Knock-in reconstitution studies reveal an unexpected role of Cys65 in regulating APE1/Ref-1 subcellular trafficking and function

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SUPPLEMENTARY INFORMATION

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

The oligonucleotides used for siRNA of APE1 were as follows: sense, 5'-CCTGCCACACTCAAGATCTGC-3'; antisense, 5'-GCAGATCTTGAGTGTGGCAGG-3'. These sequences were drawn following the empirical rules of Mittal (Mittal et al., 2004) and were designed to recognize and bind to a 21 base sequence placed 175 nucleotides after the AUG initiation codon of APE1 gene. As a control, we used the scrambled (Scr-1) oligonucleotide sequences: sense, 5'- AGTCTAACTCGCCACCCCGTA-3'; antisense, 5'- TACGGGGTGGCGAGTTAGACT-3'. These sequences were checked with BLAST (http://www.ncbi.nlm.nih.gov/blast/) for their inability to pair any human cDNA sequence. Sequences were cloned into BglII and HindIII restriction sites of the pTer vector (van de Wetering et al., 2003), which presents a tetracycline- (doxycycline-) responsive promoter to form the so called pTer/APE1 vector and pTer/Scr, respectively. In a first step, HeLa cells were transfected with pcDNA6/TR to generate stable Tet-repressor expressing cell clones, which were further selected for the acquired resistance by incubation with blasticidin (5 μg/ml) (Invitrogen, Milan, Italy) for 14 days. Individual clones were isolated by using cell cloning cylinders (Sigma, Milan, Italy), transferred and grown stepwise into 24-well, 12-well, 6-well plates for expansion to 10^7 cells. Clone TR5 was shown to express the Tet-repressor at higher levels and was therefore selected for transfection with pTer/APE1 vector previously linearized with Bst1107I (Fermentas, St.Leon Rot, UK) and subjected to selection with zeocine (200 μg/ml) (Invitrogen) for 14-21 days. Thirty single clones were isolated by using cell cloning cylinders transfected and grown stepwise for expansion to 10^7 cells. As a control, we used cell clones transfected with the empty pTer vector or with pTer/Scr vector. For inducible siRNA experiments, doxycycline (Sigma) was added to the culture medium at the final concentration of 1 μg/ml and cells were grown for 10 days. Total cellular extracts were analyzed for APE1 expression by immunoblotting.

For generation of APE1 knock-in cell lines, an APE1 expression vector was generated by cloning an EcoRI-BamHI fragment from pFLAG-CMV-5.1/APC1 (Sigma) into p3XFLAG-CMV-14 vector (Sigma). To avoid the degradation of the ectopic APE1 mRNA by the specific siRNA sequence described above, two nucleotides of the APE1-cDNA coding sequence were mutated by using the Site-Directed Mutagenesis Kit (Stratagene), leaving unaffected the APE1 amino acid sequence: siRNA: 5'-CCTGCCACACTCAAGATCTGC-3'; APE1: 5'-CCTGCACCGCTCAAGATCTGC-3'. The Site-Directed Mutagenesis Kit was used to generate the APE1 mutants K6K7/R6R7, 31-34A, C65S, H309N, C310S and H309N+C310S. All the mutants were confirmed by DNA sequencing (MWG, Ebersberg, Germany). Then, the APE1 siRNA clone was transfected with p3XFLAG-CMV/APC1 WT and mutants, previously digested with ScaI (Fermentas). Forty-eight h after transfection, the cells were subjected to selection with
geneticin (Invitrogen) for 14 days and selected for the acquired resistance. Individual clones were isolated by using cell cloning cylinders (Sigma), transferred and grown for expansion to $10^7$ cells in the presence of selective antibiotics. As control, the siRNA control clone was transfected with the p3XFLAG-CMV-14 empty vector. After 10 days of doxycycline treatment at the final concentration of 1 $\mu$g/ml, total or nuclear and cytoplasmic cellular extracts were analyzed for APE1 expression by immunoblotting, thus revealing the silencing of the endogenous APE1 and the expression of the ectopic flagged WT and mutant forms of the protein.

