Poly(ADP-ribose) Polymerase-1 Is a Nuclear Epigenetic Regulator of Mitochondrial DNA Repair and Transcription

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ABSTRACT

Poly(ADP-ribose) polymerase-1 (PARP-1) is a NAD-consuming enzyme with an emerging key role in epigenetic regulation of gene transcription. Although PARP-1 expression is characteristically restricted to the nucleus, a few studies report the mitochondrial localization of the enzyme and its ability to regulate organelle functioning. Here, we show that, despite exclusive nuclear localization of PARP-1, mitochondrial homeostasis is compromised in cell lines exposed to PARP-1 pharmacological inhibitors or small interfering RNA. PARP-1 suppression reduces integrity of mitochondrial DNA (mtDNA), as well as expression of mitochondria-encoded respiratory complex subunits COX-1, COX-2, and ND-2. Accordingly, PARP-1 localizes at promoters of nuclear genes encoding both the mtDNA repair proteins UNG1, MYH1, and APE1 and the mtDNA transcription factors TFB1M and TFB2M. It is noteworthy that poly(ADP-ribosyl)ation is required for nuclear gene expression of these mitochondrial proteins. Consistent with these findings, PARP-1 suppression impairs mitochondrial ATP production. Our results indicate that PARP-1 plays a central role in mitochondrial homeostasis by epigenetically regulating nuclear genes involved in mtDNA repair and transcription. These data might have important implications in pharmacology of PARP-1 inhibitors as well as clinical oncology and aging.

Introduction

Poly(ADP-ribosyl)ation is a post-translational modification of proteins operated by poly(ADP-ribose) polymerases (PARPs) (Amé et al., 2004). This a family of enzymes able to transform NAD into polymers of ADP-ribose (PAR) that can be targeted to the enzymes themselves and to a large number of proteins (Schreiber et al., 2006). Because of the chemical aspects of the reaction catalyzed, the classification of PARPs as ADP-ribose transferases has been proposed (Hottiger et al., 2010). Nuclear PARP-1 is the oldest member of the PARP family and is typically involved in DNA repair and cell death. These pathophys-iological properties stem from the ability of PARP-1 to be hyperactivated and alter pyridine and adenine nucleotide homeostasis upon genotoxic events that follow a large array of stress conditions in different cell types and organs (Chiarugi, 2002). Accordingly, a great deal of effort has been focused on developing potent PARP-1 inhibitors that were efficacious in numerous models of human disorders (Jagtap and Szabó, 2005) and recently reached the clinical arena (Annunziata and O'Shaughnessy, 2010). Although data from patients with neoplasms indicate a safety profile of PARP-1 inhibitors, their pharmacodynamic effects still need to be fully identified at the molecular level.

In contrast with the old dogma that PARP-1 gets activated only in the presence of DNA damage, accumulating evidence indicates that PARP-1 activity takes place in resting cells at sites of complex chromatin architecture and active transcription (Lonskaya et al., 2005; Potaman et al., 2005). This basal poly(ADP-ribosyl)ation is thought to contribute to mainte-

nance of nuclear homeostasis and cellular functions (Asher et al., 2010; Krishnakumar and Kraus, 2010). Current knowledge about PARP-1-catalyzed reactions allows us to identify the enzyme as a key epigenetic regulator of gene expression thanks to the covalent and noncovalent modification of chromatin architecture-regulating proteins and ensuing assembly of transcriptionally active supramolecular complexes by PAR formation (Kraus, 2008; Krishnakumar and Kraus, 2010). Remarkably, although these properties have drastically changed the physiological role of PARP-1 within the nucleus, they have also widened the pharmacodynamic properties of PARP-1 inhibitors.

Despite the impressive advancement in the field of poly(ADP-ribosyl)ation, the pathophysiological relevance of PARP-1 and PAR formation within mitochondria is still debated. Despite its canonical nuclear localization, indeed, several studies report that PARP-1 and poly(ADP-ribosyl)ating proteins also localize in mitochondria (Scovassi, 2004; Pankotai et al., 2009). Mitochondrial PARP-1 is reported to actively participate in maintenance of functional integrity of the organelles (Rossi et al., 2009) and to play a detrimental role when hyperactivated (Du et al., 2003; Lai et al., 2008). Nevertheless, although there is ample agreement that PAR formation is sensed by mitochondria (Wang et al., 2009), skepticism persists about the mitochondrial presence of PARP-1 and/or additional PAR-forming enzymes.

In an attempt to reconcile these biochemical and pathophysiological interpretations, we speculated that PARP-1 can *indirectly* regulate mitochondrial functions through its epigenetic regulation of nuclear gene expression. In the present study, although we were unable to detect PARP-1 within mitochondria, we present the first evidence that the enzyme localizes at promoters of nuclear genes coding for mitochondrial proteins of key relevance to mtDNA repair and transcription. Accordingly, prolonged exposure to PARP-1 inhibitors prompts mtDNA damage and impairs mitochondrial bioenergetics.

Materials and Methods

Cell Culture Conditions and Transfection. Human neuroblastoma SHSY5Y or cervical carcinoma HeLa cell lines were purchased from the European Collection of Cell Cultures (Porton Down, Wiltshire, UK) and maintained in Ham's F12/minimal essential medium (1:1) supplemented with 10% fetal bovine serum, 1% nonessential amino acids, and 1% glutamine in 100% humidity, 37°C, and 5% CO_2 atmosphere. Pharmacological inhibitors of PARP-1 were 6(5H)-phenanthridinone (PHE), benzamide (both from Sigma-Aldrich, Milan, Italy), and N-(6-oxo-5,6-dihydrophenanthridin-2-yl)-(N,N-dimethylamino)acetamide hydrochloride (PJ34) (Enzo Life Sciences/Vinci-Biochem, Vinci, Italy). Transient transfection of SHSY5Y cells with plasmids or siRNA against human PARP-1 (Dharmacon RNA Technologies, Lafayette, CO) or GFP (QIAGEN, Milan, Italy) were carried out with jetPRIME reagent (Polyplus-transfection SA, Illkirch, France) using 50 nM siRNA according to the manufacturer's instructions. The pEGFP-hPARP-1, pCDNA3/mit-HA1-GFP, pCDNA3/mit-HA1-RFP, pCMV5-PARP1-Flag, pGL3-hTFB1M, pGL3-hTFB2M, pRL-TK and pGL2-PB (the last two plasmids are control vectors coding for *Renilla reniformis* luciferase) plasmids or empty vectors were used.

