (Fig. 4A). We therefore investigated the functional effect of APE1 silencing in controlling the expression of one of the most well-known Egr-1 target genes, the tumor suppressor PTEN, which is induced upon UV- and oxidative stress-cellular damage (5, 137). An impairment of Egr-1 expression upon APE1 silencing affected the inducible expression of PTEN gene with a transcriptional mechanism, confirming the dual nature of APE1 itself (Fig. 4B and C). Thus, the lack of APE1 impairs the adaptive cellular response to damaging agents. These findings further enlighten the double nature of APE1 to be involved in both the processes of cell growth and in growth arrest upon cellular damage. Interestingly, this kind of dual role nature in transcriptional regulation and DNA repair for proteins is consistently present in the five major DNA repair pathways, that is, homologous recombinational repair (HRR), nonhomologous end joining (NHEJ), nucleotide excision repair (NER), BER and mismatch repair (MMR), as in the cases of BRCA1, ATM, and p53 itself (for review, refer to Ref. 6). The existence and the correct regulation of a mechanism shifting cells from DNA repair to apoptosis is central to avoid progression to cancer, preventing clonal expansion of cells in which unrepaired damage would lead to mutation and to carcinogenesis. In this regard, it is interesting to note that a number of DNA repair genes (*i.e.*, GADD45, BRCA1) were downregulated as a consequence of APE1 silencing, suggesting the existence of a cross-regulation of the expression between individual partners of different pathways and underlining the central role of APE1 in DNA repair processes with a different function besides the well-known AP-endonuclease activity. This hypothesis will require further investigation.

The interesting finding that, besides its activity as a redox coactivator for Egr-1 transcriptional activity (64, 107), APE1 is also able to regulate Egr-1 expression levels reinforces the previously suggested hypothesis (107) of the existence of a functional loop between APE1 and Egr-1 in reciprocally modulating each other. This finding strengthens the biological complexity typical of this multifunctional protein and could well be a molecular tool by which APE1 may regulate the expression of DNA repair genes such as GADD45 (130).
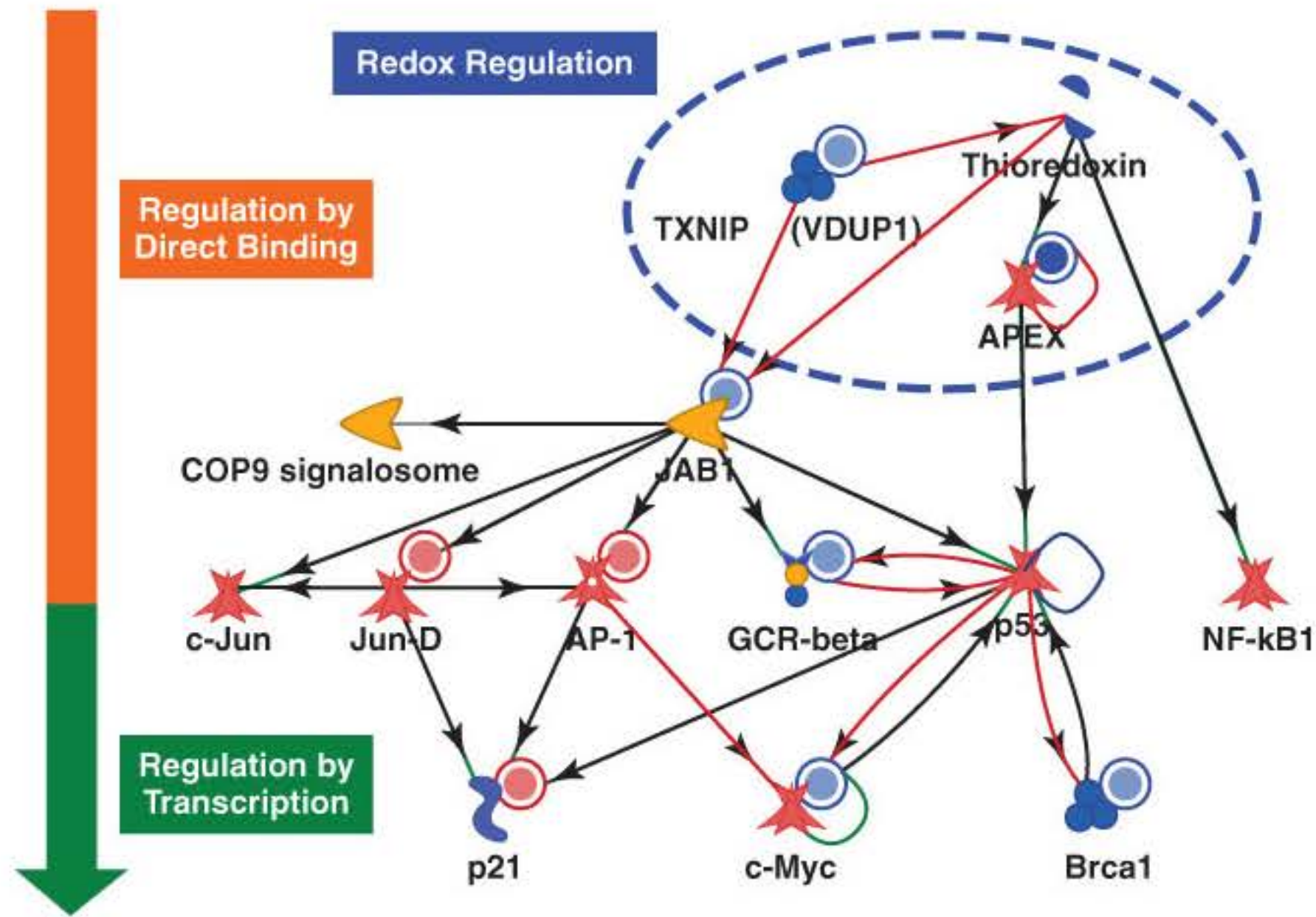
Egr-1 activates the transcription of genes in response to a variety of mitogenic and nonmitogenic stimuli, including growth factors and hypoxia (75) and it is able to form redox-modulated transcriptional complexes with the AP-1 as well as with APE1 and Trx (76). Accordingly, in line with the data obtained in our cell model, the role of Egr-1 seems to be pivotal. The central effect of APE1 on Egr-1 biological functions is also reinforced by the concomitant downregulation of additional Egr-1 target genes such as C/EBPβ, VEGF and c-Myc (Vascotto *et al.*, unpublished observations; 135). Based on these findings the networking model depicted in Fig. 4D was assembled.

