Role of the unstructured N-terminal domain of the hAPE1 (human apurinic/apyrimidinic endonuclease 1) in the modulation of its interaction with nucleic acids and NPM1 (nucleophosmin)

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INTRODUCTION

The hAPE1 (human apurinic/apyrimidinic endonuclease 1) is an essential enzyme, being the main abasic endonuclease in higher eukaryotes. However, there is strong evidence to show that hAPE1 can directly bind specific gene promoters, thus modulating their transcriptional activity, even in the absence of specific DNA damage. Recent findings, moreover, suggest a role for hAPE1 in RNA processing, which is modulated by the interaction with NPM1 (nucleophosmin). Independent domains account for many activities of hAPE1; however, whereas the endonuclease and the redox-active portions of the protein are well characterized, a better understanding of the role of the unstructured N-terminal region is needed. In the present study, we characterized the requirements for the interaction of hAPE1 with NPM1 and undamaged nucleic acids. We show that DNA/RNA secondary structure has an impact on hAPE1 binding in the absence of damage. Biochemical studies, using the isolated N-terminal region of the protein, reveal that the hAPE1 N-terminal domain represents an evolutionary gain of function, since its composition affects the protein’s stability and ability to interact with both nucleic acids and NPM1. Although required, however, this region is not sufficient itself to stably interact with DNA or NPM1.

Key words: apurinic/apyrimidinic endonuclease 1 (APE1), nucleophosmin (NPM1), protein–DNA interaction, protein–protein interaction, phylogenesis.
PCDNA5.1-hAPE1 (full-length or amino acids 1–49) plasmids were transfected with 6 μg at a density of 3 × 10⁵ cells/plate. Cells were then transiently seeded in 10-cm plates (diameter × depth) or 60-mm dishes. The indicated amount of GST-tagged protein was mixed, together with the prey protein or 10 μg of total RNA, with 10 μl of glutathione-Sepharose 4B beads (GE Healthcare). RNA was extracted using the TRIzol® Reagent (Invitrogen). Binding was performed in PBS supplemented with 5 mM DTT (dithiothreitol) and 0.5 mM PMSF for 2 h under rotation at 4°C. The beads were washed three times with washing buffer [PBS supplemented with 0.1% Igepal CA-630 (Sigma), 5 mM DTT and 0.5 mM PMSF]. Beads were then resuspended in Laemmli sample buffer for Western blotting analyses or in TRIZol® Reagent for RNA extraction and rRNA quantification. The levels of 28S and 18S rRNA transcripts were determined as described previously [17] by using the iScript cDNA synthesis kit (Bio-Rad Laboratories) and the respective full-length constructs as templates, following the manufacturer’s instructions and using standard subcloning procedures.

**Plasmids and expression of recombinant proteins**

The constructs pGEX-3X-hAPE1 and pGEX-3X-zAPE1, encoding for the GST-fused hAPE1 and zAPE1 (zebrafish APE1) full-length proteins respectively, were provided by Dr Mark R. Kelley (Indiana University, Indianapolis, IN, U.S.A.). The plasmid encoding for full-length GST–NPM1 was provided by Dr P.G. Pellicci (European Institute of Oncology, Milan, Italy). The pTAC-MAT/hAPE1 and the pEJM-20/zAPE1 constructs, encoding for C-terminal-tagged MAT (metal-affinity tag)–hAPE1 and His₆–zAPE1 respectively, were generated by subcloning. All proteins were expressed in E. coli BL21(DE3) cells, induced with 1 mM IPTG (isopropyl β-D-thiogalactopyranoside) and then purified on an AKTA Puriﬁer FPLC system (GE Healthcare) by using a GSTrap HP column (GE Healthcare) for the GST-tagged proteins, or a HiTrap HP column (GE Healthcare) for the His₆ and MAT-tagged proteins. The quality of purification was checked by SDS/PAGE analysis. Extensive dialysis against PBS was performed to remove any trace of imidazole from the HisTrap-purified proteins. Accurate quantification of all recombinant proteins was performed by colorimetric Bradford assays (Bio-Rad Laboratories) and confirmed by SDS/PAGE and Western blotting analysis. To remove the GST tag, GST–hAPE1 was further hydrolysed with Factor X, as described previously [17]. The zAPE1 K27 ‘swapping mutant’ and the hAPE1 NΔ43 and zAPE1 NΔ36 deletion mutants, as well as the hAPE1 1–48 and 1–49 constructs, were generated using the QuikChange Mutagenesis kit (Stratagene) and the respective full-length constructs as templates, following the manufacturer’s instructions and using standard subcloning procedures.

**GST pull-down assays with RNA or NPM1 and co-immunoprecipitation**

The indicated amount of GST-tagged protein was mixed, together with the prey protein or 10 μg of total RNA, with 10 μl of glutathione–Sepharose 4B beads (GE Healthcare). RNA was extracted using the TRIZol® Reagent (Invitrogen). Binding was performed in PBS supplemented with 5 mM DTT (dithiothreitol) and 0.5 mM PMSF for 2 h under rotation at 4°C. The beads were washed three times with washing buffer [PBS supplemented with 0.1% Igepal CA-630 (Sigma), 5 mM DTT and 0.5 mM PMSF]. Beads were then resuspended in Laemmli sample buffer for Western blotting analyses or in TRIZol® Reagent for RNA extraction and rRNA quantification. The levels of 28S and 18S rRNA transcripts were determined as described previously [17] by using the iScript cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer’s instructions. Quantitative PCR was then performed in an iQ5 multicolour real-time PCR detection system (Bio-Rad Laboratories) according to the manufacturer’s protocol. GST-tagged proteins were detected with an HRP (horseradish peroxidase)-conjugated anti-GST antibody (Sigma), whereas the prey proteins were detected using the indicated antibodies. Co-immunoprecipitation analyses were performed as described previously [12].

**DSP (dithiobis(succinimidyl propionate)) cross-linking**

The indicated amounts of recombinant NPM1 and hAPE1 (either full-length or the amino acids 1–48 peptide), were allowed to

**EXPERIMENTAL**

**Cell culture and transient transfection experiments**

HeLa cells were grown in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% FBS (fetal bovine serum), 100 units/ml penicillin and 10 mg/ml streptomycin sulfate. At 1 day before transfection, cells were seeded in 10-cm plates at a density of 3 × 10⁵ cells/plate. Cells were then transiently transfected with 6 μg of either pCDNA5.1 (empty vector) or pCDNA5.1-hAPE1 (full-length or amino acids 1–49) plasmids per dish using the Lipofectamine® 2000 Reagent (Invitrogen) according to the manufacturer’s instructions. Cells were harvested at 24 h after transfection.