Immunocytochemistry and Fluorescence Image Acquisitions. Cells (2 × 10^5) were immobilized onto glass coverslips (15 × 15 mm), transiently cotransfected with PARP1-Flag and mit-GFP or EGFP-PARP-1 and mit-RFP vectors. For immunocytochemistry, cells cotransfected with PARP1-Flag and mit-GFP were washed twice with ice-cold phosphate-buffered saline (PBS) and fixed for 10 min in 4% paraformaldehyde in PBS. After three 2-min washes with PBS, the cells were incubated in 1 ml of blocking buffer (3% bovine serum albumin and 0.1% Triton X-100 in PBS) for 1 h at room temperature and then incubated overnight at 4°C with the primary mouse-monoclonal anti-FLAG M2 (Sigma-Aldrich) antibody (1:250) in blocking buffer. Later, cells were washed three times for 15 min each in washing buffer (0.1% Triton X-100 in PBS), incubated with the secondary Cy-3-conjugated anti-mouse antibody (Millipore Bioscience Research Reagents, Temecula, CA) (1:800) for 60 min at room temperature, and washed three times with 1 ml of washing buffer for 5 min each at room temperature. Cells on coverslips were then dried, mounted onto glass slides, and examined with fluorescence microscopy using a Nikon Eclipse TE2000-U. Fluorescence images (1024 × 768 pixels) were obtained using a 60× objective lens. Cells cotransfected with EGFP-PARP-1 and mi-RFP cells were washed in PBS twice and directly analyzed by fluorescence microscopy.

Direct PCR Assay. For direct PCR, SHSY5Y cells were treated with vehicle, PHE, PJ34, or PARP-1 siRNA, and DNA was extracted by NucleoBond Xtra Midi Kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions. Amplification products of 5.6 kbp or 157 bp of mtDNA were obtained by PCR using specific primers as described previously (Rossi et al., 2009) the resulting bands were analyzed by ImageJ software (http://imagej.nih.gov/ij/) for densitometric analysis.

Real-Time PCR Assay. One microgram of total RNA isolated from treated or untreated cells was retrotranscribed using iScript kit (Bio-Rad Laboratories, Hercules, CA) and amplified with the specific primers shown in Table 1. Real-time PCR assays were performed by Rotor-Gene SYBR Green PCR Kit (QIAGEN) and analyzed using the Rotor-Gene 3000 cycler system (Corbett Research, Mortlake, NSW, Australia).

Chromatin Immunoprecipitation Assay. SHSY5Y cells transiently transfected with PARP1-Flag were fixed by 1% formaldehyde, and fixation was stopped by adding 0.125 M glycine for 10 min at room temperature. Cells were washed with ice-cold PBS; scraped; lysed in 5 mM EDTA, 50 mM Tris-HCl, pH 8.0, and 1% SDS supplemented with protease inhibitors; and rocked on ice for 10 min. Total lysates were sonicated to obtain chromatin fragments of an average length of 100 to 500 bp and centrifuged at 10,000 rpm for 10 min at 4°C. The sonicated supernatant fractions were 10-fold diluted with 5 mM EDTA, 50 mM Tris-HCl, pH 8.0, 0.5% IGEPAL CA-630, 200 mM NaCl supplemented with protease inhibitors. Then, 150 μg of chromatin were incubated with ANTI-FLAG M2 magnetic beads (Sigma-Aldrich) or beads without antibody overnight with gentle rotation at 4°C. Before washing, an aliquot of the supernatant from each sample was collected and considered the input. The remaining supernatant was centrifuged at 10,000 rpm for 10 min, and then the pellet was washed with low-salt wash buffer (0.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl), high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 500 mM NaCl), LiCl wash buffer (1% Nonidet P-40, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 250 mM LiCl, and 1% sodium deoxycholate), and Tris-EDTA buffer (10 mM Tris-Cl, pH 7.4, and 1 mM EDTA). Pellets were dissolved in 300 μl of elution buffer (1% SDS and 0.1 M NaHCO$_3$). The samples were treated with RNase A for 10 min at room temperature than incubated at 65°C for 4 h to reverse the protein-DNA cross-linking. After treatment with proteinase K, the DNA was extracted with phenol-chloroform, precipitated with isopropyl alcohol, and resuspended in distilled water. Output DNA concentrations were determined spectrophotometrically at 260 nm. The primers used for PCR amplification are given in Table 1. PCR products were analyzed by agarose gel electrophoresis and visualized by UV light apparatus. Real-time PCR analyses of the ChIP samples were carried out in triplicate using 100 ng of the output or input DNA samples and amplified with the specific primers describe above.

Measurement of Luciferase Activity. For TFB1M or TFB2M promoter activity, 3×10^4 SHSY5Y cells/well were cotransfected in triplicate in 96-well plates with equal amounts (200 ng) of the plasmids pGL3-hTFB1M and pGL3-hTFB2M and pGL4 internal standard and were subjected to different treatments. For UNG1 promoter activity, cells were cotransfected with pGL2-PB and pRL-TK internal standard. Forty-eight hours after transfection, cells were lysed, and the Firefly and *R. reniformis* luciferase activities were measured sequentially from a single aliquot of cell lysate using the Dual-Glo Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions by using the TopCount NXT luminometer (PerkinElmer Life and Analytical Sciences, Milan Italy).

Western Blotting. For Western blotting, cells were scraped, collected in Eppendorf tubes, centrifuged (1500g for 5 min at 4°C) and resuspended in lysis buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 4 μg/ml aprotinin and leupeptin, and 1% SDS). Twenty to forty micrograms of protein were loaded on each lane. After 4–20% SDS-polyacrylamide gel electrophoresis and blotting, membranes (Immobilon-P; Millipore, Billerica, MA) were blocked with PBS containing 0.1% Tween 20 and 5% nonfat milk (PBST/5% milk) and then probed overnight with primary antibodies (1:1000 in PBST/5% milk). The anti-PAR monoclonal antibody (10H) was from Alexis Bioschemicals (Vinci, Italy) and the anti-β-actin was monoclonal from Santa Cruz Biotechnology (Santa Cruz, CA), whereas the polyclonal anti-histone H3 acetylated Lys-18 was from Cell Signaling Technology (Danvers, MA). Membranes were then washed with PBST and incubated for 1 h in TPBS/5% milk containing the corresponding peroxidase-conjugated secondary antibody (1:2000). After washing in TPBS, enhanced chemiluminescence (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) was used to visualize the peroxidase-coated bands.