Future Perspectives in Elucidating the Pleiotropic Function of APE1 in Mammalian Cells at the Molecular Level. Looking at the Roles of the Mitochondrial and the Subnuclear Distribution of APE1. Could the N-Terminal Unconserved Domain Make the Difference?
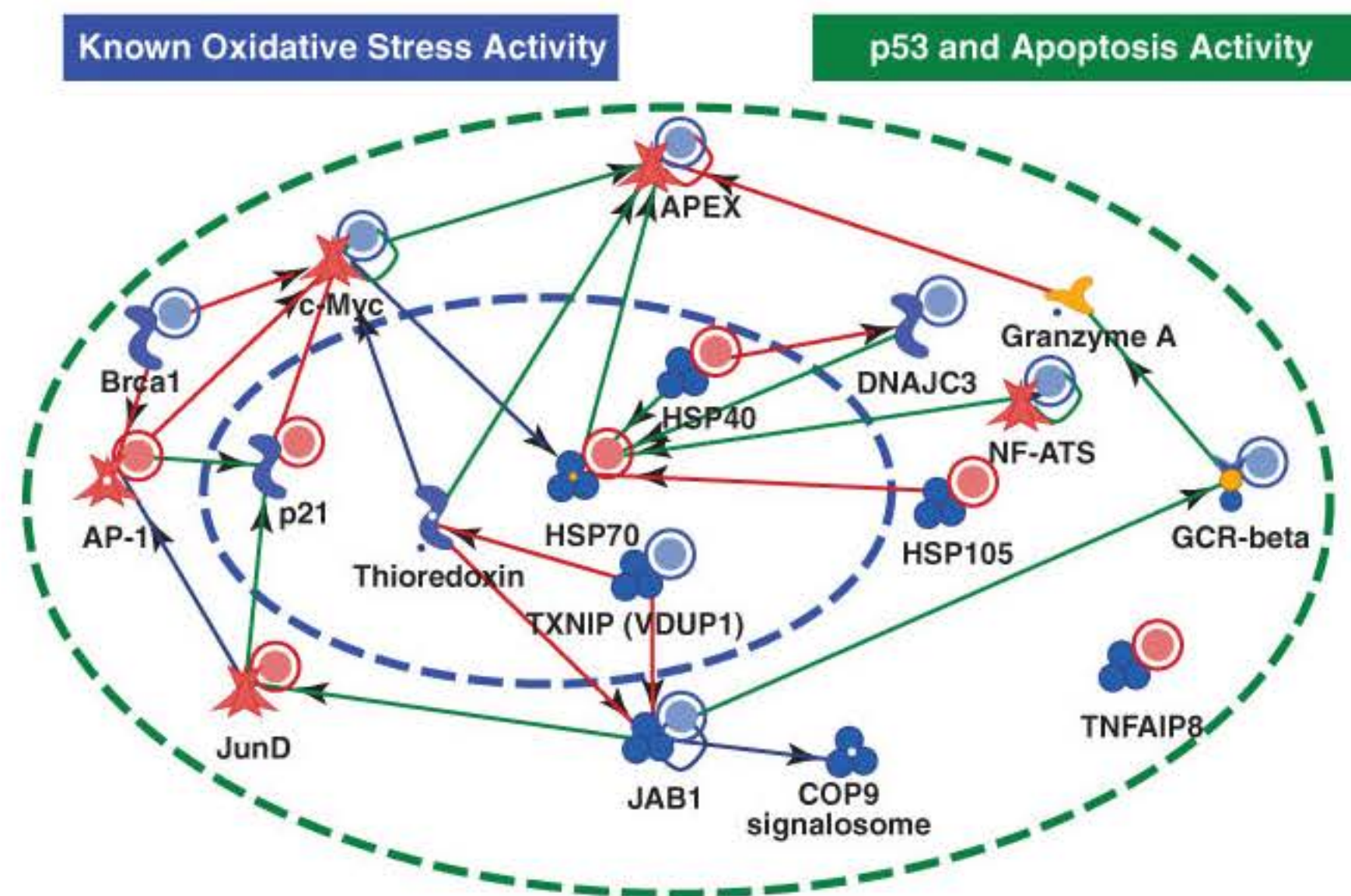
As mentioned above, removal of APE1 NLS through proteolysis controls the amount of APE1 present within the nuclear compartment and would constitute an elegant tool to control APE1 alternative functions in noncanonical subcellular compartments, as mitochondria. Unfortunately, in non-immune cells, neither the identity of the specific protease responsible for this cleavage nor the mitochondrial localization signal (MTS) have been determined yet. The relatively high molecular weight of APE1 is not fully compatible with a passive mechanism of translocation through the outer membrane of mitochondria, and therefore it may require the presence of a specific regulatory transport mechanism. Whereas a large majority of proteins synthesized in the cytoplasm localize into mitochondria by means of an N-terminal MTS, a significant fraction of mitochondrial proteins lack this recognition signal. In the case of APE1, it has been suggested that the MTS may reside in the C-terminal 69 amino residues of the protein (110). Mitochondrial localization of APE1 may be associated to a potential role in DNA repair of oxidized bases in the mitochondrial genome (15, 88, 126). However, since it is not clear whether, *in vivo*, NΔ33APE1 maintains its DNA repair activity (29) or, as previously suggested, may acquire an aspecific endonuclease activity for dsDNA *in vitro* (150), at present it is impossible to derive any definite conclusion. Moreover, since generation of truncated NΔ33APE1 form is associated with the occurrence of an apoptotic phenotype (52; Vascotto *et al.*, unpublished observations), it cannot be excluded that this APE1 form may be causatively involved in the cytotoxic effect driving pro-apoptotic triggering directly from mitochondria. Preliminary data obtained in our laboratory in cells expressing a noncleavable mutant APE1 protein (*i.e.*, 31–34A mutant) seem to support this hypothesis. Should this be confirmed, strategies to modulate the pro-

A

Redox Regulation

Regulation by Direct Binding

Regulation by Transcription

TXNIP (VDUP1)

Thioredoxin

APEX

COP9 signalosome

JAB1

c-Jun   Jun-D   AP-1   GCR-beta   p53   NF-kB1

p21   c-Myc   Brca1

B

Known Oxidative Stress Activity

p53 and Apoptosis Activity

APEX

c-Myc

Brca1

DNAJC3

Granzyme A

HSP40

NF-ATS

AP-1   p21

HSP70

GCR-beta

Thioredoxin

TXNIP (VDUP1)