**Immunofluorescence analysis**

HeLa or SF767 cells in the number of $10^5$ per 24 X multiwall plate were seated on glass coverslips and left to attach overnight. Then cells were fixed in 4% (w/v) paraformaldehyde in PBS for 20 min, at room temperature, permeabilized for 5 min with PBS–0.25% (w/v) Triton X-100 and incubated with 5% normal fetal bovine serum in PBS–0.1% (v/v) Tween-20 (blocking solution) for 30 min, to block unspecific binding of the antibodies. Cells were then incubated with the mouse monoclonal anti-APE1 (IgG2b) antibody previously conjugated with Alexa Fluor-568 Zenon Labeling Kit (Molecular Probes, Invitrogen) following manufacturer instructions. A final anti-APE1 dilution of 1:30 was prepared in blocking solution and cells were incubated for 3 h, at 37°C, in a humid chamber. After washing three times with PBS-0.1% (v/v) Tween-20 (washing solution) for 5 min, a second blocking step of 15 min was performed in the dark, followed by three washes with washing solution for 5 min. Then, cells were mounted on microscope glass with Duolink Mounting Media (Olink Biosciences). In the case of colocalization experiments with nucleophosmin (NPM1), instead of mounting the coverslips on microscope glass, cells were incubated with anti-NPM1 polyclonal antibody (Abcam) diluted 1:200 in blocking solution, at 4°C, overnight. After washing, cells were incubated with secondary antibody anti-rabbit FITC conjugated (Jackson Immuno Laboratories, West Grove, PA, USA) for 2 h. The preparations were then washed three times with washing solution for 5 min, in the dark.

**Cell growth assays and apoptosis studies**

For proliferation assays, cells were harvested at indicated times, stained with Trypan blue (Sigma), plated in triplicate and counted. Colony survival assays were performed as already described (Plumb, 1999; Vascotto et al., 2009a). Briefly, an equal number (500) of control (Scr-1), siRNA and APE1-knock-in cells were plated in petri dishes and grown with medium containing or not doxycycline (1 $\mu$g/ml). On day 10, the medium was removed and colonies were stained with 2
ml of cristal violet solution (10% w/v in 70% aqueous ethanol) for 2 min. Then, dye was poured off and plates were rinsed with tap water and allowed to dry. Colonies were counted by using ImageQuant TL software (GE Healthcare, Milan, Italy). For each experimental point, the mean, SD and statistical significance were calculated by performing three independent experiments of cell colony count.

Cell cycle studies were performed by flow cytometry by using a FACScan apparatus (Becton Dickinson, Franklin Lakes, NJ, USA). The number of apoptotic cells in control and APE1 siRNA cells after 0 and 10 days of treatment with 1 μg/ml of doxycycline was determined as previously reported (Nicoletti et al., 1991), by evaluating the number of cells with subdiploid DNA content through flow cytometric determination. Briefly, 2 x 10⁶ cells were harvested and washed once with cold PBS/0.1% sodium azide solution, resuspended in 1 ml of low-salt stain solution [4 mM sodium citrate, 3% polyethylene glycol 8000, 1 mg/ml propidium iodide solution (Invitrogen, Carlsbad, CA, USA), 180 U/ml RNAse A (Sigma) and 1% Triton X-100 in PBS/sodium azide solution] and then incubated, in the dark, at 37°C, for 20 min, with gentle mixing every 5 min. Then, 1 ml of high-salt stain solution (0.4 M sodium chloride, 3% polyethylene glycol 8000, 1 mg/ml propidium iodide solution and 1% Triton X-100 in PBS/sodium azide solution) was added by gentle pipetting and samples were stored at 4°C, overnight. Cells were centrifuged at 5000 x g for 5 min, at 4°C, the supernatant was removed, pellet resuspended in 500 μl of low-salt solution and then cells were analyzed on a Becton-Dickinson Canto using an Ar laser (excitation 488 nm). For each sample, 25,000 single events were detected and data analysis was performed by using both WinMDI 2.8 (written by Joseph Trotter, Scripps Research Institute, La Jolla, CA) and ModFitLT V3.0 software program.