Cellular ATP Production. ATP production from cells cultured in the presence or absence of glucose and exposed to or not to oligomycin, rotenone, and antimycin was measured in total cell lysates by means of the ATPlight kit (PerkinElmer Life and Analytical Sciences), as described previously (Formentini et al., 2009).

Results

Intracellular Localization of Human PARP-1. Because of prior work showing the mitochondrial presence of PARP-1 (Du et al., 2003; Scovassi, 2004; Rossi et al., 2009), we first attempted to determine the intracellular localization of the enzyme by means of immunocytochemistry and/or cell transfection with fluorescent proteins. Prior work from our group reports no evidence of colocalization between PARP-1 and the mitochondrial proteins cytochrome *c* or apoptosis-inducing factor (Cipriani et al., 2005). Because the apparent lack of mitochondrial PARP-1 might be due to organelle expression levels not reaching the antibody detection limit, we now overexpressed Flag-PARP-1 in neuroblastoma cells also transfected with mitochondrially targeted GFP. By means of anti-Flag antibodies, we found exclusive nuclear Flag-PARP-1 localization (Fig. 1A). To further corroborate our findings, we also investigated the intracellular distribution of GFP-PARP-1 and checked whether it showed any colocalization with mitochondrially targeted red fluorescent protein (RFP). Data shown in Fig. 1B demonstrated that GFP-PARP-1 did not colocalize with the network of mitochondria depicted by mitochondrially targeted RFP. Taken together, these findings provide evidence for exclusive nuclear localization of PARP-1.

PARP-1 Contributes to mtDNA Integrity and Gene Expression. Next, we checked whether PARP-1 has a role in maintenance of mtDNA integrity and gene expression in SHSY5Y neuroblastoma cells. To this end, by means of PCR, we amplified fragments of mtDNA in cells exposed to two different PARP-1 inhibitors. Comparison of PCR amplification efficiency of large (5.6 kbp) with small (157 bp) mtDNA sequences is a classic index of genomic integrity, because decreased amplification of the large fragments is indicative of polymerase stalling at DNA damage sites (Rossi et al., 2009). Exposure to the PARP-1 inhibitors PHE (30 μM) or PJ34 (20 μM) (concentrations consistent with their IC$_{50}$ on PARP-1) for up to 24 h did not alter PCR efficiency (not shown). Conversely, selective reduction of amplification efficiency of the large mtDNA fragment was found when drug exposure was pronged to 72 h (Fig. 2, A and B). Similar results were found with 300 μM benzamide, a less potent PARP-1 inhibitor (Fig. 2C). To rule out possible nonspecific effects of the chemicals, we then attempted to confirm the findings suppressing PARP-1 by means of siRNA. Seventy-two hours after siRNA exposure, PARP-1 transcripts were reduced by 72% as revealed by real-time PCR (not shown). As shown in Fig. 2, D and E, silencing of PARP-1 for 72 h reduced the 5.6 kbp mtDNA amplification to an extent similar to that achieved by means of pharmacological inhibition.

Next, considering the impact of PARP-1 suppression on mtDNA integrity, we quantitated the transcripts for respira-

TABLE 1
Primers and sequences

| Primer	Sequence
COX-1	
Forward	5′-TACCAGGCTTCGGAATAATCTC-3′
Reverse	5′-GATAGCGATGATTATGGTAGC-3′
COX-2	
Forward	5′-CTCCTTGACGTTGACAATCG-3′
Reverse	5′-CCACAGATTTCAGAGCATTGA-3′
ND-2	
Forward	5′-AGGTGGATTAAACCAAACCCAG-3′
Reverse	5′-CGAGATAGTAGTAGGGTCGTGG-3′
UNG1	
Forward	5′-CAGCTCTTGAGCCGCCTCTG-3′
Reverse	5′-GTCCAACTGCTCGGCACTC-3′
MYH1	
Forward	5′-GAGCCTGCTAAGCTGGTACG-3′
Reverse	5′-TGCAGCATGACCTCTGAGAC-3′
APE1	
Forward	5′-GTGCCCACTCAAAGTTTCTTAC-3′
Reverse	5′-CGGCCTGCATTAGGTACATATG-3′
TFB1M	
Forward	5′-TGCAAGCAGCGAAGCAGCTATC-3′
Reverse	5′-TTCAACCACCAGAAGTTCAGCG-3′
TFB2M	
Forward	5′-CGATCGGAGATTGGCTGAGAC-3′
Reverse	5′-TCACTTTCGAGCGCAACCAC-3′
18S ribosomal RNA	
Forward	5′-CGGCTACCACATCCAAGGAA-3′
Reverse	5′-GCTGGAATTACCGCGGCT-3′
TFB1M promoter	
Forward	5′-CCTAGTCCACCCGGCTCT-3′
Reverse	5′-GAGGAACCTGCGAGACCTAA-3′
TFB2M promoter	
Forward	5′-ACGGTCCACTCACAATCCTC-3′
Reverse	5′-CCCACGTGGAACATTTTCTG-3′
UNG1 promoter	
Forward	5′-CCAATGGGAACGCGTCTCGG-3′
Reverse	5′-TTGTGGAACAGGTCCCAGCAAAAG-3′
MYH1 promoter	
Forward	5′-ACGCTCAATCCACTCCACTG-3′
Reverse	5′-CCGCCGACAGTGACGATGGCGCA-3′
Mitochondrial D-loop	
Forward	5′-CCCCTCACCCACTAGGATAC-3′
Reverse	5′-ACGTGTGGGCTATTTAGGC-3′

tory chain subunits encoded by mtDNA in neuroblastoma cells exposed to PARP-1 inhibitors or siRNA. As shown in Fig. 2F, mRNA levels of cytochrome oxidase subunits COX1 and COX2, as well as those of complex I ND2 subunit, were significantly reduced by a 72-h exposure to PHE and PJ34. Again, a 24-h exposure to the compounds exerted no change in the transcript levels (not shown). It is noteworthy that reduction of mRNAs for COX1, COX2, and ND2 was also found 72 h after PARP-1 silencing (Fig. 2G). To rule out the possibility that the effects of PARP-1 suppression were not cell line-specific, we attempted to reproduce our experiments in HeLa cells. Figure 2, H and I, show that, akin to SHSY neuroblastoma cells, mtDNA integrity as well as transcript levels for mitochondrially encoded respiratory chain subunits were reduced in HeLa cells exposed to PARP-1 inhibitors for 72 h.