HSP105

JunD

JAB1   COP9 signalosome

TNFAIP8

C

The p53 network

Thrombospondin   S100A2   FZD2   Staufen   LONP mitochondrial   EGF2   CDCAB   VEG-A   RAIGI   COL4A1   OGT (GlcNAc transferase)

CDC25A   MCMS   ANKRD10   Ku60   Caldesmon

HNRPUL1   StarD4   OR5 (TNFRSF10B)

GADD45 alpha   E1B-AP5   MDS009   ERp29

XBP1   HERP   SNK

TUBBS   Tubulin beta 3   NF-ATS   Cyclin E2

Tubulin beta 1   Tubulin beta   Tubulin beta 2   TMEM33

Tubulin meterodimer (in microtubules)   14-3-3   TNFA1P8   RFC4

JAB1   1-4-3-3 zeta/delta   1-4-3-3 eta   Gas alpha   FAK1   NOXA

EGR1   HSP70   GLUT1   GLIPR

Guanine deaminase   Ca-ATPase2

ADNP   SUMO-4   JAB1   C/EBPbeta   APEX

CRMP2   RNF11   TXNIP (VDOP1)   SMRT   c-Myc   HSPA1A   P21

TACC3   SMUR2   LBC   Zac1   Hx-5   GCR-beta   CREM (activators)   Brca1   Mx11   Karyopherin alpha 2
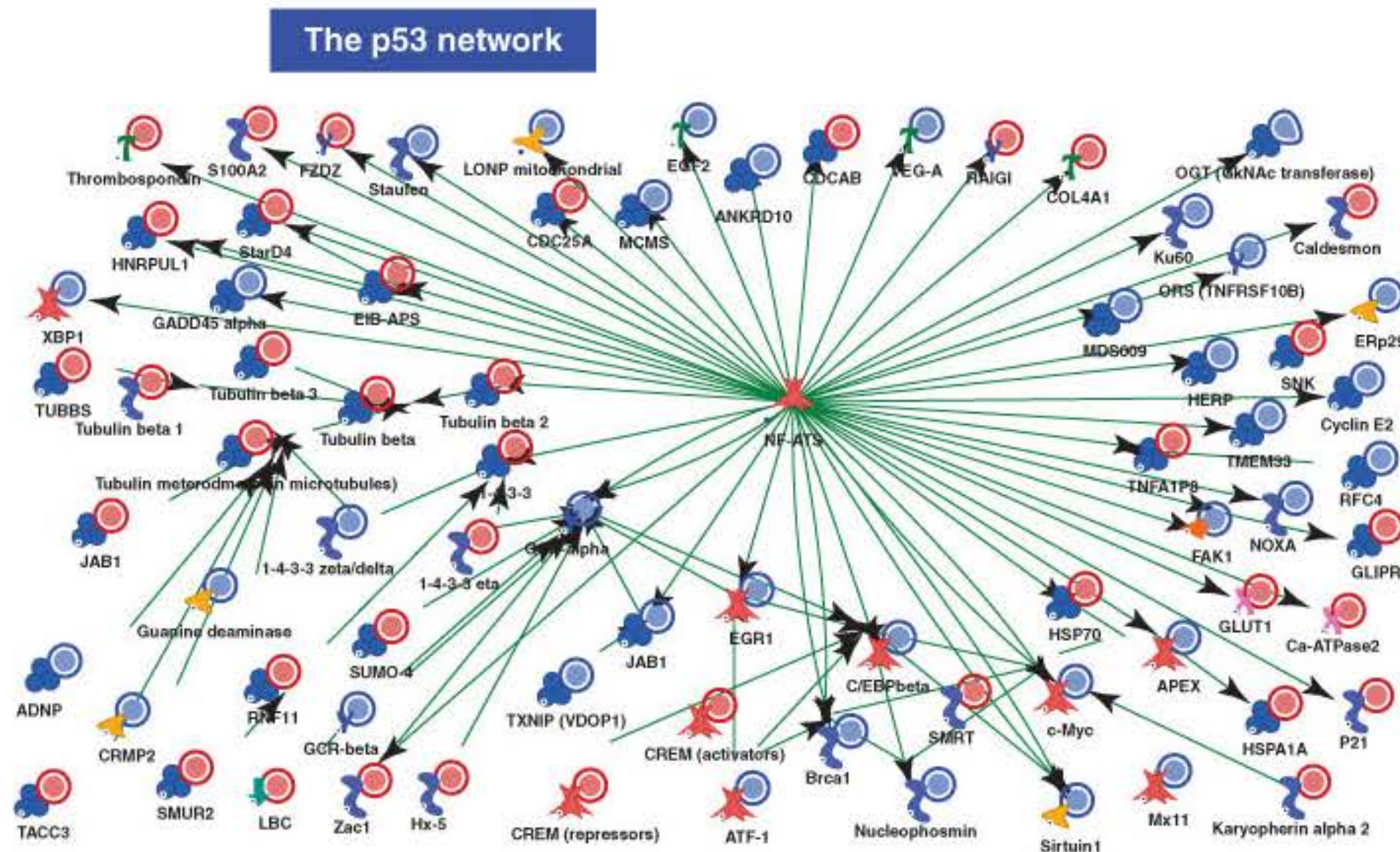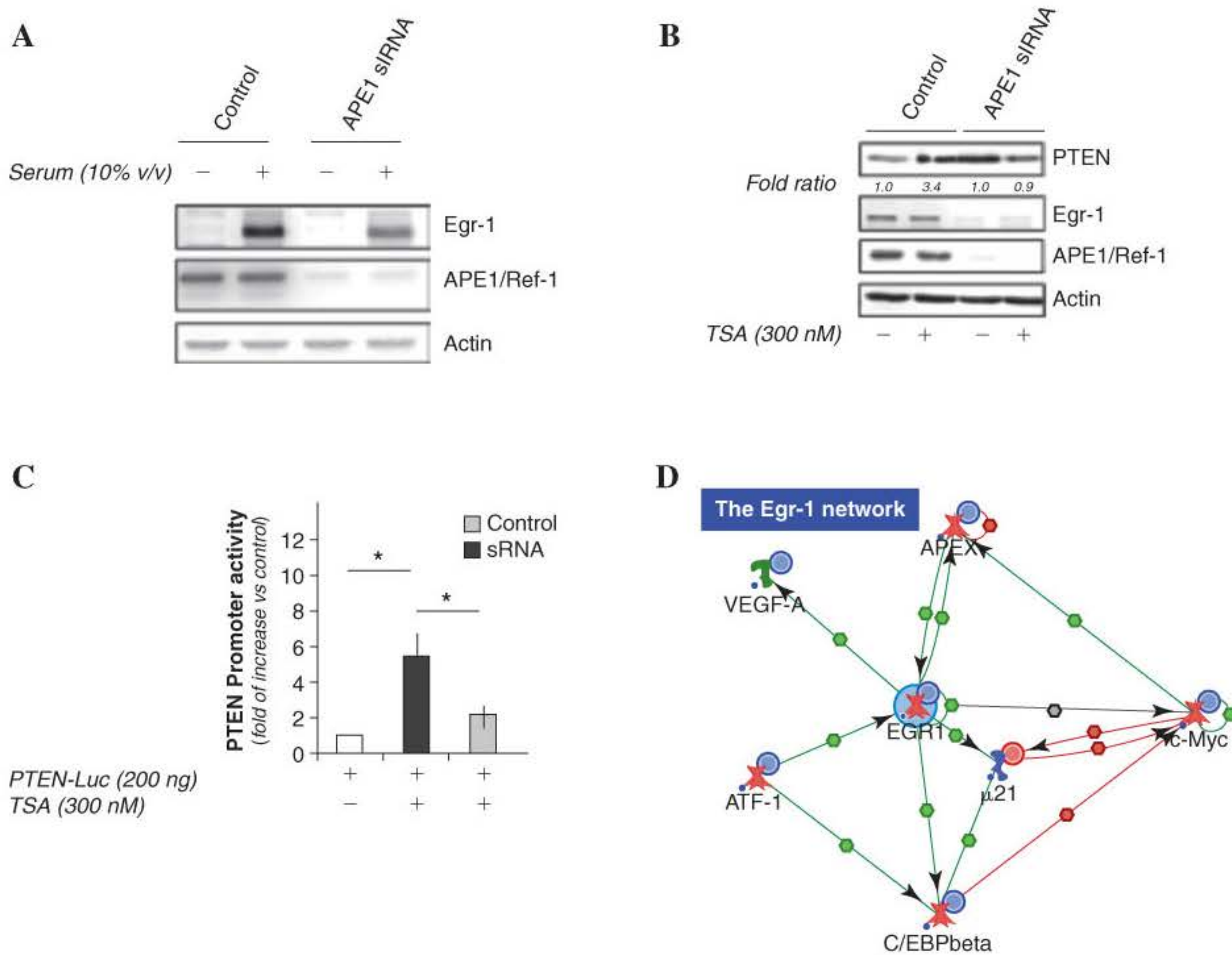
CREM (repressors)   ATF-1   Nucleophosmin   Sirtuin1

