

**Poly(ADP-ribose) polymerase-1 is a nuclear epigenetic regulator of mitochondrial DNA
repair and transcription**

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Running title: Nuclear PARP-1 and mtDNA homeostasis

Number of text pages: 25

Number of Figures: 5

Number of References: 39

Number of words in:

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List of Abbreviations: ChIP, chromatin immunoprecipitation; GFP, green fluorescent protein; mtDNA, mitochondrial DNA; PARP-1, poly(ADP-ribose) polymerase-1; PAR, poly(ADP-ribose); PHE, 5(6H)-phenanthridinone; PJ34, N-(6-Oxo-5,6-dihydrophenanthridin-2-yl)-(N,N-dimethylamino)acetamide hydrochloride; RFP, red fluorescent protein.

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 classically 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, in spite of exclusive nuclear localization of PARP-1, mitochondrial homeostasis is compromised in cell lines exposed to PARP-1 pharmacological inhibitors or siRNA. PARP-1 suppression reduces integrity of 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. Notably, 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. Data might have important implications in pharmacology of PARP-1 inhibitors as well as clinical oncology and aging.

Introduction

Poly(ADP-ribose)ylation is a post-translational modification of proteins operated by poly(ADP-ribose)polymerases (PARPs) (Ame *et al.*, 2004). This a family of enzymes able to transform NAD into polymers of ADP-ribose (PAR) which can be targeted to the enzymes themselves and to a large number of proteins (Schreiber *et al.*, 2006). Recently, because of the chemical aspects of the reaction catalyzed, the classification of PARPs as ADP-ribose transferases (ARTs) has been proposed (Hottiger *et al.*, 2010). Nuclear PARP-1 is the oldest member of the PARP family and classically involved in DNA repair and cell death. These pathophysiological 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 at developing potent PARP-1 inhibitors which proved of efficacy in numerous models of human disorders (Jagtap and Szabo, 2005), and recently reached the clinical arena (Annunziata and O'Shaughnessy, 2010). Although data from neoplastic patients 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-ribose)ylation is thought to contribute to maintenance of nuclear homeostasis and cellular functions (Krishnakumar and Kraus, 2010; Asher *et al.*, 2010). Current knowledge about PARP-1-catalyzed reactions allows to identify the enzyme as a key epigenetic regulator of gene expression thanks to the covalent- and non-covalent modification of chromatin architecture-regulating proteins and ensuing assembly of transcriptionally active supramolecular complexes by PAR formation (Kraus, 2008; Krishnakumar and Kraus, 2010). Remarkably, if on the one hand these properties have drastically changed the physiological role of PARP-1 within the nucleus, on the other they have widened the pharmacodynamic properties of PARP-1 inhibitors.

Despite the impressive advancement in the field of poly(ADP-ribose)ylation, the pathophysiological relevance of PARP-1 and PAR formation within mitochondria is still debated. In spite of its

canonical nuclear localization, indeed, several studies report that PARP-1 and poly(ADP-ribose)ating proteins also localizes in mitochondria (Scovassi, 2004; Pankotai *et al.*, 2009). Reportedly, mitochondrial PARP-1 actively participates to maintenance of functional integrity of the organelles (Rossi *et al.*, 2009), and plays a detrimental role when hyperactivated (Du *et al.*, 2003; Lai *et al.*, 2007). 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 unable to detect PARP-1 within mitochondria, we originally report 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 cervicocarcinoma 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% CO₂ 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) (Alexis Biochem., Vinci, Italy). Transient transfection of SHSY5Y cells with plasmids or siRNA against human PARP-1 (Dharmacon, Denver, USA) or GFP (QIAGEN, Milan, Italy) were carried out with jetPRIME™ reagent (Polyplus, 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 plasmid are control vectors coding for renilla luciferase) plasmids or empty vectors were used.

Immunocytochemistry and fluorescence image acquisitions

Cells (2×10^5) were immobilized onto glass coverslips (15x15mm), transiently co-transfected with PARP1-Flag and mit-GFP or EGFP-PARP-1 and mit-RFP vectors. For Immunocytochemistry cells co-transfected with PARP1-Flag and mit-GFP were washed twice with cold PBS and fixed for 10 min in 4% paraformaldehyde in PBS. Following three washes for 2 min with PBS, the cells were incubated in 1 ml of blocking buffer (3% bovine serum albumin, 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, Milan, Italy) antibody (1:250) in blocking buffer. Later on, cells were washed three times for 15 min in washing buffer (0.1% Triton X-100 in PBS), incubated with the secondary Cy-3-conjugated anti-mouse antibody (Chemicon, Billerica USA) (1:800) for 60 min at room temperature, and washed three times with 1 ml of washing buffer for 5 min 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 x 768 pixels) were obtained using a 60 X objective lens. Cells co-transfected with EGFP-PARP-1 and mi-RFP cells were washed in PBS twice and directly analyzed by fluorescence microscopy.