PARP-1 Regulates Expression of Nuclear Genes Coding for mtDNA Repair Factors. Lack of mitochondrial PARP-1 along with the enzyme's ability of regulating mtDNA integrity and transcription prompted us to hypothesize that these functional effects might be indirect (i.e., due to nuclear PARP-1). We reasoned that epigenetic regulation of nuclear expression of mtDNA repair factors by PARP-1 might underlie, at least in part, the enzyme's role in maintenance of mtDNA integrity. The long-term exposure (72 h) required for PARP-1 suppression to affect mtDNA integrity was consistent with an indirect effect. To confirm our hypothesis, we investigated the effect of PARP-1 inhibition or silencing on expression of proteins involved in the base excision repair pathway of mtDNA. We found that transcripts for the mtDNA repair factors investigated (the glycosylases UNG1 and MYH1 and the endonuclease APE1) (Weissman et al., 2007) were reduced by neuroblastoma cells exposed to PHE or PJ34 for 24 h (Fig. 3A). The effects of the inhibitors was concentration-dependent (3–300 μM PHE, 1–100 μM PJ34; not shown). Similar decreases in mRNAs for APE1, UNG1, and MYH were found 24 h after PARP-1 silencing (Fig. 3B), thereby confirming specificity of the PARP-1 inhibitors' effects.

There is ample agreement that PARP-1 protein per se and PARP-1 protein activity exert key epigenetic regulation by direct binding to both DNA promoter regions and transcription-activating supramolecular protein complexes (Kraus, 2008). On this basis, we next investigated whether PARP-1 is present at promoters of the nuclear-encoded mtDNA repair factors under scrutiny. By means of ChIP assay we found that Flag-PARP-1 localizes at promoters of UNG1 and MYH1 (Haug et al., 1998) but not at that of APE1 (Fig. 3C). ChIP also indicated lack of Flag-PARP1 bound to the D-loop of mtDNA (Fig. 3C), further indicating its absence in mitochondria. Remarkably, pharmacological inhibitors of PARP-1 reduced the amount of the enzyme present at the MYH or UNG1 promoter regions (Fig. 3D). These findings, taken together suggest that PARP-1 assists nuclear transcriptional activation of specific mtDNA repair factors by poly(ADP-ribosyl)ation at their respective promoters. This interpretation is corroborated by evidence that, in neuroblastoma cells, PARP-1 inhibitor PHE or PJ34 reduces expression of luciferase driven by UNG1 promoter (Fig. 3E).

PARP-1 Regulates Expression of Nuclear-Encoded Mitochondrial Transcription Factors. In analogy with our reasoning on the indirect effect of nuclear PARP-1 in maintenance of mitochondrial genome integrity, we also hypothesized that expression of mitochondrial transcription factors typically encoded by nuclear genes might be epigenetically regulated by PARP-1. TFB1M and TFB2M are master activators of the mitochondrial transcriptional machinery typically encoded by genomic DNA (Scarpulla, 2008). We found that transcript levels for both transcription factors were reduced in neuroblastoma cells exposed for 24 h to pharmacological inhibition or silencing of PARP-1 (Fig. 4, A and B). Accordingly, ChIP revealed that Flag-PARP-1 transfected in neuroblastoma cells localized at TFB1M and TFB2M promoters (Gleyzer et al., 2005) (Fig. 4C), and that chemical inhibitors of the enzyme drastically reduced its presence at promoters (Fig. 4D). To corroborate these findings, we then took advantage of plasmids encoding luciferase driven by TFB1M or TFB2M promoter. Figure 4, E and F shows that luciferase expression is inhibited by both PARP-1

A

| Flag-PARP1 | mit-GFP | Merge |

**B**

| EGFP-PARP-1 | mit-RFP | Merge |
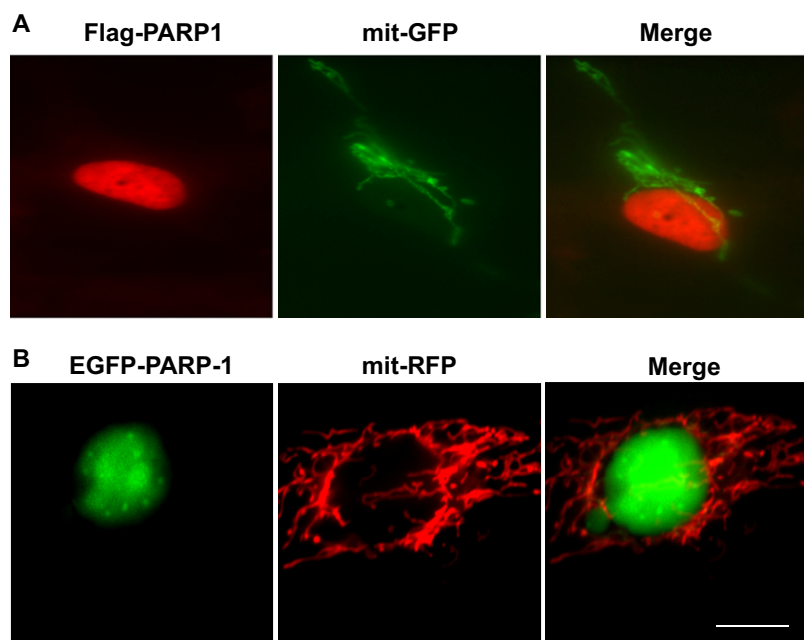
Fig. 1. Intracellular distribution of human PARP-1. Intracellular distribution of PARP-1 in SHSY5Y cells cotransfected with PARP1-Flag and mit-GFP (A) or EGFP-PARP-1 and mit-RFP (B). Images are representative of at least three independent experiments. Note the absence of colocalization between PARP-1 and mitochondrial fluorescence. Scale bar, 5 μm.

inhibitors and silencing, again indicating the key role of the enzyme in promoting nuclear transcriptional activation of mitochondrial transcription factors TFB1M and TFB2M.

Overall, findings point to a key role of PARP-1 in epigenetic regulation of mitochondrial gene expression at the nuclear level. In keeping with this, a large body of literature indicates that PARP-1 activity is in accord with the architectural organization of chromatin and ensuing genome functions. In particular, steric hindrance and electrostatic charge transfer as result of PAR binding to proteins significantly alter functioning of DNA-interacting factors in either a positive or negative fashion (Kraus and Lis, 2003; Kraus, 2008; Krishnakumar and Kraus, 2010). Thus, to substantiate the hypothesis that PARP-1 inhibitors altered epigenetic homeostasis in SHSY neuroblastoma cells, we evaluated the chemicals' effects on PAR content and histone H3 acetylation levels. As shown in Fig. 4G, a 72-h treatment with PHE or PJ34 reduced both the amount of PAR and histone H3 acetylation at Lys-18 in neuroblastoma SHSY cells. It is noteworthy that reduced acetylation levels at Lys-18 correlate with decreased transcriptional activity at specific gene promoters in neural cells (Faraco et al., 2006, 2009).