FIG. 3.

FIG. 4. Functional inactivation of Egr-1 transcriptional activity on PTEN target gene. HDACs inhibitors-induced PTEN upregulation requires APE1 expression. (A) Inducible expression of Egr-1 by 1 h serum treatment after o/n starvation is significantly reduced in APE1-silenced cells. Endogenous APE1 silencing was obtained by inducible RNAi strategy (30). Expression of Egr-1 and APE1 proteins was evaluated by Western blot analysis on 10 μg of nuclear extracts as previously described (30). (B) Induction of endogenous PTEN expression by HDACs inhibitors is inhibited in HeLa cells in which Egr-1 is diminished upon constitutive stable APE1 knock-down. To evaluate the endogenous PTEN levels upon HDACs inhibitors treatment, control and knocked-down HeLa cells were treated with TSA for 30 h and further collected and lysed to obtain total extracts that were subsequently used for Western blot analysis. Ten μg of extracts were separated onto a 10% SDS-PAGE, blotted onto nitrocellulose membranes, and assayed for the presence of APE1 protein by using the monoclonal anti-APE1 antibody, for the presence of PTEN protein by using the monoclonal anti-PTEN antibody and for the presence of Egr-1 protein by using a specific polyclonal antibody. Actin was also measured, as loading control. Values represent the relative amount of PTEN with respect to control untreated cells, after normalization for actin content. The mean value of two independent experiments is reported. (C) Induction of PTEN promoter activity by HDACs inhibitors is prevented in constitutive stable APE1 knocked-down HeLa cells in which Egr-1 is significantly diminished. APE1 silencing was obtained as described in legend to (A). After 10 days of doxycycline treatment, HeLa cells were transfected with plasmid containing the PTEN promoter-Luc sequence and then treated with TSA for 30 h. Twenty-four hours after treatment, cells were harvested, and luciferase and β-galactosidase activities were measured. Bars indicate the mean value ± SD of three independent experiments. (*$p < 0.05$ by Student's t-test). (D) Model network involving APE1-Egr 1 functional interaction. This model network is accessible at this site: https://portal.genego.com/pub/network/n-839812191.html. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

FIG. 3. Different model networks for APE1-mediated signaling as derived by gene expression and proteomic data. Direct interaction network for genes dysregulated upon APE1 knock-down, involved in redox control, transcription regulators and chaperones (A) and (B) and in p53-signaling (C). Colors of the symbols indicate inhibition (blue) and activation (red). Here, the name APEX is used to refer to APE1. This model network is accessible at this site: https://portal.genego.com/pub/network/n-860173808.html. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

teolytic removal of the APE1 N-terminus will constitute a possible good candidate target for future drug development. On the other side, it is not completely clear whether truncation of APE1 N-terminus may affect APE1 redox activity (29, 52), leaving open the debate on the biological meaning of the process. Since the N-terminal region of the protein seems to be a result of phylogenetic evolution, it is intriguing that this part of the protein could account for the difference in modulating the various functions of APE1.