**Multiple sequence alignments**

For the multiple sequence alignments full-length hAPE1 orthologous metazoan sequences (human, Pan troglodytes, Macaca mulatta, Equus caballus, Mus musculus, Rattus norvegicus, Canis lupus familiaris, Sus scrofa, Bos taurus, Ornithorhyncus anatinus, Salmo salar, Gasterosteus aculeatus, Danio rerio, Xenopus tropicalis and Xenopus laevis) were retrieved from the RefSeq protein database using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/blast.cgi) and aligned using the ClustalW2 program with default parameters (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

**Western blotting analyses**

The protein samples were electrophoresed by SDS/PAGE (10–18% gels) or discontinuous Tris/Tricine SDS/PAGE [24]. Proteins were then transferred on to nitrocellulose membranes (PerkinElmer), developed as described previously [12] using an ECL (enhanced chemiluminescence) procedure (GE Healthcare) and then quantified by using a ChemiDoc XRS video-densitometer (Bio-Rad Laboratories).
interact at 37 °C for 15 min in a total volume of 10 μl; DSP (2 μg in DMSO) was added and cross-linking was carried out for 30 min at room temperature (22 °C). The reactions were quenched by adding 0.5 volumes of ice-cold 0.4 M ammonium acetate and incubating on ice for 10 min. Non-reducing Laemmli sample buffer was immediately added and the samples were separated by SDS/PAGE (8 % gel).

**Immunofluorescence confocal analyses and PLA (proximity ligation assay)**

Immunofluorescence was carried out as described previously [12]. FLAG-tagged 1–49 hAPE1 was labelled using an anti-FLAG antibody and stained with a secondary anti-mouse Alexa Fluor™ 488-conjugated antibody (Jackson ImmunoResearch). Nuclei were counterstained with DAPI (4,6-diamidino-2-phenylindole). For the PLA analyses we used the Duolink II® reagent kit (Olink Bioscience); cells were fixed in 4 % (w/v) paraformaldehyde, permeabilized with 0.25 % PBS-Triton (PBS with Triton X-100) and saturated with 10 % (v/v) FBS. Slides were incubated with primary antibodies (mouse anti-FLAG, 1:100 dilution and rabbit anti-NPM1, 1:200 dilution) and then reactions were carried out following the manufacturer’s instructions. Microscope slides were mounted and visualized through a Leica TCS SP laser-scanning confocal microscope.

**hAPE1–nucleic acid binding through EMSA**

hAPE1 binding to nucleic acids was assessed as described previously [1] with some modifications. Briefly, in order to reduce unspecific binding and to obtain more defined bound complexes, recombinant proteins or peptides were incubated at 37 °C with 250 pmol of unlabelled poly(dT) for 15 min and then 2.5 pmol of 32P-labelled probe was added. The reactions were then incubated for further 15 min and separated by native PAGE (6–10 % gel) at 150 V for 4 h.

**Southwestern assays**

Bait proteins were separated using a pre-cast SDS/PAGE (Bio-Rad Laboratories), electrotransferred on to a nitrocellulose membrane and subsequently denatured and renatured by washing six times in 1:1 serial dilutions of 6 M guanidinium/HCl in EMSA buffer [40 mM Hepes (pH 8.0), 50 mM KCl, 2 mM EDTA, 10 % (v/v) glycerol and 1 mM DTT]. The membrane was then saturated in 5 % (w/v) BSA in EMSA buffer and incubated with 50 pmol of the indicated 32P-labelled DNA probe at 37 °C, washed in EMSA buffer to remove unspecific bound probe and finally subjected to autoradiography.

**SPR**

Real-time binding analyses were performed on a Biacore T-100 SPR instrument (GE Healthcare). Streptavidin was immobilized on to a research-grade CM5 sensor chip using amine-coupling chemistry. The immobilization steps were carried out at a flow rate of 10 μl/min in Heps buffer [20 mM Heps, 150 mM NaCl, 3.4 mM EDTA, 0.005 % P20 surfactant and 0.1 mM TCEP [tris-(2-carboxyethyl)phosphine]]. All surfaces were simultaneously activated for 30 s with a mixture of NHS (N-hydroxysuccinimide; 0.05 M) and EDC [N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide; 0.2 M]. Streptavidin was injected at a concentration of 20 μg/ml in 10 mM sodium acetate (pH 4.5) for 1 min. Ethanolamine (1 M, pH 8.5) was injected for 7 min to saturate the remaining activated groups. An average of 5000 RU (response units) of streptavidin was immobilized on each flow cell. Biotinylated oligonucleotides were injected at a concentration of 500 nM until the desired level of immobilization was achieved (average value of 300 RU). Proteins were serially diluted in running buffer (80 nM–2.0 μM) and injected at 20 °C at a flow rate of 60 μl/min for 1 min. Disruption of any complex that remained bound after a 5-min dissociation was achieved using a 30 s injection of 1 M NaCl at 100 μl/min. When the experimental data met the quality criteria, the kinetic parameters were estimated assuming a 1:1 binding model and using version 4.1 Evaluation Software (GE Healthcare). Conversely, an affinity steady-state model was applied to fit the RU_{max} data compared with the protein concentrations and fitting was performed with GraphPad Prism v4.00 [17].

**CD**

Far-UV CD spectra were recorded on a Jasco J-810 spectropolarimeter (JASCO) in a 195–260 nm interval. Experiments were performed employing a protein concentration of 5 μM in 10 mM phosphate buffer (pH 7.0), supplemented with 1 mM DTT and using a 0.1 cm path-length cuvette. Thermal denaturation profiles were obtained by measuring the temperature dependence of the signal at 225 nm in the 20–100 °C range with a resolution of 0.5 °C and 1.0-nm bandwidth. A Peltier temperature controller was used to set the temperature of the sample with the heating rate set to 1 °C/min. Data were collected at a 0.2-nm resolution, 20 nm/min scan speed and a 4 s response and were reported as the unfolded fraction against the temperature.