Direct-PCR assay (DPCR)

For DPCR SHSY5Y cells were treated with vehicle, PHE, PJ34 or PARP-1 siRNA and DNA extracted by NucleoBond® Xtra Midi Kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions. Amplification products of 5.6 Kbp or 157 opb of mtDNA were obtained by PCR using specific primers as previously described (Rossi *et al.*, 2009) the resulting bands were analyzed by ImageJ software for densitometric analysis.

Real-time PCR assay

One μg of total RNA isolated from treated or untreated cells treated was retro-transcribed using iScript™ kit (Bio-Rad, Hercules, USA) and amplified with the following specific primers: Cytochrome oxidase subunit 1 (*COX-1*): Forward 5'-TACCAGGCTTCGGAATAATCTC-3' and Reverse 5'- GATAGCGATGATTATGGTAGCG-3'; Cytochrome oxidase subunit 2 (*COX-2*): Forward 5'- CTCCTTGACGTTGACAATCG-3' and Reverse 5'-CCACAGATTTTCAGAGCATTGA-3'; NADH dehydrogenase subunit 2 (*ND-2*): Forward 5'-AGGTGGATTAAACCAAACCCAG-3' and Reverse 5'-CGAGATAGTAGTAGGGTCGTGG-3'; Uracil-DNA glycosylase variant 1 (*UNG1*): Forward 5'-CAGCTCTTGAGCCGCCTCTG-3' and Reverse 5'-GTCCAACCTGCTCGGCACTC-3'; MutY homolog (E. coli) transcript variant 1 (*MYH1*): Forward 5'-GAGCCTGCTAAGCTGGTACG-3' and Reverse 5'-TGCAGCATGACCTCTGAGAC-3'; APEX nuclease multifunctional DNA repair enzyme transcript variant 1 (*APE1*): Forward 5'-GTGCCCACTCAAAGTTTCTTAC-3' and Reverse 5'-CGGCCTGCATTAGGTACATATG-3'; Mitochondrial transcription factor *TFB1M*: Forward 5'-TGCAAGCAGCGAAGCAGCTATC-3' and Reverse 5'-TTCAACCACCAGAAGTTCAGCG-3'; Mitochondrial transcription factor *TFB2M*: Forward 5'- CGATCGGAGATTGGCTGAGAC-3' and Reverse 5'-TCACTTTTCGAGCGCAACCAC-3'; 18S ribosomal RNA: Forward 5'-CGGCTACCACATCCAAGGAA-3' and Reverse 5'-GCTGGAATTACCGCGGCT-3'. Real-time PCR assays were performed by Rotor-Gene™ SYBR® Green PCR Kit (Qiagen, Milan, Germany) and analyzed using the Rotor-Gene 3000 cyclor system (Corbett Research, Mortlake, Australia).

Chromatin Immunoprecipitation assay (ChIP)

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 cold PBS, scraped, lysed in 5 mM EDTA, 50 mM Tris-HCl (pH 8.0), 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 100–500 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% Nonidet P-40, 200 mM NaCl supplemented with protease inhibitors. Then, 150 μg of chromatin were incubated with ANTI-FLAG® M2 Magnetic Beads (Sigma-Aldrich, Italy) 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/10 min and the pellet washed with a low salt wash buffer (0.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl), LiCl wash buffer (1% Nonidet P-40, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 1% sodium deoxycholate), and Tris-EDTA buffer (10 mM Tris-Cl, pH 7.4, 1 mM EDTA). Pellets were dissolved in 300 µl of elution buffer (1% SDS, 0.1 M NaHCO₃). The samples were treated with RNase A for 10 min at room temperature than incubated at 65°C for 4 hours 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. Outputs DNA concentration were determined spectrophotometrically at 260 nm. The following primers were used for PCR amplification: Forward, 5'-CCTAGTCCACCCGGCTCT-3' and Reverse, 5'-GAGGAACCTGCGAGACCTAA-3' (TFB1M promoter); Forward, 5'-ACGGTCCACTCACAATCCTC-3' and Reverse, 5'-CCCACGTGGAACATTTTCTG-3' (TFB2M promoter); Forward, 5'-CCAATGGGAACGCGTCTCGG-3' and Reverse, 5'-TTGTGGAACAGGTCCCAGCAAAG-3' (UNG1 promoter); Forward, 5'-ACGCTCAATCCACTCCACTG-3' and Reverse 5'-CCGCCGACAGTGACGATGGCGCA-3' (MYH1 promoter); Forward, 5'-CCCCTCACCCACTAGGATAC-3' and Reverse 5'-ACGTGTGGGCTATTTAGGC-3' (mitochondrial D-Loop) (Rossi *et al.*, 2009). 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 × 10⁴/well of SHSY5Y cells were co-transfected in triplicate in 96-well plates with equal amounts (200 ng) of the following plasmids, pGL3-hTFB1M, pGL3-hTFB2M, and pGL4 internal standard and subjected to different treatments. For UNG1