Apoptosis was also assessed by staining of phosphatidylserines exposed on cell membranes with FITC labeled Annexin V, according to manufacturer instruction (Roche Diagnostic Italia, Monza, Italy). Samples were analyzed by flow cytometry using a FACScan apparatus. In addition, caspases activation was also measured; caspase-3/7 activation was measured by an immunofluorescent assay (Apo-ONE homogeneous caspase assay by Promega Corp., Madison, WI, USA) according to manufacturer’s instructions.

**Microarray analysis**

Total RNA was extracted in triplicate by using TRIzol reagent kit (Invitrogen), and quantified. Total RNA quality was determined by using the Agilent Bioanalyser (Agilent Technologies, Stockport, Cheshire, UK). Human genome HG U133 PLUS2 microarrays
were run according to manufacturer’s instructions (Affymetrix Inc., Santa Clara, CA, USA) and submitted in MIAME (Minimum Information About a Microarray Experiment)-compliant format to the ArrayExpress database (www.mged.org/Workgroups/MIAME/miame.html, accession number E-MEXP-1315). Array data were normalized and summarized using RMAExpress (Bolstad et al., 2003). Differential expression in response to siRNA treatment was calculated using Cyber-T (Baldi and Long, 2001). A false-discovery correction was applied to p-values to produce a q-values (Storey and Tibshirani, 2003). Validation of gene expression analysis was performed through Q-PCR, as previously reported (Vascotto et al., 2009b).

Quantitative real-time PCR

RNA expression levels of selected genes from microarray experiments were quantified by quantitative real-time PCR (Q-PCR) using an Applied Biosystem ABI 7300 (Applied Biosystems, Foster City, CA, USA) on independent biological replicas. Genes of interest were validated by TaqMan Gene Expression assays (Applied Biosystems) and specific sequences are available on request. The results were normalized to the relative amounts of β2-microglobulin (B2M). PCR was carried out in a final volume of 25 µl with the Platinum Quantitative PCR SuperMix-UDG with Rox (Invitrogen) using 5 µl of cDNA and 1.25 µl each assay-on-demand, in triplicate. Relative gene expression levels were calculated according to the comparative Ct method of relative quantitation vs B2M. To normalized data, ΔΔCt was calculated for each sample by using the mean of its ΔCt values subtracted of the mean ΔCt value of HL60 cells, considered as a calibrator; relative quantization (RQ) value was expressed as 2−ΔΔCt.

Visualization of the F-actin cytoskeleton

HeLa cells were grown on coverslips at a density of 80,000-100,000 cells per coverslip. One day after plating, cells were fixed in fresh 4% paraformaldehyde for 20 min, at room temperature, washed three times, and permeabilized in 0.1% (v/v) Triton X-100 for 5 min. Cells were then washed twice, blocked with 5% goat serum in PBS (blocking solution) for 15 min and incubated for 90 min in 0.25 U/coverslip of Alexa 488-phalloidin diluted in blocking solution. After three washings, the microscope slides were mounted onto slides in Mowiol® 4-88 supplemented with DABCO (1,4-diazabicyclo[2.2.2]octane) (4:1) to retard photobleaching. All incubation and washing steps were performed at room temperature in PBS, unless otherwise indicated. Coverslips were visualized through a Leica TCS SP laser-scanning confocal microscope (Leica Microsystems,
Wetzlar, Germany) equipped with a 488 nm Ar laser, a 543 nm HeNe laser and a 63x oil fluorescence objective.