**Statistical analyses**

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

**RESULTS AND DISCUSSION**

**The binding affinity of hAPE1 for undamaged nucleic acids is dependent on their secondary structure**

Several studies have thoroughly characterized hAPE1 structure and its enzymatic activities on abasic DNA, examining its substrate specificity and recognition mechanisms [7,11,25–28]. However, when considering undamaged structured nucleic acids, the knowledge of hAPE1 substrate selectivity is still scarce. In the present study we sought to determine whether some particular structural features were favoured by hAPE1 when binding to nucleic acids. Hence, we used a biophysical approach exploiting the SPR technique to quantitatively measure the binding affinity of hAPE1 for intact oligonucleotides [29,30]. We compared the binding affinity of recombinant purified full-length hAPE1 toward different single-stranded DNA probes, either intrinsically lacking secondary structure (i.e. 34dT), or having a stem and loop-like folding (e.g. Stem10 and Stem20) as predicted by bioinformatics analyses (Figure 1A and Table 1). The results of the SPR experiments (representative sensograms in Figure 1B and Supplementary Figure S1C at http://www.biochemj.org/bj/452/bj4520545add.htm) are summarized in Table 2. As already observed by Beloglazova et al. [11], an increased length in the stem region of the oligonucleotides augmented the affinity of hAPE1 for undamaged DNA (compare the Stem10 and the Stem20 sequences). This observation was confirmed by direct EMSA experiments (Figure 1C) and by competition EMSA
Figure 1 The secondary structure of the substrate determines the binding selectivity of hAPE1

(A) The predicted structures for the oligonucleotides used in the present study. Calculations were performed using either the Mfold web server (http://mfold.rna.albany.edu/?q=mfold) or the DINAmeit web server (http://mfold.rna.albany.edu/?q=DINAMelt). The calculated minimum free energy for folding ($\Delta G$) at 37 $^\circ$C and 50 mM Na$^+$ is also reported. The arrow indicates the absence of loop in the Stem20 no loop probe. (B) Representative overlay sensorgrams relative to a typical SPR experiment for the binding of hAPE1 to immobilized Biot-Stem20. The kinetic parameters were measured injecting purified recombinant hAPE1 solutions at increasing concentrations (i.e. 87.5, 175, 350, 700, 1050, 1400 and 1750 nM). The $K_d$ values were estimated for each concentration using the BIAevaluation v.4.1 software. (C) Positive effect of the stem length on ability of hAPE1 to bind undamaged DNA as evaluated through EMSA using the Stem10 and Stem20 oligonucleotides (Table 1). For each reaction the indicated amount of purified recombinant hAPE1 was used. The results confirm a different binding capacity of hAPE1 towards double-stranded oligonucleotides of different lengths. The arrow indicates the hAPE1–DNA complexes, whereas F denotes the position of the free oligonucleotide probe. wt, wild-type.
two oligonucleotides. A lower stability of the stem region in the loop side as the stem side remains invariant between the double-stranded region bound by hAPE1 might accelerate the expansion of the protein–DNA complex then was investigated by comparing the affinities of the Stem20 and the Stem20 no loop sequences. The effect of the presence of an intact loop structure on the stability of the protein–DNA complex then was investigated by comparing the affinities of the Stem20 and the Stem20 no loop sequences. The difference in the unfolding free energy of the two molecules (Figure 1A). We finally investigated whether the position of the unstructured region might affect the binding capacity of hAPE1 (compare Bulge, Stem20 and Stem20_2). Despite the differences in both the dissociation and the association rates, the overall affinity of hAPE1 for the oligonucleotides remained unchanged, suggesting that a local denaturation of the double helix, rather than its location within the double-stranded region, is a feature that enhances binding of hAPE1 to undamaged DNA. To compare our measurements on synthetic oligonucleotides with a physiologically relevant undamaged DNA substrate, we investigated the binding properties of hAPE1 towards an nCaRE duplex sequence, namely nCaRE_B2 (G. Antoniali, L. Lirussi, C. D’Ambrosio, F. Dal Piaz, C. Vascotto, D. Marasco, A. Scaloni, F. Fogolari and G. Tell, unpublished work). This sequence displayed a $K_d$ value akin to that of the unstructured Stem20 no loop sequence (Table 2). It is worth pointing out that our in vitro assays, performed with the isolated purified recombinant protein, might underestimate the real affinity of hAPE1 for this sequence, since interactions with other protein partners [e.g. Ku70/80 and hnRNPL (heterogeneous nuclear ribonucleoprotein L)] significantly increase the protein–DNA complex stability, as already demonstrated previously (G. Antoniali, L. Lirussi, C. D’Ambrosio, F. Dal Piaz, C. Vascotto, D. Marasco, A. Scaloni, F. Fogolari and G. Tell, unpublished work) [31].

EMSA analyses were also performed on RNA substrates to test whether the nature of the sugar backbone might affect the binding activity of hAPE1 toward structured nucleic acids. The results obtained confirmed that the structural requirements for hAPE1 binding to undamaged substrates are not influenced by the composition of the sugar backbone (results not shown). These observations suggest that hAPE1 shows no apparent preference for DNA or RNA substrates, as long as the nucleic acid is undamaged and is endowed with a secondary structure. The results of the present study are in agreement with experimental data from other laboratories that have estimated $K_d$ values for the interaction of hAPE1 with structured RNA that is similar to our values for DNA (∼0.9 μM) [32]. It must be noted, however, that these experiments do not allow the exclusion of the existence of a differential affinity of hAPE1 for abasic site-containing DNA or RNA molecules, as has been suggested by others [12,26].

In summary, hAPE1 binding to undamaged nucleic acids appears to be strongly affected by the presence of secondary structural elements. In particular, the presence of a double-stranded region is a primary element required for hAPE1 binding. The occurrence of a single-stranded local distortion, moreover, greatly enhances the interaction with nucleic acids. These factors are corroborated by studies reporting a significant reduction in the APE activity of the protein on single-stranded unstructured (or poorly structured) substrates (e.g. [7]). The requirement of a double-stranded DNA region is conceivable, on the basis of the observation that hAPE1 contacts both of the DNA strands [27,28]. The presence of a loop or a bulge within a double-stranded stem could mimic a local denaturation region that resembles a baseless spot, hence explaining the increased affinity for loop-containing structures displayed in vitro by hAPE1.

### The N-terminal domain contributes to nucleic acid-binding by hAPE1

We have shown previously that the N-terminal 33 amino acids of hAPE1 is able itself to bind RNA oligonucleotides in solution [17]. Sequence alignment analyses, performed on different metazoan