promoter activity cells were co-transfected with pGL2-PB and pRL-TK internal standard. 48 hours post-transfection cells were lysed and the Firefly and Renilla luciferase activities measured sequentially from a single aliquot of cell lysate using the Dual-Glo Luciferase Reporter Assay System (Promega, Madison, USA) according to the manufacturer's instructions by using the TopCount NXT luminometer (PerkinElmer, Milan, Italy).

Western Blotting

For Western blotting, cells were scraped, collected in eppendorf tubes, centrifuged (1500 g/5 min/4°C) and resuspended in lysis buffer [50 mM Tris pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 4 µg/ml aprotinin and leupeptin, 1% SDS]. 20-40 µg of protein/lane were loaded. After 4-20% SDS-PAGE and blotting, membranes (Immobilon-P, Millipore, MA, USA) were blocked with phosphate buffered saline (PBS) containing 0.1% Tween-20 and 5% skimmed milk (TPBS/5% milk) and then probed overnight with primary antibodies (1:1000 in TPBS/5% milk). The anti-PAR monoclonal antibody (10H) was from Alexis (Vinci, Italy), the anti-β-actin was monoclonal from Santa Cruz Biotechnology (Santa Cruz, CA, USA) whereas the polyclonal anti histone H3 acetylated Lys-18 was from Cell Signaling (Beverly, MA, USA). Membranes were then washed with TPBS and incubated 1 h in TPBS/5% milk containing the corresponding peroxidase-conjugated secondary antibody (1:2000). After washing in TPBS, ECL (Amersham, 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 or not to oligomycin, rotenone and antimycin was measured in total cell lysates by means of the ATPlight kit (Perkin Elmer, Milan, Italy), as previously described (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; Rossi *et al.*, 2009; Scovassi, 2004), 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 (AIF) (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 (mtGFP). 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 (mtRFP). Data shown in Fig. 1B demonstrated that GFP-PARP-1 did not colocalize with the network of mitochondria depicted by mtRFP. 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 μ M or PJ34 20 μ M (concentrations consistent with their IC_{50} on PARP-1) for up to 24 hrs did not alter PCR efficiency (not shown). Conversely, selective reduction of amplification efficiency of the large mtDNA fragment was found when drug exposure was prolonged to 72 hrs (Fig. 2A and B). Similar results were found with 300 μ M benzamide, a less potent PARP-1 inhibitor (Fig. 2C). To rule out possible non specific 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

transcript were reduced by 72% as revealed by real-time PCR (not shown). As shown in Fig. 2D and E, silencing of PARP-1 for 72 hrs 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 respiratory 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 did not exert any change in the transcript levels (not shown). Of note, reduction of mRNAs for COX1, COX2 and ND2 was also found 72 h after PARP-1 silencing (Fig. 2G). To rule out that the effects of PARP-1 suppression were not cell line specific, we attempted to reproduce our experiments in HeLa cells. Fig. 2H 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 hrs.

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, at least in part, underlie the enzyme's role in maintenance of mtDNA integrity. The chronic 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 (BER) pathway of mtDNA. We found that transcripts for the mtDNA repair factors investigated (the two glycosylases UNG1 and MYH1, and the endonuclease APE1) (Weissman *et al.*, 2007) were reduced by neuroblastoma cells exposed to PHE or PJ34 for 24 hrs (Fig. 3A). The effects of the inhibitors was concentration-dependent (3-300 μ M PHE, 1-100 μ M PJ34, not shown). Similar

decreases of 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 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 expression of luciferase driven by UNG1 promoter is reduced by PARP-1 inhibitors PHE or PJ34 in neuroblastoma cells (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 24 h to pharmacological inhibition or silencing of PARP-1 (Fig. 4A 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 this findings, we then took advantage of plasmids encoding luciferase driven by TFB1M or TFB2M promoter. Fig. 4E and F shows that luciferase expression is inhibited by both PARP-1 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 concurs to the architectural organization of chromatin and ensuing genome functions. Specifically, steric hindrance and electrostatic charge transfer due to 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 hrs treatment with PHE or PJ34 reduced both the amount of PAR and histone H3 acetylation at Lys-18 in neuroblastoma SHSY cells. Of note, reduced acetylation levels at Lys-18 correlate with decreased transcriptional activity at specific gene promoters in neural cells (Faraco *et al.*, 2006; Faraco *et al.*, 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 DMEM (basal ATP content was 21 ± 8 nmol/mg prot). 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 only dependent 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 OXPHOS inhibitors oligomycin, rotenone and antimycin (all at 10 μ M, not shown), confirming the mitochondrial origin of residual