In addition to the canonical 37 kDa APE1 protein, and the truncated NΔ33APE1 form, in some circumstances the appearance of a lower mobility band of ~50 kDa has been detected (our unpublished data). This form would account for a PTM that introduces a substantial modification to the wild-type protein. A recently identified PTM, typically occurring on Lys residues of several nucleoproteins, is sumoylation (40, 56). The addition of a small ubiquitin-like modifier (SUMO) molecule to the target protein accounts for an increase of ~12 kDa of the apparent molecular mass of the target protein itself. Many SUMO-modified proteins function in regulation of transcription, chromatin structure, maintenance of the genome stability, and signal transduction. Upon sumoylation, interactions that are dependent on other post-translational modifications or on unmodified lysine are lost. Sumoylation also promotes novel interactions, in some cases associated with a conformational change in the target protein. The effects of PTM by SUMO to compete for target lysines enhance or inhibit interactions with other proteins (or other binding partners, such as DNA) or induce conformational changes. These effects are not mutually exclusive, but might not all occur on the same substrate. Thus, we investigated the possibility that APE1 may undergo sumoylation. APE1 protein contains 29 Lys residues, 15 of them lie in the N-terminal domain, and 14 residues lie in the C-terminal domain of the protein. Protein sequence analysis using three different softwares for prediction of sumoylation sites (SUMOPlot, SUMO sp, and SUMO PSFS) revealed several "low probability" putative sumoylation sites and a "high probability" putative sumoylation site, which contains a canonical sumoylation ψ-K-X-D/E motif (Fig. 5A and B). Putative sumoylation sites do not distribute homogenously along the whole sequence, but concentrate at the N-terminal domain, which is not present in functionally related proteins from other organisms and is required for the redox activity of APE1. Co-localization experiments on cells transfected with GFP-SUMO-1 clearly demonstrated that endogenous APE1 can be modified by wild-type SUMO-1 but not by a deletion mutant (i.e., Δ6SUMO-1), which looses its ability to modify target proteins (Fig. 5C). In vitro sumoylation experiments also clearly demonstrated that APE1 may undergo sumoylation (unpublished data). Our inability to identify the Lys residue target of sumoylation, by mutagenizing the putative sumoylation sites identified during in silico analysis (unpublished data), may suggest that multiple Lys residues could be the simultaneous target of this PTM. Additional work, which is difficult as sumoylated APE1 represents a very tiny fraction in comparison to the nonsumoylated protein, is required to understand the role of sumoylation in controlling APE1 functions.

In summary, all of these studies have confirmed the dual role nature of APE1, as a prototypical example of an apparent biological paradox. While a number of reports clearly demonstrated the antiapoptotic roles as well as the positive effect on cell proliferation (for a review, see Ref. 126, and 35, 67, 88), other data underlined its potential role in controlling proapoptotic functions through p53-mediated activation of p21 (38, 54, 117), leading to the arrest of cell cycle by inhibiting cyclin-dependent kinase function (11), and cyclin G, having pro-apoptotic activity (97). However, it is becoming increasingly evident that the antiapoptotic roles of APE1 are ascribable to its DNA repair functions (35) rather than to its activities as a transcriptional co-activator. Thus, it is tempting to speculate that the latter function—activation of transcription factors (i.e., p53 and Egr-1) ensuring efficient cell-cycle arrest—may act in concert with the previous (repair of DNA damage) to protect cells from accumulation of oxidative damage (Fig. 6) and be a later evolutionarily acquired function since only mammals and not just vertebrates appear to have the redox signaling function (39). This may have been an advantageous addition to APE1's interactions and functions that benefited mammalian cells, given the potentially toxic nature of their environment. This may have subsequently led to additional signaling and regulatory interactions and functions. Obviously, for a proper modulation of these two interconnected functions, a fine-tuned regulation of APE1 activities is required. Thus, a better understanding of the processes controlling APE1 subcellular distribution, of the post-translational modifications occurring on the protein itself, of the mechanisms controlling protein half-life, and of the different interacting partners recruited as a function of cellular response, is required to fully address this paradoxical issue.

Clinical Perspectives: Altered Expression/Distribution of APE1 and Human Pathology

Accumulating evidence has demonstrated that the heterogeneity of APE1 expression pattern is linked to different pathological conditions ranging from metabolic to differentiative disorders, including cancer and neurodegenerative diseases. Different kinds of human tumors were characterized by alterations in subcellular distribution of APE1 with respect to nontumoral tissue (73; for review, see 126). Generally, APE1 localization is eminently nuclear, while in several carcinomas a nuclear, cytoplasmic, and nuclear/cytoplasmic staining was observed (126). This peculiar distribution correlates well with the aggressiveness and prognosis of the tumor as nuclear localization was always associated with a better prognostic feature together with a higher degree of cellular differentiation, low angiogenesis, and negative lymph node status. As in hepatocellular carcinoma (HCC), a cytoplasmic localization of APE1 was associated with a significant lower degree of differentiation and with a shorter survival time, the localization of APE1 in liver biopsy is of prognostic value (22). Noteworthy, alteration in subcellular distribution of APE1 is not functionally related to the ability of cancerous tissue to repair abasic sites, suggesting that DNA repair by BER may not be affected (8, 113, 115). Therefore, it appears that the extranuclear roles of APE1 are responsible for its association with cancer.

Whether the alterations of APE1 subcellular localization are causally responsible or only associated with tumor progression is not clear at present. However, our recent findings in HCC, in which a chronic cellular oxidative