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<th>$k_{on}$ (M$^{-1}$·s$^{-1}$·10$^{3}$)</th>
<th>$k_{off}$ (s$^{-1}$)</th>
<th>$K_d$ (μM)</th>
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<td>34dT</td>
<td>0.004</td>
<td>0.112</td>
<td>308 ± 3.00</td>
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<td>0.270</td>
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<tr>
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<tr>
<td>Stem20 Loop8</td>
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<td>0.004</td>
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</tr>
<tr>
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<td>0.019</td>
<td>0.23 ± 0.02</td>
</tr>
<tr>
<td>nCaRE_B2</td>
<td>0.270</td>
<td>0.105</td>
<td>3.90 ± 0.80</td>
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| Stem20 Loop8 sequence could also reflect the decreased binding affinity; bioinformatic predictions, however, do not suggest any major difference in the unfolding free energy of the two molecules (Figure 1A). We finally investigated whether the position of the unstructured region might affect the binding capacity of hAPE1 (compare Bulge, Stem20 and Stem20_2). Despite the differences in both the dissociation and the association rates, the overall affinity of hAPE1 for the oligonucleotides remained unchanged, suggesting that a local denaturation of the double helix, rather than its location within the double-stranded region, is a feature that enhances binding of hAPE1 to undamaged DNA. To compare our measurements on synthetic oligonucleotides with a physiologically relevant undamaged DNA substrate, we investigated the binding properties of hAPE1 towards an nCaRE duplex sequence, namely nCaRE_B2 (G. Antoniali, L. Lirussi, C. D’Ambrosio, F. Dal Piaz, C. Vascotto, D. Marasco, A. Scaloni, F. Fogolari and G. Tell, unpublished work). This sequence displayed a $K_d$ value akin to that of the unstructured Stem20 no loop sequence (Table 2). It is worth pointing out that our in vitro assays, performed with the isolated purified recombinant protein, might underestimate the real affinity of hAPE1 for this sequence, since interactions with other protein partners [e.g. Ku70/80 and hnRNPL (heterogeneous nuclear ribonucleoprotein L)] significantly increase the protein–DNA complex stability, as already demonstrated previously (G. Antoniali, L. Lirussi, C. D’Ambrosio, F. Dal Piaz, C. Vascotto, D. Marasco, A. Scaloni, F. Fogolari and G. Tell, unpublished work) [31].

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species, highlight that the phylogenetic conservation of the amino acid sequence among APE1 proteins from different organisms starts at position 48 in the human protein, and is significantly lower in the region spanning amino acids 1–48 (Figure 2A). In order to better clarify the relevance of the non-conserved N-terminal domain in modulating the binding ability of hAPE1 to nucleic
acids, we cloned and expressed in *E. coli* cells the 1–48 amino acid region of hAPE1 in fusion with a C-terminal MAT (Supplementary Figures S2A and S2B at http://www.biochemj.org/bj/452/bj45205454add.htm) and evaluated the binding properties of the recombinant purified peptide toward different substrates, including DNA and RNA. We indirectly estimated the rRNA-binding activity of the peptide exploiting a GST pull-down competition strategy. In these experiments, the full-length GST–hAPE1 was used as the bait and total HeLa RNA as the prey, while either the recombinant purified 1–48 peptide or its synthetic untagged 1–33 counterpart were used as soluble competitors. The data obtained revealed that both soluble peptides were able to compete with full-length hAPE1 for RNA binding, independently of the presence of a tag (Figure 2B).

In order to compare the nucleic acid-binding activity of the 1–48 peptide and that of the full-length hAPE1 protein, we used EMSA analyses. Under our experimental conditions we observed a low-affinity binding activity for the hAPE1 N-terminal peptide towards the nCaREB_2 duplex sequence (Figure 2C and Supplementary Figure S2C). Although at a reduced affinity, however, the interaction with DNA was specific, as demonstrated by both Southwestern assays (Figure 2D and Supplementary Figure S2D) and Western blotting performed after the EMSA analysis (Supplementary Figure S2E). These results, along with the notion that the N-terminal 1–33 amino acid portion of hAPE1 is absolutely required for the stable interaction of the protein with nucleic acids [12,17], suggest that this lysine-rich region might act by stabilizing the association of hAPE1 with nucleic acids through electrostatic interactions, whereas the remaining C-terminal domain accounts for the specific binding to DNA/RNA. Our data support a two-step model to describe the mechanism through which hAPE1 contacts nucleic acids. The unstructured N-terminal domain would be required for the preliminary low-affinity binding process in search of the proper lesion to be repaired, as suggested for other DNA-binding proteins [33]. Recognition of a local distortion (or an abasic site) involves and is stabilized by a higher-affinity binding event. The whole hAPE1 molecule would, at this point, become necessary for the recognition of the structured nucleic acid region. Intriguingly, this model nicely fits previous hypothesis proposed by Masuda et al. [34] more than a decade ago.

The N-terminal domain of hAPE1 is necessary, but not sufficient, for a stable binding to NPM1

In our previous work, we demonstrated that the N-terminal 1–33 amino acid domain of hAPE1, besides being essential for stabilizing its binding to nucleic acids, is also required for a stable interaction with NPM1 [12] and that lysine residues spanning the region 27–35 of hAPE1 are directly involved in stabilizing this interaction [17]. However, we did not check whether the N-terminal domain of hAPE1 was able to directly bind to NPM1 itself. In an effort to better characterize the protein–protein interaction features of the hAPE1 N-terminal domain and its relative contribution to the overall binding, we analysed the ability of the 1–48 peptide to directly interact with NPM1. We expressed both the full-length hAPE1 and the 1–48 peptide in fusion with a MAT. The recombinant proteins were affinity purified and tested for their binding ability toward GST-tagged NPM1 through GST pull-down assays (Figure 3A). Although the full-length hAPE1 protein was able to directly interact with NPM1 (Figure 3A, left-hand panel), we failed to detect any stable peptide–NPM1 association in vitro (Figure 3A, right-hand panel), even under less stringent binding conditions (results not shown). This negative result prompted us to hypothesize that, if present, any peptide–NPM1 association could be too weak to be detected through a direct pull-down assay. We confirmed this hypothesis through a competition GST pull-down assay, in which the 1–48 peptide was used to squelch the binding occurring between the full-length hAPE1 and GST–NPM1 (Figure 3B). The peptide, as well as an untagged full-length hAPE1, was able to displace the association between the MAT-tagged hAPE1 and GST–NPM1. However, whereas the competitor untagged hAPE1 was also efficiently recovered in the pulled-down fraction, the competitor 1–48 peptide was not. This indicates that the peptide was able to mask the hAPE1-binding site on NPM1, thus competing with the hAPE1–NPM1 association, although its interaction with the protein was too weak to be maintained during the washing steps. The observed competition effect could also be interpreted by assuming a masking effect of the peptide on the full-length hAPE1 N-terminal region, however, to the best of our knowledge, no oligomerization ability has ever been described for hAPE1.

To definitely prove the ability of the N-terminal peptide to directly associate with NPM1, we performed an *in vitro* stabilization of the complex exploiting the bifunctional cross-linker DSP (Figure 3C). As expected, treatment of NPM1 alone with DSP resulted in the stabilization of slow migrating oligomers, as documented previously [35]. Moreover, the full-length hAPE1 protein and its N-terminal peptide were detected as slow migrating signals only in the presence of both NPM1 and DSP, indicating a direct association for both with NPM1. The lower affinity of the 1–48 peptide toward NPM1, in comparison with that of the full-length protein, indicates that the C-terminal domain of hAPE1 contributes to the stabilization of the protein–protein interaction.