ATP. Of note, cells growing in the absence of glucose and exposed to PHE or PJ34 showed a more drastic reduction of ATP contents when compared to 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 BER machinery and transcriptional apparatus. Consistently, PARP-1 is 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 respiratory 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 (Rossi *et al.*, 2009; Gilbertson *et al.*, 1991). Notably, however, we have been unable to find evidence for mitochondrial localization of PARP-1. Indeed, both Flag- and GFP-tagged PARP-1 appears 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 terminal. This notion, along with knowledge that the N-terminal of proteins is that recognized by the mitochondrial import apparatus (Schmidt *et al.*, 2010), suggests that GFP or Flag should not alter mitochondrial migration. Also, intracellular visualization of PARP-1 in living cells by means of GFP tagging allowed to avoid 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 keeping with a large number of prior studies, and with recent reports on mitochondria-targeted PARP-1 (Niere *et al.*, 2008; Dolle *et al.*, 2010). Remarkably, in these two

studies Ziegler and co-workers tagged mt-PARP-1 with GFP at the N-terminal, 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 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 be easily envisaged that targeting of highly electronegative polymers to DNA-binding proteins profoundly alters chromatin architecture and function, at present the exact molecular mechanism underlying PAR-dependent epigenetic regulation still waits 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 inhibitors-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 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 (Pirrota, 2003). Further, 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. Of note, 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 Figs. 3A 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 (Virag and Szabo, 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 chronic exposure to chemicals inhibiting PARP-1 impairs mitochondrial homeostasis and related bioenergetics. On the other, given the key relevance of perturbations of nuclear-mitochondrial intergenomic cross-talk 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 non-transformed cells. If this hypothesis holds true, then impairment of mitochondrial functions by PARP-1 inhibitors represents an additional mechanisms through which these chemicals exert tumor-specific cytotoxicity. Finally, accumulation of mtDNA damage has been repeatedly 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 explaining why its activity positively correlates to extended life span expectancy (Beneke and Burkle, 2007).

Acknowledgments

We are indebted to Valerie Schreiber, W. Lee Kraus, Richard C. Scarpulla, Hans Krokan for providing us with the pEGFP-hPARP-1, pCMV5-PARP1-Flag, pGL3-hTFB1/2M, and pGL2-PB reporter plasmids, respectively.

Author contribution

Participated in research design: Lapucci, Moroni, Chiarugi

Conducted experiments: Lapucci, Pittelli, Felici

Contributed new reagents or analytic tools: Rapizzi

Performed data analysis: Lapucci

Wrote or contributed to the writing of the manuscript: Lapucci, Chiarugi

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Footnote: This study was supported by grants from Fondazione Giuseppe Tomasello [15-09], Italian Ministry of Health and Research [PRIN 2007], Fondazione Italiana Sclerosi Multipla [2009/R/6].

Legends to Figures

Figure 1. Intracellular distribution of human PARP-1. Intracellular distribution of PARP-1 in SHSY5Y cells co-transfected with (A) PARP1-Flag and mit-GFP, (B) EGFP-PARP-1 and mit-RFP. Images are representative of at least 3 independent experiments. Note the absence of colocalization between PARP-1 and mitochondrial fluorescence. Bar = 5 μ m.

Figure 2. PARP-1 contributes to mtDNA integrity and gene expression. (A) Evaluation of mtDNA integrity by semi-quantitative PCR analysis of 5.6kbp or 157-bp of regions of mtDNA extracted from control, PHE (30 μ M) or PJ34 (20 μ M) exposed cells for 72 hours. (B) densitometric analysis of data shown in (A). (C) Evaluation of mtDNA integrity by semi-quantitative PCR analysis of 5.6kbp or 157-bp of regions of mtDNA extracted from control cells, or cells transfected for 48 hrs 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). In (H) and (I) the effects of a 72 hrs exposure to PHE and PJ34 on mtDNA integrity or mRNA levels of mitochondrially-expressed respiratory factor subunits in HeLa cells is shown. Columns represent the mean \pm SEM of at least three experiments conducted in duplicate. * $p < 0.05$, ** $p < 0.01$ vs control, ANOVA plus Tukey's post hoc test.