PARP-1 Suppression Impairs Mitochondrial Bioenergetics. Next, we sought to understand the functional relevance of PARP-1-dependent epigenetic regulation of nuclear-encoded mitochondrial proteins involved in mtDNA integrity and transcription. We therefore evaluated ATP content as a parameter of mitochondrial bioenergetics in neuroblastoma cells undergoing PARP-1 suppression. As shown in Fig. 5, a 72-h exposure to PHE or PJ34 reduced the content of ATP in cells cultured in complete Dulbecco's modified Eagle's medium (basal ATP content was 21 ± 8 nmol/mg of protein). To clarify whether total ATP reduction in PARP-1 inhibitor-exposed cells was due to impairment of mitochondrial ATP production, we then quantified ATP in cells cultured in a medium containing pyruvate and glutamine but lacking glucose. Under these experimental conditions, cellular ATP contents do not derive from glycolysis, being dependent only on mitochondrial production, were less than 25% of those of cells growing in the presence of glucose, and further decreased to undetectable levels when exposed to the oxidative phosphorylation inhibitors oligomycin, rotenone, and antimycin (all at 10 μM, not shown), confirming the mitochondrial origin of residual ATP. It is noteworthy that cells growing in the
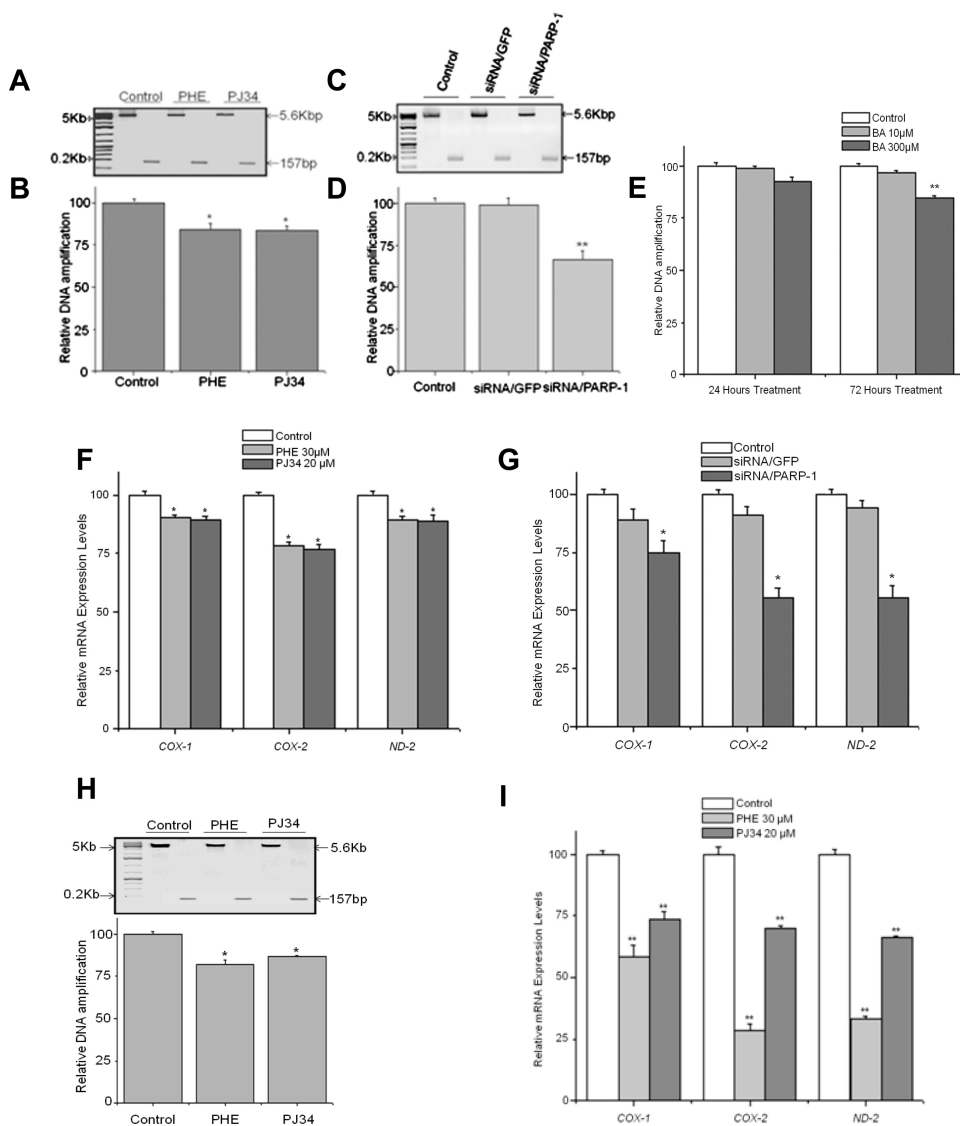
Fig. 2. PARP-1 contributes to mtDNA integrity and gene expression. A, evaluation of mtDNA integrity by semiquantitative PCR analysis of 5.6-kbp or 157-bp regions of mtDNA extracted from control cells or those exposed to PHE (30 μM) or PJ34 (20 μM) for 72 h. B, densitometric analysis of data shown in A. C, evaluation of mtDNA integrity by semiquantitative PCR analysis of 5.6-kbp or 157-bp regions of mtDNA extracted from control cells or cells transfected for 48 h with siRNA against GFP (used as negative control) or PARP-1. D, densitometric analysis of data shown in C. E, densitometric analysis of the effects of benzamide (BA) on mtDNA integrity according to the method described in A. Effects of PHE and PJ34 (F) or PARP-1 silencing (G) on the mRNA expression levels of *COX-1*, *COX-2*, and *ND-2* mitochondrial genes evaluated by real-time PCR (18S rRNA was used as housekeeping gene). H and I, effects of a 72-h exposure to PHE and PJ34 on mtDNA integrity or mRNA levels of mitochondrially expressed respiratory factor subunits in HeLa cells. Columns represent the mean $\pm$ S.E.M. of at least three experiments conducted in duplicate. $*$, $p < 0.05$; $**$, $p < 0.01$ versus control, ANOVA plus Tukey's post hoc test.

absence of glucose and exposed to PHE or PJ34 showed a more drastic reduction of ATP contents compared with those challenged with the PARP-1 inhibitors but having glucose in their growth media (Fig. 5).