To evaluate whether the interaction between the hAPE1 N-terminal extension and NPM1 also occurs *in vivo*, we created a HeLa cell line expressing a FLAG-tagged 1–49 hAPE1 peptide upon treatment with doxycycline, as already described previously [36]. As assessed using Western blotting, the peptide expression increased after the addition of doxycycline to the culture medium (Figure 4A, upper panel). Interestingly, the ectopically expressed peptide showed a peculiar localization pattern, being cytoplasmic and nuclear, but excluded from nucleoli (Figure 4A, lower panel), in contrast with the nuclear/nucleolar accumulation observed for the wild-type protein both under basal conditions [12,21] and when ectopically expressed (Figure 4A, lower panel). This result confirms that the C-terminal domain of hAPE1 contributes to the nucleolar accumulation of the protein within cells, despite the fact that the N-terminal domain retains the nuclear localization signal of the protein [37]. This phenomenon may be explained by the relevance that the C-terminal domain has in stabilizing both rRNA and NPM1 binding. By exploiting this cell model, we performed co-immunoprecipitation experiments and demonstrated the occurrence of a molecular interaction between NPM1 and the isolated hAPE1 N-terminal peptide. However, the FLAG-tagged 1–49 peptide was only able to co-precipitate NPM1 poorly when compared with the full-length protein (Figure 4B). This result parallels our *in vitro* results with recombinant purified proteins and confirms that the unstructured N-terminal region of hAPE1 does indeed interact with NPM1, albeit with a lower affinity in the absence of the remaining C-terminal domain. We further confirmed the presence of an interaction occurring between the FLAG-tagged 1–49 hAPE1 peptide and endogenous NPM1 using a PLA [21], which allows the quantification of physical proximity between molecules at a distance lower than 40 nm (Supplementary Figure S3A at http://www.biochemj.org/bj/452/bj45205454add.htm) [38]. The PLA signal was present in the nucleoplasmic compartment only in the presence of both the anti-FLAG and the anti-NPM1 antibodies.
Figure 3  The unstructured N-terminal domain of hAPE1 is required, but not sufficient, for stable binding to NPM1 in vitro

(A) A representative GST pull-down experiment showing the absence of a stable interaction, in vitro, between recombinant GST-tagged NPM1 (bait) and the MAT-tagged 1–48 hAPE1 N-terminal peptide (prey) (right-hand panel). The same assay using full-length MAT-tagged hAPE1 is reported as a positive control (left-hand panel). The assay was performed as described in the Experimental section using the indicated amounts of recombinant proteins. (B) The recombinant 1–48 hAPE1 peptide is able to compete, in vitro, with the full-length hAPE1 protein for the binding to NPM1. Competition GST pull down using GST–NPM1 as the bait and MAT-tagged full-length hAPE1 as the prey. The hAPE1–NPM1 interaction was evaluated in the presence of a 10-fold excess competitor (comp.; i.e. untagged full-length hAPE1 as a positive control or MAT-tagged 1–48 peptide). The extent of interaction was assessed using an anti-MAT antibody to recognize the pulled down hAPE1 prey. The competing untagged hAPE1 was visualized using an anti-APE1 antibody. The histogram shows the average of three independent experiments with the error bars representing the S.D. *P < 0.05. ¥, Residual GST contaminant in the untagged hAPE1 preparation. (C) The low-affinity binding between the N-terminal recombinant hAPE1 peptide and NPM1 is stabilized by in vitro cross-linking. Recombinant purified NPM1 was incubated alone or together with either the 1–48 peptide or full-length hAPE1 as indicated. The bifunctional cross-linker DSP was also added where indicated. The presence of high-molecular-mass complexes was assessed by Western blotting using an anti-NPM1, an anti-APE1 or an anti-MAT antibody. The formation of high-molecular-mass complexes in the presence of DSP denotes the ability of the 1–48 peptide to interact weakly with NPM1. MT, Mat; WB, Western blot; wt, wild-type.

being absent when using either the anti-FLAG or the anti-NPM1 alone. This demonstrates the existence of an interaction between the isolated hAPE1 N-terminal domain and endogenous NPM1 only in the nucleoplasmic compartment. We also measured, using PLA, the extent of endogenous hAPE1–NPM1 interaction in the presence or absence of expression of the 1–49 peptide. In this case, the expression of the N-terminal region resulted in a poor, but statistically significant, competing effect (about 10% loss in interaction, Figure 4C). Altogether, these results confirm that the unstructured hAPE1 N-terminal domain alone is required, but not sufficient, for a stable binding of hAPE1 to NPM1 both in vitro and in vivo. Notably, several studies have demonstrated that hAPE1, targeted by granzymes A and K, undergoes proteolysis in living cells and its truncation products (hAPE1 N/D1 or 35) have been proposed to take part in the apoptotic process [18,39,40]. In light of the results of the present paper, it may be tempting to speculate that the N-terminal proteolytic products (the 1–31 or 1–35 peptides) might also exert an active regulatory function within cell, for example by competing with the full-length protein for binding to nucleic acids or protein partners, such as NPM1, XRCC1 (X-ray repair complementing defective repair in Chinese hamster cells) [41], DNA polymerase β [42] or CSB (Cockayne syndrome B) [43] and thus possibly acting as decoy peptide.

The evolutionarily acquired N-terminal domain of hAPE1 influences the stability and the binding properties of the protein

Given the importance of the unstructured N-terminal region of hAPE1 in tuning many of the protein’s functions, the poor evolutionary conservation of this domain appears remarkable. hAPE1 belongs to the exonuclease III family of endonucleases, being 27% identical with the archetype E. coli ExoIII (xth) [14]. Although the C-terminal domain of the human protein is conserved from prokaryotes to mammals, the N-terminal portion of hAPE1, comprising the redox domain, is not [17,44]. Metazoan hAPE1 orthologues, in fact, share a unique basic N-terminal extension that heavily affects the isoelectric point of the whole protein. Interestingly, the overall positive charges number within this basic region increased during evolution (from nine in fishes...
Multiple roles of unstructured APE1 N-terminal domain

Figure 4  The hAPE1 N-terminal peptide interacts weakly with endogenous NPM1 in HeLa cells

(A) Upper panels, Western blotting analysis of the expression kinetics of the FLAG-tagged 1–49 hAPE1 peptide following the doxycycline treatment of HeLa. The peptide expression is induced after 2 days of treatment. Lower panel, immunofluorescence confocal analysis on HeLa cells after 5 days of doxycycline treatment shows the lack of nucleolar accumulation of the 1–49 peptide (white arrowheads), in contrast with the nucleolar positivity shown by the full-length hAPE1. (B) The recombinant 1–49 peptide binds poorly to endogenous NPM1 in vivo. NPM1 co-immunoprecipitation carried out using HeLa cells transfected either with an empty vector (mock) or a FLAG–full-length hAPE1- or a FLAG–1–49-expressing vector as shown. The amount of immunoprecipitated NPM1 was evaluated using Western blotting with an anti-NPM1 antibody. (C) The competition effect of the 1–49 hAPE1 peptide on endogenous hAPE1–NPM1 interaction. The extent of endogenous hAPE1–NPM1 interaction was measured by PLA of HeLa cells either expressing the 1–49 hAPE1 peptide or mock transfected. The hAPE1–NPM1 association was estimated by scoring the interaction signals in transfected cells. Peptide expression resulted in a significant competing effect, leading to a 10 % reduction in hAPE1–NPM1 interaction. The boxplot shows the relative amount of hAPE1–NPM1 interaction in 1–49-expressing cells with respect to the mock-transfected cells +− S.D. (n = 70). *P < 0.05. IP, immuoprecipitation; WB, Western blot; wt, wild-type.

to 11 in mammals; Figure 2A; [17]), this event has possibly been paralleled by an improved ability of APE1 to cope with a progressively complex cellular environment.