A

B
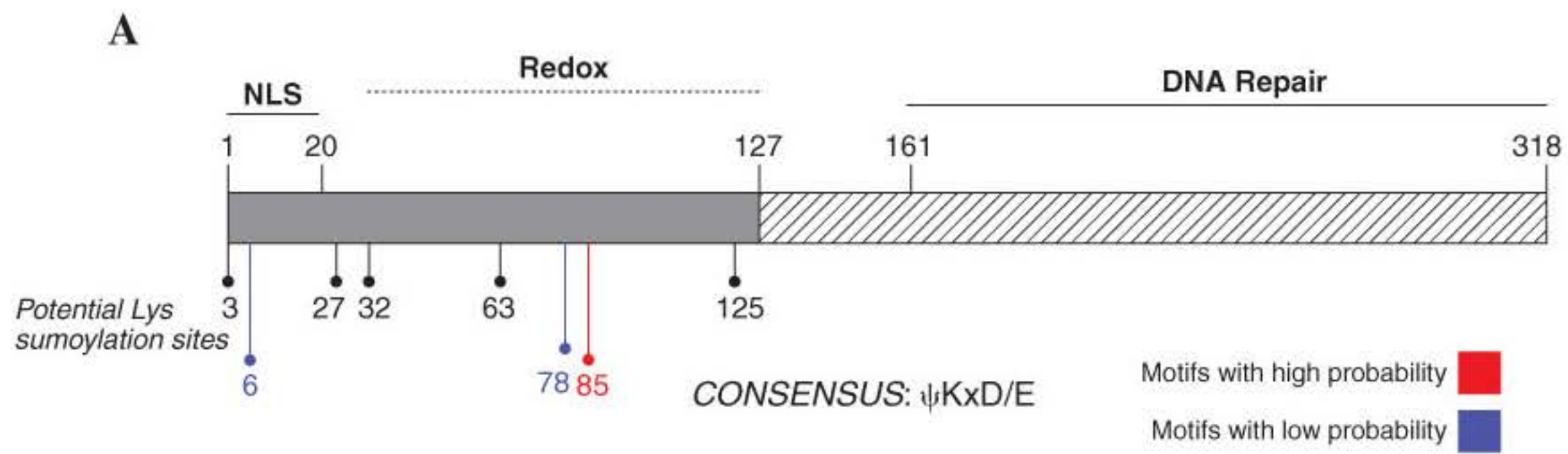
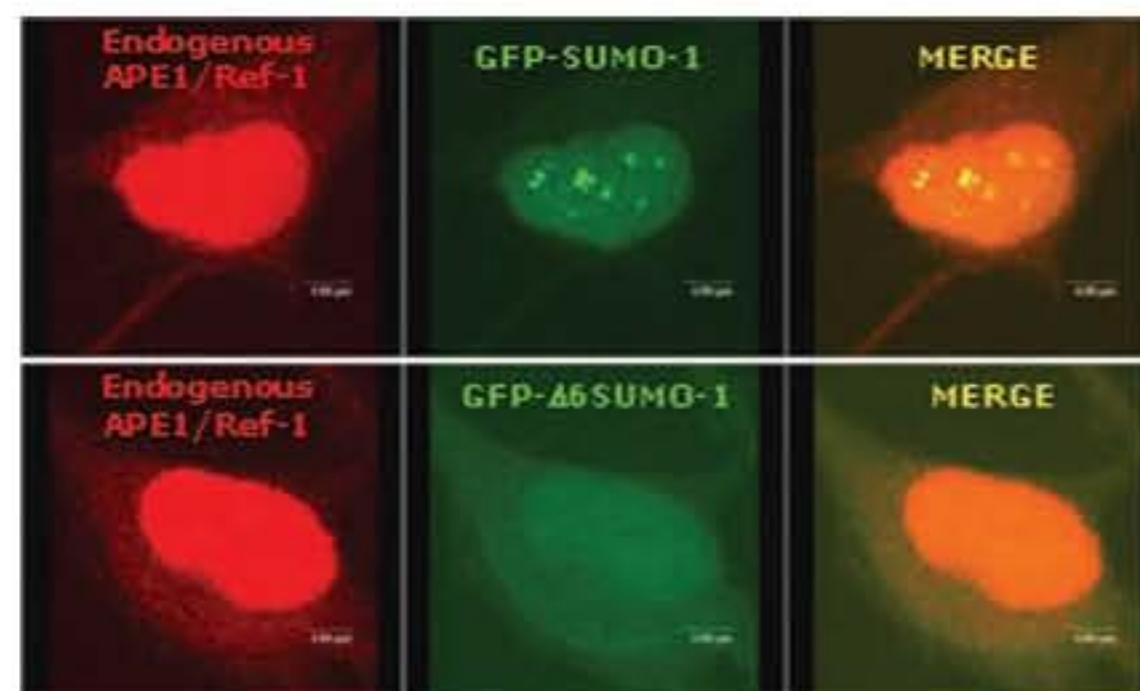|	SUMOPlot[a]		SUMO sp[b]		SUMO PSFS[c]	
- APE1/Ref-1 -						
		score		score		score
K 3	+	0,43	–	1,19	–	–
K 6	+	0,50	–	2,00	–	–
K 27	–	–	+	7,00	–	–
K 32	+	0,48	–	2,01	+	0,11
K 63	–	–	+	14,00	–	–
K 78	+	0,31	+	4,00	–	–
K 85	+	0,93	+	2,95	+	1,20
K 125	+	0,33	–	0,25	–	–

[a]Gramatikoff K et al. (45a).
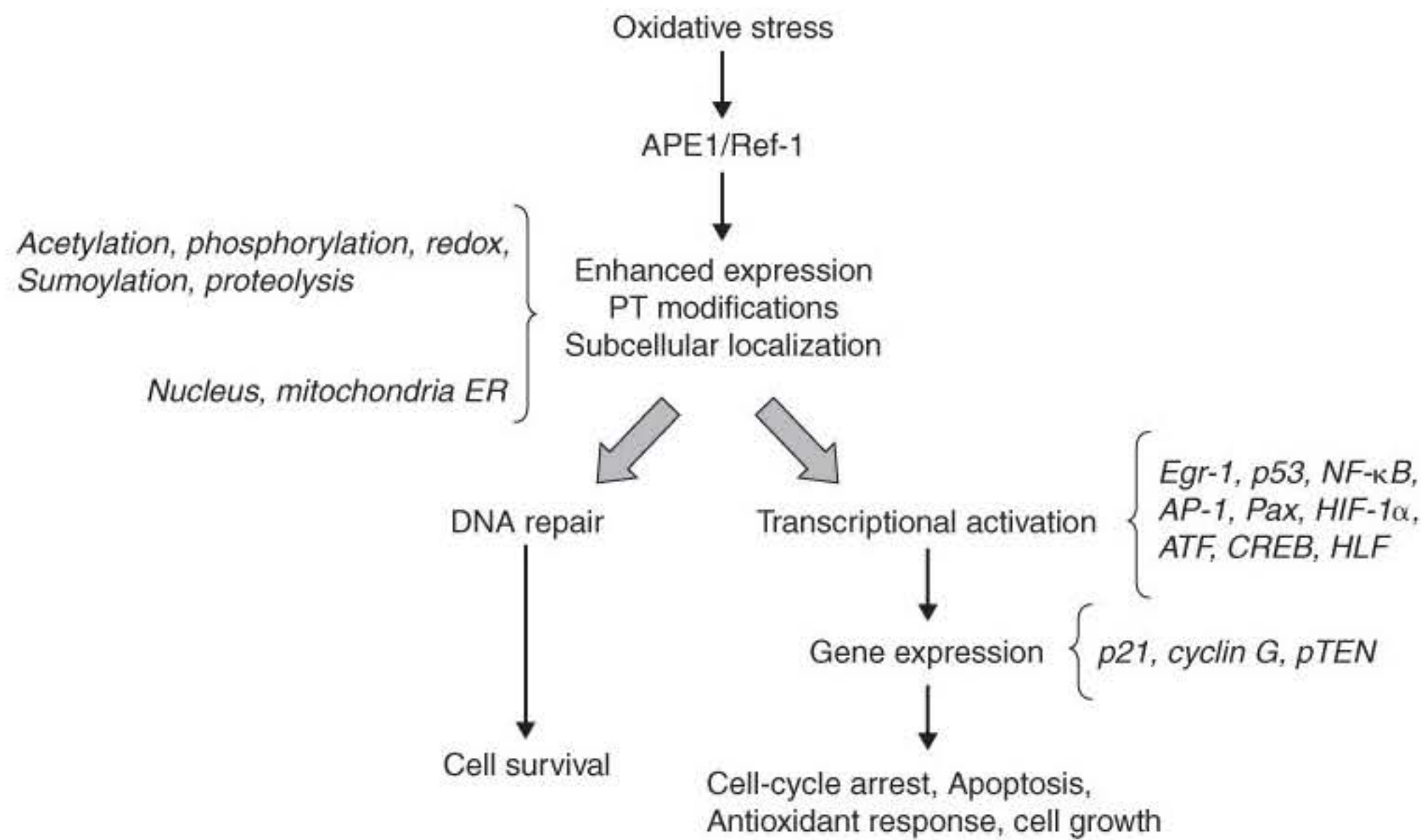[b]Xue et al. (147a).
[c]Liu et al. (79a).