Discussion

In the present study, we originally report a key role of nuclear PARP-1 in expression of genes coding for mitochondrial proteins involved in maintenance of organelle homeostasis. Specifically, we demonstrate that PARP-1 enzymatic activity is necessary to allow transcriptional activation of nuclear genes coding for components of the mitochondrial base excision repair machinery and transcriptional apparatus. PARP-1 is consistently present at nuclear promoters of these genes, and its pharmacological or genetic suppression results in reduced expression of genes coded by mtDNA, such as COX1, COX2, and ND2. Overall, the present study points to PARP-1 as a key epigenetic regulator of nuclear-encoded mitochondrial proteins and is in keeping with a recent study reporting that PARP-1 activity is necessary for nuclear re-spiratory factor (NRF)-1-dependent trans-activation and cytochrome c promoter activity (Hossain et al., 2009).

Our data are in line with prior studies reporting a pivotal role of PARP-1 in maintenance of mtDNA integrity (Gilbertson et al., 1991; Rossi et al., 2009). It is noteworthy, however, that we have been unable to find evidence for mitochondrial localization of PARP-1. Indeed, both Flag- and GFP-tagged PARP-1 seem to be exclusively nuclear when expressed in SHSY5Y neuroblastoma cells. We acknowledge that intracellular localization of tagged enzymes may be misleading with respect to that of normal proteins because of tag-dependent alteration of protein trafficking. Yet in the PARP-1 vectors, both GFP and Flag were present at the C terminus. This notion, along with knowledge that the N terminus of proteins is that recognized by the mitochondrial import apparatus (Schmidt et al., 2010), suggests that GFP or Flag should not alter mitochondrial migration. In addition, intracellular visualization of PARP-1 in living cells by means of GFP tagging allowed the avoidance of cell fixation and antibody utilization and the ensuing potential, technical artifacts. It is worth noting that lack of mitochondrial localization of PARP-1 is in
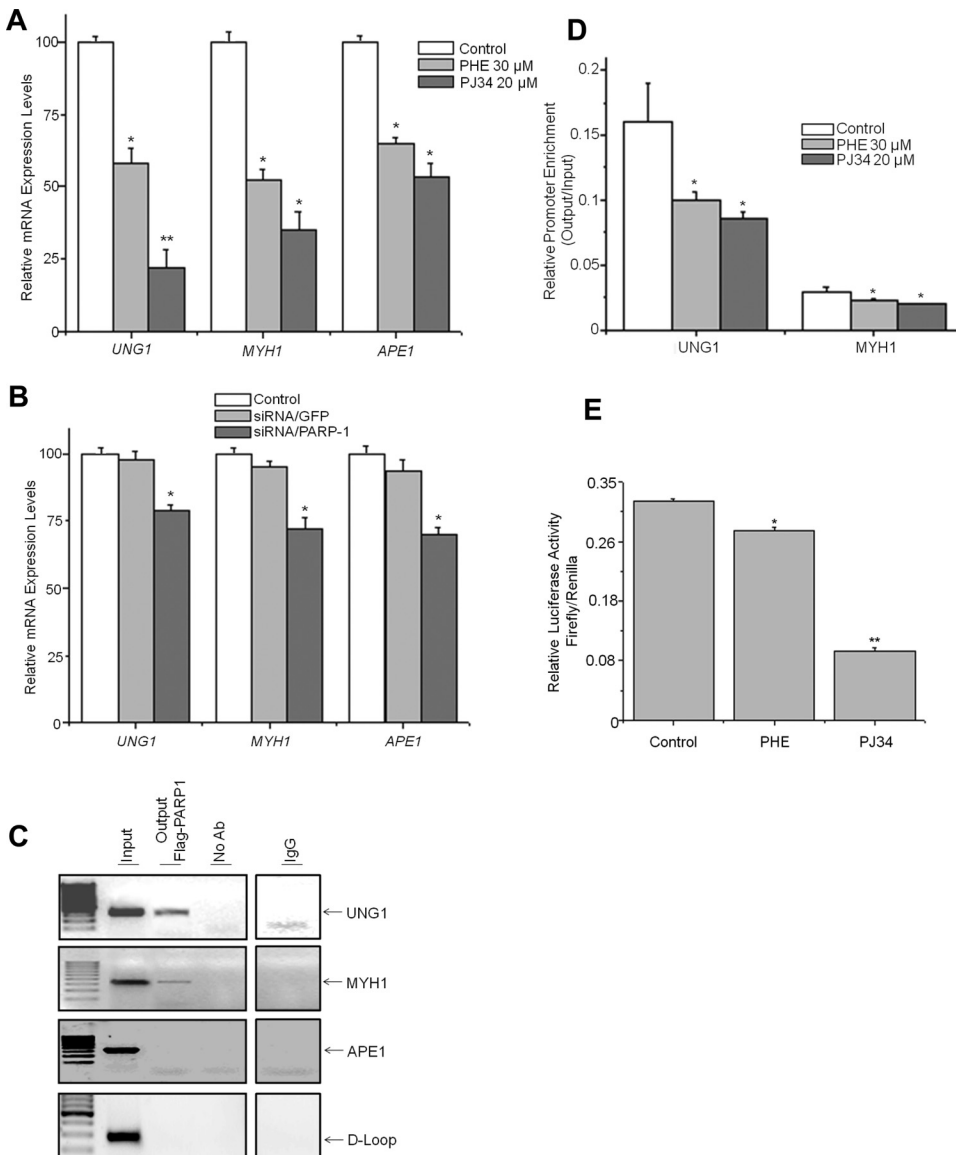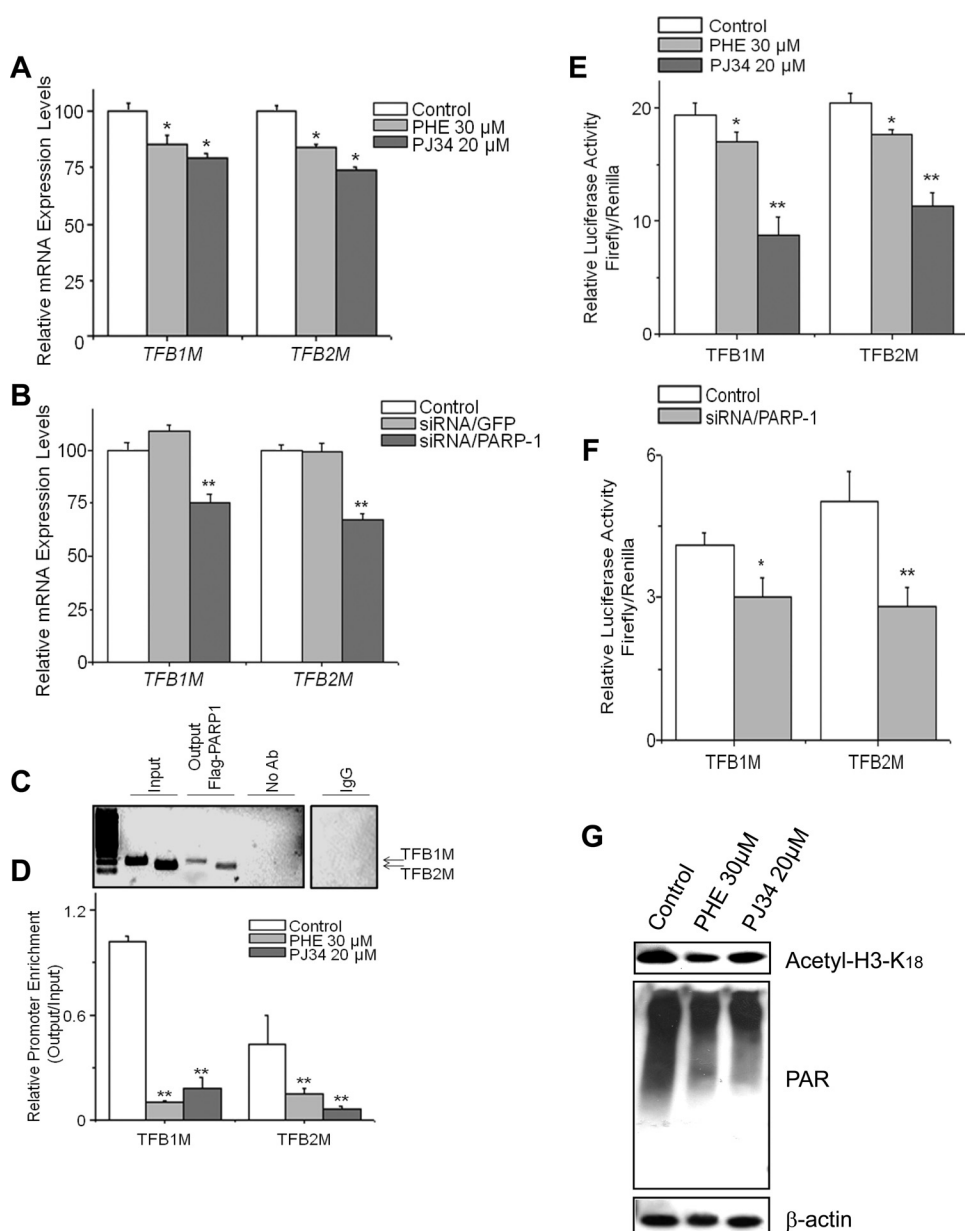