Our recent work showed that the DNA-binding activity of hAPE1 is substantially higher than that of the orthologous protein from a phylogenetically distant organism, such as zebrafish (zAPE1) [17]. In the present study, we hypothesized that evolutionarily acquired amino acids within the N-terminal domain of the protein could account for the increased binding properties of hAPE1 towards both nucleic acids and NPM1. As a proof of concept, we performed interaction experiments with recombinant purified hAPE1 and zAPE1 enzymes. Initially, through GST pull-down experiments, we confirmed that zAPE1 has a lower binding activity towards both NPM1 (Figure 5A) and rRNA (Figure 5B). We then used insertion mutagenesis to generate a mutant recombinant zAPE1 protein (namely, zAPE1 K27) bearing, after Glu26, a lysine residue followed by a TAA (threonine–alanine–alanine) spacer spanning amino acids 28–30, in order to mimic the local charge density and distribution present within the wild-type hAPE1 N-terminal 24–35 region (Figure 5C). Interestingly, the theoretical isoelectric point, calculated for the whole unstructured N-terminal domain, is identical in both the wild-type human protein and the zAPE1 K27 mutant (Table 3).

The NPM1-binding activity of the purified recombinant proteins was then analysed using GST pull down with equimolar amounts of each protein (Figure 5D and Supplementary Figure S4A at http://www.biochemj.org/bj/452/bj4520545add.htm). The data obtained indicate that, whereas the wild-type zAPE1 is less able to interact with NPM1 than hAPE1, the full NPM1-binding activity of the ‘swapping mutant’ is clearly restored. Notably, both the hAPE1 NΔ43 and the zAPE1 NΔ36 deletion mutants, lacking the entire N-terminal extension, showed neglectable interaction ability. Although recognizing the limitations of this approach when evaluating the binding of zAPE1 to a human NPM1 protein (even though both human and zebrafish NPM1 share extensive similarity and have a nearly identical pI value), our ‘domain swapping’ approach supports the notion that the reduction in binding affinity might be a consequence of a decreased
Figure 5 The amino acidic composition of the N-terminal region enhances the affinity of the human APE1 protein for nucleic acids and NPM1

(A) zAPE1 has a lower affinity than hAPE1 for NPM1. GST pull-down assay using equimolar amounts of recombinant GST-tagged hAPE1 or zAPE1 as baits and His-tagged NPM1 as the prey. The amount of pulled-down NPM1 was quantified through densitometric analysis (right-hand panel). The histogram show the results from three independent experiments with the error bars representing the S.D. (n = 3). ¥, Residual GST contaminant in the GST–hAPE1 preparation. (B) zAPE1 has lower affinity than hAPE1 for rRNA. The APE1–RNA-binding capacity was evaluated through RNA pull down using GST-tagged full-length hAPE1 or zAPE1 as the bait and whole HeLa RNA as the prey. To quantify the amount of residual hAPE1-bound rRNA the pulled-down RNA was then retrotranscribed and 28S or 18S rRNAs were amplified by real-time PCR. The affinity for the 18S RNA was similar to what shown for the 28S (results not shown). The histogram reports the relative affinity to rRNA with respect to the GST–hAPE1 sample. Error bars represent the S.D. (n = 3). ¥, Residual GST contaminant in the GST–hAPE1 preparation. (C) Multiple sequence alignment of the N-terminal region of wild-type hAPE1, zAPE1 and the mutant zAPE1 K27. The four-amino-acid insertion mutation in zAPE1 K27 is boxed. (D) The NPM1-binding affinity of the ‘domain swapping’ mutant measured using GST pull down. GST-tagged NPM1 was used as the bait, whereas wild-type or mutant APE1 proteins were used as preys. The amount of pulled-down preys was assessed by Western blotting using an anti-APE1 antibody and is shown as the percentage of input prey. Whereas wild-type zAPE1 and the deletion mutants NΔ143 hAPE1 and NΔ36 zAPE1 show a loss of function phenotype, the NPM1-binding capacity of the zAPE1 K27 mutant is restored. (E) Binding affinity of the ‘domain swapping’ mutant for the nCaRE_B2 (left-hand panel) or the Stem20 (right-hand panel) oligonucleotides as measured using EMSA. The nucleic acid-binding activity of the proteins reflects what was observed using SPR (Table 4): the wild-type zAPE1 protein and the deletion mutants NΔ143 hAPE1 and NΔ36 zAPE1 show a reduced binding ability, whereas the zAPE1 K27 mutant displays a gain of function. F, the position of the free oligonucleotide probe. Arrows indicate the bound complex. (F) Overlay of thermal melting profiles for recombinant hAPE1, zAPE1 and zAPE1 K27 in the 25–65°C range shown as the unfolded fraction against temperature, measured by CD at 222 nm. The difference in thermal stability observed between the wild-type hAPE1 and zAPE1 is clearly restored by the N-terminal insertions in the zAPE1 K27 mutant. WB, Western blot; wt, wild-type.
number of positive charges within the N-terminal region of zAPE1.

A similar functional rescue for the zAPE1 K27 mutant was also observed when analysing the DNA-binding activity of the proteins (Figure 5E). EMSA experiments carried out with either a nCaRE_B2 duplex (Figure 5E, left-hand panel) or the Stem20 sequence (Figure 5E, right-hand panel) revealed a reduced binding activity for wild-type zAPE1 and both the hAPE1 NA43 and the zAPE1 NA36 deletion mutants, with a restored functionality in the case of the zAPE1 K27 protein. EMSA analyses showed clear differences in the electrophoretic mobility of the hAPE1 and zAPE1 in complex with the nucleic acid substrates, suggesting the existence of possible conformational differences between the two recombinant proteins when bound to the cognate DNA. The formation of a DNA–protein complex between the hAPE1 NA43 mutant and the Stem20 substrate (Figure 5E, right-hand panel), but not with the nCaRE_B2 DNA, is probably owing to the presence of a highly stable stem-loop structure within the Stem20 sequence, absent in the case of the nCaRE_B2 duplex (also see the SPR data below). This observation again supports the concept that the C-terminal domain of hAPE1 may account for the high-affinity binding to structured nucleic acid substrates. Moreover, the complete lack of nucleic acid-binding activity observed for the hAPE1 NA33 mutant [12,17] suggests that the acidic residues in the 33–42 region might have a negative effect on the overall nucleic acid-binding capacity of the protein.