Preparation of cell extracts and anti-Flag coimmunoprecipitation

For preparation of total cell lysates and coimmunoprecipitation, cells were harvested by trypsinization and centrifuged at 250 x g for 5 min, at 4°C. Supernatant was removed, and the pellet was washed once with ice-cold PBS and then centrifuged again as described before. Cell pellet was resuspended in lysis buffer containing 50 mM Tris HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, and 1% (w/v) Triton X-100, supplemented with protease inhibitor cocktail (Sigma), 0.5 mM PMSF, 1 mM NaF, and 1 mM Na3VO4, at a cell density of 10⁷ cells/ml, and rotated for 30 min, at 4°C. After centrifugation at 12,000 x g for 10 min, at 4°C, the supernatant was collected as total cell lysate. Protein concentration was determined by using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Samples were then coimmunoprecipitated for 3 h using anti-Flag M2 affinity gel (Sigma) following the manufacturer’s instructions. Proteins were eluted by incubation with 0.15 mg/ml 3X Flag peptide in Tris-buffered saline (TBS) and subjected to Western blotting analysis.

For cell nuclear and cytoplasmic extracts, cells were collected as previously reported and the pellet was resuspended in buffer A (10 mM Tris-HCl pH 7.5, 1.5 mM MgCl₂, and 10 mM KCl, supplemented with protease inhibitor cocktail, 0.5 mM PMSF, 1 mM NaF and 1 mM Na3VO4) at a cell density of 3 x 10⁷ cells/ml, and incubated on ice for 10 min. Nuclei were collected by centrifugation at 2,000 x g for 10 min, at 4°C. The supernatant was considered as the cytoplasmic fraction. Nuclei were washed twice with the same volume of buffer A to minimize cytoplasmic contamination and then resuspended in buffer B (20 mM Tris-HCl pH 7.5, 0.42 M KCl, 1.5 mM MgCl₂, 20% v/v glycerol, supplemented with protease inhibitor cocktail, 0.5 mM PMSF, 1 mM NaF, and 1 mM Na3VO4) and incubated on ice for 30 min. The suspension was centrifuged at 15,000 x g for 30 min, at 4°C, and the supernatant was collected as nuclear protein extract. Nuclear extracts were then analyzed for protein content (Bradford, 1976), stored at –80°C in aliquots and used for endonuclease assay and Western blotting analysis.

Two-dimensional polyacrylamide gel electrophoresis of immunoprecipitated material

Fifteen-twenty μg of immunoprecipitated material were loaded onto 13 cm, pH 3-10 L IPG strips (GE Healthcare). IEF was conducted by using an IPGPhor II system (GE Healthcare) according to the manufacturer’s instructions. Focused strips were equilibrated with 6 M urea, 26 mM DTT, 4% w/v SDS, 30% v/v glycerol in 0.1 M Tris-HCl (pH 6.8) for 15 min, followed by 6 M
urea, 0.38 M iodoacetamide, 4% w/v SDS, 30% v/v glycerol, and a dash of bromophenol blue in 0.1 M Tris-HCl, pH 6.8, for 10 min. The equilibrated strips were applied directly to 10% SDS-polyacrylamide gels and separated at 130 V. Gels were fixed and stained by ammoniacal silver (Shevchenko et al., 1996). Gels were scanned with an Image Master 2-D apparatus and analyzed by the Melanie 5 software (GE Healthcare), which allowed estimation of relative differences in spot intensities for each represented protein.

Identification of the differentially-expressed spots by mass spectrometry analysis