Fig. 3. PARP-1 regulates expression of nuclear genes coding for mtDNA repair factors by direct interaction on their promoters. Real-time PCR evaluation of transcript levels for *UNG1*, *MYH1*, and *APE1* genes in SHSY5Y cells treated with PHE (30 μM) or PJ34 (20 μM) for 72 h (A) or PARP-1 siRNA for 48 h (B). B, siRNA against GFP was used as a negative control (18S rRNA was used as housekeeping gene). C, ChIP analysis of the presence of PARP-1 on promoters of UNG1, MYH1, and APE1 or mitochondrial D-loop in SHSY5Y cells. Input, total lysate before antibody immunoprecipitation (positive control). Output, immunoprecipitation with Flag-PARP1. No antibodies or IgG are shown as negative controls. D, ChIP analysis of the presence of PARP-1 on promoters of UNG1 and MYH1 in SHSY5Y cells treated or not with PHE or PJ34 quantitated by real-time PCR. E, effects of PHE or PJ34 on UNG1 promoter activity in cells transfected with the UNG1 promoter-driven luciferase pGL2-PB vector. Columns represent the mean $\pm$ S.E.M. of at least three experiments conducted in duplicate. ∗, $p < 0.05$; ∗∗, $p < 0.01$ versus control; ANOVA plus Tukey's post hoc test.

keeping with a large number of prior studies and with recent reports on mitochondria-targeted PARP-1 (Niere et al., 2008; Dölle et al., 2010). Remarkably, in these two studies, the researchers tagged mt-PARP-1 with GFP at the N terminus, thereby strengthening our hypothesis that the fused fluorescent protein does not impair per se mitochondrial PARP-1 import.

Despite lack of mitochondrial localization of PARP-1, we show here that the enzyme plays a key role in functional integrity of the organelles. Our findings demonstrate that this role is merely indirect and is due to epigenetic regulation of nuclear genes. Accordingly, prolonged exposure to PARP-1 inhibitors is necessary to impair mtDNA integrity or transcription. Present data are consistent with the emerging relevance of PAR formation in epigenetic regulation of gene expression. Although it can easily be predicted that targeting of highly electronegative polymers to DNA-binding proteins would profoundly alter chromatin architecture and function, at present, the exact molecular mechanism underlying PAR-

dependent epigenetic regulation remains to be unequivocally identified. It is likely, however, that PARP-1 and its activity regulates gene expression through several mechanisms, such as modulation of chromatin compaction, enhancer binding, transcriptionally active supramolecular complex formation, and DNA insulation (Kraus, 2008; Krishnakumar and Kraus, 2010). It is also worth noting that PARP-1 inhibitor-dependent reduction of cellular PAR contents correlates to diminished histone H3 acetylation (Fig. 4G). This finding is in keeping with the view that reduced poly(ADP-ribosyl)ation leads to chromatin compaction (Schreiber et al., 2006; Krishnakumar and Kraus, 2010), which, in turn, limits histone acetylation, two epigenetic events shifting the chromatin status toward gene silencing. PARP-1 inhibitor-dependent hypoacetylation, however, does not indicate that poly(ADP-ribosyl)ation regulates acetylation levels at all gene promoters. Indeed, PARP-1-dependent transcriptional regulation has been shown to be gene-specific (Kraus, 2008; Krishnakumar and Kraus, 2010). The present data showing that PARP-1 is