Figure 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 μ M) or PJ34 (20 μ M) for 72 hrs (A), or PARP-1 siRNA for 48 h (B). In (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

prior to antibody pull-down (positive control); Output: pull-down with Flag-PARP1. No Ab 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 SEM of at least three experiments conducted in duplicate. * $p < 0.05$, ** $p < 0.01$ vs control, ANOVA plus Tukey's post hoc test.

Figure 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 μ M) or PJ34 (20 μ M) for 72 hrs (A) or PARP-1 siRNA for 48 hrs (B). In (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 prior to antibody pull-down (positive control); Output: pull-down with Flag-PARP1. No Ab 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 hrs and quantitated by real-time PCR. Effects of PHE or PJ34 (72 hrs) (E) or siRNA against PARP-1 (48 hrs) (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 hrs 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 SEM of at least three experiments conducted in duplicate. In (G) a representative blot of 3 independent experiments is shown. * $p < 0.05$, ** $p < 0.01$ vs control, ANOVA plus Tukey's post hoc test.

Figure 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 hrs. Glucose was withdrawn 12 hours before ATP content analysis.

Columns represent the mean \pm SEM of three experiments conducted in duplicate. * $p < 0.05$,

** $p < 0.01$ vs control, ANOVA plus Tukey's post hoc test.

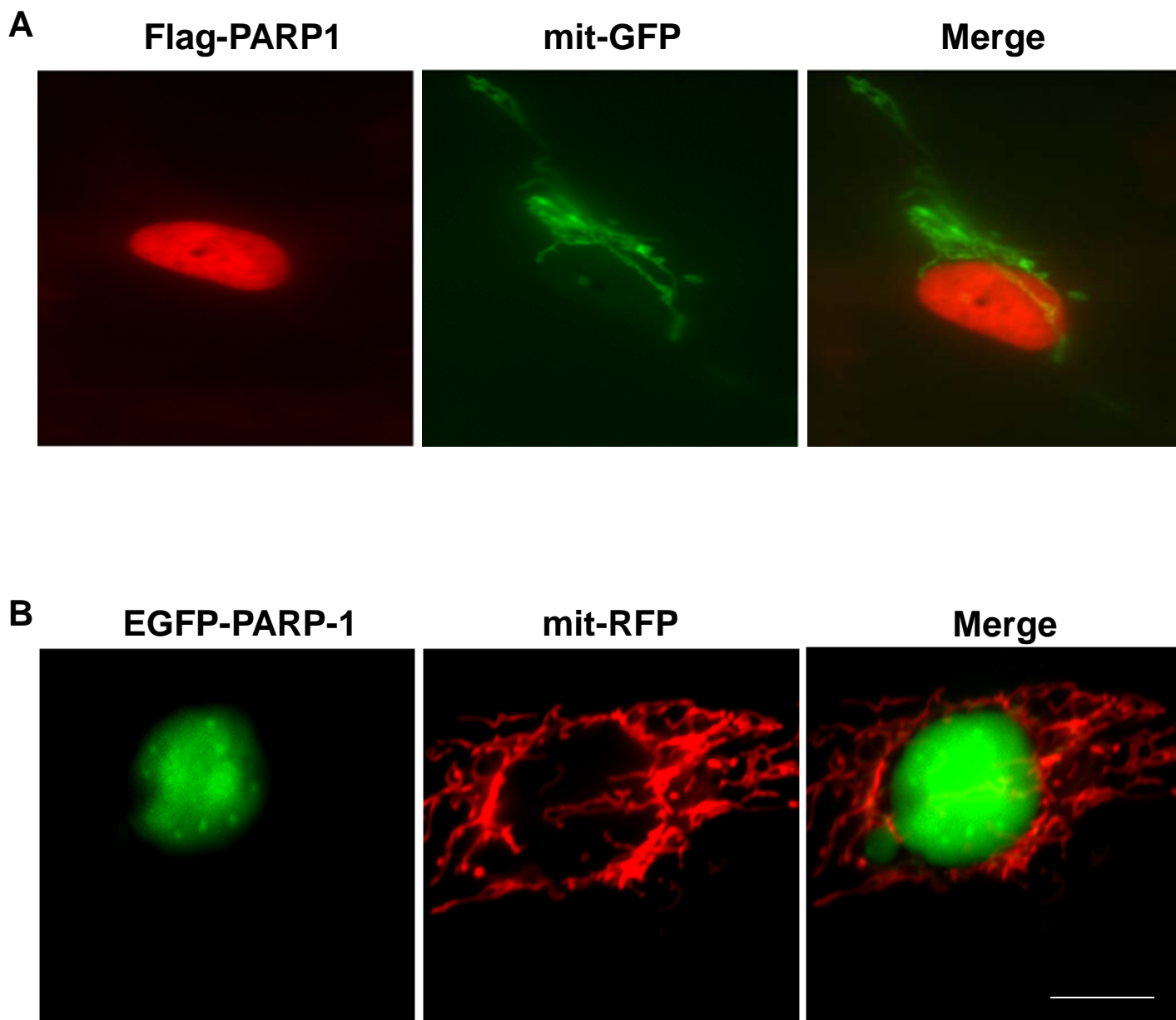


Figure 1

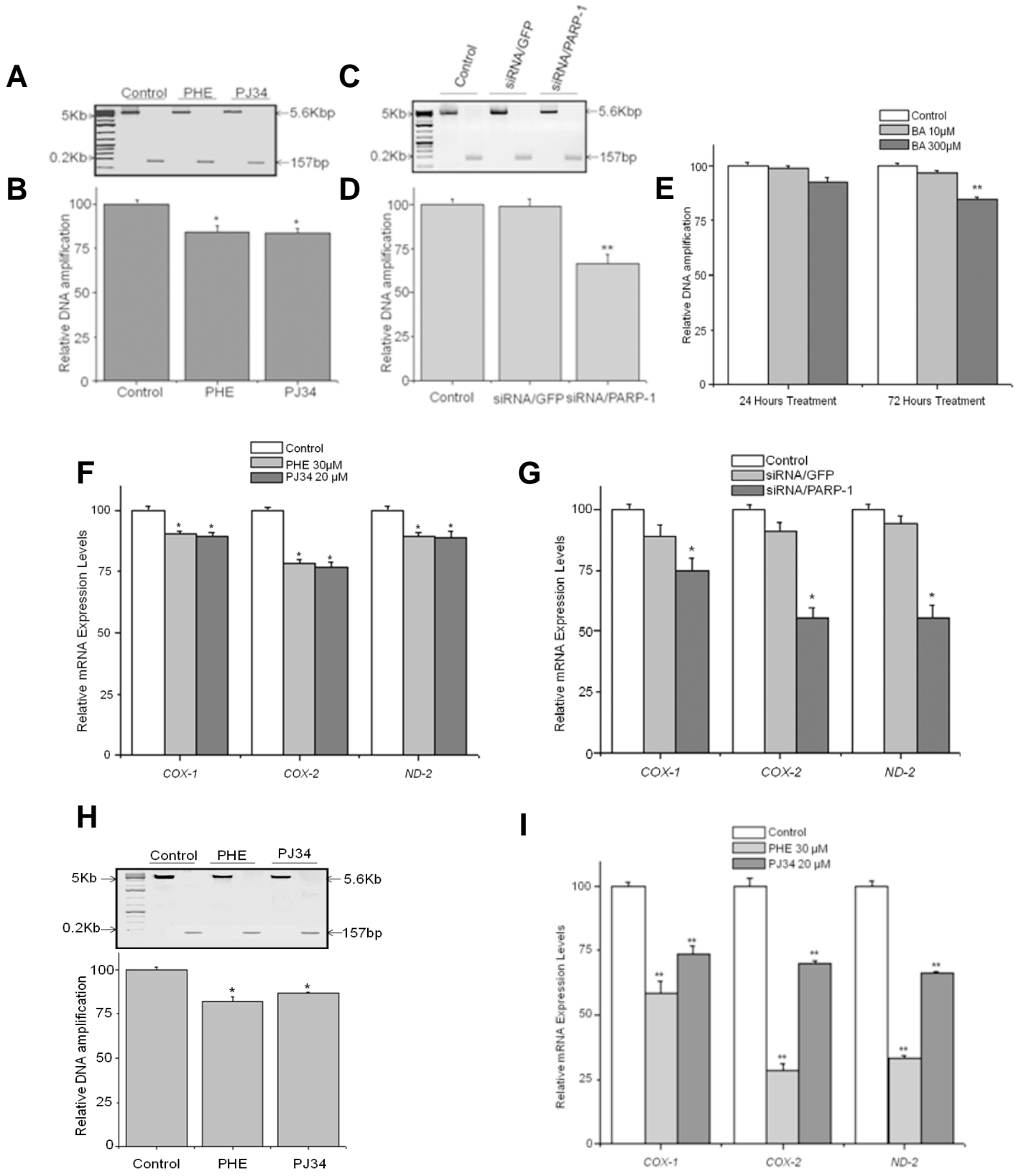


Figure 2

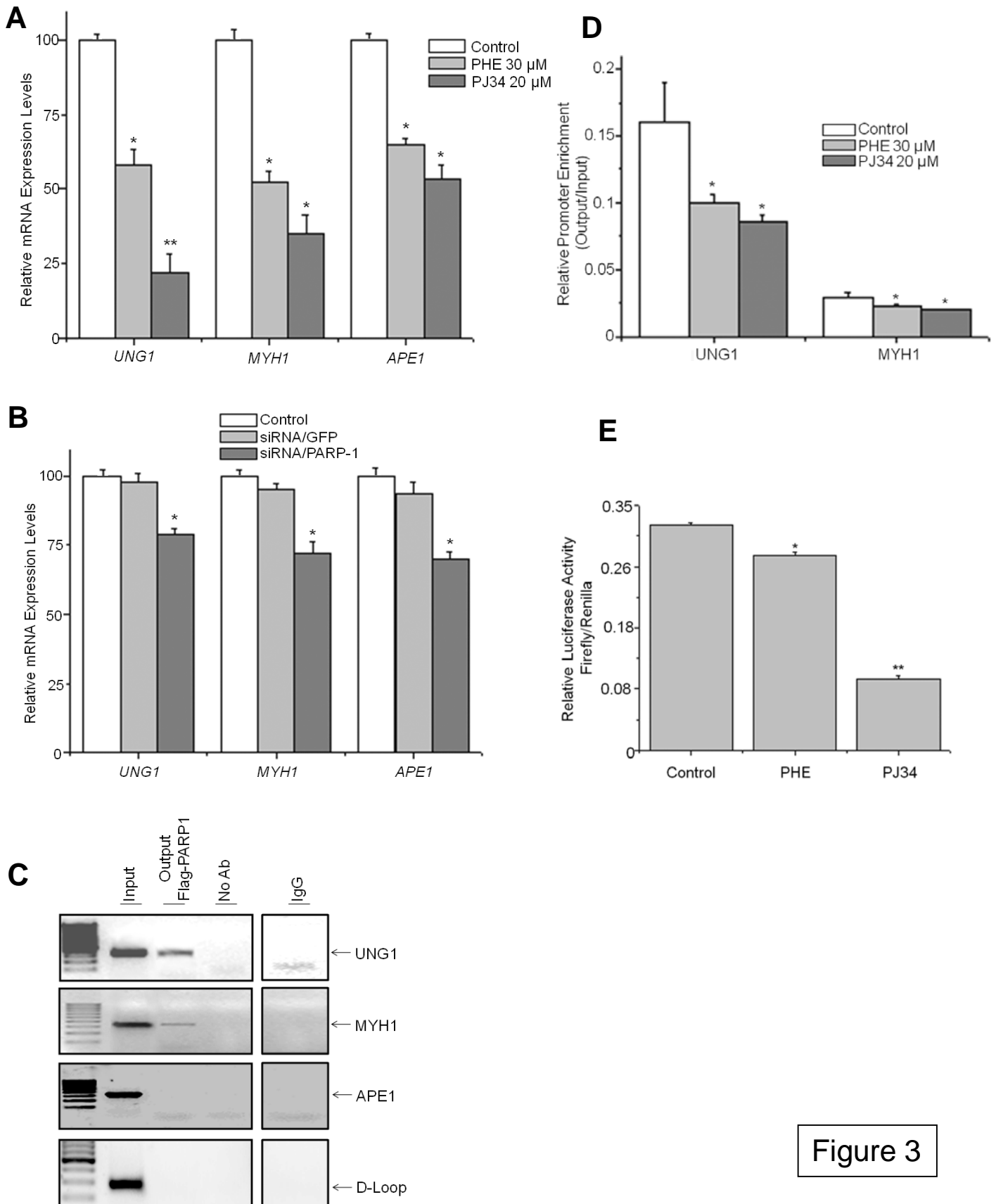


Figure 3

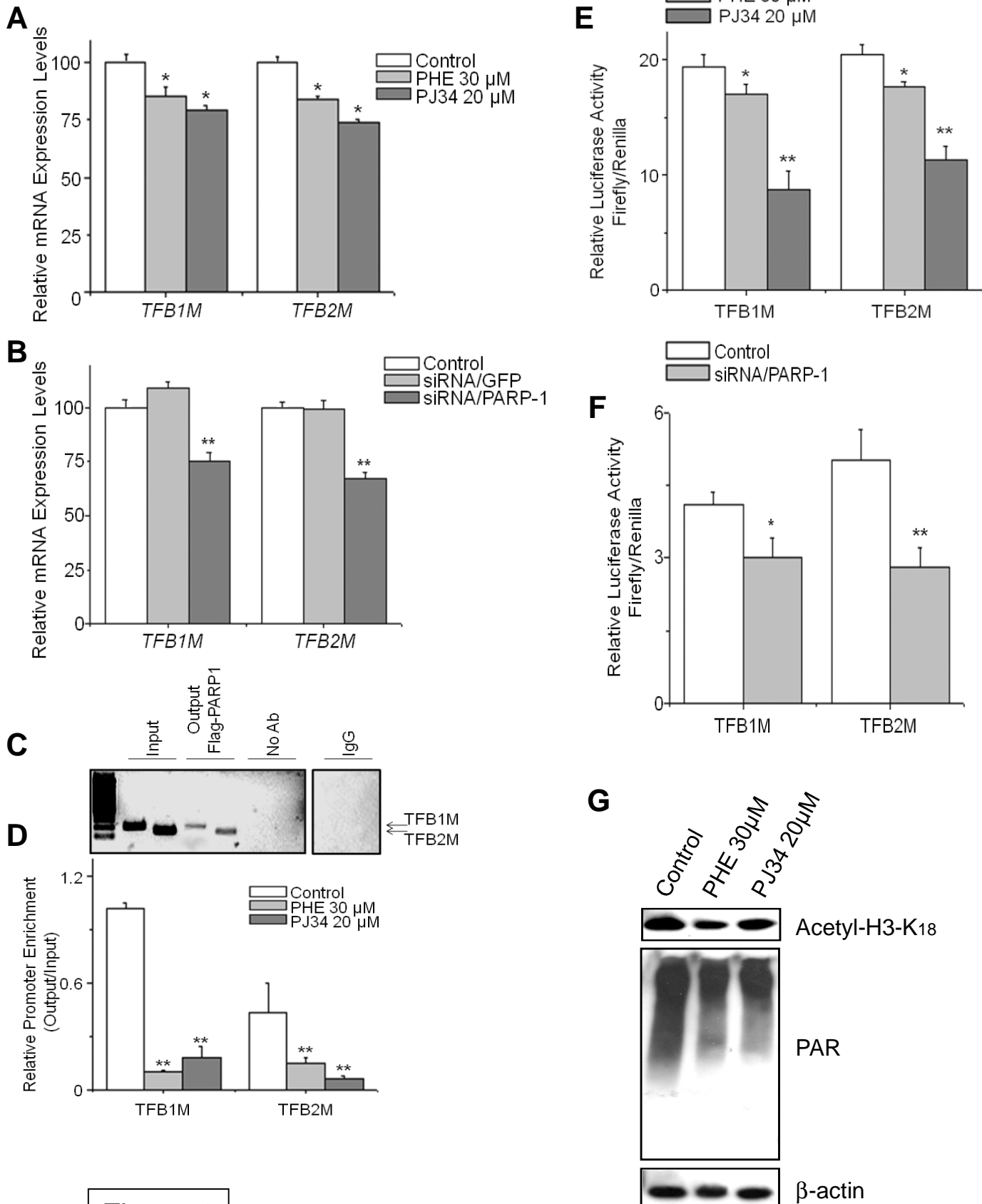


Figure 4

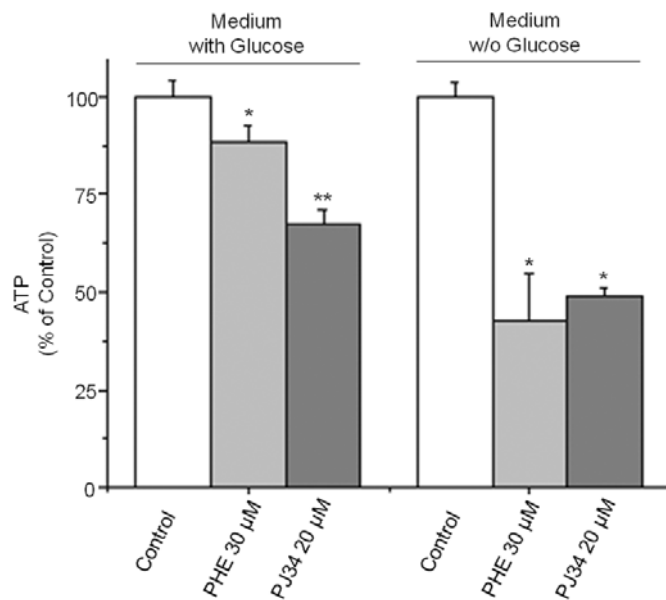


Figure 5