Differential spots from 2-DE were excised from the gel, triturated and washed with water. Proteins were in-gel reduced, S-alkylated and digested with trypsin as previously reported (Vascotto et al., 2009b). Digest aliquots were removed and subjected to a desalting/concentration step on μZipTipC₁₈ (Millipore Corp., Bedford, MA, USA) by using acetonitrile as eluent before MALDI-TOF-MS analysis. Peptide mixtures were loaded on the MALDI target, using the dried droplet technique and α-cyano-4-hydroxycinnamic acid as matrix, and analyzed by using Voyager-DE PRO mass spectrometer (Applied Biosystems, Framingham, MA, USA). Spectra were elaborated using the DataExplorer 5.1 software (Applied Biosystems) and manually inspected to get the peak lists. Internal mass calibration was performed with peptides deriving from enzyme autodigestion. Mascot software package was used to identify spots unambiguously from updated all taxa NCBI non-redundant sequence database (NCBI nr 20080610, containing 6573034 protein sequences) by using a mass tolerance value of 50-70 ppm, trypsin as proteolytic enzyme, a missed cleavages maximum value of 2 and Cys carbamidomethylation and Met oxidation as fixed and variable modification, respectively. Candidates with Mascot’s scores greater than 81 (P < 0.05 for a significant identification) (Supplementary Table S2) were further evaluated by the comparison with their calculated mass and pI using the experimental values obtained from 2-DE.

Endonuclease assays

Determination of AP endonuclease activity of APE1 was performed as already reported (Vascotto et al., 2009a). Same amounts of nuclear extract from APE1WT and APE1C₆₅S expressing cells were incubated with a 5’-³²P-end-labeled 26-mer oligonucleotide containing a single tetrahydrofuranyl artificial AP site at position 14 (here called dsFDNA), which is cleaved to a 14-mer in the presence of AP endonuclease activity. Samples from reaction mixtures (10 μl) containing the protein of interest, 2.5 pmol of the 5’-³²P end-
labeled oligonucleotide dsFDNA, 50 mM HEPES, 50 mM KCl, 10 mM MgCl2, 1 μg/μl bovine serum albumin, and 0.05% (wt/vol) Triton X-100 (pH 7.5) were allowed to proceed for 15 min, at 37°C. Reactions were halted by adding 10 μl of 96% (v/v) formamide and 10 mM EDTA, with xylene cyanol and bromophenol blue as dyes. AP assay products (10 μl) were separated on a 20% polyacrylamide gel containing 7 M urea. Gels were then exposed to film for autoradiography and quantification was performed as described previously (Vascotto et al., 2009a).
**Supplementary Table 1. Comparative gene expression profile of APE1\textsuperscript{WT} and APE1\textsuperscript{C65S}-expressing cells reveals differential expression of cell cycle- and growth factor-related species.**

Out of 19,235 genes in the database 2,924 contain disulphide bonds (15%). Interestingly, among the 38 significant genes found as differentially expressed between APE1\textsuperscript{WT} and APE1\textsuperscript{C65S} expressing cells, 13 contain disulphide bonds (34%).
### Supplementary Table S2. Protein identification as ascertained by mass spectrometric analysis.

**SpotUP matches to: sp|P30101|PDIA3_HUMAN**

**Score:** 108 Expect: 8.2e-006

- **Protein disulfide-isomerase A3** OS=Homo sapiens GN=PDIA3 PE=1 SV=4
- **Nominal mass (Mr):** 57146
- **Calculated pI value:** 5.98

**NCBI BLAST search of sp|P30101|PDIA3_HUMAN against nr**

Unformatted sequence string for pasting into other applications

**Cleavage by Trypsin:** cuts C-term side of KR unless next residue is P
**Number of mass values searched:** 9
**Number of mass values matched:** 8
**Sequence Coverage:** 23%
**Matched peptides shown in Bold Red**

| Start - End | Observed | Mr(expt) | Mr(calc) | ppm  | Miss | Sequence |
|-------------|----------|----------|----------|------|------|----------|
| 74 - 94     | 2317.0540| 2316.0467| 2316.1930| -63  | 2    | R.LKGIVPLAKVDCTANTNTCNK.Y |
| 105 - 119   | 1652.7550| 1651.7477| 1651.7590| -7   | 1    | K.IFRDGERAGAYGDPR.T |
| 148 - 173   | 2938.3180| 2937.3107| 2937.3709| -20  | 1    | K.FISDKDASIVGFDGEXSEASEFLK.A |
| 305 - 329   | 2703.2790| 2702.2717| 2702.3915| -44  | 1    | R.KTFSSHLSDFGLESTAGEIPVVAIR.T |
| 306 - 329   | 2575.3430| 2574.3357| 2574.2966| 15   | 0    | K.TFSHELSDFGLASTEIGPVVAIR.T |
| 336 - 344   | 1172.5390| 1171.5317| 1171.5332| -1   | 0    | K.FVMQEEFSR.D |
| 352 - 363   | 1515.7540| 1514.7467| 1514.7518| -3   | 1    | R.FLQDYFDGNLKR.Y |
| 472 - 482   | 1370.7340| 1369.7267| 1369.6878| 28   | 0    | R.ELSDFISYLQREATNPVFQEEKPKKKK |