Fig. 4. PARP-1 regulates expression of nuclear-encoded mitochondrial transcription factors. Real-time PCR evaluation of transcript levels for *TFB1M* and *TFB2M* genes in SHSY5Y cells treated with PHE (30 μM) or PJ34 (20 μM) for 72 h (A) or PARP-1 siRNA for 48 h (B). B, siRNA against GFP was used as a negative control (18S rRNA was used as housekeeping gene). C, ChIP analysis of the presence of PARP-1 on promoters of TFB1M and TFB2M genes in SHSY5Y cells. Input, total lysate before antibody immunoprecipitation (positive control). Output, immunoprecipitation with Flag-PARP1. No antibodies or IgG are shown as negative controls. D, ChIP analysis of the presence of PARP-1 on promoters of TFB1M and TFB2M genes in SHSY5Y cells treated or not with PHE or PJ34 for 72 h and quantitated by real-time PCR. Effects of PHE or PJ34 (72 h) (E) or siRNA against PARP-1 (48 h) (F) on TFB1M and TFB2M promoter activity in cells transfected with the TFB1M or TFB2M promoter-driven luciferase pGL3 vectors. G, Western blotting evaluation of the effects of a 72-h challenge to PHE or PJ34 on PAR content and histone H3 acetylation at lysine (K)-18. β-actin is shown as loading control. Note that Western blotting evaluation of poly(ADP-ribose) appears as a typical smear because of the different molecular weights of the proteins bound to the polymer. Columns represent the mean $\pm$ S.E.M. of at least three experiments conducted in duplicate. G, a representative blot of three independent experiments is shown. *, $p < 0.05$; **, $p < 0.01$ versus control, ANOVA plus Tukey's post hoc test.

present at promoter regions of nuclear genes coding for mtDNA repair enzymes and transcription factors is in keeping with the ability of the enzyme to assist proper assembly of the transcriptional machinery and render chromatin architecture permissive for transcription (Pirrotta, 2003). Furthermore, present evidence that suppression of PARP-1 activity reduces the amount of PARP-1 bound at promoters as well as transcription of the downstream genes underscores the relevance of nuclear PAR formation, in addition to PARP-1 protein per se, to expression of mitochondrial genes. These latter findings also suggest that PAR is necessary to recruit PARP-1 at transcriptionally active loci and ensuing assembly of the transcriptional machinery. This interpretation is in keeping with a recent report showing that inhibition of PARP-1 reduces expression of BRCA1 and RAD51, two factors essential for homology-dependent repair of nuclear DNA. It is noteworthy that evidence that PARP-1 inhibitors allow binding of repressor complexes at promoters of *BRCA1* and *RAD51* genes, further strengthens the key role of PARP-1-dependent PAR formation in promoter activation (Hegan et al., 2010). In this regard, it is worth noting that we have been unable to localize PARP-1 at APE1 promoter (Fig. 3C), despite evidence for reduced levels of APE1 transcripts in cells exposed to PARP-1 inhibitors (see Fig. 3, A and B). These findings suggest that PARP-1 can regulate promoter activity even indirectly, likely poly(ADP-ribosyl)ating promoter-interacting proteins without direct binding to the promoter region itself.

In light of the pleiotypic therapeutic potential of PARP-1 inhibitors (Virág and Szabó, 2002) as well as their current clinical trials (Fong et al., 2009), our findings might have important therapeutical implications. On the one hand, present data suggest that long-term exposure to chemicals inhibiting PARP-1 impairs mitochondrial homeostasis and related bioenergetics. On the other hand, given the key relevance of

perturbations of nuclear-mitochondrial intergenomic crosstalk in the pathogenesis of mitochondrial disorders in childhood (Spinazzola and Zeviani, 2009), we speculate that positive modulation of PARP-1-dependent regulation of mtDNA integrity and respiratory chain gene expression can be harnessed for therapeutic interventions. It is worth noting, however, that cancer cells, such as those used in the present study, undergo dramatic bioenergetic changes during transformation because of gene expression alterations due to epigenetic reprogramming (Vander Heiden et al., 2009; Locasale et al., 2010). It is possible, therefore, that relevance of PARP-1 and its inhibition to epigenetic regulation of mitochondrial functions is quantitatively and/or qualitatively different in nontransformed cells. If this hypothesis holds true, then impairment of mitochondrial functions by PARP-1 inhibitors represents an additional mechanism through which these chemicals exert tumor-specific cytotoxicity. Finally, accumulation of mtDNA damage has repeatedly been proposed as a mechanism contributing to aging in different organisms, including humans. In this light, our data showing that PARP-1 assists mtDNA repair and transcription emphasize the relevance of PARP-1 and PAR formation to aging and help explain why its activity positively correlates to extended life span expectancy (Beneke and Bürkle, 2007).

Authorship Contributions
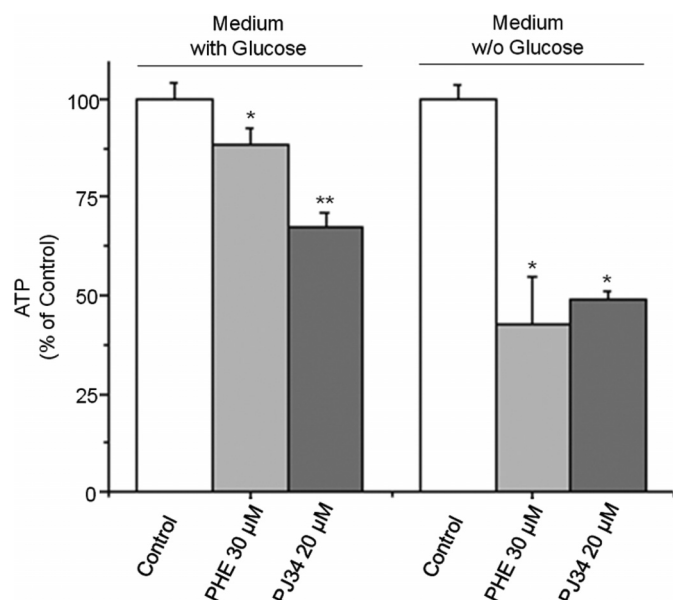
Participated in research design: Lapucci, Moroni, and Chiarugi.
Conducted experiments: Lapucci, Pittelli, and Felici.
Contributed new reagents or analytic tools: Rapizzi.
Performed data analysis: Lapucci.
Wrote or contributed to the writing of the manuscript: Lapucci and Chiarugi.

Fig. 5. PARP-1 inhibition reduces mitochondrial ATP production. Effects of PHE or PJ34 on total (medium with glucose) or mitochondrial (medium without glucose) ATP production in SHSY5Y cells treated for 72 h. Glucose was withdrawn 12 h before ATP content analysis. Columns represent the mean ± S.E.M. of three experiments conducted in duplicate. *, $p < 0.05$; **, $p < 0.01$ versus control, ANOVA plus Tukey's post hoc test.

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