In order to corroborate these findings, we carried out SPR experiments on zAPE1 and its mutants (Table 4) using both the Stem20 and nCaRE_B2 sequences, which showed different affinities for hAPE1 (Table 2). Interestingly, both the association and dissociation rate constants could be determined when the Stem20 was used as ligand; whereas, they were too high to be determined with the nCaRE_B2 (in this case affinities were determined through Langmuir isotherm fitting (Supplementary Figure S4B)). These results further point to the relevance of the presence of a stable stem-loop secondary structure within the oligonucleotide for a robust APE1–nucleic acid interaction. This result is corroborated by the presence of a detectable binding activity of the N-terminal truncated proteins (hAPE1 NA43 and zAPE1 NA36) only for the stem-loop substrate (i.e. Stem20). Similarly to what was observed in the EMSA experiments (Figure 5E) with both DNA substrates, the measured $K_d$ values were higher for zAPE1 than for hAPE1 (for the kinetic parameters with the human protein see Table 2). The zAPE1 K27 mutant, in turn, displayed an intermediate behaviour, confirming the restored binding activity of the ‘swapping mutant’ (Table 4).

In order to correlate the solution stability with binding ability, we performed CD analyses on wild-type, mutant and truncated APE1 proteins. The crystal structures of two vertebrate redox-inactive enzymes C65A hAPE1 and zAPE1, in their N-terminally truncated variants (C65A 40–318 hAPE1 and 33–310 zAPE1), revealed no substantial differences in the structural organization of the globular region of APE1 [44]. This was confirmed by our analyses as the CD spectra of all our proteins exhibited minima at 225 and 208 nm, typical of $\alpha$-helices and $\beta$-strands (results not shown). This indicates that, as expected, the C-terminal globular region of the protein predominantly accounts for the folded portion of the APE1 proteins. Thermal denaturation experiments were carried out to investigate protein stability: the sigmoidal profile of folded fraction against temperature (Figure 5F) shows that the unfolding process was fully cooperative for all of the proteins tested. By comparing the $T_m$ values, however, an influence of the N-terminal extension on protein stability could be highlighted. Indeed, whereas hAPE1 has a $T_m$ value of 41.5°C, the $T_m$ value measured for zAPE1 was 46.5°C. Interestingly, the mutations and insertions in the zAPE1 K27 mutant effectively reduced the stability of the protein, since the measured $T_m$ value (39.5°C) was closer to that of hAPE1 (Figure 5F). These results suggest a direct correlation between the ability to recognize different oligonucleotides or protein substrates and the flexibility of the N-terminal region of APE1. Moreover, these data also support the hypothesis that the altered mobility observed in the EMSA experiments between hAPE1 and zAPE1 bound to the cognate DNA sequences (Figure 5E) might be ascribed to substantial conformational differences in the two protein–DNA complexes. Similar CD analyses carried out on both the hAPE1 NA43 and the zAPE1 NA36 deletion mutants indicated that the differences in thermal stability between human and zebrafish proteins are already present in their globular portions (the $T_m$ values were 44°C and 50.5°C respectively), despite the similar structural organization (Supplementary Figure S4C).

Taken together, these results indicate that the amino acid composition and the local charge distribution within the N-terminal domain of hAPE1 are responsible for its evolutionarily acquired binding properties towards both nucleic acids and NPM1 and have an impact on the flexibility of the N-terminal region of APE1. Therefore the results of the present study point to the insertion of human Lys37, as well as the proper charge distribution, as central steps in the phylogensis of this protein. From a phylogenetic perspective, the acquisition of additional lysine residues could have provided the human protein with novel ways to fine-tune its different activities by the means of new ‘hot spots’

### Table 3  Predicted biophysical characteristics of the recombinant proteins used in the present study

The table reports the theoretical molecular masses and isoelectric points calculated using the Exasy pI/Mw tool (http://web.expasy.org/compute_pi/). The pI value of the N-terminal region was calculated restricting the analysis to the human AIA6 or the homologue Ile6 in fish. Note the identical pI values between the wild-type (wt) hAPE1 and the zAPE1 K27 N-terminal extensions.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular mass</th>
<th>Isoelectric point (whole protein)</th>
<th>Isoelectric point (N-terminal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hAPE1 wt</td>
<td>35564</td>
<td>8.33</td>
<td>9.10</td>
</tr>
<tr>
<td>zAPE1 wt</td>
<td>34881</td>
<td>5.77</td>
<td>8.04</td>
</tr>
<tr>
<td>hAPE1 NA43</td>
<td>31217</td>
<td>7.59</td>
<td>N/A</td>
</tr>
<tr>
<td>zAPE1 NA36</td>
<td>31207</td>
<td>5.54</td>
<td>N/A</td>
</tr>
<tr>
<td>zAPE1 K27</td>
<td>35252</td>
<td>5.96</td>
<td>9.10</td>
</tr>
</tbody>
</table>

### Table 4  Dissociation constants and kinetic parameters for the interaction of the mutated APE1 proteins with the Stem20 and nCaRE_B2 oligonucleotides

Results were obtained using SPR analyses using different APE1 mutants as analytes on the indicated biotinylated oligonucleotide ligands. The affinity values for the nCaRE_B2 sequence were determined through Langmuir isotherm fitting (see the text for details). wt, wild-type.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_\alpha$ (M$^{-1}$·s$^{-1}$·$\times$10$^3$)</th>
<th>$k_{on}$ (s$^{-1}$)</th>
<th>$K_d$ (µM)</th>
<th>$K_f$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>zAPE1 wt</td>
<td>0.174</td>
<td>0.053</td>
<td>3.28 ± 0.07</td>
<td>5.7 ± 0.2</td>
</tr>
<tr>
<td>hAPE1 NA36</td>
<td>0.232</td>
<td>0.530</td>
<td>23.0 ± 0.5</td>
<td>No binding</td>
</tr>
<tr>
<td>zAPE1 NA43</td>
<td>0.841</td>
<td>0.733</td>
<td>10.3 ± 0.3</td>
<td>No binding</td>
</tr>
<tr>
<td>zAPE1 K27</td>
<td>0.419</td>
<td>0.067</td>
<td>1.60 ± 0.02</td>
<td>2.4 ± 0.9</td>
</tr>
</tbody>
</table>
that can undergo acetylation or ubiquitination [17, 19–21] and regulate substrate accessibility, on the basis of protein plasticity in solution. In conclusion, in light of the results of the present study and others [17, 21, 42, 45], we suggest that the hAPE1 unstructured N-terminal domain might represent a flexible device that has been selected during evolution to specifically modulate the nucleic acid- and NPM1-binding functions of the protein. It would be interesting to explore novel pharmacological strategies aiming at the functional modulation of the protein using small molecules and/or peptides that target its N-terminal region.