C

FIG. 5. (A) APE1 protein sequence bears several putative sumoylation sites, as from *in silico analysis*. APE1 protein contains 29 Lysine residues, 15 of them lie in the N-terminal domain of the protein. Protein sequence analysis using three different softwares for prediction of sumoylation sites (SUMOPlot, SUMO sp, and SUMO PSFS) revealed several "low probability" putative sumoylation sites and a "high probability" putative sumoylation site, which contains a canonical sumoylation ψ-K-X-D/E motif. Putative sumoylation sites do not distribute homogenously along whole sequence, but concentrate at N-terminal domain, which is not present in functionally related proteins from other organisms and is required for the redox activity of APE1. (B) Of the 29 Lys residues, 8 of them are identified as potential sumoylation sites with the three different softwares. Lys85represents a classical consensus sequence, recognized by all progr ams. Lys78 is recognized as low-probability consensus sites by two different programs, while five Lys residues (K3, K6, K27, K63, and K125) are recognized as potential sumoylation sites by only one program. Interestingly, all potential sumoylation sites reside within the Redox transactivation domain. (C) APE1 colocalizes with SUMO-1, but not with Δ6 SUMO-1, into nuclear subdomains, presumably nuclear bodies. HeLa cells were transfected with either pGFPSUMO-1 or pGFPΔ6SUMO-1, as a negative control. The inactive Δ6SUMO-1 is a deletionmutant lacking the 6 C-terminal aminocid residues, including double glycines being the site for the isopeptidic bond with the target protein, whichcannot sumylate target proteins located in the nuclear bodies. Thus, this mutant relocalizes within nucleus and cytoplasm in a diffuse way. APE1 staining was analyzed using a monoclonal anti-APE1 antibody. Primary antibody and GFPSUMO-1 staining were revealed by incubation with Alexa Fluor 546 conjugated secondary antibody or by intrinsic green fluorescence of GFP, respectively. Merging of the two colors results in a yellow signal, corresponding to co-localized proteins. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

Oxidative stress

↓

APE1/Ref-1

↓

Acetylation, phosphorylation, redox,
Sumoylation, proteolysis
⎱ Enhanced expression
PT modifications
Nucleus, mitochondria ER ⎰ Subcellular localization

DNA repair   Transcriptional activation ⎰ *Egr-1, p53, NF-κB,*
AP-1, Pax, HIF-1α,
ATF, CREB, HLF

↓

Gene expression ⎰ *p21, cyclin G, pTEN*

↓   ↓

Cell survival   Cell-cycle arrest, Apoptosis,
Antioxidant response, cell growth

FIG. 6.   Model of APE1 multifunctional roles in coordinating cell response to oxidative stress. APE1 has dual functions 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.

stress conditions (91) in a milieu of inflammation (106) plays a central role in tumor progression implying functional activation of pro-survival transcription factors such as NF-κB and STAT3 (43, 84), supports the latter conclusion. To definitely address this point, it is mandatory that the interactome network of APE1 as well as the role of PTM in controlling the cytoplasmic localization of the protein must be unveiled. An interesting working hypothesis for a role of APE1 in the inflammatory process comes from recent works on APE1-mediated IL-8 production in *Helicobacter pylori* infections (96). These authors demonstrated that APE1 is required for *H. pylori* infection-mediated induction of IL-8 production by gastric cells through the involvement of NF-κB and AP-1 transcription factors (96). Thus, APE1 seems to play also a leading role in the production of inflammatory cytokines. Future work will be needed to extend these observations and to open new applicative perspectives in molecular medicine.

Other than in proliferative disorders mentioned above, APE1 deregulation has also been demonstrated in other pathologies, in particular degenerative disorders. Neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS), are characterized by a condition of chronic oxidative stress that primarily contributes to the pathogenesis through apoptosis of neuronal cells (33). APE1 is highly expressed in selected regions of the central nervous system (99, 100, 142). A reduction in APE1 expression, followed by an increase in the apoptotic rate, occurs in the hippocampus after a hypoxic–ischemic injury (41), in the cortex after compression injury (79), and in the spinal cord after ischemia (116). The hippocampus of patients with Alzheimer shows an increased expression of APE1 levels in senile plaques and plaque-like structures (123). Our recent unpublished data demonstrated an increased nuclear expression of APE1 in neuronal and glial cells of the cere-

bral cortex in both familial and sporadic Alzheimer (Marcon *et al.*, unpublished data). These findings, together with the observation of an increased DNA repair mechanism in Alzheimer (18), may be associated with the cellular adaptive response to the oxidative stress condition typical of Alzheimer, and may be involved in the pathogenesis of the disease. Despite the abundance of APE1 in neurons and the correlations between alterations in APE1 levels and various neuropathologies, few studies have addressed the role of APE1 in preventing neurotoxicity. Recent data demonstrate that APE1 protects primary cultures of hippocampal and sensory neurons from oxidative damage induced by H_2O_2 (134). However, despite its fundamental importance for future therapeutic development, the precise mechanism of the protective effect is still poorly understood.