**No match to:** 1164.6100

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**SpotDOWN matches to: sp|P30041|PRDX6_HUMAN**

**Score:** 131 Expect: 4.1e-008

- **Peroxiredoxin-6** OS=Homo sapiens GN=PRDX6 PE=1 SV=3
- **Nominal mass (Mr):** 25133
- **Calculated pI value:** 6.00

**NCBI BLAST search of sp|P30041|PRDX6_HUMAN against nr**

Unformatted sequence string for pasting into other applications

**Cleavage by Trypsin:** cuts C-term side of KR unless next residue is P
**Number of mass values searched:** 8
**Number of mass values matched:** 7
**Sequence Coverage:** 37%
**Matched peptides shown in Bold Red**

| Start - End | Observed | Mr(expt) | Mr(calc) | ppm  | Miss | Sequence |
|-------------|----------|----------|----------|------|------|----------|
| 2 - 22      | 2098.1060| 2097.0987| 2097.0855| 6    | 0    | M.PGGLLGDVAPNFENATTVEGRIFHFDLG DSWGILFSHHPRDFTPVCTTE |
| 42 - 53     | 1395.6780| 1394.6707| 1394.6500| 15   | 0    | R.DFTPVCTTELGR.A |
| 57 - 64     | 931.5490 | 930.5417 | 930.5287 | 14   | 1    | K.LAPEFAKR.N |
| 85 - 106    | 2649.3420| 2648.3347| 2648.2428| 35   | 1    | K.DINAYCEPEKLFPIIDDR.N |
| 98 - 106    | 1085.6150| 1084.6077| 1084.5917| 15   | 0    | K.LPPFIDDR.N |
| 133 - 142   | 1135.6740| 1134.6667| 1134.6438| 20   | 1    | R.VVFVFDPDKK.T |
| 145 - 155   | 1191.6620| 1190.6547| 1190.6659| -9   | 0    | K.LSILYSATGR.N |

**No match to:** 2210.1060
Supplementary Figure Legends

Supplementary Figure S1.
Immunofluorescence analysis to characterize the localization of the ectopic Flag-tagged APE1 proteins. HeLa cells stably transfected with the siRNA-resistant Flag-tagged APE1 cDNA encoding plasmids were fixed and stained for anti-Flag-APE1 (green). Nucleus was stained with propidium iodide (PI). Merged images (yellow) show the localization of APE1 within nucleus of transfected cells.

Supplementary Figure S2.
APE1 colocalizes with nucleophosmin and lose its nuclear localization after E3330 treatment. APE1 and NPM1 subcellular localization on SF767 cells was analyzed through confocal microscopy after treatment with 140 μM E3330 for 6 h. A specific APE1 monoclonal antibody derivatized with Alex Fluor 568 was used in combination with a polyclonal anti-NPM1, followed by incubation with an anti-rabbit FITC-conjugated antibody. Nuclear compartment was stained with DAPI. Control sample represents cells treated with DMSO. In control, APE1 and NPM1 colocalize within nucleoli, while E3330 treatment determinates APE1 cytoplasmic relocalization, but does not affect nucleolar structure.
Supplementary FIGURE 1

| α-FLAG | PI | Merged |
|--------|----|--------|
| WT     |    |        |
| K6K7/R6R7 |   |        |
| 31-34A |    |        |
| C65S   |    |        |
| C310S  |    |        |
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