AUTHOR CONTRIBUTION
Mattia Poletto, Daniela Marasco and Gianluca Tell provided substantial contributions to conception and design of the study, substantial contributions to acquisition of data or to analysis and interpretation of data, drafting the article or revising it critically for important intellectual content, and approved the final version. Carlo Vascotto and Lisa Lirussi provided substantial contributions to acquisition of data or to analysis and interpretation of data. Pasqualina Scognamiglio provided substantial contributions to conception and design, and substantial contributions to acquisition of data or to analysis and interpretation of data.

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SUPPLEMENTARY ONLINE DATA

Role of the unstructured N-terminal domain of the hAPE1 (human apurinic/apyrimidinic endonuclease 1) in the modulation of its interaction with nucleic acids and NPM1 (nucleophosmin)

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RESULTS

Purification of the recombinant hAPE1 1–48 peptide

The recombinant MAT-tagged hAPE1 1–48 peptide (Figure S2A) was purified to homogeneity by metal-affinity followed by cation-exchange chromatography. The purity of the peptide was verified by SDS/PAGE separation and silver staining (Figure S2B). The predicted molecular mass for the tagged peptide is 6.3 kDa; however, its migration pattern on SDS/PAGE is quite peculiar as its apparent molecular mass is between 11 and 17 kDa. We hypothesize that this phenomenon could be ascribed to the high content of positive charges of the molecule (predicted isoelectric point = 8.04) that are not efficiently neutralized by SDS treatment. The exact molecular mass of the peptide was confirmed through LC/MS (liquid chromatography MS) analysis (results not shown).

Affinity of the recombinant hAPE1 1–48 peptide for nucleic acids

We investigated the nucleic acid-binding activity of the recombinant MAT-tagged 1–48 peptide in order to confirm the presence of the hAPE1 N-terminal domain in the low affinity complexes seen in our EMSA experiments. To this aim, in addition to the Southwestern assays (Figure 2D of the main text and Figure S2D), we performed an EMSA experiment coupled to a Western blotting using an antibody directed against the hAPE1 N-terminal domain (amino acids 1–14; Novus; Figure S2E, right-hand panel). A signal showing the same migration pattern observed in the silver-stained EMSA (Figure S2E, right-hand panel) confirmed the presence of the 1–48 hAPE1 peptide in the shifted complex.

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Figure S1 The presence of a single-stranded loop enhances hAPE1-binding capacity

(A) The positive effect of the presence of a loop on the ability of hAPE1 to bind undamaged DNA as evaluated by EMSA using the Stem20 and Stem20 no loop oligonucleotides (Table 1 of the main text). For each reaction the indicated amount of purified recombinant hAPE1 was used. The results highlight the lower binding capacity of hAPE1 in the absence of a stem and loop structure. The arrow indicates the hAPE1–DNA complexes, whereas F shows the position of the free oligonucleotide probe. wt, wild-type. (B) Annealing control for the Stem20 no loop duplex used in the EMSA in (A). Equimolar amounts of the indicated individually 32P-labelled probes were separated by native PAGE (15% gel). As expected, the Stem20 no loop duplex migrates similarly to the Stem20 probe, but differently from its own forward strand (Stem20 no loop_for, which is the labelled in the duplex probe). (C) A representative plot of residuals of the SPR data fitting (related to the experiment reported in Figure 1B of the main text). The adopted 1:1 Langmuir binding model provides low residuals levels both in the association and dissociation phases.
Figure S2  Characterization of the 1–48 hAPE1 recombinant peptide

(A) Amino acid sequence of the MAT-tagged hAPE1 1–48 peptide. The two extra residues from the cloning procedures are underlined, whereas the C-terminal MAT is shown in italic font.  
(B) Silver-stained Tris/Tricine SDS/PAGE showing the purified MAT-tagged hAPE1 1–48 peptide. M, molecular mass markers. The molecular mass of the markers is shown on the left-hand side in kDa.  
(C) EMSA dose–response analysis showing that the 1–48 peptide binds undamaged DNA with a low affinity when compared with the full-length hAPE1 protein. For each reaction the indicated amount of purified recombinant protein was used. F, the position of the free oligonucleotide probe.  
(D) Southwestern assay showing a dose–response binding of the Stem20 oligonucleotide to the 1–48 peptide. The indicated amounts of protein or peptide were separated by SDS/PAGE, electrotransferred on to nitrocellulose membranes (left-hand panel) and probed with 32P-labelled Stem20 (right-hand panel). Note the difference in binding affinity between the full-length hAPE1 protein and the isolated peptide. Molecular mass is shown on the left-hand side in kDa.  
(E) EMSA coupled to Western blotting to confirm the presence of the N-terminal hAPE1 peptide in the bound complex. The indicated amounts of hAPE1 protein or peptide were incubated with different unlabelled DNA probes and separated on two parallel native gels. The first one was silver-stained, to reveal the presence of shifted DNA–protein complexes (left-hand), whereas the second one was subjected to Western blotting followed by immunorecognition with an anti-hAPE1 N-terminal antibody (right-hand panel). MT, MAT; wt, wild-type.
Figure S3  In vivo interaction of the 1–49 hAPE1 peptide with endogenous NPM1

(A) PLA analysis of 1–49 hAPE1-expressing HeLa cells using an anti-FLAG and an anti-NPM1 antibody reveals the interaction occurring in vivo between the hAPE1 N-terminal peptide and endogenous NPM1 (red dots in the upper middle panel). The lower panels represent the technical control where the immunoreaction was carried out while omitting the anti-NPM1 antibody.
Figure S4  Evolutionary characterization of the hAPE1 N-terminal region

(A) Coomassie Blue-stained gel showing the recombinant purified APE1 proteins used in the present study. M, molecular mass marker. The molecular mass of the markers is shown on the left-hand side in kDa. (B) Representative SPR analysis on the wild-type zAPE1–nCaRE_B2 interaction. Recombinant zAPE1 was used as the analyte and biotinylated nCaRE_B2 was used as the ligand. Plot of RU_{max} from each binding against concentration of wild-type zAPE1 is shown. The data were fitted by non-linear regression analysis. (C) Overlay of thermal melting profiles of recombinant hAPE1 NΔ43 and zAPE1 NΔ36 in the 25–65°C range, reported as unfolded fraction against temperature measured using a CD signal at 222 nm. wt, wild-type.