Translational, Clinical Applications of APE1 Redox Inhibition for Cancer or Other Areas

As discussed in the previous sections, APE1 has two primary and major activities: DNA BER and redox signaling of downstream transcription factor targets. While previous studies have demonstrated that altering APE1 levels leads to blockage of cell growth and increased cancer cell sensitivity (8, 9, 16, 26, 31, 32, 55, 59, 65, 78, 80–83, 87, 98, 120, 124, 131, 138, 140, 149), these studies have either used overexpression of APE1, APE1 antisense oligonucleotides, or APE1 siRNA. The dilemma with this approach, while valid, is that each of these procedures changes the total cellular content level of APE1 and removes all of APE1's functions, not just the repair or redox activities. Because APE1 has multiple functions, as well as interactions with many other proteins (belonging to DNA repair, signaling and to the Egr-1 pathway), the increase or decrease of APE1 protein may result in multiple effects in which the APE1's specific role cannot be easily de-

picted. Furthermore, recent studies tested the hypothesis that APE1 is responsible for mediating production of single-strand DNA (ssDNA) breaks in gene promoters during repair of targeted base oxidation lesions caused by oxygen radicals generated during physiologic signaling (10, 153, 154). Production of ssDNA breaks is believed to play a key role during transcription by imparting substantial flexibility to promoter sequences, enabling them to bend in a manner that establishes the chromatin architecture needed for gene expression. In addition, APE1, besides forming the ssDNA break, is also required for high-fidelity repair of the break (104, 105, 119). Thus, defects in the APE1-mediated step in BER pathway could be linked to altered gene expression besides altering transcription factor state.

Use of specific small molecule inhibitors, such as one that blocks APE1 redox, but not repair, will be important to delineate the distinct roles of APE1 in various cancers, other diseases, and normal cellular functions. Likewise, an APE1 specific repair inhibitor will help to elucidate that role (4). Ultimately, using APE1 redox inhibitors with APE1 specific endonuclease repair inhibitors will give a clearer picture of the multiple activities of APE1.

APE1 was originally identified as the primary target of E3330 (3-[5-(2,3-dimethoxy-6-methyl-1,4-benzoquinoyl)]-2-nonyl-2-propionic acid), a small molecule redox inhibitor (155). E3330 was immobilized on beads and APE1 was identified from a nuclear extract of a leukemia cell line as a protein that specifically bound to E3330. Using surface plasmon resonance (SPR), an equilibrium constant (K_D) of 1.6 nM was obtained for the binding of E3330 to APE1, suggesting a specific interaction. E3330 was also shown to block the ability of APE1 to reduce NF-κB, thus interfering with the redox activity of APE1 (45, 60, 89, 118). The proposed binding site on APE1 is somewhat puzzling. The amino acid residues 72–80 form a ridge on the surface of the molecule with no obvious cavities or binding pockets that are large enough to bind E3330. Currently, we are pursuing studies to delineate the binding site or region of E3330 on APE1.

Our recent data have demonstrated that E3330 blocks the redox function of APE1 with AP-1 as the downstream target *in vitro* (86). Additionally, using a transactivation assay for AP-1 or HIF-1α targets in ovarian cancer cells, increasing amounts of E3330 led to decreased activation of a luciferase reporter downstream of AP-1 and HIF-1α (86). While E3330 blocked APE1's redox function, it had no effect on APE1 repair endonuclease activity nor other members of the BER pathway (86). These studies demonstrate the specificity of E3330 for APE1's redox, but not for its repair function. This is supported by our recently published data in ovarian cancer studies in xenografts demonstrating that the knockdown of APE1 results in the blocking of cell growth and proliferation, but not necessarily cell death (31). This is the first time that cancer cell killing has been reported using a small molecule inhibitor of APE1 redox function.

E3330 was also shown to have single agent inhibition of cell growth using a variety of cancer cell lines including, ovarian, colon, lung, breast, brain, pancreatic, prostate, and multiple myeloma cancers (Kelley *et al*. unpublished observations). In stark contrast, we do not see significant growth inhibition in our studies with normal cells such as hematopoietic embryonic cells (155) or in human CD34+ progenitor cells (Kelley *et al*. unpublished observations). These data

are novel in that they implicate the redox role of APE1 in cancer, but not "normal", cell survival.

Recent studies using this APE1 redox inhibitor in angiogenesis model systems have been instructive. Proliferation of endothelial cells (EC) is an important index for angiogenic ability of EC *in vitro*. E3330 inhibited retinal vascular endothelial cells (RVEC) growth in a dose-dependent manner (86). Additionally, an *in vitro* angiogenesis assay was also used to determine the effect of E3330 on RVEC formation of capillary-like structures on Matrigel. The inhibitory effect of E3330 was similar to the proliferation assay with the complete loss of tube formation at low micromolar concentrations. These results demonstrate that blocking APE1's redox function attenuates RVEC proliferation and capillary formation *in vitro* and these findings implicate the use of an APE1 redox inhibitor in antiangiogenic translational studies. Further mechanistic studies as to how this is occurring are in progress, although preliminary studies indicate the redox inhibitory effect is not necessarily related to cell killing, but to a block in cell proliferation or cytostatic effect similar to that observed using APE1 siRNA *in vivo* (31).

In conclusion, APE1 is a multifunctional protein with both important DNA repair and redox capabilities. However, in order to demarcate the various functions of APE1, small molecule inhibitors of each function will be necessary to ultimately conclude which function is required in normal and cancer cell function. Recent findings with redox inhibition of APE1 have potential clinical translational significance such that a redox inhibitor could be used as a single agent, in combination with current treatments or as a potential antigrowth, cytostatic agent. Furthermore, new APE1 redox analogues could play a role in antiangiogenic therapies.

Acknowledgments

Abbreviations

AP, apurinic/apyrimidinic; AP-1, activator protein-1; BER, base excision repair; CKI and CKII, casein kinase I and II; CREB, cAMP response element binding protein; Egr-1, early growth response protein-1; FEN1, flap endonuclease I; GSK3, glycogen synthase kinase 3; GzmA, granzyme A; GzmK, granzyme K; HIF-1α, hypoxia inducible factor-1α; MTS, mitochondrial targeting sequence; MPG, methylpurine DNA glycosylase; nCaRE, negative calcium-responsive regulatory element; NF-κB, nuclear factor-κB; Ogg1, 8-oxoguanosine DNA glycosylase; P2Y, purinergic receptors (G-proteins coupled); Pax, paired box containing genes; PCNA, proliferating cell nuclear antigen; PEBP-2, polyoma virus enhancer-binding protein-2; PKC, protein kinase C; PTH, parathyroid hormone; PTM, post-translational modification; RFC, replication factor C; ROS, reactive oxygen

species; SPR, surface plasmon resonance; ssDNA, single-stranded DNA; SUMO, small ubiquitin-like modifier; TFs, transcription factors; Trx, thioredoxin; TTF-1, thyroid transcription factor-1; XRCC1, X-ray cross-species complementing 1.

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